Sunita Borkar *Editor*

Bioprospects of Coastal Eubacteria

Ecosystems of Goa

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To Our Mentor Prof. Saroj Bhosle

Foreword

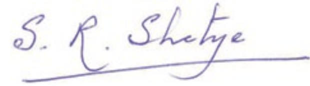
Microorganisms have been shown to be the most versatile living organisms on Earth due to their multifunctional abilities. The promise of these organisms as biological resources was recognized by the discoverer of these organisms, *Antonie Philips van Leeuwenhock*, often called “The Father of Microbiology”. Eubacteria, an important group of microorganisms, embodies diverse organisms that colonize and survive in a variety of habitats in natural environment, and are an important source of primary and secondary metabolites that are valuable to many industries. These organisms also play an important role in recycling of organic matter, biodegradation, bioremediation, waste management, and many other applications.

India’s terrestrial, marine, and other aquatic ecosystems offer high diversity of biological forms. Goa, a tiny state on the central west coast of India, with an area of 3702 sq. km, harbors many coastal and estuarine ecosystems, including low-nutrient sand dunes, nutrient-rich mangroves, beaches, rivers, etc. These ecosystems support an impressively diverse array of microorganisms.

Professor Saroj Bhosle, who joined the Goa University as a faculty in the 1980s, focused her research career at the university on Eubacteria found in the upper ecosystems. Her research on isolation, characterization, and industrial applications of the Eubacteria made her laboratory into an important center of learning in this field in India. The 15 doctoral students whose research was supervised by her with commitment, dedication, and innovation are now experts in various fields associated with Eubacteria by their own right. This book “Bioprospects of Coastal Eubacteria” brings together their expertise to provide new and useful insights into the working of coastal ecosystems of Goa. I compliment and congratulate Dr. Sunita Borkar for her notable venture in compilation of various research articles as chapters in the book.

I am sure that the book, which is being published as Dr. Bhosle steps down from her position as Professor in the Department of Microbiology at Goa University on superannuation, will benefit graduates, postgraduates, and researchers interested in

the field of microbiology of coastal ecosystems. I wish Dr. Bhosle all the best as she takes on new challenges after retirement from the university.



Dr. Satish Ramnath Shetye
Vice Chancellor
Goa University

Preface

Microbiology is an inherently valuable, fascinating branch and useful discipline of biological sciences with its own orientation and emphasis that offers an intimate view of an invisible world.

Microorganisms have promising potential in different fields as reflected in their diversity and unique characteristics. The role of microbes in the generation of biological resources is immense and has been well appreciated. The observation made by the renowned microbiologist, Louis Pasteur, about 160 years ago that "Life would not remain possible in the absence of microbes" has proved "The role of infinitely small as infinitely large". Not only natural products of microbial origin known earlier are better understood now but also newer ones are being added to the list. Their multifarious applications in all aspects of human life and environment are of immense importance.

Among several microorganisms, Eubacteria plays a very important role in our lives in recycling of organic matter, biodegradation, bioremediation and waste management and have tremendous potential in biodiscovery, soil fertility, crop protection, nutrition, etc. Pathogens are of concern to human health as they can cause fatalities. But, despite their recognized importance less than 5% of the world's Eubacteria are on record, and their meaningful exploitation is possible only if the perspectives of the cultures are properly documented with readily accessible information.

India is immensely rich in its diversity of biological forms, which includes all living organisms from ecological niches like terrestrial, marine, and other aquatic ecosystems. These niches provide natural enrichment for an impressively large array of microorganisms, and are an important repository having unique microbial flora suitable to their unique and highly variable environment. However, as stated by Edward Wilson, "Biodiversity is the earth's most important but least utilized resource".

Coastal zones of Goa have been exclusively used for agriculture, shell fishing, and traditional fishing and need to be explored for isolation and characterization of Eubacteria. Broad-based studies of characterization and the promising potential of Eubacteria from coastal econiches of Goa have been investigated and explored in research laboratory under the able guidance of reputed microbiologists of India,

Prof. Saroj Bhosle, Goa University, Taleigao Plateau, Goa. She has been working in this field for more than three decades with commitment, dedication, and innovative approach.

I strongly believe that the research we plan and perform must yield results that are of global significance in relevance and applicability. For this purpose, I requested my co-researchers to contribute articles for this book. "Bioprospects of Coastal Eubacteria" therefore, is an attempt to survey and consolidate the research on the potentials of Eubacteria mostly isolated from coastal ecosystems of Goa on varied microbiological aspects. It is not intended to be an exhaustive catalog of everything that has been done. Rather, it is an attempt to give the reader an overview of potentials of Eubacteria and an insight into research that can be oriented in different fields of applications in microbiology and biotechnology.

The chapters put together in this book, written by experts in their own field, will hopefully provide useful insights on ecology and applied aspects of Eubacteria from diverse coastal ecosystems of Goa and open new avenues of research for scientific communities. Written in a lucid language and illustrative manner for easy understanding, this book will benefit graduates, postgraduates, and researchers interested in the field of microbiology and scientists working at different levels and disciplines.

Sunita Borkar

Acknowledgements

It gives me immense pleasure to express my deep sense of gratitude to my mentor, Professor Saroj Bhosle, for her invaluable guidance, stimulating discussion, and constant inspiration during the progressive development of this endeavor. She has been a rich source of suggestions about content, order, depth, organization, and readability. I, as an editor, and we, as authors, have been fortunate to have an exceptional guide who meets every challenge with patience, positive disposition, insight, and professionalism. Her expertise as a microbiologist has proved authenticity with this worthy contribution.

I gratefully acknowledge the scholarly contributions of my fellow researchers to the world of microorganisms who have added highly informative chapters to this book. I am indebted to them for their time and patience during the process of rigorous edition. Thank you for being so accommodating and prompt.

I place on record my deep sense of gratitude to The President Shri Ravi S. Naik, the management members and the Principal, Dr. Anil Dinge of P. E. S's S. R. S. N. College of Arts and Science, Farmagudi, Ponda, Goa. I am indebted to the Department of Higher Education, Government of Goa, for relieving me of my regular duties to take up this endeavor. I am grateful to the Vice Chancellor, Goa University, for writing the foreword to this book.

I thank Dr. Nimali Prabhu, Dr. Maria Celisa Santimano, Dr. Trelita DeSouza, Dr. Rasika Gaokar, Dr. Teja Gaonkar, Dr. Naveen Krishnamurthy, Dr. Rupali Bhagat, Ms Neha Prabhu and Prof. Sushanta Basu, in preparing the manuscript for publication and everyone who directly or indirectly contributed to bring out this book.

I am indeed indebted to my family for their unconditional support and cooperation during the innumerable hours taken from them to carry out this work. Thank you for caring about me.

Special thanks are due to the production staff of Springer, Netherlands, especially, Juliana Pitanguy, Mariëlle Klijn, and Megha Koirala for their timely suggestions and for shouldering the responsibility of printing and publishing.

I think that readers' satisfaction is the best reward for the editor, contributing authors, and the publisher.

Sunita Borkar

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About the Editor

Dr. Sunita Borkar Associate Professor and Head, Department of Microbiology, P. E. S. S. R. S. N. College of Arts and Science, Farmagudi, Ponda, Goa, started with the Department of Microbiology at the same college in 1989 as Lecturer after an MSc from Bombay University and became Associate Professor in 2006. She obtained her Doctoral degree in 2003 during her teaching tenure from Goa University. She worked on "Studies on Alkaliphiles—An Industrially Significant Group of Extremophiles" under the able guidance of Professor Saroj Bhosle. She has 27 years of teaching and research experience, and has guided project work of 40 undergraduate students, two PhD scholars as a coguide, and one MPhil student. She has to her credit three minor research projects funded by UGC and one major research project funded by the Ministry of Earth Sciences, Goverment of India, New Delhi. Her major research interests encompass diversity of alkaliphilic bacteria, bioremediation, phosphate solubilization, and PGPR. Dr. Borkar has participated and presented several research papers at national and international conferences and symposia, and published research papers and articles related to her research interest. She is a life member of "Association of Microbiologists of India" and has received Carmelite Award for the best outgoing graduate student.

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Chapter 1
Coastal Sand Dunes: A Potential Goldmine of Bioresources

Aureen L. Godinho

1.1 Introduction

The first impression one gets when one views coastal sand dunes is utter barrenness; sand, sand, and more sand everywhere with very little vegetation growing on the dunes. Yet, one finds a certain group of vegetation growing on the dunes and stabilizing the dunes (Boorman 1977). The coastal dune ecosystem experiences severe stresses in the form of salt spray, sand burial, dryness, high light intensity, wind exposure, soil salinity, and nutrient deficiency. Sand dune plants or psammophytes as they are called are naturally adapted to these stress conditions. They are usually tough in nature. These plants are capable of retaining fresh water and can resist salt sprays, temperature, and tides (Desai and Untawale 2002). Little is known about the bacterial communities associated with sand dune vegetation, i.e., in the rhizosphere and endosphere of the plants. It was therefore of interest to study the different communities of bacteria inhabiting this ecosystem and further screen the predominant isolates for industrially significant biomolecules and plant-growth-promoting metabolites.

1.2 Coastal Sand Dune Vegetation

Among all the dune plants, the dune grass *Spinifex littoreus* and the creeping herb *Ipomoea pes-caprae* are dominant as they have been very well adapted to the extreme stress conditions encountered in coastal sand dune ecosystem (Fig. 1.1a, b). *I. pes-caprae* always occupies the foreshore region and has long creeping branches. Next to it, is a thick patch of *S. littoreus,* which has long rhizomes which spread

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Fig. 1.1 **a** *Spinifex littoreus* plant growing on the sand dunes on the Aswem Mandrem beach in North Goa. **b** *Ipomoea pes-caprae plant* growing on the sand dunes on the Miramar beach in North Goa

horizontally on the sand and long roots which go obliquely below the sand and may extend upto 62 cm. The leaves are rigid with an acute spine at the tip which show the deposition of sand and salt. Due to this specific feature, they are not grazed upon.

The aerial parts of the vegetation obstruct the wind and absorb wind energy. Wind velocity near vegetation is thus reduced below that needed for sand transport, and hence the sand deposits around the vegetation. A characteristic of dune vegetation, particularly the grasses growing under these conditions, is its ability to produce upright stems and new roots in response to sand covering. The development of vegetation cover on newly formed dunes, if undisturbed, creates conditions which suit the colonization and growth of a wider range of plant species. Dead plants and litter from these plants add humus to the sand. The accumulation of humus results in improved moisture and nutrient-holding capacity of the developing dune soils. Thus, with lower surface temperature and increased moisture and nutrient content, the sand is able to support a great variety of plants (Desai and Untawale 2002).

The dune vegetation helps in keeping the coastal land free from erosion and also prevents internal desertification. It also plays an important role in the formation and stabilization of coastal sand dunes. Erosion of the beach and unvegetated frontal dunes results in coastline recession. The dune plants act as obstruction, increase surface roughness, and cause reduction in the surface speed of sand carrying wind. The reduction in wind movement results in the deposition of sand around the plants. The dune system with vegetation on them acts as a buffer to the main inner land (Desai and Untawale 2002).

1.3 Bacterial Communities Associated with Sand Dune Vegetation

Plant communities in sand dunes are controlled by the interaction between biotic and physicochemical components of the sand matrix. Interactions with microbes appear crucial in obtaining inorganic nutrients or growth-influencing substances. In addition, human activities may also be an important factor, as they will certainly affect the vegetation as well as plant–microbe interactions. Plants play an important role in selecting and enriching the types of bacteria by the constituents of their root exudates. Thus, depending on the nature and concentrations of organic constituents of exudates, and the corresponding ability of the bacteria to utilize these as sources of energy, the bacterial community develops in the rhizosphere.

Bacteria living in the soil are called free living as they do not depend on root exudates for their survival. Rhizospheric bacterial communities, however, have efficient systems for uptake and catabolism of organic compounds present in root exudates. Several bacteria have the ability to attach to the root surfaces (rhizoplane) allowing these to derive maximum benefit from root exudates. Some of these are more specialized, as they possess the ability to penetrate inside the root tissues (endophytes) and have direct access to organic compounds present in the apoplast. By occupying this privileged endophytic location, bacteria do not have to face competition from their counterparts as encountered in the rhizosphere or in soil. Bacteria associated with plants can be harmful and beneficial. Plant-growth-promoting bacteria (PGPB) may promote growth directly, e.g., by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, production of siderophores that solubilize and sequester iron, or production of plant growth regulators (hormones). Some bacteria support plant growth indirectly, by improving growth restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens. Since associative interactions of plants and microorganisms must have come into existence as a result of coevolution, the use of latter group as bioinoculants must be preadapted, so that it fits into a long-term sustainable agricultural system. A number of bacterial species associated with the plant rhizosphere belonging to genera *Azospirillum, Alcaligenes, Arthrobacter, Acinetobacter, Bacillus, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Pseudomonas, Rhizobium,* and *Serratia* are able to exert a beneficial effect on plant growth (Tilak et al. 2006).

Plant–microbe interactions have been made use of in sand dune restoration programs. Arbuscular mycorrhizal fungi are important to some sand dune plants used in restoration projects of coastal sand ecosystems (Sylvia and Burks 1988; Beena et al. 2000). McCoy (2000) investigated the presence of alkane monooxygenase gene from bacterial isolates from coastal sand dunes from the Guadalupe sites, California. Some of the bacterial isolates contained alkane hydroxylase biodegradative enzyme capable of degrading short-chain *n*-alkanes present in petroleum. The 16S rDNA GenBank matches for the six isolates were all expected genera for soil microorganisms. Three of the isolates are common soil microorganisms (*Alcaligenes, Bacillus,* and *Rhodococcus*), and the other three are known soil microorganisms (*Microbacterium, Proteobacteria,* and *Phyllobacterium*). Dalton et al. (2004) suggested that the nitrogen-fixing bacteria isolated from the rhizosphere and root of *Ammophila arenaria* may contribute to the prolific success of these plants in nutrient-poor sand. Despite the important role played by bacterial diversity in sand dune plant communities, little is known on the distribution and abundance of root or rhizosphere associated bacteria.

Park et al. (2005) were the first to report on bacterial diversity associated with sand dune plants using a culture-dependent approach. A number of bacterial strains were isolated from root samples of two sand dune plant species, *Calystegia soldanella* (beach morning glory) and *Elymus mollis* (wild rye), which are found as the dominant plant species along the coastal sand dune areas in Tae-An, Chungnam Province, Korea. Members of the phylum Gamma proteobacteria, notably the *Pseudomonas* species, comprised the majority of both the rhizospheric and endophytic bacteria, followed by members of Bacteroidetes and Firmicutes in the rhizosphere and Alpha proteobacteria and Bacteroidetes in the root. (Park et al. 2005). Two strains designated PSD1-4T and PHA3-4vT, forming yellow colonies on R2A agar (Difco) were isolated from the roots of *C. soldanella* (beach morning glory) and *E. mollis* (wild rye) respectively, and subjected to further taxonomic investigation. Analysis of the 16S rRNA gene sequences showed that both of the isolates could be placed within the phylogenetic clade encompassed by the genus *Chryseobacterium* of the family Flavobacteriaceae (Park et al. 2006).

Further, Lee et al. (2006) studied the bacterial diversity in the rhizosphere of *C. soldanella* and *E. mollis* by the analysis of community 16S rRNA gene clones. The amplified rDNA restriction analysis (ARDRA) of the clones using Hae III not only exhibited significant differences in the community composition between the two plant species as well as regional differences but also identified a specific ARDRA pattern that was most common among the clones regardless of plant species. Subsequent sequence analysis indicated that the pattern was that of *Lysobacter* spp., which is a member of the family Xanthomonadaceae, class Gamma proteobacteria. The *Lysobacter* clones composed of 50.6 % of the clones derived from *C. soldanella* and 62.5 % of those from *E. mollis*. Other minor patterns included those of *Pseudomonas* spp., species of *Rhizobium, Chryseobacterium* spp., and *Pantoea* spp. among *C. soldanella* clones, and *Pseudomonas* sp. and *Aeromonas hydrophila* among *E. mollis* clones.

1.4 Adaptations of Bacterial Communities in Coastal Sand Dune Ecosystem

1.4.1 Resting Stages

Prokaryotes have evolved numerous mechanisms of resistance to stress conditions. For example, many microorganisms have an inherent ability to form resting stages (e.g., cysts and spores). Alternatively, other bacteria exhibit a metabolic versatility in order to cope with fluctuations in the chemical conditions of their environment. Bacteria have evolved a number of mechanisms that allow them to survive under nutrient starvation conditions. Some bacteria, such as *Bacilli, Clostridia,* and *Azospirilli,* undergo major differentiation programs leading to the formation of highly stress-resistant endospores or cysts (Van veen et al. 1997).

However, even without the formation of such elaborately differentiated cells, bacteria enter starvation-induced programs that allow them to survive long periods of nongrowth and to restart growth when nutrients become available again. This often leads to the formation of metabolically less active cells that are more resistant to a wide range of environmental stresses. This adaptation to starvation conditions is often accompanied by a change in cell size as well as the induction of genes and the stabilization of proteins that are essential for long-term survival. The best-studied examples of starvation-survival in nondifferentiating bacteria are *Escherichia coli, Salmonella typhimurium,* and *Vibrio* sp. strain S14, which show qualitative similarities in their survival responses. Most carbon in soil is present as recalcitrant compounds, such as humic substances and lignins, that may also complex available compounds, and so soil can be considered an oligotrophic environment. The resulting low amount of available carbon in soil generally precludes bacterial growth, and it is estimated that soil microbes typically receive sufficient energy for just a few cell divisions per year (Van veen et al. 1997).

1.4.2 Polyhydroxyalkanoates

The accumulation of intracellular storage polymers is another bacterial strategy that increases survival in a changing environment. PGPB serve as an endogenous source of carbon and energy during starvation. Polyhydroxyalkanoates (PHAs) are members of a family of polyesters that include a wide range of different D-hydroxyalkanoid acids. Since poly-β-hydroxybutyrate (PHB), one of the most abundant PHAs, was first described in *Bacillus megaterium,* several studies have demonstrated the production of PHAs by a wide variety of prokaryotes and also by several plants and animals. However, only prokaryotes accumulate high-molecular-weight PHAs in cytoplasmic granules. The granules are coated with a monolayer of phospholipids and proteins. These granule-associated proteins play a major role in the synthesis and degradation of PHAs and in granule formation. In bacteria, PHAs

constitute a major carbon and energy storage material, which accumulates when a carbon source is provided in excess and another nutrient (such as nitrogen, sulfur, phosphate, iron, magnesium, potassium, or oxygen) is limiting. The polymerization of soluble intermediates into insoluble molecules does not change the osmotic state of the cell, thereby avoiding leakage of these nutrient-rich compounds out of the cell. In addition, PHA-producing bacteria have the advantage of nutrient storage at a relatively low maintenance cost and with a secured return of energy (Berlanga et al. 2006). PHB exists in the cytoplasmic fluid in the form of crystalline granules about 0.5 μm in diameter and can be isolated as native granules or by solvent extraction. PHB has been identified in more than 20 bacterial genera including *Alcaligenes, Azotobacteer, Bacillus, Beijerinckia, Pseudomonas, Rhizobium,* and *Rhodospirillum* (Aslim et al. 2002).

1.4.3 Exopolysaccharide

Bacteria also produce exopolysaccharide (EPS) as one of the mechanisms to overcome desiccation. An EPS matrix may slow the rate at which a bacterial colony equilibrates with the surrounding soil. Slowing the rate of drying within the colony microenvironment could increase bacterial survival by increasing the time available for metabolic adjustment. Clays, which slow the rate of drying of the soil, have been shown to increase the ability of bacteria to survive desiccation in the soil. An EPS matrix may provide another advantage to bacteria living within it by decreasing the water content of the soil and thereby restricting the diffusion of nutrients to microorganisms. Polysaccharides are hygroscopic and therefore may maintain higher water content in the colony microenvironment than in the bulk soil as water potential declines. This increase in water content could increase nutrient availability within the bacterial colony. There is some evidence in plants that polysaccharides increase survival and activity during drying. Plants with high levels of EPS showed higher transpiration rates than low-level-EPS plants during mid-day water stress. Roberson and Firestone (1992) revealed that bacteria respond to desiccation by channelling energy and nutrients into polysaccharide production.

1.4.4 Microbial Extracellular and Degradative Enzymes

Extracellular enzyme activity is generally recognized as the key step in degradation and utilization of organic polymers by bacteria, since only compounds with molecular mass lower than 600 Da can pass through cell pores (Fabiano and Danovara 1998). Microbial enzymes such as protease, amylase, lipase, cellulase, xylanase, rennin, glucoamylase, glucose oxidase, pullulanase, invertase, and tannase, which are used widely in several industries, are mainly derived from terrestrial microorganisms, particularly from bacteria and/or fungi (Chandresekaran 1997).

Coastal sand dune ecosystem is low in nutrients and the humus contributes to the organic matter on decomposition of the vegetation litter. Besides naturally occurring tidal waves, oil spills bring in nutrients for the microorganisms. Microorganisms in such ecosystem utilize detrital matter and other available nutrients including petroleum hydrocarbons breaking these into simpler compounds. Hydrocarbon pollution of estuarine and marine environment occurs frequently. Removal of these pollutants by biodegradative processes has been a subject of extensive interest, owing partly to their recalcitrance to biodegradation in the natural environment. Recent work has indicated that the stimulation of microbial activity in the rhizosphere of plants can also stimulate biodegradation of various toxic organic compounds. This general "rhizosphere effect" is well known in terrestrial systems. The rhizosphere soils have been described as the zone of soil under the direct influence of plant root surface and is a dynamic environment for microorganisms. Microbial activity is thus generally higher in the rhizosphere due to readily biodegradable substrate exuded from the plant (Daane et al. 2001). Petroleum products contain hazardous organic chemicals such as benzene, toluene, naphthalene, and benzopyrene some of which are recognized carcinogens. Oily sludge is a complex mixture of alkene, aromatics, NSO (nitrogen, sulfur, oxygen)-containing compounds, and asphaltene fractions, and a single bacterial species has only limited capacity to degrade all the fractions of hydrocarbon present. Many microbes are endowed with metabolic properties enabling them to degrade these compounds. These include the presence of mono and dioxygenase enzyme for the oxidation of organic compounds (Khan et al. 2006). Microbial activities allow the conversion of some petroleum components into CO_2 and H_2O, and microbial transformation is considered a major route for the complete degradation of petroleum components (Prince 1993). The toxic effects of aromatic and aliphatic hydrocarbons, phenols, and alcohols due to the interaction of these compounds with the membrane and membrane constituents have been studied for many years. Small hydrophobic molecules are highly toxic for microorganisms due to their partitioning into the cytoplasmic membrane. They interrupt the protein–lipid and lipid–lipid connections in the membrane and, as a result, cause functional disturbances and increase membrane fluidity and passive diffusion of the hydrophobic compounds into the cell (Sikkema et al. 1994 1995).

1.4.5 Secondary Metabolites Produced by Sand Dune Rhizobacteria

Microbial secondary metabolites include pigments, siderophores, organic acids, indole-3-acetic acid (IAA), 1-aminocyclopropane-1-carboxylate (ACC) deaminase, hydrogen cyanide, ammonia, toxins, effectors of ecological competition and symbiosis, antibiotics, pheromones, enzyme inhibitors, immuno modulating agents, receptor antagonists and agonists, pesticides, antitumor agents, and growth promoters of animals and plants. They have a major effect on the health, nutrition, and economics of our society. They often have unusual structures and their formation

is regulated by nutrients, growth rate, feedback control, enzyme inactivation, and enzyme induction (Demain 1998). Rhizosphere bacteria are present in large numbers on the root surface, where nutrients are provided by plant exudates and lysates. Certain strains of rhizosphere bacteria are referred to as plant-growth-promoting rhizobacteria (PGPR), because their application can stimulate growth and improve plant development under stressful conditions (Gomez et al. 2003). Increased plant productivity results in large part from the suppression of deleterious microorganisms and soilborne pathogens by PGPR. Fluorescent *Pseudomonas* spp. are among the most effective rhizosphere bacteria in reducing soilborne diseases in disease suppressive soils, where disease incidence is low, despite the presence of pathogens and environmental conditions conducive to disease occurrence.

1.4.5.1 Diffusible Pigments

Pigments have a great commercial value and are used immensely as a colorant in numerous industries such as plastics, gums, food industry as dye and stains, etc. (Nelis and Leenbeer 1991). However, the nature of these compounds is largely unknown. There have been reports on the analysis of carotenoids from psychrotrophic bacterium *Micrococcus roseus* (Strand et al. 1997) and from *Staphylococcus aureus* (Hammond and White 1970). These pigments form an integral part of the complex membrane structure of a range of mesophilic and thermophilic microorganisms and influence membrane fluidity, by increasing its rigidity and mechanical strength (Armstrong 1997). It has been suggested that the presence of carotenoids may change the effectiveness of the membrane as a barrier to water, oxygen, and other molecules (Britton 1995). Microorganisms accumulate several types of carotenoids as part of their response to various environmental stresses (Bhosale 2004).

1.4.5.2 Siderophores

Iron is made biologically available by iron-chelating compounds called siderophores that are synthesized and secreted by many bacteria and fungi under conditions of iron limitation (Neilands 1995). Siderophores are water-soluble, low-molecular weight molecules that are secreted by bacteria and fungi. The term siderophore stands for "iron carriers" or "iron bearers" in Greek. This is an appropriate term because the siderophore binds iron with an extremely high affinity and is specifically recognized by a corresponding outer membrane receptor protein, which in turn actively transports the complex into the periplasm of the cell (Braun and Braun 2002; Faraldo-Gómez and Sansom 2003). The role of these compounds is to scavenge iron from the environment and to make the mineral available to the microbial cell which is almost always essential (Neilands 1995). Studies on suppression of Fusarium wilt of carnation and radish, caused by *Fusarium oxysporum* f. sp. *dianthi* (Fod) and *F. oxysporum* f. sp. *raphani* (For), respectively, established competition for iron as the mechanism of disease reduction by *P. putida* strain WCS358. Under iron-limiting

conditions in the rhizosphere, WCS358 secretes a pyoverdin-type siderophore (pseudobactin 358) that chelates the scarcely available ferric ion as a ferric siderophore complex that can be transported specifically into the bacterial cell. Siderophores released by Fod or For under these circumstances are less efficient iron-chelators than pseudobactin 358. Hence, iron available to the pathogens can become limiting in the presence of WCS358. Due to iron deficiency, fungal spore germination is inhibited and hyphal growth restrained, effectively lowering the chance that the plants become infected, and reducing disease incidence and severity. The plant, in contrast, does not appear to suffer from iron shortage (Loon et al. 1998).

1.4.5.3 Organic Acid Production for Phosphate Solubilization

A large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and become unavailable to plants. Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields. These reactions take place in the rhizosphere, and because phosphate-solubilizing microorganisms render more phosphates into soluble form than is required for their growth and metabolism, the surplus gets absorbed by plants (Mehta and Nautiyal 2001).

1.4.5.4 Indole-3-Acetic Acid

Auxins are a class of plant hormones and one of the most common and well characterized is indole-3-acetic-acid (IAA), which is known to stimulate both rapid (e.g., increases in cell elongation) and long-term (e.g., cell division and differentiation) responses in plants (Glick 1995). Some of the plant responses to auxin are: (a) cell enlargement, (b) cell division, (c) root initiation, (d) root growth inhibition, (e) increased growth rate, (f) phototropism, (g) geotropism, and (h) apical dominance (Frankenberger and Arshad 1995; Leveau and Lindow 2005). Most notably, exogenous auxin production by bacteria has been associated with altered growth of the roots of plants on which they were inoculated. While many PGPB, which stimulate the growth of roots, can produce at least small amounts of the auxin IAA, high IAA producers are inhibitory to root growth (Lindow et al. 1998). Various authors have identified the production of IAA by microorganisms in the presence of the precursor tryptophan or peptone. Eighty percent of microorganisms isolated from the rhizosphere of various crops have the ability to produce auxins as secondary metabolites (Kampert et al. 1975; Loper and Schroth 1986). Bacteria belonging to the genera *Azospirillum, Pseudomonas, Xanthomonas,* and *Rhizobium* as well as *Alcaligenes faecalis, Enterobacter cloacae, Acetobacter diazotrophicus,* and *Bradyrhizobium japonicum* have been shown to produce auxins which help in stimulating plant growth (Patten and Glick 1996).

1.4.5.5 1-Aminocyclopropane-1-carboxylate Deaminase

1-Aminocyclopropane-1-carboxylate (ACC), the cyclopropanoid amino acid, is a precursor in the biosynthetic pathway of the plant hormone ethylene. Plant-growth-promoting soil bacteria have been found to contain ACC deaminase (ACCD), a pyridoxal 5'-phosphate (PLP) dependent enzyme that converts ACC to a ketobutyrate and ammonium. The possibility of a close mutualistic relationship between the plants and the soil bacteria and the role of ACCD in ensuring low levels of ethylene at critical stages of root growth has been proposed (Hontzeasa et al. 2005). The enzyme ACCD is important as this enzyme can cleave the plant ethylene precursor ACC, and thereby lowers the level of ethylene in a developing or stressed plant (Glick 2005).

1.4.5.6 Hydrogen Cyanide

Hydrogen cyanide (HCN) is a potential inhibitor of enzymes involved in major plant metabolic processes including respiration, CO_2 and nitrate assimilation, carbohydrate metabolism and may also bind with the protein plastocyanin to block photosynthetic electron transport (Grossman 1996). HCN is a potent inhibitor of cytochrome c oxidase and of several other metalloenzymes, some of them involved in respiratory processes. HCN biosynthesis is catalyzed by HCN synthase, from glycine with stoichiometric production of CO_2. HCN affects sensitive organisms by inhibiting the synthesis of Adenosine triphosphate (ATP) mediated by cytochrome oxidase and is highly toxic to all aerobic microorganisms at picomolar concentrations (Pal and McSpadden Gardener 2006). No role is known for HCN in primary bacterial metabolism, and it is generally considered as a secondary metabolite (Blumer and Haas 2000). HCN-producing bacteria can help plants in their defence against fungal pathogens (Blumer and Haas 2000; Voisard et al. 1989). This property was predominantly described among *Pseudomonas* strains (Kremer and Souissi 2001). Therefore, depending on the target organisms, HCN-producing microorganisms are regarded as harmful when they impair plant health and beneficial when they suppress unwanted components of the microbial community (Bellis and Ercolani 2001).

1.4.5.7 Ammonia

Biological N_2-fixation (BNF) by soil microorganisms is considered one of the major mechanisms by which plants benefit from the association of micropartners. One of the benefits that diazotrophic microorganisms provide to plants is fixed nitrogen in exchange for fixed carbon released as root exudates (Glick 1995). Many of the PGPR described to date are free-living diazotrophs that can convert molecular nitrogen into ammonia in a free state by virtue of the nitrogenase enzyme complex (Postgate 1982; Saikia and Jain 2007).

1.5 Bioprospects of Rhizosphere Bacteria Associated with Coastal Sand Dune Vegetation, *Ipomoea pes-caprae* and *Spinifex littoreus*

1.5.1 Biodiversity of Bacteria in the Coastal Ecosystem

Plant and soil samples were collected from two coastal sand dune areas of North Goa during July 2003, December 2003, and May 2004. These areas included actively growing zones of *I. pes-caprae* and *S. littoreus,* which vigorously stand on the seaward dune faces. *I. pes-caprae* was collected from Miramar while *I. pes-caprae* and *S. littoreus* were collected from Aswem Mandrem. Soil samples collected from individuals of a species were mixed to form a composite sample. These composite soil samples were used for microbiological analysis.

Rhizosphere sand from *I. pes-caprae* and *S. littoreus* was collected and the sand was dispensed in 0.85 % saline and dilutions were prepared. The dilutions were plated on specific media such as nutrient agar medium for the total viable count, polypeptone yeast extract glucose agar (PPYG) for isolating alkaliphiles, nitrogen-free mannitol agar for isolating diazotrophs, sodium chloride tryptone yeast extract agar for isolating halophiles, and nutrient agar with pH 4 for isolating acidophiles. The plates were incubated at 28 ± 2 °C and the colonies were counted. The endophytic bacteria of the vegetation were isolated by taking 1 g of roots and washing it well in sterile distilled water. The roots were then treated with 0.01M EDTA and centrifuged at 5000 rpm for 10 min, and this process was repeated three times to remove any sand particles attached to the root surface. The roots were then transferred to a sterile mortar and homogenized. The extract obtained was diluted upto 10^{-6}, and the appropriate dilutions were plated on respective media as mentioned above.

Sand and root samples of two different dune plants (*I. pes-caprae* and *S. littoreus*) collected from different sand dunes in North Goa showed the presence of a large number of bacteria both in the rhizosphere as well as endophytes. Further, the seasonal variation was also detected within different groups of bacteria. The variation in the three seasons was observed with postmonsoon showing higher bacterial counts followed by monsoon and premonsoon. The total viable counts ranged from as high as 10^7 cfu/g to as low as 10^3 during premonsoon period, from 10^7 cfu/g to 10^3 during monsoon period and from as high as 10^8 cfu/g to as low as 10^5 during postmonsoon period. The different communities of bacteria observed were acidophiles, alkalophiles, halophiles, and diazotrophs.

Overall, it was observed that endophytic bacterial counts were higher than rhizosphere bacterial counts among the different bacterial groups. Interestingly, the total viable counts in unvegetated areas of sand dunes were lower than the vegetated areas as seen from the analysis of the samples collected from unvegetated area (10^3 cfu/g). However, the counts of acidophiles and alkaliphiles in the unvegetated areas were similar to the counts obtained in vegetated areas of dunes. The soil here

is influenced by various factors and has incident pockets or niches with varied pH. The bacterial counts obtained on various media reflect the population of the different groups of bacteria surviving in the sand dune ecosystem, a nutrient-limited ecosystem. The viable counts of general heterotrophic neutrophilic bacterial populations and alkaliphilic bacteria were found to be comparatively lower in contrast to marine sediment counts or soil counts (Desai et al. 2004). Colonies obtained from different media showed wide variation in cultural characteristics with, neutrophiles and alkaliphiles showing consistent growth.

The majority of the neutrophiles and alkaliphiles surviving were Gram positive. It was interesting to note that in all three seasons viz. premonsoon, monsoon, and postmonsoon periods, Gram positive isolates were predominant. In the postmonsoon period, nearly all the neutrophilic and alkalophilic isolates showed Gram positive character as compared to the premonsoon and monsoon period. In premonsoon period, neutrophiles showed more Gram negative isolates both in rhizosphere and endophytic isolates from *I. pes-caprae* from Miramar. Among the alkaliphiles, the majority of the isolates were Gram positive, nonsporulating rods compared to neutrophilic isolates which were sporulating rods. The alkaliphiles were categorized into facultative alkaliphiles and alkalitolerant. It was observed that there were no obligate alkaliphiles detected among the sand dune rhizosphere and endophytic bacteria. A few of them were alkalitolerant but interestingly, the majority of the alkaliphiles were facultative alkaliphiles.

The bacterial community of the coastal sand dune ecosystem possesses hydrolytic enzymes involved in degradative processes. Amongst the neutrophilic (Fig. 1.2a plates 1–4) and alkalophilic (Fig. 1.2b plates 1–3) bacteria screened for protease, cellulase, amylase, tannase, and chitinase enzymes, the number of cellulose and protein degraders were highest in the premonsoon period, due to their involvement in the degradation of the shedded foliage. Among the neutrophilic isolates, the premonsoon and monsoon bacterial isolates showed good enzymatic activity as compared to postmonsoon period. Interestingly, the bacterial isolates from coastal sand dune ecosystem also exhibited multiple enzyme activities, e.g., amylase, tannase, protease, and cellulase which reflects their role in the stressful ecosystem. Besides, such isolates also play an important role in nutrient recycling and maintaining the biogeochemical cycles.

To increase survival and stress tolerance in changing environments and in competitive settings, microorganisms are known to accumulate PHAs where carbon and energy sources may be limited, such as those encountered in the soil and the rhizosphere (Fig. 1.3). Accumulation of PHAs can provide the cell with the ability to endure a variety of harmful physical and chemical stresses, either directly linked to the presence of the polyester itself (PHA granules) or through a cascade of events concomitant with PHA degradation and the expression of genes involved in protection against damaging agents.

To sequester and solubilize ferric iron, many microorganisms utilize an efficient system consisting of low-molecular mass (<1000 Da) compounds with high iron affinity termed,"siderophores" (Guerinot et al. 1990; Neilands 1995). According

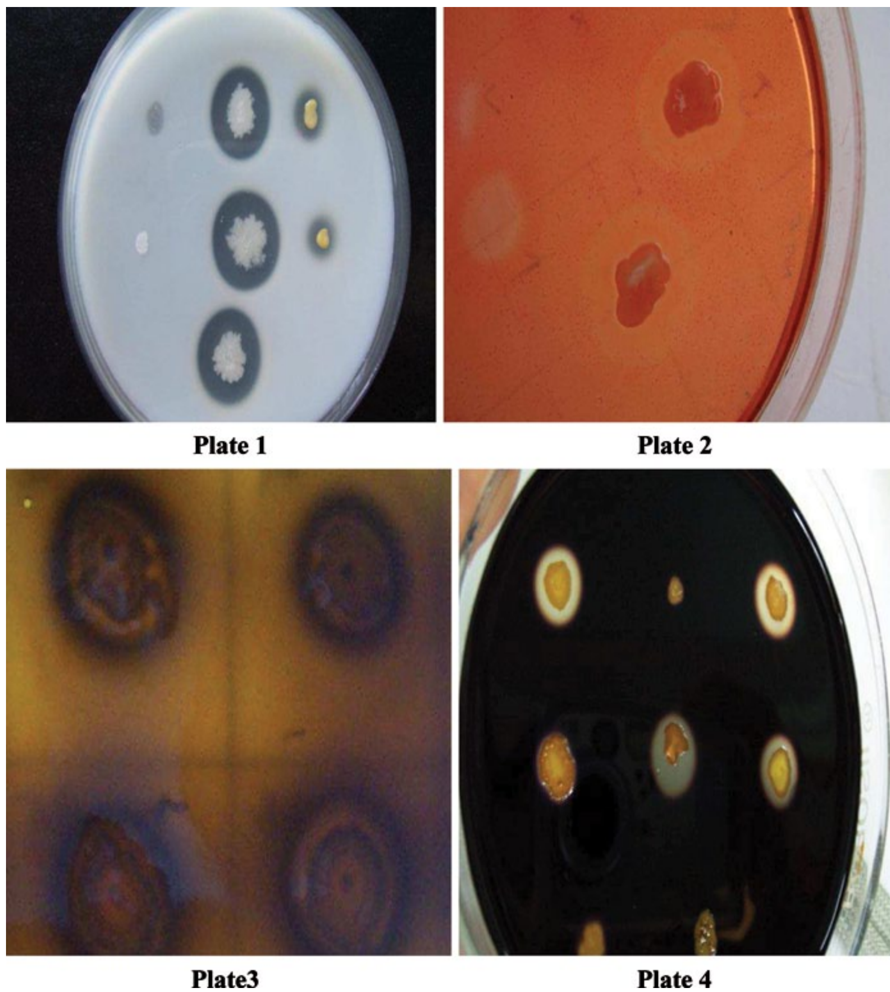

Fig. 1.2 **a** Neutrophilic bacterial isolates showing production of enzymes protease (*plate 1*), cellulase (*plate 2*), tannase (*plate 3*), and amylase (*plate 4*). **b** Alkalophilic bacterial isolates showing production of enzymes protease (*plate 1*), amylase (*plate 2*), and cellulase (*plate 3*)

to the generally accepted definition, siderophores are ferric-specific microbial iron-chelator compounds whose biosynthesis is regulated by the availability of iron in the surrounding medium, and under conditions of high iron concentrations, the production of these compounds is repressed. A majority of the sand dune bacteria isolates (rhizosphere and endophytic bacteria) were scored positive for siderophores. On the chrome azurol sulphonate (CAS) plate assay, the isolates showed orange to yellow orange color halo around the colonies. Among the neutrophilic isolates, the majority of the premonsoon isolates were scored positive for siderophore production followed by postmonsoon and monsoon isolates (Fig. 1.4).

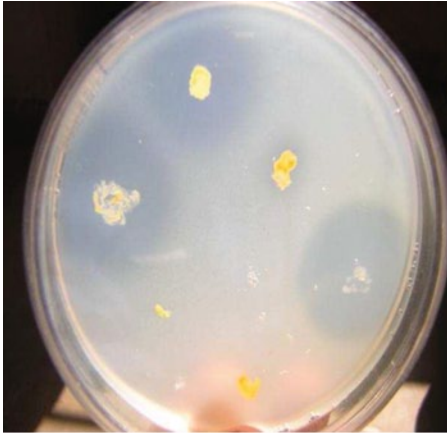

Plate 1

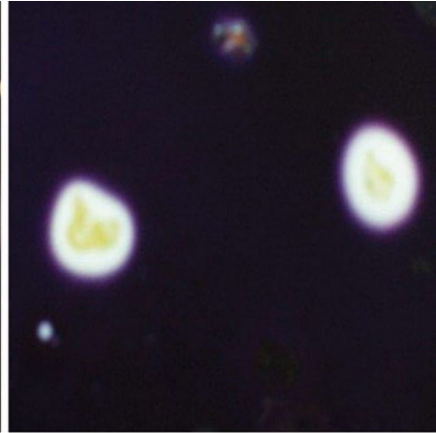

Plate 2

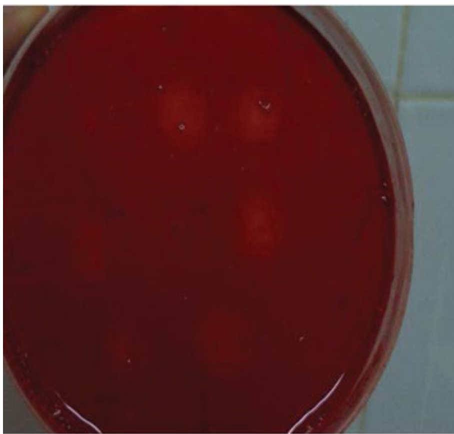

Plate 3

Fig. 1.2 (continued)

Fig. 1.3 Fluorescence exhibited by neutrophilic bacterial isolates grown on E2 medium plate on staining with *Nile blue* A

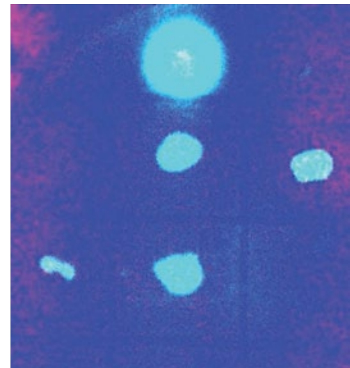

Fig. 1.4 The *yellow orange* halo surrounding the bacterial colony is indicative of the production of an Fe-binding compound such as siderophore, which removes Fe (III) from the Fe (III)–CAS HDTMA complex in the plate and turns the blue dye to *yellow* color. (*Color figure online*)

Besides these characteristics, another important characteristic of such isolates which helps the plant is the phosphate solubilization either by enzymes or by production of organic acids. Phosphate-solubilizing bacteria were routinely screened by a plate assay method using Pikovskaya (PVK) agar incorporated with 0.4% bromothymol blue (Fig. 1.5 plates 1 and 2). The test of the relative efficiency of isolated strains was carried out by selecting the microorganisms that were capable of producing a halo/clear zone on a plate owing to the production of organic acids into the surrounding medium. The majority of the neutrophilic and alkalophilic rhizosphere and endophytic isolates were found to solubilize inorganic phosphate. Among the neutrophilic isolates, the majority of the P solubilizers were isolated in the premonsoon season followed by postmonsoon and monsoon period.

Based on the cultural, physiological, and biochemical characteristics, it was observed that among the neutrophiles, the majority of the isolates belonged to *Bacillus* genus, while among the alkaliphiles, the majority of the isolates were Gram positive irregular rods belonging to genera such as *Brevibacterium, Brochothrix, Cellulomonas,* and *Microbacterium*. Interestingly, the alkalophilic genera like

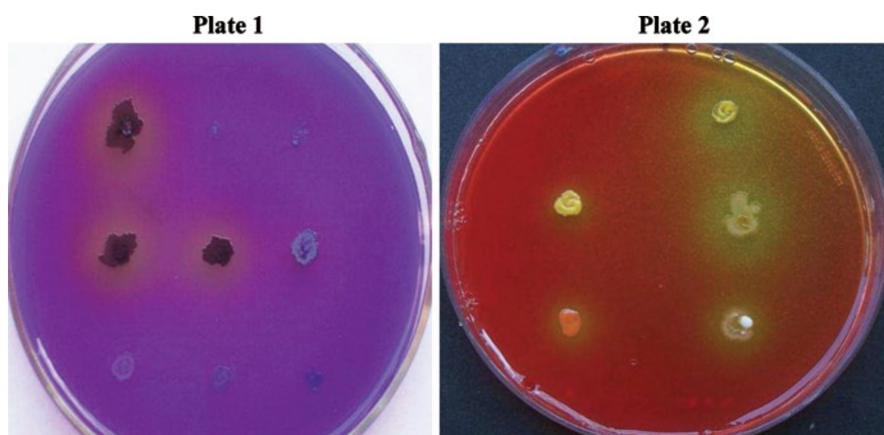

Fig. 1.5 P solubilizing bacteria producing yellow halo/clear zone on PVK's agar due to production of organic acids (plate 1: neutrophilic isolates, plate 2: alkalophilic isolates). (Color figure online)

Brochothrix spp., *Cellulomonas* spp., and *Renibacterium* spp. were found to be potent producers of protease and amylase enzymes as compared to the neutrophilic isolates. They were also found to be good P solubilizers. The neutrophilic *Bacillus* genus was found to be better siderophore producers as compared to the alkaliphilic genera.

Since the beaches in Goa are found to have contact with hydrocarbons washed to the shore, the ability of these isolates to grow in the presence of different aromatic compounds and survive in the presence of solvents was determined. It was interesting to note that the majority of these cultures isolated showed the ability to grow on hydrocarbons. The coastal sand dune ecosystem is low in nutrients and the humus contributes to the organic matter, on decomposition of the vegetation litter. Besides naturally occurring tidal waves, oil spills bring in nutrients for the microorganisms. Microorganisms in such ecosystem utilize detrital matter and other available nutrients including petroleum hydrocarbons breaking these into simpler compounds. Hydrocarbon pollution of estuarine and marine environment occurs frequently. Removal of these pollutants by biodegradative processes has been a subject of extensive interest, owing partly to their recalcitrance to biodegradation in the natural environment. Polycyclic aromatic hydrocarbons are of environmental concern because of their toxic, mutagenic, and carcinogenic properties. Certain polyaromatic hydrocarbons (PAH) although persistent mainly due to their hydrophobicity, can be degraded by a variety of microorganisms (bacteria and fungi).

Among the neutrophilic isolates, nearly 100 % of the premonsoon rhizospheric and endophytic isolates were found to grow on hydrocarbons such as phenanthrene, biphenyl, naphthalene, and sodium benzoate followed by postmonsoon isolates and monsoon isolates. Among the alkaliphilic isolates, 80% of the rhizosphere and endophytic bacterial isolates were found to degrade the hydrocarbons in the monsoon period followed by postmonsoon and premonsoon period. The majority postmonsoon rhizosphere and endophytic bacteria were found to tolerate solvents such as cyclohexane, toluene, benzene, and hexadecane followed by the premonsoon bacterial isolates while the monsoon bacterial isolates were found to tolerate only benzene (Godinho and Bhosle 2013a).

1.5.2 Sand Aggregation by Exopolysaccharide-Producing Microbacterium arborescens-AGSB

In the rhizosphere, exopolymers are also known to be useful to improve the moisture-holding capacity. An isolate from this ecosystem, *Microbacterium arborescens*-AGSB, a facultative alkalophile showing very high production of exopolysaccharide (EPS), was studied for exopolymer production (Fig. 1.6a). The isolate, a Gram positive nonspore-forming slender rod (Fig. 1.6b a–c) was catalase positive, oxidase negative, and survived on 12 % sodium chloride. The isolate was found to produce exopolymer which showed good aggregation of sand which has an important role in the stabilization of sand dunes (Fig. 1.6c). The exopolymer

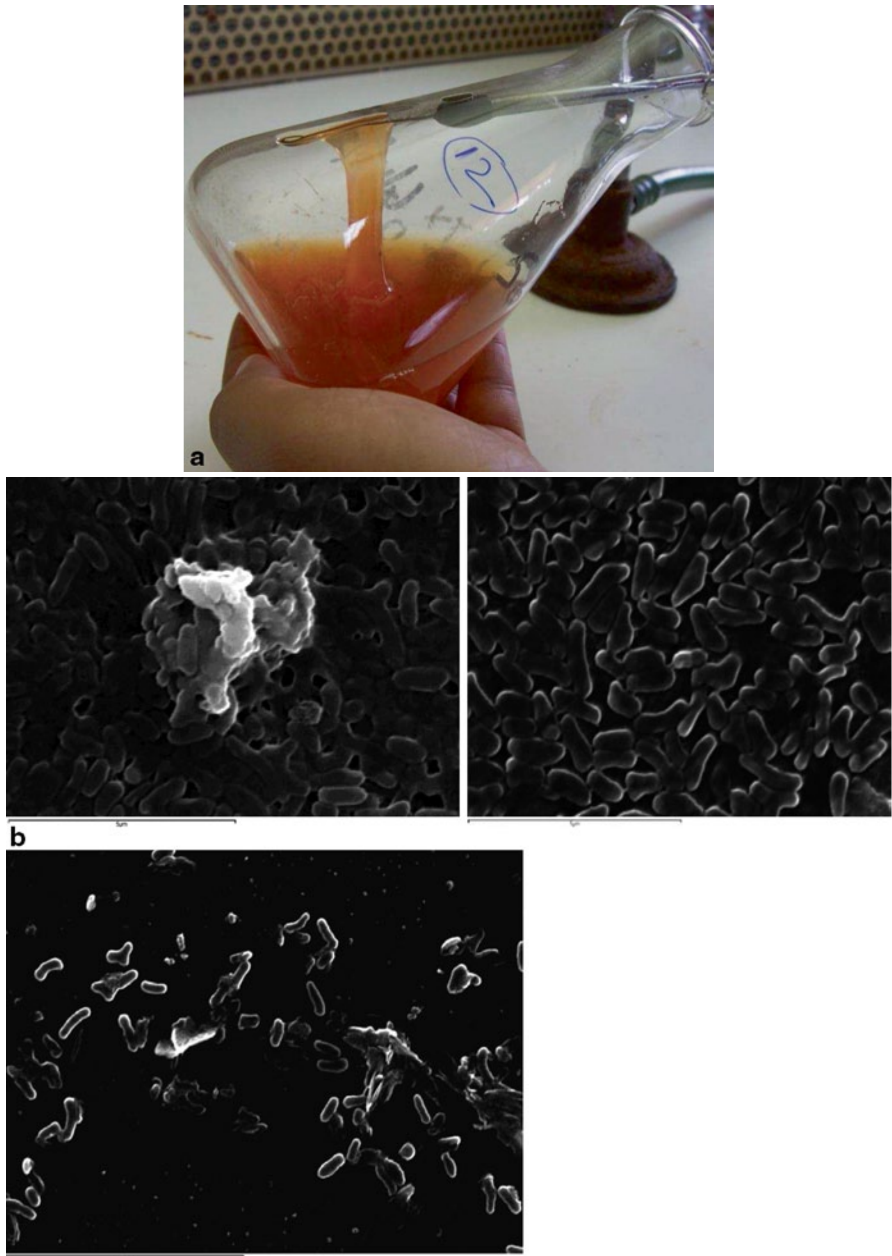

Fig. 1.6 **a** Viscous exopolysaccharide produced by *Microbacterium arborescens*. **b** Scanning electron micrographs of exopolysaccharide formation by *Microbacterium arborescens* (**a**, bar, 5 μm). Most of the surface has been colonized with actively dividing rod cells, and finger-like projections of extracellular polymeric material are present (**b**, bar, 5 μm). High magnification indicates the presence of extracellular polymeric materials on the surfaces of bacterial cells (**c**, bar, 8 μm). **c** Aggregation of soil by *Microbacterium arborescens*

Fig. 1.6 (continued)

was further analysed. The cold isopropanol precipitation of dialysed supernatants grown in polypeptone yeast extract glucose broth produced partially soluble exopolymer with glucose as the sole carbon source. Chemical analysis of the EPS revealed the presence of rhamnose, fucose, arabinose, mannose, galactose, and glucose. On the optimization of growth parameters (sucrose as carbon source and glycine as a nitrogen source), the polymer was found to be a heteropolysaccharide containing mannose as the major component. It was interesting to note that the chemical composition of the exopolymers produced from both unoptimized and optimized culture conditions of *Microbacterium arborescens*-AGSB is different from those of other species from the same genera. This study shows that marine coastal environments such as coastal sand dunes, are a previously unexplored habitat for EPS-producing bacteria, and that these molecules might be involved in ecological roles protecting the cells against desiccation especially in nutrient-limited environments such as the coastal sand dunes, more so in the extreme conditions of pH. Such polysaccharides may help the bacteria to adhere to solid substrates and survive during the nutrient limitations (Godinho and Bhosle 2009).

1.5.3 Microbacterium arborescens-AGSB sp. nov. from the Rhizosphere of Sand Dune Plant, Ipomoea pes-caprae

Phenotypic and phylogenetic studies were performed for the facultative alkalophile from the rhizosphere of *I. pes-caprae,* a plant growing on coastal sand dunes. The isolate was Gram positive and showed optimum growth at pH 10.5. Chemotaxonomic analysis revealed that the isolate contained type B1 peptidoglycans with L-lysine as the diamino acid; rhamnose and galactose were the cell wall sugars and belonged unambiguously to the genus *Microbacterium*. The major menaquinones were MK-11 and MK-12. The 16S rDNA sequence of the *Microbacterium*

arborescens isolate has been deposited in the GenBank with an accession number DQ287961. The phylogenetic and phenotypic distinctiveness of the strain indicates it could be a novel *Microbacterium* sp., named as *M. arborescens*-AGSB (Godinho and Bhosle 2013b).

1.5.4 Carotenes Produced by Alkaliphilic Orange Pigmented Strain of Microbacterium arborescens-AGSB

Collections of Gram positive bacteria from coastal sand dune vegetation, *I. pes-caprae* showed a predominance of orange pigmented colonies of *Microbacterium arborescens*-AGSB (Fig. 1.7, plates 1 and 2). The pigment was identified using a combination of UV/visible spectral data and high-performance liquid chromatography (HPLC) retention time as a lycopene type carotenoid pigment with λmax at 468 nms. These bacteria may be accumulating carotenoids as part of their responses to various environmental stresses and thus aiding their survival in this stressed habitat (Godinho and Bhosle 2008).

1.5.5 Bacteria from Sand Dunes of Goa Promoting Growth in Solanum melongena (Eggplant)

PGPR are known to influence plant growth by various direct or indirect mechanisms. Given the negative environmental impact of chemical fertilizers, the use of PGPR

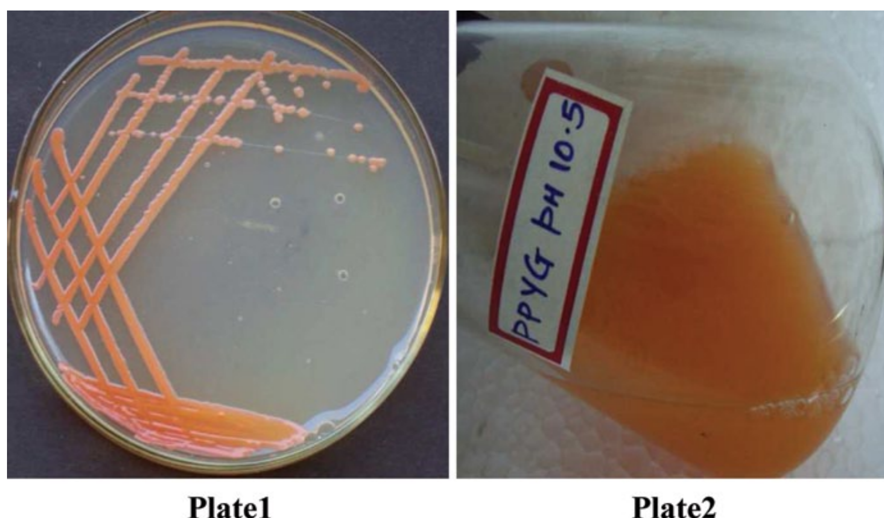

Fig. 1.7 Orange pigment produced by *Microbacterium arborescens* on PPYG agar (*plate 1*) and in broth (*plate 2*). (Color figure online)

as natural fertilizers is advantageous for the development of sustainable agriculture. Four predominant isolates were screened for the production of siderophores, hydrogen cyanide (HCN), IAA, ACCD and inorganic phosphate solubilization. All the four isolates showed significant production of these traits. These isolates were further characterized and identified based on biochemical, morphological and 16sRNA studies as *Microbacterium arborescens, Bacillus subtilis, Bacillus sp MF-A4* and *Kocuria rosea.* The production of siderophore was maximum by *B. subtilis,* HCN was produced by Bacillus *sp. MF-A4* and *M.arborescens,* while IAA and ammonia were produced significantly by *Bacillus subtilis*. ACCD activity and phosphate solubilization were shown by all the isolates.

The growth-promoting effect of these four isolates was tested on eggplant. In this study among the four sand dune bacterial isolates, *B. subtilis, K. rosea,* and *M.arborescens* were found to be good plant growth promoters in neutral (pH 7) soil conditions (Fig. 1.8a–e). All the four sand dune bacterial cultures were found to have ACCD activity and other attributes like IAA production, HCN production, siderophore production, and phosphate solubilization. Chelation of iron by microbial siderophores and phosphate solubilization has been reported earlier to increase crop yield (Glick 1995). These traits might have helped in better nutrient mobilization, availability, and thus uptake, which in turn increased plant biomass, N and P content. Also, ACCD activity might have produced better root growth in the initial stages of crop growth by reducing the level of ethylene in the roots of the developing plants thereby increasing the root length and growth. This resulted in healthy plant due to balanced nutrient availability and uptake, which in turn increased plant biomass.

Although ACCD activity in enhancing plant growth cannot be ruled out, coordinated expression of multiple growth-promoting traits could have been responsible in the overall plant growth promotion of eggplant by these sand dune bacterial isolates. The study has also indicated that individual cultures as bioinoculants have a better effect on eggplant growth as compared to the consortium. The present study has, therefore, confirmed the bioprospects of using these sand dune bacteria as biofertilizers for agricultural crops (Godinho et al. 2010).

1.6 Conclusions and Future Prospects

This chapter reveals the diverse group of microorganisms in the coastal sand dune ecosystem and signifies them as potential PGPR which are widely distributed in this nutrient-limited ecosystem. The importance of PGPR and its potential for plant growth promotion and enhancement of agriculturally important crop, eggplant in Goa has been elaborated in this chapter. Most research work so far has largely focused on arbuscular mycorrhizal fungi in coastal sand dunes in India. This work is one of the first attempts to study the bacterial communities in the coastal sand dunes

Fig. 1.8 Growth-promoting effects of sand dune bacteria in normal soil (pH 7) on *Solanum melongena* (eggplant) seedling (**a** control + *M. arborescens*, **b** control + *consortium*, **c** control + *K. rosea*, **d** control + *B. subtilis*, **e** control + *Bacillus* sp. MF-A4)

with the aim to use potential PGPR exhibiting direct and indirect effects on the plant as bioinoculants in agriculture to improve crop productivity. More studies need to be carried out on understanding this aspect and isolating novel species of PGPR which can be applied to crops in Goa.

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Chapter 2
Eubacterial Siderophores and Factors Modulating Their Production

Teja Gaonkar

2.1 Introduction

2.1.1 Need for Iron

Iron is essential for the growth and development of almost all living organisms. It acts as a catalyst in some of the most fundamental enzymatic processes, including oxygen metabolism, electron transfer and deoxyribonucleic acid (DNA)/ribonucleic acid (RNA) synthesis (Guan et al. 2001). However, despite its abundance on earth and the micromolar concentrations required for microbial growth, iron is biologically unavailable in most environments. In aerobic inorganic environments, iron is present in the oxidized ferric form Fe^{+3}, which aggregates into insoluble oxyhydroxide polymers (Wandersman and Delepelaire 2004).

The most obvious effect of iron deficiency on microbial cells is the decrease in quantity of cellular biomass. Iron deficiency has been reported to induce changes in activities of certain enzymes. For example, iron deficiency has been reported to result in 24-fold stimulation of NADase activity and is known to decrease the concentrations of cytochromes, peroxidase and catalase. Such a decrease in cytochrome concentration has been reported in *Torula utilis*; decrease in peroxidase, catalase and oxidase activity has been reported in *Candida guilliermondii*. Nicotinamide adenine dinucleotide (NADH) dehydrogenase is a membrane-bound enzyme with iron-sulfur centres which brings about NADH oxidation in the terminal electron transport systems of aerobes. Its enzyme activity and polypeptide chain composition have been found to be affected by iron limitation. Aconitase activity has also been found to diminish due to iron deficiency as opposed to aldolase whose activity is found to increase. Diminution in alcohol dehydrogenase activity has been observed in *C. guilliermondii* with decrease in iron concentration in the growth

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medium. Iron exerts an effect on enzymes involved in the biosynthesis and degradation of iron-chelating agents, siderophores (Light and Clegg 1974).

Besides affecting enzyme activity, iron deficiency results in morphological changes in microorganisms. In *Mycobacterium smegmatis*, an increase in cell length has been observed as compared with the cells grown in iron-sufficient medium. This could be due to inhibition of DNA synthesis without proportional inhibition of cell growth or due to a direct effect on cell division or cell separation (Light and Clegg 1974). Hence, to acquire this iron from natural ecosystems, bacteria have evolved multiple parallel pathways, the most important of these is, siderophore production.

2.1.2 Siderophores

Siderophores are low-molecular-weight ligands (20–2000 Da) produced by bacteria, fungi and plants to solubilize and take up iron (Chu et al. 2010; Hider and Kong 2010). Siderophores chelate iron with an affinity constant of almost $10^{30}M^{-1}$. More than 500 different siderophores mostly from bacteria have been described. The iron ligation groups have been tentatively classified into three main chemical types: hydroxamate, catecholate and hydroxycarboxylates; however other varieties of siderophore structures have been resolved which include oxazoline, thiazoline, hydroxypyridinone, α- and β-hydroxy acids and α–keto acid components. The most important property of siderophores is their denticity (number of iron coordinating atoms per molecule) which ranges from bidentate to hexadentate (Raymond and Dertz 2004). The peptide backbone of siderophores is usually made of several nonprotein amino acid analogs including both modified and D-amino acids. Some bacteria produce one type of siderophore while many produce multiple types of siderophores-permitting microorganisms to grow in different environments.

2.1.3 Marine Siderophores

Marine bacteria are known to require micromolar concentration of iron. However, iron concentration in the surface waters of the oceans is only 0.01–2 nM (Sandy and Butler 2009). Therefore, many of the marine bacteria belonging to alpha and gamma proteobacteria produce siderophores. Marine siderophores can be structurally categorized into two major groups: (1) Siderophores that are produced as suites of amphiphiles and differ in the chain length of a fatty acid appendage (Homann et al. 2009; Ito and Butler 2005; Martin et al. 2006; Martinez et al. 2003) and (2) siderophores that are produced with an α-hydroxy carboxylic acid moiety, which is photoreactive when coordinated with Fe^{+3} (Barbeau 2006; Barbeau et al. 2001, 2002, 2003; Kupper et al. 2006; Hickford et al. 2004). The aquachelins, marinobactins, ochrobactins and synechobactins coordinate Fe^{+3} by both oxygen atoms of each hydroxamate group and both oxygen atoms of the α-hydroxy carboxylate group. The amphibactins coordinate Fe^{+3} with the three hydroxamate groups.

The alterobactins and pseudoalterobactins coordinate Fe^{+3} via the two β-hydroxy aspartate moieties and one catecholate group, whereas petrobactin and petrobactin sulfonate coordinate Fe^{+3} with the two catecholates and the α-hydroxy acid portion of the citrate backbone.

The most characteristic and distinguishing feature of marine siderophores is their photoreactivity (Barbeau et al. 2001). Siderophores that contain α-hydroxycarboxylate moiety when in complex with Fe^{+3} undergo oxidation and Fe^{+3} is reduced to Fe^{+2}. Fatty acid-containing marine siderophores have the important property of amphiphilicity, the degree of which varies in marine siderophores. Such variations in amphiphilicity arise due to the differences in the head-group composition relative to the fatty acid chain length. Amphibactins and ochrobactins are highly hydrophobic and are extracted from the bacterial pellet (Martin et al. 2006; Martinez et al. 2003) whereas aquachelins are isolated from aqueous supernatant which indicates their hydrophilic nature. Synechobactins are isolated from the supernatant as well as the pellet (Ito and Butler 2005). Hydrophobic amphibactins have smaller peptide portion consisting of four amino acids while fatty acid chains are longer. Marinobactins and aquachelins have longer peptide chain consisting of 6–7 amino acids and a shorter fatty acid chain. The ochrobactins are also quite hydrophobic as a result of two fatty acid appendages, and a small head group, whereas the synechobactins, with a similarly small head group, are less hydrophobic than the ochrobactins as they have only one fatty acid (Vraspir and Butler 2008).

Other marine organisms that have been studied for siderophore production are: *Marinobacter hydrocarbonoclasticus* (petrobactin), *M. aquaeolei* (petrobactin sulfonate(s)), *Aeromonas hydrophila* (amonabactins) and fish pathogens such as *Vibrio anguillarum*, which produces vanchrobactin and anguibactin. Petrobactin produced by *M. aquaeolei* is different from the petrobactin produced by *B. anthracis* in that it is a mono or di sulphonated derivative. Sulphonation of the catechol group has also been observed in pseudoalterobactin which is structurally related to alterobactin (Sandy and Butler 2009).

2.2 Factors Affecting Siderophore Production

2.2.1 Iron Concentration

Iron concentration is the most important factor which regulates siderophore production, since they are produced under iron-limiting conditions (Budzikiewicz 1993; Vasil and Ochsner 1999; Visca et al. 1993). Iron availability depends upon the environment in which the microorganism is growing. In an intensively aerated liquid medium, iron exists mainly in the form of Fe^{+3} which is extremely insoluble at neutral pH. Thus, the increase of oxygen pressure in the culture broth can reduce iron availability (Kim et al. 2003). With laboratory culture media, the amount of siderophores produced by iron-sufficient growth can be as little as 0.1% of that

produced under iron-deficient conditions. It is therefore necessary to remove the traces of iron from the culture medium for a successful production of siderophores (Messenger and Ratledge 1985).

The influence of iron on siderophore production has been studied by many authors (Braud et al. 2006; Ochsner et al. 2002). Studies carried out with different *Pseudomonas* strains confirm that siderophore production is associated with iron concentration in the medium. Siderophore production is suppressed at Fe^{+3} concentration of 100 µM in *Pseudomonas fluorescens* (VTE94558), *P. fluorescens* (VTT/ELT 116) and *Pseudomonas chlororaphis* (VTT-E-94557) (Laine et al. 1996). Iron concentrations of about 10 µM are considered high enough and generally result in excellent cell-mass accumulation with only modest yields of siderophores (Neilands 1984); even so, *P. fluorescens* 94 produces siderophores at an iron concentration of 50 µM (Manninen and Mattila-Sandholm 1994). On the other hand, siderophore production in *Pseudomonas syringae* B30ID is gradually repressed at Fe^{+3} concentrations from 1 to 10 µM (Bultreys and Gheysen 2000). Villegas et al. (2002) have observed that cell growth of *Pseudomonas aeruginosa* PSS reaches a maximum at 10 µM Fe^{+3}, however, the biosynthesis of siderophores is lowered at this concentration, since cell growth and siderophore production are inversely proportional under such conditions. *P. aeruginosa* NCCB 2452 and ATCC 15692 produce the siderophores pyoverdine and pyochelin. The concentration of these was found to increase in iron-limited culture, although the level of pyochelin was ten times lower than that of pyoverdine (Kim et al. 2003).

2.2.2 Nature of Nitrogen and Carbon Source

Nitrogen is one of the major components of important cellular elements including proteins, nucleic acid and cell wall. Amino acids are particularly good sources of nitrogen, generally inducing an increased growth rate. Glutamic acid as the sole source of carbon and nitrogen has been reported to improve the production of siderophores (Casida 1992). Villegas et al. (2002) have studied siderophore production by *P. aeruginosa* PSS in a glutamic minimum medium with glutamic acid as the sole carbon source and siderophore production as high as 140 µM has been obtained. Different nitrogen sources have been reported for siderophore production including the supplements of other amino acids (Albesa et al. 1985). Bultreys and Gheysen (2000) have reported high siderophore production in strains of *P. syringae* with all the 20 amino acids when used as the sole source of both carbon and nitrogen.

Another amino acid commonly present in such media is asparagine, which is used as carbon and nitrogen source by almost all fluorescent *Pseudomonas* (Palleroni 1984). Solid and liquid culture media containing asparagine are reported to be highly effective for the induced production of siderophores by strains of *P. syringae* (Bultreys and Gheysen 2000). This amino acid is usually combined with sucrose in the medium known as sucrose-asparagine (SA) for the production of siderophores in *Pseudomonas* (Laine et al. 1996; Morris et al. 1992).

Siderophore production can also be achieved with several organic substrates (Meyer and Abdallah 1978). Glycerol used as the carbon source in different media (Nowak-Thompson and Gould 1994), for example, the King's medium B (King et al. 1954), stimulates pyoverdine production. Duffy and Defago (1999) have reported increased pyochelin production in *P. fluorescens* CHAO with glucose but not with glycerol. However, salicylic acid production was found to increase significantly with glycerol. Use of sodium succinate has also been reported for increase in siderophore production (Boruah and Kumar 2002; Meyer and Abdallah 1978; Sharma and Johri 2003).

2.2.3 Metal Ions

Metals other than iron also stimulate or inhibit siderophore production in a number of bacteria, even in the presence of high iron concentrations. For example, Duhme et al. (1998) have studied the effect of molybdenum on siderophore production by *Azotobacter vinelandii* and found that at concentrations upto 100 mM of molybdenum, azotochelin production is activated, whereas at higher metal concentrations the synthesis of the siderophore is completely repressed. Reports also show that high concentrations of aluminium increase the production of schizokinen and *N*-deoxyschizokinen (two hydroxamate siderophores) in iron-limited cultures, but not in iron-rich cultures of *Bacillus megaterium* (Hu and Boyer 1996). Pyoverdine production is induced by 10 mM aluminium, copper, gallium, manganese and nickel when grown in the iron-limited succinate medium (Braud et al. 2009a). Teitzel et al. (2006) have made an interesting observation wherein under iron-limited conditions, exposure to 10 mM copper upregulates genes involved in the synthesis of pyoverdine and downregulates those involved in the synthesis of pyochelin. Increase in pyoverdine even in the presence of iron (100 mM) has been reported by Braud et al. (2010) when 10 and 100 mM copper and nickel (290 and 380 %, respectively) are added in growth medium. In all these experiments, metals were added at the beginning of the cultures. Most of these studies have been carried out with only a single or in some cases two metal concentrations and not for a large range of concentrations. It is probable that as iron concentration regulates siderophore production, it can be both activated and inhibited by many of these metals, depending on their concentrations. Therefore, studies involving varied concentrations of each metal are necessary to establish their exact roles in the regulation of siderophore production.

It is not clear how metals other than iron stimulate siderophore production. One possible explanation is that the free siderophore concentration in the medium is reduced in the presence of other metals as a result of complex formation. Such a decrease in the siderophore concentration may be sufficient to activate further secretion of additional siderophore into the medium. Induction of siderophore production by heavy metals indicates their role in bacterial heavy metal tolerance. Toxic metals enter the periplasm of Gram-negative bacteria mostly by diffusion across the porins (Li et al. 1997; Lutkenhaus 1977; Pugsley and Schnaitman 1978). The binding of

metals to siderophores in the extracellular medium reduces the free metal concentration, affecting diffusion (the molecular mass of the resulting siderophore–metal complex is too great for diffusion via porins) and therefore their toxicity. Growth assays have also shown that *P. aeruginosa* strains capable of producing pyoverdine and pyochelin are more resistant to metal toxicity than a siderophore nonproducing strain (Braud et al. 2010).

The presence of siderophores is apparently highly beneficial for bacteria in an environment contaminated with toxic metals and bacteria able to produce siderophores are more resistant to heavy metals than those not producing siderophores. Though siderophores provide an extracellular protection for bacteria by sequestering heavy metals outside the bacteria and avoiding their diffusion through porins into the bacteria, however, it is possible that siderophore production in response to heavy metal exposure can also have detrimental effects. If the siderophore uptake receptor does not distinguish between the metal–siderophore and the ferri–siderophore complexes, siderophores may provide a secondary mechanism for the uptake of toxic metals. Therefore, the ferri–siderophore pathways must have high metal specificity to transport or accumulate only the appropriate metal(s).

2.3 Occurrence of Siderophore-Producing Bacteria in Coastal Ecosystems of Goa

During our study on siderophore-producing bacteria from coastal ecosystems of Goa, such bacteria were isolated from two distinct ecosystems, one, a low-nutrient sand dune ecosystem and the other a nutrient-rich mangrove ecosystem. Sand dunes represent large amounts of shifting sand barren to plants. Sand dunes are largely categorized into two types. The first kinds are extremely dry interior deserts such as Sahara in Africa or Rajasthan in India. Coastal sand dunes occur along the coasts of the Atlantic, Pacific, North America and Australia. In Asia, coastal sand dunes occur in Japan, India and several other countries (Boorman 1977; Carter 1998; Desai and Untawale 2002). Vegetation plays an important role in determining size, shape and stability of the dunes. Dead plants and humus from sand dune vegetation adds humus to the sand. Furthermore, microorganisms growing in these sand dunes help reduce the nutrient stress.

Mangroves are highly reproductive ecosystems which host a wide range of coastal and off-shore marine organisms. Mangroves provide a unique ecological environment for diverse bacterial communities (Ramanathan et al. 2008). Tidal variations, salinity and intense human activities such as transportation through ships, barges and accidental spills result in diverse microflora in the marine coastal ecosystem (Rawte et al. 2002). Bacteria largely influence nutrient cycling in mangroves and thus contribute to soil and vegetation patterns (Hossain et al. 2012). Environments wherein microorganisms grow are highly variable with respect to temperature, pH,

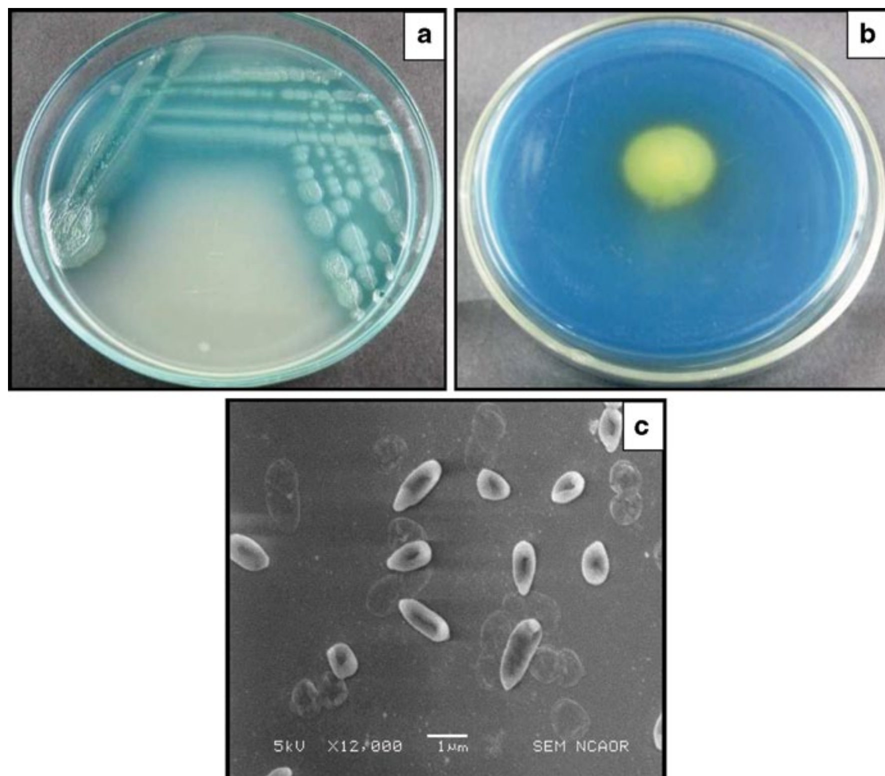

Fig. 2.1 *Pseudomonas aeruginosa* TMR2.13. **a** Colony morphology. **b** Siderophore production on a chrome azurol sulfonate (CAS) agar plate. **c** Electron microscopic view

redox potential, osmotic pressure, water activity, salinity and general and essential nutrients. Previous studies have shown the presence of a large number of bacteria in these two ecosystems (Godinho and Bhosle 2002).

During our study, 13 siderophore positive isolates from sand dunes and mangrove which showed the optimum siderophore production were subjected to routine biochemical tests and 16S rDNA sequencing for tentative identification. Of the thirteen isolates selected, ten belonged to the *Bacillus* spp, two were *Streptomyces* spp. and one was identified as *P. aeruginosa* (Gaonkar et al. 2012).

Amongst the many siderophore-producing organisms, the two isolates TMR2.13 (*P. aeruginosa*) (Fig. 2.1a, b, c) and NAR38.1 (*Bacillus amyloliquefaciens*) (Fig. 2.2a, b, c) were selected to study various aspects of siderophore production. Functional group tests proved that the siderophores produced by TMR2.13 and NAR38.1 were of carboxamate and catecholate type, respectively.

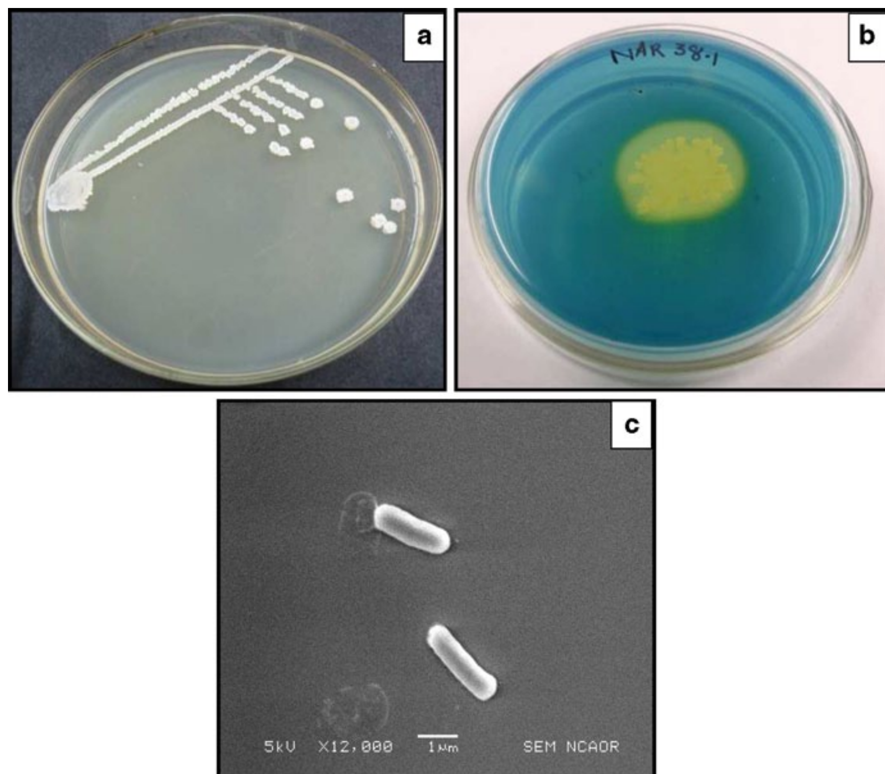

Fig. 2.2 *Bacillus amyloliquefaciens* NAR38.1. **a** Colony morphology. **b** Siderophore production on a CAS agar plate. **c** Electron microscopic view

2.3.1 Effect of Iron on Siderophore Production

Iron is one of the most important micronutrients required by bacteria for their metabolism as a cofactor for a large number of enzymes and iron-containing proteins (Harrington and Crumbliss 2009). However, its availability in the environment may not be sufficient to support microbial growth. Studies with NAR38.1 showed the production of siderophore up to 1 μM of Fe^{+2} and up to 30 μM of Fe^{+3}, suggesting that higher levels of Fe^{+3} are required to suppress siderophore production as compared to the Fe^{+2}. This could possibly be because of the well known fact that Fe^{3+} form is not the highly soluble form of iron as opposed to Fe^{2+} ion which has a much better solubility (Chu et al. 2010). However, the effect of iron with *P. aeruginosa* TMR2.13 showed a significant siderophore production up to 54 μM which was suppressed at 108 μM irrespective of it being divalent or trivalent. The fact that pyoverdine binds to both the forms of Fe, as has been reported earlier (Xiao and Kisaalita 1998), results in the suppression of siderophore production with the same concentration of Fe^{+2} and Fe^{+3}.

2.3.2 Effect of Sodium Benzoate on Siderophore Production

Pseudomonas is known to be versatile in its ability to metabolize various organic compounds. A number of natural and anthropogenic compounds are known to be degraded, transformed or cometabolized by organisms belonging to this genus (Zeyaullah et al. 2009). Many of the reactions involve the enzyme oxygenase which is the key enzyme involved in the opening of the aromatic ring structure.

Oxygenases are enzymes which catalyse the incorporation of molecular oxygen into various substrates. The cofactors involved in oxygenase reactions are heme, nonheme iron, copper, flavin etc. However, the most frequent one is iron. Nearly 100 oxygenases are known till date, of which almost 50 % have iron built into their structure or require iron for their activity (Nozaki and Ishimura 1974). When a benzene ring is cleaved by a dioxygenase reaction, hydroxylation of the benzene ring proceeds with the formation of catechol or phenol derivatives (Song et al. 2000). Catechol derivatives are further cleaved by dioxygenases which are further divided into intradiol and extradiol. Intradiol is red in colour and contains trivalent iron while extradiol enzymes are colourless containing divalent iron (Nozaki and Ishimura 1974). Hence, metabolism of aromatic compounds is expected to impose a specific iron requirement on cells due to the involvement of oxygenases. An attempt was therefore made in this study to understand the effect of the aromatic compound, sodium benzoate, on siderophore production in the organisms' capability of utilizing sodium benzoate as the sole source of carbon.

Sodium benzoate was found to have a remarkable effect on siderophore production in *P. aeruginosa* TMR2.13. It was noted that while the production of siderophore was inhibited above 54 μM of added iron in Mineral salts medium (MSM) with glucose without affecting growth, in the presence of sodium benzoate, siderophore was produced even up to the presence of 108 μM of added iron (Gaonkar et al. 2012).

A report on the effect of trace element requirement has shown that the iron demand in bacteria increases during the expression of alkane hydroxylase (Staijen and Witholt 1998). A study on the effect of iron concentration on degradation of toluene by *Pseudomonas* strain reports a reduction in the efficiency of the culture when the iron concentration is low (Dinkla et al. 2001). Observations from this study also suggest the requirement of a higher concentration of iron to sustain growth in a benzoate medium.

2.3.3 Effect of Metal Ions on Siderophore Production

Studies further carried out with *B. amyloliquefaciens* NAR38.1 to understand the effect of the presence of both biotic and abiotic metals in the growth medium, depicted following four responses (Gaonkar and Bhosle 2013):

1. In the presence of zinc, siderophore production increased, but no effect was seen on the growth while the presence of cobalt and manganese increased siderophore production with decrease in growth.

2. In the presence of molybdenum and arsenic, siderophore production decreased, but there was no effect on growth.
3. Decrease in siderophore production at lower concentrations but increase at higher concentration without affecting growth was manifested in the presence of lead and aluminium.
4. Decrease in growth as well as siderophore production was brought about by the presence of cadmium and copper.

Such effects indicate that the potential of the organism to combat metal toxicity varies with the type of metal it is in contact with. Although bacteria are known to possess different mechanisms to alleviate the effects of toxic metals, the concentration of the metal plays an important role in the manifestation of such mechanisms. Production of siderophores in the presence of metals can be a useful trait for plant growth promoting organisms as the soils which are contaminated with metals are often iron deficient (Tank and Saraf 2009). In fact, siderophore-producing bacteria have been considered important for inducing metal tolerance in plants and for promotion of metal accumulation in plants, especially in the phyto-extraction technology for remediation of metal-contaminated soils (Ahmed and Holmstrom 2014). The effects of siderophore-producing bacteria on the uptake of metals by hyperaccumulator plants have been the focus of increased attention (Dimkpa et al. 2008; Braud et al. 2009b). Braud et al. (2009b) have reported an increase in the bioavailability of Chromium and Lead in soils inoculated with *P. aeruginosa*. Bacterial siderophores can also provide iron to various plants, which helps in reducing the metal toxicity (Bar-Ness et al. 1991; Hussain and Joo 2014; Reid et al. 1986; Wang et al. 1993). Such beneficial effects exhibited by siderophore-producing bacteria implicate that the inoculation with metal-resistant siderophore-producing bacteria may help in improving the process of phyto-extraction in metal-contaminated soils.

2.4 Conclusions and Future Prospects

This study has offered an insight into the response of siderophore-producing bacteria to the aromatic compound sodium benzoate and to metal ions. The present work can be extended to natural ecosystems exposed to the influx of such pollutants. Inoculation with metal-resistant siderophore-producing bacteria may help in improving the process of phyto-extraction in metal-contaminated soils.

Moreover, it would also be interesting to probe deeper into the results of the present studies and study effect of iron limitation on the enzymes involved in utilization of sodium benzoate in *P. aeruginosa* TMR2.13 and the role of siderophores in metal resistance in *B. amyloliquefaciens* NAR38.1.

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Chapter 3
Denitrifying Bacteria: Physiological Response to Hydrocarbons

Trelita de Sousa

3.1 Introduction

Denitrification is a major component of the nitrogen cycle with significant implications to coastal and marine systems. The process is a natural sink for nitrate removal in estuaries, counteracts eutrophication in coastal systems, helps maintain the marine productivity in the ocean and is crucially associated with global warming and climate change (De Sousa and Bhosle 2012c). Although, denitrification is efficiently used in municipal/industrial wastewater treatment (Zhao et al. 2009) and ground water remediation (Hunter and Shaner 2010), interestingly, its role in petroleum bioremediation has gathered much interest over the last few years (Cao et al. 2009; De Sousa and Bhosle 2012c).

Environments rich in hydrocarbons, especially coastal/estuarine systems generally also have a high nitrate load (Bae et al. 2009). These systems, being exposed to an inexorable input of anthropogenic nutrients, including petroleum hydrocarbons, are inevitably susceptible to varying oxygen fluxes (Bae et al. 2009; Cao et al. 2009). Denitrifying bacteria use nitrate as their terminal electron acceptor when oxygen is unavailable (Seitzinger et al. 2006). These ubiquitous, mostly heterotrophic organisms are essentially aerobic, but their facultative mechanism of respiration allows them to have an extensive range of habitats with varying oxygen levels as compared to other microbial groups (Bae et al. 2009; Wilson and Bouwer 1997). Because of their ubiquity, unique, flexible respiration and efficient ability to degrade a wide range of aliphatic, aromatic and polycyclic aromatic hydrocarbons, denitrifiers fashion an ideal clean-up approach towards petroleum bioremediation (Cao et al. 2009; Wilson and Bouwer 1997).

In natural environments, hydrocarbon breakdown is correlated with the functional composition of the autochthonous microbial communities and their ensuing

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adaptive response to the presence of these organic pollutants (Van Hamme et al. 2003). Denitrifying bacteria when exposed to hydrocarbons exhibit several metabolic adaptations that not only enable them to overcome the stress but also facilitate the uptake of hydrocarbons, thereby ensuring an effective degradation process (Chayabutra and Ju 2000; De Sousa and Bhosle 2012a, b). This review critically examines the response of denitrifying bacteria, indigenous to coastal ecosystems of Goa, to hydrocarbons in the hope of understanding the influence of these organic pollutants on the metabolic activities of the microorganisms for their potential application in petroleum remediation. Such a comprehensive understanding of the physiological responses in the predominant indigenous bacteria rendered by the presence of hydrocarbons would shed light on the overall productivity in natural ecosystems.

3.2 Fate of Hydrocarbons Released into the Marine Environment

Petroleum or crude oil is a complex mixture of aliphatic, aromatic and polycyclic aromatic hydrocarbons with widespread anthropogenic usage and global economic importance. However, when this commodity is callously dumped into an ecosystem, it unleashes a series of events with dire consequences to the environment. When oil enters the sea, it initially floats forming a film that blocks sunlight penetration and impedes air exchange within the system. Due to intense wind and wave action, the oil becomes weathered and spreads across a wider spectrum, simultaneously gaining access to the deeper reaches of the water column. The volatile components escape into the atmosphere while the relatively water-soluble aromatic and polycyclic aromatic hydrocarbons are leached into the water. These petroleum hydrocarbons spread throughout the marine environment, water column, bottom sediments and shoreline habitats, ensuing severe short-term and long-term effects on the vulnerable marine ecosystem, including deep-sea, coastal and estuarine systems (Ingole and Sivadas 2007; Peterson and Holland-Bartels 2002). Biodegradation of hydrocarbons by natural populations of microorganisms has long been recognized as an ideal strategy for mitigating petroleum contamination and has stimulated considerable research towards biotransformation, biodegradation and bioremediation of hydrocarbons. Numerous hydrocarbon-degrading organisms (bacteria, yeasts, fungi and algae) have been isolated, identified and characterized for potential application in petroleum bioremediation designs (Bae et al. 2009; De Sousa and Bhosle 2012c; El-Sheekh et al. 2012).

Hydrocarbons, including petroleum compounds are organic substrates that support microbial growth and microorganisms have developed well-advanced pathways to break down these otherwise recalcitrant substances (Van Hamme et al. 2003). Biodegradation of hydrocarbons proceeds via both aerobic and anaerobic processes (Cao et al. 2009; Pérez-Pantoja et al. 2010; Philipp and Schink 2011; Wentzel et al. 2007). Aerobic degradation of straight chain aliphatic hydrocarbons (alkanes) uses a membrane-bound monooxygenase together with soluble rubredoxin and rubredoxin

reductase to shunt electrons through NADH (Nicotinamide adenine dinucleotide dehydrogenase) to the hydroxylase, in order to convert the alkane to alcohol, which is then oxidized to aldehyde and acid before proceeding to the β-oxidation and tricarboxylic acid cycles (Van Hamme et al. 2003; Wentzel et al. 2007). During aerobic biodegradation of aromatic compounds (benzoate, benzene, toluene), oxygen not only acts as an electron acceptor, but is also involved as a highly reactive cosubstrate in the initial hydroxylation reactions catalyzed by mono and dioxygenases. The monooxygenases transform the benzene ring into a few central intermediates such as catechol, protocatechuate, gentisate and hydroxyl benzoquinols. These are then further cleaved by different dioxygenases to the tricarboxylic acid intermediates via the ortho or meta pathways (Cao et al. 2009; Pérez-Pantoja et al. 2010). In the case of polycyclic aromatic hydrocarbons like naphthalene, acenaphthene, anthracene, fluoranthene, pyrene and chrysene, molecular oxygen is introduced into the aromatic nucleus by multicomponent nonheme iron oxygenase enzyme systems, like the naphthalene dioxygenase, forming a dihydrodiol which then proceeds via the normal aromatic breakdown pathways (Van Hamme et al. 2003).

Although the importance of oxygen in hydrocarbon breakdown is very well apparent, it must be remembered that systems accumulating petroleum hydrocarbons are also inevitably exposed to varying oxygen fluxes (Wilson and Bouwer 1997). Hydrocarbon-polluted aquifers, aquatic sediments and submerged soils invariably become anoxic (Cao et al. 2009; Wilson and Bouwer 1997) favouring microbial processes like denitrification, iron (III) reduction, sulphate reduction and methanogenesis, capable of utilizing alternative electron acceptors such as nitrate (NO_3^-), iron (Fe^{3+}), sulphate (SO_4^{2-}) and carbon dioxide (CO_2), respectively, for intrinsic and engineered bioremediation designs (Cao et al. 2009; Pérez-Pantoja et al. 2010; Philipp and Schink 2011; Wilson and Bower 1997). The anaerobic mechanism for alkane degradation is proposed to transpire either by alkane carboxylation leading to the formation of fatty acids, as seen with sulphate reducers or by the addition of alkane to the double bond of fumarate giving succinate substituted with alkane-derived alkyl chains as in the case of denitrifiers (Wentzel et al. 2007). In the anaerobic biodegradation of aromatic compounds, the peripheral pathways catalyzed by the oxygenases converge to benzoyl-CoA (occasionally to resorcinol or phloroglucinol). Specific multicomponent energy-requiring reductases catalyze the de-aromatizing reactions (Cao et al. 2009; Philipp and Schink 2011). Polycyclic aromatic hydrocarbons are also anaerobically metabolized in a similar way via carboxylation or by the addition to fumarate (Van Hamme et al. 2003).

3.3 Why Denitrifying Bacteria?

The addition of nitrate to oil-contaminated sites has been regarded as a potential means of enhancing bioremediation efforts. The ability of denitrifying bacteria to efficiently switch between O_2 and NO_3^- as the electron acceptor, favours their proliferation in transition redox zones, where microaerophilic conditions prevail

(Van der Zaan et al. 2012; Wilson and Bouwer 1997). In terms of energy acquisition, NO_3^- is the preferred electron acceptor because its yield is close to that of O_2 compared to Fe_3^+, SO_4^{2-} and CO_2 (Wilson and Bouwer 1997) and gives the highest rates for anaerobic degradation of benzene in comparison to SO_4^{2-}, chlorate and Fe^{3+}(Van der Zaan et al. 2012).

Degradation, especially of the aromatic compounds like benzoate and toluene by denitrifying bacteria is enhanced by the presence of readily consumable carbon sources like succinate and acetate (Kariminiaae-Hamedani et al. 2004; Martínez-Hernández et al. 2009). Chénier et al. (2003) also reported the enhancement of hexadecane mineralization and denitrification with nutrient amendment through the addition of glucose, ammonium chloride and phosphorous (K_2HPO_4). Denitrifying bacteria utilize a wide range of hydrocarbon substrates, including alkanes, toluene, xylene, phenols, cresols, phthalate, cyclohexanol, benzenes (including halobenzenes), benzoate and other aromatic acids, alcohols, aldehydes and polycyclic aromatic hydrocarbons (Chayabutra and Ju 2000; Shinoda et al. 2004; Zhang et al. 2011).Hydrocarbon-degrading denitrifying bacteria encompass several genera such as *Pseudomonas, Azoarcus, Thiosphaera, Thauera*, *Rhodopseudomonas, Bradyrhizobium, Ochrobactrum, Paracoccus, Mesorhizobium, Ensifer* and *Acidovorax* (Chayabutra and Ju 2000; De Sousa and Bhosle 2012c; Shinoda et al. 2004; Song and Ward 2005). Research on *Azoarcus vansii*, *Pseudomonas aeruginosa* and *Thauera aromatica* have led to substantial mechanistic advances in understanding the anaerobic degradation of hydrocarbons (Schmeling and Fuchs 2009; Song and Ward 2005).

Denitrifying bacteria possess highly evolved mechanisms that enable them to use aerobic degradation pathways to metabolize hydrocarbons in the presence of molecular oxygen and switch to nitrate respiration when oxygen levels diminish. (Pérez-Pantoja et al. 2010; Wilson and Bouwer 1997). Chayabutra and Ju (2000) proposed such a sequential hydrocarbon bioremediation strategy for the degradation of hexadecane by *P. aeruginosa*. The initial oxygen-requiring transformation of the alkane was performed using aerobic resting cells in order to reduce the oxygen demand and the oxygenated metabolites were degraded even under anaerobic conditions using nitrate as the alternative electron acceptor. Shinoda et al. (2004) also elucidated a similar mechanism in a denitrifying *Thauera* sp. growing on toluene. The initial degradation of toluene occurred through a dioxygenase-mediated pathway in the presence of oxygen and by the benzyl-succinate pathway under denitrifying conditions. Such mechanisms also exist in *Azoarcus* (Song and Ward 2005).Denitrifying bacteria can thus utilize hydrocarbons under both aerobic and anaerobic conditions by suitably modifying their pathways in adaptation to the availability of oxygen in the environment.

3.4 Prevalence of Denitrifying Bacteria in Coastal Ecosystems of Goa

The Arabian Sea contains one of the major denitrification zones in the world (Naqvi et al. 2006). Goa, located on the west coast of India, lies along the oil tanker route which is accident-prone due to its proximity to two major oil choke points of the

world—Strait of Hormuz and Strait of Malacca. Because of its precarious location, Goa has already succumbed to some major oil spills in recent times (Sivadas et al. 2008). The coastline of Goa is evidently highly vulnerable to oil pollution (Ingole and Sivadas 2007; Sivadas et al. 2008), and this greatly influences the dynamics within the coastal and estuarine systems in the state through the constant tidal action (Shetye et al. 1995).

The rivers of Mandovi and Zuari have their origin in the Western Ghats of India and traverse the entire breadth of Goa, meandering for about 75 and 70 km, respectively, before emptying into the Arabian Sea. The rivers are connected by a narrow channel called the Cumbarjua canal. The width at the mouth of the Mandovi and Zuari is approximately 3.2 and 5.5 km, respectively, and the estuaries formed experience strong tidal variations (De Sousa et al. 2013; Shetye et al. 1995). These estuaries, each about 50 km long and 5 m deep (Shetye et al. 1995), are fringed on either side by luxuriant mangroves. The rivers provide an excellent and extensive water route efficiently accommodating a heavy movement of barges (carrying iron and manganese ore), boats and passenger ferries besides several water sports and fishing activities. Several jetties, wharfs, docks and boat-repair workshops are located all along the two estuaries. The rivers also flow along fertile agricultural lands that support rice cultivation and horticulture, activities that inevitably involve the abundant use of nitrate-based fertilizers. The banks of the estuarine channels harbour vast human and industrial settlements. These anthropogenic undertakings allow an influx of nutrients, including hydrocarbons and nitrates into the coastal/estuarine ecosystems ensconced by the Mandovi and Zuari rivers (De Sousa 1983; De Sousa et al. 2013; Maya et al. 2011; Sardessai and Sundar 2007).

Reports have indicated that denitrification activity is found to be higher in nutrient-rich systems such as the Mandovi and Zuari estuaries (De Sousa et al. 2013; Sardessai and Sundar 2007). These ecosystems showed a proliferation of nitrate-reducing and denitrifying bacteria attributed to the abundant availability of organic compounds, including hydrocarbons, the constant oxygenic fluxes and the profuse influx of nitrates from mining activities, surrounding paddy fields and atmospheric precipitation (De Sousa et al. 2013). Amongst the many denitrifying bacterial cultures isolated from these systems are two hydrocarbon-utilizing strains, *Pseudomonas nitroreducens* TSB.MJ10 (De Sousa and Bhosle 2012b) and *P. aeruginosa* TMR2.13 (De Sousa and Bhosle 2012a; Gaonkar et al. 2012).

P. nitroreducens TSB.MJ10, isolated from the mangroves at Merces (Fig. 3.1a, b) in the vicinity of a petroleum pump, showed interesting characteristics like polyhydroxyalkanoate production, feruloyl esterase activityand siderophore formation (Fig. 3.2a, b, c), besides exhibiting strong and stable hydrocarbon-emulsifying activity (Fig. 3.3a, b, c) which has been rarely explored in this species (De Sousa and Bhosle 2012b). Its ability to utilize a wide range of hydrocarbons, including hexadecane, sodium benzoate, 1-naphthol, crude oil and weathered crude oil as sole sources of carbon can be significantly capitalized on, in hydrocarbon bioremediation studies. *P. nitroreducens* is an aerobic denitrifying bacterial species affiliated to the *P. aeruginosa* subgroup (Anzai et al. 1997) and was first isolated from oil brines in Japan (Iizuka and Komagata 1964). It is ubiquitously found in oil-contaminated

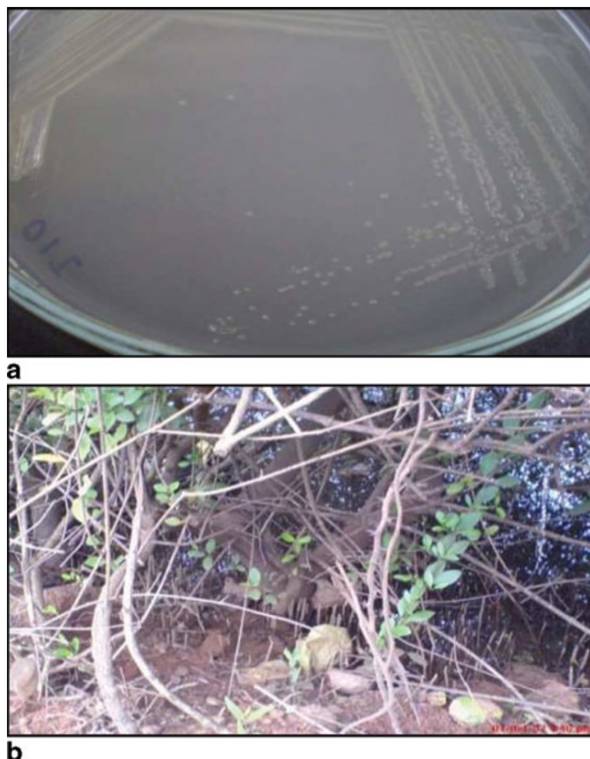


Fig. 3.1 **a** *Pseudomonas nitroreducens* TSB.MJ10 isolated from **b** the mangrove ecosystem in the backwaters of River Mandovi at Merces, Goa

systems (Zhang et al. 2010). Although the work on this species is limited, previous findings have attributed robust characteristics to it, such as production of polyhydroxybutyrate, degradation of surfactants, herbicides and insecticides and biotransformation of eugenol and isoeugenol to vanillin (De Sousa and Bhosle 2012b).

P. aeruginosa strain TMR2.13, isolated from the sand-dunes at Miramar (Gaonkar et al. 2012) (Fig. 3.4a, b), showed luxuriant growth between 0.1 and 1.5 % and tolerated up to 3 % benzoate (Fig. 3.5) (De Sousa and Bhosle 2012a). *P. aeruginosa* is a well-documented denitrifying bacterium (Brenner et al. 2005; Chayabutra and Ju 2000) and is known to play a typically active and functional role as a denitrifier (De Sousa and Bhosle 2012a). This ubiquitous species has unique and attractive pigment characteristics and an ability to transform a wide variety of natural and unnatural organic compounds, including aliphatic, aromatic and polycyclic aromatic hydrocarbons (Chayabutra and Ju 2000; De Sousa and Bhosle 2012a; Zhang et al. 2011).

The persistent accumulation of hydrocarbons in a system, especially the aromatics, will inevitably render an effect on the important physiological activities of the autochthonous microbial communities (Vázquez et al. 2009), such as membrane transport (Sikkema et al. 1995), denitrification (Gilbert et al. 1997) and pigment production (Domínguez-Cuevas et al. 2006). Such significant physiological changes can consequently influence the overall productivity of the marine ecosystem

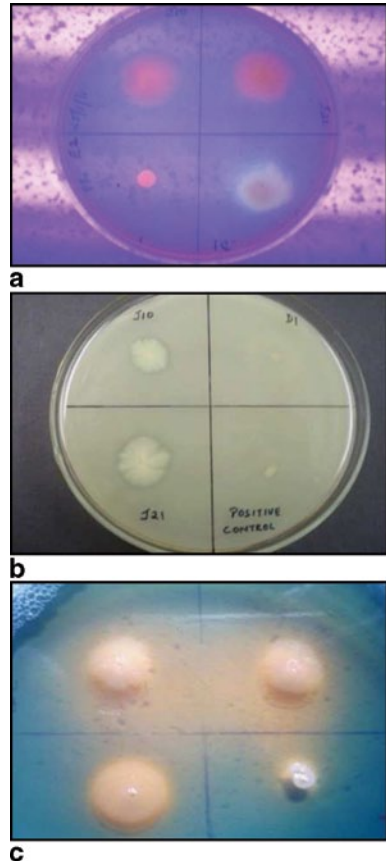


Fig. 3.2 **a** Orange fluorescence indicating polyhydroxy alkanoate production. **b** Zone of clearance due to presence of feruloyl esterase activity. **c** Yellow halo formation on chrome azurol-S (CAS) agar depicting siderophore production

(Zehr and Ward 2002). These metabolic changes in the organisms which manifest in terms of altered growth, morphology and production of extracellular products like biosurfactants, exopolysaccharides and pigments, facilitate their adaptability and survival (De Sousa and Bhosle 2012a, b) and will in turn affect the overall degradation of hydrocarbons (El-Sheekh et al. 2012). Therefore, in order to generate an effective bioremediation design employing indigenous denitrifiers, it is important to understand the effect of such hydrocarbons on their physiological processes.

3.5 Effect of Hydrocarbons on Growth and Cell Morphology

Cells exposed to an influx of hydrocarbons are reported to undergo changes in the structure and permeability of the cell membrane (Sikkema et al. 1995). This helps them overcome the toxicity or adapt to the altered carbon source. These effects

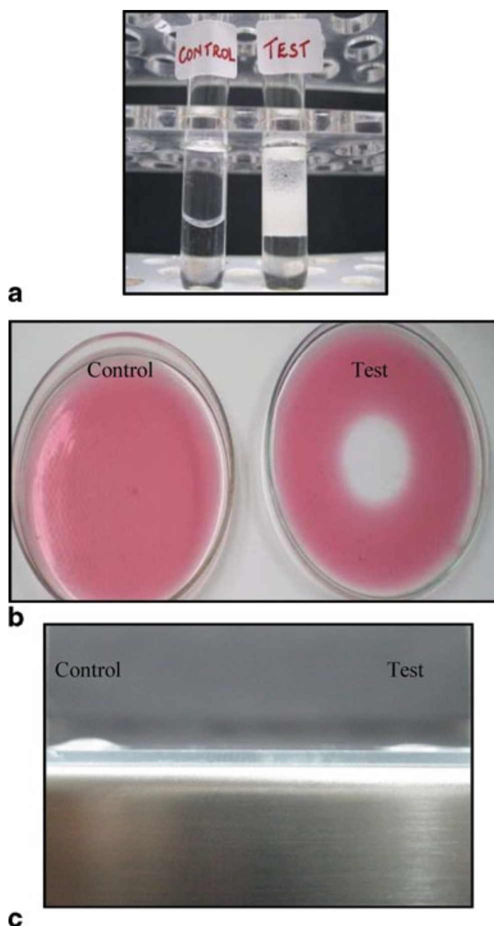


Fig. 3.3 Surface activity exhibited by TSB.MJ10. **a** Emulsification activity. **b** Oil displacement. **c** Drop collapse

are invariably reflected on the cell morphology in terms of size reduction, destructive openings, wrinkling and other deformations (Domínguez-Cuevas et al. 2006). Cells of TMR2.13 were also prominently reduced in size in the presence of aromatic hydrocarbons (De Sousa and Bhosle 2012a) suggestive of the adaptive response of the culture to the presence of hydrocarbons (Neumann et al. 2005). On the other hand, the presence of benzoate depicted effective aggregation and clustering of cells of TSB.MJ10 (Fig. 3.6a, b) (De Sousa and Bhosle 2012b). Cell aggregation is favoured by the production of surface-active compounds like biosurfactants and/or bioemulsifiers that facilitate cell-to-cell communication, thereby promoting microbial interactions, quorum sensing, biofilm formation and swarming motility (Franzetti et al. 2012).

Furthermore, TSB.MJ10 showed enhanced growth with sodium benzoate than with glucose, a relatively simpler carbon source irrespective of whether the cells were previously exposed to the hydrocarbon or not. Preferences for complex

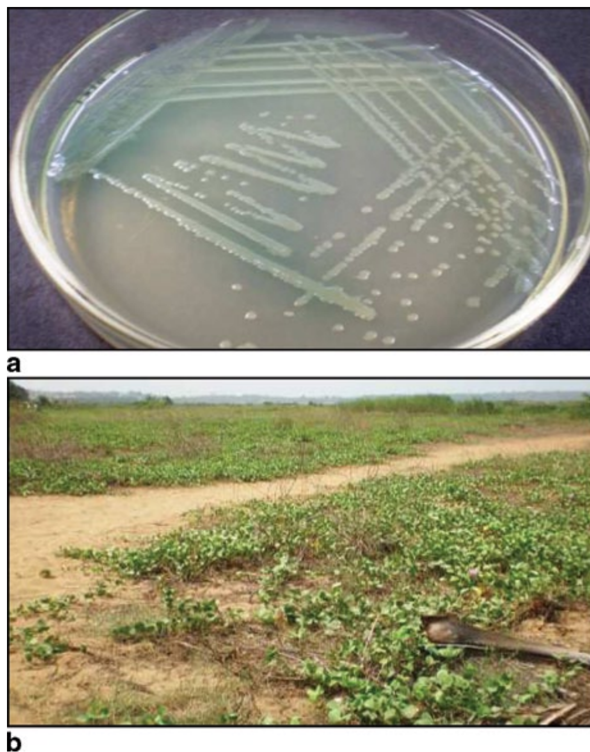


Fig. 3.4 **a** *Pseudomonas aeruginosa* TMR2.13 isolated from **b** the sand dune ecosystem at Miramar beach, Goa

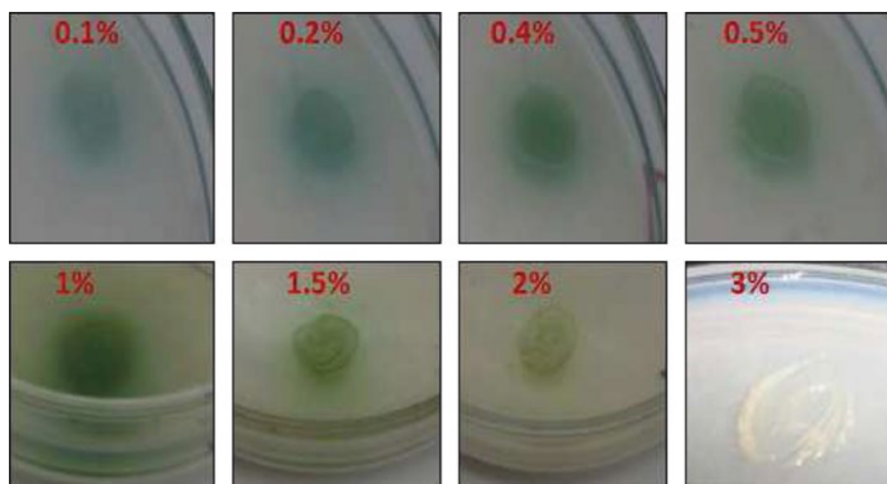


Fig. 3.5 Growth and pigmentation exhibited by TMR2.13 with increasing concentrations of sodium benzoate

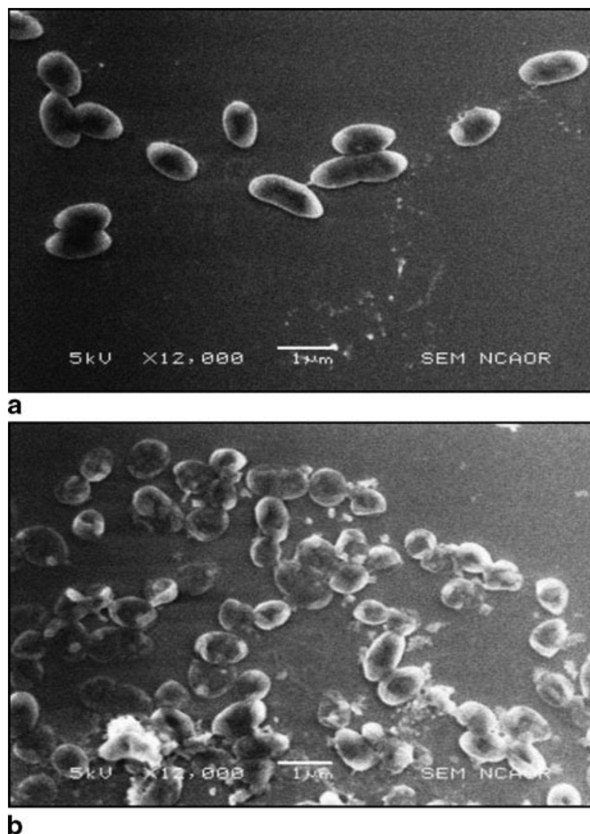


Fig. 3.6 Scanning electron micrographs of TSB.MJ10 grown in a mineral salts medium with 0.5 % **a** glucose or **b** sodium benzoate as the sole carbon source

compounds over simpler counterparts have been previously reported in *Pseudomonas putida* strains. Reber and Kaiser (1981) showed that a strain of *P. putida* showed better and faster growth in benzoate than in glucose. Recently, Basu et al. (2006) reported the preferential utilization of aromatic compounds over glucose in *P. putida* wherein the naphthalene-degrading organism exhibited a diauxic growth pattern with utilization of the aromatic in the first log phase and of glucose in the second log phase. Basu and Phale (2008) also described the preferential utilization of salicylate, benzyl alcohol and methylnaphthalene over glucose by the naphthalene-degrading *P. putida* strain with the possible transfer of the preferential degradation property by conjugation. The occurrence of such a phenomenon of preferential utilization of aromatic compounds over simpler sugars may be attributed to the possible suppression of glucose utilization enzymes by organic acids produced during the degradation of the hydrocarbon or due to the inability of glucose to suppress aromatic-degrading enzymes (Basu et al. 2006).

3.6 Influence of Hydrocarbons on Denitrification

Gilbert et al. (1997) reported that hydrocarbons either stimulate or inhibit denitrification depending on their concentration and correlated it with several factors like availability of organic compounds and nitrate. However, this stimulation or inhibition of denitrification has more to do with the oxygen requirement during degradation of the hydrocarbons, especially in the case of aromatic compounds. This can be explained by citing the example of *P.aeruginosa* TMR2.13 which degrades sodium benzoate via the aerobic ortho mode of ring cleavage (β-ketoadipate pathway) mediated by catechol 1,2-dioxygenase (De Sousa and Bhosle 2012a). The culture, therefore, needs oxygen for this enzymic reaction (Wang et al. 2006) resulting in oxygen depletion during growth, which favours denitrification. However, the presence of the aromatic compound puts an excessive demand on the oxygen requirement of the aerobic culture, which increases progressively with the increase in the hydrocarbon concentration. This limits the growth of the culture and subsequently delays its denitrification activity. Notably, aeration at 100 rpm simulates ideal conditions for growth and denitrification to occur simultaneously (De Sousa and Bhosle 2012a).

Furthermore, increasing benzoate levels caused a prominent decrease in transitional nitrite levels while increasing hexadecane levels advanced the rate of nitrate reduction inducing a gradual increase in intermediate nitrite levels during growth of TMR2.13 (De Sousa and Bhosle 2012a). Her and Huang (1995) also reported a similar decrease in intermediate nitrite levels in the presence of aromatic compounds in a batch reactor system. Brezonik (1977) explained that benzoate during denitrification gets converted to nitrosobenzoate followed by the release of nitrogen gas as a consequence of tautomerism. This phenomenon induces a decrease in intermediate nitrite accumulation in the presence of the aromatic hydrocarbon. Accordingly, as the benzoate concentration is increased in the medium, the nitrite formed binds to the excess benzoate available, thereby preventing its accumulation in the medium (De Sousa and Bhosle 2012a). Hexadecane on the other hand, when added to a liquid medium, forms an immiscible layer at the surface which impedes air penetration resulting in scarcity of oxygen levels available for the culture, thus instigating the denitrification pathway of the culture at a faster rate than otherwise (Wilson and Bower 1997). Such a mechanism could find relevance in alleviating nitrite toxicity in natural ecosystems contaminated with aliphatic and aromatic hydrocarbons as well as excessive nitrates.

3.7 Production of Extracellular Metabolites in Response to Hydrocarbons

Microbial communities are dynamic and are greatly influenced by the metabolic activities of the individual organisms. Thus, factors like nutrient availability and oxygen concentration which affect their composition profile would invariably cause

shifts in their overall functioning and response to environmental stresses such as hydrocarbon contamination (Norman et al. 2004). This is why, in any given bacterial community, cells growing in association with each other generally produce extracellular metabolites which are essential to both, the individual bacterium and the entire microbial population, and therefore boost their survival (Vignesh et al. 2011). So also, denitrifying bacteria, frequently isolated from petroleum-contaminated sites, produce several extracellular substances, including surface-active compounds, bioactive metabolites and pigments that enhance individual competitiveness and overall community functioning (De Sousa and Bhosle 2012a, b; Norman et al. 2004; Pierson and Pierson 2010).

3.7.1 Surface-Active Compounds

The low solubility of hydrocarbons in water makes it essential for bacteria to come in direct contact with the hydrocarbon substrate for effective breakdown. This may be facilitated by the production of amphiphilic substances by the bacteria called biosurfactants which may be produced on microbial cell surfaces or excreted extracellularly (Makkar et al. 2011). These potent surface-active agents contain hydrophobic (unsaturated or saturated fatty acids) and hydrophilic (amino acids or peptides; mono, di or polysaccharides) moieties that reduce surface tension and interfacial tension between individual molecules at the surface and the interface of the immiscible liquids (Makkar et al. 2011; Vignesh et al. 2011). Biosurfactants thereby expedite the degradation of hydrocarbons by enhancing their diffusion into the bacterial cell via dispersion and/or emulsification (Anyanwu et al. 2011).

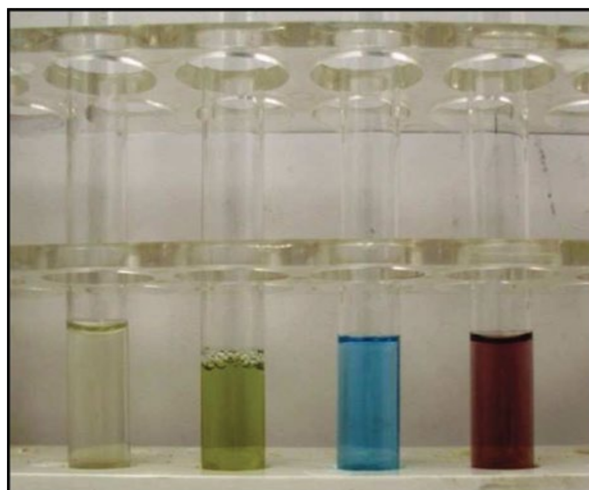
Bioemulsifiers are a sub-class of biosurfactants that stabilize dispersions of one liquid in another by inducing stable emulsions (De Sousa and Bhosle 2012b; Nerurkar et al. 2009). Bioemulsifiers do not necessarily reduce surface tension, but because these are, in any case, surface-active compounds that form stable emulsions between liquid hydrocarbons and water mixtures, they are also often referred to as biosurfactants (Franzetti et al. 2012). Biosurfactants encompass structurally distinct molecules like glycolipids, lipopeptides, lipolysaccharides, polysaccharide-protein complexes, lipoprotein, phospholipids, fatty acids, lipids, polymeric biosurfactant and particulate biosurfactants (Makkar et al. 2011; Vignesh et al. 2011). Bioemulsifiers are mostly low-molecular-weight compounds like lipopeptides and glycolipids while bioemulsans are high-molecular-weight emulsifiers which include polymers of polysaccharides, lipopolysaccharides, proteins or lipoproteins (Franzetti et al. 2012). Lipopeptides are known to have better surface-active properties than glycolipids (Janek et al. 2010) and include some of the most efficient biosurfactants, such as surfactin produced by *Bacillus subtilis*, viscosin produced by *Pseudomonas fluorescens*, arthofactin produced by *Arthobacter* sp., lichenysin A produced by *Bacillus licheniformis*, serrawettin produced by *Serratia marcescens* and rubiwettin produced by *Serratia rubidaea* (Peng et al. 2008).

Biosurfactant production is widespread in hydrocarbon-utilizing bacteria. *P. nitroreducens* TSB.MJ10 produced a lipopeptide bioemulsifiers during growth with 0.5 % sodium benzoate as the sole carbon source. TSB.MJ10 is the first strain of *P. nitroreducens* reported to produce a lipopeptide bioemulsifier during growth on benzoate (De Sousa and Bhosle 2012b). It showed higher emulsification activity (70 %), greater oil displacement activity (3.5 cm^2) and better adherence to hydrocarbons (32 %) when 0.5 % benzoate was the carbon source in a mineral salts medium at pH 7.2, temperature 28 °C and 150 rpm (De Sousa and Bhosle 2012b). The bioemulsifier was stable over a pH range of 5–11 and a temperature range of 20–90 °C and displayed emulsification in the presence of relatively high NaCl concentrations (up to 25 %). The surface-active compound formed stable emulsions with aliphatic (hexadecane, *n*-heptane, cyclohexane), aromatic (xylene, benzene, toluene) and petroleum (petrol, diesel, kerosene, crude oil) compounds. It exhibited maximum emulsification activity with weathered crude oil (97 %) and was capable of transforming the rheological behaviour of the pseudoplastic to a Newtonian fluid (De Sousa and Bhosle 2012b).

Reduction in viscosity by biosurfactants and bioemulsifiers is a cost-effective method that can be employed as an alternative to heating, blending and lubrication to facilitate handling and transportation of crude oil/weathered crude oil (Anyanwu et al. 2011; De Sousa and Bhosle 2012b; Franzetti et al. 2012). Biosurfactants are a desirable alternative to synthetic counterparts because of their selectivity, biodegradability, low toxicity and stability at extreme temperatures, pH levels and salt concentrations (Nerurkar et al. 2009; Vignesh et al. 2011). The lipopeptide bioemulsifier produced by TSB.MJ10 enhanced the uptake of hydrocarbon and can be suitably employed in hydrocarbon bioremediation, oil spill management and enhanced oil recovery (De Sousa and Bhosle 2012b).

3.7.2 Bacterial Pigments

Bacteria produce a variety of pigments which confer a number of biological functions, influence their metabolism and therefore enhance their competitiveness and survival under stressed conditions (Mavrodi et al. 2006; Norman et al. 2004; Pierson and Pierson 2010; Wang et al. 2010). The most well-studied pigments are those produced by the denitrifying bacterium *P. aeruginosa* (Pierson and Pierson 2010). *P. aeruginosa* produces a wide array of colourful extracellular pigments: the yellow–green fluorescent pyoverdin, the bright safire blue pyocyanin, the light to dark brown pyomelanin and the brilliant red pyorubrin (Moss 2002). Pyoverdin is a pteridine-derivative with a polyhydroxy moiety in the side chain and acts as a siderophore in iron-limiting conditions (Gaonkar et al. 2012; Moss 2002; Wasielewski et al. 2008). Melanin pigments are water-soluble and safeguard the cells from desiccation and ultra violet (UV) irradiation (Yabuuchi and Ohyama 1972). Pyocyanin and pyorubrin are both phenazine pigments (Mavrodi et al. 2006) which are effective antibiotic agents, serve as electron shuttles to alternate terminal acceptors, modify cellular redox states, act as cell signals that regulate patterns of



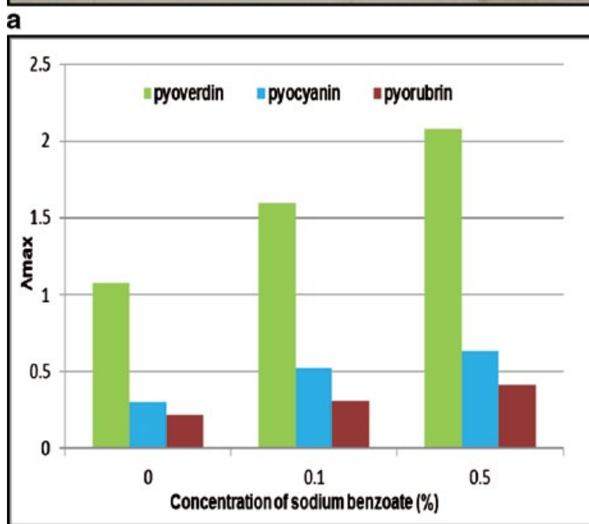


Fig. 3.7 **a** Pigments extracted from TMR2.13 (*yellow–green* fluorescent pyoverdin, bright safire blue nonfluorescent pyocyanin and brilliant red nonfluorescent pyorubrin). **b** Enhanced production of pigments with increasing benzoate concentrations

gene expression and contribute to cell adhesion, biofilm development and enhanced bacterial survival (Pierson and Pierson 2010). Because of their significant characteristics, these pigments can also influence the diversity of microbial communities thereby affecting the ecological structure in natural environments (Norman et al. 2004; Wang et al. 2010). In a hydrocarbon-contaminated system, such alterations would have major implications on the overall degradation and bioremediation processes (Norman et al. 2004).

P. aeruginosa TMR2.13 produces three extracellular pigments: pyoverdin, pyocyanin and pyorubrin (Fig. 3.7a). Interestingly, the amounts of all three pigments were significantly enhanced by the presence of the aromatic hydrocarbon, sodium benzoate (Fig. 3.7b). These pigments are greatly influenced by the oxygen levels in

a medium and stimulated by low oxygen like those developed during denitrification (De Sousa and Bhosle 2012a). Pyoverdin is a water-soluble aromatic chromophore-derivative and acts as a siderophore in low-iron media (Wasielewski et al. 2008). Benzoate augments pyoverdin production since benzoate-grown cells have a specific iron requirement. Iron acts as a cofactor for the enzyme catechol 1,2-dioxygenase involved in the degradation of the aromatic compound. Benzoate-grown cells, therefore, readily become iron deficient (De Sousa and Bhosle 2012a; Gaonkar et al. 2012). Pyoverdin being a siderophore supports iron chelation and renders it available to the cells. Pyocyanin and pyorubrin on the other hand, are phenazine pigments which are known to be stimulated under low aeration conditions (Cox 1986). Pierson and Pierson (2010) demonstrated that phenazines are essential for the bacterial survival under oxygen-deficient conditions. Rao and Sureshkumar (2000) also reported enhanced production of pyocyanin by *Xanthomonas campestris* induced by oxidative stress and explained that the pigment enhances the bioavailability of oxygen by acting as its carrier. Ferguson et al. (2007) have reported that pyorubrin is involved in the protection of the bacterium producing it against oxidative stress.

3.8 Conclusions and Future Prospects

Research devoted to the influence of stress agents like hydrocarbons, especially the aromatics on the critical aspects of microbial physiology, although limited, is ecologically valuable especially for toxicity studies. In natural ecosystems exposed to an influx of hydrocarbons, such regulation in physiological activities would affect the overall community structure and consequently the productivity of the system. The current study sheds light on the various adaptive mechanisms undertaken by denitrifying bacteria in response to hydrocarbons and provides an insight into the influences of these pollutants on the efficiency of denitrifiers thereby endorsing their exploitation in bioremediation designs. However, there are still several loopholes in our understanding of the intriguing denitrifiers and methodological inadequacies continue to impede studies on denitrification. Many facets of the process are insufficiently understood such as the shuffling of electrons between O_2 and NO_3^- and the interaction between denitrification and the other processes of the nitrogen cycle. Novel techniques and instrumentation need to be devised to advance our understanding of this fascinating process.

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Chapter 4
Alkaliphilic Bacteria: Diversity, Physiology and Industrial Applications

Sunita Borkar

4.1 Introduction

Microorganisms are ubiquitous in nature occupying not only different moderate habitats on earth but are also found to adapt and colonize certain harsh environments, once assumed to be sterile by man. Such organisms thriving in extreme environments are called extremophiles and alkaliphiles are a diverse group of organisms that thrive in highly alkaline environments with pH optima for growth of 9.0 or above. They are represented in a large number of bacterial genera and physiological types, but share common challenges that include cytoplasmic pH homeostasis, associated problems of bioenergetic work and function of surface-associated and secreted proteins that must function at very high pH (Krulwich and Guffanti 1989). Alkaliphilic microorganisms have made a large impact with their application and manufacture of mass-market consumer products in industries, especially with enzymes active at high pH. The immense potential of alkaliphiles and a remarkable versatility regarding the production of their primary and secondary metabolites has resulted in their exploitation for use in industries.

4.2 Ecological Niches of Prevalence of Alkaliphiles

Alkaliphilic microorganisms, particularly prokaryotes are widely distributed extremophiles some of which grow in alkaline niches where the pH is above 12. These niches include alkaline soda lakes, which are found throughout the world, providing natural enrichments for an impressively diverse array of alkaliphiles. They

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can be found not only in alkaline environments but also in nonalkaline environments such as neutral and acidic soils, although counts of alkaliphiles are higher in alkaline environments (Grant 2006). In addition, alkaliphiles can be isolated from more specialized alkaline niches such as alkaline ground water (Roadcap et al. 2006), termite gut (Thongaram et al. 2003, 2005), the guts of insect larvae (Aizawa et al. 2010), man-made alkaline environments such as indigo dye balls (Aino et al. 2008; Ghauri et al. 2006) and alkaline effluents of various industries (Borkar et al. 2003; Blanco et al. 2012) as presented in Table 4.1.

4.3 Genesis of Natural Alkaline Environments

The most stable naturally occurring alkaline environments such as eutrophic soda (Na_2CO_3) lakes, soda deserts and alkaline ground waters are caused by a combination of geological, geographical and climatic condition (Grant et al. 1990). Lake Magadi in Kenya, Lake Wadi Natrum in Egypt of the East African rift valley and the western soda lakes of the USA are probably the most stable and highly alkaline environments on the earth with a consistent pH of 10.5–12.0. These environments are characterised by the presence of large amounts of sodium carbonate and sodium chloride, formed by the evaporative concentration, giving rise to environments that are both alkaline and saline. In India, such alkaline and saline lakes are Lake of Lonar situated in Maharashtra having an average pH 10.5 (Joshi et al. 2008; Kanekar et al. 1999) and Deodani Kyars salt lake in Rajasthan (Upsani and Desai 1990).

The genesis of alkaline lakes is due to volcanism, contributing to a source of sodium carbonate to the surface environment or biological activity and weathering, which produce CO_3^{2-} charged surface waters, thus forming a bicarbonate–carbonate solution that leaches surrounding minerals. In most environments, such ground waters rapidly become saturated with Ca^{2+} and Mg^{2+}, resulting in the precipitation of calcite ($CaCO_3$), magnesite ($MgCO_3$) and dolomite ($MgCa(CO_3)_2$). Carbonate is removed from the solution and the genesis of alkaline brine is inhibited, as it is the case for most ground waters. However, when the CO_3^{2-} concentration exceeds that of Ca^{2+} and Mg^{2+}, alkalinity develops, usually with Na^+ as the dominant cation (Fig. 4.1).

Bacterial sulphate reduction in surrounding swamps has also been proposed to contribute towards the alkalinity of Wadi Natrum depression and Hungarian soda lakes (Grant et al. 1990). Transient localised alkaline conditions can also be generated due to animal excreta and biological activities such as proteolysis, ammonification, sulphate reduction or photosynthesis (Horikoshi 1991).

Table 4.1 Ecological niches of prevalence of alkaliphiles

Source	Reference	Source	Reference
Natural environments		High-organic-content ecosystems	
Alkaline springs	–	Biogas plants	Horikoshi (1991)
Aqua de Ney, Siskiyou country, California	Nimura et al. (1987)	Bioteriorated casein-containing building material	Karlsson et al. (1988)
Black bird valley, Stanislaus country, California	Nimura et al. (1987)	Chicken manure digester	Lowe et al. (1993)
Ground water calcium springs in Oman	Grant et al. (1990)	Compost	Horikoshi (1991)
Hyperthermal springs in Bulgaria	Dimitrov et al. (1997)	Alkaline soil	Gupta et al. (2000)
Yellow Stone National Park	Wang et al. (1995), Li et al. (1994)	Japanese forest soil	Nakamura et al. (1993)
Hveragerdi Island	Hansen and Ahring (1997)	Decomposed manure	Horikoshi (1991)
Hot water spring in Bombay–Thane India	Chauthaiwale and Rao (1994)	Dung	Gordon and Hyde (1982)
Alkaline ground water	Roadcap et al. (2006)	Faeces of man and animals	Horikoshi (1991)
Soda lakes	–	Horn meal	Gordan and Hyde (1982)
Alkaline Soda Lake, Ethiopia	Gessesse (1998)	Indigo dye ball	Aino et al. (2008)
Big Soda Lake, Nevada	Grant et al. (1990)	Lime-treated garden soil	Guffanti et al. (1980)
Hungarian Soda Lakes	Borsodi et al. (2008)	Horn meal	Gordon and Hyde (1982)
Lake Ashanti, Bosumatwi, Ghana, Africa	Grant et al. (1979)	Neutral garden soil	Ghosh et al. (2007)
Lake Bogoria, Kenya	Grant et al. (1979)	Sewage	Boyer and Ingle (1972)
Lake Hannington, Kenya	Grant et al. (1979)	Termite-infested mound soil	Rajaram and Varma (1990)
Lake Magadi, Kenya	Florenzano et al. (1985)	Deep-sea mud of Mariana trench	Georganta et al. (1993), Takami et al. (1997)
Lake WadiNatrun, Kenya	Florenzano et al. (1985)	Sandy soil	Lee et al. (2008)

Table 4.1 (continued)

Source	Reference	Source	Reference
Lake Canyan Diable, Arizona, USA	Horikoshi (1991)	Mangolian soda soil	Sorokin et al. (2008)
Lake Nakuru (Pine Lake)	Grant et al. (1990)	Industrial effluents	–
Lake Quebec, Labrador, Canada, North America	Horikoshi (1991)	Battery-cell-manufacturing units	Horikoshi (1991)
Lonar Lake, Maharashtra, India	Kanekar et al. (1999)	Cement manufacturers and casting	Grant et al. (1990)
Owenn's Lake	Horikoshi (1991)	Detergent factories	Horikoshi (1991)
Soda Lake of Kulunda steppe	Horikoshi (1991)	Electro plating	Grant et al. (1990)
Soda Lake Baikal region	Zhilina et al. (2005)	Lye treatment of animal hide	Grant et al. (1990)
Soda Lake in Taiwan	Nomoto et al. (1988)	Paper-processing factories	Grant et al. (1990)
Tibetan soda lake	Xu et al. (1999)	Potato-/food-processing plants	Gee et al. (1980)
Soda Lake of Tuva	Khmelenina et al. (1997)	Textile mills	Horikoshi (1991)
Southwestern Siberian soda lake	Banciu et al. (2008)	Cassava starch waste water	Blanco et al. (2012)
Alkaline salterns	–	Agrochemical factory	Borkar et al. (2003)
–	–	Other environments	–
Great Salt Lake, Utah	Lowe et al. (1993)	Air contaminants	Gordon and Hyde (1982)
Sambhar Salt Lake, Rajasthan, India	Upsani and Desai (1990)	Acidic soil	Horikoshi (1991)
–	–	Deep-sea water	Gordon and Hyde (1982)
–	–	Gut of higher termites	Thongaram et al. (2005)
–	–	Guts of Japanese horned beetle larvae	Aizawa et al. (2010)

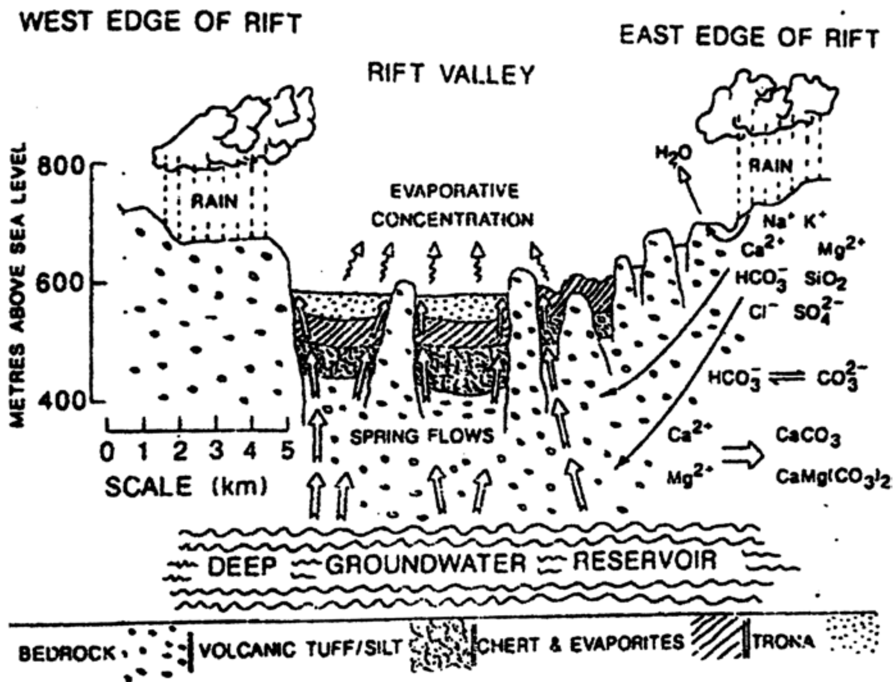

Fig. 4.1 Schematic representation of the possible mechanisms involved in the function of alkaline soda lakes. (Source: Grant et al. 1990)

4.4 Biodiversity and Physiological Groups of Alkaliphiles

4.4.1 Biodiversity

The alkaliphilic microorganisms are known as a diverse group consisting of a heterogeneous collection of eubacteria and archaeobacteria (Yumoto 2007) often adapted to multiple environmental challenges such as high/low temperature, high pressure or high sodium concentrations in addition to high pH. Koki Horikoshi, 'The Explorer of Extremophiles', has isolated a large number of alkaliphiles since 1969 and purified many alkaline enzymes (Horikoshi 1991; Horikoshi and Bull 2011). Extreme alkaliphilic eubacteria are known to be members of Gram-positive group belonging to genera such as *Bacillus* (Aizawa et al. 2010; Blanco et al. 2012; Georganta et al. 1993; Ghosh et al. 2007; Lee et al. 2008; Nogi et al. 2005; Sorokin et al. 2008; Takimura et al. 2007; Yumoto et al. 2003), *Micrococcus* (Kimura and Horikoshi 1990), *Staphylococcus* (Gupta et al. 2000), *Vagococcus* (Joshi and Kanekar 2011), *Arthrobacter* (Horikoshi 1991), *Corynebacterium* (Bahm et al. 1998), *Streptomyces* (Garg et al. 1998), *Actinomyces* (Borgave et al. 2012; Tsujibo et al. 1988),

Clostridium (Zhang et al. 2005; Zhilina et al. 2005), *Exiguobacterium* (Collins et al. 1983), Spirochaetes (Zhilina et al. 1996), magnetotactic bacteria (Lefevre et al. 2011), including cyanobacteria (Florenzano et al. 1985) to Archaeobacteria (Jangir et al. 2012; Khmelenina et al. 1997) with reports also on eukaryotes growing at high pH (Bhushan et al. 1994).

Reports are also available on Gram-negative alkaliphiles belonging to the genera like *Aeromonas* (Krulwich and Gufantii 1989), *Pseudomonas* (Horikoshi 1991; Kanekar et al. 1999), *Vibrio* (Krulwich and Gufantii 1989), *Alkaliflexus* (Zhilina et al. 2004), *Nitrobacter* (Sorokin et al. 1998); *Nitritalea halalkaliphila* (Jangir et al. 2012), photosynthetic *Ectothiorhodospira* (Grant et al. 1979); sulfur-oxidizing bacteria (Banciu et al. 2008), methanotrophic bacteria (Khmelenina et al. 1997) and *Flavobacterium* (Horikoshi 1991).

4.4.2 Pysiological Groups

Alkaliphiles consist of two main physiological groups:

4.4.2.1 Alkaliphiles

These organisms require an alkaline pH of 9.0 or more for growth with optimal growth pH of 10.0. Based on pH preference, such alkaliphiles are grouped into two broad categories: Alkali-tolerant organisms that show optimal growth in the pH range of 7.0–9.0 but cannot grow above pH 9.5 and alkaliphilic organisms that show optimal growth between pH 10.0 and 12.0. The extreme alkaliphiles are further subdivided into facultative alkaliphiles, which show optimal growth at pH 10.0 or above but can grow well in neutral pH range and obligate alkaliphiles which show optimal growth above pH 10.0 but do not grow below pH 9.0. They can be isolated on alkaline media with alkalinity generated using sodium carbonate, sodium bicarbonate, sodium hydroxide, trisodium phosphate or sometimes sodium borate. These salts are added in concentration of about 0.5–2.0% depending on the microorganism used, giving a pH of 8.5–11.0 (Gee et al. 1980; Krulwich and Guffanti 1989; Krulwich and Ito 2013; Horikoshi 1991). Although most of the alkaliphiles are aerobic or facultatively anaerobic, there are reports of alkaline tolerant strictly anaerobic strains such as *Clostridium* and *Methanobacterium* (Ferguson et al. 2006; Karlsson et al. 1988). *Clostridium proteolyticum* was isolated from a chicken manure digester and *Clostridium collagenovorans* from a sewage sludge digester (Lowe et al. 1993). *C. bifermentans* and *C. sporogenes* have been isolated from biodeteriorated casein containing building materials having maximal pH tolerance of 12.2 and 11.7, respectively, (Karlsson et al. 1988). Kevbrin et al. (1998) isolated alkaliphilic obligately anaerobic *Tindallia magadi* from deposits of lake Magadi Kenya.

There are alkaliphiles reported to grow at high temperature under aerobic (Kitada et al. 1987) and anaerobic (Hansen and Ahring 1997) conditions. Enzymes from these thermophilic alkaliphiles are both alkalostable and thermostable, used for protein engineering and production of thermostable enzymes.

Anaerobic alkalithermophiles have been isolated from alkaline hot spring in Yellowstone National Park in North America (Li et al. 1994). Interestingly, Yumoto et al. (2004) have isolated several strains of bacteria which can grow at high pH and temperatures as low as 0 °C. Alkaliphilic and barophilic bacteria have also been reported from deep-sea sediments collected from depths up to 10,898 m of Mariana trench (Georganta et al. 1993; Horikoshi 1999; Takami et al. 1997).

4.4.2.2 Haloalkaliphiles

These organisms isolated from alkaline and highly saline environments such as alkaline soda lakes of Wadi Natrum in Egypt (Mesbah et al. 2007, 2009), lake Magadi in Kenya (Rees et al. 2004; Vargas et al. 2005), Mongolian soda soil (Sorokin et al. 2008), Hungarian soda lake (Borsodi et al. 2008), Lonar Lake, India (Joshi et al. 2007, 2008; Joshi and Kanekar 2011) and Sambhar lake, India (Upsani and Desai 1990) also require high salinity achieved by addition of 20 % NaCl to the isolation medium. *Natronobacterium* and *Natronococcus* are two well-recognized genera of haloalkaliphilic bacteria growing only at pH values above 8.0–9.0 and extremely high salt concentration (Tindall et al. 1980). Xu et al. (1999) isolated two haloalkalophilic archaea from the soda lake in Tibet belonging to a new genus *Natronorubrum*. Complete genome sequence of the anaerobic, halophilic alkalithermophile *Natranaerobius thermophilus* has been studied by Zhao et al. (2011). A number of anaerobic halophilic eubacteria have been isolated from hypersaline alkaline environments such as Big Soda Lake, Aqua de Ney spring (Nimura et al. 1987). Haloalkaliphilic sulfur-oxidizing bacteria have been reported by Sorokin and Kuenen (2005) and Sorokin et al. (2011).

4.5 Molecular and Biochemical Adaptations in Alkaliphiles

Organisms that grow at extreme pH values are faced with a central problem of pH homeostasis. The mechanisms by which these organisms solve and develop survival strategies have been extensively studied and investigated during the past decades. Yet, complete understanding of any of the specific adaptation in all extreme alkaliphiles needs more attention. Some adaptative and regulatory mechanisms have been elucidated in aerobic, alkaliphilic *Bacillus* sp. and have been characterised as passive and active mechanisms.

4.5.1 Passive Mechanisms

Alkaliphilic bacteria are known to change external pH values to a pH suitable for growth, thereby creating their own environment. From an ecological point of view, this phenomenon explains the survival strategies of alkaliphiles in nonalkaline and acidic soils by creating a microcosm favourable for their proliferation.

Alkaliphilic bacteria also show significantly higher external (Bo) and internal cytoplasmic (Bi) buffering capacities under alkaline conditions in comparison to the other nonalkaliphiles (Krulwich et al. 1985, 2011a, 2011b; Ruis and Loren 1998). It is postulated that this property is conferred by high levels of basic proteins or polyamines in alkaliphilic cells (Gilmour et al. 2000). The cell wall components of alkaliphiles also play a key role in protecting the cell from alkaline environments since the protoplasts of alkaliphilic *Bacillus* strains were found to lose their stability in alkaline environments (Ito and Aono 2002). In addition to the peptidoglycan, alkaliphilic *Bacillus* sp. grown at pH 10.0 contain acidic polymers such as galacturonic acid, glucuronic acid, glutamic acid, aspartic acid, phosphoric acid and teichuronopeptides which are more negatively charged than in cells grown at pH 8.0. The negative charges on the acidic nonpeptidoglycan components adsorb sodium and hydronium ions and repulse hydroxide ions assisting cells to grow in alkaline conditions/environments/econiches.

The cytoplasmic membranes of alkaliphiles have a higher concentration of membrane lipid: membrane protein ratio with high cardiolipin content and fatty acid composition consistent with a very fluid membrane (Padan et al. 2005). *Bis* monoacyl-glycero phosphate (BMP), a novel lipid, absent in neutrophiles, is found in alkaliphiles (Horikoshi 1991). Like other *Bacilllus* spp., the alkaliphilc bacilli possess a diverse group of branched chain fatty acids in their membrane lipids and also contain variable amounts of saturated and unsaturated straight chain fatty acids. Further, all alkaliphilic bacilli have squalene and dehydro or tetrahydro squalene with substantial quantities of C_{40} and smaller quantities of C_{50} isoprenoids in the neutral lipid fraction of cell membrane along with phosphatidyl ethanolamine and phosphatidyl glycerol (Krulwich et al. 1998).

Facultative alkaliphiles, however, are found to contain much lower content of unsaturated fatty acids and branched chain fatty acids than obligate alkaliphiles. Studies indicate that fatty acid composition of obligate alkaliphile membrane is a factor precluding growth at low pH values (Yumoto et al. 2000). Facultative alkaliphiles lack desaturase activity and lose their ability to grow at near neutral pH when supplemented with an unsaturated fatty acid (Dunkley et al. 1991). The proteins secreted into the external environment by alkaliphilic bacteria consist mainly of acidic amino acid moieties (Krulwich 1995). A salient feature of alkaliphiles is that their cytoplasmic membranes contain a high concentration of respiratory chain components such as cytochromes including a-, b- and c-type and are red in colour (Krulwich et al. 1988), believed to be crucial to life at high pH. This high concentration of respiratory chain components may provide the mechanism by which the alkaliphiles maximise productive proton transferring collisions between respiratory chain components and the ATPase (Fujisawa et al. 2010; Hicks et al. 2010; Krulwich et al. 1998, 2011a; Yumoto et al. 2003).

4.5.2 Active Mechanisms

A typical alkaliphile, which grows at an alkaline pH range of 9.0 to 11.0, maintains an internal pH 1–2 units lower than the external pH (Horikoshi 1999; Ito and Aono 2002). This mechanism is found to be present in obligate as well as facultative alkaliphiles but is absent in alkali-tolerant bacteria which cannot grow at pH values above 9.0 (Guffanti et al. 1980). As a result of this, the pH gradient is reversed (acid in) with respect to a chemiosmotic driving force. Yet, the cells of alkaliphiles like other aerobic bacteria and mitochondria, extrude protons during respiration acidifying a narrow region near the membrane. In alkaliphiles, however, primary proton extrusion is followed by a Na^+ dependent proton accumulation resulting in a proton gradient (pH) H^+ in > H^+ out and a Na^+ gradient Na^+ in < Na^+ out. This function is perfomed by Na^+/H^+ antiporter, which exchanges internal Na^+ for external H^+, both the ions, transported against their concentration gradients at high pH (Janto et al. 2011; Krulwich 1995; Krulwich et al. 2009, 2011a, 2011b; Krulwich and Ito 2013). Most alkaliphilic bacteria therefore require the presence of Na^+ not only for growth (Pogoryelov et al. 2003) but also for motility, (Fujinami et al. 2009; Ito et al. 2005) and sporulation (Kosono et al. 2000). When alkaliphilic bacilli are either transferred to Na^+ free buffers or subjected to a shift in external pH from near neutral to highly alkaline conditions in the absence of Na^+, cytoplasmic pH rapidly equilibrates to the alkaline external pH, thereby resulting in a complete failure of pH homeostasis. This alkalinisation of the cytoplasm is immediate in spite of the observation that, the cytoplasmic buffering capacity of the bacilli is relatively high in alkaline range (Krulwich et al. 1985). All alkaliphilic bacilli growing at alkaline pH possess F_1 F_0 ATPase localised within the cytoplasmic membrane which helps in the translocation of protons (Ferguson et al. 2006; Hicks et al. 2010; Krulwich et al. 1998, 2011b; Mesbah and Wiegel 2011; McMillan et al. 2007).

A diagrammatic summary of properties of alkaliphilic bacilli relating to their bioenergetics is as represented in Fig. 4.2.

4.6 Industrial Applications

Alkaliphilic microorganisms have made a large impact with their application and manufacture of mass-market consumer products in industries (Nogi et al. 2005; Sarethy et al. 2011). Indigo fermentation is the first industrial application of alkaliphilic bacteria in the world where reduction of indigo from indigo leaves is brought about by alkaliphilic *Bacillus* sp. (Aino et al. 2008; Grant et al. 1990; Horikoshi 1999; Krulwich and Guffanti 1989). The so-called biological detergents contain enzymes obtained from alkaliphilic and alkali-tolerant bacteria. The traditional craft industry of leather tanning uses a series of highly alkaline and hostile processes where the application of alkaline enzymes has brought significant process improvements. Paper and textile processing industries also require enzymes for enzymatic pulping process under alkaline conditions, which help the plant tissues

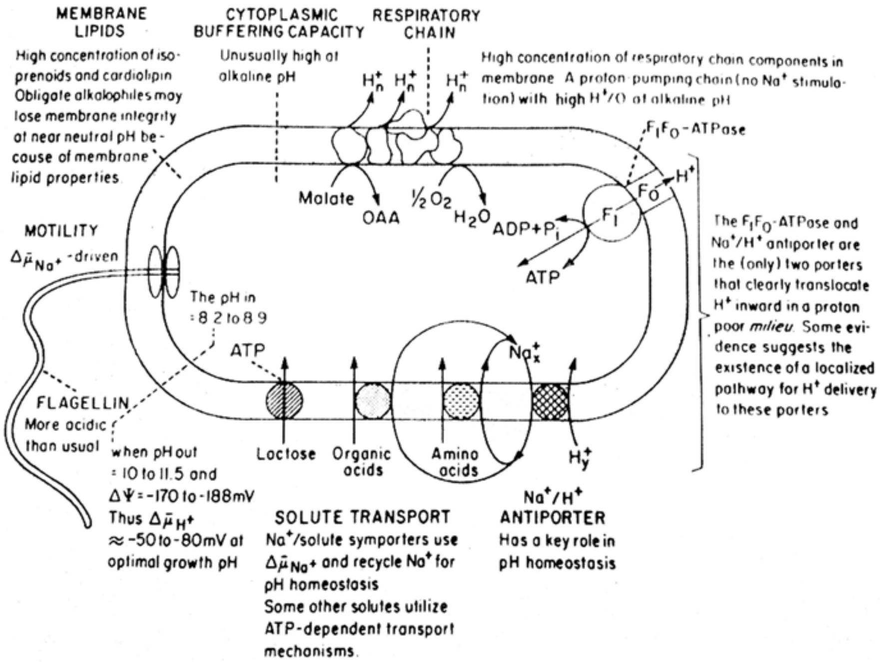

Fig. 4.2 Diagrammatic summary of properties of alkaliphilic bacilli relating to their bioenergetics. (Source: Krulwich et al. 1988)

to swell and facilitate the degradation of pectic substances. Alkaliphiles have also been used for the production of cyclodextrins from starch by the action of cyclomaltodextrin gluconotransferases. These cyclodextrins are used as emulsifying, foaming, stabilizing agents and as molecular capsules to wrap up fragrances or drugs for slow release and hence find wide applications in food, fine chemicals and pharmaceutical industries. Alkaline enzymes have significantly been used in waste treatment (De Graaff et al. 2011), retting process (Yoshihara and Kobayashi 1982), degradation of aromatic compounds (Yumoto et al. 2003) and appear as useful candidates for bioremediation of sites contaminated with toxic chlorinated compounds (Kanekar et al. 1999), since increase in pH can increase the bioavailability of these compounds with the resultant decrease in the toxicity.

The use of microbial enzymes in laundry processes is not a new idea, e.g. Biotex®, a prewash laundry detergent containing an alkaline protease called Alcalase® was launched in the early 1960s (Grant et al. 1990). During the 1980s, there was a dramatic revival of detergent enzymes due to remarkable technical innovations and improvements such as the exclusion of sodium tripolyphosphates (STPP) from formulations, resulting from the environmental concerns of phosphate pollution.

Since detergent enzymes account for 30 % of the total worldwide enzyme production, the continuous screening of improved alkaline enzymes from alkaliphilic

microorganisms is a good example of a successful commercial exploitation of biology by industry.

The most commonly used enzymes in detergent formulations are alkaline amylases and proteinases with the recent introduction of cellulases and lipases, as discussed below:

4.6.1 Amylases

Amylases are one of the most widely exploited enzymes to date in the laboratory and in industry. These enzymes hydrolyse starch, including raw starch and have tremendous potential in different industries like food, detergent, textile, paper, adhesives, fine chemicals, pharmaceutical and fermentation. Although amylases produced by members of the plant and animal kingdom have been traditionally used for various commercial purposes , microbial intracellular or extracellular amylases are preferred for commercial exploitation due to their multifold properties, easy extraction procedure, lower production cost and unlimited supply (Ray and Nanda 1996).

Amylases as detergent additives at high pH remove starch-based stains often working synergistically with proteinases to dissolve protein–starch combinations in food stains. Products include Termanyl® (Novo) and Maxamyl® (IBIS), which are effective up to 100 °C and exhibit activity up to pH 10.0. Rapidase® is used in desizing and softening of denim (Horikoshi 1999). The first alkaline amylase was produced in Horikoshi II medium by cultivating alkaliphilic *Bacillus* sp. A-40-2 (Horikoshi 1971). Many alkaliphilic strains have now been reported to produce alkaline amylases (Shirai et al. 2007).

4.6.2 Proteases

These are the most commonly used enzymes in detergent formulations and have the largest market segment obtained from alkaliphilic microorganisms (Grant et al. 1990; Krulwich and Gufffanti 1989; Saeki et al. 2007). Serine proteinases produced from alkaliphilic *Bacillus* spp. are endopeptidases with a reactive serine moiety at the active site. Commercial products include Alcalase® and Esperase® (Novo industries) and Maxatase®and Maxacal® (International Biosynthetics—IBIS). Maxatase® is an alkaline protease active between pH 7.0 and 11.0 with an optimum activity at pH 9.5–10.0, the pH of many detergents. In their encapsulated form, they are added as 0.4–0.6 % to detergents for the hydrolysis of proteins and removal of proteinaceous stains such as blood, egg, grass and body secretions. Besides lifting proteinaceous soil, proteinases ensure that coagulated protein is not redeposited on the fabric during the wash, which gives a grey unclear appearance to whites. (Horikoshi 1991).

Alkaline proteases have also been used in the hide dehairing process, where dehairing is carried out at pH values between 8.0 and 10.0 (Horikoshi 1999). An interesting application of alkaline protease was developed by Fujiwara et al. (1991) for the removal of the gelatinous coating of X-ray films for the recovery of silver.

4.6.3 Cellulases

Alkaline cellulases with an optimum pH of 10.0 for activity were first reported by Horikoshi et al. (1984) who found two *Bacillus* sp. strains N4 and 1139 producing extracellular alkaline carboxy methyl cellulases. This discovery led to an industrial application of cellulases as laundry detergent additives. Cellulase 103, introduced by Genecor International (Rochester, NV) and launched as 'Attack®' detergent with added cellulases, isolated from alkaliphilic bacterium is the first large-scale commercial application of an extreme molecule.

Cellulases exhibit fabric-softening and colour-brightening properties, besides removing soil (Horikoshi 1991) by removing or opening up the microfibrils that appear on the surface of the cotton fabrics due to wear and repeated washing, thereby restoring the original appearance and smooth fibre structure. Cellulases can also be used in waste treatment, since the undigested material is mainly cellulose (Shikata et al. 1990). Ito (1997) has extensively studied the use of alkaline cellulases in detergents.

4.6.4 Lipases

These enzymes degrade fats into more hydrophilic fatty acids. This property makes them desirable as detergent additives, since hydrophobic stains caused by cosmetics, body fats and oil-based foods are more difficult to remove. Introduction of alkaline lipases as detergent additives is only recent, although their market value is comparable to proteases. Wang et al. (1995) reported purification and characterisation of thermophilic and alkaliphilic tributyrin esterase from *Bacillus* A-30-1, isolated from a hot spring area of Yellow Stone National Park. Lesuisse et al. (1993) have characterised an extremely pH-tolerant lipase while Bhushan et al. (1994) found a lipase produced from an alkaliphilic *Candida* sp. by solid state fermentation.

In addition to these enzymes, there are reports on alkaline xylanases (Aizawa et al. 2010; Gessesse 1998; Gupta et al. 2000), pectinases (Cao et al. 1992), phosphatases (Nomoto et al. 1988), chitinases (Bhushan 2000) , cyclomaltoglucono transferases (Salva et al. 1997) and urease (Singh 1995).

Alkaliphiles also exhibit remarkable versatility in regards to the production of their secondary metabolites such as 2-phenylamine (Hamasaki et al. 1993), carotenes (Aono and Horikoshi 1991), siderophores (McMillan et al. 2010), antibiotics (Tsujibo et al. 1988) and exopolymers (EPs) (Joshi and Kanekar 2011; Kumar et al. 2004).

4.7 Bioprospecting Alkaliphilic Bacteria from Varied Econiches of Goa

4.7.1 Isolation

For the isolation of alkaliphilic bacteria, samples representing neutral, acidic and alkaline econiches were serially diluted in carbonate-bicarbonate buffer (pH 10.0) and plated on alkaline media: (1) polypeptone yeast extract glucose agar (PPYG) (Gee et al. 1980), (2) Horikoshi I and (3) Horikoshi II (Horikoshi 1991). The plates were incubated for 24–48 h at room temperature and colonies were counted to estimate cfu/ml or g.

All the samples were found to yield the presence of alkaliphilic bacteria and the counts were higher on PPYG agar as compared to Horikoshi I and Horikoshi II media (Table 4.2). Interestingly, sample with acidic pH also showed the existence of alkaliphiles with higher counts of 2.06×10^5 cfu/g on PPYG, 1.2×10^3 cfu/g on Horikoshi I and 1.5×10^1 cfu/g on Horikoshi II media.

Predominant isolates (126) from all the samples were purified and characterised. The isolates had varied cultural and morphological features varying from sporulating rods to cocci and pleomorphic forms. A large number of organisms were found to be filamentous with varying degrees of branching. All organisms were found to be Gram-positive except one. The isolates further varied in their tolerance to high pH and temperature. Equal distribution of obligate alkaliphiles (61) and facultative alkaliphiles (65) was observed. It was interesting to note that 39.3 % of obligate alkaliphiles and 29.23 % of facultative alkaliphiles were also tolerant to high temperature (55 °C). A wide variety of organisms were pigmented with the colour ranging from buff-yellow-orange-pink-red-green.

Table 4.2 Total viable cell counts of Alkaliphiles from acidic, neutral and alkaline econiches

Samples analysed	pH	c.f.u./ml/g		
		PPYG	Horikoshi I	Horikoshi II
Acidic soil	6.00	2.06×10^5	1.2×10^3	1.5×10^1
Garden soil	7.00	7.00×10^8	6.20×10^8	6.50×10^8
Termite mound soil	7.00	2.0×10^4	1.1×10^4	1.56×10^5
Marine water	7.354	1.20×10^7	4.0×10^6	2.0×10^7
Mangrove sediments	8.00	4.50×10^3	3.25×10^2	2.1×10^2
Mangrove water	7.80	1.00×10^2	2.50×10^1	1.0×10^1
Salt pans	8.00	2.57×10^6	1.55×10^4	1.2×10^4
Alkaline soil	8.50	2.50×10^4	1.2×10^2	–
Mining effluent	8.00	1.60×10^8	1.8×10^5	2.0×10^4
Agro chemical factory effluent	10.00	5.30×10^9	1.50×10^5	1.3×10^4

4.7.2 Enzyme Profiles of the Isolates

The enzyme activities of the isolates from PPYG agar (pH10.5) were further determined qualitatively by spot inoculating on media with specific substrate: example, Horikoshi II agar for amylase, skimmed milk agar for protease, carboxymethylcellulose (CMC) agar for cellulase and tributyrin/tween 80 agar for lipase, all at pH 10.5.

The studies on enzyme production showed the presence of extracellular enzymes by obligate and facultative alkaliphiles at high pH. Presence of cellulase was found in 79.35 % of the total isolates. Amylase production was shown by only 58.72 % and protease by 47.61 %. However, only 29.35 % of the total isolates exhibited lipase activity. Further, 16 % of the isolates showed the production of multiple enzymes as has also been reported by Nihalani and Satyanarayan (1992). The two isolates, a mesophilic obligate alkaliphile (SB-D) and, a thermophilic, facultative alkaliphile (SB-W) produced maximum amylase activity at alkaline pH in comparison with other isolates. Hence, these isolates were selected for further studies.

Enzymes from both the isolates were found to belong to the group of α-amylases. The temperature for optimum enzyme activity was found to be 25 °C for amylase from isolate SB-D, whereas amylase from SB-W showed maximum activity at 55 °C, irrespective of the pH being either 7 or 10.3. Interestingly, the enzyme from isolate SB-W was found to be stable between pH 7.3 and 10.3 and temperature up to 65 °C, while isolate SB-D had more thermolabile enzyme being stable only up to 35 °C and pH between 9.3 and 10.3.

The activity of the enzymes in the presence of organic solvents was also monitored. It was interesting to note that the purified enzyme from isolate SB-W retained almost 80 % activity in butanol, isopropanol and diethyl ether. However, α-amylase from isolate SB-D showed no activity in the presence of organic solvents. Interestingly, both the enzymes obtained from isolates SB-D and SB-W were found to be stable in the presence of detergent additives.

4.7.3 Characterisation of Potential Isolates

Isolates SB-D and SB-W were found to be spore forming, catalase positive, motile and aerobic rods. Scanning electron micrographs of the cells (Figs. 4.3 and 4.4) also revealed a rod shaped morphology. Both the strains showed acid production from glucose, arabinose, xylose and mannitol, hydrolysed starch and casein and were oxidase positive. Based on Bergey's Manual of Systematic Bacteriology (Sneath et al. 1986) and 16 S rRNA sequencing, the organisms were found to belong to genus *Bacillus* with isolate SB-D identified as *Bacillus lehensis* and isolate SB-W as *Bacillus halodurans*.

The effect of sodium ions was determined as per the procedure followed by Koyama and Nosoh (1995). Both the isolates demonstrated the necessity of sodium ions for growth at pH 10.5. Interestingly, the presence of sodium was

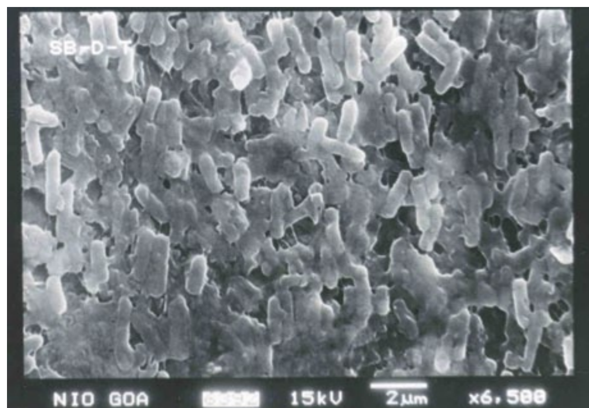

Fig. 4.3 SEM of *Bacillus lehensis* SB-D

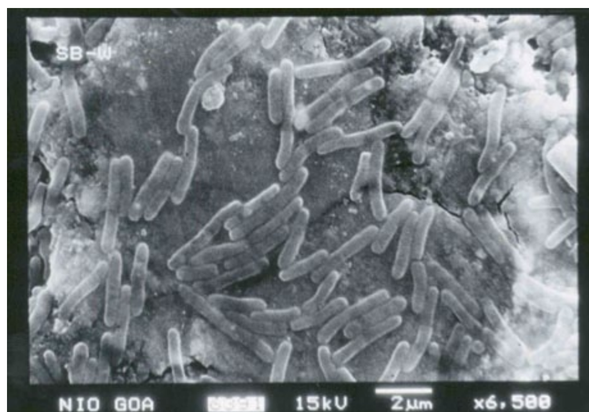

Fig. 4.4 SEM of *Bacillus halodurans* SB-W

also needed for growth of facultative alkaliphile at not only pH 10.5 but also at pH 7.4. The absence of sodium failed to support the growth of both the isolates at alkaline pH.

Determination of intracellular pH as performed by the method of Cook et al. (1996) showed that the intracellular pH of *B. halodurans* SB-W was only 7.7 as compared to the obligate alkaliphile *B. lehensis* SB-D which exhibited a pH of 8.02 when grown at pH 10.5.

In the present study, the buffering capacities of *B. lehensis* SB-D and *B. halodurans* SB-W and two neutrophilic isolates were determined (Borkar and Bhosle 2003; Krulwich et al. 1985). It was interesting to note that the alkaliphiles, SB-D and SB-W required 920 and 490 μl of 0.05 M KOH respectively for change of 1 pH unit. In comparison, the neutrophiles *S. aureus* and *B. subtilis,* however, needed only 40 and 85 μl, respectively, for 1 pH unit change to occur. Alkaliphilic cultures, however, were found to lose this property on treatment with Triton X-100 as a drastic reduction in the KOH requirement (30–350 μls) was observed.

Fig. 4.5 Viscosity of culture broth of *Bacillus lehensis* SB-D in Horikoshi II medium

4.7.4 Exopolymer Production by B. Lehensis Strain SB-D

During the studies on purification of amylase from *B. lehensis* SB-D, it was found that the viscosity of Horikoshi II medium increased when the culture was grown under shaking conditions for 16 h and kept stationary for 4 h (Fig. 4.5) indicating the production of EP. It was, therefore, envisaged to isolate and characterise the EP using analytical methods.

Preliminary screening for cell-free/cell-bound EP was carried out using alcian blue adsorption assay (Bar-or and Shilo 1987). For isolation, purification and characterisation of EP from viscous culture supernatant, isopropanol was added in the ratio of 1:1 and the precipitate obtained after spooling on a bent glass rod was purified by dialysis and then lyophilised. Emulsification property of the fermentation broth and polymer, metal adsorbtion and adhesive properties of EP were also studied.

It was interesting to note that the alcian blue was adsorbed on the cells obtained from the medium and viscous supernatant indicating the presence of cell-associated and extracellular EP. Physicochemical analysis of dialysed and lyophilised EP revealed an organic content of 76% with 212 µg/mg of carbohydrates and 130 µg/mg of lipids. The compound also contained uronic acids, pentoses, phosphates and pyruvate. The product appears to be a glycolipid complex as the major components are carbohydrates and lipids.

Prior to the addition of isorpanol, 24-h culture broth supernatant (Fig. 4.6) as well as the pelleted cells demonstrated a strong water-in-oil emulsification activity. This property was not evident in the EP removed by the glass rod after the addition of isopropanol as well as the precipitated polymer. The low activity of the polymer but the high activity of the supernatant appears to indicate the requirement of water soluble component for optimum activity. The EP possessed strong metal binding affinity towards copper, as reported for EP (Mc Lean et al. 1990). The most significant feature of EP was that this polymer possessed strong adhesive property, which

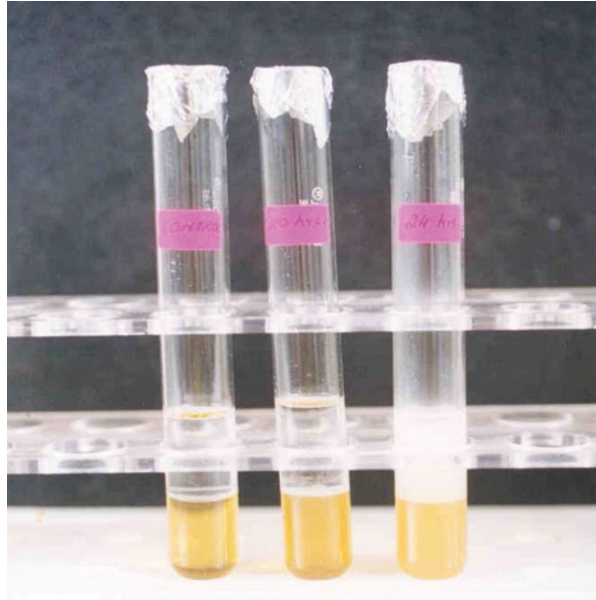

Fig. 4.6 Emulsification activity of culture broth of *Bacillus lehensis* SB-D

Fig. 4.7 Adhesive property of viscous EP

could be demonstrated by its ability to adhere glass-on-glass as well as glass-on-paper as seen in Fig. 4.7.

4.8 Conclusions and Future Prospects

From the results of the present study, it can be conclusively inferred that the alkaliphiles are a diverse group of organisms present in varied econiches. They exhibit an interesting phenomenon of maintaining their internal pH more than two units

less than the external pH and possess high cytoplasmic buffering capacity. In addition, they are a potential source of several enzymes not only active at pH 10.3 but also other harsh conditions such as high temperature, presence of organic solvents and detergent additives, therefore having wide industrial applications. They are also a versatile source of other new metabolites/bioactive compounds. Hence, future research on this group of organisms may be directed towards the role of membrane proteins/components in the mechanism of pH tolerance. Studies on hydrolytic extracellular enzymes such as cellulase, amylase, protease, lipase, xylanase, etc., of industrial application for potential use in organic matter degradation and bioremediation of toxic effluents can be investigated. The production of biopolymer obtained from *B. lehensis* SB-D strain including adhesive and metal scavenging property for industrial exploitations can be undertaken.

Thus, alkaliphiles, by virtue of several unique characteristics, hold immense potential for the production of the enzymes active at alkaline pH with wide applications in various industries. Since alkaliphilic bacterial strains are more tolerant to toxic compounds and have better bioavailability, efficiency of biodegradation and removal of pollutants from waste treatment systems can be undertaken. The five major extreme groups of microorganisms, i.e. *Thermophiles, Halophiles, Acidophiles, Barophiles and Alkaliphiles* have been rightly termed by Horikoshi in 1991, as *Super Bugs* on account of their potentiality. Basic research, directed towards isolation of extremophiles producing unique extremozymes and bioactive products need to be continued, strengthened,and pursued with renewed scientific vigour so that their potentials are revealed and exploited.

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Chapter 5
Biodegradation of Aromatic Compounds by Alkaliphilic Bacteria

Rasika Desai Gaokar

5.1 Introduction

Mangroves, one of the specialized ecosystems of the tropical areas are nutritionally rich due to the continuous shedding of foliage which gets decomposed to form detritus matter. These are highly reproductive ecosystems which host a wide range of coastal and offshore marine organisms and provide a unique ecological niche for diverse bacterial communities (Ramanathan et al. 2008). These organisms, though have continuous nutrients, are affected by tidal variations, salinity and by anthropogenic substances added through run offs from the terrestrial ecosystem such as excess of fertilisers, pesticides and by the activities of various industries such as mining, shipbuilding, etc. The interaction of microbial flora with such substances has resulted in the proliferation of physiologically diverse microflora in marine ecosystems. Among these, are also the bacteria which have the ability to degrade hydrocarbons and xenobiotics. Mangrove ecosystem is also found to develop specialized niches having specific conditions such as high pH, increase in salinity and high nutrient content in the form of nitrogen and phosphorus (Robertson 1992).

5.2 Biodegradation of Aromatic Compounds

Biodegradation is the metabolic ability of microorganisms to transform or mineralise organic compounds into less harmful, nonhazardous substances which are then integrated into natural biogeochemical cycles. Organic compounds are often classified as biodegradable, persistent or recalcitrant, depending on their behaviour in the environment. Biodegradation means the biological transformation of an organic

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chemical to another (Blackburn and Hafker 1993; Liu and Suflita 1993). The monocyclic aromatic substances such as benzoate, toluene and phenol are more easily attacked than polycyclic aromatic hydrocarbons such as naphthalene, benzopyrene, anthracene, substituted aromatics, chlorinated biphenyls, etc. The intensity of biodegradation is influenced by several factors such as availability of nutrients, oxygen supply, pH value, composition, concentration and bioavailability of the contaminants, chemical and physical characteristics and the pollution history of the contaminated environment (Margesin and Schinner 2001).

5.3 Incidence of Aromatic Compounds

The ever growing list of chemical contaminants released into the environment on a large scale includes numerous aromatic compounds such as petroleum hydrocarbons, halogenated and nitroaromatic compounds and phthalate esters. These compounds enter the environment through various paths such as (1) components of fertilisers, herbicides, pesticides, fungicides and insecticides that are used to increase food production, (2) pharmaceuticals used for health reasons (3) organic solvents used in industries and (4) synthetic organic xenobiotic chemicals. Combustion processes release others such as polycyclic aromatic hydrocarbons (PAHs), dibenzo-p-dioxins and dibenzofurans. The local concentration of a contaminant depends on:

a. The amount present and the rate at which the compound is released
b. Its stability in the environment under both aerobic and anaerobic conditions
c. The extent of its dilution in the environment
d. The mobility of the compound in a particular environment
e. Its rate of biological or non-biological degradation (Ellis 2000; Janssen et al. 2001)

5.4 Bacterial Transformation of Aromatic Hydrocarbons

Due to the hazardous effect of aromatic hydrocarbons, it is necessary to keep a check on the concentration of these substances in the environment. The control measure followed nowadays is the degradation of these substances by microorganisms. Degradation may occur either during the process of utilisation of the hydrocarbon as a carbon source or as a co-metabolic transformation by microorganisms. When the organism utilises the hydrocarbon as a carbon or energy source during its growth, the original compound is largely incorporated into the cell mass or is destroyed as carbon-dioxide. The parameters required for a degradation process to occur are:

a. A capable organism must be present
b. An opportunity must exist for requisite enzymes to be synthesized
c. Environmental conditions must be sufficiently suited for the enzymatically catalysed reaction to proceed at a significant rate

Microorganisms with the ability to degrade a wide variety of compounds like benzene, phenol, naphthalene, atrazine, nitroaromatics, biphenyls, polychlorinated biphenyls (PCBs) and chlorobenzoates have been isolated and characterised (Dickel et al. 1993; Sangodkar et al. 1989). Biotransformations of organic pollutants in the natural environment have been extensively studied to understand microbial ecology, physiology and evolution for their potential in bioremediation (Bouwer and Zchendir 1993; Chen et al. 1999; Johan et al. 2001; Kiran 2009; Mishra et al. 2001; Watanabe 2001). List of bacteria degrading the various aromatic compounds is as presented in Table 5.1.

5.5 Mechanism of Degradation of Aromatic Compounds by Bacteria

Aerobic degradation is dependent on the presence of molecular oxygen and is catabolized by enzymes that have evolved for the catabolism of natural substrates and exhibit low specificities (Dagley 1971). Oxygenases are a group of enzymes which catalyse the incorporation of molecular oxygen into various organic and inorganic substrates (Hayaishi 1966). Depending upon the type of enzyme catalysing the reaction, either one or two oxygen atoms are inserted into the molecule via an electrophilic attack on an unsubstituted carbon atom (Janssen et al. 2001). These oxygenases are classified into two groups:

a. ***Monooxygenases*** These enzymes catalyse the addition of a single atom of oxygen per molecule of substrate, e.g. Tyrosinase enzyme on substrate tyrosine.
b. ***Dioxygenases*** These enzymes catalyse the addition of two atoms of molecular oxygen per molecule of substrate, e.g. catechol 1,2 dioxygenase and catechol 2,3 dioxygenase on substrate catechol.

Aromatic compounds are ubiquitous growth substrates for microorganisms. Like some aliphatic compounds, aromatic compounds have chemical inertness which makes them difficult substrates. Benzoate is the most studied monocyclic aromatic compound. The aerobic degradation of benzoate follows various routes in microorganisms. Most of the bacteria aerobically degrade benzoate initially to catechol which then follows the *ortho*, *meta* or *gentisate* mode of ring cleavage (Murray and Williams 1974) (Fig. 5.1). Microorganisms utilise these compounds by dissimilating them in a step-wise manner as follows:

5.5.1 Initial Hydroxylation of Aromatic Ring Structure

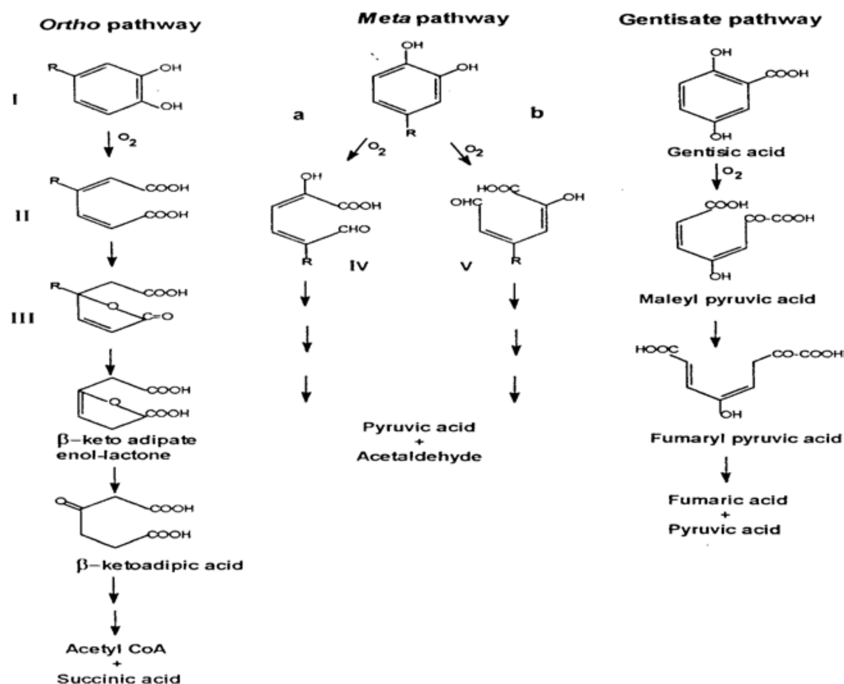
Aerobic organisms introduce hydroxyl group in the aromatic ring using molecular oxygen as obligatory cosubstrate. The position whereby the group is incorporated decides the type of intermediate formed such as catechol, protocatechuate or gentisate (Fig. 5.1) (Bayley and Barbour 1984; Chapman 1972; Gibson and Subramanian

Table 5.1 Microorganisms involved in the degradation of various aromatic compounds

Aromatic compound	Application	Microorganisms involved in degradation	Reference
Various xenobiotics	Herbicides, pesticides	*Rhodococci*	Poelarends et al. (2000)
o-, m-, p-Cresols	–	*Pseudomonas putida*	Chapman (1972)
		Pseudomonas alcaligenes	Poh and Bayley (1980)
		Alcaligenes eutrophus	Hughes et al. (1984)
Xylenol	–	*Pseudomonas putida*	Chapman (1972)
		Pseudomonas alcaligenus	Poh and Bayley (1980)
Benzoate	Bacteriocide, fungicide	*Pseudomonas putida mt-2*	Williams and Murray (1974)
			Nakazawa and Yokoto (1973)
		Pseudomonas aeuroginosa	Kataeva and Golovlea (1990)
		Pseudomonas putida NCIB10015	Sala-Trepat et al. (1972)
		Azotobacter	Sala-Trepat and Evans (1971)
		Alkaliphilic *Bacillus*	Yumoto et al. (2003)
		krulwichiae	
Nitrophenols	Starting material in manufacture of pharmaceuticals, pesticides, explosives	*Arthrobacter sp.*	Zylstra et al. (2000)
Chlorinated ethylenes	Industrial solvents	*Pseudomonas stutzeri* OX	Ryoo et al. (2000, 2001)
Trichloroethylene			
Tetrachloroethylene	Polyaromatics hydrocarbons found in petroleum	*Geobacter*	Kazumi et al. 1995
	Kazumi et al. (1995)		
Monochlorinated & nonchlorinated aromatic compounds			Kiran (2009)
–	–	*Dietzia* sp	Wang et al. (2011)

Table 5.1 (continued)

Aromatic compound	Application	Microorganisms involved in degradation	Reference
Triclosan (5-chloro-2-2,4 dichlorophenoxy phenol)	Antimicrobial agent used in a wide range of consumer products like soaps, toothpastes, handcreams and plastics	Bacterial consortium	Hay et al. (2001)
Atrazine	Herbicide for control of broad leaf and grassy weeds	*Pseudomonas sp.* ADP	De Souza et al. (1998)
			Wackett et al. (2002)
Phenols and their derivatives	Used for manufacture in synthetic organic compounds	*Thauera aromatic*	Whiteley and Bailey (2000)
		Pseudomonas	Feist and Hegeman (1969)
		Pseudomonas putida MTCC	Bandhyopadhyay et al. (1999)
		Alcaligenus eutrophus	Hughes et al. (1984)
		Halomonas campisalis	Alva and Peyton (2003)
		Acinetobacter	Abd-El Haleem et al. (2003)
Nonyl phenol	used mostly in agricultural fertilisers	*Sphinogomonas sphinobium*	
	Tanghe et al. (1999)		
		Sphinogomonas amiense	Ushiba et al. (2003)
Mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid]	Herbicide	*Alcaligenes Ralstonia sp. CS2*	Smejkal et al. (2001)
2,4-dichlorophenoxyacetic acid	–	*Alcaligenes*	Clement et al. (2001)
3-chlorobenzoate	–	*Ralstonia eutrophus*	
Naphthalene	Used in fungicides, pesticides, detergents, dyes, moth balls, motor fuel and resins	*Pseudomonas sp*	Fuenmayor et al. (1998)
Aniline & p-chloroaniline	Production of polyurethanes, rubber, azo dyes drugs, photographic chemicals varnishes and pesticides	*Moraxella sp.* strain G	Zeyer et al. (1985)
		Pseudomonas putida	Fukumori and Saint (1997)
		Pseudomonas acidovorans	Loidl et al. (1990)
Anthracene	–	*Bacillus badius*	Ahmed et al. (2012)



R=H, I=Catechol; II=Cis,cis-muconate; III=Muconolactone;
IV= α-Hydroxymuconic semialdehyde
R=COOH, I=Protocatechuate; II=β-Carboxy-cis,cis-muconate;
III= γ-Carboxy-muconolactone; IV= γ-Carboxy-muconic semialdehyde;
V= α-Hydroxy-γ-carboxy-muconic semialdehyde

Fig. 5.1 Modes of ring cleavage in bacteria. (Adapted from Murray and Williams 1974)

1984; Stanier and Ornston 1973). The hydroxyl group is introduced either in *ortho* or *para* position. In the case of compounds such as resorcinol, gallic acid, etc. wherein two hydroxyl groups are already present, a third hydroxyl group must either be present or introduced before ring cleavage can occur (Bayley and Barbour 1984).

Cleavage of the aromatic ring is a key reaction in the oxidation of aromatic compounds. Dioxygenases capable of cleaving the aromatic ring occur widely in bacteria but differ in their mode of aromatic ring cleavage, specific inductions and substrate specificity (Kataeva and Golovlea 1990; Ornston 1971). Certain bacteria of the genus *Pseudomonas* synthesize several aromatic ring-cleaving enzymes that enable them to oxidize various aromatic compounds. For example, *Pseudomonas aeuroginosa* which grows on a wide set of aromatic substrates including p-xylene, synthesizes four dioxygenases namely pyrocatechase (Catechol 1,2 dioxygenase), metapyrocatechase 1 and 2 (Catechol 2,3 dioxygenases) and protocatechuate 3,4 dioxygenase (Kataeva and Golovlea 1990). The usual prerequisite for the oxidative cleavage of the aromatic ring by the action of dioxygenases where O_2 is used as a substrate is that, the ring contains two hydroxyl groups that are either *ortho* or *para* to one another.

5.5.2 Modes of Ring Cleavage

There are three distinct modes of ring cleavage

5.5.2.1 Ortho-Ring Cleavage

Cleavage of the bond between adjacent carbon atoms that carry hydroxyl groups is known as "*Ortho*" or "intradiol" cleavage and the pathway by which the product of such cleavage is metabolized is known as the *ortho* or β-ketoadipate pathway (Fig. 5.1). This kind of cleavage is brought about by catechol 1,2 dioxygenase which catalyses the cleavage of the aromatic ring of catechol to *cis*, *cis* muconic acid with the consumption of 1 ml of oxygen (Nakazawa et al. 1969; Nakazawa and Yokoto 1973).

5.5.2.2 Meta-Ring Cleavage

In the second mode of cleavage of the benzene nucleus, attack occurs between two carbon atoms, only one of which carries a hydroxyl group, the other carbon atom being either unsubstituted or substituted with other than a hydroxyl group (Fig. 5.1). This kind of cleavage is known as "*Meta*" or extradiol cleavage. In this case, the hydroxyl groups may be either *ortho* or *para* to one another and the enzymes catalysing such cleavages are designated as catechol 2,3 dioxygenases, which catalyse the cleavage of the aromatic ring of catechol to 2-hydroxymuconic semialdehyde (HMS) (Kojima et al. 1961). This enzyme catalyses the critical ring-cleaving step in the biodegradation pathways of many complex aromatic compounds. This enzyme has been reported in various microorganisms for catechol and cleaves the bond between carbon atoms 2 and 3 to yield the stoichiometric quantity of 2-hydroxymuconic semialdehyde (Evans 1971; Hayaishi 1966; Sala-Trepat et al. 1972).

5.5.2.3 Gentisate Pathway

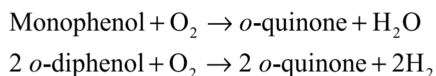
This is the third mode of aromatic ring cleavage seen in compounds having the hydroxyl groups *para* to one another as in gentisic acid (2,5-dihydroxy benzonic acid). Oxidative cleavage is catalysed by gentisate 1,2 dioxygenase and the subsequent pathway is the gentisate pathway. Gentisate 1,2 dioxygenase is heat stable and inhibited by *o*-phenanthroline and 2,2′ bipyridyl (Crawford et al. 1975). Gentisate formed from a number of aromatic compounds is cleaved by this pathway (Fig. 5.1) (Harpel and Lipscomb 1990; Poh and Bayley 1980).

Benzoate ring cleavage is thus a central core for degradation of substituted and polycyclic aromatic compounds. Such ring cleavage could occur either by the *meta* or *ortho*-pathway. Oxygenative cleavage of catechol is then followed by a series of reactions leading to the formation of succinate and acetyl coenzyme A in case of

ortho-pathway, acetaldehyde and pyruvate in the *meta*-pathway and fumaric acid with pyruvic acid in the gentisate pathway (Nakazawa and Yokoto 1973; Murray and Williams 1974).

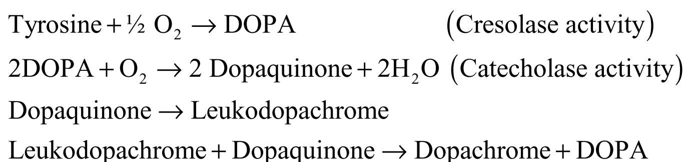
5.6 Degradation of Tyrosine

Tyrosine is a nonessential or sometimes called semiessential amino acid produced from phenylalanine. It is a constituent of aminosugars and aminolipids. It is known to increase the suppressant effects of phenyl propanolamine, ephedrene and amphetamine and helps to reduce body fat. It is the precursor of neurotransmitters like 3,4 dihydroxy phenylalanine (L-DOPA), dopamine, nerepinephrine and epinephrine. Tyrosine enters the mainstream pathways vital for the cells routine functioning. Besides this, it can also enter side pathways wherein the products produced may not be very harmful to the cell under normal conditions. Such a pathway is termed as the melanogenesis pathway. Here, tyrosine is first converted to DOPA and then dopaquinone, the intermediate metabolites in the production of melanin via the enzyme tyrosinase. Tyrosinase is fairly ubiquitous and was previously called monophenol monooxygenase with the terminology changed to phenolase, monophenol oxidase and as cresolase. Tyrosinase is a copper-containing monooxygenase, catalysing both *o*-hydroxylation of monophenols and the oxidation of *o*-diphenols to *o*-quinones (Lerch 1987).



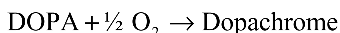
$$\text{Monophenol} + O_2 \rightarrow o\text{-quinone} + H_2O$$
$$2\ o\text{-diphenol} + O_2 \rightarrow 2\ o\text{-quinone} + 2H_2$$

It has been reported that the active site of the enzyme contains a pair of antiferromagnetically coupled Cu^{+2} ions. Its number in the standard enzyme nomenclature is (EC 1.14.18.1). Tyrosine is first *ortho*-hydroxylated to DOPA (Cresolase activity). L-DOPA is oxidized to dopaquinone (Catecholase activity), which undergoes further nonenzymatic oxidation and polymerization, yielding melanin. (Kelley et al. 1990).



$$\text{Tyrosine} + ½\ O_2 \rightarrow \text{DOPA} \qquad (\text{Cresolase activity})$$
$$2\text{DOPA} + O_2 \rightarrow 2\ \text{Dopaquinone} + 2H_2O \quad (\text{Catecholase activity})$$
$$\text{Dopaquinone} \rightarrow \text{Leukodopachrome}$$
$$\text{Leukodopachrome} + \text{Dopaquinone} \rightarrow \text{Dopachrome} + \text{DOPA}$$

The overall reaction for tyrosinase activity is:



$$\text{DOPA} + ½\ O_2 \rightarrow \text{Dopachrome}$$

The oxidation of tyrosine is preceded by a short lag which can be eliminated by the addition of a trace of DOPA as the oxidation of DOPA goes rapidly without a detectable lag.

Dopaquinone is converted to dopachrome through spontaneous autooxidation and finally to dihydroxyindole or dihydroxyindole-2-carboxylic acid (DHICA) to form eumelanin (brown–black pigment). The hydroxylation of tyrosine to DOPA can also be catalysed by the enzyme tyrosine hydroxylase which is found in the nerve cells. However, there are major differences between the two enzymes being:

a. They can be easily distinguished on the basis of their cofactor requirements. Tyrosinase requires DOPA, the product of the initial reaction to act as a cofactor for the reaction while tyrosine hydroxylase requires a tetrahydroprotein as cofactor. Neither enzyme can utilise the other cofactor as they are extremely specific (Hearing 1987; Pomerantz 1966).
b. Tyrosinase is a copper-containing enzyme while tyrosine hydroxylase contains iron and thus, they can be distinguished on the basis of inhibitors specific for either of these metals (Hearing 1987).

5.7 Products of Degradation of Aromatic Compounds

During the biodegradation of aromatic compounds, the number of products formed either as byproducts or intermediates include:

5.7.1 Quinones

Aromatic compounds which have two hydroxyl groups oriented either in *ortho* or *para* position can easily be oxidized to ketone like compounds called quinones (O'Leary 1976). Quinones are non-aromatic conjugated cyclo-hexadienones (Goodwin 1979). Varieties of quinones exist and are found in bacteria, fungi and higher plants. Four types of quinones described are: (1) Benzoquinones, (2) Naphthoquinones, (3) Anthraquinones and (4) Isoprenoid quinones.

5.7.2 Melanins

Melanin pigments occur widely in plants and animals and often in bacteria (Mason 1953). Apart from imparting intense pigmentation, which in certain cases is of taxanomic value, melanins play a role in increasing resistance of fungi towards lytic enzymes, detoxification of polyphenolics in root nodules, detoxification mechanism for pathogen *Cryptococcus neoformans* creating anaerobic conditions in nitrogen-fixing cultures and protecting cells due to antioxidant, antiradical and superoxide scavenging properties (Banulescu 2000; Barnett and Hageman 1983; Djordjeric et al. 1987; Margalith 1992; Mayer and Hazel 1979; Sadasivan and Neyra 1987; Shivprasad and Page 1989). Four basic kinds of melanins are:

5.7.2.1 Eumelanin

It is a black to brown insoluble material, consisting of highly polymeric cross-linked structures of several hundred monomeric units found in mushrooms and potatoes. The two types of eumelanin monomers known are dihydroxy indole (DHI) and dihydroxy indole carboxylic acid (DHICA) (Banulescu 2000).

5.7.2.2 Phaeomelanin

It is a yellow to reddish brown alkali soluble material and is found in the hair and feathers of fowls, encysted forms of certain strains of *Azospirillum brasilene*, black spot of potato, human hair, squid ink etc. (Sadasivan and Neyra 1987; Stevens et al. 1998).

5.7.2.3 Neuromelanin

DOPA is too hydrophilic to cross the blood–brain barrier and hence is converted to hydrophobic L-DOPA which can cross the blood–brain barrier, where it is reconverted to DOPA. This is then oxidatively converted to neuromelanin. Neuromelanins can act as chain-breaking antioxidants (Shivprasad and Page 1989).

5.7.2.4 Allomelanin

It is formed from nitrogen-free precursors such as acetate, catechol-1,8-dihydroxynaphthalene, etc. No tyrosine is involved in the synthesis of this pigment. The pathway for allomelanin formation is known as the polyketide biosynthetic pathway. Allomelanin is seen in watermelon seeds and in the filamentous fungus *Alternaria alternata* (Kimura and Tsuge 1993).

5.8 Degradation of Aromatic Compounds at Alkaline pH

Study of microbial degradation of aromatic compounds at alkaline pH is important in natural ecosystems where the fate and toxicity of these contaminants are unknown, but the existence of alkaliphilic microorganisms thriving at alkaline pH would support the functioning of the carbon cycle. Use of these microorganisms in the removal of aromatic compounds from alkaline and/or industrial wastewater will support the environmental concern of industries and environmentalists. The microbial degradation of aromatic compounds is important not only for the industrial applications of the respective enzymes involved, but also for studying struc-

tural, functional and genetic aspects of aromatic compound oxygenases. However, there is little information concerning alkaliphiles that degrade aromatic compounds, or concerning aromatic compound oxygenases isolated from them. Isolation of aromatic compound degrading alkaliphiles might enable the acquisition of new information not only on the taxonomy, physiology and ezymology of alkaliphiles, but also on aromatic compound oxygenases (Gibson and Subramanian 1984). Hydrocarbon biodegradation at alkaline pH is of interest for the bioremediation of industrial waste waters which are contaminated with aromatic or chlorinated hydrocarbons (Margesin and Schinner 2001).

Although much is known about the degradation of aromatic compounds at neutral pH, relatively very little information is available about such biotransformations occurring at an alkaline pH, more so with alkaliphilic bacteria. The term "Alkaliphilic" is used for microorganisms that grow optimally or very well at pH values above 9, often between 10 and12, but cannot grow or grow only slowly at the near-neutral pH value (Horikoshi 1991). Alkaliphilic bacteria have been extensively studied with regard to their biotechnologically relevant extracellular enzymes and as model organisms to solve bioenergetic problems (Garcia et al. 1983; Krulwich and Guffanti 1989; Krulwich 1995). However, very little information is known about their capabilities of degrading aromatic compounds. One of the few available reports is on degradation of 4-chlorobenzoate by a facultatively alkaliphilic *Arthrobacter* sp. strain SB8 (Shimao et al. 1989). The bacterium dehalogenated 4-chlorobenzoate in the initial stages of degradation and metabolized it via 4-hydroxybenzoate and protocatechuate. The substrate concentration tolerated by this bacterium was as high as 150 mM. The degradation of phenol at pH 10 by bacteria isolated from a 2.6 g/l NaCl Lonar lake has been reported (Kanekar et al. 1999). To determine the feasibility of aromatic compound biodegradation in saline and alkaline conditions, the effect of pH and salinity on the biodegradation of phenol as a model aromatic waste compound by the haloalkalophilic bacterium *Halomonas campisalis* has been examined (Alva and Peyton 2003). Yumoto et al. (2003) have reported a halotolerant obligate alkaliphile *Bacillus krulwichiae* sp.nov.capable of utilising benzoate and m- hydroxybenzoate. Prior to this report, the degradation of 2,4 Dichlorophenoxyphenol by an alkaliphilic, moderately halophilic soda lake isolate *Halomonas* sp. EF 43 has been reported (Kleinsteuber et al. 2001). Three kinds of alkaliphilic bacteria able to utilise thiocyanate (CNS^-) at pH 10 were found in highly alkaline soda lake sediments and soda soils. Genetic analysis has demonstrated that both the heterotrophic and autotrophic alkaliphiles that utilised thiocyanate as a nitrogen source belonged to the gamma subdivision of proteobacteria (*Halomonas* group for heterotrophs and *Thioalkali vibrio* for autotrophs) (Sorokin et al. 2001). The heterotrophic and autotrophic alkaliphiles that grew with thiocyanate as a nitrogen source possessed a relatively high level of cyanase activity which converted cyanate (CNO^-) to ammonia and CO_2. Since it is relatively stable under alkaline conditions, cyanate is likely to play a role as a nitrogen buffer that keeps the alkaliphilic bacteria safe from inhibition by free ammonia which otherwise would reach toxic levels during dissimilatory degradation of thiocyanate.

Removal of aromatic compounds from alkaline or industrial waste water is an environmental concern for the industry. In addition, aromatics may be accumulating in soda lakes and unique natural systems where the fate and toxicity of these contaminants are unknown. It was, therefore, envisaged to review the degradative studies performed on aromatic compounds under alkaline conditions. Since benzoate is a central core for the degradation of substituted and polycyclic aromatic compounds, it was of interest to study the degradative pathway by alkaliphilic bacteria in the presence of sodium benzoate and tyrosine and note the various intermediates formed during their metabolism.

5.9 Degradation of Aromatic Compounds by Alkaliphilic Bacteria from Mangrove Ecosystems of Goa

5.9.1 Isolation of Alkaliphilic Bacteria and Screening for Aromatic Compound Degradation

Alkaliphiles from mangrove ecosystems of Goa namely Banastari, Merces, Panaji, Ribandar and St. Cruz, situated on the west coast of India, were studied with respect to biodiversity and their ability to biodegrade aromatic compounds under alkaline conditions. Samples collected from mangrove ecosystems were plated on polypeptone yeast extract glucose agar (PPYG) (pH 10.5) (Gee et al. 1980). Interestingly, all the samples were found to yield the presence of alkaliphilic bacteria (Desai et al. 2004). Predominant isolates (141) obtained on PPYG agar (pH 10.5) were purified, maintained and replica plated on four sets of PPYG agar plates of pH 7, 8.5, 10.5 and 12 for selection of obligate alkaliphiles. The plates were incubated for 48 h at room temperature and the isolates growing only at pH 10.5 and 12 were selected to be obligate alkaliphiles. Significantly, 20 % isolates were found to be obligate alkaliphiles while 80 % isolates were considered as facultative. Identification studies done by morphological, biochemical, chemotaxonomical and phylogenetic analysis revealed that 98 % of the cultures were Gram-positive with 54 % of the alkaliphilic cultures belonging to the genus *Bacillus*, 21 % to *Corynebacterium*, 7 % each to *Micrococcus* and *Actinomycetes*. Only one culture, designated as A-131 in the study was found to be Gram-negative short rods (Fig. 5.2) and was identified to belong to the genus *Flavobacterium* by 16S rRNA analysis. Further, 28 obligate alkaliphiles were screened for aromatic compound degradation by growing them in Mineral salts medium (MSM) with the aromatic compounds such as benzoate, tyrosine, phenylalanine, phenol, cresol, aniline, resorcinol, quinol and parachloroaniline. Interestingly, all the isolates grew luxuriantly when supplemented with benzoate, phenol, tyrosine and phenylalanine as sole source of carbon, whereas a few isolates exhibited the capability of utilising aniline, cresol and other phenolic compounds such as resorcinol, quinol and parachloroaniline.

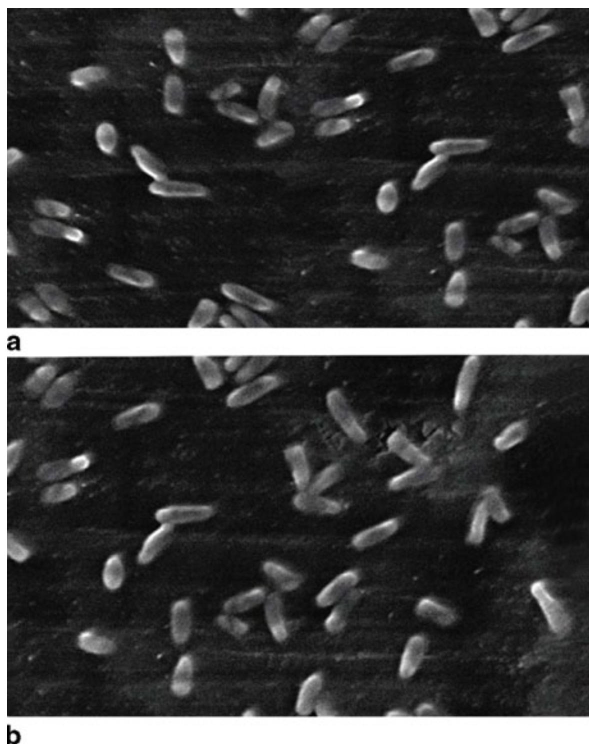


Fig. 5.2 Scanning electron micrographs of *Flavobacterium* A-131 in (**a**) MSM with 0.3 % benzoate and (**b**) MSM with 0.2 % tyrosine

5.9.2 Degradation of Sodium Benzoate by Flavobacterium Strain A131

Flavobacterium strain A-131 was inoculated in MSM at pH 10.5 containing sodium benzoate as the sole source of carbon at different concentrations (0.1–1 %). It was noted that the isolate could grow upto the concentration of 0.7 % sodium benzoate with the optimum concentration of 0.3 %. *Flavobacterium* A-131 grown in the presence of 0.3 % benzoate and extracted in ether showed a distinct spot of Rf of 0.9 of on thin layer chromatography (TLC) plate which turned black in colour and was corresponding to standard catechol, further confirmed by the addition of lead acetate to the supernatant which gave a white precipitate. Ring cleavage of aromatic substrate was studied by the modified Rothera's test (Offlow and Zolg 1974) which indicated that the *meta*-ring cleavage pathway was present in the *Flavobacterium* A-131 grown in benzoate as sole carbon sourceTherefore, the enzyme catechol 2,3 dioxygenase was assayed using whole cells as well as cell-free extract, and their specific activities were determined. Whole cells showed an activity of 0.38 U/mg as

compared to 1.08 U/mg shown by the cell-free extract. During the growth in MSM-containing benzoate, the culture showed very little growth and the initial colour of the medium was yellow, and later turned orange-red. The yellow and orange-red products formed during the metabolism of benzoate were analyzed further.

A distinct peak at 375 nm in the UV-visible scan was obtained with the appearance of a yellow colour in the medium, with the absorbance found to increase with deepening of the colour during growth. The orange-red supernatant formed during the metabolism of benzoate by *Flavobacterium* A-131 was tested for the presence of quinone (Finley 1974). The tests done showed that the orange-red product in the supernatant could probably be a quinone. Catechol is thus an important intermediate formed from benzoate metabolism by *Flavobacterium* A-131 which gets converted to a yellow-coloured intermediate and subsequently forms an orange-red product probably a quinone. From the above observations, we propose catechol to be the central metabolite in sodium benzoate biodegradation by *Flavobacterium* culture A-131 under alkaline conditions linking two pathways, namely the *meta*-cleavage pathway and the quinone formation, both of which operate simultaneously during benzoate biodegradation. The proposed pathway for the benzoate metabolism by *Flavobacterium* culture A-131 is as presented in Fig. 5.3.

Yumoto et al. (2003) have reported the utilisation of benzoate and *m*-hyroxybenzoate under alkaline conditions by the alkaliphilic bacterium *Bacillus krulwichiae*. The intermediates and the final product formed during the metabolism, however have still not been characterised and reported. *Rhodococcus* sp. K-37 and HA-99

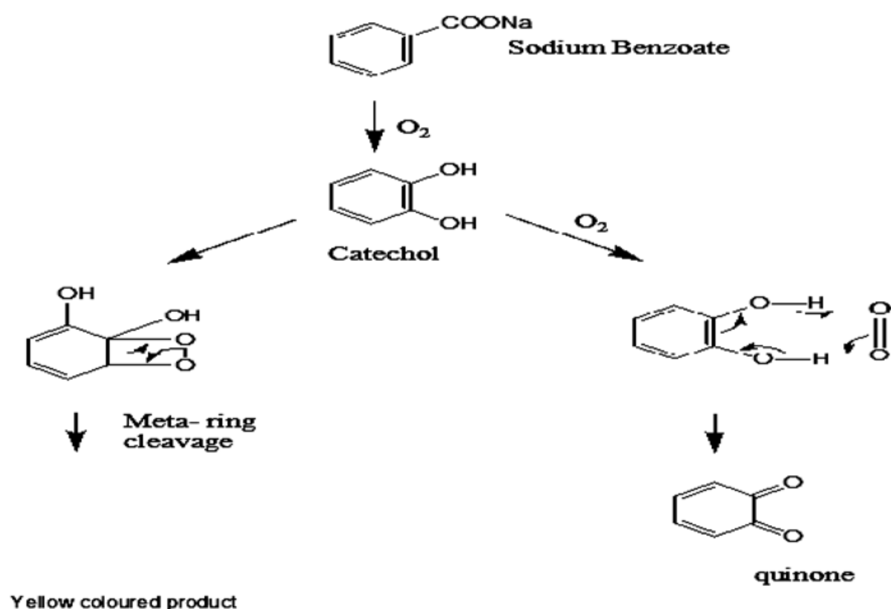


Fig. 5.3 Proposed pathway for metabolism of benzoate by *Flavobacterium* A-131

have also been reported to utilise benzoate and 3-hydroxybenzoate, phenol, biphenyl, 4-chlorobiphenyl and naphthalene under alkaline conditions (Maeda et al. 1998), but the intermediates or products formed during utilisation have not been discussed.

5.9.3 Degradation of Tyrosine by *Flavobacterium* Strain A-131

To check the degradation of tyrosine by *Flavobacterium* A-131, it was inoculated on MSM agar (pH 10.5) containing 0.1–1 % concentrations of tyrosine and incubated for 24 h at the room temperature. Interestingly, zones of clearance were observed depicting growth and tyrosinase activity (Fig. 5.4).

In liquid MSM medium with varied concentrations of tyrosine, growth and turbidity varied from pink colour within 16 h which deepened to a brown colour at 40 h (Fig. 5.5).

From the qualitative tests performed (Kelley et al. 1990) (Table 5.2), the pink-coloured compound could be a quinone, probably dopaquinone and the black-coloured product is similar to melanin which could be eumelanin as reported by Kelley et al. (1990). Degradation of tyrosine is known to occur by the production of tyrosinase, a copper-containing monooxygenase. From the qualitative tests performed, it can be concluded that there exists an enzyme which produces DOPA from tyrosine and further oxidizes it to dopaquinone. DOPA autooxidation and polymerization then converts dopaquinone to melanin which is the brown-coloured product turning black in colour. The tyrosinase (oxidase) activity was found to be present in whole cells and cell-free extract with an activity of 0.23 U/mg with 0.55 U/mg, respectively. Many reports are available which support the above data (Lerch

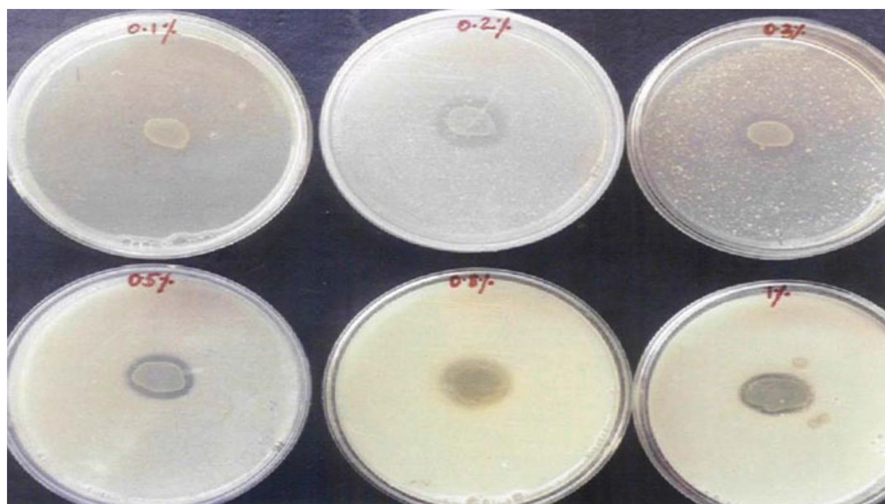
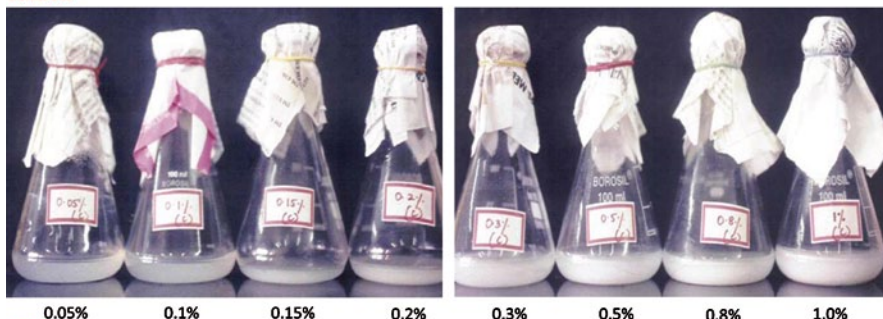


Fig. 5.4 Demonstration of tyrosinase activity by *Flavobacterium* A-131



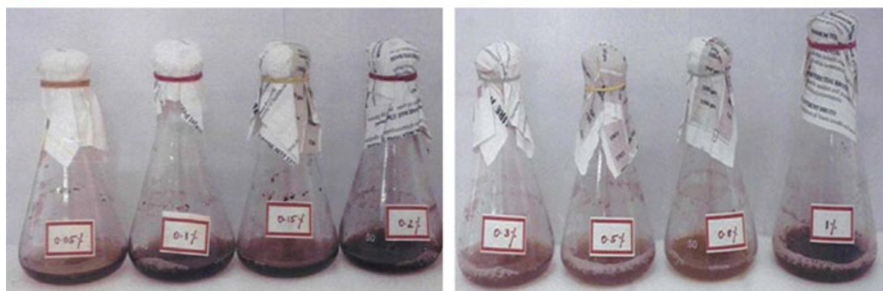


Fig. 5.5 Growth of *Flavobacterium* A-131 in MSM with tyrosine as a sole source of carbon (40 h incubation)

Table 5.2 Qualitative tests for the detection of metabolic products of tyrosine

S. no	Tests	Control*	Culture supernatant	Black
		(0.1 % DOPA)	Pink	
1	Supernatant + 6N HCl (pH 2)	Pink to colourless	Pink to colourless	Black to colourless
2	(1) + 10N NaOH (pH 12)	Colourless to pink	Colourless to pink	Colourless to black
3	Supernatant + 2 % sodium dithionite	Pink to colourless	Pink to colourless	Black to colourless
4	(3) + when aerated for 2 h	Colourless to pink	Colourless to pink	Colourless to black
5	Supernatant + pinch of sodium borohydride	Pink to colourless	Pink to colourless	Black to colourless
6	Supernatant + H_2O_2	Pink to colourless	Pink to colourless	Black to colourless

* Control (0.1 % DOPA) was incubated at the room temperature till the pink colour appeared

1987; Kelley et al. 1990). Thus, the pathway of tyrosine → DOPA → dopaquinone → dopachrome → melanin is followed by *Flavobacterium* A-131 for degradation of tyrosine and is carried out by the enzyme tyrosinase. Reports have shown ex-

tensive studies of aromatic compound degradation with Gram-positive bacteria at neutral pH. However, limited work has been done with Gram-negative bacteria (Kelley et al. 1990). In view of this, the culture *Flavobacterium* A-131 is a novel isolate from the mangrove ecosystem showing growth and metabolism of aromatic compounds only at alkaline pH. It is a versatile alkaliphilic bacterium having the ability to degrade a wide range of aromatic compounds, including monoaromatic, polyaromatic, chlorinated and nitrocompounds exhibiting the formation of coloured intermediates when grown in sodium benzoate and tyrosine as a sole source of carbon. The culture has been deposited at the National Chemical Laboratory, Pune, with the accession number NCIM 5243.

5.10 Conclusions and Future Prospects

The mangrove ecosystems harbour obligate alkaliphilic bacteria which are capable of producing a wide range of enzymes with the potential of degradation of aromatic compounds. The presence of these bacteria indicates that they play an important role in the biogeochemical cycles as well as pollution abatement. Search for these isolates from such ecosystems using various techniques and media could result in isolating highly potential obligate alkaliphiles including *Flavobacterium* A-131. Further research in this area can, therefore, be directed towards:

a. Derivitisation, purification and characterisation of the yellow- and orange-coloured products obtained from benzoate degradation and the dopaquinone obtained from tyrosine degradation.
b. Purification and characterisation of the enzymes catechol 1,2 dioxygenase, 2,3 dioxygenase and tyrosinase obtained from *Flavobacterium* A-131 with respect to enzyme assays and substrate specificities.
c. Utilisation of *Flavobacterium* A-131 in the treatment of industrial effluents containing aromatic compounds for bioremediation.

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Chapter 6
Response of Alkaliphilic Bacteria to Aromatic Amines

Naveen Kumar Krishnamurthy

6.1 Introduction

Aromatic compounds constitute a major portion of organic compounds following the glucosyl ones in nature. Anthropogenic activities have led to the release of man-made complex aromatic compounds into the environment through effluents from industries and unregulated use of pesticides. Such compounds alien to the environment are termed as xenobiotics.

The manufacture of xenobiotics involves the use of various substituted aromatic compounds of which aromatic amines are one such group. Some of the aromatic amines include aniline—the simplest aromatic amine, chloroanilines, nitroanilines, aminobenzoates, polycyclic aromatic amines etc., which are widely used in the manufacture of drugs, dyes and pesticides. Anilines are known to form coloured compounds on coupling with other aromatic amines, collectively known as azo dyes. These dyes are used in fabric and leather dying industries, food and drink, pharmaceutical, paper, plastics, lacquer, paints and wood staining (Combes and Haveland-Smith 1982; Doulati et al. 2007; Wainwright 2008). Substituted anilines are potential contaminants of aquatic environments because of their large-scale use as precursors in the industrial synthesis of plastics, dyes, polyurethanes, rubber, drugs, photographic chemicals, varnishes, and pesticides (Boon et al. 2000; Rădulescu et al. 2008; Struijs and Rogers 1989). Diphenylamine (DPA) is the most commonly used stabilizer for nitrocellulose-containing explosives and propellants. As a result of nitration of DPA, the products N-nitroso-DPA, mononitro-DPAs, dinitro-DPAs and to a minor extent trinitro-DPAs and nitro-N-nitroso-DPAs are formed (Drzyzga et al. 1996).

Besides their biodegradability, aromatic amines are also gaining importance for their carcinogenic properties. A total of 80 different aromatic amines were tested

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for their mutagenecity by carrying out the Ames test involving various strains of *Salmonella typhimurium* (Chung et al. 1997). A transformed product of an azo dye, an aromatic amine p-phenylenediamine, which is extensively used in hair dyes, was found to be the most potent carcinogen. Crebelli et al. (1981) reported that p-phenylenediamine is not a carcinogen but gets transformed to a potent carcinogen when activated by the action of enzymes within a biological entity due to its ability to form nitrenium ion (Brennan and Schiestl 1997; Wild 1990). Compounds such as aniline, *m* and *p*-aminophenol, were found to be nontoxic but in the late nineteenth century, carcinogenicity in workers (urinary bladder cancer) at a dyestuff industry was related to aromatic amine toxicity. Aromatic amines have, therefore, been gaining importance as the intermediates formed during the transformation of complex aromatic amino compounds could be potent carcinogens.

Biodegradation of such aromatic amines is therefore of considerable importance to protect the ecosystems and so is the management of industrial effluents prior to their disposal in the environment. Although, a large number of reports are available on bacteria that can degrade aromatic compounds at neutral pH (Ajithkumar and Kunhi 2000; Annweiler et al. 2002; Bhat et al. 1998; Cartwright and Cain 1958; Harwood and Gibson 1997; Herbes and Schwall 1978; Konopka et al. 1989), very little information is available on the degradation of such compounds by bacteria which can survive and grow under alkaline condition (Sarnaik and Kanekar 1995; Yumoto et al. 2008).

6.2 Sources of Aromatic Compounds

6.2.1 Natural Compounds

The major contributor of organic compounds in the natural world is plant based, which includes cellulose, hemicellulose, which are glucosyl based and lignin, which has an aromatic backbone structure. The benzene ring is the next widely distributed structure in nature after the glucosyl residue (Díaz et al. 2001). Other aromatic compounds present in nature include aromatic amino acids (tyrosine, tryptophan, phenylalanine), alkaloids (nicotine, quinine, cocaine), hormones (epinephrine, acetylcholine), vitamins (thiamine, biotin), steroids (Flavanoids, quinones), pigments (chlorophyll), nucleic acids, etc. Commonly found aromatic compounds at the plant roots include benzoate, phenols, 1-carvone, cymene, limolene (Gilbert and Crowley 1997; Hegde and Fletcher 1996) containing homocyclic, heterocyclic and polycyclic ring structures. Lignin being an aromatic-based polymer, is the major contributor of aromatic compounds comprising up to 25 % of the land-based biomass on the earth (Díaz et al. 2001), and its recycling along with other plant-derived aromatic compounds is vital for maintaining the Earth's carbon cycle. Some of the resistant aromatic compounds include tannins, a plant-based polyhydroxy aromatic compound, second most abundant group of plant phenolics after lignin. The presence of a number of phenolic hydroxyl groups enables them to form large complexes, mainly with proteins, and other macromolecules like cellulose and

pectin (Bhat et al. 1998). Gribble (2003), has reviewed the presence of various organohalogenic compounds in nature produced by diverse species such as marine and terrestrial plants, marine sponges, bacteria, fungi, algae, lichens, animals and humans. Abiogenic methods of introduction of these compounds include biomass fires, volcanoes and other geothermal processes, including crude oil which comprises benzenes, toluenes, ethylbenzenes, xylenes (BTEX), polyaromatic hydrocarbons and resins.

6.2.2 Anthropogenic Compounds

Since industrialization, aromatic compounds are produced in large amounts and are released into the environment by human activities. Various industries from which the pollutants are released include (1) chemical and pharmaceutical industries that produce a variety of synthetic compounds and polymers, (2) paper and pulp bleaching industries, (3) coal and petroleum industry and (4) agricultural practices where pesticides are extensively used (Diaz 2004). In 1988, the US Environmental Protection Agency listed a number of chemicals as priority pollutants which included pesticides, halogenated aliphatics, nitroaromatics, chloroaromatics, polychlorinated biphenyls, phthalate esters, polycyclic aromatic hydrocarbons and nitrosamines (Fewson 1988). Nitroaromatic compounds are used worldwide as explosives, pesticides, and as precursors for the manufacture of many products including dyes, pharmaceuticals and plastics. Nitroaromatic compounds are well-known toxins; some are mutagenic/carcinogenic or teratogenic and others are un-couplers of cellular phosphorylation reaction (Crawford 1995). Xenobiotic compounds (organohalogens) such as polychlorobiphenyls (PCB), dichlorodiphenyl trichloroethane (DDT) are recalcitrants and their lipophilic property enhances their bioaccumulation and biomagnifications (Gray 2002; Goerke et al. 2004; Nfon and Cousins 2006; Richter and Nagel 2007; Vettery 2002).

Though the concentration of xenobiotics is present in sublethal levels, their long-term exposure causes significant damage to marine population. Animals such as seals, bald eagle and seabirds showed disrupted hormonal cycle, leading to reproductive dysfunction such as reduction in fertility, hatch rate, alternation of sex behaviour and viability of offspring (Crews et al. 1995). The grey seal (*Halichoerus grypus*) population in the northern Baltic has shown a marked increase since the ban of DDT and PCB in the Baltic region (Wu 1999).

Degradations of such compounds are brought about by microorganisms either through metabolic or cometabolic processes.

6.3 Metabolism and Cometabolism

Microorganisms can assimilate aromatic compounds as a source of carbon and energy or they may be biotransformed thereby reducing their toxicity or convert them to an inactive form. Such metabolism normally occurs in the presence of additional

carbon sources, which support the growth of organisms and simultaneously degrade them. In some metabolic cases the organic compound is similar to a substrate and therefore gets metabolised by a mechanism called gratuitous metabolism (Grady 1985). With various pathways present in the nature to bring about degradation of aromatic compounds, many microorganisms utilize these compounds as a sole source of carbon based on the activity of enzymes. Glucose, abundantly present in nature also plays an important role in the degradation of certain xenobiotic compounds. Tharakan and Gordon (1999) showed that trinitrotoluene (TNT), a chemical used in explosives can be removed in the presence of glucose by bacteria. Glucose also facilitated a *Pseudomonas* sp. (Ziagova and Liakopoulou-Kyriakides 2007) in the degradation of 1,2-Dichlorobenzene, a known xenobiotic without which it hardly grew. Raymond and Alexander (1971) reported that bacteria resistant to *m*-nitrophenol utilized it only in the presence of *p*-nirophenol, which was used as a source of carbon and energy. Benzopyrene, one of the polyaromatic hydrocarbons (PAH) was seen to be removed by *Sphingomonas* JAR02 when incubated with benzopyrene in the presence of root products. The cometabolism of benzopyrene was facilitated during utilization of other aromatic compounds present (Rentz et al. 2005). Van Herwijnen et al. (2003) indicated that the isolate *Sphingomonas* LB216 could cometabolise various PAHs such as phenanthrene, fluoranthene, anthracene, dibenzothiophene only when initially grown in the presence of fluorene.

Cometabolism and gratuitous metabolism thus play a vital role in biotransformation/biodegradation of various xenobiotic compounds which otherwise would only be persistent or recalcitrant and this depends on various factors.

6.4 Role of Environmental Factors in Biodegradation of Aromatic Compounds

Biodegradation is the metabolic ability of microorganisms to transform or mineralise organic compounds to simpler forms which are then integrated into natural biogeochemical cycle. Organic contaminants also enter the biogeochemical cycle after getting biodegraded. Biodegradation of such organic compounds or contaminants depends on various factors. Some of the important factors responsible for biodegradation of the aromatic compounds include—positive chemotactic behaviour of the bacterium towards the pollutant, bacterial ability to produce specific enzymes, physical characteristics, pollution history of the contaminated environment, the nature of the pollutant, concentration and bioavailability of the contaminants. Environmental factors such as oxygen availability (electron acceptor), temperatures (low or high), pH (acidic, neutral or alkaline), salt concentration and pressure are also involved under which degradation is required to be carried out. Effects of certain stress conditions such as high salinity and pH are discussed below.

6.4.1 Salinity

One of the natural extremities includes environment with high salinity which varies with ecosystems. Freshwater ecosystem shows negligible or no salinity as compared to open seas where salinity with 30–35 % salt concentration is seen. Salt pans have salt concentrations at saturation levels. Estuarine ecosystem shows a fluctuation in salt concentration due to constant mixing of seawater with freshwater. The general term given to organisms that survive, tolerate and thrive in highly saline environment is halophiles and are included in a separate domain called Archaea (Woese et al. 1990). Whereas, organisms that tolerate high salt concentrations but can even grow in environments with no or less salt concentrations are called halotolerant and belong to eubacterial domain. Some of the members of halophiles that were isolated from Dead Sea belonged to the family Halobacteriaceae, such as genus *Haloferax, Haloarcula, Halorubrum* (formerly *Halobacterium)* (Arahal et al. 1996). Pasic et al. (2005) reported bacteria belonging to some of the genera mentioned above thriving in salterns. These organisms belonged to obligate halophiles as they could not survive in the medium with salt concentration below 15 %. Halophilic archaea maintain an osmotic balance with the hypersaline environment by accumulating high salt concentrations, which require salt adaptation of the intracellular enzymes (Hough and Danson 1999; Oren 1999; Ventosa et al. 1998). Halophilic bacteria have adapted to the osmotic stress of high-salinity environments by actively accumulating K^+, glycerol, betaine and ectoine within the cytoplasm (Galinski et al. 1985; Jebbar et al. 1992; Peyton et al. 2002; Woolard and Irvine 1995). Eubacteria are more promising degraders than archaea as they have a much greater metabolic diversity. Their intracellular salt concentration is low and their enzymes involved in biodegradation may be conventional (i.e. not salt-requiring), which can be compared with enzymes similar to those of nonhalophiles. The use of microorganisms able to degrade organic wastes in salt could prevent costly dilution to lower the salinity, or the removal of salt by reverse osmosis, ion exchange or electro-dialysis before subjecting to biological treatment. These organisms thus play a vital role in the biodegradation and biomineralisation of organic residues in saline environment. Nicholson and Fathepure (2004) have reported to have isolated a mixed population of halophiles from brine soil which was dominated with the culture *Marinobacter* spp. able to degrade benzene, toluene, ethylbenzene or xylenes (BTEX components). Emerson et al. (1994) isolated a halophile *Haloferax* D1227 that utilized aromatic compounds such as benzoate, cinnamate, and phenylpropanoate in the presence of 1.7–2.6 M NaCI at 45 °C. Garcia et al. (2004) isolated a halophile *Halomonas organivorans*, a moderate halophile able to utilize a variety of aromatic compounds which include benzoic acid, *p*-hydroxybenzoic acid, cinnamic acid, salicylic acid, phenylacetic acid, phenylpropionic acid, phenol, *p*-coumaric acid, ferulic acid and *p*-aminosalicylic acid. Li et al. (2010) demonstrated the degradation of aniline by halophilic *Erwinia* spp.

6.4.2 pH

The hydrogen ion concentration (pH) plays an important role in the degradation of any compound as it determines the ability of the compound to dissociate. It has been observed that certain organic compounds such as chlorophenols and nitrophenols dissociate with an increase in pH, thereby lowering their toxicity (Holcombe et al. 1980; Kishino and Kobayashi 1995; Kulkarni and Chaudhari 2006) which makes them vulnerable to microbial attack leading to their degradation. The optimum level of degradation is observed in the neutral condition where the neutrophilic bacteria are abundant and get acclimatized to the newly introduced pollutant. The most important criteria for the degradation of such organic compounds is the ability of microbes to survive and grow in alkaline environments. Organisms requiring alkaline condition as a prerequisite for their growth are called alkalophiles/alkaliphiles and are able to tolerate pH values within a range of 8.0–12.0. Based on their pH requirements, they are called as obligate alkaliphiles (grow at pH values of 10.5–12.0) and alklitolerant (tolerate pH from 8.0 to 12.0 but have an optimal pH requirement of 8.0). Alkaliphilic bacteria are presently gaining importance in the degradation studies of aromatic pollutants, especially for their use in wastewater treatment (Margesin and Schinner 2001). Horikoshi (1991) reported that the majority of bacteria belong to genus *Bacillus*, *Micrococcus*, *Corynebacterium*, *Pseudomonas, Flavobacterium*, Actinomycetes such as *Streptomyces, Nocardiopsis* and Yeasts and grow in the absence of high concentration of salt. However, halotelerant alkaliphiles isolated from Lake Magadi in Kenya belonging to the family Halomonadaceae under genus *Halomonas*, with various species such as *H. elongata*, *H. halodenitrifican*, *H. desiderata*, *H. cupids* and *H. Magadii* were found to grow at high pH and high salt concentration of upto 15 % (Duckworth et al. 2000). References available from other parts of the world report bacterial species such as *H. organivorans* from Spain (Garcia et al. 2004), *H. boliviensis* from Bolivia (Quillaguaman et al. 2004), *H. Koreensis* from Korea (Lim et al. 2004), *H. glade* from Antartica (Reddy et al. 2003) that require high pH along with 15 % salt as their optimal growth condition. Haloalkaliphilic archaea like *Natronococcus* and *Natronobacterium*, from the salt lake of Magadi, Kenya (Mwatha and Grant 1993; Tindall et al. 1984) on the other hand require high pH and 25 % salt concentration for their growth.

Halotolerant alkaliphilic bacteria as mentioned by Garcia et al. (2004), have the capability to degrade many aromatic hydrocarbons and thus can be utilized to degrade various pollutants under extreme conditions (Satyanarayana et al. 2005).

6.5 Degradation of Aniline

Aniline, the simplest form of aromatic amine is used as raw material in the manufacture of pesticides, herbicides and fiungicides such as phenylurea, phenylcarbamate and acylanilide including azo dyes (Boon et al. 2001; Engelhardt

et al. 1977). Aniline is usually easily metabolised and undergoes detoxification by chemical, physical and biological methods. However, insertion of groups such as bromo, chloro, methyl, methoxy, nitro or cyano in the aromatic ring increases the resistance to degradation by microorganisms (Paris and Wolfe 1987). The rate of transformation of these compounds decreases in the order: aniline > 3-bromoaniline > 3-chloroaniline > 3-methylaniline > 3-methoxyaniline > 3 -nitroaniline > 3-cyanoaniline. Several researchers have reported the degradation of aniline by various neutrophilic organisms, especially *Pseudomonas* sp. (Ahmed et al. 2001; Anson and Mackinnon 1984; Fukumori and Saint 1997; Konopka et al. 1989; Liu et al. 2002; Peres et al. 1998; Shanker et al. 2006; You and Bartha 1982; Walker and Harris 1969; Zeyer and Kearney 1982), *Nocardia* sp. (Bachofer and Lingens 1975), *Commanomonass* sp. (Boon et al. 2001; Peres et al. 1998), *Acinetobacter* sp. (Fujii et al. 1997), *Phanerechaete chryso sporium* (Sandermann et al. 1998), *Bacillus* sp. (Zissi et al. 1997), *Rhodococcus* sp. (Obinna et al. 2008; Zhuang et al. 2007) *Burkholderia* sp. (Takenaka et al. 2003) and *Delftia sp* (Liu et al. 2002; Sheludchenko et al. 2005; Xiao et al. 2009) with few reports on degradation by halophiles (Li et al. 2010) and haloalkaliphiles (Jin et al. 2012).

Degradation of aniline and its simple derivatives occurs systematically by initially removing the amino group and insertion of two hydroxyl groups in the aromatic ring to form an intermediate-catechol via the catechol-1,2 or catechol-2,3 dioxygenase pathway. This is followed by the cleavage of the benzene ring either at *ortho*- or *meta*-positions of the hydroxyl group, thereby mineralising the aromatic hydrocarbon. Lyons et al. (1984) proposed various interactions of aniline in the environment, which explained its degradation and polymerization (Fig. 6.1) and have reported the aniline transformation via catechol, 1,2-dioxygenase. Other responses observed in bacteria include detoxification mechanisms, wherein aniline is transformed to an anilide or condensed with other aromatic or aliphatic compounds to give a high-molecular-weight compound.

6.6 Studies on Response of Alkaliphilic Bacteria to Aromatic Amines from Marine Ecosystem

6.6.1 Isolation and Characterisation of Alkaliphilic Bacteria

Sediment and water samples were collected from mangrove ecosystems in Panjim (Banks of river Mandovi, Goa) and beach ecosystem at Palolem, Goa (Fig. 6.2). Bacterial counts were determined by standard dilution plate count method on nutrient agar (pH 7.0) for neutrophilic bacteria and polypeptone yeast extract glucose agar (PPYG) (pH 10.5) (Gee et al. 1980) for alkaliphilic bacteria.

Results revealed higher colony counts on nutrient agar as compared to PPYG medium, indicating the dominance of neutrophilic population over alkaliphilic

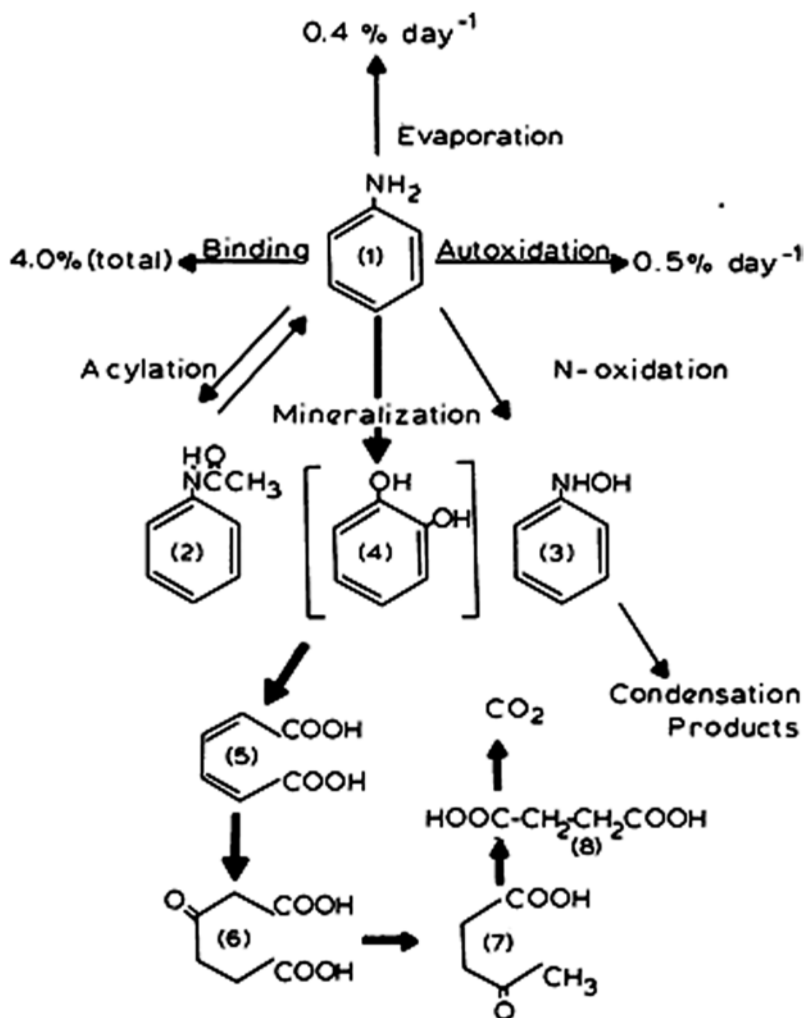


Fig. 6.1 Interactions of aniline in the environment. Key: (*1*) aniline; (*2*) acetanilide; (*3*) phenylhydroxylamine; (*4*) catechol; (*5*) *cis,cis*-muconic acid; (*6*) beta-ketoadipic acid; (*7*) levulinic acid; (*8*) succinic acid. (Lyons et al. 1984)

bacteria at the sampling sites. Further, sediment samples had a higher count of alkaliphiles as compared to the water samples (Table 6.1).

Predominant alkaliphiles growing on PPYG agar at pH 10.5 were screened for their ability to grow at various levels of alkalinity by replica plating the isolates from the master plate onto PPYG agar (Gee et al. 1980) with pH values 6.0, 8.5, 10.5 and 12.0. Alkaliphiles able to tolerate the pH range from 6.0 to 12.0 were grouped as alkalitolerant; those that were able to grow in the pH range 8.5–12.0 were alkaliphiles, while those strictly growing between 10.5 and 12.0 were considered as obligate alkaliphiles. Interestingly, the population of alkalitolerant bacteria

Fig. 6.2 Samples collected from mangrove ecosystems Goa

Table 6.1 Total bacterial counts obtained from mangrove sediment and water samples

Sediment samples	pH	Neutrophiles	Alkaliphiles
		TVC × 10^3	TVC
Palolem	8.3	69	1020
Panjim	8	680	1240
Water stations	pH	Neutrophiles	Alkaliphiles
		TVC × 10^3	TVC
Palolem	7.9	1000	30
Panjim	7.6	2415	20

TVC total viable count (per ml/g)

showed dominance in the mangrove ecosystems with 47 %, followed by obligate alkaliphiles with 28 % and the alkaliphilic population of 25 %.

Forty six obligate alkaliphiles were spot inoculated on mineral salts medium agar (MSMA) (pH 10.5) (Sangodkar and Mavinkurve 1991) incorporated with various aromatic compounds such as aniline, *p*-amino phenol, diphenylamine, N, N-Dimethyl-1-Naphthylamine (NND) and *p*-chloroaniline at a concentration of 0.05 mM.

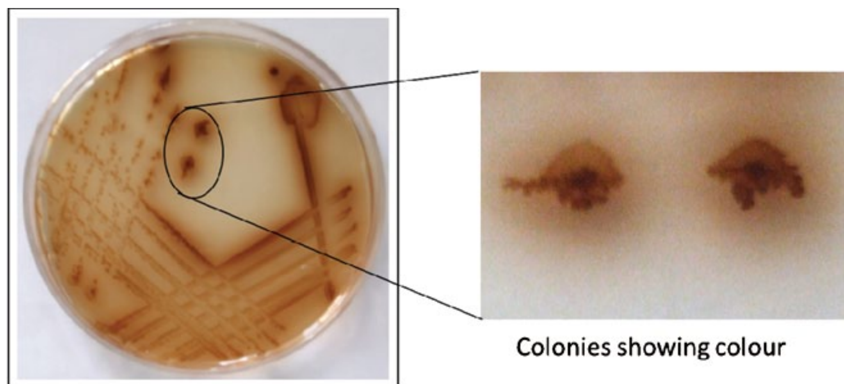


Fig. 6.3 Colour development during growth of *H. campanieinsis* NRS-01 in the presence of aniline

52 % of the isolates were found to grow in the presence of aniline, followed by 26 % each in NND and *p*-chloroaniline and 9 %in *p*-aminophenol. None of the isolates were able to grow in the presence of diphenylamine. These results show the toxicity level of the aromatic amines with an increase in the order of aniline <NND<*p*-chloroaniline <*p*-aminophenol <diphenylamine. It has been reported that the toxicity of the aromatic compounds increases with the increase in substitutions of groups onto the aromatic ring and increase in the length of the side chain (Sierra-Alvarez and Lettinga 1991).

Isolate No. 27 (designated as NK2 in this study) enriched from the mangrove sediment of the Panjim mangrove ecosystem was found to grow in the presence of most of the aromatic amines under consideration and showed tolerance and ability to remain active on a solid medium with NND. Isolate No. 24 (designated as NRS-01) was found to produce red colouration on the plate containing aniline during its growth (Fig. 6.3). These isolates were identified using physiological and biochemical tests as described by Gee et al. (1980), followed by molecular identification method based on 16s rRNA gene sequencing. The isolates were found to belong to genus *Halomonas*, with the isolate NK2 (Bankit EF 080950) closely related to *H. pacifica* (similarity 98 %) and NRS-01 (Accession no. DQ202277) closely related to *H. campanieinsis* (similarity 97 % through the Basic Local Alignment Search Tool (BLAST) analysis).

The uniqueness of these isolates is their ability to tolerate aniline and NND, respectively, with no reports on haloalkaliphilic isolates tolerating these toxic pollutants. A few reports are however available on haloalkaliphile *H. organivorans* showing its ability to utilize various simple aromatic acids (Garcia et al. 2004) including the degradation of phenol compounds through the catechol 1,2- dioxygenase pathway and protocatechuate 3,4- dioxygenase (Garcia et al. 2005; Hinteregger and Streichsbier 1997). Maltseva and Oriel (1997) reported the ability of a haloalkaliphilic *Nocardioides* sp. strain M6 able to utilize 2,4,6-Trichlorophenol.

Response of the isolates to substrates NND and aniline were further studied.

6.6.2 Growth Response Studies of *H. pacifica* Strain NK2 to NND

Growth response of *H. pacifica* NK2 to NND was carried out by growing the isolated culture in liquid and solid PPYG, half-strength Polypeptone yeast extract medium (PPY) medium and mineral salts medium (MSM) in the presence of NND (0.05 %/0.1 %). Growth was observed as turbidity coupled with visual disappearance of NND globules.

Interestingly, the growth of NK2 on PPYG agar plate containing 0.1 % NND was better as compared to growth on defined MSM agar with 0.1 % NND. Further, supplementing MSM broth with 0.05 % yeast extract enhanced growth to a significant level along with production of a biosurfactant leading to the formation of emulsion and thereby resulting in the disappearance of NND.

6.6.3 Growth Response Studies of *H. campanieinsis* Strain NRS-01 to Aniline

In the present study, *Halomonas campanieinsis* strain NRS-01 produced a red-coloured product in the presence of aniline which diffused into the agar medium (Fig. 6.3). It was therefore of interest to further study the correlation of growth response of the organism to the concentration of aniline.

Culture was inoculated in two sets of flasks with MSM broth (pH 10.5) with one set of flasks having sodium acetate (easily utilizable 3-carbon compound) and the other with glucose. 0.1 % aniline was added to one of the flasks in each set. All the flasks were incubated on a rotary shaker (180 rpm) for a period of 18 h at room temperature (30 °C ± 2). Growth was monitored as turbidity (600 nm) and the time at which the red colour appeared was noted. The addition of a simpler carbon source was aimed to study the transformation or degradation process by inducing cometabolism of aniline in the presence of sodium acetate or glucose.

Isolate *H. campanieinsis* NRS-01 showed difference in growth characteristics when incubated in different media. The flask where aniline served as a source of nitrogen and carbon did not support good growth while the flask containing glucose as an additional source of carbon in the presence of 0.1 % aniline showed good growth with marked changes with respect to colour formation occurring in the culture broth, indicating tolerance to high concentrations of aniline. Sodium acetate, on the other hand, did not support good growth in the presence of aniline as compared to glucose, though it was utilized as a sole source of carbon in the absence of aniline. These studies indicate the inability of the culture to utilize aniline as a sole source of carbon or nitrogen and require an additional carbon source to facilitate good growth in the presence of high concentration of aniline. Similar observations were demonstrated by Aranda et al. (1999) where *Pseudomonas paucimobilis* utilized glucose for the enhancement of growth and facilitated the degradation of aromatic compound 2,4,6-tricholorphenol. Cometabolisation was also demonstrated by *Pseudomonas putida* with other aromatic compounds in the presence of glucose (Horvath 1973; Reber and Kaiser 1981).

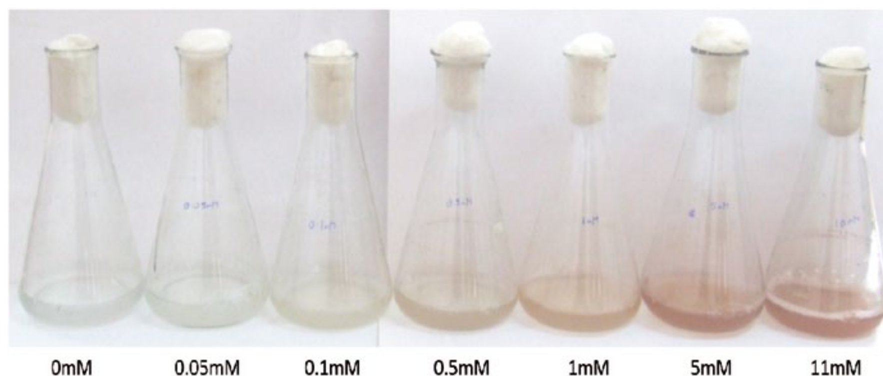


Fig. 6.4 Production of red compound in the medium with increasing aniline concentrations

The dependence of the alkaliphile *H. campanieinsis* NRS-01 on pH and salinity was monitored by growing the culture in liquid medium with pH values of 7.4, 8.5, 9.0 and 10.5 and in salt concentrations of 0, 5, 10, 15, 20 and 25 % in MSM medium incorporated with 0.1 % glucose. *H. campanieinsis* NRS-01 responded characteristically to its alkaliphilic nature, showing excellent growth in the medium with pH value 9.0 and 10.5, below which growth was grossly retarded. Although optimum growth was observed at pH 9.0 as compared to 10.5, high intensity of red colour was formed at pH 10.5. Further, the culture was found to be halotolerant and showed optimum growth at 10 % salinity.

Growth response of *H. campanieinsis* strain NRS-01 to various concentrations of aniline was undertaken by growing the cells in MSM broth (pH 10.5) having aniline at concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0,11.0 and 15.0 mM, with 0.1 % glucose.

Luxuriant growth in the form of turbidity with aniline concentrations of 0.01 mM to 11 mM was observed while further increase in the aniline concentration retarded its growth. Interestingly, although best growth was observed with aniline at a concentration of 0.05 mM, the intensity of red-coloured compound (designated as TP2 in the study) increased with an increase in aniline concentration, maximum being at 11 mM (Fig. 6.4). Significantly, an extracellular fluorescent compound (designated as FP in the study) was also detected in the culture medium until the production of TP2 by the cells. The fluorescent compound disappeared completely on further incubation butTP2 concentration was found to increase. Hence, it was of interest to monitor the production of TP2 and FP during degradation/transformation of aniline.

6.6.3.1 Degradation/Transformation Studies of Aniline

Growth of *H. campanieinsis* NRS-01 in MSM medium with 0.1 % glucose, in the presence of aniline could be divided into seven stages—(1) 0 h—experimental initiation stage, (2) 18 h—growth (as turbidity) initiation stage, (3) 28 h—a

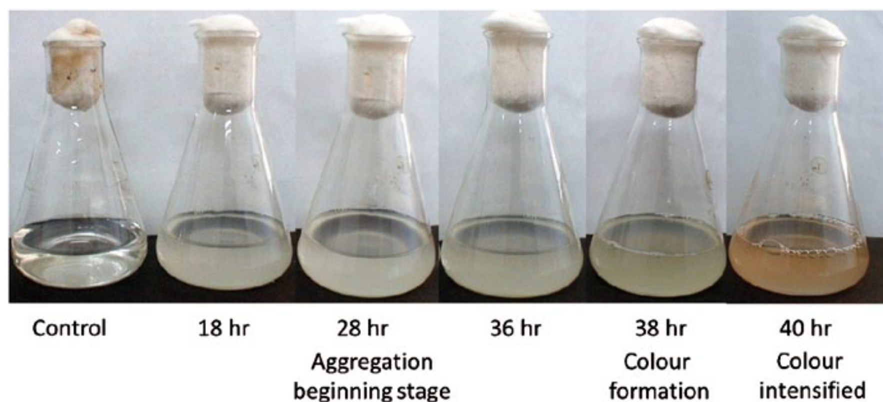
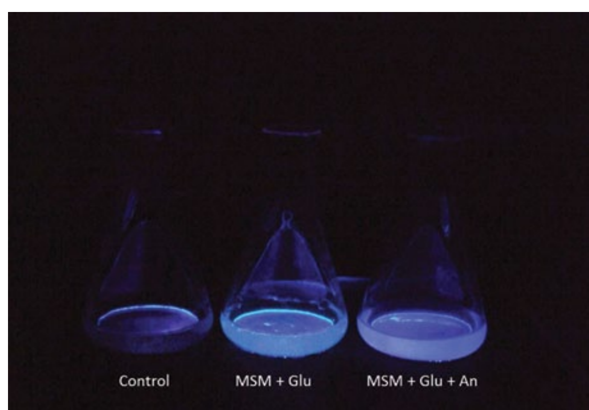


Fig. 6.5 Stages of growth of *H. campanieinsis* NRS-01 in MSM with aniline and glucose

Fig. 6.6 Flasks showing the difference in fluorescence in the presence and absence of aniline



stage where the turbidity was found to be highest (onset of stationary phase), (4) 28–30 h—bright blue fluorescence, (5) 30 h—beginning of aggregation of cells, (6) 36 h—pink colour formation and (7) 40 h—prominent red colour formation (Fig. 6.5). On centrifuging the culture broth, the colour appeared on the cells during 36th h of incubation which intensified further. After 40th h of incubation, a deep red colour was found on the cell pellet with no pink colouration visible in the supernatant thereby projecting the role played by the cells in the production of the red compound. Further, the culture medium, in which the isolate *H. campanieinsis* NRS-01 was grown with 0.01 or 0.05 Mm aniline, did not show the presence of the fluorescent or red compound, indicating their production specifically at high concentrations of aniline. Growth in glucose alone gave a greenish fluorescence as compared to bright blue fluorescence (Fig. 6.6) when grown in the presence of high concentration of aniline. Comparing glucose concentration in the supernatant with that of aniline suggested the use of glucose as a primary source of carbon.

Results thus suggested nonutilization of aniline at a high concentration (11 mM), but inducing the cells to form aggregates and produce a blue fluorescent and red hydrophobic compound as a defence strategy towards aniline. Further, these products were observed only in the presence of cells indicating the role of *H. campanieinsis* NRS-01 in their formation. Such compounds are known to be produced during the growth of organisms in response to various stress conditions as signature molecules (Borchardt et al. 2001; Camara et al. 1998; Chen et al. 2004) and quorum-sensing molecules (Atkinson et al. 1999; Burgess et al. 2002; Miller and Bassler 2001), especially in the microbial community structure.

6.6.3.2 Characterisation of Compounds (FP and TP2) Produced in Response to Aniline

For production and characterisation of fluorescent compound (FP), isolate *H. campanieinsis* NRS-01 was inoculated in 1.0 litre MSM with 11 mM aniline supplemented with 0.1 % glucose and incubated at 30 °C on a rotary shaker at 180 rpm. Cells were harvested before the formation of TP2, i.e. between 30 and 32 h of incubation and culture broth was centrifuged at 10,000 rpm. Fluorescent blue colour observed on the incidence of UV-light confirmed the presence of FP in the broth. The supernatant was filtered through a 0.22 μm Millipore membrane filter and used for extraction of FP by evaporation-solvent extraction technique involving concentration of the filtrate at 60–70 °C to yield a resultant volume of 15 ml which was repeatedly extracted with isopropanol. Isopropanol extracts were pooled, dried over sodium sulphate and vacuum evaporated to dryness. The resultant compound was dissolved in a minimum amount of acetone and stored at 4°C.

Purification of fluorescent compound was carried out by column chromatography (Shriner et al. 1980) using petroleum ether: acetone with a sequential increase of acetone concentration from 2 to 30 %. The molecular weight of the isolated, purified FP was determined by carrying out filtration through Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-3 membrane which had a cut-off molecular weight of 3 KDa. Characterisation was done using various methods including Bradford's (Bradford 1976) and the bicinchoninic acid assay (BCA) (Smith et al. 1985) method.

The product, FP, was found to be highly water soluble and tolerant to high temperatures of 60–70 °C without losing its profound fluorescence. Characterisation of the compound indicated it to be a protein as per Bradford's and BCA method. Further, the molecule was found to be less than 3 kDa indicating it to be a low-molecular-weight protein. The role of stress-induced proteins has been significantly established in response to various pollutants present or under stressful environmental conditions. Stress-induced protein produced by *Burkholderia xenovorans* LB400 (Denef et al. 2005) has been attributed to the presence of benzoates and biphenyls and brings about detoxification of these aromatic compounds. Low-molecular-weight proteins have also been reported to be produced

by bacteria in response to stress conditions such as toxicity due to heavy metals (Narender and Prasad 1990). Higham et al. (1986) also reported cadmium-binding proteins produced by *P. putida* having a low molecular weight of 3.5–7 kDa. Su (2005) has studied the presence of low-molecular-weight fluorescent proteins in nature and their applications in biotechnology. Green fluorescent protein (GFP) isolated from a jellyfish *Aquorea victoria* is used as an important tool to study recombinant proteins. Studies on aniline toxicity in organisms have revealed various mechanisms of detoxification which involve either acetylation of the amino group converting aniline to acetanilide and formanilide analogues or transformation of aniline to indole analogues, which complex to give high-molecular-weight compounds (Shanker et al. 2006; Tweedy et al. 1970).

In the present study, the production of FP appears to be a mechanism of protection for the cells towards the exposure of toxic aniline since the appearance of FP is seen prior to aggregation. Bacterial aggregation is an important process which gives protection to the cultures from toxic chemicals and antimicrobials. This has been demonstrated in *Pseudomonas* in response to antibiosis and cold shock (Farrell and Quilty 2002; Hoffman et al. 2005; Klebensberger et al. 2006).

For the production and characterisation of TP2 from cells, isolate *H. campanieinsis* NRS-01 was inoculated in 1.0 litre MSM broth with 11 mM aniline supplemented with 0.1 % glucose and incubated on a shaker at room temperature (30 °C) at 180 rpm for 72 h. Cells were harvested by centrifuging the culture broth at a speed of 10,000 rpm, washed twice with 0.1 M phosphate buffer (pH 8.0) and resuspended in the same buffer.

Extraction of TP2 was carried out by layering the cell suspension with toluene in a conical flask and incubating the flask on a rotary shaker (60 rpm) for a period of 48 h. The organic layer containing the red compound was separated, dried over sodium sulphate and evaporated using a combination of evaporation and drying under Nitrogen. Purification was brought about using preparative thin-layer chromatography (TLC) (Dhar and Rosazza 2000) and flash chromatography. Compound TP2 was analysed for carbon, hydrogen and nitrogen using an automated elemental Carlo-Erba 1100 analyser and spectroscopic techniques such as UV1601 shimadzu spectrophotometer, BRUKER AC-200 instrument for nuclear magnetic resonance (NMR) spectrum, Shimadzu FTIR-8000S for infrared (IR) spectrum, Gas Chromatography-Mass Spectrometry (GC-MS) Shimadzu.

Compound TP2 was found to be a water insoluble product and changed to yellow-green when acidified with 1N HC1 and turned back to light red when neutralized with 1N NaOH. This reaction is normally used as a spot test for quinones (Finley 2010). Further, the elemental analysis showed 68.86 % C, 4.94 % H, and 13.48 % N. Spectrophotometric scan showed an absorbance maximum of the red colour at 520 nm. The possible empirical formula derived from the elemental analysis and the mass spectrum was found to be $C_{21}H_{18}N_3O_3$. Furthermore, on comparing the mass distribution on the chromatogram with the mass spectra present in the library, the compound TP2 was found to show a 61 % similarity to a basic steroidal structure.

Reports on aniline transformation resulting in (3E)-14-Hydroxy-3,7,11-trimethy1-3,5-tetradecadieny1-2-(hydroxymethyl)-1Hindole-3-carboxylate, a high-molecular-weight compound of 443 KDa has been reported by (Shanker et al. 2006). Thorn et al. (1996) reported that in the absence of catalysts, aniline undergoes nucleophilic addition reactions with the carbonyl functionality of the fulvic and humic acids and becomes incorporated in the form of anilinohydroquinone, anilinoquinone, anilide, heterocyclic and iminenitrogens. Characterisation of the red compound (TP2) produced by *H. campanieinsis* NRS-01 in response to aniline during growth on glucose also appears to follow a similar path. It is interesting to note that the reactions taking place under alkaline and neutral conditions are not different. The general mechanisms of detoxification by developing resistance and tolerance to aniline appear to be similar. However, based on the various analytical spectral data procured, further research on complete characterisation of this compound is in progress and the complete structure of the compound is yet to be elucidated.

6.7 Conclusions and Future Prospects

The studies with biotransformation of N, N-Dimethy1,1-Naphthylamine and aniline by bacteria under alkaline conditions have shown that the microorganisms detoxify pollutants in the manner, which is common for the cellular systems irrespective of the environmental conditions. To our knowledge so far, there have been no reports on alkaliphiles tolerating high concentrations of NND and aniline.

Alkaliphiles are a diverse group of bacteria having an enormous potential to survive and grow at pH conditions likely to kill other neutrophilic bacteria. Such characteristics are best suited for developing industrially important bacterial strains for the production of commercially important molecules. From the present work carried out, two such potential organisms able to grow at pH 10.5 and high salt concentration have been isolated, showing the production of biosurfactant and coloured compounds on their interaction with NND and aniline, respectively. Future research on these cultures may be directed towards:

1. Characterisation of biosurfactant produced by *H. pacifica* NK2
2. Use of the blue fluorescent compound (FP) produced by *H. campanieinsis* NRS-01 as a bioindicator for early detection of hydrocarbon pollution
3. Identification of the red compound TP2 produced by NRS-0 1 as a medically important compound as quinones are used as anticancer drugs

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Chapter 7
Insights into Organic-Solvent-Tolerant Bacteria and Their Biotechnological Potentials

Yogita N. Sardessai

7.1 Introduction to Organic-Solvent-Tolerant Bacteria

Organic solvents are known to be extremely toxic to microbial cells, even at low concentrations of 0.1 % (v/v). Solvents are known to accumulate in and disrupt the bacterial cell membrane, thus affecting the structural and functional integrity of the cell (Inoue and Horikoshi 1989). Although there are some microorganisms which can assimilate these toxic organic solvents, they do so only when the solvent concentrations are very low (Ramos et al. 1997). Any medium containing organic solvents seems to be an extreme environment for microorganisms and hence for many years, it was believed that no microorganism could withstand such a harsh environment (Bar 1987; Cartwright 1986). The discovery of several *Pseudomonas* species not only able to withstand the severe stress caused by solvents but also capable of thriving in such environments led to the coining of the term "OSTB" or organic-solvent-tolerant bacteria.

7.1.1 History

Organic-solvent-tolerant bacteria (OSTB) were historically reported first by Inoue and Horikoshi in 1989 who isolated a strain of *Pseudomonas putida* named IH-2000 from mud samples which could actively grow and multiply in the presence of 50 % v/v toluene. This surprising observation was confirmed by several other scientists who discovered more microbes with amazing levels of solvent tolerance and a new, academically interesting and industrially significant category of extremophiles was created (Aono et al. 1994; De Bont 1998; Heipeiper et al. 1994; Huertas and Duque 1998; Isken and de Bont 1998a; Kobayashi et al. 1998; Pinkart et al. 1996; Sardessai and Bhosle 2002a, b).

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Studies done on the toluene tolerance of certain *Pseudomonas* strains led to an understanding of the mechanisms of organic solvent tolerance, including novel adaptations such as toluene efflux pumps, *cis-trans* isomerisation of membrane fatty acids, rapid membrane repair mechanisms among others (Ramos et al. 1997, 1998; Weber et al. 1993). In addition to the scientific significance of these microbes, they are also known to have tremendous potential in industrial and environmental biotechnology (De Bont 1998; Sardessai and Bhosle 2004). This is because their enzymes preserve function in the presence of organic solvents.

Incidentally, the first few OSTB to be reported were species of *Pseudomonas* such as *P. putida* IH-2000*, P. aeruginosa* ST-001*, P. putida* S12*, P. putida* DOT-T1 (Heipeiper et al. 1994; Huertas and Duque 1998; Isken and de Bont 1998a; Pinkart et al. 1996) which led to a misconception that only *Pseudomonas* species and Gram-negative bacteria were capable of exhibiting such tolerance. However, it has now been established that a wide variety of Gram-positive as well as Gram-negative bacteria exhibit solvent tolerance (Alex and Bhat 2012; Sardessai and Bhosle 2002b; Segura et al. 2012). In fact, recent reports suggest that Gram-positive bacteria dominate in benzene, toluene, ethyl benzene and xylene group of compounds (BTEX) degradation in hydrocarbon saturated sites (Alvarez 2012). Other solvent-tolerant genera now known include *Acinetobacter, Aromatoleum aromaticum, Arthrobacter, Bacillus, Brevibacillus, Burkholderia, Enterobacter, Escherichia coli, Moraxella, Providencia, Rhodococcus* and *Staphylococcus hemolyticus* (Torres et al. 2011). Recent reports confirm the belief, that OSTB have a stunningly versatile and resistant repertoire of enzymes which can play a vital role in enzymic processes and revolutionize areas such as nonaqueous biocatalysis, environmental and industrial biotechnology (Nicolau et al. 2012). Some reported strains of OSTB and their characters are as presented in Table 7.1.

7.1.2 Occurrence and Distribution of OSTB in Nature

OSTB have been isolated from forest, garden and humus soils. Soil and marine environments have been contaminated for decades with hydrocarbons and hence have yielded many OST strains. Kato et al. (1996) have reported that OSTB are over 100 times more abundant in deep sea mud samples than in terrestrial soils. In their studies, incubation of artificial sea water containing deep sea sediment with 50% v/v benzene led to the isolation of OSTB on plates from the benzene layers after incubation. Using this procedure, several strains of Gram-positive bacteria were isolated such as *Bacillus* DS994, *Bacillus* DS 1906, *Arthrobacter* ST1, etc., which were found to degrade sulphur compounds, polyaromatic hydrocarbons and cholesterol, respectively (Kato et al. 1996). Species of *Staphylococcus hemolyticus, Enterococcus feacalis* and *Clostridium sporogenes* have been isolated from the larval guts of oil flies (*Helaeomyia petrolei*) which grow submerged in oil and ingest large quantities of oil and asphalt (Kadavy et al. 2000).

Table 7.1 Some reported strains of OSTB and their characteristics. (Horikoshi 2011; Kato et al. 1996; Kadavy et al. 2000; Sardessai and Bhosle 2002a, 2003)

Psuedomonas putida IH-2000	First reported OSTB, isolated from mud samples, multiplying in 50% v/v toluene
Pseudomonas putida DOT-T1E	Degradation of toluene and its metabolism at 0.1 to 90% v/v in culture medium
Pseudomonas putida S12	Degradation of styrene, octanol and heptanol
Pseudomonas putida ST-491	Production of steroid hormone precursors from lithocholic acid
Burkholderia cepacia ST-200	Oxidative modification of cholesterol in the presence of 10% solvent
Acinetobacter ST-550	Produces indigo from indole in the presence of 3–30% diphenylmethane
Staphylococcus hemolyticus	From oilfly larval gut
Providencia rettgeri	From oilfly larval gut resistant to organic solvents and antibiotics
Morganella morganii	From oilfly larval gut resistant to organic solvents and antibiotics
Bacillus DS994 and DS1906	Isolated from deep sea sediment growing in 50% v/v benzene, degrade polyaromatic hydrocarbons
Bacillus BC1	Isolated from cholesterol agar from mangrove sediment, degrades cholesterol, tolerant to chloroform
Bacillus SB1	Isolated from mangrove sediment by butanol enrichment, the lowest index value of 0.8

7.1.3 Adaptation of Laboratory Strains to Organic Solvent Tolerance

Several laboratory strains have been found to be adapted to high solvent concentrations such as *P. putida* Idaho to p-xylene and *P. putida* S12 to styrene (Cruden et al. 1992; Kato et al. 1996). It has been found that *Pseudomonas* strains growing in the presence of short fatty acids like acetates have a lower membrane fluidity which prepares them for growth in the presence of saturated levels of nonmetabolisable solvents like toluene. *Clostridium thermocellum* strain 27405 was able to adapt to alcohol when sequentially transferred into media containing increasing quantities of ethanol (Islam et al. 2009). Using this adaptation, cells tolerating up to 80% v/v to ethanol were created. Construction of *E. coli* mutants tolerant to cyclohexane and p-xylene has been done from parents having solvent-sensitive phenotype (Aono and Kobayashi 1997; Asako et al. 1997; Doukyu et al. 2012; Watanabe and Doukyu 2012).

7.1.4 Analysis of Organic Solvent Tolerance

Traditionally, solvent tolerance plate assays have been used to determine solvent tolerance. Other methods generally used include the use of C14-labelled toluene to determine efflux used in *P. putida* S12, gene cloning and mutagenesis in *E. coli* strains, electron microscopy to detect the phenomenon like solvent accumulation, filamentation and changes in the cell envelope. Efflux inhibitors and ionophors have been used to study the effect of efflux pump inhibition. Bacterial adherence to hydrocarbon (BATH) assays has been used to determine cell surface hydrophobicity. Scientists have proposed a cell growth assay using tetrazolium violet and image analysis to determine solvent tolerance in *E. coli* OST3410 and have studied surface characteristics of cells exposed to organic solvents using contact angle measurements, Z potential and fluorescence microscopy analysis (Nicolau et al. 2012; Torres et al. 2011).

7.2 Physiological Basis of Solvent Toxicity

Tolerance to toxins by genetic adaptations or acclimatization as a response to living in contaminated environments has been observed in microbes exposed to different chemicals. Traditionally, alcohols and phenols in natural oils have been used as antimicrobials (Cartwright 1986, Ingram and Buttke 1984).

To predict organic solvent tolerance based on physicochemical properties, polarity and molecular size of the solvent are two important properties in a biphasic system. Polarity can be determined by the Hildebrand solubility parameter and size by molar volume or molecular weight. It was proposed that in this system, high biocatalytic rates could be obtained using organic solvents with polarity <8 and high molecular weight $M>150$. Later, the log P concept was devised to explain organic solvent toxicity (Banerjee et al. 1980; Brink and Tramper 1985; Inoue and Horikoshi 1989).

In bacterial cells, the cell membrane, a phospho-lipid bilayer is a matrix in which various enzymes and transport proteins are embedded. It plays a vital role in solute transport, maintaining energy system of the cell, regulation of the intracellular environment, turgor pressure, signal transduction and energy-transducing processes. Solvents partition into and disrupt the lipid bilayer compromising cell viability (Sikkema et al. 1995). It has been proved that, not only the chemical structure of the solvent, but also the degree to which it accumulates in the cell membrane plays a crucial role in cellular toxicity (Heipeiper et al. 1994; Sikkema et al. 1995).

Physiological investigations of microbes have revealed a correlation between solvent tolerance and its log P value. The parameter log P is defined as the partition coefficient of the given solvent in an equimolar mixture of octanol and water (Inoue and Horikoshi 1989, 1991; Isken and de Bont 1998b; Torres et al. 2011).The greater the polarity of the solvent, the lower the log P value and greater the toxicity.

Table 7.2 Organic solvents and their log *P* values. (Sikkema et al. 1995)

Organic solvent	Log *P* value
n-decane	5.6
Decalin	4.8
Diphenyl ether	4.3
Cyclooctane	4.2
Propyl benzene	3.8
Tetralin	3.8
Hexane	3.5
Cyclohexane	3.2
Ethyl benzene	3.1
p-xylene	3.0
Styrene	3.0
Octanol	2.9
Toluene	2.5
Benzene	2.0
Chloroform	2.0
Butanol	0.8

This is because the more polar solvents partition into the aqueous phase to a greater extent, penetrate into and accumulate in the lipophilic cell membranes of bacteria causing lethal damage (De Bont 1998). Generally, solvents with log *P* values below 4 are extremely toxic as their degree of partitioning into the cell containing aqueous layer and into the lipid membrane bilayer is high. Every organism has its own intrinsic tolerance level for organic solvents which is determined genetically and is also influenced by environmental factors (Kobayashi et al. 1998). In 1994, Sikkema et al. proposed an equation to correlate the log PO/W of a solvent and its partitioning between membrane and water as log P m/w = 0.97 × log P o/w-0.64. Hydrophobic solvents having log $P>4$ do not reach high membrane concentrations due to low water solubility though they accumulate. Log *P* 1–4 solvents present higher water solubility levels and partition to biomembranes (Table 7.2). The organic-solvent-tolerant strains reported in literature have index values of 3 (*E. coli* mutants tolerant to xylene) (Aono and Kobayashi 1997), 2.5 (*Pseudomonas* strains tolerant to toluene) and 2 (some Gram-positive strains tolerant to benzene; Kato et al. 1996).

The tolerance level of each microorganism is represented by two terms: index solvent and index value. The index value is the log *P* value of the most toxic organic solvent (called index solvent) among those that can be tolerated by the organism. Each bacterium can grow on agar medium overlaid with any of the solvents having log *P* greater than the index value. However, under such conditions, bacterial growth will be suppressed by organic solvents having a log *P* value close to the index value (Heipeiper et al. 1994; Isken and de Bont 1998b; Kobayashi et al. 1998; Sardessai and Bhosle 2002a, b). Mechanisms of solvent tolerance are listed out in Table 7.3.

Table 7.3 Mechanisms of organic solvent tolerance. (Sardessai and Bhosle 2002b; Torres et al. 2011)

1	Active efflux pumps for solvent removal
2	Modification of cell membrane by cis-trans isomerisation of fatty acids and changes in membrane proteins
3	Faster rate of membrane repair
4	Cell surface modifications such as increased or decreased hydrophobicity
5	Unusual extracellular capsules which may promote clumping and aggregation
6	Presence of solvent deactivating enzymes and emulsification of the solvent
7	Changes in cellular morphology such as decrease in cell surface to volume ratio and cell filamentation
8	Formation of membrane vesicles attached to solvent molecules for exclusion
9	Involvement of stress regulons and production of stress response proteins

7.3 Biotechnological Potential

OSTB have tremendous scope in the bioremediation of petroleum contamination and other toxic chemicals, biofuel manufacture, nonaqueous biocatalysis, whole-cell biphasic (organic-aqueous) biotransformations, particularly of poorly soluble compounds like steroids and many other areas which require withstanding a solvent-saturated environment (Liszka et al. 2012).

7.3.1 Role of OSTB in Bioremediation—Their Ecological Impact

P. putida strains are among the most tolerant bacteria with high tolerance to toluene and other solvents. Different putida strains have been reported to grow on toluene via the benzyl alcohol pathway (*P. putida* Idaho) or the toluene deoxygenase pathway (*P. putida* DOT-T1E; Huertas and Duque 1998; Isken and de Bont 1998a).

Recently, several extraordinary strains of *Bacillus* isolated from estuarine and petroleum-contaminated habitats have emerged displaying great potential in bioremediation (Edwards et al. 2012). Photosystem II (PSII) strain of *Bacillus thermophilus* has been reported which showed adaptation based on the reorganization of the cell membrane and metabolic transformation of the hydrophobic solvents to less toxic compounds (Sarkar and Ghosh 2012). *Bacillus aquimarius* decontaminates organic pollutants showing excellent functional stability on enzymes in benzene, methanol, acetone, toluene and heptanes (Trivedi et al. 2011). It has been recently proposed that *Bacillus subtilis* is more tolerant than *P. putida* towards fullerene-based nanomaterials. It is also stated that Gram-positive bacteria dominate in benzene, toluene, ethyl benzene and xylene group of compounds (BTEX) degradation in contaminated sites (Alvarez 2012). Several strains of *Bacillus, Burkholderia, Deinococcus, Deinococcus geothermalis* T27*, Enterobacter* and *Nocardiodes* are shown to have excellent solvent tolerance. *Rhodococcus erythropolis* strain DCL14 can tolerate up to 52% v/v toluene (Nicolau et al. 2012; Torres et al. 2011).

7.3.2 Role in Biofuel Production

When the cell physiology is routinely exposed to certain toxic chemicals, programmed responses are expected as part of the cells evolutionary adaptation and it is believed that such responses would provide tolerance and protection to the cells. Studies have been done on *Clostridium acetobutylicum* ATCC824 to improve solvent stress. Screening of plasmid-based genomic DNA libraries and microarray analysis has resulted in the increase of up to 13 % and 81 % butanol tolerance based on batch culture and serial enrichment. In another study in the same culture, overexpression of groESL in a plasmid under the thiolase promoter has led to decreased inhibition by butanol and increased solvent titres also allowed prolonged metabolism under butanol stress. In *E. coli* cells, global transcription machinery engineering (gTME) of sigma factor 70 increased ethanol tolerance of the culture up to 60 g/l. Detailed understanding of ethanol and butanol tolerance traits can play an important role in boosting biofuel production. In this context, OSTB can serve as role models in metabolic engineering and synthetic biology (Islam et al. 2009; Nicolau et al. 2012)

7.3.3 Medical Significance as Potential Superbugs

It is interesting to note the medical significance of the versatility of efflux pumps and their impact on conferring a survival advantage to OSTB by conferring antibiotic resistance to them. *Helaeomyia petrolei* (oil fly) larvae inhibit the asphalt seeps of Rancho La Brea in Los Angeles, California. Kadavy et al. (2000) examined the culturable microbial gut contents of larvae collected from the viscous oil where nine of fourteen strains were identified as *Providencia* sp. These nine strains classified as *Providencia rettgeri* exhibited dramatic resistance to tetracycline, vancomycin, bacitracin, erythromycin, novobiocin, polymixin, colistin and nitrofurantoin. All 12 isolates were sensitive to nalidixic acid, streptomycin, norfloxacin, aztreonam, pipericillin and cefotaxime. On preexposure to 20 mg of tetracycline per ml, seven of nine oil fly bacteria tolerated overlays of 50 % xylene and 50 % cyclohexane. The correlation between antibiotic resistance and organic solvent tolerance is explained by an active efflux pump maintained in oil fly bacteria owing to the constant selective pressure of La Brea's solvent rich environment. Hence, the oil fly bacteria and their genes for solvent tolerance appear to offer a microbial reservoir of antibiotic genes (Kadavy et al. 2000).

Edwards et al. (2012) have proposed that, due to the dual presence of organic solvents and heavy metals in petroleum-contaminated sites, tolerance mechanisms can be inter-related and activation of one will increase the chances of multiantimicrobial extrusion and disease-causing abilities. Further, antibiotic and heavy metal potentiation studies for solvent tolerance have revealed that antimicrobials can activate the efflux system which in turn enhances solvent tolerance. This was determined by incubating the cultures with antimicrobials below their minimum inhibitory concentration values for 6 h at 37 °C in microtitre plates. Krist et al. (2008)

Table 7.4 Examples of some extremely organic solvent stable enzymes (Liszka et al. 2012)

Protease subtilisin E from *Bacillus subtilis*—activity increase 470-fold in the presence of 60% dimethyl sulfoxide
Protease subtilisin BPN *from Bacillus amyloliquefaciens*—active in neat organic solvents
Protease from *Bacillus licheniformis* RSP-0937—shows high transesterification activity in neat organic solvents
Esterase from *Pyrobaculum calidifontis* VA1—stable in up to 80% of organic solvents
Lipase A from *Bacillus subtilis*—exhibits increased half-life 100-fold in 50% organic solvents
Protease PST-01 from *Pseudomonas aeruginosa*
Protease PT-121 from *Pseudomonas aeruginosa* isolated from the organic solvent host—shows high peptide synthesis

have demonstrated that inactivation of efflux pumps abolishes bacterial biofilm production. These studies prove that organisms in contaminated sites could use efflux pumps to survive solvent shock and develop antibiotic resistance. The simultaneous activation of solvent tolerance and antimicrobial resistance may thus lead to the development of superbugs. The promiscuity of multidrug efflux pumps and their role in OST of *P. putida* had been reported by Li et al. in 1998. The fact that a bacterial single colony transforms to confluent growth in the presence of organic solvents confirms that pretreatment of bacteria with tetracycline antibiotics greatly enhances solvent tolerance. This interlinked phenomenon is vital as exposure of pathogens to organic solvent contamination could promote resistance to antibiotics resulting in a need to redesign disinfection protocols (Oeithinger et al. 1998).

7.3.4 Biocatalysis in Extreme Conditions: Enzymes of OSTB

Extremophilic native enzymes have evolved naturally under extreme stress conditions and are designed to function efficiently in the most hostile environments (Table 7.4; Liszka et al. 2012; Pandey and Singh 2012; Sardessai and Bhosle 2003, 2004). Bioprospecting for such microorganisms has thus led to the discovery of new enzymes with high tolerance to nonnatural conditions (Pandey and Singh 2012; Trivedi et al. 2011). However, there are evolutionary constraints to bioprospecting. Protein engineering has been successfully used to generate extremophilic enzymes by rational mutagenesis and directed evolution. The trait of solvent tolerance also imparts tolerance to end products such as ethanol, acetone, butanol, butane-diol and organic acids produced during pentose metabolism. When such strains are used in fermentation, the adverse effects of end products on the performance of these economically significant bioprocesses are automatically resolved (Brink and Tramper 1985; Nicolau et al. 2012).

A classic example is lipase LST-03 isolated from solvent-tolerant *Pseudomonas aeruginosa* which is stable in the presence of 25% organic solvents at 30°C and stabilized tenfold by directed evolution. The gene has also successfully been cloned into *E. coli* for recombinant expression (Liszka et al. 2012).

P. putida strain S12, a well-studied solvent-tolerant bacterium has been proposed as a platform strain for production of many chemicals (Fei et al. 2012). Extensive research have been done in the field of nonaqueous biocatalysis and enzymes in biphasic systems (Brink and Tramper 1985; De Bont 1998; Liszka et al. 2012; Nicolau et al. 2012; Sardessai and Bhosle 2004; Torres et al. 2011) There is a lot of interest towards designing a semisynthetic stress response system to engineer solvent tolerance and bioprospecting extremophiles with such interesting properties is gaining tremendous importance (Ni et al. 2013; Nicolau et al. 2012; Oh et al. 2012; Torres et al. 2011; Zingaro and Paptsakis 2013).

7.3.5 Role of OSTB in Nonaqueous Biocatalysis and Whole-Cell Biotransformations in Organic-Aqueous Biphasic Systems

The vast majority of synthetic chemistry reactions are performed in synthetic media and nonaqueous biocatalysis offers several unique advantages such as increased solubility of nonpolar substrates and products, reversal of thermodynamic equilibrium in favour of synthesis as opposed to hydrolysis, drastic changes in enantio-selectivity of the reaction when the organic solvent is changed, suppression of unwanted water-dependent side reactions and elimination of microbial contamination. Unfortunately, most enzymes exhibit diminished activity in the presence of organic solvents. Studies have been conducted to enhance the OST status of *E. coli* as a strategy to improve usability of whole-cell biocatalysts in a 2-phase system using organic solvents. Whole cells of OSTB have also been used to catalyse reactions in organic-aqueous biphasic systems for steroid transformations.

7.3.6 Biotransformation of Steroids and OSTB

For a biotransformation to occur, the ideal substrate should be soluble in the fermentation medium and pass the cell membrane without being toxic to the organism. The major rate-limiting factor in the biotransformation process is the extremely poor dissolution of steroids in water (10^{-2}–10^{-3} g/ml), which lowers the transformation rates and increases costs. It is, therefore, desirable that solubility conditions be improved during biotransformation (Arima et al. 1969; Arinbasarova and Koskcheenko 1983).

Steroids are a diversified class of oxygenated tetracyclic isoprenoid derivatives bearing a ring system consisting of four fused rings that are vital in many ways to the life of a eukaryotic organism. Although it is possible to produce nearly all molecular weight compounds by organic synthesis, production routes of stereo-specific compounds are always very complicated and expensive. To meet such a large demand for a diverse range of products, microbiological transformations play a vital role and are employed when a given reaction step is not easily accomplished by chemical means (De Bont 1998; Doukyu and Aono 1998; Sardessai and Bhosle

2004). Today, an increasing number of pharmacologically active steroids are annually produced in the industry through the initial microbial transformation of sterols (Smith 1984) and biotransformation of steroids is now a multimillion dollar industry having numerous pharmaceutical uses.

Steroids like cholesterol are completely soluble in organic solvents such as *n*-alkanes, cyclohexane, toluene, benzene, carbon tetrachloride, chloroform, methylene chloride, ethyl or butyl acetate, diethyl or dibutyl ether, butanol, etc., and have been used with intact cells and enzymes. An ideal organic solvent (1) should be practically immiscible in water (2) should exhibit high solubility for the product (3) should not exert an inhibitory effect on the biocatalyst and (4) nonflammable solvents are preferred for reasons of safety. Hence, an organic-aqueous biphasic bioconversion system, wherein the cells are present in the aqueous phase and steroids dissolved in the organic phase is an ideal set-up. The major problem here is the fact that most bacteria and their enzymes are inactivated or destroyed in the presence of these toxic organic solvents even in low concentrations (Aono et al. 1994; Moriya et al. 1995). This problem can be overcome by using OSTB which can carry out the desired biotransformations in an organic solvent-saturated system. There are several advantages in using OSTB to catalyse steroid transformations in bi-phasic organic-aqueous bioconversion systems, since the problems arising from the poor water solubility of steroids can be solved by using an organic phase, and there is ample proof to show that enzymes of OSTB can perform stably in the presence of an organic solvent (Aono et al. 1994; De Bont 1998; Doukyu and Aono 1998; Ogino et al. 1995; Sardessai and Bhosle 2004).

7.4 Studies on OSTB from Tropical Estuarine Ecosystem of Goa

When our work on organic-solvent-tolerant microorganisms from coastal marine and estuarine habitats began in 1999, the only OSTB known were predominantly strains of *Pseudomonas* and certain *E. coli* strains. Toluene tolerance of *Pseudomonas* strains was a well-documented fact.

Gram-positive OSTB were rare and a few reports of *Bacillus*, *Arthrobacter* and *Rhodococcus* were just beginning to emerge (Moriya et al. 1995; Paje et al. 1997). Kato et al. (1996) had demonstrated that the marine sediments are a rich source of novel OSTB, particularly Gram-positive species. The Indian state of Goa has a very extensive tropical mangrove forest association with rich biodiversity. Hence, estuarine samples collected from Mandovi estuary, Goa were used in this study (Fig. 7.1). The aim was the isolation and identification of *n*-butanol-tolerant bacteria since butanol is one of the most toxic solvents and bacteria tolerant to it were not reported earlier. The existence of cholesterol transforming isolates for the development of a suitable organic-aqueous biphasic fermentation system was also probed. Since organic solvents are a good dissolution medium for cholesterol, and OSTB can not only withstand solvent toxicity but also transform cholesterol in the presence of

Fig. 7.1 Sampling site in the Mandovi estuary

organic solvents, this work was carried out. In addition, since Gram-positive OSTB were extremely rare at that point and no information was available on their possible resistance, investigations were also carried out to reveal the mechanisms of organic solvent tolerance.

7.4.1 Isolation and characterization of Estuarine n-Butanol-Tolerant Bacteria

The relative toxicity levels of solvents are designated by the parameter log *P* which depends on the polarity of the given solvent and its tendency to penetrate into and disturb the cell membrane. Butanol, an amphiphilic alcohol having a log *P* value of 0.8 is one of the most toxic solvents for bacterial cells. Hence, an attempt was made to isolate *n*-butanol- tolerant bacteria by enrichment culture of mangrove sediment soaked with butanol.

This procedure led to the isolation of a single bacterial culture designated as SB1 in the study which was purified, maintained on Luria agar, characterized and identified (Sardessai and Bhosle 2002a). The isolate produced capsulated, large, wrinkled, mucoid, creamish white colonies. The colony appearance changed with age, with mucoid colonies acquiring a dry wrinkled creamish appearance on Luria agar supplemented with magnesium and glucose (LBMG). Significantly, the isolate showed growth at 50 °C with 7 % NaCl, tolerating direct exposure to undiluted butanol in the plate assay (Kobayashi et al. 1998; Ogino et al. 1995) where cultures are streaked on LBMG agar and submerged with the respective solvent and incubated.

Appearance of colonies signifies resistance to the solvent and 50 % (v/v) butanol in liquid medium. Identification studies revealed that SB1 is a Gram-positive, aerobic, catalase positive, endospore-producing rod, identified as *Bacillus subtilis* on the basis of morphological, physiological and biochemical characteristics, as per Bergey's *Manual of Systematic Bacteriology*. The estuarine isolate *Bacillus* SB1 eventually isolated by butanol enrichment of mangrove sediment has the lowest ever reported index value for OSTB (0.8) which is a unique trait.

Effect of butanol concentration on the growth of *Bacillus subtilis* SB1 indicated good growth up to a concentration of 3 % of butanol above which there was a severe retardation. The minimum inhibitory concentration of butanol for *B. subtilis* SB1 was found to be between 3 and 4 % (v/v) butanol, as there was 97.2 % reduction in growth at 3 % butanol and no growth was seen at 4 % (v/v). The decrease in yield with increase in butanol concentration was attributed to damage induced by butanol and involvement of energy-consuming adaptations to counter solvent stress (Sardessai and Bhosle 2002a). It has been proved that it is not the chemical structure of the solvent, but the dose to which it accumulates in the cell membrane which is the deciding factor governing lethality of the solvent (Isken and De Bont 1998b). It is clear from the studies done here that, above a concentration of 3 % (v/v), the dose of butanol accumulated in the membrane of *B. subtilis* SB1 is capable of inducing significant damage, thereby causing retardation of growth.

Since *B. Subtilis* SB1 was an endospore-producing culture, the role of spores in butanol tolerance was investigated. The process of sporulation was found to be sensitive to the presence of butanol (Sardessai and Bhosle 2002a).This is in accordance with earlier reports on other strains of *Bacillus* which have proved that sporulation process in bacteria is sensitive to the presence of alcohols, with spores surviving in alcohol for long durations (Ingram and Buttke 1984).

7.4.2 Factors Affecting Butanol Tolerance in B. subtilis SB1

The culture was grown in Luria broth (LB) and in LB with supplements Luria broth supplemented with magnesium salts (LBM) (having 10 mM $MgSO_4$), LBG (having 0.1 % glucose) and LBMG (having both supplements; (Inoue and Horikoshi 1989) and also in mineral medium with butanol as the sole carbon source. Butanol tolerance and growth rate was found to be the best in LB supplemented with 0.1 % glucose and 10 mM $MgSO_4$ which is incidentally the most nutritive medium, as opposed to broth without supplements and minerals. The role of Mg ions in solvent tolerance is believed to be significant as Mg is the cofactor for several enzymes involved in membrane repair, and this activity has to be enhanced to promote growth in the presence of solvents. The effect of various factors such as temperature, pH, inoculum density and adaptation to butanol on the growth of SB1 in the presence of butanol was further determined.

Interestingly, the growth of *B. subtilis* SB1 in the presence of butanol was adversely affected by the increase in temperature with no growth in the presence of butanol at 48 °C while growth in the absence of butanol at the same temperature

was found to be excellent as *B. Subtilis* SB1 is a facultative thermophile. This could be attributed to changes in membrane fluidity. Similarly, it was found that *B. subtilis* SB1 is incapable of growth at pH 5 when grown in buffered media. However, growth does occur in the presence and absence of butanol in unbuffered broth as the culture is found to make the medium alkaline. Higher inoculum density was found to promote faster growth in the presence of butanol. Significantly, *B. subtilis* SB1 does not appear to depend upon prior adaptations to butanol to withstand butanol exposure. Even after 12 repeated subcultures in the absence of butanol, it was found that *B. subtilis* SB1 could grow well in LBMG medium with butanol. This indicates that butanol tolerance is a stable phenotypic property of the culture. This is unlike certain strains of *Pseudomonas* where prior cultivation in the presence of small concentrations of organic solvent is a prerequisite for solvent tolerance (Isken and De Bont 1998a).

During the Bacterial adherence to hydrocarbons (BATH) assay, it was found that the cells of *B. subtilis* SB1 as well as the cell-free broth contain a butanol emulsifying substance, since the absorbance was found to increase on shaking the sample with butanol. *B. subtilis* SB1 is a butanol degrader as well as a solvent-tolerant culture. It is possible that the emulsification of butanol may play a role in butanol tolerance. Many hydrocarbon degrading bacteria are known to produce hydrocarbon emulsifying substances. One of the mechanisms to increase solvent tolerance as found in *E. coli* and *Pseudomonas* strains is to decrease cell surface hydrophobicity, so that solvent molecules cannot adhere to the cells (Aono and Kobayashi 1997). The higher the number of solvent molecules adhering to the cell, the greater the damage inflicted, thus increasing cell fragility. Solvent-tolerant bacteria bring about subtle changes in the chemical composition of the cell surface by changing the type and amount of certain proteins, carbohydrates or lipids which decrease cell surface hydrophobicity. In the case of *B. subtilis* SB1, both log phase and stationary phase cells do not adhere to butanol, probably on account of changes in the cell surface or even due to the production of capsule and a butanol emulsifying substance.

Electron micrographs of *B. subtilis* SB1 when grown in the presence of its index solvent butanol and compared with those of cells grown on LBMG agar in the absence of any organic solvent, indicated a decrease in cell size when grown in the presence of *n*-butanol, from 3 to 1.8 µm in length (Figs. 7.2 and 7.3). It has been reported that solvent tolerance is a complex multigenic phenotype and a long-term adaptive response to deal with solvent stress involves morphogenetic and biosynthetic differentiation programs including alterations at a membrane and the cell wall level. It is a multifactorial process that involves a range of genetic and physiological changes to overcome the cellular damage and death (Torres et al. 2011).

7.4.3 Butanol Tolerance: A Unique Trait of B. Subtilis SB1

The estuarine isolate *B. subtilis* SB1 has one of the lowest index values of 0.8 reported for butanol, indicating survival in the harshest solvent stress conditions. In addition, it tolerates various other solvents like benzene, toluene and chloroform.

Fig. 7.2 SEM of *B. subtilis* SB1 cells grown in absence of *n*-butanol

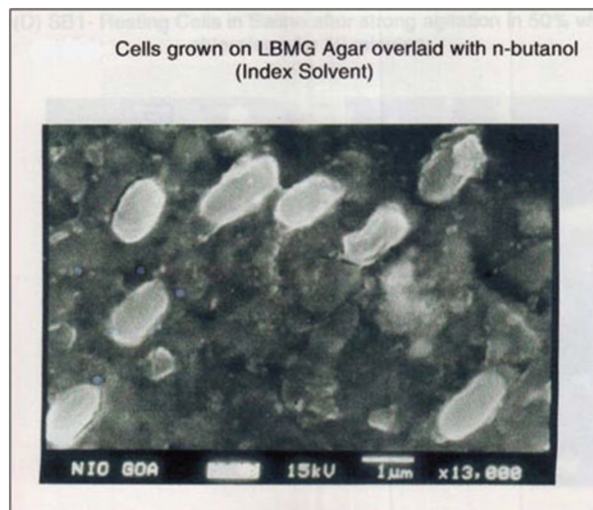

Fig. 7.3 SEM of *B. subtilis* SB1cells grown on LBMG agar overalid with index solvent *n*-butanol

Solvent tolerance is a stable phenotypic property of the cell as it does not require prior subculture or exposure in the presence of solvent before exposure to solvent stress. Additionally, effect of butanol on *B. subtilis* SB1 is bacteriostatic and not bactericidal as viable cells have been isolated from butanol saturated (99 %v/v) flasks even after a month of incubation. *n*-butanol, which is an amphiphilic alcohol is one of the most toxic organic solvents and is known to damage the cell membrane, cause leakage of potassium ions, protons and cell components and affect

inter-dependent activities like pH homeostasis, respiration and cell death (Ingram and Buttke 1984). The only other culture generally reported to be tolerant to this solvent is the solventogenic *Clostridium acetobutylicum*, the producer in acetone–butanol fermentation. But even this culture shows 99 % reduction in growth yield in the presence of 1.23 % v/v butanol, and butanol toxicity is known to be the limiting factor in acetone–butanol fermentation (Bowles and Ellefson 1985; Islam et al. 2009; Sardessai and Bhosle 2002b).

7.5 Studies on Cholesterol Transforming Bacteria in Presence of Chloroform

Tolerance to toxic chemicals has a wide range of applications ranging from production of solvents as biofuels, commodity chemicals, bioremediation with a major role in biotransformations, particularly of poor soluble compounds like steroids. An attempt was, therefore, made to isolate bacteria capable of cholesterol transformation in an organic-aqueous biphasic system from marine/estuarine habitats, identify a potential isolate and develop a suitable organic-aqueous biphasic fermentation system for cholesterol transformation.

7.5.1 Isolation and Identification

Coastal sediment samples and estuarine sediment sample from Mandovi estuary were screened for the presence of bacteria utilising cholesterol as a carbon source in the presence of 20 % (v/v) chloroform. Of each sample, 1 g was suspended in 8 ml mineral salts medium which was then supplemented with chloroform (2 ml) containing dissolved cholesterol as a carbon source. The cholesterol concentration was 1 mg/ml of the medium and the organic solvent concentration was 20 % v/v. The tubes were incubated on a rotary shaker (180 rpm) at 28 °C for 2 days. Aliquots of 0.2 ml from each sample tube were plated out on mineral medium agar containing cholesterol as the sole carbon source. The plates were incubated at 28 °C for one week and the colonies obtained were purified by streaking on mineral medium with cholesterol. Cultural and morphological characteristics of pure colonies obtained were recorded and the cultures maintained on mineral medium agar with cholesterol as carbon source followed by identification of a potential culture.

Interestingly, all the marine sediment samples and estuarine sample showed the presence of cholesterol-degrading bacteria. Eleven bacterial cultures were obtained, most of which were aerobic and Gram-positive rods. Cholesterol degradation was confirmed by the production of pure colonies on mineral salts agar with cholesterol as carbon source. Of these cultures, BC1, an aerobic Gram-positive endospore-forming rod, identified as *Bacillus megaterium* was found to show the fastest growth on cholesterol agar (within 48 h) and produce relatively larger colonies and hence selected for further studies.

7.5.2 Transformation of Cholesterol by Resting cells of Bacillus megaterium BC1 in a Biphasic System with 50 % Chloroform

7.5.2.1 Determination of Growth Profile of *B. megaterium* BC1 in Mineral Medium with Cholesterol as Sole Carbon Source and in LB

Culture grown in mineral medium with cholesterol for 8 h was harvested by centrifugation at 10,000 rpm for 20 min. The cells were washed and resuspended in saline. 10 % (v/v) of this inoculum was added to 100-ml side-arm flasks having 20 % (v/v) of mineral medium with 0.5 mg/ml cholesterol. The flasks were incubated on a rotary shaker (120 rpm) at 30 °C. Viable counts were taken on nutrient agar at 0 min and at periodic time intervals. Growth profiles were plotted and growth rate and generation time of the culture in cholesterol medium was determined.

B. megaterium BC1 was found to utilise cholesterol as the sole source of carbon and energy when grown in mineral salts medium with a growth rate of 0.37 h and doubling time of 162 min. Viability assays to monitor the survival of the resting cells in the presence of 50 % (v/v) chloroform indicated a decline in cell number from 10^6 to 10^5 within 4 h of incubation. Since the first step of the conversion of cholesterol occurs within 3 h and the cell number is substantially high during this time, it was considered as an ideal system for transformation. A large number of viable cells were also found to be present even on 24 h incubation, during which the second intermediate of chloroform transformation is known to appear, indicating chloroform tolerance of the culture.

7.5.2.2 Development of a Biphasic Organic-Aqueous Bioconversion System for Cholesterol Transformation

Resting cells of the organic-solvent-tolerant cholesterol-degrading bacterium *B. megaterium* BC1 were prepared by inoculating the culture in LB and incubating the flasks overnight at 30 °C on a rotary shaker (120 rpm). The cells were harvested by centrifugation at 10,000 rpm for 20 min, washed with sterile phosphate buffer (pH 7) as aqueous phase and suspended in 50 ml of sterile phosphate buffer (pH 7) and supplemented with 2 % (v/v) mineral salts medium (prepared without a nitrogen source) as a source of cofactors/stimulators for the required enzymes in a 250-ml flask. The flask was overlaid with 50 ml chloroform (50 % v/v; found as an ideal organic phase after several trials) which contained dissolved cholesterol such that the concentration of cholesterol in flask was 1 mg/ml. The flasks were incubated on a rotary shaker (120 rpm) for a period of 2 days. The cellular viability in the presence of 50 % (v/v) chloroform was continuously monitored by spread plating 0.1 ml of the medium layer from each flask on Luria agar at periodic time intervals.

7.5.2.3 Detection, Extraction and Purification of Cholesterol Degradation Intermediates

For detection of intermediates produced due to degradation of cholesterol, aliquots were taken from the chloroform layer at periodic time intervals and used to perform thin layer chromatography (TLC) with appropriate controls using chloroform–acetone (9:1) as the solvent system. The preferred developing agent used was vanillin phosphoric acid as it gives a violet colour only with steroids (Vessal and Rasti 1998).

After bioconversion by resting cells, the chloroform layer was removed by transferring the contents of the flask in a separating funnel and concentrated by evaporation. Separation of the intermediates was done by preparative TLC (Janozka et al. 2000) and silica gel was scraped from the layers for recovery of compounds by filtration with chloroform which was evaporated to give a fine powder.

The first transformed product was found to be formed which appeared as a spot below cholesterol (more polar intermediate-p1) in the chloroform–acetone solvent system in the early stages of transformation (30 min to 5 h). Further, this appeared to be a transient intermediate, which was not detected on further incubation of the flask overnight. The more polar steroids are known to bind more tightly to the silica gel layer and hence have a slower rate of migration (lower Rf values) as compared to the less polar steroids. However, after overnight incubation (even 7–10 h), there was the appearance of a clear spot above cholesterol (less polar product—p2), which was found to persist in the system even when incubated for over a week. The TLC pattern suggests that p1 is probably a hydroxylated derivative of cholesterol with greater polarity. On account of the transient nature of the compound, sufficient quantity could not be extracted for further analysis.

The second product, p2, was purified by preparative chromatography and extracted for analysis which gave the typical violet-grey colour with vanillin–phosphoric acid spray, which was used to discriminate between the interfering cellular origin compounds such as free fatty acids.

Further, TLC, UV absorption spectra and proton-NMR studies revealed that the product p2 is cholest-4-ene-3,6-dione (Fig. 7.4) and *B. megaterium* BC1 has the potential of effectively transforming cholesterol into cholest-4-ene-3,6-dione in a biphasic system of 50% chloroform and phosphate buffer (Sardessai and Bhosle 2003). Moriya et al. (1995) have used *Arthrobacter* strain ST1 to convert cholesterol into androsta-1, 4-diene-3, 17 dione in biphasic system with *n*-decane or *n*-dodecane. *Pseudomonas* ST-200 has also been used in oxidative bioconversion of cholesterol in water-organic 2-phase system (Aono et al. 1994; Doukyu and Aono 1998). This work conclusively proves that OSTB with the required enzymes can be effectively used to transform water-insoluble substrates in a biphasic system comprising an organic solvent as one of the phases. *B. megaterium* BC1 is a unique culture in terms of its chloroform tolerance and chloroform transformation which are novel traits.

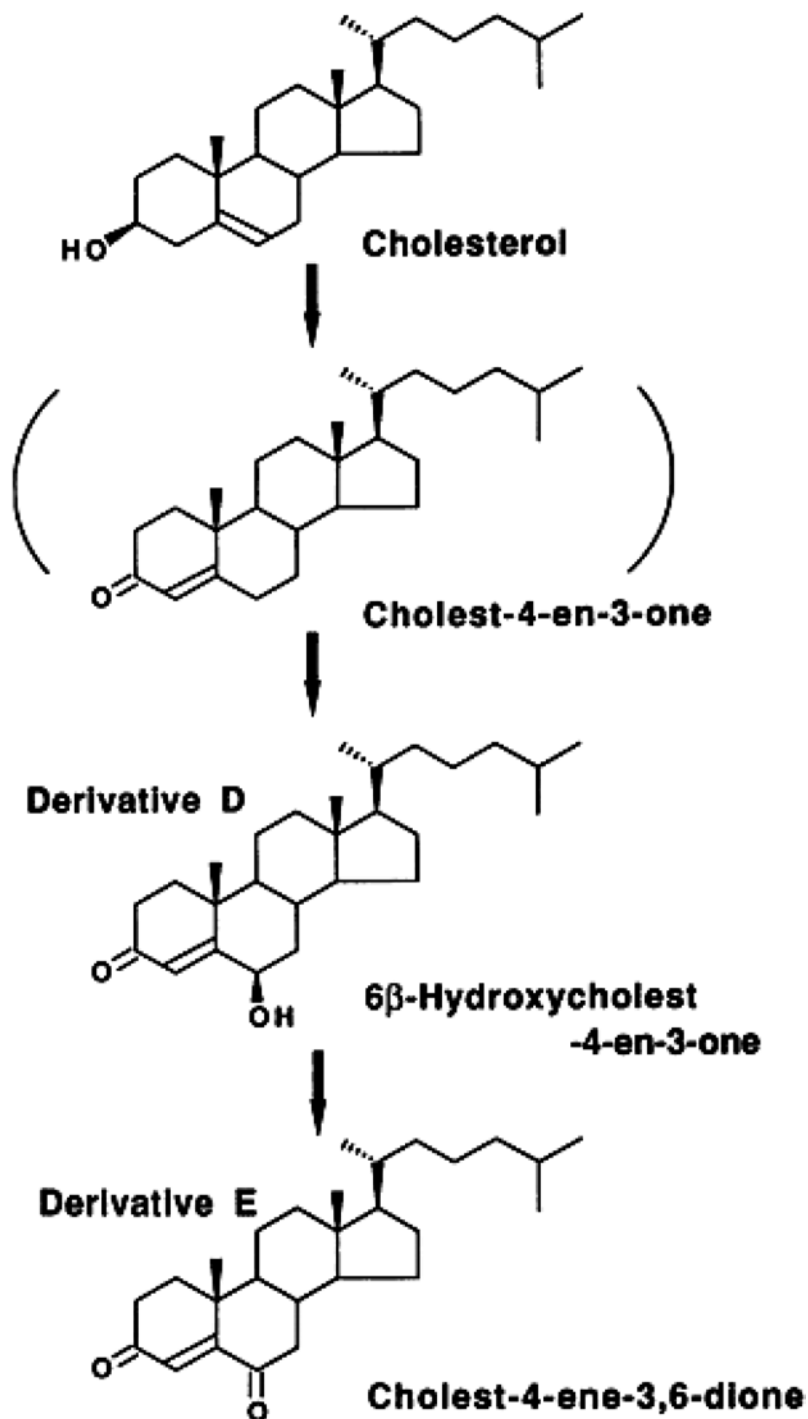

Fig. 7.4 Putative scheme for cholesterol transformation by *B. megaterium* BCI

7.6 Conclusions and Future Prospects

It is encouraging to note that since the discovery of the first OSTB in 1989 till date, a plethora of these interesting extremophiles are being studied for their cellular adaptations, enzymes and cell components. Novel species of OSTB with unique characteristics and tremendous potential are being reported at a high frequency and the interest generated by these organisms has only increased with time. Bioprospecting for such native species and use of these as role models in protein engineering and synthetic biology can have a tremendous impact on several fields of biotechnology. The academic, industrial and environmental impact of OSTB is immensely enriching and holds a lot of promise for the future of biotechnology and microbiology.

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Chapter 8
Heterotrophic Bacteria Producing Polyhydroxyalkanoates: A Biodegradable Polymer

Nimali N. Prabhu

8.1 Introduction

Conventional synthetic plastics derived from petroleum have become an inevitable part of our day to day life. With the exponential growth in human population, the unconditional use of these plastics has led to the accumulation of large amounts of nonbiodegradable waste in the environment. The disposal of these harmful wastes is a serious global problem. Many countries are now trying to overcome this problem by conducting programmes such as solid waste management (Rohini et al. 2006). These include the development of biodegradable plastics to reduce the plastic wastes in the environment. Secondly, the enticement to use biodegradable plastics is also related to the rapid depletion of nonrenewable crude oil resources (Leong et al. 2014; Naik et al. 2008; Philip et al. 2007; Reddy et al. 2008; Suriyamongkol et al. 2007).

Biodegradable plastics are biological polymers that are enzymatically degraded to carbon dioxide and water under aerobic conditions, and to methane and inorganic compounds anaerobically (Naik et al. 2008). They are largely divided into three categories: chemically synthesized polymers, starch-based biodegradable plastics and polyhydroxyalkanoates (PHAs).

Among the several biodegradable polymers, PHAs, a class of naturally occurring, optically active, aliphatic biopolyesters are currently receiving tremendous attention from both academic and industrial point of view. These microbial bioplastics possess material properties similar to petro-based synthetic plastics such as polypropylene but unlike petroplastics they are completely biodegradable, biocompatible, nontoxic and can be produced using renewable carbon sources (Madison and Huisman 1999; Sheu et al. 2009; Valappil et al. 2007).

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PHAs are therefore considered suitable for commercial exploitation and have gained applications in several fields such as medicine, pharmacy, agriculture, food and packaging industry, as raw materials for synthesizing enantiomerically pure chemicals and in the production of paints (Anderson and Dawes 1990; Rawte and Mavinkurve 2001; Rehm 2007; Sudesh et al. 2000).

8.2 Bacterial Polyhydroxyalkanoates and Their Chemical Structure

PHAs are structurally simple macromolecules synthesized by a wide variety of Gram-positive and Gram-negative bacteria including members of the family halobacteriaceae of the archaea (Anderson and Dawes 1990; Brandl et al. 1990; Hezayen et al. 2002; Madison and Huisman 1999; Philip et al. 2007). Marine prokaryotes accumulate PHAs up to 80% of their dry cell weight (DCW) especially when present in "high-nutrient" econiche (Philip et al. 2007; Valappil et al. 2007; Weiner 1997). Synthesis of PHA occurs when a carbon source is present in excess and one of the essential growth nutrients is limiting (Anderson and Dawes 1990; Madison and Huisman 1999; Rehm 2007).

Polymer production is initiated when acetyl coenzyme A (CoA) is restricted from entering the tricarboxylic acid (TCA) cycle due to nutrient limitation. This results in shunting the acetyl units from the TCA cycle into PHA production (Lenz and Marchessault 2005). The polymer acts as a sink for carbon and reducing equivalents which is mobilized by intracellular depolymerases as soon as the supply of limiting nutrient is restored (Anderson and Dawes 1990; Byrom 1994; Gao et al. 2001; Madison and Huisman 1999).

PHA is deposited in the cell cytoplasm as discrete, insoluble "inclusions or granules" (Fig. 8.1) (Anderson and Dawes 1990; Rehm 2007). Being highly refractile, the granules can be easily visualized using phase contrast light microscope (Dawes and Senior 1973). These granules are lipidic in nature and therefore stained with Sudan black B (Burdon 1946). A more specific dye that binds to PHA is the oxazine

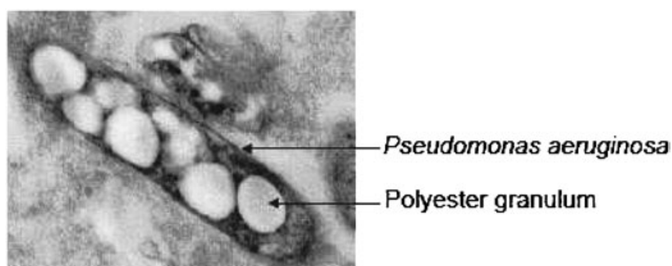


Fig. 8.1 Electron microscopy image of *Pseudomonas aeruginosa* accumulating polyester granules. (Rehm 2007)

as well as the fluorescent oxazone form (Nile red) of Nile blue A. Both Nile blue A and Nile red can also be used to detect PHAs inside the growing bacterial cells (Ostle and Holt 1982; Spiekermann et al. 1999; Wu et al. 2003).

In vivo, PHA is present in an amorphous state (Revol 1989). Extraction of the polyester using organic solvents makes it highly crystalline. The extracted polymer exhibits material properties similar to synthetic plastics such as high molecular weight. The molecular weight of PHA synthesized usually ranges from 2×10^5 to 3×10^6 Daltons (Da) and depends upon the microorganism, carbon source used as well as the growth conditions (Byrom 1994; Madison and Huisman 1999; Ojumu et al. 2004; Sudesh et al. 2000).

8.2.1 Chemical Structure

PHAs are made up of 3-hydroxyfatty acid (3HA) monomers that are arranged in a linear, head-to-tail fashion, i.e. the ester bond is formed between the carboxyl group of one monomeric unit with the hydroxyl group of the adjacent monomeric unit. The HA monomers that are incorporated into the polyester through the native cell metabolism are strictly in the (R)-configuration due to the stereospecificity of PHA synthase (Sudesh et al. 2000). This stereoregularity makes the polymer optically active.

The general structure of PHA is shown in Fig. 8.2 (Rehm 2007). The hydroxyl substituted carbon in the (R)-configuration contains an alkyl group that can vary from methyl to tridecyl. The basic unit and most abundant PHA in nature is poly-3-hydroxybutyrate, (PHB). It is the simplest PHA with respect to the chemical structure having a methyl ($–CH_3$) group in the alkyl side chain. The PHB homopolymer is made up of repeating units of (R)-3-hydroxybutyrate (3HB). It is hard and brittle. Incorporation of 3-hydroxyvalerate (3HV) monomers along with (3HB) yields a copolymer P(3HB-co-3HV), which is an elastomer similar to polypropylene. Due to the flexibility of the copolymer, it can be melt processed into a variety of consumer products including plastics, films and fibres (Anderson and Dawes 1990; Madison and Huisman 1999; Rawte and Mavinkurve 2001; Rehm 2007).

The saturated alkyl side chain of PHA can also be substituted with aromatic, unsaturated, halogenated, epoxidized and branched monomers. Similarly, the position of the hydroxyl group can also be varied to obtain 4-, 5- or 6-hydroxyacid. Some eubacteria are also capable of synthesizing polythioesters using mercaptoacids as

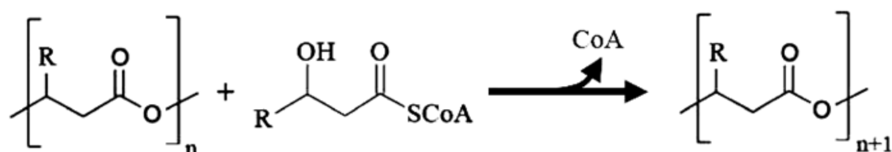


Fig. 8.2 Reaction catalyzed by the polyester synthases. (Rehm 2007)

a carbon source (Lutke-Eversloh et al. 2001). Overall, the size of the alkyl side chain as well as the monomeric composition determines the material properties of PHA. Therefore, by manipulating these features, new polymers with desired material properties can be synthesized (Anderson and Dawes 1990; Madison and Huisman 1999).

8.2.2 Classification of PHA

PHAs are divided into two broad groups based on the substrate specificity of PHA synthase to accept 3HAs of a certain carbon chain length (Naik et al. 2008; Philip et al. 2007):

- Short-chain-length (SCL) PHAs
- Medium-chain-length (MCL) PHAs

The SCL PHAs consist of HA monomers which are composed of 3–5 carbon atoms. These polymers are stiff and brittle. They possess a high degree of crystallinity, lack toughness and show thermoplastic material properties similar to polypropylene. MCL PHAs consist of 6–14 carbon-containing HA monomers and are generally flexible, have low crystallinity, tensile strength and melting point. They are elastomeric in nature, hence opening new opportunities for their application as rubbers and elastomers (Anderson and Dawes 1990; Gagnon et al. 1992; Ojumu et al. 2004; Suriyamongkol et al. 2007).

Recently, bacteria able to synthesize PHAs containing both SCL and MCL monomeric units consisting of 3–14 carbon atoms have been reported. These copolymers have properties in between that of SCL and MCL PHAs depending on the mole ratio of SCL to MCL monomers, further improving their physical and thermal properties. For example, the incorporation of small amounts of MCL monomer, 3-hydroxyhexanoate (3-HHx) into PHB backbone alters the material properties of PHA. The resulting PHA formed is similar to that of low-density polyethylene and therefore suitable for commercial applications (Madison and Huisman 1999; Philip et al. 2007; Yu 2007).

8.3 PHA Biosynthesis

Biosynthesis of PHA in bacteria is divided into three phases (Fig. 8.3) (Steinbuchel and Valentin 1995):

- **Phase I**: Entry of the carbon source from the surrounding environment into the cell either by simple diffusion or a specific membrane transport system.
- **Phase II**: Conversion of the carbon source into hydroxyacyl Coenzyme A (CoA) thioesters.
- **Phase III**: Polymerization of hydroxyacyl CoA thioester precursors by the key enzyme, PHA synthase to produce PHA.

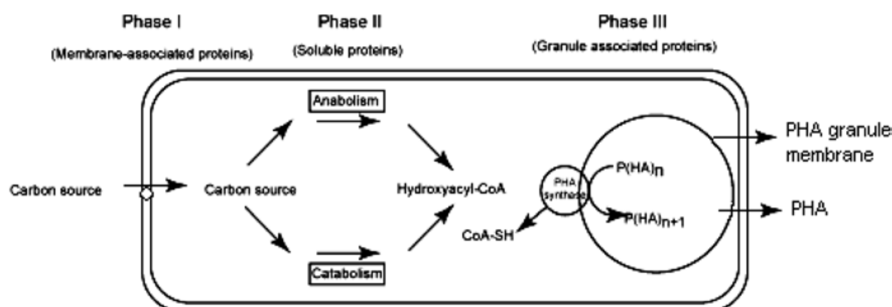


Fig. 8.3 Phases of PHA biosynthesis in bacteria. (Steinbuchel and Valentin 1995)

8.4 Distribution, Isolation and Identification of Bacteria Producing PHA

The biodegradable and biocompatible biopolymer, PHA, has been long since recognized as the potential substitute for the petro-based synthetic plastics (Anderson and Dawes 1990). However, synthesis of this biopolymer at an industrial scale has been limited owing to its high production cost, depending mainly on the bacterial strain used. In addition, the chemical composition of the polymer which greatly influences its material properties is also determined by the type of bacteria synthesizing PHA (Chien et al. 2007; Rawte et al. 2002). Therefore, attempts are now being made to isolate efficient and high yielding bacterial strains which synthesize PHAs with novel monomeric composition within a short incubation period thus cutting down on the overall production cost (Chien et al. 2007).

In the recent years, studies are directed towards exhaustive screening of samples from diverse environments such as soil (Anil Kumar et al. 2007; Halami 2008), activated sludge (Borah et al. 2002; Omar et al. 2001; Reddy et al. 2009), but till date, only a few reports on the isolation of bacteria from marine and mangrove ecosystems are available (Arun et al. 2009; Chien et al. 2007; Rawte et al. 2002; Sathiyanarayanan et al. 2013; Wei et al. 2011). The sediments obtained from these econiches are rich in bacterial flora that can utilize diverse carbon compounds (formed as a result of breakdown of the decomposing detrital matter) for PHA production (Bhosle and Mavinkurve 1980; Matondkar et al. 1980). Interestingly, the PHAs thus synthesized possess novel chemical composition that can be exploited for commercial applications (Weiner 1997). However, the marine environment which provides such a virtually untapped resource for the isolation of novel PHA-producing bacteria has not been explored adequately as yet.

Hence, the present study was undertaken in the quest of isolating potential PHA producers specifically from marine and coastal ecosystems. The sites included coastal beaches, mangrove area and plant leaf litter area in Goa. The sediments collected from these econiches were processed for the determination of total viable count (TVC) of the heterotrophic bacteria. The results of TVC obtained for sediments of coastal beaches were hundred-fold lower than that of mangrove as well as

plant leaf litter area. The highest heterotrophic bacterial count of 13.8×10^6 colony forming unit per gram (cfu g^{-1}) dry weight was obtained for the sediment collected from the mangrove area while the lowest count of 16.6×10^4 cfu g^{-1} sediment dry weight was obtained for one of the coastal beach sediment sample (Caranzalem). Such low heterotrophic bacterial counts in the beach sediment samples as compared to that of marine (Palaniappan and Krishnamurthy 1985) and mangrove sediments (Matondkar et al. 1981) have also been reported by Prabhu et al. (1990).

So far, there is only one report on the isolation of PHA-producing bacteria isolated from marine and mangrove ecosystems in Goa (Rawte et al. 2002). In this report, higher heterotrophic bacterial load has been demonstrated in the mangrove sediments and lower bacterial counts in the coastal beach sediment samples. The fluctuation of the bacterial population in these ecological niches could be attributed to various environmental factors in such econiches, widely differing from each other (Nair and LokaBharathi 1980). For example, in the mangrove area, there is a continuous leaf fall, which is being degraded and mineralized resulting in the source of nutrients for microorganisms. This is reflected as high load of bacteria possessing diverse hydrolytic enzymes which are involved in the degradation process (Bhosle and Mavinkurve 1980; Matondkar et al. 1981; Rawte et al. 2002). Hence, such an ecosystem can be expected to be rich in PHA-accumulating bacteria. The heterotrophic bacterial population present in the marine sediments plays a significant role also in nutrient and energy cycle but the bacterial load is reported to decline with depth, lowest counts being obtained at a depth below 1000 m (Nair et al. 1989; ZoBell 1963). The lower TVC obtained in the case of sediments collected from various coastal beaches could be attributed to the sandy nature of these sediments. Higher bacterial counts have been reported by Nair et al. (1978) in clay and clayey-sand sediments rather than fine sand, suggesting that the bacterial population possess a negative relationship with the particle size and a significant direct relation to the organic matter.

Further, random selection of the culturally dissimilar colonies obtained from the respective sediments was carried out. The percentage variation of the total isolates selected from different econiches was found to be the highest in samples obtained from marine sediments and minimum in coastal beach sediments (Fig. 8.4a). Similar observation on the percentage distribution of Gram-positive isolates indicated maximum number of these organisms obtained from marine sediments and minimum number from coastal beach sediments (Fig. 8.4b). The predominance of Gram-positive bacteria in the marine sediments has also been reported by Palaniappan and Krishnamurthy (1985) and Rawte et al. (2002). The percentage distribution of the bacterial population in the beach sediment samples has also been reported to be greatly affected by factors such as the moistening of beaches by rain water, river influence and human activities (Nair and LokaBharathi 1980).

Finally, screening of all the isolates obtained from these diverse econiches for PHA production was carried out. Interestingly, the percentage distribution of the total number of PHA producers showed a slightly different trend. In this case, the highest percentage of PHA producers was obtained from the marine sediments and that of the mangrove sediment was found to be the lowest (Fig. 8.4c) perhaps

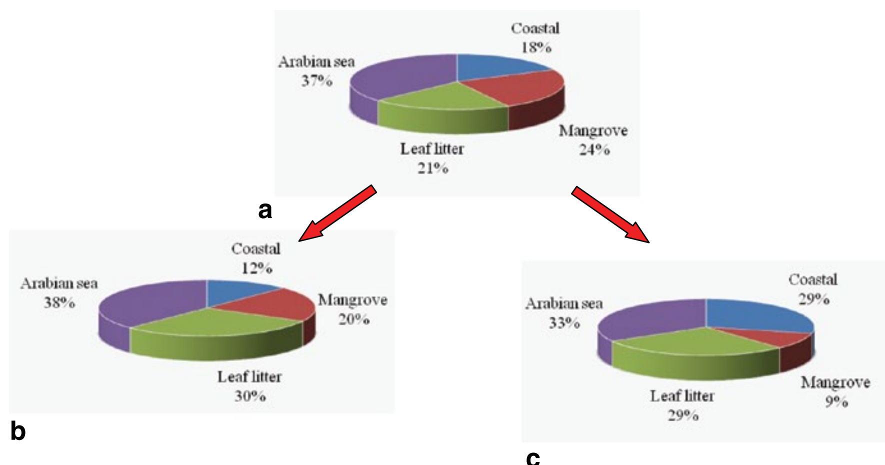


Fig. 8.4 Percentage distribution of the total number of isolates obtained from various marine and coastal econiches. **a** Morphologically and culturally distinct isolates, **b** Gram-positive isolates, **c** PHA accumulators

because mangroves are a nutrient-rich ecosystem (Matondkar et al. 1980; Rawte et al. 2002). PHA serves as a carbon and energy reserve, the accumulation of PHA offers a selective advantage for the survival of bacteria in samples with low nutrients (Lopez et al. 1995). The higher percentage of PHA producers in the marine sediments suggests the scarcity of nutrients in this ecosystem creating unbalanced nutritional conditions ideal for the growth of PHA-accumulating bacteria.

The bacterial isolates that exhibited maximum PHA accumulation using glucose as a sole source of carbon and for a long duration of time were selected for further studies. These included five Gram-positive, rod-shaped, sporulating bacterial strains each from coastal beaches and plant leaf litter sediments and only one Gram-positive, rod-shaped, sporulating bacterial isolate from the mangrove sediment.

Identification of all these isolates was carried out as per the cultural, morphological and biochemical tests described in Priest et al. (1988) and *Bergey's Manual of Systematic Bacteriology* (Sneath et al. 1986). The data were analyzed numerically, using the simple matching coefficient (SSM). Clustering was achieved by unweighted pair group average linkage (UPGMA). The computations were performed by using Probiosys program.

From the results obtained, all the selected isolates were tentatively identified to species level, with eight of the isolates closely related to *Bacillus megaterium* and one isolate to *Bacillus licheniformis*. However, the remaining two isolates were identified only up to the genus level, i.e. *Bacillus* sp. Microscopic staining of the isolates using Nile blue A was carried out to detect the presence of intracellular PHA granules. These granules exhibited bright orange–red fluorescence when viewed under fluorescent light (Fig. 8.5). This microscopic staining method has also been used for the preliminary screening of bacterial isolates accumulating PHA by

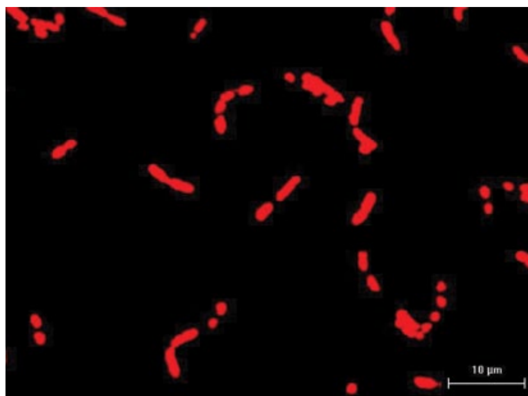
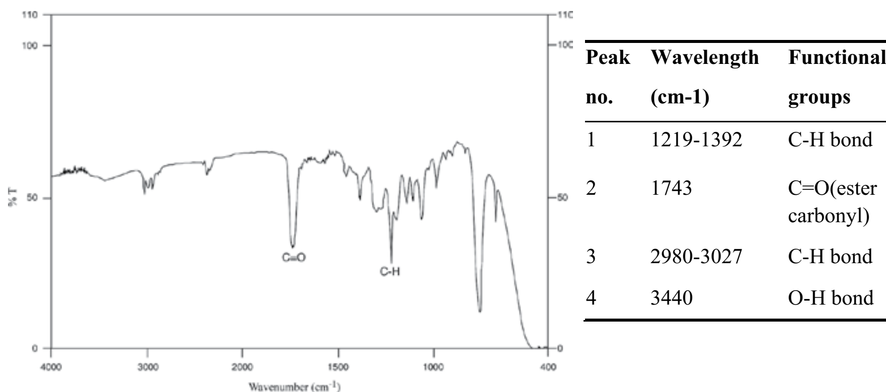


Fig. 8.5 Micrograph showing the presence of PHA granules inside the cells on staining with Nile blue A

Rawte et al. (2002) as well as for visualization of intracellular PHA inclusions during the kinetic studies of growth (McCool et al. 1996). McCool et al. (1996) have also determined the PHA quantity inside the cells of *Bacillus megaterium* during exponential and stationary phases of growth and indicated this staining method to be a reliable technique for PHA estimation.

Fourier transform-infrared (FTIR) spectra of PHA purified from these *Bacillus* isolates exhibited a strong band at 1743 cm^{-1} corresponding to ester carbonyl (C=O) stretching frequency (Fig. 8.6). The bands at 2980–3027 and 1219–1392 cm^{-1} represented the typical C–H stretching and bending vibrations of the aliphatic portion of the compound, respectively. A distinct broadband at 3440 cm^{-1} indicated the free O–H stretching of the polymer end groups. Moreover, the infrared (IR) spectra of these polymers were found to be super-imposable with that reported previously (Pal and Paul 2001). Hence, the FTIR analysis revealed that the polymer produced in these *Bacillus* isolates was aliphatic in nature. Characterization of PHA using FTIR analysis has also been reported by Arun et al. (2009);



Peak no.	Wavelength (cm-1)	Functional groups
1	1219-1392	C-H bond
2	1743	C=O(ester carbonyl)
3	2980-3027	C-H bond
4	3440	O-H bond

Fig. 8.6 Infrared spectrum of PHA polymer extracted from the bacterial cells

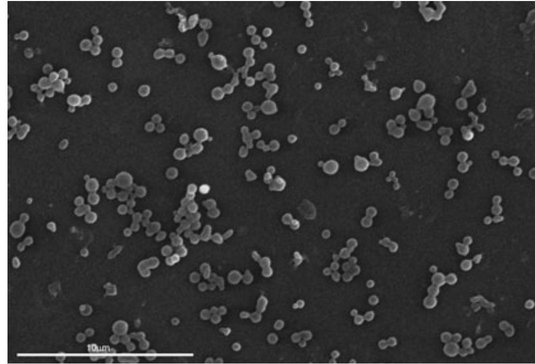


Fig. 8.7 Scanning electron micrograph of purified native PHA granules from isolate NQ-11/A2

Prabhu et al. (2009) and Rohini et al. (2006). Further, purification of the PHA polymer along with the intact bilayer membrane, i.e. the "native" PHA granules was achieved using sucrose density gradient. Figure 8.7 reveals the scanning electron micrograph (SEM) of purified native PHA granules from isolate NQ-11/A2 (Prabhu et al. 2010).

8.5 Conclusions and Future Prospects

The present study was initiated to isolate bacteria from different marine and coastal arenas with the hope of obtaining PHA producers, potentially useful for industrial applications. Out of the several isolates obtained, five Gram-positive, rod-shaped sporulating isolates each from coastal beaches and plant leaf litter area and one Gram-positive, rod-shaped sporulating isolate from mangrove area. Biochemical identification of these isolates revealed that all of them belonged to the genus *Bacillus*, with the majority being *Bacillus megaterium*. Nile blue A staining of all the 11 isolates revealed the presence of intracellular PHA granules. FTIR analysis confirmed the aliphatic nature of the polymer produced by these bacteria.

The major application of PHA being in the medical field, especially in tissue engineering needs the polymer to be free from any contaminating substances like the endotoxins. Lipopolysaccharide (LPS) is one of the major contaminants known to copurify along with PHA when extracted from Gram-negative bacteria. Hence, in the recent years, Gram-positive organisms such as *Bacillus* and *Streptomyces* species have been recognized as potential strains for commercial scale PHA production. Different strains of *Bacillus* species obtained in the present study could be exploited for the production of PHA in biomedical applications.

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Chapter 9
Bacterial Synthesis of Polyhydroxyalkanoates Using Renewable Resources

Maria Celisa Santimano

9.1 Introduction

Synthetic plastics have replaced natural materials and become an integral part of the everyday life. They are being extensively used because of their desirable physical and chemical properties such as low cost, durability, lightweight, ease in processing and high resistance to chemical and biological degradation. The very properties of plastics that have contributed immensely towards their popularity are causing grave environmental and societal concerns. Their xenobiotic and recalcitrant nature could be attributed to their large molecular size and the inability of nature's existing in-built mechanisms to degrade these novel unfamiliar pollutants (Reddy et al. 2003). New enzymes capable of degrading synthetic polymers are yet to be evolved since their presence in nature has increased enormously only during the recent years (Mueller 2006; Reddy et al. 2003). Increasing environmental and societal concerns have put pressure on the development of sustainable and eco-friendly plastic materials. Biodegradable polymers, especially polyhydroxyalkanoates (PHA) are ideal candidates to replace petroleum-based synthetic plastics. They are a class of biopolyesters synthesized and accumulated by a wide range of microorganisms as reserve food material. These polymers are produced in response to the limitation of any essential nutrient required for cell growth and the presence of a generous supply of carbon (Anderson and Dawes 1990). PHA are gaining tremendous importance as these are the only plastics produced exclusively by microorganisms and hence are completely degraded to benign compounds (Anderson and Dawes 1990; Yu 2007). They are nonpolluting as they do not need catalysts or additives to promote their degradation. The common microflora present in soil, water, compost or sewage is capable of degrading this polymer. These polymers completely degrade to carbon dioxide and water when disposed in aerobic environments (Lee 1996; Luzier 1992).

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In anaerobic conditions, the degradation of PHA can result in methane formation which can be trapped and resold as fuel (Budwill et al. 1992). Biocompatibility of PHA and its manufacture from inexpensive resources are some other key features which have generated global interest in this polymer (Shah et al. 2008).

The market price of commercially available PHA (approximately 10–12 €/kg) is comparatively higher than that of conventional synthetic plastics (Castilho et al. 2009). The high manufacturing costs involved during the production of these polymers is mainly responsible for hampering the commercialization of biodegradable plastics. The most important factors affecting the overall economics of PHA production are PHA productivity, PHA content and PHA yield. These are dependent on the bacterial strains employed, raw materials used for production, fermentation strategies and recovery processes. The widespread use of this polymer is restricted due to its high production cost and finds limited applications in the medical field.

9.2 Use of Efficient Bacterial PHA-Producing Strains

In PHA production process, the final quality and quantity of the product greatly depends on the strain, metabolic pathway involved, fermentation parameters, PHA synthesis phase (either stationary or throughout growth), carbon source as well as nutrient limitation conditions necessary for PHA production (Somashekara et al. 2009).

Selection of an ideal bacterial strain is very crucial for PHA production. There are several factors that determine the suitability of the organism for large-scale production of the polymer such as the cell's ability to utilize an inexpensive carbon source, growth rate, polymer synthesis rate and the maximum extent of polymer accumulation (Khanna and Srivastava 2005). The factors that directly affect the productivity include growth rate, polymer synthesis rate and the maximum extent of polymer accumulation which should be as high as possible. Further, the cost of PHA can be significantly reduced using cheap carbon sources. In addition, the microorganisms that produce extracellular polysaccharides (EPS) besides PHA should be avoided since the cells not only use up the carbon source for their synthesis but they also make the recovery process inefficient (Lee and Chang 1995). Fermentation strategies targeting high productivity of PHA are absolutely essential in reducing the fermentation and purification costs of the process. Since PHA is synthesized and accumulated under unfavourable growth conditions, cultivation strategies that stimulate these conditions and allow efficient production of PHA should be employed (Lee and Chang 1995). Higher PHA content in the cells will allow more fermentation runs to be carried out for the given total annual operating time, thereby resulting in the reduction of the fermenter size and fixed cost (Choi and Lee 1997). Equipment-related costs are also reduced with increasing PHA content as smaller amount of cells needs to be produced to obtain the same amount of PHA.

High PHA content also reduces the amount of carbon substrate needed to produce PHA and hence the feedstock will not be wasted on other cellular materials

and/or metabolites. This will help in lowering the cost of the carbon substrate, a major contributor to the overall total raw material cost (Kim 2000). Higher productivity has a profound influence on the purity and yield of the PHA produced. High PHA yield not only reduces the recovery costs by allowing the processing of less amount of non-PHA cellular material to obtain the same amount of PHA, but also reduces the costs associated with waste disposal (Choi and Lee 1999).

PHA synthesis has been described in a large number of bacteria, but only a few bacteria have been employed for the production of PHA at an industrial scale. Based on the culture conditions required for PHA synthesis, these bacteria can be divided into two groups. The first group which includes bacteria such as *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), *Pseudomonas oleovorans* and several methylotrophs require unbalanced growth conditions for PHA synthesis. The presence of excess carbon with the simultaneous limitation of an essential nutrient triggers the synthesis and accumulation of the polymer. PHA production is growth-associated in bacteria belonging to the second group (e.g., *Alcaligenes latus*, a mutant of *Azotobacter vinelandii* and recombinant *Escherichia coli*) and does not depend on nutrient limitation (Lee 1996; Khanna and Srivastava 2005). These characteristics have to be taken into consideration during strain selection for PHA production (Khanna and Srivastava 2005). In addition, the cyclic nature of PHA metabolism, synthesis and degradation also needs to be considered since prolonged metabolism inside the cells can result in PHA degradation by depolymerases (Jendrossek 2001).

9.2.1 The Genus Bacillus and PHA Production

Currently, Gram-negative microorganisms such as *C. necator*, *A. latus* and recombinant *E. coli* are used for commercial polymer production (Valappil et al. 2007a). The lipopolysaccharide (LPS) tainted polymers extracted from these bacteria require additional purification procedures before they can be safely used in mammalian systems, thereby increasing production costs (Chen and Wu 2005).

Studies have shown the genus *Bacillus* to be one of the most versatile PHA producers since it can accumulate PHA from a variety of substrates (Table 9.1). These Gram-positive bacteria have an added advantage as potential candidates for industrial scale PHA production due to the lack of LPS layer. Members of this genus are also known to grow rapidly, possess various hydrolytic enzymes and produce copolymers from structurally unrelated carbon sources (Halami 2008; Valappil et al. 2007b). These very characteristics of *Bacillus* spp. can be exploited for the production of PHA with desirable material properties from various low-cost agricultural feedstocks.

Table 9.1 Studies on PHA production carried out using various *Bacillus* spp. and low-cost feedstocks

Organism	Carbon source	PHA content (%)	Reference
Bacillus cereus M5	Beet molasses	73.8	Yilmaz and Beyatli (2005)
B. cereus (5 strains)	Pea-shell waste	47.0–72.0	Kumar et al. (2009)
Bacillus megaterium ATCC 6748	Molasses	35.0	Chaijamrus and Udpuay (2008)
B. megaterium BA-019	Molasses	55.5	Kulpreecha et al. (2009)
B. megaterium	Dairy waste + sea water	11.3	Pandian et al. (2010)
Bacillus sphaericus	Cornflour	3.3	Ramadas et al. (2009)
	Wheat bran	6.8	
	Cassava bagasse	6.4	
	Jackfriut seed powder	46.0	
	Potato starch	47.0	
B. sphaericus	Sesame oil cake	14.6	Ramadas et al. (2009)
	Groundnut oil cake	18.7	
Bacillus thuringiensis R1	Molasses	23.1	Rohini et al. (2006)
	Table sugar	28.2	
B. thuringiensis EGU45	Pea-shell waste	55.0	Kumar et al. (2009)
Bacillus sp. JMa5	Molasses	35.0	Wu et al. (2001)
Bacillus sp.	Soytone	25.4	Full et al. (2006)
Bacillus sp. 256	Mahua flower extract	51.0	Anil Kumar et al. (2007)
Bacillus sp. COL1/A6	Hydrolysed coconut oil cake	41.92	Santimano et al. (2009a)
Bacillus sp. COL1/A6	Hydrolysed wafer residue	62.4	Santimano et al. (2009b)
	Cane molasses	54.7	
	Hydrolysed citrus pulp	47.5	

9.3 PHA-Producing *Bacillus* Strains Isolated from Marine and Coastal Ecosystems of Goa

Sixteen bacterial strains belonging to the genus *Bacillus* previously isolated and identified in the laboratory were used for the study (Prabhu 2010). These strains were isolated from sediment samples collected from diverse econiches in and around Goa. Preliminary investigations revealed the isolates to be potential candidates for PHA production. Kumar et al. (2009) have also observed that among the different bacteria belonging to various genera, *Bacillus* species were found to possess high PHA-producing ability.

Submerged cultivation of the different *Bacillus* species used in the present study revealed that these isolates grew rapidly on glucose as sole carbon source (Fig. 9.1). The

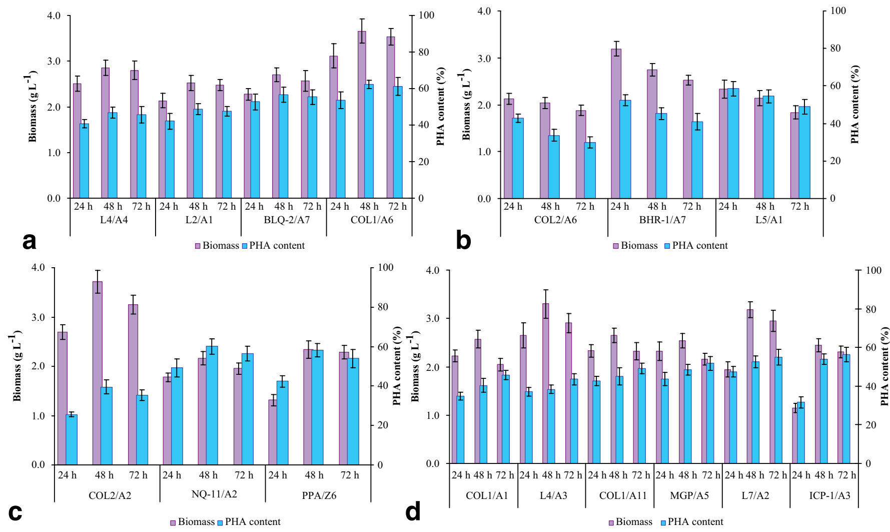

Fig. 9.1 Temporal variation in PHA-accumulating ability among different *Bacillus* species using glucose as sole carbon source. **(a)** Consistent PHA yield after maximum accumulation, **(b)** maximum PHA yield at 24 h, **(c)** maximum PHA yield at 48 h, **(d)** maximum PHA yield at 72 h

PHA content accumulated by these isolates, which is calculated as the ratio between the polymer extracted and the cell biomass, both in terms of dry cell weight (DCW) ranged between 39.6 % (COL2/A2) to 62.3 % (COL1/A6). Generally, members of the genus *Bacillus* are known to produce PHA content ranging from 6.53 to 48.2 % (Aslim et al. 2002; Chen et al. 1991; Shamala et al. 2003). Recently, few researchers have reported higher amounts of accumulated PHA in different *Bacillus* spp. such as *Bacillus megaterium* NQ-11/A2 (61 % DCW), *Bacillus thuringiensis* R1 (64.1 % DCW), *Bacillus mycoides* RLJ B-017 (69.4 % DCW) and *Bacillus* sp. CL1 (90 % DCW) (Borah et al. 2002; Full et al. 2006; Prabhu et al. 2009, 2010; Rohini et al. 2006).

On comparing the PHA yield produced for three consecutive days of incubation, it was observed that the time of incubation influenced PHA production. It was interesting to note that these *Bacillus* species could be categorized into four groups based on the PHA content produced.

Most of the isolates showed a reduction in the accumulated PHA content on prolonged incubation whereas only about one third of the isolates were capable of consistently maintaining the maximally accumulated PHA till the termination of the experiment which included isolates BLQ-2/A7, L2/A1, L4/A4 and COL1/A6 (Fig. 9.1a). With a further incubation till 72 h, the intracellular PHA content accumulated by these strains was found to be stable, indicating that the PHA depolymerase enzyme of these strains remains inactive over a longer period of time. *Bacillus cereus* SPV is also `reported to exhibit similar pattern for PHA production (Philip et al. 2009; Valappil et al. 2007c).

The isolates belonging to the second group, namely, BHR-1/A7, L5/A1 and COL2/A6 produced maximum PHA at 24 h of incubation (Fig. 9.1b). Further

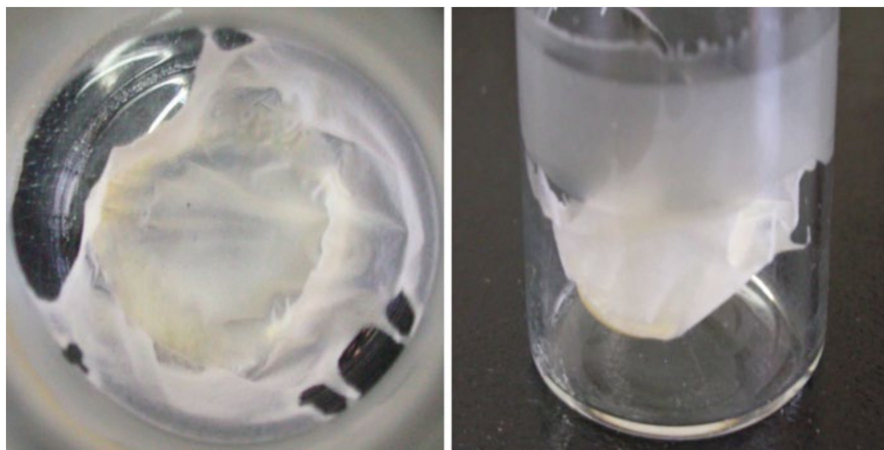

Fig. 9.2 PHA polymer extracted from isolate COL1/A6 after 72 h of growth

increase in the incubation time reduced the PHA content in the cells suggesting that the accumulated PHA was possibly utilized for growth or spore formation as observed by Wu et al. (2001). A slightly different trend was observed with isolate NQ-11/A2, PPA/Z6 and COL2/A2 (belonging to the third group) which exhibited maximum PHA content at 48 h and further incubation resulted in a decrease in the accumulated PHA content (Fig. 9.1c). These findings were consistent with that reported by Shamala et al. (2003). They observed maximal production of PHA at 24 h (or 48 h in case of *B. megaterium*) and the amount of accumulated PHA decreased on further incubation in fermentations employing various *Bacillus* species. The amount of PHA accumulated in six isolates belonging to the fourth group namely, ICP-1/A3, L4/A3, L7/A2, MGP/A5, COL1/A1 and COL1/A11 increased with increase in the incubation time (Fig. 9.1d). Halami (2008) has also reported a similar observation using *B. cereus* CFR06 wherein the PHA content increased on prolonged incubation of 72 h. This trend could be attributed to the decline in the cell biomass consequently resulting in increased PHA content. The polymer extracted from isolate COL1/A6 after 72 h of growth is shown in Fig. 9.2.

9.4 Use of Alternative Substrates for Microbial PHA Production

In the manufacturing process, the raw material cost, especially the carbon source greatly influences the overall cost of the final product. Synthesis of PHA using expensive carbon sources such as glucose renders the process economically nonviable. Therefore, for economical PHA production inexpensive substrates that can be used as carbon sources for bacterial strains to synthesize large quantities of intracellular PHA are necessary. By-products such as molasses, straw, bagasse generated in the agricultural sector are available abundantly and are generally used as cattle feed

since they have little economic value. Their use as a carbon feedstock for PHA production can contribute to as much as 40–50 % reduction in the overall manufacturing cost (Kim 2000). These agricultural residues are rich in carbohydrates and the use of such materials for the synthesis of value-added products can be advantageous and also contribute significantly to the reduction of their disposal costs (Thomsen 2005; Castilho et al. 2009).

Members of the genus *Bacillus* are ubiquitous in nature and possess innate ability to produce various hydrolytic enzymes that metabolize complex residues present in the surrounding environment. Therefore, such native *Bacillus* strains are now being explored industrially for economic PHA production from complex residues, e.g. agroindustrial by-products (Gouda et al. 2001; Kumar et al. 2009; Kulpreecha et al. 2009; Santimano et al. 2009a, b).

After the preliminary evaluation of various inexpensive and easily available agro-industrial residues, the polymer-producing ability of the selected *Bacillus* isolates on these carbon substrates was quantified by cultivation under submerged conditions. These isolates were specifically selected based on the maximum intensity of fluorescence exhibited by them on the respective substrates (Kitamura and Doi 1994).

9.4.1 PHA Production Using Submerged Cultivation

A. Molasses Molasses served as an excellent source for growth as well as polymer production. The growth of the isolates was highest on molasses (3.58–6.23 g L^{-1}) as compared with any other substrate. A PHA content as high as 68.56 % DCW was achieved with ICP-1/A3 (Fig. 9.3). All the isolates grown under these conditions accumulated more than 50 % DCW as PHA.

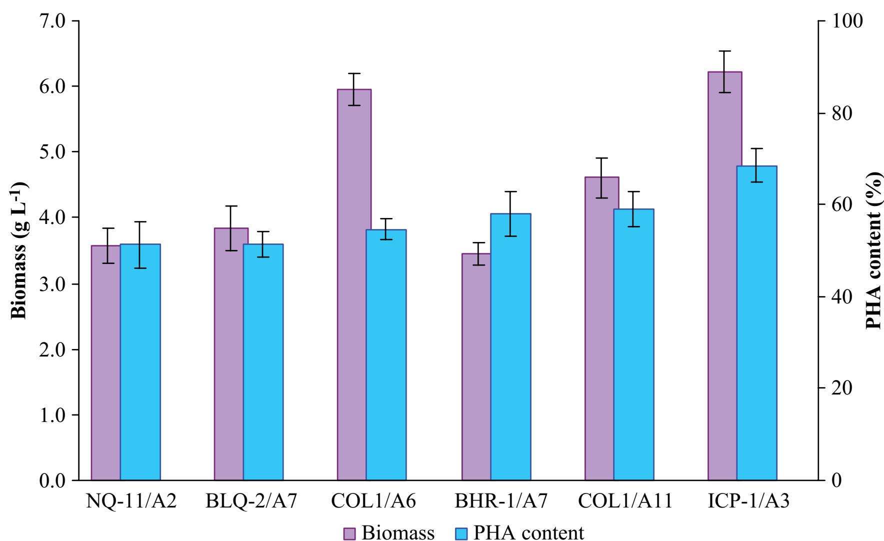

Fig. 9.3 Production of PHA in various *Bacillus* species using sugarcane molasses

The enhanced growth and PHA production of the isolates can be attributed to the additional nutrients such as vitamins and minerals found in molasses which function as growth factors (Kulpreecha et al. 2009; Oliveira et al. 2004). The PHA content accumulated by *B. megateruim* BA-019 improved significantly when molasses rather than sucrose were used as a carbon source. This isolate was able to accumulate 55.46% DCW as PHA when cultivated on molasses and urea (Kulpreecha et al. 2009). Gouda et al. (2001) reported maximum PHA production using *B. megaterium* with cane molasses and glucose as sole carbon sources (40.8 and 39.9% DCW, respectively). These authors demonstrated that higher molasses concentration (3% w/v) resulted in increased growth whereas 2% molasses yielded maximum PHA content. *B. thuringensis* R1 cells were found to accumulate 22.95 and 31.36% DCW as PHA in the presence of molasses and table sugar, respectively (Rohini et al. 2006). Wu et al. (2001) demonstrated that under fed-batch conditions, *Bacillus* sp. JMa5 could accumulate 25–35% PHA during fermentation using molasses as a sole carbon source.

B. Starch-Based Residue Even though the isolates were capable of hydrolysing starch with the enzyme amylase, acid hydrolysis of the wafer residue could not be avoided since the insoluble starch particles interfered during downstream processing.

The resulting hydrolysate of wafer residue was hence used as a carbon source for PHA production. The majority of the isolates exhibited bright fluorescence indicating excellent PHA-producing ability of these isolates on wafer hydrolysate. Isolates able to produce PHA on all the 3 days were further selected for submerged cultivation. Luxuriant growth of the isolates was observed with isolate L7/A2 producing maximum biomass of 5.24 g L^{-1} (Fig. 9.4). The PHA content accumulated in the various isolates ranged between 56.4 and 62.4% DCW.

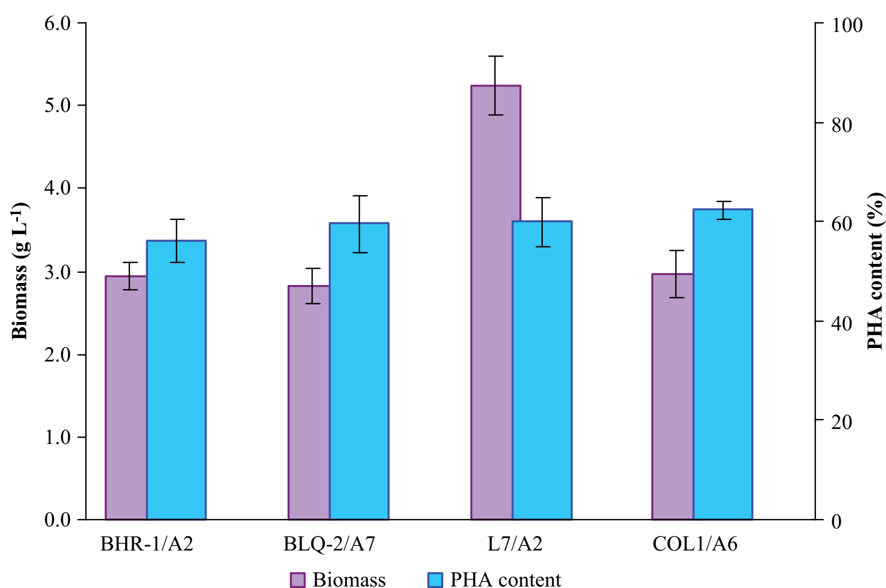

Fig. 9.4 Production of PHA in various *Bacillus* species using wafer residue hydrolysate

Use of soluble starch without hydrolysis has been reported by Halami (2008) and Kim (2000). *B cereus* strain described by Halami (2008) was able to accumulate a PHA content of 48 % DCW using starch-based medium, whereas 46 % DCW was obtained using *Azotobacter chroococcum* (Kim 2000). Lillo and Rodriguez-Valera (1990) have reported soluble starch as the ideal carbon substrate for *Haloferax mediterranei* growth and polymer synthesis. Koutinas et al. (2007) have demonstrated the use of wheat hydrolysate and fungal extract (as carbon and nitrogen source, respectively) for PHA production in *C. necator*. Under these conditions, a PHA content of 70 % DCW was achieved. Production of PHA from inexpensive extruded rice bran (ERB) and extruded corn starch (ECS) employing *H. mediterranei* was investigated by Huang et al. (2006). Repeated fed-batch fermentation with ERB resulted in a PHA content of 38.7 % DCW and on using a mixture of ERB and ECS resulted in a PHA content of 55.6 % DCW. However, *B. sphaericus* NCIM 5149 grown on hydrolysates of cornflour and wheat bran was able to produce only 3.3 and 6.8 % DCW of PHA content, respectively. It was observed that wheat bran hydrolysate favored cell growth rather than PHA synthesis (Ramadas et al. 2009). Using waste potato starch hydrolysate as the chief carbon source, Rusendi and Sheppard (1995) have reported PHA production with a yield of 77 % DCW employing *R. eutropha*. *Halomonas boliviensis* LC1 attained a PHA content of 34 % DCW when grown on wheat bran hydrolysate (Van-Thuoc et al. 2008).

C. Citrus Fruit Waste Exploiting the ability of the various *Bacillus* species to grow and produce PHA from citrus pulp waste resulted in a PHA content ranging from 38.87 % DCW (BLQ-2/A7) to 48.86 % DCW (NQ-11/A2) (Fig. 9.5). Citrus pulp waste promoted biomass production with maximal growth observed in case of isolate BLQ-2/A7 (4.5 g L^{-1}) followed by COL1/A6 (4.13 g L^{-1}).

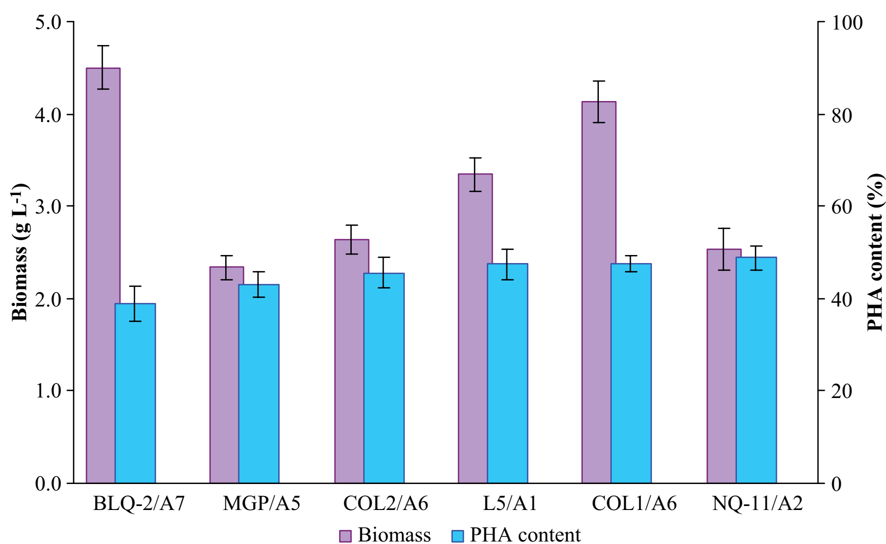

Fig. 9.5 Production of PHA in various *Bacillus* species using citrus pulp waste hydrolysate

Till date, no studies have been published reporting the use of citrus pulp waste as a carbon source. However, a number of studies are conducted using organic matter from wastes as an alternative to produce the polymer from inexpensive sources. These studies also highlight the necessity of incorporating a hydrolysis step prior to inoculation (Rebah et al. 2009). Polymer production from mahua flower extract employing fermentations with *Bacillus* sp. 256 resulted in a PHA content of 51 % DCW (Anil Kumar et al. 2007). Kumar et al. (2009) have evaluated PHA production from pea-shell waste with the help of different *Bacillus* strains. They have reported higher yields (22–65 % DCW) with the enzyme-hydrolysed substrate as compared to the unhydrolysed waste.

D. Coconut Oil Cake Preliminary evaluation of coconut oil cake, an agroindustrial residue suggested it to be a potential carbon source for PHA production. Further, studies involving submerged fermentation were also carried out. Isolates exhibiting good fluorescence intensity on staining with Nile blue A were selected for these studies. Among the five isolates tested, isolate COL1/A6 exhibited maximal biomass and PHA of 3.75 and 1.58 g L^{-1}, respectively (Fig. 9.6) whereas the highest PHA content was observed with isolate COL1/A11 (42.4 % DCW). The removal of adherent fatty acids from the coconut oil cake hydrolysate was achieved by a brief hexane wash thereby facilitating the quantification process (Lee et al. 2000; Santimano et al. 2009a).

Recently, a few studies have been conducted using oil cakes such as sesame, groundnut, mustard, palm under submerged fermentation and babassu, soy cake using solid-state fermentation (Ramadas et al. 2009; Singh and Mallick 2009; Oliveira

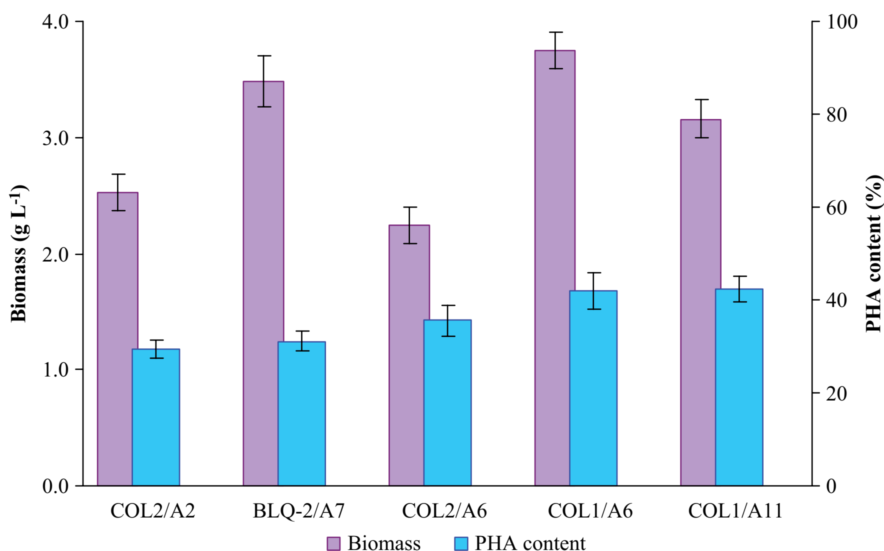

Fig. 9.6 Production of PHA by various *Bacillus* species using coconut oil cake hydrolysate

et al. 2004). The PHA content accumulated using these oil cakes varied from 14.0 to 39.2 % DCW. As seen from these studies, species belonging to the genus *Bacillus* are able to utilize oil cakes for growth better than Gram-negative organisms.

E. Polymeric Substrates Bacterial strains belonging to the genus *Bacillus* were evaluated for their PHA-producing ability using the simplest and most easily metabolizable monomeric sugar, glucose. Since these isolates exhibited excellent PHA production on glucose, polymers of glucose were then tested as sole carbon substrates. Among these, starch proved to be an excellent source for PHA production. All the *Bacillus* isolates (except isolates PPA/Z6 and COL1/A1) were able to synthesize PHA on the majority of the polymers tested. All the 16 isolates were unable to utilize bagasse and rice chaff as raw material for polymer production.

Quantitation of PHA using submerged cultivation of selected isolates was achieved using hydrolysate of wafer residue. Hydrolysis of these by-products using dilute acid not only improved the ability of the isolates to assimilate the released fermentable sugars as PHA, but also avoided the interference caused by the insolubles present in the wastes during downstream processing when grown under submerged cultivation conditions. Under these conditions, high PHA accumulation ranging from 56.14 to 62.41 % DCW was obtained.

9.5 Conclusions and Future Prospects

The present study deals with PHA production by selected *Bacillus* spp. and indicates their potential as PHA producers. These *Bacillus* spp. isolated from diverse marine and coastal econiches, exhibited temporal variation in their PHA accumulation pattern. Based on the PHA content accumulated within the cells, the isolates could be categorized into four groups. Isolates (BLQ-2/A7, COL1/A6, L2/A1 and L4/A4) belonging to one of these groups were capable of consistently maintaining the accumulated PHA without significant intracellular degradation during the entire experimentation time. This property is crucial in large-scale PHA production as variation in the time of harvest will not adversely affect the product yield.

Ability of the different *Bacillus* isolates to utilize diverse agroindustrial by-products revealed substrates such as molasses, wafer residue, citrus pulp and coconut oil cake to be potential carbon feedstock for PHA production. The majority of the isolates were capable of producing PHA using these substrates. Using molasses as the carbon source, all the isolates tested were able to accumulate PHA ranging between 51.23 % and 68.56 % DCW. Wafer residue was also efficiently utilized by the isolates as more than 55 % DCW PHA was produced within the cells. Citrus pulp was able to support PHA content ranging between 38.87 % and 48.86 % DCW in the selected *Bacillus* spp. Coconut oil cake also served as a potential carbon source for PHA production with a highest PHA content of 42.4 % DCW.

These studies emphasize the potential of *Bacillus* spp. to produce value-added product (PHA polymer) from diverse renewable low-cost feedstocks at substantially reduced production costs. The isolates were able to unequivocally produce PHA ranging from 29.45 % DCW (COL2/A2 on coconut oil cake hydrolysate) to 68.56 % DCW (ICP-1/A3 on molasses) using low-cost agroindustrial residues. Further characterization of the produced polymer is necessary as this genus is known to produce PHA with different monomer compositions from a wide variety of substrates (Valappil et al. 2007a). Based on these investigations further studies may be directed towards:

- PHA production and high-cell density fermentation with intensive studies on fed-batch mode of operation involving pH, dissolved oxygen (DO)-regulated system and glucose supplementation
- The use of solid-state fermentation (SSF) for the production of PHA

The high-cell density is vital for the economic viability of the production process while SSF is an attractive strategy that allows the use of inexpensive feedstock such as agroindustrial residues. The advantage of employing such fermentation strategies is that, they provide solutions to the disposal of agro-based residues with simultaneous production of value-added products. These residues can be directly incorporated in the fermentation media without any pretreatment unlike submerged fermentation (SMF). The fermented solids containing PHA products can be used directly without downstream processing to prepare composite materials of increased biodegradability.

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Chapter 10
Transformation of Triphenyltin by Eubacteria: Fate and Effects in Environmental System

Sangeeta Jadhav

10.1 Introduction

The earth's crust consists of nearly 80 elements. The elements, especially the metals, play a pivotal role in human life. Among several metals, tin (Sn) has been used for over 3000 years. This silvery, malleable posttransition metal is not easily oxidized in air and hence its compounds are used to coat other metals to prevent corrosion. The organic derivatives of tin are known as organotins (OTs). The estimated worldwide industrial production of OTs exceeds 50,000 t per annum. About 70 % of the total production is being used as additives in the plastic industry, for the production of polyurethane foams and silicones (Bennett 1996). Some OT compounds are highly toxic and have been used as biocides in antifouling paints and agriculture (Champ and Seligman 1996). Triphenyltin (TPT) is one such OT compound used as a biocide. TPT is also been used as a fungicidal component in agriculture, and for timber preservation (Hoch 2001). However, TPT has a harmful effect on nontarget aquatic organisms when released into the environment, even at trace levels (Harino et al. 1998, 2000). TPT pollution in aquatic systems may cause various changes in the affected fauna, such as thickening of the shell and failure of spat in oysters (Alzieu 1996), impotence in gastropods and neogastropods (Bryan et al. 1998, CICAD 13 1999), reduction of dogwhelk populations (Gibbs et al. 1991), retardation of growth in mussels (Salazar and Salazar 1991) and immunological dysfunction in fish (Suzuki et al. 1992).

The European Commission now considers OTs such as tributyltins (TBT) and TPT as priority hazardous substances in water and the maximum allowable concentration of OTs is proposed to be fixed at 0.0015 µg L^{-1} in inland surface waters (COM 2006). The International Maritime Organisation (IMO) has banned the use of OTs on ship hulls and in aquaculture since 2008. But these compounds are illegally used in many South Asian countries including India. As TPT is cheaper compared

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to TBT, it has replaced TBT in many industrial applications. This has given rise to a substantial amount of TPT pollution (Kannan et al. 1995; Kannan and Lee 1996).

10.2 Chemical and Physical Properties of TPT

TPT consists of three phenyl molecules having a covalent bond with the Sn atom (Fig. 10.1a). The covalent bond between Sn and C remains stable in the presence of water, atmospheric O_2 and heat. TPT compounds may be characterised by a general formula $(C_6H_5)_3Sn\text{-}X$, where X is an anion or an anionic group, such as chloride, hydroxide and acetate. The physical and chemical properties of TPT compounds vary depending upon the X linked to the Sn molecule (Table 10.1). These compounds are lipophilic in nature and have low water solubility (typically a few mg L^{-1} at neutral pH). Ultraviolet radiations, strong acids and electrophile agents may cleave the covalent bond between Sn and the carbon moiety. Diphenyltin (DPT) and monophenyltin (MPT) are degradation products of TPT, together called as phenyltins (PTs). OTs can be synthesized using three different routes, Grignard, Wurtz and aluminium alkyl route (Fig. 10.1b).

Environmental analysis of TPT requires methods that are sensitive enough for an accurate determination at extremely low concentrations (ng Sn L–1). Species-selective analysis of TPT compounds is performed by coupled techniques based on

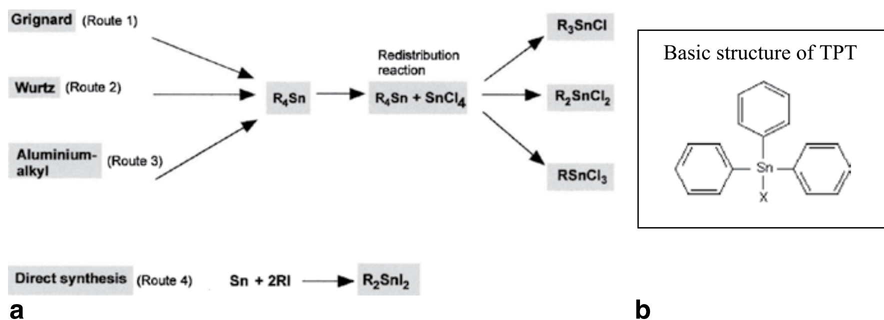

Fig. 10.1 Synthesis of organotin compounds **a** and structure of TPT **b** (Blunden and Evans 1990). Where R can be phenyl, butyl, ethyl, methyl, alkyl or aryl group and X can be any halide

Table 10.1 Physical and chemical properties of some TPT compounds (Tomlin 1997)

	TPT		
	Acetate	Chloride	Hydroxide
Molecular formula	$C_{20}H_{18}O_2Sn$	$C_{18}H_{15}ClSn$	$C_{18}H_{16}OSn$
Molecular weight	409.1	385.5	367.0
Melting point	122–124 °C	106 °C	122–123.5 °C
Solubility	9 mg L^{-1} (pH 5)	40 mg L^{-1}	1 mg L^{-1} (pH 7)
Vapour pressure	0.047 mPa (50 °C)	0.021 mPa	0.047 mPa (50 °C)

a combination of a chromatographic separation technique with a sensitive and element-selective detection method. The most common technique is gas chromatography (GC) coupled with element-specific detection methods like atomic absorption spectrometry (GC-AAS; Cai et al. 1993), mass spectroscopy (GC-MS; Jadhav et al. 2009), microwave-induced and inductively coupled plasma atomic emission spectrometry (GC-MIP-AES and GC-ICP-AES, respectively) or flame photometric detection (GC-FPD; Bhosle et al. 2004, 2006).

10.3 TPT as Source of Environmental Pollution

Any surface immersed in water adsorbs dissolved organic matter thereby leading to conditioning of the surface. Conditioned surfaces are then colonized by microorganisms followed by macroorganisms. Attachment and growth of organisms on a surface is called fouling. Fouling is of economic concern to the shipping industries because it induces frictional drag on the hulls of ships thereby increasing the fuel consumption. In order to reduce economic losses due to fouling, the ship hull is coated with paints containing antifouling agents like OTs (TBT and TPT). These biocides are slowly released from the hull when it comes in contact with water. The release of biocide prevents the settlement of fouling organisms such as barnacles, tubeworms, etc. The OT-based paint protection for 5–7 years is estimated to save the shipping industry some US$ 5.7 billion per annum (Rouhi 1998). This also results in annual fuel saving of 7.2 million tonnes per year (Bennett 1996).

Another major use of TPT lies in the agricultural industry (Kannan et al. 1995). TPT has been used as a fungicide across the globe, to treat a variety of plants such as potatoes, sugar beets, peanuts and rice. There has been considerable increase in the amount of TPT used as a fungicide in some areas over the last three decades as it is an approved pesticide for feedstock. Kannan and Lee (1996) reported a 3-fold increase in the usage of TPT as pesticide all over the world. TPT compounds are also used in the textile and paper industry (Hoch 2001). The PVC polymer becomes unstable under the influence of heat and light resulting in discolouration and embrittlement. Addition of OT compounds prevents this degradation process of the polymer.

PTs enter the aquatic environment directly by leaching through antifouling paints, through river run-off from agriculture and industrial waste, municipal sewage, etc (Fig. 10.2). TPT compounds may also enter the environment by leaching into soil and groundwater from consumer products containing PT compounds disposed off in landfills (Fent 1996; Fent and Hunn 1991). The environmental concentration of TPT varies based on where, when and how the compounds are used.

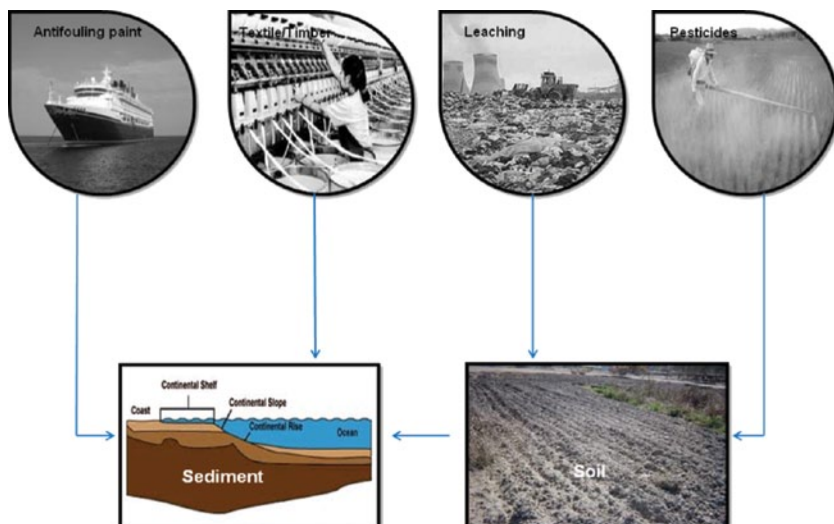

Fig. 10.2 Industrial applications and sources of PTs in the environment. (Jadhav 2013)

10.4 Fate of TPT Compounds in Environmental Systems

With the wide industrial applications, considerable amounts of TPT compounds have entered various ecosystems. The persistence of TPT compounds in polluted ecosystems is a function of physical (adsorption to suspended solids and sediments), chemical (chemical and photochemical degradation) and biological (uptake and biological degradation) removal mechanisms (Fig. 10.3). Thus, it is important to study the distribution and the degradation processes of TPT compounds under natural conditions. Degradation of TPT in the natural environment can be caused by three processes;

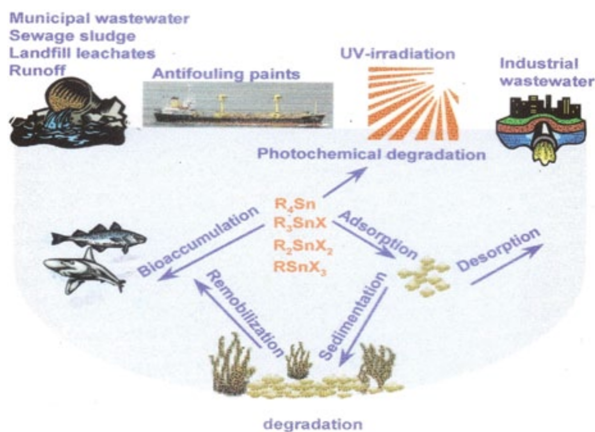

Fig. 10.3 Fate of TPT in the aquatic environment. (Hoch 2001)

1. Photolysis by sunlight appears to be the fastest route of degradation in water. But because of attenuation of sunlight with depth in the water column, photolysis is probably not important at greater depths in water, nor in sediments or soils (Hoch 2001).
2. The Sn–C bond can be attacked by both nucleophile and electrophile reagents. For example, mineral acid, carboxylic acids and alkali metals are agents which are able to cleave Sn–C bonds (Hoch 2001).
3. Barnes et al. (1973) showed stepwise decomposition of TPT acetate in soil to DPT and MPT by bacteria capable of degrading TPT such as *Pseudomonas aeruginosa*, *P. putida* and *Alcaligenes faecalis*. The study demonstrated that bacteria may play an important role in TPT degradation. However, only a limited number of such species have been identified until now and little is known about the conditions required for biological degradation (Dubey and Roy 2003).

10.5 Distribution and Effects of TPT Compounds on Various Ecosystems

Anthropogenic activities have led to an increase in TPT concentrations in water, soil, sediments and organisms. Knowledge about the environmental concentrations of any chemical compound is required to understand its effects on the system. The aquatic system is most susceptible to TPT contamination, as TPT is directly released into the aquatic environment by antifouling coatings, agricultural and municipal waste into coastal areas, marinas, bays and the open sea (Fig. 10.2). Concentrations of PTs in the marine environment can vary among seasons (Lee et al. 2006). Concentrations of TPT in organisms have been found to be greater in summer (Hung et al. 1998, 2001; Lee et al. 2005). Because antifouling paints are not the only source of PTs, this seasonal trend of PTs might be due to their use in aquaculture nets and application of PT-containing biocides in both mariculture and agriculture during the summer (Hung et al. 1998; Meng et al. 2009).

The observed distribution and variation in the concentrations of PTs are caused by different sources of contaminants. In the aquatic environment, TPT compounds have low solubility and mobility, and they are adsorbed onto suspended particulate matter (SPM). The deposition of SPM leads to TPT scavenging in sediments, where considerable amounts of TPT and its degradation products can be detected. TPT is expected to be present in the upper 2–3 cm of the sedimentary column because of its recent use in aquatic systems. Nevertheless, a core collected (107 ng Sn g^{-1}) from freshwater marina of Switzerland indicated the presence of TPT upto 11 cm of the sediment core in varying concentration (Fent and Hunn 1991). Dated sediment indicates that anoxic harbour sediments are long-term reservoirs of TPT compounds. The degradation rate of OT in sediments may range from 1.8 to 2.8 years (De Mora and Pelletier 1997). Therefore, the subject of growing concern and debate is persistence of OT, the transformation kinetics and their possible release from sediments. The presence of PT species on suspended matter or sediment makes them avail-

able to filter or sediment feeding organisms. Another possible contamination risk is resuspension and remobilisation of contaminants from sediments due to dredging, swirling or desorption due to life activities (Hoch 2001).

TPT accumulation in marine fauna was first reported by Takami et al. (1988). In general, TPTs are known to bind to amino acids, peptides lipids and proteins and this complexation may influence tissue distribution in organisms (Davies and Smith 1980, Hu et al. 2009). TPT is hazardous to aquatic life and has been proved to be more neurotoxic than TBT (Lee et al. 2006). TPT compounds are found to be potential endocrine disruptors and induce imposex in some prosobranch (snails) species (Barroso and Moreira 2002; Horiguchi et al. 1997; Santos et al. 2009; Schulte-Oehlmann et al. 2000). Laboratory experiments showed that 'imposex' is initiated and promoted by TPT at concentrations of 1 ng Sn L^{-1} in Japanese rock shell *Thais clavigera* (Horiguchi et al. 1997). Imposex gives rise to reproductive failures and, as a consequence, population decline. TPT and other OT compounds can have adverse effects on molting, growth and reproduction of crustaceans (Rodriguez et al. 2007). Widdows et al. (1995) reported that threshold values for affecting the growth of mussels was 2 µg g^{-1} dw. Individual populations may vary in their susceptibility to TPT exposure due to different genotypes, rate of metabolism, ontogenic development and environmental history. TPT affects algal cells at 5–15 µg L^{-1} and cells were totally damaged at 20 µg L^{-1} (Rumampuk et al. 2004). This study showed that reproductive cells of algae were more sensitive than somatic cells (Rumampuk et al. 2004). TPT had adverse effects on the freshwater plant *Lemna polyrhiza*, at concentrations of 2–5 µg L^{-1} (Song and Huang 2005). Due to the hydrophobicity of the PT molecules, it can easily solubilise in biological membranes and thus affect the organism. However, individual populations may vary in their susceptibility to TPT exposure due to different genotypes, rate of metabolism, ontogenic development and environmental history. The review of measured TPT concentrations in marine ecosystem can be found in Yi et al. (2012).

TPTs inhibit a number of microbial processes. TPTs affect energy transduction, solute transport and retention and oxidation of substrates. The mode of action of OT compounds has been described in terms of hydrogen bonding with the active centres of cell constituents resulting in interference with normal processes. Since the OT complexes inhibit the growth of microorganisms, it has been assumed that the production of an enzyme is being affected as a result of which the microbes are less able to metabolize the nutrients resulting in growth cessation. Those enzymes that require free sulfydryl groups (–SH) for activity, appear to be especially susceptible to deactivation by ions of the complexes (Basu Baul 2008).

In general, OT compounds are more toxic towards Gram-positive bacteria than Gram-negative bacteria, but TPT–Cl is equally toxic towards both. TPTs are known to inhibit methanogens and fermentative bacteria (Harino et al. 1997). Triphenyltin actetate (TPT–Ac) inhibited the nitrification by bacteria and fungi (Wurtz and Cooney 1989). TPT-inhibited light-induced proton uptake in *Halobacterium halobium* (Mukhohata and Kaji 1981). A good activity minimum inhibitory concentration (MIC) 3.1 µg ml^{-1} against *Staphylococcus epidermis* was noted for DPT-Cl. PTs are known to inhibit several Gram-positive bacteria like *Bacillus subtilis*,

Staphlococcus aureus and several bacilli. The recommended dose for TPT and DPT is given as 3 μg L^{-1} for bactericidal action (Basu Baul 2008).

10.6 Microbial Transformation of TPT

Bacteria have developed very efficient and very different mechanisms for tolerating OTs (Trevors et al. 1985). Often, normal toxic levels of OT have no effect on cell growth of these tolerant organisms. The mechanisms responsible for the tolerance of OT by bacteria could be because of the following reasons:

1. Exclusion of OTs from cell-mediated by a multidrug efflux pump
2. Bioaccumulation into cell without breaking down
3. Degradation/utilization of OTs as a carbon source
4. Transformation of OTs to less toxic compounds (Cruz et al. 2007)

The microbial transformation and degradation of OT compounds with special reference to TBT and TPT are summarized in this section, Barug (1981) reported that *P. aeruginosa* was able to degrade 40% TBT–Cl when grown in presence of 2.5 mg L^{-1} TBT–Cl. *Pseudomonas diminuta* isolated from polluted river, Osaka, Japan, was able to degrade 20 μg L^{-1} TBT (Kawai et al. 1998). *P. aeruginosa* capable of utilizing TBT–Cl as a sole source of carbon was isolated from marine water samples (Roy and Bhosle 2006).

Suzuki et al. (1992) found that nearly 90% of the bacterial flora mostly belonging to *Vibrio* species of natural seawater was resistant to TBT–Cl. One of the cultures, *Alteromonas* sp. was found to be tolerant to 250 nm TBT–Cl (Suzuki et al. 1992). According to most of the reports, OT resistance in bacteria is rendered by chromosomal genes (Suzuki et al. 1994, Suzuki and Fukagawa 1995). *Pseudomonas stutzeri* 5MP1 isolated from sediments of Arcachon harbour (France) resisted 1000 mg L^{-1} of TBT which was attributed to the presence of operon TbtABM homologus to resistance-nodulation-division (RND) multidrug efflux pump (Jude et al. 2004). *Aeromonas veronii* isolated from an estuarine environment of Portugal was able to resist 3 mM TBT (Cruz et al. 2007). However, OT resistance in some bacteria can be plasmid-mediated (Miller et al. 1995).

A study conducted on TPT acetate degradation in soil revealed that *P. aeruginosa*, *P. putida* and *A. faecalis* were responsible for degradation of TPT to DPT, MPT and inorganic Sn (Barnes et al. 1973). Visoottiviseth et al. (1995) isolated *P. putida* no.C from soil samples collected from the dockyards of Thailand. *P. putida* no. C was able to transform 97% of TPT in minimal medium supplemented with glucose and 7 mg L^{-1} TPT. Inoue et al. (2000) isolated *Pseudomonas chlororaphis* CNR 15 from coastal sediment. The culture was able to transform 47% of TPT when grown in minimal medium containing 40 mg L^{-1} TPT. Later, they identified TPT degrading factor as suc-pyoverdine (succinic acid derivative of siderophore) in *P. chlororaphis* CNR 15. (Inoue et al. 2003). Immobilized cells of *P. chlororaphis* with calcium alginate were able to adsorb and transform TPT. The transformation activity was influenced by temperature and pH and showed maximum at 30 °C and

pH 8.8 (Osamu et al. 2003). Yet another fluorescent pseudomonad, *P. aeruginosa* CGMCC 1.860 was able to transform nearly 50 % of TPT when grown in succinate M9 medium with 100 µM of Fe (III) and 77 mg L^{-1} TPT (Sun et al. 2006). Sun and Zhong (2006) demonstrated that the presence of TPT–Fe–pyochelin complex was instrumental in TPT degradation by *P. aeruginosa*. Pyochelin is a secondary siderophore present in the culture along with pyoverdine. So far, only siderophores like pyoverdine and pyochelin have been reported to be instrumental in TPT transformation process of TPT by bacteria.

Other than bacteria, few algal and fungal species have also been reported to carry out OT degradation. Reader and Pelletier (1992) reported that microalgal species *Skeletonema costatum* is capable of degrading TBT at even 4 °C. Maguire et al. (1984) demonstrated that the green algae *Ankistrodesmus falcatus* was capable of degrading TBT to dibutyltins (DBT). Unicellular green algae, *Chlorella vulgaris* and *Chlorella* sp. degrade TBT–DBT and monobutyltins (MBT) intracellularly with the help of a TBT-metabolizing enzyme (Tsang et al. 1999). *Cunninghamella elegans* IM 1785/21Gp, a fungus was able to degrade 70 % of TBT (added at 10 mg L^{-1}) to less toxic derivatives such as DBT and MBT when grown over a period of 7 days in Sabouraud medium and M3 medium involving cytochrome P-450 system (Bernat and Długonski 2002, 2006).

Bacteria can be used as an effective tool in bioremediation processes as they are able to tolerate and transform OT compounds at higher concentration and the transformation period is considerably reduced with bacteria as compared to fungi and algae. However, the process of TPT transformation by bacteria is poorly understood. The question is whether only fluorescent bacteria are capable of TPT degradation/transformation and whether the transformation is by enzymes or due to chelation process. In view of this, there is a need to search for bacteria which can tolerate and transform/degrade TPT, especially at environmental concentrations present in harbours and marinas. These TPT-tolerant bacteria can be used as an effective tool to transform TPT to nontoxic forms.

10.7 TPT-Transforming Bacteria from Coastal Sediments

Studies were undertaken to isolate TPT-transforming bacterial cultures from marine sediments collected from Sancoale shipyard (South Goa), Betim fish jetty (North Goa), and Visakhapatnam harbour, east coast of India.

10.7.1 Enrichment Technique

Enrichment was carried out by adopting two methods. In the first method, sediment sample was collected from Sancoale shipbuilding industrial area in Goa. Sediment sample (100 g) was transferred to a 500 mL beaker and 500 mg of TPT-chloride,

nitrates and phosphates (9:1 ratio) were added and the sample was thoroughly mixed. The beaker was incubated at room temperature (28 ± 2 °C) in the dark. After 30 days, 1 g sediment was appropriately diluted and plated on ZoBell marine agar (ZMA) plates containing 100 mg L^{-1} of TPT. Plates were incubated at room temperature (28 ± 2 °C) for 48 h. The individual colonies were collected and restreaked on ZMA plates to check their purity. The cultures thus obtained were maintained on ZMA slants containing TPT (100 mg L^{-1}) at 4 °C.

In the second method, enrichment was carried out by inoculating 10 g of sediment collected from Betim fish jetty (North Goa) and Visakhapatnam harbour to Basal Salt Solution (BSS) medium supplemented with TPT-chloride (100 mg L^{-1}; Bhosle 1981). The flask was incubated on a rotary shaker (100 rpm) at room temperature (28 ± 2 °C) in the dark for one week. The culture was subcultured twice using the same but fresh medium. After obtaining consistent growth, 1 mL of the culture was appropriately diluted and plated on ZMA containing 100 mg L^{-1} of TPT. These plates were incubated for 48 h at room temperature (28 ± 2 °C). Individual colonies were picked, purified and maintained as described above.

Using enrichment technique, 24 bacterial cultures were isolated from marine sediments collected from Sancoale shipbuilding yard (South Goa), Betim Fish Jetty (North Goa) and Visakhapatnam harbour.

10.7.2 Growth of Bacterial Isolates in BSS with Varying Concentrations of TPT

Bacterial isolates were tested for their ability to grow or transform various concentrations of TPT (0, 25, 50, 75 and 100 mg L^{-1}) in BSS medium. The growth of bacterial cultures was slow and minimal in BSS medium supplemented with 25 and 50 mg L^{-1} of TPT. Only 4 cultures (SG 01, SG 04, SV 04, and SV 14) showed visible turbidity when grown with 75 and 100 mg L^{-1} of TPT. Hence, additional carbon sources in the form of sodium succinate and glycerol were used in BSS medium to boost the growth of the bacteria and the medium was designated as BSS-SG. Bacterial cultures were grown by preparing an inoculum in BSS, BSS with 100 mg L^{-1} of TPT, BSS with succinate (0.4 %) glycerol (0.1 %) and BSS with succinate (0.4 %), glycerol (0.1 %) and 100 mg L^{-1} of TPT, individually. Growth was monitored using UV-VIS spectrophotometer. The successive transfers of bacterial cultures in BSS-SG medium supplemented with TPT produced luxuriant growth; therefore, BSS-SG medium supplemented with 100 mg L^{-1} of TPT was used for the screening of potential bacterial cultures.

To check for transformation ability, aliquots of culture broth (100 µL) were withdrawn at 0 days and 7 days following inoculation, and transferred to clean stoppered test tubes. The culture broth was then analysed for TPT using GC-MS-EI (70 eV) as described by Jadhav et al. (2009). Out of 24 isolates, 17 were able to grow and transform TPT to varying extents (5–65 %), while the rest of the isolates did not transform TPT but were able to tolerate TPT (Table 10.2).

Table 10.2 Growth and transformation of TPT by bacteria isolated from marine sediments

Isolate	Culture optical density (OD) at 600 nm	% of TPT transformation	Isolate	Culture OD at 600 nm	% of TPT transformation
SG 01	0.163	5	*SV 10*	0.202	0
SG 02	0.464	30	*SV 11*	0.517	12
SG 03	0.353	7	*SV 12*	0.434	7
SG 04	0.551	65	*SV 13*	0.439	10
SV 01	0.501	5	*SV 14*	0.462	35
SV 02	0.417	12	*SV 15*	0.169	0
SV 03	0.408	8	*SV 16*	0.192	0
SV 04	0.405	0	*SV 17*	0.561	5
SV 05	0.199	5	*SV 18*	0.256	0
SV 06	0.455	5	*SV 19*	0.399	12
SV 07	0.516	15	*SV 20*	0.375	0
SV 08	0.370	12	*SV 21*	0.339	10

10.7.3 Characterisation and Identification

The cultures consistently growing in the presence of TPT (100 mg L^{-1}) were examined for morphological, biochemical and physiological characteristics to identify the isolates upto generic level using the standard techniques (Kreig and Holt 1994). Among Gram-positive organisms, *Bacillus* sp. were dominant while among Gram-negative isolates it was found that *Pseudomonas* sp. were predominant and accounted for 48 % of the total bacterial isolates. The permeable architecture of cell membrane in Gram-negative bacteria allows the mobilization of metal ions (Cruz et al. 2007). Moreover, the presence of multidrug efflux pumps in these bacteria makes them more resistant to metals and pollutants compared to Gram-positive bacteria (Jude et al. 2004).

Most (81 %) of the isolates belonging to *Pseudomonas* genus were able to transform TPT. One of the *Pseudomonas* isolate, designated as SG-04 was able to degrade ~ 65 % of TPT and was identified as a strain of *P. stutzeri*. Further, 16S rDNA molecular sequencing followed by NCBI-BLAST search also confirmed the isolate SG-04 as a strain of *P. stutzeri* (Fig. 10.4). The 16S rDNA fragment from the strain has been deposited in the GenBank database (Accession no. JF509451.1). *P. stutzeri* is a ubiquitous bacterium in the environment (both in water and sediment) with a high degree of physiological and genetic adaptability. It is present in different natural environments. *P. stutzeri* is involved in environmentally important metabolic activities (Lalucat et al. 2006). Members of this species can metabolize a wide range of organic substrates, including environmental contaminants like naphthalene, carbon tetrachloride, etc. (Baggi et al. 1987; Dybas et al. 1995; Rossello-Mora et al. 1994). They can also be used as model denitrifier in marine environments (Lalucat

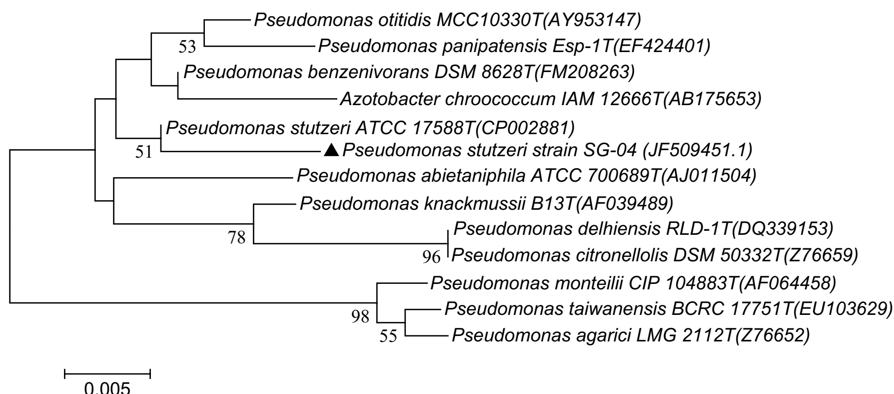

Fig. 10.4 Neighbour-joining tree based on analysis of the 16S rRNA partial gene sequence of *P. stutzeri* and other close strains

et al. 2006). *P. stutzeri* strains have been widely studied as a model for natural transformation processes (Sikorski et al. 1998, 2002; Lorenz and Sikorski 2000).

Marine strains of *P. stutzeri* are located in the water column and in sediment. The most relevant strains studied in detail are ZoBell, AN10, NF13, MT-1 and HTA208 (Lalucat et al. 2006). The ZoBell strain was isolated from the water column of the Pacific Ocean, and studied as a model denitrifier in marine environments. AN10 was isolated from polluted Mediterranean marine sediment and studied as a naphthalene degrader. NF13 isolated from a sample taken at 2500–2600 m depth in the Galapagos rift from near a hydrothermal vent was studied as a strain that oxidizes sulphur chemolithotrophically and strains MT-1 and HTA208 were isolated from deep-sea samples taken at the Mariana Trench at 10,897 m depth (Lalucat et al. 2006).

P. stutzeri strain isolated from black sea was able to oxidize thiosulphate to tetrathionate (Lalucat et al. 2006). *P. stutzeri* 5MP1 is a tributyltin-resistant strain (MIC 1000 mg L^{-1}) isolated from the sediment of Arcachon harbour (France). Tributyltin resistance was found to be associated with the presence of the operon *tbtABM*. It is a member of the resistance-nodulation-cell division efflux pump family (Jude et al. 2004).

10.8 Conclusions and Future Prospects

The review indicates the presence of microbes in the coastal ecosystem which have the potential of transforming TPT and other oganotins. The study undertaken in Goan coastal waters also showed the presence of such bacterial cultures capable of transforming/degrading TPT. A nonfluorescent *Pseudomonas* spp. identified as *P. stutzeri* was obtained which transformed ~65 % of TPT when grown in BSS–SG medium with 100 mg L^{-1} TPT. To the best of our knowledge, this is the first report

of the nonfluorescent *Pseudomonas* carrying out the transformation of TPT. TPT transformations have been reported so far by only fluorescent *Pseudomonas* spp. with the help of siderophores called pyoverdine and pyochelin.

The major application of TPT-transforming bacteria lies in bioremediation of pollutants from the contaminated sites. The bacterial strains having the capability of transforming and withstanding high concentration of pollutants is a prerequisite for such in field bioremediation experiments. The bacterial strain obtained in the present study showed the ability to survive and transform TPT at environmentally relevant concentrations and could be an ideal vector for carrying out bioremediation at contaminated sites. This study opens new avenues for TPT transformation by nonfluorescent bacteria which might lead to new information on TPT transformation mechanism.

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Chapter 11
Bacteria Adhered to Particulate Matter and Their Role in Plant Litter Mineralization

Amrita Kharangate-Lad

11.1 Introduction

Mangrove ecosystem is the Earth's most unique ecosystem. It forms the interface region between the aquatic and terrestrial ecosystems. It is biodiverse and productive as it is rich in flora and fauna. It is economically important as it provides crustaceans such as shrimp, crabs and other fishes while ecologically it is valuable as it acts as a barrier to storm surges, tsunamis and soil erosion (Dittmar et al. 2006; Jennerjahn and Ittekkot 2002). It is also rich in microbial communities which play a major role in recycling of organic matter (Abbasnezhad et al. 2011).

Many bacterial groups utilise the organic particulate matter from the mangroves which primarily consists of plant litter and contains complex polymers such as cellulose, hemicellulose, starch, lignin, chitin, etc. (Britto et al. 2006). Bacteria are known to utilise these complex natural polymers by adhesion to the particulate organic matter containing these polymers. Bacteria such as *Halomonas* (Garcia et al. 2004), *Sagittula stellata* (Gonzalez et al. 1997b), *Serratia marcescens* (Perestelo et al. 1990), *Marinobacter hydrocarbonoclasticus*, *Microbulbifer hydrolyticus*, *Marinobacterium georgiense* (Gauthier et al. 1992; Gonzalez et al. 1997a), *Vibrio* sp. (Gao et al. 2010) have been reported from coastal marine and mangrove ecosystem that degrade such complex natural polymers by attaching to the organic particulate matter containing these polymers.

11.2 Mechanism of Adhesion

Many bacteria in the mangroves grow attached to plant litter. This surface is often the substrate to which the bacteria initially adhere and use as a source of energy for their metabolic activity and help in degradation by a combination of heterotrophy

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and autotrophy. Some bacteria from the coastal, marine, estuarine and mangrove ecosystems known to show adherence include *Halomonas* sp., *Marinobacter* sp., *Vibrio* (Gulig et al. 2005), *Pseudoalteromonas, S. stellata, M. hydroliticus, M. georgiense* (Gonzalez et al. 1997a, b).

For adhesion to occur, the bacterial cell comes in contact with the substrate by either physical or chemical forces or a combination of both. The physical forces of interaction include the Brownian motion, Van der Waals' forces due to the surface electrostatic charge and hydrophobic interactions. Chemical process such as chemotaxis (chemoattractant is diffused forming a concentration gradient) and haptotaxis (when the chemoattractant is bound to the surface) are also responsible in some cases. These chemoattractants can be amino acids, oligopeptides sugars or any other biomolecule. Chemotaxis is seen in almost all microbes and can mediate bacterial growth on surfaces by regulating cellular adhesion components and preparing cells for cell–cell and cell–surface interactions (Katsikogianni and Missirlis 2004).

11.2.1 Physical Phase in Adhesion

The physical interactions are further classified as long-range and short-range interactions. The long-range interactions are nonspecific when the distance between the bacterial cell surface and the substrate surface is >50 nm. When the bacterial cell and substrate is separated by a distance of 10–20 nm, Van der Waals' forces come into play. Short-range interactions become effective when the bacterial cell surface and the substrate surface come into close contact about a distance of <5 nm. This involves chemical bonds such as hydrogen bonding, covalent bonding and hydrophobic interactions. The mechanism of interaction between the bacterium and the surface of the substratum is shown in the Fig. 11.1 (Gottenbos et al. 2002; Katsikogianni and Missirlis 2004).

This mechanism forms the basis of the interaction that occurs in the first step of adhesion of bacteria to their substrate in the long process of degradation. Thus, a bacterial cell is brought in contact with the substrate by long-range nonspecific interactions and kept in contact initially until the short-range interactions take over.

11.2.2 Molecular/Cellular Phase of Adhesion

The second phase involves specific molecular interaction between the bacterial extracellular moieties and the surface of the substratum and is practically irreversible. It can be specific like ligand-receptor (lectin-carbohydrate, protein-protein and hydrophobin-lipid) interaction or chemical bond formation or nonspecific such as hydrophobic or electrostatic interaction. The bacterial cell binds more firmly to the substratum by means of bacterial surface polymers, appendages such as fimbriae, pili, flagella or capsules and slime layers (Dworkin et al. 2006b; Katsikogianni and Missirlis 2004; Prescott et al. 2005; An and Friedman 2000; Bhaskar and Bhosle 2005; Kokare et al. 2009).

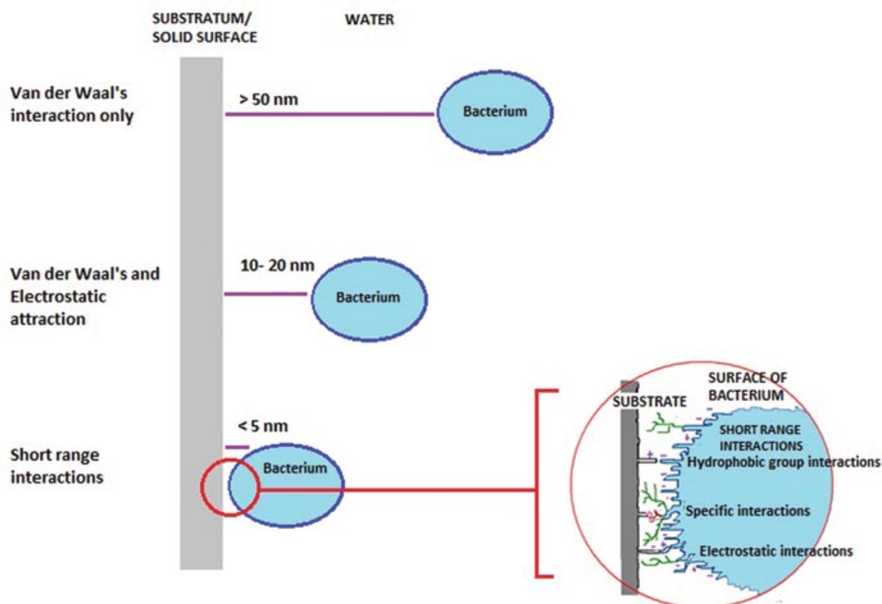

Fig. 11.1 Schematic representation of interaction between bacterial cell and substratum in the initial phase of bacterial adhesion. (Gottenbos et al. 2002)

It is believed that the functional part of these structures is the presence of adhesin—a protein component that helps the bacterium to bind to the substrate (An and Friedman 2000; Gottenbos et al. 2002; Katsikogianni and Missirlis 2004; Prescott et al. 2005). Bacterial adhesins involved in adhesion include lectins, fibronectin-binding proteins, glycolipid and lipid-binding protein.

11.3 Theories of Adhesion

Two theories are put forth to explain the mechanism of adhesion. The Derjaguin, Landau, Verwey and Overbeek (DLVO) theory and the thermodynamic approach to understand the mechanism of adhesion.

11.3.1 The DLVO Theory of Adhesion

The basic understanding of the interaction and attachment process of bacteria to a surface can be explained by the DLVO theory. It explains that the overall net interaction between the bacterial cell and the substrate surface is the result of balance between two additive forces. One, the electrostatic attractive force, i.e. Van der Waal's interaction and two, the repulsive interactions such as Coulomb interactions

that arises due to the electrical double layer of the bacterial cell and the substratum (Hayashi et al. 2001; Hermansson 1999; Katsikogianni and Missirlis 2004).

The DLVO theory explains the low attachment of the bacteria to negatively charged substratum, i.e. it explains the ability of the bacterial cell to overcome any electrostatic barrier. However, the theory has its limitations as it does not explain the molecular interactions that are likely to occur between the bacterial surface polymers and the substrate surface molecules in terms of cell–substrate distance and the type of interaction. It also does not account for the surface roughness of the substrate.

11.3.2 The Thermodynamic Approach to Attachment

This physicochemical approach was put forth by Morra and Cassinelli 1997. It takes into account attractive and repulsive forces such as Van der Waal's, electrostatic and dipole interactions and expresses them collectively as free energy. The approach uses numerical estimates of surface-free energy of the bacterial cell and the surface-free energy of the substratum to calculate Gibbs adhesion energy for bacterial adhesion. Adhesion of the bacterial cell to a surface is preferred if the free energy per unit surface area is negative when adhesion occurs, i.e. the attachment is accompanied by a decrease in the free energy of the system, as per the second law of thermodynamics (Katsikogianni and Missirlis 2004).

This theory has helped to explain the fact that a hydrophobic substrate or cell surface show increased attachment or adhesion. However, it is not possible to accurately calculate the surface-free energies of the bacterial cell due to their complex nature and hydration properties. Thus, the free energy change calculations during adhesion may be incorrect. This theory is applicable only to closed systems where no energy is put in from the outside. However, bacteria being a living entity that can convert substrate into energy, it fails to consider that the adhesion may be driven by physiological mechanisms and synthesis of adhesive bacterial surface polymers.

While the above theories fail to take into account the role of cell surface polymers or appendages that are produced that aid the bacterial cell to attach to the substrate, studies show numerous bacteria that are known to adhere to their substrates by means of these polymers and appendages which include extracellular polymeric substances (EPS), glycocalyx, capsule, fimbriae, pili and holdfast (Bhaskar and Bhosle 2005; Bouchotroch et al. 2001; Dworkin et al. 2006a, b; Gulig et al. 2005; Quintero et al. 1998; Romanenko et al. 2002; Toh et al. 2008).

11.4 Factors Influencing Bacterial Adhesion

The interactions between the bacterial cell and the substrate are influenced by various factors such as the environmental factors and the surface properties of the substrate/material and that of the bacterium. Environmental factors include pH, ionic

strength of the surrounding medium (Bitton and Marshall 1980), the flow conditions of the surrounding medium and presence of inhibiting (antibiotics, albumin) and promoting (fibronectin, fibrinogen) factors in the surrounding medium. Substrate/material composition includes the substrate chemical composition (charge and hydrophobicity of the surface), roughness of the surface, porosity and density of the surface while bacterial cell characteristics include bacterial hydrophobicity and bacterial cell surface charge (Katsikogianni and Missirlis 2004).

11.5 Structures and Appendages that Participate in Adhesion Process

Various surface structures such as EPS, glycocalyx, capsule, holdfast and surface appendages such as pili, fimbriae, flagella are known to play a crucial role in adhesion of the bacterial cell to the substrate.

11.5.1 Extracellular Polymeric Substances

EPS produced by bacteria are of two types. Capsular EPS is tightly bound to the bacterial cell and more organized as seen in *Halomonas maura, Hyphomonas* strain MHS-3 and *Thiohalomonas denitrificans* (Quintero et al. 1998), *Halomonas organivorans* (Garcia et al. 2004). The slime EPS is diffused and loosely bound to the cell. When the bacterial cell is very close to the surface of the substrate at the final stage of adhesion, it produces EPS that helps the cell to attach to the substrate surface irreversibly (Bhaskar and Bhosle 2005; Vu et al. 2009; Mancuso et al. 2004).

The glycocalyx encompasses both the capsules and slime layers. It is often described as a network of polysaccharides extending from the surface of bacteria and helps it to adhere to surfaces. The EPS structure is said to aid the bacteria in adherence to substrates, other bacteria, animal tissue and other inert substances apart from being protective (Kokare et al. 2009; Mata et al. 2006). Literature survey shows halophilic bacteria such as *Halomonas* species and *Salipiger mucescens* producing EPS aiding the cell in adhesion (Martinez-Canovas 2004a, b, c; Martinez-Checa et al. 2005)

11.5.2 Fimbriae or Simple Pili

Most Gram-negative bacteria exhibit the presence of short hair-like or nonflagellar filamentous projections external to the cell wall. These are called fimbriae, and they aid the cell to adhere to other cells and substrates. In the attachment phase, it has been seen that lectins of fimbriae act as adhesins permanently attaching the bacterial cell to the substrate (An and Friedman 2000; Prescott et al. 2005). The marine

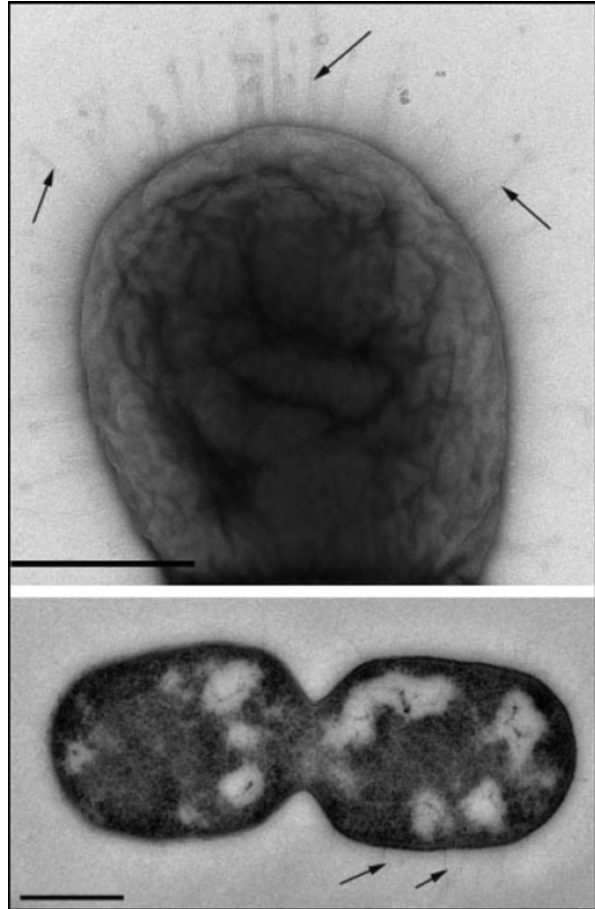

Fig. 11.2 *Halomonas halocynthiae* Fimbriae seen through an electron microscope. (Romanenko et al. 2002)

bacteria *Hyphomonas* strain MHS-3 which is the primary colonizer of surfaces in the marine environment synthesises two structures that mediate adherence to the solid substrate—a capsular polysaccharide and fimbriae. Their attachment is important as it paves the way for attachment of other members of adherent community such as protozoa, fungi by rendering the surface enriched and thus suitable. Interestingly, it is seen that the EPS produced attaches to both, the hydrophobic and hydrophilic surfaces. It is also believed that the fimbriae mediate long-range primary adherence to surfaces as they extend beyond the EPS capsule of the cell and bring bacteria into contact with the surface (Quintero et al. 1998). *Vibrio vulnificus*, a moderate halophile and *Halomonas halocynthiae* (Fig. 11.2) are known to produce fimbriae (Gulig et al. 2005; Romanenko et al. 2002).

Fimbriation as it is often called is widely distributed in Enterobacteriaceae and Pseudomonadaceae. However, it is not restricted to these two families. Ecologically, it is important as it initiates attachment to solid surfaces mainly with solubil-

ity substrate and contact with other members of the community which may aid in biofilm formation.

Bacteria such as halotolerant *Caulobacter* sp. and some *Pseudomonas* sp. form star-shaped aggregates called rosettes as a consequence of polar fimbriae and slime production by these bacteria.

11.5.3 Holdfasts

These are extracellular bridging structures that are produced by some bacteria to attach themselves to flat surfaces. They are usually polysaccharide in nature. The holdfast has strong adhesive properties at the end tip of its stalk (Ong et al. 1990). Bacteria known to form holdfast structures are *Thiothrix sp, Flexibacter sp, Seliberia stellata* and *Sagittula stellata* (Cytryn et al. 2006; Gonzalez et al. 1997b, Quintero et al.1998). Apart from these, other bacteria which were stalked, prosthecate or holdfast forming have been reported such as *Caulobacter* sp. (Fig. 11.3) (Levi and Jenal 2006; Merker and Smit 1988; Ong et al. 1990; Toh et al. 2008; Tsang et al. 2006) and *Roseisalinus antarcticus* (Labrenz et al. 2005). In the marine environment *Leucothrix mucor* is known to attach to seaweed by means of holdfast structures (Dworkin et al. 2006b).

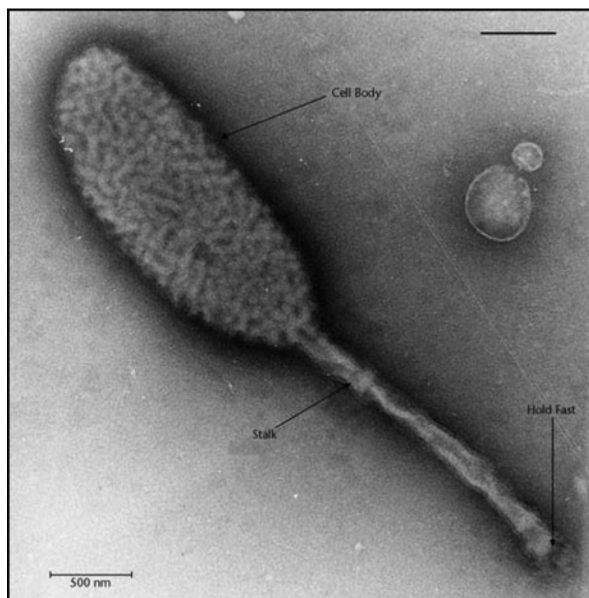

Fig. 11.3 *Caulobacter* sp. holdfast structure seen through an electron microscope. (Toh et al. 2008)

11.5.4 Flagella

Bacterial flagellae play an important role in bringing the bacteria in physical contact with substratum. A recent report on pathogenic bacteria such as *Pseudomonas aeruginosa*, *Clostridium difficile* and *Escherichia coli* shows that flagella are responsible in aiding adhesion of these bacteria to hospital medical instruments and medically implanted devices (Haiko and Westerlund-Wikström 2013). Flagella facilitate motility of the bacterium and also aid it to hook onto crevices of surfaces. The flagellin in the flagella aids adhesion and allows the cell to anchor itself to the substrate.

11.6 Benefits of Adhesion

Adhesion is a way for a bacterium to establish and sustain in a particular habitat. The ability to adhere has been conferred by certain fine structures on the bacterial cell surface such as polysaccharide fibres. This fibre network extends from the surface as glycocalyx and mediates adhesion to abiotic components, substrates, other cell hosts and prey. In the case of medically important bacteria such as *Stapylococcus aureus*, *Staphylococcus epidermides* (Katsikogianni and Missirlis 2004) and *Streptococcus sangui* (Ofek et al. 2003) adhesion is an important factor for colonization and virulence and thus confers the bacterium with pathogenecity.

Sessile or adhered bacteria are able to exploit the nutritional opportunities of habitat better than nonattached bacteria. Adherence of bacteria to substrate prevents the cell from being washed off by flow conditions while nonadhered bacteria get washed off into another ecosystem and no nutrient exchange is facilitated by the movement of medium past the bacterial cell. It helps to provide the bacterial cell with a continuous supply of nutrients and prevents starving conditions. A structure such as the EPS that helps in attachment also protects the cell from desiccation and predators such as bacteriophages and toxins. Attachment of the bacterial cell ensures close proximity with the substrate. It does not matter if the surface is organic or inorganic as by the law of physics, nutrient molecules adsorb at interfaces and the surface is the interface. This means that in any environment, the concentration of any particular nutrient molecule is very likely to be higher on, or in close proximity to, a surface.

Utilisation of certain substrates requires close contact between the bacterium and the substrate as in the case of *Cytophaga* and *Sporocytophaga* cells that adhere closely to cellulose fibres. Also, many bacteria digest complex polymers such as starch and chitin by means of adherence to starch grains and chitin components. It results in optimum degradation by the extracellular enzymes produced by the cell. As in the case of the bacterium *S. stellata*, close binding to the substrate with the holdfast structure ensures that optimum degradation occurs by hydrolytic enzymes present in the blebs and vesicles that are produced externally (Gonzalez et al. 1997b). Thus, it has now been established that bacterial adhesion helps in the colonization of a solid surface and is the first step in biofilm formation (Anderson et al. 2007).

11.7 Adhered Bacteria from Mangrove Ecosystem from Goa

In the marine, estuarine and mangrove ecosystem the bacteria experience liquid-solid interface interaction. The colonization of a solid surface of substrate is a pre-requisite for exploitation of the habitat and its substrates. In running water of rivers, streams, estuaries and mangrove areas where there is constant tidal variation, the adhered bacteria receive organic matter from upstream and during influx at high tides, respectively. Thus, in such an environment, free-living bacteria would not be able to compete with fast growing bacteria if they were not attached to the substrate or a solid surface where food is available in concentrations for sufficient growth (Crump et al. 1998). Thus, growth in such habitats is restricted to those bacteria that can attach themselves to solid surfaces. In rivers and sea waters small floating particles such as silt, clay or detritus which form the particulate matter has remarkable growth-promoting effects on the bacteria. In environments where nutrient concentration is low, the presence of particulate matter favours growth of bacteria as they absorb nutrients from particle surface.

Our studies on the adhered bacteria from the mangrove samples from Manxer mangroves and Shirdona mangroves in Goa have shown a variation in the total viable count (TVC) on different media (Fig. 11.4). The bacterial isolates were isolated on three different media, Zobell Marine A (ZMA), 15 % NaCl tryptone yeast extract agar (NTYE) and 25 % NTYE agar. Out of the 34 isolates obtained, 73 % were found to grow on ZMA, 21 % on 15 % NTYE and 6 % on 25 % NTYE agar. 68 % of these bacteria were halotolerant and 32 % were halophilic in nature, indicating that halotolerant bacteria are predominant in the mangrove ecosystem (Kharangate-Lad and Bhosle 2014).

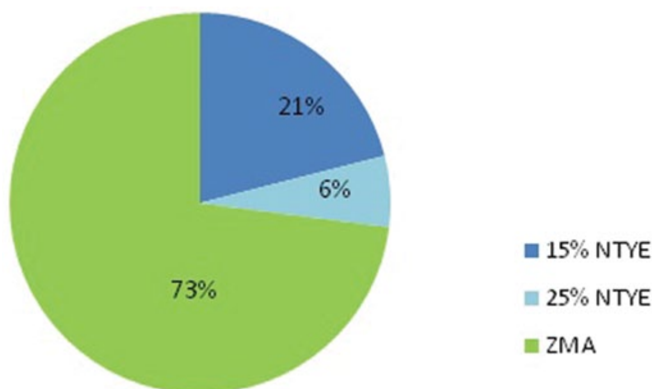

Fig. 11.4 Total viable count (TVC) of adhered bacteria on different isolation media. (*NTYE* NaCl-Tryptone Yeast Extract Agar, *ZMA* Zobell Marine Agar)

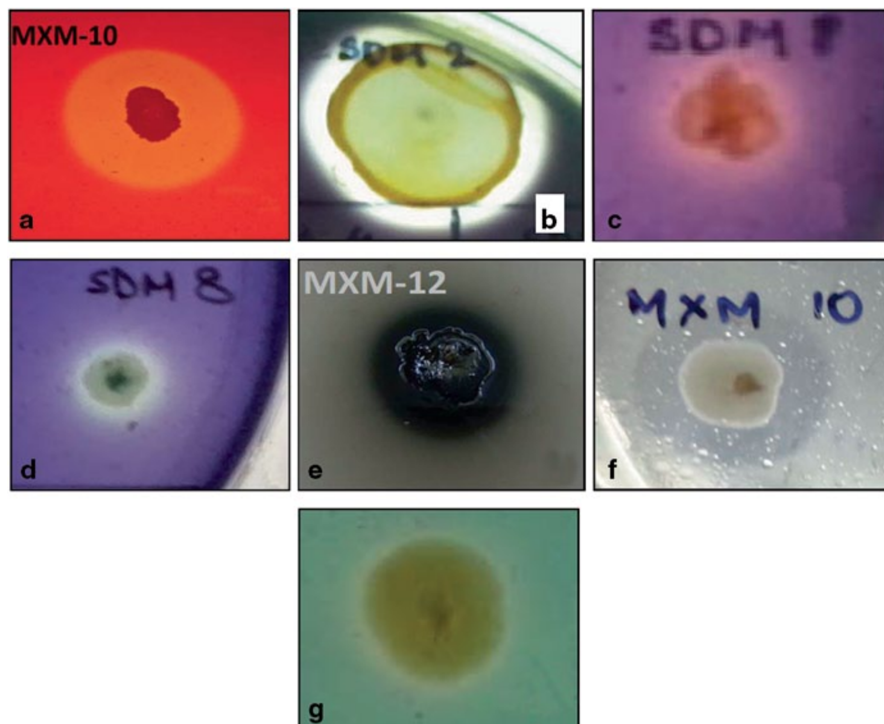

Fig. 11.5 Enyme activities of bacterial isolates. **a** Cellulase activity, **b** amylase activity, **c** and **d** lignolytic activity, **e** tannase activity, **f** lipase activity, **g** siderophore production. (Kharangate-Lad and Bhosle 2014)

These bacteria also showed varied enzyme activities like cellulose, starch, tannin, lignin and lipid degradation (Fig. 11.5a, b, c, d, e and f). It was seen that 48 % of the halotolerant bacteria showed multiple enzyme activities while no halophilic bacteria showed multiple enzyme activities. Some bacteria that showed multiple enzyme activity production were identified as *Halobacillus* sp., *Brevibacterium casei* and *Acinetobacter schindleri* (Kharangate-Lad and Bhosle 2014). This was a significant observation as it indicated that halotolerant bacteria are responsible for most of the degradation as compared with the halophilic bacteria of the mangrove ecosystem owing to their ability to degrade various natural plant polymers. Interestingly, halophilic and halotolerant bacteria from mangroves, estuaries and coastal ecosystems also have shown the production of siderophores (Fig. 11.5g) (Kharangate-Lad and Bhosle 2014). Bacteria often produce siderophores which are Fe (III) ion carriers. Iron is required by bacteria for their biological functions and the bioavailability of this is limited in the environment as most of it occurs as complexes such as oxides and hydroxides (Balagurunathan and Radhakrishnan 2007).

11.8 Conclusions and Future Prospects

This study has demonstrated the presence of halophilic and halotolerant-adhered bacteria in the mangrove plant litter. It has also offered insight into the presence of various enzyme activities and siderophore production by these bacteria. Present work can be extended to other coastal and estuarine ecosystems. Such bacteria can be useful in increasing the efficiency of degradation of organic particulate matter in iron deficient ecosystems. Furthermore, they can also be industrially important due to their ability to produce commercially important enzymes such as cellulases, lignolytic enzymes, tannases, etc. Siderophores are now generating a lot of interest in environmental bioremediation of soils contaminated with heavy metals (Gaonkar and Bhosle 2013; Godinho and Bhosle 2013). Thus, these halophilic and halotolerant bacterial siderophores can be studied for their ability to sequester heavy metals. From the present studies, it can be inferred that halophilic and halotolerant bacteria producing varied enzymes and siderophores can prove to be a very important tool for degradation and nutrient recycling in saline ecosystems like marine and coastal ecosystems which are iron deficient.

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Chapter 12
Feruloyl Esterase: A Principal Biodegradative Enzyme

Cristabell Pinto

12.1 Introduction

12.1.1 Plant Cell Walls—The Natural Substrate

The biodegradation of plant cell walls is a key component in the global cycling of photosynthetically fixed carbon. This decay process is continuous, starting with fresh plant litter and leading to the formation of soil organic matter. Plant cell walls are made up of three essential components namely lignin, hemicellulose and cellulose. Cellulose makes up the bulk of the dry matter of around 35–50 % that are polysaccharides, followed by hemicellulose that are heteropolysaccharidic in nature, the compositions of which vary based on the origin or type of plant in question, forming 25–50 % of the total dry mass. Lignin represents 10–35 % of the dry matter. Proteins, ash and oils form the remaining fraction of the lignocellulosic biomass (Wyman 1994). Lignin is a heterogenous and irregular arrangement of a polymeric molecule that resists chemical or enzymatic degradation to protect cellulose and is, therefore, considered as recalcitrant, hence limiting the rate of carbon recycling. The nature of plant cell wall components coupled with the cross linkages between lignin, polysaccharides and the phenolics to form three-dimensional matrices limit their degradability (Iiyama and Lam 2001; Wang and McAllister 2002). Hydroxycinnamates exist as monomers of ferulate and *p*-coumarate and have been known to be linked to plant polysaccharides through ester linkages and to lignin through ester and/or ether linkages. Hydroxycinnamates are also known to link cell wall proteins with other structural polymers. Further, the presence of ferulate and dehydrodiferulates also reduce the rate and extent of the plant cell wall degradation. One such hyrdoxycinnamate is ferulic acid (FA) which is found to be linked to polysaccharides via a covalent ester bond and to lignin via an ether linkage (Fig. 12.1; Borneman et al. 1991; García et al. 1998; Iiyama and Lam 2001; Ishii 1997; Jeffries

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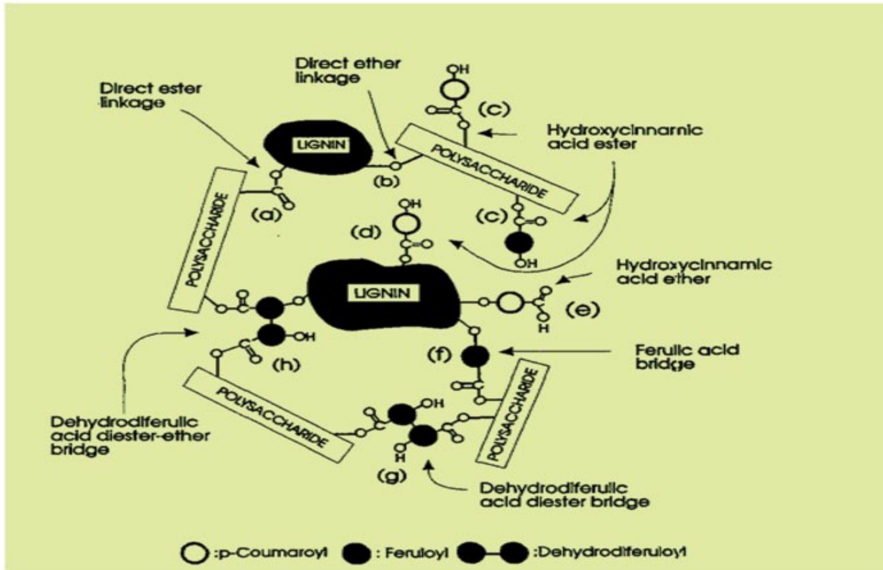

Fig. 12.1 Schematic diagram showing possible crosslinks between polysaccharides and lignin in walls. *Open circles* represent p-coumarolyl (PCA), *closed circles* ferulolyl (FA) and *double closed circles* dehydroferuluic acid. **a** Direct ester-linkage, **b** direct ether linkage, **c** hydroxycinnamic acid esterified to polysaccharides, **d** hydroxycinnamic acid esterified to lignins, **e** hydroxycinnamic acid etherified to lignin, **f** ester-ether bridge, **g** dehydrodiferulic acid diester bridge, **h** dehydrodiferulic acid diester-ether bridge. (Iiyama et al. 1994)

1990; Scalbert et al. 1985; Tenkanen et al. 1991). Ferulic acid also dimerises due to oxidative coupling by peroxidases (Bartolomé et al. 1997a, b; Brézillon et al. 1996; Iiyama and Lam 2001; Kroon et al. 1999; Ralph et al. 1994) or trimmerises as demonstrated in maize (Bunzel et al. 2003; Rouau et al. 2003). As a result, polysaccharides become extensively cross-linked by FA dimerisation or trimerization and its incorporation in lignin (Bunzel et al. 2003; Grabber et al. 1995; Grabber et al. 2000; Rouau et al. 2003). In its monomeric linkages, FA is known to be linked to the C-5 hydroxylgroup of arabinofuranose residues of arabinoxylans in monocotyledonous plants and esterified to the C-2 hydroxyl group of arabinofuranose or to the C-6 hydroxyl group of galactopyranose residues of the pectic side chains in dicots.

12.1.2 Feruloyl Esterase

Feruloyl esterase (FAE) forms part of the enzymatic spectrum that is involved in the breakdown of the crosslinks in plant cell walls. FAEs (EC 3.1.1.73) are a subclass of carboxylic esterases (EC 3.1.1) and belong to the hydrolase family. In very simple terms, the enzyme catalyses the hydrolysis of the substrate feruloyl-polysaccharide in order to yield ferulate and polysaccharide as the products.

Fungi are known to produce these enzymes more than the bacteria. The activity of FA esterase was first discovered in culture filtrates of *Streptomyces olivochromogenes* (Mackenzie et al. 1987) and *Schizophyllum commune* (MacKenzie and Bilous 1988). The first purified FA esterase was reported by Faulds and Williamson (1991). Several microbial FAEs have been isolated and characterized. Fungi such as *Anaeromyces mucronatus* (Qi et al. 2011), *Aspergillus awamori* (Fazary and Ju 2008; Kanauchi et al. 2008), *Aspergillus niger* (Faulds and Williamson 1994; McAuley et al. 2004; Hermoso et al. 2004), *Aspergillus oryzae* (Koseki et al. 2009b), *Aspergillus phoenicis* (Topakas et al. 2007), *Aspergillus tubingensis* (De Vries et al. 1997), *Aureobasidium pullulans* (Rumbold et al. 2003), *Fusarium oxysporum* (Topakas et al. 2005), *Fusarium proliferatum* (Shin and Chen 2006), *Humicola grisea* var. *Thermoidea* (Mandalari et al. 2008), *Humicola insolens* (Hatzakis et al. 2003), *Neurospora crassa* (Crepin et al. 2004a), *Penicillium brasilianum* (Panagiotou et al. 2007), *Penicillium pinophilum* (Castanares and Wood 1992), *Penicillium funiculosum* (Knoshaug et al. 2008), filamentous bacteria such as *Streptomyces ambofaciens* (Kheder et al. 2009), *Streptomyces avermitilis* (García et al. 1998), *S. olivochromogenes* (Faulds and Williamson 1991), *Talaromyces stipitatus* (Crepin et al. 2003a), and eubacteria such as *Bacillus subtilis* (Donaghy et al. 1998), *Cellvibrio japonicas* (McClendon et al. 2011), *Butyrivibrio proteoclasticus* (Goldstone et al. 2010), *Clostridium stercorarium* (Donaghy et al. 2000), *Clostridium thermocellum* (Tarbouriech et al. 2005), *Dickeya dadantii* (Hassan and Hugouvieux-Cotte-Pattat 2011), *Lactobacillus acidophilus* (Wang et al. 2004b), *Lactobacillus fermentum* (Bhathena et al. 2008), *Prevotella ruminicola* (Dodd et al. 2009), *Pseudoalteromonas haloplanktis* (Aurilia et al. 2007), *Pseudomonas fluorescens* (Bartolomé et al. 1997a) are reported. These enzymes are mostly extracellular and are released in the culture medium with the exception of the enzyme from the plant-pathogenic bacterium *D. dadantii*, which produced two enzymes (Hassan and Hugouvieux-Cotte-Pattat 2011); one, FaeD, was an extracellular enzyme and the other, FaeT, encoded by *faeD* and *faeT* genes, of cytoplasmic origin. The molecular weight ranges from 11 kDa isolated from the *Neocallimastix* sp. (Borneman et al. 1991) to 210 kDa from *Aureobasidium pullula* (Rumbold et al. 2003). FAEs are known to be stable and display activity in a wide range of pH and temperature. *A. oryzae* demonstrated activity at a very low extremely acidic pH of 3, while *A. niger* exhibited activity at a pH optimum of 9 in the alkaline range (Hegde and Muralikrishna 2009), while pH values range from 3 in *A. niger* to 9.9 in *F. oxysporum*. FAE from psychrophilic bacterium *P. haloplanktis* (Aurilia et al. 2007) was active at an optimal temperature of 20 °C. However, *P. funiculosum* demonstrated a thermal stability at a temperature of 70.4 °C (Knoshaug et al. 2008).

Ferulic acid esterases were initially classified into two groups namely A and B based on their substrate specificity for aromatic moieties and their varying ability to release diferulic acids from esterified substrates as per a study conducted by Crepin et al. (2003b), where the expression of the inducible enzyme activities were regulated in response to the initial substrate and the degree of substrate degradation as a function of time. However, in 2004, there was an elaboration in the classification into types A, B, C and D based on substrate utilities supported by primary sequence

identity (Crepin et al. 2004b). Synthethic substrates mimic the ester bonds between polysaccharide and phenolic acids. The four standard substrates are methyl ferulic acid (MFA), methyl sinapic acid (MSA), methyl caffeic acid (MCA) and methyl p-coumaric acid (MpCA; Puchart et al. 2007).

12.2 Applications

FAEs have a large number of potential applications in biotechnological processes, in animal feed, fuel, chemicals, pulp and paper, textile and laundry, food and agriculture, and pharmaceutical industries. Ferulic acid, a by-product of the enzymatic reaction on agro-industrial waste materials, also has a variety of applications. Hence, these enzymes have an important role, resulting in increasing amount of research being carried out.

12.2.1 Agriculture

Genetic modification of the phenolic composition and digestibility of monocot cell walls by vacuolar targeting of *A. niger* FA esterase in plants led to its use in the agriculture industry (Buanafina et al. 2006). Cross linkages existing in plant cell walls hinder cell wall degradation by ruminant microbes, reducing plant digestibility. The targeted expression of FAE type A (FAEA) was claimed as an effective strategy for improving wall digestibility in *Festuca* and, potentially, other grass species used for fodder or cellulosic ethanol production (Buanafina et al. 2008).

12.2.2 Biofuel Production

Ferulic acid esterases effectively degraded corn fibre and released substantial amounts of FA and sugars such as glucose and xylose in the incubation medium, thus aiding cellulase in the bioethanol production (Akin and Rigsby 2008).

12.2.3 Biotechnology

Chimeric enzyme consisting of FAEA from *A. awamori* and *Aspergillus kawachii* family 42 carbohydrate-binding modules could be used as an innovative enzymatic tool for biotechnological applications and biotransformation of plant biomass by degrading plant polysaccharides (Koseki et al. 2009a). An alkaline FAE from *A. niger* CFR 1105, had its use in biotechnological applications and especially in the treatment of alkaline wood pulp due to the ability of the enzyme to be active in

alkaline pH (Hegde and Muralikrishna 2009). Release of dietary FA in the gut by the gut microorganisms is not concentrated enough to result in the physiological benefits. The in vivo physiological importance of FA depends on its availability for absorption. Bhathena et al. (2008) showed that microencapsulated *L. Fermentum* 11976 cells could efficiently breakdown FA-containing substrate and established the biotechnological basis for their use in supplementing the bioavailability of dietary FA in the intestine.

12.2.4 Degradation

Agro-industrial by-products are a potential source of added-value phenolic acids. FAE type B (FAEB) was used as a tool for the release of phenolic compounds from agro-industrial by-products (coffee pulp, apple marc, wheat straw, sugar beet pulp and maize bran) and FAEAs, for the release of phenolic compounds from agro-industrial by-products (wheat straw, sugar beet pulp and maize bran). The ability of these enzymes to hydrolyze quinic esters and ester linkages between phenolic acids and lignin monomer had promising applications in the food and pharmaceutical industries (Benoit et al. 2006). The degradation of xylans by *Penicillium pinophilum*, were commercially important.

12.2.5 Food Industry

A. awamori G-2, showed stable FAE activity at pH 3 and 50 °C, thus being acid and heat tolerant, making this enzyme useful for food production (Kanauchi et al. 2008). The production of low-cost cell-wall-deconstructing enzymes on agro-industrial by-products could lead to the production of low-cost enzymes for use in the valorisation of food-processing wastes as described by Mandalari et al. (2008). FA is known to be an effective antidiscolouring agent in food industries preventing discoloration of Green Tea, and prevent oxidation of banana turning black color, thus reducing bacterial contamination. FA, which is a vanillic acid precursor, is used in the production of vanillin, an important flavourant used extensively in foodstuffs. Apart from its use in flavouring, vanillin is also required for the synthesis of pharmaceutical drugs and is used extensively in the perfume and metal plating industries. The enzyme FAE, together with a number of glycanases and oxidases have been implicated in the improvement of bread quality and cereal processing (Schwimmer 1981).

12.2.6 Cosmetic Industry

An alkaline and thermostable FAE from *Caldanaerobacter subterraneus* subsp. *tengcongensis* MB4T was used in the bioproduction of FA from triticale bran (Abokitse et al. 2010). FA has been shown to inhibit melanin formation by competing with tyrosine. It also has photo-protective properties thus being used as an ingredient in sunblock creams (Murray et al. 2008; Tournas et al. 2006).

12.2.7 Pharmacology and Medicine

FA is a powerful antioxidant and scavenges free radicals and is known to induce a robust cell stress response through the upregulation of cytoprotective enzymes such as heme oxygenase-1, heat shock protein 70, extracellular signal-regulated kinase 1/2 and Akt also called protein kinase B that play a key role in multiple cellular processes. Cytotoxic enzymes such as inducible nitric oxide synthase, caspases and cyclooxygenase-2 were inhibited by FA. They could either affect their expression and/or activity, thus showing anticancer, antidiabetic, antineurodegenerative potential. FA is also known to decrease blood glucose and lipid levels (Jung et al. 2007). Ardiansyah et al. in 2008, reported that single administration of FA (9.5 mg/kg) may lower blood pressure in rats by inhibiting *angiotensin-converting enzyme* (ACE), an enzyme known to increase blood pressure. FA supplements significantly lowered total cholesterol and triglyderide concentrations in the blood, as compared to the control. It is also being used in order to increase good cholesterol (Wang et al. 2004a). Cancers such as that of breast, liver, lung, prostate, intestine, stomach, esophagus, colorectum (Hudson et al. 2000), etc. are suppressed with FA supplementation based on a study on the substance conversion of FA using an organic synthetic method by the Ministry of Education, Culture, Sports, Science and Technology (1998–2000). Diseases such as Alzheimers, muscular degeneration, Parkinsons and retinal cell proliferation are also other important areas in which this substance is of medical use (Jin et al. 2005, 2006). Tissue culture experiments have shown that FA stimulates the production of white blood cells and increases the secretion of IFN-γ, an immune system stimulatory protein. Hot flashes in menopausal women are also known to be prevented with sufficient FA supplements. FA, a precursor for the synthesis of cinnamic acid and its derivatives serve as anticancer drugs, antimicrobial, antiviral and anti-inflammatory agents (Ou and Kwok 2004). Phenolic acids such as *p*-coumaric acid, FA and phydroxybenzaldehyde, are known to inhibit the growth of rumen microorganisms (David et al. 2010) and phenolic acids derived from plant cell walls have also been used as food preservatives to inhibit microbial growth. Bone degeneration leading to osteoporosis can also be restricted with FA.

12.2.8 Paper Production

Enzymatic treatments with FAE and xylanase, increased the amount of phenolic compounds released, destructure hemicellulose and lignin, and improve the soda cooking conditions of pulps with the reduction of chemical charge needed in the papermaking process thus enhancing the cost effectiveness of papermaking from annual plants (Tapin et al. 2006). The potential application of recombinant FAEA in combination with laccase and xylanase for use in pulp bleaching, for efficient delignification of wheat straw pulp was demonstrated (Record et al. 2003). An alkalitolerant type C FAE from *F. oxysporum* showed broad pH stability making it an important candidate for alkaline applications such as pulp treatment in the paper industry (Moukouli et al. 2008).

12.3 Studies on Eubacteria Producing FAE from Coastal and Estuarine Ecosystems of Goa

12.3.1 Isolation and Identification

Sand dune and mangrove plant litter samples were used for the isolation of eubacteria on Nutrient agar and Zobell Marine agar by the serial dilution method. A total of 66 isolates obtained from mangroves in Nerul, 52 isolates from plant litter samples from Benaulim sand dune and 146 isolates obtained from mangroves in Siridao were spot inoculated on mineral salt medium containing ethyl ferulate (EF) as the sole source of carbon. Plates were incubated at room temperature and observed for the zone of clearance. Interestingly, 14 bacterial isolates showed distinct and prominent zones of clearance around the colonies (Fig. 12.2) indicating the production of FAE enzyme.

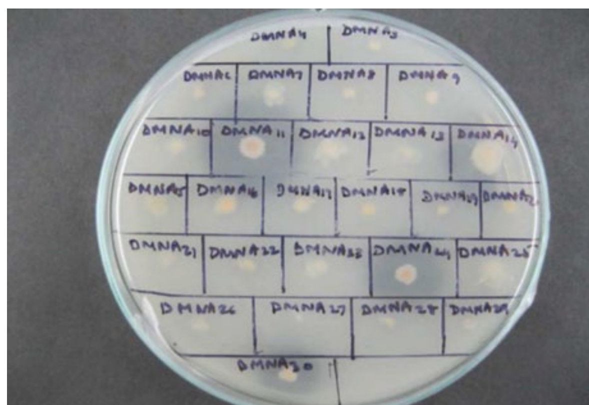

Fig. 12.2 Screening of coastal isolates for the production of feruloyl esterase enzyme on a mineral salts medium with ethyl ferulate as the carbon source. Zones of clearance indicate the production of the enzyme by the isolate

Of the 14 cultures isolated, eight isolates were found to be Gram-positive, endospore-forming rods, catalase positive, oxidase negative, facultative anaerobes, tentatively identified as belonging to the genus *Bacillus*. Isolate LMNB25 was Gram-positive, endospore-forming, oxidase and catalase negative and facultatively anaerobic which was tentatively identified as *Sporolactobacillus*. Isolates DMZA3 and DMZA7 were Gram-positive cocci that are facultative anaerobes, oxidase negative, catalase positive with no acid or gas formation from sugars tested, i.e. glucose, fructose, sucrose, galactose and lactose, thus tentatively identified as belonging to the genus *Stomatococcus*. Isolate LMNB26 and DMZA10 were Gram-positive nonsporing, oxidase negative, catalase positive facultatively anaerobic short irregular rods thus tentatively identified as *Cellulomonas*. Isolate DMNA29 was a Gram-positive nonsporing regular rods, oxidase negative, catalase positive, facultatively anaerobic short regular rods thus tentatively identified as *Brochothrix*.

12.3.2 Screening of Eubacterial Isolates for the Utilization of Standard Substrates

The isolates were spot inoculated on the medium containing the standard substrates namely, methyl sinapate (MSA), methyl caffeate (MCA), methyl *p*-coumarate (MpCA) and methyl ferulate (MFA). The plates were incubated at room temperature and observed for growth. Most of the isolates were able to grow on all the substrates as shown in Table 12.1 indicating the potential of these cultures in plant litter decomposition.

Table 12.1 Growth of isolates on synthetic methyl esters as the sole source of carbon

Isolates	Methyl sinapate	Methyl caffeate	Methyl *p*-coumarate	Methyl ferulate
DMNA11	+	+	+	+
DMNA14	+	+	+	+
DMNA24	+	–	+	+
DMNA30	+	+	+	+
LMNA5	+	+	+	+
LMNB25	–	+	+	+
LMNB26	–	+	+	+
DMZA29	+	+	+	+
DMZA10	+	+	+	+
DMZB7	+	+	+	+
LMZB1	+	+	+	+
LMZB5	–	–	+	+
LMZA3	+	–	+	+
LMNA7	–	–	+	+

+ growth, − no growth

12.3.3 Screening of DMZB7 Isolate for the Location of Enzyme

Isolate DMZB7 showing the highest zone of clearance was chosen for further studies. DMZB7 was grown in 100 mL mineral medium containing 0.3 mL of ethyl ferulate (10 % v/v dimethyl formamide (DMF), incubated under shaker conditions at 150 rpm for 48 h. The sample was centrifuged at 9000 rpm for 10 min. Culture supernatant was used to check enzyme activity according to Ralet et al. (1994), with slight modifications, where the decrease in the substrate absorbance was recorded spectrophotometrically at 324 nm. Culture pellet was washed with sterile saline and then suspended in buffer (0.01 M TRIS/HCl pH 7.0; Nicotinamide adenine dinucleotide phosphate (NADP) 0.038 %w/v and EDTA 0.4 %w/v) with glass beads and sonicated for 3 min (10-s bursts with 30-s intervals). The crude enzyme supernatant was recovered by centrifugation at 9000 rpm for 10 min and enzyme activity determined as above. The studies indicated that the enzyme is present in the culture supernatant indicating the extracellular production of FAE by culture DMZB7.

12.3.4 Response of the Isolates to Metal Salts and Dimethyl Sulfoxide (DMSO)

Fourteen isolates obtained were spot inoculated on the medium containing the substrate ethyl ferulate, with varying 0, 5 and 10 mM concentrations of metal salts (copper, nickel, cobalt and zinc) and solvent 0.1 % DMSO, incubated at room temperature (RT) and the growth and zones of clearance compared.

Metals were introduced in the medium to access their effect on the enzyme. Significantly, all the isolates showed growth in the presence of all the metals tested. 5 mM concentration of Cu^{2+} was found to cause a decrease in the zone size, hence enzyme action (Fig. 12.3a). Complete inhibition of the enzyme action was observed in six cultures at 5 mM concentration and all cultures at 10 mM concentration. An increase in the zone size was observed in two isolates at a 5 mM concentration of Ni^{2+} (Fig. 12.3b). Growth of all the other isolates was inhibited at 10 and 5 mM concentration. Zn^{2+} at 5 mM completely inhibited the zone of clearance whereas a 10 mM concentration resulted in an increase in the activity in one of the isolates (Fig. 12.3c). Co^{2+} however, either partially or completely inhibited enzyme action at both the tested concentrations of 5 and 10 mM (Fig. 12.3d). Solvent DMSO showed results comparable to the control with very slight decrease in the zone size in six isolates (Fig. 12.4). Two isolates demonstrated an increase in activity, thus playing a role of an activator. Whereas solvent DMSO had no effect on some cultures as zone sizes in both the control and test measured the same.

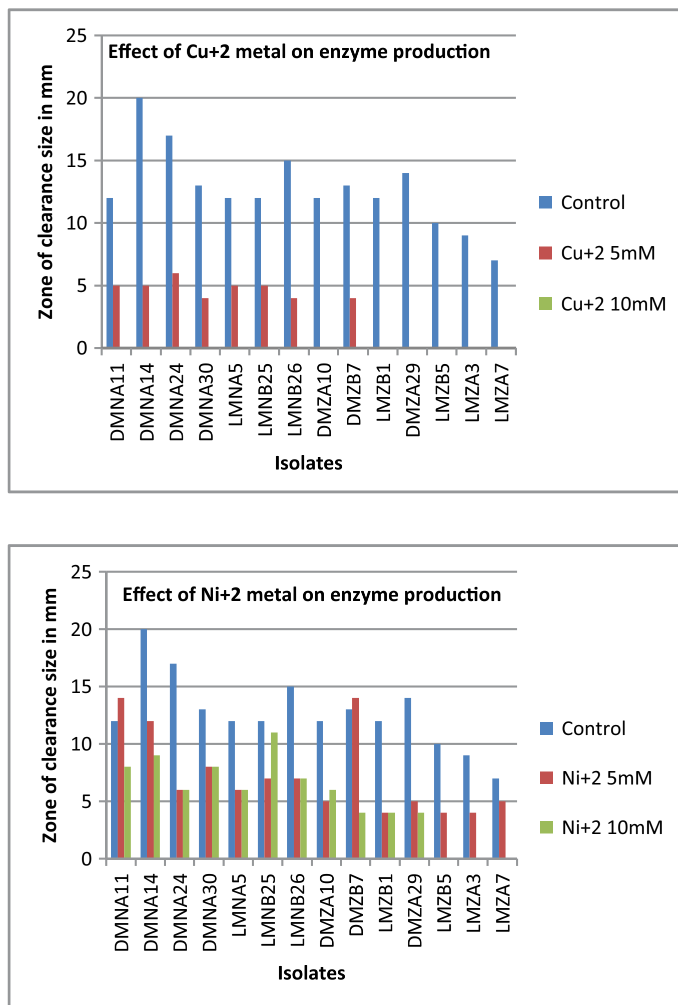

Fig. 12.3 **a** Effect of Cu^{2+} metal on enzyme production, **b** effect of Ni^{2+} metal on enzyme production, **c** effect of Zn^{2+} metal on enzyme production and **d** effect of Co^{2+} metal on enzyme production

12.3.5 Screening of Isolates for the Utilization of the Product Ferulic Acid

The isolates were spot inoculated on medium containing the product of enzyme action FA. The plates were incubated at room temperature and observed for growth. All the 14 isolates were able to utilize the product FA as sole source of carbon, thus indicating that these eubacterial isolates not only utilize ethyl ferulate, but were also able to hydrolyze FA as the sole source of carbon, thus being promising candidates in industrial applications.

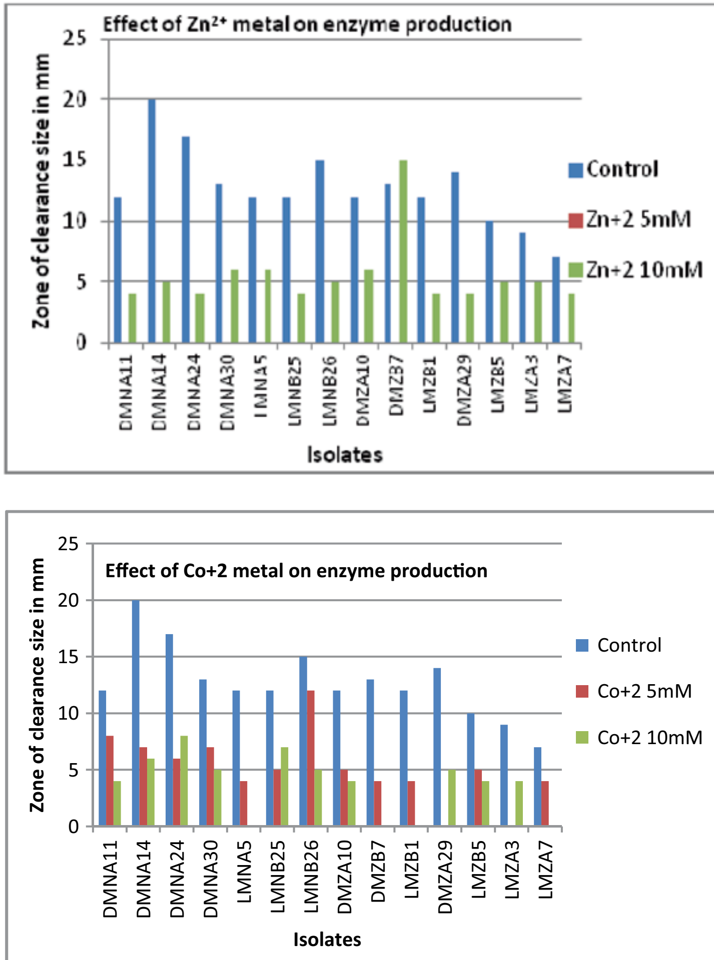

Fig. 12.3 (continued)

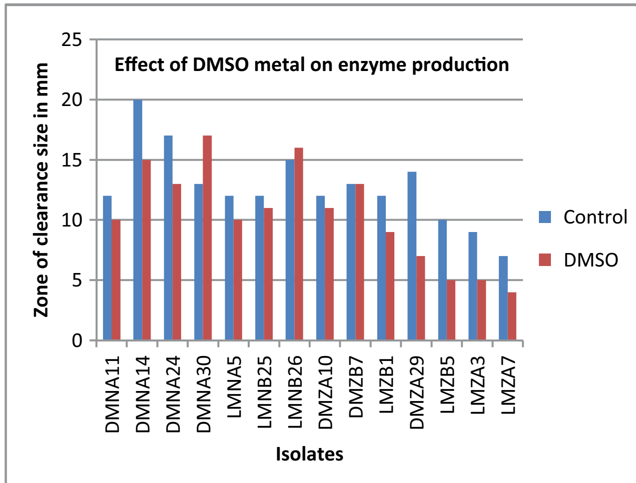

Fig. 12.4 Effect of DMSO metal on enzyme production

12.4 Conclusions and Future Prospects

FAE enzymes that have been isolated from a number of microorganisms display diverse specificity for synthetic methyl hydroxy-cinnamate substrates as well as plant cell wall-derived feruloylated oligosaccharides releasing valuable compounds as the products. These enzymes have been mostly studied from fungi, however their bacterial counterparts also need to be explored for the production of these enzymes. Ferulolyl esterase enzymes can be greatly exploited for use in various industries in the development of value-added products for food and drugs. However, a greater understanding with respect to enzyme structure as well as the mechanism of action needs to be thoroughly explored in order to utilize this enzyme to its full potential.

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Chapter 13
Bio-processing of Coir—A Natural Fibre for Diversified End Use

Anita Das Ravindranath

13.1 Introduction

Man has been dependent on plant fibres in the form of structural and building materials, utilised in various industries such as paper, textiles, packaging, furniture, etc. The coconut palm, found by Megasthenes, the ambassador of Seleucus Nicator in Sri Lanka as far back as 300 B.C., is one of the most remarkable renewable bioresources. The early Arabs knew of coir, and the coconut palm has been a source of wealth for centuries (Mukherjee 1996).

The coconut fruit is unique, as nature has used its utmost skills to design it in such a way that despite being very heavy, it is buoyant and has a water repellent surface, which enables it to float for long distances. The reason of this buoyancy is the packing material inside the fruit which has been designed to protect its seed that is the nut. The cushions provided around the nut comprise of fibre and pith which are designed to be light but strong reinforcement material and the outer coating is a poly-phenolic substance known as lignin, which is hydrophobic in nature. The lightness of the fruit is due to these fibres and pith which are perforated to the extent of 40 %, thereby lowering the specific gravity to the minimum.

In India, about 75 % of the coir produced in terms of value is consumed in the Indian domestic market itself, while the remaining 25 % of coir is exported (Table 13.1). Countries in Europe and America together consume about 90 % of coir exports from India. Coir is marketed in different forms such as fibre, yarn, mat, matting, rug, carpet, geo-textile, curled coir and rubberised coir. The factors that contribute to the increase in export opportunities for coir are the rising cost of synthetic substitutes, a shift in tastes and preferences in favour of natural materials due to greater appreciation of the environmental implications. The 100 % biodegradable nature of coir floor coverings has resulted in a steady increase in demand for them as compared with the synthetic materials which create problems of recycling, fire/health hazards and biodegradability. Another development of interest to the coir

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Table 13.1 Export of coir products from India March 2012–April 2013. (Source: Coir Board Agenda Notes 2014)

Item	Qty. (tons)	Value (Rs. Lakhs)
Coir fibre	140,692.93	20,707.66
Coir yarn	4202.31	2387.22
Coir mats	61,441.38	56,386.16
Coir matting	1418.31	1702.76
Coir rugs and carpets	94.83	133.37
Coir rope	419.62	282.41
Rubberised coir	321.47	495.01
Curled coir	8883.14	2112.46
Coir geotextiles	3597.3	2628.74
Coir other sorts	30.37	39.32
Coir pith	208,399.28	24,727.61
Total	*429,500.94*	*111,602.72*

industry is the growth of the market in Europe and America with consumers demanding materials that can be used to prevent soil erosion and promote re-vegetation (Coir Board Report 1996).

13.2 Coir Fibre

Coir fibre is lignocellulosic in nature, with lignins and hemicelluloses forming the cementing materials of the fibre cells. The source of coir fibre is coconut husk obtained after taking the nut. Husk is embedded in the matrix of coir pith where coir pith is present to the extent of 70 % and fibre up to 30 %.The physical characteristics of coir fibre such as length, fineness, strength and elongation determine its utility. Coir has a high extensibility (about 37 %) and high lignin content of 40 % which distinguishes it from other cellulosic fibres. The high extensibility of the coir fibre is primarily because of the microfibrils in the cell wall which lie in perfect helical spirals, extension of the fibre being related to the changes of the spiral angle, that is, the angle which a microfibril element makes with the fibre axis. Coir is in great demand on account of its natural resilience, durability, resistance to dampness and eco-friendliness. Physical and chemical characteristics of coir fibre are presented in Table 13.2 and Table 13.3, respectively.

Table 13.2 Physical properties of the coir fibre. (Source: Ravindranath 1999)

1.	Ultimates	
	Length in mm	0.6
	Diameter/width (microns)	16
2.	Single fibre	
	Length in inches	6–8
	Density (gm/cc)	1.40
	Tenacity (gm/tex)	10.0
	Breaking elongation %	30
	Moisture regain at (65 % R.H. (%))	10.5
	Swelling in water (dia)	5 %

Table 13.3 Chemical composition of coir fibre. (Source: Ravindranath 1999)

Water solubles	5.25 %
Pectin and related compounds	3.00 %
Hemicellulose	0.25 %
Lignin	45.84 %
Cellulose	43.44 %
Ash	2.22 %
	100.00 %

13.3 Natural Retting of Coconut Husk

The coir fibre can be extracted by the retting process by steeping the husks in the backwaters for 10 months. During this period, the husks become soft by microbial activity following which the husks are taken out of the water and beaten gently to release the fibre and pith. The natural coconut husk retting process has been studied by various scientists to understand the involvement of microorganisms and the biochemical changes occurring during the process.

The problems associated with the natural retting process have been a concern of the environmentalists. Besides, the low availability of the fibre as compared to the demand has been a point of focus of the coir industrialists. An established fact known to cause the delay in the retting of coconut husks is the presence of the high percentage of polyphenols (Jayasankar and Bhat 1966; Pandalai et al. 1956; Varrier and Moudgil 1947). Polyphenols from the coconut husks get constantly leached out into the surrounding steep liquors and significantly influence the retting process, thereby resulting in a delay in extraction of the fibre (Jayasankar and Bhat 1966). Retting is also a cause of environmental pollution (Aziz and Nair 1978) as the pH of the environmental waters in a retting zone is lowered from neutral to the acidic range, indicating the release of acidic substances. The biochemical oxygen demand

(BOD) levels increase considerably leading to the deterioration in the quality of the backwaters which is detrimental to the aquatic life. Recommendations have been made for adopting fibre pretreatments by improved retting and biobleaching (Sarma and Ravindranath 2005a; Van Dam 1999). It is therefore imperative to develop ecofriendly methods for coir extraction from coconut husks. Alternative measures, like the development of Coirret, have limitations such as insufficient production capacity to meet the requirement of all coconut-growing regions and its high cost. Therefore, a process which could overcome these shortcomings would be useful for economic utilisation of the husk potential in any coconut-growing region. Bacteria are the most versatile organisms dissimilating an array of aromatic compounds with catechol as the key intermediate involved in the oxidative cleavage of the aromatic ring (Evans and Fuchs 1988). Some important degradative bacteria that occur in water and soil environments belong to the genera such as *Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Azotobacter, Bacillus, Enterobacter, Escherichia, Flavobacterium, Methylomonas, Methylococcus, Moraxella, Proteus, Pseudomonas, Rhizobium, Serratia* and *Xanthomonas* (Cork and Krueger 1991). Growth of specific types of microorganisms and their physiological activities are a response to the physicochemical environment. The steeping of coconut husks for retting leads to the establishment of such a unique ecosystem for proliferation of specific microorganisms degrading polyphenols (Dwyver et al. 1986). The biological retting of coconut husks differs from that of other fibrous materials in that it is not confined to pectin decomposition alone but extends also to the disintegration of the phenolic cement binding the fibres together (Jayasankar and Menon 1966b).

The present studies have hence been carried out with a view to explore the possibility of developing a consortium that can survive and proliferate on the leachates from coconut husk which are rich in phenolic compounds in a confined environment. An advantage of such a consortium would be that, it can be developed at any site where coconut husk retting needs to be carried out. It would lead to increasing the supply of raw material from India and establish coir industry, without high investments in states where natural facilities for retting do not exist. This would generate employment opportunities and increase the economy of the region. An attempt is also made in this work to reduce the period of retting of husks for coir extraction, increase the utilisation of the husk potential in coconut-growing regions and address the environmental problems arising during retting.

13.4 Microbiological Studies on Retting of Coconut Husk

Retting of coconut husks involves biodegradation mainly of polyphenols and pectins which bind the fibre in the husk (Bhat and Nambudiri 1971). The efficiency of this process, therefore, depends on the rate and extent of degradation of these binding components. During the retting process, these polyphenols from the coconut husks leach out into the steep liquors, in significantly high concentrations, resulting in the delay of the retting process due to their toxicity. Inoculation of

selected strains of phenol and pectin degrading bacterial cultures have resulted in improving "retting" in a poor retting areas where dull-coloured fibre is produced after 10 months of retting and reducing the retting period to two and a half months (Ravindranath 1991).

Since retting of coconut husk for extraction of coir also leads to the release of phenolic compounds into the retting environment, it was envisaged that inoculation of microorganisms which can degrade the components in the husk leachate would accelerate the retting process. Studies were undertaken to develop a consortium of indigenous bacteria from coconut husks and estuarine water capable of growing on husk leachate, isolate and characterize bacteria growing on phenolic compounds from consortium and to study the effect of seeding of consortium for the coir extaction process in the laboratory system (Ravindranath and Bhosle 1999a, b).

13.4.1 Development of the Consortium, Isolation and Identification of the Bacterial Cultures

One husk was steeped in 1.5 L of estuarine water of salinity 6 ppt in a 5 L beaker. To this, 1.5 L of sterile distilled water was added and the salinity adjusted to 6 ppt with sodium chloride. The mixture was allowed to stand for 30 days. During this period, the phenolic compounds leaching out from the husk would act as a carbon source for the indigenous organisms present in the husk and estuarine water. These organisms would get enriched and proliferate utilising the carbon sources leached out and multiply to give the growth in substantial numbers.

1.5 L of the first enriched culture with the husk was transferred to another beaker and was supplemented with 1.5 L of distilled water with salinity adjusted to 6 ppt and kept for 30 days to yield the second enriched culture. This second enriched culture was also subjected to the above treatment to give a third enriched culture of the consortium growing on husk leachate. The viable count in all the enriched cultures were studied on nutrient agar (Hi Media) and 0.05 % resorcinol mineral medium (RMM). The bacterial isolates were purified on nutrient agar and RMM and the cultural and biochemical characteristics of the bacterial isolates were studied using standard methods.

Interestingly, an increase in the total count as c.f.u./ml was observed on both nutrient and resorcinol media during the transfers. On nutrient agar, the counts changed from 23×10^2 to $85 \times 10^2$and 120×10^3 whereas on resorcinol agar they changed from 43×10^2 to 73×10^2 and 141×10^2. Plating of the consortium on nutrient agar showed different types of colony morphology and out of the nine isolates, four were Gram-positive and five were Gram-negative. On the basis of their biochemical characteristics, the organisms were tentatively identified as belonging to the genera of *Actinomycetes*, *Azotobacter, Bacillus, Micrococcus,* and *Pseudomonas*. Interestingly, on plating the consortium on 0.05 % RMM, only one type of pinnate colonies were observed. When streaked on nutrient agar, the colonies were observed to be bigger in size with a translucent blue sheen formed in 24 h which

disappeared on further incubation after 24 h. This culture, which was isolated from both the subcultures and the final consortium was Gram-negative coccobacilli, oxidase positive, catalase positive and motile. On the basis of the biochemical tests, this isolate was identified as *Pseudomonas* (Ravindranath and Bhosle 1999a, b).

13.4.2 Retting of Husks in Tanks Using Bacterial Consortium

13.4.2.1 Preparation of Inoculum

One surface-sterilised husk was taken in each of the four round bottom flasks (5 L capacity). To this, 2 L of sterile mineral medium was added and allowed to stand for 48 h. During this period, the leachate containing phenolic and other compounds would comprise the nutrient medium for the proliferation of the consortium. The medium was decanted aseptically into sterile flasks and was inoculated with 5 % (v/v) of the consortium prepared in nutrient broth and incubated at room temperature for 24 h. This was used as inoculum for the laboratory scale retting experiment.

13.4.2.2 Retting of Husks in Tanks

Mature coconut husks from 11-month-old nuts, which are normally utilised for coir extraction, were used for the laboratory scale study. Three tanks A, B and C were set up with ten husks immersed in tap water. After 24 h of soaking, tanks A and B were inoculated with the consortium in concentrations of 5 and 10 %, respectively. Tank C was maintained as the untreated control. In all the three sets, the final husk: liquor ratio was maintained at 1:5. A periodic flushing of the water in all the three tanks was carried out by removal of the steep liquor and refilling with tap water at fortnight intervals. This was done to simulate the flushing action in the environment which gives a brightening effect on the fibre and also exerts a beneficial influence in retting. In order to supplement the loss of organisms due to flushing, tanks A and B were reinoculated with the consortium in the concentration of 5 and 10 %, respectively after 1 month of the first inoculation.

Water samples drawn out from the three retting tanks at intervals of 30 days were plated on nutrient medium and mineral salt medium with 0.05 % resorcinol. The water sample collected after 60 days of retting was also plated on mineral medium with 0.05 % pectin. The initial count in the tanks inoculated with the consortium was observed to increase from 83×10^5 and 300×10^4 to 5×10^7 and 21×10^5 in tanks A and B, respectively, as cfu/ml, whereas the count in the control tank was observed to decrease from 196×10^5 to 120×10^4 cfu/mL in a period of 30 days. There was growth on 0.05 % RMM in samples from the two inoculated tanks in 30 days. A sample from the control tank C during the same period showed no growth and colonies appeared only after 90 days of incubation. Plating of the 60-day water samples on 0.05 % pectin mineral medium showed the emergence of 43 cfu/mL and

55 cfu/mL from tanks A and B, respectively. However, no pectin degraders could be isolated from the control tank. These pectin degraders comprising of four different types of colonies were purified isolated and identified as *Micrococcus, Alcaligenes, Arthrobacter* and *Escherichia* on the basis of biochemical characteristics.

13.4.2.3 Analysis of Ret Water in Lab-Scale Retting

The phenolic compounds leached out from the coconut husks steeped for retting into the ret water in the lab-scale retting tanks were analysed using the high-performance liquid chromatograph (HPLC). The mobile phase consisted of an isocratic solvent system of acetonitrile/water (20:80), and the flow rate was set at 0.5 mL/min. In the first 2 months, the peaks were found to be similar to the retention time (RT) ranging from 2.7 to 2.8, 3.4 to 3.5 and 4.3 to 4.8 min. The RT of standards used such as catechol, resorcinol and pyrogallic acid indicated that the first peak represents pyrogallic acid and the third represents resorcinol. The second peak however could not be identified. During the third month, these peaks were not present, however there were other peaks with RT 0.68, 5.07 and 6.41. The peak at 5.07 corresponded to catechol when used as standard.

13.4.2.4 Analysis of the Retted Husks

Three husks were drawn out from each of the three tanks at periodic intervals of 1 month (30 days) to monitor the progress in retting. The parameters analysed by standard methods (Nazareth and Mavinkurve 1987; Ravindranath 1991; Ravindranath and Bhosle 1999a, b) were as follows:

A. Pectin and polyphenol content in the husk samples
B. The change in the texture of the husk by physical touch and feel method
C. The lignin content, lightfastness rating and the degree of softness by Xenotest of the fibre from the husks

Interestingly, significant changes were observed in the polyphenol content of the husk which decreased in the inoculated as well as the control tanks. The decrease in the inoculated tanks ranged from 90 % to almost less than 10 %, whereas in the control tanks, the polyphenol content remained at 30 % after 3 months. The pectin content was lowered from 7 % to less than 1 % in all the three tanks in 30 days and to negligible thereafter. The husks drawn out from the inoculated tanks were softer and the exocarp could be peeled off easily, indicating completion of retting after 90 days. A significant difference was observed with the husks drawn out from the control (untreated tank) which exhibited a hard nature and the exocarp would not peel off easily indicating incomplete retting.

The fibre extracted from tanks A and B exhibited less pith content as compared to the fibre from tank C which was comparatively inferior having a dull colour. Further, the Xenotest rated the fibre from consortium-treated tanks as grade II, whereas

the fibre from the untreated husk showed a rating of grade I. The flexural rigidity of the fibre extracted from tanks A, B and C tested for the degree of softness were found to be 1.19, 1.13 and 2.00 g/cm^2, respectively. The lignin content in the fibre from the consortia treated husk was 38 %.

The principal change brought about in the plant tissue during retting is the breakdown of pectic substances which form the chief constituent of the middle lamellae between the fibre cells and the cementing material (Bhat and Nambudiri 1971). The consortium degrading the husk leachates is therefore postulated to contain bacteria belonging to different physiological groups, particularly, metabolizers of phenolic compounds and pectin degraders. The growth of colonies on resorcinol and pectin could substantiate the presence of such bacteria which are involved in coconut husk retting. Preliminary evidence of bacterial growth on resorcinol suggests the presence of bacteria that are able to use the husk leachates as a carbon source.

The study could thus establish the fact that retting of coconut husks could be carried out in tanks by inoculating the consortium to yield fibre in 3 months. This fibre is comparable in quality with that obtained by natural retting in 9–11 months (Ravindranath and Sarma 1998). The potentials of the consortium in biosoftening of the coir could therefore be established. This method eliminates the environmental pollution caused by retting and also provides an alternate method for tapping the husk potential available in all coconut-growing regions for setting up of coir industries (Ravindranath and Bhosle 1999a, b; Ravindranath 2001).

Today, the fibre and pith from the retted husk can be mechanically extracted in a matter of just 10 s using a mobile coir fibre extraction machine known as "Swarna" (Fig. 13.1). The fibre treatment has now been upgraded to a new retting process using a biochem spray (Fig. 13.2) which results in a soft coir fibre which can be used to manufacture good quality traditional products (Fig. 13.3).

The Coir Board has been successful in developing a fully automatic versatile coir spinning machine (Fig. 13.4) which can spin a wide variety of yarn such as sisal-blended yarn (Fig. 13.5) with a productivity of at least 50 kg of single yarn per 8 h and is convenient to be operated by the women workers (Ravindranath and

Fig. 13.1 Mobile coir fibre extraction machine

Fig. 13.2 Biochem spray on coir fibre

Fig. 13.3 Coir floor covering products

Fig. 13.4 Automatic coir spinning machine

Fig. 13.5 Coir sisal-blended yarn

Chitralekha 2010). Coir fibre on blending with jute, sisal, cotton and silk in 80:20 ratio produces fibre yarn of runnage up to 1300 m/kg. Such yarn is used to weave fabrics to make diversified products such as umbrellas, winter jackets, conference bags, acupressure footwear, curtains, lampshades and novel gift articles.

13.5 Coir Pith

Coir pith (Fig. 13.6) which is present to the extent of 70% in the coconut husk is the byproduct of the coir industry and accumulates in the form of hillocks in various places in the country wherever the coir fibre extraction activity takes place. It is one of nature's major lignocellulosic byproducts and its direct application on soil results in reduction in the soil microbial population, soil bio-polysacchases, soil dehydrogenases and soil respiration. Further, coir pith contains 8–12% soluble tannin related phenolics which apparently inhibit plant and microbial growth and also immobilize nutrient nitrogen in the soil during polymerization. Since 1995, Tamil Nadu in South India has started exporting this product and during the last year its export has exceeded 140,000 MT for a value of approximate Rs. 200 crores. (Coir Board Export Data 2014).

Coir Board has carried out research on coir pith for the synthesis of nanocellulose (Subha and Ravindranath 2012) and to convert into compost using Pithplus, an edible mushroom spawn to establish its utility as a potential organic manure (Babu et al. 2008).

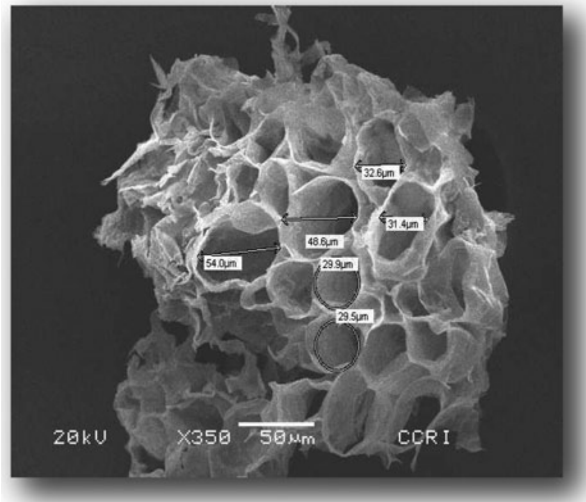

Fig. 13.6 SEM image of a single coir pith particle. (Shubha and Ravindranath 2012)

Pithplus, an edible mushroom spawn speeds up the composting process of coir pith and leads to 42 % reduction in volume. This mushroom belongs to the fungal group Basidiomycetes, capable of detoxifying phenolics and producing lignolytic enzymes. Cellulosic compounds present in coir pith support the initial growth of this fungus and act as cosubstances for lignin degradation. The fungal mycelia spread to the surface of the coir pith particles and degrade the lignocellulosic content. In addition, the growth of nitrogen-fixing bacteria is also known to enhance the biodegradation process (Reghuvaran and Ravindranath 2011, 2013a, b).

The standard method of composting of coir pith is to select an area of 5 × 3 m in a sheltered place followed by spreading uniformly 100 kg of coir pith on the marked area. A total of 400 g of fungus, Pithplus on the coir pith is further spread and this layer is covered with another 100 kg of coir pith over which 1 kg urea is applied. This process of sandwiching the Pithplus and urea alternatively with 100 kg coir pith is repeated so that the heap reaches a height of 1 m. To compost one ton of coir pith, 2 kg Pithplus and 5 kg urea are required which can also be replaced by Azolla and soya hulls (Radhakrishnan et al. 2011, 2012). Water is sprinkled on the heap for sufficient moisture and allowed to decompose for 25 days, following which the coir pith is transformed into coir pith organic manure with the nutrient status as detailed in Table 13.4. The composted coir pith obtained is 100 % organic manure, with increased nutrient status, reduced C:N ratio, pH and electrical conductivity, most suitable for rooting and plant growth (Ravindranath 2008; Reghuvaran and Ravindranath 2011, 2013a, 2014; Reghuvaran et al. 2008).

Coir in the form of pith, has gained ground as an ideal potting and growth medium for horticulture applications. Coir pith has readily available nutrients like nitrogen, phosphorous and potassium suitable for plant growth. The organic matter content of soil/substrate is an indicator to its fertility and nutrient availability and coir pith has a higher organic matter content as compared to peat moss (Ghosh et al.

Table 13.4 Nutrient status of composted coir pith. (Source: Reghuvaran and Ravindranath 2014)

Lignin	4.8%
Cellulose	10.20
Organic carbon	24.4%
Nitrogen	1.26%
Phosphorous	0.06%
Potassium	1.20%
C:N ratio	19:1
Volume	0.58 cu m
Calcium	0.50%
Magnesium	0.48%
Iron (ppm)	0.09
Manganese (ppm)	25.00
Zinc (ppm)	15.80
Copper (ppm)	6.20
Cation Exchange Capacity	40–90 meq/100 g of sample
Electrical conductivity	>0.25 millimhos/cm

2007). This leads to improvement in plant growth and production when the coir pith is added to the soil. Today, coir pith is an internationally acclaimed medium for horticulture and floriculture and widely exhibited at the world's largest Horti Fair the annual event held at Amsterdam, The Netherlands.

13.6 Tender Coconut Husk

A large proportion of the coconuts produced in India are prematurely plucked for utilisation as a nutritious refreshing drink sold along national highways and kiosks in all coconut-growing states of India. The residue husk forms a bulky agrowaste which is a source of environmental pollution. It is estimated that out of total production of coconuts in the country, 10% are plucked as tender coconuts (CDB Project Report 2010). In West Bengal, 60% of the coconuts are plucked at a very early stage of growth. Studies were conducted by Coir Board (Fig. 13.7), and the overall assessment revealed that suitable machinery & equipment can be used to chop the residue tender husk into chips. The biodegradation of the chips using Pithplus, urea, and a consortium of microorganisms could bestow it with properties for use as a plant nutrient source. A significant increase in the nitrogen, phosphorus and potassium (NPK) content was observed after biodegradation and the potential of microbial decomposition of tender coconut husk into valuable compost could be confirmed. Adoption of this finding in outlets selling tender coconuts would solve the problem of accumulation of tender coconut husk creating environment pollution and composting the husk would give it value addition for use as green manure.

Fig. 13.7 Composting of tender coconut husk

Considerable research effort was also made trying to utilise the tender coconut husk fibre with other agrowaste biomass from agricultural and forestry residues (Sarma and Ravindranath 2005a; 2006a, b). The fibre from the husk of tender nuts is inferior in quality and unsuitable for spinning into coir yarn. In this context, an environmental friendly organosolv process was developed based on the use of organic solvents for delignification, where it is possible to break up the lignocellulosic biomass to obtain cellulose fibre for paper making, high-quality hemicelluloses and other lignin degradation products (Fig. 13.8). This process also avoids emissions and effluents making it an ecofriendly one. Organosolv pulping of tender coconut fibre (cooking liquor to material ratio 10:1) at 121 °C for 60 min could yield a good pulp for the preparation of handmade paper after bleaching. High-quality lignin

Fig. 13.8 Tender coconut fibre after organosolv pulping and bleaching. (Sarma and Ravindranath 2006a, b)

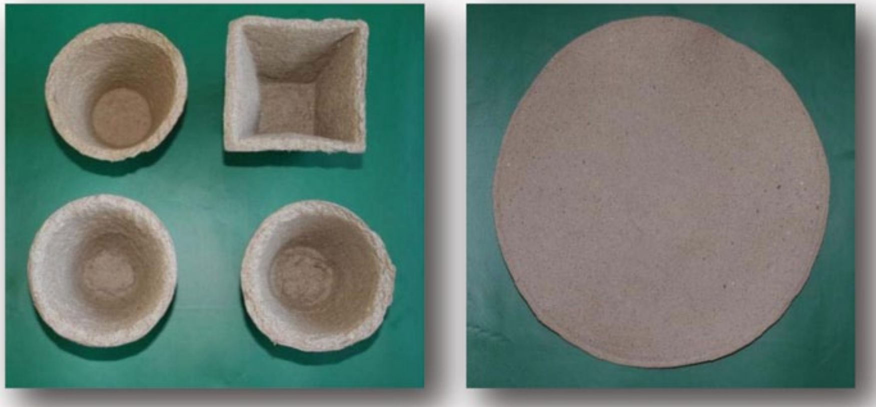

Fig. 13.9 Garden articles and handmade paper from tender husk fibre. (Sarma and Ravindranath 2006a, b)

from the spent black liquor was also extracted thereby opening up new possibilities for alternative uses for lignin. The unbleached organosolv pulp could be moulded into various products like disposable egg cartons, paper plates, garden articles like paper pots (Fig. 13.9). Blending bleached organosolv pulp with paper waste long-fibred pulp in suitable proportions improves strength and surface smoothness of the handmade paper. The results obtained indicate the promising potential of the organosolv process for the pulping of tender coconut fibre to produce high-quality pulp and value-added products for industrial end use.

13.7 Conclusions and Future Prospects

The husk of the coconut is an abundantly available renewable bioresource from which fibre and pith is derived. The coir fibre and pith are lignocellulosic substrates which have great potential for the development of cost effective ecofriendly products to substitute nonbiodegradable ones. Tender coconut husks which accumulate causing environmental pollution can be tapped as raw material for the development of products for human benefit. These lignocelluloses can also be harnessed as substrates for biofuel production in all coconut-growing regions of the world.

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Chapter 14
Role of Microbes in Vermicomposting: A Review

Prakash Mallappa Munnoli

14.1 Introduction

The subject of solid waste (SW) management is important with an objective to minimize the adverse environmental effects caused by the indiscriminate disposal of SWs (Munnoli 2007). Further, the disposal of SW through vermiculture biotechnology (VBT) upgrades the value of original waste materials and gives final product free of chemical or biological pollutants (Divya 2001; Munnoli 1998; Munnoli and Bhosle 2013, 2014). SWs increase in their quantity and the mixing of biodegradable and nonbiodegradable wastes at the generation point makes it complicated to handle them with limited resources.

Studies on the earthworm microbes reflect that the soil macro fauna, especially earthworms, is a major component, together with microbes in a wide variety of soils and climates, which are involved directly or indirectly in organic matter decomposition and soil stabilization (Edwards and Lofty 1977; Lavelle and Spain 2001; Munnoli 2007; Munnoli and Bhosle 2011; Singh 1997a). These processes are associated with the symbiotic relationships between earthworms and microorganisms, which mainly occur in the earthworm gut, casts, burrows and pasture land. Casting is very important, considering the high rate at which casts are produced and strong modifications in biochemical properties with respect to ingested material (Aira et al. 2003; Munnoli and Bhosle 2008, 2009, 2013, 2014). It is generally accepted that microbial biomass and respiration are greater in earthworm casts than in the parent soil (Aira et al. 2002, 2003; Munnoli 2007; Tiunov and Scheu 2000). However, earthworms can feed on these selectively (Edwards 2004; Moody et al. 1995), resulting in an increase in culturable aerobic microorganisms in the gut contents of earthworms, as seen with studies on *Lumbricus terrestris* and *Lumbricus rubellus* (Fischer et al. 1995; Schönholzer et al. 1999) and also increase in cast microorganisms as noticed in *Eisenia fetida, Eudrilus eugeniae* and *Megascolex megascolex* when press mud was fed as a substrate. Such earthworms are, therefore, considered potential for use in SW management through the use of VBT.

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14.2 Vermiculture Biotechnology

Vermicomposting involves biooxidation and stabilization of organic material through the interactions between earthworms and microorganisms. Although microorganisms are mainly responsible for the biochemical degradation of organic matter, earthworms play an important role in the process by fragmenting and conditioning the substrate, increasing the surface area for growth of microorganisms, and altering its biological activity (Domínguez 2004; Domínguez and Edwards 2004; Edwards and Lofty 1977; Munnoli 2007).

Earthworms are well-known soil-inhabiting animals, having a cylindrical body and marked external and internal metameric segmentation which facilitates in burrowing activity, and they breathe through skin keeping the system aerobic in nature (Munnoli 2007; Munnoli and Bhosle 2009). Earthworms are hermaphrodites and sexually matured worms with a distinctive epidermal ring-shaped clitellum, which has gland cells that secrete materials to form a cocoon (Edwards and Lofty 1977; Gajalakshmi and Abbasi 2004; Munnoli and Bhosle 2011). Earthworms are the major components of the soil fauna in a wide variety of soils and climates and are involved directly or indirectly in biodegradation in association with the microbes like bacteria, fungi, actinomycetes, yeasts, etc., which are all involved in humification and soil processes (Jeevanrao and Ramalakshmi 2002; Lavelle and Spain 2001; Munnoli 2007; Munnoli et al. 2002, 2010). SW management leading to the production of biofertilizers through VBT has a bright future. Suitable species of earthworms, capable of consuming organic-rich matter are efficient decomposers and stress-resistant so as to sustain adverse environmental conditions, and have high fecundity rates (Edwards and Lofty 1977).

14.3 Earthworm Biodiversity

More than 4200 species of Oligochaetes are known in the world. Of these, 280 are microdrili and about 3200 belong to megadrili (earthworms). In the Indian subcontinent, earthworms also form the bulk of the Oligochaete fauna (Thomas and Trivedy 2002) and are represented by 509 species and 67 genera (Julka 1993). Commonly adopted worms in VBT are *Bimastos parvus*, *Dendrobaena rubida*, *Dendrobaena veneta*, *E. fetida*, *Eisenia hortensis* and *Eisenia andrei* belonging to the family Lumbricidae; *Eudrilus eugeniae*: family Eudrilidae; *Amynthas diffringens*, *Amynthas morrisi*, *Lampito mauritii*, *Metaphire anomala*, *Metaphire birmanica*, *Perionyx excavatus*, *Perionyx sansibaricus*, *M. megascolex*, *Notoscolex*, *Pontoscolex corethrurus*, *Octochaetona serrata*, *Octochaetona surensis*, *Pheritima elongata*, *Pheritima posthuma* belonging to Megascolecidae family.

There are several reports indicating the efforts made to explore the possible potential use of earthworm biodiversity (Edwards and Lofty 1977; Greg Smith et al.

1992; Julka and Senapati 1993; Meyer and Bouwman 1997). Wide biodiversity has been reported from around the world by several groups: Sims and Gerard (1985) for the British Isles (44 species); Bouché (1972) for France (180 species); Easton (1980) for Japan (over 70 species); Reynolds (1977) for Ontario, Canada (about 20 species); Lee (1959) for New Zealand (192 species); Shahnaz et al. (2002) for Gujranwala, Pakistan (15 species); Nair et al. (2005) for Libya (3 species); Jamieson (1981) for Australia (300 species). The other species are *Drawida willsi*, *Dichogaster bolaui* and *L. rubellus* (Dash and Senapati 1985; Singh 1997a). The deep burrower *Pheretima elongata* has been used for treatment of industrial wastes (Singh 1997b). The species identified for economic multiplication of earthworms and vermicomposting are (1) *E. fetida*—European worm, (2) *E. eugeniae*—African worm, (3) *P. excavatus*—indigenous to India are prolific breeders, have a high multiplication rate, are voracious feeders and are easy to handle, having 1–3 years longevity, and survive under aberrant weather conditions (Kale 1994). The latter were successfully employed for both household and industrial organic waste (OW) (Divya 2001; Munnoli 2007). In contrast, Gautam and Choudhary (2002) and Singh (2005) suggested *P. excavatus*, *P. corethrurus*, *Dichogaster modiglianii* and *P. elongata* as continuous breeders with high fecundity, *L. mauritii* and *Drawida nepalensis* as semicontinuous and *Eutyphoeus gammiei* as discrete breeders.

The management of organic SWs through composting is a time-consuming process. VBT utilizes earthworms as versatile natural bioreactors to convert OW into value-added products, the vermicasts (Munnoli 2007), at a faster rate and can be applied to industries producing OWs through the synergistic effect of microorganisms and earthworms (Bhattacharya et al. 2000; Bhawalkar 1995; Roig et al. 1993; Surekha and Mahadev Kumar 2007).

The treatment of OWs was realized in the late 1970s in USA. In India, the importance of OW management has only been realized in the last 20–25 years (Munnoli 2007; Munnoli and Bhosle 2011, 2013, 2014; Piccone et al. 1986; Ranganathan and Christopher 1996). The application of different types of species is not reliable unless it is compatible with the specific substrate being studied. The species good for one region may not necessarily be good in another. This is especially true for the deep burrowers as they require more time to acclimatize to a new environment (Singh 1997b). As such, deep burrowers undergo diapause when subjected to shocks of vibrations (Munnoli 1998). It is therefore advisable to transport the vermicompost (VC) containing earthworms and cocoons in the new environment. In the case of surface feeders, the biomass of earthworms or even cocoons can be easily transported and placed in a new environment.

The use of different organic substrates and species employed and their suitability in vermicomposting is presented in Table 14.1, and a sample of fresh SW from the sugar industry is placed in Fig. 14.1

Table 14.1 Various species employed for vermiprocessing of organic wastes

Sl No	Waste/substrate	Species employed	Reference
1	Potato peels	*Pheretima elongata*	Munnoli et al. 2000
2	Press mud	*P. elongata*	Singh 1997b
		Eudrilus eugeniae, *Eisenia fetida*	Munnoli 2007, 2010
		Megascolex megascolex	Munnoli and Bhosle 2008, 2013, 2014
3	Canteen waste	*E. fetida*	Kale 1994; Narayan 2000
4	Tomato skin seed	*P. elongata*	Singh 1997a
5	Onion residue	*E. fetida/E. eugeniae*	White 1996
6	Sericulture waste	*Perionyx excavatus*	Guthilingaraj and Ravignanam 1996
7	Sericulture waste	*Phanerochaete chrysosporium*	Kallimani 1998
8	Board mill sludge	*Lumbricus terrestris*	Butt et al. 2005
9	Sugar cane residues	*P. elongate*	Bhawalkar 1995
10	Gaur gum	*E. eugeniae*	Suthar 2006, 2007
11	Agricultural residues	*E. eugeniae*	Kale 1994
12	Municipal wastes	*Megascolex mauritii*	Ravichandran et al. 2002
13	Municipal solid wastes	*E. eugeniae*, *P. excavatus*, *Perionyx sasibaricus*	Reddy et al. 2002
14	Distillery sludge	*E. fetida*	Munnoli 1998
		E. eugeniae	Suthar 2008b, d
		P. elongata	Munnoli 1998
15	Tannery waste	*P. excavatus*	Hameed et al. 2002
16	Kitchen waste	*P. excavatus*	Hameed et al. 2002
17	Spent straw	*P. excavatus*	Hameed et al. 2002
18	News paper	*P. excavatus*	Hameed et al. 2002
19	Wood shavings	*P. excavatus*	Hameed et al. 2002
20	Forest litter: *Tectona grandis* (teak), *Madhuca indica* (mahua) and *Butea monosperma* (palas)	*E. fetida*, *P. excavatus* and *Dicogaster bolaui*	Manna et al. 2003
21	Domestic/municipal sewage sludge mixed with sugarcane trash	*E. fetida*	Suthar 2008a, b, c
		Lampito mauritii	
22	Sago waste	*L. mauritii*	Rajesh Babu 2008
23	Onion waste	*E. eugeniae*	Mishra et al. 2009
24	Garlic waste	*E. fetida*	Mishra et al. 2009
24	Source-separated human faeces	*E. fetida*	Yadav et al. 2010
25	Cow dung with biogas slurry	*E. fetia*	Munnoli and Bhosle 2008

Table 14.1 (continued)

Sl No	Waste/substrate	Species employed	Reference
26	Cow dung with jackfruit peels	*E. fetia*	Munnoli et al. 2010
27	Biogas slurry	*E. eugeniae*	Munnoli and Bhosle 2013
28	Neem Cake	*E. eugeniae*	Kale et al. 1986
29	Rice polish	*E. eugeniae*	Bano et al.1987
30	Green gram barn	*E. eugeniae*	Bano et al. 1987
31	Wheat barn	*E. eugeniae*	Bano et al. 1987
32	Cow dung	*E. fetia*	Edwards and Lofty 1977
		E. eugeniae	Edwards and Lofty 1977; Satchell 1955
		M. megascolex	Munnoli 2007; Munnoli and Bhosle 2009
33	Sheep, horse, cow dungs	*E. eugeniae*	Bano et al. 1987
34	Rubber leaf litter	*P. excavatus*	Choudhary et al. 2001
35	Rubber leaf litter	*E. euggeniae*	Choudhary et al. 2001
36	Rubber leaf litter	*E. fetida*	Choudhary et al. 2001
37	Soil + cellulose waste	*E. fetida*	Zajonc and Sidor 1990
38	Pulp and paper industrial waste	*E. fetida*	Piccone et al. 1986
39	Activated sludge	*E. fetida*	Mitchell and Horner 1980; Hartenstein et al. 1979
40	Activate sludge with dead leaves	*E. eugeniae*	Jayashankar 1994
41	Primary sewage sludge	*E. fetida*	Hait and Tare 2011
42	Sewage sludge	*L. mauritii*	Yang et al. 2014
43	Tea factory coal ash	*L. mauritii*	Goswami et al. 2014
44	Apple pomace waste	*NA*	Hanc and Chadimova 2014
45	Paper mill waste water sludge	*E. fetida*	Renu and Suthar 2013
46	Milk industry sludge	*E. fetida*	Suthar et al. 2012
47	Herbal pharmaceutical waste	*E. fetida*	Garg et al. 2012

14.3.1 Earthworm Gut Morphology and Contents

The earthworm gut is basically a straight tube extending from the mouth to the anus. Its different regions are the muscular pharynx, oesophagus, intestine and associated digestive glands. The oesophagus may be further differentiated into two bulbous chambers, a muscular gizzard and a thin-walled crop. There are various modifica-

Fig. 14.1 Fresh sample of press mud/filter mud

tions in the digestive system in different worms depending upon the food eaten (Meglitsch and Schram 1991). There may be more than one gizzard depending upon the species. The gizzard is generally absent or rudimentary in earthworms which thrive on a liquid or semiliquid diet. Litter-feeding species lack a typhlosole which is well developed in soil-feeding worms (Senapati 1993).

The gut contents usually comprise of mucus, organic and mineral matter. An analysis of the gut contents in earthworms revealed the occurrence of different kinds of symbiont-like microfungi, bacteria, protozoa, etc. Most microfungal species are found in the foregut, gradually decreasing in number in the mid- and hindgut with the least in freshly laid casts (Dash et al. 1980a; Munnoli 1998, 2007; Munnoli et al. 2010).

It is well established that the earthworm gut provides suitable conditions for the development of bacterial colonies since earthworm casts contain significantly higher counts of bacteria than in the surrounding soil (Bhattacharyya et al. 2000; Edwards and Lofty 1977; Munnoli 1998, 2007; Singh 1997b). Microorganisms may constitute an important part of the diet of earthworms, which can feed on them selectively (Edwards 2004; Moody et al. 1995). A 13.76; 572.96-fold increase in actinomycetes and a 700.8; 927.78-fold increase in bacteria in the mid- and hindgut as compared with the foregut, respectively, have been noted (Edwards and Lofty 1977; Munnoli 2007).

The presence of digestive enzymes like amylase, cellulase, protease, lipase, chitinase, gelatinase and a variety of others have also been reported from the alimentary canal of earthworms. These enzymes in the intestine are usually related to the preferred diet of the organism (Wallwork 1984) signifying the digestive ability of earthworms (Abbasi and Ramasamy 2001) and operate in a medium with remarkably stable pH ranging between 6.3 and 7.3 (Wallwork 1984). Zhang et al. (1993) found strongest enzyme activities in the fore- and mid-gut. The activity of enzymes digesting cellulose, starch, glucomannan and glactomannan predominated. They also concluded that cellulase and mannase activities were mainly due to microor-

ganisms. Dash and Senapati (1986) also reported the gut enzymes of earthworms such as amylase cellulase, chitinase, protease and urease. The gizzard and the intestine work as a "Bio-reactor". Worms secrete enzymes like amylases, proteases, lipases, cellulases and chitinases in their gizzard and intestine which bring about rapid biochemical conversion of the cellulosic and the proteinaceous materials in the OWs (Rajiv et al. 2010). They ingest the food materials, cull the harmful microorganisms, and deposit them mixed with minerals (Munnoli and Bhosle 2011; Singh and Suthar 2013) and beneficial microbes as "vermicasts" in the soil. The literature pertaining to enzyme activities associated with gut/tissue/VC/earthworm skin has been reported in several studies (Table 14.2).

High population densities of earthworms in vermin-composting systems result in a rapid turnover of fresh organic matter into earthworm casts or VCs (Aira et al. 2003, 2006). These casts can be deposited both inside and outside of a fresh organic matrix, thereby affecting the decomposition rates in their proximity because of their different microbial composition. During the vermin-composting process, earthworms can modify the diversity and abundance of the micro flora directly, by selective feeding, or by stimulation of a particular group of microorganisms (Tiunov and Scheu 2000; Wolter and Scheu 1999). Moreover, earthworms exert other indirect effects on microbial communities, such as microbial dispersion and the release of additional food resources in their casts. For all these reasons, better knowledge of the changes in the chemical and biochemical properties of OWs during the vermin-composting process is required to understand the effect of the earthworms' activities on the process of biodegradation. Plaza et al. (2004) used pig slurry as the OW since the number of pig-breeding farms in Spain is increasing and because most of the pig slurry produced is applied without any treatment to the soil. The main objective of their study was to monitor the short-time changes (fresh manure to casts) of pig manure, after passing through the gut of the epigeic earthworm *E. eugeniae* under controlled environmental conditions. They also monitored the changes in available pools of carbon and nitrogen of pig slurry and analysed microbial biomass (respiration and substrate utilization patterns, biolog ecoplate) and enzyme activities, since these have been shown to be reliable indicators of the response of microbial communities to variations in environmental conditions (Carreiro et al. 2000) and are very important in regulating soil properties (Dick 1992)

The gut environment is anoxic having pH of 6.9 with about 50 % water content. The gut bacteria are enriched in total carbon, organic carbon and total nitrogen with a carbon to nitrogen ratio of 7 (Horn 2003). In a study conducted at Sanjeevani Sugar factory, Goa, vermireactors were set up using *M. megascolex* and *Eudrilus eugenae* as depicted in Fig. 14.2a and b, respectively. VC was harvested as placed in Fig. 14.3. The sources of bacteria isolated were earthworm skin including gut, vermicasts and VC. Interestingly, bacterial counts in gut and VC were higher than the vermicasts and surrounding soil (Munnoli 1998, 2007; Munnoli et al. 2000), as also reported by Edwards and Lofty (1977); Nechitaylo et al. (2010) and Suthar (2008a). Significantly, most of the bacteria isolated were endospore-forming Gram-positive *Bacilli* showing amylase and gelatinase activity, indicating biochemical changes of the organic matter in the gut brought about by gut-inhabiting bacteria (Munnoli 2007).

Table 14.2 Enzyme activity associated with earthworm gut/vermicasts/tissue bacterial isolates

Earthworm species		A	C	Ch	P	U	I	E	AP	NR	G	L	Reference
Detochaetona surensis	Gut	+	+	–	+	+	–	–	–	–			Mishra and Dash 1980
Dichogaster balaui	Gut	+	+	–	+	+	–	–	–	–			Mishra and Dash 1980
Drawida celebi	Gut	+	+	–	+	+	–	–	–	–			Mishra and Dash 1980
Perionyx millardi		+	+		+	+							Dash et al. 1981
Hemienchytraeus khallikotosus	Tissue	+	+		+	+	+						Dash et al. 1981
Enchytraeus berhampurosus	tissue	+	+		+	+	+						Dash et al. 1981
Fridericia kalinga	Tissue	+	+		+	+	+						Dash et al. 1981, 1980b
Dichogaster bolaui	Gut	+	+	–	+	+							Dash and Senapati 1986
Drawida calebi	Gut	+	+	–	+	+							Dash and Senapati 1986
Drawida willsi	Gut	+	+	–	+	+							Dash and Senapati 1986
Eutyphoeus sp.	Gut	–	–	–	+	+							Dash and Senapati 1986
Lampito mauritii	Gut	+	+	–	+	+							Dash and Senapati 1986
Dendrobaena octoedra	Gut	–	+	–	–	–							Dash and Senapati 1986
Eisenia fetida	Gut	–	–	–	+	–							Dash and Senapati 1986
E. fetida	Vc	+	+		+								Munnoli 1998
Eudrilus eugeniae	Vc	+	+		+								Munnoli 1998
Pheritima elongate	Vc	+	+		+								Munnoli 1998
L. mauritii	Vc	+	+		+		+						Parthasarathi and Ranganathan 2001
E. eugeniae	Vc	+	+		+		+						Parthasarathi and Ranganathan 2001

Table 14.2 (continued)

Earthworm species		A	C	Ch	P	U	I	E	AP	NR	G	L	Reference
E. eugeniae	Gut	+	+					+	+	+			Prabha et al. 2007
E. fetida	Gut	+	+					+	+	+			Prabha et al. 2007
E. eugeniae	Gut/vc	+	+	–	+	+	+			+			Munnoli 2007
E. fetida	Gut/vc	+	+	–	+	+	+			+			Munnoli 2007
Megascolex megascolex	Gut/vc	+	+	–	+	+	+			+			Munnoli 2007
E. eugeniae	Skin	+	+	+	+								Munnoli 2007
E. fetida	Skin	+	+	+	+						+	+	Munnoli 2007
M. megascolex	Skin	+	+	–	+						+	+	Munnoli 2007

A amylase, *AP* acid phosphatise, *C* cellulase, *Ch* chitinase, *E* endogluconase, *G* gelatinase, *L* lipase, *NR* nitrate reductase, *P* protease, *U* urease,+ positive, – negative, *vc* vermicast

Fig. 14.2 **a** Field trials bed covered with wet jute bags with deep burrower species; **b** field trials showing bed covered with jute bags with surface feeder

The genetic makeup of the strains and environment has a profound influence on the efficiency of earthworms in the bioconversion process (Giraddi 2009) and in the assessment of diversity and community composition (Thakuria et al. 2010).

The extraction of genomic DNA from various parts of the adult *E. eugeniae* entire worm, gut, clitellum, prostomial and tail region using the CTAB method with few modifications (Murray and Thompson 1980) suggested that the clitellum was the best region to get high quality DNA as also reported by Giraddi et al. (2009) during their studies on *E. fetida* and *P. exavatus*. This technique is also suitable for extraction of DNA from other earthworm species such as *L. terrestris* (El Adloun et al. 1995) and *Lumbricid* earthworms (Pop et al. 2003). The extraction of DNA from *L. terrestris* (Hammond et al. 1998), *L. rubellus* (Morgan et al. 1999) and *Octolaseum lacteum* (Erseus et al. 2000) has also been carried out. The high quality of DNA suggests the efficiency of *E. eugeniae* in the bioconversion process. Our study on bioconversion of press mud also supports the superiority of *E. eugeniae* over *E. fetida* and *M. megascolex* (Munnoli 2007).

The diversity of eight bacterial groups from fresh soil, gut and casts of the earthworms *L. terrestris* and *Aporrectodea caliginosa* was studied by single-strand

Fig. 14.3 Vermicompost samples

conformation polymorphism (SSCP) analysis using both newly designed 16S rRNA gene-specific primer sets targeting *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* and *Firmicutes* and a conventional universal primer set for SSCP, with ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) as templates. *Bacteroidetes*, *Alphaproteobacteria* and *Betaproteobacteria* were predominant in communities from the soil and worm cast samples. It has been reported that some specific bacterial taxonomic groups maintain their diversity and even increase their relative numbers during transit through the gastrointestinal tract of earthworms (Nechitaylo et al. 2010).

Bacterial communities were detected using automated ribosomal intergenic spacer analysis of 16S and 23S genes and ribotype data were used to assess diversity and community composition. Soil and earthworm samples collected from adjacent wheat–barley and grass–clover fields showed that the anecic *L. terrestris* and *Lumbricus friendi*, the endogeic *A. caliginosa* and *Aporrectodea longa* (classically defined as anecic, but now known to possess endogeic characteristics) contain ecological group-specific gut wall-associated bacterial communities. The abundance of specific gut wall-associated bacteria (identified by sequence analysis of ribotype bands), including *Proteobacteria*, *Firmicutes* and an *Actinobacterium*, was dependent on the ecological group (Thakuria et al. 2010). Thus, there is a greater role played by the DNA of microorganisms isolated from earthworm species and studies

Table 14.3 Composition of good quality vermicompost and rate of application for various crops. (JeevanRao and Ramalakshmi 2002; Purakayastha and Bhatnagar 1997)

Parameter	Vermicompost	Crop	Rate/Th^{-1}
pH	7–8.5	Cereals	5
Organic carbon (%)	20–30	Pulses	5
Nitrogen (%)	1.5–2.0	Oil seeds	12.5
Phosphorus (%)	1–2	Spices	10
Potassium (%)	1–2	Vegetables	12.5
Calcium (%)	1–3	Fruits	7.5
Manganese (pap)	1–2	Cash crops	15–17.5
Sculpture (%)	< 1	Plantations	7.5
Moisture (%)	15–20	Horticulture crops	100–200 g/tree
C/N ratio	15–20:1	Kitchen garden and pots	50 g/pot
Micronutrients (pap)	200		

on DNA extraction from earthworms and bacteria isolated from various body parts will be of significance in bioconversion of OWs and in increasing the efficiency of vermicomposting systems (Munnoli 2007).

14.3.2 Soil Fertility

Soil micro and macroorganisms play an important role in improving soil fertility and crop productivity due to their capability of fixing atmospheric nitrogen, solublizing insoluble phosphate and decomposing farm wastes resulting in the release of plant nutrients (Binet et al. 1998; Tewatia et al. 2007). There are varying reports on the nutrient contents of vermicasts (Manna et al. 2003; Narayan 2000; Ranganathan Reddy et al. 2002; Shahul Hameed 2002) whereas Rao and Lakshmi (2002) ascertained nutrient values for a good VC based on their study on urban wastes and suggested the rate of application of VC (Table 14.3).

Along with soil microorganisms, the addition of VC also benefits soil fertility in terms of nutrients and microbial population (Binet et al. 1998; Munnoli 2007; Singleton 2003).

14.3.3 Microbial Population

Teotia et al. (1950) reported a 3.4- to 5.4-fold increase in bacteria compared to the surrounding soil on application of VC. Ghilarov (1963) claimed that the number of microorganisms in earthworm casts was 1.64-, 1.35- and 1.97-fold higher than in regular soil in three different fields, namely oak forest, rye and grass, respectively.

A 5- and 40-fold higher level of bacterial counts was reported in vermicasts more than the surrounding soil in the case of potato peel waste (Munnoli 1998) and dairy effluent (Kavian and Ghatnekar 1996), respectively. An increase in hydrolytic microflora in vermicomposting of organic SWs was reported by Munnoli (2007) and Singh (1997b). The moisture content of VC is an essential environmental condition for the survival of beneficial microorganisms, irrespective of whether earthworms continue to live or not. The decrease in moisture content brings down the level of colony forming units and organic carbon (Parthasarathi 2006; Parthasarathi and Ranganathan 2001).

A study on microbes in the gut of earthworms revealed an increase in the number of bacteria and actinomycetes compared to soil, following an exponential law (Edwards and Lofty 1977). In general, the level of microorganisms in the gut and vermicasts of earthworms can be used as one of the measures to evaluate VCs, allowing us to say that earthworms are important in inoculating the soil and their casts are the foci for dissemination of soil microorganisms, which elevates the overall fertility of soil. Monson et al. (2007) reported an increase in nutrients of kitchen waste vermicomposted by *E. eugeniae*: in N, from 1.31 to 2.12%; in P, from 0.121 to 0.7%; in K, from 0.45 to 0.48% and the C: N ratio decreased from 32.45 to 13.66%. A significantly higher number of microbes were observed in experimental plots treated with VC. Nitrogen-fixing bacteria were also higher in plots to which VC was applied after harvest of the crop. A higher microbial load was observed in paddy fields to which VC was applied (Kale et al. 1992). An increase in the microbial population was recorded with potato waste using *P. elongata* (Munnoli 1998) and with press mud waste using *E. fetida*, *E. eugeniae* and *Megacolex megascolex* when compared with the surrounding soil (Munnoli 2007). Meena and Renu (2009) reported an increase in nutrients when press mud was blended with sawdust and treated using three different earthworm species *E. fetida*, *E. eugeniae* and *P. excavatus* individually (monocultures) and in combination (polycultures).

The effect of VC on the microbial population in a soil environment was reported to be best with VC prepared out of a combination of leaf litter; straw, grass and water hyacinth (VC1) compared to VC of leaf litter (VC2), home garbage (VC3) and partially decomposed cow dung (VC4) when applied at a rate of 5% (w/w). The fold increase was 2.16, 1.83, 1.71 and 1.69 in bacteria, 1.49, 1.30, 1.52 and 1.40 in actinomycetes, 2.89, 2.76, 2.38 and 2.47 in fungi for VC1, VC2, VC3, VC4, respectively (Sahu et al. 2000). Fragoso et al. (1993) also reported similar findings. Kale et al. (1992) reported that earthworm burrows lined with earthworm casts are an excellent medium for harbouring N-fixing bacteria; Loquet et al. (1977) and Bhattacharya et al. (2000) also recorded an increase in the microbial count of VCs compared to traditional compost (Table 14.4). The characteristics of source-separated human faeces and VC prepared out of source-separated human faeces by precomposting faeces using bulking materials (VC) using *E. fetida* showed that pathogens were eliminated and nutrients were enhanced (Yadav et al. 2010). The studies carried out on sugar industry waste have also revealed the presence of microbial population input (press mud); process (skin-adhered microbes) and output

Table 14.4 Comparison of microbial counts of traditional compost and vermicompost. (Bhattacharya et al. 2000)

Type of microbes	Traditional compost	Vermicompost
Bacteria	$143 \times 10^{+7}$ g^{-1}	167.29×10^{7} g^{-1}
Fungi	39.61×10^{5} g^{-1}	96.25×10^{5} g^{-1}
Actinomycetes	365.27×10^{6} g^{-1}	419.62×10^{6} g^{-1}
PP solution	195.61×10^{5} g^{-1}	168.20×10^{5} g^{-1}
N_2-fixing bacteria	92.58×10^{5} g^{-1}	96.62×10^{5} g^{-1}
Thiosulphate oxidizer	315.38×10^{5} g^{-1}	569.29×10^{5} g^{-1}

VC (Table 14.5). The isolated strains were successfully utilized in the bio conversion of press mud (Munnoli 2007).

14.4 Vermiwash (VW)

Providing a proper bed slope to vermibeds, facilitates the collection of vermiwash (VW) (Munnoli 2007) which is essentially a bioliquid consisting of colloidal matter/particles of VC in suspension that can be used as a spray which will act as an insecticide or can be applied in the form of a liquid fertilizer (Munnoli 2007; Ravikumar et al. 2008). Zambre et al. (2008) reported the presence of various enzyme activities associated with VW like amylase, protease, urease and phosphatase and nitrogen-fixing bacteria like *Azotobacter* sp., *Agrobacterium* sp. and *Rhizobium* sp., having the ability to also solubilise phosphate. The nutrient content and different physiochemical parameter in VW obtained from VC of sheep, cow and horse dung using *E. fetida* are reported by (Gorak Nath and Singh 2009). The VW of press mud subjected to detection of pathogens using Mc Conkey agar showed nil pathogens/*Escherichia coli* (Munnoli 2007)

Although there is limited literature on the use and quality of VW, many vermicomposting rural projects are being implemented in various parts of India, (Munnoli 2007) and Chennai (Ismail 1997). The beneficiaries are being well trained to collect VW, which is then used as a liquid biofertilizer.

Increasing OWs due to human activities in rural and urban areas and industries is globally a serious constraint in the maintenance of a clean and healthy environment. Earthworms are effective converters of these wastes. Experiments have been successfully conducted for recycling OWs (Munnoli 2007; Singh 1997b; Suthar 2007). Press mud is one such waste that, though a good organic amendment, contains an appreciable amount of toxicants and heavy metals although there is the potential to use it to extract nutrients (Pagaria and Totwat 2007). The use of biofertilizer prepared from press mud VC and VW will have beneficial effects on soil without any build-up of toxicants (Munnoli 2007). The biofertilizer prepared out of OWs which has higher microbial population provides several advantages as listed in Table 14.6.

Table 14.5 Microbes in vermicompost system and isolates using *Eisenia fetida, Eudrilus eugeniae* and *Megascolex megascolex*

Inputs	Process/earthworm activity	Outputs
Pressmud cfu/g	Skin-adhered microorganisms [cfu/ml/g]	Vermicasts cfu/ml/g
Fungi 108×10^4	*Eisenia fetida* 19×10^5	*E. fetida*
Yeasts 35×10^4	Seven isolates	Fungi 11×10^4
Bacteria 7×10^4	*Eudrilus eugeniae* 27×10^5	Yeast 85×10^4
	Three isolates	Bacteria 29×10^8
	Megascolex megascolex 21×10^5	6 Isolates
		E. eugeniae
	Six isolates	
		Fungi 24×10^4
	Soil processes	
	Fungi 11×10^5	Yeast 138×10^4
	Yeast 6×10^4	Bacteria 77×10^8
	Bacteria 1.28×10^7	23 Isolates
		M. megascolex
		Fungi 30×10^4
		Yeast 56×10^4
		Bacteria 82×10^7
		8 Isolates
0 Isolates	Total 16 Isolates	37 isolates

Table 14.6 Benefits derived out of vermicompost with microbial population. (Munnoli 2007, 2010; Rodale 1967)

Increases water-holding capacity	The insect menace is reduced to the minimum
Eliminates valuable watering time	Eliminates pathogens
Reduces soil erosion and flood hazards	Higher residual nutrients leading to continuous enrichment of soil
Increases the soil layer thickness every year	Microbes tolerate adverse environmental conditions
Multiplies the microbial population	Weed and insect resistant
Increases aeration of soil	
Soil made darker by humus, absorbs heat more quickly and effectively	
Acts as an antibiotic	

14.5 Conclusion and Future Prospects

As far as scientific communities are concerned, there is an urgent need to develop research in the field of understanding biodegradation of organic matter by earthworms and microorganisms. The microbes associated with virgin wastes needs to be understood thoroughly and their lifetime assessment becomes an essential factor in deciding the storage time for the waste before it is subjected to biological conversion. The microbes isolated from VCs could separately be tested for biodegradation. The use of consortia developed based on enzyme activities and characteristics of particular industrial wastes could be used for designing an industrial waste treatment unit which is yet a new field. Although this review is primarily aimed towards presenting information on the dynamics of earthworms-microbes–soil-vermicompost interface, accurate experimental studies are needed to standardise the microbial technologies.

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Chapter 15
Prevalence of *Listeria* in Milk from Farm to Table

Dilecta D'Costa

15.1 Introduction

Food safety is at the centre of one's health as food is an excellent vehicle by which many pathogens can reach an appropriate colonization site in a new host. Many, if not most, of all important zoonoses relate in some way to animals in the food production chain. One of the major issues in food safety over the latest decennial has been the lack of cross-sectoral collaboration across this chain (Wielinga and Schlundt 2012).

Dairying plays a dynamic role in agro-based economy and milk is a high value source of nutrients for the urban and rural population. Milk as it is secreted by the gland of the mammals is free of microorganisms but can get contaminated with bacteria during or after milking. Mastitis is one of the most economically important diseases affecting the dairy industry and reduces milk production and alters milk composition (Bansal and Gupta 2009). Although many studies are reported on the analysis of milk collected at different stages of processing, data are lacking on the analysis of milk in production chain, i.e. from farm to table (Anon 2011). Hence, there is an urgent need from the dairy industry to understand the introduction of emerging food-borne pathogens in milk and thus in turn control and prevent milk-borne epidemics and outbreaks.

Human illness from milk-borne pathogens is usually associated with consumption of raw milk or products made from raw milk. Occasionally, this has also been linked to pasteurised milk products but these cases usually have been a result of contamination of the product after pasteurisation or improper pasteurisation (Oliver et al. 2005). In the past 20 years, food-borne illnesses from dairy product consumption have been predominantly associated with *Salmonella enterica, Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*.

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The microbiological safety of food remains a dynamic situation heavily influenced by multiple factors. Given the recent spate of food-borne illness outbreaks, *Listeria monocytogenes* is once again in the spotlight as an important bacterial pathogen (Newell et al. 2011). Although the number of people infected by food-borne *Listeria* is comparatively small, this bacterium is one of the leading causes of death from food-borne illness due to its toxin listeriolysin O which is rightly referred to the Swiss army knife of *Listeria* (Hamon et al. 2012).

15.2 *Listeria* spp. and *Listeria monocytogenes*

Listeria spp. are ubiquitous bacteria widely distributed in the environment (Liu et al. 2006). They are Gram-positive, nonsporulating, facultatively anaerobic rods that measure 1–2 µm in length and 0.5 µm width. Growth occurs between 3 and 50 °C but the optimum temperature is 30–37 °C and they show typical tumbling motility at 20–25 °C (Topley and Wilson 1990) which is a characteristic feature of the organism.

The genus *Listeria* includes various species such as *L. monocytogenes*, *Listeria ivanovii*, *Listeria innocua*, *Listeria welshmeri*, *Listeria seeligeri*, *Listeria grayi*, *Listeria marthii* (Graves et al. 2010), *Listeria rocourtiae* (Leclercq et al. 2010), *Listeria weihenstephanensis* (Lang et al. 2013), *Listeria fleischmannii* (Bertsch et al. 2013), *Listeria floridensis*, *Listeria aquatica*, *L. cornellensis*, *L. riparia* and *L. grandensis*. (Bakker et al. 2014). Among the 15 species of *Listeria*,only *L. monocytogenes* is pathogenic for humans whereas *L. ivanovii* mainly affects ruminants.

L. monocytogenes is a food-borne opportunistic pathogen of great concern for the food industry and milk-producing companies. Due to its physiological characteristics, such as resistance to acidic and sodium chloride stress, ability to grow at low temperature and possibility to form biofilms (Harvey et al. 2007), it can persist and/or recontaminate food products, thereby representing an important risk for the safety of the consumers (Gardan et al. 2003; Liu et al. 2002; Olesen et al. 2009; Pan et al. 2006). The term "*Listeria hysteria*" was coined towards the end of 1980s following a series of listeriosis outbreaks due to the consumption of soft cheese and ready-to-eat (RTE) meats in the UK. Recently, this emerged again in the large outbreaks in Canada caused by deli meats (Warriner and Namvar 2009) and also in USA (Anon 2011). Although human listeriosisoccurs only sporadically (Farber and Peterkin 1991; Schuchat et al. 1991) several outbreakshave been observed during the last two decades (McLauchlin et al. 2004).It is established that food-borne transmission constitutesthe main route of acquisition of listeriosis (Farber and Peterkin 1991). Although the incidence of the first human case of listeriosis was reported by Nyfeldt (1929), it is only since 1981, after the three well investigated listeriosis epidemics, first caused by coleslaw (Schlech et al. 1983), second caused by whole and 2 % fat milk (Fleming et al. 1985) and third caused by consumption of soft Mexican-style cheese (Linnan et al. 1988) that this organism came to be considered as a food-borne pathogen. Multinational outbreak from dairy products was reported

by Fretz et al. (2010). *L. monocytogenes* is composed of at least 12 serovars, i.e. 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Liu 2006); All 12 serovars of the pathogen are known to cause human listeriosis, but serovars 1/2a, 1/2b and 4b are associated with most of the cases.

15.3 Listeriosis

Listeriosis is a serious invasive bacterial zoonotic disease characterised by neural, visceral and reproductive disorders. It is usually manifested as septicaemia, abortion, stillbirth, meningitis, and meningo-encephalitis in a variety of animals including humans; especially in immunocompromised individuals and persons in contact with animals. It is an important food-borne bacterial disease and a nagging public health hazard caused by ingestion of contaminated food and water. To manage the problem of food-borne listeriosis, it requires an understanding of the burden of the disease on a worldwide scale as food that are prone to contamination are eaten widely domestically and many are traded globally (Todd and Notermans 2011). *Listeria* spp. including *L. monocytogenes* are isolated from diverse environmental sources including soil, water, sewage, vegetation (e.g. grass, meadows, forests, silage), wild animal faeces, as well as on the farm and in food-processing facilities (Barbuddhe and Chakraborty 2009; Doijad et al. 2011; Sauders and Wiedmann 2007).

Listeriosis was first recognized as a disease in 1926 during a spontaneous outbreak of infection among laboratory rabbits and guinea pigs in Cambridge characterised by mononuclear leucocytosis (monocytosis) and the isolated organism was named as "*Bacterium monocytogenes*" (Murray et al. 1926). Subsequently, in 1927, Pirie isolated a similar bacillus from the liver of infected gerbils and named it *Listerella hepatolytica* in honour of Lord Lister (Gray and Killinger 1966). Finally, the genus was named as *Listeria* in 1940 for taxonomic reasons (McLauchlin 1987).

Although Listeriosis was first recognized as a disease of animals and the link between silage feeding and infection in farm animals has been known for decades, it was the recognition in the 1980s of listeriosis as a food-borne human disease that promised intense research activity (Low and Donachie 1997).

Human listeriosis is a public health problem of low incidence but high mortality, requiring prompt diagnosis and adequate antibiotic therapy. Antibiotic resistance and inefficient empirical treatment of *Listeria* infections could be responsible for this increased mortality (Rodas-Suárez et al. 2006).

Milk-Borne Listeriosis

The food-borne pathogens in raw milk originate from the farm environment and direct excretion from animals' infected udder and poor silage quality, whereas, in dairy plants the pathogens may enter via contaminated raw milk, colonize the dairy plant environment and consequently contaminate dairy products. Important sources of contamination during the handling and processing might be the workers as well (Bemrah et al. 1998; Kousta et al. 2010), with *Listeria* also being shed in the faeces (Van Kessel et al. 2004). The prolonged excretion of the organism in milk, the

apparently normal appearance of the milk in majority cases and the consumption of raw milk, especially on farms, could be important factors in the transmission and epidemiology of *Listeria* infection. The sources of contamination of *Listeria spp*. in raw milk are probably insufficient hygiene during milking, storage and transport of milk. *L. monocytogenes* may directly contaminate milk as a consequence *of listerial mastitis*, encephalitis or *Listeria*-related abortion in cattle. Rawool et al. (2007) reported overall occurrence of *L. monocytogenes* in 0.55 % of 243 cattle and buffaloes with subclinical mastitis in India. The contamination of food by *L. monocytogenes* occurs along the food chain from farm-to-fork (Farber and Peterkin 1991). The ability of *L. monocytogenes* to grow at low temperatures is important in the bacterium's persistence in food-processing environments. Further, biofilm-forming abilities (Di Bonaventura et al. 2008) and sanitizer resistance (Lundén et al. 2003) also contribute to the persistence of *L. monocytogenes*.

Globally, cases of human listeriosis are on the increase which is evident from the major outbreaks recorded in various countries. The first proof that milk products could be responsible for listeriosis outbreaks was corroborated by Fleming et al.in 1985which involved 49 cases, seven of them in the fetus and 42 in immunocompromised adults. Listeriosis outbreaks have mostly been linked to consumption of raw milk or cheese made of unpasteurized milk (Fleming et al. 1985; Linnan et al. 1988; Lyytikäinen et al. 2000; Rebagliati et al. 2009).

When cattle are infected with *L. monocytogenes*, the organism is excreted in the milk. *L. monocytogenes* is quite resistant to heat and milk's postpasteurisation storage at a refrigeration temperature might allow the selective growth of the remaining organisms (Dalton et al. 1997). Extensive work has been ongoing in many countries during the last decade to prevent outbreaks and decrease the incidence of listeriosis (Rossi et al. 2008).

The occurrence of listeric infections in the Indian subcontinent has been extensively reviewed by Malik et al. (2002). In Indian context, few studies have been carried out to study the incidence of *Listeria* in food. *L. monocytogenes* was isolated from 8.1 % of raw milk samples (Bhilegaonkar et al. 1997). *L. monocytogenes* could not be isolated from pasteurised bulk milk tanks. Isolation of pathogenic *L. monocytogenes* strains was reported from milk of 1.56 % goats (Barbuddhe et al. 2000) and 6.25 % buffaloes (Barbuddhe et al. 2002). In an extensive study involving central India, *Listeria* spp. were isolated from 139 (6.75 %) samples out of 2060 samples collected from dairy cows; 105 (5.1 %) were positive for *L. monocytogenes* (Kalorey et al. 2008). Aurora et al. (2008) analysed milk (471) and RTE indigenous milk products (627) and detected *L. monocytogenes* isolates. *L. monocytogenes* has been isolated from cases of mastitis, reproductive disorders and septicaemia in animals (Shakuntala et al. 2006; Rawool et al. 2007).

15.3.1 Symptoms of Listeriosis

L. monocytogenes causes two forms of listeriosis: noninvasive gastrointestinal listeriosis and invasive listeriosis (Allerberger and Wagner 2010). *L. monocytogenes*

infects normally sterile parts of the body such as liver, spleen, cerebro-spinal fluid and blood, and most cases end up being hospitalized (Todd and Notermans 2011). Persons with a predisposed condition, linked to decreased level of cell-mediated immunity such as individuals with cancer malignancies, organ transplant, liver disease, HIV/AIDS and diabetes are more prone to infection and can develop sepsis, meningitis and serious infections affecting the nervous system (Allerberger and Wagner 2010).

In healthy adults, disease is mainly diarrhoea and fever. In pregnant women fever, diarrhoea, abortion or stillbirth are common; newborns get sepsis, pneumonia or meningitis (Todd and Notermans 2011). Most cases of confirmed listeriosis fall into the ageing category, especially over 65 years old. Case-fatality rates can be high (20–30 %). It is estimated that 10 % of the population in the developed world carry the bacterium in their gastrointestinal tract (Swaminathan and Gerner-Smidt 2007).

15.4 Isolation of *Listeria* Species

Meat, poultry and dairy products have been most frequently implicated as vehicles of transmission. Large outbreaks are usually linked to errors in food-processing plants, such as contaminated slicing machines, followed by opportunities for growth of the pathogen (Todd and Notermans 2011). A number of methods and media currently exist for the detection and enumeration of *L. monocytogenes*. For the isolation of *L. monocytogenes* from foods, animals/human clinical samples and environmental samples, the use of enrichment cultures followed by selective plating is required (Curtis and Lee 1995). The Food and Drug Administration (FDA) Bacteriological and Analytical Method (BAM), and the International Organization for Standardization (ISO) 11290 method (Barbuddhe et al. 2008) are the most widely used culture reference methods for detection of *Listeria* in all foods. Other methods, like the United States Department of Agriculture (USDA) and the Association of Analytical Chemists (AOAC) are also used.

15.4.1 Enrichment Procedure/Media

Isolation of *Listeria* from complex samples, such as food, environmental and stool samples, containing abundant background flora and a low number of *Listeria,* requires enrichment. The earliest method available was the cold enrichment technique (Gray et al. 1948). This required inoculation of the sample into a nutrient broth lacking selective agents, followed by incubation at 4 °C for long periods. However, the method was time consuming and has subsequently been replaced by methods involving selective enrichment and selective plating based on the inhibition of the growth of background flora by adding inhibitory agents such as lithium chloride, nalidixic acid, acriflavine, cefotetan, ceftazidime, colistin, cycloheximide, fosfomycin and polymyxin B (Gasanov et al. 2005).

A number of media such as polymixin acriflavin lithium chloride ceftazidime aesculin mannitol egg yolk (L-PALCAMY; Van Netten et al. 1989), Lovett (FDA; Lovett et al. 1987), University of Vermont Medium (UVM; Donnelly and Baigent 1986) and Fraser broth (Fraser and Sperber 1988) were developed.

The ISO 11290 method employs a two-stage enrichment process: the first enrichment in half Fraser broth (Fraser and Sperber 1988) for 24 h, followed by transfer of an aliquot to full-strength Fraser broth for further enrichment. In the FDA BAM method, the sample (25 g) is enriched for 48 h at 30 °C in *Listeria* enrichment broth (LEB; Lovett et al. 1987) containing the selective agents acriflavin and nalidixic acid, and the antifungal agent cycloheximide. The USDA and the AOAC/International Dairy Federation (IDF) methods use a modification of UVM (Donnely and Baigent 1986) containing acriflavin and nalidixic acid for primary enrichment. The USDA method was designed and has been officially recommended primarily for meat and poultry products and the FDA method was designed for processing dairy products (Brackett and Beuchat 1989).

15.4.1.1 Selective or Differential Plating Media

A number of media have been developed which include Oxford agar (Curtis et al. 1989); lithium chloride–ceftazidime agar (LCAM; Lachica 1990); polymixin–acriflavin–lithium chloride–ceftazidime–asculin–mannitol (PALCAM) agar (Van Netten et al. 1989); Dominguez-Rodriguez isolation agar (DRIA; Dominguez-Rodriguez et al. 1984); Dominguez-Rodriguez *Listeria* selective agar medium, modified (LSAMm agar; Blanco et al. 1989); modified Vogel Johnson agar (MVJ; Buchanan et al. 1989) and MVJ modified further (MVJM; Smith and Buchanan 1990). *L. monocytogenes* blood agar (LMBA) is a a very useful tool to detect *L. monocytogenes.*

15.4.1.2 Chromogenic Media

The chromogenic media commercially available include Agar *Listeria* according to Ottaviani and Agosti (ALOA), the BCM *L. monocytogenes* detection system, CHROM agar, and rapid *L. monocytogenes*. Chromogenic media are simple, cost effective and easy to interpret (Gasanov et al. 2005). ALOA is both a selective and differential medium for the isolation of *Listeria* spp. and presumptive identification of *L. monocytogenes* (Ottaviani et al. 1997). ALOA was found to be superior to Oxford and PALCAM when samples containing both *L. monocytogenes* and *L. innocua* were examined (Vlaemynck et al. 2000).

15.4.2 Differentiation of Listeria Species

Conventionally, the identification of *Listeria* spp. have relied on the results of fermentation of sugars and haemolytic reactions (Seeliger and Jones 1986), and the commercially available API *Listeria* identification kit (Bille et al. 1992).

Differentiation of *innocua* and *monocytogenes* (DIM), a test based on the detection of acrylamidase present in *L. innocua* strains and in majority of other non- *L. monocytogenes* listerial strains but absent in *L. monocytogenes* can easily and clearly differentiate *L. innocua* and other *Listeria* strains from *L. monocytogenes* (Bille et al. 1992). All species of *Listeria* except *L. monocytogenes* produce amino acid peptidase activity on alanine substituted substrates (Kämpfer 1992). This reaction has been modified by using DL-alanine ß-napthylamide (DLABN) as the substrate and has successfully been carried out for identification of *Listeria* within 5 h (Clark and McLaughlin 1997; McLauchlin 1997). However, despite the availability of alternative identification techniques, conventional and haemolytic reactions are most commonly used (McLauchlin 1997).

15.4.3 Detection of Listeria Species

L. monocytogenes and other *Listeria* species closely resemble morphologically and biochemically and the clinical manifestations of listeriosis are nonspecific (Vázquez-Boland et al. 2001). Therefore, rapid, specific and sensitive diagnostic tests capable of distinguishing *L. monocytogenes* from other *Listeria* species are essential for the effective control of the disease (Liu 2006).

Detection and enumeration of *L. monocytogenes* from environments such as food, which can be heavily contaminated with other organisms, are often difficult (Golden et al. 1988). The *L. monocytogenes* demonstrates strain variations in virulence and pathogenicity (Liu et al. 2003; Roche et al. 2003). Therefore, the ability to determine accurately and rapidly the pathogenic potential of *L. monocytogenes* isolates is essential to limit the spread of listeriosis and reduce unnecessary recalls of food products. The pathogenic potential of *Listeria* isolates can be assessed by in vitro pathogenicity tests like β-haemolysis on sheep or horse blood agar (Schonberg 1989), phosphatidylinositol-specific phospholipase C (PI-PLC) assay (Notermans et al. 1991b) and by the use of chromogenic media (Greenwood et al. 2005), and by in vivo methods namely, chick embryo and mouse inoculation (Menudier et al. 1991). The in vivo methods remain objectionable from an ethical point of view and need skilled personnel to perform. Therefore, the application of molecular techniques has facilitated the identification and characterisation of *L. monocytogenes* (Liu 2006). Among the several approaches to nucleic acid amplification, The polymerase chain reaction (PCR) was the first and remains the most widely applied technique in both research and clinical laboratories (Liu 2006).

A number of factors have been shown to be associated with the virulence of *L. monocytogenes* (Portnoy et al. 1992). The haemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. Subsequently, the virulencegene cluster in which most of the genetic determinants requiredfor the intracellular life cycle of pathogenic *Listeria* spp. residewas discovered(Vázquez-Boland et al. 2001). The listeriolysin O(LLO)-encoding gene (*hlyA*) is present only in virulent strains of the species and is required for virulence. LLO is a secreted protein toxin that can be detected easily with the use of blood agar or haemolysis

assays, and it is well characterised and understood (Churchill et al. 2005). A strong correlation between haemolytic activity and pathogenicity in the genus *Listeria* has been demonstrated (Seeliger and Jones 1986; Skalka et al. 1982).

Differentiation of *L. ivanovii* from *L. monocytogenes* and other *Listeria* species can be achieved by its production of a wide, clear or double zone of haemolysis on sheep or horse blood agar, a positive Christie–Atkins–Munch-Petersen (CAMP) reaction with *Rhodococcus equi* but not with haemolytic *Staphylococcus aureus* (Rocourt and Catimel 1985). A positive CAMP reaction or fermentation of rhamnose and nonfermentation of xylose can be used to identify pathogenic *Listeria* spp. with the exception of *L. seeligeri* which is haemolytic but nonpathogenic (Seeliger and Jones 1986).

Pathogenic *Listeria* spp. exhibit three different enzymes with phospholipase C (PLC) activity, PlcA and PlcB activities are shown by *L. monocytogenes* and SmcL is specific to *L. ivanovii* (Vázquez-Boland et al. 2001). The activity of virulence factor called phosphatidylinositol-specific phospholipase C (PI-PLC), encoded by the *plc*A gene, has been reported to be expressed by the pathogenic species of *Listeria* (Notermans et al. 1991b). The *plcA*-deficient strains have been reported to be less virulent in mice (Camilli et al. 1991).

A chromogenic medium (ALOA) has been developed based on PI-PLC activity (Ottaviani et al. 1997). ALOA medium has proven to be a useful and significantly better assay than other media for the differentiation of *L. monocytogenes* from nonpathogenic *Listeria* species (Vlaemynck et al. 2000; Beumer and Hazeleger 2003). All the *Listeria* species form bluish green colonies due to the presence of a chromogenic compound X-glucosidase which detects β-glucosidase. Further, pathogenic *Listeria* spp. can be distinguished from other *Listeria* species through the production of opaque halo around the colonies (Ottaviani et al. 1997).

Virulence of *L. monocytogenes* for humans has been correlated with pathogenicity in mice (Mainou-Fowler et al. 1988), particularly in immuno-compromised mice (Stelma et al. 1987). Mice inoculation is capable of providing an in vivo measurement of all virulent determinants; therefore, it is regarded as the gold standard for any newly developed tests for *L. monocytogenes* virulence (Liu et al. 2003; Roche et al. 2001). The mouse virulence assay is conducted by inoculating mice with various doses of *L. monocytogenes* via the oral, nasal, intraperitoneal, intravenous or subcutaneous routes. The virulence of a given *L. monocytogenes* strain is determined by the mouse mortality resulting from infection, or by the number of *L. monocytogenes* bacteria that reach the spleen following experimental infection (Liu 2006).

Inoculation of chick embryos with pathogenic *Listeria* species through chorioallantoic (CAM) route may cause death of embryo within 72 h while nonpathogenic species fail to do so (Terplan and Steinmeyer 1989), and the test has been reported to agree with mouse bioassay (Notermans et al. 1991a). Because of nonspecific deaths, yolk sac route inoculation has been found to be less suitable than the CAM challenge for assessing virulence (Notermans et al. 1991a).

An array of virulence-associated genes associated with the pathogenicity of *Listeria* spp., include *plc*A encoding phosphatidylinositol phospholipase-C (PI-PLC),

*plc*B encoding phosphatidycholine phospholipase-C, *hly*A encoding a haemolysin, *mpl* encoding a metalloprotease and *act*A encoding the surface actin polymerisation protein ActA. All these genes are physically linked in a 9 kb chromosomal island referred to as *Listeria* pathogenicity island-1 (LIPI-1; Vázquez-Boland et al. 2001).

One of the biggest problems associated with the detection of *L. monocytogenes* is the low numbers at which the bacteria are normally found in contaminated food samples (Hoffman and Weidmann 2001). DNA-based methods of detection employ ways of amplifying the specific genetic signals from a few cells. PCR is the basis of many nucleic acid-based detection systems (Churchill et al. 2005). Among the target genes for PCR detection of *L. monocytogenes* are the *hlyA* gene (Norton et al. 2001; Thimothe et al. 2004), the *iap* gene (Cocolin et al. 2002), *inl*B (encoding internalin B; Lunge et al. 2002) and 16S rRNA (Call et al. 2003). Among these genes, the *hlyA* gene has been most commonly used (Aznar and Alarcón 2002).

Multiplex PCR is a variation of the traditional PCR. This method makes use of multiple sets of primers to amplify a number of genes or gene fragments simultaneously (Churchill et al. 2005). An mPCR assay employing four genes, the *hly*A, *plc*A, *iap* and *act*A for the detection of *L. monocytogenes* from clinical samples has been developed (Kaur et al. 2007). Rawool et al. (2007) detected multiple virulence-associated genes (the *plcA, prfA, hlyA, actA* and *iap*) in *L. monocytogenes* isolated from bovine mastitis cases. The development of PCR-based serotyping procedures, such as the use of group-specific PCR primers, has provided additional tools for the identification and grouping of *L. monocytogenes* (Borucki and Call 2003; Doumith et al. 2004).

Use of real-time PCR in a 96-well PCR format eliminates the need for agarose gel electrophoresis. In this method, a fluorescent dye, such as SYBR Green I is used to follow the PCR amplification in real-time and can be used to detect the amplified products from a number of genes at the same time (Bhagwat 2003). Primers for real-time PCR can be designed to simultaneously detect both *Listeria* spp. and *L. monocytogenes* by amplifying the 23S rRNA gene (conserved in all *Listeria* spp.) at the same time as the *hlyA* gene (Rodríguez-Lázaro et al. 2004). Real-time PCR can be used with the proper primers to quantify the number of pathogens present in a sample by measuring the level of fluorescence as compared to a standard. The adaptation of conventional PCR to the reverse transcription PCR (RT-PCR) format also permits the detection of viable *L. monocytogenes* organisms in specimens (Liu 2006).

The development of a 10-min assay based on **m**atrix-**a**ssisted **l**aser **d**esorption/**i**onisation-**t**ime **o**f **f**light (MALDI-TOF) spectroscopy directly from colonies on agar plates has been reported. The method allows not only discrimination between pathogenic and nonpathogenic *Listeria* spp. but also permits resolution up to the level of the PCR serotype analysis (Barbuddhe et al. 2008). Nevertheless, high cost of the capital equipment involved, despite being offset by cheap running costs of the assay, puts this method beyond the means of smaller diagnostic laboratories.

15.5 Subtyping of *Listeria* Species

L. monocytogenes is a ubiquitous organism and exhibits diversity of strains. The subtyping procedures are used to track individual strains involved in listeriosis outbreaks, and to examine the epidemiology and population genetics of *L. monocytogenes*. The subtyping is integral to control and prevention programmes aimed at listeriosis. Two major subtyping approaches are in common use: phenotypic and genotypic (molecular or DNA subtyping).

The choice of a method depends most on the performance criteria of the method, such as typeability, discriminatory power, reproducibility, rapidity and ease of use and the purpose of subtyping, e.g. phylogenetic analysis, epidemiological surveillance, outbreak investigations or food-processing contamination analysis (Struelens et al. 1996).

L. monocytogenes can be classified into four lineages: lineage I encompasses serotypes 1/2b, 3b, 4b and 3c; lineage II includes serotypes 1/2a, 1/2c, 3a, lineage III comprises serotypes 4a, 4b and 4c and lineage IV comprises 4a, 4b, 4c (Orsi et al. 2011). Several subtyping procedures including serotyping, multilocus enzyme electrophoresis (MLEE), DNA restriction endonuclease analysis, ribotyping, DNA sequencing-based subtyping techniques, e.g. multilocus sequence typing (MLST) and PFGE have been developed for *L. monocytogenes* (Borucki and Call 2003; Liu 2006). Serotyping may potentially be useful for tracking *L. monocytogenes* strains involved in disease outbreaks. Indeed, it has been observed that *L. monocytogenes* serotypes 1/2a, 1/2b and 4b are responsible for 98 % of documented human listeriosis cases, whereas serotypes 4a and 4c are rarely associated with outbreaks of the disease (Wiedmann et al. 1996; Jacquet et al. 2002). The development of PCR-based serotyping procedures has provided additional tools for the identification and grouping of *L. monocytogenes* (Borucki and Call 2003; Doumith et al. 2004).

15.5.1 Phenotypic Methods

Phenotypic methods often have a low power of discrimination in strains, suffer from biologic variability (e.g. phage typing), and may not be applicable to all strains (Graves et al. 1999). Serotyping has been a classical tool in subtyping of *L. monocytogenes.* Based on somatic (O) and flagellar (H) antigens, *L. monocytogenes* strains are divided into 12 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). The major drawbacks of serotyping include cost, availability and standardization of reagents, as well as the technical expertise needed to perform the assay (Borucki and Call 2003). The usefulness of serotyping in epidemiological investigations is limited as more than 95 % of strains isolated from human cases and foods belong to serotypes 1/2a, 1/2b and 4b (Liu 2006). The enzyme-linked immunosorbent assay (ELISA) method of serotyping described by Palumbo et al. (2003) is a cost-effective method. The reduced variability of the antiserum quality by using a commercially available antisera and also the reduction of

inconsistencies in judgement associated with weakly agglutinating antigen–antiserum combinations are overcome by this method (Palumbo et al. 2003). Studies have found that serotype 1⁄2a was the predominant serotype of *L. monocytogenes* food and environmental isolates (Corcoran et al. 2006; Gilbreth et al. 2005; Lukinmaa et al. 2003).

Phage typing has been shown to be an efficient method for large-scale subtyping of *L. monocytogenes* (Audurier and Martin 1989), and a standard phage set with a standardized method has been described (McLauchlin et al. 1996). Even though phage typing shows high discrimination power, the high number of strains remains nontypeable (Rocourt and Catimel 1985).

Multi locus enzyme electrophoresis (MLEE) is a protein-based, isoenzyme typing method that correlates specific protein band patterns with genotypes (Liu 2006).

15.5.2 Genotypic Methods

PFGE is a molecular subtyping method that has been successfully used to characterise *L. monocytogenes* isolates (Kerouanton et al. 2010; Mammina et al. 2009) and is considered to be the gold standard subtyping method because of the documented reproducibility in previous epidemiological studies and its high discriminatory power (Autio et al. 2002; Graves and Swaminathan 2001). Ribotyping is based on the use of nucleic acid probes targeting ribosomal genes after restriction enzyme analysis of chromosomal DNA (Grimont and Grimont 1986). Automated ribotyping was previously used for rapid subtyping *L. monocytogenes* for source tracking, population genetics-based studies, and epidemiological investigations (Wiedmann 2002); however, it is expensive and not as discriminatory as PFGE (Inglis et al. 2003). PFGE provides sensitive subtype discrimination and is often considered the standard subtyping method for *L. monocytogenes* (Graves and Swaminathan 2001). However, this method is not automated and is labour intensive. Even recently developed rapid protocols take approximately 30 h to perform (Graves and Swaminathan 2001). Computer-assisted data analysis of large and diverse PFGE type databases can improve the correct interpretation of subtyping data in epidemiological studies and in tracing routes and sources of contamination in the food industry (Neves et al. 2008).

The application of PFGE in the characterisation of *L. monocytogenes* isolated from the food can provide a significant insight into the presence of endemic strains and valuable information on potential sites of cross-contamination. Epidemiological tracking of strains over a period of time is required to enable more precise identification of sites of cross-contamination, or critical control points, and to enable to take some measures to avoid the persistence of individual strains within the processing environment.

Several typing methods involving PCR have been developed. The methods employ either just PCR amplification or random amplified polymorphic DNA-PCR (RAPD-PCR) amplification is performed either before polymerase chain reaction-restriction enzyme analysis (PCR-REA) or after amplified fragment length polymorphism (AFLP) restriction enzyme analysis. RAPD is more economical and

faster than other typing methods and is particularly suitable for testing fewer than 50 strains (Liu 2006). It employs short (9–10 bp) primers with sequences chosen at random, thus prior sequence knowledge of template DNA is not needed. A total of 30 *L. monocytogenes* isolates from human patients and food originated from two different geographic locations without any epidemiological relations showed 92–99 % genetic homogeneity and contained virulence genes, *inlA, inlB, actA, hlyA, plcA* and *plc*B (Jaradat et al. 2002).

A rapid multiplex PCR serotyping assay has been developed which separated the four major *L. monocytogenes* serovars (1/2a, 1/2b, 1/2c and 4b) into distinct groups (Doumith et al. 2004, 2005). Serotyping of 145 *L. monocytogenes* isolates revealed serovar 1/2a to be the most frequent (57.4 %) followed by 4b (14.1 %, 1⁄2b (9.7 %) and 4c (4.4 % and 1⁄2c (6.7 %).

15.6 Studies on *Listeria* spp. from Milk Samples in Goa

15.6.1 Isolation and Characterisation

A total of 767 milk samples from dairy cows were taken at different levels of collection and processing (udder, from milking utensils/cans, dairy cooperative society, receiving dock and market) and were processed for the isolation of *Listeria* as per the USDA method described by McClain and Lee (1988) after making necessary modifications. Double enrichment of the samples was carried out using UVM medium. The enriched inoculum from UVM-2 was streaked on PALCAM agar (Himedia Labs, Mumbai, India). The inoculated plates were incubated at 37 °C for 24–48 h. From the isolation media, suspected colonies of *Listeria* were subcultured on 5 % sheep blood agar. Morphologically typical colonies were verified by Gram's staining, catalase and oxidase reaction, tumbling motility at 25 °C, methyl red-Voges Proskauer (MR-VP) reactions, fermentation of sugars (rhamnose, xylose, mannitol and α-methyl- D-mannopyranoside), hemolysis and Christie-Atkins Munch Petersen (CAMP) test with *S. aureus* and *Rhodococcus equi*.

The grey green colonies with black sunken centres from PALCAM (Fig. 15.1), Gram-positive, coccobacillary forms (Fig. 15.2) with characteristic tumbling motility at 20–25 °C were considered as to be of *Listeria*. Overall, 10.56 % of the samples (81 of 767) were positive for *Listeria* species. The catalase positivity and oxidase negativity was observed in all the 81 isolates. On further testing, 37 isolates produced acid from rhamnose and α-methyl D-mannopyranoside but not from xylose, and therefore were tentatively designated as *L. monocytogenes.*

On streaking of 81 confirmed *Listeria* isolates onto 5 % sheep blood agar, a varying degree of haemolysis was observed (Fig. 15.3) and a total of 38 isolates showed haemolysis. Unlike a typical β-haemolysis with broad and clear zones exhibited by the isolate of *L. ivanovii*, the degree of haemolysis shown by *L. monocytogenes* isolates was moderate.

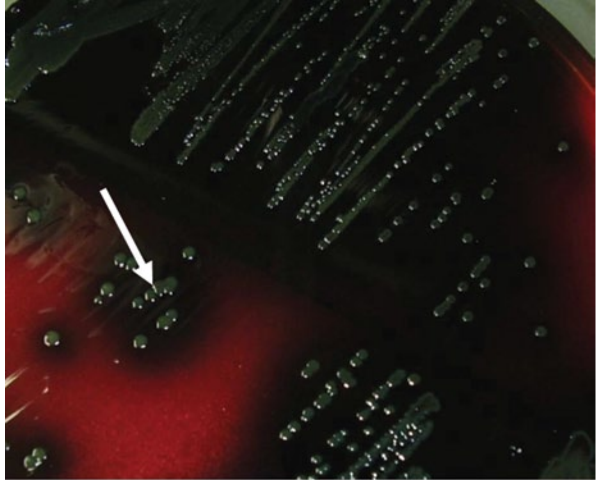

Fig. 15.1 Colonies of *Listeria* species on PALCAM agar

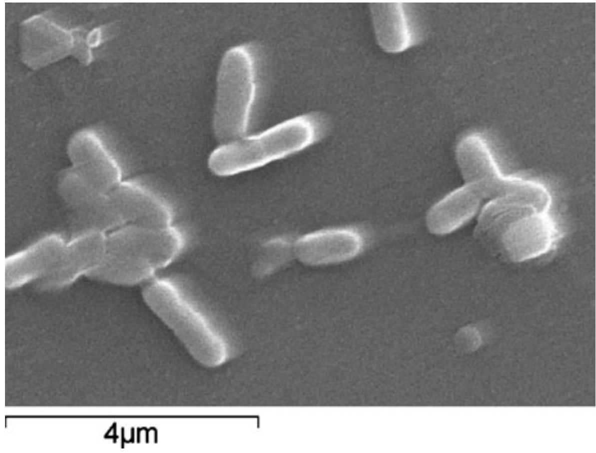

Fig. 15.2 Scanning electron microscopy (*SEM*) of *L. monocytogenes*

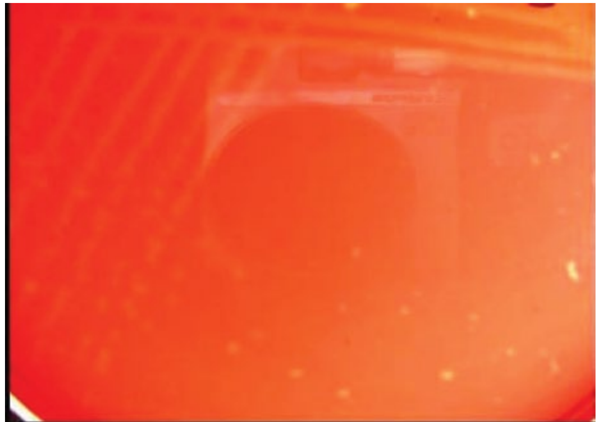

Fig. 15.3 *L. monocytogenes* hemolysis on sheep blood agar

The 81 *Listeria* isolates from milk were then analysed by CAMP test. 37 isolates showed characteristic enhancement of haemolytic zone with *S. aureus* indicating the presence of *L. monocytogenes* while one isolate showed enhanced haemolytic zone typically with *R. equi* confirming the presence of *L. ivanovii* (Fig. 15.4).

Agar *Listeria* according to Ottaviani and Agosti (ALOA) assay, an alternative way to assess phophatidyl inositol phospho lipase C activity (PI-PLC) was carried out using chromogenic ALOA (Himedia, Mumbai, India) medium which helps to differentiate pathogenic *Listeria* spp. From nonpathogenic ones. (Ottaviani et al. 1997). Out of the 81 isolates of *Listeria*, 38 isolates exhibited halo formation on ALOA (Fig. 15.5). The enzymatic activity expressed by *L. monocytogenes* isolates on ALOA agar was reckoned as high (with 8–9 mm zones), moderate (with 5–6 mm zones) and low (with > 4 mm zone) in case of 11, 18 and 8 isolates, respectively. The only isolate of *L. ivanovii* showed a low enzymatic activity.

Among the virulence genes of *Listeria*, hemolysin gene (*hly*A) of *L. monocytogenes* has been used most commonly for confirmation of the isolates (Aznar and Alarcón 2002). All the isolates were subjected to PCR assay for amplification of the *hly*A gene as per the method described by Paziak-Domańska et al. (1999) and Notermans et al. (1991b). Out of 81 *Listeria* isolates, the *hly*A gene was detected in 37 isolates while all the other *Listeria spp*. were negative in PCR analysis.

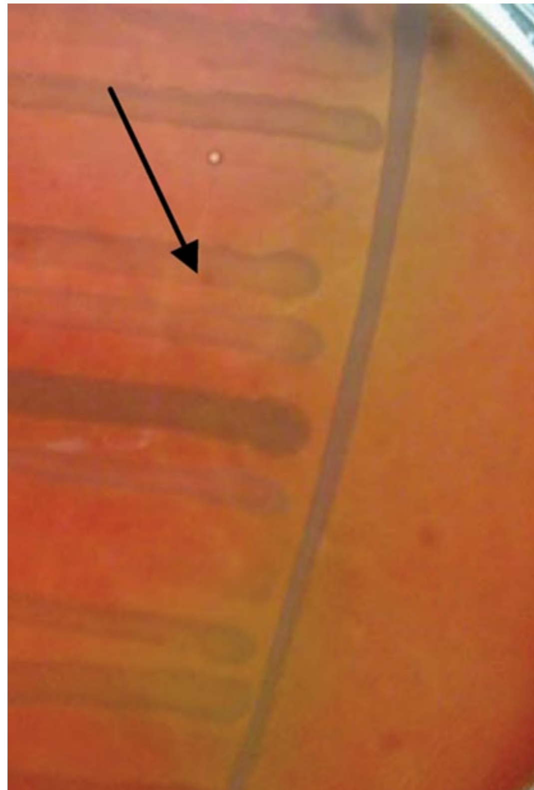

Fig. 15.4 *L. monocytogenes* showing positive CAMP test

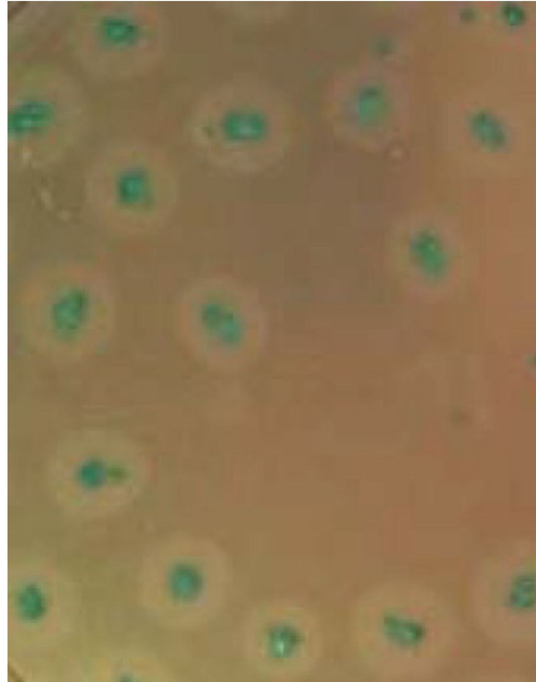

Fig. 15.5 *L. monocytogenes* positive PI–PLC activity on ALOA agar

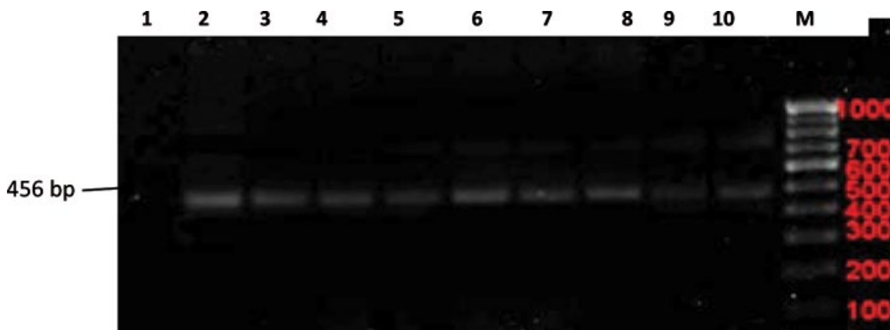

Fig. 15.6 Amplification of the *hly*A gene in *L. monocyotgenes* isolates. *Lane 1*: Negative control; *Lanes 2–9: Listeria monocytogenes* isolates; *Lane 10: Listeria monocytogenes* MTCC 1143

Amplification of the *hlyA* gene of *L. monocytogenes* to its respective 456 bp product represented by a single band in the corresponding region of the DNA marker ladder is as presented in Fig. 15.6.

15.6.2 Serotyping by Multiplex PCR

Typing of *L. monocytogenes* is important in epidemiological studies for investigation of food-borne outbreaks and in the food-processing environment, to identify

the sources of contamination and routes of dissemination. Serotyping by multiplex PCR (mPCR) has been developed which separates the four major *L. monocytogenes* serovars into distinct groups (Doumith et al. 2004, 2005). This assay was employed in the present investigation to serotype *L. monocytogenes* isolates recovered from milk. The genomic DNA of all the isolates was extracted using bacterial DNA extraction kit (Chromous Biotech, Bangalore, India) and were subjected to mPCR-based serotyping. The five primer sets for target fragments from genes *lmo0737, lmo1118,* ORF2819, ORF2110 and *prs* were synthesized by Sigma Aldrich, USA (Table 15.1).

Out of 37 *L. monocytogenes* isolates, a larger proportion of isolates (26) belonged to the group corresponding to serovars 1/2a, 1/2c, 3a, and 3c. Serogroup corresponding to serovars 4b, 4d and 4e was detected in two strains while serogroup 1/2b, 3b, 4b, 4d, and 4e was detected in nine strains (D'Costa et al. 2012). The profiles of multiplex PCR serotyping of standard *Listeria* strains and *L. monocytogenes* isolates recovered from milk are as depicted in Fig. 15.7. Our data showed that most of the isolates belonged to 1/2a, which was considered as a sporadic cause for human listeriosis (Liu 2006). Studies have also found that serotype 1⁄2a was the predominant serotype of *L. monocytogenes* food and environmental isolates (Corcoran et al. 2006; Gilbreth et al. 2005; Lukinmaa et al. 2003). Our earlier studies indicated predominance of *L. monocytogenes* serotype 4b in human clinical isolates (Kalekar et al. 2011) and 1/2a in isolates from milk-processing environments (Doijad et al.

Table 15.1 Primer sequences for *L. monocytogenes* used in multiplex PCR serotyping

Target gene	2. Primer sequence	3. Product size (bp)
lmo0737	2. Forward 5′-AGGGCTTCAAGGACTTACCC-3′	3. 691
	Reverse 5′-ACGATTTCTGCTTGCCATTC-3′	
lmo1118	Forward 5′-AGGGGTCTTAAATCCTGGAA-3′	906
	Reverse 5′CGGCTTGTTCGGCATACTTA-3′	
ORF2819	Forward 5′-AGCAAAATGCCAAAACTCGT-3′	471
	Reverse 5′-CATCACTAAAGCCTCCCATTG-3′	
ORF2110	Forward 5′-AGTGGACAATTGATTG-GTGAA-3′	597
	Reverse 5-CATCCATCCCTTACTTTGGAC-3′	
prs	Forward 5′-GCTGAAGAGATTGC-GAAAGAAG-3′	370
	2. Reverse 5′-CAAAGAAACCTTG-GATTTGCGG-3′	

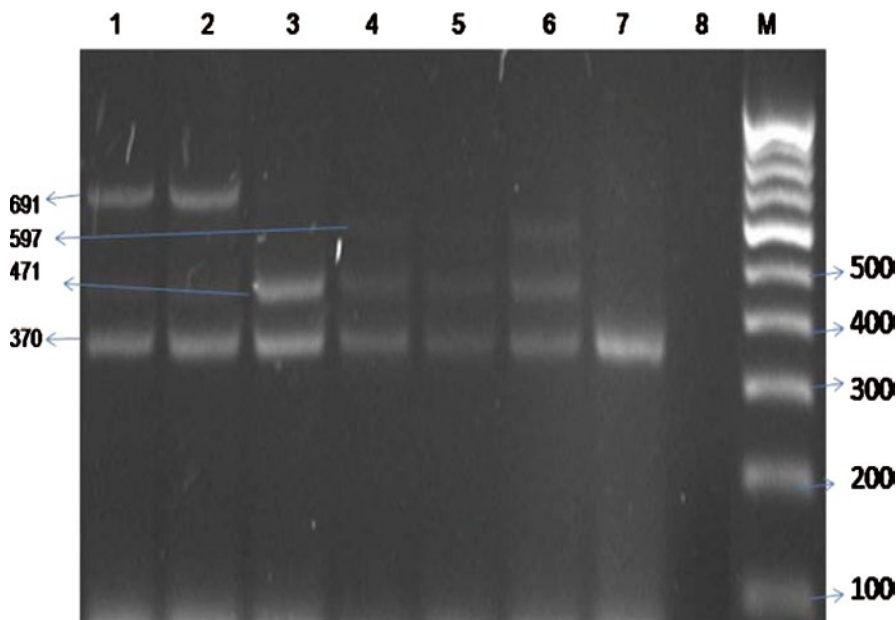

Fig. 15.7 Serotype profile of *Listeria* species by multiplex PCR serotyping. *Lanes 1–5 L. monocytogenes* isolates, *Lane 6 L. monocytogenes,* 4b (NCTC 11994), *Lane 7 L. innocua, Lane 8* Negative control, M – DNA ladder

2011). The observation indicates the potential of milk and milk products to serve as vehicles of transmission of virulent *L. monocytogenes*.

15.6.3 Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is considered the gold standard method for subtyping food-borne pathogens, because of its high discriminatory power and reproducibility and is currently used by several public and private laboratories for subtyping and serogrouping *L. monocytogenes*. A total of 36 *Listeria* isolates were subjected to PFGE analysis to cover different sampling areas and different species. PFGE was performed according to the CDC PulseNet standardized procedure (Graves and Swaminathan 2001) used for typing *L. monocytogenes* by using the CHEF-DRII apparatus (Bio-Rad Laboratories, Hercules, USA).

PFGE discriminated the *L. monocytogenes* isolates into 5 *Apa*I and 4 *Asc*I PFGE patterns (pulsotypes) at 80 % similarity, but could differentiate serovars within multiplex PCR (MPCR) serogroups, in which isolates from different serovars displaying the same pulsotype were found.

Dendrogram analysis showed that PFGE yielded a good binary division into genetic lineages I (serotypes 1/2b, 3b, 4b, and 4e) and II (serotypes 1/2a, 1/2c, 3a and 3c) a result that is consistent with previous studies (Gilbreth et al. 2005; Nadon et al.

2001) and further confirm that these two lineages represent distinct subgroups. Our data also showed that there was a nearly complete correlation between pulsotypes and serotypes with identical PFGE patterns belonging to the same serotype.

15.7 Conclusions and Future Prospects

Based on the results, it can be concluded that enzymatic assays like CAMP and ALOA tests simultaneously with PCR targeting *hlyA* gene, serotyping by multiplex PCR and PFGE dendrograms can be used for confirming and comparing the pathogenic strains of *L. monocytogenes.*

Farm animals can be asymptomatic or suffer from encephalitis, septicaemia and abortions and thus may be a source of *L. monocytogenes* in the farm environment. Managing the problem of milk-borne listeriosis requires an understanding of the burden of the disease on a worldwide scale as milk that is prone to contamination is consumed widely domestically and traded globally. Surveillance of the disease, caused by *L. monocytogenes*, is typically restricted to developed countries, but many of these do not consider listeriosis as a notifiable disease and estimate the numbers by other means.

Studies on microbiological surveillance of milk and milk products at farm level may help in management and stamping of herds as clean**.** Human illness attribution has been recently recognized as an important tool to better inform food safety decisions. All dairy farmers, suppliers to dairy farmers, milk carriers, dairy product and food manufacturers, distributors and retailers should be part of an integrated food safety and quality assurance management system. Good farming practices underpin the marketing of safe, quality-assured milk-based products. Good dairy-farming practices should contribute to ensuring that milk and milk products are safe and suitable for their intended use.

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