BIOPROSPECTS OF RHIZOSPHERE BACTERIA ASSOCIATED WITH COASTAL SAND DUNE VEGETATION, *IPOMOEA PES-CAPRAE* AND *SPINIFEX LITTOREUS*

Thesis submitted to the

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DOCTOR OF PHILOSOPHY

In

MICROBIOLOGY

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by

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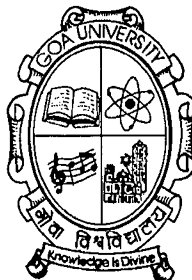
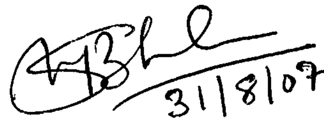


AUGUST 2007

Certificate

This is to certify that **Ms. Aureen Remedios Lemos Godinho** has worked on the thesis entitled **"Bioprospects of rhizosphere bacteria associated with coastal sand dune vegetation, *Ipomoea pes-caprae* and *Spinifex littoreus*"** under my supervision and guidance. This thesis, being submitted to the Goa University, Taleigao Plateau, Goa, for the award of the degree of Doctor of Philosophy in Microbiology, is an original record of the work carried out by the candidate herself and has not been submitted for the award of any other degree or diploma of this or any other University in India or abroad.



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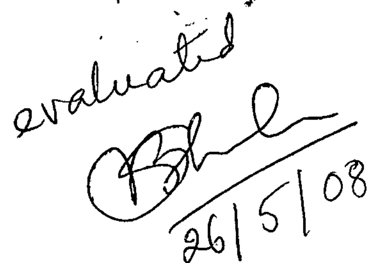

Prof. K. S. Jagadeesh

Declaration

I hereby state that this thesis for the PhD degree in Microbiology on **"Bioprospects of rhizosphere bacteria associated with coastal sand dune vegetation, *Ipomoea pes-caprae* and *Spinifex littoreus*"** is my original contribution and that the thesis and any part of it have not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

ALgodinho 31/8/07

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Aureen Godinho
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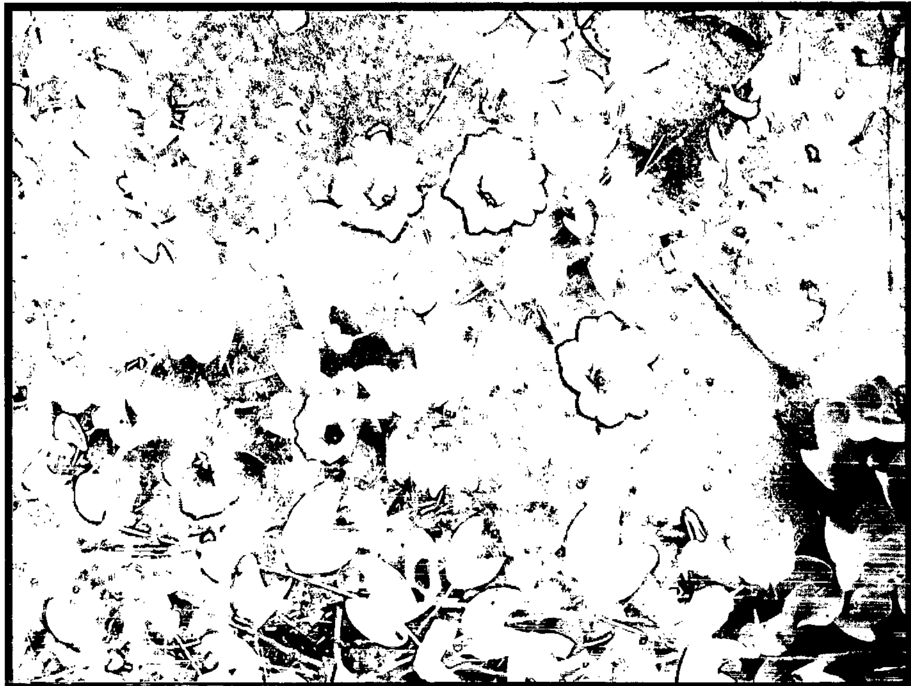
Dedicated

To my parents

With love and gratitude



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LIST OF ABBREVIATIONS

Da	Daltons	EPS	Exopolysaccharide
ACC	1-aminocyclopropane-1-carboxylic acid	bp	Base pairs
EDTA	Ethylenediaminotetraacetic acid	^{0}C	Degree Celsius
HPLC	High-performance liquid chromatography	Fig	Figure
IAA	Indole-3-acetic acid	g	Gram
IAM	Indole-3-acetamide	h	Hour (s)
SAM	S-adenosylmethionine	ul	Microliter
TRP	Trytophan	uM	Micromolar
PGPR	Plant growth-promoting rhizobacteria	mg	Milligram
CFU	Colony forming units	ml	Milliliter
DNA	Deoxyribonucleic acid	mm	Millimeter
UV	Ultraviolet	mins	Minute (s)
w/v	Weight per volume	ns	Nonsignificant
ANOVA	Analysis of variance	rpm	Revolution per minute
%	Percent	spp.	Species (plural)
-	Negative	sp.	Species (single)
CAS	Chrome S azurol	PCR	Polymerase chain reaction
HCN	Hydrogen cyanide	N	Nitrogen
PHA	Polyhydroxyalkanoates	GC	Gas chromatography
PPYG	Polypeptone yeast extract glucose agar	OD	Optical density
MW	Molecular weight	PVK	Pikovskaya medium
ACCD	ACC deaminase	Fe	Iron
TLC	Thin layer chromatography	FMM	Fiss glucose minimal medium
DHBA	2,3 - dihydroxy benzoic acid	SDS	Sodium dodecyl sulphate
DF	Dworkin and Forster minimal medium	PAGE	Polyacrylamide gel electrophoresis
NCBI	National center of biotechnology and information	RNA	Ribonucleic acid
PNPP	p- nitrophenyl phosphate	PNP	p- nitrophenol
KDa	Kilodalton	Rf	Resolution factor
λ	Wavelength	PSM	Phosphate solubilizing microorganism

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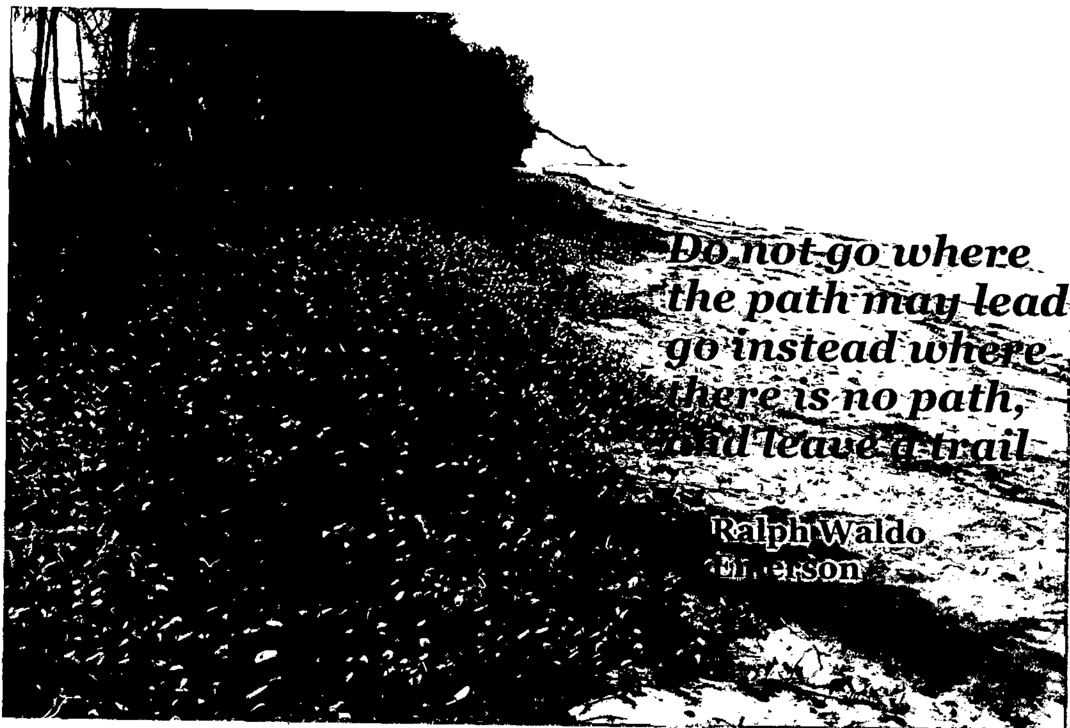

Do not go where
the path may lead -
go instead where
there is no path,
and leave a trail
Ralph Waldo
Emerson

Introduction

➢ Coastal Sand Dune Ecosystem

The word sand dune reflects the images of vast amount of shifting sand barren to plants and hostile to human habitation. Sand dunes are generally of two types. The first type is the extremely dry interior deserts such as Sahara in Africa or Rajasthan in India and the other type is known as the coastal Sand dunes which occur along the coasts of the Atlantic, Pacific, North America and Australia. In Asia the coastal dunes occur in Japan, India and several other countries (Desai & Untawale, 2002; Boorman,1977; Carter,1998).

➢ Sand Dune vegetation

Vegetation plays a dominant role in determining the size, shape and stability of fore dunes (Fig1.). 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 deposit 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 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 & Untawale, 2002).

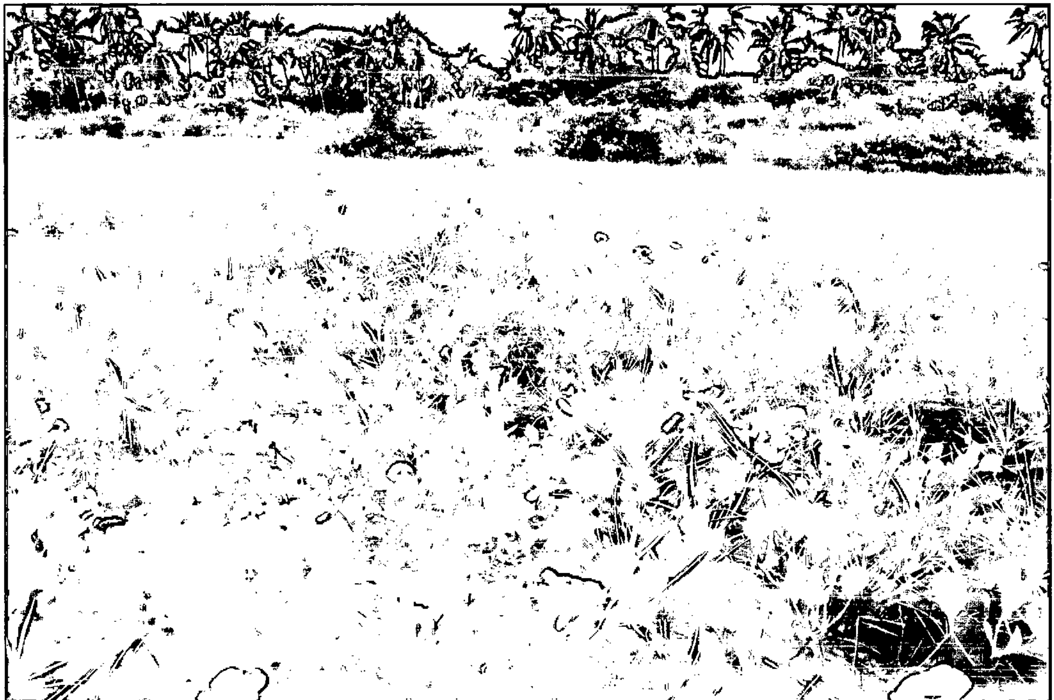


Fig. 1 Beach grasses and shrubs growing on sandy dunes

- **Classification of sand dune vegetation**

The oldest classification given by Turner, Carr and Bird (1962) describes 5 well defined zones of vegetation (Fig. 2).

Zone I:- The Embryonic dune which is the zone nearest to the sea and is unvegetated.

Zone II:- The Fore dune is the one which runs parallel to the first beach ridge and has sand binding grasses like *Spinifex littoreus* and creepers like *Ipomoea pes-caprae* growing on it.

Zone III:- Dune scrub is the one close to the fore dune and is higher than the fore dunes and forms the main part of the dune.

Zone IV:- Shrub woodland is a long narrow sandy ridge running parallel and separated by mud flats with fringing slat marshes which are indundated at high tide.

Zone V:- Dune woodland is made up of the stable sand dunes with vegetation community similar to that found in the neighboring coastal region of the main land (Desai & Untawale, 2002).

Among all the dune plants, dune grass that is *Spinifex littoreus* and creeping herb that is *Ipomoea pes-caprae* are dominant as they have been very well adapted to these extreme stress conditions. *Ipomoea pes-caprae* always occupies the fore shore region and has long, creeping branches (Fig. 3a). Next to it, thick patch of *Spinifex littoreus* grows, which has long rhizomes which spread horizontally on the sand and long roots which go obliquely below the sand and may extend upto 62cms also (Fig. 3b). The leaves are rigid with a acute spine at tip which shows the deposition of sand and salt. Due to this specific feature, they are not grazed upon. During rainy season,

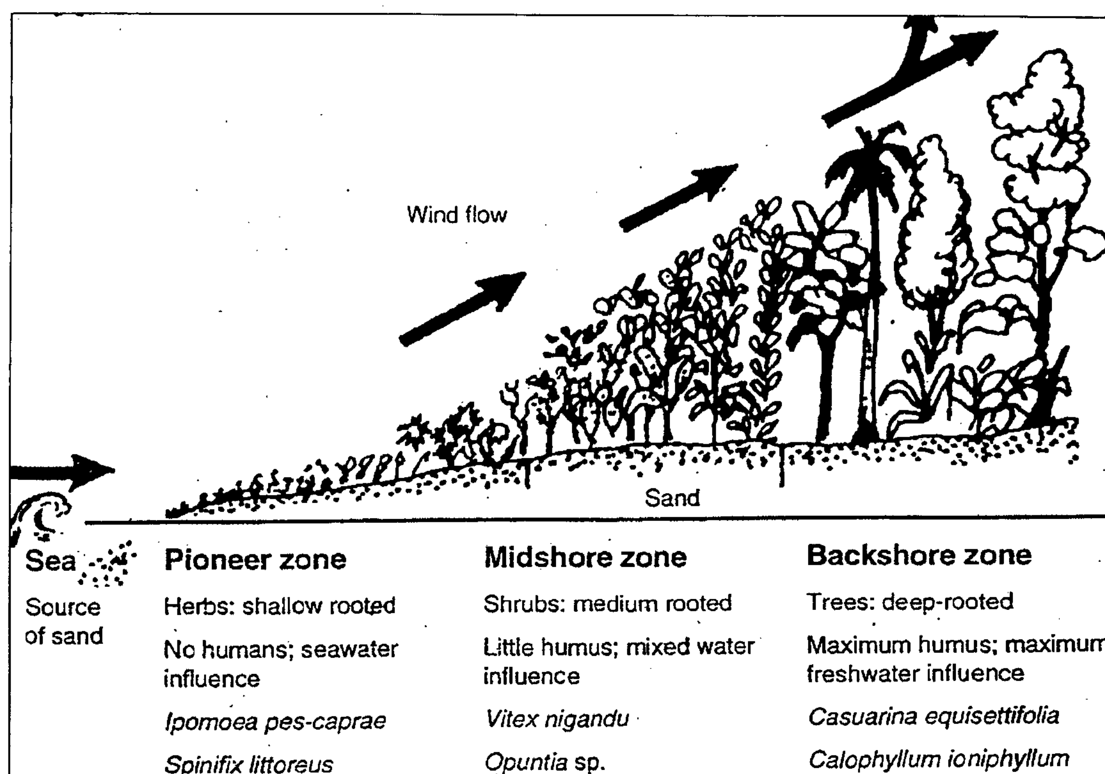


Fig. 2 Sand dune vegetation zones (Untawale, 1994)

Fig. 3a ***Spinifex littoreus*** **plants growing on the sand dunes on the Aswem Mandrem beach in North Goa**

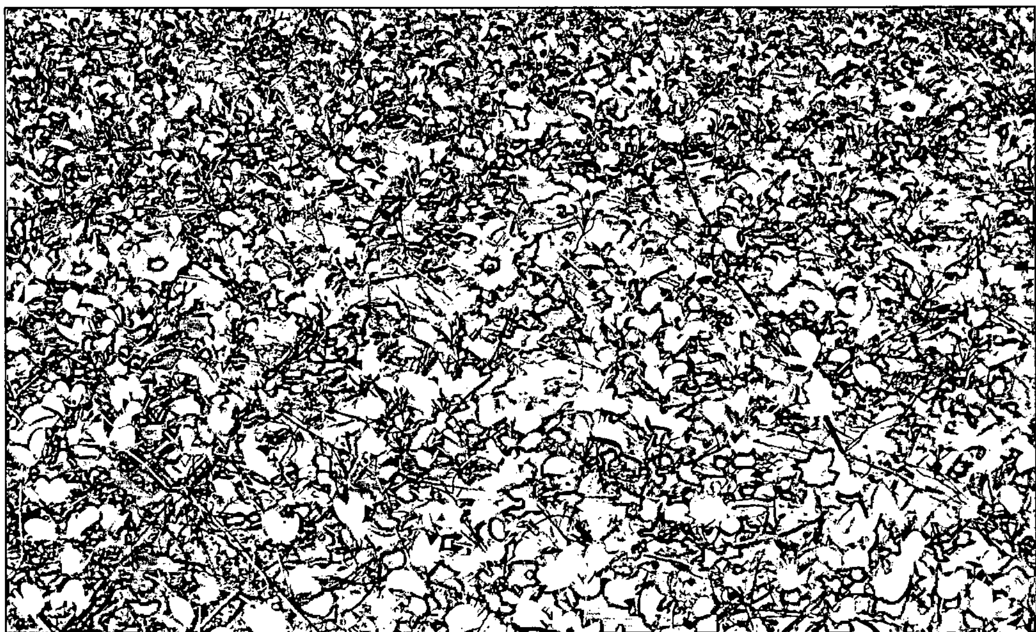


Fig. 3b ***Ipomoea pes-caprae plants*** **growing on the sand dunes on the Miramar beach in North Goa**

lot of waste gathers in the region of their growth, but with all its special adaptations of this plant it can grow on dunes. The outer surface of the leaf is covered with thick cuticle which helps to protect the underneath tissues from excessive heat and also to store water to prevent transpiration (Desai & Untawale, 2002).

- **An introduction to the species: *Ipomoea pes- caprae* and *Spinifex littoreus***

Ipomoea pes-caprae, commonly referred to as railroad vine, is a trailing vine that routinely colonizes sand dunes. It grows just above the high tide line along coastal beaches, forming large mats that assist in stabilizing sands (Devall 1992). *Ipomoea pes-caprae* of family Convolvulaceae is an indigenous sand dune pioneer from tropical areas which has been introduced for sand stabilisation on coastal dunes. Vegetatively, *Ipomoea pes caprae* is an extensively climbing or trailing perennial herb with thick long root. The leaves are fleshy and bilobed. The flowers are purplish pink, large and funnel shaped. It is one of the most common and most widely distributed salt tolerant plants and provides one of the best known examples of oceanic dispersal. Its seeds float and are unaffected by salt water. This species can be found on the sandy shores of the tropical Atlantic, Pacific and Indian Oceans. It grows on almost all parts of the dune but is usually found on the seaward slopes sending long runners down towards the toe of the dune. This plant grows in association with sand *Spinifex* grass and is a useful sand binder thriving under conditions of sand blast and salt spray. The genus *Ipomoea pes-caprae* in the family Convolvulaceae, is centered on the entire coast of Goa (Desai &Untawale, 2002). *Spinifex littoreus*, in the family Poaceae is a pale dioecious grass, forming impenetrable bushes. The stems and the leaves are long. This hardy grass is useless as fodders, but is an excellent sand

binder. This grass is extensively cultivated on the coast to control the movement of sand. It is distributed all over the world, Asia-temperate: China and eastern Asia. Asia-tropical: India, Indo-China, Malaysia, and north Indian Ocean. Australasia: Australia. Pacific: southwestern (Desai & Untawale, 2002; Desai, 1995; Packham,1977).

Besides the adaptations to environmental conditions, the nutrient stress is reduced due to the presence of microorganisms in the rhizosphere. Our preliminary studies (Godinho and Bhosle, 2002) have shown the presence of a large number of bacteria including diazotrophs in the close proximity of roots and in the rhizosphere.

- **Plant growth promoting rhizobacteria (PGPR)**

The plant growth promoting rhizobacteria (PGPR) play a significant role in supporting growth of plants. These bacteria possess traits which help in either improving the availability of the nutrients or inhibiting the pathogenic bacteria, The availability of nutrients is facilitated by production of siderophores, exopolysaccharides (EPS) and polyhydroxyalkanoates (PHA). Further the enzymes involved in degradation of macromolecules (amylase, cellulase, lipase, protease etc.) release low molecular weights in the rhizosphere which are available for plants. Microorganisms living within plant tissues for all or part of their life cycle without causing any visible symptoms of their presence are defined as endophytes. They inhabit majority of healthy and symptomless plants, in various tissues, seeds, roots, stems and leaves (Johri, 2006). Plants benefit extensively by harbouring these endophytic microbes; they promote plant growth (Compant *et al,* 2005) and confer

enhanced resistance to various pathogens by producing antibiotics. Endophytes also produce unusual secondary metabolites of plant importance It has been suggested that the presence of a mutualistic endophyte acts as a "biological trigger" to activate the stress response system more rapidly and strongly than nonmutualistic plants (Bandara *et al*, 2006).

The plant growth promoting traits include the production of chelating compounds such as, siderophores, a iron chelating compound produced at low iron concentration by several rhizobacteria ; phosphate solubilization, a characteristic shown by bacteria in releasing the inorganic phosphates ; production of IAA helps in increasing root growth ; HCN decreases the growth of phytopathogens and other deleterious microorganisms ; ACC deaminase production has been a focus of agricultural microbiologists, as it is important for reduction of ethylene concentration in the root thus increasing root elongation.

Besides the biocontrol of pathogens by rhizobacteria have shown effective results in control of phytopathogens. Such bacteria have also evolved numerous mechanisms of resistance to stress conditions and nutrient limitations. For example, many microorganisms have an inherent ability to form resting stages (e.g., cysts and spores). Even without the formation of such elaborately differentiated cells, bacteria enter starvation-induced programs that allow them to survive long periods of non growth 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 non differentiating bacteria are *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio* sp. strain S14, which show qualitative similarities in their survival responses.

Nutrients may become available locally, for example, in decaying plant and animal material or via plant roots, which are one of the major sites of carbon input into soil. The rhizosphere therefore is a soil region with a transiently high availability of carbon in a form readily available to soil bacteria. Soil bacteria that have evolved in close association with plants, such as rhizobia and pseudomonads, benefit from being able to quickly escape the starvation state and colonize the plant root. The accumulation of intracellular storage polymers is another bacterial strategy that increases survival in a changing environment. Poly(3-hydroxyalkanoates) (PHAs) are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as a sink for carbon and reducing equivalents. The bacterial origin of PHAs make these polyesters a natural material and microorganisms have evolved ability to degrade these macromolecules (Madison & Huisman, 1999).

In bacteria, PHA constitute a major carbon and energy storage material, which is 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). PHAs produced by these bacteria are important due their biodegradability, water resistance and oxygen permeability. Their applications are varied, they are used for all sorts of biodegradable packaging materials (Thakor *et al*, 2006).The levels of biological organization in the coastal ecosystem are depicted in Fig 4.

Production of exopolymeric substances especially EPS by bacteria as one of the mechanisms to overcome dessication. The rate of drying within the colony microenvironment is slower with EPS and helps could increase bacterial survival by increasing the time available for metabolic adjustment. Further an EPS matrix provides another advantage to bacteria living within it as decreasing water content of soil restricts diffusion of nutrients to microorganisms. Polysaccharides being hygroscopic, maintain higher water content in the colony micro environment than in the bulk soil as water potential declines. This increase in water content could increase nutrient availability within the bacterial colony. Roberson and Firestone (1992) revealed that bacteria respond to desiccation by channeling energy and nutrients into polysaccharide production. Soil is an extremely heterogeneous environment, and wetting and drying may not proceed uniformly throughout it and any microbial processes in soil depend on this heterogeneity.

It is envisaged that the rhizobacteria from the rhizosphere and the sand dune plants along with endophytic bacteria could play an important role in helping the survival of these vegetation (*Ipomoea pes-caprae* and *Spinifex littoreus*) in the sand dunes.

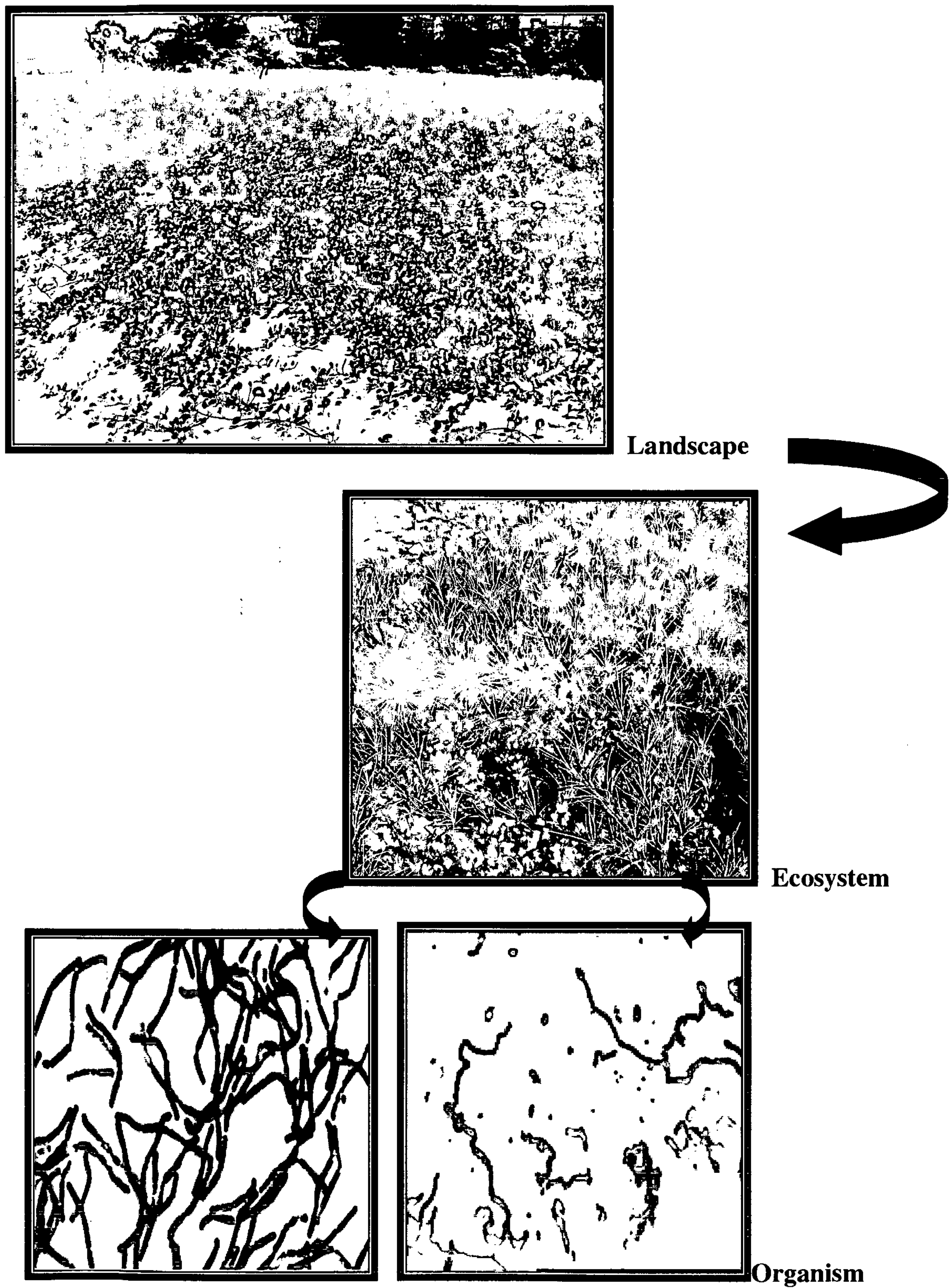


Fig. 4 Levels of biological organization range from organism to biosphere in the coastal sand dune ecosystem.

➢ The present study was therefore undertaken with the following objective -

1. Viable counts of bacteria associated with *Ipomoea pes-caprae* and *Spinifex littoreus* sand dune vegetation during premonsoon, monsoon and postmonsoon seasons under normal and extreme conditions such as temperature, pH, salinity and nitrogen free conditions
2. Screening of predominant bacteria with relation to degradation of cellulose, starch, xylan, tannins, hydrocarbons, and phosphate solubilizers under alkaline and neutral conditions
3. To study the bacteria for production of exopolysacharides, polyhydroxyalkanoates and siderophores under alkaline and neutral conditions
4. Screening, selection and identification of selected culture producing significant amount of EPS and Optimization of conditions for maximum EPS production
5. Role of selected EPS producing bacteria in aggregation of sand particles, mine reject soils and effect of isolates on growth of agriculturally important crop, eggplant.

Chapter I
Literature Survey

1.1 Biodiversity of bacteria in the coastal ecosystem

The different types of microorganisms belonging to various genera and species and their number in a selected ecosystem constitutes its biodiversity. This biodiversity of an ecosystem is influenced by the various environmental factors. A nutrient rich detrital type of ecosystems such as mangroves has diverse bacterial populations as compared to an ecologically low nutrient econiche. Mangrove ecosystem in the coastal region is an unique inter-tidal ecosystem of the tropics. This ecosystem is ideally situated at the inter-phase between the terrestrial and marine environment and supports a rich and genetically diverse group of microorganisms playing an important role in biogeochemical cycles, recycling nutrients and forming detritus which result in the high productivity (Das *et al,* 2006). The studies on mangrove microflora from the mid-west coast of India have shown the production of diverse enzymes especially nitrogen fixers and cellulase producers. The roots associated bacteria and rhizobacteria also support the growth of plants. The microflora of these ecosystems also showed a high potential to accumulate important polymers such as polyhydroxyalkanoates (PHA) (Rawte *et al,* 2002). A large number of organisms were found to be alkalotolerant producing various enzymes (Desai *et al,* 2004). Further these isolates have also shown remarkable solvent tolerant capacity (Sardessai & Bhosle, 2002), suggesting the wide potential of the bacterial community associated with mangrove ecosystem.

In contrast to mangrove ecosystem the sand dune ecosystem is also in the coastal belt and in its environmental condition is unique. Plant communities growing on sand dunes are controlled by the interaction between biotic and physico-chemical components of the sand matrix. The diversity of culturable bacteria associated with

two major 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 was studied by Park *et al*,(2005). Many of their isolates belonged to *Pseudomonas* genera in both the rhizosphere soils and roots of the two plants. Further, the bacterial communities associated with the rhizosphere and roots differed significantly between the two plant species. *Agrobacterium* spp. (5.1%) were isolated from *C. soldanella*, but not from *E. mollis*. In contrast, *Klebsiella* (9.2%) and *Brevibacillus* (5.2%) were isolated only from the latter. *Pseudomonas* strains have been found in rhizosphere and roots of *Calystegia soldanella*, but the bacterial community in the root of *C. soldanella* was not dominated by any one genus or species. *Acinetobacter, Pseudomonas, Paenibacillus, Microbacterium, Agrobacterium* and *Chryseobacterium* were commonly isolated in the root of *C. soldanella*, each comprising 11 ~ 13% of the total isolates (Park *et al*, 2005). Further bacterial diversity in the rhizosphere *C. soldanella* and *E. mollis*, was studied by the analysis of community 16S rRNA gene clones. Regardless of plant species, *Lysobacter* spp., which is a member of the family Xanthomonadaceae, class Gamma proteobacteria was found to be predominant. *Lysobacter* clones comprised 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 (Lee *et al*, 2006).

Studies on the bacterial communities associated with creeping bent grass in soil and sand root zones showed that both soil-based and sand-based roots contain a

relatively high level of bacterial diversity, and bacterial communities in soil based root zones differed significantly from those in sand-based root zones in species composition, and in species diversity. Based on 16S rDNA analysis of the isolated strains it was observed that sand root zone communities were composed largely of *Arthrobacter*, *Microbacterium* and *Bacillus* species, many of these with 100% sequence identity. Furthermore, the soil root zone community was composed almost exclusively of *Bacillus* species with a few *Arthrobacter* species, both root zones were dominated by *Pseudomonas* and *Bacillus* species (Karp & Nelson, 2004). The differences in the microbial communities between plants species have also been observed in many other studies, which are due to differences in the amount and composition of root exudates as well as the root cell components at the root tip and in the mature root zone. Root exudates are widely reported to control rhizosphere populations. The presence of diverse communities in the mangroves has been a subject of study in the Indian scenario however not much work is done on these expects in the sand dune ecosystem except for mycorrhizae (Beena *et al,* 2000 & 2001; Kulkarni *et al,* 1997; Arun *et al,* 1999).The association of the bacteria with plant roots appears to be an essential part for sustainability of the plants in these ecosystems and for homeostasis of the rhizosphere of these plants.

Plant roots release a wide variety of compounds into the surrounding soil, including ethylene, sugars, amino acids, organic acids, vitamins, polysaccharides and enzymes. These materials create unique environments for the microorganisms living in association with plant roots, in the rhizosphere. The rhizosphere was first described by Hiltner (1904) as the volume of soil surrounding plant roots influenced by the living root. Bacteria respond differently to the compounds released by the plant root,

and thus different compositions of root exudates are expected to select different rhizosphere communities. On the other hand, rhizosphere bacteria also influence plant growth as these can promote plant growth via chemical agents such as auxins, gibberellins, glycolipids, and cytokinins.

The plant microbe interaction in the rhizosphere therefore has an important impact on survival of microbes and plants.

1.2 Rhizosphere as a site of Plant – Microbe interactions

The rhizosphere is that portion of the soil under the direct influence of the roots of higher plants. It is considered the most intense ecological habitat in soil in which microorganisms are in direct contact with plant roots. The root system of all higher plants is associated with a distinct, diverse community of metabolically active soil microbiota that carry out biochemical transformations. Rhizosphere microorganisms may have specific associations with plants through which they exert their influence on plant growth. The production of biologically active metabolites, particularly the plant growth regulators by rhizosphere microbiota are considered one of the most important mechanisms of action through which the rhizosphere microbiota affect plant growth directly after being taken up by the plant or indirectly by modifying the rhizosphere environment. The plant rhizosphere is a dynamic environment in which many factors may affect the structure and species composition of the microbial communities that colonize the roots. Microbial communities associated with the rhizosphere also vary depending on the plant species, the soil type and cultural practices such as crop rotation or tillage (Frankenberger and Arshad, 1995, Davison, 1988).

Bacteria can form close associations with roots within the root tissue itself, on the root surface (rhizoplane), and within the soil immediately adjacent to the root (rhizosphere). Inhabitants of these sites rely heavily for their energy supply on organic substances provided by the roots, and their growth is therefore related intimately to the metabolic activity of the plants involved (Gaskins *et al*, 1985) (Fig 1.1). While many bacteria found in soil are bound to the surface of soil particles and are found in soil aggregates, a number of soil bacteria interact specifically with the roots of plants. Infact, the concentration of bacteria(per gram of soil) that is found around the roots of the plants (i.e. in the rhizosphere) is generally much greater than the bacterial density, or concentration, that is found in the zone around the roots and can be used to support bacterial growth and metabolism (Glick, 1995; Alexander,1977). The rhizobacteria respond to plant signals, exchange nutrients with plant cells, suffer damage due to plant defense responses and colonize or even evade root tissues, creating pathologies or symbiosis as compared to the bacteria present in bulk soil. Mucigel provides the immediate environment for rhizobacteria, it consists of plant mucilage, bacterial exopolymers and soil particles. Plant roots sheathed with mucigel have higher relative water content than do bare roots and thus mucigel protects the root and associated microflora from dehydration (Miller *et al,* 1996).

The constituents of root exudates play an important role in selecting and enriching the types of bacteria. Depending on the ability of the bacteria to utilize these as sources of energy, the bacterial community develops in the rhizosphere. Plant root exudate components serve as a source of carbon substrate for microbial growth, in addition they also contain chemical molecules that promote chemotaxis of soil

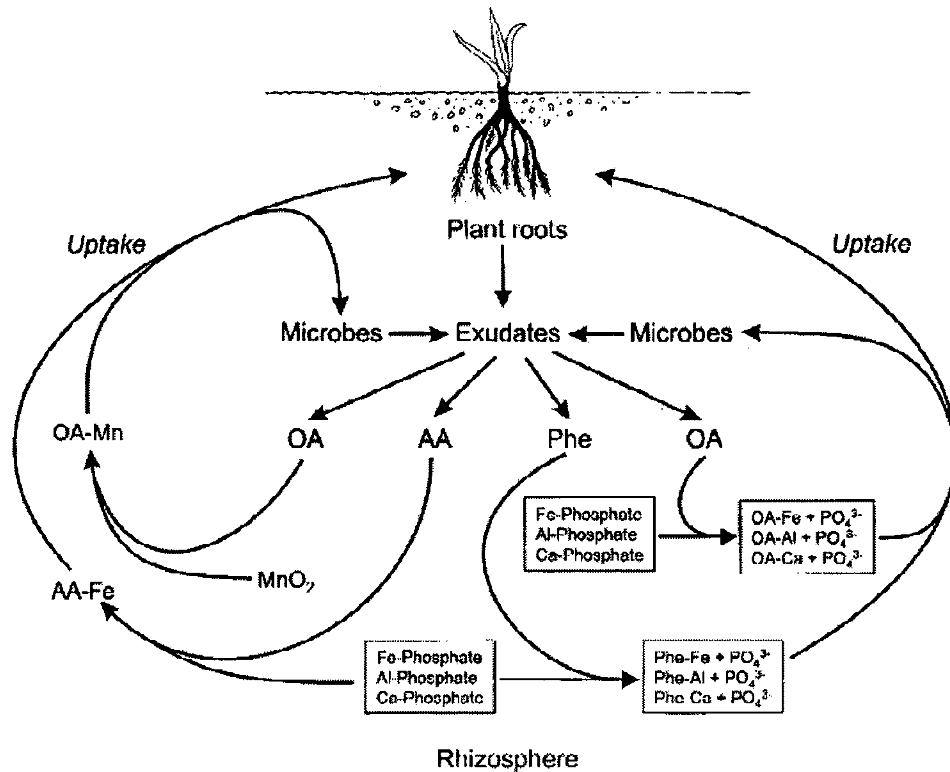


Fig 1.1 Effects of root exudates components on nutrient availability and uptake by plants and rhizosphere microbes OA=organic acids; AA=amino acids including phytosiderophores, Phe = phenolic compounds (Dakora & Phillips, 2002).

microbes to the rhizophere. Root exudates are supplemented in maintaining a steady concentration of flavonoids and mineral nutrients in the rhizosphere by the compounds released from the decomposition of organic matter such as dead roots and fallen leaves (Dakora & Phillips, 2002). 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 while rhizospheric bacterial communities 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. It is also known that some of the PGPR strains can colonize inside plant tissues, and bacterial strains that naturally exist in healthy plant tissues are referred to as "endophytes." Halmann *et al*, (1997) defined endophytic bacteria as "bacteria that can be isolated from surface disinfested plant tissue or extracted from within the plant, and that do not visibly harm the plant." Most of the endophytes reported previously were isolated by maceration of surface-sterile plant tissues. Various endophytes have been isolated from agronomic crops and prairie plants (Halmann, 1997; Weller, 1988), and many of them have been utilized as microbial inoculants to control plant pathogens and promote plant growth. By occupying this privileged endophytic location, bacteria do not have to face competition from their counterparts as encountered in the rhizosphere, or in soil. Such bacteria which influence the plant

growth either directly or indirectly are termed as plant growth promoting bacteria (PGPB).

1.3 Plant growth promoting rhizobacteria (PGPR)

Plant growth promoting rhizobacteria (PGPR) are naturally-occurring, free-living soil bacteria that are capable of colonizing roots and enhancing plant growth when added to seeds or roots (Kloepper and Schroth, 1978; Arshad and Frankenberger, 1995) (Fig 1.2). Beneficial free living soil bacteria are usually referred to as plant growth promoting rhizobacteria or PGPR or by the Chinese as "yield increasing bacteria" or YIB. Several mechanisms have been postulated to explain how PGPR stimulate plant growth, and these can be broadly categorized as either direct or indirect (Kloepper, 1993). There are several ways in which plant growth-promoting bacteria can directly facilitate the proliferation of their host plants. They may: fix atmospheric nitrogen and supply it to plants; solubilize minerals such as phosphorus; produce siderophores, which can solubilize and sequester iron and provide it to plants; synthesize phytohormones, including auxins, cytokinins, and gibberellins, which can enhance various stages of plant growth. Indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a pathogenic organism by any one or more of several different mechanisms including improving growth restricting conditions either via production of antagonistic substances or by inducing resistance against plant pathogens (Tilak *et al,*2005). For example, production of antibiotics can interfere directly with growth and activity of deleterious soil microorganisms (Glick and Bashan, 1997), whereas induction of resistance in the plant increases the plants defense capacity (Van Loon *et al,*1998). In addition,

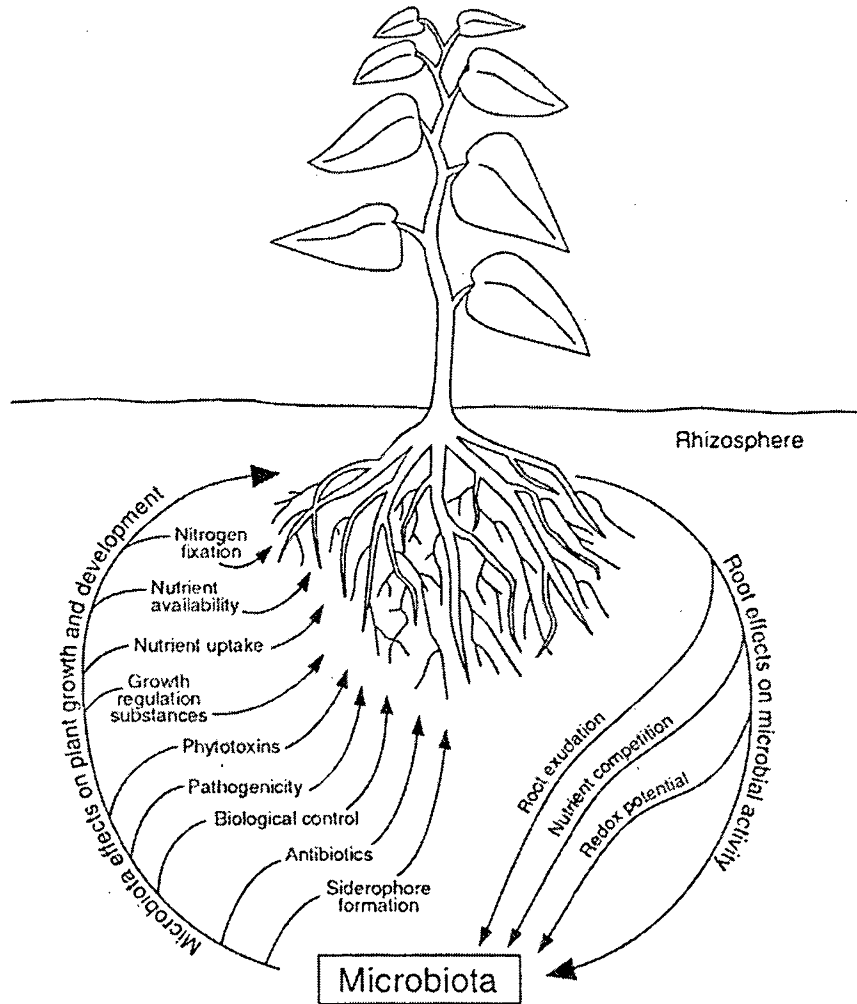


Fig. 1.2 Possible plant-microbe interactions affecting plant growth (Frankenberger & Arshad, 1995)

bacteria may reduce stresses resulting from the presence of toxic wastes by sequestering heavy metals or degrading organic pollutants.

There are 20 different biocontrol PGPR strains commercially available in the market at present. Traits associated with the biocontrol of plant pathogens include antibiotic synthesis, secretion of iron binding siderophores to obtain soluble iron from the soil and provide it to a plant, thereby deprive fungal pathogens in the vicinity of soluble iron, production of low molecular weight metabolites such as hydrogen cyanide with antifungal activity, production of enzymes including chitinase B-1,3-glucanase,protease or lipase which can lyse some fungal cells, out-competing phytopathogens for nutrients and niches on the root surface and enzyme ACC deaminase (Penrose and Glick, 2003). A particular bacterium may promote plant growth and development using any one, or more of these mechanisms. For example, following seed germination a PGPR may lower the plants ethylene concentration thereby decreasing the ethylene inhibition of seedling root length. Once the seedling has depleted the resources that are contained within the seed, the same PGPR may help to provide the plant with iron and phosphorus from the soil. The impact of the mechanisms by which the bacteria provides a compound or nutrient such as fixed N, P or Fe to the plant varies considerably depending upon the soil composition. Thus PGPR often have little or no measurable effect on plant growth when the plants are cultivated in nutrient rich soil and grown under optimal conditions.

Further root associated bacteria capable of fixing nitrogen occur regularly in diverse soils which vary widely in nitrogen content. Common genera capable of fixing nitrogen include *Azospirillum, Azotobacter, Bacillus, Clostridium, Derxia* and *Klebsiella*. These are commonly designated “free living” bacteria, since they are able

to exist in the soil and reduce nitrogen without entering into symbiotic association with plants (Gaskins *et al*, 1985). Denitrification which transforms reduced nitrogen compounds into gaseous nitrogen allows return of nitrogen to the atmosphere from the soil. *Alcaligenes, Bacillus* and *Pseudomonas sps* are common types of denitrifying bacteria. The removal of soil nitrogen by denitrifying bacteria is normally considered detrimental to crop production, because in most instances nitrogen is the element which most severely limits plant growth. However, these bacteria are useful since they prevent nitrogen compounds from accumulating to toxic levels, particularly in poorly drained areas. Also, denitrification activity beneath the root zone is beneficial, since it reduces the nitrate load in ground water. Denitrification tends to maintain a balance between soil and atmospheric nitrogen (Gaskins *et al*, 1985). Also the mechanism most often invoked to explain the various effects of plant growth promoting bacteria on plants is the production of phytohormones most notably auxin. Auxins, are a class of PGPRs known to stimulate both rapid (e.g., increases in cell elongation) and long term (e.g., cell division and differentiation) responses in plants. Diverse soil microorganisms including bacteria, filamentous fungi and yeasts are capable of producing physiologically active quantities of auxins and which have pronounced effects on plant growth and development. L-Tryptophan (L-TRP) is considered as a physiological precursor of auxins biosynthesis in both higher plants and microorganisms (Arshad and Frankenberger, 1998). Since plants as well as plant growth promoting bacteria can synthesize indole acetic acid (IAA), it is important when assessing the consequences of treating a plant with a plant growth promoting bacterium to distinguish between the bacterial stimulation of plant auxin synthesis on the one hand and auxin that is synthesized by the bacterium on the other. The level of

auxin produced by a bacterium in the rhizosphere determines its effect on the host plant; high levels induce developmental abnormalities and stimulate formation of lateral and adventitious roots, while low levels promote root elongation (Van Loon & Glick, 2004).

A number of different bacteria considered to be PGPR include *Azotobacter* spp., *Azospirillum* spp., *Pseudomonads, Acetobacter* spp., *Burkholderia* spp., *Bacillus, Alcaligenes, Klebsiella, Enterobacter, Herbaspirillum, Xanthomonas* (Glick,1995).. 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*, 2005).

1.4 Implications of rhizobacteria

1.4.1 Biofertilizers

Biofertilizer can be defined as a substance which contains living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant. This definition is based on the logic that the term biofertilizer is a contraction of the term *biological fertilizer*. As biology is the study of *living* organisms, biofertilizer should contain living organisms which increase the nutrient status of the host plant through their on-going existence in association with the plant. Chemical fertilizers have played a significant role in the Green Revolution but excessive use of them has led to reduction in soil fertility and to environmental degradation. Moreover, the use of chemical fertilizers is reaching the theoretical

maximum use beyond which there will be no further increase in yields. Nitrogen (N) is the most limiting nutrient for crop yields, and N fertilizers are an expensive input in agriculture costing more than US$45 billion per year globally. After N, phosphorus (P) is the major plant growth limiting nutrient despite being abundant in soils in both inorganic and organic forms. However, many soils throughout the world are P-deficient because the free phosphorus concentration (the form available to plants) even in fertile soils is generally not higher than 10 μM even at pH 6.5 where it is most soluble (Vessey, 2003).

On an average, most mineral nutrients in soil solution are present in millimolar amounts, however, phosphorus is present only in micromolar or lesser quantities (Ozanne, 1980). These low levels of P are due to high reactivity of soluble P with calcium (Ca), iron (Fe) or aluminum (Al) that lead to P precipitation. Inorganic P in acidic soils is associated with Al and Fe compounds whereas calcium phosphates are the predominant form of inorganic phosphates in calcareous soils. Phospholipids and nucleic acids form a pool of labile P in soil that is easily available to most of the organisms present there. To circumvent the problem of P deficiency, chemical fertilizers are added to the soils. The production of chemical phosphatic fertilizers is a highly energy intensive process requiring energy worth US$4 billion per annum in order to meet the global need. The situation is further compounded by the fact that almost 75–90% of added P fertilizer is precipitated by Fe, Al and Ca complexes present in the soils (Kapoor *et al*, 1989).

Phosphorus is second only to nitrogen as a mineral nutrient required by both plants and microorganisms, its major physiological role being, in certain essential steps, the accumulation and release of energy during cellular metabolism. Phosphorus in soils is immobilized or becomes less soluble either by absorption, chemical precipitation, or both. Plants can absorb only inorganic phosphorus, and the concentration of inorganic phosphate in soil is very low because most of the phosphorus in soils is present in insoluble forms. This, combined with the relative immobility of the ion in the soil, can cause the phosphate supply to be the limiting factor for plant growth. Organic phosphate can constitute 4-90% of the total soil phosphate. Therefore organic phosphate mineralization is an important soil process because it results in releases of inorganic phosphorus to the soil solution for its availability to plants and soil microbes. Microorganisms are known to solubilize insoluble phosphate through the production of organic acids and chelating oxo acids from sugars. Seed or soil inoculation with phosphate solubilizing bacteria (PSB) is known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields. The use of rock phosphates as phosphate fertilizer and its solubilization through microbes have become a valid alternative to expensive chemical fertilizers (Johri *et al,* 1999).

The soil environment surrounding plant roots is the zone of intense microbial activity. A large number of microorganisms capable of solubilizing insoluble phosphates have been isolated from the root region of crop plants. Population of phosphate dissolving microorganisms is more in the rhizosphere (20-40% of the total population) compared to non-rhizosphere (10-15%) of the total population) (Swaby and Sperber, 1958 ; Gaur,1990;). The higher population of phosphate solubilizers in

the rhizosphere is of great relevance to plants especially in P deficient soils as it helps in mobilization of insoluble P. Important genera of phosphate solubilizing bacteria are *Bacillus* and *Pseudomonas* (Ilmer and Schinner,1992 ; Morsara *et al,* 1995). Phosphate solubilizing bacteria are common in the rhizosphere and secretion of organic acids and phosphatases are common methods of facilitating the conversion of insoluble forms of P to plant-available forms (Kim *et al,* 1998).The solubilization of insoluble phosphates by microorganisms is caused by the production of organic aids although chelating substances also have an important role. The type of organic acids produced and their amounts differs with different microorganisms. The type of organic acid has a significant effect on the solubilization. Trio and dicarboxylic acids are more effective as compared to monobasic and aromatic acids (Kapoor *et al,* 1989).

Studies involving plants inoculated with PSMs showed growth enhancement and increased P contents but large variations were found in PSMs effectiveness (Kucey *et al,* 1989; Subbarao, 1982). *Penicillium bilaii* and *Bacillus megaterium* are considered the most effective PSMs according to field experiments (Kucey, 1989). *Bacillus megaterium* has been shown to release P from organic phosphates, but does not solubilize mineral phosphates. The PSM-plant inoculations resulted in 10–15% increases in crop yields in 10 out of 37 experiments (Tandon, 1987). PSMs can also increase the growth of plants by mechanisms other than P solubilization, e.g. production of phytohormones such as Indole-acetic acid (Arshad and Frankenbergar Jr., 1998; Datta *et al,* 1982). Mustafa *et al,* (2005) evaluated the efficiency of novel P-solubilizing and N_2-fixing bacterial strains isolated from barley and wheat rhizosphere soils to assess their possible use as inoculants for increasing productivity of agricultural crops by minimizing the need for chemical fertilizers. They reported

that inoculation of barley with the *Bacillus* RC01 and M-13 significantly increased concentration of P in plants and in soil. Also, bacteria inoculation increased the N content in soil and plant. The higher total N and P uptake of barley indicated that *Bacillus* RC01 and M-13 were able to fix N and solubilize P, with consequent promotion of plant growth. Increased P uptake by plants and plant growth as the result of the PSB inoculation have been already reported (Pal, 1998; Rodriguez and Fraga 1999; Puente *et al,* 2004). *Azospirillum brasilense* and *Azospirillum irakense* strains stimulated overall plant growth, including root development and grain yield of spring wheat and maize, but both rhizobacteria did not change the N concentration in plants or grains. By contrast, plants inoculated with the PGPB generally have a higher N content than the uninoculated plants (Puente *et al,* 2004).In the case of *Bacillus RC02* and RC03 inoculants, stimulation of barley growth probably occurred through the release of plant growth substances by these bacteria since an increase in the available P content of soil was not observed.

The production of hormones has been suggested to be one of the mechanisms by which PGPR including *Bacillus species* stimulate plant growth. *Bacillus megaterium* and *Paenibacillus polymyxa* were able to enhance growth and yield but not the P uptake of canola, indicating that P solubilization is not the main mechanism responsible for positive plant response. Their results have also showed that root length and root and shoot weight of plants were reduced by soil compaction due to increased resistance to root penetration and decreased the effects of fertilizer and bacterial strains. The results suggest that the bacterial strains tested in their study have a potential to be formulated and used as inoculants in sustainable and organic agriculture.

The solubilization of P in the rhizosphere is the most common mode of action implicated in PGPR that increase nutrient availability to host plants (Richardson, 2001). Examples of recently studied associations include *Azotobacter chroococcum* and wheat, *Bacillus circulans* and *Cladosporium herbarum* and wheat, *Bacillus* sp. and five crop species (Pal, 1998), *Enterobacter agglomerans* and tomato (Kim *et al,* 1998), *Pseudomonas chlororaphis* and *P. putida* and soybean (Cattelan *et al,* 1999), *Rhizobium* sp. and *Bradyrhizobium japonicum* and radish (Antoun *et al,* 1998), and *Rhizobium leguminosarum* bv. *Phaseoli* and maize.

Phosphate-solubilizing bacteria are common in rhizospheres (e.g., Nautiyal *et al,* 2000 ; Vazquez *et al,* 2000). However, the ability to solubilize P by no means indicates that a rhizospheric bacterium will constitute a PGPR. For example, Cattelan *et al,* (1999) found only two of five rhizospheric isolates positive for P solubilization actually had a positive effect on soybean seedling growth. Likewise, not all P solubilizing PGPR increase plant growth by increasing P availability to the hosts. For example, De Freitas *et al,* (1997) found a number of P-solubilizing *Bacillus* sp. isolates and a *Xanthomonas maltophilia* isolate from canola (*Brassica napus* L.) rhizosphere which had positive effects on plant growth, but no effects on P content of the host plants (Vessey, 2003).

There are a few other points of interest that relate to agricultural uses of PGPR. For example, it has been shown that some PGPR strains are able to counter act irrigation problems by reducing the negative effect of irrigation of crops with highly saline water. This may reflect the lowering of plant ethylene levels elevated by salt stress by means of 1-amino-cyclopropane-1-carboxylate (ACC) deaminase-containing PGPR. Also, it has been observed that PGPR numbers decline rapidly in the

rhizosphere after inoculation, although their effects last throughout the growing season. Several studies show that growth promotion effects are seen early in plant development, and these subsequently translate into higher yields (Glick *et al,* 1997; Kloepper *et al,* 1988). Evidence for late season grain weight increases have also been reported in studies with PGPR and rice. Free living PGPRs can be administered to crops in some formulations that are available commercially. The majority of these products are biocontrol agents which contribute indirectly to the growth promotion of crops (Glick *et al,* 1999). Understanding the mechanisms of plant growth promotion is important when deciding what type of bacteria to use with a plant in a given situation. For example, *Pseudomonas putida* GR12-12 contains the gene for ACC deaminase, which inhibits ethylene synthesis, ethylene being a product of stress. This mechanism is most effective on plants that are more susceptible to the effects of ethylene, such as dicotyledonous plants (Lucy *et al,* 2004).

1.4.2 Biological Control Agents

Collectively phytopathogens can reduce crop yields by 25-75 %, which is an enormous potential loss of productivity. At present this loss is dealt with the use of chemical agents (pesticides), although fumigation, steam treatment and solarization of soils have also been employed. With the realization that these chemicals persist and accumulate in natural ecosystems the alternative approaches were looked for. The biological approaches employed to control phytopathogenic agents include development of transgenic plants that are resistant to one or more pathogenic agents (Glick, 1995).

Some strains of nonpathogenic rhizobacteria can suppress disease by inducing resistance in plants such strains can induce resistance against multiple pathogens in the same crop. This induced resistance has been termed 'induced systemic resistance" (ISR) (Somers *et al,* 2004; Hammerschmidt and Kuc, 1995; Van Loon *et al,* 1998). ISR is dependent on colonization of root system by sufficient number of PGPR, which can be activated by coating seeds with high number of bacteria or by adding bacterial suspension to soil before sowing or transplanting. PGPR bring about ISR through fortifying the physical and mechanical strength of the cell wall as well as changing the physiological and biochemical reactions of the host leading to the synthesis of the defense chemicals against the challenge pathogen.

Direct growth promotion occurs when a rhizobacterium produces metabolites that directly promote plant growth without interactions with native soil microflora. In contrast antibiotics, siderophores and HCN, which decrease activities of pathogens or deleterious microorganisms and, thereby, increase plant growth, are examples of indirect growth promotion by biological control. Microbial interactions in the rhizosphere of plants are now believed to be of considerable importance to agriculture. In some known interactions, the formation of antibiotic substances by ectomycorrhizal fungi suppresses growth of pathogenic species; in others free living microorganisms produce antibiotics that inhibit pathogens (Vining,1990)

One of the major mechanisms postulated for the biological control of plant root diseases is the production of antimicrobial compounds by the disease control agent. Antimicrobial compounds may act on plant pathogenic fungi by inducing fungistasis, inhibition of spore germination, lysis of fungal mycelia, or by exerting fungicidal effects. The fluorescent *Pseudomonads* have been shown to produce a large

number of antibiotics. *P.fluorescens* 2-79 produces a phenazine antibiotic with potent antifungal activity, which has been used to control take-all disease of wheat caused by fungus *Gaeumannomyces graminis var.tritici*. The antibiotic has subsequently been identified as phenazine-1-carboxylic acid. Another important class of antibiotics produced by bacteria are the bacteriocins. The bacteriocins are usually proteins produced by many gram positive and gram negative bacteria, and they are inhibitory to other related strains of the same species because of their high degree of specificity. One of the first commercial applications of biological control for root diseases has been the use of *Agrobacterium radiobacter* K84 to control the crown gall disease off dicotyledons plants caused by *Agrobacterium tumefaciens*. *Pseudomonas fluorescens* is well known for its production of various diffusible metabolites including different pigments. The mechanism by which *Pseudomonas fluorescens* suppresses plant pathogen production is the production of antibiotics such as 2, 4-diacetylphloroglucinol, pyrrolnitin, pyoluteorin and phenazine 1-carboxylate. 2, 4-diacetylphloroglucinol plays a major role in the suppression of variety of soil borne phytopathogens by fluorescent *Pseudomonas* strains (Keel *et al*, 1992; Shanahan e*t al*, 1992).

Even though iron is one of the most abundant minerals on earth it is not available freely for direct assimilation by microorganisms.Fe^{+3} or Fe^{+2} is sparingly soluble, the amount of iron that is available in the soil is much too low to support microbial growth. In order to overcome this microorganisms secrete low molecular mass iron binding molecules (siderophores) that bind Fe^{+3}, transport it back to the microbial cell and then make it available for microbial growth. One way the PGPR can prevent the proliferation of phytopathogens and thereby facilitate plant growth, is

through the production and secretion of siderophores with a very high affinity for iron. The secreted siderophore molecules bind most of the Fe^{+3} that is available in the rhizosphere and as a result effectively prevent any pathogens in this immediate vicinity from proliferating because of lack of iron. The bacterium that originally synthesized the siderophore takes up the iron-siderophore complex by using a receptor that is specific for the complex and is located in the outer cell membrane of the bacterium (Glick, 1995). Unlike microbial phytopathogens, plants are not generally harmed by the localized depletion of iron caused by PGPR. Most plants can grow at much lower (about 1000-fold) iron concentrations than microorganisms and a number of plants have mechanisms for binding the bacterial iron-siderophore complex, transporting it through the plant, and then reductively releasing the iron from the siderophores so that it can be used by the plant. Fluorescent *Pseudomonads* form a line of siderophores comprised of a quinoline moiety responsible for the fluorescens and a peptide chain of variable length bearing hydroxamic acid and alpha hydroxyl acid function. Capacity to form pseudobactin and pyoverdin type siderophores has been associated with improved plant growth either through a direct effect on plants, through control of pathogens in soil or by some other route. Siderophores may indirectly stimulate the biosynthesis of other antimicrobial compounds by increasing availability of minerals to the biocontrol agent. Siderophores and antibiotics may function as stress factors and signal inducing local systemic host resistance (Duffy and Defago, 1999).

Fluorescent *Pseudomonads* also promote plant growth by producing and secreting auxins, gibberellins and cytokinins. Enhanced plant growth caused by these strains is often accompanied by reductions in populations of deleterious

microorganisms. (Xiao and Kisaalita, 1995) PGPR compete with the deleterious rhizobacteria for infection sites and nutrients. Suslow (1982) reported that PGPR prevented the deleterious rhizobacteria from colonizing sugar beet to their full potential, may be because the PGPR occupied and excluded deleterious rhizobacteria from cortical cell junctions at which exudation and nutrients us maximal (Kloeppler,1993).

One of the most effective mechanisms that a PGPR can employ to prevent proliferation of phytopathogens is the synthesis of antibiotics. PGPR can also inhibit phytopathogens in different ways like

1) It has been suggested that the ability of some *Pseudomonad*s to synthesize hydrogen cyanide may be linked to the ability of those strains to inhibit some pathogenic fungi.
2) In at least one instance it has been demonstrated that several different microorganisms including strains of *Cladosporium werneckii*, *Pseudomonas cepacia* and *Pseudomonas solanacearum* are able to hydrolyze the compound fusaric acid. Fusaric acid is the causative agent of the damage to plants that occurs upon *Fusarium* infection. As a consequence of the ability to hydrolyze fusaric acid, these bacterial strains can prevent plant diseases that are caused by various species of the fungus *Fusarium.*
3) Many plants respond to pathogenic attack by synthesizing enzymes that can hydrolyze the cell walls of some fungal pathogens. Similarly, some PGPR strains have been found to produce enzymes that can lyse fungal cells. For example, Lim *et al,* (1991) isolated a strain of *Pseudomonas stutzeri* that produced extracellular chitinase and laminarinase. These enzymes could digest

and lyse *Fusarium solani* mycelia thereby preventing the fungus from causing crop loss owing to root rot. In addition, Fridlender *et al,* (1993) were able to reduce the incidence of plant diseases caused by phytopathogenic fungi *Rhizoctonia solani, Sclerotium rolfsii* and *Pythium ultimum* by using a beta-1,3-glucanase-producing strain of *Pseudomonas cepacia* that was able to damage fungal mycelia

4) Competition for nutrients and suitable niches on the root surface is a overlooked mechanism by which PGPR may protect plants from phytopathogens. In one study Stephens *et al,* (1993) concluded that the major factor influencing the ability of a pseudomonad isolate to act as a biocontrol agent against *Pythium ultimum* on sugar beets in soil, is their ability to metabolize the constituents of seed exudates in order to produce compounds inhibitory to *P. ultimum*. They also observed that there was not necessarily any relationship between the ability of a bacterium to inhibit a fungal pathogen when the bacterium was was grown in the laboratory on media that favoured the production of either antibiotics or siderophores and the biocontrol activity of the bacterium *in vivo*.

5) In many plants long lasting and broad spectrum systemic resistance to disease-causing agents including fungal pathogens can be induced by treating the plant or seed with a PGPR. In this case the PGPR appears to turn on the synthesis of some antipathogenic metabolites within the plant in a mechanism that does not involve any direct interaction between the PGPR and the pathogen (Glick, 1995).

1.5 Significant plant growth promoting metabolites produced by rhizobacteria

Microbial secondary metabolites include antibiotics, pigments, toxins, effectors of ecological competition and symbiosis, pheromones, enzyme inhibitors, immuno modulating agents, receptor antagonists and agonists, pesticides, antitumor agents and growth promoters of animals and plants. Secondary metabolism is brought on by exhaustion of nutrient biosynthesis or addition of an inducer, and/or by a growth rate decrease. 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). PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellin, ethylene etc.), siderophores, HCN and antibiotics.

1.5.1 ACC deaminase

Ethylene, which is produced in almost all plants, mediates a range of plant responses and developmental steps. Ethylene is involved in seed germination, tissue differentiation, formation of root and shoot primordia, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and senescence, fruit ripening and degreening, production of volatile organic compounds responsible for aroma formation in fruits, storage product hydrolysis, leaf and fruit abscission and the response of plants to biotic and abiotic stresses (Frankenberger and Arshad, 1995). In some instances ethylene is stimulatory, while in others it is inhibitory. The increased level of ethylene formed in response to trauma inflicted by temperature extremes, water stress, ultraviolet light, chemicals, mechanical wounding,

insect damage and disease can be both the cause of some of the symptoms of stress (e.g., onset of epinastic curvature and formation of aerenchyma) and the inducer of responses, which will enhance survival of the plant under adverse conditions(e.g., cell wall strengthening, production of phytoalexins and synthesis of defensive proteins.

1-aminocyclopropane-1-carboxylate (ACC), the cyclopropanoid amino acid is a presursor in the biosynthetic pathway of the plant hormone ethylene. Plant growth promoting soil bacteria have been found to contain ACC deaminase (ACCD), a PLP-dependent enzyme that converts ACC to a ketobutyrate and ammonium. Introduction of ACCD in higher plants by gene modification technology reduced the production of ethylene and delayed ripening of fruits. *Pseudomonas putida* UW4, a novel ACCD containing bacteria has been shown to promote plant growth under different environmental stresses to promote plant growth under different environmental stresses including flooding, drought and the presence of heavy metals and phytopathogens. It has been suggested that the possibility of a close mutualistic relationship between the plants and the soil bacteria has been suggested and the role of ACCD in ensuring low levels of ethylene at critical stages of root growth has been proposed (Hontzeasa *et al,* 2004). The enzyme ACC deaminase 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. A burst of ethylene is required to break seed dormancy for many plants, but following germination a sustained high level of ethylene would inhibit root elongation. PGPR that contain the enzyme ACC deaminase when bound to the seed coat of a developing seedling act as a mechanism for ensuring that the ethylene level does not become elevated to the point where crucial root growth is impaired. By facilitating the formation of longer roots these bacteria may enhance the

survival of some seedlings especially during the first few days after the seeds are planted. In addition, plants that are treated with ACC deaminase containing PGPR are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding, heavy metals, the presence of phytopathogens and drought and high salt. In each of these cases the ACC deaminase containing PGPR markedly lowered the level of ACC in the stressed plants thereby limiting the amount of stress ethylene synthesis and hence the damage to the plant. These bacteria are beneficial to plant growth since in the natural environment plants are often subjected to ethylene producing stress. ACC deaminase containing PGPR facilitate plant growth to a much greater extent with plants that are ethylene sensitive such as canola, peppers and tomatoes (Penrose and Glick, 2003).

In higher plants ethylene is produced from l-methionine via the intermediates, S-adenosyl-l-methionine (SAM) and 1-aminocyclopropane-1-carboxylicacid (ACC) (Yang and Hoffman, 1984). The enzymes involved in this metabolic sequence are SAM synthetase, which catalyzes the conversion of methionine to SAM, ACC synthase, which is responsible for the production of ACC and 5¢-methylthioadenosine from SAM and ACC oxidase, which further metabolizes ACC to ethylene, carbon dioxide and cyanide. While ACC deaminase activity has been found only in microorganisms, there are no microorganisms that synthesize ethylene via ACC. Glick *et al*, (1997) proposed a model for lowering of plant ethylene concentrations by plant growth-promoting rhizobacteria based on the presence of the enzyme ACC deaminase. The model suggests that uptake and subsequent hydrolysis of ACC by the PGPR decreases the quantity of ACC outside the plant, which must exude higher amounts of ACC to maintain a balance between the internal and external

ACC levels. Lowering of ACC concentrations within the plant also reduces the amount of ethylene produced in the plant, decreasing the inhibitory effect of ethylene on root elongation and leading to longer roots (Patten and Glick 1996). In order to test the model canola seeds were imbibed in the presence of a chemical ethylene inhibitor, various PGPR and a strain with ACC deaminase (Penrose *et al*, 2001). The results demonstrated that, in the presence of the ethylene inhibitor or ACC deaminase-expressing strains, the growth of canola seedling roots was enhanced and the levels of ACC in the roots were lowered. The bacterium utilizes ACC as a nitrogen source, thereby lowering the internal plant ethylene concentration, which leads to increased root elongation (Cartieaux *et al*, 2003).

A model has been proposed in which plant growth-promoting bacteria can lower plant ethylene levels and in turn stimulate plant growth (Fig 1.3). In this model, the plant growth-promoting bacteria bind to the surface of either the seed or root of a developing plant. In response to tryptophan and/or other small molecules in the seed or root exudates , the plant growth- promoting bacteria synthesize and secrete IAA, some of which is taken up by the plant. This IAA, together with endogenous plant IAA, can stimulate plant cell proliferation and elongation or induce the activity of ACC synthase to convert SAM to ACC. Much of the ACC produced by this latter reaction is exuded from seeds or plant roots along with other small molecules normally present in seed or root exudates. Imbibed canola seeds exuded about 10 mol ACC per seed per h (Penrose and Glick, 2001), whereas root systems of 55-day-old tomato plants released micromolar concentrations of ACC upon flooding stress. The ACC in the exudates may be taken up by ACC deaminase containing bacteria and subsequently cleaved by the enzyme to ammonia and a-ketobutyrate, thereby

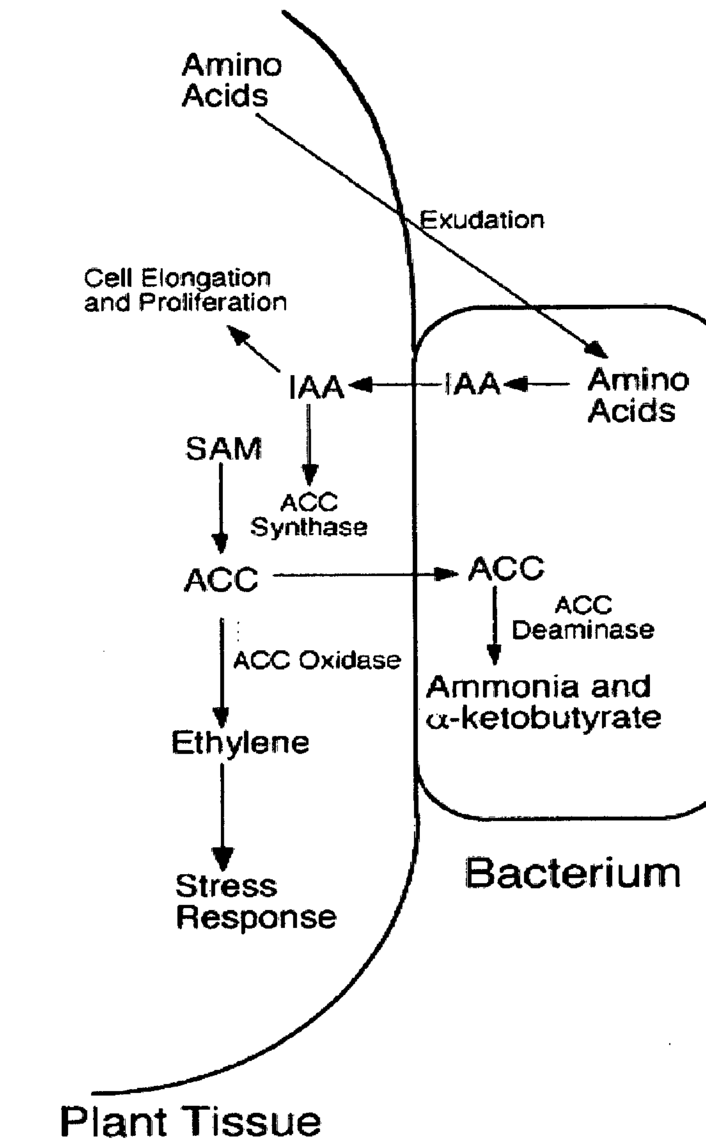


Fig. 1.3 Model explaining PGPR stimulation of plant root elongation (Arshad and Frankenberger, 1995)

decreasing the amount of ACC outside the plant. Subsequently, increasing amounts of ACC are exuded by the plant in order to maintain the equilibrium between internal and external ACC levels. The net result of these interactions is that the bacteria induce the plant to synthesize more ACC than it would otherwise, and as well stimulate the exudation of ACC from the plant (Van Loon & Glick, 2004).

Thus, plant growth-promoting bacteria are supplied with a unique additional source of nitrogen in the form of ACC that enables them to proliferate under conditions in which other soil bacteria may not flourish, for instance, when nitrogen availability is low and competition for nutrients is intense. As a result of lowering the ACC level within the plant, either the endogenous level or the IAA-stimulated level, the amount of ethylene in the plant is also reduced. Plant growth-promoting bacteria that possess the enzyme ACC deaminase and are bound to seeds or roots of seedlings can reduce the amount of plant ethylene and the extent of its inhibition on root elongation. Thus, these plants should have longer roots and possibly longer shoots as well, in as much as stem elongation is also inhibited by ethylene, except in ethylene-resistant plants. Consistent with the model, when the ACC deaminase gene (*acdS*) from *Enterobacter cloacae* UW4 was replaced by homologous recombination with aversion of the same gene with a tetracycline resistance gene inserted within (Van Loon & Glick, 2004).

1.5.2 Auxins

One of the direct mechanisms by which PGPR promote plant growth is by production of plant growth regulators or phytohormones (Glick, 1995). Frankenberger and

Arshad, (1995) have discussed in detail the role of auxins, cytokinins, gibberellins, ethylene and absicisic acids (ABA) which, when applied to plants, help in increasing plant yield and growth. Microbial production of individual phytohormones such as auxins and cytokinins has been reviewed by various authors over the last 20 years (Pilet *et al*, 1979; Hartmann *et al*, 1983; Fallik and Okon 1989; Barbieri and Galli 1993; Patten and Glick 1996; Patten and Glick 2002). Auxins are a class of plant hormones and one of the most common and well characterized is indole-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 as follows: a) cell enlargement b) cell division c) root initiation d) root growth inhibition e) increased growth rate f) phototropism g) geotropism h) apical dominance (Frankenberger and Arshad 1995; Leveau & 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 plant growth-promoting bacteria, which stimulate the growth of roots, can produce at least small amounts of the auxin indole-3-acetic acid(IAA), high IAA producers are inhibitory to root growth (Lindow *et al,* 1998). Bacterial IAA producers (BIPs) have the potential to interfere with any of these processes by input of IAA into the plant's auxin pool (Leveau & Lindow, 2005).

IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR. Promotion of root growth is one of the major markers by which the beneficial effect of plant growth-promoting bacteria is measured. Rapid establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as

it increases their ability to anchor themselves to the soil and to obtain water and nutrients from their environment, thus enhancing their chances for survival. Most root-promoting bacteria synthesize IAA, and their effect on plants mimics that of exogenous IAA. Plants generally grow one or more primary roots from which lateral roots emerge by division of specific pericycle cells. Adventitious roots are a type of lateral root that arise from nonroot tissue, such as the tissue at the base of the stem or on cuttings. Whereas lateral and adventitious roots are induced by high concentrations of exogenous IAA, a feature exploited in horticulture by applying natural and synthetic auxins, primary root growth is stimulated by application of relatively low levels of IAA, typically between 10^{-9} and 10^{-12} M and is inhibited by higher IAA concentrations, likely via auxin-induced ethylene (Patten & Glick, 2002). A root is one of the plants organs that is most sensitive to fluctuations in IAA, and its response to increasing amounts of exogenous IAA extends from elongation of the primary root, formation of lateral and adventitious roots to growth cessation (Leveau & Lindow, 2005).While many plant growth promoting bacteria, which stimulate the growth of roots, can produce atleast small amounts of the auxin indole-3-acetic acid (IAA), high IAA producers are inhibitory to root growth (Lindow,1998).

Various authors have identified the production of indole-3-acetic acid 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). Various metabolic pathways such as a) indole-3-acetamide pathway b) indole-3-pyruvic acid pathway c) tryptophan side chain pathway d) tryptamine pathway and e) indole-3-acetonitrile pathway are involved in the production of IAA (Fig 1.4). Phytopathogens such as *Agrobacterium tumefaciens*, *A. rhizogenes* and *P. syringaepv. savastanoi* synthesize IAA via the indole-3-acetamide pathway (Liu *et al*, 1982;Offringa *et al*, 1986). Koga *et al*, (1991b) suggested that *E. cloacae*, isolated from the rhizosphere of cucumber, synthesized IAA via the indole pyruvic acid pathway and promoted growth of various agricultural plants. *P. fluorescens* demonstrated the ability to convert L-tryptophan directly into indole-3-acetaldehyde (Narumiya *et al*, 1979). Strains such as *B. cereus* and *A. brasilense* produced IAA by the tryptamine pathway (Hartmann *et al*, 1983). Bacterial production of IAA suggests that the pathways involved in IAA production may play an important role in defining the effect of the bacterium on the plant (Patten and Glick 1996).

Glick *et al,* (1999) reported that most of the pathogenic strains of bacteria synthesized IAA via the indole acetamide pathway while plants use the indole pyruvic acid pathway. This helps the bacteria to evade plant regulatory signals and thus the IAA produced induces uncontrolled growth in plant tissues. In contrast the beneficial bacteria such as PGPR synthesize IAA via the indole pyruvic acid pathway and the IAA secreted is thought to be strictly regulated by the plant regulatory signals. Differences in the production of IAA among bacterial strains can be attributed to the various biosynthetic pathways, location of the genes involved, regulatory sequences, and the presence of enzymes to convert active free IAA into conjugated forms. It is also dependent on environmental conditions (Patten and Glick, 1996). The

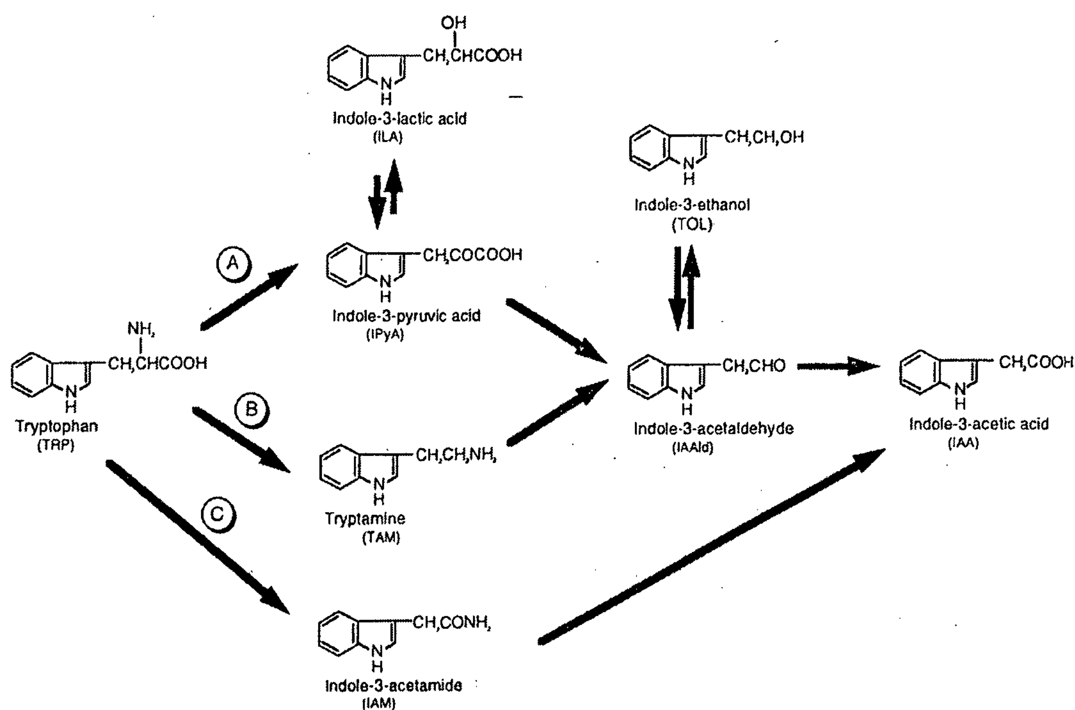


Fig. 1.4 The biosynthesis pathway leading to indole-3-acetic acid (IAA)(Either or both the indole-3-acetamide(IAM) and the indole-3-pyruvate acid(IPA) biosynthesis pathways of IAA are found in different microorganisms. The IPA pathway is considered the major IAA synthesis pathway in plants (Cohen *et al.*, 2002)

physiological effect of microbial IAA on plant growth depends ultimately on the amount of hormone that is available to the plant, which is based on the interaction between the plant and bacterium (Patten and Glick,1996). Three general types of possible association between the plant and bacterium are important in order to exert a positive effect. These are a) transfer of IAA genes directly into the host genome as is the case in *Agrobacterium* species b) infection of internal regions of the plant and secretion of IAA into the surrounding tissue and c) colonization of the external surface and secretion of IAA as an exogenous source to plants. In case of beneficial rhizobacteria the effect is primarily thought to be advantageous when the bacteria are colonizing the external surface of the plant (Del Gallo and Fendrik, 1994)

Eighty percent of microorganisms isolated from various crops have the ability to produce auxins as secondary metabolites (Kampert *et al,* 1975 ; Loper and Schroth,1986).Bacteria belonging to the genera *Azosprillum, 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).

1.5.3 Hydrogen cyanide

Cyanide is a potential inhibitor of enzymes involved in major plant metabolic processes including respiration, CO_2 and nitrate assimilation, and 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

1.5.4 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). Raj Kumarand Lakshmanan (1995) suggested that ammonia excretion seems to be the result of nitrogenase activity. In symbiotic associations where relatively large amounts of atmospheric N reach the plant as ammonia released by the bacteroids. By contrast, most of the ammonia produced in PGPB by the nitrogenase-catalysed N_2 fixation would be assimilated by the rhizobacteria through the glutamine synthetase / glutamate synthase (GS/GOGAT) pathway. Also plant growth-promoting bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase and that this enzyme can cleave the ethylene precursor ACC to α-ketobutyrate and ammonia and thereby lower the level of ethylene in developing or stressed plants (Hontzeas *et al,* 2005).

1.5.5 Exopolysaccharide

Exopolysaccharide is a term first used by Sutherland to describe high molecular weight carbohydrate polymers produced by marine bacteria. EPSs can be found as capsular material that closely surrounds a bacterial cell or as a dispersed slime in the surrounding environment with no obvious association to any one particular cell (Sutherland, 1982; Decho, 1990). In the natural environment bacteria occur mostly in

aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substance. Thus EPS production seems to be important for their survival limited (Sutherland, 1982).

Marine microbes grown under laboratory conditions produce EPS when nutrients such as nitrogen, phosphorus, sulfur and potassium are limited (Sutherland, 1982).The composition of polysaccharide is generally independent of the nature of the limiting nutrient. There was little variation observed in the polysaccharide composition of *Pseudomonas* NCIB11264 when grown in continuous cultures, irrespective of pH, temperature or nitrogen, carbon phosphate content of the growth media.The polymer yield was high at suboptimal temperatures, high carbon-to-nitrogen ratios, and during stationary phase. A deep sea hydrothermal vent strain of *Alteromonas* produced EPS at the beginning of stationery phase and during nitrogen limitation, suggesting EPS synthesis for this strain was also induced by restricted growth conditions. Most bacteria use carbohydrates as a carbon and energy source and amino acids or an ammonium salt as a nitrogen source(Sutherland,1982).The composition of EPSs and the chemical and physical properties of these biopolymers can vary greatly (Decho,1990) but it is generally independent of the carbon substrate (Sutherland, 1982).

Most bacteria produce the most quantity of EPS during stationery phase of growth in laboratory conditions (Decho, 1990; Manca *et al,* 1996). The composition of EPS may also vary according to the growth phase of the culture (Christensen *et al,* 1985). Although culture conditions generally do not affect the types of monosaccharides in an EPS, they do affect the functional properties of the polysaccharide such as molecular weight, conformation and monosaccharide ratios

(Arias *et al,* 2003). Extracellualr polymers aid the microbes to compete and survive in changing environmental conditions by altering the physical and biogeochemical microenvironment around the cell. In the marine environment bacterial exopolymers and EPS are essential in the production of aggregates, adhesion to surfaces and other organisms, biofilm formation and sequestration of nutrients and thus provide protection and ecosystem stability.

1.5.6 Siderophores

Iron is the fourth most abundant element on earth, but in the presence of oxygen and at neutral pH, it is not sufficiently available to microbes due to the rapid oxidation of Fe^{+2} to Fe^{+3} and the formation of ferric hydroxides and oxyhydroxide polymers (Neilands, 1995). Concentration of free iron in soil under these conditions is as low as 10^{-17M}, which is much less than that required for optimal growth of soil microflora (Guerinot, 1994). A large number of proteins require iron for their activity, which underlines the importance of iron for living organisms. The iron that is present in proteins can exist in several different forms: heme, iron-sulfur, iron-nickel, di-iron and mononuclear iron (Andrews, 1998). Iron containing proteins participate in various processes such as: (1) respiration where cytochromes, ferredoxins, and other iron-sulfur proteins play a role; (2) activation of oxygen where cytochrome oxidase plays a role; (3) degradation of hydrogen peroxide and hydroxyl radicals where heme-catalase, iron-superoxide dismutase, and peroxidase play a role; (4) amino acid and pyrimidine biosynthesis where glutamate synthase and dihydro-orotate dehyrogenase play a role; (5) the citric acid cycle where fumarase, aconitase, and succinate dehydrogenase play a role; (6) DNA synthesis where ribonucleotide reductase plays a

role; (7) nitrogen fixation where nitrogenase plays a role (8) carbon fixation metabolism where methane monooxygenase plays a role (9) photosynthesis where light-harvesting complexes and ferredoxin play a role; and (10) oxygen binding where globins play a role (Andrews, 1998).

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 & Gomez and Sansom, 2003) or, which imparts specificity of uptake and works in association with periplasmic iron-binding proteins and cytoplasmic membrane-associated proteins (Gomez and Sansom, 2003). The molecular weights of siderophores range from approximately 600 to 1500 daltons, and because passive diffusion does not occur for molecules greater than 600 daltons, siderophores must be actively transported (Ishimaru, 1993). The role of these compounds is to scavenge iron from the environment and to make the mineral, which is almost always essential available to the microbial cell (Neilands, 1995).There are more than 500 different types of siderophores produced by bacteria, yeasts, and fungi. Siderophores are produced and secreted only when the amount of iron is low in the growth environment. The genes involved in siderophore production regulate siderophore production based on the concentration of iron in the environment. That is, siderophore

production is shut off when iron is present at sufficient concentration and vice versa. Siderophores specifically bind to ferric ion with high affinity. The binding power of the siderophore for iron has a stability constant range from 10^{22} to 10^{50}(Ratledge and Dover 2000). This range is sufficiently high for the removal of iron attached to molecules like ferritin and transferrin by siderophore, but not high enough for the removal of iron present in heme proteins. Siderophore molecules display considerable structural variation but can be classified as either hydroxamates or catechols. Structurally, 20 siderophores are ring or semi-ring shaped structures containing oxygen atoms. Siderophores show high affinity for ferric ion, since the oxygen atoms present can form coordination bonds with a single Fe (III) ion (Neilands, 1995). The production of siderophores has been reported in aerobic and facultative anaerobic microbes, but their production has not yet been reported in strict anaerobes, lactic acid bacteria, or in higher organisms such as plants and animals. The main function of siderophores is involved in the high affinity acquisition and receptor dependent transport of ferric ion. Siderophores are also associated with growth or germination factors and virulence factors.

The concentration of iron required for optimum growth of cells is at least one micromolar (μM). Though iron is required by a majority of microorganisms, there are some exceptions like the lactic acid bacteria. Their growth is not enhanced by the addition of iron because they do not contain heme enzymes and the iron containing ribonucleotidereductase (Neilands, 1995). The efficiency of siderophores in microbial metabolism is based mainly on a) siderophores contain iron binding ligand types consisting of hydroxamate, catecolate or a hydroxycarboxylate ligands that form hexadentate Fe(III) complexes, satisfying the six coordination sites on ferric ions

b) regulation of siderophore biosynthesis s an economic means of spending metabolic energy, but it also allows for the production of high local concentration of siderophore in the vicinity of microbial cells under iron limitation. This kind of overproduction may also be operating in host adapted bacterial and fungal strains, leading to increased virulence c) besides their ability to solubilize iron and to function as external iron carriers, siderophores exhibit structural and conformational specificities to fit into membrane receptors and/or transporters (Winkelmann, 2001).

Competition for iron in the soil is believed to occur at two stages. Firstly, the competition between the excreted siderophores for the available iron and secondly competition between microorganisms for the iron-siderophore complexes formed (Jurkevitch *et al,* 1992). Siderophore cross feeding is a widespread phenomenon in the soil, and rhizosphere bacteria with the ability to scavenge the siderophores produced by other microorganisms have an ecological advantage for survival in the iron-limited soil medium (Jurkevitch *et al,* 1992; Bellis and Ercolani, 2001; Buyer and Sikora, 1990 & Plessner *et al,*1993). Siderophores produced by several fluorescent *Pseudomonas* spp. play a role in the biological control of plant pathogens and in plant growth promotion through competition for iron (Loper and Henkels, 1999). Since these plant growth promoting rhizobacteria produce siderophores with higher Fe^{+3} affinity than the siderophores produced by deleterious rhizosphere microorganisms, the latter microorganisms are out-competed due to iron unavailability (Loper and Henkels, 1999 & Bellis and Ercolani, 2001). For example, *Pseudomonas aeruginosa* synthesizes and utilizes two of its own siderophores along with heterologous microbial siderophores like enterobactin, aerobactin and ferrioxamine B and a number of siderophores produced by other *Pseudomonads* (Dean and Poole, 1996).

Bradyrhizobium japonicum 61A152 releases citric acid as a siderophore under conditions of iron deficiency (Guierinot and Plessner, 1990) but is also able to utilize iron from two fungal siderophores, rhodotorulic acid and ferrichrome (Plessner *et al,* 1993).

In gram-negative bacteria, the siderophore ferric complex is internalized through an outer membrane channel that displays ligand specificity and energy dependency. The transport across the inner membrane is mediated by less specific ABC-type transporters. In the rhizosphere, siderophores released by bacteria and fungi can capture iron from natural chelates, thus depriving of iron microorganisms that produce siderophores in lower concentrations or with a lower affinity for this metal (Expert,1999).

1.5.6.1 Siderophore Mediated Iron Acquisition

A high affinity system for the uptake of iron from the external medium is present in many microorganisms (Fig 1.5). This system has three parts: (1) a siderophore that acts as a high affinity ferric-ion specific ligand that is usually released to the extracellular environment by microbes; (2) a membrane receptor for iron bound-siderophore (ferri-siderophore) complex that transports the chelated iron across the microbial membrane; and (3) an enzymatic system that is present within the cell that can release ferric ion bound to the siderophore. The other mechanisms that microbes have used to acquire iron are: (1) reduction of extremely insoluble forms of ferric ion to soluble forms of ferrous ion that can be used easily (2) use of iron present in hemoglobin by the destruction of erythrocytes and hydrolysis of hemoglobin (3) direct use of the iron stored in ferritin (Ferritin, found in animals, plants [phytoferritins] and even microorganisms [bacterioferritin], are complexes that store

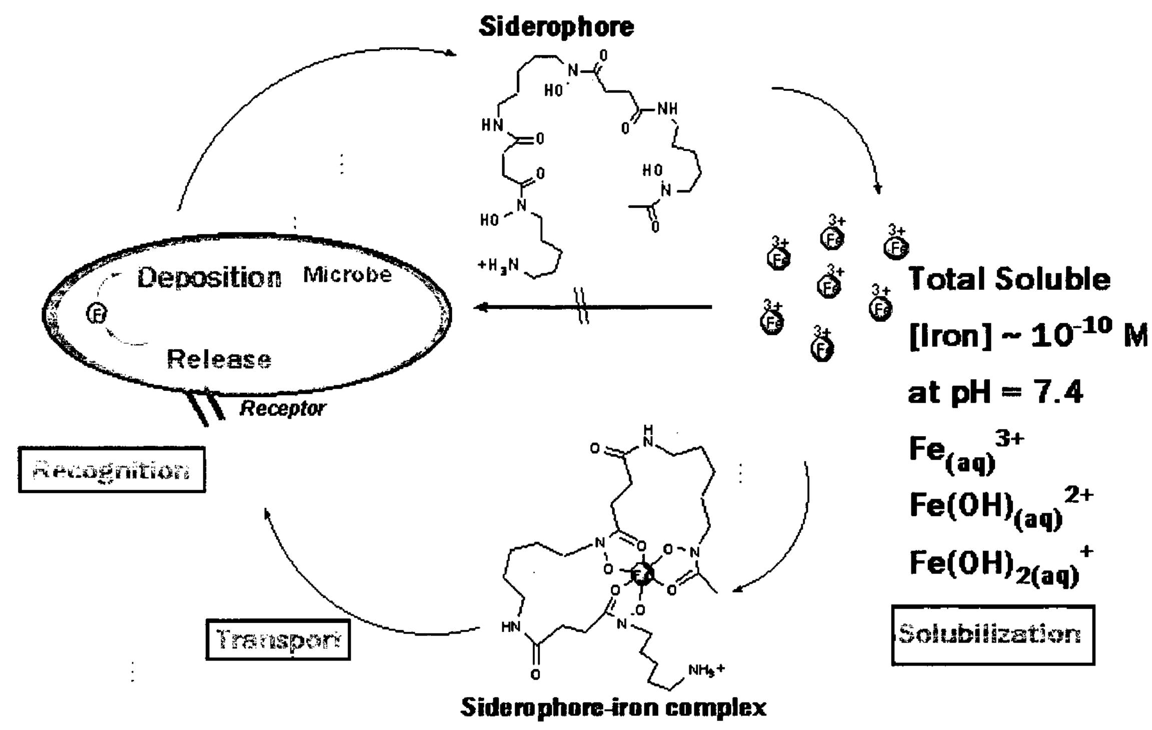


Fig. 1.5 Siderophore mediated uptake of iron into the bacterial cell

iron in a form that is soluble, bio-available,and non-toxic and (4) enzymatic degradation of compounds that bind iron like transferrin (Vasil and Ochsner, 1999).

In gram negative bacteria, Fe^{+3} siderophores bind to highly specific receptor proteins and are then transported into the cytoplasm (Faraldo-Gomez and Sansom, 2003) while in Gram positive bacteria, which lack an outer membrane, the receptors are binding proteins that are anchored to the cytoplasmic membrane by a covalently linked lipid. A periplasmic transport protein and several inner membrane associated proteins complete the transport of iron into the cell. This arrangement of proteins from periplasm to cytoplasm is similar to other bacterial periplasmic protein dependent systems, termed ABC transporters (for ATP binding cassette-type transport), which transport amino acids, peptides and sugars into the cell(Braun & Killman, 1999; Clarke *et al,* 2001; Fatht, and Kolter, 1993).

1.5.6.2 Siderophores in relation to plant growth and disease

Studies on Fe nutrition of plants have resulted in the division of higher plants into two groups (strategies I and II) in relation to their Fe uptake mechanisms. Strategy I plants (dicots and nongrass monocots) rely mainly on membrane reductase activity and on chemical modifications of the rhizosphere (e.g. excretion of reductants and protons) and a subsequent Fe^{+3} reduction. Strategy II plants (grasses) possess a unique system for Fe acquisition based on phytosiderophore excretion and a specific membrane-bound receptor for the ferrated phytosiderophores (Ness *et al,* 1991).

Beneficial or plant growth promoting rhizobacteria have been isolated and demonstrated to protect the roots of certain root crop plants. Fluorescent

Pseudomonas spp. have emerged as the largest and potentially most promising group of plant growth promoting rhizobacteria involved in the biocontrol of plant diseases. These bacteria are ideally suited as soil inoculants because of their potential for rapid and aggressive colonization. One of the disease suppression mechanisms includes the siderophore mediated suppression. Soil pseudomonads generally produce fluorescent, yellow, green, water-soluble siderophores with both a hydroxamate and phenolate group; these siderophores have been classified as either pyoverdins or pseudobactins. Fluorescent siderophores, which have a very high affinity for ferric iron, are secreted during growth under low-iron conditions. The resulting ferric-siderophore complex is unavailable to other organisms, but the producing strain can utilize this complex via a very specific receptor in its outer membrane. In this way, fluorescent pseudomonas strains may restrict the growth of deleterious bacteria and fungi at the plant root. These iron starvation conditions may also prevent the germination of fungal spores (O'Sullivan and O' Gara, 1992). The capacity to u tilize siderophores is important to the growth of bacteria in the rhizosphere and on plant surfaces. Specific siderophore-producing *Pseudomonas* strains rapidly colonize plant roots of several crops, and this colonization can result in significant yield increases. Enhanced plant growth caused by these strains often is accompanied by reduction in the populations of fungi and other bacteria on the roots. These beneficial *Pseudomonas* strains suppress some soil-borne fungal pathogens, and there is convincing evidence to support a direct role of siderophore-mediated iron competition in the biocontrol ability exhibited by such isolates.

1.5.6.3 Examples of siderophores produced by microorganisms

Organism	Siderophore
Escherichia coli	Enterobactin, Enterochelin
Mycobacterium tuberculosis	Mycobactin, Exochelins, and Carboxymycobactins.
Ustilago sphaerogena	Ferrichrome
Pseudomonas sps	Pseudobactin, Pyoverdines, Aerobactin
Agrobacterium tumefaciens	Agrobactin
P. stutzeri	Desferrioxamine B
Salmonella sps	Enterochelin

Antagonism depends on the amount of iron available in the medium: siderophore production by the biocontrol agent and sensitivity by target pathogens are expressed only under iron-limiting conditions. *Agrobacterium rhizogenes* strain K84 (formerly called *A. radiobacter*) is used worldwide as a commercial agent for the biocontrol of crown gall disease caused by tumorigenic *Agrobacterium* strains. It is reasonable to hypothesize that the capacity of this bacterium to colonize the roots of treated plants is an important factor in the successful biological control of crown gall disease. The ability of soil-borne bacteria to produce and utilize siderophores confers an ecological advantage in colonizing the rhizosphere. Moreover, pyoverdine-type siderophores produced by certain *Pseudomonas* spp. are involved directly in the ability of these strains to colonize the rhizosphere in presence of other soil-borne bacteria. Production of this hydroxamate siderophore by strain K84 could represent a trait important to the successful interaction between this microbe and its plant host. In addition, siderophores produced by certain isolates of *Pseudomonas* spp. play a direct

role in biocontrol of some plant pathogens. Production of ALS84 and the siderophore may, in a similar fashion, contribute to the biocontrol of crown gall by strain K84, especially under conditions of iron limitation (Penyalver *et al,* 2001). Siderophores can also function in antibiosis; i.e., siderophores produced by rhizobacteria can inhibit the growth of pathogenic organisms in the rhizosphere and enhance plant growth (Kloepper *et al,* 1980 ; Xu *et al,*1986). Siderophore activity was detected in cultures of *P. luminescens* (Bintrim &Ensign 1984) but its role in nematode symbiosis or insect virulence was not studied.

The modes of action of siderophores in suppression of disease were thought to be solely based on competition for iron with the pathogen. Interestingly, siderophores can induce systemic resistance (ISR). One may therefore consider whether the mode of action of other bacterial metabolites that have been implicated in disease suppression also involves triggering of systemic resistance mediated by rhizobacteria (Bakker *et al,* 2003). *Pseudomonas putida* and *Pseudomonas fluorescens* are common rhizosphere and soil inhabitants, and certain strains promote plant growth and health by suppressing diseases caused by soil borne pathogens. The capacity of these strains to produce pyoverdines is linked, in some cases, with disease suppression; mutants deficient in pyoverdine production can be less effective than parental strains in biological control. Pyoverdines produced in situ by *Pseudomonas* spp. are thought to chelate iron in a form that is unavailable to pathogens, thereby preventing the pathogens' access to the already limited pool of soluble iron in the rhizosphere (Loper & Henkels, 1999).

1.5.6.4 Applications of siderophores

1.5.6.4.1 Biotechnological applications

The importance of these siderophores extends beyond their immediate role in microbial physiology and their applications in biotechnology. Siderophores and their substituted derivatives have a lot of applications. For example, haemochromatosis is a disorder wherein there is a progressive increase in body iron content causing iron deposits in liver, heart or pancreas. Primary haemochromatosis is caused by increased absorption of iron from the gastrointestinal tract without any associated anemia. Desferrioxamine in the form of Desferal. Siderophore is used in the treatment of haemochromatosis (Mohandas, 2005).

1.5.6.4.2 Sideromycins

Sideromycins are iron-chelating antibiotics produced by *Streptomyces*. Albomycin and Ferrimycin are two examples of this group being closely related to ferrichrome. Both antibiotics contain an extra chemical group attached to the basic siderophore structure. Thus, they use the siderophore transport system to gain access to the cell, but once inside the cell their mechanism of action is not against iron transport or its acquisition. Instead these antibiotics exert their effect by inhibiting protein synthesis, similar to many other antibiotics. When the functional group is removed, the molecules lose their antibiotic activity but their growth promoting activities as siderophores are retained. There are of course, other antibiotics that probably fall in to this category but these two are probably the best understood examples (Mohandas, 2005).

1.5.6.4.3 Biomedical application of siderophores

The development of electricity generation by nuclear energy has led to increased human exposure to transuranic elements such as aluminum. Investigations have been carried out to evaluate the capacity of siderophores in removing such elements from the body. Administration of desferrioxamine lowers the level of aluminum in the body and relives the symptoms of the disease. Desferrioxamine has also been used to remove vanadium. In rats Desferal reduced the vanadium content in kidney by 20%, in lung by 25% and in liver by 26% when administered at 100 μ mol kg^{-1} following a dose of 5 μmole kg^{-1} of Na48 VO3. Both urinary and faecal excretion increased at this dose. Desferrioxamine has proved successful in the treatment of dialysis encephalopathy, which is a major complication of long-term dialysis. It is caused by the accumulation of aluminum in the brain from the dialysis water supply. Siderophore from *Klebsiella pneumoniae* has been used as an antimalarial agent, and in cosmetics as deodorants (Mohandas,2005). Siderophores and their analogues can also behave as antibiotic agents. Very effective chelators may deprive pathogenic microbes of the iron essential for growth. This deprivation could be accomplished by competitive chelation of iron or by the blocking of ferric siderophore receptor sites with a nonfunctional siderophore analog. The natural siderophore can also be presented to the microbe bound to another metabolically less useful metal to provide another alternative method of iron starvation. This idea has been successfully demonstrated by mycobactins, family of siderophores isolated from *Mycobacteria* including *M. tuberculosis* and *Mycobacterium phlei.* Natural examples of siderophore antibiotics have been reported, such as albomycins. One of the important practical applications of this active transport system is development of species-selective active

drug transport (the "Trojan Horse" approach) to potentially treat infections due to drug resistant strains of microbes. Siderophore-drug conjugates have shown great potential in active drug delivery to target pathogenic microbes (Roosenberg *et al,* 2000).

1.5.6.4.4 Agricultural uses of siderophores

In agriculture, inoculation of soil with *Pseudomonas putida,* which produces pseudobactin, increases growth and yield of various plants. Electricity generation by nuclear energy has resulted in human exposure to transuranic elements like aluminum. In view of this, investigations have been carried out to assess the capability of siderophores to remove such elements from the body. Powell *et al*, (1980) suggested that hydroxamate siderophores are present in soil at concentrations high enough (10^{-7} to 10^{-8}) to be taken up by plant roots. In many ways, iron is involved in nitrogen fixing by soil bacteria. Recently, an iron and molybdenum cofactor for nitrogenase has been discovered (Mohandas, 2005).

Chapter III

Biodiversity of bacteria associated with sand dune vegetation, *Ipomoea pes-caprae* & *Spinifex littoreus*

2.1 Introduction

Exploration of microbial diversity is a topic of considerable importance and interest. Biological diversity or biodiversity "is the variety of life and its processes". It includes the variety of living organisms, the genetic differences among them, and the communities and ecosystems in which they occur. Besides, analysis of microbial biodiversity also helps in isolating and identifying new and potential microorganisms (Das *et al,* 2006). Little is known about the bacterial communities associated with the plants inhabiting sand dune ecosystems. Coastal sand dunes are stressed ecosystems, the plants found in these habitats have to adapt to survive extreme conditions of temperature, low moisture content and limiting levels of nutrients. It is envisaged that rhizosphere microorganisms may help such plants to overcome the environmental extremes.

In 1904, Hiltner first defined the rhizosphere as "... that zone of soil in which the microflora are influenced by plant roots" (Kang and Mills, 2004). The rhizosphere effect is primarily due to the influx of mineral nutrients to the plant roots through diffusion and the accumulation of plant root exudates. Microbial communities in the rhizosphere are primarily plant-driven, responding with respect to density, composition and activity to the abundance and diversity of plant-derived exudates, eventually leading to plant species-specific microflora. A substantial portion of the root exudates consist of carbon and energy sources readily available for microbial growth; by now it is clear that plant roots excrete amino acids, proteins, sugars, organic acids, vitamins and other bacterium-beneficial substances affecting growth, development and physiology of a microbial population. Low molecular weight plant-derived exudates, mainly amino acids, organic acids and sugars commonly found in

most plants are rapidly utilized by microorganisms. In addition, high molecular weight root mucilage, consisting of approx. 95% sugars and 5% amino acids in the form of heteropolysaccharides and glycoproteins, also serve as a source of energy for rhizosphere bacteria. In fact, most rhizosphere bacteria and fungi are highly dependent on associations with plants that are clearly regulated by root exudates (Bais *et al,* 2004), and in the rhizosphere the numbers of microorganisms can reach 10^{10} to 10^{12} organisms g^{-1} soil. Plant-microbe symbioses have been exploited in programs of sand dune restoration. Plant-associated bacteria increase the ability of plants to utilize nutrients from the soil by increasing root development, nitrate uptake or solubilizing phosphorus, and to control soil-borne pathogens (Smith and Read, 1997; Whipps, 2001).

In order to understand the effects of plant-bacteria interactions, it is essential to study the bacterial diversity associated with plants, and there have actually been a number of studies characterizing the structures and functions of rhizosphere and root bacterial communities (Hallmann *et al,* 1997; Maloney *et al,*1997 ; Germida *et al,*1998). Plant communities in sand dunes are controlled by the interaction between biotic and physico-chemical components of the sand matrix (Read, 1989). Interactions with microbes appear crucial in obtaining inorganic nutrients or growth-influencing substances. 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.

In this study an attempt was made to understand the biodiversity of the rhizosphere and endophytic bacteria associated with *Ipomoea pes-caprae* and *Spinifex littoreus*, the viable counts of different groups of bacteria on specific media and the identification of bacterial isolates based on cultural, morphological, biochemical and chemotaxonomic analysis. The neutrophiles and alkaliphiles were screened for their plant growth promoting traits such as production of siderophores, phosphate solubilization, and enzymes involved in degradation of macromolecules. Further, the isolates were also tested for hydrocarbon tolerance, degradation and PHA accumulation.

2.2 Materials and Methods

2.2.1. Collection of samples

Plants and soil samples were collected from two coastal sand dune areas of North Goa during July 2003, December 2003 and May 2004(Fig 2.1).These areas included actively growing zones of *Ipomoea pes-caprae* and *Spinifex littoreus*, which vigorously stand on the seaward dune faces. *Ipomoea pes caprae* was collected from Miramar while *Ipomoea pes-caprae* and *Spinifex 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 the microbiological analysis.

2.2.2. Isolation of rhizosphere and endophytic bacteria from sand dune vegetation

2.2.2.1 Rhizosphere bacteria

Rhizosphere sand from *Ipomoea pes-caprae* and *Spinifex 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 medium was used for isolating alkaliphiles, nitrogen free mannitol agar for isolating diazotrophs, sodium chloride tryptone yeast extract agar for isolating halophiles, nutrient agar pH 4 for isolating acidophiles .The plates were incubated at 28 ± 2^0 C and the colonies counted.

2.2.2.2 Endophytic bacteria

The endophytic bacteria of the vegetation were isolated by taking one gram of roots and washing it well in sterile distilled water. The roots were then treated with 0.01M EDTA and centrifuged at 5,000 rpm for 10 mins and this process was repeated 3 times to remove any sand particles attached to the root surface. The roots were then transferred to a sterile mortar and homogenized (Agate *et al*, 1988). The extract obtained was diluted upto 10^{-2} and 10^{-6} and the dilutions were plated on respective media as mentioned above.

2.2.3. Purification and maintaineance of the cultures

Cultures were subcultured successively on the respective media to obtain pure cultures, maintained on agar slants, sealed with parafilm and preserved at 4^0C. The

isolates were periodically checked for purity. Stock cultures were subcultured every three months and stored at 4°C.Working cultures were subcultured from the stock cultures every month or when required.

2.2.4 Selection and characterization of bacterial strains

Among the groups of bacteria isolated alkalophilic and neutrophilic bacterial isolates were chosen for further studies as on repeated subcultures the other groups of bacteria did not survive. The isolates from polypeptone yeast extract glucose agar (PPYG) (pH10.5) were replica plated on PPYG agar plates with pH 7.0, 9.0 and 10.5 to categorize the alkaliphiles as alkali-tolerant, facultative or obligate alkaliphiles.

2.2.5 Identification of prominent bacterial isolates based on cultural, morphological, biochemical and chemotaxonomic analysis

A. Identification of the selected isolates using biochemical methods

The selected isolates were classified using routine biochemical methods/techniques as per the Bergey's Manual of Systematic Bacteriology (Sneath *et al*,1986; Krieg and Holt, 1984). Colony characters such as size, shape, colour, opacity, elevation and consistency of the isolates were studied by streaking the isolates on nutrient agar and PPYG agar, gram staining and motility of the isolates was studied(Appendix C).

2.2.6 Identification of the selected isolates using chemotaxonomic tools

I. Cellular Lipids

A. Polar and Non-polar lipids

i) Extraction of polar and non-polar lipids

The method involves treatment of organisms with a biphasic mixture of methanolic saline and petroleum ether (BP 60-80°C) followed by extraction of the cells and residual aqueous phase with monophasic chloroform-methanol-saline according to Bligh & Dyer (1959) procedure (Appendix C). The petroleum-ether extract from the first stage is composed of readily extractable non polar lipids and addition of chloroform and saline to the second stage extract gives the remaining more polar lipids (Minnikin and Goodfellow, 1985; Collins *et al*, 1982).

B. Isoprenoid Quinones

i. Extraction of Quinones

Approximately 50-100 mg of lyophilized cells were extracted with a small volume (20-40 ml) of chloroform/methanol (2:1v/v) for approximately 2h using a magnetic stirrer. The cell/solvent mixture is passed through a filter funnel (Whatman filter paper) to remove cell debris, collected in a flask, and evaporated to dryness under reduced pressure. Dried cells should not be stored for too long a time before extraction because exposure of dried cells to air stimulates oxidation of the quinone. The lipid extract was resuspended in a small volume of chloroform/methanol and applied to silica gel TLC plate. The TLC plate was developed in hexane/diethyl ether (85:15v/v) and purified quinones were revealed as dark brown/purple bands on a green fluorescent background by brief irradiation with UV light.

ii. Reverse phase chromatography of ubiquinones and menaquinones

The quinone sample was spotted on a TLC plate prerun in the solvent system paraffin / acetone (10:90v/v). The plate was then developed in acetone / acetonitrile

(80;20,v/v) for ubiquinones, while acetone/water (99:1) for menaquinones. Quinones were revealed by irradiation with UV light as dark spots on a blue fluorescent background (Komagata & Suzuki, 1987; Collins, 1985; Minnikin & Goodfellow, 1985).

II. Cell-wall analysis

i. Reading method

a. Preparation of cell wall material by alkali treatment

About 200 mg wet weight of cells were dispersed in 5 % (w/v) KOH with a rotamixer. The suspension was divided between two Bijou bottles, one-third for amino acid analysis and two-third for sugar analysis. The bottles were sealed tightly with polypropylene caps and then heated at 100°C in an oven. Colour change was observed in the bottles from yellow to brown until they just began to clear. At this point, the portion for sugar analysis was removed from the oven and cooled immediately while the portion for amino acids was allowed to continue to clear. Treatment times are usually 15-40 min for sugar portion and 2-30 min longer for amino acid portion. Next an equal volume of distilled water was added to the cooled suspensions which were then centrifuged and supernatants were discarded and the deposits washed twice in 10 ml amounts of distilled water (Minnikin & Goodfellow, 1985; Schleifer and Kandler,1972).

b. Hydrolysis of cell-wall material

The wall deposit for sugar analysis was hydrolysed in about 0.5ml of 2 M HCl at 100°C for 2 h and evaporated to dryness in vacuum over silica gel in a desiccator

containing a small dish of solid NaOH. The deposit for amino acid analysis was hydrolysed in about 0.5 ml of 6M HCl at105°C for 18h and evaporated to dryness over a boiling water bath.

Thin layer chromatography for sugars was carried out in n-butanol/acetic acid/water (12:3:3) and visualized by spraying with aniline phthalate. Thin layer chromatography for amino acids was carried out in n-butanol/acetic acid/water and visualized by spraying with ninhydrin solution (Minnikin & Goodfellow, 1985; Schleifer and Kandler,1972).

2.2.7 Enzymatic potential of the sand dune bacterial flora

The enzyme activities of the isolates from PPYG agar (pH 10.5) were determined qualitatively by spot inoculating on media with specific substrates; starch agar for amylase, skimmed milk agar for protease, carboxymethylcellulose agar for cellulose, tributyrin agar for lipase, xylan degrading agar for xylanase, tannin degrading agar for tannase, chitin degrading agar for chitinase, all at pH 10.5 (Appendix A).The plates were incubated for 48 h at room temperature and checked for enzyme activity. The enzyme activities of the isolates from nutrient agar (pH 7) were determined as mentioned above, but for all the media the pH was maintained at pH 7 (Appendix A). Starch, xylan and cellulose hydrolysis was determined by flooding the plates with iodine solution, 1 % congo red solution (Clarke *et al*, 1991) and 0.1 % congo red solution (Teather and wood, 1982) respectively (Appendix A). Proteolytic, lipolytic, chitinolytic and tannase activities were directly observed as colonies surrounded by a halo/clearance on specific medium. Lipase production was confirmed by streaking the lipase producing strains on agar plates containing Rhodamine B using olive oil

as substrate at pH 10.5 (Appendix A). Lipase productions was monitored by exposing the plates to UV light at 245 nm after incubation for 24 h (Kouker and Jaeger, 1987).

2.2.8 Accumulation of PHA in bacteria (plate assay method)

Bacteria accumulating PHA were screened by the plate assay method (Shirokitamura and Doi,1994).The bacterial isolates were replica plated on E2 mineral medium agar, with 2% (w/v) glucose(Appendix A). The colonies were allowed to grow till a diameter of 5 mm. The plate was then flooded with ethanolic nile blue A for 20 min with intermittent shaking at room temperature. The staining solution was decanted and the plate allowed to dry.The plate was then exposed to UV light keeping a distance of 10 cm from the light source. Colonies exhibiting an orange fluorescence were scored as PHA accumulators

2.2.9 Detection of siderophores by Chrome Azurol S (CAS) Agar Plate Assay

The CAS assay (Appendix A) is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, whereby ferric iron bound to dye, is complexed and released from the dye. The blue color of the medium is due to the dye complexed with iron. When siderophore is added, the siderophore binds the ferric iron, releasing the free dye, which is orange in color.

Fe^{3+} dye (blue) + siderophore $\longrightarrow$ Fe^{3+} siderophore + dye (orange)

Hence, the presence of siderophore is indicated by a color change from blue to orange (Schwyn and Neilands, 1987).

2.2.10 Qualitative estimation of phosphate solubilization

Qualitative estimation of phosphate solubilization was conducted using petridish assays on modified Pikovskaya agar containing tricalcium phosphate as a source of inorganic phosphate and using 0.4 % bromophenol blue (Appendix A). The spot inoculation was carried out using a sterile toothpick and the petridishes were incubated at 28°C. The halo (zone of solubilization) around the bacterial colony and colony diameter were measured after incubation (Gupta *et al,* 1994 & Nautiyal, 1999).

2.2.11 Screening bacterial isolates for degradation and solvent tolerance to hydrocarbons

The bacterial isolates were screened for their aromatic hydrocarbon degrading ability on mineral salt medium (MSM) containing 0.1% each of phenanthrene, naphthalene, biphenyl and sodium benzoate(Appendix A). Plates were incubated for 5-7 days and the growth was checked after every 24h interval. Bacterial isolates were replica plated on PPYG agar pH 10.5 and the plates were overlaid with organic solvents such as benzene, hexane, toluene and hexadecane (3ml), sealed with parafilm, wrapped in paper and incubated at room temperature for 24h (Aono *et al,* 1991: Kobayashi *et al,* 1998 & Ogino *et al,* 1995). This procedure slows down the rate of evaporation of the solvents and all the solvents used in this study were found to persist on the plate for at least 24 - 48h. Survival on direct exposure to solvents and appearance of growth on a plate overlaid with undiluted (100%) organic solvent was considered as an indication of tolerance.

2.2.12 Screening of bacterial cultures for EPS production

Bacterial cultures were grown in 50 ml of nutrient broth and PPYG broth in 250 ml flask. The flasks were incubated at room temperature on a rotary shaker at 160 rpm for 48 hrs. The culture broth were centrifuged at 8,000 rpm at 4°C for 10 min and EPS was estimated using the phenol-sulfuric acid method (Dubois *et al*, 1956).

Results & Discussion:

Samples collected from different sand dunes (Fig 2.1) with two different plants (*Ipomoea pes-caprae & Spinifex littoreus*) showed the presence of 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 (TVC) ranged from as high as 10^7 cfu/g to as low as 10^3 during premonsoon period (Table 2.1A), from 10^7 cfu/g to 10^3 during monsoon period (Table 2.1B) and from as high as 10^8 cfu/g to as low as 10^5 during postmonsoon period (Table 2.1C). It was observed that the TVC of rhizospheric bacteria associated with *Ipomoea pes caprae* was maximum (21×10^8 cfu/g) in plants collected from Aswem Mandrem beach (Postmonsoon) followed by the plants collected from Miramar beach (400×10^7, monsoon), whereas the TVC of the endophytic bacteria associated with *Ipomoea pes caprae* was maximum (25×10^7 cfu/g) in plants collected from Miramar beach (Premonsoon). The rhizospheric bacteria associated with *Spinifex littoreus* showed maximum TVC in the premonsoon

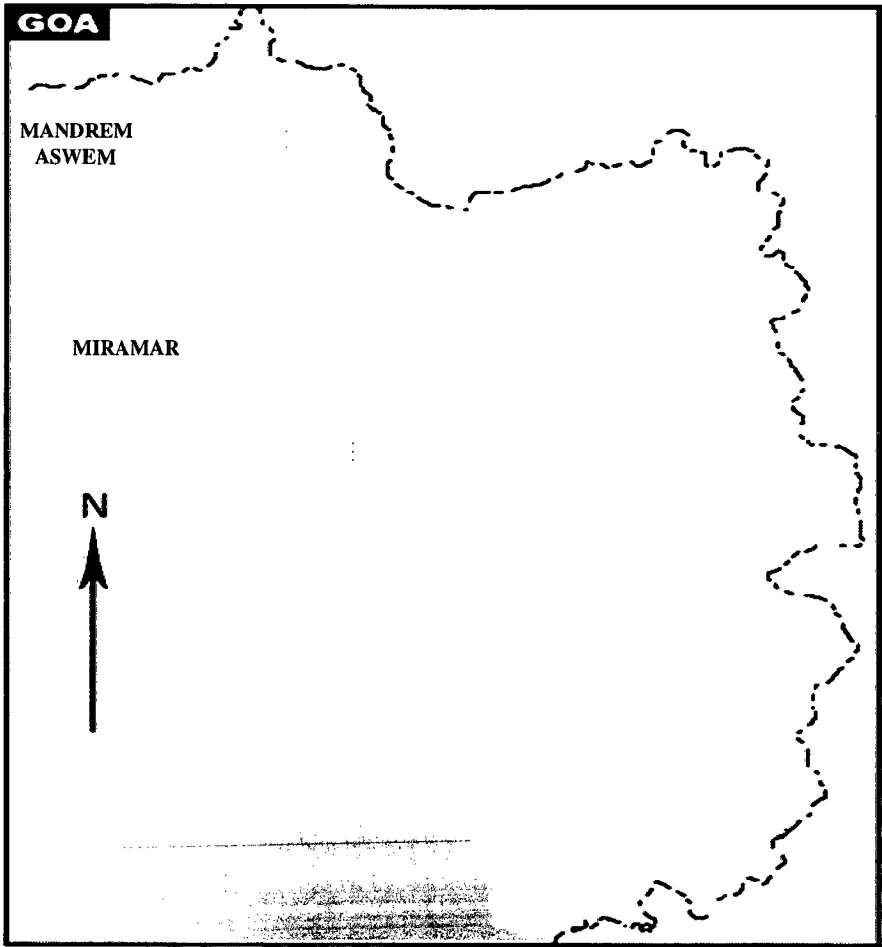


Fig. 2.1 Map showing the sampling sites

Table 2.1 Total viable counts of bacterial groups associated with sand dune vegetation, *Ipomoea pes caprae* and *Spinifex littoreus* in the three seasons.

Table A Premonsoon

Expected bacterial groups*	Miramar beach		Aswem Mandrem beach			
	Ipomoea pes caprae		*Ipomoea pes caprae*		*Spinifex littoreus*	
	Rhizosphere	Endophytic	Rhizosphere	Endophytic	Rhizosphere	Endophytic
	cfu/g		cfu/g		cfu/g	
Neutrophiles	70×10^3	25×10^7	20×10^5	835×10^5	245×10^6	150×10^5
Acidophiles	365×10^4	21×10^5	122×10^3	168×10^3	131×10^3	18×10^3
Alkalophiles	40×10^2	25×10^2	585×10^3	40×10^2	16×10^2	26×10^2
Haloalkalophiles	NC	NC	NC	NC	NC	NC
Halophiles	2×10^2	1×10^3	6×10^2	4×10^4	745×10^3	7×10^2
High salt tolerant	3×10^2	5×10^3	NC	NC	130×10^2	10×10^2
Salt tolerant	135×10^2	122×10^4	8×10^2	1×10^2	73×10^3	300×10^3
Diazotrophs	200×10^2	150×10^3	35×10^3	300×10^4	55×10^3	40×10^3

NC – no counts

*** Media used for isolation of respective bacterial groups**

Neutrophiles – Nutrient agar pH 7.0
Acidophiles - Nutrient agar pH 4.0
Alkaliphiles – Polypeptone yeast extract glucose agar (PPYG), pH 10.5
Haloalkaliphiles – PPYG with 25 % NaCl
Halophiles – Sodium chloride tryptone yeast extract agar (NTYE), pH 7.0
High salt tolerant – Nutrient agar with 25 % NaCl
Salt tolerant – Nutrient agar with 15 % NaCl
Diazotrophs – Nitrogen free mannitol agar

Table B Monsoon

Expected Bacterial groups	Miramar beach		Aswem Mandrem beach			
	Ipomoea pes caprae		*Ipomoea pes caprae*		*Spinifex littoreus*	
	Rhizosphere	Endophytic	Rhizosphere	Endophytic	Rhizosphere	Endophytic
	cfu/g		cfu/g		cfu/g	
Neutrophiles	400 x 10^7	143 x 10^6	1004 x 10^4	960 x 10^6	600 x 10^3	610 x 10^4
Acidophiles	104 x 10^3	434 x 10^5	110 x 10^4	148 x 10^6	343 x 10^3	280 x 10^4
Alkaliphiles	112 x 10^3	113 x 10^4	198 x 10^3	949 x 10^2	280 x 10^2	210 x 10^2
Haloalkaliphiles	NC	NC	20 x 10^1	20 x 10^1	60 x 10^1	NC
Halophiles	40 x 10^4	310 x 10^2	192 x 10^3	800 x 10^3	620 x 10^2	600 x 10^2
High salt tolerant	400 x 10^3	240 x 10^3	360 x 10^3	600 x 10^4	513 x 10^3	496 x 10^3
Salt tolerant	200 x 10^4	150 x 10^5	286 x 10^3	390 x 10^4	440 x 10^2	320 x 10^2
Diazotrophs	220 x 10^4	400 x 10^4	800 x 10^4	600 x 10^5	200 x 10^3	600 x 10^3

Table C Post monsoon

Expected Bacterial groups	Miramar beach *Ipomoea pes caprae*		Aswem Mandrem beach *Ipomoea pes caprae*		Aswem Mandrem beach *Spinifex littoreus*	
	Rhizosphere	Endophytic	Rhizosphere	Endophytic	Rhizosphere	Endophytic
	cfu/g		cfu/g		cfu/g	
Neutrophiles	900×10^6	1600×10^6	21×10^8	108×10^6	65×10^5	920×10^6
Acidophiles	300×10^3	210×10^3	187×10^2	433×10^2	230×10^2	300×10^4
Alkalophiles	30×10^2	20×10^2	501×10^2	28×10^2	1031×10^2	52×10^2
Haloalkalophiles	NC	NC	NC	NC	NC	NC
Halophiles	21×10^2	744×10^2	19×10^2	50×10^2	616×10^2	14×10^2
High salt tolerant	22×10^2	250×10^3	36×10^2	10×10^2	88×10^2	20×10^2
Salt tolerant	260×10^3	300×10^2	84×10^4	80×10^4	75×10^4	44×10^4
Diazotrophs	300×10^5	200×10^5	300×10^5	600×10^4	100×10^4	100×10^6

(245×10^6 cfu/g) while the endophytic bacterial counts were maximum during postmonsoon period (920×10^6 cfu/g). Rose (1988) reported that the populations of bacteria in marine sand dune ecosystem were higher in winter than during summer sampling periods in all communities. In the monsoons, the heavy rainfall leads to continous wash out of nutrients leading to low counts of bacteria. However heavy leaf litter during monsoons provides a good medium for the growth of decomposers like bacteria and fungi. After monsoons the accumulation of degraded foliage with particulate matter, utilized by the heterotrophic bacteria, leads to an increase in bacterial counts in the postmonsoon period. Thus the ecological changes in the environment are reflected on the bacterial population of the ecosystem.

Further, acidophiles showed higher counts in monsoon followed by premonsoon and postmonsoon. The rhizosphere of *Ipomoea pes caprae* were found to harbour maximum counts of acidophiles (365×10^4 cfu/g) in plants collected from Miramar beach (premonsoon) followed by plants collected from Aswem Mandrem beach (110×10^4 cfu/g, monsoon) whereas the endophytic counts of acidophiles were maximum (148×10^6 cfu/g) in plants collected from Aswem Mandrem(monsoon) followed by plants collected from Miramar beach (434×10^5 cfu/g, monsoon). The rhizospheric counts of acidophiles associated with *Spinifex littoreus* were maximum in monsoon (343×10^3 cfu/g) while endophytic counts were maximum in postmonsoon (300×10^4 cfu/g).

Alkalophilic counts ranged from 10^2-10^3 in premonsoon period, 10^2 -10^4 cfu/g in monsoon and 10^2 in postmonsoon period while halophilic count ranged from 10^2 to 10^4 cfu/g in premonsoon period, 10^2 to 10^4 cfu/g in monsoon period and 10^2 cfu/g in postmonsoon period. The rhizospheric counts of alkaliphiles associated with *Ipomoea*

pes caprae was maximum (585 x 10^3 cfu/g) in plants collected from Aswem Mandrem (premonsoon) whereas the endophytic count of alkaliphiles associated with *Ipomoea pes caprae* was maximum (113 x 10^4 cfu/g) in plants collected from Miramar (monsoon). The rhizospheric count of alkaliphiles associated with *Spinifex littoreus* was maximum (1031 x 10^2 cfu/g) in postmonsoon. It has been reported that the ratio of alkaliphiles to neutrophiles found in soil is about 1:10 to 1:100 (Horikoshi, 1995; Grant *et al*, 1990).

The rhizospheric counts of halophiles associated with *Ipomoea pes caprae* was found to be maximum (40 x 10^4 cfu/g) in plants collected from Miramar (monsoon) followed by plants collected from Aswem Mandrem (192 x 10^3 cfu/g)(monsoon). The rhizospheric counts of halophiles associated with *Spinifex littoreus* were found to be maximum (745 x 10^3 cfu/g) in premonsoon while the endophytic counts were maximum in monsoon (600 x 10^2 cfu/g). High counts of salt tolerant bacteria were obtained in the postmonsoon period probably due to the rich nutrients available during the season through foliage degradation, which help the bacteria withstand the saline stress. In another study, restricted to offshore and nearby shore sediments off the Mangalore Coast in the south-west coast of India, marine sediments were found to have a high counts (10^5–10^7/g) of halotolerant eubacterial microorganisms (Raghavan & Furtado, 2004). Bacterial counts ranged between (1.5 ± 0.2) x 10^8 cells g^{-1} and (53.1 ± 16.0) x 10^8 cells g^{-1} in sandy and muddy sediments, respectively (Luna *et al*,2002). Haloalkaophiles were not detectable in premonsoon and postmonsoon period however in monsoon period the count was seen but as low as (10^1cfu/g) from Aswem Mandrem. During the monsoon, heavy rainfall leads to considerable accumulation of plant litter. The degradation of this plant litter gives rise to

degradation products resulting in the change in the alkalinity. Such variation gives rise to salt tolerant organisms growing in presence of high pH in the coastal sand dunes.

Amongst the other physiological groups it is reflected that the nitrogen fixing organisms play an important role in maintaining the biogeochemical cycles. Besides the free living organisms which can fix nitrogen are important in such stressful ecosystems. Preliminary studies done in this laboratory have shown the presence of diazotrophs(Godinho and Bhosle, 2004). It was therefore of interest to see the presence of free living diazotrophs in the samples collected. It was interesting to note that this ecosystem not only harbours free living nitrogen fixers in the rhizosphere to an extent of 300 x 10^5 cfu/g but also showed the presence of these bacteria as endophytes with counts ranging from 40 x 10^3 cfu/g to 100 x 10^6 cfu/g. The diazotrophic count ranged from 10^2 cfu/g to 10^4 cfu/g in premonsoon, 10^3 cfu/g to 10^5 cfu/g in monsoon and 10^4 cfu/g to 10^6 cfu/g in post monsoon period. The rhizospheric count of diazotrophs associated with *Ipomoea pes caprae* was maximum (300 x 10^5 cfu/g) in plants collected from Miramar and Aswem Mandrem(postmonsoon) whereas the endophytic counts of diazotrophs associated with *Ipomoea pes caprae* were maximum (600 x 10^5 cfu/g) in plants collected from Aswem Mandrem(monsoon). The rhizospheric counts of diazotrophs associated with *Spinifex littoreus* was maximum (100 x 10^4 cfu/g) in postmonsoon whereas the endophytic counts of diazotrophs was maximum(100 x 10^6 cfu/g) in postmonsoon. It is reported that in normal fields a large number of diazotrophs are present, however not many are reported to be found in extreme environments. The sand dune ecosystem being unique to the fact that it supports the growth of certain type of vegetation which has

rhizosphere colonizing microorganisms, such bacteria perhaps are more relevant as rhizosphere bacteria which support plant growth (Beena *et al*, 2000). Dalton *et al,* 2004, reported the presence of large populations (10^5 to 10^6 cfu/fresh weight) of nitrogen fixing bacteria associated with sand dune grasses *Ammophila arenaria* and *Elymus mollis.* These grasses from temperate climates contain endophytic diazotrophic bacteria that may contribute to the phenomenally successful growth of these grasses on nutrient poor sand by contributing to the nitrogen nutrition of the host plant.

Overall, it was observed that endophytic bacteria 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) (Table 2.2). 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 were found to be comparatively lower in contrast to marine sediment counts or soil counts (Desai *et al,* 2004). Colonies obtained on different media showed wide variation in cultural characteristics with, neutrophiles and alkaliphiles showing consistent growth (Table 2.3).

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 (Table 2.4a & b). In

Table 2.2 Total viable counts of bacterial groups associated with unvegetated areas of sand dunes

Bacterial groups	Miramar beach	Aswem Mandrem beach	
		S1	S2
Total viable count	14×10^3	3×10^3	4×10^3
Acidophiles	265×10^3	NC	1×10^3
Alkaliphiles	2×10^2	NC	1×10^2
Haloalkaliphiles	NC	NC	NC
Halophiles	NC	NC	NC
High salt tolerant	5×10^2	NC	NC
Salt tolerant	53×10^2	1×10^2	6×10^2
Diazotrophs	5×10^3	300×10^3	NC

S1- sampling site 1 **S2-sampling site 2**

Table 2.3 Neutrophiles and alkaliphiles in rhizosphere and endosphere

Sand dune vegetation		PREMONSOON		POSTMONSOON		MONSOON	
		N	A	N	A	N	A
		(cfu/g)		(cfu/g)		(cfu/g)	
Miramar							
Ipomoea pes-caprae	**R**	70 X 10^3	40 x 10^2	900 x 10^6	30 x 10^2	400 x 10^7	112 x 10^3
	E	25 X 10^7	25 x 10^2	1600 x10^6	20 x 10^2	143 x 10^6	113 x 10^4
Aswem Mandrem							
Ipomoea pes-caprae	**R**	20 x 10^5	585 x 10^3	21 x 10^8	501 x 10^2	1004 x 10^4	198 x 10^3
	E	835 x 10^5	40 x 10^2	108 x 10^6	28 x 10^2	960 x 10^6	949 x 10^2
Spinifex littoreus	**R**	245 x 10^6	16 x 10^2	65 x 10^5	1031 x 10^2	600 x 10^3	280 x 10^2
	E	150 x 10^5	26 x 10^2	920 x 10^6	52 x 10^2	610 x 10^4	210 x 10^2

R-Rhizosphere bacteria E-Endophytic bacteria

N – Neutrophiles
A – Alkaliphiles

Table 2.4a Characterization of neutrophilic bacteria

Sand dune vegetation		Premonsoon		Postmonsoon		Monsoon	
		Gram positive	Gram negative	Gram positive	Gram negative	Gram positive	Gram negative
Miramar							
Ipomoea pes- caprae	R	18	0	3	0	4	2
	E	7	3	4	0	19	7
Aswem Mandrem							
Ipomoea pes- caprae	R	10	0	3	0	17	0
	E	11	2	6	3	19	0
Spinifex littoreus	R	12	0	5	0	2	0
	E	12	0	9	0	29	0

R-Rhizosphere bacteria E-Endophytic bacteria

Table 2.4b Characterization of alkaliphilic bacteria

Sand dune vegetation		Premonsoon		Postmonsoon		Monsoon	
		Gram positive	Gram negative	Gram positive	Gram negative	Gram positive	Gram negative
Miramar							
Ipomoea pes- caprae	R	4	2	9	0	4	0
	E	4	0	10	0	12	5
Aswem Mandrem							
Ipomoea pes- caprae	R	27	2	6	0	5	1
	E	12	0	1	0	10	2
Spinifex littoreus	R	6	1	3	0	3	0
	E	4	2	3	0	1	0

R-Rhizosphere bacteria E-Endophytic bacteria

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 *Ipomoea pes caprae* from Miramar. Among the alkaliphiles majority of the isolates were gram positive non sporulating rods compared to neutrophilic isolates which were sporulating rods. The alkaliphiles were categorized into alkalitolerant, facultative alkaliphiles and alkalitolerant (Table 2.5). 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 majority of the alkaliphiles were facultative alkaliphiles.

In mangrove ecosystem the abundance and biomass of organisms living in the canopy, on or beneath the forest floor and in associated waterways often vary seasonally in relation to rainfall (Alongi, 2002). Mangroves provide a unique ecological environment for diverse bacterial communities and are fundamental to the functioning of these habitats. Bacterial counts are generally higher on attached mangrove vegetation than they are on fresh leaf litter. This is probably because attached, undamaged leaves leak amino acids and sugars but the leaf litter does not release much. Shome *et al,* (1995), isolated thirty-eight distinct bacteria from mangrove leaf litter and sediments in south Andaman which were generally gram-positive (76.3%). Bacterial colonies appear shortly after the litter has been colonized by fungi. The bacteria grow quickly and can reach very high densities. Zhuang and Lin (1993) measured bacterial densities from 2 x 10^5 to 10 x 10^5 g^{-1} on *Kandelia candel* leaves that had decomposed for 2-4 weeks. Mini Raman and Chandrika, 1993 reported that the total microflora shows a seasonal cycle in their counts. The bacterial

Table 2.5 Percentage distribution of facultative and alkalitolerant rhizosphere and endophytic bacteria in three seasons

Season	Sand dune vegetation	Facultative Alkaliphiles		Alkalitolerant		Pigmented strains	
		R	E	R	E	R	E
MONSOON	MIRAMAR						
	Ipomoea pes caprae	80	81	ND	19	80	94
	ASWEM MANDREM						
	Ipomoea pes caprae	100	100	ND	ND	100	91
	Spinifex littoreus	100	100	ND	ND	33	60
POST MONSOON	MIRAMAR						
	Ipomoea pes caprae	ND	80	86	ND	43	80
	ASWEM MANDREM						
	Ipomoea pes caprae	100	100	ND	ND	67	ND
	Spinifex littoreus	100	100	ND	ND	33	25
PREMONSOON	MIRAMAR						
	Ipomoea pes caprae	89	100	ND	ND	70	17
	ASWEM MANDREM						
	Ipomoea pes caprae	67	50	33	50	50	ND
	Spinifex littoreus	100	100	ND	ND	ND	ND

R-Rhizosphere bacteria E-Endophytic bacteria

ND –Not detected

Facultative alkaliphiles – Bacteria which show optimal growth at 10.0 or above but which can grow well in the neutral pH range

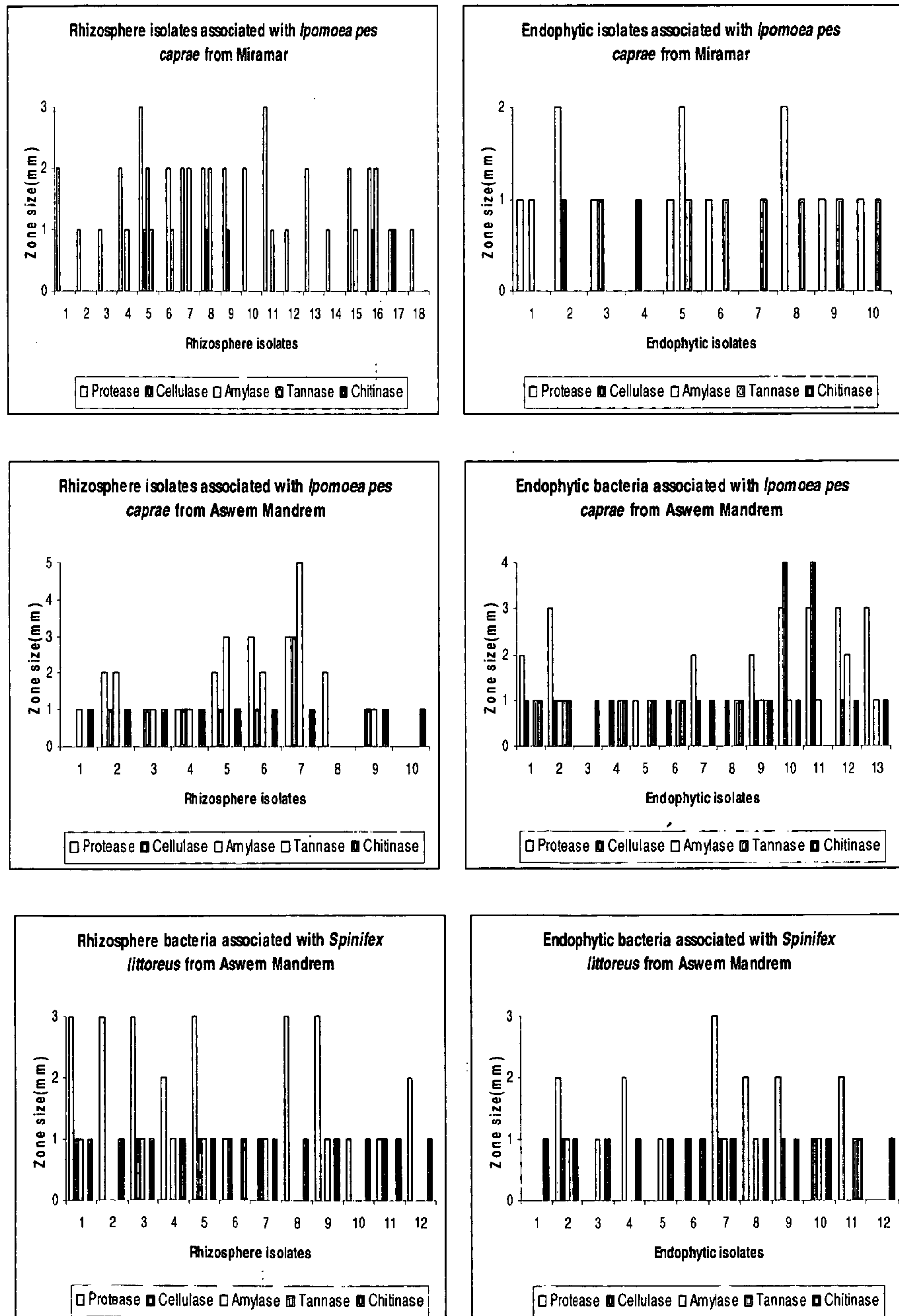
Alkalitolerant – Bacteria which show optimal growth in the pH range of 7.0-9.0 but cannot grow above pH 9.5

counts are maximum during the postmonsoon months and the counts of the fungi and actinomycetes are maximum during the monsoon months. In sand dunes, the survival of the bacterial communities is dependent on the plant litter and the stress conditions making it favourable for the isolates which can withstand these conditions and degrade available organic matter.

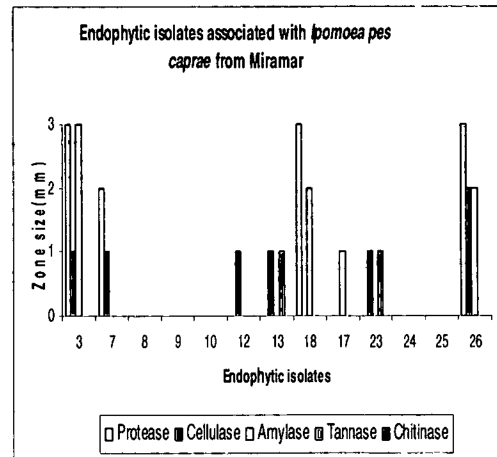
Enzymatic potential of the sand dune bacterial isolates was therefore determined. The bacterial flora of the coastal sand dune ecosystem possess hydrolytic enzymes involved in degradative processes. Extracellular enzyme activity (EEA) is generally recognized as the key step in degradation and utilization of organic polymers by bacteria, since only compounds with molecular mass lower than 600 Da can pass through cell pores (Fabiano and Danovara, 1998). Amongst the neutrophilic and alkalophilic bacteria screened, 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 (Figs 2.2 A, B & C and Fig. 2.3), the premonsoon and monsoon bacterial isolates showed good enzymatic activity as compared to postmonsoon period. The bacteria were screened for protease, cellulase, amylase, tannase and chitinase enzymes. Neutrophilic rhizosphere isolates from Miramar associated with *Ipomoea pes caprae* were found to be good producers of protease (1-3mm), cellulase (1mm) and amylase (1-2mm) enzymes in premonsoon and postmonsoon period while the endophytic isolates associated with *Ipomoea pes caprae* from Miramar were good producers of protease (1-3mm), cellulase (1-2mm),

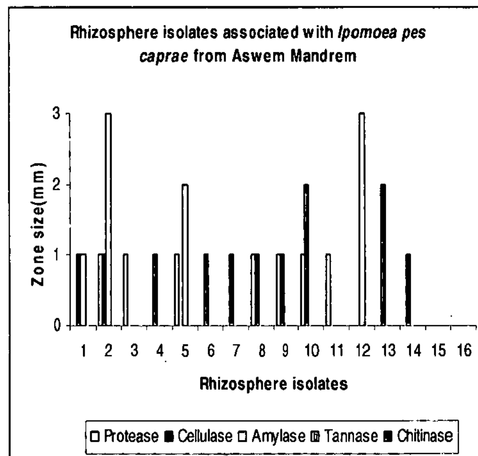
Fig 2.2 Comparative activity of multiple enzyme production by rhizosphere and endophytic neutrophiles associated with *Ipomoea pes caprae* and *Spinifex littoreus.*

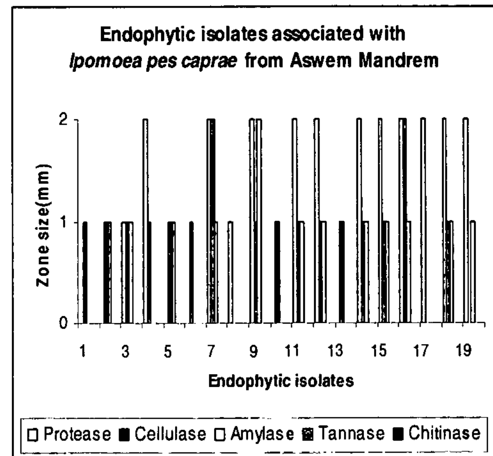
A. PREMONSOON

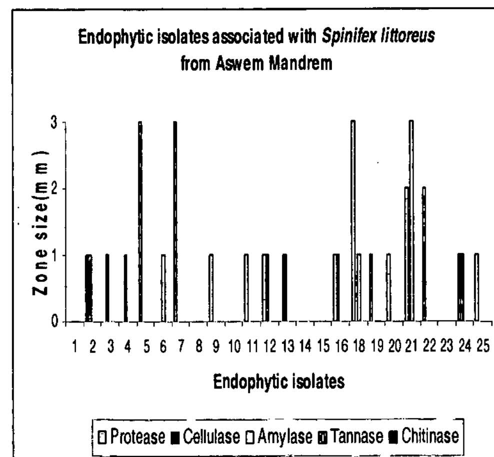


B. MONSOON

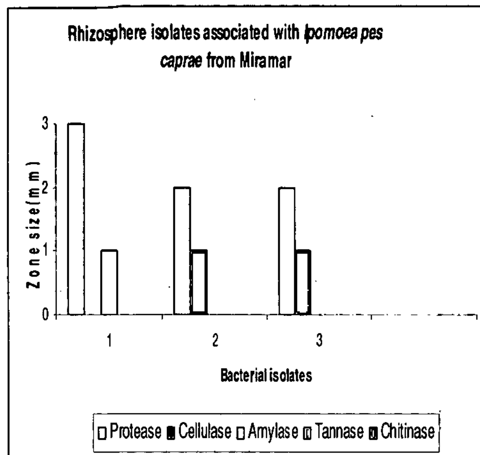


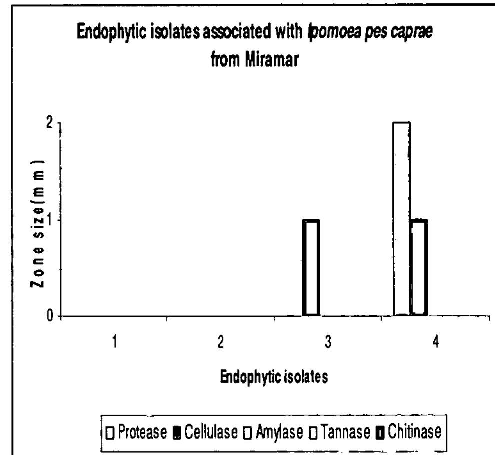


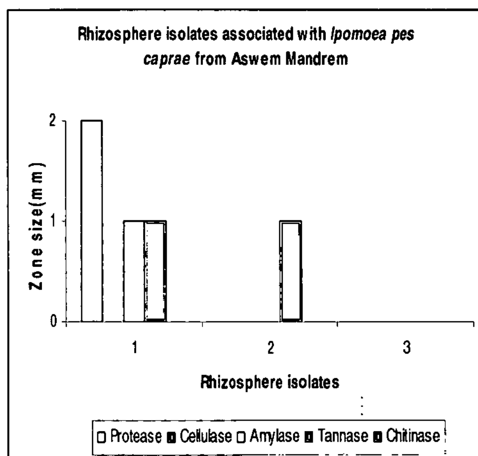


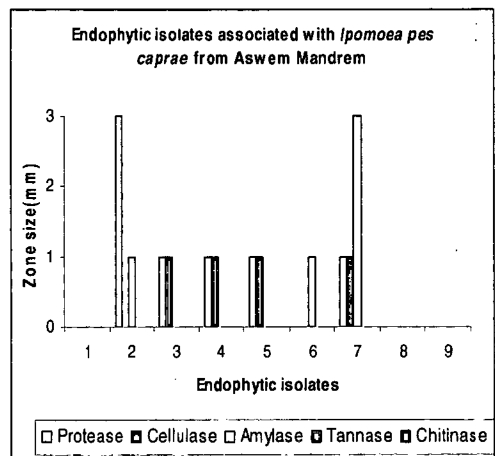


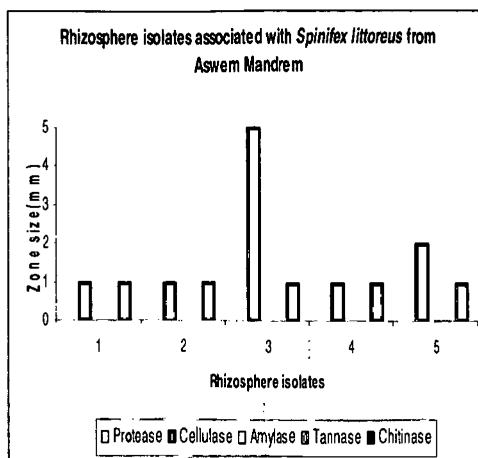
C. POSTMONSOON

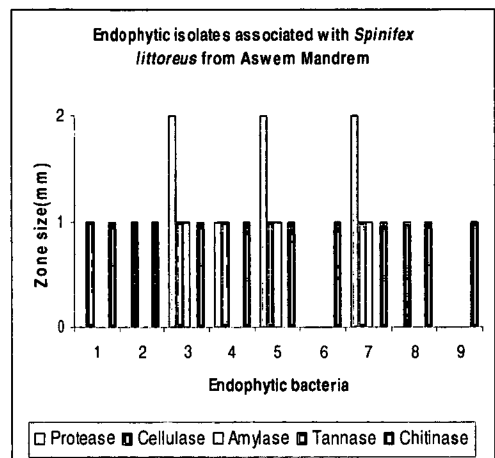












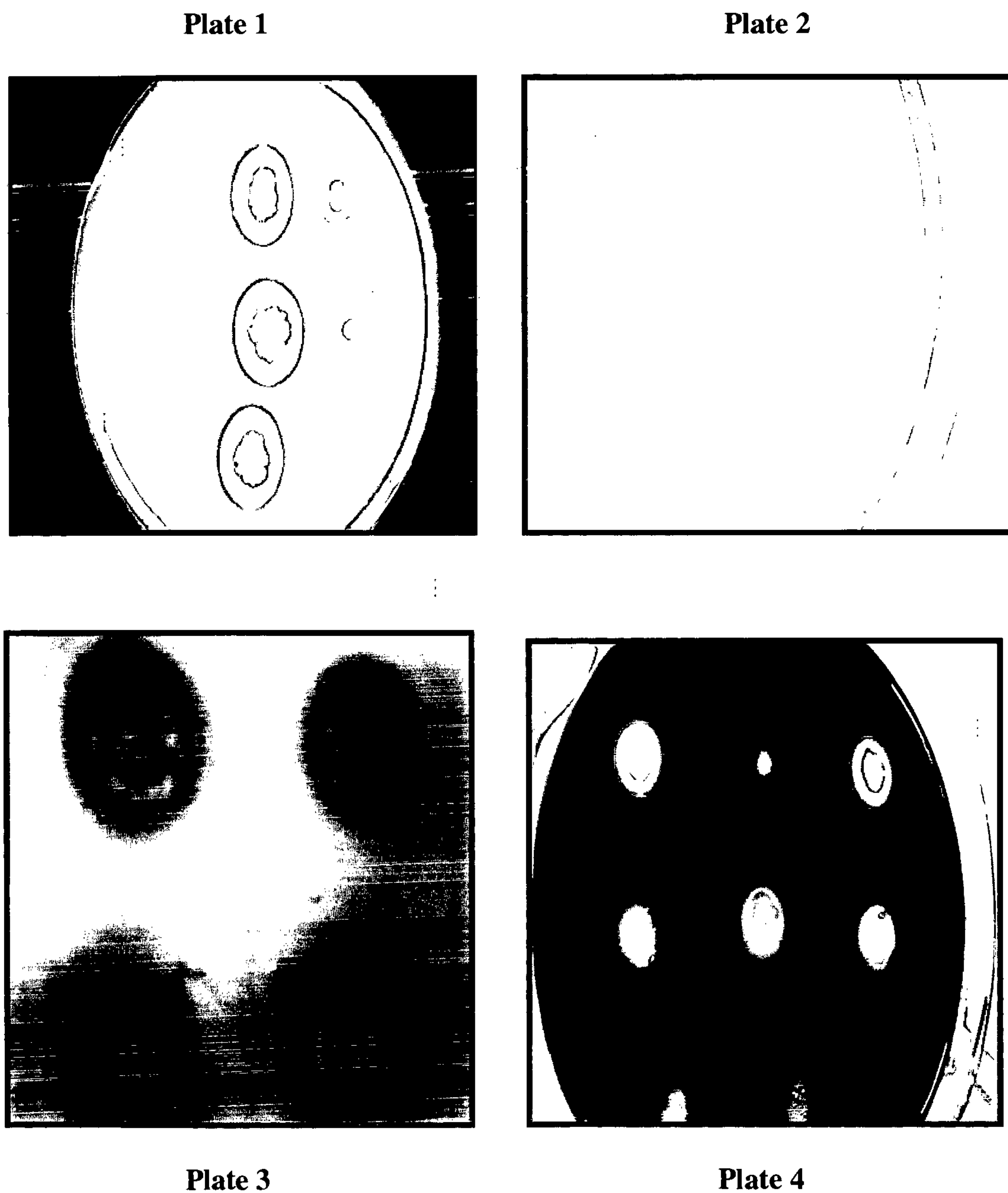


Fig 2.3 Neutrophilic bacterial isolates showing production of enzymes, Protease (plate 1), Cellulase (plate 2), Tannase (plate 3) and Amylase (plate 4) enzymes.

amylase (1-3mm) and tannase (1mm) in premonsoon and monsoon period. In contrast neutrophilic rhizosphere isolates from Aswem mandrem associated with *Ipomoea pes caprae* were good producers of amylase (1-5mm), protease (1-3mm), cellulase (1-3mm) and chitinase (1mm) enzymes in the premonsoon and monsoon period while neutrophilic endophytic isolates were good producers of cellulase (1-4mm), protease (1-3mm), amylase (1-2mm) , tannase (1mm) and chitinase (1mm) in premonsoon period. The neurophilic rhizosphere isolates associated with *Spinifex littoreus* are potent producers of protease (1-3mm), cellulase (1mm), amylase (1mm) and chitinase (1mm) in the premonsoon period. Isolates with multiple enzyme activity though small in number are important in such ecosystems. Interestingly the bacterial isolates from coastal sand dune ecosystem also exhibited multiple enzyme activities e.g amylase, tannase, protease, 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.

Among the alkalophilic isolates(Figs 2.4 A, B & C and Fig 2.5) screened for the above enzymes, the premonsoon isolates showed good enzymatic activity followed by postmonsoon and monsoon period including lipase and xylanase activity. Alkalophilic rhizosphere isolates from Miramar associated with *Ipomoea pes caprae* were found to be good producers of protease (1-8mm), xylanase (1-4mm), amylase (1-5mm), cellulase (1mm) enzymes in the premonsoon and postmonsoon period while the alkalophilic endophytic isolates associated with *Ipomoea pes caprae* were good producers of protease (1-6mm) and amylase (1-9mm) in premonsoon and postmonsoon. Among the alkalophilic rhizosphere isolates from Aswem Mandrem associated with *Ipomoea pes caprae* potent producers of protease (1-10mm), xylanase

Fig 2.4 Comparative activity of multiple enzyme production by rhizosphere and endophytic alkalophilic associated with *Ipomoea pes caprae* and *Spinifex littoreus*

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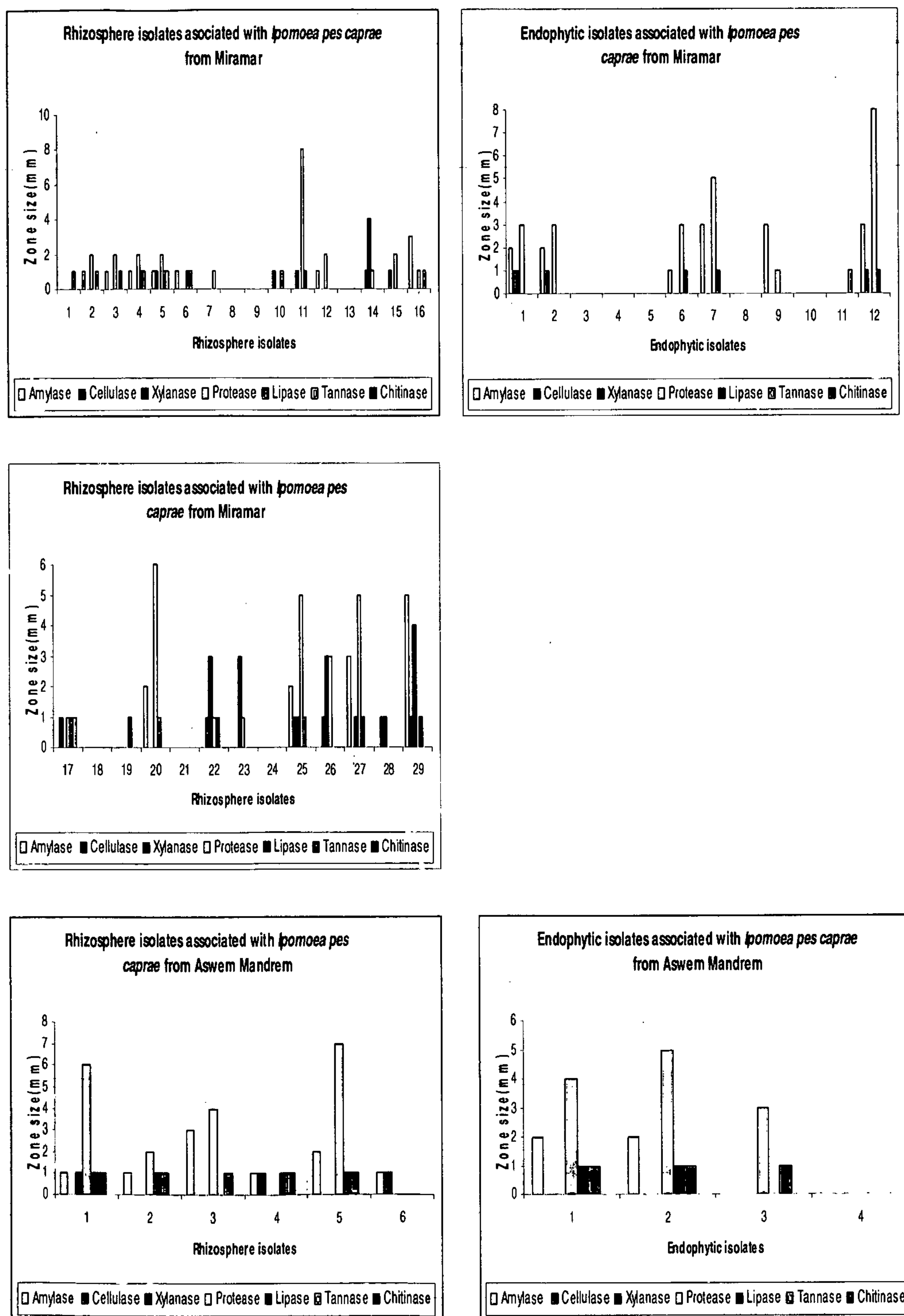
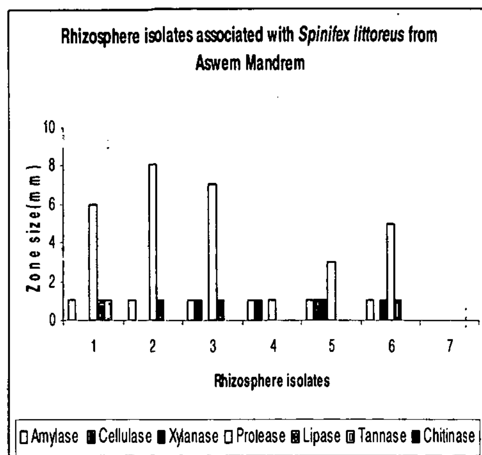
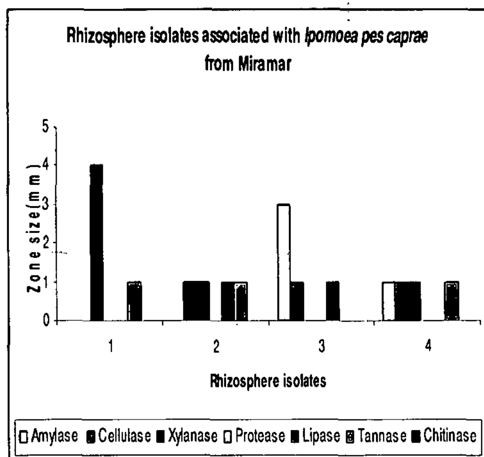
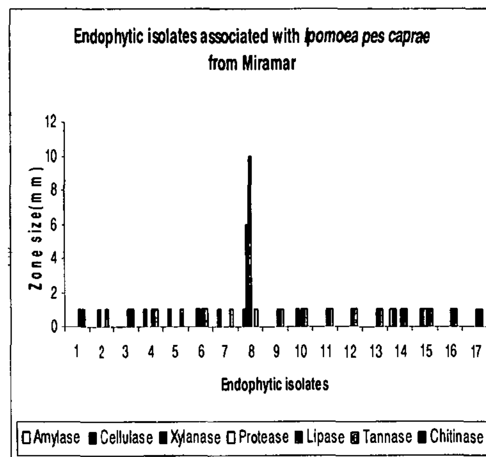
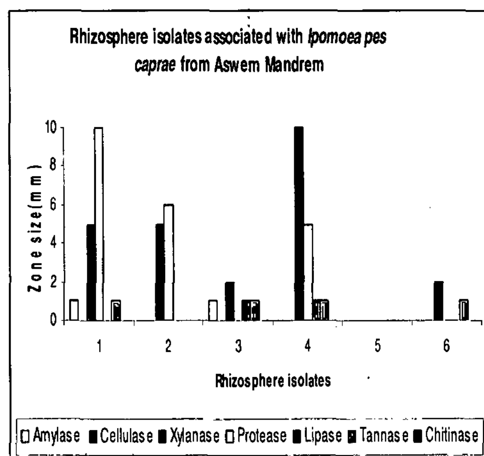


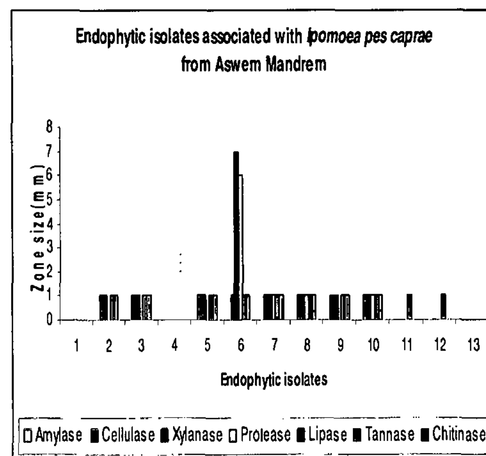

Rhizosphere isolates associated with *Spinifex littoreus* from Aswem Mandrem
Zone size (m m)
10
8
6
4
2
0
1
2
3
4
5
6
7
Rhizosphere isolates
Amylase Cellulase Xylanase Protease Lipase Tannase Chitinase

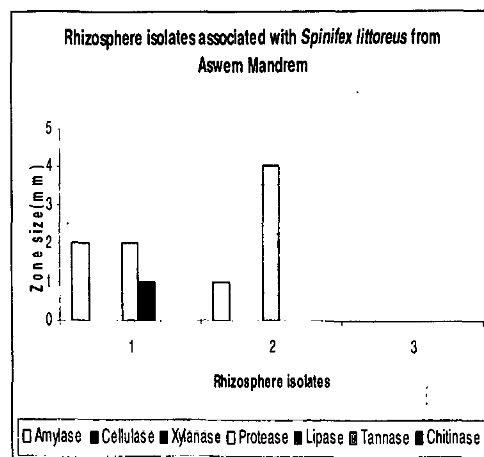
B.MONSOON

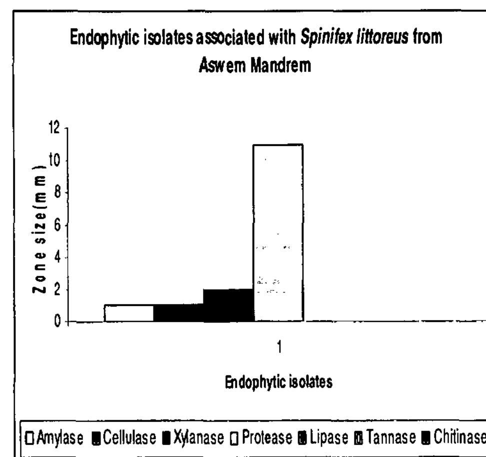




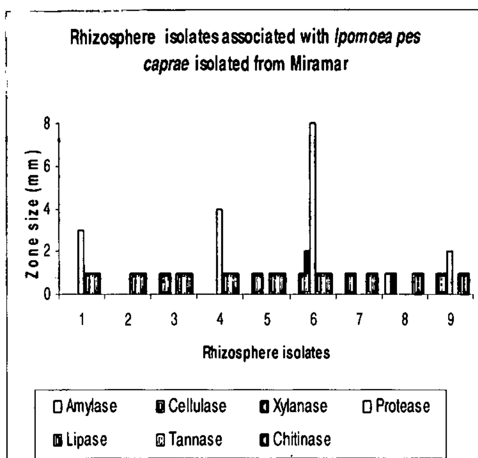


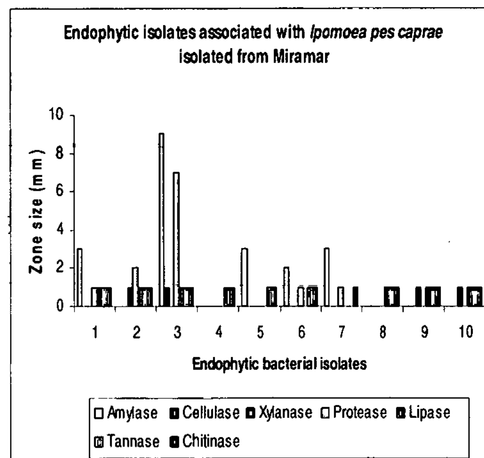


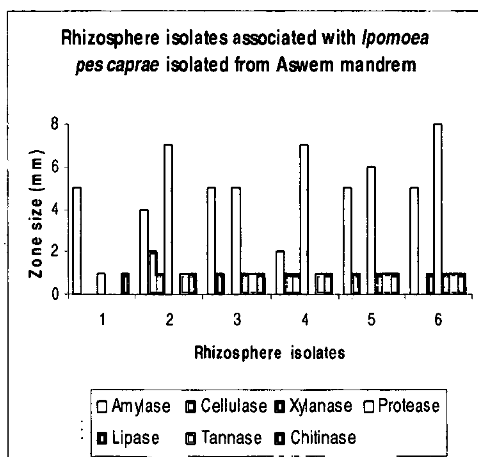


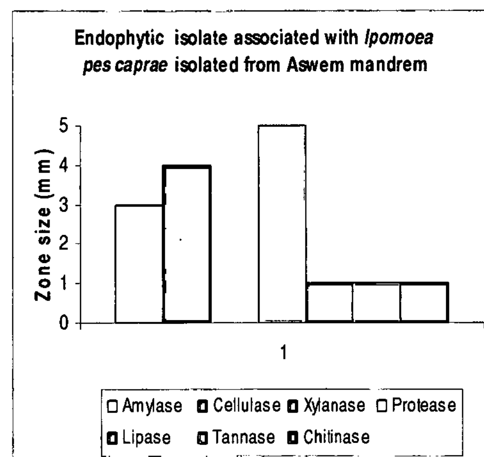


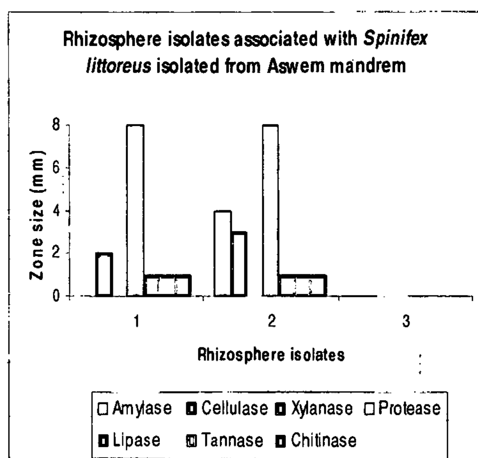
C. POSTMONSOON











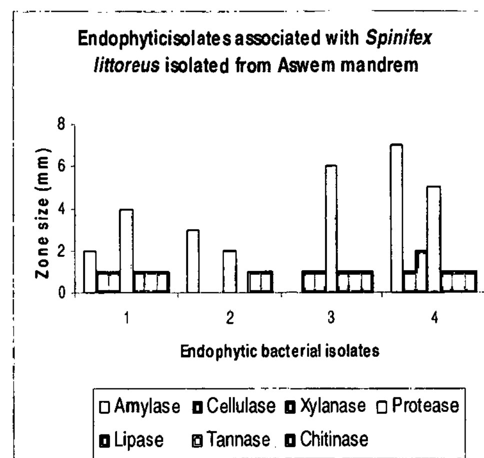
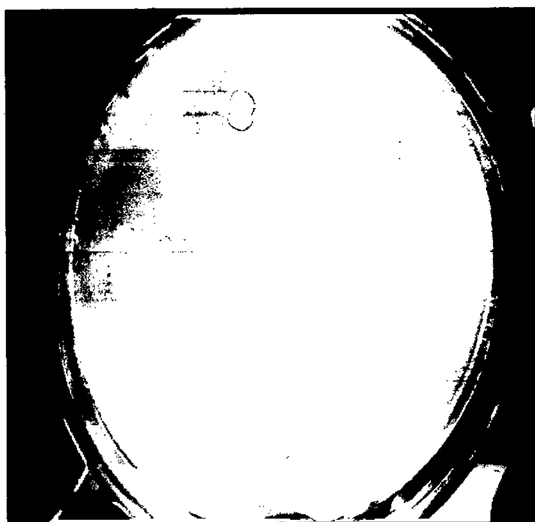


Plate 1 **Plate 2**



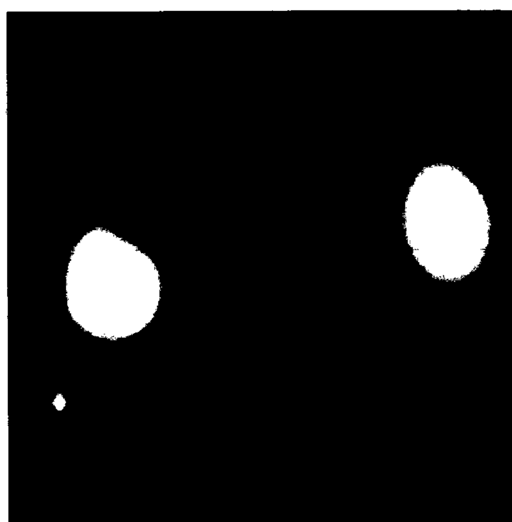


Plate 3

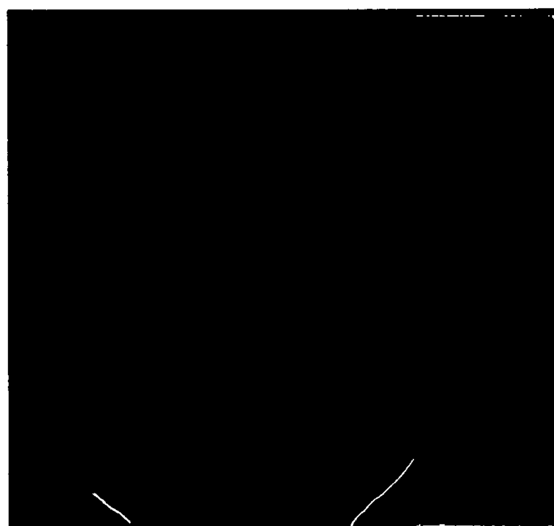


Fig 2.5 Alkalophilic bacterial isolates showing production of enzymes, Protease (plate 1), Amylase (plate 2) and Cellulase (plate 3)

(1-10mm) and cellulase (1-3mm) enzymes were detected in premonsoon, monsoon and postmonsoon period while among the endophytic isolates protease (1-7mm) and cellulase (1-3mm) enzymes were detected in premonsoon. Among the alkalophilic rhizosphere isolates from Aswem Mandrem associated with *Spinifex littoreus* potent producers of protease (1-8mm) were detected in premonsoon while endophytic isolates associated with *Spinifex littoreus* were good producers of protease (2-6mm) and amylase (2-7mm) in postmonsoon period. Desai *et al,* 2004 in their studies on akaliphiles from estuarine mangrove regions of Goa, India reported that the alkaliphiles produced amylase (39%), protease (50%) and lipase (100%) enzymes.

Microbial enzymes are relatively more stable than corresponding enzymes derived from plants and animals (Chandrasekaran, 1997). Lokabharathi *et al,* (1977) reported that the proteolytic and starch hydrolyzing microflora and strains that expressed dehydrogenase activity were found to be abundant from sediments from beaches of Goa on the west coast of India. Shanta Nair *et al,* (1978) reported that marine sediments on the west coast contained cellulase, lipase, protease and urease producing bacteria in high numbers. In the mangrove ecosystem there are different groups of bacteria which get nourished by detritus and in turn help the mangrove ecosystem in different ways. These bacteria perform various activities in the mangrove ecosystem like photosynthesis, nitrogen fixation, methanogenesis, agarolysis, production of antibiotics and enzymes such as arylsuphatase, L-glutaminase, chitinase, L-asparaginase, cellulase, protease and phosphatase etc. which result in the high productivity (Das *et al,* 2006). Rawte *et al,* 2002, reported that the tropical marine and mangrove microflora from the mid-west coast of India were found to be diverse with respect to enzymatic activities. The bacterial counts and the enzyme

activities of the mangrove microflora were found to be influenced by the seasonal variations, as also seen during this study with the bacterial isolates from coastal sand dunes. The microorganisms when subjected to an ecosystem with detritus are known to accumulate certain elements which would help them to survive through nutrient limiting conditions. The isolates were therefore screened for the accumulation of PHA using a standard method.

To increase survival and stress tolerance in changing environments and in competitive settings, microorganisms are known to accumulate polyhydroxyalkanoates (PHAs) where carbon and energy sources may be limited, such as those encountered in the soil and the rhizosphere. Accumulation of PHA 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. It has been proposed that the accumulation and degradation of PHA is one such strategy by which bacteria can improve establishment, proliferation, and survival in competitive settings such as soil and rhizosphere. They found that soil bacteria within the pseudomonads, coryneform and bacilli produce PHA. PHA has been identified in more than 20 bacterial genera including *Azotobacter, Bacillus, Beijerinckia, Alcaligenes, Pseudomonas, Rhizobium* and *Rhodospirillum* (Aslim, 2002). The plate assay method of Shirokitamura and Doi was used to screen bacteria accumulating PHA (Fig 2.6 & Table 2.6)). Interestingly neutrophilic bacteria were found to be rich sources of PHA, since a wide variety

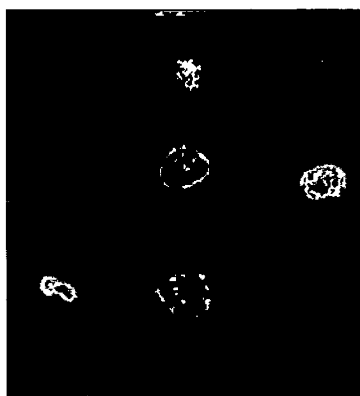


Fig 2.6 Fluorescence exhibited by neutrophilic bacterial isolates grown on E2 medium plate on staining with Nile Blue A.

Table 2.6 Percentage of neutrophilic PHA producers

Sand dune Vegetation	*Ipomoea pes caprae*	*Spinifex littoreus*
Premonsoon		
Aswem Mandrem	15	17
Miramar	43	ND
Monsoon		
Aswem Mandrem	26	35
Miramar	12	ND
Postmonsoon		
Aswem Mandrem	23	ND
Miramar	25	ND

ND – not detected

of heterotrophic bacteria were scored positive for PHA accumulation as compared to alkaliphiles which did not show presence of PHA. However among the isolates screened the maximum PHA accumulators were isolated from rhizosphere and endophyte of *Ipomoea pes caprae* from Miramar (43%) followed by *Spinifex littores* from Aswem Mandrem (35%).

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 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) scored positive for siderophores. On the CAS plate assay the isolates showed orange to yellow orange colour halo around the colonies (Fig. 2.7).

Among the neutrophilic isolates(Figs 2.8 A,B & C), the majority of the premonsoon isolates scored positive for siderophore production followed by postmonsoon and monsoon isolates. Majority of neutrophilic rhizosphere isolates from Miramar associated with *Ipomoea pes caprae* were found to be good producers of siderophores in premonsoon and postmonsoon period (1-2mm) while majority of endophytic isolates were found to produce siderophores in premonsoon and monsoon period (1-2mm). In contrast, majority of neutrophilic rhizosphere isolates from Aswem Mandrem associated with *Ipomoea pes caprae* produced siderophores in monsoon (1-3mm)while majority of the endophytic isolates associated with *Ipomoea*

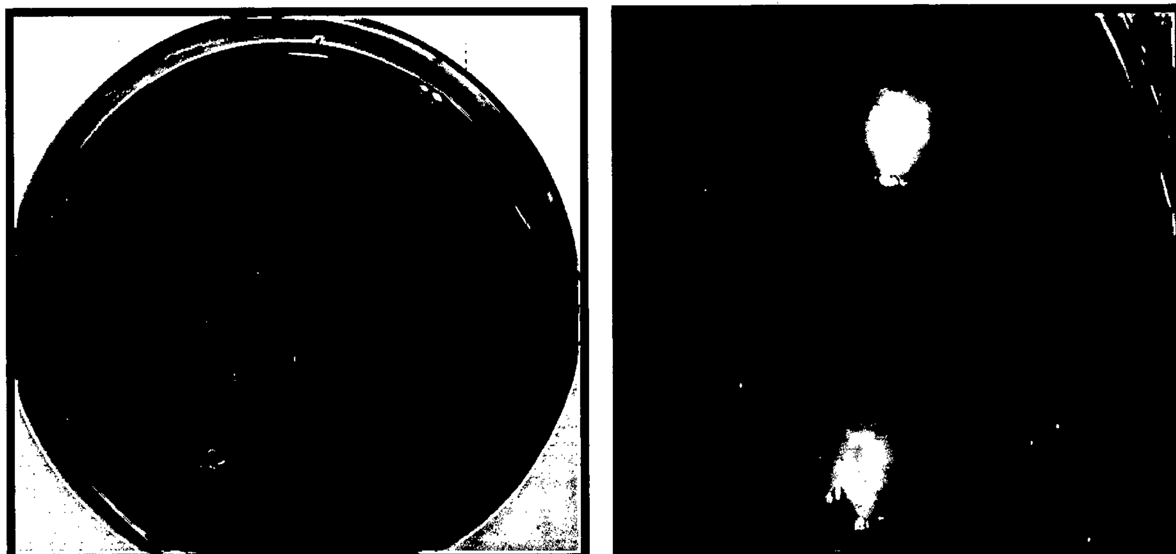


Fig 2.7 The yellow orange halo surrounding the bacterial colony is indicative of the production of an Fe binding compound such as siderophore, which removes Fe (III) from the Fe (III)–CAS HDTMA complex in the plate and turns the blue dye to yellow color.

Fig 2.8 Screening of neutrophilic rhizosphere and endophytic bacteria for production of siderophores

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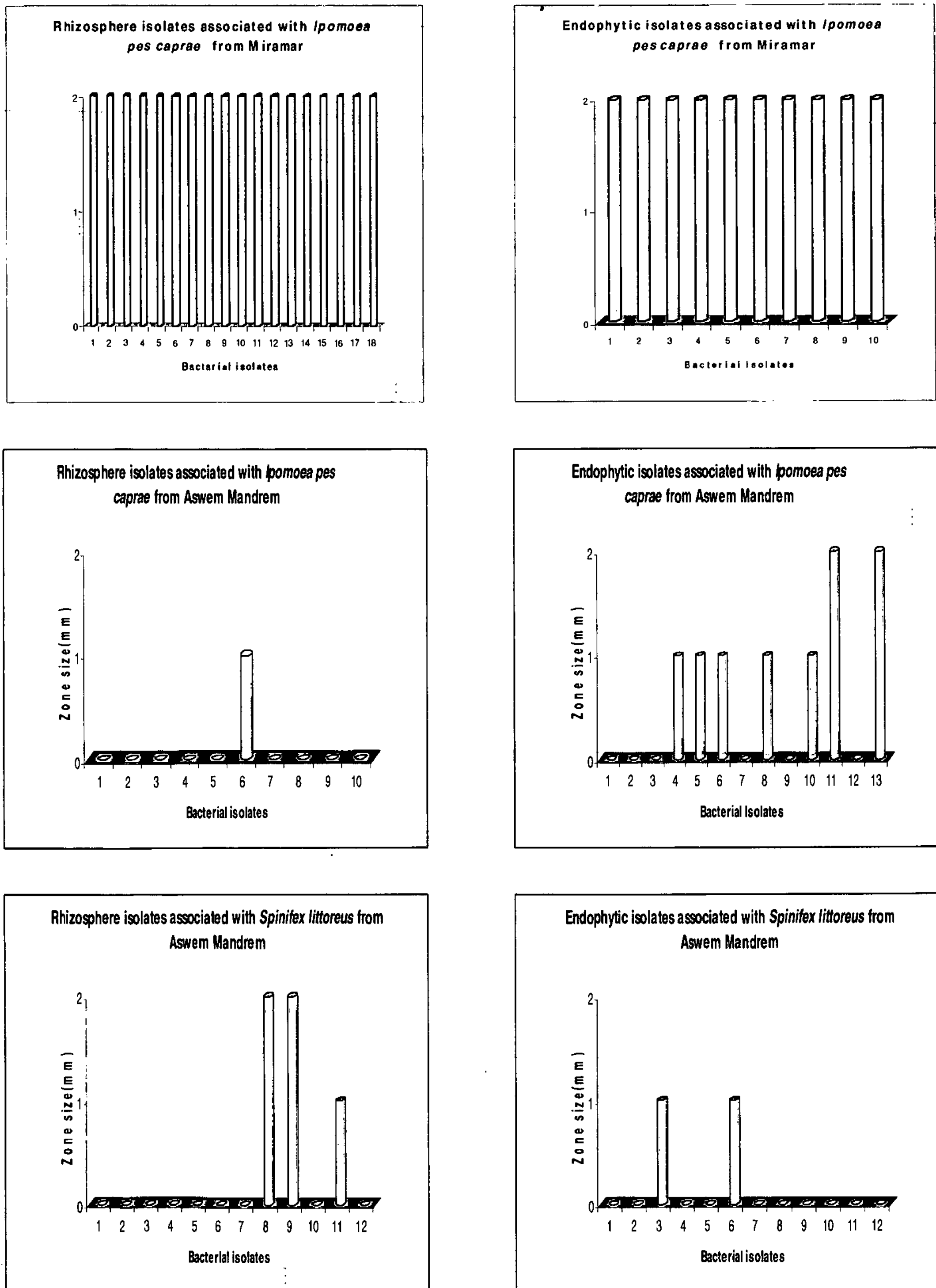
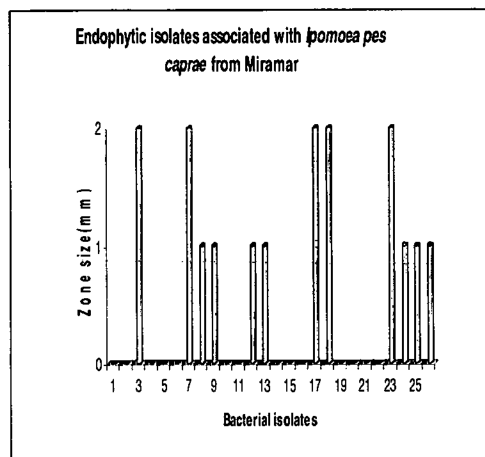
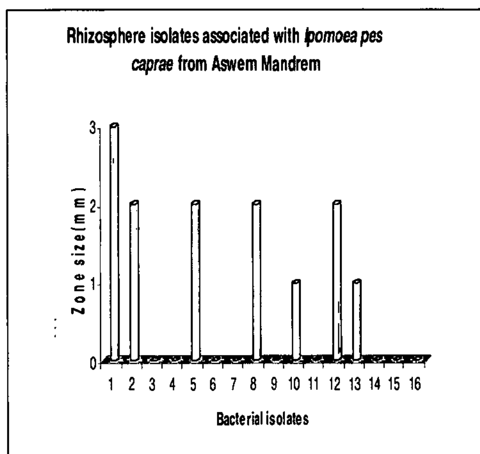
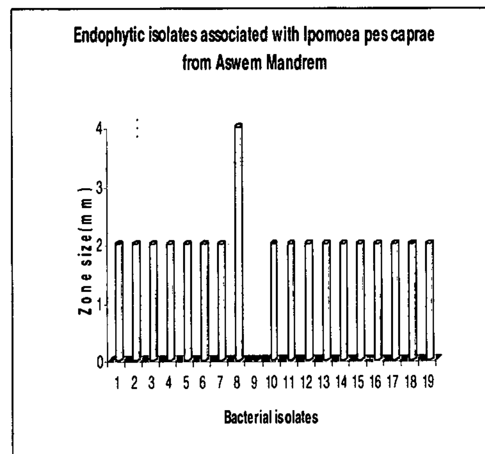
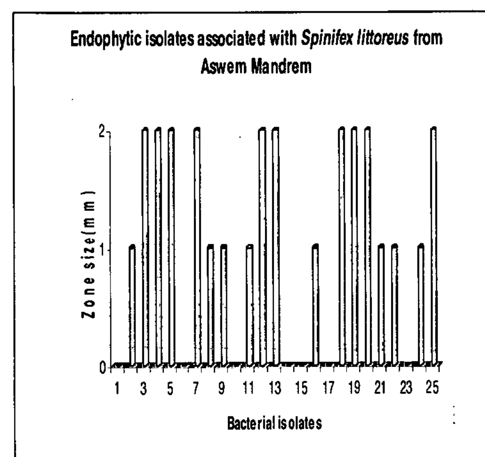


B. MONSOON

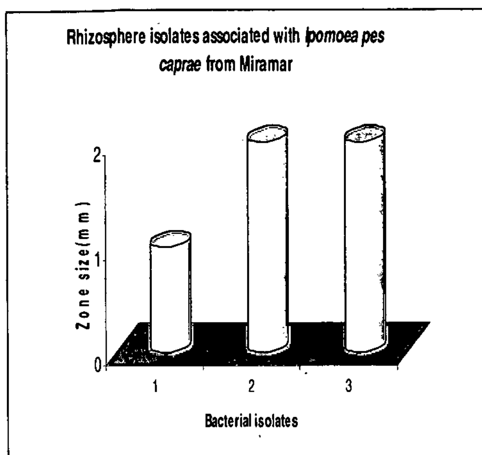


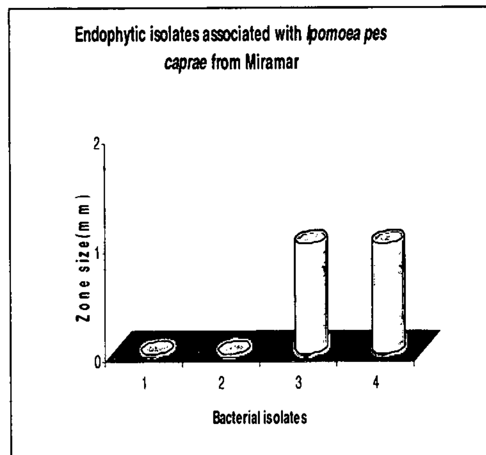


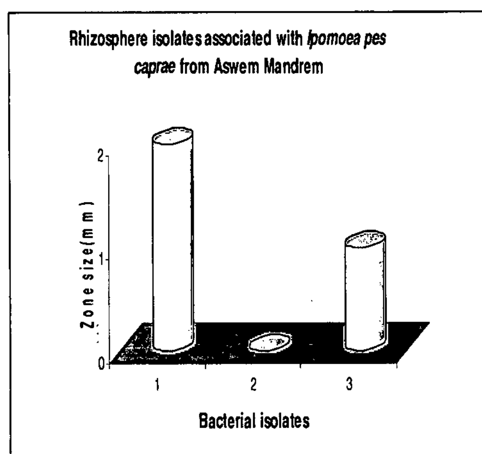


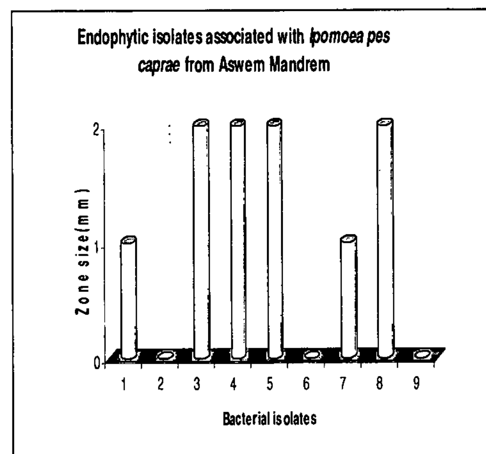


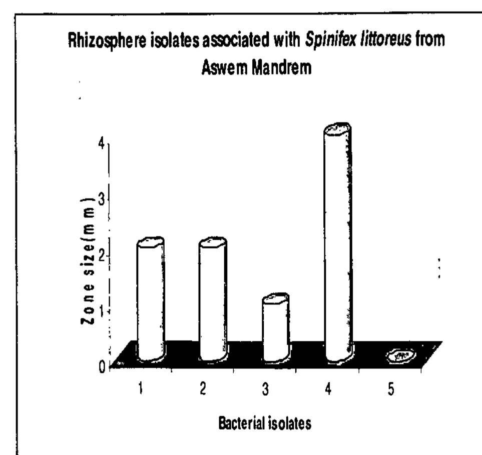
C. POSTMONSOON

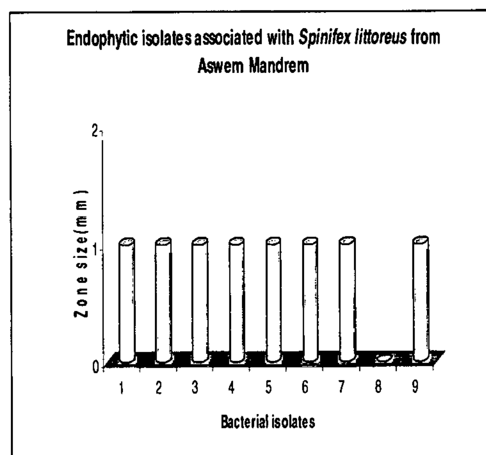












pes caprae isolated in premonsoon, monsoon and post monsoon periods produced siderophores (1-4mm).The neutrophilic isolates from Aswem Mandrem associated with *Spinifex littoreus* isolated in postmonsoon period were also found to be good siderophores producers (1-4mm) while majority of endophytic isolates from Aswem mandrem associated with *Spinifex littoreus* and isolated in the monsoon and post monsoon produced siderophores.

Among the alkaliphiles (Fig 2.9 A,B & C), there was a comparatively less number of isolates producing siderophores as against the neutrophilic isolates. Siderophores are known to be produced by different bacteria especially in iron limiting conditions. It has been reported that the most predominant isolates producing siderophores is *Escherichia coli*, *Bacillus megaterium*, *Pseudomonas* spp., *Mycobacterium* sp. and the plant pathogen *Agrobacterium tumefaciens* (Vandenbergh *et al*, 1983). Such organisms in the rhizosphere are therefore useful for supporting the growth of plants. 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. The ability of these isolates to solubilize inorganic phosphates was therefore screened.

Fig 2.9 Screening of alkalophilic rhizosphere and endophytic bacteria for production of siderophores

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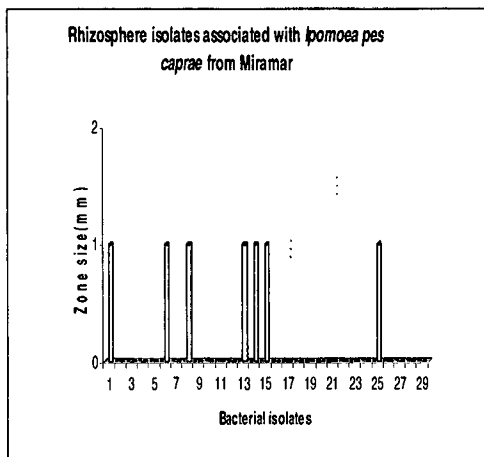

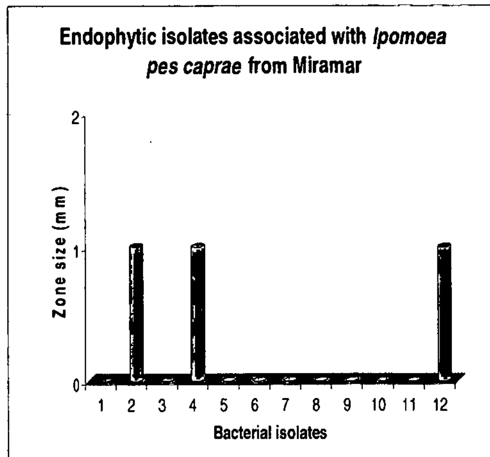

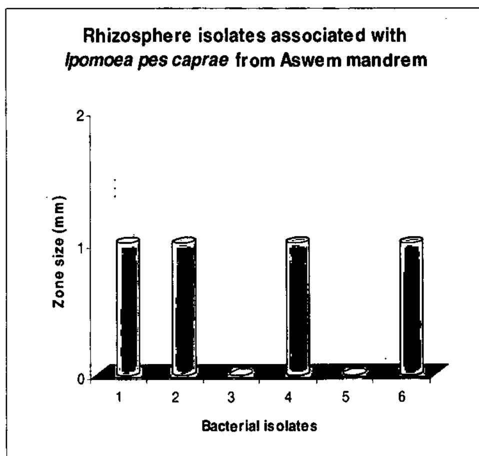

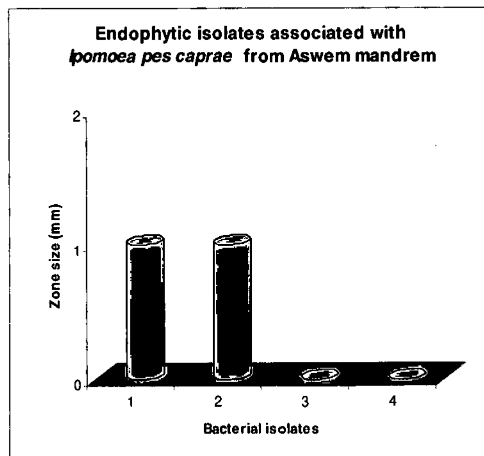

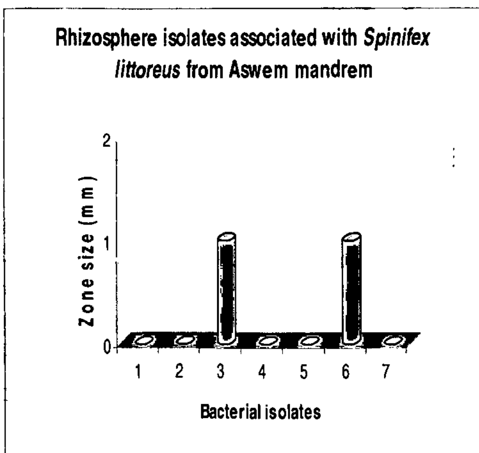

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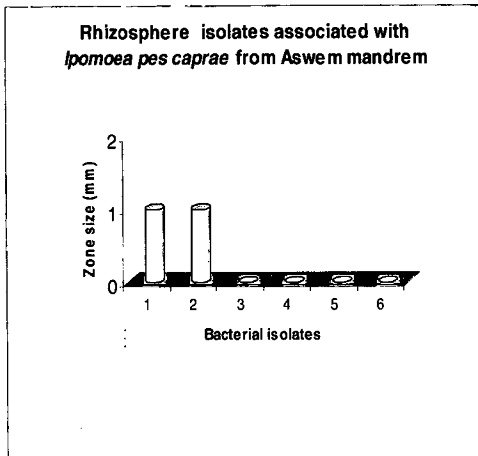
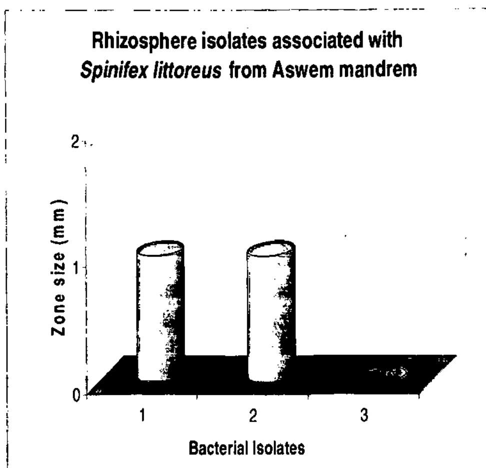
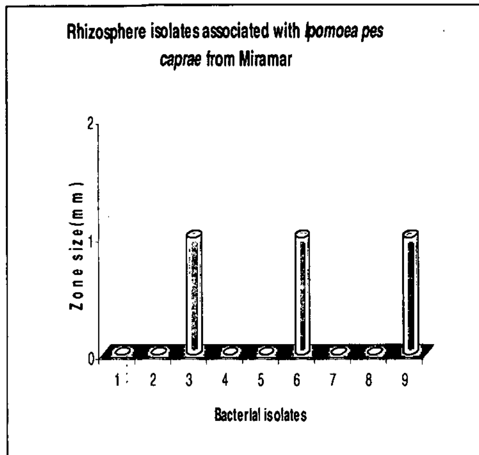
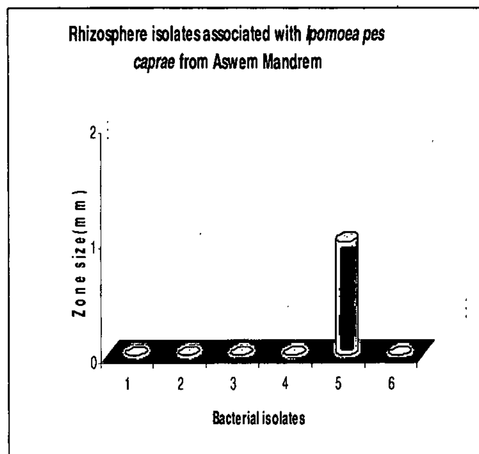
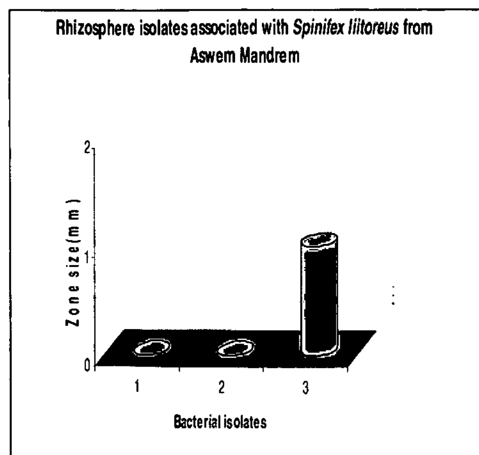




C. POST MONSOON







Phosphate-solubilizing bacteria were routinely screened by a plate assay method using Pikovskaya (PVK) agar. The test of the relative efficiency of isolated strains is carried out by selecting the microorganisms that are capable of producing a halo/clear zone on a plate owing to the production of organic acids into the surrounding medium. Majority of the neutrophilic and alkalophilic rhizosphere and endophytic isolates were found to solubilize inorganic phosphate (Fig 2.10). A large portion of inorganic phosphates applied to soil as fertilizer are 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).

Among the neutrophilic isolates (Figs 2.11 A, B & C), majority of the P solubilizers were isolated in the premonsoon season followed by postmonsoon and monsoon period. Among the rhizospheric isolates associated with *Ipomoea pes caprae* from Miramar P solubilizing activity was less in all the seasons as compared to endophytic isolates, majority of which showed P solubilizing activity (1-2mm). Among the rhizosphere isolates associated with *Ipomoea pes caprae* from Aswem mandrem, P solubilizers were detected in premonsoon while the majority of endophytic isolates showed P solubilizing activity in premonsoon and monsoon period. Majority of rhizosphere isolates associated with *Spinifex lttioreus* from Aswem Mandrem were

Plate 1 **Plate 2**

Fig 2.10 P solubilizing bacteria producing yellow halo/clear zone on Pikovskya agar due to production of organic acids (Plate 1, Neutrophilic isolates, Plate 2 Alkalophilic isolates)

Fig 2.11 Screening of P solubilizing neutrophilic Rhizosphere and Endophytic bacteria associated with *Ipomoea pes caprae* and *Spinifex littoreus*

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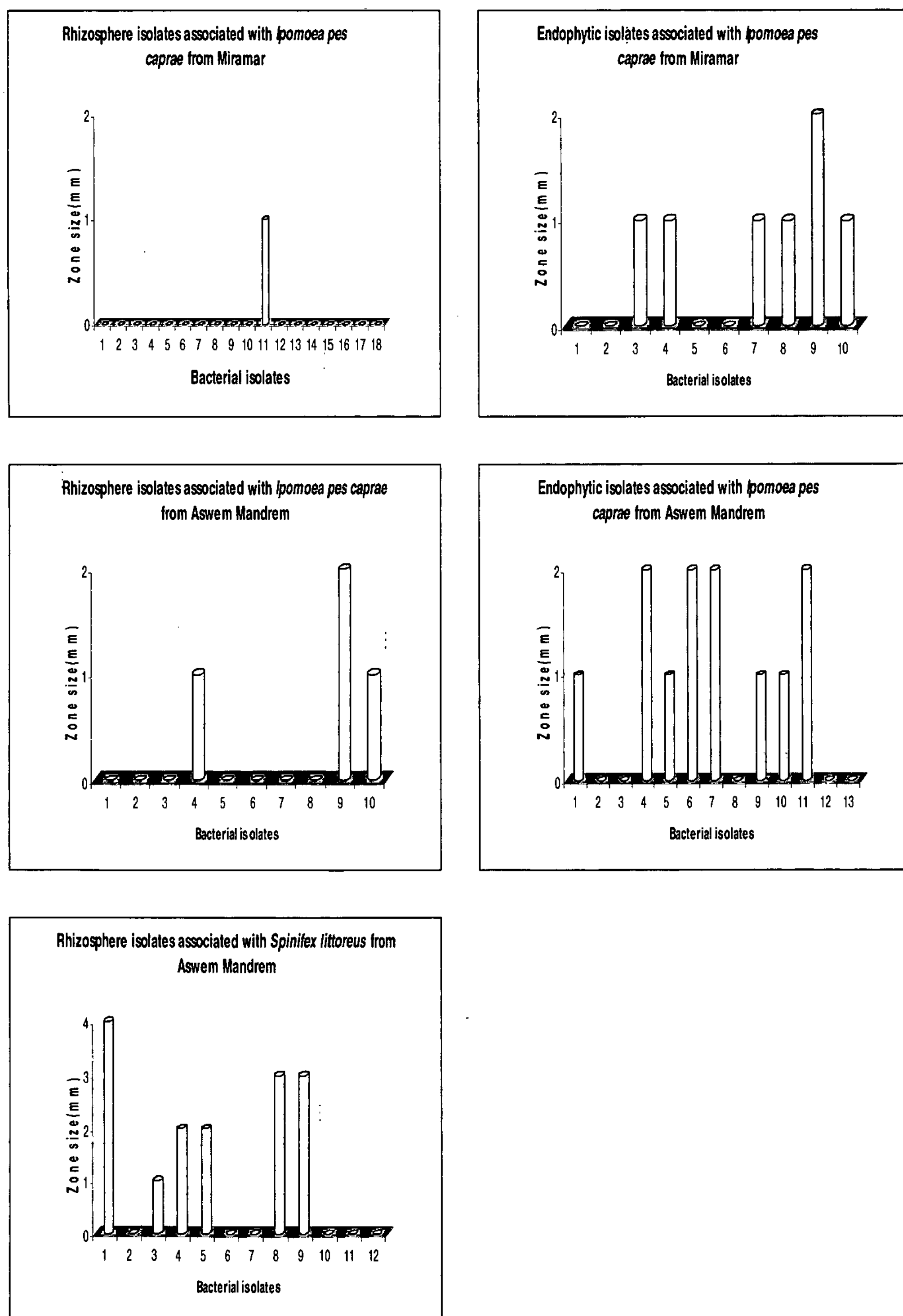

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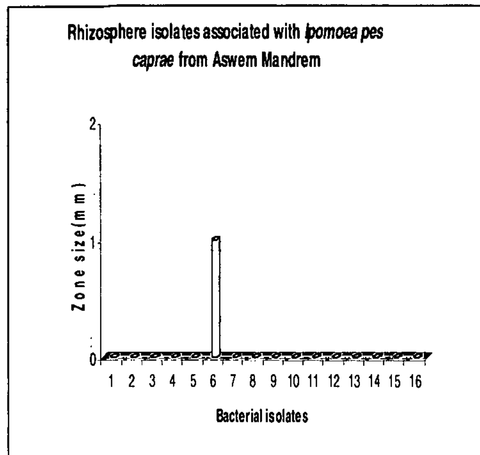

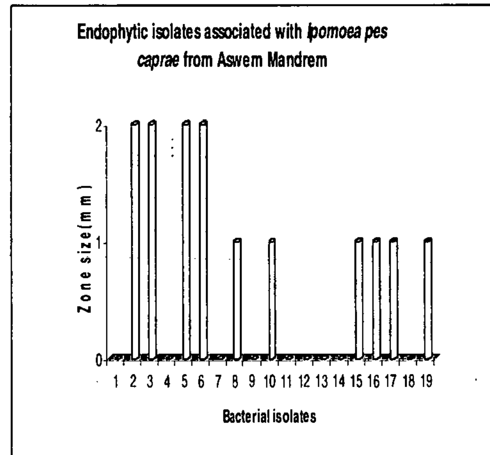

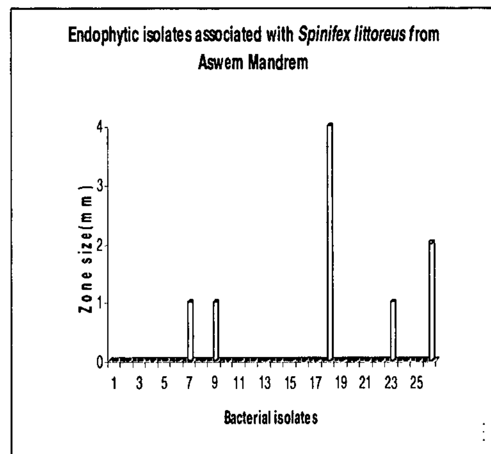

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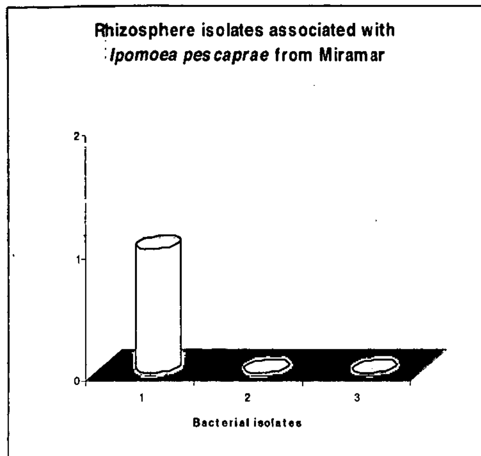
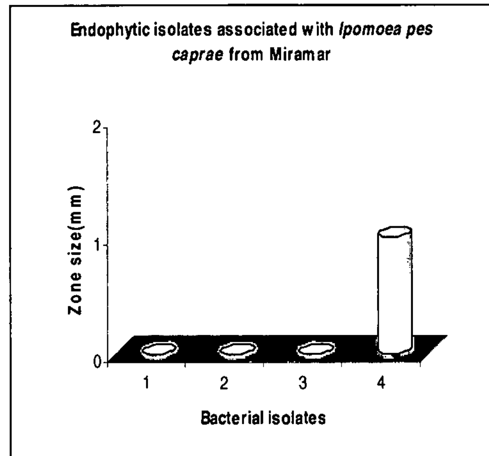
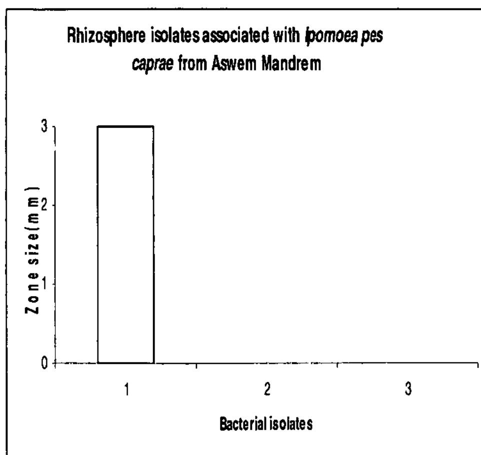
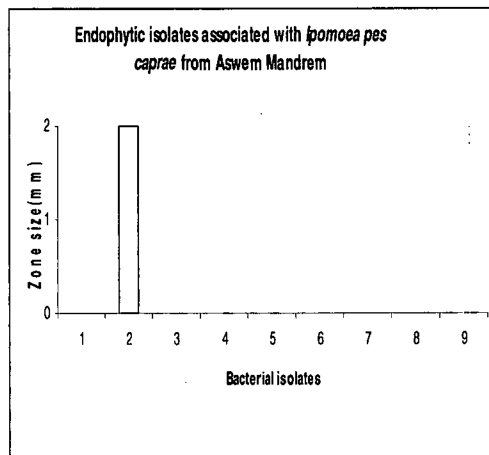
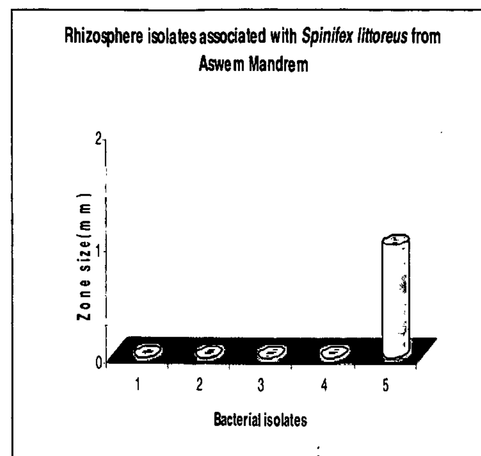
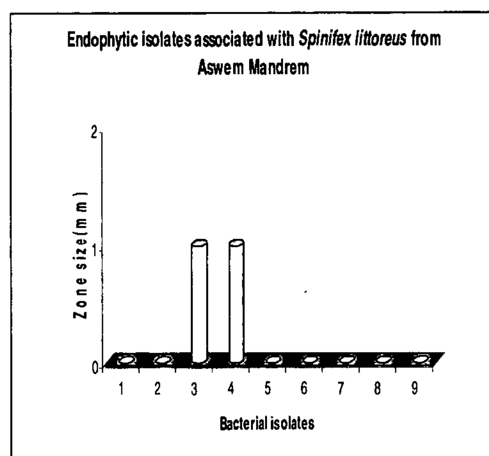










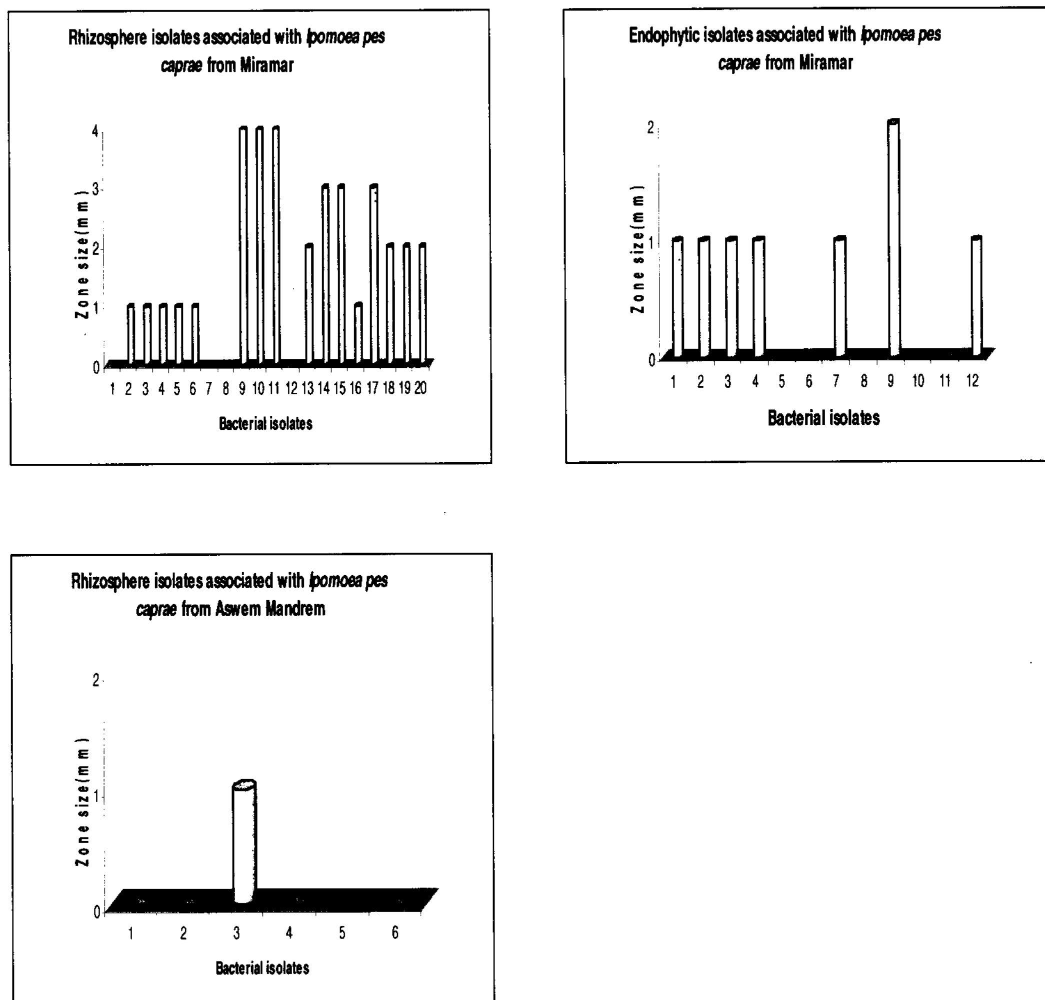


good P solubilizers (1- 4 mm) in the premonsoon while the endophytes showed good P solubilization (1-4mm) in the monsoon period.

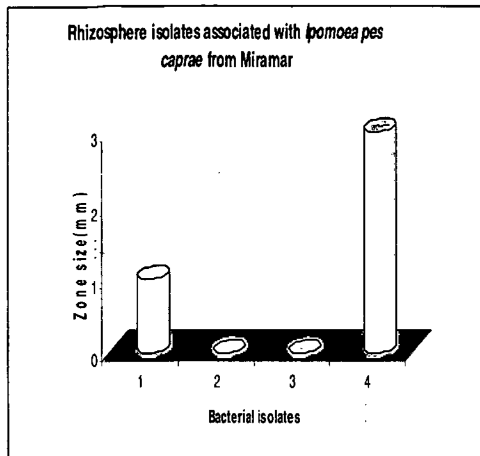
Among the alkaliphilic rhizosphere isolates associated with *Ipomoea pes caprae* from Miramar (Figs 2.12 A, B, C), majority of them were P solubilizers (1-4mm) in all the seasons while majority of endophytic isolates also showed good P solubilizing activity (1-4mm) in all the seasons. The alkalophilic rhizospheric isolates associated with *Ipomoea pes caprae* from Aswem Mandrem had comparatively lesser number of P solubilizers, although majority of endophytic isolates showed P soulubilizaton (1-4mm) in monsoon. There were few isolates showing P solubilization among the alkaliphiles associated with *Spinifex littoreus* from Aswem Mandrem. De Souza *et al,* (1999) studied the occurrence and physiological characteristics of marine phosphate solubilizing bacteria (PSB) around the Indian peninsula. A higher percentage of phosphate solubilizing bacteria was seen in the islands and coastal areas as compared to the offshore and sandy beaches. Most of the PSB belonged to *Bacillus* spp. In order to know the potential characteristics of these isolates supporting plant growth it is desired that the taxonomic identification of these bacteria be known to understand their distribution in this ecosystem in terms of rhizosphere and endophytic isolates. The isolates were therefore subjected to routine biochemical tests and chemotaxonomic analysis.

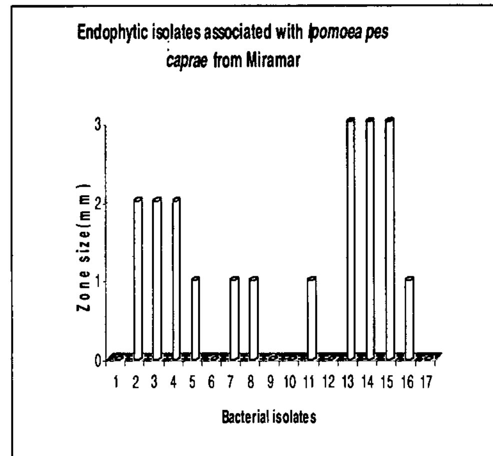
Fig 2.12 Screening of P solubilizing alkalophilic rhizosphere and endophytic bacteria associated with *Ipomoea pes caprae* and *Spinifex littoreus*

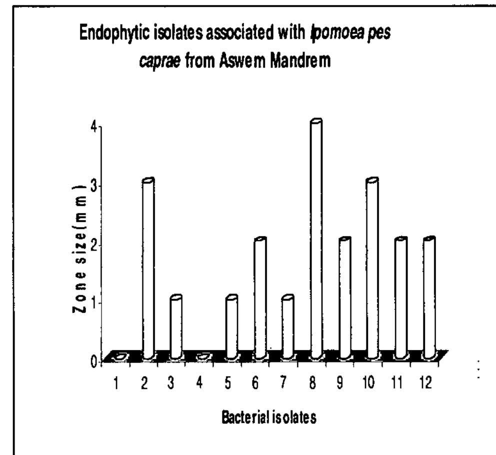
A. PREMONSOON

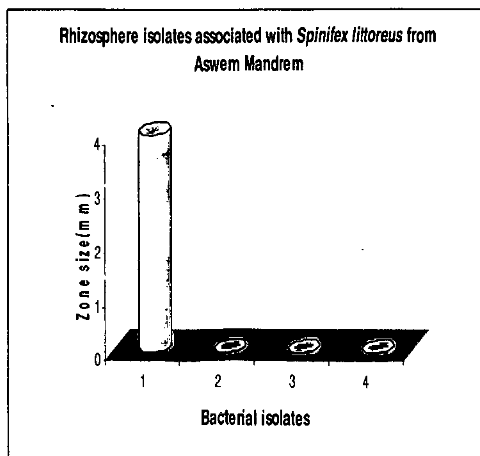


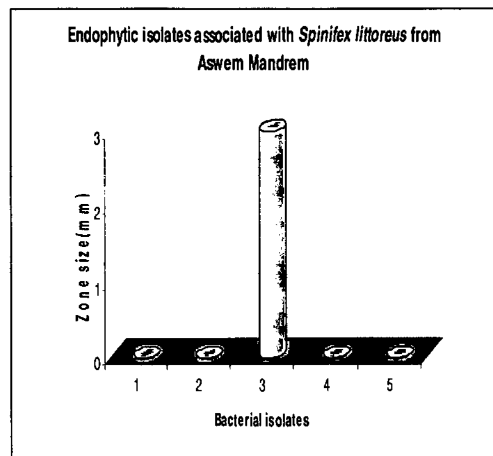
B. MONSOON



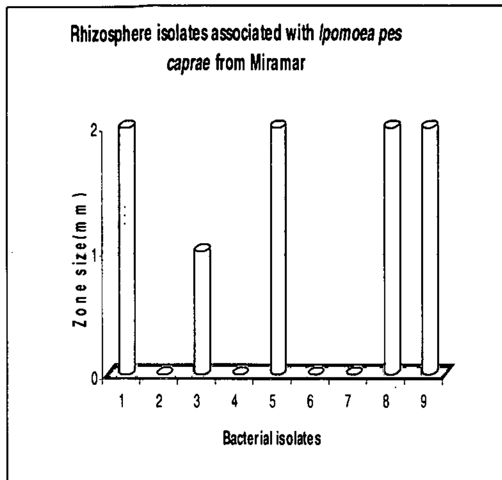


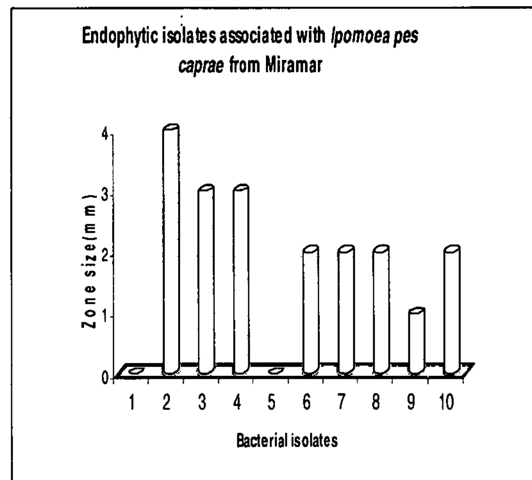


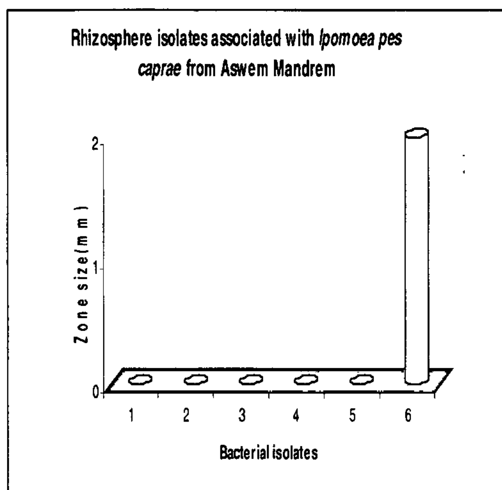


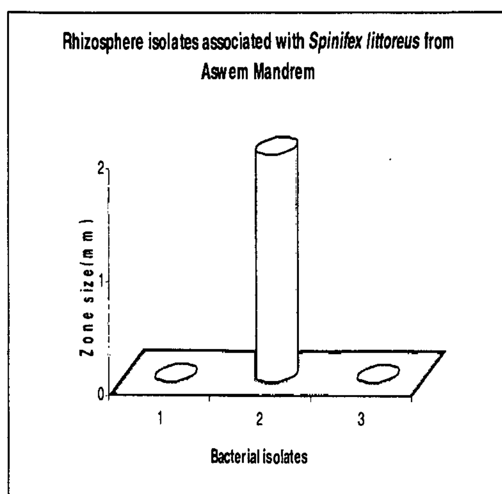


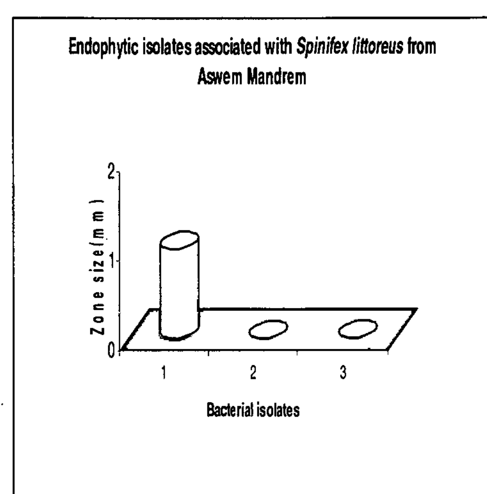
C. POSTMONSOON











Based on the cultural, physiological and biochemical characteristics, it was observed that among the neutrophiles, 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 *Brochothrix, Cellulomonas Microbacterium, Brevibacterium* (Table 2.7a & b). Zinniel *et al,* (2002) identified *Cellulomonas, Clavibacter, Curtobacterium* and *Microbacterium* as the most promising colonizing strains with four agronomic crop species. Karp & Nelson, (2004) reported that the sand and soil root zones were dominated largely by gram positive species e.g *Arthrobacter, Bacillus* and *Microbacterium* species, as also observed during the present study. Soil rhizosphere communities consisted almost entirely of *Actinobacterium*, *Arthrobacter* and *Bacillus* isolates, whereas sand root zones contained clones of a few gram negative genera such as *Aminobacter*, *Chelatobacter*, *Ensifer*, and *Pseudomonas.* Smit *et al,* (2001) studied the bacterial diversity and dynamics in Lovinkhoeve, soil samples, the most dominant bacterial genera detected by plating appeared to be *Micrococcus* and *Arthrobacter.* These genera are often found in various soils, such as those of wheat fields, deciduous woodlands, grasslands, and sand dunes. Tiago *et al,* (2004) investigated the bacterial diversity in a nonsaline alkaline environment and reported that the majority of the isolates were related to *Microbacteriaceae* family members while another set of isolates represented populations related to different species in the lineage of the *Micrococcaceae*, namely, *Micrococcus luteus*, *Citrococcus muralis*, and *Rothia dentocariosa*, and others were related to various species of the genera *Kocuria* and *Nesterenkonia.*

Table 2.7a Distribution of representative neutrophilic bacterial taxa in the rhizosphere and endophyte of sand dune plants

Isolate No	Sand dune vegetation	Source	Rhizosphere / Endophyte	Tentative identification
MIRT 5	*Ipomoea pes caprae*	Miramar	Rhizosphere	*B.polymyxa*
MIRT 11	*Ipomoea pes caprae*	Miramar	Rhizosphere	*B.macerans*
AMIRT 4	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*B.alvei*
MIRT 1	*Ipomoea pes caprae*	Miramar	Rhizosphere	*B.firmus*
MIRT 2	*Ipomoea pes caprae*	Miramar	Rhizosphere	*B.globiosporus*
AMIRT 1	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*B.licheniformis*
AMIRT12	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*B.circulans*
AMSRT8	*Spinifex littoreus*	Aswem Mandrem	Rhizosphere	*B.cereus*
AMIAT10	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.subtilus*
MIAT 4	*Ipomoea pes caprae*	Miramar	Endophyte	*B.laterosporus*
AMIAT 2	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*Staphylococcus*
AMIAT 3	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.laterosporus*
AMIAT 7	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.anthracis*
MIAT 3	*Ipomoea pes caprae*	Miramar	Endophyte	*B.mycoides*
MIAT 7	*Ipomoea pes caprae*	Miramar	Endophyte	*Stomatococcus*
AMIAT3	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.anthracis*
AMIAT8	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.brevis*
AMIAT15	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.mycoides*
AMIAT16	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*B.panthothenticus*
MIAT 3 AMSAT 3	*Spinifex littoreus*	Aswem Mandrem	Endophyte	*Flavobacterium sp.*
AMSAT 7	*Spinifex littoreus*	Aswem Mandrem	Endophyte	*B.lentimorbus*
AMSAT18 AMSAT21	*Spinifex littoreus*	Aswem Mandrem	Endophyte	*B.firmus*

Table 2.7b Distribution of representative alkaliphilic bacterial taxa in the rhizosphere and endophytes of sand dune plants

Isolate No	Sand dune vegetation	Source	Rhizosphere /Endophyte	Tentative identification
MIRA 9	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Alcaligens sp*
AMIRA 2, AMIRA 5	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*Brochothrix sp*
MIRA 2	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Corynebacterium sp*
MIRA 4	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Cellulomonas sp*
MIRA 3 MIRA 4 MIRA 14, MIRA 15	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Microbacterium arborescens*
MIRA 25	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Brochothrix sp*
MIRA 27	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Brevibacterium sp*
MIRA 3	*Ipomoea pes caprae*	Miramar	Rhizosphere	*Micrococcus roseus*
AMIRA1	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*Brevibacterium sp*
AMIRA 3	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*Pseudomonas sp*
AMIRA 6	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*B.laterosporus*
AMIRA1	*Ipomoea pes caprae*	Aswem Mandrem	Rhizosphere	*Cellulomonas sp*
AMSRA 1	*Spinifex littoreus*	Aswem Mandrem	Rhizosphere	*Brochothrix sp*
AMSRA 2	*Spinifex littoreus*	Aswem Mandrem	Rhizosphere	*Brochothrix sp*
AMSRA 3, AMSRA 6	*Spinifex littoreus*	Aswem Mandrem	Rhizosphere	*Brevibacterium sp*
MIAA 3	*Ipomoea pes caprae*	Miramar	Endophyte	*Microbacterium sps*
AMIAA 1	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*Renibacterium sp*
MIAA 2	*Ipomoea pes caprae*	Miramar	Endophyte	*Brochothrix sp*
MIAA 9, MIAA12	*Ipomoea pes caprae*	Miramar	Endophyte	*Cellulomonas sp*
AMIAA 2	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*Brevibacterium sp*
MIAA 8	*Ipomoea pes caprae*	Miramar	Endophyte	*Pseudomonas sp*
MIAA 14	*Ipomoea pes caprae*	Miramar	Endophyte	*Brochothrix sp*
AMIAA 6	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*Staphylococcus sp*
AMIAA 8	*Ipomoea pes caprae*	Aswem Mandrem	Endophyte	*Brochothrix sp*
AMSAA 1	*Spinifex littoreus*	Aswem Mandrem	Endophyte	*Cellulomonas sp*

Interestingly, the alkalophilic genera like *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 (Table 2.8 a & b). The neutrophilic *Bacillus* genus were found to be better siderophore producers as compared to the alkalophilic 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 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. Ceratin polyaromatic hydrocarbons (PAH) although persistent mainly due to their hydrophobicity, can be degraded by a variety of microorganisms (bacteria & fungi).

Table 2.8a Neutrophilic isolates showing production of enzymes, siderophores and P solubilization

Culture no	Tentatively identified as	Enzymes produced (mm)			Siderophore production (mm)	Phosphate solubilization (mm)
		Amylase	Cellulase	Protease		
Premonsoon						
MIRT 5	*B.polymyxa*	2	1	3	2	-
MIRT 11	*B.macerans*	1	-	3	2	1
MIAT 3	*Flavobacterium sp.*	-	-	1	2	1
AMIRT 4	*B.alvei*	1	1	1	-	1
AMIAT 4	Unidentified	-	1	-	1	2
AMIAT 10	*B.subtilus*	1	4	3	1	1
AMSRT 8	*B.cereus*	-	-	3	2	3
AMIAT 8	*Pseudomonas sp.*	-	1	-	1	-
Postmonsoon						
MIAT 4	*B.laterosporus*	-	1	2	1	1
MIRT 1	*B.firmus*	1	-	3	1	1
MIRT 2	*B.globiosporus*	-	1	2	2	-
AMIRT1	*B.licheniformis*	1	-	2	2	3
AMIAT 2	*Staphylococcus sp.*	1	-	3	-	2
AMIAT 3	*B.laterosporus*	-	1	1	2	-
AMIAT 7	*B.anthracis*	3	1	1	1	-
AMSAT 3	*Flavobacterium sp.*	1	1	2	1	1
AMIAT 5	*B.macerans*	-	1	1	1	-
AMIAT 3	*B.macerans*	-	1	1	1	1
Monsoon						
MIAT 3	*B.mycoides*	3	1	3	2	-
MIAT 7	*Stomatococcus sp.*	-	1	2	2	-
AMIAT 3	*B.anthracis*	1	-	1	2	2
AMIRT 12	*B.circulans*	-	3	-	2	-
AMIAT 8	*B.brevis*	-	-	1	4	1
AMIAT 15	*B.mycoides*	1	1	2	2	1
AMIAT 16	*B.pantothenticus*	1	2	2	2	1
AMSAT 7	*B.lentimorbus*	-	-	3	2	1
AMSAT 18	*B.firmus*	1	-	3	2	4
AMSAT 21	*B.firmus*	3	-	2	1	-
MIAT 1	*Planococcus sp.*	1	1	2	-	1
MIAT 9	*B.marinus*	-	-	-	1	1
MIAT 13	*Stomatococcus sp.*	-	1	-	1	1
MIAT 15	*B.megaterium*	1	1	2	1	1
MIAT 26	*B.megaterium*	2	-	3	1	1
MIAT 16	*B.pumilus*	3	2	1	1	1
MIAT 25	*Staphylococcus sp.*	-	-	-	1	-

Table 2.8b Alkalophilic isolates showing production of enzymes, siderophores and P solubilization

Culture no	Tentatively identified as	Enzymes produced(mm)			Siderophore production (mm)	Phosphate solubilization (mm)
		Amylase	Cellulase	Protease		
Postmonsoon						
MIRA 3	*Microbacterium sp.*	-	1	-	1	1
MIRA 9	*Alcaligens sp.*	-	1	2	1	2
AMIRA 5	*Brochothrix sp.*	4	2	7	-	-
AMIRA 6	*Brochothrix sp.*	5	-	8	-	2
AMIAA 1	*Renibacterium sp.*	3	4	5	1	-
MIAA 3	Unidentified	9	1	7	-	3
AMIRA 2	Unidentified	4	2	7	-	-
AMSRA 2	*Brochothrix sp.*	4	3	8	-	2
Monsoon						
MIRA 3	*Micrococcus sp.*	3	1	-	-	-
MIRA 4	*Microbacterium sp.*	1	1	-	-	3
MIAA 8	*Pseudomonas sp.*	-	1	10	-	1
MIAA 14	*Brochothrix sp.*	1	1	-	-	3
AMIAA 8	*Brochothrix sp.*	-	1	1	-	4
AMSRA 1	*Brochothrix sp.*	2	-	2	1	4
AMIRA 1	*Cellulomonas sp.*	1	-	10	1	-
AMSAA 1	*Cellulomonas sp.*	1	1	11	-	1
AMIAA 6	*Staphylococcus sp.*	-	1	6	-	2
Premonsoon						
MIAA 2	*Brochothrix sp.*	2	1	3	1	1
MIRA 25	*Brochothrix sp.*	2	1	5	-	1
MIAA 9	*Cellulomonas sp.*	3	-	1	-	2
MIAA 12	*Cellulomonas sp.*	3	1	8	1	1
MIRA 4	*Cellulomonas sp.*	1	1	2	-	1
AMIRA 3	*Pseudomonas sp.*	3	-	4	-	1
MIRA 2	*Corynebacterium sp.*	1	-	2	-	1
MIRA 14	*Microbacterium arborescens*	1	1	1	1	4
MIRA 15	*Microbacterium arborescens*	-	1	2	1	4
MIRA 26	Unidentified	-	1	3	-	3
MIRA 27	*Brevibacterium sp.*	3	-	5	-	2
AMIAA 2	*Brevibacterium sp.*	2	-	5	1	-
AMIRA 1	*Brevibacterium sp.*	1	-	6	1	-
AMSRA 3	*Brevibacterium sp.*	1	1	7	1	-
AMSRA 6	*Brevibacterium sp.*	1	-	5	1	-
AMIRA 6	*B.laterosporus*	1	1	-	1	-

Recent work has indicated that the stimulation of microbial activity in 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. The rhizosphere microbial community is comprised of microorganisms with different types of metabolism and adaptive responses to variation in environmental conditions. The production of mucilaginous material and the exudation of a variety of soluble organic compounds by the plant root play important part in root colonization and maintenance of microbial growth in rhizosphere. Microbial activity is thus generally higher in 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, sulphur, 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 (Khan *et al*, 2006; McCoy, 2000). 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).

Among the neutrophilic isolates (Table 2.9 a), nearly hundred percent of the premonsoon rhizospheric and endophytic isolates were found to grow on hydrocarbons such as phenanthrene, biphenyl, naphthalene and sodium benzoate

Table 2.9a Percentage growth of neutrophiles associated with *Ipomoea pes caprae* and *Spinifex littoreus* on hydrocarbons.

Sand dune vegetation		Premonsoon				Monsoon				Post monsoon			
		Phn	**Biph**	**Naph**	**Na benz**	**Phn**	**Biph**	**Naph**	**Na benz**	**Phn**	**Biph**	**Naph**	**Na benz**
MIRAMAR													
Ipomoea pes caprae	**R**	94	100	100	88	100	100	100	100	100	100	100	100
	E	90	50	100	100	83	91	91	75	75	50	75	50
ASWEM MANDREM													
Ipomoea pes caprae	**R**	100	100	100	100	56	56	50	62	80	80	100	20
	E	100	100	100	100	100	84	100	94	77	77	88	88
Spinifex littoreus	**R**	91	100	100	100	60	60	60	60	ND	ND	ND	ND
	E	100	91	100	91	ND	ND	ND	ND	ND	ND	ND	ND

Phn –Phenanthrene, Biph – biphenyl, Naph-Naphthalene, Na benz - sodium benzoate, ND –Not detected

followed by postmonsoon isolates and monsoon isolates. In premonsoon and postmonsoon period the rhizosphre and endophytic bacteria were found to tolerate the solvents such as cyclohexane, benzene, toluene and hexadecane (Table 2.9b). Solvent toxicity is graded by log P values, where P is the partition coefficient of a given solvent in an equimolar mixture of octanol and water. The lower the log P value, the greater its polarity and hence its toxicity. The isolates tolerate cyclohexane (log P=3.2), Toluene (log P= 2.5), Benzene (log P= 2) and hexadecane. The toxic effects of aromatic and aliphatic hydrocarbons, phenols and alcohols due to 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 partition into the cytoplasmic membrane (Sikkema *et al*, 1994, 1995), they accumulate in the cytoplasmic membrane of bacteria causing disorganization of the cell membrane structure and the impairment of vital membrane functions such as loss of ions, metabolites, lipids and proteins, the dissipation of the pH gradient and electrical potential or the inhibitions of membrane functions. It was interesting to note that sand dune bacteria were tolerant to toxic solvents like Benzene and toluene. The possible mechanisms of tolerance to aromatic hydrocarbons may be attributed to a) metabolism of the toxic hydrocarbons, which can contributed to their transformation into non toxic compounds, b) rigidification of the cell membrane via alteration in composition of phospholipids and c) efflux of the toxic compound in an energy dependent process(Segura *et al*, 1999).

Among the alkaliphilic isolates (Table 2.10 a), eighty percent of the rhizosphere and endophytic bacterial isolates were found to degrade the hydrocarbons in monsoon period followed by post monsoon and premonsoon period. Majority of the

Table 2.9b Percentage distribution of solvent tolerant rhizosphere and endophytic neutrophilic bacteria in premonsoon, monsoon and postmonsoon period

Sand dune vegetation		Premonsoon				Monsoon				Post monsoon			
		Hex	Benz	Tolu	Hexad	Hex	Benz	Tolu	Hexad	Hex	Benz	Tolu	Hexad
MIRAMAR													
Ipomoea pes caprae	R	83	11	94	88	ND	ND	ND	ND	100	100	ND	66
	E	50	50	100	80	16	41	91	83	25	25	25	25
ASWEM MANDREM													
Ipomoea pes caprae	R	50	ND	80	50	43	50	31	31	60	60	60	60
	E	38	38	92	92	68	57	57	73	77	77	44	88
Spinifex littoreus	R	58	41	91	100	60	40	40	52	ND	ND	ND	ND
	E	75	25	75	91	ND	ND	ND	ND	ND	ND	ND	ND

Hex- Cyclehexane, Benz-benzene, Tol-Toluene, Hexad-Hexadecane

Table 2.10a Percentage growth of alkaliphiles associated with *Ipomoea pes caprae and Spinifex littoreus* on hydrocarbons

Sand dune vegetation		Premonsoon				Monsoon				Post monsoon			
		Phn	Biph	Naph	Na benz	Phn	Biph	Naph	Na benz	Phn	Biph	Naph	Na benz
MIRAMAR													
Ipomoea pes caprae	R	48	55	27	62	83	66	66	66	66	44	44	66
	E	91	66	41	83	52	70	76	70	40	60	30	30
ASWEM MANDREM													
Ipomoea pes caprae	R	83	10	66	100	66	66	83	83	66	66	83	83
	E	100	100	75	50	53	46	46	53	100	ND	100	100
Spinifex littoreus	R	28	42	42	71	50	50	50	0	66	100	33	66
	E	66	100	33	100	40	40	20	60	66	33	100	33

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 (Table 2.10b).

Organic solvent-tolerant micro-organisms have the potential to be utilized for degrading pollutants in natural ecosystems (Sardessai and Bhosle, 2002). Organic solvents with a $\log P_{OW}$ value (logarithm of the partition coefficient of the target compound in a mixture of octanol/water) between 1.5 and 3 are extremely toxic to microorganisms, a characteristic that has been well documented for toluene ($\log P_{OW}$ 2.5). Oil pollutants undergo natural biodegradation by the native microbial flora over long periods of time (Roy *et al,* 2002). During the last few decades, a variety of bacteria capable of degrading polycyclic aomatic hydrocarbons (PAHs), particularly low-molecular weight compounds e.g. naphthalene and phenanthrene have been discovered. Such bacteria belong to the genera *Agmenellum*, *Aeromonas*, *Alcaligenes*, *Acinetobacter*, *Bacillus*, *Berjerinckia*, *Burkholderia*, *Corynebacterium*, *Cyclotrophicus*, *Flavobacterium*, *Micrococcus*, *Moraxella*, *Mycobacterium*, *Nocardioides*, *Pseudomonas*, *Lutibacterium*, *Rhodococcus*, *Streptomyces*, *Sphingomonas*, *Stenotrophomonas*, *Vibrio*, *Paenibacillus*, and others (Zhang *et al,* 2004). Roy *et al,* (2002) reported that the coastal water of Sunderban Biosphere Reserve has a substantial presence of naturally occurring oil-degrading bacteria. The efficiency of oil degradation of the naturally occurring strains studied under laboratory conditions revealed that they are quite efficient in degrading crude oil. The hydrophobic nature of PAHs prevents significant uptake and translocation within plants so these contaminants generally remain in the root zone.

Table 2.10b Percentage distribution of solvent tolerant rhizosphere and endophytic alkalophilic bacteria in premonsoon, monsoon and postmonsoon period

Sand dune vegetation		Premonsoon				Monsoon				Post monsoon			
		Hex	Benz	Tolu	Hexad	Hex	Benz	Tolu	Hexad	Hex	Benz	Tolu	Hexad
MIRAMAR													
Ipomoea pes caprae	R	34	17	34	20	0	83	0	0	66	55	77	77
	E	33	58	83	41	0	64	0	0	30	70	100	80
ASWEM MANDREM													
Ipomoea pes caprae	R	33	66	83	33	0	83	0	0	0	0	83	66
	E	100	75	75	75	0	76	0	0	0	0	100	100
Spinifex littoreus	R	14	71	42	71	0	25	0	0	66	33	66	100
	E	33	0	0	0	0	60	0	0	66	33	66	100

Hex- Cyclehexane, Benz -benzene, Tol-Toluene, Hexad-Hexadecane

This suggests that plant roots stimulate microbial populations for enhanced biodegradation. Potential plant–microbe interactions affecting PAH degradation include: (i) prolific microbial (ii) catabolic enzyme induction (iii) co-oxidation of high-molecular-weight PAHs and (iv) improved bioavailability (Rentz *et al,* 2004). Miya and Firestone (2000) observed greater percentages of phenanthrene-degrading bacteria in rhizosphere soil than in bulk soils and suggested that the rhizosphere be selected for PAH degraders. Hydrocarbon utilizers were found to be more in samples collected from *Ipomoea pes caprae* from Miramar and Aswem Mandrem as compared to *Spinifex littoreus* from Aswem Mandrem. However the distribution of hydrocarbon utilizing alkaliphiles were more in *Ipomoea pes caprae* from Aswem Mandrem as compared to Miramar. Interestingly, most of the isolates from *Spinifex littoreus* showed the ability to grow in the presence of hydrocarbons. Such bacteria which are capable of growing on hydrocarbons have the mechanisms of either directly emulsifying or adhering to the insoluble droplets. One of the most studied factors for such organisms is the production of exopolymers. In the rhizosphere the exopolymers are also known to be useful to improve the moisture holding capacity. The ability of these isolates to produce exopolymers was determined.

The alkalophilic isolates showed significant production of exopolysaccharide as measured by the phenol sulphuric acid method. Interestingly, the neutrophiles although capable of growing on hydrocarbons did not show such significant production of exopolymer. A significant observation was that the culture MIRA 15 showed very high production of EPS (Table 2.11) and its growth on sodium benzoate and solvent tolerance was also significant. It appears that this organisms could be

Table 2.11 Screening of alkalophilic bacteria for EPS production

Bacteria	OD (600 nms)	EPS (ug/ml)
MIRA 14	1.12	180
MIRA 15	**1.54**	**260**
MIRA 29	1.99	120
AMIAA 8	0.42	20
AMIRA 6	1.99	95
MIRA 27	1.75	60
MIRA 28	1.01	90
MIRA 26	1.03	175
MIAA 5	0.932	10.30
MIAA10	1.349	33.46
AMIRA5	1.242	7.08
AMIRA7	2.0	14.37
MIRA18	1.059	229
MIRA26	1.43	28.00
MIRA5	1.23	121.12
AMIRA8	1.84	17.66
AMSRA 2	0.52	16.02
AMSRA5	1.605	17.73
AMSRA8	1.54	30.60
AMSRA10	1.678	78
AMSRA12	1.603	8.22
AMSRA 4	1.431	34.23
AMSAA1	1.075	8.08
AMSAA 6	0.978	25.31
AMSAA5	1.765	15.88
AMSAA8	0.548	120.1
AMSAA2	1.078	30.60
AMSAA 4	1.77	38.90
MIRA 22	1.00	115

representative of the rhizosphere flora which help the plant by producing the EPS and improving the moisture holding capacity and formation of aggregates in soil. Amongst the isolates MIRA 15 was selected for further studies on EPS production and its role in the formation of aggregates. The following chapter compiles results obtained on this aspect.

The present study indicated that a large number of bacteria are associated with rhizosphere and as endophytes with plants growing on sand dunes. Such organisms are envisaged to play a role in promoting growth of plants by making the soils available with nutrients. It was interesting to understand this significance of the isolates obtained through enzymatic activities shown largely by neutrophilic and alkaliphiles obtained during this study. The distribution of activities among the different genera reflected that the most predominant neutrophilic isolates belonged to genera *Bacillus* while the alkaliphiles belonged to genera such as *Brochothrix, Cellulomonas Microbacterium, Brevibacterium.* Among the isolates four highly promising isolates were selected for further studies on their plant growth promoting traits and field studies.

Chapter III

Isolation, Extraction and Characterization of exopolysaccharide production by *Microbacterium arborescens*

3.1. Introduction

3.1.1 Bacterial Exopolysaccharide

Exopolysaccharide (EPS) is a term first used by Sutherland (1972) to describe high molecular weight carbohydrate polymers produced by many marine bacteria. EPS can be part of the capsular material that closely surrounds the bacterial cell or released into the surrounding environment as dispersed slime with no obvious association to any one particular cell (Sutherland 1982 ; Decho 1990). In the natural environment, EPS production seems to be essential for survival since most bacteria occur in microbial aggregates whose structural and functional integrity is based on the presence of a matrix of extracellular polymeric substances (Sutherland 1982).

3.1.2 Physical and chemical nature of the EPS

Most bacteria use carbohydrates as a carbon and energy source and amino acids or an ammonium salt as a nitrogen source (Sutherland, 1982).The composition of EPS and the chemical and physical properties of these biopolymers can vary greatly (Decho, 1990), but it is generally independent of the carbon substrate (Sutherland, 1982). Uptake of substrate is one of the first limitations on EPS production and the presence of a carbohydrate substrate such as glucose results in optimal EPS yields (Sutherland, 1979). Most bacteria release the largest quantity of EPS during stationary growth phase in laboratory culture (Decho, 1990, Manca *et al,* 1996). The composition of EPS may also vary according to the growth phase of the bacteria (Christensen *et al,* 1985). Although culture conditions generally do not affect the types of monosaccharides in an EPS, they impact on the functional properties of the polysaccharide such as molecular weight, conformation and monosaccharide ratios

(Arias *et al,* 2003). In natural systems where nutrients levels in close proximity to the bacterial cell may vary considerably, shifts in the physiological state of the cell probably result in variable EPS compositions (Geesey, 1982).Most EPS produced by marine bacteria are heteropolysaccharides consisting of three of four different monosaccharides arranged in groups often or less to form repeating units (Decho, 1990). The monosaccharides maybe pentoses, hexoses, amino sugars or uronic acids. Most polymers are linear overall and of varying lengths with an average molecular weight of $1x10^5$ to $3x10^5$ daltons (Sutherland, 1977). Branches of one or more monosaccharides are often attached at regular intervals (Decho, 1990). Organic or inorganic (sulphate, phosphate) substituents may also be present. Some are neutral macromolecules, but the majority are polyanionic due to the presence of either uronic acids (D-glucuronic acid) being the commonest, although D-galacturonic and D-mannuronic acids are also found) or ketal-linked pyruvate. Inorganic residues, such as phosphate or rarely sulphate, may also confer polyanionic status (Sutherland, 1990).The species-specific structural heterogeneity and the many roles EPS play in the natural environment are reflected in the numerous existing and potential applications for these bio-polymers (Weiner,1997). Xanthan gum, the most well known microbial polysaccharide, is produced by the plant pathogen *Xanthomonas campestris* pv. campestris. Because of its physical properties it is commonly used as a thickener in both food and non-food industries (Becker *et al*, 1998). Bacterial cellulose, produced by *Acetobacter xylinum* and other, mainly Gram-negative bacterial species, has a high water binding capacity. This EPS is used to make a type of wound dressing for patients with burns, chronic ulcers or extensive tissue loss. Several *Agrobacterium* and *Rhizobium* species produce curdlan

and this improves the texture of tofu, bean jelly and fish pastes in Japan (Sutherland,1998). The study of EPS produced by bacteria from the marine environment provides additional opportunities for novel uses of these biopolymer. The EPS plays an important role in cell aggregation, cell adhesion, biofilm formation, and protection of cells from hostile environments (Cescutti *et al,* 1999 & Looijesteijn *et al,* 2001). Microbial polysaccharide with important mechanical properties have significant impact in commercial applications (Petersen *et al,* 1989) for example xanthan gum is an anion polysaccharide produced by the bacteria *Xanthomonas campestris*. Because of its high viscosity, pseudoplasticity and the rheological property over wide range of temperature and pH, it has a wide variety of applications (Sandford and Baird, 1989). It is commonly used as a thickening agent in food product, for mobility control in secondary and tertiary oil recovery, in drilling fluids and in paint, pharmaceutical and cosmetic industries (Niel and Kristiansan, 1990).

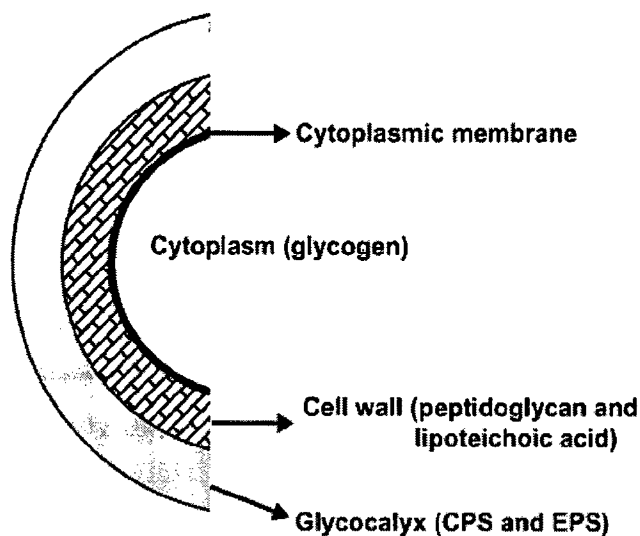
The composition and structure of the polysaccharides determines their primary conformation. Further, ordered secondary configuration frequently takes the form of aggregated helices. In some of these polymers, the backbone composition of sequences of 1, 4-*b*- or 1, 3-*b* linkages may confer considerable rigidity, as is seen in the cellulosic backbone of xanthan from *Xanthomonas campestris*. Other linkages in polysaccharides may yield more flexible structures. These can be exemplified by the1, 2-*a*- or 1, 6-*a*-linkages found in many dextrans. The transition in solution from random coil to ordered helical aggregates is often greatly influenced by the presence or absence of acyl substituents such as *O*-acetyl or *O*-succinyl esters or pyruvate ketals (Sutherland, 1997). In most natural and experimental environments, the EPS will be found in the ordered configurations which are found at lower temperatures and

in the presence of salts. The polysaccharides are essentially very long, thin molecular chains with molecular mass of the order of 0±5±2±0¨10' Da, but they can associate in a number of different ways. In several preparations, the polysaccharides have been visualized as fine strands attached to the bacterial cell surface and forming a complex network surrounding the cell. Mayer *et al*,(1999) suggested that electrostatic and hydrogen bonds are the dominant forces involved. Ionic interactions maybe involved, but more subtle chain to chain complex formation in which one macromolecule ` fits ' into the other may result in either floc formation or networks which are very poorly soluble in aqueous solvents. Another result may be the formation of strong or weak gels. The polysaccharides can thus form various types of structures within a biofilm. However, in biofilms the polysaccharides do not exist alone but may interact with a wide range of other molecular species, including lectins, proteins, lipids etc., as well as with other polysaccharides. The resultant tertiary structure comprises a network of polysaccharide and other macromolecules,in which cells and cell products are also trapped.

3. 1. 3 Functions of EPS

Most but not all, of the functions ascribed to EPS are of a protective nature. The ability of a microorganism to surround itself in a highly hydrated EPS layer may provide it with protection against dessication and predation by protozoans. Also, the presence of a gelled polysaccharide layer around the cell may have significant effects on the diffusion properties, both into and out from the cell. Cells buried within a polymer matrix for instance, be inaccessible to antibacterial agents such as antibiotics. Anionic EPS may also affect the penetration to the cell surface of both useful and

toxic metal ions and this type of interaction is of considerable practical importance in the corrosion of metallic surfaces (Whitfield,1988).Polysaccharides are either used as stabilizers to maintain oil and water containing colloidal particles or as gelling agents to alter the texture of food. As stabilizers polysaccharides are widely used in dried products which are reconstituted by the addition of water. Microbial polysaccharides such as xanthan and alginate are used as gel formers to change the texture of the food. The widest non-food industrial application of bacterial derived polysaccharides is the use of xanthan in the oil industry. Xanthan has unique rheological properties demonstrating pseudo-plastic flow and visco-elasticity, retaining its physical properties over a broad temperature range. As such xanthan has been exploited in drilling fluids to both lubricate the drill head and remove rock cuttings and in enhanced oil recovery. Polysaccharides such as succinoglycan, which has relatively low transition temperature have also been used I these processes.Polysaccharidse are used as thickeners in the printing and textile industry. The rheological properties of the polysaccharide are important in restricting the flow of the dye and maintaining the coating of dye prior to fixation. Both xanthan and succinoglycan are widely used particularly in the manufacture of paints and pigmenst. The most biomedically important polysaccharide is heparin with its widespread use as an effective anticoagulant. In addition, heparin and heparin sulfate may have important clinical applications in mediating inflammatory reactions and in reverting the transformed state of certain tumour cells. The most significant biomedical exploitation of bacterial polysaccharides has been in their use as vaccine agents. Capsular polysaccharides, with few exceptions, are immunogenic in non-infants, generally non-toxic and have



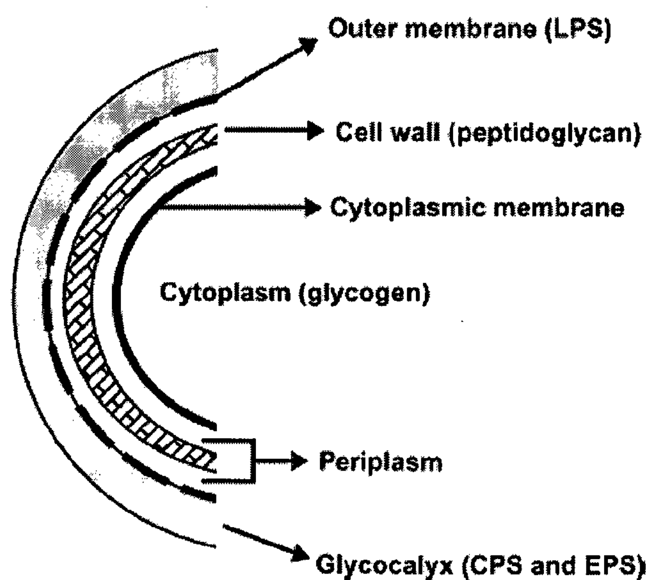


Figure 3.1 Cell location of polysaccharides produced by gram-positive and gram negative bacteria.

CPS = Capsular polysaccharides (capsule), EPS = exopolysaccharides (slime layer)

(Ruas-Madiedo & Reyes-Gavila´n,2005)

none of the deleterious side-effects associated with whole-organism vaccines (Roberts,1995).

Preliminary studies on rhizosphere and endophytic bacteria from sand dune vegetation have shown the production of exopolysaccharide by a promising culture tentatively identified as *Microbacterium arborescens* AGSB. Such exopolysacharide are found to be important in the soil for improving the moisture holding capacity and serve as additional nutrients for microorganisms. The studies on the isolate obtained from sand dune with respect to extraction, characterization and its role in aggregation are compiled in this chapter.

3.2 Materials and Methods

3.2.1 Media and growth conditions

A loopful of the isolate from slant was inoculated into sterile polypeptone yeast extract glucose (PPYG) media (pH 10.5) and incubated at 28°C on a rotary shaker at 160 rpm overnight. This culture broth was used as inoculum for all the experiments

3.2.2 Alcian blue staining and adsorption assay of the EPS

The culture was smeared onto a slide and flooded with an aqueous solution of Alcian blue 8G X (pH 2.5) and incubated for 30 mins and carefully washed with distilled water. Microscopic observation revealed that the transparent exopolymer particles were stained blue(Passow & Alldredge 1994).For the alcian blue adsorption assay for cell free/cell bound exopolymers, the 24hr culture broth was centrifuged and the pellet was resuspended in 4 ml carbonate-bicarbonate buffer(0.2 M,pH10). The assay was carried out by adding 1 % aqueous alcian blue solution (8 µls)(8G X Sigma) to 4ml of

24 hr old culture broth and the cell suspension. The mixture was centrifuged after 5 mins and the colour retained in the supernatant was measured at 606 nm. Aqueous and carbonate-bicarbonate controls without cells and negative control of cells were also tested by the assay method. The decrease in the reading of the absorbance in the supernatant, after the cells were removed by centrifugation, determined the amount of the dye bound/adsorbed to the cells/exopolymer (Bar-orand Shilo,1987 &Vandevivere and Kirchman.1993).

3.2.3 Scanning Electron Microscopy (SEM)(Lee *et al*, 1973)

Specimen Preparation Methods from SEM, JEOL application note

The culture was centrifuged at 5000 rpm for 5 min and the supernatant discarded. The pellet was dispersed in 0.05M phosphate buffer (pH 7) and smeared onto the stub. This was fixed in 2ml of 2.5% glutaraldehyde fixative (pH 7.2-7.4), overnight at R.T. The stub was placed in 0.05M phosphate buffer and then in 30% acetone. It was allowed to stand for 10 min. The dehydration procedure was repeated likewise with 50%, 70% and 90% acetone for 10 min, each and finally in 100% acetone for 30 min. The stub was then put in the critical point drying device wherein the acetone gets replaced by liquid carbon dioxide at high pressure. This was evaporated by raising the temperature to 45°C and liquid carbon-dioxide gets converted to gaseous carbon-dioxide and escapes. The process takes 1 h. The stub was placed on the sputter coater (spi-module) specimen holder, after drying. The position of the stage is set such that the specimen is approximately 50mm from the bottom of the sputter head. After sputtering the specimen with 10-15nm thin film of gold, the stub was placed onto the electron microscope sample chamber and observed with JEOL- 5800 LV SEM.

3.2.4 Optimization of growth medium for maximum EPS production by *Microbacterium arborescens*

To elucidate the optimal growth medium for maximal EPS production, various factors influencing EPS production by the culture *Microbacterium arborescens* were assessed.

3.2.4.1 Effect of shaker conditions on EPS production

250 ml Erlenmeyer flasks containing 100 ml of PPYG media were inoculated with 1%v/v) of an overnight grown culture and incubated at static, shaker and shaker + static conditions. Growth, appearance of viscosity and total sugar content in the medium were determined

3.2.4.2 Carbon source

The effect of carbon source on the production of EPS was studied using PPYG supplemented with 1% of glucose, galactose, maltose, sucrose and xylose as carbon and energy source. Further sucrose concentration in the growth medium was varied from 0.25 to 5 %. The culture was grown for 2 days and the cells were removed by centrifugation at 8,000 rpm for 10min at 4°C. The supernatant was passed through 0.2 µm pore size filter (Nucleopore) and the filtrate was dialyzed against distilled water at 4°C using dialysis bags (MW cut-off of 12,000 g). The dialysate was used to estimate EPS by the phenol sulphuric acid method.

3.2.4.3 Yeast extract

The PPYG growth medium was inoculated with the overnight grown *Microbacterium arborescens* -AGSB culture grown in the same medium and the culture incubated at room temperature for 2 days on rotary shaker at 160 rpm and the exopolysaccharide formed was estimated.

3.2.4.4 Nitrogen source

The PPYG containing 1 % sucrose and 0.5 % of nitrogen either as ammonium chloride, ammonium sulphate, sodium nitrate, glycine or urea was used to assess the effect of nitrogen source on EPS production. Further glycine concentration in the growth medium was varied from 0.5 to 3 %. The growth medium was inoculated with the overnight grown *Microbacterium* sp. culture grown in the same medium, incubated at room temperature for 2 days on rotary shaker at 160 rpm. The exopolysaccharide was estimated in the dialysed supernatant described above.

3.2.4.5 Rate of agitation and size of inoculum

The growth medium was inoculated with the overnight grown *Microbacterium* sp. culture grown in the same medium. The culture was grown at room temperature for 2 days on rotary shaker and the exopolysaccharide in the cell free supernatant was estimated.

3.2.5 Growth and EPS production of *Microbacterium arborescens*

The culture *Microbacterium arborescens* was grown in 500ml conical flask containing 100 ml optimized PPYG medium containing 1% sucrose as carbon source.

Culture was grown on rotary shaker (160 rpm) at room temperature and 5ml aliquots were removed at regular intervals. Growth was monitored by measuring optical density at 600 nm. Samples were centrifuged (8000 rpm for 10 min) and 1 ml supernatant was dialysed, and a suitable aliquot was used to estimate EPS concentration using phenol sulphuric acid method.

3.2.6 Isolation of EPS

Large scale production of EPS was studied in batch cultures. *Microbacterium* sp. AGSB was grown in 2 liter conical flasks containing 500 ml of the optimized PPYG medium (1% sucrose as carbon source) and PPYG medium (1% glucose as carbon source).The medium was inoculated with 1% of the overnight grown culture broth. Flasks were incubated at room temparature on a rotary shaker (160 rpm) and removed after 2 days. Bacterial exopolymer was extracted from the culture broth by centrifuging at 10,000g for 20 mins at 4°C, the supernatant was then filtered through Whatman GF/F (pore size 0.47 µm) glass filter followed by filteration through 0.22 um millipore filters and the filtrate concentrated using rotary vacuum evaporator. EPS was precipitated by adding 3 volumes of chilled isopropanol, the threads formed collected on a glass rod and left overnight at 4°C for complete precipitation. The precipitated EPS was collected after decanting the isopropanol, dissolved in small volume of distilled water and dialysed using dialysis bags (MW cut-off 13,000 Da) at 4°C to remove any low molecular weight sugars or salts present (Decho,1990 ; Decho and Lopez,1993). The tubes containing the polymer suspension were frozen and hydrolyzed to powder form using Labconco lyophilizer. The purified, lyophilized polymers were stored at –20°C for subsequent analysis.

3.2.7 Chemical analysis of EPS

Lyophilized EPS was hydrolyzed with 2N HCl for 2 hrs at 100°C in ampoules flushed with N_2 before sealing (Read and Costerton, 1987). The components were assayed for total carbohydrates (Dubois *et al*, 1956), uronic acids (Tullia *et al*, 1991; Blumenkrantz and Asboe-Hanzen, 1973), proteins(Smith *et al*, 1985), pyruvates (Slonecker and Orentas, 1962)(Appendix D) and inorganic content was estimated by gravimetric method. The pre-weighed EPS sample was ashed at 400°C for 4h and cooled to room temperature. The resulting ash quantifies the inorganic content of the EPS and the organic material lost as CO_2 was estimated from the difference in weight before and after ashing. Each value is a mean of three determinations.

3.2.8 Sugar composition of the EPS

In order to determine monosaccharide composition of the EPS, 2 mg of the polysaccharide was hydrolyzed with 2 ml of 2N HCl for 3h at 100°C in ampoules flushed with nitrogen before sealing. After hydrolysis the HCl was removed by repeated evaporation (three times) at 40°C under reduced pressure(dissolved in 2 ml distilled water and evaporated to dryness under reduced pressure)The sample was dissolved in 1 ml distilled water and internal standard was added (100 µg).A drop of triethylamine was added and pH raised to 8.6 to 9. The mixture was left to stand for 30 min to allow the hydrolysis of lactones. The samples were treated with excess of sodium borohydride (20 mg of $NaBH_4$) at room temperature in dark for 3 h to reduce aldoses to alditols. Excess of borohydride was destroyed by adding glacial acetic acid untill effervescence ceases completely. The samples were evaporated to dryness in rotary evaporator bath at 40°C. 8 ml of methanol was added and evaporated to

dryness using rotary evaporator at 40°C.This was repeated thrice to remove boric acid as ethyl ester of boric acid. The pear shaped flask containing the samples were dried overnight at room temperature in a dessicator with a silica gel and KOH after applying vacuum.0.3 ml of pyridine and acetic anhydride were added to the reaction mixture and kept overnight at room temperature for acetylation. The resulting mixtures containing alditol acetates were evaporated to dryness and dessicated overnight under vacuum over silica gel and KOH. The samples were dissolved in 4 ml of water and extracted thrice with 4 ml of dichloromethane each time. Extracts were pooled and dried by adding anhydrous sodium sulphate. The filtrate was evaporated to dryness under reduced pressure. The alditol acetate mixture was transferred into vial by dissolving with a small amount of dichloromethane. The sample was dried and dissolved in small volume of dichloromethane and subjected to analysis by capillary gas chromatography (Bhosle *et al,* 1995).

3.2.9 Chromatographic separation

Capillary chromatography of alditol acetates was performed using a capillary gas chromatogarph (GC)(Chrom pack, Middleburg, The Netherland) using a fused silica capillary column coated with CP Sil-88(L=25m,i.d.=0.32mm,df=0.12um) and equipped with flame ionization detector(FID) and on column injector. Nitrogen was used as carrier gas. 1 μl of the sample was injected at 70°C. The oven temperature was then rapidly raised to 150°C and further programmed to raise at 3°C/minute to 230°C and maintained at this temperature for 40 min. The alditol acetates were identified by comparing the retention time with authentic standard. Quantification was done by peak area integration of the GC results. Response factor for the alditol acetates were

calculated using a standard sugar alditol acetates and were compared to the response for the inositol internal standard for absolute quantification.

3.2.10 X-ray diffraction analysis

For X ray diffraction analysis lyophilized polymer samples were mounted on a slide with a cavity for the sample and XRD was performed using Rigaku X-ray diffractometer (Japan).

3.2.1 Characteristic properties of the polymer

3.2.11.1 Emulsification

a) Emusification properties of the culture broth supernatant

5 ml benzene was added to 5ml of the supernatant obtained after centrifugation of the culture broth after 48 hrs. The tubes were vortexed vigorously for 10mins and left undisturbed for 1 hr. Absorbance of the emulsion formed was measured at 610 nms using uninnoculated PPYG as blank. Increase in turbidity was taken as a measure of emulsification activity. The dye oil red O and crystal violet was added to monitor visual distinction between the oil and water phases and determine the type of emulsification Cooper *et al*,1987).

b) Emulsification properties of the cell bound polymer

The cell pellet obtained after centrifugation of the culture broth after 48 hrs was suspended in phosphate buffered saline so as to give an absorbance of 0.54. To 5ml of this suspension was added 5ml of benzene and emulsification property determined as above (Cooper *et al*, 1987).

c) Emulsification activity of the polymer

5 mg of the lyophilized polymer was suspended and dissolved in 1 ml of deionised water by heating in a bioling water bath for 30 mins. Phosphate buffered saline was added to the sample to make up the volume to 5ml and emulsification activity was determined as described (Cooper *et al*, 1987).

3.2.11.2 Metal adsorption by the polymers

10 mg of purified polymer were suspended for 1 h at room temperature in 10mM $CuSO_4$ solution (1 mg/ml). After 1 h of equilibration at room temperature, the polymer-metal mixture was observed for the metal bound to the polymer. A process control was maintained and monitored as described.

3.2.12 Extraction of Plasmid DNA from *Microbacterium arborescens*

A) Extraction of Plasmid DNA from cells (Maniatis *et al*, 1989)

The culture was inoculated in PPYG broth pH 10.5 and incubated on the shaker, 160 rpm for 24h. Cells from 1.5ml broth (OD = 1.0) were centrifuged at 10,000 rpm for 30 secs at 4°C in an eppendorf tube. The pellet was suspended in 100ul of ice cold Solution I(Appendix E) and vortexed vigorously. To this 200 µl Solution II(Appendix E) was added and mixed thoroughly by inverting gently for 5-6 times. The eppendorf was then stored in ice and 150 µl of ice cold solution III(Appendix E) was added to it and vortexed gently for 10 sec. The eppendorf was stored in ice for 3-5min and further centrifuged at 12,000g for 5min at 4°C. The supernatant was then transferred into a fresh eppendorf tube and equal volume of phenol-chloroform solution was added to it and centrifuged at 12,000g for 5min at 4°C. The aqueous phase was

collected using a pasteur pipette and two volumes of ice cold ethanol was added to it and mixed by inverting and centrifuged at 12,000g for 5 min at 4°C.The pellet was collected and 40 µl TE buffer(Appendix E) containing RNase was added to it and tapped gently. It was stored at -20°C untill further processing.

B) Agarose Gel Electrophoresis of plasmid DNA (Maniatis *et al*, 1989)

Agarose gel 0.8% (w/v) was prepared in 1X-TAE buffer (pH 8.0) (Appendix E) by heating in a microwave oven for 2 min. The platform for electrophoresis was sealed on open sides with leucoplast. To molten agarose (50ml), 5µl of ethidium bromide (10mg/ml) (Appendix E) was added to a final concentration of approximately 4 µg/ml in molten agarose, poured into the platform to a thickness of 0.5 cm and allowed to set at room temperature. After setting, the comb and leucoplast were carefully removed. The gel slab was placed in the electrophoretic chamber and 1X TAE buffer was poured into the chamber till the gel was just below the buffer.DNA sample (10µl) was mixed with 2µl of tracking dye and added in the sample slots of the agarose gel using micropipette.The lid of the electrophoretic chamber was closed. The electrodes were connected to the power supply by means of connecting wires. The voltage was adjusted to 72V and the electrophoresis was carried out at constant voltage for 2h.After electrophoresis the gel was observed on a UV-photodyne transilluminator and the plasmid DNA was visualized

3.2.13 Effect of culture broth on aggregation of soils

The EPS producing, *Microbacterium arborescens* culture was grown in PPYG broth (pH 10.5) and a 10 % inoculum was inoculated in 1 kg of sandy soil, agricultural soil

and mine reject soil and left undisturbed for 45 days. A control was also maintained for each. After incubation period of over a month, the soils were sieved in sieves with different mesh sizes to check for aggregate formation.

Results & Discussion :

The isolate tentatively identified as *Microbacterium* showed a potential of producing exopolysaccharide as visually observed on its cultural characteristics when picked up from the plate for subculturing (Fig. 3.2). Further confirmation has been carried out using the alcian blue staining and the adsorption assay.

Alcian blue is a polysaccharide specific anionic stain which stains extracellular material but not the cells as revealed by light microscopy (Vandevivere & Kirchman, 1993). The alcian blue side chains react with the acidic groups of polysaccharides yielding an insoluble non-ionic pigment. The selectivity of the stain can be controlled by varying either the pH or the salt content of the dye solution. An aqueous dye solution at a pH of 2.5 without extra electrolyte will stain both carboxyl and sulfated polysaccharides instantaneously, but not neutral sugars while an aqueous dye solution at pH 0.5 stains only uronic acids present in the polymer (Passow & Alldredge,1995). In the present study, when the isolate was stained with Alcian blue pH 2.5 it depicted granulated forms indicating the presence of sulphates and carboxylates(Fig 3.3a) while when stained with alcian blue pH 0.5 it depicted thread like forms indicating the presence of uronic acids(Fig 3.3b).

Further it was interesting to note that alcian blue was also adsorbed on the cells obtained from media indicating the presence of cell associated exopolymer. Alcian blue adsorption assay is a semiquantitative assay for cell bound exopolymers

Fig. 3.2 Viscous exopolysaccharide produced by *Microbacterium arborescens*

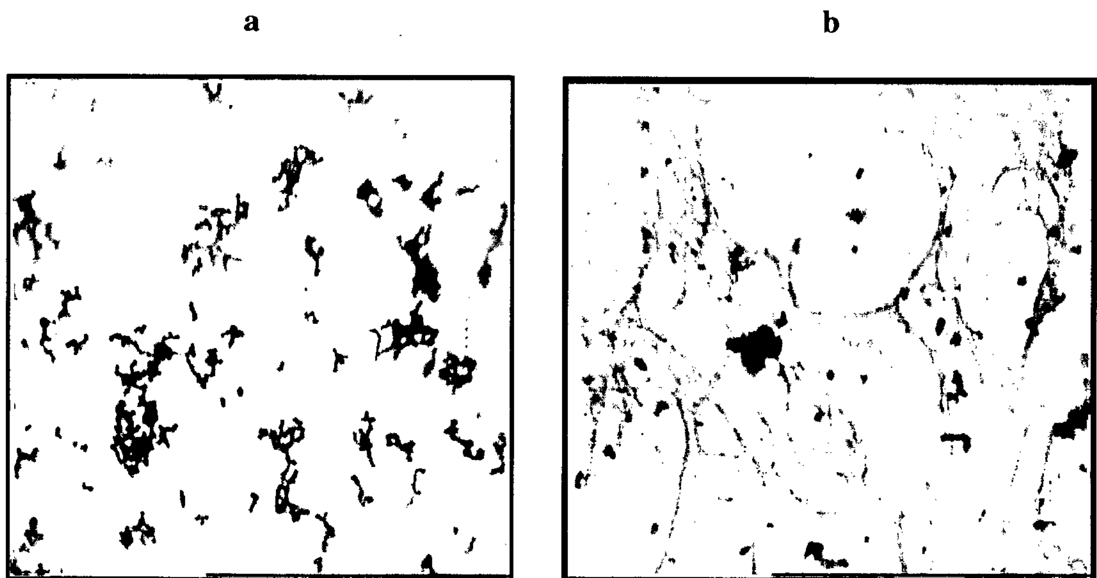


Fig. 3.3 a) Sulphates stained with Alcian Blue (pH 2.5), b) Uronic acids stained with Alcian Blue (pH 0.5)

based on the adsorption of alcian blue, a dye known to bind polymers. This assay estimates the amount of exopolymer by decrease in absorbance of supernatant(Vandevivere & Kirchman, 1993). As seen in Table 3.1 the absorbance of the supernatant of cells/entire culture broth showed a marked reduction as compared to the controls (Vandevivere and Kirchman, 1993). Further, scanning electron micrographs of the isolate has also confirmed the presence of exopolysaccharide in the form of fibrils which help in aggregating the cells together (Fig 3.4). It was therefore of interest to understand the relation between exopolysaccharide and the growth phase of this isolate

A characteristic growth curve in PPYG medium of the exopolysaccharide producing bacterium *Microbacterium arborescens* is depicted in Fig 3.5. The lag phase of the culture was for about 4h, thereafter the exponential phase lasted for 14h followed by the stationery phase. EPS production was observed at all stages of culture growth, however, the maximum EPS production was seen during stationary phase of growth with concentration being maximum at 38h of growth. The results have indicated that although exopolysaccharide is produced as a secondary metabolite during the stationery phase (Read & Costerton,1987; Majumdar *et al*, 1999; Torino *et al*, 2000 : Duenas *et al*, 2003), this culture also has the ability to produce such polymers during the exponential phase depending upon the availability of nutrients such as the carbon source.

A number of physiological conditions such as aeration and nutrient composition of the medium are reported to influence EPS production by bacteria. The culture was therefore grown under shaker, static and shaker + static conditions to understand the effect of aeration on EPS production. It was interesting to note that

Table 3.1 Alcian blue adsorption assay for exopolymer

Sample	0.D at 606 nms
A (cell pellet)	0.13
B (culture broth)	0.44
Control	
Carbonate-bicarbonate buffer pH 10	0.60
Distilled water	0.55

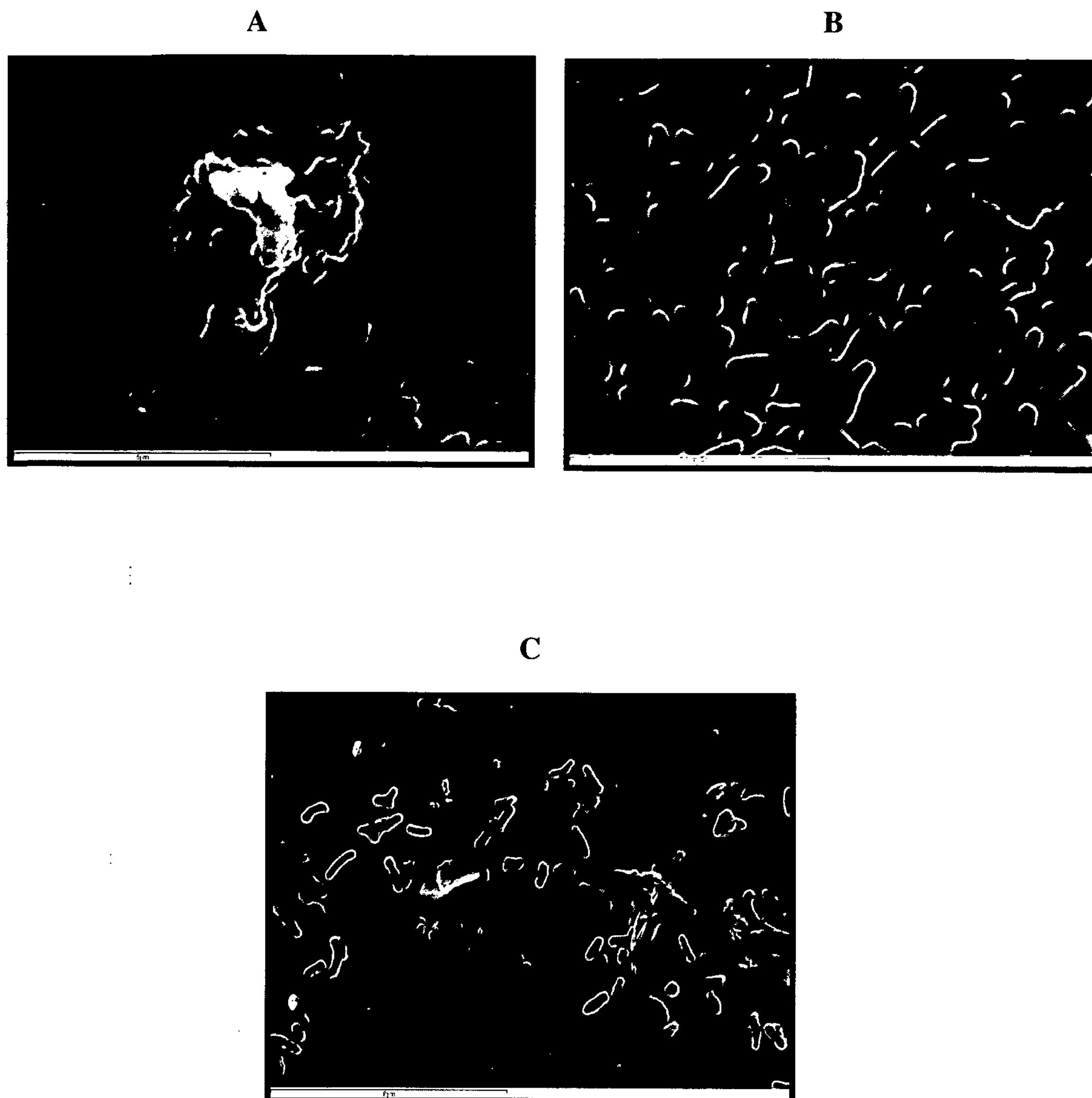


Fig 3.4 Scanning electron micrographs of Exopolysaccharide formation by *Microbacterium arborescens*(Fig A, Bar, 5um). Most of the surface has been colonized with actively dividing rod cells, and finger-like projections of extracellular polymeric material are present(Fig B, Bar, 5um). High magnification indicates the presence of extracellular polymeric materials on the surfaces of bacterial cells (Fig C, Bar, 8 um).

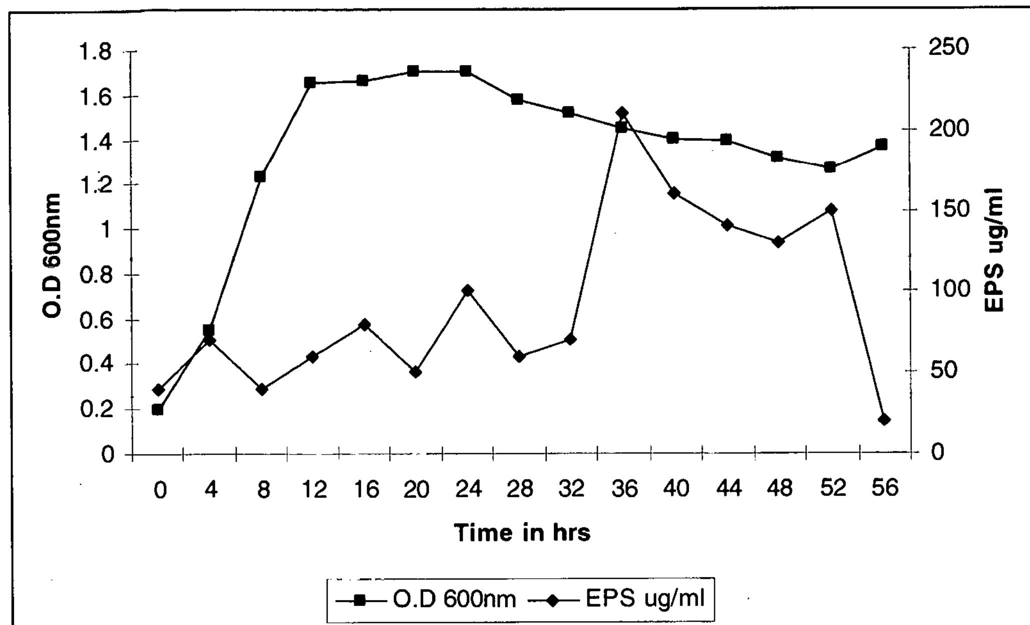


Fig 3.5 Growth curve and EPS production by *Microbacterium arborescens* grown in PPYG with glucose (1%) as carbon source

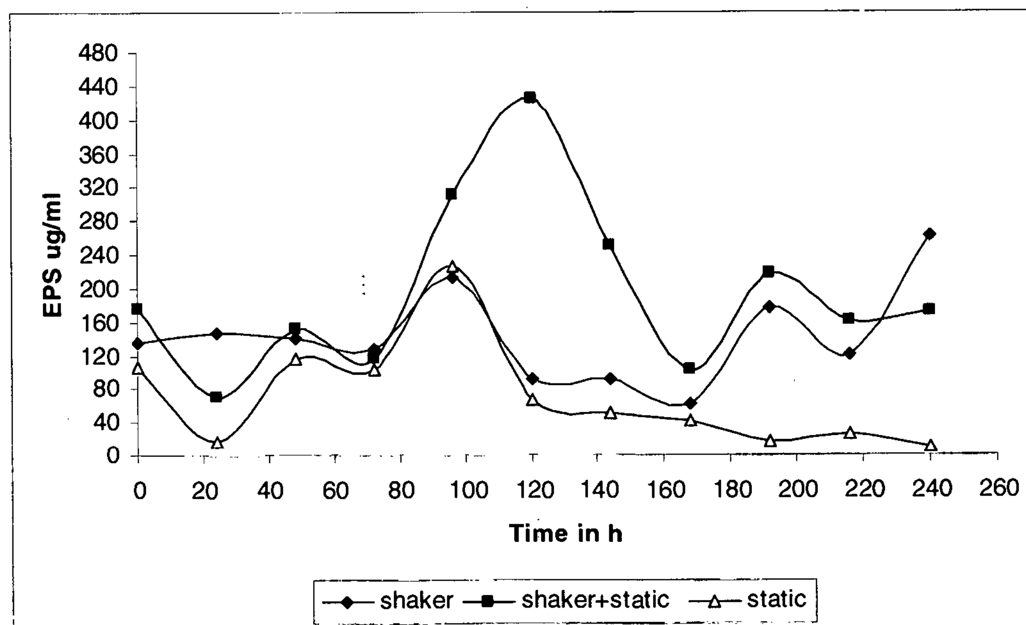


Fig 3.6 EPS production by *Microbacterium arborescens* in PPYG under shaker, shaker+static and static conditions

maximum EPS was produced when the culture was shaken for 120h and then kept stationary. The EPS production during this period was 430 µg/ml (Fig 3.6). However further incubation (50h) was found to reduce the quantity to 120 µg/ml. thereafter the pattern was followed similar to shake conditions. Such effect reflects on the aerobic nature of the isolate and its utilization of the carbon source under shaker conditions which is reduced when subjected to stationery conditions. Further the shaker conditions with respect to rpm were varied and it was found that maximum EPS production as at 160 rpm.

The total yield of EPS produced by *Microbacterium arborescens* depends on the composition of the medium and the composition of EPS was found to be dependent on the carbon source present in the medium. During the present study the culture was grown in the presence of various carbon sources viz. glucose, sucrose, maltose, galactose, xylose and mannitol, maximum EPS was seen with sucrose carbon source (Fig 3.7a). Further the sugar concentration had a marked effect on EPS yield as increase in the sucrose concentration resulted in increased EPS production(Fig 3.7b), the maximum EPS production occurred with 10 g of sucrose per liter in the medium (1%), above 2% the production of EPS reduced. The effect of sucrose on production of EPS has also been reported by Ko *et al*, (2000) for the marine strain *Hahella chejuensis*. Further it was noted that the concentration dependent increase in EPS production was not correlated with growth. It has also been reported that EPS is produced even when little or no growth occurs (Cerning *et al,* 1994). In the present study the culture broth was found to be extremely viscous as the EPS was being produced and therefore the relation of growth is not depicted with reference to the concentration. As PPYG has 0.15 % of yeast extract, the yeast extract concentration

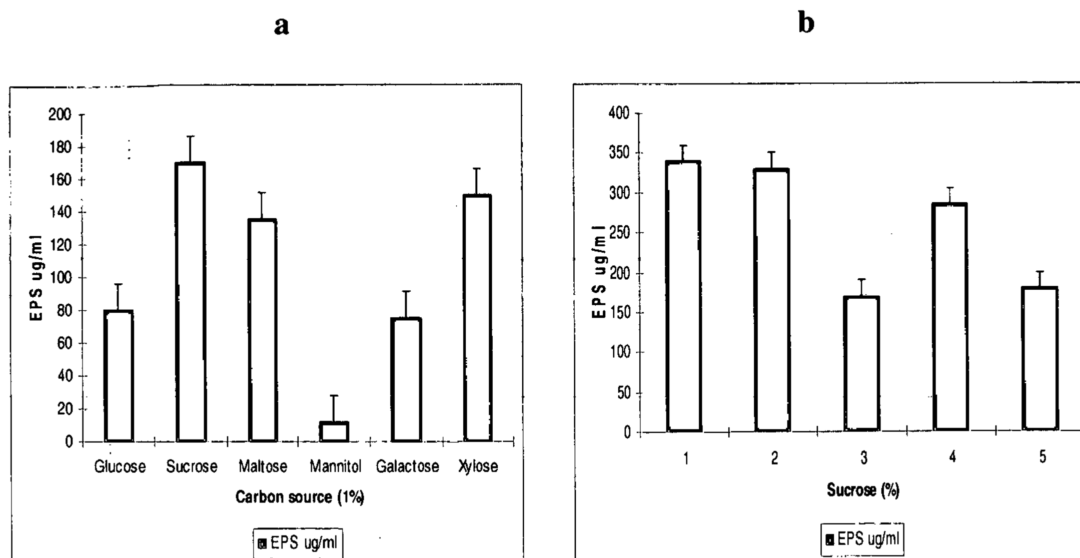


Fig 3.7 Effect of a) different carbon substrates and b) sucrose concentration on exopolysaccharide production by *Microbacterium arborescens*

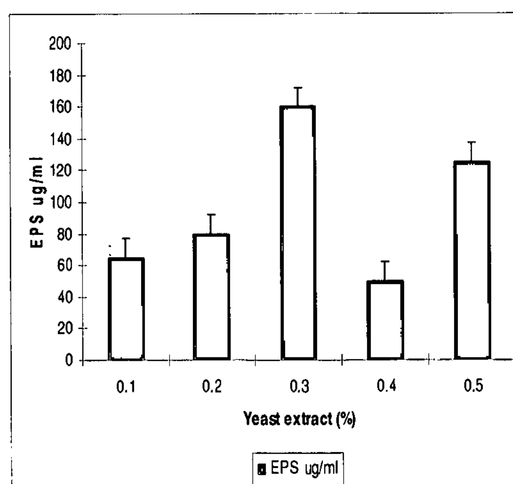


Fig 3.8 Effect of yeast extract concentration on EPS production by *Microbacterium arborescens*

was varied from 0.1 to 0.5 %. It was noted that 0.3% yeast extract concentration was found to produce maximum EPS (Fig 3.8).

When the culture was grown on different concentration of nitrogen (0.5-3%), at lower nitrogen concentration (0.5-1%) there was decrease in exopolysaccharide production (Fig 3.9a). The exopolysaccharide yield varied at higher concentrations (1.5-3%) and showed maximum production at 1.5% nitrogen . Further studies on the effect of various nitrogen sourcs showed maximum production with glycine as nitrogen source with 1.5% concentration of glycine showing maximum production(Fig 3.9b). The effect of amino acids on growth as well as production of secondary metabolites has always been reflected to the fact that readily available amino acids save energy and carbon source and therefore the effect on production of EPS is a secondary metabolite. Inorder to understand this, the culture was also subjected to growth on aspartic acid, glutamate and no significant increase in EPS was noted. This therefore confirms that glycine plays a different metabolic role in the formation of the exoplymers. It has been reported that some bacteria produce high EPS yields when grown on complex nitrogen sources (Souw & Demain, 1979; De Souza & Sutherland, 1994)

Various methods are used for isolation of exopolymers from supernatants. For the polymers to be released from the cells, the polymer needs to be released into the supernatant and precipitated. In the present work, culture broth was centrifuged and the polymer from the cells was found to be released into the supernatant depicting a weak bondage between the cell surface and the polymer. The supernatant was filtered through a millipore filter to remove residual bacterial cells and further filtered through GF/C filters to remove other macromolecules from the supernatant. Further the

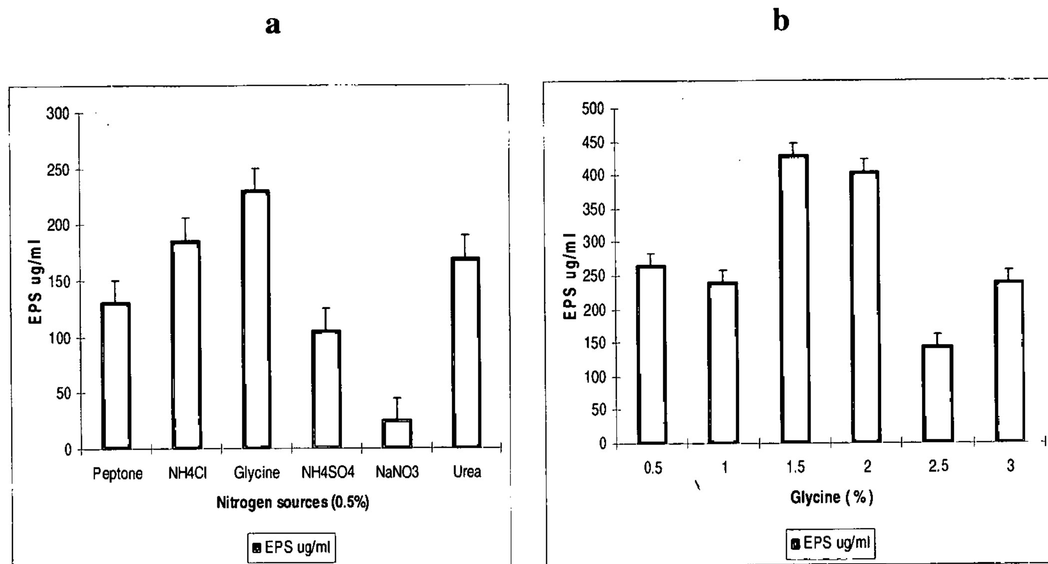


Fig 3.9 Effect of a) nitrogen concentration and b) glycine concentration on EPS production by *Microbacterium arborescens*

polymer was precipitated using ice cold isopropanol. *Microbacterium arborescens* isolated from rhizosphere of coastal sand dune vegetation was found to produce 86 mg/l of exopolymer in PPYG broth medium while on optimization of growth parameters it produced 286 mg/l of exopolymers. The optimized medium was found to produce 3-fold increase (280 mg/l) in polymer as compared to 86 mg/l in PPYG. A significant characteristic of the isolate with reference to the exopolymer was that in the unoptimized medium a single polymer which was orange in colour and flaky in appearance, could be precipitated out however under optimized conditions two polymers were observed both white in colour, one powdery in appearance and the other granular/brittle in appearance, such variations in the polymers from a single isolate reflects on the diverse metabolic potential which is triggered by the external factors for example available carbon.The solubility of the polymers in different solvents is listed in Table 3.2 and the UV-Vis scans of the polymers are listed in Table 3.3. The UV absorption spectrum of a 10mg/ml solution did not absorb strongly at 260 nm wavelength specific for proteins or nucleic acids. A marked peak was found at 288 nm. Matsuyama *et al,* (1999) in their studies on exopolysaccharide producing *Microbacterium kitamiense* sp. nov., isolated from the wastewater of a sugar-beet factory reported that these organisms produced both insoluble and soluble EPS. Ashraf *et al.,*(1999) reported that strains of *Microbacterium sp.* isolated soil and rhizosphere of saline soils produced the most abundant exopolysaccharide.

The chemical composition of the polymer showed significant difference with respect to the carbohydrate, protein, uronic acid and inorganic content as shown in Table 3.4. The cold isopropanol precipitation of dialysed supernatants grown in PPYG produced a single polymer (PP1) while the optimized /defined medium

Table 3.2 Solubility of the polymers isolated from *Microbacterium arborescens*-AGSB in different solvents

Solvents	Normal PPYG	Optimised PPYG
Distilled water	partially soluble	partially soluble
1N HCl	Insoluble	Insoluble
2N HCl	Soluble	Soluble
1N NaOH	partially soluble	partially soluble
Chloroform	Insoluble	Insoluble
Methanol	Insoluble	Insoluble
Diethyl ether	Insoluble	Insoluble
Boiling water for 30 mins	Soluble	Soluble

Table 3.3 UV-Vis absorption maxima of the polymers PP1 and OP1

Polymer dissolved in	PP1	OP1
2N HCl	288, 258 nm	291, 261 nm
Boiling water for 30 mins	288, 250 nm	291, 230 nm

Table 3.4 Chemical composition of the polymers isolated from *Microbacterium arborescens* – AGSB

Chemical composition	Normal PPYG(PP1)	Optimised PPYG(OP1)	Optimised PPYG Supernatent (OP2)
	μg/ml	μg/ml	μg/ml
Carbohydrate	18.3	124.125	286.5
Protein	70.04	39.9	37.49
Uronic acids	6.593	24.38	35.26
Pyruvates	30.59	30.85	33.38
Inorganic content	794	568	428

produced two polymers (OP1 & OP2). Polymer PP1 had high levels of proteins while polymer OP1 had lower amounts of proteins. Another important differing feature between both EPS was the higher concentration of neutral sugars in polymer OP1. All the polymers possessed pyruvates and uronic acids, although concentration of later was higher in OP1 and OP2 polymers. It is interesting to note that the chemical composition of EPS from both polymers of *Microbacterium arborescens* is different from other species from the same genera. Non sugar components including sulfate and protein make up a relatively smaller portion of exopolysaccharide on a per weight basis. However, they may be extremely important to the tertiary structure and physical properties of the exopolysaccharide. The occurrence of these non sugar components imparts acidic nature to the polymer. The presence of high uronic acid confers an overall negative surface charge and acidic properties to the exoplysachharide as it contains an acidic carboxyl group that is ionisable at seawater pH (Iyer *et al,* 2005 ; Decho 1990).The EPS from PPYG broth medium had approximately 6% uronic acid while the EPS from the optimized PPYG medium had 24% and 35 % uronic acids. Exopolymers produced by marine bacteria generally contain 20-50% of the polysaccharide as uronic acid (Kennedy and Sutherland, 1987). Bouchotroch *et al,* (2000) on their studies on exopolysaccharide from *Halomonas* reported that in general the carbohydrate content of the polymers was low. Uronic acids confer anionic characteristics upon the EPS and are responsible for two important biotechnological applications; their use in the biodetoxification of environments polluted with heavy metals, and in waste water treatments. Matsuyama *et al,* (1999) reported that *M. kitamiense* EPS contained neither protein non uronic acids, significantly differing from the EPS produced by *Microbacterium* MC3B-10. Similarly, polymer produced

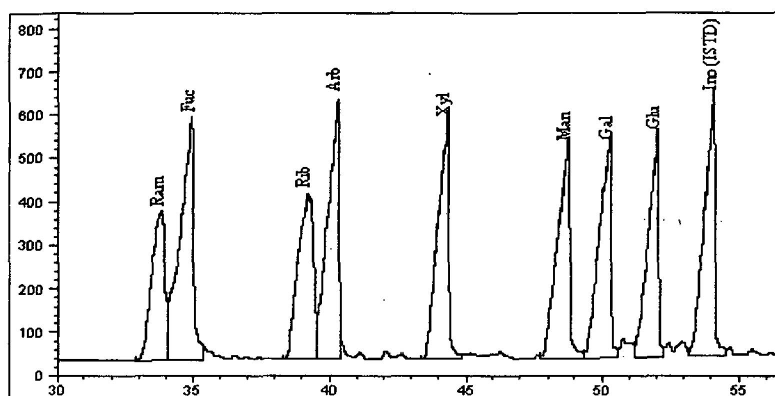
by *Bacillus* sp MC6B-22 contained amino sugars and uronic acids.

The sugar composition of the EPS examined in this study showed that pentoses (ribose and xylose), hexoses (rhamnose, fucose , galactose, mannose and glucose) were present with glucose, galactose and mannose being the most abundant monosaccharides (Table 3.5). The GC analysis of polymers OP1 & OP2 revealed the presence of unusually high amount of mannose (58-74%) (Fig 3.10). Rhamnose was found in large amounts in PP1 polymer (56%) while was detected in small amounts in OP1(9%) and OP2(3%). These sugars are typically found in bacterial EPS (Kenne and Lindberg, 1983).The calculated molar ratio (rhamnose: fucose: arabinose: mannose: galactose: glucose) based on the percent recovery for PP1 polymer (PPYG) was 34:4:1:8:3:6 while for OP1 & OP2 (optimized medium) was 12.5:1.5:1:80:27.5:12.5 (rhamnose : fucose : arabinose: mannose: galactose: glucose) and 10:9:1:2:410:50:50 (rhamnose: fucose: ribose: arabinose: mannose: galactose: glucose) respectively. The presence of the sugar mannose as a major component is common in other exopolymers, xanthan, for example (Anton *et al,*1988; Nichols *et al,*2005; Corsaro *et al,* 2004). Mucoid strains of *Rhodococcus rhodochrous* S-2 produce an extracellular polysaccharide of several million daltons in size, which consists of D-glucose, D-galactose, D-mannose, D-glucuronic acid, stearic acid, palmitic acid and oleic acid(Iwabuchi *et al,* 2002). Exopolysaccharide producing bacteria from sugar beet showed glucose as dominant sugar in the polysaccharides and fructose was second most dominant sugar. Galactose, mannose, and small amounts of rhamnose, fucose and arabinose were present in most polymers, 71 % was glucose and 18 % fructose (Tallgrenn *et al,*1999). Extracellular low molecular weight polysaccharide isolated from *B. japonicum* M1E7 mutant was found to contain glucose, mannose,

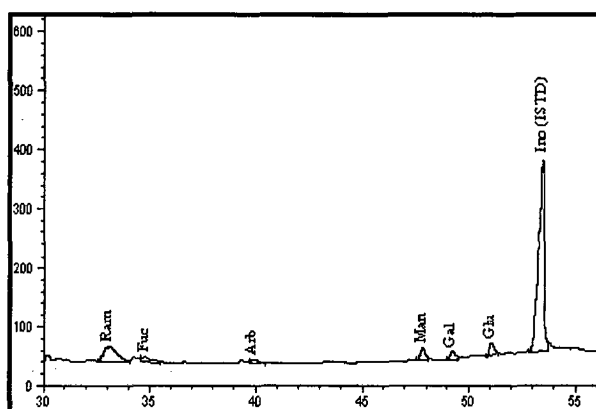
Table 3.5 Monosaccharide composition (%) of the polymers produced by *Microbacterium arborescens* – AGSB

Polymers	Rhamnose	Fucose	Ribose	Arabinose	Xylose	Mannose	Galactose	Glucose
A1	56.8	6.7	0	2.4	0	14.9	6.6	12.4
B2	9.2	1	0	0.6	0	58.5	20	10.3
C2	2.9	1.5	0.2	0.42	0	74.8	9.5	10.3

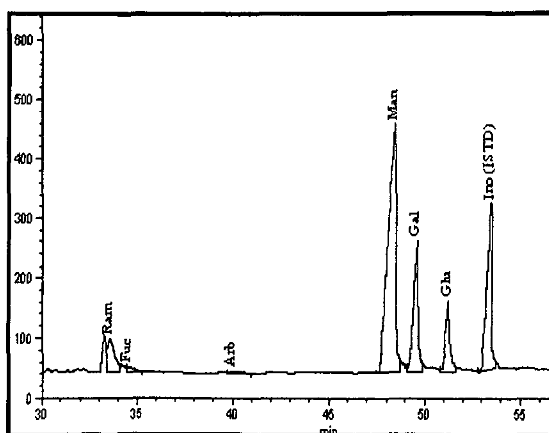
Aldoses Standard



A1 (Polymer PP1)



B2 (Polymer OP1)



C2 (Polymer OP2)

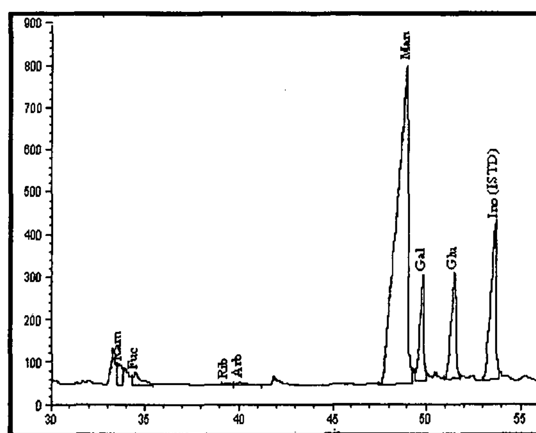


Fig 3.10 GC analysis profiles of a) Aldoses standard and b) Bacterial EPS(A1,B2 & C2). A mixture of 8 aldoses sugars at 1ug/ml each was used as a standard for the caliberation. The sugars estimated are Ram-rhamnose, Fuc-fucose, RIB-Ribose, Ara-Arabinose, Xyl-Xylose, Man-Mannose, gal-Galactose, Glu-Glucose and Ino(ISTD)-Inositol(Internal Standard).

galacturonic acid and galactose in a 2:1:1:1 ratio(Louch and Miller,2001) while Manca *et al,* (1996) reported the synthesis of a sulfated heteropolysaccharide composed exclusively of mannose and glucose in a *Bacillus thermoantarcticus* strain. Morales *et al.,* 2006 reported that *Microbacterium* MC3B-10 polymer is not a polysaccharide but a glycoprotein in nature as it had high levels of protein (36%) and lower concentration of neutral sugars. Rodrigues and Bhosle (1991) reported that the EPS produced by *V. fischeri* consisted of glucose,galactose, mannose, arabinose and rhamnose in the ratio of 3.4:3.7:1.5:0.6:0.7.

Read and Costerton (1987) have reported the presence of glucose and galactose in the ratio of 1:1 in addition to pyruvate and acetate in the EPS produced by *P. flourescens*. The sugar constituents of soluble EPS of *Microbacterium kitami* C2T were 14%(w/w) rhamnose, 19% mannose, 25% galactose and 42% glucose, while those of insoluble EPSs were 27% rhamnose, 13% mannose, 9% galactose and 51% glucose. The sugar constituents of the soluble EPS of Kitami A1 were 31% rhamnose, 19% mannose and 50% glucose, while those of insoluble EPS were 25% rhamnose, 11% mannose, 21% galactose and 43% glucose (Matsuyama *et al,* 1999). Iyer *et al,* (2005) in their studies on characterization of EPS from *E. cloacae* revealed that the GLC analysis showed presence of unusually high amount of fucose, which is quite rare for bacterial exopolysaccharide. It also contained galactose,glucose and glucuronic acid. The molar ratio of the monosaccharide i.e fucose, galactose, glucose and glucuronic acid present in EPS was expressed as 2:1:1:1 while the EPS produced by *Lact. rhamnosus* C83 contained glucose and galactose as the major monosaccharides and traces of other sugars were also detected (Nourani *et al.,* 1998). Bouchotroch (2000) in his studies on EPS from *Halomonas* indicated the presence of

glucose, mannose and galactose in EPS S1 and S32 in ratios 1:4:2.5 and 5:1:1.6, respectively. Muralidharan and Jarachandran,(2003) in their studies on EPS produced by *V. alginolyticus* showed the presence of glucose,aminoarabinose ,aminoribose and xylose in the ratio 2:1:9:1. Royan *et al,(*1999) on their studies on EPS from *Ps .mendocina* P_2d showed the presence of hexoses(rhamnose,50.79%; fucose3.33% and glucose,7.23%) and pentoses (ribose,6.53% ; arabinose,0.76% and mannose,19.21%). Two different EPS's i.e EPS1 and EPS2 were produced by the same bacterial species *B. thermoantarcticus.* EPS 1 was composed of D-mannose and D-glucose whereas D-mannose was present in EPS 2(Manca *et al,* 1996).

Many of the EPS physico-chemical properties are closely linked to the three dimensional structure of the biopolymer network. Small-angle X-ray scattering techniques have also been frequently used in studying polysaccharide structures (Dogsa *et al,* 2005). In order to obtain more information about the bonding of the carbohydrate backbone in the crystalline part of the sheaths, X-ray crystallography was used. X ray diffraction analysis revealed the partially crystalline structure of the polymers (Fig 3.11). Hoiczyk, (1998) studied the X-ray powder diffraction pattern of the isolated and purified sheaths of *Phormidium uncinatum* wherein the recorded sharp signals indicated a substantial degree of crystallinity of the sheath material.

The polymers isolated were found to have significant chemical composition therefore their characteristics in terms of emulsification and metal chelation were studied.

1) The emulsification activity was checked using hexadecane and it was noted that the polymer PP1 showed 16% emulsifying activity while OP1 and OP2 showed 79

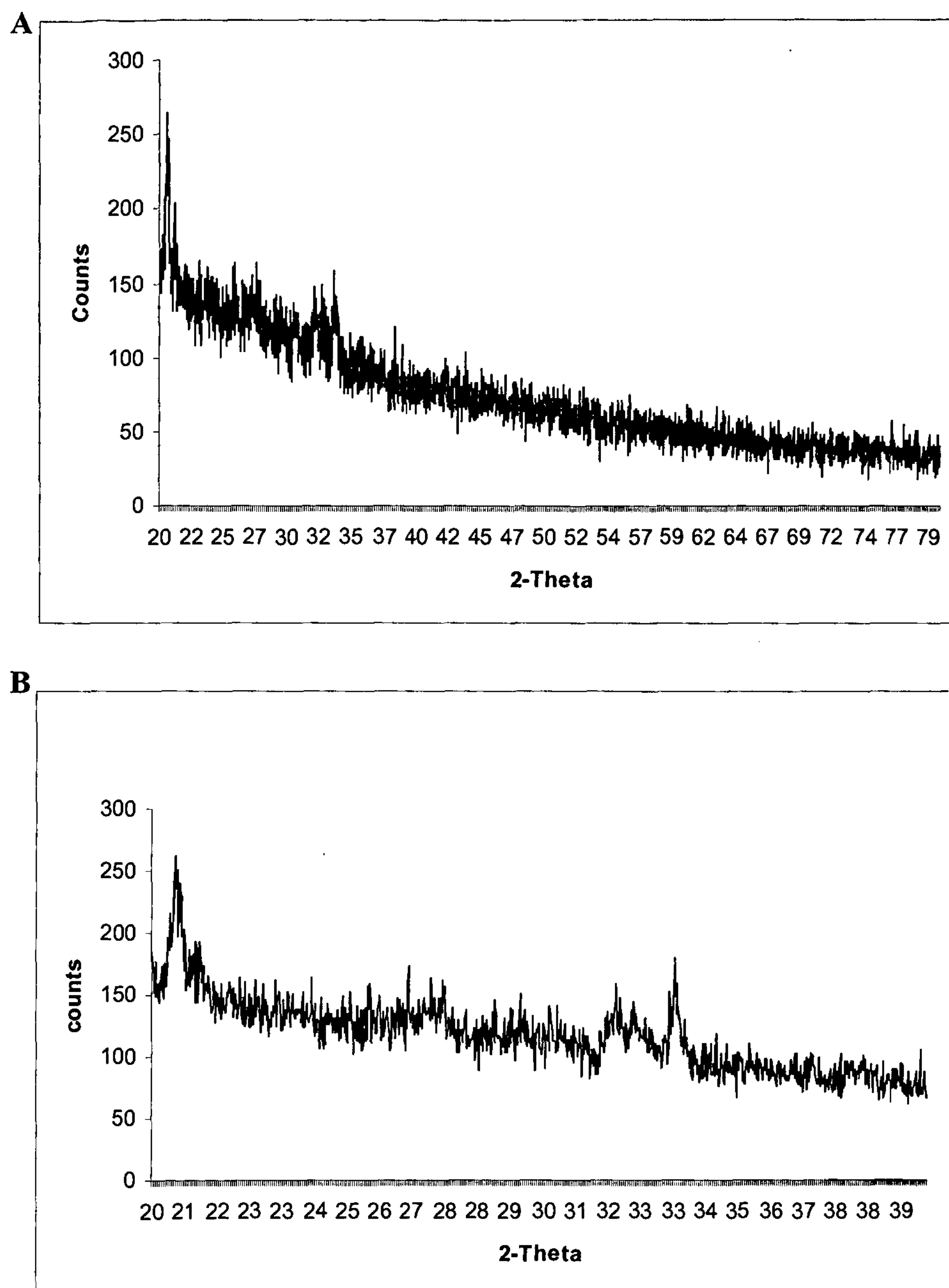


Fig 3.11 X-ray diffraction patterns of the exopolymers A) OP1 and B) PP1

and 83 % emulsifying activity respectively(Fig 3.12). Bouchotroch *et al,* (2000) in their studies reported that strains of *Halomonas* were capable of emulsifying pure hydrocarbons at higher percentages than the controls, which showed their potential for use in the oil industry.

2) The polymers showed metal binding affinity towards copper. Alginates produced by *Azotobacter vinelandii* and *Pseudomonas aeruginosa* which are negatively charged and rich in uronic acid, exhibit a higher metal chelating capacity. Such EPS have been reported to exhibit high copper binding capacity which widens their application in the field of biodetoxification and waste water treatment(Iyer *et al,* 2005). Significantly, *Microbacterium arborescens* from sand dunes also has potential for chelation of metals such as copper.

Plasmids are commonly found in bacteria isolated from a variety of natural habitats Their prevalence suggests that they play a significant role in bacterial ecology and it is now recognized that the well-characterized plasmids represent only a small percentage of those occurring in the environment. In our study a 23 Kb plasmid DNA was detected in *Microbacterium arborescens* (Fig 3.13). Several research groups have associated EPS biosynthesis with plasmids of various sizes: 4.5, 17, 18.5, 26.5, and 30 MDa (Knoshaug *et al*, 2000). Peiris and Dlamini, 2004 reported the involvement of a plasmid in exopolysaccharide production by *Klebsiella oxytoca.* Polysaccharide production in few bacteria has been shown to be controlled by plasmid(Vedamuthu and Neville, 1998).

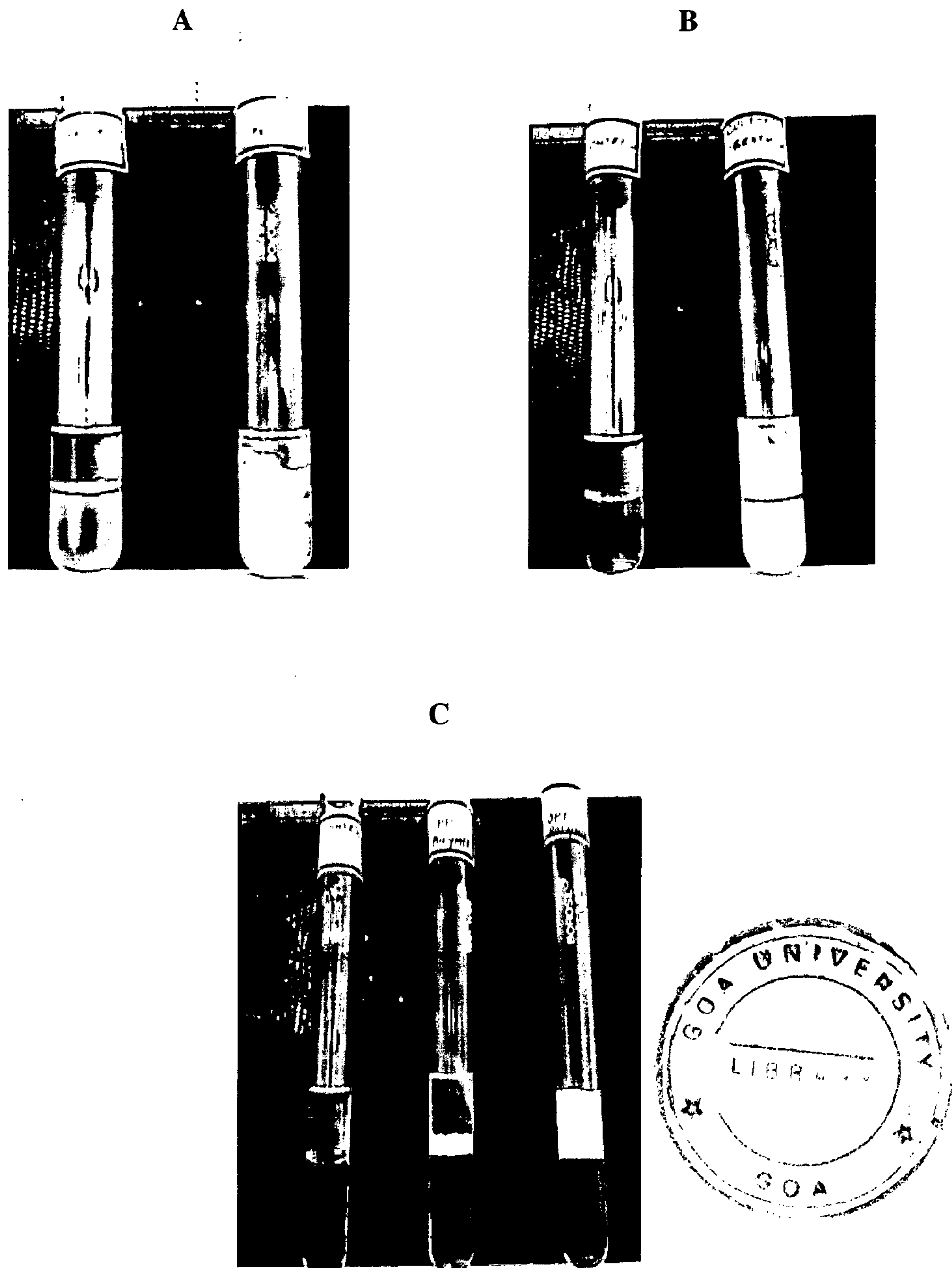


Fig 3.12 Emulsification activity shown by *M. arborescens* A- Culture broth. B - Cell pellet C- Exopolymer

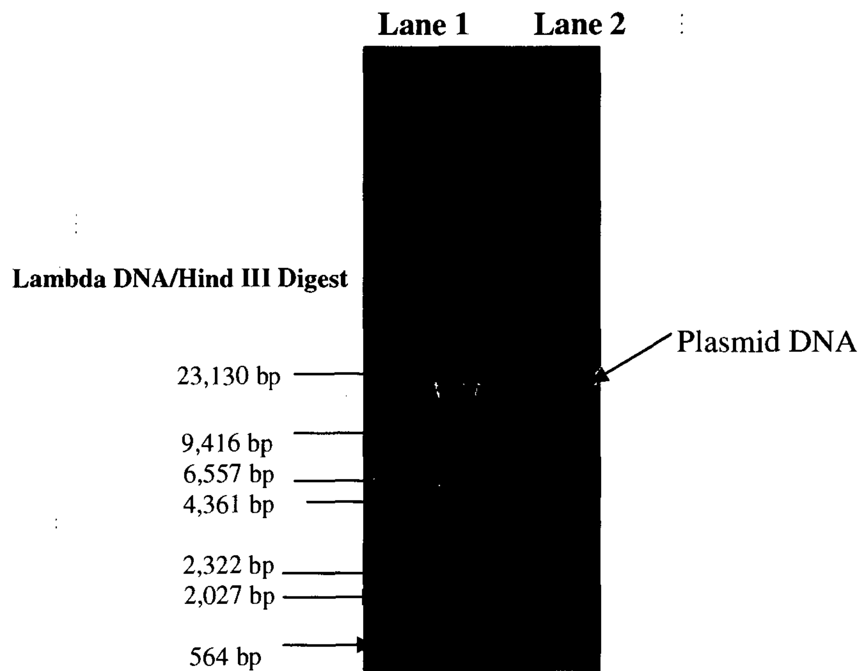


Fig 3.13 Agarose Gel Electrophoresis of Plasmid DNA from *Microbacterium arborescens* (Lane 1 – Mol.wt marker, Lane 2- Culture)

Aggregation and aggregate stability are of fundamental importance to soil profile characteristics and in determining agricultural capacity. The soils when inoculated with culture broth and incubated showed the formation of aggregates of varying sizes. On sieving these aggregates it was observed that the aggregates recovered from sand ranged in size from about 1.5- 3 cm in length and 0.7- 2 cm in breadth. The smaller aggregates were characteristically spherical in shape, but large aggregates were subspherical or flattened. Microrganisms are more important as aggregating agents than either roots or debris and of particular importance in aggregating beach sand. There is strong evidence that soil polysaccharide contribute to soil aggregate stability (Martin, 1971). Bacteria have been shown to cement soil particles together by forming polysaccharide substances. In their free state polysaccharides are easily degraded by other microorganisms but they appear to be protected from such degradation once they are firmly bound within an aggregate or incorporated into a clay lattice. Forster (1979) in her studies on microbial aggregates of sand in an embryo dune system showed the involvement of bacteria in the aggregation and stabilization of sand prior to colonization by higher plants. The production of binding material of a polysaccharide nature by bacteria would cause sand particles to adhere and build up an aggregate. There is strong evidence that microbial polysaccharides contribute to soil aggregate stability and Forster (1979) has stressed the importance of bacteria in the aggregation of dune sand. Aggregation of sand by microorganisms plays an important role in the stabilization of sand dunes with over 2 % of the sand being aggregated by microorganisms. Both free living and plant associated microorganisms are important in this process (Forster and Nicolson, 1981).

After a 45 days period of incubation, aggregate formation was observed in the sandy soils as observed in the plates (Fig 3.14) and Tables 3.6 to 3.8 but the agricultural soils and mine reject soils did not show good aggregate formation and these aggregates were also found to be fragile as compared to the strong binding shown by the *Microbacterium* culture towards sand. It has been reported that *Microbacterium* genera do not aggregate agricultural soils however our isolate seems to have adapted to the sandy soil(Caesar *et al.*, 2004). Perhaps the culture has adapted to the ecosystem to enable it to survive under low moisture and nutrient conditions.

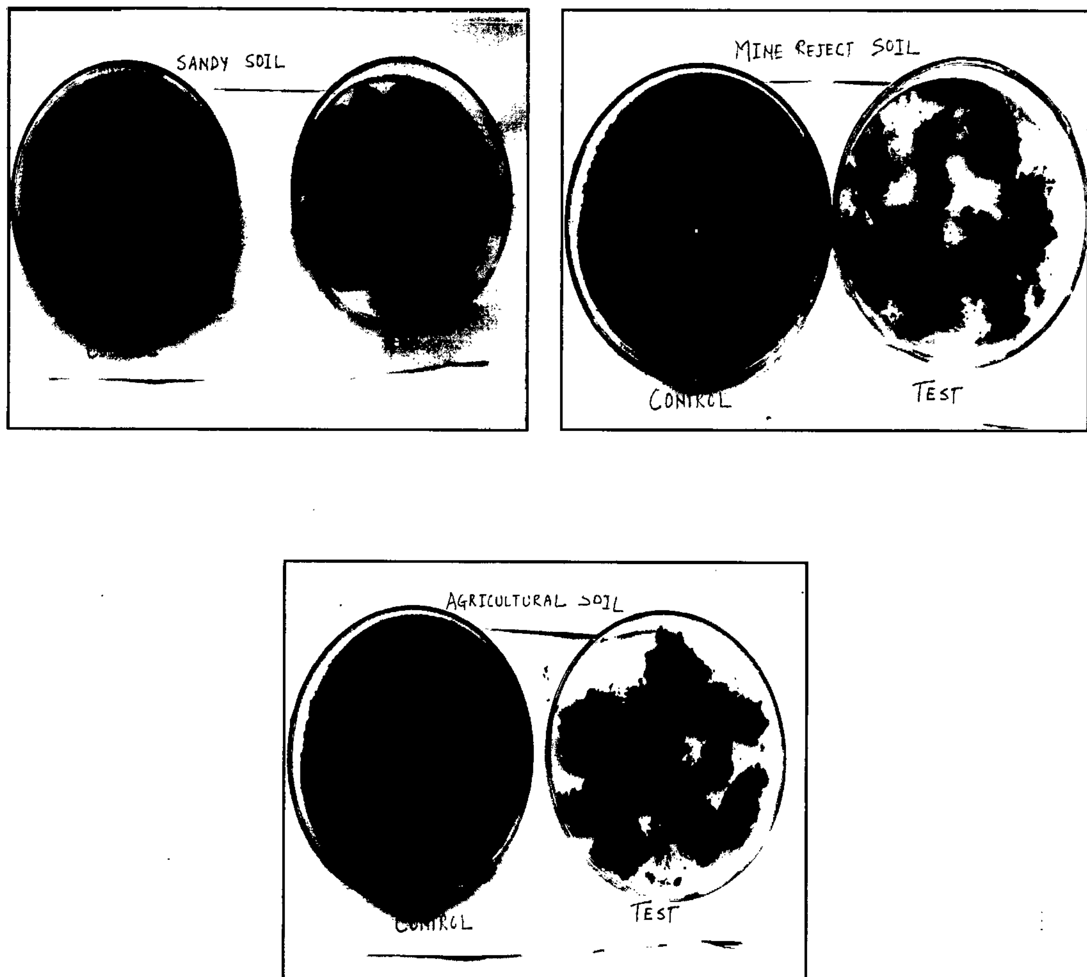


Fig 3.14 Aggregation of soils by *Microbacterium arborescens*

Table 3.6a Sieve analysis of uninoculated sandy soil

Sieve size	Weight of soil retained (g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil (% finer)
mm	g	g	g	%
4.75	0	0	0	100
2.36	0	0	0	100
1.18	0	0	0	100
850u	0	0	0	100
600	0	0	0	100
425	2	2	0.2	99.8
300	6.5	8.5	0.85	99.15
75	989	997.5	99.75	0.25
Pan	0	0	0	0

Table 3.6b Sieve analysis of inoculated Sandy soil

Sieve size	Weight of soil retained (g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil (% finer)
mm	g	g	g	%
4.75	15	15	1.5	98.5
2.36	14	29	2.9	97.1
1.18	782	817	81.7	18.3
850u	61	878	87.8	12.2
600	91	969	96.9	3.1
425	30	999	99.9	0.1
300	0	0	0	0
75	0	0	0	0

Table 3.7a Sieve analysis of uninoculated agricultural/field soil

Sieve size	Weight of soil retained(g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil(% finer)
mm	g	g	g	%
4.75	86.5	86.5	8.65	91.35
2.36	201	287.5	28.75	71.25
1.18	320	607.5	60.75	39.25
850u	48	655.5	65.55	34.45
600	86	741.5	74.15	25.85
425	95.5	837	83.7	16.3
300	45	882	88.2	11.8
75	116.5	998.5	99.85	0.15
Pan	1	999.5	99.95	0.05

Table 3.7b Sieve analysis of inoculated agricultural/field soil

Sieve size	Weight of soil retained(g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil(% finer)
mm	g	g	g	%
4.75	123	123	12.3	87.7
2.36	190	313	31.3	68.7
1.18	260	573	57.3	42.7
850u	50	623	62.3	37.7
600	95	718	71.8	28.2
425	115	833	83.3	16.7
300	14	847	84.7	15.3
75	140	987	98.7	1.3
Pan	11.5	998.5	99.85	0.15

Table 3.8a Sieve analysis of uninoculated mine reject soil

Sieve size	Weight of soil retained(g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil(% finer)
mm	g	g	g	%
4.75	72	72	7.2	92.8
2.36	102	174	17.4	82.6
1.18	135	309	30.9	69.1
850u	29.5	338.5	33.85	66.15
600	52	390.5	39.05	60.95
425	281	671.5	67.15	32.85
300	5	676.5	67.65	32.35
75	310	986.5	98.65	1.35
Pan	12.5	999	99.9	0.1

Table 3.8 b Sieve analysis of inoculated mine reject soil

Sieve size	Weight of soil retained(g)	Cumulative weight retained	Cumulative weight retained as % of total soil	Cumulative weight of % passing as % total soil(% finer)
mm	g	g	g	%
4.75	314.5	314.5	31.45	68.55
2.36	103.5	418	41.8	58.2
1.18	105	523	52.3	47.7
850u	16	539	53.9	46.1
600	42	581	58.1	41.9
425	62	643	64.3	35.7
300	15	658	65.8	34.2
75	256	914	91.4	8.6
Pan	85	999	99.9	0.1

This can also be seen from the particle size distribution curve for sandy soil in Fig 3.16 . Which clearly separates out the sandy soil aggregation from the agricultural soil(Fig 3.17) and mine reject(Fig 3.18) soil when compared to the standard particle distribution curve shown in Fig 3.15. Further studies on different organisms and their role in aggregation would reveal the difference in the polymers produced by the isolates from field conditions and dune conditions.

The studies have revealed the production of two exopolymers from *Microbacterium arborescens* in the presence of sucrose and glycine. These are composed of mannose as the maximum monosaccharide and uronic acids. These polymers showed emulsifying activity and chelation of the metal , copper. Further they were also found to be aggregating sand particles which shows their potential to improve the moisture holding capacity of sand and indirectly support the plant growth. Therefore it was envisaged to also study other extracellular growth promoting traits of these isolates along with three other selected isolates as described in the following chapter.

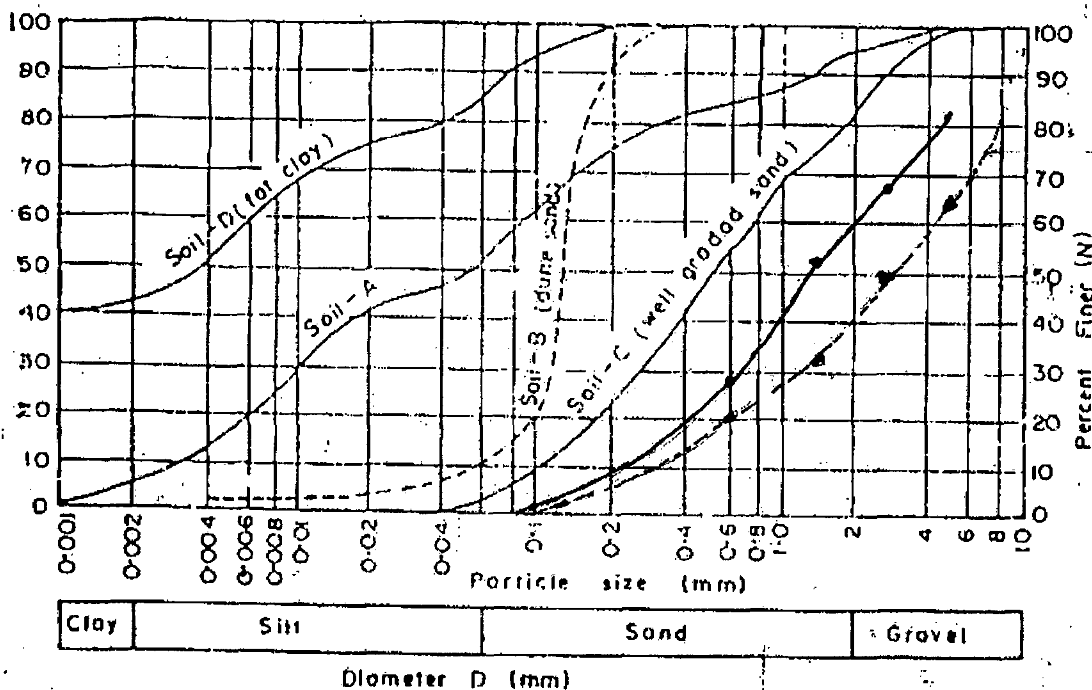


Fig 3.15 Standard particle size distribution curves (Pummia,1987)

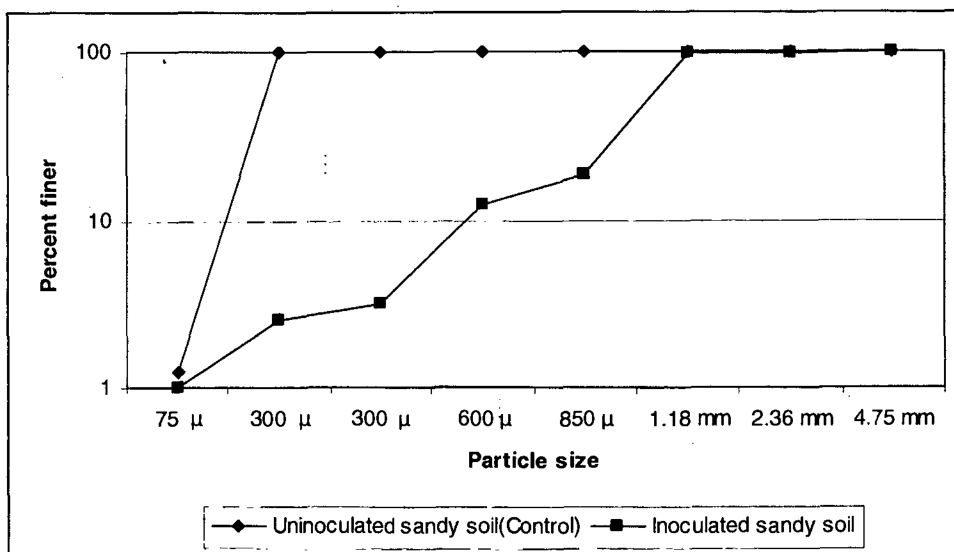


Fig 3.16 Particle size distribution curves of uninoculated and inoculated sandy soil with *Microbacterium arborescens*

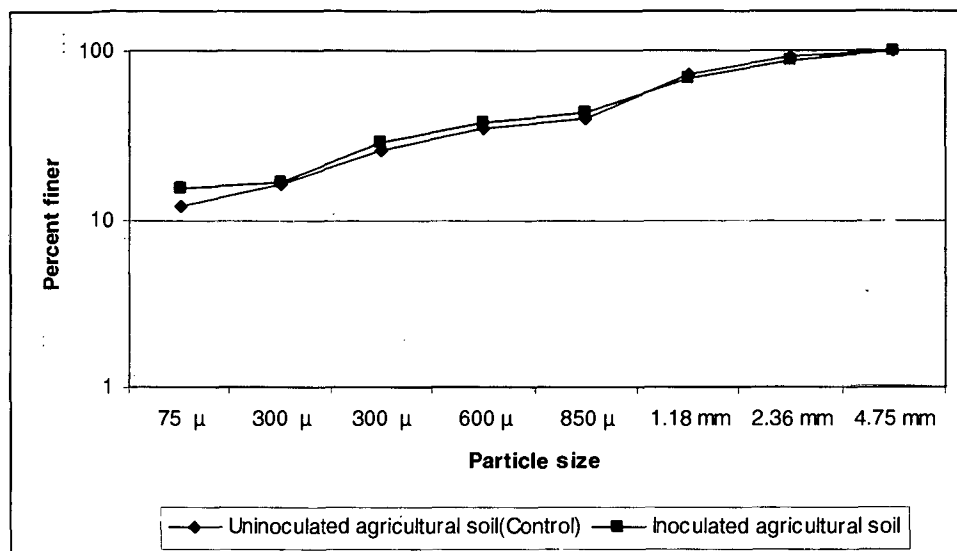


Fig 3.17 Particle size distribution curves of uninoculated and inoculated agricultural soil with *Microbacterium arborescens*.

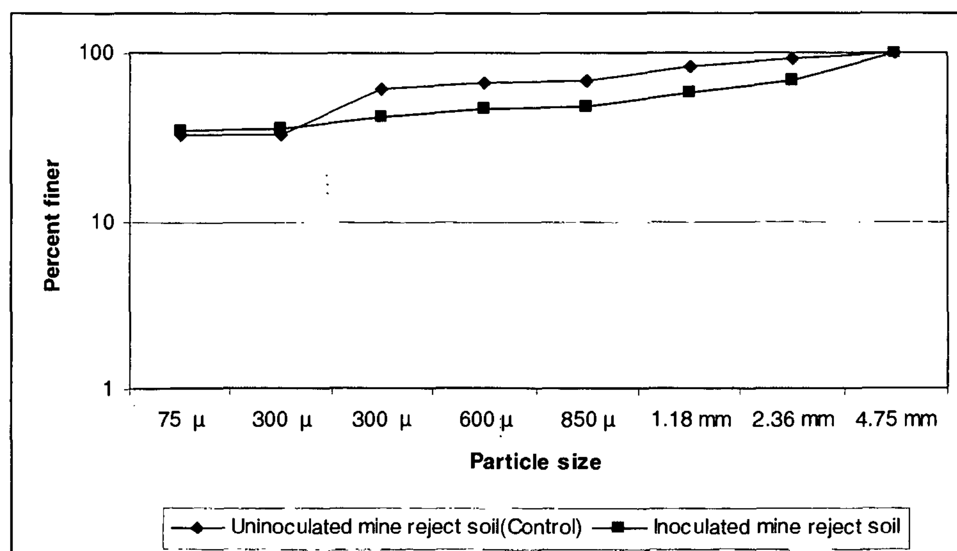


Fig 3.18 Particle size distribution curves of uninoculated and inoculated mine reject soil with *Microbacterium arborescens*.

Chapter IV
Plant growth promoting characteristics of four potential sand dune rhizobacterial isolates

4.1 Introduction

PGPR can help the improvement of plant growth, plant nutrition, root growth pattern, plant competitiveness, and responses to external stress factors. They can also inhibit soil borne plant pathogens by producing growth-promoting chemical substances and inducing plant resistance (Lifshitz, 1987). Different plant-growth promoting rhizosphere bacteria, including associative bacteria such as *Azospirillum*, *Bacillus*, *Pseudomonas* and the *Enterobacter* group have been used for their beneficial effects on plant growth (Kloepper & Beauchamp 1992). Several studies clearly showed the effect of plant growth-promoting bacteria on growth of different crops at different climates, soils and temperatures (Arshad and Javed, 1997).

Plant growth-promoting rhizobacteria (PGPR) are free-living or root-associated bacteria that can increase plant growth and productivity (Kloepper and Schroth, 1978). PGPR are also used as biological control agents owing to their capacity to reduce the development of plant diseases caused by plant pathogenic fungi, bacteria, viruses, and nematodes (Compant, 2005; Kloepper, 2004, Ryu *et al.,* 2005). Various mechanisms by which PGPR promote plant growth and elicit plant defense have been proposed, and some of these have been described in detail. Production of phytohormones such as auxins, gibberellins, cytokinins, and bacterial volatiles (e.g., 2,3-butanediol and acetoin) as a mechanism of plant growth promotion by PGPR has been demonstrated (Bottini, 2004 ; Chanway, 1997 ; Glick, 1995 & Ryu, 2003). Direct suppression of the growth of plant pathogenic fungi by PGPR through the synthesis of antimicrobial compounds such as antibiotics, siderophores, and hydrolytic enzymes has been studied extensively (Compant *et al.,* 2005 ; Weller, 1988).

Microorganisms inhabiting rhizospheres of various plants are likely to affect the growth of plants due to production of secondary metabolites, and on inoculation are responsible to increase the dry weight of leaves and roots of several plant species following root treatment e.g *Azotobacter paspali* (Barea & Brown, 1974). It was found that inoculation of wheat seedlings with *Azospirillum brazilance* increased the number and length of lateral roots (Barbieri, 1986). Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produces low levels of IAA, resulted in a 2 - or - 3 fold increases in the length of seedling roots. It is presumed that PGPR producing plant growth regulators play a critical role in plant growth promotion. Today numerous microbial inocula are available for enhancing crop performance. The oldest and most tested of these is "Nitragin", a preparation containing nodule bacteria (*Rhizobium*) of leguminous plants. Inoculants containing *A. chroococcum* or *Bacillus megaterium va. Phosphaticum* were formulated by soviet scientists under trade names of Azotobacterin and Phosphibacterin, respectively. During the last decades, many new inoculants consisting of the aforementioned and other growth promoting microbial cultures have been commercialized under different trade names in different parts of the world, with claims that these strains improve plant performance.

Work conducted in India and Pakistan on *Azotobacte*r inoculations in soil showed significant increased yield in most experiments. Well designed pot and field experiments have confirmed the positive effects of these bacteria on cereals and vegetables. Also *Azospirillum*, a free living nitrogen fixing bacterium, has increased crop yields significantly under green house and field conditions. Some investigators have suggested nitrogen fixing by this bacteria to be responsible for enhanced plant growth, whereas others have reported that the effects on plant morphology may be

due to production of plant growth promoting substances by the colonizing bacteria or by the plant in reaction to colonization. Phosphobacterin composed of *B.megaterium var. phosphaticum* was first produced commercially in the USSR in 1947, widely used in the country as a soil and seed inoculant. It was claimed that the mechanism of action with this inoculant was enhancement of P solubilization, with a subsequent increase in P uptake by plants. The production of plant growth regulators (PGRs) as microbial metabolites in soil is often directly linked to substrate availability, including plant exudates and animal residues. Microbial production of PGRs may benefit the microorganism as well as the plant by synthesizing phytohormones. Microrganisms play a role in control of their own environment by affecting the plant metabolites. The microbe, in turn, affects the chemical composition of the plant exudates released and hence its nutritional supply. PGR metabolites may function as a) active PGRs b) translocation forms or c) storage products or d) a mixture thereof (Arshad and Frankerberg, 1995; Metting, 1993).

During this study on the rhizosphere bacteria from *Ipomoea pes caprae* and *Spinifex littoreus*, 400 bacterial isolates were obtained. These were further screened for their plant growth promoting traits such as production of siderophores, phosphate solubilization, production of enzymes, IAA, etc. among these four PGPR isolates were selected for further studies and characteristics of siderophore production, phosphate solubilization activities and evaluation of the plant growth promoting traits in soil plant systems in field studies. Studies on growth, characteristics of the siderophore, the phosphate solubilizing activity of the four isolates are compiled in this chapter.

4.2 Materials and methods

4.2.1 Identification of the selected isolates using 16S rRNA sequencing

A single isolated colony of the selected bacterial cultures were taken from agar plate and suspended in 50µl of colony lysis solution (10mM Tris–HCl, pH 7.5, 10mM EDTA and 50µg /mL of proteinase K). The reaction mixture was incubated at 55°C for 15 min followed by proteinase K inactivation at 80°C for 10 min. The reaction mixture was centrifuged at 15,000rpm at 4°C for 15 min. The supernatant containing genomic DNA was directly used as template in PCR reaction. PCR amplification of almost full length 16S rRNA gene was carried out with eubacteria specific primer set 16F27N (5'CCAGAGTTTGATCMTGGCTCAG3') and 16R1525XP (5'TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC-3'), in a 25µl final reaction volume, containing about 10ng of genomic DNA, 1X reaction buffer (10mMTris–HCl, pH 8.8 at 25°C, 1.5mM MgCl2, 50mM KCl and 0.1%Triton X-100), 0.4mM (each) deoxynucleoside triphosphates (Invitrogen), 0.5U of DNA polymerase (New England Labs, UK) and the final volume was made 25µl by adding sterile nuclease free water. The PCR was performed in an automated Gene Amp PCR system 9700 thermal cycler (Applied Biosystems, Foster City, USA) under the following conditions: The amplification conditions were as follows 94°C for 1 min (denaturation), 55°C for 1 min (annealing), 72°C for 10 min (final elongation), 72°C for 10 min final elongation. Expected PCR product of around 1.5 kb was checked by electrophoresis of 5µl of the PCR product on 1% agarose gel in 1X TBE buffer and stained with ethidium bromide (0.5µg/ml). The PCR product was precipitated by PEG–NaCl (20%PEG in 2.5MNaCl) precipitation at 37°C for 30 min. The reaction mixture was centrifuged at 12,000 rpm for 30 min at room temperature. The

supernatant was discarded and the pellet was washed twice with 70% ethanol. After drying the pellet it was resuspended in 5µl of sterile nuclease free water. One microliter (50 ng) of purified 16S rRNA PCR product was sequenced by 16S rRNA specific primer i.e. 16F27N, 530F (GTCCCAGCMGCCGCGG), and 16R1525XP. The sample was sequenced by using BIG DYE Terminator cycle sequencing ready reaction kit (v3.1) in ABI PRISM 3730 Genetic Analyzer (Applied Biosystems, USA).

4.2.2 Phylogenetic analysis

The 16S rDNA sequences were aligned using Clustal X with respective culture nucleotide sequences derived from GenBank. The phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987) with Clustal X software (Thompson *et al*, 1994). Tree topologies were evaluated by bootstrap analysis of the neighbour-joining tree using the original dataset and 1000 bootstrap datasets. The PHYLIP package (Felsentein, 1993) was used to generate trees with the four algorithms and the trees were viewed using the TREEVIEW package (Page, 1996 & Felsenstein, 1981; Pytel and Hedrick, 2006).

4.2.3 Siderophore production by the isolates

4.2.3.1 Detection of siderophores

The bacterial isolates were screened for production of siderophore by chrome azurol S (CAS) plate assay method (Schwyn & Neilands, 1987). Hydroxamate; catecholate and carboxylate nature of the siderophores was examined by the following tests:

A. Hydroxamate nature:

i) Neilands spectrophotometric assay: To 1 ml of cell free supernatant, 1-5ml of freshly prepared 2 % aqueous $FeCl_3$ solution was added. The resultant mixture was scanned between 400-600nm. A peak between 420-450 nm indicated the hydroxamate nature of the siderophores (Yeole *et al,* 2001).

ii) Tetrazolium salt test:

To 0.5ml of cell free supernatant, a pinch of tetrazolium salt and few drops of NaOH were added. Instant appearance of a deep red color indicated the presence of a hydroxamate siderophore(Dave *et al,* 2006).

ii) Csaky assay

Cell free supernatant (1ml) was hydrolyzed with 1 ml of 6N H_2SO_4 in a boiling water bath at 130°C for 30 min. The solution was buffered by adding 3ml sodium acetate solution and 1 ml sulfanilic acid solution, 0.5 ml iodine solution was added. After 3-5min, excess iodine was destroyed with 1 ml sodium arsenite solution(Appendix D). To this 1 ml alpha naphthylamine solution was added and the volume made upto 10ml with water. The absorbance of the color, developed after 20-30 min, was measured at 526nm. (Payne, 1994; Gilliam *et al*, 1981).

B. Catecholate nature

i) Neilands spectrophotometric assay: 1-5ml of freshly prepared 2 % aqueous $FeCl_3$ was added to 1 ml of the test sample. The absorbance of wine coloured complex formed that absorbed maximally at 495 nm, indicated catecholate nature of siderophores (Yeole *et al,* 2001).

ii) Arnow assay

To 1 ml of supernatant, the following reagents were added, mixing after each: 1 ml HCl, 1 ml nitrite-molybdate and 1 ml NaOH(Appendix D). The colour change from yellow to red indicated the presence of catechol in the reaction mixture (Payne, 1994).

C. Carboxylate nature

i) Vogel's chemical test: 3 drops of 2N NaOH and 1 drop of phenolphthalein was added to 5 ml of water to give a light pink colour. Dissapearance of pink colour on addition of test sample, indicated carboxylate nature of siderophores (Yeole *et al,* 2001).

ii) Spectrophotometric assay(Shenkers test): To 1 ml of cell free culture supernatant 1 ml of 250µm $CuSO_4$ and 2 ml of acetate buffer (pH 4) was added. The copper complex formed was scanned from 180 to 800nm and the absorption maxima was noted between 190-280nm as there is no specific wavelength to observe the peak absorption of siderophores(Yeole *et al,*2001).

4.2.3.2 Growth and siderophore production

The cultures were grown in Fiss Glucose Minimal medium (Vellore, 2001)(Appendix A) and monitored at 2hr intervals for siderophore production and growth. Arnow test was performed on the supernatant to check for catechol production and absorbance was taken at 600nm. To study the effect of iron on growth and siderophore production on *Bacillus subtilus*, autoclaved FMM medium, with or without added iron, was used. Iron sufficient media was supplemented with 20 µm of iron from a filter (Millipore,

0.45μM) sterilized $FeSO_4$ stock solution. When examining the effect of increasing concentrations of Fe on siderophore production, various concentrations of $FeSO_4$ (0, 0.25, 0.5, 1, 2, 5, 10, 20, 100 μM) were added to the FMM medium. To study the effect of iron on growth and siderophore production, by the isolate, autoclaved FMM medium with or without added iron was used. Iron sufficient media was supplemented with 20 μM of iron from a filter (Millipore, 0.45 μM) sterilized $FeCl_3$ stock solution.

4.2.3.3 Extraction and Characterization of siderophore

The 24h old culture broth of *B. subtilis* was centrifuged at 8,000 rpm and extracted with ethyl acetate (3:1v/v). The extract was then run on a thin layer chromatograph in a solvent system containing benzene:toluene:acetic acid (2:2:1 by vol.). The siderophore of *B. subtilis* was spotted along with dihydroxy benzoic acids and salicylic acid. The plates were sprayed with Hathway's reagent(Appendix B). The band corresponding to standard 2, 3-DHBA was scraped out and isolated by preparative TLC using the same solvent system and the identity of the compound was confirmed by UV spectroscopic analysis (Shah *et al*, 1993).

4.2.3.4 Effect of iron limitation on the membrane proteins composition of *Bacillus subtilis*

The effect of iron starvation on the outer membrane protein composition of *Bacillus subtilis* was determined by comparing the SDS-PAGE profiles (Maniatis *et al*, 1989) obtained when cells were grown with or without adequate iron present.

Preparation of samples for SDS-PAGE

A 2ml culture broth of *B. subtilis* was inoculated in 50ml Fiss-glucose minimal medium with no added iron, and 50 ml Fiss-glucose medium with 20 µM $FeSO_4$. Cultures were grown for 24 hrs on a rotary shaker at 28°C. Cells were harvested by centrifugation at 7,000 rpm for 10 minutes and the supernatant was discarded. The pellet was suspended in 10 ml of 10 mM Tris buffer (pH 8.0) and sonicated in an ice bath. Sonicated samples were centrifuged at 7000 rpm for 10 minutes.

SDS-PAGE analysis of samples

A 10 % separating gel was prepared (Appendix E) and the ingredients were deaerated for 10 minutes before addition of 10% ammonium persulfate solution that polymerizes the gel. The separating gel was pipetted into a gel-casting unit and was allowed to polymerize for 30 minutes. A stacking gel was prepared (Appendix E) and also deaerated for 10 minutes before adding 10% ammonium persulfate. This gel was pipetted on top of the solidified separating gel and a comb was placed. This gel was allowed to polymerize for 30 minutes and then the gel was placed into an electrophoresis unit. The upper and lower chambers were filled with tank buffer. The pellets were prepared by adding equal volume of 2X gel loading buffer in an eppendorf tube. A protein molecular weight standard was prepared by adding 2ul SDS-PAGE broad-range molecular size marker with 8ul of 2X gel loading buffer. All samples were then placed in a boiling water bath for five minutes. Samples were loaded onto the gel and the gel was run. The gel was then placed in Coomassie Blue stain for 30 minutes, destained, and stored in 5% acetic acid.

4.2.4 Phosphate solubilization activity of the isolates

4.2.4.1 Qualitative and quantitative estimation of phosphate solubilization

Qualitative estimation was conducted using petridish assays on Pikovskaya agar (Gupta *et al*, 1994). Spot inoculation of cultures was carried out using sterile toothpicks and the petridishes were incubated at 28°C. The halo (zone of solubilization) around the bacterial colony and colony diameter were measured after incubation for 48 h. Solubilizaton index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter. Quantitative estimation of tricalcium phosphate solubilization in broth was carried out at 28°C using Erlemmeyer flasks (250ml) containing 100 ml of Pikovskaya broth inoculated with 5% of the respective bacterial inoculum. Autoclaved uninoculated medium was used as the control, one ml of culture broth was withdrawn aseptically at 2h intervals from each flask and centrifuged (10,000 rpm, 20 min), and the supernatant obtained was analyzed for inorganic phosphate content by Fiske and Subbarow method(1925). pH of the supernatant was measured with the help of pH meter, organic acids produced were detected using paper chromatography (Isherwood,1987) using the solvent system n-propanol:conc ammonia (70:30,v/v) for lower fatty acids and n-propanol : water : concentrated ammonia (60:20:20.v/v) for dicarboxylic acids e.g tartaric and malic acids. The spots were visualized by spraying with 0.15% Thymol blue in NaOH, pH10. Ammonium salts of the acids showed up as yellow spots on a deep blue background, cations other than ammonium showed spots with a deeper blue colour than the background (Jones *et al*, 1953, Stark *et al*, 1951).

4.2.4.2 Detection of phosphatase enzyme

1 ml of filter sterilized PNPP (p-nitrophenyl phosphate) (10mM) was added to nutrient agar and PPYG plates and spot inoculated with *Bacillus subtilus*, *Microbacterium arborescens* , *Bacillus sp.* MF-A4 and *Kocuria rosea.* Plates were incubated at room temperature for 24 hr and the yellow halo around the colony was indicative as positive for phosphatase enzyme.

4.2.5 In vitro screening of bacterial isolates for their plant growth promoting (PGP) metabolites

a. Production of indole acetic acid (IAA)

Production of IAA was detected by using the method described by Viswanathan (1999). The isolates were grown in nutrient both containing 0.5% tryptophan for 30h and filtered through a Millipore filter. 1ml of cell free culture filtrate was reacted with two ml of Salkowsky's reagent (Glickmann and Dessaux, 1995)(Appendix B), the tubes incubated for 30 mins and the absorbance at 530 nm was noted. Standard graph was drawn using pure IAA and presence of IAA in the culture filtrate was quantified as μg/ml.

b. Hydrogen cyanide production

Production of hydrogen cyanide by strains was determined using the method of Kloepper *et al*, (1991) with few modifications. The alkaliphilic isolates were spread plated on to PPYG agar supplemented with 4.4 g /l- of glycine. Filter paper strips soaked in picric acid solution (Appendix B) were placed in the lid of each plate.

Plates were sealed with parafilm and incubated for 72h. Production of HCN was indicated by the change in colour of the filter paper strips from orange to red colour. The intensity of the colour was recorded visually (Haas and Defago, 2005).

C. Ammonia production

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10ml peptone water in each tube and incubated for 48–72 h at 28°C. 0.5 ml of Nessler's reagent was added to each tube, and the development of brown to yellow colour was noted indicating a positive test for ammonia production (Cappuccino and Sherman, 1992).

D. ACC deaminase

Bacterial isolates were screened for the ability to use ACC (1-aminocyclopropane carboxylate, Sigma chemical) as a sole nitrogen source. The isolates were streaked on Dworkin and Foster (DF salts) minimal medium (Appendix A) containing 3.0 mM solution of filter sterilized ACC as the sole source of nitrogen. The inoculated plates were incubated at 28°C and growth on the plates was observed (Penrose and Glick, 2003).

E. Pigment

E.1 Extraction and characterization of carotenoids from *Microbacterium arborescens*

Cells were harvested by centrifugation (8,000 x g, 10 min) and the pellet extracted with acetone by sonication using 0.5 pulses for 3 sec interval for 10 mins. The extraction was repeated if necessary until all pigment had been extracted. Extraction was carried out in the dark to avoid light isomerization. Separation of carotenoids was achieved by HPLC on a C-18 a reverse phase column (Waters Spherisorb ODS2 5 ìm, 4.6mm x 250 mm) column, using acetonitrile : water (solvent A) and ethyl acetate (solvent B) at a flow rate of 1 ml/min. Peaks were monitored with a HPLC-WATERS equipped with Waters 2996 Phase diode array detector. The gradient for separation was 0 – 100% ethyl acetate in acetonitrile/water (9:1) over 25 min with flow rate of 1.2 ml/min (Liaaen-Jensen and Andrewes, 1985)

E.2 UV – Vis Spectrophotometry

UV/visible scanning spectra of the acetone extracts containing carotenoids were recorded between 200 and 800 nms on a UV-Visible spectrophotometer.

E.3 Thin layer chromatography of pigment

Pigments were also purified by thin layer chromatography using silica gel plates developed in petroleum ether and ethyl acetate (90:10 v/v). Different solvent systems were used for separation of carotenoids. Petroleum : benzene (98:2 v/v) for separation of carotenoid hydrocarbons, benzene for separation of carotenoid ketones and benzene : methanol (49:1) for separation of carotenoid hydroxylated (Stahl,1969).

E.4 Photosynthetic activity of the culture

The *Microbacterium arborescens*- AGSB culture was grown in broth and analyzed for its photosynthetic activity using a chlorophyll fluorometer (PAM 101, Walz, Effelrich, Germany). Light intensity was supplied and peak production was observed.

Results & Discussion:

Rhizosphere soil and roots of the vegetation (*Ipomoea pes-caprae* & *Spinifex littoreus*) were collected from Miramar, Aswem and mandrem beach in North Goa. The selected isolates were characterized with reference to 16S rRNA sequencing and metabolites produced by them which would help plant growth were tested.

In recent years, microbial taxonomy has been strengthened with the modern techniques developed with molecular tools. One of the important tools is the analysis of 16S rRNA which is a good marker as it has both the regions which are highly conservative and evolutionary. This has been sequenced in a vast array of organisms with an attempt to determine the phylogenetic placement of newer isolates. In the present study, an attempt was made to sequence the 16S r RNA of four selected sand dune bacteria in order to study their relationship to other species of the same genus (Fig 4.1 A, B, C & D). The phylogenetic analysis of the 16S rDNA sequence was accompanied by PCR amplification of approximately 1500 base pairs using universal primers. The resulting PCR segments were sequenced and optimally aligned and phylogenes were constructed using alogorithms available in the PHYLIP site of phylogenetic analysis programs. Phylogenetic trees was constructed by neighbour joining method (bootstrap method) as shown in Fig 4.2 to Fig 4.5. The sequence data of the four selected sand dune bacterial isolates were compared with available 16S

Fig 4.1 16S rRNA base sequence of the selected isolates

A. ***Microbacterium arborescens*** **(GenBank ACC No DQ287961)**

ATCAGTGGCGAACGGGTGAGTAACACGTGAGCAATCTGCCCCTGACTCTG
GGATAAGCGCTGGAAACGGCGTCTAATACCGGATACGAGCTGCGACCGCA
TGGTCAGTAGCTGGAAAGAATTTCGGTCAGGGATGAGCTCGCGGCCTATC
AGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGTCGACGGGTAGCCGGCC
TGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CAACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTATCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGTCTGTCGCGTCTGCTGTGAAAACCC
GAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTA
GGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG
GAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGA
GCGAAAGGGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACCCCG
TAAACGTTGGGAACTAGTTGTGGGGTCCATTCCACGGATTCCGTGACGCA
GCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGCGGAGCATGCGGATTAA
TTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATATAGAGGAAACG
TCTGGAAACAGTCGCCCCGCAAGGTCTCTATACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCA

B. ***Bacillus sp. MF-A4*** **(GenBank ACC NoDQ287962)**

TTGCTCCCGGACGTTAGCGGCGGACGGGTGAGTAACACGTAGGTAACCTG
CCCCTTAGACTGGGATAACTCCGGGAAACCGGAGCTAATACGGGATAATA
AAGAGAATCACCTGATTTTCTTTTGAAAGACGGTTTCGGCTGTCACTAAGG
GATGGGCCTGCGGCGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGG
CGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAA
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATG
GACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAGGAAGGCCTTCGGG
TCGTAAAGCTCTGTTGTGAGGGAAGAACAAGTACCGGGGTTTCGATGCC
CGTAGTGCCGAAGTAAACACATTAAGCACTCCGCCTGGGGAGTACGGCCG
CAAGGCTGAAACTCAAAGGAATTGACGGGGACCCGCACAAGCAGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACAT
CCTTTGACCACTCTGGAGACAGAGCTTCCCCTTCGGGGGCAACGAGCGCA
ACCCTTGATCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTT
ATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCAGCGAA
ACCGCGAGGTGGAGCCAATCCCATAAAGCCATTCTCAGTTCGGATTGTAG
GCTGCAACTCGCCTACATGAAGCCGGAATTGCTAGTAATCGCGGATCAGC
ATGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACC
ACGAGAGTTTGTAACACCCGAAGTCGGTGAGGTAACCTTTTGGA

C. *Kocuria rosea* (GenBank ACC No DQ287963)

GAAAGGGTTTACTGGTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGT
GGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTG
ACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAG
CAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCG
TGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGC
CACAAGTGACGGTACCTGCAGCTGGGTACGGGCAGACTAGAGTGCAGTAG
GGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGG
AACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCTGAGGAGC
GAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTA
AACGTTGGGCACTAGGTGTGGGGGACATTCCACGTTCTCCGCGCCGTAGC
TAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATT
CGATGCAACGCGAAGAACCTTACCAAGGCTTGACATCCACCGGACCGCAC
TGGAGACAGTGCTTCCCTTCGGGGCTGGTGGACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAAC
CCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGC
CGGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCT
TATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAAAGGGTTGCGA
TACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAG
GTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAG
CAACGCTGCGGTGAATACGTTCCCGGGCCTTG

D. *Bacillus subtilis* (GenBank ACC No DQ287964)

GCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGG
CTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAG
TAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGT
ACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC
GGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTG
AAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGA
GATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGAC
GCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTA
GTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAG
ACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCT
GACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGT
GCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAC
GAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGT
GACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGG
GCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCG
GATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCG

rRNA gene sequences in the GenBank using the BLAST program of the National Center for Biotechnology and Information (NCBI), to determine the approximate phylogenetic affiliation. The 16S rRNA gene sequences of the neutrophilic isolate comprising approximately 1500 nucleotides were determined. Fig. 4.2 shows the phylogenetic position of isolate (in bold) clustering on the branch of spore-forming bacteria was more closely related to the genus *B. subtilis*. The 16S RNA gene sequence similarity values of neutrophilic isolate to other strains of the genus *Bacillus* are 100% (Table 4.1). These values and the phylogenetic position shown in the phylogenetic tree (Figure 4.2) indicate that neutrophilic isolate belongs to the genus *Bacillus*, and is closely related to Uncultured bacterium clone 9-sw-su5-2 (DQ981833), with 100 % sequence similarity.

In case of the alkaliphilic isolates, Figure 4.3 shows the phylogenetic position of isolate (in bold) was more closely related to the genus *Kocuria*. The genus *Kocuria* was created from the genus *Micrococcus* on the basis of the phylogenetic and chemotaxonomic dissection of the genus *Micrococcus*. The 16S RNA gene sequence similarity values of the isolate to other strains of the genus *Kocuria* in the range of 97 to 100% (Table 4.2). These values and the phylogenetic position shown in the phylogenetic tree (Figure 6) indicate that the isolate belongs to the genus *Kocuria*, and is closely related to *Kocuria* sp. CNJ787PL04 (DQ448773), with 100 % sequence similarity. Figure 4.4 shows the phylogenetic position of isolate (in bold) was more closely related to the genus *Bacillus*. The 16S RNA gene sequence similarity values of the isolate to other strains of the genus *Bacillus* in the range of 99 to 100% (Table 4.3). These values and the phylogenetic position shown in the phylogenetic tree (Figure 4.4) indicate that the isolate belongs to the genus *Bacillus*, and is closely

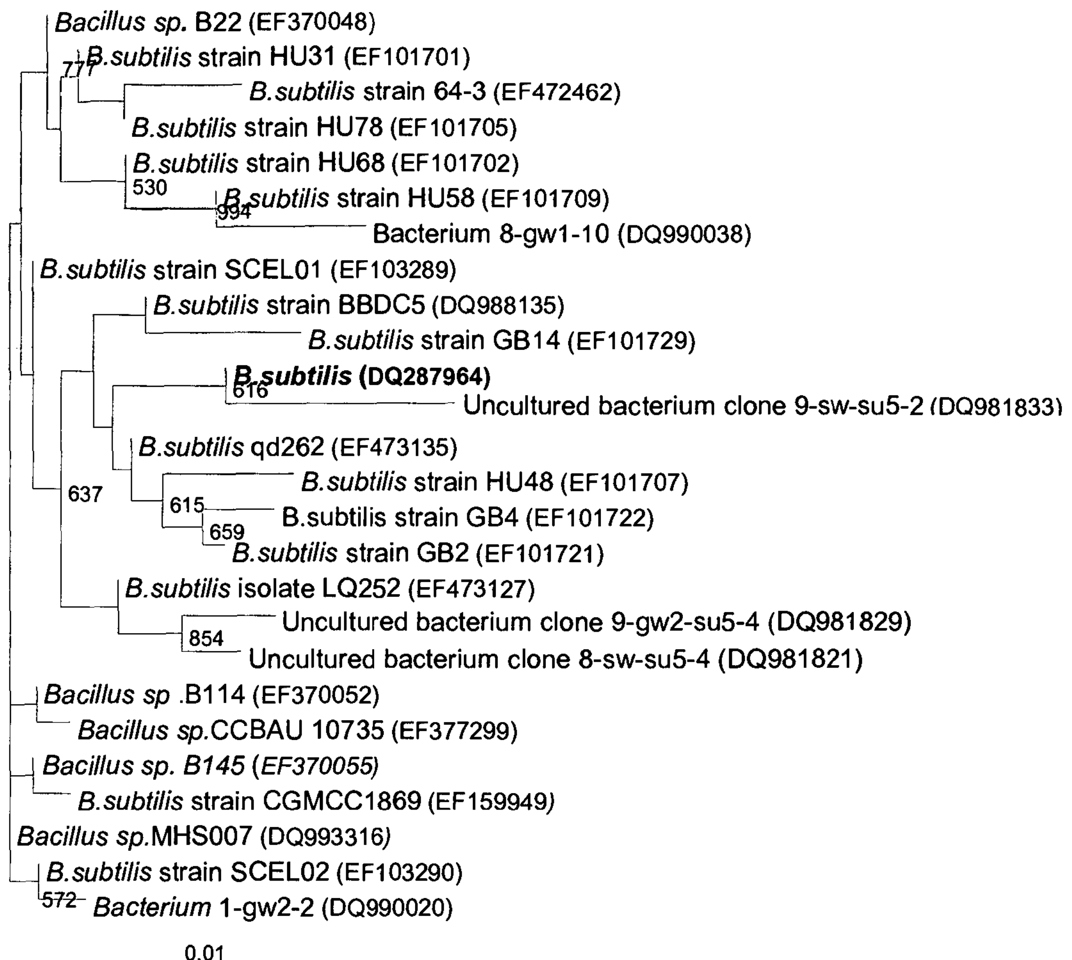


Fig 4.2 Unrooted tree showing the phylogenetic relationships of *B. subtilis* (DQ287964) and members of the genus *Bacillus* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1500 nt. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches. Accession numbers are given in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

Strain	Similarity (%)
Bacillus subtilis strain 64-3 (EF472462)	100
Bacillus subtilis isolate qd262 (EF473135)	100
Bacillus subtilis isolate LQ252 (EF473127)	100
Bacillus sp. CCBAU 10735 (EF377299)	100
Bacillus sp. B145 (EF370055)	100
Bacillus sp. B114 (EF370052)	100
Bacillus sp. B22 (EF370048)	100
Bacillus subtilis strain CGMCC 1869 (EF159949)	100
Bacillus subtilis strain BBDC5 (DQ988135)	100
Bacillus subtilis strain SCEL02 (EF103290)	100
Bacillus subtilis strain SCEL01 (EF103289)	100
Bacillus subtilis strain GB14 (EF101729)	100
Bacillus subtilis strain GB4 (EF101722)	100
Bacillus subtilis strain GB2 (EF101721)	100
Bacillus subtilis strain HU58 (EF101709)	100
Bacillus subtilis strain HU48 (EF101707)	100
Bacillus subtilis strain HU78 (EF101705)	100
Bacillus subtilis strain HU68 (EF101702)	100
Bacillus subtilis strain HU31 (EF101701)	100
Bacillus sp. MHS007 (DQ993316)	100
Bacillus subtilis strain BCRC 10058 (DQ993674)	100
Bacillus subtilis subsp. subtilis (AB271744)	100
Bacillus subtilis (DQ645478)	100
Bacillus licheniformis strain B425 (DQ523501)	100
Bacillus subtilis subsp. subtilis (AM237342)	100

Table 4.1 Percentage 16S rRNA sequence similarities between *Bacillus subtilis* and some closely related *Bacillus* spp.

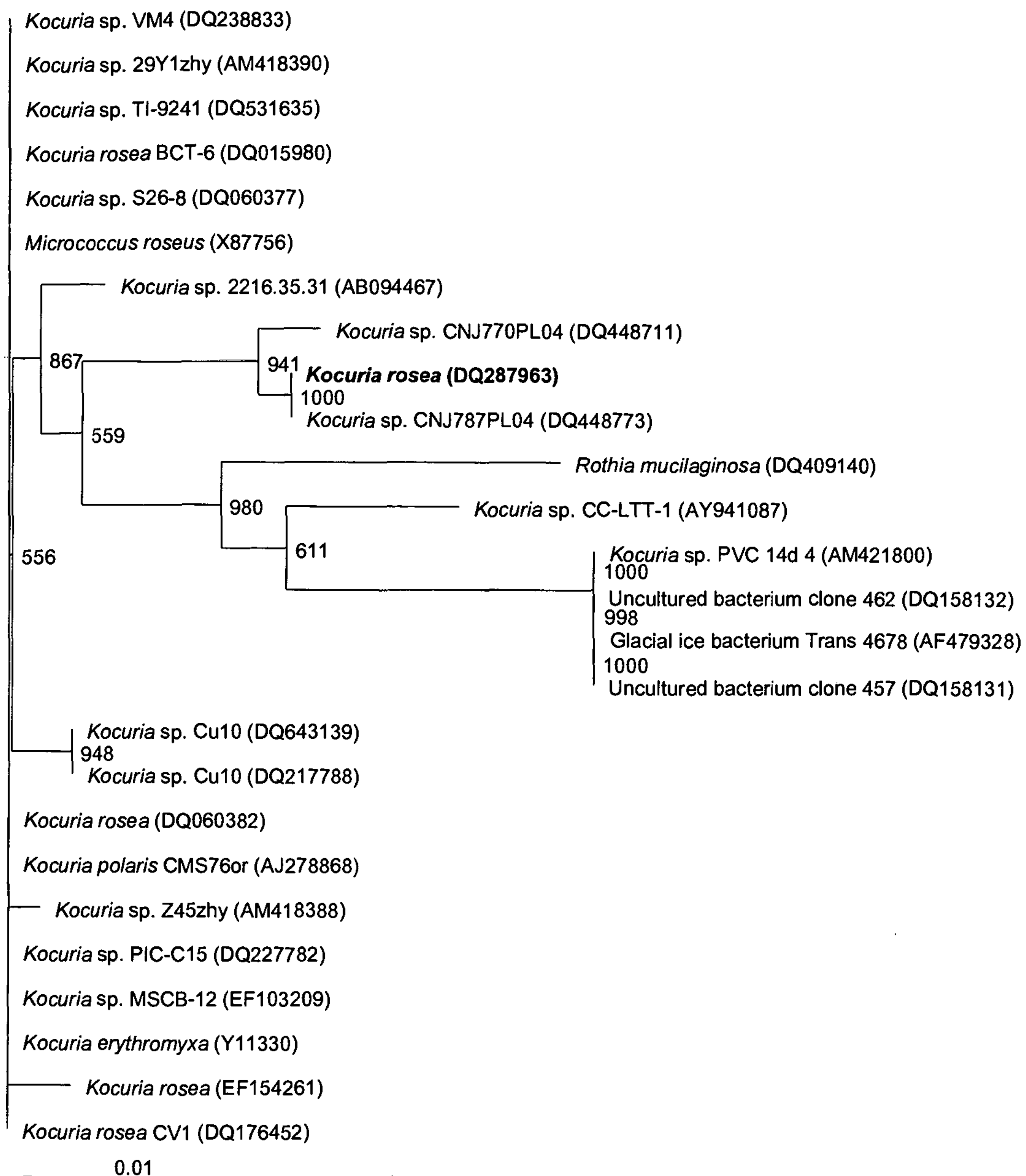


Fig 4.3 Unrooted tree showing the phylogenetic relationships of *Kocuria rosea* (DQ287963) and members of the genus *Kocuria* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1500 nt. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches. Accession numbers are given in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides

Strain	Similarity (%)
Kocuria sp. CNJ787 PL04 (DQ448773)	100
Kocuria sp. CNJ770 PL04 (DQ448711)	99
Kocuria sp. MSCB-12 (EF103209)	98
Kocuria sp. 29Y1zhy (AM418390)	98
Kocuria sp. TI-9241 (DQ531635)	98
Kocuria sp. PIC-C15 (DQ227782)	98
Kocuria rosea strain CV1 (|DQ176452)	98
Kocuria rosea (DQ060382)	98
Kocuria sp. S26-8 (DQ060377)	98
Kocuria rosea isolate BCT-6 16S (DQ015980)	98
M.roseus (X87756)	98
Kocuria polaris (AJ278868)	98
K.erythromyxa (Y11330)	98
Kocuria sp. VM4 (DQ238833)	98
Kocuria sp. Z45zhy (AM418388)	98
Kocuria sp. 2216.35.31 (AB094467)	98
Kocuria sp. Cu10 (DQ643139)	98
Kocuria sp. Cu10 (DQ217788)	98
Kocuria sp. CC-LTT-1 (AY941087)	97
Kocuria rosea (EF154261)	97
Kocuria sp. PVC(14d)4 (AM421800)	97
M.kristinae (X80749)	97
M.mucilaginosus (X87758)	97

Table 4.2 Percentage 16S rRNA sequence similarities between *Kocuria rosea* and some closely related *Kocuria* spp.

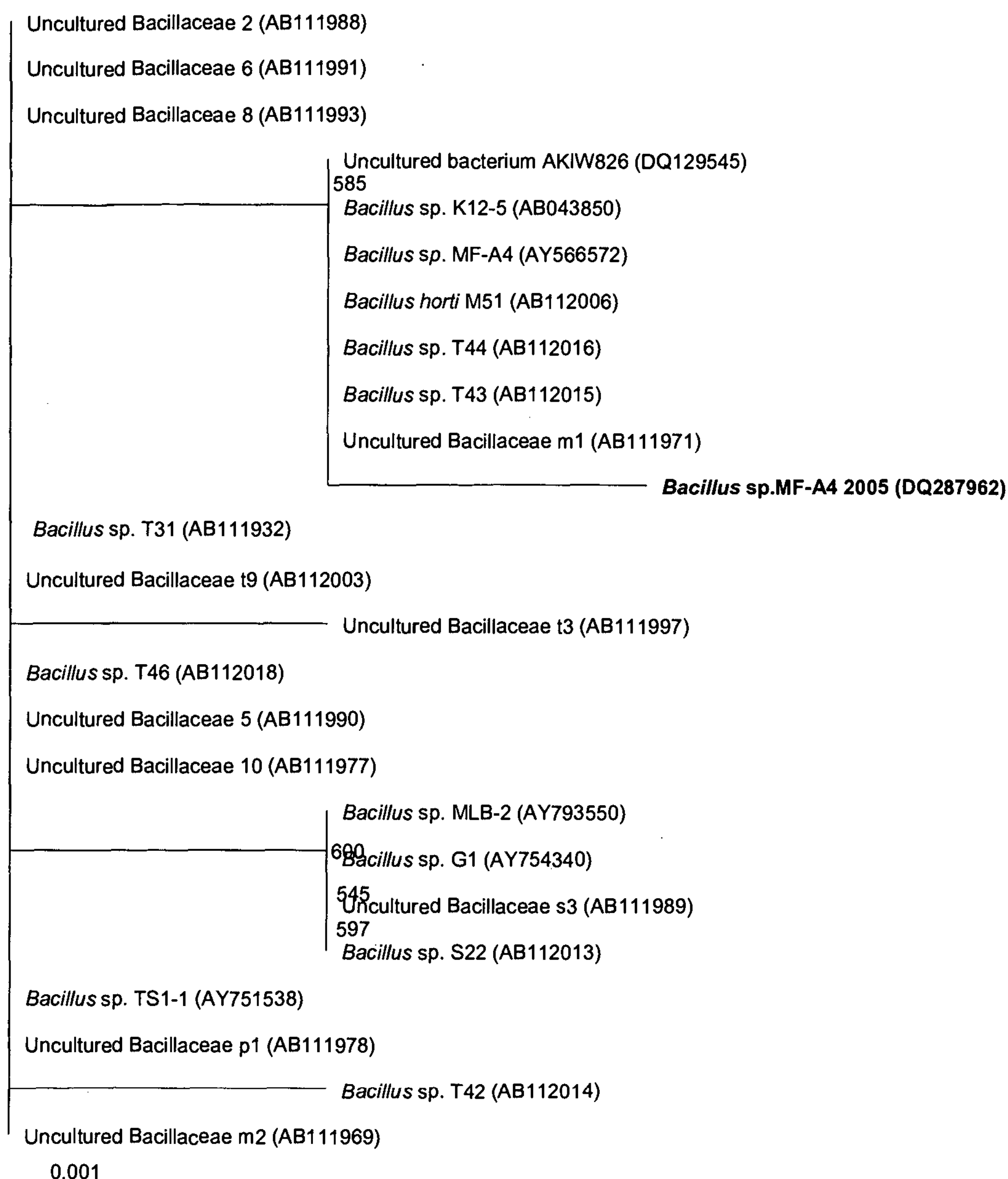


Fig 4.4 Unrooted tree showing the phylogenetic relationships of *Bacillus sp. MF-A4* (DQ287962) and members of the genus *Bacillus* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1500 nt. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches. Accession numbers are given in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

Strain	Similarity (%)
Bacillus sp. MF-A4 (AY566572)	99
Uncultured Bacillaceae bacterium (AB111971)	99
Bacillus sp. T44 (AB112016)	99
Bacillus sp. T43 (AB112015)	99
Bacillus horti (AB112006)	99
Bacillus sp. K12-5 (AB043850)	100
Bacillus sp. TS1-1 (AY751538)	100
Uncultured Bacillaceae bacterium (AB112003)	99
Uncultured Bacillaceae bacterium (AB111993)	99
Uncultured Bacillaceae bacterium (AB111991)	99
Uncultured Bacillaceae bacterium (AB111990)	99
Uncultured Bacillaceae bacterium (AB111988)	99
Uncultured Bacillaceae bacterium (AB111978)	99
Uncultured Bacillaceae bacterium (AB111977)	99
Uncultured Bacillaceae bacterium (AB111969)	99
Bacillus sp. T31 (AB111932)	100
Bacillus sp. T46 (AB112018)	99
Bacillus sp. G1 (AY754340)	100
Uncultured Bacillaceae bacterium (AB111989)	99
Bacillus sp. MLB-2 (AY793550)	100
Bacillus sp. S22 (AB112013)	99
Uncultured Bacillaceae bacterium (AB111997)	99
Uncultured Bacillaceae bacterium (AB111996)	99
Uncultured Bacillaceae bacterium (AB112004)	99
Uncultured Bacillaceae bacterium (AB112001)	99

Table 4.3 Percentage 16S rRNA sequence similarities between *Bacillus sp MF-A4* and some closely related *Bacillus* spp.

related to Uncultured bacterium AKIW826 (DQ129545) *Bacillus* sp. K12-5 (AB043850) *Bacillus sp.* MF-A4 (AY566572) *Bacillus horti* M51 (AB112006) *Bacillus* sp. T44 (AB112016)*Bacillus* sp. T43 (AB112015) Uncultured Bacillaceae m1 (AB111971), with 50 % sequence similarity. Figure 4.5 shows the phylogenetic position of the isolate (in bold) was more closely related to the genus *Microbacterium.* The 16S RNA gene sequence similarity values of the isolate to other strains of the genus *Microbacterium* in the range of 96 to 100% (Table 4.4) (Takeuchi and Yokota, 1994). These values and the phylogenetic position shown in the phylogenetic tree (Figure 4.5) indicate that the isolate belongs to the genus *Bacillus*, and is closely related *Microbacterium arborescens* SE14 (AY649756) with 60 % sequence similarity.The data on the four isolates have shown that they belong to *Bacillus, Kocuria and Microbacterium* genera. It was therefore of interest to study the plant growth promoting traits of the four selected isolates. The isolates were spot inoculated on specific media and the zones formed were observed.

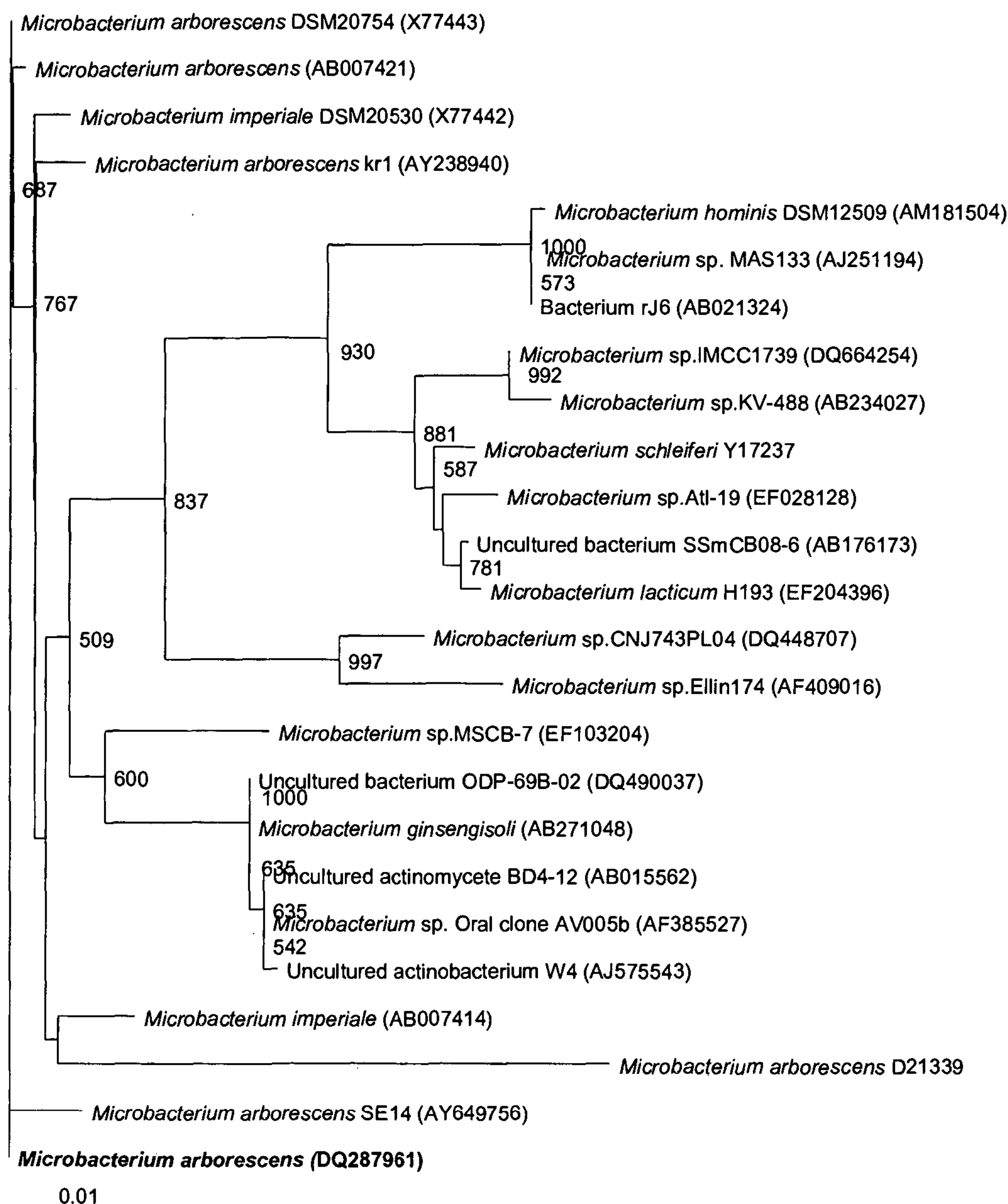


Fig 4.5 Unrooted tree showing the phylogenetic relationships of *Microbacterium arborescens*(DQ287961) and members of the genus *Microbacterium* based on 16S rDNA sequences. The tree, constructed using the neighbour-joining method, was based on a comparison of approximately 1500 nt. Numbers at the nodes indicate the percentages of bootstrap samplings, derived from 1000 samples, supporting the internal branches. Accession numbers are given in parentheses. Bar, 1 nucleotide substitution per 100 nucleotides.

Strain	Similarity (%)
Microbacterium arborescens (DSM 20754) (X77443)	100
M.imperiale (DSM 20530) (X77442)	99
Microbacterium arborescens (AB007421)	99
Microbacterium arborescens SE14 (AY649756)	99
Microbacterium arborescens kr10 (AY238940)	99
Microbacterium imperiale (AB007414)	98
Microbacterium ginsengisoli (AB271048)	98
Microbacterium sp. oral clone AV005b (AF385527)	97
Microbacterium sp. MSCB-7 (EF103204)	97
Microbacterium sp. CNJ743 PL04 (DQ448707)	96
Microbacterium sp. IMCC1739 (DQ664254)	100
Microbacterium sp. MAS133 (AJ251194)	100
Microbacterium schleiferi (Y17237)	100
Microbacterium arborescens (D21339)	99
Microbacterium hominis (AM181504)	100
Microbacterium sp. Atl-19 (EF028128)	100
Microbacterium sp. Ellin174 (AF409016)	96
Microbacterium sp. KV-488 (AB234027)	100
Microbacterium lacticum isolate H193 (EF204396)	98
Microbacterium lacticum isolate D84 (EF204392)	98
Microbacterium lacticum (AB007415)	100
Microbacterium hominis (AB004727)	100
Microbacterium sp. KV-483 (AB234026)	100
Microbacterium sp. 62NP10 (AB242734)	98
Microbacterium lacticum (DSM20427) (X77441)	100

Table 4.4 Percentage 16S rRNA sequence similarities between *Microbacterium arborescens* (DQ287961) and some closely related *Microbacterium* spp.

All the isolates produced orange halos on CAS agar plates indicating the production of siderophores viz. *Bacillus subtilus*, *Microbacterium arborescens*, *Bacillus sp*. MF-A4 and *Micrococcus roseus* (Fig 4.6). Further the Arnow's test was positive for *B. subtilus* and *Bacillus* sp. MF-A4 indicating the presence of catechol type siderophore (Fig 4.7). The carboxylate test was positive for *Microbacterium arborescens* and *Micrococcus roseus* cultures (Table 4.5). Dave *et al*, 2006 reported the presence of carboxylate siderophores in extreme halophiles. The thin layer chromatography of ethyl acetate extracts of culture supernatant of *B. subtilis* and *Bacillus* sp MF-A4 showed fluorescence under UV indicating the presence of dihydroxybenzoic acid in the structure of the siderophore (Alakhov, 2003). Dihydroxybenzoic acids show a characteristic fluorescence under UV light and a blue colour when sprayed with Hathways reagent. Peyton & Apel (2005) reported siderophore producton in *Halomonas campisalis* (ATCC 700597) a halotolerant alkaliphile which was not stimulated by increasing pH. The low iron medium increased siderophore production and increased the lag phase of bacterial growth. Production of siderophores by isolates from saline and alkaline environments suggests that when evaluating important heavy metal transport mechanisms and remediation strategies for mixed wastes of high pH and salinity siderophore production by indigenous microbial communities should be considered.

Aiken *et al,* (2003) suggested that because of the scarcity of iron under alkaline conditions, alkaliphilic bacteria will have a stronger affinity for binding and solubilize ferrous iron than siderophores produced by mesophilic bacteria. In *H. campisalis,* a negative response to Arnow assay indicated that the siderophore produced did not contain any catechol moieties in its chemical structure and was

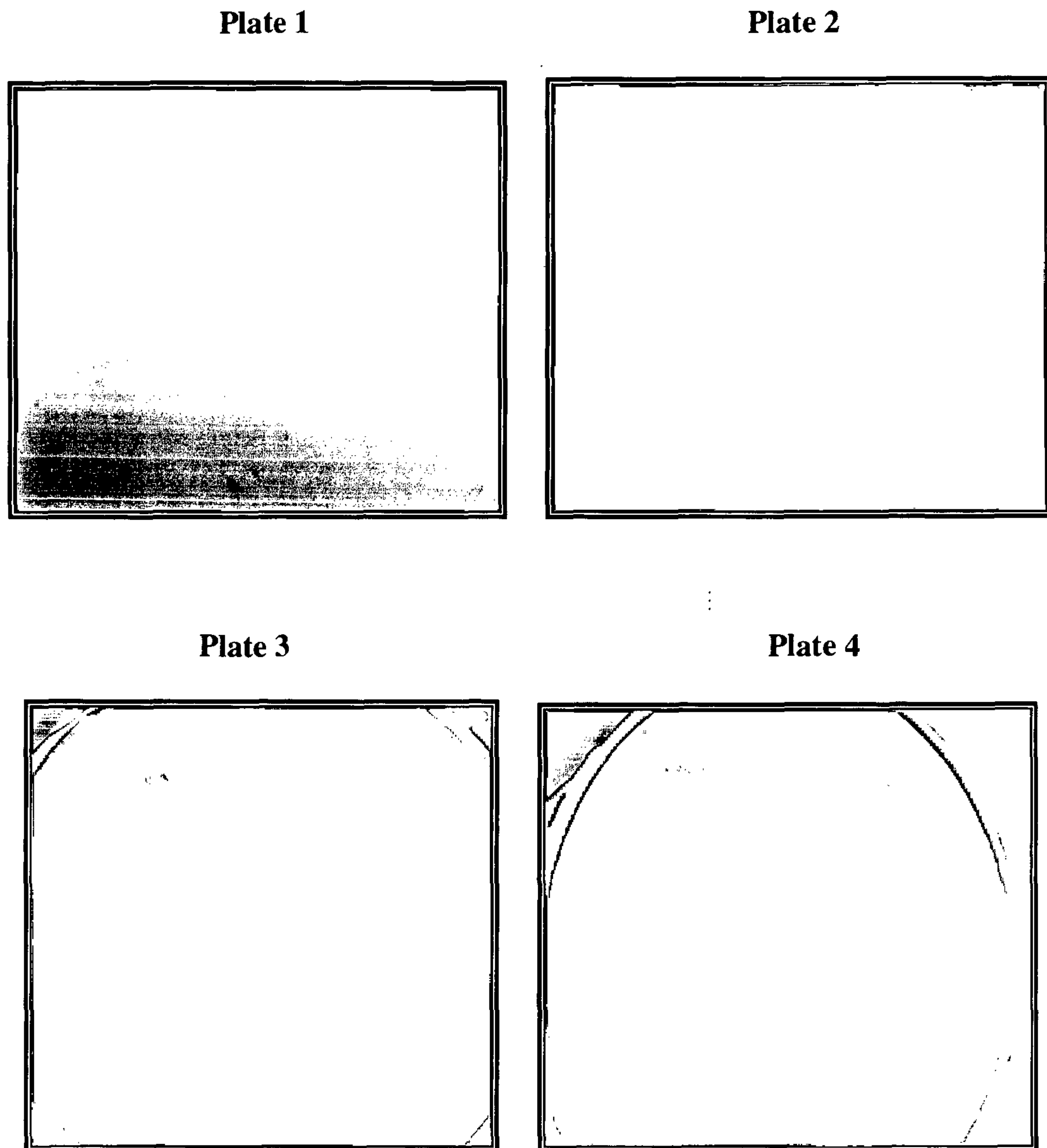


Fig 4.6 CAS plate assay showing production of orange halos around the colonies of the isolates indicating the presence of siderophore production Plate 1 & 2- *Bacillus subtilis* Plate 3 - *M .arborescens* (Mb), *K. rosea* (Mc) & *Bacillus* sp. MF-A4 (Bac 10)

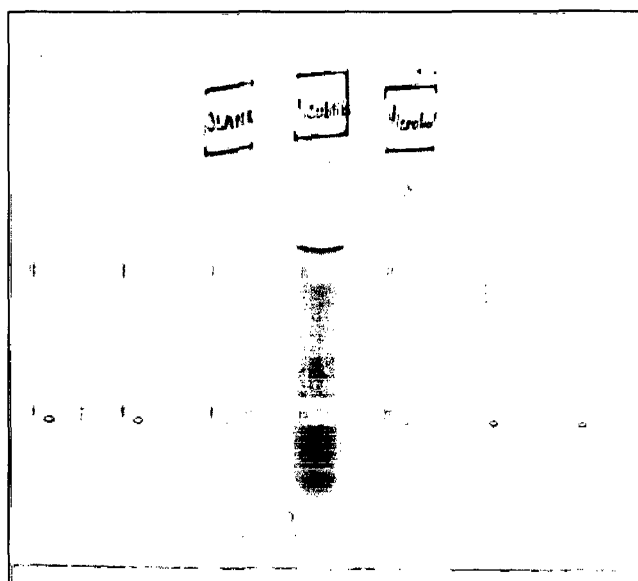


Fig 4.7 Reddish colouration obtaind in Arnows test in the supernatant of *B. subtilis*

Table 4.5 Nature of the siderophores produced by the sand dune bacterial cultures

S. No	Cultures	Nature of siderophore
1	*B.subtilis*	Catecholate
2	*Bacillus* sp. MF-A4	Catecholate
3	*Kocuria rosea*	Carboxylate
4	*Microbacterium arborescens*	Carboxylate

found to produce maximum siderophore during mid stationery phase. Chakraborty *et al*, (1990) reported that *Pseudomonas stutzeri* RC 7 grown under iron-deficient conditions produced catechol type siderophore. The concentration of siderophore in the culture supernatant was maximal after 24 h of growth. Addition of iron to the medium increased bacterial growth but repressed the production of siderophore. Temirov *et al*, (2003) reported that thermoresistamt *Bacillus licheniformis VK21* strain produced catecholic siderophore whose production was maximum at the beginning of the stationery growth phase of the culture. The UV spectrum of the siderophore showed absorption maxima at 248 and 315 nm.

The results have indicated that all the four isolates produced siderophores with one of the alkaliphilic isolate producing catechol type, the other two alkaliphilic isolates producing carboxylate type while the neutrophlic isolate producing catechol type of siderophore. It was therefore envisaged that the relationship of siderophore production and growth would throw light on the phases to identify it as a secondary metabolite. Studies were therefore carried out with neutrophilic isolate producing catechol type of siderophore.

B. subtilus was grown in Fiss minimal medium with no added iron and the supernatant was collected at various time intervals. The growth was measured at 600 nm wavelength using the culture, and the supernatant was tested using Arnow's assay to estimate catechol type siderophore concentration. The results indicate that optimum siderophore production is achieved after 20 h of growth, while the organism entered stationery phase at 24 hrs (Fig 4.8). The fact that siderophores were produced late in the exponential growth phase of *Bacillus subtilis* suggests that siderophores might be used for scavenging iron and sustaining growth and survival of the microorganism.

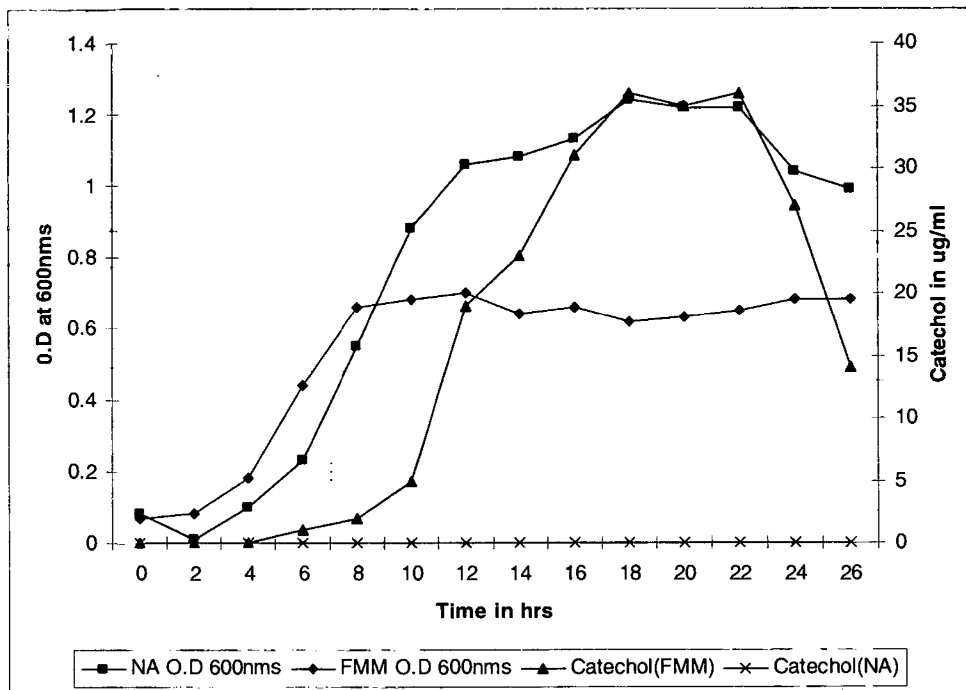


Fig 4.8 Growth curve and siderophore production by *B.subtilis*

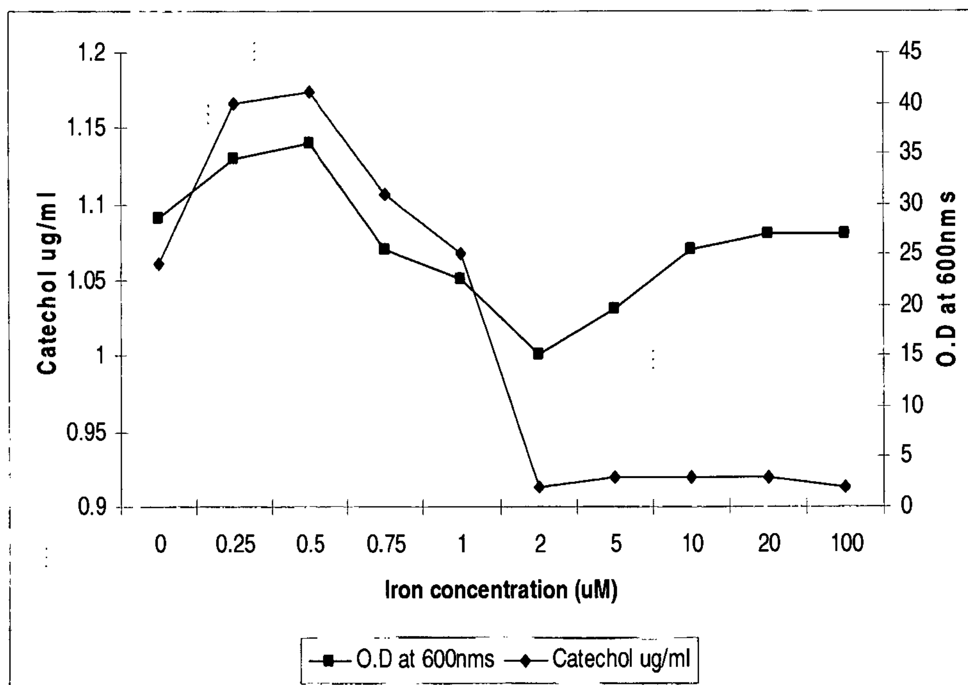


Fig 4.9 Effect of increasing iron concentration on siderophore production by *B.subtilis*

The genes controlling the iron siderophore transport system are regulated by the concentration of iron in the medium. To examine the effect of the concentration of ferric iron on the catechol type siderophore production, the culture was grown in the presence of varying concentrations of ferric iron ($FeCl_3$) in the Fiss medium for 24 h at 28°C, growth was measured, supernatants collected and siderophore concentrations were estimated using Arnow's assay. Data in Fig 4.9 show that an optimal siderophore production occurred when the culture medium was supplemented with 0.5μM of iron. At this concentration, the strain excreted about twice as much siderophore than when grown in an iron deficient medium. A concentration of 5μM (and above) caused total inhibition of siderophore production(Arahou *et al*, 1998).

This iron repressibility of siderophore biosynthesis is a common characteristic of many microorganisms producing these compounds (Neilands *et al,* 1987 ; Lesueur *et al,*1993). It is known that siderophore expression is repressed under high iron conditions, but growth of the organism does require some iron in the medium (Neilands *et al,* 1987). Shah *et al,* (1993) in their studies on siderophores from *Azospirillum lipoferum* have shown that siderophore synthesis was repressed by addition of iron (50μM $FeCl_3$) and induced by iron starvation. Siderophore extract from culture supernatant of iron starved *A. lipoferum* M showed presence of salicylic acid, 2,3-dihydroxybenzoic acid and 3,5-dihydroxybenzoic acid (DHBA) when analysed by TLC.

Almost all catecholic siderophores are known to fluoresce in UV light, due to the presence of the residue of 2, 3-dihydroxybenzoic acid in their molecules, and their emission wavelength depends on the structure of a particular compound (Temirov *et al,* 2002). De Villegas *et al,* 2002 found that concentrations of iron > 10 μM had a

negative effect on siderophore production, although cell growth reached a maximal value above 10 μM added Fe(III). Siderophore biosynthesis was lowered at this concentration, since cell growth and the siderophores production are inversely proportional responses. Iron concentration of 10 μM is considered to be high and generally results in excellent cell growth with only modest yields of siderophores. Manninen and Sandholm (1993) reported that siderophore production occurred only at an iron concentration of > 50uM. Rachid and Ahmed (2005), in their studies on effect of iron chelator on siderophore production reported that the iron chelator ά ά-dipyridyl diminished the contaminating iron content of the medium and growth yield increased significantly with the amount of siderophore production and with 8-hydroxyquinoline, both parameters diminished.

Iron is present in the ferric form (iron III) in nature, which is not soluble and thus not readily available to the bacteria. To overcome this problem, microorganisms have evolved specific iron uptake systems consist of a) the production and excretion of an iron ligand molecule called a siderophores, and ii) a protein, located in the outer membrane of gram negative bacteria that serves as receptor for the iron-siderophore complex. It is reported that under iron limiting conditions, two proteins of 84 and 76 kilodaltons were overexpressed by *E. amylovora* (Vanneste and Expert, 2001). Iron deprivation caused conspicuous changes in the membrane protein composition of *Bacillus*. Two protein bands are visible in all low iron samples with molecular weights of approximately 50,000 and 60,000 KDa and the bands are absent in the high iron samples (Fig 4.10). This indicates repression of these proteins under high iron conditions, which is consistent with the behavior of the iron-regulated genes of iron transport systems *Staphylococci* respond to iron deprivation *in vitro* and *in vivo* by

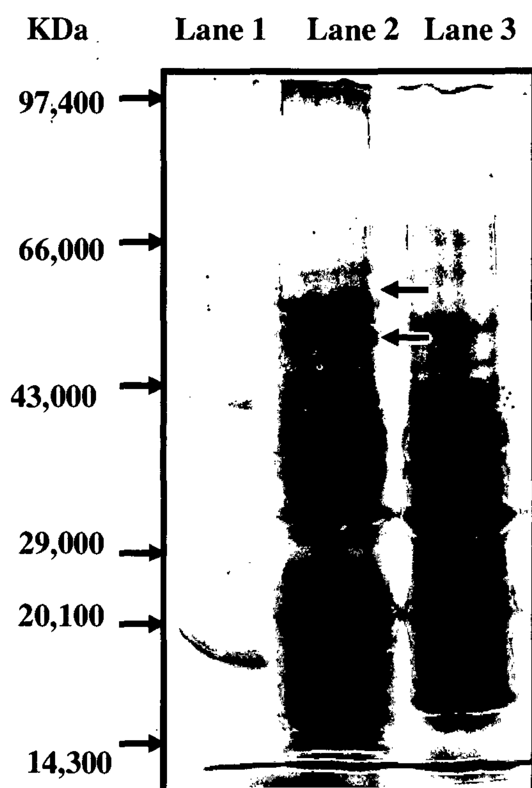
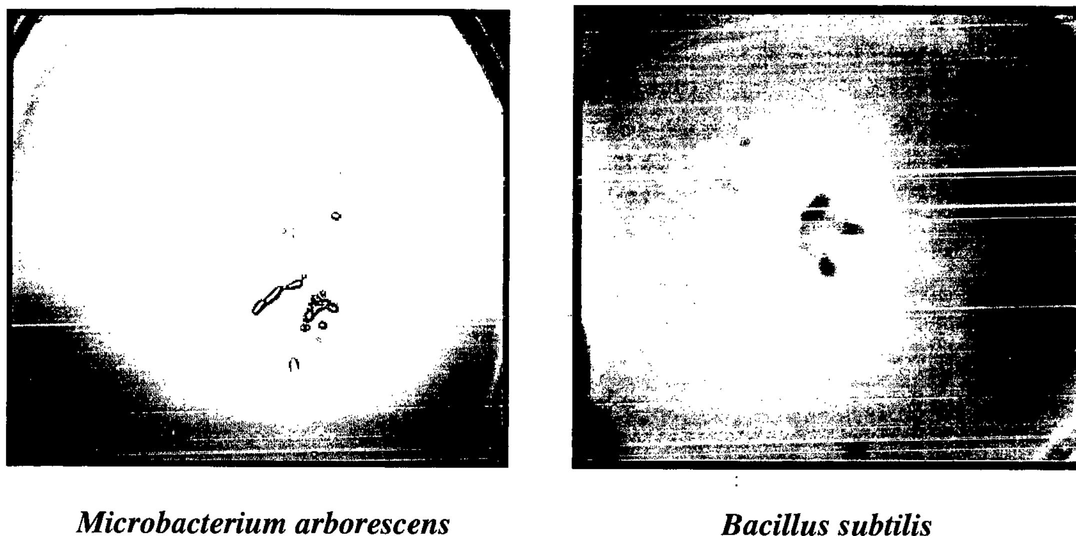


Fig 4.10 SDS-PAGE of possible receptor proteins of *B. subtilus*.

An 8 % SDS PAGE of cell pellets grown in low and high iron fiss minimal medium. Lanes 1. Lane 1 Molecular weight marker 2. Lane 2 - Low iron Bac 7 Lane 3 - High Iron

increasing the expression f a umber of iron-regulating proteins. These include a 42 KDa cell wall associated transferin binding protein and two cytoplasmic membrane proteins of 32 and 36 KDa (Cockayne *et al*, 1998). *S. aureus* contains a ferrichrome uptake system which exists with a cytoplasmic membrane anchored binding proteins and an ABC-type membrane permease (Jin *et al*, 2006). Lin *et al*, (2006) suggested that ABC-type permeases are the principal components of gram positive bacterial iron uptake systems. Gram positive organisms like *S. aureus* (skin infections), *Streptococcus pyogenes* (scarlett fever), *Bacillus anthracis* (anthrax) and *Listeria monocytogenes* (meningitis) do not contain an outer membrane. Instead their inner membrane is covered by a thick layer of peptidoglycan, in which proteins and lipids are anchored and extend to the cell surface. It is therefore confirmed that when bacteria are deprived of iron, they turn on their cell envelope iron acquisition systems, these iron-regulated membrane proteins appear in SDS-PAGE analyses of the gram positive bacterial cells (Klebba, 2001).

Another important trait checked with these isolates was the phosphate solubilization using the plate assay method on Pikovskaya medium(PVK). It was interesting to observe the yellow zones around the colonies of all four isolates indicating their P solubilizing activity (Fig 4.11). The yellow halo is attributed to the production of organic acids by the isolate to bring about P solubilization. The phosphate solubilization efficiency was highest in *B. subtilus* followed by *Bacillus* sp. MF-A4, *Microbacterium arborescens* and *Kocuria rosea* (Table 4.6). *Microbacterium arborescens* and *B. subtilis* hydrolyzed PNPP to PNP indicating phosphatase enzyme activity (Fig 4.12 & Table 4.7).



Microbacterium arborescens *Bacillus subtilis*

Fig 4.11 Plate assays of P solubilizers *B. subtilus* (Pikovskaya medium incorporated with bromothymol blue dye) and *Microbacterium arborescens* (Pikovskaya medium incorporated with phenol red dye)

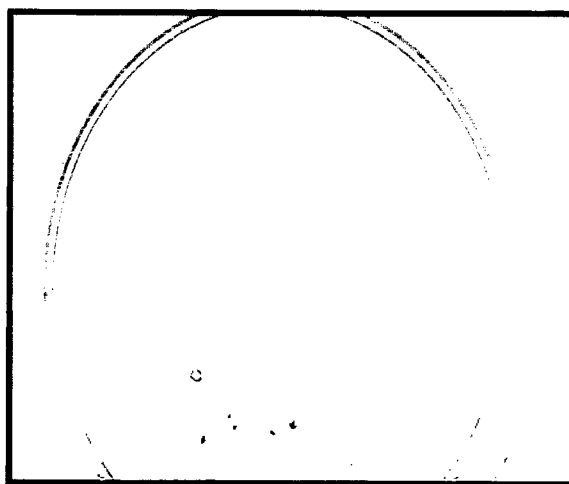


Fig 4.12 *B. subtilus* shown hydrolyzing PNPP (p-nitrophenyl phosphate) to PNP (p-nitro phenol)

Table 4.6 Phosphate solubilization efficiency (SE) of the bacterial cultures

Cultures	SE
Microbacterium arborescens	25
Kocuria rosea	20
Bacillus sp MF-A4	57.14
B. subtilus	71.4

Table 4.7 Phosphatase enzyme in the bacterial isolates

Cultures	Phosphatase
Microbacterium arborescens	Present
Kocuria rosea	Absent
Bacillus sp MF-A4	Absent
B. subtilus	Present

Quantitative estimation of phosphate solubilization in all the four cultures, estimated after incubation, is presented in Figs 4.13, 4.14 and 4.15. The pH of the broth was found to decline in each case due to bacterial activity; lowering of the pH coincided with increase in the efficiency of phosphate solubilizing activity. The pH was found to decline from 7 (control) to 5 in the case of P solubilization by *B . subtilis* (Fig 4.14). The drop in the pH can be attributed to the production of organic acids (Kucey *et al,*1980; Bar-yosef, 1991) and the presence of enzyme phosphatase in *Microbacterium arborescens* and *Bacillus subtilus.* In this study, pH values decreased gradually in PVK broth during early days of incubation, and no revival was observed in later days for all the tested bacterial strains. It is concluded from the present study that all the isolated phosphate solubilizing strains produce organic acids by utilizing the carbon of given substrates in their broth medium. This supports the major role of organic acid production in mineral phosphate solubilization. The pH drop in PSM liquid cultures have been reported in several researches which supports the pH change in the present study (Cunninghamj, 1992 ; Motsara,1995; IIlmer *et al,*1995; Bar Yosef *et al,*1999). El-Komy (2004), indicated that the P concentration in PVK broth increased gradually, achieving a peak on the 6th day and then declined slowly during the later days. In general, the bacterial activity was initially slow, and then increased gradually followed by a decline at the end of incubation period. Decrease in P during initial stages in PVK medium can be attributed to the utilization of the existing P for growth development of the organism, in a later phase the bacteria would have started acting on the substrate for the need of nutrients, thus releasing P from poorly soluble sources.

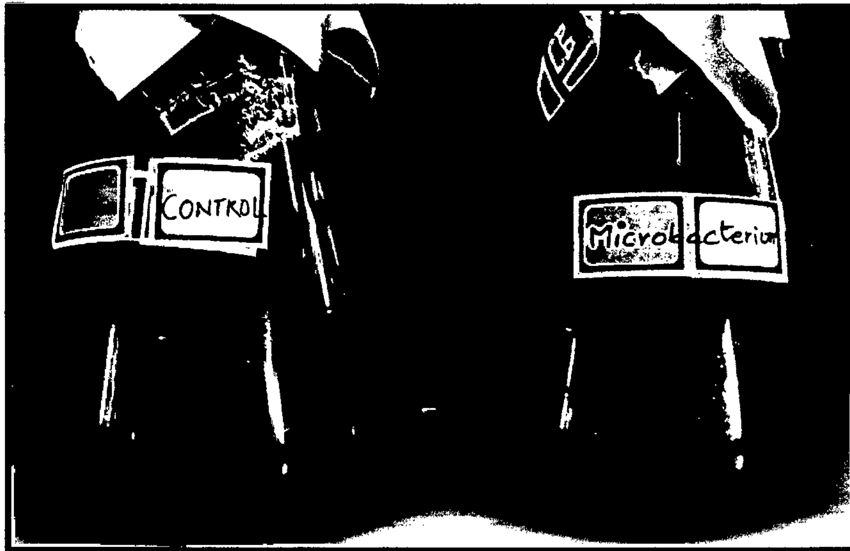


Fig 4.13 ***Microbacterium arborescens*** **solubilizes inorganic phosphate, colour change in phenol red dye from pink to yellow, indicating a drop in pH due to release of organic acids into the medium.**

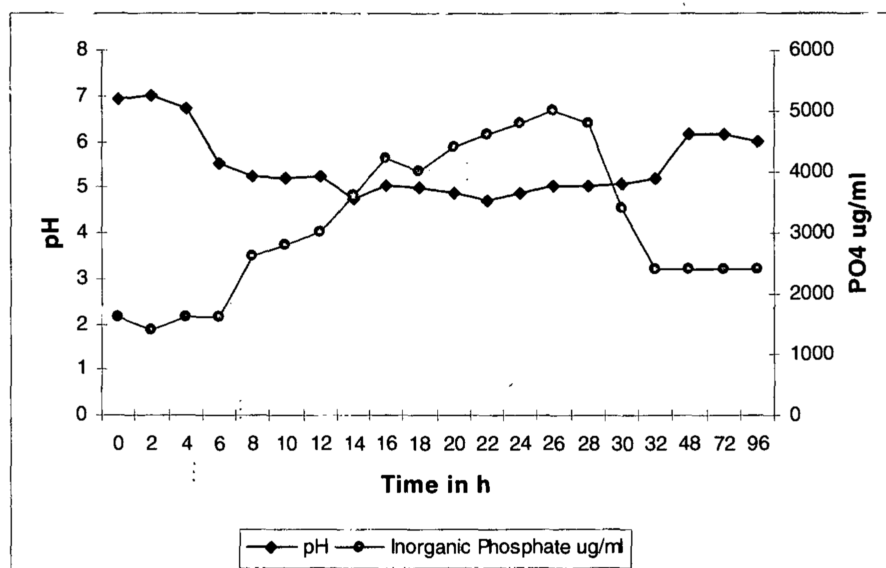


Fig 4.14 P solubilization and lowering in pH of broth due to phosphate solubilizing activity of *B. subtilus*

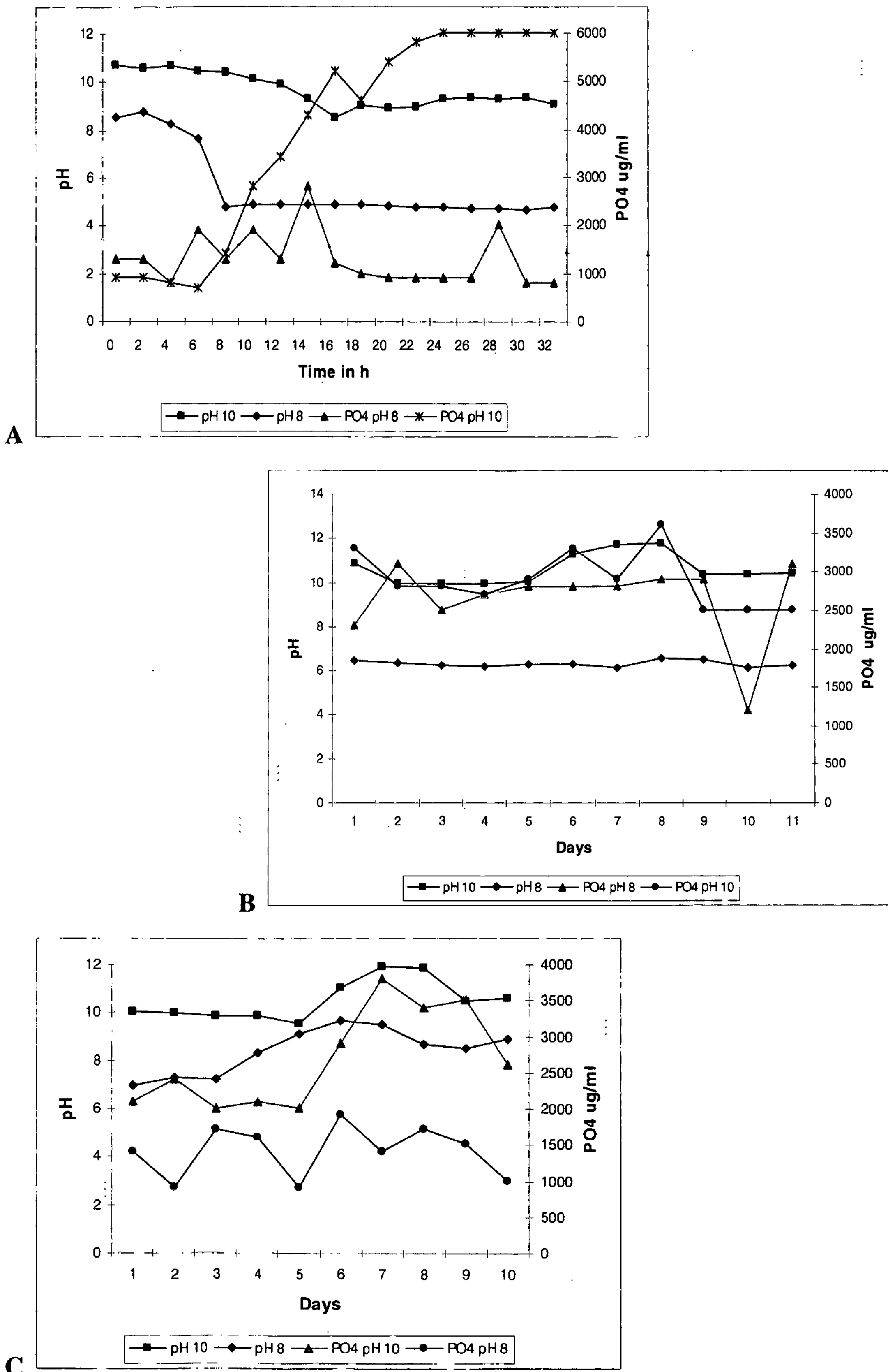


Fig 4.15 P solubilization by A - *M. arborescens* , B- *K . rosea* & C- *Bacillus* sp. MF-A4

Phosphate solubilization is considered an important attribute of plant growth promoting rhizobacteria. Phosphate-solubilizing bacteria can play an important role in plant nutrition through an increase in phosphorus uptake by plants and their use as plant growth-promoting rhizobacteria may be an important contribution to biofertilization of agricultural crops. It has been observed by many investigations that a high proportion of P solubilizing microorganisms are concentrated in the rhizosphere of plants (Gaur,1990). Pandey *et al,* (2006) studied the phosphate solubilizing ability of *Ps putida* strain isolated from a sub-alpine location in Indian central Himalaya and observed maximum activity of 247 ug ml-1. The phosphate activity coincided with a concomitant decrease in pH of the medium. Singh and Kapoor (1999) in their studies on inoculation with phosphate-solubilizing microorganisms and a vesicular-arbuscular mycorrhizal fungus on wheat grown in a sandy soil attributed the significant increase in grain and straw yields to a greater absorption of nutrients, particularly P. The P-solubilizing organisms dissolve unavailable forms of P by excreting organic acids and chelating substances. Plant roots also exert their influence on P availability through processes such as secretion of organic acids and the production of chelating substances. Sheshadri *et al,* 2000 in their work on solubilization of inorganic phosphates by *Azospirillum halopraeferans,* reported that the better performance of bacteria in Pikovskaya's medium by all strains indicates the role played by the substrate to trigger the microbial action.

There are several potential mechanisms reported for phosphate solubilization that include modification of pH by secretion of organic acids and protons or cation dissociation. These PSM strains are also capable of solubilizing and immobilizing inorganic phosphorus provided in the broth medium (Rashid *et al,* 2004). Rivas *et al,*

(2004) for the first time reported a *Microbacterium* strain that could solubilize inorganic phosphate. These data suggest that the biodiversity of phosphate solubilizing bacteria is still poorly understood and that isolation of these micro-organisms is not restricted to the soil. Poberejskaya *et al,* 2002 showed that the inoculation of phosphorus with PSB increased dry matter accumulation, N and P uptake, yield of cotton and soil P content. Phosphorus uptake efficiency and yield increased with phosphorus application and with inoculation. In previous works, *Bacillus* sp. mobilise phosphate from organic hard soluble phosphoric compounds and increased growth and yield of cotton in Calcisol soil. Their results suggest that PSB are able to mobilise more P, where hard soluble phosphates are presented in soil and increased yield and growth.

Rashid *et al,* (2004) screened a large number of PSM from rice rhizosphere on the basis of SI and pH drop. All the PSM strains utilized the carbon glucose for the production of organic acids with a drop in pH. Glucose is the main carbon source for the growth of PSM but other carbon sources can also be utilized (Ilmer and Schinner, 1992;Motsara *et al,*1995). The P-solubilizing ability of PSMs also depends on the nature of N source used in the media, with greater solubilization in the presence of ammonium salts than when nitrate is used as N source. This has been attributed to the extrusion of protons to compensate for ammonium uptake, leading to a lowering of extracellular pH. In some cases, however, ammonium can lead to decrease in P solubilization. In addition, other media components were also found to affect the P solubilization ability (Cunningham and Kuiack, 1992). Schinner (1992) reported that sometimes the culture filtrate pH was relatively high and yet in the medium high P solubilization occurred,this may occur due to the chelation of organic acids with Ca^{++}

ion in tricalcium phosphate. Peix, (2003), isolated *Pseudomonas rhizosphaerae sp.* nov., a novel species that actively solubilizes phosphate in vitro from rhizospheric soil of grasses growing spontaneously in Spanish soil, actively solubilized phosphates *in vitro* when bicalcium phosphate was used as a phosphorus source. De Souza *et al,* (2000) reported that the phosphate solubilizing bacteria are widely distributed in different niches with the coastal area having a high density. Important genera of phosphate solubilizing bacteria are *Bacillus* and *Pseudomonas* (Ilmer and Schinner, 1992).

In our study the presence of organic acids i.e oxalic acid, citric acid, lactic acid, gluconic acid, etc in liquid culture filtrates were determined by paper chromatography (Table 4.8). Gyaneshwar *et al,* (2002) reported that the culture supernatant of *Bacillus coagulans* contained succinic, lactic, citric and acetic acids. Phosphobacteria have been found to produce some organic acids such as monocarboxylic (acetic, formic), monocarboxylichydroxyl (lactic, gluconic, glycolic), ketogluconic, dicarboxylic (oxalic, succnic), dicarboxylic hydroxyl (malic, maleic) and tricarboxylic hydroxyl (citric) acids in order to solubilize inorganic phosphate compounds (Ponmurugan and Gopi, 2006). Organic acid production is the principal mechanism for mineral phosphate solubilization in bacteria. Gluconic acid is the principal organic acid produced by *Peudomonas sp., Erwinia herbicola, Peudomonas cepacia* and *Burkholderia cepacia,* . *Rhizobium leguminosarum, Rhizobium meliloti* and *Bacillus firmus* produce 2-ketogluconic acid. Other organic acids such as lactic, isovaleric, isobutyric, acetic, glycolic, oxalic, malonic and succinic acids are also generated by different PSB (Igual *et al,* 2001). The role of organic acids produced by PSM in solubilizing insoluble P may be due to the lowering of pH, chelation of

Table 4.8 Paper chromatography of organic acids produced by cultures

Solvent system	Culture	Rf	Organic acids
$CHCl_3$:Ethanol:Formic acid(2:1:2)	*B. subtilis*	0.9	Maleic acid
NH_3:Propanol(70:30)	*M. roseus*	0.37	Malonic acid
	Bacillus sp. MF-A4	0.14	Succinic acid
	B. subtilis	0.14	Succinic acid
Propanol:NH_3:H_20(60:20:20)	*M. roseus*	0.37	Citric acid
	Bacillus sp. MF-A4	0.87	Maleic acid
		0.46	Tartaric acid
	B.subtilis	0.37	Citric acid
		0.4	Tartaric acid
	M.arborescens	0.46	Tartaric acid
		0.37	Citric acid

cations and by competing with P for adsorption sites in the soil. It has also been investigated that organic acids may also form soluble complexes with metal ions associated with insoluble P (Ca, Al, Fe) and thus P is released (Kepert *et al,*1979 ; Omar,1998). Glucose is the main carbon source for the growth of PSM but other carbon sources can also be utilized. IIlmer and Schinner (1992) reported that sometimes the culture filtrate pH was relatively high and yet in the medium high P solubilization occurred, this may occur due to the chelation of organic acids with Ca++ ion in tricalcium phosphate The solubilization of insoluble phosphates by microorganisms is caused by the production of organic aids although chelating substances also have an important role. The type of organic acids produced and their amounts differs with different microorganisms. The type of organic acid has a significant effect on the solubilization. Tri and dicarboxylic acids are more effective as compared to monobasic and aromatic acids (Kapoor *et a.,* 1989). Ryan *et al,* (2001) stated that, among the carboxylic acids identified, dicarbozylic acid (oxalic, tartaric, malic, fumaric, malonic acids) and tricarboxylic (citric) acids were more effective for phosphorus mobilization. IIlmer and Schinner (1992) reported that gluconic acid may be the most frequent agent for mineral phosphate solubilization.

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms including PGPR (Barry *et al,* 2002; Asghar *et al,* 2002). Microorganisms inhabiting rhizospheres of various plants are likely to synthesize and release auxin as secondary metabolites because of the rich supplies of substrates exuded from the roots compared with non rhizospheric soils. Plant morphogenic effects may also be a result of different ratios of plant hormones produced by roots as well as by rhizosphere

bacteria. Diverse soil microorganisms including bacteria, fungi and algae are capable of producing physiologically active quantities of auxins, which may exert pronounced effects on plant growth and establishment (Khalid *et al*, 2004 ; Okon and Vanderleyden, 1997 ; Glick, 1995; Leveau and Lindow, 2005). The positive effects of PGPR on plant growth are always correlated with remarkable changes in root morphology. It is generally assumed that these developmental responses are stimulated by phytohormones like IAA produced by the bacteria (Botelho and Hagler, 2006). All the four bacteria produced appreciable quantity of IAA (*Bacillus subtilus*, 50 µg/ml ; *M.arborescens*, 33 µg/ml; *M. roseus,* 26 µg/ml and *Bacillus sp,*30 µg/ml).Ahmad *et al*, (2005) reported that isolates of *Azotobacter* and fluorescent *Pseudomonas sp.* isolated from rhizospheric soil of different crops (wheat, berseem, mustard, cauliflower) in the vicinity of Aligarh city, UP, India varied greatly in their intrinsic ability to produce IAA. The range of IAA production in *Azotobacter* isolates without tryptophan was 2.68-10.80 mg/ml. *Pseudomonas* isolates were able to produce IAA without tryptophan in the range 5.34 to 22.4 mg/ml. Yasmin *et al,* (2007) reported that in their studies the rhizobacterial islates from sweet potato produced IAA of a concentration between 3.84 to 13.33 ug/ml. Under natural condition, plant roots excrete organic compounds including L-TRP which can be utilized by the rhizobacteria for IAA biosynthesis (Yasmin *et al,* 2007) *Azotobacter paspali* secreted IAA into culture media and significantly increased the dry weight of leaves and roots of several plant species following root treatment. It was found that inoculation of wheat seedlings with *Azospirillum brazilance* increased the number and length of lateral roots. Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produces low levels of IAA, resulted in 2 - or - 3 fold increases in the length of

seedling roots. It is presumed that PGPR producing plant growth regulators play a critical role in plant growth promotion (Ahmad, 2005).

M. arborescens and *Bacillus sp. MF*-A4 showed intense orange colour indicating high HCN production while *B. subtilis* showed light orange colour while *Kocuria rosea* was not found to produce HCN (Fig. 4.16). It has been suggested that production of volatile metabolites like HCN by *Pseudomonas* spp. may be a part of their antagonistic activity against plant pathogens. Also HCN is known to inhibit plant metabolism and root growth (Adam and Zdor, 2001). HCN inhibits plant pathogens by interfering with their respiratory mechanisms. (Voisard *et al*, 1989). In *Pseudomonad* species, hydrogen cyanide is released by the decarboxylation of glycine (Wissing, 1974; Bakker and Schippers, 1987). Kloepper *et al,* (1991) described the HCN formed a brownish red compound with sodium picrate and the intensity of the colour increased with the amount of HCN. Defago *et al,* 1990 demonstrated by mutational analysis and complementation that production of HCN by *Pseudomonas fluorescens* strain CHAO accounted for about 60 % of the biocontrol activity. They suggested that since CHAO also was found to colonize the root cortex the strain may produce a stress effect in the plant leading to cyanide resistant respiration and possible modification of tobacco metabolism resulting in enhanced host resistance mechanisms.

All the cultures had the ability to grow in minimal medium with ACC as the sole nitrogen source. ACC-deaminase activity of PGPR strains is known to enhance the root length and growth by sequestering and hydrolyzing ACC from germinating seeds and thereby increasing the active rhizosphere zone(Dey *et al*, 2004; Glick, 2005). The ability to use ACC as sole nitrogen source can be linked to the model

Fig 4.16 Production of hydrogen cyanide (HCN) (Orange-red colour) by the sand dune bacterial isolates

proposed by Glick *et al*, where the bacteria utilize the plant-exuded ACC and decrease the concentration of ethylene, thereby breaking down ACC to ammonia. This helps in promoting plant growth by increasing root elongation, which may be a possible mechanism of action. Ammonification, an important step in the transformation of organic nitrogen to ammoniacal form, would enhance soil nitrogen content by the ammonifying character of the PGPR isolates (Dey *et a*l, 2004 ; Brown and Dilworth, 1975). *M. arborescens, Kocuria rosea* and *Bacillus* sp. MF-A4 were also found to produce volatile substances like ammonia while *B. subtilis* did not produce ammonia.

Collections of Gram positive bacteria from coastal sand dune vegetation showed a predominance of orange pigmented isolates (Fig 4.17). 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 & 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).The pigment may be aiding the bacteria to survive in this stressed habitat.

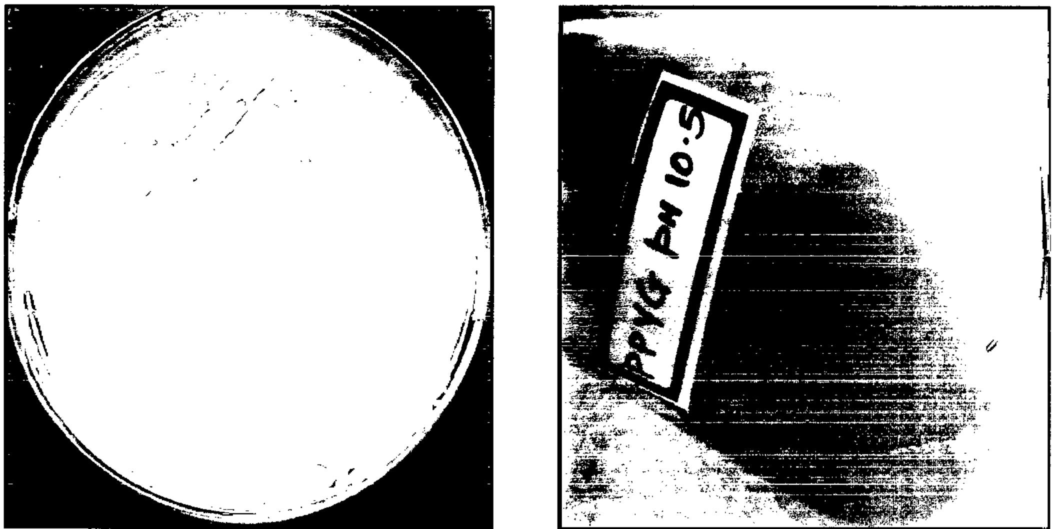


Fig. 4.17 Orange pigment produced by *Microbacterium arboresecens* on PPYG agar medium and in broth

The orange colour of *Microbacterium arborescens* is due to the presence of C_{40} carotenoids. *Microbacterium* species produce yellow, orange, light yellow and whitish yellow pigments. *M. aurantiacum, M. chocolatum, M. imperiale* and *M.testaceum* produce orange pigments (Takeuchi& Hatano, 1998). Trutko *et al,* (2005) reported that pigmented *Microbacterium* strains viz.yellow, orange, and red pigments were found to have absorption spectra typical of C_{40} carotenoids. These compounds were identified using a combination of UV/Visible spectral data and HPLC retention times. Polar organic solvents such as acetone and methanol have been extensively used for the extraction of carotenoids from bacteria (Jagannadha *et al,* 1991).

UV-visible absorption spectra of carotenoid pigments are of immense importance since they aid a great deal in determining the structure of carotenoids (Jagannadha *et al,* 1991). Table 4.9 gives the absorption maxima of the pigment in various solvents. In the present investigation, two extractions with acetone liberated all the pigment from the alkaliphilic *M. arborescens* strain. The pigment was completely extractable in acetone and partially extractable in the other solvents. The UV-visible absorption spectra of the pigment showed absorption maxima at 533,468 and 341 nm in acetone (Fig 4.18). The clear three-band shape of the absorption spectrum of the pigment is characteristic of carotenoids and further reflects its purity (Umeno *et al,* 2005). Carotenoids absorb maximally at three wavelengths (533, 468, 341nm) resulting in three peak spectra which is characteristic of carotenoid pigments. The polyene chromophores of carotenoids, which absorb light in the 400 to 550-nm range, provide the basis for their characteristic yellow-to-red colors and their ability to quench singlet oxygen.

Table 4.9 Absorption characteristics of pigment in various solvents

Solvent	**λ max(nm)**
Acetone	533,468,341,294
Methanol	493,466,441,389
Petroleum ether(60-80°C)	497,468,444,389
Diethyl ether	497,469,371,278

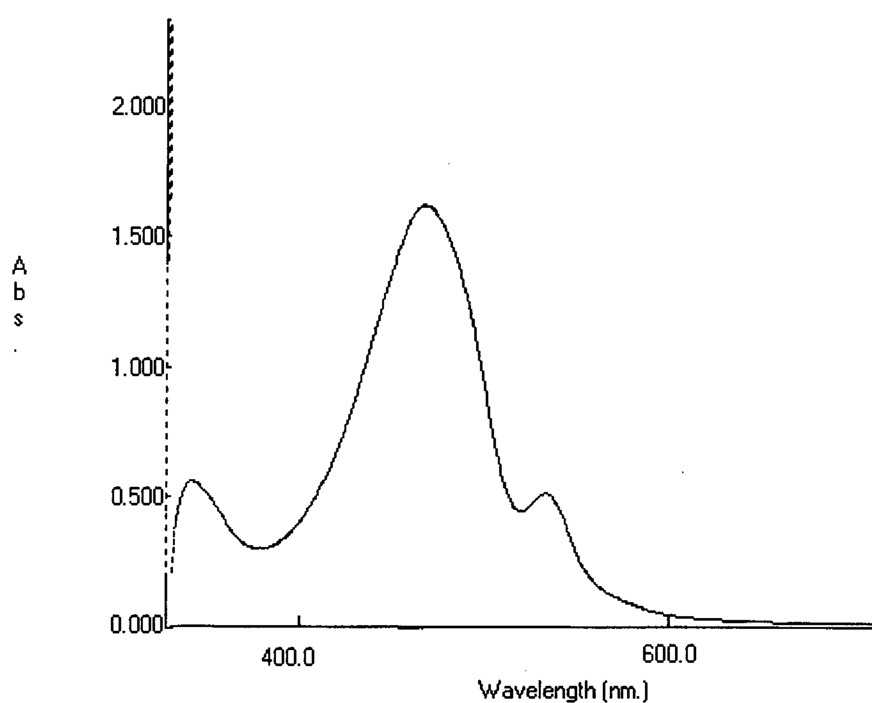


Figure 4.18 Absorption spectrum of pigment from Alkaliphilic *Microbacterium arborescens* – AGSB in acetone

Thin layer chromatography of the acetone extracts were developed in petroleum ether and ethyl acetate showed the separation of the pigment into four spots with different migration rates: Yellow spot (Rf 0.15 cms), pink spot (0.20 cms), light yellow spot (0.47 cms) and dark yellow spot (0.77 cms). Carotenoid hydrocarbons showed two spots Rf 0.6cms and Rf 0.86 cms while carotenoid ketones showed 3 spots. Intense red (Rf 0.05 cms), Pink (0.31) and peach (0.75cms) carotenoid hydroxylated showed 3 spots pink (Rf 0.52 cms), yellow (Rf 0.67) and yellow (Rf 0.77 cms). The thin layer chromatographic analysis of the pigments revealed the presence hydroxylated, hydrocarbon and ketones compounds in the carotenoid pigment. The thin layer chromatographic analysis of the pigments revealed the presence hydroxylated, hydrocarbon and ketones compounds in the carotenoid pigment.

The HPLC profile of *Microbacterium arborescens* pigment can be ascertained from Figs. 4.19 & 4.20. All major peaks in Fig. 4.19 could be tentatively identified on the basis of their absorption spectra. The principal pigment of *Microbacterium arborescens* were found to be Lycopene (Peak 2, Fig. 4.20). Carotenes are readily soluble in petroleum ether, hexane, and toluene; xanthophylls dissolve better in methanol and ethanol. (Schiedt and Liaaen-Jensen 1995). HPLC has been used in the past for the purification of carotenoids from bacteria by employing normal-phase chromatography. However, since normal-phase chromatography is more variable than reverse-phase chromatography, the latter method has now become more popular and has been used in conjunction with a photodiode array detector. Reverse-phase chromatography was used in the present investigation to purify the major carotenoid pigment from a alkaliphilic *M. arborescens* strain from coastal sand dunes. The carotenoids resolved into distinct pigments three peaks and the absorption spectra

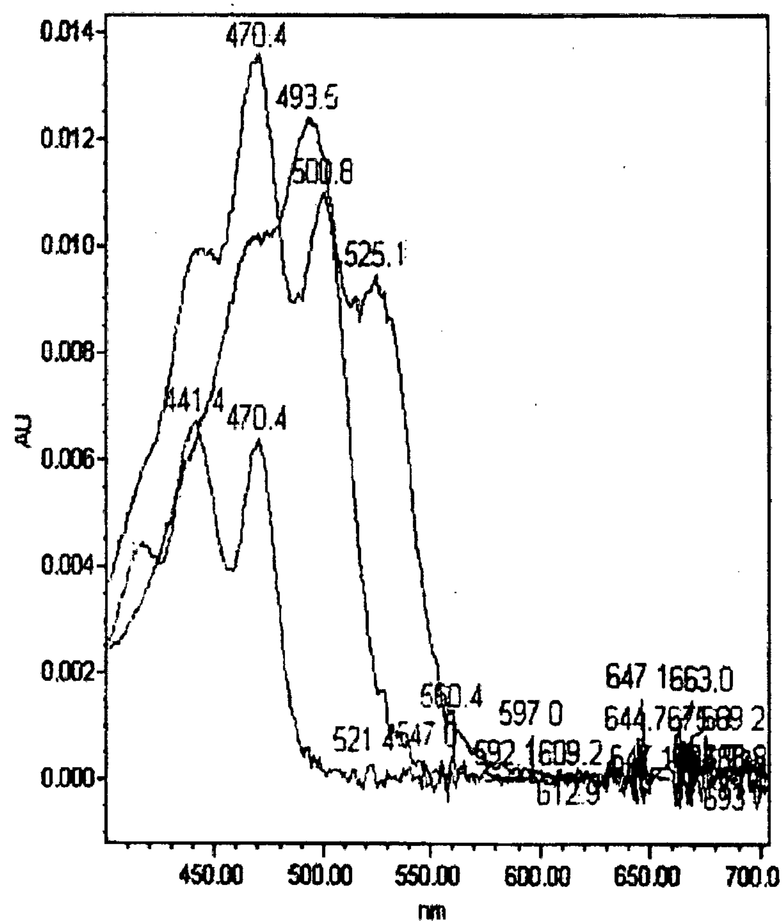


Figure 4.19 HPLC chromatogram showing carotenoid fractions separated from the crude pigment extract of *Microbacterium arborescens*. Separation achieved using a C-18 Reverse Phase column

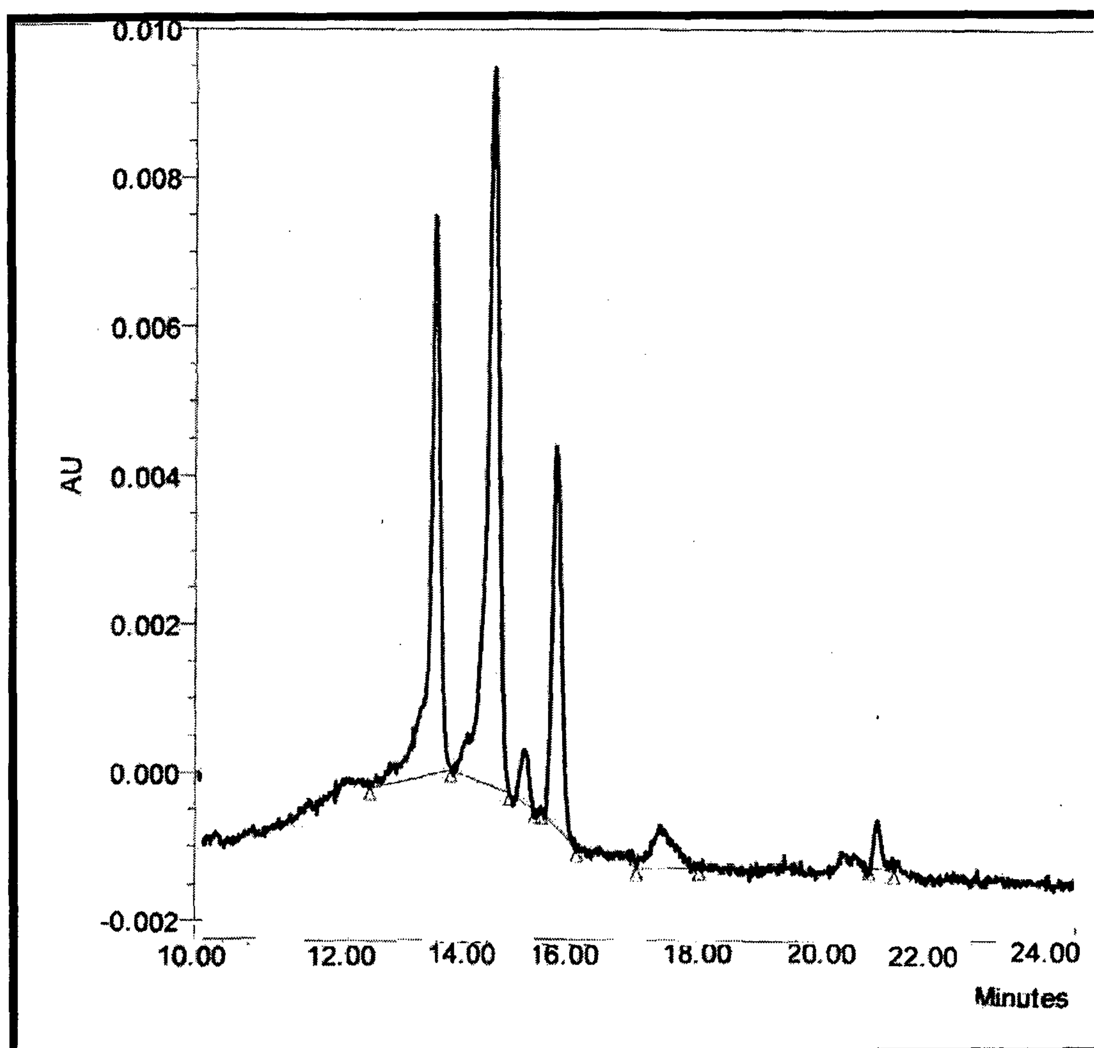


Figure 4.20 HPLC profile of the pigment of *Microbacterium arborescens*

of the pigments were simultaneously recorded with the help of the on-line photodiode array detector. The greater the number of conjugated double bonds, the higher the λ_{max} values. Thus, the most unsaturated acyclic carotenoid lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λ_{max} at 444, 470, and 502 nm.

Interestingly this bacterium was found to show photosynthetic activity. Scientists at the Oregon State University have studied a bacterium known as SAR11 that can switch to using light much the way plants do to stay alive (Giovannoni *et al,* 2005). The biosynthesis of carotenoids in some representatives of the genera *Agromyces, Leifsonia*, and *Microbacterium* is induced by light (Trutko *et al,* 2005). When a light intensity of 4 units was supplied to the *Microbacterium arborescens* culture, no peak of excitation was observed but on increasing the light intensity to 8 units a peak was observed. The F_0 (initial fluorescence) was calculated as 7 units from the graph suggesting photosynthetic activity of the pigment. The spectral scan and the HPLC profile, identified the pigment produced by *Microbacterium arborescens* is a lycopene type of carotenoid pigment with λ_{max} at 468nms. The present study is important in view of identification of a native bacterial strain with a strong potential for development as a bioinoculant. Given the negative environmental impact of chemical fertilizers, the use of PGPR as natural fertilizers is advantageous for the development of sustainable agriculture.. An organism with properties such as phosphate solubilization, disease control potential and rhizosphere colonization would seem ideal for selection as well as a suitable bioinoculant. The plant growth promoting traits of the four isolates were promising mainly with siderophore production and P solubilization. The effect of these isolates on the agricultural crop, eggplant in nursery was therefore studied.

Chapter V
Growth promoting ability of sand dune bacteria in eggplant

5.1 Introduction

Sand dune ecosystem is unique and the bacterial community in this ecosystem has not been sufficiently explored for beneficial bacteria. In this study, we isolated 400 bacterial isolates from sand dune ecosystem of Goa. The bacteria were screened for useful traits like production of enzymes, siderophores, and solubilization of inorganic phosphates. The effective four isolates were identified as *Bacillus subtilis*, *Microbacterium arborescens*, *Bacillus sp.* MF-A4 and *Kocuria rosea.* Since the bacteria were isolated from coastal ecosystem, we evaluated their growth promoting ability under a similar ecosystem. Eggplant was selected as a model plant as it is popularly grown in our area and is a valuable vegetable species.

In the past, Park *et al,* (2005) studied the bacterial populations associated with two major sand dune plant species, *Calystegia soldanella* (beach morning glory) and *Elymus mollis* (wild rye), growing along the coastal areas in Tae-An, Chungnam Province and identified majority of the rhizospheric and endophytic bacteria belonging to *Pseudomonas* species which were useful in plant growth promotion. In an other study by Park *et al, (*2006) bacteria belonging to genus *Chryseobacterium* of the family *Flavobacteriaceae* were isolated from two sand-dune plant species inhabiting coastal areas in Tae-an, Korea. The results from their study suggests that other bacterial taxa, such as *Chryseobacterium, Acinetobacter, Arthrobacter* and *Microbacterium* may have potential for their application in plant growth facilitation.

In the present study the cultures were inoculated on the seeds and in soil and the effect on the growth of eggplant was studied. The methodology and the results are detailed out.

5.2 Materials and methods

5.2.1 Bioassay of isolated sand dune bacteria against plant pathogens, *Ralstonia* and *Sclerotium* under *in vitro* conditions

Four effective bacterial isolates (*Bacillus subtilus, Microbacterium arborescens, Bacillus sp.* MF-A4 and *Kocuria rosea*) were tested for their ability to inhibit the plant pathogens and to improve the plant growth. The plant pathogens used in the study are *Ralstonia solanacereum*, a bacterial pathogen on solanacious crops and *Sclerotium sp.*, a soil borne, root rot pathogen of cucurbits. 2 days old culture of the respective pathogen was added to molten Kings B medium, PDA, PPYG and Nutrient agar medium respectively, poured to plates and allowed to solidify. Wells were bored into the agar and the culture broth of respective bacteria were centrifuged and the supernatant was put into the well. Inhibition of growth of pathogen indicated antagonistic activity of the culture.

5.2.2 Growth promoting effect of the sand dune bacteria (SDB) on eggplant by the Roll Towel method

24h old cultures were centrifuged at 8,000 rpm for 10min and the cell pellet was suspended in phosphate buffered saline (pH 7) and mixed with sterile powder to form slurry. Eggplant seeds of Agassaim were coated in the culture slurry and placed on the germination paper. The paper was loosely rolled and tied with thread and placed in a beaker of water (2cm depth). The four culture isolates cell pellets viz. *Microbacterium arborescens, Kocuria rosea, Bacillus* sp MF-A4 and *Bacillus subtilis* were mixed together in equal proportions to form the consortium and treated as above. After a

week, the parameters like the number of seeds germinated, shoot length and root length were recorded. In order to test the effect of the culture on pregerminated eggplant seeds, the seeds were allowed to germinate for 3 days, and then treated in a similar way as mentioned above.

5.2.3 Growth promoting ability of sand dune bacteria in eggplant nursery.

An experiment was conducted in split plot design where soil sterilization was main treatment and bacterial isolates were sub-treatments. *B. subtilus* was grown in nutrient broth at pH 7 while *M. arborescens*, *Bacillus sp. MF-A4* and *K. rosea* were grown in poly peptone yeast extract glucose broth at pH 10. Seeds of eggplant cultivar Agassaim were soaked in the 48 hr old grown culture of the above isolates for 30 mins and were allowed to dry under shade for 30 min. The treated seeds were sown in pots filled with pot mixture which was treated with 48 h old grown culture of the respective isolates to a final concentration of 5 percent. The four culture isolates viz. *Microbacterium arborescens, Kocuria rosea, Bacillus* sp MF-A4 and *Bacillus subtilis* were mixed together in equal proportions to form the consortium. Suitable control was maintained without any treatment while a positive control was also maintained with the recommended dose of fertilizers. Regular crop production practices were followed. Observations were recorded at frequent intervals on number of germinated seedlings; shoot length, root length and plant wet weight upto the seedling attained growth stage suitable for transplanting. In another experiment, the normal non-sterile field soil was only used in pots and all the isolates were grown at pH 7 and the

experiment was conducted as described above. This experiment was repeated under same conditions once again. All the data were statistically analyzed using ANOVA.

5.2.4 Change in population of sand dune bacteria inoculated to soil

Soil samples were collected from the pots on day one and 35 days after sowing to check the population of bacteria present in the soil. The dilutions were plated on nutrient agar, pH7 to observe the population of *B. subtilus* while Polypeptone Yeast extract Glucose agar , pH 10 was used to identify the *M. arborescens*, *K. rosea* and *Bacillus sp.* MF-A4 The isolates were identified based on their growth at pH10 on PPYG, cultural characteristics and typical pigment produced by the isolates on PPYG.

5.2.5 Analysis of C, N and P content in the inoculated soils

Soil samples were taken from the pots on day one immediately after the treatments and 35 days after sowing to analyse carbon content, available nitrogen and phosphate content of the soils. Carbon content of the soils was determined using the Walkley and Black's rapid titration method (Gali *et al*, 2000). Available nitrogen content of soil was estimated using alkaline potassium permanganate method (Jackson, 1973) and phosphate content of soil was determined by the Olsen method (Olsen and Sommers, 1982).

Results and Discussion :

The efficacy of sand dune bacteria against plant pathogens, *Ralstonia* and *Sclerotium* sp. was studied using the well method. It was interesting to note that out of the four cultures *B. subtilis, Bacillus* sp. MF-A4 inhibited the growth of *Sclerotium* sp. on PPYG medium while *B. subtilis* inhibited the growth of *Sclerotium* sp. on PDA with 2 % chitin (Fig 5.1). *M. arborescens* did not show significant inhibition of *Sclerotium sp.* on PPYG, however inhibition was observed on PDA medium under *in vitro* conditions. Further no inhibition or overgrowth of the isolates was observed when *R. solanacearum* was tested on KB medium.

Having seen the effect of the four selected isolates on plant pathogens, the growth promoting effect of these four isolates was tested on eggplant by the roll towel method (Fig 5.2). Seeds were coated with the isolates and the effect on the shoot and root length was observed. Early vigour of the seedlings is important for better establishment, which can be assessed by increasing shoot and root length. Ten seeds were treated with 24h old grown cultures of selected isolates and kept for 7 days in dark. When compared with control, the seedlings that showed increased shoot length include those treated with cultures *Bacillus sp. MF-A4* (8.13 cm) and *K. rosea*(8.01 cm) while the root length was highest (4.62 cm)in *K.rosea* (Table 5.1). When the bacterial cells re-suspended in PBS and pre-germinated seeds were used, the growth promotion effect was very much evident in all the treatments. When germinated seeds were treated with talc slurry the highest shoot length (6.89 cm) and root length(8.22 cm) was recorded in *K.rosea*. The reason attributed may be increased contact between

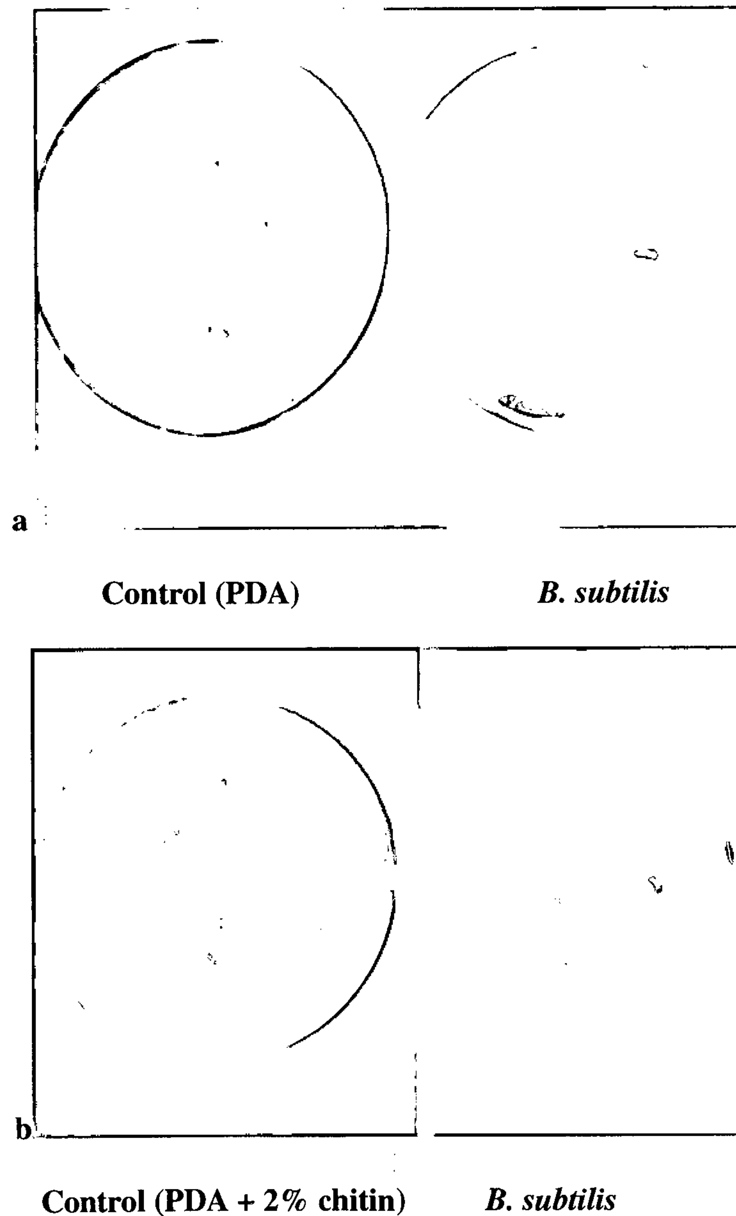


Fig 5.1 ***In vitro*** **inhibition of growth of** ***Sclerotium*** **sp. by antagonistic** ***B. subtilis*** **on a) PDA medium and b) PDA + 2% chitin**

Fig 5.2 Germination of Eggplant seedlings by Roll Towel method

Table 5.1 Effect of sand dune bacteria on germination on eggplant seeds

S. No.	Sand dune bacteria	Seed treatment with SDB		Pre-germinated seeds treated with SDB	
		Shoot length (cm)*	Root length (cm) *	Shoot length (cm) *	Root length (cm) *
1	*Bacillus subtilis*	6.97 ± 0.20	4.22 ± 0.12	6.69 ± 0.23	6.17 ± 0.18
2	*Microbacterium arborescens*	5.97 ± 0.80	4.11 ± 0.30	6.50 ± 0.22	6.77 ± 0.23
3	*Kocuria rosea*	8.01 ± 0.31	4.62 ± 0.03	6.89 ± 0.11	8.22 ± 0.07
4	*Bacillus sp. MF-A4*	8.13 ± 1.05	4.28 ± 0.14	6.68 ± 0.15	6.83 ± 0.411
5	Consortium	6.46 ± 0.21	4.42 ± 0.13	6.59 ± 0.10	6.00 ± 0.46
6	Control	6.38 ± 0.31	4.53 ± 0.18	6.51 ± 0.20	5.91 ± 0.28
	CD(0.05)	1.51	NS	0.211	1.012
	CV	14.4	7.86	2.104	10.09

SDB- sand dune bacteria, * Mean of triplicates, NS- not significant

the bacteria and the rhizosphere region of the germinated seeds. Our results indicated theability of isolates to colonize the seeds/roots to improve the growth parameters, however, root colonization by cells during the initial stages was found to be more efficient than the seed colonization alone. It has been shown under controlled experimental conditions, that initial bacterial binding to seed, not necessarily the roots after germination, is most important for enhanced plant root elongation (Lucy *et al*, 2004).

Further having seen the effect of the isolates on increase in shoot and root length the study was carried out under competitive (non-sterile) and non competitive soil (sterilized soil) conditions, to understand the effect of the inoculum in the presence and absence of native microorganisms. Among sterilized and non sterilized soil, the effect was observed at different days after sowing. There was significant difference in three parameters (root length at 30 DAS, shoot length and wet weight at 35 DAS). The highest number of seeds germinated in *B. subtilis* treatment (86.0 and 80.0 in non-sterilized and sterilized soil respectively) (Table 5.2, Figs 5.3 a & b). The results indicate that at 25DAS the highest shoot length(6.17 cm) and root length (4.73 cm) was recorded in positive control in non sterilized soil while the maximum shoot length (4.70 cm) was recorded in consortium treatment of sterile soil and the maximum root length (3.07 cm) was recorded in the control. The maximum wet weight was recorded in consortium both in non sterile and sterile soil conditions. At 30 DAS, the maximum shoot length was shown by *K. rosea* in nonsterilized (9.30 cm) soil and sterilized (10.35 cm) soil treatments followed by *Bacillus* sp.MF-A4 in non sterilized (9.20 cm) soil. The maximum root length (4.96 cm) was recorded in *B. subtilis* treatment in non sterilized soil and *K. rosea* (4.35 cm) in sterilized soil

›le 5.2 Growth promoting effects of sand dune bacteria in sterilized and un-sterilized soil on eggplant seedlings

. o	Treatments	No. of seeds germinated / 150 seeds (8DAS)		Growth at 25 DAS						Growth at 30 DAS						Growth at 35 DAS					
				Shoot length*		Root length*		Wet weight*		Shoot length*		Root length*		Wet weight*		Shoot length*		Root length*		Wet weight*	
		I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
	B.subtilis	86.0	80.0	5.34	3.49	4.03	2.77	0.93	0.73	8.07	6.82	4.96	2.40	2.10	1.42	9.50	7.90	4.40	2.85	3.08	2.80
	M.arborescens	52.5	48.5	4.81	4.13	3.09	2.58	1.05	0.68	8.55	7.50	3.90	2.55	2.24	2.25	9.10	9.20	5.80	2.75	3.38	1.78
	K.rosea	75.0	80.0	4.85	4.67	2.44	3.04	1.33	1.07	9.30	10.35	4.00	4.35	2.16	1.79	8.15	8.45	7.35	4.40	5.47	2.94
	Bacillus sp. MF A-4	59.0	41.0	5.30	4.25	2.22	2.63	1.84	0.86	9.20	7.13	4.00	2.82	2.57	2.55	10.60	9.60	4.20	4.35	2.33	2.27
	Consortium	67.5	70.0	4.91	4.70	3.10	2.11	1.92	1.24	7.60	9.00	4.05	2.85	2.25	2.22	9.10	9.85	4.50	3.65	1.90	2.65
	Control	70.0	51.5	5.36	4.29	3.77	3.07	1.82	1.09	6.80	6.70	4.25	3.45	1.45	2.51	7.00	7.45	6.50	4.30	2.09	2.15
	+ve control	26.5	64.0	6.17	3.23	4.73	2.72	1.82	1.16	7.20	6.76	4.50	3.50	1.46	2.61	7.10	11.70	6.00	2.90	2.20	5.79
	CD (0.05)																				
	Main	11.8		5.09		1.89		1.94		2.60		0.86		3.18		2.36		3.36		0.51	
	Sub	17.9		1.08		0.68		0.42		1.96		0.84		0.98		1.54		1.06		0.83	
	MXS	25.4		1.52		0.96		0.59		2.77		1.20		1.39		1.17		1.41		1.17	

›n-sterilized soil
terilized soil
ot length, Root lengths are in cm
weight of 10 plants at 25 DAS and 5 plants at 30 DAS, 35DAS (g)
- Days after sowing
›an of triplicates

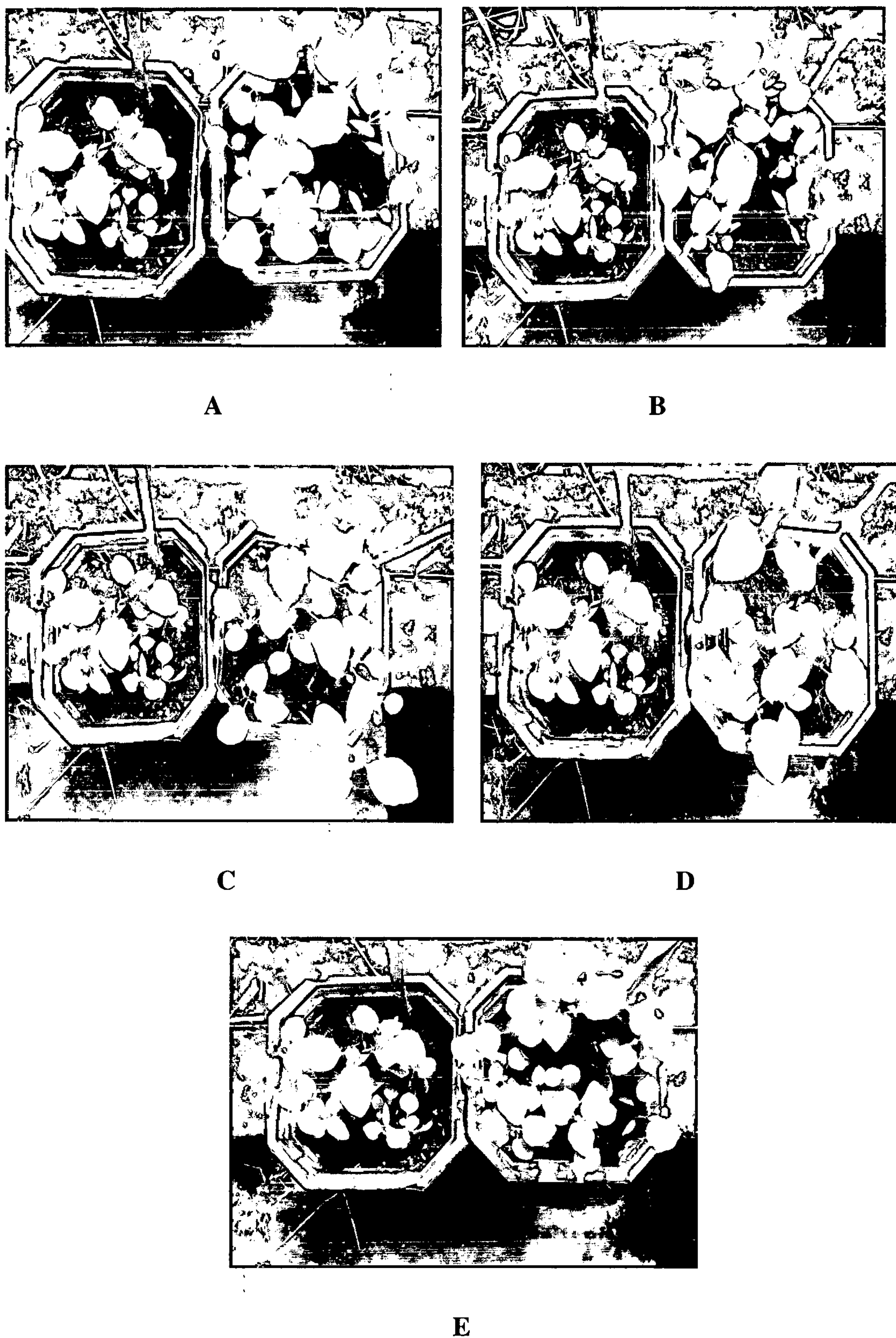


Fig 5.3a Growth promoting effects of sand dune bacteria in non sterilized soil on brinjal seedlings (A – Control + *M. arborescens*, B - Control + Consortium, C- Control + *K. rosea*, D – Control + *B. subtilis*, E – Control + *Bacillus* sp MF-A4).

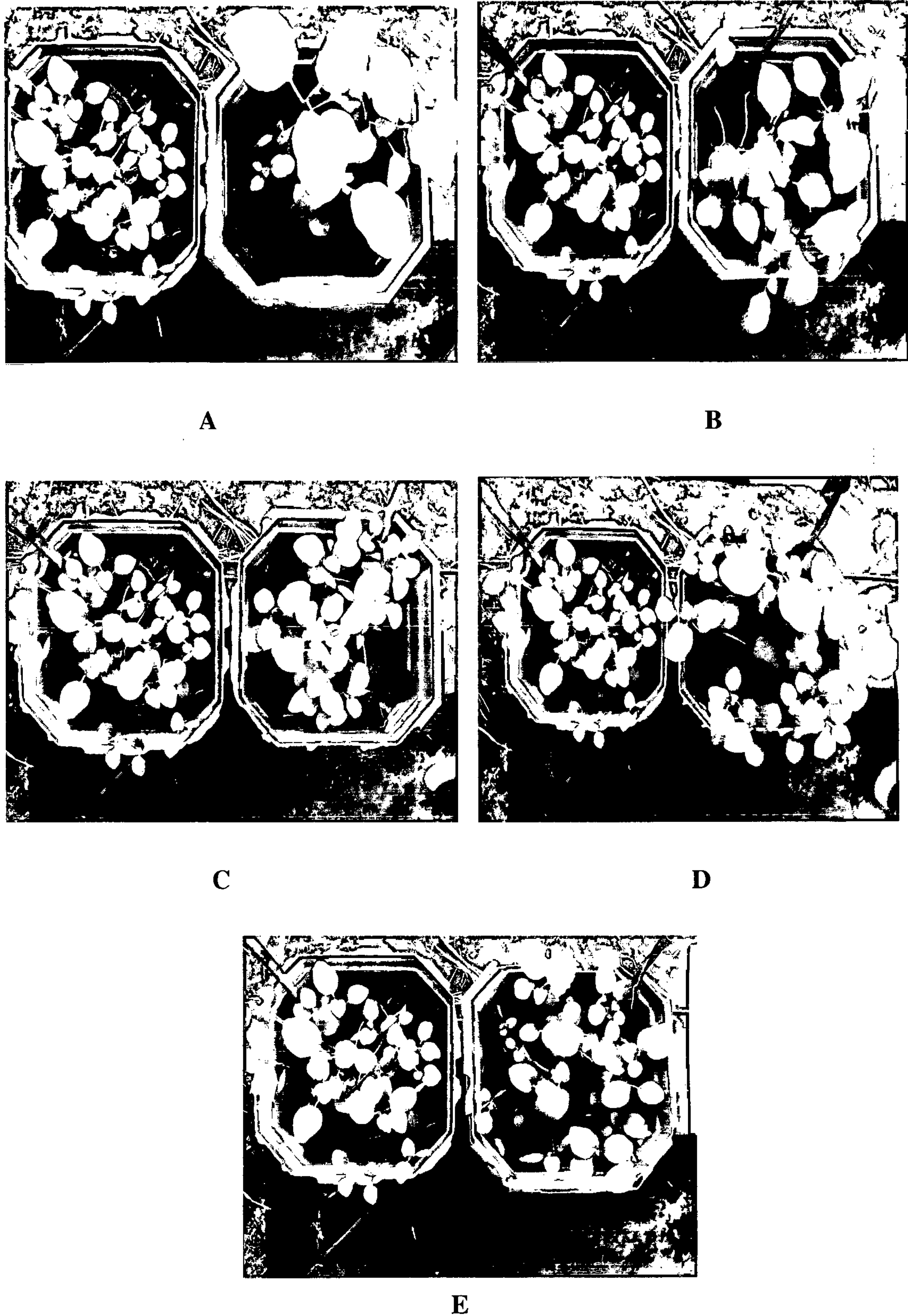


Fig 5.3b Growth promoting effects of sand dune bacteria in sterilized soil on brinjal seedlings (A – Control + *M. arborescens, B-* Control + Consortium, C- Control + *K. rosea*, D – Control + *B. subtilis*, E – Control + *Bacillus* sp MF-A4).

treatment. *Bacillus* sp. MF-A4 showed the maximum increase in wet weight in non sterilized and sterilized soil. At 35 DAS *Bacillus* sp. MF-A4 showed the maximum shoot length (10.60 cm) in nonsterilized soil treatment while consortium recorded the maximum shoot length (9.85 cm) in consortium treatment. The maximum root length was recorded in *K. rosea* treated non sterilized (7.35 cm) and sterilized soil (4.40 cm). Also *K. rosea* showed the maximum wet weight in non sterilized and sterilized soil treatments.

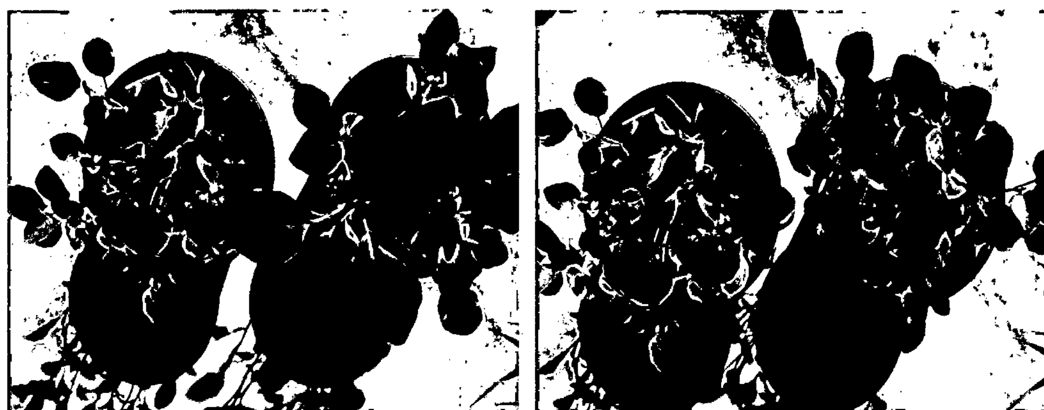
The results indicated no significant difference in growth parameters between sterilized soil and non sterilized soil in most of the observations upto 35 DAS. Hence it was concluded that the efficacy of SDB to be tested in normal soil i.e competitive conditions. It is assumed that since the pH of the soil is near neutral it was decided to grow the SDB under neutral pH conditions in respective media in order to adapt to the soil conditions when treated. All the cultures grew at neutral pH and their population was assessed immediately after application to the soil. Mean of two season data showed that *K. rosea* and *B. subtilis* increased shoot length and weight of the plants consistently upto 44 DAS. However *Bacillus sp* MF-A4 increased the growth parameter significantly from 37 DAS after sowing. *M. arborescens* was effective in the latter stages (at 44DAS) (Table 5.3 and Fig 5.4). At 30 DAS, *B. subtilus* and *K.rosea* recorded maximum shoot length in the first (11.68 cm & 11.08 cm) and second (10.52 cm and 10.43 cm) experiment. Consortium treatment recorded maximum root length (4.74 cms) in the first experiment while *B. subtilus* recorded maximum root length (5.47 cm) in the second experiment. *K. rosea* recorded maximum plant weight (3.86 g) in the first experiment while *B. subtilis* recorded maximum plant weight (5.68 g) in the second experiment. At 37 DAS, *Bacillus sp.*

ble 5.3 Growth promoting effects of sand dune bacteria in normal soil on brinjal seedling

S.No	Treatments	Growth at 30 DAS									Growth at 37 DAS								
		Shoot length*			Root length*			Wet weight*			Shoot length*			Root length*			Wet weight*		
		I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean	I	II	Mean
1	*B.subtilis*	11.68	10.52	11.10	3.52	5.47	4.49	2.48	5.68	4.08	11.79	12.8	12.29	4.23	8.406	6.31	1.69	8.30	4.99
2	*M.arborescens*	10.19	10.17	10.18	4.53	4.54	4.53	2.41	4.71	3.56	11.35	13.0	12.17	4.30	7.66	5.98	2.06	4.06	3.06
3	*K.rosea*	11.08	10.43	10.75	3.20	4.78	3.99	3.86	4.97	4.41	12.81	12.4	12.6	5.43	8.20	6.81	3.83	6.88	5.35
4	*Bacillus sp.MF A-4*	10.4	9.72	10.06	4.47	4.55	4.51	2.55	4.89	3.72	14.40	11.9	13.15	6.52	6.533	6.52	5.47	5.90	5.68
5	Consortium	10.61	9.88	10.24	4.74	5.03	4.88	2.24	4.54	3.39	13.42	11.6	12.51	7.70	8.34	8.02	5.47	6.06	5.76
6	Control	7.12	8.65	7.88	2.75	4.82	3.78	2.1	2.94	2.52	11.60	10.5	11.05	3.56	7.22	5.39	4.03	3.55	3.79
7	+ve control	7.94	9.36	8.65	4.01	4.74	4.37	1.64	3.96	2.80	10.21	11.7	10.95	6.34	8.12	7.23	2.56	5.52	4.03
	CD (0.05)	2.21	1.044		NS	NS		NS	NS		1.72	1. 32		1.34	NS		2.38	NS	
	CD (0.01)	3.10									2.41			1.88					

S.No	Treatments	Growth at 44 DAS								
		Shoot length*			Root length*			Wet weight*		
		I	II	Mean	I	II	Mean	I	II	Mean
I	*B.subtilis*	12.15	14.36	13.25	6.27	7.68	6.97	2..20	9.00	5.60
2	*M.arborescens*	13.59	13.94	13.76	7.54	7.20	7.37	4.03	8.73	6.38
3	*K.rosea*	13.8	14.08	13.94	6.48	6.90	6.69	3.18	8.29	5.73
4	*Bacillus sp.MF A-4*	13.17	13.48	13.32	8.56	7.20	7.88	3.31	9.72	6.51
5	Consortium	13.04	13.41	13.22	7.17	7.53	7.35	3.67	6.43	5.05
6	Control	11.99	12.03	12.01	4.89	8.33	6.61	5.58	6.52	6.05
7	+ve control	10.57	13.68	12.12	7.13	9.55	8.34	2.64	8.35	5.49
	CD (0.05)	1.65	1.332		NS	NS		NS	NS	

* Mean of triplicates , I,II – experiment repeated twice



A B

C

D

E

Fig 5.4 Growth promoting effects of sand dune bacteria in normal soil (pH 7) on eggplant seedling (A – Control + *M.arborescens, B*- Control + *consortium*, C- Control + *K.rosea*, D – Control + *B.subtilis*, E – Control + Bacillus sp MF-A4)

MF-A4 and consortium treatment recorded the maximum shoot length in the first (14.40 cm and 13.42 cm) experiment while *B. subtilis* and *Kocuria rosea* recorded maximum shoot length (12.8 cm and 12.4 cm) in the second experiment. Consortium treatment and *Bacillus sp.MF-A4* recorded maximum root length (7.70 cm and 6.52 cm) in the first experiment whereas *B. subtilus* and consortium treatment recorded maximum root length(8.4 cm and 8.34 cm) in the second experiment. *Bacillus* sp.MF-A4 and consortium treatments recorded maximum plant weight (5.47 g) in the first experiment while *B. subtilus* and *Kocuria rosea* recorded maximum plant weight (8.30 g and 6.88 g) in the second experiment. At 44 DAS, K. *rosea* and *M. arborescens* recorded the maximum shoot length(13.8 cm and 13.59 cm) in the first experiment while *B.subtilus* and *Kocuria rosea* recorded maximum shoot length(14.36 cm &14.08 cm) in the second experiment. *Bacillus sp.* MF-A4 and *M. arborescens* recorded maximum root length(8.56 cm and 7.54 cm) in the first experiment while positive control and control recorded maximum root length(9.55 cm and 8.33 cm) in the second experiment. Positive control and *M. arborescens* recorded maximum plant weight (5.58 g and 4.03 g) in the first experiment while *Bacillus sp MF-A4* and *M. arborescens* recorded maximum plant weight (9.72 g and 8.73g) in the second experiment. The difference in the time of growth promotion may be due to the time taken for initial establishment and colonization of root zones by the bacteria and the rhizosphere competence of respective bacteria.

Bacillus are spore-forming gram positive rod shaped bacteria which are highly tolerant of adverse ecological conditions. Common physiological traits important to their survival include production of multilayered cell wall structure, formation of stress resistant endospores and secretion of peptide antibiotics, peptide

signal molecules and, they probably bring about growth promotion through biocontrol of disease and insect pests (McSpadden Gardener, 2004; Woitke, Jung *et al*,2004). *Paenibacillus polymyxa* (previously *Bacillus polymyxa;* Ash *et al,* 1993), a common soil bacterium, belongs to this group. A range of activities has been found to be associated with *P. polymyxa* treatment, some of which might be involved in plant growth promotion (Timmusk *et al,* 1999, and references therein). The mechanism by which *P. polymyxa* exerts its beneficial effect is not understood: e.g., in certain *P. polymyxa* strains (Lindbergand Granhall, 1984), atmospheric nitrogen fixation ability does not correlate with the observed growth-promoting effect (Lindberg *et al,* 1985).

Growth promoting effects of rhizobacteria have been reported by several workers in many crops viz. *B. benzoevorans* on growth of tomato (Medina and Antoun, 2005), *Bacillus subtilis* on potato and maize (Woitke *et al*, 2004), *Bacillus* sps on barley (Canbolat *et al*,2006) and pine and spruce seedling growth(Shishido,1996), *Azospirillum* on wheat seedlings (Tilak *et al.*, 2005; Rai, 2006), *Rhizobium* on pigeon pea (Tilak, Reddy, 2006) and on non-legumes(Antoun *et al*, 1998; Matiru and Dakora,2004), *Microbacterium* on cotton, wheat and maize (Egamberdiyeva et al ,2003). *P. fluorescens* is a major member of PGPR and has been reported to enhance crop growth, yield, and reduce disease in many crops (Roesti, 2005; Tilak *et al*, 2005; Deshwal *et al*, 2006). The plant growth promoting bacteria are known to produce many growth promoting substances like, siderophores (Alexander and Zuberer, 1991;Kloepper, 1980), IAA (Ahmad F, 2005 ; Tsavkelova, 2005) and solubilization of inorganic phosphate (Nautiyal *et al*, 2000;Ponmurugan and Gopi, 2006) in soil to enhance plant 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 beneficial effect on plant growth. (Tilak *et al*, 2005).

Different plant-growth promoting rhizosphere bacteria, including associative bacteria such as *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter* group have been used for their beneficial effects on plant growth. In addition to the well known *Azotobacter* and *Azosprillum spp.*, and a number of other bacteria may be considered as PGPR, including various species of *Pseudomonas, Acetobacter, Burkholderia, Alcaligenes, Klebsiella, Enterobacter, Herbaspirillum, Xanthomonas* and *Bacillus* (Arshad & Frankenberger,2004). Microorganisms that colonize roots and stimulate plant growth have been applied to a wide range of agricultural species for the purposes of growth enhancement, including increased seed emergence, plant weight, crop yields, and disease control (Kloepper *et al*, 1991). For example, emergence increases of 10–40% resulted for canola when seeds were coated with PGPR before planting (Kloepper *et al*, 1991 & 1980), and plant weight of tuber-treated potatoes increased by 80% on average by midseason (Kloepper and Scroth, 1980). Yield increases between 10% and 20% with PGPR applications have been documented for several agricultural crops (Kloepper *et al*, 1991).Proposed mechanisms for plant-growth promotion by PGPR include bacterial synthesis of the plant hormones indole-3-aceticacid (Loper, & Schroth, 1986), cytokinin (Timmusk *et al*, 1991), and gibberellin ; breakdown of plant produced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate deaminase ; and increased mineral and N availability in the soil . Egamberdiyeva *et a,l* (2003), isolated the bacterial strains

from calcareous Calcisol soil and determined their effect on plant growth of cotton, wheat and maize under conditions of high temperature and nutrient deficient soil. Their work demonstrated that independent of the origin, selected growth-stimulating bacteria isolates are able to increase the growth cotton, wheat and maize in nutrient poor Calcisol soil. The bacterial isolates *Microbacterium M12M, Bacillus sp. B10M, Pseudomonas sp.P29M, Pseudomonas sp. P12M* had a significant effect on cotton, wheat and maize in nutrient-poor Calcisol soil, while non-treated plants by comparison performed poorly under such conditions.

In our studies on effect of sand dune bacteria on the growth of eggplant, the rhizobacteria showed appreciable results. In the nursery studies the pots containing non sterilized soil *B. subtilus* and *Kocuria rosea* proved to be effective as bioinoculants to improve plant growth while in the sterilized pot experiments *Kocuria rosea* was found to be effective. In the nursery experiments where all the inoculants had a neutral pH, it was seen that *Bacillus sps, Microbacterium* and *Kocuria* were found to increase plant growth to a great extent. In similar studies the effects of three Plant Growth-Promoting Rhizobacteria (PGPRs) on tomato and pepper seedlings grown in two types of peat (brown and black, each sterilized or unsterilized) were studied by Garcia, (2003). All three bacteria had the capacity to modify the growth, but tomato seedlings responded better than pepper seedlings, possibly indicating better root colonization in the former. All bacteria increased total stem length of tomato seedlings in nonsterilized brown peat, nonsterilized black peat and sterilized black peat.Fluoresent *Pseudmonads* having ACC deaminase activity, phosphate solubilizing ability, IAA and siderophore producing characters have been found to enhance peanut growth under potted conditions (Pal *et al*, 1999.) Stimulation of

different crops by PGPR has been demonstrated in both laboratory and field trials. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* have increased root and shoot elongation in canola, lettuce, and tomato as well as crop yields in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants, and wheat. Wheat yield increased up to 30% with *Azotobacter* inoculation and up to 43% with *Bacillus* inoculants, and a 10–20% yield increase in the same crop was reported in field trials using a combination of *Bacillus megaterium* and *Azotobacter chroococcum. Azospirillum* spp. have increased yield in maize, sorghum, and wheat, and *Bacillus* spp. has increased yield in peanut, potato, sorghum, and wheat (Rodrigues and Fraga, 1999).

Population sizes of bacteria decline more or less rapidly following introduction into a natural soil, it has been attributed to the scarcity of available nutrient sources to microbes in soil and the hostility of soil environment to incoming microbes due to a myraid of adverse abiotic and biotic factors. The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in the soil as observed after introduction of a genetically marked *Bacillus subtilis* strain into field soils, the inoculant populations were shown to decline to levels approximately similar to the number of spores initially present in the inoculum. The populations remained in this level and mainly in the spore form over a period of 120 days (Van veen, 1997). In our study, change in total viable counts of sterilized and non sterilized soil showed that *B. subtilis* populations survived upto 10^6 cfu/g followed by *Bacillus sp* MF-A4(10^5 cfu/g) in non sterilized soil and *B. subtilis* showed a similar survival rate in sterilized soil as well followed by *K. rosea* (10^5 cfu/g) Comparatively, *M. arborescens* was found to survive at lower counts of 10^4

cfu/g in non sterile soil and 10^3 cfu/g in sterile soils. *Kocuria rosea* was found to survive counts of 10^4 cfu/g in non sterile and 10^5 cfu/g in sterile soil while *Bacillus* sp MF-A4 survived at 10^5 cfu/g in nonsterile and 10^4 in sterile soil conditions. Consortium survived at 10^4 cfu/g in nonsterile soil conditions and was not detected in sterile soil conditions after 35 DAS (Table 5.4). In normal soil conditions where pH of the growth medium was maintained at pH 7, *Bacillus* sp MF A4, *B. subtilis, K. rosea* and *M. arborescens* showed a consistent survival rate of 10^5 cfu/g (Table 5.5). Population reduction may be due to the competition from native microflora and depletion of nutrients over a period of time. This bacterial cell decrease and ultimate death can be due to depletion of essential nutrients and accumulation of toxic wastes products as described by Tate, (1985). *Ps. pv. tomato* survived in autoclaved soils for 3 months in their study. Devash *et al,* (1980) reported survival of the bacterium in autoclaved soils for 6 months. In Switzerland the bacterium survived for 120 days approximately 4 months (Bosshard Heer and Vogelsanger, 1977).

In addition, the effect of inoculants on soil pH and nutrient availability was studied once the crop was removed. In all SDB inoculated treatments pH changed from slightly acidic to near neutral or slightly alkaline. There was increase in pH at 35 DAS with the treatments in the non-sterile soil conditions. The pH of the soil increased from 6.03 at the time of inoculation to 7.50 at the end of the experiment after 35 days of inoculation in in *K. rosea* treated soil (Table 5.6). Similar observations were recorded for sterile soils as well. Soil pH affects the solubility of soil minerals, the availability of plant nutrients and the activity of microorganisms. In general, pH values between 6 and 7.5 are optimal for crop growth. It has been shown that soil pH controls the microbial community and that bacteria will decrease at low

Table 5.4 Change in total viable count of sand dune bacteria inoculated under nursery pot conditions in sterilized and nonsterilized soil conditions (NS- non sterilized soil, SS- sterilized soil)

Sand dune bacteria	Total viable counts (cfu/g of soil)		
	Day 1	35 DAS (NS)	35 DAS (SS)
Bacillus subtilis	$1.4 x 10^9$	$3.4 x 10^6$	$5.88 x 10^6$
M. arborescens	$4.400 x 10^{11}$	$2 x 10^4$	$4.0 x 10^3$
K. rosea	$2.60 x 10^{10}$	$6 x 10^4$	$8.20 x 10^5$
Bacillus sp MF-A4.	$5.40 x 10^{10}$	$2.6 x 10^5$	$2.00 x 10^4$
Consortium	$1.25 x 10^8$	$1.20 x 10^4$	-

Table 5.5 Change in total viable count of sand dune bacteria inoculated under nursery pot conditions in normal field soil.

Sand dune bacteria	Total viable count (cfu/g of soil), pH7	
	Day 1*	46 DAS*
Bacillus subtilis	$2.76 x 10^9$	$2.5 x 10^5$
M.arborescens	$2.85 x 10^9$	$3.5 x 10^5$
K.rosea	$1.38 x 10^9$	$3.5 x 10^5$
Bacillus sp MF-A4.	$2.95 x 10^9$	$7.7 x 10^5$
Consortium	$1.375 x 10^9$	$1.75 x10^5$

Table 5.6 Change in the soil properties of sterilized and non sterilized soils inoculated with sand dune bacteria

Treatments		pH		Carbon content %		Available N content %		PO_4- P content (mg/kg soil)	
		Day 1	**Day 35**	**Day 1**	**Day 35**	**Day 1**	**Day 35**	**Day 1**	**35 DAS**
B. subtilis	NS	6.75	7.30	0.012	0.042	0.020	0.033	30	50
	SS	6.60	7.20	0.011	0.039	0.020	0.031	28	48
M. arborescens	NS	6.53	7.25	0.012	0.032	0.023	0.045	25	35
	SS	6.44	7.20	0.011	0.022	0.021	0.042	24	34
K. rosea	NS	6.03	7.50	0.012	0.045	0.025	0.054	24	32
	SS	6.01	7.56	0.012	0.040	0.024	0.053	22	30
Bacillus sp.MF-A4	NS	6.43	7.23	0.013	0.064	0.035	0.052	28	40
	SS	6.33	7.32	0.014	0.059	0.032	0.051	26	38
Consortium	NS	6.54	7.21	0.014	0.020	0.025	0.048	32	46
	SS	6.60	7.25	0.012	0.021	0.024	0.046	30	44
Control	NS	6.80	6.85	0.012	0.013	0.020	0.030	30	31
	SS	6.78	6.9	0.014	0.012	0.023	0.028	28	30

NS – Non-sterile soil; SS- Sterile soil

pH (Arias *et. al,* 2005). The carbon content increased 2-4 folds during the crop period in SDB treatments in sterilized and nonsterilized soil conditions. The plant residues entering the soil are the primary source of soil organic matter and come from litter and root material. The increase in carbon in the soil is due to microbial activity in soil (Killham, 1994;White, 1997;Wardle, 1992; Paul and Clarke, 1989) which breaks down the plant and leaf residues thus increasing carbon in the soil. The available N content and phosphate content showed increasing trend during crop period. Nitrogen is especially important, if it is reduced then plant growth is also reduced, as the soil microorganisms will utilize the nutrients in dead organic matter before roots can. (Miller & Donahue, 1990).There was an increase in the nitrogen content of the treated soils over a period of time which could be due to breakdown of humus and other plant debris, When inorganic P content was analysed all SDB treatments increased P availability with *B. subtilis* maximum increase in phosphate content from 30 to 50 mg per kg of soil after 35 DAS in non sterilized soil whereas in sterile soil there was an increase in phosphate content from 28 to 48cmg per kg of soil. This increase in the phosphate content of the bacteria treated soils over a period can be attributed to the P-solubilizing ability of the bacterial inoculants which solubilize inorganic phosphates present in the soil to readily utilizable forms of phosphate by producing organic acids such as citric acid, oxalic acid and thus increasing the available phosphates in soil. Seed or soil inoculation with PSB such as *Bacillus* spp. can solubilize fixed soil P and applied phosphates, resulting in higher crop yields and also in increased inorganic P availability to plant by mineralization of organic P solubilization of insoluble compounds is due to the excretion of microbial metabolites such as organic acids.

In normal soil conditions, where inoculated cultures were grown at neutral pH, the pH of the soil increased from 6.13 to 7.62 in *K. rosea* treated soil (Table 5.7). A marked increase in organic carbon content was observed in all the SDB treatments. There was a 4-fold increase in *Bacillus sp MF-A4* treated soil. There was also an 1-2 fold increase in available N content in *K. rosea, Bacillus sp MF-A4* SDB treatment except *B. subtilis* and *M. arborescens* treated soils. The phosphate content of the soil increased drastically in *B. subtilis* (34 to 49 mg phosphate per kg of soil) treated soils.

soil pH which plays a major role in solubilization of some nutrients (Canbolat *et al*, 2006). The competitiveness of a P solubilizing microorganisms in natural environment will depend upon its stability to survive and multiply. In general, the population sizes of the introduced microbe decline rapidly upon the introduction in soils. The survival of the inoculant strain depends upon various actors such as soil composition, temperature and the presence of the recombinant plasmids.

The biotic factors that affect the survival of the inoculated microbes include competition, predation and root growth that provides the substrates to the microbes. The abiotic factors include texture, pH, temperature, moisture content and substrate availability in the soils. The biotic factors play a very important role in the survival of the inoculated strains as the decline observed in non sterile soils can often be abolished in sterile soils (Gyaneshwar *et al*, 2002). Phosphate solubilizing bacilli are important as inoculants for crops. In growth chamber studies *B.circulans* and *B.megaterium var phosphaticum* inoculants increased plant weight and P-uptake of millet and pea, respectively. Gaind and Gaur, (1991) reported that a *B.subtilis* inoculant increased biomass, grain yield and P and N–uptake of mung bean grown in a P-deficient field soil amended with rock phosphate.

Table 5.7 Change in soil properties due to treatments with SDB grown at neutral pH

Treatments	pH		Carbon content %		Available nitrogen %		PO4-P(mg/ kg soil)	
	Day 1	46 DAS	Day 1	46 DAS	Day 1	46 DAS	Day 1	46 DAS
B. subtilis	6.02	6.49	0.014	0.013	0.0035	0.0035	34	49
M. arborescens	6.32	6.54	0.014	0.023	0.0045	0.0035	30	36.5
K. rosea	6.13	7.62	0.015	0.018	0.0035	0.0055	32	37
Bacillus sp MF-A4.	6.16	7.06	0.016	0.03	0.003	0.005	29	47
Consortium	6.55	7.44	0.009	0.015	0.006	0.002	31	50.5

Among the four SDB, *B. subtilis* increased C, available N content significantly, and increased P availability significantly which could be the reason for increased growth by *B. subtilis*. *Kocuria rosea* was also found to increase plant growth significantly. It is reported that growth enhancement by *Bacillus* may be associated to its ability to produce hormones, especially IAA (Sheng and Yu, 2005), and siderophore (Hu and Boyer 1996). There is also report of the presence of plant growth-promoting *Bacillus* strains in the root nodules of soybean plants (Yu Ming *et al*, 2002). *Bacillus* species used as biofertilizers may have a direct effect on plant growth through the synthesis of plant growth hormones, N_2-fixation and synthesis of the enzymes modulating the level of plant growth promoting rhizobacteria(Fikretin *et al.,* 2004). *K. rosea, M. arborescens* and *Bacillus sp. MF-A4* isolates also increased C, available N and P content which manifested in increased plant growth. The consortium of the SDB did not increase the growth significantly compared to individual application and it was identified that some degree of antagonism exists among them when tested under *in vitro* conditions. Hence consortium application using the above cultures may not be recommended.

In this study among the four sand dune bacterial isolates, *B. subtilis*, *K. rosea* and *M.arborescens* found to be good plant growth promoters in neutral soil conditions. All the four sand dune bacterial cultures were found to have ACC deaminase activity and other attributes like IAA, 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 contents in plants. Also ACC

deaminase 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 ACC deaminase 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.

Summary

This research work contributes significantly to our knowledge of the wide occurrence of effective PGPR bacteria associated with sand dune vegetation in the ecosystem. A large number of bacteria are associated with rhizosphere and as endophytes with vegetation growing on coastal sand dunes. Such organisms are envisaged to play a role in promoting growth of plants by making the soils available with nutrients. It was interesting to understand this significance of the isolates obtained through enzymatic activities shown largely by neutrophilic and alkalophiles associated with the sand dune vegetation, *Ipomoea pes-caprae* and *Spinifex littoreus*. The distribution of activities among the different genera reflected that the most predominant isolates belonged to *Bacillus, Microbacterium, Brochothrix, Cellulomonas* and *Brevibacterium* genera. Among the isolates four highly promising isolates were selected for further studies on their plant growth promoting traits and field studies.

This study has also revealed the production of two exopolymers from *Microbacterium arborescens* in the presence of sucrose and glycine. These are composed of mannose as the maximum monosaccharide and uronic acids. These polymers showed emulsifying activity and chelation of the metal, copper. Further they were also found to be aggregating sand particles which shows their potential to improve the moisture holding capacity of sand and indirectly support the plant growth.

Further plant growth promoting characteristics of four promising isolates was studied. Native plant growth promoting microorganisms with properties such as phosphate solubilization, disease control potential, rhizosphere colonization would seem ideal for selection as a suitable bioinoculant. The cultures were found to produce siderophores, solubilize inorganic phosphates, ammonia, hydrogen cyanide

and indole-3-acetic acid. All these metabolites are important for plant growth promotion. They were all found to utilize ACC as a sole source of nitrogen further confirming the presence of ACC deaminase enzyme.

Among the four sand dune bacterial isolates, *B. subtilis* , *K. rosea and M. arborescens* were found to have an significant effect on plant growth promotion of eggplant, a agricultural important crop, in neutral soil conditions. Plant growth promoting sand dune rhizobacteria therefore present an alternative to the use of chemicals for plant growth enhancement in many different applications. Our research has demonstrated that sand dune rhizobacteria could have an important role in agriculture and horticulture in improving crop productivity.

Appendices

Appendix – A

Media composition

A.1. Nutrient Agar

Ingredients	g/L
Peptone	1g
NaCl	0.5 g
Meat Extract	0.3 g
Agar	2.0 g
Distilled water	100 ml
PH	7.4

A.2. Polypeptone Yeast Extract Glucose Agar (PPYG) (Gee *et al* 1980; Horikoshi, 1991)

Ingredients	g/L
Peptone	5
Yeast extract	1.5
Glucose	5
$Na_2HPO_4.12H_2O$	1.5
NaCl	1.5
$MgCl_2.6H_2O$	0.1
Na_2CO_3	5.0
Agar	15
Final pH	10.5

Solutions of glucose and Na_2CO_3 were sterilised separately as 10% solutions and then added to the cold basal molten medium to avoid precipitation of salts.

A.3. Media used for enzyme activities for neutrophiles

Ingredients g/L	Protease	Amylase	Cellulase	Lipase	Tannase	Chitinase
Soluble starch	-	10	-	-	-	-
CMC	-	-	10	-	-	-
Tributyrin	-	-	-	10	-	-
Skimmed-milk	20	-	-	-	-	-
Tannic acid	-	5	5	5	1 %	1%
Yeast extract	-	5	5	5	-	-
KH_2PO_4	1	1	1	1	-	-
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2	-	-
Beef extract	-	-	-	-	3	3
peptone	-	-	-	-	10	10
NaCl	-	-	-	-	5	5
Agar	20	20	20	20	20	20
d/w	1L	1L	1L	1L	1L	1L
Final pH	7.2	7.2	7.2	7.2	7.2	7.2

Starch hydrolysis was determined by flooding the plates with Iodine solution (1% I_2+1.5% KI). Xylan and cellulose hydrolysis was determined by flooding the plates with 1 %(w/v) aqueous Congo red and destained with 1M NaCl(Clarke et al,1991) and 0.1 % congo red solution for 15 mins and then rinsed with 1M NaCl (Teather and wood, 1982) .

A.4. Basal medium for Haloalkaliphiles PPYG :

Ingredients	g/L
Polypeptone	5
Tryptone	1
Tri sodium citrate	0.3
KH_2PO_4	13.7
K_2HPO_4	0.05
$MgSO_4$	-
KCl	-
Na_2CO_3	5.0
NaCl	25
Agar	20
Final pH	10.5

25% crude salt was added to the above medium

A.5. Media used for enzyme activities for alkaliphiles (Horikoshi 1991)

Ingredients g/L	Protease	Amylase	Cellulase	Xylanase	Lipase
Soluble starch	-	10	-	-	-
CMC	-	-	10	-	-
Xylan				5	
Tributyrin	-	-	-	-	10
Skimmed milk	20	-	-	-	-
Polypeptone	-	5	5	10g	5
Yeast extract	-	5	5	5g	5
KH_2PO_4	1	1	1	1g	1
$MgSO_4.7H_2O$	0.2	0.2	0.2	0.2g	0.2
Na_2CO_3	10	10	10	10g	10
Agar	20	20	20	20g	20
d/w	1L	1L	1L	1L	1L
Final pH	10.5	10.5	10.5	10.3	10.5

10% Na_2CO_3 and 10% carbon source sterilized separately and added just before pouring the medium in plates.

A.6. Basal medium for Halophiles NTYE (NaCl- Tryptone Yeast Extract Agar) (Kushner, 1985)

Ingredients	g/L
$MgSO_4.7H_2O$	20
KCl	5
$CaCl_2.2H_2O$	0.2
Yeast extract	3
Tryptone	5
Crude salt	250
D/w	1L

pH was adjusted to 7 using 1M NaOH.

A.7. Pikovskaya (PVK) medium (Pikovskaya, 1948)

Ingredients	Neutrophiles	Alkaliphiles
	g/L	
Glucose	10	10
$Ca_3(PO_4)_2$	5	5
$(NH_4)_2SO_4$	0.5	0.5
NaCl	0.2	0.2
$MgSO_4$	0.1	0.1
KCl	0.2	0.2
Yeast extract	0.6	0.6
$MnSO_4$	0.002	0.002
$FeSO_4$	0.002	0.002
Agar	20	20
Bromophenol blue	0.4 %	-
Phenol red	0.4%	10ml
Na_2CO_3	-	1ml
D/w	1L	1L
Final pH	7	8

A.8. Mineral medium (MM) (Double Strength)

a. $FeSO_4$. $7H_2O$	60 mg, Dissolved in 250ml d/w
b. Stock solutions added as follows:	
K_2HPO_4 (12.6%)	50 ml
KH_2PO_4 (18.20%)	10 ml
NH_4NO_3 (10%)	10 ml
MgSO4 (1%)	10 ml
$Na_2MoO_4.2H_2O$ (0.6%)	0.1 ml
$MnSO_4.H_2O$ (0.6%)	0.1 ml
$CaCl_2.2H_2O$ (0.1%)	7.5 ml

Add $CaCl_2$ drop by drop and stir make volume to 500ml with d/w. Store the medium in amber coloured bottle. Sterilize the medium in pressure cooker for 10 minutes. Adjust pH of the medium to 10.5 using 10% Na_2CO_3 solution, sterilized separately.

A. 9. Chrome azurol Agar for detection of siderophores (Vellore, 2001)
Preparation of Chrome Azurol S Assay Medium

Preparation of CAS Indicator Solution
60.5 mg of chrome azurol S was dissolved in 50 ml of ddH2O. 10ml of Fe III solution (27 mg FeCl3·6H2O and 83.3 µL concentrated HCl in 100 mlddH2O) was added, along with 72.9 mg hexadecyltrimethyl ammonium bromide (HDTMA) dissolved in 40 ml ddH2O. The HDTMA solution was added slowlywhile stirring, resulting in a dark blue solution (100 ml total volume), which wasthen autoclaved.

Preparation of CAS Agar Plates

Peptone	1g
NaCl	0.5 g
Meat Extract	0.3 g
CAS Indicator Solution(separately sterilized)	10 ml
Agar	2.0 g
Distilled water	90 ml
pH	7

A.10. Fiss Glucose Minimal medium (Vellore, 2001)

KH_2PO_4 + L-asparagine	96 ml
50 % Glucose solution	1ml
0.005 % Zinc chloride solution	1ml
0.001 % Manganese chloride solution	1ml
0.4 % Magnesium sulfate solution	1ml
pH	6.8

A.11. Nitrogen Free Mannitol Agar

Ingredients	g/L
Mannitol	10 g
K_2HPO_4	0.5 g
$MgSO_4$	0.2 g
NaCl	0.2 g
$MnSO_4.4H_20$	0.005 g
Agar	25 g
$FeCl_2$	0.005 g
$CaCO_3$(sterilize separately)	5 g
Distilled water	1000 ml

A.12. E2 mineral medium for PHB producers (Rawte, 2001)

Ingredients	g/L
$NaNH_2HPO_4.4H_2O$	3.5 g
$K_2HPO_4.3H_2O$	7.5 g
KH_2PO_4	3.7 g
$MgSO_4.7H_2O$(100mM)	10 ml
MT microelement stock	1 ml
Agar	1.5 %
Distilled water	890 ml
Glucose (Sterilized)	1 %
PH	7

MT Microelement stock

$FeSO_4.7H_2O$	2.78 g
$MnCl_2.4H_2O$	1.98 g
$COSO_4.7H_2O$	2.81 g
$CaCl_2.2H_2O$	1.47 g
$CuCl_2.2H_2O$	0.17 g
$ZnSO_4.7H_2O$	0.29 g
Distilled water	1000 ml

A.13 Dworkin and Fosters minimal salt medium

Ingredients	g/L
KH_2PO_4	4g
$NaHPO_4$	6g
$MgSO_4.7H_2O$	0.2g
$FeSO_4.7H_2O$	0.001g
Glucose	2g
Gluconic acid	2g
Citric acid	2g
Distilled water	1000 ml
Micronutrients	0.1ml/L
ACC	3 mM

Micronutrient stock solution

H_3BO_3	10mg
$MnSO_4$	11.2mg
$ZnSO_4$	124.6mg
$CuSO_4$	78.2mg
MoO_3	78.2mg
Distilled water	1000 ml

A.14 Kings B medium (KB)

Ingredients	**g/L**
Peptone	20g
K_2HPO_4	1.5g
$MgSO_4$	1.5g
Glycerol	10ml
Agar	18g
Distilled water	1000ml
pH	7.2

Appendix - B

Composition of stains, buffers and reagents

B.1. Stains

(i) Gram stain reagents

(a) Crystal violet

Solution A - 2g of crystal violet dissolved on 20ml ethanol.

Solution B - 0.8g ammonium oxalate dissolved in 80ml d/w.

Mixed solution A and B and filtered through Whatman paper No. 1

(b) Gram's iodine

Dissolved 1g iodine and 2g potassium iodide in 300 ml d/w. Filtered through Whatman filter paper No. 1 (diameter = 12.5 cm).

(c) Safranine

2.5 g Safranine was dissolved in 10 ml ethanol made the volume to 100ml with d/w and filtered through Whatman filter paper No. 1.

Procedure

Prepared smear of the organism on a slide and heat fixed it. Flood the smear with crystal violet for a min. Washed with tap water and flooded with gram's iodine for a min. Washed with tap water and decolorized with 60% ethanol prepared in d/w. Counter stained with safranine for 45 seconds. Washed with tap water, blot dried with tissue paper and examined under oil immersion.

(ii) Endospore staining (Schaeffer and Fulton's method)

(a) Malachite green solution:

Dissolved 5g of malachite green in 100ml d/w. Filtered through Whatman filter paper no.1.

(b) Safranine:

Dissolved 1g of Safranine in 100ml d/w. Filtered through Whatman filter paper no.1.

Procedure:

Prepared smear of the organism and heat fixed it. Flooded the smear with 5% malachite green solution. Hold the slide over a boiling water bath for 15 min to allow the penetration of the stain through calcium dipicolinate of spores. Washed with tap water and counterstained with safranine for 2min, washed with tap water and blot dried with tissue paper and examined under oil immersion objective. Endospores appeared green with cells colored red.

iii) Nile Blue A stain for visualizing fluorescense in PHB producers

Nile blue A 0.05

Ethanol 100ml

B. 2. Buffers

1. Phosphate buffer (0.05M)

(a) Solution A : (0.05M monobasic hydrogen phosphate): 6.0g of NaH_2PO_4 dissolved in 1000ml d/w.

(b) Solution B : (0.05M dibasic hydrogen phosphate): 7.1g of NaH_2PO_4 dissolved in 1000ml d/w.

X ml of A + **Y** ml of B mixed to obtain buffers of the desired pH

X	**Y**	**pH**
87.7	12.3	6
39	61	7
5.3	94.7	8
2	98	9.5

2. Phosphate buffered saline (PBS)

X ml	**1X**
NaCl	8 g
KCl	0.2 g
$Na_2HPO_4.2H_20$	2.9 g
KH2PO4	0.2 g
Distilled water	1L

3. Tris-HCl buffer (0.05M)

(a) **Solution A :** (0.05M Tris): 6.0g of Tris dissolved in 1000ml d/w.

(b) **Solution B :** (0.05M HCl): 4.4ml of 11.35 N HCl added to 1000ml d/w.

70ml of A and 30ml of B mixed to obtain buffer of pH 9.0

4.Tris-HCl buffer (0.1M)

Add Xml of 0.2 mol/lit HCl to 50 ml of 0.2 mol/lit Tris-base and make volume upto 100ml.

X ml	pH
43	7.2
24	8.2
6	9

Add 6ml of 0.2 mol/lit HCl to 50ml of 0.2 mol/lit Tris and make volume upto 100 ml.

5. **Carbonate-bicarbonate buffer (0.2 M)**

(a) **Solution A :** (0.2 M anhydrous sodium carbonate): 21.2g of anhydrous sodium carbonate dissolved in 1000ml d/w

(b) **Solution B :** (0.2 M sodium bicarbonate): 16.8g of sodium bicarbonate dissolved in 1000ml d/w

27.5ml of A + 22.5ml of B, diluted to a total volume of 200ml with d/w to obtain buffer of pH 10.0

Carbonate-bicarbonate buffer (pH10) was sterilized by autoclaving for serial dilution technique.

B.3. Biochemical test reagents

a. Kovac's reagent

Isoamyl alcohol	150ml
p-dimethyl aminobenzaldehyde	10g
Concentrated HCl	50ml

b. Methy Red indicator

Methyl Red	0.1 g
Ethanol	300ml
D/w	200ml

c. Omeara's reagent

KOH	40g
Creatine	0.3g
D/w	100ml

e. Reagents for nitrate reduction :

(a) Solution A (Sulfanilic acid) :

Sulfanilic acid	8g
Acetic acid (5N)	One part of glacial acetic acid added to 2.5 parts of d/w

(b) Solution B (α-naphthylamine)

α-naphthylamine	5g
Acetic acid (5N)	1L

Before use mix equal volumes of solutions A & B to give test reagent.

B.4 Hathways reagent

0.1M $FeCl_3$ in 0.1N HCl	1ml
Distilled water	100ml
0.1M Potassium ferricyanide	1ml

B.5 Picric acid solution

Picric acid	2.5g
Na_2CO_3	12.5g
Distilled water	1L

B. 6 Salkowsky's reagent

0.5M $FeCl_3$	40g
35% $HClO_4$	50%

Appendix – C

Biochemical media composition and identification charts of the organisms

C.1. Biochemical media used for identification of alkaliphiles (Gee *et al* 1980 and Sneath *et al* 1986)

(a) Carbohydrate fermentation

Peptone	5.0g
Beef extract	3.0g
*Sugar	0.5g
D/w	To make 1L
O-Cresol red	0.01g
pH	9.5 adjusted by using 10% Na_2CO_3 solution

* arabinose, glucose, mannitol and xylose (10 % stock)

Autoclaved at 15 psi for 20 minutes. Tubes inoculated and incubated at R.T. for 24-48h. Change in color and presence or absence of gas bubble was noted. Uninoculated tubes serve as the control.

(b) Nitrate reduction test :

Peptone	5g
Beef extract	3g
KNO_3	1g
Sodium carbonate	10g
D/w	Make volume to 1L
pH	10.3

Nitrate broth is inoculated and incubated at R.T. for 24-48h. After incubation, 5 drops of sulfanilic acid and 5 drops of α-naphthylamine were added. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5mg of zinc. Uninoculated tubes served as the control.

(c) Citrate utilization test :

Simmons citrate agar

Ammonium dihydrogen phosphate	1g
Diammonium phosphate	1g
Sodium chloride	5g
Magnesium sulphate	2g
Sodium carbonate	10g
Agar	20g
D/w	Make volume to 1L
pH	10.3

Inoculate Simmon's citrate agar slants by means of stab inoculation and incubate for 24-48h at R.T. Observe the slants for presence or absence of growth. Citrate utilizers were indicated by the presence of growth on the slant.

(d) Catalase test :

Three or four drops of 3% (v/v) hydrogen peroxide were mixed with a loopful of culture in a plate. Evolution of gas bubbles caused by liberation of free oxygen was indicative of catalase positive organisms.

(e) Oxidase test :

A filter paper strip was soaked in Tetramethylparaphenylenediamine (TMPD) dye. A loopful of fresh bacterial culture was smeared on the moist filter paper. Production of a deep purple colour in 5-10 seconds indicated a positive oxidase test.

(f) Gelatin liquefaction :

Peptone	5g
Beef extract	3g
Gelatin	120g
d/w	To make 1L
Sodium carbonate	10g
pH	10.5

Inoculate tubes and incubate at R.T. for 24-48 h, the tubes were refrigerated for 30 min and the medium were observed. Liquid medium after refrigeration showed a positive test.

(g) Starch hydrolysis:

Starch agar medium

Peptone	5g
Beef extract	3g
Soluble starch	2g
Agar	20g
D/w	Make volume to 1L
Sodium carbonate	10g
pH	10.5

Inoculate starch agar plates by spot inoculation. Incubate the plates at R.T. for 24-48 h, then flood the plates with Gram's iodine for 1 min and pour off the excess stain. Clear zone surrounding the colony indicated a positive test.

(h) Casein Hydrolysis :

Inoculate milk agar plates and incubate at R.T. for 24-48 h, then examine the plates for the presence or absence of a clear area around the colony. A clear area around the bacterial colony indicates a positive proteolytic activity.

(i) Hugh and Leifson's test :

Peptone	2g
NaCl	5g
K_2HPO_4	0.3g
Glucose	10g
O-cresol red	0.01g
Sodium carbonate	10g
D/w	Make volume to 1L
pH	10.3

Heat in boiling water bath and cool immediately. Inoculate young culture in the medium and dispense into two tubes. The medium of one tube was overlayered with sterile liquid paraffin. Growth and color change of the indicator dye was noted in the two tubes. Strict aerobes grow only in aerobic conditions. Facultative anaerobes grow in both aerobic and anaerobic conditions. The anaerobic organisms grow only in anaerobic conditions.

C.2. Biochemical media used for identification of Neutrophiles

a. Sugar fermentation media

Peptone	10 g
NaCl	5 g
Distilled water	1L
Phenol Red	50 ml of 0.2 %

Glucose	(10% solution)
Fructose	(10% solution)
Sucrose	(10% solution)
Mannitol	(10% solution)
Mannose	(10% solution)
Arabinose	(10% solution)
Rhamnose	(10% solution)
Xylose	(10% solution)
Lactose	(10% solution)

Dispense 4.5 mls of the medium with inverted durhams tube and add 0.5 mls of each 10 % sugar after sterilization to give the final concentration of 1 %.

b. Methyl Red test & Voges-Proskauer test

Glucose phosphate peptone medium

Peptone	5 g
K_2HPO_4	5 g
Distilled water	1000 ml
pH	7.6
Glucose	50 ml

Dispense in 5 ml amounts in test tubes and sterilize at 121oC, 15 mins. Add 0.25 ml of separately sterilzed glucose to each tube (final conc 0.5%)

c. Tryptone water

Tryptone	1 g
NaCl	0.5 g
Distilled water	100 ml
pH	7.2

d. Normal Saline

NaCl	0.85 g
Distilled water	100 ml

e. Hugh Leifson's medium

Peptone	2 g
NaCl	5 g
K_2HPO_4	0.3 g
Bromothymol Blue (1% aqueous)	3 ml
Agar	4 g
Glucose	10 %
Distilled water	1000 ml
pH	7.1

Dispense 0.5 ml in small test tubes and sterilize. sterilize 10 % glucose separately. Sterilize paraffin oil separately in McCartney bottle and heat I the oven. 1% Bromothymol blue grind with mortar and pestle and dissolve in distilled water.

f. Nitrate reduction test

Peptone	5 g
KNO_3	19 g
Beef extract	3g
NaCl	5g
Distilled water	1L
pH	7.2

g. Indole test

Tryptone	10 g
Nacl	5 g
Distilled water	1L
pH	7.4

h. Simmon's Citrate agar

NaCl	5 g
$NH_4H_2PO_4$	1 g
$MgSO_4.7H_2O$	0.2 g
KH_2PO_4	1 g
Sodium citrate	5 g
Agar	20 g
Distilled water	1L
Bromothymol Blue,0.2%	40 ml
pH	6.8

i. Gelatin liquefaction
Nutrient broth 1L
Gelatin 120g

j. Oxidase reagent
1 % (w/v) aqueous solution of tetra methyl para phenylene diamine solution, kept in a dark bottle.

k. Catalase reagent
10 % H_2O_2

l. Phenylalanine deaminase

Yeast extract	3g
DL-phenylalanine	2g
Disodium hydrogen phosphate	1g
Sodium chloride	5g
Agar	20g
Distilled water	1L
pH	7.3

m. Tyrosinase medium

L- Tyrosine	0.5g
Nutreint agar	90 ml
Distilled water	100 ml
pH	7.3

C.3 Lipid extraction and analysis:

400 mg wet weight of cells were stirred for 1 min in 27ml f 0.3% aqueous methanol-NaCl solution(10ml 0.3% w/v aqueous NaCl in 100ml CH_3OH)+14ml of petroleum ether. The mixture after shaking for 20 min was transferred into a separating funnel to obtain two layers. The upper layer was collected and marked as I. To the lower layer was added 14ml petroleum ether and separated into 2 layers again. Top layer was collected and marked as II. Fractions I and II were pooled, dried by evaporation of petroleum ether and weighed as non polar lipids.

The lower layer was kept in boiling water bath for 5 min and 30ml of $CHCl_3$-CH_3OH-NaCl in the ratio of 90:100:30 was added. After shaking for 1h, mixture was centrifuged and the supernatant was collected.10ml of $CHCl_3$-CH_3OH-NaCl(50:100:40) was added to the residue and kept on shaker for half hr, centrifuged and supernatants were pooled which contained polar lipids. Polar lipids were washed with a mixture of 18ml $CHCl_3$+18ml NaCl(0.3%) and after shaking, the layers were separated in a funnel. Lower layer containing purified polar lipids was collected and evaporated by a stream of oxygen free nitrogen.

The dried preparation of polar and non-polar lipids were redissolved in small amount of $CHCl_3$-CH_3OH (2:1v/v).Material was spotted on TLC and developed in a solvent system, acetone:water:benzene (91:30:8) and visualized using UV/I_2 chamber.

C.4 Identification tables of the neutrophiles and alkaliphiles

Table1 Identification charts of gram positive sporulating rods

Culture	Neutrophile	Neutrophile	Neutrophile	Neutrophile	Neutrophile
Sampling	Monsoon	Monsoon	Monsoon	Monsoon	Monsoon
Isolate No.	AMIAT 16	AMSAT 7	AMSAT 18	AMSAT 21	MIAT 9
Isolated from	AM	AM	AM	AM	Miramar
Associated	Roots of	Roots of	Roots of	Roots	Roots of
with vegetation	*Ipomoea*	*Spinifex*	*Spinifex*	of *Spinifex*	*Ipomoea*
Morphology	Gram +ve rods	+ve rods	+ve rods	+ve rods	+ve rods
Pigmentation	-	+	-	-	-
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Hugh Leifson's	O/F	O/F	O/F	O/F	O/F
VP	-	-	-	-	-
Acid from					
D- Glucose	+	+	+	+	+
L - Arabinose	-	-	-	-	-
D- Xylose	-	-	-	-	+
D- Mannitol	-	-	+	+	-
Gas from	-	-	-	-	-
Glucose					
Hydrolysis of					
Casein	+	+	+	+	-
Gelatin	+	-	+	+	+
Starch	+	-	+	+	-
Utilization of					
Citrate	-	-	-	-	-
Propionate	-	-	-	-	-
Degradation of	-	-	-	-	-
Tyrosine					
NO_3-NO_2	-	-	+	+	+
Growth in NaCl					
2%	+	-	+	+	+
5%	+	-	+	+	+
7%	+	-	+	+	+
10%	+	-	+	+	-
Quinones	MK	MK	MK	MK	MK
Lipids	PG, Glycolipids	Glycolipids	PG,Glycolipids	DPG, Glycolipids	Glycolipids
Identified as	***B. pantothenticus***	***B. lentimorbus***	***B. firmus***	***B. firmus***	***B. marinus***

Table1 Identification charts of gram positive sporulating rods contd

Culture	Neutrophile	Neutrophile	Neutrophile	Neutrophile	Neutrophile
Sampling	Monsoon	Monsoon	Postmonsoon	Postmonsoon	Postmonsoon
Isolate No.	MIAT 15	MIAT 26	AMIAT 3	AMIAT 5	AMIAT 7
Isolated from	Miramar beach	Miramar	AM	AM	AM
Associated	Roots of	Roots of	Roots of	Roots of	Roots of
with vegetation	*Ipomoea*	*Ipomoea*	*Ipomoea*	*Ipomoea*	*Ipomoea*
Morphology	Gram +ve rods	+ve rods	+ve rods	+ve rods	+ve rods
Pigmentation	-	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Hugh Leifson's	O	O	O/F	O/F	O/F
VP	-	-	-	-	-
Acid from					
D- Glucose	+	+	+	+	+
L - Arabinose	-	-	+	+	+
D- Xylose	+	+	+	+	+
D- Mannitol	-	-	+	+	+
Gas from	-	-	+	+	+
Glucose					
Hydrolysis of					
Casein	+	+	-	-	-
Gelatin	+	+	+	+	+
Starch	+	+	+	+	+
Utilization of					
Citrate	+	+	+	+	+
Propionate	+	+	-	-	-
Degradation of	-	-	-	-	-
Tyrosine					
NO_3-NO_2	-	-	-	-	-
Growth in NaCl					
2%	-	-	-	-	-
5%	-	-	-	-	-
7%	-	-	-	-	-
10%	-	-	-	-	-
Quinones	MK	MK	MK	MK	MK
Lipids	Glycolipids	Glycolipids	Glycolipids	Glycolipids	Glycolipids
Identified as	***B. megaterium***	***B. megaterium***	***B.macerans***	***B.macerans***	***B. macerans***

Table1 Identification charts of gram positive sporulating rods contd.

Culture	Neutrophile	Neutrophile	Neutrophile	Neutrophile	Neutrophile
Sampling	Postmonsoon	Postmonsoon	Premonsoon	Premonsoon	Premonsoon
Isolate No.	AMIAT 3	AMIAT 7	AMIRT 4	AMIAT 10	AMSRT 8
Isolated from	AM	AM	AM	AM	AM
Associated with vegetation	Roots of *Ipomoea*	Roots of *Ipomoea*	Rhizosphere of *Ipomoea*	Roots of *Ipomoea*	Rhizosphere of *Spinifex*
Morphology	+ve rods	+ve rods	+ve rods	+ve rods	+ve rods
Pigmentation	+	-	-	+	+
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Hugh Leifson's	O/F	O/F	O/F	O/F	O
VP	-	+	+	+	+
Acid from					
D- Glucose	+	+	+	+	+
L - Arabinose	-	-	-	+	-
D- Xylose	-	-	-	+	-
D- Mannitol	+	-	-	+	-
Gas from Glucose	-	-	-	-	-
Hydrolysis of					
Casein	+	+	+	+	+
Gelatin	-	-	+	+	+
Starch	-	+	+	+	+
Utilization of					
Citrate	+	-	-	+	+
Propionate	-	-	-	-	-
Degradation of Tyrosine	+	+	-	+	+
NO_3-NO_2	+	+	-	-	+
Growth in NaCl					
2%	-	-	-	+	-
5%	-	-	-	+	-
7%	-	-	-	+	-
10%	-	-	-	+	-
Quinones	MK	MK	MK	MK	MK
Lipids	Glycolipids	Glycolipids	PG/Glycolipids	PG, Glycolipids	PG, Glycolipids
Identified as	***B.laterosporus***	***B.anthracis***	***B.alvei***	***B.subtilus***	***B.cereus***

Table1 Identification charts of gram positive sporulating rods contd.

Culture	Neutrophile	Neutrophile	Neutrophile	Neutrophile	Neutrophile	Neutrophile
Sampling	Postmonsoon	Postmonsoon	Postmonsoon	postmonsoon	Premonsoon	Premonsoon
Isolate No.	MIAT 4	MIAT 1	MIAT 2	AMIRT 1	MIRT 5	MIRT 11
Isolated from	Miramar	Miramar	Miramar	AM	Miramar	Miramar
Associated with vegetation	Roots of *Ipomoea*	Roots of *Ipomoea*	Roots of *Ipomoea*	Rhizosphere of *Ipomoea*	Rhizosphere of *Ipomoea*	Rhizosphere of *Ipomoea*
Morphology	+ve rods	+ve rods	+ve rods	+ve rods	+ve rods	+ve rods
Pigmentation	-	-	-	+	-	-
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+
Hugh Leifson's	O/F	O/F	Alkaline	O/F	O/F	O/F
VP	-	-	-	+	+	-
Acid from						
D- Glucose	+	+	+	+	+	+
L - Arabinose	-	-	-	-	+	+
D- Xylose	+	-	-	+	+	+
D- Mannitol	+	+	-	+	+	+
Gas from Glucose	-	-	-	-	+	+
Hydrolysis of						
Casein	+	+	+	+	+	-
Gelatin	-	+	+	+	+	+
Starch	-	+	-	+	+	+
Utilization of						
Citrate	-	-	-	+	-	+
Propionate	-	-	-	+	-	-
Degradation of Tyrosine	+	-	-	-	-	-
NO_3-NO_2	+	+	+	+	+	+
Growth in NaCl						
2%	-	+	+	+	-	-
5%	-	+	-	+	-	-
7%	-	+	-	+	-	-
10%	-	+	-	+	-	-
Quinones	MK	MK	MK	MK	MK	MK
Lipids	PG, Glycolipids	PG, Glycolipids	PG, Glycolipids	PG, Glycolipids	PG, Glycolipids	PG, Glycolipids
Identified as	***B. laterosporus***	***B. firmus***	***B. globisporus***	***B. licheniformus***	***B. polymxa***	***B. macerans***

Table2 Identification charts of gram positive cocci

Culture	Neutrophile	Neutrophile	Neutrophile	Neutrophile
Sampling period	Monsoon	Monsoon	Monsoon	Postmonsoon
Isolate no.	MIAT 1	MIAT 13	MIAT 25	AMIAT 2
Isolated from	Miramar	Miramar	Miramar	Aswem Mandrem
Associated with	Roots of *Ipomoea*	Roots of *Ipomoea*	Roots of *Ipomoea*	Roots of *Ipomoea*
vegetation				
Morphology	Gram +ve cocci	Gram +ve cocci	Gram +ve cocci	Gram +ve cocci
Pigmentation	+	+	+	-
Catalase	+	+	+	+
Oxidase	-	-	-	-
Hugh Leifson's	O	O/F	O/F	O/F
VP	-	-	-	-
Acid from				
Glucose	+	+	+	+
Maltose	+	+	+	+
Glycerol	-	+	-	-
Galactose	-	-	-	-
Sucrose	-	+	+	+
Lactose	-	-	+	-
Mannose	-	+	+	+
Hydrolysis of				
Gelatin	+	-	-	+
Starch	-	-	-	+
NO_3-NO_2	-	+	-	+
CW amino acids	DAP	Lys, Ala	Lys, Ala	Lys, Ala
Quinones	MK	MK	MK-6, MK-8	MK-6, MK-8
Identified as	***Planococcus sp***	***Stomatococcus sp***	***Staphylococcus sp***	***Staphylococcus sp***

Culture	Neutrophile	Alkalophile	Alkalophile
Sampling period	Monsoon	Monsoon	Monsoon
Isolate no.	MIAT 7	MIAA 3	AMIAA 6
Isolated from	Miramar	Miramar	Aswem Mandrem
Associated with	Roots of *Ipomoea*	Roots of *Ipomoea*	Roots of *Ipomoea*
vegetation			
Morphology	Gram +ve cocci	Gram +ve cocci	Gram +ve cocci
Pigmentation	+	+	+
Catalase	+	+	-
Oxidase	-	-	-
Hugh Leifson's	O/F	O	O/F
VP	-	-	-
Acid from			
Glucose	+	+	-
Maltose	+	-	-
Glycerol	-	-	-
Galactose	-	-	-
Sucrose	+	-	-
Lactose	-	-	-
Mannose	-	-	-
Hydrolysis of			
Gelatin	+	+	-
Starch	-		
NO_3-NO_2	+	+	+
CW amino acids	Lys, Ala	Ala	Lys, Ala
Quinones	MK	MK	MK-6, MK-8
Identified as	***Stomatococcus sp***	***Micrococcus sp***	***Staphylococcus sp***

Table 3 Identification charts of gram positive non sporulating rods

Culture	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile
Sampling period	Premonsoon	Premonsoon	Premonsoon	Premonsoon	Premonsoon
Isolate no.	MIAA 9	MIAA12	MIRA 2	MIRA 4	MIRA 15
Isolated from	Miramar	Miramar	Miramar	Miramar	Miramar
Associated with	Roots of	Roots	Rhizosphere	Rhizosphere	Rhizosphere
vegetation	Ipomoea	of Ipomoea	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*
Morphology	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods
Pigmentation	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	+	-	-	-
Hugh Leifson's	O/F	O/F	O/F	O/F	O
Methyl red	-	-	-	-	-
VP	-	-	-	-	-
Utilization of	+	+	+	+	+
citrate					
Acid from					
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	-	-	-	-	-
Sucrose	+	+	+	+	+
Arabinose	-	-	-	-	-
Xylose	+	+	+	+	+
Lactose	+	+	-	+	+
Galactose	+	+	+	+	-
NO_3-NO_2	+	+	+	+	+
CW sugars	Arab	Arab	Arab,Gal	Arab	Gal,Mann,Rham
CW amino acids	Orn	Orn	DAP	Orn	Lys
Quinones	MK - 9	MK - 9	MK -9,MK- 11	MK - 9	Mk-11,MK-10
Lipids	PE	PE	PE	PE	PG, Glycolipid
Identified as	***Cellulomonas sp***	***Cellulomonas sp***	***Corynebact. sp***	***Cellulomonas sp***	***Microbact. sp***

Culture	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile
Sampling period	Premonsoon	Premonsoon	Premonsoon	Premonsoon	Premonsoon	Premonsoon
Isolate no.	MIRA 25	MIRA 27	MIRA 26	AMIRA 1	AMIRA 6	AMIRA8
Isolated from	Miramar	Miramar	Miramar	Aswem Mandrem	Aswem Mandrem	Aswem Mandrem
Associated with	Rhizosphere	Rhizosphere	Rhizosphere	Roots of	Rhizosphere	Rhizosphere
vegetation	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*
Morphology	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods
Pigmentation	-	+	+	-	+	-
Catalase	+	+	+	+	+	+
Oxidase	+	-	+	+	+	+
Hugh Leifson's	O/F	O	O/F	O/F	O/F	O/F
Methyl red	+	-	-	-	-	-
VP	+	-	-	-	-	-
Utilization of	-	+	+	+	+	+
citrate						
Acid from						
Glucose	+	-	-	-	-	-
Maltose	+	-	-	-	-	-
Mannitol	+	-	-	-	-	-
Sucrose	+	-	-	-	-	-
Arabinose	-	-	-	-	-	-
Xylose	+	-	-	-	-	-
Lactose	+	-	-	-	-	-
Galactose	-	-	-	-	-	-
NO_3-NO_2	-	+	+	+	+	+
CW sugars	Arab	ND	ND	ND	ND	ND
CW amino acids	DAP	DAP	DAP	DAP	DAP	DAP
Quinones	MK -7	MK - 8, MK - 7	MK - 8, MK - 7	MK - 8, MK - 7	MK - 8, MK - 7	MK - 8, MK - 7
Lipids	PE	PE	PE	PE	PE	PE
Identified as	***Brochothrix***	***Brevibact. Sp***	***Brevibact. sp***	***Brevibact. sp***	***Brevibact.sp***	***Brevibact. sp***

Table 3 Identification charts of gram positive non sporulating rods contd.

Culture	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile
Sampling period	Premonsoon	Postmonsoon	Postmonsoon	Postmonsoon	Postmonsoon	Postmonsoon
Isolate no.	AMSRA 6	MIAA 1	MIRA 3	AMIRA 2	AMIRA 5	AMIRA 6
Isolated from	Aswem Mandrem	Miramar	Miramar	Aswem Mandrem	Aswem Mandrem	Aswem Mandrem
Associated with	Rhizosphere	Roots of	Rhizosphere	Rhizosphere	Rhizosphere	Rhizosphere
vegetation	of *Spinifex*	Ipomoea	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*	of *Ipomoea*
Morphology	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods
Pigmentation	+	+	+	+	-	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	-	+	+
Hugh Leifson's	O/F	O/F	O	O/F	O/F	O/F
Methyl red	-	-	-	-	+	+
VP	-	-	-	-	+	+
Utilization of	+	+	+	-	-	-
citrate						
Acid from						
Glucose	-	+	+	+	+	+
Maltose	-	-	+	-	+	+
Mannitol	-	+	-	-	+	+
Sucrose	-	+	+	+	+	+
Arabinose	-	+	-	+	-	-
Xylose	-	+	+	+	+	+
Lactose	-	+	+	-	-	-
Galactose	-	+	-	-	-	-
NO_3-NO_2	+	-	+	-		-
CW sugars	ND	ND	Gal,Mann,Rham	ND	ND	ND
CW amino acids	DAP	Lys	Lys	DAP	DAP	DAP
Quinones	MK - 8, MK - 7	MK	MK -12, MK - 10	MK	MK -7	MK -7
Lipids	PE	PE	PE	PE	PE	PE
Identified as	***Brevibact. sp***	***Unidentified***	***Microbact. sp***	**Unidentified**	***Brochothrix sp***	***Brochothrix sp***

Culture	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile	Alkalophile
Sampling period	Postmonsoon	Postmonsoon	Monsoon	Monsoon	Monsoon	Monsoon
Isolate no.	AMIAA 1	AMSRA 2	MIRA 4	MIAA 15	AMIRA 1	AMIAA 8
Isolated from	Aswem Mandrem	Aswem Mandrem	Miramar	Miramar	Aswem Mandrem	Aswem Mandrem
Associated with	Roots of	Roots of *Spinifex*	Rhizosphere	Roots of	Roots of	Roots of
vegetation	Ipomoea		of *Ipomoea*	Ipomoea	Ipomoea	Ipomoea
Morphology	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods	Gram +ve rods
Pigmentation	+	-	+	-	+	+
Catalase	+	+	+	+	+	+
Oxidase	+	+	+	-	+	+
Hugh Leifson's	O	O/F	O	O/F	O/F	O/F
Methyl red		+	-	+	-	-
VP		+	-	+	-	-
Utilization of		-	+	-	+	+
citrate						
Acid from						
Glucose	-	+	+	+	+	+
Maltose	-	+	+	+	+	+
Mannitol	-	+	-	+	-	-
Sucrose	-	+	+	+	+	+
Arabinose	-	-	-	-	-	-
Xylose	-	+	+	+	+	+
Lactose	-	-	+	-	+	+
Galactose	-	-	-	-	+	-
NO_3-NO_2		-	+	-	+	+
CW sugars	Gal, Rham	ND	Gal,Mann	ND	ND	Gal,Mann
CW amino acids	Lysine	DAP	Lys	DAP	Orn	Lys
Quinones	MK - 9	MK -7	MK- 12,MK-10	MK -7	MK - 9	MK- 12,MK-10
Lipids	DPG	PE	PG,DPG	PE	PE	PG,DPG
Identified as	***Renibacterium***	***Brochothrix sp***	***Microbact. sp***	***Brochothrix sp***	***Cellulomonas sp***	***Microbact. sp***

Table 3 Identification charts of gram positive non sporulating rods contd

Culture	Alkalophile	Alkalophile	Alkalophile
Sampling period	Monsoon	Monsoon	Premonsoon
Isolate no.	AMSRA 1	AMSAA 1	MIAA 2
Isolated from	Aswem Mandrem	Aswem Mandrem	Miramar
Associated with vegetation	Roots of *Spinifex*	Roots of *Spinifex*	Roots of *Spinifex*
Morphology	Gram +ve rods	Gram +ve rods	Gram +ve rods
Pigmentation	+	+	+
Catalase	+	+	+
Oxidase	+	+	+
Hugh Leifson's	O/F	O/F	O/F
Methyl red	+	-	+
VP	+	-	+
Utilization of citrate	-	+	-
Acid from			
Glucose	+	+	+
Maltose	+	+	+
Mannitol	+	-	+
Sucrose	+	+	+
Arabinose	-	-	-
Xylose	+	+	+
Lactose	-	+	-
Galactose	-	+	-
NO_3-NO_2	-	+	-
CW sugars	ND	ND	ND
CW amino acids	DAP	Orn	DAP
Quinones	MK -7	MK - 9	MK -7
Lipids	PE	PE	PE
Identified as	***Brochothrix sp***	***Cellulomonas sp***	***Brochothrix sp***

Table 4 Identification charts of gram negative rods

Culture	Alkalophile	Alkalophile	Alkalophile	Neutrophiles	Neutrophiles	Neutrophiles
Sampling period	Postmonsoon	Monsoon	Premonsoon	Premonsoon	Postmonsoon	Premonsoon
Isolate no.	MIRA 9	MIAA 8	AMIRA 3	AMIAT 8	AMSAT 3	MIAT 3
Isolated from	Miramar	Miramar	AM	AM	AM	Miramar
Associated with vegetation	Rhizosphere of *Ipomoea*	Roots of *Ipomoea*	Rhizosphere of *Ipomoea*	Roots of *Ipomoea*	Roots of *Spinifex*	Roots of *Ipomoea*
Morphology	Gram -ve rods	Gram -ve rods	Gram -ve rods	Gram -ve rods	Gram -ve rods	Gram -ve rods
Pigmentation	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Oxidase	-	-	-	+	+	+
Hugh Leifson's	O	O	O	O	O	O
VP	-	-	-	-	-	-
Methyl Red	-	+	-	-		
Acid from						
D- Glucose	-	+	+	-	+	+
L - Arabinose	-	-	-	-	+	-
D- Xylose	-	+	+	+	+	+
D- Mannitol	-	-	-	-	+	-
Maltose	-	+	+	+	+	+
Galactose	-	-	+	+	+	+
Sucrose	-	+	+	+	+	+
Lactose	-	-	-	-	+	+
NO_2-NO_3	+	+	-	+	-	-
Simmons Citrate	-	-	+	-	-	+
Identified as	*Alcaligenes*	*Pseudomonas sp.*	*Ps. sp*	*Ps. sp*	*Flavobacterium sp.*	*Flavobact.*

Appendix-D

Chemical Estimations and standard graphs

D.1 Folin Lowry's method for Proteins (Lowry *et al*, 1951)

Reagent A: 2% Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5% $CuSO_4$ in 1% potassium sodium tartarate.

Reagent C: Alkaline Copper solution-Mixed 50ml of A and 1ml of B prior to use.

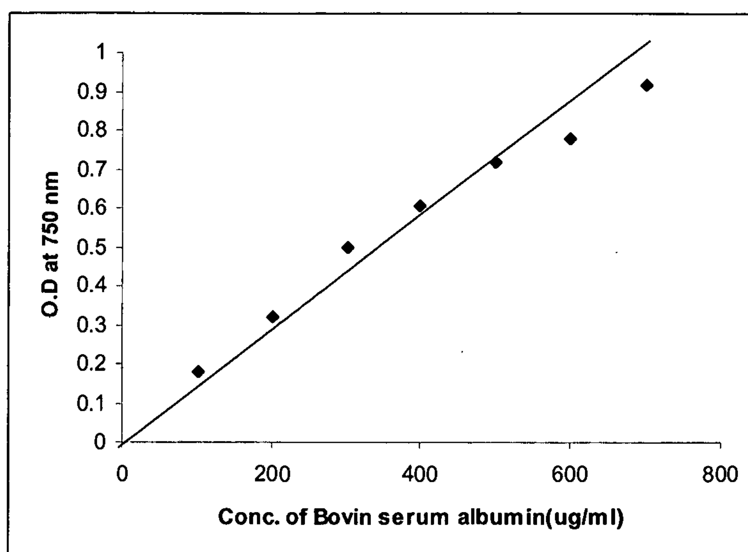
Reagent D: Folin and Ciocalteau's phenol reagent

Commercially available reagent diluted with equal volume of d/w on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solution: 0.1mg of BSA dissolved in one ml of d/w

Procedure: To 1ml of the sample, 5 ml of copper sulphate solution was added and kept at room temperature in the dark for 10 minutes. 0.5 ml of Folin and Ciocalteau's phenol reagent was then added and kept in the dark for 20 minutes. Absorbance was measured at 750 nm against reagent blank and the concentration of the samples detemined from standard graph and Factor F calculated using Bovine serum albumin as the standard (0-100 μgms/ml).

Standard graph of protein



D.2 Estimation of Total carbohydrate(Sadasivam & manickam,1996)

The total carbohydrate of polymer was assessed by phenol sulphuric acid method(Dubois et al,1956) using D glucose as standard

Phenol 5 %

50g phenol dissolved in water and diluted to 1litre.

Concentrated sulphuric acid, 96 %

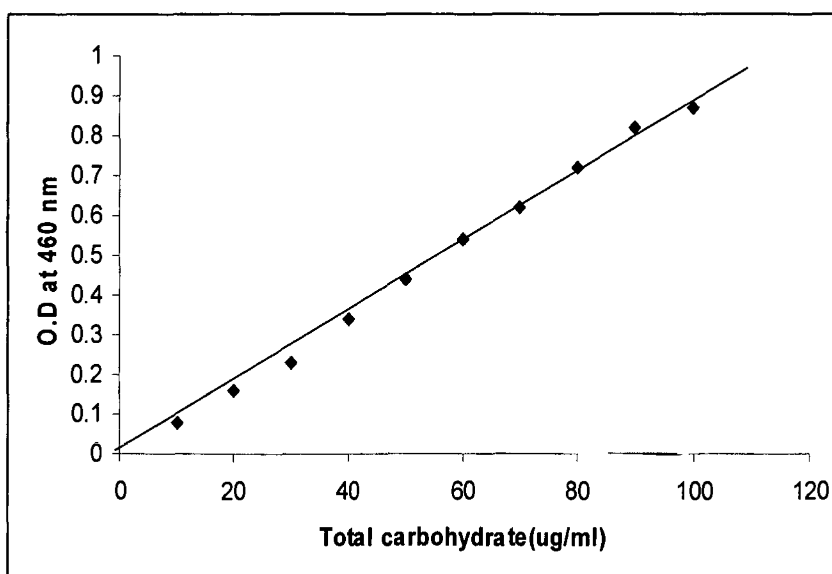
Standard glucose

Stock-100mg in 100ml distilled water

Working standard -10ml stock diluted to 100ml with distilled water

Procedure

Standard graph of Total carbohydrate

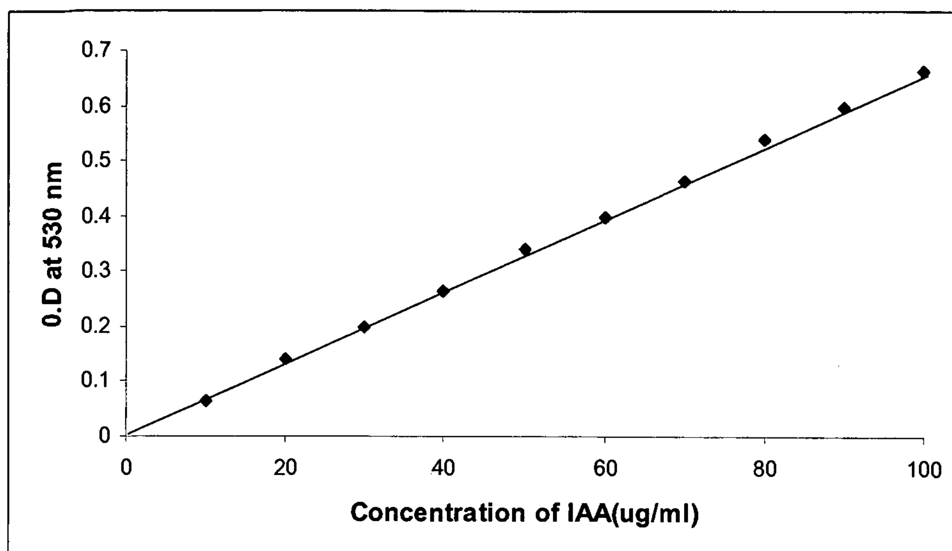


D.3 Estimation of proteins by the Bicinchoninic acid method

The proteins were estimated by method of smith et al (1985)

2 mg of sample was dissolved in 0.1ml of distilled water.2ml of Bicinchoninic acid reagent (1ml of 4% $CuSO4.5H2O$ mixed with 50ml Bicinchoninic acid) was added.the mixture was vortexed and incubated at 37oC for 30min.Optical density was measured at 562nm analytical standard was prepared using Bovine serum albumin

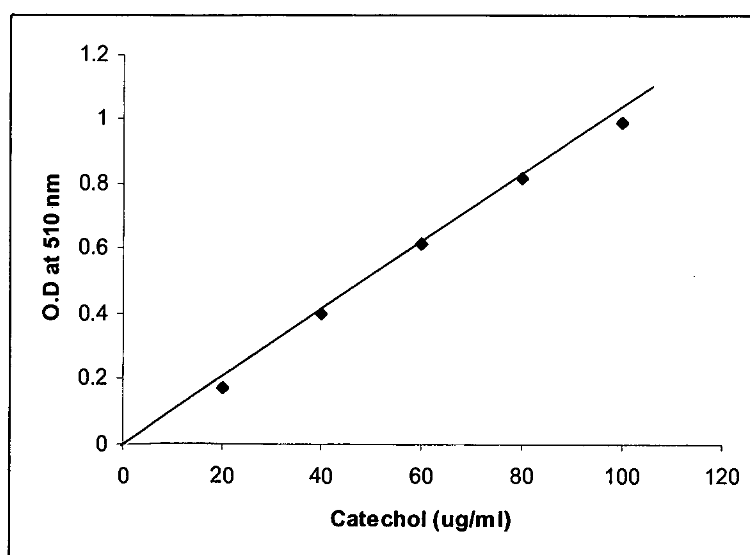
D.4 Standard graph of IAA



D.5 Arnows Assay Reagents

a. Nitrite-molybdate reagent
10 g sodium nitrite + 10 g sodium molybdate dissolved in 100 ml ddH2O
b. 1 M NaOH
4.0 g NaOH dissolved in ddH_2O to make a final volume of 100 ml

Standard graph of catechol



D.6 Reagents for Csaky Assay

a. Sulphanilic acid

1 g dissolved by heating in 100ml of 30% acetic acid(v/v)

b. Iodine solution

1.3g of iodine in 100ml of glacial acetic acid

c.Sodium arsenite

2g of sodium arsenite in 100ml of ditlled water

d.Sodium acetate

35g of sodium acetate in 100ml of distilled water

e.α–naphthylamine

3g of α–naphthylamine was dissolved in 1000ml of 30% acetic acid

D.7 Estimation of inorganic phosphate(Fiske and subbarao, 1925)

1. Solution A(sulfuric acid solution)

9N solution of H2SO4 was prepared by diluting 24.8ml of 98% concentrated H2SO4 to 100 ml with double distilled water

2. Solution B(Ammonium molybdate solution)

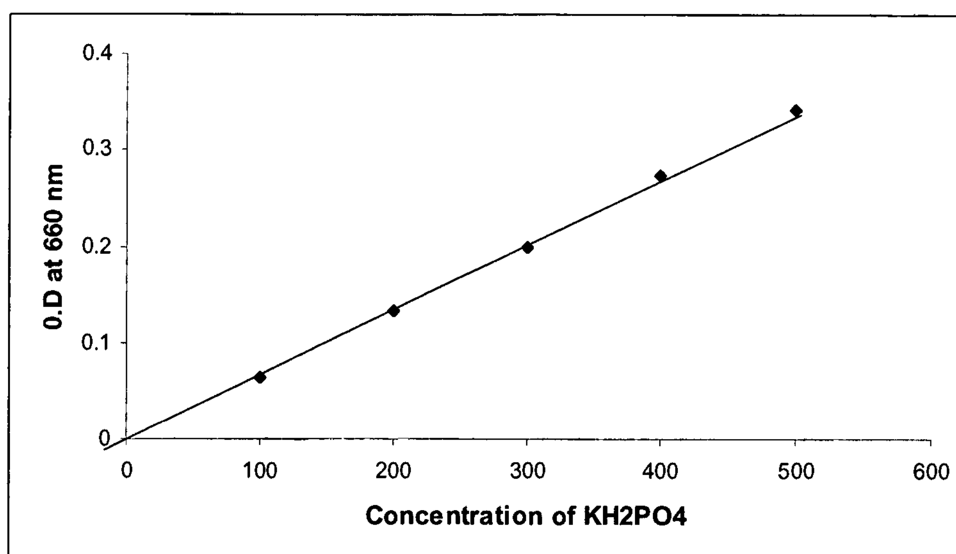
Dissolve 6g of ammonium molybdate in distilled water to a final volume of 100ml.

3. Solution C(Reducing agent solution)

8 g of ferrous sulphate was dissolved in minimal volume of distilled water in a 100 ml standard volumetric flask. To this solution, 1ml of solution A was added and mixed thoroughly and the volume was ,made up to 100ml with double distilled water

Procedure:

3.5 ml of the diluted sample was treated with 0.5ml of solution A and solution B(2-3mins in dark) and solution C sequentially and mixed thoroughly and incubated in dark. After 20 min, the absorbance was taken at 660 nms.

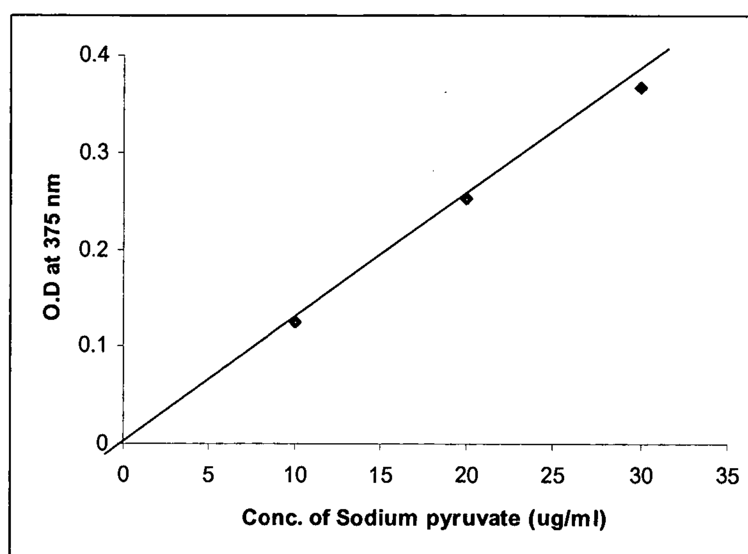


D.8 Estimation of pyruvates

Pyruvate was determined by method of Slonecker and Orentas(1962).

Procedure:

2 mg of sample were weighed and transferred to glass ampoule. 2ml of 1N HCl was added. The ampoule was flushed with nitrogen and sealed. The hydrolysis of polymer was carried out by keeping the ampoule in oven maintained at 100°C for 3 h. 2ml of aliquot was removed and mixed with 1ml of 2,4-dinitrophenylhydrazine reagent(0.5% DNPH in 2N HCl) for 5 min. The reaction mixture was extracted with 5 ml ethyl acetate and the aqueous fraction discarded. The ethyl acetate was extracted thrice with 5 ml of 10% Na_2CO_3 (each time) and the extracts pooled.The volume was made upto 25 ml with 10%Na_2CO_3. Optical density was measured at 375nm. Analytical standard was prepared using sodium pyruvate.

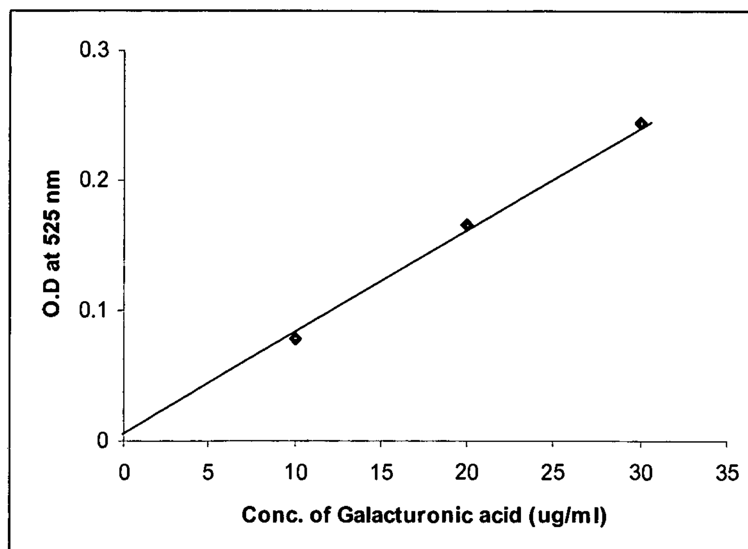


D.9. Estimation of Uronic acid

Uronic acids were estimated by the method of Filisetti and Carpita(1991).

Procedure:

To 1 mg of sample 0.4ml of distilled water was added and 40ul of sulfamic acid reagent. 94M sulfamic aicid dissolved in distilled water by dropwise addition of saturated KOH(pH=1.6) was added and mixed thoroughly. To this 2.4ml sodium tetraborate reagent (7.5mM sodium tetraborate in conc.H_2SO_4) was added. the solution was stirred thoroughly by vortex mixing.solution was heated for 20 min at 100°C in boiling water bath in stoppered tubes.the tubes were then placed in ice bath to stop the reaction. After cooling, 80ul of m-hydroxy-diphenyl reagent(0.15 % in 0.5 % NaOH solution) was added. The mixture was vortexed and left to stand for 10 min. Optical density was measured at 525nm. Analytical standard was prepared using galacturonic acid.



D.10. Carbon content

Reagents

Potassium dichromate 1 N

Dissolve 49.040 g of $K_2Cr_2O_7$(A.R) in distilled water to prepare 1 litre of solution.

Ferrous ammonium sulphate 1 N

Dissolve 393.130 g of Fe (NH4)2 (SO4)2.6H2O in distilled water adding simultaneously 15 ml of concentrated H2SO4 to prevent the hydrolysis of the salt .Dilute to 1 litre by adding distilled water and standardize by titration with K2Cr2O7 solution.

Sulphuric acid , Concentrated

Take H2SO4(sp.gr.1.84) and add 1.25 g of silver sulphate to each 100ml of acid.

Diphenylamine Indicator

Dissolve 0.5g of diphenylamine in a mixture of 100ml concentrated sulphuric acid and 20ml distilled water.

Phosphoric acid

H3PO4, 85%(sp.gr. 1.71)

Carbon content was determined by Walkley and Black's Rapid titration method .

Organic carbon was oxidized to carbon dioxide by potassium dichromate. Excess potassium dichromate leftover after oxidation was titrated against ferrous ammonium sulphate with diphenylamine indicator.

Procedure :

10 g of perfectly dried soil was weighed and transferred to a dried 500ml conical flask . 10 ml of 1N K2Cr2O7 and 20 ml concentrated sulphuric acid containing silver sulphate was added and mixed by gentle swirling .The flask was allowed to stand for about 30 mins and after reaction was over , the contents were diluted with 200 ml of distilled water. 10 ml of phosphoric acid and 1 ml of diphenylamine indicator was added to give a bluish purple colour. The contents were titrated with ferrous ammonium sulphate carefully until the blue colour changed to brilliant green.

Calculation :

$$\text{Carbon (\%)} = \frac{V_1 - V_2}{W} \times 0.003 \times 100$$

V_1 – volume of $K_2Cr_2O_7$

V_2 – volume of ferrous ammonium sulphate
W-weight of soil taken (Jackson ,1973 ; Trivedi et. al,1998)

D. 11. Nitrogen content

$KMnO_4$(0.32%)
Dissolve 3.2 g of $KMnO_4$ in distilled water and make up the volume to one liter in volumetric flask.

Sodium hydroxide (2.5%)
Dissolve 2.5 g NaOH in one liter of distilled water

Boric acid , 2% solution with mixed indicator

Dissolve 20 g of pure boric acid in 900ml of distilled water to which add 20 ml of mixed indicator and make up the volume to one liter.

Mixed indicator
Dissolve 0.1 g of Bromocresol green + 0.07 g of Methyl red in 100 ml of ethanol.
Standard Sulphuric acid (0.01 N or 0.1 N)
Liquid paraffin
Available nitrogen was determined by Alkaline Potassium Permanganate method.
The organic matter in soil is oxidized with hot alkaline $KMnO_4$ solution .The ammonia (NH_3) evolved during oxidation is distilled and trapped in boric acid – mixed indicator solution. The amount of ammonia trapped is estimated by titrating against standard acid.
Procedure:
Weigh exactly 20 g of air-dried 2mm sieved soil in a Kjeldalh flask. Add 20 ml of distilled water to moisten the soil and 1 ml of liquid paraffin and a few glass beads to avoid frothing and bumping respectively. Add 100 ml of 0.32 % $KMnO_4$ solution followed by 100 ml of 2.5% NaOH solution. Close the flask immediately. Distill the contents in Kjeldalh assembly at a steady rate and collect the liberated NH_3 in a receiving flask containing 20 ml boric acid solution with mixed indicator. With absorption of ammonia, the pink colour of boric acid solution turns green . Distill till about 100 ml of distillate is collected in receiving flask. Titrate the distillate against standard acid till the colour changes from green to pink.

Calculation :

$$\text{Available Nitrogen (\%)} = \frac{TV \times N\ H_2SO_4 \times 0.014}{\text{Weight of soil}} \times 100$$

TV – Titre value
N- Normality of H_2SO_4 (Jackson , 1973 ; Gali et. al , 2000)

D. 12. Available Phosphate

Sodium bicarbonate ($NaHCO_3$) solution, 0.5M

Adjust the pH of this solution to 8.5 with 1*M* sodium hydroxide (NaOH). Add mineral oil to avoid exposure of the solution to the atmosphere. Prepare a fresh solution before use if it has been standing over 1 month in a glass container. Store the solution in a polyethylene container for periods of >1 month, but check the pH of the solution each month.

Sulfuric acid (H_2SO_4), 5N

Add 141 ml of conc. H_2SO_4 to 800 ml of distilled water. Cool the solution and dilute to 1000 ml with distilled water.

Standard phosphate solution Place 0.4393 g of potassium dihydrogen phosphate (KH_2PO_4) in a 1 liter volumetric flask. Add 500 ml of distilled water, and shake the contents until the salt dissolves. Dilute the solution to 1 liter with distilled water. Add 5 drops of toluene to diminish microbial activity. This solution contains 0.1 mg of phosphorus (P) ml^{-1}.

Dilute phosphate solution: Dilute 20 ml of the standard phosphate solution to 1 liter with distilled water. This solution contains 2 □g of P ml^{-1}.

Ammonium paramolybdate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$] Dissolve 12 g of ammonium paramolybdate in 250 ml of distilled water. Dissolve 0.2908 g of potassium antimony tartarate ($KSbO.C_4H_4O_6$) in 100 ml of distilled water. Add these dissolved reagents to 1 liter of 5*N* H_2SO_4 (141 ml of conc. H_2SO_4 diluted to 1 liter), mix thoroughly, and dilute with distilled water to 2 liters. Store in a Pyrex glass bottle in a dark and cool compartment (Reagent A).

Ascorbic acid Dissolve 1.056 g of ascorbic acid in 200 ml of reagent A and mix. This ascorbic acid (Reagent B) should be prepared as required because it cannot be kept for more than 24 h.

Procedure

Take 5 g of soil in a 250 ml Erlenmeyer flask and add 100 ml of the sodium bicarbonate solution. Shake the flask for 30 min with a suitable shaker. Filter the suspension through Whatman no. 40 filter paper. Add activated charcoal (P free) to obtain a clear filtrate, but this step is not necessary in most soils when the ascorbic acid method is used. Shake the flask immediately before pouring the suspension into the funnel.

Place 5 ml aliquot of the extract in a 25 ml volumetric flask, and acidify with 5*N* H_2SO_4 to pH 5. This can be done by taking 5 ml of 0.5*M* extracting solution and determining the amount of acid required to bring the solution to pH 5 using p-nitrophenol indicator. Then add the required acid to all the unknowns. Add 20 ml of distilled water, and then add 4 ml of reagent B. The color will be stable for 24 h, and maximum intensity is obtained in 10 min. The absorption maximum of the blue color formed in the presence of antimony is at 882 nm. Calibrate the method using a standard P solution. Prepare a blank with distilled water and 4 ml of reagent B.

Appendix-E

E.1. Stock Solution for SDS – PAGE:

Acrylamide-bis-acrylamide solution [Monomer solution]: 29% acrylamide and 1% (w/v) N,N methylene *bis* acrylamide was dissolved in warm d/w. Checked pH to be 7.0 and stored in dark bottles at 4°C and used within 30 days.

Resolving gel buffer [1.5 M Tris, pH 8.8]: Prepared by dissolving 18.615g Tris, in 70 ml d/w water and added 100 µls of 10% SDS in d/w. The pH of the solution was adjusted to 8.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Stacking gel buffer [1.0 M Tris pH 6.8]: Prepared by dissolving 12.11g Tris, in 70 ml d/w water and added 50 µls of 10% SDS in d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was build up to 100ml with d/w. The solution was stored at 4°C.

Ammonium per sulfate (APS, 10% w/v): Prepared by dissolving 0.1g of APS in 1.0 ml d/w. The solution was prepared fresh each time and stored at 4°C.

Electrophoresis buffer:

Composition of 1X buffer is as follows:

Tris	3.0g
Glycine	14.4g
SDS(10%)	10ml
D/w	to make 1000ml
pH	8.4

Sample buffer:

Composition of 4X buffer is as follows:

TriS- HCl (1 M pH 6.8)	0.04ml
Glycine	0.04g
SDS	0.004g
β-Mercaptoethanol	0.004ml
d/w	to make 10ml

Tracking dye:

50% sucrose	10 ml
Bromophenol blue	10mg

Staining Solution:

Coomassie Brilliant Blue G - 250 solution was prepared by dissolving 0.25g Coomassie Brilliant Blue G - 250 in 100ml of 25% methanol, 10% glacial acetic acid and 65% d/w.

Destaining Solution I

Methanol 40ml

Acetic acid 10ml

d/w 50ml

Destaining Solution II

Methanol 5ml

Acetic acid 7ml

d/w 88ml

Preparation of gel monomer

The composition of the resolving and stacking gels is as follows:

Solution	Resolving gel (10%) (ml)	Stacking gel (5%) (ml)
Monomer	2.5	0.33
1.5M Tris, pH 8.8	1.875	-
1.0M Tris, pH 6.8	-	0.625
10% SDS	0.075	0.025
10% APS	0.0375	0.025
D/w	0.003	1.525
TEMED	0.005	0.005

(a) **Preparation of sample:** 100 µls of cell pellet (containing 100 mg of protein) was mixed with 10µls of 25% SDS and boiled for 2 minutes at 100°C. 50µls of sample buffer was then added and boiled for 5 minutes at 100°C. After cooling, 20 µls of bromothymol blue was added and 50µls of the samples were loaded in the gel with SDS PAGE molecular weight markers (sigma-St.Louis, MO USA).

(b) **Procedure:** SDS - PAGE were carried out in a Bangalore Genei apparatus. After a pre-run for 10 minutes, 30µls of the samples containing 50µgms of proteins along with the standard molecular weight markers were loaded in the gel. The electrophoresis was carried out at a constant voltage of 80 V for stacking gel and 120 V for resolving gel till tracking dye (Bromothymol blue) reached the bottom of the gel. At the end of the run, the gel was stained by Coomassie blue.

(c) Staining and destaining procedure:

i) Coomassie blue staining: The gel was stained in Coomassie Brilliant Blue G-250 solution Staining was carried out overnight; followed by destaining under mild shaking using de staining solution I for 3-4 hours and destaining solution II for several hours till the protein bands became clearly visible with no background colour. The gels were dried and preserved between cellophane sheets .

E.2 Solutions for Plasmid DNA extraction and agarose gel electrophoresis(Alkaline lysis method) :

1) Solution I(pH 8)

Glucose	0.9g
Tris-HCl	0.394g
EDTA	0.292g

Double distilled water 100ml

store at - 4°C

2) Solution II

0.2N	100ml
SDS	1g

3) Solution III

5M Potassium acetate	60 ml
Glacial acetic acid	11.5 ml
Distilled water	28.5ml

Ethidium bromide: 10mg: 1ml distilled water

Stock solution was prepared and kept in cool and dark place.The final concentration used for agarose gel was 5 ug/ml.

Tracking dye: Bromophenol blue 0.25g

Sucrose	40g
0.1 M EDTA	10ml
1% SDS	10ml
Distilled water	100ml

Dye stored at 4oC

Tris acetate EDTA(TAE) buffer(pH 8)(1X)

50X: Tris base 2.42g

0.5 M EDTA 1ml

Tris base and 0.5M EDTA was dissolved in 10ml of double distilled water and the pF adjusted to 8 with glacial acetic acid(0.57ml) and a final volume was made up to 500ml.

E.3 Siderophore Detection Using Thin Layer Chromatography (TLC)

Siderophore can also be detected by using thin-layer chromatography. Culture supernatant or concentrated samples of siderophore (see later sections) are spotted on Selecto Scientific 10 x 20 silica gel plates and spots are allowed to dry. The plates are then run in an n-butanol:acetic acid:dH_2O(12:3:5) solvent system until the solvent front reaches the top of the plate. Plates are then dried and sprayed with 0.1 M $FeCl_3$ in 0.1 N HCl. The formation of a wine-colored spot indicates a hydroxamate-type siderophore, while a dark gray spot indicates production of a catechol-type siderophore. Siderophores are separated on the basis of hydrophobicity using these plates.

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Conferences Attended/Publications

CONFERENCES ATTENDED

- Paper entitled **"Studies on free living and adhered bacteria from coastal sand dune ecosystem"** presented at the ***Conference on Microbiology of the Tropical Seas*** held at NIO from 13-15 December, 2004.

- Paper entitled **"Exopolysaccharide producing bacteria from coastal sand dunes"** presented at the ***National Conference on Frontiers in Environmental Sciences and Engineering in India*** held at Bharathiar University, Coimbatore from 15-17 September, 2005.
  - Awarded the **"AQUAGUARD YOUNG SCIENTIST AWARD"** for the same.

- Paper entitled **"Diversity of bacteria associated with *Ipomoea pes caprae* and *Spinifex littoreus* sand dune vegetation of coastal dunes"** presented at the ***46th AMI Conference, MICRO-BIOTECH 2005*** held at Osmania University, Hyderabad from December 8-10, 2005.

- Paper entitled "**Bacteria associated with vegetation on coastal sand dunes"** accepted at the ***11th International Symposium on Microbial Ecology*** (ISME-11) held at Vienna, Austria from August 20-25, 2006.

PUBLICATIONS

1) Paper entitled **"Carotenes produced by alkaliphilic orange-pigmented strain of *Microbacterium arborescens*- AGSB isolated from coastal sand dunes"** communicated to ***The Indian Journal of Marine Sciences*** **(In Press).**

2) Paper entitled **"Bacterial exopolysaccharide produced by *Microbacterium arborescens* - AGSB, isolated from coastal sand dune habitat of Goa"** communicated to ***Research in Microbiology Journal.***

3) Paper entitled **"Bacteria from sand dunes of Goa promoting growth in eggplant"** communicated to the ***Plant and Soil Journal.***

4) Paper entitled **"Ecological implications of rhizosphere and endophytic bacteria associated with *Ipomoea pes-caprae* and *Spinifex littoreus* growing on sand dunes an unique ecosystem"** communicated to the ***ISME Journal.***

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BACTERIA ASSOCIATED VEGETATION ON SAND DUNES AT BEACHES OF GOA, INDIA.

S. Bhosle, A. Godinho, C. De Souza

Department of Microbiology, Goa University, Goa, India

Coastal sand dunes have low moisture content, fluctuating temperatures and low nutrients making this ecosystem a stressful one for biological survival. It is however interesting to note that in such conditions also there are plants growing. We therefore concentrated our studies on the biodiversity of the rhizobacteria both free living and those adhered to the roots of these plants, screening of the enzymic potentials of these bacteria and their ability to solubilise phosphate , produce siderophores and exopolysaccarides. Sand samples were collected from the rhizosphere of the vegetation. Sampling sites were identified from North Goa and South Goa beaches. The samples were plated on nutrient media for the total viable counts and on media with the specific characteristics to isolate diazotrophs and extremophiles. It was observed that the total viable counts of bacteria adhered to the roots were higher(2x106 cfu/g to 307x109 cfu/g) than the free living in the rhizosphere, (7x103 cfu/g to 544x107 cfu/g).Amongst adhered bacteria, 47% were amylase producers, 14% produced cellulases , 69% showed protease and 24% were tannase producers. Among the freeliving bacterial isolates, 21% were amylase producers, 28% produced cellulases, 57% showed production of protease and 5% showed tannase activity. The screening of these bacteria to produce siderophores exopolysaccharides and solubilize inorganic phosphates showed that 60% could produce siderophores while 40% showed solubilization of inorganic phosphates. These bacterial isolates show promising characteristics which directly or indirectly would help in the growth of plants on sand dunes and in the biogeochemical cycles.

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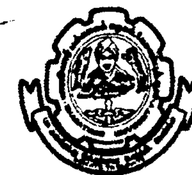

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'AQUAGUARD YOUNG SCIENTIST AWARD – 2005'

This is to certify that Aureen Godinho, Research scholar *has presented a* ~~*paper*~~*/poster entitled* " Exopolysaccharide producing Bacteria from ..... " *during the National conference on Frontiers in Environmental Sciences and Engineering in India, held on 15th - 17th September 2005 at Bharathiar University, Coimbatore and has selected for the 'Aquaguard Young Scientist Award - 2005'*

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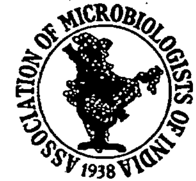

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Certificate

This is to certify that Prof. / Dr. / Mr. / Mrs. /Miss Aureen Godinho.

Dept. of Microbiology, Goa University, Goa.

has participated, presented a paper in ~~Oral~~ / Poster session at 46th Annual Conference of Association of Microbiologists of India "MICRO -BIOTECH 2005" during 8th - 10th December, 2005 organised by the Department of Microbiology, Osmania University, Hyderabad.

L. Venkateswar Rao
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P.A. Loka Bharathi
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This is to certify that ~~Dr~~/~~Mr~~/Ms AUREEN REMEDIOS LEMOS GODINHO has attended

the conference on Microbiology of the Tropical seas and presented ~~Oral~~/Poster on

"Studies on free-living and adhered bacteria from Coastal Sand dune ecosystem."
- Aureen Godinho and Saroj Bhosle.

P.A. Bharathi
[Dr. P.A. Loka Bharathi]