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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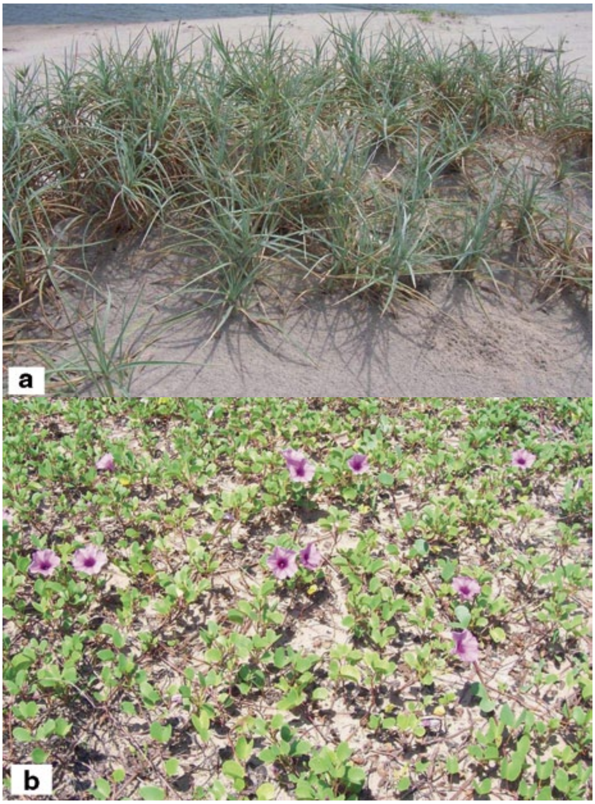


Fig. 1.1 **a** *Spinifex littoreus* plant growing on the sand dunes on the Aswem Mandrem beach in North Goa. **b** *Ipomoea pes-caprae plant* growing on the sand dunes on the Miramar beach in North Goa

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

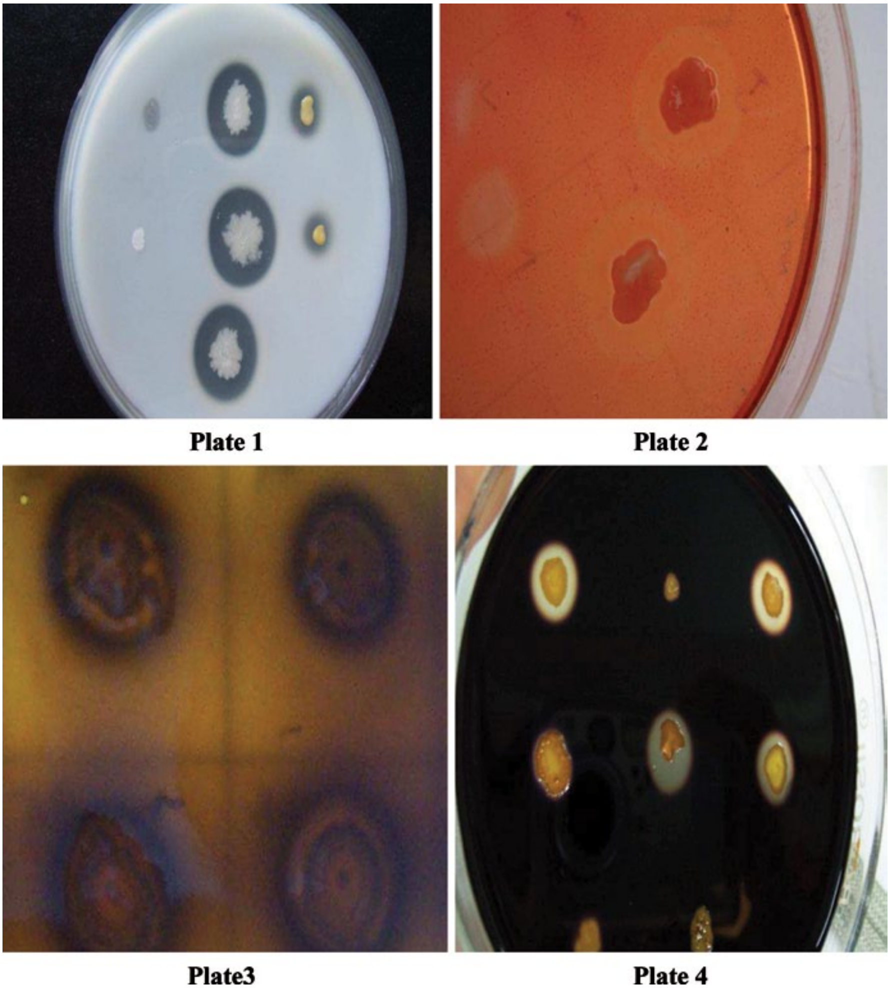


Fig. 1.2 **a** Neutrophilic bacterial isolates showing production of enzymes protease (*plate 1*), cellulase (*plate 2*), tannase (*plate 3*), and amylase (*plate 4*). **b** Alkalophilic bacterial isolates showing production of enzymes protease (*plate 1*), amylase (*plate 2*), and cellulase (*plate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

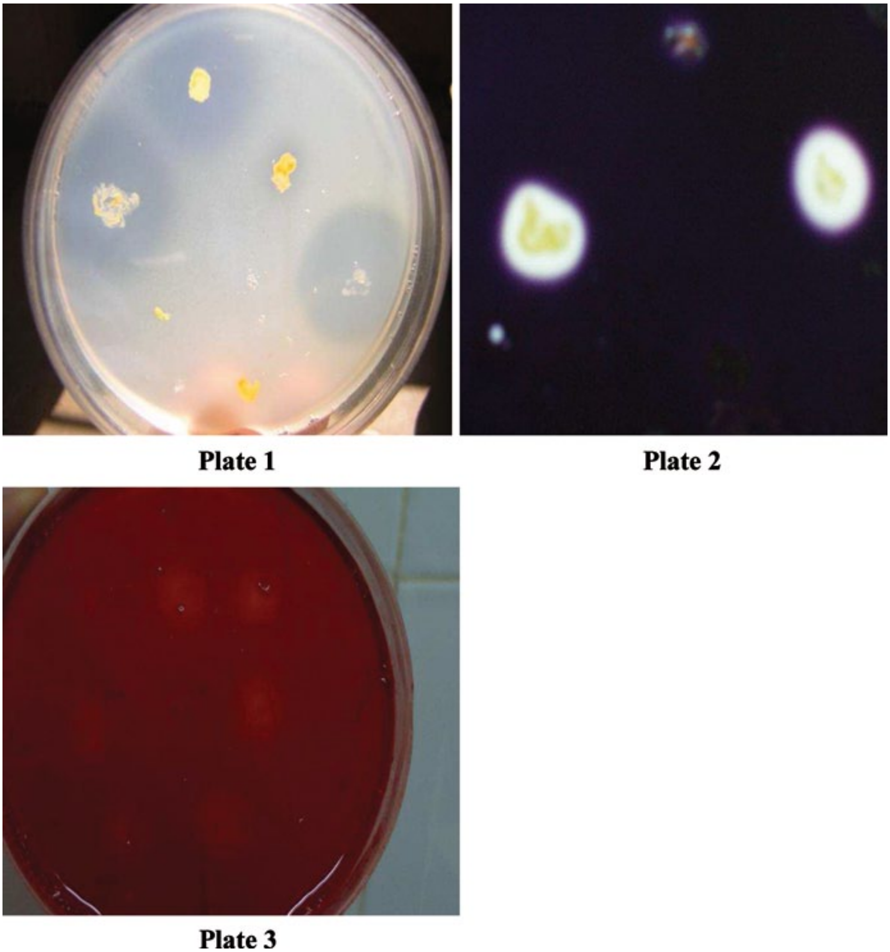


Fig. 1.2 (continued)

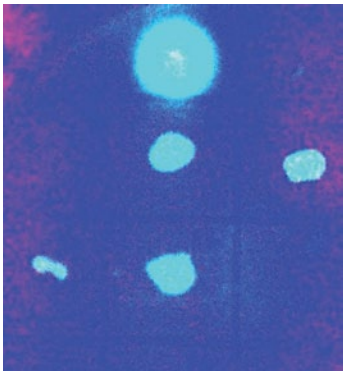
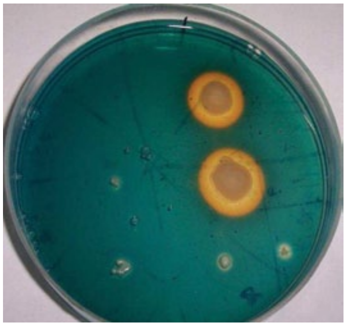


Fig. 1.3 Fluorescence exhibited by neutrophilic bacterial isolates grown on E2 medium plate on staining with *Nile blue* A

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

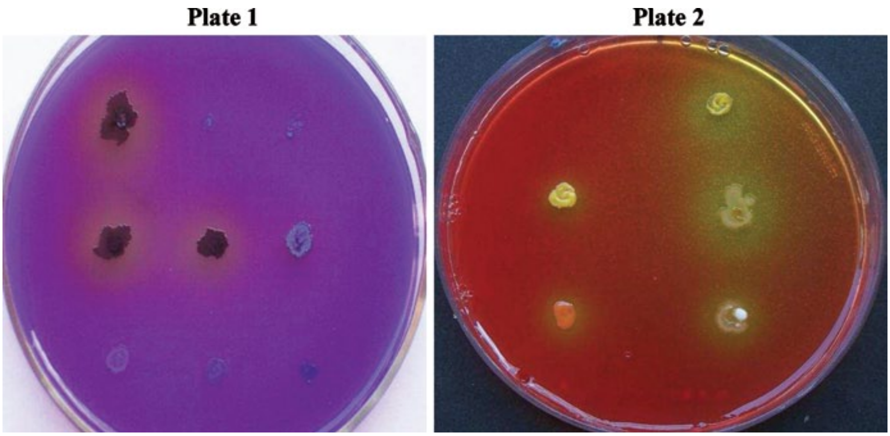


Fig. 1.5 P solubilizing bacteria producing yellow halo/clear zone on PVK's agar due to production of organic acids (plate 1: neutrophilic isolates, plate 2: alkalophilic isolates). (Color figure online)

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

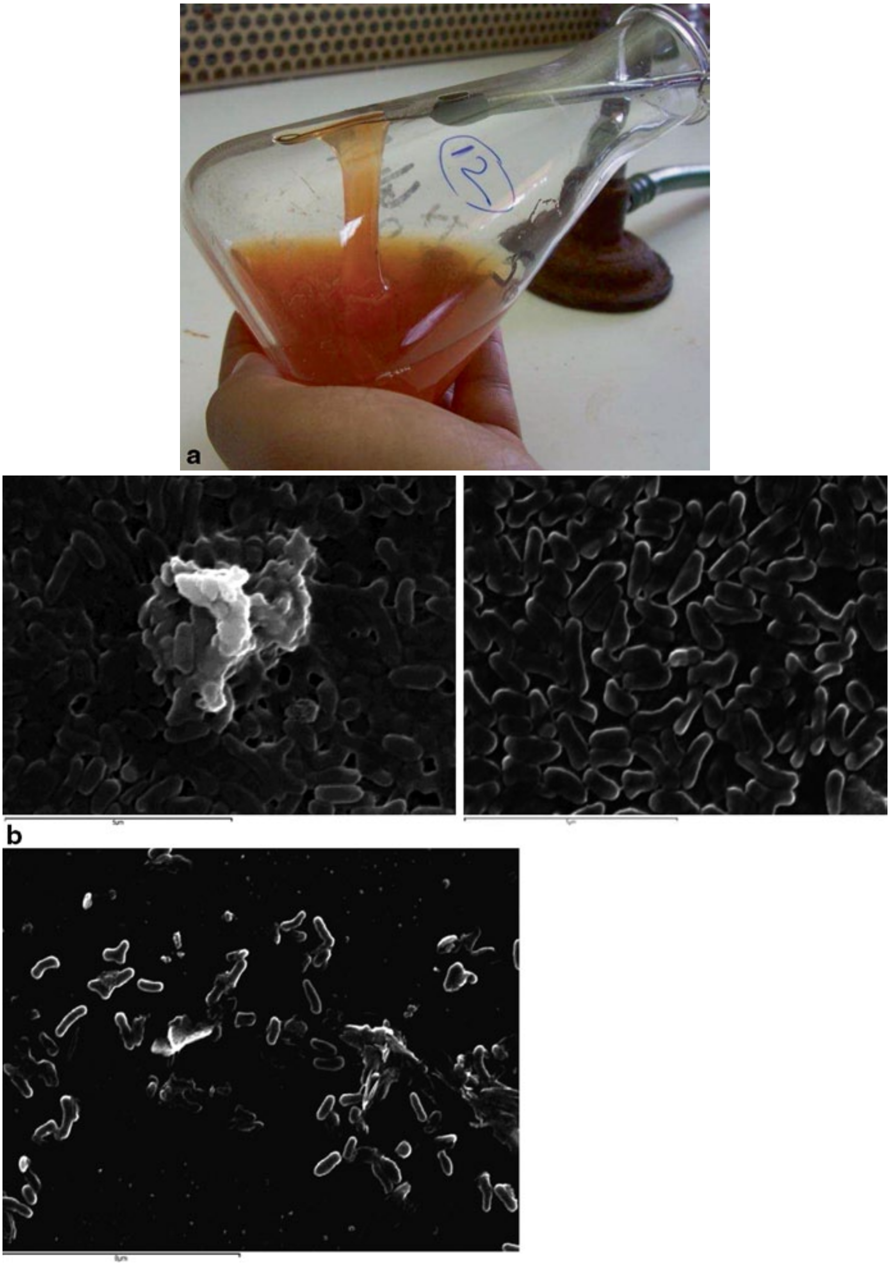


Fig. 1.6 **a** Viscous exopolysaccharide produced by *Microbacterium arborescens.* **b** Scanning electron micrographs of exopolysaccharide formation by *Microbacterium arborescens* (**a**, bar, 5 µm). Most of the surface has been colonized with actively dividing rod cells, and finger-like projections 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

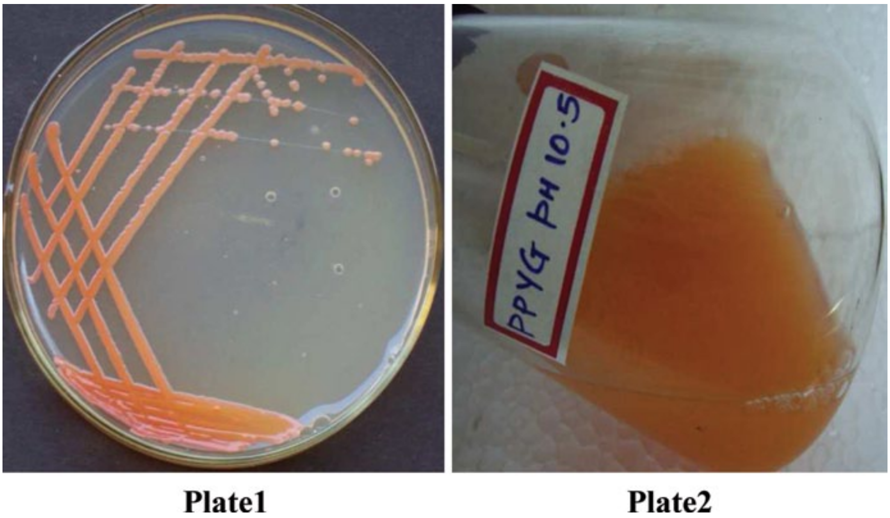


Fig. 1.7 Orange pigment produced by *Microbacterium arborescens* on PPYG agar (*plate 1*) and in broth (*plate 2*). (Color figure online)

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

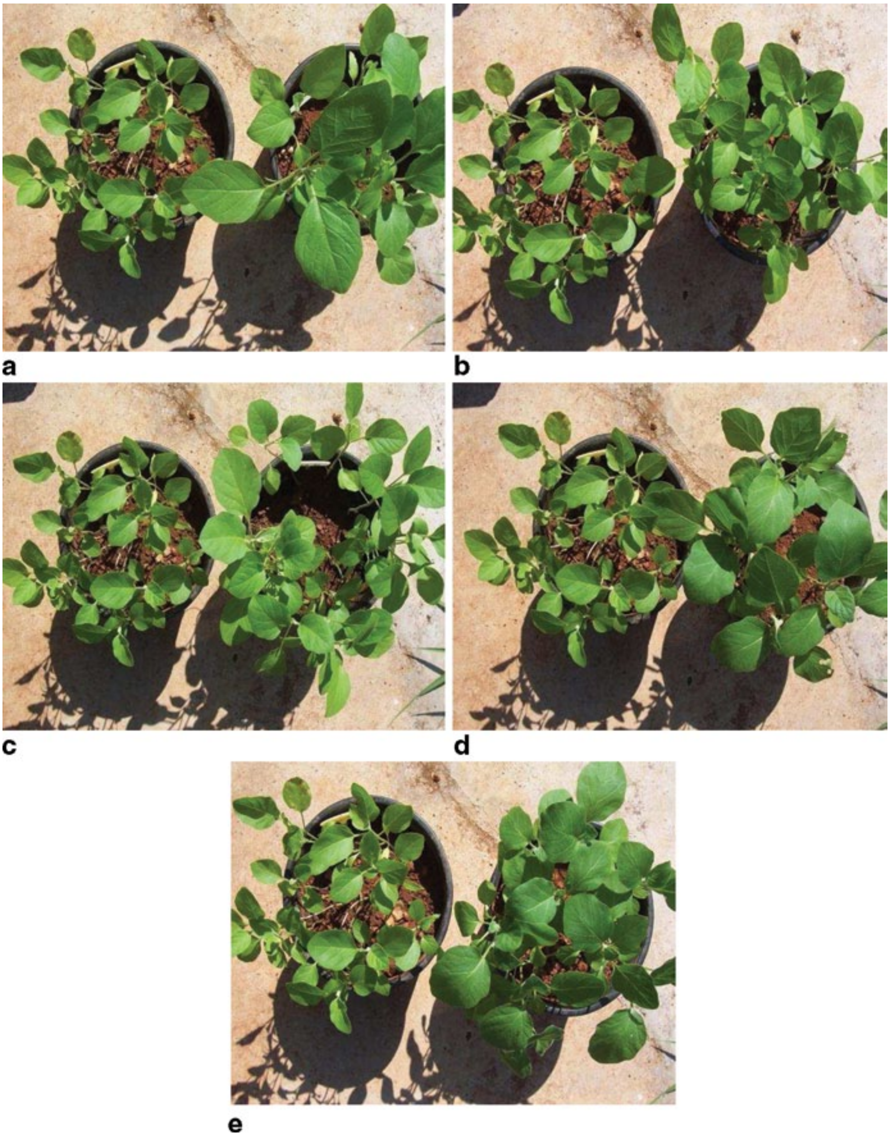


Fig. 1.8 Growth-promoting effects of sand dune bacteria in normal soil (pH 7) on *Solanum melongena* (eggplant) seedling (**a** control + *M. arborescens*, **b** control + *consortium*, **c** control + *K. rosea*, **d** control + *B. subtilis*, **e** control + *Bacillus* sp. MF-A4)

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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