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Anil Prakash *Editors*

Microorganisms in Sustainable Agriculture and Biotechnology

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Foreword

Microbes are ubiquitous and occur in a great variety of normal and extreme, and natural and man-made environments on the Earth. The existence and continuance of other organisms in various ecosystems may not be possible without the activities of these tiny microbes. With approximately 1% culturable microbial diversity known till date, understanding the remaining 99% in different ecosystems is wide open to investigation.

Although the significance of culture-dependent approach is remarkable due to its invaluable contribution and reliability, the large figure of uncultured, and thus, untouched microbes cannot be ignored. Since the introduction of the concept of direct cloning of community DNA in 1985 by Norman Pace, metagenomic approaches applied in a variety of natural habitats have led to understanding the diversity of uncultured microbes, and discovery and characterization of novel genes and gene products.

The application of fossil fuel based inputs such as chemical fertilizers, pesticides, herbicides and others has resulted in increased production. This has, however, led to growing awareness and concern over their adverse effects on soil productivity and environmental quality. The high cost of chemical fertilizers and pesticides, increasing gap between supply and demand and adverse effect on the environment has encouraged scientists to develop alternate strategies for enhancing productivity. An approach in this direction is utilizing soil microbes as bioinoculants for supplying nutrients and/or stimulating plant growth. Some of the rhizospheric microbes are known to synthesize plant growth promoting substances, siderophores and antibiotics, and aid in enhancing the availability of phosphorus. The global research efforts have enabled us to understand some of the difficult problems related to microbial sources by using powerful tools of microbial genetics, molecular biology and biotechnology.

Large quantities of over 100 industrial products such as ethanol and butanol, organic acids, amino acids and others are produced annually. Although a number of these compounds are produced from petrochemicals, all are obtainable by microbial fermentations. Any shift in future towards greater productivity by a biomass-based chemical industry will depend strictly on economics rather than feasibility.

The book includes a collection of reviews on various aspects of sustainable agriculture and biotechnology. Elegant attempts have been made to summarize the developments achieved till date and problems and prospects with further suggestions for future course of action. I believe that the book would provide an overview of developments in sustainable agriculture and biotechnology with some new ideas, which could serve as inspiration and challenge for researchers in the field.

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Preface

The culture-dependent approach has contributed significantly towards understanding the role of microorganisms in the environment and production of a wide variety of products in the welfare of mankind. Approximately 1% of the extant microbial diversity is culturable, and therefore, most of the knowledge of microbes and their applications has originated from this very small fraction of the microbial diversity in different ecosystems. In order to access the large figure of unculturable, and thus, untouched microbes, metagenomic approaches have now been used quite extensively for understanding microbial diversity and exploitation of useful genes for novel metabolic products.

Microorganisms have served the mankind in solving intractable problems through their biosynthetic capabilities in medical technology, human and animal health, food processing, food safety and quality, genetic engineering, environmental protection, agricultural biotechnology and more effective treatment of agriculture, and municipal and industrial waste treatment, which provide a most impressive record of achievements. Most of these technological advancements would not have been possible using conventional straightforward chemical and physical engineering methods, or if they were, they would not have been practically or economically feasible and could have caused considerable environmental degradation.

For many years, soil microbiologists and microbial ecologists have differentiated soil microbes as beneficial, harmful or neutral according to their function, and how they affect soil quality, plant growth and yield, and plant health. Beneficial microorganisms are those that can fix atmospheric nitrogen, decomposes organic waste and residue, detoxify pesticides, suppress plant diseases and soil-borne pathogens, enhance nutrient cycling and produce bioactive compounds such as vitamins, hormones and enzymes that stimulate plant growth. Harmful microorganisms are those that can induce plant disease, stimulate soil borne pathogens, immobilize nutrients and produce toxic and putrescent substances that adversely affect plant growth and health. Beneficial naturally occurring microorganisms can be utilized as inoculants to increase their population in soil, and thus utilize them in sustainable agricultural systems.

The conceptual designs are important in developing new technologies for utilizing beneficial and effective microorganisms for a more sustainable agriculture and

production of useful bioactive compounds. An idealized system based on this should maintain and improve human health, be economically beneficial to both producers and consumers, actively protect the environment, be self contained and regenerative and produce enough food for ever increasing world population on a long term and sustainable basis.

This book is aimed at reviewing the recent developments in understanding the role of microorganisms in sustainable agriculture and biotechnology. Towards this end, we have requested scientists who have expertise in different areas of agriculture and industrial microbiology and/or biotechnology to review the progress made in these areas till date. We wish to place on record our heartfelt thanks to all the contributors of the book. We thank Mr. Ankit Kumar for support with the background work. We wish to also express our thanks to Springer for publishing the book.

T. Satyanarayana
B.N. Johri
Anil Prakash
(Editors)

Contents

About the Editors

After completing M.Sc. and Ph.D. at the University of Saugar (India), **T. Satyanarayana** had post-doctoral stints at the University of Bhopal and France. In 1988, he joined the Department of Microbiology, University of Delhi South Campus as Associate Professor and became Professor in 1998. His research efforts have been focused on understanding the diversity of yeasts, and thermophilic fungi and bacteria, their enzymes and potential applications, heterotrophic carbon sequestration and metagenomics, and cloning and expression of yeast and bacterial genes encoding industrial enzymes. He has published over 160 scientific papers and reviews, and edited three books. He is a fellow of the National Academy of Agricultural Sciences, Association of Microbiologists of India, Mycological Society of India and Biotech Research Society of India, and a recipient of Dr. G.B. Manjrekar award of the Association of Microbiologists of India in 2003 and Dr. V.S. Agnihotrudu Memorial award of MSI in 2009 for his distinguished contributions. He is one of the Editors for Indian Journal of Microbiology and a member in the editorial board of Bioresource Technology and Indian Journal of Biotechnology. He has over 37 years of research and teaching experience.

Bhavdish N. Johri obtained his doctorate degree from the University of Alberta, Edmonton and carried out Post-Doctoral Research at the University of British Columbia, Vancouver (Canada). He has teaching experience of microbiology of over 35 years and research experience of nearly 40 year. He was Chairperson, Department of Microbiology at G.B. Pant University of Agriculture & Technology (Pantnagar) for considerable period, and was Dean, College of Basic Sciences & Humanities. During this long tenure of academics, he has won several accolades such as INSA Young Scientist Award (1974), UGC Carrer Award (1980), KN Katju Award (1985) and the prestigious Rafi Ahmed Kidwai Award of ICAR (2005). He is a Fellow of National Academy of Sciences (FNASc) and National Academy of Agricultural Sciences (FNAAS). He is the Past President, Association of Microbiologists of India. He has to his credit three edited volumes in the domain of microbiology and over 100 refereed and other publications. Currently he is Acharya PC Ray Silver Jubilee Fellow of the Madhya Pradesh Council of Science & Technology. His major research interests encompass rhizosphere biology and microbial ecology.

Dr. Anil Prakash obtained his B.Sc., M.Sc., M.Phil. and Ph.D. from Barkatullah University, Bhopal. Presently he is working as Associate Professor at the Department of Biotechnology and Coordinator of Bioinformatics Centre. He is fellow of Academy of Science, Engineering and Technology. During 25 years of research and teaching career, he has guided more than 20 Ph.D. students. He has completed a number of projects funded by UGC, ICAR, DBT (Govt. of India), and others. Dr. Anil Prakash is member of several Indian scientific bodies. He has published several research papers and articles on PGPR and mycorrhiza in national and international books and Journals, and published two books.

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Part I
Microbes in Sustainable Agriculture

Chapter 1
Plant Growth Promotion by Phytases and Phytase-Producing Microbes due to Amelioration in Phosphorus Availability

Bijender Singh and T. Satyanarayana

Abstract Phosphorus is a major and critical component of cell and its constituents. It is also an important macronutrient for plant growth and development. Phytic acid, a major form of organic phosphorus in soil, is not readily available to plants either due to the formation of complex with cations or adsorption to various soil components. Phosphate solubilizing microorganisms are ubiquitous in soils and play an important role in making P available from insoluble sources. Phytate hydrolyzing microorganisms are present in cultivated soils as well as in wetland, grassland and forest soils. Several fungi and bacteria hydrolyze the organic insoluble forms of phosphorus by secreting phytases and phosphatases, and thus promote the growth of plants. Insoluble phytates are a major problem in soil, as these are not readily hydrolyzed by all phytases. A large number of transgenic plants have been developed that are able to grow on phytate as sole source of phosphorus. Phytate-phosphorus utilization could be improved by enhancing phytase/phosphatase activity in the rhizosphere. Furthermore, genetic engineering approach could be employed for enabling plants to utilize accumulated forms of organic phosphorus in soils.

Keywords Phytase • Phytic acid • Organic phosphorus • Plant growth promotion • Transgenic plants

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1.1 Introduction

The plant roots in the rhizosphere interact with various physical, chemical and biological properties of soil (Rodríguez and Fraga 1999; Richardson et al. 2009a, b). Plant roots interact with soil microorganisms, which have impact on plant nutrition either directly by influencing nutrient availability and uptake, or indirectly through plant growth promotion (Rodríguez and Fraga 1999; Richardson et al. 2009a, b). Organic phosphorus constitutes 30–80% of soil phosphorus (P) that plays an important role in the agricultural soils (Dalal 1977). The predominant organic form of P is phytate (*Myo*-inositol hexa-phosphates) (Fig. 1.1). The organic form of P is poorly utilized by plants due to the lack of adequate levels of phytases (Mudge et al. 2003; Yadav and Tarafdar 2003, 2007a, b; George et al. 2009; Wasaki et al. 2009). This form of P must be dephosphorylated by phytases and/or phosphatases before its uptake by the plants (Rodríguez and Fraga 1999; Richardson 2001; Richardson et al. 2009a, b; Singh et al. 2011) (Table 1.1). Phytates also form complexes with minerals and proteins thus making them unavailable to plants.

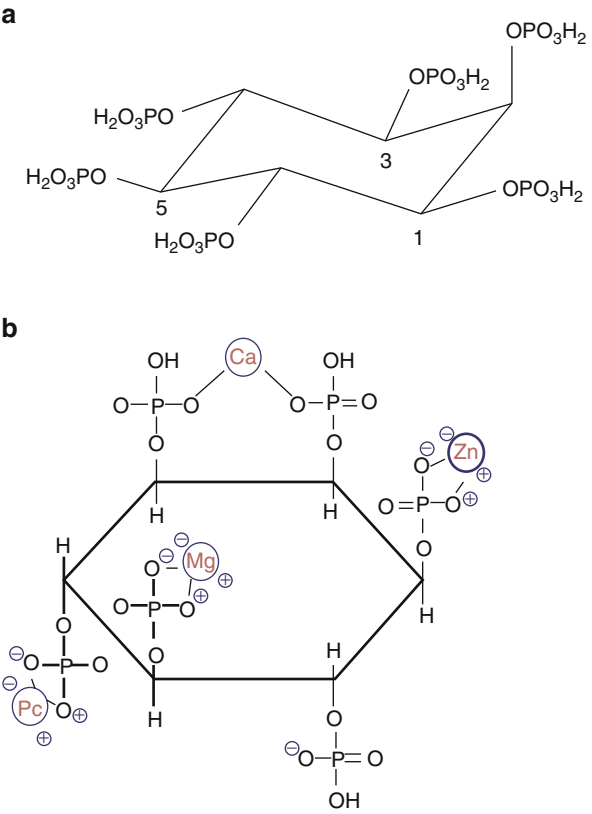

Fig. 1.1 Structure of phytic acid (**a**) and its interaction with metal ions (**b**)

Table 1.1 Hydrolysis of various organic phosphorus compounds by different microorganisms

Substrate	Microbial source	Reference
Non-specific	*Pseudomonas fluorescens*	Ryu et al. (2005)
	Pseudomonas sp.	Richardson et al. (2001a)
	Burkholderia cepacia	Unno et al. (2005)
	Enterobacter aerogenes	Thaller et al. (1995)
	E. cloacae	Thaller et al. (1995)
	Citrobacter freundi	Thaller et al. (1995)
	Proteus mirabalis	Thaller et al. (1995)
	Serratia marcenscens	Ryu et al. (2005), Hameeda et al. (2006)
	Emericella rugulosa	Yadav and Tarafdar (2007b)
Phytates	*Bacillus subtilis*	Ryu et al. (2005)
	B. pumilus	Ryu et al. (2005)
	B. mucilaginous	Li et al. (2007)
	B. amyloliquefaciens	Idriss et al. (2002)
	Pseudomonas putida	Richardson and Hadobas (1997)
	P. mendocina	Richardson and Hadobas (1997)
	Sporotrichum thermophile	Singh and Satyanarayana (2010)
	Emericella rugulosa	Yadav and Tarafdar (2007b)
	Aspergillus niger	Hayes et al. (2000)
	A. fumigatus	Tarafdar and Marschner (2005)
	Discosia sp.	Rahi et al. (2009)
	Chaetomium globosum	Tarafdar and Gharu (2006)
	A. rugulosus	Tarafdar and Rao (1996)

Microorganisms play an important role in P cycle in nature. In the rhizosphere, organic substances exuded from plant roots are utilized by microorganisms as readily available sources of carbon and energy for their growth and reproduction (Rodríguez and Fraga 1999). Phosphatase activity in the rhizosphere is responsible for the hydrolysis of organic P and making available for plants (Tarafdar and Junk 1987; Tarafdar and Claassen 1988). Phytase is an acid phosphatase, which is responsible for the sequential hydrolysis of phytates to a series of *myo*-inositol phosphate derivatives and inorganic phosphate (Singh et al. 2011). Most of the plant species cannot utilize this organic source of P due to the lack of adequate levels of extracellular phytase. When phytase is added exogenously (Singh and Satyanarayana 2010; Idriss et al. 2002; Richardson et al. 2001b; Hayes et al. 2000), or when the phytase gene from microbial origin was expressed in transgenic plants (Richardson et al. 2001a; Wang et al. 2007; Li et al. 2007, 2009), the plants are able to grow on phytate P as sole source of P. Therefore, the presence of phytase in the rhizosphere might enhance plant growth under field conditions (Singh and Satyanarayana 2010). The soil microbes such as *Sporotrichum thermophile* (Singh and Satyanarayana 2010), *Discosia* sp. FIHB 571 (Rahi et al. 2009), *Pseudomonas* sp. (Richardson et al. 2001b) and *Bacillus amyloliquefaciens* (Idriss et al. 2002) have been shown to improve P availability for plant growth promotion due to the activity of phytases.

Table 1.2 List of microorganism involved in acquisition of organic phosphorus for plant growth promotion

Source	Microorganism	Reference
Fungi	*Sporotrichum thermophile*	Singh and Satyanarayana (2010)
	Emericella rugulosa	Yadav and Tarafdar (2007b)
	Aspergillus niger	Hayes et al. (2000)
	A. fumigatus	Tarafdar and Marschner (2005)
	A. rugulosus	Tarafdar and Rao (1996)
	Discosia sp.	Rahi et al. (2009)
	Chaetomium globosum	Tarafdar and Gharu (2006)
	Glomus mosseae	Tarafdar and Marschner (2005)
Bacteria	*Pseudomonas fluorescens*	Ryu et al. (2005)
	Pseudomonas sp.	Richardson et al. (2001a)
	Burkholderia cepacia	Unno et al. (2005)
	Enterobacter aerogenes	Thaller et al. (1995)
	E. cloacae	Thaller et al. (1995)
	Citrobacter freundi	Thaller et al. (1995)
	Proteus mirabalis	Thaller et al. (1995)
	Serratia marcenscens	Ryu et al. (2005), Hameeda et al. (2006)
	Emericella rugulosa	Yadav and Tarafdar (2007b)
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	B. pumilus	Ryu et al. (2005)
	B. mucilaginous	Li et al. (2007)
	B. amyloliquefaciens	Idriss et al. (2002)
	Pseudomonas putida	Richardson and Hadobas (1997)
	P. mendocina	Richardson and Hadobas (1997)
	Actinomycetes	Yadav and Tarafdar (2007a)

Microorganisms utilize the P by secreting phosphatase and phytase in the rhizosphere (Tarafdar and Junk 1987; Tarafdar et al. 1988; Tarafdar and Rao 1996; Richardson 2001; Yadav and Tarafdar 2003; Hayes et al. 2000; Yadav and Tarafdar 2007a, b; George et al. 2009; Wasaki et al. 2009) (Table 1.2).

Besides the soil microbes, plant growth promoting rhizobacteria (PGPR) have been shown to affect the growth of plants (Bloemberg and Lugtenberg 2001; Patel et al. 2010). This effect of PGPR on plant growth promotion has been attributed either due to the production of plant growth regulating substances (Steenhoudt and Vanderleyden 2000) or due to the enhancement of nutrient availability (Nautiyal et al. 2000). There are many reports on the applications of phytases in food and feed industries (Pandey et al. 2001; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007; Rao et al. 2009; Singh et al. 2011). But none of them has described the role of phytases and phytase producing microorganism in plant growth promotion. This chapter is aimed at reviewing the role of phytases and the phytase producing microbes in plant growth promotion due to the amelioration in P availability.

1.2 Phosphorus Availability in Soil

Phosphorus levels range between 0.40 and 1.2 g per kg of soil (Rodríguez and Fraga 1999), and the amount of soluble P in soil is usually very low (Goldstein 1994). The rocks, a biggest reserve of P, provide a cheap source of phosphate fertilizers for agriculture (Rodríguez and Fraga 1999). There are almost 40 million tons of phosphatic rock deposits in India (Rodríguez and Fraga 1999). A considerable part of soil P in agricultural soil is contributed by the application of P fertilizers (Richardson 1994). Although a large portion of soluble inorganic phosphate is applied to soil as chemical fertilizer, it becomes unavailable to plants due to its rapid immobilization after application (Rodríguez and Fraga 1999; Tang et al. 2006). The type of soil and its pH greatly influences the fixation and precipitation of P in soil. For example, in acidic soils, P is fixed by free oxides and hydroxides of aluminum and iron, while in alkaline soils it is fixed by calcium such as super calcium (Jones et al. 1991). The organic form of P is the second major component of soil P, which is mainly present in the form of *myo*-inositol phosphate (phytates) (Singh and Satyanarayana 2010; Tang et al. 2006).

1.3 Organic Phosphorus Acquisition by Plants

Soil contains a wide range of organic P substrates for the growth of crop plants that must be hydrolyzed to inorganic P before its assimilation by the plants. The microbial hydrolysis of organic P is highly influenced by environmental factors as well as physicochemical and biochemical properties of the molecules (Ohtake et al. 1996; McGrath et al. 1995, 1998; Rodríguez and Fraga 1999). Phytic acid, polyphosphates, and phosphonates are decomposed more slowly as compared to nucleic acids, phospholipids, and sugar phosphates. The hydrolysis of most of the organic phosphorous compounds is carried out by means of phosphatase enzymes. The phosphatases may be acidic or alkaline depending upon the optimal pH for their activity. On the basis of substrate specificity, they are also classified as specific or nonspecific acid phosphatases (Cosgrove et al. 1970; Rossolini et al. 1998). Microbial phosphatase activity has been detected in different types of soils (Tarafdar and Junk 1987; Garcia et al. 1992; Kirchner et al. 1993; Xu and Johnson 1995). A large portion of soil and rhizosphere microorganisms is able to utilize phytates as carbon and P source (Richardson and Hadobas 1997). The utilization of phytates by crops and microbes is generally limited under field conditions due to the formation of insoluble phytates and adsorption to soil particles (Hayes et al. 2000; Tang et al. 2006; Singh et al. 2011). This hypothesis is supported by the fact that phytase activity could be stimulated by supplementation with organic acids such as oxalic acid, citric acid and malic acid (Singh and Satyanarayana 2010; Tang et al. 2006). The secretion of organic acids from plant roots and microorganisms enhances P availability by chelating cations such as Fe, Al or Ca that are involved in the formation

of insoluble phytates (Tang et al. 2006; Singh and Satyanarayana 2010). Adams and Pate (1992) proved this hypothesis in white lupin (*Lupinus albus* L.), a high organic acid producer, which exhibited vigorous growth as compared to other species using sodium phytate as the sole P source.

1.4 Phytase Producing Microbes and Their Effect on Plant Growth

The insoluble forms of organic P are converted to an accessible soluble form by many soil microorganisms. These microbes are called as plant growth promoting microorganisms (PGPM). Organic P compounds are dephosphorylated by phosphatases before assimilation by the plants, because plants acquire P as inorganic P (Richardson 2001). A list of microorganisms, which showed growth promotory effect on plants, is given in Table 1.2.

A fungus, *Discosia* sp. FIHB 571 isolated from tea rhizosphere showed plant growth promotory effects due to the solubilization of inorganic phosphates by producing phytase, and siderophores and auxins (Rahi et al. 2009). The fungal inoculum significantly increased the root length, shoot length and dry matter in maize, pea and chickpea over the uninoculated control. A phosphatase and phytase producing fungus *Emericella rugulosa* was tested under field conditions using pearl millet as a test crop in a loamy sand soil having 68% organic phosphorous as phytate (Yadav and Tarafdar 2007b). The fungal contribution was significantly higher in the hydrolysis of various organic P compounds as compared to plant. The fungal inoculation resulted in a significant improvement in plant biomass, root length, seed and straw yield and P concentration of root and shoot as compared to the control plants.

An extracellular HAP-phytase producing thermophilic mould *Sporotrichum thermophile* promoted the growth of wheat seedlings (Singh and Satyanarayana 2010). The growth and inorganic phosphate content of the plants were higher than the control.

The compost prepared by the combined action of native microflora of wheat straw along with phytase producing *S. thermophile* promoted the growth of wheat seedlings. The inorganic phosphate content of the wheat plants was higher than those cultivated on the compost prepared either with only native microflora or *S. thermophile*. *Sporotrichum thermophile* is known to efficiently decompose plant residues by secreting an array of different enzymes such as amylase, xylanase, phosphatase, protease, lipase and phytase (Singh and Satyanarayana 2006). Similarly, an extracellular HAP-phytase of *S. thermophile* promoted the growth of wheat seedlings (Singh and Satyanarayana 2010). The growth and inorganic phosphate content of the plants were higher than the control.

Sodium phytate (5 mg per plant) was adequate for liberating the amount of P required for the growth of the seedlings. The plant growth, root/shoot length and inorganic phosphate content of test plants were better than the control plants. An

enzyme dose of 20 U per plant was sufficient to liberate enough amount of inorganic phosphate required for supporting plant growth.

Phytase and phosphatase producing fungi were used as seed inoculants to ameliorate P nutrition of plants in the soils containing high phytate P (Yadav and Tarafdar 2003). The efficiency of hydrolysis of different organic P compounds by various fungi was beneficial to exploit native organic P for plant nutrition. The seedlings of the six species (three legumes and three grasses) grew well as the plants supplied with inorganic P, while phytate was a poor source for plant growth (Hayes et al. 2000). Addition of *A. niger* phytase in the medium liberated sufficient P to enable *T. subterraneum* seedlings to grow comparable with plants supplied with inorganic P. Yadav and Tarafdar (2007a) studied the effect of phytase and phosphatase producing actinomycetes on the growth of cluster bean (*Cyamopsis tetragonoloba* (L.) Taub.). The organic phosphorus source was made available to the plant by the action of phytase and phosphatase secreted by actinomycetes.

A phosphatase and phytase producing fungus, *Chaetomium globosum* was tested as an inoculant for wheat and pearl millet crops (Tarafdar and Gharu 2006). A significant improvement in plant biomass, root length, plant P content, seed and straw yield and seed P content was observed after fungal inoculation. Among various *Aspergillus* spp., *A. rugulosus* was found to be superior to others that resulted in improved dry matter and grain yield in wheat and chick pea due to acquisition of phosphorus by plants (Tarafdar and Rao 1996). Tarafdar and Marschner (2005) studied the effect of co-inoculation of a phytase producing fungus (*A. fumigatus*) and a VAM fungus (*Glomus mosseae*) on wheat grown in two heat-sterilized low-phosphorus soils supplied with sodium phytate. Seed inoculation with the *A. fumigatus* or soil inoculation with *G. mosseae* resulted in increased shoot and root dry weight and root length, phosphatase activity in the rhizosphere and shoot content of P and to a lesser extent K and Mg. However, the co-inoculation of both fungi resulted in better plant growth.

The presence of organic phosphate hydrolyzing bacteria in soil has been investigated in the rhizosphere of pasture grasses (Greaves and Webley 1965) and rice plants (Raghu and MacRae 1966; Bishop et al. 1994; Abd-Alla 1994). Soil microbes expressing a significant level of acid phosphatases/phytases include strains from the genus *Rhizobium* (Abd-Alla 1994), *Enterobacter*, *Serratia*, *Citrobacter*, *Proteus, Klebsiella* (Thaller et al. 1995), *Pseudomonas* (Richardson et al. 2001a, b; Ryu et al. 2005), *Bacillus* (Li et al. 2007), and *Sporotrichum thermophile* (Singh and Satyanarayana 2010), *Emericella rugulosa* (Yadav and Tarafdar 2003, 2007b), *Discosia* sp. FIHB 571 (Rahi et al. 2009) and some other fungi. Unno et al. (2005) isolated over 300 phytate-utilizing bacterial strains from the rhizosphere of *Lupinus albus* (L.) and these were identified as *Burkholderia* based on 16S rDNA sequence analysis. These isolates were able to hydrolyze insoluble phytates when co-cultured with *Lotus japonicus* seedlings. Some isolates resulted in improved growth of seedlings as compared to control seedlings.

Plant growth promoting rhizobacteria (PGPR) are well known to exert beneficial effects on plant growth and development. Ryu et al. (2005) studied the effect of PGPR on plant growth promotion. Some PGPR strains such as *Bacillus subtilis*

GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* SE-34, *B. pumilus* T4, *B. pasteurii* C9, *Paenibacillus polymyxa* E681, *Pseudomonas fluorescens* 89B-61, and *Serratia marcescens* 90–166 increased foliar fresh weight of *Arabidopsis* as compared to control plants. *In vivo* studies showed that all bacterial strains promoted foliar fresh weight in a greenhouse study. Some plant hormone mutants of *Arabidopsis* were generated to study the effect of bacterial strains in signal transduction pathways both *in vitro* as well as *in vivo*. The stimulation of plant growth promotion by PGPR strains *in vitro* involved signaling of brassinosteroid, IAA, salicylic acid and gibberellins, while *in vivo* ethylene signaling pathway was involved (Ryu et al. 2005).

Various phytate-mineralizing bacteria (PMB) and phosphate-solubilizing bacteria (PSB) were isolated from the rhizosphere of perennial ryegrass (*Lolium perenne*), white clover (*Trifolium repens*), wheat (*Triticum aestivum*), oat (*Avena sativa*), and yellow lupin (*Lupinus luteus*) growing in volcanic soil in Chile (Jorquera et al. 2008). Among 300 isolates, 6 bacteria were selected based on their ability to utilize both Na-phytate and Ca-phosphate as sole source of P on agar media. These isolates were identified as strains of P*seudomonas*, *Enterobacter*, and *Pantoea*. All the selected strains exhibited production of phosphatases that resulted in a higher P liberation as compared to the controls.

Hariprasad and Niranjana (2009) isolated bacteria from rhizospheric soil of tomato, which were able to solubilize both inorganic and organic forms of phosphorous. In a green house study, all the isolates showed improvement in shoot length, root length, fresh weight, dry weight and P content of tomato seedlings as compared to the control. The analysis of rhizosphere soil samples of 30 day old seedlings revealed that the available P content was high in soil samples from the plots where plants have been raised from bacterial inoculated seeds. Some isolates had also shown protection against *Fusarium* wilt.

Among various isolates of plant growth-promoting bacteria (PGPB) from farm waste compost (FWC), rice straw compost (RSC), *Gliricidia* vermicompost (GVC), and macrofauna associated with FWC, seven significantly increased shoot length, while ten showed significant increase in leaf area, root length, density, and plant weight (Hameeda et al. 2006). Maximum increase in plant weight was caused by *Serratia marcescens* EB 67, *Pseudomonas* sp. CDB 35, and *Bacillus circulans* EB 35. All the three composts significantly increased the growth of pearl millet. The inoculation of composts with bacteria further improved plant growth.

1.5 Transgenics and Plant Growth Promotion

Plants are not able to utilize organic P directly due to the lack of adequate levels of phytases and phosphatases. The microbial sources are promising and have been explored for phytase production (Pandey et al. 2001; Vohra and Satyanarayana 2003; Vats and Banerjee 2004; Kaur et al. 2007; Rao et al. 2009; Singh et al. 2011). These microbial phytases have been characterized, and the phytase-encoding gene from microbial sources could be utilized for generation of transgenic plants. There

are various reports where microbial phytase genes have been cloned and expressed in crop plants (Yip et al. 2003; Lung et al. 2005; Shengfang et al. 2007; Wang et al. 2007, 2009) (Table 1.3). The transgenic white clovers harboring *A. niger* phytase gene were established from cotyledon using *Agrobacterium tumefaciens*-mediated transformation method (Shengfang et al. 2007). The phytase activity in root of the transgenic lines was higher as compared to the control when phytate was used as sole source of P.

The P concentration of plants, the P amount per plant, plant fresh weight, and plant dry weight were much higher in transgenic lines than in controls. *Aspergillus fumigatus* phytase was expressed in tobacco using *Agrobacterium* mediated transformation (Wang et al. 2007). The recombinant protein accumulated in leaves up to 2.3% of total soluble protein and it was highly thermostable. A phytase gene from *A. niger* was expressed in soybean that resulted in improved growth and P acquisition by the transgenic plants (Li et al. 2009). The transgenic *Arabidopsis* plants harbouring *A. niger* phytase gene secreted phytase only from roots when grown on medium with low phosphate (Mudge et al. 2003). The transgenic plants were able to grow on medium containing phytate as a sole source of P. The growth rates and shoot P concentrations of these plants were similar when grown on phytate or phosphate as the P source. The growth and P nutrition of *A. thaliana* plants supplied with phytate improved significantly after the introduction of phytase gene from *A. niger* (Richardson et al. 2001a). Growth and P nutrition of the transformed plants were improved and were equivalent to the control plants supplied with inorganic phosphate as sole source of P. This suggested the extracellular phytase activity of plant roots as a significant factor in the utilization of P from phytate and opportunity for using genetic engineering for enabling plants to utilize accumulated forms of soil organic P.

A β-propeller phytase from *Bacillus subtilis* was constitutively expressed in tobacco and *Arabidopsis*, which was secreted from roots (Lung et al. 2005). In transgenic tobacco, phytase activities in transgenic leaf and root extracts were seven to nine times higher than those in the wild-type extracts, while the extracellular phytase activities of transgenic plants were enhanced by four to six times. The transgenic tobacco lines accumulated more shoot biomass than the wild-type plants after 30 days of growth with concomitant increase in shoot P concentration in hydroponic culture using 1 mM Na-IHP as the sole P source. Similar observations have been recorded in the transgenic *Arabidopsis;* this explains the conversion of soil phytate into inorganic phosphate for uptake by plants (Lung et al. 2005). Yip et al. (2003) observed phenotypic changes in the tobacco lines transformed with a neutral *Bacillus* phytase. The transgenic tobacco showed an increase in flower and fruit numbers, small seed syndrome, lower seed IP6/IP5 ratio, and enhanced growth under phosphate-starvation conditions compared with the wild type. These observations suggested that the over-expression of *Bacillus* phytase in the cytoplasm of tobacco cells shifts the equilibrium of the inositol phosphate biosynthesis pathway, thereby making more phosphate available for primary metabolism.

Transgenic *Arabidopsis* plant expressing phytase gene from *Medicago truncatula* exhibited significant improvement in organic P utilization and plant growth (Xiao et al. 2005). When phytate was supplied as the sole source of P, dry weight of

Table 1.3 Plant growth promontory effect of recombinant and wild type phytases from various microorganisms

S. No.	Phytase source	Recombinant host	Plant growth promotion	Reference
1.	*Burkholderia* sp.	Wild type	Lotus	Unno et al. (2005)
2.	*Discosia* sp.	Wild type	Maize, Chickpea	Rahi et al. (2009)
3.	*Emericella rugulosa*	Wild type	Pearl millet	Yadav and Tarafdar (2007b)
4.	*Chaetomium globosum*	Wild type	Wheat, Pearl millet	Tarafdar and Gharu (2006)
5.	*Sporotrichum thermophile*	Wild type	Wheat	Singh and Satyanarayana (2010)
6.	*Aspergillus niger*	White clover	White clover	Shengfang et al. (2007)
7.	*A. rugulosus*	Wild type	Wheat, Chick pea	Tarafdar and Rao (1996)
8.	*A. niger*	Soybean	Soybean	Li et al. (2009)
9.	*A. niger*	Wild type	*T. subterraneum*	Hayes et al. (2000)
10.	*A. niger*	Arabidopsis	Arabidopsis	Mudge et al. (2003); Richardson et al. (2001a)
11.	*A. fumigatus*	Tobacco	Tobacco	Wang et al. (2007)
12.	*A. fumigatus*	Wild type	Wheat	Tarafdar and Marschner (2005)
13.	*Bacillus subtilis*	Tobacco, Arabidopsis	Tobacco, Arabidopsis	Lung et al. (2005)
14.	*Bacillus* sp.		Tobacco	Yip et al. (2003)
15.	*Medicago truncatula*	Arabidopsis	Arabidopsis	Xiao et al. (2005)
16.	*A. niger*	*Bacillus mucilaginosus*	Tobacco	Li et al. (2007)
17.	Actinomycetes	Wild type	Cluster bean	Yadav and Tarafdar (2007a)
18.	*Bacillus subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. pasteurii*, *Paenibacillus polymyxa, Pseudomonas fluorescens*, *Serratia marcescens*	Wild type		Ryu et al. (2005)
19.	P*seudomonas* sp., *Enterobacter* sp., *Pantoea* sp.	Wild type	Perennial Ryegrass, White clover, Wheat, Oat, Yellow lupin	Jorquera et al. (2008)
20.	Rhizobacteria	Wild type	Tomato	Hariprasad and Niranjana (2009)

the transgenic lines were 3.1–4.0-fold higher than the control plants, and the total P contents were 4.1–5.5-fold higher than the control, suggesting the great potential of heterologous expression of phytase gene for improving plant P acquisition and for phytoremediation. Greenhouse and field experiments have shown that both wild (*Bacillus mucilaginosus*) and transgenic (containing phytase gene) strains promoted the tobacco plant growth (Li et al. 2007).

1.6 Future Perspectives and Conclusions

With increased concerns over P pollution in the areas of intensive livestock and availability of organic P in insoluble form, phytases have immense potential in commercial and environmental applications. The moulds such as *S. thermophile* and *A. niger* that are known to play an important role in organic matter decomposition and mineralization could be used as inoculants during composting in order to achieve solubilization of organic P for promoting growth of crop plants and improving productivity. Modern science techniques could be utilized for development of foods with a higher iron and zinc content and improved bioavailability of the minerals and proteins. Furthermore, the transgenic plants harboring the phytase gene from microorganisms could also be used to improve soil fertilization and nutrient uptake by plants. The phytic acid content in grains could be reduced using enzymatic approach in order to increase mineral absorption from the diets. Transgenic plants with low phytic acid or expressing recombinant phytase could be a novel approach for reducing micronutrient malnutrition and animal waste P. Further research efforts are, however, needed to understand the biology of phytic acid accumulation during seed development and its pros and cons in human health (Mendoza 2002). With the collaborative efforts of scientists from all over world, effective solutions to the biotechnological development of an ideal phytase for animal nutrition, human health, and environmental protection are expected to be available in the near future.

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Chapter 2
Environmental and Nutritional Benefits of Biopreservation of Animal Feed

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Abstract Biopreservation of moist animal feed provides an alternative to conventional conservation methods and is often beside of saving energy and antimicrobial chemicals a way to improve feed quality. In regions with temperate climate drying of cereal grains can require approximately 60% of the total energy input during plant husbandry. In tropical regions, drying can be incomplete due to high air humidity and improper handling of the material, resulting in a risk for contamination with mycotoxine forming moulds or pathogenic microorganisms. Microbial enzyme activities on feed and activation of intrinsic enzymes in plant material stored wet, increase the bioavalability of minerals and nutrients. For instance phytases can decrease the phytate content. Phytate is the main phosphate storage molecule in plants. It is only partially degraded in non-ruminants, resulting in phosphate release and eutrophication of water environments. Phytate is also an antinutrient, binding minerals and proteins and its degradation will improve the bioavailability of those substances. Biopreservation biomass can also be a locally produced protein source, which can replace soya. Soya production is frequently associated with negative environmental and socioeconomic consequences, and its worldwide trading requires energy for transport and handling. Translocation of nutrients between countries also precludes nutrient recirculation on arable land. Biopreservation often relies on spontaneous microbial developments, thus storage stability, feed hygiene, palatability and nutritional value may vary depending on the microbial composition. Microbial populations in feed are often monitored by traditional plate counting methods, but species identification of isolates demonstrated substantial changes in microbial populations even when plate counting indicated a stable flora. From economical, environmental and animal welfare perspectives it is of great interest to

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obtain a stable and predictable microbial population in the feed, which can be achieved by developing starter cultures.

Keywords Cereal grain • Feed microbiology • Yeast • Biopreservation • Biocontrol • Phytase • Protein

2.1 Introduction

Microbes on animal feed may interfere with feed hygiene, storage stability, palatability of the feed and bioavailability of minerals and proteins depending on the composition of the microbial population. On the other hand microbial species present in or added to animal feed may also do the opposite and contribute to increased hygiene, stability, protein and mineral availability (Olstorpe and Passoth 2010). Cereal grains such as corn, barley, oat, wheat, triticale, sorghum, or millet are used in different parts of the world as animal feed (Hammes et al. 2005). Different cereals can be included in animal diets in varying proportions, depending on cereal cultivar, species and production capacity of the animal. Calves, pigs and poultry depend upon cereal grains for their main source of energy, and as much as 90% of their diet may consist of cereals and products derived from cereal grain, like hulls or distillers' grain (McDonald et al. 2002). When storing cereal grain, the objective is to minimise losses and maintain nutritional value. Improper handling of harvested cereal grain causes significant quantitative and qualitative losses, ranging from 9% to 50% (Sinha 1995). In temperate climates cereals normally have a water activity (a_w) of 0.86–0.97, corresponding to a water content of 15–25% at harvest. Thus preservative measures are needed to secure safe storage. The main preservation methods are drying, acid treatment and airtight storage (Jonsson 1996). The water content of the cereal grain at harvest determines how quickly the cereal grain needs to be preserved to avoid deterioration (Flannigan 1987; Magan et al. 2003). The water content can vary substantially in different years, depending on the local weather conditions before and during harvest (Ekström and Lindgren 1995; Olstorpe et al. 2010b).

2.2 Survey About Common Grain Conservation Methods

2.2.1 Drying

To achieve safe storage, the grain has to be dried to a water content of 13% ($a_w < 0.65$) where there is generally no growth of microorganisms. Insufficient drying may lead to deterioration in grain quality during storage. Different drying methods may cause an uneven drying zone, leaving zones with cereal grain of higher moisture content. Especially in humid climates due to poor storage equipment or improper handling of

the material moisture increases after drying can also happen. This can result in wet spots (also called hot spots), where microbial growth and grain deterioration can occur. The most effective drying technique would be with hot air. Cereal grains dried with heat addition or with hot air usually show much lower moisture content than those dried with surrounding temperature air (Lacey and Magan 1991; Jonsson 1996). On the other hand, hot air drying consumes much energy. In temperate climates, approximately 60% of the energy used during total plant husbandry operations was calculated to be spent on grain drying (Pick et al. 1989). A large proportion of the required energy input is met by fossil fuels such as diesel or gasoline. The use of fossil fuel needs to be reduced, because their combustion gives rise to pollution that contributes to eutrophication, acidification and climate change (SJV 2008). One way to reduce energy consumption in agriculture is to use safe and energy-efficient long term storage methods for moist cereal grain. Preserving cereal grain while moist dramatically reduces the consumption of fossil fuels used for drying of the grain.

2.2.2 Acid Treatment

Acid treatment of cereal grain is a preservation method applicable to moist feed grain. The preservation method inactivates the sprout and interferes with the baking process which precludes it to be used as storage method to cereals intended to baking or seeding. Acid application is a delicate process and needs to be monitored accurately. Uneven distribution of acid over the kernel surface may permit mould growth during storage. Initial mould growth may then affect surrounding grains, as acid is less effective on actively growing microorganisms (Jonsson 1997). Addition of the correct concentrations of acid depends on the water content of the cereal grain (Lacey and Magan 1991) and it is very critical to obtain the correct amount. It has for instance been shown that too low concentrations of propionate can even stimulate the production of aflatoxins (Al-Hilli and Smith 1979). Balanced concentrations of propionic acid may be sufficient to inhibit the normal spoilage moulds associated with cereals in temperate climates, but not *Aspergillus flavus*. Even though growth of this fungus has been partially inhibited, it can still produce aflatoxin B_1 at enhanced levels in these conditions. The production of acids is very energy consuming and has been estimated to represent 15–20% of the energy consumed during silage production (Strid and Flysjö 2007). Formic acid has been widely used but is now forbidden in some countries due to the risk of aflatoxin production in the event on inaccurate dosage (Clevström et al. 1989).

2.2.3 Airtight Storage

Airtight storage of grains is a preservation method that relies on a perfectly airtight silo with modified atmosphere, enabling storage of the cereal grain at higher

moisture content. Airtight storage is not suitable for grain intended for baking, as the gluten protein is adversely affected, and the germination capacity impaired. However, further environmental benefits could be gained by using moist cereals during bioethanol production. Apart from the considerably lower energy demands during grain storage, moist stored grain also supported fermentation with a substantially improved ethanol yield of more than 10% (Passoth et al. 2009). The moist grain storage method only requires about 2% of the energy that is consumed in high-temperature drying (Pick et al. 1989). Modified atmosphere with reduced levels of O_2 and increased levels of CO_2 is received by respiration of the grain and the endogenous microflora (Lacey and Magan 1991; Magan et al. 2003). The control of spoilage microorganisms depends on maintaining the modified atmosphere. However, temperature fluctuations may, in turn, generate pressure fluctuations in the silo (Druvefors et al. 2002). Imperfect sealing and feed outtake also lead to gas leakage that may alter the modified atmosphere needed. Feed outtakes also result in a continuously diminishing grain bulk, making it difficult for microbial and grain respiration to sustain the modified atmosphere. Deteriorative microbial development and spontaneous heating may then occur (Lacey and Magan 1991).

2.2.4 Moist Grain Crimping

Moist grain crimping is ensiling of feed grain, a storage method that is now becoming widely used (Finch et al. 2002). Moist crimping of cereal grain allows the harvest of grain at higher moisture content. This may protect the crops from prolonged exposure to inclement weather, which might otherwise lead to weathering and mould infections of the grain in the field (Lacey and Magan 1991). Moist crimped cereal grain cannot be stored in silos, as moisture content above 25% impedes the feed outtake system (Jonsson 1996). However, other structures could be used, such as permanent clamps or bunkers, or plastic tubes. The use of plastic tubes has increased in the last few years. This storage system is interesting from an economical point of view because increasing oil prices also result in increasing costs of hot air drying. In addition, capital and maintenance costs for permanent storage space have been replaced by mobile costs (Sundberg 2007). Life cycle analyses have also shown that local production of cereal grains i.e. on individual farms, reduces the environmental impacts of grain production (Cederberg and Ericsson 2007). Cereals intended for crimping should preferentially be harvested during grain yellow ripeness, while the kernels have moisture content of 30–45%. Prior to storage, the cereals are preferably rolled to facilitate packing and thereby reduce air-space between the kernels. The precise mechanism of conservation is still not completely understood. It is assumed that at sufficiently high moisture content, a spontaneous fermentation starts with the endogenous microbial flora resulting in ensiled cereal grain. It is believed that the stability of moist crimped cereal grain is due to acid production by the natural microbial flora on the cereals (http://www.kelvincave.com). However, recent investigations demonstrated that

the pH of the grain does not substantially decrease, even if the moisture content is close to the recommended value.

The LAB and yeast populations were very different in the storage systems of different farms, even in those situated close to each other and partially using the same equipment for harvest and storage (Olstorpe et al. 2010a, b). It is getting clear that more research is required to understand the stabilisation of the crimped grain in airtight storage, with the aim to minimise economical risks for farmers and health risks for consumers.

2.3 Microbial Population in Cereal Grain

Microorganisms habitat the cereal grain as a mixed consortium of bacteria, yeasts and filamentous fungi (Magan et al. 2003). The colonisation of plants by microorganisms starts almost as soon as leaves are exposed to the air. Bacteria usually colonise first, rapidly followed by yeasts, and then by pathogenic and saprophytic fungi. Filamentous fungi usually continue to develop at all stages of plant growth, including seed ripening (Flannigan 1987; Lacey and Magan 1991; Magan et al. 2003).

Microbial populations in feed are traditionally determined by spreading according dilutions of microorganisms on media selective for a certain group of microbes and counting the colony forming units (cfu). However, results from this methods should be taken with care. At first, it is a well known phenomenon, that only a part of microbes present in the material will grow on the cultivation plates (Amann et al. 1995). Secondly, it is not commonly known that antibiotics have to be included when certain groups are counted, which can for instance result in bacterial growth on Malt Extract Medium, overestimating the number of yeasts. Moreover, bacteria can also grow on selective media of other groups. We found substantial growth of lactic acid bacteria on medium specific for total aerobic bacteria (see below). Finally, species composition can change within a microbial group during storage or feed fermentation. Recent studies have shown that populations were apparently stable according to plate count methods, but identification of the organisms revealed ongoing changes (Olstorpe et al. 2008, 2010a).

2.3.1 Bacteria

In the field, forages normally contain bacteria at approximately 6.5 log units g^{-1} fresh material. However, these numbers may vary substantially between production location and different microbial groups (Table 2.1, Olstorpe et al. 2010a, b). The minimum a_w supporting active growth of most Gram-negative and Gram-positive bacteria are 0.97 and 0.90, respectively (Adams and Moss 2000). The dominant bacterial flora on plant surfaces belongs to Gram-negatives such as species of *Erwinia*, *Pseudomonas* and *Xanthomonas* (Flannigan 1987) and a smaller number

Table 2.1 Log colony forming units of different bacterial groups and moisture content (%) in cereal grain at harvest

Farm	Lactic acid bacteria	Total aerobic bacteria	*Enterobacteriaceae*	Moisture content (%)
1[a]	5.5	7.8	6.6	14.9
2[a]	6.6	9.1	8.4	15.2
3[a]	4.3	7.8	7.3	16.4
4[a]	4.6	7.5	6.4	15.5
5[a]	3.1	9.6	7.8	21.4
6[a]	5.7	8.8	8.3	23.3
7[a]	6.2	8.8	6.9	29.8
1[b]	5.6	7.9	5.2	16.4

[a]Values from Olstorpe et al. (2010b)
[b]Values from Olstorpe et al. (2010a)

of Gram-positive bacteria, including species of *Lactobacillus* and *Leuconostoc* (Kaspersson et al. 1988; Adams and Moss 2000).

When storing grain moist another set of organisms may habitat the feed and may become important in the production of fermented feed. Olstorpe et al. (2010a, b) found *Lactobacillus* and *Leuconostoc* but also species of *Bacillus*, *Enterococcus*, *Lactococcus*, *Pediococcus* and *Weissella* on cereal grain at harvest. Bacterial status is usually evaluated by determining the total counts of aerobic bacteria on general substrates and *Enterobacteriaceae* on bile containing substrates, as an indication of the hygiene status of the feed. High numbers of these bacterial groups do not necessarily indicate a feed hazard, but suggest that the risk is increased. Unexpectedly, Olstorpe et al. (2010b) found a substantial presence of *Enterobacteriaceae* on cereal grain at harvest, far exceeding Swedish guideline values for silage (*Enterobacteriaceae* guideline values for cereal grain are even not established, since they are not regarded a problem, see above). This indicates the importance of including this microbial group during evaluation of feed hygiene of stored cereal grain using different technical systems. LAB are often enumerated in feed cereal storage systems as these organisms are usually considered desirable. The numbers of total aerobic bacteria were often high in moist grain. However, it was recently shown that the number of total aerobic bacteria has only limited value for evaluation of feed quality as LAB were also able to grow in the agar pouring assay on Tryptone Glucose Extract Agar used for the quantification of total aerobic bacteria (Olstorpe et al. 2010a). All bacterial groups were found in cereal grain even with a_w values below 0.70 (Olstorpe et al. 2010b).

2.3.2 Yeast

Yeasts are best known for their contribution to society through their fermentation of bread dough and alcoholic beverages, and other products. However, they are also known as spoilage organisms of food and feed (Middelhoven and van Balen 1988;

Table 2.2 Yeast species identified on cereal grain at harvest

Yeast species	References
Aureobasidium pullulans	Olstorpe et al. (2010b)
Cryptococcus albidus	Flannigan and Campbell (1977)
Cryptococcus flavescens	Olstorpe et al. (2010b)
Cryptococcus laurentii	Flannigan and Campbell (1977)
Cryptococcus macerans	Olstorpe et al. (2010a, b), Flannigan and Campbell (1977)
Cryptococcus tephrensis	Olstorpe et al. (2010a)
Cryptococcus victoriae	Olstorpe et al. (2010b)
Cryptococcus wieringae	Olstorpe et al. (2010a, b)
Kazachstania aerobia	Olstorpe et al. (2010b)
Metschnikowia aff. fructicola	Olstorpe et al. (2010b)
Pichia anomala	Olstorpe et al. (2010a)
Rhodosporidium babjevae	Olstorpe et al. (2010b)
Rhodotorula glutinis	Olstorpe et al. (2010b), Flannigan and Campbell (1977)
Sporobolomyces ruberrimus	Olstorpe et al. (2010b)
Sporobolomyces roseus	Flannigan and Campbell (1977)
Torulopsis ingeniosa	Flannigan and Campbell (1977)
Trichosporon cutaneum	Flannigan and Campbell (1977)

Fleet 1992; Loureiro and Malfeito-Ferreira 2003; Fleeth 2007). Isolates belonging to yeast genera such as *Candida*, *Cryptococcus*, *Pichia*, *Rhodotorula* and *Sporobolomyces* have been isolated from grains at harvest (Table 2.2, Flannigan and Campbell 1977; Flannigan 1987; Olstorpe et al. 2010a, b).

The significance of their presence in cereal grains has not been examined, as filamentous fungi are usually considered to be the main agents of pre- and postharvest spoilage of grain (Lacey 1989; Lacey and Magan 1991). This may explain that there are currently only a few studies where yeasts on grain are identified. Yeasts associated with pre- and postharvest cereal grain need further investigation as it is evident that yeasts play a significant role in the production and spoilage of fermented grain (Fleet 1990). Yeasts may compete with LAB for fermentable growth substrates, and by this reduce acid formation and thus conservation power. At air ingress, some yeasts can metabolise lactic and acetic acid, causing the pH to increase and encouraging the growth of spoilage bacteria and moulds (Fleet 1992). On the other hand, yeasts may also contribute to the conservation of feed, by inhibition of undesirable microorganisms (Passoth and Schnürer 2003; Olstorpe and Passoth 2010).

When describing a yeast population in cereal storage systems, the currently rather confusing situation of yeast taxonomy may result in some misinterpretations. For instance, if several isolates are identified as *Candida* sp. one might get the impression that these yeasts are related to pathogenic yeasts like *Candida albicans*. However, *Candida* is an artificial genus, comprising ascomycetous imperfect species or anamorphs. For instance, *Candida robusta* is the anamorph of bakers' yeast *Saccharomyces cerevisiae* and *Candida kefyr* that of the milk yeast, *Kluyveromyces marxianus* (Kurtzman and Fell 1998). Similarly, the genus *Cryptococcus* contains non-sexual species and anamorphs of basidiomycetous yeasts. It consists of four

orders and only recently an attempt was started to rename one group within this genus to generate a phylogenetic system (Wuczkowski et al. 2011). When identifying yeasts to our opinion the use of anamorph names should be avoided. There is also an ongoing development in re-naming sexual yeast genera. Recent investigations have shown that the genus *Pichia* is polyphyletic, requiring nomenclature changes for of a variety of yeast species (Kurtzman 2010). This will also impact many yeasts connected to feed systems, like for instance the biocontrol yeast *P. anomala*. Currently there is a debate how to correctly name this species, either to use the older name *Hansenula anomala* or to use a new name, *Wickerhamomyces anomalus* (Kurtzman 2010; Passoth et al. 2010). In this chapter we will use the designation *P. anomala*, since we used it in most of our publications, but it is clear that this name will be changed in the near future.

2.3.3 Moulds

Generally, grain stored at a moisture content equivalent to less than a_w 0.70 will not be subjected to fungal spoilage and mycotoxin production (Aldred and Magan 2004). However, cereals at harvest normally have a_w 0.86–0.97, and are often traded on a wet weight basis. Certain technological problems associated with bulk drying and storage of grain, and cases of poor practices and negligence, result in a significant risk for mould growth and mycotoxin production in the postharvest situation. Moulds may have additional undesirable effects such as loss of dry matter, discoloration, reduced nutritional value and digestibility, or production of off-flavours (Lacey 1989; Magan et al. 2003). Mould formation also results in an increased dust fraction, containing substantial numbers of fungal conidia. The dust fraction has for example been associated with chronic and recurrent airway disease in horses. There are no comparable investigations in cattle, but inhalation of mould spores comprises a continuous pro-inflammatory challenge to the upper airways of cattle as well as horses (Fink-Gremmels 2008). Fungi present on plants before harvest are traditionally termed 'field fungi'. Typically, these include species of *Cladosporium*, *Alternaria*, *Epicoccum* and *Fusarium* (Magan and Lacey 1984; Flannigan 1987; Lacey and Magan 1991). *Cladosporium* species are among the most abundant components of daytime summer air-borne spores. Species of this genus are widespread on the ears of cereals at harvest. Spores of *Alternaria alternata* are, after *Cladosporium* spp., probably the most common airborne fungal spores and may penetrate the kernel sub-epidermally. This hides them from the action of fungicides, which may be one of the reasons that *Alternaria* species can be isolated from most grains at harvest (Lacey 1989). *Fusarium* spp. may cause various plant infections such as scab, ear rot or head blight. They may also produce mycotoxins, such as deoxynivalenol and various trichothecenes, in the grain, both pre- and post harvest (Lacey et al. 1999; Aldred and Magan 2004). Pigs are more sensitive to trichothecenes than other farm animals. Symptoms generally shown are reduced feed intake and weight gain, but impairment of the immune system has also been observed in pigs (Smith 1992; Eriksen and Pettersson 2004).

Depending on the storage conditions of cereal grain, growth of typical storage fungi may occur. These fungi are present at low levels before harvest, and belong in most cases to *Aspergillus* or *Penicillium*. *Penicillium roqueforti*, a species also used in cheese manufacture, is an important spoilage fungus in airtight storage systems (Lacey and Magan 1991). Dairy cows partaking of *P. roqueforti* infested feed displayed symptoms such as lack of appetite, ketosis, paralysis and spontaneous abortions (Häggblom 1990). *P. roqueforti* has been isolated from acid-preserved cereals, as well as from airtight stored grain with insufficient oxygen exclusion (Kaspersson et al. 1988). *Aspergillus* spp. are characteristic colonisers of stored products, and different species vary considerably in their growth requirements; thus, the dominance of certain species may be indicative of previous storage conditions (Lacey 1989). However, the concept of field and storage flora should not be carried too far. The terminology was first used in northern temperate regions, whereas in warmer, more humid climates, the species distribution between field and storage fungi differs (Lacey and Magan 1991). Species composition of field and storage flora may also vary with grain storage method. For example, observations of partial persistence of the field fungi *Cladosporium* and *Fusarium* were noted in moist storage systems (Kaspersson et al. 1988; Olstorpe et al. 2010b).

2.4 Biopreservation

Biopreservation refers to extended storage life and enhanced safety of feeds using the antibacterial and/or mould inhibiting activity of microorganisms. Biopreservation of moist animal feed provides an alternative to conventional conservation methods and is often beside of saving energy and antimicrobial chemicals a way to improve feed quality.

Different forages can be stored moist, but feed hygiene is then easily impaired due to growth of deteriorative and/or hazardous microorganisms. However, addition of biopreservative organisms may ensure feed hygiene (Olstorpe et al. 2010a). Securing feed hygiene and quality in cereal grain may be achieved by inoculation of the biocontrol yeast *Pichia anomala* to the storage systems. *P. anomala* strongly reduces growth and sporulation of several mould species on agar plates. The mould inhibition was regulated by inoculation level of *P. anomala* in a clear dose dependent manner (Björnberg and Schnürer 1993; Petersson and Schnürer 1995). The anti-mould-activity of the yeast was also demonstrated in moist grain storage systems with air leakage (Petersson and Schnürer 1995). Mould inhibition studies have been done both in small (approximately 17 g) and large (approximately 160 kg) scale silos containing moist grain (Petersson and Schnürer 1995; Petersson and Schnürer 1998; Petersson et al. 1999). *P. anomala* can grow over a wide range of temperatures (3–37°C) and pH (2–12), at low a_w (0.85) and even in anaerobic environments (Fredlund et al. 2002). Several different mechanisms of fungal inhibition have been suggested for *P. anomala*. Generally, competition for limited nutrients and space (Janisiewicz and Korsten 2002), production of killer proteins (Walker

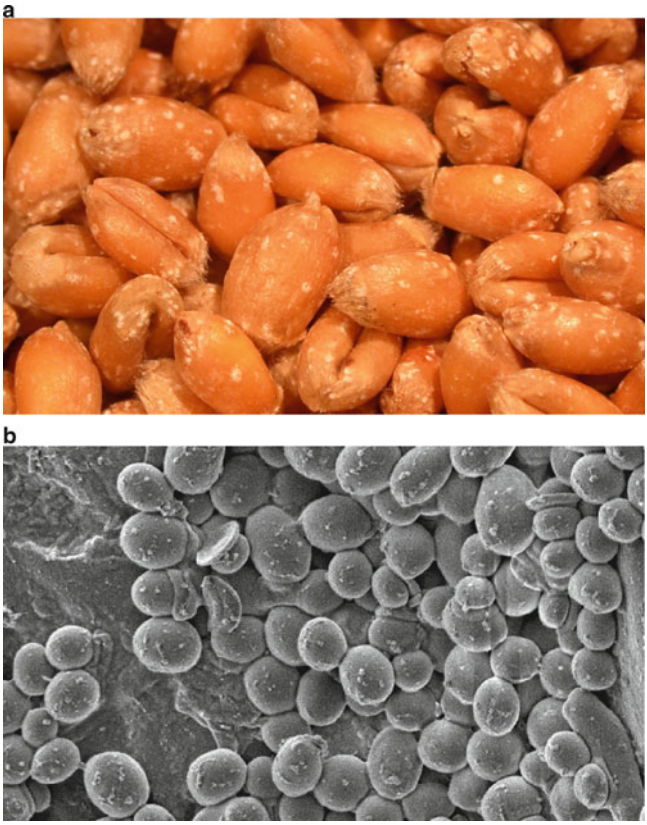

Fig. 2.1 *P. anomala* growth on moist cereal grain, a_w 0.98, initial inoculation 10^3 cfu g^{-1} grain and incubation at 25°C for 4 days. (**a**) Yeast colonies on moist cereal grain (Reproduced from Olstorpe 2008). (**b**) Morphology of yeast cells situated on the kernel surface. There were "typical" rounded yeast cells forming buds, but also elongated cells and possible hat shaped ascospores. Cryoscan and microphotograph by Dr Jan Dijksterhuis (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands)

et al. 1995) and cell wall degrading enzymes (Jijakli and Lepoivre 1998) are seen as important strategies deployed by yeasts. However, these may not be the main mode of action of biocontrol by *P. anomala* (Druvefors et al. 2005; Druvefors and Schnürer 2005). The biocontrol activity against moulds is most probably due to products of glucose metabolism, mainly ethyl acetate and ethanol (Druvefors et al. 2005). This was confirmed in a study with a diploid and haploid strain of *P. anomala*. The two yeast strains grew and inhibited mould growth equally well and showed comparable ethyl acetate production at a_w 0.98. At a_w 0.95, growth, biocontrol performance as well as ethyl acetate formation were reduced in the haploid strain. A bioassay with the pure ethyl acetate compound concluded the inhibitory effect, as reduced biomass production of mould was confirmed (Fredlund et al. 2004). Inoculating the biocontrol yeast *P. anomala* to moist crimped cereal grain (Fig. 2.1) stored in plastic

tubes in an on farm study (comprising 48 tonnes of feed grain) substantially reduced numbers of undesirable moulds, confirming earlier laboratory results.

A surprising result was that in *P. anomala* inoculated grain the number of *Enterobacteriaceae* decreased from 5.2 log cfu g^{-1} grain below detection level (10 cfu/g grain) during storage (Olstorpe et al. 2010a). This finding is of great importance to both feed and food hygiene as it has been shown that reducing the number of *Enterobacteriaceae* in feed, in turn, decrease the *Enterobacteriaceae* present later in the food chain (Brooks et al. 2001).

2.4.1 Phytase

Biopreservative organisms may also improve the nutritional value of the feed due to different enzymatic activities that in turn may increase the bioavailability of minerals. Approximately 60–90% of the phosphorus in cereal grains, a major constituent of animal feeds worldwide, as well as in oilseeds and legumes, is present in the form of phytic acid or phytate (Nelson 1967; Reddy et al. 1989; Al-Asheh and Duvnjak 1995; Mitchell et al. 1997). Phytate is an antinutrient, binding minerals and proteins and its degradation will improve the bioavailability of those substances. Phytate is indigestible for monogastric animals such as poultry, pigs and fish, as these animals lack the required gastrointestinal tract enzymes for the dephosphorylation of the phytate complex (Cromwell et al. 1995), but even for ruminants a positive effect of phytate degradation is discussed (McDonald et al. 2002). Non degraded phytate accumulates in the manure and liquid effluent, which in turn leads to phosphorus pollution and eutrophication of water environments (Balander 1998). To ensure that the phosphorus needs of animals are met, diets are commonly fortified with additional inorganic phosphates, which may further increase phosphorus excretion (Jongbloed and Lenis 1992; Balander 1998). Moreover, inorganic phosphorus is a non-renewable substance that is produced from mined rock phosphate. Major reserves of mineral phosphorus are for instance found in the West Sahara region where its production is correlated with both political and environmental problems. Resources of mineral phosphorus are limited and peak phosphor is estimated to 2030 (Cordell et al. 2009). The bioavailability of other minerals is considerably reduced by the presence of phytate, which forms insoluble complexes with numerous cations, such as copper, zinc, calcium, magnesium, iron and potassium (Reddy et al. 1989). Phytate may also negatively influence the functional and nutritional properties of proteins (Cheryan 1980). The phytate content may be reduced by phytase via supplementing the feed with pure enzymes or with phytase-producing microorganisms, and/or via activation of endogenous phytase present in the cereal grain. This leads to improved growth rates and feed conversion ratios in monogastric animals (Simons et al. 1990; Beers and Jongbloed 1991). Pre-treatment of animal feed with phytase can decrease the phosphorus load in animal manure by up to 50% (Lei et al. 1993; Kornegay and Qian 1996; Han et al. 1997; Vohra et al. 2006). The high price of commercial phytase currently restricts widespread application of

phytase as feed supplement. The enzyme is produced by conventional submerged fermentation, an expensive high technology process. A more economical alternative for phytase addition would be solid substrate fermentation (SSF) of the feed, whereby phytase is produced in situ during SSF by growth of yeasts on selected feed components (Lonsane and Ghildyal 1993; Tengerdy 1996; Pandey et al. 1999; Vohra et al. 2006). *P. anomala* grows well on cereal grain, increasing in cfu numbers during incubation on both rolled and whole grain and at different a_w independent of inoculum level (10^3, 10^5 and 10^7 cfu g^{-1} grain). The maximum cfu number reached in tested conditions was about 8 log units (Olstorpe 2008). *P. anomala* is reported to have high activity of intracellular phytase, and an insignificant extracellular phytase activity (Vohra and Satyanarayana 2001). However, in a recent study a strain dependent extracellular phytase activity was observed in *P. anomala*. In general, large inter- and intra species specific differences in intra- and extracellular phytase activities in a variety of yeasts were demonstrated (Olstorpe et al. 2009). During storage of moist crimped cereal grain a reduction in phytate content was detected. Phytate was reduced during storage with 1.3 and 0.54 μmol/g dry matter in *P. anomala* inoculated grain and non inoculated grain respectively (Olstorpe et al. 2010a). The phytate degradation may be due to exogenous activity of the microbial flora present on the grains. The more pronounced decrease in the inoculated grain was probably due to the phytase activity of the inoculated *P. anomala* (Vohra and Satyanarayana 2001; Olstorpe et al. 2009). Recently, a phytase gene of this yeast was cloned and sequenced (Kaur et al. 2010). *P. anomala* has a great potential for phytate degradation in cereal grain, either by using the cloned gene to produce phytase to an acceptable price in a heterologous expression system for feed addition, or by adding whole yeast cells as "cell bound phytase" (Vohra et al. 2010).

2.4.2 Protein

Cereals have high contents of starch, dietary fibre, vitamins and minerals, but typical amounts and qualities of protein present do not fulfil the nutritional requirements of animals (McDonald et al. 2002). Cereal proteins are particularly deficient in essential amino acids, e.g. lysine and methionine. Thus, additional feed protein sources are required to meet the animals' needs. Different legumes are often used to increase the protein content. However, there are practical limitations due to acceptability, anti-nutritional substances and influences on carcass quality. Peas have fairly high crude protein and lysine contents, but their amino acid composition is not considered to be adequate (Odal 2000). Furthermore, due to the acrid taste, animals often reject peas in the fodder. Peas also contain tannins that impede the protein turnover (Simonsson 1995). Rapeseeds have high protein content and an advantageous amino acid composition. However, their use is limited due to the high content of highly unsaturated fatty acids (Odal 2000) and glycosides (McDonald et al. 2002). The glycosides are degraded by the metabolism of the animals and the intermediates may inhibit growth of the production animals (Simonsson 1995). Europe

is currently less than 30% self-sufficient in the production of protein feed. The world market for protein feed has grown continuously over the last few years, with over 200 million tons traded annually and with soya as the primary cultivar (Chudaske 2007). Soya meal is generally regarded as one of the best sources of protein available to animals. Soya protein contains all the essential amino acids, although the concentrations of cysteine and methionine are suboptimal (McDonald et al. 2002). The production of soya is associated with negative environmental and socioeconomic issues (Bertrand 2006). Export may further increase the negative impact on the environment as translocation of nutrients between countries also precludes nutrient recirculation on arable land. Fossil fuels used in transporting soybeans further increase the environmental load. Addition of microorganisms to the feed can increase the protein content and quality of the feed, thus, providing a more local (national) protein feed base. Biomass production of biopreservative microorganisms such as *P. anomala* can be a locally produced protein source as single cell protein (SCP). Currently, SCP is produced from many species of microorganisms, including algae, fungi and bacteria. These are cultured on abundantly available agricultural and industrial wastes (Yang et al. 1993; Jin et al. 1999; Villas-Bôas et al. 2002, 2003; Leathers 2003). Although these organisms are grown primarily to increase the protein content of the feed, microbial cells also contain carbohydrates, lipids, vitamins, minerals, and non-protein nitrogen material, such as nucleic acids. One disadvantage of SCP is that it frequently has a low content of sulphur containing amino acids. Nutritionists often assume that microbial growth conditions have a limited influence on amino acid content (Giec and Skupin 1988). However, recent findings have demonstrated that the protein and lipid contents in the SCP are determined by the composition of the medium. Fungi have higher lipid and lower protein contents when grown on media rich in carbon sources and poor in nitrogen. When the yeast *P. anomala* was grown on different liquid substrates, freeze dried and then analysed for its essential amino acid content, fairly large differences in the amount of crude protein and the composition of amino acids were observed (Table 2.3) (Olstorpe 2008).

Traditionally microbial biomass is produced by submerged or solid state fermentation. After fermentation biomass is harvested and subjected to downstream processing (Villas-Bôas et al. 2002). Growth of microorganisms *in situ* on the feed material would be more cost effective as no further processing is needed. During storage of moist crimped cereal grain the amino acids and total protein levels increased over time for both *P. anomala* inoculated and non inoculated grain. A significant increase of total amount of protein and four individual amino acids was measured in inoculated grain compared to the non inoculated, which thus may be due to the increased yeast growth (Olstorpe et al. 2010a). Different strategies, efforts and nutrient amendments have been tested to increase the SCP yield of *P. anomala* growth *in situ* on cereal grain. Nitrogen sources as urea, ammonium-sulphate and ammonium-phosphate were added in different concentrations to the grain, also yeast inoculation level, storage time, air admission or using hole or rolled cereal grain were tested without increasing the cfu/g grain (Olstorpe 2008). Adding glucose to the grain moistening solution also did not significantly affect the final yeast cfu levels (Druvefors et al. 2005). This indicates the existence of a cell density

Table 2.3 Crude Protein and amino acid composition of lyophilised *P. anomala* cells grown in liquid substrates Yeast Nitrogen Base or Malt Extract

Amino acid	Yeast nitrogen base	Malt extract
Crude protein	353	123
Cysteine	3.9	1.4
Methionine[a]	5.3	1.1
Aspartic acid	29.2	10.7
Threonine[a]	15	6.4
Serine	18.9	7.2
Glutamic acid	56.2	16.2
Proline	10.4	4.3
Glycine	14.6	5.5
Alanine	18.9	6
Valine[a]	16.6	6.1
Isoleucine[a]	16.5	5.9
Leucine[a]	22.2	7.7
Tyrosine (calculated)	11.1	3.7
Phenylalanine[a]	12.6	4.6
Histidine[a]	6.6	2.4
Ornitine	1	0.1
Lysine[a]	22.7	7.8
Arginine	26.4	4.7
Hydroxiproline	0.1	0.1

Data are given as g amino acid kg^{-1} dry matter. The measuring tolerance given by the contract laboratory (Eurofins, Lidköping, Sweden) is 8% for each amino acid (Reproduced from Olstorpe 2008)
[a]Essential amino acid

dependent growth inhibition of the yeast, as maximum *P. anomala* levels on grain, regardless of treatments, never exceeded 8.5 log units (Olstorpe 2008). The amount of protein generated is insufficient for the feed to be acknowledged as a protein feed (Swedish standards). However, the yeast addition improved the total content of protein and amino acid in the grain and feeding animals with *P. anomala* inoculated grain did not adversely affect performance, it rather (although only marginally) improved weight gain (Olstorpe 2008).

2.5 Future Perspectives and Conclusions

Biopreservation often relies on spontaneous microbial developments, thus storage stability, feed hygiene, palatability and nutritional value may vary depending on the microbial composition. Drying of cereal grain currently provides the safest method for feed conservation. However, increasing energy prices and environmental concerns generate a demand for alternatives. Biopreservation may provide such an alternative. The potential of a variety of microorganisms to prevent mould growth

has frequently been shown. Microbes that grow during fermentation may even have potential to improve the nutritional value of the feed with increasing protein levels and bioavailability of minerals. However, the microbial ecology in feed biopreservation is often only poorly understood. Most of the established processes build on spontaneous microbial developments, and recent investigations in which microbial species were identified have shown that microbial populations differ substantially between different feed batches with the same preservation technology. This uncertain output of the spontaneous biopreservation processes represents a risk not only for impaired feed hygiene, but also for the introduction of pathogenic organisms into the food chain. It is, therefore, necessary to study microbial interactions in the different storage systems, and to generate appropriate starter cultures to ensure a predictable feed storage flora. The some times misleading results from cultivation dependent microbial quality tests clearly indicate the demand for developing new, culture independent microbial detection methods. Moreover, culture dependent analyses are time consuming, as results are only available after several days. Molecular methods can be an interesting alternative, for instance using hybridisation or (q) PCR techniques. However, those methods also require knowledge about the genome of common organisms in the storage ecosystem, thus, further studies of storage ecosystems in different regions of the world are required. It should also be taken in account that in many agricultural systems there is a demand for "low tech" analytics. In so far, culture dependent analyses will still play a great role. More research is required to understand the microbial ecology in storage system. It has pointed out that pH decrease is not necessarily the mechanism of microbial inhibition in cereals. Similar observations have been made for other feed materials as well, e.g. haylage, where LAB growth is detected but acids are not sufficiently formed. Therefore new biopreservatives are required that function over a broad range of water content. The yeast *P. anomala* may be one example for such new bioconservation agents.

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Chapter 3
Pivotal Role of Organic Acid Secretion by Rhizobacteria in Plant Growth Promotion

G. Archana, A. Buch, and G. Naresh Kumar

Abstract Organic acid secretion by rhizobacteria has been major factor responsible for phosphate solubilization. Efficacy as phosphate biofertilizers is determined by the nature and amount of organic acids secreted by rhizobacteria. Amongst the organic acids, gluconic and 2-ketogluconic acids are most commonly secreted by rhizobacteria. Many efforts have been made to understand the factors responsible for the variable efficacy of these bacteria as inoculants in field conditions. Nature of the soil, type and abundance of sugars available in the root exudates and nature of the available phosphate complexes contribute to the variations in efficacy of these rhizobacteria between the laboratory and field conditions in terms of the nature and amount of organic acid secretion. Phytate is a predominant organic phosphate present in many soils. The utilization of phytate as a P source appears to be dependent not only on the characteristics of the phytases but also on the organic acid secretion. Rhizobacteria are also gaining prominence in providing potassium from mineral ores to plants. Potassium solubilization by rhizobacteria could also be determined by the organic acids. Acidification of the rhizosphere by organic acid secretion could also be important in providing iron to plants specifically which are grown in alkaline soils. Citric acid is known to be siderophore in *Bradyrhizobium*. In addition to the plant growth promotion by enhancing nutrient availability, organic acid secretion by rhizobacteria could be helpful in the amelioration toxic effects caused by heavy metals. All these aspects are limited by the inherent properties of rhizobacteria. Alternative strategies of coupling beneficial properties of organic acid secretion

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with other well established plant promotion properties may have high potential in agriculture. Metabolic engineering approaches may be very effective in achieving these goals.

Keywords Root colonizing bacteria • Low molecular weight organic acids • Mineral phosphate solubilization • Phytate mineralization • Potassium solubilization • Metal mobilization

3.1 Introduction

Rhizosphere comprises of the thin soil layer surrounding the plant roots and its unique microflora is selected and their metabolic activity influenced by the plant root exudates. This zone is known to be richer in microbial diversity as compared to the surrounding bulk soil with total counts of rhizospheric microbes being 10 times greater than those of the bulk soils. Rhizobacteria, root colonizing bacteria, are an assorted group consisting of gram negative as well as gram positive bacteria including actinomycetes, belonging to various phyla.

Certain rhizobacteria influence plant health through their beneficial activities and artificial introduction of highly beneficial species is an important aspect of sustainable agriculture. Plant beneficial traits of rhizobacteria include their role in increasing nutrient availability, phytohormone secretion and biocontrol of fungal pathogens by antagonism. Recent interest is in plant-microbe interaction for phytoremediation and promotion of plant growth in stressed or polluted environments.

One of the important mechanisms by which rhizospere-dwelling bacteria participate in many of these activities is by the secretion of wide range of low molecular weight organic acids. Most secreted organic acids are formed as end products or byproducts of fermentation of sugars and polysaccharides and biochemical and molecular basis of the organic acid secretion is well established in several genera. However, organic acid secretion in rhizosphere depends not only on the metabolic potential of the organism but also on the plant physiology since root exudates are the major C-source in root zone. The ability to predict and manipulate organic acid secretion by rhizospheric bacteria *in situ* is complicated by the fact that root exudates composition varies from plant to plant and is controlled by various environmental and edaphic factors (Dakora and Phillips 2002). Besides, root exudates comprise of a complex mixture of nutrients, each in low amounts, and bacterial physiology and organic acid secretion during growth on root exudates cannot be easily predicted based on simple laboratory studies usually done using single C-source.

The present chapter highlights recent developments regarding the role of low molecular weight organic acids in various plant growth promotion mechanisms, rhizobacterial secretion of organic acids, the metabolism underlying their production, and most importantly the factors influencing the organic acid secretion under soil conditions. It is well established that certain plants exude organic acids from roots and benefit from this process irrespective of the rhizobacterial secretion of organic acids

(Jones 1998). Nonetheless, rhizobacterial secretion of organic acids is an important aspect for the large majority of plants or cultivars which do not secrete sufficient quantities of organic anions. Another important aspect to be considered is that the nature of organic acids that can be secreted by rhizobacteria is far wider than the acids released by plant roots. The ability to biotechnologically manipulate the bacterial metabolism towards secretion of appropriate quantity and quality of organic acid suitable for the particular process to be operative under soil conditions is an interesting prospect.

3.2 Role of Organic Acids Produced by Rhizobacteria in Plant Growth Promotion

Organic acids are low-molecular weight CHO containing compounds characterized by the possession of one or more carboxyl groups. Depending on the number of these carboxylic groups and their dissociation constants, organic acids can complex with cations and result in their sequestration with simultaneous release of anions associated with minerals as well as cause the displacement of anions from the soil matrix. Both these properties (sequestration of cations as well as release of anions) are important in the diverse roles that organic acids play in the rhizosphere including several important soil bioprocesses. Organic acids released into the rhizosphere can potentially participate in many soil reactions which can be broadly divided in to three categories including phosphorus (P) mobilization, uptake of cations by plant roots and alleviation of toxicity of phytotoxic cations (Fig. 3.1). The efficacy of the organic acid in these processes depends to a large extent on the amount and the type of organic acids released as well as on the physico-chemical properties of soils (Jones 1998).

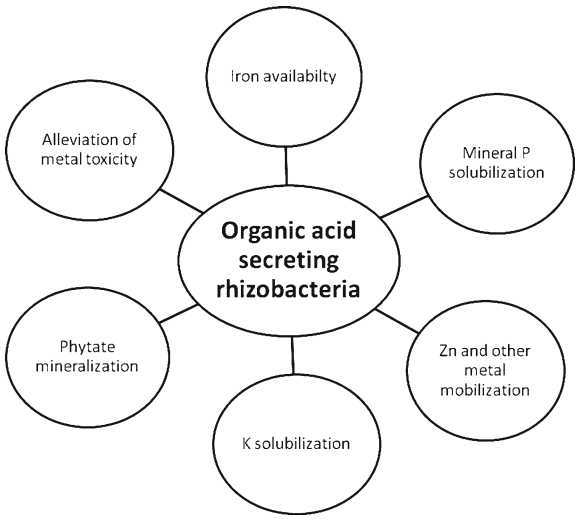

Fig. 3.1 Roles of organic acid secreting rhizobacteria in rhizosphere processes beneficial to plant growth

3.2.1 Mineral Phosphate Solubilization

One of the well-studied processes involving rhizobacteria that secrete organic acids is mineral phosphate solubilization. Phosphorous (P) is a macronutrient element in natural and agricultural ecosystems, however, throughout the world most soils lack available P because it is easily converted into insoluble complexes such as iron and aluminum hydrous oxides, and calcium phosphate complexes. Soluble P fertilizers applied to soils are rapidly fixed allowing only a small fraction (10–15%) of the P in fertilizers and manures to be taken up by plants. Consequently, a large reservoir of fixed P is present in agricultural soils which can be rendered available by rhizobacteria involved in insoluble phosphate mineralization, known as P solubilizing bacteria (PSB). Organic acid secretion is the predominant mechanism involved in P solubilization. Organic acids in the rhizosphere derived from plant sources may also be involved in P solubilization e.g. for white lupin it is well established that the release of large amounts of citrate from cluster roots results in the solubilization of soil P and hence an increase in the availability of P to plants (Dinkelaker et al. 1995; Hocking 2001).

Rhizospheric bacterial strains belonging to *Achromobacter, Acinetobacter, Agrobacterium, Alcaligenes, Arthrobacter, Azospirillum, Azotobacter, Bacillus, Burkholderia, Citrobacter, Enterobacter, Erwinia, Flavobacterium, Micrococcus, Paenibacillus, Pseudomonas, Rhizobium and Serratia* are reported as PSB (Rodriguez and Fraga 1999; Whitelaw 2000; Compant et al. 2005; Haas and Défago 2005; Khan et al. 2006, Alikhani et al. 2006).The organic acids produced by the PSB include mono-, di- and tri-carboxylic acids like acetic, lactic, oxalic, tartaric, succinic, citric, gluconic, 2-ketogluconic, formic, malic, pyruvic, glyoxalic acids, etc (Table 3.1). Some of the other uncommon acids like trans-aconitic acids, maleic, propionic, isobutyric, isovaleric, ketobutyric, itaconic, isocaproic, malonic, gulonic and glycolic acids are also secreted by various P-solubilizing bacteria (Park et al. 2010). As seen from the Table 3.1, majority of organic acid secreting bacteria are gram negative and phylogenetic distribution (Fig. 3.2) shows them to primarily belong to *Proteobacteria*. Within this phylum, A (*Rhizobium, Phyllobacterium, Xanthobacter, Azospirillum, Acetobacter*) and *Gammaproteobacteria* (*Pseudomonas, Acinetobacter, Enterobacter, Klebiella*, *Erwinia, Serratia, Citrobacter, Pantoea. Rahnella, Vibrio)* are most predominant. *Betaproteobacteria* is represented solely by *Burkholderia* sp. Only a few genera of gram positive organisms are reported as organic acid secreting; these belong to *Firmicutes* (*Bacillus, Paenibacillus*) and *Actinobacteria* (*Arthrobacter, Rhodococcus, Micrococcus*). Gluconic acid is largely produced by members of *Gammaproteobacteria*. Data compiled using information from Khan et al. 2006, Rodriguez et al. 2004; Chen et al. 2006; El-Aiat 2010; Hameeda et al. 2006; Buch et al. 2008; Gyaneshwar et al. 1998; Prijambada et al. 2009; Lin et al. 2006; Vikram et al. 2007 and other references cited in the text.

Organic acid addition in acidic form leads to rhizosphere acidification when organic acids bind cations (e.g. Ca^{2+}), causing the release of 2 H^+ (in the case of dicarboxylates, for example) from the organic acid, which consequently lowers

Table 3.1 Organic acids secreted by phosphate solubilizing rhizobacteria

Organic acid	Gram negative rhizobacteria	Gram positive rhizobacteria
2-ketogluconic	*Acetobacter pasteurianus, Enterobacter intermedium, Klebsiella aerogenes. Pseudomonas cepacia, P. fluorescens* RAF15, *P. trivialis, Rhizobium leguminosarum, R.. meliloti*	*Bacillus firmus, B. subtilis*
Acetic	*Cirobacter, Klebsiella, Pantoea, Vibrio proteolyticus*	*Bacillus amyloliquefaciens*
Citric	*Chryseobacterium* sp., *P. fluorescens*, *P. poae*, *P. trivialis*, *Serratia marcescens,*	*Arthrobacter ureafaciens, Bacillus polymyxa, B. licheniformis, B. megaterium, Rhodococcus erythropolis,*
Formic	*P. fluorescens*, *P. poae*, *P. trivialis*, *P. fluorescens* AF15	*B. megaterium*
Gluconic	*Acinetobacter calcoaceticus, A. rhizosphaerae, Azospirillum brasilense, A. lipoferum, Burkholderia cepacia, Citrobacter, Enterobacter asburiae* PSI3*, Erwinia herbicola, Klebsiella aerogenes, Phyllobacterium myrsinacearum, Pseudomonas cepacia, P. aeruginosa, P. fluorescens*, *P. poae*, *P. trivialis*, *P. fluorescens* RAF15 *P. corrugata, Rahnella aquaticus, Serratia marcescens*	*Rhodococcus erythropolis, Bacillus* sp.
Lactic	*Pseudomonas* spp., *P. fluorescens*, *P. poae*, *P. trivialis*, *Xanthobacter agilis, Serratia marcescens, Vibrio proteolyticus*	*Arthrobacter sp, Bacillus amyloliquefaciens,B. licheniformis, B. megaterium, Penibacillus macerans*
Malic	*P. fluorescens*, *P. poae*, *P. trivialis*, *P. striata*	
Oxalic	*P. fluorescens*, *P. poae*, *P. trivialis*.	*Arthrobacter* sp., *Bacillus polymyxa, B. licheniformis, B. subtilis, B. megaterium, Micrococcus* spp.
Pyruvic	*Citrobacter, Enterobacter, Klebsiella*	
Succinic	*Delftia* sp., *Pseudomonas striata, Serratia marcescens*	*Bacillus subtilis*
Tartaric	*Pseudomonas striata, P. fluorescens* RAF15	*B. megaterium*
Mixture of acetic, citric, fumaric, lactic, maleic, malonic, oxalic, propionic, pyruvic, succinic, tartaric and trans-aconitic acids[a]	*Chryseobacterium, Enterobacter, Klebsiella, Pantoea, Phyllobacterium, Serratia*	*Arthrobacter, Bacillus, Gordonia, Rhodococcus*
Fumaric, glyoxalic, isovaleric, isobutyric, Itaconic, ketobutyric, malonic, propionic	*Chryseomonas luteola, Enterobacter aerogenes, E. taylorae, E. asburiae, Kluyvera cryocrescens, Pseudomonas aerogenes, P. striata, Xanthobacter agilis, Vibrio proteolyticus,*	*Bacillus amyloliquefaciens B. atrophaeus,, B. licheniformis, Penibacillus macerans,*

[a]Organic acids were measured as mixture of all the acids (Park et al. 2010)

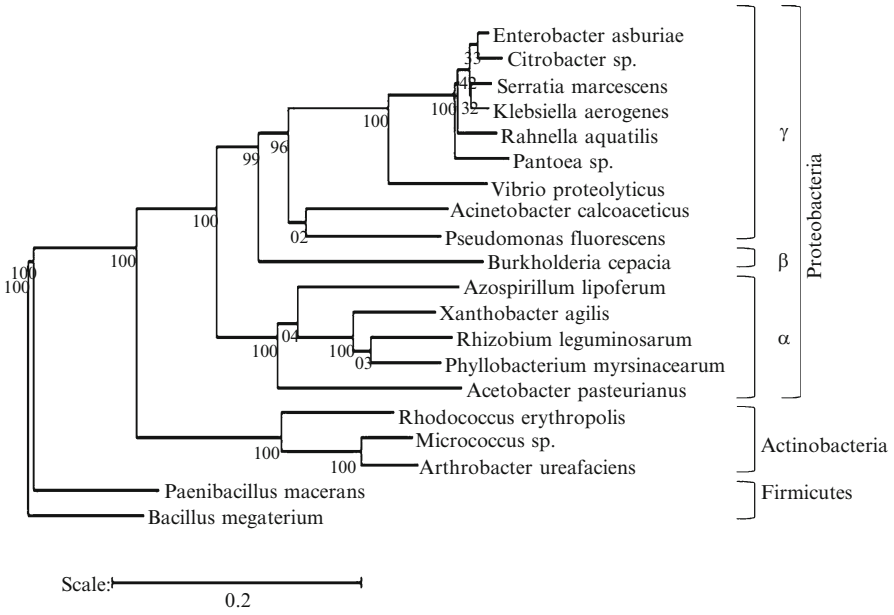

Fig. 3.2 Phylogenetic distribution of representative organic acid producing rhizobacteria. Tree was made using using the 16 S ribosomal sequences of the type strains and tree builder tool from ribosomal database project release 10 (http://rdp.cme.msu.edu/). The sequences of the following accession numbers were used to create the tree: AB004744, AB073196, AJ536198, AJ233408, AJ233423, AJ233426, AJ233431, AJ888983, AY785315, D84013, D16273, U29386, X94198, X71863, U96927, X87276, X74723, X79289, X80744, Z29619. The numbers at the nodes denote bootstrap values for 100 replicates.

solution pH causing acidification (Gyaneshwar et al. 1998). The chelation of bivalent and trivalent cations such as Ca^{2+}, Al^{3+} and Fe^{3+}, that are commonly associated with precipitated forms of soil P, can increase P availability from these mineral forms. In contrast, the addition of potassium salt of the organic acids tends to cause a rise in soil solution pH, however the chelation properties of the acid anion can be exerted even when salts are added. The addition of organic acid salts to soils could displace adsorbed phosphate through ligand exchange reactions (Palomo et al. 2006). Thus the release of organic acids either as acids or salts could cause an acidification or alkalization of the soil which in turn has a profound effect on their P solubilization capacity, depending on soil characteristics. Strom et al. (2002) have reported that the addition of citrate and oxalate as acids or salts to soil microcosms brought about an increase in P content of maize plants, with oxalate being more effective. Recently, Khademi et al. (2010) have shown similar results could be obtained with wheat plants grown in calcareous soil. These results provide direct evidence that externally provided organic acid to soils can increase P availability to plants.

The acidification of soil by organic acids depends on both the nature and quantity of the organic acid for e.g. acetic, lactic and succinic at 100 mM bring about a drop in

pH of a soil solution from around 9.0 to about 6.0; a similar drop is brought about by only 20 mM of gluconic acid, 10 mM of oxalic acid and even lesser amount of citric and tartaric acids (Gyaneshwar et al. 1998). Another aspect to be considered is that P released by different organic acids does not depend entirely on the acidification, for instance, addition of either 100 mM acetic, 100 mM succinic and 10 mM oxalic acides each individually resulted in a solution pH of around 6, but P release was highest in case of oxalic acid than the others. This indicates importance of chelation effect of the acids in addition to pH drop. In general, the di- and tri-carboxylic acids are more effective for chelation, with those containing one or more α-hydroxyl groups (e.g. citric acid) being the most effective for P mobilization (Gyaneshwar et al. 1998; Ryan et al. 2001). This is also substantiated by an observation that in a mildly acidic soil amended with rock phosphate, 50 mM lactic, acetic and succinic could bring about a drop in pH to below 4.0 (Srivastava et al. 2007). However, this was not sufficient to bring about substantial release in P, which was brought about by 10 mM oxalate. Similar observation has been reported by Khademi et al. (2010) that at an added concentration of 10 mM oxalate extracted significant P in the soluble phase irrespective of whether it was added as acid or salt.

Although many reports show plant growth promotion and P content increase in plants inoculated with P solubilizing bacteria and fungi (Gyaneshwar et al. 2002), the implication of the role of organic acid has been primarily demonstrated in laboratory media containing insoluble phosphates or by adding pure acids to soils. Ogust et al. (2010) have recently demonstrated enhanced acidification of rhizosphere by PSB in inoculated wheat seedlings using organic acid secreting *Pseudomonas* and *Bacillus.* The best acidification obtained in their results was a modest drop in pH of 0.6 units with a gluconic acid secreting *Bacillus* strain. The authors found increased proton extrusion by the plant roots and conclude that plant cell metabolism could be altered by the inoculations. It remains to be seen whether the gluconic acid or other acids that were reported in culture filtrates of the laboratory grown bacteria were also found in rhizospheric soils.

3.2.2 Mineralization of Phytates

Phytates (salts of myo-inositol hexakisphosphate IHP) represent a significant pool of organic phosphorus (Po), which may account for 29–65% of total soil P and is largely unavailable to plants. Since general soil phosphatases are unable to initiate hydrolysis of the phosphomonoester bonds of phytate, specialized enzymes known as phytases, are required for hydrolyzing phytate to a series of lower phosphate esters of myo-inositol and liberating phosphate (Vohra and Satyanarayana 2003). Since the roots of most plants cannot directly utilize phytate-P (Hayes et al. 1999; Richardson et al. 2000), phytate-mineralizing (PM) microorganisms inhabiting the rhizosphere are considered important in increasing plant available P under conditions where phytates are the major form of phosphate (Richardson et al. 2000; Unno et al. 2005). Precipitation of phytates as insoluble salts of calcium, aluminium or

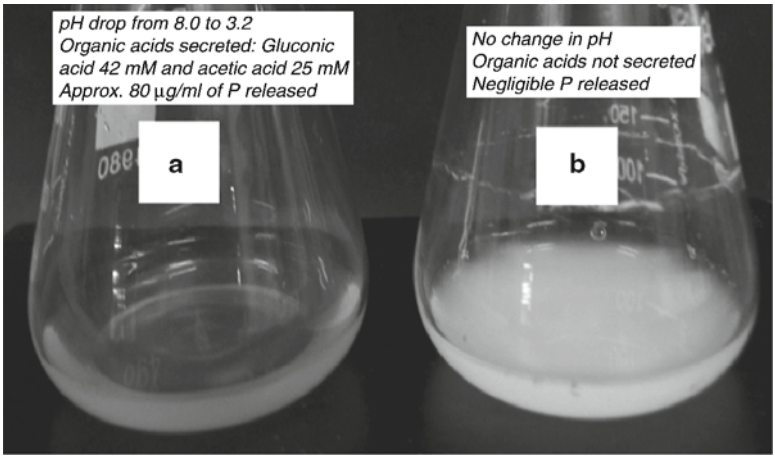

Fig. 3.3 Ca-phytate dissolution by rhizobacterial isolate *Pantoea* sp. *PP1* in minimal medium with carbon sources Glucose (**a**) and Glycerol (**b**). Note the insoluble Ca-phytate in flask B (Source: Patel, K. J., Naresh Kumar, G. and Archana, G. Unpublished data)

iron as well as adsorption of phytate ions to soil particles (Turner et al. 2002) makes them resilient to enzymatic hydrolysis by all the major types of phytases (Tang et al. 2006). Organic acid secretion by bacteria possessing phytase activity enhances the release of P from Ca-phytate, a P source largely unavailable to plants (Patel et al. 2010a). The secretion of organic acids depends on the C source and this in turn determines Ca-phytate mineralization (Fig. 3.3). The release of free phosphate from Ca-phytate correlated well with drop in pH in case of native rhizobacterial strains as well as genetically modified rhizobacteria that over-expressed the phytase *appA* gene (Patel et al. 2010b) indicating that high levels of phytase are insufficient to hydrolyse insoluble or poorly soluble phytates without organic acids. Gluconate was one of the effective organic acids supporting Ca-phytate solubilization along with acetate (Patel et al. 2010a).

3.2.3 Iron Availability

By lowering the pH of the rhizosphere, organic acids bring about simple dissolution of metal complexes particularly for metals like Fe, Zn, Mn that become more soluble with decreasing pH (Jones 1998). In addition, the organic acid anions can form organo-metallic complexes with metal ions, and bring about dissolution of minerals as well as desorption of metal ions adsorbed to soil matrix. Citrate secretion is a major mechanism adopted by *Bradyrhizobium japonicum* for sequestering Fe under Fe starvation conditions (Carson et al. 1992). Gyaneshwar et al. (1998) demonstrated the release of Fe in soil solutions from an alkaline vertisol upon shaking for 15 min with organic acid solutions. Fe could be extracted in solution in the range of

27–143 μM concentrations by different organic acids in the order citrate (20 mM) > citric (10 mM) ≈ tartaric (20 mM) > gluconic (50 mM) > gluconic (20 mM) ≈ tartaric (10 mM) > lactic (100 mM). The soil solution (1 g/ml) pH in case of all of the acids was in the range of 4–6.5. Jones et al. (1996) reported that at pH values < 6.8, citrate formed stable complexes with Fe and dissolution of Fe-oxides proceeded rapidly. An interesting observation was that oxalic acid, which was most effective at P release from the same soil, failed to bring about Fe release, even though there was a significant drop in soil pH.

3.2.4 Potassium Solubilization

Potassium (K) in soils predominantly exists in silicate forms *viz* microcline, muscovite, orthoclase biotite, fieldspars (Bertsch and Thomas 1985). Bacterial strains secreting organic acids solubilized rock K mineral powder, such as mica, illite, feldspar and orthoclases (Friedrich et al. 1991; Ullman et al. 1996; Girgis et al. 2008). Phosphate solubilizing *Bacillus mucilaginosus, Bacillus edaphicus* and several *Bacillus* strains release K from minerals (Sheng et al. 2002; Sugumaran and Janarthanam 2007; Girgis et al. 2008). *Bacillus* strains produced oxalic, citric, fumaric acids in low amounts (~1 mM) but tartaric acid was secreted upto ~10 mM (Girgis et al. 2008). These organic acids were secreted in higher amounts when grown in the presence of CaP minerals as compared to K minerals. K amount in soil solution and plants were increased by *P. mucilaginosus* (Sheng et al. 2002). *P. mucilaginosus* inoculation increased the availability of P and K in soil and in pepper, egg plant and cucumber tissues leading to their improved growth (Han and Lee 2005, 2006; Hu et al. 2006).

3.2.5 Alleviation of Metal Toxicity

Mechanism for overcoming the metal stress is by chelating the harmful metal ions by low molecular weight organic acids. Citric, malic and oxalic acids in plant exudates have been implicated in the sequestration and detoxification of phytotoxic metal ions particularly Al^{3+} (Dakora and Phillips 2002). Gluconic acid secreting P-solubilizing *Enterobacter asburiae* PSI3 could enhance growth of mung bean seedlings in the presence of phytotoxic levels of Cd^{2+} (Kavita et al. 2008). In addition to gluconic acid, oxalic, malic and succinic acids were also demonstrated to ameliorate Cd toxicity. Indirect beneficial effects are exerted by several PGPR which promote plant growth by secreting siderophores thereby helping to overcome the metal stress (Amico et al. 2008; Jing et al. 2007). While citric and oxalic acids have been implicated in effective chelation of Cu (Gadd 1999), gluconic acid secreted by *E. asburiae* PSI3 isolated from alkaline vertisols reported in conferring Cu tolerance to *Vigna radiata* (mung bean) (Srivastava 2003).

3.2.6 Zinc and Other Metal Mobilization

Rhizobacteria can bring about dissolution of zinc (Zn) from the nonlabile phase and enhanced Zn accumulation by *Thlaspi caerulescens* (Whiting et al. 2001). They also promoted accumulation and volatilization of selenium (Se) by Indian mustard (De Souza et al. 1999). Li et al. (2010) have recently shown that *Burkholderia cepacia,* isolated from the rhizosphere of plant growing in metal contaminated habitats was able to mobilize Zn and Cd from various insoluble forms in the order $ZnCO_3 > CdCO_3 > ZnO >> PbCO_3$. The dissolution of minerals was associated with a large drop in pH due to the production of formic, acetic, tartaric, succinic and oxalic acids. These results show that the combination of metal hyperaccumulating plants and associated rhizobacteria helps to achieve better metal accumulation in the plants and to remediate soils contaminated with heavy metals.

3.3 Isolation of Organic Acid Secreting Rhizobacteria

Procedures for isolation of organic acid producing PSB from soils of diverse geographical locations are simple and exploit acidification of the medium observed as a zone of dissolution/clearance of the insoluble forms of calcium phosphate (CaP) complexes or monitor the change in the colour of pH indicator (Fig. 3.4). Most laboratory media are formulated using dicalcium phosphate, tricalcium phosphate or hydroxyl-apaptites as complex CaP source. Pikovaskaya's (PVK) agar (Pikovskaya 1948) is one of the classical medium that allows isolation of any microorganism that can solubilize simple dicalcium phosphate. However, the limitation with this qualitative assay is that those bacteria which did not form clear halos with PVK agar could solubilize various types of insoluble inorganic phosphates in liquid medium, a problem which could not be overcome even by modifying the composition of PVK agar to include the pH indicator bromophenol blue (to visualize yellow-colored

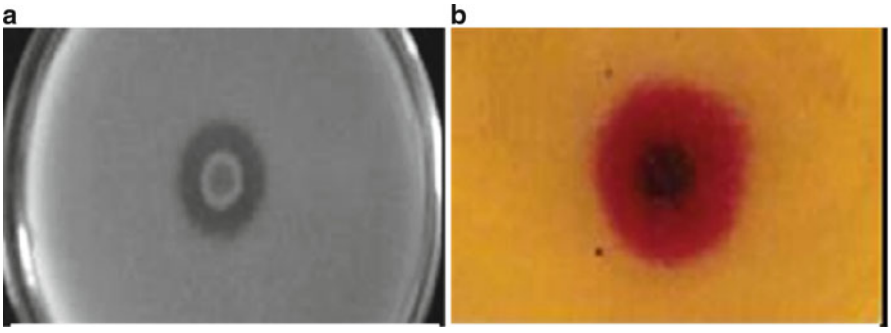

Fig. 3.4 P-solubilizing phenotype on different screening media. (**a**) PVK agar (**b**) Buffered TRP agar (Source: This laboratory)

halo) to improve the clarity of the assay results. Another disadvantage with PVK agar was use of yeast extract in the medium which makes the medium composition undefined. To overcome this and to have an effective though qualitative screen for isolation of efficient P-solubilzing microorganisms, NBRIP medium was formulated which contained $Ca_3(PO_4)_2$ as insoluble phosphate and glucose as carbon source in addition to other minimal salts (Nautiyal 1999). Although on agar plate assays the efficiency of NBRIP was similar to that of PVK agar, NBRIP showed 3 fold higher efficiency as compared to PVK in broth assay.

Two major constraints of both PVK and NBRIP media are that the sources of insoluble P were the easiest to solubilize and that media were unbuffered. Because of both these factors, bacteria secreting low amount of weak acids could also be selected. Gyaneshwar et al. (1998) demonstrated the importance of buffering of the screening medium in ascertaining the efficacy of PSB. Two PSB strains *Citrobacter koseri* and *Bacillus coagulans* selected using conventional screening media could solubilize both rock phosphate and di-calcium phosphate in unbuffered medium but failed to solubilize rock phosphate in buffered medium. Furthermore, these PSB could not release any detectable P from alkaline Indian vertisol soils supplemented with carbon and nitrogen sources. The organic acids secreted by the two PSB (*C. koseri*: 0.1 mM oxalic, 0.8 mM succinic and 1.2 mM citric acids and *B. coagulans*:1.3 mM succinic, 1.4 mM lactic, 1.4 mM citric and 4.7 mM acetic acids) were 20–50 times less than that required to solubilise phosphorus from alkaline soil. Hence, a PSB demonstrated to be efficient using PVK/NBRIP medium might not be able to perform efficiently under soil conditions since most soils contain more complex mineral phosphates like rock phosphates, FeP and AlP, and have high buffering capacity.

Gyaneshwar et al. (1999) proposed a novel medium for the isolation of PSB which was a defined minimal medium buffered with 100 mM Tris Cl (pH 8.0) (mimicking the buffering capacity of alkaline vertisols), nitrate as N source and Senegal RP as the P source with glucose as C source along with other essential salts (TRP medium). The P-solubilization phenotype on TRP medium, indicated by formation of a red zone around the microbial growth (Fig. 3.4b), would be seen only if the microorganism secreted sufficient organic acids in amount/strength high enough to overcome the buffering and result into colour change of pH indicator dye methyl red. Hence, this buffered medium not only gives a clear qualitative analysis of P-solubilizing phenotype but also helps to isolate most efficient PSB as compared to PVK and NBRIP media. Using this medium, the PSM count was found to be 2 orders of magnitude lower than that obtained using unbuffered medium (Gyaneshwar et al. 1999). *Enterobacter asburiae* obtained using this screening medium could release soluble P, although transiently, from alkaline vertisol accompanied with a large drop in the pH of the soil solution when supplemented with C and N.

Thakuria et al. (2004) have demonstrated that there is no statistically significant relation between the pH decrease in the PVK broth and its soluble P content, which indicates that the solubilizing activity may occur of because of factors other than the production of organic acids. In another study, Johri et al. (1999) reported an increase in the solubilizing activity by the NBR14 and NBR17 strains cultured in NBRIP medium when NaCl was replaced by $CaCl_2$ or KCl and incubated at 30°C. Also they

found an increase in the P solubilizing activity when bacterial strains where incubated at 37°C rather than at 30°C. These data clearly indicate the influence of the salt source and the incubation temperature on the phosphate-solubilizing activity on the NBRIP medium. Several studies have shown that the composition of the medium affects the phosphate-solubilizing activity (Gibson and Mitchell 2004) as well as nature of C and N source (Reyes et al. 1999; Nahas 2007), the type of soil (Nautiyal et al. 2000), and the location from where the bacteria were isolated (Coenye and Vyame 2003). On the other hand, *Enterobacter asburiae* PSI3, *Pseudomonas aeruinosa* P4 and *Serratia marcescens* EB 67 isolated using TRP medium are reportedly the most efficient P-solubilizing bacteria, secreting high amounts of gluconic acid (Buch et al. 2008).

3.4 Pathways of Organic Acid Secretion by Rhizobacteria

Rhizobacteria secrete organic acids as end products or by-products of primary metabolism. In most cases, sugars are catabolized by glycolytic or Entner-Doudroff pathways. Some of the bacteria have phosphotransferase system involved in the uptake-coupled with phosphorylation of various sugars and sugar alcohols utilizing phosphoenol pyruvate as the high energy phosphate (Deutscher et al. 2006). The nature and amount of organic acid secreted by rhizobacteria are governed by the presence of pathway, the regulatory circuits that control the fluxes of metabolic pathways, the availability of electron acceptors, and presence of unique pathways (Table 3.2). Hence, the nature and amount of organic acid secreted by a particular rhizobacterium is not only dependent on its metabolic potential but also on the metabolism of the plant since the availability of carbon sources and electron acceptors depends upon root exudation. Environmental conditions also determine the type of acid secreted by the rhizobacteria. For example, *Enterobacteriacae* members are known to secrete acetic, formic, lactic, fumaric and succinic acids under anaerobic conditions but secrete acetic and pyruvic acids under aerobic conditions (Fuhrer et al. 2005; Sauer and Eikmanns 2005). The amount of the organic acid secretion may differ between members of the same genus and sometimes between strains of the same species (Vyas and Gulati 2009) due to presence or absence of enzymes (Buch et al. 2010). Organic acids of aerobic or anaerobic respiration such as gluconic acid, 2-ketogluconic acid are directly formed extracellularly or in the in

Table 3.2 Major metabolic routes of organic acid secretion by rhizobacteria

Fermentation	Anaerobic respiration	Aerobic respiration	High Metabolic Flux	Unique pathways
Acetic, Formic, Isovaleric, Isobutyric, Ketobutyric, Lactic, Malonic, Propionic, Succinic	Fumaric, Isobutyric	Gluconic, 2-ketogluconic	Acetic, trans- Aconitic, Citric, Glyoxalic Maleic, Malic, Oxalic, Pyruvic	Tartaric, Itaconic

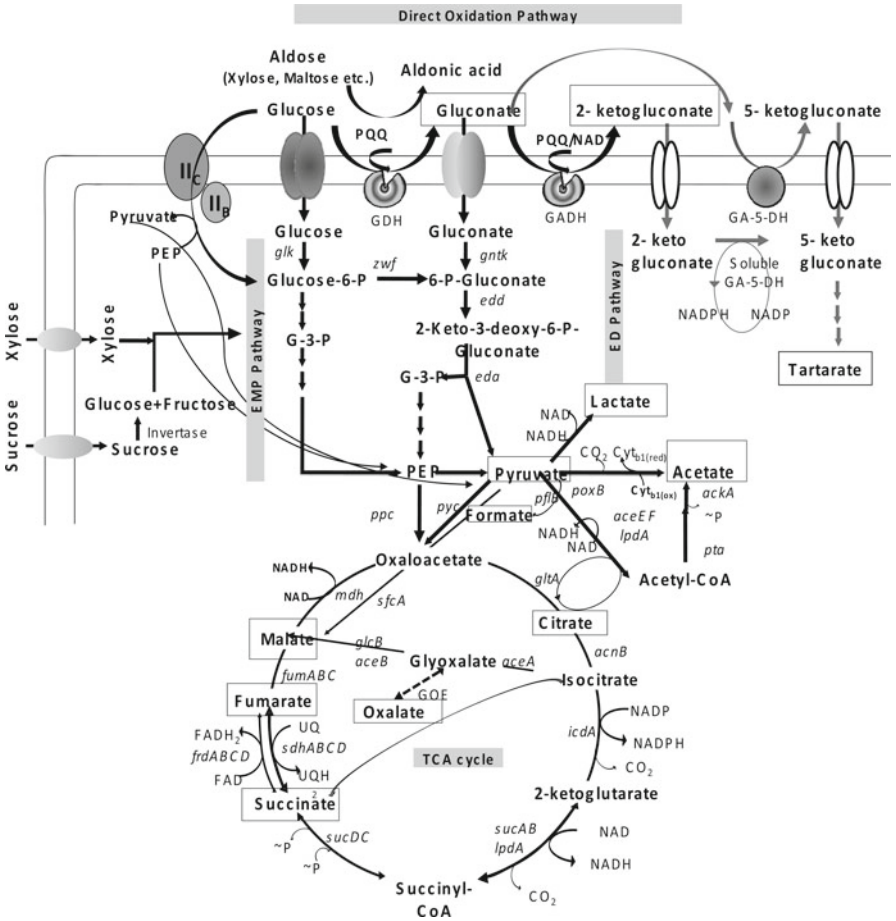

Fig. 3.5 Pathways and enzymes involved in organic acid biosynthesis by rhizobacteria. The organic acids secreted are depicted in boxes. The diagram depicts a comprehensive set of pathways – all may not be present in any given organism. Abbreviations: *GDH* glucose dehydrogeanse, *GADH* gluconate dehydrogenase, *GA-5-DH* gluconate-5-dehydrogenase, *glk* Glucokinase, *zwf* Glucose-6-phosphate dehydrogenase, *gntk* Gluconate kinase, *edd* 6-phosphogluconate dehydratase, *eda* 2-keto-3-deoxy-6-phosphogluconate aldolase, *ppc* phosphoenolpyruvate carboxylase, *pyc* pyruvate carboxylase, *gltA* citrate synthase, *acnB* Aconitase, *icdA* Isocitrate dehydrogenase, *icl* Isocitrate lyase, *sucAB* α ketoglutarate dehydrogenase, *sucDC* succinyl-CoA synthetase, *sdhABCD* succinate dehydrogenase, *fumABC* Fumarase, *frdABCD* fumarate reductase, *mdh* Malate dehydrogenase, *sfcA* malic enzyme, *aceA* Isocitrate lyase, *aceB/glcB* Malate synthase, *GOE* Glyoxalate oxidizing enzyme, *ldh* Lactate dehydrogenase, *aceEF-lpdA* pyruvate dehydrogenase, *pta* phosphotransacetylase, *ackA* acetate kinase A, *poxB* pyruvate oxidase, *pfl* pyruvate formate lyase

the periplasm by the membrane bound enzymes (Fig. 3.5). However, organic acids formed by intracellular enzymes require specific transport proteins that aid in their extracellular secretion. Mono-, di- and tri-carboxylate transporters are located in the plasma membrane mediate their secretion.

3.5 Factors Affecting Organic Acid Secretion by Rhizobacteria and Their Ability to Function in Soils

3.5.1 Nature of Soils

Addition of organic acids decreases the pH of the alkaline vertisol soil solution in the order Acetic = Succinic = Lactic << Gluconic << Oxalic < Tartaric = Citric and results in P release in a similar order excepting that oxalic acid is better than tartaric (Gyaneshwar et al. 1998). Fe is also released, although to a much lesser extent, correlated with the acidification. On the other hand, in alfisols, with buffering capacity much lesser than alkaline vertisols, acidification was much higher by these organic acids but P release was negligible. When CaP rich rock phosphates were amended to alfisol, P was released although to a much lesser extent than that found with vertisol (Srivastava et al. 2007). The influence of buffering on phosphate solubilizing ability could be mimicked in the laboratory medium for detecting the solubilization of CaP complexes by incorporating 100 mM Tris-Cl buffer of pH 8.0 (Gyaneshwar et al. 1998; Joseph and Jisha 2009). Under the buffered conditions the phosphate solubilizing ability of PSB diminished (Gyaneshwar et al. 1998).

3.5.2 Availability of Sugars

The bacterial population in the rhizosphere is 10 times higher than that in bulk soil, but it is 100-fold lower than during growth in the average laboratory medium. Therefore, the lifestyle of rhizobacteria *in situ* can be best characterized as starvation and depends on nutrients from the plant root exudates which mainly consist of sugars, sugar alcohols, organic acids and amino acids (Dakora and Phillips 2002). However, the nature and abundance of the root exudates are highly variable depending on the plant species concerned, type of soil, age, and physiological state of the plant, and nutrient availability to the plant (Bais et al. 2006). Rhizosphere also contains sugars which are derived from cellulose and hemicellulose. Thus glucose, fructose, sucrose, xylose and L-arabinose are some of the sugars available for rhizobacteria.

Glucose dehydrogenase (GDH) located in the periplasm has been postulated to be responsible for the secretion of gluconic acid by rhizobacteria. Because GDH of certain bacteria can be of broad substrate specificity and can act on many aldose mono- and disaccharides, it can help in production of corresponding aldonic acids from these sugars. Cumulative acidity formed from the direct oxidation of mixture of aldoses by GDH can help release P from rock phosphates under buffered conditions when each of the sugars individually is present in too small quantities for producing sufficient acidity (Sharma et al. 2005). Gluconic acid has not been reported to be secreted by plants under any conditions and thus GDH mediated pH

changes and organic acid secretion in the rhizosphere provides an attractive aspect of rhizobacteria for biotechnological applications.

The role of organic acids of rhizobacterial fermentation such as acetic, lactic, succinic, is not clear as plant roots also secrete some of these acids (Jones 1998). However, it is interesting to note that nature of organic acids secreted by bacteria can depend on the availability of carbon sources. *Citrobacter* DHRSS secretes high amount of gluconic acid in the presence of glucose and maltose (Patel et al. 2008). On the contrary, acetic acid is high and gluconic acid was absent when this bacterium was grown on sucrose and fructose.

3.5.3 Catabolite Repression and Other Regulations of Organic Acid Production

Gluconic and 2-ketogluconic acids are formed by the action of periplasmic GDH and GAD enzymes. Gluconic acid secretion in *Enterobacter asburiae* PSI3 was enhanced under P deficient conditions by increased GDH activity indicating this pathway to be regulated by phosphate starvation response (Gyaneshwar et al. 1999). Similarly GDH mediated gluconic acid production in *Erwinia herbicola* was also induced under phosphate starvation (Goldstein and Liu 1987). Fluorescent pseudomonads also secrete gluconic and 2-ketogluconic acids by direct oxidative pathway involving GDH which in turn is dependent on the glucose concentration and nature of electron acceptors (Lessie and Phibbs 1984). Unlike *Enterobacteriaceae* members, pseudomonads have oxidative mode of metabolism rather than fermentative and preferentially catabolize organic acids over sugars. This is reflected in the existence of a 'reverse' catabolite repression control (as compared to *Enterobacteriaceae*) where certain organic acids such as succinate, citrate are utilized as preferred carbon sources and repress the utilization of sugars such as glucose. The catabolite repression is mediated by small RNA molecules regulating the translation of the transcripts depending on the presence of carbon and nitrogen sources (Sonnleitner et al. 2009). Rhizosphere contains various nutrients derived from the root exudates and organic acids are released 0.5-165 pmol g^{-1} root fresh weight s^{-1} (Jones 1998). On the other hand, organic acids present in the soil solution are in the range of 0.5–10 μM. Thus, gluconic and 2-ketogluconic acid secretion by fluorescent pseudomonads could be influenced by the presence of organic acids in the root exudates. Phosphate solubilizing ability of gluconic acid secreting *Pseudomonas aeruginosa* strains was repressed by the presence of weak organic acids like succinate and malate (Patel et al. 2011). This was correlated with a nearly 80% decrease in the activity of the GDH but not gluconate dehydrogenase (GAD) in both the isolates. This is of relevance in rhizospheric conditions where both sugars and organic acids are expected to be available (Dakora and Phillips 2002), and is a new explanation for the lack of field efficacy of such PSB.

3.5.4 Nature of Nitrogen Source

Acidification is attributed to organic acid secretion by rhizobacteria. However, nature of nitrogen source can contribute towards acidification. Uptake of ammonium ion is associated with extrusion of protons (Roos and Luckner 1984). Phosphate solubilization due to acidification significantly decreased in the presence of KNO_3 as compared to that on NH_4Cl (Gyaneshwar et al. 1998; Sulbara´n et al. 2009). Soils, even those supplemented with nitrogenous chemical fertilizers, have nitrate but not ammonia due to highly efficient nitrification by soil bacteria.

3.5.5 Nature of Phosphate Complexes

Vyas and Gulati (2009) studied the organic acid secretion by several *Pseudomonas* strains when grown on tricalcium phosphate and three types of rock phosphates and found significant variation in the nature and amount of organic acids produced. Gluconic acid was commonly produced in high quantities on all the mineral phosphate sources accounting for 80% or more of the total acidity. Minor acids produced differed in amounts, most notable being malic, succinic and 2-ketogluconic acids which were found to be secreted by the strains in relatively higher amounts on tricalcium phosphate as compared to the rock phosphates. On the other hand, all three types of rock phosphates brought about the stimulation of oxalic acid secretion by most of the strains; this acid was below detection limits on tricalcium phosphate. Similar findings have been reported for *Acenitobacter rhizosphaereae* (Gulati et al. 2010).

3.5.6 Environmental Factors

Phosphate solubilizing *Acinetobacter* CR 1.8 growth was not much affected up to 15% NaCl concentration in nutrient rich medium (Chaiharn and Lumyong 2009). *Enterobacter asburiae* PSI3 also could show P-solubilization ability up to 750 mM NaCl in buffered conditions (Gyaneshwar et al. 1998). However, in other bacteria, phosphate solubilization decreased even at 2.5% NaCl (Malboobi et al. 2009). Thermotolerant phosphate solubilizing bacteria retained their ability to solubilize tricalcium phosphate, Al phosphate, Fe phosphate and rock phosphates at 50 °C and in some cases their efficacy was better than that at 25 °C (Chang and Yang 2009). However, *Acinetobacter* CR 1.8 P-solubilization on tricalcium phosphate, rock phosphate and aluminum phosphate was significantly affected by increase in temperature. Maximum solubilization was found at 25 °C, decreased upto 2 fold at 37 °C and very less at higher temperatures (Chaiharn and Lumyong 2009; Malboobi, et al. 2009).

3.6 Future Perspectives

Nature and amount of organic acids in the rhizosphere is determined by the plant root exudates as well as the by rhizobacteria. Since both plant exudation as well as rhizobacterial secretion of organic acids is variable, it will be interesting to determine the organic acids levels in the rhizosphere in the presence and absence of natural microflora and upon inoculation of different rhizobacteria secreting organic acid. In spite of being one of the abundant groups of rhizobacteria, the role of actinobacteria in organic secretion is not known. Actinobacteria may also determine the diversity of rhizobacteria as they are known to secrete efficient antibacterial and antifungal compounds. Many rhizobacteria are known to secrete variety of organic acids. However, the biochemical pathways involved in many of these are yet to be deciphered and their regulation is unknown. Unraveling the pathways would enable us to design strategies for manipulating the rhizobacteria to secrete high levels of organic acids. Although P and K solubilization, and iron availability are known to be determined by acidification, PSB are not been well-investigated for their role in K solubilization and improving iron availability to plants.

3.7 Conclusions

Several rhizobacteria secrete a large variety of organic acids. These organic acids promote plant growth predominantly by providing phosphate from inorganic and organic soil phosphates. Role of organic acids in solubilizing K minerals is becoming significant. The organic acids improve iron availability by acidification of the soil and some of them chelate iron as siderophores. Organic acid mediated changes in metal mobility are important for alleviation of phytotoxicity. Organic acid secretion by rhizobacteria is influenced by many soil components. The nature of organic acid secretion appears to be associated with bacterial physiology. Overall, the organic acids are gaining more importance in plant growth promotion.

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Chapter 4
Organic Farming: For Sustainable Production and Environmental Protection

M.N. Sreenivasa

Abstract Organic farming, an age old and traditional agriculture system of India is being practiced by several lakh farmers in our country. However it requires scientific out look to get fruitful results. High cost of chemical fertilizers and indiscriminate use of pesticides has already resulted in environmental pollution. This inturn affected the human health, biodiversity and soil health. Farming community is enthusiastic to use chemical fertilizers instead of organic manures as their preparation is time consuming and laborious. The chemical fertilizers can supply 2–3 nutrients while crop requires 20–25 nutrients for its growth and yield. The organic manures and bioinoculants can meet the crop requirements. Many scientists have come out with the efficient inoculants which can supply not only nutrients to crops but also protect them from pest and disease attack. This chapter deals with the importance of different organic manures, microbial inoculants and bioagents in crop production and crop protection.

Keywords Organic farming • Environmental protection • Organic inputs • Microbial inoculants • Biocontrol

4.1 Introduction

Organic farming introduces a change in the farming system that aims at maximum output in the cropping pattern and take care of optimal utilization of resources. The soil fertility can be maintained and improved in a sustainable manner by a system which optimizes soil biological activity in organic agriculture. The pest and disease

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management is attained by means of crop selection, rotation, water management and tillage in addition to use of biopesticides. The organic agriculture encourages a balanced host/predator relationship through augmentation of beneficial insect population.

Organic farming is practiced in more than 120 countries in the world covering an area of 30 Mha. In India, it is around 13 lakh hectares cultivated by 7,15,000 farmers using organic principles and practices accounting for production of 19 lakh tones of rice, wheat, pulses, cotton, oilseeds, spices, tea, coffee, fruits and vegetables during 2008–2009 (Yadav 2009). The area under organic agriculture is gradually increasing and it may take 2–3 years to achieve sustainability under organic production system.

Organic farming should be taken as means of enhancing soil fertility, soil health and productivity without causing environmental pollution. High cost of chemical fertilizers, indiscriminate use of pesticides which affected human health, biodiversity in addition to environmental pollution and global warming due to rise in carbon pool, methane *etc* are causing major concern to the society in general and farmers in particular.

Today, India requires nutritional security in addition to food security. It is a fact that the nutritional requirement has been neglected grossly in an anxiety to enhance food production in the country. India stands at 124th position in the developmental Index because of poor/malnutrition. In addition to poor health of human beings and animals, Indian soils too are starving for want of nutrients especially micronutrients. The country's poverty lies in the poverty of our soils. The nation's wealth lies in the fertility of its soils. The cosmetic approach to enhance food production through the application of higher amount of fertilizers, though, was successful in the beginning but later not only failed to meet the target but also has done considerable damage to the health of soil, human beings and animals.

Based on the research data generated at CRIDA, Hyderabad, from past two to three decades, it is evident that chemical fertilizers have significantly contributed to improved productivity of rainfed crops. However, the sustainability of higher yields over a period of time was possible only when optimum nutrients were supplied through organics or in a combination of organics and chemical fertilizers but not when supplied as chemical fertilizers alone (Hegde et al. 1998).

4.2 Crop Diversification as a Tool for Sustainability

Crop diversification is the essence of cropping system which is a practice of introducing a change in crop combinations and is an effective strategy for achieving food and nutritional security, income growth, poverty alleviation, employment generation, judicious use of land and water resources and, improvement in environment.

The crop diversification is one of the key principles of organic farming as it enhances soil fertility whenever legumes are included in addition to avoiding several pests and diseases.

The cropping system influences the soil microflora. The dynamics of soil microflora and soil enzymes was studied is a long term integrated nutrient management

experiment in a fixed site in two cropping systems at UAS, Dharwad. Both soil microflora and soil enzymes were highest in groundnut-sorghum cropping system as compared to sorghum- safflower cropping system. The organic C, available N, available P, soil microflora and enzyme activities increased significantly with the application of organic manures along with inorganic fertilizers. Positive correlation was observed between soil microflora, soil enzymes and nutrient availability. These results highlight the significance of application of organic matter in maintenance of soil fertility and bio-dynamics (Sreenivasa 2009).

4.3 Principles of Organic Farming

- Soil building by conservation and management of organic matter through integrated nutrient supply systems viz., crop rotation, organic manures (enriched compost, vermicompost), green manures, crop residues, oil cakes, bio-fertilizers etc.
- Building healthy soil by nourishing the living components of soil and, better holding, release, transformation and transfer of nutrients.
- On-farm conservation, development and efficient utilization of natural resources.
- Prevention of pests and diseases through good plant nutrition, management, healthy plants and safe treatment *viz*., biopesticides, traps, barriers etc.
- Crop rotation/intercropping/multiple cropping, change in the field ecology, effectively disrupting habitat of weeds, insects and diseases.
- Crop diversification to change the field ecology, effectively disrupting habitat of weeds, insects and diseases.
- Working in harmony with nature and agro-eco system management.
- Enriching the soil, encouraging bio-diversity and reduction of the toxic bodies.
- Employing sound cultural production practices.
- Replacing synthetic fertilizers, chemicals and pesticides.
- Providing attentive care for farm animals.
- Handling the agricultural products without the use of extraneous synthetic additives or processing in accordance with the act and the regulations.
- Creation of a safe environment for people and wild life.
- Production of nutritious food of high quality.
- Generation of more income and employment opportunities for rural people.
- Crop production according to the National Standards for Organic Production.

4.4 Organic Inputs

Today, more than 2,000 million tones of animal wastes, 350 million tones of crop and agricultural wastes, 100 million tones of sugar industrial wastes etc are available in India in addition to a large quantity of other agro industrial wastes. By the year 2015, about 25 MT of chemical fertilizers are required and certainly there

would be a short fall of 10 MT of chemical fertilizers. In this situation, organic manures *viz* compost, vermicompost, biofertilizers, green manures *etc*, can be used as better alternatives.

4.4.1 Compost

Composting is a controlled biooxidative process and compost is the stabilized and sanitized product of composting. Compost making include three important and vital scientific principles namely the narrowing down C:N ratio to a satisfactory level (12:1 or 10:1), the destruction of harmful pathogens and weed seeds due to high temperature evolved during decomposition and stabilization (humification)

There are several methods of composting: Indore method, windrow method, NADEP method, Coimbatore method, Japanese method etc.

The heterotrophs play a key role in composting. Most of the agro residues are lignocellulolytic in nature. Of late, several efficient lingocellulolytic microorganisms have been identified. Among them, *Phanerochete chrysosporium, Trichoderma harzianum, Pleurotus sojarcaju, Paecilomyces fusisporus, Pycnoporus, Trichurus spiralis etc* are important in reduction of C: N ratio and biomass in addition to enhancement in humic acid content of composted material.

Several efforts have been successfully made to improve the quality of compost with free living nitrogen fixers and phosphate solublizers. The simple sugars produced during decomposition by cellulolytic microorganisms from carbohydrates present in agroresidues are used by free living nitrogen fixers and P solubilizers for their growth. The rock phosphate is also used to enrich P content in compost. In general, compost contains 0.5–1% N, 0.2–0.4% P and 1–1.5% K in addition to several micronutrients. However this composition varies slightly with the ingradients used for composting.

4.4.1.1 Enrichment of Compost

The enrichment of compost by adding low grade rock phosphate offers a potential preposition for the effective utilization of insoluble P. The enriched organic manure can be prepared in the farmers field by mixing compostable material (90 parts agroresidues:10 parts animal residues) plus 25% rock phosphate and kept for 90 days with mixing at regular intervals (15 days). About 50% of the insoluble P is converted into soluble P (6–8% P_2O_5) in the phospho-compost. The field trials conducted with phospho-compost on pearl millet, wheat, pigeonpea, green gram and cluster bean showed comparable results with recommended dose of super phosphate even in neutral to alkaline soils. The use of *Aspergillus awamori* was found beneficial in improving the quality of rock phosphate amended compost (Gaur 2001).

Thyagarajan and Arulraj (1993) reported the enrichment of pressmud by microorganisms.In a pot culture experiment, enrichment of pressmud with *Aspergillus*

fumigatus,Bacillus subtilis Pluerotus sp and *Trichoderma viride* with additives like urea and cow dung was done for 45 days. Soil physical properties like water holding capacity, bulk density and pore space improved with enriched pressmud as compared to untreated pressmud. More fungal and bacterial population was observed. Total nitrogen per cent, available N and total P content also increased and had narrow C:N ratio as compared to untreated press mud.

Jisha and Alagawadi (1996) studied nutrient uptake and yield of sorghum inoculated with phosphate solubilising bacteria and cellulolytic fungus in a cotton stalk amended vetisol. The treatment with dual inoculation of *Trichoderma harzianum* and *Pseudomonas striata* with cotton stalks as an organic amendment and P in the form of rock phosphate showed better growth, nutrient uptake and yield of sorghum over the standard practice of supplying P in the form of super phosphate.

Senthilkumar et al. (2004) reported the effect of Zn enriched organic manure and Zn solubilizer application on the yield, curcumin content and nutrient status of soil under turmeric cultivation. A field experiment was conducted to study the effect of FYM and Zn enriched coir pith with Zn solubilizers like *Bacillus sp*. The yield increased by 21.6% than by using FYM alone. The Zn solubilizing bacteria proved to have favorable effect on the availability of N, P, K, Mn and Ca availability.

Dayananda and Mallesha (2005) reported the effect of microorganisms enriched mushroom spent coir pith on the growth of tomato. The *Pleurotus florida* (oyster mushroom) spent coir pith enriched with microbial consortia (*Azotobacter chroococcum, Bacillus megaterium* and *Trichoderma harzianum*) along with soil and sand was found to be a better nursery mix for the growth of tomato seedlings.

Kavitha and Subramanian (2007) reported the effect of bioactive compost which is a value added compost with microbial inoculants and organic additives. The normal compost was transformed into bioactive compost by the addition of *Azotobacter, Pseudomonas,* Phosphobacteria, poultry manure, rock phosphate and diluted spent wash. This enrichment increased the nutritive value of compost. The highest N content (1.75%) and P content (1.61%) was observed. The plant growth promoters like IAA and GA were more in this treatment. All these factors lead to increase in crop yield.

Rajarajan et al. (2009) worked on the effect of enriched organic wastes on available nutrients in soil and yield of coconut trees. The organic farm waste enriched with *Azospirillum* and phosphobacteria along with green manure and coir pith compost enriched with rock phosphate was applied to the crop. They observed increase in yield and reduction in cost of cultivation in both the cases. Intercropping with pulse gave additional income.

4.4.2 Vermicompost: Wealth from Waste

Earthworms are the best known soil inhabiting animals and regarded as farmer's friend. Through there are 12 families in earthworm, the Indian subcontinent has representative genera from 9 families and Family Megascolicidae is predominant in the tropical and subtropical soils.

Use of earthworms for waste management is one of the recent developments in biological sciences. Earthworms feed on various organic wastes, break complex residues in to simpler water soluble substances. They ingest wastes, digest them in the gut and defecate out fecal matter which is termed as "vermicompost". This vermicompost is rich in plant nutrients (1% N, 0.86% P_2O_5, 0.98% K_2O, several micronutrients) in addition to plant growth promoting substances, beneficial microflora *etc*.

There are two methods in vermicomposting: (1) surface vermicomposting, (2) subsurface vermicomposting (Giraddi 2007).

Here also several scientists successfully developed a technology for enrichment of vermicompost on similar lines of enrichment of compost using free living nitrogen fixers and P-solubilizers (Rajendran et al. 2000). Working on the enrichment of vermicompost by using *Azospirillum* and *Pseudomonas striata,* Sreenivasa (2010) reported the shelf life of enriched vermicompost to be 2 months after its enrichment. Both these inoculants were mixed with residues @ 1 kg each after 1 month during vermicompost preparation. The use of enriched vermicompost resulted in improvement in growth and yield of tomato and chilli (Sreenivasa 2010).

Neethasharma (1994) reported the recycling of organic wastes through earthworms as an alternate source of organic fertilizer for crop growth in India. She used maize and wheat as test crops. Increase in yield and improved status of N, P, K and micronutrients availability was observed.

Sailajakumari and Ushakumari (2002) reported the effect of vermicompost enriched with rock phosphate on the yield and uptake of nutrients in cow pea. They observed that the treatment given with enriched vermicompost showed maximum yield and uptake of nutrients like N, P, K, Ca and Mg. The maximum yield of 1,072 kg/ha was recorded with enriched vermicompost as compared to vermicompost only (877 kg/ha).

4.4.2.1 Vermitechnology in Pest Management

On organic nourishment, crop gets balanced nutrition with major, minor and secondary nutrients besides other substances such as PGRS, antibiotics *etc* which make the plants to develop defensive mechanisms against crop pests. Acceleration in enzyme activity (peroxidase, catalase, alkaline phosphatase *etc*) and secretion of plant metabolites (proteins, sugars, phenols, tannins *etc*) make the plant resistant (induced resistance) and more vigorous (tolerance) so that it is well placed in fighting against pest menace as compared to inorganically nourished crop (Giraddi 2000).

4.4.2.2 Vermiwash

Generally vermiwash is collected by spraying clean water on vermicompost and is stored in plastic/earthen containers.

Of late, vermiwash is very much used by organic growers for spray @ 20% concentration to different crops during flowering stage as it contains nutrients and plant growth harmones. It is known to impart phytoresistance against pest and disease attack.

Table 4.1 Percentage of nutrients in different oil cakes

Sl. no	Name of oil cake	N (%)	P_2O_5 (%)	K_2O (%)
1	Honge cake	3.97	0.94	1.27
2	Neem cake	5.22	1.08	1.48
3	Castor cake	4.37	1.85	1.39
4	Cotton seed cake			
a	Decorticated	6.41	2.89	2.17
b	Undecorticated	3.99	1.89	1.62
5	Groundnut cake	7.80	1.90	1.30
6	Niger cake	4.73	1.83	1.31
7	Sesamum cake	6.22	2.09	1.26
8	Safflower cake			
a	Decorticated	7.88	2.20	1.92
b	Undecorticated	4.92	1.44	1.23

4.4.3 Cakes

Generally cakes are prepared from botanicals which are not used for human consumption. Ex. Neem cake, pongamia cake *etc* are being applied to the field @ 250–500 kg per hectare to supply nutrients (Gupta 1999) in addition to imparting phytoresistance against crop pests and diseases. The nutrient content of different cakes is given in Table 4.1.

4.4.4 Green Manure

The role of green manures in improving soil fertility and supplying a part of nutrient requirement of crops is well known as early as 1134 BC from China.

Green manure refers to fresh plant matter which is added to the soil largely for supplying the nutrients contained in biomass. Green manures must be grain legume plants such as green gram, cowpea, soybean *etc* or perennial woody multipurpose legumes like *Crotolaria, Sesbania, Centrosema, Stylosanthus, Desmodium etc*. Leguminous plants are preferred as green manures due to their symbiotic N_2 fixing capacity.

The effect of green manures on crop yield varies considerably according to chemical composition and soil characteristics. Generally, soils with poor fertility respond better to the application of green manures with

4.4.4.1 Advantages of Green Manuring

Green manuring results in the improvement of physical, chemical and biological processes in soil through nutrient conservation especially those nutrients which are susceptible for leaching losses. The plants used for green manuring have deeper root system thus make nutrients to concentrate in the surface layers of soil in addition reduction in losses of surface soil by wind and water erosion. Because of deeper root

system, subsoils will become porus. Green manuring also helps in the reclamation of saline and alkaline soils by release of organic acids. Majority of studies revealed that green manure can provide N to an extent of 50–100 kgs. The best examples in this regard are Dhaincha, *Sesbania rostrata, Aeschynomene* spp which are used extensively in rice production. In water logged soils, green manure increases availability of P through mechanism of reduction, chelation and favourable changes in soil pH and this effect is more pronounced in acidic and sodic soils. Many scientists have reported higher availability of K (10–12%) Ca, Mg *etc* in soils due to green manuring.

All these additive positive effects of green manuring resulted in higher yield of chilli (Hiremath 2008) and rice (Hiremath and patel 1996).

4.4.5 Other Organic Manures

4.4.5.1 Biogas Spent Slurry

It contains nutrients @ 2% N, 0.8% P_2O_5 and 1.5% K_2O in addition to several micronutrients. Hence, it is being used as organic manure in crop production. Several scientists have developed technologies for its enrichment using low grade rock phosphate which when used for crop production has resulted in higher growth and yield.

Poultry manure and piggery manure too are being used @ 2 tones/ha for crop production. However it is suggested that poultry manure may be allowed for aerobic decomposition for atleast 1 week before its application to field.

4.4.6 Liquid Organic Manures

4.4.6.1 Animal Urine

Animal urine (especially cow urine) contains uric acid, sodium chloride, calcium sulphate, magnesium sulphate *etc*. and its spray at 20% concentration enhances plant nutrition. It is also used to suppress the pathogens like *Sclerotium*, *Fusarium etc.*

At the Institute of Organic Farming, UAS, Dharwad, the influence of different concentrations of cow urine on the growth of *Sclerotium* and *Fusarium* was studied using potato dextrose agar. The biological deterrent activity of cow urine on these pathogens was clearly noticed at 20% concentration. However, cow urine spray at 20% did not inhibit the beneficial fungi, *Trichoderma* and *Verticillium* (Sreenivasa 2010) (Tables 4.2 and 4.3).

4.4.6.2 Panchagavya

It is prepared by mixing cowdung (7 kg) and Ghee(1 kg) and, stirring for 2 days (2–3 times daily). Later, cow urine (3 L) and water (10 L) have to be mixed to this

Table 4.2 Growth of organisms (diameter in cm) on Potato Dextrose Agar supplied with different concentrations of cow urine

	Cow urine concentration					
Test organism	0%	5%	10%	15%	20%	CD at P = 0.01
Fusarium	7.67	7.17	5.00	3.27	0.60	0.45
Trichoderma	7.97	8.03	8.03	7.97	7.73	NS
Verticillium	6.93	7.23	6.83	6.87	6.93	NS

Table 4.3 Percent inhibition is calculated considering the growth of organism at 0 percent cow urine as 100

	Cow urine concentration			
Test organism	5%	10%	15%	20%
Fusarium	6.51	34.81	57.36	92.17
Trichoderma	Nil	Nil	Nil	0.50
Verticillium	Nil	1.44	0.86	Nil

and stirred twice a day for 15 days. To this mixture, sugarcane juice (3 L) or Jaggery(250 g), cow milk(2 L), curd(2 L), tender coconut water (2 L), yeast(100 g) and ripened banana (12 no's) have to be added and stirred twice a day for a fortnight (Swaminathan et al. 2007; Sreenivasa 2010). Later, this mix can be fillired and used as spray @ 3% concentration @ 500 L per hectare twice in the cropping period *i.e.,* during flowering and 15 days after first spray. This is also being used @ 500 L per hectare along with irrigation water for irrigated crops. It will supply nutrients to the crops in addition to stimulation of plant growth as it posses plant growth promoting substances. Its spray enhances flowering and fruiting in crop plants inturn crop yield because of the following reasons.

- Coconut water is the source of kinetin which increases the chlorophyll content of the plant
- Sugarcane juice facilitates easy fermentation
- Cow milk contains plant growth promoters IAA, GA
- It has an excellent sticker spreader property, Proline present in milk induce resistance in plants, milk has antiviral property also
- Curd and buttermilk increase microbial activity
- Cow urine contain uric acid, sodium chloride, calcium sulphate, magnesium sulphate. (Sreenivasa et al. 2010)

4.4.6.3 Jeevamrutha

It is prepared by adding cowdung (10 kg), cow urine (10 L), jaggery (2 kg), pulse flour (2 kg) and 500 g rhizosphere soil to 200 L water and stirring 5–6 times a day for one week. It is used @ 500 L per hectare as spray on soil before sowing or it is also used along with irrigation water.

Table 4.4 Nutrient status of different organic liquid manures

Parameter	Panchagavya	Beejamrutha	Jeevamrutha	Biodigester
pH	6.82	8.2	7.07	7.29
Soluble salt (EC)	1.88 dsm^{-1}	5.5 dSm^{-1}	3.40 dSm^{-1}	1.09 dSm^{-1}
Total Nitrogen	1,000 ppm	40 ppm	770 ppm	255 ppm
Total Phosphorus	175.4 ppm	155.3 ppm	166 ppm	79 ppm
Total Potassium	194.1 ppm	252.0 ppm	126 ppm	42 ppm
Total Zinc	1.27 ppm	2.96 ppm	4.29 ppm	0.52 ppm
Total Copper	0.38 ppm	0.52 ppm	1.58 ppm	1.24 ppm
Total Iron	29.71 ppm	15.35 ppm	282 ppm	9.60 ppm
Total Manganese	1.84 ppm	3.32 ppm	10.7 ppm	8.30 ppm

It is known to enhance biochemical activities in soil in addition to supply of nutrients to crops.

4.4.6.4 Beejamrutha

It is prepared by tieing cowdung (5 kg) in a cloth and hanging the same in a bucket of water (50 L) over night (8–10 h). Later the dung tied in cloth has to be squeezed frequently by dipping in water for 4–5 times. Then cow urine (5 L) and 50 g calcium chloride has be added to this mix (Sreenivasa et al. 2009).

The seeds/setts/root system of seedlings have to be dipped for 5 min in this mix before sowing/transplanting.

4.4.6.5 Biodigester

It requires a cement tank having 16 ft length, 10 ft width and 6 ft height. The botanicals mainly neem, calotropis, vitex, lantana, adathoda, Ipomea, custard apple and agave (5 kg each) have to be added to the biodigester tank along with cow urine (10 L), dung (10 kg), little quantity of rhizosphere soil (1 kg) and water (200 L). This mixture should be allowed for fermentation for 3 weeks. The filterate can be used @ 500 L per hectare for spraying on soil or along with irrigation water. It can also be used @ 10% for spraying on the plants. It provides nutrients and growth promoting substances to plants in addition to suppression of pests and diseases (Sreenivasa 2010).

At the Institute of Organic Farming, UAS, Dharwad scientific validation of these liquid organic manures has been done (Tables 4.4 and 4.5). The results indicated the presence of nutrients, beneficial microflora (free living nitrogen fixers, P-solubilizers, plant growth promoting rhizobacteria *etc*). Further the isolates have been screened for production of plant growth promoting substances, biological deterrent activities *etc*. Few isolates were found to posses both these beneficial traits (Table 4.6). The bacterial isolate PB 9 was found to produce growth promoting harmones in addition

Table 4.5 Microbial population in different organic products

	Panchagavya	Beejamrutha	Jeevamrutha	Bio-digester
Bacteria (cfu/ml)	26.1×10^5	15.4×10^5	19.7×10^5	12.6×10^5
Fungi (cfu/ml)	18.0×10^3	10.5×10^3	13.4×10^3	9.6×10^3
Actinomycetes (cfu/ml)	4.20×10^3	6.8×10^3	3.5×10^3	2.8×10^3
N_2 – fixers (cfu/ml)	2.7×10^2	3.1×10^2	4.6×10^2	0.4×10^2
Phosphate solubilizers (cfu/ml)	5.7×10^2	2.7×10^2	4.2×10^2	1.9×10^2

Table 4.6 N_2-fixing and phosphate solubilisation capacity of bacterial isolates of panchagavya

N_2-fixing isolates	Amount of N_2 fixed mg/g carbon	Phosphate solubilising isolates	Per cent Pi-released
AzP1	12.31	PPS1	4.32
AzP2	9.71	PPS2	2.13
AzP3	8.14	PPS3	2.63
AzP4	11.13	PPS4	8.22
AzP5	12.81	PPS5	7.70
AzP6	10.89	PPS6	1.64
AzP7	9.13	PPS7	2.78

Table 4.7 IAA and GA production and biocontrol potential of general bacteria isolated from panchagavya

			Biocontrol effect	
General bacterial Isolates	IAA (µg/25 ml)	GA (µg/25 ml)	Result	Per cent inhibition of *Sclerotium rolfsii*
PB1	6.54	1.18	−ve	5.5
PB2	4.80	2.09	−ve	5.5
PB4	3.12	−ve	−ve	nil
PB5	4.37	−ve	+ve	87
PB6	−ve	−ve	+ve	74
PB7	−ve	−ve	+ve	83
PB8	9.27	−ve	+ve	81
PB9	12.18	3.81	+ve	83
PB10	−ve	−ve	−ve	5.2
PB11	−ve	2.54	−ve	nil
PB12	3.81	−ve	−ve	nil
PB13	−ve	1.54	−ve	nil
PB14	5.18	−ve	+ve	76
PB15	9.09	1.27	+ve	80

to suppression of *Sclerotium* (Table 4.7). Further these isolates improved seedling length and vigour index in addition to germination percentage in chickpea and wheat (Tables 4.8 and 4.9) (Sreenivasa et al. 2009).

Similarly isolate BJ-5 from beejamruth was found to produce both IAA and GA in addition to inhibition of *Sclerotium* to an extent of 88% (Table 4.10). Isolate BJ-5

Table 4.8 Effect of inoculation of bacterial isolates of panchgavya on seed germination, seedling length and vigour index in chickpea

	Germination percentage	Seedling length (cm)	Seedling vigour index
T1 – inoculated with PB1	94	26.13	2457
T2 – inoculated with PB2	92	26.21	2424
T3 – inoculated with PB3	88	23.95	2107
T4 – inoculated with PB4	90	25.06	2255
T5 – inoculated with PB5	92	24.41	2244
T6 – inoculated with PB6	88	19.82	1743
T7 – inoculated with PB7	88	22.63	1991
T8 – inoculated with PB8	96	27.61	2650
T9 – inoculated with PB9	99	27.69	2741
T10 – inoculated with PB10	89	25.77	2292
T11 – inoculated with PB11	95	23.10	2194
T12 – inoculated with PB12	96	25.08	2407
T13 – inoculated with PB13	93	27.07	2519
T14 – inoculated with PB14	90	25.48	2292
T15 – inoculated with PB15	98	27.55	2699
T16 – uninoculated control	86	18.33	1575
SEM ±	1.08	0.76	79.81
CD	4.22	2.98	310.34

Table 4.9 Effect of inoculation of bacterial isolates of panchgavya on seed germination, seedling length and vigour index in wheat

Treatments	Germination percentage	Seedling length (cm)	Seedling vigour index
T1 – inoculated with PB1	92	22.24	2046
T2 – inoculated with PB2	88	20.6	1814
T3 – inoculated with PB3	91	20.48	1864
T4 – inoculated with PB4	91	24.4	2221
T5 – inoculated with PB5	88	19.44	1711
T6 – inoculated with PB6	87	24.29	2113
T7- inoculated with PB7	92	20.29	1867
T8 – inoculated with PB8	95	26.5	2517
T9 – inoculated with PB9	99	28.5	2822
T10 – inoculated with PB10	95	23.64	2251
T11 – inoculated with PB11	91	26.81	2440
T12 – inoculated with PB12	92	27.48	2528
T13 – inoculated with PB13	98	18.19	1783
T14 – inoculated with PB14	90	23.65	2129
T15 – inoculated with PB15	99	25.02	2477
T16 – uninoculated control	85	16.5	1403
SEM ±	0.55	0.34	36.79
CD	1.61	1.00	106.27

Table 4.10 IAA and GA production and bio-control potential of bacterial isolates of beejamruth

			Bio-control effect	
Isolate	IAA (μ g/25 ml)	GA (μ g/25 ml)	Result	Per cent inhibition of *Sclerotium rolfsii*
BJ-1	6.12	1.06	+ve	75
BJ-2	8.45	1.27	+ve	66
BJ-3	−ve	2.23	−ve	Nil
BJ-4	4.13	−ve	−ve	Nil
BJ-5	11.36	3.13	+ve	88

also enhanced seed germination, seedling length and seeding vigor index in chick pea and wheat (Tables 4.11 and 4.12) (Sreenivasa et al. 2009, 2010). Nileema and Sreenivasa (2010) reported the effect of these organic liquid manures on the growth and yield of tomato. The results clearly showed the improvement in soil biological indicators such as microbial population and enzyme activities (dehydrogenase, urease, phosphatase) in addition to growth, yield, lycopene content in tomato with the application of liquid organic manures.

Several field trials conducted on similarly lines from past 5 years at the Bio-farm, Institute of Organic Farming, University of Agricultural Sciences, Dharwad clearly proved the beneficial effects of these liquid organic manures when used along with other organic manures (FYM, compost, vermicompost, green leaf manures) in terms of growth and yield of soybean, groundnut, cotton, redgram, wheat, jowar, sugarcane, chilli, brinjal *etc* (Sreenivasa et al. 2010).

4.5 Microbes-Wheels of Organic Farming

In organic farming system, soil microorganisms play a pivotal role as the main driving force. The use of soil microbial technologies to improve the efficiency of farming and to ensure the safe management of the environment is an important development because the ultimate success of human kind and the health of planet relies on the development of efficient and sustainable agricultural system and improved environmental stewardship. An acre of living topsoil contains approximately 900 lb of earthworms, 2,400 lb of fungi, 1,500 lb of bacteria, 133 lb of protozoa, 890 lb of arthropods and algae. Soil microbial biomass can be regarded as a sink and a source of plant nutrients (Sreenivasa 2010).

4.5.1 Use of Microbial Inoculants in Organic Farming

Microbial inoculants are generally used in agriculture as biofertilizers to provide nutrients to crop plants or as bioagents (biofungicides, biopesticides) to suppresswpathogens or crop pests. The success of inoculation always depend upon the ability

Table 4.11 Influence of bacterial isolates of Beejamruth on seed germination and seed vigor index of chickpea

	Dipping period											
	5 min			10 min			15 min			20 min		
Treatments	Germn %	Seedling length (cm)	Seeding vigour index	Germn %	Seedling length (cm)	Seeding vigour index	Germn %	Seedling length (cm)	Seeding vigour index	Germn %	Seedling length (cm)	Seeding vigour index
BJ1	94	36.73	3453	96	37.30	3581	94	36.08	3392	94	36.71	3452
BJ2	93	34.98	3253	93	38.07	3541	91	37.88	3447	92	34.11	3138
BJ3	90	34.78	3107	91	37.18	3383	90	38.63	3477	90	37.32	3358
BJ4	91	36.44	3316	91	37.21	3390	91	36.17	3291	91	32.27	2936
BJ5	98	41.13	4031	99	41.25	4084	98	39.67	3887	98	39.39	3860
CD	1.76	7.18	679.75	4.30	5.52	628.90	1.76	7.11	628.51	2.15	8.83	836.50
SEm±	0.45	1.83	173.12	1.10	1.40	160.17	0.45	1.81	160.07	0.55	2.25	213.04

Control: Seed germination percentage: 90%, seedling length: 28.48 cm and seedling vigour index: 2563

Table 4.12 Influence of bacterial isolates of Beejamruth on seed germination and seed vigor index of wheat

	Dipping period											
	5 min			10 min			15 min			20 min		
Treatments	Germn %	Seedling length (cm)	Seeding vigour index	Germn %	Seedling length (cm)	Seedling vigour index	Germn %	Seedling length (cm)	Seedling vigour index	Germn %	Seedling length (cm)	Seedling vigour index
BJ1	96	20.39	1957	97	21.94	2128	96	20.60	1977	94	20.00	1880
BJ2	91	18.95	1724	91	19.26	1748	89	19.23	1710	90	19.18	1724
BJ3	93	18.49	1719	93	21.83	2030	90	20.17	1815	91	18.47	1680
BJ4	90	18.88	1702	90	19.58	1762	90	17.20	1548	89	18.11	1612
BJ5	99	21.39	2117	99	22.73	2250	99	22.23	2200	98	21.44	2101
CD	2.78	5.92	567.73	1.76	10.48	989.95	1.76	5.56	544.75	2.15	7.06	646.38
SEm±	0.71	1.51	144.59	0.45	2.67	252.12	0.45	1.42	138.74	0.55	1.80	164.62

Control: Seed germination percentage: 86%, seedling length: 16.39 cm and seedling vigour index: 1409

of microorganisms to multiply and survive in the introduced environment. Further, inoculants will be useful only when the organism is viable and sufficient in number to bring out the desired changes.

4.5.2 Microbial Inoculants as Biofertilizers

4.5.2.1 Symbiotic Nitrogen Fixers

Several procaryotic organisms posses the ability to reduce the gaseous atmospheric nitrogen to ammonical form with the help of the enzyme "Nitrogenase" present on their cell membrane. The best and commonly known example is the inoculation of rhizobia to legumes. Due to host specificity problem, rhizobiologists still follow cross inoculation grouping to inoculate legumes. *Rhizobium* fixes atmospheric nitrogen under symbiotic conditions in the root nodules of legumes and inturn will be benefited from supply of photosynthates from leguminous plants.

Many a times, rhizobiologists stress the importance of inoculation of rhizobia when native rhizobia are absent or ineffective or harmful. Some times rhizobia fail to recognize the host and unable to bind and colonise the root surface or may be killed by bacteriophages or due to other bacterial/fugal toxins. In addition to this, few species of *Bradyrhizobium* are known to produce toxins and hence found to be harmful.

For successful results, rhizobia must be infective, competitive and effective (ICE). The competitiveness' is influenced by several factors *viz.* inoculum size, host compatibility, aeration, pH, temperature *etc*.

4.5.2.2 Associative Symbiotic Nitrogen Fixers

Azospirillum, Acetobacter diazotrophicus (Glucanobacter diazotrophicus), Herbaspirillum are few associative symbiotic nitrogen fixers that enter the host cortical cells to reduce gaseous atmospheric nitrogen in addition to production of growth promoting substances.

4.5.2.3 Free Living Nitrogen Fixers

Azotobacter, Beijerinckia, Derxia, Azomonas etc. reduce gaseous atmospheric nitrogen within their cell system and release the same to the soil for plant root absorption.

Several cyanobacterial genera *Anabaena, Nostoc, Aulosira, Cylindrospermum, Calothrix, Tolypothrix, Stigonema etc.* normally fix atmospheric nitrogen but also produce several vitamins and growth promoting substances (ascorbic acid, auxins, vitamin B_{12}) and they also add organic matter to soil which improve plant growth

and yield. Few genera of cyanobacteria fix atmospheric nitrogen in symbiotic association with an aquatic fern, *Azolla* which itself contain N (4–6%), P (1–2%), K(3–7%) in addition to several micronutrients. Generally, it is also used as green manure in paddy cultivation in India, China, Thailand, Vietnam, Phillippines *etc*. Thus paddy crop gets dual benefit from the incorporation of *Azolla*. In several places, it is also used as cattle feed (*Azolla* is used @ 25% of cattle feed) and fish feed.

4.5.2.4 P-Solubilizing Microorganisms

The availability of P in soil to plants is dependent on soil pH. Many a times, soil phosphorus is in the bound form and thus unavailable to plants. Soil phosphorus will be precipitated as calcium or megnesium phosphate under alkaline pH conditions. Several bacteria and fungi (*Bacillus megatherium, B.polymyxa, Pseudomonas striata, Aspergillus awamori, Penicillium funiculosum Burkholderia, Serratia marcescens etc*) are known to produce organic acids like citric, succinic, glutamic, maleic, L-ketoglutaric, fumaric and tartaric acids which are helpful in solubilization of phosphates. These organic acids have been shown to chelate cationic portions of insoluble phosphate compounds. Of the several mechanisms that have been proposed in solubilization of phosphate, production of organic acids is considered to be the most significant but other products such as CO_2, H_2S, chelating agents, humic substances, siderophores and protons are also reported to be involved in the process (Cunningham and Kuiack 1992; Gaur 1990).

Several factors influence P solubilization under field conditions which include soil type, nature of insoluble phosphatic compounds, ability of phosphate solubilizing microorganisms and plant genotype. The physical condition of soil, pH, organic matter and plant nutrients directly or indirectly influence P-solubilization process (Vikram et al. 2007). The environmental conditions such as temperature, moisture, aeration, humic acids *etc.* control the growth and activity of P-solubilizers.

Several studies conducted by scientists clearly indicated the possibility of getting better crop yields with the inoculation of P-soulbilizers along with the application of rock phosphate as compared to super phosphate (Babana and Antoun 2006)

4.5.2.5 P-Mobilizers

Mycorrhizal fungi are known to mobilize several nutrients from soil with the help of radiating hyphae. However, uptake and translocation of immobile nutrients have been given importance in mycorrhizal studies. These fungi also are helpful in mobilizing moisture from soil, production of growth promoting substances and suppression of soil borne root infecting pathogens. The major bottleneck in mycorrhizal research is its inoculum production. Being obligate symbionts, Arbuscular mycorrhizal (AM) fungi are maintained as pot cultures using a suitable host and substate

(Sreenivasa and Bagyaraj 1989). Significant increases in plant growth and yield of several crops due to AM inoculation have been reported by many scientists. P-response studies with the inoculation of efficient strains of AM fungi indicated the possibility of a net saving of 20–50% of recommended P in many crops (Sreenivasa et al. 1993; Sreenivasa 1992)

4.5.2.6 Potassium Mobilizing Bacteria

Infact Indian soils does not have any problem in K availability. Hence very little work on K mobilizing microorganisms has been carried out by the scientists. Chandra and Greep (2006) isolated K mobilizing bacterium *Frateuria aurentia* from banana rhizosphere in Orissa and this bacterium is found to enhance nutrition and growth of several crop plants.

4.5.2.7 Plant Growth Promoting Rhizomicroorganisms (PGPR)

The plant growth promoting rhizomicroorganisms (PGPR) improve plant growth through production of phytoharmones, enhanced nutrient uptake and/or suppression of plant diseases. The PGPR are otherwise referred to as plant health promoting rhizomicroorganisms (PHPR). The extensively studied PHPR are *Pseudomonas fluorescens, P. aeruginosa, Arthrobacter, Methylobacterium, Bacillus subtilis, Burkholderia cepacia* in addition to several nitrogen fixers and P-solubilizers.

4.5.2.8 Decomposers

In the earlier part of this chapter, the importance of decomposers in compost preparation has been discussed. Inoculation of such organisms reduce the time taken for decomposition of agricultural and industrial wastes.

4.5.3 Microbial Inoculants in Disease and Pest Management

Plant diseases and crop pests are contributing 23–30% of losses in crop production throughout the world. The chemical control of plant diseases and crop pests is spectacular but this is relatively a short term measure as it is causing ecological problems in addition to accumulation of harmful chemical residues in soil, water, food grains, animal feed *etc*. Hence biological control of plant diseases and crop pests is gaining importance.

Several microorganisms have been successfully used to control soil borne diseases (Table 4.13) and crop pests (Table 4.14).

Table 4.13 Microbial inoculants used for biocontrol of plant diseases

Pathogen/Disease	Crop	Biocontrol/agent
Sclerotium rolfsii	Tomato, beans	*Trichoderma viride*
	groundnut	*T. harzianum* and *T. hamaturn*
Fusarium udum (wilt)	Redgram	*T. viride*
Borytis cinerea (Grey mold)	Grapes	*T. viride*
Rhizoctonia solani (Damping off, root rot)	Greengram, cotton	*T. harzianum* + *Pseudomonas fluorescens* + VA mycorrhiza
Phytophthora capsici (root rot)	Black pepper	*T. harzianum* + VA mycorrhiza
Gaemannomyces graminis var. *tritici*	Wheat	*Pseudomonas fluorescens*
Pythium (Damping off)	Brinjal	*T. harzianum*
Fusarium oxysporum	Bengal gram (wilt)	*T. harzianum*
	Banana (panama disease)	*P. fluorescens*
Meloidogyne incognita	Tomato	
M. javanica (Root knot)	*Capsicum*	*Pochonia clamydosporia*
	Brinjal	*Paecilomyces lilacinus*
Ralstonia solanacearum	Potato	*Bacillus cereus B. subtilis*

Table 4.14 Microbial inoculants used for biocontrol of crop pests

Pest	Crop	Bioagent
Bollworm	Coffee	*Beauveria bassiana*
Shoot borer	Sugarcane	NPV, GV
Berry borer	Cotton, pulses	
Fruit borer	Brinjal	*Bacillus thuringiensis*
Dimond blackmoth	Tomato	
	Cabbage	
Borers, pyrilla, White grub	Groundnut	*Metarrhizium anisopliae*
	Rice	
	Sugarcane	
Spodoptera litura *Helicoverpa armigera*	Cotton, pulses, castor, soybean	*Nomuraea rileyi*
Aphids, Mealy bugs	Oilseeds, grapes	*Verticillium lecani*
Mites	Citrus, coconut	*Hirsutella thmpsonii*
Leaf hoppers, Beetles	Coconut	*Metarrhizium anisopliae*
Rice borer	Rice	Granulosis virus
codling moth	potato	
Mosquito larvae	–	*Bacillus sphaericus*

4.5.3.1 Advantages of Using Biocontrol Agents

1. Avoid adverse effects on the beneficial microbes including antagonists in soil.
2. Avoid pollution of soil, air and water
3. Cost effective (less expensive)
4. Avoid the development of resistant strains in pathogen.

4.5.3.2 Mechanisms of Plant Disease Control by Bioagents

There are several mechanisms followed by bioagents in plant disease control. The important mechanisms are (1) Competition (2) Antibiosis (3) Antifungal enzyme production (4) Hyperparasitism (5) Induced systemic resistance

4.5.3.3 Desirable Characters of Biocontrol Agents

1. It should grow easily on the available nutrients (adaptability) and survive in the rhizosphere or spermosphere.
2. The antibiotics produced by the bioagent should not cause damage to host plants or other associated antagonists
3. They should have better tolerance/adaptability to varied environmental extremities.
4. The spore germination should be rapid and prolific. The bioagents also should have better adaptability for large scale production and handling.

There are approximately 280 biopesticides available in the market involving bacteria (37%), fungi (5%), viruses (3%) *etc*. In addition to these microbial insecticides, several botanicals (plant extracts), neem based products, natural enemies, trap crops, pheromone traps, agronomic practices *etc* are being used to avoid chemical insecticides which cause environmental pollution (Fouche 2007; Paul 2007)

4.5.3.4 Key to Success in Biocontrol

1. Selection of an efficient strain of the antagonist
2. Adequate growth and sporulation on mass culture media
3. Advance application of bioagents to provide enough time for interactions with pest/pathogen
4. Favourable soil temperature, moisture, pH *etc*. which influence the growth and development of the bioagent
5. Competency of bioagents.

4.6 Future Perspectives

Though many farmers are following organic farming principles and practices since several years, they lack scientific knowledge. There is a greater need for scientific validation of the indigenous technologies followed by organic growers. The need of the hour is to evolve methodologies for enrichment of organic manures, develop efficient location specific strains of microbial inoculants and other bioagents with better shelf life, to workout economics (Cost : Benefit ratio) *etc.* Also there is a greater need to document soil organic carbon, beneficial microbial population, pest and disease scoring *etc.* in long term organic experiments across different agro ecological zones.

4.7 Conclusions

Organic farming practices improve soil fertility, soil health and crop production without causing environmental hazards. The nation's wealth lies in the fertility of its soils. India requires not only food security but also nutritional security. Indian soils are starving for want of micronutrients. The use of organic manures is the only possible solution to enhance micronutrient supply to crops. At the same time it is the need of the hour to protect our environment from hazardous chemicals, global warming etc. Organic farming principles and practices will certainly deliver the required goods to the society.

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Chapter 5
Utilization of Psychrotolerant Phosphate Solubilizing Fungi Under Low Temperature Conditions of the Mountain Ecosystem

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Abstract The use of bioinoculants, in recent times, has received greater attention largely due to increased preference for the natural "organic" products across the globe, as well as to reduce the load of synthetics. One of the prerequisites for developing a microbe-based technology is proper understanding of the diversity of microorganisms in any given ecosystem with particular reference to their function and efficiency. Microorganisms play a fundamental role in the biogeochemical cycling of phosphorus in the natural ecosystems. Temperature, pH and biomass are important factors for such microbial activities. Since phosphate solubilization, and thereby making it available to plants, is pivotal for growth, the critical importance of phosphate solubilizing microorganisms is self evident.

While microorganisms are ubiquitous in nature, their distribution is largely governed by environmental and edaphic specificities. Several species of fungi, belonging mainly to *Aspergillus*, *Paecilomyces* and *Penicillium*, survive and dominate in low temperature environs of the Indian Himalayan Region (IHR). Occurrence of such fungal communities is likely to mediate important ecological function in low temperature environments, generally associated with low nutrient status and low decomposition rates. The phosphate solubilization efficiency of various species of the three fungal genera, isolated from the Himalayan soils, has been investigated in some detail. The experiments were performed to examine their phosphate solubilization potential with particular reference to a number of associated factors, such as temperature, pH, biomass production, and the role of phosphatases. The findings are likely to have implications in respect of the possible applications of cold tolerant microbial communities in environment management, with reference to mountain ecosystems.

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5.1 Introduction

The Green Revolution in agriculture is rightfully considered as one of the most successful human achievements of the last century. This revolution resulted in food security and also played an important role in the economic transformation of developing countries, such as India, from being food deficient to food surplus nations. The world today needs a second green revolution so as to affect 50% increase in food production in the next 20 years to sustain the ever increasing population (Vasil 1998; Leisinger 1999). The era of green revolution is associated with the use of high yielding varieties and high inputs by way of irrigation and enhanced use of chemicals, both as fertilizers to supplement plant nutrition and as agents to provide protection against damage caused by the pathogens. Chemical fertilizers being readily available, ease of handling and predictable increase in yield have been a preferred source of nutrition for a variety of crop plants for the past several decades. There is, however, a perceptible change in favour of increasing the use of biological fertilizers largely due to enhanced awareness of the ill effects of long term and continued use of chemical fertilizers, such as the contamination of surface as well as ground water, loss of soil productivity and overall damage to ecology, including health hazards to humans and animals due to leaching of chemicals from crop field and crop residues (Pandey and Kumar 1989; Nautiyal 2000; Pandey et al. 2004).

Deficiency of phosphorus in the Indian soils is a major problem for sustained agricultural productivity. Phosphorus replenishment, particularly for farmers with small land holdings, remains a formidable challenge. While the use of soluble mineral phosphate fertilizers is an obvious means to combat phosphate deficiency, it rapidly becomes unavailable to plants, accumulating in inorganic phosphorus fractions fixed by adsorption, precipitation, and organic phosphorus fractions that are immobilized in the soil organic matter. The inorganic forms are compounds of Ca, Fe, Al and Mg. The organic phosphorus containing compounds are derived from plants and microorganisms and are composed of nucleic acids, phospholipids and phytin (Subba Rao 1977; Illmer and Schinner 1992; Illmer et al. 1995). In mountainous regions of IHR, under most farming conditions, the soil is acidic in nature and rather poor in organic matter. Water soluble phosphorus, applied in the acidic soils is rapidly fixed in to unavailable forms and accounts for the low phosphate use efficiency (Sarkar and Uppal 1994). Most of the applied P accumulates in the fine soil fractions, which are readily transported along with the surface waters through runoff, especially in the hilly regions (He et al. 1995).

Microorganisms play a fundamental role in the biogeochemical cycling of phosphorus in the natural ecosystems. The major microbiological processes, by which insoluble phosphorus compounds are mobilized, is through the production of organic acids. Several bacteria and fungi release organic acids, such as citric, gluconic, and

keto-gluconic acids, to liberate phosphates in the soil (Sperber 1957; Cunningham and Kuiack 1992; Goldstein 1995; Vassilev et al. 1997; Vazquez et al. 2000; Wahid and Mehana 2000; Hwangbo et al. 2003). Besides organic acid production release of proton accompanying respiration or NH_4^+ assimilation also contribute towards phosphate solubilizing activity (Illmer and Schinner 1992). Phosphatases and phytases are also known to play important role in phosphate solubilization through catalyzing the hydrolysis of phosphatic compounds (Michael and Robert 1984; Tarafdar and Jungk 1987).

5.2 Diversity of Phosphate Solubilizing Fungi in the Himalayan Soils

Although phosphate solubilizing microorganisms are commonly found in most soils, their establishment and performance are affected largely by the environmental factors, especially under stress conditions (Gupta et al. 1986; Tilak 1991). Characterization of the microorganisms isolated from the regions facing harsh climatic conditions of IHR, with a view to elucidate their diversity and potential applications, has started receiving attention. The findings in general indicate that these microorganisms are able to tolerate a wide range of temperature and pH that in turn help them survive under the harsh climatic conditions found at the higher altitudes. Dominance of species of *Bacillus*, *Pseudomonas* and some other pigmented bacteria has been reported at the higher altitudes. Similarly, in respect of fungal genera, dominance of *Aspergillus*, *Paecilomyces*, *Penicillium* and *Trichoderma* has been recorded from high altitude regions of IHR (Pal 1998; Pandey and Palni 1998; Pandey et al. 2004, 2006; Gulati et al. 2008). A total of 246 fungi representing 36 genera and 72 species were isolated from the soil samples collected from various forest sites, namely *Abies pindrow*, *Betula utilis*, *Cedrus deodara*, *Pinus* spp., *Quercus* spp., *Rhododendron* spp., and *Taxus baccata* covering a wide altitudinal range (1,800–3,610 m above mean sea level) in the IHR, representing regions of temperate and alpine climatic conditions. Dominance of the genus *Penicillium* was reportedly more prominent in samples taken from forests with increasing altitude (Pandey and Palni 2007; Pandey et al. 2008). Diversity of phosphate solubilizing fungi isolated from the Himalayan soils and their accession numbers, assigned by the National or International Culture Collections in India, has been presented in Table 5.1 and Fig. 5.1.

5.3 Tolerance of Phosphate Solubilizing Fungi to Extreme Temperature and pH Conditions

The phosphate solubilizing species of *Aspergillus*, *Paecilomyces* and *Penicillium* were investigated for their temperature and pH tolerance (Pandey et al. 2008; Rinu and Pandey 2010, 2011). These species exhibited tolerance to a wide range of pH,

Table 5.1 Temperature, pH and salt tolerance of fungal isolates

Fungal species	Temperature range (°C)	pH range	Salt tolerance (%)	Accession no.
Aspergillus species				
A. candidus	4.0–42.0 (21.0)	2.0–12.0 (7.0)	12.0	ARIFCC774
A. deflectus	9.0–42.0 (28.0)	3.0–12.0 (8.0)	12.0	ITCC5016
A. flavus	9.0–35.0 (28.0)	2.0–12.0 (7.0)	12.0	ARIFCC1161
A. fumigatus	9.0–42.0 (21.0)	2.0–12.0 (7.0)	15.0	ITCC3717
A. glaucus	9.0–42.0 (21.0)	2.0–12.0 (7.0)	12.0	ARIFCC771
A. nidulans	9.0–42.0 (28.0)	2.0–12.0 (7.0)	15.0	ARIFCC772
A. niger	9.0–42.0 (28.0)	2.0–12.0 (9.0)	15.0	ITCC2546
A. parasiticus	4.0–42.0 (28.0)	2.0–12.0 (7.0)	12.0	ITCC4239
A. sydowii	9.0–42.0 (35.0)	2.0–12.0 (9.0)	15.0	ITCC4210
A. wentii	9.0–42.0 (28.0)	2.0–12.0 (8.0)	12.0	ARIFCC773
Penicillium species				
P.aurantio-griseum	4.0–35.0 (21.0)	3.0–12.0 (5.0)	20.0	ITCC4394
P. citrinum	9.0–50.0 (28.0)	3.0–12.0 (9.0)	20.0	ITCC4212
P. janthinellum	9.0–50.0 (28.0)	3.0–12.0 (5.0)	15.0	ITCC4242
P. oxalicum	4.0–35.0 (21.0)	3.0–12.0 (6.0)	15.0	ITCC3891
P. pinetorum	9.0–42.0 (28.0)	3.0–12.0 (6.0)	15.0	ITCC3893
P. pinophilum	4.0–35.0 (28.0)	3.0–12.0 (6.0)	15.0	ITCC5354
P. purpurogenum	9.0–50.0 (28.0)	2.0–12.0 (6.0)	05.0	ITCC3684
P. raistrickii	4.0–35.0 (21.0)	1.5–11.0 (6.0)	15.0	ITCC4243
Paecilomyces species				
P. hepiali	4.0–35.0 (21.0)	2.0–13.5 (9.0)	12.0	MTCC9621
P. liliacinus	4.0–35.0 (21.0)	3.0–13.0 (9.0)	15.0	ITCC2557
P. variotti	4.0–35.0 (21.0)	3.0–12.0 (8.0)	12.5	ITCC5719

Values in parenthesis indicate optimal temperature and pH for growth
Temperature, pH and salt tolerance was done in potato dextrose agar/broth medium at 21°C
ARIFCC Agharkar Research Institute Fungal Culture Collection, Pune, India; *ITCC* Indian Type Culture Collection, Indian Agricultural Research Institute, New Delhi, India; *MTCC* Microbial Type Culture Collection, Chandigarh, India

between 1.5 and 13.5. The best tolerance to lowest pH (1.5) was exhibited by a species of *Penicillium*, i.e., *P. raistrickii*. Most species of *Aspergillus* tolerated pH as low as 2. The tolerance to highest pH was exhibited by a species of *Paecilomyces* (*P. hepiali*; 13.5 followed by *P. liliacinus*; 13.0). The fungal species also exhibited tolerance to a wide range of temperature, between 4–9°C (minimum) and 42–50°C (maximum). Three *Penicillium* spp., viz. *P. citrinum*, *P. janthinellum*, and *P. purpurogenum* tolerated temperatures up to 50°C, while none of the species of *Aspergillus* could tolerate temperatures above 42°C. The species of *Paecilomyces* showed less tolerance to thermophilic range of temperature; they could tolerate temperatures up to 35°C. The range of pH and temperature tolerance of these phosphate solubilizing fungal species, along with the optimal values, are presented in Table 5.1. These findings are indicative of the presence of fungal species possessing ability to tolerate and survive wide range of abiotic conditions. Such species can, therefore, be referred as pH and temperature tolerant, rather than true acidophiles, alkaliphiles or psychrophiles.

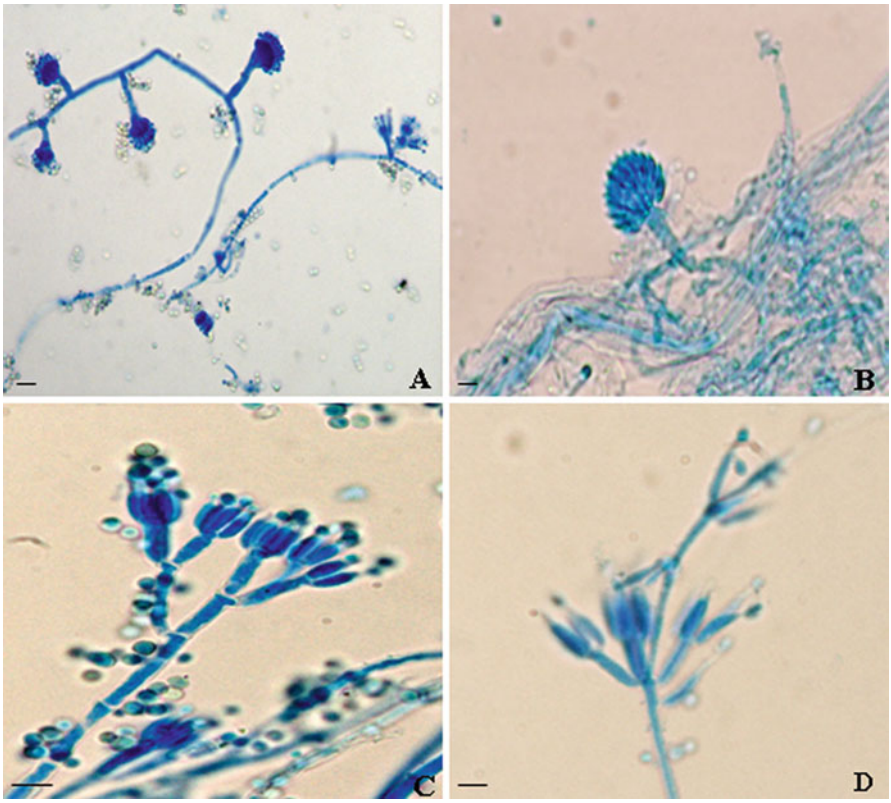

Fig. 5.1 Microscopic features of selected phosphate solubilizing fungi: (**a**) *Aspergillus candidus*, (**b**) *Aspergillus wentii*, (**c**) *Penicillium aurantiogresium*, (**d**) *Paecilomyces hepiali*; bar = 3 μm

5.4 Temperature Dependent Phosphate Solubilization by Psychrotolerant Fungi

Phosphate solubilization efficiency of fungal species was examined at different temperatures (viz. 4°C, 9°C, 14°C, 21°C and 28°C), following a longer period of incubation time. also interesting that the pigments were not secreted at the lower temperature (9°C), Qualitative estimations, performed on Pikovskaya's agar plates, indicated that most fungal cultures formed very little and some times no clear zones. Contrary to this, the same cultures were found to be efficient solubilizers when Pikovskaya's broth was used (Rinu and Pandey 2010). Figure 5.2a–c show the zones of solubilization by the representative fungal species on Pikovskaya's agar plates at 21°C. Some cultures (e.g., *A. flavus* and *A. wentii*) secreted pigments in the agar medium around the colony at 14°C, 21°C and 28°C, thus adversely affecting the visibility of the zone of solubilization. It was allowing the full visibility of the zone of solubilization around the fungal colony. The production of large amounts of a

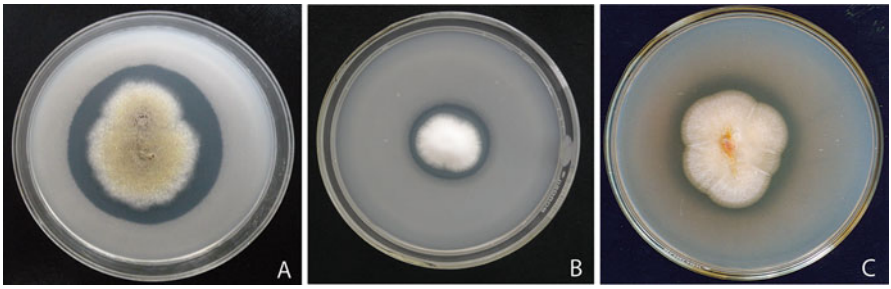

Fig. 5.2 Zone of solubilization on Pikovskaya's agar plates by different fungi (**a**) *Aspergillus niger*, (**b**) *Paecilomyces hepiali*, (**c**) *Penicillium raistrickii*

thick polysaccharide-like compound in liquid culture resulting in lower P levels has been reported by Wakelin et al. (2004). On the contrary, in flasks, where the production of polysaccharide was minimal, P level in the solution was recorded to be much higher. These observations indicate that the physiological status of fungal cultures plays an important role in the P levels during the process of solubilization.

The efficiency of phosphate solubilization of various fungal species was found to be temperature dependent. The level of soluble P in the solution cultures was found to fluctuate over time. Estimation of P levels at different time intervals, over the entire duration of experiments is, therefore, preferable. The importance of this parameter has been previously reported in case of *Penicillium radicum*, and some other phosphate solubilizing microorganisms (Illmer and Schinner 1992; Whitelaw et al. 1999; Wakelin et al. 2004), which is in tune with the findings of the present study. The slow growth and persistence of phosphate solubilizing efficiency at low temperatures render these organisms beneficial under ecological conditions of the low temperature environments.

A. sydowii and *P. hepiali* solubilized maximum tricalcium phosphate (TCP) at 14°C, while the maximum biomass production by these fungal isolates was found to be at 21°C. Five species of *Aspergillus* viz. *A. deflectus*, *A. flavus*, *A. nidulans*, *A. parasiticus* and *A. wentii* solubilized maximum TCP at 28°C, and their maximum biomass production was recorded at 21°C. This could be explained by the fact that the suboptimal conditions for the growth and biomass production may become optimal for the production of metabolites (Rinu and Pandey 2010, 2011). Similarly, higher P solubilizing activity has been reported from medium containing suboptimal carbon concentration, as compared to the medium containing optimum requirements of the microbes (Illmer and Schinner 1992). Figure 5.3 shows a comparison of the optimal temperatures for phosphate solubilization and biomass production by three representative fungal species. Generally, the best phosphate solubilization was recorded when the biomass production values were well below maximum.

Phosphate solubilization by fungal species of tropical origin, mainly belonging to the genus *Aspergillus* and *Penicillium*, has been reported by several workers.

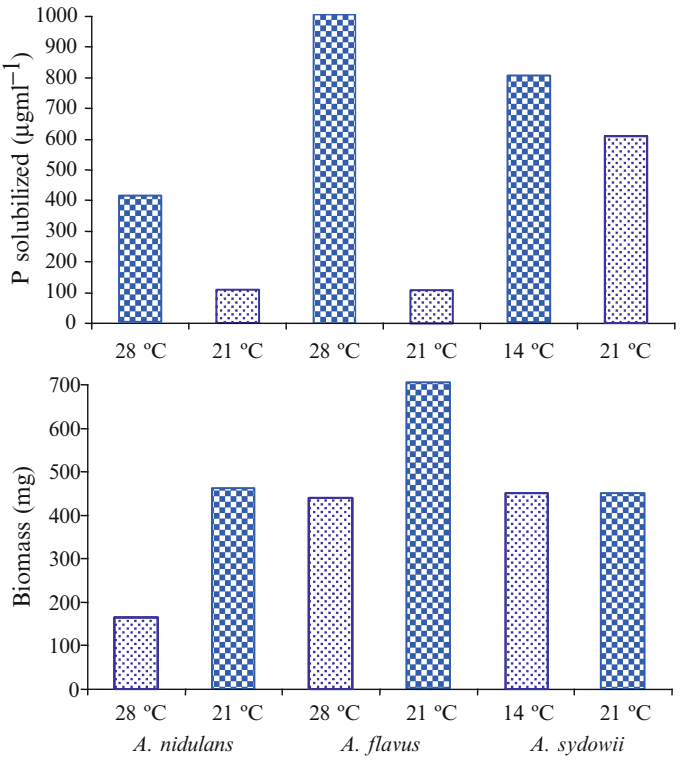

Fig. 5.3 Phosphate solubilization of selected species of *Aspergillus* at optimal (▩) and suboptimal (⁘) growth temperatures. Biomass was calculated by dry weight basis

These studies have reported maximum phosphate solubilization during the second week, followed by a decline or fluctuation in phosphate solubilization on further incubation (Vassileva et al. 1998; Goenadi et al. 2000; Wakelin et al. 2004). Pandey et al. (2008) have found maximum phosphate solubilization during the 3rd week of incubation at 21°C by eight species of *Penicillium*; the persistence of activity was found up to 42 days of incubation further at lower temperatures for some of the fungal species (Rinu and Pandey 2010, 2011). The solubilized TCP was found to be at par with the maximum solubilization occurred at 21°C after second and third week of incubation, at 14°C and 9°C, and showed persistence of the activity up to day 42 (Rinu and Pandey 2011). *Paecilomyces lilacinus* and *P. variotii* also exhibited phosphate solubilization for a longer period of incubation (42 days at 14°C). In a recent study, Gupta et al. (2007) have reported the phosphate solubilization efficiency of *Aspergillus* and *Penicillium* spp., isolated from the heavy metal mines of Orissa (India), where the average temperature ranged between 32°C and 40°C. These fungi preferred the pH range of 7.0–9.0 for maximum phosphate solubilization, and the same was found on day 12 of incubation.

5.5 Production of Organic Acids by Psychrotolerant Fungi

The phosphate solubilization by a variety of microorganisms is caused by lowering the pH of the medium, either by H^+ extrusion (Illmer et al. 1995) or by the secretion of organic acids, such as, citric acid, gluconic acid, oxalic acid, and malic acid, etc., and by way of chelating metabolites (Asea et al. 1988; Salih et al. 1989; Gaur 1990; Cunningham and Kuiack 1992; Narsian et al. 1995). The principle underlying the mechanism of action of chelators is through formation of unionized association compounds with Ca^{++}, Fe^{++}, Al^{+++}, thus increasing the soluble phosphate concentration by scavenging phosphate from mineral phosphates. The ability of low molecular weight organic acids to release P from ores or rocks is related to their ability to form stable metal complexes (Mattey 1992).

Decrease in pH was recorded throughout, in all the present experiments. In case of ten species of *Aspergillus*, maximum decrease in pH was recorded at 21°C and 28°C, resulting in maximum phosphate solubilization. In case of *A. candidus*, *A. fumigatus* and *A. parasiticus*, the decrease in pH was less at 14°C; this was reflected in terms of the phosphate solubilization as well. The decrease in pH of the medium was less at 14°C or 9°C, indicating lesser production of organic acids, relatively at lower temperatures (Rinu and Pandey 2010). At the higher temperatures (21°C or 28°C), after reaching the maximum decrease in pH during 2–4 weeks of incubation the pH began to increase without increase in phosphate solubilization, which may be due to cell death and/or lysis. However, in case of *A. niger* (at 21°C and 14°C) and *A. glaucus* (at 28°C), the P concentration in the medium also increased, probably due to cell lysis and P liberation in the culture suspension (Illmer and Schinner 1992; Rinu and Pandey 2010). In case of *Penicillium* species similar observations were recorded at 21°C. The initial increase in the P concentration is on account of acid production and the later by altered metabolism due to lack of C in the medium, and may result in the formation of an organo-P compound. Consequently, with alteration in the medium composition, the cells may utilize this compound as a source of energy, resulting in subsequent release of P (Illmer and Schinner 1992). The decrease in pH was comparatively less at 14°C and almost negligible at 9°C in case of *Aspergillus* spp., except *A. nidulans* and *A. sydowii*. These two species exhibited maximum phosphate solubilization at 14°C. Minimal activity was also seen in case of *A. niger* at 9°C, indicating towards the involvement of some other mechanism. In general, production of organic acids has been considered as the main reason responsible for the solubilization of phosphate in most studies conducted in respect of mesophilic temperature range (Illmer et al. 1995; Omar 1998; Whitelaw et al. 1999; Reyes et al. 2001). *P. hepiali* (Rinu and Pandey 2011), *P. liliacinus*, and *P. variotti* was also able to reduce the pH of culture medium to acidic conditions resulting in considerable solubilization of TCP.

The High Performance Thin Layer Chromatography (HPTLC) analyses of organic acids showed the involvement of α-keto glutaric acid, citric acid, gluconic acid, malic acid, oxalic acid and succinic acid (Fig. 5.4). Along with these acids some unidentified acids were also produced by *A. niger* (Rf = 0.92), *A. candidus* and *A. sydowii* (Rf = 0.51), *P. hepiali* (Rf = 0.45), and *P. variotti* (Rf = 0.45). The % P solubilized and the total acid concentration as well as total number of acids produced by

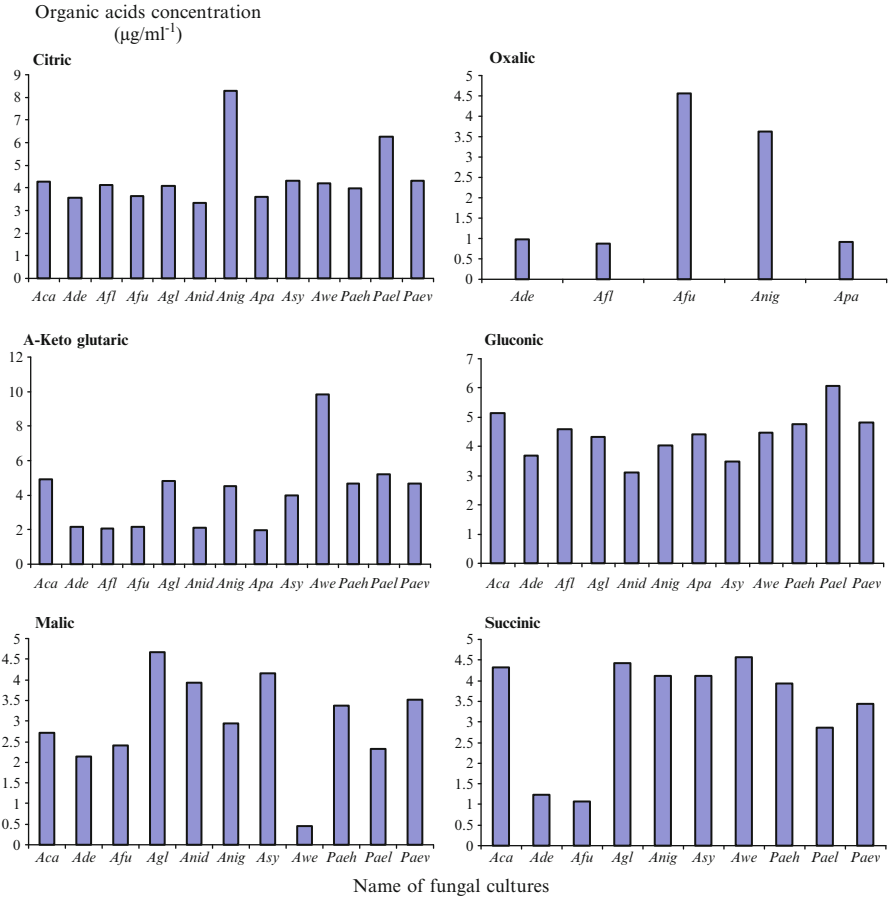

Fig. 5.4 Concentration of organic acids produced by fungal isolates following 7 days of incubation in static Pikovskaya's broth culture at 21°C after 7 days of incubation (Aca *Aspergillus canduds*, Ade *A. deflectus*, Afl *A. flavus*, Afu *A. fumigatus,* Agl *A. glucus*, Anid *A. nidulans*, Anig *A. niger*, Apa *A. parasiticus*, Asy *A. sydowii*, Awe *A. wentii*, Paeh *Paecilomyces hepiali*, Pael *P. liliacinus*, Paev *P. variotti*)

any fungal culture showed insignificant correlation. These results suggest that while the production of organic acids is an important factor in phosphate solubilization some other mechanism(s) is also likely to be responsible for phosphate solubilization (Table 5.2).

5.6 Role of Posphatases in Phosphate Solubilization

Mineralization of many of the organophosphorous compounds that account for up to 30–50% of the total phosphorus in most soils is carried out enzymatically by phosphatases, primarily acid phosphatases. These catalyze dephosphorylating reactions

Table 5.2 Summary of phosphate solubilization, number of acids produced and the total concentration of acids produced by the fungal isolates

Fungal species	% P solubilized	Number of acids produced	Total conc. of acids produced ($\mu g ml^{-1}$)
Aspergillus			
A. candidus	7.48	6	21.39
A. deflectus	9.68	6	13.74
A. flavus	18.28	4	11.65
A. fumigatus	10.60	5	13.84
A. glaucus	15.46	5	22.28
A. nidulans	8.52	4	12.45
A. niger	47.08	7	27.50
A. parasiticus	7.88	4	10.92
A. sydowii	14.60	6	20.05
A. wentii	9.24	5	23.53
Paecilomyces			
P. hepiali	3.28	6	20.74
P. liliacinus	5.40	5	22.75
P. variotii	6.24	6	20.73

involving the hydrolysis of phosphoester or phosphoanhydride bonds (Richardson et al. 2000; Tarafdar et al. 2003). Phosphate solubilizing microorganisms are known to produce phosphatases which are hydrolytic enzymes responsible for the breakdown of insoluble phosphate containing compounds. The experiments conducted in this regard resulted in the production of intracellular enzymes in most of the species examined, while extracellular phosphatase activity was detected only in *A. niger* and three species of *Paecilomyces*. The absence of extracellular phosphatase activity may be due to the presence of insoluble phosphorus in the suspension culture (the experiments were conducted in Pikovskaya's medium containing 0.5% TCP). The effect of sublethal concentrations of insoluble phosphate on the activity of phosphatases has been reported by Ramalingam and Prasanna (2006). Tarafdar et al. (2003) reported the production of extracellular phosphatases in Czapek Dox broth (lacking insoluble phosphorus). The presence of adequate levels of solubilized phosphorus in the medium, as a result of fungal activity, may also lead to a feedback inhibition. This was also evident from the results of extracellular acidic and alkaline phosphatase activity in PD broth (Rinu and Pandey 2010). Phosphatases are known to be activated when the phosphorus availability is low (Shieh et al. 1969; Micheva-Viteva et al. 2000; Aleksieva et al. 2003). However, this was not in tune with the results obtained with *A. niger,* P. *hepiali*, *P. liliacinus* and *P. variotii*. Braibant and Content (2001) have also reported that the expression of phosphatase in *Mycobacterium bovis* is not regulated by the environmental inorganic phosphate concentration.

Intracellular phosphatases are also well known for the mineralization of phosphates (Gaur 1990). Higher intracellular acidic (rather than alkaline) phosphatase activity is probably due to acidic conditions in the medium. The intracellular acidic and alkaline phosphatase activity was found to be considerably higher in all the cultures. These results support the findings of Tarafdar et al. (2003) who reported, on an

average, 1.7 times higher intracellular acid phosphatase activity over the extracellular acid phosphatase secretion. The minimal extracellular alkaline phosphatase activity of *A. niger*, in comparison to much greater extracellular acidic phosphatase activity, could be explained in relation to the observed maximum acidification of the culture medium. The acidic and alkaline phosphatases are classified due to their optimal activities in the acidic or alkaline ranges, respectively.

5.7 Psychrotolerant Microbes Vis-à-vis Environment Management

The importance of microorganisms in respect of their biotechnological applications, in agriculture, industry and medicine is well recognized. Documentation of biodiversity at the global level and the importance of conservation of the biological gene pool are receiving much needed attention in the recent times (Satyanarayana et al. 2005; Pandey et al. 2006, 2010). An important document, on the initiative of the Ministry of Environment and Forests on National Biodiversity Strategy and Action Plan (NBSAP) has been prepared. In this document Johri and co-workers (2005) have summarized the objectives of the proposed Microorganism Diversity: Strategy and Action Plan. These objectives are based on the existing knowledge of microbial diversity recorded in the country, documentation of microbial processes developed for various applications, identification of gaps, limitations and problems in demonstrating microbial diversity, and drafting an Action Plan to improve the existing scenario.

The IHR is characterized by the presence of extreme environments, and thereby hosts a unique biodiversity. The extraordinary diversity of microorganisms is based on their remarkable metabolic plasticity and genetic adaptability resulting in the production of unique, often novel biologicals. The microbial communities, referred as extremophiles, have evolved to colonize such extreme environments that are too harsh for the animals and plants to survive. With a view to address the unique features of the hill states of IHR, a special Task Force was constituted by the Planning Commission, Govt. of India. The main terms of reference of this Task Force were "To look into problems of hill states and hill areas and to suggest ways to ensure that these states and areas do not suffer in any way because of their peculiarities". The Task Force proposed 12 recommendations in respect of the mountain states of India, including a note on organic farming (Anonymous 2010a). It further suggested for the ecofriendly packaging support for branding and selling along with suitable institutional arrangements for the promotion and marketing of the organic products from the mountains. Organic fertilizers namely city-based compost, vermin compost, and biofertilizers (particularly *Rhizobium*, *Azotobacter*, *Azospirillum* and phosphate solubilizing microorganisms), have been recognized and incorporated in Fertilizer Control Order (FCO) 1985 promulgated under the Sect. 3 of the Essential Commodity Act 1955 (Anonymous 2010b). The concept of increasing the use of biological fertilizers has been particularly appreciated by the mountain states, and also received a mention in the recent guidelines (Anonymous 2009) published jointly by the

Ministry of Environment and Forests, Govt. of India and GB Pant Institute of Himalayan Environment and Development, Almora, India. The hill agriculture is generally rain fed, hence the microbe based formulations (as organic fertilizer) are expected to be of high impact in times to come. In this context, availability of location specific microbial inoculants, suitable for field applications is essential. The Department of Biotechnology, Govt. of India, has also identified "Biofertlizers" as a distinct area for financial support to the relevant research projects, and emphasised the importance of carrier based native microbial fertilizers (Anonymous 2007).

5.8 Conclusions

Microorganisms are ubiquitous in nature and their distribution is largely governed by environmental specificities. High altitudes of IHR represent the low temperature environments, including the glaciers and cold deserts. These sites are expected to harbour a variety of microorganisms, mainly the psychrophiles. Documentation of these microorganisms along with their characterization and bioprospecting are needed to generate knowledge of both basic and applied value. GB Pant Institute of Himalayan Environment and Development, Almora, India, has taken up initiatives along these lines. A culture collection of 'high altitude microorganisms' is being maintained in the laboratory. Several species of *Aspergillus*, *Paecilomyces* and *Penicillium* have been isolated from the cold environs and are being investigated for their applied value. Microorganisms growing under extreme environments are likely to possess active or passive mechanisms for survival under such conditions. The slow and steady, but effective and prolonged activities, such as production of organic acids, enzymes, etc., have implications in the nutrient cycling under mountain ecosystems. These organisms, therefore, need focussed attention on their diversity, phylogeny, survival strategies and bioprospecting.

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Chapter 6
Antimicrobial Lipopeptides of *Bacillus*: Natural Weapons for Biocontrol of Plant Pathogens

Ankit Kumar and B.N. Johri

Abstract *Bacillus* species are ubiquitous bacteria in agricultural soils and possess many traits that make them well suited as biocontrol agents of various soil borne pathogens. They are aerobic, Gram-positive bacteria with remarkable ability to produce highly resistant endospores and are well adapted to grow in the rhizosphere. Until today, hundreds of strains of genus *Bacillu*s, including *Brevibacillus* and *Paenibacillu*s, have been identified that produce a variety of antimicrobial substances, e.g., antibiotics. *Bacillus subtilis*, a model organism of this group is known to produce over two dozen molecules with antibiotic properties and an amazing variety of structures. Lipopeptides are the most frequent antibiotic compounds produced by bacilli exhibiting antibacterial and/or antifungal actions against a spectrum of pathogenic bacteria and fungi with surfactant activities in exceptional cases. Among these, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin families have well known potential in biotechnology and bio-pharmaceutical applications. Structurally, these amphiphilic molecules share a common cyclic structure comprised of a β-amino or β-hydroxy fatty acid integrated into a peptide moiety and have been classified based on the difference in amino acid sequences and fatty acid branching. Besides, lipopeptides have also been reported to contribute to the ecological fitness of the producing strain thus helping them to adapt to their ecological niche. Numerous reports covering *in vitro* production of antibiotic molecules from different *Bacillus* spp. are available however the number reduces dramatically only to few wherein production and/or recovery of these molecules could be demonstrated successfully under natural or greenhouse conditions. The aim of this review is to summarize the recent findings of *Bacillus* lipopeptides with antibiotic properties, regulatory pathways involving their production and their role in the ecological fitness of *Bacillus*.

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Keywords *Bacillus subtilis* • Lipopeptides (LPs) • Iturin • Surfactin • Fengycin • Biocontrol • Rhizobacteria • Induced systemic resistance

6.1 Introduction

Indeed, the worldwide use of agro-chemicals has reduced the outbreak of fungal diseases, but this has also contributed to the emergence of pathogens resistant to the pesticides available in the market (Raposo et al. 2000). Furthermore, these chemicals may have toxic effects on the beneficial microflora, residing in the rhizosphere or in soil; may enter into the food chain and subsequently accumulate in the human body as a result of bio-magnification (Bartlett et al. 2002). Moreover, their excessive use in agriculture has resulted in deterioration of soil fertility. To cope with these problems, biological control agents including microorganisms or microbial products and biofertilizers have received global attention as promising alternatives to the chemical inputs (pesticides and fertilizers) (Mizumoto et al. 2006). Large number of reports are available wherein application of microorganisms as successful biocontrol agents has been extensively investigated (Raaijmakers et al. 2002; Ongena and Jacques 2008; Romero et al. 2007; Chen et al. 2009; Kim et al. 2010). Application of bacterial strains with proven ability to suppress plant pathogens seem to be the best choice as biocontrol agents, due to their versatile nature and physiological diversity coupled with the possibility of combined application together with other control measures (Shoda 2000; Kondoh et al. 2001; Fogliano et al. 2002; Omar et al. 2005; Baehler et al. 2006; Cazorla et al. 2006; Nofal and Haggag 2006). Species of *Bacillus* are ubiquitous with proven excellent colonization aptitude along with plant growth promoting activities, dynamic lifestyle with sporulation ability and versatile mechanisms to antagonize the pathogens effectively (Shoda 2000; Kloepper et al. 2004; Romero et al. 2004, 2007; Schallmey et al. 2004). *Bacillus* species are often considered as microbial factories owing to the production of a diverse array of bioactive compounds some of which are potentially lethal against phytopathogens (reviewed in Emmert and Handelsman 1999; Stein 2005; Ongena and Jacques 2008).

6.2 Bacilli as Producers of Lipopeptides

Hundreds of different peptide antibiotics have been described during the past 50 years (Hancock et al. 1995; Hancock and Chapple 1999; Stein 2005). They can be categorized into two classes, non-ribosomally synthesized peptides (NRPs) such as bacitracins, polymyxins, glycopeptides etc., and ribosomally synthesized peptides such as subtilin, subtilosin A, TasA and sublancin. While NRPs are largely synthesized and modified by bacteria, ribosomally synthesized peptides are produced by almost all the living species as components of natural defense mechanisms.

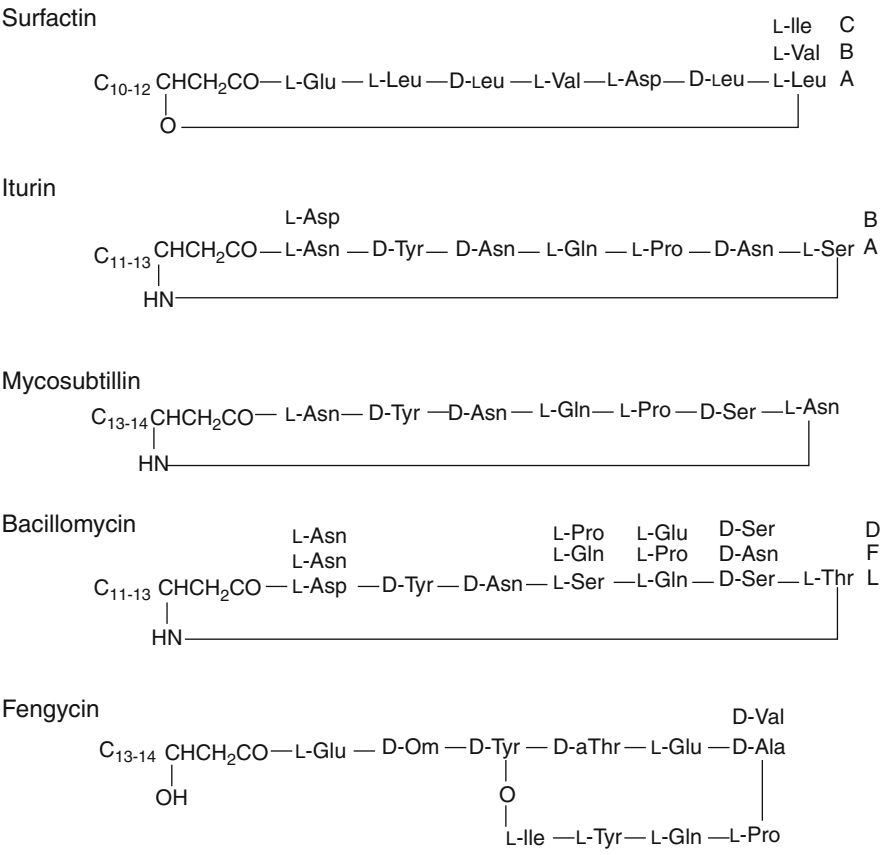

Fig. 6.1 Structure of some important representative molecules from the three families of lipopeptides/biosurfactants synthesized by different *Bacillus* spp. (Vater et al. 2002)

Species of *Bacillus* synthesize a large number of lipopeptides but a major fraction of them in case of *B. subtilis* has been found to be produced in a non-ribosomal manner via multi-domain enzymes (Non-ribosomal peptide synthetases, NRPSs and polyketide synthetases, PKSs) and most of the antifungal peptides have a molecular weight of less than 2,000 Da (Finking and Marahiel 2004). Among these, cyclic lipopeptides (LPs) of the surfactin, iturin and fengycin (or plipastatin) families have well known potential in biotechnology and bio-pharmaceutical applications due to their surfactant properties (Romero et al. 2007; Chen et al. 2009). Structurally, these amphiphilic molecules share a common cyclic structure comprised of a β-amino or β-hydroxy fatty acid integrated into a peptide moiety and have been classified based on the difference in amino acid sequences and fatty acid branching (Fig. 6.1). The potential of *Bacillus* species to synthesize a wide variety of metabolites with antibacterial and/or antifungal activity has been intensively exploited in medicine and industry and is thus one determinant of their ability to combat plant pathogens when applied as biocontrol agents.

6.3 Biosynthesis of Non-ribosomal Peptides in *Bacillus subtilis*

In spite of the structural heterogeneity, the non-ribosomal peptide antibiotics share a common mechanism of biosynthesis, the multicarrier thiotemplate mechanism (Stein et al. 1996). The biosynthesis of non-ribosomal peptide antibiotics is prevalent in bacteria and fungi, and is catalyzed by the modularly arranged multi-domain enzyme complexes, the NRPSs (Sieber and Marahiel 2003; Finking and Marahiel 2004; Walsh 2004; Stein 2005). Three basic domains are known to be involved in non-ribosomal peptide biosynthesis (Fig. 6.2), (i) A domain (adenylation domain), that selects its cognate amino acid leading to formation of aminoacyl adenylation in a way similar to the aminoacylation of tRNA synthetases in ribosomal peptide biosynthesis, (ii) PCP domain (peptidyl carrier domain) which is operational with a 4′-phosphopantetheine (PPant) prosthetic group to which the adenylated amino acid substrate is transferred and bound by a thioester bond and, (iii) The condensation domain 'C' which catalyzes the formation of a new peptide bond. The linear organization of such core units (1–3) ensures the coordinated elongation of the peptide product. Mostly, biosynthesis of NRPSs stops by macrocyclization of the peptide product, wherein parts of the molecule distant in the constructed linear peptide chain are covalently linked to one another (Kohli and Walsh 2003). A detailed account of peptide biosynthesis has been reviewed earlier (Stein et al. 1996).

6.4 Antimicrobial Lipopeptides of *Bacillus* sp.

6.4.1 The Iturin Family

The iturin family, encompassing iturin A & C, bacillomycin D, F, L, & LC, and mycosubtilin are the seven main variants within iturin family. These are heptapeptide molecules with a β-amino fatty acid chain, comprised of 14–17 carbons and exhibit strong antifungal activity against a wide range of yeast and fungi (Duitman et al. 1999; Tsuge et al. 2001; Moyne et al. 2004; Ongena and Jacques 2008). However, the antibacterial action of iturin compounds is limited with no antiviral activity (Moyne et al. 2001; Hiradate et al. 2002; Yu et al. 2002). Molecular studies of these molecules shows that peptide portion contains a tyrosine amino acid residue in D-configuration at second amino acid position and two additional amino acids at positions 3 and 6 (Chen et al. 2009). It is perhaps for this reason-the occurrence of unusual D-amino acids, that iturins are resistant to the action of proteases and peptidases (Lebbadi et al. 1994).

The mode of action of iturin group molecules includes disruption of plasma membrane by forming small vesicles and by aggregating membrane-spanning particles. They also release electrolytes, high molecular mass products and degrade phospholipids. Numerous studies have shown that members of iturin family are potential alternative to antifungal agents. For example, the antifungal property of *B.*

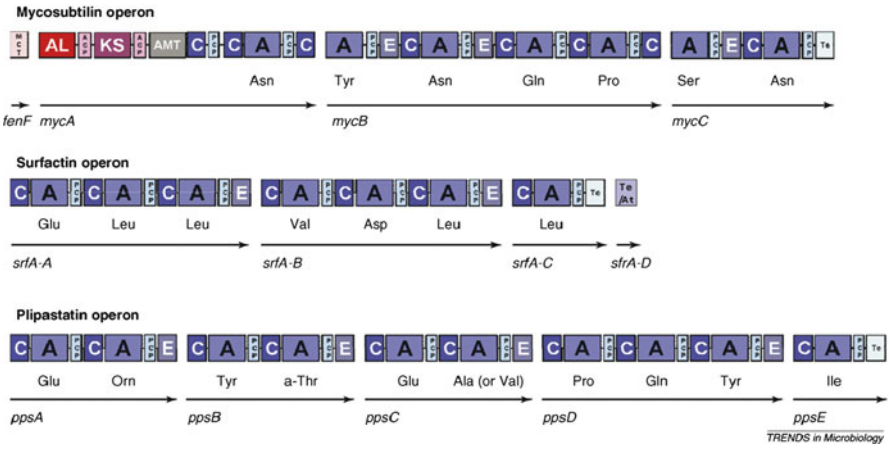

Fig. 6.2 The operons of mycosubtilin, surfactin and plipastatin synthetases in *Bacillus* species. Schematic illustration of operons (ORFs, domains of NRPSs or PKSs and amino acids incorporated by different modules) that encodes the catalytic machinery responsible for the biosynthesis of representative members of each family of lipopeptides produced by *Bacillus subtilis*: mycosubtilin for the iturin family, plipastatin for the fengycin family and surfactin (Ongena and Jacques 2008)

amyloliquefaciens strain B94 which suppressed *Rhizoctonia solani* and other fungal plant pathogens was due to iturin production and, isomers of iturin A purified from culture broth were found to be responsible for inhibition of *R. solani in vitro* (Yu et al. 2002). Similarly, another study documented the production of bacillomycin D, a variant of iturin group of molecules, by *B. amyloliquefaciens* strain A_1Z as principle inhibitory component against soil borne fungal pathogen, *Sclerotinian sclerotiorum* (Kumar et al. unpublished). Previously, chromatographic (HPLC) and mass spectrometric (MALDI-TOF) studies revealed the presence of iturin A molecule in the crude mixture, however further purification and high resolution electrospray ionization mass spectrometry (HR-ESI-MS) clearly showed bacillomycin D as a major antagonistic component.

Available reports suggest that iturin production is more common among strains of *B. subtilis* and *B. amyloliqufaciens*, however the trait does not seem exclusive to these two species as several other species are also iturin producing (Athukorala et al. 2009). Nevertheless, *B. subtilis* strains appear to be the most promising candidates for iturin production. An average of 4–5% of the total genome is devoted to the biosynthesis of antibiotics in rhizobacterium *B. subtilis*, one of the most widely studied organisms, with a potential to produce more than two dozen structurally diverse antimicrobial compounds (Stein 2005). While biosynthesis of surfactin or fengycin is mediated through NRPSs, biosynthesis of iturin or its derivatives involves PKS-NRPS hybrid complex (Tsuge et al. 2001; Moyne et al. 2004).

Bacillomycin is another member of iturin family produced by bacilli, with strong antifungal spectrum (Ramarathnam et al. 2007; Chen et al. 2009). The antifungal activity of plant growth promoting rhizobacterium *B. amyloliquefacines* FZB42 has

been attributed mainly to bacillomycin D production and this has been shown to suppress the plant pathogenic fungus *Fusarium oxysporum* (Koumoutsi et al. 2004). The *bmy* gene cluster (37.2 kb) which directs the synthesis of bacillomycin in FZB42, is an insertion within the genome and is comprised of four genes *bmy*D, *bmy*A, *bmy*B, and *bmy*C (Fig. 6.2). Molecular studies have revealed that the *bmy* gene cluster is separated by just 25 kb from the neighbouring fengycin gene cluster (Chen et al. 2009). Expression of bmy operon depends upon a σA-dependent promotor, Pbmy and is favoured in its natural host by the small regulatory protein DegQ. The global regulator DegU and ComA are required for the full transcriptional activation of bmy. Moreover, a trans-membrane protein with unknown function, Ycz E, functions at a later stage of gene expression and exerts post-transcriptional effects with DegQ (Koumoutsi et al. 2007). The first ORF of the bmy operon i.e., *bmy*D encodes for an enzyme malonyl coenzymeA transacetylase which participates in fatty acid synthesis; the enzyme has been shown to be indispensable for iturin production (Tsuge et al. 2001).

Mycosubtilin is another variant of iturin family and is produced by strains of *B. subtilis* (Leclere et al. 2005). Genetically, mycosubtilin operon consists of four open reading frames, designated as *fenF*, *mycA*, *mycB*, and *mycC*, all controlled by the same promoter P_{myc} (Fig. 6.2) (Duitman et al. 1999). The subunits encoded by the three *myc* genes contain seven modules essential for the synthesis of peptide moiety of mycosubtilin. Overproduction of mycosubtilin by a recombinant *B. subtilis* strain BBG100 has been found to show significant antagonistic properties against various fungal pathogens, *Botrytis cinerea, Fusarium oxysporum* and *Pythium aphanidermatum*, and yeasts, *Pichia pastoris* and *Saccharomyces cerevisiae* (Leclere et al. 2005). The strain BBG100 was derived from *B. subtilis* ATCC6633 and showed a 15-fold higher mycosubtilin production over the parental strain. Experimental results showed that pre-treatment of tomato seeds with vegetative cells of the mycosubtilin overproducing strain prior to planting in soil infected with *Pythium aphanidermatum* led to increased seed germination in comparison to the treatment with wild-type strain ATCC6633. Intriguingly, mycosubtilin is also believed to be involved in spreading of cells in *Bacillus* colonies and overproduction of mycosubtilin has been reported directly relevant to the enhanced invasive behaviour of *B. subtilis* cells (Leclere et al. 2006). Interesting evidence came from the findings of Julkowska et al. (2004) wherein addition of purified lipopeptide to the medium resulted in an enhancement of swarming motility of *B. subtilis* strain 168, which otherwise is a non-spreading strain. These observations clearly indicate that certain lipopeptides such as mycosubtilin not only act as antibiotic, but also play an important role in swarming/mobility behaviour, thus contributing to the ecological fitness of the producer strain.

6.4.2 The Surfactin Family

Surfactin is the most studied family of lipopeptides. Chemically, surfactins are heptapeptides with an LLDLLDL chiral sequence linked by a β-hydroxy fatty acid

(comprised of 13–15 'C' atoms) to form a cyclic lactone ring structure. Surfactin is synthesized by three NRPSs, SrfA-C and the enzyme thioesterase/acyltransferase wherein SrfD is known to initiate the process (Peypoux et al. 1999; Steller et al. 2004). The mechanism of regulation of surfactin biosynthesis is closely connected with the competence development pathway (Marahiel et al. 1993; Hamoen et al. 2003). These are probably the most powerful biosurfactants described with exceptional emulsifying and foaming properties; just 20 μM solution lowers the surface tension of water from 72 to 27 mN m^{-1} and are believed to act like a detergent on biological membranes (Carrillo et al. 2003; Pagadoy et al. 2005). Due to their amphiphilic nature surfactin molecules rapidly integrate into the lipid bilayers very tightly and interfere with the membrane integrity in a dose-dependent manner. Studies have shown that at low conc. surfactin molecules insert in the outer layer of the membrane only, thereby inducing limited perturbation. At intermediate conc. they induce reversible transient permeabilization however higher conc. results in irreversible pore formation in the membrane due to integration of surfactin rich clusters in the membrane. Addition of more surfactins leads to complete disruption of lipid bilayers resulting in formation of mixed micelles (Carrillo et al. 2003; Heerklotz and Seelig 2007). However, cholesterol has been shown to counteract the destabilizing effect of surfactins indicating that variations observed in the susceptibility of biological membranes towards surfactants might depend upon the sterol content in the membrane of target organism (Carrillo et al. 2003).

Though, surfactin is required for formation of aerial structures on the colony surfaces of *B. subtilis*, its production is correlated with the inhibition in growth and development of aerial hyphae/sporulation in co-cultivated *Streptomyces coelicolor* (Straight et al. 2006). Chen et al. (2009) proclaimed that surfactin synthesized by *B. amyloliquefaciens* FZB42 not only protects it against other bacteria but also enables it to form biofilms, thus equipping the bacterium with powerful antagonistic advantage during surface colonization. Surprisingly it has been found so, as mutants of *B. amyloliquefaciens* deficient in surfactin biosynthesis were found severely impaired in biofilm formation (cited in Chen et al. 2009). Similarly, Bais et al. (2004) reported the protective action of surfactins under *in situ* conditions and successfully demonstrated that surfactin was essential for root colonization and reduction in severity of disease caused by *Pseudomonas syringae* on *Arabidopsis* plants. The disease control was found to be associated with the inhibitory quantities of surfactin produced at the root level. However, it remained unclear whether reduction in disease incidence was due to the direct bactericidal activity or indirectly as a result of inhibition of adherence of pathogenic cells to the root surface and subsequent inhibition of biofilm growth. Intriguingly, surfactins have also been reported to inhibit biofilm formation in other bacteria and some pathogens (Mireles et al. 2001; Bais et al. 2004).

From a clinical perspective, some of the biosurfactants possess antibiotic activity, and that at least one biosurfactant, rhamnolipid produced by *Pseudomonas aeruginosa*, plays an important role in pathogenesis of this opportunistic pathogen (Vollenbroich et al. 1997; Singh et al. 2000; Bodour and Maier 2002). Many groups have shown that biosurfactants are important for microbial growth and survival in the environment, for example, surfactin is necessary for fruiting body formation by

B. subtilis and rhamnolipid is essential for normal biofilm development in *P. aeruginosa* (Branda et al. 2001; Davey et al. 2003). Mohammadipour et al. (2009) isolated surfactin producing strains of *B. subtilis* from different climatic regions of Iran and evaluated their antagonistic characteristics against *Aspergillus flavus* and *Colletotrichum gloeosporioides* following biochemical and molecular techniques. Fourteen bacterial strains were found positive for surfactin production based on molecular studies and surfactin production was also confirmed by HPLC. All surfactin producing strains harboured *sfp* gene.

Biosynthesis of surfactin is a property of the members of group *Bacillus*. The naturally occurring surfactin is a mixture of molecules which differ in chain length and branching of its β-hydroxy fatty acid and amino acid sequences (Kowall et al. 1998). Surfactin prevents fibrin clot formation by inhibiting the aggregation of platelets, thus are haemolytic in nature (Lim et al. 2005). Besides, it also helps in removal of heavy metals from contaminated soil and sediments in addition to solubilization and degradation of hydrophobic compounds thus may be an effective tool in bioremediation (Mulligan 2005). Recently, Leclere et al. (2006) showed that colonization behaviour and biofilm formation of *B. subtilis* strains depend upon the kind of lipopeptides produced and the architecture of the colony, the floating ability as well as thickness of the pellicle formed at air/liquid interface which is greatly influenced by the nature of lipopeptides produced.

Biologically, surfactin appears to play an important role in colonization of surfaces and acquisition of nutrients through their surface wetting and detergent properties. Surfactins, in fact increase the wettability and decrease the surface tension of the medium. This dual activity may be considered as a synergistic effect of biocontrol agents towards phytopathogenic fungi as a result of an increase in the ability of bacteria to colonize the target surfaces together with antifungal action. Surfactins display strong antibacterial and antifungal properties, probably because of their capability of making cell membranes permeable (Heerklotz and Seelig 2007). Thus, in a way, surfactins display an array of amazing activities however the underlying mechanisms remain unclear and need to be explored further.

6.4.3 The Fengycin Family

Members of fengycin family including the related plipastatin, are cyclic lipodecapeptides containing a β-hydroxy fatty acid (saturated or unsaturated) with a side chain length of 16–19 carbon atoms. The peptide part of fengycin contains four D-amino acids and an unusual amino acid, ornithine. Structurally, fengycin A is comprised of 1 D-Ala, 1 L-Ile, 1 L-Pro, 1 D-allo-Thr, 3 L-Glx, 1 D-Tyr, 1 L-Tyr, 1 D-Orn, while in fengycin B the D-Ala is replaced by D-Val. The biosynthesis of fengycins or plipastatins is mediated through NRPSs encoded by an operon consisting of five open reading frames fenA-E (or psA-E) (Fig. 6.2) (Chen et al. 1995; Steller et al. 1999). However, unlike iturin or surfactin biosynthesis, mechanism of fengycin regulation is completely unknown. Fengycins are comparatively less haemolytic than iturins and

surfactins but are strongly antifungal in nature, particularly against filamentous fungi (Koumoutsi et al. 2004; Hofemeister et al. 2004). Although the mechanism of action of fengycins is less well understood compared to other lipopeptides but they are also believed to interact with the lipid bilayers and alter the membrane stability and permeability in a dose dependent manner (Deleu et al. 2005).

Fengycins together with iturins have been shown to be responsible for biocontrol activity of *Bacillus* species against several pathogens *in vitro* as well as *in vivo* in different plant species (Kim et al. 2004, 2010; Ongena et al. 2005; Ramarathnam et al. 2007; Romero et al. 2007). Romero et al. (2007) showed that both iturin and fengycin families were the key components of antagonistic activity of *B. subtilis* strains against *Podosphaera fusca*, a pathogen infecting melon leaves. The fungal inhibition observed was attributed to antibiotic compounds produced which inhibited the conidial germination of *P. fusca*. More importantly, *in situ* recovery of these lipopeptides from bacteria treated melon leaves, provided interesting evidence of their putative involvement in the antagonistic activity. Further studies involving site directed mutagenesis targeted to suppress the biosynthesis of different lipopeptides, confirmed the above findings as mutants impaired in genes concerned with production of iturins and fengycins were unable to produce the respective lipopeptides thus failed to exhibit biocontrol action.

Direct evidence of the role played by fengycins in disease reduction comes from a study involving *B. subtilis* S499 which produces lipopeptides very efficiently, especially different types of fengycins (Jacques et al. 1999; Ongena et al. 2005). Strain S499 displayed strong antifungal activity against *Botrytis cinerea*, a causal agent of gray mold disease on apple fruits. The culture extract enriched with lipopeptides efficiently suppressed the fungal pathogen on wounded apple and the inhibition was due to *in situ* production of fengycins in inhibitory concentration (Ongena et al. 2005).

6.5 Rhizospheric Competence and Ecological Fitness of *Bacillus*

Surface mobility is an important mechanism of bacterial colonization of new environments. Some root exudates viz., sugars, organic acids or amino acids stimulate a positive chemotactic response in bacteria such as *Bacillus* and help to move over the root surface (Somers et al. 2004). Nevertheless motility is advantageous for plant growth promotory rhizobacteria also, that helps to establish a stable relationship with the plant surface as it favours rapid and effective colonization. Considering that phytopathogenic fungi and other soil dwelling competitors of PGPR are highly motile organisms, motility seems to be a crucial pre-requisite for survival in the environment. Rapid colonization on host surface means winning competition with the antagonists inhabiting the same niche (Shapiro 1998; Kinsinger et al. 2003). Furthermore, it helps bacterial colonies to move over the root surface to reach

nutrient rich new sites. Reports are available wherein *Bacillus* lipopeptides have been shown indispensable for surface motility (Kinsinger et al. 2003).

Root colonization by bacteria is a complex process that may involve lipopeptides at different levels. Bacterial attachment and aggregation in the form of microcolonies is the basis of root colonization by rhizobacteria and such microcolonies may be considered as a type of biofilm (Lugtenberg et al. 2001). Biofilms, in this regard are viewed as highly structured multispecies communities, a prevalent form of existence of microorganisms in every ecosystem. Among the bacterial communities, species of *Bacillus* are believed to form a robust biofilm over the biotic and abiotic surfaces. Under laboratory conditions the most common form of this structure is pellicle formation, a robust form of biofilm, at air/liquid interface. Studies have shown the involvement of surfactins in the pellicle formation at air/liquid interface (Branda et al. 2001; Kinsinger et al. 2003; Hofemeister et al. 2004; Leclere et al. 2006). *B. amyloliquefaciens* FZB42, a well known PGPR strain forms robust pellicles at air/liquid interface in liquid culture without shaking. Lipopeptide surfactin together with a protein were believed to be essential for biofilm formation and swarming motility of strain FZB42 because mutants defective in surfactin biosynthesis were found impaired in biofilm formation (Chen et al. 2007). Bais et al. (2004) confirmed the role of surfactins in biofilm formation by *B. subtilis* 6051 on the roots of *Arabidopsis* plant and showed that genetically modified bacterial strain with impaired surfactin expression failed to synthesize robust biofilms and displayed reduced colonization ability. More recently, Chollet-Imbert et al. (2009) showed that culture media/constituents which enhanced the lipopeptide production in *B. subtilis* strains 21332 and 9943 resulted in increased thickness and robustness of the pellicle synthesized by these strains. Moreover, the robustness or thickness of the pellicle was found to vary with the culture medium that favoured lipopeptide production.

Even today, *Bacillus* species are believed to be less rhizospheric competent than *Pseudomonas* species therefore most research is aimed at the development of biocontrol agents based on *Pseudomonas* species. But several reports are available wherein *Bacillus* spp. have been documented as predominant members of microbiota (Mavingui et al. 1992; Milus and Rothrock 1993). This has consistently changed the viewpoint and now a days, it is believed that rhizosphere competent genotypes do occur in genus *Bacillus*. Arias et al. (1999) studied the abundance and spatiotemporal distribution of Gram positive bacteria on the phylloplane of soybean under field conditions and most bacterial isolates belonged to the genus *Bacillus*. Another study documented the root colonization potential of plant growth promotory strains *B. amyloliquefaciens* strain A_1Z and *Pseudomonas* spp. strain GRP_3 under greenhouse conditions (Kumar et al. unpublished). Strain A_1Z was isolated from soybean (*Glycine max* L.) rhizosphere and GRP_3 from rhizoplane. *Pseudomonas* spp. strain GRP_3 is a potential root colonizer known to produce rhamnolipids (biosurfactant) and siderophore and has been shown to reduce the occurrence of pre- and post-emergence damping off in chile and tomato (Sharma and Johri 2003a, b; Sharma et al. 2007). Intriguingly, strain A_1Z multiplied and colonized the plant rhizosphere more efficiently than GRP_3 (Table 6.1). As evident, the initial bacterial counts were same for both A_1Z and GRP_3 however after 2 weeks the bacterial population of

Table 6.1 Root colonization of soybean (*Glycine max* L.) by PGPR strains

	Bacterial population (CFU ml^{-1})	Rhizobacterial population (CFU g^{-1})
Strain	Inoculum (t_0)*	14 DAI*
B. amyloliquefaciens A_1Z	1.2×10^6a	2.4×10^7A
Pseudomonas spp. GRP_3	1.2×10^6a	1.4×10^7B

DAI days after inoculation
*Data represents the average of three replicates. Values with different letters (lower-case and capital) were significantly different ($p < 0.05$)

strain A_1Z was found to be considerably higher compared to GRP_3. This clearly indicates the rhizospheric competent aptitude of strain A_1Z, a necessary pre-requisite for competitive root colonization. Indeed, pseudomonads are the key players in rhizospheric microenvironment and the population of GRP_3 increased significantly ($p < 0.05$) when compared to initial population counts used as inoculum, however bacterial population of strain A_1Z was reported significantly higher than GRP_3. Although, both A_1Z and GRP_3 were isolated from the soybean plant however the two strains differed in their ecological niche and it was envisaged, that being indigenous to the soybean rhizosphere, strain A_1Z was able to catabolize the root exudates from soybean more efficiently than strain GRP_3 as nutrient source with the result the prevailing rhizoenvironment favoured the growth and multiplication of strain A_1Z. Since the microenvironment of the rhizosphere is believed to be more competitive than rhizoplane, it could be speculated that strain A_1Z had adopted better competitive aptitude than GRP_3. Both A_1Z and GRP_3 are producers of biosurfactants (bacillomycin D and surfactin and, rhamnolipids, respectively) that help in formation of biofilms and hence root colonization but what accounts for the notable root colonization potential of strain A_1Z, is yet unclear. Nevertheless, the lipopeptides as biocontrol determinants can affect the ecological fitness of *Bacillus* and given their production under natural conditions is regulated, they can possibly be engineered into competent root colonizers and effective biocontrol agents against a wide spectrum of pathogens. But this is a challenging issue that needs the applications of advanced molecular tools to resolve the complexity of nutritional requirements imposed by the host plant, understanding the dynamics of co-habiting microbial communities and the unpredictable variations of physico-chemical factors (pH, temperature, oxygen requirement and mineral content) inherent to the soil which govern the production of lipopeptides.

6.6 Lipopeptides as Inducers of Disease Resistance

Although, in current scenario commercialization of PGPR is proceeding with emphasis on *Bacillus* species rather than pseudomonads, the preponderance of research on PGPR as elicitors of plant growth or induced systemic resistance (ISR) employs pseudomonads as PGPRs (Kloepper et al. 2004). However, there are some

published reports on ISR elicitation by *Bacillus* spp. suggesting that specific strains of the species *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides* and *B. sphaericus* could act as elicitors of ISR (Choudhary and Johri 2009; Kloepper et al. 2004). Experimental studies have shown that elicitation of ISR by these strains led to a significant reduction in severity or incidence of various diseases on a diversity of hosts under greenhouse or field conditions (Kloepper et al. 2004). For example, elicitation of ISR in sugar-beet was found to be associated with increased peroxidase activity coupled with enhanced production of chitinase and β-1,3-glucanase by *B. mycoides* strain BacJ and *B. pumilus* 203-6, respectively (Bargabus et al. 2002, 2004).

Induction of plant defense system by fengycins and surfactins was shown in a study conducted on bean and tomato (Ongena et al. 2007). Mutants with overproducing lipopeptides generated from wild type *B. subtilis* 168, a strain unable to synthesize these compounds, displayed a macroscopic reduction in severity of disease on plants. The protection was attributed to the metabolic changes associated with the plant defense responses. Lipopeptides such as fengycins can interact with plant cells as bacterial determinants and mount an immune response through induction of systemic resistance. In a study on potato tubers, treatment with pure fengycins resulted in accumulation of plant phenolic compounds which either were involved in or derived from, phenylpropanoid metabolism (Ongena et al. 2005). A strong evidence for the involvement of lipopeptides as potential inducers of plant resistance comes from the work of Tran et al. (2007) on massetolide A. The purified molecules of massetolide A, a cyclic lipopeptide produced by *Pseudomonas fluorescens*, successfully elicited ISR in tomato plants against *Phytophthora infestans* and concomitantly mutants deficient in massetolide A production, failed to protect the plants.

Podile and Dube (1988) isolated *B. subtilis* strain AF1 from soils suppressive to pigeon pea (*Cajanus cajan*) wilt caused by *Fusarium udum*. This strain was reported to cause lysis of *Aspergillus niger* thereby reducing the incidence of crown rot of peanut caused by *Aspergillus* (Podile and Prakash 1996). Later on, strain AF1 was found to induce the production of enzymes, phenylalanine ammonia lyase (PAL) and peroxidase, which are associated with elicitation of ISR (Podile et al. 1995). Further studies by Sailaja et al. (1997) demonstrated that biological control of *Aspergillus niger* by strain AF1 was associated with the induction of lipoxygenase activity in pea seedlings indicating that strain AF1 elicited ISR in peanut. However, Some authors proclaimed that experimental results of Sailaja et al. (1997) did not confirm that biological control exhibited by AF1 is a result of ISR, given that a conclusive demonstration of ISR requires a spatial separation of pathogen from eliciting bacterium (Kloepper et al. 2004). Moreover, after it has been confirmed that a bacterium displays biolocontrol action against a soil/root borne pathogen, how does one prove that ISR accounts for the biological control observed? Given there is found an increase in the production of defense related compounds, should this information be considered sufficient to state that the bacterial strain elicits ISR? These questions need to be addressed among researchers and a demonstration of systemic reduction in disease incidence together with spatial separation of pathogen and the inducer could probably answer the above questions.

6.7 Factors Affecting Production of Lipopeptides

Nutrient availability is one of the major limiting factors in the rhizosphere that can greatly influence the production of biocontrol determinants by rhizobacteria (Van Rij et al. 2004; Ongena et al. 2007). Several studies have shown the effects of different substrates (carbon, nitrogen and iron source) and physiological conditions (temperature, pH and oxygenation) on lipopeptide production by *Bacillus* strains (Cosby et al. 1998; Peypoux et al. 1999; Guez et al. 2008). The type of culture medium has been reported affecting production of lipopeptides by the bacteria. In a study, *Bacillus amyloliquefaciens* strain RC-2 was incubated in different culture media: Potato sucrose (PS), Potato semisynthetic (PSS), Luria Bertani (LB), potato dextrose (PD), King's B (KB) and peptone potato dextrose (PPD) media, for production of antifungal lipopeptides (Yoshida et al. 2001). The lipopeptide production was found to be higher in PSS and PPD media. The undiluted and even diluted (eight-fold) culture filtrates from PSS and PPD were found to completely inhibit the mycelial growth of *Colletotrichum dematium*. Likewise, the undiluted culture filtrates from PS and PD also suppressed the mycelial growth but antimycotic activity was lost when filtrate was diluted (four-fold to eight-fold). Although culture supernatant from KB and LB displayed antifungal action against the screening pathogen, however the antimycotic activity was significantly lower than other media used. Moreover, increased concentration of peptone (a mixture of several kinds of amino acids) resulted in increased production of antifungal compounds, suggesting that peptone is a key nutrient for biosynthesis of antifungals by strain RC-2. This presumption was also supported by findings of Emilianus et al. (1997) who reported enhanced lipopeptide antibiotic production by *B. amyloliquefaciens* 383B using peptone as nitrogen source, but a mixture of yeast extract and peptone in a fixed proportion led to enhanced production of antibiotics. This indicates that medium composition/constituents greatly influence the production of antimycotic lipopeptides. Similarly carbon is also a vital component of living organisms and without any carbon source no antibiotic production was reported while the best carbon source for antibiotic production was found to be glucose (Emilianus et al. 1997). Although, use of galactose and lactose as carbon source resulted in increased bacterial growth however no significant increase in antibiotic production was observed. In another study, depletion of carbon source in the medium had been correlated with decreased lipopeptide production in the culture medium (Chollet-Imbert et al. 2009). Conclusively both nitrogen and carbon source are essential for biosynthesis and production of antibiotic lipopeptides.

The physical variables (pH, temperature and agitation) affecting the biological processes, indirectly influence the production of antifungal lipopeptides (Cosby et al. 1998; Jacques et al. 1999). Emilianus et al. (1997) studied the effect of physicochemical parameters on production of bioactive compound(s) from *B. amyloliquefaciens* strain 383B. A pH range between 6.0 and 7.0 and temperature 28°C were found optimum for production of antibiotics from strain 383B. Low pH (3–4) and high pH (8–9) reduced the bacterial growth significantly resulting in poor antibiotic

production while at higher temperature, a pH drop was observed coupled with reduced antibiotic production. Similarly, Vater et al. (2002) found higher biosurfactant production from *B. subtilis* C-1 at 25°C and 30°C than at 45°C. Since pH and temperature, both affect the enzyme activity, necessary for growth and production of antibiotic lipopeptides, therefore it can be inferred that pH and temperature have significant effects over production of antibiotic lipopeptides. However, different strains behave differently with respect to lipopeptide production under varied conditions. For example, Chollet-Imbert et al. (2009) studied the lipopeptide production from two strains of *B. subtilis*, viz. 21332 and 9943 under repetitive batch cultures and continuous culture conditions. While the lipopeptide productivities were very low for strain 21332 under continuous conditions, strain 9943 showed a relatively higher surfactin and better fengycin productivity.

The physiological conditions prevailing in the rhizosphere can also transform the antibiotic gene expression, in addition to the development of bacterial population owing to change in quantity and composition of root exudation (Lugtenberg et al. 2001; Bais et al. 2006). Subsequently, the microbiota adhering to the roots, imposed under nutrient starvation would be affected such that expression of genetic elements responsible for production of antibiotics is altered and it could favour the production of one specific family of LP inhibiting the production of other LP family. Transcriptional mechanisms, such as quorum sensing have also been shown to play an important role in regulation of LP production *in situ* (Hamoen et al. 2003; Duitman et al. 2007). Although production of LPs from all three families (iturin, surfactin and fengycin) has been reported infrequently, quantities produced in the rhizosphere are difficult to estimate. Nevertheless, improvements in the analytical techniques such as spectrometric methods viz., matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), eletrospray ionization-mass spectrometry (ESI-MS) have provided us with tools to detect/estimate the LP quantities in whole cells or culture extracts directly (Vater et al. 2002; Athukorala et al. 2009; Kim et al. 2010).

6.8 Lipopeptides: Weapons for Biological Control

In most of the cases, the antibiotics have been shown to be effective at suppressing growth of the target pathogen *in vitro* and/or *in sit*u. To be effective, antibiotics must be produced in sufficient quantities near the pathogen to result in a biocontrol effect. *In situ* production of antibiotics by several different biocontrol agents has been measured (Thomashow et al. 2002); however, the effective quantities are difficult to estimate due to: the small quantities produced relative to the other, being less toxic, and abundance of other organic compounds in the phytosphere. While methods have been developed to ascertain when and where biocontrol agents may produce antibiotics (Notz et al. 2001), detecting expression in the infection court is difficult because of the heterogeneous distribution of plant-associated microbes and the potential sites of infection. In a few cases, the relative importance of

antibiotic production by biocontrol bacteria has been demonstrated, where one or more genes responsible for biosynthesis of the antibiotics have been manipulated. For example, mutant strains incapable of producing phenazines (Thomashow and Weller 1988) or phloroglucinols (Keel et al. 1992; Fenton et al. 1992) have been shown to be equally capable of colonizing the rhizosphere but much less capable of suppressing soil borne root diseases than the corresponding wild-type and complemented mutant strains.

Several biocontrol strains are known to produce multiple antibiotics which can suppress one or more pathogens. For example, *Bacillus cereus* strain UW85 is known to produce both zwittermycin (Silo-Suh et al. 1994) and kanosamine (Milner et al. 1996). The ability to produce multiple antibiotics probably helps to suppress diverse microbial competitors, some of which are likely to be plant pathogens. The ability to produce multiple classes of antibiotics, that differentially inhibit different pathogens, is likely to enhance biological control. *Pseudomonas putida* WCS358r strains genetically engineered to produce phenazine and DAPG displayed improved capacities to suppress plant diseases in field-grown wheat (Glandorf et al. 2001; Bakker et al. 2002). More recently, *in situ* production and recovery of both iturin and fengycin families of lipopeptides provided a direct evidence of their putative involvement in suppression of cucurbit powdery mildew disease (Romero et al. 2007).

6.9 Biocontrol Activity of Mutants Deficient in Lipopeptide Production: Experimental Evidences

Bacillus lipopeptides play a pivotal role in biological control of pathogens (Table 6.2) and several authors have proved it experimentally through studies conducted on mutants generated by gene replacement/elimination strategy. Koumoutsi et al. (2004) evaluated the biological activity of *B. amyloliquefaciens* FZB42 (a producer of surfactin, fengycin and bacillomycin D) and mutants of FZB42, following direct growth tests and bioautography. Conversely, wild type strain FZB42 was found to inhibit the mycelial growth of several plant pathogenic fungi viz., *Gaeumannomyces graminis*, *Rhizoctonia solani*, *Alternaria alternatae*, *Pythium aphanidermatum*, and *Fusarium* spp. including *F. oxysporum* while mutant strain deficient in bacillomycin D synthesis were severely impaired in antimycotic activity implying that bacillomycin D contributed significantly towards the antifungal activity of strain FZB42. Intriguingly, strains with double mutations in *srf* and *feng* gene clusters still retained antifungal activity. Moreover strains harbouring mutations in *bmy* and *feng* gene clusters could not inhibit the mycelial growth of pathogens, suggesting the synergistic action of bacillomycin D and fengycin against the target pathogens (Koumoutsi et al. 2004). Similarly, protective action of iturin and fengycins was apparent from the reports of Romero et al. (2007) who demonstrated the *in situ* production of iturin and fengycin lipopeptides from strains of *B. subtilis* on melon leaves against *Podosphaera fusca*. While the wild type strains retained antifungal activity, mutants

Table 6.2 Role of *Bacillus* lipopeptides in management of plant pathogens

Sr. no.	Antibiotic	Producer organism	Pathogen(s)	Reference(s)
1	Iturin A	*B. amyloliquefaciens* PPCB004	*Alternaria citri* (Penz.) Mussat *Botryosphaeria* sp. *Colletotrichum gloeosporioides* (Penz.) Penz. & sacc. *Fusicoccum aromaticum* (Sacc.) petr. & Syd. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl *Penicillium crustosum* Thom *Phomopsis persea* Zerova	Arrebola et al. (2009)
2	Iturin A/Iturin A_2	*Bacillus* spp.	*Paecilomyces lilacinus*	Lee et al. (2007)
		B. amyloliquefaciens RC-2	*Colletotrichum dematium*	Yoshida et al. (2001)
3	Bacillomycin D	*Bacillus* spp.	*Fusarium graminearum*, *Sclerotinia sclerotiorum*	Ramarathnam et al. (2007)
		B. subtilis AU195	*Aspergillus flavus*	Moyne et al. (2001)
4	Bacillomycin F	*B. subtilis* I164	*Penicillium chrysogenum*	Mhammedi et al. (1982)
5	Bacillomycin L	*B. subtilis*		Volpon et al. (1999)
6	Mycosubtilin	*B.subtilis* BBG100	*Pythium aphanidermatum*	Leclere et al. (2005)
7	Surfactin	*B. subtilis*	*Aspergillus flavus*, *C. gloeosporioides*	Mohammadipour et al. (2009)
8	Fengycin	*B. subtilis, B. thuringiensis* CMB26	*Botrytis cinerea*	Ongena et al. (2005)
			C. gloeospoioides	Kim et al. (2004)
9	Plipastatin	*B. subtilis* YB8	*Fusarium oxysporum, Pyricularia oryzae, B. cinerea*	Tsuge et al. (1995), Yamada et al. (1990)
10	Zwittermicin A	*B. cereus* UW85	*Phytophthora medicaginis*	Silo-Suh et al. (1994), Emmert et al. (2004)
11	Kanosamine	*B. cereus* UW85	*Phytophthora medicaginis, Pythium spp., Aphanomyces euteiches*	Milner et al. (1996)
12	Bacilysin	*Bacillus* sp. CS93	*Absidia* sp.	Phister et al. (2004)
13	Bamylocin	*B. amyloliquefaciens* LP03	*B. cinerea*, *Rhizoctonia solani, F. oxysporum*	Lee et al. (2007)
14	Iturin, surfactin, fengycin	*B. subtilis* RP24, *B. subtilis* CMB32	*Macrophomina phaseolina, C. gloeosporioides*	Grover et al. (2010), Kim et al. (2010)

deficient in production of iturin and fengycins were unable to suppress the pathogen, clearly indicating that the biocontrol activity of strains was due to production of antifungal lipopeptides.

6.10 Conclusions and Future Perspectives

Several reports have described *Bacillus* strains worthy of use as biocontrol agents of phytopathogens (Shoda 2000). One of the most important reasons being an amazing battery of antibiotics produced which exhibit antimicrobial spectrum against a wide variety of pathogens and contributes towards the survival of the producing strains in their ecological niche (Stein 2005). The 'hay bacterium' *B. subtilis* is known to produce over two dozen antibiotics, requiring more than 350 kb of the total genome that accounts for a remarkable 10% of the total annotated ORFs. The potential of a given *B. subtilis* strain for antibiotic synthesis is comparable with *B. amyloliquefaciens* encompassing six operons of 306 kb corresponding to 7.5% of the genome (Koumoutsi et al. 2004). Altogether it seems to be that *B. subtilis* is outstanding in the genus *Bacillus* with regard to its potential to produce so many different antibiotics. Nevertheless, other bacilli such as *B. brevis* or *B. amyloliquefaciens* also produce a couple of antibiotics although their number seems to be minor as compared to *B. subtilis*.

Lipopeptide antibiotics are by far among the most frequently produced *B. subtilis* antibiotics. They along with some other amphiphilic compounds act as low molecular mass surfactants which alter the physical/chemical properties at interfaces probably either by increasing the surface area of hydrophobic water insoluble growth substrates or by increasing the availability of hydrophobic substrates or by modifying the ability of microorganisms to attach to the surfaces (Rosenberg and Ron 1999). The antifungal action of lipopeptides, in addition to surfactin (fengycin, iturin or bacillomycin) seems to be advantageous for the producing *Bacillus* strains as it helps to eliminate the competitors inhabiting the same ecological niche. Although antibiotics are produced by a wide array of bacterial strains, they are not obligatory for the general survival of the producing genera. Therefore, it has been speculated why antibiotics are synthesized and if they play any biological role other than being antimicrobials (Stein 2005).

The comprehensive studies based on *in vitro* or *in situ* biocontrol action of *Bacillus* spp. proved the occurrence of three different lipopeptide antibiotics; surfactin, fengycin and iturin A or bacillomycin compounds as major determinants of their antimicrobial property against different necrotrophic phytopathogenic fungi. These findings enlisted in this microreview together with the fact that the biocontrol efficacy of each strain is closely associated with the lipopeptide production, strongly supports the relevant role of antibiosis as a major factor involved in the protective effect of these strains (Stein 2005; Ongena and Jacques 2008; Chen et al. 2009). Basically, in this review we have tried to highlight the competitive advantage of efficient production of surfactin, fengycin and iturin with their specific action and

targets. Within each family, some of the structural homologues seem to be more active than others and it is probably for this reason that some *Bacillus* strains efficiently control pathogens/diseases than others.

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Chapter 7
Frankia and Actinorhizal Symbiosis

Arnab Sen and Arvind K. Misra

Abstract The actinobacterial member *Frankia* has a special significance as nitrogen fixing partner in symbiosis with some non-leguminous plants. *Frankia* seems to have entered into symbiotic association with many diverse dicotyledonous plants belonging to eight families independently several times during the course of evolution of this important symbiosis. Since this association confers a special ability to colonize soils low in nitrogen, actinorhizal plants are able to act as pioneers in the regeneration of *Jhoom* fallows, land slide affected areas, mine spoils, etc. They are also useful as wind breaks and as sand dune stabilizers. The accessibility of the complete genome sequences for three *Frankia* strains has provided new evidences pertaining to its evolution and structure, interactions between actinorhizal plants and their diversity. The establishment of the structure of NifH proteins from *Frankia* provided significant insights into the structure-function relationships. Analysis of the *nif* genes and whole genomes of *Frankia* using nucleotide triplet based phylogeny highlighted the roles of lateral gene transfer and gene duplications.

Keywords *Frankia* • Morphology • Taxonomy • Genomics • Proteome • Phylogenetics

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7.1 Introduction

In the general perception symbiotic nitrogen fixation only means legume–rhizobia symbiosis. However, another exceedingly important symbiotic association between actinorhizal plants and *Frankia* is a substantial contributor of reduced nitrogen in nature. *Frankia* is a prokaryote that fixes atmospheric di-nitrogen in a manner similar to other free living or symbiotic nitrogen fixers. Unlike the relatively narrow range of hosts for rhizobia, *Frankia* is able to nodulate about 24 genera belonging to 8 diverse flowering plant families (Table 7.1). Surprisingly, the association is genus specific and not family specific. It means that not all genera belonging to a family form root nodules with *Frankia*. For example, while genus *Alnus* of family Betulaceae forms symbiotic root nodules with *Frankia*, the type genus *Betula* of the same family does not. This and other observations lead many workers to hypothesize multiphyletic origin of this symbiosis (Swensen and Mullin 1997; Ritchie and Myrold 1999).

All actinorhizal plants are perennial dicots, found in all continents except Antarctica and except *Datisca* are woody trees. They are predominantly found in temperate regions. Some species are tropical. Actinorhizal plants abound in areas

Table 7.1 List of known actinorhizal plant genera and their families

Family	Genus
Casuarinaceae	*Allocasuarina*
	Casuarina
	Ceuthostoma
	Gymnostoma
Betulaceae	*Alnus*
Myricaceae	*Comptonia*
	Myrica
Elaeagnaceae	*Elaeagnus*
	Hippophae
	Shepherdia
Coriariaceae	*Coriaria*
Rhamnaceae	*Ceanothus*
	Colletia
	Discaria
	Kentrothamnus
	Retanilla
	Talguenea
	Trevoa
Datiscaceae	*Datisca*
Rosaceae	*Cercocarpus*
	Chamaebatia
	Cowania
	Dryas
	Purshia

close to the poles (Scandinavia, Canada, New Zealand, etc.). In Indian subcontinent and China, they are found in mountainous regions (Himalayas, Nilgiri Hills, etc.). In India, they are found in higher reaches of Himachal Pradesh, Jammu and Kashmir, Arunachal Pradesh, Meghalaya, Sikkim, West Bengal, Nagaland and in coastal areas. The genera found in India are *Alnus*, *Casuarina, Coriaria, Elaeagnus, Hippophae* and *Myrica*. *Hippophae* is found in both west and east Himalayas at altitudes above 2,000 m. *Casuarina* is found in coastal regions of the country.

Actinorhizal plants are pioneers on nitrogen poor soils including sandy and gravelly sites, shores of streams and lakes, wetlands and exposed raw mineral soils. Their ability to harbour nitrogen fixing *Frankia* in root nodules enables them to colonize nitrogen depleted soils and soils disturbed by landslides, etc. Therefore, they are used in land reclamation, sand dune stabilization and as wind breaks. Some yield timber for making packaging chests and fuel wood. *Myrica* and *Hippophae* fruits are taken raw or in processed form. Both have high nutritional value. *Myrica* is used in traditional Indian system for treating coughs and cold. Its fruit has high vitamina C content. *Hippophae* is gaining in importance lately with the Defence Research and Development Organization, Leh, popularising technology for production of packaged drink from its fruits. Indian soldiers are given this as nutritional supplement in higher reaches of Himalayas. Its fruit drink has been used by Russian astronauts as a nutritional supplement in space.

7.2 Morphology of *Frankia* in Culture

Frankia is a filamentous actinomycete with hyphae ranging from 0.5 to 1.5 μm in diameter. It is microaerophilic (Burggraaf and Shipton 1982) and forms submerged colonies below the surface of culture medium. In culture the hyphae get compacted and produce thalli and grow radially. The cell walls are composed of two layers of electron dense material (Horriere et al. 1983). Numerous rosette shaped granular glycogen bodies have been detected inside the hyphae (Benson and Evenleigh 1979). A large number of cytoplasmic tubules averaging 45 nm in diameter are present around the periphery of the hyphal cell cytoplasm (Lancelle et al. 1985).

7.3 Morphology of *Frankia* in Nodules

Nodule sections have revealed the presence of *Frankia* hyphae in cortical cells (Fig. 7.1). Electron microscopic studies of nodule sections show the presence of hyphae and vesicles such that the vesicles are radially arranged outward. However, *Casuarina* compatible *Frankia* do not form any vesicles within nodules, although the same do form vesicles in culture. Possibly the thickening of host tissues prevents diffusion of oxygen in the nodules of *Casuarina*. Therefore, the formation of vesicles does not occur in such nodules.

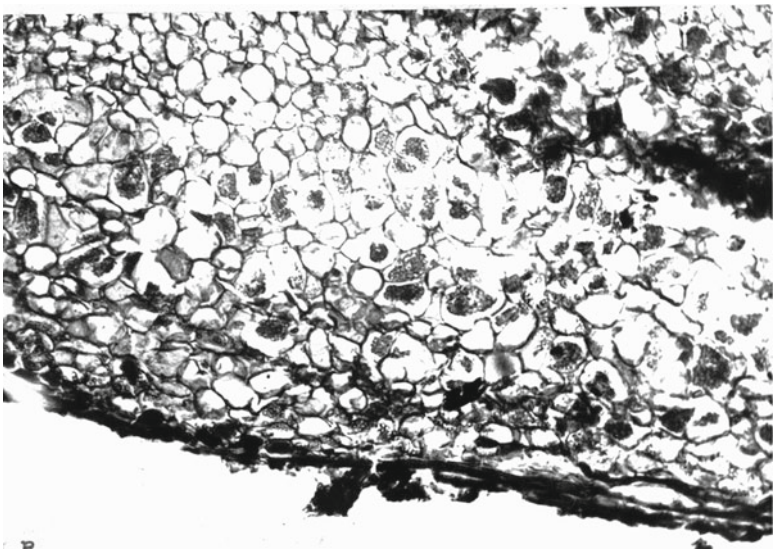

Fig. 7.1 TS of root nodule of *Alnus nepalensis* showing *Frankia* hyphae in cortical cells (Ganesh et al. 1994)

7.4 Spores and Sporangia

Spores are borne in sporangia that may be as large as 60 μm (Tjepkema et al. 1980). The sporangia may be intrahyphal or terminal (Newcomb and Wood 1987) and may be pear shaped or columnar (Horriere 1984) (Fig. 7.2). They are attached to submerged filaments by a sporangiophore (Baker et al. 1979). In some isolates, intercalary elongated sporangia like structures are formed that can get disrupted into spore like units (Diem et al. 1983). Sporangia develop by hyphal thickening and formation of septum originating from inner layer of a double layered sporangial cell wall (Horriere et al. 1983). Segmentation within the enlarging sporangia may produce multicellular sporangia containg many spores. Spores are about 1 μm in diameter and may be spherical or oblong in shape (Tjepkema et al. 1980). Mature spores show evenly dispersed cytoplasm without tubules (Lancelle et al. 1985) They germinate at variable rate with 1–3 germ tubes (Lechevalier and Lechevalier 1989).

Some strains of *Frankia* do not seem to produce spores within the nodule while others do. On the basis of this two types of strains have been classified. They have been called as Sp^+ and Sp^- depending on whether they produce spores within nodules or not. The so called Sp^- strains do produce spores in culture. It is difficult to culture Sp^+ stains (Simonet et al. 1994). In fact the rare cultures obtained from Sp^+ strains do not sporulate once reintroduced into the host. Further investigations are required to understand this behaviour of *Frankia* stains.

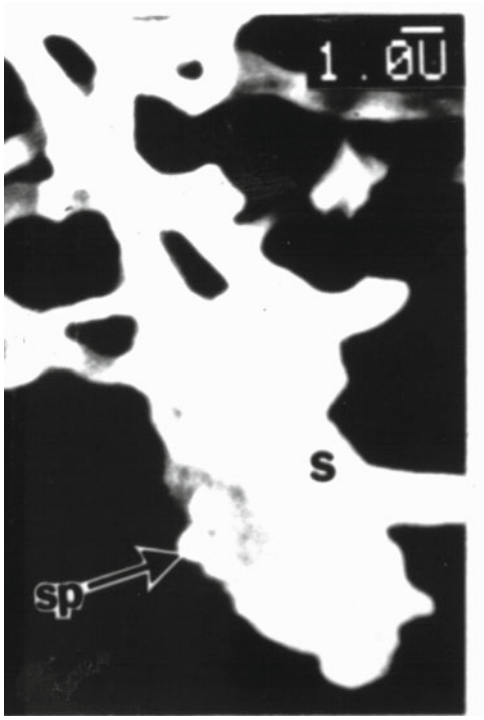

Fig. 7.2 Electron micrograph of cultured *Frankia* isolate showing sporangium (*s*) and spores (*sp*)

7.5 Vesicles

Hyphae may form thickened spherical club shaped structures called as vesicles (Fig. 7.3) that are sites for nitrogen fixation (Baker et al. 1979). The nitrogenase enzyme responsible for nitrogen fixation is located within the vesicles (Meesters et al. 1987). Under Normarski interference phase optics, vesicles show thickened walls (Tjepkema et al. 1980) that may protect nitrogenase enzyme from oxygen. Thus *Frankia* vesicles functionally resemble heterocysts found in cyanobacteria. Vesicles are produced under nitrogen stress and are suppressed in nitrogen abundance. Some strains may produce vesicles even in presence of available nitrogen (Gauthier et al. 1981). The vesicles range between 2 and 4 μm in diameter and are laminated with several layers of lipid mono-layers (Torrey and Callaham 1982).

7.6 Taxonomy of *Frankia*

Frankia belongs to family Frankiaceae of the order Actinomycetales. Initially, ten species of *Frankia* were created based on the host specificity studies (Lechevalier 1994). However, subsequent studies showed that the host specificity of *Frankia* is

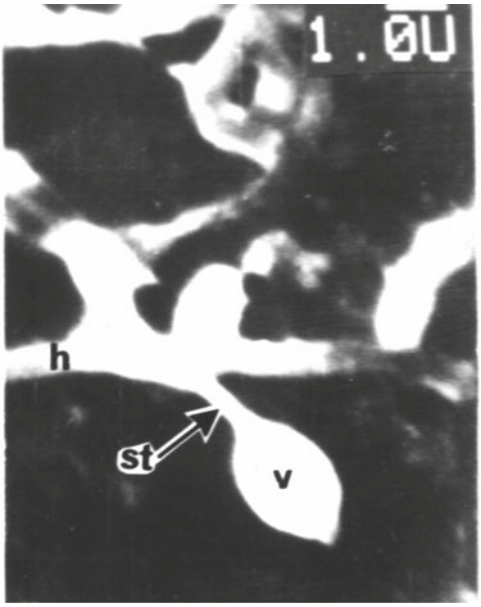

Fig. 7.3 Electron micrograph of cultured *Frankia* isolate showing hyphae (*h*) and vesicle (*v*) with stalk (*st*)

not as rigid as that of *Rhizobium* since strains isolated from one host species could nodulate other host species (Bosco et al. 1992). Therefore, this criterion for defining species may be flawed. The availability of molecular methods resulted in fresh look at defining species in *Frankia* genus. Normand et al. (1996) emended the family Frankiaceae based on rDNA studies. They have defined four major groups called as clusters based on the rDNA Phylogenetic tree. Group 1 comprises of *Alnus, Casuarina* and *Myrica* compatible isolates, Group 2 comprises of *Dryas* compatible isolate and unisolated microsymbiont of *Coriaria nepalensis* and *Datisca canabina*, Group 3 comprises of Elaeagnaceae isolates and Group 4 comprises of atypical isolates that are non-infective and non-nitrogen fixing. They have gone on to put together the characteristic features of members of family Frankiaceae as below-

'Hyphae extensive, aerial mycelium absent, hyphal diameter varies from 0.5 to 2.0 μm. Branching is limited to extensive, The mycelium may bear terminal or lateral thick-walled diazovesicles. A part of the thallus is composed of irregularly shaped cuboid to oval cells that divide in more than one plane. These cells are non-motile, possess an outer membrane and may germinate to give filaments. Gram positive to Gram variable. Aerobic to microaerophilic. The nitrogen sources used are amino acids and ammonia; atmospheric nitrogen is fixed *in vivo* and *in vitro* by most strains. The carbon sources used include carbohydrates, organic acids and fatty acids. Found in soil and as symbionts of higher plants. The cell wall type is type III (*meso*-diaminopimelic acid, glutamic acid, alanine, glucosamine and muramic acid). The whole-cell sugar patterns include patterns B (3-*O*-methyl-D-galactose, madurose), D (xylose), and E (fucose). 2-*O*-methyl-D-mannose is a diagnostic sugar.

Phospholipid pattern PI. The major menaquinone is MK-9(H_4). Fatty acid pattern 1. All strains tested contain hopanoid lipids (25a). The family comprises the single genus *Frankia*.' *Frankia* genus therefore comprises of soil microorganisms that are difficult to isolate and study.

7.7 *Frankia* Research in the New Era

Like other diazotrophs the research on the actinobacteria, *Frankia* underwent a sea change in the post genomics era. This was significant being a symbiotic partner with a number of non-leguminous plants. Although a lot of work has been performed by *Frankia* biologists in the pre-genomic era with respect to its culture characteristics, biochemistry, physiology, molecular characterization, taxonomic and phylogenetic analysis exceedingly modest attention was given to its genomic comparisons (Ventura et al. 2007). The sequences for 16S rRNA and *nif* genes came to be available in the public domain in the 1990s and most of the scientists concentrated around determination of meaningful interrelationships using sequence alignment methods for bioinformatics analysis. McEwan and Gatherer (1999) used some codon indices to predict gene functionality in a *nif* operon of *Frankia*. Sur et al. (2006) investigated the codon usage and intergenic associations in the *nif* genes *Frankia* EuIK1 and compared the results with *Bradyrhizobium*. They reported high bias and interplay of mutational pressure as well as translational selection. However, this was very little and comprehensive comparative sequence studies became exceedingly important so as to get insights into the molecular nature of the organism.

7.8 *Frankia* Genomes and Their Comparative Analysis

It is well known that *Frankia* is a very difficult bacterium to isolate in pure culture and consequently its sequencing too was not that easy. It was in the year 2007 that Normand et al. (2007) in their classic paper published the complete genome sequences of three *Frankia* strains CcI3, ACN14a and EAN1pec isolated from three different host plants. These sequences yielded considerable data with respect to their interrelationships and evolution especially the size difference. This size variation was reported to be the largest amongst closely related soil bacteria (Normand et al. 2007). Normand and his co-workers were instrumental in putting forth the concept that the differences in size amongst the *Frankia* genomes were due to the biogeographic history of the host plants for each strains. Events like gene deletions, duplications and acquisitions were vital. They also put forward the concept that host plant diversification was directly proportional to genome expansion and the reverse for genome contraction. Strain ACN14a was the most stable genome compared to CcI3 and EAN1pec. Benson and Dawson (2007) reported that geographical isolation

owing to continental drift resulted in the development of varied affinities of *Frankia* for host plants. Normand et al. (2007) compared the three sequenced *Frankia* genomes and reported that 2,810 genes were common in all the three genomes.

The post genomic era threw up a concept that each genome had its own particular characteristics (Sur et al. 2010). The genetic code and its degeneracy has been one of the most intriguing things for biologists. Peden (1999) highlighted that usage of codons vary amongst the species as well as genes in the same organism. While some workers (Grantham et al. 1981) reported that variation in codon usage was linked to tRNA content and translational selection and mutational pressure manipulated codon usage bias to a greater extent.

Sen et al. (2008) used indices like GC content, GC3 content, effective number of codons (Nc), relative synonymous codon usage (RSCU), codon adaptation index (CAI) and Fop (frequency of optimal codons) to study codon usage patterns of the complete genome sequences for three *Frankia* strains with special reference to those associated with nitrogen fixation.

The GC content is an estimate of the quantity of the guanine cytosine in sequences (Sur et al. 2006, 2007, 2008; Mondal et al. 2008). GC3 content is the percentage of G or C nucleotides in the third codon position. Effective number of codons (Nc) measure overall codon bias of codons (Wright 1990). The values for effective number of codons always range from 20 to 61. Nc values are predisposed by mutational biases and selection for specific codons. During the calculation of the effective number of codons, primarily *F*^ (F caret) values are calculated in each synonymous group using the equation:

$$\hat{F} = \frac{\left(n_{aa}\sum_{i=1}^{j} p^2\right) - 1}{n_{aa} - 1} \tag{7.1}$$

where, p symbolize the portion of usage of a codon i contained by its synonymous cluster of size j, and n_{aa} the whole usage of that synonymous group. The common of *F*^ for synonymous groups of same size (i.e. 2, 4, and 6) is also calculated. Nonetheless, in absence of the isoleucine residues *F*^ is calculated as mean of $F^{\wedge av2}$ and $F^{\wedge av4}$ and Nc is determined using the equation:

$$Nc = 2 + 9 / \hat{F}^{av2} + 1 / \hat{F}^{3} + 5 / \hat{F}^{av4} + 3 / \hat{F}^{av6} \tag{7.2a}$$

To incorporate the effect of G+C bias in Nc values the following equation has been commonly used to determine expected value of Nc under random usage:

$$Nc = 2 + S + \{29 / [S^2 + (1 - S)^2]\} \tag{7.2b}$$

where, S represents GC3 values.

Codon adaptation index (CAI) is generally used to resolve codon usage in prokaryotes over and above eukaryotes (Sharp and Li 1987). The calculation of relative synonymous codon usage (RSCU) values from a set of highly expressed

genes in an organism as determined by Sharp and Li (1987) has been a pre-requisite for calculating codon adaptation index values. RSCU has been determined using the equation:

$$RSCU_{ij} = \frac{x_{ij}}{1/n_i \sum_{j=1}^{n_i} x_{ij}} \tag{7.3}$$

Where, x_{ij} signified occurrence of the *j*th codon for *i*th amino acid, and n_i symbolizing synonymous group for *i*th amino acid (i.e., 2, 3, 4 or 6). CAI value is calculated by determination of geometric mean for relative adaptiveness values in codons present in genes. CAI is determined by the equation:

$$CAI = \exp\left(\frac{1}{L}\sum_{k=1}^{L} \ln \omega k\right) \tag{7.4}$$

where, ω*k* signified relative adaptedness of kth codon while L represented number of synonymous codons in gene. CAI values varied from 0 to 1 and higher CAI values indicated that the particular gene of interest had codon usage pattern similar to highly expressed genes.

Codon bias index (CBI) (Bennetzen and Hall 1982) determined codon bias and the extent to which gene used optimal codons. CBI values vary from 0 to 1. It is determined by the formula:

$$CBI = N_{opt} - N_{ran} / N_{tot} - N_{ran} \tag{7.5}$$

where N_{opt} = number of optimal codons; N_{tot} = total number of synonymous codons; N_{ran} = expected number of optimal codons in cases where codons are assigned randomly.

Frequency of optimal codons (Fop) (Ikemura 1985) is the percentage of synonymous codons that are optimal. Original Fop Eq. 7.6a and modified Fop index Eq. 7.6b are used when rare codons were identified. Fop is generally determined using the equations:

$$Fop = N_{optimalcodons} / N_{synonymouscodons} \tag{7.6a}$$

$$Fop_{(\mathrm{mod})} = N_{optimalcodons} - N_{rarecodons} / N_{synonymouscodons} \tag{7.6b}$$

Where, N symbolized the proportion of each codon type used. Fop values ranged from 0 to 1.

Sen et al. (2008) used correspondence analysis (Peden 1999) to find out the degree of associations between different genes and amino acids for the studied strains. Difference was observed between the strains. Using CAI calculator (Wu et al. 2005) potentially highly expressed genes were predicted and the analysis revealed that *Frankia* CcI3 had a different profile compared to ACN14a

and EAN1pec. CcI3 had fewer predicted highly expressed genes in COGs in contrast to strains EAN1pec and ACN14a. A number of genes associated with nitrogen fixation in the potentially highly expressed category for the studied *Frankia* genomes signified the importance of nitrogen fixation. Sen et al. (2008) postulated that these differences reflected the fact that CcI3 was a symbiotic specialist while the other two were facultative symbionts demonstrating their ability to exist as free-living soil dwellers. The authors' group has also analysed the TTA codon containing genes in three *Frankia* strains. The codon usage patterns of those genes have been studied to look into their nature. Majority of the potentially highly expressed TTA codon containing genes were associated with metabolism. Kosawang (2009) performed comparative genomics analysis to understand the regulation of hydrogenases in the nitrogen-fixing *Frankia*. He hypothesized that uptake hydrogenase function is expressed in *Frankia* strains CcI3, ACN14a and R43. Kosawang (2009) further stated that uptake hydrogenase probably acted in reverse direction and involved hydrogen evolution in R43 and bidirectional hydrogenase function is lacking in CcI3 and ACN14a.

7.9 *Frankia* Proteome and Secretome Research

Technological know-how has spurred proteomics research for prokaryotes in this era (Sur et al. 2010). Development of new techniques coupled with robust bioinformatics tools has improved the science of biological nitrogen fixation. Most of the research however had dealt with legume-microbe interactions focusing on identification of genes and proteins induced during host-bacteria relations (Mathesius 2009) using a cocktail of tools like 2D gel, peptide mass fingerprinting and bioinformatics (Rolfe et al. 2003).

Alloisio et al. (2007) studied the proteome of *Frankia alni* under nitrogen fixing and nitrogen depleted conditions. The characterization of its proteome led to the discovery of 126 proteins linked to nitrogen assimilation and oxidative defense system that were up regulated during growth in nitrogen fixing and nitrogen replete conditions. Mastronunzio et al. (2008) investigated the genome based secretome of three *Frankia* strains coming from different host plants. The predicted secretomes were small including few hydrolases and reflected the adaptation to symbiotic lifestyle. Mastronunzio et al. (2008) hypothesized that lack of proper secreted polysaccharide-degrading enzymes in *Frankia* was a policy to prevent eliciting host responses. They reported a number of esterases, lipases, and proteases in the core *Frankia* secretome and postulated that these probably assisted in hyphal penetration, release of carbon sources and modification of chemical signals. Niemann and Tisa (2008) investigated the genome of *Frankia* CcI3 and postulated that it housed two truncated hemoglobin genes (*hboN* and *hboO*). They found that nitric oxide caused increase in *hboN* gene expression and virtually was ineffective for *hboO* expression. They reported that lower oxygen conditions increased *hboO* gene expression levels but not in *hboN* implying that HboN functioned in protection from nitrosative stress

while HboO acted as an oxygen transport molecule for respiration in hypoxic habitats. The publication of the whole genomes of three *Frankia* strains opened the opportunity to use proteomics techniques to understand the exoproteome from *Frankia* (Mastronunzio et al. 2009). This analysis supported previous bioinformatics studies that predicted hydrolytic enzymes secreted in *Frankia* proteomes provided indication that symbiosis accomplishes partly owing to benign association. Mastronunzio and Benson (2010) characterized proteomes of *Frankia* isolated from *Alnus incana subsp. rugosa*, *Ceanothus americanus* and *Elaeagnus angustifolia* using a combination of two dimensional liquid chromatography and mass spectrometry. They identified 1,300 proteins of *Frankia* isolated from *Alnus incana* and 1,100 proteins from *E. angustifolia* nodules. Moreover, using one dimensional liquid chromatography and mass spectrometry 100 proteins were identified in *Ceanothus americanus*. These studies highlighted that nitrogenase iron proteins were the most abundant proteins thus substantiating their role in symbiotic nitrogen fixation. In proteomics of nitrogen fixers most researchers have dealt on functional annotations and amino acid compositions and focus on common physical properties have been lacking and *Frankia* was no exception. We analyzed the proteomes of *Frankia* strains ACN14a, CcI3 and EAN1pec using protein isoelectric point (unpublished) utilizing bioinformatics tools. We found that in spite of actinobacterial *Frankia* being host associated, their isoelectric point was acidic. CcI3 was less acidic compared to the other two. We hypothesized that since CcI3 was biogeographically restricted compared to EAN1pec and ACN14a having worldwide distributions, the capability of ACN14a and EAN1pec to survive as facultative symbionts probably shifted their isoelectric point towards acidity. We also analyzed the isoelectric point in COGs functional groups for the strains and reported elevated levels of amino acids in metabolism and poorly characterized groups for ACN14a for acidic category portraying the role played by metabolism in influencing the lifestyle of ACN14a. The abundance of amino acids in the cellular processes group towards acidity for CcI3 and EAN1pec revealed that COGs linked to cellular processes increased the ability of these strains to respond to signals in the soil and the environment of the cells modified the shift towards acidity. Lower percentage of amino acids in the cellular processes group for CcI3 probably indicated its obligate symbionts nature.

7.10 Phylogenetic Studies of *Frankia* in the Post Genomic Era

Like other groups of bacteria, *Frankia* biologists too studied evolutionary relationships using morphological as well as chemotaxonomic characteristics in the pre-genomic era. However, new impetus came with the advent of 16S rRNA based phylogenies (Ventura et al. 2007). The whole genome sequences of three *Frankia* strains (Normand et al. 2007) made it possible to reconstruct phylogenies using larger dataset thus making it more reliable. Efforts are on by different scientists to construct genome based phylogenetic trees using alignment free methods, gene

content methods, based on average sequence similarity, based on chromosomal disorder and trees based on shared gene families. Leul et al. (2009) studied the phylogeny of uptake hydrogenases in *Frankia* strains. They reported that the structural subunits of hydrogenase syntons 1 and 2 of *Frankia* sp. EAN1pec were closely related to *Streptomyces avermitilis* and *Anaeromyxobacter* sp., respectively, compared to other *Frankia* strains, suggesting lateral gene transfer. It was found that accessory Hyp proteins of hydrogenase syntons 1 and 2 of *F. alni* ACN14a and *Frankia* sp. CcI3 were phylogenetically more close to each other compared to *Frankia* EAN1pec. Sur et al. (2009) developed nucleotide triplet based condensed matrix technique and we applied it for studying evolution of core *nif* genes as also whole genomes in *Frankia* strains and other diazotrophs (unpublished). Gene, duplications and lateral gene transfers shaped the evolution of these genes. We are also actively engaged in applying structure based phylogenetic study to *Frankia nif* genes.

7.11 Structural Bioinformatics Studies of *Frankia* Proteins and RNAs

Contrary to genomics and proteomics research, structural bioinformatics studies on *Frankia* macromolecules have been in the backburner for a long time. This might have been attributed to difficulties associated with protein purifications. Very recently, Sen et al. (2010) provided the first ever three dimensional structures of *Frankia* nitrogenase iron proteins from CcI3, ACN14a and EAN1pec. The structures were determined using homology modelling technique. Homology modelling is dependable methodology for efficiently envisaging the three dimensional structure of the proteins having precision levels parallel to ones achieved at low-resolution via experimental technologies (Martin-Renom et al. 2000). The methodology aligned amino acid sequence of *Frankia* NifH with that of the most suitable template protein 1G5P obtained from *Azotobacter vinelandii*. They identified the metal binding sites and functionally important residues. It was found that thiol ligands and active sites helped in functioning of the protein. Recognition of structurally important nests was carried out and clefts and cavities in protein housed the residues that are significant for functioning during nitrogen fixation. *In silico* site-directed mutagenesis demonstrated that mutations of functional residues indeed hampered nitrogen fixation ability rendering it ineffective. 3D structures clearly illustrated the structure-function relationship of the proteins. These studies have spurred an interest amongst the *Frankia* researchers to look at the structural aspects of other important proteins in *Frankia* so as to get a more comprehensive picture of *Frankia*. The authors' group had also studied the structure of 16S rRNA and tRNAs from TTA codon containing genes in the three *Frankia* strains (unpublished). Structure of tRNAs in highly expressed TTA codon containing genes were correlated with their expression levels and important motifs were identified (unpublished).

Bioinformatics studies are expected to provide more novel information in *Frankia* biology with different workers taking interest in this field. The genome sequencing of five more *Frankia* strains are on and are expected to be complete soon. Many novel pathways, enzymes, genes and proteins are anticipated to be discovered that would provide a new understanding of *Frankia* biology as well as interrelationships between strains coming from different habitats, ecological niches and varied host associations.

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Chapter 8
Microbes in Agrowaste Management for Sustainable Agriculture

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Abstract Agro residues, a precious source of nutrients are being disposed off because of their bulky nature and non-availability of management practices. Besides loss of essential plant nutrients and organic matter important for sustainability of the system, it also causes environmental pollution leading to animal and human health problems. It is important to recycle these crop residues for restoring soil health and sustainability of the production system for national food and nutritional security. Composting can be adopted for large scale recycling of these agrowastes to enriched compost using efficient microorganisms. Apart from being a source of nitrogen, phosphorus, potassium, and other nutrients for plants, compost is also believed to suppress soil-borne diseases in plants. These virtues make composting an ideal option for processing the enormous quantities of agro wastes that are generated in the world. Besides this agrowastes may be utilized as a resource for production of animal feed, biofuel and enzyme to generate additional income.

Keywords Agroresidues • Compost • Soil health • Cellulases • Sustainable agriculture

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Table 8.1 Estimated amounts of crop residues generated annually in India

Crop	Residue	Production (m. ton)
Rice	Straw	220.08
Wheat	Straw	121.23
Millet	Stalks	18.37
Maize	Stacks	19.60
Cassava	Stalks	0.37
Cotton	Stalks	20.51
Soybean	Straw + Pods	18.72
Jute	Stalks	4.50
Tobacco	Stalks	1.24
Sugarcane	Bagasse	45.00
Cocoa	Pods	0.01
Other		99.00
Total		**568.63**

(Source: Kaushik et al. 2005)

8.1 Introduction

In view of the energy and power crises besides the high cost of agricultural inputs such as fertilizers, pesticides and irrigation water, agricultural wastes are now considered quite an important component of farming especially those based on organic systems including crop residues. Organic amendments in the form of agricultural wastes and crop residues activate the autochthonous microorganisms of the soil, indirectly stimulate the biogeochemical cycles therein and provide various minerals (e.g. N, P, and S) essential for plant nutrition. Crop residues are plant materials left behind in the farm after removal of the main crop produce. The quantity of agricultural residues produced differs from crop to crop and is affected by seasons, soil types, and irrigation conditions. Production of agricultural residues is directly related to the corresponding crop production and ratio of residue and produce, which varies from crop to crop and, at times, with the variety of the seeds in one crop itself. Some data for various agroresidues at the national level are given in Table 8.1. These materials at times have been regarded as waste materials that require disposal, but it is increasingly being realized that they are important natural resources and not wastes. The recycling of crop residues has the advantage of converting the surplus farm waste into useful product for meeting nutrient requirement of crops. It also maintains the soil physical and chemical condition and improves the overall ecological balance of the crop production system.

8.2 Need for Bioconversion

Burning agroresidues in the field is considered a cheap and easy means of disposal of excess agroresidues. This practice appends to air pollution, increases soil erosion and decreases the efficacy of soil applied herbicides like isoproturon (Walia et al. 1999).

According to a survey conducted by Gajri et al. (2002), burning of paddy straw in the fields, farmers in Punjab (India) lose 38.5 lakh tons of organic carbon, 58,000 tons of nitrogen, 1,900 tons of phosphorous, 34,000 tons of potash and 4,600 tons of sulphur every year. Direct incorporation of these residues in field solves the problem of air pollution but it involves additional cost of labour, irrigation and extra tillage (Sidhu et al. 1998). Moreover, observations of long term experiments indicate that though incorporation of these residues in soil improves soil health significantly (Sidhu and Beri 1989; Beri et al. 1992; Beri et al. 1995), it decreases the subsequent crop yields due to production of microbial phytotoxins (Rao and Mikkelson 1977) and immobilization of the available nitrogen (Kimber 1973). Besides, direct incorporation increases the CH_4 emission especially from rice field (Chidthaisong et al. 1996; Denier van der Gon and Neue 1995), which in turn adds to the malice of global warming.

Agroresidues can be alternatively converted into protein rich animal feed or to compost through lignocellulolytic microorganisms by solid state fermentation. The increase in the protein content of cellulosic residues to improve their nutritional value has been proposed by several investigators (Han and Callihan 1974; Moo-Young et al. 1983). This process is potentially useful in reducing the environmental impact of these residues and in enhancing animal feed and human food supplies.

Thus the biodegraded product of these agroresidues has enormous potential in agriculture to recycle nutrients and maintain soil fertility. It is estimated that about 33 mt of nutrients are removed by the crops annually in India. The nutrient addition by chemical fertilizers has reached 25 mt by 2009. Still there is a gap of 8 mt which can be narrowed down by bioconversion of agrowastes to compost using efficient lignocellulolytic microorganisms as the agroresidues has potential to supply 4–6 mt of NPK annually.

8.3 Role of Lignocellulolytic Microorganisms in Bioconversion

Composting is the biological degradation and stabilization of organic substrate under conditions that allow development of thermophilic temperature as a result of biologically produced heat (Gaur 1999). During composting, mesophilic population builds up initially by the utilization of simple nutrients, which raises the temperature in the pits. Thermophilic microbes proliferate in the second phase. The final product is stable, free of pathogens and plant seeds and can be beneficially applied to land. Microbes cannot directly metabolize the insoluble lignocellulolyitc agroresidues and all the biochemical reactions during biodegradation are catalyzed by enzymes (Ayuso et al. 1996; Garcia et al. 1992; Godden et al. 1983; Vuorinen 1999, 2000). The microbes produce hydrolytic extracellular enzymes like cellulases, xylanases, amylases, ligninase, laccase etc. to depolymerize the larger compounds (i.e., plant polymers, cellulose, hemicellulose, and lignin) to smaller fragments that are water-soluble (Hankin et al. 1976b). Different groups of microbes participate in bioconversion process.

8.3.1 Fungi

Hundreds of species of fungi are able to degrade lignocellulose. There are mainly three types of fungi that preferentially degrade one or more wood components viz. soft rot fungi, brown rot fungi and white rot fungi (Kirk 1983). Soft rot fungi (Ascomycetes and fungi imperfecti) can efficiently decompose cellulose but are reported to degrade lignin slowly and incompletely. The brown rot fungi (Basidiomycetes) generally exhibit preference for the carbohydrate components of wood (Ander and Eriksson 1978; Janshekar and Fiechter 1983; Kirk 1983) with activity towards lignin largely confined to demethylation (Kirk 1983). White rot fungi are capable of degrading both lignin and cellulose. The most commonly isolated species of celluloytic fungi in composting materials are *Aspergillus*, *Penicillium, Rhizopus, Fusarium, Chaetomonium, Trichoderma, Alternaria, and Cladosporium h*owever, the most extensively studied lignocellulolytic fungi are *Trichoderma* and *Phanerochaete.*

The role of fungi starts when simple, easily degradable substances such as sugar, starch, and protein are acted upon by bacteria and the substrate is predominated by cellulose and lignin, which normally occurs toward the later stages of composting (curing process) (de Bertoldi and Vallini 1983; Golueke 1992; Tiquia et al. 2002a). Lignolytic and cellulolytic *Trichoderma* were greater in soil under trees but there were no apparent qualitative differences among other systems (Rao and Venkateswaralu 1983). Lynch et al. (1981) observed that *Cladosporium* sp., *Alternaria* sp. and *Fusarium* sp. were more active decomposers than *Phoma* sp. Bisen et al. (1982) isolated *Fusarium solani* from soil and reported that it possessed good cellulolytic activity. Nigam and Parvu (1985) reported the cellulolytic activity in *Pleurotus ostreatus* and *Polyporus versicolor*. *Myriothecium verrucaria* is reported to produce extracellular cellulase to depolymerize cotton fibre cellulose (Halliwell 1961).

Thermophilic fungi that have been isolated from lignocellulose substrate or hot compost piles are *Taloromyces emersonii*, *T. thermophilus*, *Thermoascus auranticus*, and *Thermomyces lanuginosus*. Some white rot fungi like *Phanerochaete chyrsoporium* (*Sporotrichum pulverulentum*), *Ganoderma colosum* are capable of growing at 45°C and has an optimum temperature of 35–42°C (Adaskaveg et al. 1995).

8.3.2 Bacteria

Cellulolytic bacteria are ubiquitous in nature. Under appropriate conditions bacteria degrade cellulose and hence many bacterial strains are known to solubilize and modify the lignocellulosic structures extensively. But their ability to mineralize lignin is limited (Ball et al. 1989; Eriksson et al. 1990; Godden et al. 1992). Among bacteria that occur commonly in aerobically decomposing substrate are species of *Cytophaga, Bacillus*, *Cellulomonas*, *Pseudomonas*, *Klebsiella,* and *Azomonas* (Nakasaki et al. 1985; Strom 1985a, b)

Cytophaga (Marshall 1973; Chang and Thayer 1977) *Sporocytophaga* (Christensen 1977) are dominant cellulolytic microorganisms in all types of soil. *Cellulomonas* and *Cytophaga* are the aerobic mesophilic bacteria able to degrade cellulose. All species of *Cellulomonas* tested effect degradation of a variety of celluloses (Choi et al. 1978; Kim and Wimpenny 1981; Nakamura and Kitamura 1983; Thayer et al. 1984; Rajoka and Malik 1986).

More than one-half of the *Bacillus* spp. examined to date produces extracellular cellulases. Mesophilic aerobic and anaerobic forms of *Bacillus, B. subtilis* (Emi and Yamamoto 1972), *B. polymyxa, B. licheniformis, B. pumilus, B. brevis, B. firmus, B. circulans, B. megaterium* and *B. cereus* are known to be cellulose and hemicellulose degraders. The lignin degrading eubacteria can be divided into erosion, cavitation and tunnelling bacteria (Eriksson et al. 1990; Blanchette 1995). Erosion bacteria grew towards the middle lamella of the wood cells and cause erosion of the fibre wall, while tunnelling bacteria grow within the cell wall. Bacteria of several genera such as *Pseudomonas, Alcaligenes* and *Arthrobacter* can degrade single ring aromatic compounds. The role of bacteria may be significant in consuming the small molecular weight intermediate compounds produced by fungi (Ruttimann et al. 1991).

Kawakami and Shumiya (1983) studied the degradation of lignin and lignin related compounds by alkalophilic bacteria. Haider et al. (1978) showed that the *Bacillus* strain was able to convert ^{14}C (side-chain) lignin of spruce to $^{14}CO_2$. The rate was comparable to that of fungi upto 35 days. Deschamps et al. (1981) demonstrated delignification of bark chips by a mixed culture of *Bacillus* and *Cellulomonas,* which were insufficient alone.

Bacteria related to *B. schlegelii*, *Hydrogenobacter* spp., and particularly to the genus *Thermus* (*T. thermophilus, T. aquaticus*) are the main active microbes in hot compost (65–80°C) (Beffa et al. 1996). Similarly, thermophilic cellulolytic *B. stearothermophilus*, *B. brevis, B. sphaericus, B. subtilis* and two other species of *Bacillus* were isolated by Strom (1985a, b) from soil waste composter. Bacterial survival in high-temperature composting material is possible through formation of microcolonies and endospores. Mesophiles are likely to contribute little to compost degradation at these temperatures (Nakasaki et al. 1985).

8.3.3 Actinomycetes

Actinomycetes are filamentous spore forming bacteria, thus they resemble fungi. Majority of them are strict aerobic saprophytes, and are common in many environments including extreme environments of salinity and temperature. There are adapted to these extreme environments due to their ability to utilize a wide range of carbon sources and to sporulate prolifically. But actinomycetes colonize more slowly than both bacteria and fungi. Colonization is minimal in areas that are poorly aerated. They appear during the thermophilic phase as well as the cooling and maturation phase of composting, and can occasionally become so numerous that they are visible as a white film on the surface of the compost.

Thus, actinomycetes are important agents of lignocellulose degradation during peak heating as they tolerate higher temperatures and pH than fungi. Though actinomycetes can solubilise cellulose and modify the lignin structure extensively, their ability to mineralize lignin is limited (Eriksson et al. 1990; Godden et al. 1992). Alexander (1961) and Fergus (1969) remarked that cellulose breakdown by actinomycetes is slow. In neutral and alkaline environment, *Streptomyces viridosporus* is likely to be dominant over fungi as a decomposer of lignin and cellulose (Pomettoa and Crawford 1986). *Thermoactinomyces cruenta* was isolated by Stutzenberger et al. (1970) from the municipal compost.

The genera of the thermophilic actinomycetes isolated from compost include *Nocardia*, *Streptomyces*, *Thermoactinomyces,* and *Micromonospora* (Waksman et al. 1939; Strom 1985a). From Indian desert soil of Jodhpur, Rao and Venkateswaralu (1983) isolated *Streptomyces, Micromonospora* and *Thermoactinomyces*. These organisms were found to depolymerize crystalline celluloses by two cellulase enzyme systems and β-glucosidase. Strom (1985a, b) isolated thermophillic and highly cellulolytic *Streptomyces, Thermoactinomyces* sp. from solid waste composter. Jang and Chan (2003) isolated 18 strains of actinomycetes from the compost of agricultural wastes (vegetable residues supplemented with corncob, straw and rice hull) and cultivated them at 50°C for the thermostable cellulase production. Bardar and Crawford (1981) reported that *Streptomyces badius* can degrade milled wood lignin and degradation was enhanced when organic nitrogen and carbon substrate were added to the medium. Antai (1985) selected three *Streptomyces* strains which were lignolytic, out of which one was the most rapid lignocellulose decomposer depleting 42% of lignin and 50% of carbohydrate of the lignocellulose after 12 weeks incubation.

S. viridosporus, which was grown in lignocellulose supplemented medium, released coumaric acid and vanillic acid, which are intermediates of lignin degradation. Zimmermann and Broda (1989) used *S. cyaneus, T. mesophila* and *Actinomadura* sp. MT-809, for the degradation of barley straw lignocellulose. Zimmermann and Broda (1989) also reported lignin degrading ability of several mesophilic and thermotolerant strains of *Streptomyces*. Pasti et al. (1990) reported the lignocellulose degrading abilities of 11 novel actinomycete strain isolated from termite gut.

8.3.4 Consortium of Microorganisms for Bioconversion of Agrowaste

Different microorganisms such as fungi, bacteria and actinomycetes play unique and important roles during composting. Therefore mixed cultures of microorganisms enhance the rate of lignocellulose degradation. Gaur et al. (1982) reported that inoculation with mesophilic fungi lowered the C/N ratio of agricultural wastes and recommended the use of microbial inoculum for accelerating the process of composting. Zayed and Motaal (2005) reported the use of *Trichoderma viride* 104 and *Aspergillus niger* 111 to produce compost from rice straw. The inoculation effect of

mixed culture of *T. reesei, Phanerochaete chrysosponium, Aspergillus nidulans* and *Aspergillus awamori* were studied on decomposition of wheat straw, paddy straw, pearl millet, chickpea stover, bagasse mustard stover by Lata et al. (2005) and Gaind et al. (2006). The mixed inoculum was effective in accelerating the process of decomposition of paddy straw, wheat straw and pearl millet and mature and stable product was obtained within 60 days of composting. Pretreatment of mushroom substrate with mixture of fungi, actinomycete and bacteria can substantially improve the degradation process (Adhikari et al. 1992). This enhanced degradation could be due to synergistic activity through utilization of intermediate degradation products (Kanotra and Mathur 1994). Coinoculation of *S. aureofaciens* and *T. viride* were found to accelerate the decomposition of sugarbeet haulms (El Din and Abo Sedera 2001). Recently, Beary et al. (2002) observed that inoculation of fungal-bacterial consortium accelerated decomposition of sugarcane crop residue. In recent times, effective Microorganisms (EM) technology is also gaining popularity for preparing compost from variety of substrates including kitchen waste, MSW, industrial effluents and radio active wastes. A consortium of selected microbes like lactic acid and photosynthetic bacteria, yeast and filamentous fungi is being used in the form of Bokashi (fermented biomass). This can be made from any organic matter. The use of effective microorganism result in compost from biomass like coir-pith, coconut shell, pine needles, crop wastes, weeds, tree leaves etc. which are otherwise difficult to degrade and take a much longer period for decomposition. Lot of labor is saved due to *in situ* composting of fresh harvest wastes (Aggarwal 2004). Inoculation with effective microorganisms can enhance the microbial turnover in compost and used to produce nutrient rich organic compost.

8.4 Composting Process

It is an age old practice meant to utilize solid wastes of animal and plant origin. Composting is the strategy for improving management of solid wastes by minimization of the amount of the solid wastes generated and maximization of waste recycling as well as resource recovery. The formation of compost from the degradation of organic matter depends on the abilities of microflora to produce and excrete specific degradative enzymes (Hankin et al. 1976a).

Several reports are available where inoculation with mesophilic fungi improved the quality of compost. Gaur et al. (1982) reported that inoculation of four mesophilic fungi, *A. niger, Aspergillus* sp., *T. viride* and *Penicillium* sp. had increased total nitrogen, available phosphorus and humus content of composted jowar stalk and wheat straw (5:3). Inoculation of cellulolytic fungi and actinomycetes increased the temperature faster and accelerated the process of rice straw composting (Hang Won et al. 1995).

Several workers have dealt with rice straw composting after supplementation with rock phosphate, glue waste, basic slag and inorganic nitrogen sources (Jhorar et al. 1991; Wahyono and Sahwan 1998; Abdel Azeem 2001). The use of cattle

manure rather than relatively expensive microbial catalyst could be recommended as decreasing the composting degradation period of rice straw (Bhumibhamon et al. 1988). The inoculation of *Azotobacter* to 1 month old decomposed rice straw increased the nitrogen content and composting with rock phosphate increased both citrate and water soluble phosphorus (Tiwari et al. 1988). The two step process of composting of rice straw was effective in accelerating humification, cation exchange capacity of the composted material (Kakezawa et al. 1992). Nandi et al. (1996) proposed a two step process for composting of rice straw, which results in better humus formation.

The treatment of shredded rice straw supplemented with chemical accelerator, sheep manure and EM solution (mixture of microbial culture, commercially known as EM solution) gave the most desirable characteristics of the final product particularly narrow C/N ratio and high WHC (Abdel-Azeem 2001). Two white rot fungi, *Pleurotus ostreatus* and *Lentinus edodes* delignified and increased digestibility of corn straw (*Zea mays*) under solid state fermentation (Sermanni et al. 1994). Under fed-batch process of saccharification of short fibre waste material from the paper industry by *Penicillium* sp., *T. reesei,* combined preparation of *T. reesei* and *Aspergillus* showed highest saccharification activity (Castellanos et al. 1995). Cellulyolytic fungi such as *T. reesei* QM9414, *T. viride*, *P. sajor-caju*, *Coprinus cinereus* lowered the C/N ratio of paddy straw during composting (Kantora and Mathur 1995). Although the inoculated microbes generally enhance the degradation process, it doesn't alter the community structure of resident microflora. Phospholipid fatty acid (PLFA) analysis study of compost samples collected from bench-scale grape pomace and rice straw composting showed that inoculation had little effect on the microbial community structure of the compost when temperature peaked (Lei-Fei et al. 1998).

8.4.1 Factors Affecting Composting

Factors affecting the rate of composting and acting as major determinants of compost quality can be categorized into physicochemical, environmental and biological factors. The principal physicochemical components of considerations associated with optimal composting are temperature, pH, Electrical conductivity (EC), C and N content, the C: N ratio and C: P ratio, moisture content, amount of humic fractions and aeration. Similarly, the environmental and biological factors influencing the course of composting include the composition of agro-waste and type of microorganisms present in the composting environment at different stages of composting.

Temperature: Temperature controls the microbial activity during the composting process (McKinley et al. 1985). There are three phases in composting: an initial degradation phase, a thermophilic phase, and a curing or stabilization phase. In the initial phase of composting, degradation of sugars and proteins by the mesophilic microbial community results in increased CO_2 production (Hellmann et al. 1997).

As temperatures reach 40–45°C, the second or thermophilic stage commences during which temperatures rise to 70^0 C. It is during this stage, that weed seeds and pathogens present in the compost are killed, and the majority of cellulose, hemicellulose and lignins are degraded (Cook and Zentmyer 1986). As temperature declines, the third or curing and stabilization phase commences, during which mesophiles re-colonize pile and decomposition continues. As temperature declines, it results in exhaustion of readily available substrates, biomass decreases compared to initial, methane production decreases, and N_2O production increases (Hellmann et al. 1997). Breakdown of recalcitrant components is mediated by a shift in microbial community function of cellulose, hemicellulose and lignin decomposers (Horwath and Elliott 1996; Herrmann and Shann 1993).

pH: Optimum pH is near neutral for most microorganisms and slightly alkaline for actinomycetes in composting. This translates into a range of about 5.5–8.5 but the pH extremes should be avoided. A pH of 6.5–7.2 is ideal, because in this pH range, there will be less volatilization of NH_3, reduced odor and balanced microbial population (Eberhardt and Pipes, 1974). A consortium of three thermophilic fungi namely *Aspergillus nidulans* (Th4), *Scytalidium thermophilum* (Th5) and *Humicola sp.* (Th10) was effective in converting a mixture of paddy straw and soybean trash into nutrient rich compost within 90 days (Kumar et al. 2008).

EC: Electrical conductivity measures the amount of soluble salt in the compost sample and most desired values range from 3 to 5. The values lower than this indicates the lack of available minerals while the values higher than this will inhibit the biological activity (Gaind et al. 2005).

C:N ratio: Since agro-waste decomposition is a microbial process C, N, P is needed for synthesis of microbial cells. Therefore, nutrient content available for microbial growth is generally measured by the carbon to nitrogen and carbon to phosphorus ratio within the agricultural residues. The higher ratio (100:1) may cause immobilization of N and lower C: N ratio (20:1) would cause N loss through leaching and volatilization (Gaind and Lata 2005). In living organisms the C:N ratio is about 30:1 and theoretically this should be the ideal ratio in the composting material. But C:N ratio 40:1 is also appropriate. C:P ratio of 100:1 to 150:1 is recommended (Hagerty et al. 1973). The progress and maturity of compost is adjudged by monitoring the C:N ratio throughout the process. Hirai et al. (1983) opined that C:N should not be taken as the sole parameter of maturity since its value may vary with the characteristics of substrate and amendments. Actually C: N depends on the relative content of carbon and nitrogen in the initial matrix and on the presence of organic fraction refractory to biodegradation in initial substrate. According to California Compost Quality Council (CCQC), a compost sample must have a C: N ratio of less or equal to 25:1, to qualify for further maturity testing (California Compost Quality Council 2001).

Moisture: Adequate moisture is critical for composting. The optimal moisture content is 50–60%. If moisture content is more, O_2 diffusion impedes and creates anaerobic condition and slow decomposition rates. Ideal moisture helps to cool the compost and helps in microbial multiplication and growth. Microbial decomposition of

agro-waste proceed fastest under aerobic condition, oxygen content may be maintained at a level above 10% by turning the heap. Aeration requirement may be considered to be within the range of 0.2–0.5 m^3/min/ton of dry weight (Gaur 1999). Under farm condition this can be achieved by periodic turning of composting material.

Microbial inocula comprising lignocellulolytic microorganisms and decomposers of different substrates (proteolytic, lipolytic, amylolytic) are necessary for decomposition and humification of agricultural residues (Gaur 1987). The presence of efficient hydrolytic flora at the beginning is a prerequisite for reducing the lag time and resulting in an efficient process. The use of simple and natural inocula e.g. cattle dung slurry, organic soil, fresh compost is advantageous and should be encouraged. Likewise, consortia of mesophilic fungi may also be used as an inoculant for rapid composting (Gaur et al. 1982; Gaind et al. 2005; Lata and Pandey 2005; Gaind and Lata 2005).

8.4.2 Practical Process for Rapid Composting

Several bacteria and fungi have been successfully used to decompose plants residue and evaluated for their biodegradable activity on waste materials (Roane et al. 2001). Though, most of the organisms are mesophilic fungi belonging to genus *Aspergillus, Trichoderma, Trichurus, Paealomyces, Penicillium, Phanerochatae* etc. (Mishra et al 1979, Gaind and Lata 2004 and Gaind et al. 2005) but a few thermophilic fungi as *Aspergillus fumigatus* and *Humicola lanuginosa* also proved beneficial as inoculants. *Aspergillus awamori* and *A. niger* had shown twin advantage of being a cellulose decomposer and phosphate solubiliser and is frequently used as inoculant for bio-augmented composting. *Paecilomyces fusisporus* was found to be an efficient degrader of ligno-cellulosic wastes on the basis of cellulase activity and humus formation (Kapoor et al. 1978).

Lignin and cellulose is the most common constituent of agro-industrial waste and highly resistant to microbial attack. Therefore, lignolytic fungi as *Pleurotus sajor caju*, *Polyporus versicolor* and *Phanerochaete chrysosporium* are commonly preferred as inoculants for biological delignification of agrowastes.

8.5 Application of Lignocellulolytic Microorganisms for Value Addition to Agrowastes

8.5.1 Solid State Fermentation for Commercial Enzyme Production

The term solid-state fermentation (SSF) denotes cultivation of microorganisms on solid, moist substrates in absence of a free aqueous phase; that is, at average water activities (defined as the relative humidity of the gaseous phase in equilibrium with

Table 8.2 Industrial applications of enzymes produced by SSF processes

Process	Enzyme
Enzyme-assisted ensiling	Fungal cellulases and hemicellulases
Bioprocessing of crops and crop residues	Fungal cellulases and hemicellulases
Fibre processing (retting)	Fungal pectinases, cellulases and hemicellulases
Feed supplement	Amylases, proteases, lipases, cellulases, hemicellulases
Biopulping	Xylanases
Directed composting	Hydrolytic enzymes
Soil bioremediation	Laccases, ligninases
Post harvest residue decomposition	*Trichoderma harzianum* cellulases
Biopesticide	*T. harzianum* cellulose for helper function

the moist solid) significantly below 1 (Pandey 2003). The agrowastes can be used as substrates for the commercial production of various hydrolytic enzymes by using lignocelluloses degrading microorganisms. Currently, industrial demand for various enzymes is being met by production methods using submerged fermentation (SmF) processes. The cost of production in SmF systems is however high and it is uneconomical to use them in many of the aforesaid processes (Tengerdy 1996). This therefore necessitate reduction in production cost by deploying alternative methods, for example the SSF systems which can utilize cheap agroresidues as substrates. An efficient production method, employing a cheap, easily available substrate and a good cellulolytic organism, will reduce the cost of enzyme preparation and improve the economy of the overall cellulose bioconversion.

SSF processes simulate the living conditions of many higher filamentous fungi. Ascomycetes, basidiomycetes and deuteromycetes can be seen developing in terrestrial habitats on wet substrates. Higher fungi and their enzymes, spores or metabolites are well adjusted to growth on solid wet substrates. Table 8.2 lists some of the possible applications of the enzymes produced in SSF systems. This system offers numerous advantages over submerged fermentation (SmF) system, including high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipments, etc. (Hesseltine 1977; Pandey 1992; Chahal and Moo-Young 1981; Nigam and Singh 1994; Aidoo et al. 1982; Pandey 1991; Doelle et al. 1992).

Ideally, almost all the known microbial enzymes can be produced under SSF systems. Although most of the enzymes are produced by fungi, yeast as well as bacteria, filamentous fungi are preferred for commercial production using SSF as the levels of the enzyme produced by fungal cultures are higher than those obtained from yeast or bacteria.

8.5.2 Mushroom Cultivation and Spent Mushroom Substrate

Edible mushrooms have been cultivated since 600 A.D. when *Auricularia auricular* was first cultivated in China on wood logs but the biggest advance in mushroom cultivation came in France about 1600 A.D. when *Agaricus bisporus* was cultivated

upon a composted substrate. However, It is only over the past two to three decades that there has been major development in basic research and practical knowledge for the creation of a significant worldwide industry (Chang and Miles 1989). About 39 mushroom species (Chang 1999) have been cultivated all over the world commercially, and of these, around 20 are cultivated on an industrial scale. The most cultivated mushroom worldwide is *Agaricus bisporus* (button mushroom) followed by *Lentinus edodes* (shiitake), *Pleurotus spp* (oyster mushrooms), Auricula auricula (wood ear mushroom), *Flamulina velutipes* (winter mushroom), and *Volvariella volvacea* (straw mushroom). Techniques for the culture of some other less popular mushrooms were developed in China between 600 AD and 1800. Mushroom enzymes can break down lignin, cellulose and hemicellulose present in these organic materials into simpler molecules, which the mushrooms use for their growth and metabolism.

The ability of the different mushroom species to utilize various agroresidues depends on both mushroom-and substrate-associated factors. For example, examination of the lignocellulolytic enzymes profiles of the three important commercially cultivated mushrooms exhibit varying abilities to utilise different lignocellulosics as growth substrate. *Lentinula edodes* is cultivated on highly lignified substrates such as wood or sawdust, produces two extracellular enzymes (manganese peroxidase and laccase) which have been associated with lignin depolymerisation. Conversely, *Volvariella volvacea* prefers high cellulose- low lignin-containing substrates such as paddy straw and cotton wastes which have relatively low lignin content, and produces a family of cellulolytic enzymes including at least five endoglucanases, five cellobihydrolases and two ß-glucosidases, but none of the recognised lignin-degrading enzymes. *Pleurotus sajor-caju* is the most adaptable of the three species and can be grown on a wide variety of agricultural waste materials of differing composition in terms of polysaccharide/lignin ration, because it is able to excrete both kinds of cellulose- and lignin-degrading enzymes.

The lignocellulosic substrate used for mushroom production and which is left after harvesting of the mushrooms can be used as animal feed, soil conditioner, for mushroom recultivation, and for bioremediation, among other applications. Spent mushroom substrate besides being rich in nitrogenous material contains partly degraded lignocellulosic components, which when combined with animal dung or human excreta in a biogas digest would yield not only biogas but also a good quality organic nitrogenous fertilizer in the form of sludge. The sludge from the biogas plant as a nitrogenous fertilizer is far more beneficial than the compost from which it has been derived. Part of the biogas that is produced in the vicinity of the mushroom house can also be conveniently used for pasteurization of the mushroom bed material and maintenance of the optimal temperature in the mushroom house as well.

Some studies have been done on the use of spent mushroom substrate in vegetable and flower greenhouses (Lohr et al. 1984; Verdonck 1984; Steffen et al. 1994, 1995; Szmidt 1994; Söchting and Grabbe 1995; Celikel and Tuncay 1999), in field vegetable and fruit crops (Male 1981; Delver and Wertheim 1988; Pill et al. 1993; Stewart et al. 1998; AntSaoir et al. 2000; Batista et al. 2000), in nursery and landscape

gardening (Chong and Wickware 1989; Chong and Rinker 1994; Chong 1999), and in soil amendment (Wuest and Fahy 1991; Stewart et al. 2000). Polat et al. (2009) conducted a study to determine the effects of spent mushroom compost (SMC) on cucumber (*Cucumis sativus* L.) growth as an organic matter source for greenhouse soil and found that application of SMC (40 tons/ha) improves the cucumber growth and productivity significantly. Currently, there are some industries that manufacture and sell different kinds of compost based on spent mushroom substrate (http://www.nutrasoils.com, http://www.southmill.com, http://www.americanmushroom.org, http://www.laurelvalleysoils.com).

The potential of spent mushroom substrate to degrade organopollutants and its importance in the environmental bioremediation have also been reported (Kuo and Regan 1998; Eggen 1999; Semple et al. 2001; Webb et al. 2001; Lau et al. 2003; Law et al. 2003; Xawek et al. 2003).

Spent mushroom substrate has also been used as animal feed, since its degradation by the mushroom can improve its nutritional quality (Jalc et al. 1996a, 1996b; Adamovic et al. 1998; Díaz-Godínez and Sánchez 2002) and digestibility (Zadrazil 1977, 1996). Díaz-Godínez and Sánchez (2002) found that when maize straw spent compost generated after mushroom cultivation was added to the diets of sheep, it increased the weight gain of the sheep. Fresh spent mushroom substrate has high Na and K concentration which when applied to field crops sensitive to high salt content may cause phytotoxicity so the application of fresh SMS should be avoided in sensitive crops.

8.5.3 Nutrient Enriched Compost

Rapid process for preparation of a nutrient enriched compost can be achieved by using efficient lignocellulolytic fungi (e.g., *Trichoderma sp., Aspergillus awamori*, *Polyporous versicolor*, *Penicillium funiculosum*, *Phanerochaete chrysosporium* etc.), as compost accelerators (Gaur et al. 1982; Gaind et al. 2005; Lata and Pandey 2005). Bioinoculants such as P-solubilizers and free-living N_2 fixers can be employed to prepare phospho-compost using rock phosphate (12.5%) and pyrite (10%) along with plant residues. Zinc enriched compost can be prepared by incorporation of $ZnSO_4$ (0.1%) in crop residues before composting along with rock phosphate (1%). Likewise, fertilizer enriched compost can also be prepared by addition of urea (1%), pyrite (10%) and rock phosphate (12.5%). Addition of pyrite increases solubilization of rock phosphate by *A. awamori* and decreases loss of added N. In this manner, balanced manure with 2% N and 6% total P_2O_5 can be produced within 12 weeks of decomposition.

NADEP phospho-compost method is a process to prepare phosphorous enriched compost using farm waste, rock phosphate and phosphate solubilizing bacteria. Insoluble phosphorous present in rock phosphate is transformed into soluble form through action of P-solubilizing microorganisms during process of composting. Compost is prepared using farm wastes, cow dung and soil (8:1:0.5). Rock phosphate

is added to this mixture @ 12.5% w/w. This mixture is filled either in pit, or NADEP tank. Mixture is plastered with a mixture of dung and soil after adding sufficient water to moisten the decomposing mixture. The material is turned after 15 days and thereafter at an interval of 30 days. At each turning, water is added to maintain sufficient moisture. Compost becomes ready within 3–4 months and contains N 1%, P_2O_5 2–4% and K_2O 1–2%. On equal quantitative P_2O_5 basis, this compost @ 5 t/ha can substitute the use of phosphatic fertilizer in cereal crops.

Besides the use of cellulolytic microorganisms, inclusion of surface dwelling earthworm *Eisenia foetida* for bioconversion of waste is an efficient method of vermicomposting. Earthworms improve nutrient availability, increase the density of useful microorganisms and also produce plant growth regulators (Tomati et al., 1988, Pandey and Chaturvedi 1993; Doube et al. 1994).

8.6 Use of Compost for Sustainable Agriculture and Improving Soil Health

8.6.1 Effect on Soil Health

Proper use of composted organic residues on agricultural soils is known to improve nitrogen, phosphorus and sulfur essential for plant nutrition. Their role in improving soil organic matter content is well established (Gaind and Lata 2007; Magdoff and Van Es 2009; Marinari et al. 2000). Application of organic amendments not only influences soil health in terms of physical, chemical and biological parameters (Beffa et al. 1995; Yang et al. 2003), but also increases the size, biodiversity and activity of microbial populations (Perucci 1990; Bandick and Dick 1999; Peascock et al. 2001). Therefore, changes in the activity of microbial communities as a result of soil management practices can also be used as an indicator of soil health and its quality. All these parameters exert cumulative effect on soil health and these are discussed below.

8.6.1.1 Rhizosphere Microbial Population

Composts are increasingly used as environmentally safe bio-fertilizers in sustainable agriculture all over the world. They also contribute to soil vitality and sustainability and in the enhancement of microbial community or population level (Gomez et. al. 2006). Ammonia oxidation by autotrophic bacteria is a key process in agricultural and natural ecosystem as it plays an important role in the global nitrogen cycle. The presence of *Nitrosomonas* spp. has been reported in soil treated with compost (Innerebner et al. 2006). An increased bacterial population in the rhizosphere soil of cowpea has been reported by Zayed and Motaal (2005). The composts produced by inoculation of paddy straw with FYM when applied to soil resulted in proliferation

of total soil bacteria. The highest phosphate dissolving fungi were reported in rhizosphere soil of cowpea fertilized with composts inoculated with *A. niger* and *Trichoderma viride* (Zayed and Abdel-Motaal 2005). Similar results have also been reported by Badr El-Din et al. (2000), who found that application of compost inoculated with phosphate solubilizing fungi enriched the rhizosphere with fungi more than the other manure treatments. The highest phosphate dissolving bacterial population was found in rhizosphere soil of cowpea plants fertilized with composts inoculated with FYM. This showed that FYM was a good source of phosphate dissolving bacteria (Zayed and Abdel-Motaal 2005). Long term application of compost to soil does have effect on soil biota (Rose et al. 2006; Niemi et al. 2002).

The addition of organic material to soil may encourage microbial activity to an extended period that is closely related to the amount and nature of organic matter added Total bacterial population increased in the rhizosphere of tomato plants growing in soil treated with organic manure (FYM/compost), compared to control (Badr El-Din et al. 2000). Soil treated with compost produced by *T. viride* NRC_6 enriched the rhizosphere of tomato plants with total fungi as compared to mineral fertilization (Badr EL-Din, 2000).

8.6.1.2 Microbial Activity

Another measure of soil health is the activity of soil enzymes involved in the transformation of the principal nutrients and biodegradation of organic materials (Crecchio et al. 2001). Dehydrogenase activity which is considered as a marker of soil microbial activity has been reported to increase significantly with the addition of composted residue (Garcia et al. 1993; Tiquia et al. 2002a, b & 2005) This shows that application of composted residue induce the synthesis of enzyme without affecting the overall microbial activity (Nannipieri et al. 1990). The addition of compost improved the physical properties of soil including its porosity. Dehydrogenase activity is positively influenced by the high porosity of soil. Marinari et. al. (Marinari et al. 2000) reported high dehydrogenase activity in soil treated with manure and vermicompost. By using compost in moderate quantity on the same soil for 8 years the soil structure was moderately improved especially when the compost was applied on soil surface and after sowing of crop. The surface was thereby protected against the compressing effect of raindrops and a rapid drying afterwards similarly, water in-filtered into soil much faster in compost protected soil (Jakobsen 1995).

Application of 20 g compost per pot for growing *Faba bean* increased the soil respiration from 3.8 to 6.3 $\mu mol.m^2$ indicating increased microbial activity from compost addition (Abdelhamid et al. 2004). This may be due to decreased particle density, improved organic matter content and total organic carbon and nitrogen. Garcia et al. (2000) reported 200% increase in dehydrogenase activity of soil treated with compost @ 80 t/ha indicating an increase in the microbial metabolism in the soil. This may be due to mineralization of biodegradable carbon fractions contained in the amendments.

Similar reports of increasing dehydrogenase and microbial population have been made by Chang et al. (2007) and Pascual et al. (1998). They studied the effect of different doses of compost on vegetable crops for 3 consecutive years. A significant improvement in microbial activity was reported in compost treated soil compared to chemical fertilizer treated soil. However, the compost application rate higher than 540 kg N/ha/year did not result in any significantly increase in soil enzyme activities.

A significant and positive correlation of dehydrogenase activity with organic carbon and total nitrogen suggested that addition of organic manure to soil increased carbon turn over. Nitrogen availability and microbial activity which in turn led to greater enzyme synthesis and accumulation in soil matrix was reported by Dinesh et al. (1998). Enzyme activities such as arylsulphatase, dehydrogenase, and L-asparaginase have also been observed to increase with the addition of MSW compost, with application rates up to 90 Mg ha_1, while the activities of phosphodiesterase and phosphomonoesterase increased linearly with increasing application rates (Giusquiani et al. 1994). The enzyme activities of β-glucosidase and nitrate reductase have also been reported to increase with the addition of MSW compost when compared to a control (Crecchio et al. 2001).

8.6.1.3 Microbial Biomass

The application of organic amendment in the form of composted residue may help to increase soil organic matter and improve soil quality. Addition of organic matter improves soil structure by decreasing bulk density and increase microbial activities of soil (Giusquiani et al. 1995; Perucci 1992). Therefore, maturity of compost may significantly affect the carbon turnover in amended soil (Albiach et al. 2000). The other factors affecting carbon turn over are soil texture and pH. Microbial biomass was higher with application of compost and FYM @ 40 t/ha. An increase in microbial biomass is reported in the beginning of crop growth compared to maturity stage (Bouzaine et al. 2007). Compost application @ 20 and 80 t/ha increased microbial biomass by 10 and 46% respectively in soil under barely crop (Garcia-Gil et al. 2000). This is attributed to the microbial stimulation by added organic compost. However, the microbial biomass carbon (C_{mic}) is usually higher at the end of growing season (summer/autumn) than at the beginning. This could be due to elevated temperature and/or higher amount of rhizo-deposition and drying plant roots at the end of growing period. This also indicates that C_{mic} depends up temperature and available organic substrates (Powlson 1994). Application of compost prepared by using banana residue and effective microorganism technology also has shown encouraging results in improving the C_{mic} of soil under banana crop (Formowitz et al. 2008). Goyal et al. (2009) studied the effect of application of rice straw compost @5 t/ha along with half of the recommended dose of inorganic fertilizer on yield of rice and found that compost application increased the microbial biomass C from 136 to 258 mg/kg soil, dehydrogenase activity from 66 to 118 mg TPF/kg soil/24 h and alkaline phosphatase from 370 to 680 mg PN/kg soil/h. It also resulted in buildup of soil organic C and N from 0.471 and 0.039% to 0.545 and 0.064% respectively.

8.6.2 Enhancement of Crop Yields by Compost Application

Incorporation of compost in soil will not only protect the environment, but also take advantage of the nutrients and organic matter contained in the compost to enhance soil fertility and crop production. This is mainly attributed by improvement in soil physical, chemical and biological activities of soil due to compost application (Wander et al. 1994; Roberson et al. 1995). Large number of studies has been conducted to establish the beneficial effects of composts prepared from different agroresidues on the yields of different field and horticultural crops.

Onion (*Allium cepa* L.) yield on a sandy loam soil increased with increasing rate of organic matter application, when the organic matter was biosolids/straw compost, or digested or raw biosolids (Smith et al. 1992). Similarly, low rates of a vegetable waste and manure compost (3 Mg·ha–1) with fertilizer N at 75 kg·ha–1 significantly improved broccoli crop response and N use efficiency when compared to a fertilizer-only treatment of 150 kg·ha–1 N plus 50 kg·ha–1 P (Buchanan and Gliessman 1991). Increasing applications of compost alone (3, 7.5, and 30 Mg·ha-1) tended to increase broccoli yield and N accumulation, but decreased N use efficiency.

Maynard (1994) reported that yields of broccoli and cauliflower (*Brassica oleracea* L. Botrytis group) from unfertilized plots amended with a mixed compost (poultry manure, horse manure, spent mushroom compost, and sawdust) at 56 or 112 Mg ha^{-1} were similar to or greater than yields from plots fertilized with 150 N- 66P-125 K (kg ha^{-1}). They also reported that tomato and bell pepper fruit yields from plots amended with compost produced from poultry manure with other agricultural wastes were similar to or greater than yields from fertilized plots, except in one crop of tomatoes where they were lower.

Hue et al. (1994) conducted a pot study using a highly weathered Ultisol, for which it had been determined that P availability was the main plant nutritional limitation. Rates of yard trimming compost at 75% (by volume) or higher mixed with the soil increased corn growth, but lower rates did not have an effect as compared to corn grown in unamended pots.

Lawson et al. (1995) reported that soybeans (*Glycine max* L.) grown in acid or saline soil amended with 4% wood waste compost had improved nodulation and shoot growth when compared with those in unamended soil. In a study conducted by Kostov et al. (1996), application of compost (derived from vine branch, rice husks, and flax) significantly increased the yield of tomatoes and the quality of fruits compared to the soil treated with mineral fertilizers and manure. Shiralipour et al. (1996) observed that application of compost increased the height and dry weight of broccoli shoots and dry weight of lettuce shoots in several different soil textures tested by them.

A greenhouse pot study was conducted by Ribeiro et al. (2000) to evaluate the use of a municipal solid waste compost (MSWC) as a fertilizer for potted geranium. MSWC was mixed with a peat-based growing media at rates of 0%, 10%, 20%, 30%, 40%, and 50% by volume. Plants grew in those forms for 90 days, with no additional fertilization. Shoot dry weight, number of leaves per plant, number of

flower stems per plant, and number of flowers per flower stem were significantly affected by the percentage of MSWC, with greatest growth occurring at 10% and 20% MSWC. Experiments were conducted by Smith et al. (2001) to monitor the effects of compost application and organic fertilizer addition on the growth of two vegetable crops – Swiss chard (*Beta vulgaris* L. var. flavescens) and common bean (*P. vulgaris* L. var. nanus). In case of both plants, the soil–compost mixtures outperformed the soil alone irrespective of the amount of fertilizer added. Swiss chard produced the highest total leaf fresh mass on composts made from market and garden refuse. The yield was further significantly improved when the composts had been turned or when the proportion of compost was increased from 25% to 50%, but the addition of fertilizer had no significant effect on the total yield. The influence of maize-stover compost and nitrogen fertilizer on growth, shoot yield, and nutrient uptake of Amaranth (*Amaranthus cruentus* L) was studied by Akanbi and Togun (2002) over a period of 2 years. The plant growth parameters increased significantly with increasing levels of applied compost from 0 to 4.5 t/ha soil. Goyal et al., (2009) studied the application of rice straw compost @ 5 t/ha along with half of the recommended dose of inorganic fertilizer on yield of rice and found that the average of 3 years grain and straw yield of rice (Basmati and CSR-30) was comparable to the recommended dose of inorganic fertilizers.

Salem et al. (2010) reported that the marketable tuber yield, plant height and specific gravity were greater in compost amended soil than in non-amended soil even if inorganic fertilizers were added but application of 120 ton compost per hectare gave the highest total tubers number, marketable tuber yield, height and specific gravity. Similarly, Ravishankar et al. (2010) observed that the soils under different organic modules had significantly higher microbial population (bacteria, fungi and actinomycetes) and activities of urease, phosphatase, dehydrogenase and cellulases as compared to that under recommended dose of fertilizers (T_1) and a significant positive association between organic matter status, microbial populations and enzyme activities in soil was recorded. They concluded that application of FYM 20 kg/plant (T_2) was the best organic module with regard to higher microbial populations and enzyme activities in soil.

8.6.3 Restoration of Soil Organic Matter and Carbon Pool

During composting Carbon dioxide is emitted first in relatively large quantities during active phase of process, and then at a slower rate in the maturation phase. After application of mature compost to soil, inter-converting pools of carbon takes place and turnover rate is determined by local factors such as soil types, temperature and moisture of soil. Although a great deal of valuable information now exist on the turnover of soil organic carbon, the contribution of compost application to soil organic carbon pools remain somewhat problematic.

Smith et al. (1997) reported retention of 6–9% carbon from compost for over 100 years. Moreover, organic fertilization did not result in the permanent and

irreversible locking up of all the carbon in compost. But the application of organic matter/compost on a regular basis improved the level of soil organic carbon (SOC) in long term (Favoino and Hogg 2008). In a study carried out in Himalayan region of India by Verma and Sharma (2007), integrated use of NPK fertilizers along with organic amendments improved the sustainability of different cropping systems in the terms of total and labile carbon and soil aggregation. Several studies by Lal (2004a, b) on soil carbon sequestration and climatic change advocates use of compost/manure and other system of sustainable management to restore the SOC pools in marginal lands.

In china, Wu et al. (2004, 2005) studied the influence of fertilization and organic amendments on organic carbon fraction for 20 years. When manure was applied alone and in combination with N and P fertilizers, the light fraction of organic carbon (LFOC), and salty solution soluble organic carbon (SSOC) and microbial biomass carbon was increased significantly. A long term investigation was also carried out by Rudrappa et al. (2006) to study the influence of manure addition on different carbon fraction under intensive cropping sequence of maize-wheat crops in semi and sub tropical region of India. Integrated use of FYM with 100% NPK emerged as the most efficient management system in accumulating largest amount of organic carbon (72.1 Mg C ha^{-1}) in soil. Nevertheless, this treatment also sequestered highest amount of organic carbon (731 kg C ha^{-1} per year). Particulate organic carbon, a physically protected carbon pool in soil, could well be protected in subsurface soil layers as a means of carbon aggregations. Similarly, Majumdar et al. (2008) carried out an experiment for 11 years in rice wheat agro system to understand the influence of organic amendment in carbon pools. Results suggested that labile carbon fraction is a useful indicator for assuring soil health and balanced fertilization with FYM as suitable management practices for sustaining crop productivity of the rice-wheat system.

A long term field study in rice under flooded condition by Nayak et al. (2007) resulted in the stimulation of microbial biomass and soil enzymes. A 39% mean seasonal increase was observed in microbial biomass under treatment with compost application. Soil organic carbon content showed highly significant positive correlations with soil enzymes (dehydrogenase, urease, cellulase, FDA hydrolysis and β glycosidase).

The effects of organic manure and chemical fertilizers on total microbial biomass (C_T), water soluble organic carbon (C_{WS}), microbial biomass carbon (C_{MB}), labile carbon (C_L) was studied under wheat maize cropping system in long term experiments in North China plains by Gong et al. (2009). Application of organic manure was found to be more effective for increasing C_T, C_{NS}, C_{MB} and C_L as compared with application of chemical fertilizer alone. However, NPK treatment was important for increasing crop yield. It indicates that sustainability of the system can be maintained by integrated use of organic amendments and chemical fertilizers.

Organic manure addition in form of manure/compost/straw either alone or in combination with chemical fertilizers, appears to be more effective in maintaining or restoring organic matter and soil organic carbon pool in soil than chemical fertilizer alone (Jagadamma et al. 2009).

8.7 Conclusion

Agro wastes are the renewable resource which may be used economically for multiple purposes; Recycling of agro wastes in agriculture is one of the important options to improve soil health and fertility. Composting through efficient lignocellulolytic microorganisms offers an opportunity to recover and reuse the portions of organically bound nutrients present in the agro wastes. Composting is a dynamic process involving rapid succession of mixed microbial population which brings out mineralization of biomass components using different enzymes besides killing harmful pathogens and weed seeds. Novel approaches to improve the process of composting include amendment with nitrogen rich animal litter; organic/inorganic compounds and consortia of effective microorganisms have improved the composting process. Recent wave of organic farming has revolutionized the use of compost in diverse spheres of agriculture and made it an essential component in crop-production, crop-protection and natural resource management.

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Chapter 9
Genetic and Antigenic Diversity of Ruminant Pestiviruses: Implications for Diagnosis and Control

Niranjan Mishra

Abstract Pestiviruses are pathogens of major economic importance for the livestock industry worldwide and have significant influence in cattle, pig and sheep production. The genus *Pestivirus* in the family *Flaviviridae* contains four approved species, Bovine viral diarrhoea virus type 1 (BVDV-1), BVDV-2, Border disease virus (BDV) and Classical swine fever virus (CSFV) and one tentative species, giraffe pestivirus. The phylogenetic analysis of nucleotide sequences obtained from various region of genome has led to identification of 16 subtypes within BVDV-1, 2–4 subtypes within BVDV-2 and at least 7 subtypes within BDV species. Moreover, several new pestiviruses or new subtypes of pestiviruses have been identified recently both in domestic and wild ruminants thereby enhancing the genetic and antigenic diversity repertoire. This chapter will focus mainly on research involving genetic and antigenic diversity of ruminant pestiviruses since the wide genetic and antigenic differences between ruminant pestivirus isolates pose considerable challenges in diagnosis and control. Considering the large number of publications in recent years, it is possible to cite only selected reports. In the first part of the chapter I will provide an overview of pestivirus epidemiology, transmission, genome structure and current methods of diagnosis and control. In the following part of the chapter, critical analysis of genetic and antigenic diversity of existing and new ruminant pestiviruses will be carried out. Finally, I will discuss future perspectives in relation to ruminant pestivirus diversity vis-à-vis its implications for diagnosis and control.

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9.1 Introduction

Pestiviruses are animal pathogens of major economic importance for the livestock industry and have significant influence in cattle, pig and sheep production worldwide. The genus *Pestivirus* belongs to the family *Flaviviridae* and includes four recognized species: Bovine viral diarrhoea virus type 1 (BVDV-1), BVDV-2, Border disease virus (BDV) and Classical swine fever virus (CSFV) and a tentative species, Giraffe pestivirus (Thiel et al. 2005). However, several new pestiviruses and their subtypes have been reported since the seventh report of the International Committee on Taxonomy of Viruses, 2005 awaiting their formal classification. These include, atypical pestiviruses; HoBi (Schirrmeier et al. 2004), Brz/buf/9 (Stalder et al. 2005) and Th/04_KhonKaen (Stahl et al. 2007), Tunisian sheep (Thabti et al. 2005), caprine pestivirus (De Mia et al. 2005), pronghorn antelope (Vilcek et al. 2005), Bungowannah (Kirkland et al. 2007) and new subtypes within BDV, BDV-4, BDV-5, BDV-6, Turkey and BDV-7 (Arnal et al. 2004; Dubois et al. 2008; Ogozoglu et al. 2009; Giammarioli et al. 2010). Pestiviruses are not very strictly host specific and able to infect a wide range of ruminants, both domesticated and wild. The domesticated ruminants include cattle, buffalo, sheep, goats and yaks (Becher et al. 2003; Mishra et al. 2007a, 2008a) while free ranging ruminants include buffalo, eland, Canadian bison, alpaca, pudu, bongo, deer, roe deer, mousedeer, reindeer, giraffe, European bison, chamois and ponghorn antelope (reviewed in Vilcek and Nettleton 2006). Although under natural conditions BVDV infects mainly cattle, sheep and goats it also infects pigs, yaks and free-ranging ruminants. BDV infection is common in sheep but has also been found in goats, cattle, pigs and wild ruminants (Becher et al. 1997; De Mia et al. 2005). Except CSFV and newly recognized Bungowannah virus that infect pigs, other pestiviruses infect mostly ruminants. There is no conclusive evidence that pestiviruses spread from free-ranging animals to domestic ruminants while sufficient evidence exists regarding the spread in the opposite direction. Bovine viral diarrhoea virus causes mainly alimentary, respiratory and reproductive problems like pneumonia, diarrhoea, mucosal disease or abortion in cattle and small ruminants besides haemorrhagic syndrome and thrombocytopenia (Baker 1987). BDV infects sheep and goats causing mainly reproductive disease (Nettleton et al. 1998). Moreover, pestiviruses such as BVDV and BDV interact with their hosts differently as displayed by transient infection with immune response and persistent infection with immunotolerance (Baker 1987). Thus, pestiviruses are heterogeneous in genetic and antigenic properties, host spectrum, virulence and clinical signs providing a challenge in diagnosis and control. The aim of this chapter is not only summarise the available evidence in this field but also critically analyze the data to address problems with regard to pestivirus diagnosis and control.

9.2 Epidemiology, Diagnosis and Control of Ruminant Pestivirus Infections

9.2.1 Structure of Pestivirus Genome

The family *Flaviviridae* consists of three genera: *Flavivirus*, *Pestivirus* and *Hepacivirus*. Within *Flaviviridae*, pestiviruses show more similarity in the genome structure and mechanisms of translation initiation to the hepatitis C virus (HCV). The pestivirus genome consists of a positive-sense single-stranded RNA of about 12.3 kb in length that lacks a 5′cap and 3′ poly-A tract (Brock et al. 1992). The viral genome acts as a messenger RNA for protein translation, as a template during replication of RNA and as genetic material within virus particles. A long ORF flanked by untranslated regions at both ends (5′ and 3′ UTR) is translated into a poly-protein of about 4000 amino acids and is cleaved into four structural [capsid (C) and three envelope (E^{rns}, E1 and E2) proteins] and 7–8 nonstructural proteins (N^{pro}, P7, NS2-3 or NS2 and NS3, NS4A, NS4B, NS5A and NS5B) by viral and host cell proteases (Meyers and Thiel 1996). Cap-independent translation initiation of pestivirus genome is mediated by an internal ribosome entry site (IRES) located towards the end of 5′ UTR and initial part of N^{pro} (Poole et al. 1995). Since the 5′ UTR is the most highly conserved region in the pestivirus genome, nucleotide sequence analysis of this region has been widely used for pestivirus diagnosis and genetic typing. In addition, entire N^{pro} and E2 genes of the pestivirus genome are used for more defined phylogenetic analysis due to high level of nucleotide sequence variation (Ridpath et al. 1994; Wolfmeyer et al. 1997; Nagai et al. 2004; Vilcek et al. 2001; Hurtado et al. 2003; Becher et al. 1997, 2003; Mishra et al. 2007a, 2008b; Jackova et al. 2008).

The first protein in the ORF N^{pro}, a non-structural autoprotease is unique to pestiviruses only and is responsible for cleavage at the N^{pro}/C site (Stark et al. 1993). With the exception of the nucleocapsid protein (C), all the other structural proteins E^{rns}, E1 and E2 are glycoproteins and are part of the BVDV envelope. E2 is responsible for virus attachment, entry and generation of neutralizing antibodies (Donis and Dubovi 1987; Krey et al. 2005), while E^{rns} has the ability to bind to glycosaminoglycans and E1 is assumed to be a membrane anchor for E2 (Rumenapf et al. 1993). E^{rns} and E2 form disulfide-linked heterodimers, whereas E1 is found as heterodimers in association with E2 (Rumenapf et al. 1993). The humoral immune response allows detection of antibodies against the structural envelope proteins E2 and E^{rns} as well as against the highly conserved non-structural protein NS3 (p80).

Two biotypes of pestiviruses, cytopathic (cp) and noncytopathic (ncp) are recognized based on their effect in cultured epithelial cells (Gillespie et al. 1960). Biotypes do not correlate to the virulence of the virus in field, as BVDV strains associated with severe acute outbreaks are all ncp biotypes (Carman et al. 1998). The ncp biotypes are preponderant in nature and responsible for majority of disease syndromes including establishment of persistent infection. The cp viruses are relatively rare and cytopathogenicity is correlated with the appearance of non-structural protein

NS3 (p80) (Meyers and Thiel 1996). However, this hypothesis needs reconsideration in light of the recent studies providing evidence that NS2-3 gets cleaved into NS3 also in leukocytes of animals infected with ncp isolates (Kameyama et al. 2008; Mishra et al. 2010). The generation of a cp strain in an animal already persistently infected with ncp BVDV is considered as the crucial step for pathogenesis of mucosal disease.

9.2.2 Epidemiology

Among ruminant pestiviruses BVDV/BDV infections occur worldwide, while atypical pestivirus and other pestivirus infections occur sporadically. BVD is one of the economically important diseases of cattle and occurs worldwide. It has thus been listed recently under OIE list of priority cattle diseases for international trade. According to the latest data (WAHID Interface and OIE Animal Health Information 2010) outbreaks of BVD have been reported in the countries of Austria, Chile, Chinese Taipei, Colombia, Cuba, Cyprus, Denmark, Germany, Guatemala, Honduras, Hungary, Iran, Israel, Italy, Japan, Nicaragua, Russia, Spain and Switzerland in 2008. The number of BVD outbreaks recorded was 7,053 in Switzerland, 358 in Spain, 38 in Russia and 19 outbreaks in Iran. The combined economic impact of BVDV has been estimated at a 20–57 million dollar loss per million calving in the US. Based on the 2005 USA calf crop of 38 million, the cost of BVDV to USA producers was 760 million to 2.2 billion dollars in that year.

The manifestations of BVD infections are complex due to diversity in virulence of strains and host species (Baker 1987). Acute infection in seronegative immunecompetent cattle is common which ranges from subclinical to severe disease with mortality. Semen from bulls persistently infected with BVDV is infective to cows and causes repeat breeding, abortion and teratogenic defects in foetus. It can also cause immunosupression leading to increased susceptibility to co-infecting pathogens and vaccine failures against prevalent diseases. Persistently infected animals are the main source of transmission. Mucosal disease occurs when persistently infected animals are superinfected with antigenically related cytopathic strains or due to genetic rearrangements. The virus spreads between cattle mostly by direct contact. But vertical transmission plays an important role in the epidemiology of the disease. Although most of the transmissions occur from cattle to other species of ruminants, transmissions from PI sheep and goat to cattle have also been demonstrated. BVDV is also an important risk factor due to its presence in contaminated foetal calf sera, cell lines, biologicals and vaccines. Outbreak of BVD due to administration of a BVDV-2 contaminated live infectious bovine rhino-tracheitis (IBR) vaccine has been reported (Falcone et al. 1999, 2003).

BD, caused by BDV is principally a reproductive disease of sheep that shares many characteristics similar to BVD in cattle and occurs in several sheep rearing countries worldwide. Pestivirus infections in small ruminants can cause variety of clinical syndromes including reproductive failure, abortion, still birth, respiratory

disease, poor growth rate, diarrhoea, nervous signs and muscular tremor resulting in economic losses (Nettleton et al. 1998). Transmission of infection between small ruminants and cattle in both ways has been demonstrated though usually it is from cattle to sheep or goats. However, BD in sheep and goats can be caused by BDV, BVDV-1 and BVDV-2. Additionally, BDV has recently been detected in cattle under natural conditions in Austria (Hornberg et al. 2009) and U.K. (Strong et al. 2010). Different species of pestiviruses predominate in different countries depending on the close contact among ruminants. Border disease is caused by BVDV-1 in Norway and Sweden (Sandvik et al. 2002), BDV in Australia, New Zealand and Spain (Valdazo-Gonzalez et al. 2008; Vilcek et al. 1998), BDV and BVDV-1 in U.K. (Willoughby et al. 2006), BDV, BVDV-1 and BVDV-2 in USA (Sullivan et al. 1997).

In addition, atypical pestivirus infections have been detected in foetal calf serum from Brazil (Schirrmeier et al. 2004), Brazilian buffalo (Stalder et al. 2005) and cattle in Thailand (Stahl et al. 2007). A new pestivirus, called pronghorn antelope has been detected from a diseased pronghorn antelope in U.S.A. (Vilcek et al. 2005). Though pestiviruses do not cause human infections, inter animal species transmission is common and due to close relationship with Hepatitis C virus in humans BVDV has often been used as a surrogate model for studying antiviral drugs and unraveling the mysteries of persistent infection pathogenesis.

9.2.3 Current Methods of Diagnosis

Presently diagnosis of pestivirus infections is carried out on the basis of identification of agent or by demonstrating seroconversion in infected animals. Persistently viraemic animals resulting from congenital infection can be readily identified by isolation of noncytopathogenic virus in cell cultures from blood or serum. Immune-labelling methods such as immunoperoxidase test or immunofluorescence test are then used to detect the growth of virus in the cultures. Alternatively, direct detection of viral antigen in leukocytes is carried out by pestivirus antigen capture ELISA (PACE) or in ear notch by immunohistochemistry or ELISA. Microplate immunoperoxidase assay is usually used for mass screening of animals for persistent infection. But these methods have several limitations such as varying sensitivity and long time required for test results. Moreover, colostral antibodies can mask the presence of virus in PI animals and complicate their detection. Persistence of virus should be confirmed by resampling after an interval of at least 3 weeks and P.I. animals usually have no or low levels of antibodies to BVDV/BDV. Acute infections can be detected in leukocytes by virus isolation or by RT-PCR and rarely by PACE. Antibody to BVDV can be detected in sera of animals by virus neutralization test or ELISA. Acute infection with BVDV is best confirmed by demonstrating seroconversion using sequential paired samples (acute and convalescent) from several animals in the group.

In comparison to virus isolation in cell culture, the viral nucleic acid can be detected earlier after infection and for a longer period in BVDV infected cattle.

RT-PCR using pooled serum and milk samples has been found useful to identify PI animals in dairy and beef herds in many countries. The viral RNA in leukocytes, serum and tissues can also be detected by RT-PCR and real time RT-PCR. A large number of standard RT-PCR protocols for diagnosis of BVD have been published and are in use in many laboratories. The most widespread protocol uses primers 324/326 (Vilcek et al. 1994) that amplifies a 288 bp fragment of the 5′ un-translated region (5`-UTR) and is pestivirus specific. Then a nested PCR is used to differentiate between BVDV-1, BVDV-2 and BDV as all the three can infect ruminants and produce similar clinical manifestations. Though a number of RT-PCR and nested RT-PCR assays have been developed targeting highly conserved 5′ UTR of the genome for use in ruminants, the post processing analysis such as agarose gel analysis of amplification products increases the risk of contamination. The real time RT-PCR system is based on the detection and quantitation of a fluorescent dye. So the quantitation and genotyping of pestiviruses can be achieved simultaneously. As the results can be obtained faster due to real time visualization of data, it is useful not only during disease outbreak in herds of valuable livestock but also for routine diagnosis. Several real time PCR assays, both in uniplex or in duplex formats, are now available commercially and real time RT-PCR is now increasingly being used for diagnosis and genetic typing of ruminant pestiviruses with primers and probes targeted to 5′ UTR (Baxi et al. 2006; Willoughby et al. 2006; Liu et al. 2008).

Genetic typing of ruminant pestiviruses is important for the purpose of control and epidemiology. The more precise genetic typing of pestiviruses has been obtained from the application of molecular genetic methods such as, reverse transcription-polymerase chain reaction (RT-PCR) and sequencing coupled with computer-assisted phylogenetic analysis. Although sequence analysis of 5′UTR can be used for the segregation of pestivirus isolates into the four established species, segregation into types and subtypes is better accomplished by sequence analysis of complete genes coding N^{pro} and E2 proteins (Becher et al. 1999, 2003; Vilcek et al. 2001, 2004). Antigenic typing of ruminant pestiviruses can be achieved by using monoclonal antibodies specific to various species (Paton et al. 1995). Antigenic relationship of pestiviruses studied by cross neutralization using defined serological reagents has also been used as an additional criterion for demarcating pestivirus types. However, there is little correlation between pestivirus subtypes and course of the disease.

9.2.4 Control and Surveillance

The control of ruminant pestivirus infections depends on their economic impact and varies according to their pathogenicity and economic conditions of the country in question. The major economic damage due to BVDV is caused by reproductive problems and losses while some acute infections lead to pronounced disease signs and mortality. There are only few countries having official guidelines regarding BVD control while none for BD. There is a concern regarding control of BVD in cattle in industrialized nations while most developing and underdeveloped countries live with the infection.

BVD control is broadly implemented by two methods, preventive vaccination and/or removal of PI viraemic animals from the herd. Vaccination has still remained a major practical measure against BVD and several modified live and inactivated vaccines are available commercially in industrialized nations. Live vaccines should not be used in pregnant animals as the virus can cross the placenta and infect the fetus. As most of the live virus vaccines use cp strains, they should not be used in PI animals. For inactivated BVDV vaccines, there is also need of a booster vaccination for providing adequate immunity thereby increasing the cost of control. Vaccination is primarily aimed at preventing diaplacental and fetal infections effectively by vaccination of seronegative female animals. But even with absolute fetal protection by vaccination, PI animals still can pass BVDV to their offspring (Zimmer et al. 2002). Hence, control of BVD by vaccination of immunologically naïve animals has not been very successful. The considerable degree of antigenic diversity among BVDV strains provides another challenge to effective vaccination. The pronounced antigenic differences between BVDV-1 and BVDV-2 isolates is important for diagnosis and control as prior exposure with BVDV-1 either through infection or through vaccination does not protect against infection with BVDV-2.

As the most important aspect of BVD control is identification and removal of PI animals, Scandinavian countries have been extremely successful in reducing the infection by following this method of BVD control (Lindberg et al. 2006). With continuous improvement in performance of the diagnostic tests and development of diagnostic tests for large scale testing this method of control may be feasible in other countries not practicing vaccination. The Scandinavian model of BVD control envisages identification of infected and non-infected herds, surveillance and certification of non-infected herds followed by removal of infected animals. However, this model has taken more than a decade to reach the final phase of BVD eradication. An alternate recent model of BVD control is in practice in Switzerland with support from farmers' associations where antibody prevalence levels are high (Presi and Heim 2010). The model proposes individual identification and removal of PI animals simultaneously across the country, movement restrictions, education of farmers without undertaking initial antibody screening. Within 3 years of its implementation, the Swiss programme aims to reach the final phase of BVD eradication.

9.3 Genetic and Antigenic Diversity

9.3.1 Genetic and Antigenic Diversity of BVDV-1

The highly fatal outbreaks of cattle in USA and Canada during 1980s led to the identification of BVDV-2 and consequently classical BVDV was renamed as BVDV-1 and both BVDV-1 and BVDV-2 were considered as two separate species within pestivirus genus (Ridpath et al. 1994; Pellerin et al. 1994). BVDV-1 occurs worldwide and more frequently. Initially, two subtypes of BVDV-1, BVDV-1a

(NADL like) and BVDV-1b (Osloss like) were reported (Pellerin et al. 1994; Harasawa 1994). As more isolates in different countries around the world were characterized genetically and antigenically, the diversity of BVDV-1 subtypes expanded further. On the basis of phylogenetic analysis, Baule et al. (1997) and Becher et al. (1999) reported 3–5 subtypes within BVDV-1. A more comprehensive study of genetic analysis of BVDV-1 isolates originating from different countries revealed that BVDV-1 could be divided into 11 subtypes (Vilcek et al. 2001). More recently, 15 subtypes (BVDV-1a to BVDV-1o) within BVDV-1 have been reported with another new probable subtype (BVDV-1p) reported from China revealing extensive genetic heterogeneity (Vilcek et al. 2004; Yesilbag et al. 2008; Jackova et al. 2008; Xue et al. 2010) (Fig. 9.1).

Distinct pattern of distribution of BVDV-1 subtypes have been reported in diverse geographical areas and environments around the world. BVDV-1a and BVDV-1b subtypes have predominantly been found circulating in Americas, U. K., Ireland, Spain, Korea and India (Graham et al. 2001; Vilcek et al. 2001; Arias et al. 2003; Mishra et al. 2004; Fulton et al. 2005; Oem et al. 2009). BVDV-1c is the predominant subtype in Australia (Mahony et al. 2005) but has also been found in Germany (Becher et al. 1997), Spain (Arias et al. 2003), Japan (Nagai et al. 2008) and India (Mishra et al. 2008a). Similarly, BVDV-1d and BVDV-1f subtypes have predominantly been found in some European countries (Toplak et al. 2004; Uttenthal et al. 2005) while BVDV-1g is restricted to Slovenia (Toplak et al. 2004). At least five subtypes of BVDV-1, BVDV-1a, 1b, 1d, 1e and 1l have been identified in France, with subtype BVDV-1e predominating (Jackova et al. 2008). BVDV-1l subtype has been predominantly found in Turkey along with other BVDV-1 subtypes commonly found in Europe, BVDV-1a, BVDV-1b, BVDV-1d, BVDV-1f and BVDV-1h (Yesilbag et al. 2008). In contrast to other countries in Europe, all the cattle isolates from Switzerland to date belong to BVDV-1 only with subtypes of BVDV-1b, BVDV-1e, BVDV-1h and BVDV-1k evenly distributed (Bachofen et al. 2008). Interestingly, the degree of genetic diversity differed significantly between different subtypes as viruses of BVDV-1e were found to be genetically more diverse in comparison to viruses of BVDV-1h. High genetic diversity of BVDV-1 viruses has been found in Japan with six BVDV-1 subtypes (1a, 1b, 1c, 1j, 1n, 1o) including two new subtypes, BVDV-1n and BVDV-1o (Nagai et al. 2008). Till date, the highest genetic diversity of BVDV-1 isolates have been found in Austria where at least eight subtypes, BVDV-1a, 1b, 1d, 1e, 1f, 1g, 1h and 1k have been identified with subtype BVDV-1f and BVDV-1h predominating (Hornberg et al. 2009). BVDV-1m subtype has been predominantly found in China alongwith a new probable subtype, BVDV-1p (Xue et al. 2010). A number of strains belonging to various BVDV-1 subtypes have also been identified in wild ruminants. The most predominant subtype, BVDV-1b has been found in Canadian bison, alpaca, pudu and bongo while BVDV-1a has been identified in Canadian bison, BVDV-1c and BVDV-1j in deer, BVDV-1d in roe deer and BVDV-1f in mousedeer (reviewed in Vilcek and Nettleton 2006). However, more definitive genetic diversity of BVDV-1 isolates will be revealed when more isolates are characterized genetically in future as phylogenetic grouping is useful in evolutional and epidemiological history of pestiviruses, to trace the origin of new outbreaks and provide a basis for effective control programmes.

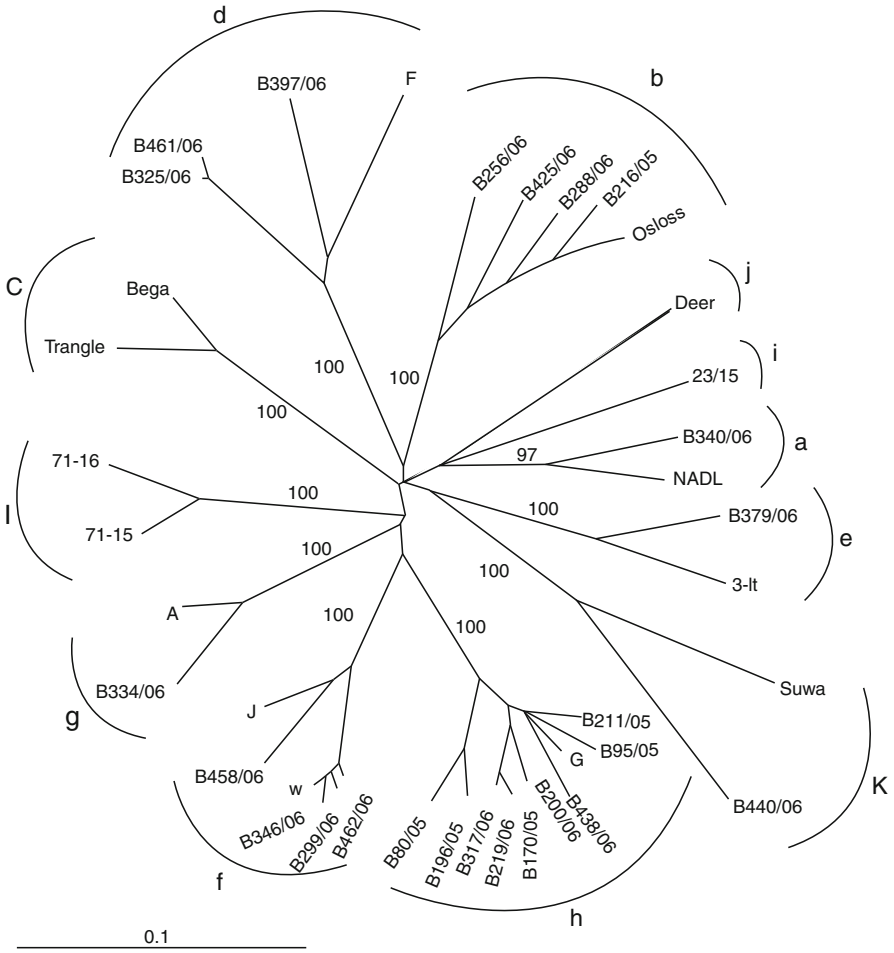


Fig. 9.1 Genetic diversity of BVDV-1 isolates (The phylogenetic tree was constructed from 392 bp nucleotide sequences from the N-terminal part of N^{pro}. The Austrian isolates analysed in this work are labelled in bold. Other sequences were taken from the NCBI GenBank with the following accession numbers: BVDV-1a – NADL (M31182); BVDV-1b – Osloss (M96687); BVDV-1c – Bega (AF049221), Trangie (AF049222); BVDV-1d – F (AF287284); BVDV-1e – 3-It (AF287282); BVDV-1f – J (AF287286), W (AF287290); BVDV-1g – A (AF287283); BVDV-1h – G (AF287285); BVDV-1i – 23–15 (AF287279); BVDV-1j – Deer (U80902); BVDV-1k – CH-Suwa (AY894998). The tree was constructed using PHYLIP Dnadist, Neighbor and Consense programmes (Felsenstein 2005). The bootstrap values presented in percentage supporting particular branch were computed with the Seqboot programme for 1,000 replicates. Reprinted from Veterinary Microbiology, 135 (3–4), Hornberg et al. 2009, Genetic diversity of pestivirus isolates in cattle from Western Austria, pp. 205–213 (2008) with permission from Elsevier)

Results of cross neutralization tests employing antisera raised to different pestivirus strains have shown that BVDV-1 strains are poorly or not neutralized by antisera raised against BVDV-2, BDV, CSFV and Giraffe (Becher et al. 2003). In general, several studies have shown that BVDV-1 isolates are antigenically closely

related to each other than to BVDV-2. However, in some cases, the antigenic differences between BVDV-1 strains of different subtypes can be similar to that found between BVDV-1 and BVDV-2 and such strong antigenic differences should be accounted for in development of vaccines and implementation of control program. Significant antigenic differences between BVDV-1a and BVDV-1b subtypes have been reported (Avalos-Ramirez et al. 2001; Becher et al. 2003). This was further substantiated by a study in U.S.A. showing that current vaccines containing only BVDV-1a strains induce lower BVDV-1b neutralizing antibody titre that may lead to inadequate protection against other subtypes (Fulton et al. 2003). Significant antigenic differences among some of the BVDV-1 subtypes prevalent in Chile (BVDV-1a, BVDV-1b, BVDV-1c) and Switzerland (BVDV-1e and BVDV-1k) but lower than that between BVDV-1 and BVDV-2 have been reported (Pizarro Lucero et al. 2006; Bachofen et al. 2008). Similarly, a marked antigenic difference was also observed between strains of subtype BVDV-1n, BVDV-1o and other subtypes BVDV-1a, BVDV-1b, BVDV-1c and BVDV-1j in Japan (Nagai et al. 2008). Antigenic differences between BVDV-1a, BVDV-1b and BVDV-1c strains prevalent in USA and Australia have also been demonstrated recently (Ridpath et al. 2010). But comprehensive cross neutralization data among all genetic subtypes of BVDV-1 are still lacking.

9.3.2 Genetic and Antigenic Diversity of BVDV-2

BVDV-2, first detected in cattle of USA and Canada was found later in several other countries of South America, Europe and Asia (Ridpath et al. 1994; Pellerin et al. 1994; Wolfmeyer et al. 1997; Vilcek et al. 2001; Couvreur et al. 2002; Flores et al. 2002; Nagai et al. 2004; Oem et al. 2009). On the basis of phylogenetic analysis, BVDV-2 has been divided into four (2a–2d) subtypes (Becher et al. 1999; Giangaspero et al. 2001; Tajima et al. 2001; Flores et al. 2002; Novackova et al. 2008). However, the subdivision of BVDV-2 into four subtypes on the basis of palindromic nucleotide substitution method of 5′ UTR sequences (Giangaspero et al. 2001) has not been validated by structural and nonstructural protein coding gene sequence analysis. Moreover, three German BVDV strains were classified as BVDV-2c on the basis of partial E2 gene sequence analysis (Tajima et al. 2001). Hence, on the basis of complete N^{pro} and E2 gene sequence analysis, only two subtypes (BVDV-2a & 2b) have thus far been identified (Becher et al. 1999; 2003; Mishra et al. 2008b; Novackova et al. 2008). BVDV-2 isolates in America, Europe and Asia are mostly of BVDV-2a subtype while members of BVDV-2b have been detected mainly in South America and sporadically in Europe and Asia (Becher et al. 1999; Vilcek et al. 2001; Flores et al. 2002; Ridpath et al. 2006; Barros et al. 2006; Mishra et al. 2008b; Novackova et al. 2009). In cattle, strains of BVDV-2b subtype were earlier found prevalent only in South American countries of Brazil and Argentina and later in Slovakia and Portugal whereas subtype BVDV-2a was

found prevalent in several countries of North America, Europe and Asia. Majority of the ovine BVDV-2 isolates belong to subtype a (Sullivan et al. 1994; Pratelli et al. 2001) while occurrence of BVDV-2b subtype in sheep has been reported from India (Mishra et al. 2008b) and Turkey (Yesilbag et al. 2008), which provides the evidence that both the subtypes of BVDV-2 can occur in sheep as in cattle. However, in goats only BVDV-2a subtype has yet been identified (Mishra et al. 2007a). It appears that BVDV-2 isolates are genetically less diverse compared to BVDV-1. Moreover, BVDV-2 has not been detected in wild animals unlike BVDV-1 and BDV. In contrast to the BVDV-1, significant antigenic diversity has not been found between BVDV-2 subtypes. Continued surveillance and characterization of BVDV-2 isolates in various countries will reveal additional diversity in future.

9.3.3 Genetic and Antigenic Diversity of BDV

Historically, the pestiviruses are named after the host species and the diseases they cause. The natural hosts of BDV are sheep and goats while natural infection of cattle and pig has also been reported (Nettleton et al. 1998; Strong et al. 2010). BDV was identified initially in sheep and when more BDV strains were analyzed, it led to reclassification of BDV strains into at least seven subtypes within BDV species (Vilcek et al. 1998; Becher et al. 2003; Arnal et al. 2004; Dubois et al. 2008; Ogozoglu et al. 2009). The more defined phylogenetic analysis classified the earlier identified BDV strains in U.K., U.S.A., Australia and New Zealand, including the BDV reference strains as BDV-1 subtype (Becher et al. 2003). A pestivirus identified in reindeer was typed initially as a separate genotype, but when more isolates were analyzed it was reclassified into BDV-2 subtype along with other German ovine strains (Becher et al. 2003). Furthermore, additional diversity was displayed when BDV-1 isolates could be divided into BDV-1a and BDV-1b and BDV-2 isolates into BDV-2a and BDV-2b (Becher et al. 2003; Strong et al. 2010). BDV-3 was initially identified in sheep in Germany, but also found in sheep in Switzerland and Austria and in cattle in Austria (Stalder et al. 2005; Krametter-Froetscher et al. 2007; Dubois et al. 2008). The BDV-4 subtype was first identified in Pyrenian chamois while investigating causes of decline in chamois population and later also in ovines in Spain (Arnal et al. 2004; Valdazo-Gonzalez et al. 2007). Recent studies have identified BDV-5 and BDV-6 subtypes in France (Dubois et al. 2008) and isolates from Turkey have been proposed to belong to a new subtype that awaits full characterization (Ogozoglu et al. 2009). In France, different subtypes of BDV have been identified even in same rearing area. In addition, pestiviruses belonging to an intermediate group, which is genetically close to CSFV but antigenically related to BDV, have been isolated from sheep in Tunisia (Thabti et al. 2005). Some of the French isolates have been classified recently also into this group. A novel caprine BDV isolate identified in Italy in 2005, was assigned to a novel pestivirus subgroup. However, recently analyzed ovine BDV strains in Italy alongwith the earlier caprine

strain have been classified into a new subtype, BDV-7 (Giammarioli et al. 2010). Among various subtypes of BDV, BDV-1 and BDV-3 subtypes have also been identified in cattle. Interestingly, BDV has also been isolated from wild animals. These include BDV-2 from reindeer and European bison in Germany and BDV-4 from chamois in Spain. Hence, the genetic diversity of BDV is greater than other species of pestiviruses and needs more efforts for their authentic classification (Fig. 9.2).

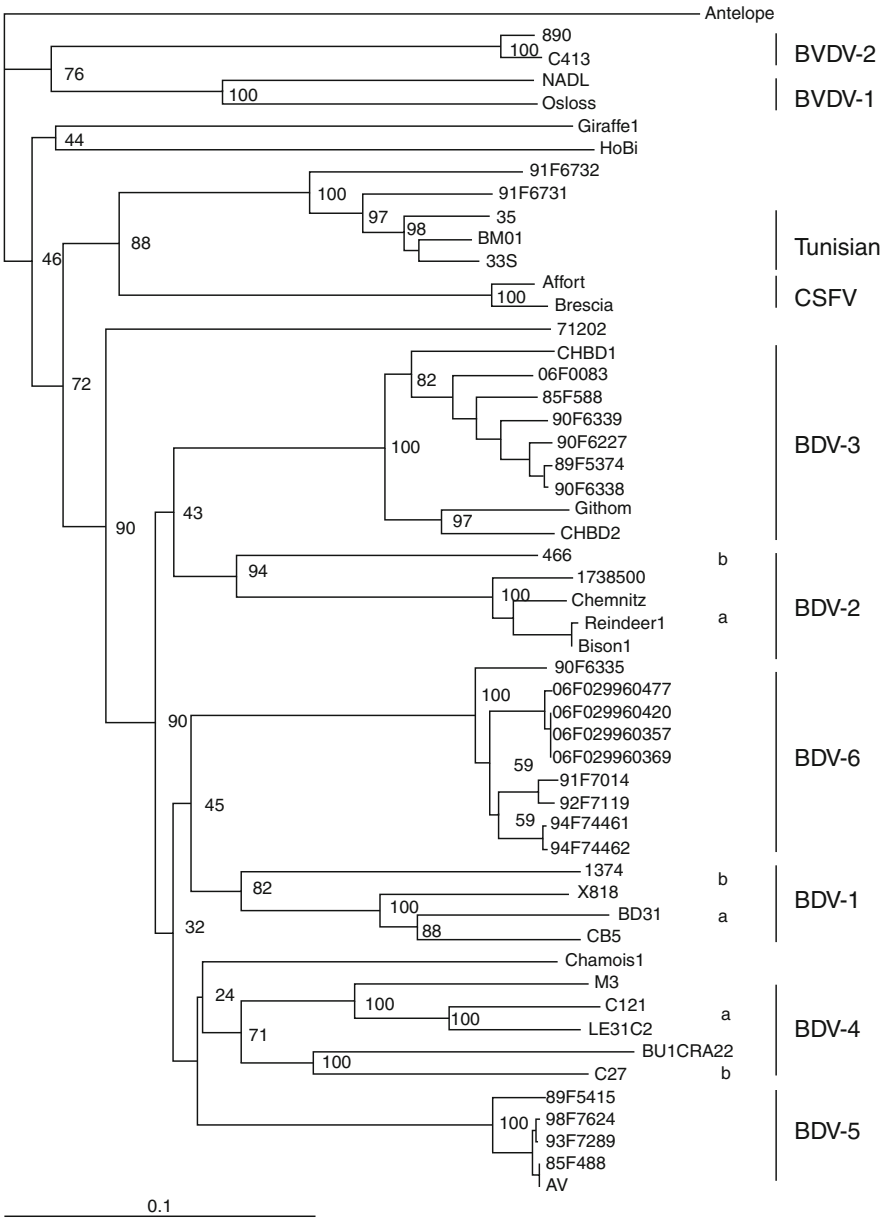
In concert with genetic diversity, significant antigenic differences have been found between BDV-1, BDV-2 and BDV-3 subtypes and also between subgroups within BDV-1 (Becher et al. 2003). The antisera raised against BDV-1, BDV-2 and BDV-3 neutralized heterologous BDV strains and CSFV to same extent while BVDV-1 and BVDV-2 were poorly or not neutralized. Similarly, a higher homologous neutralizing titre was found in chamois naturally infected with BDV-4 in comparison to heterologous viruses, BDV-1 and BDV-2. But antigenic relationship between BDV-4, BDV-5, BDV-6 and BDV-7 and other BDV subtypes is yet to be determined.

9.3.4 Diversity of Atypical and New Pestiviruses

When the complete nucleotide sequence data of Giraffe-1 isolate (H 138) became available, the results suggested that it represented a new pestivirus species, pestivirus of Giraffe. A further virus strain (PG-2) isolated from a bovine cell culture was also clustered into the phylogenetic lineage represented by the Giraffe isolate (Becher et al. 2003). This novel group of pestivirus was also supported by studies on antigenic relationship (Becher et al. 2003) and hence pestivirus of Giraffe was recognized as a tentative species within pestivirus genus (Thiel et al. 2005).

Additional pestivirus diversity became apparent when a preliminary antigenic study of a virus isolated from a dead pronghorn antelope, a new world wild animal found in USA indicated the possible involvement of a pestivirus. Then phylogenetic analysis of 5′UTR, N^{pro} and E2 genes clearly demonstrated that it belonged to an additional highly

Fig. 9.2 Genetic diversity of BDV isolates (Neighbour-joining phylogenetic tree constructed using 489 nt from the Npro region of the pestivirus sequences found during this study and from the database. Sequences taken from GenBank database with the following accession numbers: Antelope, AY781152; 890, U18059; C413, AF002227; NADL, NC001461; Osloss; Giraffe, NC003678; HoBi, AY735486; 35, AF462014; BM01, AY452482; 33S, AF462015; Alfort, X87939; Brescia, AF091661; 71202, AJ829444; CHBD1, AY895008; Gifhorn, AY163653; CHBD2, AY895009; 466, AY163650; 1738500, AY163651; Chemnitz, AY163652; Reindeer1, NC003677; Bison1, AF144476; 1374, L05402; X818, AF037405; BD31, U70263; CB5, AF145358; Chamois1, AY738083; M3, DQ273163; C121, DQ273159; LE31C2, DQ273161; BU1CRA22, DQ273155; C27, DQ273156. The tree was outgrouped to the sequence of the Antelope pestivirus. The numbers close to the major nodes indicate the bootstrap values (in %; 1,000 replicates). Bar: number of substitutions per site. Nomenclature of species, genotype and subtype were described according to Becher et al. (2003) and Valdazo-González et al. (2007). Reprinted from Veterinary Microbiology, 130 (1–2), Dubois et al. 2008, Genetic characterization of ovine pestiviruses isolated in France, between 1985 and 2006, pp. 69–79 (2008) with permission from Elsevier)


Antelope
890
C413
BVDV-2
NADL
Osloss
BVDV-1
Giraffe1
HoBi
91F6732
91F6731
35
BM01
33S
Tunisian
Affort
Brescia
CSFV
71202
CHBD1
06F0083
85F588
90F6339
90F6227
89F5374
90F6338
Githom
CHBD2
BDV-3
466
1738500
Chemnitz
Reindeer1
Bison1
b
a
BDV-2
90F6335
06F029960477
06F029960420
06F029960357
06F029960369
91F7014
92F7119
94F74461
94F74462
BDV-6
1374
X818
BD31
CB5
b
a
BDV-1
Chamois1
M3
C121
LE31C2
BU1CRA22
C27
a
b
BDV-4
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98F7624
93F7289
85F488
AV
BDV-5
0.1

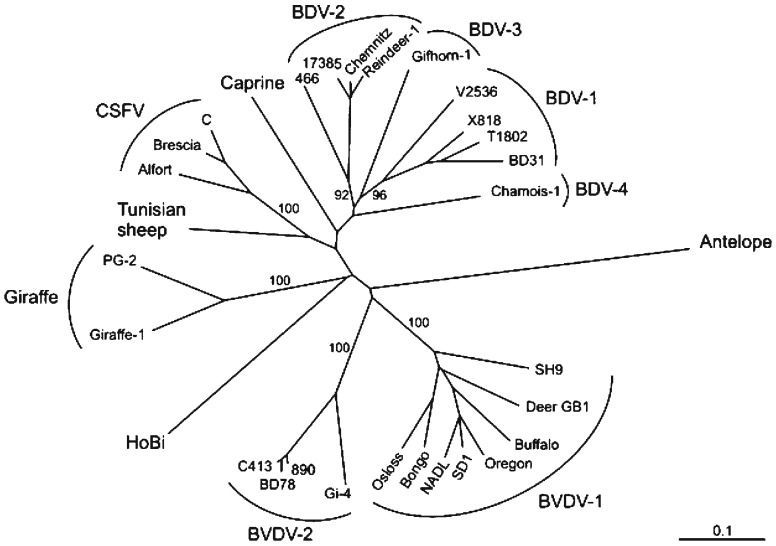

Fig. 9.3 Genetic diversity of pestiviruses (The phylogenetic tree was prepared with nucleotide sequences of the entire N^{Pro} region using a neighbour-joining method. Reprinted from veterinary microbiology, 116 (1–3), Vilcek and Nettleton, Pestiviruses in wild animals, pp. 1–12 (2006) with permission from Elsevier)

divergent pestivirus genotype (Vilcek et al. 2005). However, cross neutralization between the pronghorn isolate and BVDV-1, BVDV-2 and BDV-1 was noticed.

Novel atypical pestiviruses have been detected recently in batches of foetal calf serum and in cattle infected naturally. An atypical pestivirus, "HoBi" was isolated from a batch of FCS originating from Brazil and was proposed to be member of sixth pestivirus species (Schirrmeier et al. 2004) (Fig. 9.3). Interestingly, this virus could not be identified using well known pan-pestivirus primer pair 324/326. Additionally, Brz buf 9 has been isolated from a Brazilian buffalo and CH-KaHo/cont has been isolated from a contaminated cell culture (Stalder et al. 2005), while Th/04_KhonKaen was isolated from naturally infected cattle in Thailand (Stahl et al. 2007). The phylogenetic analysis of these atypical pestiviruses showed that they belong to a monophyletic clade, that is closely related to BVDV-1 and BVDV-2 clades and proposed to be members of BVDV-3 (Liu et al. 2009). Additionally, two subtypes within BVDV-3, BVDV-3a and BVDV-3b could be recognized (Liu et al. 2009). Interestingly, all three members of BVDV-3a subtype were derived from foetal calf serum while member of BVDV-3b subtype were derived from a naturally infected calf. However, no clinical signs have been observed in calves naturally or experimentally infected with atypical pestiviruses. The evidence of atypical pestiviruses as a separate group within pestivirus genus was also provided when the results of cross neutralization tests performed on sera from cattle in the herd of the atypical pestivirus infected calf in Thailand showed a higher neutralization titre against atypical pestivirus HoBi than BVDV-1, BVDV-2 and BDV.

9.4 Future Perspectives

9.4.1 Challenges for Pestivirus Diagnosis

The genetic and antigenic diversity of pestiviruses, diverse host range and clinical outcomes provide a real challenge for both laboratory diagnosis and clinical diagnosis in future. Furthermore, pestiviruses are commonly found as contaminants in foetal calf serum, cell lines, live attenuated vaccines and other biological products prepared for human and animal use. Virus isolation is also at risk from cross contamination from laboratory handling. Hence, identification of any new pestivirus or pestivirus in an unusual host should be reported with caution. Moreover, the serological cross reactivity complicates differentiation of pestivirus infections through serological assays. No uniform approach is currently in practice with regard to selection of correct virus for use in serological studies, selection of commercial antigen ELISA kits for identification of all ruminant pestiviruses, selection and correct use of monoclonal antibodies in antigenic typing and the use of correct cells for virus isolation. The problem has further been compounded by failure of some antigen ELISA kits or immunohistochemistry that use Mabs against E^{rns} antigen in detecting all the ruminant pestiviruses. Again, many ELISA kits used for screening of PI animals have not been validated for all ruminant pestiviruses and in different hosts. Tests for pestivirus diagnosis should also be assessed for their ability to distinguish between acute and persistent infections. These problems need to be addressed in future for accurate and quick pestivirus diagnosis.

Although genetic methods such as RT-PCR and real time RT-PCR have increasingly been used for pestivirus diagnosis, problems have also been encountered in selection of primers and probes to detect existing and new pestiviruses. It has been reported that due to the genetic differences, RT-PCR using pan-pestivirus specific primer pair 324/326 failed to detect atypical pestiviruses such as HoBi, although these primers could recognize all pestiviruses analyzed so far (Schirrmeier et al. 2004). At least three different sets of primers and probes are required currently in real time RT-PCR for accurate genetic typing of BVDV-1, BVDV-2, BDV and atypical pestiviruses in ruminants. The resultant enhanced cost of real time RT-PCR for routine laboratory diagnosis has been a limiting factor in carrying out surveillance specially in developing and less developed countries. Hence, a general pestivirus PCR followed by species specific PCR should be used for identification and differentiation of pestiviruses. The cross contamination during PCR is another major problem that should be considered while interpreting RT-PCR results in pestivirus diagnosis. Another disadvantage in PCR based assay is the decreased amplification or negative results due to mismatches in primers and probes. Therefore, evaluation and updation of primers and probes should be carried out at regular intervals to rule out newly emerging pestiviruses. The validation, standardization and quality control of PCR based diagnostic methods should be carried out in all

diagnostic laboratories instead of using in-house PCR assays. Development and validation of microarray technique in future may contribute identification of a range of pestiviruses simultaneously. Another important aspect is to study the antigenic and biological properties of pestivirus isolates and their role in pathology of infection.

Besides nucleotide sequence relatedness, antigenic relatedness is another important criterion that is considered important for demarcation of pestiviruses into species and subtypes within species (Thiel et al. 2005). Moreover, antigenic differences are important for diagnosis and vaccination. While nucleotide sequences can be quickly generated and phylogenetic analysis can be performed to identify new subtypes or species, comprehensive studies on antigenic relatedness are more demanding but have often been lacking. Therefore, antigenic differences should always be performed during subtyping of pestiviruses to have better practical utility. It needs to be emphasized that genetic typing and subtyping of pestiviruses is often based on sequence analysis of 5′UTR, N^{pro} and E2 gene regions while antigenic typing is based on the neutralizing epitopes present in the envelope protein E2. Many times it becomes difficult to correlate the results if both genetic and antigenic typing is carried out for different gene regions. Hence, a unified approach should be agreed in future to circumvent these problems.

9.4.2 Challenges for Control Strategies

From disease control point of view, antigenic diversity of ruminant pestiviruses imposes serious challenges. First of all, as recent studies suggest, BDV can infect cattle naturally while sheep and goats can be infected naturally with BVDV-1 and BVDV-2 (Strong et al. 2010). Hence, it is necessary to assess ability of BVDV diagnostic tests currently in use to detect all three pestiviruses (BVDV-1, BVDV-2 and BDV) including a range of subtypes specifically in cattle. Moreover, it needs to be ascertained to what extent inactivated BVDV vaccines are able to provide protection against BDV. Secondly, since all domestic inter ruminant contact can favour pestivirus transmission, control strategies in various countries need redesigning of surveillance strategies and ensure optimal performance of laboratory diagnostic tests in identifying PI animals. Thirdly, an atypical pestivirus should also be included in serological screening assays of cattle used in BVDV control programmes. Fourthly, since wildlife may be infected with more pestiviruses than presently identified, continual surveillance of wild ruminants is necessary especially in geographic areas, where intermingling of domestic and wild ruminants takes place. Fifthly, current and new BVDV vaccines must be able to protect against a variety of BVDV-1 and BVDV-2 subtypes as most of the current vaccines contain only BVDV-1a strains that may not be adequate in protecting against other BVDV-1 strains (Fulton et al. 2003). Sixthly, Successful BVD control programme should aim at elimination of the source of PI animals and complete protection of the foetus.

9.5 Conclusions

Pestiviruses are highly diverse in several aspects including complex interactions with host, although all pestiviruses share the same genome structure. Based on the increasing use of molecular epidemiological tools such as nucleotide sequencing and computer-assisted phylogenetic analysis, several novel and atypical ruminant pestiviruses have recently been identified in domestic and wild animals and their heterogeneity in genetic and antigenic properties have been elucidated. However, the classification and nomenclature of ruminant pestiviruses has been problematic that needs to be resolved soon and additional parameters like antigenic relationship, disease pathology and host range should also be considered for taxonomy. The critical analysis of the data discussed in this chapter, suggest that ruminant pestiviruses are highly successful in infecting many animal species with an efficient strategy to survive and outmaneuver the host. Given the large genetic and antigenic diversity of ruminant pestiviruses, laboratory diagnostic tests and control strategies need to be reevaluated. With increased surveillance, a further surge in ruminant pestivirus diversity can be anticipated in future and hence development of newer diagnostics and vaccine formulations should be continued in a similar pace.

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Chapter 10
Cyanobacteria-PGPR Interactions for Effective Nutrient and Pest Management Strategies in Agriculture

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Abstract Soil microorganisms are known to play an active role in increased crop yields and soil fertility through a diverse array of mechanisms and such organisms are termed as PGPR (Plant Growth Promoting Rhizobacteria). This enhancement has been attributed to their involvement in the cycle of nutrients like carbon and nitrogen or in the decomposition of the organic matter, or production of allelopathic metabolites or enzymes influencing the pathogenic flora/fauna which indirectly promotes plant growth. Cyanobacteria are a ubiquitous group of organisms which have been relatively less investigated as PGPR, although their role in nitrogen dynamics of paddy based cropping systems is well investigated. Cyanobacteria are known to produce compounds with a wide range of activities, including phytohormones, biocidal metabolites or nutraceuticals. The interactions between agriculturally useful heterotrophic bacteria and autotrophs such as cyanobacteria can be effective and environment friendly options as biocontrol agents and biofertilizers. Plant-microbe partnerships are increasingly being focussed for not only nutrient management, but also for improving biomass production and remediation of polluted/inhospitable environments. This compilation provides an overview of the developments on this aspect and projections for the future.

Keywords Cyanobacteria • Disease severity • Nutrient Management • PGPR • Soil fertility • Yield

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10.1 Introduction

Plant associated microorganisms fulfil important functions for plant productivity and soil health as they participate actively in almost every chemical transformation taking place in soil. In particular, they play an active role in soil fertility, as a result of their involvement in the nutrient cycles of carbon and nitrogen, which are essential for plant growth. The balanced availability of both inorganic and organic matter in the soil determines the soil fertility. The presence of enormous numbers of microbial populations and species in the soil, especially in the rhizosphere and their intensive and extensive interactions with these flora and fauna and plant roots, leads to plant growth promotion by rhizosphere microorganisms (Bashan 1998; Khalid et al. 2004). However, the beneficial plant-microbe interactions have been ignored in the on-going efforts for enhancing plant productivity, although microorganisms fulfil important ecosystem functions for plants and soils (Persello-Cartieaux et al. 2003). Current crop production methods, employing improper use of chemical pesticides and fertilizers have created serious environmental and health problems. Further, emerging, re-emerging and endemic plant pathogens continue to challenge our ability safeguard plant growth and health globally. This emphasizes the demand for sound and ecologically compatible strategies in agriculture.

Plant associated bacteria generally include endophytic, phyllospheric and rhizospheric bacteria (Fig. 10.1) which mediate several processes during plant growth, development and soil geochemical cycles. Among them, the endophytic bacteria exhibit tremendous diversity in plant hosts and bacterial taxa (Raaijmakers et al. 2009). The Psuedomonadaceae, Burkholderiaceae and Enterobacteriaceae are among the most common families of cultivable endophytic species. Bacteria residing in the phyllosphere, mainly the leaves, comprise only a few taxa, but with relatively large number of individuals or a number of taxa with only a small number of individuals each. However, extensive research has focussed on plant growth promoting rhizobacteria (PGPR) which through their close interactions with plant roots, not only exert significant effects on plant growth, but also minimize fertilizer inputs by promoting biological nitrogen fixation and enhancing acquisition of phosphorous and iron in the rhizosphere (Zahir et al. 2003; Vessey 2003; Kennedy et al. 2004; Welbaum et al. 2004). PGPR include bacteria mainly belonging to the genera *Arthrobacter, Bacillus, Burkholderia, Enterobacter, Klebsiella, Proteus, Pseudomonas, Xanthomonas* and *Serratia.*

The success of cyanobacteria or blue-green algae, as a group in a wide range of habitats has been attributed to their unique physiological characters and high adaptive ability under a wide range of environmental conditions. They are a ubiquitous group of prokaryotes, whose evolution (amply supported by fossil and molecular evidence) can be traced back 3.5 billion years (Mundt et al. 2001). Although cyanobacteria are globally important primary producers, exhibiting a worldwide distribution in diverse ecosystems and contribute significantly to the fertility of rice fields; their role as PGPR is less investigated (Karthikeyan et al. 2007; Prasanna et al. 2009a, b, 2011b).The potential of cyanobacteria as a source of a variety of

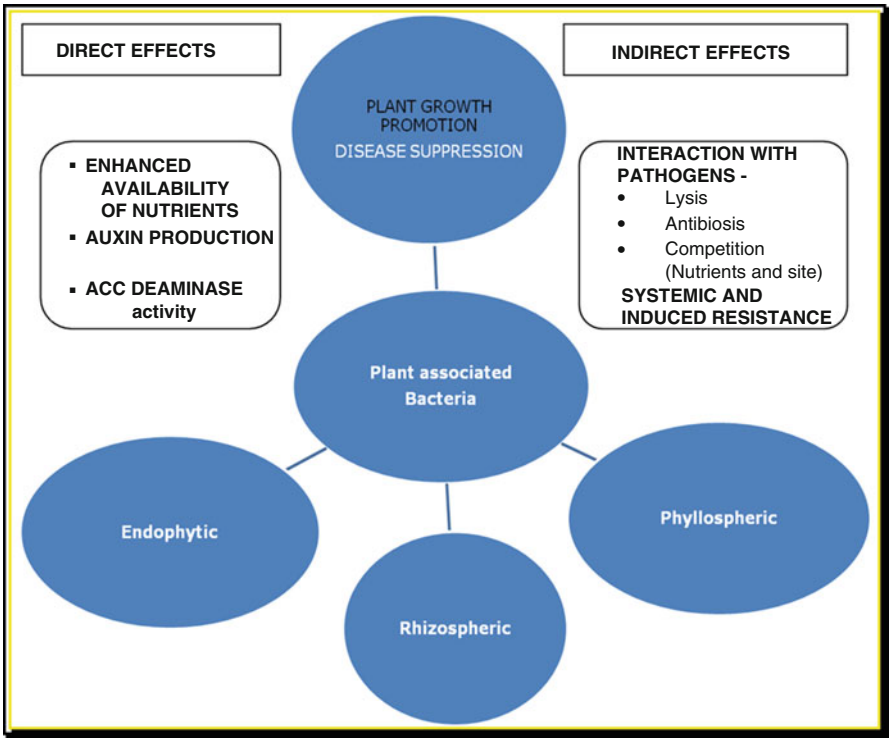

Fig. 10.1 Overview of multifaceted roles of plant associated bacteria

compounds such as polysaccharides, lipids, proteins, vitamins, sterols, enzymes, pharmaceuticals and other fine chemicals is well recognized, and research efforts are currently focussed on exploiting this biochemical diversity. The rhizosphere is considered common ground for ecologists, molecular biologists and plant biologists, besides microbiologists and with genomic sequencing initiatives of several PGP bacteria, including endophytes (http://genome.jgi-sgf.org/mic_home.html), a comprehensive understanding of the "below ground" interactions can be envisaged in the years to come. Our compilation focuses on the multifaceted roles of plant associated bacteria, with emphasis on PGP rhizobacteria and cyanobacteria and provides an overview of salient findings available globally.

10.2 Rhizosphere Interactions and Chemical Signalling

The rhizosphere is the playground and infection court for soil borne pathogens and also a battlefield, where both microflora and microfauna interact with soil borne pathogens. However, our knowledge regarding the chemical cues between the various

players is less known. As Leonardo da Vinci quoted "*We know better the mechanics of celestial bodies than the functioning of the soil below our feet*". Plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. Such bacteria have been applied to a wide range of agricultural plant species for the purpose of growth enhancement, including increased seed emergence, plant weight, crop yields and disease control (Kloepper et al. 1991). They facilitate plant growth and development, both directly and indirectly (Glick 1995). Direct stimulation may include providing plants with fixed nitrogen, phytohormones or iron (that has been sequestered by bacterial siderophores) and solubilized phosphate, while indirect stimulation of plant growth includes preventing phytopathogens (biocontrol) through production of antibiotics/siderophores and hydrogen cyanide and thus promoting plant growth and development (Kloepper et al. 1991). However, the effects are often overlapping and it becomes difficult to assign a function for each trait exhibited by the PGPR.

Plant growth promoting rhizobacteria (PGPR) display a set of positive features, which, as a rule, includes auxin synthesis (Belimov et al. 1999). Phytohormones play an important role as signals and regulators of growth and development in plants. They also contribute to the coordination of diverse physiological processes in plants, including the regulation of quiescence and seed germination, root formation, florescence, branching, tillering and fruit ripening. They increase plant resistance to environmental factors and induce or suppress the expression of genes and the synthesis of enzymes, pigments and metabolites. The group of phytohormones include auxins, cytokinins, gibberellins and ethylene like substances. The synthesis of auxins is often regarded as an important condition for associative interaction between rhizobacteria and plants (Katsy 2005).

Auxins control the plant vegetative growth, flowering and fruiting and influence the photosynthesis, pigment formation, biosynthesis of various metabolites and plant resistance to various environmental stress factors. Among auxins, indole-3-acetic acid (IAA) is the most studied plant growth regulators, in terms of physiological, biochemical and genetic aspects (Sergeeva et al. 2002). IAA, the most abundant naturally occurring auxin, has been implicated in regulating a variety of developmental and cellular processes such as cell extension, cell division, vascular differentiation, root formation, apical dominance, and tropisms (Napier and Venis 2005). The production of IAA is widespread among soil and plant-associated prokaryotes (Costacurta and Vanderleyden 1995).

Enhanced plant growth may result directly from microbial production of plant-growth regulators, including indole-3-acetic acid (Bano and Musarrat 2004; Donnell et al. 2003). Rhizospheric bacteria showed relatively more potential for IAA synthesis as compared to histoplane and phyllosphere isolates. The IAA produced by microbes colonizing the seed or root surfaces is proposed to act in conjunction with endogenous IAA in plants to stimulate cell proliferation and/or elongation and enhance the uptake of minerals and nutrients from the soil by the hosts. IAA producing bacteria affect growth and development, led to plant root system development and subsequently increased nutritional uptake by the plants. The ability to synthesize IAA was detected in many rhizospheric and epiphytic bacteria

Azospirillum sp., *Agrobacterium* sp., *Azotobacter* sp., *Alcaligenes* sp., *Enterobacter* sp., *Erwinia* sp., *Acetobacter* sp., *Rhizobium* sp., *Bradyrhizobium* sp. and *Herbaspirillum* spp. (Datta and Basu 2000). Park et al. (2005) reported the widespread synthesis of IAA among the genera *Pseudomonas*, *Bacillus* and *Xanthomonas*. Although it is relatively easy to measure the concentration of IAA produced in the laboratory; it is difficult to assess and determine the levels of IAA produced in rhizosphere as expression of IAA genes is controlled by both genetic and environmental factors. Moreover, five different pathways are identified in bacteria for biosynthesis of IAA (Glick et al. 1998).

Cyanobacteria are generally considered to be obligate phototrophs, despite several reports on their photoheterotrophy, and their ability to grow in the dark with simple sugars. Our reports revealed the taxonomic and metabolic diversity of cyanobacteria in the rhizosphere of rice and wheat (Misra and Kaushik 1989; Karthikeyan et al. 2007, 2009; Prasanna et al. 2009a; Jaiswal et al. 2008a). Sergeeva et al. (2002) reported IAA biosynthesis in free living and symbiotic cyanobacteria of the genera *Nostoc*, *Chlorogloeopsis*, *Calothrix*, *Plectonema*, *Anabaena*, *Cylindrospermum* and *Anabaenopsis*. Recently, IAA production has been observed in several *Anabaena* species and rhizo-cyanobacterial isolates from rice and wheat rhizosphere (Karthikeyan et al. 2007; Prasanna et al. 2008a, b, 2009a, 2010b, 2011a, b; Manjunath et al. 2011). Karthikeyan et al. (2007) evaluated the potential of plant growth promoting activity of the cyanobacterial species such as *Calothrix, Hapalosiphon* and *Nostoc* from the rhizosphere of Wheat, which was a first time report for this crop. Evaluation of their plant growth promoting activity and close interactions with the plant roots in pot culture experiments, revealed them as suitable PGPR candidates. The biosynthesis of IAA in *Anabaena* strains was observed to be significantly influenced by incubation under light–dark regimes in the presence or absence of tryptophan (Prasanna et al. 2010b) and the role of different levels of tryptophan revealed the significance of light–dark conditions and tryptophan levels in regulating IAA production. Interactions between PGPR-cyanobacteria were evaluated using IAA (indole-3-acetic acid) producing proteobacterial and cyanobacterial strains on the growth and yield of wheat (variety PBW343). Two proteobacterial (WRB4 *Providencia* sp and WRB10 *Alcaligenes* sp.) and two cyanobacterial (WRC3 *Anabaena oscillarioides* and WRC4 *Anabaena torulosa*) strains were used individually and in combination. A positive interaction among the proteobacterial and cyanobacterial strains especially WRC3 and WRB4 was also observed by way of enhancement of plant growth parameters. Significant enhancement in soil microbiological activities such as FDA hydrolysis and dehydrogenase activity were recorded in the treatments especially in those inoculated with cyanobacterial strains, when compared to fertilizer controls. This is a first time report on the potential of selected combinations of proteobacterial genera such as *Providencia* and *Alcaligenes* and cyanobacteria such as *Anabaena* as plant growth promoting organisms in wheat crop (Manjunath et al. 2011).

Cyanobacteria are known to characteristically liberate substantial quantities of extracellular nitrogenous compounds into the medium. Physiological attributes of a set of cyanobacterial strains, isolated from the rhizosphere of wheat (var. HD 2687),

identified as belonging to the genera – *Calothrix, Westiellopsis* , *Hapalosiphon* and *Nostoc* were analyzed (Karthikeyan et al. 2009). The concentrated culture filtrates of three cyanobacterial strains – *Calothrix ghosei*, *Hapalosiphon intricatus* and *Nostoc* sp. were able to enhance germination percentage, radicle and coleoptile length in imbibition studies with wheat seeds. TLC analyses of the filtrates revealed the presence of several amino acids, such as histidine and auxin-like compounds. Co-culturing experiments with selected cyanobacterial strains recorded significant enhancement in plant chlorophyll. Electron microscopic observations of root sections of such wheat plants revealed the colonisation and mode of entry and intracellular presence of short filaments and single cells of *Anabaena*, *Nostoc* sp.; emphasizing the promise of these cyanobacterial strains as PGPR (Prasanna et al. 2009b; Jaiswal et al. 2008a). Root sections of wheat seedlings co-cultured with *Calothrix ghosei,* revealed the presence of short filaments inside the root hairs and cortical region (Karthikeyan et al. 2009). Such strains can be promising candidates for developing plant growth promoting associations for wheat crop, besides serving as model systems for understanding the metabolic interactions of cyanobacteria with host plant, such as wheat.

Ethylene is a gaseous plant growth substance that has been shown to be crucial in many aspects of plant development as well as for their response to stress (Arshad and Frankenberger 1998). One of the mechanisms that a number of PGPR uses to facilitate plant growth and development is the lowering of plant ethylene concentration through the action of the enzyme 1-aminocyclopropane – 1 – carboxylate (ACC) deaminase (Glick 1995; Glick et al. 1998). ACC deaminase hydrolyse ACC, the immediate biosynthetic precursor of ethylene in plant and thus growth and development of plants are modified. Bacterial strains containing ACC deaminase can in part at least alleviate the stress induced ethylene mediated negative impact on plants (Glick 2005). ACC deaminase has been widely reported in numerous microbial species of Gram negative/ positive bacteria, rhizobia, endophytes and fungi (Saleem et al. 2007).

The most substantial experimental evidence to support this PGPR ACC deaminase mediated model was conducted by Glick et al. (1998). *Pseudomonas putida* GR 12-2 promoted growth of canola seedling and elongation of its root; but mutants of *P. putida* GR 12-2 lacking ACC deaminase activity were unable to promote the growth of canola seedling roots under gnotobiotic conditions implicating the role of ACC deaminase in plant growth promotion. The model suggests that PGPR strains binds to seed coat and during imbibition, the bacterium sequesters and then hydrolyzes ACC into ά ketobutyrate and ammonia by the action of ACC deaminase, thereby lowering the level of ethylene in developing plants. ACC deaminase has been widely reported in numerous microbial species of PGPR. Inoculation with PGPR containing ACC deaminase activity could be helpful in sustaining plant growth and development under stress condition by reducing stress induced ethylene production. Lately, efforts have been made to introduce ACC deaminase genes into plants to regulate ethylene level in plants for optimum growth particularly under stressed conditions (Saleem et al. 2007) like flooding, presence of organic toxicants, metals, drought, salt and flower wilting (Glick 2005).

10.3 Integrated Nutrient Management Through PGP Partnerships

Biological Nitrogen fixation by PGPR is considered one of the major mechanisms by which plant benefit from this association (Glick 1995). Ladha et al. (1998) reported that nitrogen fixation by PGPR may stimulate the growth of low land rice plants. Malik et al. (1977) and Biswas et al. (2000) also reported rice growth promotion by diazotrophic PGPR. Rhizobial and *Azospirillum* strains are well known PGPR which are used a biofertilizers due to their ability to fix nitrogen in the crop rhizosphere. The effect of PGPR inoculation to enhance growth of tissue-cultured banana plantlets under nitrogen (N) free hydroponics condition and N yield (94–144%) was reported by Baset et al. (2010). Several experts have suggested that PGPR stimulate plant growth by facilitating the uptake of minerals in the plant, particularly phosphate by releasing organic acids, (Kloepper et al. 1991, 1999; Glick 1995; Chabot et al. 1996).

PGPR have been found to participate in the solubilisation of inorganic phosphate, and majority of these were species of *Pseudomonas* and *Bacillus*. Phosphorus uptake was increased (13–23%) significantly in response to rhizobial inoculation of rice compared to a non inoculated control (Biswas et al. 2000). Toro et al. (1997) evaluated the interactive effect of phosphate solubilizing bacteria (*Enterobacter* sp. and *Bacillus subtilis*) and Arbuscular mycorrhizal (AM) fungus *Glomus intraradices* on onion with a soil of low P content. Inoculation of *B. subtilis* and AM fungi significantly increased the vegetative biomass, N and P accumulation in plant tissues. Further studies indicated that PGPR could create an acidic environment to promote mineral nutrient solubilisation (Moghimi et al. 1978). Pot culture experiments conducted at National Phytotron Facility, IARI and glasshouse with variety HD2687 and the cyanobacterial strains (applied singly or in combination) showed a significant enhancement in microbial biomass carbon and statistically at par values with full dose of NPK, in terms of plant height, dry weight and grain yields (Karthikeyan et al. 2007). Synergistic interactions among the PGPR strains (bacteria-cyanobacteria – *Bacillus* sp. + *Brevundimonas diminuta* + *Anabaena* sp.; *Bacillus* sp. + *Calothrix* sp. + *Anabaena* sp.) were observed which was manifested in terms of significant enhancement in the soil microbiological and plant growth/yield parameters in wheat pot experiments (Nain et al. 2010). A comparative assessment of promising PGPR-cyanobacteria interactions, based on our studies (Manjunath et al. 2011; Nain et al. 2010) undertaken at the Division of Microbiology, Indian Agricultural Research Institute is given in Table 10.1. Field level evaluation of these strains and testing under different agro-climatic conditions is in progress to further evaluate their agronomic efficiency and utility in integrated nutrient management of wheat crop.

Phosphate solubilising bacteria are common in rhizosphere; however, the ability to solubilise P by no means indicates that a rhizospheric bacterium will constitute a PGPR (Vessey 2003). For example, Cattelan et al. (1999) found only two among the five rhizospheric isolates were positive for P solubilisation, and actually had a positive effect on soybean seedling growth. Likewise, not all P solubilising PGPR

Table 10.1 Influence of PGPR-cyanobacteria on biometric and microbiological parameters of wheat crop

S. No.	Treatments	Crop biomass (%)[a]	Grain wt. (%)[a]	Dehydrogenase (%)[a]	FDA (%)[a]
With 2/3 NPK ($N_{80}P_{60}P_{60}$)					
1	*A. oscillarioides* + *Providencia* sp.	7.6	6.5	102.1	14.6
2	*A. oscillarioides* + *Alcaligenes* sp.	10.6	−5.8	76.2	2
3	*A. torulosa* + *Providencia* sp.	3.3	5.7	89.3	22
With 1/2 NPK ($N_{60}P_{60}P_{60}$)					
4	*Bacillus* sp. + *Brevundimonas diminuta* + *Anabaena* sp. (PW1+ PW7 + CW3)	77.1	36.1	65.5	153.3
5	*Bacillus* sp. + *Calothrix* sp. + *Anabaena* sp. (PW1 + CW2 + CW3)	71.6	30.4	−23.6	126.6
6	*Providencia* sp. + *Brevundimonas diminuta* + *Anabaena* sp. (PW5 + PW7 + CW1)	38.5	25.5	−10.1	−6.6

[a]Percent increase as compared to application of full dose of $N_{120}P_{60}K_{60}$

increase plant growth by increasing P availability to these roots. De Freitas et al. (1997) recorded a number of P solubilising *Bacillus sp*. isolates and a *Xanthomonas maltophilia* isolate from Canola (*Brassica napus* L.) rhizosphere which had positive effects on plant growth but no effect on P content of the host plants.

De (1939) attributed the natural fertility of flooded rice field soil and its maintenance to the process of biological nitrogen fixation by cyanobacteria. This was the first report, which recognized the agronomic potential of cyanobacteria in India. Watanabe and Roger (1984) demonstrated that the N fertility of soil is sustained well through cyanobacteria under flooded condition than under dry land conditions. The favourable conditions of rice fields for biological nitrogen fixation by such cyanobacteria is considered to be one of the reasons for relatively stable yield of rice under flooded conditions (Venkataraman 1972). They have been utilized mainly as biofertilizers in agriculture and extensive reviews on their distribution in rice fields/nitrogen – fixing potential exist, which reveal their generic, genetic and functional diversity (Venkataraman 1972; Nayak and Prasanna 2007). A wide range of N_2-fixing cyanobacteria exists in rice field ecosystems (Watanabe and Roger 1984). Nitrogen fixed by the symbiotic association of cyanobacteria (cyanobionts in *Azolla*) is transferred to and used by various plant groups other than rice. The high fertilizer (especially nitrogen) requirements make the use of biofertilizers imperative for using cyanobacterial inoculants in integrated nutrient management practices for the rice wheat cropping system. Nitrogen-fixing cyanobacteria are more widespread among the filamentous, heterocyst forming genera (e.g. *Anabaena, Nostoc*). However, there are also several well documented examples of dinitrogen fixation among cyanobacteria not forming heterocysts (e.g. *Trichodesmium*). Under predominantly nitrogen limited conditions, but when other nutrients are available,

nitrogen fixing cyanobacteria may be favoured and gain growth and reproductive success. Cyanobacterial inoculation has shown to enhance growth, root associated nitrogen fixation and yields of rice (Singh 1961; Roger et al. 1993; Mandal et al. 1998; Prasanna et al. 2003; Nayak et al. 2004). However, their role in plant growth promotion, especially in relation to their functioning in the rhizosphere, has not been explored in depth

10.4 Biocontrol Mediated by PGPR

Biological control offers an alternative, attractive approach, without the negative impact of chemical control measures and has become an important approach facilitating sustainable agriculture, as biocontrol agents are easy to deliver, safe for the applicator, non polluting, compatible with conventional and low-input agricultural practices and activate plant resistance mechanisms like systemic /induced resistance and in many cases, improve the plant growth and yield. Biocontrol of soil borne diseases is particularly complex because these diseases occur in the dynamic environment i.e. rhizosphere. The rhizosphere is typified by rapid change, intense microbial activity, and high populations of bacteria compared with non rhizosphere soil. Plants release metabolically active cells from their roots. It is the dynamic nature of the rhizosphere that makes it an interesting platform for the diverse interactions that lead to both disease and their biocontrol (Hawes 1991).

Fungal plant diseases are one of the major concerns to agricultural production. They are one of the important causative agents of plant diseases and more than 60% of the literature in plant diseases is devoted to the fungal infections. Almost all plant pathogenic fungi spend part of their lives on their host plants and partly in soil or on plant debris. Plant pathogens are represented in all the major groups of fungi and symptoms caused by fungal diseases can be broadly classified as necrosis or death of tissue (cf. anthracnose, blight, canker, dieback, damping off, scab, soft rots and dry rots); wilting or drooping of leaves due to loss of turgor; hyperplasia or overgrowth (e.g. galls, witches' broom); and hypoplasia: dwarfing and chlorosis. Plant diseases need to be controlled to maintain the quality and abundance of food, feed, and fibre produced by growers around the world. Different approaches are used to prevent, mitigate or control plant diseases. Beyond good agronomic and horticultural practices, growers often rely heavily on chemical fertilizers and pesticides. Such inputs to agriculture have contributed significantly to the spectacular improvements in crop productivity and quality over the past 100 years. However, the environmental pollution caused by excessive use and misuse of agrochemicals, as well as fearmongering by some opponents of pesticides, has led to considerable changes in the attitude of people towards the use of pesticides in agriculture. Consequently, some pest management researchers have focused their efforts on developing alternative biological inputs to synthetic chemicals for controlling pests and diseases.

A variety of biological controls are available for use, but further development and effective adoption will require a greater understanding of the complex interactions of

these agents with plants and the environment. Fungi and bacteria are the chief biological agents that have been studied for the control of plant pathogens, particularly soil-borne fungi. Biocontrol formulations, involving *Trichoderma*, fluorescent *Pseudomonads* have been developed and successfully used at field with a number of crops. In addition, viruses, amoebae, nematodes, and arthropods have been mentioned as possible biocontrol agents (Whipps and Mac Quilken 1993). The basis of antibiosis as a biocontrol mechanism has become increasingly better understood over the past two decades (Anjaiah et al. 2003). A variety of antibiotics have been identified, including compounds such as amphisin, 2, 4-di acetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, troplone, and cylic lycopeptides produced by pseudomonads, and oligomycin A, kanosamine, zwittermicin A, Xanthobaccin produced by *Bacillus* and *Stenotrophomonas* spp. (Kim et al. 1999).

The significance of cyanobacteria as producers of cyanotoxins and other novel bioactive molecules is globally recognized (Kumar et al. 2005), however, their chemical potential is less explored in agriculture, especially as biocontrol agents. A number of cyanobacteria and eukaryotic algae produce various biologically active compounds which have ecological roles as allelochemicals (Jaiswal et al. 2008b, 2010b; Kulik 1995; Prasanna et al. 2008a; 2010c; Chaudhary et al. 2010; Natarajan et al. 2011), and could be employed for the commercial development of agents with application as algicides, herbicides and insecticides. Welch (1962) reported that filaments of *Lyngbya majuscula* when ground up and placed on filter paper disc inhibited *Candida albicans* and *Penicillium* spp. It was also found to inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis* and *B. typhosus*. De Caire et al. (1987) evaluated the effect of cell free extracts of *Nostoc muscorum* on the growth of *Panicum miliarum* in pots. They observed that the extract prevented an outbreak of damping-off disease in their seedlings. De Mule et al. (1977) reported that culture extracts of *Nostoc muscorum* inhibited the mycelial development of *Cunnighamella blakesleeana* in a liquid culture.

Cyanobacteria produce a number of secondary metabolites exhibiting various bioactivities such as inhibitory properties against microorganisms (bacteria, cyanobacteria, algae, viruses and fungi) and toxicity to invertebrates and vertebrates. These metabolites may be used for the development and application as algicides, fungicides, herbicides and insecticides (Berry et al. 2008). They are also known to excrete bioactive compounds into the environment, which are important determinants of allelopathic activity in water and soil. Allelochemicals are secondary metabolites or non – nutritional primary metabolites that affect growth, reproduction or behaviour of individuals other than the ones producing them or influence the structure and dynamics of populations or communities of either plants or animals or microbes. Allelopathic chemicals play a role in the interactions between the emitter organisms and their direct competitors or predators; they are categorized according to their toxic stimulatory effect on several organisms, including some that may not be present in their immediate environment. Allelopathic compounds include alkaloids, cyclic peptides, terpenes and volatile organic compounds. Allelopathic compounds have various modes of action, from inhibition of photosynthesis to oxidative stresses or

cellular paralysis. Suikkanen et al. (2004) investigated the allelopathic effects of three cyanobacterial species (*Nodularia spumigena, Aphanizomenon flos-aquae* and *Anabaena lemmermannii*) that frequently form mass occurrences in Baltic Sea. They exposed monocultures of three phytoplankton species (*Thalassiosina weissflogii, Rhodomonas* sp and *Prymnesium parvum*) to cell free filtrates of the three cyanobacteria and quantified allelopathic effects with cell counts. All tested cyanobacteria inhibited the growth of *Rhodomonas* sp. but none of them affected *P. parvum*. The allelochemicals inhibited phytoplankton and epiphytes. Asthana et al. (2006) extracted pharmaceutically important γ-linolenic acid from *Fischerella* spp. using chloroform and methanol (1:2) which also exhibited allelopathic activity.

Many cyanobacterial strains, belonging to genera – *Microcystis, Anabaena, Nostoc, Oscillatoria, Nodularia, Aphanizomenon, Cylindrospermum* are known to produce a number of cyclic peptide hepatotoxins and alkaloid neurotoxins exhibiting algicidal, antifungal, pesticidal, cytotoxic, immunosuppressive and enzyme inhibitory activities. In recent years, the number of reports trickling in, on the production of antifungal compounds from cyanobacteria (Siddhanta and Shanmugam 1999) is on a steady increase. However, most reports on antifungal activity from cyanobacterial compounds are with respect to their pharmaceutical applications. At the global level, a few antifungal molecules derived from cyanobacteria have been patented for agricultural use, but in India, research on this topic is scarce (Moore et al. 1991). The extracts of cyanobacteria are known to reduce the incidence of *Botrytis cinerea* on strawberries, *Erysiphe polygoni* (Powdery mildew) on turnips and damping off in tomato seedlings (Kulik 1995; Manjunath et al. 2010a), besides reducing the growth of saprophytes – *Chaetomium globosum, Cunninghamella blakesleeana* and *Aspergillus oryzae* and plant pathogens such as *Rhizoctonia solani* and *Sclerotiana sclerotium*. Their rapid growth rate and proliferation in diverse environments – due to which they can be grown in mass culture, makes them suitable candidates for exploitation as biocidal agents of plant pathogenic bacteria and fungi. Limited information on their use as biocontrol agent exist (Manjunath et al. 2010a; Tassara et al. 2008) in agriculture. However, the ecological information on potential applications of such compounds is limited and a new approach is necessary to evaluate the ecological implications of these secondary metabolites as biocides as well as for other purposes. Furthermore, physicochemical properties that control the production of these matabolites could be used to improve biosynthetic approaches for commercial production. Eighteen new indole alkaloids with antifungal and antibacterial activity, hapalindoles C-Q and T-V were isolated from *Hapalosiphon fontinalis* by Moore et al. (1987). Moore et al. (1989) isolated and identified six indole alkaloids from *Hapalosiphon fontinalis* which showed antifungal activity. De Cano et al. (1990) found that phenolic compounds from the extracts of cells of *Nostoc muscorum* checked the growth of *Candida albicans* and *Staphylococcus aureus*. Moon et al. (1992) isolated a broad-spectrum fungicide Calophycin from *Calothrix fusca* and identified to be a cyclic decapeptide. Its total structure, including absolute stereochemistry was determined by a combination of spectral and chemical studies including synthesis of unusual β-amino acid. This compound was effective against *Aspergillus oryzae, Candida albicans, Penicillium*

notatum, Saccharomyces cerevisiae and *Trichophyton mentagrophytes*. A diverse range of compounds (indoles, alkaloids, peptides) have been isolated from different cyanobacterial genera (Smitka et al. 1992; Pergament and Carmeli 1994). Volk and Furkert (2006) evaluated the antifungal activity of two cyanobacterial strains of *Nostoc insulare* and *Nodularia harveyana* against *Candida albicans* and the extracellular extracts showed antifungal activity at 32–40 μg ml^{-1} concentration. Manjunath et al. (2010a) demonstrated the biocidal efficacy of fungicidal compounds produced by cyanobacterium *Calothrix elenkenii* against damping-off disease caused by *Pythium aphanidermatum* in solanaceous vegetable crops. Natarajan et al. (2011) attributed the fungicidal activity of *Calothrix elenkinii* can be attributed to the presence of 3-acetyl-2-hydroxy-6-methoxy-4-methyl benzoic acid. This is the first time report of a benzoic acid derivative having fungicidal activity in cyanobacteria.

The role of hydrolytic enzymes such as chitinases, exoglucanases is well established in interactions involving pathogenic bacteria/fungi and plants. Among several chitinolytic bacteria and fungi, *Trichoderma* spp. has received the most attention as biological control agents of soilborne fungal pathogens. Several recent studies reported the purification and characterization of chitinases and beta – 1, 3 – glucanases produced by *Trichoderma* spp. and *Talaromyces flavus* and highlighted their role in the mycoparasitism of soil borne pathogens such as *Sclerotium rolfsi, Rhizoctonia solani* and *Fusarium* sp. (Harman et al. 1993). Prasanna et al. (2008a, 2010a) and Gupta et al. (2010) revealed for the first time, the activity and homologues of hydrolytic enzymes in several *Anabaena* strains and their correlation with fungicidal activity.

Hydrolytic enzymes such as chitinases and beta – 1, 3 glucanases, are one such group of proteins, which have been purified and characterized from several plants/bacteria/fungi (Yaboah et al. 1998). The excretion of hydrolytic enzymes is known to be a common trait of plant pathogens/symbionts, which promotes a closer association with plant roots/target organisms and improve the stability of such associations. Chitinases are known to selectively degrade chitin by hydrolysis of the β – 1, 4 – glycosidic bonds that link N -acetyl glucosamine residues of chitin and form the basis for antifungal activity. Many species of bacteria, streptomycetes, actinomycetes, fungi and plants produce chitinolytic enzymes (Nelson et al. 1986). Bacteria from the genera *Acromonas* (Inbar and Chet 1991) and *Serratia* (Ordentlich et al. 1988) and fungi from the genera *Gliocladium* and *Trichoderma* (Elad et al. 1982), all of which produce chitinolytic enzymes, have been shown to be potential agents for the biological control of plant diseases caused by various phytopathogenic fungi. The chitinase produced by *S. plymuthica* C48 inhibited spore germination and germ-tube elongation in *Botrytis cinerea*. The ability to produce extracellular chitinases is considered crucial for *Serratia marcescens* to act as antagonist against *Sclerotium rolfsii*, and for *Paenibacillus* sp. strain 300 and *Streptomyces* sp. strain 385 to suppress *Fusarium oxysporum* f. sp. *cucumerinum* (Singh et al. 1999). A variety of microorganisms also exhibit hyperparasitic activity, attacking pathogens by excreting cell wall hydrolases, which has been a subject of intensive research in recent years. Several isolates of *Enterobacter cloacae* are known to be biocontrol

agents for different rots and pre-emergence damping off of pea, beet, cotton and cucumber plants incited by *Pythium* spp. as well as of *Fusarium* wilt of cucumber and some other plant diseases caused by fungal pathogens (Nelson 1988) which showed the complexity and diversity of the chitinolytic enzyme system, with its complementary modes of hydrolyzing chitin. Some of the fluorescent pseudomonads produce lytic enzymes (Chitinase, beta – 1, 3 – glucanase, and protease) which are responsible for the lysis and hyperparasitism of antagonistic against deleterious fungal pathogens. In these mechanisms, chitin, beta – 1, 3 – glucan and protein components of the fungal cell wall are digested by these extracellular enzymes which cause inhibition of plant pathogenic fungi and deleterious rhizobacteria with a significant increase in root colonization and plant growth. Lim et al. (1991) isolated a strain of *Psuedomonas stutzeri* that produced extracellular chitinase and laminarase, which could digest the mycelia of *Fusarium solani*. A variety of PGPR also exhibit hyperparastic activity attacking pathogens by excreting cell wall hydrolases e.g. chitinase, laminarase, β1, 3 glucanase, protease etc. Application of *Pseudomonas*, *Paenibacillus* as PGPR suppress the growth of pathogens- *Fusarium oxysporum, Sclerotium rolfsii* and *Pythium* etc. due to production of lytic enzymes (Chernin and Chet 2002).

With the growing realization that chemical based agriculture is unsustainable and is slowly leading to ecological imbalance, the latter part of the last century witnessed the emergence of the concept of "organic agriculture" advocating minimum use of chemical fertilizer and increasing dependence on biological inputs like compost, farm yard manure, green manure and biofertilizers. In recent years, composts amended container media have been investigated as a part of the integrated biological control practices. This phenomenon has studied on wide range of pathogens such as *Pythium aphanidermatum, P. ultimum, Rhizoctonia solani, Fusarium oxysporium, Sclerotium rolfsii*, and *Phytophthora cinnamori* using compost originated from waste material such as hardwood or pine bark municipal sludge grape marc or cattle manures (Nelson and Hoitink 1983). By using composts, it is possible to cut back on pesticides and inorganic fertilizers and reduce soil deterioration and erosion associated with intensive farming systems. Compost tea is increasingly being used as alternative plant disease control measure in commercial horticulture, especially for range of foliar diseases (Scheuerell and Mahalfe 2004; Welt Zien 1991). However, very less information is available on its use as soil drench or for seed coating (Scheuerell and Mahalfe 2004). Earlier studies by Trankner (1992) showed that pea seeds soaked in compost tea reduced disease symptoms caused by *Pythium ultimum*. Commercial preparations of compost tea were found to suppress damping off of cucumber in soil less container media (Scheuerell and Mahalfe 2004); however, such preparations were produced from yard trimmings as blends using vegetative or vermicompost or animal manures.

At The Division of Microbiology, Indian Agricultural Research Institute, New Delhi, the efficacy of microbial antagonists' (*Anabaena* strains ± *Bacillus subtilis*) amended paddy straw compost preparations for suppressing diseases caused by plant pathogenic fungal consortium- (*Fusarium oxysporum*, *Pythium debaryanum*, *P. aphanidermatum* and *Rhizoctonia solani*) were evaluated in tomato

Fig. 10.2 Influence of different treatments on the growth of the Tomato seedlings challenged with fungal consortium with (*1*) Chemical control (*2*) Biological control (*3*) *Anabaena variabilis* amended compost (*4*) Unamended compost (*5*) *Anabaena oscillarioides* amended compost (*6*) *Anabaena oscillarioides* amended compost formulation

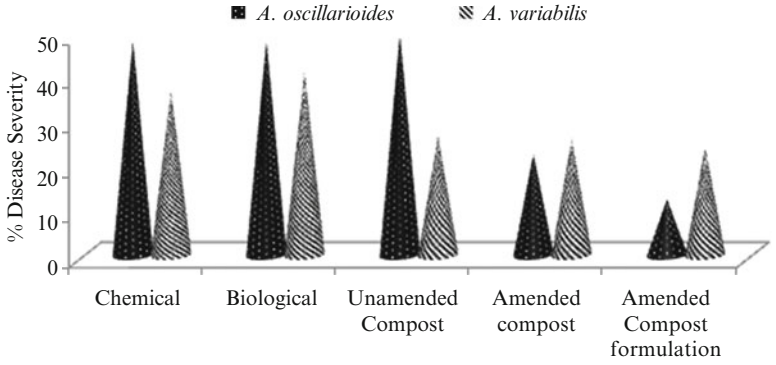

Fig. 10.3 Effect of compost formulations on disease severity (%) of fungal consortium challenged tomato seedlings

(Dukare et al. 2011). Comparative performance of the fungi challenged and control (uninoculated) treatments revealed the superiority of the compost formulations in enhancing seed germination, seedling length and biomass (Fig. 10.2), with 40–50% enhancement in plant parameters. The amended composts also led to significantly better control in terms of 29–37% reduction in disease severity (Fig. 10.3) over biological control (*Trichoderma* formulation) and chemical control (Thiram-Carbendazim). Such biocontrol agents, which provide multiple benefits, may provide useful options for improving afforestation practices and establishment of plants in diverse inhospitable/barren habitats, besides their promise as multifaceted bioinoculants in organic farming practices popular in present day agriculture.

10.5 Indirect Traits Involved in Their PGP Activities

PGPR also indirectly enhance plant growth by different mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance.

Root colonisation is an important trait involved in the successful proliferation of the inoculated strain. Root exudates and mucilage derived nutrients are known to attract deleterious and beneficial bacteria, fungi and other organisms (Compant et al. 2010). It is generally assumed that PGPR stimulation of plant growth requires the binding of the bacterium to the plant root. The successful use of either rhizobial or PGPR inoculants in agriculture depends upon the delivery of viable bacteria to the root zone which is most frequently accomplished by inoculating seeds with a preparation of dormant bacterial cells, by means of coated seed or bulk inoculants. Selected strains of PGPR are used as seed inoculants (Sahin et al. 2004) and the colonization of roots by the bacteria possessing several beneficial traits has been shown to promote and stimulate plant growth and development (Sindhu et al. 1999). Such bacteria have been applied to a wide range of agricultural crop species for the purpose of growth enhancement, including increased seed emergence, plant weight, crop yields and disease control (Kloepper et al. 1980).

10.5.1 Production of Siderophores

Iron is an essential nutrient of plants, but it is relatively insoluble in soil solutions. Therefore, availability of iron, one of the most important nutrient requirements for growth of almost all plants; is extremely limiting in the rhizosphere. The predominant form of iron in aerated soils is ferric ion, which is sparingly soluble and the concentration of iron is rather low, and insufficient to support microbial growth. To survive in such an environment, organisms were found to secrete Fe binding ligands called siderophores having higher affinity ($K_D = 10^{-20}$ to 10^{-50}) to sequester iron from the microenvironment (Neilands 1981). Siderophores are ferric ion specific ligands of low molecular weight. The secreted siderophore molecules find most of the ferric ion that is available in the rhizosphere; and as a result effectively prevent any pathogen in its immediate vicinity from proliferating because of lack of iron. It should be noted that the plant host is unaffected by depletion of iron caused by PGPR. Plants are able to grow at much lower iron concentration (~1,000 fold) than microbial phytopathogens (O'Sullivan and O'Gara 1992)

Kloepper et al. (1980) were the first to demonstrate the importance of siderphore production as a mechanism for plant growth promotion and biological control of pathogens. They demonstrated the siderophore mediated antagonism of *Erwinia carotovora* by plant growth promoting fluorescent *Pseudomonas* strains and yield increase in the case of potato, sugarbeet and radish. Evidence for siderophore mediated PGPR model was further proved by the isolation of a fluorescent siderophore was

isolated from PGPR *Pseudomonas* B10 strain (Kloepper et al. 1980, 1999). The soil Pseudomonads generally produce fluorescent yellow-green water soluble siderophore with both a hydroxamate and phenolate group (O' Sullivan and O' Gara 1992). These siderophores have been classified into two main types, pyoverdins and pyochelins (Glick et al. 1998). A third siderophore type has been isolated from *P. fluorescence* WCS374 called fluorebactin (Mercado-Blanco et al. 1977). Apart from *Pseudomonas*, other genera include *Bacillus, Rhizobium, Agrobacterium, E. coli* and many fungi which also produce a wide range of iron chelating compounds. Numerous plants are capable of using bacterial – Fe siderophore complexes as a means of obtaining iron from soil (Wang et al. 1993).

10.5.2 Antibiotics

One of the most effective indirect mechanisms of PGPR involves its ability to synthesize antibiotics. Through the synthesis of antibiotics plant growth promoting rhizobacteria can prevent the proliferation of many phytopathogens and thereby enhance plant growth in the process. A variety of antibiotics have been identified including compounds such as amphisin, 2,4-diacetylphloroglucinol (DAPG), hydrogen cyanide, oomycin A, phenazine, pyoluteorin, pyrrolnitrin, tensin, troplone and cyclic lipopeptide produced by *Pseudomonas* and oligomycin A, kanosamine, Zwrittermicin A and Xanthobaccin produced by *Bacillus, Streptomyces* and *Stenotrophomonas sp*. (Compant et al. 2005). Inoculation with antibiotic negative mutants in the rhizosphere, revealed no desirable activity, indicative of the significance of antibiotic production for effective PGP activity (Haas and Defago 2005).

10.5.3 Detoxification and Degradation of Virulence Factor

Another mechanism of biological control is the detoxification of pathogen virulence factors e.g. detoxification of allbicidin toxin produced by pathogen *Xanthomonas albilineans* (Zhang and Birch 1996) as well as irreversible detoxification of albicidin mediated by an esterase produced by *Pantoea dispersa* (Zhang and Birch 1996, 1997). Recently, it has been discovered that certain PGPR quench pathogen quorum sensing capacity by degrading auto-inducer signals, thereby blocking expression of several virulence genes (Molina et al. 2003; Newton and Fray 2004; Dong et al. 2004).

10.5.4 Volatile Compounds

PGPR strains release a blend of volatile organic compounds (2, 3 butanediol and acetone) that promote growth and induce resistance against pathogen (Ryu et al. 2004) Hydrocyanic acid (HCN) produced by many rhizobacteria is postulated to play a role in biological control of pathogens (Schippers 1988). HCN secreted by *Pseduomonas fluorescens* strain CHAO has been demonstrated to stimulate root

hair formation and suppress back root rot caused by *Thielaviopsis basicola* in tobacco plant (Voisard et al. 1989). Howell and Stipanovic (1979) reported that volatile compounds such as ammonia produced by *Enterobacter cloacae* are involved in the suppression of *Pythium ultimum* induced damping off of cotton.

10.5.5 Induction of Systemic Resistance

Plant growth promoting rhizobacteria can suppress diseases through antagonism between the bacteria and soil borne pathogens as well as by inducing a systemic resistance in the plant against both root and foliar pathogens (Ramamoorthy et al. 2001; Compant et al. 2005). Rhizobacteria mediated induced systemic resistance (ISR) has been demonstrated against bacteria, fungi and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco and tomato as evidenced by enhanced defensive capacity upon challenge inoculation. Although some bacterial strains are equally effective in inducing resistance in different plant species, others show specificity indicating specific recognition between bacteria and plants at the root surface. Bacterial determinants of ISR include lipopolysaccharides, flagellin, siderophore and salicyclic acid (Bakker et al. 2003). Bacterially produced salicylic acid mediates induced systemic resistance (ISR) in plants and works as an antimicrobial agent against various pathogens (Indiragandhi et al. 2008). Salicylic acid induces phenotypically similar systemic acquired resistance (SAR). Jasmonic acid and ethylene signaling in the plant produced by rhizobacteria also induces ISR (Ton et al. 2002). Most reports of PGPB mediated ISR involve the living rhizobacterial strains; but endophytic bacteria have also been observed to have ISR activity. The bacterial plant response induced after challenge with a pathogen resulted in the formation of structural barriers such as thickened cell wall papillae due to deposition of cellulose and the accumulation of phenolic compounds at the site of pathogen attack. (M'Piga et al. 1997; Benhamou et al. 1998). Biochemical and physiological changes in plants include induced accumulation of peroxidases, pathogenesis related proteins (PR proteins); phenylalanine ammonia lyase, phytoalexins, polyphenol oxidases and/or chalcone synthase. Recent evidence indicates that induction of some of these plant defense compounds (e.g. chalcone synthase) may be triggered by the same N- acyl homoserine lactones (HSL) that bacteria use for intraspecific signaling (Compant et al. 2005). Such PGPR perform well against specific pathogens, insects and nematode pests under field conditions (Ramamoorthy et al. 2001).

10.5.6 Promotion of Symbiosis/Enhancement of Legume Nodulation

Free living rhizobacteria may also influence the symbiosis between microorganisms and plants and thereby stimulate plant growth indirectly. Some PGPR can positively

interact with various plant symbiotic microbes such as *Rhizobium, Bradyrhizobium, Frankia, Azospirillum* and mycorrhizal fungi (Kloepper et al. 1987; Zahir et al. 2003; Figueiredo et al. 2008).

10.5.7 Soil Quality Improvement

Cyanobacteria are known to contribute to macro-aggregation and result in improved resistance to soil erosion, because as primary producers, they contribute to the enrichment of soil with SOM and to the improvement of biological activity (Acea et al. 2003). Cyanobacterial EPS secretions are dominated by polysaccharides which can bind soil particles (Belnap and Gardner 1993; Eldridge and Greene 1994; Malam Issa et al. 2001), besides aiding in the protection of the cyanobacteria against environmental conditions (De Winder 1990) and in assisting cyanobacterial motility (Stal 1995). Inoculating soil with cyanobacteria has been reported to improve the aggregation of the top soil (Rao and Burns 1990; Malam Issa et al. 2001) and to increase water retention, and ecosystem regeneration (Eldridge and Greene 1994). The potential positive effects of cyanobacteria, however, are not restricted to soil physical properties. Soil inoculation with N_2 fixing cyanobacteria, has also been shown to induce increases in SOC, total N and available nutrients in the topsoil. Rogers and Burns (1994) demonstrated that inoculation of a poorly structured silt loam soil with *Nostoc muscorum* led to a pronounced effect on soil chemical properties, with total C increasing by 50–63% and total N increasing by 111–120%. In a laboratory experiment, Acea et al. (2003) also showed that soil inoculation with different cyanobacterial strains induced great microbial proliferation as well as high increases in SOC and available nutrients, the efficacy of the treatment depending on the type of soil. Observed increases in soil total N following cyanobacterial inoculation is attributed to the ability of cyanobacteria to fix atmospheric nitrogen. Soil inoculation with cyanobacteria with these attributes may therefore represent a simple and low-cost method for improving the productivity of degraded lands in developing countries where very little or no inorganic fertilizers are usually applied.

Cyanobacteria have the ability to aggregate bare areas of rocks and soil and play an important role in revegetation in coastal and Usar soils (Singh 1950). Generally Usar soil exhaust the productivity of soil and reclamation of such soils requires the removal of ions using some chemical methods, followed by leaching which is expensive and does not result in complete removal of the salts. Jaiswal et al. (2010a) recorded the bioameliorating role of *Nostoc calcicola* and providing conducive conditions for the growth of plants and microorganisms.

10.6 Conclusions and Future Perspectives

A large body of literature exists about rhizosphere interactions as they represent the key to our better understanding of how to improve plant fitness and sustain soil health. The root system, traditionally thought to provide anchorage and uptake of

nutrients and water, is now well recognized as a metabolically active factory, mediating numerous underground interactions through chemical dialogues with neighbouring plants, microflora and microfauna. Future research needs to be undertaken towards enhancing our understanding of ecology of PGPR, i.e. colonisation, niche adaptation and interactions with diverse members of the rhizosphere. A combination of recent technologies in the area of 'omics' such as proteomics, metabolomics, transcriptomics and secretomics will allow us to strengthen our capability to visualize a complete picture of these complex multispecies interactions. This can be effectively and efficiently exploited through better predictions of how bacteria interact with plants, whether they are likely to establish themselves in the plant rhizosphere after field application as biofertilizers/biocontrol agents and help in engineering the rhizosphere for greater agricultural benefits.

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Chapter 11
Regulation of Antibiotics Production in Biocontrol Strains of *Pseudomonas* spp.

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Abstract *Pseudomonas* are ubiquitous aerobic, gram-negative rod-shaped motile bacteria. Their ability to survive in a variety of environments, and metabolic versatility, make them organisms of choice to explore a metabolic function. Among the myriad functions, their ability to suppress the pathogens (biological control) has made them highly popular. *Pseudomonas* spp. produce a range of secondary metabolites including antibiotics, siderophores, and HCN, a function often hypothesized to confer a selective advantage in the bacterial persistence in soil and the rhizosphere. Secondary metabolite production is regulated through multi-tier mechanisms. Feasibility of molecular genetic analysis and availability of genome sequences make them attractive model system to study the antibiotic production and its regulation. Secondary metabolism is regulated through various mechanisms acting at transcriptional and post-transcriptional levels. The highly conserved GacA/GacS two-component signal transduction is a universal global regulatory mechanism in fluorescent *Pseudomonas* spp. Stationary-phase gene expression is interwoven with this regulatory circuit, wherein stationary-phase sigma factor, RpoS is a central regulator controlling the stress tolerance and environmental fitness of a strain. A third pathway, consisting of small RNAs ensures secondary metabolism and biocontrol at post-transcriptional level. They scavenge small RNA binding proteins thereby relieving the translational repression of the target genes. Several functions and their regulatory network feed directly into the broad framework of quorum sensing (QS). In Pseudomonads, identified regulatory elements of QS include the RpoS, the GacS/GacA, and other two-component regulatory systems, the small RNA-binding regulator and many others. The discovery of new regulators of QS will help in elucidating the signal transduction mechanism in bacteria as a whole,

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and understanding the regulation of biocontrol function in Pseudomonads in particular. This is a pre-requisite for predicting the optimal environmental conditions for such bacteria to materialize their desired functions.

Keywords Antibiotics • Biocontrol • *Pseudomonas* • GacA/GacS • Regulation • Quorum sensing • Rhizosphere

11.1 Introduction

Disease suppressive soils are known to occur worldwide. In such soils, plants do not suffer from some diseases, or the severity is lost, even when a pathogen may be present (Haas and Defago 2005). The subsequent studies carried out on natural disease suppression suggested that such soils may be inhabited by soil microorganisms that act as pathogen antagonists. This led to the idea that such microorganisms could be used as biocontrol agents. Extensive work carried out since then has identified a long and diverse list of bacteria such as Plant Growth Promoting Bacteria/Rhizobacteria (PGPB/PGPR), that can serve this important ecological function. The genetic dissection of these functions in many PGPR has led to successful application of this environment-friendly technology both in the field as well as controlled conditions (Lugtenberg and Kamilova 2009). Rhizosphere represents one of the most complex ecosystems on Earth (Jones and Hinsinger 2008). Lorenz Hiltner is recognized as the first scientist to coin the term "rhizosphere" in 1904, described it as the layer of soil influenced by root metabolism, and is the site of unique interactions between beneficial and pathogenic ('uninvited guests') microorganisms. These microbes are attracted by root exudates, which can have a dramatic impact on plant nutrition and plant health (Hartmann 2005).

The rhizosphere and its inhabiting microorganisms fulfil important ecological functions, e.g. nutrient recycling, synthesis of useful biomolecules and bioprotection against pathogens, and thus are responsible for plant growth and health (Sørensen 1997). Additionally, this microenvironment is described as 'microbial hot-spot' where diverse interactions between organisms, beneficial as well as pathogenic, take place (Whipps 2001). The number and diversity of deleterious and beneficial microorganisms are related to the quantity and quality of the rhizodeposits and to the outcome of the microbial interactions that occur in rhizosphere (Somers et al. 2004). Bacteria, which are highly competitive, for example, due to the production of antibiotic substances, can colonize the rhizosphere better. Many rhizobacteria like the fluorescent Pseudomonads and *Streptomyces* species (Fravel 1988; Raaijmakers et al. 2002; Weller et al. 2002) produce an extended list of antibiotics. Antibiotics produced by rhizobacteria include 2,4-diacetylphloroglucinol, pyrrolnitrin, phenazine, pyoluteorin, and herbicolin A, all of which have also been detected directly in the rhizosphere (Thomashow et al. 1997).

The occurrence and production of diverse antibiotic substances in the rhizosphere explains the frequent detection of bacteria with multiple antibiotic resistances in this microenvironment (Whipps 2001) and also explains the natural disease

suppression. Understanding the processes that determine the composition, dynamics, and activity of the rhizosphere microflora has attracted the interest of scientific community encompassing multiple disciplines and can be exploited for the development of new strategies to promote plant growth and health (Tilak et al. 2005; Tilak and Reddy 2006; Raaijmakers et al. 2009). Bacteria inhabiting the rhizosphere can have a neutral, pathogenic or beneficial interaction with their host plant. In healthy plants, the occurrence of pathogenic bacteria is low and can infact be controlled by the plant defence system and plant beneficial bacteria enriched by the rhizosphere conditions. The latter group comprises the PGPR, which influence plant growth by producing phytohormones or enhancing the availability of nutrients, inducing systemic resistance in plants, and truly antagonistic bacteria (Van Loon et al. 1998; Whipps 2001). PGPR were first identified by the work of Kloepper and Schroth (1978) who described them as soil bacteria that colonize the roots of the plants and enhance the growth by several means. Root colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development by direct or indirect mechanisms have thus been defined as plant growth promoting rhizobacteria or PGPR (Nelson 2004). Among them are the strains from diverse genera such as *Pseudomonas, Azospirillum, Burkholderia, Bacillus, Enterobacter, Rhizobium, Erwinia, Serratia, Alcaligenes, Arthrobacter, Acinetobacter* and *Flavobacterium* (Rodriguez and Fraga 1999).

The mechanism by which these PGPR promote growth of plants can be either:

1. Direct Mechanism (Biofertilizer and Biostimulator activity) or
2. Indirect Mechanism (Biocontrol activity).

Direct: Direct promotion of plant growth by PGPR generally entails providing a compound to the plant that is synthesized by a bacterium or facilitating the availability of a nutrient and its uptake from environment. The rhizobacteria produces the secondary metabolites, which are directly utilized by the plants thus promoting plant growth (Bashan and de-Bashan 2005). There are several ways the PGPR may directly facilitate the proliferation of their plant hosts.

They can:

- Fix atmospheric nitrogen and supply it to the plant.
- Synthesize phytohormones like auxins (IAA) that trigger plant cell growth and proliferation.
- Solubilize minerals like phosphates in a form that can be used by the plant.
- Synthesize enzymes that can modulate plant hormone levels.

Indirect: The indirect stimulation of plant growth occurs when a beneficial microbial strain prevents the growth of a phytopathogenic soil microorganism that could otherwise interfere with the normal plant growth and development. This action is called antibiosis and can be either due to the depletion of a scarce resource, required by the pathogen, or to the production and release of a compound that impedes the growth of the phytopathogenic organism (Mercado Blanco and Bakker 2007). Thus, the plant growth promotion by beneficial bacteria through indirect mechanisms includes:

- Production of antibiotics that kill the phytopathogens i.e., biocontrol.
- Production of siderophores that limit the availability of iron to the pathogen.

- Synthesis of other compounds like toxic lipodepsipeptides, hydrogen cyanide HCN and enzymes that may lyse the fungal cell walls.
- Induction of systemic resistance (ISR).

Thus, while indirect mechanisms involve suppression of the deleterious effects of phytopathogens on crop yield, direct mechanisms involve the utilization of microbial functions/products by plants. PGPR strains are known to employ one or more of these mechanisms in conjunction in the rhizosphere (Bashan and de-Bashan 2005).

Biological control of root diseases with microorganisms is a forward-looking concept in the overall trend towards a more sustainable agriculture. Biocontrol broadly refers to the use of a living organism to curtail the growth and proliferation of another undesirable one. Rhizosphere inhabitants may provide a front line defense against pathogen attack and are ideal for use as biocontrol agents. These bacterial populations form microcolonies preferably at root regions where there are abundant root exudates secreted by the plant and initiate the quorum sensing signals that help in cell to cell communication. These signals are readily sensed by the signal specific biosensors and henceforth help in better association of microbes to the plant. The host plant root exudates and mucilage serve as a pool of sugars utilized by these bacteria as the main carbon source. It has been shown that as much as 20% of the carbon allocated to roots is deposited in the rhizosphere, for the benefit of these bacteria, suggesting a highly evolved relationship between the plants and microorganisms in the rhizosphere. Also this rhizodeposit is rich in other nutrients like amino acids and polyamines (Singh et al. 2006; Raaijmakers et al. 2009; Choudhary et al. 2009). Certain antimicrobial compounds are also produced by plant, which allow only resistant organisms to populate. Association of many *Pseudomonas* strains with its host plant roots has also been known to involve the O-antigen of lipopolysaccharides. (Compant et al. 2005). Since these bacteria produce various biocontrol and growth promoting agents in addition to host plant defense mechanisms, the plant becomes defiant to parasites and phytopathogens creating a dynamic environment in the rhizosphere that leads to biocontrol of diseases. Biocontrol microorganisms may adversely affect the population density, dynamics (temporal and spatial) and metabolic activities of soil-borne pathogens via mainly three types of interactions: competition, antagonism, and hyperparasitism (Raaijmakers et al. 2009). A variety of substances produced by biocontrol Pseudomonads have been implicated in the mechanism(s) used by these organisms to limit the damage to plants by the phytopathogens (Upadhyay and Srivastava 2008). These include siderophores, antibiotics, other small molecules and a variety of enzymes. In the case of naturally suppressive soils, the biocontrol effect has been attributed mostly to resident populations of beneficial root-colonizing bacteria and to their interaction with certain soil edaphic factors (Cook and Baker 1983; Defago and Haas 1990; Defago and Keel 1995).

During the past several years, numerous reports have illustrated the beneficial plant growth-promoting effects of several rhizosphere-colonizing bacteria in protecting the plants from the deleterious effects of plant pathogens, but one group which has gained immense importance constitutes the fluorescent Pseudomonads (Weller 1988; Defago and Haas 1990; Cook 1993). These biocontrol, fluorescent

Psuedomonas spp. have been broadly studied for their ability to reduce the development of various soil-borne plant pathogens because many strains may trigger systemic resistance in host plants and produce antifungal compounds and exoenzymes. Members of the genus *Pseudomonas* are rod-shaped Gram-negative bacteria characterized by extensive metabolic versatility, aerobic respiration (some strains also have anaerobic respiration), motility owing to one or several polar flagella, and a high genomic G+C content (59–68%) (Dubuis et al. 2007; Palleroni 2008). Many Pseudomonads are known to live in a commensal relationship with plants, thriving on root exudates secreted from plant surfaces. Pseudomonads have an exceptional capacity to produce a wide variety of metabolites, including antibiotics that are toxic to plant pathogens (Raaijmakers et al. 2002; Haas and Keel 2003).

Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents, (Weller 1988). These include the ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plants; (iv) produce a wide spectrum of bioactive metabolites (i.e., antibiotics, siderophores, volatiles, and growth-promoting substances); (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses (Weller 2007).

The production and activity of bacterial metabolites involved in pathogen suppression is significantly affected by environmental conditions that prevail in the rhizosphere. The environmental factors include climate, local weather conditions, soil characteristics, or the composition or activity of the indigenous microbial flora of the soil. Signals that influence the expression of biocontrol traits in Pseudomonads can emanate from the biocontrol bacteria themselves, as well as from other soil bacteria or fungi, or from host plants. To achieve the maximum growth promoting function of PGPR, it is important to understand how the rhizobacteria exert their effects on plant and whether the effects are altered by various environmental factors, including the presence of other micro-organisms (Bent et al. 2001; Dubuis et al. 2007). The research over the last decade has resulted in the introduction of several well-characterized *Pseudomonas* spp. that helps in understanding regulation and organization of the biosynthesis gene clusters involved in the production of antibiotics. *Pseudomonas* spp., as a model PGPR system, have thus been extensively studied as effective biocontrol agents against a wide range of phytopathogenic fungi. The better understanding of the regulation of antibiotics production is likely to result in the development of PGPR with improved reliability and efficacy. Moreover, molecular communication between different genera and species of PGPR might help in the selection of compatible strains to be released under field conditions (Fernando et al. 2005).

11.2 Production of Antibiotics by *Pseudomonas* spp.

Pseudomonas fluorescens are known for their biological control capabilities against soil-borne pathogens, aggressive root colonization, efficient rhizosphere competence, and production of a range of useful secondary metabolites. Most biocontrol

strains of *Pseudomonas* spp. with a proven effect in plant bioassays produce one or several antibiotic compounds that are unrelated to typical siderophores. Antibiotics are secondary metabolites produced by microorganisms and it has been speculated that they do confer some competitive advantage to the producer (Katz and Demain 1977), such as improved colonization of the plant surfaces and therefore niche exclusion of the pathogen (Mazzola et al. 1992) or have more subtle effects as well as direct antagonism (Dowling and O'Gara 1994). Interaction of PGPR with host plants is an intricate and interdependent relationship that involves not only the two partners but also other biotic and abiotic factors of the rhizosphere region (Dutta and Podile 2010). The production and activity of bacterial metabolites involved in pathogen suppression may be significantly affected by environmental conditions that prevail in the rhizosphere. For example, the available sources of carbon, nitrogen and micronutrients, temperature, and availability of oxygen can all influence the functions of biocontrol strains. The biocontrol abilities of such strains depend essentially on aggressive root colonization, induction of systemic resistance in the plant, and the production of diffusible or volatile antifungal antibiotics. Evidence that these compounds are produced *in situ* is based on their chemical extraction from the rhizosphere and on the expression of antibiotic biosynthesis genes in the producer strains colonizing plant roots. *In vitro*, these antibiotics inhibit fungal pathogens, but they can also be active against many bacteria and, in some cases, against nematodes (Haas and Défago 2005; Upadhyay and Srivastava 2010). Well-characterized antibiotics with biocontrol properties include phenazines and its different derivatives, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide (Haas and Keel 2003). The list of the antibiotics produced by PGPR is growing steadily and besides the more common ones, described above, include oomycin A, viscosinamide, butyrolactones, kanosamine, zwittermycin-A, aerugine, rhamnolipids, cepaciamide A, ecomycins, pseudomonic acid, azomycin, antitumor antibiotics FR901463, cepafungins and antiviral antibiotic karalicin etc. These antibiotics are known to possess antiviral, antimicrobial, antihelminthic, phytotoxic, antioxidant, cytotoxic, antitumour and plant growth promoting activities (Fernando et al. 2005). The modes of action of many of these secondary metabolites are only partly understood.

The first clear-cut experimental demonstration that a *Pseudomonas* antibiotic can suppress plant disease in an ecosystem was made by Thomashow and Weller (1988). They identified a phenazine, phenazine-1-carboxylic acid (PCA), as a biocontrol factor produced by *P. fluorescens* 2–79. Phenazines are heterocyclic compounds that are colorful diffusible bacterial metabolites produced naturally and substituted at different points around their rings in different bacterial species. A number of naturally-occurring, broad spectrum, colored phenazines have been reported in different studies. *Pseudomonas aureofaciens* produces the orange and brick-red phenazine compounds 2-hydroxyphenazine-1-carboxylic acid and 2-hydroxyphenazine (Pierson and Thomashow 1992; Delaney et al. 2001). *Pseudomonas fluorescens* are typically known to produce yellow compound phenazine-1-carboxylic acid (PCA). A cherry-red colored phenazine compound, from *P. fluorescens* Psd has been identified in the study by Upadhyay and Srivastava (2008).

One of the primary factors governing phenazine production is population density, and in *P. aeruginosa* this dependency is affected by at least three quorum-sensing systems (Whiteley et al. 1999; Deziel et al. 2004). The dependence of phenazine biosynthesis on cell density has also been demonstrated for many biocontrol Pseudomonads, and in these species it is mediated by a seemingly less complex quorum-sensing network. At the transcriptional level, phenazine biosynthesis is controlled by the PhzR-PhzI quorum sensing system (Pierson et al. 1994; Chin-A-Woeng et al. 2001; Khan et al. 2005; Dubuis et al. 2007). However, in addition to being regulated by cell-cell communication, phenazines themselves can act as intercellular signals (Price-Whelan et al. 2006; Dubuis et al. 2007). The environmental factors that affect the regulation of phenazine biosynthesis include oxygen, iron and phosphate concentration, nature of the carbon and nitrogen source and amino acids availability (Van Rij et al. 2004; Price-Whelan et al. 2006). *Pseudomonas chlororaphis* PCL1391 produces the secondary metabolite phenazine-1-carboxamide (PCN), which is an antifungal metabolite required for biocontrol activity of the strain. Decreasing the pH from 7.0 to 6.0 or decreasing the growth temperature from 21°C to 16°C decreased PCN production dramatically. In contrast, growth at 1% oxygen as well as low magnesium concentrations increased PCN levels. Salt stress, low concentrations of ferric iron, phosphate, sulphate, and ammonium ions reduced PCN levels. Different nitrogen sources also greatly influenced PCN levels. In *Pseudomonas* spp., the phenazine biosynthetic pathway branches off from shikimic acid of aromatic amino acid biosynthesis pathway, which is also the source for metabolites such as, siderophores and quinones (Dewick 1984; McDonald et al. 2001; Vandenende et al. 2004). The increase in PCN level was found to be between 8- and 23-fold after the addition of the aromatic amino acids phenylalanine, tyrosine, and tryptophan. Fusaric acid, a secondary metabolite produced by the soi-borne fungus *Fusarium* spp., also reduced PCN levels. A phenazine regulator, RpeA (repressor of phenazine expression) was shown to regulate PCN production in minimal medium, but not in complex medium (Whistler and Pierson 2003).

Phenazine production may not be regulated in the same way in various *Pseudomonas* spp. Although the results obtained for one strain is difficult to compare with others because of different culture conditions which, can have an enormous impact on phenazine production. There are some striking similarities and differences between the results in literature. The production of phenazine-1-carboxylic acid (PCA) by *P. fluorescens* 2–79 (Slininger and Shea-Wilbur 1995) and PCN production by *P. aeruginosa* (Kanner et al. 1978) and *P. chlororaphis* PCL1391 (Van Rij et al. 2004) were found to be stimulated by glucose and glycerol. This suggests a similar response of phenazine-producing Pseudomonads to these carbon sources. As in PCL1391, phenazine production in *P. aeruginosa* also is regulated by nitrogen sources in that NH_4^+ supported a higher production of PCN than urea, asparagine, or peptone (Kanner et al. 1978). PCA production by *P. fluorescens* 2–79 was not found to be affected by different nitrogen sources (Slininger and Shea-Wilbur 1995). As in strain PCL1391, aromatic amino acids stimulated phenazine production in *P. aureofaciens* 517 (Labeyrie and Neuzil 1981); in *P. aeruginosa* A237, only tyrosine and phenylalanine were reported to stimulate PCA production (Korth 1973). The pH optimum

for PCN production in PCL1391 differed from that in *P. fluorescens* 2–79, in which the optimum is pH 7.0, with only a small reduction at pH 6.0, and a severe reduction at pH 8.0 (Slininger and Shea-Wilbur 1995). Similar to strain PCL1391, Fe^{3+} and magnesium ions had a positive effect on the production of PCA in *P. fluorescens* 2–79 (Slininger and Jackson 1992). An examination of the relationship between iron availability and the regulation of phenazine biosynthesis, however, presents a complicated picture that neither refutes nor supports a role for these compounds in iron acquisition. Although in many cases, it has been reported that phenazine production is enhanced in iron-deprived cultures, other studies have demonstrated a requirement for iron in media optimized for phenazine biosynthesis (King 1954; Cox 1986; Van Rij et al. 2004). The production of pyocyanin, a phenazine derivative produced by *P. aeruginosa,* is stimulated by low phosphate concentrations (Turner and Messenger 1986), which is different from PCN production in PCL1391. From these comparisons among *Pseudomonas* strains, we can conclude that some environmental factors have similar effects whereas others may have opposite effects in different strains.

Pyrrolnitrin and phenazine are known to be most diverse antibiotics with broad-spectrum antifungal activities. They have been described to be involved in suppression of seedling diseases (Kirner et al. 1998; Mavrodi et al. 2006; Upadhyay and Srivastava 2010). The ability of biocontrol bacteria to produce pyrrolnitrin [3-chloro-4- (29-nitro-39-chlorophenyl)-pyrrole] has been correlated with biocontrol activity of fungal plant pathogens (Howell and Stipanovic 1979). Pyrrolnitrin (Prn) and its production by *Pseudomonas* species was first described by Arima et al. (1964). Pyrrolnitrin is a tryptophan-derived secondary metabolite produced by a narrow range of gram-negative bacteria. Pyrrolnitrin biosynthesis by rhizobacteria presumably has a key role in their life strategies and in the biocontrol of plant diseases. Pyrrolnitrin, described as an inhibitor of fungal respiratory chains (Tripathi and Gottlieb 1969) has been used as an antimycotic topical antibiotic in human medicine. Synthetic analogues of pyrrolnitrin have been developed for use as agricultural fungicides (Ligon et al. 2000).

Among the various extracellular metabolites produced, the polyketide antibiotic, 2,4-diacetylphloroglucinol (Phl or DAPG) is of prime importance in plant protection. Interestingly, Phl is known to positively controls its own biosynthesis (Haas and Keel 2003; Fernando et al. 2005). It is a phenolic molecule and a broad-spectrum antibiotic produced by many fluorescent Pseudomonads that exhibits antifungal, antibacterial, antihelmenthic and phytotoxic activitites (Mavrodi et al. 2001). Phloroglucinol from *P. fluorescens* CHA0 has been demonstrated earlier as a key component in biological control of take-all disease of wheat by *Gaeumannomyces graminis tritici*, and black root rot of tobacco caused by *Thielaviopsis basicola*. DAPG from *P. fluorescens* F113 can prevent damping off in sugarbeet caused by *Pythium ultimum* (Raaijmakers and Weller 1998; Picard et al. 2000). Many biotic and abiotic factors influence the expression of DAPG gene. Biotic factors such as plant species, plant age, cultivar and pathogens alter the expression of the gene *phlA* (Notz et al. 2001). DAPG production is also influenced by abiotic factors such as carbon sources and various minerals. While iron (Fe^{3+}) and sucrose increases DAPG

production in *P. fluorescens* F113, glucose has similar stimulatory effect on DAPG production in *P. fluorescens* Pf-5 and CHA0 (Nowak-Thompson et al. 1994; Duffy and Defago 1999). In *P. fluorescens* strain S272, highest DAPG yield was obtained with ethanol as the sole source of carbon. Micronutrients Zn^{2+}, Cu^{2+} and Mo^{2+} stimulated DAPG production in *P. fluorescens* CHA0 (Notz et al. 2001).

Pyoluteorin (Plt) is a phenolic polyketide with resorcinol ring. It was first isolated from *P. aeruginosa* (Takeda 1958) followed by *P. fluorescens* Pf-5 and CHAO (Bencini et al. 1983; Bender et al. 1999). Plt has bactericidal, herbicidal and fungicidal properties. Application of Plt to cotton seeds suppressed damping-off disease (Howell and Stipanovic 1980).

DAPG acts as a signal molecule to trigger the gene expression in the related species of *Pseudomonas*. But at the same time, the presence of antibiotic like pyoluteorin suppresses the expression and production of DAPG by fluorescent Pseudomonads. Though DAPG and pyoluteorin belong to the same class, namely polyketides, the expression of one type suppresses the other. Apart from it, the communication and interaction of soil-borne pathogens with PGPR also may suppress the expression of the gene in fluorescent Pseudomonads for the production of DAPG (Fernando et al. 2005).

Some of the antibiotic metabolites have remarkably diverse functions, besides their toxic activity. DAPG, PLT, and phenazines can function as signal molecules that affect gene expression not only in the producer bacteria, but also in other organisms (Schnider-Keel et al. 2000; Maurhofer et al. 2004; Brodhagen et al. 2004; Baehler et al. 2005; Dietrich et al. 2006; Price-Whelan et al. 2006). DAPG has been described as an inducer of systemic plant resistance (Iavicoli et al. 2003; Weller et al. 2007) and as a stimulant of amino acid exudation from roots (Phillips et al. 2004). Phenazines, in their reduced form, might enable the producing bacteria to mobilize micronutrients such as iron (Fe^{3+}) from the rhizosphere environment (Hernandez et al. 2004; Price-Whelan et al. 2006). Besides these antibiotics, plant beneficial Pseudomonads are also known to produce HCN, and cyclic lipopeptides. These compounds are important as they can make major contribution to biocontrol of root diseases. HCN is a general inhibitor of metalloenzymes and particularly affects the terminal oxidases. Cyclic lipopeptides as surfactant influence the surface motility of the producer organisms and at the high concentrations are detrimental to the integrity of the phospholipid membranes (Raaijmakers et al. 2006; Dubuis et al. 2007). Comprehensive list of secondary metabolites that are involved in biocontrol have been compiled in Table 11.1.

11.3 Regulatory Mechanisms for Antibiotic Production in Biocontrol PGPR – *Pseudomonas* spp.

Biosynthesis of different antibiotics by *P. fluorescens* strains varies and has a strong genetic basis necessitating the characterization of different strains. The biosynthetic pathways involved in their production, as well as regulation and the signals involved

Table 11.1 Production of biocontrol metabolites from *Pseudomonas* spp.

Pseudomonas fluorescens strains	Antibiotics					Siderophores	HCN	Lipo-depsipeptide	References
	Phloroglucinol	Phenazine	Pyoluteorin	Pyrrolnitrin	DAPG				
Psd	–	+	–	+	–	+	+	+	Upadhyay and Srivastava (2008, 2010, 2011)
Pft-1	–	–	–	–	–	+	+	–	Upadhyay and Srivastava (2008)
Q 2-79	–	+	–	–		+	–	–	Raaijmakers et al. (1997), Raaijmakers and Weller (2001), and Mavrodi et al. (2006)
Q 2-87	+	+	–	–	+	+	+	–	Vincent et al. (1991), Mavrodi et al. (2001), and Moynihan et al. (2009)
Pf-5	+	–	+	+	+	+	+	+	Howell and Stipanovic (1979), Paulsen et al. (2005), and Moynihan et al. (2009)
CHA0	+	–	+	+	+	+	+	–	Laville et al. (1992), Keel et al. (1992), Schnider-Keel et al. (2000), and Moynihan et al. (2009)

therein have received extensive attention in different studies/reports. Various studies are providing new insights into the complex regulatory network controlling important biocontrol traits. The Pseudomonads are ubiquitous bacteria found in diverse environments, and as a consequence they exhibit versatile nutritional and metabolic capabilities. Rapid detection of, and adaptation to, environmental conditions that can change swiftly are essential for efficient growth, colonisation and survival. At the genetic level such adaptive mechanisms can operate at the transcriptional, translational and post-translational levels, and global gene regulatory networks may incorporate elements operating at any or all of these levels.

Antibiotics production by these bacteria plays an important ecological role and it is not surprising to find a multi-tier regulatory network controlling this function. Signal transduction pathways coupling the sensing of an environmental signal to modulation of the expression of the relevant target genes frequently involve the use of two-component systems, consisting of sensor histidine kinases coupled to their respective response regulators (Perraud et al. 1999; Pirrung 1999; Hoch 2000; Stock et al. 2000). Thus, the global regulators known to control antibiotic production by *Pseudomonas* spp. includes a two-component regulatory system comprising the sensor kinase GacS (previously called ApdA or LemA) and GacA, a member of the FixJ family of response regulators. The stationary-phase sigma factor σ^s is another global regulator of antibiotic production in *P. fluorescens* (Whistler et al. 1998) as also the quorum sensing (Haas and Keel 2003).

Within the regulatory hierarchy of *Pseudomonas* spp. biocontrol traits, the top controlling mechanism is likely the GacS/GacA two-component system. Downstream to this one, Quorum Sensing (QS) systems and RNA-binding proteins (such as RsmA or RsmE) and small regulatory RNAs would play leading roles in the GacS/GacA signal transduction pathway (Haas and Keel 2003). The *rpoS* encodes the sigma-38 transcription factor that regulates the expression of genes during times of starvation and stationary growth.

11.4 Mechanism of Two-Component Regulatory System

Two-component regulatory systems in bacteria are responsible for sensing and responding to environmental stimuli. This widely conserved system of Gram-negative bacteria regulates the formation of extracellular biocontrol factors or pathogenicity factors, depending on the species. It comprises the sensor kinase, GacS, and its response regulator, GacA, and is involved in regulation of secondary metabolism and many other aspects of bacterial physiology (Haas and Keel 2003; Workentine et al. 2009). The GacS/GacA two-component regulatory system is found in many Gamma-proteobacteria and although the signal it responds to is yet to be determined, this system regulates a wide variety of physiological processes (Haas and Defago 2005). The GacS/GacA system has been studied primarily in Pseudomonads, particularly *P. aeruginosa* for its role in virulence, quorum sensing, and biofilm formation (Parkins et al. 2001; Goodman et al. 2004; Kay et al. 2006;

Ventre et al. 2006) and *P. fluorescens* for its role in the production of biological control factors (Haas and Defago 2005).

Gac is an acronym for global activator of antibiotic and cyanide synthesis. The sensor kinase gene g*acS*, also called *apdA, lemA, repA, or pheN* (Corbell and Loper 1995; Kitten et al. 1998), was first described in *P. syringae* pv. *syringae* strain B728a as an essential factor for lesion manifestation by this pathogenic strain on bean leaves (Hrabak and Willis 1992; Kitten et al. 1998). The response regulator, GacA was first described in *P. fluorescens* biocontrol strain CHA0 as a global activator of antibiotic and cyanide production (Laville et al. 1992). In both *P. aeruginosa* and *P. fluorescens*, the GacS sensor kinase, on perceiving a signal, is assumed to autophosphorylate a conserved histidine residue and transfer this phosphate through a tripartite mechanism to the cognate response regulator, GacA (Workentine et al. 2009). Evidence that GacS and GacA are partners of a two-component system was first obtained genetically in *P. syringae* pv. *syringae* (Rich et al. 1994) and subsequently confirmed for several other bacteria (Whistler et al. 1998; Aarons et al. 2000; Ligon et al. 2000; Bull et al. 2001; Pernestig et al. 2001).

It is suggested that the GacS/GacA system operates a switch between primary and secondary metabolism, with a major involvement of post-transcriptional control mechanisms. GacS senses a still-unknown signal and activates, via a phosphorelay mechanism, the GacA transcription regulator, which in turn triggers the expression of target genes (Heeb and Haas 2001). The GacS sensor kinase has an autophosphorylation domain (phosphoryl transmitter) around His-294, a phosphoacceptor domain (a receiver) around Asp-717 and a histidine phosphotransfer (Hpt, output domain) around His-863 (Perraud et al. 2000). These sequences are proposed to interact with each other when an environmental signal triggers the activation of the protein, inducing a conformational change of the cytoplasmic C-terminal part and thereby favoring autophosphorylation (Williams and Stewart 1999; Robinson et al. 2000). On interaction with bacterial signal molecules, GacS is autophosphorylated at His-294 and initiates a phospho-relay mechanism transfering a phosphate residue to the acceptor domain at Asp-717, and then to a Hpt domain at His-863. The Hpt domain acts as a secondary transmitter by transferring the phosphoryl group to a conserved aspartate (Asp-54) of response regulator, GacA. Phosphorylated GacA is the key regulator of several gene functions including the biocontrol traits (Haas and Keel 2003; Zuber et al. 2003; Lapouge et al. 2008) (Fig. 11.1).

The activation of the sensor kinase, GacS requires a signal, whose nature though is unidentified, may be as diverse as abiotic (e.g. pH, temperature, or osmolarity) or biotic. While some signals may be produced by the host, the others are synthesized by the resident population itself including biocontrol and other microorganisms (Raaijmakers and Weller 1998; Heeb and Haas 2001; Haas and Keel 2003; Dubuis et al. 2007). In the latter case, the signals may be produced in coordination with the cell density of the population and thereby ensuring regulatory mechanisms commonly known as quorum sensing (Bassler 1999; Pirrung 1999; Rice et al. 1999; Hoch 2000; Holden et al. 2000; Stock et al. 2000). The chemical nature of signal molecules that activate the GacS/GacA cascade in bacteria has not been identified nor are the signal biosynthesis genes known. In both *P. fluorescens* CHA0 and

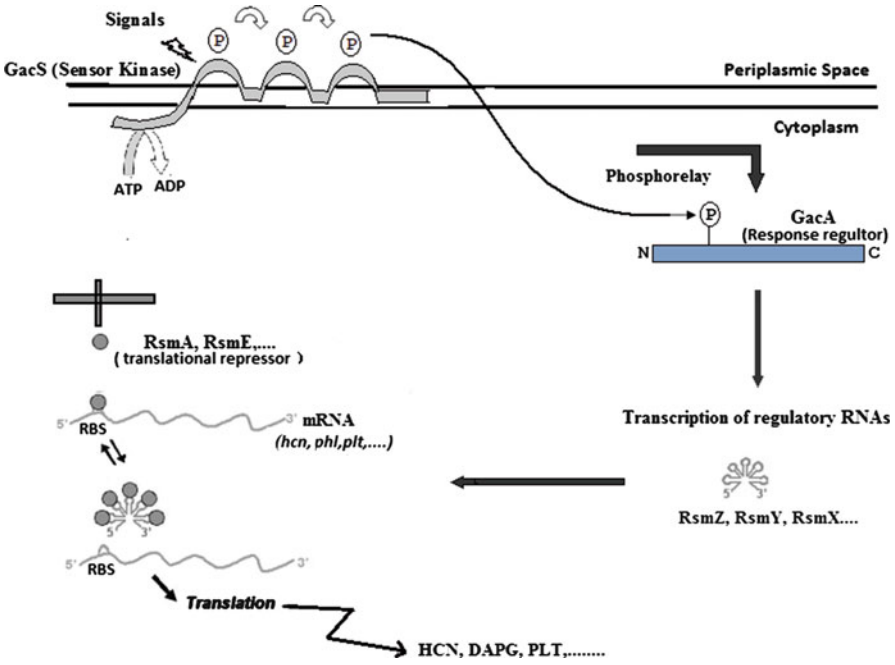

Fig. 11.1 Overview of the Gac/Rsm signal transduction pathway in Gamma-proteobacteria (Adapted from Haas and Keel 2003; Lapouge et al. 2008)

P. aeruginosa PAO1, the signals activating the GacS/GacA cascade are produced at high cell population densities and are under positive GacA control (Kay et al. 2005, 2006). Acyl homoserine lactones (AHLs), as described later, are not the only QS signals that different species or genera of microorganisms can share to establish a cross-talk (Riedel et al. 2001). A survey of beneficial and pathogenic plant-associated Pseudomonads, as discussed later, reveals that many species produce and release signal molecules activating the Gac/Rsm cascade in *P. fluorescens* and that this signal activity does not always correlate with AHL production (Dubuis et al. 2007).

In a biocontrol system, phosphorylated GacA then activates – directly or indirectly – the transcription of the three small RNA genes, *rsmX*, *rsmY, rsmZ*. Overexpression of these small RNAs is believed to titrate translational repressors like RsmA and RsmE, thereby relieving the translational repression exerted by these proteins at, or near, the ribosome binding site (RBS) of the target mRNAs (for example, those of the antibiotic biosynthesis genes *hcn* (hydrogen cyanide, HCN), *phl* (2,4-diacetylphloroglucinol, DAPG), and *plt* (pyoluteorin, PLT)) and making them accessible for translation (Heeb and Haas 2001; Haas and Keel 2003; Haas and Défago 2005; Lapouge et al. 2008).The GacA/Rsm signal transduction pathway

operates at post-transcriptional level, unlike transcriptional level control by AHL-dependent signal transduction pathway. The former is also known to favour biofilm mode of growth (Dubuis et al. 2007).

11.5 Small Regulatory RNAs (sRNA)

Regulatory RNAs or riboregulators are small, untranslated or non-coding transcripts which regulate gene-expression of target genes at post-transcriptional level. These can be grouped into two classes:

1. One that displays antisense base-pairing activity, thereby, regulating mRNA translation or stability. DsrA (87 nt) and RyhB (90 nt) are two such examples in *E.coli*.
2. Second that antagonize small, regulatory, mRNA-binding proteins of the CsrA (for carbon storage regulator in *E.coli*) family. By binding to the leader region of target mRNA they block translation and destabilize the mRNA. RsmA (for regulation of secondary metabolism), is the CsrA homolog in the biocontrol organism, *P. fluorescens* CHAO. Such translational repression can be alleviated, as described below, by the action of the small regulatory RNAs, whose expression is controlled by the GacS/GacA system in response to signal molecules produced by CHAO at the end of exponential phase (Heeb et al. 2002; Valverde et al. 2003, 2004).

Phosphorylated GacA positively activates a regulatory network which acts post-transcriptionally and involves the three small regulatory RNA called, RsmX, RsmY, and RsmZ. The activation involves the binding of GacA to a conserved upstream sequence (UAS) in the promoter. This 18 bp palindromic UAS generally occurs in the promoters of GacA-activated sRNA genes not only in the biocontrol strains but also in a variety of Gamma-proteobacteria (Humair et al. 2010). In *P. fluorescens* CHAO, a poorly conserved linker region located between the UAS and −10 promoter sequence is also essential for GacA-dependent expression. In addition, the integration host factor (IHF) also binds to the *rsmZ* promoter region. These studies suggest that besides UAS, auxiliary transcription factors and DNA binding play an important role in regulated expression of small RNAs (Humair et al. 2010). This highly complex regulatory network is a part of quorum sensing in many Gamma-proteobacteria. In many Pseudomonads, the Gac/Rsm system controls the production of N-acyl-homoserine lactones (Chin-A-Woeng et al. 2005; Kay et al. 2006). Once formed these regulatory RNAs are known to interact with RNA binding proteins or repressor proteins, RsmA and RsmE in *P. fluorescens* CHAO and thus, jointly execute post-transcriptional regulation of biocontrol factor synthesis (Kay et al. 2005; Reimann et al. 2005; Burrowes et al. 2006; Valverde 2009). These RNAs share one feature in that they scavenge small RNA-binding proteins termed RsmA and RsmE in *P. fluorescens* and CsrA in *E. coli* (Haas and Keel 2003), thereby relieving the translational repression of the target genes. These translational repres-

sor proteins (~7 kDa) are strongly conserved in Pseudomonads, enterics, and other bacteria and act by obstructing the access to the Shine-Dalgarno sequences of target mRNAs. Expression/over-expression of regulatory RNAs, therefore, titrate these translational repressors (Pessi et al. 2001; Haas and Defago 2005) thereby freeing the ribosome binding site (RBS) of target mRNA, e.g. those of the antibiotic biosynthesis genes *hcn*, *phl* (2,4-diacetylphloroglucinol), and *plt* (pyoluteorin), making them accessible for translation.

In biocontrol strains grown *in vitro*, the GacS/GacA system is activated during the idiophase coinciding with the time when RsmY and shortly afterwards, RsmZ are maximally produced. RsmX later discovered from *P. fluorescens* CHAO forms a triad of GacA-dependent small RNAs all involved in the regulation of antibiotics production (Kay et al. 2005; Valverde 2009). Though the overall sequence homology between RsmX, RsmY, and RsmZ is not significant but their structure shows 5–7 hairpins consisting of trinucleotide motif GGA exposed in the unpaired region. These GGA motifs are essential for recognition and binding of RsmA and RsmE; the deletion of five GGA repeats in RsmY results in the loss of recognition (Valverde et al. 2004; Valverde 2009; Lapouge et al. 2008).

The *hcnABC* operon responsible for the biosynthesis of the biocontrol factor HCN is positively regulated by GacA. Extensive studies on RsmY sRNA and 5'leader of *hcnA* mRNA have identified the critical contact points between these RNAs and the RsmA/RsmE proteins in *P.fluorescens* (Valverde et al. 2004; Lapouge et al. 2007). The *hcnA* 5' leader has five GGA motifs, of which most distal GGA motif overlaps the SD sequence. These studies have shown that RsmA/RsmE/CsrA proteins bind to the latter and strong binding is further favoured by the additional GGA motifs in the 5'leader of mRNA. These interactions hinder ribosome access to mRNA and thus translation initiation as well. GacA controlled sRNAs prevent this translational blockade by virtue of their multiple GGA motifs (Lapouge et al. 2008).

Furthermore, Irie et al. (2010) showed that *psl* mRNA (Psl polysaccharides are critical for biofilm formation) has an extensive 5' untranslated region, to which the post-transcriptional regulator, RsmA binds and represses *psl* translation. Their observations suggested that upon binding RsmA, the region spanning the ribosome binding site of *psl* mRNA folds into a secondary stem-loop structure that blocks the Shine-Dalgarno (SD) sequence, preventing ribosome access and protein translation. This constitutes a novel mechanism for translational repression by this family of regulators. RsmA repression of *psl* mRNA is novel, in that the high affinity target binding site of RsmA does not appear to overlap the ribosome binding site (RBS), as it is located 12 bases upstream of the SD sequence. The predicted structure contains a double-stranded RNA base-pairing event between the SD sequence and an anti-SD sequence, which forms the base of the stem. Disruption of the anti-SD-SD pairing leads to an increase in *psl* translational activity, which is no longer responsive to RsmA (Fig. 11.2). These findings suggest that the RsmA-mediated repression of *psl* translation involves the anti-SD sequence.

The study by Kay et al. (2005) provided evidence for an important feedback mechanism operating in the Gac/Rsm signal transduction pathway of *P. fluorescens*. The circuit diagram depicted in Fig. 11.3, summarizes the understanding of the

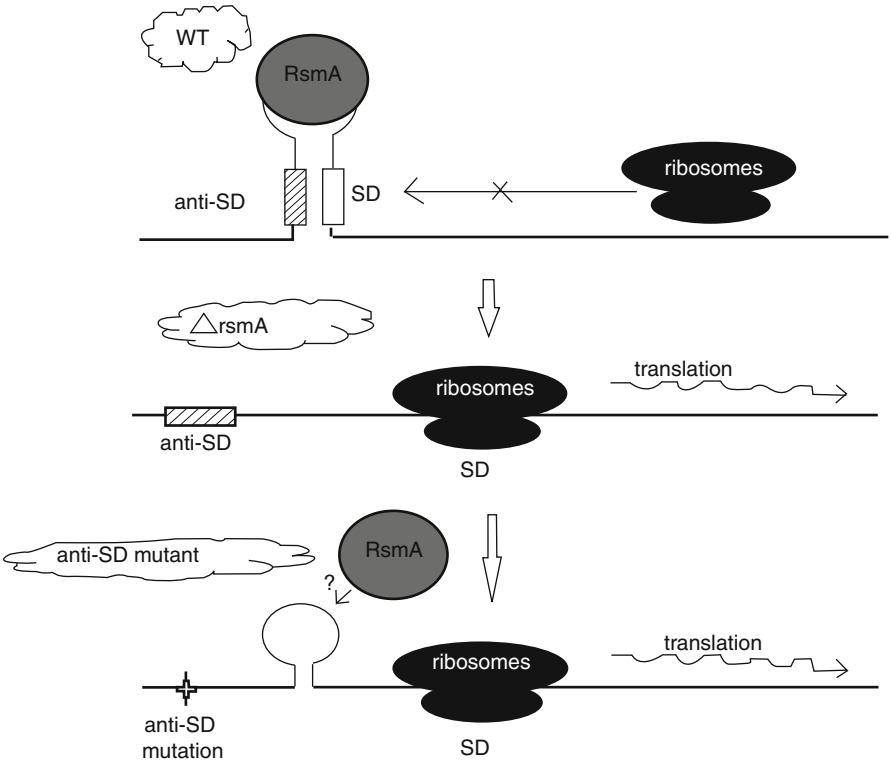

Fig. 11.2 Mechanism of translational repression: RsmA-*psl* repression model (Adapted from Irie et al. 2010)

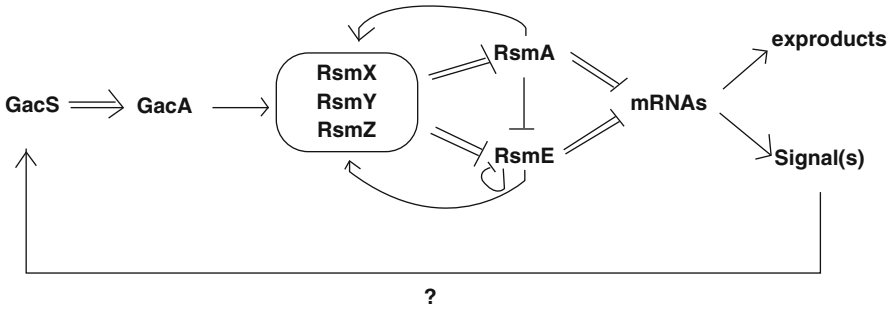

Fig. 11.3 Model for gene regulation in the Gac/Rsm system of *P. fluorescens*. —, direct or indirect regulation; =, physical interaction; →, positive effect; ⊣, negative effect (Adapted from Kay et al. 2005)

regulatory interactions. The synthesis of the signal molecules that lead to activation of target gene expression in the Gac/Rsm cascade (Heeb et al. 2002; Zuber et al. 2003) depends on GacA and on the three small RNAs. The signal activates transcription of *rsmX*, *rsmY*, and *rsmZ* (Heeb et al. 2002) and perception of the signal

needs functional GacS (Zuber et al. 2003). RsmX, RsmY, and RsmZ bind multiple copies of RsmA and RsmE and antagonize the regulatory effects of these RNA binding repressor proteins on secondary metabolite production (Heeb et al. 2002; Valverde et al. 2003). RsmE expression is regulated negatively by RsmA and RsmE and positively by GacA. It appears that RsmA and RsmE are required for RsmX, RsmY and RsmZ transcription and stability also (Reimann et al. 2005). Biocontrol factors and signals are produced in parallel at the end of exponential growth. By this mechanism, the Gac/Rsm cascade positively autoregulates its activity as a function of increasing cell population densities (Kay et al. 2005).

Therefore, the influence of the GacS/GacA signal transduction system on production of secondary metabolites and exoenzymes is thought to be mediated largely through these sRNA molecules (Kay et al. 2005).

11.6 Sigma-Factor RpoS (σ^s)

A panoply of sigma factors provides the bacterial cells with a primary level of genetic control by directing RNA polymerase to specific promoter sequences in response to changing environmental conditions (Ishihama 2000). The stationary-phase sigma factor σ^s, RpoS is one such important regulator that controls a large number of genes as the cell progresses from exponential to stationary phase. Since many of these functions are associated with stress resistance, RpoS is also referred as stress and stationary phase sigma factor. The regulation and functions of σ^s (also known as σ^{38}) have been studied in a variety of Gram-negative bacteria, especially in *E. coli* (Loewen et al. 1998; Hengge-Aronis 2002) and *Pseudomonas* spp. (Tanaka and Takahashi 1994; Sarniguet et al. 1995; Jørgensen et al. 1999; Kojic et al. 1999; Suh et al. 1999). The term stationary phase refers to a fixed physiological state regardless of what factors and/or environmental conditions lead to cessation of growth (Venturi 2003).

Under conditions of cellular starvation, σ^s accumulates, binds, and directs the RNA polymerase holoenzyme to many genes with diverse functions, including stress response. In *Pseudomonas* spp., σ^S is required for optimal survival of stationary-phase cells exposed to various environmental stresses (Sarniguet et al. 1995; Miura et al. 1998; Ramos-González and Molin 1998; Jørgensen et al. 1999; Suh et al. 1999; Whistler et al. 2000; Miller et al. 2001a, b; Heeb et al. 2005), and is involved in regulating the production of many antifungal/biocontrol agents (Haas and Keel 2003). The RpoS, is thus a global regulator, required for expression of a large number of genes involved in cross-protection to various stresses, including nutrient limitation, osmotic pressure, oxidative stress, heat shock, and growth during the stationary phase (Loewen et al. 1998). In *P. fluorescens* Pf-5, antibiotics production and biocontrol activity is influenced by RpoS (Sarniguet et al. 1995).

The *rpoS* mutants of *Pseudomonas* spp. generally exhibit diminished survival in the stationary phase when subjected to environmental and physiological stresses, including carbon starvation, UV irradiation, desiccation, and osmotic

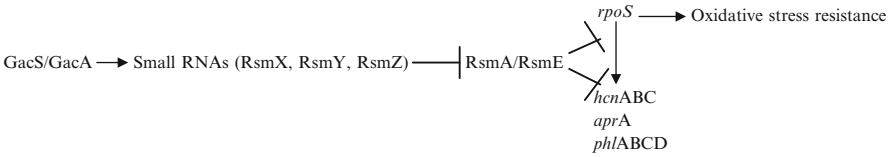

Fig. 11.4 Proposed pathway depicting the role of RpoS in Gac/Rsm controlled secondary metabolism and resistance to oxidative stress in *P. fluorescens* CHA0. →, positive effect; –|, negative effect(Adapted from Heeb et al. 2005)

stress (Sarniguet et al. 1995; Miura et al. 1998; Ramos-González and Molin 1998; Jørgensen et al. 1999; Suh et al. 1999; Miller et al. 2001a, b; Heeb et al. 2005; Stockwell and Loper 2005). Furthermore, *rpoS* mutants of *P. fluorescens* Pf-5 exhibit diminished capacity to colonize the rhizosphere of plants grown in dry soil (Sarniguet et al. 1995; Stockwell and Loper 2005), establishing *rpoS* as a fitness factor of this bacterium.

A mutation in *rpoS* of *P. fluorescens* differentially affects antibiotic production besides the capacity of stationary-phase cells to survive exposure to oxidative stress (Whistler et al. 1998). In *P. fluorescens* Pf-5, which produces multiple antibiotics, inactivation of *rpoS* stops pyrrolnitrin production, but enhances production of pyoluteorin and 2,4-diacetylphloroglucinol and improves disease control (Sarniguet et al. 1995). On the other hand, a similar mutation in strain CHAO, favours pyoluteorin synthesis but not of 2,4-DAPG and pyrrolnitrin (Haas and Keel 2003). The work by Heeb et al. (2005) suggests the role of stress sigma factor in Gac/Rsm signal transduction pathway controlling secondary metabolism as well as also shows its involvement in Gac/Rsm-mediated resistance to oxidative stress. As described earlier, the signal transduction complex pathway involving the GacS/GacA two-component system also involves RpoS. The expression of *rpoS* is controlled positively by GacA and negatively by RsmA. RpoS, positively controls the resistance to oxidative stress, and negatively affects the expression of the *hcnA* and *aprA* genes, presumably indirectly by competition with RpoD (σ^{70}) for RNA polymerase core (Fig. 11.4). The links between RpoS and GacS/GacA was revealed by the observation that both *gacS* and *rpoS* null mutants of *P.fluorescens* CHAO are more sensitive to H_2O_2 in stationary phase. Moreover, over expression of *rpoS* and *rsmZ* restore the peroxide resistance in *gacS* mutant (Heeb et al. 2005).

It has been reported that the GacS/GacA two-component system positively controlled the expression of *rpoS* in *P. fluorescens* Pf-5 (Whistler et al. 1998). Similarly, a positive regulation of GacA was observed on the transcription of *rpoS* gene in *P. fluorescens* 2P24. Together with these results, the existence of a negative feedback relationship between RpoS and the Gac system was proposed, and hence induction of RpoS by the two-component system may provide a mechanism to lower the level of the Gac when environmental cues favourable for hyperactivity of this system no longer are available (Yan et al. 2009).

11.7 Quorum Sensing

It has been a common observation that the resident microflora as well as the plants can strongly influence the expression of antibiotic biosynthesis genes in biocontrol Pseudomonads (Howie and Suslow 1991; Kraus and Loper 1995; Wood et al. 1997; Notz et al. 2001, 2002). Besides the complex regulatory network described earlier, another widespread regulatory mechanism identified in different bacterial species is referred as quorum sensing (QS). Bacterial populations in natural ecosystem communicate with each other through chemical signals, released in a cell density-dependent manner, which means a minimum cell number is needed to express a particular function. The nature of this signal, also known as autoinducer, has been identified to be amino acids, short peptide hormones, and fatty acid-derivatives such as *N*-acylhomoserine lactones (AHLs). The latter though may differ in the length of the acyl chain moiety, and the substitution at the C3 position, is the most common QS signal in Gram-negative bacteria. The bacteria reach a high population density in the rhizosphere and often form a biofilm. In these communities, the accumulation of fatty acid-derivative, AHL takes place which regulates various developmental, physiological, and environmental responses. (Chin A-Woeng et al. 2003; Gera and Srivastava 2006). Thus, quorum sensing is the ability of cells to detect relative population densities and respond appropriately.

Quorum sensing-dependent regulation is commonly based on a pair of proteins consisting of an AHL synthesising protein and a cognate transcriptional regulator. These have been identified as those belonging to the prototype LuxR/LuxI regulatory family, with LuxR being a transcriptional regulator that acts in conjunction with an AHL produced by the LuxI synthetase. Once activated by binding of AHL, the LuxR protein typically binds as dimer to a 20-bp promoter element, known as the *lux* box, and participates in the positive global regulation of a variety of cellular functions in response to the hormone gradient, and thereby activating transcription of target genes including further expression of *luxI*. The genes encoding the LuxR-type regulators and the corresponding LuxI-type AHL synthases are often adjacent to each other on bacterial chromosomes, perhaps facilitating their swift operation. AHLs once produced are released outside the cell where their concentration increases in consonance with the cell density. At a threshold concentration, being lipid soluble AHL traverses the membrane by simple diffusion and combine with LuxR (Fuqua et al. 2001; Swift et al. 2001; Whitehead et al. 2001). A screen of soil and plant isolates of different *Pseudomonas* spp. indicated that AHL production is more common among plant-associated bacteria than among those from the bulk soil (Elasri et al. 2001). Lots of details on QS system and underlying regulation are available in the literature (Pierson III et al. 1998; Venturi 2006). AHL-dependent cell–cell communication operating in the rhizosphere over a distance of up to 60 μm has been reported (Hartmann et al. 2004). AHL-signalling can be disrupted by microorganisms that enzymatically degrade AHLs. For instance, in *P. chlororaphis* strain PCL1391, the expression of phenazine-1-carboxamide depends on AHLs (Chin-A-Woeng et al. 2001); the biocontrol activity of this strain is strongly reduced

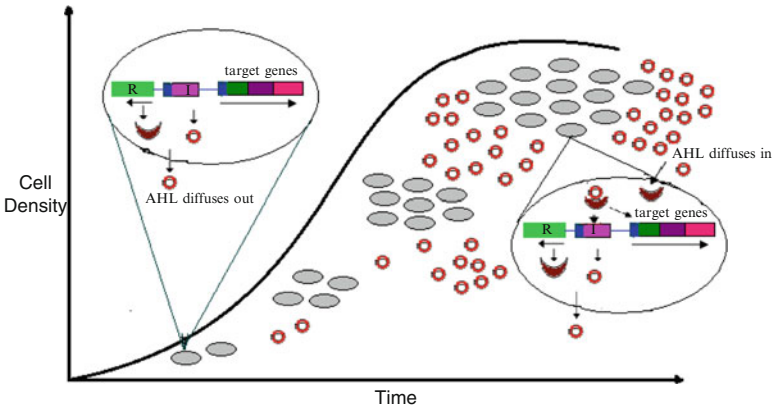

Fig. 11.5 Schematic representation of bacterial quorum sensing. At low population densities, basal-level production of autoinducer molecules results in the rapid dilution of the autoinducer signals in the surrounding environment. At high population densities, an increase in bacterial number results in accumulation of autoinducers beyond a threshold concentration, leading to the activation of the response regulator proteins, which in turn initiate the quorum-sensing cascade

when AHL-degrading bacteria are applied simultaneously to roots (Molina et al. 2003). Whether AHL degraders, which are common among rhizobacteria (Uroz et al. 2003), have an important role in rhizosphere ecology remains to be seen (Fig. 11.5).

Several studies have shown that QS in *Pseudomonas* is integrated with certain aspects of cell physiology and that it responds to various environmental signals. Thus the overlap of the AHL-QS regulon with regulons of other global regulators necessitates a high degree of interconnectivity among different signalling networks (Venturi 2006). In Pseudomonads, such regulatory elements include the stationary phase sigma factor, RpoS (Whiteley et al. 2000; Aguilar et al. 2003; Bertani and Venturi 2004), two-component regulatory system GacS/GacA (Reimmann et al. 1997; Chatterjee et al. 2003; Bertani and Venturi 2004), small RNA binding regulator, RsmA (Pessi et al. 2001), LuxR-family member, VqsR (Juhas et al. 2004), and TetR-family member, RsaL (Rampioni et al. 2007). The integration of QS as an additional regulatory circuit increases the range of environmental and metabolic signals that affect gene expression beyond cell density as well as further fine tuning the timing of the QS response (Venturi 2006; Yan et al. 2009).

The role of RpoS in AHL-QS regulation was re-addressed using transcriptional profiling (Schuster et al. 2004). During this study ~800 genes were found to be regulated by RpoS in stationary phase, including a high percentage (30–40%) of the genes previously determined to be regulated by AHL-QS. This suggested that there is a considerable regulon overlap between QS and RpoS. While more than 40% of the genes controlled by quorum sensing are also controlled by RpoS, conversely, 18% of all RpoS-regulated genes were regulated by quorum sensing as well. It is

most probable that most genes belonging to the two regulons are independently regulated by both RpoS and AHL-QS, while some are indirectly regulated through either AHL or RpoS. Considerable additional experimentation is required in order to formulate more precisely the mechanisms of how the regulon overlap is controlled by the two systems and whether at the molecular level there is indeed cross-regulation between RpoS and AHL-QS genes. It is probable that the two systems act independently, because AHL QS is a cell-density-related response whereas RpoS is a stationary-phase response, which in nature in most cases does not involve high cell densities but other stressful growth conditions such as limitation of an essential nutrient (Schuster et al. 2004).

The relationship between QS and RpoS is intriguing but confusing, largely because of the distinct regulatory features reported among different bacteria. A quorum sensing (QS) locus PcoI/PcoR has been identified in the biological control bacterium, *P. fluorescens* 2P24 (Wei and Zhang 2006). Yan et al. (2009) have shown that RpoS acts as a negative regulator of the transcription of *pcoI*. However, they suggested that in strain 2P24, RpoS could play its negative regulatory role on the *pcoI* gene under a functional GacS/GacA system background. They have further gone on to demonstrate that GacA is a positive regulator of *rpoS* gene. These results have led them to propose that a negative feedback relationship exists between RpoS and Gac system and such a mechanism would help the cell in modulating the gene expression under different environmental conditions (Yan et al. 2009).

The LasR-LasI and RhlR-RhlI quorum-sensing systems are global regulators of gene expression in the opportunistic pathogen, *P. aeruginosa*. Whiteley et al. (2000) investigated the influence of quorum sensing on *rpoS* expression in *P. aeruginosa*. Their data indicate that *rpoS* transcription is not regulated by quorum sensing. This is in contrast to a previous report that RhlR-RhlI is required for the growth phase-dependent expression of *rpoS* (Latifi et al. 1996). They further suggested a relationship between RpoS and quorum sensing, in which RpoS appears to regulate *rhlI*. Regardless of the explanation for the conflicting results, a novel relationship between *rpoS* and quorum sensing in *P. aeruginosa* involving the repression of *rhlI* transcription by *rpoS* has been reported (Whiteley et al. 2000).

It has been observed that in Plant Growth Promoting Rhizobacteria like *P. aeruginosa*, N-acylhomoserine lactones are capable of regulating a set of genes by sensing the cell density and developing an intercellular communication. An addition of AHL in the exponential growth phase, regardless of cell density, induces a repression of cell growth, an expression of stationary phase sigma factor, σ^s *in vivo* and also leads to morphological change into smaller spherical shape which is indistinguishable from that by the sigma factor expressed in the usual stationary phase. It is demonstrated that AHL can trigger the entry of bacteria into stationary phase as a growth controlling signal (You et al. 1998).

Extracellular polysaccharides are important components of biofilms. In non-mucoid strains of *P. aeruginosa*, Pel and Psl polysaccharides have been shown to be critical for biofilm formation (Friedman and Kolter 2004; Jackson et al. 2004; Matsukawa and Greenberg 2004). Irie et al. (2010) in their study, demonstrated that the alternative sigma factor RpoS is a positive transcriptional regulator of *psl* gene expression.

As discussed earlier, GacS/GacA modulates the expression of exoenzymes, antibiotics and HCN when cells are in transition from exponential to stationary phase. This two-component system also exerts a positive impact on cell density-dependent gene regulation mediated by signal molecule, AHL in *P. aeruginosa*, *P. syringae* and *P. aureofaciens* (Fuqua et al. 1994; Sacherer et al. 1994; Blumer et al. 1999; Chancey et al. 1999; Elasri et al. 2001; Heeb and Haas 2001). The *phz* operon of *P. fluorescens* 2–79, which produces phenazine-1-carboxylate, is preceded by two genes, *phzR* and *phzI*, that are homologs of quorum-sensing gene pairs of the *luxR-luxI* family (Khan et al. 2005). In *P. chlororaphis* PCL1391, the production of the antifungal metabolite, phenazine-1-carboxamide (PCN) is through expression of the biosynthetic *phzABCDEFGH* operon (Chin-A-Woeng et al. 1998, 2003). Regulation of PCN production in this strain involves the two-component signalling system GacS/GacA, the quorum-sensing system, PhzI/PhzR, and the regulator PsrA. The *phzI* gene is responsible for the synthesis of the autoinducer, of which N-hexanoyl-L-homoserine lactone (C6-HSL) is the main product (Chin-A-Woeng et al. 2001). C6-HSL is believed to bind to PhzR, thereby activating it. Subsequently, the PhzR–C6-HSL complex binds to the *phz* box(= *lux* box) upstream of the *phz* biosynthetic operon, and results in initiation of the transcription of the *phz* operon. The PhzR–C6-HSL complex also upregulates *phzI* via a second consensus sequence or *lux* box. A similar regulation of phenazine synthesis by quorum sensing was shown in *P. aureofaciens* 30–84 (Pierson et al. 1994). PhzR/PhzI quorum sensing system has been described from many Pseudomonads, such as *P. fluorescens* 2–79, *P. chlororaphis* PCL 1391 and *P. aureofaciens* 30–84 (Khan et al. 2005). Phenazines themselves have been implicated as late QS signal that may regulate several genes not related to phenazine biosynthesis genes (Dietrich et al. 2006).

P. aureofaciens 30–84 is a soil-borne bacterium that colonizes the wheat rhizosphere and produces three phenazine antibiotics. These are involved in suppressing take-all disease of wheat by inhibition of the causative agent *Gaeumannomyces graminis* var. *tritici*. Phenazines also enhance survival of *P. aureofaciens* 30–84 within the wheat rhizosphere endowing high level competitiveness. Expression of the phenazine biosynthetic operon in *P. aureofaciens* 30–84 is also controlled by the *phzR/phzI, N*-acyl-homoserine lactone (AHL) response system (Pierson et al. 1994; Wood and Pierson 1996; Wood et al. 1997). By using high-pressure liquid chromatography coupled with high-resolution mass spectrometry, the AHL produced by PhzI was identified as *N*-hexanoyl-homoserine lactone (HHL) which is also known as C6-HSL. Moreover, the expression of the quorum sensing regulatory pair PhzR-HHL in the biocontrol strains *P. chlororaphis* PCL1391 and *P. aureofaciens* 30–84 depends on the two-component system GacS/GacA (Chancey et al. 1999).

AHL type QS molecules produced by DAPG producers such as *P. fluorescens* F113 and 2P24 do not contribute to the regulation of antibiotic biosynthesis (Laue et al. 2000; Wei and Zhang 2006). In fact, in *P. fluorescens* CHAO and Pf-5, DAPG and PLT act as signal molecules, which induce their own biosynthetic genes while strongly repressing the biosynthetic genes of other antibiotics (Baehler et al. 2006). The use of Pseudomonads as PGPR and/or biological control agents requires precise understanding of the interactions between the plants and bacteria, among

bacteria and microbiota, and how biotic and abiotic factors influence these relationships (Choudhary et al. 2009). The potential for species of *Pseudomonas* to colonize and protect plants from infection by pathogenic microorganisms has long been recognized. The identification of several antifungal metabolites produced by many of these bacteria capable of inhibiting plant pathogenic microorganisms is the first step in understanding, and ultimately utilizing these bacteria for effective biological control in the field. Additionally, the identification of genetic systems involved in the production of these metabolites is beginning to offer insights into their biosynthesis and regulation. The complex intertwining of regulatory circuits governing the expression of the genes involved in the production of these antifungal metabolites reflects the important roles these products serve not only in biological control but also in the normal life cycles of the producing bacteria.

11.8 Future Perspectives and Conclusions

Our current understanding of the regulation of antifungal metabolite gene expression in *Pseudomonas* is beginning to offer some insight into how soil-borne microorganisms regulate the expression of genes involved in both competition and biological control. The identification of regulatory circuit analogous to *gacS/gacA* in many biological control bacteria will allow the identification of the environmental signals recognized by these systems. The identification of an RpoS homolog that controls gene expression strongly indicates that the events that regulate gene expression during stationary phase are important in biological control and that the effect of nutrient deprivation and other factors that stimulate stationary phase growth need to be examined further.

Thus, the regulatory picture governing the expression of the genes responsible for the production of antifungal metabolites appears to be more complicated than originally envisioned. Although specific mechanisms of regulation appear to have been conserved among bacteria, it is doubtful whether each mechanism will function identically in each system. Therefore, it will be important to ascertain the hierarchial role of specific regulatory mechanism within each system so that an understanding of the importance of each mechanism under different environmental conditions can be studied and compared. Even subtle differences in signal transduction pathways between bacteria could have a large influence on the success of the bacteria as a biological control agent. In addition, many of the regulatory loci identified regulate multiple operons involved in diverse pathways affecting multiple aspects of the cell's ability to interact with its environment, its host, or its competitors. Thus, studies on regulatory genes must be performed in the context of the complex and varied effect that such alterations may have on the expression of genes in the cell responsible for its ability to interact with its environment. Biocontrol agents from *P. fluorescens* and closely- related species have become prominent models for analysis of plant protection mechanisms and the role of secondary metabolism. In order to understand the genetic basis of these functions, it becomes

imperative to isolate structural gene/s and regulatory mutants, and test them in laboratory conditions to be followed by field applications.

In conclusion, *Pseudomonas* spp. are of agricultural and economic importance as a biological control agent largely because of their plant association and production of secondary metabolites. Thus, antibiotic production by fluorescent *Pseudomonas* spp. is continuously getting recognized as an important feature in plant disease suppression.

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Chapter 12
Exploiting Plant Growth Promoting Rhizomicroorganisms for Enhanced Crop Productivity

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Abstract The increasing pressure on land resources has made it imperative for vertical growth through enhanced crop intensity and productivity. To meet this challenge, appropriate integrated nutrient and pest management packages must be configured for different agro-ecological conditions. By 2050, the crop nitrogen demand is expected to reach 40–45 million tonnes. To meet such enormous nitrogen requirements through chemical fertilizers, would not only be expensive but also could severely degrade soil health. Similar is the situation with other macro- and micro-nutrients. The rhizosphere environment, at the interface between root and soil, is a major habitat for soil processes. Rhizosphere biology is approaching a century of investigations, wherein growth-promoting rhizomicroorganisms such as *Rhizobium*, *Azotobacter, Pseudomonas, Bacillus, Azospirillum, Frankia* and mycorrhizal fungi have attracted special attention on account of their beneficial activities. Plant growth promoting rhizomicroorganisms (PGPR) include diverse microbes that influence plant health by colonizing roots, enhancing plant growth, reducing plant pathogen populations and activating plant defenses against biotic stresses. PGPRs promote plant growth in different ways such as influencing plant hormonal balance, antagonistic to pathogens through various modes, stimulation of plant resistance/defense mechanisms, effects nutrient uptake by secretion of organic acids or protons to solubilize nutrients, atmospheric N_2 fixation and by modifying rhizospheric soil environment by exo-polysaccharides production. Though research was going on in isolation in the above areas, with the advent of a core group for PGPR research, the pace in this direction has significantly increased. The primary emphasis on exploiting the vast biodiversity of microorganisms to identify the

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beneficial strains has yielded very good results. However, most of the research is yet to reach the end-users. For effective transfer of these technologies, there is a need for functional networking of research, industry and extension systems. In this paper, we describe the recent advances in PGPR research and the future needs to strengthen PGPR research and development that will transfer the benefits to the end-users for enhanced and sustainable farm productivity hence contributing towards food security challenges.

Keywords Plant growth promoting rhizomicroorganisms • *Pseudomonas* • *Bacillus* • *Trichoderma* • Bioinoculants • Crop productivity

12.1 Introduction

Agriculture is the predominant occupation in India that employs more than 50% of the population directly or indirectly. The statistical estimation for the year 2008 shows that Indian agriculture contributed 17.2% of the `46936.02 billions national Gross Domestic Productivity (GDP). Of late to meet the growing demand for food, adequate production and even distribution of food has become a high priority global concern. With the present agricultural scenario and global competition, there is a need for enhancing the farm productivity through integrated plant health management. In India, during the year 2008–2009, the total area under cultivation was 8.74 million ha with a production of about 8.83 million tonnes reflecting a productivity of 1,011 kg.ha^{-1}. A meticulous approach towards improved crop varieties, better soil productivity, supply of balanced crop nutrients, improved plant protection, efficient water management, post-production management for value-addition and marketing can contribute to enhanced farm productivity.

As opportunities in India for further agricultural growth through increase in area under irrigation are relatively less, food security and productive growth in agriculture has to be focused in rainfed regions. The total cropped area in India has remained static at around 140 million ha since 1970s. For maintaining food security at the current nutritional levels, 100 million tonnes of food grains need to be produced additionally by 2020. Rainfed agriculture faces challenges in abiotic and biotic stresses, degraded soils with poor nutritional status and low organic content, depletion of natural resources, depletion of ground water and poor economic base of the farmers. The increasing pressure on land resources has led to an imperative vertical growth by increasing crop productivity and intensity. Integrated nutrient and pest management packages should thus be appropriately configured to meet the specific demands of different agro-ecological conditions. The 6 billion world population today consumes about 25 million tonnes of protein nitrogen each year. By 2050, it is expected to reach 40–45 million tonnes. To meet such enormous nitrogen requirements, chemical fertilizers would not only be expensive but also can severely degrade soil health and environment.

Chemical fertilizers can be substituted with biofertilizers as a low cost inputs for sustainable agriculture. However, in India there is a persistent demand in nitrogen fertilizers. This demand can be reduced with the help of biofertilizers. There is a huge gap between the demand and production of these biofertilizers due to increasing interest in the usage of microorganisms for crop health management.

Plant growth results from interaction of roots and shoots with the environment. The environment for roots is the soil or planting medium, which provides structural support as well as water and nutrients to the plant. Rhizosphere biology is approaching a century of investigations wherein plant growth promoting rhizobacteria (PGPR) such as *Rhizobium*, *Frankia* and mycorrhizal fungi have attracted special attention on account of their beneficial activities. Bacteria that aggressively colonize roots are now referred to as "rhizobacteria"; predominant among these are fluorescent pseudomonads. Fluorescent pseudomonads have been recovered from the rhizoplane and rhizosphere of not only crop species but also from wood tree seedlings and fruit trees. Roots also support the growth and functions of a complex of microorganisms that can have a profound effect on growth and survival of plants. These microorganisms constitute rhizosphere microflora and can be categorized as deleterious, beneficial or neutral with respect to root/plant health. Beneficial interactions between roots and microbes do occur in rhizosphere and can be enhanced. Increased plant growth and crop yield can be obtained upon inoculating seeds or roots with certain specific root-colonizing bacteria- 'plant growth promoting rhizobacteria'.

Microbes have been always associated with plants and the interactions between them can be beneficial or hostile. Some of the prominent beneficial interactions of agricultural importance include symbiotic nitrogen fixation, nutrient mobilization, induction of resistance mechanisms against invading pests, biological control of pests that attack the plants and plant growth promotion.

12.2 Plant Growth Promotion by Microbes

Plant growth-promoting rhizobacteria (PGPR) are free-living, soil-borne bacteria, isolated from the rhizosphere, that enhance the growth of the plant and reduce the damage from soil-borne plant pathogens (Kloepper et al. 1980) when applied to seeds or crops. The potential negative impact of chemical fertilizers on the global environment and the cost associated with production has led to research with the objective of replacing chemical fertilizers with bacterial inoculants.

Bacterial inoculants that help in plant growth are of two types (a) symbiotic and (b) free-living (Kloepper et al. 1988; Frommel et al. 1991). Beneficial free-living bacteria referred to as PGPR are found in the rhizosphere of the roots of many different plants (Kloepper et al. 1989). Breakthrough research in the field of PGPR occurred in the mid 1970s with studies demonstrating the ability of *Pseudomonas* strains capable of controlling soil-borne pathogens and indirectly enhances plant growth and thereby increased the yield of potato and radish (Kloepper et al. 1980;

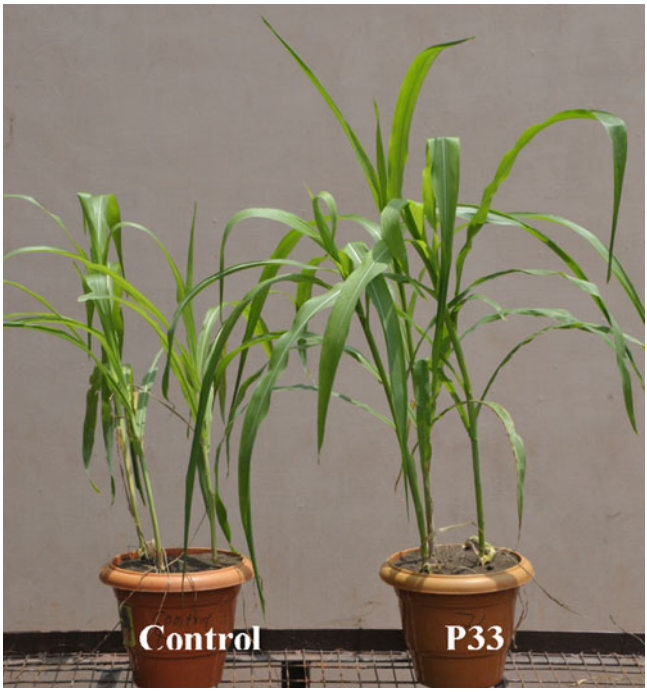

Fig. 12.1 Plant growth promotion of maize by *Pseudomonas* sp. (60 DAS)

Howie and Echandi 1983). Strains which exhibited the potential to be PGPRs were identified as *Pseudomonas putida* biovar B, *P. fluorescens*, *Arthobacter citreus* and *Serratia liquefaciens* (Lifshitz et al. 1986; Kloepper et al. 1988). Salamone (2000) reported the growth-promoting effect of *P. fluorescens* strain G20-18 on wheat and radish plants by production of cytokinin phytohormones. As the effect of PGPR on plants was demonstrated, the concept of PGPR began to gain importance and a large number of bacterial strains have been isolated, screened (Cattelan et al. 1999; Bertrand et al. 2001) and evaluated for plant growth promotion (Bent et al. 2001; Salamone 2000).

The PGPRs have different modes of action and they include production of growth promoting substances, release of nutrients that are in fixed state, production of siderophores, mycoparasitism and antibiosis. Research results indicate that the PGPRs can have one or more modes of action. PGPR may induce plant growth promotion by direct or indirect modes of action. Direct mechanisms include the production of stimulatory bacterial volatiles and phytohormones (Fig. 12.1), lowering the ethylene level in plant, improvement of the plant nutrient status (liberation of phosphates and micronutrients from insoluble sources Fig. 12.2; non-symbiotic nitrogen fixation) and stimulation of disease resistance mechanisms (induced systemic resistance). Indirect effects originate when PGPR acts like biocontrol agents in disease reduction, stimulation of other beneficial symbioses and protects the plant

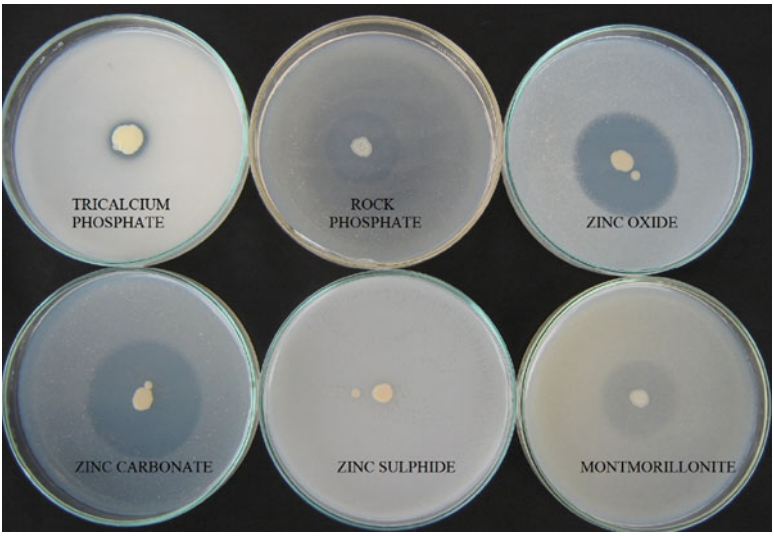

Fig. 12.2 Solubilization of macro- and micro-nutrients *in vitro* by *Bacillus* sp.

by degrading xenobiotics in contaminated soils (Jacobsen 1997). Based on their activities, Somers et al. (2004) classified PGPR as biofertilizers (increasing the availability of nutrients to plant), phytostimulators (plant growth promoting, usually by the production of phytohormones), rhizoremediators (degrading organic pollutants) and biopesticides (controlling diseases, mainly by the production of antibiotics and antifungal metabolites). Bashan and Holguin (1998) proposed the division of PGPR into two classes: biocontrol-PGPB (plant growth promoting bacteria) and PGPB. This classification may include beneficial bacteria that are not rhizosphere bacteria but it does not seem to have been widely accepted. Furthermore, in most studied cases, a single PGPR will often reveal multiple modes of action including biological control (Vessey 2003).

Biofertilizers are preparations containing live microorganisms that help in nutrient availability through fixation, solubilization or mobilization. There are many biofertilizers for application in crop production. Their scope and importance can be realized from the fact that more than 43 million ha under paddy, 35 million ha under coarse cereals, 23 million ha under pulses, 25 million ha under groundnut and 4 million ha under soybean can be benefited by using one or other types of biofertilizers. Biofertilizers benefit the crop by way of increased N fixation, enhanced availability of nutrients through solubilization or increased absorption and stimulation of plant growth through hormonal action.

Agriculturally important microorganisms (AIMs) are used in a variety of agroecosystems both under natural conditions and as manipulated inoculation for augmentation of nutrient supply, biocontrol, bioremediation and rehabilitation of degraded lands (Vessey 2003). Stressed ecosystems are however, the most challenging

to realize optimum performance from AIMs. Ecosystems with sub-optimal performance of soils and other resources in productivity are termed stressed ecosystems (Sehgal and Mandal 1994). The outstanding stress factors in India are drought or soil moisture, which affect nearly two third area forming part of the arid and semi-arid ecosystems. The other important abiotic stresses are high temperature, soil salinity/alkalinity, low pH and metal toxicity.

In recent years under controlled conditions, the beneficial effects of microorganisms such as *Pseudomonas*, *Bacillus*, *Arthrobacter*, *Pantoea*, *Burkholderia*, *Rhizobium* etc. enhanced the tolerance of crops such as sunflower, maize, wheat, chickpea, groundnut, spices and grapes towards drought, salinity, heat stress and chilling injury factors (Ait Barka et al. 2006; Arshad et al. 2008). The inoculated microorganisms in the rhizosphere enhance soil aggregation by production of exo-polysaccharides and improved the water availability to plants during dry periods (Sandhya et al. 2009). Also induced the synthesis of heat shock proteins and osmoregulants such as proline, glycine-betaine, help in maintenance of cell membrane integrity (Bano and Fatima 2009), all of which contribute to improved stress tolerance in plants. The introduced organisms also form biofilm in the rhizosphere which protect plants from harsh environment in the vicinity. These researches open up new and exciting possibilities of utilizing microorganisms as inoculants for enhancing tolerance of plants to climate change induced abiotic stresses.

Phosphorus is one of the major nutrients that limit plant growth. Most of the soils around the world are P deficient (Batjes 1997). The use of rock phosphate as a phosphate fertilizer and its solubilization by microbes (Kang et al. 2002), through the production of organic acids (Maliha et al. 2004), has become a valid alternative to chemical fertilizers. Several studies have shown that phosphate soulubilizing microorganisms (PSMs) solubilize the fixed P in the soil resulting in higher crop yields (Gull et al. 2004). The combined application of *Rhizobium* and PSM (Perveen et al. 2002) or PSM and arbuscular mycorrhizal (AM) fungi (Zaidi et al. 2003) showed enhanced plant growth as compared to their individual inoculation in 'P' deficient soils. Phosphate solubilizing bacteria are ubiquitous (Gyaneshwar et al. 2002) and *Bacillus*, *Enterobacter, Erwinia* and *Pseudomonas* spp. are among the most potent strains.

Direct evidence supports the conclusion that PGPR surviving on plant roots can induce resistance in plants to foliar or systemic pathogens, was published independently for three pathosystems: cucumber and anthracnose (Wei et al. 1991); carnation and *Fusarium* wilt (van Peer et al. 1991); bean and halo blight (Alström 1991). Systemic resistance induced by PGPR has been termed 'induced systemic resistance' (ISR) (Kloepper et al. 1992; Pieterse et al. 1996). ISR is dependent on colonization of the root system by sufficient numbers of PGPR, and this has been achieved by coating seed with high numbers of bacteria or by adding bacterial suspensions to soil before sowing or at transplanting (Kloepper 1996). Studies to elucidate the plant biochemical pathways associated with induction by PGPR were reviewed by van Loon et al. (1998).

Pseudomonas is a diverse genus containing a large number of species with a variety of catabolic and metabolic abilities. This diversity allows for the colonization

of an array of environmental niches and interactions with a wide range of eukaryotic hosts as saprotrophs, endophytes, commensals, plant pathogens and opportunistic human pathogens. *Pseudomonas* spp. are of great interest to researchers in the fields of plant, soil and as such the genomic sequences of a range of *Pseudomonas* strains have been determined or are currently in progress. *Pseudomonas*–plant interactions are found ubiquitously in nature and encompass a growing number of plant species, many of which are important in commercial horticulture. These interactions fall into two general groups: those that are beneficial and those that are detrimental to the host plants health. Species of *Pseudomonas* that successfully interact with plant hosts ultimately gain an advantage over the competing microorganisms in the immediate environment. The development of such interactions between *Pseudomonas* and host plants usually involves avoidance, subversion and sometimes even stimulation of the host plant defenses. In return the plant host can respond to the presence of the *Pseudomonas* spp. by modulating defense mechanisms and engaging in signaling between itself and the infecting bacterium in an attempt to further promote beneficial interactions or suppress pathogenic ones.

Numerous examples of plant growth stimulation by fluorescent *Pseudomonas* spp. have been reported (Kloepper et al. 1980; Bashan 1986; Suslow 1980). Significant increases in growth and yield of potatoes up to 367% were reported in greenhouse experiments by Burr et al. (1978) with specific fluorescent *Pseudomonas* strains. Van Peer and Schippers (1988) documented increases in root and shoot fresh weight for tomato, cucumber, lettuce and potato as a result of bacterization with *Pseudomonas* strains. Biological control with fluorescent pseudomonads offers an effective method of managing plant pathogens (Ramamoorthy et al. 2001). These bacteria inhibit the fungal pathogens by producing antibiotics, lytic enzymes and by inducing resistance systemically in the plant by activating defense genes such as chitinase, β-1,3-g1ucanase, peroxidase and phenylalanine ammonia lyase (Ramamoorthy et al. 2001; Viswanathan and Samiyappan 2001). Chitinases are well known to lyse the fungal cell wall (Chet 1987). The ubiquitous distribution of fluorescent pseudomonads in the rhizosphere of crop plants has broad spectrum of action in the suppression of fungi, bacteria and nematodes (Haas and Keel 2003).

Strains of the genus *Bacillus* are another most commonly reported PGPR (Compant et al. 2005; Vessey 2003). The secondary metabolites produced by certain species and strains of *Bacillus* have antibacterial or antifungal activity against phytopathogenic microorganisms (Asaka and Shoda 1996). Products for plant disease biocontrol containing *B. subtilis* and other *Bacillus* species have been used in the past as seed dresser in several crops (Schisler et al. 2004). *Bacillus* strains have the advantage of forming endospores that confer them high stability as biofungicides or biofertilizers (Schisler et al. 2004). Several mechanisms have been proposed to explain the inhibition of fungal pathogens by *Bacillus* spp., including production of antimicrobials, secretion of hydrolytic enzymes, competition for nutrients, or a combination of mechanisms (Compant et al. 2005).

The treatment of soybeans with *Bacillus cereus* has been shown to improve soybean yield in field (Osburn et al. 1995). Systemic resistance in sugar beet was

elicited by a non-pathogenic, phyllosphere colonizing *Bacillus mycoides* (Bargabus et al. 2002). Different strains *of B. subtilis, B. cereus, B. mycoides* and *B. thuringiensis* exhibit antifungal activity (Stabb et al. 1994). These strains has shown to produce zwittermicin-A and/or kanosamine that are effective against damping-off disease caused by *Phytophthora medicagenis, P. nicotianae, P. aphanidermatum or Sclerotinia minor* (Milner and Stohl 1996).

Trichoderma spp. were extensively studied over the past several decades all over the world. It is an antagonistic soil borne fungus that can effectively control soil-borne plant pathogens. The mode of action of its antagonism, are primarily by competing for substrates, chitinase production, production of antibiotics (trichodermin, viridin etc.) and mycoparasitism. Among several spp., *T. viride* is very popular as it has been accepted world over as a component in integrated disease management.

12.2.1 Stress Management Through Rhizobacteria

Application of rhizobacteria for the management of different kinds of stresses esp. root pathogens is not new. The phenomenon of biological control of soil borne- and foliar-pathogens through many bacteria has been commercially exploited. The PGPRs produce a variety of enzymes such as chitinases, glucanases, cellulases, and proteases that are useful in biological control. Biological control in India is now gaining significance as people are becoming more health conscious and there is no other economically viable option for controlling soil-borne phytopathogens. The technology should be available at a cheaper cost for all the small and marginal farmers to make use of it.

The expansion of irrigation has been one of the key strategies in achieving self-sufficiency in food production. This has been achieved through the use of ground water. In almost all cases the groundwater table, that is several meters deep prior to the introduction of irrigation has risen and has played a very significant role in the increasing salinization. One of the major implications of this processes is the increase in the concentration of soluble salts in the root zone of soils, which affects the rhizosphere populations thereby affecting the plant productivity. This is more evident in the coastal agro-ecosystems. The presence or absence of soil microbes in the soil can be used as indicators of soil health and soil productivity as they are capable of having a direct effect on plant growth. A high association between soil salinity and the distribution of *Azospirillum* genotypes reveals that soil salinity should be taken into consideration while developing biofertilizers specifically for the coastal agro-ecosystem. Phosphorus is the second critical plant nutrient next to nitrogen. Although total P pool of soil is high, only a part is available to the plants depending on the solubility and availability of the microbial load. In stressed soils it is observed that their numbers dwindled very fast.

12.3 Efficient Strain Selection and Formulation

The efficient strain selection relies on isolation and characterization of competent isolate with the ability to outgrow the native rhizospheric microbe population, physiologically active and tolerance to biotic and abiotic stresses. Initial *in vitro* study for the selection of promising strain involves a seedling test where in basic parameters like root, shoot length and dry mass are recorded and the best isolate is selected for pot study under glass house conditions in sterile soil. Finally, large scale production of the selected isolate requires basic research on the ideal pH, temperature range, agitation, aeration, media requirements etc. This basic information could help in refining the ideal conditions for large scale production.

Formulation is the process of mixing the bioinoculant with carrier material so as to enhance the shelf-life and viability. Formulation of the bioinoculant also plays a major role in consistency of performance by improving or maintaining bacterial survival following application. A suitable formulation should provide a protective habitat for the introduced bacteria, thereby increasing the potential for survival and successful colonization. Several carrier materials such as peat, bentonite, vermiculate, talc and lignite have been reported. Bacterial inoculant may be either liquid or carrier based formulation including those that are oil-based, aqueous-based, polymer-based, or combinations thereof. Aqueous-based formulation requires few more amendments other than fermenting bacteria in a liquid medium and adding components, such as stabilizers, stickers, surfactants, colouring agents, antifreeze compounds and additional nutrients (Boyetchko 1996). Alternatively, the ferment can be processed (e.g., concentrated or dried) and then re-suspended in a liquid medium. Some of the available commercial products with government organizations in India are listed in the following Table 12.1.

12.3.1 Integrated Bioresources Centre Model

A novel concept of Integrated Bioresources Centre (IBRC) was implemented at Central Research institute for Dryland Agriculture initially with the financial assistance from Andhra Pradesh - Netherlands Program. The basic objective of the concept was to provide various bio-inputs of assured quality under one roof at an affordable price to the farmers and also impart training programs to different clientele on multiplication, formulation and use of various bio-inputs. The unit is established on a self-sustainable basis and trains farmers, trainers and young entrepreneurs on various issues related to bio-inputs. The production facility also has an in-house research unit that undertakes refinement of technologies to meet the farmers' needs based on the feed-back obtained during the on-farm demonstration programs conducted by the IBRC or other researchers. The unit not only absorbs the research outputs of CRIDA but also ripe technologies from other research organizations for the overall benefit of the farmers. The product portfolio of the centre includes

Table 12.1 Commercially formulations of bioinoculants with an ability to promote plant growth and disease suppression

S. No.	Bio-product	Source	Address
1	*Trichoderma viride*	Tamilnadu Agricultural University	Coimbatore-641003, Tamilnadu
2	*Pseudomonas fluorescens*	-do-	-do-
3	*Trichoderma viride*	Directorate of Oilseeds Research	Rajendranagar-500030, Hyderabad, AP
4	*Bacillus thuringiensis* var kurstaki	-do-	-do-
5	*Trichoderma harzianum*	Indian Institute of Horticultural Research	Bengaluru-560089, Karnataka
6	*Paecilomyces lilacinus*	-do-	-do-
7	PSB, *Azospirillum, Azotobacter, Rhizobium, Gluconoacetobacter*	National Centre of Organic Farming/ Regional Centre for Organic Farming	Ghaziabad-201002, UP
8	*Trichoderma harzianum*	Regional Research Laboratory	Jammu-180001, J & K
9	*Trichoderma viride*	Central Research Institute for Dryland Agriculture	Hyderabad-500059, AP

Fig. 12.3 Bioinoculants commercialized at Integrated Bioresources Centre of CRIDA

biofertilizers, biopesticides and tissue-culture saplings. The model is also standardized for duplication at other places by young entrepreneurs so that the bio-inputs are produced under technical supervision of qualified personnel and made available at the nearest place possible and thereby reducing transportation costs and other logistics. To set up such unit the approximate cost that incur will be around 3–5 lakh rupees for 5–8 MT production capacity per annum. The basic requirements for the unit are orbital shaker, laminar air flow cabinet, autoclave, refrigerators, glassware and chemicals. The expected breakeven can be attained after 3 years of initiation of the unit. Such village level units can be sustained for a long time by unemployed rural youth with proper training and hands on experience for the production of quality bio-products. Some of the bio-products manufactured at IBRC, CRIDA are shown in Fig. 12.3.

12.4 Commercialization, Marketing and Regulatory Requirements

In India, the commercial production and sale of biofertilizers and biopesticides must comply with the regulatory norms of State Department of Agriculture and Central Insecticides Board & Registration Committee (CIB&RC), Faridabad. Hence, they are classified under FCO and pesticide act, respectively. The risk-assessment process undertaken by the CIB&RC as part of its decision to register a pesticide relies on on-farm bio-efficacy data generated for two seasons on target crops or pests conducted under three agro-climatically diverse areas, chemistry data that includes mode of action and standard procedures for the evaluation of quality, chemical composition etc. The toxicological data comprises effect of toxicity to birds, fishes, earthworm, mice and rabbits, where as container content compatibility data includes shelf life of formulated product under three varied temperature regimes, compatibility of the product with packing materials, manner of packing, labeling and leaflets in accordance with the products. The report should also contain data on the impact on natural enemies, predators, phytotoxicity on plants and residual effects. The term “residues” applies not only to the parent molecule but also to toxicologically significant metabolites, or other products that might arise by alteration of the parent molecule. Under legal data, the concerned company has to furnish set of affidavits for the assurance in quality maintenance and a responsible representative in case of failure to meet the standards. The regulations provide substantial discussion of the data required by the CIB for the risk assessment and approval process. The CIB&RC initially awards a temporary registration called 9(3)b registration which is valid for 2 years. Several recognized R&D organizations have generated data for 9(3)b registration. Tamil Nadu Agricultural University (Coimbatore), Indian Institute of Horticultural Research (Bengaluru), Directorate of Oilseeds Research (Hyderabad) and Regional Research Laboratory (Jammu) are some of the sources for obtaining data for registration of biopesticides on payment basis. For

obtaining permanent license, a separate application has to be made with data on shelf-life, bioefficacy and toxicology data. The registration certificate obtained from the CIB should be submitted to the concerned State Department of Agriculture to obtain the manufacturing and marketing license. Other statutory requirements for obtaining the manufacturing license includes.

- Product registration with State Department of Agriculture/CIB & RC
- Obtaining manufacturing license from the state department of agriculture
- SSI registration
- VAT registration
- NOC from pollution control board and fire department
- Central excise
- Labour department
- Weights and Measures certification

12.5 Transfer of Technology and Awareness Building

The transfer of technology from laboratory to field mainly relies on front line demonstrations in the field condition that can be accomplished by adopting villages through Farm Science Centres (KVKs), NGOs and other non-profit organizations. Creating awareness on the usage of microbial based agri-products is an important factor in promoting this technology because they are highly sensitive to various factors like, dosage, time and method of application, compatibility with chemical fertilizers or pesticides, expiry date, authentication of the product by the government agency, advantages and benefits of the product to the farmer are some of the important issues that the farmer should be made aware of.

Often, it is very difficult to convince the farmers about the beneficial effects of the bioinoculants as their positive effects can be realized over seasons. For making the farmers to adopt such "Slow-transfer rate technologies", constant efforts are required to build awareness among the farmers through both on- and off-farm knowledge transfer/skill enhancement programs. Also, microbial based products are not the complete replacement for chemical fertilizers.

12.6 Future Perspectives

Excess usage of fertilizers and pesticides has lead to the deterioration of soil quality, loss of natural soil microbial biodiversity and pollution. Fertilizers pose great risk due to water contamination with nitrates that acts as a potent carcinogenic agents. An ideal example is a recent update on nitrate poisoning in Punjab in India. In 2009, under a Greenpeace Research Laboratories investigation, Dr Reyes Tirado, from the University of Exeter, UK conducted a study in 50 villages in Muktsar,

Bathinda and Ludhiana districts that revealed chemical, radiation and biological toxicity was rampant in Punjab. About 20% of the sampled wells showed nitrate levels above the safety limit of 50 mg/L. These incidents have established a need to promote biofertilizers and biopesticides for biological nutrient supplement and pest management in creation of a safe environment.

For successful exploitation of microbes for sustainable agriculture, the research efforts should get into basic, applied and strategic aspects. The basic aspects should focus on the identification of potential microbes screening, characterization and cataloguing the strains for all desirable traits enhancement of potential, understanding the mechanism of action of these microbes, composition of the rhizosphere population vis-à-vis beneficial microorganisms, the effect of cultivar on bacterial population dynamics, the influence of inoculum density on antagonistic activity, the survival of inoculum under adverse conditions and the role of environmental conditions in altering the activity of microbes. The applied and strategic research aspects should include development of cheap and viable mass multiplication protocols, identify suitable carrier systems, develop methods for prolonged shelf-life, working out the economics to demonstrate the usefulness of the agents, develop effective communication methods to disseminate the technology among the end-users to get feedback and refine technology. Despite many years of research on rhizosphere, our knowledge is fragmentary. This is mainly because of variations in methodology followed by various workers. For instance, the methods of counting soil populations do not give equal opportunity to all microbes to grow on certain media. Similarly, the variability among the microbes also makes it imperative to organize research activities to understand and develop technologies. The recent advances in the taxonomy of bacteria using BIOLOG, FAME and modern molecular tools now offer a better opportunity for classification of isolates than the conventional methods.

As a long term objective, a promising approach to improve the efficiency of rhizobacteria is combining several mechanisms of action in a single micro-organism by genetic modification after giving due consideration to the ecological safety of releasing large numbers of these genetically modified microorganisms.

In addition to plant growth promotion, the biocontrol ability of these microbes should be harnessed to manage weeds that causes enormous loss in realizable yields and degrades soil health.

Thus future thrust should be on the following lines:

1. Study the role of these microorganisms in production systems mode rather than in isolation and optimize their application systems
2. Identify the candidate microbes as biosensors to assess soil health
3. Develop methods to conserve biodiversity of the microorganisms in the wake of IPR and WTO regime immediately
4. Develop research networks and centres of excellence for the research on PGPRs
5. Develop a national road map for research on PGPRs
6. Create a transparent platform for participation of all stakeholders since the success of PGPR research is ultimately decided by the active participation of industry and research fraternity.

12.7 Conclusions

In the current scenario biofertilizers, PGPRs and biopesticides are accepted across the world. However, to attain maximum exposure, the regulations controlling bio-products have to be framed exclusively. More emphasis should be given on efficient strains selection and development, novel production and quality assessment methodologies, delivery systems and regulatory affairs to bridge the critical gaps. Implementation of production units in rural areas will act as a source of resource generation, benefit the farming community as well promote eco-friendly products for sustainable agriculture.

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Chapter 13
Tripartite Association Among Plant, Arbuscular Mycorrhizal Fungi and Bacteria

Shipra Singh and Anil Prakash

Abstract Most plant roots are colonized by arbuscular mycorrhizal (AM) fungi and their presence, generally, stimulates plant growth. In addition, AM fungi can interact with different bacterial species establishing a tripartite association and represent a vital component in the plant ecosystem. These interactions may range from loose to endosymbiotic association. In context of AM fungi, interaction with host plant is long been studied, however, information is little on the mechanisms controlling interaction of bacteria with AM fungi and host plant in the mycorrhizosphere. Understanding the interaction between AM fungi and bacteria is essential for describing the soil-plant interface. Although there are several studies concerning interactions between AM fungi and bacteria, the underlying mechanisms behind these associations are in general not very well understood, and their functional properties still require experimental confirmation. Modern tools of molecular biology and genome sequencing have solved the questions about the identity and role of bacteria associated with AM fungi. In this chapter, different aspects of tripartite association among plant, AM fungi and bacteria are discussed with greater emphasis on associated bacterial component.

Keywords Arbuscular mycorrhizal (AM) fungi • Host plant • Mycorrhization helper bacteria (MHB) • Endobacteria

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13.1 Introduction

Mutualistic association between plant and fungi, mycorhhiza (Greek *Mycos*: fungus + *Rhiza*: root), is the most wide spread terrestrial symbiosis. This association is based on the plant component providing carbohydrates and other essential organic compounds to fungi. In return the fungal component, that colonizes both root and the adjacent soil, helps the plant to take up nutrients (those of low mobility; especially P) by extending the reach of its root system. Although the original concept implies an association between fungi and plant root, these associations also includes plants with no true roots for e.g. bryophytes and pteridophytes (Smith and Read 2008).

The commonest mycorrhizal symbiosis is formed by arbuscular mycorrhizal (AM) fungi. They all are members of glomeromycota, a monophyletic group that diverge from the same common ancestor as ascomycota and basidomycota (Schussler et al. 2001). The ecological importance of AM fungi is unquestionable; they certainly have contributed to structuring the plant communities in different ecosystems. The long co-evolution period has rendered AM fungi so dependent on the symbioses that they became obligate symbiont, i.e., they are unable to grow in the absence of living host roots. However, some reports show that AM fungi can grow up to spore production phase in vitro in the absence of plant root and in presence of some spore associated bacteria (Hildbrandt et al. 2002, 2006). In contrast, host plants of AM fungi can survive if deprived of their fungal partner, this condition is virtually unknown in natural ecosystems, in which AM fungi function as true helper microorganisms, improving overall plant fitness.

The plant root-fungus symbiosis is established by inter- and intra-cellular growth of AM fungal hyphae in cortical region of root. Intracellular growth is characterized by formation of highly branched structures 'arbuscules' (site for nutrient exchange between plant and fungus) or hyphal coils (Fig. 13.1). Following root colonization, AM fungi produces runner hyphae forming the extraradical mycelium (ERM). The ERM can explore the soil for resources beyond nutrient depletion zone and is an important mean of translocation of energy rich photoassimilates from plant to soil. The immediate surrounding of the ERM is commonly termed as 'hyphosphere' and the soil compartment influenced by combined activities of root and AM fungi is known as 'mycorrhizosphere'. In the same way as the rhizosphere effect is seen for plant roots, a mycorrhizosphere effect can be seen whereby the soil surrounding fungal hyphae supports distinct bacterial communities compared to the bulk soil (Linderman 1988). Mycorrhizosphere inhabitants may include intra-hyphal bacteria in ectomycorrhizal fungi (Bertaux et al. 2003), and intra-spore bacteria in some AM fungi (Bianiciotto et al. 1996). Some mycorrhizosphere bacteria (mycorrhization helper bacteria; MHB) promote mycorrhiza formation, with a variety of Gram positive and negative strains involved (Garbaye 1994), although the precise mechanisms involved are unclear. The functioning of this ERM network is of key importance in mycorrhizal ecology because it represents not only an uptake point for soil nutrients but also a dispersal mechanism and a complex linkage network among roots within a plant community.

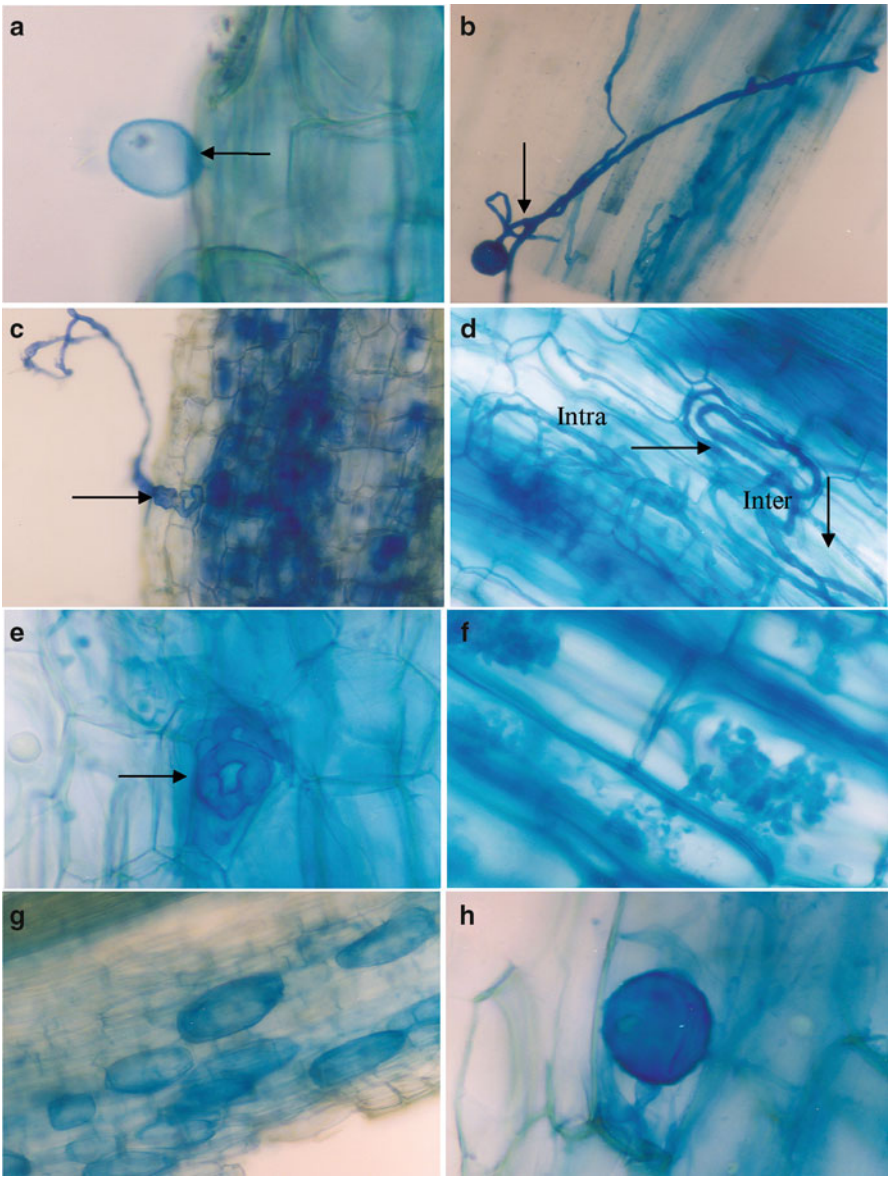

Fig. 13.1 Colonization pattern typical of AM fungi. (**a**) Attachment of spore on the root surface, (**b**) germination of spore outside the root, (**c**) appresorium formation at the root surface just before entry, (**d**) intra and inter cellular growth of hypha, (**e**) intracellular coil, (**f**) formation of arbuscules inside cortical cells, (**g**) formation of vesicles, and (**h**) intraradicle spore formation. *Arrows* show respective individual structures

Despite advancement in our knowledge of molecular basis of plant fungus interactions (Albrecht et al. 1999; Harrison 1999), several aspects of AM fungal biology, particularly their genomes, are still obscure due to their biotrophic nature, their multinuclear condition, and an unexpected level of genetic variability (Honsy et al. 1998; Lanfranco et al. 1999). Furthermore, complexity in the study of AM fungi arises due to the presence of endobacteria, most unculturable, in AM fungal spores. Modern molecular tools in combination with classical morphological approaches have stamped the presence of true bacteria (earlier known as bacteria like organisms 'BLOs') in spores of AM fungi (Bianiciotto et al. 1996).

13.2 Plant-AM Fungi Interaction: Signaling Between Symbionts

13.2.1 Presymbiotic Phase

Several plant-microbe symbioses involve detection or attraction of partners prior to direct contact. However in some instances, a molecular dialog is essential for progression to the physical stages of interaction. Till date *Rhizobium*-legume symbiosis is best characterized for their molecular dialog, in which flavonoids released from the plant signal the biosynthesis of a bacterial signal molecule called nod factor. Perception of nod factor by receptors on the legume roots triggers several initial events required for physical interaction and nodule development (Denarie and Cullimore 1993; Long 1996). Morphological aspects of AM symbiosis are well documented but information is little at the molecular level. The establishment of AM interaction and, in particular, fungus recognition by the host plant are mediated by partially characterized signaling pathway, the so called common symbiosis (SYM) pathway, partly shared with *Rhizobium*-legume symbiosis (Parniske 2008; Oldroyd and Downie 2004).

Development of AM symbiosis with plant is accompanied by significant morphological alterations at cellular level in both symbionts to create the novel symbiotic interaction. A hyphal germ tube emerges following germination from spore present in the soil and grows through the soil in search of plant root for a short distance. Upon finding root, AM fungal hyphae are encountered by plant signals present in root exudates. These signals identified as 'strigolactones' induces recursive hyphal branching increasing the probability of direct contact between the symbionts (Akiyama et al. 2005; Besserer et al. 2006). Akiyama et al. (2006) hypothesized that strigolactones are not only involved as primary AM branching signals but also as signals for the directional growth of AM fungal hyphae towards host roots. A number of flavonoids have also been reported to induce hyphal branching effect (Tsai and Phillips 1991; Phillips and Tsai 1992; Scervino et al. 2005a, b, 2006). Since flavonoid induced branching is found only in limited number of plant, their role as general signaling factors for hyphal branching as a prerequisite for a successful AM fungal root colonization is questionable.

AM fungal signals, hypothetically known as 'Myc factors', present in fungal exudates are perceived by Myc factor receptor (MFR) on root surface and thereafter trigger calcium spiking through the activation of 'SYM pathway' (Kosuta et al. 2003; Kuhn et al. 2010). Such calcium spiking is also induced by nod factors in *Rhizobium*-legume symbiosis. However, peak frequency of calcium spike induced by nod factors is regular and an irregular pattern is observed in AM fungi induced calcium spiking (Kosuta et al. 2008; Hazledine et al. 2009). Although 'Myc factors' are still unidentified, these were shown to be less than 3 kDa, partially lipophilic (Navazio et al. 2007), possess a chitin backbone (Bucher et al. 2009) and induce transcriptional activity of symbiosis related genes. Plant responses to 'Myc factors' range from the molecular to organ level and are part of the 'SYM pathway'.

An analysis of calcium spiking in *Lotus japonicum* in response to nod factor revealed that out of the seven SYM genes, five viz. *symrk*, *castor*, *pollux*, *nup*85 and *nup*133 mutants are defective for calcium spiking, whereas *CCaMK* and c*yclops* act downstream (Miwa et al. 2006; Harris et al. 2003). Similar results were obtained with *Medicago truncatula* mutants (Kosuta et al. 2008). Mutants that have common SYM genes do not form infection threads and, with the exception of c*yclops* mutants, do not initiate nodule organogenesis (Szczyglowski et al. 1998; Catoira et al. 2000). These findings suggest that common SYM gene products are involved in the early stages of symbiotic signal transduction, which involves the generation and decoding of calcium oscillations in and around the nucleus and induce early symbiosis related gene expression.

13.2.2 Formation of Prepenetration Apparatus (PPA)

A physical interaction between symbionts (hyphal tip touches the root surface) takes place on signal perception by both fungus and plant, and the plant cell actively prepares the intracellular environment for AM fungal hyphae. Upon finding the appropriate location for penetration on root surface, AM fungal hypha swell, flatten and branched repeatedly to develop hyphopodium also known as appresorium (Genre et al. 2005). Expression of several plant genes changes in the hyphopodium area including *ENOD11* (a gene coding for a putative secreted protein) during early stages of infection (Chabaud et al. 2002; Weidmann et al. 2004). During formation of PPA, new genes also become active including those involved in cell wall remodeling and defense (Siciliano et al. 2007).

Development of PPA takes place by aggregation of cytoplasm at the contact site which turns into thick cytoplasmic bridge across the vacuole of the host cell. Growth direction of PPA is guided by the movement of nucleus. Secretary machinery (abundant endoplasmic reticulum, several golgi bodies and secretary vesicles) is concentrated in PPA. Endoplasmic reticulums that decorate the PPA are ideally positioned for the synthesis of perifungal membrane that marks the appearance of symbiotic interface. This narrow intracellular compartment allows AM fungi to grow inside the plant cell without breaking its integrity (Bonfante 2001). Despite this knowledge, signals that trigger PPA formation are unknown.

13.2.3 Development of Arbuscule: Key Structure of Symbiosis

Arbuscules (Latin *arbusculum*: small tree) are characteristic structures of the symbiosis formed by dicotanomous branching of an intracellular hypha. The exact structure that is formed can vary depending on fungal and/or host genotype (Smith and Read 2008). Mechanisms associated with arbuscule development are largely unknown. However, some genes that affect arbuscule development are recently identified. A marked decrease in epidermal penetration and total block of arbuscule development was observed by RNA interference knockdown of v*apyrin* gene that codes for a cytoplasmic protein with unknown function (Pumplin et al. 2010).

At least two signaling events were suggested by Harrison (2005) in arbuscule development: cell autonomous and non autonomous. The cell autonomous signaling would be responsible for activation of the expression of certain genes (mycorrhiza specific phosphate transporters, a cellulase, a chitinase and aproton ATPase) and occurs exclusively in arbuscule containing cells. This spatial expression pattern suggests that cell autonomous signals activate expression of these genes. Whereas, cell non-autonomous signals are involved in activation of specific genes in cells containing arbuscules and their immediate vicinity (a GST, a chitinase and a β-13 endoglucanase). Reorganization of microtubule cytoskeleton in cortical cells adjacent to arbusculated cells can also be considered as a evidence for this signaling pathway (Blancaflor et al. 2001). Using in situ hybridization, Lambais and Mehdy (1998) showed an induction in the accumulation of transcripts encoding an acidic chitinase in cells containing arbuscules and in their immediate vicinity suggesting systemic signals operating in AM roots containing arbuscules.

The estimated lifespan of arbuscules is of 4–10 days (Sanders et al. 1977); after this short period, AM fungal wall collapse in fine branches following septa formation. Eventually, this senescence extends to trunk of hypha collapsing the whole structure. Consequently, arbuscule disappear and plant cell regains its normal physiology and organization with a large central vacuole (Bonfante 1984).

13.3 Function of AM Fungi: Nutrient Exchange

Mutualistic associations are based on bidirectional nutrient exchange and, as such, are beneficial to both partners (Fig. 13.2). This concept applies to AM symbiosis: fungal hyphae explore the soil substratum to efficiently take up nutrients and water to improve plant nutrition and in return obtain plant carbohydrates for successful completion of the AM fungal life cycle.

13.3.1 Carbon Uptake and Translocation

It is generally assumed that phosphate and carbon transfer occurs at the arbuscule/cortical cell interface, although direct evidence for carbon transfer at this interface

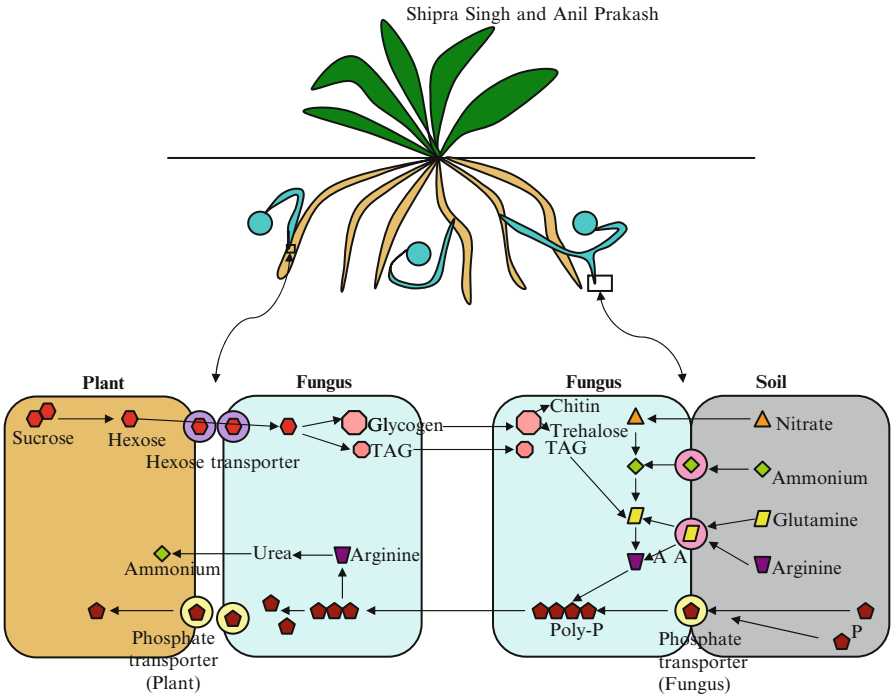

Fig. 13.2 Scheme summarizing nutrient uptake and translocation processes in AM symbiosis

is lacking (Javot et al. 2007). The assimilate transfer includes breakdown of sucrose into glucose and fructose, their export across plant plasma membrane and active uptake by hexose transporters across fungal plasma membrane, driven by an increased H^+ATPase activity at the arbuscular membrane (Gianinazzi-Pearson et al. 2000). NMR spectrometry combined with isotopic labelling showed that intraradical hyphae of AM fungi obtain and use hexoses, mainly glucose from plant (Shachar-Hill et al. 1995; Bago et al. 2003). Elevated levels of host extracellular (acid) invertase activity also suggest that hexose is the dominant form of taken up carbon (Denhe 1986). AM fungi convert hexoses into glycogen and lipids for long distance transport to the ERM (Bago et al. 2002, 2003). Although reports of carbon transfer from host plant to AM fungi came in 1960s (Smith and Read 2008), underlying molecular mechanisms are still unclear and requires identification and location of the membrane protein involved. The only hexose transporter of glomeromycota described so far has been reported in a non-arbuscule producing fungus *Geosiphon pyriforme* (Schussler et al. 2006). The major forms of stored carbon in hyphae and spore are glycogen, lipids and trehalose (Pfeffer et al. 1999).

It is stated earlier that there is no carbon transfer from fungus to plant in an AM symbiosis (Pfeffer et al. 2004). However, mycoheterotrophic plants growing in vicinity of plant associated with AM fungi present an exception to this phenomenon and likely to obtain carbon from AM fungi. Bidartondo et al. (2002) showed that

non-photosynthetic plants associate with AM fungi and can display the characteristic specificity of epiparasites. This suggests that AM fungi mediate significant inter-plant translocation of carbon in nature.

13.3.2 Phosphate Uptake

The major benefit of AM symbiosis is improved phosphorus uptake. Growing roots absorb phosphates at much higher rate compared to soil based phosphate diffusion rate resulting in a formation of phosphate depletion zone around the root system. Hyphal network of AM fungi extends beyond this zone of depletion and explore a new pool of available phosphates (Smith and Read 2008). Additionally, hyphal network contributes to the release of phosphates from inorganic complexes of low solubility by influencing directly or indirectly, the physicochemical properties of the soil (Finlay 2008). A breakthrough in understanding of phosphate uptake by AM fungi was provided by Harrison and van Buuren (1995). They identified a cDNA encoding a high affinity transmembrane phosphate transporter (Km = 8 μM) GvPT from *Glomus versiforme*. Later, Maldonado-Mendoza et al. (2001) also reported gene for phosphate transporter GiPT from *Glomus intraradices*. These genes (GvPT and GiPT) are predominantly expressed in the ERM of AM fungi (the site for phosphate uptake from the soil) exposed to micromolar phosphate concentrations. Accumulated phosphates, in the form of polyphosphate, are then rapidly translocated along the aseptate mycelium to the intraradical mycelium (Viereck et al. 2004; Smith and Read 2008). Although arbuscules are known as the site for release of phosphates into plant cells, the mechanism involved is presently unknown. It is predicted that specific carriers, pumps or channels facilitate transfer of phosphate ion through fungal plasma membrane since a concentration gradient is followed by passing phosphate ions inside the root. However, AM fungal inducible plant phosphate transporters involved in phosphate transfer into plant cortex cells have been identified in several plant species (potato: StPT3, StPT4; rice: ORYsa;Pht1;11 and *Medicago truncatula*: MtPT4) using gene expression studies (Rausch et al. 2001; Harrison et al. 2002; Paszkowski et al. 2002). Functions of these transporters were confirmed by functional complementation using yeast mutants.

13.3.3 Nitrogen Uptake and Transfer

Nitrogen uptake and transfer by AM fungi is not well understood. However, some studies highlighted the capacity of the ERM to import nitrogen from organic and inorganic sources (Hodge et al. 2001; Govindarajulu et al. 2005) through protein such as amino acid and ammonium transporters (Lopez-Pedrosa et al. 2006; Siciliano et al. 2007). Taken up nitrogen is probably incorporated into amino acid, mainly

arginine, and translocated to intraradical hyphae, but transferred to plant without carbon (Govindarajulu et al. 2005; Cruz et al. 2007). Although ammonium is considered the form that is released into plant cell, ammonium transporters in the symbiotic interface membranes have not yet been identified.

13.4 AM Fungal Interactions with Bacteria

AM fungi are key component of soil microbiota and obviously interact with other microorganisms in the rhizosphere (Bowen and Rovira 1999; Artursson et al. 2006). In this context, establishment of AM symbiosis changes plant physiology and certain nutritional and chemical properties of the rhizosphere soil. The ERM provide an interface for interaction with other soil microorganisms in the soil through active or passive exudation of plant derived carbon into the surrounding environment. This carbon is utilized by soil microorganisms as a source of energy and, in turn, affects colonization patterns of soil microorganisms in the rhizosphere region by the so called 'mycorrhizosphere effect' (Gryndler 2000). In addition, living or dead hyphae and/or compounds released by AM fungal hyphae may be used as nutrient source or substratum by soil microorganisms. On account of nutrient richness, mycorrhizosphere harbours a great bacterial diversity. Apart from a great and complex bacterial diversity, mycorrhizosphere is a very influential zone for biological system and definitely deserves more scientific attention.

Conversely, bacterial community is known to affect plant and AM fungal formation and functioning, markedly, in various ways. Both saprophytes and symbiotic bacteria interact with AM fungi in mycorrhizosphere and these two groups are potentially consisting of detrimental, neutral and beneficial bacteria (Barea et al. 2002a; Johansson et al. 2004). A number of studies have classified some interactions between populations of bacteria and fungi with AM fungi as parasitism, generating discussion about its consequences at both 'parasite' and host population levels. A review by Purin and Rillig (2008) presented potential consequences of AM fungi parasitism at the population/community level and discussed applied aspects. Deleterious rhizosphere bacteria and mycoparasitic relationships have been found to interfere with development of AM fungi whereas several bacteria (plant growth promoting rhizobacteria; PGPR) can stimulate formation and/or functioning of AM fungi (Gryndler 2000; Barea et al. 2002b). It leads to possibility that the beneficial effects of such PGPR on plant growth are due to stimulatory effect on the growth of AM fungi. For example, PGPR are known to affect the presymbiotic stages of the AM fungal development, such as spore germination and germ tube development (Azcon-Aguilar and Barea 1992; Carpenter-Boggs et al. 1995). In addition, biologically active substances such as amino acids, plant hormones, vitamins, other organic compounds and volatile substances (CO_2), produced by soil microorganisms, can stimulate the growth rates of AM fungi (Becard and Piche 1989).

13.4.1 Mycorrhization Helper Bacteria

Several PGPR possess a variety of direct mechanisms that increase the ability of the root to establish symbiotic interaction with mycorrhizal fungi. For such bacteria, Garbaye (1994) proposed the term 'mycorrhization helper bacteria' (MHB). This concept was initially proposed in the context of ectomycorrhizal fungus; however, several reports have also demonstrated enhanced AM fungal colonization in roots in the presence of PGPR (Meyer and Linderman 1986; Sanchez et al. 2004). The helper effect of these bacteria was suggested to include stimulation of root development, enhanced susceptibility of the root to mycorrhizal fungal colonization, enhancement of the recognition process between root and fungus, production of growth factors that stimulate fungal spore germination, mycelial growth, reduction of soil mediated stress through detoxification of antagonistic substances, and inhibition of competitors and antagonists (Frey-Klett et al. 2007). A classical example of helper effect is presented by rhizobia producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase; the enzyme modulating plant ethylene levels, increasing plant tolerance to environmental stress and stimulating nodulation (Ma et al. 2002). An ACC deaminase producing strain *Pseudomonas putida* UW4 was shown to provide helper effect with AM fungi *Gigaspora rosea* when inoculated into cucumber plants (Gamalero et al. 2008). Reports are also available showing that the helper effect is accompanied with the change in gene expression. In *L. bicolor* S238N, activation of genes potentially involved in recognition process, transcription regulation, and synthesis of primary metabolism protein was observed in the presence of MHB *Pseudomanas flourescence* BBc6R8 (Deveau et al. 2007).

Taken together, these studies suggest the role of active diffusible molecules and physical contact between bacteria and AM fungi for the establishment of their interaction. This can be correlated with signaling events identified in rhizobia-legumes symbiosis and in AM fungi-plant interaction as well. As discussed earlier, both share common SYM pathway and partners release active diffusible molecules that are perceived reciprocally. Ca- mediated responses are also activated whereas a physical contact between fungus and plant is required to elicit several plant responses for successful AM fungal colonization (Genre et al. 2005, 2008; Navazio et al. 2007). On the other hand, volatile organic compounds are also considered to play important role for communication between organisms. Bacterial volatiles are known to affect soil fungi including the mycorrhizal ones (Tarkka and Piechulla 2007). Although poorly understood, they are important determinants for the establishment of symbiosis. MHB are currently the most investigated group of bacteria interacting with mycorrhiza, but there is still much to be investigated about the molecules that lay the foundation of interaction between MHB and their fungal and plant hosts.

13.4.2 Endobacteria

Only a limited number of fungi are reported to host bacteria in their cytoplasm, out of which AM fungi are unique. In 1970s, several reports showed the presence of

BLOs inside the cytoplasm of field collected AM fungi and the number of such reports is continuously increasing (Mosse 1970; MacDonald and Chandler 1981; Scannerini and Bonfante 1991; Bonfante et al. 1994). Further investigation of these BLOs, including their prokaryotic nature, was long hampered due to unculturability of these organisms. However, use of combined morphological (electron and confocal microscopy) and molecular techniques allowed us to identify BLOs as true bacteria and their symbiotic relationship with AM fungi could be demonstrated (Bianciotto et al. 1996).

Family *Gigasporaceae* is most studied for the presence of endosymbiotic bacteria; however, endosymbionts are detected in several other AM fungal species such as *Glomus versiforme*, *Acaulospora laevis* etc. (Mosse 1970; MacDonald and Chandler 1981). These endosymbiotic bacteria were detected through all the steps of fungal life cycle: spores, germ tubes and extra and intraradical hyphae, except arbuscules (Bianciotto et al. 1996). *Gigaspora margarita* isolate BEG 34 is the most extensively studied AM fungus and is currently being used as model system to study AM fungi-endobacteria interaction. *G. margarita* cells were reported to harbour a large number of bacteria (on an average 20,000 bacteria per cell) and these bacteria were initially assigned to genus *Burkholderia* on the basis of their 16S rDNA sequence (Bianciotto et al. 1996). Further studies of the 16S rDNA sequences of endobacteria isolated from *Scutellospora persica*, *S. castanea* and *G. margarita* shown a strongly supported clade located close to genus *Burkhoderia* and to genera *Ralstonia* and *Pandorea* as well. In spite of several attempts, these bacteria could not be cultured and therefore, a new bacterial taxon "*Candidatus* Glomeribacter gigasporarum" was proposed (Bianciotto et al. 2003).

Functional significance of AM fungal endobacteria is still unknown, as is their potential role in the establishment of mycorrhizal symbiosis (Jargeat et al. 2004). Genomic library developed from *G. margarita* spores was shown to also represent the genome of endobacteria and helped us to identify some of such roles (van Buuren et al. 1999). Among the bacterial genes indentified so far, the most interesting are those involved in nutrient uptake: a putative phosphate transporter gene, *pst* (Ruiz-Lozano and Bonfante 1999), in host cell colonization events by enteroinvasive, pathogenic bacteria *Shigella flexneri* and *Escherichia coli*, *vacB* (Ruiz-Lozano and Bonfante 2000), in chemotaxis, *mcpA* (Minerdi et al. 2002b) and *cheY* (Minerdi et al. 2002a), a kinase, *prkA* and a *spoVR* gene involved in coat formation of bacterial endospores (Minerdi et al. 2002a). Three *nif* genes (*nifH, nifD* and *nifK*) were also found (Minerdi et al. 2001) but have not yet been demonstrated to belong to the genome of *Candidatus* Glomeribacter gigasporarum.

The mode of transmission of endobacteria to succeeding generations is not well established. However, two alternatives permanent (remain stable over time) and cyclic endosymbiosis (involve regular reassociation events) have been proposed (Bianciotto et al. 2000). Bianciotto et al. (2004) demonstrated vertical transmission of *Candidatus* Glomeribacter gigasporarum through five fungal vegetative generations of *G. margarita* spores and active bacterial proliferation was demonstrated in fungal mycelium. Transmission of endobacteria from spore to hyphae may be facilitated by the asexual reproduction typical of AM fungi and coenocytic nature of

their hyphae. On the basis of these findings, authors suggested that these bacteria are obligate endocellular component of their AM fungal host.

13.5 Economical and Ecological Significance

Natural activities of soil microorganisms may contribute to the maintenance of crop and production health by improved nutritional status and biological control of plant pathogens in low input agriculture systems. Therefore, understanding of these microbial interactions and the mechanisms involved is essential for the progress of sustainable agriculture. A breakthrough was reported by van der Heijden et al. (1998); they showed that the below ground biodiversity of AM fungi is a major factor contributing to the maintenance of plant diversity and ecosystem functioning. They have emphasized the need to protect and consider the role of these fungi in the management of diverse ecosystems. Read (1998) has further pointed out that conservation of the fungal gene pool is likely to be a prerequisite for maintenance of floristic diversity in terrestrial ecosystems, wherein the "mycorrhizal web" is known to influence natural resources. Changes in AM fungal communities may be observed with both plant community succession (Janos 1980; Johnson et al. 1991) and with changing land use intensity (Oehl et al. 2003). The more diverse assortment of host plants may support more diverse community of AM fungi (Johnson et al. 2004). In a study by Singh et al. (2008a), higher number of AM fungi was recorded in natural tea site (35 morphospecies) compared to cultivated tea site (27 morphospecies). The most obvious difference between the two AM fungal communities lied in the single dominant AM fungus at the cultivated site and the six AM fungi at the natural site, being either less frequently detected or absent in the cultivated site. Oehl et al. (2003) have noted that some AM fungi present in the natural ecosystems get strongly depressed under conventional high-input farming practices, indicating loss of at least some ecosystem function in the latter. In mixed *Araucaria* forest ecosystems, higher diversity index was recorded in native stand without any anthropogenic interference as compared to planted stand (Moreira et al. 2007). In long term field trials, Madar et al. (2000) found that root colonization by AM fungi in organic farming systems was 30–60% higher than in conventional systems. Overall higher biomass and biodiversity of soil organisms, and higher microbial activity were recorded in organic farming systems (Mader et al. 2002). However, crop yields were lower in the organic system when inputs of fertilizers, energy and pesticides were reduced. They concluded that enhanced soil fertility and higher biodiversity found in organic plots may make these systems less dependent on external inputs. Consequently, monetary loss in production may be compensated for by a reduced need of chemical fertilizers and pesticides.

The high significance of AM associations lies in the supply of mineral nutrients, in particular phosphorus, to their host plants (Miller 2000; Nielsen et al. 2002; Tiwari et al. 2004; Singh et al. 2008b). In this context, possible role of extracellular phosphatases of AM fungi in mineralization of organic phosphorus pools in soil has

attracted attention (Feng et al. 2003). Improved growth of crop plants associated with AM fungi is well documented (Tanu et al. 2004; Prakash et al. 2004) but reports are scanty for the effect of AM fungi on active principles of economically important plants. Analysis of tea plants following inoculation with AM fungi revealed that these inoculations may be effective for improved quality of tea (Singh et al. 2010).

Biofertilizer properties of some AM fungi associated bacteria have been documented and they have been found to synergistically affect nitrogen fixation and mycorrhizal development. Toro et al. (1997) demonstrated that both *Enterobacter* sp and *Bacillus subtilis* promoted the establishment of *Glomus intraradices* and increased pant biomass as well as tissue nitrogen and phosphorus contents. In addition to general plant nutrition (Sahgal et al. 2004; Sharma et al. 2009), microbial interactions may have implications for biological control of phytopathogens (Choudhary et al. 2009). A number of studies have indicated that some AM fungi and associated bacteria as well (Barea et al. 1998; Budi et al. 1999) exhibit biocontrol properties against root pathogens. However, practical use of AM fungi in single and co-culture with bacteria possessing biocontrol properties remains to be explored.

13.6 Conclusions and Future Perspectives

Use of genomics and functional genomics shed light on the advances in the understanding of the mechanisms controlling AM development and symbiosis with plants. Evidences are available that the plant and AM fungi perceive signal molecules prior to their physical interaction. Identities of several molecules of the signaling pathway such as 'Myc factor' are unknown and expected in near future. AM fungi use 'common SYM pathway' for colonization similar to rhizobia-legume symbiosis. Apparently, the signature of Ca^{2+} spikingin the cytoplasm is one of the events discriminating rhizobia from AM fungal signals, even though there is an alternative transduction pathway still to be detailed. Similarly, some transporters involved in nutrient translocation at symbiotic interface are still to be identified.

Mycorrhizal technology seems to be an unavoidable tool for developing new plant management systems in agriculture in order to ensure adequate levels of food production with satisfactory reduction in chemical fertilizers and pesticides. In the context of current global challenges, AM fungi may be crucial in several fields such as environmental change, ecosystem conservation and food safety issues. AM fungi mobilize P and N, and are an important C sink in the soil, having therefore considerable impact on the cycling of these elements. As biofertilizers, they may provide an effective alternate to the synthetic chemicals thus promote sustainable agriculture and protection of the environment. In this frame, insights on the contribution of AM fungi to the nutritional quality of the edible plant parts become a priority. Another long term issue is to identify or design crop-AM fungus combinations with optimized AM fungal performance leading to reduced fertilizer and energy inputs.

Another interesting field is the study of roles of bacteria associated with AM fungi as third component of symbiosis. Their cultivation and determination of potential in terms of improved health/growth of AM fungi and/or plants will be helpful in sustainable agriculture. Answers of these basic questions will make us able to exploit tripartite interaction among plant-AM fungi- and bacterial community for benefiting plants and humans.

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Part II
Microbes in Biotechnology

Chapter 14
Metagenomics: A Relief Road to Novel Microbial Genes and Genomes

Jyoti Vakhlu, Sheetal Ambardar, and B.N. Johri

Abstract A huge quantum of the genetic and metabolic diversity of the biosphere is locked up within the microbial communities present in and on earth, as majority of earth's biomass comprises microbes. Ironically we cannot access more than 1% of this bioresource through routine culturing techniques as we have underestimated the power of microbes to resist culturing. This 1% of the microbial diversity accessed so far contributes to more than 80% of the industrial biotechnology at present and what miracles rest of the 99% can perform is anybody's guess. Metagenomics (also known as e-genomics, community genomics and environmental genomics) is clutch of molecular biology techniques used to hunt the culture resisting, yet- to- be cultured microbes. Metagenomics is a fruit born of the wedlock between modern molecular techniques and the idea, that microbial diversity can be analyzed by direct DNA isolation from environmental samples (metagenomic DNA), it's cloning, screening and sequencing. It would not be farfetched to conclude that after invention of microscope and culturing techniques, metagenomics is third big revolution in microbiology. Metagenomic revolution would not have been possible without the availability of low cost DNA sequencing services, bioinformatics tools and high through put screening techniques. This chapter will deal with the techniques used in metagenomics and achievements made so far along with future applications and challenges.

Keywords Metagenomics • Biodiscovery • Biodiversity • Functional screening • Sequence based screening • Bioprospecting

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14.1 Introduction

The next big discovery in the field of microbiology would be to find the place where microbes are not able to survive as it has been amply demonstrated that microbes are present at all the impossible places, hot springs to Antarctic soil, geysers to glacier ice, deep sea vents to clouds and from gut of the insect to human gut. During last 10 years microbiologists have been able to convince researcher with the help of a budding technique called metagenomics that so far we have not been able to cultivate more than 1% of total microbes present on and in earth. It would not have been possible to establish their presence by routine microscopic and cultivation based techniques though the first indication, that we are not able to characterize majority of the microbes, was given by combination of microscopic and culturing techniques, "The great plate count anomaly". "The great plate count anomaly" is the discrepancy between sizes of population estimated by dilution plating and by microscopy (Handelsman 2004). If we compare direct microscopic cell count after 4-6-diamidino-2-phenylindole (DAPI) staining of bacteria with the number of the microbial colonies growing on nutrient agar, it appears that in natural samples less than one cell in thousand produces a colony. Amann and his coworkers have reported that 0.001–0.01% of microorganisms in sea water, 0.25% in fresh water, 0.25% in sediments and only 3% of soil microorganisms are found to be cultivable (Amann 1995). Cataloguing the microbes on the basis of DNA and not on cellular identification generated a great debate among the scientific community and finally in 1998 Jo Handelsman coined the term "Metagenomics" for culture-independent study of microbes in any environment. Metagenomics can be used interchangeably with e-genomics and community genomics, etc. Metagenomics is drawing attention not only in microbiology but also in other branches of science like chemical sciences, earth science, agriculture, environment remediation, human health, biodefense and microbial forensics.

14.2 Achievements and Application

Microbiologist have focused on the isolation, purification and study of single species in laboratory but lately ample evidences suggest that most of the life supporting activities are carried out by complex microbial community-integrated, intricate and balanced. Our understanding of microbial community has lagged behind and metagenomics can bridge the gap. Metagenomics in our view is the third big revolution in microbiology after discovery of microscope and culturing techniques. It is not only going to influence microbiology but all life forms, as microbes are present and perform vital functions in all life forms and habitats.

Metagenomics is a genomic technique born out of wedlock between a new idea of direct isolation of DNA from any environmental sample and various sophisticated molecular biology techniques. It includes massively parallel sequencing without cloning, high throughput assay system targeting metabolic pathways. It also

includes artificial transcriptional regulators activating reporter genes to indicate enzymatic substrate conversion and cDNA cloning from extracted mRNA to directly clone actively expressed genes from a microbial consortium. Thus, metagenomics not only provides us new insights into microbial (taxonomic/genetic diversity) life but also access to genes (metabolic diversity) producing novel biomolecules.

14.2.1 Biodiversity

The total number of prokaryotic cells on earth has been estimated to be $4–6 \times 10^{30}$ comprising of 10^6 to 10^8 separate species (Simon and Daniel 2009). More than 95% of the microbial wealth is uncharacterized and therefore is a treasure of unexplored genetic and metabolic diversity. The metagenome consists primarily of the yet to be cultivated bacterial genome and a minor fraction of the culturable bacterial genome as in most microbial habitats, unculturable bacteria dominate the total microbial community. Microbial diversity is explored usually by two approaches, i.e. by sequencing and analysis of phylogenetic anchors or reconstruction of whole genomes, after shotgun sequence (Fig. 14.1).

14.2.1.1 Assessment of Microbial Diversity by 16S/18S rRNA and Its Region

Conventional methods to establish the presence and identity of microbes are by colony characteristics, microscopy, biochemical tests and 16S ribotyping, but in metagenomic analysis the DNA is directly isolated and analyzed. Hence the identification in the latter is based only on 16S ribosome, ITS region sequence analysis and is cultivation independent. Microbial diversity is explored usually by several approaches like metagenomic library construction, mass sequencing and amplification of 16S/18S/ITS region from metagenome as shown (Fig. 14.2) but the most common so far is analysis of conserved ribosomal RNA (rDNA) gene sequences (Woese 1987). The metagenomics-based bacterial diversity analysis largely depends on the sequence analysis of small subunit ribosomal RNA (16S rRNA) genes which are amplified using broad range (universal) PCR primers based on the sequences that are well-conserved among prokaryotes. The 16S rRNA genes amplified from

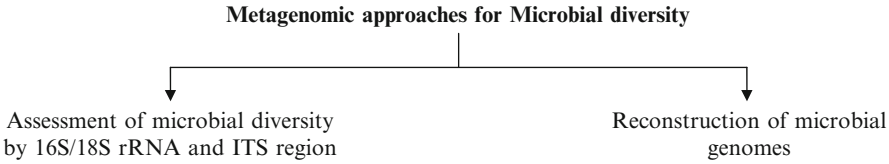

Fig. 14.1 Two different ways to explore uncultivable microbial diversity

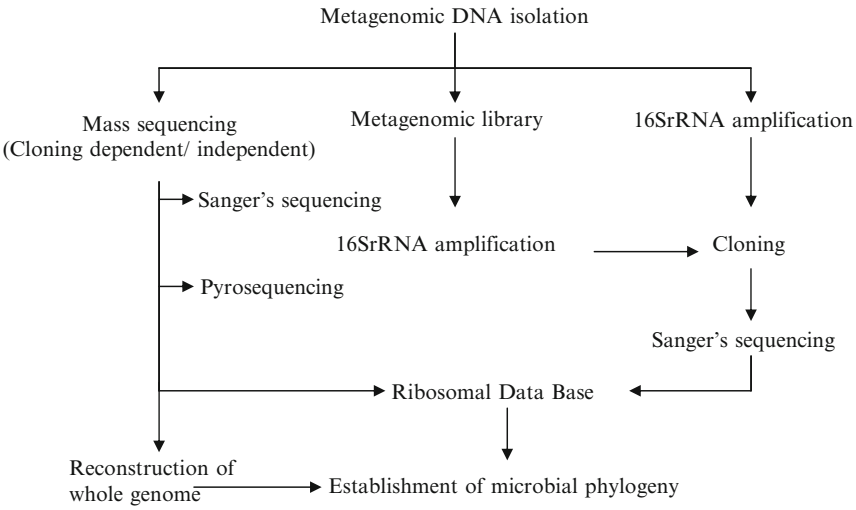

Fig. 14.2 Metagenomic approach for evaluation of microbial diversity

metagenome are used to make libraries of clones, where each clone represents a 16S rRNA gene from a prokaryotic species. Individual clones are sequenced and the 16S rRNA gene sequence similarity analysis is used to identify the species level phylogenetic types or phylotypes (Chung et al. 2008; Rajendhran and Gunasekaran 2009).

In recent years significant progress is being made in the field of metagenomics due to development of high throughput next generation sequencing technologies (Simon and Daniel 2009). Large amount of sequencing data has been generated using such techniques and analysis of this data has changed our view of microbial world. Extensive sequencing of ribosomal gene has resulted in generation of several large reference databases such as ribosomal data base project (RDP) II and Greengenes or Silva (Simon and Daniel 2009). These comprehensive databases allow classification of environmental 16S rRNA gene sequences. The sequences which do not have their counterpart in databank or show limited similarity percentage with known sequences are referred to as novel sequences.

As an example, microbial diversity of four extreme environments, explored by different culture independent (metagenomics) approaches is summarized in Table 14.1.

The advent of metagenomics has greatly accelerated our understanding about the evolution of various domains of prokaryotes and eukaryotes and provides a much broader ability to access diversity within different phyla, such as the following:

Bacteria

Twelve major divisions (phyla) have been identified in the Bacterial domain on the basis of rRNA sequence data by Woese in 1987. The analyzed bacteria represent

Table 14.1 Metagenomic approach to monitor microbial diversity of four extreme environments

S. No.	Environmental samples	Approach	Diversity	References
1	Acid mine drainage	Shotgun sequencing and **Genome reconstruction**	Leptospirillum group II (L. ferriphilum) Leptospirillum group III Sulfobacillus, Ferroplasma A-plasma and G-plasma	Tyson et al. (2004)
2	Sargasso Sea	16S r RNA analysis of Metagenomic DNA and pyrosequencing	*Proteobacteria* (subgroups *Alpha*, *Beta* and *Gamma*), *Firmicutes, cyanobacteria* and species in the CFB (phyla *Cytophaga, Flavobacterium* and *Bacteroides*)	Venter et al. (2004)
3	Yellowstone park	Linker amplified shotgun library techniques	Thermophilic crenarchaeal viruses *Acidianus rod-shaped virus* (ARV), *Sulfolobus islandicus rod-shaped virus* 1 (SIRV1) and SIRV2, and *Sulfolobus islandicus filamentous virus* (SIFV). *Pyrobaculum spherical virus* (PSV)	Schoenfeld et al. (2008)
4	Antartica	16S r RNA analysis of Metagenomic DNA and pyrosequencing	*Bifidobacterium* (phylum *Actinobacteria*), *Arcobacter* (phylum Proteobacteria) and *Faecalibacterium* (phylum Firmicutes)	Teixeira et al. (2010)

almost all major cultured groups of Bacteria. In just over a decade, culture-independent surveys have identified 40 well-resolved major bacterial divisions which show that there are about 30 major bacterial divisions with few or no cultured representatives (Xu 2006).

Archaea

Archaea were thought to be only present in extreme habitats but culture-independent methods have identified that archaea are also widespread in diverse nonextreme habitats such as garden and forest soils, water and sediments in marine and freshwater lakes, as well as extreme habitats such as hot springs, saline lakes and deep-ocean thermal vents (Xu 2006). The culture-independent methods have also revealed major new types of archaea. Phylogenetic analyses has suggested that domain

archaea contains at least 50 distinct phylogenetic groups with 33 from the current Euryarchaeota, 13 from Crenarchaeota, 1 each from Korarchaeota, Nanoarchaeota, and the ancient archaeal group (AAG). Among these 50 phylogenetic groups, only 13 have cultured representatives.

There has been significant interest in applying metagenomics to learn more about the members of the archaea in soil and as planktonic organisms in seawater (Riesenfeld et al. 2004). The discovery of 16S rRNA gene sequences that affiliate with the archaea in diverse terrestrial and marine environments on Earth has significantly altered the microbiologist's image of archaea.

The study of the symbiotic community of an *Axinella* sp., a sea sponge, has been an important application of metagenomics to archaea. In a culture-independent 16S rRNA gene survey, 65% of the symbiotic community associated with the sponge was represented by a single archaeal 16S rRNA gene sequence which was named as *Cenarchaeum symbiosum.* The complete genome sequence of *C. symbiosum* will contribute to understanding its biology and symbiotic relationship with its sponge host (Riesenfeld et al. 2004).

Virus

Viruses are known to be the most numerous biological entities on the planet and are known to infect microorganisms but little is known about their diversity in this environment. Diversity of virus has been explored more in aquatic environment as compared to the soil. Transmission electron microscopy has estimated the viral abundance in soil to be of the order, 1.5×10^8 g^{-1} (Kim et al. 2008). Molecular analysis is essential to investigate the diversity of virus because majority of viruses are difficult to cultivate due to lack of suitable host; less than one percent of microbial hosts have been cultivated. Moreover, a PCR based approach could not be employed in case of viruses as there are no universal conserved genes or markers for viruses like 16S rRNA gene for bacteria. These limitations are being circumvented by metagenomic analyses of uncultured viral communities and can provide insights into the composition and structure of environmental viral communities (Edwards and Rohwer 2005).

The metagenomics of viruses began in 2002 with the publication of two uncultured marine viral communities (Breitbart et al. 2002). In both cases, the small size of viral genomes approximately 50 kb on average was an advantage because less sequencing was required (Wommack et al. 1999; Steward et al. 2000). In this approach, viruses are purified and concentrated by sequential filtration and ultracentrifugation as in most water samples, it is necessary to concentrate virions from several hundred litres to obtain enough DNA for cloning. The whole genome of virus is further extracted, amplified, shotgun cloned and pyrosequenced (Edwards and Rohwer 2005; Angly et al. 2006).

Linker amplified shotgun library techniques has been reported to amplify viral DNA using linkers in PCR amplification. This technique is limited to double stranded DNA viruses as the linkers can only ligate to double stranded DNA and not to single stranded DNA virus (Edwards and Rohwer 2005). This limitation is

circumvented by multiple displacement amplification (MDA) which has helped in acquiring the sequence of both double and single stranded DNA viruses (Angly et al. 2006). MDA is based on whole genome amplification method which uses Ø29 DNA polymerase and random hexamers to amplify the minute amount of DNA to ~80 μg (Dean et al. 2002). Ø29 DNA polymerase amplifies the DNA via rolling circle replication in which short DNA amplifies more efficiently than linear DNA (Kim et al. 2008).

Viral classification is based on characteristics of the virions and host range and not on sequence data as universal ribosomal DNA (rDNA) sequences cannot be used for deriving the phylogenetic and taxonomic relationships due to the lack of single sequence common in all viral genomes. The most common sequence-based approach is to use a single gene locus, such as a capsid or DNA polymerase gene, to characterize a specific viral group which could be done by designing PCR primers for these genes; the diversity of specific genes in the environment can be assessed by cloning and sequencing of DNA products amplified directly from environmental samples. This single-locus approach has been used to show that there are many groups of uncultured viruses in the environment.

Recent culture independent studies have shown that both DNA-based and RNA-based viruses are common in terrestrial as well as freshwater and marine environments (Edwards and Rohwer 2005). For example, in an analysis of picorna-like viruses (a group of positive-sense single-stranded RNA viruses that are major pathogens to plants and animals), Culley et al. (2003) identified high, unexpected diversity in the sea. Indeed, all of the picorna-like sequences from marine samples were different from known picorna-like viruses in the databases. Of specific note is a virus isolated in this study that is lytic pathogen to a toxic-bloom-forming alga *Heterosigma akashiwo*. This data suggests that picorna-like viruses may be important contributors in the regulation of marine phytoplankton population dynamics.

Bacteriophages

Phages are the most abundant and diverse group of biological entities on the planet. Pedulla and coworkers (2003) have described ten new mycobacteriophage genomes using metagenomic approach. Over 50% of the open reading frames (ORFs) in the genomes are unrelated to anything in GenBank and only one of the new mycobacteriophage is significantly related to a previously sequenced phage. These findings are surprising, because all of the new phages belong to the most thoroughly studied group of ds DNA phage, the Siphoviruses.

Eukaryotes

Direct amplification and cloning of 16S rRNA genes from environmental samples has considerably expanded the information about prokaryotic diversity and sequencing of these environmental rRNA gene libraries has revealed new, often uncultivated,

groups of the *Bacteria* and *Archaea* which may be present in great abundance. In a similar way, molecular analysis of small subunit 18S rRNA genes has revealed a wide diversity among eukaryotes (Grant et al. 2006).

Photosynthesis in marine environment is basically due to four major phytoplankton lineages of eukaryotes namely prymnesiophytes, alveolates, stramenopiles, and prasinophytes. Genomes of only two lineages (stramenopiles, and prasinophytes) have been analyzed by culture dependent approaches whereas "picoplanktonic" members of the prymnesiophyte lineage remain poorly characterized but, have long been inferred to be ecologically important. Eukaryote targeted metagenomic approach was used to analyze uncultured pico-prymnesiophytes sorted out by flow cytometry from subtropical North Atlantic waters (Florida Straits, the Sargasso Sea, the Pacific Ocean). Using 18S rRNA gene analysis, the evolutionary history and ecology of pico-prymnesiophyte was examined wherein a vast majority of sequences were from uncultured prymnesiophytes. This showed that picoprymnesiophytes belonged to broadly distributed uncultivated taxa. On an average, picoprymnesiophytes form 25% of global picophytoplankton biomass, with differing contributions in five biogeographical provinces spanning across tropical to subpolar systems (Cuvelier et al. 2010).

14.2.1.2 Reconstruction of Microbial Genomes

A novel insight into community structure was provided by Tyson and coworkers (2004) by first reconstructing the multiple genomes directly from a natural sample of acid mine drainage biofilm by random shotgun sequencing. In the conventional shotgun sequencing approach, all shotgun fragments are derived from clones of the same genome but in this modest approach the shotgun fragments were derived from multiple genomes (Tyson et al. 2004).

Acid mine drainage (AMD) is a worldwide environmental problem driven by microbial activity that leads to extremely acidic outflows from metal mines. The acid is produced by oxidation of sulfide minerals that are exposed to air as a result of mining activity. In order to understand the mechanisms by which the microbes tolerate the extremely acidic environment and to evaluate how the tolerance mechanisms affect the geochemistry of the environment, reconstruction of microbial genomes was done so as to explore the distribution and diversity of metabolic pathways involved in AMD (Allen and Banfield 2005; Tyson and Banfield 2005; Ram et al. 2005; Tyson et al. 2004). Metagenomic DNA was extracted from biofilm of AMD and sheared into small fragments to construct a small insert plasmid library (average insert size 3.2 kilobases (kb)) for random shotgun sequencing. A total of 76.2 million base pairs (bp) of DNA sequence were generated from 103,462 high-quality reads (averaging 737 bp per read) and the shotgun data set was assembled with JAZZ, a whole-genome shotgun assembler. This modest sequencing effort resulted in the construction of complete genome of

(a) *Leptospirillum* group II bacteria (*L. ferriphilum* and *L. ferrooxidans23*)
(b) *Ferroplasma* group II archaeon (uncultured/novel)

(c) *Leptospirillum* group III bacteria (uncultured)
(d) *Ferroplasma* group I archaeon

Not only were the genomes reconstructed but their metabolism was also reconstructed and their role in the function of the community was also understood. Analysis of the gene complement for each organism revealed the pathways for carbon and nitrogen fixation and energy generation, and provided insights into survival strategies in an extreme environment (Tyson et al. 2004).

14.2.2 Biodiscovery

14.2.2.1 Proteorhodopsin Function and Phylogeny

The most dramatic discovery from metagenomics to date, sequencing of a clone isolated from seawater that was initially identified because it carried a bacterial 16S rRNA gene, revealed a sequence with high similarity to bacteriorhodopsin genes (Beja et al. 2000a). This provided the first indication that rhodopsins are not limited to the archaea, as previously thought. Subsequent heterologous expression of the bacteriorhodopsin gene in *E. coli* produced a functional biochemical characterization of the protein, completing the full spectrum of studies that link phylogeny to function (Schloss and Handelsman 2003).

Discovery of the rhodopsin-like photoreceptors in marine Bacteria exemplifies the type of biological surprise that can be revealed through metagenomic analysis. Previously, rhodopsins had been found only in archaea, not in members of the domain Bacteria. Beja and coworkers (2000a) sequenced a 130-kb fragment that contained the 16S rRNA operon of an uncultured γ-Proteobacterium and discovered a bacteriorhodopsin, which indicated a novel taxon of marine phototroph (Riesenfeld et al. 2004). Bacteria that harbour proteorhodopsin variants are widespread and also the class of proteorhodopsins previously observed, is a small subset of the total proteorhodopsin diversity which has been proved by the work of Venter and coworkers (2004) using a shotgun sequencing approach.

14.2.2.2 Gutless Worm

The inability to cultivate most host-associated microbe associations hampers our understanding of the intricate interactions. Comprehensive analysis of the symbiotic microbial community in the eukaryotic host *Olavius algarvensis* was performed by metagenomic approach. *Olavius algarvensis* is a gutless oligochaete which belongs to an unusual group of marine worms having symbiotic association with a highly specific consortium of phylogenetically diverse bacteria. This symbiosis has lead to the complete reduction of the worm's digestive and excretory systems. Two nearly complete and two partial genomes of the oligochaetes' predominant symbionts were assembled using shotgun sequencing of a bacteria-enriched sample combined with nucleotide-signature based binning. Metabolic pathway reconstruction from

the sequenced genomes revealed that all the four symbionts are capable of autotrophic carbon fixation and provides multiple sources of organic carbon to their host as two of them are sulfur-oxidizing and two sulfate-reducing bacteria (Giere and Erseus 2002).

14.2.2.3 Human Microbiomics

Human microbiomics is an emerging discipline which deals with micro-organisms that live in and on humans and their impact and their role in human physiology and human health. The genomes of the microbes that live in and on humans make up the "human microbiome" and; these provide traits that humans do not have. Therefore, human beings are considered to be super organisms with trillions of associated microorganisms (Jones et al. 2008). The human microbiota is expected to outnumber human cells at least by one order of magnitude and is composed of more than 1,000 different species level phylotypes. The total number of microbial genes may exceed the total number of human genes by two orders of magnitude (Rajendhran and Gunasekaran 2009; Qin et al. 2010; Lee et al. 2010).

Microbes are reported to be living in plenty in various human body parts like skin, oral cavity, oesophagus, stomach, colon, vagina etc. and this microbial diversity has been revealed by the sequence analysis of 16S rDNA genes amplified from the metagenomic samples from various human body parts. The core microbiome was shown to exist at a level of genes rather than at organismal level. It has opened way for new thoughts that we must try to study the link between host metabolism and gut microbiota genes rather than looking at species identification (Rajendhran and Gunasekaran 2009).

The human metagenome-based 16S rRNA gene analyses have also been used to detect uncultivated organisms that cause disease (Gao et al. 2007). The first novel pathogen to be identified by sequence-based method was *Rochalimaea henselae* (redesignated as *Bartonella henselae*), an organism responsible for bacillary angiomatosis (cat scratch disease) (Weng et al., 2006). *Ehrlichia chaffeensis* causing a febrile illness associated with tick bites and *Tropheryma whipplei* causing the Whipple's disease are other examples of pathogens identified using this approach (Gao et al. 2007).

14.2.2.4 Microbes and Obesity

Microbes decide the health and predisposition to various non-infectious diseases of humans by the genes encoded as in case of obesity; a strong association between the microbiota and obesity was revealed by the metagenomic sequence data of faecal specimens from obese and lean individuals (Ley et al. 2005). This is an interesting discovery possible with metagenomics which showed that certain individuals are predisposed to obesity as they have gut microbiota that has increased capacity to harvest energy from given diet as compared with the lean individuals.

Reports have shown that more than 90% of the colonic bacteria in humans and mice are Firmicutes and Bacteroidetes belonging to bacterial domain (Rajendhran and Gunasekaran 2009) and it was observed that the percentage of bacteroidetes reduces (50%) with a proportional increase in Firmicutes in obese mice as compared with lean mice.(Ley et al. 2005).

This was further complemented by a transplantation experiment by Turnbaugh and coworkers (2006) that had colonized germ-free mice with an obese microbiota which resulted in a significantly greater increase in total body fat than colonization with a lean microbiota.

14.2.3 Bioprospecting

The fact that most of the biocatalysts employed for biotechnological or industrial purposes are microbial in origin however so far we have not been able to tap more than 5% of the genetic and metabolic resources (MacNeil et al. 2001). Therefore, the amount of metabolic diversity present in nature to harness novel genes encoding is vast which could be explored with the help of metagenomic techniques. Metagenome represents the genomes of uncultured microbes as a rich source for isolation of many novel genes. Several different laboratories have successfully isolated novel genes encoding different enzymes and secondary metabolites from microbial communities and their metagenomes without cultivation of the microbes (Beja et al. 2000a; Henne et al. 1999; 2000; MacNeil et al. 2001; Rondon et al. 2000). The microbial niches studied have been highly diverse and ranged from moderate environments, such as river soil (Henne et al. 1999), to rather extreme environments, like the deep sea (Beja et al. 2000a). Already, the novel antibiotic, antibiotic resistance, vitamin and bioremediating genes have been isolated with metagenomic approach (Gillespie et al. 2002; Handelsman 2004; Zeyaullah et al. 2009).

The list of genes such as biocatalyst, antibiotics isolated by metagenomic technique is quite impressive and for easy comprehension has been presented in Table 14.2.

14.3 Metagenomic-Techniques

14.3.1 Sampling Sites and Enrichment of Microbes

Metagenomics aims at studying yet to be cultivated organisms to understand the true diversity of microbes, their functions, cooperation and evolution, in environments such as soil, water, ancient remains of animals, or the digestive system of animals and humans (Huson et al. 2009). Various environments for metagenomic DNA extraction could be broadly classified as biotic and abiotic habitats. Biotic habitat comprises of living sampling sites like animals, plants and insects. Animals are

Table 14.2 Bioprospecting of different genes from varied habitats by metagenomic approach

S.No	Name of Gene	Vector/Host	Methodology	Site	References
1	Glycerol dehydratase Diol dehydratase	*E. coli*/Plasmid	PCR Based amplification (sequence driven) Screening of genomic library constructed	a) Sediment if river grone (Germany) b) Sugar beet field gothein – gon (Germany) c) Sediment soil solar lake Egypt	Knietsch et al. (2003)
2	Type I polyketide synthase	–	Genomic library & functional Screening	Soil	Osburnis and his group cited from Handelsman (2004)
3	Oxido- reductases	*E.coli DH5ά*/plasmid pblue script Sk+	Genomic library & functional Screening	Sediment 1. River Grone 2. Sugar beet field (Germany) 3. Solar lake Egypt 4. Sediments from gulf Eilol (Israel)	Knietsch et al. (2003)
4	Violacein, Deoxyviolacein	*E. coli*/cosmid	Genomic library	Soil	Brady et al. (2002)
5	Amidase	*E. coli*/cosmid	Genomic library	Soil	Knietsch et al. (2003)
6	Turbomycin	*E. coli*/BAC	Genomic library	Soil	Gillespie et al. (2002)
7	Bacteriorhodpsin, Photeorhodpsin	Genomic library	Genomic library	Ocean	Beja et al. (2000b)
8	Biotin	*E. Coli*/plasmid	Genomic library and functional screening	Forest Soil, Agricultural site, Horse excreme, Meadow, Beach- Germany, Naent hood in oregon	Entcheva et al. (2001)

9	Lipolytic activity	Lipolytic activity *E. coli* DH5ά/ pblue script Sk+	Environmental libraries and functional screening	1. Meadow near north Germany 2. Sugar beet field Germany. Mieme river Germany	Henne et al. (2000), Rondon et al. (2000) , Bell et al. (2002), Voget et al. (2003), Rhee et al. (2005), Jiang et al. (2006), Hardeman and Sjoling (2007), Wei et al. (2009)
10	Nitrilase	*E. coli*/λ-*ZAP* -	Genomic library	Various samples	Robertson et al. (2004)
11	Cellulase	Cosmid		1. Rabbit cecum contents 2. Buffalo rumen 3. Aquatic community and soil Germany	Feng et al. (2007), Duan et al. (2009), Pottkämper et al. (2009)
12	Amylase	Plasmid		Soil and compost from the surface layer of a private garden	Lammle et al. (2007)
13	Protease	Plasmids		Compost soil (Germany), soil from mining shaft (Germany), and mixed soil sample (Germany, Israel, and Egypt)	Waschkowitz et al. (2009)
14	Esterase	Fosmids		1. Desert soil (Antarctica) 2. Surface water microbes from Yangtze river China	Heath et al. (2009), Wu and Sun (2009)

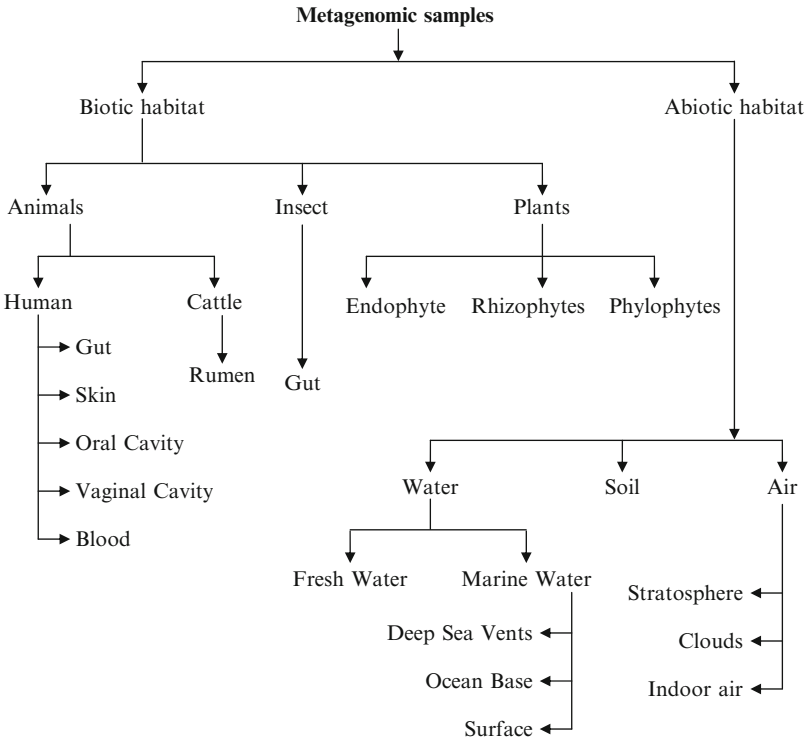

Fig. 14.3 Habitats on which a metagenomic approaches has been applied

the reserviors of the microbes which can either be present in rumen of cattle or on the skin and in blood, gut, oral cavity and vaginal cavity of humans. Plants represent a unique kind of environmental niche and serves as complex habitats like rhizosphere, phylosphere and endosphere for colonization by different kinds of microbes. Rhizosphere represents the area surrounding the root directly influenced by microbes (Pandey and Palini 2007) whereas phylosphere is the surface of leaves colonized by beneficial microbes (Kadivar and Stapleton 2003). Endophytes reside inside the plant tissue and the region colonized by them is referred to as endosphere (Backman and Sikora 2008). In few plants, microbes are also found inhabiting particular sites such as nodules and leaf galls. In addition, microbes are also known to chew on the nitrogen-based fertilizers applied to the crops. Much of this fertilizer is not directly used by the plants but is instead consumed by the microbes and may be lost forever from the plant food cycle (Edwards 2009). Different types of environment from where metagenome can be isolated are summarized in Fig. 14.3. Metagenomic studies have been done in various habitats, including sea water (Venter et al. 2004), ice cores (Bidle et al. 2007), deep mine communities (Edwards et al. 2006) and also from the organisms, which harbour various symbionts, such as unknown and

unculturable bacteria, protozoa or viruses. For example, the symbiont communities of honey bees (Cox-Foster et al. 2007), the guts of mice (Turnbaugh et al. 2008) and humans (Booijink et al. 2007), marine sponges (Schmitt et al. 2007) and plant-rhizobacteria (Kennedy et al. 2007) have revealed many new symbiont taxa (Weihong et al. 2009).

Water, soil and air comprises the abiotic habitat which are flooded with microbes and they are fundamental constituents of our soil, turning over the dead vegetable and animal matter and returning the nutrients therein back into the soil (Edwards 2009). Different marine environments have been explored for total metagenomic analyses like ocean surface waters, mesopelagic waters, the deep sea, water columns and sea subfloor sediments (Kennedy et al. 2010). Microbes are tenacious, able to survive in seemingly inhospitable environments. Recently, bacteria have been found living inside rocks deep in the Earth; these remarkable creatures use uranium radiation instead of sunlight or heat as an energy source. Microbes have adapted to extremes of acidity or temperature as well; some can live in solid ice at or below 0°C, forming small channels with highly-concentrated solutes that prevent them from freezing and allow them to move around.

Air is another source of metagenome as airborne microbes are often attached to dust particles or water droplets from sneezes and coughs. On evaporation of water in aerosols, the microbes become droplet nuclei and clumps and thus can stay airborne and drift with air flows (Tringe et al. 2008). Low concentrations of airborne microbes often challenge their metagenomic extraction so such particles enriched with microorganisms can be collected by sampling large volumes of air through air handling units (AHU) in modern building ventilation systems.

14.3.1.1 Enrichment of Microbes

Enrichment of microorganisms with special traits and the construction of metagenomic libraries by direct cloning of environmental DNA have great potential for identifying genes and gene products for biotechnological purposes. Enrichment can be of two types: one by enrichment in laboratory and other by in-situ enrichment before isolation. In laboratory, enrichment could be done by supplementing the environmental samples with some specific substrate or media for selective growth of desired microbes whereas in-situ enrichment approach involves the enrichment of environmental matrix at its own sampling site followed by DNA extraction. Both of these can be put in metagenomics as the first case deals with cultivated metagenome and later with cultivable and yet-to-be cultured microbes.

The first approach comprises the construction of metagenomic libraries by direct extraction and cloning of metagenomic DNA from environmental samples which can be screened for the targeted genes. In one example, enrichment technology and metagenomics was combined for the identification of a large number of diverse genes which confer the ability to oxidize short-chain polyols or reduce the corresponding carbonyl compounds. The resulting DNA libraries were screened for the presence of genes conferring a carbonyl compound-producing phenotype on

Escherichia coli ECL707 during incubation in the presence of short-chain (C2 to C4) polyols with adjacent hydroxyl groups (Knietsch et al. 2003). It has been reported that the number of positive clones in a screen can be increased by with environmental samples which exhibit an enrichment of microorganisms containing the desired activity for library production (Daniel 2002), though the total microbial diversity assessed will decrease in this approach.

In the second approach, selective medium can be employed for the culture enrichment by growing the target microorganisms. Out of all the inherent selection pressures which are based on nutritional, physical or chemical criteria, substrate utilization is most commonly employed. For example, culture enrichment on carboxymethylcellulose resulted in four-fold enrichment of cellulase genes in a small insert expression library (Rees et al. 2003).

Enrichment culture on agar plates was exploited for metagenomic isolation of genes encoding a variety of enzymes like agarases, amylolytic enzymes, cellulases, and lipases. The selected cosmid clones were sequenced in detail which resulted in identification of the sequences of four conserved clusters encoding agarases, a cluster of two lipase genes, and many other enzymes with high biotechnological potential (Voget et al. 2003). The poor quality of the isolated DNA hinders the construction of environmentally derived DNA libraries with large inserts. This has led to the isolation of DNA from the metagenome of a microbial community after precultivation in the laboratory. Laboratory enrichment cultures are expected to have only limited biodiversity but this technique has proven to be highly efficient for rapid isolation of large DNA fragments for cloning of operons and genes with great biotechnological value. Additionally, laboratory enrichment allows pre-selection of microbes that already carry the desired traits, resulting in high frequency of gene detection and isolation (Cowan et al. 2005).

The limitation of this technique is that enrichment of particular trait leads to the loss of microbial diversity as in this approach the enzymes and the corresponding genes are recovered from the identified organisms. In this manner, a large fraction (>99%) of the microbial diversity in an environment is lost due to difficulties in enriching and isolating microorganisms in pure culture. While pre-enrichment increases the likelihood of finding genes that encode the target trait, this must be balanced against the concurrent decrease in genomic diversity. It is also important that the pre-enrichment step may limit the chances of finding an enzyme that displays optimal activity outside the range of conditions chosen for the pre-enrichment (Elend et al. 2006).

14.3.2 Isolation, Enrichment and Normalization of Metagenomic DNA

Cell recovery and direct lysis are the two principal strategies for the recovery of metagenomic DNA and its extraction is a compromise between the vigorous extraction and the minimisation of DNA shearing along with less coextraction of inhibiting

organic acid contaminants. Mechanical bead beating has been shown to recover more diversity compared with chemical treatment (Cowan et al. 2005). However, a major difficulty associated with the methods employed is related to contamination of purified DNA with polyphenolic compounds which are copurified with the DNA. These compounds are difficult to remove, and it is well known that polyphenols also interfere with enzymatic modifications of isolated DNA.

14.3.2.1 Enrichment

The pre-enrichment of the sample provides an attractive means of enhancing the screening hit rate by applying one of several enrichment options ranging from whole-cell enrichment, to the selection and enrichment of target genes and genomes. For example, the eukaryotic cell population was effectively removed in the Sargasso Sea genome sequencing project by size-selective filtration (Cowan et al. 2005).

Gene enrichment could be done using various enrichment strategies like Suppressive subtraction hybridisation (SSH) and Differential expression analysis (DEA) (Cowan et al. 2005). SSH is a powerful technique for specific gene enrichment which identifies genetic differences between microorganisms. In this approach, adaptors are ligated to the DNA populations and DNA fragments which are unique to each DNA sample are selected by subtractive hybridization and one can thus analyze genetic differences between two closely related bacteria. Recently, this technique has been used to identify differences between complex DNA samples from the rumen of two different animals (Galbraith 2004).

DEA is a particularly effective enrichment tool which was successfully applied to identify bacterial genes upregulated in the absence of iron (Cowan et al. 2005). This approach compares the expression profile of metagenomic sample, pre- and post- exposure to a specific substrate or xenobiotic which identifies the expression of genes up-regulated for the specific activity.

In viral metagenomic DNA analyses, the presence of free and cellular DNA hinders the DNA isolation. The viral DNA signal will be lost if the free DNA is not removed and the presence of any cellular contamination will overwhelm the viral signal as the average viral genome (~50 kb long) is about 50 times smaller than the average microbial genome (2.5 Mb). A combination of differential filtration with tangential flow filters (TFF), DNase treatment and density centrifugation in caesium chloride (CsCl) is used to separate the intact viral particles from the microorganisms and free DNA (Edwards and Rohwer 2005).

14.3.2.2 Normalisation

An even representation of the population's genomes within the sample is not found when total DNA is extracted directly from environmental samples as rare organisms will contribute a relatively low proportion and the genome population might be overshadowed by a limited number of dominant organisms. This could lead to a selective bias in downstream manipulations such as PCR.

The selective bias can be partially resolved by means of experimental normalisation (Short and Mathur 1999) which includes separation of genotypes by CsCl gradient centrifugation in the presence of an intercalating agent, such as bis-benzimide, for the buoyant density separation of genomes based on their % G and C content. Equal amounts of each band on the gradient are combined to represent a normalised metagenome.

Normalisation can also be achieved by denaturing fragmented genomic DNA, and re-annealing under stringent conditions (e.g. 68°C for 12–36 h). Abundant ssDNA will anneal more rapidly to generate double-stranded nucleic acids than rare DNA species. Single-stranded sequences are then separated from the double-stranded nucleic acids, resulting in an enrichment of rarer sequences within the environmental sample (Short and Mathur 1999).

14.3.3 Analysis of Isolated Metagenomic DNA for Taxonomic and Metabolic Diversity

Metagenomics has been defined as function based or sequence based cultivation independent analysis of the collective microbial genomes, present in a given habitat (Simon et al. 2009; Riesenfeld et al. 2004). Analysis of metagenomic bounty basically depends upon two types of screening procedures: function-based and sequence-based approach. Function based screening is based on construction of metagenomic expression libraries which are further screened for target enzyme activities. After an active clone is identified, the sequence of the clone is determined, the gene of interest and its respective products are further analyzed, and explored for their biotechnological potential (Craig et al. 2010; Simon and Daniel 2009; Schmeisser et al. 2007; Streit and Schmitz 2004; Uchiyama and Miyazaki 2009). The advantage of directly screening for enzymatic activities from metagenome libraries is that the sequences and enzyme activities are functionally guaranteed and researchers access previously unknown genes and their encoded enzymes (Fig. 14.4).

In sequence based approach, target genes are amplified from metagenomic DNA using polymerase chain reaction with conserved sequences as primers and are cloned in the appropriate expression system (Simon and Daniel 2009; Streit and Schmitz 2004). The sequence-based metagenomics approach relies on the prior knowledge of proteins possessing the activity of interest, and the screening is performed toward the genes that are predicted to encode proteins with specific function. Prior knowledge regarding specific type of a protein or a functional pathway can be easily obtained due to the availability of vast gene databases, including metagenomic databases that continue to grow exponentially (Kyrpides 2009).

14.3.3.1 Sequence Based Screening of the Metagenomic Bounty

Sequence-driven analysis relies on the use of conserved DNA sequences to design hybridization probes or PCR primers to screen metagenomic libraries for clones

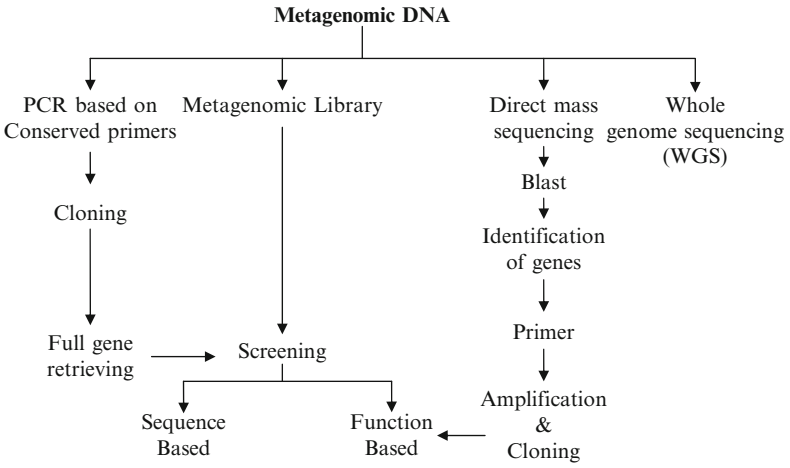

Fig. 14.4 Metagenomic approach for discovery of genes

that contain sequences of interest (Schloss and Handelsman 2003). Target genes are identified either by PCR-based or hybridization-based approaches employing primers and probes derived from conserved regions of known genes and gene products (Daniel 2005; Handelsman 2004). Thus, only genes harbouring regions with similarity to the sequences of the probes and primers can be recovered by this approach. The sequence-based metagenomics approach relies on the prior knowledge of proteins possessing the activity of interest, and the screening is performed toward the genes that are predicted to encode proteins (Chistoserdova 2010). The sequence-based screening approach is limited to the identification of new members of known gene families as dependent on known conserved sequences, and cannot uncover non-homologous enzymes. Although some members of the field view this approach as too undirected to yield biological understanding, others stress that there is so little known about some divisions of Bacteria that any genomic sequence is helpful in guiding the design of experiments to reveal their biology (Schloss and Handelsman 2003). The advantage of this screening strategy is the independence on gene expression and production of foreign genes in the host library (Lorenz et al. 2002). In addition, sequence-driven screening is not selective for full-length genes and functional gene products thus can disclose target genes, regardless of gene expression and protein folding in the host, and irrespective of the completeness of the target gene's sequence.

Several novel functional enzymes have been recovered by employing sequence driven approaches such as chitinases, alcohol oxidoreductases, diol dehydratases, and enzymes conferring antibiotic resistance (Simon et al. 2009). Two novel glycopeptide-encoding gene clusters were isolated from desert soil by a PCR-based screen which

was important for the development of novel glycopeptides and novel glycopeptides analogs, which can serve as substitutes of currently used antibiotics such as vancomycin (Banik and Brady 2008).

Genes encoding polyketide synthases (PKSs) and peptide synthetases, which contribute to synthesis of complex antibiotics, are two hotly pursued examples. The PKSs are modular enzymes with repeating domains containing divergent regions which are flanked by highly conserved regions. The conserved regions have provided the basis for designing probes to identify PKS genes among metagenomic clones (Courtois et al. 2003). Mixing and matching PKS domains from different sources has yielded new antibiotics, stimulating interest in the discovery of new PKS genes.

New approaches are directed toward identifying sequences that are unique to uncultured microorganisms or those specific to a particular environment. These methods involve:

1. Profiling clones with microarrays that identify previously unknown genes in environmental samples,
2. Subtractive hybridization to eliminate all sequences that hybridize with another environment or subtractive hybridization to identify differentially expressed genes
3. Genomic sequence tags which will enhance the efficiency of screening and aid in identifying minor components in communities and genes that define community uniqueness. (Riesenfeld et al. 2004)

Metagenomic diversity can also be accessed using sequence based screening by sequencing the phylogenetically relevant genes like 16S/18S rRNA and ITS region. So the function based screening cannot help us identifying these regions.

Direct Analysis by Sequencing

The recent development of next generation sequencing technologies, which do not require cloning or PCR amplification, and can produce huge numbers of DNA reads at an affordable cost, has boosted the number and scope of metagenomic sequencing projects.

The original Human Genome Project cost $3 billion (U.S.) and was completed in a little over 13 years. Currently, commercial sequencing of a human genome costs about $350,000 and takes about 6 months. Our understanding of the world around us, and particularly microbes that inhabit it has been revolutionized by cheaper, higher-throughput sequencing technique namely “Pyrosequencing” which has dramatically affected all aspects of sequence-based biology (Edwards 2009).

Pyrosequencing is the most advanced technology of the next-generation sequencers which uses an enzymatic reaction to generate a pulse of light each time a particular letter is read from a DNA strand. In this technique, a single piece of DNA is attached to a spherical bead 28 μm in diameter which could easily fit in 54 μm wells in glass plates thus allowing only single bead to fit in a well and ensuring only one piece of

DNA is read at a time. The current capacity of this machine, called a 454 FLX, is to sequence about 200 consecutive letters from as many as 500,000 pieces of DNA in just a few hours.

Analysis After cloning

Cloning by PCR

Cloning by PCR is one of the applications of metagenomics to gene discovery. Metagenomic library are constructed by isolating metagenomic DNA from the selective environment and amplifying the target gene using specific primers. The amplicons thus obtained are further ligated in appropriate vector and transformed into the suitable host to form the metagenomic library. The vectors can be selected depending on the size of fragments (amplicon)for cloning, plasmids (optimal range of DNA fragments 0.5–2 kb, upper limit, ~10 kb), bacteriophages (7–10 kb, ~20 kb), cosmids or fosmids (35–40 kb; ~45 kb) and bacterial artificial chromosomes (BAC, 80–120 kb, ~200 kb). The field of metagenomics has benefitted significantly from the large insert capacity vectors like cosmid, BAC and YAC cloning systems as they are ideal for studying genome organizations of unculturable microorganisms in the environment (Xu 2006). Thus, either single genes and primary gene products, or secondary metabolites could be targeted from the expression of complete operons depending on the choice of vector and host.

Sets of putative gene fragments have been amplified from metagenomic DNA extracts using primer sets specific to bacterial lipase and nitrile hydratase α-subunit sequences. The amplicons are cloned and sequenced and are aligned with known databases. The nitrile hydratase genes show very high homology to each other and known full-length genes whereas the putative lipase gene amplicons show very low levels of homology with known sequences (Cowan et al. 2004).

Inverse PCR (I-PCR), a method that can be used for cloning the upstream and downstream flanking regions of known sequences for the amplification of known gene families, has found little utility when applied to metagenomes. The reason could be the potential increased complexity and low copy numbers of target sequences in the metagenome which could be overcome by Pre-amplified I-PCR or PAI-PCR (Kennedy et al. 2010). In PAI-PCR, pre-amplification of the template DNA is done, prior to I-PCR, in order to enrich the target DNA sequences by isothermal DNA amplification, for example, by using phi29 DNA polymerase, on the basis of rolling-circle amplification (RCA) (Rector et al. 2004) or multiple displacement amplification (MDA) (Gonzalez et al. 2005). The selective amplification of a specific DNA region by the isothermal amplification is generally difficult because the specificity of the isothermal amplification is not high, especially when the reaction temperature is low or the initial amount of target DNA is small. To reduce nonspecific amplification derived from miss-priming, primers containing locked nucleic acids (LNAs) may be useful. Locked nucleic acids are DNA analogues in which the furanose ring in the sugar-phosphate backbone is chemically locked. Locked nucleic

acids obey the Watson–Crick pairing rules, but have an increased specificity and a high affinity to complementary DNA (Yamada et al. 2008). This pre-amplified I-PCR (PAI-PCR) method increased the sensitivity of PCR almost 10,000 times compared with the standard IPCR in model experiments using *Escherichia coli* (Yamada et al. 2008). This approach could also be useful for marine DNA-samples where the amount of extracted DNA is low (Kennedy et al. 2010). Using PAI-PCR, DNA glycosyl hydrolase genes have been identified from vermiform appendices of horses and termite guts. The flanking sequences of the target genes were amplified and cloned successfully using PAI-PCR, whereas standard I-PCR resulted in no amplification (Yamada et al. 2008).

Metagenomic PCR amplification methods being used successfully to identify families of homologous genes, suffer from the limitation that the primary PCR generates only partial gene sequences. Full-length sequences are subsequently obtained by hybridization screening of a complete metagenomics library or by genome walking (Cowan et al. 2004). Morimoto and Fujii (2009) conducted a PCR-DGGE targeting benA and tfdC, which encode the alpha subunits of benzoate 1, 2-dioxygenase and chlorocatechol 1,2-dioxygenase, respectively. The complete functional genes were recovered by metagenome walking (Morimoto and Fujii 2009). In contrast to PCR-based techniques, the subtractive hybridization approach allows the recovery of multiple gene targets in a single reaction. For example, recovery of multicopper oxidases from metagenomic DNA was done by subtractive hybridization magnetic bead capture in which conserved regions of the target genes are amplified from a metagenomic DNA sample by PCR using biotinylated degenerated primers. The resulting amplified target gene fragments are immobilized on streptavidin-covered magnetic beads, which are then used as probes for capturing the full-length genes from metagenomic DNA by hybridization. (Meyer et al. 2007) In a few cases, microarray technology has been employed for sequence-driven screening of metagenomic DNA and libraries. A recent example is the recovery of genes encoding blue light-sensitive proteins (Pathak et al. 2009).

Culture-independent techniques such as 16S ribosomal RNA gene coding DNA (16S rDNA) analysis and metagenomic sequencing provide a less biased perspective on environmental microbes because DNA is sampled directly from the environment. AHU filtration strategy was employed for air sample collection and both 16S rDNA and metagenomic analyses were done to characterize the airborne biological diversity in an indoor urban environment (Tringe et al. 2008).

Gene-specific PCR has two major drawbacks. First, the functionally similar genes resulting from convergent evolution are not likely to be detected by a single gene-family-specific set of PCR primers as the design of primers is dependent on existing sequence information and second, only a fragment of a gene will be amplified by gene-specific PCR thus requires additional steps to access the full-length genes like hybridization in which amplicons can be labelled as probes to identify the putative full-length gene and genome walking (Cowan et al. 2005).

Sequence-dependent bias could be decreased using methods requiring only one gene-specific primer as compared to standard twin-primer PCR amplification procedures for example, the use of immobilised oligonucleotides designed to target a

specific gene fragment or consensus sequence by affinity binding which is used for recovery of polyA RNA cDNA library construction (Cowan et al. 2005).

Metagenomic Library

Construction of metagenomic library from soils or sediments is one of the various metagenomic strategies that are used for targeting genes having specific catalyst characteristics such as substrate range or temperature and pH optima. Soils or sediments are known to harbour a high level of microbial diversity and wide diversity of biocatalysts (Schmeisser et al. 2007).

The basic steps of metagenomic library construction include generation of suitable sized DNA fragments, cloning of fragments into an appropriate vector and screening for the gene of interest and have been extensively and successfully used for over three decades. DNA fragmentation is a significant problem when constructing metagenomic libraries as the vigorous extraction methods which are required for high yields of environmental DNA often results in excessive DNA shearing. This highly sheared DNA (e.g. 0.5–5 Kbp fragments) cannot be restricted to generate ligatable sticky ends without significant loss of the total gene complement so an alternative approach uses blunt-end or T–A ligation to clone randomly sheared metagenomic fragments (Wilkinson 2002).

Random shotgun sequencing approaches involves direct cloning of metagenome without prior sequence knowledge for data generation. Venter et al. (2004) were the first to apply whole genome shotgun sequencing to samples of the Sargasso Sea in order to characterize the microbial community and identify new genes and species. Whole metagenome shotgun sequencing approaches have been mostly employed for the cloning and sequencing of microbial DNA from marine environments. This involves the generation of small-insert DNA clone libraries, and their subsequent analysis using Sanger dideoxy sequencing which generates sequences that can then be used to query the known databases for function or phylogenetic relationship. This approach can give read lengths ranging from 600 to 900 bp in length, which can be extended through the entire fosmid clone. (Kennedy et al. 2010)

Microarrays represent a powerful high-throughput system for analysis of genes. They are typically used to monitor differential gene expression, to quantify the environmental bacterial diversity and catalogue genes involved in key processes (Cowan et al. 2005). Microarray technology could also be used for the pre-selection of genes in metagenomic libraries before shotgun sequencing, thereby reducing the sequencing burden and reducing the proportion of sequences unassigned by database sequence similarity searches (Sebat 2003).

Depending on the ability to clone large fragments of metagenomic DNA, various metagenomic libraries are constructed like cosmid, bacterial artificial chromosome (BAC) libraries, fosmid library and phage-display expression libraries (Cowan et al. 2005). The large fragments of metagenomic DNA are able to target the entire functional operons with the possibility of recovering entire metabolic pathways. Cosmid and BAC libraries have been widely used for the construction of metagenomic libraries (Beja 2004; Daniel 2004). Fosmid vectors provide an improved method for

cloning and stably maintaining cosmid-sized (35–45 Kbp) inserts in *E. coli* whereas phage-display expression libraries provide DNA sequences of isolates by affinity selection of the surface-displayed expression product

Metatranscriptomic Library

Analysis of community transcripts isolated directly from the environment or from microcosms is referred to as metatranscriptomics (Chistoserdova 2010). Metagenomic complementary DNA (cDNA) libraries have been constructed from mRNA that has been isolated from environmental samples. Sequencing and characterization of metatranscriptomes has been employed to identify expressed biological signatures in complex ecosystems (Simon et al., 2009).

Constructing libraries derived from environmental mRNA is more challenging than generation of metagenomic DNA libraries due to difficulties associated with isolation of RNA, separation of mRNA from other RNA species, and instability of mRNA (Sjöling and Cowan 2008; Frias-Lopez et al. 2008) but at the same time is advantageous for gene discovery as it requires a much smaller sequence space compared to metagenomic DNA which focuses on the expressed subset of genes (Warnecke and Hess 2009).

14.4 Functional Screening of the Metagenomic Bounty

Construction of metagenome expression libraries can be done by inserting fragmented metagenomic DNA into expression vectors which is further cloned in a suitable host system for examining gene expression. Function-driven screening of metagenomic libraries is not dependent on sequence information or sequence similarity to known genes. Thus, this is the only approach that bears the potential to discover new classes of genes that encode either known or new functions (Heath et al. 2009; Rees et al. 2003). In addition, function-driven screening often requires the analysis of more clones than sequence-based screening for the recovery of a few positive clones (Daniel 2005). The major advantage of a function-based screening approach is that only full-length genes and functional gene products are detected. For a clone to be functionally active i.e., to correctly express an active enzyme, it must contain the complete gene sequence or even a gene cluster (where the gene sequence depends on more than one genetic subunit). This requires selection of suitable vector systems and expression hosts.

14.4.1 Expression Vectors Systems

The expression vector varies with the range of target insert DNA or size of gene required for cloning. For small target genes, plasmids or Lambda expression vectors

with inserts between 2 and 10 kilobase (kb) are used for constructing DNA fragment libraries which are further screened for enzyme expression. Larger target genes having size range between 20 and 40 kb require expression libraries in cosmids and fosmids and up to 100–200 kb in bacterial artificial chromosome vectors.

14.4.2 Expression Hosts

The incapability to discover functional gene products during function-based screens of metagenomic libraries might be a result of the inability of the host to express the foreign genes and to form active recombinant proteins. Many genes from environmental samples may not be expressed efficiently in heterologous hosts due to differences in codon usage, transcription and/or translation initiation signals, protein- folding elements, post-translational modifications, such as glycosylation, or toxicity of the active enzyme. Selection of suitable expression hosts or suitable vector systems containing appropriate transcription and translation-initiation sequences can reduce this limitation.

Although common, *E. coli* host strains have relaxed requirements for promoter recognition and translation initiation but it is not compatible with many environmental genes thus an expression host such as the *E. coli* Rosetta strains (Novagen, Madison, Wisconsin, USA) have been developed that contain the tRNA genes for rare amino acid codons or co-expression of the chaperone proteins, such as GroES, GroEL, and heat-shock proteins. In addition, host systems such as, the yeast *Pichia pastoris*, and bacterial hosts such as *Pseudomonas putida*, *Streptomyces lividans*, or *Bacillus subtilis a*re also suitable for heterologous gene expression (Li et al. 2009).

Enzymatic functions of individual clones can be identified by chemical dyes and insoluble or chromophore-containing derivatives of enzyme substrates which can be incorporated into the growth medium (Daniel 2005; Ferrer et al. 2009; Handelsman 2004). Examples of this simple activity-based approach are the detection of recombinant *E. coli* clones exhibiting protease activity on indicator agar containing skimmed milk as protease substrate (Lee et al. 2007; Waschkowitz et al. 2009) or the detection of lipolytic activity by employing indicator agar containing tributyrin or tricaprylin as enzyme substrates (Hårdeman and Sjöling 2007; Heath et al. 2009; Lee et al. 2006). Clones with proteolytic or lipolytic activity are identified by formation of clearing zone or halo on solidified indicator medium.

14.4.3 Modification of Function-Based Screening Substrate Induced Gene-Expression Screening (SIGEX)

Function-based methods are modified specifically for exploring metagenome libraries as reported by Uchiyama and colleagues (2005) who have developed substrate induced gene-expression screening to rapidly identify clones. SIGEX is based on induced gene expression and can be induced by a target substrate and display

catabolic gene expression. Catabolic operons are often adjacent to cognate transcriptional regulators and promoters that are induced by the substrate. In this approach, an operon-trap expression vector is used which contains the gene for a promoter less green fluorescent protein (gfp). This expression vector was employed for cloning of environmental DNA. If expression of a target gene is induced by the substrate, the gfp gene is co-expressed, and positive clones can rapidly be separated from other clones by fluorescent activated cell sorting (Handelsman 2005; Uchiyama et al. 2005).

14.4.4 Metabolite-Regulated Expression

A similar screening strategy termed metabolite-regulated expression has been employed in which metagenomic clones producing small molecules are identified by a biosensor that detects small diffusible signal molecules, which induce quorum sensing (QS). When a threshold concentration of the signal molecule is exceeded, GFP is produced. Subsequently, positive fluorescent clones are identified by fluorescence microscopy (Williamson et al. 2005).

14.4.5 Heterologous Complementation of Host Strains or Mutants

Simon and coworkers (2009) have reported a new approach that uses specific host strains that require heterologous complementation by foreign genes for growth under selective conditions. Only recombinant clones harbouring the targeted gene and producing the corresponding gene product in an active form are able to grow. DNA polymerase-encoding genes were identified from metagenomic libraries derived from glacier ice using this approach. An *E. coli* mutant was used as host for the metagenomic libraries which carried a cold-sensitive lethal mutation in the 5′-3′ exonuclease domain of the DNA polymerase I. Only recombinant *E. coli* strains complemented by a gene conferring DNA polymerase-activity are able to grow at a growth temperature of 20°C. In this way, a high selectivity of the screen is achieved (Simon et al. 2009).

14.5 Future Challenges

The four pillars that hold the science of metagenomics are, (i) direct Meta- nucleic acid isolation, (ii) high through put sequencing technologies (iii) Heterologous gene expression and (iv) bioinformatics tools to understand meta- data. All four are raw and need strengthening. After initial euphoria over characterization of microbial communities by direct meta genomic isolation, there are number of results that

clearly prove that no single isolation method is suitable for isolation of nucleic acid from all the cells in any environmental samples. Metagenomic isolation is basic to any Metagenome based goal hence isolation and purification of the DNA directly from soil needs to be polished. In conventional genomics the need to look beyond *E. coli* for Heterologous gene expression has been felt and voiced long ago, but for functional expression based screening of metagenomic bounty need for alternate expression hosts is indispensible. The recent development of ultra-high throughput sequencing technologies produces huge numbers of DNA reads at an affordable cost which further requires the new ways of user-friendly and powerful tools for comparative analysis of metagenomic data (Huson et al. 2009). The taxonomical content of metagenome is usually estimated by comparison against DNA and protein sequence databases of known sequences. Metagenomic studies characterize the composition as well as diversity of uncultured microbial communities for which BLAST-based comparisons have typically been used. MEGAN ("MEtaGenome ANalyzer") is a computer program that allows analysis of large metagenomic datasets in which set of DNA reads (or contigs) is compared against databases of known sequences using BLAST or another comparison tool. However, these bioinformatics tool suffers from various limitations like sampling biases, high percentages of unknown sequences, and the use of arbitrary thresholds to find significant similarities which can decrease the accuracy and validity of estimates. GAAS (Genome relative Abundance and Average Size) is a complete software package that implements a novel methodology to control for sampling bias via length normalization, to adjust for multiple BLAST similarities by similarity weighting, and to select significant similarities using relative alignment lengths. (Angly et al. 2009) GAAS provides improved estimates of community composition and average genome length for metagenomes in both textual and graphical formats. A gap is constantly created due to difference in the rates of collecting sequence data using high throughput techniques and interpretation of these sequences. This gap is being bridged by another software tool namely CAMERA project. CAMERA stands for Community Cyberinfrastructure for Advanced Marine Microbial Ecology Research and Analysis project which helps in developing global methods for monitoring microbial communities in the ocean and their response to environmental changes. CAMERA's database includes environmental metagenomic and genomic sequence data, associated environmental parameters, precomputed search results, and software tools to support powerful cross-analysis of environmental samples. The main aim of CAMERA is to create a rich, distinctive data repository and bioinformatics tools resource that will address many of the unique challenges of metagenomics and enable researchers to unravel the biology of environmental microorganisms (Seshadri et al. 2007).

14.6 Conclusions

Soil habitats contain the greatest microbial diversity of all the environments on earth and the power of metagenomics has changed the microbiologists approach to access microbial diversity to a larger extent than that has been viewed in the petridish

(Zeyaullah et al. 2009). Metagenomics can provide the tools to balance the abundance of knowledge attained from culturing with an understanding of the uncultured majority of microbial life. (Handelsman 2004) Metagenomics has unraveled the diversity of many familiar habitats, including deep sea thermal vents; acidic hot springs, temperate, desert, and cold soils; Antarctic frozen lakes; and eukaryotic host organs – the human mouth and gut, termite guts, gutless microbe and plant rhizospheres and phyllospheres (Handelsman 2004).

The potential for application of metagenomics to biotechnology seems endless as it has redefined the concept of a genome, and accelerated the rate of gene discovery (Handelsman 2004). Soil microbial communities are almost unlimited resource of new genes encoding useful products, which could be explored by the power of metagenomics. Thus, metagenomic tools facilitate the recovery of a high amount of new enzymes, antibiotics and other molecules from small fraction of the soil metagenome (Daniel 2005; Simon and Daniel 2009). It can be expected that the number of novel genes identified through metagenome technologies will exceed the number of genes identified through sequencing individual microbes (Streit and Schmitz 2004).

Although considerable progress has been made in the characterization of microbial communities by efficient high-throughput random sequencing which permit cloning-independent and low-cost sequencing analyses of metagenomes, a further improvement of sequencing technologies combined with a reduction in sequencing costs and development of appropriate bioinformatic tools for analysing the enormous amount of data produced is required (Daniel 2005; Simon and Daniel 2009).

A great challenge of metagenomics is to define the origin of a specific metagenomic clone as it is important to define the origin of the clones in terms of the utility of the genes and functional genomics of the uncultured microorganisms to really solve the enigma of culture problem of microorganisms (Lee 2005).

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Chapter 15
Metagenomics of Saline Habitats with Respect to Bacterial Phylogeny and Biocatalytic Potential

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Abstract Metagenomics, an emerging field of research, has been developed over the last several years to assess the genomes of the non-culturable microbes towards better understanding of global microbial ecology and to trap vast biotechnological potential of a given habitat. The basic strategies encompass sequence and functional based approaches. Since it is widely accepted that the majority of the microbes are not cultivable, the not-yet-cultivated microbes represent a shear unlimited and intriguing resource for the development of novel genes, enzymes and other compounds for applications in biotechnology.

One of the hurdles in the way of metagenomics is the extraction of total environmental DNA (metagenome) from a given habitat. We have explored various protocols, in terms of DNA purity, yield and humic acid content, for the isolation of metagenome from various saline soils of Gujarat, to substantiate its applications for further molecular biological work. Diversity based assessment has been elucidated on the basis of 16S rRNA amplicons – DGGE and ARDRA analysis (Molecular Fingerprinting Technique). Metagenomic library constructed from the saline habitats would provide a base to address adaptation strategies and its role in moderate saline and alkaline environment. Beside, the source would also provide a huge and comprehensive platform for capturing novel gene sequences. As an extension of our on-going work on haloalkaliphilic bacteria from the saline habitats of Coastal Gujarat, we have taken alkaline proteases as model system for the assessment of genetic diversity among these habitats by designing degenerate primers with the aid of bioinformatics tools. Successful Cloning and expression of alkaline proteases revealed unidentified gene/s with interesting features.

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Several metagenomic mega projects such as Sargasso Sea, Acid-mine drainage, Human-Microbial Gut are completed worldwide successfully. However, similar efforts have not been focused in context with saline habitats. The initial results hold significance in the light of the fact that although saline environments display enormous microbial biodiversity, it remains largely unexplored. The application of metagenomic strategies embraces great potential to study and exploit the enormous microbial biodiversity present within the saline habitats.

Keywords Haloalkaliphilic bacteria • Alkaline proteases • Metagenomics • Saline habitats

15.1 Introduction

Metagenomics is an emerging approach based on the extensive analysis of the DNA of microbial communities in their natural environment. Studies on metagenomes have revealed vast scope of biodiversity in a wide range of environments, and new functional capacities of individual cells and communities, including complex evolutionary relationships between them (Kennedy et al. 2008). Microorganisms offer huge potential for new biocatalysts for industrial and commercial applications. Of late metagenomic based strategies have recently been employed as powerful tools to isolate and identify enzymes with novel biocatalytic activities from the unculturable component of microbial communities from various terrestrial and aquatic environmental niches. Besides, attention on the diversity and phylogeny of unculturable organisms is also being focused, as marine environment has enormous microbial biodiversity, yet to be explored (Kennedy and Marchesi 2007; Kennedy et al. 2008; Purohit et al. 2010; Siddhpura et al. 2010).

The classical approach to gain access to new biocatalysts is to cultivate microorganisms from soil or any other environmental sample in different media and screen for the desired activity. An alternative method is to isolate DNA directly from the microorganisms present in the soil without prior cultivation (Desai and Madamwar 2007; Xu et al. 2008; Yamada et al. 2008; Chernitsyna et al. 2008; Purohit et al. 2010; Siddhpura et al. 2010). This DNA can be used for construction of DNA libraries and to directly clone functional genes from environmental samples (Raes et al. 2007; Acevedo et al. 2008). Through metagenomics approaches, the genetic information of theoretically all indigenous organisms can be accessed, including the predominant fraction of the organisms that are recalcitrant to cultivation.

Recovery, cloning and expression of environmental DNA without cultivation is a recent approach to analyze the population dyanamics and to explore the potential of microbial communities present in environment (Acevedo et al. 2008). It has been of growing interest to both microbial ecologist and biochemists to look for novel biocatalysts and metabolites (Risenfeld et al. 2004). The metagenomic approaches highlight on the population heterogeneity and phylogenetic status of a habitat, as a whole (Esther et al. 2003). To add impetus to these efforts, bioinformatics based tools and software have been of great significance towards the interpretation of the metagenome (Hoff et al. 2008).

The field of metagenomics, particularly with reference to marine ecosystems, although has vast potential, yet not studied and explored to a great extent. As an extension of our on-going research on haloalkaliphilic bacteria and actinomycetes (Patel et al. 2005, 2006a, b; Gupta et al. 2005; Dodia et al. 2006, 2008a, b; Thumar and Singh 2007a, b, 2009), we have recently begun to explore the saline habitats along the Coast of Gujarat, India, with metagenomics stand point. Our basic objectives are to analyze the phylogenetic status and assess the functional attributes of the organisms dwelling in saline habitats of Coastal Gujarat (Purohit and Singh 2010; Siddhpura et al. 2010).

15.2 Critical Review

The vast diversity of the biosphere's genetic and metabolic potential is being currently looked into with the focus on a staggering number of yet uncharacterized microbes (Mes 2008). It is well accepted that the diversity of microorganisms represented is highly skewed toward those taxa that are amenable to growth under laboratory conditions. This implies that our discovery of microbial genes through cultivation-dependent conventional genome sequencing is equally skewed (Green and Keller 2006) limiting to our understanding of microbial physiology, genetics, and community ecology. One of the possible ways to address this limitation is metagenomics, the culture-independent cloning and analysis of microbial DNA extracted directly from an environmental sample. Recent advancement in the field of metagenomics provided glimpses into the life of uncultured microorganisms (Liles et al. 2008; Mitchell et al. 2008; Vijayanand et al. 2010; Setati 2010).

Microbiology has gone through a transformation during the last 25 years that has altered microbiologists' view of microorganisms and the way we can get access to them. The realization that most microorganisms cannot be grown readily in pure culture (Handelsman 2004; Handelsman 2005; Hugenholt and Tyson 2008), forced microbiologists to question their belief that the microbial world had been conquered. This belief was challenged with the acknowledgment of the extent of our ignorance about the range of metabolic and microbial diversity (Xu et al. 2008; Yamada et al. 2008). In its approaches, metagenomics addresses individual genes and genomes, enabling scientists to study entire genomes in a given community.

Nowadays, the genetic and enzymatic differences across the gradients from 'neutral and pristine' to 'extreme and polluted' environments are well documented. Yet, extremophilic organisms are possibly the least understood because of our limited ability to study and understand their metabolic potential. There are at least two obstacles for reaping the fruit of the microbial diversity of extremophiles: first, in spite of the recent progress in development of new culturing techniques, most extremophiles cannot be cultured using traditional culturing techniques; and second, the problem of low population densities under the conditions hostile to most of the life, which often do not yield enough DNA and reduces the effectiveness of cloning (Castro et al. 2008; Ni et al. 2009).

In the late 1980s, the direct analysis of rRNA gene sequences had shown that the vast majority of microorganisms present in the environment could not be captured by culture-dependent methods. Even with the recent success of novel and high throughput culturing strategies; we are still unable to mimic most microbial environments sufficiently to induce growth of many environmentally relevant microbes. It takes a non-traditional focus on the genomic resource of a dynamic microbial community, rather than on individual strains of microbes or individual genes and their functions (Gilbert 2010).

Diversity of total number of prokaryotic cells presents an enormous (and largely untapped) genetic and biological pool that can be exploited for the recovery of novel genes, entire metabolic pathways and their products. Observations that culturing yields only a fraction of the microbial diversity is evident from microscopic analysis supported by the phylogenetic assessment based on community DNA preparation (Vijayanand et al. 2010; Setati 2010).

The apparent underestimation of true microbial diversity was largely due to the dependence on culture-based enumeration methods. The development of metagenomic technologies over the past 10 years has provided access to much of the prokaryotic genetic information available in environmental samples, independent of culturability.

The tools of classical genomics and microbiology largely rely on isolating individual microbial species in pure cultures. Metagenomics overcome the limitations of classical genomics and microbiology by accessing a community's genome without relying on pure cultures (Green and Keller 2006). Nevertheless, metagenomics does not replace the need of exploring the genetic makeup of individual species or small groups of microbes (Kowalchuk et al. 2006).

In the coming years, approaches of metagenomics would certainly add to our knowledge regarding understanding of the microbial ecology (Cowan et al. 2004). Development of novel techniques and approaches would also add its inputs in the development of this juvenile field. Quite possibly, in the coming years, the metagenomics would likely merge with the understanding of the microbial world. Metagenomics includes a wide variety of novel techniques and approaches, and it is likely that other new methods will be developed subsequently in the field (Fig. 15.1).

Several metagenomic mega projects such as Sargasso Sea, Acid-mine drainage, Human-Microbial Gut are successfully completed worldwide. Among them, Sargasso sea project, could be considered milestone project in the field of marine microbiology, which in total yielded of over one billion base pair of non-redundant sequences. The marine genomic sequencing project aims at sequencing of samples collected over large belt from South Africa to Canada (Ventor 2004).

15.2.1 Extraction of Metagenome (Total DNA)

Metagenomic studies begin with the extraction of total DNA from a particular environment (Mitchell et al. 2008). Mostly metagenomics projects currently focus on the microbes found in the sampled environment that have smaller amounts of

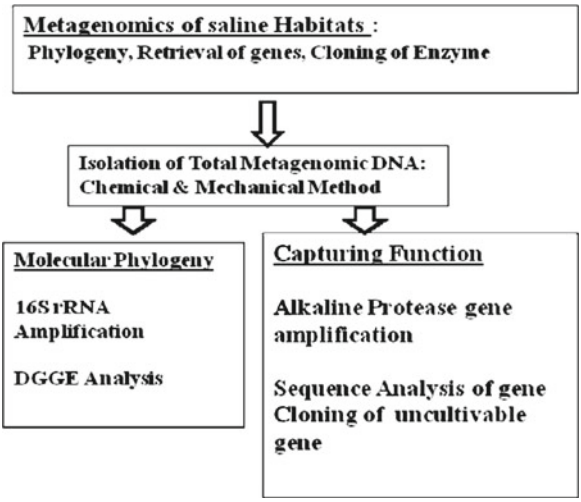

Fig. 15.1 Schematic understanding of studying metagenome from marine environments

DNA, such as bacteria and other microbes which can live in extreme environments (Risenfeld et al. 2004; Sharma et al. 2007). During the last 10 years, number of protocols for DNA extraction from environmental sample have been reported (Kauffmann et al. 2004; Handelsman 2004) and commercial soil DNA extraction kits are also available. These kits and most of the published methods have improved the original direct DNA extraction procedures mainly in terms of DNA yield and quality. The protocols for isolating total DNA from environmental sample could be broadly classified as direct and indirect methods. The variability in the outcome among the methods is viewed with respect to the degree of shearing, purity and quantity of the extracted DNA (Desai and Madamwar 2007; Purohit and Singh 2010).

15.2.2 Approaches and Techniques

The DNA is extracted from a sample followed by the construction of a genomic library containing pieces of the genomes of all the microbes. This metagenome can further be applicable by two approaches (Fig. 15.2):

15.2.2.1 Sequence-Based Metagenomics

Entire informations of genetic sequences could be determined by sequence-based approaches, which reflect DNA profile and addresses population heterogeneity in particular (Tringe and Rubin 2005; Glöckner et al. 2010). Phylogenetic and large-insert metagenomic approaches, provide access to genetic information contained within microbial populations only known to us in the form of specific phylogenetic marker gene sequences (Rondon et al. 2000).

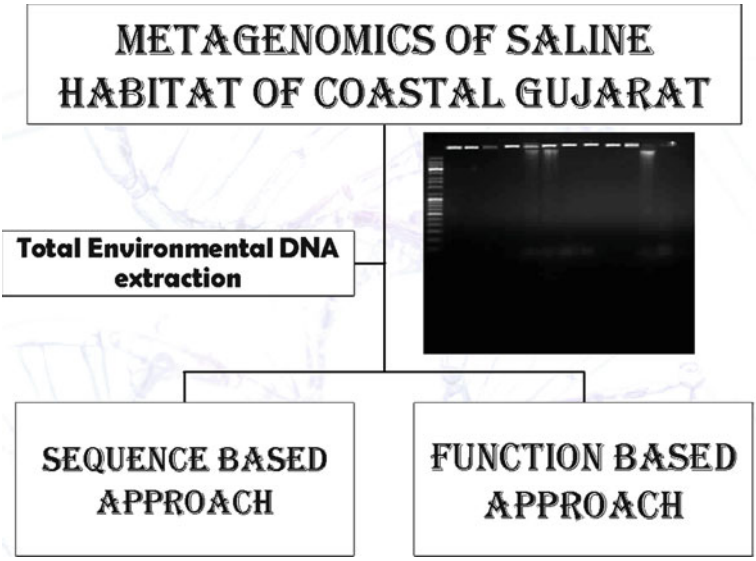

Fig. 15.2 Major approaches in metagenomics

15.2.2.2 Function-Based Metagenomics

It explores and aims at the specific products from the microbes in a community. In function-based metagenomics, researchers screen metagenomic libraries for various functions, such as biocatalysts, vitamins or antibiotic production (Raes et al. 2007). Through this approach, scientists can search and identify the functions that are largely unknown. Similarly, recent technological advances enable to directly extract and identify novel proteins and metabolites (the products of cellular processes) from a microbial community (Jeffrey 2010). Moreover, metagenomics analyses microbial communities as systems that have functional properties beyond individual genes or individual microbes (or even single-taxon populations). Metabolic cascades, for example, can be distributed over different members of multi-taxa communities (Rajendhran and Gunasekaran 2008). Product or activity-driven metagenomic studies are often approached with a more applied perspective in view, exploring useful properties encoded within the metagenome (Raes et al. 2007).

15.2.2.3 Techniques

A wide range of techniques has been employed to gain access to metagenomes, among them shotgun analysis of community genomes is a rather simple exercise (Wooley et al. 2010). Metagenomics, however, has more to offer than merely providing lots of interesting DNA sequence data. It takes a non-traditional focus on the genomic resources of a dynamic microbial community, rather than on individual strains of

microbes or individual genes and their functions. Community genomics perspectives aim to explore how horizontal transfer allows otherwise distantly related organisms. Therefore, metagenomic analyses of microbial communities focus on systems having functional properties beyond the individual genes or individual microbes (Jeroen et al. 2007). Through automated high-throughput methods, its possible to recover and sequence as many clones as necessary (Handelsman 2004; Handelsman 2005).

15.2.2.4 Metagenome Screening

An enormous variety of different biocatalysts or other functional products can be theoretically obtained using DNA extracted from a given environmental sample. An example for the impressive diversity of metagenome-encoded enzymes was provided by Diversa Corporation (San Diego). By fragmenting total DNA from an alkaline marine sample, cloning it into an expression vector, and screening for esterase/lipase activity in an easily cultivable host strain, 120 new enzymes were discovered, falling into 21 protein families (Miller 2008). During the past 5 years, cloning of genes from the metagenome has become the most popular tool for cultivation-independent enzyme discovery, leading to the recovery of a range of new biocatalysts by academic and commercial institutions (Kennedy et al. 2008). While in almost all studies, *E. coli* was used as expression host, there is an example of cloning in a broad host range vector and expression in *Streptomyces lividans* (Tringe et al. 2003; Hugenholt and Tyson 2008). Vector systems used for the cloning of environmental DNA range from small-insert cloning vectors such as plasmids or phage vectors (up to 15 kb inserts) to bacterial artificial chromosomes (BACs) that can harbor as much as 100-kb fragments. While BAC vectors are usually applied when activities are targeted on the expression of large gene clusters (e.g. metabolite formation), small-insert libraries are usually prepared for the screening of single genes or small operons (Gilbert 2010).

However, smaller cloned fragments necessitate larger gene banks required for a comprehensive and comparable coverage of the genetic information, which ultimately leads to more laborious screening procedures. For example, one amylase-expressing clone could be isolated per 450 clones screened using a BAC vector (Gilbert 2010).

15.2.3 Metagenomics Coupled with Protein Engineering

Currently, there are three major approaches in protein engineering: (1) rational design by site-directed mutagenesis techniques; (2) random introduction of point mutations, e.g. by error-prone PCR, mutator strains, UV or chemical means; and (3) recombination of orthologous genes by gene shuffling techniques. Coupling of metagenomic approaches with protein engineering, provides information about important residues, previously unknown for their role in catalysis (Lorenz and Eck 2005). By random *In-vitro* recombination of point mutants of the same gene or two or more different homologous genes (family shuffling), the diversity in the resulting

gene bank can be enormously increased. Gene shuffling techniques are among the most powerful tools for the creation of diverse sequences (Takahashi et al. 2010; Sato et al. 2010). Ideally, the creation of sequence diversity should be targeted to sites or regions that have a significant effect on the catalytic performance of the enzymes (Ferrer et al. 2009).

With the rapid development of powerful protein evolution and enzyme-screening technologies, there is a growing belief that optimum conditions for biotransformation processes can be established without the constraints of the properties of the biocatalyst (Cowan et al. 2004). These technologies can then be applied to find the 'ideal biocatalyst' for the process. In identifying the ideal biocatalyst, the processes of gene discovery and enzyme evolution play major roles. These technologies, which currently include metagenomic library screening, gene-specific amplification methods and even full metagenomic sequencing, provide access to a volume of 'sequence space' that is not addressed by traditional screening (Lorenz and Eck 2005). However, in order to expand the pool genes for *in-vitro* evolution, new technologies, which circumvent the limitations of microbial cultivability, must be applied.

15.2.4 Gleaning Information Out of the Data – Bioformatics and Data Analysis

Metagenomic approaches have the potential to generate enormously huge body of sequences. However, the knowledge gleaned from such studies is not proportional to the sequencing effort involved, and it depends on the bioinformatics interpretation of the information (Donovan 2010)

A high-throughput pipeline has been constructed to provide high-performance computing to all researchers interested in using metagenomics (Morgan et al. 2010). The pipeline produces automated functional assignments of sequences in the metagenome by comparing both protein and nucleotide databases. Phylogenetic and functional summaries of the metagenomes are generated, and tools for comparative metagenomics are incorporated into the standard views.

15.3 Analysis

Metagenomics, the analysis of DNA isolated from environmental samples, has proved particularly useful for the knowledge of uncultured bacteria. In the core of the metagenomic approaches, establishing better DNA extraction techniques is of prime significance. Detecting the rare members of a microbial community is a challenge (Voget et al. 2003). However, its equally important to know about a small number of microbes that play a critical role in the community. Improved DNA extraction techniques could help ensure that a metagenomic library adequately represents the entire community's genome and has little or no contamination. Extending the analyses beyond the DNA sequence to study the proteins and metabolites (the

products of cellular processes) generated by a community is critical for understanding how the microbial community operates and interacts within the habitat.

15.3.1 Total DNA

Among the key factors responsible for the success of metagenomics, the isolation of quality environmental DNA in appreciable amount from a given habitat holds significance (Raes et al. 2007). The isolation of total DNA appears to be of prime importance and a bottleneck step in metagenomic studies, as the extracted DNA should be of high quality to pursue molecular biological applications (Voget et al. 2003; Desai and Madamwar 2007; Purohit and Singh 2010; Gilbert 2010). Standardization of total DNA extraction technique is desirable as the composition of different habitats varies with respect to their matrix, organic and inorganic compounds and biotic factors (William et al. 1988; William 1998). Improved DNA extraction techniques should also ensure a metagenomic library adequately representing the entire community's genome without inhibitory substances (Santosa 2001).

We have assessed and compared various methods for the extraction of total environmental DNA from saline soil of Coastal Gujarat and optimized them for the quality, yield and PCR amplification ability. The applicability of various methods for the extraction of total DNA using small quantity of soil sample was explored. It was observed that mechanical method, bead beating treatment was based on the ballistic disintegration of all cells. The method varies depending on the time of agitation and bead size. The efficiency of cell disruption and the damage of the DNA strands during sonication mainly depend on the energy input. Harsh treatment can result in shearing of high molecular weight DNA leading to lower yields and small fragment sizes with a possibility of a bias in microbial community analysis.

The enzymatic lysis relies on the proteinase K and lysozyme digestion of microbial cells to release DNA. The focus on the DNA extraction methods has resulted in rapid performance of the molecular techniques, avoiding extensive purification steps (Desai and Madamwar 2007). Using the lysis buffer treatment and its combination with bead beating methods, microbial DNA was extracted from Sambhar Lake soil sample and the extracted DNA samples were suitable for successful PCR amplification (Siddhpura et al. 2010; Parks and Beiko 2010).

The described methods could allow the use of large scale preparations providing greater probability of detecting genes present in low abundance in the soil environment. These methods would be applicable to more challenging and heavily contaminated soils; therefore, microbial biodiversity assessment can now be more readily assessed and useful sequences could be retrieved.

15.3.2 Molecular Tools

Molecular tools developed during the past 20 years by molecular biologists have facilitated the extraction, cloning, screening and sequencing of genes and genomes.

Many of these approaches have also allowed microbial ecologists to access and study the microbial diversity in its totality, regardless of our ability to culture organisms. This has opened the doors of unexplored domains of non-cultivable microbes, allowing unprecedented access to the world of natural products encoded by community genomes (Gilbert 2010; Glöckner et al. 2010).

The advent of culture-independent techniques has transformed the field of microbiology and microbial ecology in particular. PCR-based techniques allow the classification of microorganisms based on particular genetic markers and the profiling of complex microbial communities on the basis of sequence diversity (Bach et al. 2001).

The most commonly used marker for profiling bacterial communities is the 16S rRNA gene. The size of this gene (1.5 kilobases) is large enough for reliable phylogenetic information. Hierarchal domain specific primers are designed, which can target broadly or with high specificity. Different functional genes can also be used in order to target specific groups of bacteria. Domain specific primers are designed on the basis of conserved residues of sequences.

One technique that is now routinely used is denaturing gradient gel electrophoresis (DGGE) and the analogous temperature gradient gel electrophoresis (TGGE) (William 1998). It is a genetic fingerprinting technique that is used to separate individual sequences from a complex mixture. In principle, this means that DNA fragments of the same length are separated on the basis of differing sequences, even by a single base (Ercolini 2004).

We have attempted to find 16S rRNA sequence/s of unculturables from saline soils of Coastal Gujarat, India by the DGGE protocols perfected in our laboratory. Towards this end, we aim to focus on identifying signature sequences of halophiles/haloalkaliphiles; based on shotgun sequencing approaches and designing specific primers for halophiles/haloalkaliphilies (Purohit et al. 2010; Siddhpura et al. 2010).

The results indicated that different proteolytic bacteria release different amounts or activities of proteases (Dodia et al. 2006, 2008a, b; Joshi et al. 2008; Purohit and Singh 2010; Siddhpura et al. 2010). The proteolytic bacterial communities may play a major role in determining the population dynamics in context with the available nutrition. In the overall scenario of the secretion of extracellular proteins by the microbes in their surrounding, the recently published idea on the economic synthesis of the proteins/enzymes by the microbes assumes significance (Smith and Chapman 2010). According to this proposed theme supported by the analysis of the data, the organisms spend minimum energy on the synthesis of extracellular proteins.

The widespread use of molecular techniques in studying microbial communities has greatly enhanced our understanding of microbial diversity and function in the natural environment and contributed to an explosion of novel commercially viable enzymes. Technological advances in sequencing and cloning methodologies, as well as improvements in annotation and comparative sequence analysis, generates information for microbial ecologists. e.g. Natural products isolated from sponges are an important source of new biologically active compounds (Raes et al. 2007; Glöckner et al. 2010). Metagenome of marine microbial communities have been shown to contain genes and gene clusters typical for the biosynthesis of biologically active natural products (Michelle et al. 2000; Kauffmann et al. 2004; Kennedy and Marchesi 2007; Kennedy et al. 2008).

Combining metagenomic approaches with heterologous expression holds much promise for the sustainable exploitation of the chemical diversity present in the marine microbial community. A PCR-based method targeting a 59-base recombination site highlighted on the diverse bacterial taxonomic groups and that flanks gene cassettes are associated with integrons. The recovered gene cassettes contained complete open reading frames, most of which did not show homology to any database entry, and which potentially encode enzymes of biotechnological interest (Glöckner et al. 2010). PCR-based cloning methods are also being employed to recover novel enzymes. In most cases, degenerate primers are used, hybridizing with conserved regions that preferentially are located close to the extremities of the target genes (Liles et al. 2008; Ni et al. 2009).

We relied on similar approach for our own studies, where degenerate primers were designed for alkaline proteases by using bioinformatics tools. Designed sets of primers were specifically based on halophilic/haloalkaliphilc alkaline proteases available from marine environment. We identified several such sequences and successfully cloned, over-expressed and characterized them in *E. coli* host system, our unpublished data (Purohit and Singh 2010). The characteristic features of native and recombinant enzymes were studied. Interestingly, we noticed that recombinant clones have maintained their nascent properties, specific activity of enzyme was found to be around five times higher than purified native enzymes.

However, this approach does not sound equally well in capturing functional attributes of sequences that share some sequence identity with already identified sequences (Craig and Venter 2004; Craig et al. 2010). Besides expression-based identification of biocatalysts, large-scale shotgun sequencing projects and in silico identification of enzyme-coding regions are currently carried out, for instance by The Monterey Bay Coastal Ocean Microbial Observatory (http://www.tigr.org/tdb/MBMO/) on marine picoplankton (Nakamura et al. 2009). While this approach would lead to fundamental insight into the genetic organization and function of non-cultivated organisms and accumulates valuable sequence information in the databases, shotgun sequencing remains a rather cumbersome and expensive approach when aimed at enzyme discovery due to its indirect nature (Nakamura et al. 2009).

15.4 Furture Perspectives

Microbiology has long relied on the diverse methods for the analysis of physiology, biochemistry and genetics of microorganism, where metagenomics can provide tools to balance the abundance of knowledge attained from culturing with the understanding of the uncultured majority of microbial life. Myriad environments on earth have not been studied with culture-independent methods other than PCR-based 16S rRNA gene analysis, and thus, they point out towards extensive work waiting for future (Gilbert 2010; Morrissey et al. 2010). Many barriers have limited the discovery of new genes that provide insight into microbial community structure and function. Alternatively, new genes could be used to provide biomolecules leading to

novel applications in medical, agricultural, or industrial fields. The potential for discovery of novel products from metagenomic approaches depends on the advancement of methods and tools that are central to library construction and analysis (Morrissey et al. 2010). Sequence-based assignment of function will also benefit from advances in detection of homology, which will increasingly rely on the tertiary structures of the predicted proteins rather than simply on primary sequence (Hoff et al. 2008; Wooley et al. 2010). Functional analysis will require further innovations in method development.

With the possibilities to access vast genetic resources in different ecosystems, the unlimited realms of microbial diversity would slowly but steadily lead to new knowledge and novel biotechnological avenues. However, the usual challenge of heterologus gene expression needs to be addressed to turn metagenomic technologies into commercial successes, particularly in applications where bulk enzyme or product have to be produced at viable cost (Kennedy and Marchesi 2007).

Given that the majority of natural products are of microbial origin, and that the vast majority of microbial genomes are yet to be explored, its quite logical that microbial metagenomes harbour a great economic potential. Due to their huge but largely unexplored diversity and history as sources of commercially valuable molecules with agricultural, chemical, industrial and pharmaceutical applications, marine environments would be among the most common habitats to explore from metagenomics view point (Morrissey et al. 2010). Improved functional screening methods would potentially provide a means to discover new variants of functions of interest.

The goals of researchers venturing into the microbial metagenome vary from directed product discovery to total community characterization and assessment of the phylogenetic complexity of the environments. Metagenomics has redefined the concept of a genome, and accelerated the rate of gene discovery. The potential for application of metagenomics to biotechnology seems endless. Metagenomics, together with *in-vitro* evolution and high-throughput screening technologies would provide unprecedented opportunities to bring new generation of biomolecules into various fields, beside adding to new knowledge in our understanding on biotic and abiotic interactions in ecosystems.

15.5 Conclusions

New approaches and technological innovations have regularly updated our knowledge and set the directions of research. There can be little doubt that the field of metagenomics offers enormous scope and potential for both fundamental microbiology and biotechnological development. With improved methods for extracting total environmental DNA, retrieval of genes, diversity and phylogenetic analysis of community structure, funding stimulated by recent developments in the field and attraction of diverse scientists to identify new problems and solve the old ones, metagenomics will expand and enrich our understanding of microorganisms and their roles in ecosystems.

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Chapter 16
Laccases: The Biocatalyst with Industrial and Biotechnological Applications

Shilpi Thakur, Hardik Patel, Shilpa Gupte, and Akshaya Gupte

Abstract Laccases (Benzenediol: oxygen oxidoreductase, E.C. 1.10.3.2) are enzymes belonging to the group of polyphenol oxidases which are monomeric, dimeric or tetrameric glycoproteins with copper atoms per monomer located at the catalytic site. It is widely distributed in wood rotting fungi and which is also found in a variety of molds, insects as well as some plants and bacteria. Laccases have received much attention from researchers in the last decades due to their ability to oxidize both phenolic and non phenolic lignin related compounds as well as highly recalcitrant environmental pollutants, which makes them very useful for their application to several biotechnological processes. As on oxidase, laccase is used in many agricultural, industrial and medicinal applications. Thus, laccases are increasingly finding applications in biotechnology in the fields of environment-friendly synthesis of fine chemicals and for the gentle derviatization of biologically active compounds e.g. antibiotics, amino acids, antioxidants and cytostatics. Oligomerization and polymerization reactions can lead to new homo- or heteropolymers and biomaterials. These may be useful in wide range of applications including the production of polymers with antioxidative properties, the co-polymerizing of lignin components with low molecular mass compounds, the coating of cellulosic cotton fibers or wool, ingredients of cosmetics. Laccase nano particles have also been used for the elimination of micropollutants from waste water in bioreactors.

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16.1 Introduction

Enzymes play a vital role in all living systems, catalyzing biochemical reactions, structural transformations, recognition and regulation with remarkable specificity and are present in all cells and tissues. Over the years tremendous progress has been made in understanding of enzyme structure and function. Although oxidation-reduction reactions are essential in several industries, most of the conventional oxidation technologies have drawbacks like non-specific or undesirable side reactions and use of hazardous chemicals. This has led to search for new oxidation technologies based on biological systems such as enzymatic oxidation. Enzymatic oxidation techniques have a large potential within a great variety of industrial fields including paper, pulp, textile and food industries.

Enzymes recycling on molecular oxygen as an electron acceptor are the most interesting Dominic (2009). Thus, Laccase is particularly promising enzyme. Laccase (E.C. 1.10.3.2) is defined in enzyme commission (EC) nomenclature as oxidoreductases which oxidize diphenols and related substances and use molecular oxygen as an electron acceptor. Laccases belong to a group of polyphenol oxidases containing copper atoms in the catalytic centre and usually called multi copper oxidases. Other members of this group are the mammalian plasma protein ceruloplasmin and ascorbate oxidase of plants. It is difficult to define laccase by its reducing substrate due to its broad substrate range, which varies from one laccase to another and overlaps with the substrate range of another enzyme – tyrosinase (E.C. 1.14.18.1). Laccase does not have the ability to oxidize tyrosine as tyrosinases do Baldrian (2006).

The chemistry, function, industrial and biotechnological applications have be reviewed from time to time by various researchers Piontek et al. (2002), Dominic (2009), Mikolasch and Schauer (2009), Majeau et al. (2010). The present work is to summarize the data that has been accumulated in the recent past from the various authors. Most laccases are extracellular enzymes, making the purification procedures very easy and laccases exhibit a considerable level of stability in the extracellular environment. Greater attention on laccase as an ecofriendly enzyme and as a green catalyst in recent past is generating information that appeared in a number of reviews in the last couple of years. The inducible expression of the enzyme in other fungal species also contributes to the easy applicability in biotechnological processes.

This review would help to define the common general characteristics of laccases, laccase producing organisms, structure, unique properties, and reaction mechanism, industrial and biotechnological applications and would contribute to the discussion of laccase in natural environment.

Table 16.1 List of bacteria and fungi produces laccase

Bacteria	
Sources	References
Bacillus subtilis	Park and Park (2008)
S. maltophilia AAP56	Mouso et al. (2007)
Fungal (Basidiomycetes)	
Pleurotus ostreatus HP1	Patel et al. (2009)
Coriolopsis caperata AGST2	Gupte et al. (Unpublished data) (GenBank accession No.HQ323692)
Trametes versicolor, T. hirsuta, P. sanguineus T. harzianum	Saparrat et al. (2007)
Ganoderma lucidum	Verma et al. (2010)
Pseudolagarobasidium acaciicola AGST3	Gupte et al. (Unpublished data) (GenBank accession No.HQ323693)
Ganoderma cupreum AG1	Gupte et al. (Unpublished data) (GenBank accession No.HQ328947)
M. parvum, Pestalotigsis sp. and *Diaporthe* sp.	Tziallae et al. (2009)
C. byrsiana	Ciullini et al. (2008)
C. unicolor	Ramesh et al. (2008)
H. cylindrosporum	Xavier et al. (2007)
Fungal (Ascomycetes)	
Fusarium incarnatum LD3	Chhaya and Gupte (2010)

16.1.1 Distribution/Occurrence of Laccase and Physiological Functions

Laccases are common enzymes in nature. The first laccase was reported in 1883 from *Rhus vernicifera,* the Japanese lacquer tree. Bertrand and Hebd (1896) demonstrated the presence of laccase in fungi for the first time. Laccases have subsequently been detected in all spp. of family *Anacardiaceae*, other plants – *Acer pserdplatanus, Pinus taeda and Populus eruamericana* Bligny and Douce (1983), De-Marco and Roubelakis-Angelakis (1997). Plant laccases are found in xylan, where they oxidize monolignols in the early stages of lignifications Mayer and Staples (2002), Gavnholt and Larsen (2001) and also participate in the radical-based mechanisms of lignin polymer formation Hoopes and Dean (2004). Laccases have been shown to be involved in the first steps of healing in wounded leaves De Marco and Roubelakis-Angelakis (1997). Since then most of the laccases have been isolated are from fungal origin especially from white rot fungi belonging to the various classes such as Ascomycetes, Basidiomycetes and Deutromycetes (Table 16.1) Desai and Nityanand (2011). Among the various fungi, white rot fungi have been found to be the most efficient producers (Fig. 16.1).

A number of brown-rot fungi have also been known to produce laccase. D'Souza et al. (1996) for the first time showed the presence of laccase gene-specific sequence as well as laccase activity in brown-rot fungi *G. trabeum*, comparable to a number of

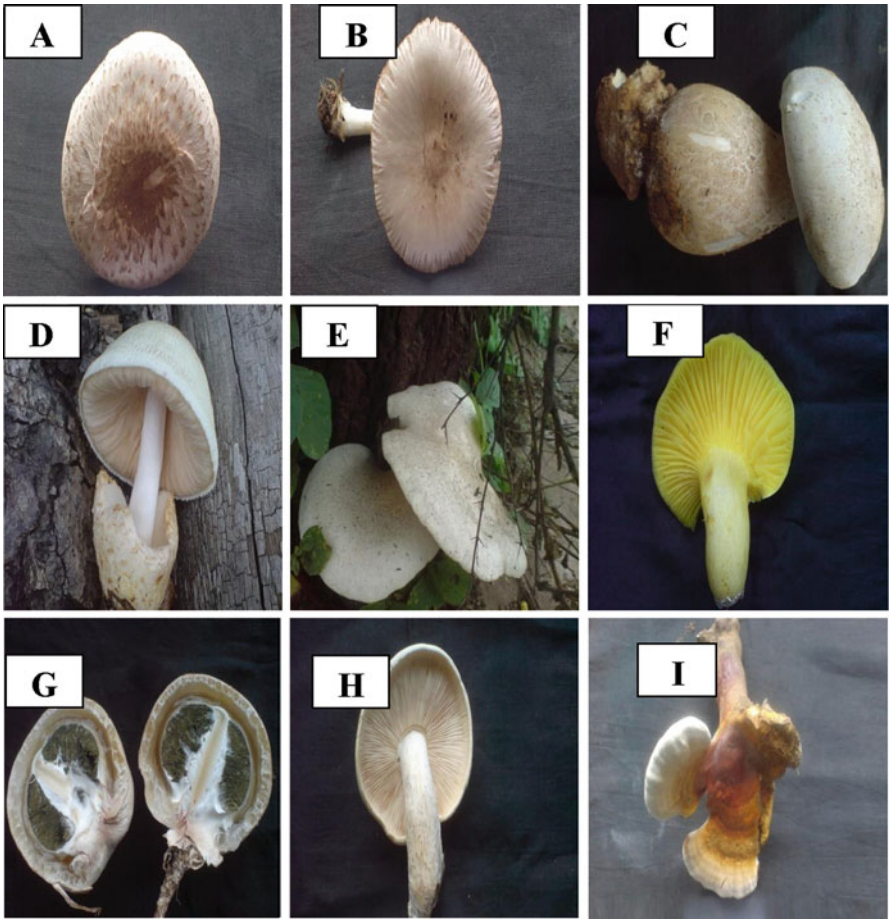

Fig. 16.1 Isolated White rot fungi (**a**). *Coriolopsis caperata* AGST2, (**b**). *Pseudolagarobasidium acaciicola* AGST3, (**c**)–(**i**) Unidentified White rot fungi

white-rot fungi. Other brown-rot fungi such as *Postia placenta, Antrodia vaillantii* have been shown to produce laccase. Laccase has also been isolated and purified from the human pathogen *Cryptococcus* (Filobasidiella) *neoforman*, basidiomycetes yeast. This yeast produces a true laccase capable of oxidation of phenols and amino phenols and unable to oxidize tyrosine Williamson (1994).

16.1.2 Structure and Catalytic Properties of Laccase

The study of purified proteins gives us an idea about the structural and biochemical/catalytic properties of fungal laccase. Fungal laccases are mostly extracellular but most of the wood rotting white rot basidiomycetes produce both extracellular and intracellular laccases with isoenzymes showing similar patterns of activity involving

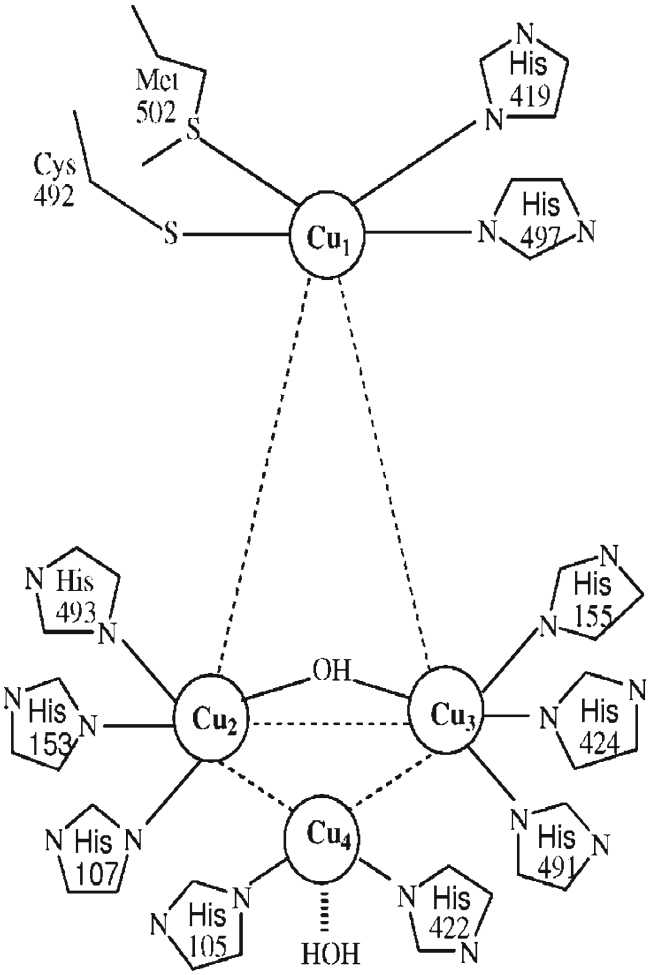

Fig. 16.2 Illustration of the active site of the laccase showing the relative orientation of copper atoms (Enguita et al. 2003)

isoelectric focusing. Most fungal laccases are monomeric, dimeric or tetrameric glycoproteins. Glycosylation of fungal laccase is believed to play a role in secretion, susceptibility to proteolytic degradation, copper retention and thermal stability. Typical fungal laccase has a molecular mass ranging from 50 to110 KDa With acidic isoelectric pH Thurston (1994), Dominic (2009). Laccases posses a high degree of glycosylation with the carbohydrate content of 10–35%. Four copper atoms are usually present but enzymes with one, two or three atoms have also been reported (Fig. 16.2). Different copper centres can be identified on the basis of their spectroscopic properties Leontievsky et al. (1997), Viswanath et al. (2008).

Many laccase producing fungi have shown to posses the isoenzymes. These enzymes have been found to originate from the same or different genes encoding for

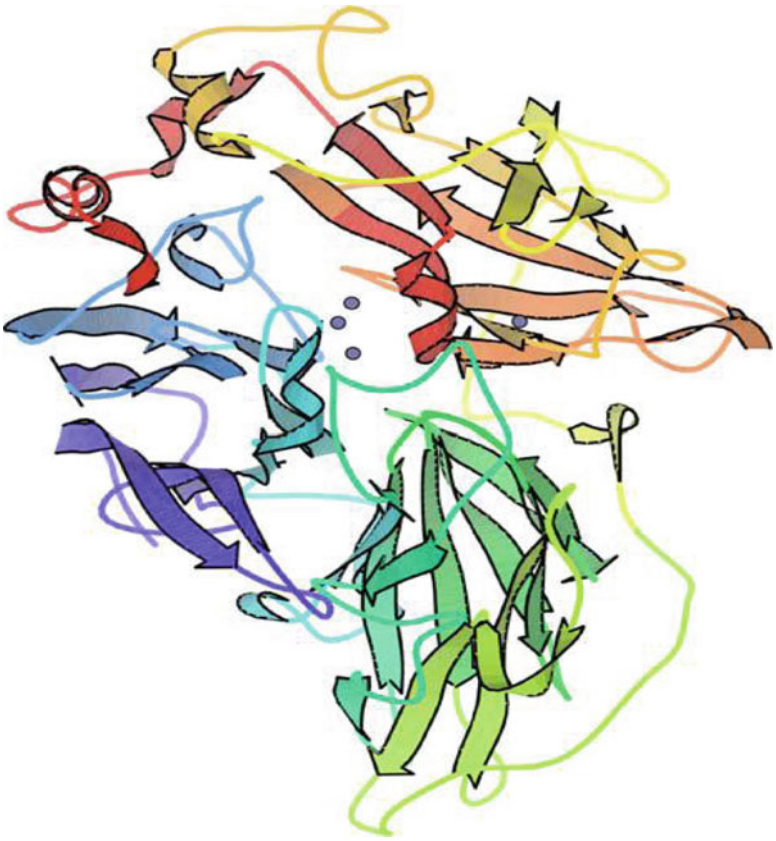

Fig. 16.3 3D structure of *Trametes versicolor* laccase (Adapted from Piontek et al. 2002)

the laccase enzyme. Palmieri et al. (2003) reported the presence of eight different laccase isoenzymes from the white rot fungus *Pleurotus ostreatus*. Isoenzymes have also been shown with different molecular weight and pI (isoelectric point) in litter decomposing fungus *M. quercophilus* D'Souza-Ticlo et al. (2009).

All fungal laccases show a similar architecture consisting of three sequentially arranged domain of a β-barrel type structure. Most monomeric laccase molecules active site contains four copper ions in their structure that can be classified in three groups using UV/visible and electron paramagnetic resonance (EPR) spectroscopy Leontievsky et al. (1997). The type 1 copper (T1) is responsible for the intense blue color of the enzymes at 600 nm and is EPR-detectable ($\Delta\varepsilon = \sim 5,000$), the type 2 copper (T2) is colorless, but EPR-detectable, and the type 3 copper (T3) consists of a pair of copper ions that give a weak absorbance near the UV spectrum but no EPR signal. The T2 and T3 copper sites are close together and form trinuclear centres that are involved in the catalytic mechanism of the enzyme, Solomon et al. (2001), Ferraroni et al. (2007). Spectroscopy combined with crystallography (Fig. 16.3) has provided a detailed description of the active site in the laccase. Magnetic circular

dichroism (MCD) and X-ray absorption spectroscopy of laccase have shown that the type-2 and 3 centres combine to function as a trinuclear copper cluster with respect to exogenous ligand interaction including reaction with dioxygen Thurston (1994).

Laccases from different sources exhibit a wide range of redox potentials. The T1 site has a high redox potential reaching 780–800 mV for the *T. versicolor* and *Neurospora crassa* enzymes, whereas the plant *R. vernicifera* enzyme has a value of 420 mV Piontek et al. (2002), Malkin and Malmstrom (1970). The redox potentials of T2 and T3 sites for the *R. vernicifera* laccase are, respectively, 390 and 460 mV at pH 7.5 Reinhammar and Vanngard (1971). The *T. versicolor* laccase T1 and T3 sites have been reported to be 785 and 782 mV, respectively Reinhammar (1972). In general, the T1 sites in fungal laccases are much higher than those of plant laccases and other blue copper oxidases, although significant differences in potentials also exist among fungal laccases Xu (1996).

The optimum pH for fungal laccases lies in the acidic range but it does depend on the substrates employed (3.0–8.0), even though many reports have reported a bell shaped profile for laccase activity Baldrian (2006). The optimal temperature of laccase can differ from strain to strain. Optimal temperature of 50°C has been reported for laccase activity. The temperature stability of laccase varies considerably. The half life of the enzymes at 50°C can be range from few minutes to hours.

16.1.3 Substrate Specificity of Laccase

Laccases of various origins differ in their substrate specificities and several substrates should be tested to assess a laccase activity. Laccase can act on wide range of substrates (Table 16.2) which are ortho, para substituted compounds along with non phenolic substrate, The catalytic constants have been reported mostly for a small group of substrates – e.g. the non-natural test substrate ABTS and the phenolic compounds 2,6-dimethoxyphenol (2,6-DMP), guaiacol and syringaldazine. These constants have been measured for a large number of laccases, and rather great variation can be observed among them. The *Km* values of Laccases are in the range of 2–5,000 μM, but differences as high as 3,500-fold can be seen in the *kcat* values between different laccases with the same substrates. The *Km* values measured for 2, 6-DMP is generally higher than those obtained with syringaldazine. The comparison of *Km* values also shows that laccases from different source organisms have different substrate preferences Xu et al. (1996). Laccases in general combine high affinity for ABTS and syringaldazine with high catalytic constant, whereas the oxidation of guaiacol and DMP is considerably slower and the respective *Km* constants higher. On the other hand, the *kcat* values for a single laccase do not generally differ more than twofold to tenfold between different substrates, which reflects the fact that the *kcat* describes the rate of the electron-transfer reactions taking place inside the enzyme after substrate binding Xu et al. (1996).

Table 16.2 Kinetic constant of different substrates of laccase

Substrates	(μM)	K_{cat} (min^{-1})	pH	Laccase	Reference
ABTS	106	1,000	4.0	*Bacillus subtilis* CotA	Martins et al. (2002)
	270	4,690	4.5	*Melanocarpus albomyces*	Kruus et al. (2001)
	90	350,000	3.0	*Pleurotus ostreatus* POXA1	Palmieri et al. (1997)
	280	57,000	3.0	*Pleurotus ostreatus* POXC	Palmieri et al. (1997)
	75	4,130	4.5	*Thielavia arenaria* Lccl	Paloheimo et al. (2006)
	14	41,400	3.0	*Trametes pubescens* LAP2	Galhaup et al. (2002)
	30	198	3.4	*Trametes trogii* POXL3	Garzillo et al. (1998)
	58	2,700	5.3	*Trametes villosa* Lcc1	Xu et al. (1996)
	45	620	5.5	*Trichophyton rubrum*	Jung et al. (2002)
2, 6-DMP	5	4,160	6.0	*Melanocarpus albomyces*	Kruus et al. (2001)
	2,100	21,000	5.0	*Pleurotus ostreatus* POXA1	Palmieri et al. (1997)
	230	430	5.0	*Pleurotus ostreatus* POXC	Palmieri et al. (1997)
	17	4,030	6.0	*Thielavia arenaria* Lccl	Paloheimo et al.(2006)
	72	24,000	3.0	*Trametes pubescens* LAP2	Galhaup et al. (2002)
	410	109	3.4	*Trametes trogii* POXL3	Garzillo et al. (1998)
Guaiacol	1,200	150	6.0	*Pleurotus ostreatus POXC*	Palmieri et al. (1997)
	36	10,800	3.0	*Trametes pubescens LAP2*	Galhaup et al. (2002)
	66	6,800	6.5	*Pleurotus sajor-caju* Lac4	Soden et al. (2002)
	5,120	115	3.4	*Trametes trogii POXL3*	Garzillo et al. (1998)
Syringaldazine	26	200	6.0	*Bacillus subtilis CotA*	Martins et al. (2002)
	1.3	4,710	6.0	*Melanocarpus albomyces*	Kruus et al. (2001)
	130	28,000	6.0	*Pleurotus ostreatus POXA1*	Palmieri et al. (1997)
	20	23,000	6.0	*Pleurotus ostreatus POXC*	Palmieri et al. (1997)
	28	n.r.	5.3	*Rhizoctonia solani Lcc4*	Xu et al. (1996)
	4.3	1,940	6.0	*Thielavia arenaria* Lccl	Paloheimo et al.(2006)
	6	16,800	4.5	*Trametes pubescens LAP2*	Galhaup et al. (2002)

16.1.4 Oxidative Mechanism of Laccase

Laccase enzymes oxidize substrates by subtracting one electron per time and generate free radicals which can thus be polymerized. The enzyme has the ability to store electrons of individual oxidation reactions and in its totally reduced state contains total of four electrons, thus enzyme can transfer these electrons to molecular oxygen to form water. The stoichiometry is as follows:

$$4RH + O_2 \rightarrow 4R + 2H_2O$$

The redox potential of the T1 copper site is directly responsible for the catalytic capacity of the enzyme. The mechanism of interaction between a laccase T1 site and its substrate seems to be identical among fungal Laccases Smirnov et al. (2001). To function, laccase depends on Cu atoms distributed among the three different binding sites. Cu plays an important role in the catalytic mechanism. In its native state, the enzyme holds copper atoms in the monovalent state as Cu^{+}. There are three

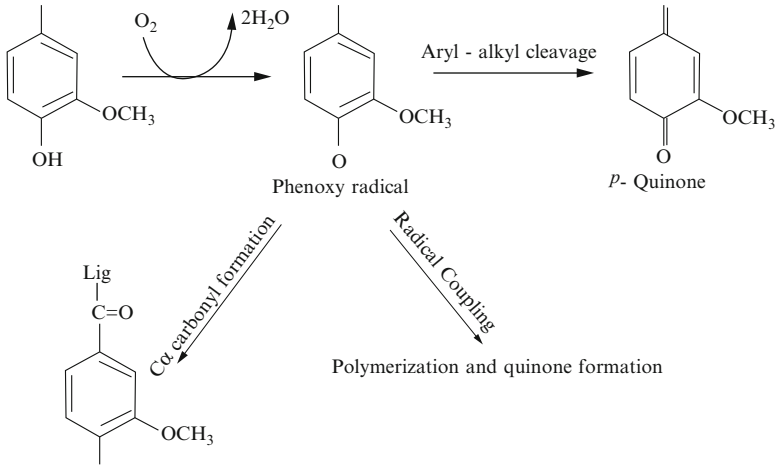

Fig. 16.4 Catalytic cycle of laccase (Adapted from Dominic 2009)

major steps in laccase catalysis. The first step of catalysis is reduction of the reducing substrate by the copper (Cu^{2+} to Cu^{+}) at the T1 site, which is the primary electron acceptor Messerschmidt et al. (1992), Huang et al. (1999). The electrons extracted from the reducing substrate are transferred to the T2/T3 trinuclear site in the second step of catalysis, resulting in the conversion of the resting form (fully oxidized) of the enzyme to a fully reduced state. The O_2 molecule is reduced to water at the trinuclear centre (Fig. 16.4). The O_2 molecule binds to the trinuclear cluster for asymmetric activation and it is postulated that the O_2 binding pockets appears to restrict the access of oxidizing agents other than the O_2. H_2O_2 is not detected outside of laccase during steady state laccase catalysis indicating that a four electron reduction of O_2 to water is occurring. Laccase can be thought to operate as a battery, storing electrons from individual oxidation reactions in order to reduce molecular oxygen. A successive $4e^-$ oxidation (from four substrate molecules) is required to fully reduce the enzyme. The e^- transfer from the substrate to the T1 copper is not a rate-limiting step in the overall reaction, but the intramolecular electron transfer from T1 to the trinuclear copper site is rate limiting in the catalytic cycle. Reduction of dioxygen takes place in two steps via the formation of bound oxygen intermediates. The dioxygen molecule first binds to the T2/T3 site, and two electrons are rapidly transferred from the T3 coppers, resulting in the formation of peroxide intermediate. The peroxide bridges between the oxidized T3 and the reduced T2 copper sites, although the configuration of the oxygen has not been fully established. The diffusion of dioxygen to the trinuclear site is rate limiting, followed by a rapid $1e^-$ transfer from T1. The peroxide intermediate decays to an oxy radical and undergoes a $2e^-$ reductive cleavage of the O–O bond with the release of a water molecule Palmer et al. (2001), Lee et al. (2002). The slow decay of the intermediate is facilitated by the final electron transfer from the T2 copper, and is accelerated with decreasing pH, with protonation from a carboxylic acid residue near the active site Zoppellaro et al. (2000).

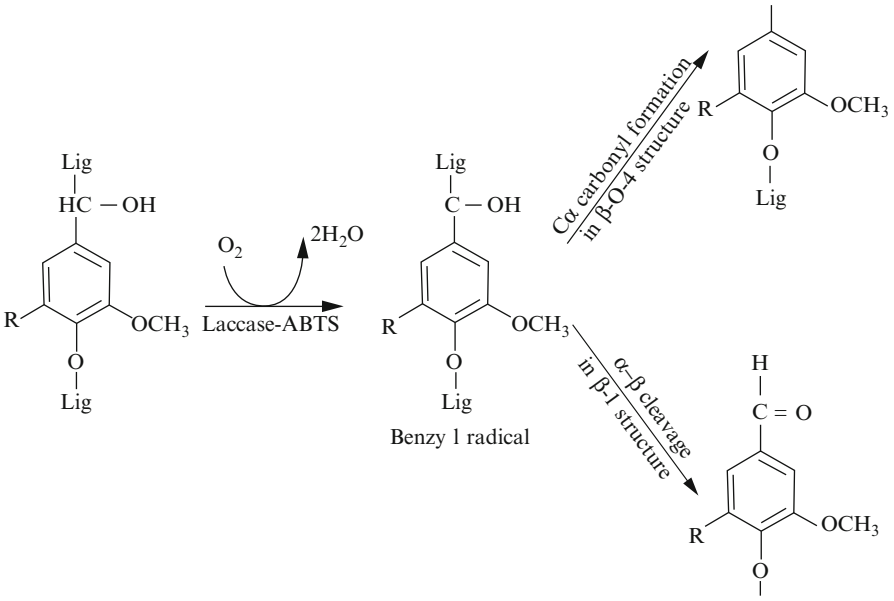

Fig. 16.5 Oxidation of phenolic sub-units of lignin by laccase

In the last step, all four copper centers are oxidized, and O_2^- is released as a second water molecule. The reoxidation of the T2 copper correlates with the decay of the intermediate in which the first water is released and the second water molecule remains bound and slowly exchanged with the bulk solution. The capture of four electrons by the substrate returns the enzyme to its native state.

For the oxidation of phenolic substrate, laccase promotes substraction of one electron from phenolic hydroxyl groups of lignin to form phenoxy radical (Fig. 16.5). The degradation of lignin proceeds by phenoxy radical that leads to either oxidation at Cα-carbon or cleavage of bond between Cα-carbon and Cβ-carbon. This oxidation results in an oxygen-centered free radical, which can then be converted in a second enzyme-catalysed reaction to quinone. The quinone and the free radicals can then undergo polymerization Thurston (1994), Viswanath et al. (2008). The presence of electron withdrawing substituents at phenoxy groups and bulky groups are more difficult to be oxidised. Laccase catalysed oxidation of phenols, anilines and benzene correlates with the redox potential difference between laccase's T1 copper site and the substrate (Xu 1996). Laccases has been found to oxidise nonphenolic compounds and lignin in the presence of mediators −2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonate) (ABTS), I-hydroxybenzotrizole (HBT) and 3 hydroxyanthranilic acid, Viswanath et al. (2008). As oxygen uptake by laccase in presence of ABTS is faster than in HBT, widening of the substrate range of laccase to non-phenolic subunits of lignin by the inclusion of a mediator such as ABTS is shown in Fig. 16.6. ABTS-mediated oxidation of nonphenolic substrates proceeds via electron transfer mechanism through formation of $ABTS^{++}$.

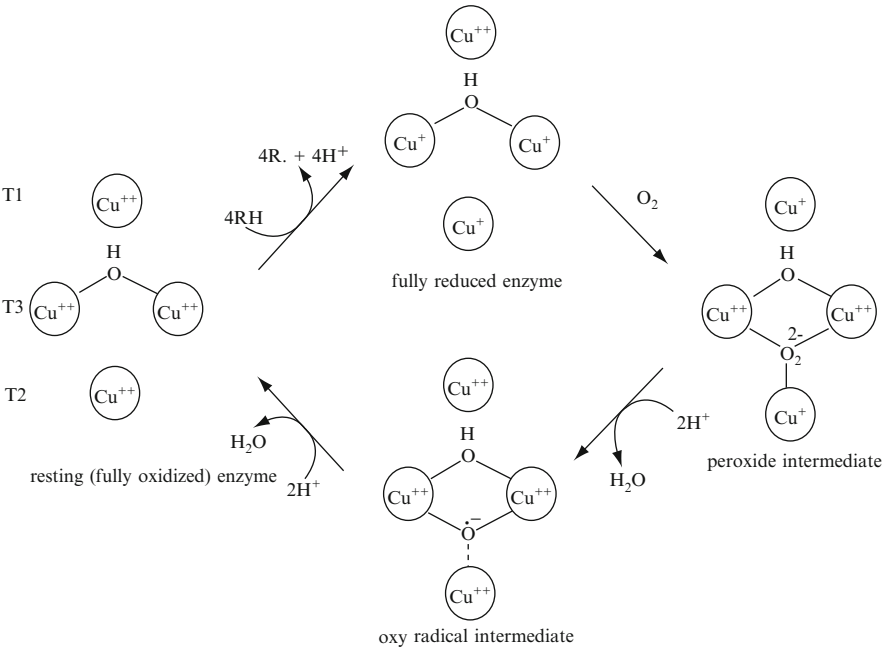

Fig. 16.6 Oxidation of non-phenolic sub-units of lignin by laccase

16.1.5 Laccase Mediator System (LMS), Inhibitors and Inducers of Laccase

White rot fungi are able to perform lignin degradation by different oxidative enzymes, including Laccases, however, the substrates of interest cannot be oxidized directly by Laccases, either because they are too penetrating into the enzyme active site or because they have a particularly high redox potential. By mimicking nature, it is possible to overcome this limitation with the addition of so-called **'Chemical mediator'**, which are suitable compounds that act as intermediate substrates for the laccase, whose oxidized radical forms are able to interact with the bulky or high redox potential substrate targets. It has been hypothesized that small redox molecules might act as a sort of 'electron shuttles' between the enzyme and the lignin and cause polymer de-branching and degradation Rochefort et al. (2004).

More than 100 mediator compounds have been described but the most commonly used are the ABTS which was introduced by Bourbonnais and Paice in 1990 and the triazole 1hydroxybenzotriazole (HBT) and 3-hyroxyanthranilic acid (HAA). The choice of a proper represents a key consideration for a given biotransformation Call (1996). The use of different mediators may yield different final products when using the same precursors. This is basically due to the fact that substrate oxidation in laccase-mediator reactions occurs via different mechanisms. The mediator radicals preferentially perform a specific oxidation reaction based on its chemical

structure and effective redox potential (or dissociation bond energy) Baiocco et al. (2003), Galli and Gentili (2004), d'Acunzo et al. (2006). For example, ABTS and HBT follow two different radical pathways: (i) electron transfer (ET) in the case of ABTS radicals ($ABTS^{\bullet+}$or $ABTS^{2+}$) and (ii) hydrogen atom transfer (HAT) for nitroxyradicals (N–O•) of HBT, Desai and Nityanand (2010). On the contrary, the stable radical TEMPO follows an ionic oxidation mechanism Baiocco et al. (2003), Fabbrini et al. (2002).

Despite all the associated advantages of LMS, there are two major drawbacks hindering the use of mediators: they are expensive and they can generate toxic derivatives. In some cases, while oxidizing the mediator, laccase is inactivated by the mediator radicals, or the latter can be transformed into inactive compounds with no more mediating capability (e.g. generation of benzotriazol from HBT by losing the hydroxyl group). This can be overcome by the use of low-cost and eco-friendly alternative mediators; in this sense, several naturally occurring mediators produced by fungi (phenol, aniline, 4-hydroxybenzoic acid and 4-hydroxybenzyl alcohol) have been identified Johannes and Majcherczyk (2000). More recently, phenolic compounds derived from lignin degradation (such as acetosyringone, syringaldehyde, vanillin, acetovanillone, ferulic acid or *p*coumaric acid) have been demonstrated to be highly-efficient laccase mediators of natural origin for dye decolorization, removal of polycyclic aromatic hydrocarbons, pulp bleaching etc. Canas et al. (2007), Camarero et al. (2007), Gutierrez et al. (2007). These natural compounds can be obtained at low cost due to their abundance in nature and also in industrial paper pulp wastes, smoothing the progress to a more environmental friendly and sustainable white biotechnology processes.

Laccase activity can be inhibited by different inhibitors. Anions such as the halides, azide, cyanide and hydroxide bind to the Type 2 and 3 Cu atoms of laccases, which disrupts the electron transfer system, resulting in enzyme inhibition Gianfreda et al. (1999). The inhibition by hydroxide generally prevents catalysis of substrates at alkaline pH Xu (1996). The inhibition of activity by hydroxide prevents auto oxidation at alkaline pH, with a resultant increase in stability at alkaline pH Xu (1996). The inhibition by halides varies according to the laccase isoenzymes, and therefore likely related to the size of the channel of the trinuclear cluster (where oxygen binds) Xu (1996). Other types of inhibitors include certain metal ions (e.g. Hg^{2+}), fatty acids, sulfhydril reagents, hydroxyl glycine, kojic acid, and cationic quaternary ammonium detergents Gianfreda et al. (1999), Desai and Nityanand (2010). These compounds may affect the laccase by chelating the Cu (II) atoms, by modifying of amino acid residues or they may elicit a conformational change in the glycoprotein.

In basidiomycete's fungi, laccase production has been found to be depended on the cultivation condition and medium components. Laccase productions are activated during the secondary metabolic phase and were triggered by nitrogen concentration or when carbon or sulfur became limiting. However, their production can be stimulated by the presence of inducers like veratryl alcohol, lignin, and 2, 3 xylidine etc. Desai and Nityanand (2010). Among various inducers used like gallic acid (1 mM), catechol (1 mM), ammonium tartarate (55 mM), hydroxybenzoic acid (1 mM) and vanillin (1 mM), it was observed that only ammonium tartarate increased the enzymatic activity reaching to 251 $U\,mL^{-1}$ of extract after 30 days in case of

laccase from *Lentinula edodes* Cavallazzi et al. (2005), Valeriano et al. (2009) obtained high activity when inducers 2, 5-xylidine was used in specific concentration in case of laccase from *Pycnoporus sanguineus*. There are compounds/substances other than the lignin related compounds which can act as inducers. Ethanol, for example, induces laccase gene expression in some fungi. Laccase production in the presence of 40 g ethanol/L by *Trametes versicolor* was 20 times higher than that without ethanol. In *Coriolus hirsutus* and *Grifola frondosa* similar stimulatory effect on laccase production was observed. Copper is another non-lignin related substances which can induce laccase production may be because this metal is part of the enzyme structure. Valeriano et al. (2009) obtained high activity when inducers ethanol was used in specific concentration in case of laccase from *Pycnoporus sanguineus* and *Pleurorus ostreatus* Palmieri et al. (2000), Desai and Nityanand (2010). Other options to increase laccase production have been the use of diverse food wastes as inducers such as apple, orange and potato under solid state fermentation Loera Corral Octavio et al. (2006).

16.1.6 Heterologous Production of Laccase

Large amount of enzymes are require for biotechnological and Environmental applications. Laccases produced from the wild type fungal organisms are too low for commercial purpose. Therefore, improving the productivity and reducing the production costs are the major goals for the current studies on laccase production. Heterologous expression should be better suited for large scale production, because of the potential of expressing different laccases in one selected optimized host. Laccases are difficult to express in non-fungal systems. The heterologous expression of active laccases has been reported mainly in filamentous fungi (*Aspergillus oryzae*, *Aspergillus niger, Aspergillus sojae* and *Trichoderma reseei*) and yeasts (*Saccharomyces cerevisiae, Pichia pastoris*, *Pichia methalonica*, *Yarrowia lipolytica* and *Kluyveromyces lactis*). There is one exception of homologous expression, in which the basidiomycete's fungus *Pycnoporus cinabarinus* was used as host to over express the active laccase (up to 1.2 $g l^{-1}$) Kunamnenei et al. (2008). Bacterial laccases from *B. subtilis, Thermus thermophilus* and *Streptomyces lavendulae* have been expressed in *Escherichia coli* but successful expression of fungal laccases in *E. coli* has not been reported, perhaps due to the requirement of glycosylation, missing chaperons and different codon usage Martins et al. (2002).

16.2 Biotechnological Applications of Laccase

Laccases of fungi are of particular interest with regard to potential industrial applications. A vast amount of industrial processes, for instance in the textile, wood processing, pharmaceutical and chemical industries, organic synthesis,

environmental, food and nanobiotechnology. Being specific, energy saving and biodegradable, laccase and laccase based biocatalyst fit well with the development of highly efficient, sustainable and ecofriendly industries.

16.2.1 Paper and Pulp Industry

The industrial preparation of paper requires separation and degradation of lignin in wood pulp. Environmental concerns urge to replace conventional and polluting chlorine-based delignification/bleaching procedures Kuhad et al. (1997). In the industrial preparation of paper the separation and degradation of lignin in wood pulp are conventionally obtained using chlorine- or oxygen-based chemical oxidants. Oxygen delignification process has been industrially introduced in the last years to replace conventional and polluting chlorine-based methods. In spite of this new method, the pre-treatments of wood pulp with laccase can provide milder and cleaner strategies of delignification that also respect the integrity of cellulose Barreca et al. (2003), Gamelas et al. (2005), Shi (2006), Xu et al. (2006).

Cellulose is the major polymer of primary interest to paper manufacturers. Different woody substrates like eucalyptus, bamboo, and agricultural residues are used for manufacturing paper depending on the type and quality of paper. Pulping and bleaching of these substrates are the two main steps for paper manufacturing. During pulping, the raw materials are reduced to the fibrous state using mechanical and chemical methods. Pulping is followed by bleaching which involves the consumption of enormous amounts of chemicals, thus leading to environmental pollution hazards. To cope up with this problem, alternative bio-bleaching systems, few enzymatic treatments exhibit the delignification/brightening capabilities of modern chemical bleaching technologies have been explored to provide environmentally safe technology which primarily involves the use of laccase Widsten et al. (2008). In addition, laccase is more readily available and easier to manipulate than both lignin peroxidase and manganese independent peroxidase and LMS has already found practical applications such as the **Lignozym® process** Call and Mucke (1997). The use of white rot fungi for the treatment of wood chips prior to mechanical or chemical pulping is called biopulping. Laccases can thus be applied for biopulping as they partially degrade lignin and loosen the lignin structures. This not only improves the strength of the paper but also reduces the kappa number in the pulp which is a measure of residual lignin. Biopulping is useful as it helps to save electrical energy, improve the strength and is environmentally compatible due to reduced effluent toxicity, also results in reduction of resins.

The laccase mediator system (LMS) is an alternative which has shown to possess the feasibility to substitute chlorine containing reagents. Laccases when used together with mediators are able to delignify pulp. Laccases are able to delignify pulp when they are used together with mediators Bourbonnais et al. (1997). Small natural low molecular weight compounds with high redox potential (>900 mV) called mediators may be used to oxidize the non-phenolic residues from

the oxygen delignification Bourbonnais et al. (1997). Although LMS has been studied extensively, there are still unresolved problems concerning with mediator recycling, cost and toxicity. Furthermore, the application of laccases in pulp-kraft bleaching may result in higher pulp yields and energy savings. Most of studies have been patented about the use of LMS in the pulp-kraft bleaching processes Xu et al. (2006a, b), Cheng et al. (2003), Hamada et al. (2006), Guo et al. (2006).

The capability of laccases to form reactive radicals in lignin can also be used in targeted modification of wood fibers. For example, laccases can be used in the enzymatic adhesions of fibers in the manufacturing of lignocellulose based composite materials such as fiberboards. Laccases have been proposed to activate the fiber-bound lignin during manufacturing of the composites, thus resulting in boards with good mechanical properties without toxic synthetic adhesives Felby et al. (1997), Huttermann et al. (2001).

In another related application, laccases can be even used for deinking and decolorizing a printed paper Shi et al. (2001), Zhao et al. (2005), Wang et al. (2007). Finally, laccases can be used for binding fiber-, particle- and paper-boards Guebitz et al. (2003), Srebotnik et al. (2005), Gadda et al. (2001). However, different wood-decaying basidiomycetes have shown a highly variable pattern of laccase formation, and this subject requires more detailed experiments Mayer et al.(2002).

16.2.2 Textile Industry

The textile industry accounts for two-thirds of the total dyestuff market and consume large volumes of water and chemicals for wet processing of textiles Riu et al. (1998). The chemical reagents used are very diverse in chemical composition, ranging from inorganic compounds to polymers and organic products Banat et al. (1996), Juang et al. (1996). Due to their chemical structure dyes are resistant to fading on exposure to light, water and different chemicals Poots and McKay (1976) and most of them are difficult to decolorize due to their synthetic origin. There is stringent government legislation in the developed countries, regarding the removal of dyes from industrial effluents. Several dyes are made from known carcinogens such as benzidine and other aromatic compounds Baughman and Perenich (1988). Most currently existing processes to treat dye wastewater are ineffective and not economical Cooper (1995), Stephen (1995). Therefore, the development of processes based on laccases seems an attractive solution due to their potential in degrading dyes of diverse chemical structure, Hou et al. (2004). Related to textile bleaching, in 1996 Novozyme (Novo Nordisk, Denmark) launched laccase enzyme in denim finishing: DeniLite. In 2001, the company Zytex (Zytex Pvt Ltd. Mumbai, India) developed a formulation based on LMS capable of degrading indigo in a very specific way. The trade name of the product is Zylite. Together with low molecular weight redox-mediator compounds, laccase can generate a desired worn appearance on denim by bleaching indigo dye Campos et al. (2001). The potential use of laccase for bleaching

has been investigated and this has even led to the esoteric suggestion of using laccase in the presence of hydroxyl stilbenes as hair dyes Onuki et al. (2000).

Laccases find potential applications for cleansing, such as cloth- and dishwashing Kirk et al. (2002), Someya et al. (2003). Laccase may be included in a cleansing formulation to eliminate the odor on fabrics, including cloth, sofa surface, and curtain, or in a detergent to eliminate the odor generated during cloth washing Wolfgang et al. (2005), Aehle et al. (2003). A patent application about the use of LMS to increase the shrink resistance of wool was published by Yoon (1998). Also, Lantto et al. (2004) found that wool fibers could be activated with LMS. Therefore, the use of laccase for anti-shrink treatment of wool seems very attractive.

16.2.3 Food Industry

Laccases can be applied to certain processes that enhance or modify the color appearance of food or beverage. In this way, an interesting application of laccases involves the elimination of undesirable phenolics, responsible for the browning, haze formation and turbidity development in clear fruit juice, beer and wine. The potential applications of laccase in different aspects of food industry such as bioremediation, beverage processing, ascorbic acid determination, sugar–beet pectin gelation, baking, and biosensor have been described Minussi et al. (2002). Many laccase substrates, such as carbohydrates, unsaturated fatty acids, phenols and thiol-containing proteins, are important components of various foods and beverages. Their modification by laccase may lead to new functionality, quality improvement, or cost reduction Minussi et al. (2002), Kirk et al. (2002). Sometimes O_2 is detrimental to the quality or storage of food/beverage because of unwanted oxidation. Laccases may be used as O_2 scavengers for better food packing Farneth et al. (2005), Adinarayana et al. (2007). The flavor quality of vegetable oils can be improved with laccase by eliminating dissolved oxygen Petersen and Mathiasen (1996), Adinarayana et al. (2007). Laccase can also deoxygenate food items derived partly or entirely from extracts of plant materials. Cacao was soaked in solutions containing laccase, dried and roasted in order to improve the flavor and taste of cacao and its products Takemori et al. (1992), Sato and Koyama (2006). The reduction of odors with laccase is documented in the patent literature Tsuchiya et al. (2000). Treatment with a fungal laccase can also be performed to enhance the color of a tea-based product Arnaut et al. (2006), Bouwens et al. (1997), Bloom et al. (2006). It can also be used to perform the cross-link of ferulic acid and sugar beet pectin through oxidative coupling to form gels for food ingredients Micard et al. (1999). Various enzymatic treatments have been proposed for fruit juice stabilization Minussi et al. (2002), Alper et al. (2004).

Wine stabilization is one of the main applications of laccase in the food industry as alternative to physical-chemical adsorbents Minussi et al. (2002), Descenzo and Irelan (2003). Musts and wines are complex mixtures of different chemical compounds, such as ethanol, organic acids (aroma), salts and phenolics compounds

(color and taste). Cinnamic acid derivatives and catechins are present in different amounts in all wines. All phenolics are subjected to various fates during shelf life of wine and some problems can arise from their modifications, which are then involved in chemical reactions. Polyphenol removal must be selective to avoid an undesirable alteration in the wine's organoleptic characteristics. Laccase presents some important requirements when used for the treatment of polyphenol elimination in wines, such as stability in acid medium and reversible inhibition with sulphite Tannoven and Eksi (2005). Laccases are also known to improve storage life of beer and are used in removal of haze formation. An alternative to the traditional treatment to remove the excess of polyphenols, laccase could be added to the wort as an attractive alternative Minussi et al. (2002), Mathiasen (1995). A laccase has recently been commercialized (Suberzyme®) for preparing cork stoppers for wine bottles Conrad et al. (2000). This enzyme is not yet allowed as a food additive, the use of immobilized laccase might be a suitable method to overcome fungal barrier as in this form it may be classified as a technological aid.

16.2.4 Pharmaceuticals Industry

Many products generated by laccases are antimicrobial, detoxifying, or active personal-care agents. Due to their specificity and bio-based nature, potential applications of laccases in the field are attracting active research efforts. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory, antibiotics, sedatives, etc. Nicotra et al. (2004), Juelich et al. (2001), including triazolo(benzo) cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline Xu (1999), Molino et al. (2004), Stahl et al. (2002).

Laccase can oxidize iodide to produce iodine, which is widely used as a disinfectant Xu (1999). Coupling activity of the enzyme can be used synthesize novel compounds like (3, 4-dihydroxy-phenyl) – propionic acid Mikolasch et al. (2002) and 1, 2, 4-triazolo (4, 1, 2) benzothiazidine-8-ones Bhalerao et al. (1994). Also, laccase has been reported to possess significant HIV-1 reverse transcriptase inhibitor activity Wang et al. (2004). Another laccase has been shown capable of fighting aceruloplasminemia (a medical condition of lacking ceruloplasmin, a multi-Cu serum oxidase whose ferroxidase activity regulates iron homeostasis) Harris et al. (2004).

A novel application field for laccases is in cosmetics. Laccase catalysis can be used to synthesize flavonoids, textile dyes, cosmetic pigments, aromatic aldehydes, pesticides, and heterocyclic compounds. Laccase-based hair dyes are less irritant than those commonly available since laccase replaces hydrogen peroxide as an oxidizing agent in the dye formulation. Recently, proteins for skin lightening have also been developed Golz-Berner et al. (2004). Wang et al. (2004) carried out the assay for HIV reverse-transcriptase inhibitor activity using *Tricholoma giganteum* laccase purified from its fruiting body. The capability of laccase to synthesize new compounds might also be used to generate new therapeutic compounds for treatment of microbiol infection or cancer.

Laccases may find use as deodorants for personal-hygiene products, including toothpaste, mouthwash, detergent, soap, and diapers Omori et al. (2004), Markussen et al. (2006), Enomoto (2005). Protein engineered laccase may be used to reduce allergenicity Roggen et al. (2001).

16.2.5 Bioremediation

One of the major environmental problems, faced by the world today, is the contamination of soil, water, and air by toxic chemicals. With industrialization and the extensive use of pesticides in agriculture, the pollution of the environment with organic compounds has become a serious problem. Laccases have many possible applications in bioremediation. Laccases may be applied to degrade various substances such as undesirable contaminants, byproducts, or discarded materials.

White-rot fungi are known to produce lignin-degrading enzymes and are considered to be the most efficient in detoxification and decolorization of effluents. Decolorization of the effluents by a marine fungal isolate (*Flavodon flavus*, *Phaeospheria spartinicola, Halosarpheia ratnagiriensis* and *Sordaria fimicola*) has also been reported Raghukumar et al. (2008), D'Souza et al. (2006). However application to real dye-laden wastewater continues to be challenging venture, as these effluents also contain some pesticides, heavy metals and pigments that might inhibit the treatment processes Zouari-Mechichi et al. (2006). Due to their chemical structure dyes are resistant to fading on exposure to light, water and different chemicals and most of them are difficult to decolorize due to their synthetic origin. Government legislation is becoming more and more stringent, especially in the more developed countries, regarding the removal of dyes from industrial effluents Poots and McKay (1976). The main concern arises, as several dyes are made from known carcinogens such as benzidine and other aromatic compounds. Currently existing processes to treat dye wastewater are ineffective and not economical. Therefore, the development of processes based on laccases seems to be an attractive solution due to their potential in degrading dyes of diverse chemical structure including synthetic dyes currently employed in the industry Hou et al. (2004), Couto et al. (2005).

Moreover, chemical and physical treatments methods, including ultrafiltration, ion exchange, and lime precipitation, are expensive, so alternative biotreatment processes are now being considered. Laccase and peroxidase oxidize phenolics to aryl-oxy radical insoluble complexes. Enzyme-mediated bioremediation processes include polymerization among pollutants themselves or copolymerization with other nontoxic substances such as humic materials, thus facilitating easy removal by such means as adsorption, sedimentation, or filtration Rabinovich et al. (2004), Gianfreda et al. (2006).

An oxidation of the olefin units by the LMS, could initiate a radical chain reaction, leading to the disintegration of the plastic, also this LMS can also be used to degrade polyurethanes Kimura and Watanabe (2004). LMS facilitates the degradation of phenolic compounds (environmental hormones) from biphenol,

alkylphenol 18 derivatives and also the decomposition of fluorescent brighteners Uematsu et al. (2001), Saito et al. (2004) Nakatani and Yoshida (2003), Chakar and Ragauskas (2001).

Another potential environmental application of laccases is the use of laccase in the bioremediation of contaminated soils, organic pollutants such as xenobiotics, PAHs, chlorophenols, and other contaminants present in the soils can be oxidized by the use of laccase and LMS Duncan (2004), Suzuki et al. (2004), Kajiuchi et al. (2006), Shirai et al. (2005). Immobilized laccase has also been found to be useful to remove phenolic and chlorinated phenolic pollutants Wang et al. (2004), Guebitz et al. (2004), Patel et al. (2009). Laccase was found to be responsible for the transformation of 2, 4, 6-trichlorophenol to 2, 6-dichloro-1, 4-hydroquinol and 2, 6-dichloro-1, 4-benzoquinone Leontievsky et al. (2000). LMSs (laccase mediator system) have been also used to oxidize alkenes, carbazole, N-ethylcarbazole, fluorene, and dibenzothiophene Niku and Viikari (2000), Bressler et al. (2000). Isoxaflutole is an herbicide activated in soils and plants to its diketonitrile derivative, the active form of the herbicide: laccases are able to convert this diketonitrile into the acid Mougin et al. (2000). Laccase can be also used to reduce the concentration of synthetic heterocyclic compound such as halogenated organic pesticides in the soil Duncan (2004). LMS has been extensively studied in the oxidation of recalcitrant PAHs, main components of several ship spills. In this sense, LMS is being included in several enzymatic bioremediation programs Alcalde et al. (2006).

Fossil fuels emit harmful sulfur-containing compounds during postcombustion or precombustion processes. The emission of such harmful chemicals can be reduced by various chemical and physical desulfurization methods but they require extreme conditions like high temperature and pressure and high maintenance costs. On the contrary, desulfurization by enzymes such as laccase can be done under milder conditions and these methods remove organic sulfurs more efficiently Villaasenor et al. (2003), Xu et al. (2006a, b).

Coal solubilization offers the possibility of converting coal to a wide range of organic chemicals which include low-grade ores and liquid fuels. However, the irregular complex structure and insoluble nature of coal make it much less susceptible to bioconversion Catcheside and Ralph (1999). In the recent years, many research efforts have been made to find microbial biocatalysts capable of depolymerizing coal into low-molecular-weight substances. Certain basidiomycetes have the potential ability to depolymerize coal humic substances and several studies indicate that ligninolytic enzymes are involved in the process Keum and Li (2004). Laccase which uses only molecular oxygen as a cofactor may prove to be economical. *T. versicolor* is able to decolorize coal-derived humic acids under conditions where laccase is active and peroxidase activity is zero or negligible Fakoussa and Frost (1999).

Phenyl-urea-based herbicides are widely used for control of grasses and broad-leaved weeds in winter cereal crops. These herbicides are potential pollutants of soil and cause oxidation reaction in soil, plant, and microbial system, including N-dealkylation, aryl, or ring substituent hydroxylation Ryan et al. (1981). As a result of oxidation process, hydroxylated metabolites are formed in soil, which appear as phenolic compounds and behaves like substrates of laccase. Jolivalt et al. (1999)

reported that laccase from white-rot fungus *T. versicolor* has an ability to transform the herbicide N,N′-dimethyl-N-(hydroxyphenyl) urea into insoluble purple phenolic compounds, p-benzoquinone, at pH 3, which can be further metabolized by other fungi. It has been observed that pH also determines the nature of reaction products. The lower pH is favorable for transformation process, as, at pH 3, p-benzoquinone (easily metabolized product) has been formed, whereas at pH 6 mixture of N, N′-dimethyl-N-(2, 5-cyclohexadiene-1-one)-4-xylidene urea (difficult to metabolize) has been formed. White-rot fungus *Phlebia brevispora* can degrade commercially produced 2, 4, 6-trichlorophenyl p-nitrophenyl ether herbicide Kamei and Kondo (2006). Laccase may also be used to eliminate odor emitted from places such as garbage disposal sites, livestock farms, or pulp mills.

16.2.6 Delignification of Lignocellulosics

Separation of lignin from cellulose fibers is an important step in processing of wood for manufacturing of paper pulp. Conventional methods involve chlorine-, sulfite-, or oxygenbased chemical oxidants which impose serious drawbacks of disposal of chlorinated and sulfide by-products or loss of cellulose fiber strength. To overcome these drawbacks, microbial or enzyme-based delignification systems can be used. Laccase is capable of degrading natural or synthetic lignin polymers. Oxidation by laccase results in breakage of aromatic and aliphatic C–C bonds and depolymerization of lignin Eggert et al. (1996). Presence of lignin in the forage used as ruminant feed affects its digestibility. Biodelignification of such agricultural lignocellulosics not only enhances the digestibility of the feed but also improves their nutritional value Okano et al. (2006), Arora and Sharma (2009).

16.2.7 Nanobiotechnology

New developments in catalysis and environment applications can be forseen in near future with the progress in nanotechnology. Nanoscience has grown rapidly in the last decade. The high potential impacts of nanotechnology almost cover all fields of human activity (environmental, economy, industrial, clinical, health-related, etc.). Nanostructures materials (nanoparticles, nanotubes, and nanofibers) have been used extensively as carrying materials for biosensoring, and biofuel cells. High surface to volume ratio in nanosystems heterogeneous enzymatic or catalytic reactions can be greatly enhanced. A biosensor is an integrated biological-component probe with an electronic transducer, thereby converting a biochemical signal into a quantifiable electrical response that detects, transmits and records information regarding a physiological or biochemical change D'Souza (2001). Nanotechnology contributes to the development of smaller and more efficient biosensor through controlled deposition and specific adsorption of biomolecules on different types of surfaces, achieving

micro and nanometer order. Some of the major attributes of a good biosensing system are its specificity, sensitivity, reliability, portability, real-time analysis and operation simplicity D'Souza (2001). Thus laccases can be applied as biosensors or bioreporters. Laccase-containing biosensors for detecting O_2, glucose, aromatic amines, phenolic compounds, and a wide variety of reducing substrates have been developed Haghighi et al. (2003).

Laccase covalently conjugated to a bio-binding molecule can be used as a reporter for immunochemical (ELISA, Western blotting), histochemical, cytochemical, or nucleic acid-detection assays Karos et al. (2006), Ju and Du (2005). The bioreporter applications are of interest for the high-sensitivity diagnostic field. Laccase that is covalently conjugated to an antibody or antigen can be used as a marker enzyme for an immunochemical assay. In this application, binding of the antibody (or antigen) to its immunological counterpart is detected by localized laccase activity on a gel or a blot membrane, much like the conventional peroxidase or phosphatase-assisted immunochemical assays Yaropolov et al. (1994). Under certain conditions, the antibody–antigen binding impairs the function of conjugated laccase, thus allowing immunochemical detection through modulation of laccase activity Messerschmidt (1994).

Two types of laccase-based O_2 sensors are widely used. One type monitors visible spectral changes (at 600 nm) of laccase resulting from the reoxidation of the type 1 copper in laccase by O_2. Another type monitors current or voltage change from a modified oxygen electrode on which O_2 reduction is enhanced under the electrocatalysis of immobilized laccase. For detecting phenols, anilines, or other reducing substrates, three types of laccase-based sensors have been reported Yaropolov et al. (1994). One type detects the photometric change resulting from the oxidation of a chromogenic substrate; the second type monitors the O_2 concentration change that is coupled to the substrate oxidation, and the third type uses an electrode that replace O_2 as the acceptor for the electrons drawn from the substrate (through laccase). Laccase-based biosensors can be used for different electrochemical measurements Cordi et al. (2007).

In addition to biosensors, laccases could be immobilized on the cathode of biofuel cells that could provide power, for example, for small transmitter systems Park et al. (2003), Palmore et al. (2004), Armand et al. (2005). Biofuel cells are extremely attractive from an environmental point of view because electrical energy is generated without combusting fuel, thus, providing a cleaner source of energy. For example, a bio-implantable electrochemical cell system for active implantable medical devices is described by Choi et al. (2005). In typical example, the fuel cell includes an electrode structure consisting of immobilized anode and cathode enzymes deposited on nano structured high-surface-area metal nanowires or carbon nanotube electrodes. The anode enzyme comprises immobilized glucose oxidase and the cathode enzyme comprises immobilized laccase. Glucose is oxidized at the surface of the anode and oxygen is reduced at the surface of the cathode. The coupled glucose oxidation/oxygen reduction reactions provide a self-generating current source. In another example, the nanowires or carbon nanotubes, along with the adjacent surface anode and cathode electrodes, are coated with immobilized

glucose oxidase and immobilized laccase containing biocolloidal substrates, respectively. This results in the precise construction of enzyme architecture with control at the molecular level, while increasing the reactive surface area and corresponding output power by at least two orders of magnitude.

Hammond and Whitesides (1995) have introduced a method to pattern ultrathin ionic multilayer films with micron-sized features onto surfaces building a patterned alkanethiol monolayer with ionic functionality onto a gold surface. Chen et al. (1998) showed a biotechnological application of such micropatterned surfaces: the production of islands of micrometer size of extracellular matrix, where the pattern of these islands could determine the position and distribution of bovine and endothelial cells. The control of the nature and the density of the groups (e.g. alkys, amides, alcohols) of a surface built with assembled monolayers has been used successfully to investigate the non-specific adsorption of proteins Sigal et al. (1998). Martele et al. (2003) have shown that micro patterning is an efficient method for the immobilization of laccases on a solid surface in order to develop a multi-functional biosensor. Also, Roy et al. (2005) found that cross-linked enzyme crystals (CLEC) of laccase from *Trametes versicolor* could be used in biosensor applications with great advantage over the soluble enzyme. More recently, Cabrita et al. (2005) have immobilized laccase from *Coriolus versicolor* on N-Hydroxysuccinimide-terminated self-assembled monolayers on gold. This procedure could be useful for the further development of biosensors. In addition, an enzyme electrode based on the co-immobilization of an osmium redox polymer and a laccase from *T. versicolor* on glassy carbon electrodes has been applied to ultrasensitive amperometric detection of the catecholamine neurotransmitters dopamine, epinephrine and norepinephrine, attaining nanomolar detection limits.

The layer-by-layer technique (LbL) shown by Decher (1997) can be used to build macromolecular structures down to nanometer control leading to surfaces of well-defined thickness (Fig. 16.7a). Recently, flat polyelectrolyte multilayers built by alternating adsorption of oppositely charged polyelectrolytes have been used to recrystallise bacterial proteins making the building of artificial cell walls possible Toca-Herrera et al. (2005). The LbL technique has also been used to build hollow polyelectrolyte capsules after core removal Donath et al. (1998). Further application of the sequential adsorption of oppositely charged polyelectrolytes onto enzyme crystal templates would permit their encapsulation. Caruso et al. (2000) showed that the encapsulated enzyme could retain 100% of its activity after incubation for 100 min with protease. The permeability properties of the wall capsule are important for the proper function of the encapsulated enzyme. Antipov et al. (2002) investigated the permeability properties of hollow polyelectrolyte multilayer capsules as a function of pH and salt concentration. It was shown that the capsule wall was closed to a pH value of 8 and higher, but at pH values lower than 6 the macromolecules permeate into the capsule interior. In this way, the authors showed how to open and close the capsule wall in a reversible way. This mechanism together with the LbL encapsulation technique permits the development of microreactors Also, colloidal particles covered with polyelectrolytes and phospholipids have been used to host and activate rubella virus Fischlechner et al. (2005) . This type of system is shown in Fig. 16.7b.

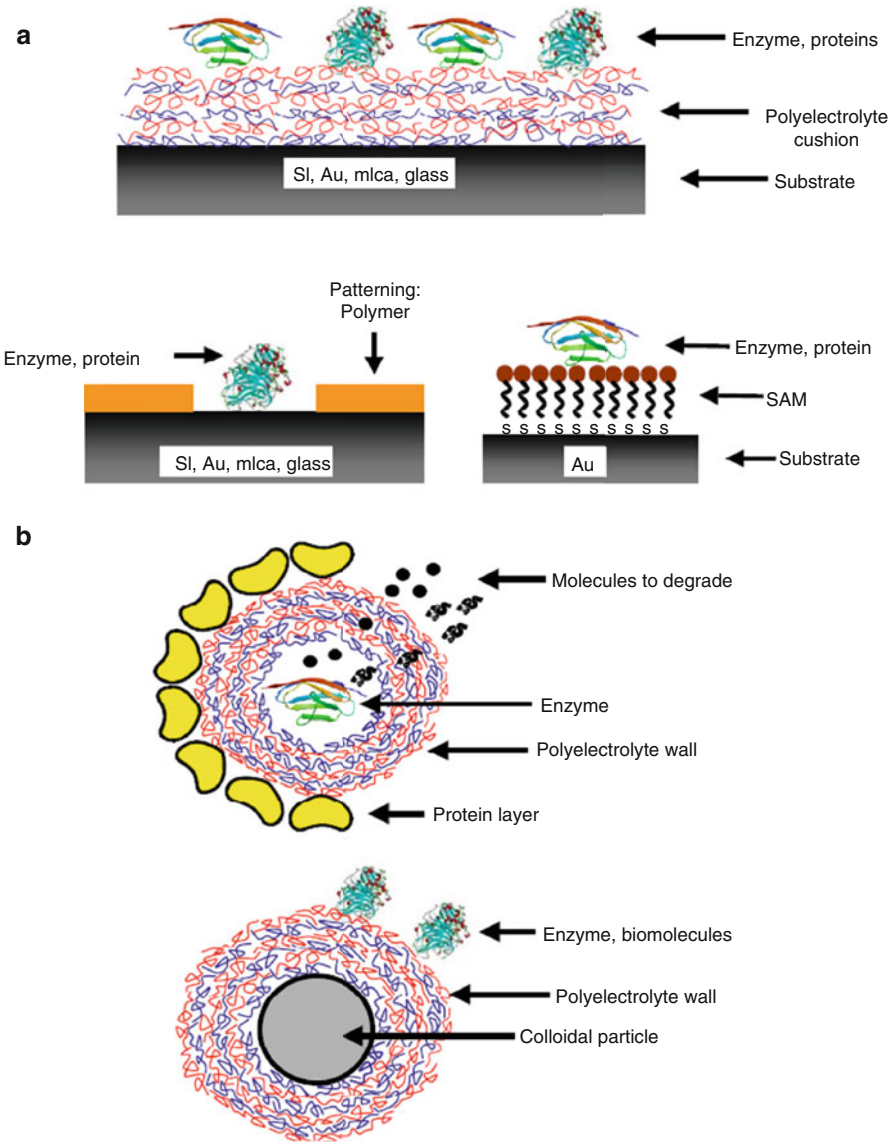

Fig. 16.7 (**a**) 2D supramacromolecular structures that can be used to immobilise biomolecules. Several structures are suitable: polylectrolyte multilayer, micropatterning and self-assembled monolayers (SAMs). (**b**) 3D supramacromolecular structures that can be used to build microreactors and immobilise biomolecules (Adapted from Couto and Herrera 2006)

Laccase catalysis can be used to assay other enzymes. In these assays, either the enzyme of interest catalyzes the production of a compound whose subsequent oxidation by laccase generates detectable physical change or a product from laccase

catalysis (whose production is accompanied by a detectable physical change) is quenched by the activity of the enzyme of interest.

In the LANCE (Laccase-nanoparticle conjugates for the elimination of micropollutants) project a nanoparticle (NP)-based technology has been under development. Cheap and resistant oxidative enzymes, i.e. laccases are immobilized onto the surface of the particles in order to produce systems possessing a broad substrate spectrum for the degradation of cocktails of recalcitrant pollutants. One of the objectives was to produce NPs that are compatible with wastewater treatment and that can be synthesized in a cost-effective and large-scale fashion, e.g. silica based NPs using flame spray pyrolysis and emulsion based techniques. The modified particles are then applied in bioreactors where they are retained, i.e. membrane bioreactors or perfusion basket reactors to eliminate pollutants from the wastewater. Such reactors allow multi-cycle use of the NPs coated with active enzymes and thus can contribute to decrease the treatment costs

16.2.8 Synthetic Chemistry

In the future laccases may also be of great interest in synthetic chemistry, where they have been proposed to be applicable for oxidative deprotection Semenov et al. (1993) and production of complex polymers and medical agents Uyama and Kobayashi (2002), Kurisawa et al. (2003) Nicotra et al. (2004). Recently, Mustafa et al. (2005) synthesized phenolics colorants by using an industrial laccase named Suberase® (Novo Nordisk A/S, Bagsvaerdt, Denmark).

16.2.9 Organic Synthesis

Organic synthesis of chemicals suffers from several drawbacks including the high cost of chemicals, cumbersome multistep reactions and toxicity of reagents Yaropolov et al. (1994), Riva (2006). Laccases can be very useful in synthetic chemistry where they have been proposed to be applicable for production of complex polymers and medical agents. Recently, increasing interest has been focused on the application of laccase as a new biocatalyst in organic synthesis Mayer et al. (2002). Laccase provide an environmentally benign process of polymer production in air without the use of H_2O_2 Kobayashi et al. (2003), Mita et al. (2003).

The laccase reactions presumably proceed by formation of a radical cation, with subsequent deprotonation of the hydroxyl group to give a radical. The radical may then undergo formation of a quinonoid derivative Manda et al. (2007) or intermolecular nucleophilic attack by itself to produce homomolecular dimers. These dimers can be C–O coupled (defined as type I dimers) or C–C coupled (defined as types II a and II b dimmers). These dimer formation reactions are referred to as “oxidative coupling,” “oxidative condensation,” and “phenolic oxidative coupling,” respectively.

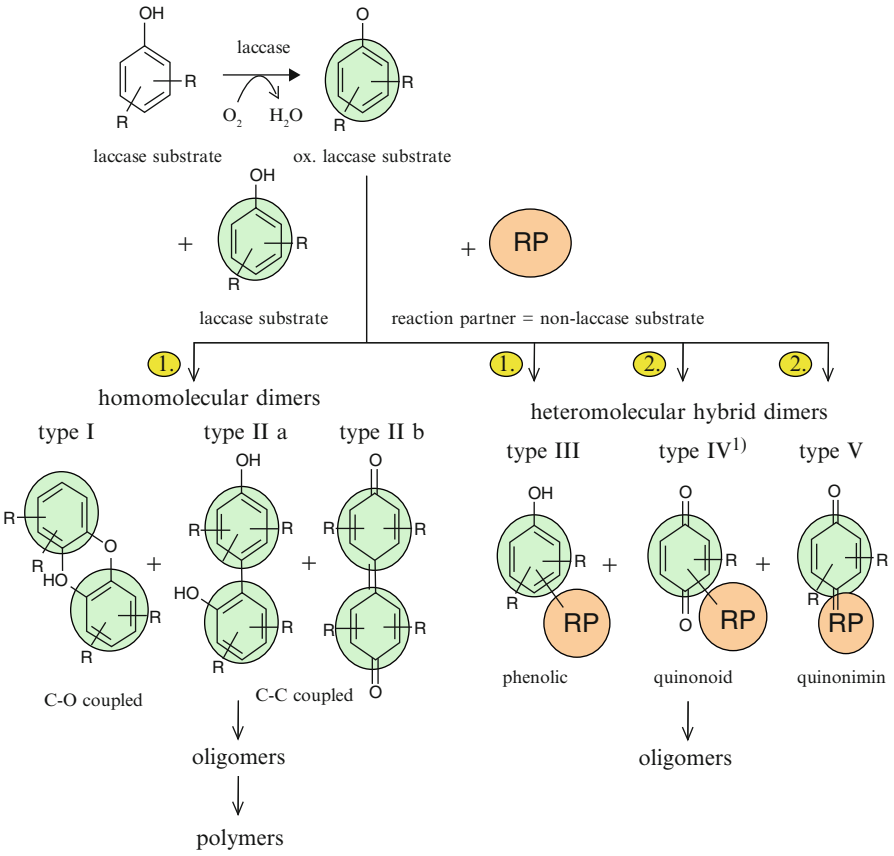

Fig. 16.8 Formation of homomo-lecular and heteromolecular dimers (simplified structure). *R* several substituents or ring systems. *1.* Reaction type = oxidative coupling, oxidative condensation or phenolic oxida-tive coupling. *2.* Reaction type = oxidation coupled with nuclear amination. *1)* The formation of type IV dimers requires dihydroxylated laccase substrates; *para*-dihydroxylated substrates result in *para*-quinonoid products; *ortho*-dihydroxylated substrates result in *ortho*-quinonoid products (Adapted from Mikolasch and Schauer 2009)

After longer reaction times, oligomers and polymers can be generated from the dimers. Apart from these homomolecular coupling activities, laccases are also able to couple a laccase substrate (aromatic with hydroxyl group or groups) and a non-laccase substrate (variable reaction partner) to create new heteromolecular hybrid molecules. The reaction can result in (1) phenolic structures (type III dimers), (2) quinonoid structures (type IV dimers), or (3) quinonimin structures (type V dimers). If the nonlaccase substrate involves a free amino group, the quinonoid or the quinonimin structures can be generated by oxidation coupled with nuclear amination. The reaction products types III, IV, and V can be C–O, C–S coupled heteromolecular dimers or trimers (Fig. 16.8).

Enzymatic transformation of antibiotics: Laccase is of great interest in the enzyme-catalyzed production of new antibiotics via phenolic oxidation, phenolic oxidative coupling Agematu et al. (1993), Anyanwutaku et al. (1994) and oxidation coupled with nuclear amination Mikolasch et al. (2006, 2007). Whereas the phenolic oxidative couplings of penicillin X derivatives combine two molecules of one antibiotic containing a phenolic moiety to form ortho-ortho C–C, ortho-para C–C/C–O, and ortho C–O coupling products, the phenolic oxidative couplings of hydroquinone and mitramycin (Anyanwutaku et al. 1994, Mikolasch et al. 2009) result in the formation of heteromolecular hybrid dimers. The antibiotic activity of the formed products was lower than the activity of the substrates. Furthermore, several novel penicillin and cephalosporin derivatives were synthesized by oxidation followed by nuclear amination forming C–N coupled aminoquinonoid heterodimers type IV Mikolasch et al. (2006, 2007) and Mikolasch and Schauer (2009). The aminoquinonoid heterodimers consist of one molecule of the oxidized laccase substrate and one molecule of the reaction partner.

Another possible use of laccase-catalyzed amination in the field of antibiotic synthesis is the derivatization of new bioactive compounds isolated from biological resources. In this regard, we reported the straightforward laccase-catalyzed amination of dihydroxylated arenes with N-analogous corollosporines Mikolasch et al. (2008). In biological assays, the amination products obtained are more active than the parent compounds. This technology for derivatization of potentially active lead compounds has many advantages over other classical synthetic technologies both because a variety of new substances can be synthesized smoothly within a short time and because sensitive natural substances can be functionalized in this way. In this respect, it is worth noting that active compounds, which are not themselves substrates of laccase, can be linked with the appropriate substrates of the enzyme Mikolasch and Schauer (2009).

Derivatization of amino acids: The enzymatic derivatization of pure amino acids by use of laccase was achieved by oxidation coupled with nuclear amination of dihydroxylated laccase substrates with amino acids. The main products were identified as heteromolecular dimmers type IV and demonstrate that laccase-catalyzed C–N coupling occurred on the α-amino group. During the incubation of L-phenylalanine with para-hydroquinones using laccase as biocatalyst, one or two main products were formed. Both the α- and the ε-amino group of the amino acid lysine participates in the laccase-catalyzed C–N-coupling Manda (2006), Mikolasch and Schauer (2009). Hence, these enzymatic reactions may be suitable to cross-link proteins which contain large amounts of lysine and may be important for developing new types of adhesives and biomaterials (Fig. 16.9).

Production and transformation of further biologically active compounds: Apart from antibiotics and amino acids a broad diversity of biologically active compounds can be transformed or produced by laccase-catalyzed reactions. In addition, the transformation of toxic substances may lead to new structurally interesting molecules. The biological activities ranges from antioxidative, hormonal, anti-inflammatory, analgesic, central stimulant, central depressant, anti-secretory, sedative to anti-proliferative, anti-neoplastic, and 5-lipogenase-suppressive effects. Although most

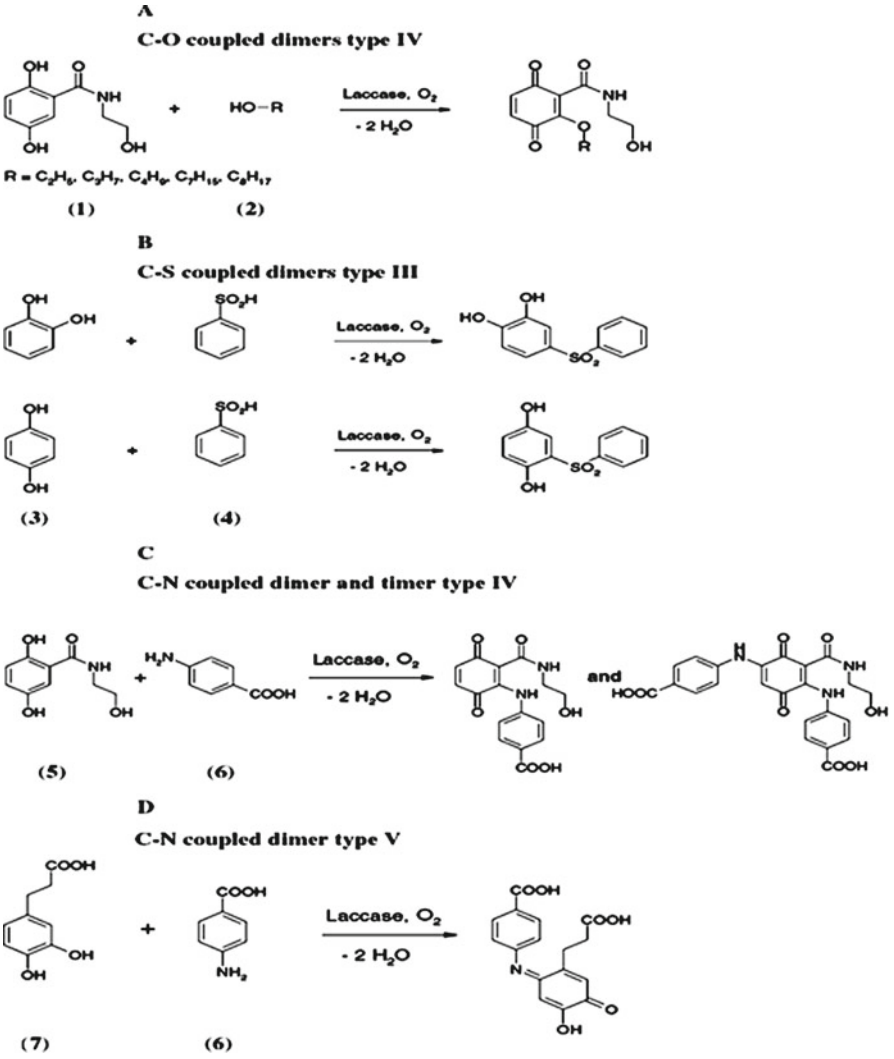

Fig. 16.9 Examples of enzymatic transformation resulting in heteromolecular dimers (**a**) from 2,5-dihydroxy-*N*-(2-hydroxyethyl)-benzamide *1* and different alcohols *2*. (**b**) coupling reaction products of hydroqui-nones *3* and benzene sulfinate *4*. (**c**) heteromolecular dimers and timers from 2,5-dihydroxybenzoic acid derivatives *5* and p-aminobenzoic acid *6* obtained by oxidation coupled with nuclear amination. (**d**) Heteromolecular dimer from dihydrocaffeic acid *7* and *p*-aminobenzoic acid *6* obtained by oxidation coupled with nuclear amination (Adapted from Mikolasch and Schauer 2009)

of the antioxidative compounds resulted from oxidative couplings, the structures of the reaction products are quite diverse. Furthermore, the synthesis of phenoxazinone derivatives has also been described Eggert et al. (1995), Mikolasch and Schauer (2009), Bruyneel et al. 2008). In these cases, the laccase generates a free radical

from the substrate and two molecules of the radical disproportionate to the laccase substrate and a quinonimin structure, which react with each other forming phenoxazinone derivatives.

Applications of laccase for the production of polymers and new biomaterials: Ligninoolytic enzymes from white rot fungi are useful for polymerization, detoxification, and diminishing of bioavailability of environmental pollutants Jonas et al. 2000, Schultz et al. 2001, Uchida et al. (2001), their application in organic and polymer chemistry remains a relative new field in fungal and white biotechnology.

The laccase-catalyzed preparation of polymeric complexes which can be carried out using simple procedures and mild conditions in the absence of toxic side products (e.g., formaldehyde) has been increasingly investigated in the past decade as an environmentally friendly alternative to the usual chemical production of these compounds. By utilizing specific enzymatic catalysis and regio- and chemoselective polymerizations have been observed, yielding functional and useful polymers, which are often difficult to synthesize by conventional methodologies Uyama and Kobayashi (2003), Mikolasch and Schauer (2009).

By using laccase and other biocatalysts (peroxidases, tyrosinases), several new polymers have been developed. Oxidative polymerization of phenol derivatives has now revisited as a clean and low-loading process for synthesis of phenolic polymers Kobayashi and Higashimura (2003). Laccase-catalyzed cross-linking reaction of new urushiol analogues for the preparation of "artificial urushi" polymeric films (Japanese traditional coating) has been demonstrated Ikeda et al. (2001). Budolfsen et al. (2004) also mentioned that laccase induced radical polymerization of acrylamide with or without mediator. Laccases are also known to polymerize various amino and phenolic compounds Aktas et al. (2003), Kunamneni et al. (2010). Recently, to improve the production of fuel ethanol from renewable raw materials, laccase from *T. versicolor* was expressed in *S. cerevisiae* to increase its resistance to (phenolic) fermentation inhibitors in lignocellulose hydrolyzates Larsson et al. (2001), Mikolasch and Schauer (2009).

Additionally, laccase has been used for polymerization processes to form melanin like colored pigments. Polyphenols are the basic building blocks of natural skin, leather, and hair pigments and can be enzymatically polymerized to create versions of natural black, brown, and orange melanins Covington et al. (2005), Mikolasch and Schauer (2009). Using dihydroxy naphthalenes as substrates, for example leather, can be colored by laccase action. The colored products are lightfast and bound to the natural collagen by a covalent tanning manner Suparno et al. (2007). In addition, wool and cellulosic cotton fibers can be dyed in the presence of laccase and suitable substrates Shin et al. (2001), Calafell et al. (2007), Kim et al. (2008). Coating of lignocellulosic material can not only color the surface but also give it antimicrobial properties Schroeder et al. (2007), Mikolasch and Schauer (2009). In general, it is important to distinguish between homopolymers synthesized from one type of laccase activated monomer and heteropolymers formed from two or more different monomers from which at least one must be a laccase substrate. Because different types of nonlaccase substrates can be integrated in laccase-synthesized heteropolymers, various types of polymers varying in their properties

can be made. In this way, the common biopolymers (proteins, polysaccharides) can be changed in quality and function resulting in new biomaterials. Examples are new adhesives, protein conjugates, or coating materials of implants Kunamneni et al. (2008), Mikolasch and Schauer (2009).

16.3 Conclusions and Future Perspectives

This article summarizes the available literature about the properties and possible industrial and biotechnological uses. Because of their versatile biochemical properties, high protein stability, broad substrate spectrum and wide range of applications, laccases are very impressive and useful biocatalyst. Laccases are promising enzyme to replace many conventional chemical processes of industries such as paper and pulp, textile, pharmaceuticals, food and nanobiotechnology. However the most important obstacles to commercialize the applications of laccase are the lack of sufficient enzyme stocks and cost of redox mediators. Laccase catalyze not only catabolic and depolymerization processes but depending upon the reaction conditions can carry out various dimerization, oligomerization and polymerization reactions.

The use of laccase in organic synthesis does show a promising green alternative. In the near future the practical use of fungal laccase for troublesome transformation (digestion of lignocelluloses to use as a carbon source, modification of lignosulfonates for production of emulsifier, surfactant and adhesives, synthesis of polymers with properties as redox films for bioelectronics devices, synthesis of antibiotic and amino acid) will expand the need for this biocatalyst. Laccase may be used in future as an important tool for combinatorial biochemistry. In order to achieve over production of this catalyst, heterologous host and also their modification by chemical means or protein engineering to obtain most robust and active enzyme.

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Chapter 17
Biotechnological Applications of Biocatalysts from the Firmicutes *Bacillus* and *Geobacillus* Species

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Abstract *Bacillus* and *Geobacillus* species are the dominant workhorses in industrial biotechnology. The ability of these bacteria to produce a variety of extracellular enzymes, such as amylases, xylanases, proteases, phytases, carbonic anhydrases, catalases, pectinases and others, has ranked them among the most important enzyme producers. These bacteria produce enzymes that are active in broad pH and temperature ranges, and thus, have led to the development of processes for large scale production of a variety of enzymes with suitable properties for industrial applications. The industrial enzymes are produced by submerged as well as solid state fermentations. Classical mutation and selection techniques, and protein engineering strategies have been used for developing enzymes with the desirable characteristics. The enzymes find applications in starch, paper, food, feed, textile and several other industries.

Keywords *Bacillus* • *Geobacillus* • Enzymes • Submerged fermentation • Solid state fermentation • Protein engineering • Directed evolution

17.1 Introduction

The genus *Bacillus* proposed by Cohn (1872) includes diverse bacterial strains that are commonly found in soil, composts and water sources like rivers, coastal waters and estuaries (Priest 1989). Bacteria of this genus are aerobic/facultatively anaerobic, endospore forming, rod shaped, straight or curved, growing solitarily or in groups, and arranged in pair or chains. The systematics of the genus is very complex and puzzling. A high phylogenetic heterogeneity was observed in the 16S rDNA

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sequences of the bacteria of this genus, and therefore, besides *Bacillus*, many new genera such as *Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus, Salibacillus, Gracilibacillus, Ureibacillus, Geobacillus, Amphibacillus, Anoxybacillus, Thermobacillus, Fiobacillus, Halobacillus* and others have been created. The ubiquitous occurrence of *Bacillus* and related genera is due to their nutritional versatility, broad tolerance for environmental extremes, and stress tolerant thick walled endospores. The microbial enzyme fermentations are dominated by *Bacillus* and *Geobacillus* species. The secretory products of the species of *Bacillus* and *Geobacillus* are considered by Food and Drug Administration (FDA) as generally regarded as safe (GRAS), and thus, led to the widespread use of their products in food, beverage and detergent industries (Satyanarayana et al. 2005). The ability of *Bacillus* and *Geobacillus* species to grow at acidic, alkaline and neutral pH, at elevated temperatures and to produce high titres of extracellular enzymes active in broad pH and high temperature ranges has positioned them among the most important industrial enzyme producers (Fogarty et al. 1974). Advanced technologies like directed evolution and protein engineering help in improving and tailoring the properties of proteins to suit various industrial applications. Besides extracellular enzymes, *Bacillus* and *Geobacillus* species are also known to produce antibiotics, nucleotides, the flavor agent ribose and poly-γ-glutamic acid (DeFuria and Claridge 1976; Ogawa et al. 1997; De Wulf and Vandamme 1997; Sauer et al. 1998). The secretory system of *Bacillus* species has made them preferred hosts for gene cloning to produce a variety of products. This chapter is aimed at reviewing the potential applications and properties of biocatalysts produced by the species of *Bacillus* and G*eobacillus*.

17.2 Starch-Hydrolyzing Enzymes

17.2.1 α*-Amylases*

α-Amylase (1, 4- α-D-glucan glucanohydrolase, E.C. 3.2.1.1) is a widely distributed endo-acting enzyme that hydrolyses α-1,4 glycosidic linkages randomly to produce linear and branched oligosaccharides in an α-anomeric configuration. These enzymes are industrially important as they have diverse applications in starch, food, textile and paper industries (Pandey et al. 2000). Among amylases derived from various sources like plants, animals and microorganisms, microbial enzymes are known to fulfill industrial demands. The accessibility of microbial amylases with required properties have completely replaced the chemical hydrolysis of starch in starch processing industries. The action of α-amylase on starch results in the quick loss of viscosity by the formation of oligosaccharides. The hydrolysis of α-1, 4 linkages on branch points of the dextrins results in the formation of α-limit dextrins (four or more glucose units). The extended action of α-amylase further results in the formation of a mixture of glucose, maltose, maltotriose and maltooligosaccharides. Amylases that form maltose in high concentrations are known as maltogenic α-amylases (Antranikian 1992).

A wide variety of liquefying and saccharifying α-amylases are known. Liquefying α-amylase rapidly reduce the viscosity of the starch, while saccharifying α-amylases reduce the viscosity slowly and relative to the concentration of reducing sugars liberated (Antranikian 1992).

Every application of α-amylase needs distinctive properties in the context of specificity, stability, temperature and pH dependence (McTigue et al. 1995). A large number of α-amylases with diverse properties ranging from thermostable to acid-stable and alkalistable are known from *Bacillus* and *Geobacillus* species (Malhotra et al. 2000; Ezeji et al. 2005; Hashim et al. 2005; Ezeji and Bahl 2006; Saxena et al. 2007; Burhan 2008; Dheeran et al. 2010; Mollania et al. 2010; Sharma and Satyanarayana 2010). Although most of the α-amylases are active at neutral pH, there are also reports of acidic and alkaline α-amylases. Thermostable α-amylases are important for various applications in food, chemical and pharmaceutical industries. Fermentation at elevated temperatures is considered to be advantageous as the cost of cooling is reduced, substrates are properly solubilized at high temperature, viscosity of the solution is reduced allowing the proper mixing and pumping, and the risk of contamination is also reduced (Pancha et al. 2010). The extracellular enzymes of genus *Bacillus* have several industrial applications (Fogarty et al. 1974). Thermostable α-amylases are important as they have diverse applications and also for studying the factors responsible for the biocatalysis and protein stability at high temperatures (Manning and Campbell 1961; Suzuki et al. 1987; Bertoldo and Antranikian 2002). Thermostability along with acid stability in α-amylase can be important characteristic for starch industry. In starch saccharification process, raw starch from wheat, maize, tapioca and others is hydrolyzed to maltodextrins by α-amylase, which are further saccharified into maltose, glucose and fructose syrups using β-amylase, glucoamylase and glucose isomerase, respectively. Presently the α-amylase used in starch industry is active at pH 6.5 and 95°C and requires Ca^{2+} for stability and/or activity. Since the pH of the native raw starches is around 3.2–3.6, the modulation of pH and removal of Ca^{2+} from the product streams in the subsequent stages adds to the process operating costs. The need for acidstable, Ca^{2+}-independent α-amylase has, therefore, been emphasized. Acid stable enzymes also have application in baking, as the pH of the dough is acidic (5.5) (Jones et al. 2008; Sharma and Satyanarayana 2010). Alkalistable α-amylases are important ingredients in detergents for automatic dishwashers and laundries (Kim et al. 1995).

Amylases are produced by submerged and solid state fermentations (SSF). There are various advantages and disadvantages of submerged fermentation like easy to control process parameters, inoculum is properly distributed in the medium and the high content of nutrients is ideal for the growth of bacteria, but this process is not cost effective. On the other hand, in solid state fermentation, microorganism on the moist solid substrate is grown in the absence of free flowing water. The solid substrates used have the capacity to absorb moisture and act as a source of nutrients necessary for the growth of microorganisms. Solid state fermentation is simple technique with less capital and energy requirements, superior productivity with better product recovery, reduced foaming and lower levels of catabolite repression. Further, this process is cheap with easily available substrates such as agriculture and food

industry byproducts (Stredansky et al. 1999). α-Amylase produced by SSF has various industrial applications (Babu and Satyanarayana 1993; Hashemi et al. 2010).

Production of α-amylase is affected by various physico chemical factors like composition of the medium, pH, temperature, inoculum age and size, carbon and nitrogen sources, aeration, agitation and phosphate concentration (Fogarty and Kelly 1979). Among various carbon sources, starch, fructose and glucose support high enzyme production (Ezeji et al. 2005). It has also been reported that the synthesis of carbohydrate degrading enzyme in *Bacillus* species is subject to catabolite repression in the presence of readily metabolizable substrates like glucose and maltose. Haseltine et al. (1996) reported the repression of α-amylase produced by *Sulfolobus solfataricus* in the presence of glucose.

The presence and absence of various amino acids, organic and inorganic nitrogen sources is correlated with the synthesis of amylase in different microorganisms. Nitrogen sources in combination significantly affect α-amylase production. Yeast extract and peptone are preferred nitrogen sources for α-amylase production by *Bacillus* spp. (Narang and Satyanarayana 2001; Saxena et al. 2007). The use of organic sources favored the α-amylase production in *Geobacillus thermodenitrificans* (Ezeji et al. 2005). Aiyer (2004) reported the combined effect of peptone and ammonium hydrogen phosphate on amylase production. Along with carbon and nitrogen sources, phosphate is important requirement for microorganisms as it regulates the synthesis of primary and secondary metabolites in microorganisms (Mertz and Doolin 1973). The presence of various metal ions in the medium like Mg^{2+}, Fe^{2+}, Na^{+}, K^{+} and Mn^{2+} stimulates enzyme production (Wu et al. 1999). The temperature and pH of the medium affects the growth of the microorganism, which in turn affects enzyme production.

The production of amylases by microbes is significantly affected by physical and chemical parameters of the medium (Babu and Satyanarayana 1993). Various methods are being used for the optimization of media components and culture variables. Statistical designs have often been used for optimizing the fermentation variables for α-amylase production (Rao and Satyanarayana 2003). Plackett and Burman design is a well-established statistical method for screening critical culture variables, while the Response Surface Methodology (RSM) provides information regarding the optimum levels of each variable, interactions among them and their effects on product yields. Statistical designs used for the selection of critical variables affecting enzyme production and the interaction among selected variables is a good and useful technique, and thus, has been used successfully for optimizing the α-amylase production by various microorganisms (Gangadharan et al. 2008; Hashemi et al. 2010; Sharma and Satyanarayana 2011). Ezeji and Bahl (2007) optimized the culture conditions for maximum α-amylase production in a bioreactor using full 2^4 and 3^2 factorial designs.

A number of amylases with varying physicochemical properties including pH, temperature optima, thermostability and pH stability, pI, effect of substrate concentration, and various cations have been purified and characterized from various *Bacillus* and *Geobacilllus* spp (Table 17.1). Various methods are used for the purification of α-amylases like anion and cation exchange, affinity and gel permeation

Table 17.1 Characteristics of α-amylases of *Bacillus* and *Geobacillus* species

Organism	Opt temp. (°C)	Opt. pH	Mol. wt (kDa)	K_m (mg ml^{-1})	V_{max} (U mg^{-1})	p*I*	References
Bacillus sp. A3-15	70	11	60.5/86	–	–	–	Arikan (2008)
Bacillus sp. PN5	90	10	–		–	–	Saxena et al. (2007)
Bacillus sp.	110	8.0	–	–	–	–	Pancha et al. (2010)
Bacillus subtilis JS 2004	70	8.0	–	–	–	–	Asgher et al. (2006)
Bacillus subtilis KCC 103	65–70	5–7	53	2.6	909	–	Nagarajan et al. (2006)
Bacillus sp. KR 8104	75–80	4.0–6.0	59	–	–	–	Sajedi et al. (2005)
Bacillus halodurans LBK34	60	10.5–11.5	107.2	–	–	4.41	Hashim et al. (2005)
Bacillus sp. I3	70	7.0	–	–	–	–	Goyal et al. (2005)
Bacillus sp. YX-1	40–50	5.0	56	–	–	–	Liu and Xu (2008)
Bacillus sp. IMD 435	65	6.0	63	–	–	–	Hamilton et al. (1999)
Bacillus sp. KR-8104	75–80	4.0–6.0	59	–	–	–	Sajedi et al. (2005)
Bacillus sp. *Ferdowsicous*	70	4.5	53	–	–	–	Asoodeh et al. (2010)
Geobacillus stearothermophilus JT2	55	7.0	–	–	–	–	Al-Qodah (2006)
Geobacillus thermodenitrificans HRO10	55	6.5–7.5	58	3.05	7.35 (U/ml)	–	Ezeji and Bahl (2006)
Geobacillus sp. IIPTN	80	5.0	97	36	222 (umol/mg/min)	–	Dheeran et al. (2010)
Geobacillus sp. LH8	80	5.0	52	–	–	–	Khajeh et al. (2009)

chromatography. The pH optima of α-amylases vary in the range between 2 and 12. The pH optima of most of the α-amylases are in the acidic and neutral range (Pandey et al. 2000). α-Amylase from *B. subtilis* AX20, *B. licheniformis*, *G. thermoleovorans* showed highest activity at pH 6.0, 6.5, and 8, respectively (Najafi et al. 2005; Malhotra et al. 2000). While α-amylases from *Bacillus* YX1 (Liu and Xu 2008), *Geobacillus* sp. IIPTN (Dheeran et al. 2010), and *B. acidicola* TSAS1 (Sharma and Satyanarayana 2010) exhibited pH optima at 4.0, 5.0 and 4.0, respectively.

Thermostability of the enzyme is important characteristic and determines primary structure of the protein. Many thermostable α-amylases have been purified and characterized from different *Bacillus* and *Geobacillus* spp., which exhibited varying physicochemical properties (Ezeji et al. 2005; Ezeji and Bahl 2006; Saxena et al. 2007; Khajeh et al. 2009; Dheeran et al. 2010; Mollania et al. 2010). Temperature optima ranging between 50°C and 100°C have been observed in these α-amylases, which are listed in Table 17.1. Thermostable and phytate-resistant α-amylases have been purified from *Geobacillus thermodenitrificans* HRO10 and *Geobacillus* sp. LH8 (Ezeji and Bahl 2006; Mollania et al. 2010). Dheeran et al. (2010) reported the purification and characterization of a protease-resistant 97 kDa α-amylase showing stability over a broad range of pH (5–10) and temperatures (40–120°C).

Various metals, substrate and other stabilizers affect thermostability of the enzyme (Vihinen and Mantasala 1989). Although α-amylase is known to be Ca^{2+}-dependent, there are reports of Ca^{2+}-independent α-amylases that do not require Ca^{2+} for stability and activity (Malhotra et al. 2000; Ezeji and Bahl 2007; Sharma and Satyanarayana 2010). Some amylases are also known to be inhibited by Ca^{2+} (Babu and Satyanarayana 1993). Besides Ca^{2+}, many other metal ions are known to stabilize α-amylase like Mg^{2+} (Ezeji et al. 2005), Mn^{2+} (Khajeh et al. 2009; Dheeran et al. 2010; Mollania et al. 2010), K^{+} (Khajeh et al. 2009; Mollania et al. 2010), Li^{+} (Ezeji et al. 2005), Ba^{2+}, Fe^{2+}, Cu^{2+} and Na^{+}. While ions like Hg^{2+}, Ni^{2+}, Zn^{2+}, Fe^{3+} have been shown to inhibit the α-amylase activity (Khajeh et al. 2009; Dheeran et al. 2010; Mollania et al. 2010). Mg^{2+}, $Ba^{2+}$and Cu^{2+} have also been shown to reduce the α-amylase activity (Khajeh et al. 2009; Dheeran et al. 2010; Mollania et al. 2010).

Genes encoding α-amylases have been cloned, expressed and purified from a number of *Bacillus* and *Geobacillus* species. Maltogenic α-amylase encoding gene with 588 amino acids from thermophilic *Bacillus* sp. WPD 616 was cloned and expressed in *E. coli*, which showed optimum activity at pH 6.0 and 50°C (Liu et al. 2006). Another α-amylase gene, Amy N, from *B. licheniformis* NH1 was also cloned, sequenced and expressed in *E. coli* using pDEST17 expression system. This recombinant α-amylase showed high thermostability at 85°C (60 min) as compared to wild type amylase (8 min) (Hmidet et al. 2008). Gene encoding a thermostable α-amylase with temperature optima of 75°C from *G. thermoleovorans* YN cloned in Bluescript ® II KS(+) vector in *E. coli* has been sequenced. The corresponding amino acid sequence showed 99% sequence similarity with the known α-amylases from different Bacilli and Geobacilli (Berekaa et al. 2007). A gene corresponding to thermo- and pH-stable maltogenic amylase in *G. caldoxylosilyticus* TK4 has been cloned into pET28a (+) vector and expressed in *E. coli* with 6xHis-tag at the N-terminus (Kolcuoglu et al. 2010).

An oxidation-resistant variant of a thermostable maltohexaose-forming α-amylase produced by *G. stearothermophilus* sp. US100 was derived by substituting methionine 197 with alanine. This mutein, known as AmyUS100DIG/M197A, showed higher resistance to oxidation as compared to Termamyl 3001, the well-known commercial α-amylase used in detergents (Khemakhem et al. 2009).

X-ray crystallographic studies revealed the presence of three domains in α-amylases. A central $(\alpha/\beta)_8$ TIM barrel also known as domain A forms the core of the molecule and contains three active site residues Asp231, Glu261 and Asp328. On the opposite side of this TIM barrel, domain B and C are located. The C terminal part of the sequence is present in domain C. The domain B is considered to be responsible for the differences in substrate specificity and stability (Svensson 1994). Structure-function relationship of α-amylase protein from *Bacillus* and *Geobacillus* species had been studied. A shift in pKa value of the catalytic residues to acidic pH was observed on studying the 3-dimentional structure of *Bacillus* sp. KR-8104. The amino acid substitutions affecting the *Bacillus* sp. KR-8104 amylase putative active site led to the formation of extra hydrogen bond between Glu261 and Arg229, thereby shifting the pH activity profile towards lower pH (Alikhajeh et al. 2007). Circular dichroism spectroscopic data of Ca^{2+}-independent, hyperthermostable high maltose-forming α-amylase from *G. thermoleovorans* (α-amylase gt) contained the 25% α-helix, 21% β-sheet, and 54% random coils. Fluorescence-quenching experiments using different quenchers revealed the importance of Trp moieties in stabilizing this α-amylase. No change in the α-helix content or the enzyme activity with an increase in temperature (60–100°C) had been observed, suggesting a critical role of the α-helix content in maintaining the catalytic activity of α-amylase (Rao and Satyanarayana 2008).

17.2.2 Amylopullulanase

Amylopullulanases (Saha et al. 1989) or type II pullulanases (Spreinat and Antranikian 1990) are unique enzymes capable of hydrolyzing α-1, 6 linkages of pullulan, as well as α-1, 4 linkages of starch, amylose, amylopectin, glycogen, and cyclodextrins leading to the release of small sugars such as glucose, maltose and maltotriose. Although amylopullulanases are most prevalent in thermophilic anaerobes, they have also been reported from some aerobic thermophiles, mesophiles and archaea (Noorwez et al. 2004). Among aerobes, certain species of *Bacillus* and *Geobacillus* are known to produce this enzyme, most of which are thermophilic. *Bacillus* strain 3183 (Shen et al. 1990), *Bacillus* sp. XAL 601 (Lee et al. 1994), *B. circulans* F-2 (Saha and Zeikus 1989; Kim et al. 1990; Kim and Kim 1995), *Bacillus* sp. TS-23 (Lin et al. 1996), *Bacillus subtilis* (Takasaki 1987), *Bacillus* sp. KSM 1378 (Ara et al. 1995a), *Bacillus* sp. DSM 405 (Brunswick et al. 1999), *G. thermoleovorans* NP33 (Noorwez et al. 2006), *Bacillus* sp. US149 (Roy et al. 2003) and *G. stearothermophilus* L14 (Zareian et al. 2009) have been reported to produce amylopullulanases.

Agitation rates in the range of 100–250 rpm are generally reported for the production of amylopullulanases from diverse bacteria (Brunswick et al. 1999).

Growth-associated production of amylopullulanases has been reported from *Bacillus* sp. 3183 (Shen et al. 1990), *G. thermoleovorans* NP33 (Noorwez et al. 2006) and *G. stearothermophilus* (Zareian et al. 2009). *Bacillus* strain 3183 grew well on monosaccharides and produced maximum titres of pullulanase in the presence of wheat flour or pullulan (Shen et al. 1990), while starch supported a high titre of amylopullulanase in *B. subtilis* (Takasaki 1987), *G. stearothermophilus* L14 and *G. thermleovorans* NP33 (Noorwez 2000). The production of amylopullulanase was induced by pullulan, but not by amylose or soluble starch. In most of the amylopullulanase producing organisms, high growth and enzyme production have been supported by yeast extract (vitamins) and trace elements. Nitrogen sources such as soybean, peptone, meat extract, and inorganic phosphate supported highest enzyme production and growth of *G. stearothermophilus* L14 (Saha and Zeikus 1989).

Most of the thermostable amylopullulanases have been found to be active at acidic or neutral pH, while amylopullulanase from *Bacillus* sp. KSM 1378 was active at alkaline pH values (Hatada et al. 1996). The amylase activity of this amylopullulanase has been completely inhibited by incubation of the enzyme at 40°C and pH 9.0 within 4 days, whereas the pullulanase activity remained at the original level under the same conditions (Ara et al. 1995a).

Dual hydrolytic activity associated with different active sites have been reported from *B. circulans* F-2 (Saha and Zeikus 1989), *Bacillus* sp. KSM 1378 (Hatada et al. 1996) and *G. thermoleovorans* NP33 (Noorwez 2000), while with single active site found in *B. subtilis* TU (Takasaki 1987), *Bacillus* sp. Strain XAL 601 (Lee et al. 1994), *Bacillus* sp. DSM 405 (Brunswick et al. 1999) and *Bacillus* sp.3183 (Saha et al. 1989). Amylopullulanase of *Bacillus* sp. KSM 138 could be cleaved by papain to generate a 114-kDa amylose hydrolyzing polypeptide and a 102-kDa pullulan hydrolyzing polypeptide (Ara et al. 1996), which was visualized after rotary shadowing by the method of Tyler and Branton (1980). The intact amylopullulanase molecules were seen as "castanet-like" or "bent dumbbell-like" shapes with a diameter of approximately 25 nm. Two globular (ovoid) heads of different sizes were clearly seen, and they were joined by a thin short linker region.

Amylopullulanase from *G. stearothermophilus* L14 was purified from culture supernatant by ammonium sulfate precipitation, anion-exchange and gel filtration chromatographies. The ratio of the pullulanolytic and amylolytic activities slightly changed as the enzyme was purified, but no considerable divergence between activities was noticed during the purification process (Zareian et al. 2009). Amylopullulanases from *Bacillus* sp. KSM-1378 was purified by chromatography on DEAE-cellulose, affinity chromatography on Sepharose 6B-α-cyclodextrin, and gel filtration on Sephacryl S-200 (Ara et al. 1995b).

The molecular cloning and sequencing of amylopullulanase gene has been reported from *Bacillus* sp. KSM 1378 (Hatada et al. 1996). The amylopullulanase gene (apuA) was cloned and expressed using the plasmid pAP101 and the host *B. subtilis* ISW1214. The product of apuA gene secreted in the culture medium was purified by chromatography on a DEAE-cellulose column and then by HPLC on a column of TSK G3000 SWXL.

Research on the amylopullulanases from thermophiles is interesting not only for understanding the mechanism of enzyme stability, but also for discovering improved enzymes with more efficient application in industrial starch hydrolysis process. Owing to its high thermal stability, the enzyme can be added before gelatinizing at the same time as α-amylase. Also because of its bifunctionality, it could reduce or even obviate the glucoamylase requirement. In the conventional enzymatic starch saccharification process, variation of parameters in different steps causes many handicaps in the industry. Due to pH variation, large amounts of salts have to be removed by ion exchangers. Besides being time consuming, these steps lead to reverse reactions and lower yields. Undesirable products like branched oligosaccharides, panose, isopanose and isomaltose are formed. Improvement in the starch-conversion process by finding new efficient and suitable enzymes with high thermostabilities, functioning in the acidic to neutral pH range, and independent of cations such as Ca^{2+} for stability/activity would significantly lower the cost of sugar syrup production (Saha and Zeikus 1989). Increasing the starch-saccharification process temperature would result in several benefits such as higher substrate concentrations, decreased viscosity and lower pumping costs, limited risk of bacterial contamination, increased reaction rates and decrease of operation time, lower costs of enzyme purification, and longer catalyst half-life, due to increased enzyme thermostability (Vieille and Zeikus 2001). Further studies are needed in order to improve the yield of enzyme production via regulation of the microbial metabolism or genetic manipulations.

17.2.3 Xylanases

Biomass is an alternative natural source for chemical feedstock with a very short replacement cycle enough to meet the demand in the world fuel market. Annually, 830 Gt of renewable plant biomass is produced that consists mainly of cellulose and hemicellulose (Rauscher et al. 2006). The naturally occurring lignocellulosic plant biomass consists on an average 23% lignin, 40% cellulose, and 33% hemicellulose by dry weight (Ahmad et al. 2009). Hemicellulose is composed of xylan as a major component that constitutes about 20–40% of total plant biomass (Ninawe et al. 2008; Nair et al. 2008) and accounts for approximately one third of all renewable organic carbon on earth (Ning et al. 2008). Xylan is a heteroglycan having a backbone made up of β-1, 4-linked D-xylopyranose residues with substitutions of L-arabinofuranose, D-glucuronic acid, and 4-O-methyl-D-glucuronic acid at 2′ and 3′ positions (Wakiyama et al. 2008). It represents an immense resource of biopolymers for practical applications, accounting for 25–30% of the dry biomass of woody tissues of dicots and lignified tissues of monocots and occurs up to 50% in some tissues of cereal grains (Moure et al. 2006). Being the major constituent of hemicellulose, it is the second most abundant renewable resource that can be degraded to useful end products. The development of inexpensive technologies based on hemicellulose is needed for various biotechnological applications. In the near future,

xylan, in combination with cellulose, will supply most of the global demand for raw materials. It is not unrealistic to foresee that biomass is likely to substitute coal and crude oil in another 50 years.

Being a heteropolymer, complete conversion of the hemicellulose requires action of several enzymes to cleave main chain as well as side chain: endoxylanase (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan 1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-L-arabinofuranosidase, E.C.3.2.1.55), and acetylxylan esterase (E.C. 3.1.1.72). Endo-β-1, 4-xylanases catalyze the hydrolysis of the backbone of xylan to produce xylooligosaccharides, which in turn can be converted to xylose by β-xylosidase (Collins et al. 2005; Zhang et al. 2007).

Microorganisms involved in carbon cycling produce a variety of carbohydrate degrading enzymes including xylanases (Yin et al. 2010). Some microorganisms produce these as free extracellular enzymes, while others produce a multi-enzyme complex (MEC) such as the cellulosome in which several enzymes such as cellulases and xylanases are combined within a complex. MECs have attracted great attention in biofuel technology as they potentially offer a solution to the more effective degradation of complex plant material into fermentable sugars (Dyk et al. 2010).

Xylanases have potential uses in the food, feed, biofuels and pulp and paper industries (Dutta et al. 2007; Bakri et al. 2008), and currently, the most promising application is in the prebleaching of pulps, mainly to reduce chlorine as a bleaching agent. The basis of this application is that the xylanases are active and stable at high temperatures under alkaline conditions (Dhiman et al. 2008). Since paper mills in India using rice and wheat straw as substrate for pulping are rich in arabinoxylans, xylanases capable of showing high activity against arabinoxylans are desirable. In addition, xylanases can be used in the hydrolysis of arabinoxylans for the production of xylooligosaccharides (XOS) that are used as FOSHU (Foods for Specified Health Use) in Japan and are being produced commercially from agro-residues in China and Japan (Sharma et al. 2010).

The biggest potential application of xylanolytic enzymes is prebleaching of kraft pulp in the pulp and paper industry that requires large amount of xylanases (Polizeli et al. 2005; Dhiman et al. 2008). There is limited understanding of the mechanism by which enzymatic prebleaching takes place. However, it was proposed that hemicellulose precipitated on the surface of the fibre during pulping were depolymerized by xylanases that opens up the pulp structure and allows access of chlorine and other chemicals employed in subsequent treatment stages (Kiddinamoorthy et al. 2008). This in turn imparts similar brightness to the paper by facilitating pulp delignification and bleach boosting. Enzymatic prebleaching is considered to be environment friendly as it reduces the amount of environmentally hazardous and toxic organochlorines in the effluent streams. While enzymatic prebleaching requires a thermostable and alkalitolerant enzyme, only a few xylanases are reported to be active and stable at alkaline pH as well as elevated temperatures (Mamo et al. 2006a, b; Sharma et al. 2007; Kiddinamoorthy et al. 2008). Most of the commercially available xylanases are of fungal origin, which show stability at acidic, neutral or very few in alkaline conditions and they have optimal activity at 30–60°C (Krisana et al. 2005).

On the other hand, xylanases produced by the species of *Bacillus* and *Geobacillus* exhibit a reasonable thermostability as well as alkalistability (Mamo et al. 2006a, b; Sharma et al. 2007). Continuing interest in *Bacillus* and *Geobacillus* xylanases is also evident in the recent work published on several aspects of enzyme production, purification, characterization, cloning and expression, and applications (Table 17.2) (Avcioglu et al. 2005; Heck et al. 2005; Sapre et al. 2005; Virupakshi et al. 2005; Battan et al. 2006; Choudhury et al. 2006; Sharma et al. 2007; Dhiman et al. 2008; Yin et al. 2010; Zhang et al. 2010a, b).

Each organism or strain has its own special physical and chemical parameters for maximum enzyme production. Cost of the medium is an important factor to be monitored while developing a production medium. The use of abundantly available and cost effective agricultural residues such as wheat bran, corncobs and wheat straw provide suitable methods to achieve higher xylanase yields and reduction of production cost (Battan et al. 2006; Kapoor et al. 2008).

In solid state fermentation (SSF) the microorganisms grow on moist substrates in the absence of free flowing water, where water is present in an absorbed or in complex form with the solid matrix and the substrate. Higher xylanase yields using SSF allows reduction of the overall cost of enzyme production. There are so many reports available on production of xylanase from *Bacillus* and *Geobacillus* sp. in SSF (Battan et al. 2006; Sindhu et al. 2006; Poorna and Prema 2007; Sanghi et al. 2008; Mohana et al. 2008; Bajaj and Singh 2010).

Although fed-batch and solid state fermentation techniques are reported to be more efficient than submerged fermentation (SmF), almost 90% of the industrial enzymes are produced by submerged fermentation, as it provide several advantages over the other methods, including reduction of contamination due to relatively short growth period, lower capital investment when compared to continuous processes for the same bioreactor volume with other techniques, higher conversion levels of the raw medium substrates due to the controlled growth period, less labor intensive production monitoring and well developed scale-up methods (Kapoor et al. 2008). Many *Bacillus* and *Geobacillus* species produce xylanases in media containing lignocellulosic agricultural residues as well as commercial xylan sources in submerged fermentation (Poorna and Prema 2006; Anuradha et al. 2007; Sharma et al. 2007; Sanghi et al. 2009; Ko et al. 2010).

Global markets for industrial enzymes have grown from €510 million in 2001 to €760 million in 2010, among which currently xylanase accounts for approximately 25–28% of total enzyme sales (Shrinivas et al. 2010). One of the most important large-scale biotechnological applications of recent years is the use of xylanases as bleaching agents in the pulp and paper industry. In conventional papermaking processes, manufacturers use large quantity of oxidizing chemicals, which has resulted in hazardous effluent disposal problems (Ayyachamy and Vatsala 2007). Treatment with xylanases facilitates the chemical extraction of lignin from pulp and leads to a significant reduction in the use of hazardous chemicals required for bleaching. For biobleaching applications, the candidate xylanase must be thermostable, alkalistable, and stable on kraft pulp, and other various properties, such as low molecular weight, net ionic properties, and specific action pattern must suit the process requirements.

Table 17.2 Characteristics of xylanases from *Bacillus* and *Geobacillus* species

Microorganism	Growth conditions	Production (IU/ml)	Optimum conditions		References
			Temperature (°C)	pH	
Bacillus circulans D1	D-maltose, smf	9.18	60	5	Bocchini et al. (2008)
Bacillus halodurans S7	0.5% wheat bran smf	0.4	70–75	9.5	Mamo et al. (2006a)
Bacillus megaterium	Wheat bran, ssf	846 U/g	–	–	Sindhu et al. (2006)
Bacillus pumilus B20	5% wheat bran, smf	313.3	60	6.5	Geetha and Gunasekaran (2010)
Bacillus pumilus MK001	wheat bran, Smf	4,000	60	7.5	Kapoor et al. (2008)
Bacillus pumilus SV-85S	1% wheat bran, Smf	2995.20	50	6	Nagar et al. (2010)
Bacillus pumilus	wheat bran, smf	430	50	6.5	Poorna and Prema (2006)
Bacillus sp.GRE7	oat splet xylan, Smf	5	70	7	Kiddinamoorthy et al. (2008)
Bacillus stearothermophilus SDX	wheat bran, Ssf	3,443 U/g	70	9.5	Dhiman et al. (2008)
Bacillus subtilis ASH	wheat bran, Smf	410	60	7	Sanghi et al. (2009)
Bacillus subtilis	Oat spelt xylan, smf	128	55	9	Annamalai et al. (2009)
*Geobacillus thermoleovorans*AP07	1% wheat bran, smf	14	80	8.5	Sharma et al. (2007)
Lysinibacillus sp. strain P5B1	Corn Straw, Smf	17.8	60	6	Prado et al. (2010)
Paenibacillus campinasensis BL11	Rice husk, Smf	10.5	50	8	Ko et al. (2010)

Moreover, to avoid damage to cellulose fiber, enzyme preparations should be free from cellulase activity as cellulases could be proving detrimental to yield and strength of pulp (Sudan and Bajaj 2007). Treatment with xylanases facilitates the chemical extraction of lignin from pulp, and helps in significant reduction of consumption of hazardous chemicals such as chlorine to achieve comparable levels of paper brightness while simultaneously preserving the important paper characteristics such as tensile strength, brightness, fibrillation, and drainage (Subramaniyan and Prema 2002; Damiano et al. 2003; Ayyachamy and Vatsala 2007; Sharma et al. 2007; Sudan and Bajaj 2007; Dhiman et al. 2008). In recent years, many investigations have demonstrated that chlorine consumption could be reduced up to 12–30% by using xylanases from *Bacillus* and related genera, without compromising with paper quality (Beg et al. 2001; Polizi et al. 2005; Dhiman et al. 2008; Ko et al. 2010). For effective biobleaching, some investigators have suggested use of other hemicellulases along with xylanases (Ahlawat et al. 2007; Dhiman et al. 2009; Kaur et al. 2010).

Another most important application of xylanases is in recycling of carton and office waste paper. When pulp fibers are treated with xylanase they underwent a peeling process that gives rise to flakes and filaments of materials detached from fiber surfaces, due to xylan hydrolysis. Enzyme treatment increases the fiber swelling that facilitates refining, which in turn results in better physical properties. Enzyme addition prior to refining can improve strength and properties of pulp and will also reduce the cost of refining (Poorna and Prema 2007).

Genes encoding xylanases have been cloned in homologous and heterologous hosts with the objective of overproducing the enzyme and altering its properties to suit commercial applications (Jamil et al. 2005). Introduction of recombinant DNA technology has resulted in the selection of xylanolytic enzymes that are more suitable for application in pulp and paper industries. Most important prerequisite for industrial usage of xylanases is their alkalistability and thermostability. Recombinant DNA technology provides a tool for cloning of the desired xylanase genes not only for incorporating the requisite properties but also to enhance the production levels even in the altered physiological conditions. Although a majority of the alkalistable and thermostable xylanases are documented to be produced from thermophilic *Bacillus* and *Geobacillus* species, thermophilic microorganisms cannot be propagated on a large scale due to extreme fermentation conditions, the desired xylanase genes from these bacteria have to be cloned into the genomes of mesophilic hosts such as *E. coli*, *Pichia pastoris* or *Bacillus subtilis*. Several genes of xylanase from various *Bacillus* spp. have been cloned by homologous as well as hetrologous cloning. Heterologous cloning of xylanase from these gram-positive bacteria to gram-negative bacteria like *E. coli* or *P. pastoris* is very common in recombinant DNA technology. In case of heterologous cloning, the selection of host plays an important role. *Escherichia coli* has been widely used as heterologous host for cloning of genes and expression of recombinant proteins including xylanases from *Bacillus* and *Geobacillus*. This is mainly due to the wide choice of cloning vectors, overproduction of recombinant enzymes, and the ease of DNA cloning, and in many cases, secretion of heterologous proteins from *E. coli* into the culture medium that reduces the efforts associated with protein purification from their natural hosts. *E. coli* is an

excellent expression system capable of producing recombinant proteins either intracellular or extracellularly (Mergulhaoa et al. 2005; Sorensen and Mortensen 2005). Over expression of recombinant enzymes in *E. coli* makes easy the studies like, engineering of the enzymes for studying structure–function relationship by site directed mutagenesis, as well as improvement of the enzyme properties using directed evolution technology (Alcalde et al. 2006; Kaur and Sharma 2006). Although *E. coli* has limitations as expression host, the main limitation is not secretion. Another important expression system for heterologous expression is *P. pastoris* that has well known capacity of high secretion efficiency, attains high cell densities in inexpensive culture media, and the scale up of industrial process is easy. It is a particularly attractive expression host due to the availability of strong and regulatory promoters, which are involved in methanol metabolism (Tsai and Huang 2008). The recombinant xylanases expressed from *P. pastoris* generally exhibit similar or even better characteristics compared to the enzymes produced from native species (Ahmad et al. 2009). It serves as an excellent mesophilic host for the expression of thermophilic xylanases (Liu and Liu 2007; Zhang et al. 2009, 2010). The xylanase genes of *Bacillus* and *Geobacillus* species cloned and expressed are listed in Table 17.3.

17.2.4 Pectinases

Pectolysis 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/pectinolytic enzymes or pectinases are widespread in nature and are produced by bacteria, fungi, yeasts, insects, nematodes, and protozoa. They comprise a group of enzymes that catalyze the breakdown of pectic substances. Pectic substances are high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) that are present in the plant kingdom. They are present as the major components of middle lamella between the cells in the form of calcium pectate and magnesium pectate. Pectic substances are classified into four main types: pectin, pectic acids, pectinic acid and protopectin. Pectin is composed of an α-(1→4)-linked D-galacturonan backbone, with substitution of a α-(1→2)-linked α-L-rhamnopyranose residues occasionally (Perez et al. 2000).

Approximately 75% of the carboxyl groups of the galacturonate units are esterified with methanol. The homogalacturonan parts of the polymer are referred to as "smooth" regions, while the rhamnose rich zones are called "hairy" regions (Perez et al. 2000, 2003). Pectic acid is a group designation for pectic substances generally composed of colloidal polygalacturonic acids and essentially free from methoxy groups. Pectinic acid contains up to 75% methylated galacturonate units. Protopectin is the water-insoluble parent pectic substance, located primarily in the middle lamella that serves as the glue to hold cells together in the cell walls. It yields pectin or pectinic acids upon restricted hydrolysis.

The pectinolytic enzymes may act on the glycosidic bonds of the backbone (polygalacturonases, pectin lyases and pectate lyases) or on the methoxy groups

Table 17.3 Xylanases of *Bacillus* and *Geobacillus* species cloned in different hosts

Microorganisms	Host	Vector	Xylanase activity of recombinant (U/mg)	References
B. polymyxa NCIB 8158/ATCC 842	*E. Coli*	pBR322	0.1	Shandhu and kennedy (1984)
B. polymyxa NRC 2822/NRRL 8505	*E. Coli*	pBR322	–	Yang et al. (1988)
B. ruminicola #49	*E. Coli*	pUC18	1.1	Whitehead and Hespell (1989)
B.circulans NRC 9024/USDA 729	*E.Coli*	pUC 19	–	Yang et al. (1988)
B.*pumilus* IPO	*E. Coli*	pBR322	0.002	Panbangred et al. (1983)
Bacillus halodurans S7	*E. coli*	pET 28b	–	Mamo et al. (2006b)
Bacillus licheniformis	*P. pastoris* strain GS115.	pPIC9 K	122.9	Liu and Liu (2008)
Bacillus licheniformis MS5-14	*E.Coli*	pET29b+	–	Lee et al. (2008)
Bacillus pumilus	*Bcillus megaterium*	pWH1520, pWG03	–	Bao et al. (2009)
Bacillus pumilus HB030	*Pichia pastoris* KM71, GS115, SMD1168	pPIC9 K	–	Jiang et al. (2003)
Bacillus sp.	*P. pastoris* strain GS115	pHBM905A	92.5	Zhang et al. (2010)
Bacillus sp. (NCIM 59)	*E. Coli*	pUC8	128	Shendye and Rao (2006)
Bacillus sp. BP 7	*E. Coli*	pUC19	–	Gallardo et al. (2004)
Bacillus sp.C125	*E. Coli*	pBR322	–	Honda et al. (1985)
Bacillus subtilis AMX4	*E.Coli*	pUC19	–	Yoon, Ki-Hong (2009)
Geobacillus stearothermophilus	*E. Coli*	pET28a	–	Zhang et al. (2009)

(pectin esterase) (Catilho et al. 2000). On the basis of their optimal pH of activity, they can be classified into acidic and alkaline pectinases. The acidic pectinases have extensive applications in the extraction and clarification of fruit juices and wine (Pretel et al. 1997; Blanco et al. 1999; Alkorta et al. 1998; Kaur et al. 2004; Ortega et al. 2004; Vaillant et al. 2005; Ingallinera et al. 2005). Alkaline pectinases are being used for the pretreatment of wastewater from vegetable food processing industries containing pectinacious material (Tanabe et al. 1987, 1988), processing and degumming of plant fibers such as ramie (*Boehmeria nivea*), sunn hemp (*Crotalaria juncea*), buel (*Grewia optiva*), flax (*Lisum usitatissimum*), and jute (*Chorchorus capsularis*) (Cao et al. 1992; Sreenath et al. 1996; Henriksson et al. 1999; Bruhlmann et al. 2000; Kapoor et al. 2001; Kashyap et al. 2001a, b; Sharma and Satyanarayana 2006; Akin et al. 2007), depolymerizing and debarking (Viikari et al. 2001), desizing and bioscouring of cotton knitted fabrics (Klug-Santner et al. 2006; Wang et al. 2007; Ahlawat et al. 2009), and development of the quality of fermented cocoa (Freire et al. 1990; Bhumibhamon and Jinda 1997; Schwan and Wheals 2004; Ouattara et al. 2011). The pectinases have also been used for biobleaching of mixed hardwood and bamboo kraft pulps (Ahlawat et al. 2007, 2008; Dhiman et al. 2009; Kaur et al. 2010).

Pectinases are produced by several *Bacillus* spp. (Dave and Vaughn 1971; Horikoshi 1972; Kelly and Fogarty 1978; Miyazaki 1991; Nasser et al. 1993; Singh et al. 1999; Kobayashi et al. 2001; Sharma and Satyanarayana 2006; Klug-Santner et al. 2006; Basu et al. 2008; Ahlawat et al. 2009). Pectinase production under submerged fermentation (SmF) as well as solid state fermenation (SSF) has been studied. *Bacillus subtilis* SS produced an alkaline and thermostable pectinase under submerged fermentation using wheat bran (Ahlawat et al. 2009). *Bacillus pumilus* dcsr1 produced a highly alkaline and thermostable pectinase in submerged fermentation. Optimization using RSM led to an overall 41-fold increase in enzyme production in the lab fermenter. The enzyme was optimally active at pH 10.5 and 50°C (Sharma and Satyanarayana 2006). Pectinase production using wheat bran (Cavalitto et al. 1996; Gupta et al. 2008), citrus pulp-pellets (Fonseca et al. 1991), and sugarcane bagasse (Solis-Pereyra et al. 1993) has been recorded in SSF. Gupta et al. (2008) reported the maximum exo-polygalacturonase production from *Bacillus subtilis* RCK under solid state fermentation, using moistened wheat bran as a substrate (with 1:7 solid substrate-to-moisture ratio).

Few pectinases have been purified to homogeneity. Klug-Santner et al. (2006) purified an extracellular endo-pectate lyase from *Bacillus pumilus* BK2, with optimum activity at pH 8.5 and around 70°C, in three steps. The molecular mass as determined by SDS-PAGE was 37.3 ± 4.8 kDa. An extracellular pectate lyase was purified from the culture filtrate of *Bacillus pumilus* DKS1, using ion-exchange and gel filtration chromatography. The purified enzyme had a molecular weight of approximately 35 kDa and exhibited maximal activity at a temperature of 75°C and pH 8.5 (Basu et al. 2008).

It has been shown that calcium ion plays an important role in the synthesis, as well as for the activity of several pectinases. Kelly and Fogarty (1978) showed an increase in production levels of polygalacturonase lyase in the presence of Ca^{2+}. The activation of enzyme was seen in the presence of Ca^{2+} (Horikoshi 1972).

Several pectate lyases have been shown to require Ca^{2+} for their activity (Dave and Vaughn 1971; Sakai et al. 1993; Basu et al. 2008), except the pectate lyase from *B. pumilus* BK2 (Klug-Santner et al. 2006), which did not require Ca^{2+} for its activity.

Gene encoding alkaline pectate lyase (PL) has been cloned and expressed in *Pichia pastoris*. The first report of cloning and expression of bacterial PL gene in *P. pastoris* was by Bin et al. (2008). PL gene from *Bacillus subtilis* WSHB04-02 was overexpressed, attaining the enzyme titres as high as 100 U/ml, which was ten times higher than those achieved when the gene was expressed in *E. coli* (Bin et al. 2008). The recombinant pectate lyase was purified to homogeneity and maximal enzyme activity was observed at 65°C and pH 9.4. The recombinant enzyme showed a wider pH and thermal stability than the purified pectate lyase from *B. subtilis* WSHB04-02.

17.2.5 Alkaline Proteases

Proteases, which accounts for the 60% of the world's total enzyme sale (Banerji et al. 1999), are among the most extensively studied hydrolytic enzymes. They not only play an important role in cellular metabolic processes, but have also gained considerable attention in the industrial community. Microorganisms account for a two-third share of commercial protease production in the world (Kumar and Takagi 1999). Proteases find application in the food, cosmetic, leather, pharmaceutical and detergent industries and are important tools in studying the structure of proteins and polypeptides (Bhosale et al. 1995).

Alkaline proteases (EC.3.4.21–24, 99) are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine center (serine protease) or are of metallo-type (metalloprotease); and the alkaline serine proteases are the most important group of enzymes exploited commercially. Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions (Maurer 2004; Saeki et al. 2007). These enzymes find applications in detergents, feather processes, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis and biotransformation (Gupta et al. 2002; Bhaskar et al. 2007; Sareen and Mishra 2008; Jellouli et al. 2009). Most of the alkaline proteases are produced by bacteria, especially *Bacillus* spp. (Bhaskar et al. 2007; Dipasquale et al. 2008; Haddar et al. 2009; Jellouli et al. 2009). The literature survey showed that alkaline proteases from *Bacillus* spp. are the best commercial sources available to date (Singh et al. 2004). Since the first alkaline protease, Carlsberg, from *Bacillus licheniformis* was commercialized as an additive in detergents in the 1960s (Saeki et al. 2007), a number of *Bacillus*-derived alkaline proteases have been purified and characterized, and significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification, and low cost have been demonstrated (Maurer 2004; Haddar et al. 2009). Currently, large portions of commercially available alkaline proteases are derived from *Bacillus* strains (Mohamed et al. 1998; Kumar and Hiroshi 1999; Gupta et al. 2002; Haki and Rakshit 2003; Ravichandra et al. 2007).

Use of alkaline proteases as active ingredients in detergents is the most important application of this enzyme (Ramnani et al. 2005; Sellami-Kamoun et al. 2008; Mukherjee et al. 2008; Hmidet et al. 2009; Rai and Mukherjee 2010; Jellouli et al. 2011). Alkaline proteases are also widely used in leather processing (Benerjee and Bhattacharyya 1980; Mukhtar and Ul-Haq 2008), medical diagnostics, recovery of silver from X-ray films (Fujiwara and Yamamoto 1987), dehairing of animal hides (Srinivas and Naik 2010; Sundararajan et al. 2011), and shrimp waste deproteinization (Jellouli et al. 2011).

Alkaline proteases from *Bacillus* spp. are thermostable, being optimally active between the temperature ranges 50–70°C. These include *B. licheniformis* (Horikoshi 1971; Sinha and Satyanarayana 1991; Ramnani et al. 2005; Hmidet et al. 2009; Li et al. 2009; Jellouli et al. 2011), *B. subtilis* (Wang and Yeh 2006; Mukherjee et al. 2008; Rai and Mukherjee 2010), *B. mojavensis* (Beg et al. 2002; Haddar et al. 2009), *B. pumilis* (Kumar 2002; Jaouadi et al. 2008), *B. clausii* (Joo et al. 2003), *B. proteolyticus* (Bhaskar et al. 2007), *Bacillus* sp. L21 (Tari et al. 2006), *B. clausii* (Oskouie et al. 2008), *B. halodurans* (Shrinivas and Naik 2011) and *B. cereus* (Manni et al. 2008; Doddapaneni et al. 2009). Recently, cold-active proteases have also attracted considerable attention to develop laundry application at lower temperatures.

Alkaline proteases of bacterial origin are generally produced by submerged fermentation; while, solid state fermentation processes have been exploited to a lesser extent for production of these enzymes. Carbon and nitrogen sources are the significant components of the medium, which affect protease production in submerged fermentation. Different carbon sources ranging from simple sugars like glucose, to complex polymers like starch, have been used. Fructose supported high protease production by *B. licheniformis* S-40 (Sen and Satyanarayana 1993), while lactose was the preferred carbon source for *B. licheniformis* ATCC 21415 and *B. brevis* MTCC B0016, respectively (Mabrouk et al. 1996; Banerjee et al. 1999). Corn starch and glycogen supported high enzyme titres by *B. licheniformis* RG1 and *B. aquimaris* VITP4, repectively (Shivanand and Jayaraman 2009), while wheat flour containing basal medium, supplemented with fructose and maltose, produced high protease titres by *Bacillus* sp. I-312 and *Bacillus clausii* I-52, respectively (Joo and Chang 2005; Joo and Chang 2006).

Both organic as well as inorganic nitrogen sources have been used for the maximum production of alkaline protease from *Bacillus* spp. *Bacillus licheniformis* S-40, *Bacillus* sp. I-312 and *Bacillus* sp. L21 showed maximum protease production in the presence of soyabean meal (Sen and Satyanarayana 1993; Joo and Chang 2005; Tari et al. 2006), while Sardinella peptone was chosen as a cost-effective nitrogen source supporting maximum protease production by *Bacillus mojavensis* A21 (Haddar et al. 2010). Casamino acid, soy flour and beef extract supported high protease production by *Bacillus* sp. Ve1, *B. licheniformis* RG1 and *B. aquimaris* VITP4, respectively (Patel et al. 2005; Ramnani et al. 2005, Shivanand and Jayaraman 2009).

Agro-industrial residues like wheat bran (Sen and Satyanarayana 1993; Aijun et al. 2005), green gram husk (Prakasham et al. 2006), grass and potato peel (Mukherjee et al. 2008), rice husk (Lulla and Subrahmanyan 1954), soyabean flour and coffee husk (Germano et al. 1998) have been used for the alkaline protease production in SSF. Low-cost medium for high levels of protease production has been developed

using RSM (Tari et al. 2006; Oskouie et al. 2008; Reddy et al. 2008; Hadder et al. 2010; Mukherjee and Rai 2011).

Several thermostable alkaline proteases from *Bacillus* spp. have been purified to homogeneity as well as characterized. They exhibit optimum activity in the temperature range 45–70°C and pH range 6.5–11 (Gupta et al. 2005; Jaouadi et al. 2008; Manni et al. 2008; Doddapaneni et al. 2009; Haddar et al. 2009; Hmidet et al. 2009; Li et al. 2009; Deng et al. 2010; Rai and Mukherjee 2010; Jellouli et al. 2011; Shrinivas and Naik 2011).

The microbial proteases are usually produced using free or immobilized cells. The use of immobilization of enzymes or cells in microbial fermentations offers many advantages over free cell fermentation systems (Kukubu et al. 1981) because such systems offer better operational stability and high efficiency of catalysis (Ramakrishna et al. 1992). It allows the continuous use of cells, making immobilization a very advantageous procedure (Linko and Haapala 1996). Cells of *B. subtilis* PE-11, *B. licheniformis* NCIM-2042, *B. circulans* MTCC 7906, *B. polymixa* and *B. subtilis* were immobilized (Adinarayana et al. 2005; Potumarthi et al. 2008; Maal et al. 2009; Kumar and Vats 2010) on different matrices. These were proposed as an effective biocatalyst for repeated usage for maximum production of alkaline protease.

A number of alkaline protease encoding genes from different *Bacillus* spp. have been cloned and overexpressed in *E. coli* (Yamagata et al. 1995; Jaouadi et al. 2008) and *Bacillus subtilis* (Zaghloul et al. 1994; Deng et al. 2011). Gene for alkaline protease (sapB, 1149 bp) from *Bacillus pumilus* CBS was cloned, sequenced and overexpressed in *E. coli*. The purified recombinant enzyme (SAPB) has the same physicochemical and kinetic properties as the native one (Jaouadi et al. 2008). Based on sequence alignments and homology-modeling studies, five amino acids Leu31, Thr33, Asn99, Phe159 and Gly182, which were putatively important for the behaviour of this enzyme (SAPB) were identified. In mutated protease generated by site-directed mutagenesis, the optimum temperature shifted the enzyme activity from 65°C to 75°C. It also increased the half life at 50°C and 60°C to 660 and 295 min from of 220 and 80 min for the wild-type enzyme, respectively (Jaouadi et al. 2010).

17.2.6 Carbonic Anhydrase

Global warming is probably the most challenging environmental threat of this century. An abrupt increase in the carbon dioxide concentration, which is a consequence of anthropogenic carbon emissions, has lead us to focus on the novel ideas that ensure a leakage-proof and cost-effective approach for long term and maximal storage of CO_2. Carbonic anhydrase (CA, EC 4.2.1.1), the ubiquitous and one of the fastest enzymes known ($K_{cat} = 1.4 \times 10^6\ s^{-1}$), catalyses the reversible hydration of carbon dioxide into bicarbonates. This fast hydration property has sparked a recent research interest in sequestering CO_2 by a promising biomimetic approach. CA can surprisingly expedite the process of calcification by catalyzing the rate determining step (step 2) in the conversion of CO_2 to $CaCO_3$ (Mirjafari et al. 2007).

$$CO_{2(g)} \leftrightarrow CO_{2(aq)} \quad (17.1)$$

$$CO_{2(aq)} + H_2O \leftrightarrow H_2CO_3 \left(K_{2a} = 2.6 \times 10^{-3}\right) \quad (17.2a)$$

$$H_2CO_3 \leftrightarrow H^+ + HCO_3^- \left(K_{2b} = 1.7 \times 10^{-4}\right) \quad (17.2b)$$

$$HCO_{3^-} + OH^- \leftrightarrow CO_{3^{-2}} + H_2O \quad (17.3)$$

$$Ca^{+2} + CO_{3^{-2}} \leftrightarrow CaCO_3 \downarrow \quad (17.4)$$

Carbonic anhydrases are encoded by six distinct types of unrelated gene families (α, β, γ, δ, ε and ζ). Prokaryotes predominantly have α and β gene families (Lee et al. 2010; Smith and Ferry 2000). Phylogenetic analysis among carbonic anhydrases of the Firmicutes has shown that *Bacillus* spp. can harbor any of the three-α, β and/or γ-type of CAs. Gene encoding α-type CA was found to be present in *B. clausii* whereas most of the other Bacilli such as *B. pumilus*, *B. subtilis*, *B. cereus, B. anthracis, B. thuringiensis*. *B. amyloliquefaciens* and *B. licheniformis* were found to contain genes encoding either β and/or γ-type CA. However, γ-type CA predominates in the genomes of *Geobacillus thermodenitrificans* and *G. kaustophilus*. CA from Bacillales are closely related to those of the Clostridia indicating a possibility of horizontal γ-type CA gene transfer between the Bacillales and the Clostridia (Nishida et al. 2009).

Despite its ubiquity in nature, this enzyme has been scarcely explored in microorganisms with GRAS status. Till date, carbonic anhydrase has been reported from just two Bacilli (*Bacillus pumilus* TS1 and *Bacillus subtilis* SA3). *B. pumilus* TS1 is able to grow at elevated levels of CO_2 (20%) producing carbonic anhydrase both in the extracellular and intracellular fractions. However the production of carbonic anhydrase was repressed when the bacterium was cultivated at higher concentrations of CO_2. Ramanan et al. (2009) have purified and characterized a novel-plant type β CA from *B. subtilis* SA3 that shows α CA like-esterase activity along with its normal hydration activity. CA from *B. pumilus* TS1 is stable over a wide range of pH (6.0–11.0) and temperature (30–65°C) and is optimally active at pH 9.0 and at 55°C. CA from *B. subtilis* SA3 has shown optimal activity at pH 8.3 and at 37°C. It retained 100% activity at pH 8.0 and 8.3, compared to 70% activity recorded at pH 7.0 and 9.0 and 20% activity at pH 11.0 (Ramanan et al. 2009). Both the enzymes were inactive within the acidic pH range. Irrespective of the type of gene family, all the carbonic anhydrases are metallozymes containing Zn^{2+} or Fe^{2+} or Cd^{2+} at its active site. CA acts by a nucleophilic attack of MOH^- on CO_2 concentrated around the hydrophobic residues in the active site cleft to form the HCO_3^-. Effect of various divalent cations on the CA activity has been studied. It was found that Zn^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+} and Mg^{2+} acted as activators of CA of *B. subtilis* SA3. However, Pb^{2+} was found to be a moderate inhibitor, whereas Hg^{2+} completely inhibited the CA activity (Ramanan et al. 2009). Carbonic anhydrases are being immobilized on different support matrices to ensure maximal activity along with its long-term use in bioreactors

for sequestration purposes. Chitosan-clay beads were found to be a better support matrix among different chitosan-based materials tested for the immobilization of whole-cells of *B. pumilus* TS1 and its carbonic anhydrase (Prabhu et al. 2009). CA from *B. pumilus* TS1 has been recently immobilized on surfactant-modified silylated chitosan (SMSC) for the carbonation reaction. Each mg of the immobilized and free CA formed 10.73 and 14.92 mg of $CaCO_3$ respectively, under a limiting concentration of CO_2 (14.5 mg of CO_2/10 ml) in the reaction mixture (Yadav et al. 2010). A single enzyme nanoparticle of carbonic anhydrase (SEN-CA) formed by the modification of the surface of this enzyme with thin layer of chitosan significantly improved the sequestration capacity by forming 147 mg of $CaCO_3$/mg of CA (Yadav et al. 2011).

17.2.7 Catalase

Catalases [EC 1.11.1.6] (Hydrogen-peroxidase: hydrogen peroxidase oxidoreductase) are the oxidoreductases, which promote the conversion of hydrogen peroxide, a potentially harmful oxidizing agent into water and oxygen (Ames et al. 1993).

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

The catalase reaction is one of the fastest (turnover number of 100,000 per second per active center) and highly efficient ($K_{cat}/K_m = 4 \times 10^8\ M^{-1}\ s^{-1}$) enzyme reactions known that requires neither ATP nor an exogenous reducing agent for its completion (Frew and Jones 1984; Fita and Rossman 1985; Laura et al. 1995). Nature has equipped most of the aerobes with highly stable catalase to combat oxidative stress caused by toxic levels of H_2O_2.

Based on the structural and functional similarities, catalases have been classified into three different classes: typical monofunctional catalases, bifunctional catalase-peroxidases and manganese catalases (Zamocky and Koller 1999). The typical monofunctional catalases are involved in the disproportionation of hydrogen peroxide into molecular oxygen and water. However, the bifunctional catalase-peroxidases exhibit peroxidase activity along with their normal catalase activity:

$$ROOH + HR'OH \rightarrow R'O + ROH + H_2O$$

where R represents a H atom, an alkyl or acyl group. R' can be an O atom, Carboxy or H $(CH_2)_n$ CH group (Where n = 1, 2 or 3) (Reid et al. 1981).

Manganese catalases comprise a minor group of pseudocatalases which utilize manganese ions instead of ferric ion in their active site (Allgood and Perry 1986). Most of the textile finishing industries preferably use hydrogen peroxide as a bleaching agent. It is also used as an effective bactericide in the treatment of raw milk during pasteurization. The cytotoxic effects of hydrogen peroxide of high concentration (≥50 μM) are well documented (Halliwell et al. 2000). Catalases have been routinely used as the most environment friendly alternative for the treatment and

recycling of textile bleaching effluents, to remove traces of hydrogen peroxide and in the process of cold sterilization of milk and cheese (Paar et al. 2001). Catalase in combination with glucose oxidase, remove glucose from egg white before drying for use in the baking industry. Additionally, catalase has also been used as a novel enzyme for use in labelling biomolecules (Hande 2003), as an immunotargetting agent to remove oxidative stress in pulmonary endothelium (Kozower et al. 2003), as a therapeutic antioxidant complex along with superoxide dismutase (SOD) for the treatment of several diseases in which oxidative injury has taken place like, myocardial ischaemia, reperfusion oxidative injury (Zughaib et al. 1994), and in the healing of gynaecological surgery wounds. Multiple catalases have been reported from *Bacillus subtilis*, which were produced at different phases of growth. The levels of a H_2O_2-inducible catalase 1, produced during logarithmic growth phase, were increased 15-fold during growth in stationary phase (Loewen and Switala 1987). A H_2O_2-inducible vegetative *katA* gene was found to be responsible for secreting high titers of extracellular catalase in *B. subtilis* during its stationary growth phase (Naclerio et al. 1995). H_2O_2-non-inducible catalase 2 (hydroperoxidase II) was produced only when the cells entered the stationary growth phase. This enzyme is produced to manage the cellular stress and adverse growth conditions. It was linked to two loci, *katB* (Loewen 1989) and *katE* (Engelmann et al. 1995), which were later shown to be identical. A third catalase, encoded by *katX* gene was found only in dormant spores of *B. subtilis*. However, H_2O_2 resistance of dormant spores was not affected by loss of *katX* gene (Casillas-Martinez and Setlow 1997). Recently, catalase 2 was found to be involved in the development of swarming colonies when the vegetative cells of *B. thuringiensis* reach high cell density (Salvetti et al. 2009; Senesi et al. 2010).

Three different isozymes of catalases were detected in the cell extract of *B. firmus* OF4 when cultivated under ambient and elevated pH. Isozyme I was found to be down-regulated, whereas isozyme II was up-regulated in early exponentially-grown cells at pH 10.5 relative to pH 7.5. The chlorine-containing isozyme III was expressed only during stationary phase. A detectable level of peroxidase activity along with the typical catalase activity was only observed in isozyme I (Hicks 1995).

Induced catalase production by 5 mM silver nitrate in the growth medium of *B. licheniformis* has been reported recently (Pandian et al. 2010). Oxidative damage to exponential cells induced by a strong oxidizing agent, menadione along with increase in dissolved oxygen had a stimulatory effect on catalase production by *Bacillus* sp. F26 (Yan et al. 2006). The crude extracts of a novel bacterium, *Bacillus* sp. N2a isolated from the Antarctic seawater was found to exhibit a cold-active psychrophilic catalase (Wang et al. 2008). Psychrophilic catalases are a better choice to treat textile effluents during winter days.

A 318-fold purification was achieved when DEAE-Sephadex along with Bio-Gel A-1.5 m and Bio-Gel HTP was used to purify catalase 1 from *B. subtilis* 168 (Loewen and Switala 1987). The 204 kDa homotetramer of catalase-peroxidase was purified to 3.8-fold using Phenyl-Sepharose CL-4B, Sephacryl S-200 HR and MonoQ HR. This catalase-peroxidase shows activity in a wide range of pH (6.4–10) and is also stable at a relatively higher temperature (30–60°C) (Ogawa et al. 2004).

The cold active catalase of *B. subtilis* IAM 1026 is optimally active between 0°C and 30°C and pH 7–10 (Fusho and Yajima 1996, 1997). Wang et al. (2008) have reported a catalase from *Bacillus* sp. N2a that is optimally active at 25°C, however high catalytic activity was retained even at lower temperatures (measured at 0°C and 5°C). Study on the stabilizing efficiency of various additives on catalase produced from *Bacillus* sp. inferred that glycerol and glutaraldehyde were the best stabilizers at 30°C and neutral pH, whereas polyethylene glycol and glycerol were the most appropriate stabilizers below pH 12 (Costa et al. 2002).

17.2.8 Phytases

Phosphorus is an important mineral nutrient in the growth and development of animals. In most feed sources used in animal production, such as cereals grains and legumes, phytate (myo-inositol hexakisphosphate) is regarded as the principal storage form of phosphorus. However, because of digestive enzyme deficiency, non-ruminant animals such as poultry, pigs, and fish do not utilize the phosphorus combined with phytate efficiently (Roy et al. 2009). Animal manures with excess amounts of undigested phytate can accelerate eutrophication in water leading to other massive environmental hazards (Abulkalam 2008). Apart from this, phytate can negatively affect nutrition absorption in animals by binding to proteins and by chelating with calcium, magnesium, and zinc or other minerals. To alleviate these problems phytase can be used.

The term phytase is used to describe phosphohydrolase enzyme, which catalyses the sequential release of orthophosphate from phytic acid and phytate. Based on carbon ring position where removal of phosphate group from phytase is initiated, the ENZYME database classifies phytases into three different groups: 3-phytases (alternative name 1-phytase; EC 3.1.3.8), 5-phytase (EC 3.1.3.72), and 6- phytases (alternative name 4-phytase; EC 3.1.3.26). On the basis of pH optimum, phytases can be broadly divided into two major classes: acid and alkaline phytases. Phytases also exhibit variation in structure and catalytic mechanism, and consequently, have been categorized into cysteine phytases, histidine acid phytases (HAPs), β-propeller phytases (BPPs) and purple acid phosphatases (PPPs) (Chu et al. 2005).

Phytases are widely distributed in nature and can be derived from a number of sources including plants, animals and microorganisms. *Bacillus* spp. producing thermostable phytases have mainly been isolated from soils. *B. amyloliquefaciens* DS11 produced a thermostable and extracellular phytase in submerged fermentation (SmF), in the presence of wheat bran and casein hydrolysate at 37°C (Kim et al. 1998a). The maximal phytase activity of 2.957 U/ml was produced by *B. laevolacticus* in the shake flasks (Gulati et al. 2007). *Bacillus* sp. KHU-10 produced the highest level of 0.2 U/ml phytase after 4 days of incubation (Elkhalil et al. 2007).

Several *Bacillus* phytases have been purified and characterized. Power and Jagannathan (1982) purified a phytase from *Bacillus subtilis* having a molecular weight of 36.5 kDa, with pH optima between 7.0 and 7.5 and a temperature optimum of 60°C.

This phytase was stable up to a temperature of 70°C. The specific activity of the purified enzyme was reported as 8.5–9.0 U/mg protein. *B. subtilis* (natto) phytase described by Shimizu (1992) was purified to homogeneity and was shown to have a molecular weight of between 36 and 38 kDa. This enzyme had a pH optima between pH 6.0 and 6.5 and an optimum temperature of 60°C. The specific activity of this purified phytase was reported to be 8.7 U/mg protein. Kerovuo et al. (1998) isolated, purified, and characterized phytase from *B. subtilis* VTT E-68013; the enzyme showed optimum activity at pH 7.0 and 55°C.

Choi et al. (2001) reported that an extracellular phytase from *Bacillus* sp. KHU-10 was purified and its molecular weight was 44 kDa. Its optimum pH and temperature were 6.5–8.5 and 40°C respectively, without 10 mM $CaCl_2$ and pH 6.0–9.5 and 60°C, with 10 mM $CaCl_2$. About 50% of its original activity was retained, after incubation at 80°C for 10 min, in the presence of 10 mM $CaCl_2$. Gulati et al. (2007) described a partially-purified phytase from *B. laevolacticus*. It was optimally active at 70°C and pH 7.0–8.0. This enzyme retained its 80% activity at 70°C and pH 8.0, for up to 3 h, in the presence or absence of 5 mM $CaCl_2$. The thermal stability of phytase is an important and useful criterion for its application as an animal additive, because of the process with high temperature and steam during pelleting (Lei and Stahl 2000). *Bacillus* phytases are quite stable at the high temperature range of 80–95°C. Elkhalil et al. (2007) revealed that *Bacillus* phytases are more resistant to heat treatments than *E. coli* and *Klebsiella* phytases. Owing to this property, *Bacillus* phytases are better candidates to be used as feed supplements.

Bacillus phytases have been found to be metal ion-dependent as they require Ca^{2+} for their activity and stability. Ca^{2+} binds two oxyanions from the phosphate groups of phytate to form an ideal positively charged distribution in the calcium–phytate complex. Because of the existence of three Ca^{2+}-binding sites at the active site cleft, Ca^{2+} serves as an essential activator to reduce the negative charge around the active site cleft, whereas excess amounts of Ca^{2+} acts as a competitive inhibitor. In addition, Ca^{2+} has an important effect on the stability against temperature and pH (Choi et al. 2001). Kerovuo et al. (1998) studied the metal ion requirement of *B. subtilis* phytase and reported that the removal of metal ions from the enzyme by EDTA resulted in complete inactivation. The loss of enzymatic activity was most likely because of a conformational change, as the circular dichroism spectra of holoenzyme and metal-depleted enzyme were different. Metal depleted enzyme was partially able to restore the active conformation when incubated in the presence of calcium. Only minor reactivation was detected with other divalent metal ions and their combinations.

Bacillus phytases exhibit highly strict substrate specificity for calcium–phytate complex and have no enzymatic activity on other phosphate esters (Idriss et al. 2002). Oh et al. (2006) reported that no detectable phytate hydrolysis was observed when the phytase was added to phytate in the Ca^{2+}-free environment at several different phytate concentrations. At fixed phytate concentration, increasing the concentration of Ca^{2+} enhanced the initial velocities of phytase in a saturating manner. This activation is mediated by a cooperative interaction of Ca^{2+} to the substrate and the Ca^{2+}-dependent increase of reaction rate followed a sigmoidal curve. Other divalent

Table 17.4 Characteristic features of phytases from *Bacillus* spp.

S. No.	Source organism	Specific activity (U/mg)	Molecular weight (kDa)	Temp. (°C)	pH optimum	$K_{m\,(\mu M)}$	Reference
1	*B. subtilis*	35	44	55	7.0	–	Tye et al. (2002)
2	*B. laevolacticus*	12.69	41–46	70	7.0–8.0	52.6	Gulati et al. (2007)
3	*Bacillus* sp. *KHU. 10*	36	44	40	6.5–8.5	50	Choi et al. (2001)
4	*Bacillus* sp. *DS11*	–	44	70	–	–	–
5	*Bacillus licheniformis*	–	47	65	7.0	–	Tey et al. (2002)
6	*Bacillus* sp.	16	41	–	–	–	Rao et al. (2008)

cations such as Co^{2+}, Cs^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} have no effect on enzymatic activation. According to these results, the calcium–phytate complex is regarded as the true substrate for the BPPs, and Ca^{2+} plays an important role as a substrate activator in the formation of the true substrate.

The crystal structure of the phytase from *B. amyloliquefaciens* DS11 demonstrated that a negatively charged active site provides a favorable electrostatic environment for the positively charged calcium–phytate complex (Ha et al. 2000; Shin et al. 2001).

Genes encoding *Bacillus* phytases from *B. amyloliquefaciens* (Idriss et al. 2002) *B. licheniformis*, and *B. subtilis* have been cloned and sequenced. These phytases are composed of 383 amino acids and encoded an extracellular monomeric protein. Their approximate molecular mass was found to be 42 kDa. The amino acid sequences of phytases from *Bacillus* are highly homologous to each other showing 90–98% sequence similarity. However, these amino acid sequences do not align with any other previously well-characterized HAPs or with PAPs. Most significantly, the active site heptapeptide motif RHGXRXP and the catalytically active dipeptide HD, which are highly conserved sequence among HAPs (Van Etten et al. 1991), are absent. Besides, *Bacillus* phytases do not carry disulfide bonds, which are necessary for the conformational stability and catalytic activity in several fungal phytases (Wang et al. 2004). Different molecular characteristics of various *Bacillus* phytase are given in the Table 17.4.

Ha et al. (1999) carried out preliminary x-ray crystallographic analysis of a novel phytase from a *B. amyloliquefaciens* strain using the hanging-drop vapor-diffusion method. The enzyme consists of five 4-stranded and one 5-stranded antiparallel β sheets aligned around a pseudo sixfold symmetry axis lying on the shaft of the propeller, which is a distinct central channel filled with many ordered water molecules. Ha et al. (1999) further determined the crystal structure of *B. amyloliquefaciens* phytase at 2.1 Å resolution in partially and fully Ca^{2+} loaded states. Two calcium ions (Ca1 and Ca2) form a biocalcium center, where the Asp308 carboxylate serves as a bridging arm and tightens the "double clasp," stabilizing the circular arrangement of the propeller structure. The binding of Ca1 and Ca2 to high-affinity calcium-binding sites results in a dramatic increase in thermal stability by tightening

of several peptide segments at the strategic location remote in the amino acid sequence. Three calcium ions (Ca4, Ca5, and Ca6) at the active site form triadic calcium center and neutralize an otherwise negatively charged calcium cage surrounded by a total of six aspartate and glutamate residues. The top of the molecular structure forms a shallow cleft that is lined predominantly with negatively charged side chains. The calcium-occupied cleft turns into a favorable electrostatic environment for the binding of phytate together with nearby Lys76, Lys77, Arg122, and Lys179. In addition, Ca4, Ca5, and Ca6 participate in catalysis directly by binding the phosphate group(s) of the substrate and stabilizing the pentavalent transition-state intermediate. Ca^{2+} reduces the negative charge around the active site cleft such that phytate neutralized by Ca^{2+} can easily fit to the active site. Furthermore, the enzyme has two low phosphate binding sites, the "cleavage site," which is responsible for the hydrolysis of a substrate, and the "affinity site," which increases the binding affinity for the atom of the substrates containing adjacent phosphate groups.

A bidentate (P3-Ca^{2+}-P4) of Ca^{2+}-InsP6 initially binds to two phosphate-binding sites in the active site of *Bacillus* phytase, which preferentially hydrolyzes the phosphate group at the d-3 position of Ca^{2+}-InsP6 to release Ins (1,2,4,5,6) P5 as an initial product (Idriss et al. 2002). After the hydrolysis of the first phosphate group, the enzyme binds another bidentate (P1-Ca^{2+}-P2) of Ins (1,2,4,5,6) P5, followed by the hydrolysis of the phosphate group at the d-1 position to release Ins (2,4,5,6)-P4, as identified by 2-D NMR analysis of reaction intermediates.

Finally, the enzyme binds a bidentate (P5-Ca^{2+}-P4) of Ins (2,4,5,6) P4 and eventually hydrolyzes the phosphate group at the d-5 position to yield myo-Ins(2,4,6) P3 as a final product (Oh et al. 2006). Greiner and Konietzny (2002) established the pathway of dephosphorylation of phytate by *B.* subtilis 168, *B. amyloliquefaciens* ATCC15841, and *B. amyloliquefaciens* 45 using a combination of HPLC analysis and kinetic studies. The result demonstrated that these enzymes dephosphorylate phytate by sequential removal of phosphate groups via two independent routes: the routes via D-Ins (1,2,4,5,6) P5 to Ins(2,4,5,6) P4 and finally, to D-Ins(2,4,6) P3. These enzymes prefer the hydrolysis of every second phosphate over that of adjacent ones. This finding does support previous phytate degradation models proposed by Kerovuo et al. (1998) and Greiner and Konietzny (2002) but seems to fit with the structural model given by Shin et al. (2001)

Tye et al. (2002) overexpressed the phytase (PhyL) from *B. licheniformis* and phytase gene (168phyA) identified from *B. subtilis* strain 168 in *B. subtilis* using a 105MU331 prophage vector system. Up to 35 U/ml of phytase was secreted into the medium. Both of these phytases exhibited a broad temperature and pH optima range, and showed high thermal stability. The phytase encoded by phyL exhibited higher thermal stability, even at a lower calcium concentration, as it was able to recover 80% of its original activity after denaturation at 95°C for 10 min. The gene-encoding phytase from *Bacillus* sp. DS11 was cloned in *E. coli* BL21 using the pET22b (+) vector with the inducible T7 promoter and produced up to 20% content of total soluble proteins (Kim et al. 1998a, b). The phytase gene with a native promoter derived from *B. amyloliquefaciens* was expressed in *B. subtilis*, which resulted in 100 times higher phytase yield in tailored Luria broth medium (Kim et al. 1999).

Plants can be used as phytase producers and carriers for animal feeding. *Bacillus* phytases would be good candidates for producing transgenic plants because of their optimum pH under 7.0. The expression of a *Bacillus* phytase in transgenic plants may create a new biochemical pathway that mobilizes inorganic phosphate from phytate, so that more phosphorus is available for plant growth or physiology (Tye et al. 2002). Engineering crop plants would produce heterologous phytase to reduce phosphate load on agricultural ecosystems with improving phosphate bioavailability. Yip et al. (2003) introduced a phytase from *B. subtilis* into the cytoplasm of tobacco cells that resulted in equilibrium shift of inositol biosynthesis pathway, thereby making more phosphate available for the primary metabolism. The transgenic line exhibited phenotypic changes like increased flowering, lower seed IP6/IP5 ratio, and enhanced growth under phosphate starvation conditions compared to wild type. Lung et al. (2005) reported that the phytase from *B. subtilis* (168phyA) was constitutively expressed in tobacco and *Arabidopsis* to generate transgenic plants capable of utilizing exogenous phytate. In tobacco, phytase activities in transgenic leaf and root extracts were seven to eight times higher than those in wild-type extracts; whereas, the extracellular phytase activities of transgenic plants were enhanced by four to six times. Similar results were observed from the transgenic *Arabidopsis*. These results may offer a new perspective in mobilizing soil phytate into inorganic phosphate for plant uptake. Chan et al. (2006) studied the biochemical properties and kinetic parameters of the recombinant phytase (t168phyA) from transgenic tobacco. The t168phyA was glycosylated with a 4-kDa increase in molecular size. Its temperature optimum shifted from 60°C to 45–50°C, and its pH optimum shifted from pH 5.5 to 6.0; whereas, the thermal stability remained unchanged. Kinetic data showed that the t168phyA had a lower K_{cat}, but a higher K_m than the native enzyme. Despite these changes, t168phyA remained catalytically active and has a specific activity of 2.3 U/mg protein. These results verify the activity of recombinant *Bacillus* phytase expressed in plants.

During the past two decades, there has been an increase in the use of microbial phytases as feed additives in diets for swine and poultry to enhance the utilization of plant based foods. Apart from being commercially valuable in feed and food industries, phytases have potential biotechnological application in various other fields such as environmental protection, aquaculture (Cao et al. 2007), processing of human food (Greiner and Konietzny 2006), synthesis of lower inositol phosphates, in pulp and paper industry, biosensor technique (Caseli et al. 2006), agriculture (Richardson et al. 2005) and soil amendment (Idriss et al. 2002). Many fundamental issues related to biotechnological application of phytases and phytates remain to be elucidated.

17.2.9 Other Enzymes

Bacillus spp. are known to produce other enzymes, which have diverse applications in biotechnology and molecular biology. Chitinolytic enzymes find application in

the biocontrol of plant pathogenic fungi and insects (Lorito et al. 1993), and in many other biotechnological areas (Patil et al. 2000). The production of chitinases has been reported in a number of *Bacillus* spp. (Woo and Park 2003; Chang et al. 2010; Shanmugaiah et al. 2008). Alkaline phosphatase is a non-specific monophosphoester hydrolase that catalyzes the elimination of phosphate groups from a range of small organic molecules and large biomolecules such as DNA and proteins (Kobori et al. 1984). Extracellular alkaline phosphatase has been reported in *B. cereus* (Cercignani et al. 1974) and *B. licheniformis* (Pandey and Banik 2010).

Tannase (tannin acyl hydrolase) hydrolyzes the ester and depside linkages of tannic acid to produce gallic acid and glucose. It is used in food, beverage, pharmaceutical and chemical industries. The enzymatic product, gallic acid, is used in dye making, pharmaceutical and leather industries (Lekha and Lonsane 1997). Tannase production has been reported in *B. licheniformis* (Mondal et al. 2000; Mohapatra et al. 2009).

Nucleases are also known from *Bacillus* spp. A large number of DNases, RNases and phosphodiesterases have been purified from *B. amyloliquefaciens* (Hartley and Barker 1972) *and B. subtilis* (Kanamori et al. 1974). The extracellular RNase insensitive to EDTA was reported from *Bacillus amyloliquefaciens* (Rushizky et al. 1964) and *Bacillus pumilus* (Hartley and Barker 1974). Various strategies are used for the large-scale production of nucleases. Kumar et al. (2010) reported the optimization of RNase production in *Bacillus firmus* VKPACU-1. Multicyclic process of the cultivation of *B. amyloliquefaciens* has been employed for obtaining Bam H1 for its scale up (Dubinina et al. 1983). Site-specific endonucleases are important tools in research and their use in analysis and manipulation of nucleic acid molecules are well accepted. A number of restriction nucleases like BciBl, BciBll (Manachini et al. 1987) and BliI (Samko et al. 1994) have been reported from *Bacillus circulans* and *B. licheniformis*, respectively.

17.3 Conclusions and Future Perspectives

Enzymes of the species of *Bacillus* and *Geobacillus* are receiving considerable attention because of their industrial applications. In spite of the availability of large number of α-amylases, the need for Ca^{2+}-independent, thermostable α-amylase active at 100°C and acidic pH for the improved starch saccharification processing has been highlighted. The cost effective production of thermostable and alkalistable xylanases and pectinases is needed in view of their applications in environment friendly processes. Although carbonic anhydrase is widespread in prokaryotes, there is a need to explore and find out the sturdy CA, and develop effective methods of enzyme immobilization for efficient sequestration of CO_2.

The species of *Bacillus* and *Geobacillus* produce a variety of biocatalysts. Further research is called for studying their diversity and to develop novel strategies for improving the performance of enzymes. Research efforts are also needed on the economic production of enzymes using efficient wild and recombinant strains, and improved fermentation processes.

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Chapter 18
Recent Trends in Valorization of Lignocellulose to Biofuel

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Abstract Bioconversion of renewable lignocellulosic biomass is globally gaining significant prominence. Market forces demonstrate a drive towards products benign to natural environment increasing the importance of renewable materials. The development of second generation bioethanol from lignocellulosic biomass serves many advantages from both energy and environmental point of views. Lignocellulose is a major structural component of woody and non-woody plants and consists of cellulose, hemicellulose and lignin. The effective utilization of all the three components would play a significant role in the economic viability of cellulosic ethanol. The huge amount of plant biomass can be used as an inexpensive feedstock for the production of various value added products including biofuels, chemicals and improved animal feeds. Biomass conversion process involves five major steps: choice of suitable biomass, effective pretreatment, production of saccharolytic enzymes such as cellulases and hemicellulases along with the accessory enzymes, fermentation of hexoses and pentoses, downstream processing. Within the context of production of fuels from biomass, pretreatment has come to denote processes by which cellulosic biomass is made amenable to the action of hydrolytic enzymes. The limited effectiveness of current enzymatic process on lignocellulose is thought to be due to the relative difficulty of pretreating the feedstocks. The present chapter is a comprehensive state of the art describing the advancement in recent pretreaments, metabolic engineering approaches, valorization with special emphasis on the latest developments in consolidated biomass processing and biorefinery concept for the production of biofuel.

Keywords Lignocellulose • Pretreatment • Cellulolytic complex • Hydrolysis • Consolidated bioprocessing • Biorefinery

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18.1 Introduction

The development of fuels from lignocellulosic biomass has many advantages over first generation bioethanol from energy and environmental concerns. First generation biofuel derived mainly from food crops creates many problems ranging from net energy losses to green house gas emission to increased food prices. The recent imbalance in oil market and hike in fuel costs have initiated a global challenge for biofuel production from lignocelluloses. Efficient conversion of lignocellulosic materials to ethanol and value added biochemical is still to day a challenging proposition. In contrast to fossil fuels, cellulosic ethanol produced through fermentation of sugars is a renewable energy source. The estimated global annual production of biomass is 1×10^{11} tons (Smeets et al. 2007) and is a significant renewable resource for the production of biofuel and value added products. It is well documented that cellulosic ethanol offers greater environmental benefits and sustainability; however the drawback remains about the economic viability. Also they relay heavily on major technological innovations centered on effective and low cost enzymes, feedstocks and conversions processes. A large amount of agricultural residues are produced and India alone generates around 600 million tons, most of which is burnt as waste disposal and small amount is used for mulching, for fuel or as fodder. Three types of energy can be produced from these residues, liquid fuels such as ethanol or pyrolysis oil, gaseous fuels such as biogas (methane) and electricity.

Biomass in general consists of 40–50% cellulose, 25–30% hemicellulose and 15–20% lignin and other extractable components (Knauf and Moniruzzaman 2004). The effective utilization of all the three components would play a significant role in economic viability of the cellulose to ethanol process (Pandey 2009). Cellulose is a linear polymer of glucose whereas hemicellulose is a branched heteropolymer of D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and D-glucuronic acid. Lignin is composed of three major phenolic components, namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. Lignin is synthesized by polymerization of these components and their ratio within the polymer varies between different plants, wood tissues and cell wall layers. Cellulose, hemicellulose and lignin form structures called microfibrils, which are organized into macrofibrils that mediate structural stability in the plant cell wall (Rubin 2008). Lignin is a complex hydrophobic, cross-linked aromatic polymer that interferes with the hydrolysis process.

Lignocellulosic biomass includes materials such as agricultural residues (eg. corn stover and wheat straw), forestry residues (eg saw dust and mill waste), municipal soild waste (eg. waste paper), and various industrial wastes. Table 18.1 illustrates the composition of various lignocellulosic feedstocks. Biomass conversion process involves five major aspects: choice of suitable biomass, effective pretreatment, production of saccharolytic enzymes such as cellulases and hemicellulases along with the accessory enzymes, fermentation of hexoses and pentoses, downstream processing. Within the context of production of fuels from biomass, pretreatment has come to denote processes by which cellulosic biomass is made amenable to the action of hydrolytic enzymes (Menon et al. 2010a). Research attention has

Table 18.1 Composition of some Lignocellulosic feedstocks

	Carbohydrate composition (% dry weight)			
Feedstocks	Cellulose	Hemicellulose	Lignin	References
Barley hull	34	36	19	Kim et al. (2008)
Barley straw	36–43	24–33	6.3–9.8	Garda-aparicio et al. (2006), Rowell (1992)
Bamboo	49–50	18–20	23	Reguant and Rinaudo (2000), Alves et al. (2010)
Banana waste	13	15	14	John et al. (2006)
Corn cob	32.3–45.6	39.8	6.7–13.9	Cao et al. (1997), Mckendry (2002)
Corn stover	35.1–39.5	20.7–24.6	11.0–19.1	Lee et al. (1994), Torget et al. (2000)
Cotton	85–95	5–15	0	Kadolph and Langford (1998)
Cotton stalk	31	11	30	Rubio et al. (1998)
Coffee pulp	33.7–36.9	44.2–47.5	15.6–19.1	Pérez-Díaz et al. (2005)
Douglas fir	35–48	20–22	15–21	Schell et al. (1999)
Eucalyptus	45–51	11–18	29	Pereira (1988), Alves et al. (2010)
Hardwood stems	40–55	24–40	18–25	Howard et al. (2003), Malherbe and Cloete (2002)
Rice straw	29.2–34.7	23–25.9	17–19	Brylev et al. (2001), Prassad et al. (2007)
Rice husk	28.7–35.6	11.96–29.3	15.4–20	Allen et al. (2001), Abbas and Ansumali (2010)
Wheat straw	35–39	22–30	12–16	Grohmann et al. (1985), Prassad et al. (2007)
Wheat bran	10.5–14.8	35.5–39.2	8.3–12.5	Miron et al. (2001)
Grasses	25–40	25–50	10–30	Stewart et al. (1997), Hon (2000)
Newspaper	40–55	24–39	18–30	Howard et al. (2003)
Sugarcane bagasse	25–45	28–32	15–25	Singh et al. (2009), Alves et al. (2010)
Sugarcane tops	35	32	14	Jeon et al. (2010)
Pine	42–49	13–25	23–29	Pereira (1988)
Poplar wood	45–51	25–28	10–21	Torget and Hsu (1994)
Olive tree biomass	25.2	15.8	19.1	Cara et al. (2008)
Jute fibres	45–53	18–21	21–26	Mosihuzzaman et al. (1982)
Switchgrass	35–40	25–30	15–20	Howard et al. (2003)
Grasses	25–40	25–50	10–30	Howard et al. (2003), Malherbe and Cloete (2002)
Winter rye	29–30	22–26	16.1	Petersson et al. (2007)
Oilseed rape	27.3	20.5	14.2	Petersson et al. (2007)

(continued)

Table 18.1 (continued)

Feedstocks	Carbohydrate composition (% dry weight)			References
	Cellulose	Hemicellulose	Lignin	
Softwood stem	45–50	24–40	18–25	Howard et al. (2003), Malherbe and Cloete (2002)
Oat straw	31–35	20–26	10–15	Rowell (1992)
Nut shells	25–30	22–28	30–40	Sinner et al. (1979)
Sorghum straw	32–35	24–27	15–21	Herrera et al. (2003), Vázquez et al. (2007)
Tamarind kernel powder	10–15	55–65	–	Menon et al. (2010c)
Water hyacinth	18.2–22.1	48.7–50.1	3.5–5.4	Nigam (2002), Aswathy et al. (2010)

been focused extensively for over two decades to enhance the digestibility of lignocellulosic biomass for the efficient conversion of cellulose to ethanol, methane and in the recent years to hydrogen. It is very necessary to understand the complex structure of cellulose to design a suitable pretreatment.

18.2 Pretreatment

The crucial step in the production of biochemicals from lignocellulosic biomass is pretreatment. Pretreatment denotes to the solubilization and separation of the major components of biomass i.e. cellulose, hemicellulose and lignin and thus render the digestibility of lignocellulosic material. The choice of pretreatment should consider the overall compatibility of feedstocks, enzymes and organisms to be applied. Pretreatment is not only costly in its own right but has a pervasive impact on the cost of virtually all other biological processing operations, including those preceding pretreatment, the handling of the liquid stream generated, the processing of the solids from pretreatment, waste treatment, and potential production of co-products. To implement successfully the bioethanol production process, the first drawback that must be solved is the efficient removal of lignin and hemicellulose through a cost effective pretreatment process.

After initial biomass processing, the production of fermentable sugars from biomass is usually approached in two steps:

1. A pretreatment process in which the cellulose polymers are made accessible for further conversion. In this step hydrolysis of hemicellulose may occur, as well as separation of the lignin fraction, depending on the process applied.
2. Enzymatic cellulose hydrolysis to fermentable sugars using cellulase enzyme cocktails produced in-house or acquired from enzyme manufacturers.

During the past few decades, several technologies have been used for low cost pretreatment approaches for generating sugar syrups from both cellulose and hemicellulose (Yang and Wyman 2008). Different pretreatment technologies published in literature are described in terms of the mechanisms involved, advantages and disadvantages, and economic assessment. Pretreatment technologies for lignocellulosic biomass include biological, mechanical, chemical methods and various combinations thereof (McMillan 1994; Hsu 1996; Wyman et al. 2005). The choice of the optimum pretreatment process depends very much on the objective of the biomass pretreatment, its economic assessment and environmental impact. There are a number of reports on pretreatment options for various biomass types. Table 18.2 illustrates some of the most promising pre-treatment technologies that can be commercialized for the biofuel industry. However, none of those can be declared a "winner" because each pretreatment has its intrinsic advantages and disadvantages. An effective pretreatment is characterized by several criteria: avoiding size reduction, preserving hemicellulose fractions, limiting formation of inhibitors due to degradation products, minimizing energy input, and being cost-effective. Except for these criteria, several other factors are also needed to be considered, including recovery of high value-added co-products (e.g., lignin and protein), pretreatment catalyst, catalyst recycling, and waste treatment (Banerjee et al. 2010). When comparing various pretreatment options, all the mentioned criteria should be comprehensively considered as a basis.

18.2.1 Physical Pretreatment

Most of the lignocellulosic biomass requires some of the mechanical processing for size reduction. Several pretreatment methods such as milling, irradiation (using gamma rays, electron beam, microwave radiations etc.), hydrothermal, extrusion and pyrolysis are commonly used to improve the enzymatic hydrolysis or biodegradability of lignocellulosic materials.

18.2.2 Physico-Chemical Pretreatment

Pretreatments that combine both chemical and physical processes are referred to as physicochemical processes. The most important processes of this group includes: steam explosion, catalyzed (SO_2 or CO_2) steam explosion, ammonia fiber explosion (AFEX), liquid hot water, microwave-chemical pretreatment (Mosier et al. 2002).

18.2.2.1 Steam Explosion

In steam explosion the biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the materials undergo an explo-

Table 18.2 Most promising pretreatment technologies

Method of pretreatment	Sugar yield	Inhibitor formation	Byproduct generation	Reuse of chemicals	Applicability to different feedstock's	Equipment cost	Success at pilot scale	Advantages	Limitations & disadvantages
Mechanical	L	Nil	No	No	Yes	H	Yes	Reduce cellulose crystallinity	High Power consumption than inherent biomass energy
Mineral acids	H	H	H	Yes	Yes	H	Yes	Hydrolysis of cellulose and hemicellulose. alters lignin structure	Hazardous, toxic and corrosive
Alkali	H	L	H	Yes	Yes	Nil	Yes	Removal of lignin and hemicellulose, increases accessible surface area	Long residence time, irrecoverable salts formed
Liquid hot water	H	H	L	No	–	–	Yes	Removal of hemicellulose making enzymes accessible to cellulose	Long residence time, less lignin removal
Organosolv	H	H	H	Yes	Yes	H	Yes	Hydrolyze lignin and hemicellulose	Solvents needs to drained, evaporated, condensed and reused
Wet oxidation	H or L	Nil	L	No	–	H	–	Removal of lignin, dissolves hemicellulose and causes cellulose decrystallization	–
Ozonolysis	H	L	H	No	–	H	No	Reduces lignin content, no toxic residues	Large amount of ozone required
CO_2 explosion	H	L	L	No	–	H	–	Hemicellulose removal, cellulose decrystallization, cost effective	Does not modify lignin

Steam explosion	H	H	L	–	Yes	H	Yes	Hemicellulose removal and alteration in lignin structure	Incomplete destruction of lignin-carbohydrate matrix
AFXE	H	L	–	Yes	–	H	–	Removal of lignin and hemicellulose	Not efficient for biomass with high lignin content
Ionic liquids	H/L	L	–	Yes	Yes	–	–	Dissolution of cellulose, increased amenability to cellulase	Still in initial stages

H High, *L* Low

sive decompression. Steam explosion is typically initiated at a temperature of 160–260°C (corresponding pressure, 0.69–4.83 MPa) for several seconds to a few minutes before the material is exposed to atmospheric pressure. The biomass/steam mixture is held for a period of time to promote hemicellulose hydrolysis, and the process is terminated by an explosive decompression (Varga et al. 2004; Ruiz et al. 2006; Kurabi et al. 2005). The process causes hemicellulose degradation and lignin transformation due to high temperature, thus increasing the potential of cellulose hydrolysis.

The difference between 'steam' pretreatment and 'steam explosion' pretreatment is the quick depressurization and cooling down of the biomass at the end of the steam explosion pretreatment, which causes the water in the biomass to 'explode'. During steam pretreatment parts of the hemicellulose hydrolyze and form acids, which could catalyze the further hydrolysis of the hemicellulose. This process, in which the in situ formed acids catalyze the process itself, is called 'auto-cleave' steam pretreatment. The role of the acids, is probably however not to catalyze the solubilization of the hemicellulose, but to catalyze the hydrolysis of the soluble hemicellulose oligomers (Hendriks and Zeeman 2009). Steam explosion and thermal pretreatments are widely investigated for improving biogas production from different dedicated materials such as forest residuals (Hooper and Li 1996) and wastes of e.g. activated sludge (Ward et al. 1998; Bougrier et al. 2007), cattle manure (Mladenovska et al. 2006) or municipal solid wastes. However, there are several investigations on combining "thermal" pretreatment with addition of bases such as NaOH, which usually give a better result than individual thermal or chemical pretreatment (Kim et al. 2003; DiStefano and Ambulkar 2006). The process of steam explosion was demonstrated on a commercial scale at the Masonite plants (Chum et al. 1985).

18.2.2.2 Ammonia Fiber Explosion (AFEX) and Ammonia Recycle Percolation (ARP)

Ammonia fiber explosion is a physicochemical pretreatment process in which lignocellulosic biomass is exposed to liquid ammonia at high temperature and pressure for a period of time, and then the pressure is suddenly reduced. The AFEX process is very similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg of ammonia/kg of dry biomass, the temperature is 90°C, and the residence time is 30 min. AFEX pretreatment can significantly improve the fermentation rate of various herbaceous crops and grasses (Zheng et al. 2009). The AFEX technology has been used for the pretreatment of many lignocellulosic materials including alfalfa, wheat straw, and wheat chaff. AFEX pretreatment results in the decrystallization of cellulose, partial depolymerization of hemicellulose, removal of acetyl groups predominantly on hemicellulose, cleavage of lignin–carbohydrate complex (LCC) linkages, lignin C–O–C bond cleavage, increase in accessible surface area due to structural disruption, and increased wettability of the treated bio-

mass (Gollapalli et al. 2002; Kumar et al. 2009). The AFEX process demonstrates attractive economics compared to several leading pretreatment technologies based on a recent economic model (Teymouri et al. 2004) for bioethanol from corn stover. An advantage of AFEX is that the ammonia used during the process can be recovered and reused. Also, the downstream processing is less complex compared to other pretreatment processes. Over 90% hydrolysis of cellulose and hemicellulose was obtained after AFEX pretreatment of bermudagrass (approximately 5% lignin) and bagasse (15% lignin). Thus, AFEX is not a very efficient technology for lignocellulosic biomass with relatively high lignin content such as woods and nut shells (Taherzadeh and Karimi 2008) Furthermore, ammonia must be recycled after the pretreatment to reduce the cost and protect the environment. However both the ammonia cost and the cost of recovery processes drive up the cost of the AFEX pretreatment (Holtzapple et al. 1992).

Another type of process utilizing ammonia is the ammonia recycle percolation (ARP) method. In this process, aqueous ammonia (10–15 wt.%) passes through biomass at elevated temperatures (150–170°C) with a fluid velocity of 1 cm/min and a residence time of 14 min, after which the ammonia is recovered (Galbe and Zacchi 2007). In the ARP method, the ammonia is separated and recycled. Under these conditions, aqueous ammonia reacts primarily with lignin and causes depolymerization of lignin and cleavage of lignin-carbohydrate linkages. The ammonia pretreatment does not produce inhibitors for the downstream biological processes, so a water wash is not necessary (Jorgensen et al. 2007). Generally, AFEX and ARP processes are not differentiated in the literature, although AFEX is carried out in liquid ammonia and ARP is carried out in an aqueous ammonia solution (10–15%). The ammonia fiber explosion pretreatment simultaneously reduces lignin content and removes some hemicellulose while decrystallizing cellulose. It can have a profound effect on the rate of cellulose hydrolysis (Sun and Cheng 2002). The cost of ammonia, and especially of ammonia recovery, drives the cost of the AFEX pretreatment.

18.2.2.3 Microwave-Chemical Pretreatment

The microwave/chemical pretreatment resulted in a more effective pretreatment than the conventional heating chemical pretreatment by accelerating reactions during the pretreatment process (Zhu et al. 2005, 2006). Zhu et al. (2006) examined three microwave/chemical processes for pretreatment of rice straw – microwave/alkali, microwave/acid/alkali and microwave/acid/alkali/H_2O_2 – for its enzymatic hydrolysis and for xylose recovery from the pretreatment liquid. They found that xylose could not be recovered during the microwave/alkali pretreatment process, but could be recovered as crystalline xylose during the microwave/acid/alkali and microwave/acid/alkali/H_2O_2 pretreatment. The enzymatic hydrolysis of pretreated rice straw showed that the pretreatment by microwave/acid/alkali/H_2O_2 had the highest hydrolysis rate and glucose content in the hydrolyzate.

18.2.3 Chemical Pretreatments

Chemical pretreatments were originally developed and have been extensively used in the paper industry for delignification of cellulosic materials to produce high quality paper products. The possibility of developing effective and inexpensive pretreatment techniques by modifying the pulping processes has been considered. Chemical pretreatments that have been studied to date have had the primary goal of improving the biodegradability of cellulose by removing lignin and/or hemicellulose, and to a lesser degree decreasing the degree of polymerization (DP) and crystallinity of the cellulose component. Chemical pretreatment is the most studied pretreatment technique among pretreatment categories. The various commonly used chemical pretreatments includes: acid, alkali, organic acids, pH-controlled liquid hot water and ionic liquids.

18.2.3.1 Ionic Liquids (ILs)

Recently a new class of solvent has emerged – ionic liquids. These solvents are often fluid at room temperature, and consist entirely of ionic species. They have many fascinating properties which make them of fundamental interest to all chemists, since both the thermodynamics and kinetics of reactions carried out in ionic liquids are different to those in conventional molecular solvents. In general, ionic liquids consist of a salt where one or both the ions are large, and the cation has a low degree of symmetry (Earle and Seddon 2000). These factors tend to reduce the lattice energy of the crystalline form of the salt, and hence lower the melting point (Seddon 1998). Ionic liquids come in two main categories, namely simple salts (made of a single anion and cation) and binary ionic liquids (salts where an equilibrium is involved). Ionic liquids have been described as designer solvents (Freemantle 1998), and this means that their properties can be adjusted to suit the requirements of a particular process.

Very recently ILs or green solvents, have been confirmed to be efficient for dissolution of lignocellulosic materials, such as cellulose, wood, or wheat straw (Liu and Chen 2006; Diego et al. 2007; Li et al. 2009). Using 1-butyl-3-methylimidazolium chloride (BMIMCl) for pretreatment, Dadi et al. (2006) found that the initial enzymatic hydrolysis rate and yield of pretreated Avicel-PH-101 were increased by 50- and 2-fold in comparison with untreated Avicel. Kuo and Lee (2009) also observed that the 1, 3-N-methylmorpholine-N-oxide (NMMO) pretreated sugarcane bagasse has twofold higher enzymatic hydrolysis yield as compared to untreated bagasse. Nguyen et al. (2010) reported the combined use of ammonia and ionic liquid ([Emim]Ac) for the recovery of bio-digestible cellulose from rice straw. The treatment exhibited a synergy effect for rice straw with 82% of the cellulose recovery and 97% of the enzymatic glucose conversion.

The cellulosic materials regenerated from ILs were found essentially amorphous and porous and were much more prone to degradation by cellulases (Li et al. 2009; Dadi et al. 2006). Furthermore, increased rate of cellulose hydrolysis via cellulase

in ionic liquids could lead to increased production of fermentable sugars that can be converted into fuels. In addition, ionic liquids involved processes are less energy demanding, easier to operate, and more environmentally friendly than current dissolution processes (Zhao et al. 2009; Rogers and Seddon 2003; Hélène and Lionel 2002). In addition, residue of ionic liquid from the pretreatment may follow the cellulose fibers to the enzymatic hydrolysis and fermentation process.

However, more in-depth research involving environment friendly IL is much needed to explore pretreatment green route for resolving the challenge of ionic liquid application (Zhao et al. 2008; Rogers and Seddon 2003). As a result, recent efforts have been focused on exploration enzyme friendly and environment-friendly ionic liquids (Roosen et al. 2008), because green solvent is promising for commercial application (Kamiya et al. 2008). Therefore, it is a key point to evaluate the biocompatibility of ionic liquids and select green IL for pretreatment process (Li et al. 2010). Application of ionic liquids has opened new ways for the efficient utilization of lignocellulosic materials in such areas as biomass pretreatment and fractionation. However, there are still many challenges in putting these potential applications into practical use, for example, the high cost of ILs, regeneration requirement, lack of toxicological data and knowledge about basic physicochemical characteristics, action mode on hemicellulose and/or lignin contents of Lignocellulosic materials and inhibitor generation issues. Further extensive research is required to address such challenges.

18.2.4 Biological Pretreatment

Biological pretreatment employs wood degrading microorganisms, including white-, brown-, soft-rot fungi, and bacteria to modify the chemical composition and/or structure of the lignocellulosic biomass so that the modified biomass is more amenable to enzyme digestion. Fungi have distinct degradation characteristics on lignocellulosic biomass. In general, brown and soft rots mainly attack cellulose while imparting minor modifications to lignin, and white-rot fungi are more actively degrade the lignin component (Sun and Cheng 2002). Present research is aimed towards finding those organisms which can degrade lignin more effectively and more specifically. White-rot fungi were considered the most promising basidiomycetes for bio-pretreatment of biomass and were the most studied biomass degrading microorganisms (Sánchez 2009). The biological pretreatment appears to be a promising technique and has very evident advantages, including no chemical requirement, low energy input, mild environmental conditions, and environmentally friendly working manner (Kurakake et al. 2007). However, its disadvantages are as apparent as its advantages since biological pretreatment is very slow and requires careful control of growth conditions and large amount of space to perform treatment. In addition, most lignolytic microorganisms solubilize/consume not only lignin but also hemicellulose and cellulose. Therefore, the biological pretreatment faces technoeconomic challenges and is less attractive commercially (Eggeman and Elander 2005).

Obstacles in the existing pretreatment processes include insufficient separation of cellulose and lignin, formation of by-products that inhibit ethanol fermentation, high use of chemicals and/or energy, and considerable waste production (Banerjee et al. 2010). Research is focused on converting biomass into its constituents in a market competitive and environmentally sustainable way. Only a small number of pretreatment methods have been reported as being potentially cost-effective thus far. These include steam explosion, liquid hot water, and concentrated acid hydrolysis and dilute acid pretreatments. An economical evaluation of five different pretreatment technologies (dilute acid, hot water, ammonia fiber explosion (AFEX), ammonia recycle percolation (ARP), and lime) is presented by Eggeman and Elander (2005). At the moment the production of ethanol from lignocellulose is growing rapidly and by looking at the industrial activities in this field more knowledge can be gained on the applied pretreatment methods. It is not possible to define the best pretreatment method as it depends on many factors such as type of lignocellulosic biomass and desired products. Pretreatments must improve the digestibility of lignocellulosic biomaterials, and each pretreatment has its own effect on the cellulose, hemicellulose and lignin fractions. Looking at industrial activities for the production of ethanol, acid-based pretreatment methods are preferred.

For systematically comparing the leading pretreatment technologies a biomass refining Consortium for Applied Fundamentals and Innovation (CAFI) was formed in early 2000.

The results of the research by this team are the first comparative data based on use of the same feedstock, the same enzyme formulations, identical analytical methods, consistent material balance approaches, and a common framework for economic comparisons (Wyman et al. 2005). All of the pretreatment in this research use chemicals including dilute acid, sulfur dioxide, ammonia, and lime but span a wide range of pH values. Among these options, low pH acidic pretreatments tend to remove and recover a large fraction of the hemicellulose in biomass, and high pH pretreatments using base typically remove a significant fraction of the lignin effectively (Yang and Wyman 2008). There are several desirable goals for pretreatment processes, however, in practice not all of them are achieved with any current treatments. Generally, the pretreatment should promote high product yields in a subsequent enzymatic hydrolysis and fermentation operations with minimum cost. Recently it has been demonstrated that the dilute acid pre-hydrolysis can achieve high reactions rates in short time and significantly improve cellulose hydrolysis. However, pretreatment operating conditions must be tailored to the specific chemical and structural composition of the various sources of biomass. Despite continuing interest in the kinetic mechanism of solid-phase acid-catalyzed hydrolysis for several type of biomass, little attention has been given to incorporating those kinetic models into the plant process modeling. For the one side, most of the techno-economic studies based on process simulations for bioethanol production from lignocellulosic biomass to produce mainly sugar monomers (glucose, xylose, arabinose, mannose) and acid-soluble lignin, but using conversion fractions at fixed operating conditions. On the other side, from batch kinetic studies, it has been revealed that the main factors affecting the acid pretreatment are the type of biomass, the type

of acid, the feed acid concentration, the reaction time and the reaction temperature. So that kinetic modeling and the operating conditions of the pretreatment unit play an important role in the design, development, and operation of the complete process of bioethanol production. Process modeling and simulation is critical and decisive for the well design of the pretreatment process of lignocellulosic biomass. For a deeper insight into technical and conceptual aspects of various pretreatments, the readers are referred to recent monographs (Taherzadeh and Karimi 2008; Kumar et al. 2009; Zheng et al. 2009; Hendriks and Zeeman 2009).

18.3 Cellulases and Hydrolysis

The hydrolysis of cellulose by cellulolytic enzymes has been investigated intensively since the early 1970s, with the objective of developing a process for the production of ethanol. Over the past decades, a great amount of research interest and effort has been generated in this area. Cellulases play a significant role in the enzymatic process by catalyzing the hydrolysis of cellulose to soluble, fermentable sugars. Cellulases are synthesized by fungi, bacteria and plants. A list of microorganisms producing cellulases are enlisted in Table 18.3**.** At least, three major type of cellulase enzymatic activities are believed to be involved in cellulose hydrolysis based on their structural properties: endoglucanases or 1,4-β-D-glucan- 4-glucanohydrolases (EC 3.2.1.4), exoglucanases, including 1,4-β-D-glucan glucanohydrolases (also known as cellodextrinases) (EC 3.2.1.74) and 1,4-β-D-glucan cellobiohydrolases (cellobiohydrolases) (EC 3.2.1.91), and β-glucosidases or β-glucoside glucohydrolasess (EC 3.2.1.21) (Lynd et al. 2002).

Enzymatic hydrolysis methods have shown distinct advantages over acid based hydrolysis methods; the very mild process conditions give potentially higher yields, the utility cost is low (no corrosion problems), Therefore this is the method of choice for future wood-to-ethanol processes. The effective hydrolysis of cellulosic biomass requires the synergistic action of cellulases such as exocellulase, endocellulase and β-glucosidase (Schulein 1988). Even though soluble substrates have been developed for measuring endoglucanase and β-glucosidase activities there are very few substrates available for the estimation of exoglucanase activity. The hydrolysis data from soluble substrates cannot yield useful information on the hydrolysis of insoluble substrates. There are different factors that affect the enzymatic hydrolysis of the cellulose namely, substrates, cellulase activity, and reaction conditions (temperature, pH, as well as other parameters). To improve the yield and rate of the enzymatic hydrolysis, research has been focused on optimizing the hydrolysis process and enhancing the cellulase activity. The yield and initial rate of enzymatic hydrolysis of the cellulose is affected mainly by the substrate concentration. The heterogeneity of the cellulosic biomass, the dynamic interactions between insoluble substrates and complex cellulase components, and result in formidable problems in extrapolating the activity measured on one solid substrate to other solid substrates, especially those with biorefinery impact. This point is critical to the eventual – improvement of

Table 18.3 Production of cellulases using microorganisms

Microorganism	Method	Enzyme activities			References
		FPase	CMCase	β-glucosidase	
Acinetobacter anitratus	SmF	ND	0.48 U/ml	ND	Ekperigin (2007)
Bacillus subtilis	SSF	2.8 IU/gds	9.6 IU/gds	ND	Tsao et al. (2000)
Bacillus pumilus	SmF	ND	1.9 U/ml	ND	Abdel-Fattah et al. (1997)
Cellulomonas biazotea	SmF	7,450 nkat/g	13,933 nkat/g	2,850 nkat/g	Rajoka and Malik (1997)
Clostridium papyrosolvens	SmF	35 IU/ml	45 IU/ml	ND	Rani et al. (2004)
Chaetomium globosum	SmF	1.4 U/ml	30.4 U/ml	9.8 U/ml	Umikalsom et al. (1997)
Streptomyces drodowiczi	SmF	4.4 U/gds	595 U/L	ND	Grigorevski de-limaa et al. (2005)
Thermomonospora sp	SmF	0.11 IU/ml	23 IU/ml	0.02 IU/ml	George et al. (2001)
Thermoascus auranticus	SSF	4.4 U/gds	987 U/gds	48.8 U/gds	Fujian et al. (2002)
Neurospora crassa	SmF	1.33 U/ml	19.7 U/ml	0.58 U/ml	Romero et al. (1999)
Thermotoga maritima	SmF	ND	ND	30 mU/ml	Jang and Chang (2005)
Trichoderma ressei	SmF	2.49 IU/ml	7.15 IU/ml	2.17 IU/ml	Pham and Halos (1990)
T ressei RUT C 30	SmF	6.2 U/ml	54.2 U/ml	0.39 U/ml	Pham and Halos (1990)
T species A-001	SmF	18 U/ml	167 U/ml	49 U/ml	Gashe (1992)
T ressei ZU 02	SmF	0.25 IU/ml	5.48 IU/ml	ND	Shen and Xia (2004)
T viridae	SmF	0.88 U/ml	33.8 U/ml	0.33 U/ml	Adsul et al. (2004)
Penicillium funiculosum	SmF	1.4 IU/ml	4.55 IU/ml	9.29 IU/ml	Pham and Halos (1990)
Penicillum pinnophilum	SmF	2 U/ml	65 U/ml	10 U/ml	Singh et al. (2009)
P janthinellum	SmF	0.55 U/ml	21.5 U/ml	2.31 U/ml	Adsul et al. (2004)
P decumbans	SSF	20.4 IU/g	ND	ND	Yang et al. (2004)
P occitanis	SmF-Fed	23 IU/ml	21 IU/ml	ND	Belghith et al. (2001)
A fumigatus IMI 246651	SmF	40 EU/ml	0.5 EU/ml	1.73 EU/ml	Stewart and Parry (1981)
A terreus	SSF	243 U/g	581 U/g	128 U/g	Gao et al. (2008)
Fusarium oxysporum	SSF	304 U/g	ND	0.140 U/g	Panagiotou et al. (2003)

cellulases for the conversion of pretreated plant cell walls in energy crops and agricultural residues. Realistic methods must be based on physically and chemically relevant industrial substrates.

The most frequently reported source of cellulases is the fungus *Trichoderma reesei,* the most studied cellulolytic microorganism during the last 60 years. Among the various microorganisms capable of synthesizing cellulase enzymes, *T. reesei* produces an extracellular, stable, and efficient cellulase enzyme system (Jana et al. 1994; Saddler and Gregg 1998). However, the low-glucosidase activity of the enzyme system from *T. reesei* leads to incomplete hydrolysis of cellobiose in the reaction mixture and, as a result, to serious inhibition of the enzymes (Holtzapple et al. 1990). The factors influencing enzymatic hydrolysis can be divided into substrate related factors and enzyme related factors. The relationship between structural features of cellulose and rates of enzymatic hydrolysis has been the subject of extensive study and several reviews have been published (Lynd et al. 2002; Zhang and Lynd 2004). Most desired attributes of cellulases for lignocellulose bioconversion are the complete hydrolytic machinery, high specific activity, high rate of turn over with native cellulose/biomass as substrate, thermostability, decreased susceptibility to enzyme inhibition by cellobiose and glucose, selective adsorption on cellulose and ability to withstand shear forces. These parameters are fulfilled through protein engineering approaches, over expression techniques and developing optimal enzyme cocktails and conditions for hydrolysis. Recently the enzymatic hydrolysis of lignocellulosic biomass has been optimized using enzymes from different sources and mixing in an appropriate proportion using statistical approach of factorial design. A twofold reduction in the total protein required to reach glucan to glucose and xylan to xylose hydrolysis targets (99% and 88% conversion, respectively), thereby validating this approach towards enzyme improvement and process cost reduction for lignocellulose hydrolysis (Berlin et al. 2005). Despite intensive research over the few past decades, the enzyme hydrolysis step remains as a major techno-economic bottleneck in lignocellulose biomass-to-ethanol bioconversion process.

18.4 Fermentation Strategies and Consolidated Biomass Processing

The pretreated biomass can be processed using variety of process configurations such as separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated biomass processing (CBP) (Fig. 18.1). SHF is a conventional two step process where the lignocellulose is hydrolysed using the enzymes to form the reducing sugars in the first step and the sugars, thus formed, are fermented to ethanol in the second step using various yeasts such as *Saccharomyces*, *Kluveryomyces*, *Debaryomyces*, *Pichia*, *Zymomonas* as well as their recombinants.. The advantage of this process is that each step can be carried out at its optimum conditions. The

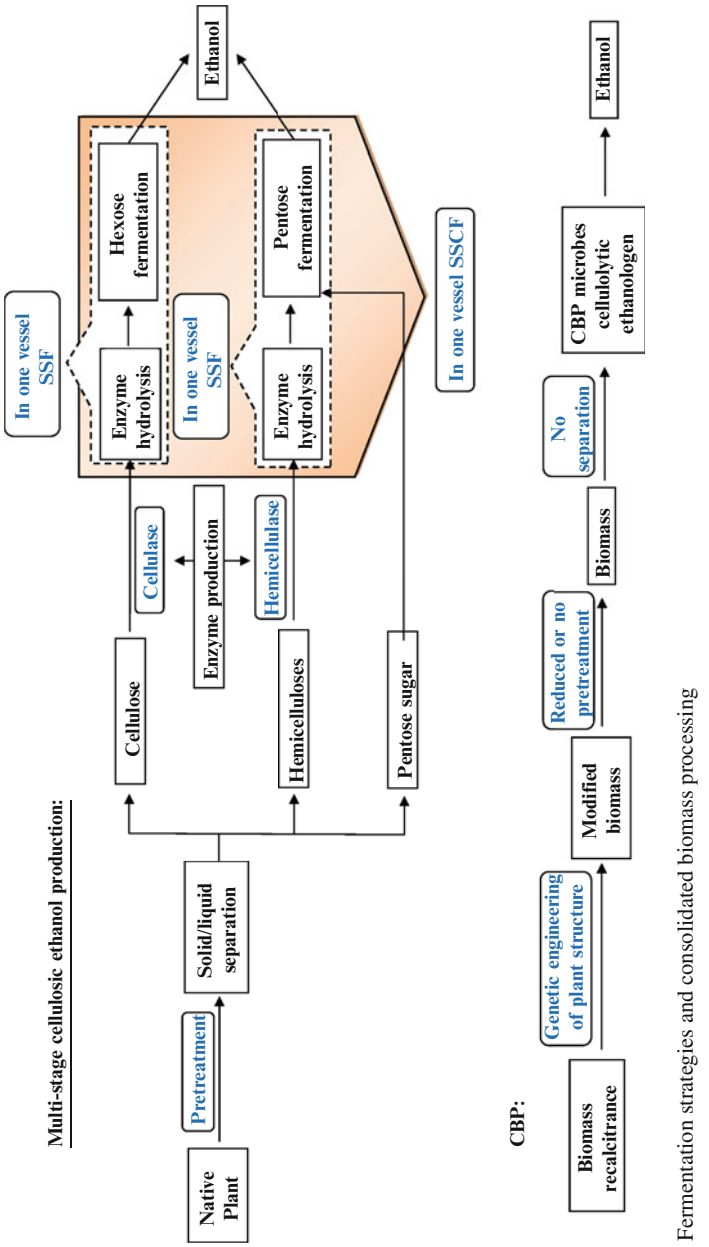

Fig. 18.1 Fermentation strategies and consolidated biomass processing

saccharification of the lignocellulosic biomass by the enzymes and the subsequent fermentation of the sugars to ethanol by yeast such as *Saccharomyces* or *Zymomonas* takes place in the same vessel in SSF. The compatibility of both saccharification and fermentation process with respect to various conditions such as pH, temperature, substrate concentration etc. is one of the most important factors governing the success of the SSF process. The main advantage of using SSF for the ethanol bioconversion is enhanced rate of hydrolysis of lignocellulosic biomass (cellulose and hemicellulose) due to removal of end product inhibition. Another factor of great importance in the fermentative processes is the cultivation conditions, which, if inadequate, can stimulate the inhibitory action of the toxic compounds (Ballesteros et al. 2004). Thermotolerant yeast is an added advantage for SSF and thermotolerant yeast strains, e.g. *Fabospora fragilis*, *Saccharomyces uvarum*, *Candida brassicae*, *C. lusitaniae*, and *Kluyveromyces marxianus*, have been evaluated for future use in SSF, to allow fermentation at temperatures closer to the optimal temperature for the enzymes. However, in all these cases saccharification of pure cellulose (e.g. Sigmacell-50) or washed fibers, in defined fermentation medium, were applied. SSF of cellulose with mixed cultures of different thermotolerant yeast strains have also been carried out (Olofsson et al. 2008). However, there is scarcity of literature from SSF experiments in which hemicelluloses have been used together with thermotolerant strains. Menon et al. (2010b) have reported for the first time the SSF experiments using hemicellulosic substrates and thermotolerant *Debaryomyces hansenii*. Maximum ethanol concentrations of 9.1 g/L and 9.5 g/L were obtained in SSF with oat spelt xylan and wheat bran hemicellulose respectively. These concentrations were attained in 36 h for OSX and 48 h for WBH from the onset of SSF. The increased ethanol yield in SSF systems is evidently due to removal of xylose formed during hydrolysis which causes end product inhibition.

Simultaneous saccharification of cellulose and hemicellulose and co-fermentation of end products glucose and xylose (SSCF) is reported to be carried out by genetically engineered microbes. Synthetic biology approach for co-fermentation of hexose and pentose sugars has been successfully demonstrated by incorporating two mutants of *E.coli* in a single system – one capable of utilizing only glucose, while the other capable of utilizing only xylose as a carbon source. In this study, catabolite repression due to the presence of glucose was made irrelevant by the use of two strains, since one cannot utilize glucose at all. Furthermore, the authors demonstrate by fed-batch experiments that the system robustly adapts to fluctuations in the feed stream, i.e., cultures actually grow in concert with the varying feed composition (Eiteman et al. 2008). Another promising alternative for simultaneous utilization of pentoses and hexoses is the conversion of xylose into xylulose by xylose isomerase or oxidoreductase. The fermentation of xylulose begins by its intracellular phosphorylation to xylulose-5-phosphate, which enters the pentose phosphate pathway and eventually forms ethanol. Yeasts like *S. cerevisiae* and *Schizosaccharomyces pombe* are capable of fermenting xylulose to ethanol, along with glucose (Chandrakant and Bisaria 1998).

Lignocellulosic bioprospecting involving enzymes or microbial systems commonly includes three major biologically mediated processes: production of saccharolytic

enzymes (cellulases and hemicellulases), hydrolysis of cellulose and hemicellulose to monomeric sugars, fermentation of hexose and pentose sugars. These three biotransformations occurring in a single process configuration is called as consolidated biomass processing (CBP). CBP offers the potential for lower cost and higher efficiency than processes featuring dedicated cellulase production. This result from avoided costs for capital, substrate and other raw materials, and utilities associated with cellulase production. In addition, several factors support the possibility of realizing higher hydrolysis rates, and hence reduced reactor volume and capital investment, using CBP. These include enzyme–microbe synergy as well as the use of thermophilic organisms and/or complexed cellulase systems. CBP requires a microbial culture that combines properties related to both substrate utilization and product formation. Desired substrate utilization properties include the production of a hydrolytic enzyme system allowing high rates of hydrolysis and utilization of resulting hydrolysis products under anaerobic conditions with a practical growth medium. Desired product formation properties include high product selectivity and concentrations. A cellulolytic culture with this combination of properties has not been described to date. Development of microorganisms for cellulose conversion via CBP can be pursued according to two strategies. The native cellulolytic strategy involves naturally occurring cellulolytic microorganisms to improve product-related properties such as yield and tolerance. The recombinant cellulolytic strategy involves engineering noncellulolytic microorganisms that exhibit high product yields and tolerance so that they become able to utilize cellulose as a result of a heterologous cellulase system (Lynd et al. 2002). Both CBP organism development strategies involve very large challenges that will probably require a substantial sustained effort to overcome. The feasibility of CBP will be fully established only when a microorganism or microbial consortium is developed that satisfies the requirements. Fundamental and applied topics relevant to CBP are comprehensively reviewed in Lynd et al. (2002) and Lynd et al. (2005).

18.5 Metabolic Engineering Approaches

One of the strategies for improving the economics and yields of lignocellulosic bioethanol process is optimization of metabolic pathways through genetic modifications for effective manipulation of metabolic capabilities of microbes. The exploitation of the diverse metabolic pathways leading to energy-rich, fuel-like hydrocarbons opens up a path to develop renewable fuels that go far beyond the restrictions of bioethanol and plant-derived biodiesel. The increasing number of sequenced genomes is a potent source of enzymes. According Rubin (2008) even though the cellulosic biomass has the potential to contribute to meet the demand for liquid fuels, however the land-use requirements and process inefficiencies causes disturbance to large scale deployment of biomass to biofuel technologies. Genomic data collected globally, including potential energy crops and cellulolytic microbes will be significant for cellulosic biofuel production. Significant progress has been made in understanding

the induction mechanism of cellulases by lactose in *Trichoderma ressei*, which involves an alternative D-galactose metabolism pathway. With the recent availability of the complete genome sequence, *T ressei* has entered the post-genomic era and the knowledge in advancement of metabolic engineering approaches will enhance the probability of obtaining new hyper active mutants strains. *Trichoderma* sp. dominantly occupies their ecological niches, it is reasonable to assume to the presence and operation of efficient machinery for the perception and interpretation of environmental signals. Thus a better understanding of signal transduction pathways initiating and/or modulating this process may help to develop new strategies for improving cellulase gene expression (Kubicek et al. 2009).

Industrial biocatalysts, such as the common yeast *Saccharomyces cerevisiae*, rarely possess native pathways able to efficiently ferment both hexoses (such as glucose) and pentoses (such as xylose) (Jeffries and Jin 2004). *E. coli* can actively metabolize a wide range of substrates, including hexoses and pentoses (Ingram et al. 1999), but its hexose metabolism is inferior to that of *Z. mobilis*, an obligate ethanologenic bacterium (Zhang et al. 1995). As a metabolic enhancement strategy, *E. coli* has been genetically engineered for cofermentation of all constitutive sugars from lignocellulose by importing the highly efficient fermentation pathway for ethanol production from *Z. mobilis* (Ingram et al. 1987). Metabolic redirection is an alternative strategy, i.e., redirection of the central metabolism of the ethanologenic organism by gene knockout to block undesirable metabolic pathways, generally in order to divert the carbon flow of pyruvate from organic acids to ethanol production (e.g., Jarboe et al. 2007; Zhu and Shimizu 2004).

Advancements in the fields of metabolic engineering, synthetic biology, and systems biology have further increased the ability to successfully implement and analyze different strategies to engineer microbes for the production of a broad range of novel biofuels through the exploitation of various metabolic pathways. The continued development of synthetic biology tools that both reduce the time required to make genetic constructs as well as increasing their predictability and reliability should greatly improve metabolic engineering techniques for the effective production of a wide variety of fuels (Lee et al. 2008) and chemicals (Carothers et al. 2009). In addition, the use of system biology tools such as genomics, transcriptomics, proteomics, metabolomics, and fluxomics will help facilitate the design, characterization, and integration of new metabolic pathways for biofuel production (Rodriguez-Moya and Gonzalez 2009; Mukhopadhyay et al. 2008). The continued use and development of strategies and tools from these fields can only serve to further increase the yield, titer, and productivity of an even greater variety of biofuels from various feedstocks representing a promising path for the viable industrial production of renewable fuels critical to lessening our dependence on fossil fuels. Furthermore, microbial fuels that are easy to recover and do not require additional chemical conversion have the best chances to be developed in cost-effective and unsubsidized commercial processes. (Rude and Schirmer 2009). The strategies, concepts and applied topics related to metabolic engineering is reviewed in (Lee et al. 2008; Clomburg and Gonzalez 2010; Peralta-Yahya and Keasling 2010; Dellomonaco et al. 2010).

18.6 Biorefinery Approach

The use of biomass as a resource for energy and fuel production will be limited by maximum production rates and the supply of biomass rather than the demand for energy and fuel. The relatively low energy content, seasonality and discrete geographic availability of biomass feedstocks have been noted as barriers to the large volume demands for energy and fuel (Lipinsky 1981). As such, there exists an economic opportunity for the development of bio-sourced chemical products since the value of the chemical industry is comparable to the fuel industry, but requires only a fraction of the biomass. Biorefinery concept is to utilize inedible lignocellulosic biomass to produce biofuels or fibers as well as byproducts, lignin and/or hemicellulose. These fractions can be used directly as desired chemicals or can be converted by chemical, enzymatic, and/or microbial approaches. Conversion of these by-products to high-value co-products will offset the cost of biofuel, improve the economy of lignocellulose biorefinery, minimize the waste discharge, and reduce the dependence of petroleum-based products. The biorefinery of biomass will offer new economic opportunities for agriculture and chemical industries by the production of a tremendous variety of chemicals, transportation fuels, and energy (Fitzpatrick et al. 2010). To develop technologically sustainable bio-refinery routes, the entire chain of biomass production, i.e., from breeding, cultivation, and harvest, its (pre)treatment, and conversion to products should be considered. In addition, it will be shown that small scale (pre)processing of the biomass may be advantageous over large-scale processing (Sanders et al. 2007). Conceptually, a biorefinery would apply hybrid technologies from different fields including polymer chemistry, bioengineering and agriculture (Ohara 2003). Biomass is separated into its component parts: sugars (as cellulose, hemicellulose or starch), lignin, protein and oils. In various current biorefinery concepts, the sugar or oil fractions are used to produce liquid transport fuel or products while lignin is most often relegated to low-value uses of combustion (Table 18.4). In fact, in the currently operating biorefineries lignin is either burned to produce process heat and recover pulping chemicals in paper mills or sold as a natural component of animal feeds in wet or dry corn mills.

The choice of feedstock and final products are important in biorefinery design due to the large-scale production implications. Initial feedstock availability and its potential use in multiple production streams both need to be considered (Mabee et al. 2005). Recently, chemical companies such as Dow Chemical Company, Huntsman Corporation, Cargill and Archer Daniels Midland Corporation, have begun to use glycerol as a low-cost building block material for conversion to higher value propylene glycol (McCoy 2007). Furthermore, Dow Chemical Company and Solvay are exploring the use of glycerol in the production of epichlorohydrin, which can be used in the manufacture of epoxy resins and epichlorohydrin elastomers (Dodds and Gross 2007). Due to its versatility, it is anticipated that glycerol could become a substitute for many commonly used petrochemicals (Pagliaro and Rossi 2008).

Table 18.4 Value added biochemicals potentially derived from cellulose, hemicellulose and lignin

Lignocellulosic biomass		
Cellulose	Polymers	
	Levulinic acid	Succinic acid, THF, MTHF, 1,4 butanediol, NMP, Lactones
	Ethanol	
	Lactic acid	Acrylic acid, Acetaldehyde 2,3-pentanedione, Pyruvic acid
	3-hydroxy-propanioc acid	
	Itaconic acid	3-methyl THF, 3-methyl pyrrolidone 2,methyl-1,4- butane diamine Itaconic diamide
	Glutamic acid	
	Glucuronic acid	
	Succinic acid	2-pyrrolidones, 1,4- butanediol, Tetrahydrofurane
Hemicellulose	Xylitol	
	Ethanol, butanol, hydrogen 2,3-butanediol	
	Ferulic acid	Vanillin, Vanillic acid, Protocatechuic acid
	Lactic acid	
	Furfural	
	Chitosan	
	Xylo-oligosaccharides	
Lignin	Syngas	
	Syngas products	Methanol/Dimethy ether, Ethanol, Mixed liquid fuels
	Hydrocarbons	Cyclohexanes, higher alkylates
	Phenols	Cresols, Eugenol, Coniferols, Syringols
	Oxidized products	Vanillin, vanillic acid, DMSO, aldehydes, Quinones, aromatic and aliphatic acids
	Macromolecules	Carbon fibres, Activated carbon, polymer alloys, polyelectrolites, substituted lignins, thermosets, composites, wood preservatives, Neutraceuticals/ drugs, adhesives and resins

The current situation indicates that the demand for food, energy, mobility, chemicals and materials will increase tremendously in the near future. 80–90% of the fossil resources are used for energy supply and mobility. Being aware that a coupling between world markets for energy and fuels, raw materials for chemicals and materials, feed and food and biomass exists one has to solve the problems of raw material supply for food production as well as for materials, fuels and energy production (Octave and Thomas 2009). Depending on regional and technological

boundary conditions the most economic solution for a biorefinery may be provided by large centralized facilities as well as by smaller decentralized plants (Lyko et al. 2009).

18.7 Current Status of Lignocellulosic Ethanol Industry

The commercialization of cellulose to ethanol technology in the current scenario is not economically viable. Currently there are special programs in a number of countries targeting production of biofuel such as biogas, bioethanol, biodiesel and fuel cells from renewable resources (Yuan et al. 2008). Global production of bioethanol increased from 17.25 billion liters in 2000 to over 46 billion liters in 2007, which represented about 4% of the 1,300 billion liters of gasoline consumed globally (Balat 2007). With all of the new government programs in America, Asia, and Europe in place, total global fuel bioethanol demand could grow to exceed 125 billion liters by 2020 (Demirbas 2007). Bioenergy ranks second (to hydropower) and accounts for 3% in renewable primary energy production in U.S. (James and Barry 2007). The United States is the world's largest producer of bioethanol fuel, accounting for nearly 47% of global bioethanol production in 2005 and 2006 (Balat and Balat 2009). The "Biofuels Initiative" in the U.S. (US Department of Energy), strives to make cellulosic ethanol cost-competitive by 2012 and supposedly to correspond and account for one third of the U.S. fuel consumption by 2030. In 2007, the U.S. president signed the Energy Independence and Security Act of 2007, which requires 34 billion liters of bio-fuels (mainly bioethanol) in 2008, increasing steadily to 57.5 billion liters in 2012 and to 136 billion liters in 2022. Brazil is the world's largest exporter of bioethanol and second largest producer after the United States. Production is expected to rise from 15.4 billion litres in 2004 to 26.0 billion litres by 2010. Ethanol from sugarcane provides 40% of automobile fuel in Brazil and approximately 20% is exported to the U.S., EU, and other markets. There are more than ten ethanol biofuel facilities either in operation or under construction in Canada and 130 plants in the United States (Parcell and Westhoff 2006; Oosterveer and Mol 2010). In eastern Canada and the U.S., corn is used as the feedstock while in western Canada wheat is used. Many Asian countries such as China, India, Japan, and Indonesia are also developing ethanol production capacity (Yang and Lu 2006; Worldwatch Institute 2006). In India, Praj Industries have recently started a demonstration plant that can process 2 tonnes per day of lignocellulosic feedstocks such as corn stover, corn cob, bagasse, agro waste and wood chips for biofuel production (Banerjee et al. 2010). A few companies have operated pilot plants (Table 18.5), however no commercial industrial scale plants for biofuel production is in operation.

Current research is directed towards identifying, evaluating, developing and demonstrating different pretreatment methods that result in efficient enzymatic hydrolysis. Several physical, physico-chemical, chemical and biological pretreatments or their combinations are under evaluation. Due to the high cost of enzyme

Table 18.5 Different technologies used worldwide for production of bioethanol

Company	Location	Products	Status	Raw material	Pretreatment/technology	Fate of lignin
Abengoa	Spain	4.000 t/a EtOH	Demo facility, start-up 2009	Wheat straw	Acid catalysed steam explosion, Enzymatic hydrolysis	As co-product, Recovered after distillation
Inbicon	Denmark	4.000 t/a EtOH C5-molasses Solid biofuel	Demofacility, start-up 2009	Wheat straw	Liquid hot water (hydro-thermal, auto catalysed)	Solid biofuel for power-plant, Recovered after distillation
Iogen	Canada	70.000 t/a EtOH	Commercial facility, start-up 2011	Straw (wheat, bar-ley, oat)	Modified steam explo-sion, Enzymatic hydrolysis	For steam and electricity generation, Recovered after enzymatic hydrolysis
KL Energy	USA	4.500 t/a EtOH	Demo facility, opera-tional since 2007	Wood waste, cardboard and paper	Thermo mechanical	For steam or electricity generation, or as wood pellet co-product, Recovered after distillation
SEKAB	Sweden	4.500 t/a EtOH	Demo facility, start-up 2011d	Wood chips or sugar-cane bagasse	Acid pre-treatment	For energy production or other uses Recovered after (enzymatic) hydrolysis
Verenium process	USA	4.200 t/a EtOH	Demo facility, opera-tional since 2009	Sugarcane bagasse, energy crops, wood products and switchgrass	Mild acid hydrolysis and steam explosion	Lignin-rich residue burned for steam generation Recovered after distillation
Range fuels	Georgia	20 million gallon/ annum EtOH	Commercial facility, start up at 2010	Woody biomass and grasses	Thermo-chemical process	For energy production

Source: Biotechnology Industry Organization (BIO), companies

the current fuel grade ethanol produced from lignocellulosic material is still not able to compete with gasoline. In a contemporary process of lignocellulosic ethanol which is being worked out for more than 2–3 decades is still not materialized into a viable technology. The permissible cost of enzymes is 15–30 cents/gal of ethanol which is still not a reality. The lignocellulosic biomass feedstock including pretreatment costs around 50–80 cents/gal of ethanol (Chapple et al. 2007). Today cellulosic ethanol still lies around approx US$ 4 per gallon of ethanol based on best estimates (Louime and Uckelmann 2008). The cellulase cost has been reduced dramatically from US$5.40 per gallon of ethanol to approximately 20 cents per gallon of ethanol; further efforts are focused on lower costs for bioconversion to below 5 cents per US gallon ethanol. The hydrolysis and subsequent fermentation of lignocellulose is complicated and the economic viability of the process is yet to be achieved.

18.8 Future Prospects

Although bioethanol production has been greatly improved by new technologies there are still challenges that need further investigations. Tremendous focus is essential for developing a detailed understanding of lignocellulose, the main structural material in plants, from cellulose synthesis and fibril formation to a mature plant cell wall, forming a foundation for significant advancement in sustainable energy and materials. Characterization, understanding and overcoming the barriers for enzymatic hydrolysis of different raw material is essential for the development of economically competitive processes based on enzymatic treatments.

Availability of feedstocks for biofuel production, their variability and sustainability are major criteria's to be addressed. Each raw material requires a different processing and pretreatment strategy which has to be tailored taking in to consideration their composition and susceptibility to such treatments. Physical/chemical/physico-chemical or combinations thereof needs to be optimized for pretreatment of each feedstocks. The pretreatment must be advanced and appropriately integrated with the rest of the process to achieve the complete potential of lignocellulosic ethanol. Another major challenging area of research is to develop low cost effective enzymes for lignocellulose saccharification of the pretreated biomass. Improvements in fermentation technology and media optimization approaches have to be performed along with genetic engineering techniques to improve the yield and efficiency of cellulases. Although enzyme costs have decreased in the last few years, this is still at its pre-mature state. The high cost of enzyme production and the requirement of higher enzyme dosage for hydrolysis of biomass are considered to be main hurdles for the economic viability of lignocellulosic bioethanol. Synthetic biology and metabolic engineering approaches has to be utilized for developing efficient microbes for SSF, SSCF and CBP processes.

Advances in the cost-effective conversion of lignocellulosic biomass are often difficult to assess accurately because of the lack of integrated process configurations.

Tremendous R&D studies are improving the conversion process but the issue of feedstock availability and revenue stability remain uncertain and subject to political risks. Keeping a realistic perspective one can conclude that several pieces still remain to be properly assembled and optimized before an efficient industrial configuration is acquired. Even though developing the technology for cost-effective motor fuel production by 2030 is challenging, the advances in scientific understanding necessary to achieve this goal appear realizable.

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Chapter 19
Probiotic Yeasts

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Abstract Probiotics are live microbial cells which on administration in adequate quantities confer health benefits to the host by stimulating the immune system, improving lactose absorption, production of enzymes and vitamins, reducing serum cholesterol, inhibition of pathogens, and antitumor properties. Recent evidence suggests that these effects are strain-specific and the dosage dependent. The most extensively studied and widely used probiotics are the lactic acid bacteria, *Lactobacillus* and *Bifidobacterium* spp. The only yeast reported to have probiotic effects is *Saccharomyces boulardii*, which has been shown to have promising effect as a probiotic due to its ability to survive during the passage through the human gastrointestinal (GI) tract, tolerating exposures to low pH and to bile salts, and its potential benefit is in getting relief from ailments such as acute diarrhoea in children and *Clostridium difficile* associated diarrhoea. The important aspects of probiotics like the characteristics of a microbe to be used as a probiotic, isolation of yeasts from different sources and their screening for probiotic properties, their mechanism of action, their potential benefits in the treatment of various diseases and various yeast probiotic products available in the market are discussed in this chapter. The safety issues concerning probiotic organisms have also been covered briefly.

Keywords Probiotic • *Saccharomyces boulardii* • Diarrhoea • *Clostridium difficile* • Fermented food

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19.1 Introduction

Human intestine harbors a complex and dynamic microbial ecosystem comprising 95% of the total number of cells in the human body. These gut microorganisms have several functions such as stimulation of the immune system, protection from pathogenic microbes, and aiding in digestion (Guarner and Malagelada 2003). A balanced gastrointestinal flora is one in which health promoting or beneficial bacteria predominate over the potentially harmful bacteria. As consumers today become increasingly aware of the processes that may be necessary for maintenance of their environment, health and nutrition, scientific research has focused on the roles that diet, stress, and modern medical practices (e.g. the use of antibiotics and radiotherapy) play in threatening human health by affecting the balance. This imbalance could result in gastrointestinal tract infections, constipation, irritable bowel syndrome, inflammatory bowel disease (Crohn's disease and ulcerative colitis), food allergies, antibiotic-induced diarrhoea, cardiovascular disease, and certain cancers (e.g. colorectal cancer) (Salminen et al. 1995). Furthermore, a serious concern has been expressed as the degree of microbial resistance to indiscriminately prescribed and misused antibiotics increases. To combat these trends directly, the World Health Organization currently advocates the implementation of alternative disease control strategies such as exploiting the prophylactic and therapeutic potential of microorganisms as probiotics (Daly and Davis 1998). The term probiotic has been derived from Greek that means 'for life'. Probiotics have been defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (Fuller 1989).

The concept of probiotics evolved around 1900, when Nobel Prize-winning Elie Metchnikoff hypothesized that the long, healthy lives of Bulgarian peasants were the result of their consumption of fermented milk products, and later he was convinced that yogurt contained the organisms necessary to protect the intestine from the damaging effects of other harmful bacteria. The first clinical trials were performed in the 1930s on the effect of probiotics on constipation. A probiotic product was licensed in 1950s by the United States Department of Agriculture as a drug for the treatment of scour (*Escherichia coli* infection) in pigs (Orrhage et al. 1994). During the last century, different microbes have been studied for their ability to prevent and cure diseases that led to coin the term probiotics (Lidbeck et al. 1992; Lee et al. 1999). The discovery by Mann and Spoerig (1974) that people who drank yogurt fermented with wild strains of *Lactobacillus* sp. had very low values for blood serum cholesterol opened up a new area for investigation. Harrison et al. (1975) reported that cells of *Lactobacillus acidophilus* added to infant formula decreased levels of serum cholesterol. In 1994, the World Health Organization considered probiotics to be the next-most important immune defense system, when commonly prescribed antibiotics are rendered useless by antibiotic resistance (Kailasapathy and Chin 2000). The use of probiotics in antibiotic resistance is termed as a microbial interference therapy.

19.2 Prerequisites of a Good Probiotic

The following are the prerequisites of a good probiotic (Fuller 1989):

- Exert a beneficial effect on the host
- Present in large numbers in food stuffs and remain viable throughout the shelf-life of the product
- Withstand transit through the GI tract such as exposure to acids and bile salts.
- Adhere to the intestinal epithelium cell lining and colonize the lumen of the tract
- Produce antimicrobial substances towards pathogens
- Stabilize the intestinal microflora and be associated with health benefits.
- Must have a good shelf-life in food or preparations, containing a large number of viable cells at the time of consumption,
- Devoid of pathogens and toxic products in them.

19.3 Microorganisms Used as Probiotics

Microorganisms most commonly used as probiotics are lactic acid producing Lactobacilli and Bifidobacteria, both of which belong to the normal microflora (Alvarez-Olmos and Oberhelman 2001). Other less commonly used probiotic microorganisms are the strains of *Streptococcus*, *Escherichia coli*, *Bacillus* and *Saccharomyces* (deVrese et al. 2001) (Table 19.1). The mechanisms by which probiotics exert their effects may involve modifying gut pH, antagonizing pathogens through production of antimicrobial compounds, competing for pathogen binding

Table 19.1 List of microorganisms used as probiotics

Lactobacillus spp.	*Bifidobacterium* spp.	*Streptococcus* spp.	*Saccharomyces* spp.	Others
L. acidophilus	*B. bifidum*	*S. thermophilus*	*S. boulardii*	*Bacillus cereus*
L. casei	*B. lactis*	*S. salivarius* subsp. *thermophilus*		*Escherichia coli*
L. fermentum	*B. breve*			*Propionibacterium freudenreichii*
L. gasseri	*B. longum*			*Enterococcus*
L. bulgaricus	*B. infantis*			
L. salivarius	*B. adolescentis*			
L. lactis				
L. plantarum				
L. reuteri				
L. paracasei				
L. johnsonii				

and receptor sites as well as for available nutrients and growth factors, stimulating immunomodulatory cells, and relieving lactose intolerance by producing lactase (Gupta and Garg 2009).

Probiotics are available as capsules of freeze-dried or lyophilized culture supernatants, dried power of heat-dried culture supernatants, mixed in diary foods (yogurt, cheese, milks, or ice cream) or other foods (kefir, chocolate, wafers) (Elmer et al. 2007).

19.4 Potential Benefits of Probiotics

19.4.1 Amelioration in Digestion

Lactic acid bacteria are known to release various enzymes and vitamins into the intestinal lumen. This improves digestion, alleviating symptoms of intestinal malabsorption. Bacterial enzymatic hydrolysis may enhance the bioavailability of protein and fat (Fernandes et al. 1987), and increase the production of free amino acids and short chain fatty acids (SCFA). When absorbed, these SCFAs contribute to the available energy pool of the host (Rombeau et al. 1990; Rolfe 2000).

19.4.2 Nutrient Biosynthesis

The nutritional content of fermented foods is improved as the lactic acid bacteria increase folic acid in yogurt, bifidus milk and kefir (Alm 1982). Similarly, niacin and riboflavin levels in yogurt are increased with fermentation (Deeth and Tamime 1981; Alm 1982).

19.4.3 Alleviation of Lactose Intolerance

Strains of lactic acid bacteria, in adequate amounts, can alleviate symptoms of lactose intolerance. *Streptococcus thermophilus*, *Lactobacillus bulgaricus* and other lactobacilli used in fermented milk products deliver enough bacterial lactase to the intestine and stomach where lactose is degraded to prevent symptoms in lactase non-persistent individuals (Kilara and Shahani 1975; Martini et al. 1991).

19.4.4 Prevention of Diarrhoeal Diseases

Probiotics are well known for diarrhoeal disease prevention and management. A number of specific strains, including *Lactobacillus* GG, *Lactobacillus reuteri*,

Saccharomyces boulardii, *Bifidobacterium* spp. and others, have been shown to have significant benefit for diarrhoea (Marteau et al. 2001; Benchimol and Mack 2004), travellers' diarrhoea (Hilton et al. 1977) and diarrhoea disease in young children caused by rotaviruses (Vanderhoof 2000).

19.4.5 Stimulation of Immune System

The majority of evidence from *in vitro* systems, animal models and humans suggests that probiotics can enhance both specific and nonspecific immune responses. These effects are believed to be mediated by increasing secretary IgA, activating macrophages, increasing levels of cytokines, increasing natural killer cell activity and/or increasing levels of immunoglobulins (Perdigon and Alvarez 1992; Ouwehand et al. 2002). Probiotic bacteria down regulate inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergy (Isolauri 2004; Pohjavuori et al. 2004). The immune response may further be enhanced when one or more probiotics are consumed together and work synergistically, as in *Lactobacillus* administered in conjunction with Bifidobacteria (Cunningham-Rundles et al. 2000).

19.4.6 Inhibition of Pathogens

Probiotics might prevent infection by competing with pathogens for binding sites on epithelial cells (O'Sullivan et al. 1992). Probiotics might also inhibit the growth of pathogenic bacteria by producing bacteriocins such as nisin (Del Miraglia and De Luca 2004). Production of lactic acid lowers the pH of the intestinal content and helps in inhibiting the development of invasive pathogens such as *Salmonella* spp. or strains of *E. coli* (Mack et al. 1999). Aiba et al. (1998) showed *Lactobacillus salivarius* capable of producing high amounts of lactic acid that inhibits the growth of *H. pylori* in vitro. It was found that the higher the level of lactic acid production by *Lactobacillus*, the more potent was the effect on reducing *H. pylori*'s urease activity.

19.4.7 Hepatic Disease

Hepatic encephalopathy (HE) is a liver disease and its effects can be life threatening. The exact pathogenesis of HE still remains unknown. The probiotics *S. thermophilus*, *Bifidobacterium*, *L. acidophilus*, *Lactobacillus plantarum*, *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, and *E. faecum* containing therapeutic effect have multiple mechanisms of action that could disrupt the pathogenesis of HE and may make them superior to conventional treatment and lower portal pressure with a reduction in the risk of bleeding (Shanahan 2001; Solga 2003).

19.4.8 Hypertension

Probiotic bacteria or their fermented products may play a role in blood pressure control, with animal and clinical studies documenting antihypertensive effects of probiotic ingestion (Nakamura et al. 1995, 1996). Elderly hypertensive patients who consumed fermented milk with a starter containing *Lactobacillus helveticus* and *S. cerevisiae* experienced reductions in systolic and diastolic blood pressure (Hata et al. 1996). Considering the current epidemic of heart disease, regular consumption of probiotics may provide a modest prophylactic effect against heart disease (Nakamura et al. 1995).

19.4.9 Cancer

Probiotic cultures can decrease the exposure to chemical carcinogens by detoxifying ingested carcinogens. Lactobacilli may bind to mutagenic compounds in the intestine, thereby decreasing the absorption of these mutagens. Probiotics may also suppress the growth of bacteria that convert procarcinogens into carcinogens, thereby reducing the amount of carcinogens in the intestine (Motta et al. 1991; Lidbeck et al. 1992; Isolauri 2004). They may alter the environment of the intestine, thereby decreasing populations or metabolic activities of bacteria that may generate carcinogenic compounds. Stimulating the immune system by probiotic microbes also helps to defend better against cancer cell proliferation (Isolauri 2004).

19.4.10 Control of Blood Cholesterol and Hyperlipidaemia

Lactic acid bacteria have been evaluated for their effect on serum cholesterol levels. Clinical studies on the effect of lowering of cholesterol or low-density lipid (LDL) levels in humans have not been conclusive. Some studies suggest blood cholesterol levels can be reduced by consumption of probiotic-containing dairy foods. In addition, it is likely that some strains may demonstrate this effect while others do not. One study in hypercholesterolemic mice showed that administration of low levels of *L. reuteri* for 7 days decreased total cholesterol and triglyceride levels by 38% and 40%, respectively, and increased the high-density lipid: LDL ratio by 20% (Taranto et al. 1998). The cholesterol-lowering potential of *L. acidophilus* has been most widely studied (Lin et al. 1989).

19.4.11 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBDs) are chronic, immune-related inflammatory diarrhoeas, which include ulcerative colitis, pouchitis and Crohn's disease. Lactic

acid bacteria may improve intestinal mobility and relieve constipation possibly through a reduction in gut pH (Sanders and Klaenhammer 2001). It has also been reported that probiotic combination therapies may benefit patients with IBD (Schultz and Sartor 2000). *Saccharomyces boulardii* in patients with Crohn's disease was found to extend remission time and reduce relapse rates. Both *S. boulardii* and *Lactobacillus* GG have been reported to increase secretory IgA levels in the gut (Gorbach et al. 1987).

19.4.12 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) is a frequent disorder characterized by a triad of symptoms (bloating, abdominal pain, and intestinal transit disturbances). Probiotics exhibit a direct effect in the gut in the treatment of inflammatory and functional bowel disorders. In one of the most common functional bowel disorders, irritable bowel syndrome, *L. plantarum* 299v and DSM 9843 strains were shown in clinical trials to reduce abdominal pain, bloating, flatulence, and constipation (Motta et al. 1991; MacFarlane and Cummings 2002). It was also observed that *S. boulardii* decreased diarrhoea in irritable bowel syndrome, but was not effective in alleviating other symptoms of the syndrome (Marteau et al. 2001).

19.4.13 Oral Candida Infections

Several elements in the immune system, such as T-lymphocytes, granulocytes, NK-cells, mast cells, and macrophages, account for the protection against *Candida* infections (Peterson 1992). *Lactobacillus* GG and *Propionibacterium* JS cause enhanced T-cell and B-cell proliferation in mice. Probiotics may also inhibit the *Candida* growth by producing antimicrobial compounds (Strus et al. 2005), and may inhibit its adhesion to epithelial cells. In an *in vitro* model mimicking gastrointestinal conditions, *Lactobacillus* suppressed the growth of *Candida* after antibiotic treatment (Payne et al. 2003), possibly by competing for the same receptor sites.

The following clinical probiotic studies have been reported as having beneficial effects (Fig. 19.1):

Normalize intestinal flora: *Lactobacillus (acidophilus, casei, plantarum) and Bifidobacterium bifidum*

Stimulate the immune system: *Lactobacillus (acidophilus, casei, rhamnosus, plantarum, delbrueckii, johnsonii) and Bifidobacterium bifidum*

Diarrhoea associated with antibiotics: *Lactobacillus (rhamnosus, acidophilus, bulgaricus), Saccharomyces boulardii, Bifidobacterium longum, and Enterococcus faecium.*

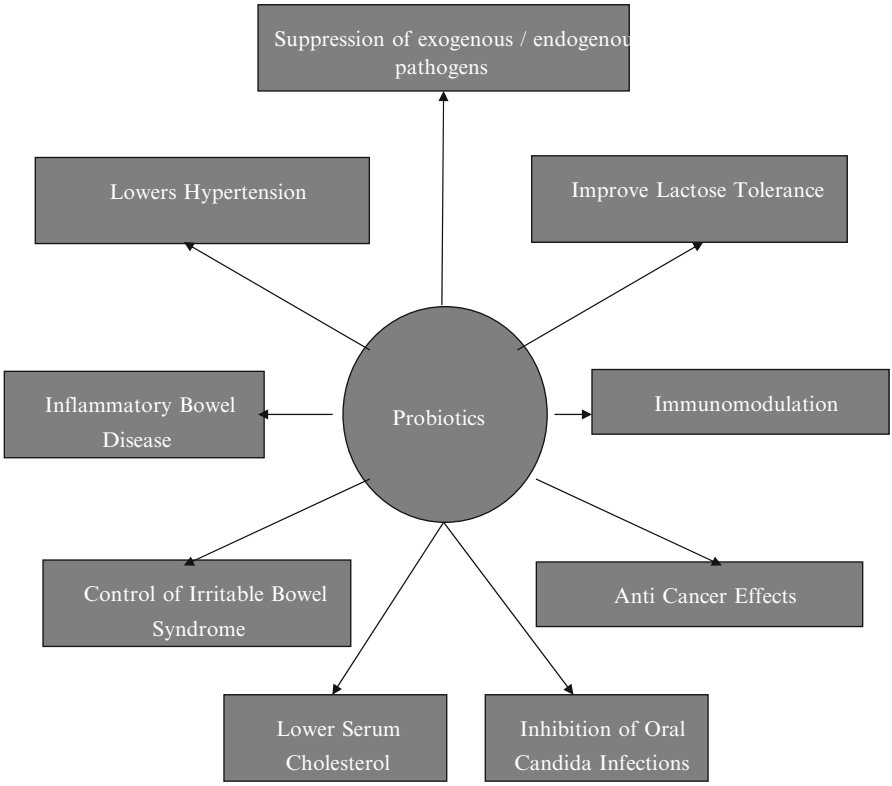

Fig. 19.1 Beneficial effects of probiotics

Diarrhoea associated with traveling: *Lactobacillus (rhamnosus, acidophilus, bulgaricus, johnsonii), Bifidobacterium bifidum, and Streptococcus thermophilus, S. boulardii*

Diarrhoea associated with the Rotavirus: *Lactobacillus rhamnosus, Bifidobacterium bifidum, and Streptococcus thermophilus*

Acute diarrhoea: *Bifidobacterium bifidum*, *Lactobacillus* (*bulgaricus, acidophilus, rhamnosusm, reuteri*) and *Streptococcus thermophilus*

Recurring Clostridium difficile colitis: *Lactobacillus rhamnosus, Saccharomyces boulardii*

Anti-tumor properties: *Lactobacillus (acidophilus, casei, plantarum, delbrueckii, gasseri) and Bifidobacterium (longum, bifidum, adolescentis, infantis)*

Reducing lactose intolerance: *Lactobacillus (bulgaricus, rhamnosus, johnsonii) and Streptococcus thermophilus*

Lowering fecal enzyme activity: *Lactobacillus rhamnosus, L. casei, L. gasseri, L. delbrueckii, L.acidophilus*

19.5 Occurrence of Yeasts in Fermented Foods

Fermentation is one of the oldest forms of food processing and preservation in the world. Since ancient times, yeasts and their metabolic products have been exploited for use by humans, the most important being baking and brewing (Moslehi-Jenabian et al. 2010). Yeasts contribute to the fermentation of a broad range of other commodities, where various species may work in concert with bacteria and/or filamentous fungi. The occurrence of yeasts has been reported in a number of Indian fermented products like idli, dosa, jalebi, warries, pappad, kanji, fruit juices, bakery products, brewery products and milk products like curd and cheese etc. In other parts of the world, fermented milk products that are manufactured using starter cultures containing yeasts include acidophilus-yeast milk, Kefir, Koumiss and Leban, cheese (Lang and Lang 1975). *Saccharomyces* spp. e.g. *S. burnetii, S. kluveri, S. byanus, S. rosinii, S. cerevisiae* and *S. boulardii* may be isolated from a variety of dairy products including milk, yogurt, cream, dahi, cheese and kefir. *Saccharomyces* spp. cannot ferment lactose so they develop in milk as a secondary flora, after bacterial growth. Lactic acid produced by lactic acid bacteria creates a high acid environment creates a selective environment for yeast growth (Fleet 1990). *Saccharomyces cerevisiae*, which according to EFSA (The European Food Safety Authority) has a QPS (Qualified Presumption of Safety) status, is the most common yeast used in food fermentation where it has shown various technological properties.

Whole yeast cells of *S. cerevisiae* have nutritive value since they are an excellent source of amino acids, good source of mineral and vitamin B complex, boosts immunity levels in the system resulting in better protection against infection, produces unidentified growth factors, augments the digestive processes by initiating the processes of fermentation, source of digestive enzymes of various kinds, degrade phytic acid-an anti-nutrient in food and feed (Yamada and Sgarbieri 2005).

19.6 *Saccharomyces boulardii* as a Probiotic

Yeasts are also part of the residual microbial system that makes up <0.1% of the microbiota. Most yeast isolates from the GI tract are *Candida albicans*, although others are occasionally found. The cell size of yeast is many times larger than that of bacteria (Giuliano et al. 1987). Different yeast species such as *Debaryomyces hansenii, Torulaspora delbrueckii* (Psani and Kotzekidou 2006)*, Kluyveromyces lactis, Yarrowia lipolytica* (Chen et al. 2010), *Kluyveromyces marxianus, Kluyveromyces lodderae* (Kumura et al. 2004) have shown tolerance to passage through the gastrointestinal tract or inhibition of enteropathogens. However, *Saccharomyces boulardii* is the only yeast with clinical effects and the only yeast preparation with proven probiotic efficiency in double-blind studies (Sazawal et al. 2006).

Saccharomyces boulardii (brand name Florastor, among others) is a tropical strain of yeast first isolated from lychee and mangosteen fruit in 1923 by French scientist Henri Boulard after he observed natives of Southeast Asia chewing on the skin of lychee and mangosteen in an attempt to control the symptoms of cholera. He was a visitor during a cholera outbreak and noticed that some people who did not develop cholera were drinking a special tea. This tea was made by taking the outer skin from a tropical fruit (lychee and mangosteens) and cooking them down to make tea. He succeeded in isolating the agent responsible. It was a special strain of yeast he named '*Saccharomyces boulardii*'. The patent for this yeast was bought by Laboratories Biocodex in 1947, which began researching and manufacturing protocols. This yeast is related to *Saccharomyces cerevisiae* but distinct from it in several taxonomic, metabolic, and genetic properties (Malgoire et al. 2005). By using comparative electrophoretic karyotyping and multivariate analysis of the polymorphism, observed in pulsed-field gel electrophoresis (PFGE), Cardinali and Martini (1994) classified *S. boulardii* outside of *S. cerevisiae* species. With the rapid development of molecular phylogenetics in recent years, there have been changes in the classification of many yeast species (Vaughan-Martini 2003). By molecular typing [species-specific polymerase chain reaction (PCR), randomly amplified polymorphic DNA–PCR, restriction fragment length polymorphic analysis of rDNA spacer region and PFGE] *S. boulardii* is classified within the species *S. cerevisiae* (Mitterdorfer et al. 2002). Using comparative genomic hybridization for whole-genome analysis, it was concluded that *S. cerevisiae* and *S. boulardii* are the members of the same species (Edwards-Ingram et al. 2007). However, genetically *S. boulardii* differs from other *S. cerevisiae*. Hennequin et al. (2001) identified a unique and specific microsatellite allele characterizing *S. boulardii* that distinguishes it from other strains of *S. cerevisiae*. Metabolically and physiologically, *S. boulardii* differs from *S. cerevisiae*. While most *S. cerevisiae* strains grow and metabolize at 30°C, *S. boulardii* is thermotolerant yeast that grows optimally at 37°C (Fietto et al. 2004; Czerucka et al. 2007).

Saccharomyces boulardii possesses many properties that make it a potential probiotic agent,

- it survives transit through the GI tract
- its has been shown to maintain and restore the natural flora in the large and small intestine
- its nonpathogenic, non-systemic (it remains in the gastrointestinal tract rather than spreading elsewhere in the body)
- its temperature optimum is 37°C, both *in vitro* and *in vivo,*
- it inhibits the growth of a number of microbial pathogens.

However, *S. boulardii* belongs to the group of simple eukaryotic cells (such as fungi and algae) and, it thus differs from bacterial probiotics that are prokaryotes. Table 19.2 lists the main properties differentiating the yeast from the bacteria that account for the specificity of *S. boulardii* as a probiotic (Czerucka et al. 2007).

Table 19.2 Major differences between yeast and bacteria and their probiotic implications (Czerucka et al. 2007)

	Bacteria	Yeast	Probiotic implication
Presence in human flora	99%	<1%	
Cell size	1 μm	10 μm	Stearic hindrance
Cell wall	Peptidoglycan, LPS (Gram-negative⁻), LTA (Gram-positive)	Chitin, mannose (PPM, PLM), glucan	Immune response via TLRs, lectin receptors
Optimal growth conditions			
pH	6.5–7.5	4.5–6.5	Different sites of action in the GI tract
Temperature (°C)	10–80	20–30	
Resistance to antibiotics	No	Yes	Safety in combination with antibiotherapy
Transmission of genetic material (e.g. resistance to antibiotics)	Yes	No	

LPS lipopolysaccharide, *LTA* lipoteichoic acid, *PPM* phosphopetidomannan, *LPM* phospholipomannan, *TLR* Toll-like receptor, *GI* gastrointestinal

19.7 Mechanisms of Action

S. boulardii is often marketed as a probiotic in a lyophilized form and is therefore often referred to as *Saccharomyces boulardii lyo*. In this form, *S. boulardii* survives gastric acidity and bile salts. It can be detected alive throughout the entire digestive system. It is also resistant to proteolysis. It is able to achieve and maintain high populations in the GI tract. It can permanently colonize the colon and does not easily translocate out of the intestinal tract (Boddy et al. 1991). *S. boulardii* is sensitive to non-absorbable antimycotics such as nystatine but can safely be administered with re-absorbable antifungal agents such as fluconazole. *S. boulardii* is naturally resistant to antibiotics, thus it can be prescribed to patients receiving antibiotics (Bergogne-Bérézin 1995). Simultaneous oral intake of amoxicillin and *S. boulardii* doubles the number of *S. boulardii* surviving in the gastrointestinal tract (Klein et al. 1993). No transfer of resistance genes is possible from the yeast to the pathogens, while the resistance genes can be transferred between the lactic acid bacteria and the pathogens. This feature makes the yeast an ideal candidate for use in patients on antibiotic therapy.

19.7.1 Antimicrobial Effects

S. boulardii have been shown to protect against various enteric pathogens and members of the family Enterobacteriaceae in animal studies (Czerucka and Rampal 2002) such as *Escherichia coli*, *Shigella*, *Salmonella typhimurium*, *Pseudomonas aeruginosa,*

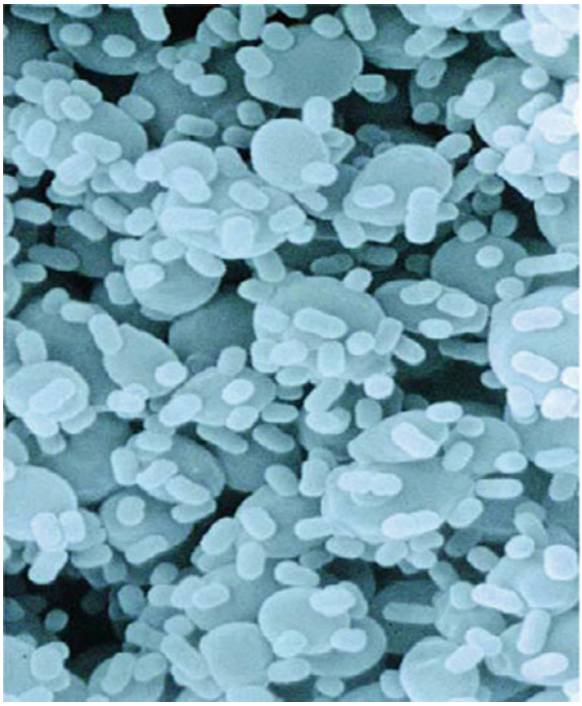

Fig. 19.2 Adherence of enterohemorrhagic *E. coli* serogroup 0157:H to the surface *of S. boulardii*. Electron microscopic photograph, magnitude × 5,000 (Gedek 1999)

Staphylococcus aureus, and *Entamoeba histolytica.* This is due to the fact that the cell wall of *S. boulardii* has the ability to bind enteropathogens such as enterohaemorrhagic *Escherichia coli* and *Salmonella enterica* serovar *typhimurium* (Fig. 19.2) (Gedek 1999).

Once the invading microbe is bound to *S. boulardii*, it is prevented from attaching to the brush border; it is then eliminated from the body during the next bowel movement. Also, the yeast inhibits adherence of *Clostridium difficile* to Vero cells (derived from kidney epithelial cells). This is because yeast cells or cell wall particles are able to modify the surface receptors involved in adhesion of *C. difficile* through a proteolytic activity and by steric hindrance (Tasteyre et al. 2002). Administration of *S. boulardii* reduces adherence of enterotoxigenic *E. coli* to mesenteric lymph node in pig intestine (Lessard et al. 2009). Ingestion of *S. boulardii* decreased the incidence of antibiotic-induced bacterial translocation in rats. The total bacteria count of fecal flora and especially the number of Gram-negative bacteria were significantly lower after intake of the yeast in addition to antibiotic (Herek et al. 2004). *S. boulardii* produces two proteins, one of 120 kDa and another of 54 kDa. The 54-kDa serine protease inhibits the enterotoxic and cytotoxic activities of *C. difficile*

by proteolysing toxin A and its receptor. This inhibits water and electrolyte secretion but has no effect on the cellular lesions caused by *C. difficile* (Castagliuolo et al. 1996; Pothoulakis et al. 1993). This leads to a reduction in the enterotoxic and cytotoxic effects of *C. difficile* infection (Castagliuolo et al. 1999). The 120 kDa protein has a non-proteolytic activity, competes specifically with the hypersecretion caused by the toxins of *Vibrio cholera* decreasing cyclic adenosine monophosphate in the intestinal cells. The metabolic changes in the intestinal mucosa caused by cholera toxin are decreased if *S. boulardii* is ingested prior to the cholera toxin. The 120-kDa protein acts directly on enterocytes and includes signal transduction pathways involved in the regulatory secretion (Czerucka et al. 1994). *S. boulardii* also synthesizes a phosphatase that dephosphorylates endotoxins such as lipopolysaccharide of *E. coli* 055B5 and inactivates its cytotoxic effects (Buts et al. 2006).

19.7.2 Antitoxin Effects

19.7.2.1 Increased Immune Response

S. boulardii induces the secretion of Immunoglobulin A (IgA) in the small intestine of the rat (Butts et al. 1990). There is a very clear and marked stimulation of IgA production as demonstrated by the increased content in the intestinal lumen and in crypt cells. This may be explained by a trophic effect exerted on the mucosa or by direct immunostimulation (Butts et al. 1990). Secretory IgA provides protection against invading microbes in the gastrointestinal and respiratory tracts.

19.7.3 Anti-inflammatory Effects

Probiotics may decrease inflammation by exerting positive effects on the epithelial cell and mucosal immune system dysfunctions that constitute the basis of the inflammatory processes. *In vitro* studies using mammalian cell cultures have shown that *S. cerevisiae* var. *boulardii* modifies host cell signalling pathways associated with pro-inflammatory response during bacterial infection. The mechanism is based on blocking activation of nuclear factor-kappa B (NF-κB) and mitogen activated protein kinase (MAPK). This leads to decreased expression of inflammation-associated cytokines such as interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) (Jonkers and Stockbrügger 2007). In addition, probiotics stimulate regulatory T cells and thus inhibit the effector T cells that would otherwise induce inflammation. Probiotic administration does lead to the increased elaboration of regulatory cytokines, and these play a major role in their protective effects. It seems likely that probiotics bring about these effects through their interactions with mucosal dendritic cells that produce regulatory cytokines or induce T cells with these capabilities (Vandenplas et al. 2009).

19.7.4 Trophic Effects on Enterocytes

The trophic effect on enterocytes has been shown to increase levels of brush border membrane enzymes, such as lactase, sucrase, maltase, glucoamylase, and N-aminopeptidase alkaline phosphatase, in the intestinal mucosa of humans and rats upon oral administration of yeast. This can have a positive influence on nutrient degradation and absorption (Buts et al. 1986). It can lead to the increased breakdown of disaccharides into monosaccharides that can then be absorbed into the bloodstream via enterocytes (Zaouche et al. 2000) This effect may improve the absorption of carbohydrates, usually defective in acute and chronic diarrhoeal disorders and help in its treatment. The hypersecretion of water and electrolytes (including chloride ions), caused by cholera toxin during a *Vibrio cholerae* infection, can be reduced significantly with the introduction of *S. boulardii* (Czerucka and Rampal 1999).

S. boulardii has also been shown to stimulate the production of polyamines in the rat ileum. These polyamines (spermine, spermidine and putrescine) enhance the expression of brush border enzymes (such as hydrolases, proteases, and transport molecules) (Buts et al. 1994). Polyamines have been theorized to stimulate the maturation and turnover of small intestine enterocytes. This could aid in the increased recovery rate of a patient from diarrhoea (Buts et al. 1994).

In pigs with a reduced gastrointestinal flora because of administrations of antibiotics, *S. boulardii* restores normal levels of colonic short chain fatty acids (SCFA) (Breves et al. 2000). SCFAs are important metabolites produced by the anaerobic flora and are relevant for colonic absorption of water and electrolytes (Schneider et al. 2005). These effects may be especially relevant in the management of antibiotic-associated diarrhoea.

19.8 Clinical Benefits of *S. boulardii*

19.8.1 Antibiotic-Associated Diarrhoea

Antibiotic-associated diarrhoea (AAD) is defined as otherwise unexplained diarrhoea that occurs in association with the administration of antibiotics. The bacterial agent most commonly associated with AAD is *C. difficile* (Bartlett et al. 1978). Among other infectious organisms *Staphylococcus aureus, Clostridium perfringens, Klebsiella oxytoca, Candida species, E. coli* and *Salmonella* species can be mentioned. Almost all antibiotics, particularly those that act on anaerobes, can cause diarrhoea, but the risk is higher with the aminopenicillins, the combination of aminopenicillins and clavulanate, the cephalosporins, and clindamycin (Barbut et al. 1997; McFarland et al. 1990). The incidence of diarrhoea in children receiving broad-spectrum antibiotics ranges from 11% to 40% (Turck et al. 2003). Randomized control trial (RCT) conducted by Surawicz et al. (2000) evaluated the efficacy of

S. boulardii administered during treatment and continued for 2 weeks, after the end of course in 180 hospitalized patients receiving antibiotics belonging to various classes. The incidence of diarrhoea was significantly reduced in patients receiving *S. boulardii* (10% vs. 22% in placebo, $P=0.038$). In another study *Saccharomyces boulardii* (at the dosage of 1 g/day) or placebo was administered to 193 patients from the beginning of antibiotic treatment and continued 3 days after the end of the course. *S. boulardii* mediated a significant preventive effect on the occurrence of diarrhoea (7% vs. 15%, $P=0.02$). Another study on the impact on AAD of *S. boulardii* in children with otitis media and/or respiratory tract infections was conducted (Kotowska et al. 2005). Children received antibiotics plus 250 mg of *S. boulardii* ($n=132$) or placebo ($n=137$) orally, twice daily for the duration of antibiotic treatment. Analyses included data from 246 children and show that patients receiving *S. boulardii,* had a lower prevalence of diarrhoea than those who received the placebo (8% vs. 23%); RR, 0.3 (95% CI: 0.2–0.7); NNT, 7 (95% CI: 5–15). *S. boulardii* is the probiotic that has been the most extensively studied with four large-scale placebo-controlled clinical studies showing a significant efficacy for preventing AAD (Vandenplas et al. 2009).

Clostridium difficile accounts for 20–25% of AAD in hospitalized patients and about 10% of AAD in community patients (Beaugerie et al. 2003). *Clostridium difficile* is responsible for 95% of pseudomembranous colitis. *Saccharomyces boulardii* is the sole probiotic that has proven a significant efficacy in treating relapsing *C. difficile*-associated diarrhoea. In a randomized, placebo-controlled trial, McFarland et al. (1990) evaluated the effect of *S. boulardii* (1 g/day for 28 days) and placebo as adjunctive therapy to metronidazole or vancomycin in 124 patients. It was the first episode of *C. difficile* infection in 64 cases and a relapse in 60 cases. In this study, after the administration of *S. boulardii,* the authors observed a 50% reduction of recurrences in patients who had previously experienced a first relapse of *C. difficile* infection.

19.8.2 Traveller's Diarrhoea

Traveller's diarrhoea is a well-known public health problem, particularly among travellers to developing countries. Enterotoxigenic *Escherichia coli,* Shigellae and Salmonellae account for about 80% of cases (Sanders and Tribble 2001). Kollaritsch et al. (1993) evaluated the efficacy of *S. boulardii* for the prevention of diarrhoea in 1,016 travellers visiting various countries in the world. The incidence of diarrhoea was 40% in patients receiving placebo, 34% in patients receiving *S. boulardii* 250 mg/day ($P=0.019$) and 29% in patients receiving *S. boulardii* 1 g/day ($P<0.005$). The more *S. boulardii* taken in prevention, starting 5 days before leaving, the higher the reduction in diarrhoea reported. In a meta-analysis of probiotics for the prevention of traveller's diarrhoea analyzing 12 different studies, McFarland (2007) concluded that two probiotics, *S. boulardii* and a mixture of *L. acidophilus* and *Bifidobacterium bifidum,* had significant efficacy.

19.8.3 Acute Diarrhoea

Diarrhoea is defined as a change in bowel movements in an individual with an increase in the water content, volume, and—usually—frequency of stools (WHO 1995). Although oral rehydration is important in treatment, it does not result in substantial shortenings of the diarrhoeal episodes nor in reductions in stool volume, prompting a growing interest in adjunctive treatments. Kurugol and Koturoglu (2005) have investigated the effect of *S. boulardii* in a double-blind randomized study involving 200 children. The duration of diarrhoea was significantly reduced (4.7 vs. 5.5 days, $P = 0.03$) as well as the number of days of hospitalization (2.9 vs. 3.9 days, $P < 0.001$). *Saccharomyces boulardii* has also been shown to be efficient, in reducing the number of children with prolonged diarrhoea (3 of 44 vs. 12 of 44; RR 0.25; 95% CI: 0.1–0.8) in a double-blind, randomized study (Villarruel et al. 2007). Also, preliminary results suggest that, *S. boulardii* might be effective in preventing the occurrence of new episodes of diarrhoea in a 2-month long-term follow-up (Billoo et al. 2006). Recently, the European Society for Paediatric Gastroenterology, Hepatology and Nutrition and European Society of Paediatric Infectious Diseases Expert Working Group addressed the clinical efficacy of probiotics in the management of acute gastroenteritis. It stated that the selected probiotics such as *Lactobacillus* GG and *S. boulardii* may be an effective adjunct to the management of diarrhoea (Guarino et al. 2008).

19.8.4 Tube-Feeding-Associated Diarrhoea

Diarrhoea is a common complication associated with enteral tube feeding and may result in a loss of nutrition in an already seriously ill patient. The frequency of diarrhoea in enteral tube fed patients has been reported as high as 50%-60% (Whelan et al. 2009) and complications may include life-threatening acidosis, increased morbidity and mortality and increased healthcare costs. Tempe et al. (1983) compared 40 enteral-fed patients randomized to either *S. boulardii* (1×10^{10}/day) or placebo for 11–21 days and found significantly fewer patients (8.7%) given *S. boulardii* developed diarrhoea compared with placebo (16.9%). Schlotterer et al. (1987) randomized 18 patients with burns who were receiving enteral nutrition to *S. boulardii* (4×10^{10}/day) or placebo for 8–28 day. Those given *S. boulardii* suffered fewer diarrhoea days (3/204 day, 1.5%) than those given placebo (19/208 day, 9.1%, $P < 0.001$). No adverse reactions associated with *S. boulardii* were reported in any of these trials.

19.8.5 AIDS

S. boulardii has been shown to significantly increase the recovery rate of stage IV AIDS patients suffering from diarrhoea versus placebo. On average, patients receiving *S. boulardii* gained weight while the placebo group lost weight over the 18 month trial.

A randomized, double-blind trial covering 35 patients with AIDS-related diarrhoea showed the efficacy of *S. boulardii* 3 g/day given for 7 days in resolving diarrhoea. Sixty-one percent of the patients were diarrhoea-free after 1 week of treatment with *S. boulardii* vs. 12% in the placebo group (Saint-Marc et al. 1995).

19.8.6 Helicobacter pylori Infection

Helicobacter pylori is well known to cause chronic gastritis and peptic ulcer in adults and children and as a risk factor for gastric malignancy in adults. Studies revealed that probiotics reduced treatment-related side effects and individual symptoms such as diarrhoea, epigastric pain, nausea, and taste disturbances. One RCT evaluated *S. boulardii* and found that it decreased the risk of diarrhoea when given concomitantly to patients receiving *H. pylori* triple eradication therapy (Duman et al. 2005). Subsequently published RCTs carried out in adults also found that *S. boulardii* supplementation reduced post-treatment dyspepsia independent of *H. pylori* status. However, *S. boulardii* had no significant effect on the rate of *H. pylori* eradication (Cindoruk et al. 2007).

19.8.7 Inflammatory Bowel Diseases

Three preliminary studies have evaluated the effect of *S. boulardii* in patients with IBD. A double-blind study of 20 patients suffering from Crohn's disease with moderate activity found that the addition of *S. boulardii* to conventional therapy with sulfasalazine or mesalazine (mesalamine) and corticosteroids significantly reduces bowel movements (Plein and Hotz 1993). Similarly, a single-blind study of 32 patients with Crohn's disease of the ileum or colon who had been in remission for ≥3 months showed that 6-month maintenance therapy with mesalazine 500 mg twice daily plus *S. boulardii* 500 mg/day was significantly more effective in preventing relapse than mesalazine 500 mg three times daily ($P = 0.04$) (Guslandi et al. 2000).

19.8.8 Irritable Bowel Syndrome

S. cerevisiae var. *boulardii* has also shown positive results in patients with irritable bowel syndrome (IBS). In a double-blind, placebo-controlled study, performed on patients with diarrhoea-predominant IBS, administration of *S. cerevisiae* var. *boulardii* decreased the daily number of stools and improved the consistency of the stools (Maupas et al. 1983). A double-blind study on the patients with Crohn's disease with moderate activity showed that the addition of *S. cerevisiae* var. *boulardii* to conventional therapy considerably reduced bowel movements (Plein and Hotz 1993). In addition, treatment with *S. cerevisiae* var. *boulardii* together with the conventional

therapy was more efficient in preventing relapse, compared to conventional therapy alone (Guslandi et al. 2000; Moslehi-Jenabian 2010).

19.8.9 Giardiasis

This condition is characterized by long lasting diarrhoea with symptoms ranging from mild to severe diarrhoea, weight loss, abdominal pain and weakness. It is also found in people enjoying outdoor hiking and camping who drink contaminated untreated water that appears clean. Besirbellioglu et al. (2006) randomized 65 adults with giardiasis in Turkey to either *S. boulardii* (1×10^{10}/day) or placebo for 10 day. Both groups also received metronidazole for the same duration. Two weeks later, both groups reported a resolution of their diarrhoea, but none of those on *S. boulardii* had detectable giardia cysts, while significantly more (17%) on placebo still carried giardia cysts.

19.9 Safety and Packaging

S. boulardii is considered to be a safe and well-tolerated probiotic. There have been sporadic reports of fungemia, in patients with severe general or intestinal disease who had an indwelling catheter. Presence of such catheters is thus, a contraindication for the administration of *S. boulardii* (Mcfarland 2010). However, *S. cerevisiae* might as well be responsible for a number of these fungemias, since identification of the strain *S. boulardii* is difficult (de Llanos et al. 2006). There is indeed confusion between fungemia with *S. boulardii* and *S. cerevisiae* (Graf and Gavazzi 2007). Once the diagnosis is made, fungemia with *S. boulardii* can effectively be treated with antimycotic medication, although treatment failure with fluconazole has been reported (Burkhardt et al. 2005).Patients with yeast allergies, immunocompromised or critically ill are not encouraged taking *S. boulardii*.

S. boulardii is usually available in capsules of either lyophilized or heat-dried preparations. Heat-dried preparations are not stable at room temperature and must be refrigerated or they lose their potency rapidly. Lyophilized products are stable at room temperature over 1 year and have the advantage of portability and convenience and maintain high viability counts over prolonged periods (Graff et al. 2008; Schwenzer 1998). A list of commercially available probiotic products containing *S. boulardii* is presented in Table 19.3.

19.10 Future Perspectives

Probiotic therapy has already made its way in the treatment of number of conditions-Infectious, inflammatory, neoplastic and allergic. But proper evaluation of these products is essential before using them routinely. There are several challenges in

Table 19.3 Examples of commercially available probiotics containing "*Saccharomyces boulardii*"

S.no.	Product name	Probiotic strain
1	Florastor® (US) Perenterol® (Germany) Reflor® (Turkey) Ultra-Levure (Asia)	*S. boulardii* lyo
2	*Saccharomyces boulardii*	*S. boulardii*
3	Protecflor® (Canada) Erce Flora® (Belgium)	*S. cerevisiae boulardii* (CNCM I-1077) + *L. rhamnosus* + *L. acidophilus* + Bifido. strain
4	MitoMix®	*S. boulardii* and *Pediococcus acidilactici*
5	Primal Defense™	*S. boulardii* with 13 other strains[a]
6	Pro-Bio Defense™	*S. boulardii* + 7 other strains[b]
7	ABX Support™	*S. boulardii* + *L. rhamnosus* + *Bifido. bifidum* + *Bifido. breve*
8	Kombucha fermented tea	*S. boulardii* + *L. bacterium* + blue-green algae

Adapted from: McFarland (2010)

[a]Other strains in Primal Defense include: 11 strains of *Lactobacilli* (*L. acidophilus, L. bulgaricus, L. lactis, L. plantarum, L. casei, L. lactis, L. leichmannii, L. brevis, L. caucasicus, L. fermenti, L. helveticus* and 1 strain each*: Bifidobacterium bifidum, Bacillus subtilis, Bacillus lichenformis*)

[b]Other strains in Pro-Bio Defense include: 5 strains of *Lactobacilli* (*L. plantarum, L. rhamnosus, L. acidophilus, L. casei, L. bulgaricus*) and *Bifidobacterium lactis* and *Streptococcus thermophilus*.

GMP Good manufacturing practices

choosing the appropriate probiotic, including the wide diversity of probiotic strains, quality control of commercially-available probiotic products and the degree of evidence-based trials for each disease and probiotic. The ability of an organism to be an effective probiotic has been found to be strain-specific and dosage dependent. Thus there is a need for standardization of dosage, strain specificity, viability and biosafety of these probiotics, before they are released in the market for usage by consumers. Thus in future, well designed placebo controlled studies with validated results are required for ascertaining the true health benefits of these products. Ongoing basic research will continue to identify and characterize existing strains of probiotics, identifying strain-specific outcomes, determine optimal doses needed for certain results and assess their stability through processing and digestion. Gene technology will certainly play a role in developing new strains, particularly with yeasts as only *S. boulardii* is the only probiotic yeast reported. Gene sequencing will allow for an increased understanding of mechanisms and functionality of probiotics. In addition, industry-centered research will focus on prolonging the shelf-life and likelihood of survival through the intestinal tract, optimizing adhesion capacity and developing proper production, handling and packaging procedures to ensure that the desired benefits are delivered to the consumer. Over time, new food products containing probiotics will emerge such as energy bars, cereals, juices, infant formula and cheese, as well as disease-specific medical foods. The establishment of standards of identity for probiotic-containing food products will serve to accelerate the development and availability of these food products.

19.11 Conclusions

"Let food be thy medicine and medicine be thy food", the age-old quote by Hippocrates, is certainly the tenet of today. The market for functional foods, or foods that promote health beyond providing basic nutrition, is flourishing with growing awareness of people. Within the functional foods is the rapidly expanding arena of probiotics – live microbial food supplements that beneficially affect an individual by improving intestinal microbial balance.

Probiotics have been extensively studied under *in vitro* and *in vivo* conditions. The main fields of research with respect to probiotics are heart diseases, allergic reaction, cancer, diarrhoea, etc. Several species of *Lactobacillus* and *Bifidobacterium* have shown promising results as probiotics.

The species of *Saccharomyces* are emerging as potential probiotic organisms. There is a marked increase in the sale of various yeast based probiotic products. Studies to elucidate the mechanisms of action of *S. boulardii* have demonstrated the existence of mechanisms such as release *in vivo* of substances that inhibit certain bacterial toxins and/or their pathogenic effects; trophic effects; antisecretory activity, immunostimulatory and anti-inflammatory effects. The antidiarrhoeal effect of lyophilized *S. boulardii* has been investigated in several forms of diarrhoeal diseases. Its role for both the prevention of AAD and the treatment of recurrent *C. difficile* disease has been clearly demonstrated. Randomized trials also support the use of this yeast probiotic for prevention of enteral nutrition-related diarrhoea and reduction of *Heliobacter pylori* treatment-related symptoms. Data on acute gastroenteritis and on traveller's diarrhoea are accumulating. *S. boulardii* shows promise for the treatment of irritable bowel syndrome, Crohn's disease, giardiasis, human immunodeficiency virus-related diarrhoea; but more supporting evidence is recommended for these indications. Probiotic yeast has been considered superior over probiotic bacteria because of the natural resistance of yeast to antibacterial antibiotics, which leaves intact their viability and probiotic properties. The use of *boulardii* as a therapeutic probiotic is evidence-based. Its safety has been established except in case of immunocompromized patients.

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Chapter 20
Biotechnological Approach to Caffeine Degradation: Current Trends and Perspectives

Swati Sucharita Dash and Sathyanarayana N. Gummadi

Abstract Caffeine (1,3,7-trimethylxanthine) is a naturally occurring, commercially important alkaloid. It is an active psychostimulant which increases alertness and sustains concentration by overcoming fatigue. This makes caffeine one of the most widely consumed dietary chemicals, with the global consumption ranging from 80 to 400 mg caffeine per person per day. During coffee processing, various byproducts like pulp water, husk etc. containing appreciably high content of caffeine are generated and are discarded as wastes. Pulp, husk and other waste products from coffee and tea industries increase toxicity of surrounding landmass due to their caffeine content resulting in soil infertility. Effluents are often discharged into water bodies, contaminating drinking and surface water with caffeine and affecting the saprophytic organisms involved in essential biotransformation in the environment. Decaffeination therefore becomes an important step in coffee processing. In this aspect, microbial cells and enzymes, which are biological and non toxic, have been found to be more beneficial than conventional techniques using chemicals and energy. Several microbial strains and enzyme systems such as N-demethylases and caffeine oxidases have been discovered over the last two decades which serve are potential candidates for development of biodecaffeination techniques. The rarity of strains and the unstable nature of caffeine degrading enzymes are some of the challenges with scope for research and development in the area of biodecaffeination.

Keywords Caffeine • Biodegradation • Decaffeination • Enzymes • Caffeine Demethylases

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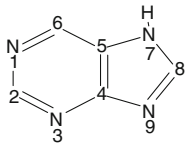
20.1 Introduction to Caffeine

Alkaloids constitute the major bulk of secondary metabolites in plants. These compounds play a key role in the plant defense mechanism against pests and herbivores and are known to have an effect on the metabolism of living organisms. This property of alkaloids has also been useful in developing a wide variety of products for the use of mankind. Caffeine (3,7-dihydro-1,3,7-trimethyl-1 H-purine-2,6 dione) is one such commercially important alkaloid which belongs to the family of purine alkaloids synthesized by plants. The word 'caffeine' came from the German word 'kaffee' and the French word 'café', each meaning coffee. The consumption of caffeine in form of foods and beverages was in practice long before its isolation in pure form in 1891 by the German chemist Friedrich Ferdinand Runge (Weinberg and Bealer 2001).

Chemically, caffeine is a hydrophilic molecule with hydrophobic methyl groups. In its pure state caffeine is an odourless, amorphous, intensely bitter white powder with a molar mass of 194.19 g/mol. Apart from caffeine, other methylxanthines also form an important group of compounds with the basic purine structure. Most of the methylxanthines are the intermediates of caffeine degradation pathway in various life forms resulting from demethylation of caffeine. These include dimethylxanthines namely theobromine (3,7-dimethylxanthine), theophylline (1,3-dimethylxanthine) and paraxanthine (1,7-dimethylxanthine); monomethylxanthines like 7-methylxanthine, 3-methylxanthine and 1-methylxanthine; and xanthine. The structure of caffeine and other methylxanthines is given in Fig. 20.1.

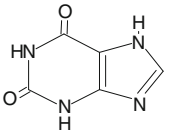
In nature, caffeine is distributed in the leaves and fruits of 13 different orders of plants, including coffee (*Coffea arabica)*, tea (*Camellia sinensis*), mate (*Ilex paraguariensis*), guarana (*Paullinia coplanar*), cola (*Cola nitida*) and cocoa (*Theobroma cacao*) (Ashihara and Crozier 2001). The world's primary source of caffeine is the coffee bean (the seed of the coffee plant), from which coffee is brewed. *Coffee arabica* (Arabica) and *Coffee robusta* (Robusta) are the widely used species of coffee where the caffeine content varies between 1 and 4% by dry weight (Martin et al. 1998; Silvarolla et al. 2000; Mazzafera et al. 1994; Campa et al. 2004). In plants, caffeine is formed by the successive methylation of xanthosine molecule. The precursor xanthosine molecule is obtained from adenine nucleotide pool, guanine nucleotide pool or obtained by *de novo* synthesis (Mazzafera et al. 1991, 1994; Ashihara et al. 1996). Anabolism of caffeine in plants is given in Fig. 20.2. The caffeine synthesis in plants comprises of sequential methylations at *N*-7, *N*-3 and *N*-1 of xanthosine ring, which are catalyzed by different *N*-methyl transferases (NMT), viz., 7-methyl transferase (methyl xanthosine nucleotidase), 3-methyl transferase (caffeine synthase) and 1-methyl transferase (caffeine synthase) (Kato et al. 1998, 1999; Mizuno et al. 2003; Ashihara and Crozier 2001; Waldhauser et al. 1996). The methyl groups required in these steps and sometimes even the adenosine backbone of caffeine are supplied by S-adenosyl-L-methionine cycle (SAM cycle) (Koshiishi et al. 2001).

Backbone molecule



7*H*-purine

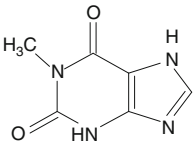
Precursor molecule



Xanthine

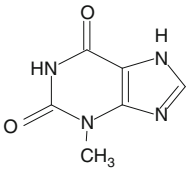
2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine

Monomethyl xanthines



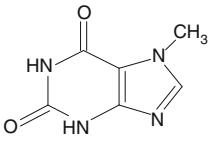
1-methyl xanthine

1-methyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine



3-methyl xanthine

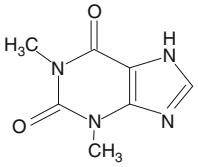
3-methyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine



7-Methyl xanthine

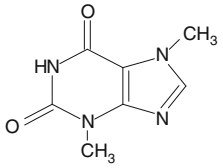
7-methyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine

Dimethyl xanthines



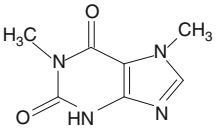
Theophylline
(1,3-dimethyl xanthine)

1,3-dimethyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine



Theobromine
(3,7-dimethyl xanthine)

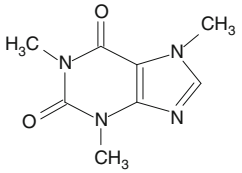
3,7-dimethyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine



Paraxanthine
(1,7-dimethyl xanthine)

1,7-dimethyl-2,6-dioxidanylidene-2,3,6,7-tetrahydro-1*H*-purine

Trimethyl xanthine



Caffeine
1,3,7-trimethyl xanthine

3,7 dihydro -1, 3, 7 trimethyl -1H-Purine -2, 6 dione

Fig. 20.1 Structure of caffeine and other related compounds

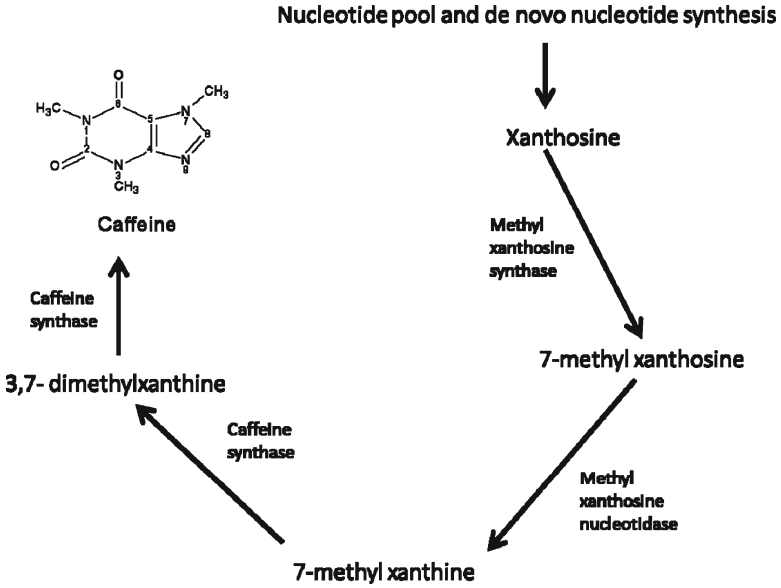


Fig. 20.2 Biosynthesis of caffeine in plants

Table 20.1 Caffeine content of beverages and food items (www.cspinet.org)

Sl.No.	Beverages and food items	Caffeine content
1.	Coffee, brewed (drip)	130–680 mg/l
2.	Coffee, instant	130–400 mg/l
3.	Coffee, decaffeinated	13–20 mg/l
4.	Espresso	3,400 mg/l
5.	Black tea, brewed	100–470 mg/l
6.	Tea, instant	120 mg/l
7.	Tea, canned iced	70–100 mg/l
8.	Soft drink (caffeinated)	100–270 mg/l
9.	Caffeine pills	100 mg/pill
10.	Chocolate, bittersweet	875 mg/kg

The global caffeine consumption has been estimated to be 120,000 tons which accounts to approximately 80–400 mg caffeine per person per day. The main source for caffeine intake is through beverages like tea, coffee, caffeinated soft drinks and numerous food products like chocolates and desserts (Gokulakrishnan et al. 2005). Caffeine is widely used in pharmaceutical preparations as it enhances the effect of certain analgesics and antipyretic drugs. It also finds application as a cardiac, neurological and respiratory stimulant and as a diuretic (Mazzafera 2002). The caffeine content of common consumables have been listed in Table 20.1.

Caffeine is a central nervous system and metabolic stimulant (Nehlig et al. 1992) and this property is primarily related to blocking the binding of adenosine to

adenosine receptors. In human and other mammals, there exists four different types of adenosine receptors A_{2A}, A_1, A_{2B}, and A_3 (Ribeiro et al. 2003) and caffeine acts as a prototypic adenosine receptor antagonist (Nehlig 1999; Daly and Fredholm 1998; Smith 2002). Activation of A_{2A} receptors by A_{2A} receptor agonists will decrease the affinity of dopamine binding to D_2 receptors. Caffeine, by blocking striatal A_{2A} receptors tonically activated by adenosine would then increase the potency of endogenous dopamine at D_2 receptors (Daly and Fredholm 1998). Thus, caffeine is able to stimulate brain neurons with adenosine receptors by blocking the effects of adenosine, which is an endogenous inhibitory neurotransmitter or neuromodulator. This stimulation helps in increasing the alertness and cognitive performance of the individual (Lorist and Tops 2003) thus making caffeine one of the most consumed dietary ingredients in today's world.

Commercial coffee is obtained from coffee cherries, 6% of which generate the coffee powder whereas the remaining 94% constitute the byproducts such as husk, pulp water etc. Therefore, caffeine-containing byproducts and effluents generated from coffee and tea-processing plants constitute a major part of the agro-industrial wastes in coffee producing nations. Solid wastes such as coffee pulp and husks pollute the surrounding landmass by increasing toxicity and bacterial contamination (Adams and Dougan 1981; Bressani 1987). The presence of caffeine in soil can also affect soil fertility as it inhibits seed germination and growth of seedlings (Friedman and Waller 1983). Caffeine-containing effluents are often discharged to the surrounding water bodies and, subsequently, caffeine has been detected in surface water, ground water and waste water effluents at a high concentration (~10 g/l) (Buerge et al. 2003; Glassmeyer et al. 2005). The ingestion of caffeine and its chlorinated byproducts (derived during chlorination of water during recycling) has severe adverse effect on the physiological system (White and Rasmussen 1998). Therefore, in order to free the natural waters and soil from this compound, decaffeination of the byproducts becomes a very necessary step in treatment of coffee and tea wastes.

20.2 Conventional Methods of Decaffeination

Decaffeination is currently being carried out widely in beverages because of the growing belief that the chronic ingestion of caffeine can have adverse effects on health. Coffee and tea pulps generated while processing tea and coffee are generally dumped in the soil and later discharged into the nearby water sources (rivers and lakes), thereby causing pollution in the water bodies located near tea and coffee industries. Coffee and tea pulps are rich in nutritional compounds such as carbohydrates and proteins and thus have good biotechnological potential (Pandey et al. 2000). However, the presence of anti-nutritional factors such as caffeine, tannins and poly-phenols restricts the use of coffee and tea pulps as domestic fodder. Conventional decaffeination strategies mostly use physical and chemical methods to remove caffeine. The three important conventional methods include solvent

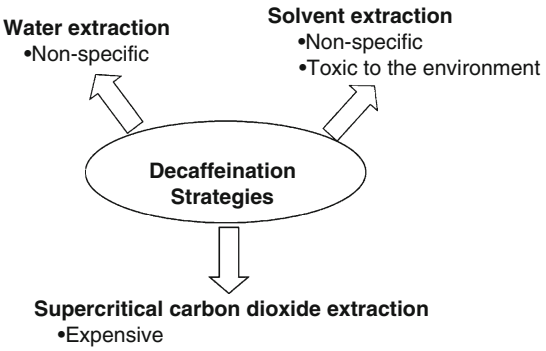


Fig. 20.3 Various methods of caffeine removal by conventional methods

decaffeination, water decaffeination and supercritical carbon dioxide decaffeination (Gokulakrishnan et al. 2005). In solvent extraction, trichloroethylene, methylene chloride or some other similar chlorinated compounds are used as solvents to remove caffeine from coffee beans. Although simple, this method has to meet stringent environmental restriction while discarding the solvent (Feldman and Katz 1977). To overcome this drawback, in a different method water is used as a solvent to remove caffeine (water extraction). This method in inexpensive and non hazardous to the environment, but results in the loss of many water soluble components of coffee and tea that contribute to flavor and aroma. The use of carbon dioxide in its supercritical state (supercritical fluid extraction) is an improved technique that is superior to the other methods in terms of environment safety and preservation of aroma and flavor. However, the major disadvantage of this method is the requirement of a huge capital cost that impedes its commercialization. The detailed description of the conventional decaffeination techniques is shown in Fig. 20.3.

Other methods of caffeine removal have been recently reported in the literature. Caffeine and polyphenols from tea have been removed much more effectively by using microwave assisted extraction process and solid phase extraction using molecularly imprinted polymer prepared with caffeine as a template (Pan et al. 2003; Theodoridis and Manesiotis 2002). These extraction methods remove other taste and aroma causing compounds from coffee and tea. The use of membranes or carbon filters in caffeine removal processes will be very expensive and the commercialization of the process becomes less viable. To overcome this problem, a specific caffeine removal process such as microbial or enzymatic degradation of caffeine is necessary.

In order to overcome the shortcomings of the conventional methods of decaffeination, microbial or enzymatic degradation of caffeine has been advocated (Gokulakrishnan et al. 2005). The use of microbes and enzymes offer advantage of being specific to caffeine, thereby preserving the aroma and flavor imparting substances in coffee and tea. As compared to the other methods, biodecaffeination strategies are more environment friendly and safe for the health of the consumers. The commercialization of such techniques is also more feasible as the capital investment required is much less as compared to the other chemical and physical methods.

The main criteria for developing such a strategy is the use of a microbial strain that is capable of tolerating and degrading caffeine at a higher concentration and faster rates at the same time being non- pathogenic and having simple growth requirements.

20.3 Degradation of Caffeine by Microbial Methods

Studies on caffeine degradation by microorganisms were not reported until 1970 probably because caffeine was regarded toxic to bacteria (Sundarraj and Dhala 1965; Putrament et al. 1972; Khilman 1974). Caffeine concentration greater than 2.5 mg/ml in the growth medium has been found to inhibit the growth of many bacterial species. Synergistic effect has been observed when caffeine is added to antimicrobial agents like chloramphenicol (Sundarraj and Dhala 1965). First report on caffeine degradation by microorganisms was in early 1970 (Kurtzman and Schwimmer 1971). Since then research has been developed on using caffeine as source for microbial growth (Woolfolk 1975; Vogels and Drift 1976; Schwimmer et al. 1971).

20.3.1 Bacterial Degradation of Caffeine

In bacteria (*Pseudomonas*), caffeine is initially converted into theobromine and paraxanthine parallely by demethylases. Further demethylation forms xanthine with 7-methyl xanthine as the intermediate. There is also an evidence for oxidation of xanthine, mono and dimethyl xanthines to their respective uric acids, which enter the purine catabolic pathway (Blecher and Lingens 1977) (Fig. 20.4a). In *Serratia marcescens*, the caffeine catabolic pathway is similar to *Pseudomonas* sp. except for the formation of methyl uric acid intermediate (Mazzafera et al. 1994). This information about pathway and different enzymes involved in the degradation of caffeine could help in developing an enzymatic process for caffeine removal.

Bacterial strains capable of degrading caffeine belonged to *Pseudomonas* and *Serratia* genus (Blecher and Lingens 1977; Mazzafera et al. 1994). Attempts were made for biological production of caffeine catabolic intermediates with the help of inhibitors. Asano and co-workers reported the production of theobromine using *Pseudomonas* strain for the first time (Asano et al. 1993). Theobromine was accumulated at different levels ranging from 5 g/l and above in the presence of 1 mM of Zn^{2+}. Fructose and tryptone were found to be the most suitable carbon and nitrogen sources. A 10 fold increase in production of theobromine has been observed in the presence of Fe^{2+} (0.04%) in the medium suggesting that Fe^{2+} may enhance the synthesis of demethylating enzymes or act as a co-factor. In another minor pathway observed in microbial species consisting of *Rhodococcus, Klebsiella* and *Alcaligenes*, caffeine is directly oxidized to 1,3,7-trimethyluric acid and consequently to uric acid (Madyastha et al. 1999; Mohapatra et al. 2006) (Fig. 20.4b).

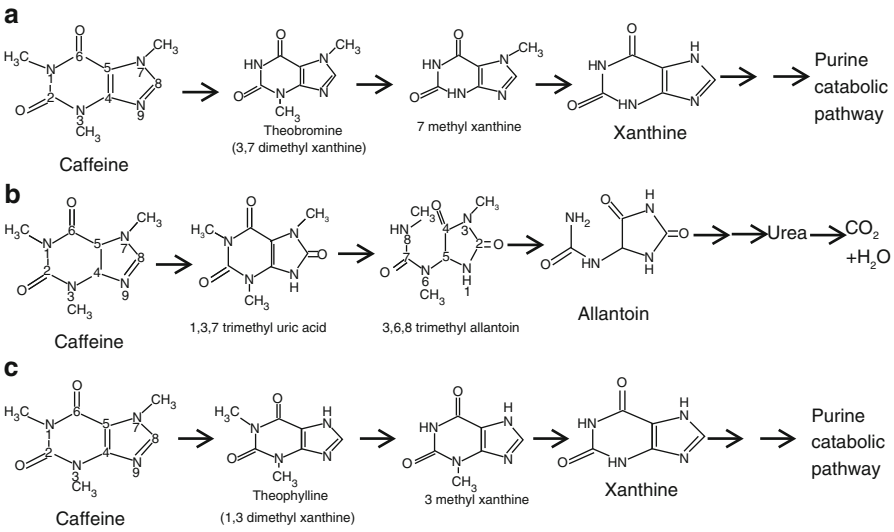


Fig. 20.4 (**a**) Catabolism of caffeine in bacteria, (**b**) Catabolism of caffeine in bacteria by direct oxidation to uric acid, (**c**) Catabolism of caffeine in fungi

Bacteria can be used in reducing the caffeine content in caffeine bearing plants. It has been found that leaf surface play a vital role in *Agrobacterium* infection in tea plants (Kumar et al. 2004). A method has been proposed for producing tea leaves with less caffeine content by growing caffeine degrading bacteria on the surface of the leaf. Ramarethinam and Rajalakshmi (2004) found *insitu* lowering of caffeine in tea leaves without affecting the quality of the other tea components when tea plants were sprayed with a suspension of *Bacillus licheniformis*. Anaerobic fermentation of coffee pulp resulted in about 13–63% reduction of caffeine in 100 days (Hakil et al. 1998). In contrast, aerobic fermentation resulted in 100% degradation of caffeine in 14 days (Rojas et al. 2003). Further studies to enhance caffeine degradation by this natural fermentation are required.

20.3.2 Degradation of Caffeine by Fungal Strains

Although fungi have been used to study about the detoxification of pulp and the products formed during caffeine degradation are known, its caffeine catabolic pathway is not yet elucidated (Hakil et al. 1998). It is assumed that the degradation of caffeine in fungi takes place via the demethylation route, with theophylline as the first formed intermediate (Fig. 20.4c). Caffeine degradation has been observed in fungal species like *Stemphyllium* sp. (Schwimmer et al. 1971), *Penicillium* sp. (Hakil et al. 1998; Brand et al. 2000; Roussos et al. 1994, 1995) and *Aspergillus* sp. (Hakil et al. 1998; Brand et al. 2000; Roussos et al. 1995). *A. tamari, A. niger,*

Table 20.2 Comparison of caffeine degradation rate in various microorganisms (Gokulakrishnan et al. 2005)

Microorganism	Initial caffeine concentration (g/l)	Carbon source (g/l)	Caffeine degradation%	Rate of caffeine degradation(g/l/h)
Klebsiella and *Rhodococcus*	0.5	Glucose (1)	100% in 10 h	0.05
Serratia marcescence	0.6	–	100% in 72 h	0.008
Stemphyllium sp.	0.19	Sucrose (30.1)	100% in 29 h	0.0053
Pseudomonas putida	5	–	95% in 50 h	0.095
Aspergillus tamari	1.2	Sucrose (28.4)	67.2% in 48 h	0.0536
Penicillium commune	1.2	Sucrose (28.4)	61.6% in 48 h	0.0521

A. fumigatus, P.commune showed appreciable growth when caffeine was used as the sole source of nitrogen (Hakil et al. 1998). *A. tamarii* and *P. commune* showed good caffeine degrading ability (about 60%) whereas others had less than 20% caffeine degradation (Hakil et al. 1998).

Bioremediation of coffee pulp to reduce the caffeine content has been studied more in fungal systems. Among the microbial community present in coffee pulp, only a few species like *Aspergillus*, *Penicillium* and *Rhizopus* could degrade caffeine. *Aspergillus* and *Penicillium* species degraded caffeine almost with 100% efficiency at 25°C, whereas the efficiency of degradation decreased to 30% at 30°C (Roussos et al. 1995). *Rhizopus* sp. produced a higher quantity of biomass, whereas *Aspergillus* sp. showed more efficient caffeine degradation (92%) (Fig. 20.4c).

20.3.3 Bioprocess Considerations in Decaffeination by Submerged Fermentation

The important parameters for the development of technological process for microbial degradation of caffeine are (i) Rate of caffeine degradation (ii) Initial caffeine content (iii) nitrogen source and (iv) pH. The rate of caffeine degraded by important microorganisms is given in Table 20.2. The bacterial degradation of caffeine was found to be greater than fungi. Since caffeine is toxic to microorganisms (Sundarraj and Dhala 1965; Putrament et al. 1972; Khilman 1974), the initial concentration of caffeine in fermentation is crucial. In *Serratia marcescens*, the critical inhibitory concentration has been found to be 1.2 mg/ml (Mazzafera et al. 1994). *Pseudomonas putida* has been found to be the best organism for degrading caffeine at higher concentration (Table 20.2). In fungi, the presence of an external nitrogen source inhibits caffeine degradation completely till the external source is depleted. The presence of urea and ammonium sulphate prevents the uptake of caffeine in *Aspergillus niger* and *Penicillium verrucosum* (Hakil et al. 1999; Roussos et al. 1994). However, in contrast to fungi, the presence of an external organic nitrogen source did not prevent

the degradation of caffeine (Asano et al. 1993). Nevertheless, care should be taken while adding an external nitrogen source during caffeine degradation.

Initial pH of the medium is also an important parameter for caffeine degradation. Though no specific reports are available on the effects of initial pH, it has been reported that the final pH of the medium becomes acidic (pH 4–3.2) for *Penicillium roqueforti* (Schwimmer et al. 1971) and basic for *Aspergillus* sp. (Brand et al. 2000). Importantly, it has been reported that there was a shorter lag phase when the inoculum was grown in caffeine, compared to that grown on xanthine or uric acid (Blecher and Lingens 1977). Hence the inoculum should be caffeine grown before fermentation. In a study of caffeine degradation by *A. niger* LPBx in a glass column reactor, yield of 3.811 g of biomass per g of oxygen and a maintenance coefficient of 0.0031 g of oxygen consumed per g of biomass was reported (Brand et al. 2001, 2002). It has been found that caffeine degradation is related to spore formation and respiration rate of the mold. Packed bed fermentation of *Rhizopus delemar* with coffee pulp as a substrate was seen to produce theophylline and 3-methyl xanthine as the major metabolites due to the degradation of caffeine in the pulp in accordance with previous reports (Tagliari et al. 2003; Hakil et al. 1998).

20.3.4 Use of Solid State Fermentation in Caffeine Degradation

The degradation of caffeine in coffee pulp and coffee husk has been studied by SSF with *Aspergillus*, *Rhizopus* and *Phanerochaete*. In *Rhizopus* and *Phanerochaete* the critical parameters affecting caffeine degradation were pH and moisture content the values of which were found to be 5.5, 65% and 6, 60% respectively. For *A.niger* the critical parameters affecting caffeine degradation were temperature and pH and the optimal values were 28°C and 4 respectively (Brand et al. 2000). An effective method has been reported for utilizing the caffeine using coffee pulp and husk as the substrate for the growth of molds (Leifa et al. 2000; Salmones et al. 2005). Caffeine was degraded during the growth of *Lentinus edode* whereas caffeine was accumulated in the fruiting bodies of *Pleurotus* sp. Hence *Pleurotus* sp. could be used to recover caffeine from coffee and tea waste.

20.4 Enzymatic Systems Involved in Microbial Caffeine Degradation

Bioconversion of caffeine to its methylxanthine derivatives is an enzymatic process involving more than one type of enzyme. Both whole cells and purified enzymes of caffeine-degrading bacterial strains have been explored in this aspect. Resting cells of *Pseudomonas putida* (Middelhoven and Bakker 1982; Gummadi and Santhosh 2006) and *Pseudomonas alcaligenes* (Sarath Babu et al. 2005) are effective in degrading caffeine in both free and immobilized form. Whole cells are

advantageous in the bulk treatment of soil, wastewater and caffeine containing by products as it is cheaper than using purified enzymes which require costly cofactors and whole cells can convert complex compounds to non-contaminating compounds such as CO_2 and H_2O. However, decaffeination using whole cells can be non-specific and when applied to food products may remove other compounds rendering flavor and aroma to coffee and tea resulting in an unpalatable product. Since several enzymes are involved in conversion of caffeine to its end products, whole cells will not be beneficial when the objective is to recover a particular methylxanthine intermediate requiring a specific enzyme of caffeine catabolic pathway. Hence for these applications, purified enzymes are more beneficial than whole cells.

20.4.1 Bacterial N-demethylases

The speculation that N-demethylation was the possible mechanism of conversion of caffeine to its methylated derivatived came from the study of Woolfolk who demonstrated methylxanthine degrading activity in cell free extracts and subcellular fractions of *Pseudomonas putida* (Woolfolk 1975). He suggested that the conversion of caffeine to its metabolites was brought about by an enzyme that hydrolytically removed all the three methyl groups with the production of methanol and free xanthine (Fig. 20.5a). The free xanthine presumably entered the purine degradation pathway to be converted to simpler compounds by the action of enzymes like xanthine dehydrogenase and uricase. The methanol formed was oxidized to CO_2 through formaldehyde by the sequential action of methanol dehydrogenase, formaldehyde dehydrogenase and formate dehydrogenase (Woolfolk 1975). The involvement of demethylase enzyme in caffeine degradation in a strain of *Pseudomonas putida* was further confirmed by Bletcher and Lingens. However they postulated that the demethylation was oxidative rather than hydrolytic, as suggested by Woolfolk, resulting in the formation of formaldehyde instead of methanol (Fig. 20.5b). Their studies also showed that the demethylase enzymes were not constitutive but inducible by the addition of caffeine and other methylxanthine that formed as a result of degradation of caffeine (Blecher and Lingens 1977). Similar NADH and NADPH dependant demethylating enzyme was demonstrated in the crude extracts of *Pseudomonas putida* (Hohnloser et al. 1980). The enzyme was found to be active at pH 6.0 and temperature of 22–24°C. It was also observed that the formaldehyde generated by caffeine demethylase is further oxidized by the action of formaldehyde dehydrogenase. Purified formaldehyde dehydrogenase had a pH and temperature optimum of 8.5–9.0 and 30–35°C respectively and required NAD^+ for activity. (Hohnloser et al. 1980). NADH/NADPH dependant caffeine demethylase was isolated by Yamaoka-Yana and Mazzafera and the optimal pH and temperature was 7.0 and 20–30°C respectively. But attempts to purify this enzyme were unsuccessful as this enzyme lost its activity rapidly in partially purified extracts due to its unstability (Yamoka-Yano and Mazzafera 1998).

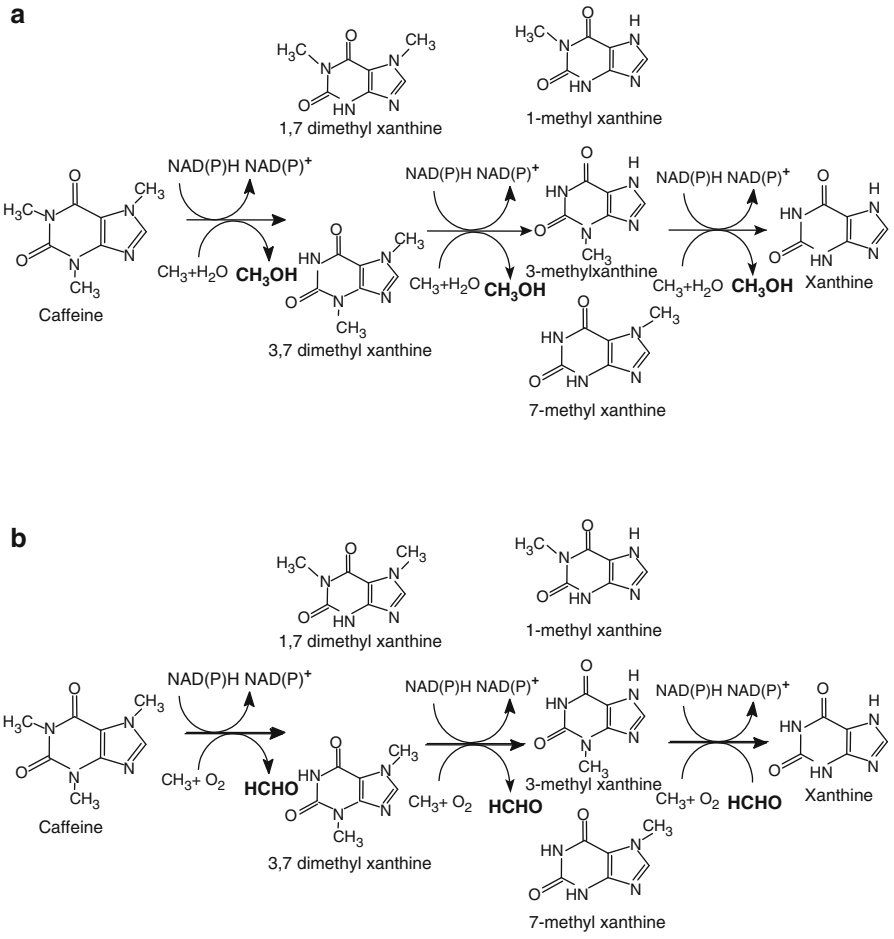


Fig. 20.5 (**a**) Schematic representation of bacterial N-demethylation by hydrolysis, (**b**) Schematic representation of bacterial N-demethylation by oxidation

Similar to caffeine demethylase, enzymes for demethylation of other methylxanthines have been reported in the literature. Asano and coworkers isolated a monooxygenase i.e. theobromine demethylase from cell free extracts of *Pseudomonas putida* and reported that this enzyme specifically brought about the demethylation of theobromine. They further investigated to find that the theobromine demethylase enzyme was inhibited by Zn^{2+} which generated the accumulation of theobromine (Asano et al. 1994). Another enzyme, heteroxanthinedemethylase isolated from *Pseudomonas putida* species has been shown to selectively convert heteroxanthine (7-methylxanthine) to xanthine by oxidative demethylation with the formation of formaldehyde (Glück and Lingens 1988). The enzyme showed maximum activity at a temperature of 30–35°C and

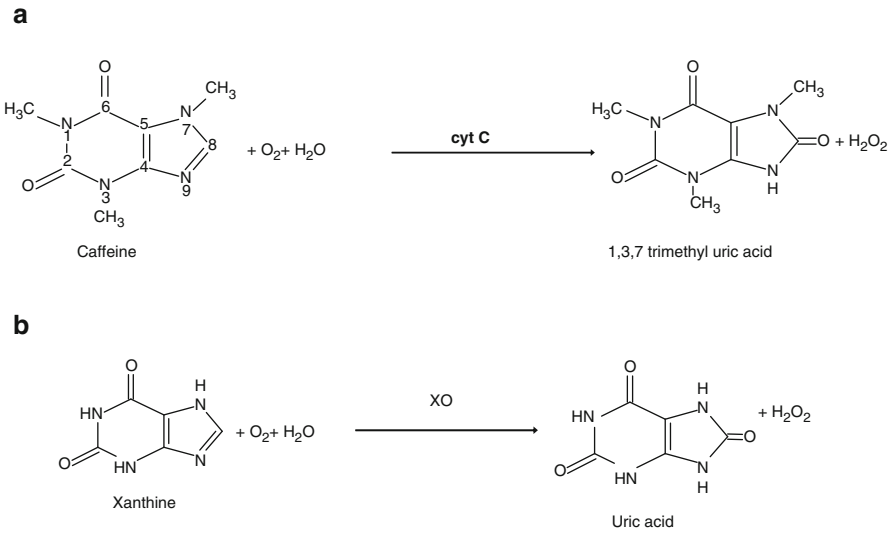


Fig. 20.6 (**a**) Schematic representation of mechanism of action of caffeine oxidase enzyme, (**b**) Schematic representation of mechanism of action of xanthine oxidase enzyme

at pH 7.5 with NADH/NADPH as cofactors. It showed no activity when either caffeine or dimethylxanthines were used as substrates. Similar to caffeine demethylase, heteroxanthinedemethylase was also found to be unstable and loss of activity occurred upon storage.

20.4.2 Bacterial Caffeine Oxidases

In certain species of bacteria, instead of caffeine being demethylated to methylxanthines, gets oxidized to 1, 3, 7- trimethyluric acid. Enzymes catalyzing this reaction have been designated as caffeine oxidases (Fig. 20.6a). Two such caffeine oxidases have been purified from bacterial species like *Rhodococcus, Klebsiella* and *Alcaligenes* (Madyastha et al. 1999; Mohapatra et al. 2006). Caffeine oxidase from *Rhodococcus* and *Klebsiella* was a 85 kDa flavoprotein that catalyzed the formation of 1,3,7-trimethyluric acid directly from caffeine in a single step. This enzyme showed substrate specificity toward caffeine and various analogues of theobromine (Madyastha et al. 1999). The other caffeine oxidase isolated from *Alcaligenes* sp. was found to be 65 kDa serine type metalloprotease which showed strict substrate specificity towards caffeine. It had maximal activity at 35°C and at pH 7.5 (Mohapatra et al. 2006). Both these enzymes were not inhibited by the known inhibitors of xanthine oxidase, thus proving that they were different from

xanthine oxidase. From the biotechnological point of view, caffeine oxidase enzymes would prove to be more useful in treatment of caffeine containing effluents or the wastes of coffee processing industries as these are more stable and can be purified to homogeneity without loss of activity. However, the use of these enzymes will not be useful in recovery of the more useful methylxanthines since caffeine is directly converted to trimethyluric acid without the formation of methylxanthine intermediates.

20.4.3 Bacterial Xanthine Oxidases

The methylxanthines formed during the course of caffeine breakdown can be further converted to the corresponding methyluric acids (Woolfolk 1975; Blecher and Lingens 1977) and this conversion is brought about by a group of enzymes called as xanthine oxidases (Fig. 20.6b). Several researchers have purified this particular enzyme from microorganisms (Bradshaw and Barker 1960; Smith et al. 1967; Woolfolk et al. 1970; Sin 1975). Isolation of the activities from diverse sources (Bray 1965) has revealed that these enzymes are all fundamentally similar with regard to molecular properties and prosthetic group content, although they may differ considerably in utilization of electron acceptors and the relationship to the economy of the cells from which they are obtained. Xanthine oxidase isolated from *Pseudomonas putida* has three subunits (71, 65.6 and 61.8 kDa) and it could bring about the conversion of various methylxanthines to methyluric acids. This enzyme had a K_m value of 169 μM and showed optimum activity at 30°C and pH of 7.0 (Yamoka-Yano and Mazzafera 1999). The xanthine oxidase enzymes have been shown to be inhibited by the methyxanthines (Kela et al. 1980).

20.5 Challenges in Biodecaffeination by Enzymatic Approach

Although numerous caffeine degrading microorganisms have been identified in the recent past, reports on the enzymatic aspects of caffeine degradation are few. The labile nature of N-demethylases hinders the purification and characterization of these enzymes. Use of chemicals such as 20% glycerol, BSA, thioagents (Glück and Lingens 1988) or cryoprotectants and freeze drying techniques (Sideso et al. 2001) have been shown to confer some degree of stability but more research in area is required to come up with an effective technique. In comparison, caffeine oxidase enzyme is more stable and hence can be utilized as biocatalysts for decaffeination purposes. But direct oxidation of caffeine to methyluric acids is a less favoured catabolic pathway, so bacterial species producing caffeine oxidase enzymes are rare. Isolation of such strains will prove beneficial from the biotechnological point of view.

20.6 Biotechnological Applications of Biodecaffeination

Though the search for caffeine degrading microorganism began nearly 35 years ago, studies conducted in this area are inadequate. Very few studies have been conducted to isolate strains capable of degrading caffeine. More microorganisms, which could degrade caffeine, need to be isolated. It is probable that an organism, which is able to grow well on coffee or tea pulp, can degrade caffeine. Coffee fermentation microflora mainly belonged to aerobic Gram negative *bacilli* (Avallone et al. 2001). If caffeine degradation ability of these organisms were checked then more caffeine degrading strains would be obtained. In bacteria, *Pseudomonas* species and in fungi *Aspergillus* and *Penicillium* species are efficient in degradation of caffeine. Degradation in bacteria occurred predominantly through demethylation route but oxidative route occurs predominantly in mammals. However, the degradation pathway in fungi is not clearly known. Biological detoxification of coffee and tea pulp is done by solid-state fermentation using fungi. Initial caffeine concentration and external nitrogen concentration are very crucial for caffeine degradation to be effective. Biological production of theobromine and other methyl xanthines from caffeine need to be optimized. Such studies will lead to development of process for caffeine degradation and as well as production of value added compounds.

20.7 Future Perspectives and Conclusions

Biological decaffeination provides an attractive alternative to the conventional methods of decaffeination. Keeping in mind the increasing demand for decaffeinated products, such methods can be carried out at a much larger scale with the proper understanding of the biological system involved in this conversion. Biodecaffeination can be used effectively for the treatment of solid caffeine wastes such as husk and pulp for use as animal feed (Roussos et al. 1995; Mazzafera 2002), for removal of caffeine in sewage (Ogunseitan 1996), for the production of other commercially important methylxanthines (Glück and Lingens 1988; Asano et al. 1993) and also for the production of decaffeinated beverages and other food products. Microbial enzymes involved in caffeine catabolism will prove more effective than microorganisms as these will be specific and economical. The isolation, purification and characterization of new caffeine degrading enzymes and development of methods for increasing the stability of existing ones are some of the areas where more studies are required. Further, identification of genes involved in caffeine catabolism will open up new avenues for gaining insight on the regulatory mechanism of these enzymes, and provide scope for their manipulation using molecular tools like recombinant DNA technology to suit various applications.

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Chapter 21
Arxula adeninivorans (*Blastobotrys adeninivorans*) – An Imperfect Dimorphic Yeast of Biotechnological Potential

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Abstract The imperfect, haploid, dimorphic ascomycetous yeast *Arxula adeninivorans* (syn. *Blastobotrys adininivorans*) exhibits some unusual properties. Being a thermo-, osmo- and salt tolerant species, it is able to assimilate and ferment many compounds as sole carbon and/or nitrogen sources. It utilises tannic acid, purines, xylose, n-alkanes and is capable of degrading starch. Due to these unusual biochemical properties *A. adeninivorans* can be exploited as a gene donor for the production of enzymes with attractive biotechnological characteristics. Examples of *A. adeninivorans* genes that have been over-expressed include the *ATAN1* gene encoding a secretory tannase, *ALIP1* gene encoding a secretory lipase, the *AXDH* encoding xylitol dehydrogenase and the *APHY* encoding a secretory phosphatase with phytase activity.

The thermo-, osmo- and salt tolerances, as well as differential morphology-dependent glycosylation pattern and the secretion characteristics, render *A. adeninivorans* attractive as host for heterologous gene expression. A transformation system has been established based on homologous and non-homologous integration of linearised DNA fragments. Successful expression examples such as the human *HSA, IL6* and *IFNα* genes, as well as the production of functional recombinant receptors, for example, human estrogen receptor add to the attractiveness of *A. adeninivorans* as host for heterologous gene expression.

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21.1 Taxonomy and Strains

Arxula adeninivorans was first cultured and described by Middelhoven et al. (1984). It was isolated from soil by enrichment culturing and designated *Trichosporon adeninovorans.* This yeast exhibited unusual biochemical characteristics and was able to assimilate a range of amines, adenine and several other purine compounds as a sole energy and carbon source. After isolation of further wild type strains from wood hydrolysate in Siberia (LS3), chopped maize herbage ensiled at 25 or 30°C in the Netherlands and humus-rich soil in South Africa, a new genus, *Arxula,* located in the *Candidaceae,* was proposed for all of these strains (Van der Walt et al. 1990). All representatives of the newly proposed xerotolerant, nitrate positive species were ascomycetous, anamorphic and arthroconidial (Middelhoven et al. 1984, 1991, 1992; Van der Walt et al. 1990; Gienow et al. 1990).

The most recent reclassification of the yeast species was carried out by Kurtzmann and Robnett (2007). This was based on phylogenetic analyses of the ascosporic yeast genera *Sporopachydermia, Stephanoascus, Trichomonascus, Wickerhamiella* and *Zygoascus* and the associated anamorphic genera *Arxula, Blastobotrys, Sympodiomyces* and *Trigonopsis* and on comparison of sequences derived from the large-subunit rDNA genes, the mitochondrial small-subunit rDNA genes, and the genes for cytochrome oxidase II. They deduced that *Arxula, Blastobotrys* and *Sympodiomyces* are members of the *Trichomonascus* clade, with the genus *Blastobotrys* having taxonomic priority for anamorphic states (Table 21.1).

The genus *Blastobotrys* includes both type species of the genus *B. terrestris* (Van der Walt and Johanssen) Kurtzman and Robnett comb. nov. (Basionym: *Arxula terrestris*) and *B. adeninivorans* (Middelhoven, Hoogkamer Te-Niet and Kreger van Rij) Kurtzman and Robnett comb. nov. (Basionym: *Arxula adeninivorans*).

Table 21.1 Taxonomic classification of the species *B. adeninivorans*

Superkingdom	*Eukaryota*
Kingdom	*Fungi*
Phylum	*Ascomycota*
Subphylum	*Saccharomycotina*
Class	*Saccharomycetes*
Order	*Saccharomycetales* (*Mitosporic Saccharomycetales*)
Genus	*Blastobotrys*
Species	*Blastobotrys adeninivorans*

Synonym: *A. adeninivorans* – Kurtzmann and Robnett (2007)

21.2 The *A. adeninivorans* genome

The relatively high frequency of auxotrophic mutants that is obtained after nitrosoguanidine mutagenesis, quantitative analysis of chromosomal DNA and determination of genome size demonstrate that *A. adeninivorans* is a haploid organism (Samsonova et al. 1996; Gienow et al. 1990).

The complexity of the nuclear genome of *A. adeninivorans* was analyzed by DNA re-association studies and karyotyping was performed by pulsed field gel electrophoresis (PFGE). With a genome sizes of 16.1×10^9 and 16.9×10^9 Da, calculated from reassociation kinetics of chromosomal DNA, *A. adeninivorans* wild type strains exhibit the highest value reported for a yeast species so far; much higher than the 9.2×10^9 Da determined for *S. cerevisiae* under identical conditions. The proportion of repetitive sequences was determined as 33.1% in LS3 and 35.9% in CBS 8,244 T. These values are also higher than those reported for other yeasts. Karyotype polymorphisms observed in the wild type strains tested demonstrated the existence of four chromosomes ranging between 1.6 and 4.6 Mb in size (Gienow et al. 1990; Kunze and Kunze 1994a).

Because no sexual cycle was observed, *A. adeninivorans* is classified as imperfect yeast. Therefore mapping techniques based on parasexual mechanisms had to be applied to establish a genetic map of the yeast. This permitted the linkage analysis of various markers resulting in 32 genes being assigned to four linkage groups, thus determining the chromosome number of the *A. adeninivorans* genome. This was confirmed by relating the 32 auxotrophic mutations identified to particular chromosomes by PFGE and subsequent DNA hybridization with specific probes (Samsonova et al. 1989, 1996; Gienow et al. 1990).

21.3 Biochemistry and Metabolism

21.3.1 Growth and Nutritional Requirements of A. adeninivorans

Soon after its identification, *A. adeninivorans* LS3 was employed in the production of single cell protein (SCP). This early process took advantage of the thermo-, osmo- and salt tolerance of this yeast that permitted the use of higher cultivation temperatures and media with higher osmolarity than those used in the cultivation of established SCP organisms.

Most observations on the growth characteristics of *A. adeninivorans* have been based on shake flask experiments. Under these conditions with yeast minimal medium (YMM) supplemented with vitamins and 2% glucose (final concentration), optimal growth of the yeast was shown to occur at pH values between 2.8 and 6.5. Within this pH range, maximal specific growth rates of ca. 0.32 h^{-1} and high biomass yields of ca. 0.55 g cell dry weight (CDW) g $glucose^{-1}$ can be achieved. Growth in

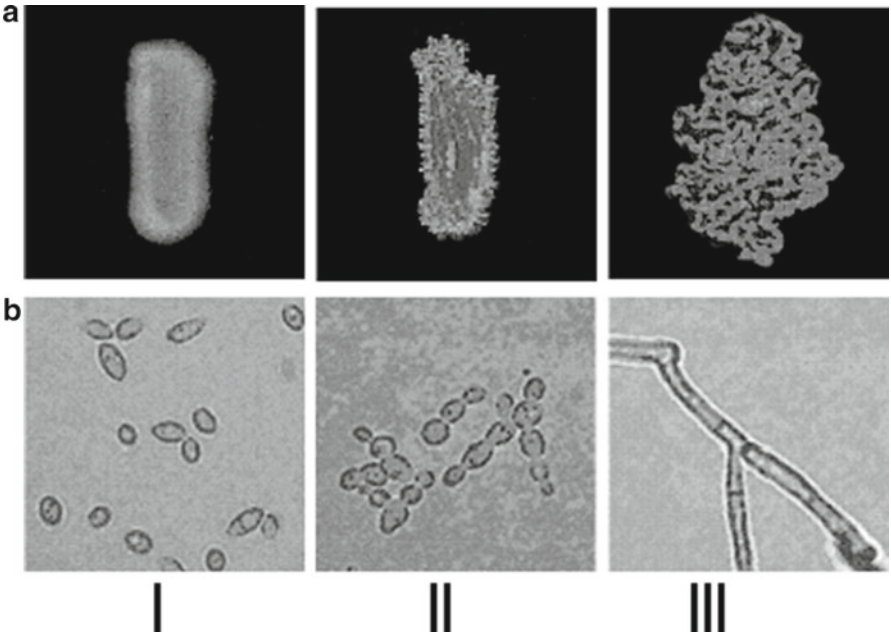

Fig. 21.1 (**a**) Colony form and (**b**) cell morphology of *A. adeninivorans* LS3 grown at 30°C (*I*), 42°C (*II*) and 45°C (*III*). The cells were cultured in YEPD medium for 18 h

a non-pressurized fed-batch 2 l bioreactor results in a high cell density of ca. 112 g l^{-1} CDW at a maximum oxygen transfer rate (OTR) of 0.15 mol l^{-1} h^{-1}. In comparison, cultivation in a pressurized system led to a 2-fold increase in cell density and a 2.7 fold maximum OTR (Minocha et al. 2007; Knoll et al. 2007; Knabben et al. 2010).

A. adeninivorans exhibits a temperature-dependent dimorphism which is especially pronounced in the Siberian wild strain *A. adeninivorans* LS3. This strain can grow at temperatures of up to 48°C without previous adaptation to elevated temperatures and is able to survive several hours at 55°C. At temperatures above 42°C a reversible transition from budding cells to a mycelial form is induced. Budding is re-established when cultivation temperature is decreased to below 42°C (Fig. 21.1 – Böttcher et al. 1988; Wartmann et al. 1995a).

In common with other dimorphic yeasts *A. adeninivorans* budding cells and mycelia differ in their content of RNA, soluble protein and in their dry weight. The synthesis of secreted proteins is greater in mycelia resulting in a twofold greater extracellular accumulation of these proteins, including the enzymes glucoamylase and invertase (Wartmann et al. 2000).

Another interesting property of *A. adeninivorans* is its osmotolerance. It can grow in minimal media with added NaCl, PEG400 and ethylene glycol up to 3.32 osmomol kg^{-1} H_2O (Yang et al. 2000; Böer et al. 2004a).

Table 21.2 Some nutrient requirements of *A. adeninivorans*

Element	Sources
Carbon	Glucose, fructose, galactose, mannose, sucrose, maltose, trehalose (fermentative and respiratory substrates), acetate, ethanol, glycerol, starch, tannic acid, xylose (respiratory substrates only)
Nitrogen	NH_4^+, NO_3^{2-} salts, urea, amino acids, peptides, purines, pyrimidines
Phosphorus	Phosphate salts
Sulphur	SO_4^{2-} salts, methionine, glutathione
Minerals and trace metals	K^+, Mg^{++}, Ca^{++}, Cu^{++}, Fe^{++}, Mn^{++}, Zn^{++}, Ni^{++} salts

21.3.2 Nutrition

A range of nutrient requirements of *Arxula* and sources that can provide these are summarized in Table 21.2.

21.3.3 Carbon and Energy Metabolism

A. adeninivorans is a Q_9-positive species that exhibits a wide substrate spectrum. It rapidly assimilates all sugars, polyalcohols and organic acids used in conventional carbon compound assimilation tests, except for L-rhamnose, inulin, lactose, lactate and methanol. Alcohols, dialcohols, carboxylic acids, dicarboxylic acids and other nitrogen-free analogous compounds are also assimilated. Other unusual sole energy and carbon sources include adenine, uric acid, butylamine, pentylamine, putrescine, soluble starch, melibiose, melicitose, propylamine and hexylamine. *A. adeninivorans* also degrades some phenols, hydroxybenzoates and tannic acid. Several nitrogen compounds such as amino acids and purine derivatives, primary n-alkylamines and terminal diamines are metabolized as sole energy, carbon and nitrogen sources (Middelhoven et al. 1984, 1991, 1992; Middelhoven 1993; Gienow et al. 1990).

A. adeninivorans produces numerous secretory enzymes including RNases, proteases, glucoamylase, lipase, tannase, acid phosphatases, trehalase, cellobiases, invertase, ß-glucosidase, xylosidase and phytase. Most enzymes exhibit a wide substrate spectrum and with the exception of lipase and tannase, a high temperature optimum (50–75°C) (Table 21.3).

21.3.4 Nitrogen Metabolism

The ammonium, nitrate and nitrite ions are optimal sources but others such as urea, uric acid, adenine, lysine, glutamine are also used (Table 21.2 – Middelhoven

Table 21.3 Properties of secretory enzymes of *A. adeninivorans*

Enzyme	Optimum			Molecular mass (Da)
	Temperature	pH	k_m value	
Glucoamylase (1.4-α-D-glucan glucohydrolase, EC 3.2.1.3)	60–70°C	4.0–5.0	1.2 g/L for starch 11.1 mM for maltose	225,000
Acid phosphatase I and II (ortho-phosphoric-monoester phospho hydrolase, EC 3.1.3.2)				
I	50–55°C	5.2–5.5	3.5 mM for p-nitro-phenylphosphate	320,000
II	50–55°C	5.2–5.5	5 mM for p-nitro-phenylphosphate	250,000
Trehalase (α,α-trehalose-gluco hydrolase, EC 3.2.1.28)	45–55°C	4.5–4.9	0.8–1.0 mM for trehalose	250,000
Cellobiase I and II (ß-D-glucosidase, EC 3.2.1.21)				
I	60–63°C	4.5	4.1 mM for cellobiose	570,000
II	60–63°C	4.5	3.0 mM for cellobiose	525,000
Invertase (ß-D-fructofuranoside fructohydrolase, EC 3.2.1.26)	50–60°C	4.5	40–60 mM for sucrose 36 mM raffinose	600,000
ß-D-xylosidase (1,4-ß-D-xylan xylohydrolase, EC 3.2.1.37)	60°C	5.0	0.23–0.33 mM for p-nitro-phenyl-ß-xylopyranoside	60,000
3-Phytase (*myo*-inositol hexakis phosphate 3-phosphohydrolase EC 3.1.3.8)	75°C	4.5	0.23 mM for phytata	n.d.
Lipase (triacylglycerol acylhydrolases, EC 3.1.1.3)	30°C	7.5	0.4 mM *p*NP-caprate	100,000
Tannase (tannin acyl hydrolase, EC 3.1.1.20)	35–45°C	5.0–6.5	0.14 mM for gallotannin	320,000

Böer et al. (2004b, 2005a, b, 2009b), Büttner et al. (1987, 1988, 1989, 1990a, b, c, 1991a, b, 1992); Büttner and Bode (1992), Kunze and Kunze (1994b), Sano et al. (1999), Wartmann et al. (1995b)

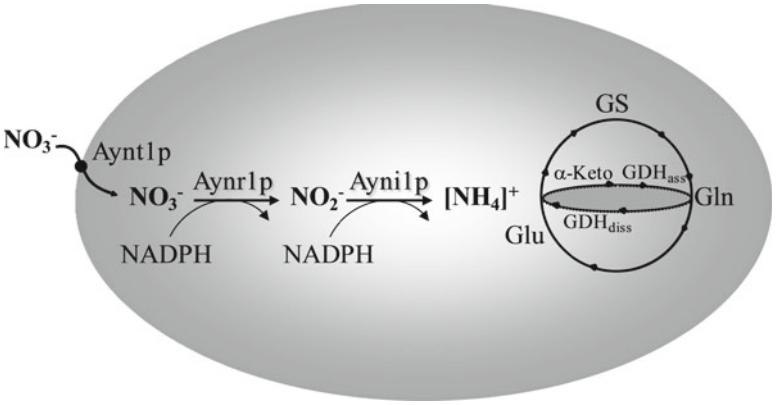

Fig. 21.2 Enzyme pathway of assimilative nitrate metabolism in *A. adeninivorans*. After nitrate transport by Aynt1p in the yeast cell, the two-step reduction of nitrate to ammonia is catalysed by the sequential action of nitrate reductase (*Aynr1p*) and nitrite reductase (*Yyni1p*). Glutamine synthetase (*GS*) fixes ammonia to an α-ketoglutarate skeleton to form glutamine, which can undergo a second ammonia fixation to form glutamate (*Glu*)

et al. 1984, 1991, 1992; Middelhoven 1993). Ammonium ions exert nitrogen catabolite repression on a number of nitrogen linked metabolic systems in the yeast including assimilative nitrate metabolism. Nitrate is transported by a specific nitrate transporter (Aynt1p) into the cell where the two-step reduction of nitrate to ammonia is catalysed by the sequential action of nitrate reductase (Aynr1p) and nitrite reductase (Ayni1p) in presence of NADPH and FAD as coenzymes (Fig. 21.2 – Böer et al. 2009a).

21.4 Biotechnological Applications

21.4.1 A. adeninivorans as a Gene Donor

A. adeninivorans has, because of its broad substrate range, a multitude of degradative pathways. Some of these are partially unknown, for example, pathways for the degradation of tannic acid and purine. For analysis of these pathways, genes such as *GAA, ALIP1, ATAN1, AINV* and *ATAL* were isolated from gene libraries containing either cDNA or chromosomal DNA from *A. adeninivorans* LS3 by PCR amplification using specific consensus primer sequences (Böer et al. 2004a, b, 2005a, b; El Fiki et al. 2007; Kaur et al. 2007; Kunze and Kunze 1996; Rösel and Kunze 1995, 1996; Steinborn et al. 2005; Stoltenburg et al. 1999; Wartmann et al. 2001, 2002a, 2003a).

The *GAA* gene encoding a thermostable glucoamylase was identified from a cDNA library using an anti-glucoamylase antibody as probe for product detection.

When heterologously expressed in *S. cerevisiae* and *Kluyveromyces lactis* more than 90% of the synthesized glucoamylase was found to be secreted. The level of secreted enzyme was 20 times higher in *Kl. lactis* than that observed in *S. cerevisiae* transformants using a similar construct for transformation (Bui et al. 1996a, b).

In addition, a number of biotechnologically important *Arxula* secretory enzymes were produced as recombinant proteins in *A. adeninivorans*. An example is the temperature-sensitive lipase Alip1p that hydrolyses ester bonds in triglycerides (optimum pH of 7.5). Fatty acids with middle-sized chains are more efficiently hydrolysed than those with short or long chains, with the highest activity occurring with the C8/C10 fatty acid esters *p*NP-caprylate, *p*NP-caprate and tricaprylin (Böer et al. 2005a).

The use of tannins such as tannic acid by *A. adeninivorans* as carbon source requires ester and depside bonds to be hydrolysed by a secreted tannase, Atan1p, to yield gallic acid and glucose. The enzyme has a temperature optimum at 35–40°C and a pH optimum at ~6.0 and is able to remove gallic acid from both condensed and hydrolysable tannins. Under inducing conditions, the wild-type strain LS3 secreted amounts of tannase equivalent to 100 U l^{-1}. To improve this level, transformant strains were constructed containing the strong, constitutively active *A. adeninivorans TEF1* promoter which over expresses the *ATAN1* gene to increase levels of tannase up to 400 U l^{-1} when grown in a glucose medium in shake flasks (Böer et al. 2009b).

The *AINV* gene provides another example of an interesting enzyme-encoding gene. The encoded invertase preferentially hydrolyzes ß-D-fructofuranosides and could be applied to the hydrolysis of sugar cane molasses or sugar beet molasses on an industrial scale. *AINV* was obtained by screening a cDNA and a chromosomal library with PCR amplification that corresponded to the gene segment. The isolated gene was fused to the strong constitutive *TEF1* promoter and expressed in transgenic *A. adeninivorans* strains. The resulting transformants were found to secrete the enzyme in high concentrations independent of the carbon source used for cultivation (Böer et al. 2004b).

A further example is the *ATAL* gene encoding a temperature-sensitive transaldolase with an acidic pH optimum. The preferred substrates for the enzyme include D-erythrose-4-phosphate and D-fructose-6-phosphate. Based on these properties, the enzyme could be applied to C-C bonding and enantio-specific synthesis of novel sugars, as previously demonstrated for the *S. cerevisiae*-derived transaldolase (El Fiki et al. 2007).

21.4.2 The A. adeninivorans-Based Transformation/ Expression Platform

A. adeninivorans provides an attractive gene transformation/expression platform based on the two different plasmid types; Xplor®1 and Xplor®2. These have multicloning sites for integration of modules such as selection, expression, rDNA, ARS and chaperone modules. Xplor®1 comprises *E. coli* and yeast derived sequences that

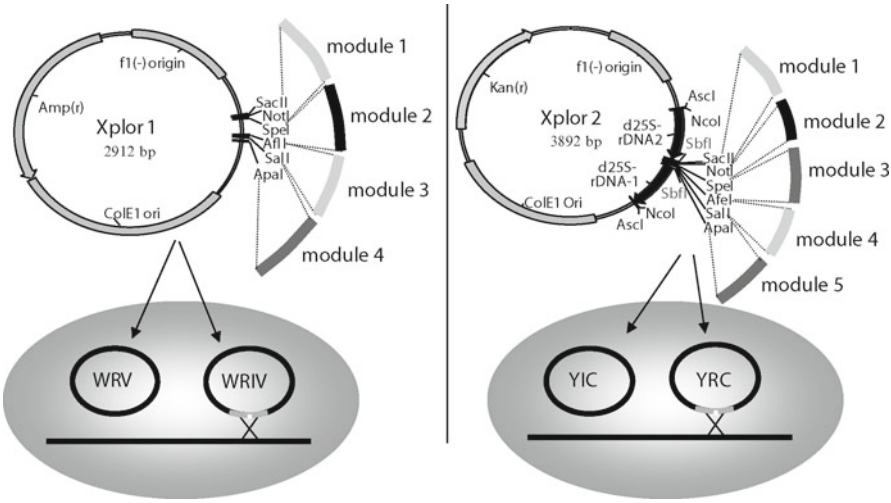

Fig. 21.3 Design, functional components and transformation strategy of the Xplor®1 and Xplor®2 basic vectors. Xplor®1 contains all *E. coli* elements for propagation in *E. coli* and a multicloning site (*MCS*) for integration of up to four ARS, rDNA, selection marker, expression and chaperone modules, flanked by appropriate restriction sites for accommodation in the MCS. Xplor®2 comprises bacterial sequence elements necessary for propagation in the *E. coli* system, together with a bipartite 25S rDNA target sequence which is interrupted by a MCS that permits the insertion of up to five modules

are used for transformation as Wide Range Vector (WRV) in *A. adeninivorans* and other yeast species. Linearization within its rDNA targeting sequences and transformation as Wide Range Integrative Vector (WRIV) favours integration into the rDNA cluster in the *Arxula* genome by homologous recombination. Consequently, the use of Xplor®1 vectors results in integration of *E. coli* sequences including the resistance marker gene into the yeast genome (Fig. 21.3, Table 21.4 – Steinborn et al. 2006, 2007a; Böer et al. 2007a, b).

The ARS modules are flanked by *Sac*II/*Spe*I or *Afe*I/*Spe*I, selection marker modules by *Afe*I/*Sal*I, expression modules by *Sal*I/*Apa*I or *Sac*II/*Spe*I and chaperon modules by *Spe*I/*Afe*I restriction sites. The rDNA module in the Xplor®1 vector is flanked by *Spe*I/*Afe*I.

After insertion of the appropriate modules, the Xplor®1 plasmids were transformed in the circular form as wide range vector (WRV) or in the linearized form using a unique restriction site localized in the rDNA module as a wide range integrative vector (WRIV). The *E. coli* sequences of plasmids based on Xplor®2 are omitted by restriction with *Asc*I or *Sbf*I respectively and the modules are transformed in a linearized form with and without rDNA as Yeast rDNA Integrative Cassette (YRC) or Yeast Integrative Cassette (YIC).

The Xplor®2 system, which is resistance marker-free, was developed to increase the acceptability of the system. It is based on a vector backbone into which yeast

Table 21.4 ARS, rDNA, selection marker, expression and chaperone modules of the Xplor®1 and Xplor®2 vector system

Region/gene	Donor organism	Reference
ARS module flanked by *Sac*II/*Spe*I or *Afe*I/*Spe*I		
• HARS	*H. polymorpha*	Kang and Gellissen (2005)
• SwARS	*Schw. occidentalis*	Piontek et al. (1998)
rDNA module flange by *Spe*I/*Afe*I (only for Xplor®1)		
• NTS2-ETS-18SrDNA-ITS1	*H. polymorpha*	Klabunde et al. (2003)
• 25S rDNA	*A. adeninivorans*	Rösel and Kunze (1998)
• 18S rDNA	*A. adeninivorans*	Steinborn et al. (2005)
• ITS-5 S-ETS-18S-ITS-5,8S-ITS	*A. adeninivorans*	Steinborn et al. (2005)
• NTS2-ETS-18SrDNA-ITS1	*A. adeninivorans*	Steinborn et al. (2005)
Selection marker module flanked by *Afe*I/*Sal*I		
• *ALEU2*	*A. adeninivorans*	Wartmann et al. (2003a)
• *ATRP1*	*A. adeninivorans*	Steinborn et al. (2006)
• *AILV1*	*A. adeninivorans*	Wartmann et al. (1998)
• *ALYS2*	*A. adeninivorans*	Kunze and Kunze (1996)
Expression modules with *A. adeninivorans* promoter and *S. cerevisiae PHO5* or *Asp. nidulans trpC* terminat flanked by *Sal*I/*Apa*I or *Sac*II/*Spe*I		
• *TEF1* promoter– *PHO5* terminator	*A. adeninivorans*	Wartmann et al. (2002b)
• *AHSB4* promoter– *PHO5* terminator	*A. adeninivorans*	Wartmann et al. (2003b)
• *GAA* promoter– *PHO5* terminator	*A. adeninivorans*	Bui et al. (1996a)
• *GAA* promoter - *trpC* terminator	*A. adeninivorans*	Wartmann and Kunze (2000)
• *ALIP* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2005a)
• *AINV* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2004b)
• *AXDH* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2005a, b)
• *ATAN1* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2009b)
• *AYNT1* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2009a)
• *AYNI1* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2009a)
• *AYNR1* promoter– *PHO5* terminator	*A. adeninivorans*	Böer et al. (2009a)
Chaperone modules with *A. adeninivorans AHSB4* promoter and *S. cerevisiae PHO5* terminator flanked by *Spe*I/*Afe*I		
• Calnexin (*AHSB4 promoter – CNE1 gene – PHO5* terminator)	*H. polymorpha*	Böer et al. (2009c)

modules such as selection, expression, replication or chaperone modules can be inserted. The multicloning site is located between two *Arxula* 25S rDNA segments which are arranged in the same orientation but in opposite order to the genomic rDNA. After insertion of the selected modules, the *E. coli* portion of the plasmid can be removed by restriction with *Asc*I or *Sbf*I. Furthermore, the choice of restriction enzyme determines whether or not the linearized fragment is targeted to the rDNA (*Sbf*I) or not (*Asc*I), allowing one to obtain a Yeast rDNA Integrative Cassette (YRC) or Yeast Integrative Cassette (YIC) (Fig. 21.3 – Böer et al. 2009c).

Table 21.5 *A. adeninivorans h*ost strains for the Xplor1® and Xplor2® transformation/expression systems

Yeast strain	Genetic markers	Reference
• LS3	Wildtype	Kunze and Kunze (1994a)
• G704	*cys1-9 lys2-41*	Rösel and Kunze (1998)
• G1342	*lys5-38 ilv1-2*	Wartmann et al. (1998)
• G1212	*aleu2 atrp1::ALEU2*	Steinborn et al. (2007b)

Using dominant and auxotrophic selection markers makes transformation possible in *A. adeninivorans* wild type and auxotrophic strains. As well as the dominant *hph* selection marker conferring hygromycin B resistance, the *AILV1, ALEU2* and *ATRP1* genes were isolated and the corresponding auxotrophic strains selected. The Xplor®1 and Xplor®2 plasmids with the respective modules were transferred into *A. adeninivorans ailv1, aleu2* or *atrp1* hosts which were then selected after nitrosoguanidine mutagenesis (*ailv1, aleu2*) or deletion by gene disruption (*atrp1*). The yeast transformants that complemented the respective auxotrophic mutation contained plasmid copies stably integrated, usually in the ribosomal DNA. For multicopy integration in *A. adeninivorans,* the selection marker consists of the *ATRP1* coding sequence under control of a truncated *ALEU2* promoter of 53 bp. After transformation with YRC´s containing this selection marker, 25rDNA targeting sequences and up to three expression cassettes, eight or more copies of the vector were found to be integrated in the genome of the recombinant strains (Table 21.5 – Wartmann et al. 1998, 2002b, 2003a; Steinborn et al. 2007b).

21.5 Heterologous Gene Expression

The construction of expression plasmids follows a two-step cloning strategy. First, the heterologous genes are inserted between an *A. adeninivorans*-derived promoter and fungal terminator elements such as *PHO5* from *S. cerevisiae* and *trpC* from *Aspergillus nidulans*. The resulting expression cassettes (*A. adeninivorans* promoter – heterologous gene - fungal terminator) are then integrated into an *A. adeninivorans* expression plasmid (Table 21.5 – Böer et al. 2009c).

An increasing number of heterologous genes have been expressed in *A. adeninivorans*. Examples are the *XylE* gene from *Pseudomonas putida* encoding catechol-2, 3-dioxygenase, and the *Green Fluorescent Protein* (*GFP*) and *Human Serum Albumin* (*HSA*) genes, encoding green fluorescent protein and human serum albumin respectively, have been expressed in both wild type and mutant *A. adeninivorans* strains. The recombinant strains contained 1–2 copies of the heterologous DNA integrated in 25S rDNA region. In the case of *GFP* expression, the recombinant protein was localized in the cytoplasm rendering the cells fluorescent. In contrast *HSA* expression was based on an ORF including the native 5′-signal sequence and resulted in more than 95% of the recombinant HSA being secreted into the culture medium. In fermentation trials of a single-copy transformant in a 200 ml

shake flask, maximal HSA product levels of 50 mg l^{-1} were observed after 96 h of cultivation. Budding cells and mycelia secreted similar levels demonstrating, in this case, morphology-independent production (Wartmann et al. 2002a, 2003a, b).

Because *A. adeninivorans* is a dimorphic yeast, recombinant proteins can be produced in cells of different morphologies. A *MFα1-IL6* fusion was expressed under control of the strong *TEF1* promoter in *A. adeninivorans* budding cells and mycelia. In cultivation at shaking flask scale, a productivity of ca. 210 mg l^{-1} was observed in budding cell cultures and 145 mg l^{-1} in mycelial cultures (Böer et al. 2007b). In contrast to other yeast species (*Saccharomyces cerevisiae, Hansenula polymorpha*) the recombinant interleukin-6 (IL-6) was correctly processed from the MFα1-IL6 precursor and more than 95% was exported to the culture medium.

New metabolic pathways were established in *A. adeninivorans* by simultaneously introducing several genes. For example, the genes *phb*A, *phb*B and *phb*C of the polyhydroxyalkanoate (PHA) biosynthetic pathway of *Ralstonia eutropha* encoding ß-ketothiolase, NADPH-linked acetoacetyl-CoA reductase and PHA synthase were introduced to construct a recombinant biocatalyst. *A. adeninivorans* strains initially transformed with the PHA synthase gene (*phb*C) plasmids were able to produce PHA. However, the maximal content of the polymer detected in these strains was just 0.003% (w/w) poly-3-hydroxybutyrate (PHB) and 0.112% (w/w) poly-3-hydroxyvalerate (PHV). The expression of all three genes (*phb*A, *phb*B, *phb*C) resulted in a small increase in the PHA content. Under controlled conditions, however, using a minimal medium and ethanol as the carbon source for cultivation, the recombinant yeast containing all three *phb* genes was able to accumulate up to 2.2% (w/w) PHV and 0.019% (w/w) PHB (Terentiev et al. 2004).

The *A. adeninivorans* transformation/expression system can also be used for promoter assessment. The expression cassettes described above containing the *GAA, AINV* and *ATAL* promoters in a reporter gene – *PHO5* terminator construct, were investigated with *lacZ* from *E. coli, GFP* from *Aequorea victoria, phyK* from *Klebsiella* species and *XylE* from *Ps. putida* employed as reporter genes. The characteristics of the selected promoters were assessed for effect on cell characteristics such as carbon source preference, osmotolerance and morphological state (Hahn et al. 2006; Böer et al. 2004a, b; El Fiki et al. 2007).

Another example of the application of recombinant strains is a novel estrogen biosensor. Recombinant *A. adeninivorans* strains were engineered to co-express the human estrogen receptor α (hERα) and a *Klebsiella*-derived phytase (*phyK*) reporter gene under control of an *A. adeninivorans*-derived glucoamylase (*GAA*) promoter modified by insertion of estrogen-responsive elements (EREs). In response to the presence of estrogenic compounds, two estrogen-hERα complexes dimerize and bind to estrogen-responsive elements (ERE) within the promoter to induce the expression of the reporter gene. The insertion of different numbers of EREs in three alternative positions within the promoter and its effect on reporter gene expression were assessed. In one construct, a detection limit of <2 ng l^{-1} and a quantification limit of 3 ng l^{-1} for 17β-estradiol equivalent activity was achieved. A convenient photometric assay enables estrogen monitoring in sewage samples within 20 h (Table 21.6 – Hahn et al. 2006; Kaiser et al. 2010).

Table 21.6 Examples of recombinant protein production in *A. adeninivorans* by shaking flask cultivation

Gene	Gene product	Donor organism	Vector type	Promoter	Recombinant protein level	Reference
HSA	Human serum albumin	*Homo sapiens*	Xplor1®	*TEF1*	50 mg l^{-1}	Wartmann et al. (2002b, 2003a)
HSA	Human serum albumin	*Homo sapiens*	Xplor1®	*AHSB4*	50 mg l^{-1}	Wartmann et al. (2003b)
AINV	Invertase	*A. adeninivorans*	Xplor1®	*TEF1*	500 µkat l^{-1}	Böer et al. (2004b)
phbA	ß-ketothiolase	*R. eutropha*	Xplor1®	*TEF1*	2.2% PHA[a]a	Terentiev et al. (2004)
phbB	Acetoacetyl CoA reductase	*R. eutropha*	Xplor1®	*TEF1*	2.2% PHA[a]	Terentiev et al. (2004)
phbC	PHA synthase	*R. eutropha*	Xplor1®	*TEF1*	2.2% PHA[a]	Terentiev et al. (2004)
AXDH	Xylitol dehydrogenase	*A. adeninivorans*	Xplor1®	*TEF1*	900 mkat l^{-1}	Böer et al. (2005b)
ALIP1	Lipase	*A. adeninivorans*	Xplor1®	*TEF1*	3,300 U l^{-1}	Böer et al. (2005a)
phyK	Extracellular phytase	*Klebsiella* sp.ASR1	Xplor1®	*GAA*	75 mkat l^{-1}	Hahn et al. (2006)
amyA	α-amylase	*B. amyloliquefaciens*	Xplor1®	*TEF1*	300 µkat l^{-1}	Steinborn et al. (2007a)
APHO1	Acid phosphatase	*A. adeninivorans*	Xplor1®	*TEF1*	70 mkat l^{-1}	Kaur et al. (2007)
ATAL	Transaldolase	*A. adeninivorans*	Xplor1®	*TEF1*	35 mkat l^{-1}	El Fiki et al. (2007)
IL6	Interleucin-6	*Homo sapiens*	Xplor1®	*TEF1*	220 mg l^{-1} (b.c.) 145 mg l^{-1} (m.)	Böer et al. (2007b)
ATAN1	Tannase	*A. adeninivorans*	Xplor2®	*TEF1*	400 U l^{-1}	Böer et al. (2009b)
IFNα2a	Interferone α	*A. adeninivorans*	Xplor2®	*TEF1*	27 mg l^{-1}	Böer et al. (2009c)
phyK	Extracellular phytase	*Klebsiella* sp.ASR1	Xplor2®	*TEF1*	250 mkat l^{-1}	Böer et al. (2009c)
phyK	Extracellular phytase	*Klebsiella* sp.ASR1	Xplor2®	*AYNI1*	210 mkat l^{-1}	Böer et al. (2009a)
PPHY	Extracellular phytase	*Pichia anomala*	Xplor2®	*TEF1*	220 U l^{-1}	Kaur et al. (2010)

n.d. not detected, *b.c.* budding cell culture, *m.* mycelial culture
[a]Percentage final product per dry weight

21.6 Conclusions and Future Perspectives

A. adeninivorans (syn. *Blastobotrys adeninivorans*) is a haploid, dimorphic, non-pathogenic, imperfect, ascomycetous, anamorphic, arthroconidial yeast. It is an attractive organism for both, basic and applied research. The broad range of substrates which can be used as carbon and/or nitrogen sources, the growth and secretion characteristics, the thermo-, osmo- and salt tolerance as well as the temperature-dependent dimorphism make this yeast an attractive organism for biotechnological application. *A. adeninivorans* is an interesting host for the synthesis of special products like recombinant glycosylated secreted proteins and a suitable biocatalyst for synthesis of biotechnological interesting products like PHB because all essential prerequisites and components for heterologous gene expression are available. In addition, these exceptional properties make *A. adeninivorans* a potential donor for genes underlying such properties to equip organisms used in current biotechnology with new attractive capabilities.

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Chapter 22
The Thermostable and Multi-functional Enzymes Catalyzing Carbohydrate Molecules Identified from Thermophilic Archaea

Yutaka Kawarabayasi

Abstract Thermophilic archaea are known to possess a number of thermostable proteins and enzymes. Therefore, we may find and characterize the potentially useful gene products from genomic data of thermophilic archaea. However, the useful information extracted from the genomic sequence is only estimated data. Thus, the attempts have been made to express the proteins from the genomic data of thermophilic archaea in *E. coli*. Among over 400 pET-based expression vectors constructed for expression of the genes from thermophilic archaea, *Sulfolobus tokodaii* strain7 and *Pyrococcus horikoshii* OT3, approximately 50% of *E. coli* harboring these vectors indicated to produce thermostable and soluble proteins. Among those successfully expressed, the proteins, activities of which are correlated with the carbohydrate metabolism, have been selected as the first target for analyzing their activities and functions. All enzymes analyzed showed the extreme thermostability, usage of the multiple substrate molecules and utilization of unusual metal cations as cofactor. Also a significant number of the novel enzymatic activities, which have not been previously found in any organism, were detected in *S. tokodaii* strain7. From these observations, it can be suggested that thermostable enzymes from thermophilic archaea retains ancestral features for the enzymes identified in higher organisms, and these have potential applications in both the industry and basic research.

Keywords Thermophilic archaea • Thermostable enzymes • Carbohydrate metabolic pathways • Functional genomics • Sugar-1-phosphate nucleotidylyltransferase • Amino-sugar-1-phosphate acetyltransferase

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22.1 Introduction

Carbohydrate molecules are used in constructing the outermost structures on a microorganism's cellar surface as component that separates the cellar inner and outer environment, and play important roles for the energy metabolism (e.g. TCA cycle), storage of energy (glycogen) and construction of heredity molecules as a part of DNA and RNA. Also carbohydrate molecule is modified the function of protein by binding to this molecule as polymer form (Udenfriend and Kodukula 1995).

As components of outermost structure of cell surface, peptideglycan contains modified form of carbohydrate (Niemetz et al. 1997; Kandler and König 1998). For obtaining energy, the metabolic pathway from glucose was well analyzed in the most model organism. In most organisms, including aerobic and anaerobic organisms, glucose is phosphorylated and degradated into pyruvate. In aerobic organisms, pyruvate is usually utilized in TCA cycle.

At present, determination of nucleotide sequence has become faster and easier than a decade ago. Especially next generation sequencer is able to produce over some tens of Giga bases-long data every week. The improvement of equipment for nucleotide sequencing makes it easier to obtain the entire genetic information from the genomic DNA of micro- and higher- organisms and the gene data from unknown microorganisms living in the environment through direct extraction and sequencing of DNA. In both cases, only estimated information is obtained, it is not the evidence of activities or functions. Also a lot of unknown genes, which is indicating no similarity with genes already deposited into the database, are detected in the entire genomic data of microorganisms. Even over some hundreds of entire microorganisms are determined, these unknown genes are continuously detected in the genomic data newly determined.

Therefore, two approaches are important for understanding the entire system of microorganism, the one is obtaining the evidence of estimated activities or functions by functional genomics and the other is direct obtaining the proteins for analyses of their actual activities or functions without using the similarity. Actually it is very difficult to obtain the trail for activities or functions of unknown genes, because of no effect for phenotype by gene disruption in every microorganism. It is also difficult to approach to their functions by more detailed analyses by informatics because of indicating no similarity to any other genes.

Because of difficultness in understanding the function from environmental samples or genomic data, the functionally predicted genes were selected as the first target of our functional genomics, especially those predicted as enzymes for carbohydrate metabolism. Because the carbohydrate and polysaccharide synthesis is not analyzed well in thermophilic archaea, these genes are selected as target to express in *E. coli* and functional analyses.

In this chapter, an attempt has been made to show the results of expression of genes identified from the genomic data of thermophilic archaea, examples of detailed analyses of their activities and functions, and to look at the possibility of finding novel metabolic pathways.

22.2 Expression of the Predicted Thermostable Genes in *E. coli*

22.2.1 Construction of Expression Vectors

The predicted genes or ORFs (Open Reading Frames) with annotation were chosen as the target to express in *E. coli*. The thermostable proteins even when expressed in *E. coli* usually retain their thermostability. Therefore, this is one of the big advantages for their purification step. By only treatment at high temperature, most *E. coli* proteins are denatured and change to insoluble form, but thermostable protein heterologously expressed in *E. coli* keeps their native form and remains in solution as soluble form. Thus, thermostable protein expressed in *E. coli* is easily purified by treatment at high temperature. After this heating step and following centrifuge, added tag at an N- or C-terminal of target protein utilized for its easy purification. The histidine tag was added at N- or C-terminal of the target proteins by designing of the appropriate primers for construction of the expression vectors. For this purpose the appropriate number of the pET vector was selected and the appropriately designed primers were synthesized and used for PCR amplification of entire gene (or ORF) region. For construction of the histidine tag containing protein, stop codon is removed from the primer designed from the sequence of C-terminal of target protein. Oppositely, if addition of histidine tag is not required, the stop codon is remained at the original position of the target protein in the PCR primer.

According to this strategy, over 400 independent expression vectors harboring different genes from *S. tokodaii* and *P. horikoshii* were constructed (Table 22.1). After evaluation of cloning of the DNA fragment with the correct nucleotide sequence by sequencing, these expression vector clones were introduced into the host *E. coli* strain cells.

Table 22.1 Summary of expression of thermostable protein in *E. coli*

Functional category	Soluble	Insoluble	ND	Total
Protein synthesis	32	13	9	54
Transcription	20	9	4	33
DNA metabolism	9	10	12	31
Energy metabolism	28	12	5	45
Purines, pyrimidines, nucleosides, and nucleotides	22	15	10	47
Cell envelope	17	5	4	26
Amino acid biosynthesis	5	18	11	34
Cellular processes	11	7	4	22
Other categories	8	4	11	23
Others	17	11	15	43
Conserved and hypothetical protein	32	8	22	62
Total	201	112	107	420
(%)	(48)	(27)	(25)	

ND not detected

22.2.2 Expression of the Thermostable Proteins in E. coli

For comparison of the codon usage (which codon is dominantly used within the codons for same amino acid) of *E. coli* and archaea, pattern of codon usages for some amino acid is strictly different between *E. coli* and archaea. This fact reveals that it is difficult for the protein containing the many number of codons rarely used in *E. coli* to heterologously express in *E. coli*. For expression of these proteins, the rare tRNA molecule supplying *E. coli* strain was constructed and purchased from company (e.g. BL21-CodonPlus(DE3)-RIL). When the archaeal thermostable protein is tried to express in *E. coli*, selection of the tRNA-supplying *E. coli* strain is necessary.

The *E. coli* harboring the expression vectors were grow up to 200 ml of LB liquid medium at 37°C and when OD_{600} becomes to 0.6, IPTG is added for induction of expression of introduced protein gene. After addition of IPTG, incubation at lower temperature (30°C or 25°C) is continued for overnight. After over production of heterologous expression, *E. coli* cells were collected and harvested by the most appropriate method. This crude extract was used for next analyses.

22.2.3 Summary of Expression of Thermostable Proteins in E. coli

As shown in the Table 22.1, over 400 pET-based expression vectors were constructed with genes encoding different proteins. Numbers of the constructed vectors are separately shown according to the functional category shown in the first column.

For estimation whether the expressed protein indicates soluble form or insoluble form, the clued extract is separated by centrifuge. The soluble protein is remaining in the solution (soluble fraction) and the insoluble protein is precipitated by centrifuge (inclusion body).

In this case if the expected thermostable proteins were successfully expressed in *E. coli*, the comparison between the supernatant (Fig. 22.1, lane 2) and precipitate (Fig. 22.1, lane 1) of the harvesting *E. coli* cells after centrifuge is performed. The supernatant was generally used for treatment at high temperature (temperature is depending on species of materials; at 80°C for *S. tokodaii* protein), then as the *E. coli* proteins are denatured into insoluble form, the protein soluble in the supernatant (Fig. 22.1, lane 4) after treatment at high temperature is the heterologously expressed protein derived from the target thermophiles in *E. coli*. The Fig. 22.1 shows the examples of comparison of these four different samples.

As shown in these observations, the success of expression is dependent on the conditions and the vector species used. However, it is very difficult to estimate which condition is optimum for some gene or protein. So trial and error is necessary for successful of expression of genes or proteins identified from thermophilic archaeal genomic data.

In the following section, the example of analyses of activities and functions has been described.

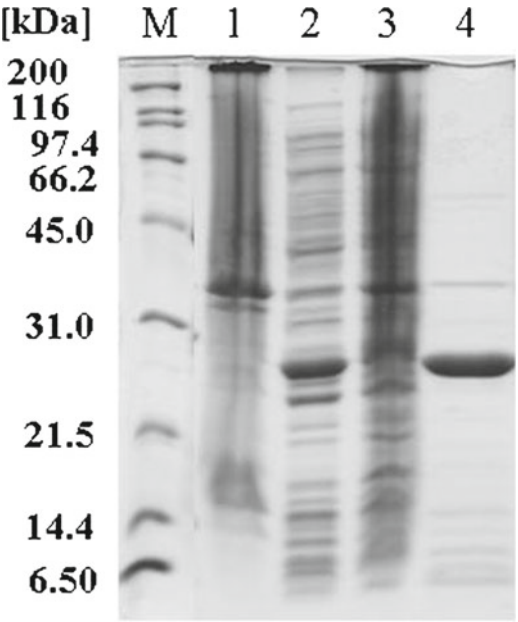

Fig. 22.1 SDS-PAGE analysis of the thermophilic protein in *E. coli*. The recombinant protein expressed in *E. coli* harboring the pET-derived expression vector was subjected to the 12% of polyacrylamaide gel containing 0.1% of SDS. *Lane 1*: the total insoluble fraction, *Lane 2*: the total soluble fraction, *Lane 3*: the insoluble fraction after treatment of the total soluble fraction at 80°C for 20 min, *Lane 4*: the soluble fraction after treatment of the total soluble fraction at 80°C for 20 min, *Lane M*: the molecular weight standard proteins. The proteins were visualized by Coomassie brilliant blue R-250 staining

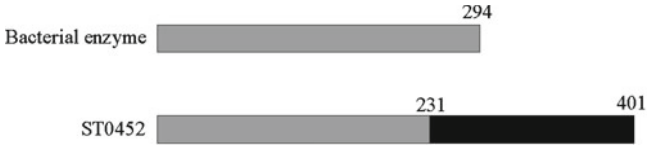

Fig. 22.2 HPLC elution profile of the products by ST0452 glucose-1-phosphate nucleotidylyltransferase activity. The HPLC elution profiles for the products incubated for the indicating period at 80°C with the ST0452 protein in the standard reaction solution. The scale is modified according to the amount of materials detected

22.3 Analyses of Activities

In the genome of *Sulfolobus tokodaii* strain7, several genes encoding proteins similar to the sugar-1-phophate nucleotidylyltransferases were detected. By comparison of these protein or gene sequences each other and versus database, the ST0452 was identified as that with longer C-terminal domain (Fig. 22.2) than the bacterial similar genes. For dissolving the role of this extra C-terminal region in the ST0452 protein, the soluble and thermostable ST0452 protein was used for the following activity and function analyses (Zhang et al. 2005).

22.3.1 Sugar-1-Phosphate Nucleotidylyltransferase Activities on the ST0452 Protein

At first the expected activity on the ST0452 protein by the sequence similarity, glucose-1-phosphate thymidylyltransferase activity, was analyzed. For evaluation of this activity, the reaction mixture containing 0.1 mM dTTP and 10 mM glucose-1-phosphate was used. Then, the reaction was progressed by incubation at 80°C, and produced nucleotide-sugar molecule was separated by HPLC and detected by absorbance at 254 nm. As shown in Fig. 22.3, before incubation or without the ST0452 protein (0 min) only dTTP substrate was detected. However, when the ST0452 protein was added, production of dTDP-glucose molecule according to the incubation period was detected (5 min to 15 min). This result indicated that the ST0452 protein possessed the activity for producing the dTDP-glucose from glucose-1-phosphate and dTTP.

Then, the optimum conditions for this activity were determined. The ST0452 protein indicates relatively high activity between 50 and 90°C and pH 4 and 9, with the maximum activity at 80°C and pH 7.5, as shown in Fig. 22.4. As shown in the Table 22.2, Co^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} can work as cofactors but Ca^{2+} inhibit the glucose-1-phosphate thymidylyltrasferase activity of the ST0452 protein.

As the next step of detailed recognition of the activities of the ST0452 protein, the substrate specificity was analyzed. The substrate specificity for the NTP and dNTP was determined on the glucose-1-phosphate nucleotidylyltransferase activity of the ST0452 protein. As shown in the Table 22.3, all dNTP and UTP were utilized as substrate on this activity. Unlikely to the glucose-1-phosphate thymidylyltransferase enzyme identified from bacteria, the ST0452 protein was not capable of utilizing glucosamine-1-phosphate as substrate, even though similarity between the ST0452 protein and *E. coli* RmlA indicated over 30% identity.

For the sugar-1-phosphate substrate on the sugar-1-phosphate nucleotidylyltransferase activity of the ST0452, *N*-acetyl-D-glucosamine-1-phosphate was capable of utilizing as substrate on this activity as well as glucose-1-phosphate. For this substrate, dTTP and UTP were acceptable as NTP substrates.

Moreover, *N*-acetyl-D-galactosamine-1-phosphate was capable of being utilized as substrate for the sugar-1-phosphate nucleotidylyltransferase activity of the ST0452 protein (Zhang et al. 2010). This activity was detected correlating with the acetyltransferase activity shown below. For detection of this nucleotidylyltransferase activity, the substrate, *N*-acetyl-D-galactosamine-1-phosphate, was not purchased from any company. Therefore, the coupling reaction with acetyltransferase activity on the same ST0452 protein was used for detection of this nucleotidylyltransferase activity. The reaction mixture containing galactosamine-1-phosphate, UTP, acetyl-CoA and the ST0452 protein was incubated at 80°C, the produced UDP-sugar molecule was detected as shown in the Fig. 22.5. The elution position of the peak was same for control UTP-*N*-acetylgalactosamine molecule, meaning that the ST0452 protein possessed the *N*-acetyl-D-galactosamine-1-phosphate uridyltransferase activity. These results indicated that the ST0452 protein is the first thermostable enzyme with both *N*-acetyl-D-glucosamine-1-phosphate and *N*-acetyl-D-galactosemine-1-phosphate nucleotidylyltransferase activities.

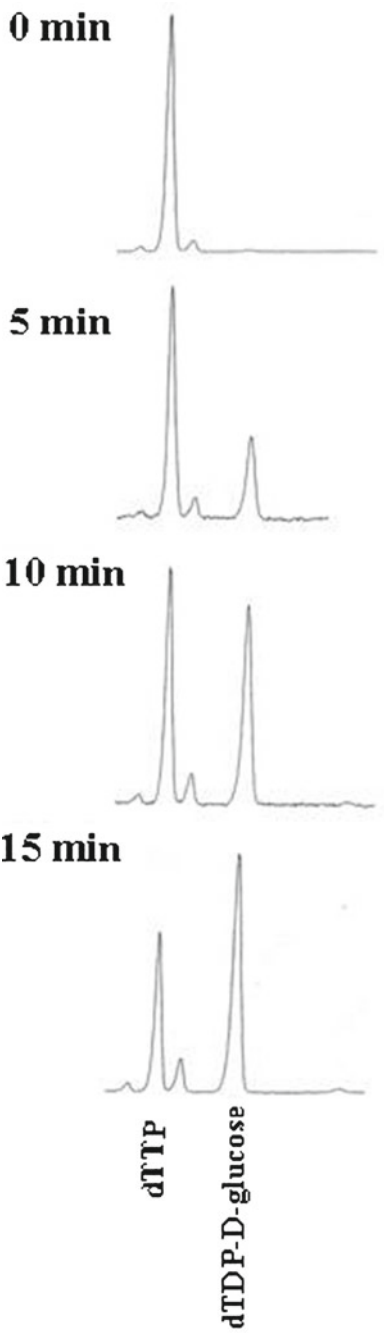

Fig. 22.3 Comparison of the structures of the ST0452 protein and bacterial similar enzyme. Hatched region indicates the similar domain between two proteins

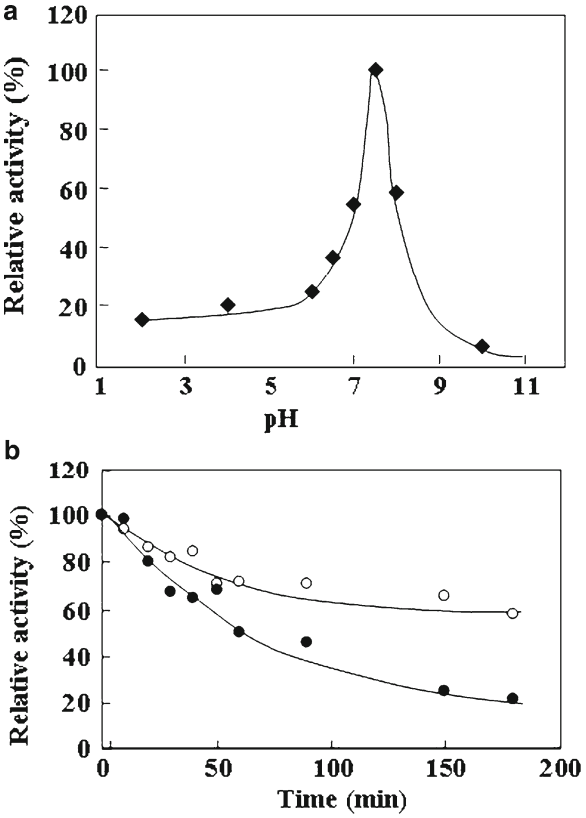

Fig. 22.4 Property of the glucose-1-phosphate nucleotidylyltransferase activity on the ST0452 protein. (**a**) The relative activity depending to the pH. The relative activity shown is expressed as a percentage of the maximum activity at pH 7.5. (**b**) The relative activity after treatment at the periods indicated at 80°C (○) and 95°C (●). The relative activity shown is expressed as a percentage of the activity without treatment at high temperature

Table 22.2 Glucose-1-phosphate thymidylyltransferase activities of the ST0452 protein in the presence of different metal ions

Metal ion	Relative activity (%)
Co^{2+}	100
Mn^{2+}	64
Mg^{2+}	47
Zn^{2+}	27
Ca^{2+}	ND
None	ND

The relative activity is shown as a percentage of the activity detected for Co^{2+}
ND not detected

22.3.2 Aminosugar-1-Phosphate Acetyltransferase Activities on the ST0452 Protein

On the long C-terminal region of the ST0452 protein, 24-times incomplete repeats of motif sequences, which were originally identified on the acetyl- or acyl-transferase (Raetz and Roderick 1995), succinyltransferase (Beaman et al. 1997) and carbonic

Table 22.3 Substrate specificity of the sugar-1-phosphate nucleotidylyltransferase activity of the ST0452 protein

Direction of reaction	Substrate A (0.1 mM)	Substrate B (10 mM)	Relative activity
Forward	dTTP	α-D-glucose-1-phosphate	100
	dATP		35
	dCTP		7
	dGTP		1
	UTP		130
	ATP/CTP/GTP		ND
	dTTP	N-acetyl-glucosamine-1-phosphate	320
		α-D-glucosamine-1-phosphate	ND
		α-D-Galactose-1-phosphate	ND
		α-D-mannose-1-phosphate	ND
	UTP	N-acetyl-glucosamine-1-phosphate	540
		α-D-glucosamine-1-phosphate	ND
		α-D-Galactose-1-phosphate	ND
		α-D-mannose-1-phosphate	ND

ND Not detected

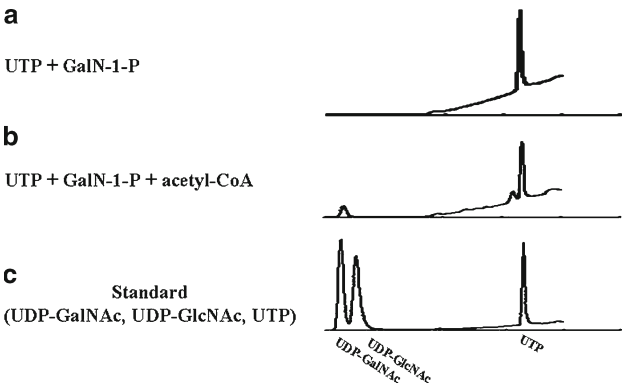

Fig. 22.5 HPLC elution profile of the standard molecules and product of the *N*-acetyl-D-galactosamine-1-phosphate uridyltransferase activity of the ST0452 protein. HPLC elution profiles of the standard UTP and UDP-acetyl-sugar and products on a CarboPac PA1 column. The reaction was progressed at 80°C for 10 min in the acetyltransferase reaction solution with 0.1 mM UTP and 2 mM GalN-1-P (**a**) and with 0.1 mM UTP, 2 mM acetyl-CoA, and 2 mM GalN-1-P (**b**). Elution profile of a mixture of the standard UTP, UDP-GlcNAc, and UDP-GalNAc (**c**)

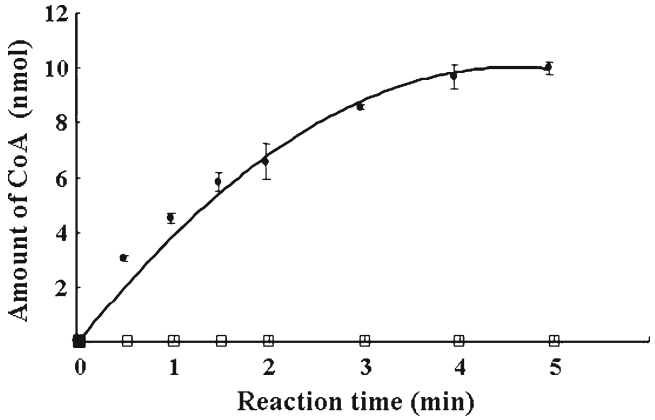

Fig. 22.6 Time course of CoA production by the ST0452 protein. Ellman's reaction was used to monitor CoA production. The amount of CoA was calculated from the absorbance measured at 412 nm according to the standard curve. The reaction progressed at 80°C in the presence of 2 mM Glucosamine-1-P (*closed circle*), or in the absence of the ST0452 protein (*open square*)

anhydrase enzymes (Kisker et al. 1996), were detected (Zhang et al. 2010). From this finding, it was expected that the ST0452 protein possessed an acetyltransferase activity. Thus, the acetyltransferase activity on the ST0452 protein was analyzed. The reaction mixture containing glucosamine-1-phosphate and acetyl-CoA was used for this analysis. In this reaction system, CoA is produced from acetyl-CoA according to the progression of reaction, thus increase of absorbance at 412 nm by DTNB solution (Riddles et al. 1983) means progression of this reaction. Before incubation or reaction without enzyme, increase of absorbance is not detected (Fig. 22.6. open squares). However, increase of absorbance was detected when the ST0452 protein was added in the reaction solution (Fig. 22.6, closed circles). This observation indicates only that acetyl group was released from acetyl-CoA. To recognize this reaction, identification of product was required. Direct detection of the expected product, *N*-acetyl-D-glucosamine-1-phosphate which was produced from glucosamine-1-phosphate and acetyl-CoA, was not performed, because the appropriate detector required for phosphor-sugar molecules was not available in my laboratory. Therefore, the coupling reaction was designed for detection of product.

The sugar-1-phosphate nucleotidylyltransferase activity on the ST0452 protein can utilize glucose-1-phosphate and *N*-acetyl-D-glucosamine-1-phosphate as sugar-1-phosphate substrate, but cannot accept the glucosamine-1-phosphate molecule as substrate (Zhang et al. 2005). This activity of the ST0452 protein can produce UDP-*N*-acetylglucosamine when acetyl group is transferred from acetyl-CoA to glucosamine-1- phosphate and UTP is included in the reaction solution.

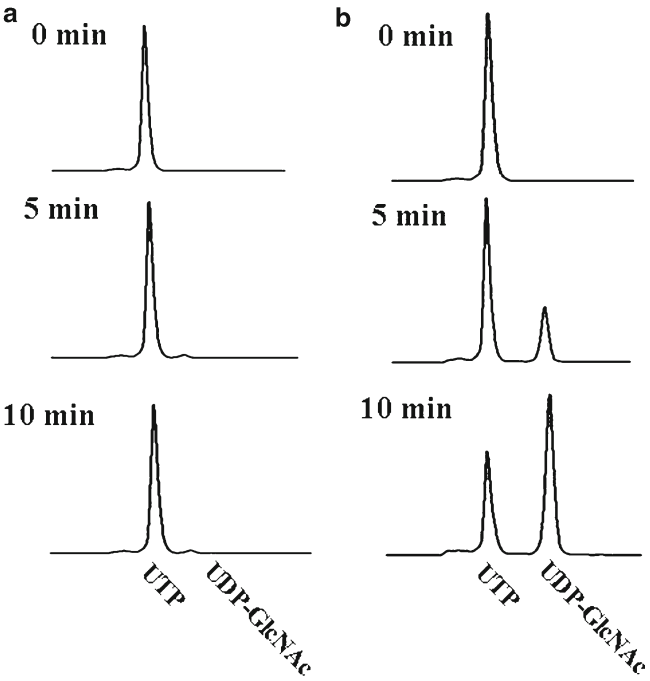

Fig. 22.7 HPLC elution profile of the product of the coupling reaction of the ST0452 protein. HPLC elution profiles of the products on a Wakosil 5C18-200 column. The reaction was progressed at 80°C for indicating period in the acetyltransferase reaction solution with 0.1 mM UTP and 2 mM *N*-acetyl-D-glucosamine1-phosphate (**a**) and with 0.1 mM UTP, 2 mM acetyl-CoA, and 2 mM *N*-acetyl-D-glucosamine-1-phosphate (**b**). The elution positions of standard UTP and UDP-*N*-acetylglucosamine (UDP-GlcNAc) are shown. The scale is automatically modified by HPLC according to the amount of materials detected

If enzyme or acetyl-CoA is not added into the reaction mixture, UDP-*N*-acetylglucosamine was not detected (Fig. 22.7a). However, when acetyl-CoA was added into the reaction mixture, the production of the final product, UDP-*N*-acetylglucosamine, was detected (Fig. 22.7b). It means that the acetyl group released from the acetyl-CoA was combined with the glucosamine-1-phosphate to produce *N*-acetyl-D-glucosamine-1-phosphate.

As shown in the Fig. 22.5, the ST0452 protein is capable of acetylating the galactosamine-1-phosphate molecule. It is the first identification of this activity from any organisms (Zhang et al. 2010). It is very important that both novel galactosamine-1-phosphate acetyltransferase and *N*-acetyl-D-galactosamine-1-phosphate uridyltransferase activities were identified on the ST0452 protein. It means that the ST0452 protein has a strong potential for industrial application.

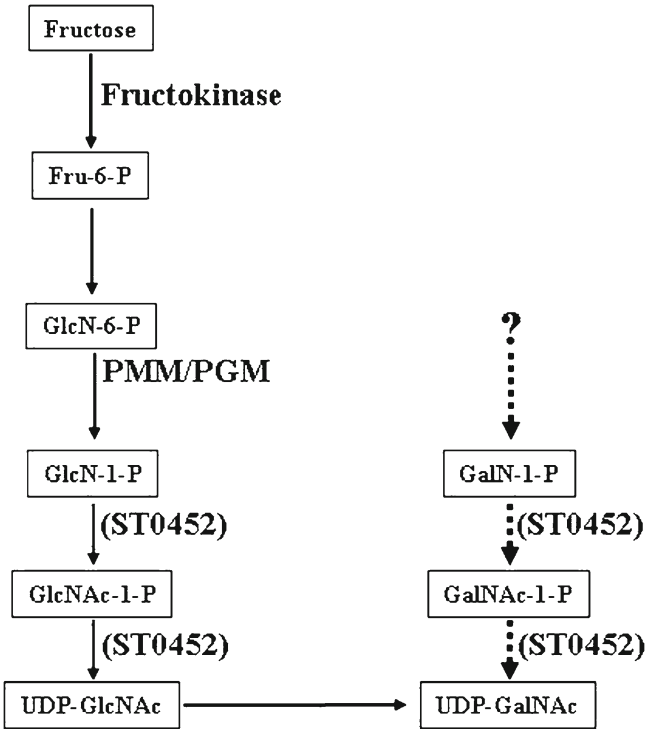

Fig. 22.8 Predicted metabolic pathway for acetyl-aminosugar nucleotides in *S. tokodaii*. Solid lines indicate the already known pathways identified in other organisms, and hatched lines indicate the metabolic pathways predicted from this work. The question mark indicates the unknown pathway not predicted by this work yet

22.3.3 Prediction of Novel Metabolic Pathway from ST0452 Activities

As shown above, the ST0452 protein possesses the sugar-1-phosphate nucleotidylyltransferase and aminosugar-1-phosphate acetyltransferase activities. For sugar-1-phosphate nucleotidylyltransferase activity, novel *N*-acetyl-D-galactosamine-1-phosphate uridyltransferase activity was identified on the ST0452 protein as well as glucose-1-phosphate thymidylyltransferase and *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activities. Also for aminosugar-1-phosphate acetyltransferase activity, novel galactosamine-1-phosphate acetyltransferase activity was identified on the ST0452 protein as well as glucosamine-1-phosphate acetyltransferase activity. These observations predict that the novel pathway to construct the UDP-*N*-acetylgalactosamine from galactosamine-1-phosphate is present in this microorganism as shown in the Fig. 22.8.

Previous analyses indicated that UDP-*N*-acetylgalactosamine was produced only from UDP-*N*-acetylglucosamine by the UDP-*N*-acetylglucosamine 4-epimerase in

Table 22.4 Kinetic properties for the *N*-acetylglucosamine-1-phosphate uridyltransferase activity of the ST0452 protein

Substrate	*Km* (μM)	*kcat* (S^{-1})	*kcat/Km* (μM^{-1} S^{-1})
ST0452 protein			
UTP	1.73 ± 0.35	3.53 ± 0.24	2.12 ± 0.51
GlcNAc-1-P	8.04 ± 0.83	2.54 ± 0.22	0.32 ± 0.043
E.coli enzyme			
UTP	17	24	1.4
GlcNAc-1-P	18	21	1.2

The kinetic parameters for *E. coli* enzyme is according to the results described by Gehring et al. (1996)

bacteria and eukarya (Bernatchez et al. 2005; Bourgeaux et al. 2005; Creuzenet et al. 2000; Guo et al. 2006; Pastuszak et al. 1996; Thoden and Holden 2005). However, no gene similar to the UDP-*N*-acetylglucosamine 4-epimerase was detected within the genomic data of *S. tokodaii*. These observations indicate that the UDP-*N*-acetylgalactosamine biosynthesis pathway summarized in the Fig. 22.8 is the novel and sole pathway in this microorganism.

As shown in this chapter, analysis of each gene activity or function should provide great information for metabolic pathway. Especially, for microorganisms for which genomic data is available, this approach is the powerful tool to recognize the actual metabolic pathway.

22.3.4 Activation of Useful Activity by the Targeted Mutagenesis

On the sugar-1-phosphate nucleotidylyltransferase activity of the ST0452 protein, *Km* values for each substrate, *N*-acetyl-D-glucosamine-1-phosphate and UTP, are smaller than that of the activity on the *E. coli* similar enzymes (Table 22.4). This observation indicates that the ST0452 enzyme can utilize ten-times lower concentrate substrate than that in bacterial enzyme. However, *kcat* value of the sugar-1-phosphate nucleotidylyl transferase activity on the ST0452 protein is approximately seven-times lower than that of the *E. coli* enzyme.

This observation indicates that turnover of the reaction progresses slower than that of the *E. coli* similar enzyme. For industrial application of this enzyme, enzyme with the more higher *kcat* is appropriate, thus construction of the mutant enzyme remaining its absolute thermostability and with more higher *kcat* value was performed (Zhang et al. 2007).

The reaction center is located within the pocket structure, therefore, it was expected that conversion of the amino acid residues located within the reaction center should not affect on the overall structure and thermostability of the protein. Also conversion of the amino acid residues within reaction center may affect on the

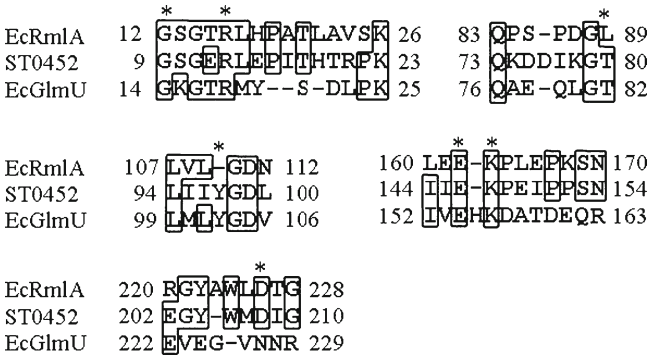

Fig. 22.9 Sequence alignment of the five highly conserved domains among the ST0452 protein and *E.coli* Glcose-1-phosphate thymidylyltransferase and *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase. EcRmlA and EcGlmU indicate the Glcose-1-phosphate thymidylyltransferase from *E. coli* (GenBank accession number P37744) and *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase from *E. coli* (NC_000913). The letters within boxes indicate the residues conserved within three proteins. The amino acid residues chosen for the construction of the mutant proteins are indicated by asterisks. The numerals indicate the coordinates of the two ends of each domain from the N-terminus of each protein

reaction parameters. Therefore, introduction of the targeted mutations were performed on the ST0452 protein for increasing its *kcat* value without decreasing its thermostability.

For this purpose, seven mutant ST0452 proteins which contain the amino acid conversion to alanine listed in the Fig. 22.9 were constructed. The mutant ST0452 proteins were expressed in *E. coli* and the soluble fraction containing the expressed mutant ST0452 protein were treated at 80°C for 20 min. All mutant ST0452 proteins were remained in the soluble fraction after treatment at high temperature. The observation indicates that thermostability of all mutant ST0452 proteins are not changed by introduction of the conversion of amino acid.

As all mutant ST0452 proteins retained their thermostability, the activities of all these mutant ST0452 proteins were analyzed. *Km* and *kcat* values for all mutant ST0452 proteins indicate that all mutant proteins exhibited the larger *Km* values than that of the wild-type ST0452 protein. It means that binding with substrates of these mutant ST0452 proteins becomes weaker than that of the wild-type ST0452 protein. However, unfortunately some number of mutant ST0452 proteins indicated the higher *kcat* values than that of the wild-type ST0452 protein. This observation reveals that some mutant proteins can turnover substrate faster than that of the wild-type ST0452 protein.

After optimization of reaction condition of these mutant ST0452 proteins, 20 and 200 times higher concentration of UTP and *N*-acetyl-D-glucosamine-1-phosphate substrates, optimize their *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activity and five mutant proteins indicate the higher relative activity than the wild-type ST0452 protein (Fig. 22.10). Also different kinds of cations affect on the maximum activity of the mutant protein. As shown in Fig. 22.11, when Zn^{2+} or Co^{2+} is added into the reaction solution, the activities detected are higher than that of the

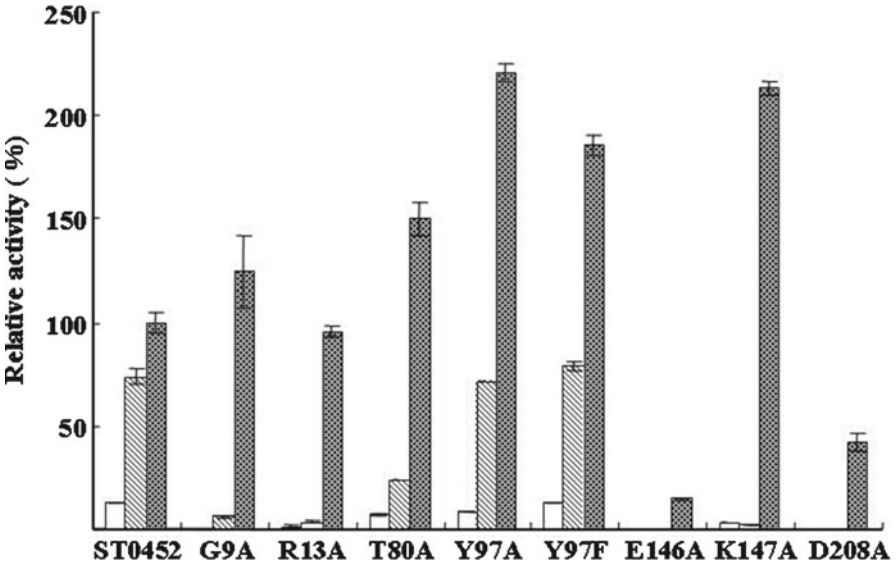

Fig. 22.10 *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activity of the mutant ST0452 proteins under three different conditions. *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activities of each mutant protein were measured in the reaction solution with 5 μM UTP plus 50 μM *N*-acetyl-D-glucosamine-1-phosphate (*open bars*), 100 μM UTP plus 50 μM *N*-acetyl-D-glucosamine-1-phosphate (*hatched bars*) and 100 μM UTP plus 10 mM *N*-acetyl-D-glucosamine-1-phosphate (*double hatched bars*). The relative activity is expressed as a percentage of the activity detected on the wild-type ST0452 protein under the condition containing 100 μM UTP plus 10 mM *N*-acetyl-D-glucosamine-1-phosphate

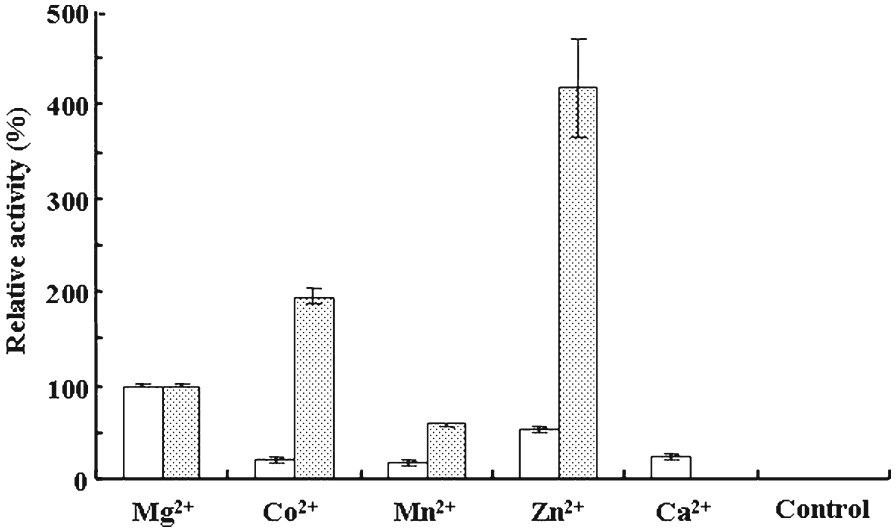

Fig. 22.11 *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activity of one mutant protein in the presence of different metal ions. The *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activities of the wild-type ST0452 protein (*open bars*) and the mutant protein (*hatched bars*) were measured in the reaction solution with 100 μM UTP, 1 mM *N*-acetyl-D-glucosamine-1-phosphate, 0.1 mM EDTA and 2 mM of the corresponding metal ion. The relative activity is expressed as a percentage of the activity detected on the wild-type ST0452 protein when Mg^{2+} was used

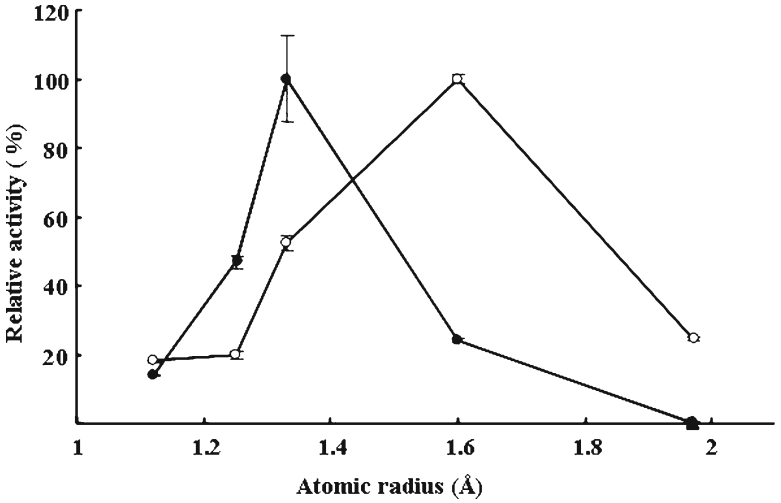

Fig. 22.12 Correlation between the atomic radius of metal ions and *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activities of the wild-type ST0452 and one mutant protein. The relative activities of the *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activities on the wild-type ST0452 protein (*open circles*) and the mutant protein (*black circles*) in the presence of different metal ions are shown according to the atomic radius. Relative activity is expressed as a percentage of the maximum activity when the most effective metal ions were used

wild-type ST0452 protein. This observation reveals that when the reaction is progressing, this mutation changed the optimum radius of the metal ions from that for the wild-type ST0452 protein (Fig. 22.12).

Any way introduction of mutation into the ST0452 protein improved its *N*-acetyl-D-glucosamine-1-phosphate uridyltransferase activity. It is indicating that the introduction of targeted mutagenesis should be powerful tool for application of the thermostable enzymes identified from thermophilic archaea.

22.4 Conclusions

A part of metabolic pathway of carbohydrate molecules in thermophilic microorganisms was well characterized. However, the details for most of carbohydrate molecules and their metabolic pathways in thermophilic archaea are not well characterized. However, as shown in this chapter, the thermostable enzymes catalyzing carbohydrate molecules identified from thermophilic archaea possess unique, unexpected and useful features, e.g. ST0452 protein. These unique and unexpected or novel activities in the thermophilic microorganisms should have big potential to application in industry.

In addition to the cultivable microorganisms, a large number of uncultivable microorganisms are present in the environment, it is usually said that 99% of microorganisms in our planet is uncultivable. Some of these uncultivable microorganisms are living in the thermal environment, hydrothermal vent, hot springs and so on. It is expected that these uncultivable microorganisms also possess useful enzymes or proteins. However, it is very difficult to identify these molecules directly from these uncultivable microorganisms. So environmental genomics techniques, direct sequencing of DNA isolated from the environment, are thought to be powerful approach into the uncultivable microorganisms living in the environment. The genomic sequencing-based and environmental genomics-based identification of novel enzymes will provide useful and powerful resources to the human beings.

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Chapter 23
Structural Biology of the Ribonuclease P in the Hyperthermophilic Archaeon *Pyrococcus horikoshii* OT3

Makoto Kimura and Yoshimitsu Kakuta

Abstract *Pyrococcus horikoshii* OT3 is a hyperthermophilic archaeaon isolated from hydrothermal fluid. Because of their genetic features as well as hyperthermophilic properties, macromolecules produced by this thermophilic bacterium have served as an excellent model for structural biology. Ribonuclease P (RNase P) is a ribonucleoprotein complex involved in the processing of the 5′-leader sequence of precursor tRNA (pre-tRNA). We found that RNase P RNA (*Pho*pRNA) and five proteins in *P. horikoshii* OT3 reconstituted RNase P activity that exhibited enzymatic properties like those of the authentic enzyme. A mutational analysis indicated that nucleotides A40, A41, and U44 in *Pho*pRNA are crucial for catalysis, strongly suggesting that *Pho*pRNA catalyzes the hydrolysis of pre-tRNA in approximately the same manner as eubacterial RNase P RNAs, even though it has no enzymatic activity in the absence of the proteins. The *P. horikoshii* RNase P proteins are predominantly involved in optimization of *Pho*pRNA's conformation, though individually they are dispensable for RNase P activity *in vitro*. This chapter summarizes structure-function relationships of the *P. horikoshii* RNase P subunits, including the high-resolution structural information that is currently available for the protein subunits.

Keywords Archaea • *Pyrococcus horikoshii* • Ribonucleoprotein • Ribozyme • RNase P • Three-dimensional structure • X-ray crystallography

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Abbreviations

C5 protein	*E. coli* RNase P protein
CD	circular dichroism
EM	electron microscopy
M1 RNA	*E. coli* RNase P RNA
PDB	protein data bank
*Pho*pRNA	RNase P RNA in *P. horikoshii*
pre-tRNA	precursor tRNA
RNase P	ribonuclease P
RNP	ribonucleoprotein
UV	ultraviolet.

23.1 Introduction

Archaea, the third phylogenetic kingdom, are bacteria living in extraordinary environments such as anaerobic, high-salt, high-temperature, and extremely acidic conditions (Woese and Fox 1977). It has been generally accepted that various components of the translation apparatus in Archaea share many sequence-related and functional features with eukaryotic counterparts (Kyrpides and Woese 1998). In addition, proteins from thermophilic organisms are more robust and crystallize better than those of mesophilic organisms. For example, Archaea possess all three subunit homologues of eukaryotic initiation factor 2 (eIF2), and three subunits in the hyperthermophilic archaeon *Pyrococcus abyssi* were overproduced in *Escherichia coli* cells, and their reconstitution to form a trimeric protein was reported (Schmitt et al. 2002). In addition, the crystal structure of the γ subunit of the *P. abyssi* IF2 was established: it displays significant similarities to elongation factors, such as EF-Tu (Schmitt et al. 2002). Thus, information on archaeal proteins will ultimately be of help in establishing the structural and functional relationships of corresponding macromolecules from Eukaryotes.

Pyrococcus horikoshii OT3, the first organism whose genome was analyzed at the Genome Analysis Center in Japan, is a hyperthermophilic archaeon isolated from hydrothermal fluid obtained in Okinawa Prefecture in 1992 by the manned deep-sea research vessel "Shinkai 2000" as part of the DeepStar project. It is an obligate anaerobe, growing at temperatures of between 88°C and 104°C with an optimal temperature of 98°C in the presence of sulfur, and possessing circular genomic DNA. The proteins and enzymes produced by this hyperthermophilic bacterium have outstanding heat resistance and may, therefore, be utilized in various industrial fields such as chemistry, food, medical supplies, etc. Analysis of the 1.74-MB genome of this bacterium revealed the presence of only one set of genes for 16S and 23S rRNA and two genes for 5S rRNA, while it is likely to harbor 11 genes/ORFs encoding intein-containing proteins and two tRNA genes with introns (Kawarabayasi et al. 1998).

Ribonuclease P (RNase P) is a ubiquitous *trans*-acting ribozyme that processes the 5′ leader sequence of precursor tRNA (pre-tRNA) (Frank and Pace 1998; Altman and Kirsebom 1999). Although the functionality of RNase P remains similar from bacteria to humans, the chemical composition of this enzyme differs in the three domains of life (Hsieh et al. 2004). Eubacterial RNase P is composed of a catalytic RNA and a single protein cofactor, both of which are required for pre-tRNA processing *in vivo*. The RNase P from *E. coli* contains a catalytic RNA subunit termed M1 RNA and a single protein cofactor known as the C5 protein. Since Altman and co-workers discovered that M1 RNA itself can hydrolyze pre-tRNA *in vitro* (Guerrier-Takada et al. 1983), biochemical and structural studies on RNase P have mainly focused on the eubacterial enzymes (Kirsebom 2007; Smith et al. 2007). These studies showed that eubacterial RNase P RNAs are composed of two domains, the substrate-binding domain (S-domain) and the catalytic domain (C-domain) (Loria and Pace 1996). These domains can fold independently and the catalytic domain alone retains RNase P activity at high Mg^{2+} concentrations (Loria and Pace 2001). Furthermore, it was found that the P1–P4 multihelix junction plays a crucial role in the optimization of Mg^{2+} interactions important for catalysis. In particular, nucleotides A65 and A66 at J3/4 and helix P4 and the *pro*-Rp and *pro*-Sp non-bridging phosphate oxygen at A67 in helix P4 were assigned as binding sites for Mg^{2+} ions required for catalysis by M1 RNA (Christian et al. 2002). The three-dimensional structure of the *Thermotoga maritima* (bacterial Type A) and *Bacillus stearothermophilus* (bacterial Type B) RNase P RNAs has been solved (Kazantsev et al. 2005; Torres-Larios et al. 2005). Several findings indicated that the main functions of the protein component in eubacterial RNase P are to interact with the 5′ leader sequence which enhances the affinity of both pre-tRNA and specific Mg^{2+} ions bound to the RNase P – pre-tRNA complex and also to promote transition from the intermediate to native conformation (Smith et al. 2007). Recently, it was reported that the *E. coli* C5 protein functions as a metabolic stabilizer of M1 RNA (Kim and Lee 2009). The three dimensional structure of the eubacterial protein subunit was established (Stams et al. 1998; Spitzfaden et al. 2000; Kazantsev et al. 2003). In addition to the processing of pre-tRNA, eubacterial RNase Ps are involved in cleavage of other RNA substrates, such as precursor 4.5S RNA (Liu and Altman 1994) and tmRNA (Komine et al. 1994), mRNAs (Alifano et al. 1994), and riboswitches (Altman et al. 2005; Seif and Altman 2008). Hence, eubacterial RNase Ps have the potential to recognize a variety of RNA substrates and cleave them in a site-specific manner.

Eukaryotic RNase Ps comprise a single RNA moiety and as many as 10 proteins; a highly purified nuclear RNase P from HeLa cells has at least 10 distinct protein subunits termed Rpp14, Rpp20, Rpp21, Rpp25, Rpp29, Rpp30, Rpp38, Rpp40, hPop1, and hPop5 (Xiao et al. 2001; Jarrous and Altman 2001; Kikovska et al. 2007). Although Rpp21 and Rpp29 are known to be strongly involved in the catalytic activity of human RNase P, the functional roles of the other subunits have not been established (Mann et al. 2003). It was recently reported that Rpp20 and Rpp25 belonging to the Alba protein family bind to each other, and the heterodimerization regulates their RNA-binding activity, subcellular localization, and expression,

though their catalytic contribution to human RNase P remains unclear (Welting et al. 2007). Furthermore, a recent study has shown that human nuclear RNase P is required for transcription of tRNA and other small noncoding RNA genes by pol III, and Rpp20 and Rpp25 are expected to play an essential role in this function as chromatin-binding proteins (Jarrous and Reiner 2007). Recently, the crystal structure of the P3 domain from *Saccharomyces cerevisiae* RNase P in a complex with Pop6 and Pop7, yeast homologues of Rpp20 and Rpp25, respectively, was determined at a resolution of 2.7 Å (Perederina et al. 2010).

As for archaeal RNase P, the presence of several protein components associated with their RNAs has been suggested (Darr et al. 1990; Nieuwlandt et al. 1991; Andrews et al. 2001; Hall and Brown 2002). Some archaeal RNase P RNAs have no enzymatic activity in the cleavage of pre-tRNA, though some have catalytic activity in the presence of unusually high salt concentrations (Pannucci et al. 1999). During the course of studying the structural genomics of the hyperthermophilic archaeon *P. horikoshii* OT3, we identified the genes PH1481, PH1496, PH1601, PH1771, and PH1877, the translational products of which show sequence similarity to those of protein subunits hpop5, Rpp38, Rpp21, Rpp29, and Rpp30 of human RNase P, respectively (Kouzuma et al. 2003; Fukuhara et al. 2006). To gain more insight into the structure-function relationship of archaeal and eukaryotic RNase P, we have been extending biochemical and structural studies to components of the *P. horikoshi*i RNase P.

The focus in this chapter is on the structure-function relationships of RNase P from the hyperthermophilic archaeon *P. horikoshii* OT3, including the high-resolution structural information that has become available for the RNase P subunits. For current progress in the study of the structure and function of archaeal and eukaryotic RNase Ps, the reader is referred to the excellent recent reviews (Esakova and Krasilnikov 2010; Jarrous and Gopalan 2010).

23.2 Identification of the Components of *P. horikoshii* RNase P

23.2.1 Reconstitution of the RNase P Activity

A search of the *P. horikoshii* OT3 genome database revealed four genes, PH1481, PH1601, PH1771, and PH1877, to have significant homology to those encoding protein subunits hpop5, Rpp21, Rpp29, and Rpp30 of human ribonuclease P (RNase P), respectively (Table 23.1). The amino acid sequences of the four proteins can be aligned to those of corresponding proteins from both human and yeast, with 15–29% identical residues. Hence, the gene products of the four genes were overproduced in *E. coli* cells and purified to homogeneity by column chromatography. In addition, the RNA subunit (*Pho*pRNA), a homologue of human H1 RNA, was transcribed *in vitro*. To characterize putative RNase P protein homologues of *P. horikoshii*, we tested whether the subunits could be assembled *in vitro* to reconstitute an active RNase P. The result indicated that the reconstituted particle (R-4P) composed of *Pho*pRNA and four proteins exhibited RNase P activity with salt dependency similar to the authentic RNase P from *P, horikoshii* (Kouzuma et al. 2003) (Fig. 23.1a).

Table 23.1 Chemical features of the *P. horikoshii* RNase P subunits

P *horikoshii*	Residues	M*r*	ρ I	*H. sapiens*	*S. cerevisiae*
Pho Pop5	120 aa	14,043	10.8	hPOP5	POP5
Pho Rpp21	120 aa	14,588	11.3	Rpp21	RPR2
Pho Rpp29	127 aa	15,053	11.8	Rpp29	POP4
Pho Rpp30	212 aa	24,693	10.4	Rpp30	Rpp1
Pho Rpp38	124aa	13,554	5.2	Rpp38	POP3
Pho pRNA	324 nt	105,370		H1 RNA	RPR1

H. sapiens and *S. cerevisiae* indicate human and yeast RNase P subunits, respectively, corresponding to the *P. horikoshii* subunits. aa and nt indicate amino acid and nucleotide residues, respectively

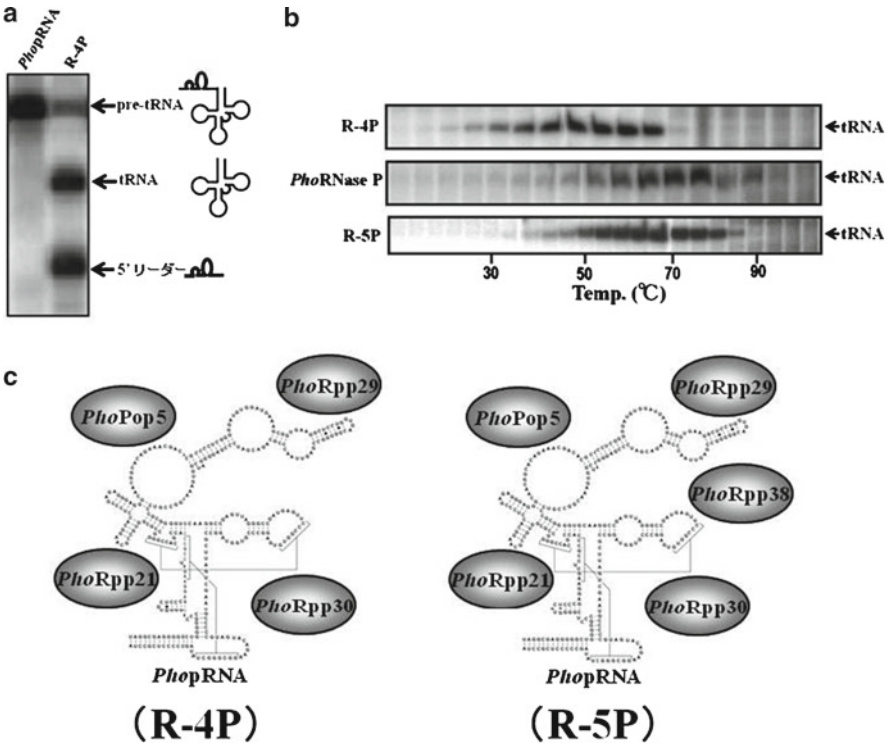

Fig. 23.1 Reconstitution of *P. horikoshii* RNase P from *Pho*pRNA and five proteins. (**a**) Cleavage of pre-tRNA by *Pho*pRNA or the reconstituted particle (R-4P) composed of *Pho*pRNA and four proteins (*Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29 and *Pho*Rpp30). (**b**) Effect of the fifth protein *Pho*Rpp38 on the optimum temperature for nuclease activity of reconstituted particles R-4P and R-5P. *Pho*RNase P indicates the nuclease activity by the authentic RNase P partially purified from *P. horikoshii* OT3. (**c**) Components in the reconstituted particles R-4P and R-5P

It was, however, found that the reconstituted RNase P (R-4P) had an optimal temperature of around 55°C, while the native enzyme was most active around 70°C (Kouzuma et al. 2003) (Fig. 23.1b, c).

This finding prompted us to re-search the *P. horikoshii* genome, on the assumption that the authentic enzyme has an additional component partly responsible for

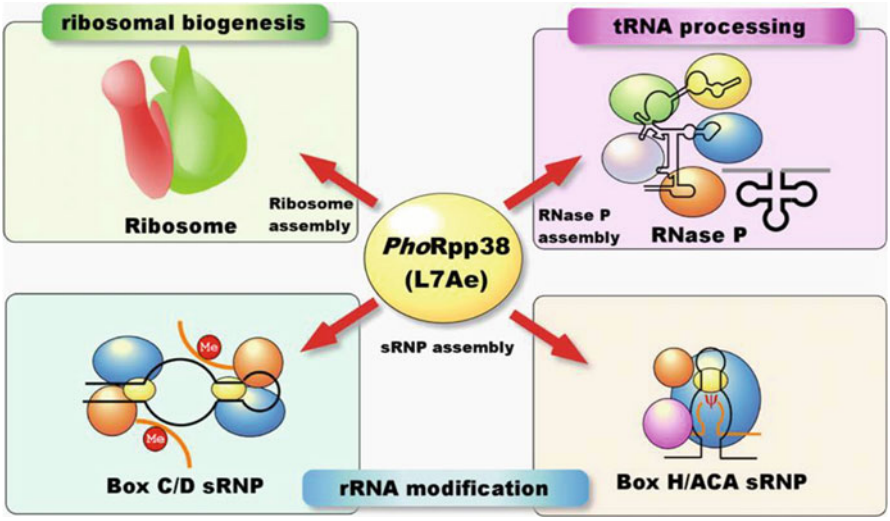

Fig. 23.2 Multifunctional protein *Pho*Rpp38. The archaeal ribosomal L7Ae is a multifunctional protein, functioning in ribosomes as well as RNP complexes, box C/D and box H/ACA. The study assigned an RNase P protein to L7Ae as a fourth function

the elevated optimal temperature for nuclease activity. We found that the gene PH1496 in the *P. horikoshii* genome, whose product is a homolog for the ribosomal protein L7Ae (Ban et al. 2000) from *Haloarcula marismortui*, has significant homology to the gene encoding subunit Rpp38 of the human RNase P protein (Eder et al. 1997). Then, we examined the optimal temperature of the reconstituted RNase P including the gene product. The result showed that addition of the protein to R-4P significantly increased the optimal temperature from 55ºC to 70ºC, which is comparable to that of the authentic RNase P from *P. horikoshii* (Fig. 23.1b, c). These results revealed that *Pho*pRNA and five proteins (the reconstituted particle was referred to as R-5P) in the hyperthermophilic archaeon *P. horikoshii* reconstituted RNase P activity that exhibits enzymatic properties like those of the authentic enzyme (Fukuhara et al. 2006). The *P. horikoshii* RNase P proteins were designated *Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, *Pho*Rpp30, and *Pho*Rpp38, according to their sequence homology with the human RNase P proteins hpop5, Rpp21, Rpp29, Rpp30, and Rpp38, respectively (Table 23.1).

We found that *Pho*Rpp38, a possible ribosomal L7Ae protein in *P. horikoshii*, is the fifth component in *P. horikoshii* RNase P and contributes to the optimal temperature for the catalytic activity of *P. horikoshii* RNase P. The archaeal ribosomal L7Ae is a multifunctional protein, active in ribosomes as well as RNP complexes (box C/D and box H/ACA) (Rozhdestvensky et al. 2003), which are involved in the modification of rRNA. Thus, the study may assign a fourth function to L7Ae, as an RNase P protein (Fig. 23.2). Very recently, Cho et al. reported that the RNA and five proteins corresponding to the components in the *P. horikoshii* RNase P could be

assembled *in vitro* to reconstitute an active RNase P from a different thermophilic archaeal variant, *Methanocaldococcus jannaschii* (Cho et al. 2010).

It was recently reported that Rpp20 and Rpp25 belonging to the Alba protein family bind to each other, and the heterodimerization regulates their RNA-binding activity, subcellular localization, and expression, though their catalytic contribution to human RNase P remains unclear (Welting et al. 2007). Furthermore, a recent study has shown that human nuclear RNase P is required for transcription of tRNA and other small noncoding RNA genes by pol III, and Rpp20 and Rpp25 are expected to play an essential role in this function as chromatin-binding proteins (Jarrous and Reiner 2007). Alba was initially identified as 10-kDa proteins present in the chromatin of crenarchaeota, particularly in *Sulfolobus* species (Reddy and Suryanarayana 1988; Dijk and Reinhardt 1986). Subsequently, it was found that Alba homologues are present not only in crenarchaeota but also in Euryarchaeota and in some Eukarya. During the course of studying the *P. horikoshii* RNase P, we identified the gene PHS053, the translational product of which shows sequence similarity to the Alba family proteins. We referred to this protein as *Pho*Alba, and tested its involvement in RNase P activity. The result indicated that although *Pho*Alba interacts with both *Pho*pRNA and pre-tRNA in *P. horikoshii*, the addition of *Pho*Alba to reconstituted particles composed of *Pho*pRNA and four or five protein subunits had little influence on either the pre-tRNA processing activity or the optimum temperature for the processing activity (Hada et al. 2008). Hence, it was concluded that *Pho*Alba contributes little to the catalytic activity of *P. horikoshii* RNase P, although we can not exclude the possibility that it might, as is the case for Rpp20 and Rpp25 in human RNase P, be involved in transcriptional regulation of tRNA and noncoding RNAs in *P. horikoshii*.

23.3 Structure and Function of *Pho*pRNA

23.3.1 Catalytic Residues in Pho*pRNA*

Nucleotides A65, A66, and U69 were assigned as binding sites for Mg^{2+} ions required for catalysis by M1 RNA (Christian et al. 2002). In addition, G292 and G293, located at the loop (L15/16) between helices P15 and P16 in M1 RNA, are known to be involved in recognition of the acceptor end (CCA) in pre-tRNA (Kirsebom and Svard 1994). Although RNase Ps in Archaea contain additional proteins, their RNAs retain an essential core of conserved sequence and secondary structure (Chen and Pace 1997) (Fig. 23.3a). It is thus likely that archaeal RNase P RNAs are directly involved in catalytic function. However, the investigation of archaeal RNase P RNAs *in vitro* has been limited by the lack of catalytic activity of the RNA alone. Since we have achieved the reconstitution of the *P. horikoshii* RNase P, we examined the involvement of conserved nucleotide residues in *Pho*pRNA in

RNase P activity. The result indicated that nucleotides A40, A41, and U44 at J3/4 and helix P4, and G269 and G270 located at L15/16 in *Pho*pRNA are, like the corresponding residues in M1 RNA, involved in hydrolysis by coordinating catalytic Mg^{2+} ions, and in the recognition of the acceptor end (CCA) of pre-tRNA by base-pairing, respectively (Terada et al. 2007) (Fig. 23.3b). The result strongly suggested that *Pho*pRNA catalyzes the hydrolysis of pre-tRNA in approximately the same manner as eubacterial RNase P RNAs, even though it has no enzymatic activity in the absence of the proteins.

23.3.2 A Minimal Region of Pho*pRNA*

A characteristic feature of *Pho*pRNA is the acquisition of terminal stem-loop structures including P12.1 and P12.2, which are absent in eubacterial counterparts (Harris et al. 2001) (Fig. 23.3a). We examined their involvement in RNase P activity by preparing successive deletion mutant *Pho*pRNAs. The RNase P activity assays showed that the mutant *Pho*pRNAs, in which nucleotides encompassing 145 to 178 (helix P12.2) were deleted, retained a level of RNase P activity comparable with that of native *Pho*pRNA (Terada et al. unpublished results). A further deletion of nucleotides 121–195 (helices P12.1 and P12.2) resulted in a slight reduction in RNase P activity. These results indicated that the unique terminal stem-loop structure containing helices P12.1 and P12.2 in *Pho*pRNA is dispensable for RNase P activity (Terada et al. unpublished results). However, it was shown that a further deletion of nucleotides 100–212, including helices P12, P12.1 and P12.2, resulted in a considerable reduction in the activity of the mutants, demonstrating a crucial role for the loop structure (L11/12) between helices P11 and P12 in RNase P activity (Terada et al. unpublished results).

Furthermore, we prepared deletion mutants, in which stem-loop structures containing helices P15 and P16 were successively deleted, and analyzed their RNase P activity. The results showed that although deletions of helices P15 and P16 modestly reduced RNase P activity, both mutants retained considerable RNase P activity. It was further shown that mutants in which both stem-loop structures containing helices P12.1, P12.2, P15, and P16 were successively deleted, still retained RNase P activity, though the double deletions significantly reduced the activity (Terada et al. unpublished results). Siegel et al. reported that the RNase P RNA from *Mycoplasma fermentans* lacks the stem-loop structure containing helix P12 and this helix is dispensable in some organisms (Siegel et al. 1996). It was further described that an engineered RNA (Micro P RNA), in which all evolutionarily variable structures were deleted from the *M. fermentans* RNA, was catalytically active *in vitro* with an approximately 600-fold decrease in catalytic efficiency relative to the native RNA (Siegel et al. 1996). The results are well

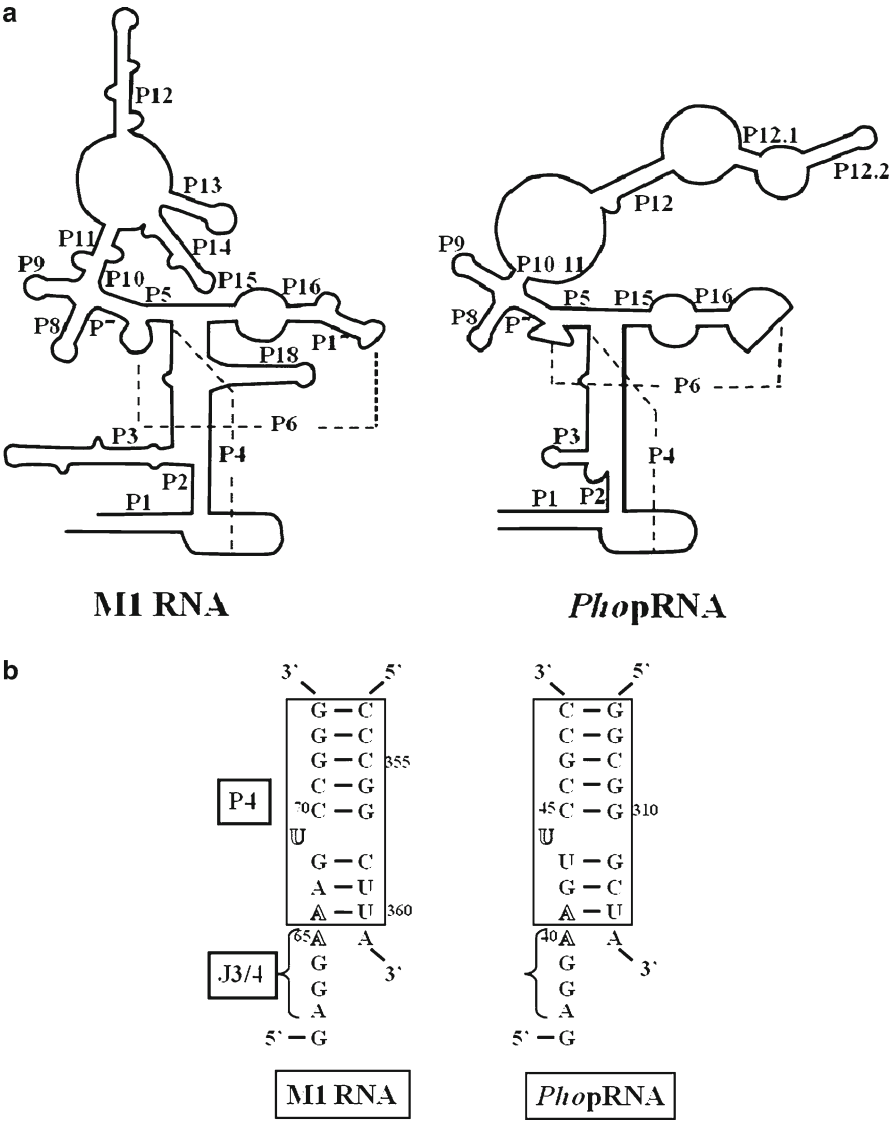

Fig. 23.3 Secondary structure of *E. coli* RNase P RNA (M1 RNA) and *P. horikoshii* RNase P RNA (*Pho*pRNA). (**a**) Secondary structure of M1 RNA and *Pho*pRNA. (**b**) Secondary structure of J3/4 and helix P4 in M1 RNA and *Pho*pRNA. Nucleotide residues at helix P4 are boxed. A65, A66, and U69 in M1 RNA and A40, A41, and U44 in *Pho*pRNA, involved in catalytic activity, are indicated

consistent with the notion (Chen and Pace 1997) that all phylogenetically variable structures in RNase P RNA are dispensable for the catalytic mechanism and led us to conclude that the structure containing helices P1–P12, like the corresponding structure in eubacterial counterparts, constitutes an essential catalytic core of *Pho*pRNA.

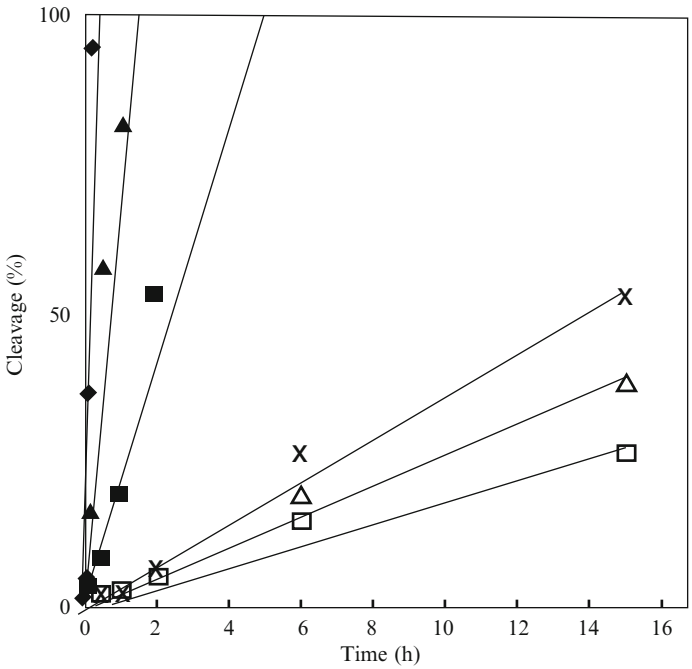

Fig. 23.4 Time course analysis of RNase P RNA activity of R-4Ps. The time course of substrate cleavage at 65°C. ♦, R-5P; □, R-4P lacking *Pho*Pop5; ▲, R-4P lacking *Pho*Rpp38; ×, R-4P lacking *Pho*Rpp21; ■, R-4P lacking *Pho*Rpp29; Δ, R-4P lacking *Pho*Rpp30

23.4 Structure and Function of the *P. horikoshii* RNase P Proteins

23.4.1 Contribution of the Proteins to RNase P Activity

We investigated involvement of the individual proteins in RNase P activity. Two particles (R-3Ps), in which *Pho*pRNA was mixed with three proteins, *Pho*Pop5, *Pho*Rpp30, and *Pho*Rpp38 or *Pho*Pop5, *Pho*Rpp30, and *Pho*Rpp21 showed detectable RNase P activity, and five reconstituted particles (R-4Ps) composed of *Pho*pRNA and four proteins exhibited RNase P activity, albeit at a reduced level compared to that of the reconstituted particle (R-5P) composed of *Pho*pRNA and five proteins. Time-course analysis of the RNase P activities indicated that the R-4Ps lacking *Pho*Pop5, *Pho*Rpp21, or *Pho*Rpp30 had reduced activity, while omission of *Pho*Rpp29 or *Pho*Rpp38 had only a slight effect on the activity (Fig. 23.4). The results indicated that the proteins contribute to RNase P activity in the order of *Pho*Pop5 > *Pho*Rpp30 > *Pho*Rpp21 >> *Pho*Rpp29 > *Pho*Rpp38. It was further found that R-4Ps showed a characteristic Mg^{2+} ion dependency

approximately identical to that of R-5P, indicating that the proteins do not participate in the stabilization of Mg^{2+} ions. It is thus suggested that the *P. horikoshii* RNase P proteins are predominantly involved in the optimization of the *Pho*pRNA's conformation, though individually they are dispensable for RNase P activity *in vitro* (Terada et al. 2006).

23.4.2 Protein-Protein Interaction

In vivo interactions between the five protein subunits of RNase P in *P. horikoshii* OT3 were examined by using a yeast two-hybrid system (Kifusa et al. 2005). *Pho*Pop5 and *Pho*Rpp21 strongly interacted with *Pho*Rpp30 and *Pho*Rpp29, respectively, whereas *Pho*Pop5 moderately interacted with *Pho*Rpp21. In contrast, no interaction was detected between *Pho*Rpp38 and the other four proteins. The results allowed us to determine the crystal structure of the complexes *Pho*Pop5-*Pho*Rpp30 (Kawano et al. 2006) and *Pho*Rpp21-*Pho*Rpp29 (Honda et al. 2008), as described below. Similar protein-protein interactions in archaeal as well as eukaryotic RNase Ps were reported (Jiang and Altman 2001; Hall and Brown 2004). It is thus likely that the two protein-protein interactions observed in *P. horikoshii* RNase P are conserved and play an important role in catalytic activity in archaeal and eukaryotic RNase Ps. Indeed, structure-based mutagenesis of *Pho*Pop5-*Pho*Rpp30 and *Pho*Rpp21-*Pho*Rpp29 demonstrated a crucial role for their interactions in the RNase P activity (Kawano et al. 2006; Honda et al. 2008).

23.4.3 Protein-RNA Interaction

The characterization using the gel shift assay, chemical probing, and the mutational analysis suggested that *Pho*Rpp38 binds two regions encompassing nucleotides 128–190 (helices P12.1 and P12.2) and 227–272 (helices P15 and P16) in *P. horikoshii* RNase P RNA (Fukuhara et al. 2006). Klein et al. identified a new RNA motif in the *H. marismortui* large ribosomal subunit, designated K-turn, with which nine ribosomal proteins (L4, L7Ae, L10, L15e, L19e, L24, L29, L32e, and L37Ae) interact (Klein et al. 2001). Although these nine ribosomal proteins do not share a common structural domain that recognizes K-turns, there is at least one homologous family of RNA-binding domains that specifically recognize K-turns. Thus, the *H. marismortui* ribosomal protein L7Ae, the yeast ribosomal protein L30e, and the human spliceosomal protein 15.5 kDa share an RNA-binding motif that binds K-turn RNA elements in the same fashion (Vidovic et al. 2000). The configurations of the amino acids responsible for binding with the K-turn motif in these homologues are conserved in *Pho*Rpp38. It is thus likely that these conserved amino acid residues are involved in interaction with the two stem-loop structures. It is known that the K-turn motif has a kink in the phosphodiester backbone that causes a sharp

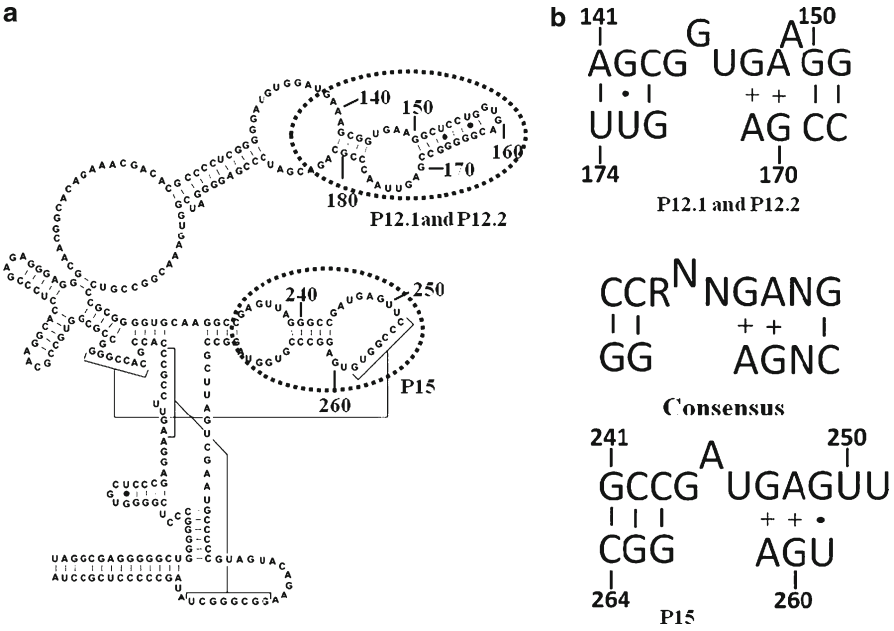

Fig. 23.5 K-turn motif in *Pho*pRNA. (**a**) Secondary structure of *Pho*pRNA. The putative binding sites of *Pho*Rpp38 are circled with dashed lines. (**b**) Possible K-turn motifs predicted in *Pho*pRNA

turn in the RNA helix, and its asymmetric internal loop is flanked by C-G base pairs on one side and sheared G-A base pairs on the other (Klein et al. 2001) (Fig. 23.5b). Based on the consensus sequence, we found possible K-turn motifs at the putative *Pho*Rpp38 binding sites, where they contain asymmetric internal loops with G-C rich pairs (Fig 23.5a, b). Interestingly, Lopez et al. reported that the K-turn motif is present in a significant fraction of the RNase P and MRP RNA sequences (Lopez et al. 2009). Further structural analysis of *Pho*Rpp38 in complexes with RNA fragments including putative binding sites should help reveal the precise molecular mechanism by which *Pho*Rpp38 recognizes *Pho*pRNA.

23.4.4 Functional Equivalence Between C5 Protein and P. horikoshii Proteins

To localize functional sites of the other four proteins (*Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, and *Pho*Rpp30) on *Pho*pRNA, we examined the functional equivalency between the *E.* coli RNase P protein (C5) and the four *P. horikoshii* RNase P proteins for RNase P activity. The C5 protein and *P. horikoshii* RNase P proteins were unable to activate non-congnate RNase P RNAs, *P. horikoshii* RNase P RNA (*Pho*pRNA) and *E. coli* RNase P RNA (M1 RNA), respectively. Two chimeric RNAs, in which

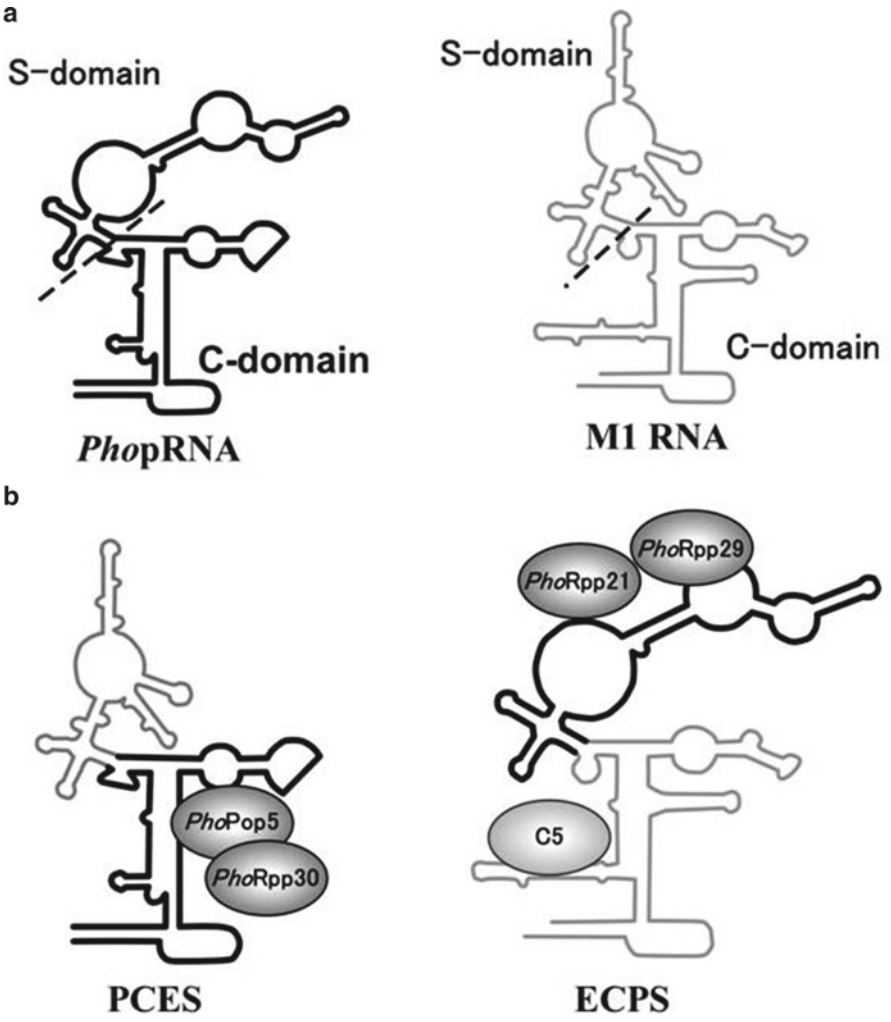

Fig. 23.6 Schematic representation of the reconstituted particles with cleavage activity. (**a**) Secondary structure of *Pho*pRNA (black line) and M1 RNA (grey line), showing S and C-domains. (**b**) The chimeric RNA PCES, comprising the *Pho*pRNA C-domain (black line) and the M1 RNA S-domain (grey line), was activated by *Pho*Pop5 and *Pho*Rpp30. The other chimera, ECPS, composed of the M1 RNA C-domain (grey line) and the *Pho*pRNA S-domain (black line), was activated by C5, *Pho*Rpp21and *Pho*Rpp29

functional C- and S-domains of M1 RNA and *Pho*pRNA were mutually exchanged, were prepared and characterized with respect to the cleavage of *P. horikoshii* pre-tRNA in the presence of either C5 or *P. horikoshii* proteins. The result suggested that *Pho*Pop5 and *Pho*Rpp30 function equivalent to the C5 protein in the *E. coli* RNase P, being involved in activation of the *Pho*pRNA C-domain (Fig. 23.6). On the other hand, *Pho*Rpp21 and *Pho*Rpp29 are implicated in the stabilization of the *Pho*pRNA S-domain (Honda et al. 2010) (Fig. 23.6).

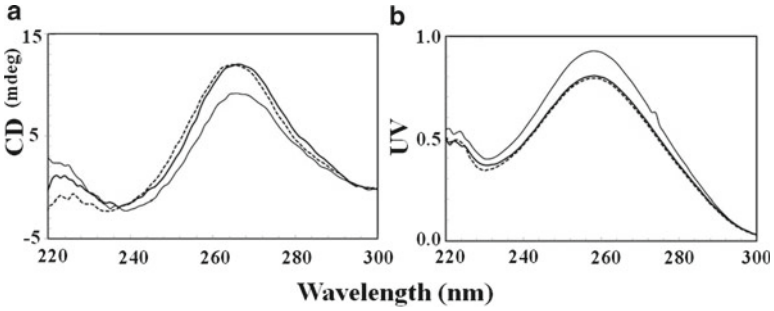

Fig. 23.7 Structural change of *Pho*pRNA by the proteins examined by photometric analysis. (**a**) Circular dichroism (CD) spectra of *Pho*pRNA in the reconstituted particles. The CD spectra of the free *Pho*pRNA and *Pho*pRNA in R-5P and R-4P were recorded on a Jasco J-720 spectropolarimeter at 55°C using a 0.1 cm-long optical path. The dashed, thin, and thick lines indicate CD spectra of free *Pho*pRNA, *Pho*pRNA in R-4P, and *Pho*pRNA in R-5P, respectively. (**b**) Ultraviolet (UV) absorbance spectra of *Pho*pRNA in the reconstituted particles. UV absorbance spectra of the free *Pho*pRNA and *Pho*pRNA in R-5P and R-4P were recorded on a UVmini-1,240 at room temperature. The dashed, thin, and thick lines indicate UV spectra of free *Pho*pRNA, *Pho*pRNA in R-4P, and *Pho*pRNA in R-5P, respectively

23.4.5 Structural Change of Pho*pRNA upon Interaction with the Proteins*

As described above, the reconstituted particle (R-4P), however, had a lower optimal temperature (around 55°C) as compared with the authentic RNase P from *P. horikoshii* (70°C). The fifth protein, *Pho*Rpp38, was found to be partly responsible for the elevated optimal temperature of the reconstituted particle (R-5P). Hence, the activated form of *Pho*pRNAs in R-5P and R-4P was characterized based on circular dichroism (CD) and ultraviolet (UV) absorbance spectra (Kosaka et al. 2010). The CD spectra showed, as for bacterial RNase P RNAs (Guo et al. 2006), a maximum signal at 265 nm. These spectra were consistent with the presence of a highly structured RNA with a significant amount of A-form helix (Pan and Sosnick 1997). Comparison of the CD spectrum for the free *Pho*pRNA with that for *Pho*pRNA in R-4P revealed a significant decrease in the CD signal at the peak and a slight shift to a longer wavelength (Fig. 23.7a). A similar change in signals was reported in the holoenzyme assembly of *Escherichia coli* RNase P (Guo et al. 2006). Since it has been reported that the decrease in the CD signal is correlated with the destabilization of base stacking in RNA, the interaction of the four proteins with *Pho*pRNA may result in unfolding. As for the CD spectra for R-5P, the binding of *Pho*Rpp38 caused a significant increase in the CD signal at 268 nm that is attributed to an increase in base stacking in *Pho*pRNA (Fig. 23.7a). It is thus assumed that *Pho*Rpp38′s binding to R-4P results in an increase in base stacking, thereby stabilizing the activated structure of *Pho*pRNA.

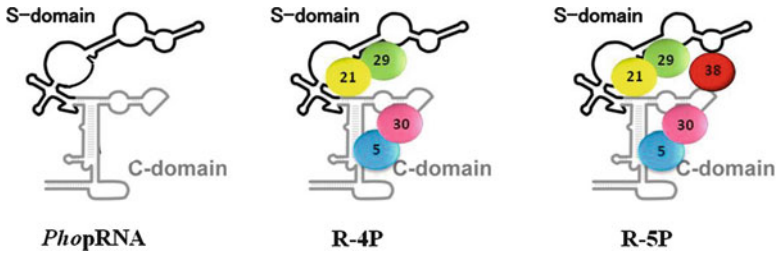

Fig. 23.8 A model of the mechanism of activation of *Pho*pRNA. The free *Pho*pRNA is misfolded, and the protein complexes, *Pho*Pop5-*Pho*Rpp30 and *Pho*Rpp21-*Pho*Rpp29, activate the *Pho*pRNA C- and S-domains, respectively, to result in the active form of *Pho*pRNA, serving as an RNA chaperon. Then, the binding of *Pho*Rpp38 to the terminal helices of the C- and S-domains stabilizes the particle, giving rise to R-5P. The numbers *5*, *21*, *29*, *30*, and *38* indicate *Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, *Pho*Rpp30, and *Pho*Rpp38, respectively

The interaction was analyzed further using UV absorbance spectra, because nucleic acids show a hyperchronic effect as a consequence of the disruption of the electronic interactions among neighboring bases. When the four proteins were added to *Pho*pRNA, the UV absorbance at 260 nm increased significantly, but when *Pho*Rpp38 was added, the absorbance decreased (Kosaka et al. 2010) (Fig. 23.7b). This result suggested that the interaction of the four proteins with *Pho*pRNA disrupts base pairing, while the presence of the fifth protein, *Pho*Rpp38, stabilizes base pairing in *Pho*RNA, which is fully consistent with the assumption predicted from the finding obtained in the CD analysis.

There are two kinds of RNA-binding proteins that can help the RNA to fold correctly and they differ in their mode of action (Schroeder et al. 2004). The first group includes specific RNA-binding proteins that force the unfolded molecule to adopt the correct structure by stabilizing the native conformation. The second group includes proteins that unfold RNAs non-specifically or impede the formation of misfolded structures; these proteins are referred to as RNA chaperones (Herschlag 1995). The four proteins (*Pho*Pop5, *Pho*Rpp21, *Pho*Rpp29, and *Pho*Rpp30) are suggested to function as an RNA chaperone, belonging to the second group, and act on the misfolded *Pho*pRNA so that it gets another chance to fold into the native structure. In contrast, *Pho*Rpp38 is known to interact specifically with two stem-loop structures: nucleotides A116-G201 in the S-domain and G229-C276 in the C-domain, and can be classified into the first group. From the results, the mechanism of activation of *Pho*pRNA is proposed in Fig. 23.8. The free *Pho*pRNA is assumed to become trapped in a misfolded structure (folding trap), as described previously (Schroeder et al. 2004). The protein complexes, *Pho*Pop5-*Pho*Rpp30 and *Pho*Rpp21-*Pho*Rpp29, activate the *Pho*pRNA C- and S-domains, respectively, to produce the active form of *Pho*pRNA (R-4P), probably by unfolding the misfolded *Pho*pRNA. Then, the specific binding of *Pho*Rpp38 to the terminal helices in the C- and S-domains stabilizes the activated structure of *Pho*pRNA, by increasing base stacking. Further study using chemical and nuclease mapping will provide more insight into the structural change of *Pho*pRNA at the nucleotide level.

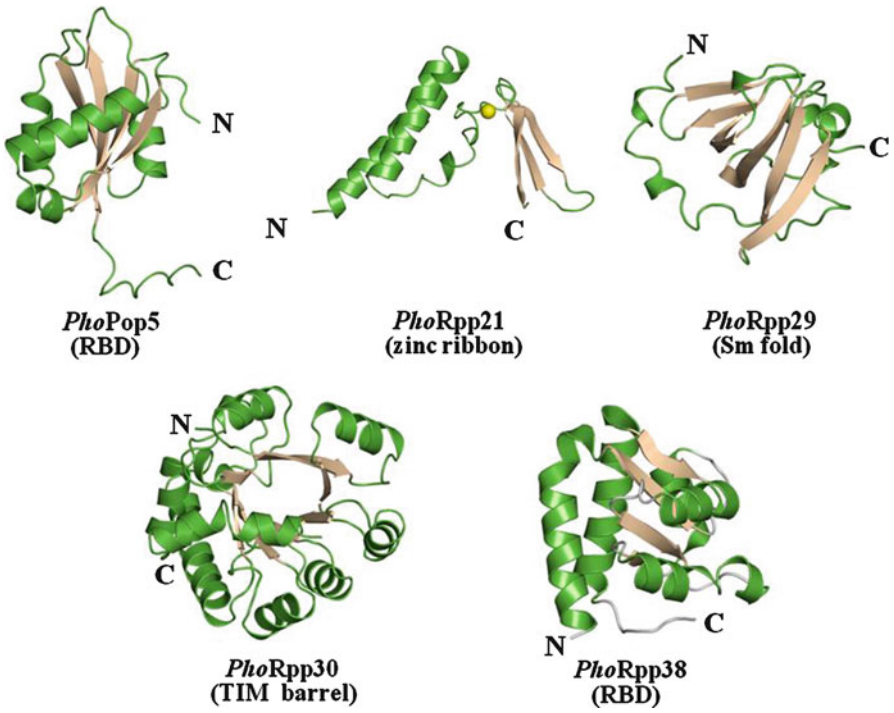

Fig. 23.9 Crystal structure of the *P. horikoshii* RNase P proteins. The proteins *Pho*Pop5, *Pho*Rpp29, *Pho*Rpp30, and *Pho*Rpp38 consist of a globular domain and a tail at the N- or C-terminus. The tail segment, which is largely devoid of secondary structure and usually rich in basic residues, may be involved in interaction with *Pho*pRNA. Structural motifs observed in the proteins are indicated in parentheses

23.4.6 Crystal Structure of the Proteins

A knowledge of the three-dimensional structure of proteins is a prerequisite for understanding their functions and how they participate in biological processes at a molecular level. We have undertaken the task of determining the crystal structures of individual proteins from *P. horikoshii* RNase P. The conditions produced crystals that diffracted to a high resolution. So far, the crystal structure of all five RNase P proteins has been determined (Fig. 23.9). In addition, the structure of *Pho*Pop5-*Pho*Rpp30 and *Pho*Rpp21-*Pho*Rpp29 has become available (Figs. 23.10 and 23.11). In this section, we summarize structural features of the proteins.

*Pho*Pop5 (PDB ID. 2CZV): Since *Pho*Pop5 was not amenable to crystallization, we determined its crystal structure in a complex with *Pho*Rpp30. *Pho*Pop5 consists of a five-stranded anti-parallel β-sheet and five helices, which fold in a way that is topologically similar to the ribonucleoprotein (RNP) domain. *Pho*Pop5 is, however,

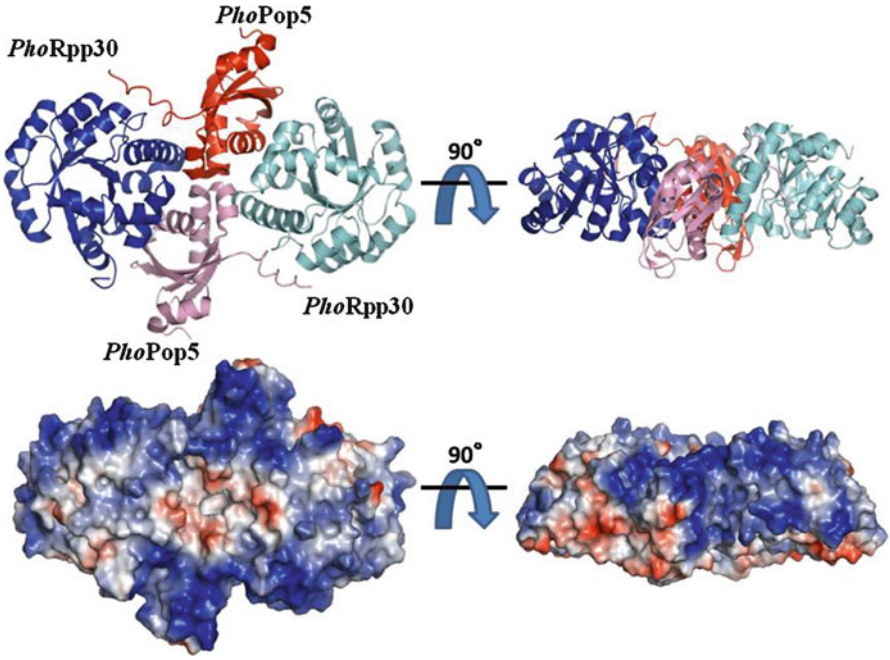

Fig. 23.10 Crystal structure of the complexe of *Pho*Pop5-*Pho*Rpp30. In the heterotetrameric structure [*Pho*Rpp30-(*Pho*Pop5)$_2$-*Pho*Rpp30], a homodimer of *Pho*Pop5 sits between two *Pho*Rpp30 monomers. Electrostatic surface potentials of the *Pho*Pop5-*Pho*Rpp30 complex are shown. Surface are colored according to their electrostatic potentials calculated by the program GRASP (Nicholls et al. 1991), The surface potential is displayed as a color gradient from red (negative) to blue (positive), showing the relative strong electro positive character of the putative RNA binding site

distinct from the typical RNP domain in that it has additional helices at the C-terminus, which pack against one face of the β-sheet (Kawano et al. 2006).

*Pho*Rpp21 (PDB ID. 1X0T): *Pho*Rpp21 comprises an N-terminal domain (residues 1–55), a central linker domain (residues 56–79) and a C-terminal domain (residues 80–120), forming an L-shaped structure. The N-terminal domain consists of two long α-helices, while the central domain and the C-terminal domain fold in a zinc ribbon domain. The electrostatic potential representation indicated the presence of positively charged clusters along the inside of the L-arms, suggesting a possible role in RNA binding. A single zinc ion binds the well-ordered binding site constituted by four Cys residues (Cys68, Cys71, Cys97, and Cys100) and appears to stabilize the relative positions of the N-and C-domains. Mutations of Cys68 and Cys71 or Cys97 and Cys100 by Ser abolished the RNase P activity, demonstrating a strong involvement of zinc ions in the structure and/or function of *Pho*Rpp21 (Kakuta et al. 2005).

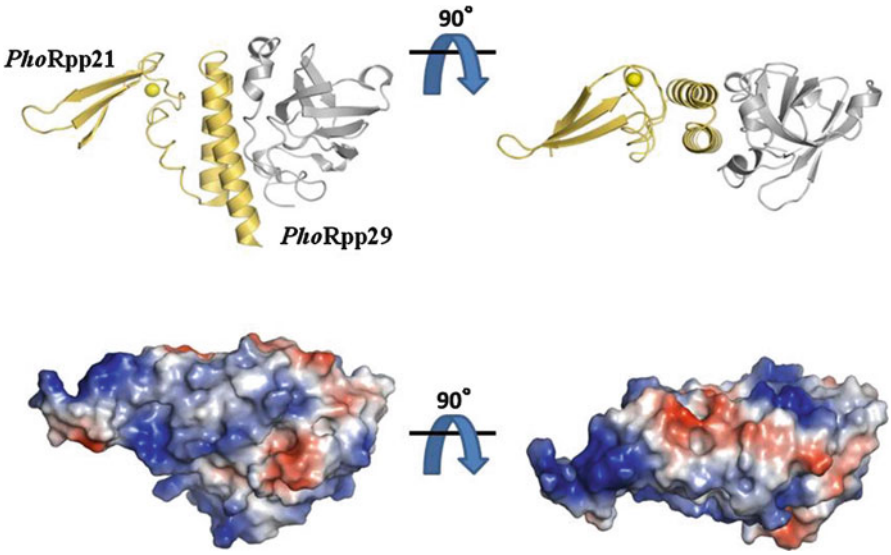

Fig. 23.11 Crystal structure of the complexe of *Pho*Rpp21-*Pho*Rpp29. *Pho*Rpp21 and *Pho*Rpp29 form a heterodimeric structure, where the N-terminal two helices in *Pho*Rpp21 predominantly interact with the N-terminal extended structure, the β-strand, and the C-terminal helix in *Pho*Rpp29. Electrostatic surface potentials of the complex are shown as described in Fig. 23.10

*Pho*Rpp29 (PDB ID. 1V76): The structure is composed of four helices and a six-stranded antiparallel β-sheet with a protruding β-strand in the C-terminal region. Structural comparison showed that the β-barrel structure of *Pho*Rpp29 has a topological resemblance to those of the *Staphylococcus aureus* translational regulator Hfq and *H. marismortui* ribosomal protein L21E, suggesting that these RNA-binding proteins have a common ancestor and then diverged to specifically bind to their cognate RNA (Numata et al. 2004).

*Pho*Rpp30 (PDB ID. 1V77): The protein forms a TIM barrel structure, consisting of ten α-helices and seven β-strands, and has closest similarity to the TIM barrel domain of *E. coli* cytosine deaminase with a root-mean square deviation of 3.0 Å. The electrostatic representation indicated the presence of several clusters of positively charged amino acids present on the molecular surface (Takagi et al. 2004).

*Pho*Rpp38 (PDB ID. 2CZW): The protein comprises five α-helices and a four stranded β-sheet. The β-sheet is sandwiched between one side by three helices and other side by two helices, forming a three-layer α−β−α sandwich structure (Fukuhara et al. 2006).

*Pho*Pop5-*Pho*Rpp30 (PDB ID. 2CZV): The presence of two complexes in the asymmetric unit related by a local two-fold symmetry suggested dimerization of the complex in the crystals. In the heterotetrameric structure [*Pho*Rpp30-(*Pho*Pop5)$_2$-*Pho*Rpp30], a homodimer of *Pho*Pop5 sits between two *Pho*Rpp30 monomers. *Pho*Pop5

dimerizes through a hydrogen bond in the loop between the N-terminal helices, and each *Pho*Pop5 interacts with two *Pho*Rpp30 molecules (Fig. 23.10). Deletion of the loop between the two helices in *Pho*Pop5 caused heterodimerization with *Pho*Rpp30, and abolished the ability to homodimerize itself and heterotetramerize with *Pho*Rpp30. Furthermore, the reconstituted particle containing the deletion mutant *Pho*Pop5 exhibited significantly reduced nuclease activity (Kawano et al. 2006). These results suggest that the presence of the heterotetramer of *Pho*Pop5 and *Pho*Rpp30 is essential for activation of the C-domain in *Pho*pRNA. The electrostatic potential on the surface on the heterotetramer showed a very unique charge distribution (Fig. 23.10). It is thus likely that the highly positively charged face may be involved in binding to the phosphate backbone of *Pho*pRNA.

*Pho*Rpp21-*Pho*Rpp29 (PDB ID. 2ZAE): *Pho*Rpp21 and *Pho*Rpp29 form a heterodimeric structure, where the N-terminal two helices in *Pho*Rpp21 predominantly interact with the N-terminal extended structure, the β-strand, and the C-terminal helix in *Pho*Rpp29. The interface is dominated by hydrogen bonds and several salt bridges, rather than hydrophobic interactions. The electrostatic potential on the surface of the heterodimer shows a positive charged cluster on one face, suggesting a possible RNA-binding surface of the *Pho*Rpp21-*Pho*Rpp29 complex (Fig. 23.11). Further, the mutational analysis demonstrated that as is the case for *Pho*Pop5-*Pho*Rpp30, the heterodimerization between *Pho*Rpp21 and *Pho*Rpp29 plays an important role in activation of the S-domain in *Pho*pRNA (Honda et al. 2008).

23.5 Future Directions

It is now becoming apparent that a variety of RNPs act as catalytic, structural, and regulatory elements in essential biological processes such as pre-RNA processing, pre-mRNA splicing, and translation control. For a full understanding of the molecular mechanisms of these molecules, it is a prerequisite to elucidate their three dimensional structure. RNase P, being ubiquitous in all three phylogenetic domains of life, is composed of a catalytic RNA and a variable number of proteins, dependent on the organism. We found that *Pho*pRNA and five proteins in the hyperthermophilic archaeon *P. horikoshii* OT3 reconstituted RNase P activity that exhibits enzymatic properties like those of the authentic enzyme. Hence, *Pho*pRNA, like eukaryotic counterparts, is deficient in functions and cooperates with five protein subunits in substrate recognition and catalysis. It is thus of interest to understand how protein subunits in eukaryotic and archaeal RNase Ps exert their roles in the catalytic activity. These investigations will aid in establishing structure-function relationships of the RNP ribozyme, and the resulting information will ultimately shed light on the transition from the proposed RNA world to the modern protein world.

Taking advantage of the intrinsic thermostability and a genetic property of the hyperthermophilic archaeon, the crystallographic analysis has established the structure of all five *P. horikoshii* RNase P protein components. Furthermore, the biochemical

data indicated that *Pho*Pop5 and *Pho*Rpp30, archaeal homologues of human hPop5 and Rpp30, function equivalent to the C5 protein in the *E. coli* RNase P, being involved in activation of the *Pho*pRNA C-domain, whereas *Pho*Rpp21 and *Pho*Rpp29, archaeal homologues of human Rpp21 and Rpp29, are implicated in the stabilization of the *Pho*pRNA S-domain. It is clear that a detailed understanding of the mechanism involved requires knowledge of the high resolution structure of the RNase P holoenzyme. However, progress in the structural determination of the holoenzyme has been retarded by two factors. The first is the difficulty in obtaining significant quantities of pure RNase P needed for crystallographic studies. The second factor is the complexity of the subunit components as compared to that of eubacterial RNase P. An alternative approach is to obtain high resolution structures of individual RNase P proteins by X-ray crystallography, and then obtain the structure of complexes of specific proteins with fragments of RNase P RNA. By this piecemeal approach, it will be possible to understand the structure of the RNase P in much greater detail than is possible now, especially when the high resolution information obtained is combined with a knowledge of the overall arrangement of the components of the RNase P analyzed by either Cryo-electron microscopy (Cryo-EM) or small-angle X-ray scattering. In particular, Cryo-EM can achieve an estimated resolution of 6–7 Å (Schuler et al. 2006). At this resolution, the major and minor grooves of RNA helices and α-helices of proteins are easily discernible, so it would be possible to fit known X-ray structures into Cryo-EM envelops. Hence, a combination of crystallography and Cryo-EM will provide important information at an increasing rate. Completion of the three-dimensional structure of the archaeal RNase P will definitely provide a framework for understanding structure-function relationships of archaeal and eukaryotic RNase Ps.

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Chapter 24
Biosynthesis of Pullulan and Its Applications in Food and Pharmaceutical Industry

R.S. Singh and G.K. Saini

Abstract Pullulan is a water-soluble polysaccharide produced by yeast-like fungus *Aureobasidium pullulans*. It is a regularly repeating copolymer with the chemical structure{→6)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→}$_n$ and viewed as a succession of α-(1→6)-linked (1→4)-α-D-triglucosides i.e. maltotriose (G_3). Pullulan has a wide range of commercial applications in biomedical and food industries. Because of its strictly linear structure, pullulan is also a very valuable tool in basic research as well as a well-defined model substance. This chapter focuses on the current literature on pullulan mainly its microbial sources, structural geometry, fermentative production, biosynthesis aspects, peculiar characteristics and varied applications.

Keywords *Aureobasidium pullulans* • Pullulan • Structure • Biosynthesis • Fermentative production • Food and biomedical applications

24.1 Introduction

Exopolysaccharides (EPSs) produced by a number of microorganisms are chemically well defined and have attracted worldwide attention due to their novel and unique physical properties. These are rapidly emerging as new and industrially important source of polymeric materials, which are gradually becoming economically competitive. Microorganisms that produce a large amount of slime have the

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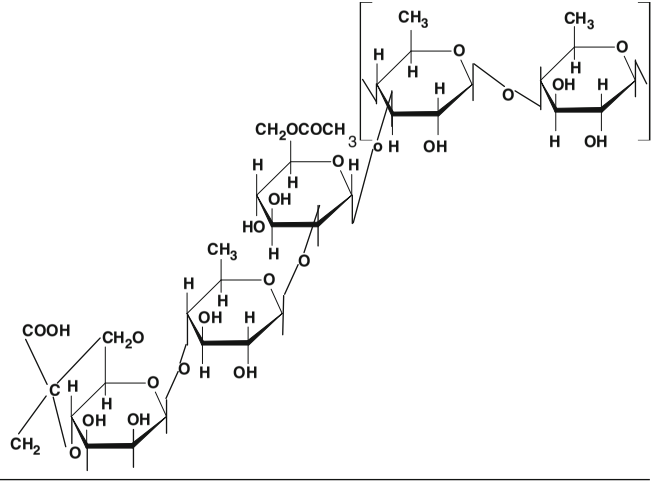
greatest potential for commercialization, since EPSs can be recovered from the fermentation broth easily. During recent years, microbial EPSs have become available for use in many applications that are not only compatible with human lifestyle but also are friendly to the environment (Steinbüchel 2001). Many microorganisms overproduce EPSs with an interesting range of chemical and physical properties presented in Table 24.1.

Although many microorganisms are known to produce EPSs, most of the research effort has been directed to the α-glucan pullulan, produced by *A. pullulans*, a yeast-like fungus with impressive metabolic and ecological versatility (Deshpande et al. 1992). Pullulan is a linear α-D-glucan with 'maltotriose trimer' i.e. α-(1→4)Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*-, as a regularly repeating structural unit, produced extracellularly by *Aureobasidium pullulans* (De Bary) G. Arnaud, a mitosporic fungus formerly known as *Pullularia pullulans* (De Bary) Berkhout (Syn: *Dematium pullulans* De Bary) (Gibbs and Seviour 1996; Leathers 2003). However, other structures particularly the tetramer or maltotetraose, α-(1→4)Glu*p*-α-(1→4)-Glu*p*-α-(1→4)Glu*p*-α-(1→6)Glu*p*-, may be present in the pullulan polymeric chain (Wallenfels et al. 1965). The maximum extent to which maltotetraose subunits have been detected so far is 7% (Catley et al. 1986). The majority of tetrasaccharide units have been found to be in non-terminal locations (Catley and Whelan 1971), with an apparent random distribution throughout the molecule (Carolan et al. 1983). This is the main reason that currently and frequently in the literature, the term 'pullulan' is used for both the "polymaltotriose" produced by *A. pullulans* and the polysaccharide varieties similar to pullulan being produced by other microbes. Bauer (1938) made early observations on extracellular polymer formation from *A. pullulans* and it was characterized by Bernier (1958). Bender et al. (1959) studied the novel glucan and named it as "pullulan". During the 1960s, the basic structure of pullulan was resolved (Wallenfels et al. 1961; Bouveng et al. 1962, 1963a, b; Sowa et al. 1963; Ueda et al. 1963). The enzyme pullulanase, which specifically hydrolyzes α-(1→6) linkages in pullulan and converts the EPS almost quantitatively to maltotriose was discovered by Bender and Wallenfels (1961). A diagrammatic presentation of the pullulan structure with maltotriose as repeating unit is shown in Fig. 24.1. This polysaccharide is viewed as a succession of α-(1→6)-linked (1→4)-α-D-triglucosides i.e. maltotriose (G_3).

Pullulan possesses several potential applications in cosmetics, diet foods, medicine and manufacturing industries as flocculant, adhesive, binder, etc. (Leathers 2003; Shingel 2004; Rekha and Sharma 2007; Singh et al. 2008c; Chi et al. 2009; Gaur et al. 2010). Since it is a biodegradable plastic, it can be used in place of package wraps and plastic containers (Yuen 1974). Pullulan is commercially exploited for its coating and wrapping potential and as a food ingredient (Sugimoto 1978). Reports are available on applications of pullulan, describing its use in meltable medical treatment contact lenses (Himi et al. 1981), as a sizing agent for paper (Japan Carlit Co. Ltd. 1983), in plywood manufacturing (Aika Industries Co. Ltd. 1982a) and in cosmetic emulsions (Aika Industries Co. Ltd. 1982b).

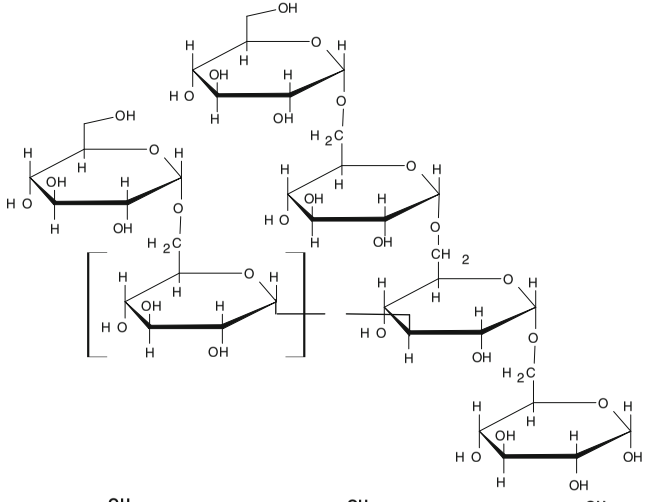
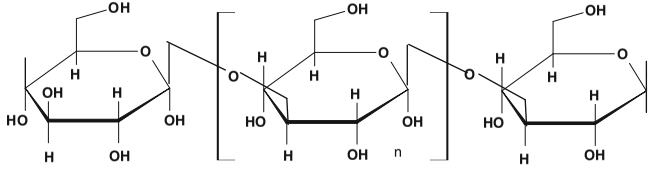
Pullulan can be formed into compression moldings that resemble polystyrene, or into fibres that resemble nylon (Yuen 1974). Pullulan serves as a model substrate for

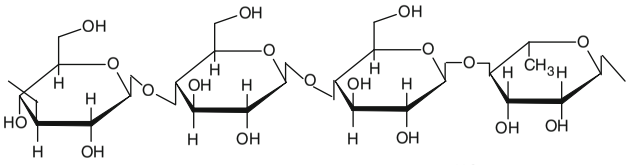
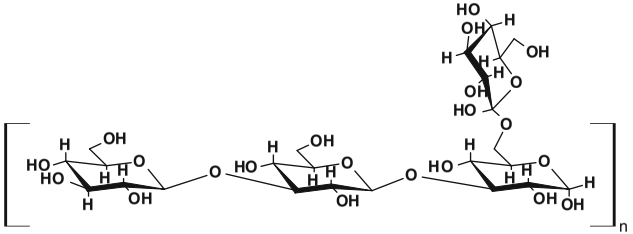
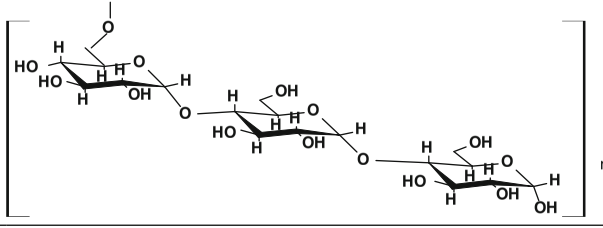
Table 24.1 Some commercially available microbial exopolysaccharides (EPSs)

EPS	Producer organism	Composition	Structure
Xanthan	*Xanthomonas campestris*	Pentasaccharide containing glucose, mannose, glucuronic acid, and acetyl and pyruvate substituents	

(continued)

Table 24.1 (continued)

EPS	Producer organism	Composition	Structure
Dextran	*Leuconostoc mesentroides* *Streptococcus mutans* *Acetobacter* sp.	Polyglucose linked by α-(1 → 6) glucosidic linkages	
Curdlan	*Alcaligenes faecalis*	β-(1 → 3) glucan; polyglucose	

Gellan	*Pseudomonas elodea*	Partially *O*-acetylated polymer of glucose, rhamnose and glucuronic acid	
Scleroglucan	*Sclerotium glutanicum*	Glucose units primarily β-(1 → 3) linked with occasional β-(1 → 6) glycosidic bonds	
Pullulan	*Aureobasidium pullulans*	Glucose units primarily α-(1 → 4) linked with occasional α-(1 → 6) glycosidic bonds	

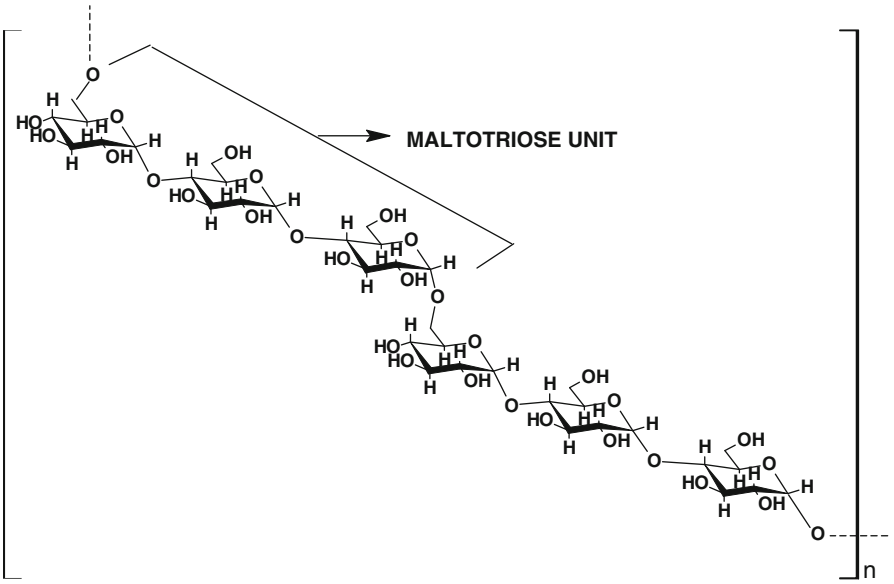


Fig. 24.1 Structure of pullulan with maltotriose as repeating unit

starch debranching enzyme pullulanase, which is industrially used in combination with glucoamylase and β-amylase in the production of glucose and maltose syrups from starch (Goldstein 1990).

24.2 Historical Background

Currently only a small number of exocellular fungal α-glucans are reported in the literature. Pullulan is the best studied α-linked glucan produced by the polymorphic fungus *A. pullulans*. *A. pullulans* was first described by de Bary in 1986 (Cooke 1959). Pullulan has never been found inside the cells of *A. pullulans* (Catley 1980). Bauer (1938) was pioneer to observe the polysaccharide production by *A. pullulans*. First isolated and characterized by Bernier (1958) from culture broths of *A. pullulans*, pullulan has become the object of an ever increasing research effort. Thorough study of this novel polysaccharide was done by Bender et al. (1959) and named as "Pullulan" by the same group. A number of research groups worked for elucidating the pullulan structure and reported that the structure of pullulan could not be narrated as consisting solely of α-(1→4)- and α-(1→6)-linkages, because a small proportion of α-(1→3)-linkages are also reported in the structure (Bouveng et al. 1962; Sowa et al. 1963). Wallenfels et al. resolved the

pullulan structure in the beginning of 1960s (Bender and Wallenfels 1961; Wallenfels et al. 1961, 1965). Catley et al. (1986) evidenced that pullulan may be branched and possess maltosyl or glucosyl groups instead of maltotriosyl residues. Wallenfels et al. (1961) reported the action of enzyme pullulanase on pullulan. The enzyme specifically hydrolyzes the α-(1→6)-linkages in pullulan yielding quantitatively the oligomers $(G_3)_n$, where G_3 represents the α-(1→4)-linked repeating trimer maltotriose, and 'n' is the number of trimeric repeating units in the oligomer. Catley et al. (1986) suspected that there is, perhaps, no unique structure of pullulan, as reported by Catley (1970). Interesting reviews on the structural elucidation of pullulan done so far included those by Gibbs and Seviour (1996), Leathers (2003), Shingel (2004) and Singh et al. (2008c).

Commercial production of pullulan began in 1976 by Hayashibara Company, Okayama, Japan (Tsujisaka and Mitsuhashi 1993). Pullulan production was a natural outgrowth of Hayashibara's original business of starch syrup production. Hayashibara adjusted the growth conditions of the source fungus to produce pullulan products of particular molecular weight and specification. These include food grade (designated as PF) and deionized (PI) products with mean molecular weight of 100,000 (PI-10 and PF-10) or 200,000 (PI-20 and PF-20). Pullulan films were commercialized by Hayashibara in 1982. Today, this company is the principle commercial producer of pullulan. Hayashibara Biochemical Labs (HBL) has granted Pfizer a worldwide license to commercialize and market film-based oral care products containing HBL's patented pullulan. It has also commenced the supply of pullulan powder to Warner-Lambert Company, a subsidiary of Pfizer Inc. Pfizer has introduced the film form oral care product in Canada under the brand name Listerine. Pullulan has been produced commercially for about 30 years and currently sold at US$ 2,000/Kg (Sigma Catalogue, USA), which is too high for many of its potential applications.

24.3 Structural Chemistry

The characteristic dimeric segments of pullulan are [→x)-α-D-glucopyranosyl-(1→4)-α-D-glucopyranosyl-(1→] and [→4)-α-D-glucopyranosyl-(1→6)-α-D-glucopyranosyl - (1→], where x may be either 4 or 6 for the (1→4)-linked segment. The trisaccharide G_3 (maltotriose), the fundamental repeating unit of pullulan contains two (1→4)-linkages and no (1→6)-linkages. For the first time pullulan was isolated from cultures of *A. pullulans* in 1958 and D-glucose was reported to be the main product of acid hydrolysis (Bernier 1958). Bender et al. (1959) evidenced the polymer to be an α-D-glucan with predominantly α-(1→4) linkages on the basis of its positive optical rotation and infra-red spectrum. Elemental analysis of pullulan suggested the compound to have chemical formula $(C_6H_{10}O_5)_n$. In the beginning of 1960s, the research group of Wallenfels and other researchers concluded that

pullulan is a linear α-D-glucan possessing (1→4) and (1→6) linkages in a ratio of 2:1. This conclusion was drawn on the basis of IR spectroscopic data, periodate oxidation and methylation data (Wallenfels et al. 1961, 1965; Bouveng et al. 1962; Sowa et al. 1963). The structural formula of pullulan corresponds to:

$$\left[\alpha-\mathrm{D}-\mathrm{Glc}_p-(1\rightarrow4)-\alpha-\mathrm{D}-\mathrm{Glc}_p-(1\rightarrow4)-\alpha-\mathrm{D}-\mathrm{Glc}_p-(1\rightarrow6)\right]_n$$

However, it was stated that the linear chain of pullulan also contains maltotetraose subunits (Wallenfels et al. 1965) that should be randomly distributed throughout the molecule (Carolan et al. 1983). The frequency of maltotetraose subunits appears to vary on a strain-specific basis. In addition, according to other authors (Taguchi et al. 1973a; Catley et al. 1986), 7% represents the maximum extent to which maltotetraose subunits have been detected. Unlike maltotriose subunits, maltotetraose residues act as potent substrates for many α-amylases. Hydrolysis of pullulan at these sites accounts for decrease in molecular weight in late cultures. Moreover, α-(1→3) and even β-(1→3) as well as β-(1→6) linkages were found in the main backbone of pullulan produced by some strains, in addition to the α-(1→4) linkages (Sowa et al. 1963; Fujii et al. 1984). So, polymaltotriose is not the only structure proposed for pullulan that might be used as generic name for the neutral α-D-glucan elaborated by *A. pullulans*. Bouveng et al. (1963a, b) isolated a β-glucan and an acidic polysaccharide containing galactose, glucose, mannose, and uronic acid in addition to pullulan from the EPS secreted by the strain of *A. pullulans*. Leal-Serrano et al. (1980) isolated a β-glucan containing 68% (1→3) linkages, 32% (1→6) linkages and malic acid. Promma et al. (1993) also reported a malic acid containing EPS in addition to pullulan in their strain but no structural detail was given.

Discovery of an extracellular enzyme from *Aerobacter aerogenes* i.e. pullulanase proved to be a critical tool for the analysis of pullulan structure (Bender and Wallenfels 1961). Pullulanase converted a yeast (*A. pullulans*) α-glucan containing α-(1→6) bonds into maltotriose, thereby describing pullulan as a polymer of (1→6) linked maltotriose subunits (Fig. 24.1). Sometimes, partial acid hydrolysis of pullulan yields isomaltose, maltose, panose and isopanose (Bender et al. 1959; Bouveng et al. 1963a, b; Sowa et al. 1963). Thus, pullulan is often viewed as a polymer of panose or isopanose (Figs. 24.2 and 24.3). Panose [*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranose] and isopanose [*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranose] is the D-glucose containing trisaccharides having (1→4)- and (1→6)-α-D-glucosidic linkages.

The black yeast *A. pullulans* (de Bary) Arnaud is known to synthesize occasionally additional aubasidan-like EPS along with pullulan. In a series of publications (Elinov and Matveeva 1972; Elinov et al. 1974, 1975), the aubasidan-like components, a related group of glucans with α-1,4-D-; β-1,6-D- and β-1,3-D-glycosidic bonds have also been reported. Aubasidan is a highly branched polysaccharide containing a main core of β-1,3-linked D-glucopyranosyl residues, to which the side chains of α-1,4-D-glucosyl residues are attached through β-1,6-D-glucosidic bonds

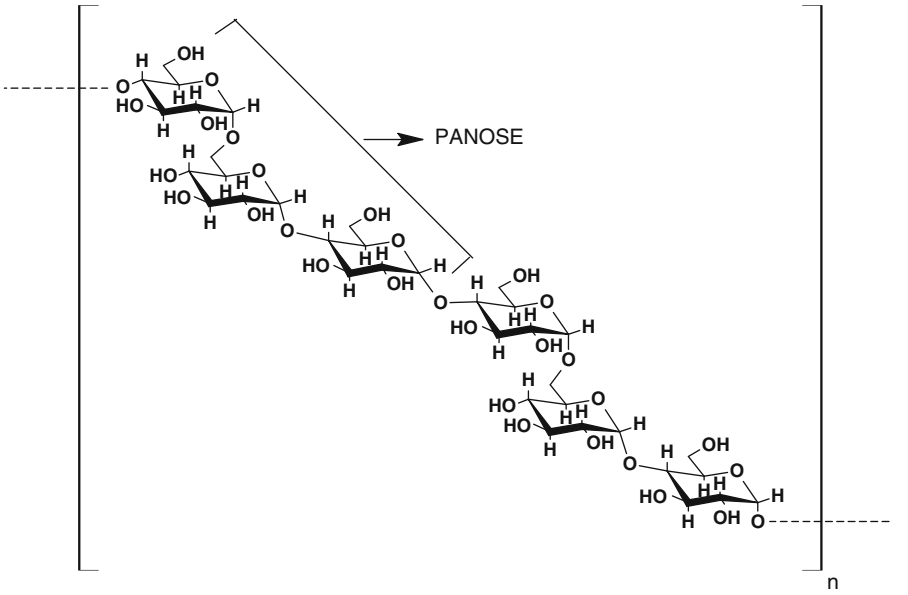


Fig. 24.2 Schematic chemical structure of pullulan with panose as repeating unit

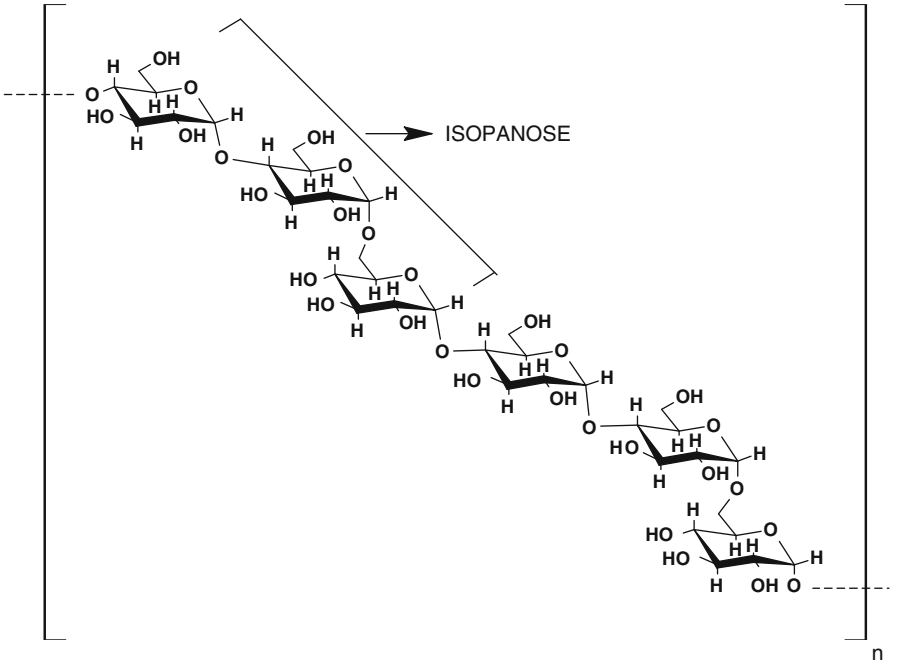


Fig. 24.3 Schematic chemical structure of pullulan with isopanose as repeating unit

(Elinov et al. 1975). The structure of the β-glucan produced by *A. pullulans* described by Elinov has the following structure:

$$\left\{\begin{array}{c} [4\alpha-\mathrm{D}-\mathrm{Glu}\ 1\rightarrow 4\alpha-\mathrm{D}-\mathrm{Glu}1]_n \\ \downarrow \\ \beta-\mathrm{D}-\mathrm{Glu}\ 1\rightarrow 3\beta-\mathrm{D}-\mathrm{Glu}1\rightarrow 3\beta-\overset{6}{\mathrm{D}}-\mathrm{Glu}1 \end{array}\right\}_m$$

Where, m = 4,409–13,900 and n = 0.5–2.0.

The main chain of the polymerized β-D-glucose residues is constant. The branches at C-6 position may involve up to four α-D-glucose units depending on the cultivation conditions (Elinov 1982). Extracellular elaboration of β-(1→3) glucan was not commonly accepted (Finkelman and Vardanis 1982). Navarini et al. (1996) reported that β-glucan possesses weak anionic properties due to the presence of malate residue linked to the EPS through ester bonds.

Yurlova and de Hoog (1997) identified a distinct taxonomic group of *A. pullulans* strains that produce both pullulan and aubasidan-like polysaccharides. Methylation and periodate oxidation data showed that pullulan-like EPS contained a high amount of 1,4-linkages (33.8–59.0%) and aubasidan-like EPS proved to contain a higher amount of 1,3-linkages. Specific rotation $[\alpha]_D$ of pullulan is positive and high, indicating the predominance of α-glycosidic bonds. $[\alpha]_D$ of aubasidan-like EPS is positive but lower than that of pullulan-like EPS, indicating a predominance of β-glycosidic bonds. Infra-red spectroscopy also confirmed the presence of α- and β-glycosidic bonds in the polymers. In IR-spectra of pullulan-like EPS, the peak at $\lambda = 850\ \mathrm{cm}^{-1}$ is significant, while in aubasidan-like EPS, a significant peak is obtained at $\lambda = 890\ \mathrm{cm}^{-1}$ (Yurlova and de Hoog 1997). Madi et al. (1997) also reported a peak at $\lambda = 859.6\ \mathrm{cm}^{-1}$ for pullulan from *A. pullulans* which is interpreted as an α-configuration. The co-existence of α-(1→4)- and α-(1→6)-glycosidic linkages in the pullulan structure can be established by the appearance of a band at $\lambda = 935\ \mathrm{cm}^{-1}$ (Petrov et al. 2002).

Due to co-existence of both α-(1→4)- and α-(1→6)-linkages in a single compound, pullulan structure is often seen as an intermediate between amylose and dextran structures. Consequently, the segmental mobility of pullulan backbone is not uniform, with the regions of increased mobility centered on the α-(1→6) linkages (Dais 1995; Dais et al. 2001). Burton and Brant (1983) reported that the pullulan macromolecule might exhibit slight helical twists on the basis of computational data. Benesi and Brant (1985) completely assigned the NMR signals for all carbon atoms in the repeating unit of pullulan. Pullulan structure has also been determined unambiguously by employing proton and carbon-13 NMR spectroscopy (McIntyre and Vogel 1993; Arnosti and Repeta 1995). The number of α-(1→4)- and α-(1→6) linkages could be quantified for pullulan by NMR spectroscopy. Arnosti and Repeta (1995) evidenced the occurrence of α-(1→6) linkage between every third glucose ring by ^{13}C NMR spectra. Raman spectroscopy has also been employed to establish pullulan structure. Bands at 543 and 480 cm^{-1} appear corresponding to the presence of α-(1→4)- and α-(1→6)-glycosidic linkages in the biopolymer 'pullulan' (Zhbankov et al. 1995).

24.4 Pullulan Producing Microorganisms

Pullulan is produced as a water-soluble, extracellular polysaccharide by certain strains of the polymorphic fungus *Aureobasidium pullulans* (de Bary) Arnaud (formerly known as *Pullularia pullulans* de Bary) *Berkhout* or *Dematium pullulans* (de Bary). The microbial production of pullulan by *Pullularia pullulans* was discovered by R. Bauer in 1938. *A. pullulans* is a ubiquitous fungus isolated commonly from the environment (Cooke 1959; Hermanides-Nijhof 1977). It is found in soil, water and as saprophyte on decaying leaf litter, wood and many other plant materials. *A. pullulans* has a role in the deterioration of both exterior and interior house paints (Brand and Kemp 1973). *A. pullulans* has also been reported as a slime-producing contaminant of paper mills and to colonize optical lenses (Webb et al. 2000). The fungus has been described to be omnivorous and its isolates produce an impressive array of degradative enzymes, including amylases (Federici 1982; Leathers 1987, 1993; Linardi and Machado 1990; Saha et al. 1993), proteases (Ahearn et al. 1968; Federici 1982), esterases (Federici 1982), pectinases (Dennis and Buhagiar 1973; Finkelman and Zajic 1978; Federici 1982) and hemicellulases including xylanase (Flannigan 1970; Biely et al. 1978, 1979; Saha and Bothast 1998) and mannanase (Kremnicky et al. 1996; Kremnicky and Biely 1997). Some of the pullulan producing color-variant strains of *A. pullulans* are also known as natural overproducers of endoxylanase (Leathers et al. 1984; Leathers 1989). The color-variant strains are differentiated from typically pigmented (off-white to black in appearance) strains of *A. pullulans* by their brilliant pigments of red, yellow, pink or purple color (Singh and Saini 2008a) and exhibit 37–44% DNA relatedness (Wickerham and Kurtzman 1975; Leathers et al. 1988). Unlike typically pigmented strains, color-variants have been found only in tropical latitudes, although they may be isolated side-by-side with typical strains.

A. pullulans is a polymorphic fungus and its three distinctive forms are: elongated branched septate [i.e. the septa, or "cross-walls," that divide the hyphae into numerous uninucleated or multinucleated cells] filaments, large chlamydospores and smaller elliptical yeast-like cells. Colonies are initially smooth that eventually become covered with a slime. Initially as yellow, cream, light pink or light brown, the colonies become blackish due to chlamydospore formation. Hyphae are hyaline, smooth and thin-walled, 2–16 μm wide, cells are often wider than long, forming a tough and compact mycelium. *A. pullulans* can be identified by straight conidia and by the presence of lobed chains of thick-walled chlamydospores. Ramos and García-Acha (1975) proposed a complex life cycle of *A. pullulans* containing six subcycles using hanging drop cultures of single cells. First subcycle explained blastospores producing new blastospores usually via polar buds. On the contrary, second described formation of pseudomycelium when these buds failed to separate from the mother cell. Third subcycle included differentiation of blastospores to larger cells (swollen cells) which can then either bud or germinate. This subcycle was inoculum concentration dependent and favored by NH_4^+ as a

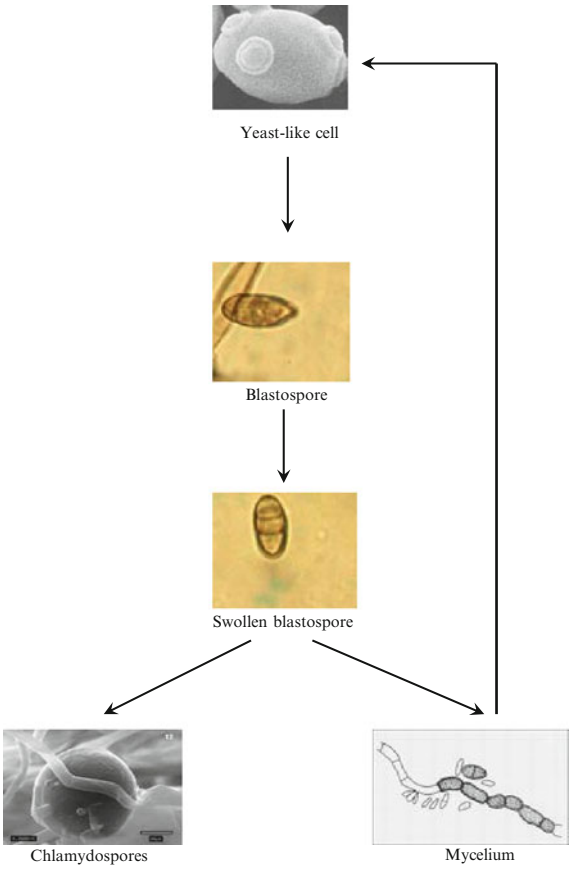
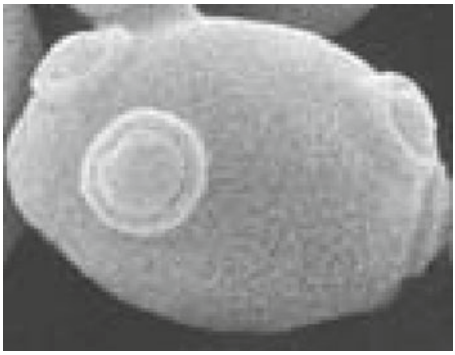


Fig. 24.4 Morphological stages of *A. pullulans* (Source: Ronen et al. 2002)

nitrogen source. The fourth subcycle detailed the formation of swollen septate cells, which could further bud, germinate or form chlamydospores. Last two subcycles explained the germination of the chlamydospores to form mycelium and the production of chlamydospores from the mycelium. This mechanism doesn't propose any direct route for mycelial production as it describes germination of either swollen cells or chlamydospores. But later studies have proposed direct mycelial production via blastospore germination (Pechak and Crang 1977; Kocková-Kratochvílová et al. 1980; Park 1982). *A. pullulans* have five cell morphological stages: yeast-like cells, young blastospores, swollen blastospores, chlamydospores and mycelia (Guterman and Shabtai 1996). The cells' morphological stages follow the subsequent path: yeast-like cell → young blastospore → swollen blastospore → mycelium (Shabtai et al. 1996) as depicted in Fig. 24.4. No telomorph has been identified for *A. pullulans*.

An undesirable characteristic feature of *A. pullulans* is the production of a dark pigment, which is a melanin-like compound and appears dark green to black in color.

Melanins are powerful antioxidant agents capable of protecting cells from damage caused by free radicals (Goncalves and Pombeiro-Sponchiado 2005; Hung et al. 2006). In *A. pullulans*, nearly half the melanins are deposited on cell walls of chlamydospores, and the rest are released into the medium as black granulates (Hung et al. 2006). The pigment 'melanin', most striking characteristic of this yeast-like fungus is insoluble in organic solvents as acetone, ether, benzene, etc. It gives a positive reaction to Nile blue indicating it to be melanin. The melanin is quite impervious to ultraviolet light. The melanin is synthesized both intracellularly as well as extracellularly by the pentaketide pathway (Siehr 1981) during the last stages of the fermentation, when the cell morphology changes from swollen cells to true chlamydospores.



Another undesirable characteristic of *A. pullulans* occurs during its submerged growth; as the fermentation progresses, the culture viscosity decreases due to a decrease in the average molecular weight of the accumulated extracellular pullulan (Catley and Whelan 1971; Kaplan et al. 1987). *A. pullulans* is genetically imperfect and traditionally has been considered to be among the Fungi Imperfecti or Deuteromycetes (Wynne and Gott 1956; Cooke 1962; Hermanides-Nijhof 1977). Recently, *Aureobasidium* has been described as a filamentous ascomycetes (Euascomycetes, order Dothideales, family Dothideaceae) capable of growing yeast-like in culture (de Hoog and Yurlova 1994; de Hoog 1998). The species is of practical interest due to production of extracellular polysaccharide present widely applied in biotechnology. Two varieties of this species i.e. var. *pullulans* and var. *aubasidani* exist which are distinguished by molecular characteristics, nutritional physiology and the structure of exopolysaccharide elaborated by them. *A. pullulans* varieties show medium-dependence differing with EPS structure. The production of pullulan is stimulated with ammonium sulpahte as nitrogen source by *A. pullulans* var. *pullulans* (Imshenetskii et al. 1981b; Bulmer et al. 1987), while sodium nitrate is optimal for the production of aubasidan by *A. pullulans* var. *aubasidsani* (Elinov et al. 1974). Key features distinguishing the varieties of *A. pullulans* i.e. *pullulans* and *aubasidani* are presented in Table 24.2.

Table 24.2 Summary of the characteristics distinguishing the varieties *pullulans* and *aubasidani*

Characteristics	var. *pullulans*	var. *aubasidani*
Exopolysaccharides	pullulan-like	aubasidan-like
Optimal N-source for EPS production	$(NH_4)_2SO_4$	$NaNO_3$
Assimilation of:		
methyl-α-D-Glucoside	+	−
Lactose	+	−
rDNA group	1, 2	3

Source: Yurlova and de Hoog (1997)

Table 24.3 Microbial sources of pullulan

Microorganisms	Reference(s)
Aureobasidium pullulans	Bauer (1938), Cooke (1959), Leathers (2003)
Tremela mesenterica	Fraser and Jennings (1971)
Cytaria harioti	Waksman et al. (1977), Oliva et al. (1986)
Cytaria darwinii	Waksman et al. (1977), Oliva et al. (1986)
Cryphonectria parasitica	Corsaro et al. (1998), Delben et al. (2006), Forabosco et al. (2006)
Teloschistes flavicans	Reis et al. (2002)
Rhodototula bacarum	Chi and Zhao (2003)

Many, but not all, strains of *A. pullulans* are capable of producing pullulan (Ueda et al. 1963; Leathers et al. 1988; Augustin et al. 1997). Apart from *A. pullulans*, a number of other microorganisms summarized in Table 24.3 are also reported as pullulan producers. Pullulan has also been isolated from the saprophytic (sometimes mycoparasitic) fungus *Tremella mesenterica* (Fraser and Jennings 1971), from obligate tree parasitic fungi *Cytaria harioti* and *C. darwinii* (Waksman et al. 1977; Oliva et al. 1986) and from the fungal agent of chestnut blight, *Cryphonectria parasitica* (Corsaro et al. 1998). It is important to mention that this was the first report of *C. parasitica* as a pullulan producer *in vitro*. Jennings and Smith (1973) confirmed the relative composition of the *T. mesenterica* glucan and obtained its sequence by ^{13}C NMR spectroscopy. The sequence information came from the sensitivity of some resonances to the nature of linkages to the next glucosyl moieties. An α- and β-glucan have been isolated from the lichenised ascomycetous *Teloschistes flavicans*. The α-glucan structure obtained has been classified as pullulan, similar to that has been isolated from the fungi *A. pullulans*, *Tremella mesenterica* and *Cyttaria hariotii*, but with different ratios of the component glycosidic linkages (Reis et al. 2002). Its 1:1 linkage ratio is different from those of known pullulans i.e. 2:1. *Rhodotorula bacarum*, an isolate from the Chinese plant leaves collected from the south China has been reported to produce a large amount of pullulan and melanin has not been produced during the fermentation (Chi and Zhao 2003). Forabosco et al. (2006) and Delben et al. (2006) also reported the production and characterization of pullulans from virulent and hypovirulent strains of *Cryphonectria parasitica* (Murrill) M.E. Barr, formerly called *Endothia parasitica* (Murrill)

P.J. Anderson and H.W. Anderson. The spectral properties of the pullulan-like exopolysaccharides produced by *C. parasitica* agree with the presence of both maltotriose and maltotetraose sequences in the polysaccharide backbone.

24.5 Biosynthesis of Pullulan

Literature published on pullulan concerns the control of pullulan elaboration by culture conditions and the relationship between pullulan production and cell morphology. Much is known about the biosynthesis of fungal β-glucans (Ruiz-Herrera 1991), than about pullulan. This is probably because β-glucans are important components of fungal cell wall.

24.5.1 Morphological State of A. pullulans vs. Pullulan Synthesis

Keen interest has been shown by researchers to relate pullulan production with a particular morphological form of yeast-like fungus *A. pullulans*. Despite of complex life cycle of *A. pullulans*, the proposal of EPS production being morphology associated has received a considerable support. Researchers have reported that the culture produce two different types of EPSs. One EPS corresponds to pullulan while, other is frequently described as a water-insoluble jelly-like material. Chlamydospores are the primary cells that are considered as the main EPS producers. Catley (1973) was first to notice that pullulan formation appeared to coincide with the shift in morphology from mycelial to unicellular forms and consequently blastospores were held responsible solely for pullulan elaboration. This proposal of unicellular morphology has been supported by frequent reports of maximum polysaccharide production under culture conditions (Gibbs and Seviour 1996; Reeslev et al. 1997). Some researchers reported that unicellular forms other than blastospores are the major pullulan producers. Simon et al. (1993) revealed that pullulan as well as the insoluble polysaccharide are localized on the outer surface of chlamydospores. The highly dense peripheral layer is ascribed to the chains of pullulan. These chains are arranged in a network covering the inner layer of β-(1 → 3)-glucan composed of glucose and mannose (Fig. 24.5).

Data is also available where swollen cells as well as chlamydospore formation parallels pullulan formation (Simon et al. 1995). Simon and coworkers also suggested that whatever polysaccharide might be synthesized by hyphal forms of *A. pullulans*, it is not pullulan-like. When a significant amount of chlamydospores are present, pullulan is always elaborated, whatever be the culture conditions. Yurlova et al. (1993) showed that hyphal cells can also produce extracellular polysaccharides, while Seviour et al. (1984) supposed that polysaccharide production did not coincide with any major morphological transformations at all. Recent developments by Campbell et al. (2004) showed polysaccharide secretion by

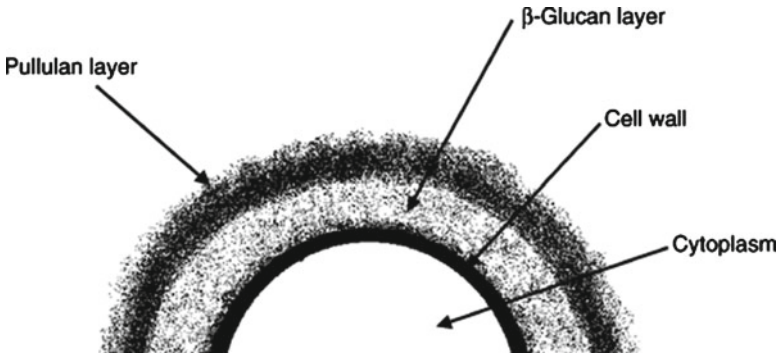


Fig. 24.5 Schematic presentation of the localization of polysaccharide layers on the outer surface of *A. pullulans* cells (Source: Shingel 2004)

chlamydospores and swollen cells but not by conidia or hyphae. Relatively little is known to how the mechanism of biosynthesis of jelly-like β-glucans is associated with the pullulan elaboration. There were indications that the elaboration of insoluble EPSs is dependent on genetic type of *A. pullulans* (Imshenetskii et al. 1983).

Till date, it is unclear why *A. pullulans* produces large amounts of EPS material though many applications for it have been proposed so far. Kikuchi et al. (1973) and Dominguez et al. (1978) suggested pullulan to be a minor cell wall component on the basis of degradation studies with α-amylase and pullulanase. Catley and Hutchinson (1981) reported the lack of association between pullulan elaboration and cell wall regeneration by protoplasts as cell walls were not degraded by pullulanases. Later, large fibrils were detected under electron microscope in the walls of chlamydospores and mycelium of *A. pullulans* which were suggested to be α-glucans but no chemical data has been reported for this theory (Takeo et al. 1993).

24.5.2 Biosynthetic Mechanism of Pullulan Synthesis

Inspite of thorough investigations on cytological as well as physiological characteristics of *A. pullulans*, biosynthetic mechanism of pullulan biosynthesis is not fully understood. Both intact and acetone dried cells have shown synthesis of the α-linked oligosaccharide monomers constituting pullulan (Ueda and Kono 1965; Yun et al. 1994). Taguchi et al. (1973b) reported the synthesis of the pullulan from sucrose by acetone dried cells and from uridine 5′-diphosphate glucose (UDPG) in the presence of adenosine 5′-triphosphate (ATP). UDPG cannot be replaced by ADPG suggesting that the UDPG is the initiator of pullulan chains or pullulan precursors. Further, it was suggested that a lipid intermediate or carrier was involved. Ono et al. (1977) confirmed the formation of glycolipids in mycelia believed to be accumulating pullulan. These observations were supported by Catley and McDowell (1982) who evidenced the crucial role of glucose-containing lipids in pullulan biosynthesis. Pullulan precursor (isopanose) biosynthesis with the aid of

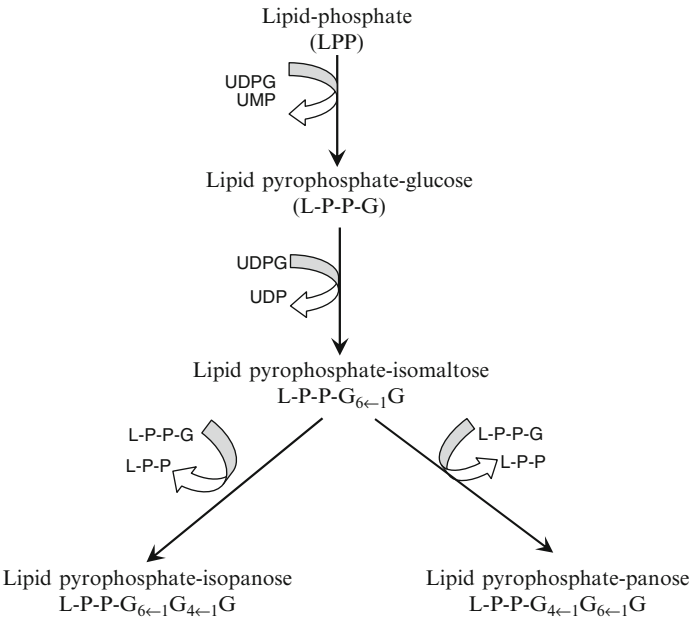


Fig. 24.6 Biosynthetic mechanism postulated for the synthesis of panose and isopanose (pullulan repeating units) involving a lipid phosphate carrier (Source: Gibbs and Seviour 1996)

the phospholipids intermediate (LPh) and its glucose conjugates (L-P-P-G) proposed by Catley and McDowell is depicted in Fig. 24.6. The pathway has two stages, the first involving UDPG-mediated attachment of a D-glucose residue to the lipid molecule (L-P-P) via phosphoester bridge. The second stage involves transfer of the D-glucose residue from UDPG to give lipid-linked isomaltose. Further, isomaltosyl reacts with lipid-linked glucose to yield an isopanosyl or pyranosyl residue that are polymerized into pullulan chain. The polymerization process of panose and isopanose into pullulan is shown in Fig. 24.7. *A. pullulans* is capable of consuming other sugars as mannose, galactose, fructose, etc., but biosynthesis of pullulan from these sources is not clear. Hayashi et al. (1994) established that the carbohydrate metabolites required for EPS formation from maltose i.e. panose [α-Glc-(1→6)-α-Glc-(1→4)-α-Glc] and/or isomaltose [α-Glc-(1→6)-α-Glc] can be synthesized through a glucosyl-transfer reaction in *A. pullulans*. An occasional direct linkage of panose and isopanose was postulated to form maltotetraose elements in pullulan. Rho et al. (1988) reported the polymerization of the carbohydrate precursors stored inside the cells apart from direct conversion of glucose residues into EPS. It is thought that cells first accumulate sugars as reserve and then use it for pullulan production in later stages of culture life cycle. Catley and Kelly (1975) gave experimental evidence for this hypothesis. Simon et al. (1998) found an inverse relation between the concentration of pullulan and the content of intracellular glycogen. The mechanism in accordance to which glycogen gets transformed into pullulan is not well understood. But this hypothesis is useful to explain the independent character of the processes of pullulan production and cell mass growth.

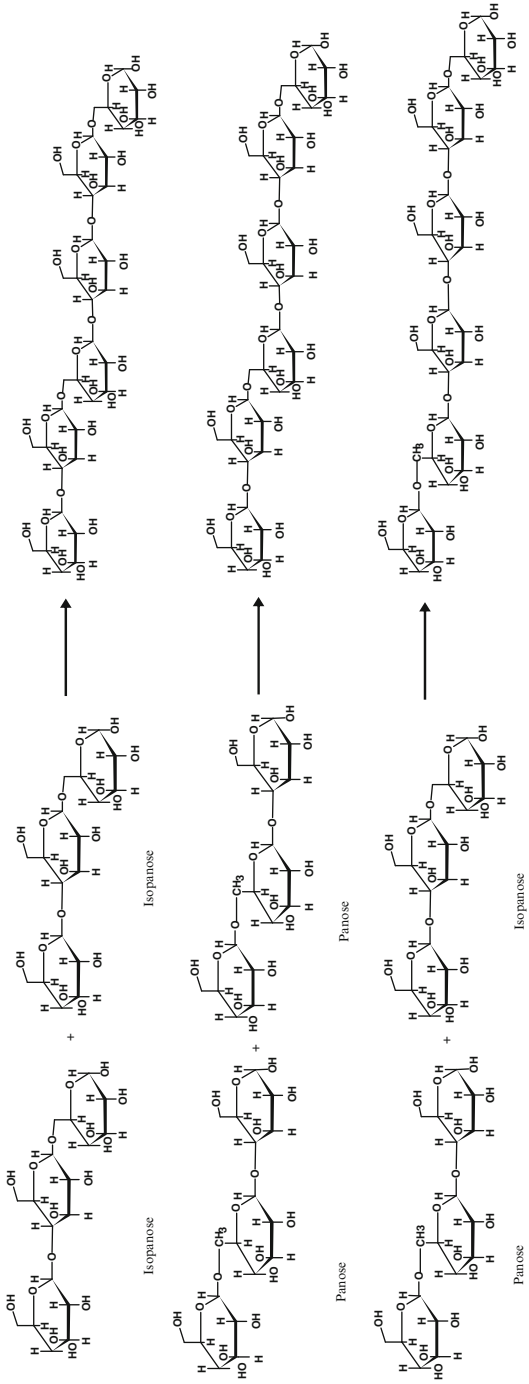


Fig. 24.7 Mechanism postulated for the assembly of panose and isopanose residues into repeating oligosaccharide present in pullulan (Source: Gibbs and Seviour 1996)

24.6 Fermentative Production of Pullulan

Microbially produced polysaccharides have properties which are extremely useful in different industrial applications. Both upstream and downstream processing are important in the production of pullulan to make it cost effective.

24.6.1 Upstream Processing

During the fermentation process/upstream processing of EPS production, the characteristics of the liquid media change drastically. At the beginning, the liquid has a Newtonian behavior with viscosity close to that of pure water but the formation of EPSs results in a rapid increase in the apparent viscosity and a change to non-Newtonian rheology. Since the microbial polysaccharide production process is aerobic, the supply of oxygen in the liquid media during the fermentation is of great importance. Most of the frequently cited work on pullulan is concerned with the control of pullulan synthesis by culture conditions and the relationship between pullulan production and cell morphology. The published accounts of pullulan production are confusing as well as contradictory, which may be due to the reason that multiple factors interact in the regulation of pullulan biosynthesis.

Extensive attention has been paid to the microbial sources for pullulan production (Guterman and Shabtai 1996), pullulan production with respect to the nutrients (LeDuy and Boa 1983; LeDuy et al. 1983; Deshpande et al. 1992), fermentation conditions as pH (Ono et al. 1977; Lacroix et al. 1985), temperature (McNeil and Kristiansen 1990), minerals as Zn^{+2}, Fe^{+2}, Mn^{+2}, Ca^{+2} and Cu^{+2} (Reeslev and Jensen 1995) and process technology for commercial pullulan manufacture (Thibault and LeDuy 1999). The considerable obstacles faced during the pullulan production include high viscosity of the fermentation broth and melanin pigmentation. At the completion of fermentation, the resulting broth consists of microbial cells and cellular debris, residual media components from culture medium and extracellular metabolites produced and excreted during the fermentation.

The comparative studies reveal that *A. pullulans* strains differ considerably with respect to growth, pullulan yield and cell morphology (Ueda et al. 1963; Cernáková et al. 1980; Kocková-Kratochvílová et al. 1980; Park 1982; Gadd and Cooper 1984; Leathers et al. 1988; Silman et al. 1990; Kremnicky et al. 1996). The specific isolates of *A. pullulans* require specific optimal conditions for pullulan production. The sugars such as sucrose, glucose, fructose, maltose, starch, or malto-oligosaccharides support pullulan production by *A. pullulans* (Bender and Wallenfels 1961; Ueda et al. 1963; Catley 1971b; Behrens and Lohse 1977; Imshenetskii et al. 1981b, 1985; Leathers et al. 1988; West and Reed-Hamer 1991; Badr-Eldin et al. 1994). The sugars such as xylose, arabinose, mannose, galactose, rhamnose and lactose, though used less frequently also support pullulan production but with reduced yields (Bouveng et al. 1962; 1963a, b; Ueda et al. 1963; Imshenetskii et al. 1981b; LeDuy

et al. 1983). Glycerol can also contribute to pullulan synthesis but only in the presence of the inducer glucose (Catley 1971b). The induction of pullulan synthesis is inhibited by cycloheximide, indicating that new protein synthesis is required. Pullulan synthesis is also inducible by glucose, fructose or saccharides, which can be broken down to these sugars (Catley 1972).

Often it has been reported that there is correlation between *A. pullulans* morphology and pullulan production, although the cause of correlation is not yet clear. It has been reported that yeast-like forms of *A. pullulans* are the primary producers of pullulan (Catley 1980; Heald and Kristiansen 1985). Pullulan is produced in the late exponential and early stationary phase of cultures primarily by yeast-like cells (Catley 1971a, 1973; Ono et al. 1977). It has also been reported that the rate of pullulan synthesis is independent of pH (Heald and Kristiansen 1985). Pullulan has been reported to be a secondary metabolite produced by yeast-like cells during ammonium limitation (Bulmer et al. 1987). In comparison, a 50% mixture of yeast-like and hyphal cells favour pullulan production under chemostat conditions. Pullulan production by batch cultures is related to the formation of yeast-like cells (McNeil et al. 1989; Reeslev et al. 1991, 1993; Reeslev and Jensen 1995). It has also been reported that bioreactor design and nitrogen sources affect pullulan production by *A. pullulans*, but its morphology has no affect (Gibbs and Seviour 1992). Ammonium and complex nitrogen sources have been found to be superior to nitrate for pullulan production from *A. pullulans* (West and Reed-Hamer 1991; Reed-Hamer and West 1994; Cheng et al. 2011). Lee and Yoo (1993) obtained optimal yields of pullulan at an initial pH of 6.0 using *A. pullulans*. It has been reported that pH 4.5 is optimal for pullulan production, though yeast-like growth is favored at pH 6.5 (Madi et al. 1996). Pullulan formation by *A. pullulans* is associated mainly with swollen cells and chlamydospores (Simon et al. 1993, 1995; Andrews et al. 1994). Osmotolerant *A. pullulans* strains have also been reported for the production of pullulan (Choudhury et al. 2011).

Optimal temperature for pullulan production vary slightly in the range of 24–30°C, on strain-specific basis (Zajic 1967; Imshenetskii et al. 1981a; McNeil and Kristiansen 1990; Tsujisaka and Mitsuhashi 1993; West and Reed-Hamer 1993). Vitamins and mineral salts have also been reported to influence the pullulan synthesis. Bender and coworkers reported that thiamine increases pullulan yields (Bender et al. 1959). Biotin, ferric chloride, manganese chloride and zinc chloride are found to enhance pullulan formation by *A. pullulans* (West and Reed-Hamer 1992; West and Strohfus 1997a). In contrast, inhibitory effects of Fe^{3+} and Zn^{2+} on the development of both yeast-like cells and polysaccharide have also been reported (Reeslev et al. 1993; Reeslev and Jensen 1995).

Several methods and conditions for pullulan production have been reported (Kato and Shiosaka 1975b; Kato and Nomura 1976, 1977; Sugimoto 1978; Kondrat'eva and Lobacheva 1990; McNeil and Harvey 1993; Thorne et al. 1993, 2000; Murofushi et al. 1998). *A. pullulans* has been cultivated batch-wise on media comprising starch hydrolysates of dextrose equivalent 40–50 at 10–15% concentration for commercial production (Tsujisaka and Mitsuhashi 1993). The medium also contained peptone,

phosphate and basal salts, and the initial culture pH was 6.5. Optimal pullulan yield was obtained within about 100 h at 30°C.

A number of complex carbon sources have been reported for pullulan production, including beet molasses (Roukas 1998; Roukas and Liakopoulou-Kyriakides 1999), carob pod (Roukas and Biliaderis 1995), cornmeal hydrolysates (Imshenetskii et al. 1985), corn syrup (West and Reed-Hamer 1991, 1993), fuel ethanol fermentation stillage (Leathers and Gupta 1994), grape skin pulp (Israilides et al. 1998), olive oil and sucrose (Youssef et al. 1998), peat hydrolysate (Boa and LeDuy 1984, 1987), hydrolyzed potato starch (Barnett et al. 1999), spent grain liquor (Roukas 1999) and spent sulfite liquor (Zajic et al. 1979). Mixed-culture techniques have been employed for the utilization of lactose (LeDuy et al. 1983) and inulin (Shin et al. 1989). Vijayendra and coworkers isolated *Aureobasidium pullulans* CFR-77 and used it for the production of pullulan employing jaggery as a carbon source (Vijayendra et al. 2001). Jaggery supported good growth of *Aureobasidium pullulans* and the pullulan produced was pigment free and more highly viscous as compared to that derived from sucrose. Pullulan production from beet molasses, via pretreatment with sulphuric acid and activated carbon has been reported (Lazaridou et al. 2002). The production of pullulan by *Aureobasidium pullulans* can be enhanced by yeast extract or soybean pomace as a nitrogen source (Seo et al. 2004). Various carbon sources (20 g/L) namely, glucose, gluconic acid, glucosamine, fructose, maltose, dextrin, and cellulose have been screened for the production of pullulan. In this study, glucose, sucrose, and dextrin have been reported to be better carbon sources for pullulan production. Optimal conditions like effect of composition of feed solution, dilution rate, and concentration of sucrose in feed solution for the continuous production of pullulan were also determined in a bioreactor (Seo et al. 2006).

The production of pullulan in batch (Vijayendra et al. 2001) and fed-batch fermentations (Shin et al. 1987; Moscovici et al. 1996) has been reported. Continuous fermentation has also been carried out for pullulan production (McNeil et al. 1989; Schuster et al. 1993; Reeslev et al. 1997). Pullulan production using immobilized *A. pullulans* cells have been studied by a number of workers (Mulchandani et al. 1989; West and Strohfus 1996, 1997b, 1998; West 2000; Ürküt et al. 2007). Ürküt et al. (2007) has reported the production of pullulan from synthetic medium by *Aureobasidium pullulans* P56 immobilized in Ca-alginate beads using batch and repeated batch fermentation systems. The results suggested that the immobilization of *A. pullulans* cells in Ca-alginate gel beads is suitable for batch and repeated batch production of pullulan. Use of a reciprocating plate bioreactor for pullulan fermentations has been demonstrated by Audet et al. (1996). Modeling of pullulan fermentation by using a color variant strain of *A. pullulans* has been reported recently (Cheng et al. 2010). Enhanced production of pullulan in *A. pullulans* by a new process of genome shuffling has been reported very effective (Kang et al. 2011).

Recently, response surface methodology (RSM) which is a collection of statistical techniques for designing experiments, building models, evaluating the

effect of factors and searching optimum conditions for desirable responses is used. The classical method involving variation of one factor at a time, does not allow evaluation of the combined effects of all the factors involved in a particular process and is a time-consuming methodology. Above mentioned restrictions can be overcome by the use of RSM, which identify and quantify the various interactions among the factors involved (Li et al. 2002; Göksungur et al. 2005). RSM is applied extensively for optimizing the culture medium conditions and other parameters in the bioprocesses. Many researchers have worked on optimizing medium and process parameters for pullulan production from *A. pullulans* (Singh et al. 2009a; Jiang 2010; Göksungur et al. 2011). Göksungur et al. (2005) described the optimization of pullulan production by RSM in a stirred tank reactor using free cells of *A. pullulans* P56. They investigated the sugar concentration, agitation, and aeration at three different levels using response surface methodology. Lin et al. (2007) investigated six factors i.e. strain, carbon source, nitrogen source, nitrogen concentration, aeration and initial pH for their effects on pullulan production by *A. pullulans* using 2-level fractional factorial design at shake flask level. Simultaneous optimization of concentration and molecular weight of exopolysaccharide was carried out. The effect of major factors together with the two-factor interactions was observed and empirical models were used for optimization of levels of six factors. Ürküt et al. (2007) worked on optimization of pullulan production using immobilized *A. pullulans* by response surface methodology in batch and repeated batch fermentation systems. RSM was used to investigate the effect of three parameters i.e. initial pH, agitation speed and incubation time on pullulan concentration. Ca-alginate beads were used consecutively for six runs without any marked loss of activity in repeated batch fermentations. Singh et al. (2009a) optimized the critical medium components for pullulan production by *A. pullulans* FB-1.

24.6.2 Downstream Processing

Downstream processing is required to obtain pure biopolymer from the fermentation broth and it comprises of cell harvesting from culture broth after cultivation, removal of melanin pigments produced during fermentation and precipitating the polymer with a suitable solvent. Melanin is one of the major obstacle in pullulan production and it is responsible for dark green to black color of the broth. Siehr (1981) reported intracellular as well as extracellular synthesis of melanin by the pentaketide pathway during the last stages of fermentation when the cell morphology changes from swollen cells to true chlamydospores. Thus, an appropriate downstream processing of the fermentation broth is required to alleviate the pigmentation problem in case of melanin producing strains. Generally from strains producing melanin-free pullulan, recovery and purification is accomplished with one precipitation step using a suitable organic solvent. The organic solvent usually used is from

the group of alcohols, esters and ethers with three or more carbon atoms. The studies and evaluations on processes for separation and purification of pullulan, led to the discovery that solvents of relatively higher molecular weight and slightly lower hydrophilicity are more suitable for such processes in comparison with those having lower molecular weight and higher hydrophilicity as methanol, ethanol and acetone. The solvents having relatively low hydrophilicity such as propyl alcohol, isopropyl alcohol, tetrahydrofuran, dioxane, etc. are capable of effecting complete precipitation of pullulan with an addition of less volume of the solvent (Wu et al. 2009). These solvents also display sufficient impurity removing efficacy (Kato and Nomura 1977). Further purification of pullulan can be achieved by ultrafiltration and ion-exchange resins (Kachhawa et al. 2003). A flow diagram showing various steps for pullulan purification is shown in Fig. 24.8.

Kikuchi et al. (1973) developed a purification procedure for separating pullulan by giving treatment to the culture media with acetyltrimethylammonium hydroxide. Pullulan purification by centrifugation, precipitation with three volumes of methanol and then by filtration has been reported (Boa and LeDuy 1984, 1987). Alternatively, centrifuged broth is treated with acetone and ethanol mixture 1:1 (v/v) to precipitate the exopolysaccharide (Shin et al. 1989). Pullulan purification by precipitation of centrifuged broth with an equal volume of tetrahydrofuran (THF) has been carried out by Leathers et al. (1988). Pullulan precipitation from the supernatant with an equal volume of ethanol has also been reported (Mulchandani et al. 1989). Pollock has recovered polysaccharide from the clarified broth by precipitation with IPA (Pollock et al. 1992). One volume of IPA was used and the precipitates were dried to a constant weight in an oven at 80°C. Roukas and Biliaderis initially precipitated crude polysaccharide with two volumes of acetone, dissolved in distilled water and reprecipitated with absolute ethanol (Roukas and Biliaderis 1995). Pullulan in the supernatant was recovered by addition of two volumes of ethanol at 4°C (Barnett et al. 1999; Youssef et al. 1999; Lazaridou et al. 2002). Vijayendra and coworkers purified pullulan by precipitation using two volumes of isopropyl alcohol to one volume of cell free fermentation broth followed by washing of precipitates with acetone and dried to a constant weight at 90°C (Vijayendra et al. 2001). Pullulan produced by a novel color variant strain of *A. pullulans* FB-1 has been purified from cell free extract using two volumes of isopropyl alcohol and purity of polysaccharide was comparable with commercially available pullulan from Sigma, USA (Singh and Saini 2008b; Singh et al. 2009b).

Pullulan has been precipitated using two volumes of absolute ethanol from cell free broth (Kachhawa et al. 2003). The precipitates obtained were dried till a constant weight in an oven at 60°C to give the crude pullulan. To separate melanin, demelanization by adsorption on activated charcoal or by use of solvent/solvent blends or by solvent/salt combinations has been used. Among all these treatments, a combination of ethanol/ethyl methyl ketone in 60:40 ratio has been reported to be the most effective. Cross-flow membrane technology for pullulan recovery by filtration has also been reported (Yamasaki et al. 1993a, b).

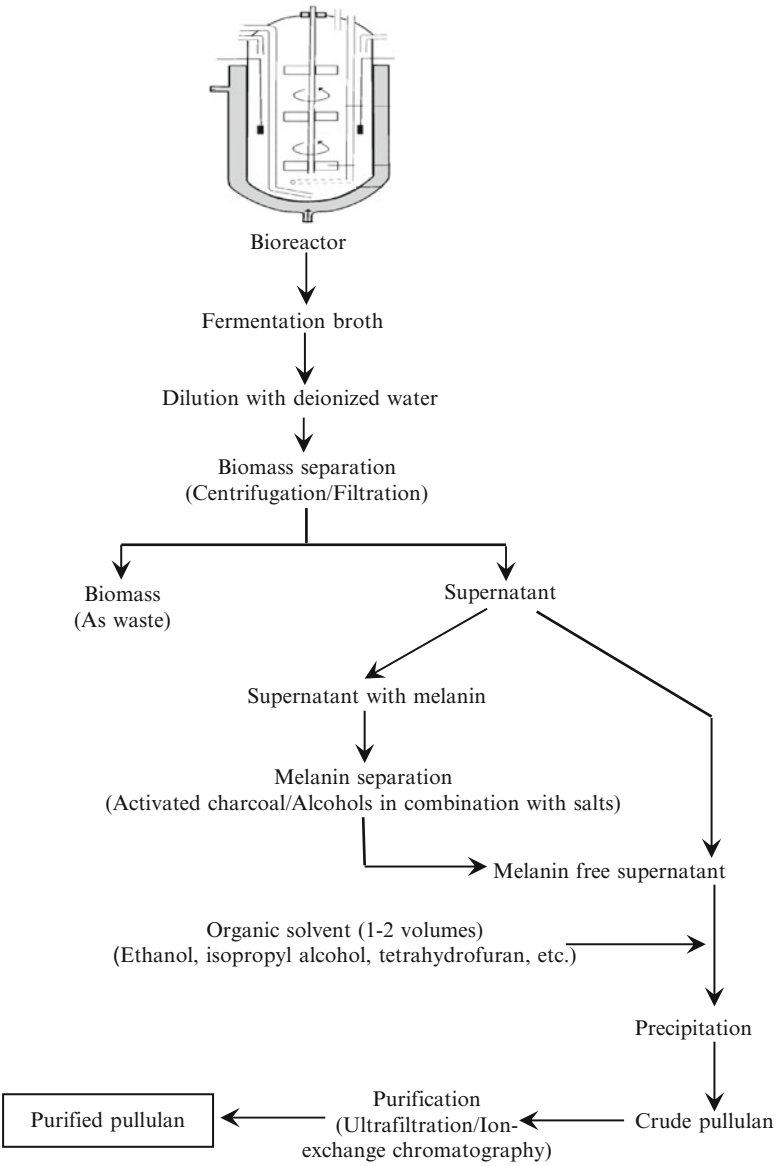


Fig. 24.8 Flow diagram for downstream processing of pullulan

24.7 Kinetics of EPS Production by *A. pullulans*

Attempts to develop models for polysaccharide production in *A. pullulans* have been made by several groups based on the early work of Klimek and Ollis (1980). An autonomous (logistic) rate equation was considered where the growth rate of the organism

increased linearly with an increase in biomass concentration. But as the concentration reached its maximum, growth rate approached zero which can be shown as:

$$\frac{dx}{dt} = \mu X\left[1 - \frac{X}{Xm}\right]$$

Where

X = biomass concentration (g/L)
dx/dt = rate of increase in biomass concentration
μ = specific growth rate constraint (h^{-1})
Xm = maximum biomass concentration

Luedeking and Piret (1959) initially proposed the product formation rate equation as given below:

$$\frac{dp}{dt} = \alpha X + \left[\frac{\beta\, dx}{dt}\right]$$

Where

α = non-growth associated product formation constant
β = growth associated product formation constant

This model was applied by Klimek and Ollis (1980) to the batch culture data published earlier (LeDuy et al. 1974). Pullulan production was suggested to be growth associated in this case. Kristiansen et al. (1983) used similar approach on their batch and continuous culture data and suggested that polysaccharide production was both growth asssociated as well as non-growth associated. The value for α and β obtained from batch and continuous fermentations agreed closely. Luedeking and Piret model was applied by Boa and LeDuy (1987) to pullulan production on peat hydrolysate. The results revealed that pullulan synthesis was mainly growth-associated in batch as well as in continuous culture but entirely growth-associated in fed batch fermentations. Values for α and β varied considerably with fermentation conditions as temperature, aeration, pH, etc.

Later, a modified logistic equation was proposed by Mulchandani et al. (1988) and Luong et al. (1988). The earlier published models were critically examined as they did not always closely predict the kinetics of microbes. A constant, θ, was incorporated that took into account any deviations from exponential growth shown by their cells. Thus,

$$\frac{dx}{dt} = \mu X + \left[1 - \frac{(X)\theta}{Xm}\right]$$

Where θ > 0, suggesting that when θ is very large, then (dx/dt) = μX. But when θ = 1, then the Klimek and Ollis (1980) logistic rate equation is obtained. All the models suggested above are simple and support the view that pullulan and other EPSs are not strictly secondary metabolites or wholly non-growth associated as proposed by Bulmer et al. (1987).

24.8 Properties of Pullulan

The regular (1 → 6) linkages in pullulan are thought to impart structural flexibility and enhanced solubility (Buliga and Brant 1987a). Recently, Angioletti (2003) has also confirmed the same. This allows pullulan to mimic the synthetic polymers derived from petrochemical-derived polymers in many aspects as biocompatibility, biodegradability, and both human as well as environmental compatibility. Pullulan powders are white, non-hygroscopic and dissolve readily in hot or cold water. It is non-toxic, non-mutagenic, odorless, tasteless and edible with a number-average molecular weight (M_n) of about 100–200 kDa and a weight-average molecular weight (M_w) of about 362–480 kDa (Okada et al. 1990). Values of M_w/M_n have been frequently reported and lie between 2.1 and 4.1 (Wiley et al. 1993; Roukas and Mantzouridou 2001; Petrov et al. 2002). These values are significantly lower in comparison to the other industrially important exopolysaccharides such as amylose and dextran. The difference in the biosynthetic pathways or the cell morphology-regulated mechanism for pullulan elaboration might be the possible reason for the same. It is insoluble in organic solvents except dimethyformamide and dimethylsulfoxide (Sugimoto 1978; Tsujisaka and Mitsuhashi 1993). Aqueous solutions of pullulan are viscous but don't form gels. The viscosity of water solutions of pullulan is proportional to the molecular weight of the pullulan. It is also stable over a broad range of pH conditions in solution form. It does not liberate any harmful gas even when burnt and is spontaneously decomposed by microorganisms even when discarded as it is. Pullulan has excellent adhesive properties when dried and excellent foam retention properties when it is dissolved in water. The excellent adhesive properties of pullulan together with its low viscosity, enables active ingredients to be effectively applied to the body. This also makes it suitable as a binder for agglomerated products, helping to improve the performance of existing manufacturing processes by providing an opportunity to create new product presentations for the consumer. Pullulan also has good oxygen barrier properties and has been developed for food-packaging applications. The main quality parameters of pullulan are depicted in Table 24.4.

Table 24.4 Main quality parameters of pullulan

Parameter	Specification
Appearance	White or yellowish-white
Water solubility (25°C)	Easily soluble
Specific optical density $[\alpha]D_2O$ (1% in water)	Minimum +160°
Polypeptides (%)	Maximum 0,5
pH of solution	5–7
Mineral residue-ash (sulphated,%)	Maximum 3
Moisture (loss on drying,%)	Maximum 6
Molecular weight (range, kDa)	100–250

24.8.1 Molecular and Hydrodynamic Properties

Detailed studies have been carried out for hydrodynamic and molecular properties of pullulan in solution (Kato et al. 1982, 1984; Kawahara et al. 1984; Nishinari et al. 1991; Nordmeier 1993). Hydrodynamic properties depict the response of the biopolymer to the action of a solvent and this depends upon the conformational flexibility of the polymer chain. Okada et al. (2002) performed NMR experiments to find the equilibrium hydration shell of pullulan chain in solution. On this basis, it was concluded that pullulan chain in solution consists of 13 water molecules per glucose residue. Kato et al. (1984) derived the molecular weight (*M*)-intrinsic viscosity relationship for pullulan with *M* greater than 1×10^5 as under:

$$[\eta] = 2.21 \times 10^{-2} M^{0.06} \left(\text{cm}^3 / \text{g}\right) \tag{24.1}$$

These were in good agreement with the results reported by the same group earlier (Kato et al. 1982) and data of Nishinari et al. (1991). Dependence of the radius of gyration (R_g) and hydrodynamic radius (R_H) on *M* obeyed the following equations given by Kato et al. (1984).

$$R_g = 1.47 \times 10^{-2} M^{0.58} \left(\text{nm}\right) \tag{24.2}$$

$$R_H = 2.25 \times 10^{-2} M^{0.52} \left(\text{nm}\right) \tag{24.3}$$

Behavior of pullulan as a random coil in an aqueous solution was confirmed from the exponents in Eq. 24.1–24.3. Fishman et al. (1987) obtained close value (0.595) of exponents in Eq. 24.2 from the size-exclusion chromatographic data. The ratio (R_g/R_H) was determined for estimating the extent perturbance of chain dimensions of pullulan by the exclusion volume effect (Kato et al. 1984). The 'ρ' ratio, a dimensionless quantity, is a very useful parameter for discussing the discrepancy between the static and dynamic radius. This ratio was smaller than the theoretically predicted value for a linear polymeric chain indicating the presence of the excluded volume effect. Tsvetkov (1986) stated the exclusion volume effect to be responsible for the non-linear logarithmic plot of the Mark-Kuhn-Hauwink dependence ($[\eta] = KM^{\alpha}$) of pullulan within a wide range of *M*. Thus, $[\eta]$ versus *M* dependence was depicted by the following equations: When *M* is greater than 3×10^4,

$$[\eta] = 1.96 \times 10^{-2} M^{0.66 \pm 0.012} (\text{cm}^3 / \text{g}) \tag{24.4}$$

When *M* is smaller than 3×10^4,

$$[\eta] = 6.16 \times 10^{-2} M^{0.56 \pm 0.042} (\text{cm}^3 / \text{g}) \tag{24.5}$$

A good correlation between Eq. 24.4 with the findings of Kato et al. (1984) has been depicted in Eq. 24.1. The value of exponent in Mark-Kuhn-Hauwink dependence for pullulan (0.65–0.67) was found to be intermediate between the exponents for dextran (0.50) and amylose (0.68–0.70).

Pullulan conformational behavior in solution of dimethyl sulphoxide (DMSO) has been extensively studied. The value of exponent 'α' in the Mark-Kuhn-Hauwink equation was found to be 0.75 and the intrinsic viscosity of pullulan in DMSO is reported to be higher in comparison to that in water (Pavlov and Korneeva 1995). It is well established that primary hydroxyl groups in the adjacent (1→4)-linked glucose residues of pullulan form hydrogen bonding by means of NMR spectroscopy. The hydrogen bonding pattern is not found in case of DMSO. After analyzing the flow birefringence, value of the thermodynamic swelling parameter (ε) for pullulan in water and DMSO has been reported to be 0.112 and 0.18, respectively. Higher value of ε for pullulan in DMSO may be attributed to the pronounced exclusion volume effect. Nakanishi et al. (1993) also observed similar chain expansion phenomenon in DMSO solution for other polysaccharides. Shingel (2002) recorded IR spectra for pullulan in water as well as in DMSO and found a pronounced increase of a band in the structure-sensitive spectral region (950 cm^{-1}). This change in the intensity of the band may be due to conformational transitions of the polysaccharide caused by rotational isomerism of the pyranose rings about the glycosidic linkage. Keilich and Bittiger (1972) investigated the conformation of the EPSs differing in the type of glycosidic linkages by circular dichroism (CD). The CD spectrum of pullulan was interpreted as a superposition of the spectra of dextran and amylose.

24.8.2 Biological Activity

Polysaccharides are known to exert inherent physiological activity due to presence of hydroxyl groups in high concentration. Tsujisaka and Mitsuhashi (1993) reported the use of a dextran-based blood plasma substitute thus explaining the importance of neutral EPSs as bioactive polymers. The blood plasma substitutes maintain the volume of circulating blood and its osmotic pressure in cases of massive blood loss. These preparations are the subject of intense investigations and are widely administered all over the world (Mehvar and Shepard 1992; Haljamäe 1993; Dubick and Wade 1994; Heller 1998). Attempts have been made to develop blood plasma substitutes based on pullulan (Seibutsu and Kenkyujo 1983). Shingel (2004) summarized all efforts made so far to understand the pharmacokinetics of intravenously applied pullulan in terms of the molar mass and concentration. The polymer should be free from low and high molar mass components. Pullulan-based intravenous preparation exhibited a superior therapeutic efficiency in comparison to dextran because of biodegradation of the polysaccharide. Pullulan also exhibits a great affinity towards the liver and is effectively endocytosed by the parenchymal liver cells (Tabata et al. 1999), but it has no mutagenic, carcinogenic and toxicological activities (Kimoto et al. 1997). Great affinity of pullulan towards liver is considered to be the reason for its short half-life period of circulation in blood (Yamaoka et al. 1993).

24.9 Pullulan Degrading Enzymes 'Pullulanases'

Pullulanases are defined as enzymes that hydrolytically cleave pullulan, an α-glucan and belong to a family of 13 glycosyl hydrolases, also called the α-amylase family (Janecek et al. 1997; Matzke et al. 2000). Pullulanases are widely distributed among animals (Lee and Forstner 1990; Witmer and Martínez del Rio 2001), plants (Renz et al. 1998; Wu et al. 2002), fungi (Nguyen et al. 2000; Aquino et al. 2001), yeasts (Wartmann et al. 1995) and bacteria (Albertson et al. 1997; Lee et al. 1997; Possot et al. 1997; Yamashita et al. 1997; Yebra et al. 1997; Kroschwitz 1998). All pullulanases can also degrade other polysaccharides in addition to pullulan reported on the basis of biochemical studies. Detailed enzymatic mechanisms of substrate degradation and the resulting final products are different in each case. Pullulan undergo enzymatic hydrolysis by both (1–6)-α-D- and (1–4)-α-D-pullulanases. The (1–6)-α-D-pullulanases cleave the (1–6)-α-D-glucopyranosidic linkages. Complete hydrolysis of pullulan using (1–6)-α-D-pullulanase yields maltotriose as major product along with traces of maltotetraose. The (1–4)-α-D-pullulanases act on (1–4)-α-D-glucosidic linkages at their reducing ends adjacent to (1–6)-α-D linkages. Complete hydrolysis of pullulan with (1–4)-α-D-pullulanase gives isopanose as the main product. Products of enzymatic pullulan degradation are used in industry.

24.9.1 Types of Pullulan Degrading Enzymes

Enzyme classification is based on two biochemical features i.e. the catalytic reaction and substrate specificity as recommended by the International Union of Biochemistry and Molecular Biology (IUBMB). Pullulan hydrolyzing enzymes have been classified into four types (Webb 1992; Kuriki and Imanaka 1999) and mode of action of each type is depicted in Fig. 24.9.

1. Glucoamylses (EC 3.2.1.3), hydrolyzing pullulan from non-reducing ends to yield glucose as product.
2. Pullulanases (α-dextrin 6-glucohydrolase, EC 3.2.1.41), which hydrolyze α-(1,6)-glucosidic linkages to produce maltotriose.
3. Isopullulanases (EC 3.2.1.57), that hydrolyze α-(1,4)-glucosidic bonds of pullulan to produce isopanose (6-*O*-α-maltosylglucose).
4. Neopullulanases (EC 3.2.1.135), which hydrolyze α-(1,4)-glucosidic bonds of pullulan to produce panose (6-*O*-α-glucosylmaltose).

The sources and localization of pullulan degrading enzymes are depicted in Table 24.5. In food industry, pullulan degrading enzymes are used in brewing process and starch hydrolysis together with β-amylase to produce starch syrup high in maltose content (Belitz and Grosch 1999).

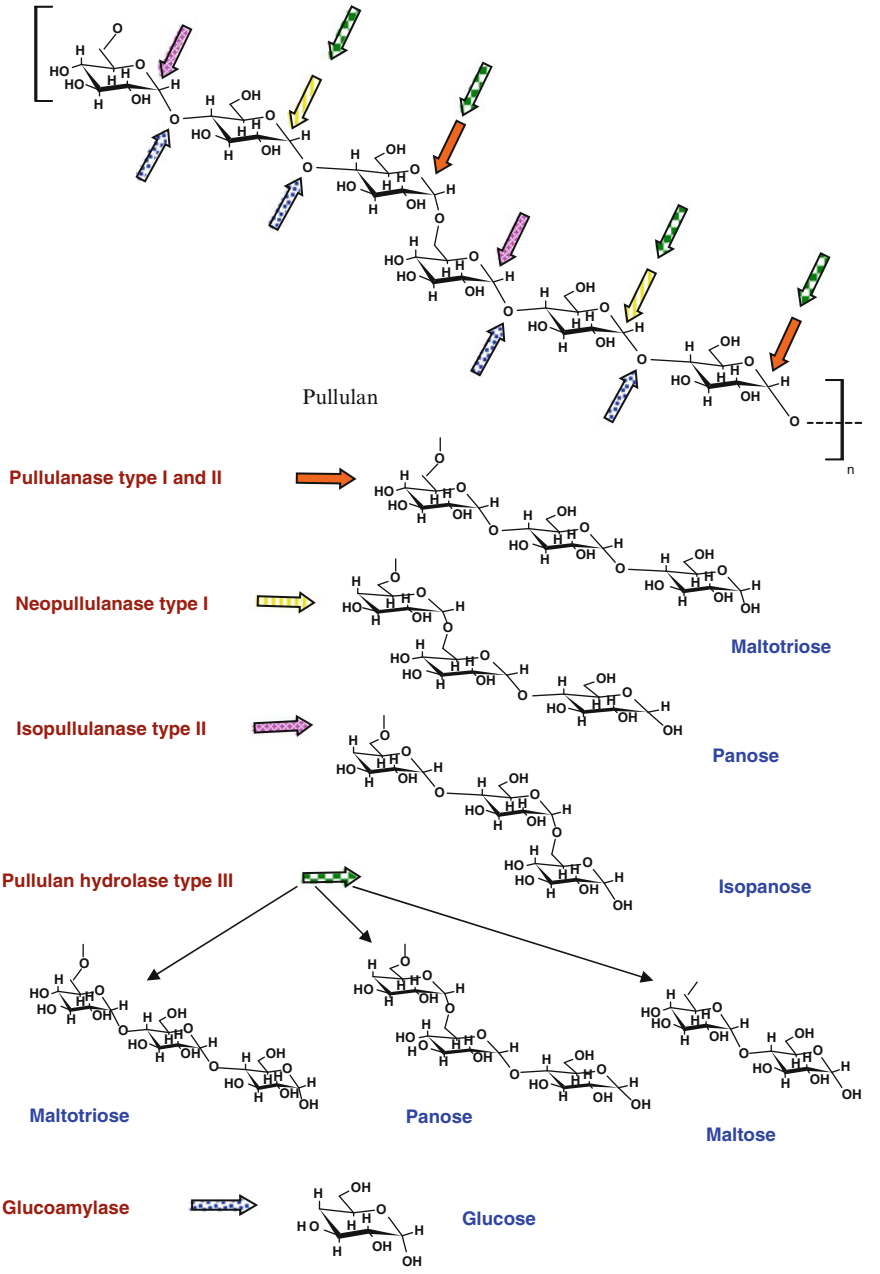


Fig. 24.9 Mode of action of different types of pullulanases

Table 24.5 Sources and localization of types of pullulan degrading enzymes

Enzyme	Producer microorganism	Molecular weight (kDa)	Localization
Pullulanase type I			
Pullulanase	*Bacteroids thetaiotaomicron*	75	Extracellular
Pullulanase	*Klebsiella aerogenes*	150	Intracellular
Thermostable pullulanase type I	*Caldicellulosiruptot saccharolyticus*	95,7	Extracellular
Amylopullulanase			
Amylopullulanase	*Bacillus* sp. KSM-1378	210	Extracellular
Pullulanase type II	*Pyrococcus woesei*	90	Cell-associated
Amylase-pullulanase	*Bacillus circulans* F-2	220	Extracellular
Neopullulanase			
Neopullulanase	*Bacillus polymyxa*	58	Extracellular
Neopullulanase-α-amylase	*Thermoactionomyces vulgaris* R-47	–	Intracellular
Pullulan hydrolase type III			
Pullulan hydrolase type III	*Thermococcus aggregans*	726	–
Glucoamylase			
Glucoamylase	*Thermoanaerobacterium therosaccharolyticum*	77,5	Extracellular
Glucoamylase	*Flavobacterium* sp.	–	Cell-bound

Source: Domań-Pytka and Bardowski (2004)

24.9.1.1 Type I Pullulanases

Type I pullulanases hydrolyze typically in an endo-wat the α-(1,6)-glucosidic linkages in pullulan and in branched oligosaccharides to yield maltotriose and linear oligosaccharides, respectively. They are also termed as true pullulanases, limit dextrinases, debranching enzymes, amylopectin 6-glucanohydrolases (Saha et al. 1989; Webb 1992) or α-dextrin 6-glucanohydrolases (EC 3.2.1.41). Wallenfels et al. (1966) discovered pullulanase in *Aerobacter aerogenes* (now reclassified and belong to genus *Klebsiella*). They are not able to hydrolyze either α-(1,4)-linkages in glucans or α-(1,6)-linkages in panose and glycogen (Kashiwabara et al. 1999). Thermoactive and thermostable pullulanases play a vital role in the starch industry, in the production of maltose syrups (Gantelet and Duchiron 1998) and in production of high purity glucose and fructose (Niehaus et al. 1999). Kusano et al. (1990) reported the use of thermostable and acidophilic pullulanase in industrial production of glucose syrup. Useful industrial substrates as maltose, amylose and glucose are being produced by type I pullulanases by debranching starch with or without α-amylase, β-amylase and glucoamylase (Takizawa and Murooka 1985). Saha and Zeikus (1989) reported the use of pullulanase type I in production of low carbohydrate (low caloric) "light beer" by adding it with fungal α-amylase or glucoamylase to wheat during fermentation.

24.9.1.2 Type II Pullulanases 'Amylopullulanases'

Amylopullulanases possess unique biochemical properties as it exhibits both α-amylase and pullulanase activities. They are known to cleave α-1,4- and α-1,6-glucosidic linkages in starch and other related polysaccharides (Mathupala et al. 1993; Ganghofner et al. 1998). The amylase and pullulanase activites of amylopullulanases are active over the same range of pH and temperature. An alkaline amylopullulanase can be used as an additive in dishwashing and laundry detergents at alkaline conditions (Yuji et al. 1996; Dong et al. 1997).

24.9.1.3 Pullulan Hydrolases Type II 'Isopullulanases'

Isopullulanases (EC 3.2.1.57) hydrolyze α-(1,4)-D-glucosidic linkages of pullulan to yield isopanose as product. They are also termed as pullulan 4-glucanohydrolase and are not able to hydrolyze starch (Webb 1992). Isopullulanaes have been found in fungi and not in bacteria (Aoki and Sakano 1997).

24.9.1.4 Pullulan Hydrolases Type III

Type III pullulan hydrolases are able to hydrolyze both α-1,4- and α-1,6-glucosidic bonds in pullulan and yield a variety of products including maltotriose, panose and maltose. They are also able to hydrolyze starch, amylose and amylopectin to form maltotriose and maltose as the major products (Niehaus et al. 2000; Bertoldo and Antranikian 2002). Being highly thermostable, they play an important role in food, chemical and pharmaceutical industries. The enzyme is not only exploited in glucose/fructose syrup production, but also used in the synthesis of nonfermentable anticarcinogenic carbohydrates and antistaling agents in bakery.

24.9.1.5 Glucoamylases

Glucoamylases (CGA, EC 3.2.1.3) consecutively split α-1,4-glucosidic linkages of α-glucans as pullulan to yield glucose. They are also termed as exoglucanases. The synonyms used for glucoamylases in literature include glucan 1,4-α-glucosidase, amyloglucosidase, γ-amylase, lysosymal α-glucosidase, acid maltase and exo-1,4-α-glucosidase (Webb 1992). The glucoamylase activity is reported in many fungi and a few bacteria. Glucoamylases have wide industrial applications, pure forms are used in degradation of dextrins to yield glucose which is an intermediate step in the production of high-fructose syrups and ethanol. Saha and Zeikus (1989) reported the use of glucoamylase together with pullulanase during starch saccharification process.

24.10 Applications of Pullulan

Though microbial biopolymers are known to possess useful physical properties, even then currently only a small number of biopolymers are produced commercially on large scale. A few fungal EPSs have been reported so far that possess appealing industrial applications. Pullulan, a water soluble biopolymer from *A. pullulans* is one of such fungal EPSs. Numerous applications of pullulan in food and pharmaceutical manufacturing have been reviewed by Leathers (2003).

24.10.1 Food Industry

Polysaccharides are widely used in food products, sometimes for technological reasons such as process aids to stabilize emulsions, giving the physical structure required for packing or distribution. They are also used to enhance (or standardize) the 'eating quality' of the product (Morris 1989). Pullulan provides few calories and is treated as dietary fiber in rats and humans (Oku et al. 1979; Yoneyama et al. 1990). This is because of its resistance to mammalian amylases. Studies indicate that dietary pullulan functions as prebiotic, promoting the growth of beneficial bifidobacteria (Mitsuhashi et al. 1990; Yoneyama et al. 1990; Sugawa-Katayama et al. 1994). Pullulan may be incorporated in solid as well as liquid food to replace starch; imparting the characteristics to food normally derived from starch as consistency, dispersibility, moisture retention, etc. Pullulan can also be used as a partial replacement for starch in pastas or baked goods. Pullulan improves the shelf life of the food as it is not a readily assimilable carbon source for bacteria, molds and fungi responsible for spoilage of food. Pullulan is also superior to starch in water retention thus retarding the spoilage of food by drying out (Yuen 1974; Hijiya and Shiosaka 1975b; Kato and Shiosaka 1975a; Hiji 1986). Yuen claimed that pullulan inhibits fungal growth in foods (Yuen 1974). Solution properties of pullulan have been studied (Kato et al. 1982; Kawahara et al. 1984; Buliga and Brant 1987b; Nishinari et al. 1991). Pullulan solutions resemble gum Arabic having relatively low viscosity (Tsujisaka and Mitsuhashi 1993). Pullulan can be used as low-viscosity filler in beverages and sauces. It can also be used to stabilize the quality and texture of mayonnaise. The viscosity of pullulan is not affected by heating, changes in pH and most metal ions, including sodium chloride. Adhesive properties are also exhibited by pullulan and its derivatives (Hijiya and Shiosaka 1975a). Pullulan can be used as a binder and stabilizer in food pastes; it can also be used to adhere nuts to cookies. Pullulan can be employed as a binder for tobacco (Miyaka 1979), seed coatings and plant fertilizers (Matsunaga et al. 1977a, 1978).

Pullulan films are clear and highly oxygen-impermeable with excellent mechanical properties. Pullulan films are prepared by drying a pullulan solution (usually 5–10%) on a smooth surface and it can be as thin as 5–60 μm (Yuen 1974). Underivatized films are readily dissolved in water, thus having the property to melt

in the mouth as edible food coatings (Conca and Yang 1993). The oxygen resistance of pullulan films is suitable for protection of readily oxidized fats and vitamins in food. Pullulan films can be employed as coating or packaging material of dried foods, including nuts, noodles, confectionaries, vegetables and meats (Krochta and De Mulder-Johnston 1997). Pullulan can be used complexed with polyethylene glycol (Hijiya and Miyake 1990) or enriched with rice protein (Shih 1996). Pullulan can be used directly to foods as a protective glaze. Pullulan substituted with cholesterol or fatty acids can be used to stabilize fatty emulsions (Yamaguchi and Sunamoto 1991).

Wolf (2005) reported the use of pullulan as a slowly digested carbohydrate and its incorporation into food products, especially beverages and meal replacement products. Pullulan is referred to as slowly digested carbohydrate as human enzymes gradually convert pullulan to glucose that results in gradual rise in blood glucose level in humans. Pullulan may be incorporated into dietetic snack foods designed for diabetics. Pullulan is also beneficial to patients who have impaired glucose tolerance. Pullulan exhibits application in frozen food. It increases the shelf-life of ice cream, improves organoleptic properties, decreases the ice and lactose crystal growth and increases the resistance to melting (Goff 1995; Carrington et al. 1996; Goff and Sahagian 1996).

Generally maltotriose rich syrups are being produced from maltose syrup by cation exchange chromatography. Maltotriose syrup can also be produced by enzymatic hydrolysis of pullulan using pullulanase. This syrup possesses many excellent properties as low freezing point depression, mild sweetness, keeps in moisture, prevention of retrogradation of starch in foodstuffs, less color formation compared with maltose syrups, glucose syrups or sucrose, good heat stability, low solution viscosity, high fermentability and favoring glassy states. These properties are useful in food industry. Maltotriose syrup has been prepared from pullulan in a batch system using soluble and immobilized pullulan (Singh et al. 2010a, b). A bench scale continuous system has also been developed for the hydrolysis of pullulan using covalently immobilized pullulanase in a packed bed reactor (Singh et al. 2011).

24.10.2 Pharmaceutical Industry

Pullulan is now extensively studied for various applications in pharmaceutical industry. This is mainly due to its non-toxic, non-immunogenic and biodegradable properties. In comparison to a similar but more popular polysaccharide dextran, the degradation rate of pullulan in serum is faster than that of dextran. The areas in which pullulan has been investigated in pharmaceutical industry are discussed in this section. Pullulan and its derivatives can be used as denture adhesive. Adhesives or pastes are prepared by dissolving or dispersing uniformly pullulan ester and/or pullulan ether in water or in a mixture of water and acetone. Adhesives and pastes containing pullulan as the main component have higher water solubility and lower

moisture resistance (Hijiya and Shiosaka 1975a). Sugar-coated pharmaceutical compositions such as tablets, pills, granules contain pullulan in the sugar layer for the purpose of preventing brownish color change of the composition with lapse of time. The solid sugar-coated preparations exhibit an enhanced impact strength and shelf life. Pullulan can also be used for pharmaceutical coatings, including sustained-release formulations (Miyamoto et al. 1986; Izutsu et al. 1987; Childers et al. 1991). Oral care products have been commercialized based on pullulan films. The colorless, transparent and edible pullulan film has also attracted a great deal of interest for other uses such as a non-polluting wrapping material (Anonymous 2001). Scott et al. (2005) reported pullulan compositions for the use in pharmaceutical products preferably for predosed formulations like soft and hard capsules. Pullulan derivatives are promising as non-toxic conjugates for vaccines (Yamaguchi et al. 1985; Mitsuhashi and Koyama 1987). Covalent attachment between the virus and pullulan remarkably enhances the inherent producibility of immonuglobulin G and immunoglobulin M antibodies and diminishes the immunoglobulin E antibody producibility as well as sufficiently inactivating and detoxifying the virus. Pullulan can provide liposome delivery (Takada et al. 1984; Sunamoto et al. 1987). Sized pullulan fractions having molecular weight 30,000–90,000 Da can be used as a blood plasma expander in place of dextran (Igarashi et al. 1983; Kulicke and Heinze 2006). There have been several attempts to develop plasma substitutes on pullulan (Seibutsu and Kenkyujo 1983). Shingel (2004) summarized all efforts that have been made so far to understand the pharmacokinetics of intravenously applied pullulan in terms of the molar mass and concentration. Nakashio et al. (1976b) has demonstrated the use of pullulan in cosmetics, lotions and shampoos. Pullulan being non-toxic and non-irritant to human body, may be applied to any cosmetics, but is preferably used as an ingredient of cosmetic lotions, cosmetic powders, cosmetics around eyes, facial packs, shampoos, specific hair dressings (set lotions and hair lacquers), and tooth powders. Excellent transparent film-forming ability, moisture absorptivity, water solubility and tackiness are the properties making pullulan suitable for use in cosmetics. In addition, pullulan is characteristically lower in aqueous solution viscosity than any of such high polymer for cosmetics. Leung et al. (2006) disclosed physiologically acceptable films including edible films prepared from pullulan. These edible films include pullulan and antimicrobially effective amounts of the essential oils as thymol, methyl salicylate, eucalyptol and menthol. These films are effective killers of plaque-producing gums that cause dental plaque, gingivitis and bad breath. Pullulan-acetate nanoparticles have been used for epirubicin chemotherapy (Zhang et al. 2009). Nanoparticles of cholesterol-bearing pullulan have been reported as a carrier of anti-cancer drugs (Satoh et al. 2008).

Owing to pullulan's non-toxic, non-immunogenic, non-mutagenic and non-carcinogenic nature, this polysaccharide has been explored for various biomedical applications including targeted drug or gene delivery, wound healing and tissue engineering (Rekha and Sharma 2007). Pullulan is gaining lot of attraction towards its role in drug delivery systems e.g., self-assembling nanoparticles from hydrophobized pullulan, pH sensitive derivatised pullulan nanoparticles, etc. (Na et al. 2003). Gene therapy is another area where pullulan is being explored extensively. Gene

therapy is thought to be a cure for inherited disorders and cancer (Hosseinkhani et al. 2002; Gupta and Gupta 2004). Pullulan exhibits specificity for liver and this property is being exploited for liver targeting. Hemocompatible pullulan-polyethyleneimine conjugates have been used for liver cell gene delivery (Rekha and Sharma 2011). Liver targeted siRNA delivery by polyethyleneimine (PEI)-pullulan carrier has also been reported (Kang et al. 2010). Self-quenching polysaccharide waste nanogels of pullulan/folate-photosensitizer conjugates have also been reported for photodynamic therapy (Bae and Na 2010).

24.10.3 Miscellaneous

Pullulan can be formed into fibres resembling nylon or rayon by wet or dry spinning. Goods resembling polystyrene or polyvinyl alcohol can be formed from pullulan by compression or extrusion. The surface of molded pullulan type resin is coated with a thermosetting resin film. Molded pullulan type resins exhibit properties as transparency, toughness, gas impermeability and non-polluting (Hijiya and Shiosaka 1974, 1975c; Nakashio et al. 1975b; Matsunaga et al. 1976, 1977b; Tsuji et al. 1976). Pullulan is also used as an industrial flocculating agent (Zajic 1967; Zajic and LeDuy 1973). Novel biodegradable flocculating agents based on pullulan have been reported recently (Ghimici et al. 2010). It can also be used in the production of paper. The invention pertains to novel paper-coating material containing pullulan, which is excellent in gloss, printing gloss, adhesive strength and viscosity stability during storage. Pullulan has an excellent property as a paper-coating adhesive. The pullulan paper is high in strength and folding resistance, is tougher than a wood pulp paper. It favors ink receptivity because of its high hydrophilic nature, hence making it suitable for printing and writing (Nakashio et al. 1976a; Nomura 1976). It can improve the characteristics of paint (Nakashio et al. 1975a). Pullulan and its derivatives also have photographic, lithographic and electronic applications (Sano et al. 1976; Tsukada et al. 1978; Shimizu et al. 1983; Sasago et al. 1988; Vermeersch et al. 1995). Lithography is the process of printing from specially prepared surfaces, some areas of which are capable of accepting lithographic ink, whereas other areas, when moistened with water, will not accept the ink. Pullulan and pullulan derivatives are superior to traditionally used gum Arabic solution in the protection of the surface of lithographic printing plate against oxidation and scumming as well as in the ability to enhance the hydrophilic character of metallic surface of a non-image area. Nagase et al. (1979) and Motozato et al. (1986) reported the use of cross-linked pullulan beads (analogous to Sephadex®) in gel permeation chromatography. Cross-linked pullulan is water-resistant without losing excellent properties such as high degree of transparency, toughness and adhesiveness. Pullulan gels have been used for enzyme immobilization. Hydrophilic pullulan gel having a three-dimensionally reticulated structure which is obtained by the reaction between pullulan and a bifunctional compound capable of forming an ether linkage with the hydroxyl

group present in glucose unit of pullulan is used as a carrier. Enzymes immobilized with pullulan gel have a high activity and good retention of activity (Hirohara et al. 1981). Onda and coworkers reported potential uses of cyanoethylated pullulan in electronic devices (Onda et al. 1982). Pullulan is readily cyanoethylated by reacting with acrylonitrile in the presence of an alkali catalyst like sodium hydroxide. The cyanoethylated pullulan possess unique properties of heat resistance, solubility in organic solvents, film-forming property, and adhesive bonding to metals. Accurately sized pullulan molecular weight species are produced commercially for their use as chromatography standards (Kawahara et al. 1984).

24.11 Outlook and Future Perspectives

Research studies in the field of polysaccharides have revealed that pullulan is a unique polysaccharide with a variety of potential industrial and medical applications. The unique properties of this polysaccharide are due to its characteristic glycosidic linkage. Pullulan membranes/films are being used as coating and packaging materials for foods such as instant food seasonings, powdered tea and coffee. Pullulan-coated papers also decompose easily and do not contaminate the environment (Domań-Pytka and Bardowski 2004). Pullulan production has been stable with its major applications in food for a number of years, but now-a-days it is also being used for formulating dietary capsules. Pullulan-based oral care products are also being commercialized. Pullulan's property of thermal stability can be utilized along with its biological properties to develop implant devices. Despite of a large number of valuable applications, the major constraint prevailing on the use of pullulan is its cost, which is three times higher than the price of other polysaccharides such as dextran and xanthan. Engineering innovations or improved production strains, particularly with reduced melanin production could be beneficial to improve the economics of the production, thereby opening new avenues for pullulan utilization. In conclusion, pullulan is a polymer with numerous unique properties to be explored extensively and should be tapped to its maximum potential.

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Chapter 25
Halophilic Microorganisms as Sources of Novel Enzymes

Ram Karan, Sumit Kumar, Rajeshwari Sinha, and S.K. Khare

Abstract Search for new enzymes endowed with novel activities and enhanced stability continues to be a desirable pursuit in enzyme research. This is fuelled by industrial requirements and necessity of enzymatic interventions therein. Halophiles, a class of extremophiles inhabiting high salt conditions, are excellent source of enzymes possessing inherent ability to function in extreme conditions viz. high salt, alkaline pH and non-aqueous medium. Their biotechnological applications have been quite promising in food processing, industrial bioconversions and bioremediations. The chapter describes some of the novel halophilic enzymes and their potential usages in facilitating catalysis under harsh operational conditions. Effect of salt on their activity and stability; current level of understanding about structure-function relationship and molecular haloadaptations have also been encompassed in the chapter.

Keywords Halophiles • Haloenzymes • Halophilic adaptations • Halophilic proteases • Halophilic lipases • Halophilic amylases

25.1 Introduction

The enzymes are nature's catalysts, which have found wide industrial applications since time immemorial because of their remarkable property of high catalytic power and substrate/reaction specificity. However, enzyme stability is a necessary prerequisite for their industrial application, as most of the enzymes are inactivated under

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harsh operational conditions employed in industrial processes. Enzyme stabilization has therefore been an extensive pursuit by variety of techniques viz. chemical modification, immobilization, mutagenesis, enzyme engineering and directed evolution. In this context, enzymes occurring in extremophiles which are inherently stable under native conditions have received considerable attention in the recent years.

Halophiles are a major class of extremophiles, which inhabit hypersaline environments. Halophilic enzymes, dubbed as "haloenzymes", are endowed with unique structural features and catalytic power to sustain the metabolic and physiological processes under high salt conditions. Haloenzymes are not only salt stable but can also withstand other extreme conditions at times. Halophiles and their enzymes are perceived to be potentially useful for variety of applications in food bioprocesses, in production of important biomolecules, in enhanced oil recovery and remediation of saline wastes.

Halophiles have been widely studied with respect to their biodiversity, cellular function and metabolic activities. However, the industrial potential of halophilic enzymes remains largely unexplored. The present chapter focuses on enzymes from halophilic microbes for novel biocatalysis and biotransformation processes under polyextreme conditions and describes the recent understandings about halophilic enzymes, especially their structure-function relationship, saline adaptation, catalysis and potential applications.

25.2 Halophilic Microorganisms

These are generally defined as the microorganisms living in the saline environment having salt above 0.5 M. Different authors resort to different definitions and classifications for halophiles. Larsen (1962) divided them into four groups according to their salt requirement, namely nonhalophiles (<2.0%), slight halophiles (2.0–5.0%), moderate halophiles (5.0–20.0%) and extreme halophiles (20.0–30.0%). The definition proposed by Kushner (1993) is widely accepted (Ventosa et al. 1998; Yoon et al. 2003; Oren 2008) wherein he classified the halophiles in five groups namely, non-halophiles (<0.2 M salt); slight halophiles (0.2–0.5 M salt); moderate halophile (0.5–2.5 M salt); borderline extreme halophiles (1.5–4.0 M salt) and extreme halophiles (2.5–5.2 M salt).

25.3 Habitats and Diversity of Halophilic Bacteria

Halophiles primarily abound in saline water, saline soil, salt lakes, sea water and salterns (Ventosa et al. 1998; Oren 1999, 2000, 2008; Baati et al. 2010). Halophiles have mainly been studied from saline water soils and salt piles in hypersaline environment of California and Nevada, Great Salt Lake, Utah, USA; Soda lake in Kenya; Japanese sea; inland and marine saltern of France, Canada, Germany, UK and many other countries (Kamekura 1998; Vreeland et al. 2002; Oren 2002b, 2008). Diversity of halophiles occurring in different saline habitat is summarized in Table 25.1.

Table 25.1 Halophilic diversity in various saline habitats

Site of isolation	Halophilic microorganism	Salt requirement (%, w/v)	Reference(s)
Sambhar Salt Lake, India	*Geomicrobium* spp.	5.0–20.0	Karan et al. (2011)
Solar saltern in Sfax, Tunisia	*Halomonadaceae* sp., *Vibrionaceae* sp., *Alteromonadaceae* sp., *Idiomarinaceae* sp., *Alcanivoracaceae* sp.	5.0–15.0	Baati et al. (2010)
El-Djerid Salt Lake, Tunisia	*Salicola* sp., *Pontibacillus* sp., *Halomonas* sp., *Marinococcus* sp., *Halobacillus* sp.	10.0–25.0	Hedi et al. (2009)
Great Salt Plains of Oklahoma, USA	*Haloarcula* sp., *Haloferax* sp., *Halorubrum* sp., *Haloterrigena* sp., *Natrinema* sp.	15.0–30.0	Caton et al. (2009)
Solar salterns in Baja California, Mexico	*Halorubrum* sp., *Haloarcula* sp., *Halomonas* sp., *Halovibrio* sp., *Salicola* sp., *Salinibacter* sp.	5.0–25.0	Sabet et al. (2009)
Lonar Lake, India	*Halomonas* sp.	15.0–25.0	Joshi et al. (2007)
Solar salterns of sea water	*Bacillus* sp.	10	Rao et al. (2006)
Solar evaporation pond, Tuticorin, Tamil Nadu, India	*Chromohalobacter* sp.	10.0–25.0	Vidyasagar et al. (2006a)
Bakhtegan lake, South of Iran	*Salinivibrio* sp.	7.5–10.0	Amoozegar et al. (2007)
Solar evaporation pond, Tuticorin, Tamil Nadu, India.	*Halogeometricum* sp.	20	Vidyasagar et al. (2006b)
Saline habitat of coastal Gujarat, India	Haloalkaliphilic strain S5	10	Dodia et al. (2006)
Soda Lakes in Mongolia, Kenya, California, Egypt & Siberia	*Thioalkalivibrio* spp.	n.s.	Foti et al. (2006)
Soda-Lake sediments	Haloalkaliphilic strain Z-7026	n.s.	Zvereva et al. (2006)
Soda Lakes	*Alkalispirillum* sp., *Alkalilimnicola* sp.	n.s.	Sorokin et al. (2006)
Saltpan from Okha, India	*Bacillus* sp.	n.s.	Nowlan et al. (2006)
Saline habitats of Gujarat, India	*Bacillus* spp.	10	Patel et al. (2005, 2006)
Soda Lakes	*Thioalkalimicrobium* sp., *Thioalkalivibrio sp.*, *Thioalkalispira sp.*	n.s.	Sorokin and Kuenen (2005)

(continued)

Table 25.1 (continued)

Site of isolation	Halophilic microorganism	Salt requirement (%, w/v)	Reference(s)
Algal mat from a mineral pool in Malvizza (Italy)	*Halomonas* sp.	25	Romano et al. (2005)
Fish sauce factories in the eastern part of Thailand	*Halobacterium* sp.	17.5–25.0	Kanlayakrit and Bovornreungroj (2005)
Soda-lake sediments	*Thialkalivibrio* sp.	n.s.	Sorokin et al. (2004)
Alkaline hypersaline lake in the Altai Steppe, Russia.	*Thialkalivibrio* sp.	1.0–25.0	Banciu et al. (2004)
Lake Zabuye in Tibet, China	*Halorubrum* sp.	n.s.	Fan et al. (2004)
Soda lake of the Southern Transbaikal region.	*Methylophaga* sp.	2.0–3.0	Doronina et al. (2003a)
Alkaline, hypersaline Mono Lake in California	*Spirochaeta* sp.	2.0–12.0	Hoover et al. (2003)
Athalassic, alkaline Mono Lake in California	*Tindallia* sp.	1.0–20.0	Pikuta et al. (2003)
East Mongolian saline soda lake	*Methylophaga* sp.	3.0–4.0	Doronina et al. (2003b)
Mono Lake, California	*Ectothiorhodospira* sp.	n.s.	Oremland et al. (2002)
Soda Lakes in South-East Siberia, Kenya and Egypt	*Thioalkalivibrio* sp., *Thioalkalivibrio* sp.	25	Sorokin et al. (2002a)
Alkaline and saline Mono Lake, California, USA.	*Thioalkalimicrobium* sp., *Thioalkalivibrio* sp.	3.0–4.0	Sorokin et al. (2002b)
Soda lake in China	*Salinicoccus* sp.	0–25.0	Zhang et al. (2002)
Soda lakes in South-East Siberia, Kenya and Egypt	*Thioalkalispira* sp.	20.0–25.0	Sorokin et al. (2002c)
Coastal lagoon mud of the highly mineralized Lake Magadi (Kenya)	*Halonatronum* sp.	3.0–17.0	Zhilina et al. (2001)
Ethiopian soda lakes in the Rift Valley area-Lake Shala, Lake Abijata, and Lake Arenguadi	*Bacillus* spp.	10	Martins et al. (2001)
From deep-sea sediment, at 1,050 m depth on the Iheya Ridge	*Oceanobacillus* sp.	0–21.0	Lu et al. (2001)
From several saline and alkaline East African soda lakes	*Halomonas* sp.	0–20.0	Duckworth et al. (2000)
Isolated from a soda lake in Tibet	*Natronorubrum* sp.	12.0–25.0	Xu et al. (1999)
Anoxic muds of Mono Lake, California	*Bacillus* sp.	n.s.	Switzer et al. (1998)

Anoxic mud of Mono Lake, California,USA	*Bacillus* sp.	2.4–6.0	Jodi et al. (1998)
Soda deposits in Lake Magadi, Kenya	*Natronoincola histidinovorans* gen. nov., sp. nov.	4.0–16.0	Zhilina et al. (1998)
Hamatai Soda Lake, China	*Natronobacterium* sp.	12.0–30.0	Tian et al. (1997)
Saline and alkaline Alkali Lake in Southwestern Oregon	*Halomonadaceae* sp.	3.5–6.0	Maltseva et al. (1996)
Lake Khatyn, Central Asia	*Spirochaeta asiatica* sp.	3.0–6.0	Zhilina et al. (1996)
Sand of Venere Lake, Pantelleria Island, Italy	*Halomonas* sp.	1.25–15.0	Romano et al. (1996)
Kenyan soda lake, Lake Magadi	*Natronococcus* sp.	n.s.	Kanai et al. (1995)
Great Salt Lake, Utah,USA	*Haloanaerobium* sp.	10	Tsai et al. (1995)
Lake Gabara in the Wadi Natrun, Egypt	*Bacillus* sp.	2.9–17.4	Weisser and Trüper (1985), Fritze (1996)

Halococcus and *Halobacterium* are most prominently occurring halophiles, which have been extensively studied for their structural and genetic diversity. In the Indian context, researchers have actively contributed to the study of halophilic microorganisms from the Lonar Lake (Maharashtra), Sambhar Salt Lake (Rajasthan), marine salterns near Bhavnagar (Gujarat), Peninsular coast, solar pond Tuticorin (Tamil Nadu), salterns and coastal regions of Kumta (Karnataka), Kandla salt pans (Gujarat), continental shelf of west coast and sea coast of Gujarat (Raghavan and Furtado 2004; Patel et al. 2005; Asha et al. 2005; Dave and Desai 2006; Joshi et al. 2007, 2008a, b; Akolkar et al. 2008; Shivanand and Jayaraman 2009; VijayAnand et al. 2010; Karan and Khare 2010). Common halophiles which have been reported from the India belong to the genus *Haloarcula, Halobacteria, Halomonas*, *Bacillus, Geomicrobium* and *Flavobacterium*.

25.4 Morphology and Adaptive Features of Halophilic Microorganisms

Halophiles are typically non-sporing rods or cocci. The cocci occur singly or in groups. In some cases, these are motile containing polar tufts of flagella. The size and shape of halophilic bacteria may be influenced by the concentration of the salt present in surrounding environment. As the concentration of the salt is varied, rod forms become elongated or bulbous, or even appear as cocci. There are some reports that the type of basal medium employed also influences the morphology of the cells (Ventosa et al. 1998). Colonies of halophilic bacteria can appear red or orange due to the presence of C-50 carotenoids (α-bacterioruberin and derivatives) (Oren 2008).

The survival of halophiles under saline/hypersaline conditions has raised much curiosity about their adaptive mechanism. Apparently, the high salt concentration in surroundings should lead to exosmosis, thereby dehydrating the cell. Halophiles resist such dehydration by employing two major strategies to prevent osmotic movement of water out of the cell. Both the strategies work by increasing the internal osmolarity of the cell.

25.4.1 Compatible-Solutes Strategy

This is commonly observed in most of the known halotolerant and moderate halophiles. Though intracellular salt concentration in these cases is low, the osmotic balance between cytoplasm and external medium is maintained by (i) active pumping of ions out of the cells and (ii) by uptake of low molecular-weight organic osmotic solutes, known as compatible solutes (Oren 2002a). Commonly four types of molecules are used as compatible solutes viz. polyols (usually glycerol), sugars (sucrose, trehalose), amino acids (proline, glutamic acid), and quaternary amines (glycine betaine, ectoine, hydroxyectoine) (Galinski and Trüper 1982;

Galinski et al. 1985; Detkova and Boltyanskaya 2007). Uptake of compatible solutes depends on the organism species and their growth environments. Sugars like sucrose, fructose, glucose, or trehalose and amino acids like glutamate, proline are usually accumulated by slight halophilic bacteria while those like glycine betaine or glutamate betaine, glycosyl glycerol, ectoine, and sometimes glutamate, K^+, trehalose, or sucrose are mainly accumulated by moderately and halotolerant bacteria (Detkova and Boltyanskaya 2007). The concentration of osmolytes in the cell ranges from millimolars to 1–2 M depending on the extracellular osmolarity. Since osmolytes are tolerated by the macromolecular machinery of the cell over a wide range of concentration, these compounds are termed as compatible solutes (Brown 1976; Lentzen and Schwarz 2006).

Osmotic balance by osmolytes gives a high degree of flexibility to halophilic microorganisms (Oren 2002a). Compatible solutes have also been found beneficial as protectants against UV-radiation (Kunte 2006) and stress, specially to protect proteins and membranes against the decreased water activity, freezing and drying (Göller and Galinski 1999; Roberts 2005).

25.4.2 "Salt-in" Strategy

This approach is mainly adapted by the halophilic Archaea and the anaerobic halophilic bacteria. The strategy involves the selective influx of K^+ ions into the cytoplasm and their accumulations (usually equilibrated with Cl^-) up to isotonic levels matching the external environment (Oren 2002a). Due to significantly low water binding, potassium ions are preferred over sodium ions (Dennis and Shimmin 1997). The concentration of chloride ions is in equilibrium with that present in the surrounding medium, while those of sodium is lower and potassium is several times higher (Detkova and Boltyanskaya 2007). The membranes of these organisms are endowed with large number of Na^+/H^+ antiporters, to create a proton gradient for the removal of Na^+ from the cytoplasm (Padan et al. 2001). It is observed that reverse potassium gradient increases with increasing medium salinity, thereby implicating the important role of K^+ in the osmoadaptation.

In order to cope with such high Na^+/K^+ concentration, intracellular and extracellular proteins of this category of halophiles undergo several structural adaptations to counter the salting-out effects (Madern et al. 2000). The cell wall glycoproteins are endowed with unusually high acidic amino acids contents (Fukuchi et al. 2003). Their excess negative charges are neutralized by sodium ions of the surrounding environment. Under low sodium ion concentrations, these negative charges on the glycoproteins remain poorly shielded. This may lead to electrostatic repulsion between the negative charges causing destabilization and lysis of cell wall.

Some halophiles e.g. *Halobacillus halophilus* which accumulate compatible solutes are also known to contain moderate intracellular concentrations of Na^+, K^+, and Cl^- and therefore, they apply both strategies (Oren 2008; Saum and Müller 2008).

25.5 Adaptive Features of Halophilic Proteins/Enzymes

It is interesting to note in the perspective of adaptations, that halophilic proteins/enzymes work under salt environment which would critically affect their structure and function. Salt is known to cause protein aggregation due to enhanced hydrophobic interactions, increased hydration of ions, decreased availability of free water and preventing intra and inter-molecular electrostatic interactions (Dennis and Shimmin 1997; Detkova and Boltyanskaya 2007). Halophilic proteins should therefore adapt to maintain native conformation and functionality in presence of high salt concentrations. In this context, surface of halophilic proteins is highly negatively charged and require positive charges for stabilization. Since, potassium is high in concentrations inside the cells, they have evolved to require potassium, rather than sodium as a counter ion. Potassium ions have an additional advantage of having low water binding nature as compared to sodium ions (Dennis and Shimmin 1997). Ions in solution interact with surrounding water molecules to create spheres of hydration. The binding of these water molecules allows these ions to become soluble. Any water bound to the ion is consequently unavailable to proteins. As the concentration of ions inside the cell increases, the amount of available water becomes scarce. Halophilic proteins also need spheres of hydration to prevent their aggregation (Lanyi 1974; Eisenberg 1995; Paul et al. 2008) for which they are adapted through decreased number of hydrophobic amino acids and increased hydrophilic amino acid content on their surface (Mohana Rao and Argos 1981; Bolhuis et al. 2008). An increased proportion of smaller hydrophobic amino acids (Gly, Ala, Val) compared to larger hydrophobic amino acid residues is another adaptive feature taken up by halophilic enzymes (Lanyi 1974; Danson and Hough 1997; Madern et al. 2000; Fukuchi et al. 2003; Kastritis et al. 2007; Tadeo et al. 2009).

Halophilic proteins therefore possess (i) high acidic amino acid content on the surface (ii) low lysine content (iii) relatively low hydrophobicity at the core of the protein and (iv) increased salt bridge. *Halobacterium salinarum* dodecin and *Haloarcula marismortui* malate dehydrogenase (HmMDH) may be cited as typical examples where high content of acidic residues play significant roles in (i) binding of essential water molecules (ii) binding of salt ions (iii) preventing protein aggregation and (iv) providing flexibility to structure of the protein through electrostatic repulsion (Mevarech et al. 2000; Siddiqui and Thomas 2008). These characteristic features are more prevalent in halophiles employing the “salt-in strategy”. On the contrary, in case of halophiles using the “compatible solute strategy” such amino acid adaptations are not observed (Oren 1999).

25.6 Enzymes from Halophiles

It is not surprising that halophilic microorganisms have been perceived as an ideal source of salt stable enzymes. The halophilic enzymes may perform the same enzyme function as their non-halophilic counterparts, yet they surpass mesophilic

enzymes in their ability to carry out catalysis under high salinity. Interestingly, some of the enzymes derived from halophiles display polyextremophilicity i.e. stability towards more than one extreme conditions e.g. high salt, alkaline pH and non-aqueous medium. The potentials of halophiles and haloenzymes have been extensively reviewed from time to time (Danson and Hough 1997; Da Costa et al. 1998; Hough and Danson 1999; Sellek and Chaudhuri 1999; Madern et al. 2000; Eichler 2001; Gomes and Steiner 2004; Oren 2002b, 2010).

Much of the interest in haloenzymes stems from their unique properties. Some of these attributes include: (i) optimum activity and stability at high NaCl concentrations, (ii) role of salt in maintaining structure, (iii) higher resistance towards denaturation and (iv) ability to catalyze in low water or non-aqueous medium (Madern et al. 2000; Tokunaga et al. 2008; Setati 2010).

Some interesting enzymes of this class that are active and stable in saline environments are listed in Table 25.2. Commonly studied halophilic enzymes are amylases, proteases, nucleases, cellulases, chitinases, xylanases, esterases and lipases. These have been mainly studied from to the genera *Acinetobacter, Haloferax, Halobacterium, Halorhabdus, Marinococcus, Micrococcus, Natronococcus, Bacillus, Halobacillus* and *Halothermothrix* (Danson and Hough 1997; Da Costa et al. 1998; Amoozegar et al. 2003; Oren 2002c, 2010).

25.7 Halophilic Proteases

Commercial proteases are mostly derived from mesophilic organisms. Proteases from halophiles present the advantage of being stable at high salinities. Kamekura (1986), Ventosa et al. (1998), De Castro et al. (2006) have extensively reviewed halophilic proteases. Some of potential proteases from halophiles are listed in Table 25.3.

Halophilic proteases, owing to their adaptability to saline environments, are more sustainable under detergent procedures (Schumacher et al. 2001). The surfactant and detergent compatible protease from *Bacillus* sp. has been shown to be advantageous in detergent ingredients (Haddar et al. 2010).

Proteases catalyze the forward reaction of peptide bond hydrolysis in aqueous medium. Exploiting this property, the halophilic protease from *Halobacterium* sp. has been employed in fish sauce preparation (Akolkar et al. 2010). However, in low water or solvent medium, the reverse reaction takes place and peptide bonds are synthesized. This necessitates proteases to be stable in solvent medium. Most of the known proteases are inactivated or show low catalytic activities in organic solvents (Vulfson et al. 2001). The reaction equilibria of these hydrolytic enzymes can be shifted toward completion of the reverse reaction of hydrolysis i.e. the synthetic reaction (peptides and esters synthesis) (Sardessai and Bhosle 2004). A number of important peptides such as the analgesic dipeptide kyotorphin (Tyr-Arg) (Jönsson et al. 1996; Sareen et al. 2004) and aspartame (Eichhorn et al. 1997) have been synthesized using proteases. Protease-catalyzed synthesis offer several advantages over chemical synthesis viz. regio- and stereo-selectivity, absence of racemization, lack

Table 25.2 Potentially useful enzymes from halophiles

Enzymes	Microorganism	Properties/projected applications	References
Amylases	*Saccharopolyspora* sp. A9	Detergent formulation	Chakraborty et al. (2011)
	Nesterenkonia sp. strain F	Starch hydrolysis	Shafiei et al. (2010)
	Chromohalobacter sp. TVSP 101	Starch hydrolysis	Prakash et al. (2009)
	Bacillus sp. strain TSCVKK	Detergent formulation	Kiran and Chandra (2008)
Chitinases	*Virgibacillus* sp., *Terribacillus* sp., *Salinivibrio* sp.	Salt stable	Rohban et al. (2009)
Cellulases	*Marinobacter* sp. MSI032	Alkaline, pH stable	Shanmughapriya et al. (2009)
	Virgibacillus sp., *Halobacillus* sp.	Salt stable	Rohban et al. (2009)
Esterases	*Haloarcula* sp.	Alkaline and salt stable	Camacho et al. (2009)
β-galactosidase	*Haloferax alicantei*	Salt stable	Holmes et al. (1997)
Lipases	*Salicola* strain IC10	Alkaline and salt stable	Moreno et al. (2009)
	Natronococcus sp.	Salt and thermal stable	Boutaiba et al. (2006)
Proteases	*Halobacterium* sp.	Alkaline, salt stable, fish sauce preparation	Akolkar et al. (2010)
	Geomicrobium sp.	Alkaline, salt and solvent stable	Karan et al. (2011)
	Halobacterium sp.	Solvent stable, antifouling coating	Akolkar et al. (2008)
	Halobacterium halobium	Solvent stable, peptide synthesis	Ryu et al. (1994)
Nucleases	*Bacillus* sp.	Alkaline, salt and thermal stable	Onishi et al. (1983)
	Micrococcus sp.	Alkaline, salt stable	Kamekura and Onishi (1974)
Xylanases	*Thalassobacillus* sp., *Gracilibacillus* sp.	Salt stable	Rohban et al. (2009)
	Halophilic bacterium CL8	pH, salt and thermal stable	Wejse et al. (2003)

of requirement of side-chain protection, milder non-hazardous reaction conditions, higher solubility of hydrophobic species, reduced microbial contamination and reduced water activity which alters the hydrolytic equilibrium (Jakubke et al. 1985; Klibanov 1986; Gill et al. 1996).

So far, a few halophilic proteases which are organic solvent stable have been reported viz. *Halobacterium salinarum* (Kim and Dordick 1997), *Haloarcula* sp. strain OMF-1 and OMF-2 (Usami et al. 2005), *Gamma-Proteobacterium*

Table 25.3 Proteases from halophiles

Halophiles	Halophilic enzymes	Purification procedure	Enzymatic characteristics	Novel properties	Reference(s)
Geomicrobium sp. EMB2	Serine protease	Ultrafiltration and Hydrophubic interaction chromatography (HIC) on phenyl sepharose 6FF	pH_{opt} 10.0. T_{opt} 50°C, Mw 38 kDa	Stable in organic solvents and detergents	Karan and Khare (2010)
Virgibacillus sp. SK33	Serine protease	HIC on Phenyl-Sepharose and Sephadex G-75 gel filtration chromatography	pH_{opt} 7.5. T_{opt} 55 m°C, Mw 32 kDa	Stable upto 4.0 M NaCl and organic solvents	Sinsuwan et al. (2010)
Bacillus mojavensis A21	Two serine protease BM1 and BM2	Acetone precipitation (40–60%), Sephadex G-75 gel filtration chromatography and DEAE-cellulose chromatography	pH_{opt} 8.0–10.0 for BM1 and 10.0 for BM2. T_{opt} 60°C, Mw 29 kDa for BM1 and 15.5 kDa	Detergent stable	Haddar et al. (2009)
Haloalkaliphilic bacterium sp. AH-6	Serine protease	Ammonium sulfate and HIC on Phenyl Sepharose 6 FF	pH_{opt} 9.0–11.0. T_{opt} 37°C, Mw 40 kDa	Stable in pH 8.0–13.0	Dodia et al. (2008)
Salinivibrio sp. strain AF-2004	Serine metall-oproteases	50–80% acetone, Q-Sepharose anion exchange chromatog-raphy and Sephacryl S-200 gel filtration chromatography	pH_{opt} 8.5. T_{opt} 55°C, Mw 29 kDa	Stable in organic solvents	Karbalaei-Heidari et al. (2007)
Haloalkaliphilic *Bacillus* sp.	Serine protease	HIC on Phenyl Sepharose 6 FF	pH_{opt} 10.0–11.0. T_{opt} 37°C, Mw 29 kDa	Stable in surfactants and detergents	Gupta et al. (2005)
Pseudoalteromonas sp. strain CP76	Metalloprotease	Ultrafiltration, Q-Sepharose, Superdex-200 gel filtration chromatography	pH_{opt} 8.5. T_{opt} 55°C, Mw 38 kDa	Stable 0–4.0 M NaCl	Sanchez-Porro et al. (2003a)
Natrialba magadii	Serine protease	Ethanol ppt, Bacitracin-Sepharose 4B affinity chromatography, Sephacryl S-200 gel filtration chromatography	pH_{opt} 8.0. T_{opt} 60°C, Mw 45 kDa	Stable in pH 6.0–12.0 and 1–3.0 M NaCl	Giménez et al. (2000)

(continued)

Table 25.3 (continued)

Halophiles	Halophilic enzymes	Purification procedure	Enzymatic characteristics	Novel properties	Reference(s)
Halobacterium halobium (ATCC 43214).	Protease	Ultrafiltration YM-10, Ammonium sulfate, Sephadex G-200 and Sephadex G-75 gel filtration chromatography	pH_{opt} 10.0. T_{opt} 30°C, Mw 66 kDa	Stable at 4.0 M NaCl	Ryu et al. (1994)
Halobacterium mediterranei	Serine protease	Ultrafiltration, bacitracin-Sepharose affinity chromatography, Sephadex G-75 gel filtration chromatography	pH_{opt} 8.0–8.5. T_{opt} 55°C, Mw 41 kDa	Activity in 2.0–5.0 M NaCl range	Stepanov et al. (1992)
Halobacterium halobium	Serine protease	Bacitracin-Sepharose affinity chromatography and Sephadex G-25 gel filtration chromatography	pH_{opt} 8.0–9.0. T_{opt} 37°C, Mw 41 kDa	Stable at high salt concentration	Izotova et al. (1983)

(Sana et al. 2006), *Natrialba magadii* (Ruiz and De Castro 2007), *Natrialba magadii* (Ruiz and De Castro 2007), *Salinivibrio* sp. strain AF-2004 (Karbalaei-Heidari et al. 2007), *Haloferax lucentensis* VKM 007 (Manikandan et al. 2009) and *Geomicrobium* sp. EMB2 (Karan and Khare 2010).

Ryu et al. (1994) isolated and partially purified an extracellular protease from the extreme halophile *Halobacterium halobium* (ATCC 43214). It is active in DMF/water mixtures. The enzyme was used for peptide synthesis. Recently, the applications of halophilic proteases have been highlighted in following processes: (i) the protease from *Halobacterium* sp. stable over a broad pH range and high salt concentration has been effectively used in fish sauce preparation (Akolkar et al. 2010) (ii) the surfactant and detergent compatible protease from *Bacillus* sp. has been shown to be advantageous in detergent ingredients (Haddar et al. 2010) and (iii) the solvent stability of *Halobacterium* sp. protease has been exploited for use in antifouling coatings (Akolkar et al. 2008).

Yet another protease from *Natrialba magadii* demonstrated efficient peptide synthesis in aqueous organic system (30%, v/v dimethyl sulfoxide). The tripeptide Ac-Phe-Gly-Phe-NH_2 was synthesized using Ac-Phe-OEt and Gly-Phe-NH_2 substrates as building blocks (Ruiz et al. 2010). This presents the basis of a new approach for peptide synthesis (Marhuenda-Egea and Bonete 2002).

In recent years, halophilic proteases have been studied on molecular level by some researchers. A halophilic serine protease gene from *Geomicrobium* sp. EMB2 has been cloned and sequenced by Karan et al. (2011). The gene encoding the protease Nep secreted by the haloalkaliphilic archaeon *Natrialba magadii* was cloned and sequenced by De Castro et al. (2008). Molecular cloning and sequence analysis of a novel zinc-metalloprotease gene from the *Salinivibrio* sp. strain AF-2004 and its extracellular expression in *E. coli* was reported by Karbalaei-Heidari et al. (2007). Shi et al. (2006) studied the cloning of an extracellular protease gene, designated SptA, from this halophilic archaeon. In addition, the SptA gene had been successfully expressed in *Haloferax volcanii* WFD11 and the recombinant SptA protease had been purified and characterized. Kwon et al. (1995) cloned and characterized the *rapT* gene encoding an extracellular SDS-resistant alkaline protease (VapT) from *Vibrio metschnikovii* strain RH530. Molecular cloning and sequencing of the gene for a halophilic alkaline serine protease (Halolysin) from an unidentified halophilic Archaeal strain (172 PI) followed by the expression of the gene in *Haloferax volcanii* was reported by Kamekura et al. (1992).

25.8 Halophilic Lipases

Isolation and characterization of salt stable lipases from halophilic sources has seen a growing interest in recent years (Kanlayakrit and Boonpan 2007; Kim et al. 2007; Guzmán et al. 2008). Availability of such enzymes would facilitate industrial processes that require activity at high salt concentrations as well as low water activity. However, the present limitation in the industrial use of this enzyme because of its

high production costs may be overcome by use of modern upcoming molecular technologies. Sanchez-Porro et al. (2003b) isolated hydrolase producing moderately halophilic and halotolerant eubacteria from Spanish salterns. Only 23% of the 892 strains showed extracellular lipolytic activity.

A moderately halophilic bacterium (strain SM19T) displaying novel lipolytic activity has been isolated and characterized. Strain SM19T is a Gram-negative rod that grows optimally in culture media containing 7.5% NaCl under aerobic conditions. The lipolytic activity was envisaged from zone of hydrolysis around bacterial colonies. This has been classified under the genus *Marinobacter* with proposed name *Marinobacter lipolyticus* sp. nov. (Martín et al. 2003). Another extremely halophilic isolate, *Salicola* strain IC10, showing lipase and protease activities has been marked for potential biotechnological applications. The strain is reported to grow under optimal conditions of 15–20% (w/v) NaCl, pH 8.0 and 37°C. Its lipase showed highest activity against p-nitrophenylbutyrate (Moreno et al. 2009). The production and characterization of an esterase from *H. marismortui* was recently reported by Camacho et al. (2009).

In a study related to Archaea, a total of 118 halophilic strains were screened for lipolytic activity. Eighteen strains were found positive on rhodamine agar plates. Highest lipase activities were detected at pH 8.0, temperature 45–65°C and NaCl 3.5–4.0 M. These results indicate the presence of salt-dependent and thermostable lipases in halophilic Archaeal groups (Ozcan et al. 2009). Fifty strains of moderately halophilic bacteria were isolated from various salty environments in Iran. A strain designated SA-2 was shown to be the best producer of extracellular lipase. Biochemical and physiological characterization along with 16S rDNA sequence analysis placed SA-2 in the genus *Salinivibrio* (Amoozegar et al. 2008).

Lipolytic activity from an extremely halophilic archaeon, *Natronococcus* sp. has been studied. The activity was optimum in presence of 4.0 M NaCl and complete loss of activity was observed in absence of salts. Unique salt dependent thermal stability was exhibited by this enzyme. High salinity appeared to influence the complete adaptability and efficiency of the enzyme at molecular level (Boutaiba et al. 2006). Lipolytic enzyme production has also been reported in *B. halodurans*, *B. alcalophilus* and *B. licheniformis* strains isolated from a Kenyan alkaline soda lake (Vargas et al. 2004). Müller Santos et al. (2009) has very recently evidenced the salt dependence of an esterase from *Haloarcula marismortui*. The corresponding gene was cloned, overexpressed in *E. coli* followed by its purification and biochemical characterization. The recombinant enzyme displayed salt dependent activity and folding characteristics as indicated by circular dichroism studies. Similar study was also carried out by Rao et al. (2009) wherein circular dichroism, dynamic light scattering and small angle neutron scattering techniques have been employed to deduce the structural and functional basis of the recombinant enzyme. Molecular and biochemical characterization of a tetrameric 160 kDa lipase from *Staphylococcus simulans* was carried out by Sayari et al. (2001) in the recent past. Report of an organic solvent stable lipase sourced from organic solvent tolerant bacterium *Staphylococcus saprophyticus* M36, isolated from soil/sea water is also available (Fang et al. 2006).

It is seen that a variety of potential lipase producers has been studied from halophiles by different researchers. But purification of halophilic lipases, their characterization and application in industrial processes is an area which still awaits considerable research.

25.9 Halophilic Amylases

Amylases are among the most significant enzymes, which find potential applications in various sectors of biotechnology. These extracellular polymer degrading enzymes, sourced from halophiles are projected to be useful in harsh industrial processes wherein high salinities may inhibit many enzymatic conversions (Mohapatra et al. 1998). Isolation and characterization of novel amylases from halophiles with desirable properties of salt, thermal, alkaline and organic solvent stability serves as a requisite approach to replenish the current dearth of industrially stable enzymes.

Alike halophilic proteases and lipases, use of halophilic amylase in bioprocesses presents the advantage to obtain optimal activities at high salt concentrations. Halophilic amylases have been known to function under low water conditions which justifies its stability in organic solvents. An organic solvent tolerant amylase from the extremely halophilic Archaea, *Haloarcula* sp. strain S-1 remained active and stable in presence of various organic solvents such as benzene, toluene and chloroform but lost its activity in presence of more polar solvents like those of ethyl alcohol and acetone (Fukushima et al. 2005).

Halophiles which have been studied for amylase production are *Halobacterium halobium* (Good and Hartman 1970; Patel et al. 1993), *Acinetobacter* sp. (Onishi and Hidaka 1978), *Micrococcus halobius* (Onishi and Sonoda 1979), *Micrococcus varians* subsp. *halophilus* (Kobayashi et al. 1986), *Micrococcus* isolates (Onishi 1972; Khire 1994), *Halomonas meridiana* (Coronado et al. 2000), *Halobacterium sodomense* (Oren 1983), *Natronococcus* sp. strain AH-36 (Kobayashi et al. 1992), *Bacillus dipsosauri* (Deutch 2002), *Halobacillus* sp. strain MA-2 (Amoozegar et al. 2003) and *Bacillus* sp. strain TSCVKK (Kiran and Chandra 2008). Some of the amylases studied from halophiles are listed in Table 25.4.

Optimum pH for halophilic amylase activity usually lies in the range of 6.5–7.5. A few exceptions arise in cases of *Chromohalobacter* sp. TVSP 101 and *Saccharopolyspora* sp. A9 where the optimum pH appears to be higher in the alkaline range (Prakash et al. 2009; Chakraborty et al. 2011). The optimal temperature range for halophilic amylase activity lie between 45°C and 65°C. Molecular weight of halophilic amylase range from 55 kDa for the amylase I sourced from *Acinetobacter* sp. (Onishi and Hidaka 1978) to 100 kDa in *Nesterenkonia* sp. strain F (Shafiei et al. 2010). Interestingly, a moderate halophile *Kocuria varians* produced six different forms of α-amylase in culture medium. Processing of a pre-pro-precursor form resulted in all the six forms of amylase. The enzyme was purified and cloned in *E. coli* as fusion protein. Amylase from *Kocuria varians* showed salt-dependent thermal reversibility (Yamaguchi et al. 2010). Amylase

Table 25.4 Purification and characterization of halophilic amylases

Halophiles	Purification procedure	Enzyme characteristics	Novel properties	Reference(s)
Nesterenkonia sp. strain F	80% ethanol precipitation, Q-Sepharose anion exchange and Sephacryl S-200 gel filtration chromatography	pH_{opt} 7.5, T_{opt} 45°C, Starch digesting. Mw 100 kDa (SDS), 106 kDa (gel filtration)	SDS and surfactant stable. Stable at 0–4.0 M NaCl, optimum 0.5 M NaCl or 1.0 M KCl	Shafiei et al. (2010)
Chromohalobacter sp. TVSP 101	HIC on Butyl Sepharose 4B (single peak), Sephacryl S-200 gel filtration chromatography (two forms of amylase)	pH_{opt} 9.0, T_{opt} 65°C, Mw amylase I 72 kDa and amylase II 62 kDa	$T_{1/2}$ 80°C = 1 h, Stable at 0–20.0% (w/v) NaCl	Prakash et al. (2009)
Bacillus sp. strain TSCVKK	Partially purified by acetone precipitation	pH_{opt} 7.5, T_{opt} 55°C	Stable in various surfactants and detergents, Maximum stability at 10% NaCl	Kiran and Chandra (2008)
Haloferax mediterranei	**NS**	pH_{opt} 7.0–8.0, T_{opt} 50°C–60°C	Stable at 2.0–4.0 M NaCl Optimum 3.0 M NaCl	Pérez-Pomares et al. (2009)
Halobacillus sp. strain MA-2	**NS**	pH_{opt} 7.5–8.5, T_{opt} 50°C	Maximum stability at 5% NaCl	Amoozegar et al. (2003)
Halothermothrix orenii	**NS**	pH_{opt} 7.5, T_{opt} 65°C	Tolerates up to 25% NaCl, T_{opt} at 5% NaCl	Mijts and Patel (2002)
Bacillus dipsosauri	**NS**	pH_{opt} 6.5, T_{opt} 60°C	Stable up to 60°C	Deutch (2002)
Micrococcus halobius ATCC 21727	Glycogen-complex formation, DEAE-cellulose chromatography and Bio-Gel P-200 gel filtration chromatography	pH_{opt} 6.0–7.0 in 0.25 M NaCl or 0.75 M KCI at 50–55°C, Mw amylase 89 kDa	Activity lost by dialysis against distilled water	Onishi and Sonoda (1979)
Acinetobacter sp.	Glycogen-complex formation, DEAE-Sephadex A-50 chromatography, and Sephadex G-200 gel filtration chromatography	pH_{opt} 7.0 in 0.2–0.6 M NaCl or KCl at 50°C–55°C, Mw amylase I 55 kDa and amylase II 65 kDa	Activity lost by dialysis against distilled water	Onishi and Hidaka (1978)

production from halotolerant bacteria *Rheinheimera aquimaris* has been very recently reported by Ghasemi et al. (2010).

The amylase gene, AmyA, from *Halothermothrix orenii*, a moderately halophilic bacterium has been cloned, overexpressed, and purified. The recombinant enzyme showed NaCl and $CaCl_2$ dependent activity and thermostability (Mijts and Patel 2002). Crystal structure of this AmyA revealed that its surface lacks conserved acidic amino acids, a feature common in halophilic proteins and essential for stability under highly saline conditions (Sivakumar et al. 2006). High resolution crystal structure of a second α-amylase AmyB from *Halothermothrix orenii* has also been reported (Tan et al. 2008).

25.10 Other Halophilic Enzymes

Besides proteases, lipases and amylases other hydrolases reported from halophiles are carboxylesterase, cellulase, xylanase, nuclease and β-galactosidase (Table 25.5). Although, these enzymes have been reported to possess novel characteristics, very few commercial applications have been designed for them.

A salt-tolerant β-galactosidase from psychrophilic *Planococcus* isolate was cloned in *E. coli*. Sequence analysis showed its similarity to the enzyme from *Bacillus circulans.* β-galactosidase remained active at high salt concentration and its potential as a reporter enzyme for halotolerant and halophilic organisms was recognized (Sheridan and Brenchley 2000). Giridhar and Chandra (2010) have recently reported the production of xylanase from *Gracilibacillus* sp. TSCPVG, stable under saline, alkaline and high temperature conditions. The β-xylanase so produced extracellularly was stable over a broad range of salt concentrations. Xylanase activity was maximum at pH 7.5 and temperature of 60°C. The bacteria also proved to be a source of high levels of β-xylosidase under submerged conditions. Endoglucanase, FPase, amylase, avicelase, acetyl esterase and protease activity were also observed.

Cellulase production from moderately halophilic bacterium *Marinobacter* sp. MSI032 has been reported recently. Enzyme production was maximum in medium containing (w/v) 1% maltose, 1% peptone or casein, 2% NaCl, 10 mM Zn^{2+} at 27°C and pH 9.0. Purification of cellulase using ammonium sulfate precipitation, Sephadex G-200 and DEAE Sepharose chromatography led to 12.5 fold purification with recovery of 37%. Pure enzyme had molecular weight of 68 kDa and was alkaline in nature with optimum activity at pH 9.0 and stability upto pH 12.0. Wang et al. (2009) recently used FPLC to purify and further characterized another "halostable" cellulase from *Salinivibrio* sp. strain NTU05. Apart from its ability to remain stable in saline environment, the enzyme is also reported to be thermostable to some extent and holds immense potential in biodegradation of cellulose.

Chitinases sourced from *Virgibacillus* sp., *Terribacillus* sp., *Salinivibrio* sp. have also been discussed by Rohban et al. (2009). An eightfold purification with 11% recovery was obtained in case of an extracellular carboxylesterase from the moderately halophile *Thalassobacillus* sp. strain DF-E4. The molecular mass of the native enzyme was approximately 49 kDa (Lv et al. 2010).

Table 25.5 Other halophilic enzymes

Halophiles	Halophilic enzymes	Purification procedure	Enzyme characteristics	Novel properties	Reference(s)
Thalassobacillus sp. strain DF-E4	Carboxylesterase	Ultrafiltration, DEAE-Sepharose A-25 chromatography, Sephadex G-75 gel filtration chromatography	pH_{opt} 8.5, T_{opt} 40°C, Mw 45 kDa	Broad pH stability in the range of 6.0–10.0, stable at temperature below 45°C and lost about 36% of its activity at 50°C, the enzyme was completely inactivated at 80°C. 90% of the original activity retained even after 12 h incubation.;displayed extreme stability up to 4.0 M NaCl	Lv et al. (2010)
Marinobacter sp. MSI032	Cellulase	Amonium sulfate precipitation, Sephadex G-200 gel filtration chromatography and DEAE-Sepharose chromatography	pH_{opt} 9.0, T_{opt} 27–35°C, Mw 68 kDa	Alkaline in nature with stability upto pH 12.0	Shanmughapriya et al. (2009)
Salinivibrio sp. strain NTU05	Cellulase	Amonium sulfate precipitation, Anion exchange Hi-Trap Q chromatography, Sephadex G-200 gel filtration	pH_{opt} 7.5, T_{opt} 35°C, Mw 68 kDa and pHopt 9.0, Topt 27–35°C, Mw 68 kDa	Highly stable between 10°C and 40°C; retained 100% of its activity at pH 7.0 and pH 8.0 for 24 h; remained 90% active in 0% NaCl, but lost activity as the NaCl concentration was increased	Wang et al. (2009)

Halophilic bacterium, CL8	Xylanase 1 Xylanase 2	HIC on Phenyl-Sepharose and anion exchange chromatography on Mono Q	pH_{opt} 6.0, T_{opt} 60°C for Xylanase 1 and 65°C for Xylanase 2, Mw 43 kDa (Xylanase I) and 62 kDa (Xylanase II), pI of 5.0 and 3.4 for Xylanase 1 and Xylanase II respectively	Xylanase 1 Stable 97 min at 60°C Xylanase 2 Stable 192 min at 60°C (Topt at 4 M NaCl) stable at pH 4.0–11.0	Wejse et al. (2003)
Haloferax alicantei	β-galactosidase	Gel filtration chromatography, Q Sepharose and IMAC-Ni	Optimally active at 4 M NaCl 180 ± 20 kDa in size, consisting of two monomers each 78 ± 3 kDa.	Stabilized by sorbitol in absence of salt	Holmes et al. (1997)
Halophilic *Bacillus* sp.	Nuclease (Both RNase and DNase activities)	Ethanol precipitation, DEAE-Sephadex A-50 chromatography and Sephadex G-200 gel filtration chromatography	pH_{opt} 8.5, T_{opt} 50°C for DNA and 60°C for RNA. Maximum activity in the presence of 1.4–3.2 M NaCl or 2.3–3.2 M KCl, Mw 138 kDa	Activity lost by dialysis against water and buffer of low salt	Onishi et al. (1983)
Micrococcus varians	Nuclease (Both RNase and DNase activities)	Alcohol precipitation, DEAE-Sephadex chromatography and Sephadex G-200 gel filtration chromatography	pH_{opt} 8.0, T_{opt} 43°C. Maximum activity in the presence of 2.9 M NaCl or 2.1 M KCl, Mw 105 kDa	Activity lost by dialysis against buffer of low salt	Kamekura and Onishi (1974)

25.11 Applications of Halophiles and Their Enzymes

Major applications of halophiles and their enzymes are envisaged in:

(i) Biotransformations at high salt concentrations
(ii) Manufacturing of solar salt from seawater
(iii) Production of:

- Antimicrobial compounds
- Traditional fermented foods
- Compatible solutes (used as stabilizers of biomolecules, enzymes, DNA, membranes and whole cells, salt antagonists and stress-protective agents)
- Novel halophilic biomolecules such as bacteriorhodopsin (used for biocomputing)
- Pigments for food colouring

(iv) Bioremediation of toxic compounds, decontamination of saline industrial wastewater.

25.12 Future Perspectives and Conclusions

Halophiles are an important class of extremophilic organisms adapted to carry out metabolic functions in high salt concentration environment. Enzymes from halophilic microbes are potential candidate for efficient catalysis under high salt and non-aqueous reaction conditions. They find important applications in the field of novel biocatalysis and biotransformation reactions. Products derived from halophiles also have wide applications in biotechnology and they promise to be a rich resource for future biotechnological and industrial demands.

Despite much of the research work focused on halophiles, they remain an untapped resource for industrial applications. Exploration of new saline environment to isolate and characterize new microbes producing desirable biocatalysts and biomolecules will provide better tool kits for future. Genetic engineering of halophilic enzymes in mesophilic hetrologous host will be economical for enhanced enzyme production. A metagenomic approach to harness the novel biocatalytic prowess of the yet unexplored halophilic microorganisms should be useful for many bioprocesses. Structure-function relationships and an increased number of resolutions of 3-D structures of halophilic enzymes may enhance our understanding of protein folding and stability in high saline and low water environment. Last but not the least, halophiles and their enzymes may also be aptly utilized in bioremediation of saline wastes produced by many modern day industries.

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Chapter 26
Sourcing the Fungal Endophytes: A Beneficial Transaction of Biodiversity, Bioactive Natural Products, Plant Protection and Nanotechnology

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Abstract Endophytes are the group of microorganisms that reside to internal and healthy tissues without causing negative symptoms to their host plant. Endophytes are extremely diverse and range from fungi, bacteria and actinomycetes. Development of drug resistance to pathogenic forms of bacteria, fungi and other microbes, emergence of lethal viruses, the perpetuating epidemics in developing and under developing countries, and multifold fungal infection, enhancement in human population globally, all shows our inability to overcome these biomedical problems. In addition to this, we are also unable to assure people towards enough food security in specific regions of the earth due to infestation of different plant diseases. Since the fungal endophytes are relatively less studied group of microbial flora, but are responsible for several prospects such as biodiversity, ecology, bioactive metabolites (metabolomics) and nanotechnology, may enable us to overcome the above mentioned problems. Fungal endophytes represent a dependable source of specific secondary metabolites and can be manipulated both physicochemically and genetically to increase yield of desired compounds and to produce novel analogues of active metabolites. In this chapter, we have discussed several bioactive compounds and classified them in to different classes as per their properties such as antifungal, antibacterial, antiviral, antimalarial, anticancer, antioxidants, antidiabetic and immunosuppressive agents derived from fungal endophytes with their hosts and made the chemical structures

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for 73 compounds using chemdraw 3D ultra version 7.0. These bioactive products are related to human health with MIC/EC/IC_{50} values less that 50 μg/mL. This article also discusses nematicidal, some antimicrobial volatile compounds (VOCs) that are related to plant protection and faecal disposal. Therefore, this chapter is not very specific and covers almost prospects of fungal endophytes which could be useful in biodiversity, agrochemicals, biotechnology, biomedical and nanotechnology in ecofriendly manner.

Keywords Endophytic fungi • Antimicrobial • Anticancer • Antioxidants • Mycodiesel • Metals nanoparticles

26.1 Introduction

Originally, the term endophyte was introduced by de Bary (1866) and was assigned to all those microbes that reside inside the living healthy tissues of the plants. Later, this term was expanded as fungi and bacteria including actinomycetes, which spend the whole or at least a part of their life cycle colonizing inter- or intra-cellularly, inside the healthy living tissue of the host plant, typically causing no apparent symptoms of disease. Many workers define the endophytes in different ways, but Bacon and White (2000) gave a conclusive and widely accepted definition of endophyte as 'microbes that colonize living, internal tissues of plant without causing any immediate, overt negative effect'. Endophytes are extremely diverse and range from fungi Carroll and Carroll (1978), Petrini (1986), Rajagopal and Suryanarayanan (2000), Gond et al. (2007), to bacteria Hallmann et al. (1997) including actinomycetes Verma et al. (2009a), but the fungi are most studied group of endophytes and among fungi the best studied endophytes are intercellular symbionts from ascomycetous family Clavicipitaceae in the grasses of temperate zone. The presence of endophytes was observed from algae to angiosperm studied till date Aly et al. (2010). The literature suggests that endophytes augment resistance in their hosts against herbivores Brem and Leuchtmann (2001), pathogenic fungi, bacteria, viruses, insects, nematodes Gond et al. (2010), illness Clay (1990), reduced seed production Rice et al. (1990), temperature and salinity Redman et al. (2002) and also against drought and minerals Malinowski et al. (1997).

Development of drug resistance to pathogenic forms of bacteria, fungi and other microbes, emergence of lethal viruses, the perpetuating epidemics in developing and under developing countries, and multifold fungal infection, enhancement in human population globally, all shows our inability to overcome these biomedical problems. In addition to this, we are also unable to assure people towards enough food security in specific regions of the earth and in India too, to support the local human population. Environmental degradation, loss of biodiversity and spoilage of land and water also added to the problems facing mankind.

The access of new disease causing agents like AIDS, SARS, Ebola and already epidemic like malaria, leismania and encephalitis requires the discovery and development of new therapeutic drugs that target them specifically within the cellular metabolism. Due to safety and the environmental problems, many synthetic agricultural agents have been and currently are being targeted for removal from the market, which creates a need to find the alternative ways to control farm pests and pathogens. In search of these new and lead molecule/or drug, we have to find out new and alternative resources, and endophytic fungi are one of them which could be explored for this purpose Kharwar and Strobel (2011). After the discovery of taxol (billon dollar anticancer drug) from an endophytic fungus *Taxomyces andreanae,* research related to fungal endophytes got a great attention for rich and novel alternative source of natural bioactive compounds Stierle et al. (1993). The number of research publications only related to secondary metabolites (115) from fungal endophytes during period of 2000–2009, itself indicates the attention of researchers to this field of study Aly et al. (2010). Since the fungal endophytes are relatively less studied group of microbial flora, but are responsible for several prospects such as biodiversity, ecology, bioactive natural products (metabolomics) and nanotechnology. Therefore, this chapter is not very specific and covers almost prospects of fungal endophytes which could be useful in biodiversity, agrochemicals, biotechnology, biomedical and nanotechnology in ecofriendly manner.

26.2 Biodiversity and Ecology of Endophytic Fungi

Endophytic fungi are hidden, highly diverse and potential entities of microbial world as they reside in a unique biotope. They have successfully been isolated ranged from host of highly water stressed desert Bashyal et al. (2005), cold stressed arctic Fisher et al. (1995), antarctic Rosa et al. (2009), ocean Wang et al. (2006), geothermal soils Redman et al. (2002), highly diverse rain forests Strobel (2002), dry deciduous and coastal forests Suryanarayanan et al. (2003, 2005) and mangrove swamps Lin et al. (2008). The significant presence of endophytes were observed in all green biota ranging from algae Kralj et al. (2006), Wang et al. (2006), Pontius et al. (2008), bryophytes Silvia et al. (2008), pteridophytes Swatzell et al. (1996), gymnosperms Hoffman and Arnold (2008) and to angiosperms Gond et al. (2007) including underground root to all aerial parts of host Kharwar et al. (2008). Our earth harbors all most 300,000 higher plants species and each species represents either one or plethora of endophytic community and it is well proved by the various studies of higher plants fungal endophytes Strobel (2002). Out of 300,000 higher plants that exist on the earth, only a few dozens, have been studied related to their endophytic biology.

It is expected that except bacteria and fungi, other microbial entity may also exist in plants as endophytes such as archebacteria, streptomycetes, mycoplasmas and rickettsia. Actually, non culturable behaviour of these microbes and lack of precise techniques of isolation may be the reasons for not reporting them frequently from plants except a few reports (Verma et al. 2009a). The most frequently isolated endophytes are the fungi. However, at outset, it is important to note that the vast majority of plants have not been studied for any endophytic association. Thus, enormous opportunities exist for the recovery of novel fungal forms, including genera, biotypes, as well as species in the myriad of plants yet to be studied in different settings and ecosystem. Hawksworth and Rossman (1987) estimated there may be as many as 1.5 million different fungal species, while only about 100,000 have been described. As more evidence accumulates, estimates keep rising as to the actual number of fungal species. For instance, Dreyfuss and Chapela (1994) have estimated at least one million species of endophytic fungi alone.

It seems obvious that endophytes are a rich and reliable source of genetic diversity and may represent many previously undescribed species. Among endophytic fungi, ascomycetous members of family clavicipitaceae were excellently recovered and studied from poaceae Clay (1989), however, the members of coelomycetes, hyphomycetes and mycelia-sterila Pereira et al. (1999), Tejesvi et al. (2005), Chareprasert et al. (2006), Gond et al. (2007) were also frequently isolated while members of basidomycetes Santos et al. (2003) and zygomycetes Spurr and Welty (1975), are not frequently isolated and studied.

26.3 Metabolomics

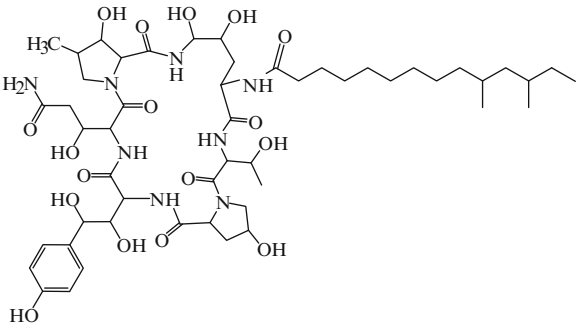
In the course of ongoing study about antimicrobials, it was found that with the evolution of antibiotics, the pathogenic bacteria and fungi also start the development of multidrug resistance, and the conventional antibiotics become failure to cure the diseases. Therefore, to overcome the problem medical sciences carry out the research for new and effective antibiotics from new sources, and in this respect fungal endophytes give the rays of hope and may fill some gaps as endophytic researches indicate that 51% of bioactive substances isolated from endophytic fungi were previously unknown compared to 38% novel compounds from soil fungi Schulz et al. (2002), and this data induce scientists to explore the possibility of antimicrobial compounds of novel entity from this relatively hidden repertoire.

26.3.1 Role of Endophytic Fungal Metabolites in Human Health

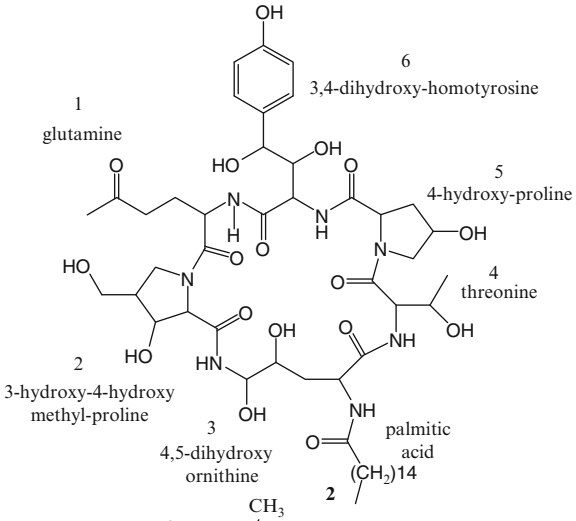
The metabolites received from fungal endophytes could be categorized in the following groups based on their bioactivity either against particular group of pathogens or against diseases.

26.3.1.1 Antifungal Agents

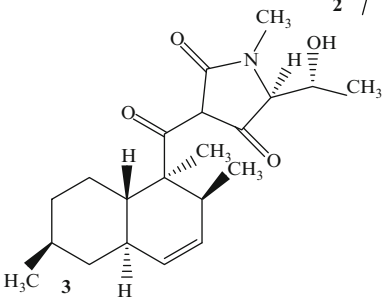
Undoubtedly, fungi are major causal organisms of various diseases in humans and there are a number of chemical (synthetic) fungicides are in use to protect the humans, but these chemicals also make an adverse impact on environment Strobel et al. (2002). Literatures suggest that 52.3% of endophytic metabolites display growth inhibition activity to at least one or more than one pathogenic microbes Gond et al. (2010). There are a huge number of antifungal compounds that have been isolated from endophytic fungi, but here we are mentioning only those having MIC value equal to or less than 50 μg/mL. An echinocandin (antifungal agent L-*671,329*) (**1**) was isolated from endophytic *Cryptosporiopsis* sp. of *Pinus sylvestris* and *Fagus sylvatica* found to have activity against *Candida albicans* and *Saccharomyces cerevisiae* Noble et al. (1991). An endophytic fungus *Cryptosporiopsis* cf. *quercina* recovered from stem of *Triptergeum wilfordii*, produces a potent antimycotic, cryptocandin (**2**) (lipopeptide) and cryptocin (**3**). Cryptocandin showed activity against human pathogenic fungi *Trichophyton rubrum* (ATCC 28188), *Trichophyton mentagrophytes* (ATCC 28185), *Candida albicans* (ATCC 90028), *Candida parapsilosis and Histoplasma capsulatum* Strobel et al. (1999). CR377 (**4**), a novel pentaketide possess anticandida activity extracted from endophytic fungus CR377 (*Fusarium* sp.) inhabited the internal tissue of *Selaginella pallescens* Brady and Clardy (2000). Cytosporones D (**5**) is trihydroxybenzene lactone that has been reported from two endophytic fungal strains, CR 200 (*Cytospora* sp.) and CR 146 (*Diaporthe* sp.) which were isolated from the tissue of *Conocarpus erecta* and *Forsteronia spicata* plants, respectively possess significant anticandida activity Brady et al. (2000). 7-amino-4-methylcoumarin (**6**) isolated from endophytic sp., residing inside *Ginkgo biloba* exhibit antifungal activity against *Candida albicans*, *Penicillium expansum* and *Aspergillus niger*, including the antibacterial activity Liu et al. (2008). *Chloridium* sp. isolated as an endophyte from *Azadirachta indica*, produced the compound javanicin (**7**) a strong antibacterial naphthaquinone had also inhibited the growth of several fungal pathogens at MIC below 20 μg/mL Kharwar et al. (2009).



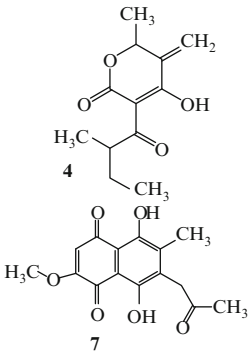
1

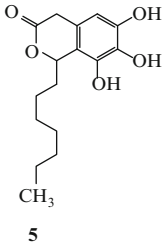

1
glutamine
2
3-hydroxy-4-hydroxy
methyl-proline
3
4,5-dihydroxy
ornithine
4
threonine
5
4-hydroxy-proline
6
3,4-dihydroxy-homotyrosine
palmitic
acid
(CH2)14

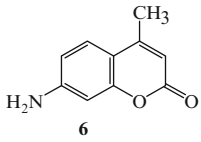
2



3



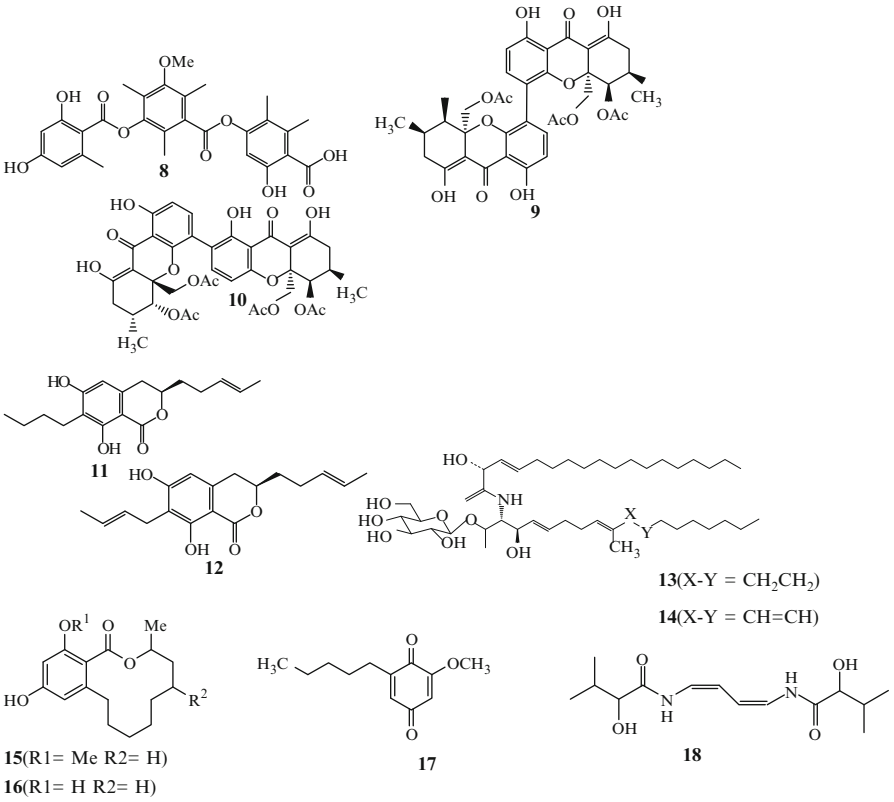




26.3.1.2 Antibacterial Agent

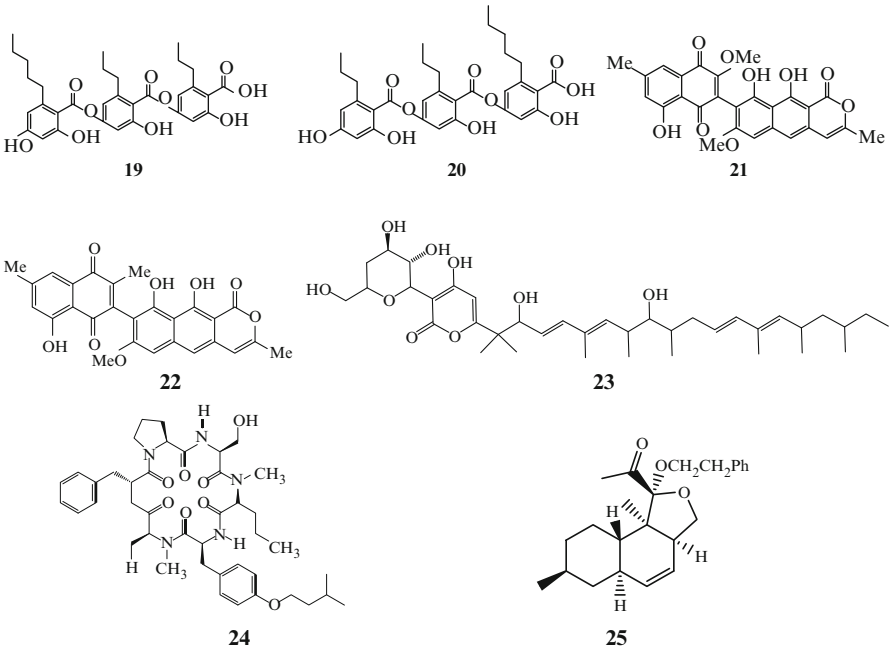
A considerable number of effective and potential antibacterial compounds have been isolated from endophytic fungi against a range of gram +ve and gram −ve bacteria. A novel secondary metabolite colletotric acid (**8**) was isolated from *Colletotrichum gloeosporioides* colonizing the tissues of *Artemisia mongolica* and inhibited the growth of *Bacillus subtilis, Staphylococcus aureus* and *Sarcina leutea* Zou et al. (2000). Phomoxanthones A (**9**) and B (**10**) are novel xanthone dimers that were isolated from the endophytic fungus *Phomopsis* sp. BCC 1323 showed antituberculosis activity Isaka et al. (2001). Cytosporones D (**5**) described earlier as antifungal also exhibited the significant activity against *Staphylococcus aureus,* and *Enterococcus faecalis*, respectively Brady et al. (2000). 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**11**) and 7-butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11- enylisochroman-1-one (**12**) extracted from endophytic *Geotrichum* sp. which inhabited *Crassocephalum crepidioides*. Both the compounds had shown anti-TB (tuberculosis) effect Kongsaeree et al. (2003). Two cerebrosides (glycosphingolipids) cerebroside (**13**) and fusaruside (**14**), the later being new were isolated from *Fusarium* sp. IFB-121, an endophytic fungus of *Quercus variabilis*. Both the compounds were recorded as antibacterial in respect to *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas fluorescens* Shu et al. (2004). Two known lactones lasiodiplodin (**15**), and de-*O*-methyllasiodiplodin (**16**) isolated from an endophytic fungus (No. ZZF36) of brown alga, possess considerable activity against *Staphylococcus aureus* (ATCC27154) and *Bacillus subtilis* (ATCC6633) while activity against *Salmonella enteritidis* only possessed by later Yang et al. (2006). The endophytic fungus *Botrytis mamane* PSU-M76 was isolated from the interior of *G. mangostana* reported to produce a known metabolite primin (**17**) which exhibits prominent antibacterial activity against *Staphylococcus aureus* ATCC 25923 and methicillin-resistant *S. aureus* SK1 Pongcharoen et al. (2007). Another compound phomoenamide (**18**) was isolated from an endophytic fungus *Phomopsis* sp. PSU-D15 an inhabitant of *Garcinia dulcis* leaf, showed the prominent activity in opposition to *Mycobacterium tuberculosis* H37Ra Rukachaisirikul et al. (2008). Javincin (**7**)

described earlier also, exhibits strong antibacterial activity against *Bacillus sp., Escherichia coli, Pseudomonas fluorescens and Pseudomonas aeruginosa* Kharwar et al. (2009).



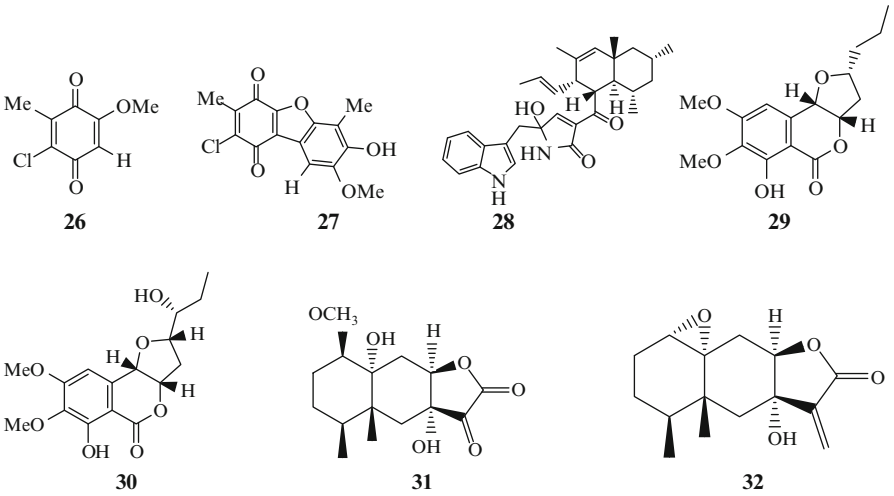
26.3.1.3 Antiviral Agent

Two novel human cytomegalovirus protease inhibitors, cytonic acids A (**19**) and B (**20**) isolated from *Cytonaema* sp., an endophyte of *Quercus* sp. aganist human cytomegalovirus (hCMV) Guo et al. (2000). Endophytic fungus *Penicillium chrysogenum* of unidentified tree in Peru found to produce xanthoviridicatins E (**21**) and F (**22**), inhibited the cleavage reaction of HIV-1 integrase Singh et al. (2003). Metabolite S 39163/F-I (**23**) extracted from an isolate of endophytic fungus strain NRRL 15684 was isolated from the leaf of *Buxus sempervirens* L., showed better activity against herpes viruses in addition to antifungal activity Tscherter et al. (1988) and Gunatilaka (2006). Pullularin A (**24**) possesses activity against herpes simplex virus type 1, isolated from endophytic fungus *Pullularia* sp. BCC 8613 of *Culophyllum* sp. Isaka et al. (2007). Oblongolide Z (**25**) (hexaketide γ-lactone) has been isolated from *Phomopsis* sp. BCC 9789 associated with *Musa acuminata* (wild banana) as an endophyte possesses activity as anti-herpes simplex virus type 1 Taridaporn et al. (2010).



26.3.1.4 Antimalarial Agent

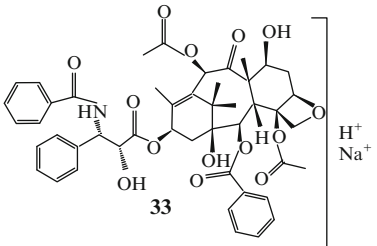
Several anti-malarial compounds have also been isolated from this group of microbes and a few of them are mentioned here as representatives. Phomoxanthones A (**9**) and B (**10**) isolated from an endophytic *Phomopsis* sp., are known to display antimalarial activity Isaka et al. (2001). Endophytic *Geotrichum* sp. collected from *Crassocephalum crepidioides* have found to produce 7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one (**11**) and 7-butyl-15-enyl-6,8-dihydroxy-3(*R*)-pent-11- enylisochroman-1-one (**12**) Kongsaeree et al. (2003). The above mentioned four anti malarial compounds are also antibacterial which have already been described in previous section. 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**26**) and xylariaquinone A (**27**) are novel benzoquinones that were isolated from an endophytic *Xylaria* sp. Tansuwan et al. (2007). Codinaeopsin (**28**) is another antimalarial agent, isolated from an endophytic fungal isolate CR127A (98% idetical to *Codinaeopsis gonytrichoides*) collected from *Vochysia guatemalensis* (a white yemeri tree) Kontnik and Clardy (2008). Monocerin (**29**) and its new analogue 11-hydroxymonocerin (**30**) were isolated from cultures of *Exserohilum rostratum*, a fungal strain endophytic in *Stemona* sp. Sappapan et al. (2008). Two new eremophilane-type sesquiterpenoids, eremophilanolides 2 (**31**) and 3 (**32**) were isolated from an endophytic fungus BCC 21097 of *Licuala spinosa* Isaka et al. (2010). All 12 copmound showed *in vitro* activity against *Plasmodium falciparum* K1.



26.3.1.5 Anticancerous Agent

About 100 anticancer compounds have been isolated from endophytic fungi yet, and among them 57% are novel, while rest are previously known Kharwar et al. (2011). Taxol (**33**) is highly functionalized diterpene and is the first compound possessing the taxane ring, isolated from the bark of the *Taxus brevifolia* (Pacific Yew) for the first time by Wani et al. (1971) and named it as 'taxol'. Its effectiveness against ovarian and breast cancers make the drug highly valuable. For the first time in history, Stierle et al. (1993) isolated this important compound from a fungus *Taxomyces andreanae* endophytically associated with *Taxus brevifolia*. After this discovery, a number of scientists isolated Taxol from different endophytic fungi associated with different hosts such as *Bartalinia robillardoides* and *Pestalotiopsis terminalia*e, the endophytic fungi of *Aegle marmelos* and *Terminalia arjuna*, Gangadevi and Muthumary (2008, 2009). Camptothecin (**34**), another important anticancer compound previously known to be isolated from wood of *Camptotheca acuminata* plant was isolated from an endophytic fungus of *Nothapodytes foetida*, *Entrophosphora infrequens* and *Neurospora* sp. for the first time by Puri et al. (2005) and Rehman et al. (2008), respectively. This very compound with two of its

analogues, 9-methoxycamptothecin (**35**) and 10-hydroxycamptothecin (**36**) also isolated from *Fusarium solani* an endophytic fungus of *Camptotheca acuminata* Kusari et al. (2009). Additionally, 9-methoxycamptothecin and 10-hydroxycamptothecin do not have the therapeutic drawbacks as it found in plant camptothecin even after showing the similar activity. The similar nature of compound is also reported with vincristine (**37**) produced by *Fusarium oxysporum*, an endophyte of *Catharanthus roseus* Zhang et al. (2000), Tung et al. (2002). Emindole DA (**38**) isolated from *Emericella nidulans* var. *acristata* an endophyte of a Mediterranean green alga showed the antitumor activity against 36 human tumor cell lines Kralj et al. (2006). Leptosphaerone C (**39**) and penicillenone are the novel anticancer polyketides isolated from *Penicillium* sp. JP-1, an endophytic fungus associated to the *Aegiceras corniculatum* (mangrove plant). Leptosphaerone C showed activity against A-549 cells, whereas penicillenone exhibits cytotoxicity against P388 cells Lin et al. (2008). An endophyte *Alternaria* sp., isolated from *Polygonum senegalense* (Egyptian medicinal plant) had produced three lactone compounds, alternariol (**40**), alternariol 5-*O*-sulfate (**41**), alternariol 5-*O*-methyl ether (**42**), and a phenol derivative altenusin (**43**) bearing cytotoxic activity against L5178Y cells Aly et al. (2008). Phomoxanthones A (**9**) and Phomoxanthones B (**10**) describe avtivity against KB, BC-1, Vero cell lines Isaka et al. (2001). Merulin A (**44**) (nor-chamigrane endoperoxide) and C (**45**) (chamigrane endoperoxides) are two new sesquiterpenes produced from an endophytic fungi XG8D, member of class basidiomycetes isolated from mangrove plant, *Xylocarpus granatum* Konig (Meliaceae). Both the compounds showed significant cytotoxicity against human breast (BT474) and colon (SW620) cancer cell lines Chokpaiboon et al. (2010). *Phomopsis longicola*, an endophytic fungus of the *Dicerandra frutescens* (endangered mint) produces three cytotoxic compounds dicerandrols A (**46**) B (**47**) and C (**48**) against two human cancer cell lines, A-549 and HCT-116 Wagenaar and Clardy (2001). Two new, Oblongolide (hexaketide γ-lactone) Y (**49**) and Z (**25**) have been isolated from *Phomopsis* sp. BCC 9789 associated with *Musa acuminata* (wild banana) as an endophyte. Oblongolide Y showed cytotoxic activity against BC line while Oblongolide Z had shown cytotoxic activities against KB, BC, NCI-H187, and nonmalignant (Vero) cell lines Taridaporn et al. (2010). Six noble benzofuranone-derived γ-lactones, photinides A-F (**50–55**) isolated from a single endophytic fungus *Pestalotiopsis photiniae* resides inside *Roystonea regia*. All six noble γ-lactones exhibit cytotoxicity against MDA-MB-2311 (human tumor cell lines) Ding et al. (2009).



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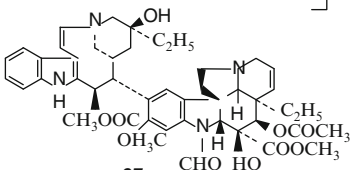

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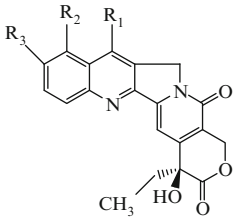
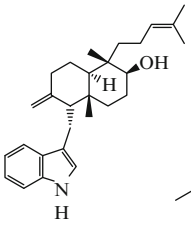


34 (R_1, R_2, R_3 = H)
35 (R_1, R_3=H; R_2=OCH_3)
36 (R_1, R_2= H; and R_3= OH)



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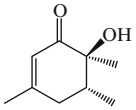

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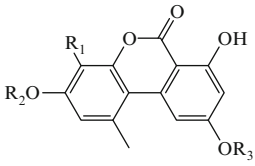
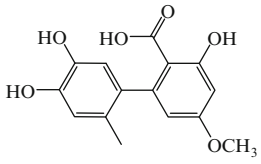


40 (R^1, R^2, R^3 = H)
41 (R^1, R^2, = H and R^3= SO_3H)
42 (R^1, R^2, = H and R^3= CH_3)



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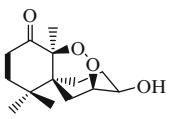

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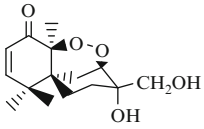

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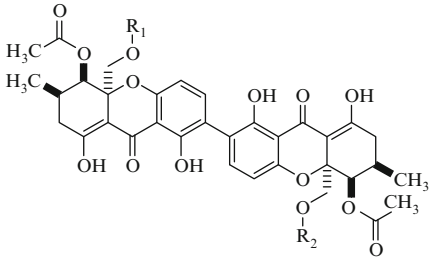
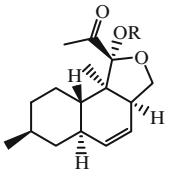


46 (R1 = R2 = H)
47 (R1 = Ac, R2 = H)
48 (R1 = R2 = Ac)



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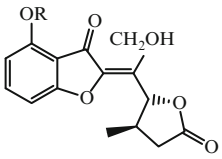
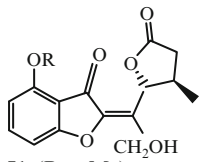
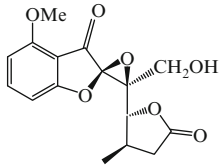


50 (R = H)
52 (R = H)



51 (R = Me)
53 (R = H)



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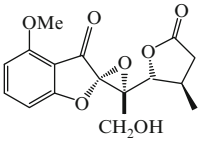
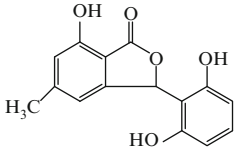


55

26.3.1.6 Antioxidant Agent

Antioxidant agents are the free radical-scavenging molecules that reduce the possibilities of aging, cancer, coronary heart problems, and Alzheimer's disease. These agents also maintain the quality of food grains during storage. Pestacin (**56**) and isopestacin (**57**) obtained from endophytic *Pestalotiopsis microspora* from interior of *Terminalia morobensis* display an antioxidant activity Strobel et al. (2002) and Harper et al. (2003). *Cephalosporium* sp. IFB-E001, an endophytic fungus harbored in *Trachelospermum jasminoides* produces graphislactone A (**58**) shows significant antioxidant activity Song et al. (2005). Chaetopyranin (**59**) and isotetrahydroauroglaucin (**61**) isolated from an endophytic fungus *Chaetomium globosum*, associated with *Polysiphonia urceolata* posseses antioxidant activity. The former compound also exhibit anticancerous activity Wang et al. (2006).



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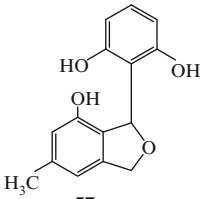

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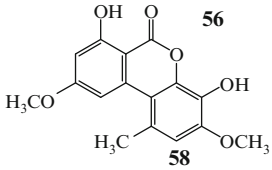

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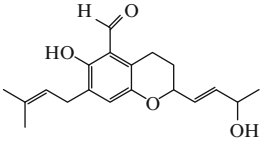

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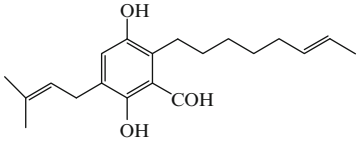

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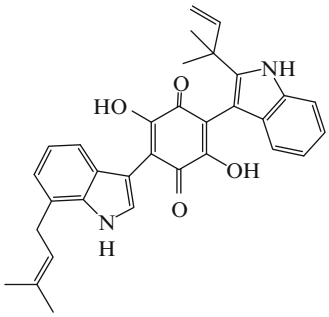
26.3.1.7 Antidiabetic Agent

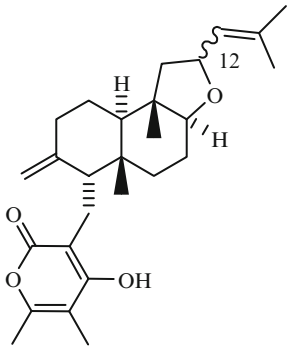
The two impressive antidiabetic agents namely L783281 (**61**) are the nonpeptidal fungal metabolites isolated from endophytic *Pseudomassaria* sp. The compound acts as an insulin mimetic, but without destroying the digestive tract Zhang et al. (1999).



61

26.3.1.8 Immunomodulator and Immunosuppressive Agents

Immunomodulator agents play a key and potential role in the treatment of patients suffering from AIDS, cancer and organ transplant. One of the dominant fungal genus of endophyte *Pestalotiopsis leucothes*, isolated from *Tripterygium wilfordii* produces three compounds designated as BS, GS, and YS. These compounds have uneven effects on T- and B-cells and monocytes, and they may partially show the immunosuppressive activity particularly, against human immune mediated diseases Kumar et al. (2005). The rejection of organ/s is the major difficulty in organ transplanted patients and therefore, there is a need of immunosuppressive drugs until transplanted organ totally adopted by the body. An endophytic fungus *Fusarium subglutinans* from *Tripterygium wilfordii* produces the immunosuppressive, but non cytotoxic diterpene pyrones subglutinol A (**62**) and B (**63**) Lee et al. (1995). Collutellin A, a novel peptide with antimycotic activity isolated from *Colletotrichum dematium* is another endophytic fungus recovered from interior of *Pteromischum* sp. Collutellin A, inhibits IL-2 indicates the potential immunosuppressive activity of this compound at such a lower concentration than other previously known compound cyclosporin Ren et al. (2008).



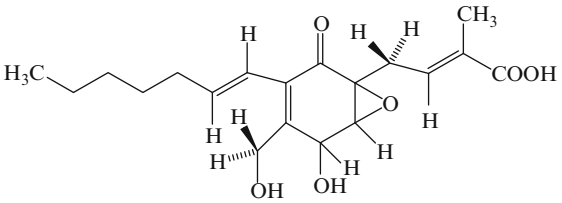
62 (12 S)
63 (12 R)

26.3.2 Endophytic Fungi and Plant Protection

26.3.2.1 Anti Fungal Agent

The role of endophytic fungi is well known in plant protection and disease control as many of their isolates have potential to produce effective antifungal compounds that have inhibited fungal pathogens growth successively. Cryptocin (**3**) possesses antifungal activity against phytopathogens like *Pythium ultimum*, *Phytophthora cinnamomi*, *Phytophthora citrophthora*, *Sclerotinia sclerotiorum*, *Pyricularia oryzae*, *Rhizoctonia solani*, *Geotrichum candidum*, *Fusarium oxysporum* Li et al. (2000). *Colletotrichum gloeosporioides* isolated as an endophyte from *Artemisia mongolica* produced colletotric acid (**8**) showed antibacterial and antifungal activity against *Helminthosporium sativum* Zou et al. (2000). Ambuic acid, (**64**) a highly functionalized cyclohexene isolated from endophytic *Pestalotiopsis* sp. and *Monochaetia* sp. showed significant antifungal property against several pathogens like *Fusarium solani, F. cubense, Helminthosporium sativum, Diploidia natelensis, Cephalosporium gramineum* and *Pythium ultimum* Li et al. (2001). Endophytic fungus *Pestalotiopsis jesteri* isolated from *Fragaraea bodenii* has produced jesterone (**65**), a highly functionalized novel cyclohexenone epoxides exhibits antifungal ability in opposition to *Pythium ultimum*, *Aphanomyces* sp., *Phytophthora citrophthora*, *P. cinnamomi*, *Rhizoctonia solani* and *Pyricularia oryzae* Li and Strobel (2001). Pestacin (**56**) and Isopestacin (**57**) are isobenzofurans isolated from *Pestalotiopsis microspora* obtained as an endophyte from *Terminalia morobensis*. Both compounds exhibit antioxidant and antifungal activity against pathogenic fungus *Pythium ultimum* Strobel et al. (2002), Harper et al. (2003). 2,4-dihydroxy-6-[(1'*E*,3'*E*)-penta-1', 3'-dienyl]-benzaldehyde (**66**) has been isolated from *Periconia atropurpurea*, endophytically obtained from the leaves of *Xylopia aromatic* showed the acute antifungal activity against *Cladosporium sphaerospermum* and *C. cladosporioides* at concentration of 1.0 and 2.5 μg/mL respectively Teles et al. (2006). A novel

solanapyrone N (**67**) along with a known compound, nigrosporalactone (**68**) were isolated from the fermentation culture of *Nigrospora* sp., YB-141, an endophytic fungus of *Azadirachta indica*. Solanapyrone N exhibits activity against *Botrytis cinerea* and *Penicillium islandicum* while nigrosporalactone showed activity in opposition to *Botrytis cinerea* Wu et al. (2008). *Chloridium* sp. isolated as an endophyte from *Azadirachta indica*, produced javanicin (**7**) a highly functionalized naphthaquinone inhibited the growth of *Cercospora arachidicola, Fusarium oxysporum, Rhizoctonia solani* and *Verticillium dahlae* Kharwar et al. (2009).



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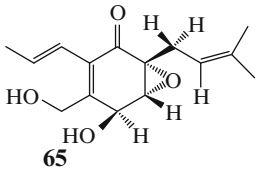

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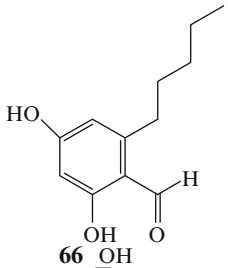

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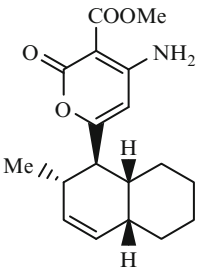

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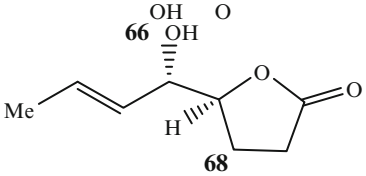

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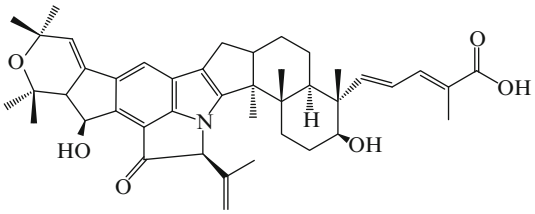
26.3.2.2 Insecticidal Agent

The potent insecticidal agent nodulisporic acid A (**69**), an indole terpene was isolated from fermentation of an endophytic fungus *Nodulisporium* sp., of *Bontia daphnoides*. Nodulisporic acid A was active against the larvae of the blowfly and mosquito at sub-part-per-million levels Ondeyka et al. (1997). Another two novel compounds 5-hydroxy-2-(1-oxo-5-methyl-4-hexenyl) benzofuran (**70**) and 5-hydroxy-2-(1-hydroxy-5-methyl-4-hexenyl) benzofuran (**71**) were isolated from unidentified endophytic fungus of *Gaultheria procumbens*. The later one exhibits toxicity to *Christoneura fumiferana* (spruce budworm cells) only, whereas the former was toxic to larvae also Findlay et al. (1997). 12 fractions were obtained from secondary metabolites of *Penicillum* sp., an enophytic inhabitant of *Derris elliptica*,

and out of these, fraction D, E and J showed the significant toxicity against adult turnip aphid, *Lipaphis erysimi* Hu et al. (2005).



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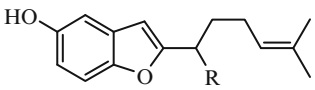
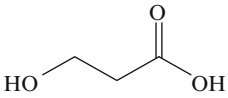


70 (R=O)
71 (R=OH)

26.3.2.3 Nematicidal Agents

The compound 3- Hydroxypropionic (**72**) acid was isolated from *Phomopsis phaseoli* endophytically present to *Betula pendula and B. pubescens* showed selective nematicidal activity against the plant-parasitic nematode *Meloidogyne incognita* Schwarz et al. (2004). The nematicidal alkaloid peniprequinolone (**73**), has been extracted from an endophytic fungus *Phomopsis janczewskii* of Chilean gymnosperm *Prumnopitys andina* with significant activity, however, initially the compound was also reported from a soil fungus *Penicillium* cf. *simplicissimum* Schmeda-Hirschmann et al. (2005).



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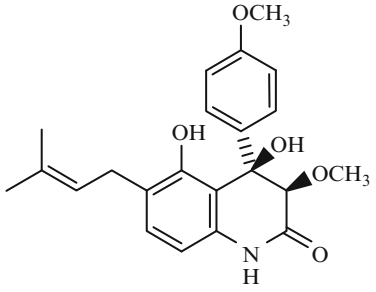

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26.3.2.4 Endophytic Fungi and Abiotic Stresses

Excess or lowering of temperature, water, metals, salts and pH cause stresses on survival of plants and literatures suggest that endophytes are also involved in protection of plants against these abiotic stresses. Redman et al. (2002) concluded that *Dichanthelium lanuginosum* infected with endophytic *Curvularia protuberate* was able to grow at temperatures as high as 57°C in the geothermal soils of Yellowstone National Park (YNP) and Lassen Volcanic National Parks (LVNP). However, the

refined research indicates that this effect is not only due to endophytic *Cuvularia*, but was also due to a *Curvularia* thermal tolerance virus (CThTV) as a third partner, which parasitized on *C. protuberate* Marquez et al. (2007). Hutton et al. (1996), observed in south-west Australia that in drought condition, the water availability could be maintained at higher levels in endophytes infected tall fescue compared to enophytes free plants. Elbersen and West (1996), Buck et al. (1997), Liu et al. (1996), observed the aluminium tolerance in endophyte infected fine fescues (*Festuca* spp.) was greater compared to non-infected plants. Malinowski and Belesky (1999) concluded that endophytes infected root of tall fescue increases the pH of limed and acidic soil faster than non infected one.

26.4 Antimicrobial Volatiles Compounds (VOCs)

Muscodor albus I-41.3a, isolated as an endophyte from interior of unidentified vine (plant I-41) produced a number of volatiles with antimicrobial property. The VOC's included tetrahydrofuran, 2-methylfuran, 2-butanone, aciphyllene, and much amounts of an unusual azuleene derivetives. The fungus showed interesting activity against *Stachybotrys charatarum* and other phytopathogen Atmosukarto et al. (2005). A volatile oil with trans-1,2,3,3a,4,7a-hexohydro-7a-methyl-5H-inden-5-one (73.1%), 2-methylene-4,8,8-trimethyl-4-vinyl bicycle [5.2.0] nonane (12.0%), and 2,6-dimethyl-6-(4-methyl-3-pentenyl) bicycle [3.1.1] hept-2-ene (4.5%) isolated from endophytic *Fusarium tricinctum* of Chinese medicinal herb *Paris polyphylla* var. *yunnanensis* exihibit anti fungal (*Candida albicans* and *Magnaporthe oryzae)* and anti bacterial activities (*E. coli, Salmonella, Bacillus etc.*) Zhang et al. (2010). The artificial mixture of volatile compounds may also have usefulness in treating seeds, fruits and plant parts in storage and while being transported. In addition, *M. albus* is already in a limited market for the treatment of human wastes. Its gases have both inhibitory and lethal effects on such faecal-inhabiting organisms as *Escherichia coli* and *Vibrio cholera* Kharwar and Strobel (2011).

26.5 Mycodiesel

Gliocladium roseum was isolated as an endophyte residing inside *Eucryphia cordifolia*, produced a series of volatile hydrocarbons and hydrocarbon derivatives. The hydrocarbon series isolated showing similarities with diesel fuel and this fungus could be used as a novel biofuel source and due to fungal source this fuel was named as a mycodiesel by Strobel et al. (2008). The another recent and interesting discovery appeared when eucalyptol, a rare compound only previously known to be found in eucalyptus bark was isolated from an endophytic fungus. Surprisingly, Dr. Strobel, has run his Honda motorbike using this eucalyptol and therefore, it could be a good and safe alternative of gasoline as its potential is much better Tomsheck et al. (2010). The emphasis is being given to drive this kind of research world wide to solve the

problem of fuel using non conventional sources, so that pressure could be reduced from conventional ores, and Dr. Strobel and his group was given a huge amount from funding agency to precise his research.

26.6 Endophytic Fungi and Nanotechnology

Nanotechnology is one of highly important and cutting edge area of scientific research refers to nano scale synthesis of metals with novel properties. Now these days, the nanotechnology shows valuable scope in communication, energy, electronics, instruments, optical engineering, defence and security, cosmetics, bioengineering, nanofabrics, agri food industry, biomedicals and drug delivery. The nano food market is the fastest growing sector where this technology could be used in the production, processing and packaging. A nanocomposite coating process may improve food packaging by placing antimicrobial agents directly on the surface of the coated film. Further, the research is carried out to the detection of chemical and biological substances for sensing biochemical changes in foods quality. As per the survey of Helmut Kaiser consultancy the nanofood market will surge from 2.6$ billion to 20.4$ billion by 2011 Verma et al. (2009b). There are many chemical and physical approaches for synthesis of nano particles, but they are highly costly and hazardous to environment. Therefore, scientific community looks for ecofriendly, cost effective and an alternative technology, and the one step biosynthesis or green synthesis is one of them, which synthesizes nanoparticles of useful metals (Au, Ag, Cd, Pt, Ti, Zn and Pd) via biological tools such as Bacteria Yong et al. (2002), fungi and actinomycetes Sastry et al. (2003), Verma et al. (2010), algae Brayner et al. (2007) and plants Narayanan and Sakthivel (2008). Recently, a number of fungi including endophytic such as *Fusarium semitectum, Verticillum* sp.*, Aspergillus*, *Fusarium and Penicillum fellutanum* were successfully employed for synthesis of metal nano particles (NPs). Endophytic *Aspergillus clavatus* isolated from *Azadirachta indica* collected from Banaras Hindu University was efficiently able to synthesize the silver NPs of 5–55 nm. These NPs showed the antifungal (*Candida albicans*) and antibacterial (*E. coli* and *Pseudomonas fluorescens*) activity Verma et al. (2010). *Verticillium* sp. isolated from *Taxus* sp., when challenged with ions of silver and gold led to biofabrication of silver and gold nanoparticles within the fungal biomass Sastry et al. (2003). These examples are enough to prove the use and role of endophytic fungi in different aspects of nanotechnology.

26.7 Conclusions

Historically, the study of endophytes has been limited more or less to botanists to observe their ecology, diversity, distribution and biologics. However, in recent years, the discovery of novel chemical compounds produced by endophytes has opened

the field to structural chemists, biochemists, and medical and industrial scientists as well. The endophytes based bioactive compounds/drugs can only be achieved through interdisciplinary cooperation of research including botanists, microbiologist, molecular biologists, structural chemist, biochemist and pharmacologists. Before dealing the endophytic research we must take care of the basics, such as (i) Location: It is one of the most important criteria as tropical and subtropical rain forests contain the largest diversity of plant species, and can be utilized to discover diverse species of endophytes for the identification of novel anti-fungal and antibacterial natural products. In addition to these habitats, researcher should also consider other habitats such as those in the desert and high elevation Alpine passes. (ii) History: The plants with ethanobotanical history and their use by indigenous people for healing and medicinal purposes may provide guidance for further the study. The discovery of "Taxol" a cytotoxic compound used in cancer therapy is made by both the fungal endophyte and the host plant (pacific yew), and also the discovery of vincristine and vinblastine from fungal endophytes of *Catharanthus roseus* are good examples of the potential bioactive compounds that may be obtained based on plant history. (iii) Communication: Very little, if any, is known about the consequences of fungal/fungal, bacterial/bacterial and fungal/bacterial associations and interactions, especially *in planta*. In some preliminary studies, it was found that the presence of one or more than one endophytes facilitate the production of bioactive compounds. Obviously, more efforts must be initiated in this area of research to be able to optimally harnessing the potential of endophytes. (iv) Distribution and abundance: Studies have shown that in some systems, the presence of endophytes is ubiquitous while in some systems very few endophytes colonize the tissues such as roots and stems. So, these studies beg one to as the question of "What are the regulatory aspects involved in the differential in vivo and *in planta* distribution(s) observed?" (v) Production: Aspects affecting the stimulation and subsequent production of bioactive compounds is an important variable to determine. Studies have indicated that the production of compounds is affected by the immediate environment. For example, when grown under *in vitro* conditions, an endophyte may successfully and constantly produce bioactive materials for a time, after which, production seizes. Additional research addressing the regulatory factors involved is required to determine how to induce the endophytes to produce the compound(s) in a steady fashion for successful commercialization to become a reality.

The past history of endophytic research in India especially with fungi is not very encouraging. It seems that investigators that have started this research in India are still actively involved in advancing their research manifesto with this 'under studied' group of microbes and yet, have not advanced the field significantly. An exception to this statement only a few people are engaged and pursuing their research in this area consistently such as Prof. T. S. Suryanarayanan (Chennai), Prof Umashaankar (Karnataka), T. Amna and Sanjana Kaul (Jammu and Kashmir), Prof. H. S. Prakash, and K. R. Sridhar (Mangalore, Karnataka) and R. N. Kharwar (BHU, Varanasi, Uttar Pradesh). Their group is actively searching the various habitats of the countryside in van attempt to investigate microbe/plant associations. They have initiated the biodiversity and distribution patterns of fungal endophytes with some medicinal

plants in India and have published several papers along this line. They have also isolated some bioactive compounds from different endophytic fungi inhabited to different hosts. Some other groups have also started research with fungal endophytes, but overall situation in India compared to countries such as China and Brazil, for exploration of bioactive compounds is still minimal. However, recently, several research groups have started displaying more attention and efforts towards the potential importance of endophytic microbes, especially fungi. About two dozen research groups throughout India are presently engaged in vigorous studies centered on biodiversity and hence natural products discovery from this untapped alternative resource. It has become apparent to many researchers that endophytic microbes have enormous potential for addressing and solving many problems of mankind. With the discovery of new compounds, collectively as a society, we may find new options to protect our interests in agriculture, medicine and nanotechnology. Actually, the status of total published work by Indian researchers hardly approaching up to hundred, which shows a small representative potential of the possibilities that exists in this realm of study. If serious efforts were to be explored in India, with its vast and enormous plant diversity, and/or worldwide with all the collective habitat potentials, it boggles the mind in the possibilities we as scientists may unravel. As such, studies of this nature truly need to be supported not only by the scientific community, but society at large that will surely reap the benefits.

Due to great variation in plant biodiversity and seasonal changes in India, we may have better opportunity to collect/isolate various types of promising endophytic fungi, especially from rainforests and mangrove swamps, which may be able to produce an enormous variety of potential bioactive natural compounds. The bioactive compounds to be obtained that are of interest are those that have activity against important plant and human pathogens/parasites (such as those causing malaria, tuberculosis, leprosy and encephalitis, etc.,), which have either become drug resistant, and/or lack drug treatment (i.e., AIDS). In recent years, India has experienced an increase in AIDS and immuno-compromised patients. A search for safe and effective drugs to enhance the resistance capacity of patients would be of great benefit and add quality to life. Discovery of novel antimicrobials against disease that can be used as weapons against virulent pathogens such as *Bacillus anthracis* would be greatly beneficial. Unfortunately, in recent times, a high incidence of scientifically advanced and remote-operated terrorist activities in countries like India, USA, UK, Germany, USSR and France is a reality that needs to be urgently addressed. Required are the developments of compounds that can effectively nullify the potential adverse effect of biochemical attacks. Yet another concern is the inordinately huge population in India (more than one billion strong), all who require a safe, reliable food source for sustenance. Such goals may only be achieved once production of disease free crops is obtainable. Management of human fecal matter is yet another alarming issue if left unmanaged will result in unhygienic and potentially lethal conditions, especially in slums and localities of poorer people. India unfortunately is still suffering from several severe human diseases (tuberculosis, malaria and leprosy, etc.,) that have been successfully eradicated from other "first world" countries. One of the primary reasons these disease persist is due to the development of

disease resistance in these microorganisms against particular drug(s). Therefore, to overcome these problems, India as well as the world in general, needs to obtain a variety of novel antimicrobial compounds from biological sources to begin to address these serious issues. In general, fungi as a group hold enormous potential as a promising source of antimicrobials and tool for nanotechnology. Our studies indicate that this group of organisms reside inside healthy plant tissue (endophytes) without causing any detectable symptoms, and are capable of producing powerful natural bioactive compounds, biofules and nanomaterials. Therefore, we strongly feel that India, as well as the world, needs to make serious efforts to address the potential of endophytes for isolation and synthesis of natural products, biofuel and nanoparticles, and in so doing, help facilitate the development of options and ultimately, the rescue of humanity from annihilation.

26.8 Future Perspectives

Endophytes are extremely diverse group of microbes that range from fungi to bacteria including actinomycetes, but the fungi are most studied group of endophytes, and among fungi the best studied endophytes are intercellular symbionts from ascomycetous family Clavicipitaceae in the grasses of temperate zone. The presence of endophytes was observed from algae to angiosperm studied till date. As per studies made world wide, endophytes augment resistance in their hosts against herbivores, pathogenic fungi, bacteria, viruses, insects, nematodes illness, reduced seed production, temperature and salinity and also against drought and minerals as well. The day by day development of drug resistance to pathogenic forms of bacteria, fungi and other microbes, emergence of lethal viruses, the perpetuating epidemics in developing and under developing countries, and multifold fungal infection, enhancement in human population globally, all shows our inability to overcome these biomedical related problems. In addition to this, we are also unable to assure people towards enough food security in specific regions of the earth and in India too, to support the local human population. Environmental degradation, loss of biodiversity and spoilage of land and water also added to the problems facing mankind. The access of new disease causing agents like AIDS, SARS, Ebola and already epidemic like malaria, leismania and encephalitis requires the discovery and development of new therapeutic drugs that target them specifically within the cellular metabolism.

Although many products can be produced synthetically, but natural bioactive products remains as an important alternative used heavily in modern medicine and agricultures. Approximately 60% of the new drugs produced during the period 1985–2005 were anticancer, antimigraine and antihypertensive agents derived from either natural products or based on natural products structure. In some instances, endophytic microorganisms have developed the biochemical ability to produce compounds similar or identical to those produced by their host plants as a result of gene recombination during the evolutionary process. Bioactive natural products from endophytic microbes have enormous potential as a source for novel medicinal

Table 26.1 Fungal endophyte's derived bioactive compounds with their hosts, activity and MIC/IC_{50} values

Host	Endophyte	Compound	Activity	Pathogen/cell line	MIC/IC_{50}
Pinus sylvestris	*Cryptosporiopsis* sp.	Echinocandin	**Antifungal**	*Candida albicans*	0.015 μg/mL
Fagus sylvatica				*S. cerevisiae*	2.0 μg/ml
Tvipterigeum wilfordii	*Cryptosporiopsis quercina*	Cryptocandin	-do-	*Trichophyton rubrum* (ATCC 28188)	0.07 μg/mL
				T. mentagrophytes (ATCC 28185)	0.07 μg/mL
				Candida albicans (ATCC 90028)	0.035 μg/mL
				C. parapsilosis	2.5 μg/mL
				Histoplasma capsulatum	0.01 μg/mL
Selaginella pallescens	*Fusarium* sp.	CR377	-do-	*Candida albicans*	30 μg
Conocarpus erecta & *Forsteronia spicata*	*Cytospora* sp. & *Diaporthe* sp.	Cytosporones D	-do-	*C. albicans*	4 μg/mL
Gingo biloba	*Xylaria* sp.	7-amino-4-methylcoumarin	-do-	*Candida albicans*	15 μg/mL
				Penicillium expansum	40 μg/mL
				Aspergillus niger	25 μg/mL
Azadirachta indica	*Chloridum* sp.	Javanicin	-do-	*Candida albicans*	40 μg/mL
Artemisia mongolica	*Colletotrichum gloeosporioides*	Colletotric acid	**Antibacterial**	*Bacillus subtilis*	25 μg/mL
				Staphylococcus aureus	50 μg/mL
				Sarcina lutea	50 μg/mL
Tectona grandis	*Phomopsis* sp.	Phomoxanthones A & B	-do-	*Mycobacterium tuberculosis*	0.50 and 6.25 μg/mL
Conocarpus erecta &	*Cytospora* sp. &	Cytosporones D	-do-	*Staphylococcus aureus*	8.0 μg/mL
				Enterococcus faecalis	8.0 μg/mL
Crassocephalum crepidioides	*Geotrichum* sp.	7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one	-do-	*Mycobacterium tuberculosis*	25 μg/mL

(continued)

Table 26.1 (continued)

Host	Endophyte	Compound	Activity	Pathogen/cell line	MIC/IC_{50}
		7-but-15-enyl-6,8-dihydroxy-3(*R*)-pent-11- enylisochroman-1-one	-do-	-do-	50 μg/mL
Quercus variabilis	*Fusarium* sp. IFB-121	Cerebroside 1 & fusaruside 2	-do-	*Bacillus subtilis*	7.8 and 3.9 μg/mL
				Escherichia coli	3.9 and 3.9 μg/mL
				Pseudomonas fluorescens	7.8 and1.9 μg/mL
Sargassum sp.	ZZF36	Lasiodiplodin & de-*O*-methyllasiodiplodin	-do-	*Bacillus subtilis*(ATCC6633)	25 and 50 μg/mL
				Staphylococcus aureus (ATCC27154)	6.25 and 12.5 μg/mL
				Salmonella enteritidis (ATCC 13076)	12.5 μg/mL
Garcinia mangostana	*Botryosphaeria mamane*	Primin	-do-	*S. aureus* (ATCC 25923)	8 μg/mL
				S. aureus SK1	8 μg/mL
Garcinia dulcis	*Phomopsis* sp. PSU-D15	Phomoenamide	-do-	*Mycobacterium tuberculosis*	6.25 μg/mL
Azadirachta indica	*Chloridum* sp.	Javanicin	-do-	*Bacillus sp.*	40 μg/mL
				Escherichia coli	40 μg/mL
				Pseudomonas fluorescens	2 μg/mL
				Pseudomonas aeruginosa	2 μg/mL
Quercus sp.	*Cytonaema* sp.	Cytonic acids A & B	**Antiviral**	hCMV	43 and 11 μmol*
Unidentified tree	*Penicillium chrysogenum*	Xanthoviridicatins E & F	-do-	HIV-1	6 and 5 μM*
Buxus sempervirens	NRRL 15684	S 39163/F-I	-do-	Herpes simplex I	3 μg/mL*
Culophyllum sp.	*Pullularia* sp.	Pullularin A	-do-	-do-	3.3 μg/mL*
Musa acuminate	*Phomopsis* sp. BCC 9789	Oblongolide Z	-do-	-do-	14 μM*

Tectona grandis	*Phomopsis* sp.	Phomoxanthones A & B	**Antimalarial**	*Plasmodium falciparum*	0.11 and 0.33 μg/mL*
Crassocephalum crepidioides	*Geotrichum* sp.	7-butyl-6,8-dihydroxy-3(*R*)-pent-11-enylisochroman-1-one	-do-	-do-	4.7 μg/mL*
		7-butyl-6,8-dihydroxy-3(*R*)-pentylisochroman-1-one -do-	-do-		2.6 μg/mL*
Sandoricum koetjape	*Xylaria* sp	2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione	-do-	-do-	1.84 μM*
		Xylariaquinone A	-do-	-do-	6.68 μM*
Culophyllum sp.	*Pullularia* sp.	Pullularin A	-do-	-do-	3.6 μg/mL*
Vochysia guatemalensis	CR127A	Codinaeopsin	-do-	-do-	2.3 μg/mL*
Stemona sp.	*Exserohilum rostratum*	Monocerin & 11-hydroxymonocerin	-do-	-do-	0.68 and 7.70 μM*
Licuala spinosa	BCC 21097	Eremophilanolides 2 & 3	-do-	-do-	8.1 and 13.0 μM*
Taxus brevifolia	*Taxomyces andreanae*	Taxol	**Anticancer**		
Terminalia arjuna	*Pestalotiopsis terminaliae*	Taxol	-do-	BT220, H116, Int407 HL251 and HLK210	0.005–5 μM*
Aegle marmelos	*Bartalinia robillardoides*	Taxol			
Nothapodytes foetida	*Neurospora* sp & *Entrophospora infrequens*	Camptothecin	-do-		
Camptotheca acuminate	*Fusarium solani*	Camptothecin 9-methoxycamptothecin 10-hydroxycamptothecin	-do-		
Catharanthus roseus	*Fusarium oxysporum*	Vincristine	-do-		

(continued)

Table 26.1 (continued)

Host	Endophyte	Compound	Activity	Pathogen/cell line	MIC/IC_{50}
Mediterranean green alga	*Emericella nidulans* var. *acristata*	Emindole DA cell lines	-do-	36 human tumor	5.5 μg/mL*
Aegiceras corniculatum	*Penicillium* sp.	Leptosphaerone C	-do-	A-549	1.45 μM*
				P388	1.38 μM#
		Penicillenone	-do-	P388	1.38 μM*
Polygonum senegalense	*Alternaria* sp.	Alternariolone	-do-	L5178Y	1.7 μg/mL*
		Alternariol 5-*O*-methyl ether	-do-	-do-	7.8 μg/mL*
		Alternariol 5-*O*-sulfate	-do-	-do-	4.5 μg/mL*
		Altenusin	-do-	-do-	6.8 μg/mL*
Tectona grandis	*Phomopsis* sp.	Phomoxanthones A & B	-do-	KB, BC-1, Vero	0.99, 0.51, 1.4 μg/mL*
					4.1, 0.70, 1.8 μg/mL*
Xylocarpus granatum	XG8D (basidiomycetes)	Merulin A & C	-do-	BT474, SW620	4.98 and 4.84 μg/mL*
					1.57 and 4.11 μg/mL*
Dicerandra frutescens	*Phomopsis longicolla*	Dicerandrols A	-do-	A549, HCT-116	7.0 μg/mL*
		Dicerandrols B	-do-	-do-	1.8 μg/mL*
		Dicerandrols C	-do-	-do-	1.8, 7.0 μg/mL*
Musa acuminate	*Phomopsis* sp.	Oblongolide Z	-do-	KB, BC,	37.0, 26.0 μM*
				NCI-H187	32.0 μM#
		Oblongolide Y	-do-	BC	48 μM*
Roystonea regia	*Pestalotiopsis photiniae*	Photinides A-F	-do-	MDA-MB-2311	10 μg/mL*
Terminalia morobensis	*Pestalotiopsis microspora*	Pestacin & isopestacin	**Antioxidant**	hydroxyl free radicals	1.7 and 0.22 mM

Trachelospermum jasminoides	*Cephalosporium* sp.	Graphislactone A	-do-	DPPH	2.9 μg/mL*
Polysiphonia urceolata	*Chaetomium globosum*	Chaetopyranin	-do-	DPPH	35 μg/mL*
		Isotetrahydroauroglaucin	-do-	DPPH	26 μg/mL*
Unidentified tree	*Pseudomassaria* sp.	L-783,281	**Antidiabetic**	–	
Tripterygium wilfordii	*Pestalotiopsis leucothes*	BS, GS, YS	**Immunomodulator**		
Tripterygium wilfordii	*Fusarium subglutinans*	Subglutinol A & B	**Immunosuppresive**		0.1 μM*
Pteromischum sp.	*Colletotrichum dematium*	Collutellin A	-do-		167.3 ± 0.38 nM
Endophytic Fungi and Plant Protection					
Triptergyium wilfordii	*Cryptosporiopsis cf. quercina*	Cryptocin	**Antifungal**	*Pythium ultimum*	0.78 μg/mL
			-do-	*Phytophthora cinnamoni*	0.78 μg/mL
			-do-	*Phytophthora citrophthora*	1.56 μg/mL
			-do-	*Sclerotinia sclerotiorum*	0.78 μg/mL
			-do-	*Pyricularia oryzae*	0.39 μg/mL
			-do-	*Pyricularia oryzae*	0.39 μg/mL
			-do-	*Rhizoctonia solani*	6.25 μg/mL
			-do-	*Geotrichum candidum*	1.56 μg/mL
			-do-	*Fusarium oxysporum*	1.56 μg/mL
Artemisia mongolica	*Colletotrichum gloeosporioides*	Colletotric acid	-do-	*Helminthosporium sativum*	50.0 μg/mL
Rain forest trees	*Pestalotiopsis* spp.	Ambuic acid	-do-	*Phythium ultimum*	7.5 μg/mL
Fragraea bodenii	*Pestalotiopsis jester*	Jesterone	-do-	*Pythium ultimum*	25.0 μg/mL
			-do-	*Aphanomyces* sp.	6.5 μg/mL

(continued)

Table 26.1 (continued)

Host	Endophyte	Compound	Activity	Pathogen/cell line	MIC/IC_{50}
			-do-	*Phytophthora citrophthora*	25.0 μg/mL
			-do-	*Phytophthora cinnamomi*	6.5 μg/mL
			-do-	*Rhizoctonia solani*	25.0 μg/mL
			-do-	*Pyricularia oryzae*	25.0 μg/mL
Terminalia morobensis	*Pestalotiopsis microspora*	Pestacin	-do-	*Pythium ultimum*	10.0 μg/mL
		Isopestacin	-do-	*Pythium ultimum*	40 μg/mL
Xylopia aromatic	*Periconia atropurpurea*	2,4-dihydroxy-6-[(10E,30E)-penta-10, 30-dienyl]-Benzaldehyde	-do-	*C. sphaerospermum*	1.0 μg/mL
			-do-	*C. cladosporioides*	25.0 μg/mL
Azadirachta indica	*Nigrospora* sp. YB-141	Solanapyrones N	-do-	*Botrytis cinerea*	31.25 μg/mL
		nigrosporalactone	-do-	*Botrytis cinerea*	31.25 μg/mL
Azadirachta indica	*Chloridum* sp.	Javanicin	-do-	Cercospora arachidicola	5.0 μg/mL
			-do-	*Fusarium oxysporum*	20.0 μg/mL
			-do-	*Rhizoctonia solani*	10.0 μg/mL
			-do-	*Verticillium dahlae*	10.0 μg/mL
Bontia daphnoides	*Nodulisporium* sp.	Nodulisporic acid A	**Insecticidal**	*Aedes aegypti* larvae	0.5 ppm
			-do-	*Lucilia seracata*	0.3 ppm
Betula pendula	*Phomopsis phaseoli*	3-Hydroxypropionic	**Nematicidal**	*Meloidogyne incognita*	12.5–15.0 μg/mL

* represent IC50 values

and agricultural product development. Thus, there is an urgent need to facilitate the identity of appropriate natural products and the subsequent development of drugs based on them. Such needs add further credence to the importance of the need to preserve natural habitats, and in so doing, preserve plant and endophyte biodiversity. A concerted effort involving multiple organizations needs to be spearheaded in order to begin the daunting task of collection and cataloguing of endophytic microorganisms throughout the world. A better understanding of the biosynthetic pathways involved in the production of bioactive endophytic compounds by chemical and biochemical means is essential. Recent progress in the field of molecular biology of secondary metabolites has given us better insights about how the genes encoding for these bioactive compounds are organized. Investigation of these microorganisms and their relationship to the host requires improved quantitative analysis.

Interestingly, 02 biofuels of fungal endophytes origin have been discussed which certainly provide an alternative to gasoline in future and an integrated approach is required to explore this source of biofuel. Role of fungal endophytes especially against abiotic stresses has also been discussed and this may be used as tool in plant growth promotion using the endophytic microbes, biofabrication of nanoparticles (NPs) of noble metals is also one of the challenging and promising area under niche of nanotechnology as it has lower environmental impact than other techniques available. Several fungal endophytes have been identified and used as potential microbes for biosynthesis (Green Synthesis) of various metals nanoparticles with different properties. Therefore, we may conclude that this group of microbes, in addition to other usages, may also play a crucial role in this cutting edge technology.

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Chapter 27
Fungi: A Potential Source of Anti-inflammatory Compounds

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Abstract Inflammation is a complex protective process that can become dysregulated and can lead to a large number of diseases, the most common being rheumatoid arthritis, inflammatory bowel diseases, psoriasis and multiple sclerosis. Inflammation also plays a key role in other complex diseases such as alzeihmers, cardiovascular disease and cancer. A range of therapies exists for the treatment of inflammation-driven diseases such as steroids, non-steroidal anti-inflammatory drugs and anti-histamines. Despite some notable success, there is still major unmet medical need in the treatment of inflammatory diseases. Current therapeutic approaches for the treatment of inflammatory diseases are centered on cyclooxygenases (both COX-1 and 2) proinflammatory enzymes but present available drugs of this category are associated with undesirable gastrointestinal and cardiovascular side effects. Recent advances in drug research are focusing on bio-molecules such as proinflammatory cytokines, components of signal transduction and matrix degrading enzymes which are playing major roles in resolving inflammatory responses, might be new targets for treatment of chronic inflammatory diseases. In the present review several metabolites obtained from fungi with potential as anti-inflammatory agents are listed. The targets covered in this chapter are inhibitor of iNOS, NF-κB, AP-1, JAK, STAT, cytokines, cyclooxygenase (COX-1 and COX-2), 3α-HSD, XO and PLA2. The mode of action of some of the metabolites and a short description of some leads discovered is reviewed.

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27.1 Introduction

Inflammation, as we know it results from a traumatic injury even a disease process. But it is a complex protective process that can become dysregulated and can lead to a large number of diseases. The earliest therapeutics like the salicylates still continue to play a major role in the pathway leading to inflammation. The discovery of aspirin-like drugs, non-steroidal anti-inflammatory drugs (NSAIDs), all act by inhibiting the enzyme involved in the generation of prostaglandin from arachidonic acid (cyclooxygenase) provided an unifying explanation of their therapeutic actions and firmly established certain prostaglandins as important mediators of inflammatory diseases. Here, it should be noted that arachidonic acid leads not only to COX products, but also to leukotrienes via the 5-lipoxygenase pathway (Chen et al. 1994; Funk 2001). In this context NSAIDs such as diclofenac and indomethacin, have emerged as the most commonly used anti-inflammatory agents, including the therapy of rheumatoid arthritis. Many of these drugs target COX-1 rather than COX-2. But due to the side effect including gastrointestinal ulceration and bleeding, renal damage and platelet dysfunction associated only with the inhibition of COX-1 (Langenbach et al. 1995; Morham et al. 1995; Dinchuk et al. 1995; Vane et al. 1998), the selective inhibition of enzyme subtype COX-2 has become an important goal. Further works have reported that NSAIDs inhibit 3α- hydroxy steroidehydrogenase (3α-HSD) (Pawlowski et al. 1991; Penning et al. 1985) and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced edema (Yasukawa et al. 1992; Yasukawa et al. 1990). More recently kinases are believed to play a significant role in the expression and activation of inflammatory mediators (Adcock et al. 2006).

A range of therapies exists for the treatment of inflammation-driven diseases. The current mainstays of anti-inflammatory therapies such as steroids, NSAIDs and anti-histamines are largely based on inhibiting the synthesis or action of inflammatory mediators. The newer biopharmaceuticals (for example, tumour necrosis factor-α-neutralizing therapies, anti-IgE and anti-CD20 antibodies) continue to this line of therapeutic intervention (Fleischmann and Yocum 2004; Holgate and Polosa 2006), although other biological agents have been designed to target the recruitment or activation of inflammatory cells that drive the host's response to injury, such as antibodies to integrin-α4β7 and CTLA-4-Ig, which disrupt T-cell activation. However, neither the established therapies nor the newer biopharmaceutical approaches are without their shortcomings (Kremer et al. 2003; Edwards et al. 2004; Sandborn et al. 2005). For example, steroids can cause osteoporosis and impair wound healing, while the ulcerogenic effects of traditional NSAIDs, which are cyclooxygenase (COX) inhibitors, and the increased risk of coronary thrombosis

and stroke associated with the use of selective COX-2 inhibitors are well documented (Wang et al. 2005). Moreover, use of biopharmaceuticals such as the tumour necrosis factor-α (TNF-α) and integrin-α4β7-neutralizing therapies have also led to several complications (Kremer et al. 2003; Edwards et al. 2004; Sandborn et al. 2005; Strand et al. 2007). Given the limitations of existing small molecule and biopharmaceuticals, there remains a clear need for identification and validation of new anti-inflammatory drug targets (O'Neill 2006). This was the subject of a focus topic organized for the Life Sciences 2007 meeting in Glasgow (July 2007) (Ward 2008).

In this review several metabolites obtained from fungi, with potential as anti-inflammatory agents are listed in Table 27.1. The mode of action of some of the metabolites is indicated followed by a short description of leads discovered.

27.2 Fungal Metabolites Interfering with Inducible Nitric Oxide Synthase (iNOS)/Nitric Oxide (NO) Function

27.2.1 Role of iNOS in Inflammation

NO is synthesised in mammalian systems *via* a five electron oxidation of the terminal guanidino nitrogen of the amino acid, L-arginine, by a family of enzymes known collectively as NO synthases. With regard to regulation, the NOS enzymes fall into two distinct categories: constitutively-expressed isoforms (endothelial constitutive NOS [ecNOS] and neuronal NOS [nNOS]) which are regulated primarily by Ca^{2+} and calmodulin and a cytokine (or endotoxin)-inducible isoform (iNOS) which is regulated primarily at the level of *de novo* protein synthesis.

iNOS inhibitors have been characterized in view of their potential as therapeutic agents in acute and chronic inflammatory conditions. Two observations have been reported that may help to explain the probable mechanisms of action of NO in inflammatory conditions. The first observation is that NO stimulates COX activity resulting in the exaggerated production of pro-inflammatory prostaglandins (Salvemini et al. 1993). The second is that NO can react with O^{2-} to form the cytotoxic radical peroxynitrite (Beckman et al. 1990). The induction of human iNOS expression also depends on activation of the signal transducer and activator of transcription 1 (STAT1) pathway and could therefore be an appropriate target for the development of inhibitors of iNOS.

27.2.2 Role of NO in Inflammation

NO is recognized as a mediator and regulator of inflammatory responses. NO reacts with soluble guanylate cyclase to form cyclic guanosine monophosphate (cGMP), which mediates many of the effects of NO. NO can also interact with molecular oxygen and superoxide anion to produce reactive nitrogen species that can modify

Table 27.1 Anti-inflammatory compounds isolated from fungi

Sr. No.	Name of Fungi	Name of compound	Mode of action	References
1	*Hypoxylon fuscum*	Daldinin C **(1)**	Inhibits NO production in RAW 264.7 cells	Hashimoto et al. (1994), Quang et al. (2004a, 2006a)
2	*H. fuscum*	Daldinin E **(2)**, F **(3)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2004a, 2006a)
3	*Hypoxylon multiforme*	Multiformin D **(4)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2005d, 2006a)
4	*Creospharia sassafras*	Sassafrins A **(5)**, B **(6)**, C **(7)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2005a, 2006a)
5	*Hypoxylon rubiginosum*	Entonaemin A **(8)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2004b, 2006a)
6	*Hypoxylon rubiginosum*	Rubiginosin A **(9)**, B **(10)**, C **(11)**,	Inhibits NO production in RAW 264.7 cells	Quang et al. (2004b, 2006a)
7	*Hypoxylon cohaerens*	Cohaerin A **(12)**, B **(13)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2005b, 2006a, b)
8	*Hypoxylon rutilum*	Rutilin A **(14)**, B**(15)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2005c, 2006a)
9	*Daldinia childiae*	Daldinal A**(16)**, B**(17)**, C**(18)**	Inhibits NO production in RAW 264.7 cells	Hashimoto and Asakawa (1998), Quang et al. (2006b)
10	*Albatrellus dispansus*	Grifolic acid **(19)**, Grifolic acid methyl ester **(20)**, Grifolin**(21)**, Neogrifolin **(22)**	Inhibits NO production in RAW 264.7 cells	Hashimoto et al. (2005), Quang et al. (2006c)
11	*Albatrellus caeruleoporus*	Grifolinones A **(23)**, B **(24)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2006c)
12	*Hypoxylon fragiforme*	Mitorubrinic acid **(25)**, Mitorubrinol **(26)**	Inhibits NO production in RAW 264.7 cells	Steglich et al. (1974), Quang et al. (2006e)
13	*Tyromyces fissilis*	Tyromycic acid **(27)**, Tyromycic acid B **(28)**, C **(29)**, E **(30)**, F **(31)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2003a, 2004c, 2006b)

14	*Annulohypoxylon cohaerens*	Cohaerins C–F **(32–35)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2006d)
15	*Thelephora aurantiotincta*	Thelephantin A–C **(36–38)** and Thelephorin A **(39)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2003b, 2006b)
16	*Paxillus cirtisii*	Curtisian H **(40)**, J **(41)**, P **(42)**, Q **(43)**	Inhibits NO production in RAW 264.7 cells	Quang et al. (2003b, c, d, 2006b)
17	*Acremonium* sp.	Ascofuranone **(44)**, Ascochlorin **(45)**, Ilicicolin F, LL-Z 1272 ε **(46)**, Ilicicolin C **(47)**	Inhibits NO production in RAW 264.7 cells	Zhang et al. (2009)
18	*Antrodia cinnamomea* (BCRC 36799)	Antrocinnamomins A **(48)**, 3-isobutyl-4-[4-(3-methyl-2-butenyloxy)phenyl]-1 H-pyrrole-2,5-dione **(49)**	Inhibits NO production in RAW 264.7 cells	Wu et al. (2008)
19	*Penicillium* sp.	Sporogen **(50)**, S14-95 **(51)**, *S*-Curvularin **(52)**	Inhibitors of iNOS-dependent NO production, acting on the Janus tyrosine kinase-STAT pathway.	Yao et al. (2003)
20	Deuteromycete strain 45–93	Cycloepoxydon **(53)**, 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene **(54)** and 1-hydroxymethyl-3-pent-1, 3-dienylbenzene **(55)**	Inhibitors of eukaryotic signal transduction, Inhibitors of NF-κB and AP-1 mediated signal transduction pathways in COS-7 cells using SEAP as a reporter gene	Gehrt et al. (1998)
21	Ascomycete strain A23-98	2-(1-chloropropenyl)-4,5-dihydroxycyclopent-2-enone (CPDHC) **(56)**,4,5-dihydroxy-2-propenylcyclopent-2-enone (DHPC) **(57)**, 5-hydroxy-2, 3-dimethylcyclopent-2-enone (HDC) **(58)** and 4-methyl-5-methylenecyclopent-3-en-1,2-diol (MMCD) **(59)**	Inhibits the IL-6-induced SEAP expression	Weidler et al. (2000a, 2001)
22	*Trichoderma harzianum*	Gliovirin **(60)**	Inhibition of inducible TNF-α expression	Rether et al. (2007)

(continued)

Table 27.1 (continued)

Sr. No.	Name of Fungi	Name of compound	Mode of action	References
23	*Pestalotiopsis jesteri*	Derivative of Jesterone **(61)**	Inhibitor of TNF-α induced activation of NF-κB DNA binding activity	Liang et al. (2003)
24	Ascomycete strain A111-95	Galiellalactone **(62)**	Inhibits the IL-6-induced SEAP expression	Weidler et al. (2000b)
25	*Trichosporiella* sp. 20-95	Trichodion **(63)**	Inhibitor of inflammatory signal transduction pathways	Erkel (2000)
26	*Lentinus crinitus*	Panepoxydone **(64)**	Inhibitor of NF-κB activation	Erkel et al. (1996, 2007)
27	*Irpex* sp.	14,15-irpexanoxide **(65)**, 14,15-dihydroxyirpexan **(66)**, 14-acetoxy-15-hydroxyirpexan **(67)**, 14-acetoxy-22,23-dihydro-15,23-dihydroxyirpexan **(68)**	Inhibitor of expression of AP-1 and NF-κB driven SEAP reporter gene	Silberborth et al. (2000)
28	*Phomopsis* sp.	Phomol **(69)**	Inhibitor of edema in the mouse ear assay	Weber et al. (2004)
29	*Gliocladium fimbriatum, Trichoderma virens, Penicillium* sp., *Aspergillus fumigatus, Pseudallescheria* sp., *Candida albicans*	Gliotoxin (**70**)	NF-κB inhibitor	Johnson et al. (1943), Anitha and Murugesan (2005), Brian (1946), Kerzaon et al. (2008), Shah and Larsen (1991), Li et al. (2006), Eichner et al. (1986), Waring et al. (1988), Sutton et al. (1994), Pahl et al. (1996), Ward et al. (1999), Fitzpatrick et al. (2000), Coward et al. (2004), Pahl et al. (1996), Kroll et al. (1999), Herfarth et al. (1998, 1999, 2000)

30	Lichen sp.	Usnic acid **(71)**	Inhibitor of TNF-α mRNA expression	Jin et al. (2008)
31	*Ganoderma lucidum, G. tsugae*	Tsugaric acid A **(72)**	Inhibitor of superoxide anion formation in fMLP/ CB-stimulated rat neutrophils.	Ko et al. (2008)
32	*Ganoderma lucidum, G. tsugae*	3-oxo-5α-lanosta-8,24-dien-21-oic acid **(73)**	Inhibitory effect on the release of β-glucuronidase from rat neutrophils. Inhibits NO production.	Ko et al. (2008)
33	*Aspergillus fumigatus*	Fumigaclavine C **(74)**	Inhibitor of several inflammatory cytokines	Zhao et al. (2004), Wu et al. (2005)
34	*Neocosmospora tenuicristata, Cylindrocarpon radicicola*, *Monocillium nordinii*, *Humicola fuscoatra, Chaetomium chiversii* and *Pochonia chlamydosporia* var.*chlamydosporia*	Radicicol **(75)**	Inhibitor of LPS-induced expression of iNOS by blocking the p38 kinase and NF-κB in macrophages	McCapra et al. (1964), Evans and White (1966), Ayer et al. (1980), Wicklow et al. (1998), Jeon et al. (2000), Sohn et al. (2007), Wang et al. (2008) and Shinonaga et al. (2009)
35	*Monascus* sp.	Citrinin **(76)**	Suppressed LPS/IFN-γ induced NO	Liu et al. (2010)
36	An unidentified endophytic fungus	Ergoflavin **(77)**	Inhibitor of human TNF-α and IL-6	Deshmukh et al. (2009)
37	*Penicillium rubrum*	Berkeleyamides A–D **(78–81)**	Inhibitor of Caspase-1 and MMP-3	Stierle et al. (2008)
38	*Antrodia camphorata*	Antrocamphin A (**82**), Antcin A (**83**) and Antcin B (**84**)	Inhibitor of fMLP-induced superoxide production	Chen et al. (2007)

(continued)

Table 27.1 (continued)

Sr. No.	Name of Fungi	Name of compound	Mode of action	References
39	*Penicillium wortmannii, P. funiculosum, Fusarium oxysporum, F. sambucinum, Myrothecium roridum*	Wortmannin (**85**)	Inhibitor of LPS-induced Akt phosphorylation, PI3K inhibitor	Brian et al. (1957), MacMillan et al. (1972), Haefliger and Hauser (1973), Abbas and Mirocha (1988), Petcher et al. (1972), Ezeamuzie et al. (2001), Schabbauer et al. (2004), Ward et al. (1995), Nakanishi et al. (1992)
40	*Penicillium decumben, Eupenicillium brefeldianum, Curvularia subulata*, *C. lunata*, *Nectria radicicola*, *Penicillium cyaneum*, *P. simplicissimum*, *P. camemberti, Phoma medicaginis* and *Alternaria carthami*	Brefeldin A (**86**)	Inhibitor of protein trafficking in the endomembrane system	Singleton et al. (1958), Harri et al. (1963), Abraham and Arfmann (1992), Nebenfuhr et al. (2002), Schuerwegh et al. (2001)
41	*Cladosporium* sp., *Trichoderma* sp., *Aspergillus wentii, A. terreus, Penicillium islandicum, Thielavia subthermophila*	Emodin (**87**)	Inhibitor of pro-inflammatory cytokines (IL-1β, IL-6), chemokines (IL-8, CCL2), NF-κB activation and IκB-α degradation in HUVECs	Anthony et al. (2009), Hasan (1998), Schimmel et al. (1998), Kawai et al. (1984), Kusari et al. (2009), Meng et al. (2010), Chiu et al. (2010), Kitano et al. (2007)

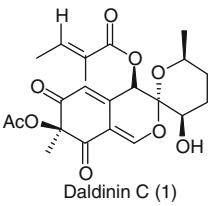
42	*Inonotus* sp.	Inonotic acid methyl ester (**88**), Inotilone (**89**), *Iso*-hispidin (**90**), (*E*)-4-(3,4-dihydroxyphenyl) but-3-en-2-one (**91**), Hispidin (**92**)	Inhibitor of COX-1, COX-2, 3α-HSD and XO	Wangun et al. (2006)
43	*Aspergillus* sp. HKI 0472	Funalenine **(93)**	Inhibitor of COX-1, COX-2 and 3α-HSD	Wangun (2006)
44	*Pholiota squarrosa*	Squarrosidine **(94)**	Inhibitor of COX-1, COX-2, 3α-HSD and XO	Wangun (2006)
45	*Phellinus pini* DSM 5238	Pinillidine **(95)**, Hypholomine B **(96)**	Inhibitor of 3α-HSD and XO	Wangun and Hertweck (2007)
46	*Thielavia terricola* RF-143	Thielocins A1α **(97)**, A1β **(98)** A2α **(99)**, A2β **(100)**, A3 **(101)**, B1 **(102)**, B2 **(103)**, B3**(104)**	PLA2 inhibitors	Yoshida et al. (1991), Matsumoto et al. (1995)
47	*Penicillium chermesinum*	Plastatin **(105)**, Luteosporin **(106)**	PLA2 inhibitors	Singh et al. (1985)
48	*Circinotrichum falcatisporum* RF-641	Cinatrins A**(107)**, B**(108)**, C2**(109)** and C3**(110)**	PLA2 inhibitors	Itazaki et al. (1992), Tanaka et al. (1992)
49	*Penicillium* sp. BM-99	Ergophilones A **(111)**, B **(112)**	PLA2 inhibitors	Hyodo et al. (1995)
50	*Poria cocos*	Pachymic acid**(113)**, Dehydrotumulosic acid **(114)**	PLA2 inhibitors	Cuéllar et al. (1996), Giner et al. (2000)
51	*Aspergillus unguis*	Folipastatin A **(115)**	PLA2 inhibitors	Hamano et al. (1992)
52	*Lactarius hatsudake*	Ergosterol peroxide **(116)**, 5α,8 α-epid-ioxy-(24 S)-ergosta-6-en-3β-ol **(117)**	PLA2 inhibitors	Gao et al. (2007)
53	*Trichoderma koningii*	Koninginins E **(118)**, F **(119)**	PLA2 inhibitors	Souza et al. (2008)

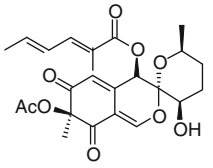
Number in the brackets corresponds to the structure of the compound

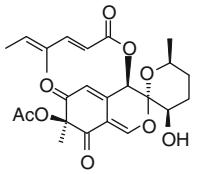
various cellular functions. These indirect effects of NO have a significant role in inflammation, where NO is produced in high amounts by iNOS susequently reactive oxygen species are synthesized by activated inflammatory cells. NO also has cytotoxic properties that can damage host tissues (Palmer et al. 1987).

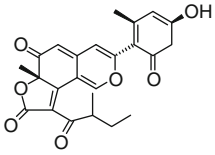
27.2.3 iNOS/NO Inhibitors

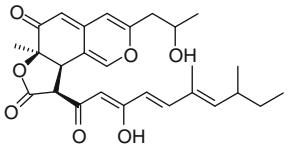
Quang et al. (2006a) tested 15 azaphilones that suppress NO production stimulated by Lipopolysaccharide (LPS) in RAW 264.7 cells. Daldinins C **(1),** E **(2)** and F **(3)** belonging to bicyclic spiro-azaphilones isolated from *Hypoxylon fuscum* (Hashimoto et al. 1994 and Quang et al. 2004a) weakly suppressed NO production with an IC_{50} of 55.66, 43.68 and 30.62 μM (Quang et al. 2006a). Multiformin D **(4)** isolated from *H. multiforme* (Quang et al. 2005d) and Sassafrins A–C **(5–7)** isolated from *Creospharia sassafras* (Quang et al. 2005a), azaphilones with a lactone ring in their molecule had an IC_{50} of 13.81 μM for Multiformin D and 14.68, 15.66, 10.02 μM for Sassafrins A, B and C respectively (Quang et al. 2006a). Entonaemin A **(8)**, Rubiginosin A **(9)** and B **(10)** were isolated from *Hypoxylon rubiginosin* (Quang et al. 2004b), azaphilones with an orsellinic acid moiety attached to the bicyclic azaphilone backbone through an ester linkage, showed an IC_{50} of 14.24 μM for Entonaemin A and 2.56 and 15.66 μM for Rubiginosin A and B respectively (Quang et al. 2006a). Rubiginosin C **(11)** isolated from *H. rubiginosin* (Quang et al. 2004b) and Cohaerin A **(12)** and B **(13)** isolated from *H. cohaerens* (Quang et al. 2005b), belonging to azaphilones with a fatty acid side chain linked with bicyclic azaphilone by an ester bond weakly suppresses NO production with an IC_{50} of 39.16, 50.76 and 54.55 μM respectively (Quang et al. 2006a). The potent inhibitors of NO production were dimeric azaphilones namely Rutilins A **(14)** and B **(15)** isolated from *H. rutilin* with IC_{50} values of 1.76 and 1.80 μM (Quang et al. 2005c, 2006a).

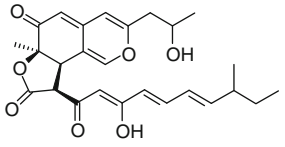

Daldinin C (1)

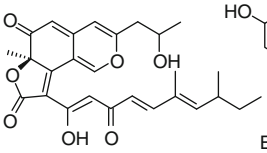

Daldinin E (2)

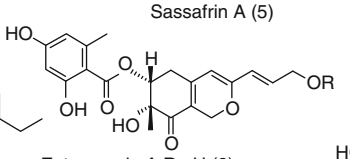

Daldinin F (3)

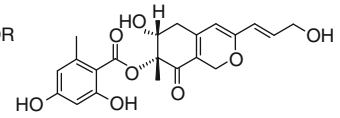

Multiformin D (4)


Sassafrin A (5)


Sassafrin B (6)


Sassafrin C (7)


Entonaemin A R= H (8)
Rubiginosin A R = Ac (9)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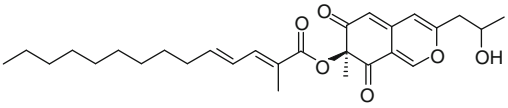
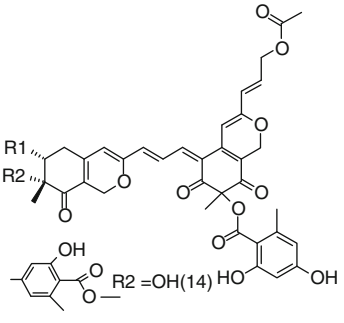
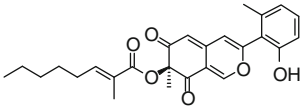
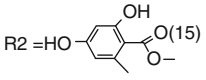

Rubinosin C (11)

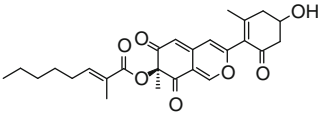


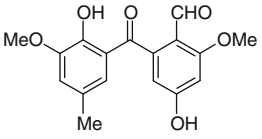

Cohaerin A (12)

Rutilin A R1 =HO

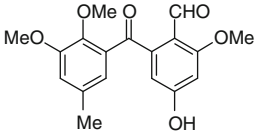
Rutilin B R1 =OH



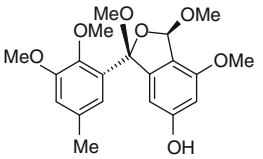

Cohaerin B (13)



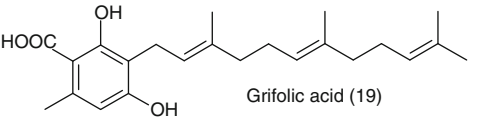
Daldinal A (16)



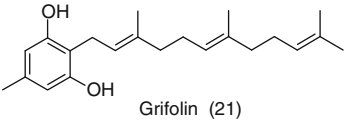
Daldinal B (17)



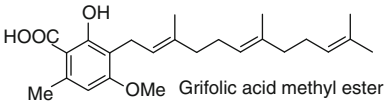
Daldinal C (18)



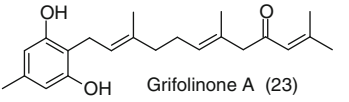
Grifolic acid (19)



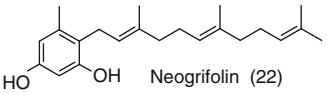
Grifolin (21)



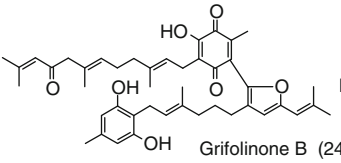
Grifolic acid methyl ester (20)



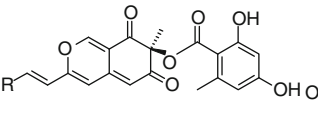
Grifolinone A (23)



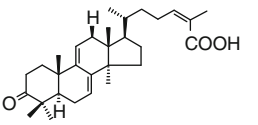
Neogrifolin (22)



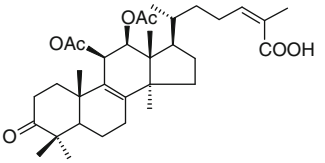
Grifolinone B (24)



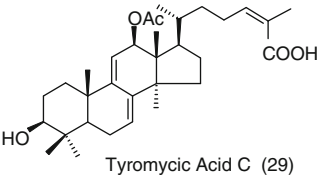
Mitorubrinol R = CH_2OH (26)
Mitorubrinic Acid R = COOH (25)



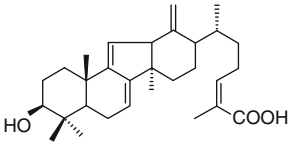
Tyromycic Acid (27)



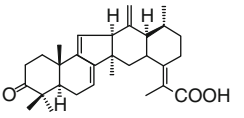
Tyromycic Acid B (28)



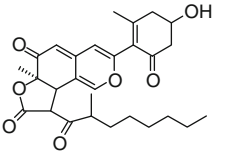
Tyromycic Acid C (29)



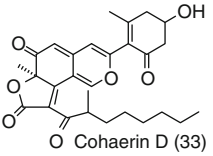
Tyromycic Acid E (30)



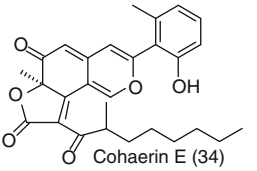
Tyromycic Acid F (31)



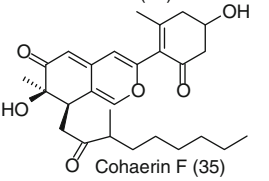
Cohaerin C (32)



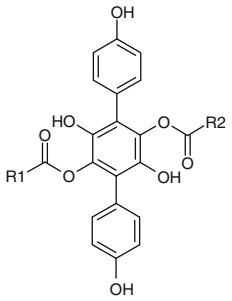
Cohaerin D (33)

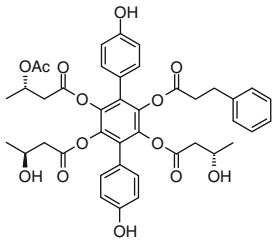


Cohaerin E (34)



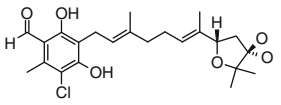
Cohaerin F (35)



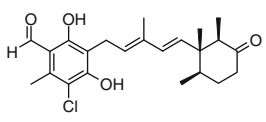


Curtisian H (40)

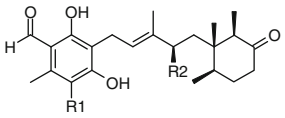
Thelephantin A R1= CH_3-CH_2-CH_2- R2 = p-HO-C_6H_4- (36)
Thelephantin B R1= CH_3-CH_2-CH_2-CH_2-CH_2 R_2 = p-HO-C_6H_4- (37)
Thelephantin C R1= CH_3 -CH(CH_3)-CH(CH_3)-CH_2- R2 = p-HO-C_6H_4- (38)
Thelephorin A R1= C_6H_5-CH_2- R2 = p-HO-C_6H_4- (39)
Curtisian J R1= CH_3-CH(OAc)-CH_2- R2= C_6H_5-CH_2-CH_2- (41)
Curtisian P R1= CH_3- R_2= C_6H_5-CH_2-CH_2- (42)
Curtisian Q R1= C_6H_5- R_2= C_6H_5-CH_2-CH_2- (43)



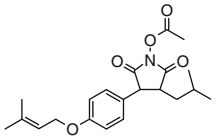
Ascofuranone (44)



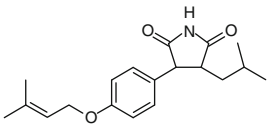
Ascochlorin (45)



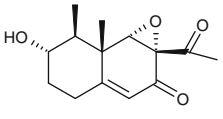
Ilicicolin F, LL-Z 1272 ε R1=H, R2=H (46)
Ilicicolin C R1=Cl, R2=H (47)



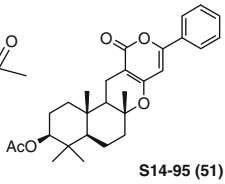
Antrocinnamomin A (48)



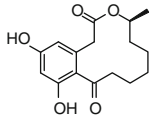
3-isobutyl-4-[4-(3-methyl-2-butenyloxy) phenyl]-1H-pyrrole-2,5-dione (49)



Sporogen (50)



S14-95 (51)



S-Curvularin (52)

Benzophenone derivatives namely Daldinal A–C **(16–18)** isolated from *Daldinia childiae* (Hashimoto and Asakawa 1998) showed an IC_{50} values of 15.2, 4.6 and 6.4 μM respectively (Quang et al. 2006b). Grifolic acid **(19)**, Grifolic acid methyl ester **(20)**, Grifolin **(21)** and Neogrifolin **(22)** isolated from *Albatrellus dispansus* (Hashimoto et al. 2005; Quang et al. 2006b) showed IC_{50} values of 68.4, 37.4, 29.0 and 23.3 μM respectively. Two farnesyl phenols named Grifolinone A and B **(23–24)** were isolated from *Albatrellus caeruleoporus* and showed IC_{50} values of 23.4 and 22.9 μM respectively (Quang et al. 2006c).

Mitorubrinic acid **(25)** and Mitorubrinol **(26)**, the bicyclic azaphilones with aliphatic side chain and aromatic ring were isolated from *H. fragiforme* (Steglich et al. 1974; Quang et al. 2006e) and inhibited NO production with IC_{50} values of 43.7 and 45.0 μM respectively (Quang et al. 2006e).

Triterpenoids namely Tyromycic acid **(27)**, Tyromycic acid B **(28)**, C **(29)**, E **(30)** and F **(31)** isolated from *Tyromyces fissilis* (Quang et al. 2003a, 2004c), inhibited the NO production with an IC_{50} of 38.2, 38.2, 39.3, 49.3 and 39.0 μM respectively

(Quang et al. 2006b). Four azaphilones namely Cohaerins C–F **(32–35)** isolated from *Annulohypoxylon cohaerens* inhibited NO production with an IC_{50} of 30.2, 19.6, 26.1 and 41.2 μM respectively (Quang et al. 2006d). p-Terphenyls, namely Thelephantin A–C **(36–38)** and Thelephorin A **(39)** isolated from *Thelephora aurantiotincta* (Quang et al. 2003b) and Curtisian H **(40)**, J **(41)**, P **(42)** and Q **(43)** (Quang et al. 2003b, c, d, e) isolated from *Paxillus cirtisii* inhibited the NO production with an IC_{50} of 44.0, 31.5 and 29.6 μM for Thelephantin A–C; 57.9 μM for Thelephorin A and 46.2, 51.1, 27.8 and 31.1 μM for Curtisian H, J, P and Q respectively (Quang et al. 2006b). The anti-inflammatory activities of Ascofuranone **(44)**, Ascochlorin **(45)**, Ilicicolin F, LL-Z 1272 ε **(46)** and Ilicicolin C **(47)** isolated from a sponge-derived *Acremonium* sp., were evaluated by measuring their inhibitory effects on the production of pro-inflammatory mediators (NO, Interleukin-6 (IL-6) and TNF-α) in RAW 264.7 murine macrophage cells. Ascofuranone and Ascochlorin significantly inhibited the production of NO and TNF-α at the concentration of 100 μM, while Ilicicolin F, LL-Z 1272 ε and Ilicicolin C showed selective inhibition of NO production at the same concentration (Zhang et al. 2009).

Antrocinnamomin A **(48)** and 3-isobutyl-4-[4-(3-methyl-2-butenyloxy) phenyl]-1H-pyrrole-2,5-dione **(49)** were isolated from *Antrodia cinnamomea* (BCRC 36799). Both the compounds were evaluated for their anti-inflammatory activity, using the RAW 264.7 cells/LPS assay. Antrocinnamomin A showed strong inhibition of NO production of macrophages consistent with anti-inflammatory activity. The high cell viability (>90%) indicated that the inhibitory activity of LPS-induced nitrite production by Antrocinnamomins A did not result in cytotoxicity. Compound (**49**) also showed strong inhibition of NO production of macrophages but the low cell viability (69.1%), suggesting the possibility of cytotoxicity (Wu et al. 2008).

Sporogen **(50)**, S14-95 **(51)** and *S*-Curvularin **(52)** isolated from different *Penicillium* species inhibited cytokine induced activity of the human iNOS promoter [a 16-kilobase (kb) fragment in stably transfected A549/8-pNOS2(16)Luc cells], cytokine-induced iNOS mRNA expression and cytokine induced NO production in a concentration-dependent manner. They also inhibited cytokine-induced activation of STAT1α in A549/8 cells. Sporogen, S14-95 and *S*-Curvularin represent new transcription based inhibitors of iNOS-dependent NO production, acting on the Janus tyrosine kinase-signal transducer and activator of transcription (JAK-STAT) pathway (Yao et al. 2003).

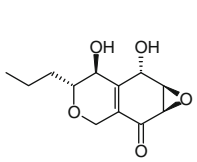
27.3 Fungal Metabolites Interfering with Major Transcriptional Factors

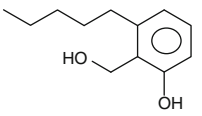
27.3.1 Roles of NF-κB, AP-1, JAK and STAT in Inflammation

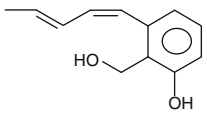
Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a transcription factor that is a major regulator of immune responses stimulated by pro-inflammatory agents such as tumor necrosis factor, viruses, interleukin-1 (IL-1) and

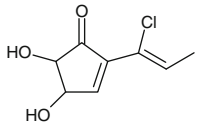
bacteria. NF-κB normally resides in the cytoplasm bound by an inhibitory protein known as IκB. Phosphorylation of IκB by IκB kinase-β (IKK-β) releases NF-κB, which then moves into the nucleus where it acts in the induction of numerous regulatory genes of the immune system. The products of these genes are pro-inflammatory factors (Hayden et al. 2006; Li and Verma 2002).

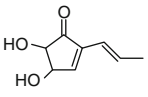
The Activating Protein-1 (AP-1) transcription factor, AP-1 elements and AP-1 activation are associated with the transcription of a variety of Th2 cytokines, as well as other inflammatory mediators. In mammals, the most abundant AP-1 proteins are the Jun (c-Jun, JunB and JunD) and Fos (c-Fos, FosB, Fra1 and Fra2) family members, which regulate gene expression at multiple levels (Zenz and Wagner 2006).

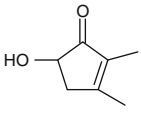

Cycloepoxydon (53)

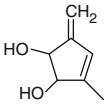

1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene (54)

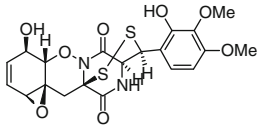

1-hydroxy-3-pent-1,3 dienylbenzene (55)

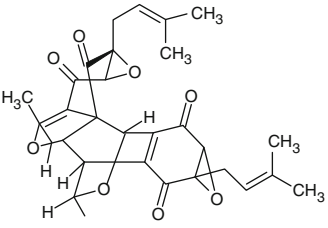

CPDHC (56)

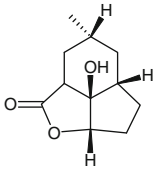

DHPC (57)

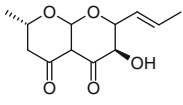

HDC (58)


MMCD (59)


Gliovirin (60)


Jesterone Dimer (61)


Galiellalactone (62)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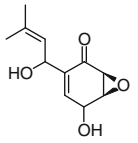
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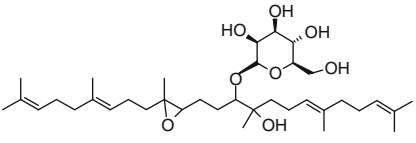
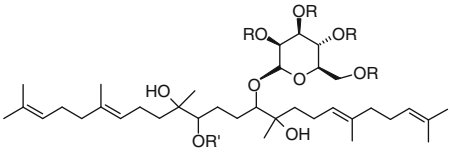
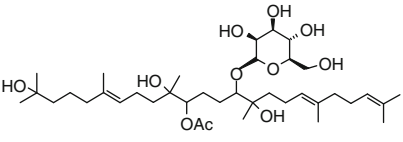
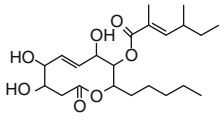
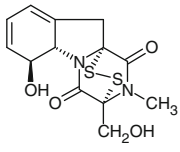

14,15-irpexanoxide (65)


14,15-dihydroxyirpexan R =R'= H (66)
14-acetoxy-15-dihydroxyirpexan R =H, R'=Ac (67)


14-acetoxy-22,23-dihydro-15,23-dihydroxyirpexan (68)


Phomol (69)


Gliotoxin (70)

Cytokines are important mediators of cardiac disease. Accumulating evidence indicates that members of the IL-6 family of cytokines promote cardiac hypertrophy through the activation of the JAK-STAT pathway. Aberrant JAK-STAT signaling may promote progression from hypertrophy to heart failure (Terrell et al. 2006). The JAK-STAT signal transduction pathway is differentially regulated in inflammatory arthritis, with changes documented in STAT1, STAT3, STAT4 and STAT6 expression. Modulation of STAT expression, either directly or through modulation of JAK or Suppressor of Cytokine Signaling (SOCS) function, represents an alternative therapeutic target to cytokine antagonists such as TNF-α and IL-1 (Finnegan et al. 2002).

27.3.2 NF-κB, AP-1, JAK and STAT Inhibitors

Cycloepoxydon **(53)**, 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene **(54)** and 1-hydroxymethyl-3-pent-1, 3-dienylbenzene **(55)** were isolated from the Deuteromycete strain 45–93. Cycloepoxydon inhibits the TPA-induced NF-κB and AP-1 mediated secreted alkaline phosphatase (SEAP) expression with an IC_{50} of 1–2 μg/ml and 3–5 μg/ml respectively whereas 1-hydroxy-2-hydroxymethyl-3-pent-1-enylbenzene inhibited it with an IC_{50} of 7 μg/ml and 5 μg/ml. Compound **(55)** showed only a weak inhibition of the AP-1 and had no influence on NF-κB dependent reporter gene expression. In COS-7 and HeLa S3 cells electrophoretic mobility shift assays showed that cycloepoxydon strongly reduced the TPA and TNF-α mediated binding of NF-κB to a high affinity consensus sequence which was due to the inhibition of phosphorylation of the protein IκB (Gehrt et al. 1998).

Four novel cyclopentenones, 2-(1-chloropropenyl)-4,5-dihydroxycyclopent-2-enone (CPDHC) **(56)**, 4, 5-dihydroxy-2-propenylcyclopent-2-enone (DHPC) **(57)**, 5-hydroxy-2, 3-dimethylcyclopent-2-enone (HDC) **(58)** and 4-methyl-5-methylenecyclopent-3-en-1,2-diol (MMCD) **(59)** were isolated from the ascomycete strain A23-98. Compound **(56)** inhibited the IL-6-induced SEAP expression with IC_{50} values of 4.0–5.3 μM. The compounds **(57)**, **(58)** and **(59)** which are structurally closely related to compound **(56)** showed no inhibitory effects on the IL-6-induced SEAP expression in HepG2 cells. Studies on the mode of action revealed that compound **(56)** affects the IL-6-dependent pathway by inhibiting the tyrosine phosphorylation of the STAT3 and STAT1 as well as the serine phosphorylation of the STAT3 transcription factor. In addition, compound **(56)** and **(57)** inhibited the AP-1 and NF-κB mediated SEAP expression in transiently transfected HeLa S3 cells with IC_{50} values of 10–15 μM and 50–100 μM respectively. Compound **(56)** inhibited the NF-κB pathway by preventing the phosphorylation and proteolytic degradation of IκB-α protein (Weidler et al. 2000a, 2001).

Gliovirin **(60)** isolated from *Trichoderma harzianum* inhibited inducible TNF-α promoter activity and synthesis in LPS/ Interferons (IFN)-γ-stimulated macrophages/ monocytes and Jurkat T-cells, co-stimulated with TPA, in a dose-dependent manner with IC_{50} values ranging from 0.21 to 2.1 μM. It suppresses TNF-α synthesis by inhibiting the activation of extracellular signal-regulated kinase (ERK), thereby

blocking the pathway leading to activation of the transcription factors AP-1 and NF-κB, the latter of which is involved in the inducible expression of many pro-inflammatory genes. It also significantly reduced TPA induced IL-2 mRNA levels and synthesis in Jurkat cells at low micromolar concentrations (Rether et al. 2007).

Jesterone was isolated from an endophytic fungus *Pestalotiopsis jesteri*, and kills tumor cells with an IC_{50} values ranging from approximately 100 to 500 μM in three different human tumor cell lines. However, a dimeric derivative of Jesterone **(61)** has approximately 10–100 fold greater antitumor cell activity than Jesterone against the same tumor cell lines (Hu et al. 2001). Jesterone dimer also blocked TNF-α induced activation of NF-κB DNA binding activity at a concentration of 20μM and in a dose-dependent manner, with half-maximal inhibition at approximately 2.5 μM and complete inhibition at approximately 5 μM. The activity of the dimer is may be due to the blocking of the induction of the NF-κB pathway by inhibiting the inhibitor of κB kinase (IKK) (Liang et al. 2003).

Galiellalactone **(62)** was isolated from the ascomycete strain A111-95 and it inhibited the IL-6-induced SEAP expression with IC_{50} values of 250–500 nM by blocking the binding of the activated STAT 3 dimers to their DNA binding sites without inhibiting the tyrosine and serine phosphorylation of the STAT 3 transcription factor (Weidler et al. 2000b).

Trichodion **(63)** was isolated from *Trichosporiella* sp. 20–95 and inhibited the IFN-γ mediated expression of the reporter gene with IC_{50} values of 21–42 μM. The NF-κB and AP-1 mediated expression of the reporter gene were inhibited with IC_{50} values of 42–84 μM and 21 μM respectively. Western blotting with COX-2 and NOS II antibodies showed that the expression of both proinflammatory enzymes was almost completely inhibited at 21–42 μM in LPS/IFN-γ stimulated J774 mouse macrophages. Mode of action of the compound revealed that the inhibition of the NF-κB dependent pathway is due to the stabilization of the IκB protein and the inhibition of the Interferon-γ (IFN-γ) dependent signaling is caused by an inhibition of the phosphorylation of the STAT1α transcription factor (Erkel 2000; Erkel et al. 2000).

Panepoxydone **(64)** was isolated from *Lentinus crinitus* (Erkel et al. 1996). It strongly inhibited the expression of 33 NF-κB dependent pro-inflammatory genes such as the chemokines CCL3, CCL4, CCL8, CXCL8, CXCL10, CXCL20, the cytokines IL-1, IL-6, TNF-α, pro-inflammatory enzymes like COX-2, and components of the REL/NF-κB/IκB family at the concentrations of 12–24 μM without significant effects on the expression of house-keeping genes. It also strongly inhibited hTNF-α, IL-8 and NF-κB promoter activity in LPS/TPA stimulated MonoMac6 cells with IC_{50} values of 0.5–1 μg/ml by blocking the phosphorylation of IκB and subsequent binding of the activated NF-κB transcription factor to the DNA (Erkel et al. 2007).

14,15-irpexanoxide **(65)**, 14,15-dihydroxyirpexan **(66)**, 14-acetoxy-15-hydroxyirpexan **(67)** and 14-acetoxy-22,23-dihydro-15,23-dihydroxyirpexan **(68)** were isolated from *Irpex* species in the course of a screening for inhibitors of AP-1 and NF-κB mediated signal transduction pathways in COS-7 cells using SEAP as a reporter gene. The expression of an AP-1 and NF-κB driven SEAP reporter gene was inhibited in a dose dependent manner with compound **(67)** being the most potent compound with

IC_{50} of 6–7 μg/ml, followed by compound **(65)** with IC_{50} of 10–15 μg/ml, compound **(66)** with IC_{50} of 15–20 μg/ml and compound **(68)** with IC_{50} of 30–40 μg/ml (Silberborth et al. 2000).

Phomol **(69)**, a polyketide lactone was isolated from *Phomopsis* sp. an endophytic fungi from the medicinal plant *Erythrina crista-galli*. The anti-inflammatory activity of Phomol was tested in different reporter gene assays (TNF-α, STAT1/STAT2 and NF-κB) and in an ear edema model in mice. In the reporter gene assays Phomol exhibited no activity, whereas it showed interesting anti-inflammatory activity in the mouse ear assay with an edema inhibition of 53.20% (Weber et al. 2004).

Gliotoxin **(70)** isolated from *Gliocladium fimbriatum* (Johnson et al. 1943), *Trichoderma virens* (Anitha and Murugesan 2005), *Penicillium* sp (Brian 1946), *Aspergillus fumigatus* (Kerzaon et al. 2008), *Candida albicans* (Shah and Larsen 1991) and a marine-derived *Pseudallescheria* sp. (Li et al. 2006) exhibited potent immunosuppressive effects both *in vitro* and *in vivo* (Eichner et al. 1986; Waring et al. 1988; Sutton et al. 1994; Pahl et al. 1996; Ward et al. 1999). Gliotoxin dose dependently inhibited cytokine production and NF-κB in macrophage and colonic epithelial cell lines (Fitzpatrick et al. 2000). Gliotoxin (1 pg/mL) reduced the generation of Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF), TNF-α and IL-8 in parallel with inhibition of NF-κB (Coward et al. 2004). Gliotoxin potently and selectively inhibit the activation of the NF-κB in T and B cells and prevents the degradation of IκB-α, which is an endogenous inhibitor of NF-κB (Pahl et al. 1996), by acting as a specific inhibitor of the 20S proteasome. This proteasome is involved in allowing the nuclear translocation of NF-κB and the subsequent regulation of inflammatory gene production by this transcription factor (Kroll et al. 1999). Herfarth et al. (1998) reported that intraperitoneal treatment of mice with gliotoxin could effectively treat the acute phase of dextran sodium sulfate (DSS)-induced colitis in mice. Recently, these investigators also found that intraperitoneal treatment with gliotoxin down-regulated the enhanced colonic NF-κB activity associated with DSS administration to mice (Herfarth et al. 1999, 2000).

27.4 Fungal Metabolites Interfering with Cytokines

27.4.1 Roles of Cytokines in Inflammation

Cytokines are key modulators of inflammation. They participate in acute and chronic inflammation in a complex network of interactions. Several cytokines exhibit some redundancy in function and share overlapping properties as well as subunits of their cell surface receptors. Better understanding of the pathways regulated by cytokines will allow the identification and/or development of agents for improved modulation

of the inflammatory response for the treatment of autoimmune, infectious and neoplastic diseases (Feghali and Wright, 1997).

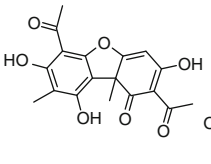
Several cytokines play key roles in mediating acute inflammatory reactions, namely IL-1, TNF-α, IL-6, IL-11, IL-8 and other chemokines, Granulocyte colony-stimulating factor (GCSF) and GM-CSF. Of these, IL-1 (α and β) and TNF are extremely potent inflammatory molecules: they are the primary cytokines that mediate acute inflammation, induced in animals by intradermal injection of bacterial lipopolysaccharide and two of the primary mediators of septic shock (Arai et al. 1991)

27.4.2 Interleukins, IFN and TNF-α Inhibitors

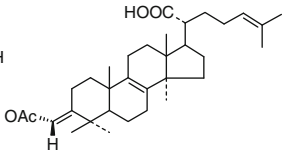
Usnic acid (UA) **(71)** is one of the most common and abundant lichen metabolites. UA decreased the TNF-α level in LPS-stimulated RAW264.7 macrophages in dose-dependent manner with an IC_{50} of 12.8 μM. RT-PCR analysis indicated that it inhibited TNF-α mRNA expression. Furthermore, it inhibited NO production in LPS-activated RAW264.7 macrophages with an IC_{50} of 4.7 μM. Western blot analysis showed that UA attenuated LPS-induced synthesis of iNOS protein and nuclear translocation of NF-κB p65 in the macrophages, in parallel. UA also inhibited LPS-mediated IκB-α degradation. Taken together, this suggests that UA has an anti-inflammatory effect by inhibiting TNF-α and iNOS expression, possibly through suppression of nuclear translocation of NF-κB- p65 and IκB-α degradation (Jin et al. 2008)

The anti-inflammatory properties of Tsugaric acid A **(72)**, 3-oxo-5-α-lanosta-8,24-dien-21-oic acid **(73)** from *Ganoderma lucidum* and *G. tsugae* were assessed *in vitro* by determining their inhibitory effects on the chemical mediators released from mast cells, neutrophils and macrophages. Compound **(73)** showed a significant inhibitory effect on the release of β-glucuronidase from rat neutrophils stimulated with formyl-Methionyl-Leucyl-Phenylalanine (fMLP)/cytochalasin B (CB) whereas compound **(72)** significantly inhibited superoxide anion formation in fMLP/CB-stimulated rat neutrophils. Compound **(73)** also exhibited a potent inhibitory effect on NO production in LPS/ IFN-γ-stimulated N9 microglial cells (Ko et al. 2008).

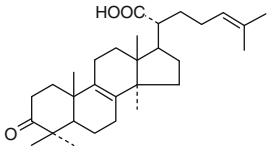
Fumigaclavine C **(74),** produced by *Aspergillus fumigatus* showed a marked inhibition on the expression of several inflammatory cytokines, including IL-1β, IL-2, IL-12α, IFN-γ, TNF-α as well as matrix metalloproteinase-9 (MMP-9) in sacral lymph node cells, colonic patch lymphocytes and colitis tissues from the TNBS (2, 4, 6-trinitrobenzene sulfonic acid) colitis mice both *in vivo* and *in vitro.* The compound also caused a dose-dependent reduction in IL-2 and IFN-γ from the lymphocytes at the protein level and MMP-9 activity suggesting that it may alleviate experimental colitis mainly via down-regulating the production of Th1 cytokines and the activity of matrix metalloproteinase. (Zhao et al. 2004; Wu et al. 2005).



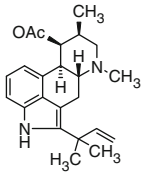
Usnic Acid (71)



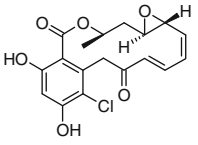
Tsugaric acid A (72)



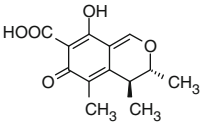
3-oxo-5-α-lanosta-8,24-dien-21-oic acid (73)



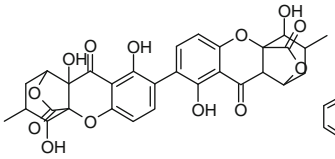
Fumigaclavine C (74)



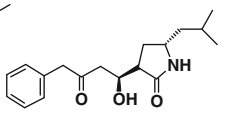
Radicicol (75)



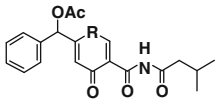
Citrinin (76)



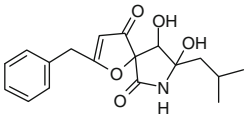
Ergoflavin (77)



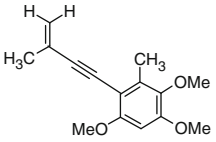
Berkeleyamide A (78)



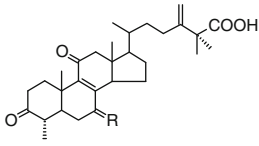
Berkeleyamide B R = O (79)
Berkeleyamide C R = NCH_2CH_2OH (80)



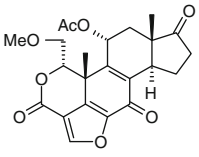
Berkeleyamide D (81)



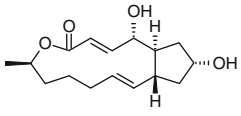
Antrocamphin A (82)



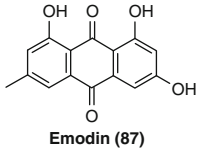
Antcin A R= H2 (83)
Antcin B R= O (84)



Wortmannin (85)



Brefeldin A (86)



Emodin (87)

Radicicol (syn. Monorden) **(75)**, an anti-fungal antibiotic was isolated from *Neocosmospora tenuicristata, Cylindrocarpon radicicola*, *Monocillium nordinii*, *Humicola fuscoatra, Chaetomium chiversii* and *Pochonia chlamydosporia* var.*chlamydosporia* (McCapra et al. 1964; Evans and White 1966; Ayer et al. 1980; Wicklow et al. 1998; Wang et al. 2008 and Shinonaga et al. 2009). It inhibits LPS-induced expression of iNOS by blocking the p38 kinase and NF-κB in macrophages (Jeon et al. 2000). Radicicol potently prevented the loss of neuronal cell bodies and neurites from LPS/IFN-γ-induced neurotoxicity in rat cortical neuron-glia cultures

with an EC_{50} value of 0.09 μM. It also inhibits the LPS/IFN-γ-induced expression of iNOS and production of NO in microglia. Additionally, it decreases the LPS/IFN-γ-induced release of TNF-α in the cultures. The inhibitory potency of radicicol against the production of NO and TNF-α was well correlated with the protection of neurons and was interpreted to be mediated via the inhibition of TNF-α release, as well as the suppression of iNOS expression in microglia (Sohn et al. 2007).

Citrinin **(76)**, isolated from *Monascus* sp. suppressed LPS/IFN-γ induced NO in glomerular mesengial cell line (MES-13) with 20% reduction in cell viability and 60% reduction in nitrite at 25 μm and 40 μm concentration respectively. Nitrite assay (Griess assay) was performed as all NO spontaneously got converted to nitrite while MTT assay portrayed cell viability results. The inhibitory effect of citrinin is solely caused by the suppression of iNOS gene and protein expressions. This is brought about by attenuation of STAT-1α phosphorylation induced by LPS/IFN-γ thereby playing a significant role in modulating various processes of cellular inflammation (Liu et al. 2010).

Ergoflavin **(77)** was isolated from an unidentified endophytic fungus, growing on the leaves of an Indian medicinal plant *Mimosops elengi*. This compound significantly inhibited human TNF-α and IL-6 with IC_{50} of 1.9 ± 0.1 μM and 1.2 ± 0.3 μM in comparison to dexamethasone with IC_{50} of 0.06 ± 0.007 μM and 0.01 ± 0.0 μM for TNF-α and IL-6 inhibition respectively (Deshmukh et al. 2009).

Berkeleyamides A–D **(78–81)**, were isolated from a deep water *Penicillium rubrum* isolated from Berkeley Pit Lake, Butte, Montana. All the four compounds were active against both Caspase-1 and MMP-3 in the low micromolar range. Berkeleyamide A and Berkeleyamide D exhibited the greatest potency, with IC_{50} values of 0.33 and 0.61 μM respectively (Stierle et al. 2008).

The anti-inflammatory effects of Antrocamphin A (**82**), Antcin A (**83**) and Antcin B (**84**) isolated from the fruiting body of *Antrodia camphorata* were evaluated by suppressing fMLP-induced production of superoxide anion, an inflammatory mediator produced by neutrophils. Antcin A was the most effective with an IC_{50} value of 8.55 ± 1.04 μM followed by Antrocamphin A (IC_{50} = 9.33 ±3.31 μM) and Antcin B (IC_{50} = 9.82 ± 4.40 μM) (Chen et al. 2007).

Wortmannin (**85**) is reported from *Penicillium wortmannii* (Brian et al. 1957; MacMillan et al. 1972), *P. funiculosum* (Haefliger and Hauser 1973), *Fusarium oxysporum*, *F. sambucinum* (Abbas and Mirocha 1988) and *Myrothecium roridum* (Petcher et al. 1972). It potently inhibited complement C5a-induced O_2^- generation and eosinophil peroxidase (EPO) release from human eosinophils, with 50% inhibition produced by a 1–10 nM concentration (Ezeamuzie et al. 2001). It also inhibited LPS-induced Akt phosphorylation in blood cells (Schabbauer et al. 2004) and CD28-mediated co-stimulation of IL-2 production in resting and activated human T cells (Ward et al. 1995). Wortmannin inhibits PI3-kinase in cell-based assays at a concentration of 100 nM. However, it also inhibits other members of the PI3-kinase superfamily. Moreover, Wortmannin was originally reported to be an inhibitor of smooth muscle Myosin Like Chain Kinase (smMLCK) (Nakanishi et al. 1992).

Brefeldin A (**86**) was first isolated from *Penicillium decumben* (Singleton et al. 1958) and was later reported from various other fungi like *Eupenicillium brefeldianum*

(Harri et al. 1963), *Curvularia subulata*, *C. lunata*, *Nectria radicicola*, *Penicillium cyaneum*, *P. simplicissimum*, *P. camemberti, Phoma medicaginis* and *Alternaria carthami* (Abraham and Arfmann 1992). It is an inhibitor of protein trafficking in the endomembrane system of mammalian cells that inhibits secretion and vacuolar protein transport (Nebenfuhr et al. 2002). It has been shown that LPS-activated monocytes treated with Brefeldin A are still able to synthesize IL-6, IL-1β and TNF-α, but due to the impaired exocytosis machinery, these cytokines are not secreted; thus, they accumulate intracellularly (Schuerwegh et al. 2001).

Emodin (**87**) was isolated from *Cladosporium* sp., *Trichoderma* sp. (Anthony et al. 2009), *Aspergillus wentii* (Hasan 1998), *A. terreus* (Schimmel et al. 1998), *Penicillium islandicum* (Kawai et al. 1984) and *Thielavia subthermophila* (Kusari et al. 2009). Emodin inhibits concentration-dependent expression of LPS-induced pro-inflammatory cytokines (IL-1β, IL-6) and chemokines (IL-8, CCL2) and in parallel, inhibits NF-κB activation and IκB-α degradation in HUVECs. However, it does not inhibit the NF-κB activation and IκB-α degradation induced by IL-1β (Meng et al. 2010). Emodin significantly inhibits the early expression (within 0.5 h) of cytokines TNF-α, IL-1β and IL-4, chemokines CCL4 and CCL8, and inflammatory modulators NFATC3 and PTGS2 (Chiu et al. 2010). It also inhibits TNF-α induced NF-κB-p65 and JNK activation but does not affect transforming growth factor- β1-induced Smad2/3 signaling. *In vivo*, Emodin inhibits proinflammatory and fibrogenic reactions (Kitano et al. 2007).

27.5 Fungal Metabolites Interfering with Cyclooxygenase (COX) and Xanthine Oxidase (XO)

27.5.1 Role of COX-2, 3α-HSD, XO in Inflammation

Nonsteroidal anti-inflammatory drugs (NSAIDs) are potent anti-inflammatory agents that act through the inhibition of the COX enzyme and the subsequent inhibition of prostaglandins at the site of inflammation. Two forms of the COX enzyme have been identified: COX-1, which is constitutively expressed in many cells and tissues and COX-2, which is selectively induced by proinflammatory cytokines at the site of inflammation. Selective inhibition of COX-2 may produce superior anti-inflammatory drugs with substantial safety over existing NSAIDs (Seibert and Masferrer 1994; Chen et al. 1994; Funk 2001). The compounds like rofecoxib, cerecoxib and others have reached the market which are COX-2 inhibitors (Prasit et al. 1999; Shishodia et al. 2004).

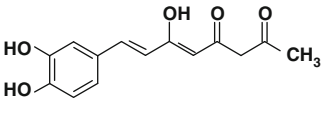
NSAIDs also inhibit 3α- hydroxysteroid dehydrogenase (3α -HSD) enzyme, a member of the NADPH-dependent oxidoreductase family, ubiquitously distributed in tissues, which catalyzes interconversion of 3-hydroxy and 3-carbonyl groups of steroids (Pawlowski et al. 1991; Penning et al. 1985).

Xanthine oxidase is found in the liver and plays a key role in the metabolic pathway of uric acid by catalyzing the oxidation of hypoxanthine to xanthine and further

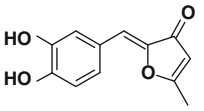
oxidation of xanthine to uric acid. XO inhibitors block the terminal step of uric acid biosynthesis (Rastelli et al. 1997; Ishibuchi et al. 2001). The search for new potent inhibitors of these enzymes could be useful lead structures for new anti-inflammatory and anti-arthritic therapeutics, plays a pivotal role.

27.5.2 COX, 3α-HSD and XO Inhibitors

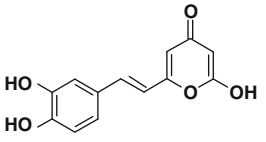
Inonotic acid methyl ester (**88**), Inotilone (**89**), *Iso*-hispidin (**90**), (*E*)-4-(3, 4-dihydroxyphenyl) but-3-en-2-one (**91**) and Hispidin (**92**) were isolated from fruiting body of *Inonotus sp.* These compounds were evaluated for their inhibitory activity in 3α-HSD, COX-1, COX-2 and XO. All the compounds tested exhibit strong COX inhibitory effects with a predominance of COX 2 in case of Hispidin, (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one, Inonotic acid methyl ester and Inotilone, with IC_{50} of 0.0008 μM, 0.01 μM, 0.21 μM and 0.3 μM respectively. All these compound except Inotilone exhibited strong 3α-HSD inhibitory effect with an IC_{50} value of 8.1 μM for Hispidin, 8.9 μM for (*E*)-4-(3,4-dihydroxyphenyl)but-3-en-2-one, 16.1 μM for Inonotic acid methyl ester and 50.4 μM for Inotilone. These compounds also exhibit moderate inhibitory effect towards XO, with IC_{50} of activity 4.4 μM, 10.1 μM, 7.1 μM, 9.1 μM and 13.8 μM for Hispidin, (*E*)-4-(3,4-dihydroxyphenyl) but-3-en-2-one, Inonotic acid methyl ester, Inotilone and *Iso*-hispidin respectively (Wangun et al. 2006).



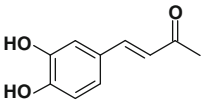
Inonotic acid methyl ester (88)



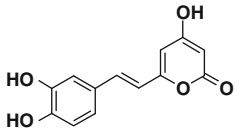
Inotilone (89)



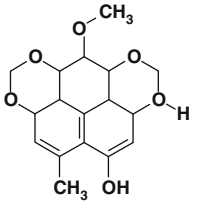
Iso -hispidin (90)



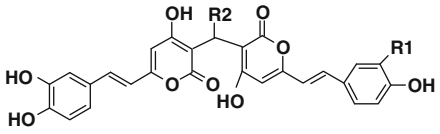
(E)-4-(3,4-dihydroxyphenyl)but-3-en-2-one (91)



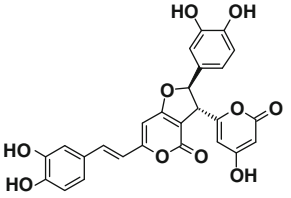
Hispidin (92)



Funalenine (93)



Squarrosidine R1 = R2=H (94)
Pinillidine R1 = OH, R2=CH_3 (95)



Hypholomine B (96)

Funalenine **(93)** isolated from *Aspergillus* sp. HKI 0472, displayed anti-inflammatory activity by inhibiting the enzyme 3α- HSD, COX-1 and COX-2 with an IC_{50} of 52, 36 and 45 μM respectively (Wangun 2006).

Squarrosidine **(94)** isolated from the fruiting body of *Pholiota squarrosa* displayed significant 3α-HSD, COX-1, COX-2 and XO inhibitory effect with an IC_{50} of 8.1, 0.02, 0.04 and 8.1 μM respectively. The ratio COX-1/COX-2 value was 2, indicating that this compound is a non-selective COX inhibitor (Wangun 2006).

Pinillidine **(95)** and Hypholomine B **(96)** were isolated from *Phellinus pini* DSM 5238. Pinillidine displayed significant 3α-HSD and XO inhibitory effect with an IC_{50} of 5.8 and 5.8 μM respectively. Similarly Hypholomine B displayed significant 3α-HSD and XO inhibitory effect with an IC_{50} values of 25.5 and 6.7 μM respectively (Wangun and Hertweck 2007).

27.6 Fungal Metabolites Interfering with Phospholipase A2 (PLA2)

27.6.1 Role of PLA2 in Inflammation

Activation of the PLA2 pathway is a key cell signaling event in the inflammatory response. The PLA2 family consists of a group of enzymes that hydrolyze membrane phospholipids, resulting in the liberation of arachidonic acid (AA), a precursor to pro-inflammatory molecules.

PLA2 catalyzes the hydrolysis of the sn-2 position of membrane glycerophospholipids to liberate Arachidonic acid, a precursor of eicosanoids including prostaglandins and leukotrienes. The same reaction also produces lysophosholipids, which represent another class of lipid mediators. So far, at least 19 enzymes that possess PLA2 activity have been identified in mammals. The secretory PLA2 (sPLA2) family, in which 10 isozymes have been identified, consists of low-molecular-weight, Ca^{2+} requiring, secretory enzymes that have been implicated in a number of biological processes, such as modification of eicosanoid generation, inflammation, host defense, and atherosclerosis (Murakami and Kudo 2002).

27.6.2 PLA2 Inhibitors

Thielocins A1α **(97)**, A1β **(98)**, A2α **(99)**, A2β **(100)**, A3**(101)**, B1**(102)**, B2 **(103)** and B3 **(104)** were isolated as a novel family of PLA2 inhibitors from *Thielavia terricola* RF-143 together with Thielavin A, B, C, D and E (Yoshida et al. 1991; Matsumoto et al. 1995). Thielocin A1β showed the most potent inhibitory activity (IC_{50} = 0.0033 μM) against rat group II PLA2 followed by B1 (IC_{50} = 0.0078 μM), B3 (IC_{50} = 0.012 μM), A1α (IC_{50} = 0.032 μM), A2β (IC_{50} = 0.038 μM), A2α

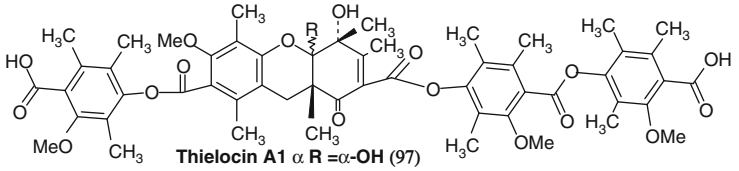
(IC_{50} = 0.051 μM), B2 (IC_{50} = 0.070 μM). Thielocin B3 was the most potent against human group II PLA2, (IC_{50} = 0.076 μM) followed by B1 (IC_{50} = 0.17 μM), A2β (IC_{50} = 0.24 μM), A2α (IC_{50} = 0.31 μM), A1α (IC_{50} = 0.39 μM), B2 (IC_{50} = 2.7 μM) and B3 (IC_{50} = 6.12 μM) (Matsumoto et al. 1995).

Thielocin A1β was also found to suppress histamine release from mast cells stimulated with secretory PLA2, which led to examine the effect of Thielocin A1β against secretory PLA2 induced paw edema. Thielocin A1β inhibited bee venom PLA2 in a dose-dependent manner (IC_{50} = 1.4 μM). Subplantar injection of bee venom PLA2 produced a rapid but transient edematous response. Coinjection of Thielocin A1β (1 μg/paw) with bee venom PLA2 resulted in a 44.7 ±4.6% reduction of edema formation. This anti-edema action was not enhanced by cyproheptadine (anti-histamine/anti-serotonin). These results suggest that Thielocin A1β shows edema-reducing activity via inhibition of the PLA2 activity which participates in histamine release by mast cells (Tanaka et al. 1995).

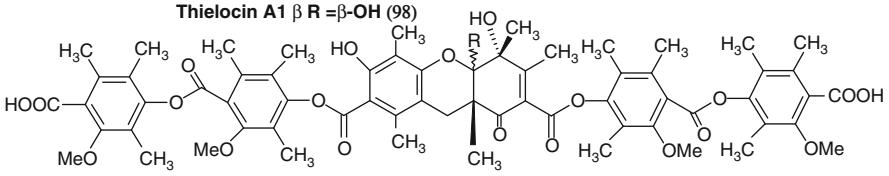
Plastatin **(105)** and Luteosporin **(106)** were isolated from *Penicillium chermesinum* as inhibitors of porcine pancreatic PLA2 with Ki values of 0.89 μM and 12.8 μM respectively. PLA2 preparations from *Naja naja* and *Crotalus adamanteus* were not significantly inhibited by Plastatin and Luteosporin (Singh et al. 1985)

Cinatrins A **(107)**, B **(108)**, C2 **(109)** and C3 **(110)** isolated from *Circinotrichum falcatisporum* RF-641 (Itazaki et al. 1992) inhibited PLA2 purified from rat platelets. Cinatrins A, B, C2 and C3 inhibited PLA2 activity in a dose-dependent manner with an IC_{50} of 320 μM for A, 120 μM for B, 800 μM for C2 and 70 μM for C3. Cinatrin C3 had the maximum inhibitory activity and was noncompetitive with a Ki value of 36 μM. Cinatrins B and C3 also inhibited both porcine pancreas and *Naja naja* venom PLA2 (Tanaka et al. 1992).

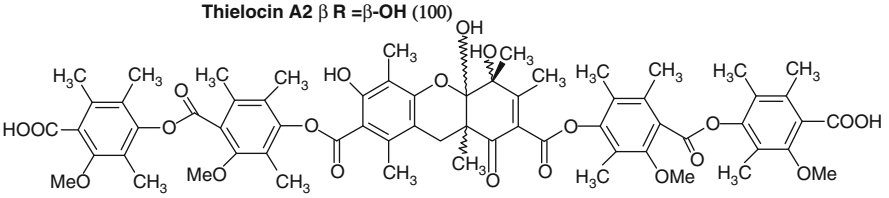
Ergophilones A **(111)** and B **(112)** were isolated from culture broth of *Penicillium* sp. BM-99 and showed inhibitory activities against the rat platelet PLA2 with IC_{50} value of 0.44 μM and 0.56 μM, respectively. However, Ergophilones A and B showed weak inhibition against the porcine pancreatic PLA2 with IC_{50} value of 51.7 μM and 39.0 μM respectively (Hyodo et al. 1995).



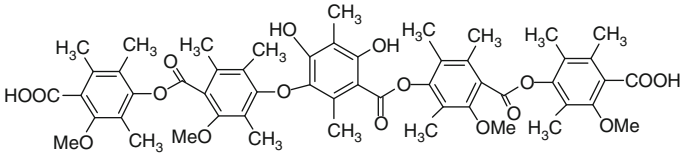
Thielocin A1 α R =α-OH (97)
Thielocin A1 β R =β-OH (98)



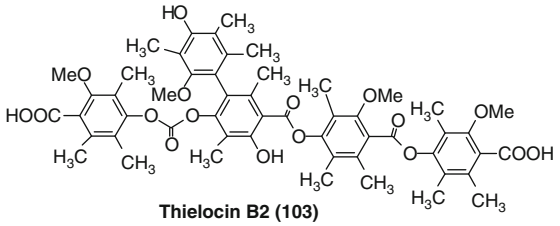
Thielocin A2 α R =α-OH (99)
Thielocin A2 β R =β-OH (100)



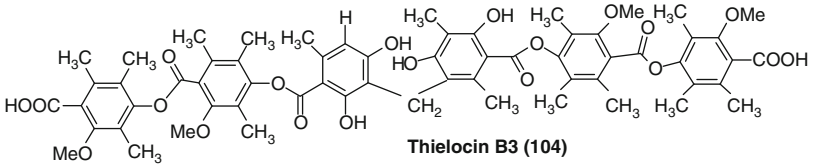
Thielocin A3 (101)



Thielocin B1 (102)



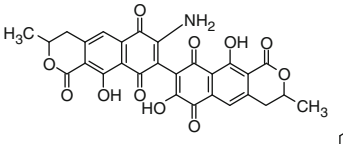
Thielocin B2 (103)



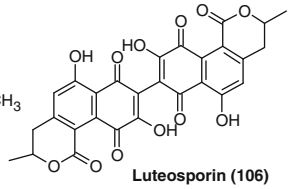
Thielocin B3 (104)

Pachymic acid **(113)** and Dehydrotumulosic acid **(114)** isolated from the hydroalcoholic extract of *Poria cocos* inhibited PLA2 from snake venom. Pachymic acid and Dehydrotumulosic acid exhibited an IC_{50} of 2.897 mM and 0.845 mM respectively. These two compounds are structurally related to certain triterpenoids from *Ganoderma* and *Schinus* that have previously been described as competitive inhibitors of PLA2 (Cuéllar et al. 1996). Pachymic and Dehydrotumulosic acids were studied in different models of acute and chronic inflammation. They proved to be active in most of the

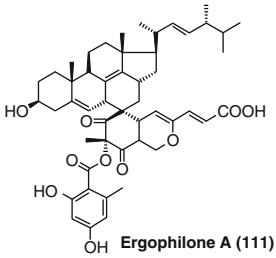
models applied. Neither of them was active against arachidonic acid-induced ear edema. Dehydrotumulosic acid significantly diminished the mouse ear edema induced by ethyl phenylpropiolate, while Pachymic acid was ineffective. When the putative corticoid-like mechanism of both compounds was explored, Pachymic acid activity was partially abolished by the glucocorticoid receptor antagonist progesterone, but the activity of Dehydrotumulosic acid was not affected. *In vivo* experiments demonstrated the inhibition by both principles of the PLA2-induced extravasation. The previous report on the effects of both compounds *in vitro* against PLA2, together with the present *in vivo* results, support the idea that the inhibition of this enzyme probably constitutes their main mechanism of action (Giner et al. 2000).



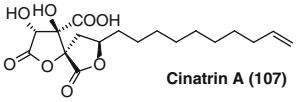
Plastatin (105)



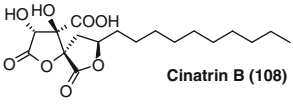
Luteosporin (106)



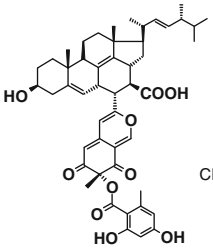
Ergophilone A (111)



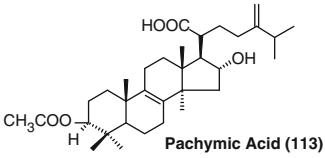
Cinatrin A (107)



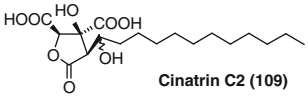
Cinatrin B (108)



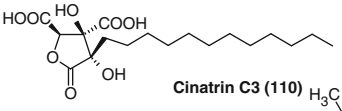
Ergophilone B (112)



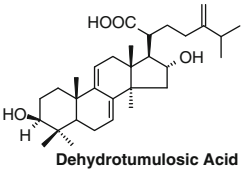
Pachymic Acid (113)



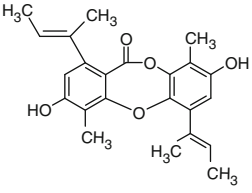
Cinatrin C2 (109)



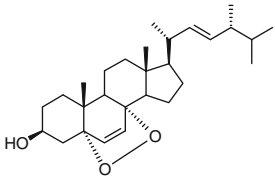
Cinatrin C3 (110)



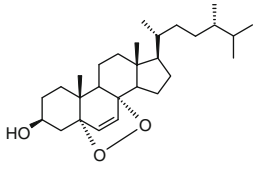
Dehydrotumulosic Acid (114)



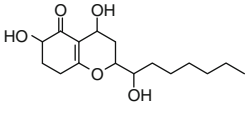
Folipastatin A (115)



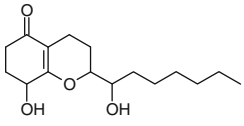
Ergosterol Peroxide (116)



5α,8α-epidioxy-(24S)-ergosta-6-en-3β-ol (117)



Koninginin F (119)



Koninginin E (118)

Folipastatin A **(115)**, isolated from *Aspergillus unguis* inhibited the PLA2 purified from rabbit peritoneal exudate with an IC_{50} of 39 μM and it suppressed the release of arachdonic acid from rat polymorphonuclear leucocyte with an IC_{50} of 24 μM (Hamano et al. 1992).

Ergosterol derivatives namely Ergosterol peroxide **(116)** and 5α,8α-epidioxy-(24 S)-ergosta-6-en-3β-ol **(117)** were isolated from a basidiomycete fungus, *Lactarius hatsudake*. These compounds exhibited selective inhibitory activity against *Crotalus adamenteus* venom PLA2 enzyme with an ED_{50} value of 100 μg/ml, but were inactive against *Apis mellifcra* bee venom PLA2 with an ED_{50} value of >400 μg/ml (Gao et al. 2007).

Koninginins E **(118)** and F **(119)** isolated from *Trichoderma koningii,* inhibited edema-inducing, myotoxic and enzymatic activities of the total venom of *Bothrops jararacussu* (jararacucu) snake analyzed, as well as one of its homolog forms of PLA2 (bjPLA2 -group IIB) and human secreted PLA2 protein fusion (hsPLA2 -group IIA). Koninginin E and Koninginin F reduced the edema provoked by the total venom to around 60%. When bjPLA2 was used for inducing the edema, they reduced the edema to around 30% and when hsPLA2 was used these compounds reduced the edema to around 40%. The lowest efficiency of compounds Koninginin E and Koninginin F in inhibiting the edema provoked by total venom might be related to the presence of other toxins in the crude venom that are also able to induce the edema formation, but that are not inhibited by Koninginin E and Koninginin F. Koninginin E and Koninginin F structures are similar to vitamin E and possibly, the mode of action these molecules is similar to the one produced by the vitamin (Souza et al. 2008).

27.7 Future Perspectives

The “Quality” of our lives has become more critical to the present generation and “pain” is a major hurdle that lowers that quality. Inflammation and pain go “hand in hand” and if that is controlled then life becomes easier. A key factor, of course, is the disease process and/or metabolic degeneration that have also to be controlled. Fungal metabolites will certainly play greater role in the restoration of pathways that have become dysregulated.

From the extensive data quoted in this paper by the authors on anti-inflammatory and earlier numerous publications on a wide variety of bioactive compounds such as anti-cancer to name just one, it becomes obvious that fungi are a rich source for the discovery of novel molecules with possible new bioactivities. New bioactivee can be generated by the development of specific targets that are ideally, most sensitive regulators of inflammatory pathways. Fungal secondary metabolites with properties unique to their chemical structure should be isolated. It should certainly be interesting to have activities comparable if not better than the classic synthetic molecule N-acetyl salisylic acid. For example, anti-inflammatory activites without the troublesome acidity side effect or cardiac problems needs to be investigated.

Oxidative stress and inflammation are major hallmarks of various chronic inflammatory diseases such as arthritis, asthma, chronic bronchitis, cardiovascular, neuro-degenerative disorders and cancer. In this review, we have presented an overall picture of several anti-inflammatory pathways which are targeted by a wide variety of novel fungal metabolites. We hope that this review which lists many metabolites with potent anti-inflammatory activity will be the start-up point for developing new scaffolds against specific anti-inflammatory targets using modern medicinal chemistry approach.

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Chapter 28
Application of Microbial Toxins for Cancer Therapy

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Abstract The principle of selective targeting of immunotoxins lies on the basis that cancer cells usually have few or specific growth factors/receptors/antigens highly over expressed on their surface. Ligands corresponding to these molecules are conjugated to modified toxins (modified to loss its native function) isolated form variety of bacterial populations. Normal cells either do not express these molecules or express at relatively low number leading to no or minimal adverse effects. The basic mechanism of action of these immunotoxins depends on the toxins employed. In this regard continuous efforts are being made to (i) Identity molecules exclusively expressed in cancer cells, (ii) to improve the specificity and efficacy (iii) reduce size effects of the drugs, (iv) Reduce immunogenicity and (v) to improve better pharmacokinetics for drugs delivery.

Keywords Cancer • Immunotoxins • Fusion toxins • Fusion proteins • Interleukins • Selective targeting • Tumor immunology

28.1 Introduction

Toxins are secreted/produced by bacteria as a byproduct has the capability to kill Tumor and normal cells. In the past these isolated toxins have been used to create a molecule, which were used to kill several types of tumors. These molecules are known as immunotoxins. Since they modulate immunoresponse also, the earlier immunotoxins was not targeted to specific types of cancer and has a capacity to

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kill both the normal as well as the cancer cells. Recently selective targeted therapy was evolved using these molecules to attack the cancer cells more specifically. This avoids in killing the normal as well as generating side effects. Tumor is an abnormal mass of closely packed cells and do not require stimuli for growth and do not respond to any inhibitory signals. Tumors can be benign or malignant; often the term cancer refers to malignant tumors. The cancer cells may or may not invade neighboring cells accordingly they are termed as invasive and noninvasive. In case of invasive they may spread to far apart tissues either through circulatory system or through lymphatic system and is referred to as metastasis. All this progression depends on molecular and chemical signals received by the transformed cell. Treatment of cancer depends on grade, stage and type of cancer. Grading of tumor indicates the level of differentiation, which is represented by roman numerals 0–IV with increasing loss of differentiation. Grading of tumor is of less clinical significance as judgment varies from clinician to clinician at the time of diagnosis. Moreover it is not an indicator of disease. Staging of tumor on other hand is more uniform it indicates the severity of the disease. Two major systems followed in staging of tumor are UICC (International Union against Cancer) and AJCC (American Joint Committee on Cancer) method. They employ TNM system where T stands for tumor; this is followed by numerical indicating the size T1–T4 with increase in size and T0 stands for no lesion. Similarly, N followed by numerical indicates extent of lymph node involvement, this is numbered N1–N3 with increase in lymph node involvement and N0 indicates no nodal involvement. M stands for metastasis; M1 indicates presence of metastasis and M0 absence of metastasis. T/N/M followed by X indicates uncertainty, this TNM method is being continuously updated to correlate to new diagnostic methods and to maintain uniformity in classification as it has a direct impact on selecting the mode of treatment to be followed (Brierley 2006; Gospodarowicz et al. 2004).

Most of the conventional therapies (chemotherapy, radiation therapy) being widely used are aimed at cell division, as it is the most important characteristic feature of transformed cell. Rate of growth of tumor depends on factors like (i) number of cells in the tumor that are capable of proliferation called growth fraction, (ii) time required to complete the cell cycle and (iii) rate at which cells within the tumor are shedding. As tumor progresses growth fraction decreases. Most chemotherapeutic agents target the proliferating pool, so rapidly growing tumors respond very well (Marchini et al. 2004; Rew and Wilson 2000). At the time of diagnosis growth fraction is remarkably lowered resulting in poor response to chemotherapeutic agents. An analogy to understand the drawback of late diagnosis is assuming doubling time for a cell is 1 min and half cup is filled in 59 min therefore it takes only one more minute to fill the cup. Half cup corresponds to palpable mass, which is diagnosis time, and full cup corresponds to size of mass causing death thus the time between diagnosis and death is less. Most chemotherapeutic agents and radiation cause damage to DNA leading to genomic instability, susceptibility to mutations that again predisposes to cancer (Benedict et al. 1977). Other conventional therapies have their own limitations like radiation therapy affects

not only tumor but also normal tissue surrounding the tumor. Surgery is applicable only in initial stages to non-hematological tumors. In most cases by the time of diagnosis, disease is in metastasis stage. All the above limitations of conventional therapies show the need for novel therapy, with wider margin for safety and good selective toxicity. New modes of therapy include proton therapy, photodynamic therapy, biologic therapy etc., most of which are targeted therapies, which selectively aim at cancerous tissue and not at normal tissue thereby minimizing toxicities and enhancing potency. Immunotoxins a class of biologic therapeutics provide an efficacious and specific treatment for cancer than other contemporary methods. Surface targeted biologic therapy such as unlabelled MAbs (monoclonal antibodies) kill cells after binding. Humanized MAbs are effective in half of the patients via mechanism of apoptosis induction, antibody dependent cytotoxicity and complement dependent cytotoxicity. In patients with malignant cells resistant to apoptosis and patients whose immune systems will not perform antibody or complement dependent cytotoxicity, the immunotoxins are a better option. Radio immunotherapy is limited by the potency of the radionuclide molecules that can be added to each MAb molecule. There are limitations of various surface targeted strategies but immunotoxins are distinct from these approaches and target the surface of cancer cells with considerable potency, using protein toxins that kill the cell with single molecule.

28.2 Targeted Toxicity in Cancer Cells

The principle behind the selective toxicity of immunotoxins lies on the basis that cancer cells usually have specific growth factors or receptors or antigens highly over expressed on their surface when compared to normal cells (Zumkeller and Schofield 1995). This is taken as the basis for selectivity. Ligands corresponding to these receptors or growth factors or antibodies raised against antigens are conjugated to toxins so that the conjugated molecule will selectively bind to these highly over expressed molecules and kill the tumor cells. However normal cells also express these receptors but are relatively less in number. Finding out the molecules exclusively expressed in cancer cells will definitely be of immense use for selectively targeting but they are several groups investigating continuously.

28.2.1 Immune Response

Immune system recognizes tumor as foreign by antigens over expressed on them. Activation of similar immune response by the use of foreign toxins (ricin toxin, diphtheria toxin, and pseudomonas exotoxin, restrictocin etc.) is one practical approach. Selective toxicity can thus be achieved by ligating toxin to growth factors

or monoclonal antibodies whose receptors or antigens are selectively over expressed in cancer (FitzGerald and Pastan 1989). Immune response is of two types i.e. non-specific and specific. Anti-tumor activity in host comes under specific immunity as it is triggered specifically to the existing tumor; in contrast non-specific immune response is general and present throughout the life. Lymphatic and immune system consisting of lymph, lymphatic vessels, lymphatic tissue, and bone marrow are important for anti-tumor activity; their other functions include transport of nutrients and draining of interstitial fluid. Lymphatic tissue contains large number of lymphocytes; two important lymphocytes that mediate immune response are B and T cells. They are referred to as immunocompetant because of their ability to fight against external invading microbes like bacteria, virus, fungi as well as abnormal tissue. Anti-tumor activity/tumor immune response involves both cell mediated and humoral immunity (Lores et al. 1998; Elisabeth et al. 1998).

Cell-mediated immunity: Cell mediated immunity is brought about by T cells. T cells become active on receiving two signals first by antigen and second by co-stimulants like IL-2. However tumor cells evade immune response due to paucity of co-stimulants and decreased expression of MHC (major histocompatibilty complex). Activation of T cells is followed by proliferation and differentiation that augments immune response immediately. T cells are classified into TC, TH, and memory T cells. $CD8^+$/TC: The most important function of Tc is immunological surveillance i.e. ability of cells to identify and eliminate transformed cells (Benchetrit et al. 2003). This is more significant in cancer caused by oncogenic viruses, as antigens are very specific. Whereas in other cancers, specific antigens are deficient hence recognition of tumor cell as non-self by immune system is difficult. TC cells are important for their cytolytic activity against tumor cells they are activated by two signals first by antigens bound to MHC I and second by co stimulators like cytokines, most important of which is IL-2. Cytolytic activity is mediated either by release of perforin that makes holes in the membrane thus lysing the cell or by release of toxin that activates DNAse. $CD4^+$/TH are not cytolytic they do not kill the tumor cells rather they help TC cells in killing transformed cells. Their action is mediated by recognizing antigen combined with MHC II on co stimulation, resulting in release of cytokines that in turn activate TC and B cells (Hung et al. 1998; Toes et al. 1999)

Humoral immunity: B cells bring about humoral immunity. They mediate their action through the release of antibodies. This type of immune response is less significant in case of tumor cells due to the deficiency of specific antigens (Old 1981). Immune response is triggered directly by antigen binding to B cell receptors, processed antigen triggers an intense response. The co-stimulators produced by TH cells help in differentiation and proliferation of B cells. B cells differentiate into plasma cells, which produce antibodies that mediate their immune response by activating phagocytosis or by activating classical pathway, which causes cytolysis by MAC (membrane attack complex).

NK (Natural killer) cells also exhibit antitumor activity. NK cells are important in situations where T cells cannot mediate their cytolytic activity due to paucity of co-stimulants or decreased expression of MHC. NK cells are cytolytic to tumor cells that specifically express abnormally high levels of proteins, to cells coated

with antibodies and to cells with MHC–antigen complex. NK cells cause cytolysis by release of perforin similar to Tc cells or by inducing apoptosis (Introna and Mantovani 1983; Herberman 2002).

28.3 Toxins

Mechanism of cytotoxicity of immunotoxins depends on the toxin employed, most of the toxins used in constructing fusion toxins are protein synthesis inhibitors and they share common features with respect to their structure, mode of action etc. in this review we elaborated diphtheria toxin.

28.3.1 Corynebacterium diphtheriae

Corynebacterium diphtheriae, a non-motile, non-capsulated, gram + ve bacillus produces 5,000 molecules of a potent exotoxin per hour after infection. A single molecule of this diphtheria toxin is lethal to the host cell. It is interesting to know that the gene for diphtheria toxin is carried by bacteriophage and not by the bacteria so the bacteria is infectious only if it is infected by phage through lysogenic pathway and not lytic so it inserts its DNA into its host i.e. bacteria, but gene regulation is under control of bacteria. Phage contains gene for toxin, its promoter and operator sequence whereas bacteria contains sequence coding for repressor. Regulation of this gene depends on iron concentration. In the presence of iron, repressor binds to iron and the complex binds to the operator thereby preventing transcription of the gene. In case of low concentration of iron, repressor is free and cannot bind to operator so transcription of gene proceeds. This is an example of negative gene regulation (Fourel et al. 1989; De Zoysa et al. 2005).

Monomeric diphtheria toxin is compact, globular and toxic whereas dimer is non-toxic. Diphtheria toxin, a Y shaped molecule is a monomer of 535 residues in two fragments A and B. Figure 28.1a A 14-residue arg rich loop connects the two fragments and the structure of this loop differs significantly between monomer and dimer. Fragment A, the amino terminal end has a catalytic domain (C-domain) consisting of residues 1–193. This C-domain has 8 β strands and 7 α helices. The C-domain is the most important domain bringing about cytotoxicity by inhibiting protein synthesis. Fragment B, the carboxy terminal end has two domains, transmembrane domain (T domain) and receptor binding domain (R domain). T-domain consisting of residues 205–379 has nine α helices. T-domain helps in translocating the C-domain from endosome to cytosol. On the T domain the first 3 helices constitute the amphipathic region that helps in stabilizing the toxin on cell surface. Helices 5, 6, 8 and 9 of this domain are apolar and protonation of anionic residues in these helices results in loss of charge and hence easy penetration of C-domain into the membrane (Hu et al. 1998). The R-domain consisting of residues 386–535 has 10 β

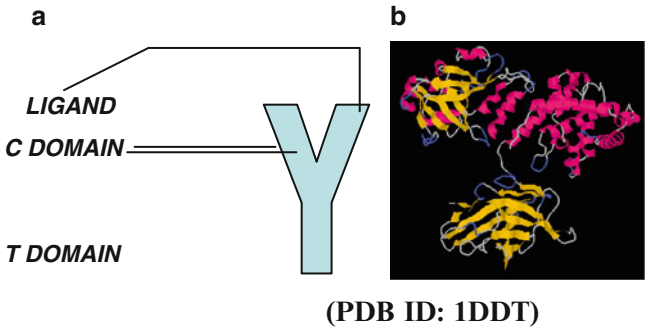

Fig. 28.1 (**a**): Diagrammatic representation of Diphtheria toxin structure and 1 (**b**): Crystal structure of Diphtheria toxin in ribbon representation (PDB ID: 1DDT)

strands. R-domain helps in binding of toxin to heparin binding epidermal growth factor receptor on the cell surface (Bennett and Eisenberg 1994). The diphtheria toxin has 4 cysteine residues, which make two internal disulphide bonds linking C186 to C201 and C461 to C471. Figure 28.1b shows the crystal structure of diphtheria toxin.

28.4 Mechanism of Action of Fusion Toxins

All diphtheria fusion toxins follow the same mechanism of action only the receptor to which it binds differs depending on ligand employed. The selective cell death of tumor cells is therefore due to protein synthesis inhibition by toxin employed. Native diphtheria toxin binds to heparin binding epidermal growth factor receptor on plasma membrane from which it is transported into the endoplasmic reticulum by receptor mediated endocytosis as a vesicle. A soluble cytoplasmic protein known as dynamin helps in pinching off the vesicle from the membrane. This vesicle is coated by cage of proteins called clathrin towards the cytosolic surface and this coat is discarded before the vesicle fuses to target. The catalytic domain is translocated into the cytosol and receptor is recycled to plasma membrane by exocytosis from early endosome and remnants are degraded. In case of receptor down regulation, receptor is also degraded. Vesicular ATPases acidify the endosomes leading to decrease in pH to about 6.0. At this acidic pH, the T domain undergoes a conformational change resulting in partial unfolding and exposing of hydrophobic sites mimicking transmembrane proteins (Ren et al. 1999a, b; Choe et al. 1992). This helps in membrane insertion and formation of channel large enough for the C domain to be translocated in unfolded state and is released into the cytoplasm (Kagan et al. 1981; Ren et al. 1999a, b). In the cytoplasm the disulphide bond is reduced by thioredoxin reductase and Hsp 90 refolds the C domain. The catalytic domain acts by transferring

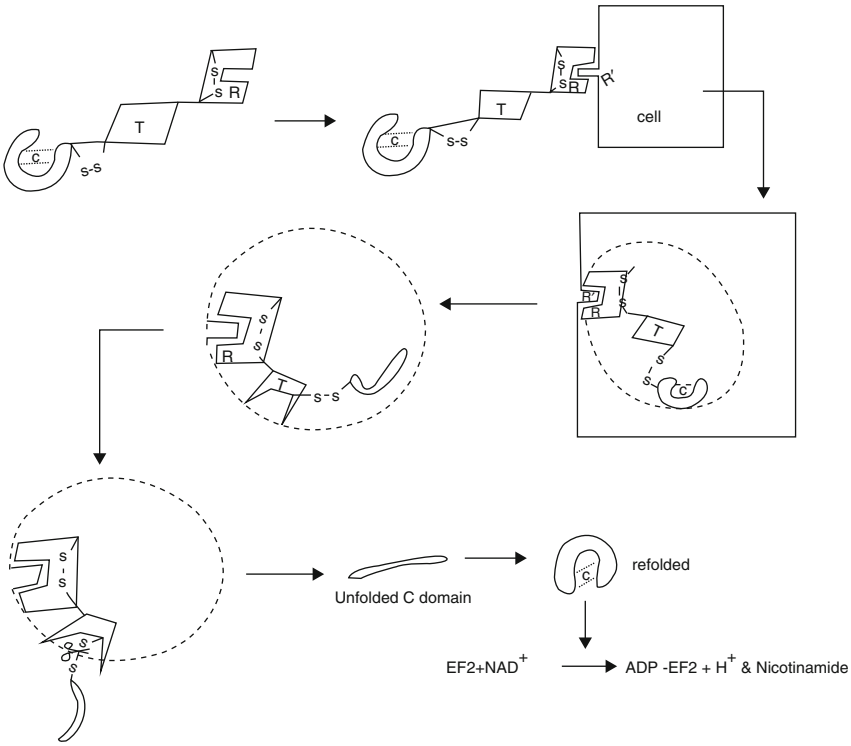

Fig. 28.2 Mechanism of action of diphtheria toxin in its native state

ADPR (adenosine Di Phosphoate Ribosyl) moiety from NAD to post transcriptionally modified histidine residue at 715 called diphthamide (2-[3-carboxyamido-3-(trimethylammonio) propyl]) (Van Ness et al. 1980; Liu et al. 2004a, b) in EF-2 (Elongation Factor 2), thus inactivating it . The role of EF-2 in translation is to help the transfer of growing peptide chain from A site to P site by moving ribosome. Inactivating EF-2 by diphtheria toxin results in inhibition of protein synthesis and cell death (Frankel 2002). This reaction is irreversible and involves an inversion in configuration of NAD from β to α anomer. This is a sequential ordered mechanism where catalytic domain first binds to NAD and then transfers ADP ribosyl moiety to EF-2. EF-2 of bacteria i.e. EF-G is not susceptible whereas EF-2 of eukaryotes, yeast and archaea are susceptible. Mutants that cannot produce diphthamide will be resistant to the toxin.

$$\text{EF2} + \text{NAD} \rightarrow \text{NICOTINAMIDE} + \text{H} + \ + \text{ADPR} - \text{EF2}$$

The mechanism of action of native diphtheria toxin has been illustrated in Fig. 28.2. The entire process starting from internalization to inhibition of protein synthesis takes about 6 h and apoptosis in induced in 3 days. The method of induction

of apoptosis by fusion toxins depends on toxin employed, method of inhibition of protein synthesis. In some diphtheria conjugates the induction of apoptosis is by activation of death receptor pathway in a receptor independent manner (Thorburn et al. 2003; Frankel et al. 1997)

Targeting angiogenesis is another selective approach as angiogenesis is almost exclusive to tumor cells. Tumors cannot grow beyond few millimeters without angiogenesis; receptors for stimulators of angiogenesis are therefore exclusively present on tumor cell surface enabling selective targeting with fusion toxins. Some stimulators of angiogenesis which can be used as ligands are epidermal growth factor, VEGF (vascular endothelial growth factor), fibroblast growth factor, granulocyte macrophage colony stimulating factor, tumor necrosis factor, some interleukins etc. (Arora et al. 1999).

28.5 Generations of Immunotoxins Using Bacterial Toxins

Immunotoxins can be classified into generations based on their time of discovery, construction method employed and potency.

28.5.1 First-Generation Immunotoxins

First generation immunotoxins are primitive. They are made up of entire toxin moiety with mutations to make the receptor binding domain non functional and are fused to ligand by chemical means using cross linking agents to introduce disulfide bonds, or establishing an amide bond between two proteins. The ligand employed is whole antibody or monoclonal antibody. The drawbacks with this first generation immunotoxins are poor tumor uptake, extended half-life, low production rates which are due to their large size.

28.5.2 Second-Generation Immunotoxins

Second generation immunotoxins are modified versions of first generation. These immunotoxins have been constructed in such a way to circumvent the drawbacks of first generation. In this type of immunotoxins receptor-binding domains is entirely deleted and are fused to ligand by genetic engineering. The ligand employed is fragment of recombinant antibody or cDNA encoding growth factors or cytokines corresponding to molecules over expressed on tumour cell surface.

28.6 Construction of Fusion Toxins

Diphtheria toxin conjugate synthesis requires 3 basic steps (i) inactivating or removing R domain (ii) identifying ligands corresponding to over expressed molecules on cancer cell surface (iii) linking ligand to toxin (Frankel 2002). Diagrammatic representation has been shown in Fig. 28.3.

28.6.1 Modifying Receptor Domain

R domain is the receptor-binding domain consisting of 386–535 amino acids. This domain needs to be removed either totally or partially to ensure loss of its binding activity. In case of diphtheria conjugates, the molecules with entire R domain removed (DT386) are reported to have higher activity than conjugates with partially removed R domain (DT486) due to higher binding affinity and tendency to remain in monomeric form. Alternatively the conjugate can be synthesized by point mutations in the binding domain mutating the residues in binding loop between 510 and 530 (Frankel 2002; Vanderspek et al. 1996) or a critical S525F

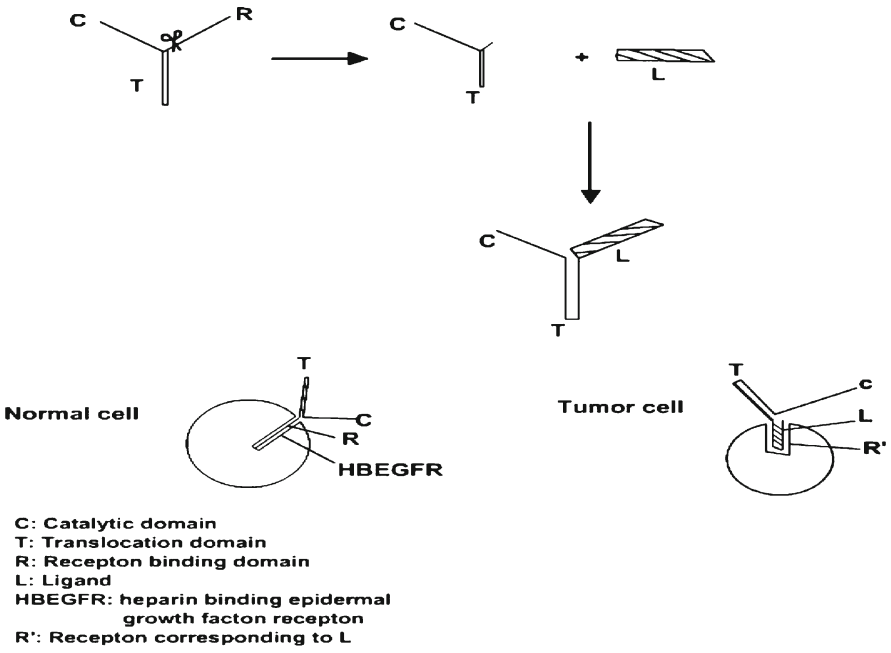

Fig. 28.3 Cloning strategy of immunotoxin

(Li and Ramakrishnan 1994) or C471Y or L390F. These mutations eliminate the binding function of the toxin to its native receptor, heparin binding epidermal growth factor receptor on normal cells in a full proof manner (Greenfield et al. 1987).

28.6.2 Ligands Attachment

Receptors for cytokines, monoclonal antibodies, proteoglycan, transferrin, granulocyte macrophage colony stimulating factor, endothelial growth factor, MSH, TRH, substance P and placental lactogen receptor are some of them found to be highly over expressed in tumor cells, ligands corresponding to these molecules are conjugated to toxins.

Cytokines are widely used as ligands due to their added advantage of cytotoxic property. IL-2 has demonstrated activity against melanoma, lymphoma (Duvic et al. 1998; LeMaistre et al. 1998) and leukemia (Frankel et al. 2003) IL-4 showed minimal cytotoxic activity it inhibits only proliferating cells. IL-6 has some activity but is found to be a growth factor for myeloma cells. Thus cytokines are useful both in hematological malignancies and immunogenic tumors. Major cytokines in use are IL-2, IL-3, IL-4, IL-12, and GMCSF.

28.6.3 Strategy for Linking Ligand to Modified Toxin

This is the most critical step in construction of fusion toxins as linking should not alter the (a) ligand's binding affinity to receptor, (b) endocytosis of toxin, (c) the translocation of B fragment, (d) the ADP ribosylation of elongation factor two, (e) endocytosis of the toxin. The ligand is placed at the carboxy terminal end of the diphtheria toxin to allow ADP ribosylation domain to translocate without ligand. This can be carried out by two ways either chemical conjugation or genetic fusion. Chemical conjugation is achieved by many ways (a) making an amide linkage between carboxyl group of modified diphtheria toxin and amine group of cytokines (b) or by cross linking using the bi-functional reagents like SDPD, MDS etc., the cross linkers add disulfide moiety into the two proteins to be fused so that a disulfide bond can be introduced, however disulphide conjugated showed little success (Chang et al. 1977) (c) or acid cleavable cross-linking agents is another potent alternative which mimics intact peptide cleavage and liberation of free toxin into cytosol (Neville et al. 1989) (d) or non reducible thioester bond is appropriate if the ligand is attached to that part of the toxin that does not translocate to the cytosol. Drawbacks of chemical conjugation are harsh conditions, batch-to-batch variation, and unwanted by products. Due to limitations of chemical methods focus has shifted to recombinant immunotoxins development where the hybrid gene is produced by the method of gene splicing by overlap extension and ligated to plasmid. After successful cloning the plasmid is transformed E.coli and the protein is harvested and purified. The stratergy

of linking ligand to toxin genetically has not only overcome the drawbacks of chemical conjugation but also offers added advantages like high precision and increased stability. Introducing a linker between toxin and the ligand increased the overall efficacy of the compound by facilitating better receptor-ligand interaction (Urieto et al. 2004 and Liger et al. 1997). This result again varies from cell line to cell line; it depends on length, composition, and location of adapter, fusion point, toxin employed, ligand used etc. (Frankel et al. 2000). Half-life can be extended for a small size molecule by ligating them to ligands with longer half-life in serum this increases stability. Immunotoxins are designed based on selective targeting of molecules expressed on tumour cell surface, which resulted in minimal or no side effects. This strategy exhibited better safety to normal tissue surrounding the tumour. The side effects can be eliminated completely if molecules present exclusively on tumour cell surface are further investigated. This experimental approach could lead to better designing of immunotoxins that can be used in future treatment of cancer with better efficacy and safety to patient.

28.6.4 ADP Ribosylating Toxins

Both PE (613 aa) and DT (535 aa) are single chain proteins with three function domains- binding domain and catalytic domain separated by an internal translocation domain T which contain furin cleavable linker that is an arginine rich loop formed by one disulphide bond.

The use of native intact protein as toxin partner in immunotoxins leads to non-specific binding to normal cells. To promote tumor specificity, the natural cell binding domain of these toxins is usually deleted, mutated or chemically inactivated. PE40 is a truncated PE derivative that has a deletion of domain Ia (1–252 amino acid) (Phan et al. 1993). Another truncated form of PE commonly used in immunotoxins is PE38, composed of amino acids 253–364 and 381–613 of PE and has a deletion of unnecessary residues in domain 1b(365–380aa). An altered carboxyl terminus from the arginine-glutamic acid-aspartic acid-leucine-lysine (REDLK) to lysine-aspartic acid–glutamic acid-leucine (KDEL) sequence in PE allows PE to bind the KDEL receptor with higher affinity and results in increased cytotoxicity (Kreitman 2009; Keppler-Hafkemeyer et al. 2000). The cleavage of furin cleavable linker within the arginine rich loop formed by disulphide loop is a prerequisite for PE and DT intoxication of cells as it enables the separation of domains and translocation to cytosol. The 35 KDa derivative of PE, PE35 is devoid of the furin cleavage site.It is conjugated to the ligand through a disulphide bond and is identical to the translocated fragment of PE38 (amino acids 280–364 and 381–613) with some exceptions(Brinkman et al. 1995). Another derivative of PE, PE38QQR is deprived of (1) domain Ia, (2) a portion of domain Ib, and (3) has three lysine residues at positions 590, 606 and 613 in domain III substituted with two glutamine residues and one arginine residue (Siegall et al. 1989).

Diphtheria toxin has been genetically modified for use in targeted toxin synthesis by either (1) a point mutation in the binding domain, altering a critical Ser525 and Leu 390 each to a phenylalanine leading to binding deficient mutant CRM107 or (2) deletion of the 147-amino acid residue cell-binding domain (DT388 or DAB389) (Kreitman et al. 1993; Kreitman and Pastan 1995; Seetharam et al. 1991). The mechanism of action of DT has been reviewed elsewhere (Potala et al. 2008). As a result of the immunization schedule against DT prevalent in developed countries, anti-DT circulating antibodies are already present in people. The antibody titers are low initially to be able to administer a few doses after which a boosted anti-DT antibody production might limit the treatment of solid tumors. Both DT and PE based immunotoxins are conveniently used to treat hematologic tumors in patients particularly when the patients are too immunosuppressed to be affected by the immunogenicity of the toxin.

28.7 Perspectives of Fusion Toxins

Most of the conventional therapies, currently in use like radiation therapy, surgery are applicable to solid tumors, whereas immunotherapy is applicable to solid tumors, hematological malignancies and metastasis. Immunotherapy causes no damage to DNA whereas chemotherapeutic agents and radiations are mutagens and hence carcinogens. Combination therapy of immunotoxins with chemotherapy or radiation therapy exhibited synergistic effects. In case of chemotherapy and immunotoxins combination, synergistic effect is due to dual targeting of cell i.e. chemotherapy targets cell division and immunotoxins target over expressed molecules on cell surface.

In case of radiation therapy and immunotoxins combination the synergistic effect is due to radiation therapy effecting signal transduction pathway resulting in change of receptor expression levels on tumour cell surface, thus making it more vulnerable to immunotoxins. (Kim et al. 2002). Fusion toxins were primarily designed to target molecules over expressed on tumour cell surface. Based upon the molecules over expressed on tumour cell surface and the ligands used to target such molecules, these fusiontoxins are used in different types of cancer. Response to these fusion-toxins varies from patient to patient and with type of disease, for example, CTCL patients are best responders to DT-IL2 conjugates. Fusion toxins are also used in treatment of psoriasis, rheumatoid arthritis, diabetes mellitus etc. Psoriasis is a chronic inflammatory skin disorder characterized by white scaly patches. T cells are directly involved in the pathogenesis of psoriasis; activated T cells express enough levels of IL2 receptors for targeting with fusion toxins (Frankel et al. 2000). DAB389IL2 showed promising results in rheumatoid arthritis and insulin dependent diabetes mellitus (Gottlieb and Bos 2002; Woodworth 1993). These chimeric molecules also exhibited activity against autoimmune disease pemphigus vulgaris. Immunotoxins are also employed in allogenic transplantation to target donor T cells that mediate immune response (graft versus host disease) (Queen et al. 1989). IL2 alone is administered as immuno-suppressant in autoimmune diseases, graft

rejection and organ transplantation. Conjugated hybrid toxins employ more than one toxin. They act on different substrates by different mechanisms hence are more potent and exhibit additive effect. Diphtheria toxin A chain on IV injection induces temporary renal damage mimicking sub-acute glomerulonephritis (Nakamura et al. 2004). Some of the modifications made in recent decade to native fusion toxins to increase efficacy are listed below:

(a) Mutating critical residues in ligand so that it enhances binding affinity, efficacy of immunotoxins and hence cytotoxicity ex: IL3 (K116W) (Liu et al. 2004a, 2004b).
(b) Receptor up regulation enhances the efficacy of these compounds. The principle behind this approach is efficacy being directly proportional to ligand receptor density. Some of the compounds used in receptor up-regulation are bexarotene, arginine butyrate, phorbol myristate acetate, phytohemagglutinin etc. (Gorgun and Foss 2002; Shao et al. 2002; Conley et al. 1998)
(c) Vitamin A and its analogues play a role in differentiation and proliferation. Rexinoids bind to either RAR (retinoic acid receptor) or RXR (retinoid X receptors) these play an important immunomodulatory role on T cells and B cells. RXR rexinoids like bexarotene can increase the expression of IL2 receptors on cell surface by increasing the expression of both α and β subunits, the exact mechanism by which up regulation is brought about is unknown. Apart from increasing the expression of IL2 receptors it also plays a role in enhancing apoptosis thus, exposing to rexinoids increases the susceptibility to IL2 linked fusion toxins (Gorgun and Foss 2002; Sidell et al. 1997)

The next generation also employed chemical conjugation but the native cell binding domains were deleted, generating much more target specific immunotoxins. The recombinant DNA techniques were applied in the production of third generation immunotoxins to promote tumor specificity and penetration, reduce production complexity and costs. The cell binding domain of the toxin is genetically removed and modified toxin fused with a ligand or with DNA elements encoding the Fv portion of an antibody in these constructs. The light and heavy chain variable fragments are either genetically linked (scFv) (Huston et al. 1988; Bird 1988) or held together by a disulfide bond (dsFv) (Reiter 1994). Compared with the single-chain toxins(scFv), disulfide-stabilized Fv (dsFv) molecules did not aggregate, were stable and also overcame the major obstacle of poor penetration into bulky tumor masses, These targeted toxins offered the problems of immunogenicity, non specific toxicity and instability (Reiter 1994).

28.8 Production and Clinical Testing of Immunotoxins

Immunotoxins are produced in bacterial or yeast expression systems. A variety of antibodies targeting specific antigens have been used to make recombinant immunotoxins. Design strategy for an immunotoxin include: confirmation of the type and

stage at which cancer cells will be the best targets, analysis of the tumor specificity of the antigen that is targeted by the recombinant antibody as well as the affinity of immunotoxins for the cell type and the ability of the immunotoxins to penetrate and enter into normal tissues and tumor cells. (Potala et al. 2008; Mrudula and Verma 2009). The stability, immunogenicity and side effects of immunotoxins should be monitored during pilot studies. Anti-immunotoxin antibodies can reduce the effectiveness of immunotoxins by accelerating their clearance from the circulation or by blocking the functional domains of the targeting module

28.9 Conclusions

Immunotoxins have shown promise in vivo and in vitro model. Till date only one agent, which contains human IL-2 and truncated diphtheria toxin, is approved for use in cutaneous T cell lymphoma. The biggest problem of these immunotoxin is their half-lives. Half-lives of immunotoxins may be too limited or short for diffusion to occur into solid tumors masses and thus hinders in the development of new and efficient drugs. Strategies should be devised to circumvent this problem which may include a immunotoxin with better pharmacokinetic property and this may be achieved by reducing the size of the immunotoxins or by using the nanotechnology. The antigen and disease targeted remains the major determinants of immunotoxins efficacy and resistance. Exciting success in future development of immunotoxins can be anticipated as combination of disease and antigen targets.

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Chapter 29
Application of Environmental DNA Resources to Create Useful DNA Polymerases with Different Properties

Sonoko Ishino and Yoshizumi Ishino

Abstract DNA polymerases use deoxynucleotide triphosphates to synthesize new DNA strands according to the template DNA during DNA replication and repair, and are essential to maintain genome integrity in DNA metabolism. In addition, these enzymes are widely used for genetic engineering techniques, including dideoxy-sequencing, PCR, DNA labeling, mutagenesis, and other *in vitro* gene manipulations. Thermostable DNA polymerases are especially useful for PCR and cycle-sequencing. We describe in this chapter a powerful strategy to use environmental DNA as a genetic resource to create useful DNA polymerases. The region corresponding to the active center of the DNA polymerizing reaction in the structural gene of well known DNA polymerases, such as Pfu DNA polymerase and Taq DNA polymerase, can be substituted with gene fragments amplified by PCR from DNAs within soil samples from various world-wide locations. The constructed chimeric *pol* genes can be expressed in *E. coli,* and the produced chimeric enzymes, possessing DNA polymerase activities with different properties, can be evaluated in terms of their processivity, fidelity, and efficiency of primer usage, to select valuable DNA polymerases for genetic engineering techniques.

Keywords Chimeric DNA polymerase • Protein engineering • Metagenomic DNA • Genetic engineering • PCR enzyme

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29.1 Introduction

DNA polymerase synthesizes a DNA strand based on the template DNA, using mononucleotide triphosphates (dNTPs) as substrates. The enzyme actually catalyzes phosphodiester bond formation between the terminal 3′-OH of the primer and the α-phosphate of the incoming triphosphate. Multiple DNA polymerases have been identified in each living organism, and these enzymes are thought to share the cellular functions in DNA transactions (DNA replication and repair processes) and maintaining genome integrity (Fig. 29.1). The DNA polymerases have been classified by amino acid sequence similarity, and seven families, A, B, C, D, E, X, and Y, are now widely recognized (Braithwaite and Ito 1993; Cann and Ishino 1999; Lipps et al. 2003; Ohmori et al. 2001). For example, *Escherichia coli* has five DNA polymerases, and Pol I, Pol II, and Pol III belong to families A, B, and C, respectively. Pol IV and PolV are classified in family Y, as the translesion DNA polymerase family. In eukaryotes, the replicative DNA polymerases, Pol α, Pol δ, and Pol ε, belong to family B, and the translesion DNA polymerases, η, ι, and κ, belong to family Y. The most studied enzyme in family X is Pol β, a gap-filling polymerase for DNA repair processes. The distribution of DNA polymerases in Archaea, the third domain of life, is interesting. Members of the Crenarchaeota, a subdomain of Archaea, have at least two family B DNA polymerases (Uemori et al. 1995; Cann et al. 1999). On the other hand, there is only one family B DNA polymerase in the Euryarchaeota, the other subdomain of the Archaea. Instead, a family D DNA polymerase, which is specific for euryarchaeota and has been never found in any other

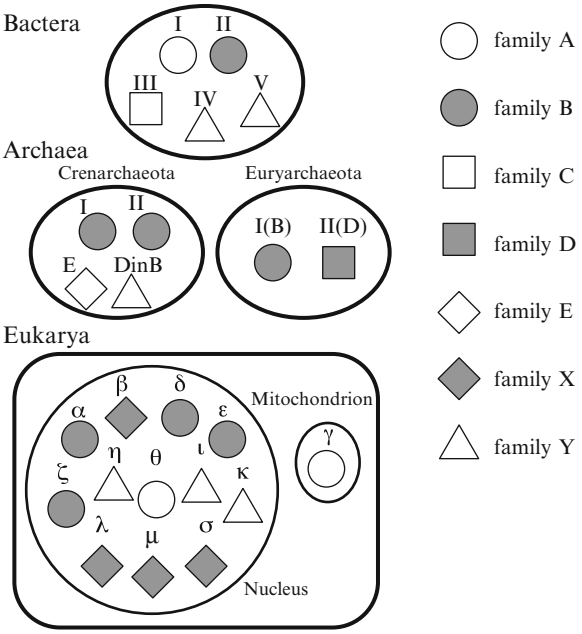

Fig. 29.1 Distribution of DNA polymerases from seven families in the three biological domains

living organisms, has been identified (Cann et al. 1998; Cann and Ishino 1999). The biochemical properties of PolB and PolD from *Pyrococcus furiosus* have been characterized (Ishino and Ishino 2001). The roles of each DNA polymerase in the archaeal cells are unknown (Ishino and Ishino 2006), although the archaeal family B DNA polymerases are useful models to understand the structure-function relationships of the eukaryotic replicative DNA polymerases (Perler et al. 1996). Many researchers are working to elucidate the physiology and enzymology of DNA polymerases.

In addition to its fundamental role in maintaining genome integrity during replication and repair (Loeb and Monnat 2008), DNA polymerase is widely used for genetic engineering techniques, including DNA cloning, dideoxy-sequencing, DNA labeling, mutagenesis, and other *in vitro* DNA manipulations. Thermostable DNA polymerases are particularly useful for PCR and cycle-sequencing. To develop DNA polymerases suitable for PCR, the enzyme's velocity, fidelity, and efficiency of primer usage for *in vitro* DNA strand synthesis are the critical factors to evaluate, and faster DNA polymerases, with better accuracy and higher efficiency, in addition to heat stability, are desired for practical usage.

Several strategies are available to obtain DNA polymerases with superior properties, in terms of the above-described characteristics, for *in vitro* DNA strand synthesis, including sequencing and PCR. Screening for a suitable DNA polymerase activity from known organisms, which can be cultured, is the most conventional way to discover useful enzymes. In this case, sufficient amounts of the cells must be cultured to prepare the extracts for biochemical analyses of the DNA polymerase activity. Alternatively, known DNA polymerases can be converted into artificial enzymes with different properties from those of the wild type enzymes, by site-specific or random mutation techniques (reviewed in Holmberg et al. 2005). The creation of DNA polymerases with novel activities by experimental artificial evolution procedures has attracted a great deal of attention (Holmberg et al. 2005; Henry and Romesberg 2005; Brakmann 2005; Ong et al. 2006). These studies have contributed to the elucidation of the detailed structure-function relationships of DNA polymerases, and have generated novel enzymes with different substrate specificities, stabilities, and activities from those of the naturally evolved counterparts.

In this chapter, we describe a useful strategy that combines the advantageous points of the above procedures. The experimental method to create artificial DNA polymerases efficiently, using environmental DNA containing various unidentified genome DNAs as useful genetic resources, includes (1) metagenomic DNA preparation from soil samples, (2) amplification of the DNA polymerase gene fragment from the metagenomic DNAs by PCR, without cultivation of the organisms, and (3) construction of chimeric *pol* genes by substituting part of the known *pol* gene with the amplified gene fragments. This experimental strategy includes both natural evolution and artificial protein engineering, and therefore has the advantageous potential to create novel DNA polymerases. This strategy also generates valuable experimental data about the structure-function relationships of the DNA polymerases, to clarify the detailed molecular characteristics of the enzymes.

29.2 Techniques to Create Chimeric DNA Polymerases

The common three-dimensional core structure of polynucleotide polymerases resembles the human right hand and consists of three distinct domains, palm, finger, and thumb (reviewed in Rothwell and Waksman 2005). The palm domain has the catalytic site of the phosphoryl transfer reaction. The finger domain is important for incoming nucleotide recognition and binding. The thumb is involved in binding the DNA strand. A comparison of the *pol* gene-encoding amino acid sequences, including not only DNA polymerase, but also RNA polymerase, reverse transcriptase, and RNA-dependent RNA polymerase, revealed three regions with highly conserved amino acid sequences, named motifs A, B, and C (reviewed in Perler et al. 1996). Motifs A and C possess catalytically essential carboxylates, and are located within the palm domain. Motif B is located in the finger domain, and is thought to bind the template strand and dNTPs. Based on these structural features, it would be possible to create degenerate primers based on the conserved amino acid sequences in motifs A and C for PCR amplification of the important region for nucleotide polymerization.

In DNA polymerases, the fundamental ability to synthesize a deoxyribonucleotide chain is conserved in relation to the structural conservation, as described above. However, the more specific properties, including processivity, synthesis accuracy, and substrate nucleotide selectivity, differ among the enzymes. The enzymes within the same family have basically similar properties. In terms of DNA polymerases as a genetic engineering reagent, only the family A enzymes are commercially available for dideoxy-sequencing, and the family A and B enzymes are practically used for PCR. None of the DNA polymerases from the other families are commercially available, because their properties are not suitable for general use in genetic engineering experiments. The main difference between the family A and family B DNA polymerases is the absence or presence of a 3′–5′exonuclease activity, which contributes to the proofreading of DNA strand synthesis. The 3′–5′ exonuclease activity is generally associated with the family B enzymes, but not with the family A enzymes, although some family A enzymes exceptionally have a weak 3′–5′ exonuclease activity (Joyce and Steitz 1994; Villbrandt et al. 2000). Based on these differences, family A is advantageous for efficient amplification of a long DNA region, and family B is generally more suitable for the precise amplification of a shorter region by PCR (Eckert and Kunkel 1991).

We focused on DNA polymerases belonging to families A and B to create useful enzymes for genetic engineering reagents. Several procedures are applicable to obtain DNA polymerases with superior properties for *in vitro* genetic engineering experiments. Screening for a suitable DNA polymerase activity from known organisms, which can be cultured, is the most conventional way to discover useful enzymes from natural resources. However, sufficient amounts of the cells must be cultured to prepare the extracts for biochemical analyses of the DNA polymerase activity (Imamura et al. 1995). It is generally not easy to find the optimal cultivation conditions for each unidentified organism. Alternatively, known DNA polymerases

can be converted into artificial enzymes, with different properties from those of the wild type enzymes, by site-specific or random mutation techniques, including direct evolutional methods (Holmberg et al. 2005). Site-specific mutagenesis is now experimentally practical; however, rational design to choose the sites where the mutations should be introduced in the target polymerase genes is necessary to obtain useful enzymes. The type of mutation, substitution, deletion, or insertion, the amino acid residues to be substituted, and the lengths of deletions or insertions also have to be considered carefully.

Over 99% of the microbial organisms in this planet have not been identified (Amann et al. 1995), and therefore, huge amounts of useful genetic resources are available in our environment. Metagenomic analysis is a revolutionary technique for microbiological ecology (Kennedy et al. 2010) and biocatalytic applications (Fernandez-Arrojo et al. 2010). Amplification of a target gene from unidentified DNA (genomic DNA, plasmid, and phage DNA) is a very powerful method to obtain new proteins. In order to create novel DNA polymerases by protein engineering, we developed a strategy to use environmental DNA samples containing various unidentified genome DNAs as genetic resources.

When we attempted to amplify part of the *pol* gene from microorganisms in the early 1990s, we looked at a bacterial Pol I multiple sequence alignment and decided to use two conserved regions, motifs A and C, to create degenerate primers. We designed degenerate primers for PCR based on the motif sequences DPNLQNIP and QVHDE(L/I)(V/L), to amplify the genes encoding the palm region of DNA polymerase from various bacteria (Fig. 29.2). Using these primers, we successfully amplified the target regions of the family A *pol* genes from the bacterial DNAs that we had available, including *E. coli, Bacillus subtilis, B. caldotenax, B. caldolyticus, Lactobacillus bulgaricus, L. homohiochii,* and *L. heterohiochii,* in addition to *Thermus aquaticus* and *T. thermophilus* (Uemori et al. 1993a). We also applied this strategy to the amplification of family B DNA polymerases. The degenerate primers were based on the sequences SLYPSII in motif A and VIYGDTD in motif C, as shown in Fig. 29.2 (Uemori et al. 1995). These experiments revealed for the first time that one archaeal organism has two different family B DNA polymerases in the cell, when we successfully amplified part of the *polB* gene from *Pyrodictium occultum* (Uemori et al. 1995). It was exciting to find plural family B DNA polymerases in one archaeal cell, since this was similar to the situation in eukaryotic cells, which possess three family B DNA polymerases (Pol α, Pol δ, and Pol ε). These results indicated that our degenerate primers actually worked for PCR to amplify the target regions. Based on these experiences, we tried the following experimental procedure, including (1) metagenomic DNA preparation from soil samples, (2) DNA polymerase gene fragment amplification from the metagenomic DNAs by PCR, without cultivation of the organisms, and (3) construction of chimeric *pol* genes, by substituting part of the existing *pol* gene with the amplified gene fragments. These experimental procedures are schematically depicted in Fig. 29.3.

To isolate DNA from thermophilic organisms, environmental specimens from hot spring areas are valuable, and we obtained samples (soil-mixed water) from representative areas in Japan, including Onikobe (Miyagi prefecture), Hachimantai

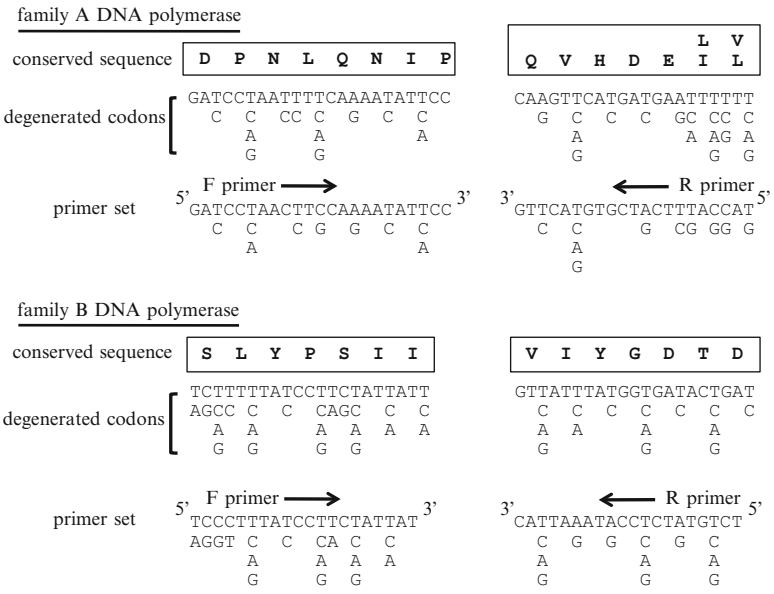

Fig. 29.2 Design of PCR primers based on the conserved sequence motifs in the DNA polymerases. The PCR primers are degenerate deoxyoligonucleotides synthesized on the basis of the universal genetic codon table

(Iwate prefecture), Kirishima (Kagoshima prefecture), Ibusuki (Kagoshima prefecture), Beppu (Oita prefecture), and Nasu (Tochigi prefecture). As another interesting place to obtain environmental specimens, we chose a coral shelf in Okinawa, because the topography of coral shelves provides diverse environments suitable for various organisms. The DNA was extracted from the environmental specimens by using the conventional phenol extraction method. Commercially available products (DNA isolation kits), such as the UltraClean Soil DNA extraction Kit (MoBio), also work quite well when used as directed. The extracted DNAs can be detected by agarose gel electrophoresis and quantified by spectrophotometrical measurement.

The methods for the construction and expression of the chimeric DNA polymerases have been described in detail (Matsukawa et al. 2009). Once the expression plasmid is constructed for a DNA polymerase, a restriction cleavage site is introduced by site-directed mutagenesis at the site where the substitution with the metagenome-derived gene fragment is desired within the *pol* structural gene. The substitution is then performed on the expression plasmid directly. For example, the expression plasmid pPF101, containing the entire structural gene encoding *P. furiosus* PolB from pET21d, was used as the basis, and site-specific mutagenesis was performed by using a QuikChange™ (Stratagene) kit to introduce a *Sac*I restriction site (GAGCTC) into the position corresponding to the 5′-cleavage site for the gene substitution (Matsukawa et al. 2009).

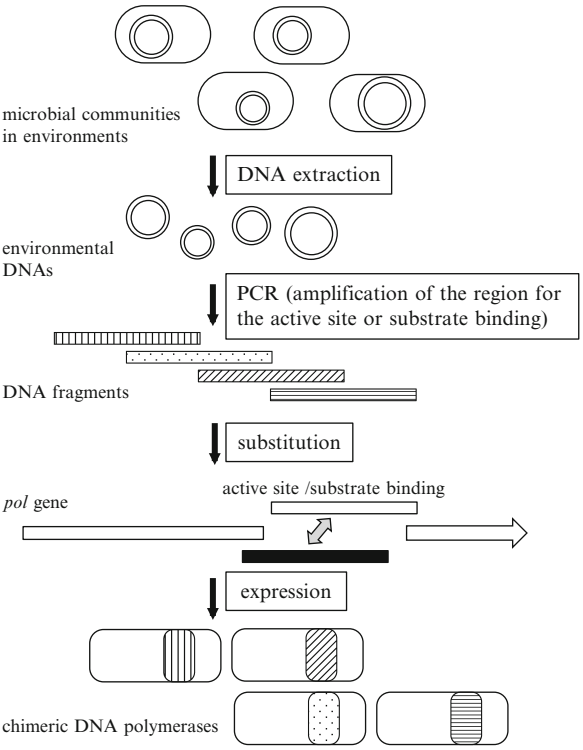

Fig. 29.3 Schematic drawing of the experimental procedure presented in this chapter to create novel DNA polymerases. Environmental DNAs isolated from various places, including hot spring areas, are used for PCR amplification of the region that is directly involved in the activity of the DNA polymerases. Each isolated gene fragment is then used to substitute for the corresponding region of the wild type *pol* gene on the expression plasmid. The resultant chimeric genes are expressed in *E. coli,* and the purified DNA polymerases are subjected to evaluations of various biochemical properties

29.3 Critical Review of Protein Engineering Studies for DNA Polymerase

29.3.1 Amplification of the pol Gene Fragments

Cloning the entire regions of the DNA polymerase genes from the unidentified genomic DNAs in the environmental specimens is not practical, because DNA polymerase is around 800 amino acids long and its gene is about 2,500 nucleotides long, which is too long to avoid degradation during the preparation after sample isolation. One practical method to utilize the environmental DNA resources

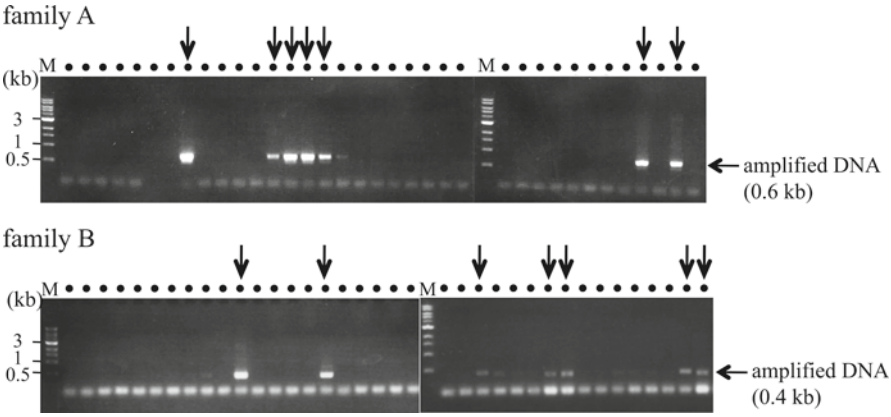

Fig. 29.4 Experimental examples showing the amplification of gene fragments from environmental DNAs. PCR was performed using the environmental DNAs and the degenerate primers shown in Fig. 29.2. The reaction mixtures were fractionated by 1% agarose gel electrophoresis, and the DNA was stained with ethidium bromide. Each lane shows a PCR reaction of environmental DNA from a different place. The expected sizes of DNA fragments, about 600 and 400 nucleotides long for the family A and family B DNA polymerase genes, respectively, were detected. The samples indicated with arrows provide a positive DNA band corresponding the *pol* gene fragment

efficiently is to use a short region of the *pol* gene as the material to replace the corresponding region in previously characterized DNA polymerase genes. The degenerate primers that we designed to amplify part of the *pol* genes by PCR from genomic DNA actually worked, as described above. Therefore, we used them to amplify the gene fragments from the environmental DNAs isolated from the soil of several hot spring locations, including Kirishima, Ibusuki, Beppu, Onikobe, Hachimantai, and Nasu, and a coral shelf of Okinawa, in Japan. As shown in Fig. 29.4, 600 bp and 400 bp gene fragments were specifically amplified from several samples by using primer sets for family A and family B DNA polymerases, respectively. We chose 40 and 42 samples, which provided clearly amplified bands from a total of 384 different metagenomic samples obtained from the places described above. These locations had various environmental conditions, including pH values of 1 ~ 7 and temperatures of 70–100°C, and thus highly diverse genetic resources were expected. The efficiency of DNA isolation may vary, and it is possible that a sufficient amount of DNA was not present in some samples. The amplified DNA fragments were excised from the gel and cloned into a plasmid vector. Twenty colonies were picked independently from each cloning experiment, and in total, 800 (20 × 40) and 840 (20 × 42) plasmids were isolated. These plasmids were subjected to sequencing analysis to confirm that the cloned DNAs actually encoded homologous amino acid sequences to the family A and family B DNA polymerases.

29.3.2 Environmental DNAs Contain DNA Polymerase Genes with Various Sequences

From our experiments described above, 189 and 172 different sequences, which exhibited conservation with known family A and family B DNA polymerases, respectively, were obtained, after deduction of the known sequences and the same sequences. These sequences have not been registered in the public databases yet, indicating that many unidentified organisms are actually present within the soil samples. The samples from the coral shelf in Okinawa had a greater variety of organisms, as compared with those from the hot springs. The detailed experimental data for the family B DNA polymerases are described in Matsukawa et al. (2009), and the data for the family A DNA polymerases will be published in the near future (Yamagami et al., unpublished).

These sequences were compared with the known sequences in the databases. In the case of the family A DNA polymerases, all of the sequences seemed to be derived from bacteria. On the other hand, the family B sequences were more diverse, and 111, 11, 49, and 1 sequences were most similar to the family B DNA polymerases from Archaea, Eukarya, Bacteria, and viruses, respectively. The sequences similar to those of archaeal DNA polymerases were from the hot spring soils in several locations, and those similar to the eukaryotic, bacterial and viral enzymes were derived from the coral shelf in Okinawa.

29.3.3 Construction of the Chimeric DNA Polymerases

Taq DNA polymerase (from *Thermus aquaticus*) and Pfu DNA polymerase (*Pyrococcus furiosus*) are representative family A and family B DNA polymerases that are used world-wide for PCR. Using these DNA polymerases as the basic enzymes, chimeric DNA polymerases were created by substituting the region surrounding the active center with the metagenome-coding sequences. We designed an experimental procedure, in which part of the Taq or Pfu DNA polymerase gene was substituted by the amplified gene fragments from the unidentified genomic DNA. The plasmids for overexpression of Taq DNA polymerase (Ishino et al. 1994b) and Pfu DNA polymerase (Uemori et al. 1993b; Komori and Ishino 2000) were used for the expression of the chimeric DNA polymerase genes. To construct a system for the gene substitution, restriction sites were needed at appropriate sites in their structural genes, and therefore, silent mutations were introduced to create recognition sites to remove the substitution region. PCR primers containing the recognition sequences for the same restriction enzymes were synthesized, so that the PCR-amplified DNA fragment could be used for substitution of the structural genes by digestion with these restriction enzymes, to construct the chimeric genes directly on the Taq or Pfu DNA polymerase expression plasmid (Fig. 29.5). The reconstructed plasmids were introduced into *E. coli,* and the chimeric genes were expressed under

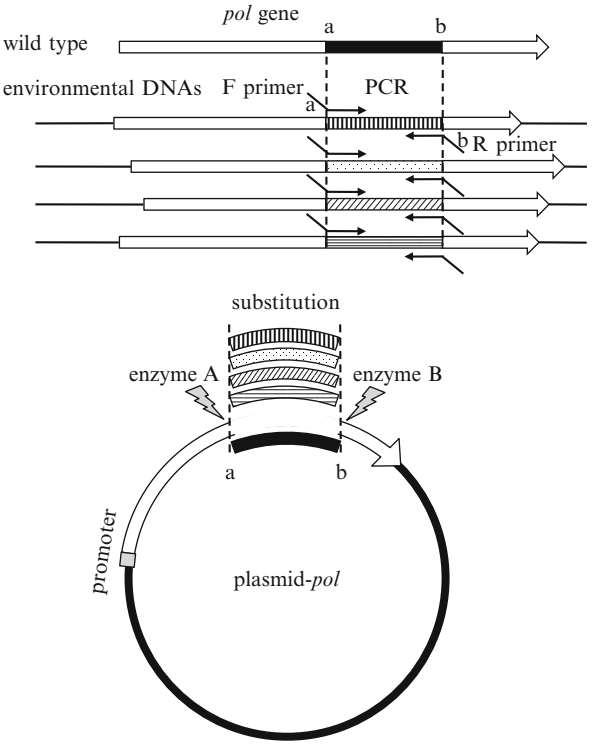

Fig. 29.5 Construction of the chimeric *pol* gene on the expression plasmid. The overexpression plasmids containing the wild type *pol* genes (for Taq or Pfu DNA polymerase) were used to replace the region containing the active site with the gene fragments that were PCR-amplified from the environmental DNAs. The sequences recognized by appropriate restriction endonucleases are indicated by *a* and *b*. The resultant plasmids can be directly introduced into the *E. coli* host strain

the sameconditions as those for the wild type Taq and Pfu DNA polymerases. In addition, the same purification procedures were used for the wild type and chimeric DNA polymerases.

29.3.4 The Chimeric DNA Polymerases Have Different Characteristics

The world-wide standard assay for DNA polymerase is to measure the incorporation of [methyl-^{3}H]TTP into the acid insoluble materials (DNA strands). The standard reaction is a 20 μl solution, containing 20 mM Tris-HCl, pH 8.8, 1.2 mM $MgSO_4$, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% (w/v) Triton X-100, 0.1 mg/ml BSA,

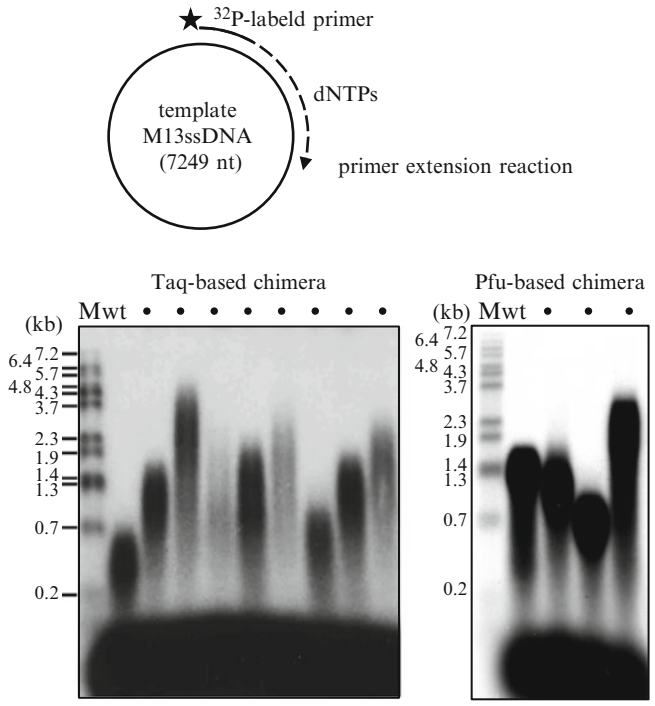

Fig. 29.6 Evaluation of the primer extension ability of the DNA polymerases. The primer extension abilities of the chimeric DNA polymerases can be compared, using M13 single-stranded circular DNA annealed with a ^{32}P-labeled oligonucleotide as the template-primer. The reaction mixtures were analyzed by 1% alkaline agarose gel electrophoresis (50 mM NaOH, 1 mM EDTA), and the products were visualized by autoradiography. The sizes indicated on the left are from BstPI-digested λ phage DNA, labeled with ^{32}P at each 5′ end

4 μg activated calf-thymus DNA, 0.2 mM dNTP, and 400 nM [methyl-^{3}H]TTP, as described previously (Uemori et al. 1995). One unit of activity is defined as the amount of enzyme catalyzing the incorporation of 10 nmol of TMP into DNA per 30 min at 75°C. Each enzymatic property was assayed in terms of the units of activity. The *in vitro* primer extension velocity is one of the important factors for DNA polymerase to be applied to *in vitro* DNA manipulation. This ability can be compared by an assay system using M13 single-stranded DNA annealed with a 5′-labeled short primer. The size of the DNA synthesized by DNA polymerase can be detected by imaging, after alkaline agarose gel electrophoresis. Taq-based and Pfu-based chimeric DNA polymerases were subjected to this experiment, and their DNA synthesis velocities were compared with those of the wild type Taq and Pfu DNA polymerases, respectively. As shown in Fig. 29.6, many Taq-based chimera enzymes that are faster than wild type Taq DNA polymerase were obtained. In the case of the Pfu-based chimeras, three DNA polymerases are shown as representatives of faster, equal, and slower enzymes, as compared with the wild type Pfu DNA polymerase.

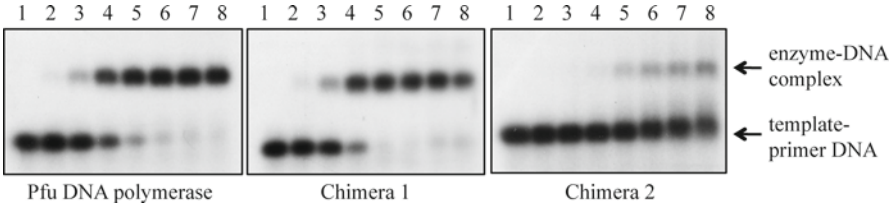

Fig. 29.7 Evaluation of the DNA binding ability of DNA polymerases. As the template-primer DNA, a 49 mer deoxyoligonucleotide was annealed with a ^{32}P-labeled 27 mer. Various amounts of the DNA polymerase proteins were reacted with the DNA (3 nM) at 40°C for 10 min, and the protein-DNA complexes were fractionated by 1% agarose gel electrophoresis in 0.1x TAE buffer. The DNA bands were visualized by autoradiography. Lanes 2 ~ 8 contain 0.4, 1, 2.5, 5, 10, 20, and 40 nM enzyme, respectively. Lane 1 has no protein

The DNA binding affinity is directly related to the DNA synthesis velocity and efficiency. This ability can be compared with a gel-retardation assay, using a labeled primed-DNA as a probe. Examples are shown in Fig. 29.7, in which a 49 mer deoxynucleotide was annealed with a ^{32}P-labeled 27 mer, to mimic a template primer. In our experience, the strength of the DNA binding ability correlates well with the DNA synthesis velocity, and the processivity is probably directly affected by DNA binding.

Evaluation of the fidelity of the DNA polymerase for DNA synthesis is another important factor. Many reports showed the differences in the fidelities among various DNA polymerases at the beginning of the PCR era (Lundberg et al. 1991: Ling et al. 1991: Cariello et al. 1991: Mattila et al. 1991). It is clear that the family B DNA polymerases possessing the 3′–5′ exonuclease activity are more accurate than the family A DNA polymerases without the exonuclease. However, it is not easy to compare the fidelity precisely, within the same family. Statistical analyses of large amounts of data obtained from a properly designed experiment are required to compare and discuss this issue. One of the well-designed experiments calculated the synthesis error in the *lacZ* (structural gene of β-galactosidase) gene by discriminating the mutations with color selection of the *E. coli* colonies on the agarose plate, after introducing a plasmid in which the *lacZ* gene is synthesized *in vitro* by the DNA polymerase sample (Kunkel and Soni 1988). However, silent mutations are neglected in this method, and the results are not precisely correct. Recent developments in DNA sequencing technology have provided a direct evaluation of the fidelity by sequencing all of the products of *in vitro* synthesized DNA. In this chapter, we described the chimeric DNA polymerases in the region containing the active site of DNA polymerizing activity, and the exonuclease domain itself is not changed from the wild type Pfu or Taq DNA polymerase. Therefore, the fidelity of the chimeric DNA polymerases is not expected to change drastically. However, the exonuclease and polymerizing domains are known to interact directly (Komori and Ishino 2000), and an ingenious mechanism seems to control the two domains during DNA strand synthesis

(Reha-Krantz 2010). Useful data will be obtained by measuring the fidelities of chimeric enzymes substituted at the polymerizing region described here. It would also be interesting to apply this strategy to create chimeric enzymes in the exonuclease domain, in which clearly conserved motifs, exo I, II, and III, are important for the expression of the exonuclease activity (Ishino et al. 1994a), and therefore, these sequences may be used to design PCR primers.

Heat stability is essential for the use of DNA polymerases in PCR and cycle sequencing, and therefore, this characteristic should be critically evaluated. The residual activities after incubations at various temperatures for 30 min are compared with the initial activity (without heat-incubation), in the standard evaluation. In our experience, the chimeric enzymes are often less stable than the wild type Taq and Pfu DNA polymerases, although the reason has not been identified. The microorganisms that provided the genetic resources for the construction of the chimeric genes may prefer lower growth temperatures, as compared to those favored by *T. aquaticus* and *P. furiosus.*

29.4 Future Perspectives

DNA polymerase is one of the most useful enzymes for *in vitro* DNA manipulation, and therefore, it is commercially valuable to produce novel DNA polymerases with superior properties to any known enzymes. The strategy to make chimeric DNA polymerases using environmental DNAs directly without isolating organisms, as described in this chapter, is practical to create DNA polymerases with properties different from those of the parental (wild type) enzymes, and we have already identified useful DNA polymerases derived from the Taq and Pfu DNA polymerases. This strategy is applicable to any other family A and family B DNA polymerases, and a huge number of unidentified organisms that can provide the genetic resources exist in this planet. It is possible that an unlimited number of chimeric DNA polymerases can be produced by this procedure. The methods used to evaluate the properties of the novel DNA polymerases are important, depending on which ability is focused on. For example, the samples should be evaluated in terms of synthesis speed, product length, primer usage efficiency, accuracy, sensitivity to various inhibitors, tolerance of contaminants in the sample, and specificity to the substrate nucleotides. The construction of a high-throughput evaluation system for each property will be helpful to identify competent DNA polymerases as commercial products.

DNA polymerase is a very important enzyme for fundamental living phenomena (DNA replication/repair) in living cells, as well as for genetic engineering applications *in vitro*. Therefore, many structural and functional investigations of DNA polymerase have been performed. In this study, we developed a method to obtain experimental data to investigate efficiently how the properties of a DNA polymerase are related to its structure. Metagenomic analysis is a revolutionary technique for microbiological ecology. Amplification of the target gene from the unidentified

genomic DNA is a very powerful method to investigate many different DNA polymerases from uncultivated microbes. Our strategy involved substituting part of the engine region of known DNA polymerases with the corresponding region derived from environmental DNAs.

In this chapter, we focused on thermophilic organisms as useful genetic resources for obtaining new, thermostable DNA polymerases, and obtained soil samples mainly from hot spring areas in Japan. In addition, we used DNAs from a coral shelf in Okinawa, as a potentially interesting place including a variety of different organisms. Actually, many mesophilic bacterial sequences, encoding both family A and family B DNA polymerases, were obtained from the coral shelf, as we expected. These results suggest that our method to amplify part of the DNA polymerase genes would be applicable to the analysis of microbial populations in any habitats. It is noteworthy that the family B DNA polymerases in Bacteria have not been well studied yet, since we found that *E. coli* DNA polymerase II belongs to family B, by determining the nucleotide sequence of the *polB* gene (Iwasaki et al. 1991) and finding that its activity is inhibited by aphidicolin, a specific inhibitor for family B DNA polymerases (Ishino et al. 1992). The comparison of the amino acid sequences deduced from the amplified DNA from Okinawa revealed that the bacterial PolBs are shorter (by about 15 amino acids) than the archaeal and eukaryotic enzymes in this family, and this sequence feature may be useful as a signature for the bacterial family B DNA polymerases, to discriminate them from the archaeal enzymes.

The experimental strategy described in this chapter is clearly different from the artificial random mutagenesis of the same region (between motifs A and C). The introduced sequences that we manipulated originated from genes that naturally evolved, and therefore, unreasonable mutations should be eliminated from the substitution experiments. We expect that many DNA polymerases with novel properties, which are different from those created by other methods, will be obtained by the procedure presented here.

As an example of more detailed analyses of structure-function relationships, it is possible to elucidate the structural determinants conferring high processivity, high accuracy, and precise discrimination of the substrate nucleotide by comparing the experimental data with the amino acid sequences. We have already produced DNA polymerases with better processivity than Taq DNA polymerase (Yamagami et al., unpublished). We also successfully converted the substrate specificity of Taq DNA polymerase by this procedure (Matsukawa et al., unpublished). The accumulated experimental data about the chimeric DNA polymerases will become a valuable database for the design of DNA polymerases with specific properties.

29.5 Conclusions

This chapter described a powerful strategy to produce novel DNA polymerases by using environmental DNAs. The regions corresponding to the active center of the DNA polymerizing reaction in the genes encoding Taq DNA polymerase and Pfu

DNA polymerase can be substituted by PCR fragments amplified from DNAs within environmental samples from various locations. The DNA polymerases produced in *E. coli,* by expression of the chimeric genes thus produced, revealed DNA polymerase activities with various properties that differed from those of the parental Taq and Pfu DNA polymerases. For this protein engineering strategy, environmental DNAs are especially valuable genetic resources.

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Chapter 30
Utilization of Tropical Fruits for Wine Production with Special Emphasis on Mango (*Mangifera indica* L.) Wine

L.V.A. Reddy, V.K. Joshi, and O.V. S. Reddy

Abstract Tropical fruits have their origin in the tropics and require rather a tropical or subtropical climate; and do not tolerate frost. The tropical zone stretches from 23.4° North latitude to 23.4° South latitude. There are hundreds of edible tropical fruits some of which have very high export potential all over the world. Most of the tropical fruits are important sources of antioxidants, vitamins and minerals; and form a very healthy part of a diet. India is the largest producer of fruits in the world with an annual production of 46 million tons, amounting to 10% of total world production. Nearly 20–30% of the produce is generally spoiled at post-harvest stages leading to losses. There is a need to preserve and utilize or transform the surplus and unutilized fruits to valuable products like wine and other processed foods. Wines form many tropical fruits like guava, banana, custard apple, litchi, pineapple, pomegranate, mango, ber and melon have been prepared and evaluated for their acceptability.

Mango (*Mangifera indica* L) is the most popular and the choicest fruit of India. A major portion (nearly 60–70%) of the total quantity produced is locally consumed and a sizable portion is exported to other countries. Ten varieties of mango, which are abundantly available in the region, were selected for wine production and the conditions for juice extraction were optimized. To prepare wine, the fruits were peeled and juice (must) was extracted immediately after crushing (control) and also after 10 h of pectinase treatment. The *Raspuri* gave the highest juice yield (600)

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followed by *Banganpalli* (570 ml/kg). The sugar content of must ranged from 15% to 18% (w/v). The recovered juice was fermented at 15°C and 20°C and the ethanol concentration of mango wine ranged from 6.3% to 8.5%. Fermentation efficacy of three yeast strains, viz. *Saccharomyces cerevisiae* CFTRI 101, Palm wine isolate and Baker's yeast was determined and highest score was obtained for *Banganpalli* wines with yeast strain *S. cerevisiae* CFTRI 101 followed by *Alphonso* and *Totapuri*. Pectinase enzyme treatment increased the yield of juice and ethanol production also. Optimization of fermentation conditions (like yeast strain, pectinase enzyme, pH and temperature) was carried out using response surface methodology (RSM). Production profiles of higher alcohols and other volatile compounds during wine fermentation were investigated. Total volatile composition of mango wine was determined using GC-MS and identified 33 compounds having fruity aroma characters. More volatiles were observed in wine produced from *Banganpalli* (343) than wine from *Totapuri* cultivar (320 mg/l). Polyphenols and carotenoids profiles were determined using HPLC, and antioxidant activity was evaluated using *in vitro* models. From physicochemical characteristics and sensory characteristics it is concluded that table wine of acceptable qualities can be prepared from mango. Further, the analyses showed that the aroma of mango wine is complex and is composed of a number of compounds, similar to grape wine.

Keywords Tropical fruits • Wine production • Fermentation conditions • Mango • Volatile compounds • *Saccharomyces cerevisiae* • Antioxidant activity

30.1 Introduction

The tropics are generally defined as the regions of the globe that lie between the Tropic of Cancer and the Tropic of Capricorn, and the environmental conditions there are quite unique, creating a habitat for incredibly diverse animals and plants. Tropical zones on earth are areas where the sun is at head top once a year and only have two seasons namely wet and dry. The tropics are warm year-round, and they are also very humid, with some areas receiving lots of rain every year. Tropical plants and their fruits have adapted to this climate. Many tropical fruits are large, brightly colored, and very flavorful so that they appeal to the animals they relay on to distribute their seeds. Tropical fruits have been used by humans for centuries, and certain fruits are in high demand all over the world. They are cultivated mostly in countries with warm climates and the only character that they share in common is frost intolerance.

Some tropical fruits are well known all over the world. Pomegranates, mangoes, papayas, avocados (alligator pears), bananas, pineapples, guavas, star fruits (or carambolas), kiwis, dates, and passion fruits are some examples of well-known tropical fruits. Production of different fruits in India is shown in Fig. 30.1. In fact, the bananas are one of the highest selling fruits around the world. They can be grown, harvested, and transported easily. Many of these fruits are available in big markets

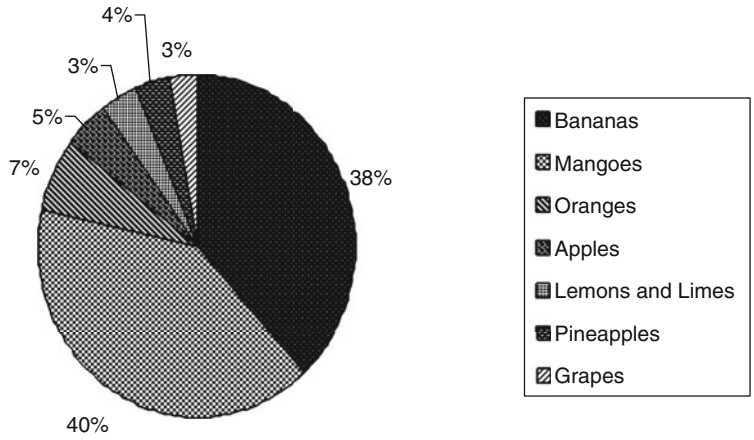

Fig. 30.1 Production of different fruits in India (Source: FAOSTAT 2007)

year-round. Other tropical fruit cultivars are more obscure. While they may be popular in specific regions of the world, they are not familiar to people outside of these areas, and some of them definitely possess an acquired taste. Some more obscure examples of tropical fruit include: soursops, cherimoyas, sugar apples, jackfruit, pawpaws, durian, acerolas, mamey, akee, breadfruit, lychees, rambutans, and mangosteens. Some of these fruits, like jackfruit and durian, are infamous for their strong odor and flavor, while others like mangosteens, lychees, and cherimoyas are quite simply delicious, but difficult to cultivate.

Wine is a popular drink being enjoyed all over the world. Historians believe that wine had been made in Caucasus and Mesopotamia as early as 6000 BC. Records to wine have been found in Egypt and Phoenicia dating as far back as 5000 BC and by 2000 BC. It was being produced in Greece and Crete colonization by Romans that spread wine-making all around the Mediterranean. By 500 BC, it was spread to Sicily, Italy, France, Spain, Portugal and Northern Africa. Cultivation of the wine also spread into Balkan states and the Romans took it into Germany and other parts of the Northern Europe, eventually reaching as far as Britain (Robinson 1994).

Production and consumption of fermented beverage like wine is an ancient practice. However, production and consumption of fruit based distilled alcoholic beverage is a later development. Different aspects of fruit based alcoholic beverages other than that from grapes have been investigated (Barnett 1980). Rigveda amply testifies that the wine is perhaps the oldest fermented product known to man. However, still the actual birthplace of wine is unknown though it had been prepared somewhere in 3500 BC (Joshi and Attri 2005). European explorers in the sixteenth century introduced the wine into the new world (Amerine et al. 1980). The early spreading and world distribution of the grape vine and winemaking technology is shown in Fig. 30.2.

Wine has been made in India as far ago as 5,000 years. It was the early European travellers to the courts of the Mughal emperors Akbar, Jehangir and Shah Jehan in

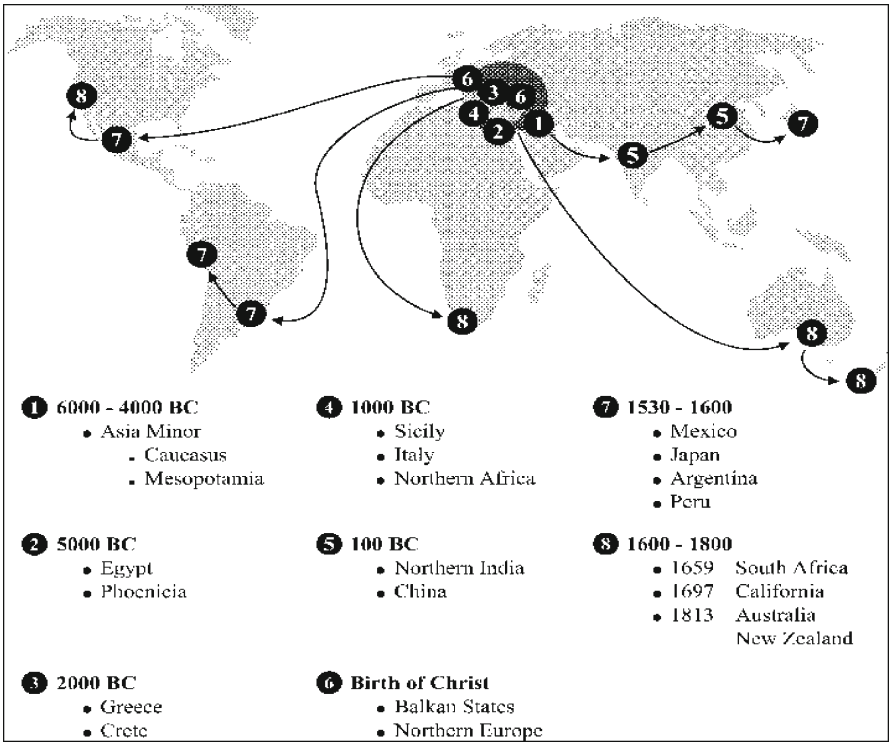

Fig. 30.2 The early spreading and world distribution of the grape vine and wine making technology (Source: Pretorius 2000)

the sixteenth and seventeenth centuries who reported tasting wines from the royal vineyards. Red wines were made from the *arkesham* grape and white wine from *arkawati* and *bhokry* grapes (Joshi and Parmar 2004).

30.1.1 Wine Production from Fruits Other than Grapes

In European usage, wine and brandy refer to exclusively fermented by-products of grapes – member of the genus *Vitis*, notably cultivars of *vinifera.* In English, especially in the new world, wines and brandies may refer to the fermented by-products of any fleshy fruit or flower. The quality and quantity of grapes depend on geographical, geological and climatic conditions in the vineyards, the grape variety and the methods of cultivation (Joshi et al. 1999).

In general, grapes are the main raw materials that have been used for wine production for the past several decades. However, many research groups have investigated the suitability of fruits other than grapes (Table 30.1). Compared to the quantity of grape wine produced and consumed in the world, the amount of wine

Table 30.1 Fruit wines produced from different fruits

Fruits/wines	Investigators
Apple	Sandhu and Joshi (1995)
Apricot	Joshi et al. (1990)
Banana	Kotecha et al. (1994)
Ber	Gautam and Chundawat (1998)
Cashew	Mandal (1997)
Custard apple	Kotecha et al. (1995)
Dates	Ali and Dirar (1984)
Jamun	Shukla et al. (1991)
Grape fruit and Kinnow	Joshi et al. (1997)
Kiwi fruit	Heatherbell et al. (1980), Soufleros et al. (2001)
Litchi	Vyas et al. (1989)
Mandarin	Selli et al. (2004)
Mango	Kulkarni et al. (1980), Onkarayya and Singh (1984), Reddy and Reddy (2005)
Marula	Fundira et al. (2002)
Muskmelon	Teotia et al. (1991)
Orange	Selli et al. (2002, 2003)
Palm sap and Coconut	Nathanael (1955)
Peach	Joshi and Shah (1998)
Pear	Attri et al. (1994)
Plum	Joshi and Sharma (1994)
Pomegranate	Adsule et al. (1992)
Sapota and Guava	Bardiya et al. (1974)
Strawberry	Pilando et al. (1985)

produced from non-grape fruits is insignificant (Amerine et al. 1980; Sandhu and Joshi 1995). However, in some countries wines from other fruits like apple in Spain, France, Belgium, Switzerland and England, plum in Germany, and cashew apple in India are very much in demand (Joshi et al. 1999).

Wines made from complete or partial alcoholic fermentation of grape or any other fruit and contain ethyl alcohol as the intoxicating agent, essential elements, vitamins, sugars, acids, and phenolics. Wines from fruits are preferable to distilled liquors for stimulatory and healthful properties (Gasteineau et al. 1979). These beverages also serve as an important adjunct to the human diet by increasing the satisfaction, and contribute to the relaxation necessary for proper digestion and absorption of food. Joshi and John (2002) have reported the antimicrobial effect of apple wine on pathogenic bacteria.

30.1.2 Fruit Wines Production and Postharvest Losses

Fruits are highly perishable commodities and have to be either consumed immediately or preserved in one or the other form. In the developed countries, a

considerable quantity of fruit is utilized, but in developing countries, lack of proper utilization results in considerable post-harvest losses, estimated to be 30–40% (Sandhu and Joshi 1995). The ever-increasing fruit production needs improvement in preserving technologies. In India, efforts have been made to utilize the surplus (after export) and small and unattractive fruits for various purposes; one of the alternatives is wine production. Fruits are utilized to produce a variety of alcoholic beverages including different types of fruit wines and their distillates are known as brandy. Rig-Veda has also mentioned the medicinal power of wines (Vyas and Chakravarthy 1971). The increased production can be utilized profitably, if wines are produced from fruits (Joshi et al. 2004). Setting-up of fruit wineries besides industrialization of the fruit growing belts could result in economic upliftment of the people, generating employment opportunities and providing better returns of their produce to the orchardists (Amerine et al. 1980; Fowles 1989; Joshi et al. 2004). Availability of technology is the single important factor determining the production though cost and type of the product is also a significant consideration in popularising the product. So production of fruit wines in those countries where fruits other than grape are grown would certainly be advantageous. The research on wine production done in India reveals that an impressive progress has been made in the development of technologies for preparation of wines of different types from various fruits. Successful marketing of grapes and apple wines in India is an indicator of potential Indian market waiting for fruit wines.

30.2 General Wine Fermentation Methodology

30.2.1 Pre-fermentative Practices

The pre-fermentative aspects are different in different places and vintages. But the main and normal aspects that are used for producing wine are:

(a) **Crushing/Stemming**: Crushing releases the juice and activates enzymes liberated from grapes. Stemming removes the fruit stalk (rachis and peduncle) and other vine parts.
(b) **Maceration**: Crushing activates a wide range of hydrolytic and oxidative enzymes in grape cells. Because many enzymes remain bound to cell fragments, their action (maceration) can be curtailed by rapid removal of the juice from the pomace (Ramey et al. 1986).
(c) **Presses and dejuicers**: Pressing separates the juice or partially vinified wine from the seeds and skins of the must.
(d) **Clarification**: It improves the fermentation performance of the yeast. Bentonite was preferable for the clarification. In large wineries, centrifugation is commonly used to speed up the clarification. Filtration and flotation are other options for rapid clarification and

(e) **Juice adjustment**: Adjustment of acidity and sugar to desirable level are permitted. If the acidity of the juice is undesirably low (<5 g/l), acids such as tartaric and citric may be added. If the acidity of the juice is high, blending with low acidity juice can be effective. It can increase sugar concentration, without addition of sugar, or by the removal of water by reverse-osmosis (Duitschaever et al. 1989), cryo-extraction (Chauvet et al. 1986) and entropie concentration (Forment 1993).

30.2.2 Fermentative Process

The must is allowed to ferment at a temperature (20–25°C) after inoculation with yeast culture. Temperature higher than 26°C should be avoided because it causes loss of volatile components and alcohol. The container in which fermentation is carried out is equipped with an air bang. The sugar content or °Brix (°Bx) is measured periodically to monitor the progress of fermentation. Normally, the fermentation is allowed to proceed till the whole sugar is consumed completely (usually °Bx of about 8). When the fermentation is completed, the bubbling due to production of CO_2 is stopped. The specific details of fermentation to produce fruit wines are discussed in subsequent sections.

30.2.3 Post-fermentative Practices

(a) **Siphoning/Racking**: After completion of fermentation, the yeast and other materials settle at the bottom of container with clear liquid separating out, which is siphoned/racked or in case of pulpy must, it is filtered through a cheese/muslin cloth followed by siphoning. Two or three rackings are usually done after 15–20 days. During inter-racking period, no headspace is kept in the bottle or container which is closed tightly to prevent the acidification.
(b) **Maturation**: The newly made wine is harsh in taste and has yeasty flavour. The process of maturation makes the wine mellow in taste and fruity in flavour besides the clarification. The period may extend up to 6 months to 2–3 years. The process of maturation is complex and the formation of esters take place thus, improving the flavour of such beverages. For details, the readers may refer to separate chapter on maturation of wines and brandy.
(c) **Clarification**: If the wine after racking and maturation is not clear it is clarified using filter aids such as Bentonite, Celite or by tannin/gelatine treatment in a filter press. These treatments usually make the wine crystal clear.
(d) **Blending**: The wine from fruits like plum is acidic may need some amount of sweetening prior to final bottling. For plum wine, the extent of sweetening to a TSS of 12°B was found to be optimum. The wine is filtered, if needed.
(e) **Pasteurization**: Wines being low alcoholic beverages are pasteurized at 62°C for 15–20 min after keeping some headspace in the bottle and crown corking

the same. Heating the wines help precipitation of tannins or other such materials which are heat sensitive besides the preservation of wines. The pasteurized wines once opened have to be kept at low temperature to prevent their spoilage. Alternately, the table wines can be preserved by the addition of preservatives like sulphur dioxide, sodium benzoate and sorbic acid (Amerine et al. 1980). The wines having higher alcohol contents like fortified wines need no preservation as the alcohol itself acts as a preservative above 15% alcohol. Carbon dioxide in conjunction with ethyl alcohol and low level of SO_2 prevents the spoilage of carbonated wines.

30.3 Types of Fruit Wines

30.3.1 Banana Wine

Bananas (*Musa paradisiaca*) are peeled manually and homogenized in a blender for about 2–3 min to obtain pulp. Potassium metabisulfite (100 ppm) is added to prevent browning and to check the growth of the undesirable microorganims. Fermentation was carried out at 18 ± 1°C and the amount of yeast inoculum added was 2%. The wines after fermentation were cold stabilized at 5–7°C for 10 days. Preliminary studies were carried out to optimize the conditions for maximum extraction of juice, using different levels of pectinase enzyme and different incubation periods at 28°C. Based on these studies, 0.2% pectinase and 4 h of incubation time were selected for obtaining the juice from the pulp. The juice was separated by centrifugation and the clear juice was used for the preparation of wine by slight modification of the method described. The juice recovery from over-ripe fruits was higher (67.6%) than from normal ripe fruits (60.2%). Good quality wine was obtained from both ripe and overripe fruits as well. Comparison of chemical composition of juice and wine made from banana is presented in Table 30.2. Addition of passion fruit concentrate and pectinase (0.15%) containing pectolytic enzyme or pectin methyl esterase and polygalacturonase with banana puree were conducted (Brathwaite and Badrie 2001; Cheirslip and Umsakul 2008). Total titrable acidity of the wine must was standardized as 0.60% citric acid to a pH of 3.3 ± 0.2. Fining agent Bentonite clay (0.1%) was used for further clarification. Wine prepared from banana puree blended with passion fruit concentrate, enzyme and concentrate, enzymes and KMS was awarded the highest score for colour and overall acceptability. Wine prepared only from banana pulp obtained highest taste and aroma score.

30.3.2 Guava Wine

The guava fruits available in abundance at low price and can be utilized for production of wine of highly acceptable quality. The fruit although has low sugar, but has characteristic flavour and golden yellow colour. Two types of wines have been

Table 30.2 Chemical composition of banana juice and banana wine

Characteristics	Juice	Wine
TSS (°B)	20.33 ± 0.87	10.2 ± 0.2
Acidity (%)	0.47 + 0.01	0.88 + 0.06
Reducing sugar (%)	10.52 + 0.05	3.18 + 0.16
Tannins (%)	0.061 + 0.04	0.044 + 0.002
Total SO_2 (ppm)	–	67.10 + 1.10
Alcohol (v/v %)	–	6.06 + 0.06
Overall score (Max 20)	–	15.00 + 0.12

(Source: Kotecha et al. 1994)

Table 30.3 Comparison of the chemical characteristics of guava wine prepared from juice, pulp and diluted pulps

Treatment	Guava juice wine	Guava pulp wine	Guava pulp (1:2 dil) wine	Guava pulp (1:3 dil) wine
Total soluble solids °B (Refractrometer reading)	17.0	17.5	15.0	17.0
pH	3.40	3.50	3.20	3.25
Volatile acidity (acetic acid/ g/100 ml)	0.033	0.046	0.052	0.063
Total acidity (g/100 ml TA)	0.901	0.796	0.750	0.627
Brix reading (Hydrometer)	9.0	9.0	6.5	10.5
Total SO_2 (ppm)	153.6	115.0	156.8	127.2
Reducing sugar (%)	7.8	10.0	8.3	9.2
Tannins (g/100 ml)	0.020	0.130	0.110	0.055
Alcohol (% v/v)	10.0	10.7	11.5	9.8
Total aldehyde (ppm)	58.1	37.4	59.8	50.6
Yield of wine (%)	60.0	60.0	–	–

(Source: Bardiya et al. 1974)

prepared from guava fruit: guava juice wine (GJW) and guava pulp wine (GPW). For making wine from pulp, dilution with water is essential and dilution level of 1:2 was found better than 1:3. Sugar (24°B), KMS (to give 125 ppm SO_2), pectinase (0.5%) and yeast inoculum (2%) are added to initiate the fermentation which is carried out at 20°C. The treatment of pulp with pectinase increased the final yield of wine to the tune of 18% (Bardiya et al. 1974). The wine prepared from guava juice obtained by treatment with pectinase for juice extraction gave wine with low tannin content, optimum colour, flavour and acceptable sensory qualities. However, fermentation of guava pulp in presence of pectinase reportedly yields a wine with high tannin, dark colour and astringent taste. The best wine was obtained by fermentation of juice. Guave pulp wine is prepared in the same way as guava juice wine. When the °B reading reached 10, the pomace is removed and more sugar is added (10%) to the fermenting materials and the mixture is allowed to ferment further (Bardiya et al. 1974). Comparison of chemical characteristics of wine fermented by juice and pulp is presented in Table 30.3. Sevada et al. (2011) have been reported a method of production of guava wine using immobilized yeast cells.

30.3.3 Orange Wine

A sweet dessert wine has been produced from oranges, as reviewed by Amerine et al. (1980). Ripe and cleaned fruits should only be used for wine making, after sorting out rotten and other fruits unfit for juice extraction. Juice for fermentation need to be extracted in FMC juice extractor. Crushing of oranges has not been found to be satisfactory for wine making since oil from the skin also comes up in the juice and inhibits the fermentation process. Since the wine from the oranges rapidly darkens the colour, higher amount of SO_2 has to be maintained during fermentation. Potassium metabisulphite (KMS) to give 150 ppm of SO_2 has been found optimum for the fermentation of must, ameliorated with sugar at 22–23°B and with 0.1% pectic enzyme, to dryness. The wine is sweetened to 10°B, adjusted to SO_2 concentration of 200 mg/l, filtered and pasteurized. However, if it is fortified to 20% alcohol and aged, it can be bottled without pasteurization. Comparison of physico-chemical and sensory quality of the citrus wines showed that the ones made from sweet orange, mandarine, kinnow, except that from lemon, were acceptable (Joshi et al. 2011). The method of wine preparation includes sweetening the respective juices (150 g/l in all the must except lemon 240 g/l sugar) and addition of KMS (100 ppm), pectinase enzyme (0.5%) and DAHP (0.1%). At the end, the wines have to be sweetened by addition of 2–3% sugar followed by pasteurization and maturation. However, bitterness characteristics of the citrus fruits remained associated with wine too (Joshi et al. 1997).

30.3.4 Custard Apple Wine

Custard apple (*Annona squamosa*) is a hardy crop and has been used for the preparation of wine (Juroszek et al. 1987). The juice was prepared by peeling (removing seeds), adding pectinase enzyme to the pulp. Both concentration of enzyme and incubation period affected the juice recovery. The juice was ameliorated to TSS of 23°B and adjusted with acidity (0.7%), phosphate (0.05%) and 125 ppm SO_2; diammonium hydrogen phosphate was also added. The must was inoculated with wine yeast, *Saccharomyces cerevisiae* and fermented at 30°C. The wine was clarified with Bentonite (0.1%). Composition of custard apple juice and wine is given in Table 30.4.

30.3.5 Litchi Wine

Litchi fruit is grown in some areas of India. It has a plenty of flavour and is a good source of minerals and vitamins and used for the preparation of alcoholic beverages in China. Utilization of this fruit to prepare low alcoholic high

Table 30.4 Chemical composition of custard apple juice and wine

Characteristics	Juice	Wine
Juice recovery (%)	47 + 1.0	–
TSS (°Bx)	21 + 0.5	7.6 + 0.2
Acidity (%)	0.32 + 0.02	0.64 + 2.20
Reducing sugars (%)	15.45 + 0.05	4.20 + 2.20
Tannins (%)	0.061 + 0.004	0.044 + 0.002
Total SO_2 (mg/l)	–	71.20 + 2.20
Alcohol (v/v %)	–	7.92 + 2.08

(Source: Kotecha et al. 1995)

flavoured beverage using the technique of partial osmotic dehydration has been made. It was found that a product containing 5–6% alcohol, 3–4% sugar and 0.35% acid can be prepared as an appetizing soft drink instead of an intoxicating liquor. With osmotic dehydration as a pre-treatment the concentration of nutrient and the flavour takes place and is reflected in the product as compared to that prepared from non-treated fruits. For the same reason, the osmotically treated juice undergoes fast fermentation and the time required for the preparation of wine is considerably less. The detailed procedure involved in preparation of this drink is described earlier (Vyas et al. 1989). The litchi fruits of optimum maturity are washed and peeled followed by dipping in the sugar solution of 70°B for 4 h at 50°C. The treated fruits are taken out, drained followed by pulping in a pulper. The sugar content is adjusted to 22°Bx by dilution with water. A 24 h-old active culture of yeast, *S. cerevisiae* prepared in the litchi juice, is added (5%) to the sterilized litchi juice to carry out the alcoholic fermentation. The fermentation is carried out as a routine practice and allowed to continue until the TSS comes to 7°Bx (Refractometer reading). The wine is matured followed by blending with equal quantities of fresh litchi juice, filtered and filled in glass bottles and closed with crown corks. The bottles are heat-processed in the water at 62.5°C for 20 min.

30.3.6 Muskmelon Wine

Muskmelon (*Cucumis melo*) is cultivated throughout India, particularly in the North-western region. The fruit has a soft sweet juicy flesh with musk like odour but is highly perishable. It was successfully converted into a fermented juice and ready-to-serve beverage (RTS), low alcoholic drink (Teotia et al. 1991). The musk melon juice recovered from fruits unfit for table purpose is fermented for 96 h at 30 ±5°C. The fermented juice contained 6.50 (w/v) alcohol and exhibited a very good sensory quality when adjusted to 10°Bx and 12°Bx levels with TSS acid ratio of 34.5 and 41.4, respectively. The RTS beverage derived from the fermented juice was excellent in sensory quality which contained 2.20% (w/v) alcohol, and adjusted to 12°Bx TSS: acid ratio of 44.4.

30.3.7 Pineapple Wine

Pineapple juice is mostly having 12–15°Bx as total soluble solids, the sugar content is raised by the addition of sugar up to 22–25°Bx to produce a wine having 12–13% alcohol. The wine is preserved by pasteurization. It can also be fortified and sweetened. However, the flavour of pineapple is not stable and oxidation can occur easily (Amerine et al. 1980). Wine from pineapple waste is made in Hawaii and Philippines to make distilled vinegar. Wine from two cultivars (cvs) *Kew* and *Queen* of pineapple was prepared and evaluated. The changes during fermentation were found to be a slight increase in acidity initially followed by a decline, and a reduction in ascorbic acid. Six month matured wine was found comparable to other fruit wines, out of two varieties cv. *Queen* was better.

30.3.8 Date Wine

Wines like *Sherbote* and *Nabit* can be prepared from dates. Both these wines are prepared from date syrup by quick fermentation taking about 36–48 h. *Dakkai* is made from whole date with a fermentation period of 96 h (Ali and Dirar 1984). The method of *Dakkai* preparation includes mixing whole date fruits with luke warm water in an earthern ware which is allowed to ferment by natural microflora followed by straining *'Dakkai'* as a liquid of pale yellow appearance. However, in *Nabbit* and *Sherbote* preparation, one part of dates is boiled with three parts of water, followed by straining, which gives sweet brown coloured syrup. Besides, in making of *Sherbote* ground cinnamon and ginger (50 g/l) are tied in a piece of cloth and dropped in the liquor to be fermented. The jar containing the syrup is closed tightly and fermentation is left to proceed for 1–3 days.

30.3.9 Apricot Wine

Apricot is a delicious fruit grown in many parts of hilly temperate countries including India. Due to its high flavour, it holds promise for conversion into wine. Wild apricot is being used locally in the high tribal hills to make liquor though the method is very crude and reportedly to be the result of natural fermentation, followed by distillation of wine. A method for the preparation of wine from wild apricot has been developed which consists of diluting the pulp in the ratios of 1:2, addition of 0.1% diammonium hydrogen phosphate (DAHP) and 0.5% pectinase, and fermentation with *S. cerevisiae*. Further, with the increase in the dilution level, the rate of fermentation, alcohol content and pH of the wines increased whereas a decrease in titratable acidity and volatile acidity, phenols, TSS, colour values and K, Na, Ca, Mg, Zn, Fe, Mn and Cu took place. Addition of DAHP at the rate of 0.1% enhanced the rate of

Table 30.5 Physico-chemical characteristics of Jamun wine

	Jamun wine	
Characteristics	With pectin esterase	Without pectin esterase
Yield (1/2.5 kg fruits)	3.62	3.70
Alcohol (% v/v)	11.23	11.61
Total acidity (CA g/100)	0.37	0.38
Volatile acidity (% A.A.)	0.036	0.032
pH	3.50	3.48
Residual sugar (g/100 ml)	Traces	Traces
Esters (mg/100 ml)	16.12	14.24
Tannins (mg/100 ml)	105	94
Total anthocyanins (mg/100 ml)	44	42
Extract (g/100 ml)	3.33	2.9
Pectin (g/100 ml)	3.33	2.9
Organoleptic scores (out of 100)	78.5	83.0

(Source: Shukla et al. 1991)

fermentation. The wine from 1:2 diluted pulps was rated as the best (Joshi et al. 1990). In apricot wine of cv. *New Castle* variety, the extraction of pulp either by hot method or addition of enzyme and water to the fruits could be adopted (Joshi and Sharma 1994). However, dilution of pulp in the ratio of 1:1 with water, addition of DAHP (0.1%) and raising the TSS to 30°Bx made the wine of superior quality.

30.3.10 Jambal Wine

The jambal fruit (*Synzygium cumini* Linn.) is liked for its refreshing pink to greyish flesh with a balanced sugar, acid and tannin contents, and the fruit is associated with therapeutic value (Khurdiya and Roy 1985). It has been used in making dry wine of acceptable quality (Shukla et al. 1991). Out of three cultivars investigated, Jamun made the best wine. The method of wine preparation included dilution of the crushed fruits in the ratio of 1:1, amelioration of the must to 23°B with cane sugar, addition of DAHP at the rate of 0.2%, sulphur dioxide 150 ppm, 0.25% pectinase enzyme. The fermentation was carried out with 2% *S. cerevisiae* inoculum followed by racking, filtration and bottling. Physico-chemical characteristics of Jamun wine made with and without pectinase enzyme are given in Table 30.5.

30.4 Mango Wine

It is a matter of astonishment to many that mango (*Mangifera indica* L.), one of the most celebrated of tropical fruits, is a member of the family Anacardiaceae, which is notorious for embracing a number of highly poisonous plants. The mango fruit is

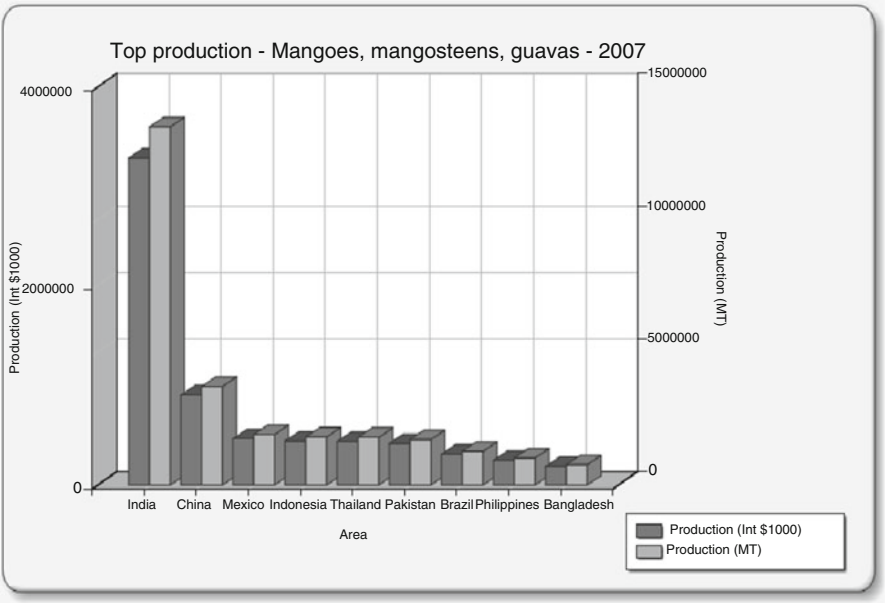

Fig. 30.3 World top production of mango (Source: FAOSTAT 2007)

one of the most highly priced desert fruits of the tropics. It has rich luscious, aromatic flavor and a delicious taste in which sweetness and acidity is delightfully blended. Mango production has experienced continuous growth in the last decades of the twentieth century (Baisya 2004).

The world's total annual mango fruit production was estimated at 22 million metric tonnes (Mmt). Global production of mangoes is concentrated mainly in Asia and more precisely in India that produced 12 Mmt per annum. Mangoes are cultivated in 85 countries. Total world production in 2004 was 26,147,900 mt (FAOSTAT 2007). Asia and the oriental countries produced around 80% of the world's total production. Major mango producing countries are India, Mexico, China and Pakistan.

Mango is the choicest fruit cultivated in India for the last 4,000 years, and is called as the king of fruits and pride fruit of India. The crop is significantly important in the fruit economy of India and is the third largest industry in the country. In India mango is grown in two million acres and it occupied 39% of total fruit production (Fig. 30.3 and Table 30.6). It is the most cultivated area occupied crop in India with 60% of the total area under fruits. More than 25 mango cultivars are available in India (Table 30.7) that are widely cultivated all over the world (Anon 1962).

Andhra Pradesh, being blessed with varied soil types and agro-climatic conditions, is better placed for cultivation of large varieties of fruit crops and is one of the largest fruit producing states in India. The major fruit crops grown in Andhra Pradesh are mango, sweet orange, banana, grape, pomegranate, coconut, and cashew. Mango occupying an area of 3.7 lakh hectares, with an annual production of 32 lakh mt, has

Table 30.6 Production of selected fruits in India

Fruit Name	Average (1992/1994) ('000 tonnes)	2002	2003	2004
Banana	9,718	16,820	16,820	16,820
Mango	10,108	10,640	10,780	10,800
Orange	1,743	3,120	3,070	3,070
Apple	1,205	1,160	1,470	1,470
Lemons and Lime	863	1,440	1,420	1,420
Pineapple	956	1,180	1,310	1,300
Grape	684	1,210	1,150	1,200
Papaya	470	700	700	700
Pear	127	200	200	200
Peache and Nectarine	83	150	150	150
Grapefruit and Pomelo	83	140	142	142
Plum	55	80	80	80
Fig	6	11	11	11
Apricot	7	10	10	10
Cherrie	4	8	8	8

(Source: www. faostat.fao.org/2007)

Table 30.7 Varieties mango produced in different Indian states

State	Varieties
Andhra Pradesh	*Banganapalli, Suvarnarekha, Neelum* and *Totapuri*
Bihar	*Bombay Green, Chausa, Dashehari, Fazli, Gulabkhas, Kishen Bhog, Himsagar, Zardalu* and *Langra*
Gujarat	*Kesar, Alphonso, Rajapuri, Jamadar, Totapuri, Neelum, Dashehari* and *Langra*
Haryana	*Chausa, Dashehari, Langra* and *Fazli*
Himachal Pradesh	*Chausa, Dashehari* and *Langra*
Karnataka	*Alphonso, Totapuri, Banganapalli, Pairi, Neelum* and *Mulgoa*
Madhya Pradesh	*Alphonso, Bombay Green, Dashehari, Fazli, Langra* and *Neelum*
Maharashtra	*Alphonso, Kesar* and *Pairi*
Punjab	*Chausa, Dashehari* and *Malda*
Rajasthan	*Bombay Green, Chausa, Dashehari* and *Langra*
Tamil Nadu	*Alphonso, Totapuri, Banganapalli* and *Neelum*
Uttar Pradesh	*Bombay Green, Chausa, Dashehari* and *Langra*
West Bengal	*Fazli, Gulabkhas, Himsagar, Kishenbhog, Langra* and *Bombay Green*

(Source: http://apeda.com/apedawebsite/SubHead_Products/Mango.htm)

placed the state in first position with a share of 20% of the India's production coupling with highest productivity (Baisya 2004).

Mango contains a high concentration of sugar (16–18% w/v) and acids with desirable organoleptic properties, and also contains antioxidants like carotene (as Vitamin A, 4,800 IU). Sucrose, glucose and fructose are the principal sugars in ripened

mango, with small amounts of cellulose, hemicellulose and pectin. The green tender fruits are rich in starch, which is hydrolyzed to reducing sugars during ripening (Anon 1962). The unripened fruit contains citric acid, malic acid, oxalic acid, succinic and other organic acids, whereas in ripened fruit, the main acid source is malic acid (Giri et al. 1953). Mango juice along with aromatics is recommended as a restorative tonic; which contains good concentrations of vitamin A and C, and is useful in heat apoplexy. Mangoes with higher initial concentration of β-carotene are helpful as cancer-preventing agents. Fruits like mango are highly perishable commodities. In developing countries like India, 20–30% of fruits produced undergo spoilage due to lack of proper utilization and undeveloped post-harvest technology (Sandhu and Joshi 1995).

An alternative and profitable method of using mangoes for wine-making could become widely accepted. Many investigators have carried out considerable research on mango composition, as well as on mango cultivation aspects. The production of wine was reported for the first time from pulp (Anon 1963) and then, Czyhrinciwk (1966) have reported the technology involved in mango wine production. Further, Kulkarni et al. (1980); Onkarayya and Singh (1984); Onkarayya (1985, 1986) screened some varieties of mango for wine making and found that mango wine had quite high acceptability. These authors however have not given details on vinification techniques and chemical composition of the wine produced from mango. The work was inadequate, particularly in the area of mango wine production and its composition. Reddy and Reddy (2005, 2007, 2008, 2009), have carried out extensive investigations on mango wine production using locally abundantly available cultivars.

30.4.1 Screening of Mango Varieties

Suitability of mango cultivars for wine production was screened based on juice quality, quantity and other properties. Mango juice was prepared by washing the mangoes with 1% HgCl, peeled and pulped manually. To get good yields of juice, the pulp was treated with Trizyme P50 (800 U/ml), a pectinase enzyme. Among the ten varieties used by Reddy and Reddy, six varieties showed promising juice yields (450–550 ml/kg). The data on juice yield, physical and chemical composition are presented in Table 30.8. Mango contains three types of sugars namely, glucose, fructose and sucrose. The totals soluble solids (TSS) of the mango juice ranged from 14.2% to 20.5%. The *Banginapalli* (20.5%) variety had high TSS. The sugar content of mango juice ranged from 15% to 18% (w/v) while titrable acidity as tartaric acid ranged from 0.310% to 0.462% (w/v). The pH of the juices was in between 3.8 to 4.5 (Reddy and Reddy 2005).

30.4.2 Enzyme Treatment

Pectolytic enzymes have been used in fruit juice processing for several purposes: extraction (Rombouts and Pilnik 1978), clarification (Pilnik 1983), liquefaction and maceration (Sreenath et al. 1987, 1995), and cloud stabilization (Askar et al. 1990).

Table 30.8 Physicochemical characteristics of mango pulp

Mango variety	Juice yield (ml/Kg)	Reducing sugars (% w/v)	Titrable acidity (%)	pH	TSS* (%)
Alphonso	570 ± 10	16.3 ± 1.32	0.33	4.1 ± 0.53	16.0 ± 1.2
Raspuri	600 ± 13	15.5 ± 2.21	0.43	3.9 ± 0.86	14.2 ± 1.8
Banginapalli	550 ± 17	18.5 ± 1.24	0.32	4.0 ± 0.6	20.5 ± 0.79
Totapuri	500 ± 22	16.0 ± 1.0	0.31	4.2 ± 1.0	16.5 ± 1.2
A. Banesha	500 ± 15	18.0 ± 0.8	0.32	4.5 ± 0.45	20.1 ± 1.42
Neelam	480 ± 20	15.5 ± 1.7	0.42	4.3 ± 0.8	15.5 ± 1.5
Mulgoa	468 ± 8	14.3 ± 1.4	0.42	4.3 ± 0.5	15.0 ± 1.24
Suvarnarekha	470 ± 12	15.0 ± 0.55	0.40	3.9 ± 1.3	14.4 ± 0.58
Rumani	475 ± 14	14.5 ± 1.0	0.39	4.2 ± 0.72	14.6 ± 1.43
Jahangir	460 ± 10	15.6 ± 1.62	0.46	4.6 ± 0.56	14.2 ± 1.3

(Source: Reddy and Reddy 2005)
* TSS total soluble solids

Maceration action of enzymes consists of a controlled action of the pectolytic enzymes (Voragen and Pilnik 1981), on the highly esterified pectins constituting cell walls rather than the pectins in the middle lamella. The use of lyases or polygalacturonases with low pectinesterase activity enabled recovery of intact cell suspensions. Conversely during liquefaction, degradation of the middle lamella is followed by cell wall lysis under the action of pectolytic enzymes, cellulases and hemicellulases. Especially the mango pulp contains high pectin and needs pectinase treatment for the clarification of juice. A decrease of 50% or more of the relative viscosity of mango pulp resulted from different times of maceration with pectolyase and with 3 of the 4 commercial enzyme preparations (Sakho et al. 1998). Preliminary studies were carried out to optimize the conditions for maximum extraction of juice, using different levels of pectinase enzyme and different incubation periods at 22 ± 2°C. Based on these studies, 0.6% pectinase and 8 h of incubation time were selected for obtaining the juice from the pulp. The juice was separated by centrifugation and the clear juice was used for the preparation of wine by slight modification of the method described. Pectinase treatment increased the juice yield and fermentability of mango pulp (Table 30.9). In addition, the pectinase treatment increased the fermentation performance, as well as chemical component production, during wine fermentation. Pectinase treatment also enhances the juice extraction and decreases the viscosity of juice that could make the wine production from mango economical. The total higher alcohol content was higher in pectinase-treated wines in comparison with control wines (Table 30.9). Finally, it is concluded that the pectinase treatment is essential to produce better quality wines from fruits like mango.

30.4.3 Fermentation

The batch fermentation was carried out typically by inoculating the actively growing yeast, prepared as above, into 100 ml of sterile mango must contained in a conical

Table 30.9 Effect of pectinase treatment on mango juice recovery and wine composition

	Banginapalli		*Totapuri*	
Character of juice	Untreated	Treated	Untreated	Treated
Juice yield (ml/Kg)	458 ± 12	550 ± 10 $p < 0.0001$	416 ± 8.0	500 ± 22 $p < 0.0001$
TSS (% w/v)	15 ± 0.60	20.5 ± 0.79 $p < 0.001$	12 ± 0.80	16.5 ± 1.2 $p < 0.001$
Reducing sugars (% w/v)	15.8 ± 1.5	18.5 ± 1.6 $p < 0.0130$	15 ± 1.0	16 ± 1.3 $p < 0.1662$
Acidity (% w/v)	0.43 ± 0.06	0.56 ± 0.08 P = 0.0097	0.31 ± 0.03	0.44 ± 0.05 $p < 0.0003$
pH	4.0 ± 0.53	3.7 ± 0.3 P = 0.2553	4.2 ± 0.5	4.0 ± 0.45 $p < 0.4831$
Wine				
Ethanol (% w/v)	6.3 ± 1.1	8.5 ± 1.4 $p < 0.0127$	5.1 ± 0.9	7.0 ± 1.2 $p < 0.0112$
Higher alcohols (mg/l)	265 ± 8.2	340 ± 10.5 $p < 0.0001$	279 ± 6.8	358 ± 12.7 $p < 0.0001$
Total esters (mg/l)	20 ± 1.3	32 ± 1.5 $p < 0.0001$	16 ± 1.2	25 ± 0.8 $p < 0.0001$
Residual sugars (g/l)	10 ± 1.6	2.5 ± 1 $p < 0.0001$	15 ± 1.0	3 ± 1.3 $p < 0.0001$
Acidity (% w/v)	0.45 ± 0.04	0.66 ± 0.08 $p < 0.0002$	0.36 ± 0.03	0.54 ± 0.05 $p < 0.0001$
pH	4.4 ± 0.50	4.7 ± 0.41 $p = 0.2823$	4.5 ± 0.5	4.2 ± 0.5 $p = 0.3232$

(Source: Reddy et al. 2008)

flask fitted with a rubber cork fixed with a bent glass tube for CO_2 release, under stationary conditions at 22°C for a period of 20 days.

30.4.4 Influence of Mango Variety on Composition of Produced Wine

In general different mango varieties have different taste and quality. Variety of mango used for wine making showed a greater influence on quality of produced wine. Higher alcohols may influence certain sensory characteristics although they constitute a relatively lesser quantity of the total substances. The concentration of higher alcohols varied significantly with the mango variety ranging from 131 to 343 mg/l (Table 30.10). The highest is in the wine produced from *Banginapalli* and lowest is in the wine produced from *Neelam* variety. Esters have a crucial importance

Table 30.10 Physicochemical characteristics of mango wine produced at 22±2°C and pH 5

Mango variety	Ethanol (%w/v)	T.A* (%v/v)	V.A* (%v/v)	pH	Residual sugars (g/l)	Higher alcohols (mg/l)	Total esters (mg/l)	Tannins % (w/v)	Colour OD at 590 nm
Alphonso	7.5	0.650	0.100	3.8	2.1	300	25	0.011	0.22
Raspuri	7	0.735	0.210	3.8	2.4	200	29	0.072	0.18
Banginapalli	8.5	0.600	0.181	3.7	2.0	343	35	0.012	0.23
Totapuri	7	0.622	0.121	4.0	2.0	230	20	0.012	0.17
A. Banesha	8	0.610	0.110	4.0	2.0	320	30	0.013	0.25
Neelam	6.5	0.826	0.234	3.6	2.5	131	15	0.014	0.21
Mulgoa	6.3	0.621	0.109	3.9	3.0	152	18	0.065	0.28
Suvarnarekha	6.8	0.630	0.153	4.1	2.3	175	22	0.025	0.19
Rumani	6.9	0.618	0.125	4.0	2.1	212	15	0.027	0.24
Jahangir	7.1	0.646	0.138	3.8	2.0	256	21	0.042	0.16

(Source: Reddy and Reddy 2005)
T.A* Titrable acidity
V.A* Volatile acidity

in wine quality as they provide pleasant aroma sensations. The formation of esters in wine depends on various factors such as type of fruit, yeast strain and fermentation conditions like temperature, pH and incubation time. Although the yeast used in fermentation was the same, the ester concentration was different in each type of mango fruit wine. In the present work, the concentration of esters varied from 15 to 35 mg/l (Table 30.10).

30.4.5 Optimization of Fermentation Conditions

Kumar et al. (2009) optimized the fermentation conditions such as temperature, pH and inoculum size using Response Surface Methodology (RSM). Reddy and Reddy (2011) have investigated the effect of fermentation conditions like temperature, pH, SO_2 and aeration on yeast growth and mango wine composition. Based on the reports, it could be said that the fermentation temperature has considerable influence on yeast growth and the wine quality compared to all tested variables. Increase in temperature increased the volatile composition and also the yeast biomass. It is also confirmed that the optimum pH is very important to produce good quality wines. SO_2 has little effect on wine quality. But high concentrations decrease the yeast growth. Presence of dissolved O_2 increased both ethanol and yeast biomass. Finally, it can be conclude that optimization of fermentation conditions are very crucial in obtaining the best quality wine from mango juice.

30.4.6 Characterization of Wine

Mango wine has been characterized in terms of ethanol and glycerol concentration, higher alcohols, carotenoids, ploy phenols and volatiles present (Reddy et al. 2009; Varakumar et al. 2011; Kumar et al. 2011).

30.4.6.1 Ethanol, Glycerol and Acids

The principal metabolite produced in highest concentration during mango wine fermentation is ethanol as compared to other metabolites. In general, the concentration of ethanol contributes to the whole characteristic quality and flavour of the produced wine. The percentage of ethanol produced in the mango wines varied between 7% and 8.5% (w/v), comparable with moderate grape wines. Glycerol concentration in mango wines is between 5.7 and 6.9 g/l. Glycerol production mainly depends upon yeast strain used, fruit variety and fermentation conditions such as temperature and aeration. Increase in temperature results in higher glycerol production (Gardner et al. 1993; Remize et al. 1999). It is reported that the optimum temperature for

maximum glycerol production by commercial wine yeast strains of *S. cerevisiae* varies between 22°C and 32°C (Scanes et al. 1998).

Another parameter, which highly influences the quality of wine, is acidity. The main organic acid present in mango musts and produced wine was malic acid; and the other acids were however less than 1 g/l.

30.4.6.2 Characterization of Mango Wine Volatiles by GC-FID/MS

Three varieties of mangoes, *Banginapalli, Alphanso* and *Totapuri* have shown better results in the course of both screening and optimizing fermentation conditions for wine. Hence the same varieties have been selected for GC-MS analyses (Reddy et al. 2009). The liquid-liquid extraction procedure was followed for the preparation of samples for GC-MS analysis. The composition of three wines is shown in Table 30.11. The three major compounds (alcohols, esters and organic acids) were present in different concentration. The isoamyl alcohol (140 mg/l) was higher in quantity as compared to all the compounds detected by GC-MS, followed by isobutyl alcohol (115.4 mg/l), n-propanol (54.11 mg/l) ethyl acetate (35.15 mg/l) and phenyl ethanol (24.15 mg/l). Ethyl hexanoate, ethyl decanoate and ethyl octanoate are produced in higher concentration in *Banginapalli* variety than the others. The volatile acids present in the mango wine are acetic acid, propanoic acid, benzoic acid. Recently, Pino and Queris (2010) reported the presence of 102 volatile compounds using advanced headspace solid-phase micro-extraction gas-chromatography mass-spectroscopy (HSME-GC-MS). According to them the mango wine contained about 9 mg/l of volatile compounds, which included 40 esters, 15 alcohols, 12 terpenes, 8 acids, 6 aldehydes and ketones, 4 lactones, 2 phenols, 2 furans, and 13 miscellaneous compounds. Isopentanol and 2-phenylethanol were the major constituents. A tentative study to estimate the contribution of the identified compounds to the aroma of the wine, on the basis of their odour activity values, indicated that the compounds potentially most important to mango wine included ethyl butanoate and decanal.

30.4.6.3 Phenol Composition

Phenolic compounds considered being basic components of wines and over 200 compounds have been identified. Two primary classes of phenolics that occur in grapes and wine are flavonoids and nonflavonoids. The most common flavonoids in white and red wines are flavonols, catechins (flavan-3-ols), and anthocyanidins, the latter being found only in red wine. Recent awareness of the role of antioxidants, which act as chemo-protective agents, has been found to play a significant role in the promotion of health (Teissedre and Waterhouse 2003). Wine polyphenols contribute to wine colour and to other sensorial characteristics of wines such as bitterness and astringency (Perez-Magarino and Gonzalez-San Jose 2004). The concentration of total polyphenols in wine varied with the variety of mango fruit

Table 30.11 Volatile compounds present in wines of three mango cultivars

S.No	Retention Time (RT)	Name of the compound	*Banginapalli* (mg/l)	*Alphanso* (mg/l)	*Totapuri* (mg/l)
Alcohols					
1	1.271	Ethanol%	8.5	7.5	7
2	1.35	Ethyl ether	Solvent	Solvent	Solvent
3	1.492	1-propanol	54.11	42.32	47.13
4	1.729	Isobutyl alcohol	102.4	115.14	98.87
5	2.581	Isoamylalcohol	125.2	108.4	140.44
6	2.85	Pentane- 2 one	1.43	1.15	1.51
7	4.823	2-furan methanol	0.123	nd	0.216
8	6.535	Hexane-1-ol	1.42	1.02	nd
9	12.9	Phenethyl alcohol	22.15	24.15	20.48
10	19.414	Cyclohexane methanol	1.13	nd	1.34
11	42.58	n-pentanedecanol	0.61	nd	tr
Esters					
12	1.665	Ethyl aetate	35.15	30.42	27.48
13	6.876	Ethyl hexanoate	0.942	0.671	0.552
14	15.92	Ethyl octanoate	1.15	1.06	1.451
15	20.124	Ethyl decanoate	2.34	1.86	1.43
16	33.62	B-phenylethyl butanoate	nd	0.62	0.92
17	19.67	Dimethyl styrene	1.11	1.34	1.09
Acids					
18	1.95	Acetic acid	0.201	0.163	0.155
19	3.292	Propanoic acid	0.145	0.217	0.184
20	3.829	Butanoic acid	0.932	0.745	0.874
21	12.655	2-furoic acid	0.91	0.548	0.745
22	15.482	Benzoic acid	1.08	1.21	1.43
23	15.75	Phenyl formic acid	0.643	0.912	0.434
24	16,723	Octanoic acid	0.735	0.427	nd
25	37.99	Decanoic acid	1.18	0.963	tr
Ketones					
26	2.85	Pentane- 2 one	1.43	1.15	1.51
27	6.245	Furanone	1.12	1.51	1.22
28	11.489	Hydroxy-dimethylfuranone	0.238	0.452	0.331
29	25.967	Phenol 2,6-bis-4- methoxy- one	0.451	0.432	0.312
Unknown					
30	15.165	Unknown	0.183	0.412	0.243
31					
32	23.377	Benzene methane –4-hydroxy	0.531	0.256	0.231
33	35.68	Unknown	0.441	0.131	nd
34	38.86	Unkonwn	0.12	tr	tr
35	46.34	Unknown	tr	tr	Nd

(Source: Reddy et al. 2010)

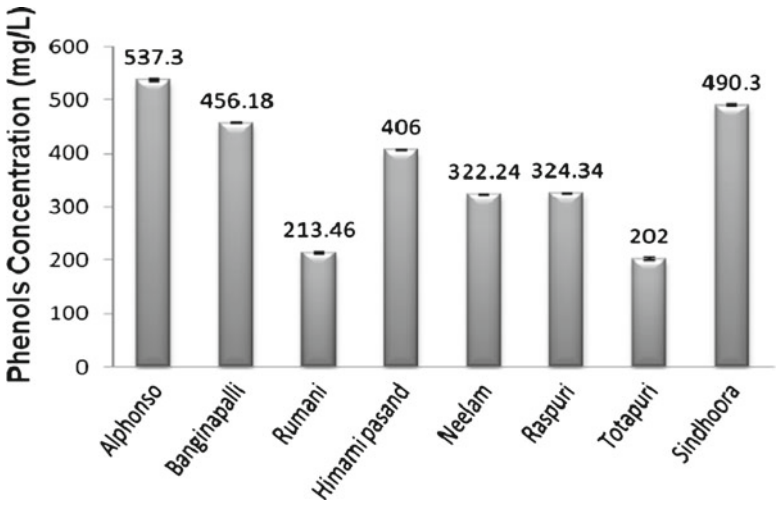

Fig. 30.4 Total phenol content in wines produced from different varieties of mango fruits (Source: Kumar et al. 2011)

Table 30.12 LC-MS determination of phenolic compounds in *Alphonso* mango wine

S.No.	Compounds	Retention time (Min)	Molecular weight	% (w/v)
1	*p*-Hydroxy benzoic acid	9.93	137	50.2
2	Caffeic acid	14.98	175	3.4
3	Vanillic acid	16.52	169	6.4
4	Ferulic acid	21.12	193	7.9
5	Sulfonic acid	22.17	187	11.21
6	Syringic acid	30.01	197	1.5
7	Protocatechuic acid	32.22	155	0.8
8	Leutolin	34.46	281	3.4
9	Sinapic acid	41.41	223	1.2
10	Quinaldinic acid	46.38	173	0.34
11	Phenylphosphoramidic acid	47.94	325	0.99

(Source: Kumar et al. 2011)

employed in fermentation. The highest concentration polyphenols of 1,050 mg/l was detected in wine produced from *Totapari* mango variety followed by *Alphanso, Banginapalli* and *Sindhoora* varieties (725, 610 and 490 mg/l respectively) (Fig. 30.4). Detailed analysis of phenolic compounds present in *Alphanso* is carried using LC-MS and 11 phenolic compounds were identified (Table 30.12). The reducing power varied from 0.71 to 2.90 mg/l ascorbic acid equivalents.

30.4.6.4 Carotenoids

The total carotenoids in the mango wines were in the range of 578–4,330 μg/100 g and the highest amount of total carotenoids was in *Alphonso* wine (4330), followed by *Sindhura* (4101), *Banginapalli* (2943), *Rumani* (2857), *Totapuri* (690), *Raspuri* (634), and *Neelam* (578 μg/100 g) (Varakumar et al. 2011). Among the carotenoids analysed in puree, β-carotene was the major carotenoid ranging from 65.4 to 94.1, followed by lutein (4.5–29.4), violaxanthin (0.6–3.8) and neoxanthin (0.7–3.1%). The relative highest total carotenoid levels in puree were found in *Sindhura* (5810), followed by *Alphonso* (5720), *Rumani* (3970), *Banginapalli* (3955), *Totapari* (1920), *Raspuri* (1080), and *Neelam* (980 μg/100 g) was the poor source of carotenoids with respect to the mango purees studied (Table 30.13). In this mango wine xanthophylls (oxygenated carotenoids) were degraded more than β-carotene (hydrocarbon carotenoid). Among xanthophylls, lutein was degraded more ranging from 78.7% to 93.9%, followed by neoxanthin (26.8–83.3%) and violaxanthin (50–74.3%) (Varakumar et al. 2011). This could be due to combined effect of acidic conditions of wine with temperature responsible for the significant degradation of xanthophylls compared to β-carotene (17.9–60.7%), which might be related to the presence of hydroxyl groups of xanthophylls. These results support the higher ratio of β-carotene/xanthophylls concentrations in all the mango wines and suggest that xanthophylls are degraded more quickly than β-carotene during wine aging. The degradation of β-carotene during carrot juice fermentation was reported by Kun et al. (2008) by using *Bifidobacterium bifidum* B3.2 strain and similarly, the losses of β-carotene and zeaxanthin in spontaneous (noninoculated) fermentation was reported by Li et al. (2007) during preparation of a fermented porridge from maize.

30.4.6.5 Immobilization Studies

A novel yeast biocatalyst was prepared by using watermelon pieces as immobilizing support for yeast, *Saccharomyces cerevisiae* 101 strain for use in wine production. Immobilization was confirmed by electron microscopy (Fig. 30.5) (Reddy 2005). The fermentation rate and other parameters were compared with free yeast cells at different temperatures. In all the cases, the fermentation time was short (22 h at 30°C and 80 h at 15°C) and produced high ethanol productivities (4 g/l/h). The volatile compounds, methanol, ethyl acetate, propanol-1, isobutanol and amyl alcohols that formed during fermentation were analyzed with the help of GC-FID. The concentrations of ethyl acetate and methanol were around 100 mg/l in all. Cell metabolism of immobilized yeast was not much affected by immobilization. It was inferred that the immobilization of yeast on watermelon pieces increased the fermentation rate, vitality and viability of yeast cells. Preliminary sensory evaluation suggested that the wine from immobilized

Table 30.13 Total carotenoid content present in varieties of mango purees and wines (μg/100 g)

	Neoxanthin		Violaxanthin		Lutein		β-Carotene		Total carotenoids	
Variety	Puree	Wine	Puree	Wine	Puree	Wine	Puree	Wine	Puree	Wine
Alphonso	41 ± 2.42	12 ± 1.4***	35 ± 2.1	9 ± 0.9***	258 ± 15	55 ± 7.8***	5,385 ± 142	4,251 ± 138**	5,720 ± 240	4,330 ± 176*
Banginapalli	56 ± 3.02	12 ± 1.3***	42 ± 1.54	13 ± 0.97***	326 ± 24	20 ± 5.5***	3,531 ± 92	2,898 ± 101*	3,955 ± 151	2,943 ± 141*
Neelam	14 ± 3.42	4 ± 0.95*	37 ± 1.21	12 ± 1.08***	288 ± 13	19 ± 6.4***	641 ± 159	543 ± 53	980 ± 152	578 ± 101
Raspuri	12 ± 0.9	2 ± 0.55***	16 ± 2.06	8 ± 0.51*	212 ± 19	42 ± 3.5***	840 ± 114	617 ± 59	1,080 ± 132	634 ± 113
Rumani	123 ± 1.7	90 ± 1.84***	30 ± 1.14	12 ± 0.67***	270 ± 15	28 ± 4.6***	3,550 ± 121	2,753 ± 91**	3,970 ± 151	2,857 ± 104**
Sindhura	102 ± 3.6	31 ± 0.84***	62 ± 1.43	21 ± 0.51***	452 ± 21	37 ± 4.1***	5,192 ± 109	4,012 ± 163**	5,810 ± 216	4,101 ± 163**
Totapuri	52 ± 2.94	25 ± 0.51***	15 ± 1.01	7 ± 0.49**	213 ± 12	13 ± 5.4***	1,642 ± 97	645 ± 74***	1,920 ± 147	690 ± 125**

***, **, * significant when compared with the carotenoids in puree to wine at $p \leq 0.0001$, 0.001 and 0.01 respectively (Source: Varakumar et al. 2011)

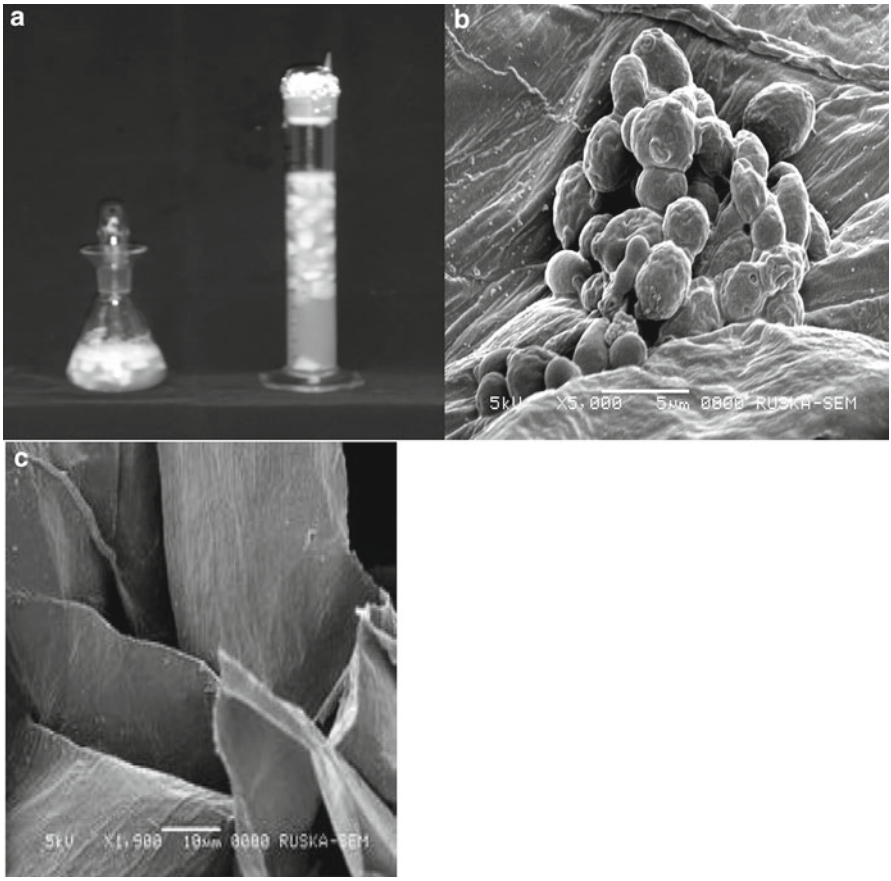

Fig. 30.5 Immobilization of yeast cells on watermelon pieces in mango wine fermentation. (**a**)Fermentation of mango juice with water melon rind pieces immobilized yeast biocatalyst, (**b**) scanning electron microscope (SEM) image of water melon rind piece Top second, (**c**) scanning electron microscope (SEM) image of yeast inside the water melon rind piece. (Source: Reddy 2005)

yeast had fruity aroma, fine taste and an overall improved quality as compared to the one produced from non-immobilized yeast.

30.4.6.6 Effect of Storage of Bottle Colour and Temperature on Wine Colour

The change in the colour of the mango wine at 8°C, 16°C and 25°C storage was monitored by the browning index. In the same way, the change in the colour of the mango wine stored in white, green and brown was studied The wine stored in darker brown bottles at low temperature indicated low browning indices as compared to wine in white bottles.

Table 30.14 Sensory evaluation studies on wines from different mango varieties

Wine variety	Flavour	Taste	Texture	Appearance	Mouth feel	Overall acceptability
Alphonso	$8.90^{a} \pm 0.14$	$8.26^{bd} \pm 0.04$	$8.59^{b} \pm 0.02$	$8.23^{ac} \pm 0.02$	$8.85^{cd} \pm 0.03$	$8.62^{cd} \pm 0.01$
Raspuri	$7.22^{bc} \pm 0.14$	$7.68^{ac} \pm 0.03$	$8.14^{ac} \pm 0.02$	$7.97^{bc} \pm 0.01$	$8^{ac} \pm 0.07$	$7.94^{ac} \pm 0.02$
Banginapalli	$8.56^{ac} \pm 0.05$	$8.35^{bd} \pm 0.03$	$8.45^{bd} \pm 0.03$	$8.76^{cd} \pm 0.00$	$8.5^{cd} \pm 0.03$	$8.3^{bd} \pm 0.03$
Totapuri	$7.84^{ac} \pm 0.05$	$7.45^{ac} \pm 0.03$	$7.98^{ac} \pm 0.14$	$8.1^{ac} \pm 0.07$	$7.6^{ac} \pm 0.02$	$7.4^{ac} \pm 0.14$
Sindhoora	$6.9^{ab} \pm 0.07$	$6.4^{b} \pm 0.03$	$7.2^{ab} \pm 0.02$	$8.45^{bd} \pm 0.03$	$7.3^{bc} \pm 0.01$	$7.2^{ad} \pm 0.07$
Neelam	$6.5^{b} \pm 0.03$	$6.65^{ab} \pm 0.15$	$6.9^{b} \pm 0.07$	$6.3^{a} \pm 0.07$	$6.7^{b} \pm 0.04$	$6.6^{b} \pm 0.07$
Rumani	$7.1^{bc} \pm 0.07$	$6.8^{bc} \pm 0.07$	$7.3^{bc} \pm 0.02$	$7.5^{ab} \pm 0.03$	$6.6^{a} \pm 0.07$	$7.0^{ab} \pm 0.14$
Himami pasand	$6.1^{a} \pm 0.07$	$6^{a} \pm 0.14$	$6.3^{a} \pm 0.03$	$6.7^{b} \pm 0.07$	$6.9^{ab} \pm 0.03$	$6.2^{a} \pm 0.07$

Values are given as mean ± SD; Values not sharing a common superscript letter significantly at $P < 0.05$ (DMRT) (Source: Kumar et al. 2011)

30.4.6.7 Sensory Analysis

Sensory properties of mango wine produced from eight different mango fruits were also performed. Trained panelists detected the significant differences among the wines in the characteristics of flavour, taste, texture, appearance, color, sweetness, and overall quality (Table 30.14). Panelists determined that the wine made from *Alphonso*, *Raspuri*, *Banginapalli* and *Totapuri* were significantly paler than the wines from *Sindhoora*, *Neelam* and *Rumani* ($p < 0.05$). The mouth feel of wines made from *Alphonso*, *Banginapalli* and *Totapari* were similar with slightly smooth to smooth consistency. Mouth feel of the wines made from *Raspuri, Rumani, Sindhoora, Neelam* and *Himami pasand* tended to be "neither smooth nor grainy". As for wines made from *Alphonso* and *Banginapalli,* the panelists indicated the mouth feel was "smooth". In overall quality, the results suggested that the wines made from *Raspuri, Rumani, Sindhoora, Neelam* and *Himami pasand* mango varieties were significantly lower in quality than the wines made from fresh *Alphonso*, *Banginapalli* and *Totapari* ($p < 0.05$). The results suggested that the wines made from *Alphonso*, *Banginapalli* and *Totapuri* received almost similar overall quality score (Table 30.14).

Correlation analysis revealed that there was a slightly positive correlation between taste and flavor ($r = 0.17$, $p < 0.01$), taste and mouth feel ($r = 0.20$, $p < 0.01$), and taste and overall quality ($r = 0.11$, $p < 0.01$). These results indicated that when the score of taste increased, the scores of flavor intensity, mouth feel, and overall quality also increased. In addition, correlation analysis results also suggested that overall quality was positively correlated with melon flavor ($r = 0.54$, $p < 0.01$) and mouth feel ($r = 0.32$, $p < 0.01$). If the scores of melon flavor and mouth feel increased, the score of overall quality increased as well (Table 30.15).

Table 30.15 Correlations of characteristics of mango wines from panelist and instrumental evaluation

	Appearance	Taste	Flavour	Texture	Mouth feel	Overall acceptability
Appearance	---	0.040	0.138	–0.114	–0.86	0.063
Taste		---	0.426**	–0.85	0.265**	0.389**
Flavour			---	–0.143	0.168**	0.365**
Texture				---	0.288**	–0.182**
Mouth feel					---	0.636**
Overall acceptability						---

**Correlation is significant at the 0.05 level (Source: Kumar et al. 2011)

30.4.7 Mango Vermouth

Vermouth is officially classified as an 'aromatized fortified wine'. This is prepared by fortification and infusion of proprietary recipe of different plants, barks, seeds, fruit peels collectively known as botanicals. This type of wine is quite popular in Europe, USA, Russia and Poland. The word vermouth derived from the German word "wermut"-means man with courage/spirit. The alcohol content in vermouth varies from 15% to 17%.

The production of aromatic wine from mango juice is known as mango vermouth. The base wine was made from the juice of *Banginapalli*. after raising to 22°B with cane sugar, adding 100 ppm SO_2, 0.5% pectinase and carrying out the fermentation at 22°C using Montrachet strain 522 of *S. cerevisiae*. The composition of mango vermouth in respect to pH, ethanol, total acidity, total phenols and aldehyde was comparable to vermouth prepared from grapes. The herbes and spices used in mango vermouth were black pepper, coriander, cumin, Bishop's weed, clove, large cardamom, saffron, fenugreek, nutmeg, cinnamon, poppy seeds, ginger, lichen and flame of forest. The sensory evaluation tests (Table 30.16) suggested that the product had very good quality as compared with grape vermouth (Onkarayya 1986; Martinez et al. 1987).

30.4.8 Cost Economics

Approximately, 500 ml of juice can be obtained from 1 kg of mangoes. In order to produce 1 l of wine, it requires about 1,250 ml juice, as there would be fermentation and evaporation losses. This means that 2.5 kg of mangoes are required costing around Rs.75.00 at the present raw material cost. And about 40% of the raw material cost would be the processing cost. Hence, 1 l of wine would cost approximately Rs. 105.00. However, scale-up studies need to be carried out for an actual assessment of the production cost of mango wine.

Table 30.16 Physico-chemical characteristics and sensory quality of mango vermouth

Herbs mixture & type of vermouth	Colour (at 420 nm)	pH	Total acidity (tartaric acid/100 ml)	Volatile acidity (g. AA/100 ml)	Alcohol (%, v/v)	Total aldehyde (ppm)	Total Phenolics (%)	Organoleptic scores (out of 20)
Dry vermouth								
Formula A	0.420	3.40	0.59	0.088	17.0	15.8	0.055	13.00
Formula B	0.658	3.50	0.60	0.087	17.5	20.9	0.064	11.50
Sweet vermouth								
Formula C	0.678	3.42	0.59	0.071	17.2	26.4	0.070	15.50
Formula D	0.690	3.50	0.61	0.091	18.0	56.3	0.075	13.60

(Source Martinez et al. 1987)

30.5 Conclusions and Future Perspectives

Fruits produced in tropics are highly perishable commodities and have to be either consumed immediately or preserved in one or the other form. In the developed countries, considerable quantities of fruits are utilized, but in developing countries, lack of proper utilization resulted in considerable postharvest losses, estimated to be 30–40%. The increased production could be utilized profitably, if fruit wines are produced. Wines made from complete or partial alcoholic fermentation of grape or any other fruit generally contain ethyl alcohol as the intoxicating agent, as well as essential elements, vitamins, sugars, acids and phenolics. Wines from fruits are preferable to distilled liquors for their stimulatory and healthful properties. These beverages also serve as important adjuncts to the human diet as they increase the satisfaction and contribute to the attributes necessary for proper digestion and absorption of food. Setting up of fruit wineries along with the fruit-growing belts would result in economic upliftment of the people, by generating employment opportunities and providing better returns to the orchardists. Availability of a suitable technology is the single important factor that determines the production cost and an acceptable product; and a significant consideration would be its popularisation. Hence production of fruit wines in those countries where fruits other than grape are grown would certainly be advantageous. Inclusion of enzymes for flavour enhancement like β-glucosidase, bioreactor technology, enzymes for clarification and juice extraction, continuous fermentation, type of sweetening agent, deacidification of the fruit acids and others would lead to a product with good consumer acceptability. However, it is essential to carry out and stimulate research and development activities on production, evaluation and marketing of non-grape fruit wines with the financial support of Government and Private sectors.

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Chapter 31
Biosystem Development for Microbial Enhanced Oil Recovery (MEOR)

Anuradha S. Nerurkar, Harish G. Suthar, and Anjana J. Desai

Abstract Conventional primary and secondary oil recovery processes recover only one third of the oil present in the oil reservoir. EOR technologies produce an additional oil recovery after these processes cease to produce oil. Microbial enhanced oil recovery (MEOR) includes the tertiary oil recovery process where microorganisms or their metabolites are used to retrieve the unrecoverable oil from oil reservoirs. Oil recovery mechanisms facilitated by microorganisms are attributed to the metabolites produced by them. Biosurfactants, biomass, biopolymers, solvents, acids, gases, etc. are notably the products of microbial growth in the reservoir. MEOR approaches are either *in situ* or *ex situ.* In *in situ* approach growth of the indigenous microbes is stimulated or isolated microbes having specific traits are injected into oil reservoir along with the nutrients where production of the metabolites causes oil release. The approach where microbes are cultivated outside in a fermentor on inexpensive raw materials and they and/or their crude metabolites, viz. biosurfactant, biopolymer, etc. are injected into oil reservoir in order to retrieve the unrecoverable oil, is known as *ex situ* oil recovery. The technical feasibility of oil recovery was assessed in porous model sand packed columns with biosystems developed separately comprising of the specially isolated *B. licheniformis* TT42, K125 and TT33. The crude biosurfactant of *B. licheniformis* K125 gave 43 ± 3.3% additional oil recovery upon application to sand column which is 13.7% and 8.5% higher in comparison to the standard strains, *Bacillus mojavensis* JF-2 and *Bacillus licheniformis* TT42 respectively. *B. licheniformis* when tested in sand packed

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column by injecting the biomass and the nutrients gave 27.7 ± 3.5% oil recovery. Lack of holistic approach allowing for a critical evaluation of economics, applicability and performance of MEOR is missing. For a thorough assessment of this process a multidisciplinary approach is warranted.

Keywords Microbial enhanced oil recovery • Biosurfactant • Bioemulsifier • Biopolymer • Biosurfactant flooding • Selective plugging • *Bacillus licheniformis* • Sand pack column

31.1 Introduction

Crude oil is an important and most sought after commodity world over since it accounts for 40% of the global energy demand that makes the economy vulnerable to fluctuating oil prices. Conventional technologies (primary and secondary oil recovery processes) recover only one third of the oil present in the oil reservoir. Hence, it becomes imperative to use alternative advanced technologies to recover maximum oil from the depleted reservoir by all possible means. These technologies called Enhanced Oil Recovery (EOR) technologies encompass the tertiary stage operations in a three stage oil recovery process viz. primary, secondary and tertiary. EOR is a generic term under the umbrella of which fall various tertiary stage processes intended to increase the oil production after the secondary stage processes cease to produce oil. Primary and secondary stage processes recover upto 35–45% of original oil in place (OOIP), a term used for total hydrocarbon content (oil) of a reservoir. EOR technologies produce an additional oil recovery after these processes cease to produce oil.

Primary stage process makes use of natural forces to drive the oil to the surface of oil reservoir which may be aided by pumping sometimes. These mechanisms include natural water displacing the oil upwards in the oil reservoir, expansion of natural gas at the top of the reservoir, expansion of gas initially dissolved in the crude oil and gravity drainage due to movement of oil within the reservoir. Secondary stage operations are employed when primary stage natural forces become insufficient to force the oil to the surface. The natural drive in the reservoir is replaced by an artificial one by injecting fluids to increase reservoir pressure. Other secondary processes like water flooding, natural gas injection and gas lift are used to mobilize the original oil in place (Tzimas et al. 2005). Hall et al. (2003) have observed that one trillion barrels of oil have been recovered globally and about two to four trillion barrels remain in oil reservoirs, a large target for EOR technologies. This implies that even after the secondary production becomes uneconomical a large amount of oil about 50–70% still remains trapped in the reservoir (called residual oil). This residual oil makes upto two thirds of the OOIP and is the target of the EOR processes (Brown 2010; Bryant 1987; McInerney et al. 1999).

31.2 Microbial Enhanced Oil Recovery (MEOR)

EOR technologies fall under two major groups, viz. thermal and chemical flooding processes. Heat reduces the viscosity of displaced oil, polymers increase the viscosity of displacing fluid (water) and chemical surfactants decrease the interfacial tension (IFT) between water and oil. These processes are environmentally hazardous, costly, and leave undesirable residues difficult to dispose off without adversely affecting the environment (Bryant 1987). A complete classification of EOR methods is given by Farouq and Thomas (1994).

Normally MEOR refers to the use of microorganisms in the oil bearing formations itself (Brown 2010; Youseff et al. 2009). However MEOR now includes the tertiary oil recovery process where microorganisms or their metabolites are used to retrieve the unrecoverable oil from oil reservoirs (Fig. 31.1). It is comparable to and capable of replacing the existing EOR technologies (Banat 1995). Recent exhaustive reviews on MEOR appraise its technical feasibility (Brown 2010; Sen 2008; Youseff et al. 2009).

MEOR offers several advantages over other EOR technologies (Khire 2010). The microbes as well as nutrients are inexpensive, easy to handle, economically attractive, give an incremental increase in oil and are ecofriendly. Implementation of MEOR needs minor modification of existing facilities and cost of injected fluids is not dependent on the oil prices. Efficiency of MEOR is particularly good in case of carbonate reservoirs (Lazar et al. 2007).

McInerney et al. (1999) have grouped MEOR processes under three main categories viz. (i) Well bore clean out processes to remove deposits from the well where hydrocarbon degrading bacteria are generally useful. (ii) Well stimulation where anaerobic bacteria and fermentable substrates are added to improve the

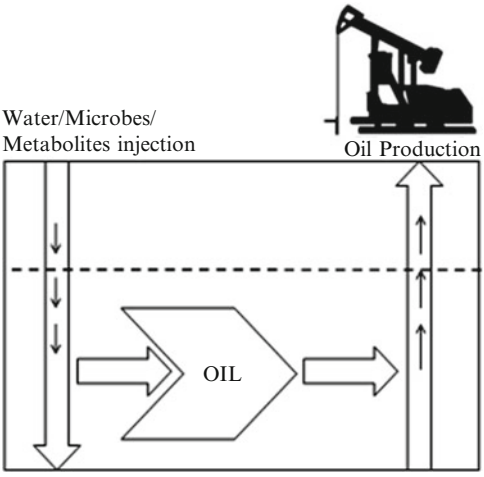

Fig. 31.1 Implementation of MEOR

wettability of rock and drainage of the oil in the well. (iii) Microbially enhanced waterflooding processes involving injection of nutrients and/or microorganisms into the reservoir. In carbonate formations acids while in sandstone formation biosurfactants are believed to cause oil release.

31.3 History of MEOR

MEOR has very much attained the technology state since it was first proposed. Beckman was the first to propose in 1926 that microorganisms could be used to release oil. In 1940s ZoBell and coworkers drew the attention of investigators and oil producers by their work. A patent was drawn by them on injection of *Desulfovibrio hydrocarbonoclasticus* along with nutrients in the oil reservoir (Bryant et al. 1989). Updegraff patented injection of a mixture of *Clostridium spp., Aerobacter aerogenes, E. coli* and *Bacillus polymyxa* with carbohydrate medium with the idea that the products of the microbial growth viz solvents, acids, gases and biosurfactants would effect oil release (Updegraff 1957 US patent). Davis reviewed the current knowledge of Petroleum Microbiology emphasizing the potential of MEOR. Investigations spanning over 1970–2000 have established the basic nature and existence of indigenous microbiota in oil reservoirs as well as reservoir characteristic essential for successful MEOR (Lazar et al. 2007). Laboratory simulation experiments and field tests were carried out in United States, former Soviet Union, Eastern European countries and China from 1990s and thus the MEOR technology developed into a new era (Zhang and Xiang 2010).To solve the problems of microbial cell plugging Hitzman proposed the use microbial spores which have small size (Bryant et al. 1989). Lappin-Scott et al. (1988) suggested the use of ultramicrobiota having less than 0.1 μ size to address this. Introduction of nitrates, nitrites and phosphates were used to control corrosion caused due to H_2S production arising out of growth of sulfate reducing bacteria (Sen 2008; Youseff et al. 2009). Thus, MEOR was developed as an alternative method for the tertiary extraction of oil from reservoirs, since after the petroleum crises in 1973, the EOR methods became less profitable. Starting from 1950s, MEOR studies were run on three broad areas, namely, injection, dispersion, and propagation of microorganisms in petroleum reservoirs, selective degradation of oil components to improve flow characteristics, and metabolites production by microorganisms and their effects (Lazar et al. 2007).

31.4 Factors Governing Oil Recovery

The major microbial activities that play a critical role in MEOR are biosurfactant, biopolymer production and selective plugging. In biosurfactant flooding approach the oil mobilization is through increasing the capillary number. The selective plugging approach aims at improving the volumetric sweep efficiency brought

about by the biopolymer or biomass production. Volumetric sweep efficiency is the fraction of total reservoir that is in contact with recovery fluid usually water. It dominates the oil recovery when there are large variations in the viscosities of water and the oil. This causes the water to move faster than the oil. Poor sweep efficiencies occur in reservoir that have large permeability zones called thief zones that conduct most of the injected water (Sarkar et al. 1989). Other reports on oil recovery studies have shown that poor oil recovery in oil reservoirs is mainly due to the capillary forces retaining oil and permeability variation in the reservoir. (Almeida et al. 2004; Banat 1995; Shah 1981; Yakimov et al. 1997).

Formidable challenge to tertiary oil recovery is posed by the fact that the oil is trapped in the reservoir pores in inaccessible manner. Several factors influence the oil recovery from these pores. The pore size of the rock determines the relative fluid saturation of water and oil. Accumulation of deposit like paraffin, asphalting, scale etc. plugs the drainage. Removal of these deposits can alter the fluid saturation making oil mobile. The oil remains trapped in the reservoir rock due to competition between two forces viz. viscous forces that mobilizes the oil and capillary forces that trap the oil. The oil displacement efficiency of a recovery process is determined by the ratio of these two forces (Shah 1981). The viscosity and capillary forces holding the oil in place are expressed as a ratio called capillary number (N_{ca}) that is inversely proportional to the interfacial tension between oil/water (Fulcher et al. 1985; Youseff et al. 2009). Lake (1989) estimated that the interfacial tension must be lowered to 10^{-2} to 10^{-3} mN/m corresponding to $N_{ca} = 10^{-3}$ to 10^{-4} before significant oil is recovered. Therefore, an EOR method has following goals: (i) Improvement of mobility ratio by decreasing the viscosity of oil or by increasing the viscosity of displacing fluid (ii) Increasing capillary number by decreasing the interfacial tension. As the interfacial tension decreases the capillary number increases making the interface between the displacing and the displaced fluids disappear (Farouq and Thomas 1994). This means steep decrease in the interfacial tension between oil/water due to surfactants is required to cause the oil to release from the rock pores.

Yakimov et al. (1997) confirmed the conditions stated by Jenneman & coworkers and Lappin-Scott & coworkers that must be met to achieve successful *in situ* bacterial growth and core plugging. These include (i) The cells must be transported throughout the rock stratum. (ii) Nutrients must be transported for growth. (iii) The microorganisms must be able to grow and reduce the permeability of the rock by biomass and extracellular polymer production. In order to minimize the risk of skin plugging in the direct viscinity of the well bore, the microbial growth must not be so rapid that results in the formation of bacterial plug further away from the well bore.

A mathematical model describing the process of MEOR has been given by Nielsen et al. (2010) which suggests a method where sensitive parameter can be estimated in order to obtain incremental oil recovery. This has been discussed in perspective of other models for MEOR suggested by different workers. Modeling of MEOR process would help to troubleshoot the existing problems in implementation of the process.

31.5 Mechanisms of MEOR

Oil recovery mechanisms facilitated by microorganisms are attributed to the metabolites produced by them (Fig. 31.2). Biosurfactants, biomass, biopolymers, solvents, acids, gases, etc. are notably the products of microbial growth in the reservoir (Banat et al. 2008; Bryant 1987; Khire 2010; Lazar et al. 2007; Sarkar et al. 1989; Youseff et al. 2009). These metabolites contribute to tertiary oil recovery variously by gas production leading to increase in pressure, acid production resulting in degradation of limestone matrices, solvent production causing decrease in viscosity, biosurfactant production leading to reduction in interfacial tension at oil/water interface and biomass accumulation leading to selective plugging of high permeability zones (Almeida et al. 2004; Banat 1995; Yakimov et al. 1997). A recently patented "MMMAP" (Multi-strain Mixed Microbial Application) consortium consisting of thermophilic, barophilic, acidophilic and anaerobic strains belonging to *Thermoanaerobacterium* sp., *Thermotoga* sp. and *Thermococcus* sp. isolated from oil reservoir water is claimed to be active in producing biosurfactants, fatty acids, alcohols, methane and CO_2 at *in situ* temperature up to 90°C. Its injection into reservoirs supplemented with specific nutrients resulted in three-fold increase in oil recovery (Lal et al. 2007 US patent).

The oil is in the form of discontinuous ganglia trapped in the pores of reservoir rocks. Biosurfactants contribute to oil recovery by reducing the interfacial tension between oil/water by affecting the capillary number which determines the microscopic displacement efficiency of oil from the pores. Biosurfactant production as a mechanism of oil recovery has been the major mechanism of MEOR studies done by Jenneman et al. (1983), Javaheri et al. (1985) and Yakimov et al. (1995).

Permeability variations in the oil field limit the oil recovery. During water flood operations the injected water moves through the high permeability zones without displacing the oil from low permeability zones. To improve the sweep efficiency, selective plugging mechanism can be used. In selective plugging, high biomass produced by selected microorganism plugs the high permeability zones, resulting in a change of direction of the water flood to oil-rich channels, consequently increasing the sweep efficiency of oil recovery with water flooding (Raiders et al. 1986, 1989).

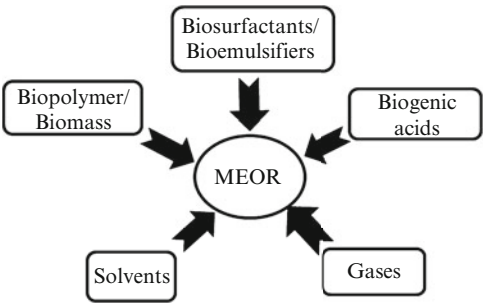

Fig. 31.2 Mechanisms of MEOR

Kalish et al. (1964) studied the effects of biomass plugging by bacteria in sandstone cores and determined that the slime-forming and larger bacteria have a much greater effect on permeability reduction than the smaller and non-slime-forming ones. Jang et al. (1983) and Jenneman et al. (1982) examined the effect of rock permeability on bacterial transport. Both concluded that nutrients such as dissolved sucrose, inorganic salts and yeast extract move easily through porous media. Jang et al. (1983) demonstrated that bacterial spores, because of their size and cell surface properties, travel faster with less adsorption to rock through sandstone cores than bacterial cells. Jenneman et al. (1982) reported that nutrients and bacterial cells are selectively transported to zones of high permeability.

Several methods have been proposed to selectively plug such high permeability zones using thickening agents like polymers, gels, clay, paraffins and resins. Use of xanthan in selective plugging has been suggested by Finnerty and Singer (1983). Bacteria injected in the reservoir are capable of plugging high permeability zones (Silver et al. 1989 US patent). Microbial permeability profile modification could be more effective by increasing the *in situ* production of polymer by indigenous microflora (Brown et al. 2002). Growth of microorganisms *in situ* can selectively plug high permeability zones and the process can be controlled by alterations in the method of nutrient injection (Raiders et al. 1986). Yakimov et al. (1997) observed that *B. licheniformis* strains produced increased oil by biogenic acid production and selective plugging MEOR.

31.6 MEOR Approaches

MEOR strategies or approaches are either *in situ* or *ex situ* (Fig. 31.1). In *in situ* approach the indigenous microbes are stimulated or specially isolated microbes are injected into oil reservoir along with the nutrients where they produce the metabolites causing oil release. The strategy where microbes are cultivated outside in a fermentor on inexpensive raw materials and they or their crude metabolites, viz. biosurfactant, biopolymer, etc. are injected into oil reservoir in order to retrieve the unrecoverable oil, is known as *ex situ* oil recovery. (Banat et al. 2008; Brown 2010; Finnerty and Singer 1983; Lazar et al. 2007; Sen 2008; Youseff et al. 2009).

McInerney et al. (1985a US patent) patented a process using *B. licheniformis* JF-2 and its surfactant produced for enhancement of oil recovery. They have also shown that strains that are enhanced biosurfactant producers could be screened for improved transport properties for application in MEOR (McInerney et al. 2005). The *in situ* MEOR is a logical and attractive approach however, microorganisms are subjected to a number of stresses like extremes of temperature and pressure which may affect the growth and metabolite production. pH, salinity and heavy metals are other factors that affect microbial growth (Kosaric 1992). For successful *in situ* approach presence of appropriate microbes in the reservoir need to be ensured. If appropriate microbes are absent, no microbial activity will be stimulated, resulting in inefficiency in oil production. The *in situ* strategy therefore is unpredictable.

In another instance specially selected strains for reservoir conditions together with required nutrients are injected to reduce the lag period in stimulation of microbial activity. Here, the microbes should be able to transport themselves and reach the oil pores inaccessible to secondary operations. Another *in situ* mechanism to modify permeability profile is to stimulate the *in situ* growth of microorganisms (Brown 1984 US patent; McInerney et al. 1985b US patent;, Yakimov et al. 1997). Biopolymers and microbial biomass are used as plugging agents to improve volumetric sweep efficiency. The plugging effect of a number of biopolymers, namely xanthan, polyhydroxybutyrate (PHB), guar gum, polyglutamic acid (PGA) and chitosan, investigated in a laboratory-pressurized pumping flow system established the correlation between biopolymer structure and plugging effect. The plugging effect is influenced by the structure of biopolymers (Khachatoorian et al. 2003). Silver et al. (1989 US patent) patented a process where a bacterial strain when injected in the reservoir is capable of plugging the high permeability zone driving the oil release.

Another attractive alternative is *ex situ* MEOR as it answers many problems faced in *in situ* approach (Kosaric 1992). The Injection of *ex situ* produced microbes or their products like biosurfactants/biopolymer is appropriate when conditions of the well are too harsh to support microbial growth. The biosurfactants/biopolymers can be produced from renewable inexpensive resources in high concentrations so that cost intensive purification is avoided. Also to ensure that proper concentration of biosurfactant is available addition of *ex situ* produced biosurfactant much more above its CMC value is possible. Loss of the injected chemical is a major concern and a problem that plagues chemical EOR approaches. The *ex situ* biosurfactant mediated oil recovery has been studied by McInerney et al. (2003) and found to be economically attractive and distinctly advantageous over *in situ* MEOR. Their studies have focused on effective biosurfactant development, its use in porous systems and computer simulation etc. Biosurfactant flooding requires mobility control and hence must be injected with a viscous agent like acrylamide and cosurfactant like 2,3-butanediol to prevent its loss (McInerney et al. 2004). Youssef et al. (2007) provided evidence that biosurfactants are produced *in situ* in concentrations needed to mobilize oil. The average concentration of lipopeptide biosurfactant in the produced fluids of the inoculated wells is 90 mg/l. This concentration is approximately nine times the minimum concentration required to mobilize entrapped oil from sandstone cores. The metabolite produced by microorganisms cause desirable changes in physicochemical properties of the crude oil and no change in the properties of the reservoir rock. In many cases biosurfactants have proven more effective than chemical surfactants. The use of biosurfactant is obvious for closed system in oil reservoir than in open system. (Banat 1995).

31.7 Biosurfactants

Microbial surfactants or biosurfactants are a structurally diverse group of compounds consisting of hydrophilic and hydrophobic domains which partition preferentially at interfaces such as oil/water and air/water interfaces (Georgiou et al. 1992; Desai and

Banat 1997). Biosurfactants are low molecular weight while bioemulsifiers are high molecular weight surfactants from microbial origin (Rosenberg and Ron 1999). Like chemical surfactants biosurfactants also are capable of reducing surface and interfacial tension. This property of partitioning at interfaces makes biosurfactants good candidates of MEOR (Javaheri et al. 1985; Youssef et al. 2004). Surfactants can increase concentraion of hydrophobic compounds in water. This is achieved through formation of oil/water emulsion above the CMC (Critical Micelle Concentration) of a given surfactant. CMC is that concentration of surfactant that favours micelle formation. Micelle formation allow partitioning enabling dispersion of a compound in solution above its water solubility limit (Youseff et al. 2009). CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant. A sharp increase in surface tension is obtained below CMC of a surfactant. Microbial culture broth or biosurfactants are diluted several-fold, surface tension is measured for each dilution, and the CMC is calculated from this value (Desai and Banat 1997).

Emulsifiers or high molecular weight surfactants can also be classified according to their HLB (Hydrophile-Lipophile Balance). The HLB classification can be used to determine the suitability of use of surfactant. In general, an emulsifier with a low HLB is lipophilic, whereas a high HLB confers better water solubility. Emulsifiers with low HLB are suitable for water-in-oil (W/O) emulsions and the ones with high HLB are suitable for oil-in-water (O/W) emulsions. Bioemulsifiers are (lipo) polysaccharides, (lipo) proteins or combinations of these. They do not reduce interfacial tension appreciably but instead bind tightly to oil–water interface and stabilize the emulsion (Christofi and Ivshina 2002).

Biosurfactants have distinct advantages over the chemical surfactants with respect to their low toxicity, ecofriendly nature due to biodegradability, biocompatibility, digestibility, economical cost of production and recovery and higher foaming, high selectivity and specific activity at extreme temperatures, pH and salinity (Desai and Banat 1997; Kosaric 1992). They have an edge over surfactants since they can be fermentatively produced from inexpensive raw materials (Lin 1996).

Biosurfactants vary greatly in their structures and properties and are reported to play various roles unique to the physiology and ecology of the producing microorganisms. The reported physiological roles of biosurfactants include (i) solubilization of hydrophobic substrates (ii) antagonism towards other microbes (iii) cell adherence (iv) cell desorption (v) gliding and swarming motility (vi) cell-cell interaction (vii) avoidance of toxic and heavy metal elements (viii) protection against high ionic strength, and (ix) byproducts released in response to environmental changes (x) adhesion/deadhesion from interfaces, (xi) regulation of cell surface hydrophobicity of bacteria (Neu 1996; Ron and Rosenberg 2001).

Biosurfactants are produced by a variety of bacteria and fungi having different chemical nature (Table 31.1). Since bacterial growth is exponential it should be possible to produce large amounts of metabolites from inexpensive and renewable resources (Youseff et al. 2009). Biosurfactants and biopolymers can be produced from cheap renewable sources in amounts sufficient for injection without extensive purification. While majority of the reports on biosurfactant production are from hydrocarbon substrate, *Bacillus* spp. produce them during growth on glucose or

Table 31.1 Some biosurfactant/bioemulsifier producing bacteria/fungi and type of their product (Compiled from Desai and Banat 1997; Banat 1995; Mulligan 2005)

Microorganism	Biosurfactant	Microorganism	Bioemulsifier
Pseudomonas aeruginosa	Rhamnolipids	*Nocardia erythropolis*	Neutral lipids
Rhodococcus eryhtropolis	Trehaloselipids	*T. thiooxidans*	Phospholipids
Mycobacterium sp.	Sophorolipids	*Acinetobacter calcoaceticus*	Emulsan
Ustilago maydis	Cellobiolipids	*Candida lipolytica*	Liposan
Bacillus subtilis	Surfactin	*A. radioresistans*	Alasan
B. licheniformis	Lichenysin	*Rhodotorula glutinous*	Polyol lipids
Serratia marcescens	Serrawetin	*Bacillus* sp.	Carbohydrate-Lipid-Protein
P. fluorescens	Viscosin	*Streptomyces tendae*	Streptofactin
Corynebacterium lepus	Fatty acids	*Penicillium spiculisporum*	Spiculisporic acid
Lactobacillus fermentii	Diglyconyl diglyceride	*Pseudomonas* sp.	Lipopolysaccharide
Arthrobacter sp.	Arthrofactin	*P. marginalis*	Particulate
Thiobacillus thiooxidans	Ornithine,lysine peptides	*A. calcoaceticus*	Vesicles, fimbriae

non-hydrocarbon media (Cooper and Goldenberg 1987; Mulligan et al. 1984). In our laboratory the biosurfactant production has been optimized using *B. licheniformis* strains TT42 from inexpensive raw materials for economizing the production by statistical media optimization method (Suthar 2010). Das and Mukherjee (2007) have reported production of biosurfactant by *Bacillus* spp. on potato as cheap carbon source to reduce the cost. Commercial production of biosurfactants is reviewed by Mukherjee et al. (2006). Makkar and Cameotra (2002) have discussed the unconventional sources of raw materials used for biosurfactant production. Application of response-surface methodology was done to evaluate the optimum medium components for the enhanced production of lichenysin by *Bacillus licheniformis* R2 in our laboratory. Biosurfactant production increased by fourfold in terms of critical micelle dilution (CMD) (Joshi et al. 2008b). With *B. licheniformis* K51 the medium optimization increased tenfold (Joshi et al. 2007). CMD is defined as reciprocal of the biosurfactant dilution at which a sharp increase in surface tension is observed (McInerney et al. 2005). Molasses and whey have been used as subtrates to produce biosurfactants by *Bacillus* spp. (Joshi et al. 2008d). By increasing the lichenysin yield via this experimental design approach, the production cost of lichenysin would markedly be reduced, enhancing feasibility of commercial application of this biosurfactant. McInerney et al. (2003) have noted that addition of peptides to nutrient medium enhances growth and biosurfactant production of *B. mojavensis* JF-2.

The largest market for biosurfactant is the oil industry for petroleum production and incorporation into oil formulations. Banat (2008) has summarized various applications of biosurfactants in the petroleum industry as wetting, coating, dispersing, solubilizing, emulsifying and surface active agent. Other applications related to

the oil industries include oil spill bioremediation, dispersion, both in land and at sea, removal/mobilization of oil sludge from storage tanks and enhancement of oil recovery (Georgiou et al. 1992). Various potential applications of biosurfactants in other industries have been listed by Banat et al. (2000) and Van Dyke et al. (1991). They find use in wide applications, important of which are in pharmaceutical, cosmetics, food, detergents and textile industries (Healy et al. 1996). Their environmental applications are in MEOR, oil pollution control and subsurface bioremediation (Banat 1995; Batista et al. 2006; Christofi and Ivshina 2002; Joshi and Desai 2010; Karanth et al. 1999; Lin 1996; Mulligan 2005; Zhang and Xiang 2010). The antibacterial, antifungal and antiviral activities of biosurfactants make them suitable for therapeutic applications (Rodrigues et al. 2006). Both organic and inorganic contaminants can be treated through desorption or biodegradation process by influencing their bioavailability (Mulligan 2005). Mixture of biosurfactants/synthetic surfactant promise to be economic approach for surface remediation of light non-aqueous phase liquids (NAPL) (Nguyen et al. 2008).

Chemically biosurfactants are heterogenous group falling under different classes like (i) Glycolipids e.g. rhamnolipids, trehaloselipids, sophorolipids, cellobioselipids (ii) Lipopeptides, e.g. Serrawetein, Visconsin, Surfactin, Subtilisin, Lichenysin etc. (iii) Fatty acids, neutral lipids and phospholipids (iv) Polymeric surfactants, e.g. Emulsan, Liposan, Biodispersan etc. (v) Particulate biosurfactants e.g. vesicles, fimbriae, whole cells etc. (Desai and Banat 1997; Gautam and Tyagi 2006; Karanth et al. 1999; Maneerat 2005). The low molecular weight biosurfactants that are of importance in MEOR are glycolipids like rhamnolipids and lipopeptides like surfactin and lichenysin.

Lipopeptides class of surfactants are of interest because of their wide range of biological activities. The cyclic lipopeptides are low molecular weight biosurfactant with a hydrophilic head made of cyclic heptapeptides and a hydrophobic tail of long chain fatty acids. They lower the interfacial tension between the oil and water phases to 0.1 mN/m, which is sufficient to increase the capillary number to mobilize oil. The critical micelle concentration of biosurfactants is lower than synthetic surfactants (McInerney et al. 2005; Youssef et al. 2004). Cyclic lipopeptides are mainly described in *Bacillus* spp. Broad spectrum of activities like antibiotic, antifungal, antiviral, antitumour, immunomodulator, toxin and enzyme inhibitor etc. are attributed to them which is due to the surface and membrane activity of these lipopeptide (Vater et al. 2002). *Bacillus* sp. isolated from oil contaminated soil produce biosurfactant over a wide range of pH, salinity and temperature (Kumar et al. 2007). Improvement in the surface activity of lichenysins is influenced by the fatty acid moiety (Youssef et al. 2005). Lipopeptides are stable at high temperatures , wide range of pH , and at high salt concentrations (Abu-Ruwaida et al. 1991; Bordoloi and Konwar 2008; Das and Mukherjee 2007; Joshi et al. 2008c, d; Makkar and Cameotra 1997; McInerney et al. 1985a). Lichenysins are named as lichenysin A (Yakimov et al. 1995), B (Lin et al. 1994), C (Jenny et al. 1991), D (Konz et al. 1999), G (Grangemard et al. 1999) and BL86 (Horowitz and Griffin 1991). A comparative account of these lichenysin as regards their chemistry , physical properties and genes involved is provided (Nerurkar 2010). Notable are the observations that

Table 31.2 *B. licheniformis* strains isolated for MEOR

Strain	Habitat of isolation	Mechanism of MEOR	Reference
B. mojavensis JF-2	Oil-field injection brine	Biosurfactant mediated	Jenneman et al. (1983)
B.licheniformis BAS50	Northern German reservoir at the depth of 1,500 m	Biosurfactant mediated	Yakimov et al. (1995)
B. licheniformis BNP29	Northern German reservoir at the depth of 866–1,520 m, 20 mPa pressure 50°C	*in situ* growth and Selective plugging	Yakimov et al. (1997)
B. licheniformis K51	Oil well, in Gujarat, India	Biosurfactant mediated	Joshi et al. (2007)
B. licheniformis TT42	Tuwa Timba hot water spring Gujarat, India	Biosurfactant mediated	Suthar et al. (2008)
B. licheniformis K125	Formation water of Kalol oil reservoir no. 125, Gujarat, India	Bioemulsifier mediated	Suthar et al. (2008)
B. licheniformis R2	Crude oil contaminated region of Kutch desert, Gujarat, India	Biosurfactant mediated	Joshi et al. (2008b)
B. licheniformis 20B	Fermented food	Biosurfactant mediated	Joshi et al. (2008c)
B. licheniformis ACO1	Petroleum reservoir in Iran	Permeability profile modification	Dastgheib et al. (2008)
B. licheniformis TT33	Tuwa Timba hot water spring Gujarat, India	Selective plugging due to biofilm formation	Suthar et al. (2009)

lichenysin B gives lowest ever interfacial tension of 0.006 mN/m against decane, reported by any biosurfactant (Lin et al. 1994) while surfactant BL86 and Lichenysin B have recorded lowest ever CMC of 10 mg/l by any biosurfactant (Horowitz and Griffin 1991; Lin et al. 1994). Enhanced biosurfactant at the concentration of 391 mg/l was produced by a mutant of *B. licheniformis* which lowers the surface tension to 26.5 mM/m and has low CMC of 10 mg/l (Lin et al. 1994). Table 31.2 depicts the *B. licheniformis* strains specially isolated for MEOR purposes in our and other laboratories. Their habitats and properties like surface tension and interfacial tension lowering abilities notable for MEOR are compared.

31.8 Microorganisms in MEOR

Youseff et al. (2009) have exhaustingly reviewed the microbial ecology of oil well. Culture dependent and culture independent approaches have been used to analyze the community structure. Anaerobes and facultative bacteria are more prevalent, while sulfate reducing, fermentative, hyperthermophilic, autotrophic, nitrate, iron and manganese reducers are the other prevalent groups. Novel lineages are reported

from culture independent studies. It is possible to alter the dynamics of natural microbial population of oil reservoir during MEOR (McInerney et al. 2003).

The conditions in the reservoir of low pH, high salt concentration and anaerobiosis create a stressful environment for microorganisms. The competition for nutrients adds to it. This imposses strict constraints and selects microorganisms accordingly. There is no dearth of biosurfactant producing microorganisms. Biosurfactant producing microorganisms are naturally present in hydrocarbon-impacted sites (Jennings and Tanner 2000) and metal contaminated and undisturbed soils (soils which are not contaminated with hydrocarbons) (Bodour et al. 2003). Isolation of biosurfactant producing bacteria from oil reservoirs of different parts of the world (Jenneman et al. 1983; Tabatabaee et al. 2005; Yakimov et al. 1995) and petroleum contaminated sites (Batista et al. 2006) have been documented. Bioemulsifier producing bacteria have been isolated from Iranian oil reservoir (Amiriyan et al. 2004). Existence of biosurfactant producing microbes in soil contaminated with diesel have good surface and emulsification activity (Menezes et al. 2005). Wang et al. (2008) based on PCR-DGGE fingerprint observed that after injection of exogenous bacteria and nutrients the indigenous bacteria were stimulated and formed a new community that contributed to increased oil yield. Pfiffner et al. (1986) have shown that halotolerant, thermotolerant, facultative polymer producing bacteria suitable for *in situ* growth and polymer production are required for selective plugging MEOR process. Biosurfactant and bioemulsifiers are also produced by marine microbes (Maneerat 2005; Nerurkar et al. 2009).

Bacillus spp. are commonly used for application in MEOR (Van Hamme et al. 2003; Youssef et al. 2007). They are reported to produce a variety of eco-friendly products, viz. extracellular enzymes, biosurfactants, biopolymers, bioemulsifiers, biopesticides, etc. (Schallmey et al. 2004). Among the *Bacillus* sp., *Bacillus licheniformis* possesses the attributes to be the most suitable candidate for biosurfactant and selective plugging mediated MEOR strategies. It is found suitable because it is (i) fast growing (ii) facultatively anaerobic (iii) spore forming (iv) motile (v) biosurfactant producer (vi) biopolymer producer (vii) producer of antibacterial substances and (viii) able to survive under extreme conditions generally prevalent in oil reservoirs (McInerney et al. 1990).

Jenneman et al. (1983) isolated biosurfactant producing *B. licheniformis* JF-2 from oil field injection water for use in MEOR. Javaheri et al. (1985) showed this strain could produce the biosurfactant anaerobically. Biosurfactant producing *P. aeruginosa* have been found to give 15% oil recovery in sand pack column (Bordoloi and Konwar 2008). Yakimov et al. (1995) isolated *B. licheniformis* BAS50 from a deep oil well which can grow and produce highly active biosurfactant under conditions that exist in oil reservoir. Jennings and Tanner (2000) showed that biosurfactant producing bacteria can be found in both, unpolluted soils and soils polluted with hydrocarbons. Similar observations with petroleum degrading bacteria have been made with respect to their occurrence in special habitats (Austin et al. 1977). Silver et al. (1989 US patent) showed enhanced oil recovery by selectively plugging the high permeability zones of reservoir rocks using individual or mixtures of oil reservoir isolates, *B. licheniformis* NRRL B-18178 and 18179, or their spores.

Yakimov et al. (1997) isolated four different *B. licheniformis* strains from Northern German oil reservoirs which were able to produce extracellular polymers, both aerobically and anaerobically over a wide range of temperature, pressure and salinity. Out of these four strains, *B. licheniformis* BNP29 producing significant amount of biomass was used for selective plugging approach of MEOR *B.licheniformis* strains isolated for MEOR purposes by different investigators are listed in (Table 31.2).

In our laboratoty *ex situ* biosystems using *B. licheniformis* were developed. Successful enrichment of the strains of interest requires an appropriate sample from an appropriate habitat. Enrichment culture technique designed based on selection criteria specific for *B. licheniformis* like sporulation, nitrate reducing ability, facultatively anaerobic growth in presence of 5% NaCl, 50°C incubation temperature and medium containing potassium nitrate was used (Slepeckey and Hemphill 1992). An easy, rapid and reliable method is required to identify biosurfactant producing bacteria. Biosurfactant production is generally detected by either of the following methods viz. measurement of cell hydrophobicity (Pruthi and Cameotra 1997), drop collapsing ability (Bodour and Miller-Maier 1998), oil spreading ability (Youssef et al. 2004), hemolytic activity (Carillo et al. 1996) and surface activity (Desai and Banat 1997). A rapid quantitative turbidometric method is reported by Mukherjee et al. (2009). A comparison of all these methods is provided by Youssef et al. (2004). In our studies 30 out of 77 of biosurfactant producing isolates from oil reservoirs and hot water springs were found to belong to *B. licheniformis* and *B. subtilis*, while high diversity was found in isolates obtained from petrol contaminated soil. This indicated the predominance of biosurfactant producing *Bacillus* spp. in oil reservoir and hot water spring environment.

Phenetic classification allows grouping of organisms based on the mutual similarity of their phenotypic (morphological, biochemical and physiological) characteristics. Cluster analysis by UPGMA was used for phenetic classification of microorganisms. The results of cluster analysis of all the 77 biosurfactant producing bacterial isolates obtained from different habitats and *Bacillus* spp. type strains based on 20 different characteristics depicted in the phenogram showed that the isolates get distributed into six main groups, viz. three in-groups and three out-groups (Fig. 31.3). *B. licheniformis* TT42 and *B. licheniformis* TT33 and 30 other strains from Tuwa-Timba hot water spring , Gujarat, India are sorted in the major ingroup with *B. licheniformis* and *B. subtilis* type strains. This is in agreement of the observation that the *B. licheniformis* and *B. subtilis* fall in the same 16S rRNA gene cluster group (Slepeckey and Hemphill 1992).

B. licheniformis TT42, isolated from Tuva-Timba hot water spring, Gujarat, India, was found to produce a highly surface active biosurfactant identified as lichenysin. The crude lichenysin lowered the surface tension of water from 72 to 27 mN/m and the interfacial tension between crude oil and water from 12 to 0.05 mN/m (Suthar 2010). *B. licheniformis* K125, a bioemulsifier producing strain, was the only isolate obtained by direct isolation from formation water of Kalol oil reservoir no. 125, Gujarat, India suggesting that *B. licheniformis* is predominant in the oil reservoir. The crude bioemulsifier produced by *B. licheniformis* K125 reduced the surface tension of water from 72 to 34 mN/m with an emulsification index (E_{24}) of 66%.

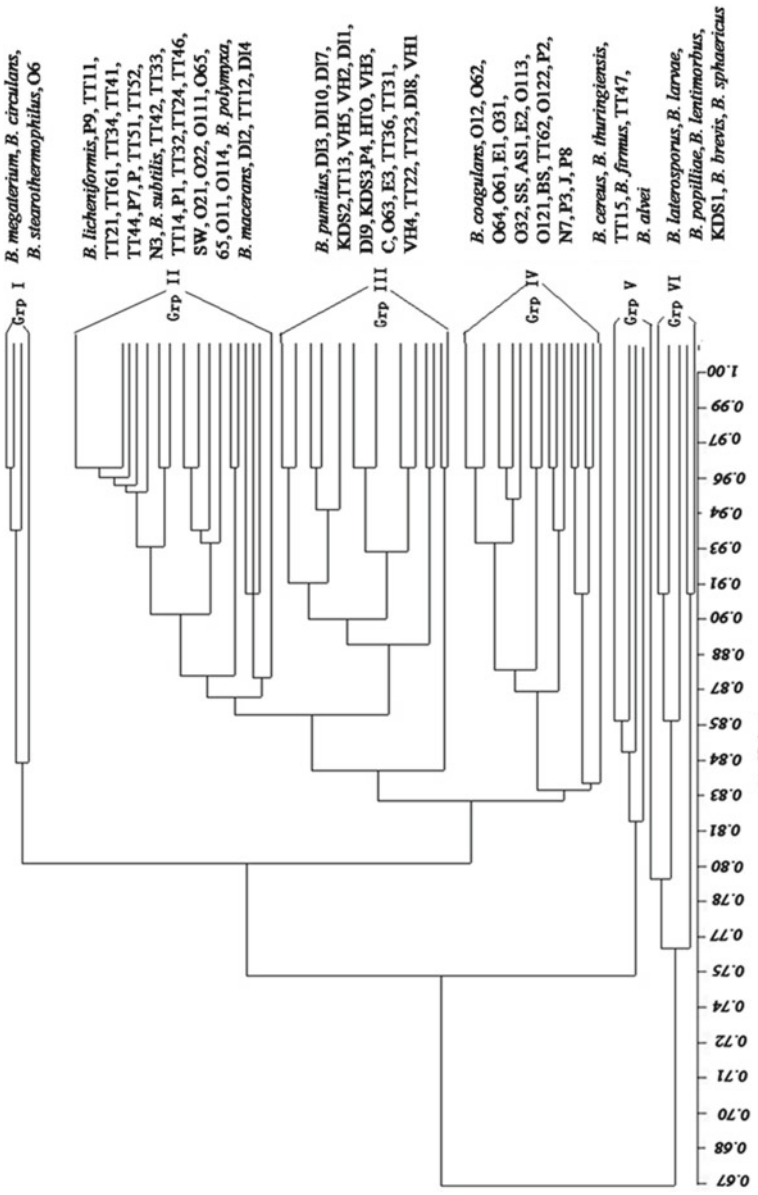

Fig. 31.3 Phenogram based on Sm coefficient and UPGMA clustering of the 77 biosurfactant producing isolates and the type strains of *Bacillus* spp. (Suthar 2010)

It also showed better interfacial tension lowering ability (4.41 mN/m) as compared to emulsan (10 mN/m), an extracellular protein-associated lipopolysaccharide emulsifier produced by *A. calcoaceticus* RAG-1 (Zosim et al. 1986). *B. mojavensis* JF-2, earlier known as *B. licheniformis* JF-2 was used as standard strain for comparative studies (Folmsbee et al. 2006). It produced lichenysin B that lowers the surface tension of water from 72 to 27 mN/m and the interfacial tension between crude oil and water from 28 to 0.1 mN/m (McInerney et al. 2005). This strain was isolated from injection brine of the water flood operation in Carter County, Oklahoma oil formation, USA. It has been reported to recover appreciable amount (3–28%) of unrecoverable oil in core flood studies (Maudgalya 2005) and in actual oil reservoirs (McInerney et al. 1999).

Selective plugging mediated MEOR requires a candidate which can survive under extreme conditions prevalent in oil reservoirs and produce high amount of biomass to selectively plug the highly permeable zones of reservoir rocks. The 77 biosurfactant producing isolates were also screened based on their growth and biopolymer production under different environmental conditions. *B. licheniformis* TT33, isolated from Tuva-Timba hot water spring, showing best growth and highest biopolymer production under aerobic, static and anaerobic conditions at 50°C was selected as the most suitable candidate for selective plugging mediated MEOR study.

31.9 Model Porous Column MEOR Studies

Core flood and sand pack column studies are the laboratory scale methods used to analyze oil recovery efficiency of microbial cultures. To evaluate the MEOR potential of a microorganism or its metabolite, laboratory experiments are conducted using cores connected to a flow system, called the Core flood studies. Such experiments have been conducted to investigate the mobilization of residual oil using biosurfactant based (Maudgalya 2005) and selective plugging based (Yakimov et al. 1997) MEOR strategies. Reports of additional oil recovery in model porous columns by *Bacillus* spp. has been summarized in Table 31.3. To simulate the MEOR processes, Sugihardjo et al. (1999) conducted core flood experiments using standard and native cores, and activated microbes from Cepu oil field at reservoir conditions. McInerney et al. (2005) tested the efficacy of biosurfactant produced by *B. mojavensis* JF-2 using berea sandstone cores and sand-packed columns which were flooded to residual oil saturation. The biosurfactant of *P. aeruginosa* (MTCC7815) and *P. aeruginosa* (MTCC7812) isolated from ONGC oilfield, Assam could release about 15% more crude oil at 90°C than at room temperature and 10% more than at 70°C in crude oil saturated sand pack column under laboratory conditions (Bordoloi and Konwar 2008). Oil recovery in sand column by bioemulsifier producing halothermophilic *B. licheniformis* ACO1 was attributed to selective plugging (Dastgheib et al. 2008). Selective plugging, has been demonstrated by Yakimov et al. (1997) using core flood experiments. Penetration of *Bacillus* strains in nutrient saturated

Table 31.3 Additional oil recovery in model porous columns by *Bacillus* spp.

Bacillus Spp.	Model porous column and biosystem	Additional oil recovery (%)	Reference
B. mojavensis JF-2 ATCC no. 39307	Sand-packed column/crude broth culture + nutrients	–	McInerney et al. (1985a US patent)
B. licheniformis BNP29	Sandstone core/biomass and nutrient medium	9.3–22.1	Yakimov et al. (1997)
B. subtilis MTCC 2423	Sand-packed column/1 mg/ml solution of purified biosurfactant	62	Makkar and Cameotra (1997)
B. brevis *B. licheniformis* *B. polymyxa*	Sand packed column/consortia in nutrient medium	11.2–18.3	Almeida et al. (2004)
B. mojavensis JF-2	Berea sandstone core/biosurfactant 10–60 mg/l + 2,3butanediol + polyacrylamide	10–40	McInerney et al. (2004)
B. mojavensis JF-2	Berea sandstone core/10–41 mg/l Lichenysin + 1,000 mg/l polymer	10–30	Maudgalya et al. (2005) Ph.D. thesis
B. subtilis	Fractured porous media/bacteria and nutrient medium	29.9	Soudmand-asli et al. (2007)
B. licheniformis TT42	Sand-packed column/crude biosurfactant	33.3–38.8	Suthar et al. (2008)
B. licheniformis K125	Sand-packed column/crude bioemulsifier	42.1–46.8	Suthar et al. (2008)
B. subtilis 20B	Sand-packed column/crude biosurfactant	30.22	Joshi et al. (2008c)
B. subtilis 20B *B. licheniformis* K51 *B. subtilis* R1 *Bacillus* sp. HS3	Sand-packed column/crude biosurfactant	25–33	Joshi et al. (2008d)
B. licheniformis R2	Berea sandstone core/crude biosurfactant	19.16	Joshi (2008a)
B. licheniformis AC01	Sand packed column saturated with paraffin/bacteria and nutrient medium	22	Dastgheib et al. (2008)
B. licheniformis TT33	Sand packed column/microbial biomass in nutrient medium	25.1–31.8	Suthar et al. (2009)
B. licheniformis TT42	Berea sandstone core/crude biosurfactant	7.94	Suthar (2010)

Berea limestone was demonstrated (Jenneman et al. 1985). Core-flooding test show that the incremental oil recoveries with indigenous strains were approximately 5–7% OOIP both in atmospheric pressure and reservoir high pressure (She et al. 2011).

While the core floods give an accurate estimate of the potential of a bacterial culture in oil mobilization, it is an expensive and a tedious process. Many replicates of the experiment cannot be run simultaneously as expensive machinery is involved. Live cores from the reservoir are needed which also require special means of preservation. This technique, therefore, cannot be suitably used as a screening method where several potential cultures need to be tested. Sand pack column studies can be an appropriate method of choice for evaluation and screening of potential microbial cultures and their products for MEOR application. Several reports have mentioned sand pack columns being used to demonstrate additional oil recovery (Abu-Ruwaida et al. 1991; Almeida et al. 2004; Banat 1995; Brown et al. 2002; Bryant 1987; McInerney et al. 2004; McInerney et al. 2005; Yakimov et al. 1997). Table 31.3 summarises additional oil recovery using model porous columns with our isolates and some important isolates of other investigators.

31.10 Additional Oil Recovery in Sand Pack Column

Our studies comprised of development of biosystems that constituted (i) crude biosurfactant in cell free spent medium for biosurfactant flooding MEOR approach and (ii) biomass in spent medium for selective plugging MEOR approach. The technical feasibility of oil recovery was assessed in porous model sand packed columns with these special *B. licheniformis* isolates obtained and the two mechanisms that significantly contribute to oil recovery i.e. biosurfactant flooding and selective plugging strategies were studied. Additional Oil Recovery was calculated as follows (Suthar et al. 2008, 2009),

Pore Volume (PV) (ml) = Volume of brine required to saturate the column
Original Oil in Place (OOIP) (ml) = Volume of brine displaced by oil saturation
S_{orwf} (ml) = Residual oil saturation after water flooding
S_{orbf} (ml) = Oil collected over residual oil saturation (in case of biosurfactant flooding)
S_{orbp} (ml) = Oil collected over residual oil saturation (in case of biomass plugging)
Initial Water Saturation = $(S_{wi})(\%) = (X/PV) \times 100$
where, X = Pore Volume – Volume of brine collected after injecting oil
Initial Oil Saturation $(S_{oi})(\%) = (OOIP/PV) \times 100$
Residual Oil Saturation $(S_{or})(\%) = (X_i/OOIP) \times 100$
where, X_i = OOIP – Volume of oil collected after water flooding
Additional Oil Recovery over S_{orwf}

$$(\text{AOR})(\%) = \frac{\text{Oil recovered using biosurfactant}}{\text{Oil in column after water flooding}} \times 100$$

Table 31.4 Oil recovery obtained in sand pack columns using crude biosurfactant/bioemulsifier of *B. mojavensis* JF-2, *B. licheniformis* TT42 and *B. licheniformis* K125 (Suthar et al. 2008)

	B. mojavensis JF-2			*B. licheniformis* TT42			*B. licheniformis* K125		
Parameter	SP 1	SP 2	SP 3	SP 1	SP 2	SP 3	SP 1	SP 2	SP 3
PV (ml)	59.0	68.0	65.0	59.0	60.0	60.0	59.0	65.0	65.0
OOIP (ml)	56.0	59.0	56.0	46.5	45.0	45.0	46.0	51.0	50.0
ROS (ml)	29.0	26.5	25.4	19.5	24.3	22.4	22.0	28.5	29.0
Sorbf (ml)	10.3	6.5	7.2	6.5	7.7	8.7	10.3	11.5	12.2
Swi (%)	5.1	13.2	13.8	21.2	25.0	25.0	22.0	21.5	23.1
Soi (%)	94.9	86.8	86.2	78.9	75.0	75.0	78.0	78.5	76.9
Sor (%)	48.2	55.1	54.6	58.1	46.0	50.2	52.2	44.1	42.0
AOR (%)	35.5	24.5	28.3	33.3	31.7	38.8	46.8	40.4	42.1

SP Sand Pack column

The *B. licheniformis* k125 bioemulsifier contains 34.3% polysaccharide, 6.9% protein and 6.4% lipid and possess both surfactant and emulsification activity. It forms oil in water emulsion which remains stable at wide range of pH, temperature and salinity. It gave 43 ± 3.3% additional oil recovery upon application to a sand pack column. This is 13.7% and 8.5% higher than that obtained by crude lipopeptide of the standard strain, *Bacillus mojavensis* JF-2 and *Bacillus licheniformis* TT42 respectively (Table 31.4). One-way ANOVA validated that the AOR obtained using the crude bioemulsifier produced by *B. licheniformis* K125 significantly differed from and was higher than the AOR obtained using the crude biosurfactants of *B. licheniformis* TT42 and *B. mojavensis* JF-2

The mechanism of oil recovery of strains JF-2 and TT42 is purely due to the lipopeptide biosurfactant. However, the increased oil recovery obtained by using the crude bioemulsifier can be attributed to its combined surface and emulsification activity. Its mechanism of oil recovery must be similar to the mechanism exhibited by surfactant–polymer flooding process of chemical enhanced oil recovery (Suthar et al. 2008).The bioemulsifier can desorb the oil from the reservoir rock by lowering the interfacial tension and also stabilize the oil/water emulsion consequently facilitating the mobilization of oil. Thus it brings the oil in water phase like a surfactant and exerts mobility of oil like a polymer.

Selective plugging strategy requires growth and production of exopolymeric substances which help in plugging high permeability zones. *B. licheniformis* biopolymer has 26% carbohydrate and 3% protein and has surface activity. When tested in sand packed column by injecting the biomass and the nutrients this strain gave 27.7 ± 3.5% oil recovery (Table 31.5). Environmental scanning microscopy showed bacterial growth adhered to sand recovered from the column after its use (Fig. 31.4) while confocal laser scanning microscopy revealed the structure of the biofilm produced by *B. licheniformis* TT33 (Fig. 31.5). The biofilm formation underlines the fact that the strain is capable of growing in a biomass cohesively bound by the biopolymer leading to oil recovery mediated by selective plugging (Suthar et al. 2009).

Table 31.5 Oil recovery obtained in sand pack columns using *B. licheniformis* TT33 (Suthar et al. 2009)

Parameters	Column1	Column2	Column3
PV (ml)	54.00	62.00	65.00
OOIP (ml)	45.50	50.00	51.00
X (ml)	8.50	12.00	14.00
ROS (ml)	25.50	25.90	26.20
Sorbp (ml)	8.10	6.50	7.00
Swi (%)	15.74	19.35	21.53
Soi (%)	84.25	80.64	78.46
Sor (%)	43.95	48.20	48.62
AOR (%)	31.76	25.09	26.72

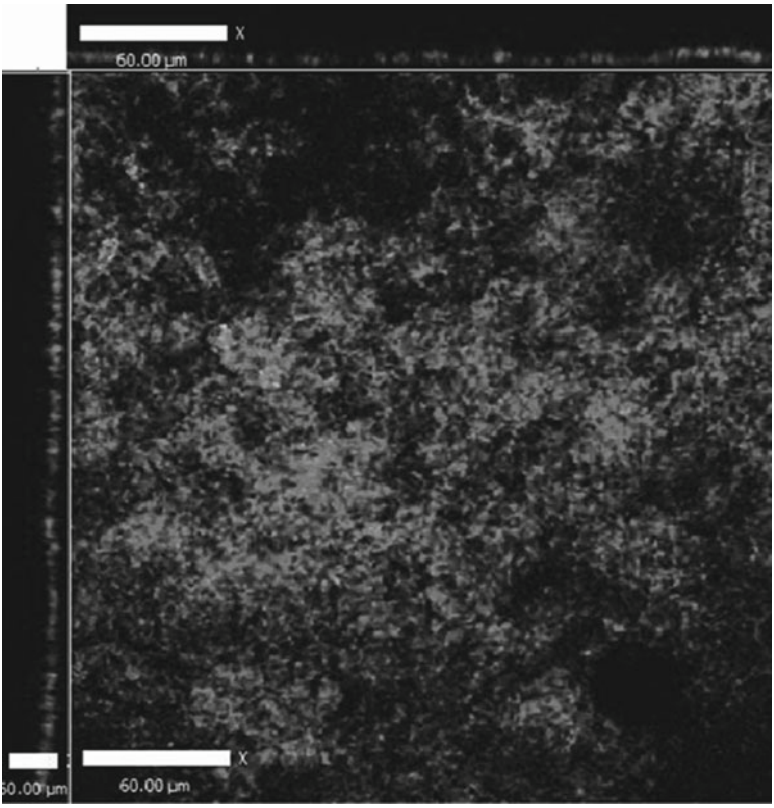

Fig. 31.4 Micrograph of *Bacillus licheniformis* TT33 biofilm obtained by Confocal laser scanning microscopy using LIVE/DEAD Baclight staining (Suthar et al. 2009)

31.11 MEOR Field Studies

Numerous MEOR field trials have been conducted in various parts of the world with varying degrees of success. Successful MEOR field trials have been conducted in USA, Russia, China, Australia, Argentina, Bulgaria, former Czechoslovakia,

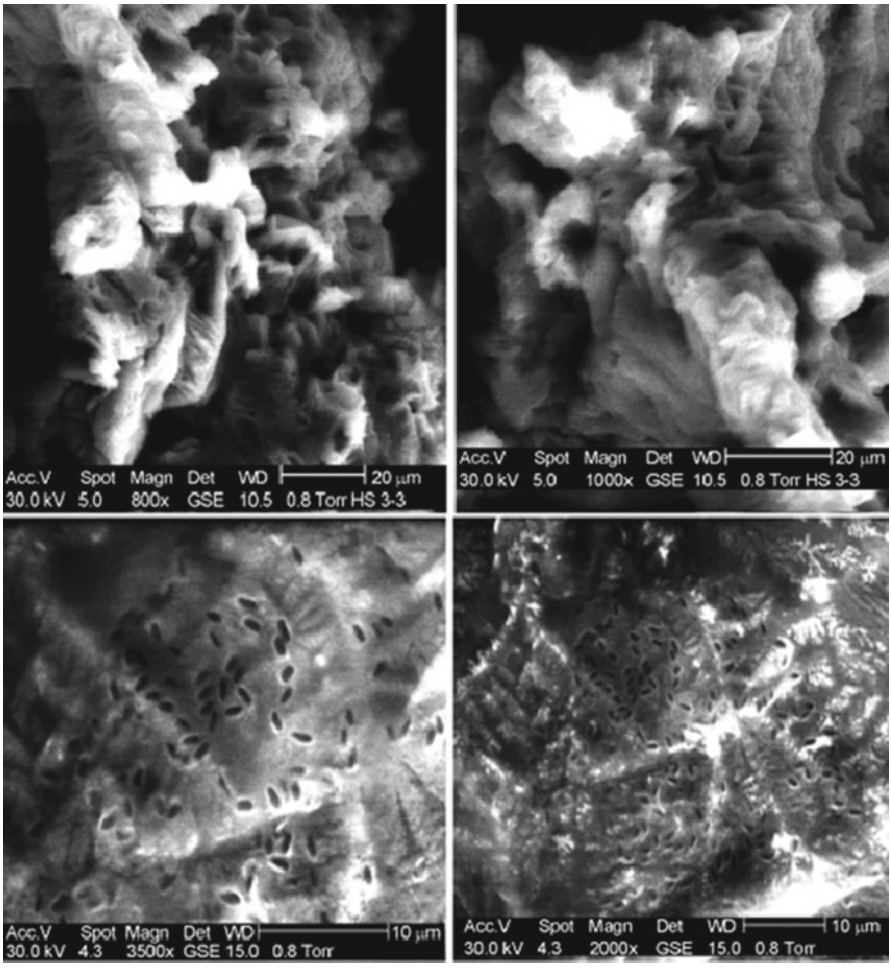

Fig. 31.5 Environmental scanning electron micrograph of sand retrieved from column showing *B. licheniformis* TT33 cells and biofilm (Suthar et al. 2009)

former East Germany, Hungary, India, Malaysia, Peru, Poland and Rumania (Awan et al. 2008; Lazar et al. 2007; Sen 2008). A process and related apparatus for enhancing the oil recovery from an oil reservoir generally include selection of a candidate reservoir, collecting formation water samples under anaerobic conditions, selecting media and enriching the microbes derived from the formation water, characterizing and identifying the selected consortium, mass scale production of the selected consortium, anaerobic preparation of a defined nutrient medium, transportation of the nutrient medium by tankers and the consortium by a specially designed field laboratory unit to the selected oil well treatment site, injection of the medium and consortium into the well, closing of the well for the proliferation of microbes for

1–3 weeks, allowing the microbes to dislodge oil in the reservoir and thereby enhance recovery of oil from the oil reservoir (Fig. 31.1) (Soni et al. 2009 US patent).

World experience on MEOR field trials in last four decades has been summarized by Lazar et al. (2007) listing field trials from eighteen countries all over the world indicating the system involved and the outcome. The first microbial-enhanced waterflood field project sponsored by the U.S. Department of Energy (DOE) conducted with the National Institute for Petroleum and Energy Research (NIPER) was initiated in the Mink Unit of Delaware-Childers field which is typical of mid-continent reservoirs in the United States. No operating and corrosion problems were encountered. Sulfate-reducing bacterial populations remained relatively low compared to the baseline counts. The microbial formulation, NIPER Bac 1 containing *B. licheniformis*, *Bacillus* spp., *Clostridium* spp., *Shewanella* sp., and molasses injection improved oil production rates by about 13% and decreased water/oil ratios for producing wells nearest the injection wells up to 35% (Bryant et al. 1990).A field test of the microbial plugging process conducted by McInerney et al. (1999) in the Southeast Vassar Vertz sandstone reservoir located in Payne County, OK, USA showed that the stimulation of *in situ* microbial growth by nutrient injection results in substantial permeability reductions in sandstone cores that leads to increase in oil recovery. Microbial technology for the enhancement of oil recovery based on the activation of the stratal microflora was tested in the high-temperature horizons Dagang oilfield, China. This allowed the recovery of more than 14,000 tons of additional oil over 3.5 years (Nazina et al. 2007). Zhang et al. (2010) have reviewed the initiation, development, mechanisms and the progress made in enhanced oil recovery with microbes and their metabolic products together with MEOR technology development in China.

31.12 Conclusions

MEOR has evolved in several stages. Initially practised as *in situ* technique where nutrients were injected to proliferate the resident microorganisms in the oil reservoir, to selective enrichment of microbes of interest such as biosurfactant/biopolymer producers with concomitant suppression of sulfate reducing bacteria and further till present generation MEOR where microbial metabolites produced outside the well are injected in the oil well as in surfactant flooding EOR.

Bryant (1987) had indicated very early that many reservoir characteristics must be determined before applying MEOR. Some of these are porosity, permeability, salinity, temperature and pressure. It is unlikely that a single MEOR method can be applied to all types of reservoirs.

Structural mathematical models are required to describe MEOR in a better way. Better understanding of MEOR processes and mechanisms from engineering standpoint based on economic applicability and performance is the key to further improve the process efficiency. Advanced MEOR technologies based on the use of genetically engineered organisms may prove to be effective (Sen 2008).

Brown (2010) has apprehensions that MEOR will remain an unproven concept rather highly desirable reality unless recommendations done by several studies are implemented to establish it as a viable method. According to him there is no question as to whether microorganisms have the capability of enhancing oil recovery by virtue of some of the products they can produce, the question is how to explore this ability in an economically practical and scientifically valid manner.

Potential of MEOR technologies has not been fully realized due to poor yield of useful microbial metabolic products, growth inhibition by accumulated toxic metabolites and longer incubation required to get the effect. The mechanisms of MEOR must be thoroughly understood to assess the efficiency and efficacy of the process so as to make it more effective (Banat 1995).

With almost a century of research and various field trials, MEOR has proven great potential in oil extraction with certain advantages. After 1990, the activity of MEOR field trials is running on the basis of conclusion that successful MEOR applications should be focused on water floods, where a continuous water phase enables the introduction of the technology or single-well stimulation (including skin damage removal) where its low cost makes it a preferred choice. At the same time, selective plugging strategies and activation of stratal microbiota remain the most promising and should be developed. Technologies such as microbial paraffin removal, microbial skin damage removal, microbial control souring and clogging, and those based on using ultramicrobiota remain of interest for the further development of the MEOR method (Lazar et al. 2007).

Oil reservoirs are home to phylogenetically and metabolically diverse microbial communities. The lack of appreciation of the microbiology of oil reservoirs often leads to detrimental consequences such as souring or plugging. However, an understanding of the microbiology can be used to enhance operations. Microbial oil recovery processes will only gain more widespread acceptance and application when quantitative measures of performance be reliably obtained (Youseff et al. 2009).

MEOR increases both the oil production rate and total oil recovery of original oil in place without affecting the reservoir rock quality. Lack of holistic approach allowing for a critical evaluation of economics, applicability and performance of MEOR is missing. A complete assessment of this process is required. No doubt it is a multidisciplinary process and requires the expertise of a Microbial biotechnologist, Reservoir engineer and Geologist. The ‘bios’ component like microbes and its metabolites contributing to MEOR mechanisms should be understood and produced taking into consideration the reservoir characteristics. The expertise of a geologist is needed to understand the reservoir characteristics and finally the implementation of MEOR needs the expertise of a reservoir engineer. Modelling the process before implementation would help to optimize it. If applied properly MEOR will emerge as a clean technology with sizable petroleum recovery in an economical manner. Considering the future demand for energy dependence on petroleum resources MEOR technologies should be refined and made application friendly.

31.13 Future Perspectives

MEOR strategies can be far more successful when based on practical hands-on experience. However, realistic practical knowledge can be obtained in MEOR only by field trials. Thorough study of the reservoir and the process to be applied is warranted to have successful field trials. Therefore multidisciplinary approach should be the aim of the studies undertaken to facilitate understanding MEOR which in turn would achieve truly sustainable technology.

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Chapter 32
Bacterial Small RNAs (sRNAs) and Carbon Catabolite Repression

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Abstract Bacterial small RNAs (sRNAs) have been shown to play a critical role in regulation of various cellular activities by modulating the expression of key genes and operons. By using highly reliable small RNA prediction tools, the existence of sRNAs is predicted for most bacterial genomes. Bacterial strains growing in the presence of more than one carbon source show diauxie. First they grow using the more preferred substrate and then use the less preferred carbon source. There are two reasons for diauxie. One of them is that the uptake of the less preferred substrate is inhibited by the presence of the more preferred substrate by a mechanism known as inducer exclusion. The second mechanism is through catabolite repression, which is essentially transcriptional repression of genes involved in degradation of the less preferred carbon compounds. Cyclic AMP (cAMP) CRP-mediated regulation of degradative traits is known for a number of years. Recently, however, the involvement of sRNA has been demonstrated as a regulatory mechanism in carbon catabolite repression. This chapter deals primarily with bacterial sRNAs by focussing on their role in carbon catabolite repression.

Keywords Bacterial small RNA (sRNA) • Carbon catabolite repression • Regulation of gene expression

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32.1 Introduction

Regulation of gene expression essentially means prevention of making product in the absence of its physiological requirement. The product need not always be a protein; it could even be an RNA molecule. Regulation of gene expression is a complex process. The cell adopts a variety of strategies to regulate expression of its gene pool depending on the physiological status as well as through the physico-chemical and biological factors found in its surroundings. Though we have a clear knowledge about some of these strategies, the discovery of sRNA-mediated gene regulation has proved to be yet another fascinating and complex regulatory circuits the cell employs to regulate gene expression.

Carbon catabolite repression is one of the best studied topics in molecular biology. It is not possible to find a molecular biology text book without having a description of Jacob and Monod's classical experiments on *lac* operon regulation. However, recent discoveries involving sRNA in carbon catabolite repression adds another unexplored dimension to this fascinating field of study. Before presenting the role of sRNA in carbon catabolite repression, a brief introduction will be given to explain the reader about the role of sRNA in regulation of gene expression both in prokaryotes and eukaryotes.

32.1.1 Eukaryotic Small RNAs

In eukaryotes, the small interfering RNA or silencing RNA (siRNAs), are a class of non-protein-coding double-stranded RNA molecules that were originally observed during transgene and virus-induced gene silencing (Hannon 2002). The RNA interference (RNAi) pathway inhibits expression of a specific gene with the help of either siRNA or (micro RNA) miRNA of 20–25 nucleotides in length. Both siRNA and miRNA were discovered in unrelated studies and have similarities in biogenesis, assembly into RNA-protein complexes and ability to regulate gene transcripts (Shabalina and Koonin 2008). However, miRNA and siRNA differ in two major aspects: firstly, the miRNAs are endogenous and are encoded by their own, distinct genes, while siRNAs are generated either by degradation of exogenous double-stranded RNA or are transcribed from transposable elements integrated in the genome, or from other types of inverted repeat sequences (Cerutti 2003). Secondly, siRNAs are fully complementary to their targets, whereas miRNAs, at least in animals, show limited complementarity to their recognition sites (Almeida and Allshire 2005; Carthew and Sontheimer 2009).

The siRNAs are generated by RNase III enzymes, also designated as Dicer enzymes. These evolutionarily conserved enzymes act on double-stranded RNA molecules and cleave them into approximately 22 bp-long double stranded RNA oligonucleotides with 5′ phosphorylated termini (Fig. 32.1). These dsRNA molecules then associate with a multi-subunit protein complex known as the RNA-induced

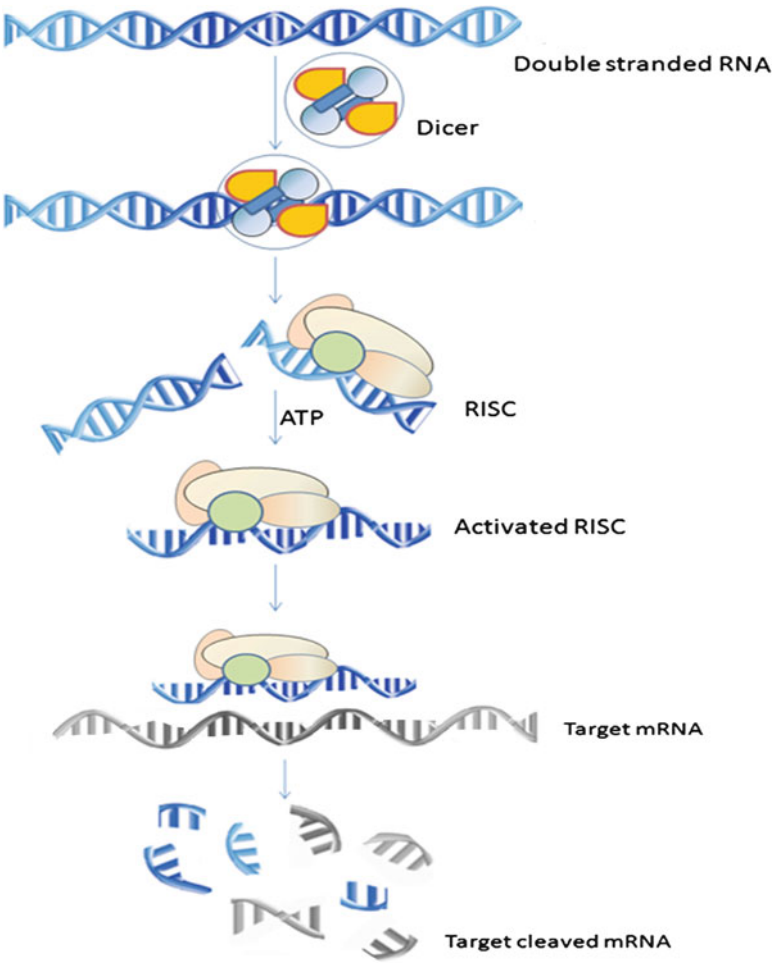

Fig. 32.1 Mechanism of siRNA interference

silencing complex (RISC). The siRNA guide strand directs the RISC to the complementary RNA target and facilitates its degradation with the help of Ago protein (Hannon 2002), which are found as part of the RISC complex (Fig. 32.1).

32.1.2 Bacterial Small RNAs

Bacteria are found ubiquitously in almost every environment on the earth. They can survive under extreme climatic conditions where other forms of life find it

difficult to survive. Bacteria have acquired such an extraordinary capability due to the plasticity of their genomes and their ability to regulate expression of their gene pool. In fact, regulation of gene expression is considered to be the prerequisite for bacteria to monitor and adapt to diverse environmental cues. Until recently gene expression was believed to be a molecular cross talk between *cis* elements of a gene, such as the promoter, operator and upstream activating sequences (UAS) and *trans* acting factors like transcription activators and repressors. However, the discovery of sRNA and its involvement in regulation of gene expression has changed that dogma.

The discovery of bacterial sRNA molecules that act as regulators dates back to 1980s. In 1981, the 108 nucleotide RNAI was found to block ColE1 plasmid replication by base-pairing with the RNA that is cleaved to produce the replication primer (Tomizawa and Itoh 1981). Similarly, the sRNA synthesized from the *copA* gene was shown to act as a replication inhibitor of plasmid R1 (Stougaard et al. 1981). The concept of sRNA as a regulatory switch was further strengthened by the identification of translation inhibition of transposase messenger RNA by a small, complementary, regulatory RNA specified by IS*10* (Simons and Kleckner 1983). The discovery of MicF sRNA underscored the concept of sRNA-mediated regulation of gene expression in bacteria. This *trans*-encoded regulator was shown to inhibit translation of *Escherichia coli ompF* mRNA (Mizuno et al. 1984). Following this discovery, a number of computational methods were developed to predict sRNAs from the bacterial genome sequences. The bioinformatic tools used for identification of sRNA are discussed elsewhere in this book chapter.

32.2 Nomenclature

Unfortunately, the nomenclature for describing small RNAs in bacteria has never been lucid or uniform. Since sRNAs represent functional RNA molecules not translated into a protein, they were originally designated as noncoding RNA (ncRNAs) molecules. As certain bacterial sRNAs, such as RNAIII of *Staphylococcus aureus*, encode a small protein the name 'noncoding' was considered inappropriate for small RNA molecules (Storz and Haas 2007). Considering the cases like SRI sRNA of *Bacillus subtilis*, where the regulatory RNA was shown to code for a small peptide destined to control gene expression, the terms functional RNA (fRNA), riboregulator, regulator RNA (rRNA) and small nonmessenger RNAs (snmRNA) were used while naming bacterial regulatory RNA molecules. Unlike eukaryotic miRNA, the bacterial noncoding RNAs are very diverse in size (ranging from 50 to 500 nucleotides in length) and show considerable diversity, in their mode of regulating the gene expression. Owing to their diverse nature, giving an apt name that could bring all bacterial regulatory RNAs under one roof was not possible. Therefore, the term small RNA (sRNA) is preferred to other names to identify noncoding functional RNA molecules.

32.3 Mode of Action

The sRNA-mediated regulatory mechanisms can be broadly classified into two categories. The first category of sRNAs regulates gene expression by base-pairing to target mRNAs. Such hybrid formation affects stability of target mRNA. The second category of sRNAs binds to the regulatory proteins that influence the expression of target genes. They possess short sequence motifs that serve as binding sites to these regulatory proteins. Existence of multiple copies of such motifs, especially in the loop regions, promotes interaction of many copies of regulatory proteins with a single sRNA molecule. These interactions sequester regulatory proteins and prevent them from acting as transcriptional modulators.

32.3.1 sRNA Interactions and Its Interactions with Target mRNA

The sRNAs base pairing with target mRNAs can be broadly classified into *cis*- and *trans*-encoded sRNAs. The *cis*-encoded sRNAs are always located in the same DNA region but on the opposite strand of the target mRNA and are fully complementary to their targets over a long sequence stretch. These are mainly present on plasmids, phages and transposons and are rarely seen on chromosomes. The *trans*-encoded sRNAs are generally present at different gene locations and often show non-contiguous target complementarity and are invariably present on chromosomes.

32.3.2 Cis-encoded sRNA

Most of the plasmid, phage and transposons have *cis*-encoded sRNAs. These constitutively expressed *cis*-encoded sRNAs are involved in the regulation of replication, conjugation or segregational stability (Brantl 2007). A majority of them are believed to regulate gene expression by direct base-pairing with mRNAs (Vogel and Wagner 2007). The base pairing of sRNA with its target can result in a variety of molecular events. The base-pairing can manifest into either attenuation of transcription or inhibition of primer maturation affecting the replication of plasmids (Tomizawa and Itoh 1981; Heidrich and Brantl 2007). In certain situations the base-pairing of sRNA can also bring about translation inhibition of target mRNA or promotion of mRNA degradation (Simons and Kleckner 1983; Dühring et al. 2006) or inhibition of mRNA degradation (Opdyke et al. 2004; Tramonti et al. 2008).

32.3.3 Trans-encoded sRNA

The majority of these *trans*-encoded sRNAs are stress regulators that allow the bacteria to adjust their physiology to environmental changes (reviewed in Storz et al. 2005; Waters and Storz 2009) or virulence gene regulators (Toledo-Arana et al. 2007). In the majority of cases, the RNA chaperone Hfq is required for productive base-pairing with the target mRNA (Zhang et al. 2002; Valentin-Hansen et al. 2004). The Hfq sequences are shown to destabilize secondary structures of the interacting RNAs to enhance interaction between the mRNA and sRNA. Hfq serves as a platform for bringing together sRNAs and target mRNAs (Valentin-Hansen et al. 2004; Brennan and Link 2007; Aiba 2007). Once sRNA is bound to the target sequence it regulates target gene expression either by preventing the translation of its cognate mRNA or by stimulating the expression of cognate genes through a mechanism known as an 'anti-antisense mechanism'. The sRNA binding sites on target mRNA sequences appear to determine the mode of action of sRNA. The sRNAs that cause down regulation of gene expression directly bind to the ribosome binding site (Vogel and Papenfort 2006) or to the 5′ untranslated regions (UTRs) (Sharma et al. 2007) or within the 5′ part of the coding region (Bouvier et al. 2008). In all these cases the sRNA-target mRNA hybrid is rapidly degraded by RNase E (Morita et al. 2005) or RNase III (Afonyushkin et al. 2005). However, the sRNA sequences that up-regulate gene expression liberate the sequestered ribosome binding site by binding to the sequences interacting with the ribosome binding site of target mRNA. This 'anti-antisense mechanism' releases the *cis* elements of mRNA needed for translation initiation and promotes its translation (Fig. 32.2).

32.3.4 sRNA and Sequestration of Regulatory Proteins

The second category of sRNAs affect gene expression in bacteria by binding and modulating the activity of regulatory proteins involved in transcription, mRNA stability or translation (Wassarman 2007; Babitzke and Romeo 2007). In principle, the sRNA-mediated regulatory protein sequestration depends on the existence of regulatory protein-binding motifs both on the mRNA target and its cognate sRNA. These motifs are found generally in the 5′ untranslated regions (UTRs) in the vicinity of the Shine-Dalgarno (SD) sequence of the target mRNA. In the absence of sRNA, the regulatory protein binds to these motifs and promotes its decay. However, the regulatory protein sequestered from the target mRNA by sRNA due to presence of multiple binding motifs for regulatory proteins (Fig. 32.2).

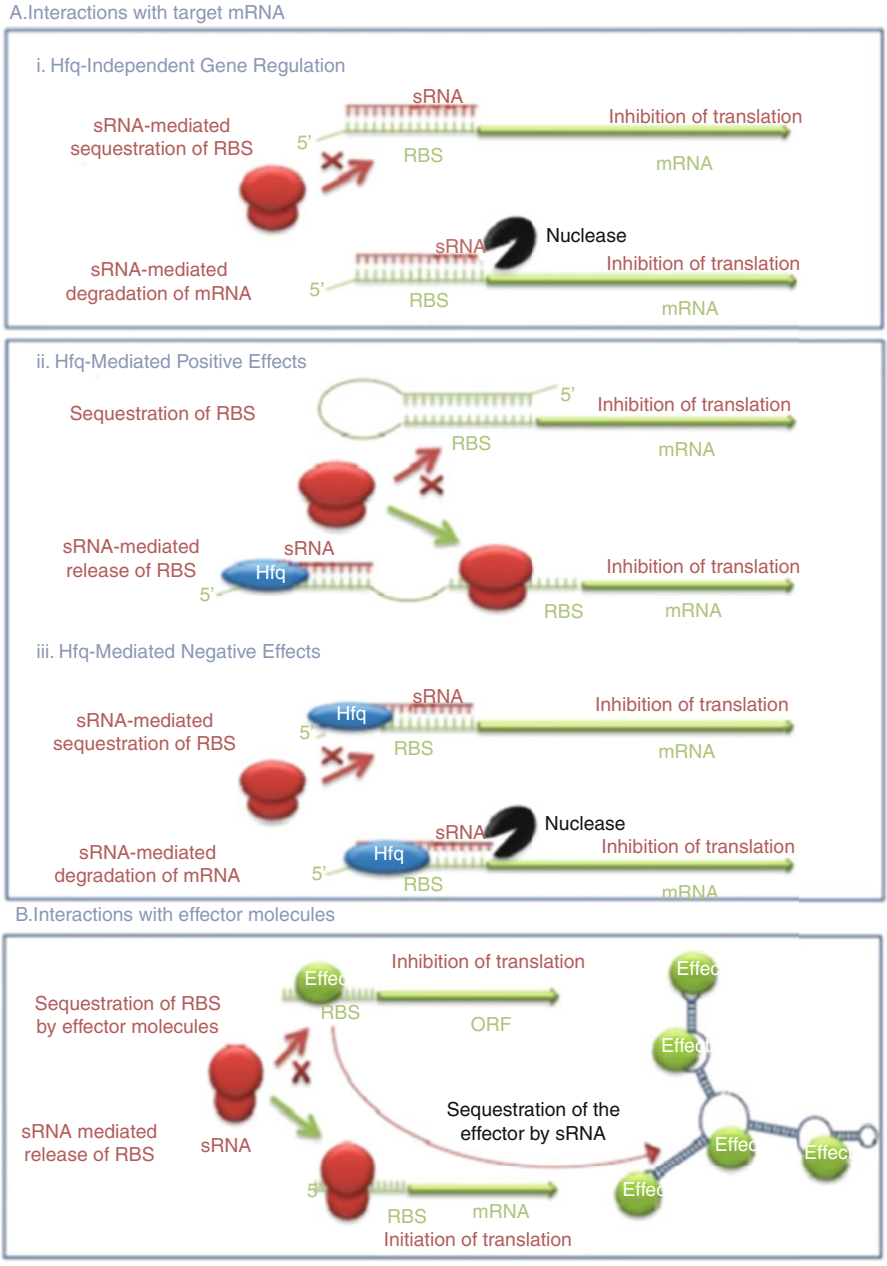

Fig. 32.2 sRNA mediated gene regulation

32.4 Identification of Small RNAs from Bacterial Genome Sequences

32.4.1 In Silico Predictions

Most of the *in silico* predictions use signature sequences, including intergenic promoter elements, sequences predicted to form stable RNA secondary structures, and intergenic rho-independent terminator elements for the prediction of candidate sRNAs (Argaman et al. 2001; Rivas and Eddy 2001; Livny et al. 2005). The following are the most widely used programmes employed in prediction of sRNAs. QRNA (Rivas et al. 2001, http://www.genetics.wustl.edu/eddy/software/) and Intergenic Sequence Inspector (Pichon and Felden 2003, http://www.biochpharma.univ-rennes1.fr/), are considered to be the very early computational tools developed for prediction of sRNA. Both of them use intergenic sequence conservation among related genomes to identify sRNAs. The RNAz (Washietl et al. 2005, http://rna.tbi.univie.ac.at/RNAz) and sRNAPredict (Livny et al. 2005; Livny et al. 2006, http://www.tufts.edu/sackler/waldorlab/sRNAPredict.html) programmes utilize estimated thermodynamic stability of conserved RNA structures and existing 'orphan' promoter and terminator annotations for sRNA predictions. Furthermore, sRNAPredict requires promoter information and rho-independent terminators predicted by TransTermHP (Kingsford et al. 2007) as input data to identify sRNAs. The upgraded version of sRNApredict, sRNAPredict2 (Livny et al. 2006) depends on input sequences and structure conservation data as identified by BLAST and QRNA, for prediction of sRNAs. PsRNA (Sridhar et al. 2010a, http://bioserver1.physics.iisc.ernet.in/psrna/) and sRNA scanner, (Sridhar et al. 2010b, http://cluster.physics.iisc.ernet.in/sRNA scanner/) are the most recent computational programmes developed for prediction of sRNAs. The PsRNA programme utilizes the KEGG Orthology (KO) numbers of the sRNA specific conserved flanking gene pairs for automated identification of putative sRNA regions in the query genomes. However, the sRNA scanner is designed based on a constructed position weight matrix (PWM), using *E. coli* K-12 MG1655 sRNA-specific transcriptional signal as positive training data, for the identification of intergenic sRNA.

32.4.2 In Vitro Validations

Various experimental strategies have been employed to validate *in silico* sRNA predictions done using the genome sequences of model organisms. Some of these studies have relied on high-density oligonucleotide arrays that include sequences either from both strands of most intergenic regions (Livny and Waldor 2007) or from sRNAs co-immunoprecipitated with the global sRNA regulator Hfq (Zhang et al. 2003). As alternatives to these molecular hybridization techniques, two genetic approaches have also been developed to screen for post-transcriptional regulators,

including sRNAs (Mandin and Gottesman 2009). These methods are target-specific and involve use of gene-specific translational fusions as screens to identify a clone form the genomic library affecting the activity of the fusion. In contrast, the shotgun cloning approach is found to be a more general method used to identify novel bacterial sRNAs (Vogel et al. 2003; Kawano et al. 2005). In this case, a cDNA library is constructed after enriching sRNA population. The sequence generated from such a library was then used to validate *in silico* sRNA predictions. This approach is widely used to gain authentic information on existence of sRNAs in bacteria.

32.5 Carbon Catabolite Repression in *E. coli*

Bacteria, like other forms of life, show preferences towards certain carbon compounds. When they are exposed to an environment where more than one carbon source is available they tune their genetic machinery to code for enzymes/proteins that facilitate transport and degradation of only preferred carbon sources. The classical experiments done on the *lac* operon and the PTS (Phosphotransferase system) system have shown that preferential transport and degradation of glucose occurs in *E. coli.* Transport of less preferred carbon sources into the cell is possible only when the concentration of glucose from the culture medium is completely depleted. Transport of alternate carbon sources into the cell depends on the elevated synthesis and stimulation of cognate permeases. The phosphorylation state of certain regulatory proteins and increased intracellular cAMP levels promote such physiological conditions in the cell. Further, these conditions also stimulate formation of the cAMP-CRP complex, which acts as a general transcriptional switch required to turn on genes needed for degradation of alternative carbon sources. The substrate that is to be catabolised itself acts as a specific transcriptional switch facilitating the transcription of genes coding for its degradative enzymes. The phenomenon, referred as carbon catabolite repression is elegantly described in any text book of molecular biology.

The sRNA influences carbon metabolism in a number of ways. Influence of sRNAs on glycogen metabolism, gluconeogenesis and on biosynthesis of sugar derivatives that serve as adhesins has been well documented. Recently the influence of sRNA was also shown for utilization of alternative carbon sources like benzoate and alkanes (Moreno and Rojo 2008). The following sections of the chapter are devoted to describe the influence of sRNA on carbon metabolism.

32.6 The Carbon Storage Regulatory (CSR) System

The carbon storage regulatory system (CSR) is one of the novel global regulatory mechanisms identified primarily in *E. coli* and numerous other bacteria. It involves multiple components, a two-component regulatory system (BarR/UvrY), an effector molecule (CsrA), which is essentially an RNA-binding protein, and a sRNA (CsrB).

The CSR system regulates glycogen synthesis and catabolism (Romeo 1998; Yang et al. 1996), gluconeogenesis (Sabnis et al. 1995) biofilm formation (Jackson et al. 2002), glycolysis (Sabnis et al. 1995), motility, flagellum synthesis (Wei et al. 2001) and acetate metabolism (Wei et al. 2000).

32.6.1 CsrB and Glycogen Metabolism

The glycogen biosynthetic enzymes, ADP-glucose pyrophosphorylase (GlgC), glycogen synthase (GlgA) and the catabolic enzyme glycogen phosphorylase (GlgP) are coded by the *glgCAP* operon. When the cell is in exponential phase of growth, CsrA binds to a conserved sequence motif (CAGGAUG) found near the SD sequence of the *glgCAP* transcript and blocks its translation contributing negatively to glycogen metabolism in *E. coli* (Liu et al. 1995; Liu et al. 1997; Baker et al. 2002). However, when cell enters the stationary phase, increased CsrB (sRNA) concentration sequesters the effector molecule, CsrA, relieving repression of the *glgCAP* operon (Liu et al. 1997; Romeo 1998). A similar mechanism is also seen with the regulation of adhesin biosynthesis. The polysaccharide consisting of ß-l,6-N-acetyl-D-glucosamine units serves as an adhesin while forming biofilms on polystyrene. The *pgaABCD* operon is involved in biosynthesis of the adhesin molecule. The 5′ UTR of *pgaABCD* mRNA has CsrA-binding sites. The CsrA cooperatively binds to the 5′ UTR overlapping the Shine-Dalgarno (SD) sequence of *pgaABCD* mRNA and competes with the 30S ribosomal subunit for binding. However, in the presence of CsrB the CsrA molecule is sequestered promoting translation of *pgaA* mRNA (reviewed in Valverde and Haas 2008).

32.6.2 Regulation of CsrB Expression

Expression of CsrB is under the control of a two-component regulatory mechanism. Sensor kinase and response regulators are shown to be involved in regulation of *csrB* expression. When the cell enters the stationary phase the histidine sensory kinase, BarA, is stimulated by an unknown sensory signal leading to phosphorylation of its cognate response regulator, UvrY. Phosphorylated UvrY acts as a transcriptional activator of the *csrB* gene contributing to elevated concentrations of CsrB (Gudapaty et al. 2001).

32.7 CbrA-CbrB-CrcZ-Crc System of *Pseudomonas*

Unlike *E. coli*, for fluorescent pseudomonads, glucose is not the preferred carbon source. The intracellular cAMP and CRP levels do not regulate carbon catabolite repression (Siegel et al. 1977; Suh et al. 2002). However, experiments conducted on a *Pseudomonas aeruginosa* PAO mutant has clearly shown involvement of a catabolite

repression control (Crc) protein in carbon catabolite repression (Wolff et al. 1991; MacGregor et al. 1996). In pseudomonads the intermediates of the tricarboxylic acid cycle usually cause repression of degradative pathways of sugars, amino acids, aliphatic and aromatic compounds (Smyth and Clarke 1975; Collier et al. 1996; Rojo and Dinamarca 2004; Moreno and Rojo 2008). Here, we present Crc and Crc-Z mediated (sRNA) carbon catabolic regulation using an aliphatic and aromatic carbon compounds as examples.

32.7.1 Regulation of the alk Operon

In *Pseudomonas putida* Gpo1, the plasmid-borne alkane-degrading genes are organized into two transcriptional units, *alkBFGHJKL* and *alkST*. Depending on the physiological state of the cell, the expression of *alkST*, which acts as repressor of its own promoter, is initiated from one of the two independent promoters, *PalkS1* and *PalkS2* found upstream of the *alkST* operon. In the absence of alkanes, expression of *alkST* is initiated from the *PalkS1* promoter with the help of RNA polymerase holoenzyme containing σ^S (Canosa et al. 1999). AlkS expressed from the *PalkS1* promoter binds to a site which overlaps the same promoter resulting in down-regulation of its own expression (Fig. 32.3a). The second promoter *PalkS2* is present 38 bp downstream of the *PalkS1* promoter. In the presence of alkanes (C3 to C13), higher expression of the *alkST* occurs from the *PalkS2* promoter which leads to an increased expression of *alkST* and *alkBFGHJKL* operons (Panke et al. 1999). Activation of the promoters, *PalkB* and *PalkS2* by AlkS is negatively modulated by a dominant global control, when cells grow in a rich medium (Yuste et al. 1998; Staijen et al. 1999; Canosa et al. 2000). This global control depends on the additive effects of Crc (Yuste and Rojo 2001) and of the cytochrome-*o*-ubiquinol oxidase (Cyo), a component of the electron transport chain (Dinamarca et al. 2002; Dinamarca et al. 2003). In order to have a focussed discussion on small RNAs, only the role of Crc and CrcZ on alkane catabolism is highlighted.

Adjacent to the start codon AUG, a Crc binding site is present on the mRNA of *alkST* (Moreno et al. 2007). In presence of preferred carbon sources, the global transcriptional regulator, Crc, binds to this site (Fig. 32.3d). As Crc binding masks the ribosome binding site (RBS) of *alkST* mRNA, translational initiation is blocked from this transcript leading to the repression of the alkane degradation pathway (Moreno et al. 2007) (Fig. 32.3b). In the presence of alkanes, the sRNA, CrcZ, sequesters Crc causing for the promotion of translation of the *alk* mRNAs (Moreno et al. 2007) (Fig. 32.3c). A detailed mechanism describing the regulation of *alk* gene expression is shown in Fig. 32.3.

32.7.2 Regulation of ben Operon

The benzoate degradation genes in *Pseudomonas putida* are organized into the *ben*, *cat* and *pca* operons, which are under the control of the the BenR, CatR and

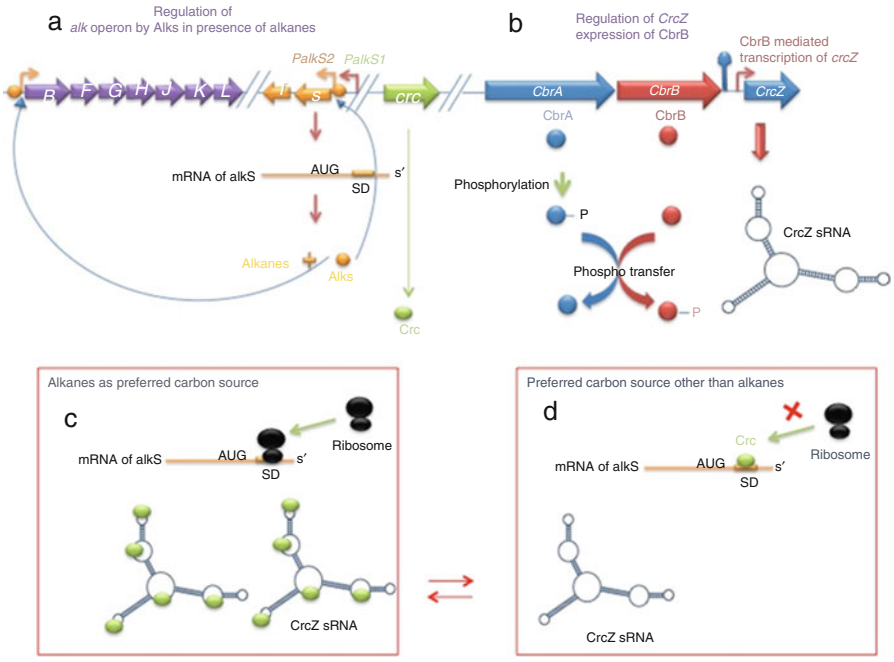

Fig. 32.3 Regulation of *alk* operon by Crc protein and the small RNA, *crcZ*

PcaR transcriptional regulators, respectively (Harwood and Parales 1996; Jiménez et al. 2002; Cowles et al. 2000; Parsek et al. 1992; Rothmel et al. 1990). The *ben* operon consists of the *benABC* and *benD* genes, which code for benzoate 1,2 dioxygenase and dihydroxybenzoate dehydrogenase, respectively (Fig. 32.4). The products of *benABC* and *benD* genes, convert benzoate to catechol, through an intermediate, dihydroxybenzoate. The *cat* operon consists of *catABC* and the gene products convert catechol to β-ketoadipate enol-lactone. The β-ketoadipate enol-lactone is converted to acetyl-CoA and succinyl-CoA by the action of *pcaDJIF* (Fig. 32.4). The activation of BenR occurs in the presence of benzoate but the utilization of benzoate in the complete medium is inhibited by a global regulatory control involving Crc protein (Morales et al. 2004). In the presence of preferred carbon sources, Crc protein binds to the *benR* mRNA to a specific site called the CA motif found at the SD sequence in the *benR* mRNA. Binding of Crc to *benR* mRNA prevents the binding of ribosome, thus affecting the formation of the translation initiation complex (Moreno and Rojo 2008; Moreno et al. 2009). This leads to repression of the *ben* operon and subsequent prevention of benzoate utilization as a carbon source. Like in *alk* operon, the repression of the *ben* operon is also relieved by the small RNA CrcZ (Fig. 32.4).

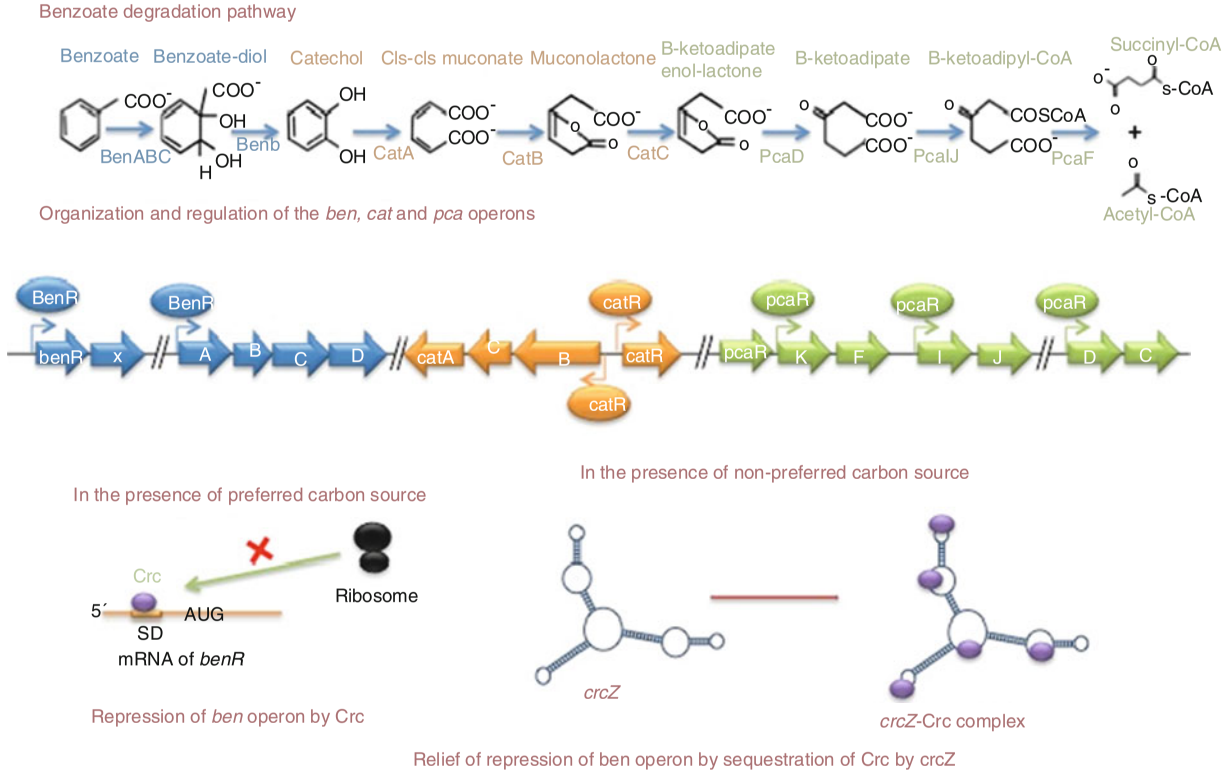

Fig. 32.4 Role of sRNA in regulation of *ben* operon

32.7.3 Regulation of CrcZ Expression

The expression of CrcZ is under the control of a two-component system consisting of a sensor kinase, CbrA and response regulator, CbrB. The CbrA-CbrB system belongs to the NtrB-NtrC family and controls specific pathways and modulates catabolism of various natural substrates in response to fluctuations in intracellular carbon-nitrogen (C:N) balance. Changes in C-N balance in the cell leads to auto-phosphorylation of CbrA protein. Phosphorylated CbrA, by a phosphor-transfer mechanism phosphorylates CbrB. CbrB-P activates the transcription of the σ^{54} dependent promoter of CrcZ by an unknown mechanism (Sonnleitner et al. 2009).

32.8 Future Perspectives

In the case of sensing the nitrogen status, the signal-transducing protein pII is uridilylated or deuridilylated depending on the intracellular glutamate and α-ketoglutarate ratio. This is in fact the key event that contributes to determining the phosphorylation state of the sensor kinase NtrB (Ninfa and Atkinson 2000). Is there such a signal-transducing protein to sense the intracellular C-N ratio? If so, what is the sequence of events that stimulate the auto-phosphorylation event in CrbA? If seen in the light of a nitrogen status sensing mechanism, whether or not prediction of such a mechanism appears logical, further work needs to be done to provide the evidence for its existence.

As described earlier, the benzoate and alkanes, like any other catabolites are capable of inducing transcription of the *ben* and *alk* operons. However, the translations of benR and alkS specific mRNAs are blocked by carbon storage regulatory protein, Crc. Such translational blockage is not seen when less preferred carbohydrates like lactose, arabinose etc. are used as the source of carbon. What is the evolutionary pressure upon bacteria to insert an additional regulatory step in its cascade when using alkanes and benzoate as sources of carbon? What advantage does the cell have with the inclusion of sRNA in its regulatory network? One possible answer to these complicated questions could be to provide a mechanism that permits the cell to use alternate carbon sources like alkanes and benzoate as a survival option in the absence of any other preferred carbon source. Such a bold statement can only be substantiated through further research on sRNA-mediated carbon catabolite repression.

32.9 Conclusions

The present chapter describes influence of sRNAs on various aspects of carbon metabolism in prokaryotes. In order to provide a comprehensive picture to the readers, especially to those who are in the beginning of their research career, a considerable

amount of information is provided on sRNA prediction methods, classification and mode of action etc. The information available on sRNA-mediated regulation of carbon catabolic operons is presented in a simplest possible terms to make the topic interesting both for general microbiologists and molecular biologists.

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Chapter 33
Lactic Acid Bacteria in Food Industry

Deeplina Das and Arun Goyal

Abstract Lactic acid bacteria (LAB) are known through ages for their wide applications in food, pharmaceutical and chemical industries. But recently LAB have aroused interest for their ability to secrete extracellular polysaccharides or glucans. These glucans have immense commercial value because of their industrially useful physico-chemical properties. The glucans derived from LAB play crucial role in improving rheology, texture, mouth feel of fermented food formulations and conferring beneficial physiological effects on human health, such as antitumour activity, immunomodulating bioactivity and anticarcinogenicity. The modulation of biochemical properties of glucans require a thorough understanding of its biosynthetic pathway and the relation between the structure of glucans and the functional effect provided by them after incorporation into the food matrix. LAB are employed in food industry for making yoghurt, cheese, sourdough bread, sauerkraut, pickles, beer, wine and other fermented foods and animal feeds like silage. LAB can also produce a variety of functional oligosaccharides that have applications as prebiotics, neutraceuticals, sweetners, humectants, drug against colon cancer and as immune stimulator. LAB are gram positive rods or cocci, non spore forming, acid tolerant, low GC containing, anaerobic or micro-aerophilic bacteria characterized by their ability to ferment sugar to lactic acid. The commonly known LAB genera are *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus* and *Streptococcus*. Besides prolonging the shelf life, lactic acid enhances the gustatory and nutritional value, imparts appetizing flavour and texture to the food. Some LAB produce proteinaceous antimicrobial compounds called bacteriocins which inhibit the growth of Gram-positive pathogenic and spoilage bacteria and used as food additives. Lactic acid bacteria as probiotics have been proven effective against diarrhoea, irritable bowel disorder, allergies, stimulation of immunity, lactose intolerance.

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Keywords Lactic acid bacteria • Exopolysaccharides • Oligosaccharides • Bacteriocin • Probiotic • Polyols

33.1 Introduction

Lactic acid bacteria are industrially important and beneficial microbes that have similar properties (gram positive and catalase negative) and all produce lactic acid as an end product of the fermentation process. The genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Melissococcus, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weissella* are recognized as LAB (Ercolini et al. 2001; Holzapfel et al. 2001). They are widespread in nature and are also found in our digestive systems. There are several potential healths or nutritional benefits possible from several species of lactic acid bacteria, among these are: improved nutritional value of food, control of intestinal infections, improved digestion of lactose, control of some type of cancer and control of serum cholesterol level. Although they are best known for their role in the preparation of fermented dairy products, they are also used for pickling of vegetables, baking, wine-making, curing fish, meats and sausages. *Lactobacillus plantarum* and *Lactobacillus sanfrancisco* are commercially available and widely used e.g. for the production of fermented milk products and for the preparation of sourdough (Sing 1977). LABs are also regarded as a major group of probiotic bacteria (Schrezenmeir and de Vrese 2001) i.e. they are a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance. LAB produces various types of exopolysaccharide which has numerous application in food and pharmaceutical industry (Naessens et al. 2005; Purama and Goyal 2005) with the help of extracellular glucansucrase enzyme. These glucansucrase produce three types of glucans based on the types of the linkages present. They are dextran, mutan and alternan. The dexran is composed of mainly $\alpha(1\rightarrow6)$ linkage and occasionally $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$ and $\alpha(1\rightarrow4)$ linkage as branching points (Monchois et al. 1999). Mutan is a type of insoluble glucan having more than 50% $\alpha(1\rightarrow3)$ linkages (Mooser 1992) and alternan composed of alternating $\alpha(1\rightarrow6)$ and $\alpha(1\rightarrow3)$ glucosidic linkages, with some degree of $\alpha(1\rightarrow3)$ branching (Seymour and Knapp 1980; Cote and Robyt 1982). LAB can also produce a variety of functional oligosaccharides synthesized by glucansucrase as a result of acceptor reaction (Demuth et al. 2002) and can be used as used as neutraceuticals, stabilizers and prebiotics (Goulas et al. 2004; Naessens et al. 2005).

33.2 LAB in Food Industry

Lactic acid bacteria is used throughout the world to produce specialty foods, particularly in fermented milk products, including yoghurt, cheese, butter, buttermilk, kefir. Some of the traditional fermented food products and their associated

Table 33.1 Fermented food products and associated bacteria

Type of fermented food	Lactic acid bacteria	Country	Sources
Kimchi	*Leuconostoc mesenteroides, Lactobacillus curvatus, Lactobacillus brevis, Lactobacillus sake* and *Lactobacillus plantarum*	Korea	Fermented mixture of Chinese cabbage, radishes, red pepper, garlic and ginger
Kefir	*Lactobacillus kefir, Lactobacillus brevis*	Russia	Fermented milk product
Swiss cheese	*Lactobacillus lactis, Lactobacillus delbrueckii*	Switzerland	Dairy product
nham	*Weissella cibaria, Leuconostoc citreum*	Thailand	Fermented fresh Pork
Magou	*Leuconostoc mesenteroides, Pediococcus cerevisiae, Streptococcus lactis*	South africa	Fermented maize porridge
Balao balao	*Streptococcus sp., Leuconostoc sp., Pediococcus sp.*	Phillipines	Fermented rice and shrimp mixture
Gari	*Lactobacillus pentosus, Leuconostoc fallax, Weissella paramesenteroides Lactobacillus fermentum*	Nigeria	Fermented cassava
Kishk	*Lactobacillus sakei, leuconostoc sp.*	Egypt	Fermented cereal and milk mixture
Laban rayeb	*Streptococcus feacalis*	Egypt	Fermented milks
Ras cheese	*Lactococcus sp., Lactobacillus sp., Enterococcus sp.,* and *Pediococcus sp.*	Egypt	Dairy product
Sauerkraut	*Leuconostoc mesenteriodes, Lactobacillus plantarum, Pediococcus acidilactici*	Western countries	Fermented cabbage
Sourdourgh	*Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus panis, Weissella cibaria*	Europe	Fermented cereals

lactic acid bacteria are listed in Table 33.1. LAB are either homofermentative or heterofermentative based on the organism's metabolic pathway. Homofermentative bacteria such as *Lactococcus and Streptococcus* yield two lactates from one glucose molecule, whereas the heterofermentative bacteria such as *Leuconostoc and Weissella* transform a glucose molecule into lactate, ethanol and carbon dioxide (Kuipers et al. 2000). Lactic acid bacterial also produce acetic acid, aroma compounds, bacteriocins and exoplysaccharide and several important enzyme. For example acetaldehyde, provides the characteristic aroma of yoghurt, while diacetyl imparts a buttery taste to other fermented milks which improve the taste quality because at the fermentation process by lactic acid bacteria produce lactic

Table 33.2 Type of dextran produced by *Leuconostoc* strain and their source (Purama and Goyal 2005)

Strain	Type of dextran	Source
Leuconostoc mesenteriodes NRRL B-512F	1,3-branched dextran	Root beer
Leuconostoc dextranicum NRRL B-1121	1,3-branched dextran	
Leuconostoc mesenteriodes NRRL B-523	1,3-branched dextran w/1,3-linear segments	
Leuconostoc mesenteriodes NRRL B-1118	1,3-branched dextran w/1,3-linear segments	Olives
Leuconostoc mesenteriodes NRRL B-1298	1,2-branched dextran	Cane juice
Leuconostoc mesenteriodes NRRL B-1299	1,2-branched dextran	
Leuconostoc mesenteriodes NRRL B-1397	1,2-branched dextran	Sugar cane
Leuconostoc mesenteriodes NRRL B-1399	1,2-branched dextrans	Cane stubble
Leuconostoc mesenteriodes NRRL B-1422	1,2-branched dextran	Orange concentrate
Leuconostoc mesenteriodes NRRL B-1424	1,2-branched dextran	Refined sugar
Leuconostoc mesenteriodes NRRL B-1297	1,2- or 1,4-branched dextran	
Leuconostoc mesenteriodes NRRL B-1402	1,2- or 1,4-branched dextran	Orange concentrate
Leuconostoc mesenteriodes NRRL B-1431	1,2- or 1,4-branched dextran	Cane juice
Leuconostoc mesenteriodes NRRL B-1433	1,2- or 1,4-branched dextran	Cane juice
Leuconostoc mesenteriodes NRRL B-1149	1,3 and 1,2- or 1,4-branched dextran	
Leuconostoc dextranicum NRRL B-1420	1,4-branched dextran	Mill slime
Leuconostoc citreum NRRL B-742	1,3-branched + 1,4-branched dextran fractions	
Streptobacterium dextranicum NRRL B-1254	1,3-branched + 1,4-branched dextran fractions	
Leuconostoc mesenteriodes NRRL B-1375	1,3-branched + 1,2- or 1,4-branched dextran fractions	
Leuconostoc mesenteriodes NRRL B-1377	1,3-branched + 1,2- or 1,4-branched dextran fraction	
Leuconostoc mesenteriodes NRRL B-1374	1,3-branched dextran	
Leuconostoc mesenteriodes NRRL B-1355	Alternan + Dextran	
Leuconostoc mesenteriodes NRRL B/11011		

(continued)

Table 33.2 (continued)

Strain	Type of dextran	Source
Leuconostoc mesenteriodes PCSIR-3		Cabbage and Carrot
Leuconostoc mesenteriodes NRRL B-1501		
Leuconostoc mesenteriodes IBT-PQ		'Pulque' (alcoholic beverage)
Leuconostoc dextranicum FPW-10		Fermenting palm wine
Leuconostoc mesenteriodes L.		
Leuconostoc mesenteriodes Lcc4		
Leuconostoc dextranicum strain elai		

acid in fermented milks which gives slightly tart taste. Some of the fermented foods produced by lactic acid bacteria are listed in Table 33.2 (Avonts et al. 2004).

33.3 Lactic Acid in Food Industry

Lactic acid is water soluble, non-volatile odorless and is classified as GRAS (generally regarded as safe) by FDA in the USA. Several lactic acid bacteria produce lactic acid at the end of carbohydrate fermentation. The presence of lactic acid, produced during the lactic acid fermentation is responsible for the sour taste and for the improved microbiological stability and safety of the food. Lactic acid is produced in the form of L(+) or D(−) lactic acid or as its racemic mixture. Organisms that form the L(+) form or D(−) form have two lactate dehydrogenases (LDH), which differ in their stereospecifity. Some *Lactobacilli* produce L(+) form, which on accumulation induces a racemase, which converts it into D(−) lactic acid until equilibrium is obtained. *Lactobacillus plantarum* produce both L(+) form or D(−) form. Of these L(+) form is of great application for its ability to tolerate high concentration of hydrogen ions and pure form of L(+) lactic acid is produced from *Lactobacillus helvicticus* by metabolic engineering (Nikkila et al. 2000).

Lactic acid is used as acidulant, flavorings and pH buffering agent or inhibitor of bacterial spoilage in a wide variety of processed foods. In contrast to other food acids it has a mild acidic taste. It is a very good preservative and pickling agent. Addition of lactic acid aqueous solution to the packaging of poultry and fish increases their shelf life. The esters of lactic acid are used as emulsifying agents in baking foods (stearoyl-2-lactylate, glyceryl lactostearate, glyceryl lactopalmitate). The manufacture of these emulsifiers requires heat stable lactic acid, hence only the synthetic or the heat stable fermentation grades can be used for this application (Sodegard 1998).

33.4 Glucans from LAB in Food Industry

Lactic acid bacteria produce a wide variety of food grade exopolysaccharides (EPS) with the help of glucosyltransferases that have nutritional and health applications. Glucansucrases are large extracellular enzymes capable of synthesizing various glucans from sucrose, such as dextran, mutan and alternan. These exopolysaccharides are potential therapeutic agents (Korakli and Vogel 2006) and are also used as viscosifying, stabilizing, emulsifying, sweetening, gelling or water-binding agents, in the food as well as in the non-food industries (Sutherland 1998; Welman and Maddox 2003). β-glucan consist of linear unbranched polysaccharides of linked β-(1→3) and β-(1→4)-D-glucopyranose units, and it is a natural water-soluble fiber that cannot be digested by human enzymes, but is degraded by bacteria in the colon into short-chain fatty acids (SCFAs). LAB strains belonging to the genera *Pediococcus*, *Lactobacillus* and *Oenococcus* isolated from cider and wine, produce a 2-substituted (1→3)-D-glucan (Dols-Lafargue et al. 2008). The plasmid *gtf* gene determinant for EPS production was cloned into *Escherichia coli* and determination of its DNA sequence revealed that it encodes a protein, named GTF glycosyltransferase. Cloning of the *gtf* gene and functional expression of its encoded glycosyltransferase in *Streptococcus pneumoniae* (Werning et al. 2006) and *Lactococcus lactis* revealed that this enzyme is indeed responsible for the synthesis of the β-D-glucan (Werning et al. 2008).

Glucan has several importances in food industry for its soluble nature which form viscous aqueous solutions and increases the viscosity of gut contents in the human alimentary tract. Now soluble fibers are very much concerned in human nutrition (Dols-Lafargue et al. 2008).

1. *Cholesterol lowering effect*: it absorbed the cholesterol, helps in lowering the blood cholesterol level, thus reduce the risk of cardiovascular disease.
2. *Lowering effect of the glycaemic index*: Glucans are very viscous in nature which makes the gastric content thicker and help in slowing down the absorption rate of glucose. ß-glucan thus spread glucose absorption over time (i.e. *reduction of glycaemic index*) and helps the body to fight against diabetes. In food industry, beside classical applications of polysaccharides as thickening agent, β-glucans have an increasing interest in the areas of edible film and as stabilizers in the manufacture of low-fat products such as salad dressings (Kontogiorgos et al. 2004), ice creams and yoghurts (Brennan et al. 2002) and cheese.

Four different genera of lactic acid bacteria, *Streptococcus, Leuconostoc, Pediococcus and Lactobacillus* are known to produce glucan. Glucan synthesis in lactic acid bacteria has been mainly studied from *Leuconostoc* spp. (Monsan et al. 2001; Majumder et al. 2009; Purama et al. 2009) with the help of glucansucrase enzyme. The culture condition of novel glucan and glucan producing glucansucrase from *Leuconostoc* spp. has been optimized by statistical approach method. Various types of glucan produced by *Leuconostoc* species and their source are given in Table 33.2. Glucansucrase (or also called dextransucrase) (E.C. 2.4.1.5) catalyzes

the polymerization of the glucopyranosyl moieties of sucrose to form dextran (Purama and Goyal 2008). Dextran are used as viscosifying, texturizing or gelling agents in food formulations (Majumder and Goyal 2009), due to its non-ionic character and good stability under normal operating condition.

L. mesenteroides NRRL B-640 is shown to produce dextransucrase that gives highly linear and soluble dextran (Uzochukwu et al. 2002). A novel dextran produced by *Leuconostoc dextranicum* NRRL-B-18242 having a slushy, applesauce-like appearance with a particulate gel-like structure was described and the dextran in particulate or dried form is used in foods and other applications where texture is important (Pucci and Kunka 1990). *Leuconostoc mesenteroides* NRRLB-512 F is also used industrially to produce dextransucrase, which synthesize extracellular homopolysaccharide dextran (Purama and Goyal 2005).

The dextran is commercialized in a powder form and as a liquid with the following specifications

1. For the powdered form (values in % of commercial product) carbohydrate 60 (with dextran 50, mannitol 0.5, fructose 0.3, leucrose 9.2), protein 6.5; lipid 0.5; lactic acid 10; ethanol traces; ash 13; moisture 10.
2. For the liquid form (values in % of commercial product): carbohydrate 12 (with dextran 6.9; mannitol 1.1; fructose 1.85; leucrose 2.15); protein 2; lipid 0.1; lactic acid 2; ethanol 0.5; ash 3.4; moisture 80.

The solubility of dextran depends upon the branched linkage pattern. Presence of 95% linear linkages makes this dextran water-soluble, which makes it suitable for various applications (Leathers 2002).

The micro-organisms used for the production of dextran (*Leuconostoc mesenteroides*, *Lactobacillus plantarum*, *Lactobacillus sanfrancisco*) are currently used in food processing without any restriction. As a result of microbial activity, dextran occurs in small amounts in naturally fermented products such as sauerkraut and cucumber and in kefir where it probably plays a role in the thickening (Roller and Dea 1992). It has been reported that the glucan produced by *Leuconostoc dextranicum* NRRL B-1146, having $\alpha(1\rightarrow4)$ and $\alpha(1\rightarrow6)$ linkages, showed non-Newtonian pseudoplastic behaviour (Fig. 33.1) indicating its branched nature and also have unique rheological properties because of its potential of forming very viscous solution at low concentration and can be used as thickening or gelling agent in food.

Further the Scanning Electron Micrograph (shown in Fig. 33.2) of the glucan produced by *Leuconostoc dextranicum* NRRL B-1146 showed small porous or web like structure, that facilitates its water holding capacity, thus can be used as a texturing agent in food industry (Majumder and Goyal 2009). The surface morphology of dried and powdered dextran from *Leuconostoc dextranicum* NRRL B-640 was also studied using Scanning Electron Microscopy and it revealed the cubical porous structure of dextran, as shown in Fig. 33.3. It was recently reported that the dextran from *Pediococcus pentosaceus* holds potential usage as gelling agent in food formulations and as drug delivery carriers (Patel et al. 2010).

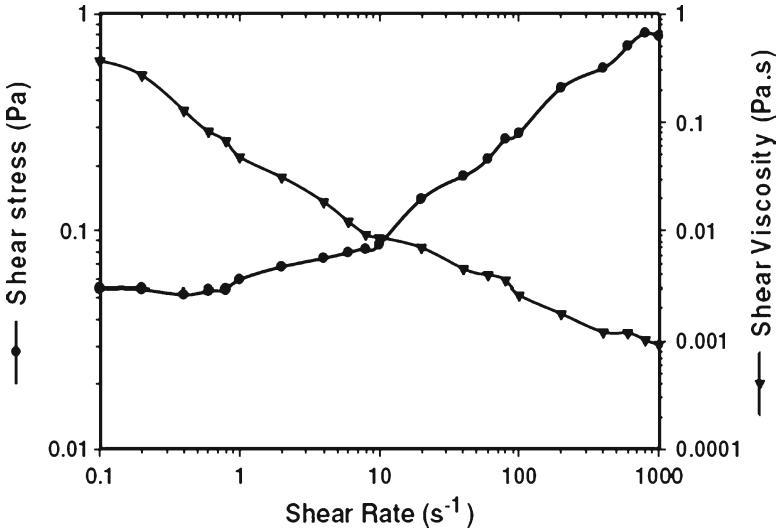

Fig. 33.1 The steady shear measurements for the semi-dilute glucan solution from *Leuconostoc dextranicum* NRRL B-1146 indicated that the viscosity (η) of the dilute polymer exponentially decreased with the increase in shear stress (τ) and exhibited a typical non-Newtonian psuedoplastic behavior (Majumder and Goyal 2009)

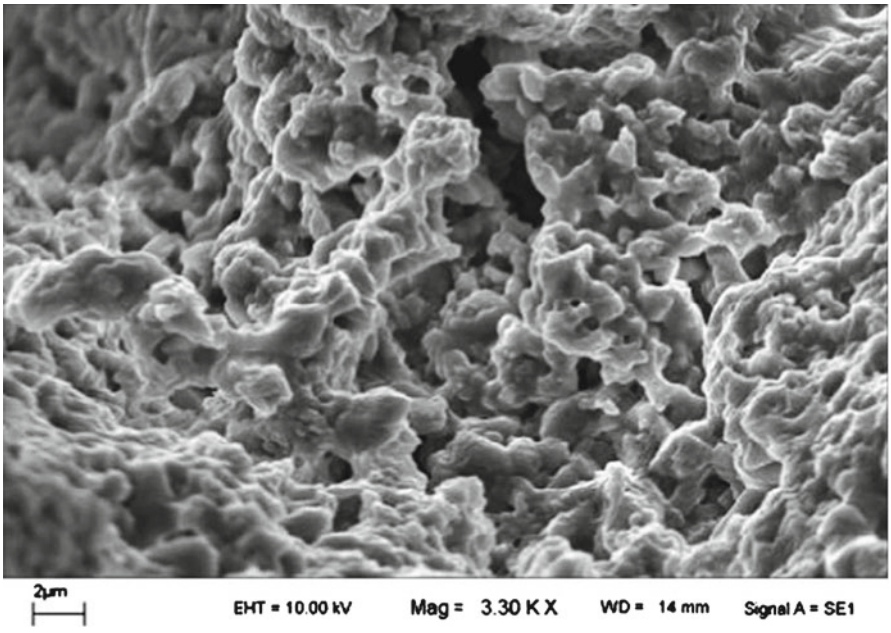

Fig. 33.2 Scanning Electron Micrograph of glucan showing surface morphology of *Leuconostoc dextranicum* NRRL B-1146 (Majumder and Goyal 2009)

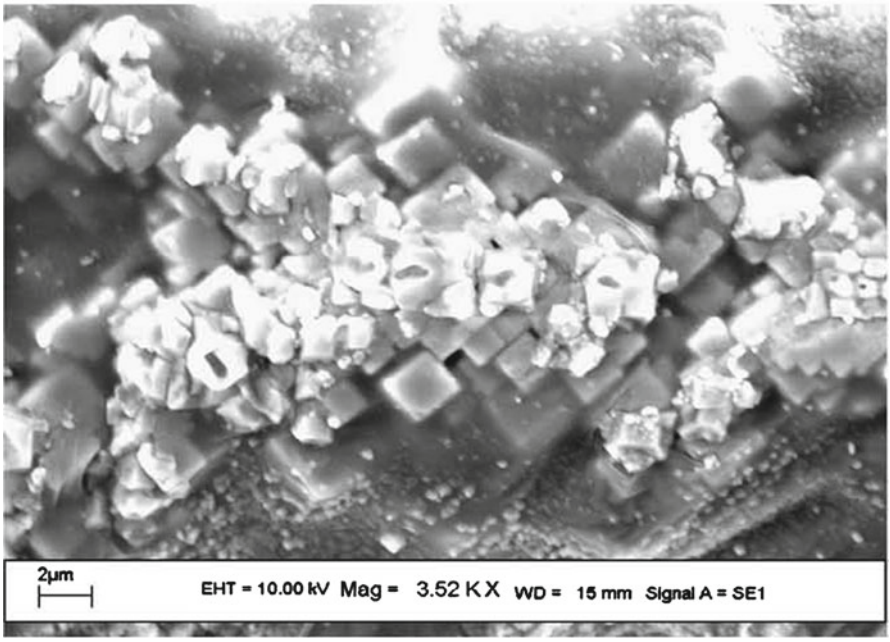

Fig. 33.3 Scanning Electron Micrograph of glucan showing surface morphology from *Leuconostoc dextranicum* NRRL B-640 (Purama et al. 2009)

The cytotoxicity test of dextran from *Pediococcus pentosaceus* was explored using human cervical cancer (HeLa) cell line and it is reported that there is no effect of dextran from *P. pentosaceus* on the viability of HeLa cells when observed for 72 h even at high concentration of 1,000 µg/ml (Fig. 33.4) (Patel et al. 2010). This revealed that the dextran is non-toxic and biocompatible, rendering it safe for drug delivery, tissue engineering and various other biomedical applications.

33.5 Oligosaccharides from LAB in Food Industry

Certain oligosaccharides (e.g. fructooligosaccharides, isomaltooligosaccharides and lactulose) and polysaccharides (e.g. fructans) are used as prebiotic foods (Monsan and Paul 1995). These oligosaccharides contains high proportion of $\alpha(1 \rightarrow 6)$ glycosidic linkages that are partly or totally resistant to attack by human's and animal's digestive enzymes. Prebiotic oligosaccharides are noncarcinogenic, nondigestible and low calorific compounds stimulating the growth and development of gastrointestinal microflora described as probiotic bacteria such as *Bifidobacteria*

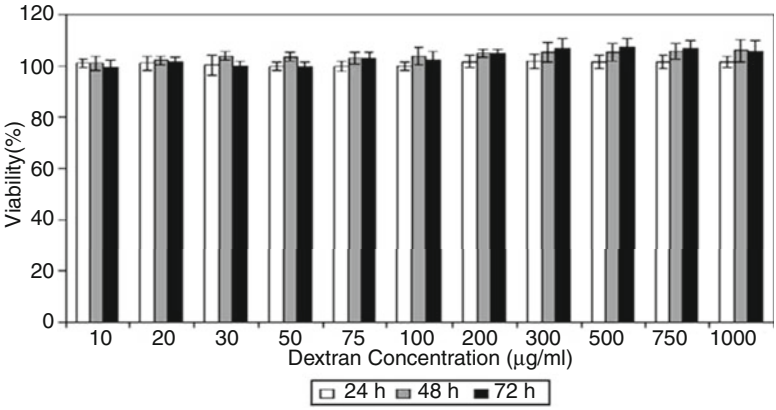

Fig. 33.4 The indirect contact based in vitro cytotoxicity assay showing the cell viability was unaltered after treatment with various concentrations of dextran (10–1,000 µg/ml) from *Pediococcus pentosaceus* over a period of 24–72 h incubation (Patel et al. 2010)

and *Lactobacilli* (Kubik et al. 2004). They are not absorbed in small intestine and in the large intestine they are metabolized by the colonic bacterial flora and fermented into short chain fatty acids. The effects of prebiotics are principally due to stimulation of the growth of bifidobacteria (bifidogenic effect). The stimulation of this growth allows a reduction in the pH of the colon, an increase in the production of short chain fatty acids (SCFAs), in particular butyrate and propionate, a prevention of the installation of pathogenic microorganisms (barrier effect), an increase in the metabolization of potentially carcinogenic aminated compounds and the production of vitamin B.

Gluco-oligosaccharides have been produced by enzymatic synthesis using glucosyl donor and an accepter molecule (Iliev et al. 2008). Gluco-oligosaccharides production from glucan by microwave assisted hydrolysis from *Leuconostoc mesenteroides* NRRL B-742 has also been reported. The peak at ~527 m/z value using MALDI-TOF-MS showed the presence of three glucose units of (m/z ~180) and degree of polymerization of 3 (DP-3) for the oligosaccharide that was obtained from the glucan sample hydrolysed for 2 min.

The oligosaccharides are used widely in various food products such as soft drinks, cookies, cereals, candies and dairy products (Barreteau et al. 2006). For instance, galactooligosaccharides have shown very promising results increasing populations of both *Lactobacilli* and *Bifidobacteria* and increasing beneficial short chain fatty acids (Smiricky-Tjardes et al. 2003). A mixture of galacto-oligosaccharides and fructo-oligosaccharides added to standard infant formulas has also been shown to increase both *Lactobacilli* and *Bifidobacteria* species in human infants (Moro et al. 2002). Other applications for oligosaccharides such as an anticariogenic agent or a low-sweetness humectant have been explored (Chung and Day 2002).

33.6 Bacteriocin from LAB in Food Industry

A large number of ribosomally synthesized bacteriocins or bacteriocin-like substances are produced by lactic acid bacteria (LAB) have been identified and characterized in recent years due to their antimicrobial activity against foodborne pathogenic, as well as spoilage bacteria. The antibacterial spectrum frequently includes spoilage organisms and food-borne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus*. For these it has raised considerable interest for their application in food preservation that help to reduce the addition of chemical preservatives and/or the intensity of heat and other physical treatments, thus satisfying the demands of consumers for foods that are fresh tasting, ready to eat and lightly preserved. Bacteriocin can be added to foods in the form of concentrated preparations as food preservatives, shelf-life extenders, additives and ingredients (*ex-situ*) or they can be produced in situ by bacteriogenic starters, adjunct or protective cultures (Galvez et al. 2007). *In situ* bacteriocin production offers several advantages compared to *ex situ* production regarding both legal aspects and costs. Bacteriocins are usually inactivated by low pH, heat and from digestive enzyme such as proteases. Nisin, the product of some strains of *Lactococcus lactis* subsp. *lactis*, which was accorded GRAS (generally recognized as safe) status and approved for food use by the U.S. Food and Drug Administration, has already found a variety of applications in food preservation (Twomey et al. 2002). Till date only nisin and pediocins have been used as biopreservatives in food systems (Ray 1992; Rodríguez et al. 2002). Up to now, bacteriocins have been isolated from the commercial probiotic strains *Lactobacillus casei* Shirota and *Lactobacillus johnsonii* La1 (Avonts et al. 2004). Immobilized bacteriocins can also find application for developement of bioactive food packaging. The effectiveness of bacteriocins requires careful testing in the food systems for which they are intended to be applied against the selected target bacteria.

33.7 LAB as Probiotic

Lactic acid bacteria *mainly Lactobacilli, Streptococci, Enterococci* and *Lactococci* can be used as probiotic (Schrezenmeir and de Vrese 2001). They possess the property by which they can be considered as probiotic such as colonization or adhesion properties, good *in vitro* development, ability of cells to produce metabolites and enzymes, stability in bile and gastric juices, production of antimicrobial substances, antagonistic action against noted pathogenic bacteria and/or viruses and no adverse interactions with host especially, in terms of pathogenicity. LAB also potentially used as starter cultures for the manufacture of dairy-based probiotic foods (Saarela et al. 2002). Probiotic acidophilus is a naturally occurring antibiotic that helps enhance digestion produces vitamin B and brings down the risk of colon cancer. In addition, antimicrobial production by probiotic LAB might play a role during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut

Table 33.3 Probiotic food and their applications

Food	Microrganism	Manufactured By	Uses
Actimel	*L.bulgaricus, S. thermophilus and L. casei.*	Dannon Company	Used as delicious sweet milk
Align	*Bifidobacterium infantis* 35624		Found in clinical studies to help build and maintain a healthy digestive system as well as benefitting those suffering from irritable bowel syndrome (IBS)
LC1	*Lactobacillus johnsonii*	Nestle	Used as baby food to reduce the risk of infant diarrhea
Lifeway Kefir	*L. lactis, L. cremoris, L. diacetylactis, L. casei*	Lifeway and is available at Wild oat markets	Used in many food products in order to improve immune system

health. Probiotics may regulate local and systemic immunity, thereby reducing allergic disease severity and susceptibilities of infants and children to allergies and atopic diseases (Hsieh and Versalovic 2008). There are several example of probiotic food that is commercially available shown in Table 33.3.

33.8 Polyols Produced by LAB

Several LAB produced polyols which are most commonly used as sugar replacement agents in food industry. Polyols are known as sugar alcohols and although they have the structure of alcohol, they mimic the sweetness of sugar when added to food. Sugar alcohols have a lower calorie count than true sugar because they are not entirely absorbed by the body and are often used in sugar-free or diet foods and drinks. One product that almost invariably contains sugar alcohols is chewing gum. They are added to gum because they are not broken down by either saliva or chewing action and therefore do not cause tooth decay. Some of the most common sugar alcohols are maltitol, sorbitol which are produced by LAB. *Leuconostoc fructosum* NRRL B-2041 produced manitol with maximum volumetric productivity of 2.36 g/l h when grown in supplemented carob syrup medium (Carvalheiro et al. 2010). All of these sugar alcohols produced by LAB have different ratings of sweetness. Sorbital is 60% as sweet as true glucose, while maltitol rates at 75%. Sorbitol is synthesised by an engineered *Lactobacillus casei* and *Lactobacillus plantarum* (Nissen et al. 2005; Ladero et al. 2007). Both homo- and heterofermentative lactic acid bacteria produce mannitol. Generally homofermentative lactic acid bacteria produce little amount of mannitol with the help of mannitol 1-phosphate dehydrogenase enzyme. Such as, in the presence of large amounts of glucose or sucrose, *Streptococcus mutans*, *Lactobacillus leichmanii* (Chalfan et al. 1975), *Lactobacillus plantarum* and *Lactococus lactis* (Neves et al. 2000) produce mannitol. Hetero fer-

mentive lactic acid bacteria produce substantial amount of mannitol with the help of mannitol dehydrogenase enzyme. In the presence of fructose or sucrose, *Leuconostoc (pseudo)mesenteroides* produces high levels of mannitol (Grobben et al. 2001). Two other heterofermentative lactic acid bacteria, *Lactobacillus sp*. and *Leuconostoc* sp. also produced mannitol from fructose and sucrose (Saha and Nakamura 2003). The amount of mannitol produced under optimal culture conditions by Lactobacillus and Leuconostoc strains were 73 and 26 g/l from 100 g/l fructose with yields of 86% and 65%, respectively (Yun and Kim 1998).

33.9 Future Perspectives

Genome sequencing and functional genomic studies of a variety of LAB are rapidly providing insight into their diversity and evolution and revealing the molecular basis for important traits like flavour formation, sugar metabolism, stress response adaptation and interaction. LAB have been investigated using biotechnological techniques, including genetically modified organisms (GMOs) that contribute to the reliability of food fermentation and better process control. New properties can be introduced that enhance quality, taste, structure and wholesomeness of food and consequently bring benefit to the consumer. Also the use of LAB as probiotics in the treatment of many forms of diarrheal disease appears especially promising. The identification of glucan production gene is important for future challenges for the construction of strains of LAB that produce Glucan with novel properties that could be applied as food additives. The yield of glucans produced by the LAB depends on the composition of the medium, the LAB strain and growth conditions like temperature, pH, oxygen tension and incubation period. The need of the day is to improve the productivity of glucans from LAB, reduce the cost of production for commercial viability and to produce custom made glucans with the desired functionality. The modulation of biochemical properties of glucans require a thorough understanding of its biosynthetic pathway and the relation between the structure of glucans and the functional effect provided by them after incorporation into the food matrix. Several investigators are working on β glucans that can promote antitumor and antimicrobial activity by activating macrophages, dendritic cells and other leukocytes.

33.10 Conclusions

Several metabolites product produced by LAB including organic acids, fatty acids, hydrogen peroxide, carbon dioxide and bioactive peptides have antimicrobial effects. As biopreservatives are more preferred than chemical preservatives, there is an increased interest in prevention of food from spoilage through these metabolites produced by LAB because of their safe association with human fermented foods. The exopolysaccharides that are produced by LAB contribute to the specific rheology and texture of fermented milk products and also have applications in nondairy

foods. When added to food products, polysaccharides function as thickeners, stabilizers, emulsifiers, gelling agents and water binding agents. Several oligosaccharides produced by LAB can act as prebiotic, increase the production of short chain fatty acid. Probiotic LAB strains might play a considerable role during *in vivo* interactions occurring in the human gastrointestinal tract, for instance towards *H. pylori, E. coli* and *Salmolella.* Bacteriocin-producing starter or co-cultures have been successfully produced on pilot-scale experiments in making cheese, fermented sausage, sourdough, etc., yielding food quality with food safety advantages.

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Chapter 34
RNA Interference and Functional Genomics in Fungi

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Abstract During the last one decade, rapid advances have been made in the field of functional genomics research in fungi using conventional and non-conventional approaches. RNA interference (RNAi), which is a sequence specific silencing of gene at the post transcriptional level, is rapidly becoming a powerful reverse genetic tool, and its potential is also being explored in fungi to validate the gene function. Since over 40 fungal genomes have been sequenced and publicly released and some more genomes are being sequenced, the functional genomics is of utmost important to discover a great deal of new information in the coming years. This review discusses the recent progress on the utilization of RNAi technology in examining gene function in fungi.

Keywords RNA interference • siRNA • Quelling • Post transcriptional gene silencing • Functional genomics • Fungi

34.1 Introduction

The kingdom fungi comprises of diverse range of eukaryotic organisms which are over 1.5 million in number and it is divided into four major groups, that is, ascomycetes, basidiomycetes, zygomycetes and chytrids. The physiology and genetics of fungi share a similarity with plants and animals, including multicellular nature, cell cycle, development and differentiation, intercellular signaling, DNA modification and methylation (Galagan et al. 2005). Fungi influence different life forms either directly or indirectly in both positive and negative ways. They colonize the roots of

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plants protecting them from diseases and provide nutrients to them by establishing the symbiotic relationship. Trappe (1987) reported that over 90% of fungi form the mycorrhizal associations with roots of plants. They are the dominant microorganisms in soil and play a crucial role in nutrient recycling. Some of the fungi are useful in food industry as the fermentation agents and while some are the producers of important secondary metabolites. Apart from these roles some of the fungi are pathogenic and infect crop plants. Fungal infections also impose a threat to human health, especially affecting the immunocompromised or therapeutically immunosuppressed patients.

All these factors emphasize on the need to understand various processes as pathogenesis, growth, development and metabolism in fungi and hence it is important to identify and functionally characterize the important genes linked to these processes by using efficient functional genomics tools. The elucidation of gene functions would make it possible to do important genetic manipulations so as to upgrade the yield of important secondary metabolites produced by them or to study their interaction with the plants and other hosts and devise the ways to control fungal diseases. The revolution in fungal genomics has been brought about by advanced genome sequencing technologies available. There are several sequencing projects which are in process of decoding the different fungal genomes. The first fungal genome to be sequenced was of *Saccaromyces Cerevisiae* (Goffeau et al. 1996) followed by *Shizosaccaromyces pombe* and *Neurospora craassa* genome (Wood et al. 2002; Galagan et al. 2003). In the year 2000, a consortium of mycologists launched the fungal genome initiative (FGI) project which aimed to sequence the genomes of fungi. At present approximately 40 fungal genomes are publicly available and over 40 are in the process of being sequenced, these include important human and plant pathogens and model organisms (Bhadauria et al. 2009). When the genome sequence is available, the important thing remains is to apply the right approach to study the functions of genes identified. The commonly used approaches in fungi are targeted gene disruption/replacement (knock out) which depends on homologous recombination. A very recent approach for manipulating fungal gene expression is RNA silencing or RNA interference (RNAi) which is gaining wide popularity as a tool to identify the functions of gene/s with known sequences, especially of genes present in multiple copies or when particular genes knock out leads to lethality in the organism (Kuck and Hoff 2010). RNAi is a RNA dependent gene silencing phenomenon present in all eukaryotes, with primary role of regulation of gene expression at transcriptional or post- transcriptional level (Denli and Hannon 2003). RNAi controls the development of an organism and physiological functions of cells and tissues and is also known to play a role in genome defense against the transposons and invading viruses in some organisms. The basic mechanism of RNAi is common to all organisms with small interfering RNAs (siRNAs) mediating the silencing mechanism (Bernstein et al. 2001). The siRNAs reduces gene expression by cleaving homologous transcripts or by translational inhibition and also at transcriptional level by chromatin modification and heterochromatin formation. This chapter mainly focuses on the basic RNAi mechanism with special reference to fungi, and its importance as an alternative tool to study functional genomics in fungi.

34.2 Basic RNAi Mechanism

Andrew Fire and Craig Mello in 2006 received the Noble prize for their contribution to the understanding of RNAi. The antisense, sense and dsRNA (mixture of sense and antisense RNA) specific to the endogenous *unc*-22 gene which encodes for myofilament protein was injected separately in a worm *Coenorhabditis elegans* to silence it and see the phenotypic effect which is visible in the form of twitching and in severe cases lack of motility and they concluded that dsRNA efficiently triggers the target mRNA silencing as compared to either sense or antisense strand alone and this signal can also move from tissue to tissue within the injected organism (Fire et al. 1998). It has also been reported that the silencing signal could be transferred to several generations in *C. elegans* through germ cells which shows the remarkability of RNAi in terms of its inheritance in the further generations (Grishok et al. 2000). In general, the RNAi pathway involves the three main steps (Fig. 34.1).

1. Induction by dsRNA,
2. Processing of dsRNA into 21–25 nt small RNA,
3. Incorporation of siRNAs into effector complexes that bind the complementary target RNA and degrade it.

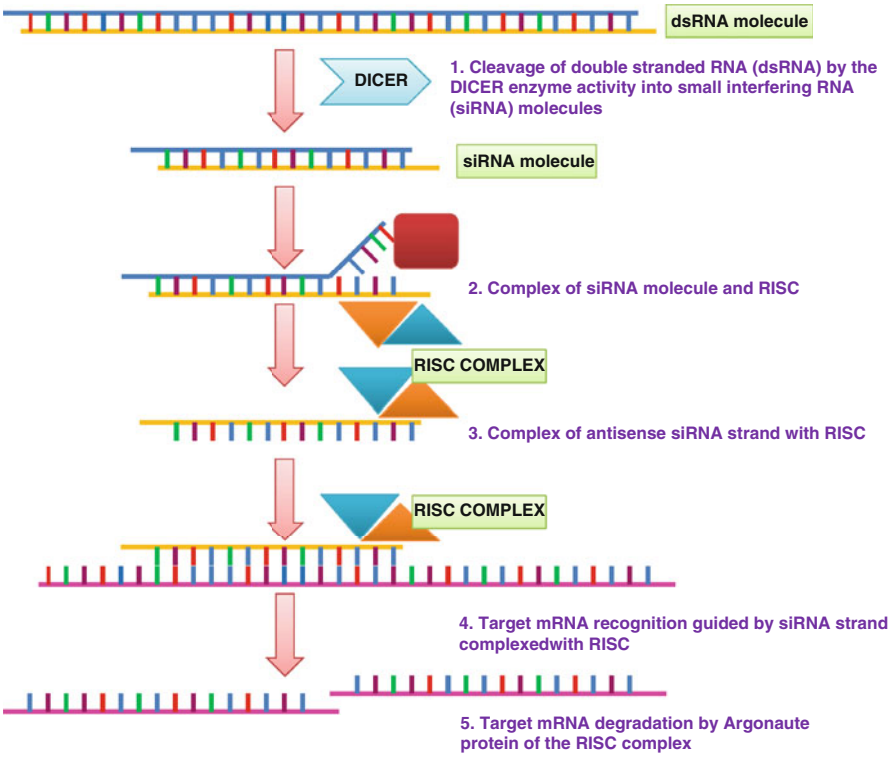

Fig. 34.1 Mechanism of RNA mediated gene silencing

The source of dsRNA could be an inverted repeat sequence or convergent transcription of transgenes, transposons, viral RNA and can also be synthesized from aberrant mRNA transcripts by RNA dependent DNA polymerases (RdRps). The basic mechanism of RNAi involves the formation of intermediates called siRNAs. The siRNAs are the dsRNA molecules of 21–25 nucleotides with characteristic two unpaired nucleotide overhangs at the 3′ end of each of the strand. The siRNAs are produced by the endonucleolytic cleavage of the exogenous dsRNA by the multidomain ribonuclease (RNase) III protein known as Dicer (Elbashir et al. 2001; Bernstein et al. 2001). The dicer comprises of three domains- the RNA helicase domain called PAZ domain (Piwi/Argonaute/Zwille), dsRNA binding domain and of RNase III domains. The slicing of dsRNA to siRNAs is the result of the RNase III activity of the dicer which is functionally active as a dimer, catalyzing four breaks in the phosphodiester backbone of dsRNA leading to the formation of single siRNA (Zamore 2001). The 3′ overhangs of dicer formed upon dicing are methylated by methyltransferases so as to protect them from oligouridylation and degradation. The siRNAs are bound by a ribonucleoprotein complex called RNA induced silencing complex (RISC) (Dalmay et al. 2000; Tijsterman et al. 2002). The enzyme component of RISC belonging to argonaute protein family (AGO) is important as it executes the effector functions leading to gene silencing. The AGO protein has two important domains, PAZ and PIWI domain (Carmell et al. 2002). The PAZ domain is involved in transfer of siRNAs to the RISC complex (Lingel et al. 2003) and PIWI domain has the nuclease activity responsible for siRNA guided cleavage of the target mRNA (Song et al. 2004). Selected siRNA strand incorporates one or several RISC that scans the cell for the complementary nucleic acids to execute their function. The siRNA directed activities include- the slicing activity/endonucleolytic cleavage of target RNA upon transportation to the cytoplasm, retention in the nucleus and DNA cytosine and histone modifications leading to methylation of the target gene sequence and thirdly, it can also cause translational repression. The RNAi is an ATP dependent process; the processing of siRNAs from dsRNA by dicer requires the ATP (Hamilton and Baulcombe 1999 and Zamore et al. 2000). Some of the important features of RNAi are its sequence specificity that the dsRNA sequence must correspond to the target mRNA sequence to be knocked down. Zamore (2001) has reported that even a short stretch of homology of 23 nucleotides between the target gene and the dsRNA can lead to gene silencing in plants and few molecules of dsRNA are sufficient to achieve the complete silencing due to amplification of the siRNA molecules by the RdRp enzyme present in worms, fungi and plants. Most of the enzymes involved in RNAi pathway are conserved in different organisms, indicating the common ancestral origin of the pathway.

34.3 RNAi in Fungi

In fungi, RNAi is commonly referred to as ‘quelling’ and was discovered by Romano and Macino (1992), in the model fungus *Neurospora crassa*. It has also been reported in many other pathogenic and non pathogenic fungi. The non pathogenic fungi

Shizosaccharomyces pombe and *N. crassa* are the commonly used model organisms to study genetic and biochemical basis of RNAi in fungi (Arndt et al. 1995; Cogoni 2001). In fungi, two RNAi related phenomena are present, quelling and meiotic silencing by unpaired DNA. Quelling was observed in *N. crassa* during the vegetative phase of fungal growth, when Romano and Macino tried to express the exogenous genes (*al*-1, *al*-2) involved in carotenoid biosynthesis in wild type strain with orange phenotype. Their experiment resulted in an albino phenotype in some of the transformants due to the silencing of transgene as well as the homologous endogenous gene. This phenomenon was found to be reversible as the transformants could revert back to the wild type phenotype, probably because of reduction in copy number of exogenous gene. Quelling is mediated post- transcriptionally, since only the gene sequence of few nucleotides could result in gene silencing without the requirement of promoter along with it. It is similar to co-suppression observed in plants, where integration of transgene in the genome leads to simultaneous silencing of both the transgene and the homologous endogenous gene and both the processes require the presence of aberrant RNA. The proteins involved in quelling and post-transcriptional gene silencing (PTGS) in plants are highly conserved suggesting origin from the common ancestral mechanism that defends the genome from foreign molecules and transposons (Fagard et al. 2000). Romano and Macino used the stably quelled *al-1* strains to isolate the 15 different quelling deficient mutants (*qde*) belonging to three distinct genetic loci- *qde1*, *qde2* and *qde3*. The genes corresponding to these loci were cloned and found to encode three important components of the pathway. The first RNAi gene identified was *QDE-1* which encodes for an RdRp and has been shown to be involved in PTGS (Fire et al. 1998). RdRP uses aberrant transgenes as templates to produce dsRNAs (Cogoni and Macino 1999). Through *in vitro* studies the RdRP activity of QDE-1 was confirmed and its secondary structure has been revealed (Fulci and Macino 2007; Laurila et al. 2005). There are evidences which suggest that QDE-1 can function both as an RdRP and as a DNA dependent polymerase. *QDE-2* gene was the second gene cloned and identified to encode for the argonaute protein and it is homologous to *rde-1* gene of *C. elegans*, involved in dsRNA mediated gene silencing (Catalanotto et al. 2000). The third important gene required for quelling is *QDE-3* which encodes for RecQ DNA helicase homologous to human Werner/Blooms syndrome. QDE-3 facilitates the binding of QDE- 1 to the single stranded DNA at the transgenic region by resolving the complex DNA structures. Another protein called RPA-1 is believed to interact with QDE-3 and helps in recruiting QDE-1 to repetitive transgenic regions. The process of siRNA production requires the dicer like enzymes (DCL) and in *N. crassa* two dicer proteins were identified, DCL-1 and DCL-2. The double mutants lacking both the dicer enzyme activities are shown to be quelling deficient. The two enzymes are redundant functionally, with presence of any of the two sufficient to produce siRNAs. DCL-2 is the major dsRNA processing enzyme since *DCL-2* mutant show reduced accumulation of siRNAs (Catalanotto et al. 2004). To execute their function siRNAs get associated with the RISC complex which has argonaute protein QDE-2 as the core component. The RISC is in its inactive form when siRNAs are associated with it in the duplex form, it is only when the passenger strand is cleaved that the RISC is activated. It has been

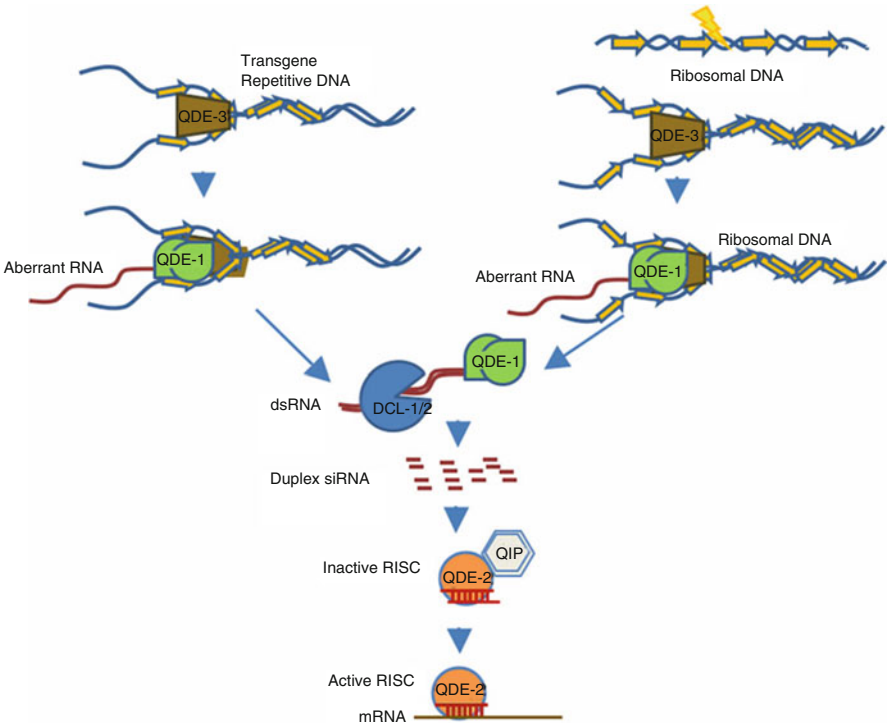

Fig. 34.2 A model for the quelling and qiRNA pathway in vegetative cells in *N. crassa* repetitive transgenes (quelling) or the rDNA locus after DNA damage induce the synthesis of aberrant RNAs by the DdRP activity of *QDE-1* facilitated by *QDE-3*. The aberrant RNA is converted into dsRNAs by the RdRP activity of *QDE-1*. The Dicer proteins *DCL-1* and *DCL-2* cleave the dsRNAs into siRNAs or qiRNAs, which are then loaded onto the RISC containing *QDE-2* and *QIP*. *QDE-2* and *QIP* convert the siRNA duplex into the mature siRNA, resulting in *RISC* activation and gene silencing of homologous RNAs (Adapted from Li et al. 2010)

reported that QDE-2 is required for both gene silencing and generation of single-stranded siRNA from siRNA duplexes *in vivo* (Maiti et al. 2007). It interacts with another protein QIP (QDE-2 interacting protein) to bring about passenger strand degradation. Gene disruption in QIP encoding gene results in accumulation of siRNA duplexes and gene silencing impairment. It acts as an exonuclease and removes nicked passenger strands from the siRNA duplex. Thus, RNA silencing pathway in fungi can be summarized into following main steps (Fig. 34.2).

1. dsRNA processing into siRNA duplexes by Dicer.
2. Association of siRNA duplexes with RISC (inactive form).
3. QDE-2 (argonaute protein) mediated cleavage of passenger strands of siRNA duplexes and removal of nicked passenger strands by QIP.
4. Single- stranded siRNA mediated cleavage of homologous mRNAs by activated RISC.

It has also been reported that treatment of *N. crassa* with DNA damage causing agents results in production of another type of small RNAs called qiRNAs. DNA damage induces significantly high levels of QDE-2 expression and this requires both QDE-1 and QDE-3 activity (Lee et al. 2009). This means during DNA damage dsRNAs are produced by QDE-1 and QDE-3 and the dsRNAs induce the expression of QDE-2. The dsRNA produced on DNA damage are cleaved to a novel class of small RNAs of ~21 nt length which have been named as qiRNA because they interact with QDE-2. The qiRNAs are mainly derived from the highly repetitive ribosomal DNA locus, have a strong 5′ uridine preference and a 3′ preference for adenine and they are produced by QDE-1, QDE-2 and Dicer enzyme activity suggesting that they are not the non-specific products of rRNA degradation (Lee et al. 2009). Interestingly, qiRNAs are derived from the aberrant RNA (aRNA) molecules that can be from both the transcribed as well as untranscribed intergenic spacer regions. Both QDE-1 and QDE-3 are required for the synthesis of DNA damage induced aRNA, since in *qde*-1 and *qde*-3 mutants aRNAs are not produced. This also proves that apart from its role in conversion of ssRNA to dsRNA QDE-1 is involved in aRNA production through its RNA polymerase activity. Importantly, qiRNA pathway and quelling share the key components, such as QDE-1, QDE-2. QDE-3 and Dicers and both require aRNA and dsRNA production. It could be said that qiRNA may contribute to the DNA damage response by inhibiting protein translation. Another RNAi related mechanism in *N. crassa* is the meiotic silencing by unpaired DNA (MSUD) which was discovered by Shiu et al. (2001). MSUD functions during meiotic phase in the life cycle of the fungus to silence the copies of unpaired gene during the pairing of homologous chromosomes. The MSUD deficient mutants led to the identification of important genes like *sad*-1 (suppressor of ascus dominance-1) which is a paralog of *qde*-1, *sms*-2 (suppressor of meiotic silencing-2) which encodes for protein homologous to argonaute proteins and another important gene is *sad*-2, product of which is required for proper localization of SAD-1 to the perinuclear region (Shiu et al. 2006; Bardiya et al. 2008). SMS-3 or DCL-1 is the Dicer protein which is also required for MSUD. All these genes were identified by analyzing the loss of function mutants; *sad-1*, *sad-2* and *sms-2* are dominant suppressor of meiotic silencing. All these proteins are found to co-localize in the perinuclear region, suggesting that it is the active center for MSUD. Quelling and MSUD require different sets of RNA- related proteins suggesting that there are two different types of RNAi pathways present in *N. crassa* (Nakayashiki et al. 2005).

34.4 Evolution of RNA Silencing Pathway in Fungi

In Eukaryotes the RNA silencing gene loss and gain are very much evident during the course of evolution and all the eukaryotic lineages possess the RNA silencing proteins (Cerutti and Casaa-Mollano 2006). It has been reported that four out of six eukaryotic supergroups have members that do not encode any of the three enzymes

of RNA silencing machinery. In the higher eukaryotic organisms, RNA silencing has the role in important biological processes like, genome defense, chromatin modification and gene regulation therefore is crucial for the growth and development processes while in fungi the importance of this phenomena is not yet clear. *N. crassa* RNA silencing mutants are sterile in homozygous crosses but they do not show any morphological abnormalities, (Lee et al. 2009; Shiu et al. 2001). In other filamentous fungi like *Magnaporthe oryzae* and *Mucor circinelloides*, the Dicer mutants show slight morphological abnormalities (Kadotani et al. 2003; Nicolas et al. 2007) and in *S. pombe* such mutants show abnormal cell cycle regulation (Carmichael et al. 2004) and cause chromosome segregation defect (Volpe et al. 2003). The RNA silencing pathway is well characterized in *N. crassa* which has two main RNA silencing pathways, Quelling and MSUD and it has been suggested that a single group of ancestral RNA silencing genes duplicated in an early ancestor of filamentous ascomycetes, leading to two paralogous groups of RNA silencing genes with evolutionary divergent functions (Borkovich et al. 2004 and Galagan et al. 2003). A number of mutants affected in quelling, PTGS and RNAi have been identified, which has led to the identification of eight genes controlling these phenomena. The comparative genetic analysis of RNA silencing proteins required for Quelling, RNAi and PTGS revealed that these three pathways are mechanistically linked. In *N. crassa*, *QDE-1*, *QDE-2* and *QDE-3* genes, in nematode *C. elegans*, *EGO-1*, *RDE-1*and *MUT*-7 genes and in *A. thaliana, SGS2* and *SGS3*, all of these genes encode for proteins similar to tomato RdRP. SGS2 and EGO-1 provide a molecular link between PTGS and RNAi but they act in different tissues. EGO-1 is required for RNAi in germline tissues while SGS2 is required for PTGS in somatic tissue as it does not occur in meristems from which germline is derived in plants. The *AGO*-1 (*A. thaliana*), *QDE*-1 (*N. crassa*) and *RDE*-1 (*Drosophila*) are all necessary for gene silencing in somatic tissues and they all have an amino acid leucine in the highly conserved domain. All these findings confirm the existence of mechanistic link amongst these pathways (Fagard et al. 2000). Nakayashiki and colleagues (2006) did phylogenetic analysis of RNA silencing proteins Argonaute, Dicer and RdRP in a wide range of fungi belonging to three main classes, ascomycetes, basidiomycetes and zygomycetes. The RNA silencing machinery seems to have undergone diversification during evolution in fungi which is evident from the fact that there are multiple RNA silencing pathways present in most of these fungi while there are some which either entirely lack them or have only some of the components present in them. *N. crassa* has three paralogues of RdRP, Argonaute and two of Dicer proteins (Galagan et al. 2003), whereas only one copy of each of these three enzymes has been identified in *Schizosaccharomyces pombe* genome (Wood et al. 2002). Interestingly, there are some fungi in which RNA silencing machinery seem to have been lost during evolution. For example, *Saccharomyces cerevisiae*, *Candida lusitaniae* and *Ustilago maydis* do not possess any gene with significant homology to any of the three RNAi proteins i.e. RdRP, Argonaute or the dicer protein, similarly *Aspergillus nidulans* possesses fewer RNA silencing proteins as compared to *A. fumigates* (Hammond and Keller 2005). Dicer and RdRP like proteins have not been reported in some fungi as *Candida albicans* and *Candida tropicalis*.

One reason for this could be loss of RNA silencing machinery in these fungi in ancestral groups since *Candida* and *Saccharomyces* are closely related or they might have been lost sporadically. Most of the members of filamentous fungi encode for the single set of proteins for quelling and MSUD but there are some exceptions like *A. oryzae* and *A. flavus* each encode three dicers and three Argonautes, might be due to duplication of genes involved in quelling. In *A. nidulans* the loss of RNA silencing gene has occurred, it has retained the genes involved in quelling, while genes *dclA* (Dicer), *ppdB* (Argonaute) corresponding to MSUD pathway in *N. craasa* are truncated at their respective 3′ and 5′ ends and these genes encode for the truncated proteins but these are not required for experimental RNA silencing, growth or developmental processes (Liande et al. 2010).

In conclusion, RNA silencing genes are either lost or have increased in number in fungi indicating that they have either evolved a new pathway or eliminated the existing pathways in response to changes in environmental conditions or with newly developed complexities in the life cycle. The role of RNA silencing pathways in the biology of these fungi is not yet very clear. But the genes involved in quelling in fungi, PTGS in plants and RNAi in animals seem to be mechanistically linked. And in plants and animals they have an important role to play in growth and development.

34.5 Functional Genomics in Fungi

The development of different transformation systems together with the efforts towards sequencing of genomes of various filamentous fungi had facilitated the rising interest in functional genomics research in filamentous fungi. Since for any functional genomics study an efficient transformation strategy is a prerequisite, in recent years many transformation strategies have been developed for a wide range of filamentous fungi. The following are the successfully used methods for fungal transformation:

1. Calcium chloride/polyethylene glycol
2. Electroporation
3. Particle bombardment
4. *Agrobacterium* mediated transformation

One of the earliest reports of transformation came in *S. cerevisiae* and the process involved the isolation of the protoplast from *S. cerevisiae* by dissolving the cell wall with glucanase preparation and incubating it with naked DNA in the presence of calcium chloride (Beggs 1978; Hinnen et al. 1978). Soon after this use of protoplasts in transformation was extended to members of Ascomycetes, *N. crassa* (Case et al. 1979) and *A. nidulans* (Tilburn et al. 1983) and to many other species. The crucial step in this method is the isolation of protoplast which depends on the choice of enzymes used for digesting the cell wall. These enzymatic preparations are the complex mixture of hydrolytic enzymes comprising chiefly of the 1, 3-glucanases and chitinases. The examples of some of the commercially available enzymes are

Novozyme 234 obtained from the fungus *Trichoderma viride* and zymolyase which is of microbilogical origin. Electroporation is also used to transform the protoplast, it involves exposing the protoplast to high amplitude of electric current which permeablizes the cell membrane thereby permitting the uptake of DNA. The method of particle bombardment depends on coating the gold or tungsten beads with the transforming DNA and bombarding it to the fungal tissue. And the more recent method is the *Agrobacterium*-mediated transformation (AMT) which has proved useful in transforming wide range of fungi and fungal tissues with high frequency even those that are recalcitrant to most other systems of transformation. Most of the functional genomics approaches are based on AMT method, since it produces stable transformants and is suitable for both gene replacement by homologous recombination (Khang et al. 2005) and insertional mutagenesis (Li et al. 2005; Combier et al. 2003) by random integration. With efficient transformation system available the only problem that remains is of the multinucleate nature of the most of the fungi and more so because techniques of gene replacement and insertional mutagenesis to achieve gene knock outs rely on the homokaryotic transformants derived from a single transformation event to study loss of functional mutants. It is possible to overcome this problem if it is possible to transform uninucleate tissue or cycle transformed tissue through a uninucleate stage (Cvitanich and Judelson 2003). The other solution to overcome this problem is the gene knock down strategy as RNAi involves inactivation of target mRNA rather than gene mutation. We will briefly discuss the conventional ways to study functional genomics in fungi before elaborating on RNAi approach to achieve the same.

34.5.1 Different Methods to Study Gene Function in Fungi

34.5.1.1 Random Insertional Mutagenesis

Random tagged mutations can be created by inserting DNA into the genome of the fungi leading to the disruption of genes, tagging of promoters or enhancers or up-regulation of genes. The phenotypic changes of interest are monitored in the transformants and the genomic region carrying the inserted genetic element is retrieved by either PCR based methods as inverse PCR and TAIL PCR or by plasmid rescue (Combier et al. 2003). Gene tagging can be achieved by direct DNA transfer in a non homologous manner and a library of thousands of tagged mutants can be obtained which could be correlated with a particular phenotype leading to the discovery of new genes. T-DNA is another form of insertional mutagenesis that relies on AMT to integrate the T-DNA at random sites in the recipient genome. AMT has various advantages over other methods of insertional mutagenesis as it leads to relatively high frequency of transformation and produces more of single copy integrations. The genome regions flanking the T-DNA could be retrieved based on general lack of major truncation or rearrangement of the T-DNA. The few drawbacks of this method are that sometimes mutations may be caused in the regions unlinked to the

site of T-DNA integration and genomic rearrangements are also observed. This necessitates the proper testing of the putatively tagged mutants to confirm if the T-DNA insertion is linked to the mutant phenotype. The loss of function mutants obtained through random insertion impose various limitations like it may not be possible to recover mutations in essential or redundant genes, loss of function provides not very confirmatory information about the mutated gene. These limitations could be overcome to some extent by promoter and enhancer trapping wherein certain elements could be added that allow the detection of promoter activity or increase the transcription of the contiguous genes. For enhancer traps a reporter gene with weak promoter is positioned near the end of the transferred DNA. The insertion of DNA near the enhancer would drive the expression of the reporter gene from the weak promoter. Similarly the promoter-less reporter gene could be used to tag the promoters. Transposon tagging provides another way to achieve insertional mutagenesis using endogenous transposons or engineered transposons. The major advantages of this method are especially while dealing with fungal systems that are recalcitrant to transformation, genetic mutations created are unlinked to the transferred DNA and there are rare chances of genomic lesions and rearrangements (Langin et al. 1995). Also, the plasmid Impala has been designed for gene tagging in fungi, it transposes by cut and paste method and does not require the host proteins.

34.5.1.2 Targeted Gene Disruption/Replacement

When the sequence of gene of interest is known, the function could be identified by gene disruption and correlated with the altered phenotype observed. The gene knock out as it is commonly known as is achieved by homologous recombination. The fungus is transformed with gene disruption cassette consisting of a selectable marker gene flanked by target gene sequences and inserted into the recipient fungal genome by homologous recombination. The efficiency of gene targeting is affected by the length of the homlogous sequences, extent of homology, transformation method and the genomic position of the target gene (Bird and Bradshaw 1997). In filamentous fungi, at least 1 kb or more of homologous sequence and that too of very high homology is required to achieve the better frequency of gene knock out (Michielse et al. 2005a). Van and Hooykaas (2003) reported that AMT is the most suitable method for achieving high frequency of gene targeting which may be because of linear and single- stranded nature of T-DNA. Particle bombardment and electroporation are also the preferred methods for gene knock out studies in fungi. The chances of ectopic integration of the gene of interest are always there which could be minimized by positive and negative selection system which leads to lethality in non viable transfomants resulting from ectopic integration. For example the inclusion of negative selection gene *amdS* in the knock out construct outside the target gene homologous sequences confers sensitivity to fluoro-acetamide if the non legitimate integration takes place (Michielse et al. 2005b). Different approaches have also been tried to overcome these limitations such as split marker technology was

developed for *S. cerevisiae* (Fairhead et al. 1996) and has been successfully applied to many filamentous fungus (Kuck and Hoff 2010). This technique requires three crossing over events to generate the functional resistance marker or auxotrophic gene, which substitutes the target gene by homologous recombination. This technique was applied in *P. chrysogenum* to obtain *lys*2 dirupted mutants using *ura*3/5 marker gene involved in uracil biosynthesis and the mutants were selected with 5- fluorotic acid (Casqueiro et al. 1999). Similar method was adopted for inactivating mecB gene that encodes for cytathionine-gamma-lyase, involved in cephalosporin C biosynthesis in *Acrmonium chyrsogenum* and 5% frequency of gene disruption in transformants was observed (Liu et al. 2001). The modified transposon based version of split marker technique has also been used in some cases like in *Colletotrichum graminicola* (Venard et al. 2008). The limitations of this method were that frequency of homologous recombination depended on the recipient strain and on the split marker gene used. So another new approach was devised using strains deficient in non homologous end joining (NHEJ) so that non homologous recombination is eliminated. A multi protein complex is required for NHEJ which comprises of DNA- dependent protein kinase, DNA ligase IV- XRCC4 complex, exonuclease Artemis and the Ku70/Ku80 heterodimer that binds directly to DNA ends and directs the DNA- Protein kinase, therby showing efficient activation (Critchlow and Jackson 1998; Hsu et al. 1999; Hefferin and Tomkinson 2005). The disruption of *Ku* genes resulted in increased homologous recombination frequency of transformation in many of the filamentous fungi. The one of the important limitations of this approach has been the increased sensitivity of *Ku* disruption mutants to various chemicals as etyl methanesulfonate and bleomycin due to reduced capability of repair systems in them. These conventional methods to study gene functions are limited by the need to prepare constructs with long stretches of homology and low transformation efficiency observed in most of the fungi, also they are not suitable for use in high throughput approach. RNAi is easy and efficient approach which provides solution to many of the problems observed with conventional approaches (Shafran et al. 2008).

34.5.2 RNAi as the Alternative Tool to Study Gene Functions in Fungi

RNAi can be used for silencing the expression of gene whose sequence is known so as to elucidate its role. RNAi could be easily induced in most of the organisms including worms, insects, fungi, plants and mammalian cells, by introducing dsRNA into their genomes. This has become the basis for RNAi as the potential reverse genetic tool in these organisms and has also proven to be an efficient tool for high throughput functional genomics studies in most of the eukaryotes. The 90% of *Drosophila melanogaster* and *C. elegans* genes were successfully targeted by RNAi for loss of function analysis (Boutros et al. 2004; Kamath et al. 2003). RNAi is gaining popularity as the upcoming tool for functional analysis in fungi. RNA silencing has also provided with ways to produce improved genetically modified strains

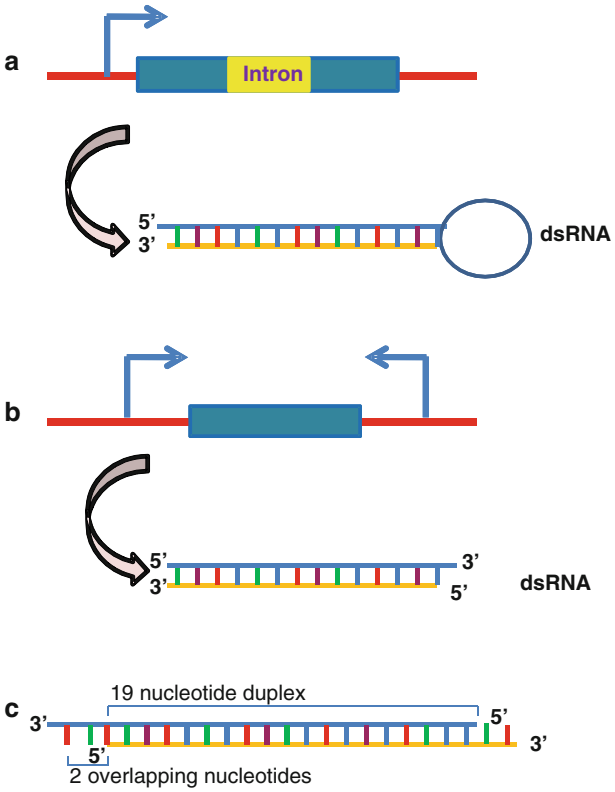

Fig. 34.3 Schematic representation of different strategies for inducing RNAi in an organism, using vectors with intron so that hairpin RNA transcripts are produced (**a**), with opposing dual promoters also dsRNA formation is facilitated (**b**), the RNAi can also be induced transiently using chemically synthesized siRNAs provided exogenously in the nutrient medium (**c**)

considering the biotechnical and pharmaceutical applications of some of the fungi, which are of industrial value being the producers of important primary and secondary metabolites (Yamada et al. 2007).

Earlier many studies were conducted using antisense mRNA which resulted in the down- regulation of transcript levels of various genes in *N. crassa* but it was only after discovery of RNA silencing pathway by Romano and Macino in 1998 that the reason for the down- regulation of gene expression was known (de Backer et al. 2002). Although there are few reports on the use of RNAi to explore gene function in fungi, the applicability of RNAi has been tested in many of the fungi, including *N. crassa*, *A. nidulans* and *Colletotrichum lagenarium* (Cogoni et al. 1996; Liu et al. 2002; Fitzgerald et al. 2004; McDonald et al. 2005; Nakayashiki et al. 2005).The efficient gene silencing has been achieved using inverted repeat transgenes when expressed in these fungi. There are various strategies by which RNAi can be induced in filamentous fungi (Nakayashiki and Nguyen 2008) (Fig. 34.3).

1. RNAi using hairpin RNA (hpRNA) expression vectors
2. RNAi using vector with opposing dual promoters
3. Direct delivery of siRNA/dsRNA into fungal cells

RNAi using Hairpin RNAi (hpRNA) expression vectors: The first successful application of RNAi was reported in pathogenic fungus, *Cryptoccus neoformans* using dsRNA (Liu et al. 2002). They achieved the significant silencing of genes involved in synthesis of polysaccharide capsule formation in the fungus leading to avirulence in the strains. The plasmid constructs expressing hpRNA or intron containing hairpin RNA are most efficient and reliable source of inducing gene silencing in fungi (Kadotani et al. 2003; Namekawa et al. 2005; de Jong et al. 2006; Takeno et al. 2004). Most of the vectors used for expressing dsRNA have the intron or spacer sequences which lie between the two inversely oriented target gene fragments so that hairpin structure is formed. The psilent-1 is one such versatile vector developed to express hairpin RNA transcripts in the Ascomycete fungi by polymerase chain reaction (PCR)- based cloning (Nakayashiki et al. 2005; Moriwaki et al. 2007). This vector has the transcriptional unit comprising of trpC promoter and terminator sequence, multiple cloning site and a cutinase intron from *M. grisea*, for selection of the transformants hygromycin resistant marker gene is present (Fig. 34.4). The vector was used to silent the *MPG1* gene, which encodes a fungal hydrophobin involved in surface interactions during infection-related development in the fungus *M. Oryzae* (Talbot et al. 1996). The 461 bp fragment of the gene was used to develop the RNAi construct and when expressed in the fungi it resulted in varying degree of silencing of *MPG1* gene in the transformants. The vector psilent1 has been tested and validated in many of the filamentous fungus, but for the high throughput functional genomics it is necessary to have the vectors as that of Hellsgate vector in plants (Wesley et al. 2001). Therefore a gateway cloning system known as pTroya based on pSilent1 was designed using invitrogen's gateway technology (Shafran et al. 2008). In pTroya two destination A cassettes of the gateway system were introduced into pSilent1 in opposite orientation in to ligation steps (Fig. 34.5). This system is commercially available and can be used for high throughput functional genomics in filamentous fungi. It uses the site- specific recombination process in bacteriophage lambda that enables the shuttling of sequences between plasmids bearing compatible recombination sites. With pTroya large scale screening could be done especially in non sequenced organisms based on partial data or the medium scale screens based on transcriptome analysis (Shafran et al. 2008). Another example of vector which has gateway technology is pFANTAi4 developed for human pathogenic fungus *Blastomyces dermatidis*. This vector has additional feature that it has green fluorescent protein (GFP) reporter system (Krajaejun et al. 2007). Importantly, the variable degree of silencing is observed with RNAi in individual trasnfromants and in some cases about 90–98% silencing takes place which is equivalent to gene knock out. Therefore, it is necessary to differentiate between the tranformants based on the extent of silencing in each one of them, to achieve this Liu and co-workers (2001) used vectors with reporter gene to drive the dsRNA expression such that the reporter gene is also down-regulated along with the endogenous target gene. *ADE2* reporter

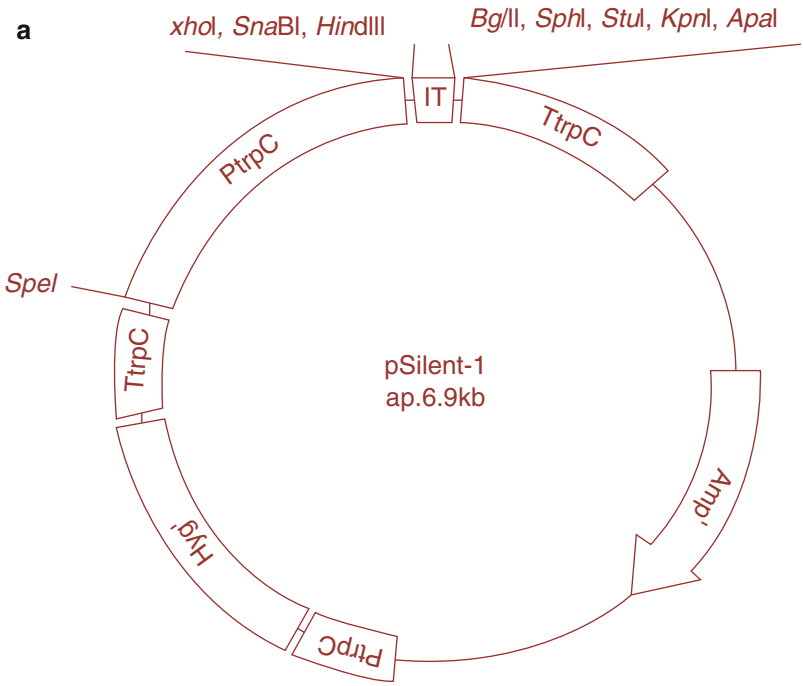

Fig. 34.4 Schematic representation of the silencing vector pSilent-1, and the sequences of multi-cloning sites and splice junctions in the vector. (**a**) A map of pSilent-1. *Amp*^{r}, ampicillin-resistant gene; *Hyg*^{r}, hygromycin-resistant gene; *IT*, intron 2 of the cutinase

gene was used by them which encodes for phosphoribosylaminoimidazole carboxylase enzyme required for adenine biosynthesis. Silencing of *ADE2* gene results in pink colonies and so the transformants with silenced genes can be recognized by the pink phenotype. Similarly, *GFP* gene has also been used for rescuing the silenced transformants by observing loss of GFP fluorescence in many of the cases like in *Magnaporthe oryzae* (Kadotani et al. 2003); *Phytophtora infestans* (Whisson et al. 2005) and *Blastomyces dermatidis* (Krajaejun et al. 2007). The other alternative is the use of *dsRed* reporter gene which imparts red phenotype to the transformants when expressed.

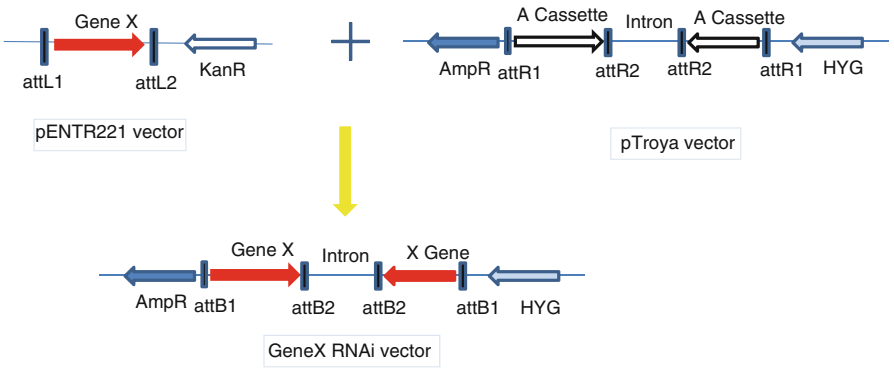

Fig. 34.5 Schematic representation of RNAi gateway vector pTroya designed for high throughput functional genomics study in fungi. The vector *pTroya* has two A-cassettes that include the recombination sites (*attR1* and *attR2*), a chlaramphenicol-resistance gene and hygromycin resistance gene. The target gene is first clone into the *pENTR221 vector* after PCR amplification using oligos that have sites *attB1* and *attB2*, this creates the RNAi entry clone. This clone and pTroya when mixed with LR clonase enzyme results in insertion of target gene in two different orientations to create the plasmid pTroya-gene X (Adapted from Shafran et al. 2008)

RNAi using Vector with Opposing Dual Promoters: Another alternative for inducing RNAi in fungi is use of RNAi vectors with an opposing dual promoter system which require just a single non oriented cloning step. In such a system the sense and antisense of the target gene which is required to produce dsRNA in the cell, are both transcribed independently under the control of two opposing RNA polymerase II promoters (Nakayashiki and Nguyen 2008). This kind of vector when used to silence eGFP gene in *Histoplasma capsulatum* induced moderate silencing of 35% on an average (Rappleye et al. 2004). The pSilent- Dual1 (pSD1) vector is based on dual promoter system; it has trpC and gpd promoters and was used in *M. oryzae*. The silencing efficiency of this vector was found to be lower than the ihpRNA expression vectors and small fraction of the transformants exhibited strong gene silencing (Nguyen et al. 2008). The similar results were obtained with dual-promoter systems in other fungi as well (Kuck and Hoff 2010). Another vector of this kind which might prove more efficient than conventional dual- promoter systems is pSuper RNAi one, initially developed for the mammalian systems. This vector has the human H1 polymerase III gene promoter which allows the cloning and expression of user defined oligonucleotide sequences to form short self complementary hairpins and was used for GFP silencing in *Coprinopsis cinerea*, a basidiomycete with quite a success (Costa et al. 2008).

Direct Delivery of siRNA/dsRNA into Fungal Cells: The direct uptake of synthetic siRNA duplexes from the nutrient medium is common phenomenon in case of mammalian cultured cells and was not normally tried in fungal systems until recently when such an application was reported in *A. nidulans*. In this report key polyamine biosynthesis gene ornithine decarboxylase (*ODC*) was specifically silenced by

treating germinating fungal spores with synthetic 23 nucleotide siRNA duplexes, which were added to the growth media. The siRNA treated fungal spores showed significant decrease in spore germination and germ-tube growth due to reduction in *ODC* mRNA transcript level and cellular polyamine level (Khatri and Rajam 2007). This method of inducing gene silencing is quite rapid and convenient and remains to be tested in other fungal species. Similarly in one of the reports dsRNA was used to induce RNAi in *Phytophtora infestans*. The dsRNA (150–300 bp) were delivered into the protoplast by lipofectin-mediated transfection and the gene silencing in this case was only transient after 15–17 days the gene expression was recovered (Whisson et al. 2005).

34.6 Advantages and Disadvantages of RNAi for Functional Genomics Study in Fungi

1. RNAi is a gene knock- down phenomenon which does not result in a complete loss of gene expression and therefore results in variation in phenotypes observed making interpretation of data difficult. This feature can be advantageous as well when targeting genes which have a crucial role to play in the growth of the organism.
2. It is quite efficient and convenient when used with inducible promoter thereby allowing silencing of gene expression at a particular developmental stage. It provides the benefit of simultaneously silencing all the members of redundant gene family using a single RNAi construct since it suppresses gene expression in a sequence specific manner.
3. It is a valuable gene analysis tool for fungal species which are difficult to analyze owing to their heterokaryotic nature.
4. RNAi could be applied in fungal species with very low efficacy of fungal recombination as well.
5. The sequence specificity of RNAi can be used to silence selectively the alternative splice variants.
6. The major disadvantage of RNAi is the possibility of “off target” effects which lead to silencing of genes which bear partial complementarity to the sense or antisense strand of the target gene.

34.7 Conclusions

RNAi is rapidly gaining popularity as the novel tool for rapid gene analysis in fungi ever since its discovery in *N. crassa*. The presence of quelling and meiotic silencing in most of the fungi demonstrates the importance of RNAi phenomenon. Many of the components of RNAi pathway had been identified and characterized in these two pathways and have contributed to our understanding of RNAi mechanism.

RNAi approach enable identification of gene functions of essential genes that too with only limited amount of sequence information available. The sequences of many of the fungi are still unknown so that gene identification and characterization is a difficult task in these fungal species but with RNAi gateway system like pTroya RNAi plasmid large scale screening is possible based on partial data. The use of RNAi is also being expanded to achieve novel traits in fungi so as to improve their industrial value as the producers of important metabolites. The one of the major drawbacks of RNAi is the incomplete repression and possibility of getting off target effects. To improve the efficacy of RNAi for gene function analysis in fungi it is important to know the extent of off target effects in fungal cells and to develop the suitable RNAi vectors with tightly controlled inducible promoters which could be used in a wide range of filamentous fungi. The use of RNAi technique is being further explored to control fungal infections in economically important crop plants as well as in humans. The host induced silencing of vital fungal genes could provide immunity against the invading fungus as the pathogen would probably ingest the siRNAs synthesized by the host plant against its own gene. Similarly the intake of chemically synthesized fungal gene specific siRNAs might lead to control of human and animal diseases caused by fungal pathogens.

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Chapter 35
Bioethanol: A Critical Appraisal

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Abstract Bioethanol production is perhaps the most successful biotechnological innovation known to human race. Presently, the renewed interest in ethanol technology is realized due to its utility not only as a chemical feedstock for synthesis of wide range of solvents, adhesives, plastics, pharmaceuticals, etc., but also as an octane enhancer in unleaded gasoline thereby, an energy feedstock. Thus, ethanol as a key tone product in the conversion of renewable biomass containing sugars is well established since the petroleum crisis of the 1970s. The economic production of ethanol from sugar and starchy materials still warrants attention on (i) availability of improved yeast strains having substrate and product tolerance, (ii) high substrate transformation rate and (iii) relatively high ethanol productivity. Although several microbes are known to produce ethanol, commercial processes mostly rely on selection of microbial strain for desirable attributes and application of economically cheaper substrates. In the light of these facts, lignocellulosic biomass for economic ethanol production has attracted attention as an alternative substrate worldwide and various efforts are being undertaken to overcome the bottlenecks. Major obstacle in large scale production from lignocellulosics for bioethanol at reasonable price lies in availability of techno-economic process and strain tolerant to edaphic conditions. Keeping this perspective in view, bioethanol production is analyzed in the present review with a focus on ethanologenic microbes, potential substrates, conventional methods of production (First generation), lignocellulosic biomass based production technologies and factor contributing to high ethanol yield etc.

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35.1 Introduction

Bioethanol production from biomass by yeast is known since time immemorial. The processes to brew beer are depicted by Mesopotamians and Egyptians as early as 2500 BC (Logsdon 2004; Niven 2005). Similarly, the art of alcohol production was known to Aryans about 2000 BC. This is evident from references to "Soma" and its medicinal values in the Vedas, written in the Indian subcontinent during 1000 to 1500 BC. The first distillery in India was setup in 1805 at Cawnpore (Kanpur).

Since then, technology of ethanol production has scaled heights in raw materials, strains, process technology, parameters and productivity. With these strides, ethanol has become an important solvent and chemical feedstock in the synthesis of a wide range of other solvents, adhesives, plastics, pharmaceuticals, detergents and a host of other industrially important chemicals (Reed and Nagodawithana 1991; Sharma 2002; Kim and Dale 2004; Balat and Balat 2009). The market size of the ethanol-based chemical industry consumed about 50% of the total production (Ghosh and Ghose 2003). The world-wide ethanol production in 2009 was about 74 bn L, while United States became the world's biggest exporter (151 mn L) of ethanol followed by Brazil (120 mn L) to export more than 330 million gallons of ethanol (RFA 2010; Licht's 2010). Thus ethanol is far the largest fermentation product with annual sale exceeding $39 bn. Now, ethanol is considered as an energy feedstock in internal combustion engines (Kosaric et al. 1980; Keim and Venkatasubramanian 1989; Wilke 1995; Kosaric and Velikonja 1995; Niven 2005; Demirbas 2007). However, with the major discoveries of petroleum sources and the steady decline in the cost of delivered petroleum products until the beginning of the last decade, alcohol lost market to petroleum-based products.

A major shift in the pattern of ethanol usage began since the petroleum crisis of 1970s. With estimated worldwide crude oil reserves at 1.33 quadrillion barrels holding good for the next 45.7 years, oil market may experience a serious crisis probably before 2015 (Tillman et al. 1994; Wilke 1995; Kosaric and Velikonja 1995; BP Statistical Review of World Energy 2010). At present, cost estimates of ethanol fall into the range of US $0.44–0.57 L^{-1}. It will be competitive with oil, until oil prices are above US $60 per bbl. Due to possible shortfall in oil supplies, environmental concerns and dwindling oil prices ($ 75 per bbl) are revising bioethanol as a gasoline substitute and an octane enhancer in unleaded gasoline (Szczodrak and Fiedurek 1996; Jackson and Moyer 2000; Niven 2005).

The interest in use of bioethanol as petrol replacement began in Brazil and USA during 1980s. In Brazil, fuel grade ethanol obtained from the fermentation of molasses is displacing petrol under National Proalcohol Programme. The programme substitutes from 15% to 25% of the domestic gasoline demand. The second largest "gasohol" programme (a gasoline blend containing 10% ethanol) is in USA, where

corn is used as the primary feedstock (Gray et al. 2006). The world ethanol production was 73.9 bn L in 2009 through fermentative route. The annual production of ethanol in Brazil, Asia, Europe was 24.9, 4.0, 3.9 bn L respectively in 2009, while estimated ethanol production in USA would reach 49 bn L by the end of 2010. In Brazil, bioethanol was totally directed into the fuel sector, whereas in the USA approx. 32 bn L was used for the domestic mix (RFA 2010). Petroleum blended with ethanol is also sold as "Cleveland Discol" in UK, "Monopolin" in Germany, "Supercarburant" in France and "Gasonol" in Philippines. Now, India has also realized the strategy of blend 5–10% ethanol in petrol. Indian alcohol industry produced 347 mn L in 2009 (RFA 2010), although installed capacity was 1.63 bn L across 170 plants. As per Ethanol Manufacturers' Association of India (EMAI), the demand for ethanol in India is 1.05 bn L, while that of crude oil is expected to reach 3.34 mn bbl day^{-1} in 2010 and 3.45 mn bbl day^{-1} by 2011 (IEA 2010). During 2009, India has imported 159.25 mn tons crude oil, costing foreign exchange worth $79.5 billion (2009–2010) (Petroleum Planning and Analysis Cell, 2010). Hence, present blending of ethanol (5%) with petrol prompted renewed interest in its production technologies. However, with an ambitious target of 10% blending, only about a third of India's 29 states have managed to mix 5% ethanol with petrol due to less availability of the biofuel.

The production of bioethanol in India is exclusively from the fermentation of sugarcane molasses, mother liquor left after sugar crystallization as like in Brazil. Molasses production varies from 4% to 4.5% of the cane crushed and average of 12 mn tons per annum is produced in India. It contains about 40–50% fermentable sugars and can be stored for long time. The conversion efficiency of fermentation by Indian distillery ranges from 80% to 82%, with a yield of 250–255 L alcohol per ton of molasses (Ghosh and Ghose 2003). On the contrary, Indian distilleries are quite reluctant to adopt newer technologies for 95% fermentation efficiency, which resulted in inconsistent production and supply (Gopinathan and Sudhakaran 2009).

35.1.1 Why Bioethanol as an Alternative Fuel Substitute?

The energy crisis has warned about possible risks of continual dependence on non-renewable fossil fuels, even after stabilization in its supply and prices and suggested for better alternative(s). Among the several alternative, interest on fermentative ethanol production has been revised for the reasons discussed below (Zaldivar et al. 2001; Chandraraj and Gunasekaran 2004; Niven 2005). Ethanol (i) has higher latent heat of vaporization (0.840 MJ/kg) than petrol (0.304 MJ/kg) hence, more power outputs, (ii) has 62.9% (29.7 MJ/kg) energy content vis a vis petrol (47.2 MJ/kg) and slightly lower (33%) energy produced by combustion during each stroke than petrol, (iii) has higher octane rating (ability to resist compression) number (107) than petrol (80–98), enabling combustion engines to run at a higher compression ratio, thereby imparting a superior net performance with no requirement and hence, no power loss or no damage to valves and piston, (iv) burnt completely with reduced hydrocarbon

emission as well as no emission of soot, CO, SO_x, NO_x, compared to petrol hence, no smog formation (v) admixed with petrol (20:80) enhances octane rating and (vi) 100% can be used as fuel with slight modification in carburetor of engines. Besides, complete combustion of ethanol facilitates to earn carbon credits as CO_2 release is recycled. However, problems associated with ethanol combustion can be alleviated by (i) electrically heating the carburetor in cool weather, and (ii) using nickel alloys instead of aluminum or magnesium for engine design.

The benefits accrued with ethanol as fuel includes (i) increased energy diversifications in transportation sector accompanied by some energy security, (ii) balance of payment benefits (iii) potential air quality improvement as a result of reduced emissions of photochemically reactive products (Jackson and Moyer 2000; Niven 2005) and (iv) addition of ethanol to gasoline enable a 4-fold reduction of tetraethyl lead supplement, without affecting automobile longevity (Szczodrak and Fiedurek 1996).

35.1.2 Production of Bioethanol

Current avenues for the commercial production of ethanol are through (i) chemical synthesis from ethylene derived from petroleum either by indirect hydration process based on absorption of ethylene in concentrated H_2SO_4 followed by dilution with water or direct catalytic hydration of ethylene using phosphoric acid supported on celite (Logsdon 2004; Chandraraj and Gunasekaran 2004) and (ii) fermentation of either sugars from sugarcane, sugar beet, hybrid sorghum or of starch/starch hydrolysate derived from either cereal crops (corn, barley, wheat, grain sorghum) or tuber crops (cassava, potato and Jerusalem artichoke) using ethanologenic microbes. The details about fermentative route of ethanol production is critically examined from industrial point of view in the following.

35.2 Ethanologenic Microorganisms

Current emphasis on ethanol production focuses upon judicious selection of microbes which can adapt to the composition of the feedstock and process employed. The microbes selected should possess (i) tolerance to high concentration of substrate, (ii) stability under adverse environmental conditions like low pH and high temperature, (iii) high fermentation rate, (iv) substantial tolerance for higher alcohol concentration, (v) high yield of ethanol per unit substrate assimilated, (vi) ability to ferment a broad range of sugars, (vii) resistance to inhibitory compounds (e.g. acetic acid, furfural, hydroxyl methyl furfural (HMF) etc.), (viii) high cell viability for a repeated cell recycling and appropriate flocculation and sedimentation characteristics to facilitate cell recycle and (ix) production of low level of byproducts (acids, glycerol, higher alcohols) (Picataggio and Zhang 1996; Chaudhari 1999; Chandraraj and Gunasekaran 2004). Ethanol fermentation from different substrates is common to a wide variety of microorganisms like bacteria, fungi and yeasts.

35.2.1 Bacteria

Amongst a number of bacteria capable of producing good yields of ethanol, only a few viz. a mesophile, like *Zymomonas mobilis,* offers a high promise of increasing productivity, as its specific ethanol production rate is six to seven times higher than that of yeasts probably due to efficient sugar uptake system (Rogers et al. 1982; Chandraraj and Gunasekaran 2004). The ethanol yield is 5% higher than yeast fermentation because less sugar is incorporated into the bacterial cell mass. *Z. mobilis* can synthesize half as much new cell mass per mole of glucose degraded as yeast does. Hence, the bacterium is used in tropical regions of America, Africa and Asia in the fermentation of plant juices (palm and agave saps) for the production of native alcoholic beverages (Rogers et al. 1982). In contrast to the yeasts, *Z. mobilis* uses Entner-Doudoroff (ED) pathway for anaerobic production of ethanol to afford net yield of 2 mol of pyruvate and 1 mol of ATP from 1 mol of glucose. *Z. mobilis* achieves 92.5–97.5% of theoretical yield which is far better than yeast (Doelle and Greenfield 1985). In spite of high substrate: product conversion efficiency, its utilization for commercial production has met with the following drawbacks. It leads to production of some lactic acid and traces of acetaldehyde, acetone, acetic acid and glycerol (Amin et al. 1983). Although, alcohol tolerance of *Z. mobilis* is similar to yeasts but ferments glucose, fructose and sucrose only and fails to utilize lactose, maltose, cellobiose and pentoses. In the presence of oxygen, it converts ethanol to acetate via TCA cycle. It grows optimally above pH 5.0, which increases chances of contamination, especially at the beginning of fermentation, leading to low yield and undesirable odor (Wayman and Parekh 1990; Kaur et al. 1993; Panesar et al. 2006).

For this purpose, *Z. mobilis* has been genetically manipulated to enhance its substrate utilization rate (Ingram et al. 1998; Davis et al. 2005). Zhang et al. (1995) introduced xylose isomerase, xylulokinase, transaldolase and transketolase genes into *Z. mobilis* to ferment xylose to ethanol. In the presence of glucose alone, the recombinant strain had a slightly lower growth rate and ethanol yield. Similarly, construction of a xylose and arabinose fermenting recombinant *Z. mobilis* with facilitated entry of xylose into cell mass was attempted by cloning the xylose transporter gene from *E. coli* in *Z. mobilis* (Conway 2000). In order to increase the industrial potential of *Z. mobilis*, its acid tolerant properties were augmented by introducing *DPS* gene (protects DNA from various assaults) from *E. coli* and a stress protection systems from *Z. mobilis* (Kasper 2000). Another microbe, *E. coli* is known to utilize major sugars and believed to be least burden with genetic modification indicated its suitability for ethanol production (Chandraraj and Gunasekaran 2004). Based on this, Hespell et al. (1996) constructed *E. coli* strains by transforming conditional lethal *E. coli* with *PET*-operon plasmid. The recombinant strains were capable of anaerobic growth and displayed no apparent plasmid losses after 60 generations. Ingram (2000) constructed a thermophilic (60°C) Gram positive ethanologen with *PD* gene from *Z. mobilis* and *AD* gene from *Bacillus stearothermophilus* for increased ethanol production.

Among the thermophilic anaerobic bacteria, *Clostridium thermocellum, Clostridium thermosaccharolyticum*, *Clostridium thermohydrosulfuricum* and

Thermoanarobacter ethanolics, T. finii and T. brockii are evaluated for ethanol production (Kosaric et al. 1987; Wayman and Parekh 1990; Chandraraj and Gunasekaran 2004). These strains have an operative Embden-Meyerhoff-Parnas glycolytic pathway. They exhibit rapid metabolic activity and high fermentation rate, resulting in high product output. Fermentation at higher temperature (i) minimizes contamination, (ii) lower cooling costs, (iii) less power input for stirring the medium, (iv) favors continuous operation and (v) assists in ethanol recovery at slightly reduced pressure. However, *Clostridium thermocellum* and *Clostridium thermosaccharolyticum* produce undesirable byproducts (Maiorella et al. 1984; Kosaric et al. 1987), rate of ethanol production is below 1 mol alcohol/mol of glucose, display poor ethanol tolerance and amenable to viral infection. However, *Thermobacter ethanolicus*, has shown a temperature optimum of 65°C and secretion of 1.8 mol of ethanol per mol of glucose (Wayman and Parekh 1990). It utilizes a wide spectrum of substrates for ethanolic fermentation and exhibits a broad range of optimum pH, 5.5–8.5 (Glazer and Nikaido 2007). Several bacteria including *Klebsiella oxytoca* and *Erwinia sp.* were genetically engineered in order to directly ferment xylose into ethanol (Szczodrak and Fiedurek 1996). Thus, genetic modifications in bacteria are possible with greater ease than yeasts. Hence, currently several laboratories are engaged to construct strains which can utilize wide range of substrates, tolerate higher temperature and salt and produce more ethanol.

In addition, a new Gram negative, facultative anaerobic bacterium *Saccharobacter fermentatus* which degrades glucose efficiently via EMP pathway had been isolated from leaves of agave in Wuhan (Yaping et al. 1990). Similarly, a novel Gram negative, non-spore forming, oxidase negative bacterium *Zymobacter palmae* T109 was isolated from palm sap and explored to produce 2 mols of ethanol from 1 mol of maltose (Okamoto et al. 1993). While, acetogenic bacterium *Clostridium ljungdahlii* grown under autotrophic and anaerobic conditions produced 11–12 mgL^{-1} ethanol at 1.6 and 1.8 atm pressure of CO after 108 h (Najafpour and Younesi 2006). Alternatively, *Synechococcous sp*. PCC 7942, a cynobacterium was genetically modified by cloning coding sequences of pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase II (*adh*) of *Z. mobilis* into pCB 4 for production of ethanol (5 mM after 4 weeks of growth) from CO_2 in presence of light (Deng and Coleman 1999).

35.2.2 Fungi

Among the mycelial fungi, *Fusarium oxysporum* has been studied as a potential candidate for ethanol production from xylose (Mamma et al. 1995; Jeffries and Jin 2000). Other mycelial fungi which ferment pentoses to ethanol are *Aspergillus*, *Rhizopus*, *Mucor* and *Monilia,* however, their rate of ethanol production is slower than that of bacteria and yeasts (Szczodrak and Fiedurek 1996; Skory et al. 1997; Sues et al. 2005). Direct fermentation of cellulose to ethanol has been reported in *N. crassa* and *Monilia* (Gong et al. 1981; Szczodrak and Fiedurek 1996). While, a fungus *Paecilomyces* NF1 strain was reported to have the ability of fermenting a wide range of substrates (starch, inulin, cellulose, lactose, xylose etc.) to ethanol (Wu et al. 1986;

Mamma et al. 1995; Lee 1997). In spite of this, mycelial fungus have not been yet explored for ethanol production due to (i) their slow fermentation rate, (ii) changes in rheology of the medium, (iii) difficulties due to their pelleted growth during submerged cultivation and (iv) low ethanol yields.

35.2.3 Yeasts

Current commercial production of ethanol employs only yeasts for desirable attributes viz. (i) homofermentative mode (EMP pathway) to produce ethanol at relatively high concentration, (ii) ferment a wide spectrum of substrates and (iii) more hardy nature (Chaudhari and Chincholkar 1996).

Yeast strains suitable for commercial production of ethanol from glucose, lactose, sucrose and molasses are *Kluyveromyces, Saccharomyces cerevisiae, Saccharomyces uvarum, Schizosaccharomyces pombe*. Although several yeasts are reported for ethanol production (Beńitez and Codón 2004), afore-mentioned strains are chosen at commercial scale for high substrate tolerance, ethanol tolerance, osmotolerance, low pH for fermentation, stability at high temperature, high fermentation rates and resistance to physical and chemical stress. High osmotolerance is particularly important where cane- or black-strap molasses constitute the raw material. In biostil process, osmotolerant peculiarities permit ease in stillage recycles (Cook 1980). Low pH during fermentation helps to reduce chances of contamination from sensitive yeast population. Further, strains have been selected for catabolite repression in oxidative pathway (crabtree effect). In the absence of oxygen, yeasts cannot synthesize ergosterol and unsaturated lipids. Such sterols and fatty acids are known to protect the strain against ethanol toxicity (Moulin et al. 1984; Hohmann 1997; Chaudhari et al. 2002). Besides this, *S. cerevisiae* can utilize xylulose but not xylose. They convert xylose to xylulose via xylitol through xylose reductase and xylose dehydrogenase. Improvement of the xylose utilizing ability of *S. cerevisiae* has been achieved by expressing heterologus genes to convert xylose to xylulose (Walfridsson et al. 1997). Whereas, cloning of xylose reductase and xylose dehydrogenase genes from *Pichia stipitis* to *S. cerevisiae* produced a recombinant strain that effectively co-fermented both xylose and glucose to yield 0.32 g ethanol per g of xylose (Ho et al. 1998). The recombinant strain gave an ethanol. Chu and Lee (2007) extensively reviewed xylose fermentation by genetically modified *S. cerevisiae*. Mitochondrial respiratory mutants (petite) have gained current spurt for the increased production of ethanol under aerobic conditions. Such "petite" (ρ^-) strains are totally committed to alcohol production (Taniguchi et al. 1997).

35.3 Factors Affecting Ethanol Production by Yeast

A variety of process variables affecting the efficiency of ethanol productivity by ethanologens are reviewed by Jones et al. (1981), Tyagi (1984) and Chandraraj and Gunasekaran (2004).

35.3.1 Effect of Sugar Substrate Concentration

Sugars fermented most often by yeasts are glucose, fructose, maltose, galactose, sucrose, lactose, trehalose, melibiose and raffinose. *Schizosaccharomyces* ferments glucose, maltose, sucrose and raffinose. Most industrial processes utilize 12–20% sugar (concentration by weight). The advantages of a high fermentable sugar in the feed are (i) reduced water requirement, (ii) suppression of osmo-sensitive contaminants and (iii) reduced distillation cost if ethanol inhibition can be limited. Optimal rates of ethanol production are generally observed at the saturation limit of 150 gL^{-1} concentration of sugars. At higher levels, plasmolysis of yeast occur (Gray 1945), and below 3 gL^{-1}, yeasts cells starve resulting in decreased productivity (Levenspiel 1980).

Pentoses like D-xylose and L-arabinose have been considered traditionally as non-fermentable by yeast. However, many types of yeast, including *Saccharomyces* and *Schizosaccharomyces,* readily ferment pentuloses such as D-xylulose to ethanol (Wang et al. 1980; Stewart and Russell 1987; Lastick et al. 1990 and Lee et al. 1995). The ability to produce ethanol from D-xylose using gene cloning has been observed in *Schizosaccharomyces pombe* (Chan et al. 1989). Some yeast, such as *Pachysolen tannophilus* (Vicente et al. 1995), *Pichia stipitis* (Wayman et al. 1987; Chamy et al. 1994), *Candida* sp. have shown to produce ethanol from D-xylose under semi-aerobic conditions (Hahn-Hägerdal et al. 1991; Devi and Singh 1997).

Starch is utilized by several amylase producing yeasts without the requirement for pre-fermentation hydrolysis. *S. diastaticus* and *S. castelli* ferment soluble starch or dextrin directly to ethanol (deMot 1990). *Schwanniomyces alluvius* also does so at conversion efficiency greater than 95% (Calleja et al. 1982). However, simultaneous enzymatic saccharification and fermentation of starch to 8.5% ethanol (v/v) has been reported by *Schizosaccharomyces pombe* (Hariantono et al. 1991). Similarly, *S. cerevisiae* produced 0.40 g ethanol per g of starch under unsterile condition (Hardaning et al. 1995). Several amylolytic strains of *S. cerevisiae* and *Schizosaccharomyces pombe* have been constructed for this purpose by gene cloning (deMot 1990; Dien et al. 2003).

Inulin from Jerusalem artichoke tuber extracts was fermented to ethanol by *Kluyveromyces marxianus, Pichia polymorpha, S. diastaticus* and *Debaryomyces phaffii* (Margaritis and Bajpai 1982; Guiraud and Galzy 1990; Galindo et al. 1995).

35.3.2 Effect of Yeast Strains

The most suitable yeast strains for ethanol production have been discussed earlier. Presently, commercial ethanol plants use strains like *S. cerevisiae, Schizosaccharomyces pombe, Zygosaccharomyces* and *S. uvarum.* The range of ethanol production among these strains varies from 4% to 12%. Although ethanol production by *S. cerevisiae* has been practiced for long, *Schizosaccharomyces pombe* appears to be equally

promising. Biostil process involves the use of *Schizosaccharomyces pombe* (Cook 1980). It converts/ferments L-malic acid to ethanol efficiently and produces ethanol in concentrations similar to *S. cerevisiae* (Ciani 1995). However, the rate of fermentation of sugars by osmophillic *Schizosaccharomyces* is slightly slower than that by *S. cerevisiae*, fermentation is accompanied by the production of low volatile acid (Slooff 1970) and tolerant to low pH (Yang 1973). Besides this, higher alcohols like byproduct formation appear to be significantly less with *Schizosaccharomyces pombe* than *S. cerevisiae* (Haraldson and Rosen 1982).

The ability to grow at high temperature (40–42°C) and low pH (below 2.5) makes it the suitable candidate for ethanol production (Reed 1982). Haraldson and Bjorling (1981) found that *Schizosaccharomyces pombe* fermented 30% sugar (50° Brix) molasses efficiently and also shown higher tolerance to dissolved solids (DS) than *S. cerevisiae.* At 25% DS the ethanol productivity of *Schizosaccharomyces pombe* was almost double than *S. cerevisiae.*

35.3.3 Effect of Nutrients

Secondary nutrient supplements are essential for cell growth maintenance and ethanol production (Jones et al. 1981). Besides sugar source, nitrogen sources such as NH_4^+ ions, urea, corn steep liquor or distiller's malt etc. constitute important constituents. Nitrogen source is used for protein, nucleotides and vitamins. Black strap molasses require supplementation with ammonium sulphate or urea only.

The role of several cations as inhibitors of growth and fermentation at different concentrations has been reviewed (Jones and Greenfield 1984; Jones and Gadd 1990). Mg^{++} activates glycolytic enzymes, stimulates fatty acid synthesis and buffers the cell against adverse conditions. Chandraraj and Gunasekaran (2004) reported addition of Mg^{++} to the medium for improved ethanol yield from 38% to 76% during fermentation using *Z. mobilis* at 40°C. Deficiency of Mg^{++} results in the synthesis of acetate rather than ethanol.

35.3.4 Effect of Temperature

Most distillery yeast strains have optimum temperature for growth between 30°C and 35°C (Gray 1945), above 32°C being detrimental, especially when ethanol begins to accumulate. The elevated temperature affects the composition and structure of the cytoplasmic membrane. The concentration of phospholipids is decreased when *Z. mobilis* is grown at 40°C, leading to loss of membrane integrity, accumulation of ethanol within the cell thereby affecting viability of cells. Wilson and McLeod (1976) reported an additional requirement of nutrients at higher temperature to forestall reduction in the sterol and fatty acid synthesis. Possibly, under high temperature,

ethanol is produced rapidly than it can traverse the plasma membrane. Such consequences lead to rise in intracellular concentration of ethanol resulting into cell death due to enzyme inhibition (Dahiya and Rose 1986; Wayman and Parekh 1990; Guerzoni et al. 1999).

35.3.5 Effect of pH

pH has profound effect on fermentation as it controls bacterial contamination, affect yeast growth, fermentation rate and byproduct formation. *Schizosaccharomyces pombe* showed constant growth over a wide pH range (pH 3.0–6.0), but *S. cerevisiae* loses its viability if pH falls below 3.0 (Reed 1982; Queiroz and Pareilleux 1990). Similarly, *Z. mobilis* grows at pH range between 5 and 7. In batch fermentation best ethanol yields are generally observed at pH 4.5–4.7 (Wayman and Parekh 1990), and inherently low pH (around 5.0) of cane molasses does not require elaborate pH control.

35.3.6 Ethanol Inhibition

Ethanol is toxic to yeasts; hence high ethanol tolerance is a pre-requisite trait for higher production of ethanol (Casey and Ingledew 1985, 1986; Nagashima 1990; Lee 1997; Chaudhari et al. 2002; Ghosh and Ghose 2003). The effect of inhibition is negligible at 2%, gradually increases (4%) as a function of ethanol concentration, and is markedly affected at 11% by weight (Bazua and Wilke 1977; Beńitez and Codón 2004). Concentrations of ethanol above 14% immediately restrict growth, fermentation rate and its production. During fermentation, the presence of ethanol hinders specific growth rate but not *Ks* and hence, growth rate is inhibited in a non-competitive manner. Hence, the effect is of major economic significance. However, with tolerant strains ethanol production is possible even in presence of 20% ethanol with tolerant stains (Kosaric et al. 1987) or addition of different supplements through molasses. Among microbes, *Saccharomyces* is the most ethanol tolerant microbe able to produce ethanol up to 25% (v/v) (Ingram and Butke 1984). Ethanol seems to have multiple effects at several levels viz. (i) modification of lipoprotein (membrane properties), (ii) solute transport system, (iii) inhibition of some enzymes (non-competitively inhibits membrane ATPase) and (iv) energy system in mitochondria. Several earlier studies demonstrated that the cell membranes (i) are highly permeable to ethanol without involvement of any carrier and (ii) outflow rate is greater than the ability of microbes to produce ethanol (van Uden 1989). In spite of this fact, most of the physiological results imply that cell membranes are key determinant of ethanol resistance. The toxic effect of ethanol has been attributed to modification of cell membrane and denaturation of key glycolytic enzymes (D'Amore et al. 1990; Nagashima 1990; Piper 1995; Chandraraj and Gunasekaran 2004).

Ethanol is a major end product of glycolysis in yeast and hence, the observed inhibition of growth in fermentation by ethanol may reflect its effect on glycolytic enzymes. Ethanol is responsible for non competitive reversible inhibition on hexokinase, pyruvate decarboxylase, alcohol dehydrogenase, α-glycerophosphate dehydrogenase. These enzymes are required in excess for maximum flux, hence, their inhibition by ethanol appeared to be major cause for decreased fermentation rate (Beńitez and Codón 2004). Factors like temperature, aeration and composition of medium influence sensitivity of cell membrane to ethanol directly or indirectly (Lee 1997). Synergy between ethanol inhibition and rise in process temperature is more toxic leading to a loss of viability of yeast cell affecting growth, fermentation accompanied by alterations in membrane lipid composition. However, high temperature and ethanol is responsible for induction of hydrophobic proteins, heat shock proteins (Hsp 104) and trehalose accumulation among ethanologen. In fact, ethanol is reported to promote mitochondrial DNA mutagenesis causing (i) ten-fold rise in spontaneous petite mutants level, (ii) mitochondrial membrane alterations leading to mtDNA loss (Bandas and Zakharov 1980; Dahiya and Rose 1986; Wayman and Parekh 1990; Guerzoni et al. 1999; Mesa et al. 2000; Chandraraj and Gunasekaran 2004; Glazer and Nikaido 2007). Thus, ethanol (i) causes exponential petite mutations, (ii) enhances thermal death and (iii) decreases the maximum temperature for yeast growth, indicating that mitochondria are major target (Beńitez and Codón 2004). Moreover, synergistic effect of ethanol and a byproduct like acetaldehyde produced during fermentation promotes DNA breaks on yeast chromosomal as well as mitochondrial DNA.

The degree of ethanol tolerance is strain dependent, apparently related to (i) increase in proportion of unsaturated fatty acids, phospholipids, composition of the plasma membrane along with a decrease in sterol/protein ratio, (ii) membrane H^+ ATPase activity, (iii) high level of ergosterol, (iv) the capacity to stabilize cellular proteins with trehalose/heat shock proteins and (v) biochemical mechanisms that destroy oxidative radicals (Agudo 1992; Piper 1995; Parks and Casey 1995; Swan and Watson 1999; Ghosh and Ghose 2003). *S. cerevisiae* seems to be more ethanol tolerant at stationary phase (Werner-Washburne et al. 1993; Piper 1995). Costa et al. (1997) reported expression of heat shock protein (Hsp104), reactive oxygen species (ROS) and mitochondrial superoxide dismutase (MnSOD) responsible for ethanol tolerance. Jeffries (2000) achieved thermotolerence, ethanol tolerance, arabinose utilization and cellulose secretion by expressing genes from yeast artificial chromosomes (*YAC*) and transferring the large blocks of genes to industrial yeasts that had high fermentation activity.

The property of ethanol tolerance in yeast is under polygenic control (Nagashima 1990). Tolerance can, therefore, be enhanced by nutritional and environmental factors. Mg^{++} supplementation increases cell mass and causes ethanol accumulation (Dombek and Ingram 1986). Similarly, improved tolerance to toxic effects of ethanol has been achieved with the medium fortified with unsaturated lipids (Ohta and Hayashida 1983; Casey et al. 1984), soy flour (Bajpai et al. 1988; Li 1995), fungal mycelium (Patil and Patil 1990) and yeast extract (Parez et al. 1992) and ergosterol (Chaudhari et al. 2002). Besides these inputs, a number of existing processes employ

a common strategy for the removal of ethanol from bioreactor environment, so that effect of feedback inhibition is minimized for higher productivity.

35.4 Overall Reaction of Ethanol Production

The overall bioconversion of glucose to ethanol (Dermirbas 2007) by yeast can be represented stoichiometrically as:

$$\underset{180\ \text{g}}{C_6H_{12}O_6} \rightarrow \underset{92\ \text{g}}{2C_2H_5OH} + \underset{88\ \text{g}}{2\ CO_2}$$

From the above Gay-Lussac's equation (1810), the theoretical yield of ethanol from each gram of glucose is calculated to be 0.51 g on weight basis while, cellulose yields (stoichiometrically) 0.568 g of ethanol. However, due to requirement of sugar for growth, metabolism and maintenance of biomass, actual yield attained does not exceed 80–90% of theoretical yield.

35.5 Potential Substrates

The principal raw materials (substrates) for the commercial production of ethanol by fermentation can be grouped in three types: viz. sugars/sugar containing materials, starchy materials and lignocelluloses (Wheals et al. 1999; Ogbona 2004; Gray et al. 2006). Their critical evaluation for ethanol production has been made by Kosaric et al. (1980). However, commercial production (90%) is based on starch (30%) and sugar crops (60%) only and to some extent on cellulose. Estimated cost of each kg of ethanol requires 0.6 kg ethylene or 2 kg sugar or 3.3 kg corn or 4 kg molasses.

35.5.1 Sugar Materials

Sugarcane (*Saccharum* sp), sweet or hybrid sorghum (*Sorghum bicolor*) and fodder beet crop are the usual saccharine raw materials. Sweet sorghum, which contains mixture of sucrose and glucose, is extensively used in Kansas and Colorado (USA) for ethanol production.

Sugarcane and sugar beet contain up to 12–20% sucrose by weight. The inherent merits of the beet crops are (i) tolerance to a wide range of soil and climatic conditions and (ii) resistance of these sugars to degradation over prolonged period of storage. Brazilian alcohol programme directly uses sugarcane juice as a substrate (Angenent 2007), while in Guatemala; solid state Ex-ferm fermentation process employs fresh or dried sugarcane pieces as a substrate (Rolz 1981).

Blackstrap molasses, the non-crystallizable mother liquor after sucrose isolation, is another raw material that may contain 65% w/w sugars. It may be clarified or refined or simply diluted before fermentation (Wheals et al. 1999). Every ton of sugar produced gives approx. 190 L of molasses. It contains between 50% and 55% fermentable sugars, yielding about 280 L of ethanol per ton of molasses (Chahal and Overend 1982).

Whey, a byproduct of cheese industry and sulfite waste liquors could be used for ethanol production using *K. fragilis* and *Candida* sp., respectively. At the end of fermentation, BOD is reduced by 85% (Wayman and Parekh 1990).

35.5.2 Starch Materials

Cereal grains (corn, wheat, rice, barley and sorghum), tuber and root plants (potato, Jerusalem artichoke, manioc, sweet potato, tapioca and cassava) have been used after converting the starch to glucose by acid or enzyme hydrolysis for ethanol production. Among these, corn is mainly used for ethanol production in USA (Gray et al. 2006; Angenent 2007). It is dehulled, gelatinized by cooking, cooled and hydrolyzed with barley malt before fermentation (Wheals et al. 1999). An alternative non-cooking process employed saccharifying amylases from *Rhizopus* sp., rendering it economically feasible at industrial scale (Matsumoto et al. 1982; Grey et al. 2006). However, corn may be a good feedstock for ethanol production only for short term as one ton of corn yields only 387 L of ethanol. Studies leading to alternate strategies have shown that the use of *Endomycopsis* amylases are more suitable for converting sweet potato to glucose for ethanol production Further, the fuel alcohol programme has spawned a major research aimed at improving the cost and efficiency of corn process. Three promising technologies for lowering operating costs are (i) substituting yeast with high temperature tolerant bacteria, (ii) using a permeable membrane to separate dissolved solids and some of the water before distillation and (iii) immobilizing the yeasts and enzymes in wet mill process to provide continuous process with higher productivities. Commercialization of these technologies might save \$0.013–0.066 L^{-1} in operating cost. While, inulin from tubers of Jerusalem artichoke may serve as an alternative source for ethanol production. Yet better, almost double amount of ethanol may be obtained compared to sugarbeet and corn (Kosaric et al. 1980). Potential of cassava root is established by Brazil through large scale production of ethanol. Novel starch obtained from unicellular green algae has been recently evaluated for ethanol production (Sharma and Swarup 2003).

35.5.3 Lignocellulosic Materials

Lignocellulosic raw materials have been suggested as an alternative futuristic substrate for ethanol production (Lee 1997). Lignocellulosic material is a more complex substrate that include, hardwood, forest residues, municipal solid wastes and

agricultural residues such as rice straw, wheat straw, corn straw and corn stovers (Kadam and McMillan 2003; Angenent 2007). It is composed of carbohydrate polymers (cellulose and hemicellulose) and lignin. The carbohydrate polymers are tightly linked to lignin by hydrogen bonds and some covalent bonds. It is composed of 50% cellulose, 25% hemicellulose and 25% lignin. Upon hydrolysis, hydrogen bonds between carbohydrate moieties of lignocelluloses yield a mixture of monomeric hexoses (glucose, mannose and galactose) and pentoses (xylose and arabinose). The global availability of cellulosic material is about 900×10^6 MT per annum (Logsdon 2004). Thus, lignocellulosic residues available for bioconversion are more abundant, have fewer competing uses, and can be harvested with less interference to food economy and hence minimal stress on environmental resources (Stephanopoulos 2007). While, India generate 390 million tons of agricultural residue per annum which have a potential of producing 170 bn L of bioethanol (Sharma and Swarup 2003).

The basic steps for conversion of lignocelluloses to bioethanol mainly depend on (i) delignification to release cellulose and hemicellulose from lignin and (ii) depolymerisation of carbohydrate polymers to produce free sugars. The delignification process is the rate-limiting and achieved with thermochemical pretreatment followed by acid or enzymatic hydrolysis or biological approach (Gray et al. 2006). In lignocellulose bioethanol technology, cellulose and hemicellulose are hydrolyzed either using enzymatically or acid to glucose, xylose, arabinose etc. and subsequently to ethanol by bacteria or yeast (Yu and Zhang 2004; Sharma et al. 2004; Ogbona 2004; Sun and Cheng 2005). Acid hydrolysis is effective but needs expensive reactor and forms reversion compounds and byproducts. While, enzymatic process is cost effective and cost reduction could be three to four times greater than acid hydrolysis (Lynd 1996). Among these, the biological delignification has several advantages over other methods viz. (i) mild reaction conditions, (ii) higher product yields, (iii) fewer side reactions, (iv) less energy intensive, and (v) less reactor resistance to pressure and corrosion (Gray et al. 2006). Lee (1997) comparatively reviewed the delignification strategy using several microbes. Cellulose stoichiometrically yields 0.568 g ethanol vis-à-vis each gm of glucose i.e. saccharified cellulose yields 11.1% more ethanol than glucose. Simultaneous saccharification and ethanol production by a consortium of mixed culture is attempted in simple operation by several researchers (Rolz 1992; Chadha et al. 1995; Lee 1997; Chandrakant and Bisaria 1998; Harikrishna et al. 2001; Kadar et al. 2004). However, pretreatment cost makes it (i) uneconomical, (ii) polluting and (iii) undesirable for industrial application. Hence, advancement in areas like pretreatment and biological conversions needs improvement to reduce the cost of production. Yet, several reviews indicated increased interest in lignocellulosic materials as a potential raw material for ethanol production (Cheung and Anderson 1997; Ghosh and Ghose 2003; Kim and Dale 2004; Hamelinck et al. 2005). The current process is based on (i) cellulase producing *Trichoderma reesei,* (ii) hexose fermenting *S. cerevisiae* and (iii) recombinant *E. coli* for pentose fermentation. Regardless of the source, lignocellulosic biomass like wheat straw, rice straw or baggasse contains (dry weight basis) cellulose (35–55%), hemicellulose (20–40%) and lignin (10–25%). Several reports explored use of wheat stillage (Davis et al. 2005), wheat bran

(Palmarola-Adrados et al. 2005), alfalfa fiber (Sreenath et al. 2001), sunflower stalks (Sharma et al. 2004) and rye straw/bermuda grass (Sun and Cheng 2005). However, with the present technology, it is still difficult to scale up at commercial scale in order to tap lignocellulose containing agricultural residues.

35.6 Industrial Fermentation Processes

A comprehensive account on current industrial processes used for ethanol production is given in this section. It is based on volumetric ethanol productivity of about 1.8–2.5 $gL^{-1}\ h^{-1}$ from conventional batch fermentation (Maiorella 1985; Bailey and Ollis 1986; Gerbsch and Buchholz 1995; Ogbona 2004). Strategies considered for improving are by (i) relieving the end product inhibition, (ii) increasing the cell concentration in the reactor and (iii) shortening the duration for economic completion of fermentation. These principles are applied in the following fermentation processes.

35.6.1 Conventional Batch Fermentation

Batch fermentation is the oldest method used for ethanol production from molasses. The process involves development of active yeast inoculum in a medium similar to the fermentation mash (Maiorella 1985). Usually the time required to completely utilize the substrate is 30–40 h. Initial pH is adjusted to 4.0–4.5 depending on the yeast strain and temperature held at 30–35°C. Depending upon the nature of the feedstock, conversion efficiency falls between 90% and 95% of theoretical, with a final ethanol concentration of 10–16% (w/v). The process has been preferred in the past due to low requirements for (i) complete sterilization, (ii) ease of operations, (iii) use of unskilled labor, (iv) easy management of feedstocks and (v) low risk of financial loss. However, fermenter productivity (1.8–2.0 $gL^{-1}\ h^{-1}$) is low because of initial growth lag and long turn-around times.

35.6.2 Melle-Boinot Fermentation Process

The process was originally developed by Boinot and Boige (1948). It reduced the initial lag period and increased the yield by recycling yeast from the previous fermentation by centrifugation in the next batch. Hence, the initial high cell density of about 80 bn cells L^{-1} initiated rapid fermentation almost immediately. This allowed reduction in overall fermentation time by 50–65% and enhancement in volumetric productivity to about 6 $gL^{-1}\ h^{-1}$ (Lagomasino 1949).

35.6.3 Continuons Fermentation

Continuous fermentation process achieves higher productivity by lowering operating costs and eliminating the downtime between two cycles. A number of large scale continuous fermentation plants are introduced for ethanol production using continuous stirred tank bioreactor (CSTBR). It comprises of outflow-inflow and overflow process to maintain the yeast population at a steady state. By this process, Cysewski and Wilke (1977) achieved cell density of 12 g dry weight per liter and ethanol productivity of 7 gL^{-1} h^{-1}.

The productivity by continuous fermentation is significantly enhanced by yeast recycle. Centrifuges are used to separate yeast from ethanol stream and the concentrated yeast slurry is recycled through the fermenter (CSTBR). Extremely high yeast cells concentration up to 100 gL^{-1} h^{-1} have been achieved (Ghose and Tyagi 1979). Alternatives to cell recycle have been suggested. These include (i) temporary thermal shock to arrest CO_2 evolution (Walsh and Bungay 1979) and (ii) use of flocculent yeast strains in specially designed fermenters with an inclined side arm (Guidoboni 1984; Maiorella 1985).

35.6.4 Biostil Process

The main features of this continuous process with yeast recycle are that fermentation and distillation are coupled. The fermenter beer is continually cycled through centrifuge for the removal of yeast and subsequent stripping of ethanol in a rectifying column (Cook 1980; Alfa-Laval 1982). Higher production is achieved through (i) concentrated feedstocks, replenishing the substrate in the fermenter (ii) use of osmophilic strain of *Schizosaccharomyces pombe* (iii) ethanol concentration in the fermenter is maintained at about 5% (w/v) to reduce its inhibitory effect. (iv) maintenance of yeast cell density at 5×10^2 bn cells L^{-1}. Because of large liquid recycle, less water is consumed and concentrated stillage is produced. This process has several merits (i) lower (25%) space requirement of the conventional process, (ii) lower consumption of molasses for yeast growth, (iii) resistance to infection, (iv) lower cost of concentrated effluent handling and disposal, (v) higher ethanol yield, and (vi) burning of stillage without evaporation for energy. However, limitation of this process is (i) gradual build up of non-fermentable components and (ii) fermentation by-products, concomitantly leading to reduced productivity.

35.6.5 Vogelbusch Cascade Process

The multistage continuous fermentation with recycle has been developed by Vogelbusch of Austria. Fermentation is carried out in a series of fermenters (numbering 5 to 7), fed with 45°Brix molasses. The mash flows from previous to the

next fermenter, with alcohol concentration rising in each fermenter (5.5–9.0% v/v). The first three fermenters are aerated and fed with molasses to obtain necessary yeast growth. The fourth and fifth fermenters are agitated with CO_2. Beer from the last fermenter is centrifuged for yeast cell recovery which is recycled back to first fermenter in series. Although, this approach is based on sound scientific principle, its commercial outcome is not known.

35.6.6 Encilium Process

The process employs highly flocculent yeast that forms hard flocs and gets retained in fermenter during continuous operation (Nene 1990). The process is operated under mild agitation and employs low sugar concentration for fermentation. High levels of ethanol at any moment restrict bacterial contamination. A sedimentation tank recovers yeast cell flocs from outlet during continuous removal of fermentation wort. The process operates for several months without any significant drop in yeast activity. It has provision for CO_2 scrubbing to remove ethanol, with fermentation efficiency of 90% and ethanol productivity of 3–4 gL^{-1} h^{-1}.

35.7 Experimental Fermentation Processes

35.7.1 Tower Fermenter

The system has been used with highly flocculent yeast strains for the production of beers (Hough et al. 1977), not for industrial production of ethanol (Maiorella 1985). It consists of a cylindrical tower (2 × 15 m), topped by larger diameter settling zone fitted with baffles. Sugar wort is pumped into the base of tower and beer rises upward through plug. The yeast tends to settle back and high cell densities of 50–80 gL^{-1} are achieved. Experimental set up has been described for glucose feeds by Ghose and Tyagi (1979) and Kuriyama et al. (1985). The major drawback is the long time required for start up and high salt in molasses exerts deflocculating effect. However, continuous stable run upto 12 months and productivity of 27 gL^{-1} h^{-1} has been achieved.

35.7.2 Novel Processes

Vacuum flash fermentation with cell recycle, membrane bioreactor and rotor fermentation constitute novel fermentation processes.

35.7.2.1 Vacuum Flash Fermentation with Cell Recycle

The Continuous stirrer tank bioreactor (CSTBR) is maintained under atmospheric pressure and connected to an external vessel under vacuum, to flash off ethanol (Cysewski and Wilke 1977; Maiorella 1985). A volumetric ethanol productivity of 82 gL^{-1} h^{-1} has been achieved through selective removal of ethanol in vacuum. The process takes advantage of the high volatility of ethanol. High cost of the process, however, is unlikely to lead to commercial application.

35.7.2.2 Rotor Fermenter and Membrane Bioreactor

These reactors employ microporous membrane filter for selective and continuous removal of ethanol at productivity of about 36 gL^{-1} h^{-1} (Margaritis and Wilke 1978). Other techniques of *in situ* ethanol recovery have included the use of liquid-liquid extraction (Larsson and Mattiasson 1984) and adsorption (Lencki et al. 1983) coupled to fermentation.

35.7.2.3 Solid State Fermentation Ex-ferm Process

The innovative process employs sugarcane stalks as raw materials for simultaneous fermentation to ethanol (Rolz 1981; Er-el et al. 1981).

35.7.3 New Emerging Lignocellulosic Biomass Based Bioethanol Technologies

For lignocellulosic conversion, additional steps viz. pretreatment, detoxification, enzyme production are needed in addition to crop-based fermentation process. Lignocellulose pretreatment should (i) yield reactive fibers, (ii) produce non-degraded form of pentoses, (iii) generate less toxic compounds, (iv) produce no solid residues, (v) be simple but robust, (vi) work in reasonable size reactors with moderate cost, (vii) be effective at low moisture content and (viii) not contribute any environmental threat. For these reasons, several pretreatment processes are available viz. (i) concentrated or dilute acid/alkaline pretreatment, (ii) steam explosion, (iii) wet oxidation, (iv) organic solvent pretreatment and (v) AFEX. Among these pretreatments, acid pretreatment is widely accepted on the merits viz. (i) release of sugar monomers from hemicellulose, (ii) exposure of cellulose fibers for further enzymatic action and (iii) more amenable for detoxification of inhibitory compounds by (a) $Ca(OH)_2$-Overliming (Martinez et al. 2000) (b) phenol-oxidase laccase and (c) addition of SO_2 to steam treatment or (d) utilization of inhibitor tolerant adapted microbial strains. There are few options available for carrying the hydrolysis and

fermentation steps viz. (i) separate hydrolysis and fermentation (SHF), (ii) simultaneous saccharification and fermentation (SSF) and (iii) direct microbial conversion using anaerobic *Clostridia* (Lynd 1996) produce cellulolytic enzymes to generate sugars which are converted to ethanol.

Current proposed processes to produce ethanol from lignocellulosics are summarized as follows

35.7.3.1 Modified Gulf SSF Process

The process comprises of acid hydrolysis followed by a solid/liquid separation step and finally treated with CaOH after washing, the stream (hemicellulose syrup). The resulting detoxified syrup can be fermented by ethanologenic engineered *E. coli* K011 (Ingram et al. 1999).

35.7.3.2 IIT Delhi Process

The process employs rice straw and bagasse (cane) as lignocellulosic biomass for saccharification followed by fermentation to ethanol (Ghosh and Ghose 2003). The lignocellulosic biomass is pretreated (cellulose 76%) by solvent (ethanol) in presence of a catalyst at 170°C for 30 min followed by mixed enzyme (*Trichoderma reesei* cellulase supplemented with *Aspergillus wentii* β-glucosidase) and finally, fermented using *Candida acidothermophilum* in solid state fermentation reactor with vacuum cycling and steep feeding. The process yield 0.23 m^3 bioethanol per ton of lignocellulosic biomass with a productivity of 4.4 gL^{-1} h^{-1} and production cost was estimated to be 54.4 cents per L ethanol provided the cost of rice straw is $10 per ton. Co-products produced during the process are lignin and animal feed.

35.7.3.3 NREL Process

The process involves dilute acid pretreatment of corn stover or poplar hardwoods followed by simultaneous saccharification using *Trichoderma* cellulase and co-fermentation of glucose and xylose to ethanol by recombinant *Z. mobilis* (Wooley et al. 1999). The hydrolysate is obtained in the continuous pretreatment reactor at 190°C with 10 min residence time. The liquid hydrolysate and solid residues are remixed and subjected to simultaneous saccharification and co-fermentation (SSCF) reactor. The reactor is fed with *T. reesei* cellulase (15 FPU/g cellulose), recombinant *Z. mobilis* and corn steep liquor. SSCF process is conducted at 30°C for 7 days. Bioethanol (100%) is recovered by distillation and molecular sieve adsorption. The process yields lignin/cell mass cake as by product and 0.27 m^3 ethanol per ton of lignocellulosic biomass. The estimated cost of ethanol production in this process was 39.5 cents per L ethanol.

35.7.3.4 Agrol Process (Agrol Ltd., Guildford, UK)

The Agrol process is based on the application of thermophilic bacteria (70°C) to ferment crude acid hydrolysate continuously to ethanol in closed loop fermenter. Each MT of feed stock produces about 400 kg of 95% ethanol and 40 kg of fresh cells as high protein animal feed.

35.7.3.5 BCI Process (BC International Corporation, Dedham)

The process employs genetically engineered *E. coli* K011 strain to ferment sugars/hemicellulose obtained using enzymatic and acid hydrolysis treatment of feed stocks (sugar cane waste, potato waste, switch grass or hay). *E. coli* K011 is genetically engineered with genes from *Z. mobilis* to convert pentoses into ethanol (Ingram et al. 1998). The process produces 20 mn gallons of ethanol.

35.7.3.6 Others

Masada Resource group has planned to construct a municipal solid-waste-to-ethanol facility in order to produce 9.5 million gallons of ethanol using Masadas patented CES OxyNol™ concentrated acid hydrolysis technology (Mielenz 2001).

The performance of above processes at pilot scale level has favored an optimistic forecast for effective utilization of lignocellulosics. Current areas proposed (Zaldivar et al. 2001) to curtail ethanol costs from lignocellulose mainly relies on (i) development of a counter current reactor for pretreatment of biomass, (ii) processing lignin residues for high value products, (iii) improved cellulase and hemicellulase enzymes, (iv) consolidated bioprocessing of hydrolysis and fermentation (v) advance pretreatments to enhance sugar yields and reduce sugar degradation and (vi) product diversification leading to coproduction of non-fuel products.

35.7.4 Continuous Fermentation with Immobilized Yeast Cell

Cell immobilization technology has been recognized since long for taking advantage of solid surfaces. In 1892; Delbruck first attempted to propose continuous ethanol production system using yeast cells retained in a porous cylinder. Barbet (1899) devised an apparatus to support yeast culture on a packing material. However, these attempts remained dormant until exploited industrially in quick vinegar process (Frings 1932) wherein microbes colonize through adhesion (Clark 1994; Gerbsch and Buchholz 1995)

It was, thus, apparent from the early work that immobilized yeast cell systems could offer attractive features like (i) high productivity by increasing the population density and (ii) high flow rate in continuous operation, without inviting the risk of

cell wash-out or deflocculation. In addition, the risk of contamination in a high density immobilized cell reactor was minimal. Moreover, immobilized systems are resistant to any accidental change in the feed composition or operational conditions, since retention of the biomass allowed restoration of fermentation activity. Ease of operation of bioreactors and potential of carrying out sequential reactions through series of reactors added versatility to the system.

Immobilized whole cells were defined at the First Engineering Conference in 1971 as "immobilized cells which are physically confined or localized in a certain defined region of space with retention of their catalytic activity and, if possible or even necessary, their viability which can be used repeatedly and continuously" (Godia et al. 1987). The use of immobilized cell systems for ethanol production has been reviewed by several authors (Linko and Linko 1984; Marwaha et al. 1986; Chaudhari and Chincholkar 1996; Kourkoutas et al. 2004). World energy crisis and phenomenal potential of yeast to ferment sugars to ethanol attracted attention in immobilized cell technology.

Cell immobilization does not grossly alter the biochemistry of cell growth and function. However, several experimental evidences reflect modifications in the metabolism, physiology and morphology of entrapped vis-à-vis free cells. Some of these, enhanced ethanol yield and specific ethanol productivity (Pundale et al. 1988; Galazzo and Bailey 1990) as a function of changes in cell morphology (Simon et al. 1990). Optimal growth conditions for immobilized cells are often different from those for free cells. Galazzo and Bailey (1990) proposed that yeast growth in gel matrix induced changes which resulted in durable modification of cell physiology. They found that larger glucose uptake rate in immobilized cells indicated improved membrane permeability. Several earlier reports have indicated increased ethanol tolerance among immobilized cells (Jirku 1991; Norton et al. 1995). Addition of 0.002 mg/L ergosterol in the medium offered 25% more CO_2 evolution concomitant with slight increase in cell density and viability of immobilized cells (Chen et al. 1990). Similarly, Hahn-Hägerdal (1990) claimed high yield of ethanol when sand was co-entrapped with yeast in alginate.

A large number of reviews have appeared on whole yeast immobilization and their application in continuous reactors (Linko and Linko 1984; Marwaha et al. 1986; Godia et al. 1987; Margaritis and Merchant 1987; Nunez and Lema 1987; Rosevear et al. 1987; Nagashima 1990; Wayman and Parekh 1990; Chaudhari and Chincholkar 1996). However, their commercial applicability remains yet to be determined.

Main methods employed for yeast immobilization have been distinguished into the following four groups (Fig. 35.1) (Kosaric et al. 1983; Scott 1987; Margaritis and Merchant 1987): (i) physical adhesion or attachment of yeasts cells to solid support, (ii) flocculation, (iii) entrapment in polymeric matrix and (iv) encapsulation (retention behind membrane). For yeast immobilization, physical adhesion and cell entrapment methods are mainly used.

Recent promising trend is in the application of immobilized cells for continuous ethanolic fermentation. Significant advances concerning the use of immobilized cell technology at laboratory, pilot and semi-commercial scale have been made

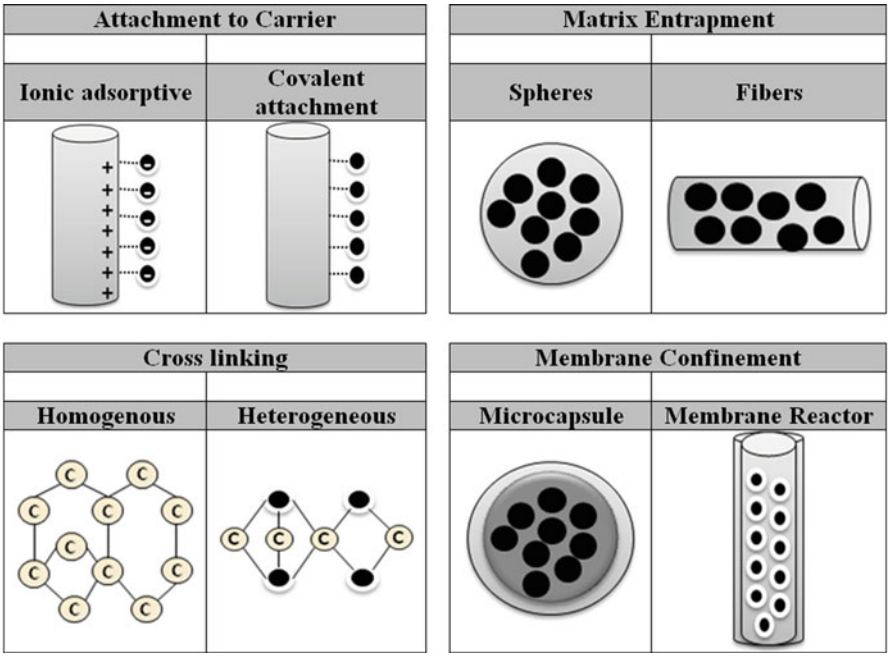

Fig. 35.1 Various techniques of whole cell immobilization for bioethanol production

(Nagashima et al. 1984; Godia et al. 1987; Nagashima 1990). These relates to (i) 1 KL reactor in series for continuous conversion of cane molasses to ethanol, (ii) use of *Saccharomyces* cells entrapped in calcium alginate gel beads and (iii) productivity of 20 gL^{-1} h^{-1} (33 gL^{-1} of gel per hour) at 8.5–10% (v/v) ethanol. Thus, 600 L of ethanol was produced per day in the 1 KL reactor. Subsequently, two 10 KL reactors in series yielded more than 8% (v/v) ethanol for more than 2,000 h. This semi-commercial plant showed 90–95% of theoretical yield. However, it showed decreased activity and limited growth on the surface of gel beads, creating difficulty in maintaining high cell density in the fermenter. The use of immobilized cell system to certain extent circumvents drawbacks of continuous fermentation by free cells.

35.8 Strategies for Improvement of Ethanol Productivity

Fermentation performance of ethanologens has been traditionally improved with (i) interplay between yeast inoculum cell density, temperature and key nutritional requirements (e.g. optimization of Mg^{++} and assimilable nitrogen availability) of growth and (ii) genetic improvement by random mutagenesis, classical breeding and genetic crossing of two strains followed by screening for mutants with enhanced properties of interest.

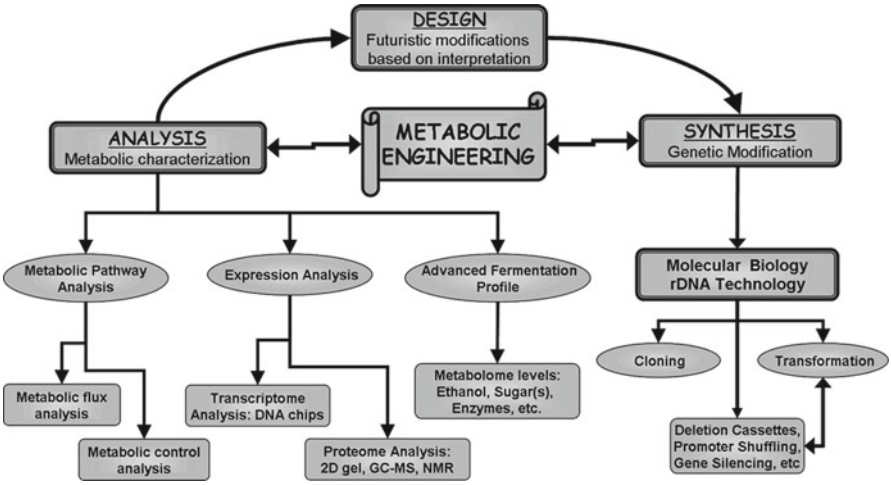

Fig. 35.2 Simplified flowchart for metabolic engineering of ethanologenic microbes

The above methods are restricted to crop-based processes but unable to improvise yeast or ethanologens (i) for utilization of lignocellulosic or xylose substrates and (ii) sensitivity to high concentration of ethanol (≧40 g/L), However, newer approach like metabolic engineering has recently transpired to address essential and desirable traits among ethanologens (Zaldivar et al. 2001). This directed approach mainly encompasses genetic engineering tools, analytical tools and better understanding of cell physiology and functions. Metabolic engineering has been defined as "the improvement of cellular activities by modification of a specific biochemical reaction(s) or the incorporation of newer one(s) with recombinant DNA technology" (Stephanopoulos 1999).

35.8.1 What Is Metabolic Engineering?

Metabolic engineering is distinctly broader in scope than rDNA technology and basically an amalgamation of an array of parameters like (i) metabolic pathways, (ii) metabolite flux and (iii) addition/deletion/modification of genes for improved cellular properties for a desired outcome. The *modus operandi* of metabolic engineering (Nielson 2001) involves (i) construction of a recombinant organism by suitable method (usually, cloning and transformations), (ii) a careful analysis of the cellular functions (i.e. physiology), genetic traits (genome) and their expression profiles (transcriptome and proteome), (iii) metabolic pathway analysis involving metabolic flux and regulation (metabolome) and (iv) further modifications of strain for more effective outcome. Thus, metabolic engineering rather operates in iterative cycles and repeated cycles of multiple modifications and analysis (Fig. 35.2).

35.8.1.1 Conceptual Requirements of Metabolic Engineering

The concept of metabolite balancing for analyzing the metabolic genotype of microorganisms has been conceived by Schilling et al. (1999) and Schilling and Palsson (2000). Preliminary efforts concentrated on sequencing of whole genomes of microbes viz. *Saccharomyces cerevisiae, E. coli, Zymomonas mobilis* and developments in bioinformatics has further accelerated the process of gene cloning and transformation.

Secondly, availability of efficient transformation vectors (e.g. high-copy number and rapid chromosomal integrative plasmids) has further facilitated genetic engineering in *S. cerevisiae, E. coli and Z. mobilis*. In case of (i) *S. cerevisiae*, availability of auxotrophic strains and a high degree of homologous recombination rendered high frequency of directed DNA chromosome integration, (ii) *E. coli*, segregation stable low-copy number plasmids enabled rapid cloning of genes (Jones and Keasling 1998) and mRNA of homologous genes containing synthetic hairpins using a 5′ cassette system for stabilization (Carrier and Keasling 1997) are available for application to new organisms with certain modifications.

In addition to constitutive and regulatory promoters (gene encoding glyceraldehyde-3-phosphate dehydrogenase of glycolytic pathway, *GAL7* promoter of *S. cerevisiae* and *lac* promoter of *E. coli*), an alternative artificial promoter have been designed for controlled gene expression over a range of 100-fold in both *Lactococcus lactis* and *E. coli* (Jensen and Hammer 1998).

Recently, a novel multiple-gene-promoter shuffling method was employed for optimized gene over-expressions. This method approaches fusion of promoters *GND2* and *HXK2* of various strengths to the genes of transaldolase (*TAL1*), transketolase (*TKL1*), and pyruvate kinase (*PYK1*) in the *Saccharomyces cerevisiae* strain FPL-YSX3 harboring integrated xylose-metabolizing genes viz. xylose reductase, xylitol dehydrogenase, and xylulose kinase. The optimal expression levels in recombinants for *TAL1*, *TKL1*, and *PYK1* were identified with volumetric ethanol productivity. The optimal combination was identified for presence of *GND2-TAL1-HXK2-TKL1-HXK2-PYK1* (Lu and Jeffries 2007).

Similarly, specific gene disruption cassette viz. the *loxP-kanMX-loxP* for gene disruption in *S. cerevisiae* have been demonstrated to loop out dominant marker and sequential disruption of several genes in polyploid industrial strains (Güldener et al. 1996).

35.8.1.2 Application of Metabolic Engineering

Currently, efforts are mainly concentrated on ethanologens (Zaldivar et al. 2001) for improvements of essential traits, viz. (i) broad substrate utilization, (ii) high ethanol yields and productivity, (iii) minimal byproduct formation, (iv) high ethanol, substrate and inhibitors tolerance and (v) tolerance to process hardiness while, desirable peculiarities for ethanologens induces (i) simultaneous sugar utilization, (ii) GRAS status, (iii) minimal nutrient supplementation and (iv) tolerance to edaphic conditions (pH, temperature) for crop based and lignocellulose fermentation. The improvement of these traits with metabolic engineering is particularly summarized in Table 35.1.

Table 35.1 Fermentative performance of a few metabolically engineered ethanologens in the presence of lignocellulose

Recombinant	Insertion of gene(s)	Substrate (gL^{-1})	Comments	Reference
E. coli K011	*Pdc, adh*B, *frd* cassette from *Z. mobilis*	Pine-steam treated hydrolysate	32.0 g ethanol L^{-1}	Barbosa et al. (1992)
S. cerevisiae	*XYL1* and *XYL2* of *P. stipitis*	Xylose (21.7)	1.6 g ethanol L^{-1}	Kotter and Ciriacy (1993)
Saccharomyces 1400 (pLNH32)	*XYL1* and *XYL2* of *P. stipitis*	Cornfiber AFEX hydrolysate	21.0 g ethanol L^{-1}	Moniruzzaman et al. (1997a)
E. coli K011 (spontaneous mutants)	*Cas AB* of *K. oxytoca* for transport and utilization of cellobiose and cellotriose	Cellobiose	90% theoretical yield	Moniruzzaman et al.(1997b)
E. coli K011	*Pdc, adh*B, *frd* cassette from *Z. mobilis*	Corn hulls and fiber hydrolysate	40.5 g ethanol L^{-1}	Ingram et al. (1998)
E. coli K011 LY01 (maintained in 50 g/L for 3 months)	*Pdc, adh*B, *frd* cassette with classic random mutation.	Xylose	60 g ethanol L^{-1} in 72 h (85% yield)	Yamano et al. (1998)
S. cerevisiae TMB3001	*XYL1-XYL2-XKS1* cassette	Glucose and xylose (5 and 15)	2.5 g ethanol L^{-1}	Eliasson et al. (2000)
S. cerevisiae	*GPD1, GPD2, GS-GOGAT*	–	40% reduction in glycerol	Nissen et al. (2000)
S. cerevisiae	Expression of laccase from *T. versicolor* and overexpression of phenylacrylic acid decarbozylase	Diluted acid hydrolysate of spruce	0.449 g ethanol/g fermentable sugar and resistant to ferulic and cinnamic acid	Larsson (2000)
S. cerevisiae IFO10150	*FAD2* of *Arabidopsis thalina* and *OLE1* of *S. cerevisiae* S288C	CM medium containing 2% glucose and 2% raffinose	Produced 54% total fatty acids as dienoic acid and 82% unsaturated fatty acid *vis-à-vis* parent strain and 4.4 times improved cell viability in 15% ethanol	Kajiwara et al. (2000)
Z. mobilis GFOR	*pZAGFβg (*glucosidase gene from *Ruminococcus albus* tagged with signal peptide GLN)	Cellobiose 22%	10.7 g ethanol L^{-1}	Yanase et al. (2005)
S. cerevisiae Xyl-gapn	*gapN*	Xylose + Glucose	Reduced glycerol by 58% and enhanced ethanol by 24%	Bro et al. (2006)

Thus, improvement of various peculiarities via metabolic engineering as shown in the Table 35.1 may provide a means of augmenting several problems associated with ethanologenic microbes for better fermentative production of ethanol from cost-effective lignocellulose or crop based fermentation.

35.9 Downstream Processing of Ethanol

Ethanol recovery from beer is energy intensive and hence, a cost center accounting for almost 50% of total energy consumption in the overall process. Effective recovery of ethanol employs various approaches which are reviewed by Larsen (1987).

Recovery of ethanol is limited because of azeotrope formation. Ethanol has a lower boiling point than water and its vapour phase in equilibrium with an alcoholic solution has a higher concentration of ethanol. Water and ethanol, therefore, form azeotrope, breakable by the addition of extraneous liquid, less volatile than the feed. The recovery is, therefore, referred to as extractive distillation. When the extraneous material is more volatile than the feed, the operation is called azeotropic distillation for better performance.

Purification of ethanol for desired purpose is achieved by (i) extractive distillation using water reflux to distil a large share of impurities and concentrate crude alcohol-water mixture, (ii) efficient fractionation to produce approximately 190° proof alcohol, (iii) hydrogenation to convert aldehyde impurities to alcohol, (iv) ion exchange resins or azeotropic distillation to dehydrate 190° proof (95% purity) to 200° proof (99.9% purity) or absolute anhydrous alcohol for fuel use (Logsdon 2004). Various distillation configurations such as vapour compression, low pressure distillations etc. have been used to make substantial savings in steam consumption profile for ethanol recovery. Recently new techniques based on non distillation approaches like selective sorption of ethanol, preferential adsorption of water and membrane separation based on reverse osmosis have been proposed to bring down the energy costs.

Solvent extraction is another alternative that relies on differential solubilities and use of additional phase. Use of chlorinated hydrocarbons, aromatic compounds and ethyl trichloroacetate is reported for high (80%) recovery.

Alternative method like Supercritical-gas extraction process is based on alterations in physical properties of the fluid (pressure or temperature) near the critical point, which enabled alteration in solvent power of the fluid from gas to liquid (Williams 1981). CO_2 is employed as a supercritical fluid at 100 atm pressure and 35°C so as to get solvent free 80–85% ethanol.

Recovery of ethanol from fermentation beer generates stillage (vinasse). Brazil employs it as a fertilizer in canfields, feedstock in the production of biomass or biomethanation system, with CO_2 and fusel oil as byproducts (Kosaric et al. 1983).

35.10 Future Perspectives

Considerable improvements in the existing fermentation processes for higher production of ethanol are warranted, particularly on account of its industrial importance. Several efforts for improvement have focused on the following problems:

- Screening/genetic alteration of suitable microbial strain(s) with high tolerance to substrate(s), temperature and product, rendering high specific ethanol productivity.
- Metabolic engineering of structural or regulatory genes responsible for unsaturated fatty acid synthesis.
- Altering functional state of heat shock proteins by phosphorylation/glycosylation (translational or post-translational modification) for enhanced thermotolerance.
- Development of fermentation process parameters, imparting high conversion efficiency.
- Economic production of bioethanol from lignocellulosic biomass.
- Enhancing the cell density in reactor and relieving the end product inhibition.
- Reduced duration for economic completion of fermentation.
- Efficient heat recovery during distillation.
- Cost-effective stillage management or effluent treatment through either biomethanation or bioearth composting.

35.11 Conclusions

Even though bioethanol production is an age old technology, the cost of its production still requires attention so as to make it more competitive with the current dwindling prices of gasoline. Novel attempts are required for development of efficient systems for economic production of bioethanol from lignocellulosic materials using efficient microbe. Priority for bioethanol production highlighted that (i) scope exists for screening novel microbes endowed with tolerance to higher substrate concentration, temperature, ethanol concentration etc., (ii) fine tuning of production parameters through physiochemical routes is in the realm of possibility and (iii) engineering aspects are required for abstraction of ethanol from cells. Efforts are being made to isolate and develop a novel ethanologen possessing requisite attributes with metabolic engineering.

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