HALOPHILIC AND HALOTOLERANT BACTERIA ADHERED TO PARTICULATE MATTER IN THE MANGROVE ECOSYSTEM

A thesis submitted to Goa University for the Award of Degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY

By

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Certificate

This is to certify that the thesis entitled **'Halophilic and halotolerant bacteria adhered to particulate matter in the mangrove ecosystem'** submitted by **Miss Amrita Pradeep Kharangate** 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 thereof has not been previously submitted for any other degree or diploma in any university or institution.

Prof. Saroj Bhosle

Research Guide

Department of Microbiology,

Goa University

Date:

Place:

Declaration

I hereby state that, this thesis for a PhD degree in Microbiology on **'Halophilic and halotolerant bacteria adhered to particulate matter in the mangrove ecosystem'** is my original contribution and that the thesis or any part of it has not been previously submitted for the award of any degree or diploma to any university or institution. To the best of my knowledge the present study is the first comprehensive work of its kind in this area.

Amrita Pradeep Kharangate

Department of Microbiology

Goa University

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...Amrita

With love and gratitude

to

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and

My best friend and husband

Pankaj Lad

Nothing great in the world has been accomplished without passion.

.....G.W.F. Hegel.

Contents

LIST OF ABBREVIATIONS

A	Adhesion	NA	Nutrient agar
ABC	ATP building cassette	NaCl	Sodium chloride
Abs	absorbance	NaOH	Sodium hydroxide
ATP	Adenosine tri phosphate	ng	Nanogram
BATH	Bacterial adherence to Hydrocarbon	nm	Nanometer
BLAST	Basic local alignment search tool	NMR	Nuclear mass resonance
Bp	Base pair	NP/np	Nanoparticles
BSA	Bovine serum albumin	NTYE	NaCl-tryptone-yeast extract
BTEX	Benzene-toluene-ethylbenzene-xylene	PBS	phosphate buffered saline
CAS	Chrome azurol sulphonate	PCB	Poly chlorinated biphenyls
Cfu	Colony forming unit	PCE	Perchloroethylene
CMC	Carboxy methyl cellulose	PCR	Polymerase chain reaction
CTAB	Cetyl Tri methyl Ammonium Bromide	pH	Hydrogen ion concentration
Da	Dalton	ppm	parts per million
DLVO	Darjaguin-Landau-Verwey- Overbeek	PRB	Permeable reactive barrier
DNA	Deoxyribonucleic acid	Rf	Resolution factor
DNAPL	Dense non-aqueous phase liquid	RNA	Ribonucleic acid
E	Emulsification	RPM	Revolutions per minute
EDTA	Ethylene diamine tetra acetic acid	SDM	Shirdona mangroves
EPS	Extracellular polymeric substances	SEM	Scanning electron microscope
EDS	Energy dispersive X-ray spectroscopy	Sq. Km	Square kilometer
Fe	Iron	sp/ spp	species (singular)/species (plural)
Fig	Figure	TAE	Tris acetate EDTA
FTIR	Fouriers transform infra- red	TE	Tris EDTA
g	Gram	TCE	Tr ichloroethyline
HCl	Hydrochloric acid	TLC	Thin layer chromatography
hr	Hour	TVC	Total Viable Count
Km	Kilometre	UV	Ultraviolet
L	Litre	V	Volt
m	Metre	Vis	Visible
mg	Milli grams	w	Week
ml	Millilitre	ZMA	Zobell marine Agar
min	Minutes	ZMB	Zobell Marine Broth
MSM	Mineral salt medium	ZVI	Zerovalent Ion
MXM	Manxer mangroves	µg	microgram
Na	Sodium	µL	microlitre

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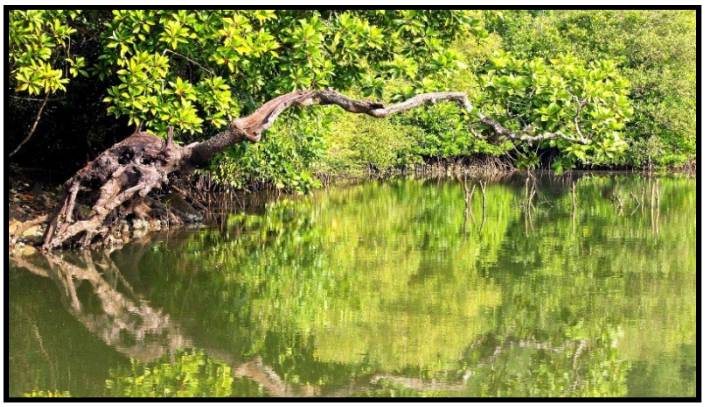
Introduction

Goa is a coastal state situated on the west coast of India and lies between the lattitudes 15° 44′ 30″ and 14° 53′ 30″ N and longitudes 73° 45′ and 74° 26′ E. The state is intersected by nine rivers namely Mandovi, Zuari, Cumbarjua, Terekhol, Chapora, Baga, Sal, Talpona and Galgibag due to which Goa has one of the most luxuriant pockets of mangrove ecosystem (Fig. 1.1). Goa has a total area of 3702 sq.km. of which 25 sq. km. is covered by the mangrove forest (Forest survey of India, 2011). It includes both, the moderately dense mangroves as well as the open mangroves. Most of these mangroves span the Mandovi, Zuari and the Cumbarjua canal areas.

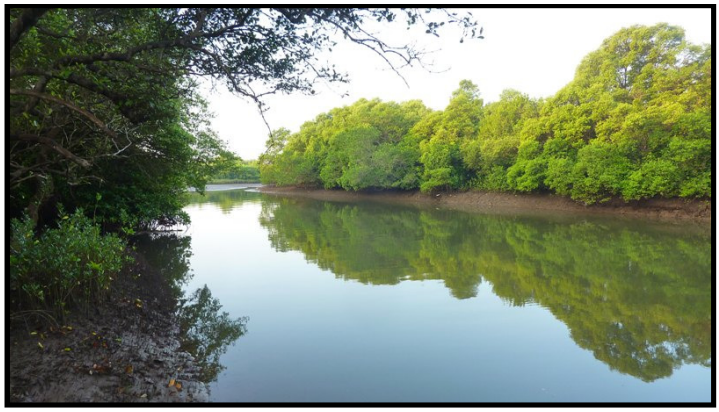
Mangroves are unique ecosystems of the earth as they are at the interface between the salt and freshwater ecosystems. They support a wide variety of macroscopic and microscopic flora and fauna and are thus biodiverse and productive. They are ecologically important as they protect the coastline against storm surges, tsunamis and erosion. Nutrients produced by the mangrove ecosystem supports the communities of mangrove ecosystem and also the surrounding estuarine and coastal ecosystem (Jennerjahn and Ittekkot 2002, Dittmar *et al*. 2006).

Particulate matter in the mangrove ecosystem primarily consists of the leaf litter from the mangrove plants and serves as a source of carbon and energy to the microorganisms of the ecosystem (Jackson and Vallaire 2007). It is composed of complex natural polymers like cellulose, hemicellulose, starch, tannin, lignin, lipids etc. These polymers are degraded by free living and adhered bacteria. Free living

***Rhizophora* sp and *Sonneratia* sp of Palolem mangroves (River Galgibag)**



***Rhizophora* sp. From Verna mangroves (River Sal)**



***Rhizophora* sp, *Avicennia* sp *and Kandelia* sp from Chorao mangroves (River Mandovi).**

Photographs of mangrove areas in Goa.

bacteria that degrade these polymers have been studied from the mangrove ecosystem such as *Microbacterium* (Yateem and Al Sharrah 2011), *Halococcus* (Sahoo and Dhal 2009), *Chromatium, Rhodopseudomonas, Rhodospirillum* (Vethanayagam 1991), *Pseudomonas, Marinobacter, Alcanivorex, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, Gordonia* (Britto *et al*. 2006, Gonzalez *et al*. 1997a,b), *Halobacillus* sp (Soto-Ramirez *et al*. 2008). Although literature survey has shown that in turbid ecosystems like estuaries, it is the adhered bacteria that bring about most of the degradation of particulate organic matter, (Gonsalves *et al*. 2009, Crump *et al*. 1999, Crump *et al*. 1998, Crump and Baross 1996, Griffith *et al*. 1994), not many reports are available on adhered bacteria from mangrove ecosystem (D'Costa *et al.* 2004).

Adhesion of a bacterial cell to a substrate or solid surface plays an important role in localising the bacterium and colonisation of the substrate. Adhesion is a way for a bacterium to establish and sustain in a particular habitat. Various mechanisms are known for adherence such as formation of extracellular cellulosomes by Gram positive cellulolytic bacteria and membrane bound proteins by Gram negative anaerobic amylolytic bacteria (Anderson 2000). Some bacteria are also known to form extracellular adhesive materials such as amorphous, granular and fibrous holdfast structures, extracellular polymers, exopolyssacharides, glycocalyx, capsule etc. (Mancuso *et al*. 2004). The ability to adhere is facilitated by cell surface appendages like pili, flagella, fimbriae (Katsikogianni and Missirlis 2004, Gottenbos *et al.* 2002).

Sessile or adhered bacteria are able to exploit the nutritional opportunities of habitat better than non attached bacteria as it helps to provide the bacterial cell with a continuous supply of nutrients and prevents starving conditions.

Various factors in the environment affect the ability of a bacterial cell to grow and survive in the environment. In the mangrove ecosystem salinity plays an important role in determining the type of community structure. In the event that the salinity is high there is an increase in halophilic and halotolerant bacteria. Such bacteria are also known to adhere to solid particles in the ecosystem and produce EPS (Qurashi and Sabri 2012, Poli *et al.* 2010). Another important factor for metabolic activities to take place at the optimum level is the availability of essential metals such as iron. Iron is a crucial metal and forms a part of many important enzyme systems in the bacteria. Owing to the aerobic atmosphere of the planet iron occurs mostly as ferric oxyhydroxide polymers which has low solubility ion. Therefore, due to the limited bioavailability of this metal bacteria have adopted strategies such as production of siderophores that (Sandy and Butler 2009) chelates iron from the environmental complexes and transports it to the bacterial cell (Neilands 1995, Hider and Kong 2010). Siderophores have generated interest as they play a vital role in efficiency of degradation in ecological habitats and phytoremediation (Gaonkar and Bhosle 2013, Gaonkar *et al*. 2012). In the mangrove ecosystem it is important that adhered bacteria should produce siderophores to be able to efficiently sequester iron from the environment to be able to mineralize/ metabolise the particulate organic matter

efficiently. It is envisaged that in a dynamic ecosystem like mangroves where there is continuous influx of tidal action resulting in variation in salinity and dilution of essential elements such as iron, the most effective mineralization would occur with halophilic/ halotolerant bacteria which are adhered and produce metal chelating ligands such as siderophores.

The present work on the thesis was therefore aimed towards understanding the role of halophilic and halotolerant bacteria adhered to particulate matter, in mineralization of plant litter in mangrove ecosystem.

These adhered bacteria were isolated and screened for growth in presence of salt, enzyme production, siderophore production and their adhesion ability. The possible application of selected halophilic and halotolerant isolates in mineralization of plant litter was determined. Further, the effect of iron nanoparticles on adhesion and siderophore production was studied. The EPS and pigment produced by a halophilic bacterial strain was extracted and characterized. The research work is compiled and presented in the thesis comprising of four chapters preceded by the literature survey.

Chapter 1:

Literature survey

1.1 The Mangrove Ecosystem

Mangrove ecosystems are found in the tropical and subtropical regions of the Earth. The mangrove forests have plants that range from prostrate shrubs to timber trees that are 60 m in height. This ecosystem is unique due to the intermingling of salt and fresh water during the tidal action. The mangrove plants are halophytes and are able to grow under both, the saline and the fresh water conditions. They support a wide variety of microscopic and macroscopic flora and fauna and are highly productive and biodiverse ecosystems. Ecologically, they serve as protective buffers against soil erosion, tsunamis and storm surges (Jennerjahn and Ittekkot 2002, Dittmar *et al*. 2006). The latter reason has lead mangroves to be termed as **"bioshields".**

1.1.1 Physical parameters of the mangrove ecosystem

Studies have been carried out on various physical parameters of the mangrove plant litter from Goa. These parameters include salinity of the leaf litter, pH of the plant litter and the dissolved oxygen content of the mangrove ecosystem (D'Costa *et al*. 2004). The salinity of the mangrove plant litter sample ranged from 11- 16% in the pre monsoon months while it reduced to 9-13% post monsoon. The pH was found to be in the acidic region of 3.0-6.5 before the monsoons and 2.0-7.0 after monsoons. This might be due to the humic acids that are produced during degradation processes. The dissolved oxygen ranged from 8.4- 22 mg/L and was found to be constant throughout the year except in areas of the mangroves that showed stagnant pools giving rise to anaerobic environment (D'Costa *et al*. 2004).

1.1.2 Bacteria in the mangrove ecosystem

Mangrove ecosystem, being a very productive ecosystem has a high rate of nutrient recycling. The mangrove plants in Goa consist of species such as *Avicennia officinalis*, *Bruguiera cylindrica, Kandelia candel, Rhizophora mucronata* etc. The plant litter consisting of leaves, twigs, pneumatophores, rhizophores, mangrove fruits and dead mangrove trees, falls on the mangrove floor and undergoes decomposition. This adds nutrients to the mangrove soil or is carried out into the sea by the sweeping tides thus contributing to the aquatic food chain. The number and type of bacteria and other microorganisms are also affected by the contents of the plant litter. For example, *Avicennia* wood is less lignified than *Rhizophora* and thus a mangrove ecosystem dominated by the later will show occurrence of more lignin degrading bacteria (Agate *et al*. 1988).

The mangroves are rich in organic and inorganic nutrients and thus support a wide spectrum of microorganisms. The efficiency of degradation in this ecosystem depends upon the rate of mineralisation by the microbial community and the nutrient turnover. The mangrove microbial community includes algae, bacteria, fungi, actinomycetes, protozoa etc. These can occur in the sediments, water, and detritus or can even be periphytonic. Autotrophs like algae and some bacteria contribute to primary production and the heterotrophs bring about nutrient recycling by transformation processes such as nitrification, denitrification, nitrogen fixation, mineralisation and

breakdown of complex particulate matter, converting them into nutrients utilisable by the microbial biomass (Fig. 1.1).

The structural components are broken into simple nutrients which enter the general pool in the biosphere to be recycled again. It is remarkable that a number of organisms are themselves sustained in the process of decomposition and degradation in the coexistence of autotrophy and heterotrophy (Agate *et al*. 1988). The variation in salinity in this ecosystem, allows halophilic and halotolerant bacteria to predominate in the mangroves. The various halophilic and halotolerant bacteria from the mangrove ecosystem studied include *Bacillus* (Van-Thuoc *et al*. 2012) *Halobacillus* (Soto-Ramirez *et al*. 2008), *Halococcus* (Kathiresan 2000). The constant influx of the tides cause the free living bacteria to be washed away thereby causing a variation in their number while the bacteria attached to particulate matter are retained behind and thus their numbers remain relatively constant.

Earlier, it was believed that since the adhered bacteria accounted for less than 10% of the pelagic bacterial population, they were considered to be of minor importance in the annual carbon budget of such ecosystems (Gonsalves *et al.* 2009). Thus most studies focused on free living bacteria. However recent studies show that in turbid ecosystems such as the estuarine and mangroves it is the adhered / attached bacteria which account for most of the degradation taking place in these ecosystems (Gonsalves *et al*. 2009, Crump and Baross 1996, Crump *et al*. 1998, Crump *et al*. 1999, Griffith *et al*. 1994). Studies on adhered bacteria from freshwater ecosystems such as pond and oceans have

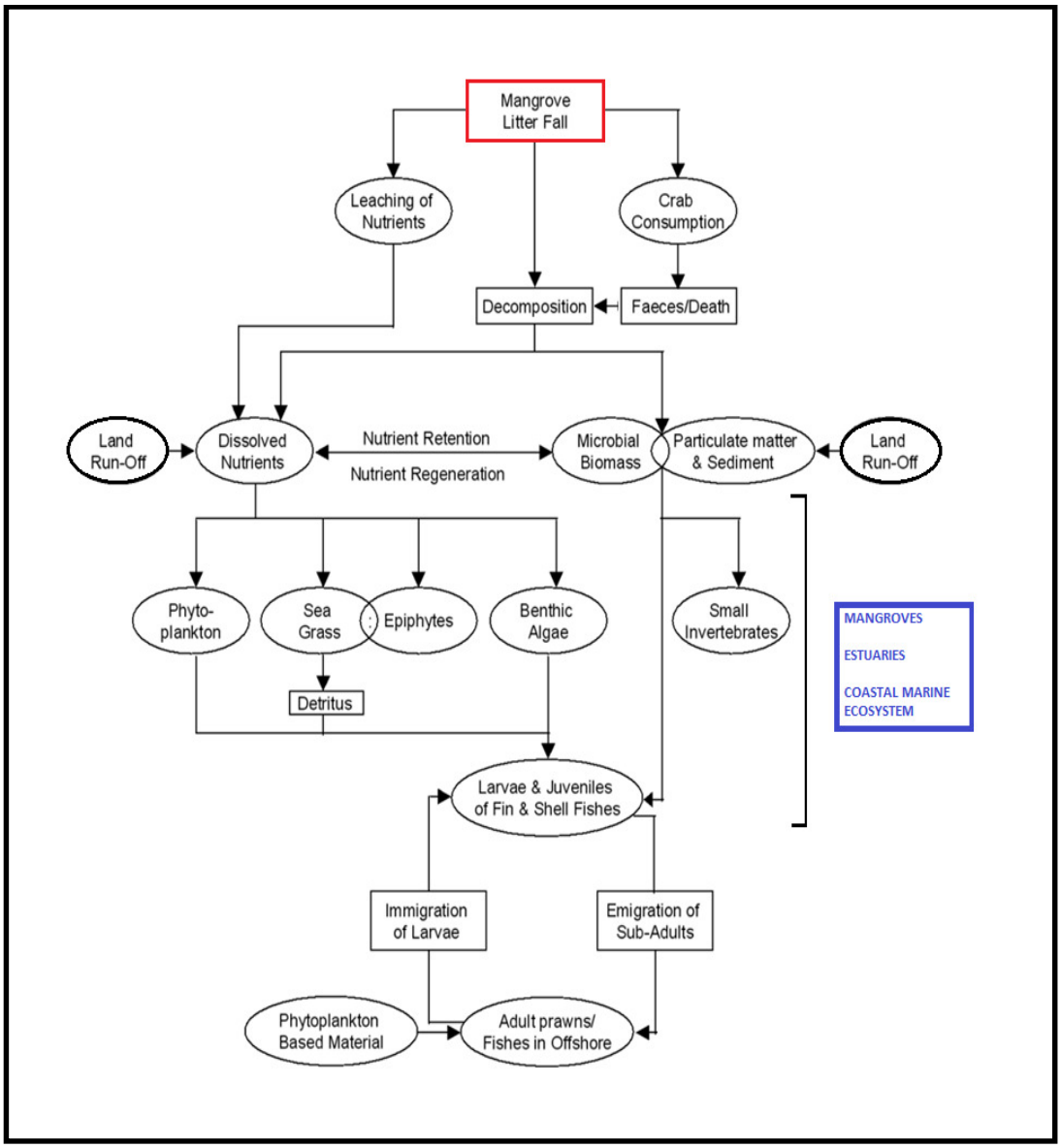


Fig.1.1: Food web of mangrove ecosystem (Kathiresan and Bingham 2001).

been reported. These studies showed that the adhered bacteria were generally larger and the cell specific uptake rates were significantly higher for monomeric carbohydrates and amino acids as compared with the free living bacteria (Simon 1987, Kirchman 1983)

The particulate organic matter in the mangrove ecosystem consists of complex natural polymers such as cellulose, hemicellulose, lignin, tannin, lipase, tannin, chitin etc. Bacteria such as *Halomonas* (Garcia *et al.* 2004), *Sagittula stellata* (Gonzalez *et al.* 1997b), *Serratia marcescens* (Perestelo *et al.* 1990), *Marinobacter hydrocarbonoclasticus*, *Microbulbifer hydrolyticus*, *Marinobacterium georgiense* (Gauthier *et al.* 1992, Gonzalez *et al.* 1997a), *Vibrio sp.* (Gao *et al.* 2010), *Microbacterium* (Yateem and Al-Sharrah 2011), *Halococcus* (Sahoo and Dhal 2009) *Chromatium, Pseudomonas, Alcanivorex, Microbulbifer, Sphingomonas, Micrococcus, Cellulomonas, Dietzia, Gordonia groups* (Britto *et al*. 2006) and *Halobacillus* sp. (Soto-Ramirez *et al.* 2008) have been reported from coastal marine and mangrove ecosystem that degrade such complex natural polymers by attaching to the organic particulate matter containing these polymers. However, very few reports are available specifically on adhered bacteria from the mangrove ecosystem.

The adhered bacteria adhere to the plant litter or the particulate matter containing these polymers, utilise it for its metabolic activities and facilitate their degradation. The adhesion of the bacterial cell to the substrate is facilitated by the presence of surface structures like capsule, EPS and holdfast and appendages such as pili, fimbriae and flagella.

1.2 Mechanism of bacterial adhesion

Bacteria prefer to grow attached to a surface. This surface is often the substrate which the bacteria initially adheres to and utilizes as a source of energy for its metabolic activity.

Bacterial attachment to a substrate occurs in 3 phases.

1.2.1 The transport phase

1.2.2 The physical interaction phase

1.2.3 The molecular or cellular phase

1.2.1 The transport phase

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

1.2.2 The physical interaction phase

The physical interactions between the bacterium and the substrate are further classified as long range and short range interactions. The long-range interactions are non specific and the distance between the bacterial cell surface and the substrate surface is >50 nm. It involves Van der Waals forces of attraction only. When the bacterial cell and substrate is separated by a distance of 10-20 nm Van der Waals forces and coulombic forces come in play and here maximum repulsion occurs due to the net negative charge on both the surfaces. The repulsive forces increase in proportion to the diameter of the two particles approaching each other thus surface appendages and structures play an important role in overcoming this barrier. Short-range interactions become effective when the bacterial cell surface and the substrate surface come into close contact at about a distance of <5nm. This

involves chemical bonds such as hydrogen bonding, covalent bonding, ionic and dipole interactions and hydrophobic interactions. The mechanism of interaction between the bacterium and the surface of the substratum is shown in the Fig. 1.2. Thus, a bacterial cell is brought in contact with the substrate by long range nonspecific interactions and kept in contact initially until the short range interactions take over. This forms basis of adhesion that makes the second phase (final phase of interaction) i.e. the molecular and cellular phase possible (Katsikogianni and Missirlis 2004, Gottenbos *et al*. 2002).

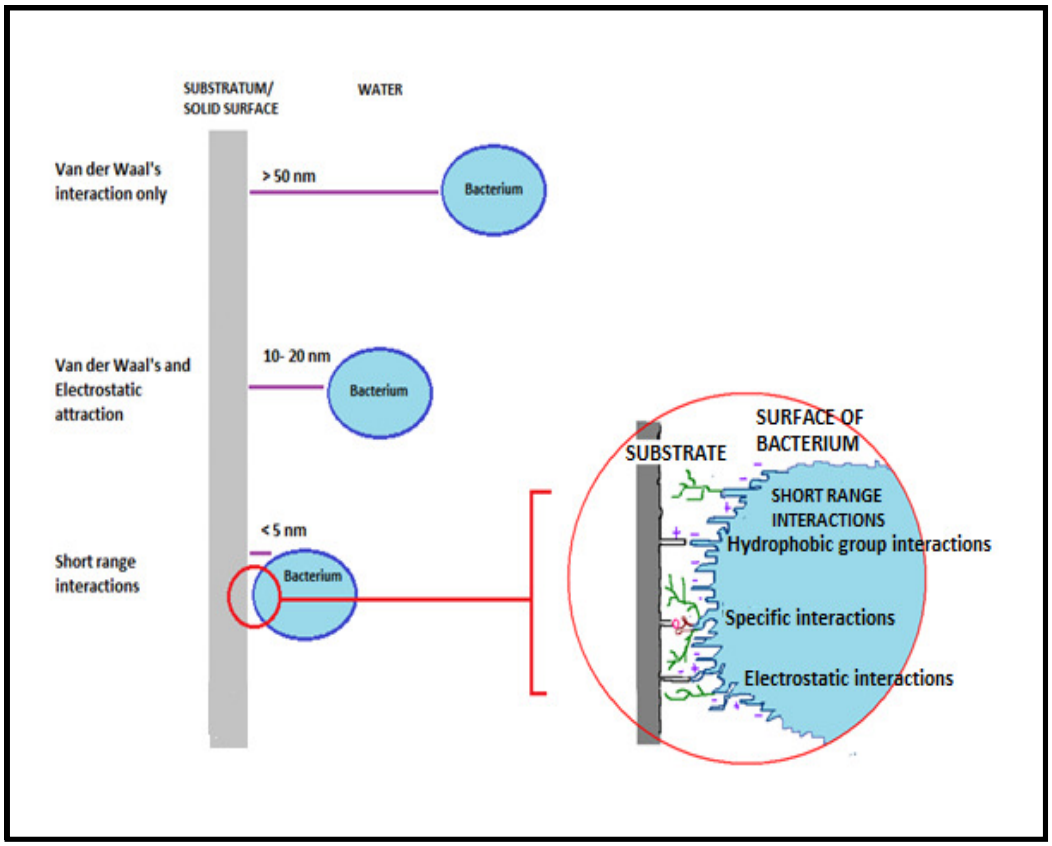


Fig 1.2: Schematic representations of interaction between bacterial cell and substrate during adhesion (Gottenbos *et al*. 2002).

1.2.3 The molecular or cellular phase

This phase involves specific molecular interaction between the bacterial extracellular moieties and the surface of the substratum and is practically irreversible. It can be specific like ligand-receptor interaction or chemical bond formation or non specific such as hydrophobic or electrostatic interaction (Ofek *et al*. 2006). The bacterial cell binds more firmly to the substratum by means of bacterial surface polymers, appendages such as fimbriae, pili, flagella or capsules and slime layers. (Marshall 2006, Schlegel and Jannasch 2006, Katsikogianni and Missirlis 2004, Prescott *et al*. 2005, An and Friedman 2000, Bhaskar and Bhosle 2005, Kokare *et al*. 2009).

Literature shows many bacteria such as *Halomonas* sp*, Marinobacter* sp*, Vibrio* sp (Gulig *et al*. 2005), *Pseudoalteromonas, Sagittula stellata* (Gonzalez *et al*. 1997b)*, Microbulbifer hydroliticus, Marinobacterium georgiense* Gonzalez *et al*. 1997a)*, Saccharophagus degradans, Hyphomonas MHS-3 strain* (Quintero *et al*. 1998)*, Halomonas maura, Caulobacter* sp (Tsang *et al*. 2006, Merker and Smit 1988, Ong *et al*. 1990)*, Roseobacter* sp (Labrenz *et al*. 2005) adhere and utilise their substrates by means of such surface structures. It is believed that the functional part of these structures is the presence of adhesin- a protein component that helps the bacterium to bind to the substrate (Katsikogianni and Missirlis 2004, Prescott *et al*. 2005, An and Friedman 2000, Gottenbos *et al.* 2002). The entire process of adhesion often forms the basis for the initiation of the biofilm formation. The entire adhesion process is

summarised in Fig. 1.3. The adhesion mechanism thus is the basis of the interaction that occurs between the bacteria and its substrate in the long process of degradation.

1.3 Theories of adhesion

Many theories have been put forth to explain the mechanism of adhesion. Out of which, the following are the most accepted ones and come quite close to explaining the exact mechanism of adhesion.

1.3.1 The DLVO theory of adhesion

1.3.2 The thermodynamic approach to attachment

1.3.1 The DLVO theory of adhesion

The basic understanding of the interaction and attachment process of bacteria to a surface can be explained by Derjaguin, Landau, Verwey and Overbeek (DLVO) theory. It explains that the overall net interaction between the bacterial cell and the substrate surface is the result of balance between two additive forces. One, the electrostatic attractive force i.e. Van der Waals interaction and two, the repulsive interactions such as Coulomb interactions that arises due to the electrical double layer of the bacterial cell and the substratum (Katsikogianni and Missirlis 2004, Hermansson 1999, Hayashi *et al*. 2001).

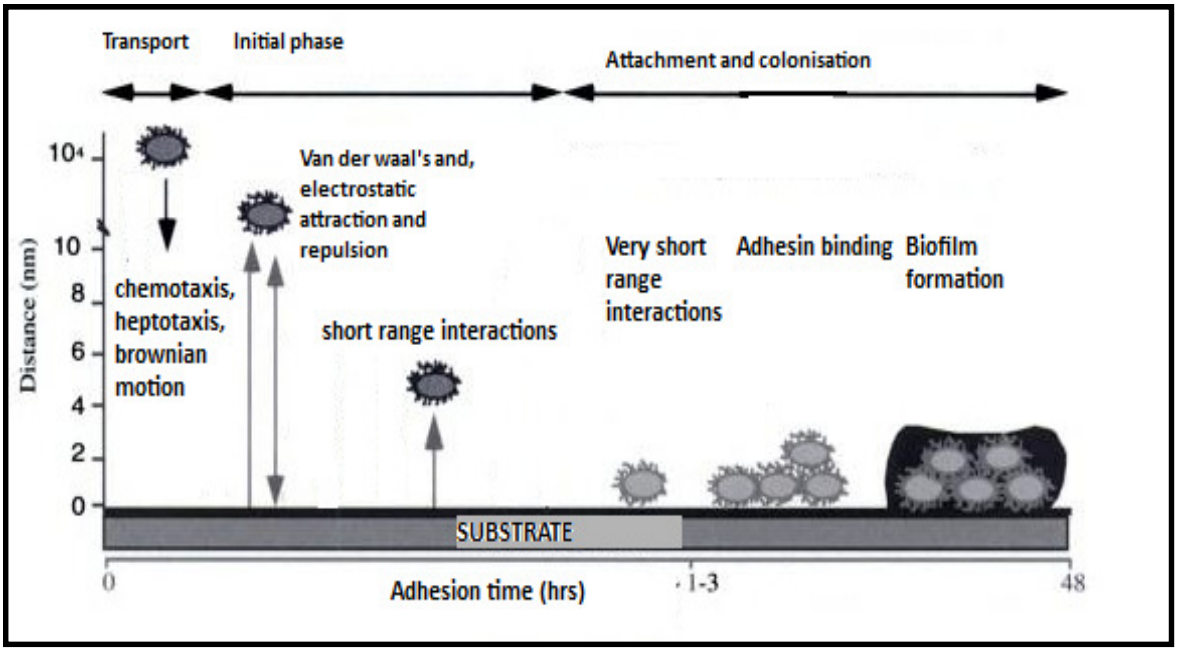


Fig.1.3: Schematic representation of transport, physical and cellular phase in adhesion process (An and Friedman 2000).

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

1.3.2 The thermodynamic approach to attachment

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

This theory has helped to explain the fact that a hydrophobic substrate or cell surface shows increased attachment or adhesion. However, it is not possible to accurately calculate the surface free energies of the bacterial cell due to their complex nature and hydration properties. Thus the free energy change calculations during adhesion may be incorrect. This theory is applicable only to closed systems where no energy is put in

from the outside. However bacteria being a living entity that can convert substrate into energy, it fails to consider that the adhesion may be driven by physiological mechanisms and synthesis of adhesive bacterial surface polymers.

1.4 Bacterial surface appendages and polymers

While the above theories fail to take into account the role of cell surface polymers or appendages that are produced that aid the bacterial cell to attach to the substrate, studies show numerous bacteria that are known to adhere to their substrates by means of these polymers and appendages which include EPS, glycocalyx, capsule, fimbriae, pili and holdfast.

1.4.1 Fimbriae or simple pili

Most Gram negative bacteria exhibit the presence of short hair-like or non-flagellar filamentous projections external to the cell wall as shown in Fig. 1.4. These are called fimbriae and they aid the cell to adhere to other cells and substrates. In the attachment phase it has been seen that fimbriae act as adhesins permanently attaching the bacterial cell to the substrate (Prescott *et al*. 2005, An and Friedman 2000)**.** The marine bacteria *Hyphomonas* strain MHS-3 which is the primary coloniser of surfaces in the marine environment synthesise two structures that mediate adherence to solid substrata- a capsular polysaccharide and fimbriae. Their attachment is important as it paves way for attachment of other members of adherent community such as protozoa, fungi by rendering the surface enriched and thus suitable. Interestingly, it is seen that

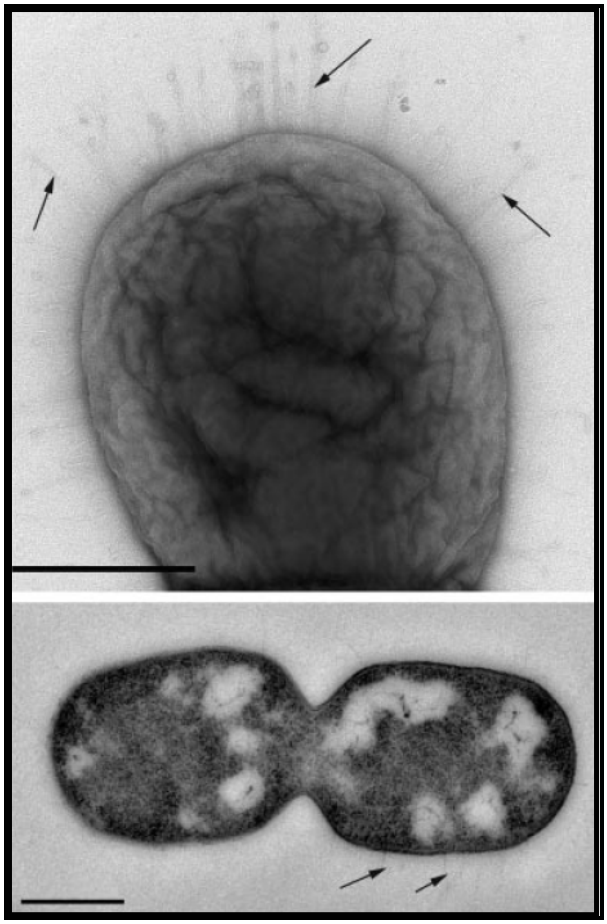


Fig: 1.4: ***Halomonas halocynthiae*** **fimbriae seen through an electron microscope (Romanenko** ***et al*****. 2002).**

the EPS produced attaches to both, the hydrophobic and hydrophilic surfaces. It is also believed that the fimbriae mediate long range primary adherence to surfaces as they extend beyond the EPS capsule of the cell and bringing it into contact with surface (Quintero *et al*. 1998). *Vibrio vulnificus* a moderate halophile and *Halomonas halocynthiae* is known to produce fimbriae (Gulig *et al*. 2005, Romanenko *et al*. 2002).

Fimbriation as it is often called is widely distributed in Enterobacteriaceae and Pseudomonadaceae however is not restricted to these two families. Ecologically it is important as it initiates attachment to solid surfaces mainly with solubility substrate and contact with other members of the community which may aid in biofilm formation. Bacteria such as halotolerant *Caulobacter sp*, and some *Pseudomonas sp* form star shaped aggregates called rosettes as a consequence of polar fimbriae and slime production by these bacteria.

1.4.2 Holdfasts

These are extracellular bridging structures that are produced by some bacteria to attach themselves to flat surfaces. They are usually polysaccharide in nature. The holdfast has strong adhesive properties at the end tip of its stalk (Ong *et al*. 1990). Bacteria known to form holdfast structures are *Thiothrix sp, Flexibacter sp, Seliberia stellata, Sagittula stellata* (Quintero *et al*. 1998, Gonzalez *et al*. 1997b, Cytryn *et al*. 2006,

Martinez-Canovas *et al*. 2004c), *Caulobacter sp*. (Fig.1.5) (Levi and Jenal 2006, Tsang *et al*. 2006, Merker and Smit 1988, Ong *et al*. 1990), *Roseisalinus antarcticus* (Labrenz *et al*. 2005).In addition to these bacteria, other bacteria which are stalked, prosthecate or slime forming were observed. In the marine environment *Leucothrix mucor* is known to attach to seaweed by means of holdfast structures.

1.4.3 Extracellular polymeric substances (EPS)

EPS produced by bacteria is of two types. Capsular EPS is tightly bound to the bacterial cell and more organised as seen in *Halomonas maura, Hyphomonas* strain MHS-3 and *Thiohalomonas denitrificans* (Quintero *et al.* 1998), *Halomonas organivorans* (Garcia *et al*. 2004). The Slime EPS is diffused and loosely bound to the cell. When the bacterial cell is very close to the surface of the substrate at the final stage of adhesion, it produces EPS that helps the cell to attach to the substrate surface irreversibly (Vu *et al*. 2009, Oren 2006, Kaiser 2006 Bhaskar and Bhosle 2005, Mancuso *et al*. 2004). The glycocalyx encompasses both the capsules and slime layers. It is often described as a network of polysaccharides extending from the surface of bacteria and helps it to adhere to surfaces.

Among the halophilic bacteria the most common genus known to form EPS is the *Halomonas* which includes *Halomonas maura*, *Halomonas eurihalina* (Bouchotroch *et al*. 2001), *Halomonas ventosa*, (Martinez- Canovas *et al*. 2004a, Mata *et al*. 2006), *Halomonas anticariensis* (Martinez-Canovas *et al*. 2004b), *Halomonas almeriensis*

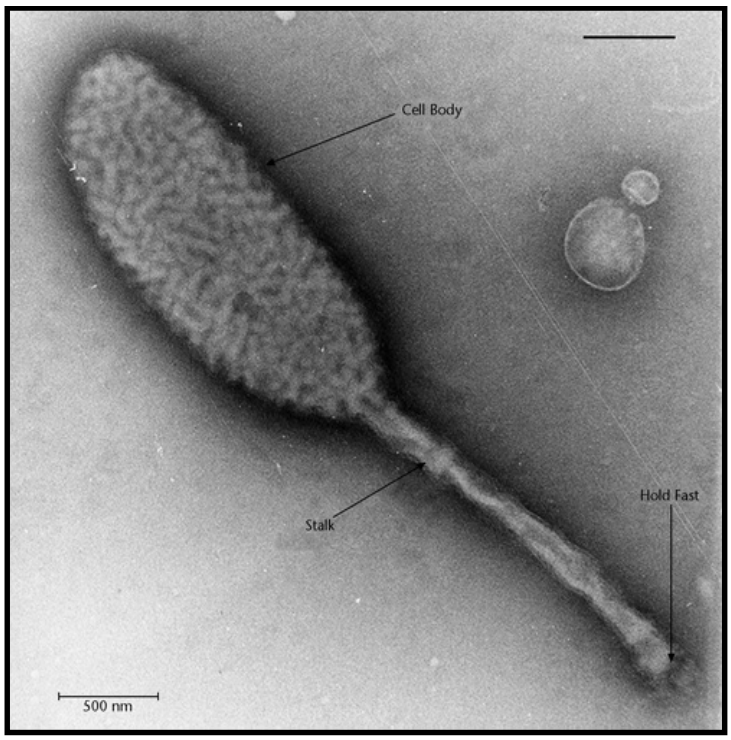


Fig. 1.5: *Caulobacter* sp. holdfast structure seen through an electron microscope (Toh *et al*. 2008).

(Martinez-Checa *et al*. 2005) *Halomonas koreensis* and *Halomonas smyrnensis* (Poli *et al.* 2013).

The EPS structure is said to aid the bacteria in adhesion to substrates, other bacteria, animal tissue and other inert substances apart from being protective (Mata *et al*. 2006, Kokare *et al*. 2009). The EPS production is the most significant mechanism of adhesion in bacteria and has been extensively studied. Interestingly, literature survey also shows the crucial role of EPS in attachment colonisation and biofilm formation (An and Friedman 2000).

1.4.4 Flagella

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

1.5 Benefits of adherence to the bacterial cell

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

In the marine, estuarine and mangrove ecosystem the bacteria experience liquid-solid interface interaction. The colonisation of a solid surface of substrate is a prerequisite for exploitation of the habitat and its substrates. In open ecosystems bacteria take advantage of being fixed to a priviledged location. Attachment to surface ensures that the bacterium remains localised in that environment to which is presumably has now adapted.

In ecosystems such as estuaries and mangroves studies have now established that attached bacteria bring about most of the degradation of the organic matter as compared to the free living bacteria (Crump and Baross 1996, Crump *et al.* 1998, Crump *et al.* 1999, Gonsalves *et al*. 2009).

In running water of rivers streams and in mangrove areas where there is constant tidal variation, receive organic matter from upstream and during influx at high tides respectively. Thus, in such an environment bacteria would not be able to compete with fast growing bacteria if it were not attached to substrate or a solid surface where food is available in concentrations for sufficient growth. Thus growth in such habitats is restricted to those bacteria that can attach themselves to solid surfaces.

In rivers and sea waters small floating particles such as silt, clay or detritus which form the particulate matter has remarkable growth promoting effects on the bacteria. In environments where nutrient concentration is low the presence of particulate matter favours growth of bacteria as they absorb nutrients from particle surface.

Though the ability to attach is considered as a means to escape low nutrient environment, attachment is beneficial as nutrients accumulate at solid-liquid interfaces. Gas bubbles get trapped at such interface region serve as vector for nutrients. Sessile or adhered bacteria are able to exploit the nutritional opportunities of habitat better than non attached bacteria.

Adherence of bacteria to substrate prevents the cell from being washed off by flow conditions while non-adhered bacteria gets washed off into another ecosystem and no nutrient exchange is facilitated by movement of medium past the bacterial cell. It

helps to provide the bacterial cell with a continuous supply of nutrients and prevents starving conditions.

Structures such as the EPS that helps in attachment also protects the cell from dessication and predators such as bacteriophages and toxins. Attachment of the bacterial cell ensures close proximity with the substrate. It doesn't matter if the surface is organic or inorganic as by law of physics nutrient molecules adsorb at interfaces and the surface is the interface. This means that in any environment the concentration of any particular nutrient molecule is very likely to be higher on, or in close proximity to, a surface.

Utilisation of certain substrates requires close contact between the bacterium and the substrate, as in case of *Cytophaga* and S*porocytophaga* cells that adhere closely to cellulose fibers. Also many bacteria digest complex polymers such as starch and chitin by means of adherence to starch grains and chitin components. It results in optimum degradation by the extracellular enzymes produced by the cell. As in case of the bacterium *Sagittula stellata* close binding to the substrate with the holdfast structure ensures that optimum degradation occurs by hydrolytic enzymes present in the blebs and vesicles that are produced externally (Gonzalez *et al.* 1997b).

In many cases the colonisation of a single type of bacteria forms a thick slimy layer which provides the best opportunity for microbes to come together and form a biofilm.

Thus it has now been established that bacterial adhesion helps in colonisation of a solid surface and is the first step in biofilm formation (Anderson *et al.* 2007).

1.6 Factors influencing bacterial adhesion

The interactions between the bacterial cell and the substrate is influenced by various factors such as the environmental factors that include pH, ionic strength of the surrounding medium (Bitton and Marshall 1980), the flow conditions of the surrounding medium and presence of inhibiting (antibiotics, albumin) and promoting (fibronectin, fibrinogen) factors in the surrounding medium; composition of substrate/ material i.e. chemical composition substrate (charge and hydrophobicity of the surface), roughness of the surface, porosity and density of the surface; bacterial cell characteristics such as bacterial hydrophobicity and bacterial surface charge (Katsikogianni and Missirlis 2004).

1.7 Applications of adhered bacteria

Adhered bacteria are more effective in bioremediation techniques targeted at pollutants such as heavy metal, benzene, toluene, ethylbenzene and xylenes (BTEX), hydrocarbons, petroleum, chlorinated and polychlorinated phenols. This is due to their localised colonisation that enables them to maintain optimum pH, localise solute concentrations and redox potential thus allowing the bacteria to improve mineralisation process (Vu *et al*. 2009).

In oil water interface adhesion of bacteria enhances the availability of the hydrocarbon to the bacterial cells and increases the rate of biodegradation. Adhesion enables rapid uptake of hydrocarbons and pollutants, allows accession to poor water soluble and non emulsified hydrocarbons. This makes adhered bacteria more efficient in oil spill bioremediation. In case of mixed communities the ability to adhere enables selective growth and enhances bioremediation with time (Abbasnezhad *et al*. 2011).

1.8 Bacterial siderophores

The mineralisation and the efficiency of degradation is dependent on the metabolic activities of the bacterial communities and other microorganisms in the mangrove ecosystem. Limitation in any essential element would mean a direct effect on the nutrient recycling. One such essential element required for respiration, DNA synthesis and metabolic activities of bacteria is Iron (Fe). Though iron is the most abundant metal in the earth's crust its bioavailability is limited, as owing to the aerobic atmosphere of the planet iron occurs mostly as ferric oxyhydroxide polymers which has low solubility iron. Therefore, due to the limited bioavailability of this metal bacteria have adopted strategies such as production of siderophores (Sandy and Butler 2009). Siderophores are low molecular weight iron binding compounds that are produced by bacteria, fungi and plants. They chelate ferric ion from the environmental complexes and transport it to the bacterial cell (Hider and Kong 2010, Neilands 1995, Leong and Neilands 1976).

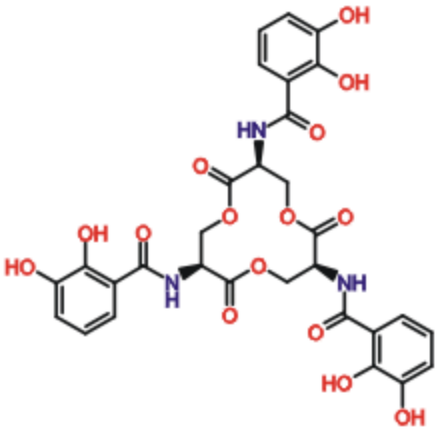
1.8.1 Classification of siderophores

The siderophores have three important iron chelating groups based on which they are classified as catecholates, hydroxamates and carboxylates.

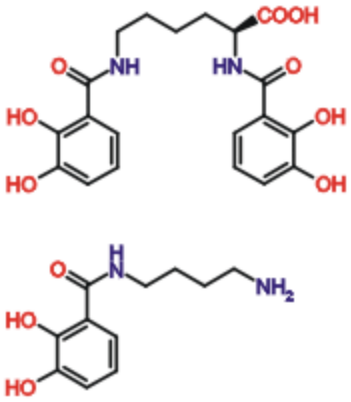
a) The Catecholate type of siderophores contain three molecules of complexing agent 2,3-dihydroxybenzoate which are attached to a cyclic trilactone made up of three serine molecules (Lengeler *et al.* 1999). They have have catechol as their functional group. The Fe(III)-catecholate siderophore complex has a positive charge. The most common examples being enterobactin from *E.coli*, azotochelin and aminochelin from *Azotobacter* (Fig. 1.6), bacillibactin from *Bacillus subtilis* and salmochelin from *Salmonella enterica*.

b) The hydroxamate type of siderophores have a basic structure consisting of three hydroxamic acids forming a cyclic structure. The Fe (III)- hydroxamate siderophore complex is uncharged (Lengeler *et al*. 1999). Ferrioximes and ferrichromes are hydroxamate type of siderophores (Fig. 1.7).

c) In the carboxylate type of siderophore the iron chelating group is a carboxylic acid. Dicarboxylic and tricarboxylic acids such as citrate and its derivatives are good siderophore complexing agents (Lengeler *et al.* 1999). Classic examples of carboxylate type of siderophores are achromobactin and vibrioferrin (Fig 1.8).

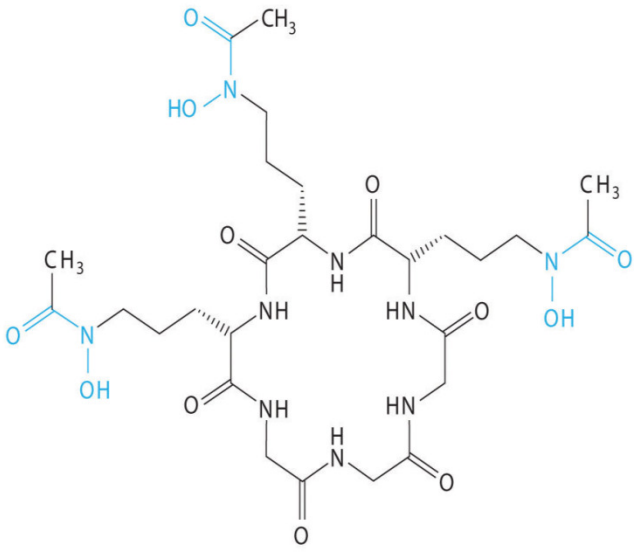


Enterobactin of *E.coli*

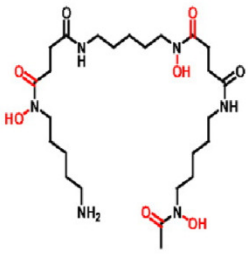


Azotochelin (top) and aminochelin (bottom) of *A. vinelandii*

Fig. 1.6: Catecholate siderophore produced by *E.coli* and *Azotobacter vinelandii.*



Ferrichrome



Desferrioxamine

Fig. 1.7: Chemical structure of hydroxamate siderophores Ferrichrome and Desferrioxamine

Interestingly it is seen that some siderophores may contain more than one type of iron binding functional group. These type of siderophores are known as **mixed ligands**. The two most common examples of mixed ligand siderophores is pyoverdin by *Pseudomonas* and azotobactin by *Azotobacter* as shown in Fig. 1.9. They posess an a-hydroxy group, a hydroxamate group and catechol. The presence of such siderophores in bacteria gives them a competitive edge over the other bacteria (Barton *et al*. 2007).

Some siderophores are also pigmented and a typical example is pyoverdine and pyocyanin produced by *Pseudomonas* spp. Bacteria produce pigments such as chlorophylls, carotenoids, phycobilliproteins etc. The most common examples being *Chromobacterium violaceum* that produces a purple pigment called violacein, *Serratia marcescens* a pink-red colour attributed to the pigment prodigiosin, *Pseudomonas aeruginosa* that produces a blue- green pigment called pyocyanin and other *Pseudomonas* spp produce pigments like pyoverdine and fluorescein. The primary function of pigment is bacteria involves photosynthesis or protection of cell from damage due to harmful oxygen radicals and light. It has reported that carotenoid pigments are produced by the bacteria in stress and aids the bacteria in order to survive in the environment (Bhosale 2004).

Carotenoids are the most widely present class of pigments in bacteria. In photosynthetic bacteria, they play a crucial role in photosynthesis while in non-photosynthetic bacteria they essentially protect the cell from the damaging effects of

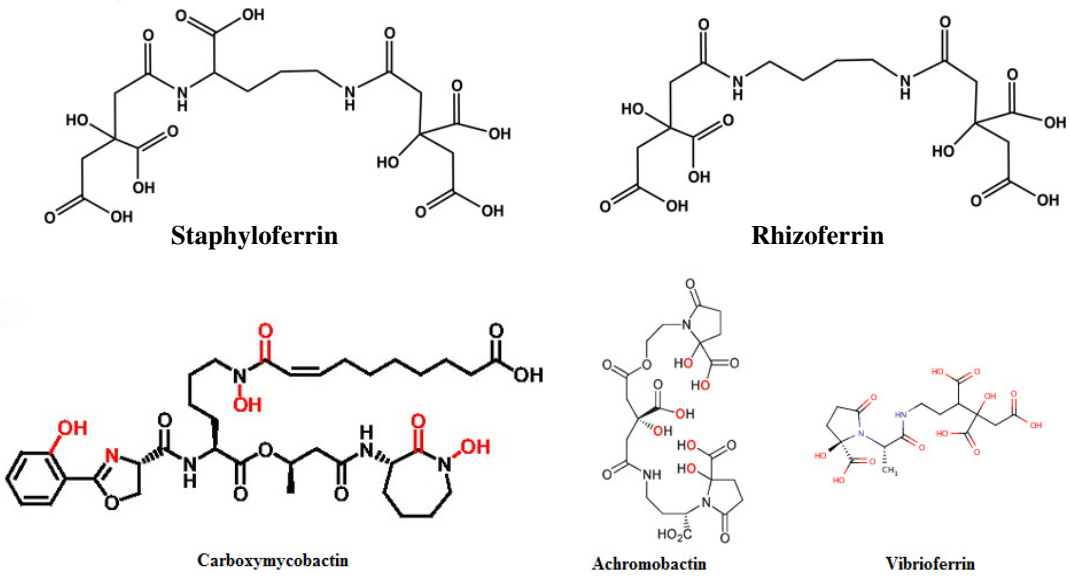


Fig. 1.8: Chemical structures of carboxylate siderophores.

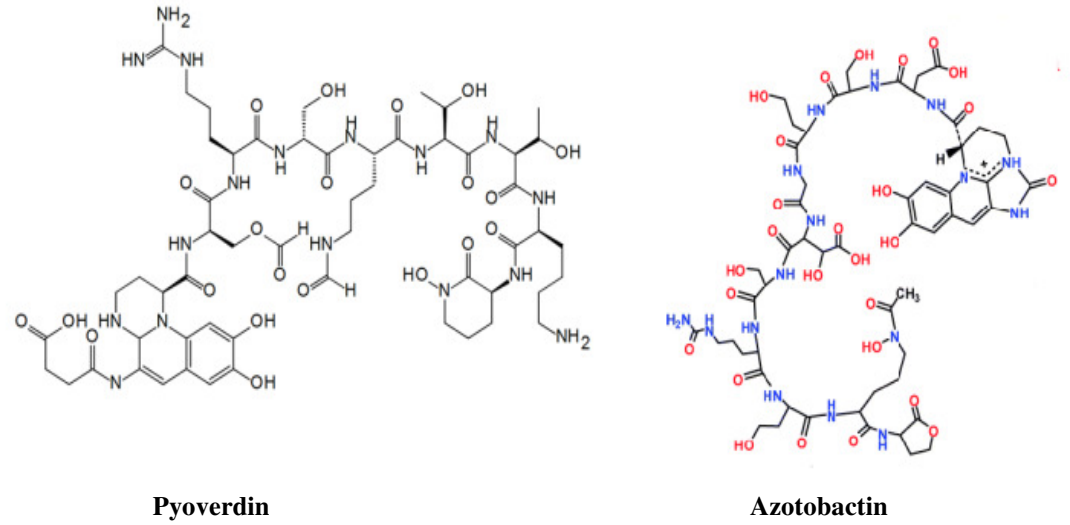


Fig. 1.9: Chemical structures of two mixed ligand siderophores pyoverdin and azotobactin.

light and oxygen. They are responsible for the red, orange or yellow colouration of the cells. Structurally carotenoids are C_{40} tetraterpenoids that are lipid soluble. The backbone of the consists of a 40-carbon polyene chain that may be terminated by cyclic groups and may be complemented with oxygen containing functional groups. Depending on the structure carotenoids are classified as carotenes and xanthophylls (Eldahshan and Singab 2013). Carotenes are hydrocarbon carotenoids containing carbon and hydrogen. e.g. β-carotene. Xanthophylls are oxygenated carotenoids that are derivatives of the hydrocarbon carotenoids. Examples include zeaxanthin, astaxanthin and antheraxanthin.

1.8.2. Iron uptake by siderophores in bacteria

The siderophores acquire the iron from the chemical and organic complexes by equilibrium displacement and thus form the iron siderophore complex. This iron siderophore complex is recognised by the binding proteins in gram positive bacteria and outer membrane receptors in case of gram negative bacteria. Interestingly the uptake and transport of the iron siderophore complex varies in these two bacteria as shown in Fig. 1.10. Although the Gram negative bacterial outer cell contains specific and non-specific binding proteins called porins, it is not sufficient to facilitate the entry of the siderophore. Hence a third type of protein called the multifunctional receptor is present that allows the passage of large molecules like the siderophores and vitamin B12. This is an energy driven process that transports the siderophore across the outer membrane to bind to the proteins in the periplasmic space.

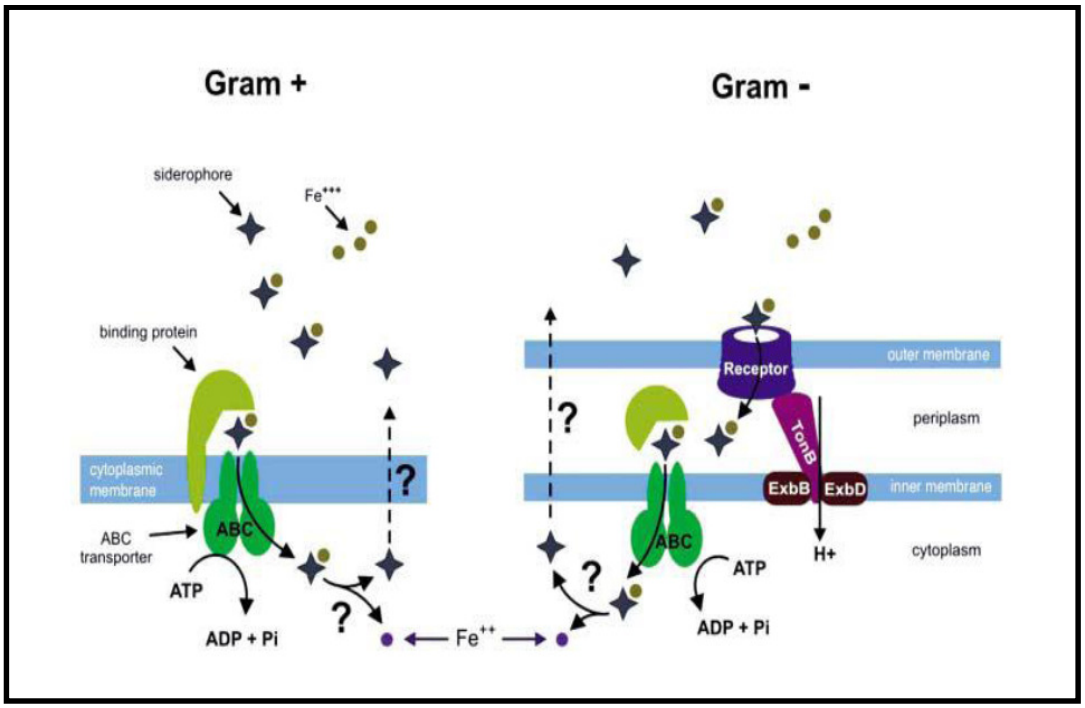


Fig. 1.10: Schematic representation of iron acquisition by siderophores in Gram positive and Gram negative bacteria (Wandersman and Delepelaire 2004).

A transmembrane protein complex called the TonB complex in the cytoplasmic membrane is in contact with the outer membrane receptors via a long periplasmic polypeptide. The transfer of the siderophore complex from the receptor to TonB occurs due to a conformational change in the inner cytoplasmic membrane as a result of an electrochemical potential. In both, Gram negative and Gram positive bacteria the transport of the siderophore complex occurs by means of the iron-ATP binding cassette (ABC) permeases driven by ATP (Adenosine tri-phosphate) hydrolysis (Wandersman and Delepelaire 2004).

1.8.3 Applications of siderophores

Microorganisms colonise mineral surfaces, and can thus chelate mineral nutrients directly from the soil minerals and can share with other soil microorganisms. Studies have shown that siderophores can play a significant role in mineral-weathering processes by accelerating the dissolution of some iron (Fe) containing minerals such as Ferrihydrate and Goethite (Hiradate and Inoue, 1998).

The recycling of iron in the ocean environment has been a subject of concern, as it is an essential micronutrient for the marine microorganisms. It also controls the productivity and community structure of phytoplanktons. Marine bacteria produce most of the siderophores for chelating Fe and hence play a significant role in the biogeochemical recycling of Fe in the marine environment. Marine siderophores include a hydroxyl–carboxylate functional group, provided either by citrate

(snychobactins, petrobactin, aerobactin and marinobactins) or by β- hydroxyaspartate (aquachelins, loihichelins and alterobactin) (Martinez and Butler 2007, Hider and Kong 2010). It is hypothesised that microbial siderophores

provide the plant with Fe required for its growth. They either do so by donating Fe from the of high redox potential microbial siderophores to the Fe transport of the plant or by transferring the chelated iron to phytosiderophores that are then taken up by the plant. Siderophores have been suggested to be an environmentally friendly alternative to hazardous pesticides (Schenk *et al*. 2012). It has been known that *Pseudomonas* species can improve plant growth by producing siderophores (pyoverdine) and/or by protecting them from phytopathogens, and thus this group of bacteria was classified as plant growth-promoting bacteria (Ahmed and Holmstrom 2014).

Siderophores control diseases in fish by fish pathogens by limiting the amount of Fe available to the pathogen for virulence and bacterial interactions. The biocontrol mechanism involves a competition between the transferring of the pathogen and the siderophore by the biocontrol agents that compete for the iron. Since the stability of the siderophore–iron complex is more than the transferring-siderophore complex the former is preferred. Studies have shown siderophore-producing bacteria *Pseudomonas fluorescens* can inhibit the growth of several fish pathogens like *Vibrio anguillarum, Vibrio ordalii, Aeromonas salmonicida, Lactococcus garvieae, Streptococcus iniae,* and *Flavobacterium psychrophilum* and is therefore used as probiotics in fish farming (Gram *et al*. 2001, Brunt *et al*. 2007, Dimitroglou *et al*. 2011).

The use of siderophores in characterisation of microbial strains is an important tool used in microbial taxonomy and is known as siderotyping. *Pseudomonas*, produce over 50 different pyoverdine siderophores in addition to a wide variety of other siderophore types. The peptide chain of the fluorescent pyoverdine varies among the different species and this variability can be easily used to determine the relatedness of these species (Chincholkar *et al*. 2005).

Siderophores are extremely effective in solubilising and increasing the mobility of a wide range of metals such as cadmium (Cd),copper (Cu), nickel (Ni), lead (Pb), zinc (Zn), and the actinides Th (IV), U(IV) and Pu(IV). Hence the siderophores are ecofriendly and effective in heavy metal bioremediation. *Azotobacter vinelandii*, which produced siderophores azotochelin and azotobactin, had the ability to use those siderophores for both Molybdenum (Mo) and Vanadium (V) acquisition.

Studies show that microbial siderophores participate in hydrocarbon degradation indirectly by facilitating the acquisition of iron for the degrading microorganisms in iron limiting conditions. Petrobactin was the first structurally characterized siderophore produced by the oil-degrading marine bacterium *Marinobacter hydrocarbonoclasticus*.

Siderophores are being used as optical biosensors in which a siderophore molecule such as pyoverdine is coupled with an electrical transmitter and the binding of the

pyoverdine with Fe (III) is monitored. This is used to determine the concentration of oceanic iron.

Pulp and paper bleaching process contribute a lot of toxic effluents into the air and waste water. Siderophores are an ecofriendly means of the treating pulp. The hydroxamate siderophores have a means to reduce the Fe by redox reactions. The reduced Fe can then react with hydrogen peroxide to generate oxygen radical species that depolymerises cellulose, hemicelluloses and lignocelluloses. This depolymerisation reaction plays the main role in bioleaching of the pulp.

Siderophores are also pigmented and one of the typical example is pyoverdine and pyocyanin produced by Pseudomonas spp.

1.9 Nanoparticles

Nanoparticles are particles with dimension between 1-100 nm**.** Nanoparticles behave in a manner very different from those of the bulk metals. Nanosize of the particle can result in a change in its colour, melting temperature, crystal structure, chemical reactivity, electrical conductivity, magnetism and mechanical strength. Due to these interesting characteristics of nanoparticles they exhibit distinct properties that influence the biological, physical and chemical interactions.

Nanoparticle research is currently an area of intense scientific research, due to a wide variety of potential applications in biomedical, optical, and electronic fields. In the

past two decades the nanoparticles have been gaining a lot of interest in the fields of engineering, medicine and environmental remediation. A wide variety of nanoparticles are being studied such as the silver nanoparticles, iron nanoparticles, titanium dioxide, aluminium and zinc oxide etc. Among these the zerovalent iron nanoparticles (ZVI) is the only nanoparticles which is currently on field application for bioremediation of environmental pollution (Lo *et al*. 2006).

1.9.1 Applications of ZVI nanoparticles

ZVI are used for groundwater remediation in case of water contaminated with TCE (trichloroethylene) and PCE (perchloroethylene) (Zhang and Elliot 2006, Wang and Zhang 1997, Senzaki and Kumagai 1988).

The ZVI nanoparticles use the same chemistry as the bulk zerovalent iron, but take advantage of the increased surface area and the rheological ability of the nanoparticles to seep in the subsurface area and permeate crevices where the contaminant is present. It uses the technology called the iron wall barrier in which the ZVI nanoparticles form a PRB (permeable reactive barrier) that filters contaminants as shown in Fig. 1.11. This PRB is highly applicable for cleaning groundwater, leaving dechlorinated ground water on the other side of the PRB.

In case of water containing mixed contaminants e.g. chlorinated organics and higher valency toxic metals, ZVI can accomplish remediation of both these contaminants

effectively. Zerovalent iron nanoparticles have been effectively used for the decontamination of ground water in the subsurface area contaminated with dense non-aqueous phase liquid (DNAPL). DNAPL refers to the contaminants in the ground water, surface water and sediments that tend to sink below the water table and stops when they reach the impermeable bedrock. Materials such as chlorinated solvents, coal tar, polychlorinated biphenyls (PCB), mercury and crude oil are DNAPLs. The remediation process may utilise ZVI nanoparticles immobilised onto a column structure or release the nanoparticles directly into the contaminated source as demonstrated in Fig 1.12 (a) and (b).

The ZVI demonstrated its ability to encapsulate heavy metal contaminant such as chromium by a combination of adsorption and reduction processes (Blowes *et al.* 1997, Blowes *et al.* 1999).

It was seen that the ZVI could reduce chromium +6 a carcinogenic form of metal, to chromium +3 a non carcinogenic state (Nuxoll *et al*. 2003, Arnold and Roberts 2000) This ability of the ZVI to act as reducing agents has significant potential in the remediation of radioactive metals like Uranium and Plutonium (Bronstein 2005).

It has been reported that, ZVI oxidation to ferrous/ ferric iron increases the pH, evolves hydrogen. Oxidizable materials are consumed resulting in reducing conditions

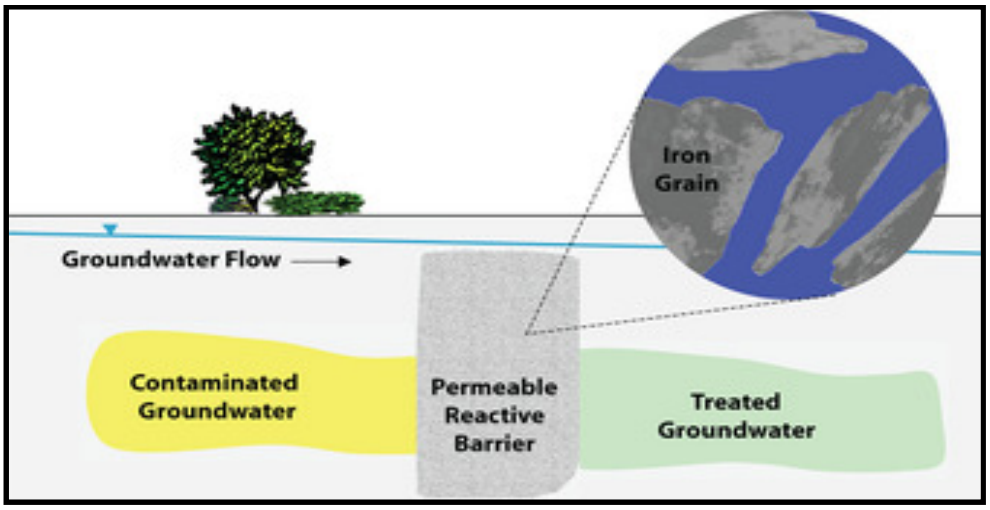


Fig. 1.11: Decontamination of ground water by formation of PRB by the zerovalent iron nanoparticles.

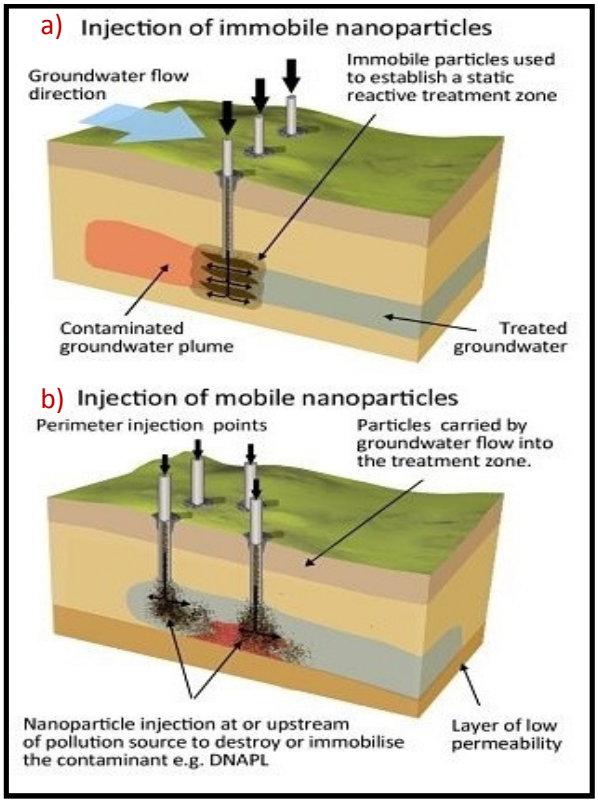


Fig. 1.12: Zerovalent iron nanoparticles technology used for treating subsurface pollutants like DNAPL in ground water (Crane 2011).

that are favourable for the pathways such as oxide-mediated electron transfer from the metal to the chlorinated organic compound.

This brings about a reduction of the chlorinated organic compound by the ferrous iron by evolution of hydrogen and results in complete dechlorination of the chlorinated compound. Eventually, ferric or ferrous iron may precipitate as a solid or remain in solution, depending on the pH and redox conditions. Additionally, the generation of strong reducing conditions and hydrogen gas fosters anaerobic microbial growth and increases the natural biological degradation in the field (Henn and Waddill 2006).

Chapter 2

Isolation, characterization and identification of halophilic and halotolerant adhered bacteria from mangrove ecosystem

2.1 Introduction

Adhesion of bacterial cell has now been recognized as an important mechanism with potential for nutrient degradation and biofilm formation (Kokare *et al*. 2009, An and Friedman 2000). Hence it is important to study adhered bacteria and their surface structures.

In recent years, studies have been directed to isolate and study adhered/ attached bacteria with medical importance such as S*taphylococcus aureus*, *Staphylococcus epidermides* (Katsikogianni and Missirlis 2004) and *Streptococcus sangui* (Ofek *et al*. 2003). These have been extensively studied for their surface proteins and attachment mechanisms. It has also been reported that attached bacteria contribute to most of the degradation in the estuarine and other turbid ecosystems (Gonsalves *et al*. 2009, Crump *et al*. 1998). Bacteria such as *Pseudoalteromonas, Sagittula stellata, Caulobacter* etc. have been studied from the marine and freshwater ecosystems for their ability to adhere to substrates by means of various surface appendages like holdfast and obtain nutrition (Gonzalez *et al*. 1997b, Tsang *et al*. 2006, Merker and Smit 1988, Ong *et al*. 1990). *Halomonas* and *Salipiger mucescens* are halophilic bacteria that have been reported to produce EPS that aids their binding to the substrate (Martinez- Canovas *et al.* 2004 a,b,c, Martinez-Checa *et al.* 2005). Studies on pathogenic *E. coli* have shown the importance of its pili, fimbriae and flagella in attachment to the uroepithelial lining and its colonization (Kaper *et al*. 2004, Rittmann and Mc Carty 2001). However, no specific studies have been carried out to study the

halophilic and halotolerant bacteria adhered to plant litter from the mangrove ecosystem.

In our quest to isolate adhered bacteria, samples of plant litter were collected from Mandovi and Zuari mangroves. Adhesion of adhered bacteria to its substrate has been proved to be aided by Magnesium ions (Chart *et al*. 1997, Takeichi and Okada 1972). Thus, ethylene diamine tetra acetic acid (EDTA), a chemical chelator of the ion, is used to destabilize the attachment of the bacteria to the substrate and dislodging the bacterial cell from it. Further studies were carried out on the ability of these bacterial isolates to grow at different sodium chloride (NaCl) concentrations. Considering that the mangrove plant litter is a rich source of natural polymers the potential of the isolates to produce enzymes to degrade these polymers was studied. Three potential isolates were selected and identified up to the species level with the help of cultural, morphological, biochemical and molecular characteristics.

2.2 Materials and methods

2.2.1 Collection of plant litter sample

a) Sampling site

Mangrove plant litter samples were collected from the mangroves of the two main rivers of Goa. The sampling sites included Manxer mangroves of the Mandovi river mangroves and the Shirdona mangroves represented the Zuari mangroves as shown in Fig. 2.1 and Fig. 2.2.

b) Mangrove plant litter sampling

The plant litter samples were collected during low tide. The mangrove plant litter was picked up by using a sterile tweezers and transferred to a sterile container with lid. The samples were immediately processed upon reaching the lab.

2.2.2 Isolation of adhered halophilic and halotolerant bacteria

10 g of mangrove plant litter sample was added to 100 ml of normal saline and kept on shaker (Remi CIS 24 BL) at 150 rpm, overnight at room temperature (28°C). It was centrifuged (Eppendorf 5804R) at 1500 rpm for 10 min at 4°C and the plant litter pellet was rinsed thrice with sterile normal saline. The pellet was ground with 20 ml of 0.1M EDTA solution (Chart *et al*. 1997) using mortar and pestle and centrifuged (Eppendorf 5804R) at 1500 rpm for 10 min at 4°C. Dilutions of the supernatant were prepared using sterile normal saline and 0.1 ml of the dilutions was spread plated on Zobell marine agar (ZMA) and 15% and 25% NaCl-tryptone-yeast extract (NTYE) agar (Appendix I) to obtain isolated colonies of adhered bacteria.

2.2.3 Subculturing, maintenance and cultivation of isolates

The isolates were maintained by subculturing on ZMA and NTYE agar slants depending on which media they had been isolated. These slants were sealed with paraffin and stored in fridge at 4°C for one to two months and at the end of which these

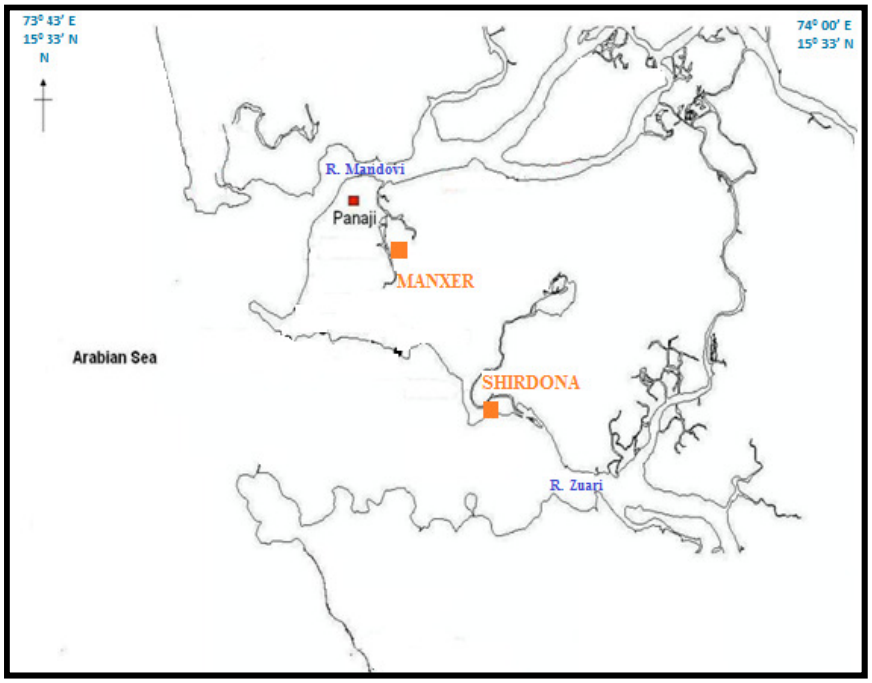


Fig. 2.1: Map illustrating the two sampling sites, Manxer mangroves and Shirdona mangroves (symbolized by solid squares) along the Mandovi and Zuari River in Goa –India respectively.

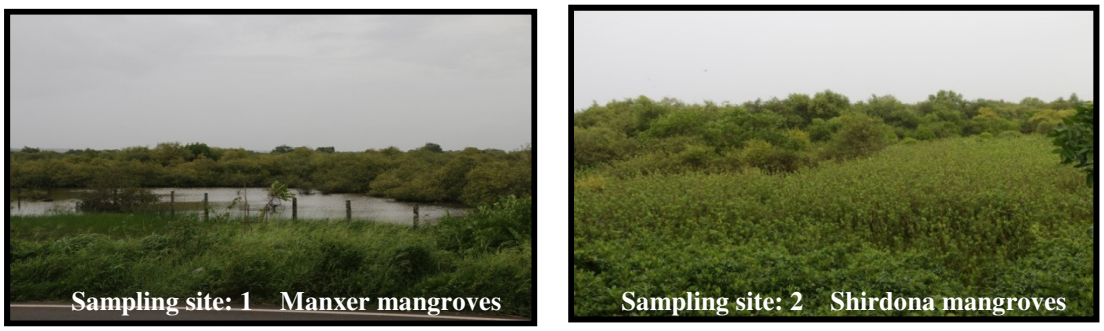


Fig. 2.2: Mangrove plant litter sampling sites along the Mandovi and Zuari river areas.

were subcultured again. Cultivation of the isolates was also done in ZMB/ ZMA and NTYE agar or broth.

2.2.4 Ability of the bacterial isolates to grow at different NaCl concentrations

The ability of the bacterial isolates to grow at different NaCl concentrations was determined by growing the isolates on media with varying NaCl concentration. 24 hr old culture of the bacterial isolates was streaked on ZMA, Nutrient agar (NA), 5% NTYE, 10% NTYE agar, 15% NTYE agar, 20% NTYE agar 25% NTYE agar (Table 2.1). The plates were incubated at room temperature (28°C) and the growth was monitored over a period of 1 week. The isolates were classified into halophilic and halotolerant bacteria with reference to the classification mentioned by Horikoshi *et al.* 2010 (Appendix IV).

2.2.5 Screening of the isolates for multiple enzyme production.

The 34 bacterial isolates were screened for the production of enzymes cellulase, amylase, lignin degrading enzymes, tannase, chitinase, protease and lipase. 24 hr culture of the bacterial isolates was used to screen for the enzymes.

a) Cellulase production

The bacterial isolates were spot inoculated on carboxy methyl cellulose (CMC) agar (Pangallo *et al*. 2007) (Appendix I) and incubated at room temperature (28°C) for 24-

48 hr. The plates were observed for zones of colour change around the bacterial colonies.

b) Amylase production

The bacterial isolates were spot inoculated on starch agar (Appendix I) and incubated at room temperature (28°C) for 24- 48 hr. The plates were flooded with Gram's iodine solution for 1 min. The excess solution was discarded and the plates were observed for zones of clearance around the bacterial colonies.

c) Lignin degrading enzyme production

24 hr culture of the bacterial isolates was spot inoculated on azure-B and toluidine blue agar (Gao *et al*. 2011) (Appendix I) and incubated at room temperature (28°C) for 24-48 hr. The plates were observed for the zones of decolourisation around the bacterial colonies and colouration of the bacterial colonies.

d) Tannase production

The bacterial isolates were spot inoculated on tannic acid agar (Illori *et al*. 2007) (Appendix I) and incubated at room temperature (28°C) for 72-94 hr. The plates were observed for zones of black colouration around the bacterial colonies.

e) Chitinase production

The bacterial isolates were spot inoculated on chitin agar (Atlas 2004) (Appendix I) and incubated at room temperature (28°C) for 1 week. The plates were observed for zones of clearance around the bacterial colonies.

f) Lipase production

The bacterial isolates were spot inoculated on tributyrin agar (Kim *et al*. 2001) (Appendix I) and incubated at room temperature (28°C) for 24-48 hr. The plates were observed for zones of clearance around the bacterial colonies.

g) Protease production

The bacterial isolates were spot inoculated on skim milk agar (Appendix I) incubated at room temperature (28°C) for 24-48 hr. The plates were observed for zones of clearance around the bacterial colonies.

2.2.6 Screening of the selected bacterial isolates for production of siderophores

Out of the 34 bacterial isolates, 16 isolates were selected based on their ability to produce multiple enzymes. The 16 isolates were screened for their ability to produce siderophores by spot inoculating on Chrome Azurol sulphonate (CAS) agar (Appendix I) as described by Schwyn and Neilands 1987. The CAS agar was prepared using nutrient agar (NA) and NTYE agar. The plates were incubated at room temperature for 24-48 hr and observed for zones of yellow colouration around the bacterial colonies.

2.2.7 Screening of the isolates for their adherence ability

The ability of the 16 selected bacterial isolates to adhere to hydrocarbons was seen using the BATH assay (Gaonkar *et al.* 2012). The isolates were grown on ZMB and NTYE broth overnight at room temperature (28°C) in shaker conditions (Remi CIS 24 BL) of 150 rpm. It was centrifuged (Eppendorf 5804 R) at 6000 rpm for 10 min to obtain pellet and supernatant. The supernatant was discarded and the pellet of each isolate was suspended in 5ml of sterile phosphate buffered saline (PBS) (pH 7.0) and the initial absorbance (A_0) was measured at 450 nm (Shimadzu UV-2450). To 2 ml of this, 0.5 ml of hexadecane was added. The tubes were vortexed (Remi CM 101) for 2 min and allowed to stand undisturbed for 30 min. The final absorbance (A_1) was measured of the aqueous layer at the same wavelength and noted for showing either adherence or emulsification (Appendix IV).

Adhesion percentage (A%) was calculated using the formula

$$A\ \% = \frac{A_0 - A_1}{A_0} \times 100$$

Emulsification percentage (E %) was calculated using the formula

$$E\% = \frac{A_1 - A_0}{A_0} \times 100$$

2.2.8 Screening of the selected bacterial isolates for the production of EPS.

Ten bacterial isolates were selected based on their ability to produce siderophores and multiple enzyme production. These isolates were screened for their ability to produce EPS by the microscopic and plate method.

a) Alcian blue staining (Microscopic method).

The bacterial isolates were grown on the respective media for 24 hr at room temperature (28°C). Smears of these isolates were prepared on clean slides and stained with Alcian blue staining protocol (Appendix III). The slides were observed under the microscope. The EPS is seen as a blue sheath while the bacteria appear red in colour.

b) Congo red-coomassie blue agar (Plate method)

The bacterial isolates were spot inoculated on congo red-coomassie blue agar (Narancic *et al.* 2012) (Appendix I). The plates were incubated for 24-48 hr at room temperature (28°C). The bacterial colonies were observed for colouration of bacterial colonies and/or smooth glossy appearance indicating the presence of EPS.

2.2.9 Characterization of halophilic and halotolerant adhered bacteria.

a) Cultural, morphological and biochemical characterization

The cultural and the morphological characteristics of the 10 selected bacterial isolates showing multiple enzyme activities and siderophore production were determined by studying the colony characteristics and Gram's staining (Appendix III) of the isolates,

followed by microscopic examination. The motility of the bacterial isolates was determined by the hanging drop test and the motility slant agar. The biochemical tests were carried out by inoculating in various biochemical media (Appendix I). The isolates were tentatively identified using Bergey's Manual of Systematic Bacteriology (De Vos *et al*. 1986).

b) Molecular characterization

i) Extraction and isolation of genomic DNA

The genomic DNA was extracted from the 3 selected bacterial isolates by the cetyl trimethyl ammonium bromide (CTAB)-proteinase K method (Ausubel *et al*. 1995). 1.5 ml of culture broth was taken in eppendorf tubes and centrifuged at 5000-6000 rpm at 4°C for 5 min. (Eppendorf 5417 R). The pellet was resuspended in 500µL of 1x Tris EDTA buffer and mixed well by vortexing (Remi CM 101) for 1 min and kept in dry bath at 98°C for 15 min. The mixture was transferred immediately to ice for 15 min. It was centrifuged at 4000-5000 rpm at 4°C for 5 min and the clear supernatant containing the crude DNA was obtained. The purity and concentration of DNA was checked by Nanodrop Spectrophotometer (ND-1000 by JH Bio-innovations) and agarose gel electrophoresis.

ii) 16S rRNA gene amplification

The extracted DNA was subjected to amplification by polymerase chain reaction (PCR) (DNA Engine Dyad by Bio-Rad). 16S rRNA gene was amplified using

eubacterial universal primer U1 5'-CCAGCAGCCGCGGTAATACG-3' and U2 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3'. The PCR products were purified by using the High Pure PCR purification kit (Roche version 16).

iii) Agarose gel electrophoresis

The amplification was confirmed by separating the PCR products through 1% agarose gel. 0.5 g of agarose powder was dissolved in 50 ml of 1x TAE buffer by boiling in a microwave for 2 min until a clear solution was obtained. The solution was cooled to 40-45°C and 5µL of ethedium bromide was added to it before it solidified. The mixture was poured into a gel casting tray with combs inserted into it. Once the gel solidified the combs were removed gently to obtain the wells and the tray was placed in the electrophoresis chamber. TAE buffer was poured in the electrophoresis chamber over the gel. 100µL of the purified PCR product was added to 2µL of bromophenol blue (tracking dye) and loaded into the wells. The electrodes were connected and the electrophoresis was carried out for 20-30 min at a voltage of 125 V to allow separation of the PCR products. Once the tracking dye reached two thirds of the gel the current was switched off. The bands obtained were visualized under UV trans-illuminator and recorded using a gel documentation system (Gel Doc XR+ by BioRad).

iv) 16S rRNA sequencing, BLAST and Phylogenetic tree of the selected bacterial isolates

The 16S rRNA gene was sequenced by Sanger automated DNA sequencing procedure using the PCR primers and the sequences of the 3 bacterial isolates were analyzed using the BLAST search program and the sequence was submitted to GenBank to obtain accession numbers for the bacterial isolates. The sequences were compared with sequences in the GenBank and the Phylogenetic tree was constructed by the neighbor joining method using the CLUSTAL-X 2.0 and Mega 4.0 software.

2.3 Results and discussion

2.3.1 Total viable count of adhered bacteria

The Manxer and Shirdona mangroves are a part of the Mandovi and Zuari river estuary. Gonsalves *et al*. (2009) and De Souza *et al*. (2003) have reported the importance of particle attached bacteria in these estuarine ecosystems. Earlier studies have also reported the physicochemical parameters like the temperature, pH and the salinity of Mandovi and the Zuari estuarine areas (D'Costa *et al*. 2004). The temperature varied seasonally and ranged from 27°C-30°C in the monsoons and 30°C-32°C in the summer season. The pH ranged from 6.6-8.1. Earlier studies have shown that regulation of the pH in this range is important for the biological processes and biological interactions occurring between the cell and the surrounding media in this ecosystem (Sylvia *et al*. 2005). The plant litter samples from the two sampling sites were collected and processed for the determination of total viable count (TVC) of

adhered bacteria. A total of 34 adhered bacterial isolates were obtained. A high percentage of 73% were isolated on ZMA agar followed by 21% on 15% NTYE and 6% on 25% NTYE agar as shown in Fig. 2.3. From the results obtained it was seen that the Manxer mangroves showed the highest viable count of 62 % on ZMA followed by 25% on 15% NTYE agar and 12% on 25% NTYE agar. The Shirdona litter sample showed a viable count of 83% on ZMA and 17% on 15% NTYE. However, no colonies were obtained from the Shirdona plant litter sample on the 25% NTYE agar. It was interesting to note that, all the adhered bacteria that were isolated on 25% NTYE agar were from the Manxer mangrove area (Fig. 2.4). The ability to grow at such high salt concentration can be a reflection of the presence of halophiles in that area owing to the saltpans in the vicinity. Presence of bacteria such as *Bacillus*, *Azospirillum*, *Azotobacter* that can tolerate such high salt concentrations have been reported from the mangrove ecosystem (Van-Thuoc *et al.* 2014, Arora *et al*. 2014, Ravikumar *et al*. 2002, Ravikumar 1995). The high number of TVC on ZMA media can be attributed to the presence of a high number of heterotrophic bacteria present in the mangrove ecosystem, owing to the presence of particulate leaf litter that contain different complex plant polymers that serve as substrates for these bacteria. Similar observation on the percentage of Gram character indicated that 76% were Gram negative adhered bacteria of which 65% were pigmented while the other 24% were Gram positive adhered bacteria of which 37.5% were pigmented as shown in Fig. 2.5. Predominance of Gram negative bacteria such as *Vibrio* sp*, Aeromonas* sp in the mangroves have been reported (Rawte *et al*. 2002).

2.3.2 Classification of bacterial isolates based on their ability to grow at different salt concentrations

Classification of bacteria based on their ability to grow in presence of different salt concentrations of NaCl is well studied and reported (Horikoshi *et al*. 2011, Oren 2006). The preliminary classification of halophilic and halotolerant has now been revised and presented into six categories based on the concentration of NaCl ranging from1%- 32% (Horikoshi *et al*. 2011) (Appendix IV). The isolates obtained during the present study were also classified into categories based on their ability to grow on different concentrations of NaCl in the solid medium as shown in Table 2.1. It was interesting to note that out of 34 isolates 68% were halotolerant bacteria and 32% were halophiles (Fig. 2.6). Although reports on free living bacteria in the mangrove ecosystem have shown the presence of halotolerant bacteria such as *Micrococcus, Microbacterium* (Yateem and Sharrah 2011)*, Halococcus* (Sahoo and Dhal 2009), *Microbulbifer* (Britto *et al*. 2006), *Chromobacterium violaceum*, *Flexibacter*, *Flavobacterium*, *Cytophaga*, *Achromobacter*, and *Pseudomonas* sp in the mangrove swamps (D'Costa *et al*. 2004), comparatively few reports are available on halophilic and halotolerant bacteria adhered to mangrove plant litter.

2.3.3 Screening of the isolates for multiple enzyme production

Production of extracellular enzymes by bacteria is considered to be one of the most crucial steps in the degradation process and the utilization of organic polymers from

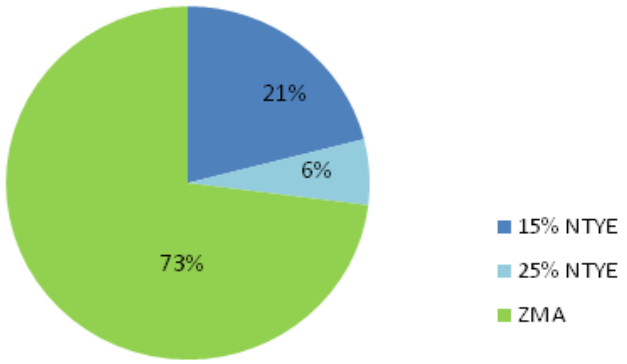
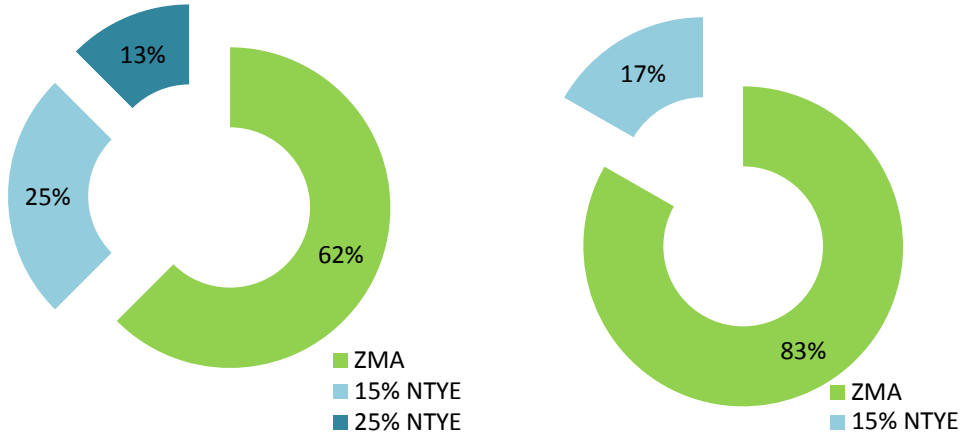


Fig. 2.3: Percentage representation of TVC of adhered bacteria on NTYE and ZMA.



Sampling site1: Manxer mangroves

Sampling site 2: Shirdona mangroves

Fig. 2.4: Total viable count obtained on isolation media (NTYE and ZMA) from the two sampling sites.

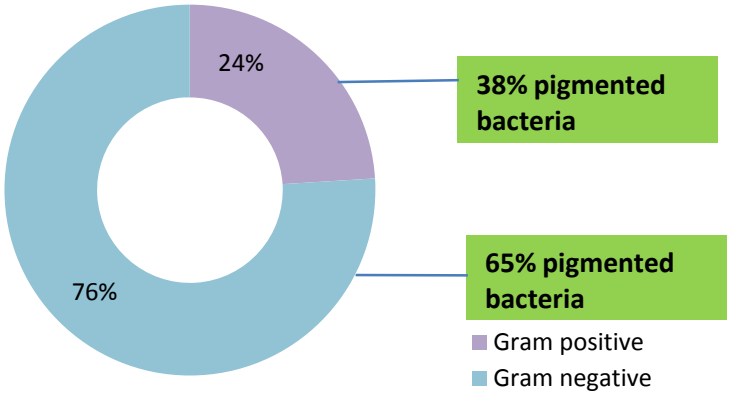


Fig. 2.5: Percentage distribution of pigmented bacteria among the Gram positive and Gram negative isolates.

the environment, since only compounds with a molecular mass of less than 600 Da can pass through the bacterial cell pores (Fabiano and Danovara, 1998). The plant litter in the mangroves is a source of complex plant polymers that require to be hydrolyzed, for them to be utilized by the bacteria. It was therefore pertinent to determine the enzymatic potential of the adhered bacterial isolates from the mangrove ecosystem.

Among the 34 isolates 50 % of the adhered bacteria showed cellulase production (Fig. 2.7), 62% of the isolates showed the presence of enzyme amylase (Fig. 2.8), 41 % showed lignin peroxidase production on Azure B agar (Fig. 2.9) and similar observations were made on toluidine blue agar, 12% showed tannase enzyme production (Fig. 2.10), 76% isolates showed lipase production (Fig. 2.11) and 41% showed the presence of the enzyme protease (Fig. 2.12 shows 3 such isolates). However, no chitinase production was shown by any of the bacterial isolates. Interestingly, all the cellulase and tannase producers were halotolerant adhered bacteria and it was also observed that 48% of the halotolerant bacteria produced multiple enzymes (> 2 enzymes) while 18% of the halophilic bacteria showed multiple enzyme production (Table 2.2). This indicated that the halotolerant bacteria played a crucial role in degradation of the particulate organic matter and reflected the role of halophilic and halotolerant bacteria in the nutrient recycling and maintaining the biogeochemical cycles in the mangrove ecosystem.

Table 2.1: Classification of bacterial isolates as halophilic and halotolerant adhered bacteria.

Isolate	NA	ZMA	5% NTYE	10% NTYE	15% NTYE	20% NTYE	25% NTYE	Result
MXM-1	-	-	+	+	+	+	+	Halophile
MXM-2	-	-	+	+	+	+	+	Halophile
MXM-3	-	-	+	+	+	+	+	Halophile
MXM-4	-	-	+	+	+	+	+	Halophile
MXM-5	+	+	+	+	+	+	+*	Halophile
MXM-6	-	+	+	+	-	-	-	Halophile
MXM-7	+	+	+	+	-	-	-	Halotolerant
MXM-8	+	+	+*	-	-	-	-	Halotolerant
MXM-9	+	+	-	-	-	-	-	Halotolerant
MXM-10	+	+	+	-	-	-	-	Halotolerant
MXM-11	+	+	+	-	-	-	-	Halotolerant
MXM-12	+	+	+	+	+	+	-	Halotolerant
MXM-13	+	+	+	+	-	-	-	Halotolerant
MXM-14	+	+	+	-	-	-	-	Halotolerant
MXM-15	-	-	-	+	+	+	+	Halophile
MXM-16	+	+	+	+	+	+	+*	Halophile
SDM-1	+	+	-	-	-	-	-	Halotolerant
SDM-2	+	+	-	-	-	-	-	Halotolerant
SDM-3	+	+	+	+	+	+	-	Halotolerant
SDM-4	+	+	+	+	-	-	-	Halotolerant
SDM-5	+	+	-	-	-	-	-	Halotolerant
SDM-6	+	+	-	-	-	-	-	Halotolerant
SDM-7	-	+	+	-	-	-	-	Halotolerant
SDM-8	+	+	-	-	-	-	-	Halotolerant
SDM-9	+	+	-	-	-	-	-	Halotolerant
SDM-10	+	+	+	-	-	-	-	Halotolerant
SDM-11	+	+	-	-	-	-	-	Halotolerant
SDM-12	+	+	+	+	+	+	-	Halotolerant
SDM-13	+	+	+	+	+	+	-	Halotolerant
SDM-14	-	+	+	+	-	-	-	Halotolerant
SDM-15	-	+	+	+	-	-	-	Halotolerant
SDM-16	+*	+	+	+	+	+	-	Halophile
SDM-17	+*	+	+	+	+	+	-	Halophile
SDM-18	+*	+	+	+	+	-	-	Halophile

*The isolates showed slow or less luxuriant growth at that concentration of NaCl.

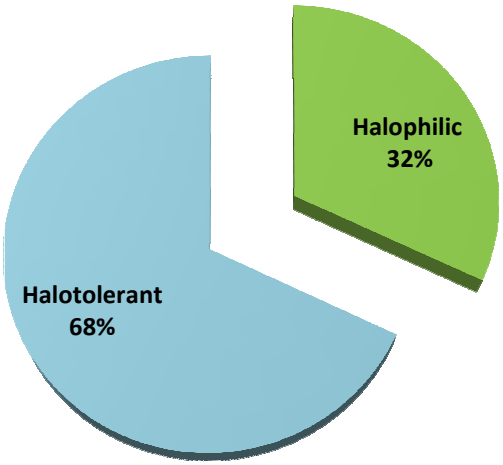


Fig. 2.6: Percentage distribution of halophilic and halotolerant bacteria among the bacterial isolates.

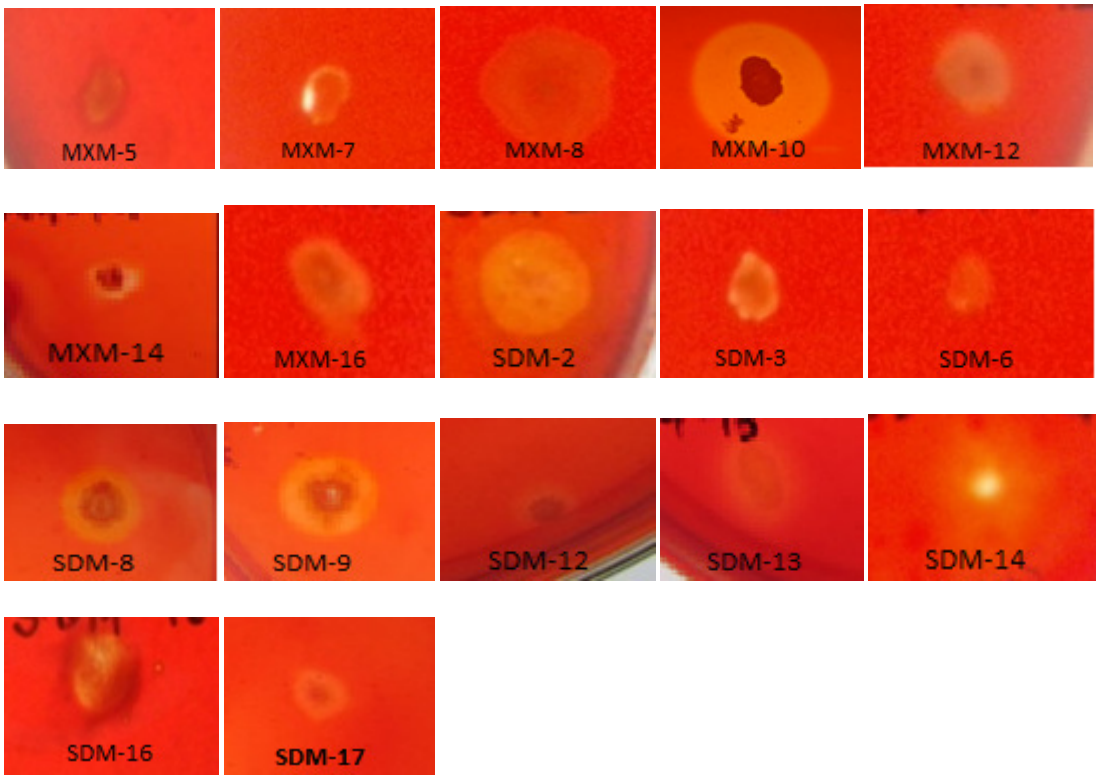


Fig. 2.7: Isolates showing yellow zones around the bacterial colonies on CMC agar.

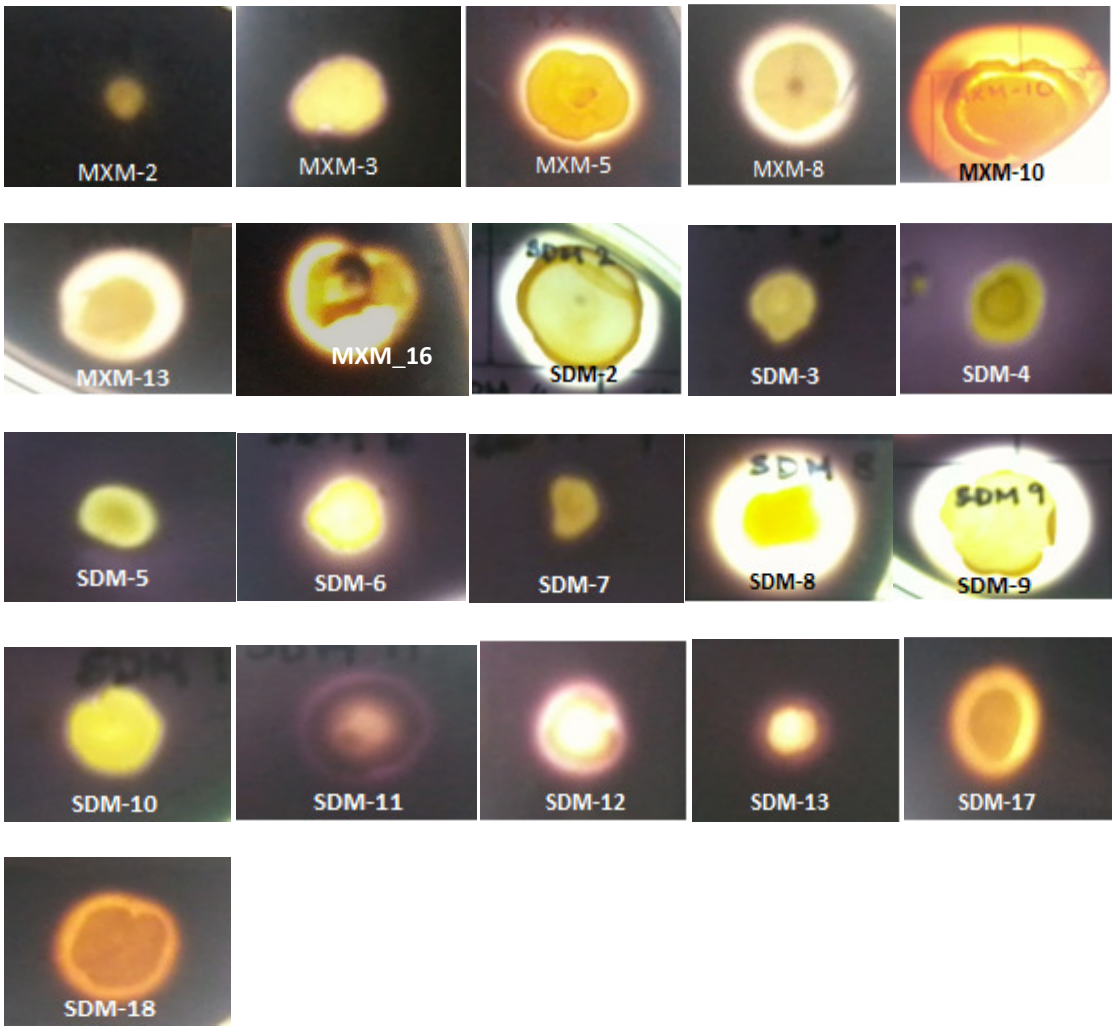


Fig. 2.8: Isolates showing zones of clearance around the bacterial colonies on starch agar.

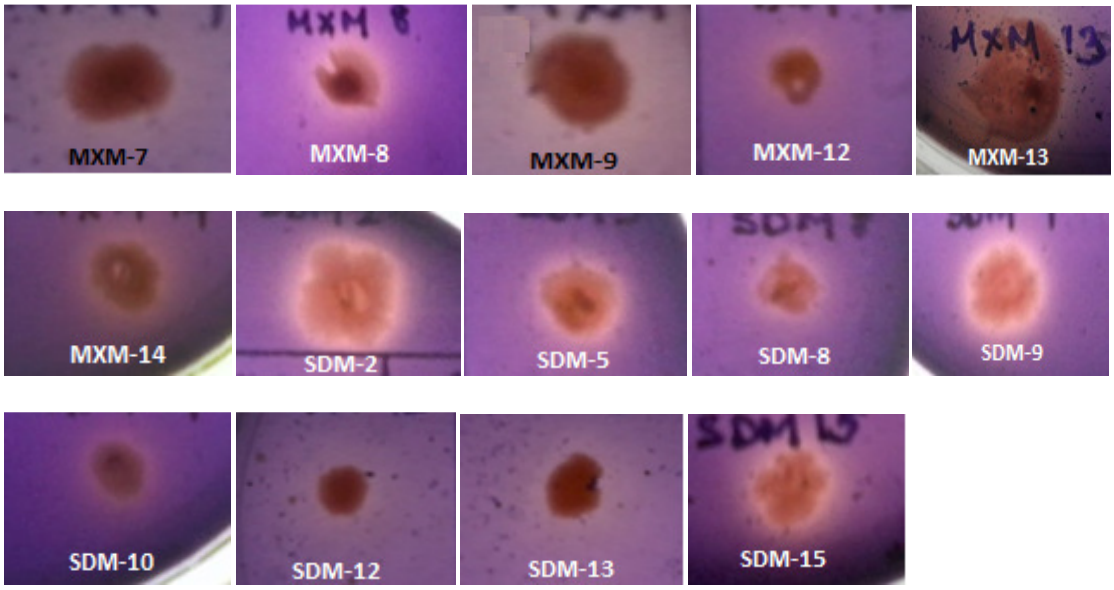


Fig. 2.9: Isolates showing zones of decolourisation around the bacterial colonies and colouration of the bacterial colony on Azure B agar.

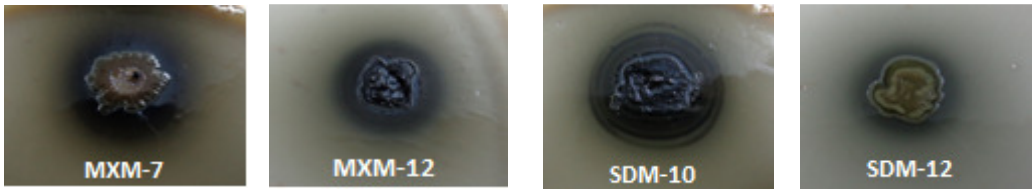


Fig. 2.10: Isolates showing zones of black colouration around the bacterial colonies on tannin agar.

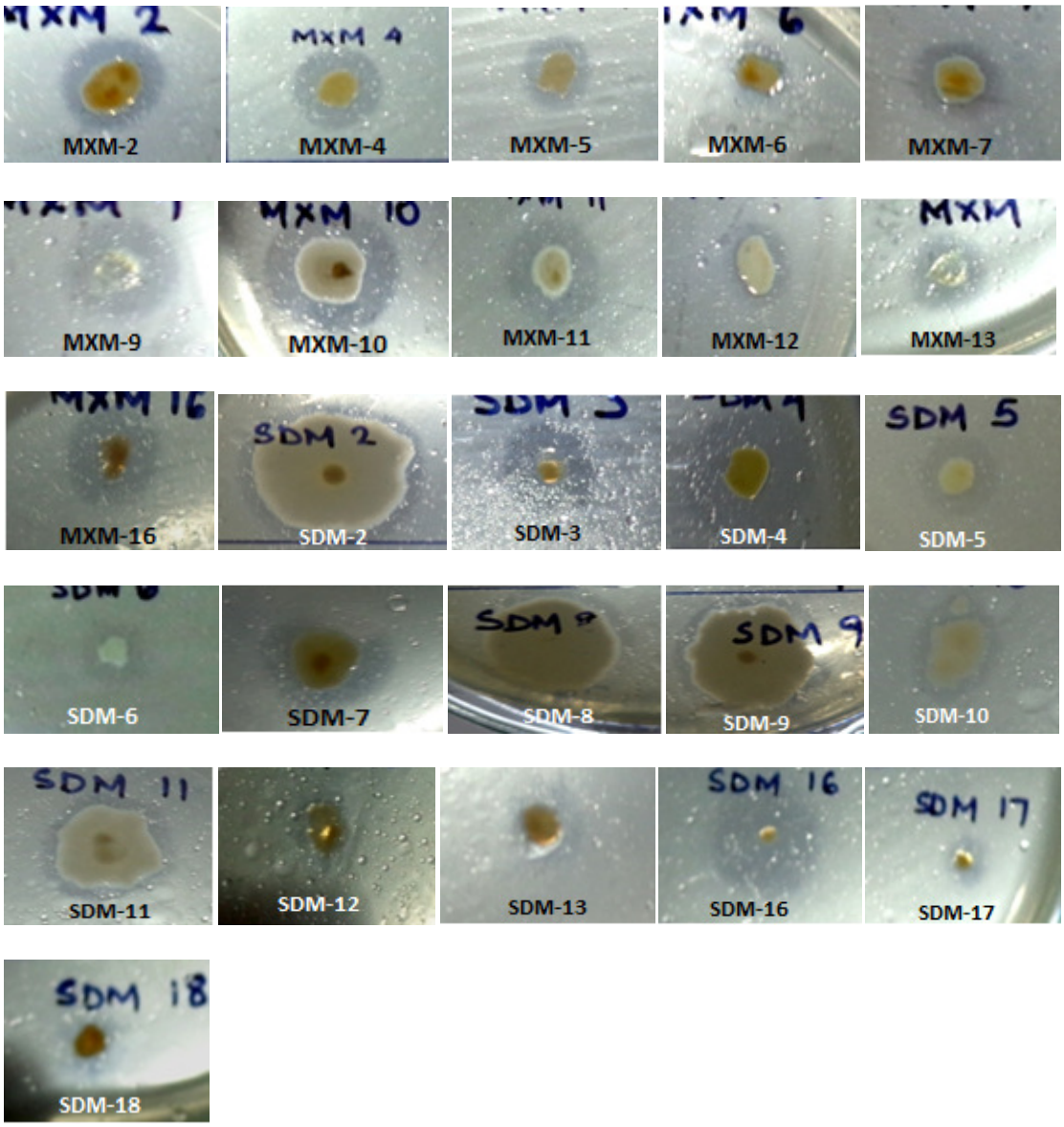


Fig. 2.11: Isolates showing zones of clearance around the bacterial colonies on tributyrin agar.

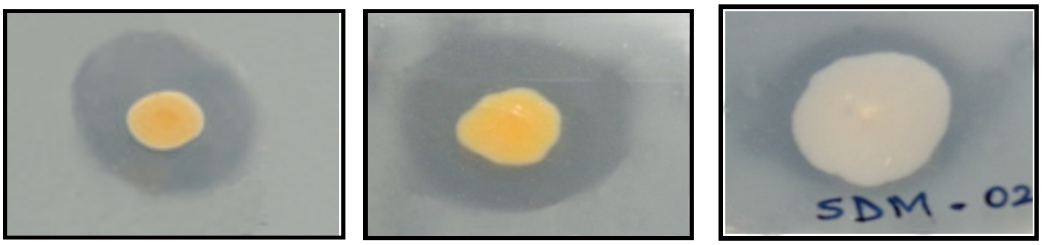


Fig. 2.12: Isolate MXM-5, MXM-16 and SDM-2 showing zones of clearance around the bacterial colonies on skimmed milk agar.

Table 2.2: Enzyme profiles of halophilic and halotolerant adhered bacterial isolates

Isolate	Classification	Cellulase	Amylase	Lignin degrading enzymes	Tannase	Lipase	Protease
MXM-1	Halophile	-	-	-	-	-	+
MXM-2	Halophile	-	+	-	-	+	-
MXM-3	Halophile	-	+	-	-	-	-
MXM-4	Halophile	-	-	-	-	+	+
MXM-5	Halophile	+	+	-	-	+	+
MXM-6	Halophile	-	-	-	-	+	+
MXM-7	Halotolerant	+	-	+	+	+	+
MXM-8	Halotolerant	+	+	+	-	-	+
MXM-9	Halotolerant	-	-	+	-	+	-
MXM-10	Halotolerant	+	+	-	-	+	-
MXM-11	Halotolerant	-	-	-	-	+	+
MXM-12	Halotolerant	+	-	+	+	+	-
MXM-13	Halotolerant	-	+	+	-	+	-
MXM-14	Halotolerant	+	-	+	-	-	-
MXM-15	Halophile	-	-	-	-	-	+
MXM-16	Halophile	+	+	-	-	+	+
SDM-1	Halotolerant	-	-	-	-	-	-
SDM-2	Halotolerant	+	+	+	-	+	+
SDM-3	Halotolerant	+	+	-	-	+	+
SDM-4	Halotolerant	-	+	-	-	+	-
SDM-5	Halotolerant	-	+	+	-	+	-
SDM-6	Halotolerant	+	+	-	-	+	-
SDM-7	Halotolerant	-	+	-	-	+	-
SDM-8	Halotolerant	+	+	+	-	+	+
SDM-9	Halotolerant	+	+	+	-	+	+
SDM-10	Halotolerant	-	+	+	+	+	-
SDM-11	Halotolerant	-	+	-	-	+	+
SDM-12	Halotolerant	+	+	+	+	+	-
SDM-13	Halotolerant	+	+	+	-	+	-
SDM-14	Halotolerant	+	-	-	-	-	+
SDM-15	Halotolerant	-	-	+	-	-	-
SDM-16	Halophile	+	-	-	-	+	-
SDM-17	Halophile	+	+	-	-	+	-
SDM-18	Halophile	-	+	-	-	+	-

Studies on the halophilic bacteria from saline environments of India have shown the presence of *Marinobacter*, *Virgibacillus*, *Halobacillus*, *Geomicrobium*, *Chromohalobacter*, *Oceanobacillus*, *Bacillus*, *Halomonas* and *Staphylococcus* (Kumar *et al*. 2012). Tropical marine and mangrove microflora having diverse enzymatic activities have been reported from the mid-west coast of India (Rawte *et al*. 2002). Halophiles and halotolerant bacteria are said to be excellent sources of enzymes as they are salt stable and can carry out reactions under extreme conditions of salinity, pH and temperature. This makes them potentially important as they can withstand the harsh operational conditions encountered in industrial processes (Kumar *et al.* 2012).

2.3.4 Screening of the selected bacterial isolates for production of siderophores

In iron limiting environments, in order to sequester and solubilize ferric ion, the bacteria produce compounds of less than 1000 Da called siderophores (Neilands 1995). Owing to their high affinity to ferric ion they form stable complexes that are taken up by the bacterial cell and utilized for various metabolic activities. Mangroves being one of the most efficient ecosystems, it was necessary to understand the ability of the bacterial isolates from this ecosystem to acquire iron and metabolize with optimum efficiency that contributes to efficient degradation and mineralization of particulate organic matter in this ecosystem. Figure 2.12 shows the isolates producing siderophores and the negative control using ferric chloride ($FeCl_3$). Among the 16 bacterial isolates that were screened, 81% showed siderophore production of which 62% were adhered halotolerant bacteria and 38% were adhered halophilic bacteria. It

was observed that 19% of the isolates that did not produce siderophores were all halotolerant bacteria while all the halophilic bacteria showed the production of siderophores as shown in Fig. 2.14. The high percentage of siderophore producers depict the iron deficiency for growth in the mangrove ecosystem which triggers the isolates to produce siderophores in order to obtain the element from the surrounding environment. Earlier studies have shown siderophore producing *Pseudomonas* sp (Matthijs *et al.* 2004, Gaonkar *et al*. 2012), *Escherichia coli, Bacillus subtilis* from coastal sand dunes (Gaonkar *et al*. 2012) and Azotobacters from the tropical mangroves (Selvam and Kathiresan, 2010, Kannapiran and Ramkumar 2011). In the coastal and marine ecosystem, the iron concentration is low and in order to metabolise organic particulate matter containing aromatic ring bacteria use the enzymes oxygenases. These enzymes have iron as their cofactor and thus the ability of bacteria to produce siderophores is an important aspect in efficient degradation of particulate organic matter and pollutants such as aromatic anthropogenic compounds and hydrocarbons (Gaonkar *et al*. 2012).

2.3.5 Screening of the isolates for their adherence ability

Adhesion is a way for a bacterial cell to localize, establish and sustain itself in a particular habitat. It has been reported that sessile or adhered bacteria exploit nutritional opportunities better than the non-attached bacteria (Schlegel and Jannasch 2006). Katsikogianni and Missirlis (2004) have reported that bacterial adhesion is influenced by the surface charge and hydrophobicity of the bacterial cell and the solid

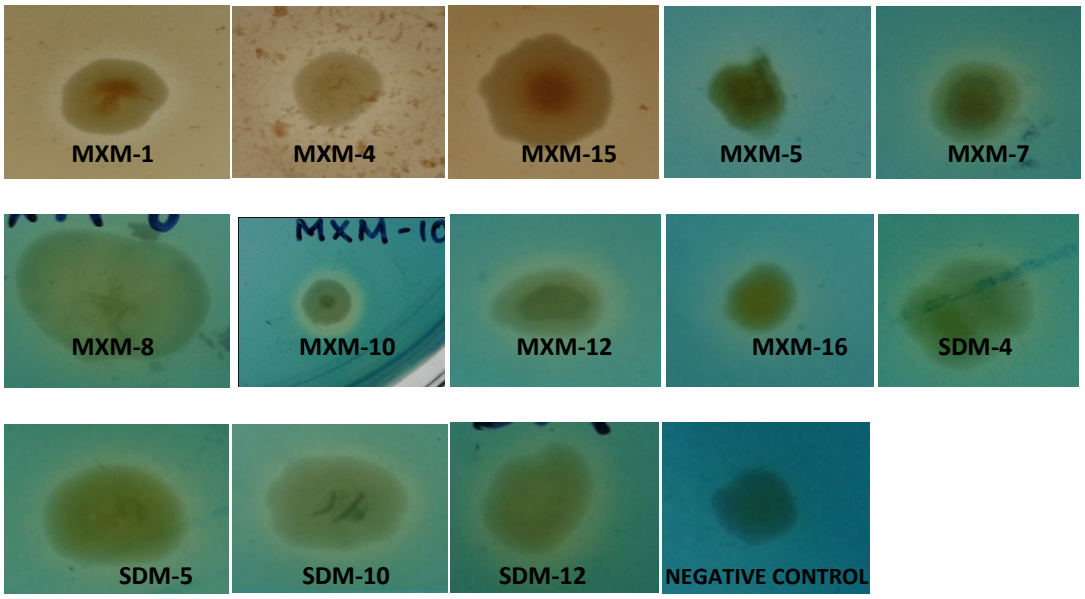


Fig. 2.13: Isolates showing zones of yellow colouration around the bacterial isolates on CAS agar.

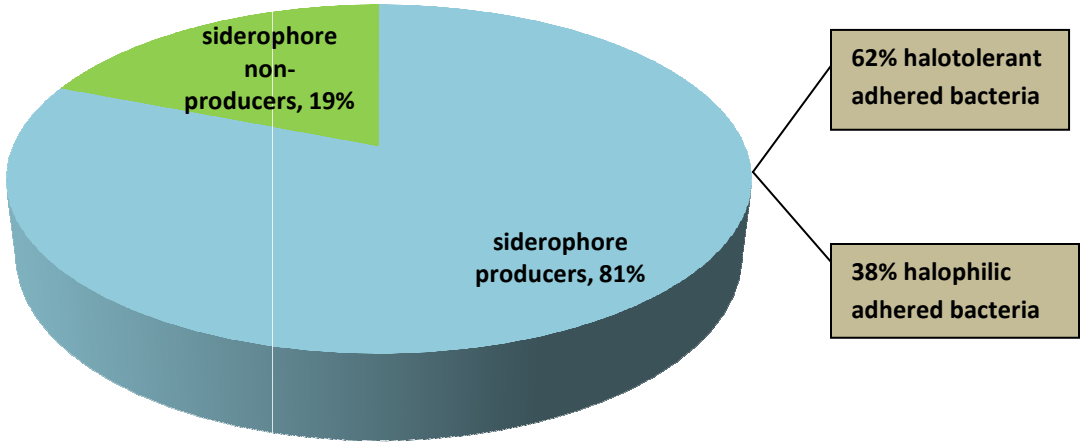


Fig. 2.14: Percentage distribution of siderophore producing halophilic and halotolerant adhered bacteria among the isolates.

surface i.e. a bacterial cell with hydrophobic cell surface will prefer to adhere to a solid surface that is hydrophobic in nature. The 16 adhered bacterial isolates were subjected to BATH assay. It was seen that 88% of the isolates showed adhesion to the hydrocarbon and 12%, all of which were halophilic isolates, showed emulsification (Appendix IV). Among those isolates that showed adhesion to the hydrocarbon 79% were halotolerant bacteria and 21% were halophilic bacterial isolates (Fig. 2.15). This indicated that the cells had a hydrophobic cell surface which helped them to adhere to the substrate. Maximum adherence was shown by giving a reduction in turbidity of almost 36% by isolate MXM-8 which was identified as *Acinetobacter schindleri* MXM-8. Literature survey has shown that hydrophobic surface of bacteria is more conducive to adhesion than hydrophilic cell surface (Katsikogianni and Missirlis, 2004, Gottenbos *et al*. 2002).

2.3.6 Screening of the selected bacterial isolates for the production of EPS.

In order to adhere, it is imperative for the bacteria to produce surface polymers like the EPS. Studies on EPS of bacteria have shown the crucial role that it plays in adhesion of bacteria and biofilm formation (Katsikogianni and Missirlis 2004). Among the 10 isolates selected for further studies, 4 were halophiles and 6 were halotolerant bacterial isolates.

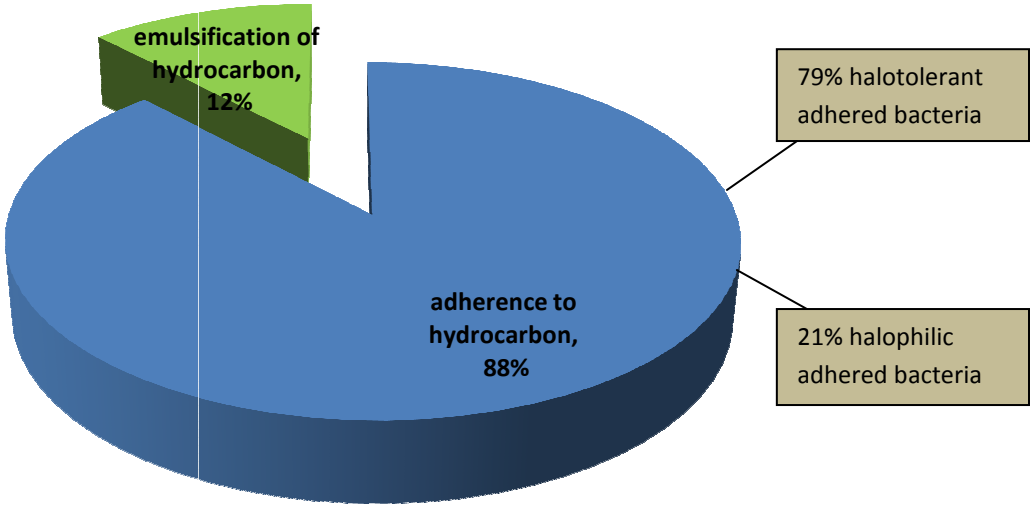


Fig. 2.15: Percentage distribution of halophilic and halotolerant bacteria adhering to hydrocarbon.

The Alcian blue showed a blue sheath against red bacterial cells in all the 10 isolates as shown in Fig. 2.16. Interestingly, the Congo red-Coomassie blue agar also showed glossy pink colonies in case of all the isolates indicating the presence of EPS in all the ten selected isolates (Fig. 2.17). Isolates producing such colonies on congo red-coomassie blue have been reported by Narancic *et al*. 2012. Halophilic bacteria such as *Halomonas* sp and *Salipiger mucescens* have been reported that produce EPS which aids the cell in adhesion to the substrate (Martinez- Canovas *et al.* 2004 a,b,c, Martinez-Checa *et al.* 2005).

2.3.7 Identification of halophilic and halotolerant adhered bacteria

Due to the interesting characteristics of the adhered bacteria it was desired that the taxonomic identification of these isolates be known, to understand their distribution and role in the mangrove ecosystem better. The 10 selected isolates after being subjected to routine cultural, morphological and biochemical characteristics (Appendix IV) were tentatively identified by Bergey's Manual of systematic bacteriology. MXM-1 as *Micrococcus* sp, MXM-4 was identified as *Aeromonas* sp, MXM-5 as *Halobacillus* sp, MXM-7 as *Brevibacterium* sp, MXM-8 as *Acinetobacter* sp, MXM-10 as *Erwinia* sp, MXM-12 as *Aeromonas* sp, MXM-16 as *Halobacillus* sp, SDM-2 as *Vibrio* sp and SDM-4 as *Staphylococcus* sp. Agarose gel Electrophoresis confirmed the extraction of DNA (Fig. 2.18) and the purity and concentration was measured by Nanodrop spectrophotometer (ND-1000 by JH Bio-innovations). The 16s rRNA gene was amplified by PCR using universal eubacterial primers U1 5'-

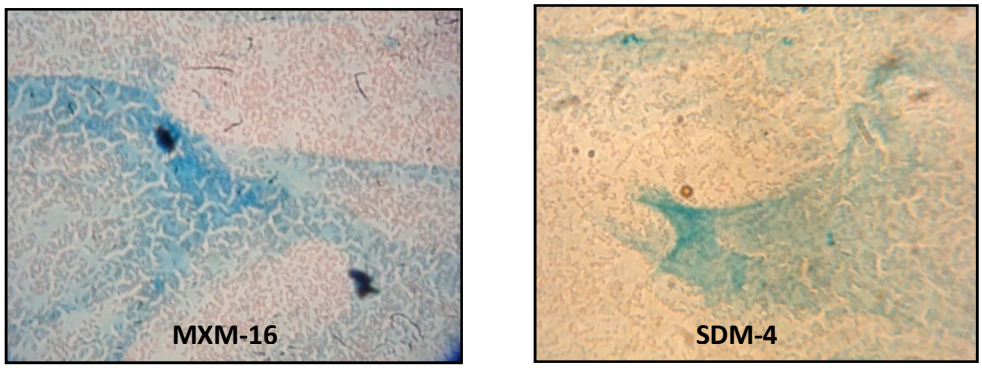


Fig: 2.16: Alcian blue staining showing blue sheath of the EPS against red bacterial cells of isolates MXM-16 and SDM-4.

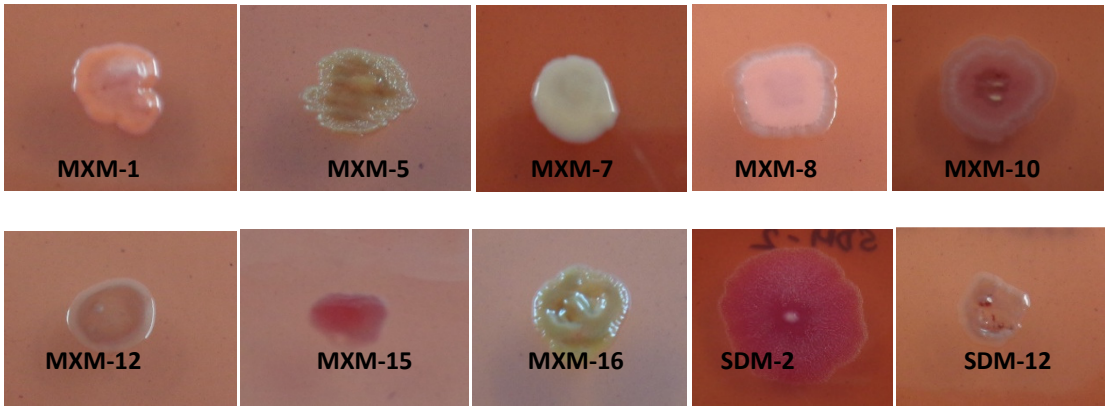


Fig. 2.17: Isolates showing EPS production on Congo red- Coomassie blue agar.

CCAGCAGCCGCGGTAATACG-3' and U2 5'-ATCGG(C/T)TACCTTGTTACGACTTC-3'.On sequencing, (Sanger's Method) the 16S rRNA gene, (Appendix VI) the 3 isolates were identified by the BLAST search tool based on the percentage similarity with other strains of the same species. Isolate MXM-5 and MXM-16 showed 99% similarity to *Halobacillus trueperi strain* MB1 and *Halobacillus trueperi* AP MSU9 while MXM-8 showed 99% similarity to *Acinetobacter schindleri strain* C47EM and *Acinetobacter schindleri strain* FSHS7. The GenBank accession numbers for the 3 isolates were *Halobacillus trueperi* MXM-5 (KF 379753), *Halobacillus trueperi* MXM-16 (KF 379752) and *Acinetobacter schindleri* MXM-8 (KF 379754) as shown in Table 2.3. The phylogenetic tree depicting the evolutionary relationship of MXM-8 with other *Acinetobacter* spp. have been shown in Fig. 2.19 while the phylogenetic tree of MXM-5 and MXM-16 showing the evolutionary relationship with other *Halobacillus* spp. is shown in Fig. 2.20.

Plant litter samples from the mangrove ecosystem showed a large number of adhered bacteria with 32% halophilic and 68% halotolerant isolates. These predominant bacteria had the ability to produce multiple enzymes responsible for the degradation of plant polymers. Further, the isolates produced EPS and siderophores and were capable of adhering to hydrocarbon. . The results depict that as mangrove ecosystem is prone to tidal action and fluctuation in salinity, it results in a predominace of halophilic and halotolerant adhered bacteria.

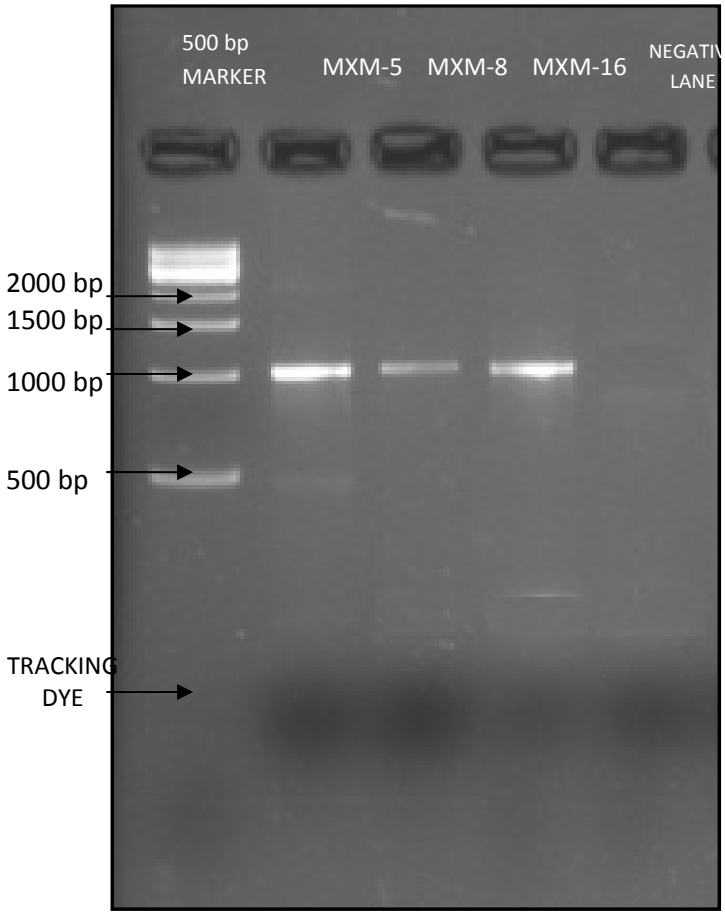


Fig. 2.18: Agarose gel electrophoresis of amplified 16S rRNA gene isolated from isolates MXM-5, MXM-8 and MXM-16.

Table 2.3: Identification of selected adhered bacterial isolates

Isolate No.	Identification by Biochemical tests	Identification by 16S rRNA	Similarity (%)	GenBank Accession number
MXM-5	*Halobacillus*	*Halobacillus trueperi*	99%	KF379753
MXM-8	*Acinetobacter*	*Acinetobacter schindleri*	99%	KF379754
MXM-16	*Halobacillus*	*Halobacillus trueperi*	99%	KF379752

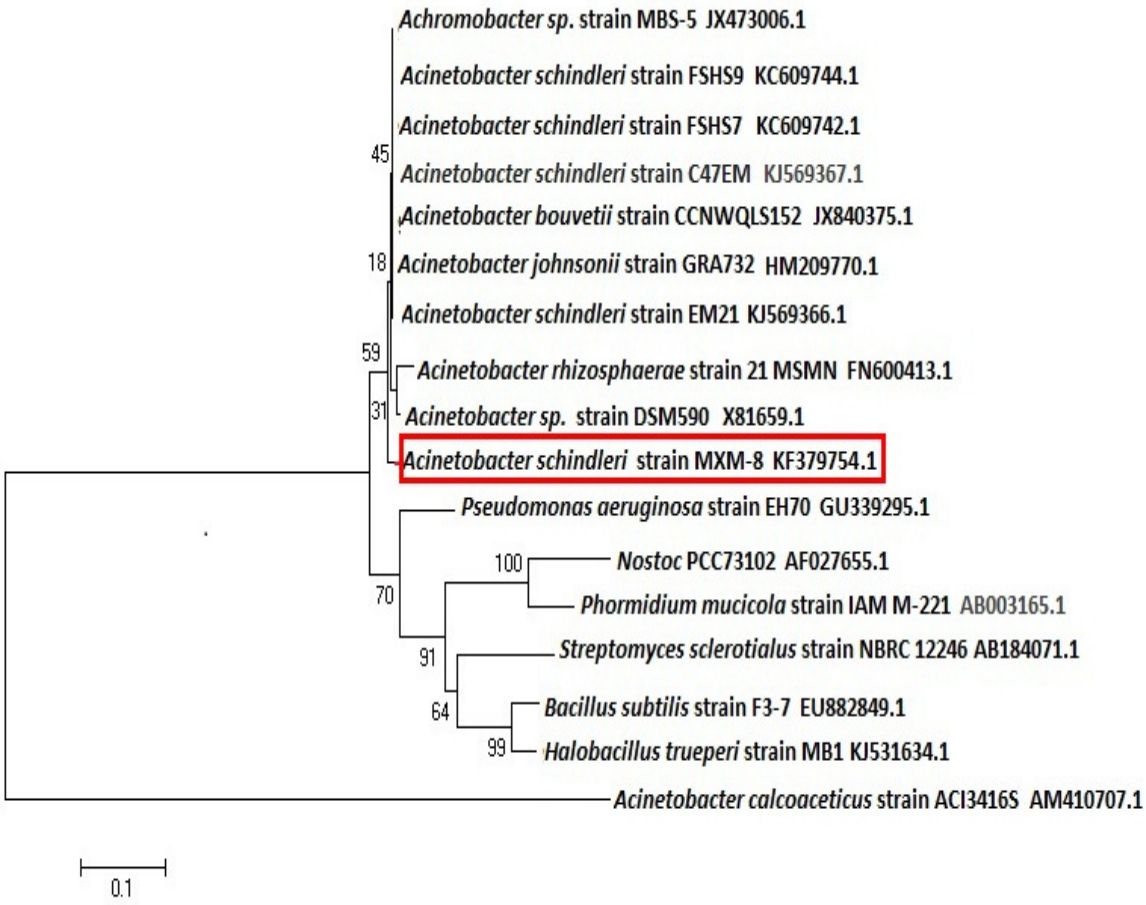


Fig. 2.19: Phylogenetic tree of *Acinetobacter schindleri* MXM-8 (GenBank accession no: KF 379754) constructed using the neighbour joining method based on the alignment of 16S rRNA gene sequences. The bootstrap values generated are shown at branch points. It shows the evolutionary relationship of *Acinetobacter schindleri* MXM-8 with other closely related *Acinetobacter* spp.

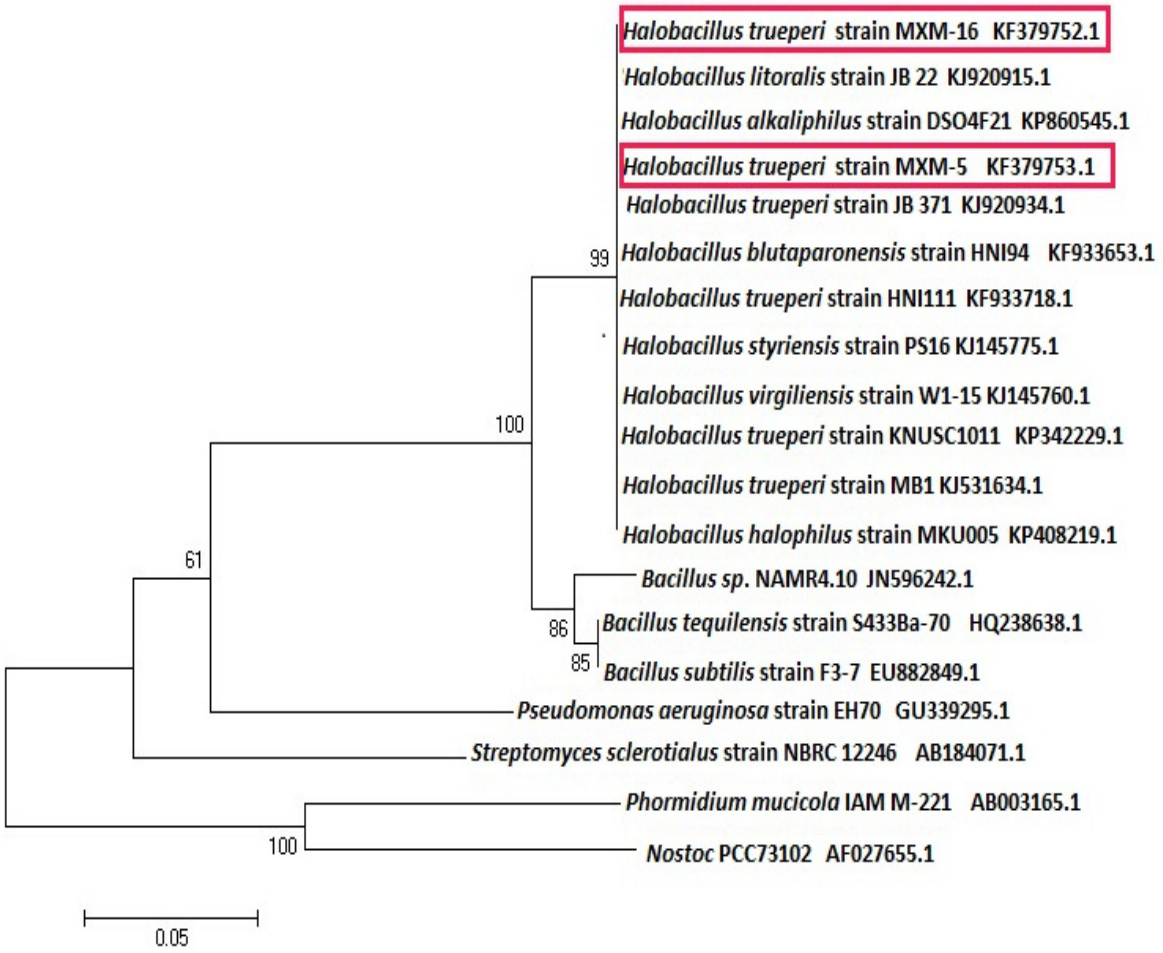


Fig. 2.20: Phylogenetic tree depicting evolutionary relationship of *Halobacillus trueperi* MXM-5 (GenBank accession no: KF 379753) and *Halobacillus trueperi* MXM-16 (GenBank accession no KF 379752) with other closely related strains of *Halobacillus* spp.

It was therefore of interest to understand the role of such isolates in the mineralization of the mangrove plant litter. Ten promising isolates were selected to prepare the consortium and inoculated in the plant litter sample. The details of the mineralization study are presented in the following chapter 3.

Chapter 3

Mineralization of mangrove plant litter using consortium of selected bacterial isolates

3.1 Introduction

The mangroves are considered one of the earth's most efficient ecosystems. The nutrients produced in the mangrove ecosystem support the communities of the mangrove ecosystem as well as the offshore communities (Kathiresan 2012, Miththapala 2008). The efficiency of this ecosystem depends upon the rate of mineralization of the particulate organic matter present in the form of mangrove plant litter.

Mineralization is an important process in degradation of plant litter as it involves decomposition of the organic matter and release of dissolved nutrients. These nutrients undergo recycling and add to the rate of nutrient turnover and efficiency of degradation of particulate organic matter in the ecosystem.

In the mangroves, the plant litter is primarily made up of complex plant polymers such as cellulose, hemicellulose, lignin, starch, tannin, etc. Studies have been carried out on fungi and bacteria associated with mangrove plant litter (Ongbonna 2011, Ananda and Sridhar 2004). Studies on plant litter degradation in the mangrove ecosystem have shown that, the degradation of these complex plant polymers such as tannin and lignin proceeds by formation of many phenolic substances (Sridhar and Ananda 2006). These tannins and other phenolic compounds form the major fraction of organic matter in the plant litter. Unless present in exceptionally high concentration they do

not inhibit microbial uptake or microbial degradation of plant litter (Benner *et al*. 1986).

The efficiency of mineralization is affected by deficiency in availability of crucial elements like iron. Iron is an important factor for metabolic activities to take place at the optimum level as it forms a part of many important enzyme systems in the bacteria. Since the bioavailability of iron is limited in the environment, to sequester it, mangrove bacteria produce siderophores that (Sandy and Butler 2009) chelates iron from the environmental complexes and transports it to the bacterial cell (Neilands 1995, Hider and Kong 2010). Siderophores have generated interest as they play a vital role in efficiency of degradation in ecological habitats and phytoremediation (Gaonkar and Bhosle 2013, Gaonkar *et al*. 2012).

The last chapter revealed the ability of the halophilic and halotolerant adhered bacteria to produce siderophores and extracellular hydrolytic enzymes that degrade natural plant polymers. This opens avenues for exploring and understanding the mineralizing potential of the bacterial isolates in the degradation of the mangrove plant litter. It was envisaged that the presence of the bacterial consortium would enhance the efficiency of degradation of the plant litter. Therefore, the mineralization studies were carried out and have been explained and presented in this chapter.

3.2 Materials and Methods

3.2.1 Preparation of bacterial consortium

The 10 bacterial isolates were grown on 50 ml ZMB/NTYE broth for 24 hr. The absorbance (Shimadzu UV 1601) at 600 nm for the isolates was maintained in the range of 1.4 to 1.8. The broth was centrifuged (Eppendorf 5804R) at 5000 rpm for 10 min at 4°C. The pellet obtained from each isolate was pooled and diluted with 20 ml of sterile normal saline. 1ml of the consortium was added to the plates in Set II and IV.

3.2.2 Mineralization study

a) Phase I: Model setup

The experiment was carried out in 4 sets. Each set had four petridishes monitored over a period of 0-3 weeks. Mangrove plant litter was washed thrice with 100 ml of sterile normal saline and surface sterilized using 70% absolute ethanol. The plant litter was dried in the oven at 60°C for 2 hr. 5g of this dry plant litter and 70 ml of sterile normal saline was added to each of the 16 petridishes of the 4 sets. 1g of sterile mangrove soil was added to all petridishes of Set I and Set II while 1g of unsterile mangrove soil was added to the petridishes of Set III and Set IV. Bacterial consortium was prepared of the ten bacterial isolates that were tentatively identified up to the generic level as explained in Chapter II. 1 ml of the bacterial consortium was added to the petridishes in Set II and Set IV and these sets served as the inoculated test sets and Set I and Set III were the uninoculated control sets. Set I and Set II containing the sterile mangrove soil also served as control for Set III and Set IV that had the unsterile mangrove soil.

The plates were monitored over a period of 0-3 weeks which was marked as phase I. In the second phase, one petridish from each set was analyzed at the end of the given time period. The plant litter was separated from the liquid medium by filtration through a filter paper and analyzed for loss in its weight (Shimadzu AX 200). The filtrate after the separation of the plant litter and the soil was centrifuged (Eppendorf 5804R) at 5000 rpm for 10 min at 4°C. 1 ml of this supernatant was used to determine the sugar, protein and the siderophore concentration and this consisted of Phase III in the mineralization studies (Fig. 3.1).

b) Phase II: Processing

1) Loss in plant litter weight

The plant litter and soil was filtered using a previously weighed filter paper on a funnel and conical flask assembly. The filter paper with plant litter and soil was transferred onto a previously weighed petridish and dried in the oven at 40°C overnight and weighed (Shimadzu AX 200). The loss in weight was calculated from the original weight of the plant litter and the soil sample.

2) pH of the mineralization medium

The pH of the supernatant was tested at the end of each week in each of the four sets over a period of 3 weeks. The sterile normal saline with pH 7.0 was the standard solution

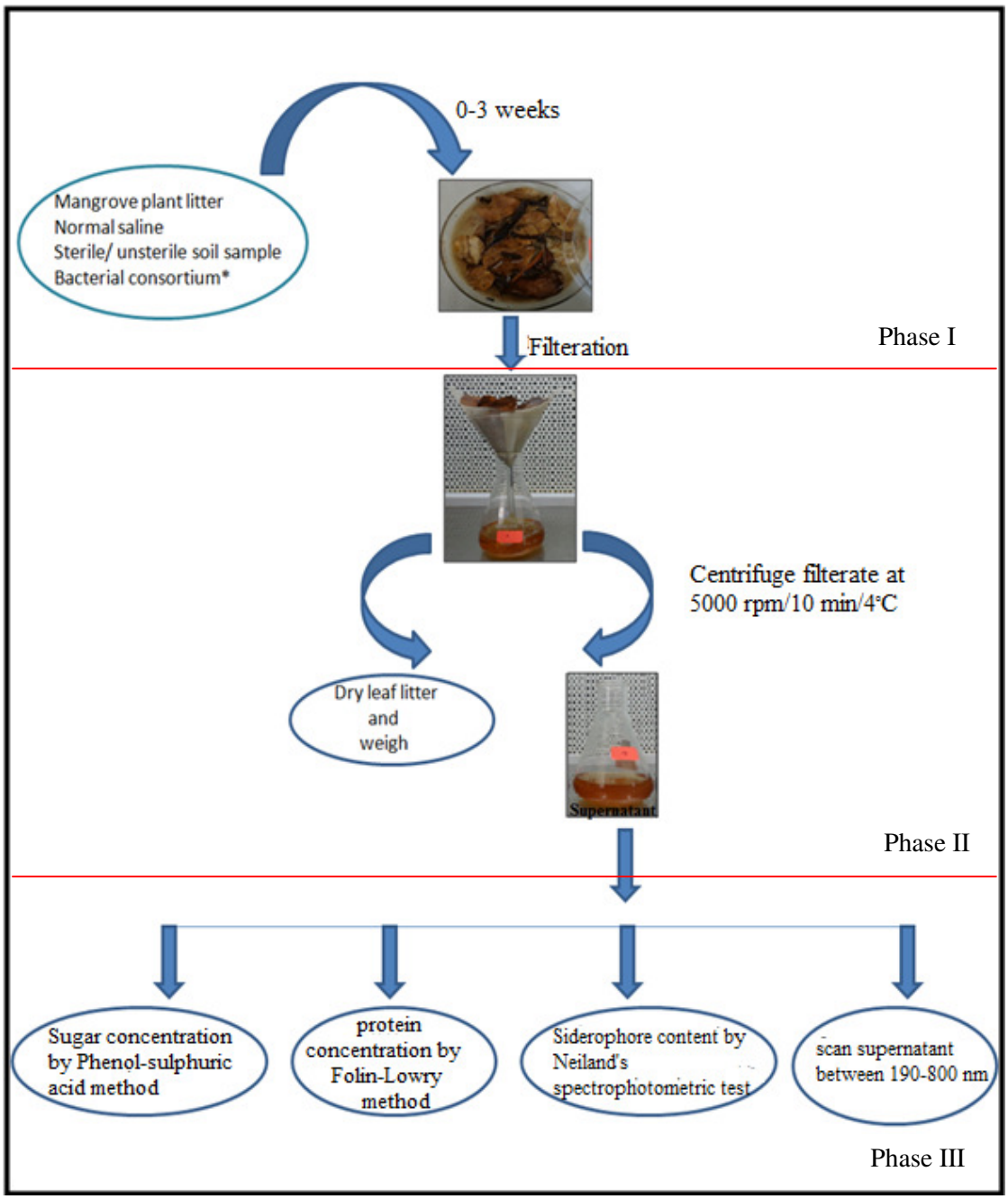


Fig. 3.1: Schematic representation of the protocol of mineralization study.

c) Phase III :Analysis

Supernatant was analyzed for sugars by the phenol-sulphuric acid method (Appendix V), proteins by the Folin-Lowry method (Appendix V), siderophores by spectrophotometry and type of siderophores by qualitative analysis (Appendix V). The supernatant was scanned from 190-800 nm using the UV-Vis spectrophotometer (Shimadzu UV 2450).

3.2.3 Results and discussion

Mineralization involves the degradation of the complex molecules in plant litter into simple molecules that can enter the nutrient pool for nutrient recycling. In mineralization, the particulate matter will reduce in size as its constituent molecules will be metabolized by the bacteria and other microorganisms in the environment. A decrease in the weight of the plant litter was noted from 0-3 weeks as shown in Fig. 3.2 (a). Set I showed a decrease in the weight of the plant litter up to 22% while the inoculated sterile Set II showed a decrease up to 26%. In the unsterile uninoculated Set III a decrease of 20% was seen while the inoculated set IV showed the highest decrease of 35% in the weight of the plant litter. Interestingly, it was observed that the inoculated sets II and IV showed more weight loss as compared with the uninoculated Set I and Set III. Although, the loss in weight was more in Set IV as compared with Set II, Set III showed weight loss less than Set II. This may be due to presence of the bacterial consortium present in Set II and Set IV that resulted in a more efficient breakdown of the plant litter.

Table 3.1 Contents of the petridishes in Set I, Set II, Set III and Set IV

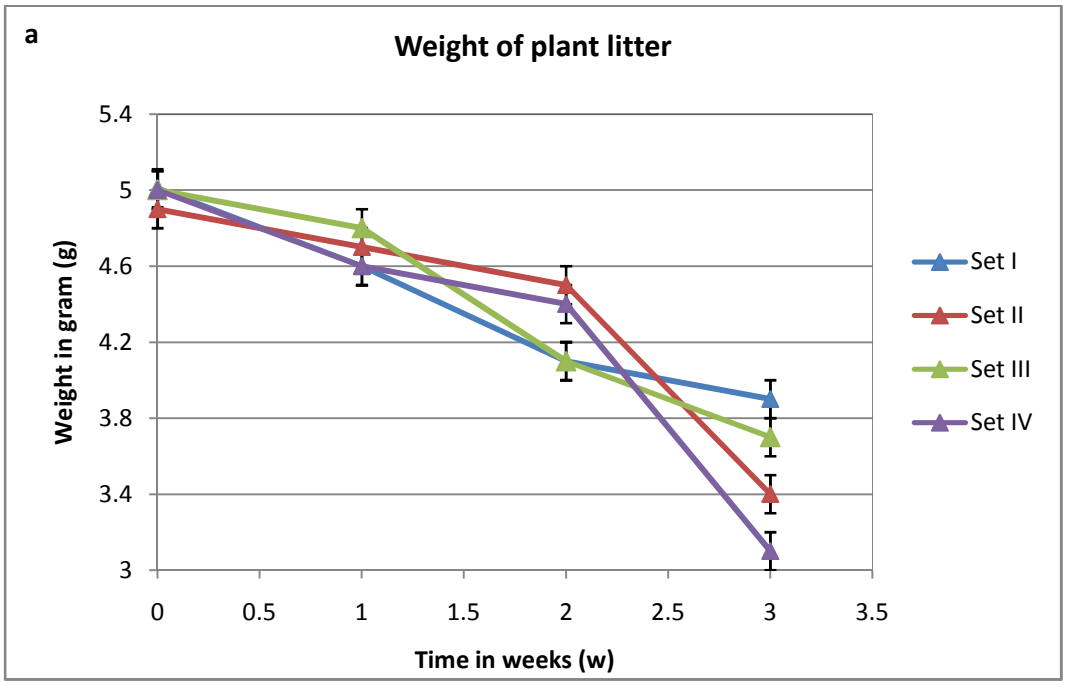
Contents	Quantity	Set I	Set II	Set III	Set IV
Plant litter	5g	+	+	+	+
Normal saline	70 ml	+	+	+	+
Sterile soil	1g	+	+	-	-
Unsterile soil	1g	-	-	+	+
Bacterial consortium	1ml	-	+	-	+
Normal Saline	1 ml	+	-	+	-

Table 3.2 Halophilic and halotolerant bacterial isolates in the bacterial consortium.

Isolate	Identification by biochemical tests
MXM-1	*Micrococcus*
MXM-4	*Aeromonas*
MXM-5	*Halobacillus*
MXM-7	*Brevibacterium*
MXM-8	*Acinetobacter*
MXM-10	*Erwinia*
MXM-12	*Vibrio*
MXM-16	*Halobacillus*
SDM-2	*Aeromonas*
SDM-4	*Staphylococcus*

Studies on mycological and bacterial degradation of plant litter from *Rhizophora apiculata* showed similar decrease in the weight in the litter sample (Raghukumar *et al.* 1995). Decomposition of plant litter produces humic acids and carbonic acids during the degradation process and decreases the pH of the surrounding soil or water. Interestingly it was noted that at the end of 3 weeks all 4 sets showed an acidic pH ranging from 6.9 to 5.1. The pH in all sets followed a decreasing trend as shown in Fig. 3.2(b). The pH dropped from 6.8 to 6.1 in set I, 6.9 to 5.8 in set II, 6.7 to 5.7 in Set III and 6.8 to 5.1 in Set II. Significantly, it was observed that set II and set IV showed the highest drop in pH as compared to the uninoculated Set I and III while Set III and IV also showed a comparatively increased drop in pH as compared to Set I and II. The changes depicted in Set I can be attributed to the presence of endophytic organisms associated with the leaf litter. Previous studies on microbial degradation of lignin and other polyphenols in organic matter have shown the production of humic acids that lower the pH of the medium turning the medium slightly acidic. (Steffen *et al.* 2002, Tuomela *et al*. 2000). It has been studied that a slight acidity is conducive for the intercellular interactions occurring between a bacterial cell and its environment such as iron uptake (Bjorn and Mc Claugherty 2008, Sylvia *et al*. 2005).

Extracellular enzymatic action on carbohydrates from the plant litter such as cellulose and hemicelluloses produces simple sugars that get released in the mineralizing medium. The sugar concentration trend in all sets showed an increase over a period of 3 weeks (Fig. 3.3a). Set I and Set II showed an increase in sugar content from 53



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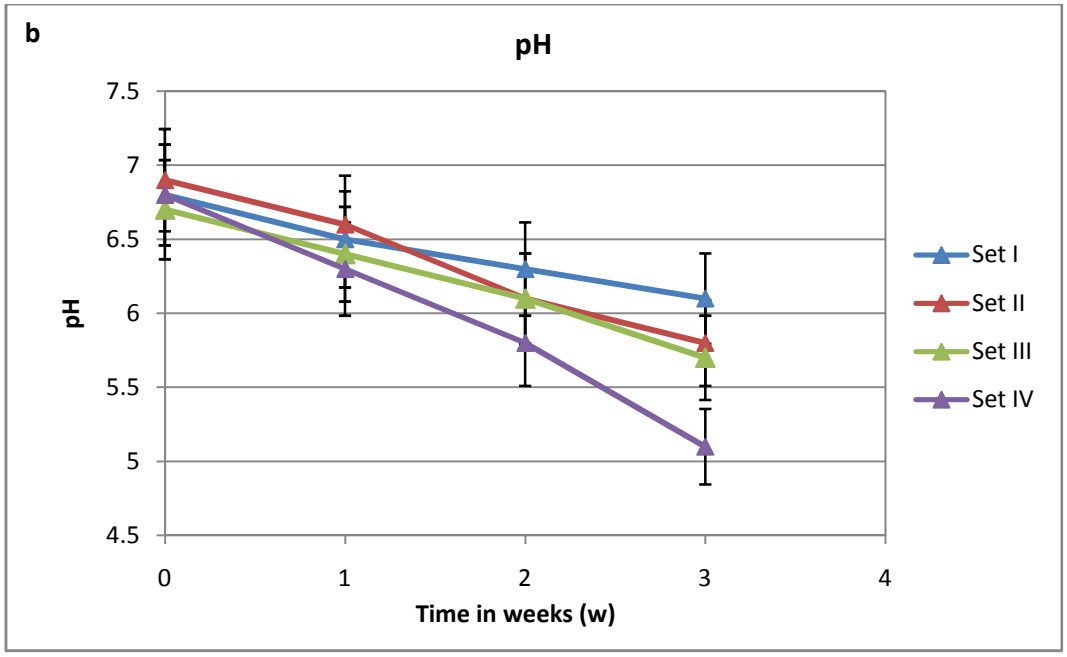

Fig. 3.2: Loss in weight of plant litter (a) and change in pH of the medium (b) during mineralization.

µg/ml and 48 µg/ml at 0 week to 115 µg/ml and 156 µg/ml respectively at the end of 3 weeks. Set III also showed an increase in the sugar concentration in the mineralization medium that increased from 47 µg/ml to 155 µg/ml by the third week. The sugar concentration of set IV increased from 53 µg/ml to 172 µg/ml from 0 to 3 weeks. Significantly, Set I showed an increase in sugar content up to 54% while Set II, III and IV showed an increase up to 69%, 70% and 69% respectively. The presence of complex polymers in the mineralizing medium triggers the bacteria to produce extracellular enzymes that degrade these polymers. Apart from these, many proteinaceous compounds are released from the particulate organic matter. Figure 3.3(b) shows the increasing trend of protein concentration in all the sets over a period of 0-3 weeks. In the uninoculated control set I and Set III the protein concentration ranged from 112 µg/ml and 154 µg/ml to 332 µg/ml and 498 µg/ml respectively. The protein increased from 148 µg/ml to 398 µg/ml in case of Set II while Set IV showed elevated protein ranging from 178 µg/ml to 980 µg/ml. Hence, the sets I and III with bacterial consortium showed an increase in protein content as compared with the uninoculated set I and III. It was also interesting to observe that the sets III and IV with unsterile soil showed a marked increase of up to 69% and 82% respectively in the protein concentration as compared with the sterile soil set I and II which showed an elevation in protein content up to 66% and 63% respectively. A remarkable increase in the protein concentration in Set IV (up to 82%) was observed after week 3, indicating that a high amount of proteins are generated during that time period in the presence of the bacterial consortium and unsterile soil.

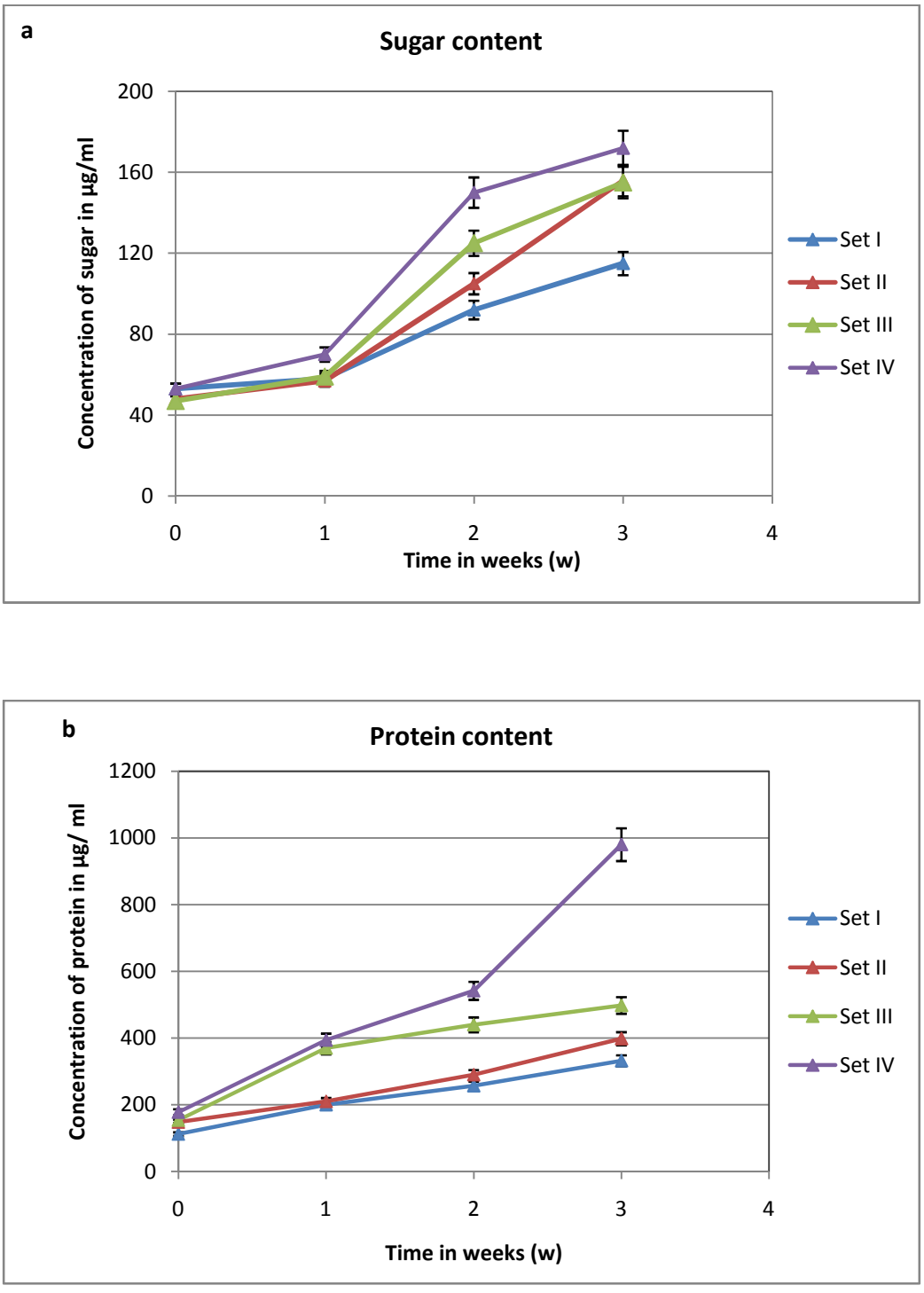

Fig. 3.3: Sugar (a) and Protein (b) concentration of the medium during mineralization.

Fig. 3.2(a), Fig. 3.3(a) and Fig. 3.3(b) indicated that the sets I and IV containing bacterial consortium showed an increase in loss of plant litter weight and a more efficient breakdown of the carbohydrates of the leaf litter and release of sugars in the surrounding medium and increase in the extracellular content of enzymes and proteins produced during the mineralization process from 0-3 weeks. This indicates an increase in efficiency of mineralization in the presence of the bacterial consortium. Set III and Set IV also showed better weight loss, sugar content and protein content in the mineralizing media indicating the presence of unsterile soil with its microorganisms increased efficiency of mineralization. Similar reports have been made on the degradation of mangrove plant litter in terms of the weight loss of plant litter, reduction in its protein and sugar content by Raghukumar *et al*. 1995. Plant litter degradation is an important step in formation of soil organic matter, mineralization of organic nutrients and carbon recycling in the ecosystem. The presence of polymers like lignin and cellulose has been studied on the degradation of plant litter from different ecosystems (Austin and Ballare 2010). Studies on leaf litter degradation in the agro forests of Bangladesh have been reported that contribute to soil fertility and nutrient recycling (Hasanuzzaman and Hossain 2014).

In the mangrove ecosystem, it is important that adhered bacteria should produce siderophores to be able to efficiently sequester iron from the environment to be able to mineralize/ metabolize the particulate organic matter efficiently. The ability of bacteria to produce siderophores provides them with the edge required to survive and

metabolize in environments that have a limited iron content such as the coastal and marine environment. The siderophore detected in the mineralization medium was hydroxamate type of siderophore. Previous studies have shown the presence of hydroxamate siderophores in degrading humus. It has been observed that in degradation process, humic acids are produced in the surrounding mineralizing medium. Studies show that bacteria that produce hydroxamate siderophores have an advantage in acidic soils due to their extreme acid stability and functionality (Winkelmann 2007). The siderophore concentration showed an increase in absorbance at 420 nm in each of the 4 sets over the period of 3 weeks as shown in Fig. 3.4. Although the increase in elevation of siderophore was more gradual in Set I, Set II and Set III, a very remarkable rise was noticed in Set IV. This can be attributed to the production of siderophores in Set IV due to the presence of bacterial consortium and the microorganisms in the unsterile soil.

The UV-Vis scans of the supernatant showed two peaks between 190-200 nm and 250-280 nm as shown in Fig. 3.5. A gradual increase in the absorbance of these peaks was observed from 0-3 weeks in all the 4 sets (Table 3.3). These peaks are characteristic of the aromatic hydrocarbons having one or two rings which show a peak between 190-200 nm due to the para band and between 260-280 nm due to the a-band (Finar 2009, Siryuk *et al*. 1979). Enzymatic degradation of heterogenous aromatic polymers lignin and tannin, result in forming compounds with a single or two aromatic ring (Martinez *et al*. 2005).

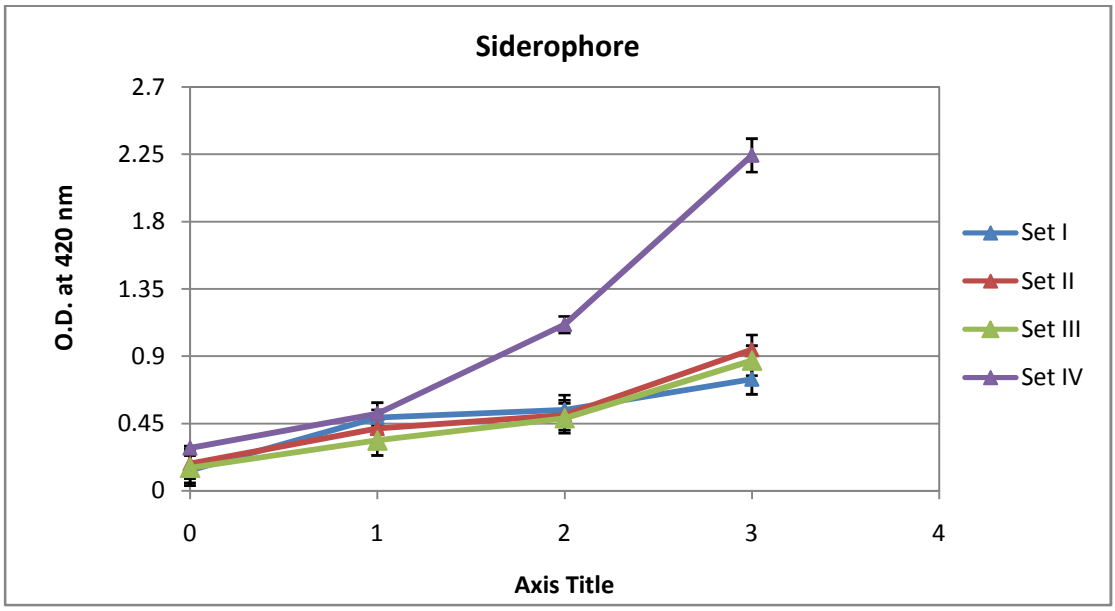

Fig. 3.4: Siderophore concentration in the mineralization medium.

Table 3.3 Absorbance of supernatant between 190-200 nm

Set	Time	Absorbance between 190-200 nm	Absorbance between 260-280 nm
Set I	0	1.23	0.25
	1	1.97	0.48
	2	2.06	0.75
	3	3.78	1.24
Set II	0	1.76	0.21
	1	2.11	0.43
	2	2.97	0.82
	3	3.80	1.20
Set III	0	1.40	0.32
	1	1.98	0.53
	2	2.57	1.03
	3	3.93	1.82
Set IV	0	2.06	0.43
	1	3.02	0.97
	2	3.87	1.42
	3	4.01	2.24

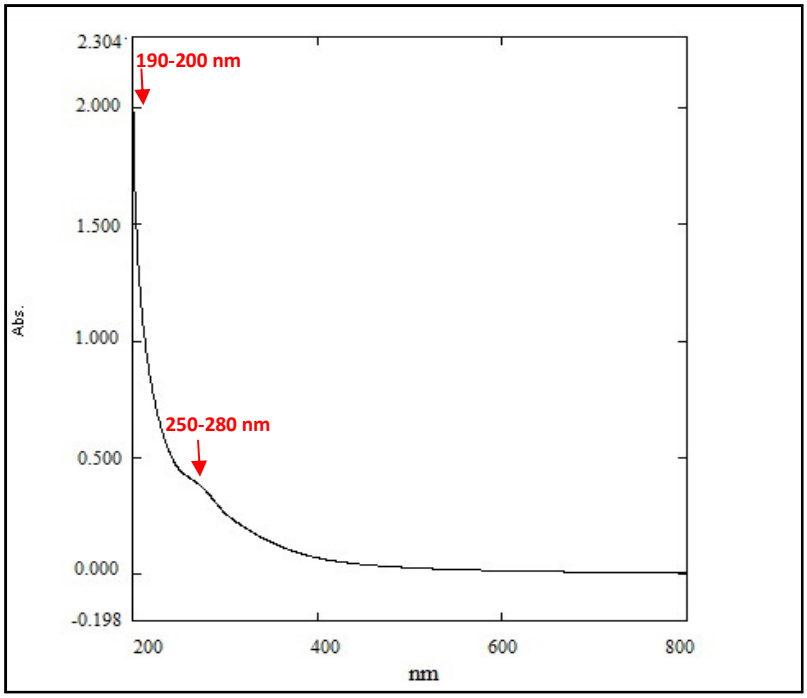

Fig. 3.5: UV-Vis scan of the supernatant of Set II after 1 week.

The mineralization studies highlighted the importance of the bacterial consortium in increasing the efficiency of degradation of the plant litter. The ability of the bacteria to enhance mineralization can be applied to agricultural and solid waste management. From the previous chapters it was seen that, a pigmented culture was isolated from the Manxer mangroves and this strain was identified as *Halobacillus trueperi* MXM-16.

The culture showed multiple enzyme production, ability to adhere to hydrocarbon, produce EPS and siderophores. Reports on this culture have been noted in Bergey's manual of systematic bacteriology and literature survey on prokaryotes, however, characteristics like production of siderophores, EPS and pigment are not detailed. The present study was undertaken to understand the type of siderophore, EPS, and pigment produced by *Halobacillus trueperi* MXM-16 and its ability to degrade aromatic compounds. These detailed studies have been presented in chapter 4.

Chapter 4

Studies on *Halobacillus trueperi* MXM-16

4.1 Introduction

The strain *Halobacillus trueperi* MXM-16 was isolated from Manxer mangroves in St. Cruz in the vicinity of the saltpans. The culture showed interesting characteristics like orange pigmentation, production of multiple enzymes such as cellulase, amylase, lipase and protease. It showed ability to adhere to hydrocarbon, produce siderophores and EPS. These characteristics of the strain indicated its potential which can be capitalized or used in bioremediation and agricultural solid waste management studies.

Bioremediation of hydrocarbons and management of agricultural waste is fast becoming an environmental concern. The oil spillage from oil tankers and oil rigs in the open sea, leakage from the transportation vessels, seepage of petrol and petroleum products from the land into the surrounding mangroves pose a serious threat to the flora and fauna of aquatic and the terrestrial ecosystem (Peterson and Holland-Bartels 2002, Fragoso dos Santos *et al*. 2011, Kingston 2002). The ability of the bacterial cell to adhere to hydrocarbon and bring about degradation plays a pivotal role in managing such pollution scenarios. Efficient methods for the management and degradation of agricultural waste are needed as the world food industry is growing at a rapid rate and largely depends on agricultural products. The ability of bacteria to adhere ensures that the bacteria will remain attached and localized and bring about efficient degradation of the solid substrate (Katsikogianni and Missirlis 2004, Ofek *et al*. 2003, Gonzalez *et al*. 1997b).

Environments like mangroves are known to harbor various types of microorganisms and bacteria producing unusual EPS of biotechnological interest (Aniszewski *et al*. 2010). EPS from bacteria like *Pseudomonas aeruginosa, Microbacterium* sp and *Marinobacter* sp have gained interest in the field of bioremediation research owing to their ability to bind to heavy metals such as copper and lead (Aniszewski *et al*. 2010, Pal and Paul 2008, Bhaskar and Bhosle 2006). Besides, the EPS of *Rhodococcus rhodochrous* has been reported to stimulate the degradation of aromatic compounds in crude oil (Iwabuchi *et al*. 2002). The EPS producing bacteria *Paenibacillus jamilae* has been studied for its efficiency in bioremediation of toxicity of olive mill effluents (Aguilera *et al*. 2008).

This chapter describes the detailed studies carried out on the ability of *Halobacillus trueperi* MXM-16 to grow on sodium benzoate and produce EPS, siderophores and pigment produced by this strain. Sodium benzoate is a monocyclic aromatic compound that requires enzymes oxygenases for its degradation. Interestingly, this enzyme requires iron as its cofactor and thus the bacteria have to produce siderophores so as to sequester this metal from the environment. However there is no literature available on the production of siderophores by this species. Therefore, it was of interest, to study the ability of this culture to produce siderophores. The characteristics and properties of the EPS, siderophores and pigment produced by the strain *Halobacillus trueperi* MXM-16 have been explored and presented in this chapter.

4.2 Materials and methods

4.2.1 Growth of *Halobacillus trueperi* MXM-16 on sodium benzoate

24 hr culture of *Halobacillus trueperi* MXM-16 was grown on minimal salt medium (MSM) with 0.2% sodium benzoate as the sole source of carbon for 48 hr at 28°C. The ability of the culture to grow on this medium was noted and the cells were subjected to Rothera's test (Appendix V), to understand the mode of ring cleavage.

4.2.2 Crystal violet adhesion assay

1 ml of 24 hr culture was added to clean grease free polystyrene petridish and glass petridish respectively such that approximately $1x10^7$ cells/ ml were obtained. The plates were incubated at 28°C for 90 min at 100 rpm on an orbital shaker (Remi CIS 24 BL) to allow attachment of cells to the plate surface. After incubation the unattached cells were removed by washing with equal volume of sterile PBS. The adhered cells were fixed with 99% methyl alcohol for 15 min. Excess solution was discarded and the plates were allowed to air dry. 1ml of crystal violet solution was added to each plate and allowed to stand undisturbed for 15 min. Excess of crystal violet was discarded and the plates were washed 3-4 times with sterile de-ionized distilled water until a clear rinse solution was obtained. 150 µl of 33% acetic acid was used to release the stain for 5 min. The solution was collected and the optical density was read at 620 nm on a UV-Vis spectrophotometer (Shimadzu UV 2450). The control was maintained with culture pellet from 1 ml culture suspension in an eppendorf centrifuge tube that was stained with the crystal violet solution. It was vortexed (Remi CM 101)

for 2 min to mix well and allowed to stand undisturbed for 15 min. After centrifugation (Eppendorf 5417C) at 5000 rpm for 10 min at 4°C the excess crystal violet solution was discarded followed by 3-4 rinse with de-ionized distilled water, distained as before. The supernatant was collected after centrifugation (Eppendorf 5417C) at 5000 rpm for 10 min at 4°C and the optical density was read at 620 nm on a UV-Vis spectrophotometer (Shimadzu UV 2450).

4.2.3 Emulsifying ability of the cell free supernatant of *Halobacillus trueperi* MXM-16.

The culture was grown on ZMB, for 48 hr at 28°C, cells were pelleted by centrifugation (Eppendorf 5804R) at 5000 rpm for 20 min at 4°C and the supernatant collected. To 2 ml of the cell free supernatant 0.5 ml of hexadecane was added and vortexed (Remi CM 101) for 2 min. A control tube with uninoculated ZMB with hexadecane was maintained. The tubes were allowed to stand undisturbed for 24 hr. The turbidity of the aqueous layer was measured using de-ionized distilled water as the blank solution at 600 nm on UV-Vis spectrophotometer (Shimadzu UV 2450).

4.2.4 Studies on EPS produced by *Halobacillus trueperi* MXM-16

a) Extraction and partial purification of EPS produced by *Halobacillus trueperi* MXM-16

The culture was grown in 1L of ZMB and incubated for 48 hr at 28°C. The cells were removed by centrifugation (Eppendorf 5804R) at 8500 rpm for 20 min at 4°C and the

supernatant collected. Twice the volume of cold ethanol was added to the supernatant and kept overnight at 4°C. The mixture was centrifuged (Eppendorf 5804R) at 5000 rpm for 20 min at 4°C and the precipitate was collected. The precipitate was dissolved in de-ionized distilled water and dialyzed in a dialysis bag with cut off value of 12,000 kDa against de-ionized distilled water for 24 hr. The dialysate was collected and dried by evaporation under vacuum.

b) Characterization of the EPS

i) Chemical composition

Total organic and inorganic composition of the EPS was determined by the ash method. 1g of dry partially purified EPS was taken in a previously weighed crucible. The compound was charred until it formed ash. The loss in weight indicated was the measure of the organic content while the weight of the ash was the inorganic component of the compound.

ii) Total carbohydrate and protein content of EPS

The carbohydrate content was analyzed by the phenol sulphuric acid method (Appendix V) for total carbohydrates/ sugars and the proteins by the Folin-Lowry method (Appendix V) for protein estimation.

iii) TLC

The purified EPS was partially hydrolysed by boiling in 0.5 N NaOH for 30 min followed by neutralization with 0.5 N HCl. The extract was spot inoculated onto thin layer aluminium sheets precoated with silica (Sd fine-chem limited aluchrosep silica gel 60/UV 254). The solvent system consisted of n-butanol, distilled water, acetic acid (12:3:6) for amino acids and sugars while chloroform, methanol, acetic acid, distilled water (25:15:4:2) for lipids. Amino acid composition was visualized by spraying with 0.2% ninhydrin prepared in acetone. Sugars were detected by phenol sulphuric acid reagent and lipids by iodine vapours.

iv) FTIR spectroscopy

The infra red spectrum of the dried EPS was recorded on FTIR spectrophotometer (Shimadzu IR Prestige-21) in 4000-400 cm^{-1} spectral region after grinding with KBr pellet.

v) ^{1}H-NMR spectroscopy

The partially purified pigment was dissolved in deuterated water and analyzed by ^{1}H-NMR spectroscopy (Brucker 300 MHz).

vi) Qualitative analysis of EPS

The EPS was analyzed for the presence of proteins by the ninhydrin test, sugars by the Molisch's test and lipids by the Sudan IV test (Appendix V).

c) Characteristics of EPS

i) Surface activity

1) Qualitative oil drop collapsing test

A drop of oil was placed on a clean grease free slide. EPS was dissolved in water and 10 µl was added as a smaller droplet onto the surface of the larger oil drop. The shape of the oil drop was observed for a minute. Flat drop indicates a positive result for surface activity while a round drop indicates negative for surface activity (Youssef *et al*. 2004).

2) Qualitative oil displacement test

10 ml mixture of oil-o-red dye in paraffin oil was poured over a petridish containing 30 ml of distilled water to form a thin red film of oil over water. EPS was dissolved in water and 10 µl was carefully placed on the centre of the film. Appearance of a clear halo within 30 seconds indicates positive test for surface activity (Morikawa *et al*. 1993).

ii) Adhesive properties

The adhesive strength of the lyophilized EPS was determined by observing the ability of the EPS to glue clean glass slides of dimension 7.5x2.5 cm together, to stick stamp onto the envelope and to clump sand particles together.

iii) Emulsification ability

To 2 ml of EPS dissolved in de-ionised distilled water 0.5 ml of hexadecane was added and vortexed (Remi CM 101) for 2 min. The tubes were allowed to stand undisturbed for 24 hr and the turbidity of the aqueous layer was measured using a UV-Vis spectrophotometer (Shimadzu UV 2450). EPS in de-ionised distilled water served as the control and de-ionised distilled water was used as a blank.

iv) Effect of EPS on Metal solutions

0.2% metal solutions were prepared of cobalt sulphate, cobalt chloride, chromium oxide, ferric chloride, copper sulphate, cadmium chloride, calcium chloride, magnesium sulphate and manganese sulphate and used for the study. 0.5 ml of the EPS dissolved in de-ionized distilled water was added to 0.5 ml of metal solution. The mixture was allowed to stand for two hours and the reaction observed.

4.2.5 Characterization of siderophore produced by *Halobacillus trueperi* MXM-16

Preliminary studies in chapter 2 showed siderophore production in the nutrient agar and NTYE agar. Therefore, the characterization of the siderophore produced was undertaken. The culture was grown in 200 ml of nutrient broth prepared in de-ionized distilled water for 48 hr at 28°C. The broth was centrifuged (Eppendorf 5804R) at 8000 rpm for 20 min at 4°C and the supernatant decanted and was used for characterization of siderophore. The type of siderophore produced was characterized

by Neiland's spectrophotometric assay and tetrazolium salt test for hydroxamate siderophore (Appendix V), Arnow's assay and Csaky's spectrophotometric assay for catecholate siderophore (Appendix V) and Vogel's test for carboxylate siderophore (Appendix V).

4.2.6 Studies on pigment produced by *Halobacillus trueperi* MXM-16

a) Extraction of pigment

The culture was grown on ZMB on shaker at 150 rpm for 48 hr at 28°C. The culture suspension was pelleted by centrifuging (Eppendorf 5804R) at 5000 rpm for 10 min at 4°C. 1g of the pellet was resuspended in 10 ml of methanol (Merck HPLC grade) and acetone (Merck HPLC grade) each and sonicated (B. Braun Labsonic U) using 0.5 pulses for 2 min. This suspension was centrifuged (Eppendorf 5804R) at 8000 rpm for 20 min at 4°C and the supernatant containing the pigment decanted.

b) UV-Vis spectrophotometry

The pigment was scanned between 190-800 nm in a UV-Vis spectrophotometer (Shimadzu UV 2450).

c) HPLC

The separation of carotenoids was achieved by using the pigment extracted in methanol by HPLC on a C-18 reverse-phase column (Waters Spherisorb ODS, 5m diameter of 4mm x 25mm) in a mobile phase of methanol. Detection of pigment was

performed at a wavelength of 342 nm at a flow rate of 1 ml/min and a pressure of 1000 psi. The peaks were monitored over a period of 20 min using an HPLC-Waters equipped with waters 2996 Phase diode array detector (Nugraheni *et al*. 2010).

d) Ability of the pigment to act as siderophore

To 3 ml of CAS solution 2 ml of the extracted pigment was added and allowed to stand for 15 min at room temperature. The control was maintained using methanol or acetone added to CAS solution. The colour change from blue to yellow indicated the ability of the pigment to chelate iron.

4.3 Results and discussion

4.3.1 Growth of (*Halobacillus trueperi* MXM-16 on sodium benzoate)

Bacteria produce the enzymes oxygenases that have iron as their cofactor. These enzymes are needed to utilize aromatic substrates such as sodium benzoate. The strain *Halobacillus trueperi* MXM-16 showed good growth on MSM with 0.2% sodium benzoate as the sole source of carbon, however, the culture did not show any pigmentation (Fig. 4.1a). Earlier studies have reported that utilization of aromatic compound puts a greater stress on bacteria for the demand of oxygen and which has an effect on formation of the pigment (Gaonkar et al. 2012). Several studies have explored the interrelation between aromatic compounds and pigments produced by the bacterial isolates. It has been seen that when *E.coli* was grown in the presence of phenol, it produced a black melanin like pigment (Diaz *et al*. 2001). While, in case of

Pseudomonas aeruginosa the absence of the pigment pyocyanin resulted in a decrease in the degradation of polycyclic aromatic hydrocarbons (Norman *et al*. 2004). Research has also shown the ability of *Halobacillus salinus* and *Halobacillus halophilus* to degrade a variety of polycyclic aromatic compounds (Logan and De Vos 2011). The Rothera's test showed a deep purple colour with a ring formation on addition of the sodium nitroprusside solution as shown in Fig. 4.1b. The purple colour was a visual indicator of production of β-ketoadipate, a result of the ortho cleavage of the aromatic ring of sodium benzoate. Haloarchaea such as *Haloferax* sp, *Halobacterium piscisalsi*, *Halobacterium salinarum* and *Halorubrum ezzemoulense* have been reported to degrade aromatic hydrocarbons such as benzene and toluene by the ortho ring cleavage pathway of degradation (Erdogmus *et al*. 2013). Sand dune bacteria, *Pseudomonas aeruginosa* TMR2.13 can grow on sodium benzoate up to 2% and *Bacillus* sp can tolerate up to 1% of sodium benzoate (Gaonkar *et al*. 2012).

4.3.2 Crystal violet adhesion assay of *Halobacillus trueperi* MXM-16

In the crystal violet assay the cells of *Halobacillus trueperi* MXM-16 showed adhesion to both, the polystyrene petridish and the glass petridish. Interestingly, it was observed that the percentage of cells showing adhesion to the polystyrene petridish was more by 7% as compared to the glass petridish in the given time period (Fig 4.2). Polystyrene is a hydrophobic surface as compared to glass which is a hydrophilic surface (Buck and Andrews 1999, Loosdrescht et al 1990). Literature survey has

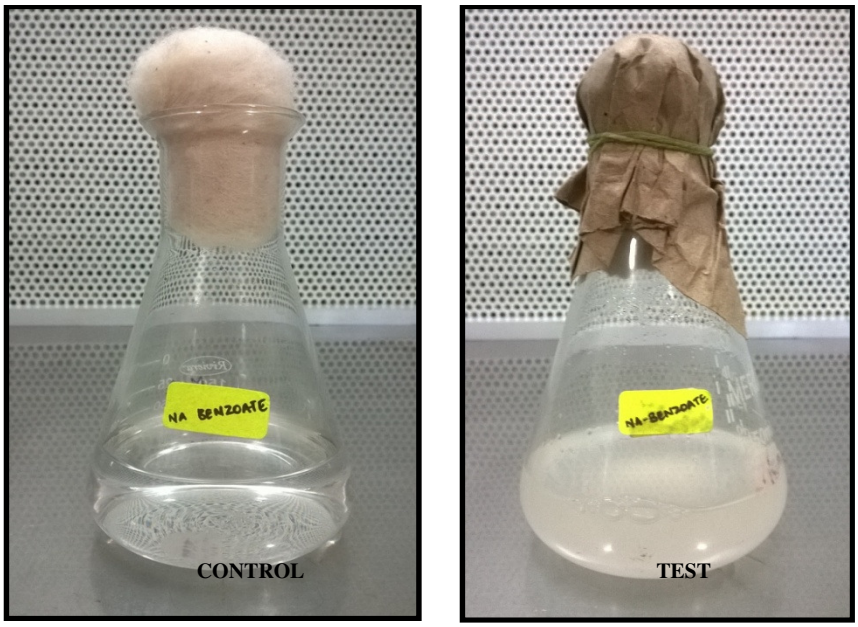

Fig. 4.1(a): Growth of *Halobacillus trueperi* MXM-16 on sodium benzoate medium.

Fig. 4.1(b): *Halobacillus trueperi* MXM-16 showing positive for ortho test.

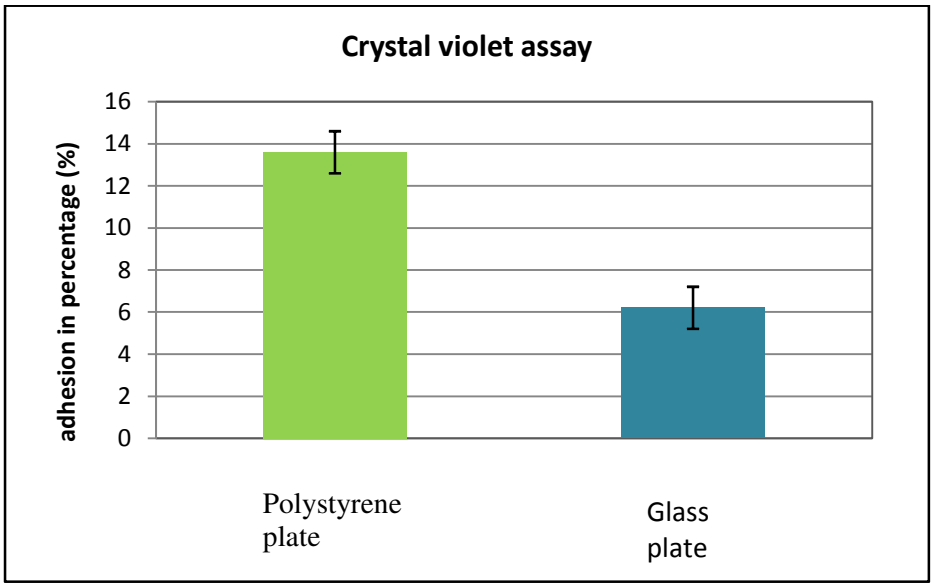

Fig. 4.2: Adhesion assay of the *Halobacillus trueperi* MXM-16.

shown that bacteria prefer to adhere to a hydrophobic cell surface in comparison to a hydrophilic cell surface (Katsikogianni and Missirlis 2004, Oliveira *et al*. 2001).

4.3.3 Emulsification test of *Halobacillus trueperi* MXM-16 supernatant.

In the emulsification test of the cell free culture supernatant it was observed that the turbidity of the aqueous layer increased by 34% than the control. This may be due to the presence of EPS in the cell free supernatant that interacts with the hexadecane, thereby increasing the turbidity of the lower layer. Reports on biodegradation of the hydrocarbon phenanthrene have shown turbidity in the lower aqueous layer that has been attributed to the emulsification of the hydrocarbon by the rhamnolipid in the aqueous layer (Henry *et al*. 2011).

4.3.4 Studies on EPS produced by *Halobacillus trueperi* MXM-16

a) Characterization of EPS of *Halobacillus trueperi* MXM-16

Preliminary qualitative analysis of the EPS showed the ninhydrin test for proteins and the Molisch's test for sugars positive concluding that the polymer was composed of proteins and sugars. The Sudan IV test for lipids however was negative indicating that the EPS did not contain a lipid moiety as indicated in Fig. 4.3. On further analysis, ash method revealed that 86% of the EPS was inorganic and 14% was organic. The EPS showed a sugar content of 400 µg/ml and a protein content of 1022 µg/ml. After partial hydrolysis the TLC showed five spots with Rf values 0.72, 0.65, 0.56, 0.27 and

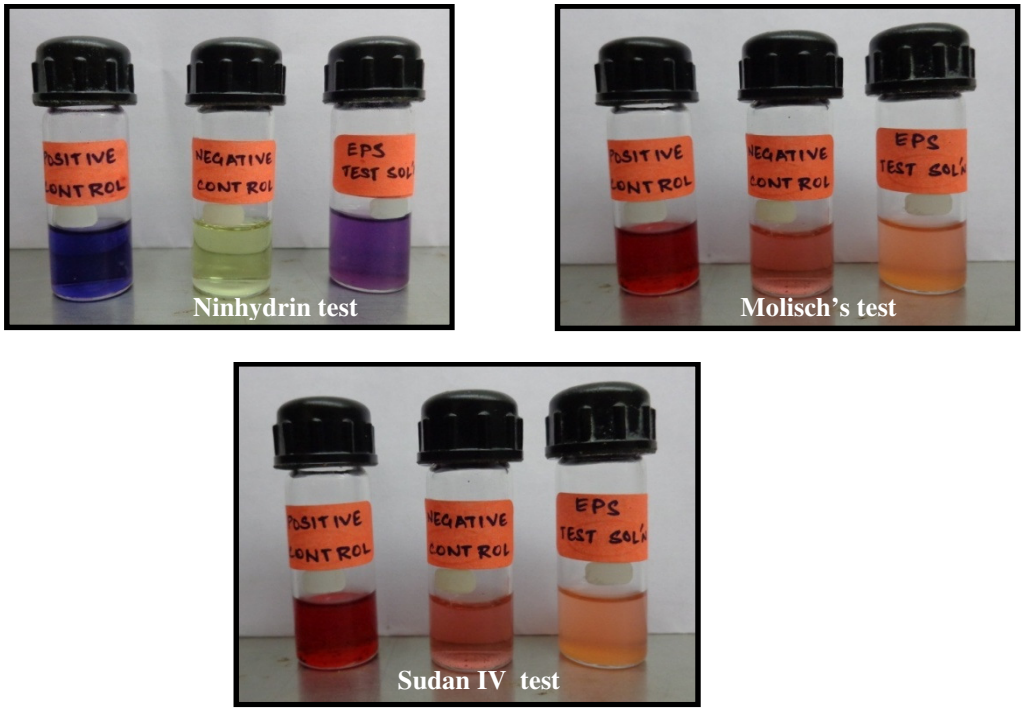

Fig 4.3: Qualitative analysis of *Halobacillus trueperi* MXM-16 EPS.

0.12 respectively with ninhydrin spray that corresponded to the amino acids tryptophan, phenylalanine, tyrosine, serine and lysine respectively. Two spots were obtained by spraying with phenol-sulphuric acid reagent with Rf values 0.47 and 0.72 corresponding to mannose and rhamnose respectively while no spots were detected on exposure to iodine vapours.

The infra red spectrum (Fig. 4.4a) of the EPS showed a symmetric stretch of O-H of polymeric compounds between 3200 and 3320 cm^{-1}. Absorption bands between 1630 and 1660 cm^{-1} were due to the C=O and C-N of amides associated with proteins. Stretching vibrations between 1400 and 1420 cm^{-1} indicated the C=O of carbohydrates. The band between 1060 and 1100 cm^{-1} were characteristic of O-H fingerprint of polysaccharides and their derivatives. Several bands were visible below 1000 cm^{-1} that indicated the presence of PO_4 and S functional group in the EPS biomolecule. Hence from the qualitative tests and the infra red spectrum it was concluded that the EPS contained proteins and sugars and that it was a glycopeptide in nature. The ^{1}H-NMR revealed non anomeric protons that were present in the narrow spectral range from 3.2 to 3.9 ppm. However the proton signal in this spectral region was hampered by signal overlap. The peaks between 2.0 and 2.9 ppm indicate R-C$\mathbf{H_2}$-structure and the peaks between 0.7-1.6 ppm indicate methyl group and a R-C$\mathbf{H_3}$ structure (Fig. 4.4b). Glycopeptide EPS have been reported from *Salmonella* (Gong *et al*. 2009) and *Clavibacter* (Van Alfren *et al*. 1987).

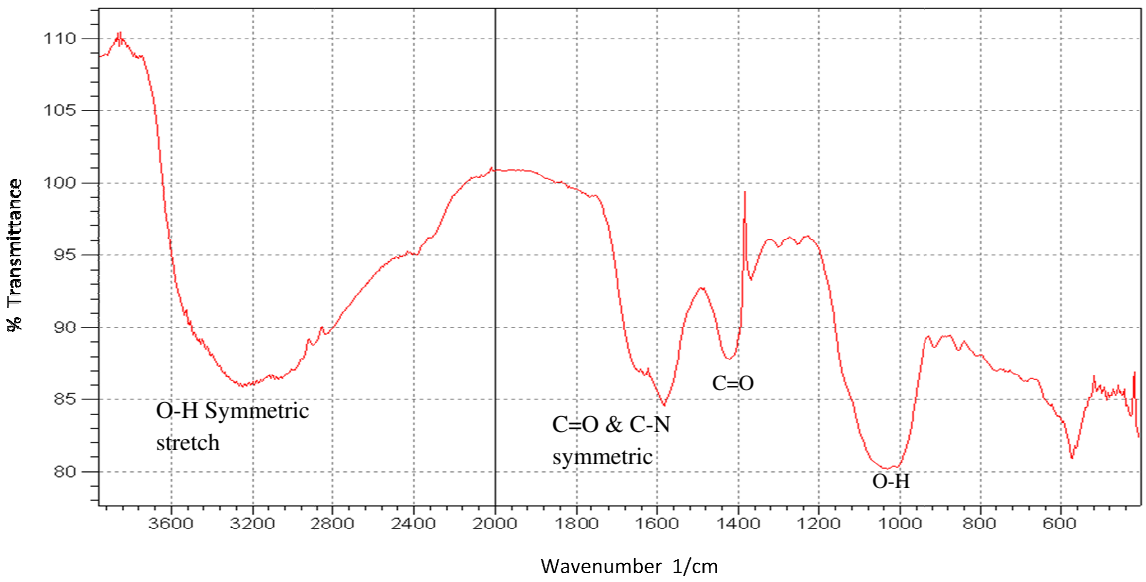

Fig 4.4 (a): Infra red spectroscopy of the EPS produced by *Halobacillus trueperi* MXM-16.

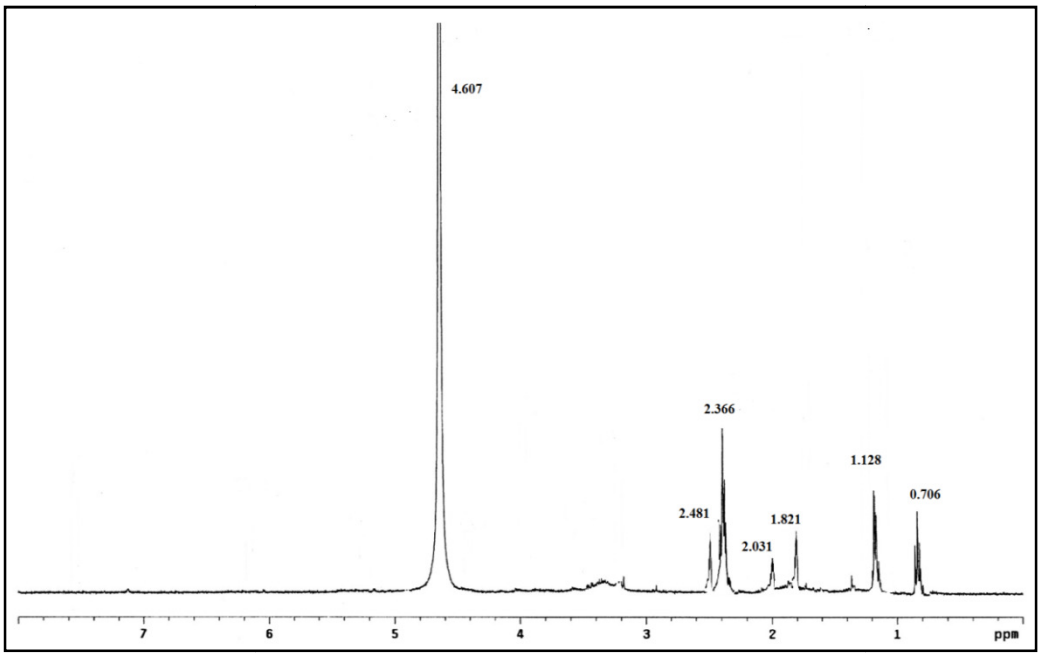

Fig. 4.4 (b): ^{1}H- NMR spectroscopy of EPS produced by *Halobacillus trueperi* MXM-16

There are a number of reports on EPS production by moderately halophilic bacteria like *Halomonas eurihalina, Halomonas maura, Halomonas ventosae, Halomonas anticariensis, Alteromonas hispanice, Idiomarina rambicola* and *Idiomarina fontisalpitosi* (Nanjani and Soni 2012).

b) Properties of the EPS produced by *Halobacillus trueperi* MXM-16

The microbial EPS are used in several biotechnological applications. The advantages of microbial polysaccharides are their novel functionality, chemical and physical properties like metal chelation, adhesion and emulsifying abilities that make them attractive for various industrial applications. The *Halobacillus trueperi* MXM-16 EPS did not show oil displacement (Fig. 4.5a) and collapsing of oil drop (Fig. 4.5b) indicating that the EPS was a bioemulsifier as it showed emulsification with hexadecane. This was seen as an increase in turbidity of the lower aqueous layer by 57% as compared to the control. Interestingly, it was also seen that the crude EPS also showed an increase in turbidity by 23% as compared to the cell free supernatant. Such emulsifying properties are crucial characteristics of bioemulsifying bacteria such as *Pseudomonas* sp that are being studied for the bioremediation of hydrocarbons and oil spills (Prakash and Irfan 2011, Moorthi *et al*. 2008).

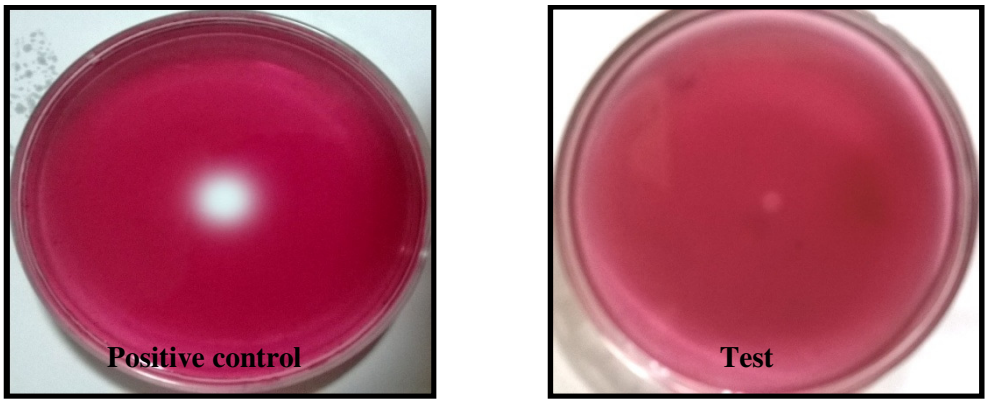

Fig 4.5(a): Qualitative oil displacement test for EPS

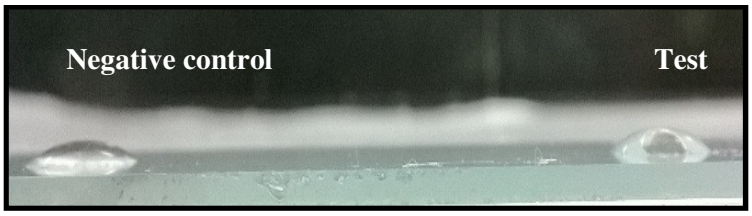

Fig 4.5(b): Qualitative drop collapse test for EPS

Studies on the adhesive ability showed that the EPS had the ability to glue the stamp to the envelope and the ability to stick the two glass slides together as shown in Fig 4.6(a) and Fig. 4.6(b). Such adhesive abilities are an important factor in the formation of biofilms on surfaces.

In the adhesion of sand particles it was observed that 61% of sand was retained behind on addition of the EPS. This was 53.5% more retention than the control sample. This observation indicated the ability of the EPS to bind together particles of sand forming particles of larger size that are unable to pass through the pores in the sieve. The ability to aggregate sand particles is an important aspect as it reflects on the ability of the EPS to bind soil particles. Such polysaccharide aggregated soils are more stable and less susceptible to erosion (Tisdall 1991, Molope *et al*. 1987).

Interaction of bacterial EPS with metals has been widely documented. EPS from *Chryseomonas luteola, Alteromonas macleodii, Pseudomonas aeruginosa, Paenibacillus polymyxa* and *Paenibacillus jamilae* has been reported to chelate metal ions from the environment and thus find a potential application in bioremediation of metal contaminated soil (Pal and Paul 2008, Bhaskar and Bhosle 2006). Therefore, it was of interest to us to see the effect the EPS would have on metal solutions. Interestingly, it was observed that on addition of EPS to the metal solutions it showed formation of precipitate within 15 min (Fig. 4.7). However in case of chromium oxide, a change in colour of the metal solution was observed. The solution turned from

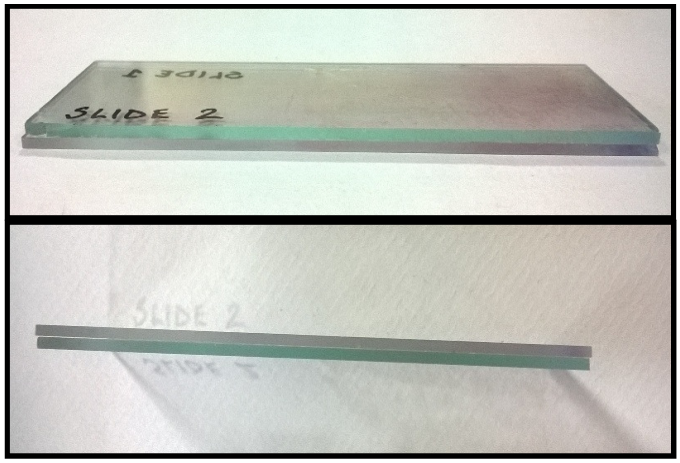

Fig. 4.6 (a): Adhesive ability of the EPS to glue glass slides.

Fig. 4.6 (b): Adhesive ability of the EPS to stick stamp on paper

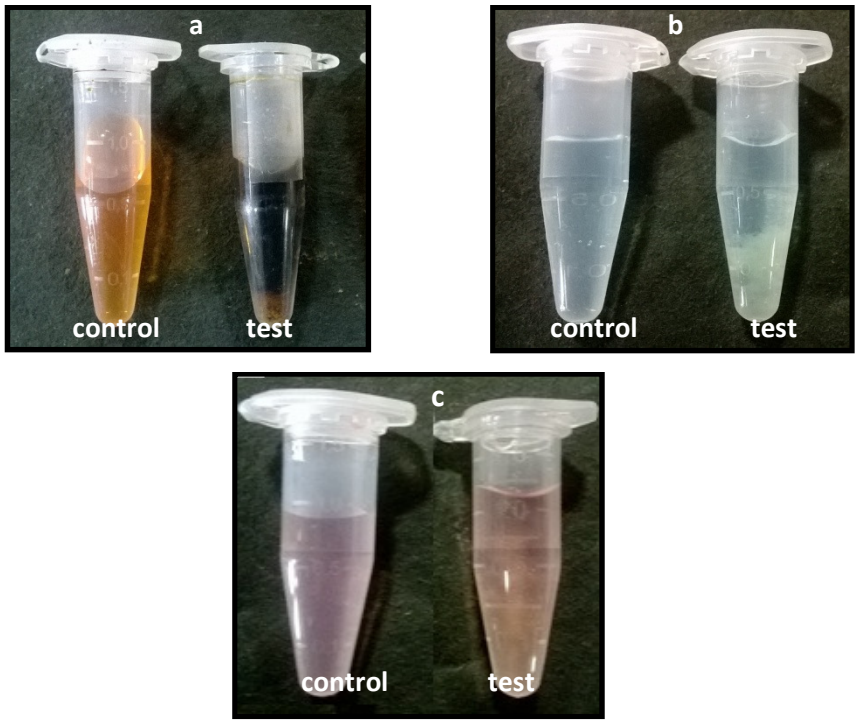

Fig. 4.7: Effect of EPS on (a) chromium oxide (b) Copper sulphate and (c) Cobalt sulphate

orange to brown indicating reduction of chromium oxide from an oxidation state of +6 in which the solution has an orange colour to +3 in which the solution has a bluish-brown colour. The EPS served as a reducing agent capable of donating electrons to chromium oxide in order for it to be reduced. This indicated that the EPS was negatively charged and was capable of interacting with the metal solution, donating or sharing electrons which resulted in the reduction and hence a change in colour. The literature survey shows that at circum-neutral pH the charge on microbial EPS is negative and therefore the sorption of cationic metal ion is always favoured (Johnson 1998). Metal uptake by such polymers results in release of protons that lowers the pH of the solution. EPS from cyanobacteria were reported to remove copper, chromium and nickel from metal solutions (Micheletti *et al*. 2008). The EPS from *Azotobacter chroococcum* strain has been studied for its ability to adsorb lead and mercury from its metal solutions (Bakhtiyor *et al*. 2013). Interestingly similar observations have been reported in case of zerovalent iron nanoparticles in which they have the potential of reducing the carcinogenic form of chromium +6 to a non-carcinogenic form of chromium +3. (Nuxoll *et al*. 2003, Arnold and Roberts 2000).

4.3.5 Studies on siderophore production by *Halobacillus trueperi* MXM-16

The ability of the bacteria to produce siderophores is crucial to the survival of the bacteria as they depend on these chelating molecules to acquire the metal iron from its surrounding environment. *Halobacillus trueperi* MXM-16 was found to produce siderophore (Fig. 4.8). Characterization of the siderophore indicated that it was a hydroxamate type of siderophore

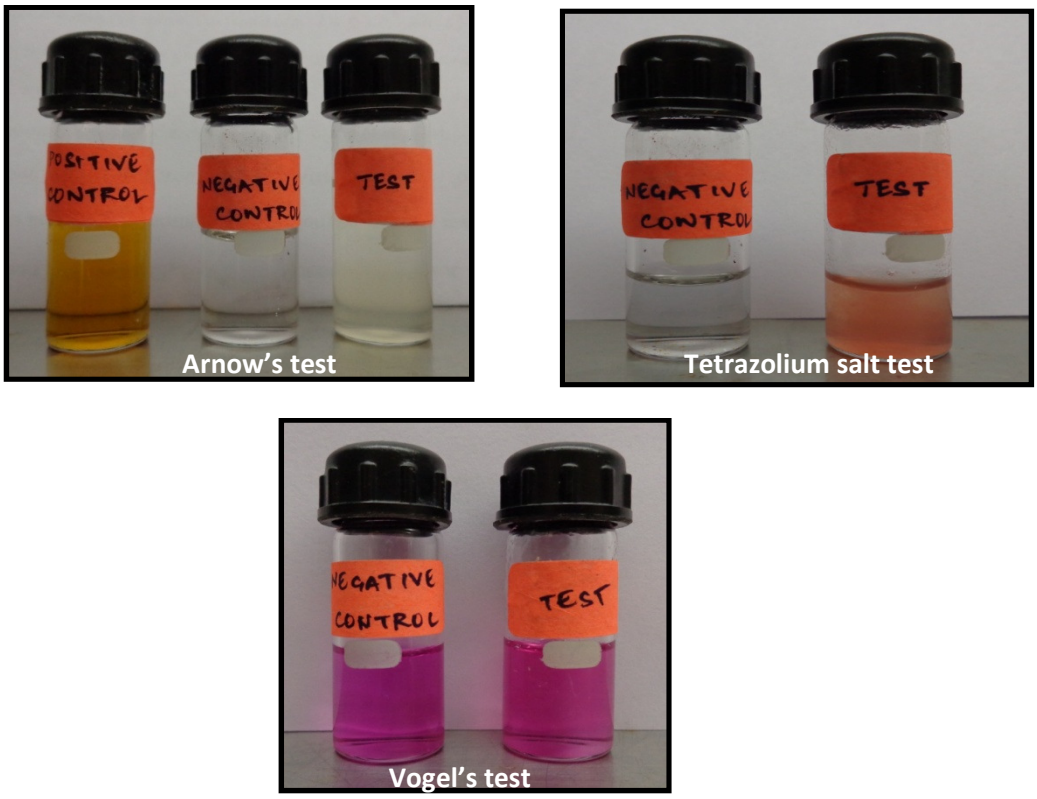

Fig. 4.8: Characteristic tests for the identification of the type of siderophore produced by *Halobacillus trueperi* MXM-16.

The Csaky's test showed a peak between 420-450 nm and the tetrazolium assay showed a reddish-pink colouration characteristic of the hydroxamate siderophore. It has been reported that hydroxamate siderophores are highly resistant to environmental degradation associated with the wide range of hydrolytic enzymes that are present in humic soil such as that of mangrove ecosystem (Winkelmann 2007). Hydroxamate siderophores produced by *Pseudomonas fluorescens* (Ali and Vidhale 2013), *Magnetospirillum magneticum* (Calugay *et al*. 2003) and root nodule bacteria such as *Rhizobium* and *Sinorhizobium* (Carson *et al*. 2000) have been reported. Bacteria from the sand dune ecosystem like *Microbacterium arborescens, Kocuria rosea* and *Bacillus* sp have been reported to produce siderophores while *Pseudomonas aeruginosa* isolated from the coastal sand dunes produced the siderophore pyoverdine (Godinho *et al*. 2010, Gaonkar *et al*. 2012).

4.3.6 Studies on pigment produced by *Halobacillus trueperi* MXM-16

The study on pigments revealed that *Halobacillus trueperi* MXM-16 produced a non diffusible orange pigment (Fig. 4.9). The orange colour of the pigment was attributed to the presence of carotenoids. These carotenoids are responsible for the cream white, pale yellow or bright orange colouration of the cells in the genus *Halobacillus* (De Vos *et al*. 1986). The carotenoid compounds in the pigment were identified by the spectral data in the UV-Vis spectrophotometer and the HPLC retention times.

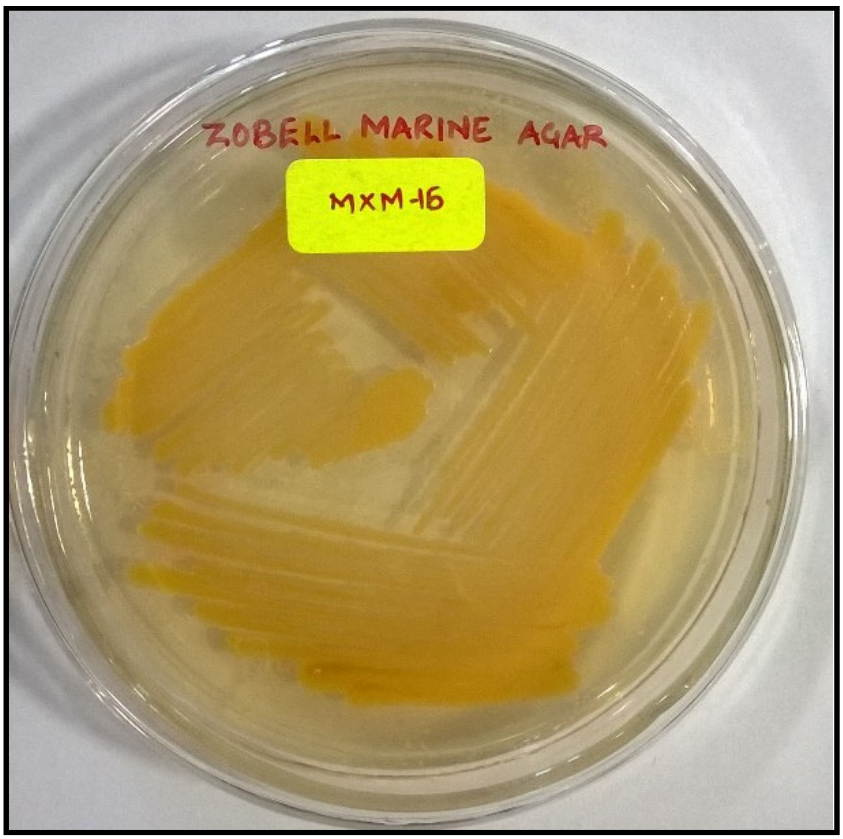

Fig 4.9 Growth of *Halobacillus trueperi* MXM-16 on ZMA medium.

The present study showed the extraction of the pigment in the solvents methanol and acetone (Sasidharan *et al*. 2013, Nugraheni *et al*. 2010). The UV-Vis scan of the pigment extracted in acetone showed the characteristic three peaks seen in case of carotenoid pigments (Fig. 4.10). These peaks were seen at 553, 468 and 341nm respectively. These wavelengths are characteristic absorbance maxima for the carotenoids owing to their polyene chromophores which absorb the light in the 400-500 nm region. This is the basis for the ability of the carotenoids to quench singlet oxygen and appear yellow to orange in colour. A number of carotenoid pigments have been reported from bacteria such as *Micrococcus roseus*, *Staphylococcus aureus* and *Microbacterium arborescens* (Godinho and Bhosle 2008). These carotenoid pigments being non-diffusible form an integral part of the bacterial cell membrane and influence the effectiveness of the membrane as a barrier to water molecules, oxygen and other small molecules (Britton 1995). Currently it has been reported that carotenoids are also converted to vitamin A (retinol) and thus is an important source of this vitamin. Other medical uses include treating sunburn, anti-inflammatory diseases and cancer therapy (Van den Berg 2000, Clinton and Libby 1992). Reports also indicate that the bacteria accumulate carotenoids as a response to environmental stress and it thus aids the bacteria to survive in such environments (Bhosale 2004). Significantly, the spectral data showed a peak at 220 nm in the UV region. Such peaks have been noticed in UV-Vis spectrum of pyoverdine and azotobactin (Tank *et al*. 2012, Sharma and Gohil 2010). Interestingly both pyoverdine and azotobactin are mixed ligand siderophores. Studies have shown that pigments are capable of acting as siderophores by chelating

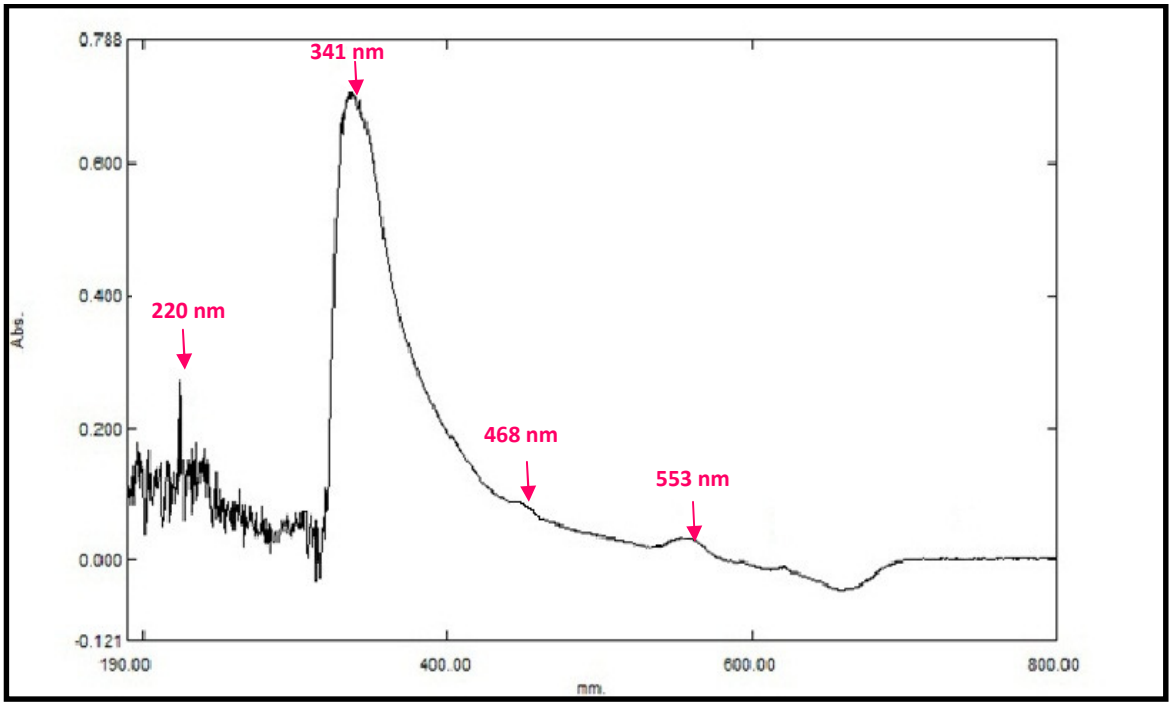

Fig. 4.10: UV-Vis profile of pigment produced by *Halobacillus trueperi* MXM-16

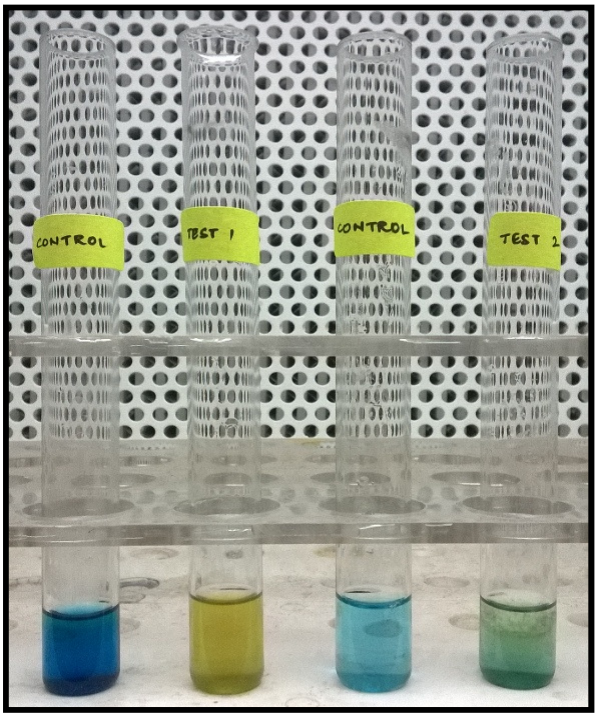

Fig. 4.11: Ability of the pigment to change colour of CAS solution.

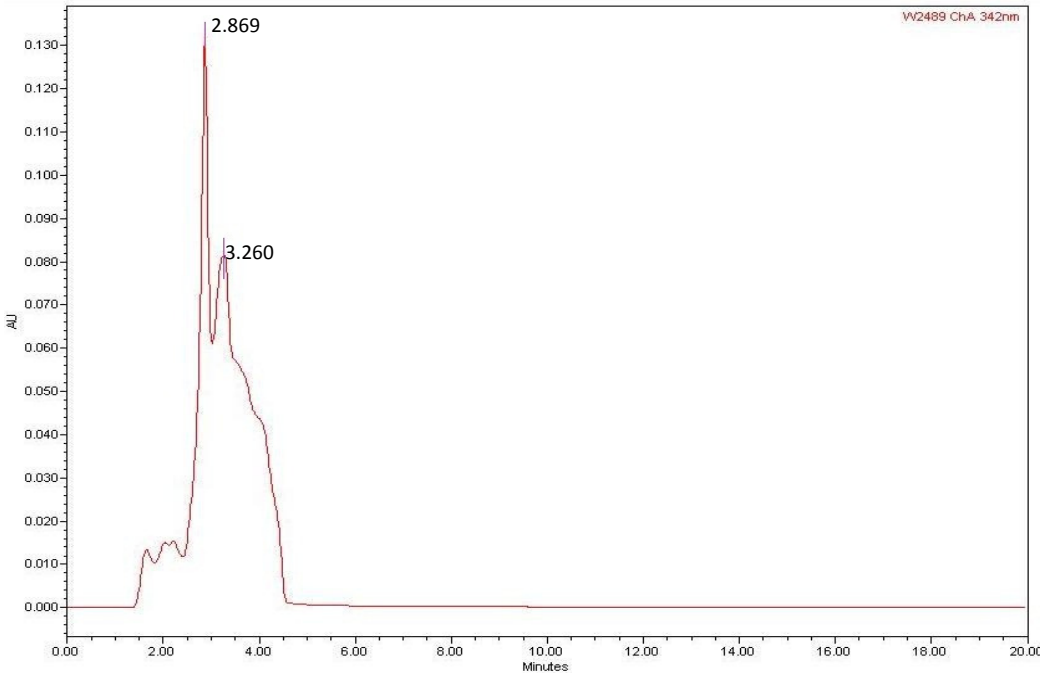

Fig. 4.12: HPLC profile of the carotenoid pigment produced by *Halobacillus trueperi* MXM-16 at 342 nm.

and sequestering iron from the environments. In the current investigation, the ability of the pigment to chelate iron from the CAS medium was studied. An interesting observation was that, the CAS solution turned yellow on addition and incubation with the pigment (Fig. 4.11).

This was a significant discovery as it indicated that the pigment aided the bacteria to acquire iron from the environment and the *Halobacillus trueperi* MXM-16 pigment may be associated with a siderophore. The fluorescent pigment pyoverdine, from *Pseudomonas aeruginosa* is one such example that is known to be a powerful scavenger and efficient transporter of Fe (III) (Gaonkar *et al*. 2012, Meyer 2000). The HPLC of the pigment revealed two prominent peaks at 342 nm as shown in Fig. 4.12. The first peak with a retention time of 2.86 min remained unidentified. However, a similar unidentified peak has also been reported in HPLC scan of from *Pseudomonas fluorescence*. The second peak with the retention of 3.26 min is indicative of a component called astaxanthin. Astaxanthin has previously been reported in *Exiguobacterium* sp (Sasidharan *et al.* 2013). Interestingly, *Exiguobacterium* and *Halobacillus* both belong to the family Bacillaceae (De Vos 1986). Astaxanthin is a keto-carotenoid that belongs to the xanthophyll family of the carotenoids. Astaxanthin is a colourful lipid-soluble pigment that is currently approved by the FDA for use as a food colourant in animal and fish feed. The colouration is due to the extended chain of alternating double and single bond at the centre of the compound. This extended chain confers the compound with the antioxidant properties (Eldahshan and Singab 2013).

This study revealed that *Halobacillus trueperi* MXM-16 has the ability to

a) grow on sodium benzoate with production of siderophores. However, it was observed that no pigment was produced when grown in the presence of this aromatic compound.

b) produce EPS which was glycoprotein in nature.

c) produce a non-diffusible carotenoid pigment capable of chelating iron from the media with one of its components being indicative of astaxanthin (retention time: 3.26, wavelength: 342 nm).

d) show a typical ability to adhere to the hydrocarbon and also produced a siderophore which helps the culture in the environment to utilize the nutrients and to chelate iron under iron limiting conditions. Such a production of siderophores by this species is not reported so far.

In this age of nanotechnology there is a growing concern on the nanoparticles being released in the environment. Such nanoparticles could affect specific characteristics of organisms in the mangrove ecosystems. Therefore it was of significant interest to study the effect of these nanoparticles on the isolates from the mangrove ecosystem. A detailed study on the effect of iron nanoparticles on adhesion and siderophore production of the adhered bacteria has been presented in the following chapter. The effect of these nanoparticles on the growth and EPS of *Halobacillus trueperi* MXM-16 was also explored and has been presented in Chapter 5.

Chapter 5:

Effect of zerovalent iron nanoparticles on:

a) Adhesion to hydrocarbon and production of siderophores by selected bacterial isolates

b) Growth and EPS of *Halobacillus trueperi* MXM-16

5.1 Introduction

Nanoparticles have dimension that measures 100 nm or less. Due to the small size of the particles it exhibits a larger surface area and hence has a high surface area to volume ratio. This allows the nanoparticles to possess physical properties different from the bulk particle e.g. gold nanoparticles having a particle size of 2.5 nm melt at a temperature of ~300°C while a block of gold requires a temperature of 1064°C for melting. Similarly, gold nanoparticles show reddish black colouration in a solution as opposed to the gold coloured solution of a large sized gold particle (Buffat and Borel 1976).

Over the past decade nanoparticles like silver, titanium dioxide, zerovalent iron and bimetallic iron have garnered a lot of interest for their use in the fields of engineering, environmental bioremediation and medicine.

Iron is the most abundant element in the Earth's crust. It is also the most important element required in trace amounts for various biological processes. Iron nanoparticles are of particular interest as they have potent magnetic and catalytic properties (Huber 2005). Research on iron nanoparticles has shown that these particles are extremely reactive than their counterpart bulk metal due to the increased surface area. Interestingly, iron nanoparticles represent the only field application of free released nanoparticles for environmental pollution. Zerovalent iron nanoparticles (ZVI) nanoparticles are used for remediation of groundwater contaminated with perchloroethylene and trichloroethylene (Senzaki and Kumagai, 1988) and currently,

ZVI nanoparticles are being studied for their role in bioremediation of other polychlorinated compounds (Fulekar *et al*. 2014, Cameotra and Dhanjal 2010, Tunqittiplakorn *et al*. 2005) and uranium contaminated effluents (Dickinson and Scott 2010).

Research has shown a correlation between bacteria and nanoparticles. Bacteria like *Bacillus licheniformis, Rhodococcus* and some filamentous cyanobacteria (Li *et al*. 2011) are reported to produce gold nanoparticles while *E. coli* and *Pseudomonas* (Saklani *et al*. 2012) produce silver nanoparticles when grown in extreme conditions of alkalinity and elevated growth temperatures. Although bacteria have garnered interest in the production of nanoparticles, reports also show the toxicity of nanoparticles on bacteria. Silver and zinc oxides show inhibitory effect on the growth and EPS production by *Pseudomonas* when used in increasing concentration (Dhas *et al*. 2014). Silver nanoparticles have been found to have toxic effects on the growth of bacteria. Studies have shown that their presence inhibits the bacteria and reduces the total viable count. Hence silver and zinc oxides are widely being used as antimicrobial agents (Dhas 2014). Reports by Mahdy *et al*. (2012) reveal the antimicrobial activity of zerovalent iron nanoparticles on *E.coli* and *Staphylococcus aureus.*

Interestingly, a recent study reported the acquisition of iron from ferrihydride nanomineral by the bacteria *Pseudomonas mendocina* by siderophores and a cell-associated metalloreductase. This study also revealed the copious amounts of EPS produced by the *Pseudomonas mendocina* which enhanced the acquisition of iron from the nanomineral (Kuhn *et al*. 2014). Nanoparticles like zinc oxide and copper

oxide are also reported to inhibit production of pyoverdine siderophore in *Pseudomonas chlororaphis* O6 (Dimkpa *et al*. 2012).

Considering the interest that the applications of zerovalent iron nanoparticles are generating and the fact that the ZVI nanoparticles are the only nanoparticles currently on field application, it is inevitable for them to be present in the surrounding soil and water. Therefore, there is a need to assess and address the eco-toxicological effects of these nanoparticles (Boxall *et al.* 2007). As in the natural ecosystem, presence of elements, their form and concentration are known to affect the metabolic activities and hence the remediation process (Gaonkar and Bhosle 2013). The present work was directed to understand the effect of the ZVI nanoparticles on adhesion ability and production of siderophores by adhered bacteria from the mangrove ecosystem. Our work also focused on the effect of these nanoparticles on the growth and EPS production by the bacterial strain *Halobacillus trueperi* MXM-16.

5.2 Materials and methods

5.2.1 Preparation of zerovalent iron nanoparticles

Zerovalent iron nanoparticles were synthesized using the borohydride method (Rashmi *et al*. 2013). For synthesis of ZVI nanoparticles, 0.5406 g of $FeCl_3.6H_2O$ was dissolved in 4:1 (v/v) ethanol/water mixture and stirred well. 0.3783g of $NaBH_4$ was dissolved in 100 ml de-ionised distilled water. The borohydride solution was poured in the burette and added drop to drop into the iron chloride solution with vigorous hand

stirring. After the addition of the first drop of sodium borohydride, black solid particles immediately appeared and the remaining sodium borohydride solution was added to completely accelerate the reaction. The mixture was left for another 10 min of stirring after adding the entire sodium borohydride solution. The vacuum filtration technique was used to separate the black nanoparticles from the liquid phase. The solid particles were washed three times with 25 ml portions of absolute ethanol to remove water. The synthesized nanoparticles were dried in the oven at 50°C overnight. The nanoparticles were stored in a thin layer of ethanol to prevent oxidation.

5.2.2 Effect of ZVI nanoparticles on adhesion of selected isolates

The ability of the bacterial isolates to adhere to hydrocarbons was seen using the bacterial adhesion to hydrocarbon assay (BATH) as mentioned in 1.2.7. The test contained 2 ml of cell pellet dissolved in PBS. To this 0.5 ml of hexadecane and 300µg of ZVI nanoparticles were added. The control tubes were prepared without the ZVI nanoparticles. The protocol for BATH assay was followed further as mentioned in 1.2.7.

5.2.3 Effect of ZVI nanoparticles on siderophore production of selected isolates

The selected 16 isolates were spot inoculated on of NA-CAS/ NTYE-CAS agar containing 0.1g/L of ZVI nanoparticles. CAS agar without ZVI nanoparticles served as the positive control for siderophore formation and CAS with $FeCl_3$ was used as the

negative control for siderophore. The plates were incubated at room temperature (28°C) for 48-72 hr and the zones of colour change around the bacterial colonies were observed.

5.2.4 Effect of ZVI nanoparticles on *Halobacillus trueperi* MXM-16

a) Growth and EPS production

Bacterial strain MXM-16 was grown on 500 ml of ZMB without ZVI nanoparticles and ZMB with 0.1g/L of ZVI nanoparticles under shaking conditions (Remi CIS 24 BL) of 150 rpm at 28°C. Aliquots of 5 ml were withdrawn from each flask every two hours for 24 hr under sterile conditions and absorbance of the culture suspension was measured at 600 nm using UV-Vis spectrophotometer (Shimadzu UV 2450). The culture broth was pelleted and the biomass was collected in a previously weighed tube, allowed to dry and weighed.

b) EPS production

The supernatant from the aliquots was used to determine the sugar content by the phenol sulphuric acid method as mentioned in section 3.2.4. The EPS content was determined by treating 4 ml of the culture suspension with twice the volume of cold ethanol overnight at 4°C. The EPS precipitated was centrifuged (Eppendorf 5804R) at 8000 rpm for 20 min, dried and weighed (Shimadzu AX 200). SEM-EDS were performed in the Jeol JSM 6360 LV microscope. The samples were prepared by progressive dehydration using acetone (Merck). The slides were fixed onto a solid

support and covered with a film of graphite and then observed under the SEM-EDS microscope.

c) Emulsification of hydrocarbon

The strain MXM-16 was grown for 24 hr on ZMB in shaker conditions (Remi CIS 24 BL) in the presence and absence of ZVI nanoparticles and the culture broth was centrifuged (Eppendorf 5804R) at 8000 rpm for 10 min at 4°C. To 2ml of the cell free supernatant, 0.5 ml of hexadecane was added and vortexed for 2 min. Control was maintained using sterile uninoculated broth with hexadecane. The tubes were allowed to settle for 24 hr and the absorbance of the lower aqueous layer was measured at 450 nm using UV-Vis spectrophotometer (Shimadzu UV-2450).

5.3 Results and discussion

5.3.1 Effect of ZVI nanoparticles on adhesion of selected isolates

The ability of bacteria to adhere, anchors the bacterial cell to a substrate in a suitable environment. This ability is a crucial aspect of the mangrove bacteria as it prevents it from being washed away into the sea by the tidal action. The 16 bacterial isolates when subjected to BATH assay, showed 88% of the isolates adhering to the hydrocarbon, among these, all halotolerant were capable of adhering to hydrocarbon but only 60% of halophilic bacterial isolates depicted this ability. However, it was observed that, when the BATH assay was carried out by addition of ZVI nanoparticles, the percentage of bacterial isolates exhibiting adhesion to hydrocarbon

decreased while there was an increase in the percentage of isolates showing emulsification. Among the 16 isolates, 56% showed adhesion and 44% showed emulsification with the hydrocarbon (Table 5.1). It was significant to note that the isolates that had earlier showed adherence to the hydrocarbon either showed a decrease in the percentage of adhesion or now exhibited emulsification with the hydrocarbon in presence of ZVI nanoparticles. The isolates that exhibited emulsification without the ZVI nanoparticles showed a greater percentage of emulsification in the presence of ZVI nanoparticles. This indicated the ability of the ZVI nanoparticles to affect the bacterial cell surface thus transforming adherent bacteria to emulsifying bacteria. Bacteria that showed maximum adhesion was MXM-8 which was identified as halotolerant isolate *Acinetobacter schindleri* MXM-8. Reports have showed the effective removal of *Pseudomonas aeruginosa* and *Bacillus cereus* cells from stainless steel using silver nanoparticles (Araujo *et al*. 2012). The mechanism could be attributed to the decrease in adhesion ability of the cells also resulting in their removal from the surface. Similar transformation of bacterial cells from adhesion to emulsification of the hydrocarbon have been reported in the presence of biosurfactant in which the biosurfactant not just increased the availability of the hydrocarbon to the cell but also increased the affinity of the cell to the hydrocarbon by altering the cell surface (Kumar *et al*. 2006).

Table 5.1 Adhesion and emulsification by the adhered bacterial isolates by BATH assay.

Isolate no	Without ZVI nanoparticles	% Adherence or % Emulsification	With ZVI nanoparticles	% Adherence or % Emulsification
MXM- 1	adherence	0.487	adherence	0.246
MXM- 4	emulsification	15.63	emulsification	21.25
MXM- 5	adherence	30.66	emulsification	4.84
MXM- 7	adherence	26.98	emulsification	50.09
MXM- 8	adherence	35.72	emulsification	10.34
MXM- 9	adherence	29.82	emulsification	6.64
MXM- 10	adherence	31.05	adherence	1.143
MXM- 12	adherence	19.56	adherence	1.057
MXM-15	emulsification	2.36	emulsification	9.615
MXM- 16	adherence	30.17	adherence	5.485
SDM- 2	adherence	24.41	emulsification	1.982
SDM- 4	adherence	27.12	emulsification	1.367
SDM- 5	adherence	20.98	adherence	3.31
SDM- 9	adherence	16.71	adherence	6.739
SDM- 10	adherence	16.63	adherence	0.947
SDM- 12	adherence	16.04	adherence	2.277

5.3.2 Effect of Fe nanoparticles on siderophore production

Iron is the basic requirement for bacterial metabolism and its concentration in the surrounding environment has significant effects on cell processes and metabolic products. The effect of ZVI nanoparticles on growth and siderophores was studied. Among the 13 siderophore producing adhered bacteria, 44% of the isolates, showed significant increase in siderophore formation in the presence of ZVI nanoparticles of which 29% were halophiles and 71% were halotolerant adhered bacteria. Two isolates, both halophiles (*Halobacillus trueperi* MXM-5 and *Halobacillus trueperi* MXM-16) showed an increase in colony diameter but no increase in the siderophore production was observed. Interestingly, one isolate MXM-10, a halotolerant isolate, showed decreased growth with increased siderophore formation. (Fig.5.1). The siderophore zone size has been mentioned in Table IV E (Appendix IV). The effect as depicted in Fig 5.2 (a-c) showed a significant increase in siderophore formation in the presence of ZVI nanoparticles in a halotolerant isolate SDM-12. However in case of halotolerant isolate MXM-10 it was seen that a significant increase in the siderophore production, was also accompanied by a decrease in the diameter of the bacterial colony (Fig 5.3 a-c). Such negative effects on the growth of the bacteria due to the presence of metals have been reported by (Gaonkar and Bhosle 2013, Wyszkowska *et al*. 2008). It was observed that although the presence of ZVI nanoparticles showed an increase in the siderophore zone size, no decrease in the siderophore zone size was observed.

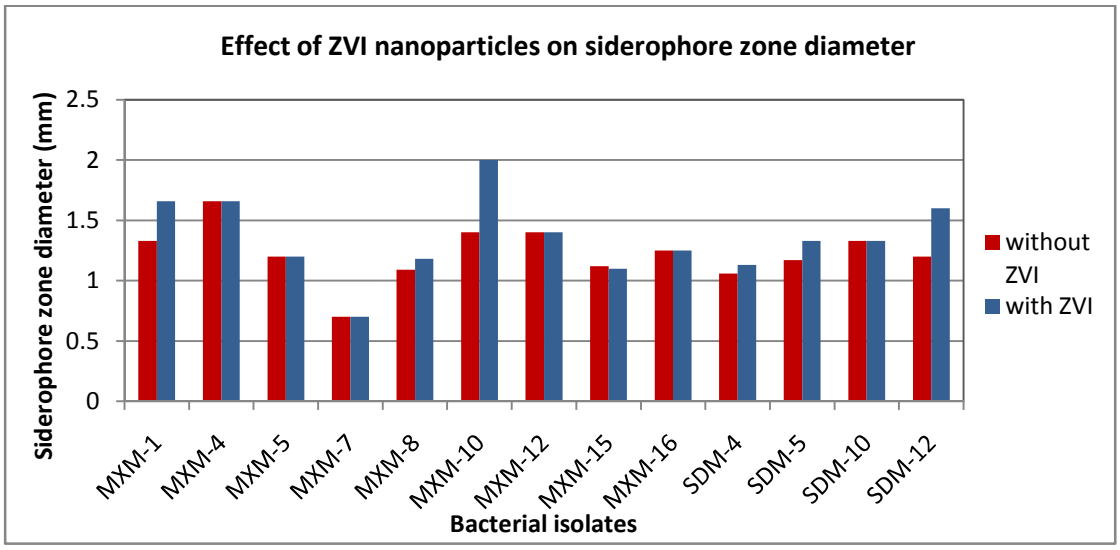

Fig:5.1: The effect of ZVI nanoparticles on the siderophore zone diameter

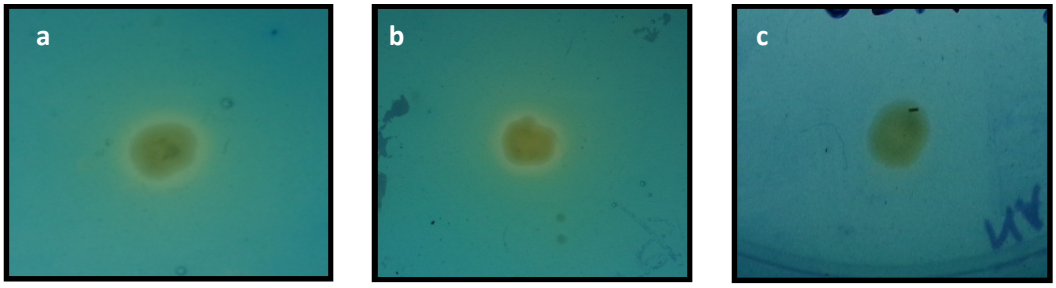

Fig 5.2: a) Isolate SDM-12 grown on a) CAS medium b) CAS medium with ZVI nanoparticles c) CAS containing $FeCl_3$.

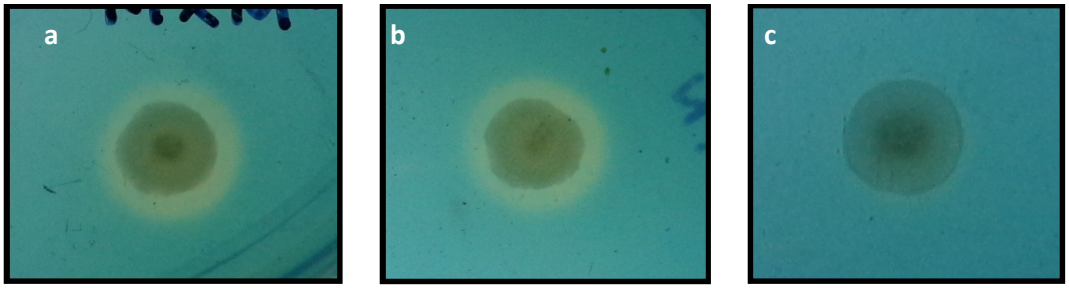

Fig 5.3: a) Isolate MXM-10 grown on a) CAS medium b) CAS medium with ZVI nanoparticles c) CAS containing $FeCl_3$.

Our studies thus indicated that the ZVI nanoparticles not only served as a source of iron to the bacteria but in many bacteria they triggered elevated production of siderophores in order to acquire the metal. Previous reports have demonstrated the ability of bacteria to acquire iron from nanominerals such as ferrihydride. *Pseudomonas mendocina* acquires iron from ferrihydride nanoparticles by producing siderophores and metalloreductases (Kuhn *et al*. 2014) while *Pseudomonas aeruginosa* has been reported to obtain iron from ferritin and ferrihydride nanomineral by siderophore dependent mechanisms (Dehner *et al*. 2013).

5.3.3 Effect of ZVI nanoparticles on growth and EPS production of *Halobacillus trueperi* MXM-16.

Halobacillus trueperi MXM-16 was a unique strain and thus considered for further studies. The strain showed a typical growth curve with lag, exponential and stationary phase (Fig 5.4). The EPS curve was also similar to the growth curve and indicated that the EPS was growth associated EPS. Such growth associated EPS have also been reported from *Lactobacillus pantosus* (Sanchez *et al*. 2006). This could also mean that the EPS played a crucial role in the adhesion of the bacterial cell. Interestingly, it was observed that when the culture was grown in the presence of ZVI nanoparticles the growth increased by 6% and the EPS production showed an increase by 8% as compared to the growth and EPS when no ZVI was present in the medium as indicated in Fig 5.4(a) and 5.4(b). Similar effects have been reported for *Paenibacillus polymyxa* showed that the presence of Fe (III) in the medium showed increase in growth of the

bacteria and also revealed that, with the increase in Fe (III) there was an increase in the intra and the extracellular protein and carbohydrate content (Raza *et al*. 2010).

5.3.4 SEM-EDS

In SEM-EDS microscopy it was observed that more number of cells and EPS were seen when the culture was grown in the presence of ZVI nanoparticles. Interestingly, in the EDS spectra (Fig. 5.6 a and b) it was observed that EDS of the EPS showed an increase in the iron content and the bacterial cell surface exhibited an increase in the carbon content when grown in the presence of ZVI nanoparticles as compared to when the ZVI nanoparticles were absent (Fig 5.6 c and d). This indicates the ability of the EPS of the bacterial strain to interact with the iron from the ZVI nanoparticles resulting in the increase in the content of iron in the EPS. Similar SEM imaging of cells has been reported in case of *Pseudomonas aeruginosa* (de Souza and Bhosle 2012) and accumulation of metals by the EPS of bacterial cells have been reported in earlier studies on *Bacillus* sp, *Serratia* and *Kocuria* (Francois *et al*. 2012), *Rhodobium marinum* and *Rhodobacter sphaeroides* (Panwichian *et al*. 2011).

5.3.5. Effect of ZVI nanoparticles on emulsification of hydrocarbon by the MXM-16 culture supernatant.

The absorbance of the aqueous layer of the cell free supernatant of the isolate MXM-16 grown in the presence of ZVI nanoparticles was found to be more as compared with that of the supernatant grown in the absence of ZVI nanoparticles. Fig 5.7 shows

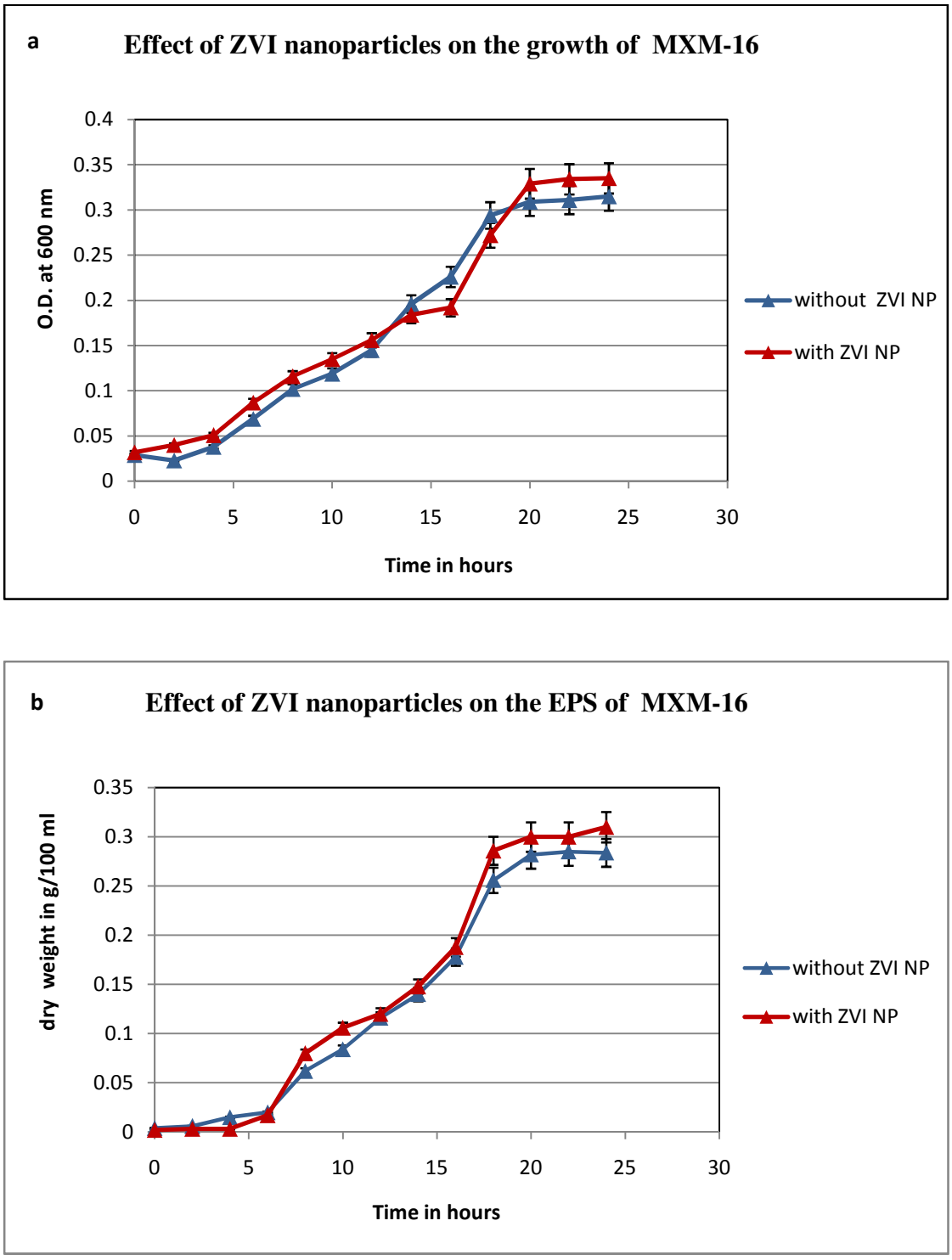

Fig: 5.4: The effect of ZVI nanoparticles on the growth (a) and EPS production (b) of *Halobacillus trueperi* MXM-16.

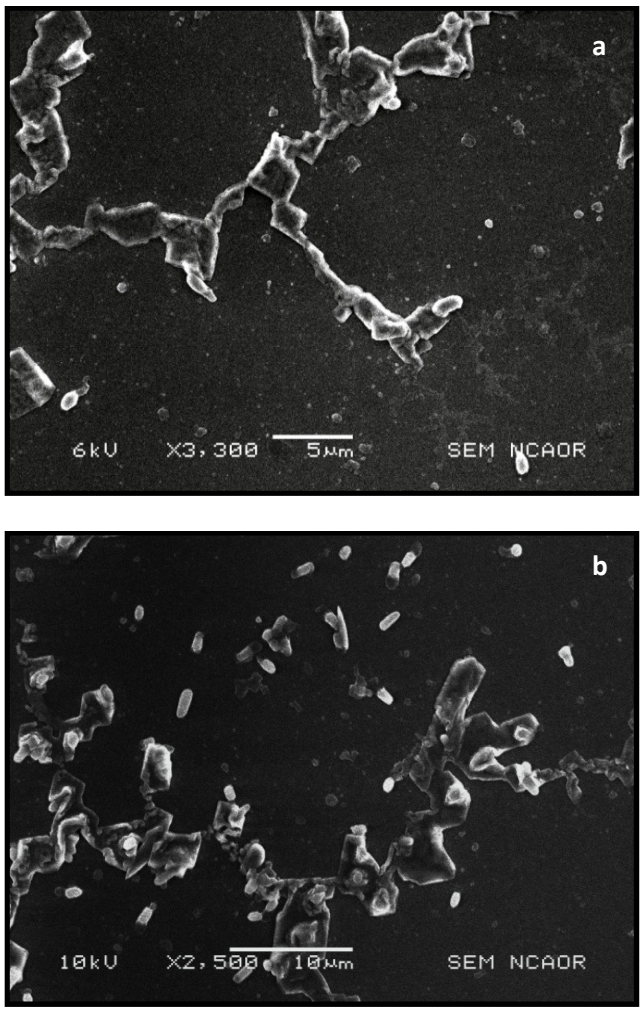

Fig. 5.5: SEM images of *Halobacillus trueperi* MXM-16 when grown in the absence (a) and presence (b) of iron nanoparticles.

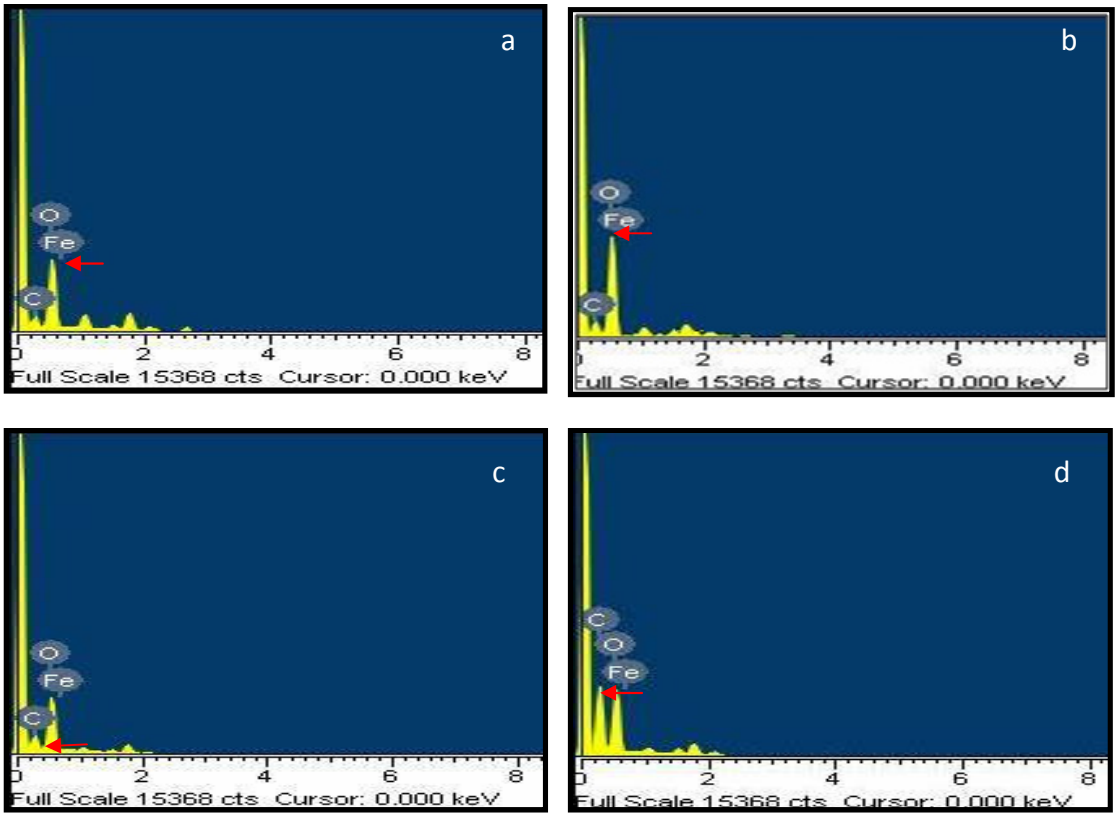

Fig. 5.6: The EDS of EPS of *Halobacillus trueperi* MXM-16 grown in the absence (a) of ZVI nanoparticles and EPS when grown in the presence (b) of ZVI nanoparticles. The EDS of cells of *Halobacillus trueperi* MXM-16 when grown in the absence (c) and presence (d) of ZVI nanoparticles.

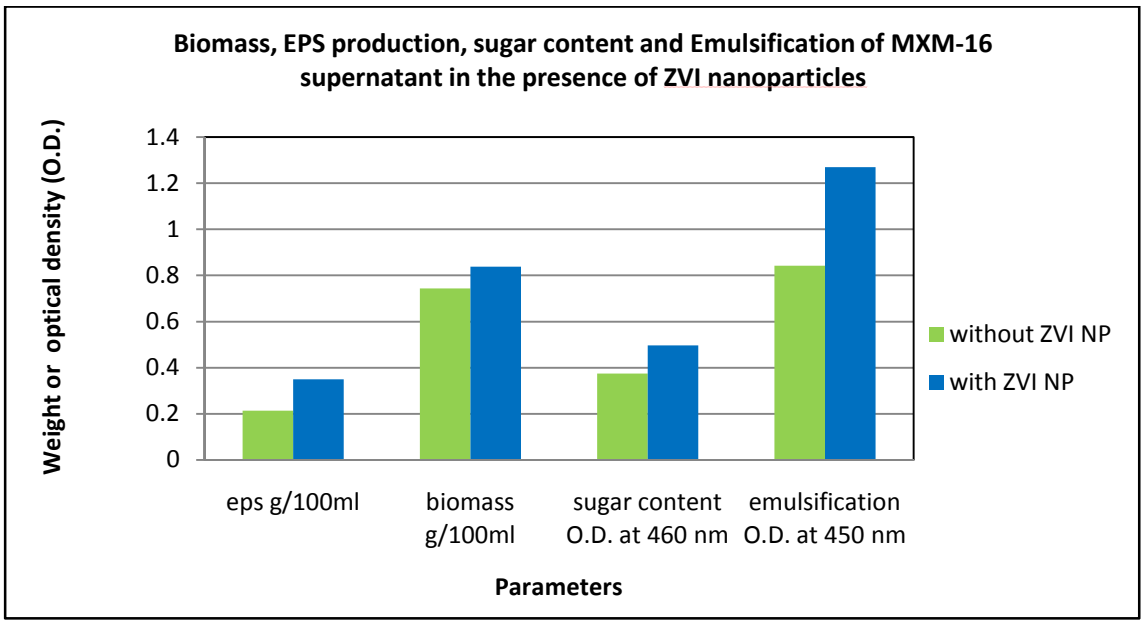

Fig. 5.7: EPS, biomass, sugar content and emulsification of the supernatant of *Halobacillus trueperi* MXM-16 when grown in the presence of ZVI nanoparticles.

the increase in the biomass and the sugar content when the culture was grown in the presence of ZVI nanoparticles . This can be attributed to the increase in metabolism of the bacteria that results in enhanced growth of the bacteria in the presence of ZVI nanoparticles which in turn increases the biomass. The increase in sugar content is due to the increase in EPS that contributes to the sugar in the supernatant. Similar findings have been reported in *Paenibacillus polymyxa* where the growth and the intra and extracellular content of the carbohydrate increased with an increase in Fe (III) concentration in the medium (Raza *et al*. 2010).

This study is the first study that revealed that ZVI nanoparticles could affect the emulsification of hydrocarbon and siderophores positively. It revealed the ability of the ZVI nanoparticles to alter cell surface properties transforming adhered bacteria to emulsifying bacteria. It was also observed that the growth and EPS in *Halobacillus trueperi* MXM-16 increased in the presence of ZVI nanoparticles.

Summary

Adhesion is a crucial phenomenon in localization of a bacterial cell and colonization of a surface. It is also the preliminary step in the formation of biofilm. Thus, adhesion has significant ecological implications in degradation of particulate organic matter and nutrient recycling in ecosystems like the mangroves. Mangrove ecosystem is one of the most productive ecosystems. However to maintain the efficiency of recycling and degradation the halophilic and halotolerant bacteria in this ecosystem has to acquire the metal iron from the environment. This iron requirement is fulfilled by producing iron sequestering molecules called siderophores.

The Mandovi and the Zuari mangroves of Goa, showed a prolific population of halophilic and halotolerant adhered bacteria in the plant litter samples. This was attributed to the fluctuation in the salinity of the mangrove ecosystem due to the influx of tides. Out of the 34 bacterial isolates isolated 68% were halotolerant and 32% were halophilic bacteria indicating the predominance of such bacteria in the mangrove ecosystem. The isolates were screened for their ability to produce multiple enzymes that could degrade complex natural polymers such as cellulose, hemicellulose, lignin, starch, tannin, lipids and chitin from mangrove plant litter.

Based on the ability of the isolates to produce multiple enzymes, 16 isolates were studied further for their ability to adhere to the hydrocarbon and produce siderophores. Interestingly, it was observed that 88% showed adhesion to hydrocarbon and 81% isolates produced siderophores. Ten isolates were selected for further studies based on their ability to adhere to hydrocarbon and produce siderophores. These isolates were screened for EPS and it was observed that all ten isolates showed EPS production.

These were tentatively identified as *Halococcus* (MXM-1), *Aeromonas* (MXM-4), *Halobacillus* (MXM-5), *Brevibacterium* (MXM-7), *Acinetobacter* (MXM-8), *Erwinia* (MXM-10), *Aeromonas* (MXM-12), *Halobacillus* (MXM-16), *Vibrio* (SDM-2) and *Staphylococcus* (SDM-4). Mineralization studies were carried out using the ten isolates. A decrease in the weight of the plant litter sample and an increase in protein, sugar and siderophore content of the mineralizing medium were observed in the inoculated sample. This indicated an overall increase in the efficiency of degradation of plant litter as compared to the uninoculated control.

Based on the tentative identification the isolates MXM-5, MXM-8 and MXM-16 were identified further by 16S rRNA sequencing as *Halobacillus trueperi* MXM-5, *Acinetobacter schindleri* MXM-8 and *Halobacillus trueperi* MXM-16. Since there were relatively few reports on EPS of *Halobacillus trueperi* and no reports on the siderophores by *Halobacillus trueperi* it was selected for further studies on siderophores and EPS.

Halobacillus trueperi MXM-16 showed growth on medium containing sodium benzoate with an ability to cleave the aromatic ring via the ortho cleavage pathway. However, no pigment was present when grown in the presence of this aromatic compound. This loss of pigmentation depicted that though the culture was capable of utilizing sodium benzoate, the culture experienced oxygen stress and thus did not produce the pigment. This alteration can find potential applications in bioremediation and monitoring studies.

The strain *Halobacillus trueperi* MXM-16 was found to produce hydroxamate siderophores which hither to is the first such report in case of this bacteria. The pigment studies showed that the pigment was a carotenoid pigment with astaxanthin as one of the components. A significant observation was the ability of the pigment to scavenge Fe from the medium thus indicating that it might be associated with the siderophore.

Furthermore, the EPS of *Halobacillus trueperi* MXM-16 was studied and was observed to contain an inorganic content of 86% and an organic content of 14%. The organic content contained approximately 1022 µg/ml of protein and 400 µg/ml of sugar. The EPS was a glycoprotein that was capable of reducing carcinogenic Cr (+6) to non-carcinogenic Cr (+3) and showed adhesive properties. However, though the EPS could emulsify hydrocarbon it did not possess any surface active properties. Thus, the EPS was a bioemulsifier and could be suitably employed in hydrocarbon remediation and oil spill management. The siderophores of MXM-16 were also studied and characterized as hydroxamate siderophores.

As reported ZVI nanoparticles are the only nanoparticles in field application, it was necessary to understand its effects on the bacteria in the environment. The influence of ZVI nanoparticles on adhesion and siderophores of mangrove bacteria revealed that, the presence of nanoparticles transformed adhering bacteria into emulsifying bacteria and increased the percentage of emulsification with the hydrocarbon of the emulsifying isolates. An increase in the siderophore production of 44% isolates was observed in the presence of ZVI nanoparticles. The growth and EPS of strain MXM-

16 was positively affected when grown in the presence of ZVI nanoparticles. This was corroborated by the increase in the sugar content and emulsification of the cell free culture supernatant. The EDS showed an increase in Fe content of EPS and carbon content of cells when grown in the presence of ZVI nanoparticles. The increase in Fe content indicates accumulation of the iron from the ZVI nanoparticles by the EPS and the increase in carbon content can be attributed to the need for more EPS production by cells in the presence of ZVI nanoparticles. This study projected that ZVI nanoparticles support the mechanisms of bacteria from the mangrove ecosystem indicating that they were safe for field trials.

Significance

The research highlights the prevalence of halophilic and halotolerant adhered bacteria in the Mandovi and Zuari mangrove ecosystems of Goa and signifies the importance of adhesion in the bacteria in such ecosystems.

The multiple enzymes produced by the halophilic and halotolerant adhered bacterial isolates indicate the crucial role played by these bacteria in the degradation of mangrove plant litter. The high percentage of halotolerant bacteria producing multiple enzymes show that the degradation of particulate organic matter in this ecosystem is brought about by such halotolerant bacteria. Moreover the ability of these isolates to adhere to hydrocarbon, produce siderophores and EPS further enforces their role in nutrient recycling.

A significant observation was the increase in the efficiency of plant litter degradation that was observed in the presence of bacterial consortium composing of these isolates.. The work reveals the importance of such bacteria in solid waste management, bioremediation and composting.

One of the significant outputs of the research has been the study on *Halobacillus trueperi* MXM-16. This is the first report on siderophore production by *Halobacillus trueperi* MXM-16 which is characterized as hydroxamate siderophore. Further the study also revealed that the pigment produced by this has the ability to scavenge iron from the medium.

It was also observed that the strain could grow on sodium benzoate cleaving the aromatic ring by the ortho pathway, thus depicting its potential in bioremediation studies of soils contaminated with aromatic compounds.

The emulsifying characteristics depicted by the EPS produced by this culture are of significant importance in bioremediation of hydrocarbon and oil spill management and its adhesive properties is of interest in commercial applications. The property of transformation of chromium +6 to chromium +3 could find potential applications in the treatment of carcinogenic chromium and needs further study.

This study also demonstrated the ability of the ZVI nanoparticles to alter bacterial cell surface thereby enhancing their ability to emulsify hydrocarbons. They showed a positive effect on the siderophore production of the isolates and was shown to positively increase growth and EPS production in *Halobacillus trueperi* MXM-16. The positive influence of these ZVI nanoparticles thus implicates the safety in use of these nanoparticles in the environment.

Future Perspectives

The work in the thesis has given some interesting results which need to be further studied to understand the mechanisms or their application in the field as described herewith.

- The mineralization ability of the isolates to bring about efficient degradation needs to be optimized and adopted to check its efficiency in degradation of agricultural solid waste.

- This work gives a perspective on the characteristics of a unique culture, *Halobacillus trueperi* MXM-16 giving an insight into the potential of the EPS, siderophore and pigment produced by this strain. Further studies on the emulsification property of the EPS and complete characterization of the pigment/siderophore, will help in understanding the potential of the strain in bioremediation to explore for application in the natural environments

- The characteristic response of the EPS to transform chromium +6 to chromium +3 needs to be explored further for the mechanism involved and its potential application in treatment of contaminated environments.

- The significant effect of the nanoparticles to change the adhesion to emulsification or increase the emulsification in number of isolates needs further exploration to understand the effect on cell surfaces.

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Appendices

Appendix I:

Media: Agars and Broths

Azure- B/ toluidine blue agar

Zobell marine agar (Himedia)	5.25 g
Or	
NTYE agar	(as per 100ml of agar preparation)
Azure B dye/ toluidine blue	0.02 g
Distilled water	100ml
pH	7.0

Carboxymethyl cellulose (CMC) agar

Zobell marine agar (Himedia)	5.25 g
Or	
NTYE agar	(as per 100ml of agar preparation)
Carboxymethyl cellulose	1.0 g
Distilled water	100 ml
pH	7.0

Chrome azurol sulphonate (CAS) agar

CAS solution	10 ml
NA (Himedia)	90 ml
Or	
NTYE agar	90 ml
pH	7.0

Chitin agar

Chitin	0.3g
ZMA	5.25g
or	
NTYE agar	(as per 100 ml of agar preparation)
pH	7.0

Congo red-coomassie blue agar

Tryptone	1.0 g
Congo red	0.004 g
Coomassie blue	0.002 g
Agar	2.0 g
Distilled water	100 ml
pH	7.0

Glucose phosphate broth

Peptone	1.0 g
K2HPO4	0.5 g
Glucose	0.5 g
Distilled water	100 ml
pH	7.0

Hugh Leifson's agar (HLA)

NaCl	0.5 g
K_2HPO_4	0.03 g
Peptone	0.2 g
Yeast extract	0.5 g
Bromothymol blue solution	0.3 ml
*Glucose solution	50 ml
Agar	0.4 g
Distilled water	50 ml
pH	7.0

* 1% glucose solution was sterilised separately at 121 lb pressure for 10 min and added to the medium after it had cooled to about 40-45^{o}C.

Anaerobic HLA tubes were prepared and stored in refrigerator for 24hrs prior to inoculation. After inoculation they were immediately over-layered with sterile paraffin oil to create anaerobic conditions. Growth and colour change of the indicator dye was noted in the two tubes after incubation. Strict aerobes grew only in aerobic conditions. Facultative anaerobes grew in both aerobic and anaerobic conditions. The anaerobic organisms grew only in anaerobic conditions.

Mannitol salt agar (MSA)

Peptone	1.0 g
Beef extract	0.1 g
D- mannitol	1.0 g
NaCl	7.5 g
Phenol red	0.0025 g
Agar	1.5 g
Distilled water	100 ml
pH	7.0

Mineral salt s medium (MSM)

$FeSO_4$. $7H_2O$	60 mg
K_2HPO_4	6.3g
KH_2PO_4	1.82g
$(NH_4)_2$ NO_3	1g
$MgSO_4$. $7H_2O$	0.1g
$MnSO_4$	0.6mg
$NaMoO_4$	0.6 mg
$CaCl_2$.$2H_2O$	75 mg
Distilled water (make final volume)	1000ml
*Agar for solid medium	

Motility agar

Beef extract	0.3 g
Peptone	1.0 g
NaCl	0.5 g
Agar	0.4 g
Distilled water	100 ml
pH	7.0

NaCl- tryptone- yeast extract (NTYE) broth

$MgSO_4.7H_2O$	2.0 g
KCl	0.5 g
$CaCl_2.2H_20$	0.02 g
Yeast extract	0.3 g
Tryptone	0.5 g
Crude solar salt	15/25 g
Distilled water	100 ml
pH	7.0

NaCl- tryptone- yeast extract (NTYE) agar

$MgSO_4.7H_2O$	2.0 g
KCl	0.5 g
$CaCl_2.2H_20$	0.02 g
Yeast extract	0.3 g
Tryptone	0.5 g
Crude solar salt	15/2 5 g
Agar	2.0 g
Distilled water	100 ml
pH	7.0

Nitrate reduction broth

NaCl	0.5 g
Peptone	2.0 g
Meet extract	0.3 g
Distilled water	100 ml
pH	7.0

The tubes were inoculated with the culture and incubated at RT for 24 hrs. After growth, a few drops of Greiss- Illoway reagent was added to the medium. Red colouration indicated positive reaction.

Nutrient agar

Nutrient agar (Himedia)	2.8g
Distilled water	100 ml
pH	7.0

Simmon's citrate agar

Ammonium dihydrogen phosphate	0.1 g
Dipotassium phosphate	0.1 g
NaCl	0.5 g
Sodium citrate	0.2 g
MgSO4	0.02 g
Bromothymol Blue	0.008 g
Agar	1.5 g
Distilled water	100 ml
pH	7.0

Skim milk agar

skim milk powder	0.5g
casein hydrolysate	0.5g
yeast extract	0.25g
dextrose	0.1 g
agar	1.5g
distilled water	100ml
pH	7.0

Starch agar

Zobell marine agar (Himedia)	5.52 g
Or	
NTYE agar	(as per 100ml of agar preparation)
Starch	1.0 g
Distilled water	100 ml
pH	7.0

Sugars in peptone water base

Peptone	1.0 g
NaCl	0.5 g
Phenol red	0.02 g
*Sugar solution	10 ml
Distilled water	90 ml
pH	7.0

*0.5- 1 gm of sugar (glucose/ lactose/ galactose/ sucrose/ fructose/ mannitol/ xylose/ raffinose) in 10 ml of distilled water. Sugar solutions were sterilised separately at 121 lb pressure for 10 min and added to the peptone water base after it had cooled to about 40- 45^{o} C.

Tannic acid agar

Zobell marine agar (Himedia)	5.52 g
Or	
NTYE agar	(as per 100ml of agar preparation)
Tannic acid	1.0 g
Distilled water	100 ml
pH	7.0

Toluidine blue agar

Zobell marine agar (Himedia)	5.25 g
Or	
NTYE agar	(as per 100ml of agar preparation)
Toluidine Blue dye	0.02 g
Distilled water	100 ml
pH	7.0

Triple sugar iron (TSI) agar

TSI agar (Himedia)	6.5 g
Distilled water	100 ml
pH	7.0

Tributyrin agar

Peptone	0.5 g
Yeast extract	0.3 g
Tributyrin	1.0 ml
Agar	1.5 g
Distilled water	100 ml
pH	7.0

Zobell marine broth (ZMB)

Zobell marine broth (Himedia)	4.25 g
Distilled water	100 ml
pH	7.0

Zobell marine agar (ZMA)

Zobell marine agar (Himedia)	5.25 g
Distilled water	100 ml
pH	7.0

Appendix II:

Reagents, solutions and stains

Alcian blue solution

Alcian blue 8GX dye	1.0 g
Glacial Acetic acid (3%)	100 ml
pH	2.5

Bromothymol blue solution

Bromothymol blue	1.0 g
NaOH (2N)	2.0 ml
Distilled water	98 ml

Chrome azurol sulphonate (CAS) solution

CAS indicator dye	60.5 mg
Fe III solution	10 ml
Hexadecyl trimethyl ammonium bromide (HDTMA)	72.9 mg
De-ionised distilled water	40 ml

Congo red solution/ stain

Congo red	1.0 gm
Distilled water	100 ml

Crystal violet solution

Crystal violet dye solution: 2.5 gm crystal violet dye in 25 ml methylated spirit.

Ammonium oxalate solution: 1.0 gm of ammonium oxalate in 100 ml distilled water.

Mix both solutions.

Di- sodium EDTA (0.1M) solution

Di sodium EDTA	3.72 gm
Distilled water	100 ml
pH	7.0

Ethanol (70%)

Ethanol	70 ml
Distilled water	30 ml

Ethedium Bromide (10 mg/ ml)

Ethedium bromide	0.2 gm
Distilled water	20 ml

CARCINOGENIC: Wrap container in aluminium foil.

Fe III solution

$FeCl_3.6H_20$	27 mg
Concentrated HCl	83.3 µL
De-ionised distilled water	100 ml

Folin Lowry reagent A

Na_2CO_3	2g
NaOH (0.1N)	100 ml

Folin Lowry reagent B

$CuSO_4$	0.5g
Potassium sodium tartarate	1g
Distilled water	100 ml

Folin Lowry reagent C (Alkaline copper sulphate solution)

Folin Lowry reagent A	50 ml
Folin Lowry reagent B	1ml

*Mix solutions prior to use.

Glucose solution

Glucose	1.0 gm
Distilled water	100 ml

Gram's iodine solution

Iodine crystals	1.0 gm
KI	2.0 gm
Distilled water	100 ml

Griess-Illoway reagent

a-naphthylamine solution : 0.5 g L-naphthylamine in 100 ml 5N acetic acid

Sulphanilic acid solution: 0.8 g sulphanilic acid in 100 ml of 5N acetic acid.

Mix both solutions in the ratio of 1:1.

Glacial acetic acid (3%)

Glacial acetic acid	3.0 ml
Distilled water	97 ml

Glacial acetic acid (5N)

Glacial acetic acid	28.6 g
Distilled water	100 ml

Hydrogen peroxide (catalase test) reagent

Hydrogen peroxide	3.0 ml
Distilled water	100 ml

Potassium hydroxide (KOH) solution (3%)

KOH	3.0 g
Distilled water	100 ml

Phenol-sulphuric acid reagent

Phenol	3g
Sulphuric acid (concentrated)	5 ml
Ethanol	95 ml

NaCl (2N) solution

NaCl	8.0 g
Distilled water	100 ml

NaCl (1M) solution

NaCl	5.86 g
Distilled water	
	100 ml

Ninhydrin reagent

Ninhydrin powder	0.2 g
Absolute ethanol	100 ml

Normal saline (NS)

NaCl	0.85 g
Distilled water	100 ml

Safranine solution

Safranine dye	0.5 g
Distilled water	100 ml

Sample loading buffer for DNA (6X)

Bromophenol blue	0.26 g
Sucrose	40 g
Distilled water	100 ml

Tetramethyl-phenylenediamine-dichloride (TMPD) oxidase reagent

TMPD	1.0 g
Distilled water	100 ml

TE buffer

Tris-Cl (1M) (pH 7.2- 8.0)	1 ml
EDTA (0.5 M) (pH 8.0)	200 µl
Distilled water	98.8 ml

Sterilised by autoclaving at 121 lb pressure for 15-20 min.

50X TAE buffer for agarose

Tris base (2M)	242 gm
Glacial acetic acid	57.1ml
EDTA (100mM)/ 0.1 M	100 ml of 0.5 M EDTA
Distilled water	1000 L

1x TAE buffer for agarose

50X TAE buffer	1ml
Distilled water	49 ml

EDTA (0.5 M) solution

EDTA	14.61 g
Distilled water	100 ml

Appendix III

Staining procedures

III A: GRAM'S STAINING

1. Clean the glass slide using detergent and wash thoroughly with water
2. Prepare a smear of the isolate and heat fix.
3. Cover the smear with crystal violet solution for ½-1 minute.
4. Throw out excess and gently rinse slide with running water for 2 min.
5. Cover the slide with grams iodine for 2 min.
6. Pour out excess iodine and decolourise using 95% ethanol solution for 5 min until solution runs clear.
7. Rinse thoroughly with water to stop action of alcohol.
8. Cover stain with Safranine solution for 1 -2 min.
9. Gently rinse off the stain with water.
10. Air dry the slide and observe under the oil immersion lens of light microscope.

III B: ALCIAN BLUE STAINING

1. Deparaffinize the slides and hydrate with distilled water.
2. Stain in alcian blue solution for 30 min.
3. Wash in running water for 2 min and rinse with distilled water.
4. Air dry the slide.
5. Counterstain with 1% congo red solution for 2-3 min.
6. Dehydrate using 95% ethanol 3 times for 3 min each.
7. Air dry the slide and observe under oil immersion lens of the light microscope.

Appendix IV
Tables

IV B: Classification of bacterial isolates based on tolerance to salinity (Horikoshi *et al.* 2011)

Category	**Properties**
Non halophile	Grows best in media containing less than 0.2M NaCl (~1%)
Slight halophile	Grows best in media containing 0.2-0.5M NaCl (~1-3%)
Moderate halophile	Grows best in media containing 0.5-2.5 M NaCl (~3-15 %)
Borderline extreme halophile	Grows best in media containing 1.5- 4.0 M NaCl (~9- 23%)
Extreme halophile	Grows best in media containing 2.5-5.2 M NaCl (~15-32%)
Halotolerant	Non- halophile that can tolerate salt. If the growth extends to above 2.5 M NaCl it may be considered extremely halotolerant.

IV C: Adhesion and Emulsification exhibited by selected isolates in BATH assay

Isolate no	conclusion	% Adherence or % Emulsification
MXM- 1	adherence	0.487
MXM- 4	emulsification	15.63
MXM- 5	adherence	30.66
MXM- 7	adherence	26.98
MXM- 8	adherence	35.72
MXM- 9	adherence	29.82
MXM- 10	adherence	31.05
MXM- 12	adherence	19.56
MXM-15	emulsification	2.36
MXM- 16	adherence	30.17
SDM- 2	adherence	24.41
SDM- 4	adherence	27.12
SDM- 5	adherence	20.98
SDM- 9	adherence	16.71
SDM- 10	adherence	16.63
SDM- 12	adherence	16.04

IV E: Effect of ZVI on siderophore zone size

Isolate no	CAS		CAS+ZVI nanoparticles		CAS+ FeCl3	
	Colony diameter	Siderophore zone size	Colony diameter	Siderophore zone size	Colony diameter	Siderophore zone size
MXM-1	0.6	1.33	0.6	1.66	0.6	No zone
MXM-4	0.5	1.66	0.5	1.66	0.5	No zone
MXM-5	0.4	1.20	0.5	1.20	0.4	No zone
MXM-7	0.6	0.7	0.6	0.7	0.6	No zone
MXM-8	1.1	1.09	1.1	1.18	1.1	No zone
MXM-9	0.5	No zone	0.5	No zone	0.5	No zone
MXM-10	0.5	1.40	0.4	2.00	0.5	No zone
MXM-12	0.5	1.40	0.5	1.40	0.5	No zone
MXM- 15	0.8	1.12	0.9	1.10	0.8	No zone
MXM-16	0.4	1.25	0.4	1.25	0.4	No zone
SDM-2	1.2	No zone	1.2	No zone	1.2	No zone
SDM-4	1.6	1.06	1.6	1.13	1.6	No zone
SDM-5	0.6	1.17	0.6	1.33	0.6	No zone
SDM-9	1.3	No zone	1.3	No zone	1.3	No zone
SDM-10	0.6	1.33	0.6	1.33	0.6	No zone
SDM-12	0.5	1.20	0.5	1.60	0.5	No zone

Appendix V

Analytical protocols

A: Sugar estimation: The sugar estimation was carried out using the phenol sulphuric acid method (Dubois *et al*. 1956)

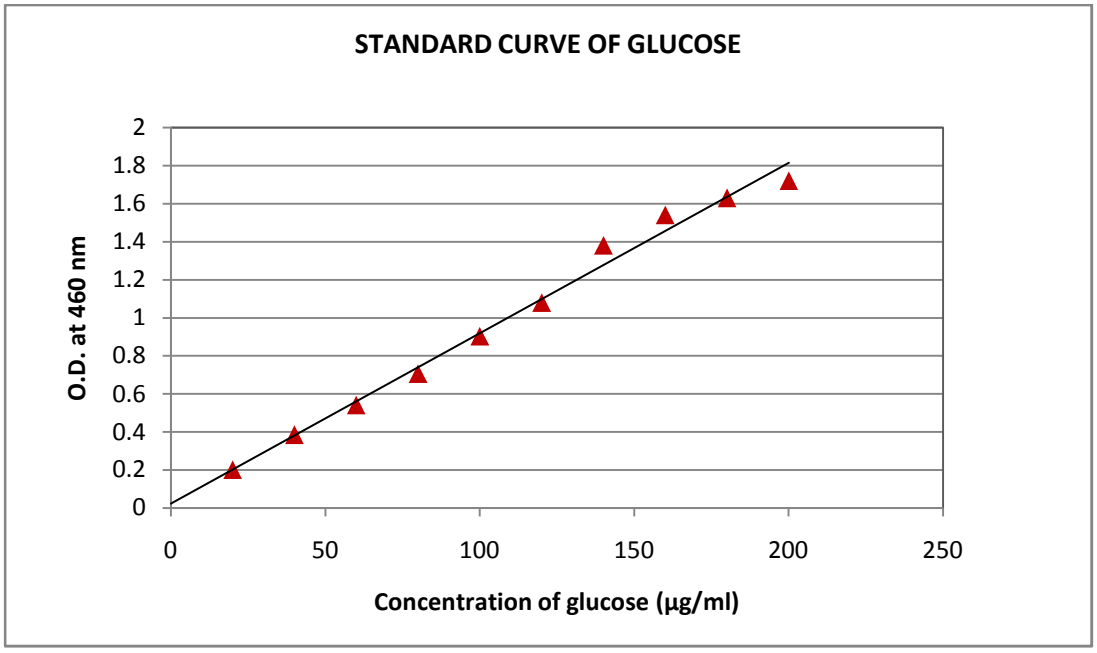
Glucose standard curve

Stock solution of 0.1mg/ml of glucose in distilled water was prepared and diluted to obtain concentrations of 20, 40, 60, 80, 100, 120, 140, 160, 180 and 200 µg/ml. To 1ml of each concentration, 1ml of 5% phenol and 5 ml of 96% sulphuric acid was added and kept for 10 min at room temperature. The tubes were then put in water bath at 25°C-30°C for 20 min. The colour developed was read in a UV-Vis spectrophotometer (Shimadzu UV 2450) at 460 nm. The readings were plotted in a graph of Abs v/s concentration of glucose to give standard glucose curve from which the concentration of the unknown sample could be deduced.

Concentration of the unknown sample

To estimate sugar in the unknown sample,1 ml of the unknown sample was used followed by the phenol sulphuric acid method protocol. The absorbance obtained was used to find the concentration of the unknown sample from the standard graph.

Standard curve for sugars and total carbohydrate



B: Protein estimation: the protein estimation was carried out by the Folin-Lowry Method (Lowry *et al*. 1951)

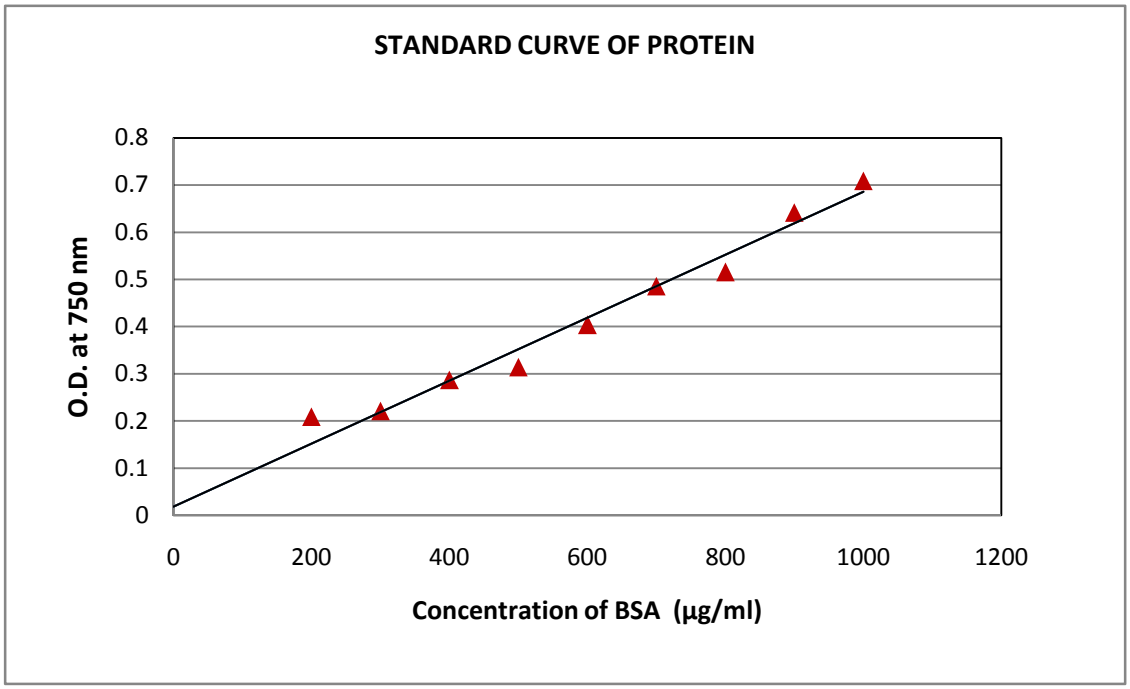
Protein standard curve:

A 1mg/ml stock solution of Bovine serum albumin (BSA) in distilled water was prepared and diluted to concentrations of 200, 400, 600, 800 and 1000µg/ml. To 1ml of each concentration 5ml of Folin-Lowry reagent C was added and kept in dark for 10 min at room temperature. Then, 0.5 ml of Folin-Ciocalteau reagent was added to each tube and incubated at room temperature for 30 min. The absorbance was measured at 750 nm using the UV-Vis spectrophotometer (Shimadzu UV 2450) and the absorbance readings were used to plot the standard calibration curve.

Concentration of the unknown sample

For estimation of protein of unknown sample, 1ml of the unknown sample was subjected to the Folin-Lowry protocol. The absorbance obtained was used to determine the concentration of the unknown sample using the standard curve.

Standard curve for protein



C: Qualitative tests for proteins, sugars and lipids

1) Ninhydrin test for Proteins

1ml of EPS solution was added to 5 drops of 0.2% ninhydrin solution prepared in acetone and boiled in water bath for 2 min. positive control was maintained using albumin solution while the negative control was with distilled water. It was cooled and observed for bluish-purple colouration that indicated a positive test for proteins.

2) Molisch's test for Sugars

1 drop of Molisch's reagent was added to 2 ml of EPS solution and 1-2 of concentrated sulphuric acid was poured along the side of the test tube so that it formed a layer at the bottom of the tube. Positive control contained glucose solution while the negative control was maintained with distilled water. Reddish purple colouration at the interface indicated a positive test for sugars.

3) Sudan IV test for Lipids

5 drops of Sudan IV dye was added to 1 ml of EPS solution. Positive control was maintained using coconut oil while distilled water served as negative control. A bright red colouration indicated presence of lipids.

D: Qualitative tests for siderophores

Hydroxamate siderophore

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

2) Tetrazolium salt test: A pinch of tetrazolium salt was added to 0.5 ml of cell free supernatant followed by a few drops of 1N NaOH. Instant deep red-pink colouration indicated hydroxamate siderophore (Snow 1954).

Catecholate siderophore

1) Arnow's assay: to 1ml of supernatant 1 ml of 0.5 M HCl, 1ml nitrite molybdenate and 1 ml of 1M NaOH was added. After addition of each reagent the tubes were vortexed. The colour change from yellow to red indicated catecholate.

2) Czaky's spectrophotometric assay for catecholate siderophore: 1.5 ml of freshly prepared $FeCl_3$ was added to 1ml of supernatant. A wine coloured complex with an absorption maxima at 495 nm indicated catecholate siderophore.

Carboxylate siderophore

Vogel's test: : In a tube 5 drops of 5N NaOH was followed by 2 drops of 0.1% phenolphthalein to give a light pink colour. To this 1 ml of supernatant was added.

Disappearance of the pink colour indicated the presence of carboxylate type of siderophore.

E: Rothera's test

The isolate was grown in MSM broth with 0.2% of sodium benzoate as the sole source of carbon for 24 hr in shaker conditions of 150 rpm at room temperature. The culture suspension was centrifuged at 5000 rpm at 4^{o}C for 10 min. 5 ml of 0.02M tris buffer (pH 8.0) was added to the pellet and vortexed. 0.2 ml of toluene was added to this mixture and vortexed again. 0.2 ml of 100mM catechol was added and allowed to stand for 10 min. Appearance of bright yellow colour indicates meta pathway. In the absence of the bright yellow colour, the tube was covered with aluminium foil and incubated on shaker for 1hr at 150 rpm. After the incubation the mixture was saturated with ammonium sulphate and 0.1 ml of NH4OH along the side of the test tube. A drop of freshly prepared 25% Na-prusside was added. Formation of a deep purple colour ring indicated an ortho pathway.

Appendix VI

Partial DNA sequence of the 16S rRNA gene of the selected isolates

Halobacillus trueperi **MXM-5 (KF379753)**

CGGGGGGTTGTCGGGATTATTGGGCGTAGGCGCGCGCAGGCGGTTCCTTA
AGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTG
GGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTTTCTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGCTTCC
ACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT

Acinetobacter schindleri **MXM-8 (KF379752)**

AGGGGGGGTTTTCGGATTTACTGGGCGTAAGCGTGCGTAGGCGGCTTTTTA
AGTCGGATGTGAAATCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGG
AAAGCTAGAGTATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAA
TGCGTAGAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTA
ATACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATACC
CTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCTTTGAGG
CTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGGAGTACGGTC
GCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGA
GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACAT
ACTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTAGATACAG
GTGCTGCATGGCTGT

Halobacillus trueperi **MXM-16 (KF 379752)**

GGGGGGACGTTCTCGGATTATTGGGCGTAAGCGCGCGCAGGCGGTTCCTT
AAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGGAACTTGAGGACAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGT
CTGTTTCTGACGCTGAGGTGCGAAAGCGTGGGTAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGGTGTTAGGGGGCTT
CCACCCCTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGAGTAC
GGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGG
TGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTT
GACATCCTTGGACCTCCCTAGAGATAGGGATTTCCCTTCGGGGACCAATTG
ACAGGTGGTGCATGGTTG

Publications

Journals

Kharangate-Lad A and Bhosle S (2014) Siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter. NeBIO: NCEER Journal of Environment and Biodiversity 5(1): 56-60.

Kharangate-Lad A and Bhosle S (2014) Studies on the bioemulsifier production by a bacterial isolate. Journal of Scientific and Industrial Research (JSIR) 73: 674-679.

Kharangate-Lad A and Bhosle S (2015) Studies on the effects of zerovalent iron nanoparticles on bacteria from the mangrove ecosystem. Environmental Science and Pollution Research. Revised and Resubmitted.

Books

Kharangate-Lad (2015) Bacteria adhered to particulate matter and their role in plant litter mineralization. In: Bioprospects of coastal eubacteria. Borkar S. (ed). Springer Switzerland, pp. 195-208.

Conferences

Oral presentation

Kharangate-Lad A and Bhosle S (2013). **Studies on halophilic and halotolerant bacteria adhered to mangrove plant litter.** Paper presented at the ***First international and third national conference on Biotechnology, Bioinformatics and Bioengineering*** by ***Society for applied biotechnology (SAB)- India*** held at Tirupathi, Andhra Pradesh- India from 29-30 July 2013.

Poster presentation

Kharangate-Lad A and Bhosle S (2013) **Studies on siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter**. Paper presented at the ***Third national conference on Environment and biodiversity of India (EBI)*** by ***North east centre for environmental education and research (NECEER)-India*** held at Pune-India on 6 October 2013.

Awards during Ph.D

Awarded the **Best Poster Award** for the poster titled **Studies on siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter** at the NCEER- EBI conference held at Pune-India on 6 October 2013.

ABSTRACTS OF THE CONFERENCES

Studies on halophilic and halotolerant bacteria adhered to mangrove plant litter

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Mangrove plant litter consists of complex natural polymers such as cellulose, starch, lignin, tannin and lipids that are degraded and recycled in the ecosystem. This ecosystem is also prone to tidal fluxes which influence the salinity of the waters and microbial communities. The degradation of litter is therefore envisaged to be effective by bacteria which remain consistently attached to the particles. The present study was therefore focused on studying such bacteria adhered to plant litter from mangrove ecosystem in Goa. The cultures were isolated from the litter particles on Zobell marine agar and NaCl-tryptone yeast extract agar. These were further classified as halophiles or halotolerant based on their growth in presence of varied salt concentration. Further, the isolates were screened for their ability to degrade the polymers using specific media. The results have shown presence of halophilic and halotolerant bacteria producing enzymes such as cellulase, amylase, lignin peroxidase, tannase and lipase . The ability of these isolates to produce EPS was observed using specific staining methods and by plating on EPS media. Further the ability of the isolate to adhere to the hydrocarbons was also determined using BATH assay. The results on the activities, identification and the role in degradation process will be presented.

Studies on siderophore producing halophilic and halotolerant bacteria adhered to mangrove plant litter

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Mangroves are unique ecosystems due to their ability to thrive in salt and saline water. In this ecosystem the nutrient recycling occurs consistently with the formation of detritus. The microbes play a major role in recycling and therefore factors affecting their growth influences the rate of mineralization. One of the factors is the availability of iron, the availability of which is limited. Under such conditions bacteria produce iron sequestering ligands called siderophores which help to get iron in soluble form that can be used by the bacterial cell for its cellular and metabolic needs. The present study was focused on studying adhered bacteria from mangrove plant litter in Goa and their ability to produce siderophores.. These bacteria were isolated on Zobell marine agar and NaCl-tryptone-yeast extract agar and were further classified as halophiles or halotolerant based on their ability to grow in presence of varied salt concentration. Further, the isolates were screened for production of siderophores using Chrome Azurol sulphonate (CAS) medium. The results of the study will be presented.

Appendix IV A: Colony characteristics of isolated bacterial isolates

Isolate	MXM-1	MXM-2	MXM-3	MXM-4	**MXM-5**	MXM-6	MXM-7	**MXM-8**	MXM-9	MXM-10	MXM-11	MXM-12
Site	Manxer	Manxer	Manxer	Manxer	**Manxer**	Manxer	Manxer	**Manxer**	Manxer	Manxer	Manxer	Manxer
Medium	NTYE	NTYE	NTYE	NTYE	**NTYE**	ZMA	ZMA	**ZMA**	ZMA	ZMA	ZMA	ZMA
Time	24-48 hr	24-48 hr	24-48 hr	24-48 hr	**24-48 hr**	24-48 hr	24-48 hr	**24-48 hr**	24-48 hr	24-48 hr	24-48 hr	24-48 hr
Temperature	28°C	28°C	28°C	28°C	**28°C**	28°C	28°C	**28°C**	28°C	28°C	28°C	28°C
Size	pinpoint	1mm	pinpoint	pinpoint	**1 mm**	1 mm	1mm	**1mm**	1mm	pinpoint	1 mm	1mm
Shape	circular	Circular	circular	circular	**circular**	circular	circular	**Circular**	spherical	circular	circular	circular
Colour	Pale white	Pale yellow	cream	white	**orange**	Pale white	Pale yellow	**Pale white**	Pale white	Pale white	Pale yellow	Pale yellow
Margin	entire	Entire	entire	entire	**entire**	entire	entire	**Lobate**	lobate	entire	undulate	lobate
Elevation	flat	Flat	convex	convex	**raised**	flat	raised	**Convex**	convex	raised	convex	raised
Surf. Tex.	smooth	Smooth	smooth	smooth	**smooth**	smooth	smooth	**Smooth**	smooth	smooth	wrinkled	smooth
Opacity	translucent	Opaque	opaque	translucent	**opaque**	transparent	translucent	**Opaque**	Translucent	transparent	translucent	Translucent
Consistency	butyrous	Butyrous	butyrous	butyrous	**butyrous**	butyrous	butyrous	**Butyrous**	Butyrous	butyrous	butyrous	Butyrous

Isolate	MXM-13	MXM-14	MXM-15	**MXM-16**	SDM-1	SDM-2	SDM-3	SDM-4	SDM-5	SDM-6	SDM-7	SDM-8
Site	Manxer	Manxer	Manxer	**Manxer**	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona
Medium	ZMA	ZMA	NTYE	**NTYE**	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24-48 hr	24-48 hr	24-48 hr	**24-48 hr**	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr
Temperature	28°C	28°C	28°C	**28°C**	28°C	28°C	28°C	28°C	28°C	28°C	28°C	28°C
Size	1mm	1mm	pinpoint	**pinpoint**	1 mm	1 mm	1 mm	1 mm	1 mm	1 mm	1 mm	1 mm
Shape	circular	Circular	circular	**circular**	circular	circular	circular	Circular	Circular	circular	spherical	circular
Colour	Pale orange	Pale yellow	cream	**Pale orange**	Pale white	white	Orange brown	Canary yellow	Pale white	Pale white	Pale brown	Pale brown
Margin	entire	Undulate	entire	**entire**	entire	entire	entire	Entire	Entire	entire	entire	entire
Elevation	convex	Convex	convex	**raised**	flat	flat	umbonate	Raised	Convex	flat	convex	convex
Surf. Tex.	smooth	Smooth	smooth	**smooth**	smooth	wrinkled	smooth	Smooth	Smooth	smooth	smooth	smooth
Opacity	translucent	Translucent	opaque	**translucent**	translucent	opaque	translucent	Opaque	Transparent	transparent	transparent	translucent
Consistency	butyrous	Butyrous	butyrous	**butyrous**	butyrous	butyrous	butyrous	Butyrous	Butyrous	butyrous	butyrous	butyrous

Isolate	SDM-9	SDM-10	SDM-11	SDM-12	SDM-13	SDM-14	SDM-15	SDM-16	SDM-17	SDM-18
Site	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona	Shirdona
Medium	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA	ZMA
Time	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr	24-48 hr
Temperature	28°C	28°C	28°C	28°C	28°C	28°C	28°C	28°C	28°C	28°C
Size	1 mm	1 mm	1 mm	1 mm	1 mm	1 mm	pinpoint	1 mm	1 mm	pinpoint
Shape	circular	Circular	circular	circular	circular	circular	circular	circular	circular	circular
Colour	white	White	white	Orange brown	Pale brown	yellow	Pink orange	cream	Pale orange	Pale white
Margin	entire	Entire	entire	entire	entire	entire	Entire	entire	entire	entire
Elevation	flat	Flat	umbonate	raised	raised	convex	convex	Flat	flat	convex
Surf. Tex.	wrinkled	Smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth	smooth
Opacity	opaque	translucent	opaque	translucent	translucent	translucent	transparent	opaque	opaque	opaque
Consistency	butyrous	Butyrous	butyrous	butyrous	butyrous	butyrous	butyrous	butyrous	butyrous	butyrous

Appendix IV D: Biochemical tests of the selected bacterial isolates

Biochemical tests	MXM 1	MXM-4	MXM-5	MXM-7	MXM-8	MXM-10	MXM-12	MXM 16	SDM-2	SDM 4
Gram character	+	-	+	+	-	-	-	+	-	+
Morphology	cocci	Short rods	Bacilli		Coccobacilli/ short rods	coccobacilli	Short rods	Bacilli	Short rods	cocci
Motility		Non motile	-	-	-	-	Non motile	+	Non motile	-
Hugh leifson's (aerobic)	+	+	+	+	+	+	+	+	+	+
Hugh leifson's (anaerobic)	-	+	+	+	-	+	+	+	+	+
ENZYMES										
Oxidase	-	+	+	-	-	-	+	-	+	-
Catalase	+	+	+	+	+	+	+	+	+	+
SUGARS										
Glucose	+	+	+	-	+	+	+	+	+	+
Lactose	-	+	+	-	+	-	+	+	+	+
Sucrose	-	+	+	-	_	+	+	+	+	+
Fructose	-	+	+	-	-	+	+	+	+	-
Mannitol	-	+	+	-	-	-	+	+	+	+
Xylose	-	+	-	-	+	-	+	-	+	-
Raffinose	-	+	-	-	+	-	+	-	+	-
Nitrate reduction	+	+	-	-	-	-	+	-	+	+
Methyl red	-	+	+	-	-	-	-	+	+	-
Citrate	-	-	_	-	+	+	-	-	-	+
TSI	Alkaline slant Acid butt, no gas and H_2S	Alkaline slant Acid butt, no gas and H_2S	Alkaline slant no gas and H_2S	Alkaline slant, no gas and H_2S	No change	Alkaline slant, no gas and H_2S	Acid butt, alkaline slant, no gas and H_2S	Alkaline slant no gas and H_2S	Alkaline slant Acid butt, no gas and H_2S	Acid butt, alkaline slant, no gas and H_2S
Tentatively identified as (Bergey's Manual)	*Micrococcus* sp	*Aeromonas* sp	*Halobacillus* sp	*Brevibacterium* sp	*Acinetobacter* sp	*Erwinia* sp	*Vibrio* sp	*Halobacillus* sp	*Aeromonas* sp	*Staphylococcus* sp *MSA +

*Mannitol salt agar