“Studies on siderophore producing bacteria from coastal ecosystems and their response to an aromatic compound (Sodium benzoate)”

A thesis submitted to GOA UNIVERSITY for the award of degree of

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in

MICROBIOLOGY

By

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Research Guide

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Taleigao, Goa

2013

CERTIFICATE

This is to certify that the thesis entitled **"Studies on siderophore producing bacteria from coastal ecosystems and their response to an aromatic compound (Sodium benzoate)"** submitted by **Ms. Teja Gopalkrishna Gaonkar** for the award of the degree of **Doctor of Philosophy** in Microbiology is based on her original studies carried out by her under my supervision. The thesis, or any part of it, has not been previously submitted for any degree or diploma to any university or institution.

Prof. Saroj Bhosle
Department of Microbiology
Goa University

Date:
Place:

DECLARATION

I hereby state that this thesis for a Ph.D. degree in Microbiology on the **"Studies on siderophore producing bacteria from coastal ecosystems and their response to an aromatic compound (Sodium benzoate)"** is my original contribution and that the thesis, or any part of it, has not been previously submitted for the award of any degree/diploma to any university or institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

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Acknowledgement

As we express our gratitude, we must never forget that the highest appreciation is not to utter words, but to live by them. ~John Fitzgerald Kennedy

I am confident that I can stand by this concept while I take this opportunity to acknowledge the people who are directly and indirectly involved in my journey for a Ph. D.

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….. Teja

Dedicated to my beloved

parents

Aaie and Baba

CONTENTS

LIST OF ABBREVIATIONS

Abs	Absorbance	NADH	Reduced form of Nicotinamide adenine dinucleotide
BLAST	Basic local alignment search tool		
bp	Base pair	NADPH	Reduced form of Nicotinamide adenine dinucleotide phosphate
CAS	Chrome azurol sulphonate		
Cfu	Colony forming units	NMR	Nuclear magnetic resonance
dI water	Deionised water	NR	Non Rhizhosphere Sample
DHBA	Dihydroxy benzoic acid	PGPR	Plant growth promoting rhizobacteria
DNA	Deoxyribonucleic acid	PCR	Polymerase chain reaction
EDTA	Ethylene diamine tetra acetic acid	rpm	Revolutions per minute
FAD	Flavin adenine dinucleotide	R	Rhizhosphere Sample
FAME	Fatty acid methyl esters	Rf	Retention factor
Fig.	Figure	RNA	Ribonucleic acid
FMN	Flavin mononucleotide	SDS	Sodium dodecyl sulphate
g	Gram	SEM	Scanning electron microscope
GSH	Glutathione	sp.	Species (Singular)
h	Hour(s)	TAE	Tris acetate EDTA
HCN	Hydrogen cyanide	TE	Tris EDTA
L	Liter	TLC	Thin layer chromatography
LCMS	Liquid chromatography-Mass spectroscopy	TVC	Total viable count
		UV	Ultra violet
µl	Micro liter	Vis	Visible
MSM	Mineral salts medium		

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Introduction

Siderophores (In Greek, Siderophores: “iron carriers”) are low molecular weight, iron chelating compounds produced by bacteria and fungi under iron limiting conditions (Neilands 1995, Boukhalfa and Crumbliss 2002). Iron is an essential compound for most living organisms. It serves as a catalytic center for enzymes involved in important cellular processes such as electron transport, activation of oxygen, peroxide reduction, amino acid and nucleoside synthesis, DNA synthesis and photosynthesis (Wandersman and Delepelaire 2004).

Despite its abundance on earth crust, it is biologically unavailable due to its presence as Fe^{+3} under aerobic and neutral to alkaline conditions (Hotta *et al*. 2010). In such iron limiting ecosystems iron availability and the efficiency of iron uptake influences microbial activity. An iron-limiting condition alters utilization patterns for various compounds. The most apparent example is the microbial degradation of aromatic compounds as oxygenases involved in hydrocarbon degradation contain iron as a cofactor, thus imposing specific iron requirement on cells (Dinkla *et al.* 2001). Environments rich in hydrocarbons, particularly, coastal and estuarine ecosystems (Bae *et al*. 2009, Borgne *et al*. 2008) are deficient in iron (Mawji *et al*. 2008, Vraspir and Butler 2008). In such environments, hydrocarbon breakdown by microbial communities depends on the adaptation of the bacteria and the strategies that they adapt to overcome nutrient deficiency such as production of siderophores under iron stress. Although iron deficiency is the key factor regulating the synthesis of siderophores, other external factors like pH, temperature, carbon source and other metals also play an important role (Saha *et al.* 2012).

While, siderophores have an extremely high affinity for ferric iron, they also form complexes with metals other than Fe^{3+}, although with a lower affinity (Braud *et al.* 2009). Metals other than iron are also reported to stimulate or repress siderophore production in bacteria (Braud *et*

al. 2010). The production of siderophore by bacteria in presence of toxic metal implicate that they may play an important role in uptake, mobilization of heavy metals or developing metal resistance. Binding of siderophores to metals also reduces free metal concentration thereby restricting their diffusion across the Porins. However, siderophores can also lead to intracellular metal accumulation in conditions where the siderophore uptake receptor does not distinguish between the metal–siderophore and the ferri–siderophore complexes (Schalk *et al.* 2011).

Besides multiple factors affecting siderophore production, structural diversity also exists among siderophores. Depending on the iron ligation groups, siderophores have been classified into three main types: hydroxamate, catecholate and carboxylate type. (Wandersman and Delepelaire, 2004). When genera wise study of siderophores is considered, the most extensively studied iron chelators are siderophores of Gram-negative bacteria, in particular, catechol and hydroxamate groups in pseudomonads and enterobacteria. Besides, siderophores of agrobacteria, *Yersinia* and vibrions have also been studied. Hydroxamates have been primarily studied from Gram-positive organisms whereas catechols were identified in a relatively small number of representatives of this group (Temirov *et al.* 2003). Amongst the *Bacillus* species, siderophores from *Bacillus anthracis* (Petrobactin and Bacillibactin) (Wilson *et al.* 2010), *B.megaterium* (Schizokinen), *B. subtilis* (Bacillibactin) (Zawadzka *et al.* 2009) and *B. licheniformis* (2, 3-dihydroxybenzoyl-glycyl-threonine) have been well studied (Temirov *et al.* 2003).

During this study, the bacteria producing siderophores were isolated from two distinct ecosystems, one, a low nutrient sand dune ecosystem and the other a nutrient rich mangrove ecosystem. Sand dunes represent large amounts of shifting sand barren to plants. Sand dunes

are largely categorized into two types. The first kinds are extremely dry interior deserts such as Sahara in Africa or Rajasthan in India. Coastal sand dunes occur along the coasts of the Atlantic, pacific, North America and Australia. In Asia, coastal sand dunes occur in Japan, India and several other countries (Desai and Untawale 2002, Boorman 1977, Carter 1998). Vegetation plays an important role in determining size, shape and stability of the dunes. Dead plants and humus from sand dune vegetation adds humus to the sand. Furthermore, microorganisms growing in these sand dunes help reduce the nutrient stress.

Mangroves are highly reproductive ecosystems which host a wide range of coastal and offshore marine organisms. Mangroves provide a unique ecological environment for diverse bacterial communities (Ramanathan *et al.* 2008). Tidal variations and salinity and intense human activities such as transportation through ships, barges and the accidental spills result in the diverse microflora in the marine coastal ecosystem (Rawte *et al.* 2002). Bacteria largely influence nutrient cycling in mangroves and thus contribute to soil and vegetation patterns (Hossain *et al.* 2012). Environments wherein microorganisms grow are highly variable with respect to temperature, pH, redox potential, osmotic pressure, water activity, salinity and general and essential nutrients. Previous studies have shown presence of large number of bacteria in these two ecosystems (Godinho and Bhosle 2002).

The important aspects of siderophore producing bacteria have instigated this investigation to explore occurrence of siderophore producers in these ecosystems and furthermore, to understand effects of various parameters on siderophore production. This study was therefore dedicated with an objective to isolate siderophore producing bacteria from sand dune and coastal ecosystem and further to study effect of pH, NaCl and iron concentrations on siderophore production in selected bacterial isolates. Furthermore, effect of sodium benzoate

(as a representative of aromatic hydrocarbons) and metals on siderophore production by *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 respectively was studied. Siderophore produced by *Bacillus amyloliquefaciens* NAR38.1 was isolated and purified to study its biochemical structure.

Chapter I

Literature Survey

1.1 Need for iron and impacts of iron deficiency

Iron is essential for the growth and development of almost all living organisms. It acts as a catalyst in some of the most fundamental enzymatic processes, including oxygen metabolism, electron transfer, and DNA/RNA synthesis (Guan *et al*. 2001). However, despite its abundance on earth and the micromolar concentrations required for microbial growth, iron is biologically unavailable in most environments (Lankford and Byers 1973). In aerobic inorganic environments, iron is present in the oxidized ferric form Fe^{+3}, which aggregates into insoluble oxy-hydroxide polymers (Wandersman and Delepelaire 2004).

The most obvious effect of iron deficiency on microbial cells is the decrease in quantity of cellular biomass. Iron deficiency has been reported to induce changes in activities of certain enzymes. For example, iron deficiency has been reported to result in 24 fold stimulation of NADase activity and is known to decrease the concentrations of cytochromes, peroxidase and catalase. Such a decrease in cytochrome concentration has been reported in *Torula utilis*; decrease in peroxidase, catalase and oxidase activity has been reported in *Candida guilliermondii*. NADH dehydrogenase is a membrane- bound enzyme with iron-sulfur centers which brings about NADH oxidation in the terminal electron transport systems of aerobes. Its enzyme activity and polypeptide chain composition has been found to be affected by iron-limitation. Aconitase activity has also been found to diminish due to iron deficiency as opposed to aldolase whose activity is found to increase. Diminution in alcohol dehydrogenase activity has been observed in *C. guilliermondii* with decrease in iron concentration in the growth medium. Iron exerts an effect on enzymes involved in the biosynthesis and degradation of iron chelating agents, siderophores (Light and Clegg, 1974).

Besides affecting enzyme activity, iron deficiency results in morphological changes in microorganisms. In *Mycobacterium smegmatis*, an increase in cell length has been observed as compared with the cells grown in iron sufficient medium. This could be due to inhibition of DNA synthesis without proportional inhibition of cell growth or due to a direct effect on cell division or cell separation (Light and Clegg, 1974). Hence to acquire this iron from natural ecosystems, bacteria have evolved multiple parallel pathways (Fig. 1.1), the most important of these is siderophore production.

1.2 Siderophores

Siderophores are low molecular weight ligands (20-2000 Da) produced by bacteria, fungi and plants to solubilize and take up iron (Chu *et al*. 2010, Hider and Kong 2010). Siderophores chelate iron with affinity constant of almost $10^{30}M^{-1}$. More than 500 different siderophores mostly from bacteria have been described. The iron ligation groups have been tentatively classified into three main chemical types (hydroxamate, catecholate and hydroxycarboxylates (Fig. 1.2), however other varieties of siderophore structures have been resolved and includes: oxazoline, thiazoline, hydroxypyridinone, α- and β- hydroxy acids, and α–keto acid components. The most important property of siderophores is their denticity (number of iron coordinating atoms per molecule) which ranges from bidentate to hexadentate (Raymond and Dertz, 2004). The peptide backbone of siderophores is usually made of several non-protein amino acid analogs including both modified and D-amino

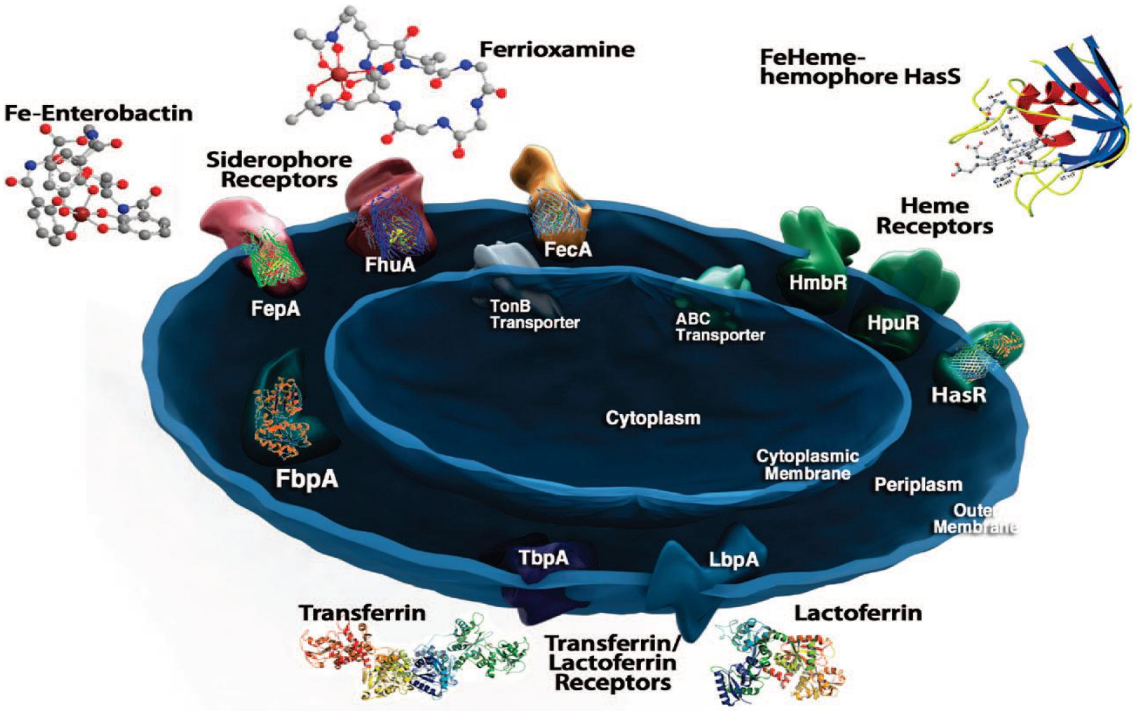

Fig. 1.1: Microbial (Gram negative) iron uptake pathways. (Ref: Sandy and Butler 2009)

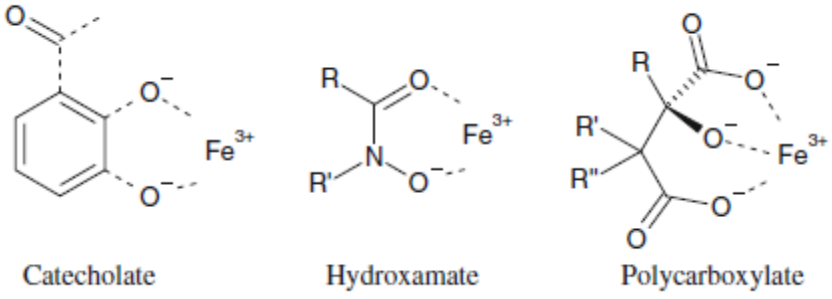

Fig. 1.2: Main structural types of siderophores as iron complexes (Ref: Mo¨llmann *et al.* 2009).

Some bacteria produce one type of siderophore while many produce multiple types of siderophores permitting microorganisms to grow in different environments.

The three important iron-chelating groups in siderophores are:

1.2.1 Catecholate

These siderophores have catechol as the functional group (Sandy and Butler 2009). catecholate type of siderophores can be detected using Neiland's spectrophotometric assay (Neilands 1981) and Arnow's assay (Arnow 1937). The classic examples of tris- catecholate are enterobactin, bacillibactin and salmochelin shown in Fig. 1.3. Enterobactin has been isolated from enteric and pathogenic bacteria including *E. coli* and it consists of three monomers of 2,3-dihydroxybenzoyl-L-serine. Salmochelin has been isolated from *Salmonella enterica* and europathogenic *E. coli*. It is a derivative of enterobactin in which one or two catechols are glucosylated at position C-5. Bacillibactin which was first isolated from *Bacillus subtilis* has now been isolated from other *Bacillus* species also. It is made of three monomers of 2, 3-dihydroxybenzoate-Gly-Thr which are cyclized. The glycine spacers elongate the three chelating arms (Wilson *et al.* 2010).

1.2.2 Hydroxamate

The basic structure consists of hydroxamic acids. The standard tests used to detect hydroxamate type of siderophores are: Neiland's spectrophotometric assay (Neilands 1981), tetrazolium salt test (Snow 1954) and Csaky assay (Gilliam 1981). The first compounds recognized as microbial iron transport compounds are ferrioximes and ferrichromes (Raymond and Dertz 2004). Ferrioxime which is an example of tris hydroxamate siderophores, consists of alternating units of *N*-hydroxycadaverine or *N*-hydroxyputrescine. Desferrioxamines B, G, E and F are examples of hydroxamate siderophores (Fig. 1.4).

Desferrioxamine E is the cyclic counterpart to the linear desferrioxamine G (Dhungana *et al.* 2001). Desferrioxamine B (DFOB) is the drug Desferal used to treat iron overload disease.

1.2.3 α-Hydroxycarboxylate, carboxylate, and mixed functional Group

The iron chelating group in carboxylate type of siderophores is a carboxylic acid (Sandy and Butler 2009). Carboxamate type of siderophores can be detected with Vogel's chemical test (Yeole *et al.* 2001) or Shenker's test (Shenker 1992). Achromobactin is a tris-α-hydroxycarboxylate siderophore (Schmelz *et al.* 2009, Franza *et al.* 2005)**.** Two α -hydroxycarboxylate groups in achromobactin are derived from α-ketoglutarate and the third α -hydroxycarboxylate comes from citric acid. Bis-α-hydroxycarboxylic acid siderophores, such as vibrioferrin, are composed of one α-hydroxycarboxylate from citrate and one from α-ketoglutarate, whereas the α–hydroxycarboxylates in staphyloferrin and rhizoferrin come from two citrate groups. Vibrioferrin has been shown to bind boron stoichiometrically through the α–hydroxycarboxylic acid groups . The simplest siderophore is citric acid. It functions in Fe^{+3} uptake as the bisferric- citrato complex, $(Fe\text{-citrate})_2$, which is recognized by the outer membrane receptor protein, FecA. Many siderophores contain more than one type of functional group moiety, such as aerobactin (Fig. 1.5). In fact, the vast majority of siderophores are comprised of different types of bidentate donor ligands.

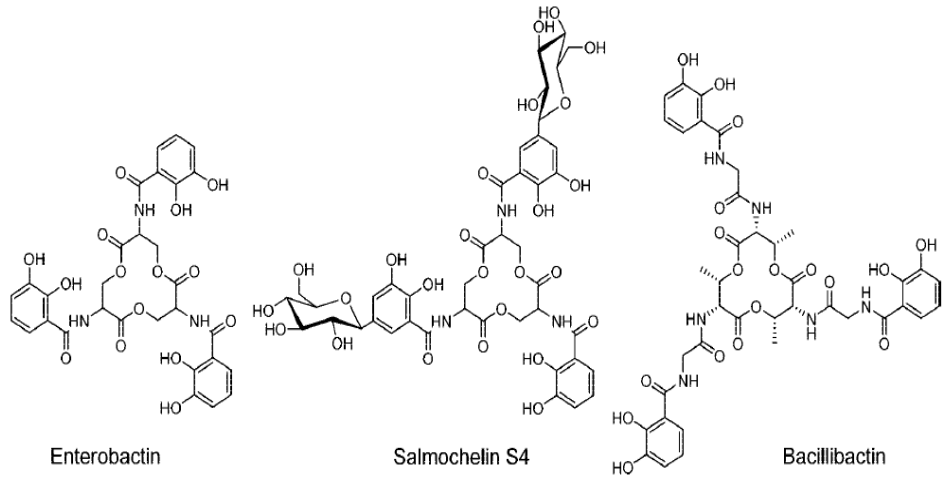

Fig. 1.3: Structures of enterobactin, salmochelin S4, and bacillibactin (Ref: Sandy and Butler 2009).

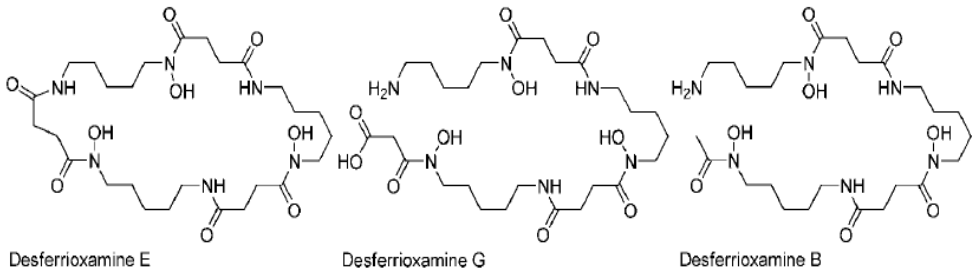

Fig. 1.4: Structures of desferrioxamines E, G, and B (Ref: Sandy and Butler 2009)

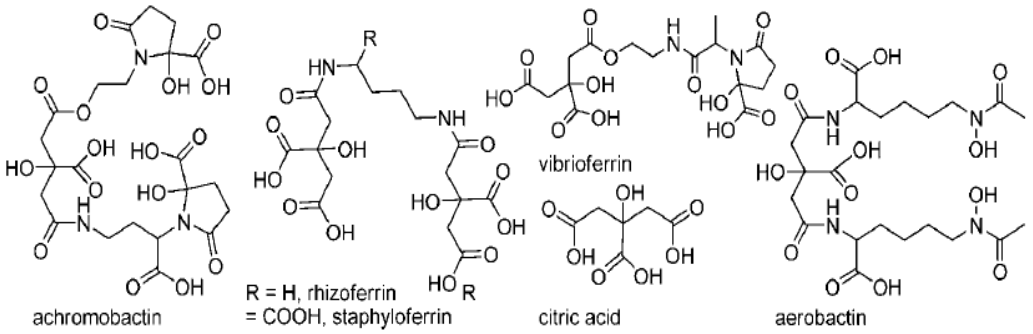

Fig. 1.5: Structures of selected α-hydroxycarboxylate siderophores (Ref: Sandy and Butler 2009).

Siderophores are assembled by non-ribosomal, cytoplasmic peptide synthetases. Genes encoding the biosynthetic enzymes are iron regulated and often clustered with genes involved in siderophore uptake.

1.3 Iron acquisition and ferrisiderophore transport

Siderophores have a high affinity for iron and they extract iron from most chemical and organic complexes by equilibrium displacement. Several bacteria use xenosiderophores as well as endogenously produced siderophores. Siderophores are transported in the cell in a form in which iron is bound to it. Such ferrisiderophores are recognized at the cell surface by the specific membrane anchored binding proteins in Gram-positive bacteria and by specific outer membrane receptors in Gram-negative bacteria (Fig. 1.6). Transport of this complex is through the pores formed by β – barrel proteins that are closed or opened depending on the requirement of the substrate by the microbial cell. Till date, three receptors have been crystallized: FepA (Buchanan 1999), FhuA (Ferguson 1998, Locher *et al*. 1998), and FecA (Ferguson *et al.* 2002, Yue *et al.* 2003) which are the receptors for enterobactin, ferrichrome, and ferric dicitrate.

These outer membrane receptors interact with a protein anchored in the cytoplasmic membrane, known as TonB (Postle 1993). TonB is associated with the ExbBD protein complex, which also resides in the inner membrane (Postle 1993, Braun 1998). The ExbBD complex couples the proton motive force sustained across the cytoplasmic membrane to bring about conformational changes in TonB (Larsen *et al.* 1999, Held and Postle 2002). Bacteria are known to use high-affinity binding proteins that are

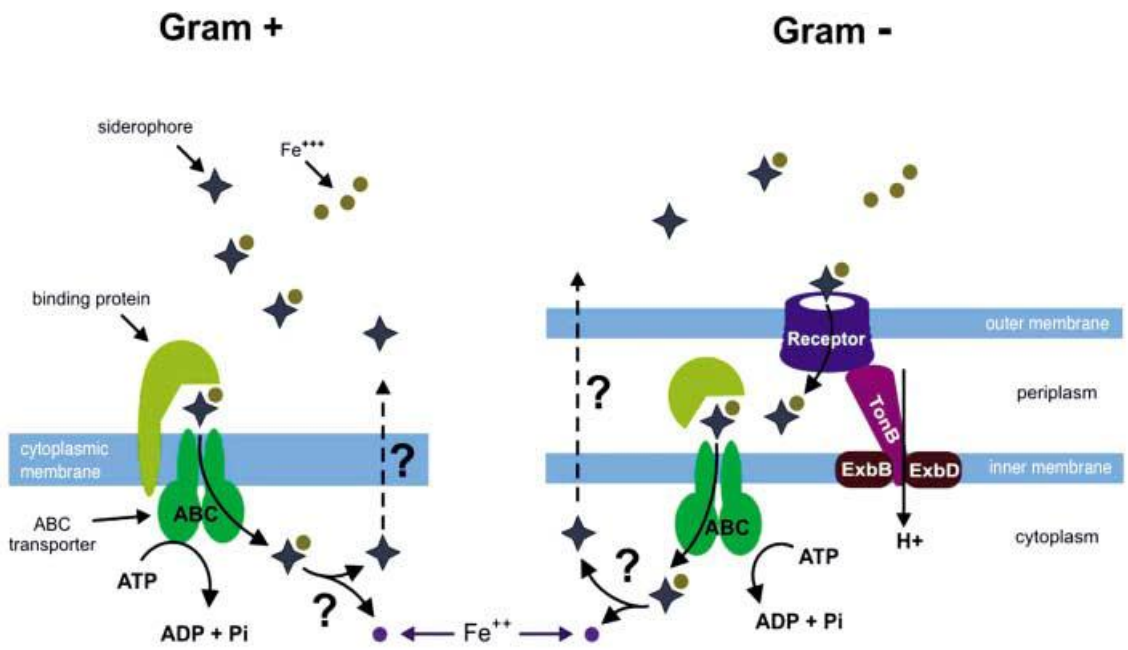

Fig. 1.6: Organization of siderophore excretion and internalization in Gram-positive and Gram-negative bacteria (Ref: Wandersman and Delepelaire 2004).

specific for their respective ligands, which include sugars, amino acids, oligopeptides, ions and other compounds. Similarly, such periplasmic binding protein (PBPs), also bind and transport ferric siderophore complex across the periplasm and subsequently deliver their ligands to specific ATP-dependent transporters or chemotaxis receptors residing in the inner membrane. Inner-membrane transporters associate specifically with loaded PBPs and pump their ligands into the cytoplasm (Higgins 2001, Davidson and Chen 2004).

Two processes have been proposed for iron release from siderophores. The first process depends on the fact that siderophores have a much lower affinity for ferrous iron than for ferric iron. Therefore, after iron reduction by free intracellular electron donors or by more specific siderophore reductases, iron release is spontaneous and the unbound siderophore can be then recycled. The second mechanism involves the intracellular breakdown of the siderophore and thus implies that siderophores are used only once and cannot be recycled.

Reduction of Fe^{+3} to Fe^{+2} occurs in the cytoplasm or on the cytoplasmic side of the inner membrane. Several reports are also available on the presence of extracellular reductases (Schroder 2003), which could provide easily assimilable Fe^{+2} to cells, however, none have been cloned or purified. Moreover, these reductases need cofactors to shuttle electrons and endogenous electron shuttles such as NAD(P)H, FAD, FMN and GSH which do not freely diffuse through membranes. Such extra cytoplasmic reductases could hence use either exogenous electron donors present or soluble quinones that freely diffuse through the envelope. Quinolic compounds have been characterized in *Shewanella putrefaciens*, and are found to be utilized in extracellular electron transfer, enabling Fe^{+3} reduction with iron as terminal electron acceptors (Newman and Kolter, 2000). Whether the resulting Fe^{+2} is used as an iron source by *S. putrefaciens* or other symbiotic organisms is not known.

Under anaerobic or reducing conditions, Fe^{+2} is the major form of iron for microorganisms. This highly soluble ion diffuses freely through the outer membrane porins of Gram-negative bacteria. It is transported through the cytoplasmic membrane by an ABC Fe^{+2} transporter conserved in many species (Kammler *et al.* 1993). *Salmonella enterica* and *Helicobacter pylori* colonize the mammalian intestine and stomach, respectively, where iron is present in Fe^{+2} form and, the ferrous iron transport system (Feo) is essential for iron acquisition. This suggests adaptation of bacteria to human tracts that these species usually colonize (Boyer *et al.* 2002; Velayudhan *et al.* 2000).

1.4 Siderophores discovered till date

1.4.1 Terrestrial siderophores: Major siderophores types produced by various terrestrial microorganisms have been discussed below:

Fluorescent pseudomonads belong to the γ proteobacteria and have diverse iron uptake systems (Cornelis and Matthijs 2002) which allows them to thrive in different econiches (Goldberg 2000). Pyoverdines are the major siderophores produced by fluorescent pseudomonads, but they are also known to produce secondary siderophores like Pyochelin, Pseudomonine, Quinolobactin/thioquinolobactin, which also bind other metals and possess antimicrobial activities. Pyoverdines are composed of a conserved chromophore and a variable peptide chain (Meyer 2000). Some *P. putida* or *P. fluorescens* strains have the capacity to utilize pyoverdines produced by other pseudomonads while others are restricted in their capacity to use heterologous pyoverdines (Raaijmakers *et al.* 1995, Ongena *et al.* 2001, Mirleau *et al.* 2000, Mossialos *et al.* 2000).

One of the well studied siderophores from enteric pathogens is Enterobactin. This catechol type of siderophore was first purified and characterized from *Salmonella enterica* serovar *Typhimurium* and *E. coli* culture supernatants and is used by most enteric pathogens (Nahlik *et al.* 1999, Pollack and Neilands 1970). Another type of siderophore used by enteric bacteria is Aerobactin. This is a hydroxamate type of siderophore produced by enteric pathogenic strains of *S. flexneri* and *Shigella boydii*. Aerobactin was first detected in *Aerobacter aerogenes* and in extraintestinal strains of *E. coli* and then subsequently also identified in a variety of enteric bacteria. Yersiniabactin is produced by many highly virulent *Yersinia* strains. It is also known to be produced by many *Salmonella* and *E. coli* strains.

Vibrio is commonly considered as an etiological agent for animal and human diseases. The most important members of the genus are *V. cholerae* and the marine pathgens *V. vulnificus, V. angiullarum, V. harveyii* and *V. parahaemolyticus*. Vibriobactin is produced by *V. cholera* (Wyckoff *et al.* 2007, Butterton and Calderwood, 1994; Butterton *et al.* 2000). While the siderophores produced by other members of the genus are anguibactin (*V. anguillarum*) (Mazoy and Lemos 1991), vulnibactin (*V. vulnificus*) and vibrioferrin (*V. parahaemolyticus*) (Lorenzo *et al.* 2004). Transferrin is known to be produced by *N. gonorrhoeae***.** Alcaligin is a dihydroxamate siderophore produced by *B. pertussis.* Petrobactin and bacillibactin are produced by *Bacillus spp***.**

Some terrestrial bacteria such as the mycobacteria are known to produce amphiphilic siderophores. Other bacteria have been reported to produce selected single or small groups of amphiphilic siderophores, including the ornibactins and corrugatin, peptide amphiphiles (Fig. 1.7), as well as rhizobactin 1021 and acinetoferrin which are citrate-based amphiphiles (Fig. 1.8). Ornibactins are produced by *Burkholderia sp.*, and corrugatin is produced by

Pseudomonas corrugata (Meyer JM *et al.* 1995, Stephan *et al.* 1993). These are hydrophilic acyl-appended peptide siderophores that possess short fatty acid tails relative to the longer hydrophilic peptide headgroup. Acinetoferrin is produced by *Acinetobacter hemolyticus* (Okujo *et al.* 1994). It is structurally related to schizokinen, rhizobactin 1021, and to the marine synechobactins by variations in the acyl appendage. While rhizobactin 1021 is thought to be synthesized from schizokinen, the biogenic precursors of the synechobactins and acinetoferrin are not yet known. Acinetoferrin, like the ornibactins, lies within the hydrophobic spectrum of amphiphilic siderophores, and it partitions into bilayer membranes like the other citrate siderophores (Fadeev *et al.* 2004).

Mycobacteria produce suites of two related siderophores, each containing the same headgroup. The mycobactins are lipophilic siderophores with long fatty acid tails that

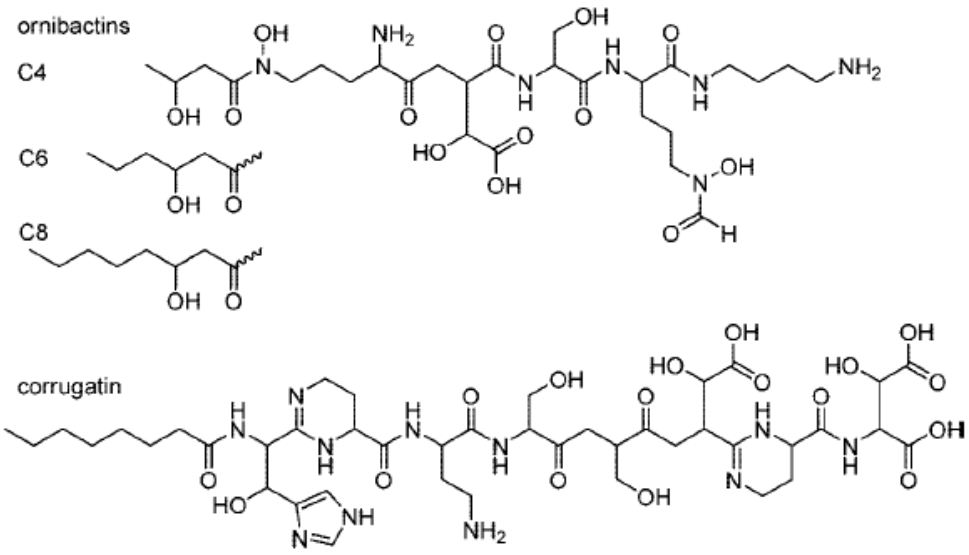

Fig. 1.7: Structures of the ornibactins and corrugatin (Ref: Sandy and Butler 2009).

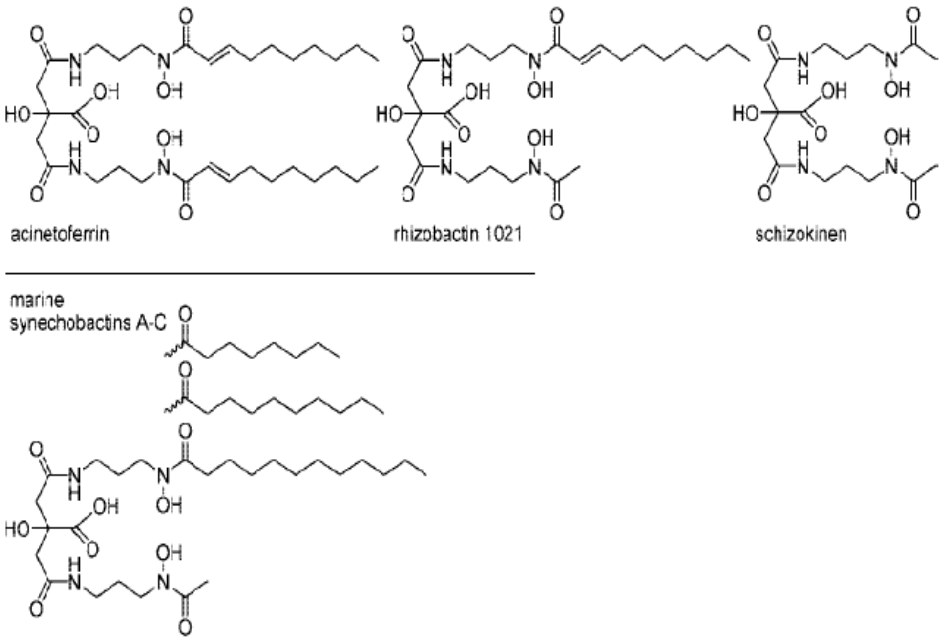

Fig. 1.8: Comparison of the amphiphilic citrate siderophores of acinetoferrin rhizobactin 1021 and the synechobactins to the hydrophilic schizokinen siderophore (Ref: Sandy and Butler 2009).

reside in the bacterial membrane, and the carboxymycobactins, which are released from the bacterium, are hydrophilic siderophores distinguished by shorter fatty acids that have a carboxylic acid at the end of the fatty acid chain, such as shown in Fig. 1.9 for mycobactin T and carboxymycobactin T produced by *Mycobacterium tuberculosis*, the causative agent of tuberculosis infections. It has been proposed that iron uptake occurs by transfer of Fe^{+3} from the carboxymycobactins to the membrane-associated mycobactin (Gobin and Horwitz, 1996). However, a new pathway for iron uptake by mycobacteria is under investigation. Mycobactin J has been shown to partition into macrophages and then to sequester Fe^{+3} from the macrophage iron pools. The presumably reduced membrane affinity of the Fe^{+3}- mycobactins would then localize them to the cytoplasm in the form of proposed self-assembled "liquid droplets" (Luo *et al.* 2005, Barry and Boshoff 2005). Of importance for bacterial growth, this form of non-membrane partitioned Fe^{+3}-mycobactins may be more readily recognized and taken up by the mycobacterium. This new strategy suggests that mycobactins although cell-associated, may under certain conditions be released from the bacterium and able to sequester iron from uninfected cells.

1.4.2 Marine siderophores:

Marine bacteria are known to require micromolar concentration of iron, however, iron concentration in the surface waters of the oceans is only 0.01-2 nM (Sandy and Butler 2009). Therefore, many of the marine bacteria belonging to alpha and gamma proteobacteria produce siderophores. Marine siderophores can be structurally categorized into two major groups: (*a*) Siderophores that are produced as suites of amphiphiles and differ in the chain length of a fatty acid appendage (Fig. 1.10) (Ito and Butler 2005,

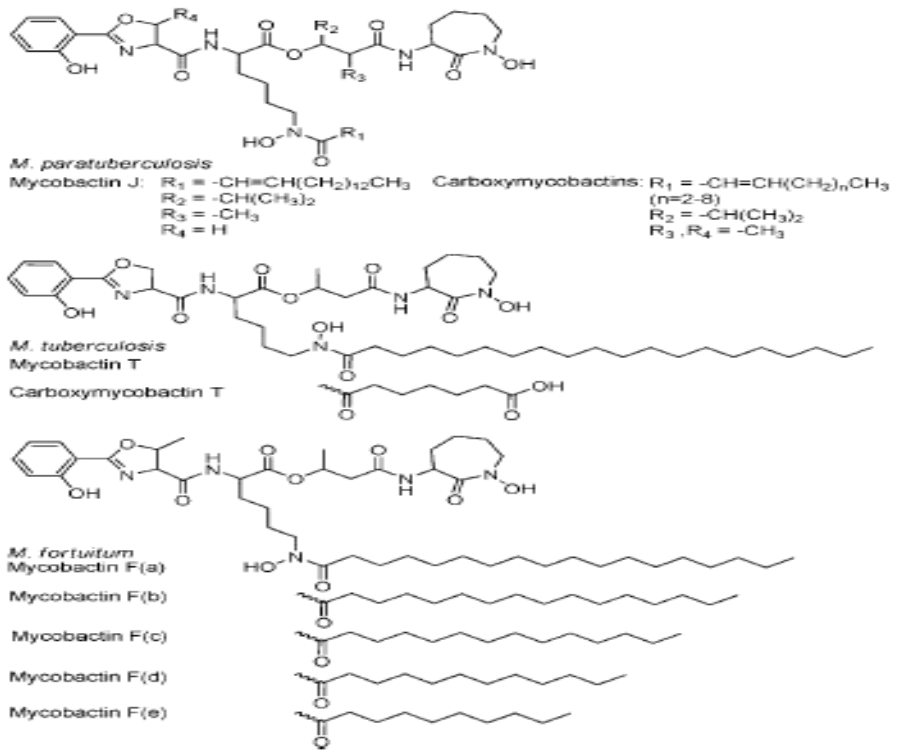

Fig. 1.9: Structures of mycobactins and carboxymycobactins produced by *Mycobacteria*(Ref: Sandy and Butler 2009) .

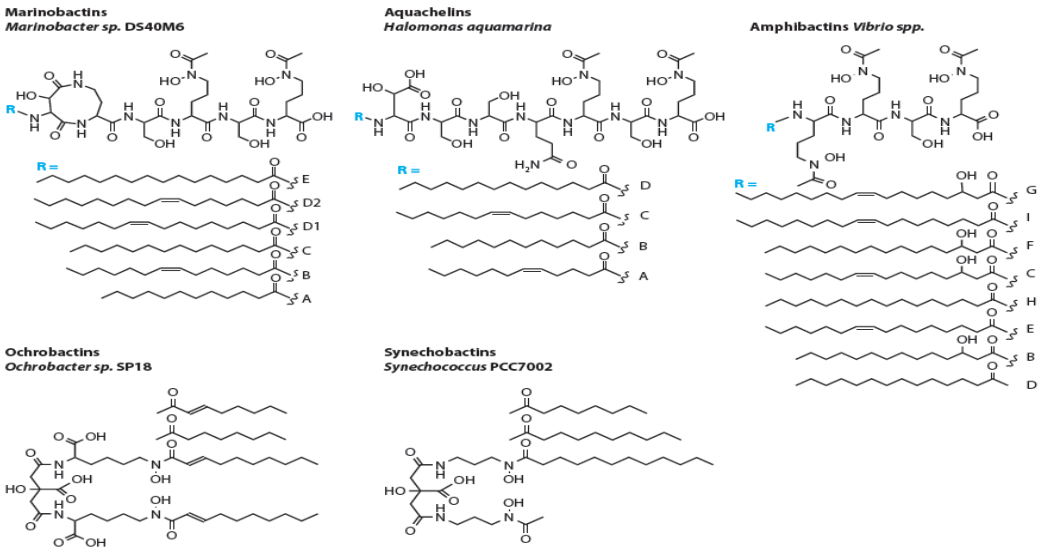

Fig. 1.10: Amphiphilic marine siderophores, including marinobactins, aquachelins, amphibactins, ochrobactins, and synechobactins (Ref: Vraspir and Butler, 2008).

Martin *et al.* 2006, Martinez *et al.* 2003, Homann *et al.* 2009), and (*b*) siderophores that are produced with an α-hydroxy carboxylic acid moiety, which is photoreactive when coordinated to Fe^{+3} (Kupper *et al.* 2006, Barbeau *et al.* 2001, Barbeau *et al.* 2003, Barbeau *et al.* 2002, Hickford *et al.* 2004, Barbeau 2006). The aquachelins, marinobactins, ochrobactins, and synechobactins in Fig. 1.10 coordinate Fe^{+3} by both oxygen atoms of each hydroxamate group and both oxygen atoms of the α-hydroxy carboxylate group. The amphibactins coordinate Fe^{+3} with the three hydroxamate groups. The siderophores in Fig. 1.11 all contain one or two α-hydroxy carboxylate groups (i.e., either β-hydroxy aspartic acid as in the alterobactins and pseudoalterobactins or citric acid as in aerobactin and the petrobactins). The alterobactins and pseudoalterobactins coordinate Fe^{+3} via the two β-hydroxy aspartate moieties and one catecholate group, whereas petrobactin and petrobactin sulfonate coordinate Fe^{+3} with the two catecholates and the α-hydroxy acid portion of the citrate backbone.

The most characteristic and distinguishing feature of marine siderophores is their photoreactivity (Barbeau *et al.* 2001). Siderophores that contain α-hydroxycarboxylate moiety when in complex with Fe^{+3} undergo oxidation and Fe^{+3} is reduced to Fe^{+2}. Fatty acid – containing marine siderophores have the important property of amphiphilicity the degree of which varies in marine siderophores. Such variations in amphiphilicity arises due to the differences in the head-group composition relative to the fatty acid chain length. Amphibactins and ochrobactins are highly hydrophobic and are extracted from the bacterial pellet (Martin *et al*. 2006, Martinez *et al*. 2003) whereas aquachelins are isolated from aqueous supernatant

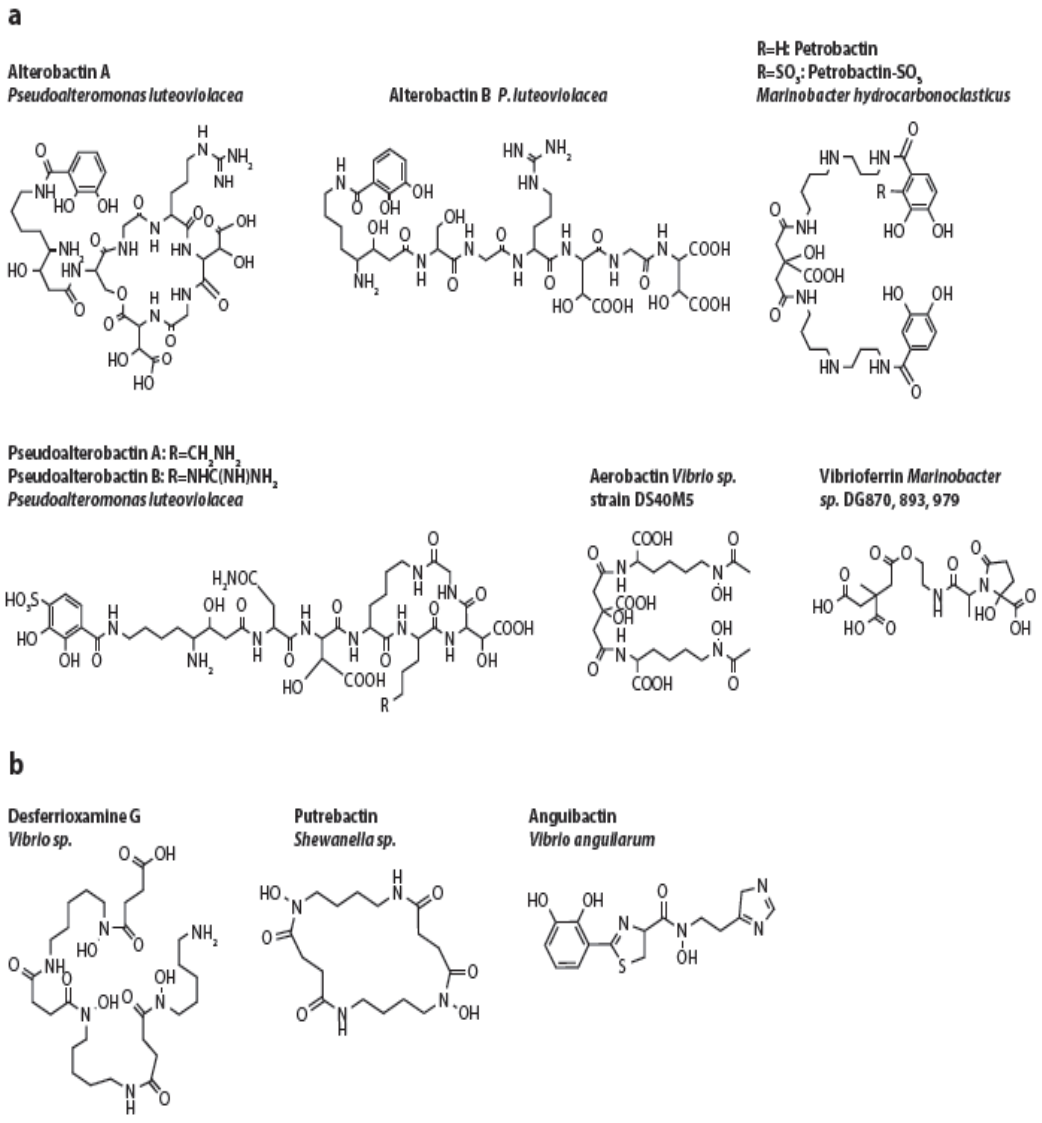

Fig. 1.11: Other marine siderophores, including (*a*) alterobactins , pseudoalterobactins, aerobactin, petrobactin, petrobactin-SO3, and vibrioferrin and (*b*) desferrioxamine G, putrebactin, and anguibactin (Ref: Vraspir and Butler, 2008).

which indicates their hydrophilic nature. Synechobactins are isolated from the supernatant as well as the pellet (Ito & Butler 2005). Hydrobphobic amphibactins have smaller peptide portion consisting of 4 amino acids while fatty acid chains are longer. Marinobactins and aquachelins have longer peptide chain consisting of 6-7 amino acids and a shorter fatty acid chain. The ochrobactins are also quite hydrophobic as a result of 2 fatty acid appendages, and a small head group, whereas the synechobactins, with a similarly small head group, are less hydrophobic than the ochrobactins as they have only one fatty acid (Vraspir and Butler, 2008).

Other marine organisms that have been studied for siderophore production are: *Marinobacter hydrocarbonoclasticus* (petrobactin), *M. aquaeolei* (petrobactin sulfonate(s)), *Aeromonas hydrophila* (amonabactins), and fish pathogens such as *Vibrio anguillarum*, which produces vanchrobactin and anguibactin (Fig. 1.12). Petrobactin produced by *M. aquaeolei* is different from the petrobactin produced by *B. anthracis* in that it a mono or di sulphonated derivative. Sulphonation of the catechol group has also been observed in pseudoalterobactin which is structurally related to alterobactin (Sandy and Butler, 2009).

1.5 Factors affecting siderophore production

1.5.1 Iron concentration

Iron concentration is the most important factor which regulates siderophore production, since they are produced under iron-limiting conditions (Budzikiewicz 1993, Vasil and Ochsner 1999, Visca *et al.* 1993). Iron availability depends upon the environment in which the microorganism is growing. In an intensively aerated liquid

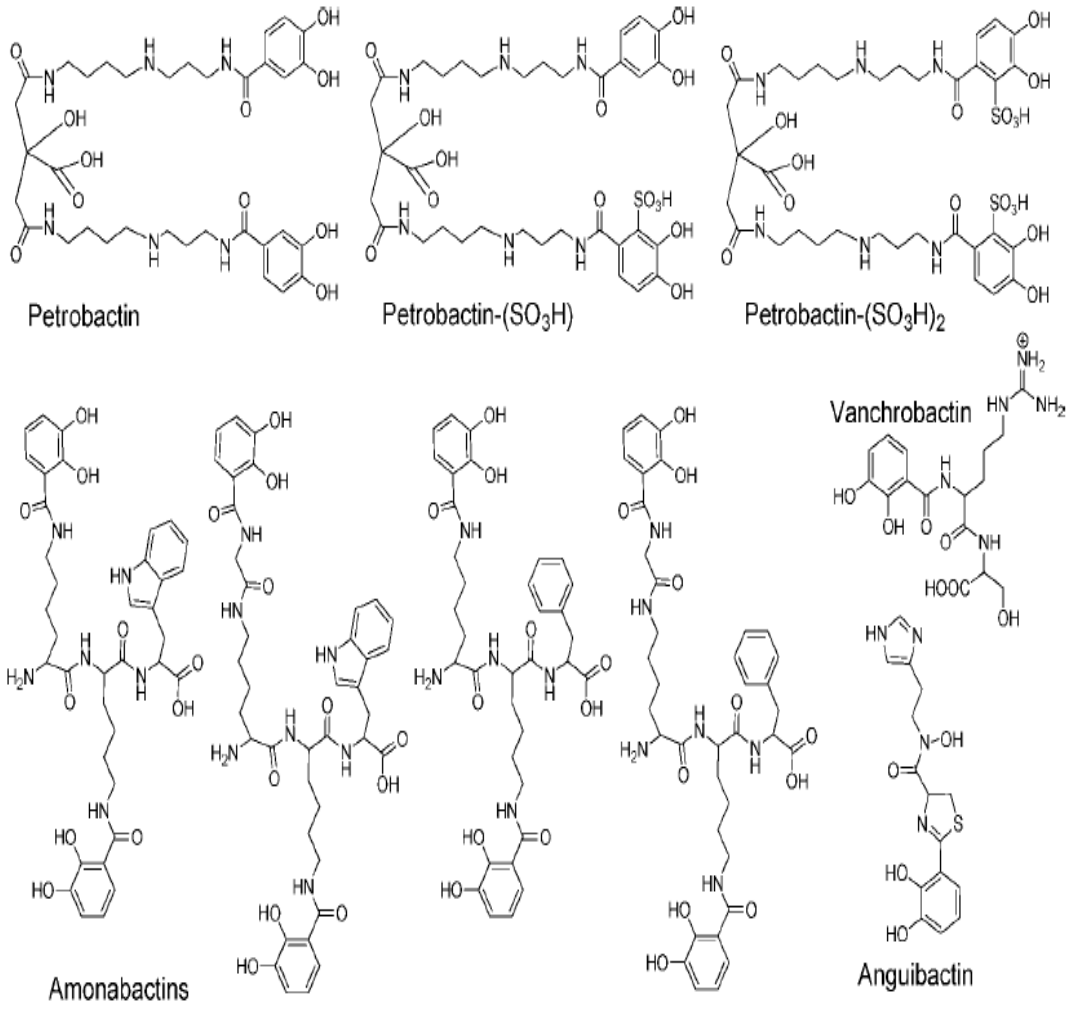

Fig. 1.12: Other siderophores produced by marine pathogens and oceanic bacteria: petrobactin, petrobactin-(SO_3H), and petrobactin- (SO_3H)$_2$ (*M. hydrocarbonoclasticus, Marinobacter aquaeolei VT8*); vanchrobactin and anguibactin (*Vibrio anguillarum*); amonabactins (*Aeromonas hydrophila*). (Ref: Vraspir and Butler, 2008)

medium; iron exists mainly in the form of Fe^{+3} which is extremely insoluble at neutral pH. Thus, the increase of oxygen pressure in the culture broth can reduce iron availability (Kim *et al.* 2003). With laboratory culture media, the amount of siderophores produced by iron-sufficient growth can be as little as 0.1% of that produced under iron-deficient conditions. It is therefore necessary to remove the traces of iron from the culture medium for a successful production of siderophores (Messenger and Ratledge 1985).

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

1.5.2 Nature of nitrogen and carbon source

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

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

Siderophore production can also be achieved with several organic substrates (Meyer and Abdallah 1978). Glycerol used as the carbon source in different media (Nowak-Thompson and Gould 1994), for an example the King´s Medium B (King *et al.* 1954), stimulates

pyoverdine production. Duffy and Defago (1999) have reported increased pyochelin production in *Pseudomonas fluorescens* CHAO with glucose but not with glycerol, however; salicylic acid production was found to increase significantly with glycerol. Use of sodium succinate has also been reported for increase in siderophore production (Meyer and Abdallah 1978, Boruah and Kumar 2002, Sharma and Johri 2003).

1.5.3 Changes in pH during siderophore production

Bultreys and Gheysen (2000) have reported marked changes in pH during the production of siderophores by *Pseudomonas* strains, grown in different media. In a medium containing asparagine, glucose and salts (GASN medium), the pH decreased from 7 to 4.6 after one day, increased to 6.6 on the second day and rose to 7.5 on the third day. The increase in pH resulted in sharp increase in siderophore concentration. Some authors (Villegas *et al.* 2002) have noted insignificant pH shift (from 7 to 7.5) despite a significant increase in siderophore production by *Pseudomonas aeruginosa* PSS when cultured in a succinate medium with sodium succinate as the carbon and energy source. pH also determines the type of siderophore produced by the microorganisms. For example, yersiniabactin and salmochelin are the dominant siderophores produced by *E. coli* under neutral to alkaline conditions, whereas, production of enterobactin and aerobactin increased under more acidic conditions (Sandy and Butler, 2009).

1.5.4 Temperature

Temperature is an important factor influencing siderophore production especially by *Pseudomonas* strains. All the known species of *Pseudomonas*, generally grow quite well at 28–30 °C (Todar 2004) while siderophores synthesis of some fluorescent *Pseudomonas* is inhibited above 33 °C (Loper and Schroth 1986). Meyer *et al.* (1996) have reported that

Pseudomonas aeruginosa ATCC 15692 (PAO1 strain) in a succinate medium shows a drastic loss in pyoverdine production when the cells are grown at temperatures ranging from 40 to 43 °C without any effect on growth. Neither pyoverdine nor pyochelin was detectable in the growth supernatants obtained from iron-starved cultures incubated at 43 °C. Digat and Mattar (1990) found that strains of fluorescent *Pseudomonas putida* from tropical origin, grew normally at 35 °C with siderophore production. At this temperature, most strains from temperate countries grew quite slowly or did not grow at all without the production of siderophores.

1.5.5 Metal ions

Metals other than iron also stimulate or inhibit siderophore production in a number of bacteria, even in the presence of high iron concentrations. For example, Duhme *et al*. (1998) have studied effect of Mo on siderophore production by *Azotobacter vinelandii* and found that at concentrations up to 100 mM of Mo, azotochelin production is activated, whereas at higher metal concentrations the synthesis of the siderophore is completely repressed. Reports also show that high concentrations of aluminum increases the production of schizokinen and *N*-deoxyschizokinen (two hydroxamate siderophores) in iron-limited cultures but not in iron-rich cultures of *Bacillus megaterium* (Hu and Boyer, 1996). Pyoverdine production is induced by 10 mM Al^{3+}, Cu^{2+}, Ga^{3+}, Mn^{2+} and Ni^{2+} when grown in iron-limited succinate medium (Braud *et al*. 2009a). Teitzel *et al.* (2006) have made an interesting observation wherein under iron-limited conditions, exposure to 10 mM Cu^{2+} up-regulates genes involved in the synthesis of pyoverdine and down-regulates those involved in the synthesis of pyochelin. Increase in pyoverdine production even in the presence of iron (100 mM), 10 and 100 mM Cu^{2+} and Ni^{2+} (290 and 380% respectively) and to a lesser extent by 100 mM Cr^{2+} (134%) has been reported by Braud *et al.* (2010). In all these experiments, metals were added at the

beginning of the cultures. Most of these studies have been carried out with only a single or in some cases 2 metal concentrations and not for a large range of concentrations. It is probable that as iron concentration regulates siderophore production, it can be both activated and inhibited by many of these metals, depending on their concentrations. Therefore, studies involving varied concentrations of each metal are necessary to establish their exact roles in regulation of siderophore production.

It is not clear how metals other than iron stimulate siderophore production. One possible explanation is that the free siderophore concentration in the medium is reduced in the presence of other metals as a result of complex formation. Such a decrease in the siderophore concentration may be sufficient to activate further secretion of additional siderophore into the medium. Braud *et al*. 2009b have reported pyoverdine concentration in an overnight culture of about 120 mM (in succinate medium), a concentration much higher than the 10 mM of metal added to the culture, indicating that the amount of siderophore is not limiting under this experimental system. The same study has revealed that all metals which result in increase in pyoverdine production (Al^{3+}, Cu^{2+}, Cr^{2+}, Ga^{3+}, Mn^{2+} and Ni^{2+}) are also able to inhibit the pyoverdine–^{55}Fe uptake. Further, such metals are reported to be transported into the cells of *P. aeruginosa* cells via the pyoverdine pathway (Braud *et al*. 2009b). It is therefore probable that these pyoverdine–metal complexes are able to interact with FpvA, at the cell surface. The stimulation of pyoverdine production by metals other than iron may involve the FpvR/PvdS signalling cascade being activated by the pyoverdine–metal complex binding to FpvA at the cell surface.

Induction of siderophore production by heavy metals indicates their role in bacterial heavy metal tolerance. Toxic metals enter the periplasm of Gram-negative bacteria mostly by

diffusion across the porins (Lutkenhaus 1977, Pugsley and Schnaitman 1978, Li *et al*. 1997). The binding of metals to siderophores in the extracellular medium reduces the free metal concentration, affecting diffusion (the molecular mass of the resulting siderophore–metal complex is too great for diffusion via porins) and therefore their toxicity. Growth assays have also shown that *P. aeruginosa* strains capable of producing pyoverdine and pyochelin are more resistant to metal toxicity than a siderophore non-producing strain (Braud *et al*. 2010). The presence of the siderophores decreases metal accumulation in bacteria as monitored by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES): pyochelin reduces the uptake by > 80% of Al^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} and pyoverdine decreases the uptake of Co^{2+}, Eu^{3+}, Ni^{2+}, Pb^{2+}, Tb^{3+} and Zn2+ by around 80% and of Al^{3+} and Cu^{2+} by around 40% (Braud *et al*. 2010). Pyoverdine is seen to be more effective than pyochelin in inhibiting the diffusion of nonbiological metals: Eu^{3+}, Ga^{3+}, Pb^{2+}, Sn^{2+} and Tb^{3+}, whereas pyochelin is more effective against biological metals: Co^{2+}, Fe^{3+}, Ni^{2+} and Zn^{2+}. These studies with *P. aeruginosa* show that siderophores are able to sequester metals in the extracellular medium and probably prevent their diffusion across the bacterial membranes.

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

mechanism for the uptake of toxic metals. Therefore, the ferrisiderophore pathways must have high metal specificity to transport or accumulate only the appropriate metal(s).

1.6 Applications of siderophores

1.6.1 Agriculture

Siderophores play an important role in the microbial interactions in a rhizosphere enhancing the growth of plants (Pandey *et al*. 2005, Mahmoud and Abdalla 2001, Dobbelaere *et al.* 2003). Siderophore production enables bacteria to compete with pathogens by removing iron from the environment (O´ Sullivan and O´Gara 1992, Persello-Cartieaux *et al.* 2003). Siderophore production is very common among pseudomonads (O´Sullivan and O´Gara 1992), *Frankia* (Boyer *et al.* 1999) and *Streptomyces* spp. (Loper and Buyer 1991). *Pseudomonas* spp. are the most studied group of rhizobacteria and have potential for rapid and aggressive colonization. Pyoverdines produced by fluorescent pseudomonads form chelates with iron and make it unavailable to pathogens. Iron starvation also prevents germination of fungal spores (O´ Sullivan and O´Gara 1992). Interestingly, siderophores are also thought to bring about disease suppression in plants by inducing systemic resistance (Bakker *et al.* 2003). A number of plants possess a heterologous iron uptake mechanism for acquisition of iron through an iron-bacterial siderophore complex (Sharma *et al.* 2003). Other mechanisms for augmenting plant growth are by plant growth promoting rhizhobacteria are: increased root development, nitrate uptake, phosphate solubilization and control of soil-borne pathogens by competing for limiting nutrients (Smith and Read 1997).

1.6.2 Medicine

Sideromycins are a complex of antibiotics which are covalently linked to siderophores. The transport process is active and energy coupled and therefore increases the efficiency of

antibiotic activity. The minimal inhibitory concentration of antibiotics required is at least 100 - fold lower than that of antibiotics which enter the cells by a mere diffusion process (Braun *et al.* 2009). The term sideromycin was coined for naturally occurring Fe^{3+}-siderophores that are covalently linked to an antibiotic moiety. Sideromycins were actually discovered prior to siderophores in a sceening for new antibiotics and were originally called sideramines or siderochromes (Nu¨esch and Knu¨sel 1967). Siderophores were later found to antagonize the activity of sideromycins. It was initially assumed that the siderophores compete with the sideromycins at the sideromycin target sites. It was then shown that siderophores and sideromycins compete for common uptake systems. Very few naturally occurring sideromycins have been found. Albomycin and salmycin are such examples. Albomycin consists of a siderophore structurally similar to ferrichrome which is connected to the antibiotic thioribosyl pyrimidine (Hartmann *et al.* 1979; Braun *et al.* 1983). Salmycin is a sideromycin which exclusively acts against Gram-positive bacteria.

The naturally occurring sideromycins have prompted scientists to design siderophores that can be conjugated to antimicrobial agents and targeted against microorganisms. A wide spectrum of siderophore–antibiotic conjugates have now been chemically synthesized. Although binding of an antibiotic brings about a drastic change in the shape of the siderophore, the complex is still recognized by the receptor proteins on the outer membrane and transported across the cell memebrane. Monodentate siderophore antibiotics consists of catechols or hydroxamic acids conjugated to β- lactam antibiotics at C-6 or C-7 position. These conjugates have excellent activity against Gram-negative bacteria even if the initial drug cannot penetrate the cell. Bi and tri dentate siderophore antibiotics consist of two or three bidentate ligands similar to natural sideromycins. Such ligands provide more stable

binding with iron and improved recognition and assimilation (Girijavallabhan and Miller, 2004).

Siderophores have also been used as therapeutic agents in treating iron overload in patients undergoing repeated blood transfusions. The most common drug to treat iron overload is siderophore desderrioxamine B (Desferal), however, it has poor oral activity, short retention time in the body, slow iron chelation and requires daily subcutaneous or intravenous treatments to remove excess iron. Therefore, new iron chelators with better pharmacological effects are being sought.

1.6.3 Taxonomy

Pyoverdines with different structures have been discovered over a period of time which necessitates a method which can determine the novelty of the pyoverdine under study. For this purpose, several analytical and biological methods have been tested and the two important methods namely Iso Electric Focusing (IEF) and pyoverdine mediated iron uptake are being extensively used (Bultreys 2007).

Siderotyping is an elctrophoretic method which has been used conventionally for discriminating proteins on the basis of their isoelectric pH values. Strains producing identical pyoverdines usually have the same IEF profile while strains producing different pyoverdines demonstrate different IEF patterns (Budzikiewicz 2004). Non-fluorescnt siderophores which are produced by fluorescent pseudomonads can be detected by an overlay of 1% melted agarose in CAS reagent which reveals siderophores as yellow to pink spots appearing at the surface of the gel. The pH can then be determined by constructing a calibration curve using commercially available standards or by slicing the electrophoresed gel into 0.5 cm bands and

incubating it in 2 ml of 10 mM KCl for 30 minutes before measurement of the pH (Bultreys A 2007). IEF patterns have been established for a small collection of pyoverdines which are now used as internal standards for pH_i determination. However, with an increasing number of pyoverdines being identified, their discrimination by IEF has become less reliable. Pyoverdines differing slightly in their structure have almost identical IEF pattern (eg. pyoverdine of *P. fluorescens* 1.3 and *P. putida* ATCC17400 which differ by a single neutral amino acid) (Fuchs *et al*. 2001). At the same time, structurally different pyoverdines have been shown to have somewhat modified IEF, differing by the number of isoform bands. Therefore this method is usually combined with pyoverdine mediated iron uptake. Iron uptake via siderophore is measured by incubating iron-starved cells in a non proliferating incubation medium in the presence of a ferrisiderophore labeled with ^{59}Fe. The cells are then filtered and counted for radioactivity.

The main goal of siderotyping is to detect novel structures of pyoverdine by analyzing strains with a particular siderotype. Strains showing identical pyoverdine pattern and 100% cross reaction are classified in the same bacterial group termed siderovar. Many siderophores differing in IEF pattern and as indicated by cross reaction are characterized by a different pyoverdine structure. Siderotyping is useful in *Pseudomonas* taxonomy. From siderotyping studies done with numerous strains belonging to taxonomically well defined new taxa (13 species and 26 clusters or genomospecies) it has been observed that all strains belonging to a given species have identical siderophore structure and produce an identical pyoverdine and that each species is characterized by a different pyoverdine (Bultreys A 2007). After determining the type of siderophore produced, the taxonomically undefined fluorescent pseudomonads can be classified in a particular siderovar. Once the siderovars are defined in a species, pyoverdins are accurate tools for identification. This can be achieved by comparing

with a reference (Bultreys *et al.* 2000, Bultreys *et al.* 2003) or a general database regrouping the characteristics of all the pyoverdins can be consulted (Meyer and Geoffroy 2004). Isoelectrophoresis is the most convenient siderotyping method, especially when numerous strains have to be analyzed. Thus, studies related to taxonomy, epidemiology, and population diversity should benefit from a technique which allows at low cost the analysis of up to 100 isolates within a day.

1.6.4 Biodegradation of petroleum hydrocarbons

Literature survey reports that not much work has been reported on the role of siderophores in biodegradation of hydrocarbons. Barbeau *et al.* (2002) have isolated an oil degrading marine bacterium, *Marinobacter hydrocarbonoclasticus,* which produces a siderophore petrobactin that undergoes a light mediated decarboxylation when bound to Fe^{+3}.They have proposed that the siderophore may play a role in the biodegradation of petroleum hydrocarbons in marine ecosystems by facilitating iron uptake by microorganisms.

1.6.5 Phytoremediation

Heavy metal pollution of soils is a serious problem and demands efficient clean up of the polluted areas (McGrath *et al.* 1995). Most of the conventional methods used for soil remediation are not economical and result in the deterioration of soil texture and its organic content (Rajkumar *et al.* 2010). One of the emerging technologies for bioremediation of metal contaminated soils is "microbial assisted phytoremediation"; a process of utilizing plants to absorb, accumulate and detoxify contaminants in soil through physical, chemical and biological processes (Prasad *et al.* 2010) in the presence of Plant Growth Promoting Rhizobacteria (PGPR). These rhizhosphere bacteria are known to support the growth and sustenance of plants. Besides, some of these bacteria are also metal resistant paving the way

for the benign technology for reclamation of metal polluted soils. An important characteristic of PGPR for use as a bio-inoculant in this technology is the siderophore production (Jing *et al.* 2007).

Production of siderophores in presence of metals can be a useful trait for plant growth promoting organisms as the soils which are contaminated with metals are often iron deficient (Tank and Saraf, 2009). In fact, siderophore producing bacteria have been considered important for inducing metal tolerance in plants and for promoting metal accumulation in plants. The effects of siderophore producing bacteria on the uptake of metals by hyper-accumulator plants have been the focus of increased attention (Dimkpa *et al*. 2008, Braud *et al.* 2009b). Braud *et al*. (2009b) have reported an increase in the bioavailability of Cr and Pb in soils inoculated with *P. aeruginosa*. Bacterial siderophores can also provide iron to various plants which helps in reducing metal toxicity (Bar-Ness *et al*. 1991, Reid *et al*. 1986, Wang *et al*. 1993). Such beneficial effects exhibited by siderophore producing bacteria implicate that the inoculation with metal-resistant siderophore producing bacteria may help in improving the process of phytoextraction in metal contaminated soils.

Chapter 2

Siderophore producing bacteria from coastal ecosystem: isolation, screening and identification of the selected isolates

2.1 Introduction

The aim of the present study was to understand the distribution and occurrence of siderophore producing bacteria in two distinct ecosystems: coastal sand dunes, which represent nutrient deficient ecosystems, and mangroves, which was chosen to represent nutrient rich ecosystems.

Coastal sand dune plants harbor a diverse community of microbes associated with their rhizhosphere and roots (Lee *et al.* 2006, Park *et al.* 2005). Sand dune ecosystems are described as mounds of sand with vegetation, found along the coastal areas (Arun *et al.* 1999). Dunes are characterized by low nutrients, drought, high salinity and sand erosion. Plants growing in sand dune habitats help in stabilization of the same and are controlled by the interaction between the biotic and physicochemical components of the sand matrix (Arun and Sridhar 2004). The interactions between plants and bacteria help plants to settle in ecosystem restoration process (Glick 1995, Egamberdiyeva 2005). Plant microbe symbioses have been exploited in programs of sand dune restoration. Arbuscular mycorrhizal fungi important to some sand dune plants have been used in restoration projects of various coastal sand ecosystems (Sylvia and Burk 1988). Despite the role of bacterial diversity in sand dune plant communities, very few reports are available on the distribution and abundance of rhizhosphere-associated bacteria.

Mangrove ecosystems are rich in organic matter in contrast to sand dune ecosystem however, they are deficient in nitrogen and phosphorous (Sengupta and Chaudhuri 1991, Holguin *et al.* 1992, Alongi *et al.* 1993, Vazquez *et al.* 2000). Efficient nutrient recycling in a mangrove

ecosystem helps to retain the scarce nutrients within the ecosystem making it the most productive ecosystem (Bashan and Holguin 2002).

This study includes the determination of viable counts of the samples obtained from sand dune and mangrove ecosystems and the isolation of bacteria which showed different cultural characteristics. The isolates were further screened for siderophore production to understand the distribution of siderophore producing bacteria in these ecosystems. Furthermore, potential isolates were identified based on their morphological, cultural and biochemical properties and on 16S rDNA sequence analysis.

2.2 Materials and Method:

2.2.1 Collection of samples

Sand dune samples were collected from Miramar beach, Goa. Four samples were collected from the rhizhosphere of a sand dune creeper, *Ipomoea pes- caprae* and four samples were collected from the non rhizhosphere region. Water and sediment samples were collected from the mangrove at Ribander and Merces.

2.2.2 Determination of the iron content of the samples

The sand samples were digested to determine the iron content as described by Jarvis and Jarvis (1985). Briefly, 0.2 g of the sand sample was digested with 10 ml of a mixture of hydrofluoric acid: nitric acid, and perchloric acid (7: 3: 1) and dried on a hot plate at 150ºC. After drying, 5 ml of the above mixture was added and dried on the hot plate for 1 hour. Further, 2 ml of concentrated HCl was added and dried completely. The residue was dissolved in 10 ml of 1:1 HNO_3 in deionised water, diluted to 25 ml with Milli Q water and

the concentration of Fe was determined by Atomic Absorption Spectrophotometer (AAS) (Shimadzu 6300).

2.2.3 Determination of the viable counts of the samples

Samples were serially diluted in physiological saline and 100 µl of appropriate dilutions of the samples were plated out on microbiological media. Sand dune samples were plated on Tryptone Yeast extract Glucose agar (TYG), Nutrient Agar (NA), NaCl Tryptone Yeast Extract Agar (NTYE) and Polypeptone Yeast extract Glucose agar (PPYG) (Appendix A) while, mangrove samples were spread plated on TYG and NA. Samples were also plated out on medium with CAS. Plates were incubated at room temperature (28 ºC) for 24 h, the colonies were counted and the viable counts of the samples were determined.

2.2.4 Purification and maintenance of the isolates

Predominant colonies were picked up and the isolates were purified by repeated streaking on the respective media. For maintenance, the isolates were streaked on slants, sealed with parafilm and stored at 4 ºC. Working stocks were maintained simultaneously and subcultured every month.

2.2.5 Screening of the isolates for siderophore production

The ability of the isolates to produce siderophores can be detected using the CAS assay described by Schwyn and Neilands (1987). CAS assay is a universal method to detect siderophore producers and is based on the principal that siderophores have high affinity for Fe^{+3}. The complex of chrome azurol sulphonate, iron (III) and hexadecyltrimethylammonium bromide, is blue in colour. When a strong chelator removes iron from the dye, the dye is rendered orange. The assay is based on this colour change from blue to orange and can be

applied to liquid cultures or agar plates. To detect siderophore production by the isolates, the growth medium of the organism was deferrated by adding 8-hydroxyquinoline to ensure complete removal of Fe (Waring and Werkman 1942); CAS solution (Appendix B) was added after autoclaving. The isolates were spot inoculated on plates, incubated for 72 h, and the isolates forming yellow orange zones were selected for further studies.

2.2.6 Utilization of sodium benzoate by selected isolates

The selected bacterial isolates were screened for their ability to utilize sodium benzoate by spot inoculation on mineral salts medium (MSM) (Appendix A) supplemented with 0.1% sodium benzoate as the sole carbon source. Selected isolates were then inoculated in MSM broth supplemented with different concentrations of sodium benzoate (0.1, 0.2, 0.5, 0.75, 1, 1.5 and 2%).

2.2.7 Identification of the selected isolates

i) Morphological and biochemical characterization

The selected cultures were streaked on respective growth media and their colony characteristics were noted after 24 h of growth. Gram staining was used to determine the Gram character of the isolates and also to reveal the morphology of the isolates. Isolates were subjected to routine biochemical tests for their tentative identification according to Bergey's Manual of Systematic Bacteriology (Sneath *et al.* 1986, Krieg and Holt 1984).

ii) Molecular characterization

a) DNA isolation

The genomic DNA was extracted using phenol chloroform isoamyl alcohol extraction method as described by Sambrook *et al.* (1989). 15 ml of overnight grown culture broth was

taken and centrifuged at 5000 rpm for 3 minutes at 4 ^{o}C. Supernatant was removed and the pellet was washed with double distilled water.

467µl of 1X TE (Tris EDTA) buffer (Appendix B) was added to the pellet. 5 µl of lysozyme (10 mg/100 µl distilled water) was added and mixed well. The mixture was incubated at 37 ^{o}C for 30 minutes followed by the addition of 30 µl of 10% SDS. It was incubated at 60 ^{o}C for 10 minutes and an equal amount of phenol : chloroform in a 1:1 ratio was added. The mixture was mixed well and centrifuged at 12,000 rpm for 10 minutes. An equal amount of phenol : chloroform in a 1:1 ratio was added to the aqueous layer to remove any contaminating proteins.

An equal amount of chloroform: isoamyl alcohol (24:1) was added to the aqueous layer. The resulting mixture was centrifuged at 12000 rpm for 10 minutes. 3M sodium acetate (one tenth the volume of aqueous layer) and 0.6 volume of isopropanol was added to the aqueous layer and centrifuged at 12000 rpm for 10 minutes. The supernatant was discarded and 1 ml of ethanol was added to the pellet. This was centrifuged at 12000 rpm for 10 minutes, the supernatant was discarded and the eppendorf tubes were left open overnight to dry the pellet. The tubes were labeled, sealed and stored at -20 ^{o}C.

b) 16S rRNA gene amplification

The 16S rRNA gene was amplified by the polymerase chain reaction (PCR) (BIOER XP cycler, China). The DNA was dissolved in 50 µl TE buffer. 2 µl of the sample was added to 98 µl of the master mix (PCR buffer = 10 µl, Primer F = 01 µl, Primer R= 01 µl, dNTP = 2 µl, Taq pol= 0.75 µl, $MgCl_2$ = 6 µl, dI water = 77.25 µl). PCR was carried out at following conditions, 94ºC → 3 Min, 94ºC → 1Min, 52ºC → 1 Min, 72ºC → 2 Min, 72ºC →

5 Min, steps 2, 3 and 4 were repeated for 35 cycles. 16S rRNA gene was amplified using standard universal forward primer (S-D-Bact-0011-a-5-17:5_-GTTTGATCCTGGCTCAG-3_) and standard universal backward primer (S-*-Univ-1392-b-A-15: 5_-ACGGGCGGTGTGTNC-3_).

Amplification was confirmed, by separating PCR product through a 0.8% agarose gel by electrophoresis (Sambrook *et al.* 1989). 0.4 g agarose was dissolved in 50 ml 1X TAE (Tris acetate EDTA) buffer (Appendix B) by boiling in a microwave for 2 min. 3 µl of ethidium bromide was added to the cooled agarose solution before it solidified. The mixture was poured into a gel casting tray with a comb inserted into it. The comb was removed once the gel solidified and the tray was placed in the electrophoresis chamber. TAE buffer was poured over the gel. 8 µl sample was mixed with 2 µl bromophenol blue (tracking dye) and loaded into the wells. The separation was carried out at 80 Volts for 30 minutes. Once the run was complete, the bands were visualized under a UV trans-illuminator and recorded in a gel documentation system (Alpha Innotech, USA).

c) BLAST and alignment of 16S rRNA gene sequences

The derived 1.5 Kb product was sequenced. The 16S rRNA gene sequence was compared with sequences in the GenBank database using the BLAST search program (Altschul *et al.* 1990) and aligned using the multiple alignments Clustal X program (Thompson *et al.* 1997).

iii) FAME analysis of isolate NAR38.1

24 hour old culture was used for FAME analysis performed by Gas Chromatography using the MIDI Sherlock Microbial Identification System software, version 6.1.

iv) SEM of the isolates TMR2.13 and NAR38.1

The two isolates, TMR2.13 and NAR38.1 were inoculated in tryptone yeast extract glucose broth and nutrient broth respectively. The flasks were incubated at 150 rpm, at 28 ºC for 24 hours. The cells were harvested by centrifugation, washed and suspended in phosphate buffered saline. The cells were fixed onto a coverslip in 2.5% gluteraldehyde fixative (pH 7.2-7.4) overnight 28 ºC and then washed using phosphate buffered saline. The cells were dehydrated with increasing concentrations of acetone (30%, 50%, 70% and 90%) for 10 minutes each and finally in 100% acetone for 30 minutes. The coverslips were placed onto stubs which were then placed in a sputter coater (JEOL JFC1600). After sputtering the specimens with platinum, the stubs were placed onto the electron microscope sample chamber and observed using JEOL JSM – 6360LV scanning electron microscope.

2.3 Results and Discussion

2.3.1 Analysis of sand dune samples

The temperature of the sand dune samples (Fig. 2.1) varied from 34-40ºC. pH of the sand dune samples was slightly alkaline and varied from 7.02-8.12. Iron content of the sand dune samples was found to be low and varied from 0.29% - 0.31% (Table 2.1). Studies on quartz grains from a range of environments from continental and coastal dune fields have reported iron-rich clay coatings on the grains (Bullard and White 2005). The present study shows that the iron level is quite low as compared to that reported the in Saharan dust which contains up to 8 percent of Fe_2O_3 (Goudie and Middleton 2001). Krishnan and Loka Bharathi (2009) have reported iron content of Tuvem mangroves of Goa to vary between 1.1 to 15.1%.

The total viable counts of the sand dune rhizhosphere samples were higher compared to the non – rhizhosphere samples. Plants are known to support microbial growth in their

rhizhosphere region by release of exudates (Phillips *et al.* 2004) or by producing compounds that mimic quorum sensing signals, thus affecting indigenous bacterial communities (Bauer and Mathesius 2004). The total viable counts of sand dune samples were found to be higher on TYG followed by NA and then NTYE. Total viable counts ranged from 9.40×10^3 to 8.96×10^5 cfu/g on TYG, 3.2×10^3 to 4.4×10^5 cfu/g on NA and 6.00×10^1 to 4.06×10^3 on NTYE, respectively (Fig. 2.2). Morphologically distinct colonies were picked up, purified and maintained on

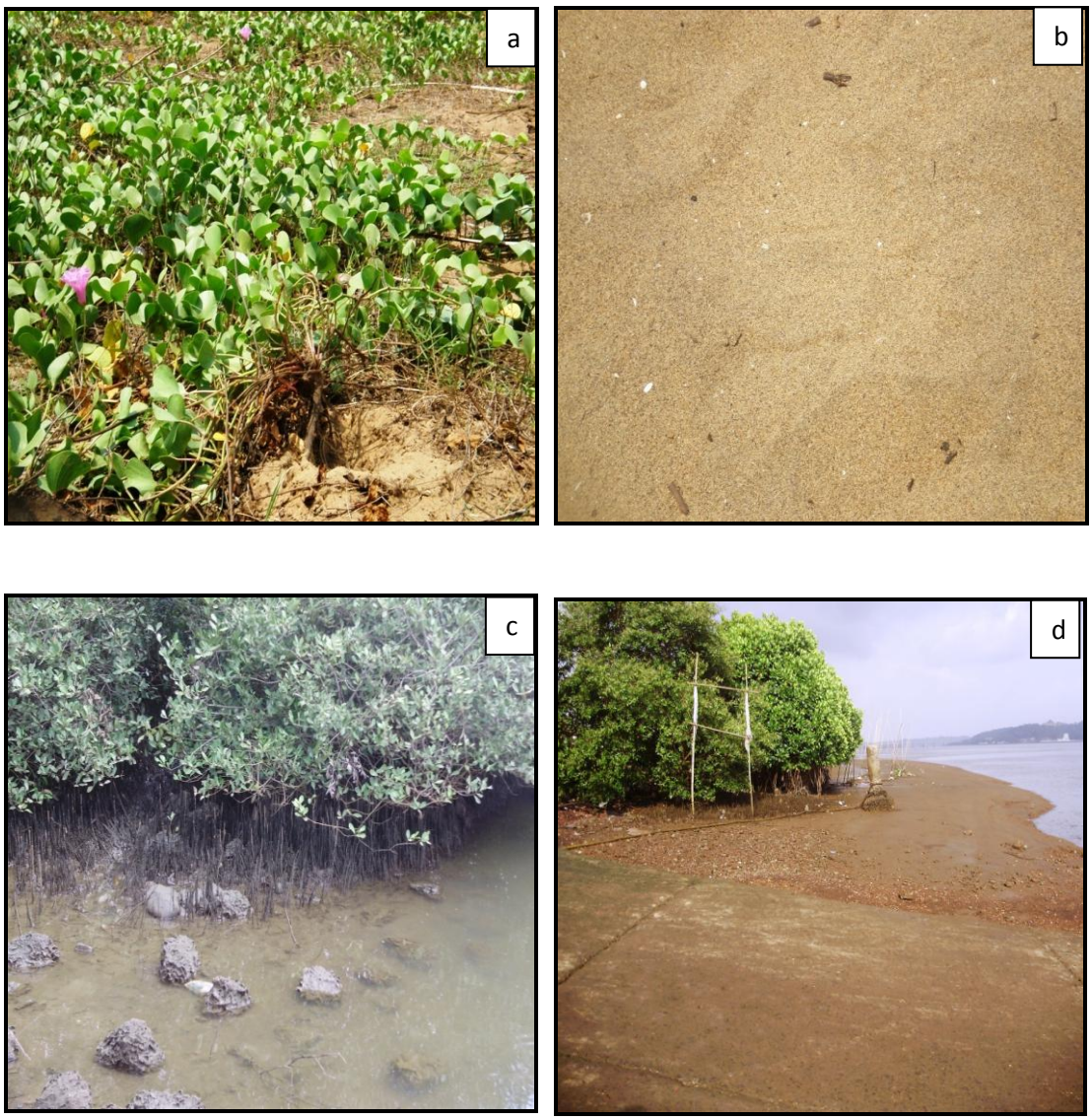

Fig. 2.1: Sampling sites a) Rhizhosphere of sand dune creeper *Ipomoea pes-caprae* at Miramar beach b) Non rhizhosphere region of sand dune at Miramar beach c) Mangrove ecosystem at Merces d) Mangrove ecosystem near Ribander Jetty

respective slants at 4 ºC. A total of 77, 96 and 40 isolates were obtained from NA, TYG and NTYE respectively.

2.3.2 Analysis of mangrove samples

The temperature of the mangrove samples (Fig. 2.1) varied between 31 and 35.6 ºC, while the pH ranged between 6.5-6.8 (Table 2.2). Higher counts were obtained in mangrove sediment samples as compared to the water samples (Fig. 2.3) as reported in earlier studies (Desai *et al.* 2004, Matondkar *et al.* 1981). Mangrove sediments are rich in organic matter due to continuous input of shed foliage which is subsequently degraded by microorganisms (Rawte *et al.* 2002) leading to the high bacterial counts.

A total of 50 and 83 isolates each were obtained from NA and TYG, respectively. An attempt to isolate siderophore producers from mangroves by plating the samples directly on CAS agar plates resulted in the isolation of very few microorganisms, probably due to the toxicity of HDTMA (Yoon *et al.* 2010). The samples were therefore plated on media without CAS and the isolates were then screened for siderophore production.

2.3.3 Screening of the isolates for siderophore production

Soil bacteria largely contribute to nutrient cycling in natural ecosystems (Toledo *et al*. 2001). Such bacteria also help in the growth of plants by supplying the essentially required elements such as C, N, P, Fe etc. Different mechanisms are present in varied bacteria to release these elements from their complex forms. One such mechanism is the production of siderophores to chelate the minimal amount of iron available in an

Table 2.1: Physico-chemical parameters of the sand dune samples

Sampling site	Sample	pH	Temperature ^{o}C	% of Fe
Miramar – Rhizosphere of *Ipomoea – pes caprae*	R1	8.12	37	0.3119
	R2	7.31	36	0.3098
	R3	7.02	35	0.3128
	R4	7.62	34	0.3137
Miramar – Non Rhizosphere	NR1	7.79	38	0.3156
	NR2	7.54	38	0.3156
	NR3	7.75	40	0.2937
	NR4	7.78	36	0.2931

Table 2.2: pH and temperature of the mangrove samples

Sampling site		pH	Temperature ^{o}C
Ribandar	Water	6.8	32
	Sediment	6.8	31
Merces	Water	6.7	35.6
	Sediment	6.5	35

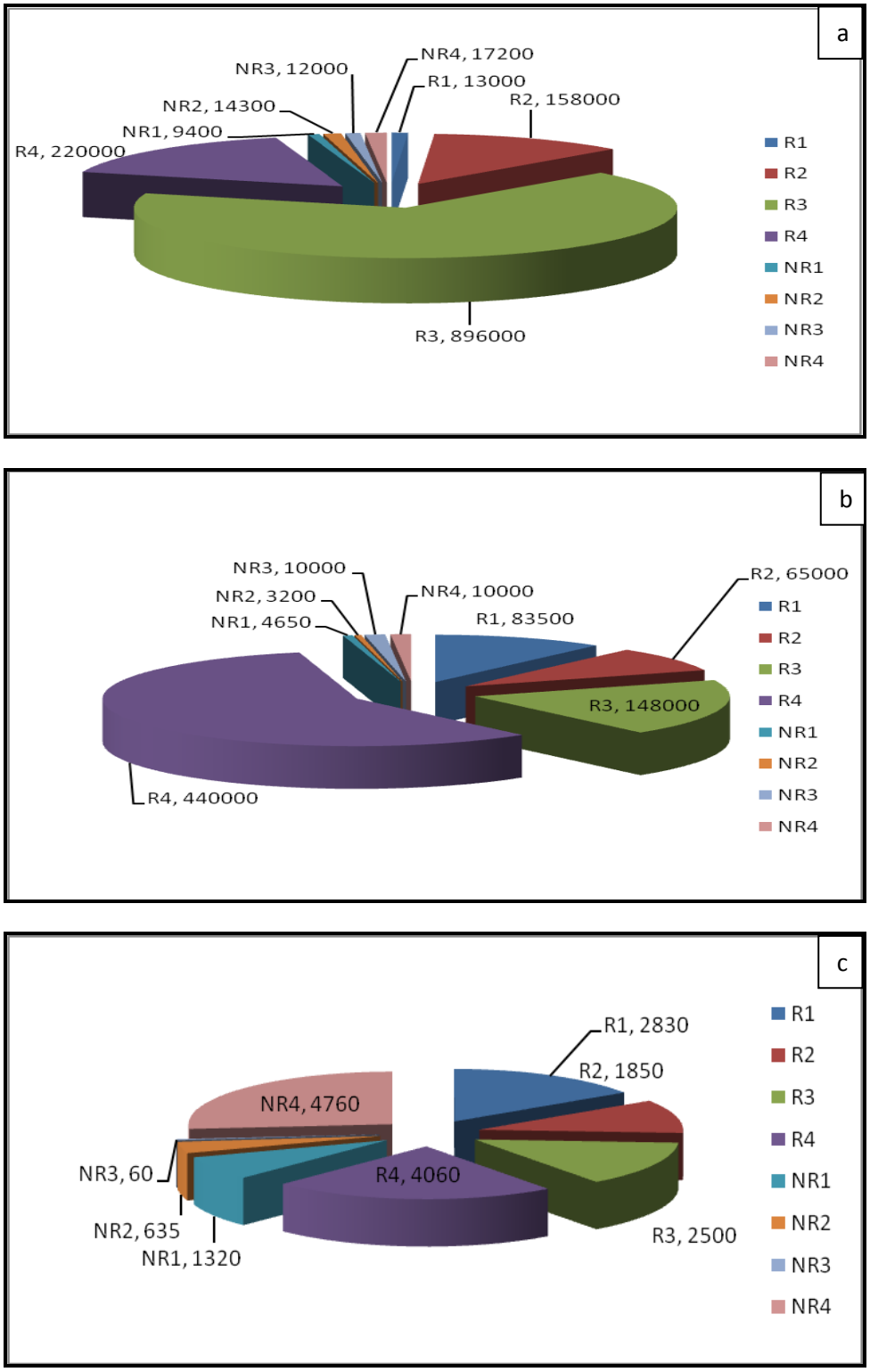

Fig. 2.2: Total viable count of sand dune samples on: a – TYG, b – NA, c- NTYE
Key: R = Rhizosphere samples, NR = Non – rhizosphere samples

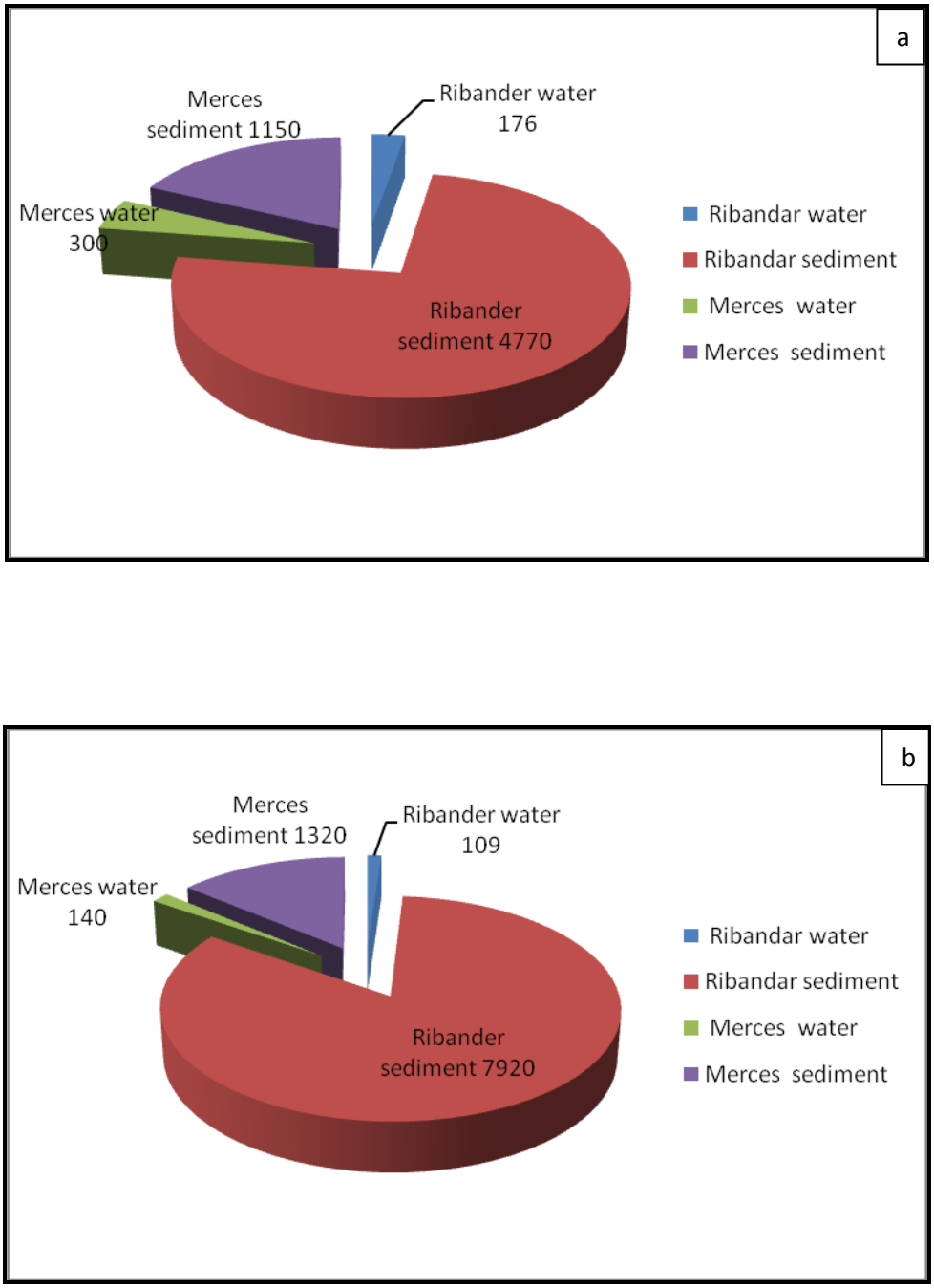

Fig. 2.3: Total viable count of mangrove samples on: a – TYG, b – NA

ecosystem. The isolates from sand dunes and mangroves were screened for production of siderophores using universal CAS assay.

The positive isolates depict a yellow zone on CAS agar (Fig. 2.4). Amongst the neutrophiles isolated on TYG and NA, it was seen that a higher percentage of siderophore producers was obtained from the non - rhizhosphere (23.7%) as compared to the rhizhosphere (14.87%) samples (Fig. 2.5a and 2.5b). Amongst the halotolerant isolates obtained on NTYE, only 2 isolates, NMR4.1 and NMR4.2, showed a positive reaction (Fig. 2.5c). From mangroves, a higher percentage of siderophore producers was obtained from water (28%) as compared to the sediment (5%) (Fig. 2.5d and Fig. 2.5e, respectively).

It was interesting to note that out of the 13 siderophore positive isolates, only one was gram negative while the remaining 12 were gram positive. Sand dune ecosystems are known to be nutrient deficient ecosystem and hence survival of these organisms depends on their ability to overcome the stress conditions. Anthropogenic material and marine fauna contribute to the organic material which supports the growth of microorganisms. The survival of such bacteria is dependent on their capacity to adapt.

The siderophore-producing organisms are just one such group of bacteria which we report from these two ecosystems especially for their ability to utilize aromatic hydrocarbons.

Iron-limiting conditions influence the efficiency of activity in microorganisms and alter their ability to utilize various compounds. For an example the microbial

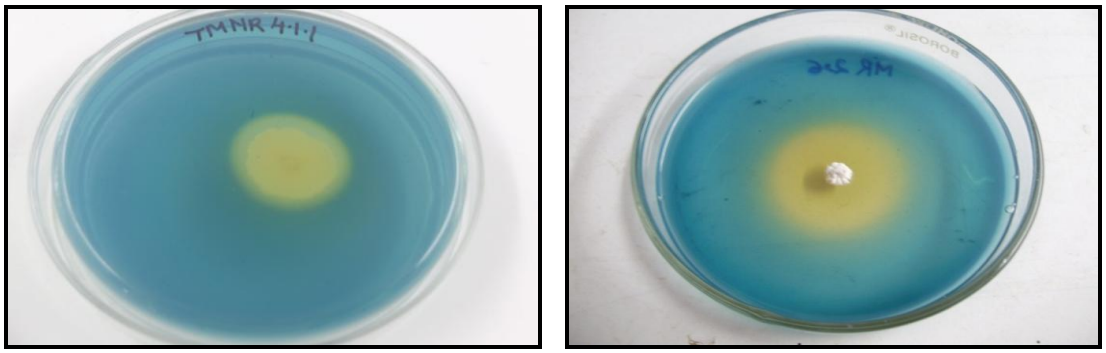

Fig. 2.4: Formation of yellow zone around the colonies of siderophore producers on CAS agar

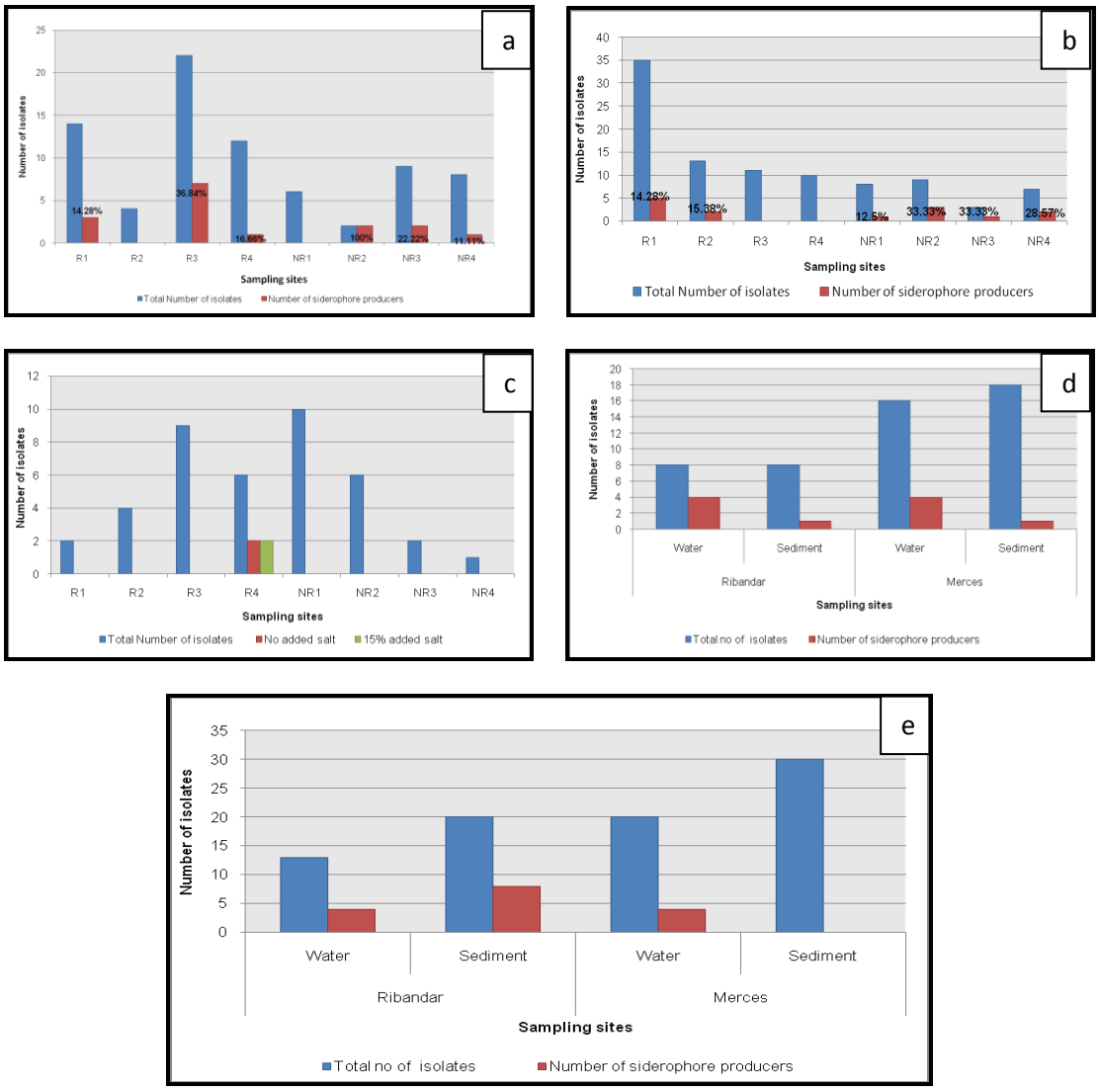

Fig. 2.5: Distribution of siderophore producers in: a) sand dunes obtained on NA b) sand dunes obtained on TYG c) sand dunes obtained on NTYE d) mangroves obtained on NA e) mangroves obtained on TYG

degradation of aromatic compounds by oxygenases requires iron as a cofactor, thus imposing a specific iron requirement on the cells. We therefore screened our cultures for their ability to utilize sodium benzoate.

2.3.4 Utilization of sodium benzoate by selected isolates

It was observed that out of the 30 siderophore producers isolated from sand dunes, only 4 showed the ability to utilize sodium benzoate, namely TMR2.13, NAMNR3.5, TMNR4.1.1 and NAMNR4.4. Isolate NAR38.1 from mangroves showed sodium benzoate utilization. These isolates were further screened on MSM with different sodium benzoate concentrations. It was observed that culture NAMNR3.5 could grow with upto 0.5% sodium benzoate concentration while cultures, TMNR4.1.1, NAMNR4.4 and NAR38.1 showed growth with upto 0.75% sodium benzoate (Fig. 2.6 b-d) as the sole carbon source. Culture TMR2.13 tolerated highest concentrations of sodium benzoate up to 2% and also produced a yellow green pigment pyoverdine which is known to be a siderophore (Fig. 2.6 e).

2.3.5 Identification of the selected isolates

13 siderophore positive isolates from sand dunes and mangrove which showed CAS zone of 8 mm and above were further subjected to routine biochemical tests to identify them. The only isolate, NAR38.1 from mangroves which showed both siderophore production and utilization of sodium benzoate was selected for identification (Table 2.3). Based on the morphological and biochemical characteristics, the isolates were tentatively identified using Bergey's Manual of Systematic Bacteriology (Table 2.4 a and b). Fig. 2.7 shows 1.5 kb PCR product

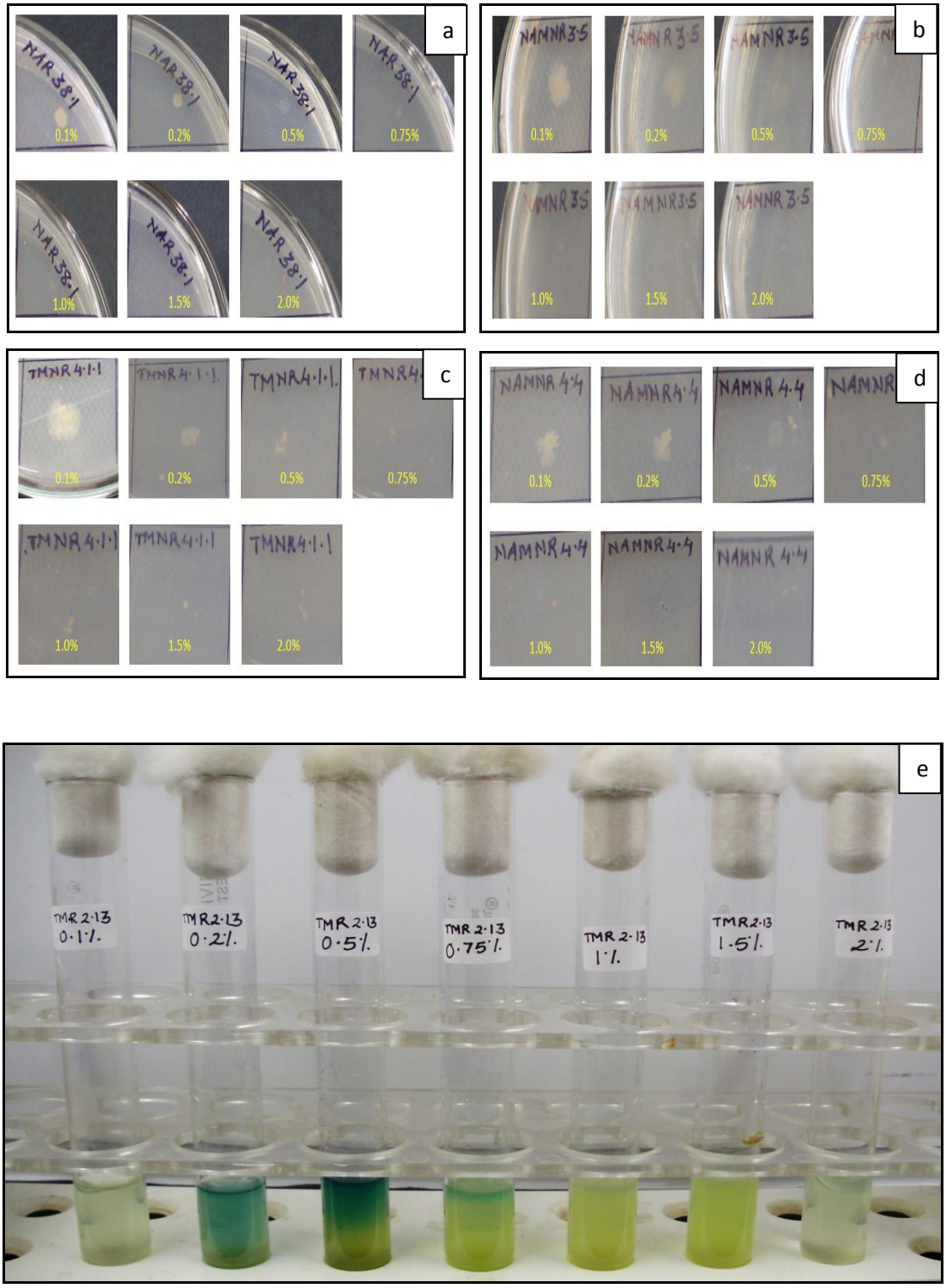

Fig. 2.6: Growth of selected isolates on different sodium benzoate concentrations a)NAR38.1 b) NAMNR3.5 c) TMNR4.1.1 d) NAMNR4.1.1 and e) TMR2.13

Table 2.3: Siderophore production by bacteria isolated from sand dunes and mangroves.

Sr. No.	Culture number	Siderophore production	Size of zone in mm on CAS plate
1	TMR1.6.1	++	6
2	TMR1.6.2	+++	8
3	TMR1.8	++	7
4	TMR1.17	+++	9
5	TMR1.18	+++	9
6	NAMR1.6	+	2
7	NAMR1.8	+	3
8	TMR2.6	+++	8
9	TMR2.13	+++	10
10	NAMR3.1	+	4
11	NAMR3.2	+	2
12	NAMR3.7	+	1
13	NAMR3.8	+	3
14	NAMR3.12	++	5
15	NAMR3.13	+	1
16	NAMR3.15	++	6
17	NAMR4.8	+	2
18	NAMR4.10	+++	9
19	TMNR1.4	++	7
20	TMNR2.7.1	+++	8
21	TMNR2.7.2	+++	8
22	TMNR2.7.3	+	4
23	NAMNR2.1	+	2
24	NAMNR2.2	+	1
25	TMNR3.3	+++	11
26	NAMNR3.3	+	5
27	NAMNR3.5	+++	9
28	TMNR4.1.1	+++	8
29	TMNR4.1.2	++	6
30	NAMNR4.4	+++	8
31	NMR4.1	+	2
32	NMR4.2	+	2
33	TM6	++	5
34	TM37	+	4
35	TM44	++	7
36	TM50	+	2
37	TR53	+	4
38	TR57	++	5
39	TR60	+	1
40	TR61	++	6
41	TR63	++	7
42	TR67	+++	5
43	TR70	++	7
44	TR75	+	4
45	TR77	+	2
46	TR79	++	5
47	TR80	++	6
48	TR82	+	4
49	NAM9	+	2
50	NAM22	+	1
51	NAM29	++	5
52	NAM30	+	3
53	NAM32	++	7
54	NAM33	++	6
55	NAR38.1	+++	8
56	NAR42	+	3
57	NAR43	++	7
58	NAR44	++	7
59	NAR47	+++	9

Key: + = <5 mm ++ = 5-7 mm +++= >7 mm

Table 2.4a: Biochemical characteristics of the selected siderophore producing bacteria

Isolate number	**TMR1.17**	**TMR1.6.2**	**TMR1.18**	**TMR2.6**	**TMNR3.3**	**TMNR2.7.1**	**TMNR2.7.2**
Gram character	+	+	+	+	+	+	+
Rod presence	+	+	+	+	+	+	+
Endospore	+	+	-	-	+	+	+
Oxidase	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+
Amylase	+	+	+	+	-	+	+
Motility	+	+	+	+	+	+	+
Nitrate reduction	+	+	-	-	+	-	+
Nitrite reduction	+	-	-	-	+	-	+
Glucose acid	+	+	-	-	+	+	+
Glucose Gas	-	-	-	-	-	-	-
Marked acidity	-	-	-	-	-	-	-
Lactose	+	-	-	-	+	-	-
VP	+	-	-	-	+	-	-
Indole	-	-	-	-	-	-	-
H_2S production	+	-	+	+	-	+	+
HL aerobic	+	+	-	-	+	+	+
HL anaerobic	+	+	-	-	+	+	+
pH	10.5	8.5	8.5	8.5	8.5	10.5	10.5
%NaCl	10	0.5	10	0.5	10	10	10
Tentatively identified as	***Bacillus* sp.**	***Bacillus* sp.**	***Streptomyces* sp.**	***Streptomyces* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus* sp.**

Table 2.4b: Biochemical characteristics of the selected siderophore producing bacteria

Isolate number	**TMNR4.1.1**	**NAMNR3.5**	**NAMR4.10**	**NAMNR4.4**	**NAR38.1**	**TMR2.13**
Gram character	+	+	+	+	+	-
Rod presence	+	+	+	+	+	+
Endospore	+	+	+	+	+	-
Oxidase	-	+	+	+	+	+
Catalase	+	+	+	+	+	+
Citrate	+	+	-	+	+	+
Amylase	+	+	-	+	+	-
Motility	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	+
Nitrite reduction	-	+	+	-	-	+
Glucose acid	+	+	+	+	+	-
Glucose Gas	-	-	-	-	-	-
Marked acidity	-	-	-	-	-	-
Lactose	-	-	+	+	-	-
VP	-	+	-	+	-	-
Indole	-	-	-	-	-	-
H_2S production	+	+	-	-	-	-
HL aerobic	+	+	-	+	+	-
HL anaerobic	+	+	-	+	+	-
pH	8.5	8.5	10.5	8.5	10.5	10.5
%NaCl	10	10	10	10	5	5
Tentatively identified as	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus* sp.**	***Bacillus amyloliquefaciens***	***Pseudomonas aeruginosa***

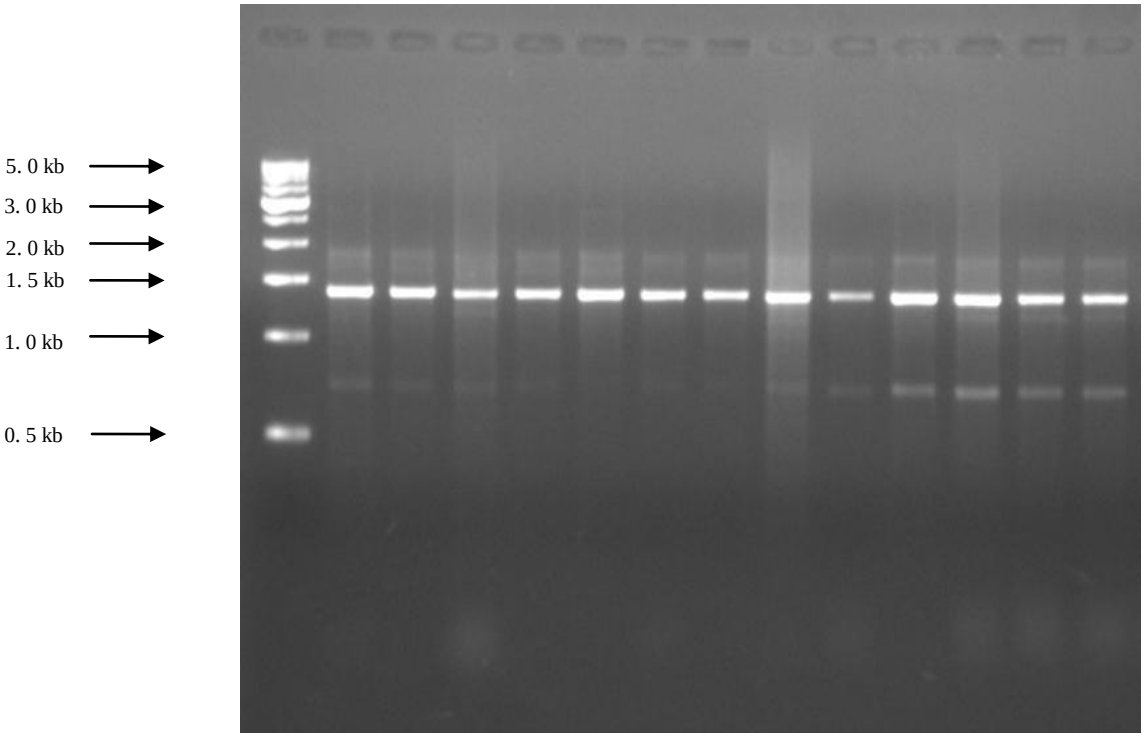

Fig. 2.7: DNA gel showing 1.5 kb band of amplified 16S rDNA

Lane 1: Marker

Lane 2: TMR1.18

Lane 3: NAMR4.10

Lane 4: TMR2.6

Lane 5: TMNR3.3

Lane 6: TMR2.13

Lane 7: TMNR2.7.1

Lane 8: TMNR2.7.2

Lane 9: NAMNR3.5

Lane 10: TMNR4.1.1

Lane 11: NAMNR4.4

Lane 12: TMR1.17

Lane 13: TMR1.6.2

Lane 14: NAR38.1

obtained on amplification of the 16S rDNA of the selected isolates. Based on 16S rDNA sequencing it was found that of the thirteen isolates selected, 10 belonged to the *Bacillus* spp, 2 were *Streptomyces* spp. and one was identified as *Pseudomonas aeruginosa* (Table 2.5). Phylogenetic trees of the selected isolates have been depicted in Fig. 2.8.

The dominant fatty acids determined in NAR38.1 by FAME analysis were 14:0 iso (0.99%), 15:0 iso (20.43%), 15:0 anteiso (41.94%), 16:0 (9.71%), 16:1 w11c (1.79%), 17:1 iso w10c (1.12%), 17:0 iso (6.11%) and 17:0 anteiso (8.47%) (Appendix D.2). FAME analysis confirmed the identification of the isolate as *Bacillus amyloliquefaciens. Bacillus amyloliquefaciens*, a gram positive spore forming rod, holds substantial importance in enzyme industry due to its ability to produce copious amounts of amylase. It has been used on an industrial scale for the production of bioactive cyclic lipopeptides, surfactin, barnase (protein toxin) and iturins (Lin *et al.* 2007).

The predominance of *Bacillus* spp. could be due to their ability to form spores and resist adverse ecological conditions characteristic of sand dune ecosystems (Godinho *et al.* 2010). *Bacillus* spp. have been reported to promote plant growth in barley (Canbolat *et al.* 2006), pine, spruce (Shishido *et al.* 1996) and in eggplant (Godinho *et al.* 2010). Shin *et al.* (2007) and Park *et al.* (2005) have reported predominance of *Pseudomonas* spp. associated with sand dune vegetation.

Table 2.5: Identification of the isolates based on biochemicals and 16S rDNA sequencing

Sr. No.	Culture number	Tentatively Identified as	Accession number	Closest Match
1	TMR1.17	*Bacillus* sp.	JN596247	HM016080.1 *Bacillus amyloliquefaciens* strain KSU-109
2	TMR1.6.2	*Bacillus* sp.	JN596242	EU882849.1 *Bacillus subtilis* strain F3–7
3	TMR1.18	*Streptomyces* sp.	JN596248	AB184071.1 *Streptomyces sclerotialus* strain NBRC 12246
4	TMR2.6	*Streptomyces* sp.	JN596249	AB184657.2 *Streptomyces djakartensis* strain: NBRC 15409
5	TMNR3.3	*Bacillus* sp.	JN596245	GU181234.1 *Bacillus amyloliquefaciens* strain SRDM2
6	TMNR2.7.1	*Bacillus* sp.	JN596243	HQ238533.1 *Bacillus tequilensis* strain S12Ba-171
7	TMNR2.7.2	*Bacillus* sp.	JN596244	HQ238638.1 *Bacillus tequilensis* strain S433Ba-70
8	TMNR4.1.1	*Bacillus* sp.	JX194168	JF894160.1 *Bacillus subtilis* strain McR-7
9	NAMNR3.5	*Bacillus* sp.	JX194166	JF411337.1 Uncultured *Bacillus* sp. clone P2
10	NAMR4.10	*Bacillus* sp.	JN596242	EU746420.1 *Bacillus* sp. A-34
11	NAMNR4.4	*Bacillus* sp.	JX194167	AB016721 strain BS62 *Bacillus subtilis*
12	NAR38.1	*Bacilllus amyloliquefaciens*	JX555984	JQ798394.1 *Bacillus amyloliquefaciens* strain Y26
13	TMR2.13	*Pseudomonas aeruginosa*	HM030825	GU339295.1 *Pseudomonas aeruginosa* strain EH70

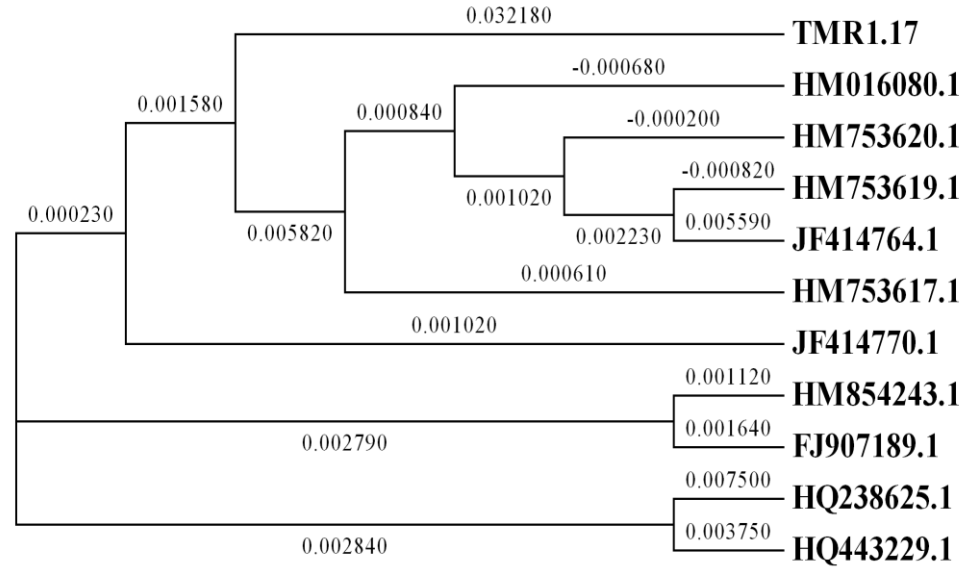

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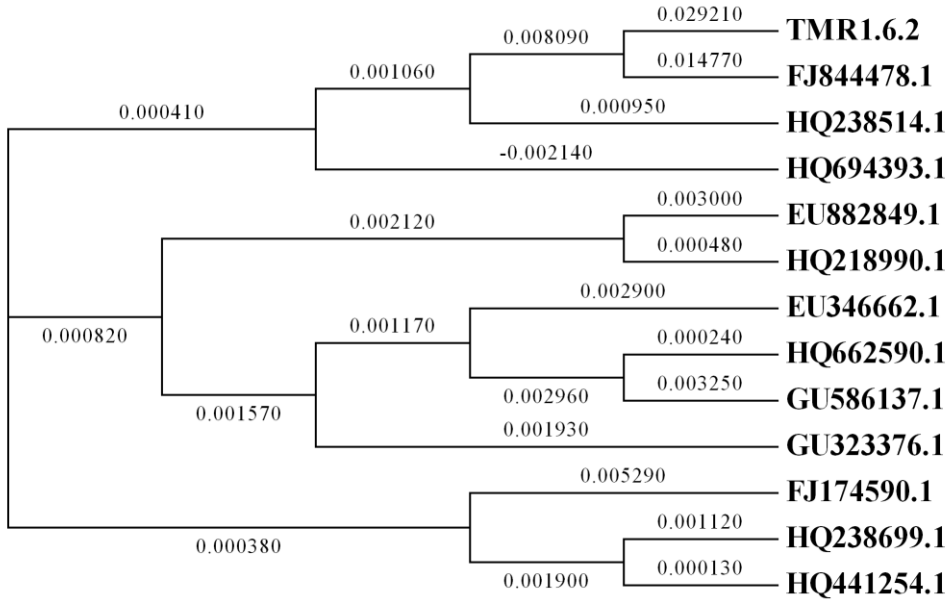
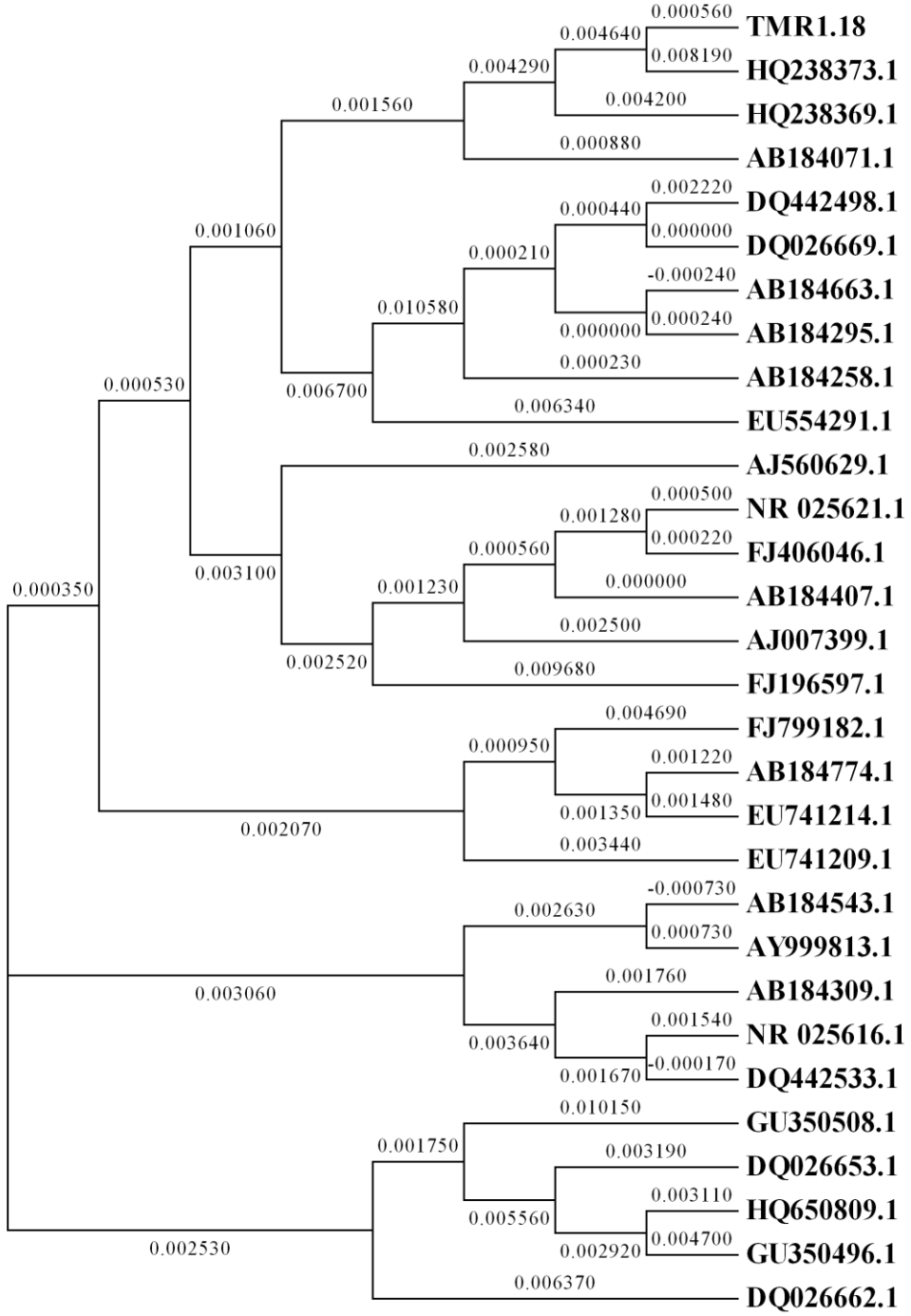
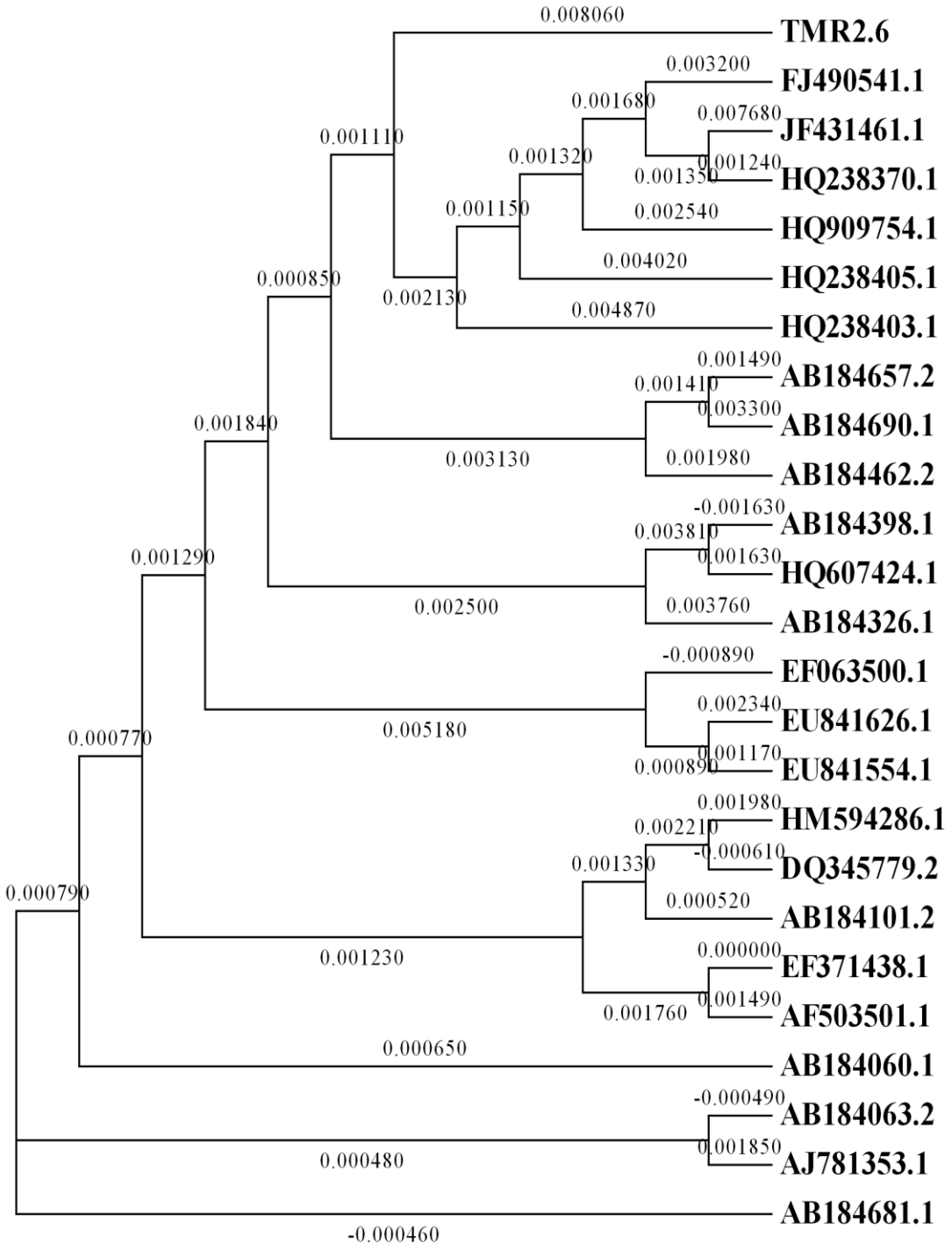
Fig. 2.8: Phylogenetic trees constructed by the neighbor – joining method based on alignment of 16S rRNA gene sequences. Bootstrap values generated from 10,000 replications are shown at branch points. a) *Bacillus* sp TMR1.17 (GenBank Acc No JN596247)



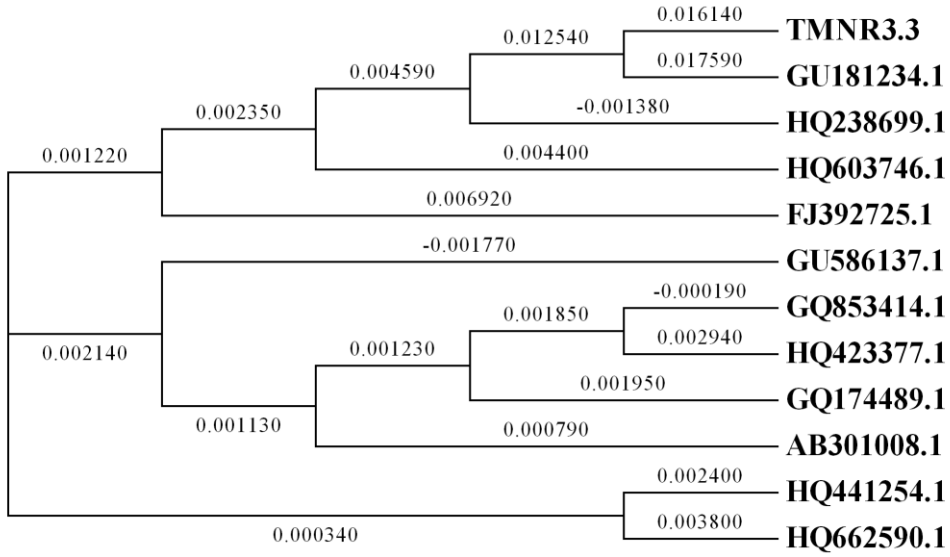
b) *Bacillus* sp. TMR1.6.2 (GenBank Acc No JN596242)



c) *Streptomyces sp.* TMR1.18 (GenBank Acc No JN596248)



d) *Streptomyces sp*. TMR2.6 (GenBank Acc No JN596249)



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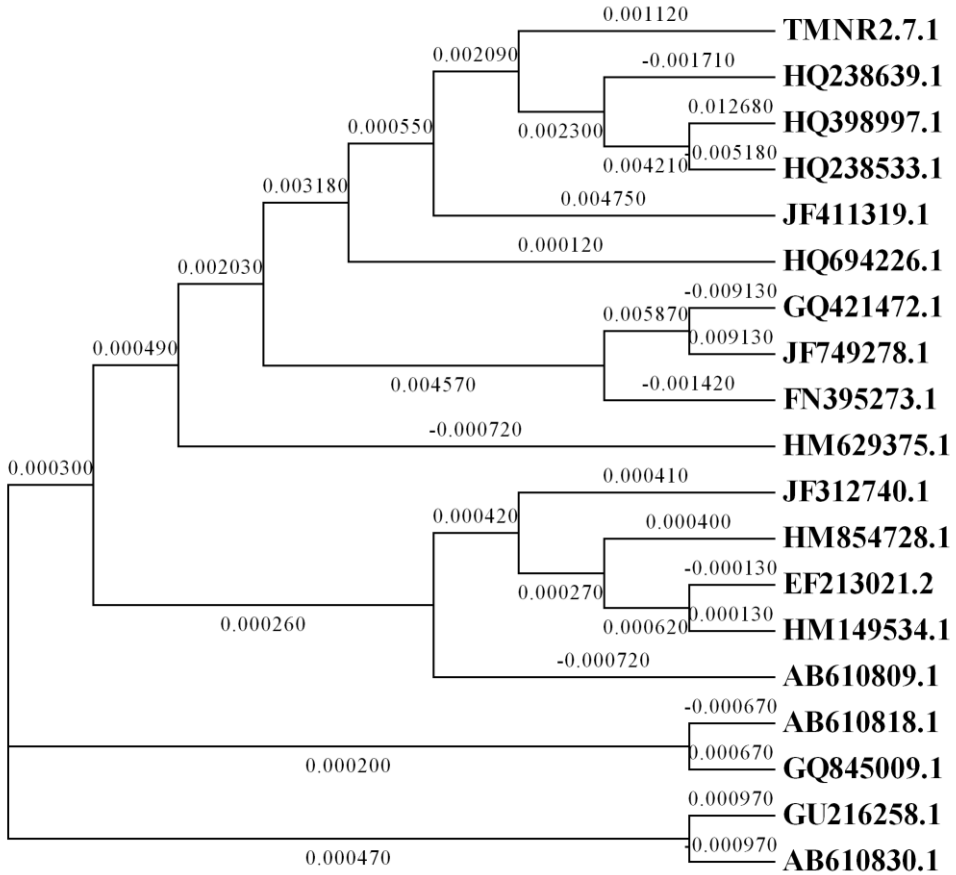
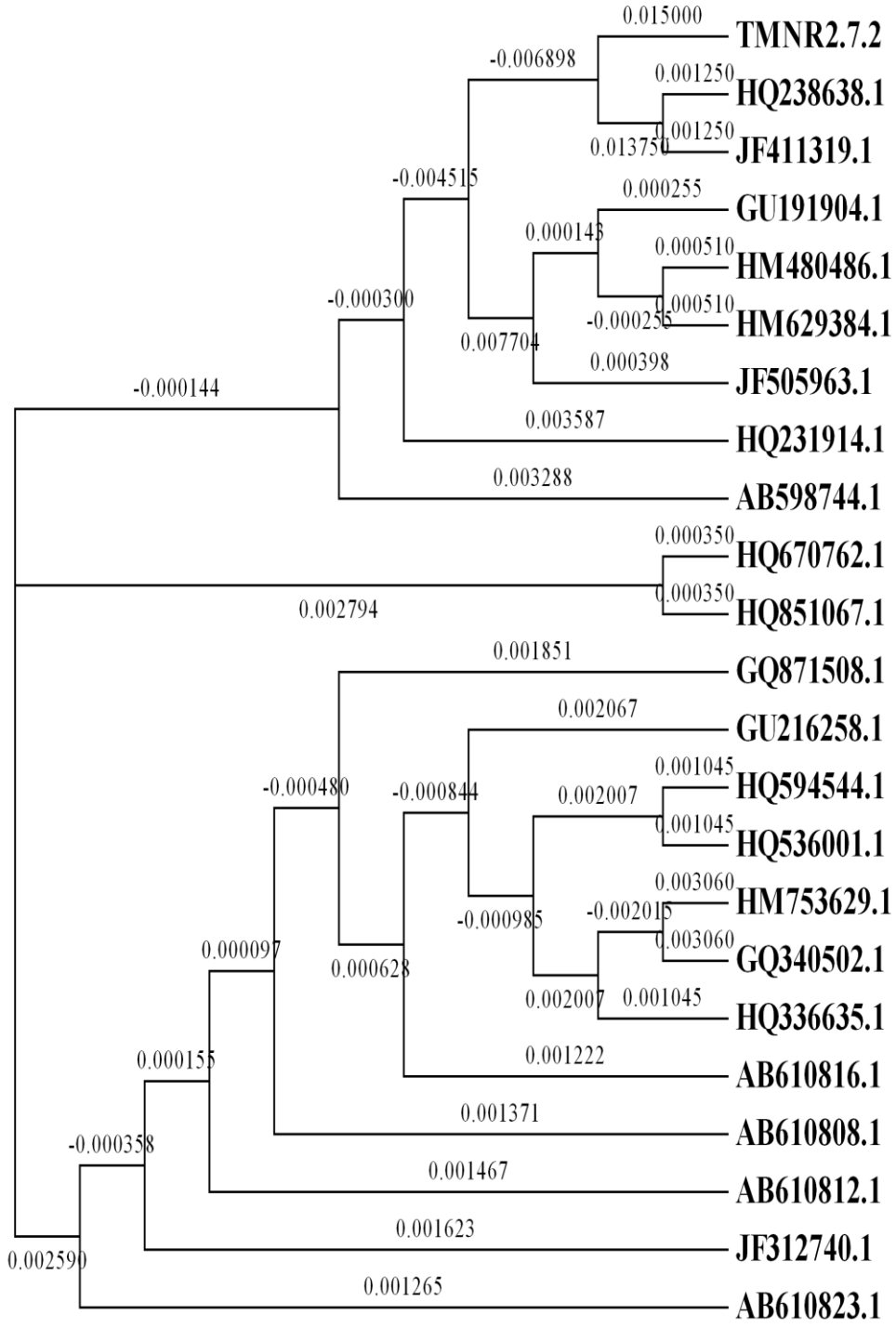
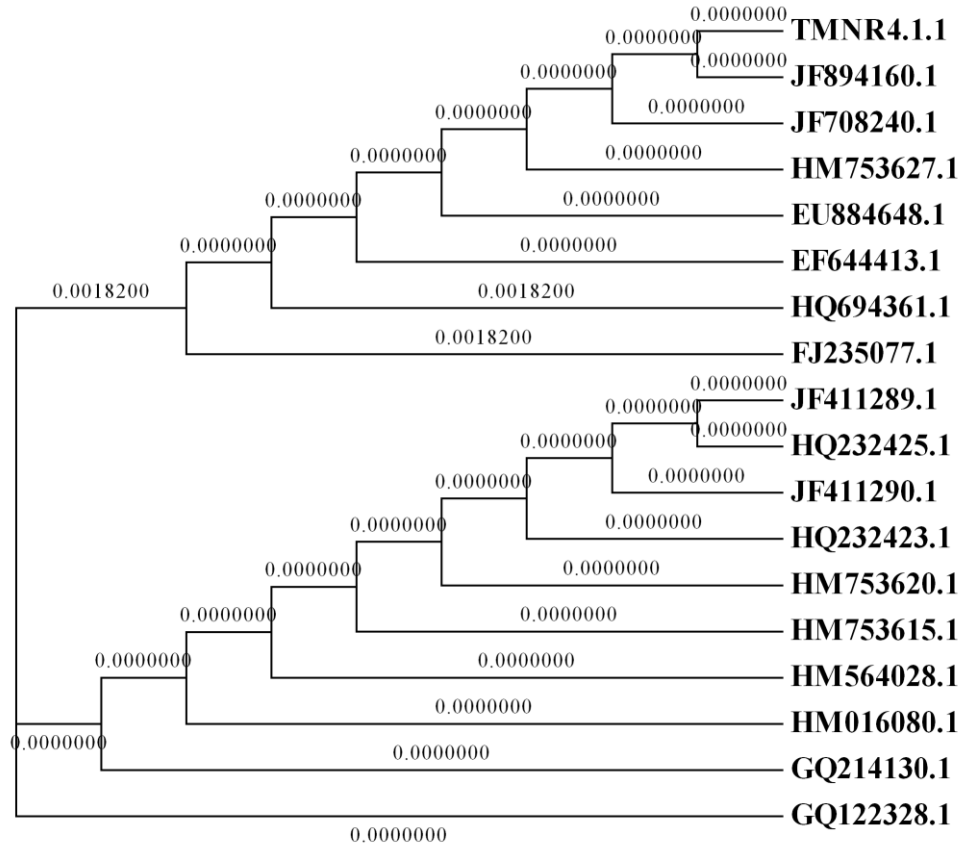


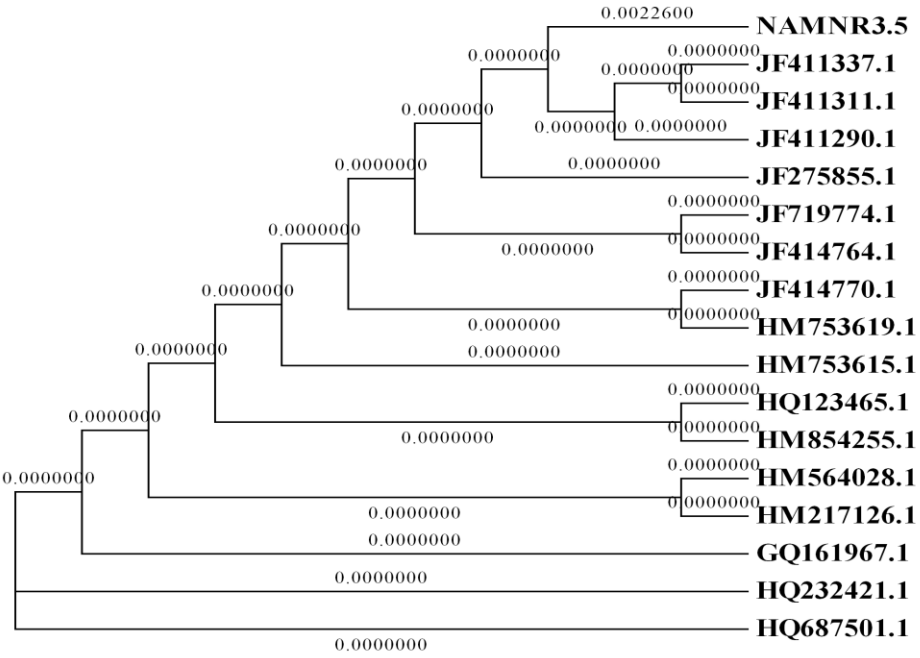
f) *Bacillus sp.* TMNR2.7.1 (GenBank Acc No JN596243)



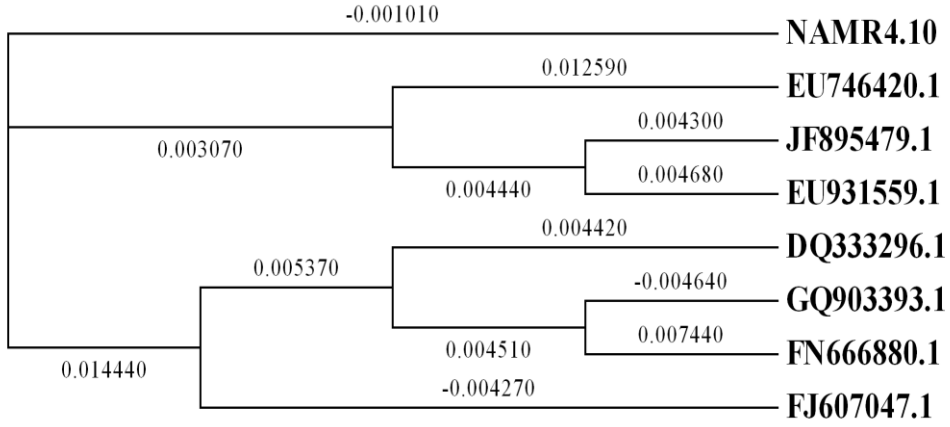
g) *Bacillus sp* TMNR2.7.2 (GenBank Acc No JN596244)



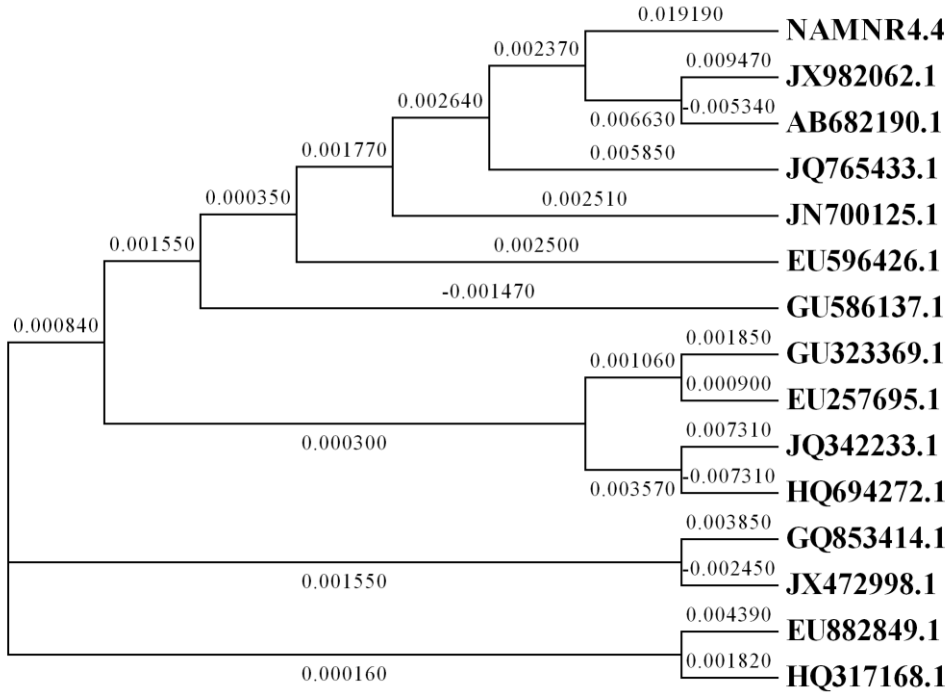
h) *Bacillus* sp. TMNR4.1.1 (GenBank Acc No JX194168)



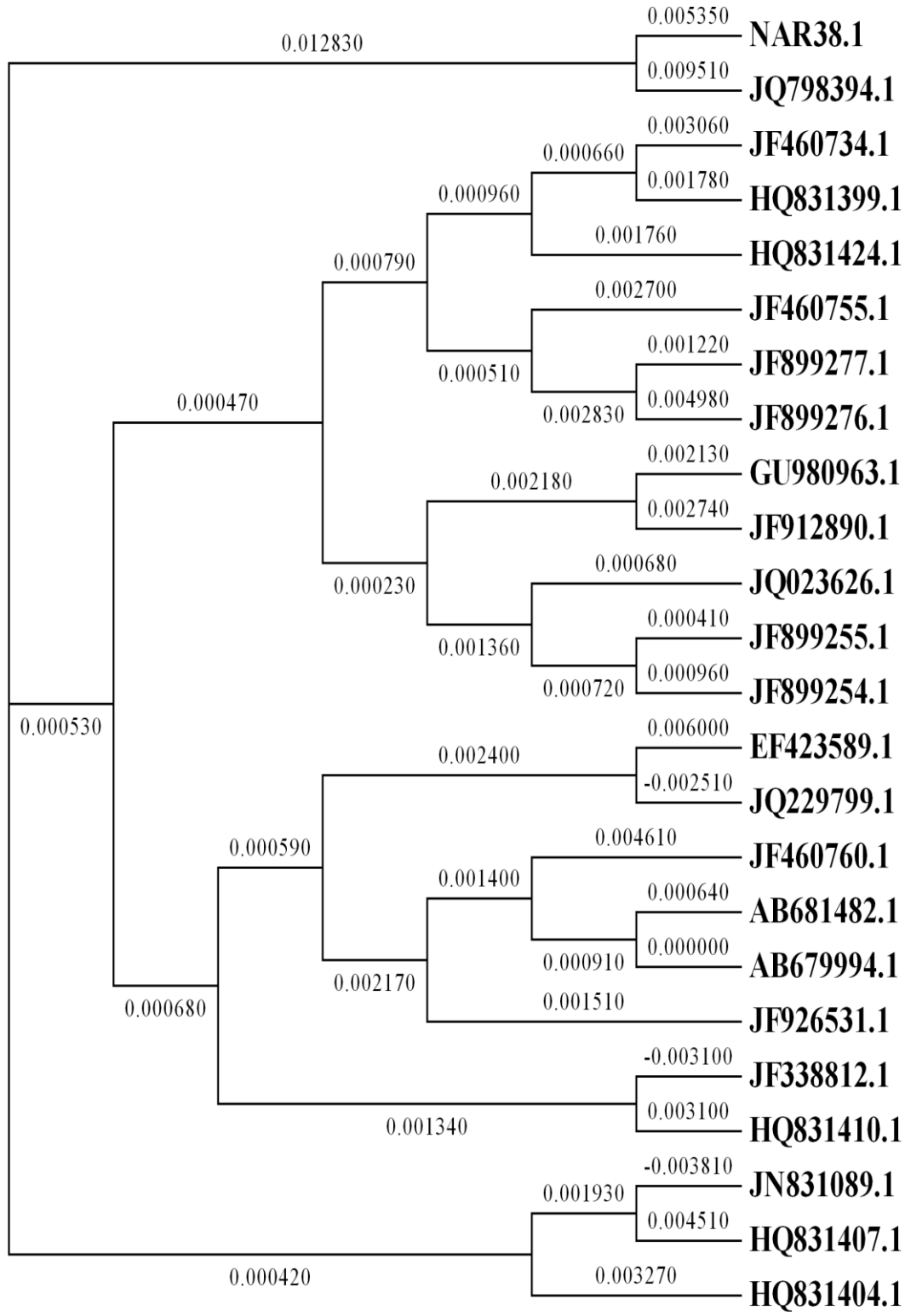
i) *Bacillus* sp. NAMNR 3.5 (GenBank Acc No JX194166)



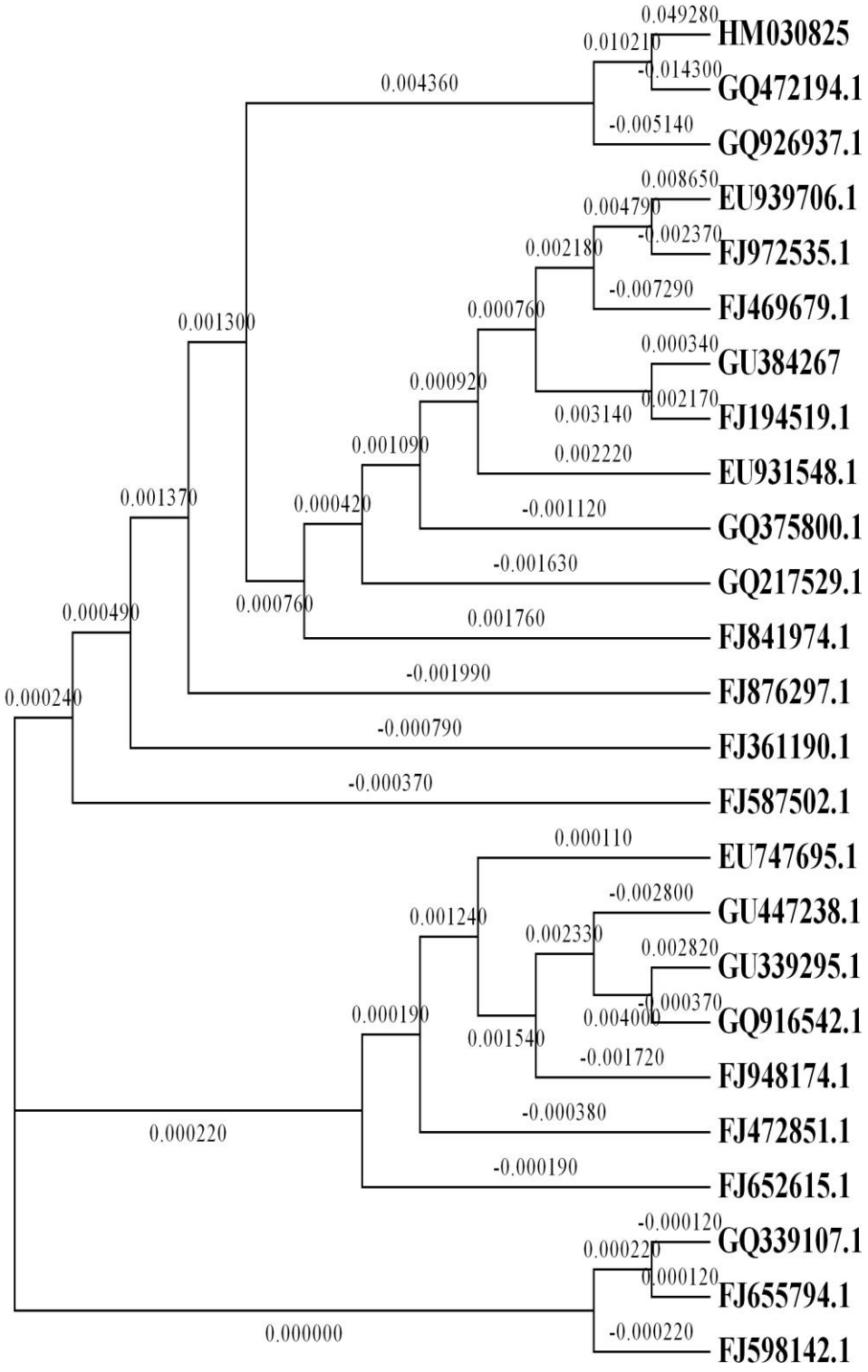
j) *Bacillus sp.* NAMR4.10 (GenBank Acc No JN596242)



k) *Bacillus* sp. NAMNR4.4 (GenBank Acc No JX194167)



l) *Bacillus amyloliquefaciens* NAR38.1 (GenBank Acc No JX555984)



m) *Pseudomonas aeruginosa* TMR2.13 (GenBank Acc No HM030825)

Based on siderophore production and sodium benzoate utilization, two isolates TMR 2.13 and NAR38.1 were selected for further studies (Fig. 2.9). Isolate TMR2.13 obtained from rhizhosphere of *Ipomoea pes-caprae* was identified as *Pseudomonas aeruginosa*. It showed growth in presence of as high as 2% sodium benzoate concentrations along with the production of a pigment pyoverdine which is known to be a siderophore. Isolate NAR38.1 isolated from mangroves showed siderophore production and consistent growth on sodium benzoate as compared to other 3 *Bacillus* spp. However, it failed to grow in liquid MSM supplemented with sodium benzoate.

This study depicts the incidence of siderophore producing bacteria in the two coastal ecosystems: sand dunes and mangroves. The total viable counts were found to be higher in rhizhosphere samples compared to the non rhizhosphere samples from sand dunes while mangrove sediments showed higher counts compared to the water samples. Identification of the selected siderophore producing isolates indicated predominance of *Bacillus* spp. in the sand dunes. Few isolates showed the ability to utilize sodium benzoate as the sole carbon source.

Production of siderophore by bacteria is an important characteristic to assist plant growth. Such isolates are also known to possess other plant growth promoting characteristics for example, IAA, HCN etc. The ability of the two selected isolates to produce such compounds was therefore tested and is described in the next chapter.

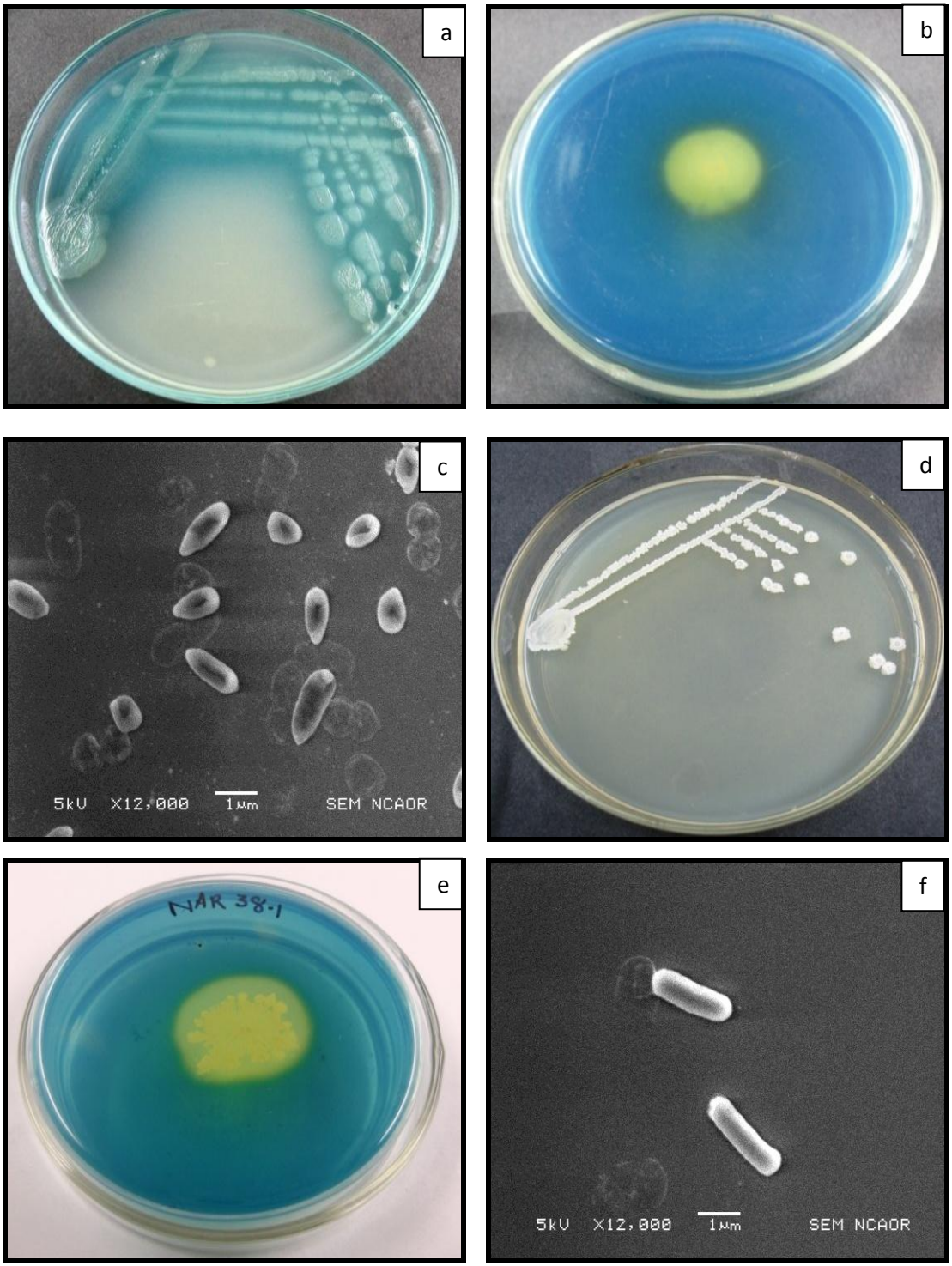


Fig. 2.9: ***Pseudomonas aeruginosa*** **TMR2.13: a) Colony morphology b) Siderophore production on CAS agar plate c) Electron microscopic view**

Bacillus amyloliquefaciens **NAR38.1 d) Colony morphology e) Siderophore production on CAS agar plate f) Electron microscopic view**

Further, the siderophore produced are also classified into three main groups the stability of which is dependent upon the pH of the econiche. It was therefore envisaged to study the effect of pH on the growth and siderophore production in these isolates and the type of siderophores produced. Besides the pH, the effect of NaCl and iron was also studied and the results are discussed in the following chapter.

Chapter 3

Studies on siderophore production by the selected isolates P. aeruginosa TMR2.13 and B. amyloliquefaciens NAR38.1

3.1 Introduction

Siderophores produced by rhizhosphere bacteria enhance plant growth by increasing the availability of Fe near the root or by inhibiting the colonization of roots by plant pathogens or other harmful bacteria (Alexander and Zuberer 1991). Plant growth promotion by microorganisms may be direct or indirect. Direct promotion is by providing the plant with growth promoting substances like indole acetic acid (phytohormone) or by making nutrients available through phosphate solubilization or siderophore production. The indirect effect is seen if plant growth promoting rhizobacteria (PGPR) lessen or prevent the deleterious effects of one or more phytopathogenic microorganisms, for example by producing hydrogen cyanide (HCN) (Voisard *et al*. 1989, Adam and Zdor 2001). The two isolates *P. aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1 obtained during this study were screened for other plant growth promoting properties like phosphate solubilization, production of HCN and production of ammonia.

Siderophores, the iron chelating agents produced by microorganisms, have been classified into three main types: hydroxamates, catecholate and carboxamate types depending on the iron ligation groups (Wandersman and Delepelaire 2004). The most extensively studied iron chelators are catechol and hydroxamate siderophores produced by Gram-negative bacteria, in particular, pseudomonads, enterobacteria, agrobacteria and related species, *Yersinia* and vibrions. Hydroxamates have been primarily studied from Gram-positive microorganisms, whereas catechols are identified from a relatively smaller number of representatives of this group (Temirov *et al.* 2003). Amongst the *Bacillus* spp. siderophores from *Bacillus anthracis* (Petrobactin and Bacillibactin) (Wilson *et al.* 2010), *B.megaterium* (Schizokinen), *B. subtilis* (Bacillibactin) (Zawadzka *et al.* 2009) and *B. licheniformis* (2, 3-dihydroxybenzoyl-glycyl-threonine) (Temirov *et al.* 2003) have been well studied. In the present study, the type of

siderophore produced by these two isolates was determined using standard tests (Neilands 1981, Snow 1954, Gilliam 1981, Arnow 1937, Yeole *et al.* 2001, Shenker 1992).

Although, iron is the key factor in regulating siderophore production as increase in iron concentration decreases siderophore production (Villegas *et al.* 2002), other factors such as pH, temperature, carbon source and other metals also play an important role (Saha *et al*. 2012). The pH of an econiche determines the type of siderophore produced by the microorganisms inhabiting it. Hydroxamate siderophores are stable over a wide pH range as compared to the catecholate type which are stable at neutral to alkaline pH. Trihydroxamates are stable over a pH range of 2 to 8; however, ferric complexes of catecholates are unstable below pH 5. Many bacteria which produce only catechol type of siderophores can grow mainly under neutral to alkaline conditions. For an example, *Azotobacter* and *Agrobacterium* produce azotochelin, protochelin and aminochelin (Winkelmann 2004).

The effect of pH, NaCl and Fe concentration on siderophore production by these isolates was determined and further, binding of siderophores to different forms of Fe was evaluated. Results obtained are discussed in this chapter along with the characterization of the functional group of the siderophore and the plant growth promoting characteristics of the selected isolates.

3.2 Materials and methods:

3.2.1 Plant growth promoting properties

i) Phosphate solubilization: The isolates were streaked on Pikovskaya's medium and incubated for 24 hours at 28 ºC (Appendix A). Zone of clearance indicating phosphate solubilisation activity was observed (Gupta *et al*. 1994).

ii) HCN production: The isolates were streaked on growth medium with 4.4 g/L glycine (Appendix A). Filter papers dipped in picric acid solution were placed on the lid of each plate. HCN production was determined from change in color of the filter paper strip from orange to red (Bakker and Schippers 1987).

iii) Indole acetic acid production: Isolates were grown in nutrient broth containing 0.5% tryptophan for 24 hours and the culture broth was centrifuged 2 ml Salkowsky's reagent (Appendix B) was added to 1 ml of supernatant. The mixture was incubated for 30 minutes at 28 ºC and the absorbance was recorded at 530 nm (Viswanathan 1999).

iv) Ammonia production: Freshly grown culture was inoculated in 10 ml of peptone water (Appendix A) and incubated for 48 hours at 28ºC. 0.5 ml of Nessler's reagent (Appendix B) was added to each tube and the development of a yellow brown color, indicating ammonia production, was observed (Cappuccino and Sherman, 1992).

3.2.2 Spectrophotometric and spectrofluorimetric characteristics of siderophore produced by the isolate TMR2.13

TMR2.13 was inoculated in MSM with 0.2% glucose, and the flask was incubated at 28ºC at 150 rpm for 24 hours. The culture broth was centrifuged, and the supernatant was scanned for peaks in UV-Vis range using spectrophotometer (Shimadzu UV-2450). Spectrofluorimetric analysis was carried out with RF-5301 PC Shimadzu spectrofluorometer at an excitation wavelength of 400 nm and the emission was recorded.

3.2.3 Determination of the functional group of siderophore

The isolates were inoculated in a mineral salts medium (MSM) without $FeSO_4$. The flasks were incubated on shaker at 150 rpm at 28 ºC for 24 hours after which the broth was

centrifuged at 10,000 rpm for 10 minutes and the supernatant was subjected to the following tests for determining the functional group:

i) Hydroxamate

a) Neiland's spectrophotometric assay: To 1 ml of cell - free supernatant, 1.5 ml of freshly prepared 2% aqueous $FeCl_3$ solution was added. The resultant mixture was scanned between 400-600 nm. A peak between 420-450 nm indicates the hydroxamate nature of the siderophore (Neilands 1981).

b) Tetrazolium salt test: To 0.5 ml of cell - free supernatant, a pinch of tetrazolium salt and a few drops of NaOH were added. Instant appearance of a deep red color indicated the presence of a hydroxamate siderophore (Snow 1954).

c) Csaky assay: The cell free supernatant (1ml) was hydrolyzed with 1 ml of 6 N H_2SO_4 in a boiling water bath at 130 ºC for 30 min. The solution was buffered by adding 3 ml sodium acetate solution and 1 ml sulfanilic acid solution followed by the addition of 0.5 ml iodine solution. After 3-5 min, excess iodine was destroyed with 1ml sodium arsenite solution. To this, 1 ml α - naphthylamine (Appendix B) solution was added and the volume was made upto 10 ml with water. The absorbance of the color developed after 20-30 minute was measured at 526 nm (Gilliam 1981).

ii) Catecholate

a) Neiland's spectrophotometric assay: 1.5 ml of freshly prepared 2% aqueous $FeCl_3$ was added to 1 ml of the test sample. The wine colored complex formed that absorbed maximally at 495 nm, indicated the catecholate nature of the siderophore (Neilands 1981).

b) Arnow's assay: To 1 ml of supernatant, the following reagents were added: 1 ml 0.5 M HCl, 1 ml nitrite molybdate (Appendix B) and 1 ml 1 M NaOH. After the addition of each reagent, the tubes were vortexed. The colour change from yellow to red indicated the presence of catechol in the reaction mixture (Arnow 1937).

iii) Carboxylate

a) Vogel's chemical test: Few drops of 5N NaOH were added to water along with 3 drops of phenolphthalein solution to give a light pink colour. Disappearance of pink color on addition of the test sample, indicated the presence of carboxylate type of siderophores (Yeole *et al.* 2001).

b) Spectrophotometric assay (Shenker's test): To 1 ml of cell - free supernatant, 1 ml of 250 µM $CuSO_4$ and 2 ml of acetate buffer (pH 4) (Appendix B) were added. The copper complex formed was scanned from 190 to 800 nm and the absorption maxima was noted between 190-280 nm (Shenker 1992).

3.2.4 Determination of the effect of pH on growth and siderophore production

Pseudomonas aeruginosa TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28ºC for 24 hours and the cultures were inoculated in MSM with pH 7 and 0.2% glucose as the carbon source. The flasks were incubated at 28ºC and 150 rpm for 24 hours. 5% of 24 hour old culture was inoculated in MSM with 0.2% glucose as the carbon source. The pH of the medium ranged from 5 to 9 (adjusted using 1 N HCl or 1 N NaOH). The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 4 ml sample was removed from all the flasks after 24 hours to determine growth and siderophore production. Growth was determined as increase in absorbance at 600 nm on a UV- Vis spectrophotometer (Shimadzu UV-2450). Siderophore (Pyoverdine) produced by *Pseudomonas aeruginosa* TMR2.13, was quantified in cell-free supernatant by noting the absorbance at 400 nm. Siderophore concentration was calculated as described by Gupta *et al.* (2007). Siderophore produced by *B. amyloliquefaciens* NAR38.1 was quantified by Arnow's assay using catechol as the standard (Appendix C).

3.2.5 Determination of the effect of NaCl on growth and siderophore production in the selected isolates

Pseudomonas aeruginosa TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28 ºC for 24 hours and the cultures were inoculated in MSM with pH 7 and 0.2% glucose as the carbon source for inoculum build - up. The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 5% of the 24 hour old culture was inoculated in MSM with different NaCl concentrations (0, 1, 2, 3, 4 and 5%) and 0.2% glucose as the carbon source. The flasks were incubated at 28 ºC and 150 rpm for 24 hours. 4 ml sample was removed from the flasks after 24 hours to determine growth and siderophore production as mentioned in section 3.2.4.

3.2.6 Growth and siderophore production in presence and absence of iron

The isolates *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 were streaked on TYG and NA respectively. The plates were incubated at 28 ºC for 24 hours and the cultures were inoculated in MSM with 60 mg/L $FeCl_3$ and without added iron. 0.2% glucose was used as the carbon source for inoculum build - up. The flasks were incubated at 28 ºC at 150 rpm for 24 hours. 5% of 24 hour old culture was inoculated in MSM with 60 mg/L $FeCl_3$ and without added iron and 0.2% glucose as the carbon source. 4 ml of sample was removed under aseptic conditions every 8 hours upto 72 hours for *Pseudomonas aeruginosa* TMR2.13 and every 4 hours upto 48 hours for *Bacillus amyloliquefaciens* NAR38.1. Growth and siderophore production were determined as mentioned in section 3.2.4.

3.2.7 Effect of iron concentrations on growth and siderophore production by *Pseudomonas aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1

Flasks containing MSM with 0.2% of glucose as the sole carbon source, supplemented with Fe^{+2} ($FeSO_4$) and Fe^{+3} ($FeCl_3$), in increasing concentrations were inoculated with 5% of exponential cells grown in the respective medium. The culture flasks were incubated at 150 rpm at 28 ºC. 5 ml sample was removed after 24 hours, and growth and siderophore production was determined as mentioned in section 3.2.4. A control was maintained without added iron.

3.2.8 Binding of siderophores produced by *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 to Fe^{+2} and Fe^{+3}

TMR2.13 and NAR38.1 were inoculated in MSM with 0.2% glucose, and the flasks were incubated at 28 ºC at 150 rpm for 24 hours. The culture broths were centrifuged at 10,000 rpm for 10 minutes to obtain the cell free supernatant. For studies with the siderophore produced by TMR2.13, fluorimetric analysis was carried out with RF-5301 PC Shimadzu spectrofluorometer at excitation and emission wavelengths of 400 and 467 nm, respectively. Fluorescence quenching was studied by adding 10 μl of Fe^{+2}/Fe^{+3} solutions to 3 ml of crude dilute culture supernatant to achieve a final concentration of 3.3 µM (Xiao and Kisaalita 1998). To determine binding of zerovalent Fe nanoparticles to the siderophore, Fe nanoparticles were added to 3 ml of diluted culture supernatant to achieve a final concentration of 0.0125, 0.025 and 0.05 M.

To determine binding of siderophore produced by NAR38.1 to Fe^{+2} and Fe^{+3}, the siderophore was dissolved in deionised water at a concentration of 10 µg/ml. To 0.5 ml of siderophore, 0.5 ml of Fe^{+2} (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) or Fe^{+3} (0.0001, 0.0005, 0.001, 0.005, 0.01 and 0.05 M) was added and the solution was centrifuged to remove the iron - bound siderophore. To determine binding of Fe nanoparticles, 3.25 µg of siderophore

was dissolved per ml of deionised water. To 0.5 ml of siderophore, 0.5 ml of 0.025 M and 0.05M Fe nanoparticles was added separately and siderophore bound to Fe nanoparticle was removed by centrifugation. Unbound siderophore in the solution was estimated using Arnow's test.

3.3 Results and Discussion

3.3.1 Plant growth promoting properties

In recent years, siderophore producing rhizobacteria have gained increased attention. Siderophores are beneficial to both, bacteria that produce them and the plant colonized by the siderophore producers. Siderophores have a dual mechanism of enhancing plant growth: either by enhancing iron availability to the host and the plant or by making iron unavailable to plant pathogens by chelating it and thus inhibiting their growth. In the previous chapter it was observed that both the isolates produced CAS detectable siderophores, therefore, these organisms were also screened for their other plant growth promoting properties such as phosphate solubilization, HCN production and ammonia production.

A large database is available to support the role of fluorescent pseudomonads in plant growth promotion (Jagadeesh *et al.* 2001). Further, pseudomonads have also been recognized as biocontrol agents against certain soil-borne plant pathogens. They are characterized by the production of yellow-green pigments termed pyoverdines, which fluoresce under UV light and function as 'siderophores' (Demange *et al.* 1987). The role of Gram negative bacteria as PGPR has been well documented in contrast to that of Gram positive bacteria especially *Bacillus* spp. Plant growth promoting effects of *B. amyloliquefaciens* have been reported only recently and PGPR activity is attributed to the production of metabolites like indole acetic acid and siderophores (Mishra and Kumar 2012).

On screening for plant growth promoting properties, both the isolates showed ammonia production. *P aeruginosa* TMR2.13 scored positive for other plant growth promoting properties like phosphate solubilization and HCN production as opposed to *B. amyloliquefacies* NAR38.1 (Fig. 3.1).

Phosphate solubilizers bring about favourable changes in the soil microenvironment which leads to solubilization of inorganic phosphate (Mehta *et al.* 2010). Indole acetic acid (IAA) is a phytohormone and induces root elongation (Fallik *et al.* 1989, Kende and Zeevaart 1997), growth of roots and shoots in response to light and gravity (Kaufman *et al.* 1995), cell division in tissues, cell differentiation, and the formation of adventitious roots (Bashan *et al.* 2008). Production of HCN by PGPR protects plants from diseases (Marques *et al.* 2010). Plant growth promoting bacteria are usually *Bacillus* or *Pseudomonas* spp. and have been applied to a large range of plant spp. (Haas and Defago 2005).

3.3.2 Determination of the functional group of siderophore

To determine the functional group of the siderophore, the supernatant of the culture grown in iron - free MSM was subjected to various tests. The supernatants containing siderophores of *P. aeruginosa* TMR2.13 showed presence of carboxamate functional group. The cell-free supernatant of TMR2.13 showed a sharp peak at 400 nm (Fig. 3.2) and emitted fluorescence at 467 nm when excited at 400 nm, characteristic of the yellow pigment, pyoverdine, frequently produced by *Ps. aeruginosa*. The pigment shows a strong affinity for iron and therefore acts as a siderophore in iron limiting media (Braud *et al.* 2009, Cornelis *et al.* 2009).

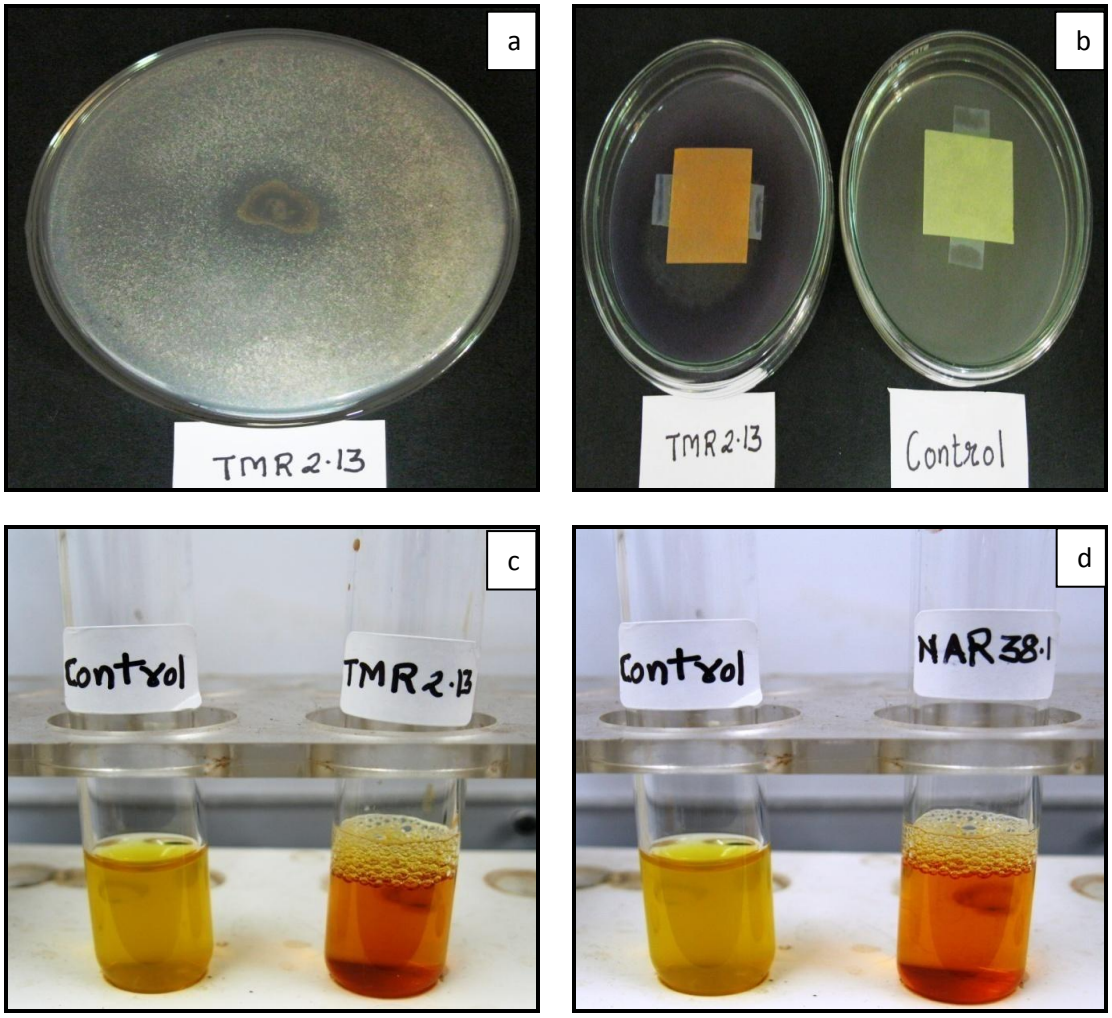


Fig. 3.1: Plant growth promoting properties of *P. aeruginosa* TMR2.13 a) Phosphate solubilzation b) HCN production c) Ammonia production d) Ammonia production by *B. amyloliquefaciens* NAR38.1

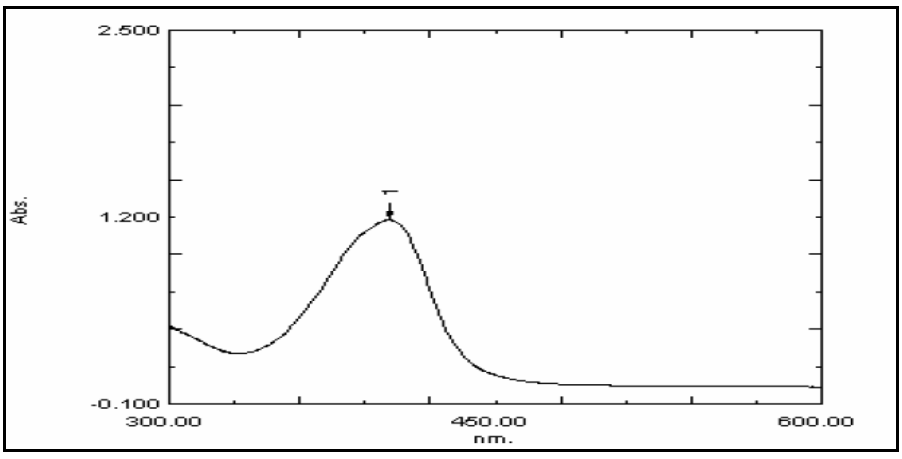


Fig. 3.2: Spectrophotometric scan of pyoverdine produced by TMR2.13

Pyoverdines are composed of three parts: i) a fluorescent chromophore based on a quinolin cycle responsible for the yellow-green color and bright fluorescence of pyoverdine; ii) a side-chain containing a carboxylic acid residue branched to an amino group of the chromophore; iii) a peptidic chain attached to a carboxyl group on the chromophore. The differences in the side chain reflects the diversity in pyoverdine structures (Meyer 2000).

B. amyloliquefaciens NAR38.1 was found to produce catechol type of siderophore. Previous studies have shown that catecholate siderophores predominate in certain Gram-negative genera, namely enterobacteria, *Vibrio*, but are also seen in the nitrogen-fixing *Azotobacteria* and the plant-associated agrobacteria. Catecholates may be common in this group because of their lipophilic nature, complex stability, high environmental pH and a weak nitrogen metabolism (Das *et al.* 2007). Catecholate siderophores are known to be produced by *Magnetospirillum magneticum* (Calugay *et al.* 2006), *Bacillus subtilis* (Corynebactin), *Corynebacterium glutamicum* (Corynebactin), *Azotobacter vinelandii* (protochelin, azotochelin), *Aeromonas hydrophila* (Amonabactins), *Chryseomonas luteola* (Cheyseomonin) and *Rhodococcus erythropolis* (heterobactins) (Winkelmann 2004).

Production of hydroxamate type of siderophores is important in an environment where a large amount of plant litter is present and is degraded by acid - producing bacteria such as *Enterobacter*, *Listeria* or *Lactobacillus* spp. In such ecosystems, iron may be present in large amounts in humic matter; however, it is not easily available to microorganisms and necessitates the production of siderophore. Ferrioxime is one such siderophore, which can extract iron from soils containing large amounts of humic acid, and is produced by streptomycetes (Winkelmann 2004). However, in the present study, although the culture NAR38.1 was isolated from a similar ecosystem, (mangrove ecosystem, where the plant litter

is being continuously degraded following a detrital cycle) the isolate produced the catecholate type of siderophore. Plant litter in mangrove ecosystems comprises of major macromolecules such as tannins, waxes, pectins etc. *Bacillus* are known to breakdown such unusual molecules thereby resulting in their proliferation (Ilori *et al.* 2007, Qureshi *et al.* 2012). This study has also shown the occurrence of *B. amyloliquefaciens* in the mangrove ecosystem at Ribander with production of the catecholate type of siderophore by the same to overcome iron stress.

3.3.3 Effect of pH on growth and siderophore production

The isolates TMR2.13 and NAR38.1 were isolated from the rhizhosphere of a sand dune creeper *Ipomoea pes- caprae* and from mangroves. The salinity in such ecosystem varies due to tidal fluxes and also results in microniches which have an alkaline/acidic pH which is a result of breakdown of plant litter (Agate *et al.* 1988). In order to understand the effect of such changes in pH and salinity on siderophore production, the isolates were exposed to varying pH and salt concentrations. For *P. aeruginosa* TMR2.13, optimum pH for growth and siderophore production was found to be pH 7 (Fig. 3.3a). Sayyed *et al*. (2004) have also reported pH 7 to be optimum for siderophore production in *P. fluorescens*. While changes in pH did not considerably affect growth, the siderophore production was decreased to a large extent at pH 5 in this isolate. In case of NAR38.1, the changes in pH also did not show any significant effect on the growth, however, the siderophore production was found to decrease under acidic and alkaline conditions (Fig. 3.3b). Optimum siderophore production as determined by Arnow's test was observed at pH 7. The decrease in siderophore production under acidic pH could be attributed to solubilization of iron at lower pH resulting in iron availability (Agate *et al.* 1988). Ferric complexes of catecholates are unstable at acidic pH, therefore, in nature, biosynthesis of the hydroxamate type of siderophore is preferred over synthesis of the catecholates (Winklemann 2004). It has also been reported that at low pH,

destruction of siderophores is noted specially in *Pseudomonas aeruginosa* and therefore an increase in pH is reported during growth (Villegas *et al.* 2002) which protects the siderophore.

3.3. 4 Determination of the effect of NaCl on growth and siderophore production in the selected isolates

During our study it was noted that the increase in NaCl concentrations increased growth of *P. aeruginosa* TMR 2.13, however, siderophore production was found to decrease (Fig. 3.4a). Siderophore production also decreased considerably with increase in salt concentrations from 0 to 5% in *B. amyloliquefaciens* NAR38.1, however, growth was not affected upto 2% of NaCl (Fig. 3.4b). Further, increase in NaCl (≥3%) showed reduction in growth which resulted in decrease in siderophore

production. Such effects of NaCl on siderophore production have been noted in *Rhizobium* strains nodulating *Macrotyloma uniflorum*. These strains showed increased siderophore production upto 8-9% of salt concentration, but siderophore production ceased when salt concentration was increased further (Prabhavati and Mallaiah 2008).

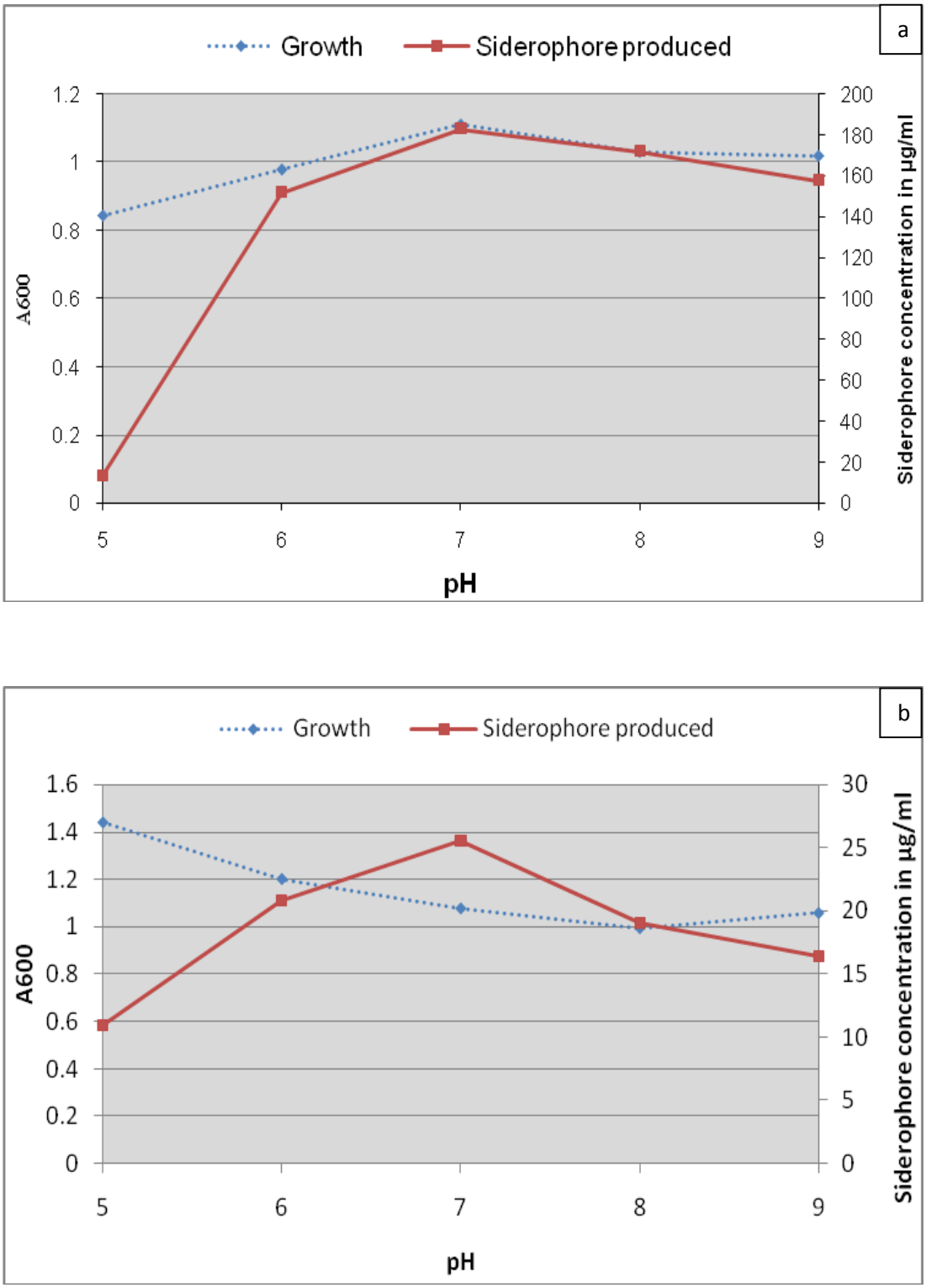


Fig. 3.3: Effect of varying pH on growth and siderophore production in a) *P. aeruginosa* TMR2.13 b) *B. amyloliquefaciens* NAR38.1

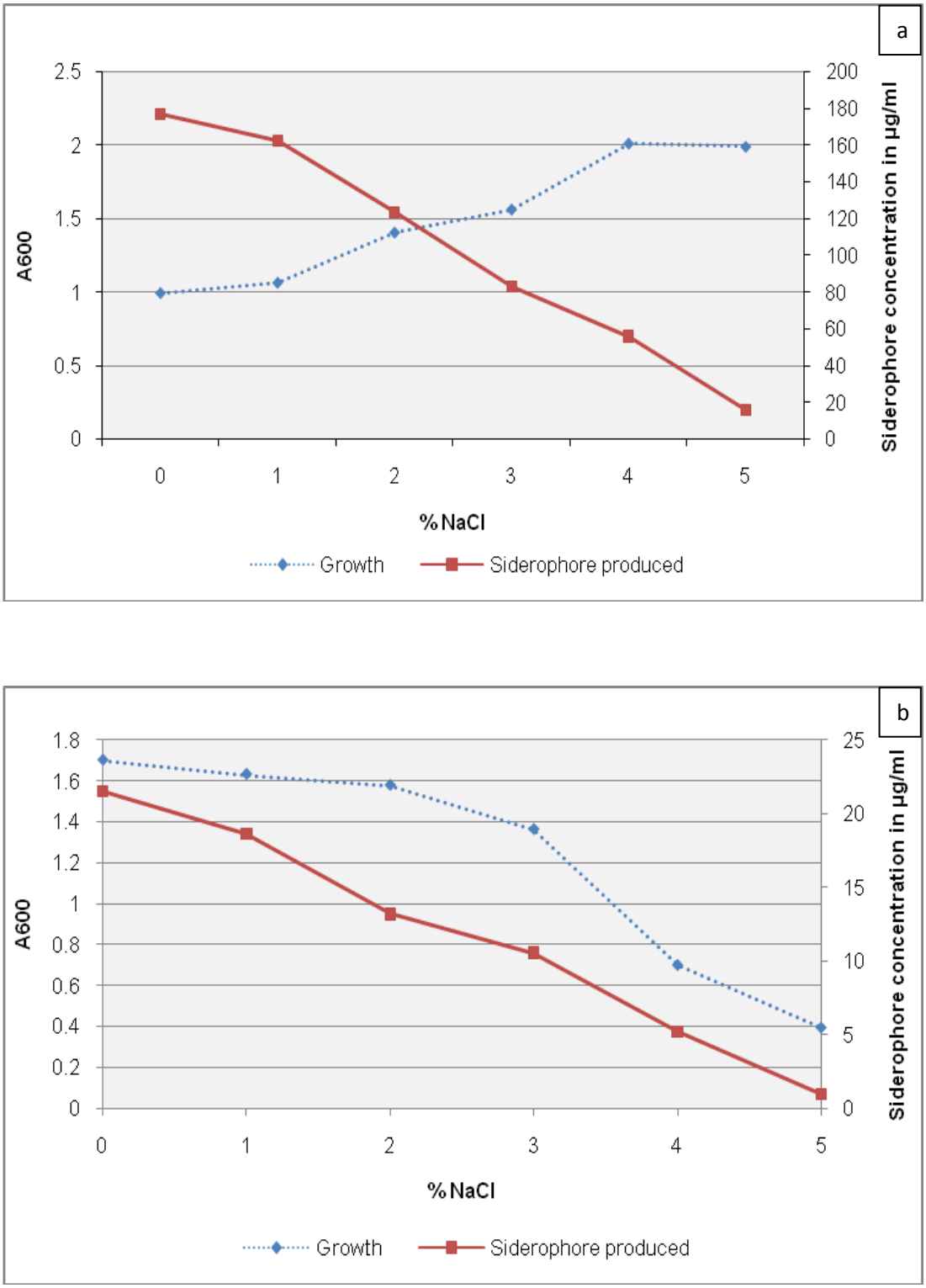


Fig. 3.4: Effect of varying NaCl concentrations on growth and siderophore production in a) *P. aeruginosa* TMR2.13 b) *B. amyloliquefaciens* NAR38.1

3.3.5 Effect of iron on growth and siderophore production in *Pseudomonas aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1

Iron is one of the most important micronutrients required by bacteria for their metabolism as a cofactor for a large number of enzymes and iron containing proteins (Harrington and Crumbliss 2009). However, its availability in the environment may not be sufficient to support microbial growth. The effect of iron on growth of the selected isolates was determined in presence and absence of added iron in MSM with 0.2% glucose as the carbon source. It was observed that presence of iron did not affect growth profiles of the cultures but completely suppressed siderophore production (Fig. 3.5 and 3.6).

Further studies with NAR38.1 showed production of siderophore upto 1 µM of Fe^{+2} and upto 30 µM of Fe^{+3} (Fig. 3.7), suggesting that higher levels of Fe^{+3} are required to suppress siderophore production as compared to the Fe^{+2}. This could be possibly because of the well known fact that Fe^{3+} form is not the highly soluble form of Fe as opposed to Fe^{2+} ion which has a much better solubility (Chu 2010).

However, the effect of iron with *P. aeruginosa* TMR2.13 showed a significant siderophore production upto 54 µM which was suppressed at 108 µM irrespective of it being divalent or trivalent (Fig. 3.8). The fact that pyoverdine binds to both the forms of Fe, as has been reported earlier (Xiao and Kisaalita 1998), results in the suppression of siderophore production with the same concentration of Fe^{+2} and Fe^{+3}.

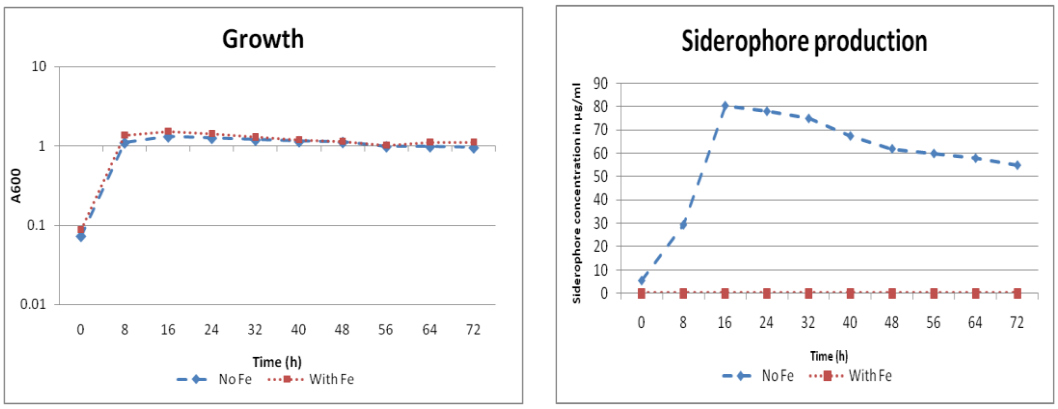


Fig. 3.5: Growth profile and siderophore production by *P. aeruginosa* TMR2.13 in presence and absence of added iron

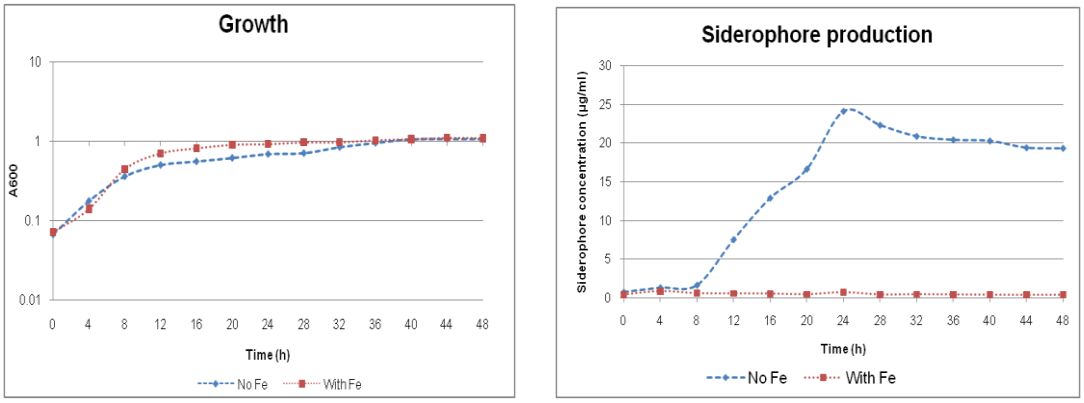


Fig. 3.6: Growth profile and siderophore production by *B. amyloliquefaciens* NAR38.1 in presence and absence of added iron

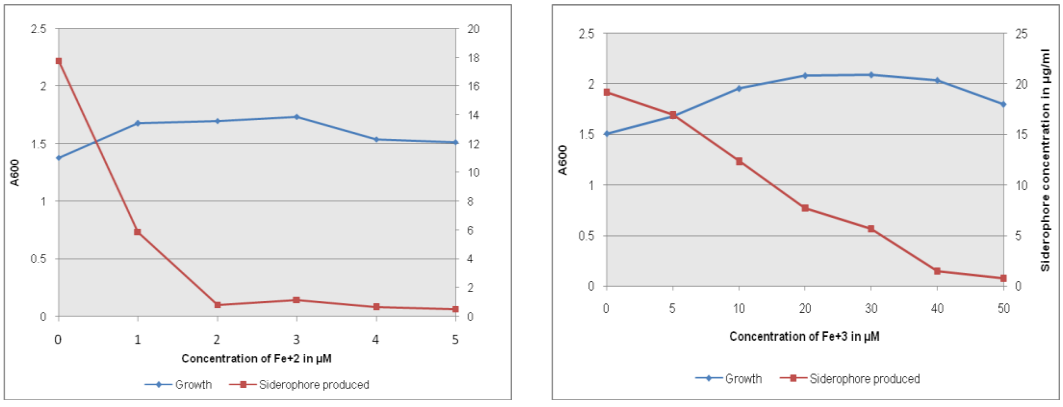


Fig. 3.7: Effect of Fe^{+2} and Fe^{+3} concentrations on siderophore production by *B. amyloliquefaciens* NAR38.1

Earlier studies on the effect of iron concentration on siderophore production by *Ps. aeruginosa* have shown siderophore production even with 248 µM of Fe^{+3} (Villegas *et al.* 2002). Rachid and Ahmed (2005) have reported Fe^{+3} levels of 200 µg/ml being inhibitory to siderophore production by *P. fluorescens*, while Sayyed *et al.* (2004) have reported suppression of siderophore production in *P. fluorescens* and *P. putida* at concentration of 20 µM. These reports and the observations made in the present study implicates that the iron requirement varies with the strains.

3.3.6 Binding of siderophores produced by *Pseudomonas aeruginosa* TMR2.13 and *Bacillus amyloliquefaciens* NAR38.1 to different forms of iron.

Amongst the different forms of iron, Fe^{+3} iron is known to have higher affinity for siderophores in contrast to Fe^{+2}. This extreme difference in the affinity is important for release of Fe^{+3} from siderophore by reducing it to Fe^{+2} (Xiao and Kisaalita 1998). The ability of siderophores produced by the selected isolates to bind to Fe^{+2}/Fe^{+3} and zerovalent iron nanoparticles was determined. It was interesting to note that the fluorescence of pyoverdine produced by the isolate TMR2.13 was quenched by 45.58% with Fe^{+2} immediately, while Fe^{+3} quenched the fluorescence by 67.08% within 30 seconds (Fig. 3.9a)**.** Xiao and Kisaalita (1998) have demonstrated that pyoverdines also bind and oxidize Fe^{+2}, with the binding of pyoverdines to Fe^{+2} being faster than to Fe^{+3} and suggested that the phenomenon could be due to the precipitation of Fe^{+3} as $Fe(OH)_3$. The most interesting observation was the binding of pyoverdine to zerovalent iron nanoparticles. Nanoparticles quenched fluorescence by 22.25, 36.56 and 62.32 % when added at concentrations of 0.0125, 0.025 and 0.05 M respectively (Fig. 3.9b).

In the present study, very low concentrations of Fe^{+3} (0.001M) resulted in complete binding of the siderophore produced by *B. amyloliquefaciens* NAR38.1 than those of Fe^{+2} (0.05M)

(Fig. 3.10). The intensity of the peak at 510 nm is directly proportional to the amount of siderophore which is not bound to Fe. Studies with zerovalent iron nanoparticles did not show any binding with siderophore confirming its affinity for trivalent form of iron.

This chapter illustrates the various aspects about the selected siderophore producing isolates. Both the isolates showed ammonia production with *P. aeruginosa* TMR2.13 being positive for other plant growth promoting properties like phosphate solubilzation and HCN production. Functional group tests proved that the siderophores produced by TMR2.13 and NAR38.1 were of carboxamate and catecholate type respectively. Optimum siderophore production for both the isolates was obtained at pH 7, 0% NaCl and with no added iron. Furthermore, addition of iron to the growth medium of both the isolates suppressed siderophore production without affecting growth profiles. Studies with NAR38.1 showed production of siderophore upto 1 µM of Fe^{+2} and upto 30 µM of Fe^{+3}. However, interestingly, siderophore production was seen upto 54 µM and suppressed at 108 µM of Fe^{+2} as well as Fe^{+3} in *P. aeruginosa* TMR2.13. Siderophore produced by *P. aeruginosa* TMR2.13 showed binding to Fe^{+2}, Fe^{+3} as well as to Fe^{0}. While, siderophore produced by NAR38.1 had much higher affinity for Fe^{+3} as compared to Fe^{+2}.

Biosynthesis and secretion of siderophores is known to be related to the requirement of iron for metabolism of specific growth substrates. The presence of aromatic aromatic compounds along with easily metabolisable co-substrates supports the production of siderophores. Iron concentration is one of the many factors influencing efficient clean-up of pollutants. In natural environments, the availability of iron is usually low, more so, in marine and coastal ecosystems as well as in arid environments where the concentration of iron can be

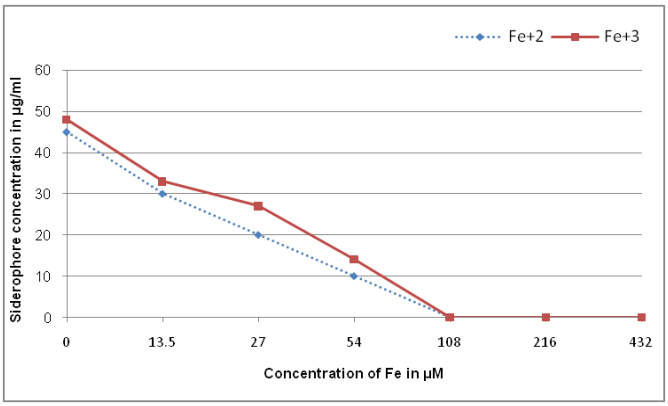


Fig. 3.8: Effect of Fe^{+2} and Fe^{+3} concentrations on siderophore production by *P. aeruginosa* TMR2.13

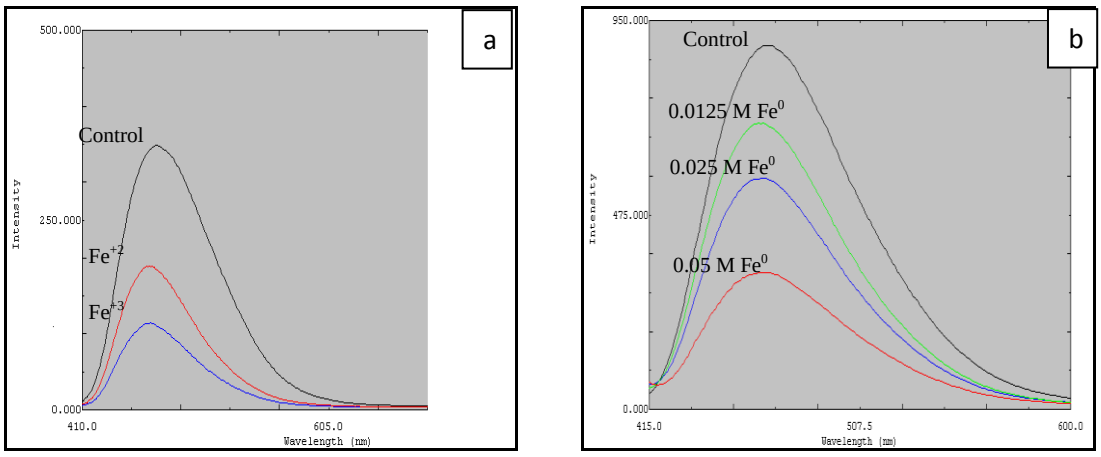


Fig. 3.9: Fluorescence spectra of pyoverdine illustrating fluorescence quenching with a) Fe^{+2} and Fe^{+3} b) Fe^{0} nanoparticles.

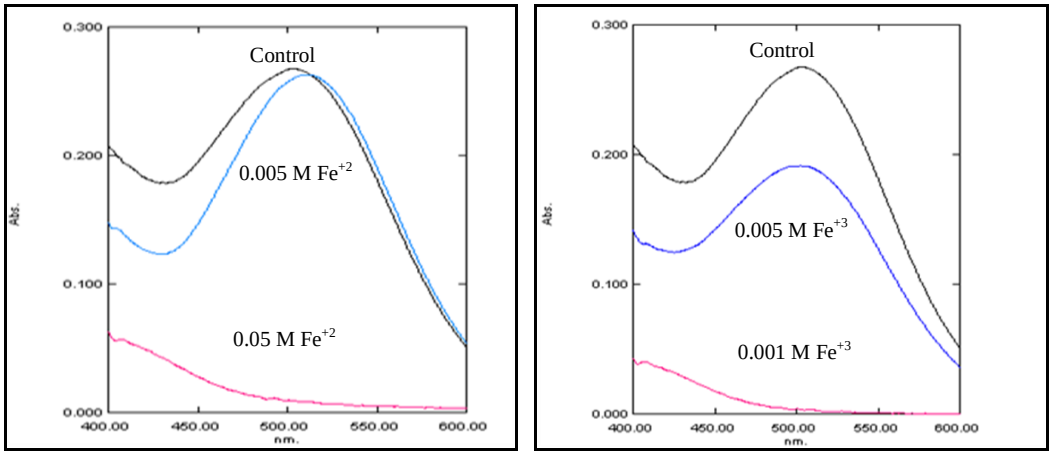


Fig. 3.10: Binding of Fe^{+2} and Fe^{+3} to siderophore produced by *B. amyloliquefaciens* NAR38.1.

as low as 10^{-18} M.Such - iron limiting conditions may have a direct implication on the siderophore production during utilization of aromatic compounds. Isolate TMR2.13 which produced siderophore and also showed growth on sodium benzoate was selected further to study the effect of sodium benzoate on siderophore production. Sand dune samples were also enriched to isolate siderophore producing organisms which could also utilize sodium benzoate as the sole carbon source. These results have been compiled in the next chapter.

Chapter 4

Response of P. aeruginosa TMR2.13 to aromatic compound - sodium benzoate

4.1 Introduction

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

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

Earlier studies with isolates from sand dunes and mangroves resulted in very few organisms (8 % of the siderophore producers) showing growth on sodium benzoate. It was therefore envisaged to enrich the isolates, especially those capable of growing on sodium benzoate as

sole source of carbon. This chapter describes the enrichment, isolation and identification of the selected isolates. Further, the response of TMR2.13 to benzoate in terms of growth and siderophore production was also studied.

4.2 Material and methods

4. 2.1 Enrichment of sand dune samples for isolation of benzoate - utilizing siderophore producing bacteria

1 g of sand dune samples, R1 and NR1, was added to 50 ml of MSM (with and without iron) in 250 ml conical flaks with 0.2% sodium benzoate. The flasks were incubated at 150 rpm at 28 ºC for 24 h. After incubation, 5 ml of sample was transferred to 50 ml of the respective medium under aseptic conditions. Flasks were incubated for 24 h at 150 rpm and 30 ºC. Sub-cultures were continued for 3 transfers to enrich the desired organisms.

The enriched samples were serially diluted and plated out on MSM with 0.2% sodium benzoate. Plates were incubated at 28 ºC for 24 hours and colonies were counted to determine the viable counts. Distinct isolates were purified by repeated sub-culturing to obtain pure colonies. These were then screened for production of siderophore in MSM and MM9 medium containing CAS. The selected isolates were identified using Hi Media kits.

4.2.2 Growth of the selected isolates in liquid MSM with varying sodium benzoate concentrations

The two selected isolates were inoculated in tubes containing liquid MSM with different sodium benzoate concentrations (0.1%, 0.2%, 0.5%, 0.75%, 1.0%, 1.5% and 2%). The tubes were incubated under stationary conditions at 28 ºC for 24 hours. Growth was checked

visually as increase in turbidity. The mode of ring cleavage in each of the organisms was determined by Rothera's test (Norris and Ribbons, 1971) (Annexure C).

4.2.3 Effect of sodium benzoate on the morphology of *P. aeruginosa* TMR2.13

24 hour old culture was inoculated in MSM with and without iron and 0.2% glucose or sodium benzoate as the sole carbon source. The flasks were incubated at 150 rpm for 24 hours. The cells were harvested by centrifugation and processed for SEM as mentioned in section 2.2.7.

4.2.4 Effect of sodium benzoate on siderophore production by *P. aeruginosa* TMR2.13 in MSM with sodium benzoate and different concentrations of iron

Flasks containing MSM with 0.2% sodium benzoate or glucose as the sole carbon source, supplemented with iron in increasing concentration (0, 13.5, 27, 54, 108, 216, and 432 μM) were inoculated with 5% of exponential cells grown in the respective medium. The culture flasks were incubated at 100 rpm at 28 °C, and growth and pigment production was monitored over a period of 72 hours. 5 ml sample was removed every 8 hours, and growth was measured as increase in turbidity at 600 nm. Siderophore was quantified in cell-free supernatant by measuring the absorbance at 400 nm using UV-Vis spectrophotometer (Shimadzu UV-2450). Siderophore concentration was calculated as described by Gupta *et al.* (2007).

4.2.5 Effect of different carbon sources and media on siderophore (pyoverdine) and pigment (pyocyanin) production in *P. aeruginosa* TMR2.13

A loopfull of 24 hour old culture was inoculated in basal medium (BM) with 0.2% glucose and incubated for 24 hours under shaker conditions at 28 ºC. 5% of the inoculum was inoculated in the following media

I) Succinate basal medium (BM) supplemented with following carbon sources

1) BM + 0.4% succinate
2) BM + 60 mg/lt $FeSO_4.7H_2O$+0.4% succinate
3) BM + 60 mg/lt $FeCl_3.6H_2O$+0.4% succinate
4) BM + 0.4% glucose
5) BM + 0.2% sodium benzoate
6) BM + 0.4% sodium benzoate
7) BM + 0.4% succinate + 0.2% sodium benzoate
8) BM + 0.4% succinate + 0.4% sodium benzoate

II) Nutrient broth (NB)

III) Typtone yeast extract glucose broth (TYB)

IV) Glutamate medium

V) Casamino acid medium (CAA)

Siderophore (pyoverdine) and pigment (pyocyanin) concentration were estimated every 24 h upto 72 h by measuring absorbance at 400 and 310 nm respectively

4.3 Results and discussion:

4.3.1 Enrichment of sand dune samples for isolation of benzoate utilizing siderophore producing bacteria

Aromatic hydrocarbons are ubiquitously found in soil and sediments due to natural and anthropogenic activities. Although some hydrocarbons, earlier considered as xenobiotics, are now known to be degraded by microorganisms (Hilyard *et al.* 2008) via aerobic and

anaerobic pathways. Aerobic degradation of aromatic compounds by bacteria proceeds via several oxidation steps catalyzed by oxygenases. Most of these oxygenases contain iron as the cofactor. In a natural environment, where the availability of iron is always low, the efficiency of the mechanism of iron uptake in bacteria uptake may influence the degradation of aromatic compounds (Dinkla *et al.* 2001). In order to understand the effect of utilization of aromatic compounds on siderophore production, sodium benzoate was used as a model compound in the present study.

Our initial attempts to isolate siderophore producers which can also utilize sodium benzoate as the sole source of carbon resulted in the isolation of a very few isolates (Chapter 2). Sand dune samples R1 and NR1 were therefore enriched in MSM with or without added iron and sodium benzoate as the sole carbon source. Enrichment technique involves providing conditions favorable for growth of bacteria on a particular substrate. A total of 18 and 22 isolates were obtained on enrichment with iron and without iron respectively It was observed that all the isolates obtained from the sample enriched in MSM with iron showed siderophore production on MSM and MM9, except for isolate EI11 which did not exhibit siderophore production on MM9. However, out of 22 isolates obtained from MSM without iron, only 16 showed siderophore production (Table 4.1a and 4.1b) (Fig. 4.1a and 4.1b).

4.3.2 Growth of the selected isolates in liquid MSM with varying sodium benzoate concentrations

Two isolates E6 and E16 which showed maximum siderophore production (Fig. 4.2) as evident from the zone size on CAS plate were selected for identification using biochemical tests. The cultures were tentatively identified as *Pseudomonas* sp. (Table 4.2) and along with the isolate TMR 2.13 were selected to check their growth in MSM with varied concentrations

of sodium benzoate. All the three isolates showed growth in MSM containing up to 2% of sodium benzoate concentration (Fig. 4.2). Rothera's test showed the ortho mode of ring cleavage (Fig. 4.3).

Interestingly, the production of siderophore in presence of benzoate was observed to be higher with TMR2.13 (chapter 2) as compared to E6 and E16. Since our objective was to understand the effect of benzoate on siderophore production, in presence and absence of iron, TMR2.13 was selected for further studies.

4.3.3 Effect of sodium benzoate on the morphology of *P. aeruginosa* TMR2.13

It was observed that the cells of TMR2.13 showed increase in length by 0.47 µM when grown in presence of sodium benzoate (Fig. 4.4b & d) as compared to when grown in presence of glucose (Fig. 4.4a & c). Reports have indicated that one of the mechanisms to overcome the toxicity of aromatic hydrocarbons is alterations in structure and permeability of the cell membrane (Sikkema *et al.* 1995).

Table 4.1: Screening of the enriched cultures for siderophore production on CAS in MM9 and MSM: a) Enriched in media with iron b) Enriched in media without iron

a)

Culture number	Siderophore screening on MM9-CAS	Siderophore screening on MSM-CAS
EI1	+	+
EI2	+	+
EI3	+	+
EI4	+	+
EI5	+	+
EI6	+	+
EI7	+	+
EI8	+	+
EI9	+	+
EI10	+	+
EI11	-	+
EI12	+	+
EI13	+	+
EI14	+	+
EI15	+	+
EI16	+	+
EI17	+	+
EI18	+	+

b)

Culture number	Siderophore screening on MM9-CAS	Siderophore screening on MSM-CAS
E1	-	-
E2	+	+
E3	-	-
E4	+	+
E5	+	+
E6	+	+
E7	-	-
E8	-	-
E9	+	+
E10	-	-
E11	+	-
E12	+	+
E13	+	+
E14	+	+
E15	+	+
E16	+	+
E17	+	+
E18	-	-
E19	+	+
E20	-	+
E21	+	+
E22	-	+

Table 4.2: Biochemical characteristics of the selected isolates

Test	E6	E16
Citrate utilization	+	+
Lysine utilization	+	+
Ornithine utilization	-	+
Urease	-	-
Phenylalanine deamination	-	-
Nitrate reduction	-	+
H_2S production	-	-
Glucose	+	+
Adonitol	-	-
Lactose	-	-
Arabinose	+	-
Sorbitol	-	-
Oxidase	-	-
Catalase	+	+
HL aerobic	+	+
HL anaerobic	±	±
Tentative identification	*Pseudomonas* sp.	*Pseudomonas* sp.

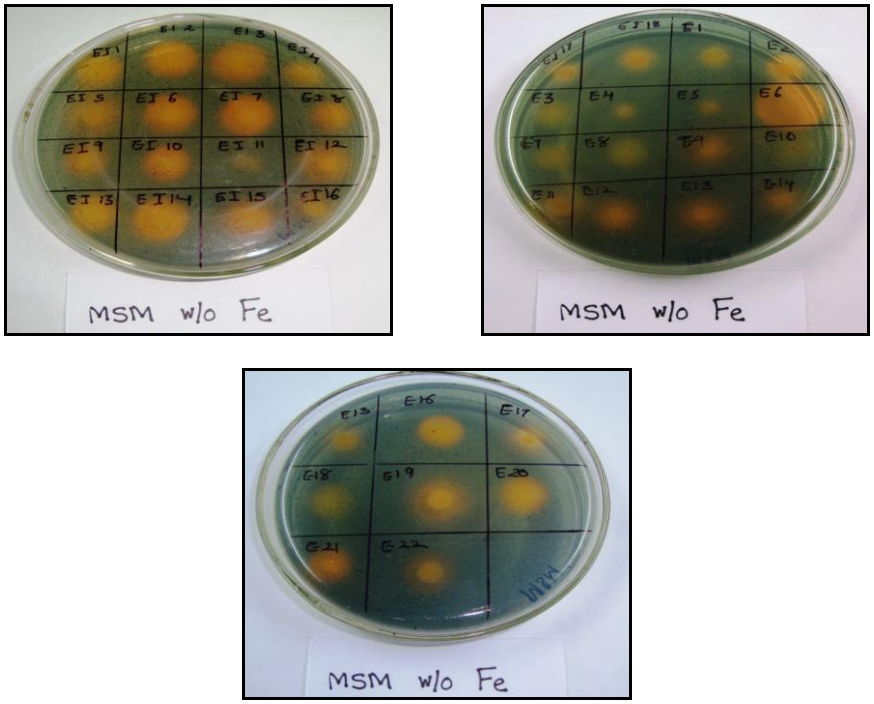


Fig. 4.1a: Screening of the enriched isolates on MSM with CAS and 0.2% sodium benzoate as the carbon source

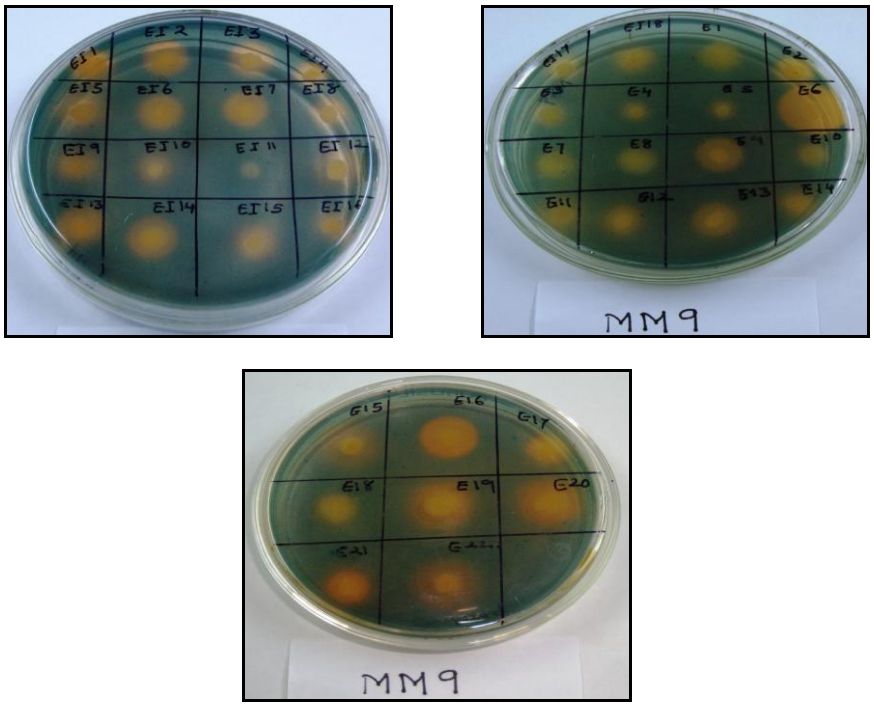
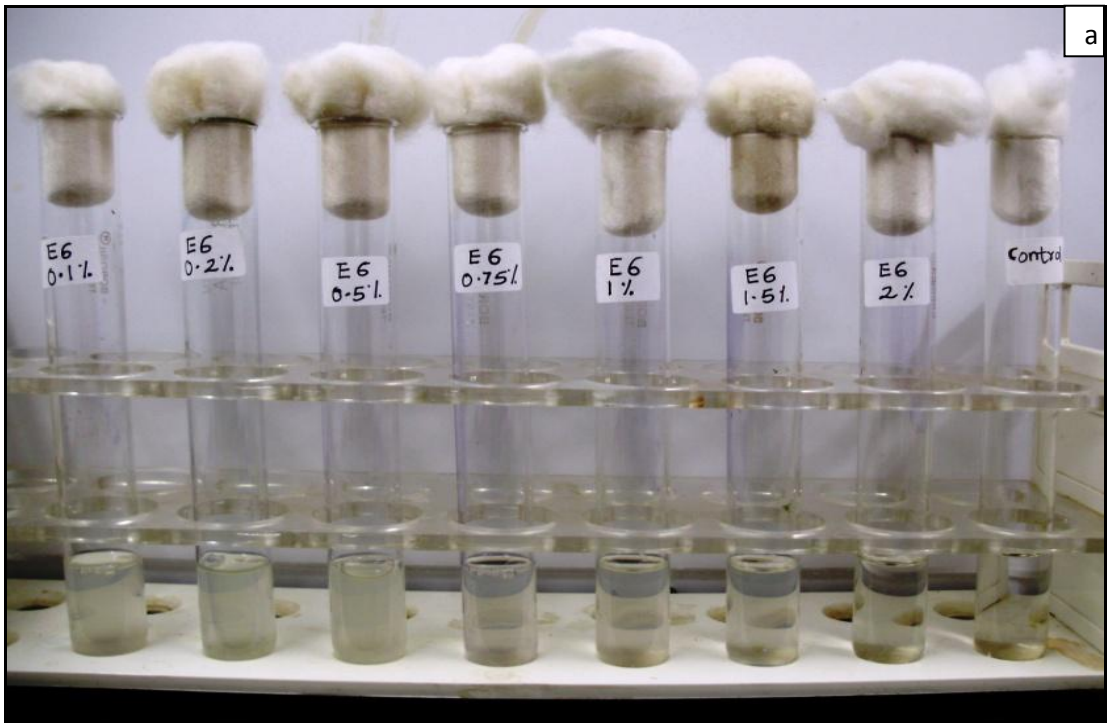


Fig. 4.1b: Screening of the enriched isolates on MM9 with CAS and 0.2% sodium benzoate as the carbon source



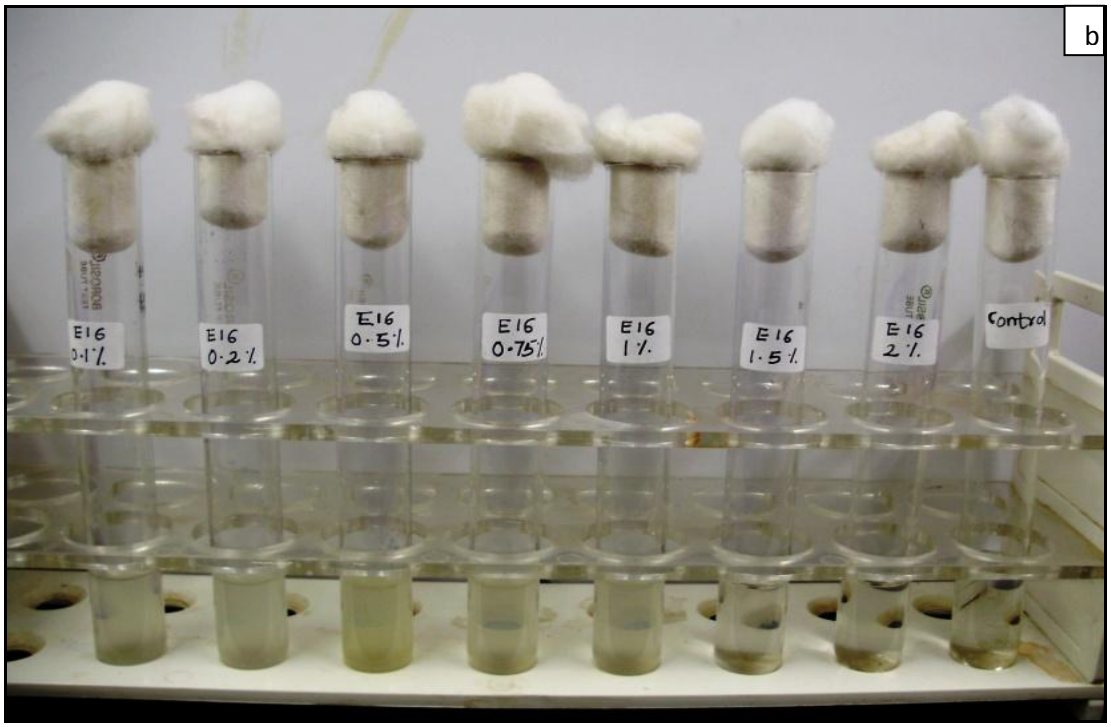


Fig. 4.2: Growth of the selected isolates a) E6 and b) E16 in MSM with different sodium benzoate concentrations

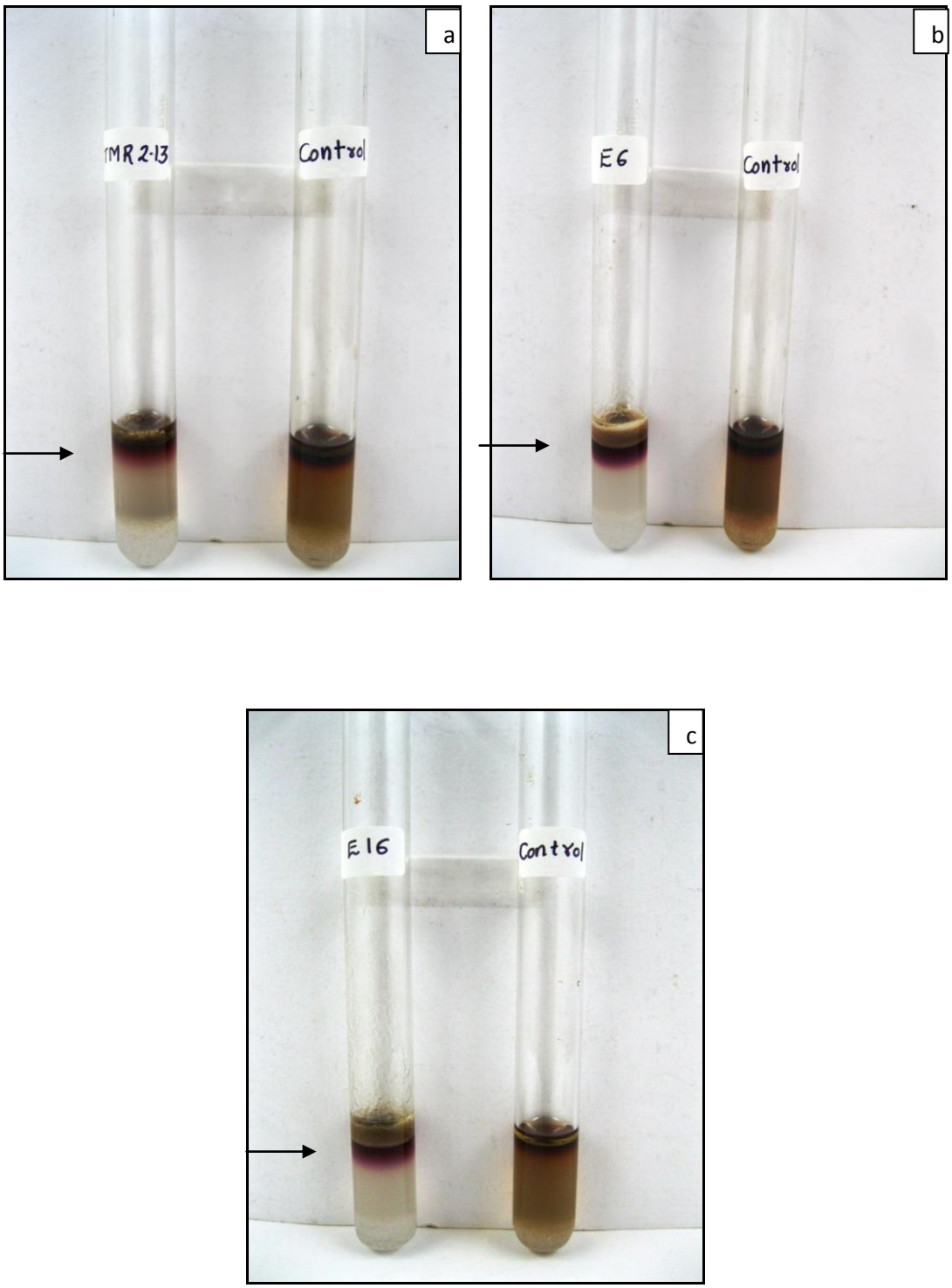


Fig. 4.3: Formation of purple ring (marked by an arrow) by the Rothera's test depicting ortho pathway in a) TMR2.13 b) E6 and c)E16

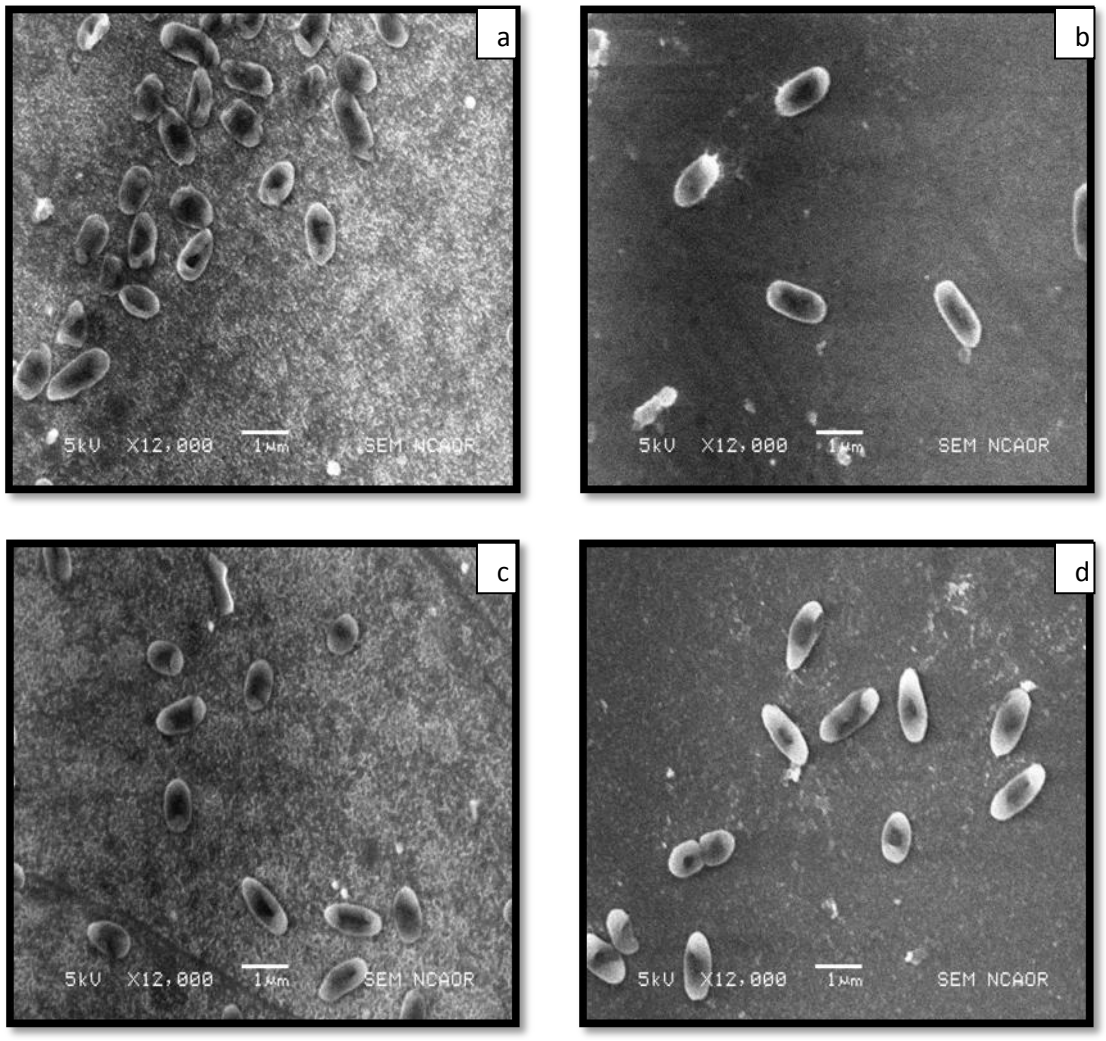


Fig. 4.4: SEM of *P. aeruginosa* TMR2.13 grown in:

a: MSM with glucose as the carbon source and without iron

b: MSM with sodium benzoate as the carbon source and without iron

c: MSM with glucose as the carbon source and with iron

d: MSM with sodium benzoate as the carbon source and with iron

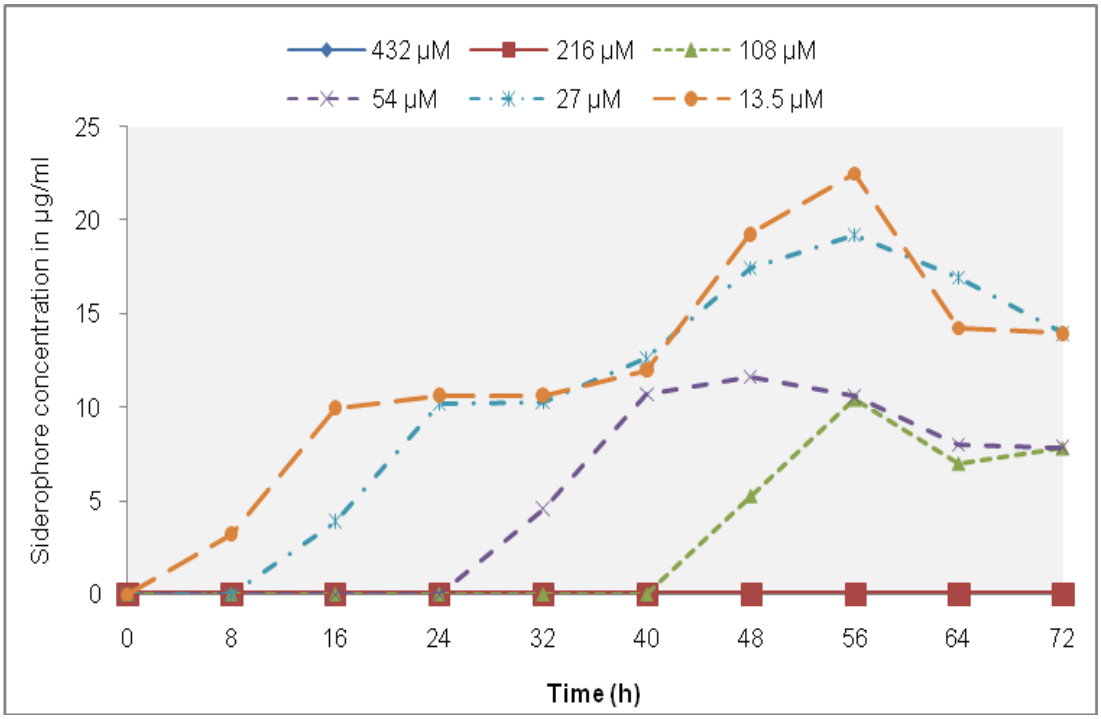


Fig. 4.7a: Siderophore production by *Pseudomonas aeruginosa* TMR2.13 in MSM with sodium benzoate at various Fe concentrations

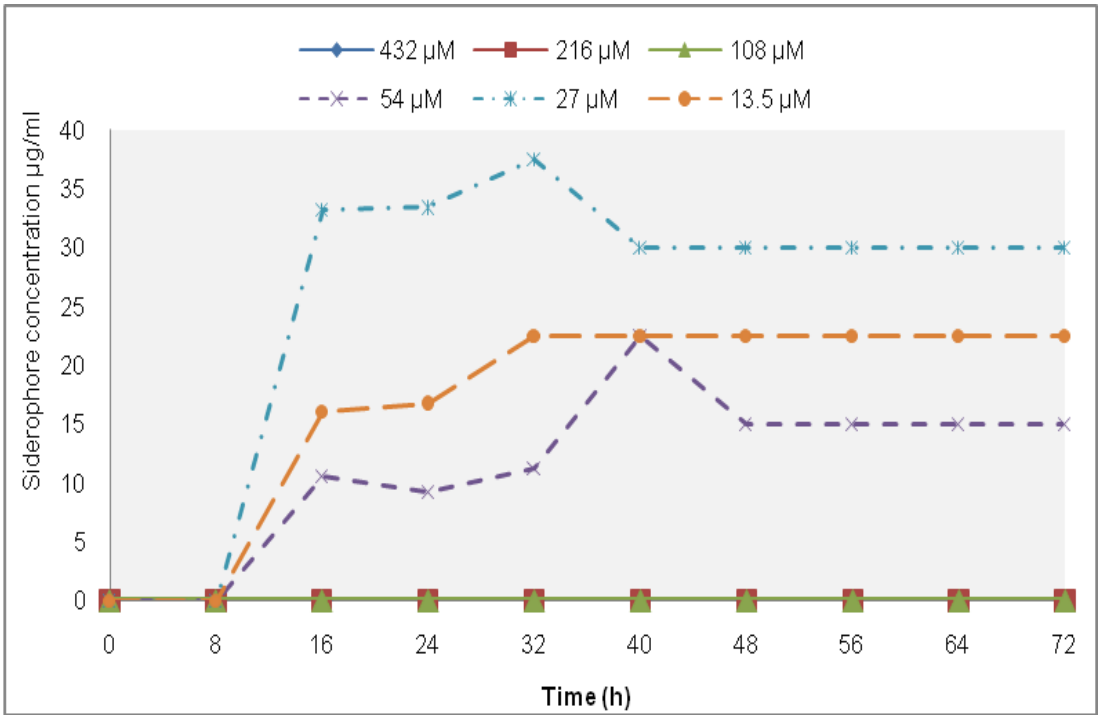


Fig. 4.7b: Siderophore production *Pseudomonas aeruginosa* TMR2.13 in MSM with glucose at various Fe concentrations

A report on the effect of trace element requirement has shown that the iron demand in bacteria increases during the expression of alkane hydroxylase (Staijen and Witholt 1998). A study on the effect of iron concentration on degradation of toluene by *Pseudomonas* strain reports a reduction in the efficiency of the culture when the iron concentration is low (Dinkla *et al*. 2001). Observations from this study also suggest the requirement of a higher concentration of iron to sustain growth in a benzoate medium. As mentioned earlier, the mono- and dioxygenases involved in degradation of aromatic hydrocarbons require iron as a cofactor (Dinkla *et al.* 2001). Earlier studies on the effect of iron concentration on siderophore production by *P. aeruginosa* have shown siderophore production even with 248 μM of Fe^{+3} (Villegas *et al.* 2002). Biosynthesis and secretion of siderophore is known to be related to the requirement of iron for metabolism of specific growth substrates. The presence of aromatic compounds along with easily metabolisable cosubstrates supports the production of siderophores.

4.3.5 Effect of different carbon sources and media on siderophore (pyoverdine) and pigment (pyocyanin) production in *P. aeruginosa* TMR2.13

Isolate TMR 2.13 was grown in presence of different carbon sources and it was observed that sodium benzoate triggered siderophore production when added as the sole carbon source or in presence of succinic acid. No siderophore production was observed when Fe was added in the succinate medium at a concentartion of 60 mg/L of $FeCl_3$ or $FeSO_4$. Siderophore production was also inhibited in NA, TYB, glutamate and casamino acid medium which could be attributed to contamination of media components with Fe (Fig. 4.8a). Peak at 310 nm corresponded to pyocyanin pigment which is produced by *P. aeruginosa*. It is known to inhibit growth of eubacteria, eukaryoytes and archaea (Salgaonkar *et al.* 2011). Pyocyanin production was observed in all media except in succinate medium supplemented with $FeCl_3$

and in NB (Fig. 4.8b). This experiment confirms sodium benzoate as the best carbon source for siderophore production by TMR2.13 amongst the various carbon sources tested.

Iron concentration is one of the many factors influencing efficient cleanup of aromatic pollutants. In natural environments, the availability of iron is usually low, more so, in marine and coastal ecosystem, also in arid environments where the concentration of iron can be as low as 10^{-18} M (Braun and Killmann 1999). In such environments, the presence of hydrocarbon will promote higher levels of siderophore production to support the hydrocarbon degradation. A direct implication of increased level of siderophores is on microbial community structure, as these siderophores can also support the growth of non-siderophore producing bacteria which may be beneficial, pathogenic or nonculturable (D'Onofrio *et al.* 2010).

This study has highlighted the impact of aromatic compound metabolism on siderophore production. Enrichment studies resulted in the isolation of siderophore producing bacteria degrading sodium benzoate. Utilization of the aromatic compound by TMR2.13 resulted in increase in its iron demand. Sodium benzoate was found to be the best carbon source for siderophore production amongst the various carbon sources tested.

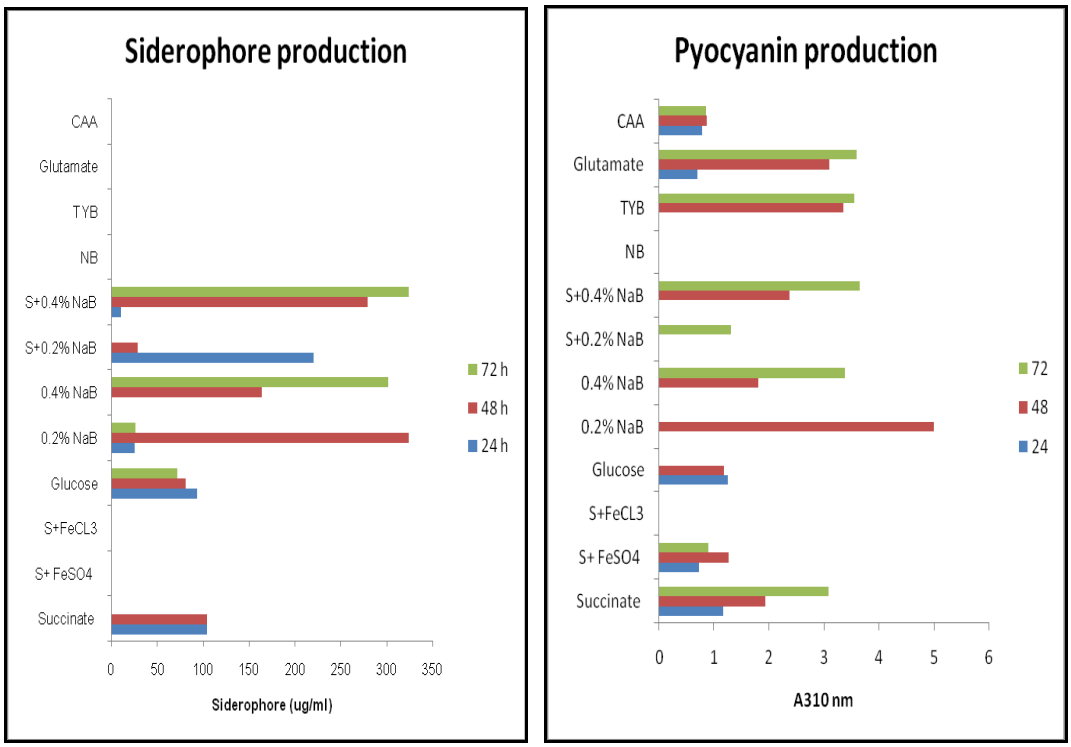


Fig. 4.8:Production of a) pyoverdine and b) pyocyanin by TMR 2.13 in presence of various carbon sources

Amongst the various factors affecting siderophore production, metal ions are now also known to have an impact. Bacteria have multiple mechanisms for accumulation and immobilization of metals (Hu and Boyer, 1996). Cell walls of Gram - positive bacteria are able to bind to metal ions (Beveridge *et al*. 1982). The cell wall of *B. subtilis* has been reported to have high metal sorption capacity (Tunali *et al.* 2006). Some bacteria produce exoploysaccharide sheaths that embed metals (Matthews *et al*. 1979). Previous studies on the effect of metal ions on siderophore production have been focused mainly on *Pseudomonas* spp. (Schalk *et al*. 2011). It was envisaged to study the effect of metal ions on siderophore production in the Gram-positive isolate *B. amyloliquefaciens* TMR2.13 and the results have been discussed in the following chapter.

Chapter 5

Response of B. amyloliquefaciens NAR38.1 to metal ions and characterization of its siderophore

5.1 Introduction:

Heavy metal pollution of soils is a serious problem and demands efficient clean - up of the polluted areas (McGrath *et al.* 1995). Most of the conventional methods used for soil remediation are uneconomical and result in the deterioration of soil texture and its organic content (Rajkumar *et al.* 2010). One of the emerging technologies for bioremediation of metal contaminated soils is "microbial assisted phytoremediation", a process of utilizing plants to absorb, accumulate and detoxify contaminants in soil through physical, chemical and biological processes (Prasad *et al.* 2010) in the presence of PGPR. These rhizhosphere bacteria are known to support the growth and sustenance of plants, besides; some of these bacteria are also metal resistant paving a way for the benign technology for reclamation of metal polluted soils. An important characteristic of PGPR, for use as a bioinoculant in this technology, is siderophore production (Jing *et al.* 2007). Therefore, for such a strategy to be viable, a better understanding of the siderophore producing bacteria and their interaction with metals is required. The study was therefore undertaken to determine the effect of various metals on growth and siderophore production on *Bacillus amyloliquefaciens* NAR38.1. The isolate is only recently known to be associated with plant growth promotion (Buensanteai *et al.* 2008, Mishra and Kumar 2012). The metals selected for the study were those which are essential for the metabolic activity eg. Zinc (Zn), Cobalt (Co), Copper (Cu), Molybdenum (Mo) and Manganese (Mn) and those which were non essential and toxic metals, termed as abiotic (Schalk *et al.* 2011), eg. Arsenic (As), Lead (Pb), Aluminium (Al) and Cadmium (Cd).

Further, it was also of interest to isolate and to characterize the type of siderophore produced by the isolate and determine its relation to different reported siderophores. Preliminary tests with the siderophore produced by NAR38.1 showed it to be a catecholate type (Chapter 3).

Since siderophores differ substantially in structure, no uniform procedure is available for their isolation. Most siderophores are water-soluble, and it is thus usually expedient to drive the siderophore into an organic solvent, such as benzyl alcohol, phenol-chloroform or ethyl acetate in order to eliminate salt. The siderophore may be isolated per se or as its iron chelate. The latter has the advantage of visual color, but the iron has to be removed before further characterization. A number of siderophores are reported to be produced by different species belonging to the *Bacillus* genus, however, there is a lack of literature on the siderophore produced by *B. amyloliquefaciens.* The only report available for siderophore production by *B. amyloliquefaciens* is that the strain isolated from rhizospheric soil samples collected from maize and wheat crop plant and its potential in plant growth promotion (Mishra and Kumar 2012), however, the chemical structure of the siderophore has not been reported hitherto. The present study was therefore focused on determining the characteristics of the siderophore produced by this isolate.

This chapter describes the effect of metals on growth and siderophore production in *B. amyloliquefaciens* NAR38.1 and characterization of the siderophore along with its antibacterial activity.

5.2 Materials and method:

5.2.1 Effect of metal concentration on growth and siderophore production

Stock solutions of the following metal forms: $MnSO_4$, $Co(NO_3)_2.6H_2O$, $ZnSO_4.7H_2O$, $Na_2HAsO_4.7H_2O$, $Pb(NO_3)_2$, $Al_2(SO_4)_3.16H_2O$, $CuSO_4$, $CdSO_4.8H_2O$ and $Na_2MoO_4.2H_2O$ were prepared and sterilized with 0.22 μM filters under aseptic condition. These stock solutions were incorporated in the sterile medium (MSM without $FeSO_4.7H_2O$) to adjust

metal concentration to predetermined levels. Mn, Mo and Zn were tested at 125, 250, 500 and 1000 µM. While, Co, Cu, Cd, As, Pb and Al were tested at lower concentrations of 10, 20, 50 and 100 µM. The flasks were inoculated with 5% of exponential cells grown in MSM without any metal ions. The culture flasks were incubated at 150 rpm at 28 $^{\circ}$C and growth and siderophore production was monitored for 24 hours as mentioned in section 3.2.4.

5.2.2 Purification of the siderophore produced by *B. amyloliquefaciens* NAR38.1

The culture was inoculated in 50 ml MSM without iron for inoculum built up. The flask was incubated at 150 rpm, at 28ºC for 24 hours. 5% of the culture was inoculated in two, 2 L flasks containing 1000 ml of MSM without Fe and 0.2% glucose as the carbon source. The flasks were incubated at 150 rpm for 24 hours at 28 ºC. The culture broth was centrifuged at 10,000 rpm at 4 ºC, for 10 minutes. The supernatant was collected and acidified to pH 2 using 6 M HCl. The acidified supernatant was extracted using 1/5th the volume of ethyl acetate. The two phases were separated in a separating funnel and the ethyl acetate phase containing the siderophore was collected. Ethyl acetate was dried in a rotary evaporator to obtain crude, dry siderophore. The siderophore was dissolved in minimal amount of methanol and purified using amberlite XAD-2 column. The column was prepared in water using amberlite XAD-2 and the siderophore, dissolved in methanol, was loaded on it. The column was washed with deionised water with double the bed volume and the siderophore was eluted using minimum amount of methanol. Fractions were collected, dried in a rotary evaporator and the partially purified siderophore was used for further analysis.

5.2.3 Characterization of the siderophore

Thin-layer chromatography of the column - purified siderophore was carried out using the solvent system: toluene : acetic acid (2:1) and visualized using iodine vapours. Standards

(Catechol and 2, 3-dihydroxybenzoic acid) were run simultaneously. Siderophore was purified using LCMS and its optical spectra recorded using UV- Vis spectroscopy. The purified siderophore was dissolved in deuterated methanol and analyzed by ^{1}H Nuclear Mass Resonance (NMR) spectroscopy (Bruker 400 MHz).

5.2.4 Antibacterial activity:

20 mg of the partially purified siderophore was dissolved in 1 ml of methanol, filter sterilized, and further diluted to 2 ml with sterile water to form a stock of 10 mg/ml. Dilutions of the siderophore were used to test for antibacterial activity: 10, 5, 2 and 0.02 mg/ml with a control consisting of 1:1 methanol : water. The antibacterial activity was tested against, *E. coli*, *P. aeruginosa* strain ATCC 9027, *B. subtilis* 3A1 and *S. aureus*. Suspensions of 24 hour old cultures were prepared in sterile saline and 200 µl of the suspension of each organism was spread plated on nutrient agar plates. Wells (7 mm) were punched into the agar medium with a sterile cork borer and each well was filled with 50 µl of the different siderophore concentrations. The plates were incubated at 28 ºC for 24 h and the size of zone of inhibition was measured.

5.3 Results and discussion

5.3.1 Effect of metal concentration on growth and siderophore production

Of the nine metals used for the study Mn, Co, Zn, Mo, As, Pb, Cd and Cu were in their divalent form while Al was in its trivalent form. The metals such as Mn, Co, Zn, Mo and Cu are known to be essential for cellular processes and are present in bacterial cell at mM or µM quantities (Heldal *et al.* 1985). However, the presence of these metals in excess may manifest toxicity due to interactions with non-specific targets (Schalk *et al.* 2011). Other metals termed as abiotic metals used for this study were Cd, As, Pb and Al and are known to be

toxic to the bacteria. It was interesting to note that all these metals showed varied effects on the growth and siderophore production in the isolate NAR38.1.

a) Effect of essential metals

Amongst the essential metals, Zn was found to increase growth by 20% at 125 µM (Fig 5.1a), with a two-fold increase in siderophore concentration. While at 250 µM (Fig 5.1b), there was a decrease in growth, but a two-fold increase was observed in siderophore production. Such an effect with Zn^{+2} has also been reported for *Azotobacter vinelandii* (Huyer and Page, 1988) when 20 or 40 µM of $ZnSO_4$ was added.

A significant observation was depicted with Mn wherein growth was reduced by more than 40% with increase in concentration, but no alteration in the concentration of the siderophore was seen as compared to the control (Fig. 5.1a-d). This indicated that at reduced growth the same amount of siderophore (approximately 8.8 µg/ml) is produced implicating an increase in siderophore production. Mn^{+2} is known to bind to siderophores like trihydroxamates, desferrioxamine B (DFOB) and desferrioxamine E with an affinity near to or more than that by Fe^{+3} (Duckworth 2009). This could result in the release of higher concentrations of siderophore even as the growth is reduced. Interestingly, Mo showed an increase in growth without any effect or increase in siderophore production (Fig 5.1a-d). Mo is an essential metal required as an active center of the redox-active enzymes in bacteria (Stiefel 1993). Its presence would therefore enhance these enzymes and thereby growth.

Co showed a 40% and 60 % decrease in growth at 10 (Fig. 5.2a) and 20 µM (Fig. 5.2b) respectively, with increase in the siderophore production in NAR38.1. At higher concentrations of 50 (Fig. 5.2c) and 100 µM (Fig. 5.2d) however, both growth and

siderophore production was decreased. Braud *et al.* (2009) have also reported growth inhibition of *Pseudomonas aeruginosa* with 100 µM of Co^{+2}. Co is required as a cofactor in enzymes such as methionine aminopeptidase, nitrile hydratase, lysine-2,3-aminomutase and bromoperoxidase (Kobayashi and Shimizu 1999). At high intracellular concentration, Co^{2+} is highly toxic. Microorganisms overcome Co stress by expressing proteins that export or chelate Co (Ranquet *et al.* 2007). Co stress has been reported to induce genes coding for iron transport proteins in *Saccharomyces cerevisiae*, a response similar to iron starvation (Stadler and Schweyen 2002).

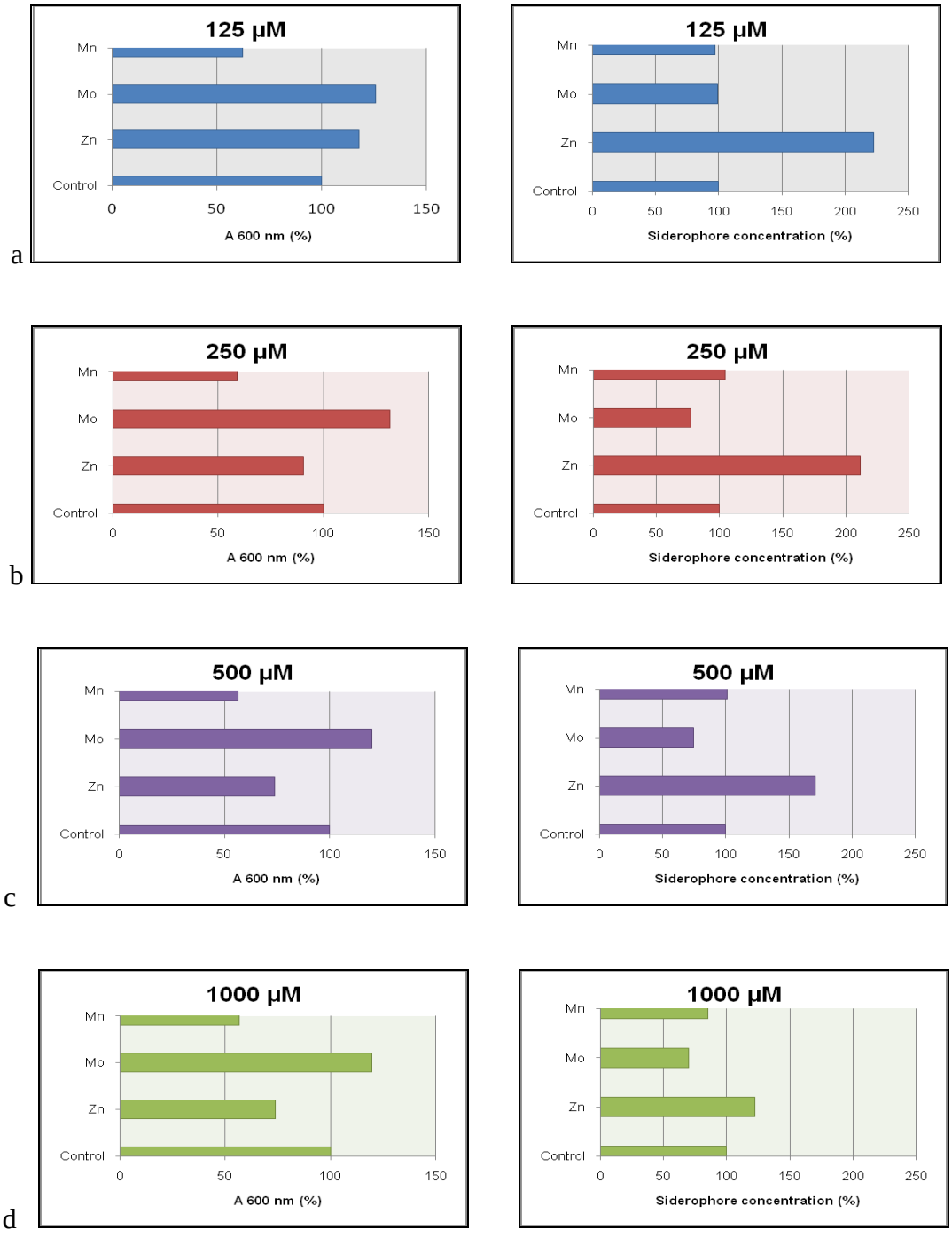


Fig. 5.1: Effect of Mn, Mo and Zn on growth and siderophore production by *B. amyloliquefaciens* NAR38.1 at concentrations of a) 125 µM b) 250 µM c) 500 µM and d) 1000 µM

The effect of Cu depicted decrease in growth by 70% at 10 µM (Fig. 5.2a) beyond which it was completely repressed. However, siderophore production was increased by 20% at 10 µM Cu (Fig. 5.2a) and decreased at 20 µM (Fig. 5.2b) and above. Decrease in siderophore production with Cu has been reported by Arceneauax *et al.* (1984) who have shown decrease in growth of *Bacillus megaterium* when Cu was added at concentrations of 25 µM. Such a change is perhaps due to the accumulation of Cu- siderophore (Schizokinen) complex resulting in cellular accumulation of bactericidal levels of Cu. Further, such high concentrations of Cu in the cells are known to cause an oxidative stress eventually leading to DNA damage (Valko *et al*. 2005).

b) Effect of abiotic metals

Amongst the abiotic metals, the most toxic element was found to be Cd, which inhibited the growth as well as siderophore production. An interesting observation was that Cd at low concentrations reduced the growth to 70 % and 40 % with 10 and 20 µM, respectively (Fig. 5.3a and 5.3b respectively), however, siderophore production was inhibited to almost 90% at 10 µM (Fig. 5.3a) with negligible production at 20 µM (Fig. 5.3b). At higher concentrations of 50 and 100 µM, both growth (Fig. 5.3c and 5.3d) and siderophore production was reduced by more than 95% (Fig. 5.3c and 5.3d). Cd has been reported to increase siderophore production in *Streptomyces* sp. as a mechanism to alleviate the toxicity by binding free metal ions in the medium. The production of the siderophores reduces uptake of Cd^{2+} by *Streptomyces tendae* F4 while supplying it with iron (Dimkpa *et al*. 2009). In the

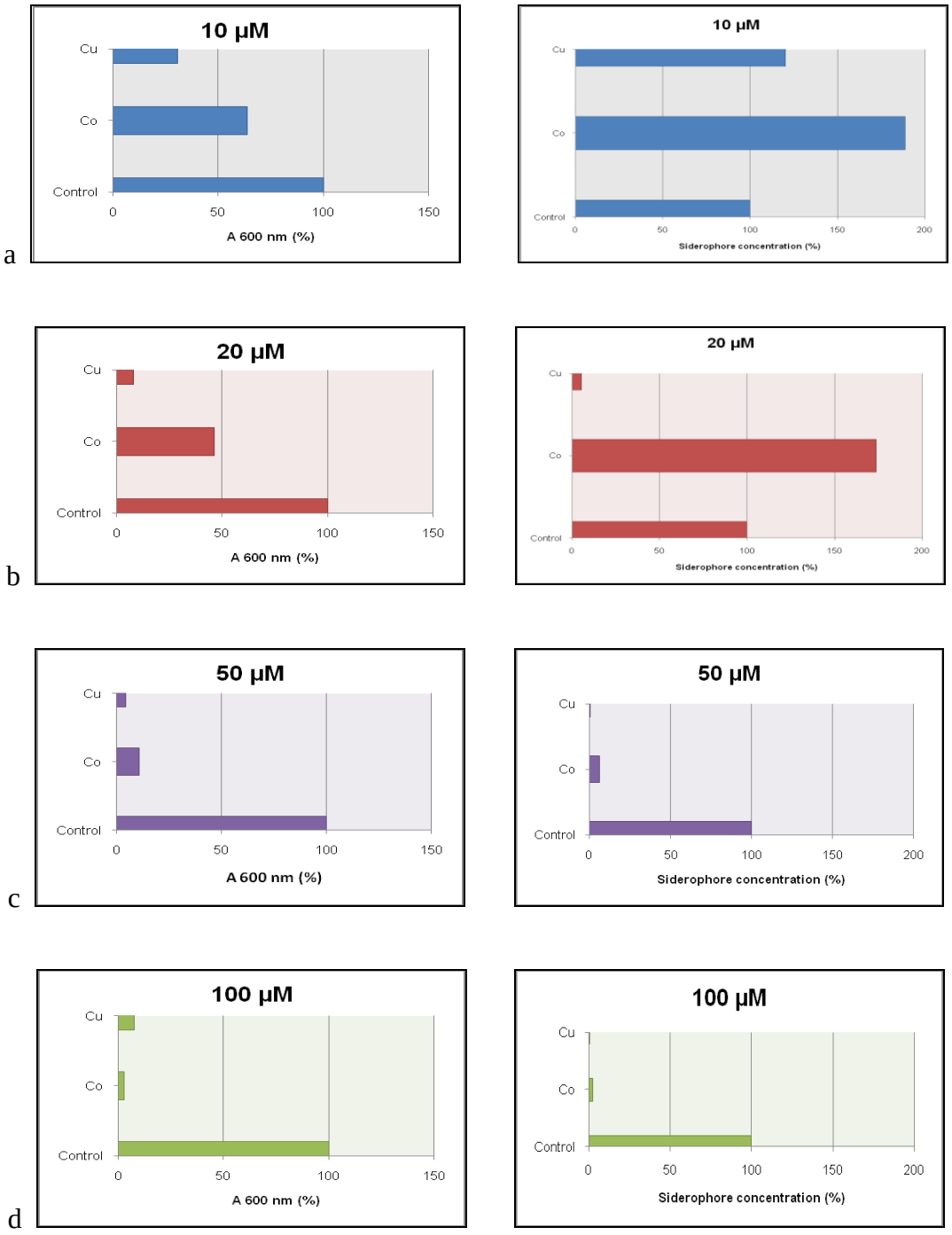


Fig. 5.2: Effect of Cu and Co on growth and siderophore production by *B. amyloliquefaciens* NAR38.1 at a) 10 µM b) 20 µM c) 50 µM and d) 100 µM

present study, Cd has decreased siderophore production which implicates that such a mechanism does not exist in the isolate thereby leading to the inhibition of growth.

It has been reported that one of the strategies for reducing the toxicity of metal elements is their binding to siderophores to form a complex which does not enter the cells and thus the toxicity is not manifested (Rajkumar *et al.* 2010). An interesting response of the isolate was seen in presence of As in the present study. It was noted that there was no significant decrease in the growth at different concentrations of Arsenic (Fig. 5.3a-d), a metal which is known to be toxic. Further, there was no siderophore detected in the extracellular medium even at 10 µM of As (Fig. 5.3a). In the present case also, the results implicated that the siderophore may be binding with As and is therefore not detectable with the test used for monitoring siderophore. Most of the As -tolerant bacteria reported to date are Gram-negative. However, *Bacillus circulans* strains isolated from polluted agricultural lands in Japan have been reported to show tolerance to As in the range of 1.88 – 2.25 g/L of As (III) in a nutrient rich medium (Hiroki 1993). Aksornchu *et al.* (2008) have isolated As resistant bacteria which can grow in medium containing 40 mM sodium-arsenite and belonged to the genera *Enterobacter*, *Neisseria*, *Pseudomonas*, *Staphylococcus*, *Streptococcus* and *Xanthobacter.* The high tolerance to As is attributed to removal of arsenite by adsorption to the extracellular polymeric-like substance produced and also by cellular adsorption in case of a few isolates.

The effect of Pb on siderophore production was found to vary significantly with the concentration of the metal. Growth was reduced by almost 30% as the concentration was increased (Fig. 5.3a-d), however, siderophore production increased with increase in metal concentration showing almost 70 % of production at 100 µM (Fig. 5.3d). Siderophores

produced by the organisms are reported to bind to lead which may prevent its diffusion into the cell (Schalk *et al.* 2011) and reduce metal toxicity (Naik and Dubey 2011).

Studies with trivalent ion of Al did not show significant effect on the growth between 10 to 50 µM (Fig. 5.3a-c), however, a 20 % decrease was observed at 100 µM (Fig 5.3d). It was interesting to note that siderophore concentration in the supernatant increased in medium containing Al, from 10 to 100 µM (Fig. 5.3a-d). The concentrations of 50 and 100 µM showed much better siderophore production as compared to the control. Such an increase has been reported by Hu and Boyer (1996) with *Bacillus megaterium* ATCC 19213. Increase in siderophore (schizokinen) production was observed from 65.7 to 140 µM in medium supplemented with 370 µM of Al with no effect on the growth of this organism. This effect is attributed to the fact that Al having similarity in size and charge as Fe^{+3}, forms complexes with siderophore which are also transported into the cell when Al is present at low concentrations (Garrison and Crumbliss 1987, Hu and Boyer 1996). Bacteria hence produce increased level of siderophores to acquire the required amount of iron for growth.

5.3.2 Purification and chemical characterization of the siderophore

In order to chemically characterize the siderophore, the siderophore was partially purified using column chromatography. Acidification of the culture supernatant prior

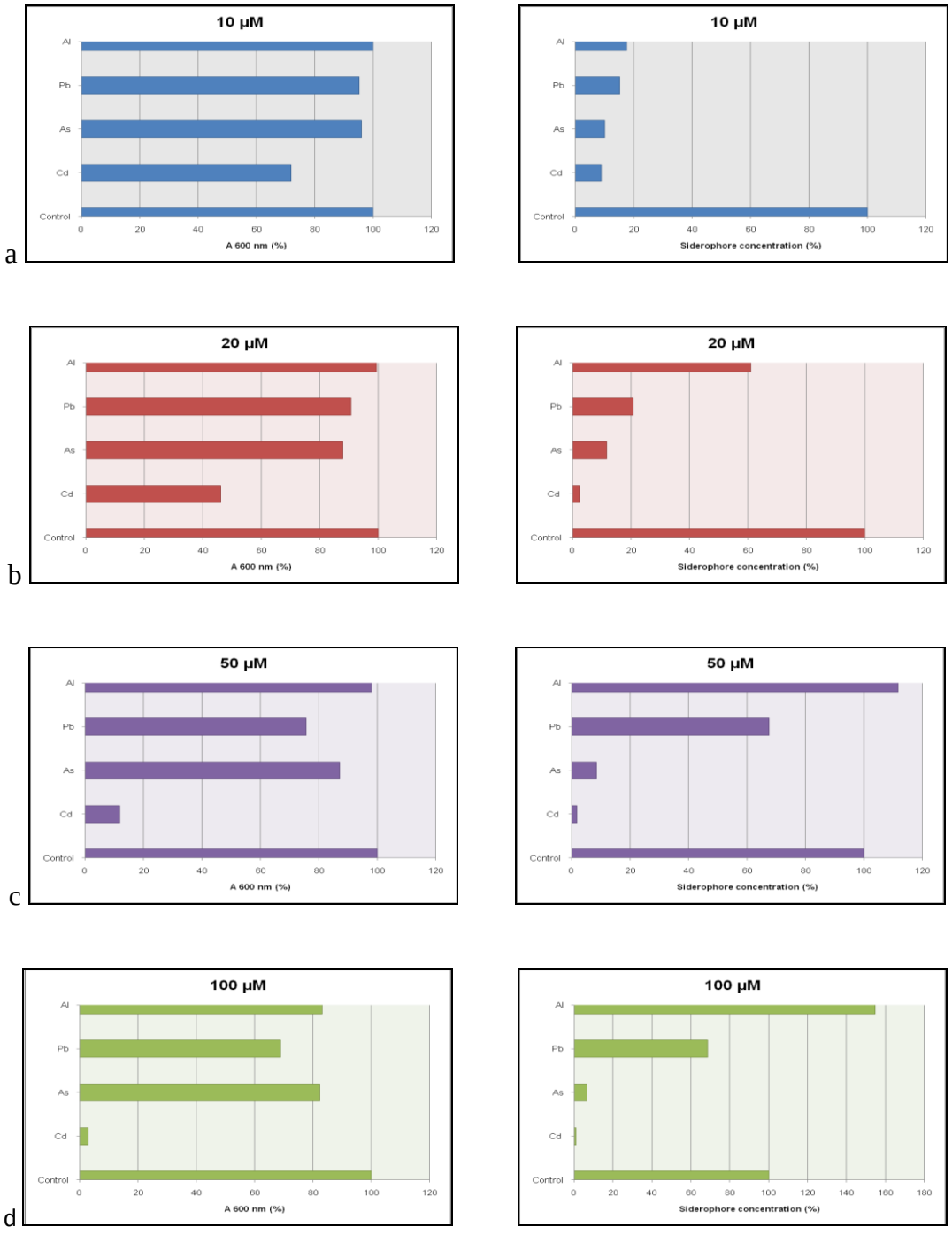


Fig. 5.3: Effect of Al, Pb, As and Cd on growth and siderophore production by *B. amyloliquefaciens* NAR38.1 at a) 10 µM b) 20 µM c) 50 µM and d) 100 µM

to ethyl acetate extraction reduces solubility of the siderophore. Amberlite binds to the ring structure of the siderophore which can be eluted with methanol. After column chromatography, a brown colored, partially purified siderophore was obtained (Fig. 5.4), which showed a single spot corresponding to catechol and 2, 3 – DHBA by TLC (Fig. 5.5). Further chemical characterization for structure determination can be performed by TLC, HPLC, NMR and MS (Van der Helm and Winkelmann 1994). The compound, hence forth arbitrarily called as *SBa,* was therefore separated on HPLC and its major peak at 1.45 minutes was found to correspond to the siderophore (Fig. 5.6 & Fig. 5.7). The UV spectrum of the purified siderophore showed absorption λ at 250 and 315 nm (Fig. 5.8). The spectral curve of *SBa* corresponded to the spectra of the known catechol siderophores (Haag *et al.* 1993, Yamamoto *et al.* 1993) in which the presence of the DHBA residue also determines the absorption in the region of 320 and 250 nm (Neilands 1995). Temirov *et al.* (2003) have reported 2, 3-dihydroxybenzoyl-glycyl-threonine from *Bacillus licheniformis* VK21 Strain which absorbs maximally at 248 and 315 nm.

The MS of the siderophore, *SBa,* showed three peaks corresponding to 374.2, 881.2 and 917.1 (Fig. 5.9). The major peak 881.2 corresponded to the molecular weight of bacillibactin Bacillibactin hitherto has been reported in *B. subtilis, B anthracis, B. cereus* and *B. thuringiensis* (http://bertrandsamuel.free.fr/siderophore_ base/siderophores.php). Further the NMR spectra (Fig 5.10) showed aromatic absorption due to hydroxyl groups as evident in the region 6.5 – 7.5 ppm, absorption in the region 0.5 – 1.5 ppm due to H from amino acid and at 8.3 ppm due to NH linked to C=O. The NMR spectra of *SBa* differs from the spectra of bacillibactin

Fig. 5.4: Partially purified siderophore obtained after column chromatography

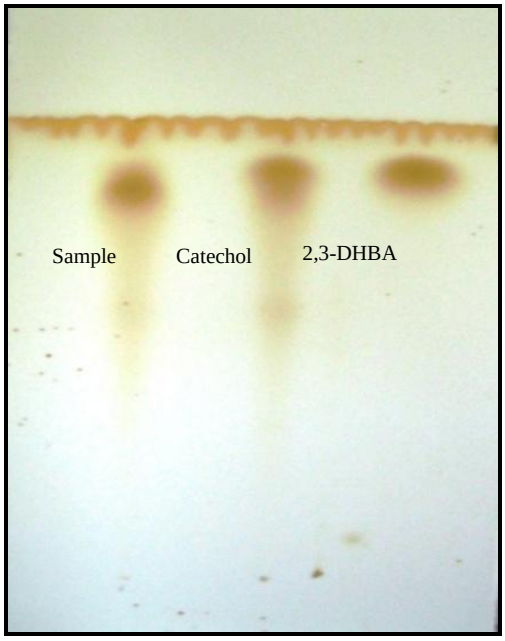
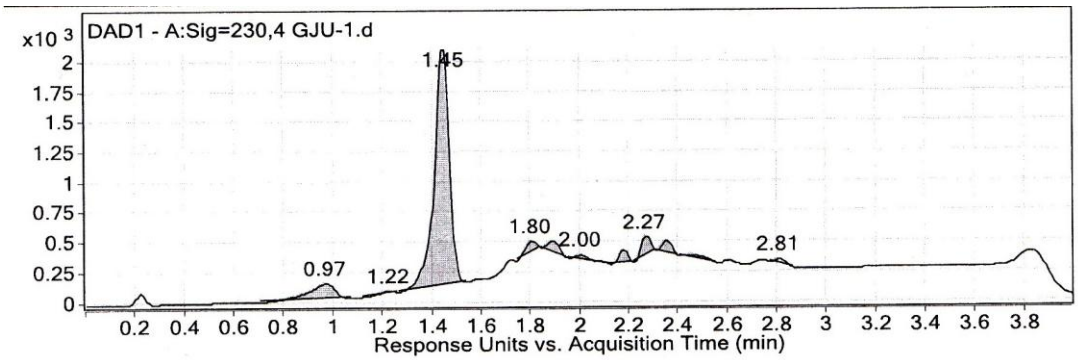


Fig. 5.5: TLC of partially purified siderophore



Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	0.71	0.97	1.07	129.84	1013.07	8.83
3	1.27	1.45	1.54	1942.83	8346.96	72.78
4	1.75	1.8	1.84	97.69	319.22	2.78
5	1.84	1.89	1.95	90.35	275.01	2.4
6	1.95	2	2.13	38.89	164.51	1.43
7	2.13	2.17	2.21	107.14	276.24	2.41
8	2.22	2.27	2.31	164.22	458.13	3.99
9	2.31	2.35	2.4	98.18	263.09	2.29
10	2.41	2.45	2.55	25.89	159.2	1.39
11	2.77	2.81	2.87	38.5	96.48	0.84

Fragmentor Voltage 100 **Collision Energy** 0 **Ionization Mode** ESI

Fig. 5.6: HPLC of the partially purified siderophore

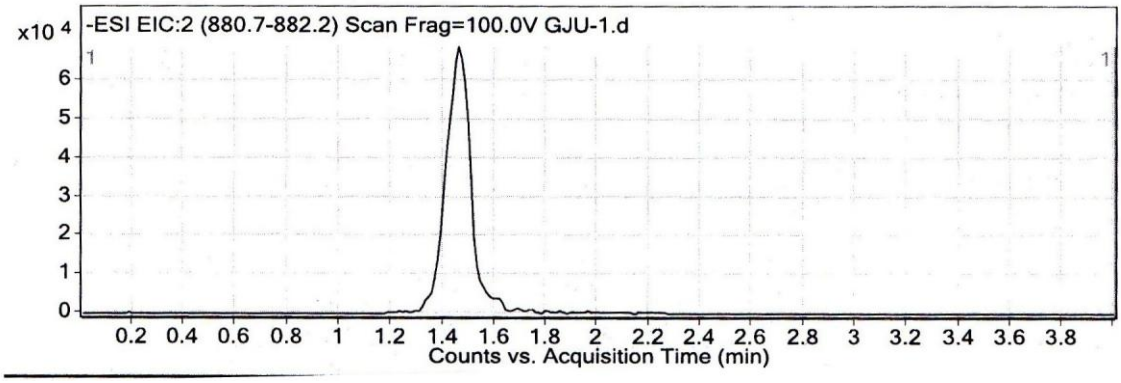


Fig. 5.7: HPLC of the purified siderophore

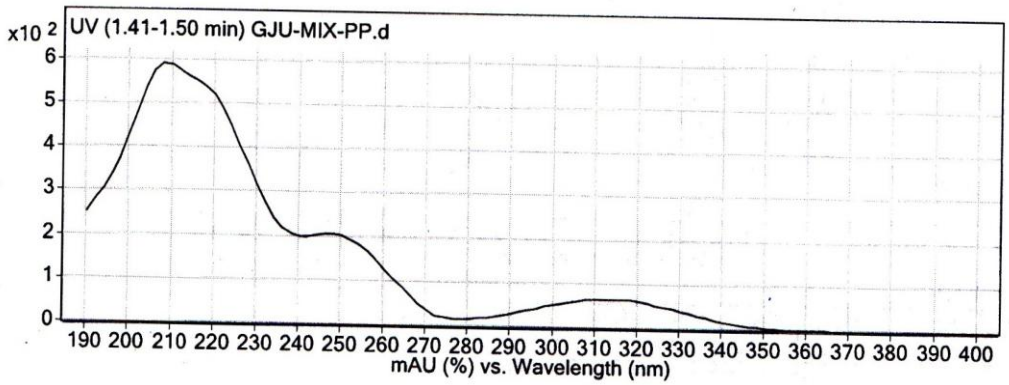


Fig. 5.8: UV spectrum of the purified siderophore

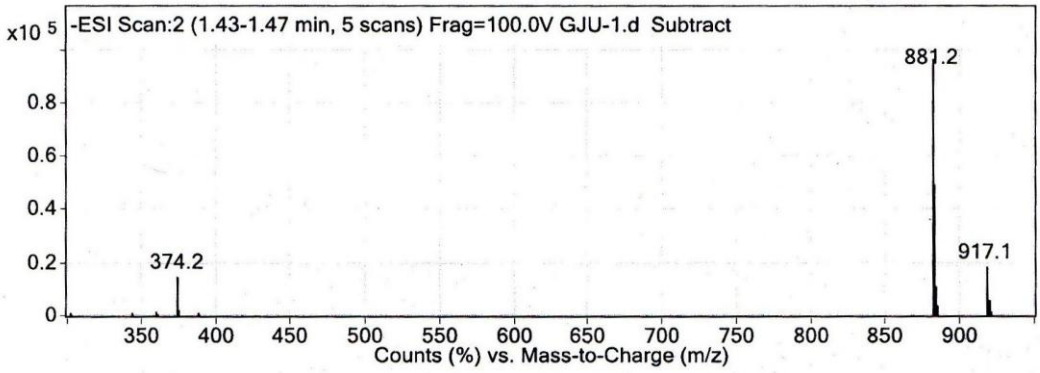


Fig. 5.9: MS of the purified siderophore

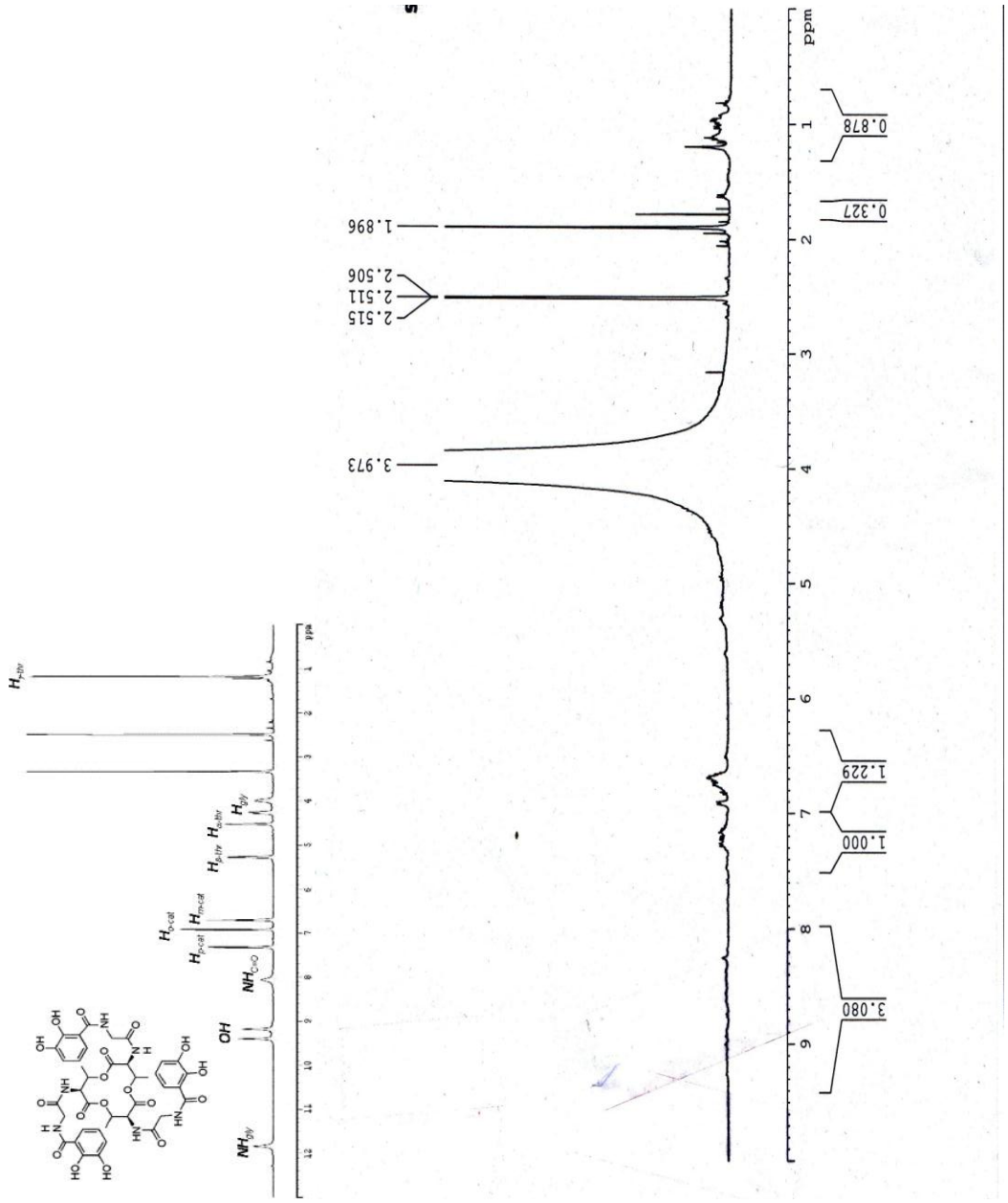


Fig. 5.10: ^{1}H NMR Spectra of amylobactin purified from *B. amyloliquefaciens* NAR38.1 in DMSO. Inset shows ^{1}H NMR Spectra of bacillibactin purified from *B. cereus* in DMSO (Ref: Wilson *et al*. 2006)

shown as inset in Fig 5.10 indicating that the structure of *SBa* could be different from that of bacillibactin although they share the same molecular weight. The difference could be due to the variation in amino acid composition of the two siderophores and needs further characterization of the siderophore *SBa.*

Bacillibactin consists of 2, 3-dihydroxybenzoate-Gly-Thr and is identical to that of corynebactin which is produced by *Corynebacterium glutamicum*. The genes required for bacillibactin biosynthesis are located within a single operon which is regulated by iron. Synthesis of bacillibactin begins with chorismate and proceeds through the intermediate 2,3-dihydroxybenzoate (Fig. 5.11) wherein, three molecules of 2, 3-dihydroxybenzoate-Gly-Thr are cyclized to form the bacillibactin (Fig. 5.12) molecule. Amongst the *Bacillus* spp. siderophores from *B. cereus sensu lato* group have been studied the most. Bacillibactin was first identified in the culture extracts of *B. subtilis* (May *et al.* 2001) and is produced by all examined strains of *Bacillus anthracis* and by the *B. cereus sensu lato* group (Koppisch *et al.* 2008, Koppisch *et al.* 2005) but not reported from *B. amyloliquefaciens*. Though the synthesis of this molecule is widely conserved amongst *Bacillus* strains with genes also found to be present in B amyloliquefaciens (Arias *et al.* 2009) it is not required for the viability of all strains (Cendrowski *et al.* 2004). However, all these reported strains are known to be isolated from terrestrial ecosystems.

The strain used for the study, NAR38.1, is isolated from a mangrove ecosystem. The culture can tolerate upto 2% NaCl and produces a siderophore *SBa*. The literature survey indicates that not many bacteria isolated from coastal ecosystems are reported

chorismate

↓ Dhbc (isochorismate synthase)

isochorismate

↓ DhbB (isochorismate lyase)

2,3-dihydro-2,3-dihydroxybenzoate

↓ DhbA (2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase)

2,3-dihydroxybenzoate

↓ DhbE (2,3-dihydroxybenzoate adenylase)

2,3 –dihydroxybenzoyladenylate

↓ DhbB (aryl carrier protein)

↓ DhbF (peptide synthetase)

(2,3-dihydroxybenzoate-glycine-threonine)$_3$ (bacillibactin)

Fig. 5. 11: Pathway for biosynthesis of bacillibactin (Ref: Heinrich *et al.* 2004).

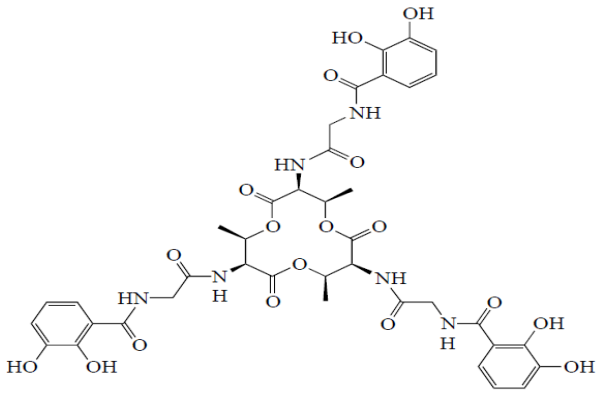


Fig. 5.12: Structure of bacillibactin (Ref: Temirov *et al.* 2003)

to produce siderophores having 2, 3 –DHBA as the core molecule. One of the well studied siderophores is from a petroleum degrading marine bacterium *Marinobacter hydrocarbonoclasticus* and is identified as petrobactin with 3,4 –DHBA as the core molecule (Barbeau *et al.* 2002). With regard to *B. amyloliquefaciens*, only reports available are those on siderophore production by the same (Mishra and Kumar 2012) however, its structure has not been elucidated.

The iron-binding affinity of bidentate siderophores is low compared to hexadentate siderophores. Such complex structures of siderophores have evolved over simple ones especially to increase the chelating effect. Such extended siderophore structures have been favored during evolution compared to the monomeric precursors. Although linear di-, tetra- and hexadentate siderophores have been found in all siderophore classes, there is a tendency for cyclization in the final biosynthetic end products. Cyclization enhances complex stability, chemical stability and improves resistance to degrading enzymes. Cyclization is a common feature of secondary metabolism and is found in microbial peptides, polyketides, macrocyclic antibiotics and other bioactive compounds. Cyclization might also be advantageous for diffusion-controlled transport processes across cellular membranes. Moreover, due to a reduction of residual functional groups, the surface of the siderophore becomes non-reactive or inaccessible to modifying enzymes (Das *et al*. 2007).

5.3.3 Antibacterial activity of the siderophore

An important characteristic of siderophores is the antibacterial activity; such an activity however, is not reported for bacillibactin. The siderophore amylobactin produced by *B. amyloliquefaciens* NAR38.1 showed strong antibacterial activity against Gram - positive organisms *B. subtilis* 3A1 and *S. aureus.* However, no activity was observed against the two

Gram - negative organisms: *E. coli* and *P. aeruginosa* strain ATCC 9027 (Fig. 5.13). This interesting property of the siderophore produced depicts the difference between bacillibactin and *SBa*.

Siderophores have been used for the selective delivery of antibiotics in drug resistant bacteria. Siderophores conjugated with antibiotics are known as sideromycins and are recognized by cellular Fe-siderophore transport systems which are delivered into the cells. However, the only literature available on siderophore acting as an antimicrobial agent against the microorganisms of same species is with regard to mycobactins M or N which can suppress the growth of *M. tuberculosis*, *M. paratuberculosis*, and *M kansasii* (Girijavallabhan and Miller 2004).

The work presented here showed that the presence of metals in the growth medium of *B. amyloliquefaciens* NAR38.1 implicated four different responses by the isolate:

1) Increase in siderophore production by Zn, Co and Mn. Zn did not have any effect on growth, while Co and Mn decreased growth.

2) Decrease in siderophore production by Mo and As without any effect on growth.

3) Decrease in siderophore production at lower concentrations but increase at higher concentration without affecting growth as manifested by Pb and Al.

4) Decrease in growth as well as siderophore production by Cd and Cu.

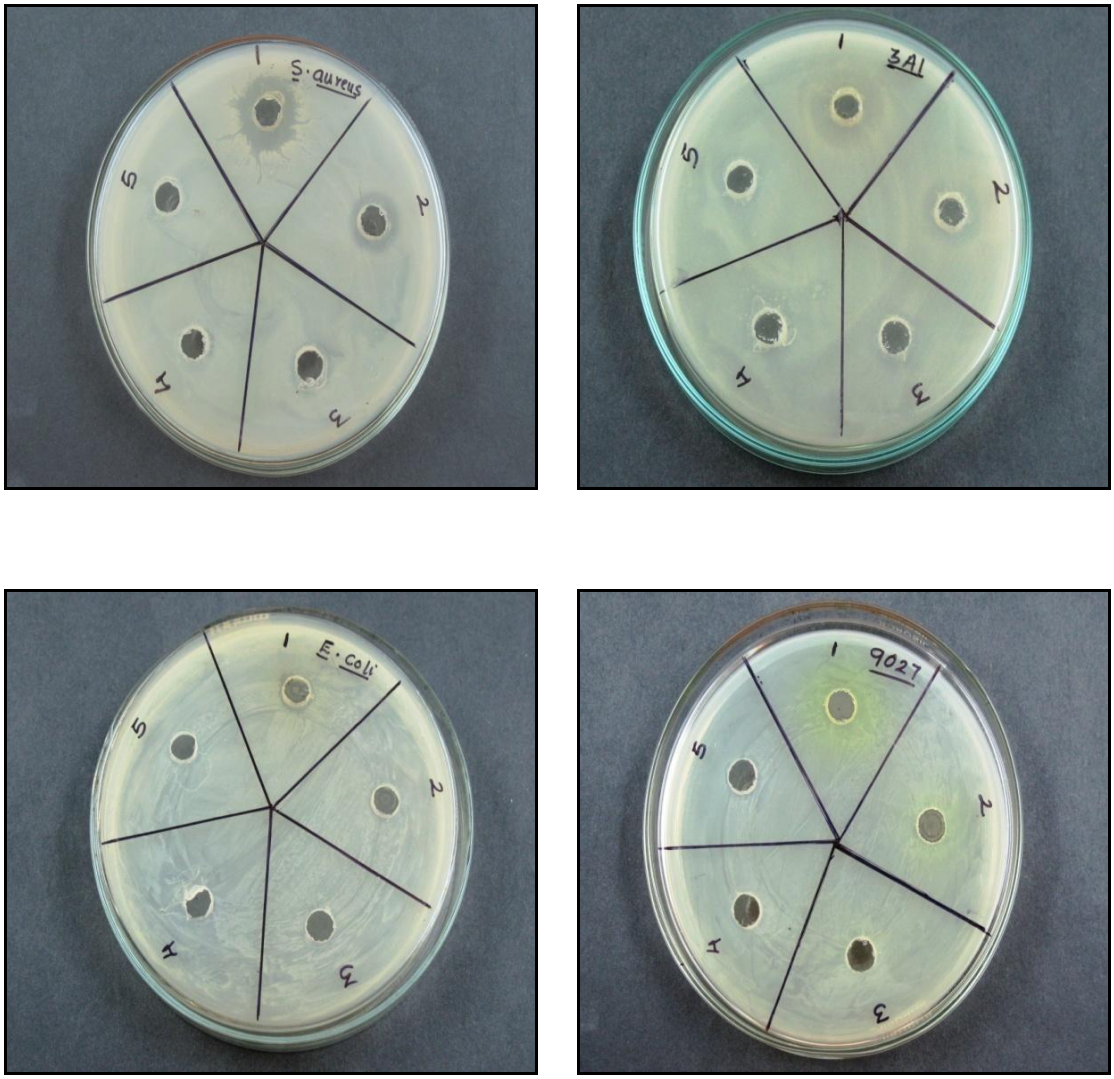


Fig. 5.13: Antibacterial activity of the siderophore produced by *B. amyloliquefaciens* NAR38.1

Concentrations used:

1. **10 mg/ml**
2. **5 mg/ml**
3. **2 mg/ml**
4. **0.02 mg/ml**
5. **Control (Methanol: water; 1:1)**

Such effects indicate that the potential of the organism to combat metal toxicity varies with the type of metal it is in contact with. Although bacteria are known to possess different

mechanisms to alleviate the effects of toxic metals, the concentration of the metal plays an important role in manifestation of such mechanisms.

Further, characterization of the siderophore, *SBa* produced by the isolate was found to be a high molecular weight compound similar to bacillibactin. Bacillibactin has been reported from *B. subtilis*, *B anthracis*, *B. cereus* and *B. thuringiensis* but not from *B. amyloliquefaciens*.

Production of siderophores in presence of metals can be a useful trait for plant growth promoting organisms as the soils which are contaminated with metals are often iron deficient (Tank and Saraf 2009). In fact, siderophore producing bacteria have been considered important for inducing metal tolerance in plants and for promotion of metal accumulation in plants. Especially in the phytoextraction technology for remediation of metal contaminated soils. The effects of siderophore producing bacteria on the uptake of metals by hyperaccumulator plants have been the focus of increased attention (Dimkpa *et al.* 2008, Braud *et al.* 2009b). Braud *et al.* (2009b) have reported increase in the bioavailability of Cr and Pb in soils inoculated with *P. aeruginosa*. Bacterial siderophores can also provide iron to various plants, which helps in reducing the metal toxicity (Bar-Ness *et al*. 1991, Reid *et al*. 1986, Wang *et al*. 1993). Such beneficial effects exhibited by siderophore producing bacteria implicate that the inoculation with metal-resistant siderophore producing bacteria may help in improving the process of phytoextraction in metal contaminated soils.

Further, characterization of the siderophore produced by *B. amyloliquefaciens* and its unique characteristic of antimicrobial activity against Gram-positive organisms may find potential application as a biocontrol agent and in the field of medicine.

Summary

This study has reported the incidence of siderophore producing bacteria in the two coastal ecosystems: sand dunes and mangroves. The total viable counts were found to be higher in rhizhosphere samples compared to the non rhizhosphere samples from sand dunes while mangrove sediments showed higher counts compared to the water samples. The isolates from sand dunes and mangroves were screened for production of siderophores using universal chrome azurol sulphonate assay.

It was seen that higher percentage of siderophore producers was obtained from non rhizhosphere (23.7%) as compared to the rhizhosphere (14.87%) samples. From mangroves, a higher % of siderophore producers was obtained from water (28%) as compared to sediment (5%) . Twelve siderophore positive isolates from sand dunes and mangrove which showed CAS zone of 8 mm and above were further subjected to routine biochemical tests to identify them. Based on the characteristics, the isolates were tentatively identified using Bergey's Manual of Systematic Bacteriology. Further, a 1.5kb PCR product of the thirteen cultures was sequenced. The 16S rDNA sequences of the isolates (Appendix D) were used to carry out BLAST (Altschul et al., 1990) with the nrdatabase of NCBI genbank database. Sequences were selected and aligned using multiple alignment software program Clustal X (Thompson et. al., 1997). Distance matrix was generated using RDP database and the phylogenetic trees were constructed using MEGA 4. 2.1 . Of the thirteen isolates selected, 10 belonged to the *Bacillus* spp, 2 were *Streptomyces* spp. and one was identified as *Pseudomonas aeruginosa*.

All the isolates were screened for their ability to utilize sodium benzoate It was observed that from sand dunes, only four showed the ability to utilize sodium benzoate, namely TMR2.13,

NAMNR3.5, TMNR4.1.1 and NAMNR4.4. and only one Isolate NAR38.1 from mangroves showed sodium benzoate utilization. These isolates when screened on MSM with different sodium benzoate concentrations showed that culture NAMNR3.5 could grow upto 0.5% sodium benzoate concentration while cultures, TMNR4.1.1, NAMNR4.4 and NAR38.1 showed growth upto 0.75% sodium benzoate . Culture TMR2.13 tolerated highest concentrations of sodium benzoate up to 2% and also produced a yellow green pigment, pyoverdine, which is known to be a siderophore. Isolate NAR38.1 isolated from mangroves showed siderophore production and consistent growth on sodium benzoate as compared to other 3 *Bacillus* spp. Based on the siderophore production and sodium benzoate utilization, two isolates TMR 2.13 and NAR38.1 were selected for further studies.

Plant growth promoting characteristics of the two selected isolates were determined and both the isolates showed ammonia production with *P. aeruginosa* TMR2.13 being positive for other plant growth promoting properties like phosphate solubilzation and HCN production. Functional group tests proved that the siderophores produced by TMR2.13 and NAR38.1 were of carboxamate and catecholate type respectively. Optimum siderophore production for both the isolates was obtained at pH 7, absence of NaCl and with no added iron.

Furthermore, addition of iron to the growth medium of both the isolates suppressed siderophore production without affecting growth profiles. Studies with NAR38.1 showed production of siderophore upto 1 µM of Fe^{+2} and upto 30 µM of Fe^{+3}. However, siderophore production was seen upto 54 µM and suppressed at 108 µM of Fe^{+2} as well as Fe^{+3} in *P. aeruginosa* TMR2.13. Siderophore produced by *P. aeruginosa* TMR2.13 showed binding to Fe^{+2}, Fe^{+3} as well as to Fe^{0}, while siderophore produced by NAR38.1 had much higher affinity for Fe^{+3} as compared to Fe^{+2}.

Sodium benzoate was found to have a remarkable effect on siderophore production in P*seudomonas aeruginosa* TMR2.13 It was note that while the production was inhibited above 54 μM of added iron in MSM with glucose without affecting growth, in the presence of sodium benzoate, siderophore was produced even up to the presence of 108 μM of added iron.

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

1) In presence of Zn, Siderophore production increased but no effect was seen on growth while presence of Co and Mn increased siderophore production with decrease in growth.

2) In presence of Mo and As siderophore production decreased but there was no effect on growth.

3) Decrease in siderophore production at lower concentrations but increase at higher concentration without affecting growth as manifested in presence of Pb and Al.

4) Decrease in growth as well as siderophore production was brought about by the presence of Cd and Cu.

Further the characterization of the siderophore produced by the isolate *B. amyloliquefaciens* NAR38.1 showed that it was a high molecular weight compound similar to bacillibactin. Bacillibactin has been reported from *B. subtilis, B anthracis, B. cereus* and *B. thuringiensis* but not from *B. amyloliquefaciens*. Further, this siderophore, which we have arbitrarily termed as *SBa*, was also found to inhibit the growth of Gram-positive organisms tested implicating its potential as biocontrol agent.

Significance

This research has established the presence of siderophore producing bacteria in the sand dune and mangrove ecosystems. The present study projects the significance of response of siderophore producing isolates *P. aeruginosa* TMR2.13 and *B. amyloliquefaciens* NAR38.1, to aromatic hydrocarbons and metal ions respectively.

Sodium benzoate has been found to be the best carbon source amongst the various carbon sources tested for siderophore production by *P. aeruginosa* TMR2.13 and increased iron demand of the organism. Siderophore produced by the same showed binding towards both the forms of Fe. Spectrofluorimetric studies have proved faster binding towards Fe^{+2} as compared to Fe^{+3}.

The production of siderophore by *B. amyloliquefaciens* NAR38.1 in presence of toxic metal signifies that it may play an important role in uptake, mobilization of heavy metals or developing metal resistance. This is a first time account of binding of siderophore produced by *B. amyloliquefaciens* NAR38.1 to Fe^{+2} and Fe^{+3}. This study has elucidated biochemical structure of siderophore produced by *B. amyloliquefaciens* and its antibiotic activity towards gram positive organisms like *S. aureus* and *B. subtilis* 3A1. Our results emphasize the implications of siderophore producing bacteria in improving the process of phytoextraction in metal contaminated soils.

Future Perspectives

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

It would be desired to apply these two isolates in field trials to study their effect on plant growth promotion as this study has evidently proved presence of plant growth promoting activities in these two organisms. Moreover, it would be interesting to probe deeper into the results of the present studies and following points are suggested for further investigation:

1) Effect of iron limitation on the enzymes involved in utilization of sodium benzoate in *P. aeruginosa* TMR2.13.

2) Role of siderophore in metal resiatance in *B. amyloliquefaciens* NAR38.1.

3) Mechanism of action of siderophore as an antibiotic.

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Appendix

Appendix – A

Media Composition

A.1 Nutrient broth

Peptone	10 g
NaCl	05 g
Beef extract	03 g
Distilled water (make to final volume)	1000 ml
*Agar (for solid medium)	25 g
pH	7.2

A.2 Tryptone Yeast Extract Glucose agar (TYG)

Tryptone	05 g
Yeast Extract	03 g
Glucose	05 g
Distilled water (make to final volume)	1000 ml
Agar	25 g
pH	7.2

10 % Solutions of glucose sterilized separately and added to the sterilized basal medium.

A.3 Polypeptone Yeast Extract Glucose Agar (PPYG)

Peptone	05 g
Yeast extract	1.5 g
Glucose	05 g
$Na_2HPO_4.12H_2O$	1.5 g
NaCl	1.5 g
$MgCl_2.6H_2O$	0.1 g
Na_2CO_3	05 g
Distilled water (make to final volume)	1000 ml
pH	10.5

10 % Solutions of glucose and Na_2CO_3 sterilized separately and added to the sterilized basal medium.

A.4 Nacl- Tryptone Yeast Extract (NTYE) (Kushner 1985)

MgSO4.7H2O	20 g
KCl	05 g

$CaCl_2.2H_2O$	0.2 g
Yeast Extract	03 g
Tryptone	05 g
Crude salt	150 g
Distilled water (make to final volume)	1000 ml
pH	7.2

A.5 Mineral Salts Medium (MSM)

$FeSO_4.7H_2O$	60 mg
K_2HPO_4 (12.6%)	100 ml
KH_2PO_4 (18.2%)	20 ml
NH_4NO_3 (10%)	20 ml
$MgSO_4$ (1%)	20 ml
$Na_2MoO_4.2H_2O$ (0.6%)	0.2 ml
$MnSO_4.H_2O$ (0.6%)	0.2 ml
$CaCl_2.2H_2O$ (0.1%)	15 ml
Distilled water (make to final volume)	1000 ml
pH	7.2

$CaCl_2.2H_2O$ added drop by drop and volume adjusted to 1000 ml. The solution stored in amber color bottle. Medium sterilized for 10 minutes before use. Carbon source sterilized separately and added.

A.8 Media for HCN production

Nutrient agar	1000 ml
Glycine	4.4 g

A.6 Pikovskaya's medium

Glucose	10 g
$Ca_3(PO)_4$	05 g
$(NH_4)_2SO_4$	0.5 g
KCl	0.2 g
$MgSO_4$	0.1 g
$MnSO_4$	0.0002 g
$FeSO_4$	0.0002 g
Yeast extract	0.5 g

A.7 Peptone water

Peptone	10 g
NaCl	5 g

A.9 Media for biochemical characterization

i) Sugar fermentation medium (peptone water base)

Peptone	10 g
NaCl	05 g
Phenol red (0.2%)	50 ml
Sugar (10%) separately sterilized	100 ml
Distilled water (make to final volume)	1000 ml
pH	7.2-7.4

ii) Hugh – Leifson's medium

Peptone	02 g
NaCl	05 g
K_2HPO_4	0.3 g
BTB aqueous solution (1%)	3 ml
Glucose (10%) separately sterilize	100 ml
Distilled water (make to final volume)	1000 ml
Agar	4 g
pH	7.2

BTB aqueous solution

BTB	1 g
2 N NaOH	2 ml
Distilled water (make to final volume)	100 ml

iii) Motility agar

Nutrient broth	1000 ml
Agar	4 g
pH	7.2

iv) Sulfide indole motility

Beef extract	3 g
Peptone	10 g
Ferrous ammonium sulphate	0.2 g

Sodium thiosulphate	25 mg
Distilled water (make to final volume)	1000 ml
Agar	4 g
pH	7.2

v) Glucose phosphate medium

Peptone	05 g
K_2HPO_4	05 g
Glucose (10%) separately sterilized	100 ml
Distilled water (make to final volume)	1000 ml
pH	7.2

vi) Starch agar

Nutrient broth	1000 ml
Soluble starch	20 g
Distilled water (make to final volume)	1000 ml
Agar	30 g
pH	7.2

vii) Simmon's citrate agar medium

$(NH)_4H_2PO_4$	01 g
K_2HPO_4	01 g
NaCl	05 g
Sodium citrate	02 g
$MgSO_4.7H_2O$	0.2g
BTB aqueous solution (0.3%)	04 ml
Distilled water (make to final volume)	1000 ml
Agar	25 g

Appendix – B

Composition of stains, reagents and buffers

B.1 Stains

B.1.1 Stains for gram characterization

i) Crystal violet

Solution A: 2 g of crystal violet in 200 ml of 45% ethyl alcohol
Solution B: 0.8 g of ammonium oxalate in 80 ml distilled water

ii) Gram's iodine solution

KI	02 g
Iodine	01 g
Distilled water (make to final volume)	300 ml

iii) Safranine solution

Safranine: 25% solution in 95% ethyl alcohol

B.1.2 Malachite green solution

Malachite green	5 g
Distilled water (make to final volume)	300 ml

B.2 Reagents

B.2.1 Reagents for nitrate reduction test

Solution A: 0.8% sulphanilic acid in 5 N acetic acid
Solution B: 0.5% α-naphthylamine in 5 N acetic acid

5 N Acetic acid

Glacial acetic acid	57.27 ml
Distilled water (make to final volume)	200 ml

B.2.2 Reagents for biochemical characterization

i) Methyl red indicator

Methyl red	0.1 g
Ethanol (95%)	300 ml

ii) O'Meara's reagent

KOH	40 g
Creatine	0.3 g
Distilled water (make to final volume)	1000 ml

iii) Kovac's reagent

Isoamyl alcohol	150 ml
p-dimethyl aminobenzaldehyde	10 g
HCl (12N)	1000 ml

B.2.3 Reagents for Csaky Test

i) Sulphanilic acid solution

Sulphanilic acid	1 g
Acetic acid (30%) (make to final volume)	100 ml

ii) Iodine solution

Iodine	1 g
Glacial acetic acid (make to final volume)	100 ml

iii) Sodium arsenite

Sodium arsenite	2 g
Distilled water(make to final volume)	100 ml

iv) Sodium acetate

Sodium acetate	35 g
Distilled water(make to final volume)	100 ml

v) α - naphthylamine

α – naphthylamine	3 g
Acetic acid (30%)(make to final volume)	1000 ml

B.2.4 Nitrite Molybdate reagent

Sodium nitrite	10 g
Sodium molybdate	10 g
Distilled water (make to final volume)	100 ml

B.2.5 Salkowsky's reagent

Perchloric acid (35%)	50 ml
0.5 M $FeCl_3$	1 ml

B.2.6 Nessler's reagent

$HgCl_2$	2 g
KI	1 g

B.2.7 CAS

CAS (in 50 ml dI water)	60.5 mg
HDTMA	72.9 mg
Fe (III) solution*	10 ml

* 27 mg FeCl3.6H2O + 83.3 µl concentrated HCl in 100 ml distilled water.

B. 3 Buffers

B.3.1 Normal saline

NaCl	0.85 g
Distilled water (make to final volume)	1000 ml

B.3.2 Phosphate buffered saline (PBS)

NaCl	0.8 g
KCl	20 mg
Na_2HPO_4	0.144 g
KH_2PO_4	24 mg
Distilled water (make to final volume)	100 ml

B.3.3 Tris buffer (0.02 M, pH 8)

Tris HCl (0.1 M)	10 ml
Distilled water	40 ml

B.3.4 Tris HCl (0.1 M)

Tris – base (0.2 M)	50 ml
HCl (0.2 N)	29 ml
Distilled water (make to final volume)	100 ml

B.3.5 Tris EDTA (TE) buffer (pH 8)

Tris – Chloride (10 mM, pH 8)	30 ml
EDTA (1 mM, pH 8)	15 ml
Distilled water (make to final volume)	50 ml

B.3.6 Tris acetate EDTA buffer (pH 8)

Tris –base	24.2 g
Glacial acetic acid	5.71 ml
EDTA (0.5 M, pH 8)	10 ml
Distilled water (make to final volume)	1000 ml

B.3.7 Acetate buffer (pH 4)

0.2 M Acetic acid	83 ml
0.2 M Sodium Acetate (make to final volume)	100 ml

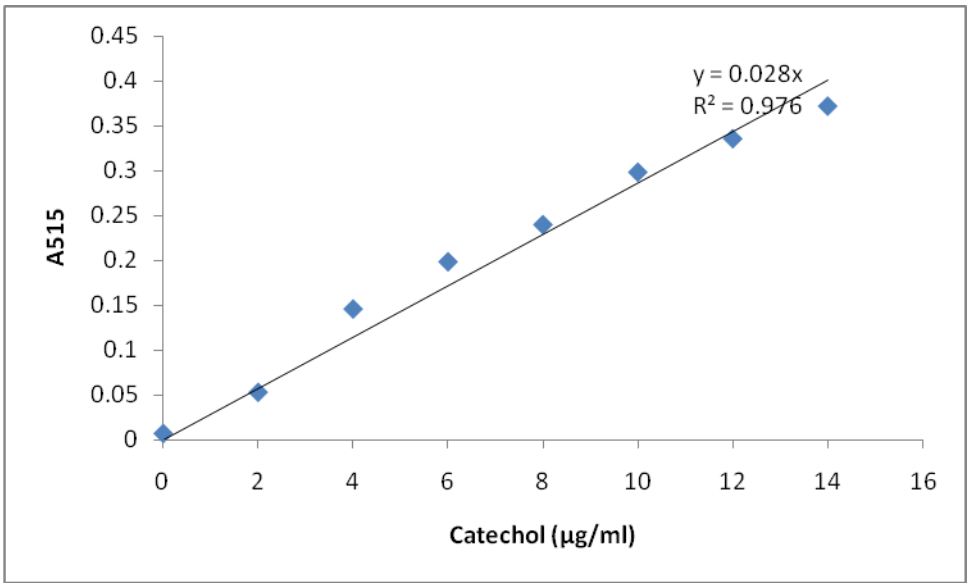
Appendix C

Standard procedure and standard graph

C.1) Rothera's test to determine the ring cleavage:

The cultures were streaked on MSM plates with 0.2% sodium benzoate and incubated at 28ºC for 24 hours. The cultures were inoculated in 50 ml MSM with 0.2% sodium benzoate broth in 250 ml conical flasks. The flasks were incubated at 28ºC and 150 rpm for 24 hours. 20 ml broth from each flask was centrifuged and the pellet was suspended in 5 ml 0.02 M Tris buffer (pH 8) to A_{600}=1. The tubes were vortexed for a minute. 0.2 ml toluene was added and vortexed again for a minute followed by addition of 0.2 ml of 100mM catechol. The tubes were vortexed and allowed to stand for 10 minutes. Formation of bright yellow colour indicates meta pathway. In the absence of yellow colour, the reaction mixture was processed for test for ortho pathway. The tubes were covered with aluminium foil and incubated for 1 hour at 150 rpm and saturated with $(NH_4)_2SO_4$. 0.1 ml of 5N NH_4OH was added along the sides of the test tube. A drop of freshly prepared 25% Na-nitroprusside was added. Formation of deep purple colour ring indicated ortho pathway

C.2) Standard graph of catechol



Appendix D

D.1) partial sequence of the 16S rRNA gene of the selected isolates.

1) ***Bacillus* sp** TMR1.17 (GenBank Acc No JN596247)

CCGGGNNGGTGCTATANATGCAGTCGAGCGGACAGATGGGAGCTTGCTC
CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT
AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGTTT
GAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGA
TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGC
GGTACCTTGACGGTACCTAACCAGAGAGCCACGGTTAAATACATGCCAA
CAGCCCTGGTAATACGTCAGTGGCAAGCGTTGTCCGAAATTATTGGGCTT
AAAGGGGTTGCAAGCGATT

2) ***Bacillus* sp.** TMR1.6.2 (GenBank Acc No JN596242)

CGNNGGCNGGGTGCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC
TGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTG
TTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACA
GATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGG
GCGGCCCCTTGCCGGTACCTAACCACAAAGCCCCGGATAACTACATGCA
TCCAGCCGGGGTAATACCTATGTGGATAGCGT

3) ***Streptomyces sp.*** TMR1.18 (GenBank Acc No JN596248)

GCGGGNNGTCTTACCATGCAGTCGAACGATGAAGCCGCTTCGGTGGTGG
ATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCT
GGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACACGGGATCG
CATGGTCTCCGTGTGGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGCC
TATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTG
ATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCT
TTCAGCAGGGAAGAAGCGCAAGTGACGGTACCTGCAGAAGAAGCGCCG
GCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGT
CCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGAT
GTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCT
AGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGC
AGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC
TGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTG
GTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTGGGCGACATTCCACG
TCGTCCGTGCCGCAGCT

4) ***Streptomyces sp.*** TMR2.6 (GenBank Acc No JN596249)

GCNGNNGGGTGCTTACCATGCAAGTCGAACGATGAACCACTTCGGTGGG
GATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTC
TGGGACAAGCCCTGGAAACGGGGTCTAATACCGGATACTGACCCTCACG
GGCATCTGTGAGGTTCGAAAGCTCCGGCGGTGCAGGATGAGCCCGCGGC
CTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAG
CCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGA
CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCT
GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTC
TTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTG
TCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTT
GTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCT
AGAGTTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGC
AGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATAC
TGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAAATACCCT
GGTAGTCCACGCCGTAAACGGTGGGGCACTAGGTGTGGGCAACATTCCA
CGTTGTCCGTGCCGCA

5) ***Bacillus sp.*** TMNR3.3 (GenBank Acc No JN596245)

GGGNGGGCGGCTAAACATGCAAGTCGAGCGGACAGATGGGAGCTTGCTC
CCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCTGT
AAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTT
GAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGA
TGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
AACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGC
GGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAATGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAACAGGA
CACCGGAATTCCATTCTTTTCGGTGCTTTGCACACAGATGGGAAC

6) ***Bacillus sp.*** TMNR2.7.1 (GenBank Acc No JN596243)

NGGGGGGGGGTGCCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTT
GCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC
CTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTT
GTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTAC
AGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTT
CGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAG
GGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGG
GCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCG
GCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGG
AACACCAGTGGCNAAGGCGACTTCTCTGGTCTGTAACTG

7) ***Bacillus sp*** TMNR2.7.2 (GenBank Acc No JN596244)

CCGGTGNNGAGCTAATACATGCAAGTCGAGCGGACAGATGGGAGCTTGC
TCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
GTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGT
TTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAG
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CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAA
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CGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
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GTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCT
CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCACAAGAGG
AGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
CACCAGTGGCAAAGCGACTCTCTGGTCTGTACTGACGCTGAGGAGCGAA
AGCGTGGGGAGCCAACCCCTTATATACTTTGGTAATCCACGCCGAATCCA
TGACTGCTGATTGN

8) *Bacillus* sp TMNR4.1.1 (GenBank Acc No JX194168)

TTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT
GCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTATACCGGATGCT
TGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTA
CAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCA
AGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG
CAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTT
TCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTG
CCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTG

9) *Bacillus* sp NAMNR 3.5 (GenBank Acc No JX194166)

ATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTG
GGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATA
CCGGATGCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCTGC
TACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAAT
GGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCA
CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG
AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGA
TGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACC

10) ***Bacillus sp.*** NAMR4.10 (GenBank Acc No JN596242)

CGGNNNNGCGTGCTATACATGCAGTCGAGCGGACGGATGGGAGCTTGCT
CCCTGAAGTCAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTGCCTG
TAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACTCTT
TTCCTCACATGAGGAAAAGCTGAAAGATGGTTTCGGCTATCACTTACAGA
TGGGCCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGC
CACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAAT
GGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG
ATCGTAAAACTCTGTTGTCAGGGAAGAACAAGTACCGGAGTAACTGCCG
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AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGCGCGCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCCCGGCTC
AACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGAA
GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAAC
ACCAGTGGCGAAGGCGACTCTTTGGTCTGTAACTGACGCTGAGGCGCGA
AAGCGTGGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AAACGATCAGTGCTAA

11) ***Bacillus*** sp. NAMNR4.4 (GenBank Acc No JX194167)

NNNGAGGGGNNGCTATACATGCAAGTCGAGCGGACAGATGGGAGCTTG
CTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCC
TGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTG
TTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACA
GATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAG
GCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTG
AGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCA
ATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAANTGCCGTTCAAATAGG
GCGGCACCTTGACGGTACCTAACCANAAAGCCCCGGCTAACTACATGCC

12) *Bacillus amyloliquefaciens* NAR38.1 (GenBank Acc No HM030825)

CGGAAGATGGGAGCTTGGTCCGTGAATAGTTAGCGGCGGACGGGTGAGT
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GGCTAATACCGGATGGTTGTTTGAACCGCAGGTTCAGACATAAAAGGTG
GCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATAGCTAGTTGGTGA
GGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGAT
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
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GAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACA
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TGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTG
ATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAA
CTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGC
GTAGAGATGTGGAGGAACACCAGTGGCGAACGCGACTCTCTGGTCTGTA
ACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACC
CTGGTAGTCCACGCCGTAACGATGAGTGCTAAGTGTTAGGGGGGTTTCCG
CCCCTTAGTGCTGCAGCTAACGCATAAGCACTCCGCCTGGGGAGTACGGT
CGCAAGACTGAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGTG
GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTG
ACATCCTCTGACAATCCTAGAGATAGGACGTCCCCTTCGGGGGCAGAGT
GACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA
AGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGG
GCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG
ACAGAACAAAGGGCAGCGAAACCGCGAGGTTAAGCCAATCCCACAAAT
CTGTTCTCAGTTCGGATCGCAGTCTGCAACT

13) *Pseudomonas aeruginosa* TMR2.13 (GenBank Acc No HM030825)

ACAGGTAACGGAGGGAGAGGGTAGTCGAGCGGTGAAGGGACTTGCTCCT
GGATCGCGGCGGACGGGTGAGCAAGCCTAGGATCTGCCTGGTAGTGGGG
GATAACGTCCGGCAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAA
AGTGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATT
AGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGT
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CAGAATAAGACCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGG
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CACCATGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCA
AAACTACTGAGCTAGAGTACGGTAGAGGGCGGGGGAATTTCCTGTGTAG
CGGTGAAATGCGTATATATAGGAAGGAACACCAGTGGCGAAGGCCCACC
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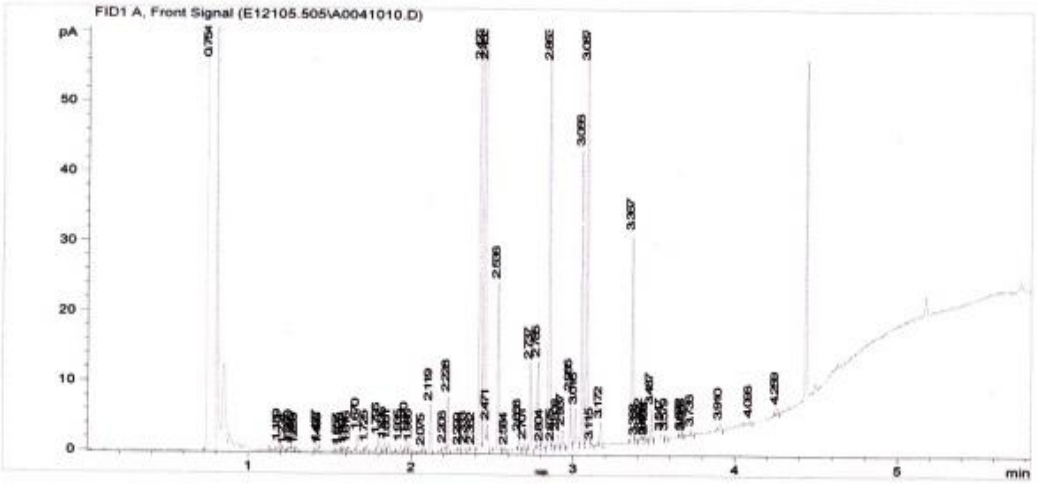
AGGATTAGATACCCTGGTAAGTCCAACGCCGTAAACGAATGTCCAACTA
GACGATTTGGCGGAATCCCTTT



D.2: FAME analysis of culture NAR38.1

Publications

PEER – REVIEWED JOURNALS

1. Gaonkar T, Nayak P, Garg S, Bhosle S (2012) Siderophore-Producing Bacteria from a Sand Dune Ecosystem and the Effect of Sodium Benzoate on Siderophore Production by a Potential Isolate. The Scientific World Journal. 2012: 1-8 **IF: 1.52**

2. Gaonkar T, Bhosle S (2013) Effect of metals on a siderophore producing bacterial isolate and its implications on microbial assisted bioremediation of metal contaminated soils. Chemosphere. http://dx.doi.org/10.1016/j .chemosphere.2013 .06.036 **IF: 3.2**

3. Nayak P, Mohanty A, **Gaonkar T**, Kumar A, Bhosle S and Garg S(2013) Rapid identification of polyhydroxyalkanoate accumulating members of Bacillales using internal primers for phaC gene of *Bacillus megaterium.* ISRN Bacteriology. 2013:1-12.

BOOK CHAPTER

1. Nayak P, Gaonkar T, Mohanty A, Kumar A, Bhosle S, Garg S (2011) Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem In Barbudhe SB, Ramesh R, Singh NP (eds) Microbial diversity and its application. NIPA, New Delhi, pp 75-82.

Conferences/Workshops

PRESENTATIONS AT CONFERENCES

Virtual Presentations

1. Paper entitled **"**Response of *Pseudomonas* to low iron concentrations in presence of sodium benzoate" presented at the III International Conference on Environmental, Industrial and Applied Microbiology, organized by BioMicroWorld, Formatex at Lisbon, Portugal from 02/12/2009 to 04/12/2009

2. Paper entitled **"**Response of a denitrifying *Pseudomonas* to sodium benzoate" co-presented at the III International Conference on Environmental, Industrial and Applied Microbiology, organized by BioMicroWorld, Formatex at Lisbon, Portugal from 02/12/2009 to 04/12/2009.

Poster presentations

1. Paper entitled **"**Siderophore producing bacteria from coastal ecosystems of Goa, West coast of India" presented at the 50th Annual Conference of Association of Microbiologists of India, AMI held at Pune from 15/12/2009 to 18/12/2009

2. Paper entitled "Diversity of polyhydroxyalkanoates accumulating bacteria isolated from coastal sand-dunes" co-presented at the 50th Annual Conference of Association of Microbiologists of India, AMI held at Pune from 15/12/2009 to 18/12/2009

3. Paper entitled **"**Isolation and characterization of polyhydroxyalkanoates producing bacteria from coastal sand-dune ecosystem" co-presented at the National Symposium on Microbial Diversity and its application in Health, Agriculture and Industry held at ICAR Research Complex Goa from 04/03/2011-05/03/2011

4. Paper entitled **"**Effect of pH, NaCl and growth medium on siderophore production by a sand dune isolate" presented at the 52nd Annual Conference of Association of Microbiologists of India (AMI) Conference held at Chandigarh from 03/11/2011 to 06/11/2011

5. Paper entitled **"**Benzoate induced variations in pigment production in a denitrifying bacterium" co-presented at the 52nd Annual Conference of Association of Microbiologists of India (AMI) Conference held at Chandigarh from 03/11/2011 to 06/11/2011.

TRAINING PROGRAMMES/WORKSHOPS

1. International workshop on Advanced Good Clinical Practices organised by Goa University on 17th September 2008.

2. A four day workshop on "Analytical Biology" at Microbiology Department, University of Pune from March 18th -21, 2009.

3. National training workshop on Bioinformatics and its applications organized by Bioinformatics Centre, Department of Biotechnology, Government of India, Unesco Mircen Marine Biotechnology, College of Fisheries, Mangalore, during March 29-31, 2011.

4. National workshop on Molecular subtyping of microbes using Pulse Field Gel Electrophoresis organized by Department of Biotechnology, Government of India during August 20-23, 2011 at ICAR Research Complex of Goa.