STUDIES ON ECOPHYSIOLOGICAL POTENTIAL OF BIOEMULSIFIER PRODUCED BY *Bacillus* SPECIES

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Declaration

Statement under O. Ph.D. 8/(iii) of M.S. University of Baroda, Vadodara, India.

The work presented in this thesis has been carried out by me under the guidance of Dr. A. S. Nerurkar, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: **Vadodara** **Anoop R. Markande**

Date: Candidate

This is to certify that the above declaration is true.

Place: **Vadodara** **Dr. A. S. Nerurkar**

Date: Research Guide

ॐ भूर्भुवः स्वः, तत्सवितुर्वरेण्यं ।

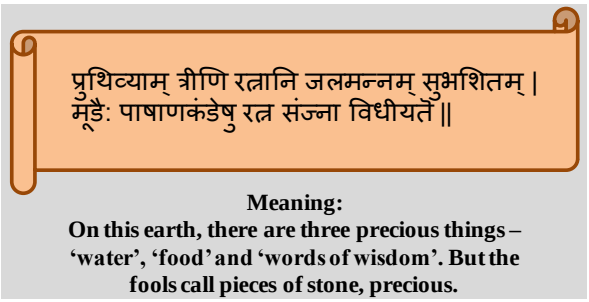
भर्गो देवस्य धीमहि, धियो यो नः प्रचोदयात् ॥

Meaning:

Salutations to that divine illumination which pervades the Bhu (physical world), Bhuva (antariksha or astral world) and Suva (swarga or the celestial world).

On that divine radiance we meditate, may that enlighten our intellect and awaken our spiritual wisdom.

Subhashitas (सुभाषितम्) in Sanskrit means "words of Wisdom". These are short verses that convey thoughtful messages or words of wisdom.



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A subhashita says,

आचार्यात् पादमादते पादं शिष्यः स्वमेधया ।
पादं सब्रह्मचारिभ्यः पादं कालक्रमेण च ॥

Which means, "a student learns a quarter from teacher, a quarter from own intelligence, a quarter from fellow students, and the rest in course of time". This is true and when I look back at five years that have passed since my research work began, there are numerous people who come into the vision without whose help; this journey would have been extremely difficult. Vadodara or Baroda, a city which enchants with its colour and diversity has made a lasting impression upon me. I had heard about the scientific pool at the Maharaja Sayajirao University of Baroda and its Department of Microbiology which I realized when I joined it, participated in its achievements. I owe a lot to this department.

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in… Among all this chaos, there was chechi (Dr. Priya) an organized, meticulous, patient, hard worker. Her presence in the lab made sure there was someone with experienced brain prevailing over all the chaos. Thank you all for letting me feel at home and helping me whenever I am stuck.

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ABBREVIATIONS

°C – degree Celsius

ANOVA – Analysis Of Variance

ARDRA – Amplified Ribosomal DNA Restriction Analysis

CFS – Cell Free Supernatant

CMC – Critical Micelle Concentration

CMD - Critical Micelle Dilution

kDa – Kilo Dalton

FAME – Fatty Acid Methyl Ester

FTIR – Fourier Transform Infrared spectroscopy

g/l – gram per liter

h - hour

LB – Luria Bertani

mg/ml – milligram per milliliter

min - minute

ml – milliliter

mN/m – milli Newton per meter

nm – nanometer

OD_{600} – Optical Density at 600 nm

PCR – Polymerase Chain Reaction

psi – pound per square inch

p-value – probability value

rpm – revolutions per minute

RSM – Response Surface Methodology

sp. – species

ST - Surface Tension

TLC – Thin Layer Chromatography

v/v – volume by volume

vvm – volume per volume per minute

w/v – weight by volume

w/w – weight by weight

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ABSTRACT

Ecophysiological group of bioemulsifier producing isolates obtained from twelve intertidal zone sampling sites spanning the entire western and one eastern coastal states of India yielded 227 isolates which were screened to acquire twelve isolates belonging to ecophysiological group of sporulating, mesophilic, heterotrophic bioemulsifier producing bacteria capable of quorum quenching with ability to form biofilm and reduce the surface tension. This is the first report of bioemulsifier production by *Solibacillus*, *Sporosarcina*, *Lysinibacillus*, *B. thuringiensis* and *B.flexus*. In this group *Solibacillus silvestris* AM1 was found to possess maximum emulsification activity of 62.5% with broad spectrum of solvent specificity and was therefore selected for further studies. This strain even though showed higher similarity with the type strain HR3-23 in FAME analysis and DNA-DNA hybridization studies, significant differences were observed in its carbon substrate utilization and transition/transversion ratio analyses suggesting evolutionary adaptations towards its niche. *S. silvestris* AM1 produced cell-bound bioemulsifier after 6h and was released into the environment after 16h. The statistical experimental design demonstrated that the bioemulsifier production was influenced by presence of peptone and yeast extract significantly in the Zobell marine medium while non-protein media cited in literature for bioemulsifier production gave negligible results. In natural environment, the organism must be producing the bioemulsifier selectively in proteinaceous but oligotrophic conditions prevailing in its niche. *S. silvestris* AM1 produced an extracellular, homo-multimeric glycoprotein bioemulsifier with a MW of more than 200 kDa and containing 30 kDa monomeric subunits comprising of minor carbohydrate components galactose and ribose/xylose which was also found to have homology with a bacterial flagellin according to Mascot analysis of LC/MS-MS data. It exhibited stability in broad pH and salinity range and also possessed resistance to moderate levels of surfactants and sensitivity to proteinase K. The bioemulsifier also exhibited an interesting feature typical of bacterial functional amyloids i.e., presence of fibrous structure with antiparallel β strand characteristics noted in TEM, CD spectrum and FTIR analysis. The emulsions formed by bioemulsifier AM1 in presence of tricholobenzene and paraffin oil exhibited pseudoplastic non-Newtonian rheological property, as observed by particle size and shear stress analysis. From the

ecophysiological studies undertaken in present work, the natural role of bioemulsifier in *S. silvestris* AM1 is envisaged as follows: It changes the cell surface hydrophobicity and acts as a protectant against the hydrocarbon toxicity. It aids in cell aggregation and adhesion to substratum and consequently helps in biofilm formation by decreasing the interfacial interaction energy. The bioemulsifier possessed the ability to influence the biofilm formation of other bacteria like *Staphylococcus aureus* and *Paracoccus* sp. In addition to emulsification activity, the bioemulsifier AM1 also exhibited biodispersant and hydrocarbon solubilization properties and therefore it has ability to facilitate other compatible hydrocarbon degrading bacteria. In microcosm studies of interaction of *S. silvestris* AM1 and its bioemulsifier with hydrocarbon degrading bacterium *Rheinheimera* sp.Co6 representing another ecophysiological group of bacteria isolated from shared niche of *S. silvestris* AM1 revealed their bioremediation potential.

Chapter 1

Introduction and Review of Literature

तदात्वे नूतनं सर्वम् आयत्यां च पुरातनं।
न दोषायैतदुभयं न गुणाय च कल्पते॥
-यादवाभ्युदय १-६

Meaning:
Everything new becomes old as time goes by. Therefore newness or oldness is not dependent on their properties.
-Yadavabhyudaya (1-6)

Chapter 1

Introduction and Review of Literature

1.1. Introduction

Water is essential for life and can be considered to be one of the most important natural resource. Aquatic environment covers more than 70% of earth's surface and most of this area is covered by oceans. Marine water, characterized by salinity of 3.3-3.7% contains diverse microbial habitats. The marine environment is the largest habitat on earth, accounting for 90% of the biosphere by volume and harbouring microorganisms responsible for 50% of the global primary production (Lauro, *et al.*, 2009). These microscopic factories are responsible for 98% of primary production and mediate all biogeochemical cycles in the oceans (Sogin, *et al.*, 2006). This microbial population and diversity depends markedly on the regional location (coastal, open seas, etc.) on earth and depth of the water column (near the surface, benthic, etc.). Microbial population near the air-water interface is considered to be more which can also be considered as the zone of primary production. At coastal waters, this system of stratification is disturbed due to mixing of water by winds, currents and temperature (Maier, *et al.*, 2000). Marine microbes are responsible for approximately half of earth's primary production and play an enormous role in nutrient cycle globally (Arrigo, 2004). According to some estimates, more than 99% of the microbes existing in nature are not amenable for detection and study due to the lack of methods to cultivate them. The study of uncultured diversity does not give clear picture of microbial function in environment (Venkataraman & Wafar, 2005).

The upper edge of World's coasts is occupied by the intertidal zone, extending over 1.6million km. Given its biological productivity and economic value, intertidal zone can be considered to be the most important coastal habitat. Owing to the daily tidal cycles resulting in gradients of moisture, temperature, wave action, UV radiation, limited access to nutrients, irregular periods drought and salinity, intertidal habitats are inhospitable environments from a microbe's viewpoint (Ortega-Morales, 2010).

World's more than half the population resides within 60km of the shoreline which is expected to rise by 2020. With rapid industrialization and aquaculture practices along the coastal areas and river systems have resulted in decline in water

quality of the estuaries and brackish waters. Adverse anthropogenic effects increase the nutrient loads by eutrophication, heavy metal, organic and microbial pollution and oil spills. Near-shore sediments are considered as repositories for many chemical species (pollutants) and also for synthesis of industrially important microbial metabolites thus increasing the recent interest in such areas (Sundaramanickam, *et al.*, 2008, Sahoo & Dhal, 2009, Kesavan, *et al.*, 2010). Vellar estuary at Parangipettai (or Porto Novo) at Tamil Nadu, India is one of the well-studied estuaries in India with respect to its chemistry and biology (Pari, *et al.*, 2008).

The main routes of oil transport from Gulf countries are across the Arabian Sea and oil pollution here normally flow towards the Indian Western coast due to surface currents during the south-west monsoon, depositing tar-like residues on these beaches giving immense environmental problems (Sengupta, *et al.*, 1989, Verlecar, *et al.*, 2006). With constant pollution threatening the coasts, search for microbes capable of producing metabolites important to industries and environment has gained importance.

Typically a microbe encounters condition of limiting food supply i.e a condition of nutrient starvation which is created predominantly by the activity of the microbes themselves (Lengeler, *et al.*, 1999). Microbial interactions in the environment result in important ecological processes. In various ecological niches, microorganisms (95-99%) are known to appear as co-aggregates of dual or multiple species populations and in the form of biofilms. They demonstrate various types of interactions between them with ability to function as multicellular associations. Competition between species plays a significant role in the structure of microbial communities. When microorganisms need to compete for a settled niche, the competition depends on the ability to produce antagonistic substances such as biosurfactants, organic acids or peroxides, quorum quenching strategies, etc (Nikolaev & Plakunov, 2007, Sadowska, et al., 2010).

High surface-to-volume ratio of microorganisms allows for efficient uptake of nutrients and release of waste products and facilitates their fast growth. Their surfaces are totally exposed to the environmental conditions. All of the components outside of the cell must be able to function under the specific conditions for ecological niche. Hence the ‘diversity of the microbial world’ as termed by Rosenberg & Ron, (1997) is said to be best expressed on the outside of the microbial cell.

Microorganisms tend to have a preference for interfaces with which they interact via the extracellular surface active compounds (SACs) elaborated by them. As the nomenclature goes these substances tend to interact with surfaces or interfaces. The phase boundary between two phases in a heterogeneous system is called an interface. 'Surface active compounds' or biosurfactants are amphipathic molecules having both hydrophobic and hydrophilic part in their structure (Figure 1.1 and 1.2). The surface-active properties are very important for a number of natural processes taking place at the interfaces. On solid interface, they are known to form a film known as a 'conditioning film', changing the properties of the original surface, influencing the interaction of bacteria with the interface (Neu, 1996).

Biosurfactants are classified into efficient surface tension reducing low molecular weight molecules generally known as Biosurfactants and high molecular weight amphiphilic or polyphilic polymers that bind tightly to surfaces and interfaces and can form effective emulsions, called as Bioemulsifiers (Ron & Rosenberg, 2001).

They are made up of ahydrophobic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids. These structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases (Neu, 1996). They exhibit an important property called Critical micelle concentration (CMC). CMC is defined as the concentration of surfactants above which they forms micelles. Micelle formation by BS/BE aggregates is depicted in Figure1.1.

The minimum concentration of biosurfactant at which micelles begin to form is represented by critical micellar concentration (CMC). If the concentration of biosurfactant is above the CMC, an increase in the concentration cannot be detected. Consequently, two cultures with very different concentrations of biosurfactant may display the same activity. This problem can be solved by serially diluting until a sharp decrease in Emulsification is observed (Walter, *et al.*, 2010), in this case by measuring Critical micellar dilution (CMD). According to (Oliveira, *et al.*, 2006) and (Makkar & Cameotra, 1997), CMD is an indirect means of measuring the surfactant production related to the range of CMC. The same concept can be applied to bioemulsifier.

There are several reviews regarding various aspects of bioemulsifires particularly its chemical nature and biotechnological applications (Rosenberg & Ron, 1997, Debnath, *et al.*, 2007, Calvo, *et al.*, 2009, Banat, *et al.*, 2010, Franzetti, *et al.*, 2010, Satpute, *et al.*, 2010). Patents granted worldwide amounted to 255 in 2006 as reported by Shete, *et al.* (2006). Surely, this must figure must now have shooted up phenomenally. Neu (1996) has emphasised the importance of bacterial SACs in interaction of bacteria with interfaces in his review and Ron and Rosenberg (1997) have reviewed their natural roles. The research in this area is bludgeoning and is currently an important thrust area. Bioemulsifier production has been reported in microorganisms like bacteria (ex: *Acenetobacter* sp., *Pseudomonas* sp., *Bacillus* sp., etc) including Actinomycetes (ex: *Streptomyces* sp., *Corynebacterium* sp., etc.), Archaebacteria (ex: *Methanobacterium* sp.) and also in eukaryotes like fungi (ex: *Aspergillus* sp., *Penicillium* sp., *Trichosporon* sp., etc,) including yeasts (ex: *Yerrowinia* sp., *Saccharomyces* sp., *Candida* sp., etc.) and even algae and cyanobacteria (ex: *Dunaliella* sp., and *Phormidium* sp.).

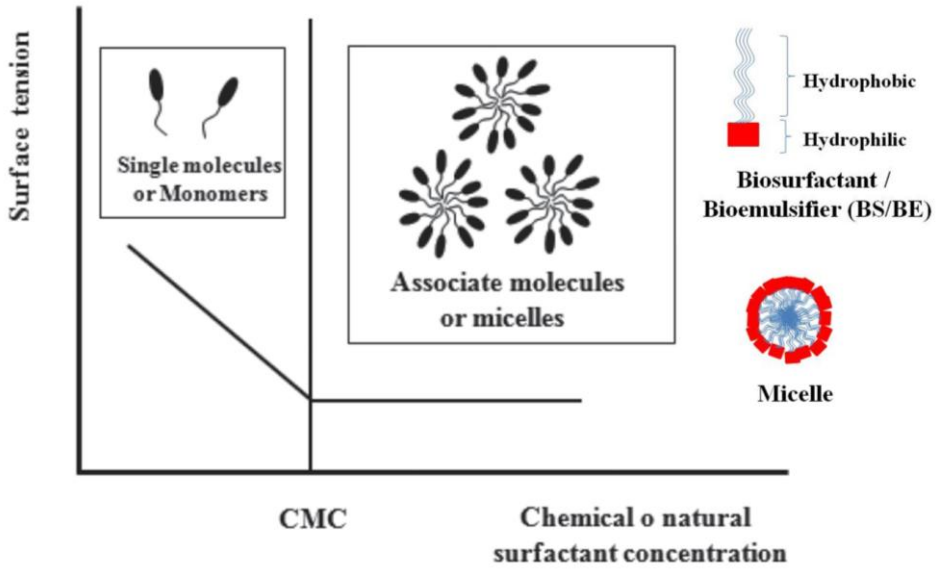


Figure. 1.1. Micelle formed by Biosurfactant/Bioemulsifier (BS/BE) and Critical Micellar concentration (CMC) (Bustamante, *et al.*, 2012).

1.2. Biochemical diversity of bioemulsifiers:

Microorganisms are able to synthesize a wide range of different bioemulsifiers. Some of the examples are lipopolysaccharides, lipoproteins, proteins, lipoteichoic acids, lipoglycans, lipomanan, lipoproteins, glycoproteins or complex

mixtures of these biopolymers. Table 1.1 lists most of the reported bioemulsifiers from different organisms and their biochemical nature.

Table1.1. Bioemulsifier producers and their Chemical nature

Bioemulsifiers produced by Eubacteria		
Exopolysaccharide	**Protein+Carbohydrate**	**Carbohydrate+lipids+ protein**
Ochrobactrum anthropi AD2 (Calvo, *et al.*, 2009)	*Enterobacter* sp. 214 (Toledo, *et al.*, 2008)	*Variovorax paradoxus* 7bCT5 (Franzetti, *et al.*, 2010)
Pseudomonas oleovorans NRRL B-14682 (Freitas, *et al.*, 2009)	*B. subtilis* 28 (Toledo, *et al.*, 2008)	*Geobacillus pallidus* XS2 (Zheng, *et al.*, 2011)
Pseudomonas putida ML2 (Bonilla, *et al.*, 2005)	*Alcaligenes faecalis* 212 (Toledo, *et al.*, 2008)	*Bacillus stearothermophilus* VR-8 (Gurjar, *et al.*, 2008)
Klebsiella sp. K32 (Bryan, *et al.*, 1986)	*Rhodotorula glutinis* (Oloke & Glick, 2005)	*Pseudomonas marginalis* ST (Rosenberg & Ron, 1997)
Halomonas eurihalina (Bouchotroch, *et al.*, 2001)	*B. licheniformis* TT33 (Suthar, *et al.*, 2009)	*Serratia marcescens* (Pruthi & Cameotra, 1997)
Acinetobacter calcoaceticus BD4 (Kaplan & Rosenberg, 1982)	**Alanine containing polysaccharide protein**	**Unknown**
A.calcoaceticus A2 (Elkeles, *et al.*, 1994)	*A.calcoaceticus* KA53 (Rosenberg & Ron, 1997)	*Lactobacillus pentosus* CECT-4023T (ATCC-8041) (Moldes, *et al.*, 2007)
Polysaccharide+protein	**Glycoprotein**	**Rhamnolipid**
A.calcoaceticus BD413 (Kaplan & Rosenberg, 1982)	*Pseudoalteromonas* sp. TG12 (Gutierrez, *et al.*, 2008)	*Pseudomonas aeruginosa* (Law, 1960)
A.calcoaceticus MM5 (Marin, *et al.*, 1996)	*Halomonas* sp. (Gutiérrez, *et al.*, 2007a)	**Actinomycetes**
Heteropolysaccharide+ fatty acids	*Antarctobacter* sp. (Gutiérrez, *et al.*, 2007b)	**Protein+sugar+lipid**
A.calcoaceticus RAG-1 (Rosenberg & Ron, 1997)	**Lipo-polypeptides**	*Streptomyces* sp. S22 (Maniyar, *et al.*, 2011)
Polysaccharide+Lipid	*Bacillus velezensis* H3 (Liu, *et al.*, 2010)	**Glycolipopeptide**
Alcanivorax borkumensis (Yakimov, *et al.*, 1998)	*Streptococcus gordonii* (Jenkinson, 1992)	*Actinopolyspora* sp. A18 (Doshi, *et al.*, 2010)
Acetylated Heteropolysaccharide	**Protein complex**	*Corynebacterium kutscheri* (Thavasi, *et al.*, 2007)
Pseudomonas tralucida (Rosenberg & Ron, 1997)	*Methylobacterium* sp. (Joe, *et al.*, 2013)	**Protein+polysaccharide**
Sphingomonas paucimobilis GS1 (Ashtaputre & Shah, 1995)		*Streptomyces* sp. S1 (Kokare, *et al.*, 2007)

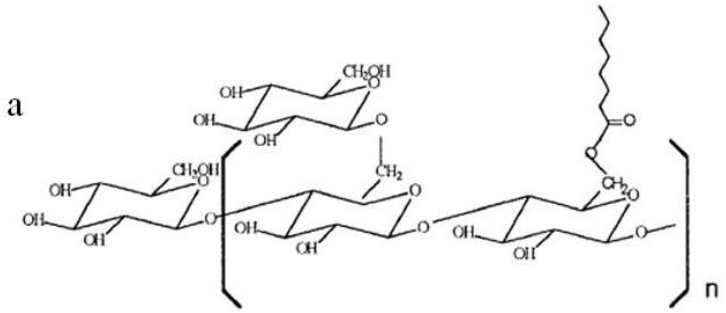
Bioemulsifiers produced by other microorganisms	
Archaea	**Fungi**
Protein complex	**Fattyacid+carbohydrate**
Methanobacterium thermoautotrophicum (Trebbau de Acevedo & McInerney, 1996)	*Trichosporon mycotoxinivorans* CLA2 (de Souza Monteiro, *et al.*, 2012)
Algae and Cyanobacteria	*Trichosporon loubieri* CLV20 (Monteiro, *et al.*, 2010)
Exopolysaccharide	**Lipids+carbohydrates**
Dunaliella salina (Mishra, *et al.*, 2011)	*Trichosporon montevideense* CLOA70 (Monteiro, *et al.*, 2010)
Sugar+fattyacid+protein	*Geotrichum sp.* CLOA40 (Monteiro, *et al.*, 2010)
Phormidium sp. strain J-1 (Bar-Or & Shilo, 1987)	**Glycolipid**
Yeasts	*Aspergillus niger* MYA 135 (Colin, *et al.*, 2010)
Fatty acid+Mannose	**Protein+polysaccharide**
Yarrowinia lipolytica, IMUFRJ 50682 (Monteiro, *et al.*, 2010)	*Curularia lunata* IM 2901 (Paraszkiewicz, *et al.*, 2002)
Protein+polysaccharide	*Syncephalastrum racemosum* (Mathur, *et al.*, 2010)
Saccharomyces cerevisiae (Dikit, *et al.*, 2010)	**Carbohydrate+lipids+protein**
Carbohydrate+protein	*Penicillium sp.* (Luna-Velasco, *et al.*, 2007)
Kluyveromyces marxianus FII 510700 (Lukondeh, *et al.*, 2003)	**Lipid+Fatty acids**
Polysaccharide+lipid	*Myroides odoratus* JCM7458 and *M. odoramitimus* JCM7460 (Maneerat, *et al.*, 2006)
Candida lipolytica ATCC 8662 (Cirigliano & Carman, 1984)	

These microbial polyphilic polymers are known to contain deoxy sugars (6-deoxy rhamnose or fucose), hydrophobic constituents like acyl-, methyl-, or other groups, and sometimes even fatty acids. There are other deoxy sugars recruited by the microorganisms which are responsible for their hydrophobic character. For synthesis and assembly of these molecules, microbes use de-novo pathway and/or assembly from substrates. The best studied and first bioemulsifiers reported are the bioemulsans

produced by different species of *Acinetobacter*, some of which are given in table-1 (Neu, 1996, Ron & Rosenberg, 2001, Satpute, *et al.*, 2010).

On the basis of chemical nature of bioemulsifiers produced by different microorganisms as listed in table 1.1, their nature can be classified into one of the following five categories.

1. Polysaccharides and its derivatives: Production of amphipathic polysaccharides and its derivatives with emulsification ability is seen not only in most reported bacteria like different species of *Acinetobacter* and *Pseudomonas*, but also in Actinomycetes like *Streptomyces* sp., algae like *Dunaliella*, fungi like *Curularia* and yeasts like *Candida lipolytica* and *Saccharomyces cerevisiae* Figure 1.2 gives the structures proposed for polysaccharide bioemulsifiers RAG-1 and BD4 .



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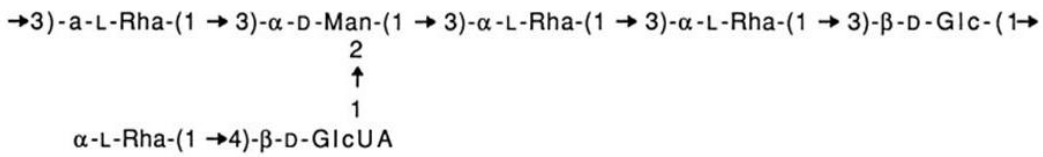

Figure 1.2. Proposed structures of polysaccharide bioemulsifiers from Acinetobacter calcoaceticus strains RAG-1 (a) and BD4 (b). (Kaplan, *et al.*, 1985, Kim, *et al.*, 1997)

2. Proteins and Protein complexes: Protein bioemulsifiers are produced by both Eubacteria and Archaea. Among eubacteria, *Methylobacterium* sp., and among archaea, *Methanobacterium thermoautotrophicum* are prominent producers of this type of bioemulsifiers. *Solibacillus silvestris* AM1, the bacterial strain

used in present studies also produces a glycoprotein bioemulsifier (Markande, *et al.*, 2013 In press).

3. Carbohydrates derivatized with proteins (and aminoacids), Lipids and Fatty acids: Carbohydrates with hydrophobic or hydrophilic nature derivatized with proteins or other hydrophobic moieties like lipids and fatty acids are major type of bioemulsifier reported. Carbohydrate-protein derivatives as bioemulsifier are reported in *A.calcoaceticus, B. subtilis*, *Halomonas* sp., *Antarctobacter* sp. and *Pseudoalteromonas* sp. in eubacteria and *Kluyveromyces marxianus* in yeasts. Carbohydrate-lipid derivatives are reported to be used as bioemulsifiers by eubacteria like *Pseudomonas aeruginosa* as Rhamnolipids and in fungi like *Trichosporon montevideense*, *Geotrichum sp.* and *Aspergillus niger*. Carbohydrate and Fatty acid derivatives are used by fungi like *Trichosporon mycotoxinivorans* and yeasts including *Yarrowinia lipolytica*.
4. Derivatives made up of Carbohydrates, Lipids and Protein/Peptides: Use of biomolecules available in the cell for producing stable extracellular moieties for survival has been a hallmark of life since it evolved. The derivatives made up of carbohydrates, lipids and proteins in different combinations make a perfect amphipathic molecule to be used by microorganisms for their surface activities. These derivatives are seen in *Variovorax paradoxus, Geobacillus pallidus*, *Bacillus stearothermophilus*, *Pseudomonas marginalis*, *Serratia marcescens*, actinomycetes like *Actinopolyspora* sp., *Corynebacterium kutscheri* and in fungi *Penicillium sp*.
5. Other derivatives: Many other derivatives of biomolecules produced by microorganisms are as bioemulsifiers. Example: Lipid-protein derivatives are seen in bacteria like *Bacillus velezensis* and *Streptococcus gordonii*. Lipid-fatty acid derivatives are reported as bioemulsifiers in *Myroides* species.

1.3. Functions of bioemulsifiers:

Although large numbers of microorganisms are known to produce bioemulsifiers indicating their significance in many aspects of growth, it is difficult to generalize on their roles in microbial physiology. Most of the concepts have been derived from a consideration of the surface properties of bioemulsifiers and many

hypotheses have emerged from these (Ron & Rosenberg, 2001). Bioemusifiers have definite functional roles in the microbes. The various roles bioemulsifiers play for microorganisms are discussed below.

1.3.1. Bioavailibilty of water insoluble substrates:

It is observed that the growth rate of the bacteria can be limited by the interfacial surface area between water and substrate/air (an interface). For bacteria growing on any interface, as the interfacial surface area becomes limiting, the biomass increases arithmetically rather than exponentially. The emulsifying agents play a natural role by increasing the surface area of insoluble substrates thereby aiding in bioremediation. When the cell is competent for micellar interaction and the emulsion occurs very close to the cell surface with no microscopic-level mixing, then each cell creates its own micro-environment and emulsification occurs, the effect of which may not be macroscopically evident (Ron & Rosenberg, 2001).

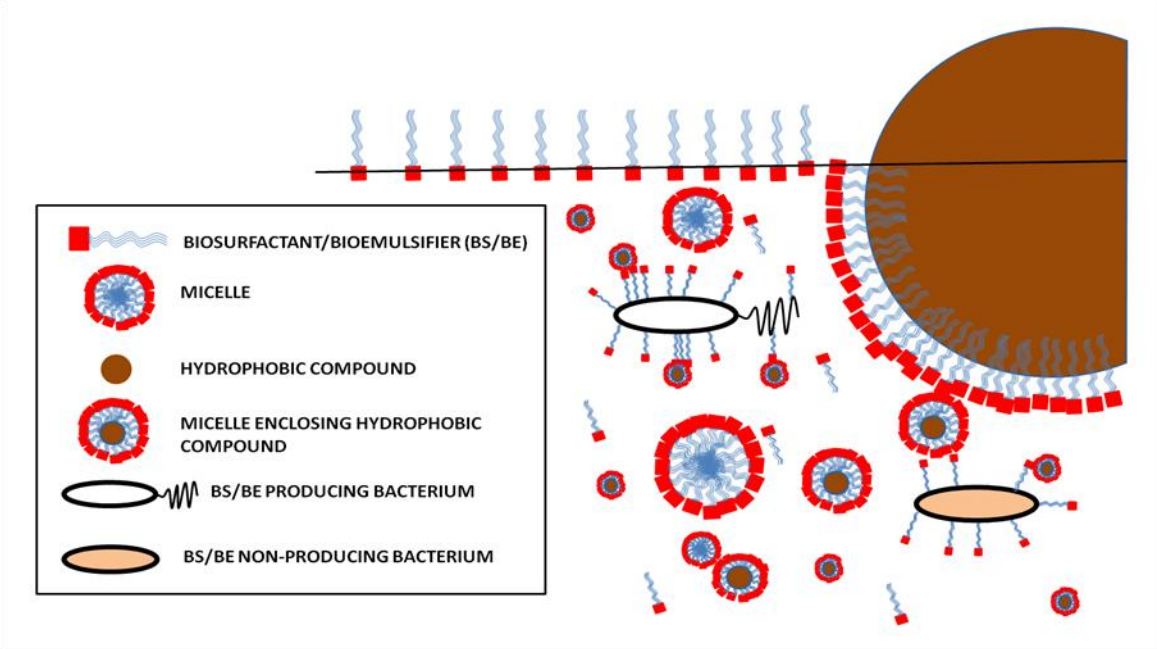

Figure 1.3. Bioavailability of insoluble/hydrophobic carbon-sources

Hydrophobic compounds, because of their low water solubility and higher sorption to surfaces, show prolonged persistence in the environment and their availability is also limited to bioremediating microorganisms. Surface active compounds like bioemulsifiers can enhance growth on bound or inaccessible substrates by desorbing them from surfaces or by increasing their water solubility

(Figure 1.3). Bioemulsifiers like Alasan are known to increase the apparent solubilities of polyaromatic compounds (PAHs) by five to twenty folds thereby increasing the biodegradation rate significantly (Miller & Zhang, 1997, Barkay, *et al.*, 1999, Rosenberg & Ron, 1999, Ron & Rosenberg, 2001).

Hydrocarbon and petroleum degrading bacteria, *Alcanivorax borkumensis, Candida lipolytica* and *A. calcoaceticus* RAG-1 are well documented to produce bioemulsifiers. *Sphingomonads* are widely distributed in soil and some are known for their bioemulsifier production and also degradation. In soil's heterogenous environment, *Sphingomonads* are hypothesized to accumulate the hydrocarbons using sphingan based bioemulsifiers in biofilms and then can access them as carbon source (Johnsen & Karlson, 2004). Calvo *et al*., (2009) reported *Halomonas eurihalina* capable of synthesizing bioemulsifier in presence of hydrocarbons though not necessarily as a direct response. Interestingly, this bioemulsifier production from *H. eurihalina* stimulated the growth of other hydrocarbon degrading bacteria such as *Bacillus, Pseudomonas, Micrococcus and Arthrobacter* species.

1.3.2. Adherence and de-adherence to surfaces:

When biosurfactants like bioemulsifiers interact with an interface, they form a conditioning film there which changes the properties of the original surface known as wettability and affect the adhesion and de-adhesion of bacteria (Neu, 1996). Although well known for working as hydrocarbon solubilisation and emulsion stabilization agent, they also act and modulate cell surface, modifying temporarily but reversibly thereby controlling the substrate access by the cells (Perfumo, *et al.*, 2010). One of the more hypothesized aspects of bioemulsifier presence on a bacterial surface is with respect to its amphipathic nature (having both a hydrophobic and a hydrophilic part) and its role in changing cell surface hydrophobicity. The bioemulsifier presence affects bacterial adhesion independent of the bacterial surface properties. The orientation of the bioemulsifier on the surface dictates the choice of bacteria capable of adhesion on the surface wetted with bioemulsifier (Figure 1.4).

Cell adherence to surfaces has been widely attributed to hydrophobic interactions. Hydrophobicity is an interfacial phenomenon and it is difficult to evaluate cell-adherence solely to hydrophobicity since many factors are involved in interfacial system of interest. As listed by Palmer et al. (2007), there have been many

reports about the enhancement and inhibition of cell adhesion to surfaces governed by cell surface hydrophobicity. Bioemulsifiers have been reported extensively to change cell surface hydrophobicity in *Serratia marcescens, Alcanivorax borkumensis, Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* (Rosenberg, *et al.*, 1983, Zhang & Miller, 1994, Neu, 1996, Pruthi & Cameotra, 1997, Yakimov, *et al.*, 1998) and affect the utilization of recalcitrant carbon sources. According to Neu (1996), lipopolysaccharides and other eubacterial common antigens are commonly seen doing such functions, however, in Gram positive bacteria lipoteichoic acid, lipomannan and other amphiphiles and in Gram negative bacteria, polysaccharides anchored to the membrane by their lipid part act as surface modulators.

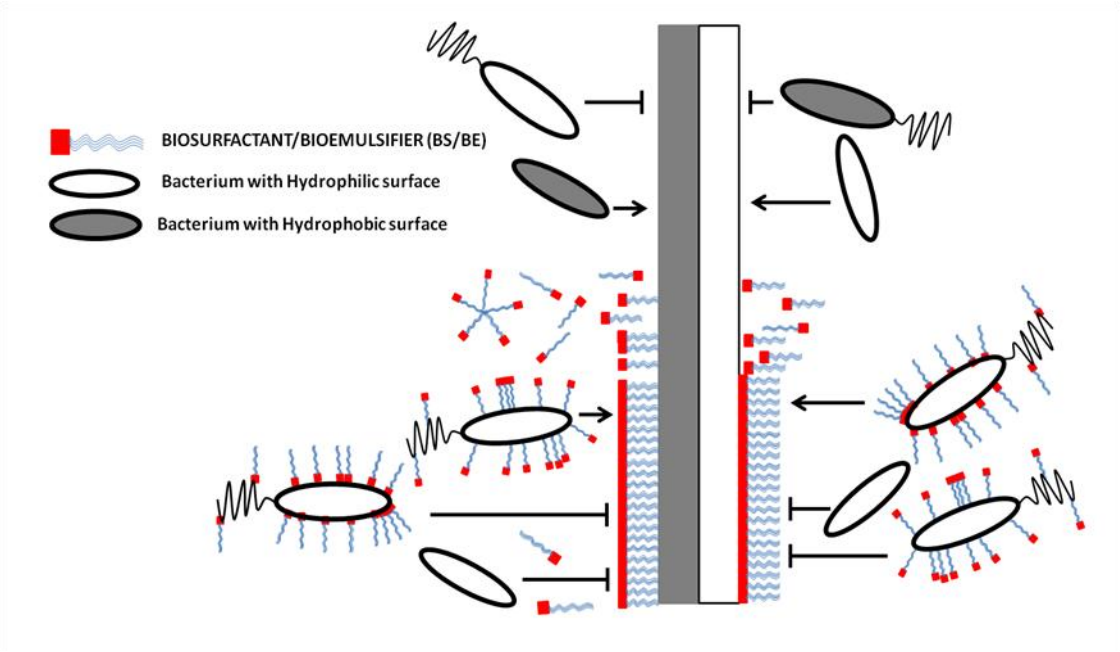

Figure 1.4. Attachment of bacterium to the surface. *The hydrophobic surface shown in grey inhibits the bacteria with predominant hydrophilic (white) surfaced bacteria and vise-a-versa from adhesion to surfaces.*

Bioemulsifiers help the microbes in forming a conditioning film at the interface changing the hydrophobic interface due to its wettability property, to hydrophilic and vise a versa. This change in interface properties helps the compatible bacteria in adherence (Figure 1.4 and 1.5). With amphipathic nature, the bioemulsifiers may help bacteria as a connecting bridge with the surface, thus microorganisms attached to an interface would be able to detach itself by releasing the polymers or parts of them on their surface (Neu, 1996). (Kaplan, *et al.*, 1987) studied

the mechanism of bioemulsifier emulsan in *A. calcoaceticus* RAG-1 and suggested that it functions as an anti-adhesion factor for hydrophobic interfaces. They further postulated that the released emulsan forms a film on the hydrophobic interface and label the substrate as being used referring microbial to it as footprints.

Bacteria are known to explore their immediate surroundings by gliding and swarming, considered to be a continuous desorption process helped by bioemulsifier along a two dimensional system of the interface with a complementary function of flagellins and flagellum (Neu, 1996, Daniels, *et al.*, 2006, Xu, *et al.*, 2012). Gliding and swarming motility of bacterium are not completely explained by sole functions of bioemulsifiers, but is seen in *Rhizobium etli* CNPAF512 (Daniels, *et al.*, 2006) and *Pseudomonas syringae* pv. syringae B728a (Xu, *et al.*, 2012). Flagellin and other factors are likely to play an important role is the lifestyle of bacteria.

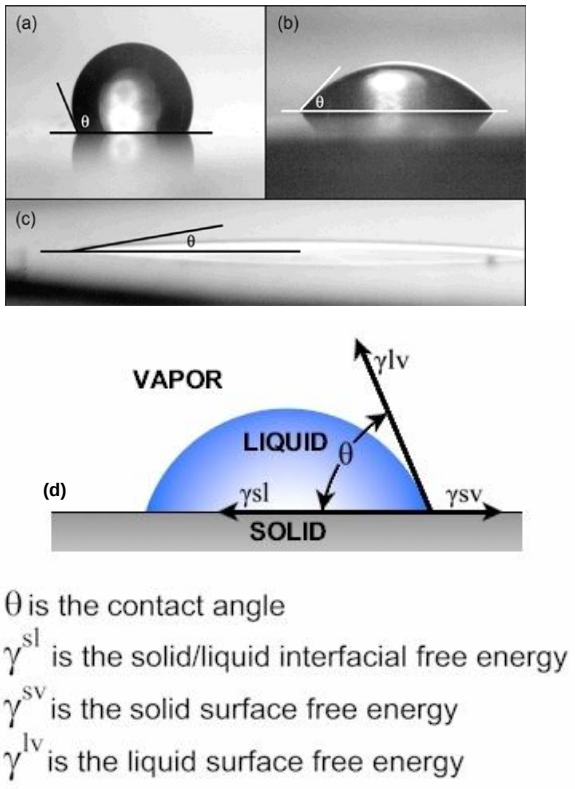

Figure 1.5. Contact angle measurements. Contact angle measurements of a water droplet on three typical borosilicate glass surfaces: (a) halocarbon wax coated using the dip method (hydrophobic surface), (b) untreated (hydrophilic surface), (c) plasma-cleaned (spreading) and (d) measurement of the contact angle of a liquid drop deposited on a solid sample (Sumner, *et al.*, 2004).

Microbial adhesion to surfaces can be calculated in several ways. Many years ago search was initiated by taking into consideration of zeta potentials, contact angles, cell surface hydrophobicities, surface free energies and other uniform physico-chemical properties, neglecting the structural complexity and chemical heterogeneity of microbial cell surfaces by applying thermodynamics or DLVO (Derjaguin, Landau, Verwey and Overbeek) theories to explain initial microbial adhesion to surfaces (Absolom, *et al.*, 1983, Pringle & Fletcher, 1983, Busscher, *et al.*, 1984, Van Oss, 1989, Palmer, *et al.*, 2007, Busscher, *et al.*, 2010, Hori & Matsumoto, 2010). At present, Contact angle measurements remains the simplest and most accurate method for characterizing the surface properties of solids and determining the interaction energy between a liquid (L) and a solid (S), at a minimum equilibrium distance (Yildirim, 2001).

θ can be defined as a measure of the competing tendencies between the energy of cohesion of the liquid molecules and the energy of adhesion between liquid and solid. When the work of adhesion between solid and liquid is exceeded by the work of cohesion between liquid molecules, a drop of liquid placed onto the solid surfaces form finite contact angle (Figure 1.5) and on the contrary, if the work of cohesion is lesser than the work of adhesion, spreading occurs. As the hydrophobicity of the surface increases, the contact angle increases. In principle, higher values of water contact angles are exhibited by solids having lower surface energies (γ_S). It is said to be a measure of hydrophobicity, thus can be used for calculating the surface free energy of a solid surface and also for free energy of interaction between two surfaces. (Bachmann, *et al.*, 2000, Yildirim, 2001, Sumner, *et al.*, 2004, Vandencasteele & Reniers, 2010).

1.3.3. Biofilm formation:

The wetting of the surface by bioemulsifier makes a conducive environment for bacterial attachment facilitating reversible adhesion of the bacteria leading to high density, attached microbial communities often embedded in extracellular matrices, also called as biofilms (Ortega-Morales, *et al.*, 2010). As given in figure 1.6, the exopolymeric bioemulsifiers, after initial conditioning of the surfaces helps bacteria in forming biofilm and are also present as integral part of the matrix which protects its inhabitants from predators, biocides, dehydration and other extreme environmental

conditions (Das *et al.*, 2009). Bacteria in these conditions are in a state of movement with the help of flagella and type IV pili (swarming/gliding motility). Bacterial adhesion to a solid surface occurs both in turbulent and immobile aqueous phase. Sedimentation and the capillary (or drainage) forces caused by pressure of the liquid flowing between the solid surface and the bacterial surface affects bacterial aggregation, movement and film formation at this interface. In various ecological niches, microorganisms usually occur as an assemblage of dual or multispecies population with various types of interaction with each other (Nikolaev & Plakunov, 2007, Sadowska, *et al.*, 2010).

The role of extracellular polymeric substance (EPS), as an important component of bacterial biofilms are studied for their role in maintaining structural integrity of biofilms, mediating cell-cell and cell-surface interactions and protection of inhabitants from dehydration, predation, biocides and other extreme conditions (Figure 1.6). The EPS, involved in bacterial adhesion and biofilm formation is known to vary from species to species, composed of polysaccharides, lipids, proteins and extracellular DNA (eDNA). Numerous exopolysaccharides are known to be influential in biofilm formation, but, not all polysaccharides can help in bacterial attachment to the surface as seen in *Vibrio* species (Das, *et al.*, 2009, Romero, *et al.*, 2011, Petrova & Sauer, 2012). Being far from mere aggregates of cells, the bacterial biofilms exhibit an ordered spatio-temporal distribution of cells with specific tasks as seen in *Bacillus subtilis.* In *B. subtilis* biofilms, cells involved are distributed into various specific functions, this may be as diverse as sporulation, motility and matrix formation (Romero, *et al.*, 2011).

Microorganisms attached to a hydrophilic or hydrophobic interface would be able to detach by releasing these expendable extracellular polymers or part of them from their surface. This mechanism is studied in detail with respect to emulsan, from *A. calcoaceticus* RAG-1 (Kaplan & Rosenberg, 1982). Emulsan present as a minicapsule on the surface of bacterium helps it in attachment. These bioemulsifier molecules are released from the cell surface by an extracellular esterase when bacteria experience starvation (Neu, 1996). There are indications that there exists a horizontal transfer of high molecular weight emulsifiers from the producing bacteria to heterologous bacteria (Osterreicher-Ravid, *et al.*, 2001, Ron & Rosenberg, 2001). This horizontal transfer of bioemulsifiers from one bacterial species to another has

significant implications in natural microbial communities, co-aggregation and may influence in formation of natural heterogeneous biofilms.

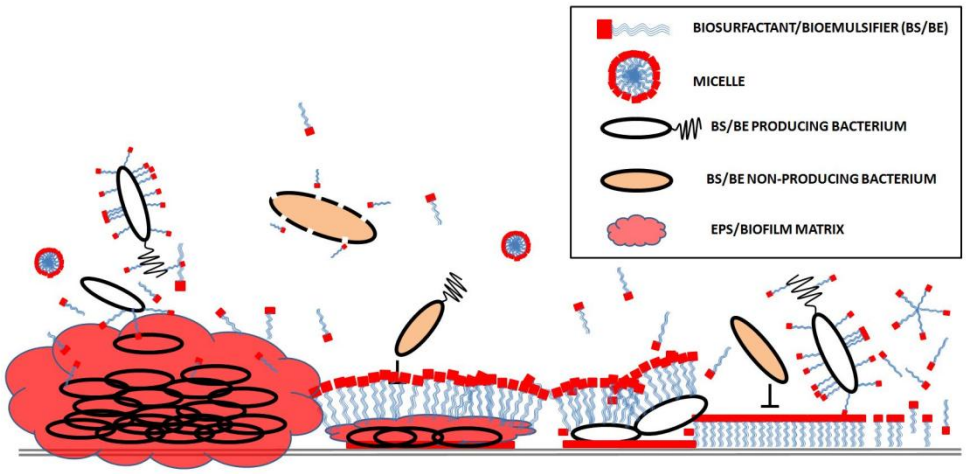

Figure 1.6. Attachment and Biofilm formation

1.3.4. Quorum sensing:

It is a generally accepted fact that bacteria produce and respond to chemical signals and this cell-to-cell communication leads to the coordination and reorganization of microbial activities (Qian, *et al.*, 2007). Quorum sensing is a ubiquitous bacterial communication mode whereby bacteria regulate their gene expressions through the presence or absence of a small signal molecule termed as autoinducer (Natrah, *et al.*, 2011). In other words, bacterial cells produce and release autoinducers and when the concentration reaches a threshold, the whole bacterial population alter their gene expression, which may lead to community assembly of biofilm formation, modulation of association with higher organisms or even in pathogenesis. Many quorum sensing systems exist in nature. In general, Gram-negative bacteria use molecules like acylated homoserine lactones (AHLs) which diffuse across the cytoplasmic membrane and bind to regulatory proteins within the cell, while Gram-positive bacteria use peptide signals which are detected through membrane bound receptors (McDougald, *et al.*, 2007).

Members of the group biosurfactants (both high and low molecular weight biosurfactants) were recently shown to influence the swarming behavior by acting as chemotactic-like stimuli. The role of rhamnolipids in swarming motility and surface motility in general is well documented (Glick, *et al.*, 2010). The Production of bioemulsifier at high cell density has a selective advantage. For the emulsifier

produced by pathogen, it has been suggested that, being virulence factors, they are produced when the cell density is high enough to cause a localized attack on the host (Sullivan, 1998). Natural environment is constantly in a war of strategies between competing organisms. Some microorganisms have devised intricate mechanisms to quench the autoinducers termed as quorum quenching (QQ) of competing microorganisms and further stoping its colonization and further nutrient shortage. (Romero, *et al.*, 2012) evaluated this mechanism that occurs abundantly among the cultivable bacteria obtained from oceanic and estuarine seawater.

Environmental cues include the production of a signal that is taken up by another, benefitting the receiver but not the producer. Quorum sensing autoinducers diffuse into the environment and can act as cues for other organisms. Release of AHL by the biofilm cells leads to the detection and preferential colonization of bacterial biofilms by higher organisms. Thus, marine biofilms are instrumental to the onset of settlement events for many sessile marine organisms and their habitat selection. Biofilms can mediate protist's colonization, the settlement of invertebrate larvae and macroalgal spores. This development of undesired microbial layer of heterotrophic organisms on the surface of substantial industrial and commercial interest is termed Biofouling (Flemming, 2002, McDougald, *et al.*, 2007, Qian, *et al.*, 2007). In order to overcome this Quorum quenching (QQ) is a usual strategy adopted among marine and coastal bacterial (and higher organisms) communities to achieve competitive advantages over AHL producing strains (Romero, *et al.*, 2011).

1.3.5. Virulence factors in bacterial pathogenesis:

Although production of bioemulsifier is often known to be enhanced in the presence of hydrocarbons, variable levels of production are maintained even in the absence of hydrophobic substrates (Klotz, 1988). The first report of production of rhamnolipids as virulence factor in *Pseudomonas aeruginosa* by (Jarvis & Johnson, 1949), is known to cause diseases like Cystic fibrosis is well characterized (Iacocca, *et al.*, 1963, Doggett, *et al.*, 1964). Since the elucidation of biosynthetic pathway of rhamnolipids by Burger, *et al.* (1963), the sequential process preceeding their production is well known (Ochsner, *et al.*, 1995). The production of rhamnolipids in *Pseudomonas aeruginosa* is under the cell density-dependent control involving

quorum sensing (QS) system. A bioemulsifier produced by *Candida albicans* enhances yeast adherence to intestinal cells (Klotz, 1988).

1.3.6. Other roles:

Polysaccharide high-molecular-weight emulsifiers interact with metals by binding them, as has been shown for the binding of uranium by emulsan of *A. calcoaceticus* (Ron & Rosenberg, 2001, Zosim, et al., 2004).The exopolymer bioemulsifier of *Pseudoalteromonas* TG12 (Gutierrez et al., 2008) was reported to be able to desorb various mono-, di- and trivalent metal species from marine sediments, thus acting as a metal-chelating agent.

The difference in chemical structures and surface properties of bioemulsifiers indicate that one group of bioemulsifiers would have an advantage in a specific ecological niche, while another group would be more appropriate for different niche. This diversity makes it difficult to generalize the natural role of biosurfactants (Ron and Rosenberg, 2001). These microbial surface active compounds including bioemulsifiers have received increasing commercial attention over the past few years as substitutes for synthetic surfactants owing to their high surfactant and emulsifying activities, stability in extreme physico-chemical conditions and other advantages (such as lower toxicity and higher biodegradability) (Franzetti et al., 2011).

1.4. Applications of bioemulsifiers:

Many functional chemicals (such as drugs, pesticides, etc.,) are insoluble in water and require organic solvents and alcohols to form solutions. Due to costly and hazardous properties of most of these solvents, their use as solutions is avoided especially in human use. Emulsifiers especially bioemulsifiers are useful due to their lower toxicity, higher stability, environment and human friendly nature. With many extraordinary benefits, they are involved in unlimited number of uses that involves every industry and every aspect of life: oil industry, pharmaceuticals, hygiene and cosmetic products, cement, beer and beverages, textiles, paint, detergents, cleaning and food processing (Gharaei-Fathabad, 2010).

High molecular weight biosurfactants like bioemulsifiers are known to have large number of reactive groups exposed, consequently with high adherence capability to surfaces and forming strong monolayers. They are known to form stable emulsions and dispersions that hardly coalesce and as it remains bound to the drops,

has the capacity to re-emulsify when fresh water is added with mixing. As listed by Rosenberg and Ron (1997), these properties confer several advantages to bioemulsifiers over low molecular weight biosurfactants like, they form very stable emulsions or dispersions that never coalesce; remain stuck to the interface even when the solvent is replaced; adhere to interfaces hence are not diluted; partitions completely to the interface therefore it is possible to treat very dilute suspensions and are biodegradable.

Utilization of bioemulsifiers for bioremediation was started in 1990s (Shete et al., 2006). Addition or *in situ* production of biosurfactant and bioemulsifier in soil can efficiently enhance biodegradation of hydrocarbons. It is observed that the degradation time and specifically adaptation time for microbes are shortened by addition of biosurfactant and bioemulsifier. The presence of biosurfactant and bioemulsifier in soil is known to produce a positive effect on soil structure, its ability with regard to water and nutrient availability by stimulation of dissolution or desorption rates, solubilization or even emulsification of hydrocarbons. Bioemulsifiers have been well reported as enhancers of hydrocarbon biodegradation in liquid media, soil slurries and water and soil microcosms and in oil contaminated fields (Ron and Rosenberg, 2002, Calvo et al., 2009).

For more than a century, emulsions are in use as drug-carriers from solubilizing drugs to controlled release. Emulsions can be used for oral, topical and parenteral routes of drug delivery. Water in oil (W/O) emulsions are reported to be more efficient in oral administration of drugs (Masuda et al. 2003). Although both W/O and O/W emulsions have been investigated for parenteral drug delivery, predominantly O/W emulsions are used. Marketed emulsion formulations are used for cancer, Alzheimer's disease, for thrombosis therapy, etc. For encapsulation of bioactive compounds, double emulsions are considered to be excellent systems. The presence of a reservoir inside the droplets of another phase can be used to sustain specific release of active compounds (Khan, *et al.*, 2006).

Emulsan and rhamnolipids are the major bioemulsifiers produced on industrial scale and are available in market. Emulsan is marketed by Petroleum Fermentations (Netherlands) for use in cleaning oil-contaminated vessels, oilspills and microbially enhanced oil recovery (MEOR), and to facilitate pipeline transportation of heavy crudeoil.

By the beginning of 2010, more than 225 patents were available with respect to microbial amphipathic agents. Highest number of patents are issued for use of biosurfactant and bioemulsifier in petroleum industry (33%), followed by cosmetics (15%), as an antimicrobial agent and medicine (12%) and for bioremediation (11%). Use of biosurfactant and bioemulsifier as replacement of chemical surfactants/emulsifiers in medicines is a relatively newer trend (Shete et al., 2006; Satpute et al., 2010). As listed in Table1, the bioemulsifier diversity does not limit to just chemical characteristics, many diverse microbes producing bioemulsifiers are reported belonging to Eubacteria, Archaea and Eukarya. Papers about biosurfactants and bioemulsifiers are being published since 1965 and patents related to their utilization in petroleum industry were issued from 1980-81 onwards (Shete, *et al.*, 2006). Correlation of natural roles of the bioemulsifier and their potential applications reported is given in Table 1.2.

Initial studies on the first bioemulsifier, Emulsan with respect to the effect of carbon source on bioemulsifier production influenced the further studies of different bioemulsifiers (Rubinovitz, *et al.*, 1982, Pines, *et al.*, 1983). There have been many reports on enhancement of bioemulsifier production optimization since early 1990s (Al-Mallah, *et al.*, 1990, Shepherd, *et al.*, 1995). Although many bioemulsifiers are reported till now as given in table 1.1, the genetics of bioemulsifier production is elucidated only for Emulsan and Alasan produced by *Acinetobacter* species. The major study done for Emulsan from *A. calcoaceticus* RAG-1 and Alasan from *A. radioresistens* KA53 includes mutagenesis by transposon mutagenesis of the strains producing them (Johri, *et al.*, 2002, Toren, *et al.*, 2002).

For the yield of all biotechnological products, the genetic study of the industrial strains is very important factor. The future research for high-level production of bioemulsifier should be with respect to the development of novel engineered strains for hyperproduction of bioemulsifiers. The extracellular bioemulsifier studied in this work demonstrates amyloid properties. Hence it is important to understand the structure and function of amyloids.

1.5. Amyloids:

Amyloids are usually known to be filamentous proteins with ~10nm width and 0.1-10μm length with a structural motif and cross- β structure. Amyloid fibers are

reported to be made up of amphipathic proteins that aggregate and since long time have been associated with neurodegenerative diseases such as Alzheimer's, Parkinsons and many other Prion diseases. Even with varied differences in their primary sequence, many proteins can assemble into amyloid folds (Soreghan, *et al.*, 1994, Gebbink, *et al.*, 2005, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). This shows that the amyloid fold has been selected multiple times during the evolution for various functions. As seen in a newly described class of 'functional' bacterial amyloids (FuBA), the amyloid formation can be an integral part of normal cellular physiology. By nucleation (aggregation) - dependent mechanism, elongation of proteins occurs into fibrils consisting of structured oligomers and protofibrils. Nucleation occurs due to conversion of monomeric precursors to amyloid fibrils. The sigmoid curve shows lag, growth and stationary phase in conversion and basic fibrillation process as shown in figure 1.6b. These oligomers and protofibrils are considered as the real cytotoxic species in relation to causing human diseases and cytotoxic bacteria (Fowler, *et al.*, 2005, Nielsen, *et al.*, 2011). This cellular toxicity of amyloids is avoided by using dedicated and highly controlled pathway for assembling amyloids and extracellular assembly of these proteins. Thus by nullifying the cytotoxic effects of amyloids, they can be used as stable protein structures for many different functions (Blanco, *et al.*, 2012). The three detailed amyloid aggregation phases of lag, exponential stationary are illustrated in figure 1.7.

Curli fibres were the first amyloids to be discovered in the 1980s on *Escherichia coli* strains that caused bovine mastitis (Olsén, *et al.*, 1989). These fimbrial proteins produced curled fibre appearance seen in electron microscope with CsgA as the main structural component. Similar fimbriae with amyloid nature have been also reported in *Salmonella* strains refered to as thin aggressive fimbriae (Tafi) (Collinson, *et al.*, 1991). These fimbrial associated FuBA are implicated in many pathological and physiological processes of *E.coli* and *Salmonella* species (Figure 1.7). These fibres help them in adhesion to surfaces, cell-aggregation and biofilm formation and internalization of bacteria into eukaryotic cells (Figure 1.7) (Barnhart & Chapman, 2006, Dueholm, *et al.*, 2010). A number of environmental factors are known to govern production of curli, but seem to change with strain type (Nielsen et al., 2011). Shortly after the discovery of curli and tafi, presences of similar structures were described from other members of *Enterobacteriaceae* such as *Citrobacter* sp and

Table 1.2. Applications of bioemulsifiers with respect to their natural functions

Sl. No	Applications	Natural roles	References
1.	Bioremediation	Bioavailibilty of water insoluble substrates	Calvo, *et al.*, (2009)
	Use in cleaning contaminated vessels and oilspills, also in Microbially enhanced oil recovery (MEOR) in petroleum industry		Satpute et al., (2010)
	Orally administered drugs and Marketted emulsion formulations used for Cancer, Alzheimer's disease, thrombosis therapy, etc.,		Masuda, *et al.*, (2003); Khan, *et al.*, (2006)
2.	Surlactin, an anti-adhesion potent bioemulsifier produced by *Lactobacillus acidophilus* RC14, used for development of anti-adhesive biological coatings for catheters.	Adherence and deadherence to surfaces	Velraeds, *et al.*, (1996)
3.	Selective plugging strategy in MEOR by use of microbes that form biofilm and produce exopolymeric substances, blocking the high permeability zones of an oil reservoir, allowing the water flow through low permeability zones increasing the oil recovery	Biofilm formation	Suthar et al., (2009)
	Suface active compound from *Brevibacterium casei* MSA19 against pathogenic biofilms *in vitro*		Kiran, *et al.*, (2010)
4.	Using emulsification in specific drug transport to infection site and as adjuvants for vaccines	Antimicrobial	Makkar & Cameotra, (2002)
	Bioemulsifier from *Acinetobacter genospecies 3* shows antimicrobial activity against human pathogens like, *Staphylococcus aureus, Salmonella typhimurium, Klebsiella pneumoniae, Aspergillus niger, Aspergillus fumigatus, Candida humicola, Candida albicans, Cryptococcus*		Bhawsar, *et al.*, (2011)
5.	Food industry: Emulsifiers are used in bakery and meat products because they influence rheological characteristics of flour or for emulsification of fat tissue. World-wide use of Lecithin-based derivatives as bioemulsifiers. Bioemulsifier from *Candida utilis* used in Salad dressing.	Other roles	Makkar and Cameotra, (2002)
	Interaction with metal ions		Zosim, et al., (2004) and Gutierrez et al., (2008)

Enterobacter sakazaki. Genetic evidence indicates the presence of genes encoding curli proteins by other *Enterobacteriaceae* (like *Shigella* species) (Gebbink, *et al.*, 2005, Larsen, *et al.*, 2007).

Only few bacterial species important in relation to human infections have been studied with respect to FuBA with curli like fibrils. This list of bacteria capable of producing FuBA is growing rapidly and encompasses representatives from *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroides* but only a few of them have been purified and investigated in depth. Thus major functions proposed for FuBA generalized for all bacterial amyloids are still speculations (Nielsen et al., 2011).

The biofilm formed by *Bacillus subtilis* are known to be stabilized by amyloid protein fibrils (TasA) with the exopolysaccharide as a mixture of exopolymeric substance released by the bacterium (Gebbink et al., 2005; Nielsen et al., 2011). The biofilm formation at the air-liquid interface and sporulationa and dispersion of spores by *B. cereus* is considered to be aided by amyloid proteins. Similar amyloid-like structures have been reported on the spores of *B. atropheus, B. mycoides* (Bowen, *et al.*, 2002, Wijman, *et al.*, 2007, Jordal, *et al.*, 2009). It is now believed that spores of many sporulating Gram-positive bacteria are covered by similar amyloids which help the bacteria in spore dispersal, attachment and pathogensity and resistance to environmental stresses.

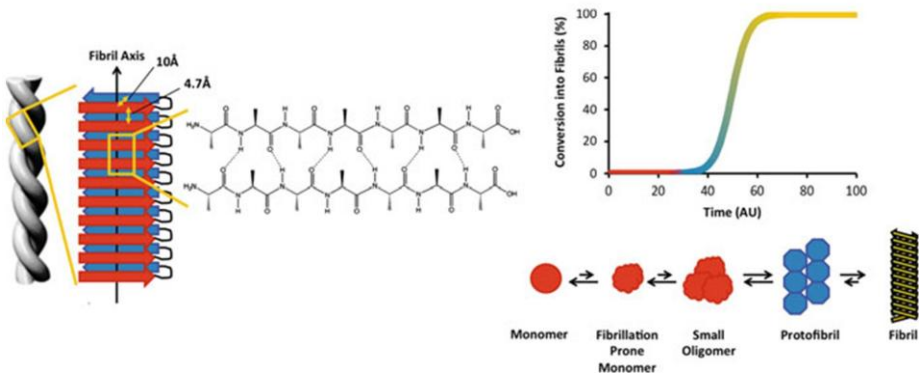

Figure 1.7. Structure and formation of amyloid fibrils. (a) Amyloid fibril composed of two intertwined protofilaments. (b) Nucleation of amyloid fibrils and conversion of monomeric precursor to amyloid fibrils (adapted from Nielsen et al., 2011).

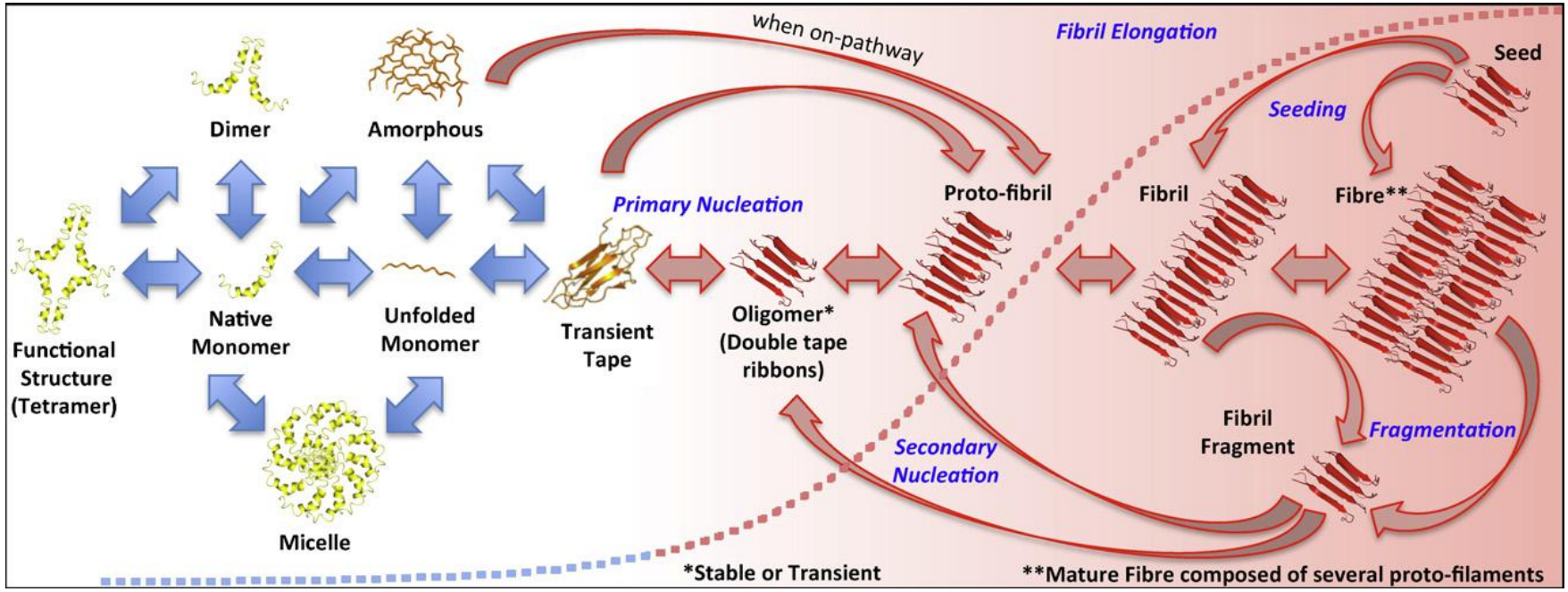

Figure 1.8. Amyloid assembly landscape (Invernizzi, *et al.*, 2012)

Some of the Hairpin proteins secreated by bacteria which elicit hypersensitive reaction in plants are known to include the toxic aspects of amyloid intermediates which include hairpins of *Xanthomonas campestris* and *Pseudomonas syringae*. *Klebsiella pneumoniae* is known to produce a bactericidal peptide, Microcin E492, which assembles (nucleation) into pligomeric pores of neighbouring bacteria belonging to *Enterbacteriaceae* species. Similarly Microcin E492 is also known to trigger apoptosis in human cell (Romero, *et al.*, 2010, Blanco, *et al.*, 2012).

Some of the amyloids are known to function as surface properties modifiers from Actinobacteria and Fungi. Chaplins, a class of amyloids produced by *Streptomyces coelicolor* and other Actinobacteria (like *Thermobifida fusca*) are known to help in morphological differentiation from submerged mycelium to growth in air by lowering the surface tension and for septae to spore conversion. Hydrophobins are kinds of amyloids known to be produced by many fungal species of ascomycetes and basidiomycetes phyla. Similar to chaplins, these structures eliminate the physical barrier of substrate-air interface, thus allowing the growth of hyphae in the air. A fungal plant pathogen *Ustilago maydis* produces repellants, which are involved in aerial hyphal attachment and penetration into host cells. These amyloids are hydrophobic in nature and play essential role of functional amyloids in fungi (Gebbink et al., 2005; Nielsen et al., 2011; Blanco et al., 2012).

All these amyloid proteins are relatively unrelated to each other at the amino acid level and indicate that horizontal gene transfer has not happened but their development has happened many times during evolution. Nielsen et al. (2011), describe this fact of amyloid variation with respect to their distribution across the living kingdom as “each species, its own amyloid”.

1.6. Ecophysiological studies:

An organism’s relation with the ecology can be distinguished into ‘aut-ecology’ and ‘syn-ecology’ where the former applies to the ecology of organisms by themselves and the latter to the ecology of habitats and all the ecosystems with respect to all inhabitant organisms. Ecophysiological studies analyze the responses of organisms to the environment and the analysis of physiological and interaction mechanisms involved from microorganism to a similar microbial group grading up to

community and ecosystem. It is autecology dedicated to the behavior of individual organisms in a particular habitat (Lüttge & Scarano, 2004).

The discipline of Ecophysiology emerged from functional studies of plant's behavior in their environment linked to ecology and physiology over a hundred years ago. Since then, with its origin as descriptive phytogeography the discipline developed into an independent functional study of plants and animals (Pardo, 2005) and eventually microorganisms. Ecophysiology and the use of a comparative approach to physiology received its due appreciation 50 years ago with establishment of Comparative Biochemistry and Physiology in 1960 (Schwarzbaum & Krumschnabel, 2011).

Plant ecophysiological studies about Mangroves (Ball, 1988), performance of trees and seedlings in the fields (Ball, *et al.*, 1991), ecophysiological process in fruiting (Barták, *et al.*, 1992), inter- and intra-specific competing plants (Lemaire & Millard, 1999), ecophysiological grouping of Mediterranean oaks and management of forest ecosystems (Pardo, 2005), Plants in dry environments (Lombardini, 2006) and life and strategy of seaweeds in polar waters (Wiencke, *et al.*, 2007) were prominent since 1985. Among animals, studies like Dopaminergic inhibition of reproduction in Teleost fishes with respect to their ecophysiological and evolutionary implications were discussed in detail (Dufour, *et al.*, 2005).

Plants dominated initial ecophysilogical studies, but recently the major involvement of microscopic organisms in ecosystems prompted the researchers in their ecophysiological studies. Häder & Figueroa (1997) studied photo-ecophysiology of marine macroalgae with respect to the light penetration into the water column in comparison to macroalgal zonation and protection against excessive light. Similarly, studies on ecophysiology of cyanobacteria, light stimulated amino acid utilization of marine Picoplankton and Stromatolitic mats were also reported (Mur, 1983, Paerl, 1991, Pinckney, *et al.*, 1995, Watkinson, *et al.*, 2005). Soil microbial ecophysiology variance as effective measures of change in response to phosphorus levels at the enriched site was studied by Corstanje, *et al.* (2007) while Anderson (2003) proposed the use of indicators including physiological performances like specific respiration (qCO2) and other parameters to assess an 'ecophysiological profile' of a site for determining the soil quality.

The ecophysiological study of filamentous bacteria from activated sludge was done recently (Kragelund, *et al.*, 2008) and protein-hydrolyzing microorganisms (*Candidatus* and *Epiflobacter* sp.) colonizing filamentous bacteria in activated sludge were identified and characterized ecophysiologically by Xia, *et al.* (2008). While Thomsen, *et al.* (2007) studied various aspects of the ecophysiology of abundant denitrifying groups from activated sludge with respect to their physiological differences. Studies on microbial whey biomethanation (Chartrain & Zeikus, 1986) and anaerobic digestion in acid bog sediments by ecophysiological adaptations of anaerobic bacteria to low pH (Goodwin & Zeikus, 1987) tried to explain the microbial ecophysiological processes involved. A new and ecophysiologically unusual group of marine obligate hydrocarbon degrading or obligate hydrocarbonoclastic bacteria were shown to play significant role in removal of hydrocarbons from polluted marine waters (Yakimov, *et al.*, 2007). While fungal isolates belonging to different ecophysiological groups (wood-degrading, litter-degrading, ectomycorrhizal, and coprophilous fungi) were checked by Casieri, *et al.* (2010) for dye decolourization and ligninolytic activity.

Using extensive phenotypic characterization of bacteria isolated from congelated, platelet, and grease ice samples, Bowmanip, *et al.* (1997) determined the type of bacteria colonizing the sea ice and their ecophysiological strategies to adapt to sea ice environment. The study encompassed specifically three ecophysiological groups of psychrophilic halophiles, psychrotolerant and halotolerant bacteria, and non-halophilic, psychrotolerant bacteria. Zdanowski & Węgleński, (2001) studied the ecophysiology of bacterial communities distributed in the vicinity of Henryk Arctowski Station, King George Island, Antarctica. Similarly, Lo Giudice, *et al.* (2005) did ecophysiological characterization of cultivable Antarctic psychrotolerant bacteria and their ability to degrade hydrocarbons.

Reports discuss the influence and potential of predictive microbiology as an aid to food safety management and also the need for understanding the ecophysiology of microbes in the growth required/non-required regions were discussed with respect to ecophysiology of food borne pathogens and the variance in carbohydrate composition of cereal cultivars alter the pig intestinal microbial ecophysiology (Bindelle, *et al.*, 2010, McMeekin, *et al.*, 2010).

Nealson & Scott (2006) elucidated the ecophysiological features common to all the physiological traits of various *Shewanella* species as a group before environments where they are common and abundant. Many more microbial ecophysiological studies have been reported like ecophysiology of magnetotactic bacteria (Bazylinski & Williams, 2007), of *Azospirillum* spp. (Hartmann, 1989), of Halophiles (Rozema & Gude, 1981), of Candida sake (Teixidó, *et al.*, 1998) and also ecophysiological studies of lithotrophic sulfur-oxidizing *Sphaerotilus* species from sulfide springs (Gridneva, *et al.*, 2009).

When studied in the same habitat, the bacterioplankton populations harbor various taxa displaying difference in their unique substrate uptake patterns. Thus ecophysiological investigations appear to be more meaningful if performed on closely related, physiologically coherent lineages of pelagic bacteria (Alonso, *et al.*, 2009). The abundance and diversity of members of Gram positive spore forming bacteria from marine environments seem to be due to their ability to grow at wide salinity ranges and do not show dependency on sea-water containing media for their growth (Stevens, *et al.*, 2007, Ettoumi, *et al.*, 2009). The ability of these bacteria to resist extreme marine environments makes them ideal for use in aquaculture and retain their beneficial properties for a long time (Natrah, *et al.*, 2011).

The intertidal zone represents a typical ecophysiological study site (Lüttge & Scarano, 2004). Studies regarding the intertidal zone sporulating bacteria with respect to the characters mentioned above are negligible infrequent and the fact that these bacteria can be utilized in aquaculture and other industrial processes and bioremediation makes their study significant. Studying them as an ecophysiological group is an alternative way to characterize this group of bacteria (Lo Giudice, *et al.*, 2005, Bodoczi & Carpa, 2010, Ramanathan, *et al.*, 2011). Phelan, *et al.* (2012) also emphasize on lack of research about microbial metabolites influencing others in an ecosystem. Among their varied characteristics, studying these Gram positive sporulating bacteria like *Bacillaceae* as an ecophysiological group is an alternative way to characterize them (Lüttge & Scarano, 2004, Lo Giudice, *et al.*, 2005, Stevens, *et al.*, 2007, Ettoumi, *et al.*, 2009, Bodoczi & Carpa, 2010, Ramanathan, *et al.*, 2011). Some of the major reports about the bioemulsifier production by *Bacillus* genus are as follows:

Patel & Gopinathan (1986) isolated two *Bacillus* strains FE-1 and FE-2 from a soil sample exposed to high doses of organophosphorus pesticide, O,O-dimethyl- 0-[3-methyl-4- (methylthio) phenyl] phosphorothioate (fenthion). *Bacillus* strain FE-1 produced a high molecular weight, lysozyme sensitive and thermostable glycolipopeptide bioemulsifier while the bioemulsifier produced by *Bacillus* strain FE-2 consisted of carbohydrate, lipid, and peptide. Two *Bacillus* strains IAF 343 and IAF 346 producing two distinct bioemulsifiers were isolated by Cooper & Goldenberg (1987) from an oil sample at Canada. While the former produced a neutral lipid bioemulsifier, the latter produced a polysaccharide bioemulsifier. They also devised a unique method of analyzing the bioemulsifier activity of the surfactant produced. *Bacillus stearothermophilus* VR-8 isolated from hot spring in 4% crude oil inducement could produce extracellular bioemulsifiers containing proteinaceous bioemulsifier with minor carbohydrate and lipid content (Gurjar et al., 1995). A homopolysaccharide bioemulsifier with 100% lipid emulsification activity was reported by Yun & Park (2000) in *Bacillus* sp.CP912.

In a study by Toledo et al., (2006), among the fifteen strains isolated from solid waste oil samples, majority were found to belong to *Bacillus* species. Many of these strains could emulsify octane, xylene, toluene, mineral oil and crude oil with ability to remove hydrocarbons. Pavitran, *et al.* (2006), in their studies encountered ten-fold increase in the marine oil degradation rate by seeding the contaminated site with agricultural runoff and fertilizers even after two years of oil-spill and majority of isolates being capable of emulsification activity. Lee, *et al.* (2007) reported a lipopeptide surface active agent from *Bacillus amyloliquifaciens* LP03 with high emulsification activity also exhibiting antagonistic activity against plant-pathogenic fungi. Kumar, *et al.* (2007) isolated a *Bacillus* sp. DHT from oil contaminated soil from Guanoco Lake, Venezuela which produced a lipoprotein bioemulsifier. It could also degrade hydrocarbons with bioemulsifier production ability in wide salinity and temperatures. *Bacillus licheniformis* ACO1 was isolated from petroleum reservoirs in Iran produced a polysaccharide rich bioemulsifier (Dastgheib, *et al.*, 2008). The bioemulsifier showed protein active part with complete loss of emulsification activity in presence of Proteinase K while it resisted heating at 121^0C for 20 min. Suthar et al., (2008) reported a novel *Bacillus licheniformis* K125 producing bioemulsifier containing substantial amount of polysaccharide, protein and lipid. It combined

surface tension (72 to 34 mN/m) and emulsification activity (66% E24) with stability at wide range of pH, temperature and salinity. They have also reported an enhancement of oil recovery upon application to a sand pack column. In two separate studies, *Bacillus* species were isolated from hydrocarbon and oil contaminated soils capable of producing bioemulsifier (Sathe, *et al.*, 2012, Klawech, *et al.*, 2013) and *Bacillus subtilis* isolated in this study could degrade waste lubricating oil (WLO) (Klawech, *et al.*, 2013).

Bacillus silvestris now called *Solibacillus silvestris*, Krishnamurthi, *et al.* (2009), reported for the first time a decade ago (Rheims, *et al.*, 1999) from a forest soil sample in Germany, and subsequently from water samples taken from the southern Baltic Sea, a brackish environment (Pettit, *et al.*, 2009)and more recently as a part of extended evaluation of terrestrial and marine microorganisms as sources of new anticancer drug candidates, collected from a marine crab on Chiloe´ Island, Chile (Pettit, *et al.*, 2009). Although there are many 16S rDNA sequences from different strains of *Solibacillus* genus available in GenBank, only one species of *Solibacillus* has been reported till now and only once strain genome is sequenced. There are no reports of *S. silvestris* producing bioemulsifier however, bioflocculant producing *S. silvestris* has been reported recently by Wan, *et al.* (2013).

1.7. Rationale and Objectives:

Since their discovery, major studies on bioemulsifiers have been with respect to their potential in applications in bioremediation and industries. Our understanding of microorganisms includes only 1% of the total marine bacteria with untapped resources and functionality. Major studies with respect to functions of bioemulsifiers have been done in genus *Acinetobacter* which has been reported for maximum number of bioemulsifier producing species. The family *Bacillaceae* is widening with many of the previously known species and strains of the genera *Bacillus* have been reassigned into new species owing to their unique properties. This family includes maximum number of strains reported for industrially and environmentally important metabolites. But bioemulsifiers from *Bacillus* genera were never studied in detail to assert their role in influencing the ecophysiology of the bacteria involved.

The type of biomolecule produced is usually dependent on its basic biochemical make-up for effective function. As discussed till now, diverse type of

bioemulsifiers are reported and studied in different microorganisms which indicate that bioemulsifiers have immense ecophysiological role and has been evolved in different microorganisms using different basic subunits. In different microorganisms, bioemulsifiers have been studied for influencing adhesion and biofilm formation. They are also known to influence the host and other organisms in environment by direct interaction and thus have ecophysiological potential to help the host in settling in a niche. The bioemulsifiers exhibit many functions as discussed before but their studies in other bacteria than *Acinetobacter* genera is lacking. There have been no studies cited in the literature to integrate the various functions/roles of bioemulsifier in a single species of bacterium.

Keeping these points in mind, the objectives of this present research work are,

1) Isolation, screening and identification of bioemulsifier producing *Bacillus* sp.
2) Characterization of the bioemulsifier
3) Ecophysiological studies of the selected isolate with respect to its bioemulsifier

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Chapter 2

Isolation and screening of bioemulsifier producing *Bacillus* sp. and characterization of the selected strain

उत्पलस्यारविंदस्य मत्स्यस्य कुमुदस्य च।
एक योनिप्रसूतानां तेषां रूपः पृथक् पृथक्॥
-सुभाषित सुधानिधी ४४.

Meaning:
A lotus, fish and lily are borne in the same water.
But their forms and characters remain different.
-Subhashita Sudhaanidhi (44)

Chapter 2:

Isolation and screening of bioemulsifier producing *Bacillus* sp. and characterization of the selected strain

2A. Isolation and screening of bioemulsifier producing Bacillus sp.

2A.1 Introduction

Intertidal zone as mentioned in chapter 1 is one of the most important marine habitats representing a typical ecophysiological site, which includes sandy beaches, mudflats, salt marshes, mangrove forests, estuaries, certain coral reefs, rocky platforms and human made infrastructures (Lüttge & Scarano, 2004). The distribution patterns, abundance and diversification of specific microorganisms or microbial groups are decided by many facts prevalent here. Pollutants like hydrocarbons, heavy metals, pesticides etc., contaminate these habitats (Menge & Branch, 2001, Ortega-Morales, *et al.*, 2010).

When microorganisms need to compete for a settled niche, the competition depends on the ability to produce antagonistic substances such as biosurfactants, organic acids or peroxides, quorum quenching strategies, etc (Sadowska, *et al.*, 2010). Production of biosurfactant/ bioemulsifier is therefore an important trait for existence of bacteria in such niches. Uptill now most of the microbial studies done in the intertidal zones of Indian peninsula have been restricted to isolation, diversity and characterization of their metabolites. Gram positive sporulating bacteria of intertidal zone as an ecophysiological group has been well characterized (Lüttge & Scarano, 2004, Lo Giudice, *et al.*, 2005, Stevens, *et al.*, 2007, Ettoumi, *et al.*, 2009, Bodoczi & Carpa, 2010, Ramanathan, *et al.*, 2011).

Isolation of bioemulsifying *Bacillus* sp is commonly cited in literature. Bioemulsifier producing *Bacillus* genus were reported by Patel & Gopinathan, (1986). Cooper and Goldenberg (1987) reported two *Bacillus* strains IAF 343 and IAF 346 isolated from an oil sample in Canada. *Bacillus stearothermophilus* VR-8 isolated from hot spring produced extracellular bioemulsifiers (Gurjar, *et al.*, 1995). A soil isolate, *Bacillus* sp. CP912 produced bioemulsifier with 100% lipid emulsifying capacity (Yun & Park, 2000). In a study by Toledo, *et al.* (2006), fifteen bacterial strains were isolated from solid waste oil samples, majority belonging to *Bacillus.*

Pavitran, *et al.* (2006) reported bioemulsifier producers in agricultural runoff water. While a lipopeptide surface active agent with high emulsification activity was reported from *Bacillus amyloliquefaciens* LP03 isolated from soil by Lee, *et al.* (2007). Kumar et al (2007) isolated a *Bacillus* sp. DHT from oil contaminated soil from Guanoco Lake, Venezuela. *Bacillus licheniformis* ACO1 and *Bacillus licheniformis* K125, isolated from petroleum and oil reservoirs respectively. Isolated from Iran and India respectively, both these isolates produced bioemulsifiers with applications in Microbial oil recovery (MEOR) (Dastgheib, *et al.*, 2008, Suthar, *et al.*, 2009).

Marine habitats are also home to many diverse group of microorganisms among which bioemulsifier producers are common. Emulsan, the first reported bioemulsifier produced by *Acinetobacter cacloaceticus* RAG-1 (further renamed to be *A. venetianus* RAG-1) and patented was isolated from a mixed population present in crude oil in sea water. (Reisfeld, *et al.*, 1972, Bach, *et al.*, 2003). Boyle & Reade, in 1983, isolated two aerobic rod-shaped bacteria producing extracellular polysaccharide from intertidal zone near Halifax, Nova Scotia. Gutiérrez, *et al.* (2007a, 2007b) in two different studies reported marine bacteria *Halomonas* species and *Antarctobacter* strain TG22 isolated from sea water supplemented with n-hexadecane. From oil-spilled water samples of harbours and docks in Thailand, Maneerat & Phetrong, (2007) isolated eight strains namely *Myroides* sp. SM1, *Vibrio paraheamolyticus* SM2, *Bacillus subtilis* SM3, *Micrococcus luteus* SM4, *Acinetobacter anitratus* SM6, *V. paraheamolyticus* SM7, *B. pumilus* SM8 and an unknown isolate SM5 which could effectively emulsify weathered crude oil. *Streptomyces* sp.S1 was found to be the best among the six potential isolates from Alibag, Janjira and Goa coastal regions of India growing on oils and hydrocarbons as substrates (Kokare, *et al.*, 2007). *Planococcus maitriensis* Anita I, an isolate from seawater of coastal Bhavnagar, India which produced an exopolymer bioemulsifier with high potential for bioremediation (Kumar, *et al.*, 2007). Similarly *Enterobacter cloaceae*, isolated from marine sediment from western coast of India produced exopolysaccharide having emulsification activity (Iyer, *et al.*, 2006).

Literature is full of several reports of bioemulsifier producing bacteria being isolated from various niches for mostly biotechnological purposes. Nevertheless, an important marine habitat like intertidal zone is likely to harbor bioemulsifier

producing bacteria due to the constant pollution of hydrocarbon and related chemicals here. Survival in such conditions must have necessitated development of unique traits in populations of bacteria like production of bioemulsifiers. However, bioemulsifier producing bacteria from intertidal zones of oceans are sparse. The implications of the bioemulsifier producing bacteria and the interactions in which they are involved has not been studied at all. Keeping this in view the isolation and screening of bioemulsifier producing bacteria belonging to *Bacillus* sp. from the intertidal zones of Indian coast was undertaken. Periodic oligotrophic nature of the intertidal zone habitat and possibility of sporulating bacteria like *Bacillus* existing there along with the fact that those of marine origin are studied moderately was the purpose of choosing them. The properties like surface tension reduction, biofilm formation and quorum quenching that give a competitive edge to the bacteria in a settled niche were also studied.

In this section an ecophysiological group of Gram positive sporulating, bioemulsifier/biosurfactant producing, biofilm forming, quorum quenching bacteria from intertidal zone of Indian coast were isolated. They were further screened to obtain a special high bioemulsifier producing strain that was well characterized. The emphasis of studies that followed in the subsequent chapters was focused on bioemulsifier characterization, its natural functions in the selected strain and the ecophysiological interactions involving it in microcosms.

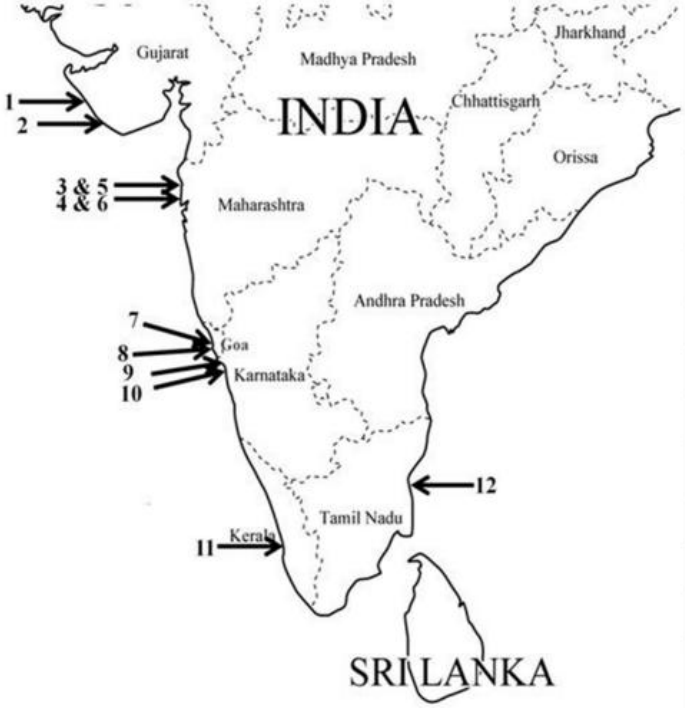
2A.2. Materials and Methods

2A.2.1. Sampling sites for isolation on bioemulsifier producing bacteria:

Sampling sites included littoral zones of coastal cities and ports of all five western and one east coastal state of India as given in the Table 2.1 and Figure 2.1. The twelve sampling sites were selected on the basis of their proximity to a constant pollution source or recent oil spill. The water and sediment samples were collected in sterile bottles and containers, kept at 4ºC till their processing by one or more of the following approaches.

2A.2.2. Isolation of bioemulsifying bacteria:

Firstly enrichment culture technique was performed for isolation. 1% of the samples were inoculated in sterile Zobell Marine medium (Appendix), and incubated



Sl. No.	Sampling sites (codes)	Sample	Location
1	Porbandar (GP)	Intertidal zone water	21.63°N; 69.58°E
2	Veraval (VG)	Intertidal zone water and sediment	20.89°N; 70.37°E
3	Bhayander (S)	Intertidal zone (saltern) water	19.31°N; 72.85°E
4	Madh-island (M)	Intertidal zone water	19.13°N; 72.79°E
5	Gorai (G)	Intertidal zone (Mudflat) sediment	19.24°N; 72.79°E
6	Versova (V)	Intertidal zone (Mudflat) sediment	19.14°N; 72.80°E
7	Panjim (P)	Intertidal zone water	15.50°N; 73.82°E
8	Vasco-da-gama (VP)	Intertidal zone water	15.41°N; 73.79°E
9	Baitkol (B)	Intertidal zone water	14.80°N; 74.12°E
10	Tadadi (T)	Intertidal zone water	14.52°N; 74.35°E
11	Kochi (K)	Intertidal zone water and sediment	9.97°N; 76.24°E
12	Parangipette (AM/CAo/CAM)	Intertidal zone (Estuary) water and sediment	11.49°N; 79.76°E

Figure 2.1. Sampling sites: Sampling sites with nature of sample and coordinates of location for sampling

at 30°C for 72h. A second approach of direct inoculation was adopted. The samples (sediments, after appropriate dilutions) were streaked on sterile Luria Bertani medium (Appendix) amended with 2.9g% NaCl and incubated at 30°C for 72h. In another direct isolation approach, pretreatment of samples was done to eliminate Gram negative bacteria without destroying spores. For this, 10% of samples were suspended in sterile N-saline (0.75% NaCl) and kept in water bath set at 80ºC for 10 minutes in aseptic conditions. After cooling to room temperature 100µl of the upper layer was spread on the ZM agar plates and incubated at 30°C for 72h. Colonies obtained were subjected to further screening.

2A.2.3. Emulsification test:

All the isolates were grown individually in ZM broth described above. 1:1 ratio of culture broth and Paraffin oil were mixed using a homogenizer (REMI RQT 127A) for 3-5 minutes for 12000RPM to get an emulsion. The tube containing the emulsion was kept for 72h to check the stability of the emulsion. Those retaining emulsion after 72h were considered positive for bioemulsifier production.

2A.2.4. Hemolysis test:

Hemolysis zone on blood agar is used as a primary screening test for biosurfactant producing bacteria (Carrillo, *et al.*, 1996). For preparation of blood agar, a basal layer of sterile Luria agar amended with 3% glucose was overlaid with sterile agar containing 5% blood. Isolates were streaked onto the medium and those giving zone of hemolysis after 48h were considered as biosurfactant producers and were selected for further screening.

2A.2.5. Emulsification index (%EI):

The isolates selected on the basis of emulsification and hemolysis test were inoculated into 50ml Zobell Marine Broth in 250ml Erlenmeyer flasks and incubated at 30°C for 72h. After incubation, 1:1 of the culture broth and paraffin oil was mixed using the homogenizer at 12000RPM and the emulsion formed was observed for 72h. After every 24h, Emulsification Index (%EI) of the 24h (E_{24}, E_{48} and E_{72}) was calculated using the formula (Cooper & Goldenberg, 1987),

$$Emulsification\ index\ (\%EI) = \frac{\text{Height of the emulsion}}{\text{Total height of the mixture}} \times 100$$

All the further tests were conducted in minimum of three replicates. %EI was used to compare quantitatively the bioemulsifier production of all the selected isolates.

2A.2.6. Surface tension reduction:

The selected isolates were grown in ZM broth at 30°C on a shaker incubator at ≈180RPM for 72h. The culture broth was then centrifuged for 10,000RPM for 10 minutes. 35ml of the cell free supernatant was then checked for the reduction in the surface tension using Tensiometer K6 model (Krűss Germany) based on DuNuoy ring detachment method, with distilled water as the standard (giving surface tension of 70±2 mN/m). Surface tension reduction was calculated by taking uninoculated sterile broth (ZM) as 100% and was considered as indirect observation of surfactant production by the isolates.

2A.2.7. 16S rDNA amplification and amplified ribosomal DNA restriction analysis (ARDRA):

16S rDNA amplification of the selected isolates was done by colony PCR method with universal eubacterial 16S rDNA primers, 27f (5' GAG AGT TTG ATC CTG GCT CAG 3') and 1541r (5' AAG GAG GTG ATC CAG CCGC 3') (Lane, 1991, Zhou, *et al.*, 1995). The thermal cycling conditions were as follows: Denaturing at 95°C for 5minutes, followed by 30 cycles at 95°C for 30 seconds; 58ºC for 45 seconds; 72°C for 30 seconds and final extension step of 72°C for 10minutes. The presence of amplicons was detected by running the PCR product on a 0.8% agarose gel containing ethidium bromide (0.5µg/ml). 16S rDNA amplicons thus obtained were digested by restriction enzymes namely *Alu*I, *Msp*I and *Hha*I (Fermentas) and restriction fragments were separated on a 2% agarose gel containing ethidium bromide (0.5µg/ml). The restriction profiles were analysed using AlphaEaseFC 4.0 and NTSYS (version 2.0) programmes and a consensus tree was plotted.

The isolates giving distinct OTUs were sent for commercial 16S rDNA sequencing (Xcelris labs, Ahmedabad, India). The sequences obtained were analysed with NCBI-BLAST. The sequence of the nearest match of each of the isolate from NCBI database was used for making a phylogenetic tree using MEGA 5 software and

the sequence chimera analysis was done with Pintail version 1.0 and were further submitted to NCBI-GenBank.

2A.2.8. Quorum quenching (QQ) activity:

Chromobaterium violaceum 026 (CV026) is a biosensor strain that produces the pigment violacein in reponse to threshold concentrations of autoinducer, AHL(McClean, *et al.*, 1997). *C. violaceum* was cultured at 30°C with shaking in nutrient broth (Appendix) in presence of Kanamycin 30µg/ml. Isolates were grown at 30°C overnight with shaking and washed with Phosphate buffered saline (Appendix) 2-3 times and resuspended in the same (PBS). A 100µl system was prepared with 20% cell suspension and 80% of 50µM AHL (N-Hexanoyl-L-homoserine lactone, HHL; Sigma) and incubated at 30°C for 2h. After incubation, 100µl of CV026 (OD 0.002) was added with 30 µl of the above system into a 96 well microtitre plate and incubated for 16-18h at 30°C in static condition. After incubation, absorbance was taken at 585nm/660nm in a microtirtre plate reader (SPECTRAmax PLUS; Molecular Devices). The violacein unit was calculated using the formula,

$$Violacein\ unit = \frac{O.D.\ at\ 585nm}{O.D.\ at\ 660nm} \times 100$$

Considering violacein produced by CV026 in presence of only HHL molecules as 0% reduction, the percentage reduction in Violacein production by the biosensor strain in presence of the isolates was calculated as QQ activity.

2A.2.9. Biofilm assay:

Isolates were incubated in 5ml ZM broth for overnight at 30°C and 2% of this inoculum was added into 24-well sterile microtitre plate (Tarsons) containing 1ml of ZM broth. The plate was incubated at 35ºC at static conditions with sterile uninoculated media as control and checked for biofilm formation as described by Srinandan, *et al.* (2010). After 48h, the free medium was decanted and each well was washed 3-5 times with sterile 1.5ml PBS. The wells were stained with 1.5ml 1% crystal violet for 30-45 minutes. Stain was removed; each well was washed 3-5 times with distilled water and treated with 1.5ml methanol for 15 minutes. Absorption of eluted crystal violet in methanol was measured at 595nm and was taken as an indirect measure of biofilm formation.

The twelve isolates were compared with student's *t* test and analyzed using GraphPad Prism 5 software.

2A.2.10. Emulsifying activity (%EI) with different hydrocarbons and oils:

For studying the emulsification ability of the selected isolates, *Acinetobacter calcoaceticus* RAG-1 (MTCC 2409, ATCC 31012) capable of producing standard emulsifier Emulsan was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. Final three isolates giving stable emulsions upto 72h were selected. These selected three isolates were then checked for their %EI as described earlier in 2A.2.5 in 14 different solvents along with standard bioemulsifier Emulsan and commercial emulsifier xanthan-gum (0.1%). Four aliphatic solvents namely- hexane, heptane, decane and hexadecane and four aromatic solvents namely- benzene, toluene, xylene and trichlorobenzene as well as six oils namely- paraffin oil, cottonseed oil, groundnut oil, silicone oil, white oil and kerosene were selected for these studies. As the commercial emulsifier xanthan gave 100% emulsification with majority of solvents, relative emulsification activity for all the four strains was calculated with respect to this standard.

2A.2.11. Nucleotide sequence accession numbers:

GenBank accession numbers of the ecophysiological group of bacteria isolated and studied in this work are *Bacillus* sp. strains (JQ582456, JQ582454, JQ582455, JQ582453), *Bacillus subtilis* strains (JQ582450, JQ582449), a *Bacillus flexus* strain (JQ582452),a *Bacillus thuringiensis* strain (HM756284), a *Sporosarcina soli* strain (HM756285) and *Solibacillus silvestris* strains (GU226320, JQ582451, JQ582448) (Table 2.2).

2A.2.12. Statistical analysis:

All experiments were conducted in triplicate and analyzed withANOVA and t-test using GraphPad Prism 5 software.

2A.3. Results and Discussion

2A.3.1. Isolation of bioemulsifier producing bacteria:

In an extreme environment like intertidal zone, a microbe must cope with exposure to high temperature, UV radiation, irregular periods of drought and limited nutrients with intense competition for nutrients and space. These habitats are also

dumping grounds and repositories for contaminants like hydrocarbons, heavy metals, pesticides and excess organic matter (Ortega-Morales, *et al.*, 2010). As mentioned by Owsianiak, *et al.* (2009), there is a possibility of enrichment of bioemulsifier/ biosurfactant (BS/BE) producing microbes in an area exposed to a previous oil spillage. With this perspective, samples were collected from marine littoral zone with oil or hydrocarbon pollutions as depicted in Figure 2.1. The water and sediment samples were collected from Intertidal zones consisting of mudflats, estuaries and ports of 12 sampling sites possessing unique characteristic features as described below.

The samples from Gujarat included water samples from Porbander (GP) and water and sediment sample from Veraval (VG). Samples from Maharashtra state show diversity, with high salt containing saltern water sample from Bhayander (S) (≈30%), and low salt content tide sample from Madh-island (M) (≈2.4%) with general marine salt content of 3.5%. Samples from Versova (V and Vh) and Gorai (G and Gh) belong to mudflats with high pollution levels. Two samples each from Karnataka and Goa state include sea water samples of ports differentially polluted by petroleum. Tadadi port (T) and Vasco-da-Gama ports (VP), where sampling was done, had oily layer floating in the sea water. Sampling sites selected in both the states had witnessed oil spills in recent past. The water samples from Panjim port (P) and Baitkol (B) form Goa and Karnataka respectively were devoid of visible oil pollution. Two samples from Kochi (Ks and Kw), Kerala state included a water sample and a sand-soil sample from intertidal zone. Parangipette, Tamil Nadu state samples included mangrove sediment and a soil sample of the intertidal zone of vellar estuary.

To obtain predominant fast growing bacteria, direct approach of isolation was preferred. And finally since bioemulsifier producing *Bacillus* species were of interest as they were reported more frequently and in oligotrophic environment by enrichment after pretreatment was adopted to select endospore forming aerobic bacteria. According to McMeekin, *et al.* (2010), an enrichment culture is a microcosm where the principles of microbial ecology apply and in ecophysiological terms, microbial population lagtime is the result of passive dispersal into a new environment occurring due to transfer of organisms into a resuscitation or enrichment medium, conducive to recovery, repair and proliferation of the cells. Furthermore, enrichment procedures are more suitable for samples containing sparse microorganisms. Therefore, enrichment

procedures and Marine medium (Zobell) were used for selective growth of sporulating marine heterotrophs since the samples were likely to be dilute.

2A.3.2. Screening of isolates for emulsification and hemolysis:

After screening, out of 227 isolates (Table 2.1) 54 isolates were found positive for test of emulsification and 40 isolates showed zone of hemolysis as an indicator of biosurfactant production (Carrillo, *et al.*, 1996, Dehghan Noudeh, *et al.*, 2003) and 17 isolates were positive for both (Figure 2.2).

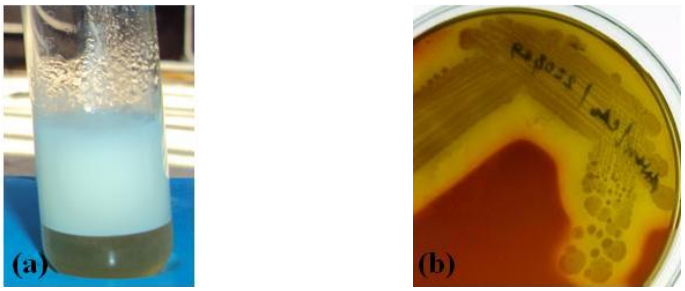


Figure 2.2. Sscreening of the total isolates a) Emulsification test and b) Hemolysis test

Codes	Sampling sites
GP	Porabandar
VG	Veraval
S	Saltern of Bhayander
M	Madh island
G	Gorai
V	Versova
P	Panjim port
VP	Vasco port
B	Baitkol port
T	Tadadi port
Kw/Ks	Kochi
AM/CA/CC/ CAM	Perangipettai

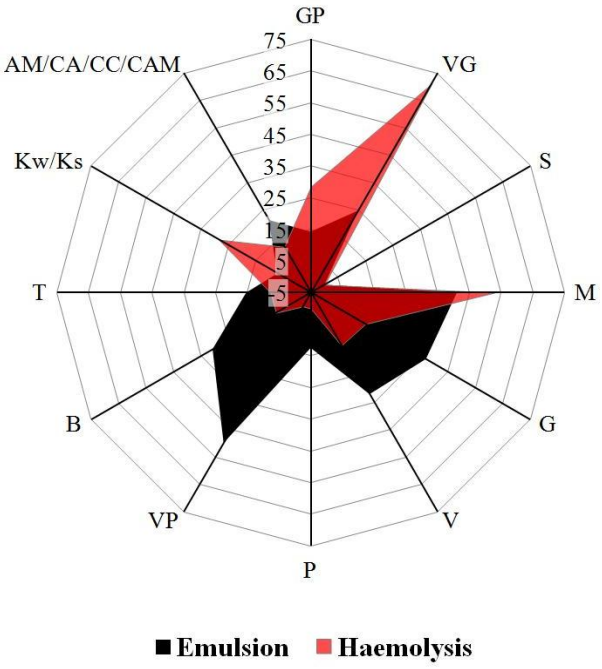


Figure 2.3. Emulsion forming and haemolytic isolates obtained from different sampling sites. Shown as percentage of total isolates obtained from each sample.

Figure 2.3 depicts the percentage of emulsion forming and/or hemolytic isolates obtained from each sampling site. Vasco port (VP) sample yielded isolates

with highest percentage of emulsifying microbes and sample from Veraval (VG) yielded maximum percentage of isolates showing haemolysis while salterns of Bhayander (S) yielded no isolates with either emulsification test or haemolysis. The hygiene standards that are followed for salterns may be the reason for lack of isolates falling into the group of interest in this study. With continuous effluent discharges from Indian metropolis like Mumbai threatening the coast line (Verlecar, et al., 2006), it was not surprising that with respect to total isolates from the Mumbai area (S, M, G and V), comparatively higher number of isolates gave positive emulsification. As the sample of Vasco port (VP) belonged to sea water next to a barge, the percentage of isolates showing emulsification is significantly high.

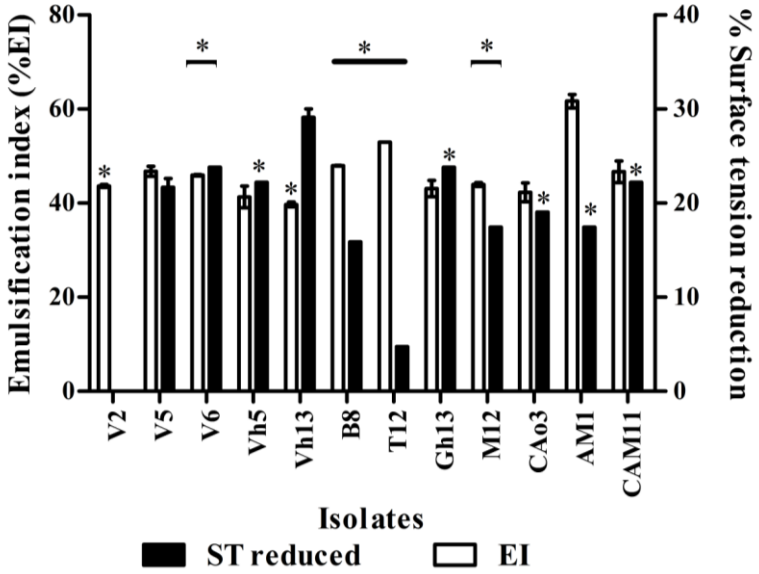


Figure 2.4. Emulsification and surface tension activities of the 12 selected isolates. Biosurfactant (BS) activities are given as % surface tension reduction in comparison to the medium and bioemulsifier (BE) activities are given as % emulsification index. ($p = *<0.001$).

2A.3.3. Quantification of emulsification and surface activities of selected isolates:

Quantification of emulsification and hemolysis is done by estimatimg the activities in terms of emulsification index or %EI and surface tension measurement respectively. Further, screening based on %EI and surface activity fourteen isolates giving more than 40% EI were selected for further characterization.

Table 2.1. Distribution of all the isolates from different intertidal zone samples belonging to six different coastal states of India

States	Sample No.	Code given	Sample	Total isolates		Isolates
				Sample	State	
GUJARAT	1.	GP	Porabandar (W)	7	35	GP1-GP7
	2.	VG	Veraval			
		VGa	Sea Water (W)	11		VGa1-VGa11
		VGb	Coral-base (S)	9		VGb1-VGb9
		VGc	Sediment (S)	8		VGc1-VGc8
Mumbai MAHARASHTRA	3.	S	Saltern of Bhayander (S)	19	91	S01-S11, S1a, S1b, S2a, S2b, S3a, S3b, S4a and S4b
	4.	M	Madh island (retreating tide) (W)	17		M1-M13, M1b, M2b, M3b and M4b
	5.	G	Gorai (mudflats) (S)	27		G1-G9, Gh1-Gh18
	6.	V	Versova (S)	28		V1-V13, Vh1-Vh15
GOA	7.	P	Panjim port (W)	8	10	P1-P8
	8.	VP	Vasco port (W)	2		VP1, VP2
KARNATAKA	9.	B	Baitkol port (W), Karwar	13	26	B1-B13
	10.	T	Tadadi port (W)	13		T1-T13
KERALA	11.	Kw	Kochi, Sea water (W)	7	14	Kw1-Kw7
		Ks	Kochi, Soil (S)	7		Ks1-Ks7
TAMIL NADU	12.	CA/AM	Coastal area (S), Perangipettai	29	51	CA/AM-1 to CA/AM-29
		CC/AMC	Chandra canal (S), Perangipettai	22		CC/AMC-1 to CC/AMC-22

Where S = sediment and W = water samples

As shown in Figure 2.4, isolate AM1 exhibited highest bioemulsification activity of 62.5% while its surface tension was 52mN/m which is not very significant if compared to the reported low mlecular weight biosurfactants from *Bacillus* and *Pseudomonas* species (Neu, 1996, Das, *et al.*, 2009, Dusane, *et al.*, 2011). Similarly isolate Vh13, which possessed 40% emulsification ability, reduced the surface tension to 44.6mN/m (~30% reduction). Isolates V5, V6, Vh5, Gh13, CAo3 and CAM11 showed 42-47% emulsification index and surface tension reduction between 48-51mN/m (reduction by 19-24%). Although isolates T9 and V2 showed 52.94% and 43.65% emulsification respectively, their surface tension reducing ability was very poor (63 and 60 mN/m surface tension viz., 0-4% reduction respectively).

As reported by Ron & Rosenberg (2001) the bioemulsifiers producers are known to show poor surface tension reduction activity which was also observed in this study. This is particularly true for bacteria producing high molecular weight bioemulsifiers which is demonstrated by isolate AM1.

2A.3.4. Amplified rDNA restriction analysis (ARDRA):

With the isolates putatively belonging to order *Bacillales* since they are sporulating aerobic rods, their 16S rDNA sequences would be almost identical and furthermore, ARDRA is able to emphasize the differences without the need for extensive 16S rDNA sequence analysis therefore ARDRA was undertaken (Figure 2.5).

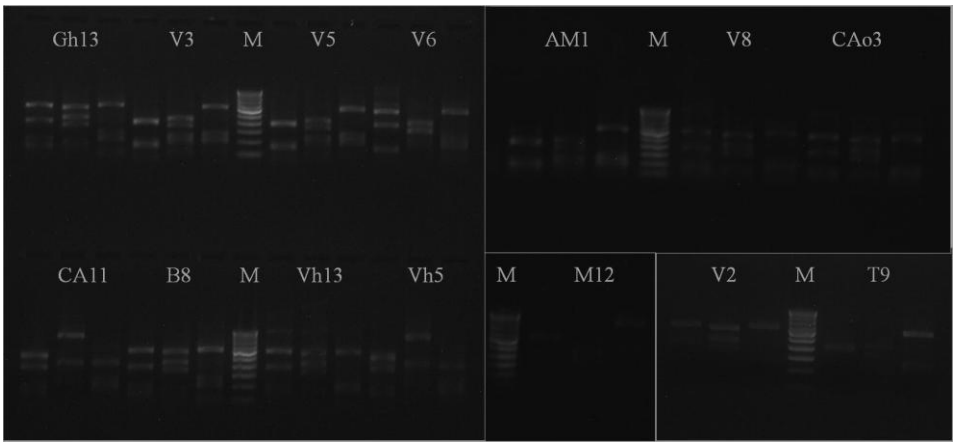


Figure 2.5. Amplified rDNA restriction analysis (ARDRA) profile gel of the 14 isolates digested by *Alu*I, *Msp*I and *Hha*I respectively. Three lanes of each isolate represent the digestion pattern of three respective enzymes.

Using colony PCR, the 16S rDNA of the isolates was amplified and amplification product of ~1.5kb was obtained for each isolate and was digested with *Alu*I, *Msp*I and *Hha*I. The banding patterns of the selected fourteen isolates obtained from all the three enzymes were grouped and depicted in a dendrogram upon analyses by NTSYSpc (Figure 2.6). Definite groups of 14 isolates were sorted by ARDRA which deemed useful for further selection.

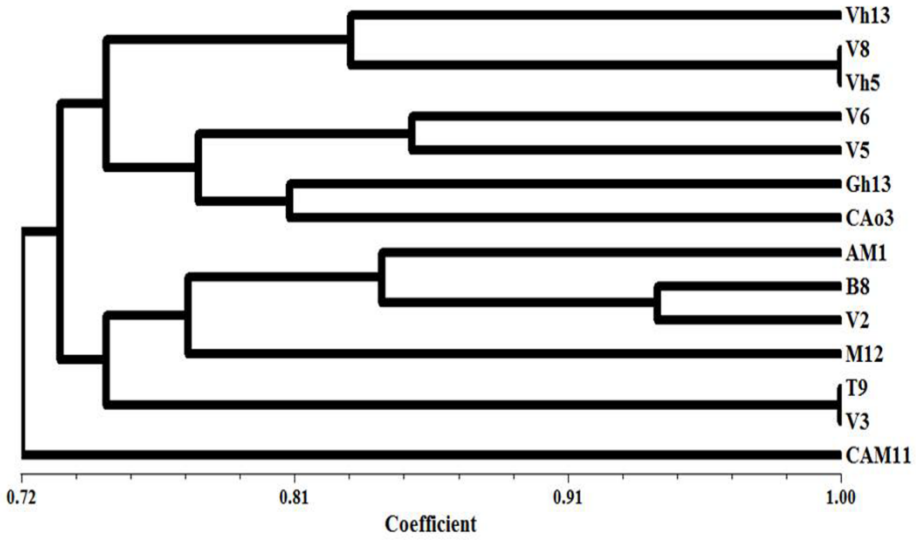


Figure 2.6. Dendrogram of selected fourteen isolates from intertidal zone representing different ARDRA groups. ARDRA band patterns were obtained after independent restriction of the amplified 16S rDNA gene with three different enzymes (*Alu*I, *Msp*I and *Hha*I). The dendrogram was constructed with software NTSYSpc Version 2.0 and grouped with Neighbour Joining method.

2A.3.5. 16S rRNA gene sequence and phylogenetic analysis of the selected twelve isolates:

One isolate each from the two groups V8-Vh5 and T9-V3 showing the same OTU), was picked since they belonged to identical ARDRA groups. The selected twelve isolates were subjected to 16S rRNA gene sequencing. Their evolutionary analyses were conducted in MEGA5 (Tamura, *et al.*, 2011). The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987) and depicted in the phylogenetic tree as shown in Figure 2.7. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura, *et al.*, 2004) and are in the units of the number of base substitutions per site. The bootstrap

consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985).

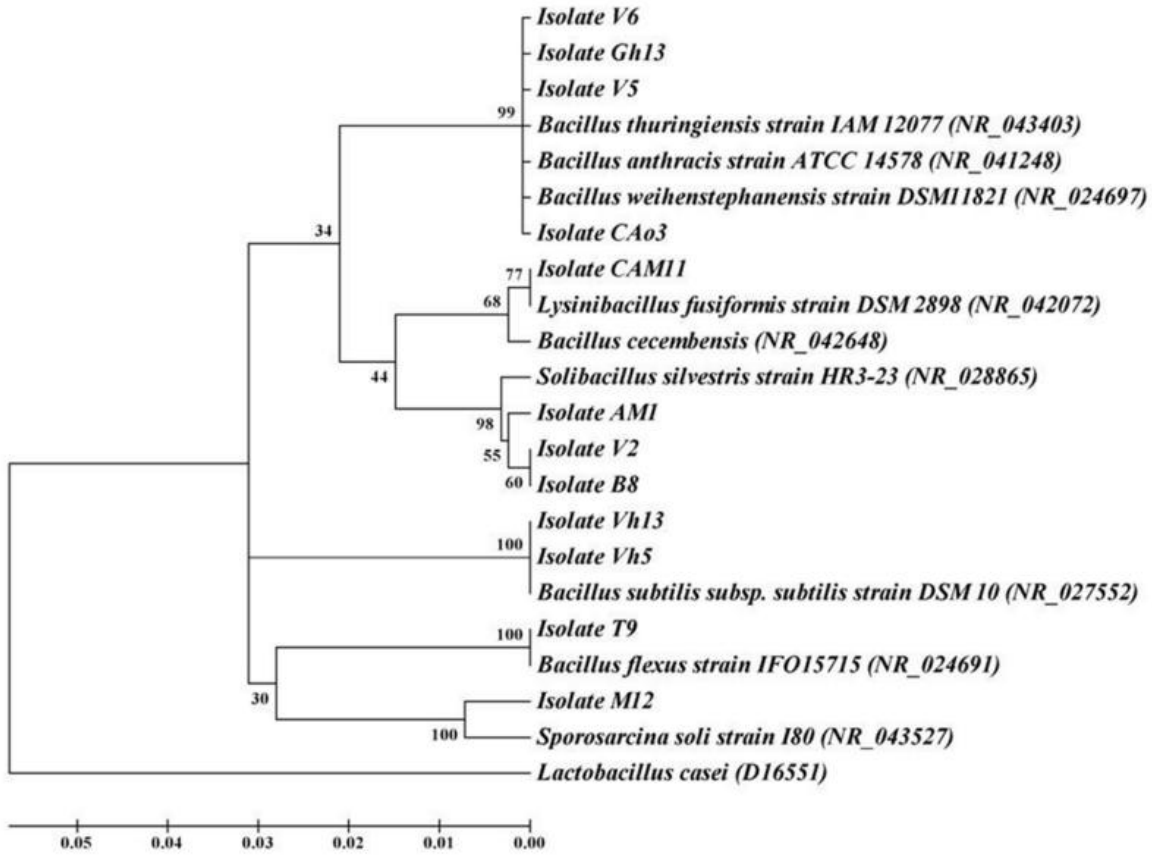


Figure 2.7. Evolutionary relationship of taxa by Neighbour-Joining method, constructed using aligned partial 16S rDNA sequences of isolates and their closest match in NCBI blast (as given in Table 2.1). The accession numbers are given for the matching sequences available in GenBank. The bootstrap values calculated from 1000 replicates using Neighbor-Joining method are shown at the nodes. *Lactobacillus casei*, being the outgroup, was used to position the root.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The sequences of the 12 isolates were submitted to GenBank and their details are given in Table 2.2. Thus 16S rDNA analysis showed that these marine sporulating isolates clustered into four major clusters of the *Bacillacieae*, namely *Solibacillus*, *Sporosarcina* and two genera of *Bacillus* (*B. cereus* group and *B. flexus* group).

The *Bacillus cereus* group cluster included four isolates, V6, V5, CAo3 and Gh13 respectively. Although isolates Vh13 and Vh5 gave higher bootstrap values with respect to *Bacillus subtilis* group, their difference in query coverage and % identity during NCBI-BLAST as given in table 2.2, shows their relative difference in strain level. Isolate T9 clustered with *Sporosarcina* group and isolate CAM11 showed a varied match and higher bootstrap with *Lysinibacillus fusiformis* and *B. cecembensis.* These results correlate with Siefert, *et al.* (2000), who reported many unique *Bacillacieae* members other than *B.subtilis, B. fusiformis* (now *Lysinibacillus fusiformis*) and round spore forming *B. sphericus* like bacteria from Gulf of Mexico.

Isolates V2, B8 and AM1 clustered within the *Solibacillus* group, with no other species mentioned in the literature, the similarity of the isolates was confined to the 16S rDNA sequence available from the type strain, *Solibacillus silvestris* HR3-23. Most of the isolates characterized in this study belong to family *Planococcaceae* and *Bacillaceae*.

2A.3.6. Biofilm formation and Quorum quenching activity of the selected isolates:

In ecological terms, microorganisms like bacteria having a small body, short generation time and highly dispersible offspring are known to be r-strategists. But when compared to higher-organisms, bacteria have evolved a wider range of growth and survival strategies. Competition dictates the terms of niche domination for a bacterium which is dependent on its ability to employ different strategies. Some of these strategies include the ability to produce compounds like organic acids or peroxides, biosurfactants or even by quorum quenching (González & Moran, 1997, Lauro, *et al.*, 2009, Sadowska, *et al.*, 2010). Thus group of bacteria under study here were also checked for other strategies like biofilm formation and quorum quenching in bacteria that give them edge in competition in such niches. Twelve isolates selected here are deemed to function in the form of an ecophysiological group.

The undesirable development of microbial layers called biofilms on the surfaces is referred to as biofouling (Flemming, 2002). As a result of biofouling and biological buildup, the frictional drag on ship increases, it smothers oceanographic equipments and bulk floating structures, promotes structural deterioration and clogs sea-water lines to power plant (Zardus, *et al.*, 2008).

Table 2.2. Coverage, identity and GenBank details of the twelve isolates selected on the basis of ARDRA.

Isolate	Closest match	Identity %	Coverage %	GenBank Accession number
V6	*Bacillus anthracis* strain ATCC 14578 NR_041248.1	99	98	*Bacillus* sp. V6 JQ582456
CAM11	*Lysinibacillus fusiformis* strain DSM 2898 NR_042072.1	99	99	*Bacillus* sp. CAM11 JQ582454
T9	*Bacillus flexus* strain IFO15715 NR_024691.1	100	100	*Bacillus flexus* strain T9 JQ582452
Vh13	*Bacillus subtilis* subsp. subtilis strain DSM 10 NR_027552.1	98	99	*Bacillus subtilis* strain Vh13 JQ582450
V2	*Bacillus cecembensis* NR_042648.1	97	99	*Bacillus* sp. V2 JQ582448
V5	*Bacillus thuringiensis* strain IAM 12077 NR_043403.1	99	98	*Bacillus* sp. V5 JQ582455
CAo3	*Bacillus weihenstephanensis* strain DSM11821 NR_024697.1	99	100	*Bacillus* sp. CAo3 JQ582453
B8	*Solibacillus silvestris* strain HR3-23 NR_028865.1	97	99	*Solibacillus* sp. B8 JQ582451
Vh5	*Bacillus subtilis* subsp. subtilis strain DSM 10 NR_027552.1	99	86	*Bacillus subtilis* strain Vh5 JQ582449
Gh13	*Bacillus thuringiensis* strain IAM 12077 NR_043403.1	97	98	*Bacillus thuringiensis* strain Gh13 HM756284
M12	*Sporosarcina soli* strain I80 NR_043527.1	99	99	*Sporosarcina soli* strain M12 HM756285
AM1	*Solibacillus silvestris* strain HR3-23 NR_028865.1	99	99	*Solibacillus silvestris* strain AM1 GU226320

The ability of the microbes to adhere and grow on any surface with optimum water has been optimized by evolution. To understand this optimized evolutionary mechanism and to develop environmentally atoxic non-fouling surfaces or making antifouling surfaces requires a integrated approach (Flemming, 2011).

Antifouling (AF) refers to preventing the accumulation of fouling organisms (Briand, 2009). In this context study of biofilm forming ability of the isolates is significant. Reports suggest that bioemulsifiers may play a role in quorum sensing and biofilm formation of some bacteria (Neu, 1996, Ron & Rosenberg, 2001). *Bacillus* sp. V2 formed maximum biofilm of 0.35 read at 595 with CV absorbance, while *Bacillus* sp. V5 and *Sporosarcina soli* M12 formed the least (0.02-0.03 at A_{595}). *Bacillus* sp. V6, *Bacillus flexus* T9 and *Bacillus* sp. CAo3 produced moderate biofilm (0.19-0.27 at A_{595}) in comparison to *Bacillus* sp. V2. The biofilm forming ability of *S. silvestris* AM1 was around 0.071 (±0.004) at A_{595}. *Bacillus* sp. V2 and *Bacillus* sp. CAM11 showed both good biofilm formation and QQ activities and show that they can dominate their ecology very easily. *Bacillus subtilis* Vh13 showed higher biofilm formation and low QQ activities (Figure 2.8).

As regards the QQ activity of the isolates *C. violaceum* 026 was used since it induced by QS inducer AHL. QQ activity was calculated as shown in Figure 2.8 with respect to the violacein production by CV026 in absence of QQ process when the isolates were co-cultured with CV026 (Teasdale, *et al.*, 2011). *Bacillus* sp. V2 showed highest QQ activity (17.88%) while *Bacillus* sp. V5 and *Bacillus subtilis* Vh13 exhibited lowest (0.9 and 1.4% respectively) and *S. silvestris* AM1 exhibited QQ of 12.59% (±0.247). *Bacillus* sp. CAM 11 showed zone of inhibition for CV026 on plate (data not shown), thus the resultant reduction in % violacein pigmentation may not be completely because of QQ activities of the isolate.

Previous studies (Qian, *et al.*, 2007, Dobretsov, *et al.*, 2011) suggest that presence of QQ strains shifts the compositions of microbial communities from Gram-negative dominates to those dominated by Gram-positive species. Many common aquaculture pathogens like *Vibrio harveyi* use AHL based QS to regulate the expression of virulence factors and have ability to form biofilms with marked resistance to disinfectants and antimicrobials (You, *et al.*, 2007, Natrah, *et al.*, 2011). There are reports of using QQ strains like CAM11 which can be used for antimicrobial protection in aquaculture and also using these QQ microbes as 'Living

paints' for inhibiting biofouling (Holmström, *et al.*, 2002, Dobretsov, *et al.*, 2006, Dobretsov, 2009, Dobretsov, *et al.*, 2011).

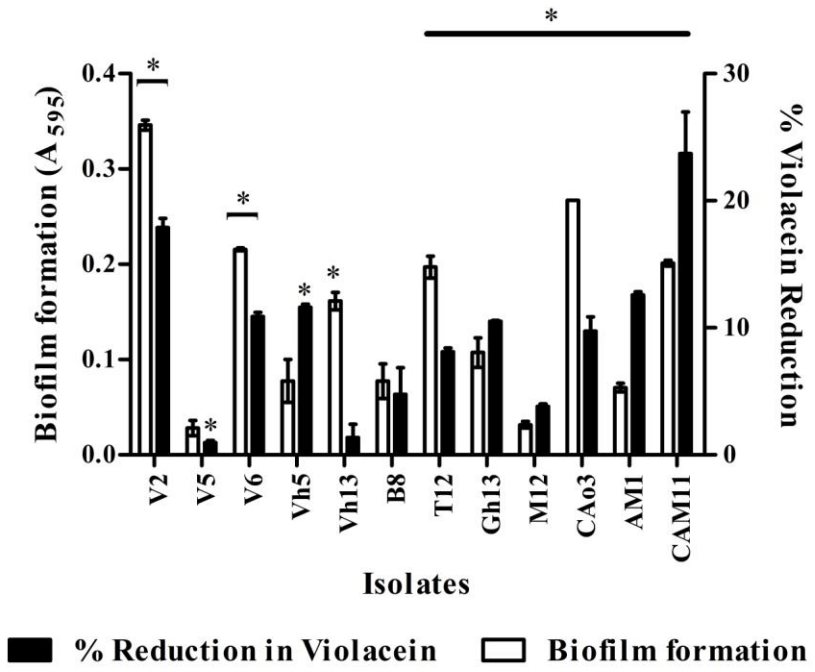


Figure 2.8. Biofilm formation and quorum quenching activities of the 12 selected isolates. Quorum quenching (QQ) activity is given as % violacein colour reduction and Biofilm formation is measured with absorption of crystal violet at 595nm (p = *<0.001).

2A.3.7. Emulsification activity of selected isolates on hydrocarbons and oils:

Three isolates identified as *Bacillus thuringiensis* strain Gh13, *Solibacillus silvestris* strain AM1 and *Sporosarcina soli* strain M12 were selected on the basis of their hemolysis, emulsifying activity and surface tension reduction for testing their %EI on different hydrocarbons and oils (Figure 2.9). Crude emulsan produced from *A. calcoaceticus* RAG-1 was included in the study along with xanthan for better comparison. Emulsification index (%EI) of the bioemulsifier from *S. silvestris* AM1 was highest in presence of aliphatics and oils, while it showed highest %EI of 68.11% with trichlorobenzene (TCB), 63.77% for decane (D), and 60.95% for paraffin oil (P) and 60.75% for White oil (W). *B. thuringiensis* Gh13 and *S. soli* M12 also showed potential activity with all the hydrocarbons tested. The bioemulsifier from *S. silvestrsis* AM1 had the ability to efficiently emulsify (> 40%EI) most solvents tested

in comparison to others showing its broad specificity. Emulsan alone was able to emulsify hexadecane effectively among the tested bioemulsifiers. Emulsification by emulsan was higher than all the other bioemulsifier producers in groundnut oil, silicone oil, cottonseed oil, kerosene and white oil.In benzene, only bioemulsifier from *S. silvestris* AM1 and emulsan could produce effective emulsion.

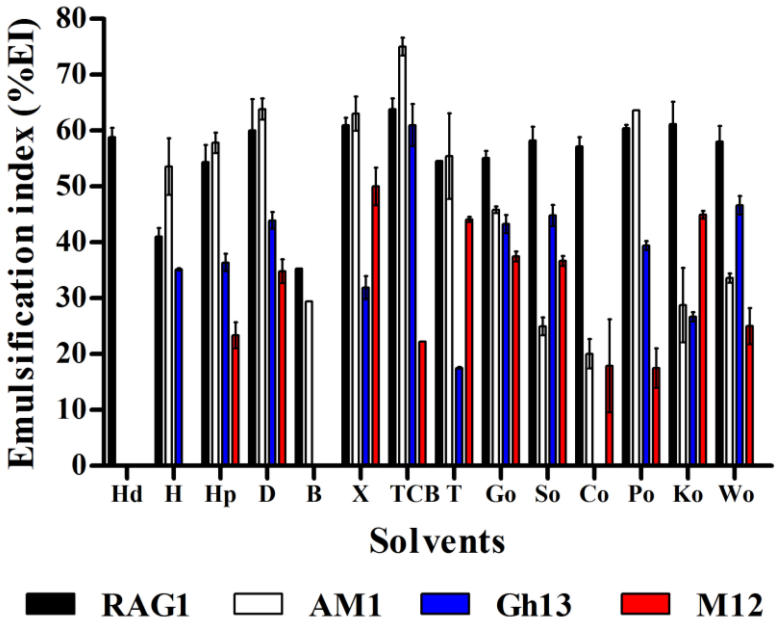


Figure 2.9. Emulsifying activity (%EI) of bioemulsifiers from isolates AM1, Gh13 and M12 with hydrocarbons and oils. Solvents used Hd, hexadecane; H, hexane; Hp, heptane; D, decane; B, benzene; X, xylene; TCB, trichlorobenzene; T, toluene; Go, groundnut oil; So, silicone oil; Co, cottonseed oil; Po, paraffin oil; Ko, kerosene and Wo, white oil.

Zuckerberg, *et al.* (1979), while studying standard emulsifier Emulasn have reported its inability to effectively emulsify low-molecular weight benzene derivatives, aromatic compounds containing more than one ring, branched chain aliphatics from pentane to octadecane and unstable emulsions formed with kerosene and gasoline. Many reported organisms, such as *Planococcus maitriensis* Anita I (Kumar et al., 2007), *Antarctobacter* sp. TG22 (Gutiérrez et al., 2007) and *Penicillium citrinum* (De Morais, *et al.*, 2006), displayed good emulsification with oils. The yeasts *Torulopsis petrophilum* (Cooper & Paddock, 1983) and *Saccharomyces cerevisiae* (Cameron, *et al.*, 1988) were reported to produce compounds that emulsify in aliphatic and aromatic compounds.

Although not as versatile as isolate AM1, the bioemulsifier from *B. thuringiensis* Gh13 (17-56%) and *S. soli* M12 (6-52%) showed emulsification in presence of aliphatics, aromatics and oils, and did not emulsify all the three types of hydrocarbons. In contrast to reports, these three isolates selected showed very low emulsification to hexadecane (Hd) and also to cotton-seed oil. Reports suggest that, the contact of BS/BE and hydrocarbons or substrate affects interaction of cell-cell and cell-substrate attachment (Neu, 1996, Ron & Rosenberg, 2001).

Presence of BS/BE, results in emulsification of hydrocarbons into microdroplets, thus increasing the surface area exposed to bacteria and making them more available to microorganisms (Baldi, *et al.*, 1999, Calvo, *et al.*, 2008, Das, *et al.*, 2009, Dusane, *et al.*, 2011). Hence, this ecophysiological group of bacteria, by emulsifying pollutants and making them available, can easily enhance the growth of other bacteria helpful in bioremediation.

The strain *S. silvestris* AM1 is a novel isolate and not hitherto reported for bioemulsifier production giving maximum %EI of 62.5% and showed versatility in growth on aliphatic, aromatic hydrocarbons as well as oil and exhibited surface, biofilm forming and QQ activities hence was specifically selected for further studies.

2B. Characterization of the selected strain of *Bacillus* sp.

2B.1. Introduction

Vellar river forms an estuarine system at Parangipettai (former Porto Novo) and opens into the Bay of Bengal (Figure 2.10). Vellar estuary is one of the prominent and best studied estuaries of India with respect to its chemistry and biology but less studied with in terms of microbial activities. Vellar estuary, with Coleroon estuary forms a Killai backwaters supporting Pitchavaram mangrove forest (Pari, *et al.*, 2008, Prasad & Ramanathan, 2008). Four irrigation channels drain into Vellar estuary and numerous shrimp farms are located throughout the estuary causing periodic eutrophication whenever the levels cross the threshold especially when tidal flushing could not neutralize the input discharges (Rajasegar, *et al.*, 2002).

According to Senthilnathan, *et al.* (2012), in last 38 years significant changes in the shore line of the Parangipettai and clear river path shift have occurred. The Indian Ocean tsunami of 26th December 2004 generated a tidal wave with run up heights of 3-6m and travelled far inside the estuaries and backwaters altering the geomorphology and physic-chemical properties of Vellar estuary. There have been numerous reports of pesticide and hydrocarbons in this estuarine environment, but most of these reports as listed and studied by Sarkar, *et al*, (2008) were before the devastating 2004 tsunami. Thus major nutrient dynamics and hydrobiological studies done prior to tsunami cannot be completely viable (Sivakumar, *et al.*, 1983, Chandran & Ramamoorthi, 1984, Chandran, 1985).

The selected isolate *Solibacillus silvestris* AM1, capable of producing bioemulsifier maximally among the isolated cultures was isolated from sediment sample of Vellar estuary, Parangipettai, Tamil nadu, India.. Although many 16S rDNA sequences from different strains of *Solibacillus* genus are available in GenBank, only one species has been reported till now. *S. silvestris* AM1 therefore is a novel strain, isolated from a special niche and in this section its characterization is presented.

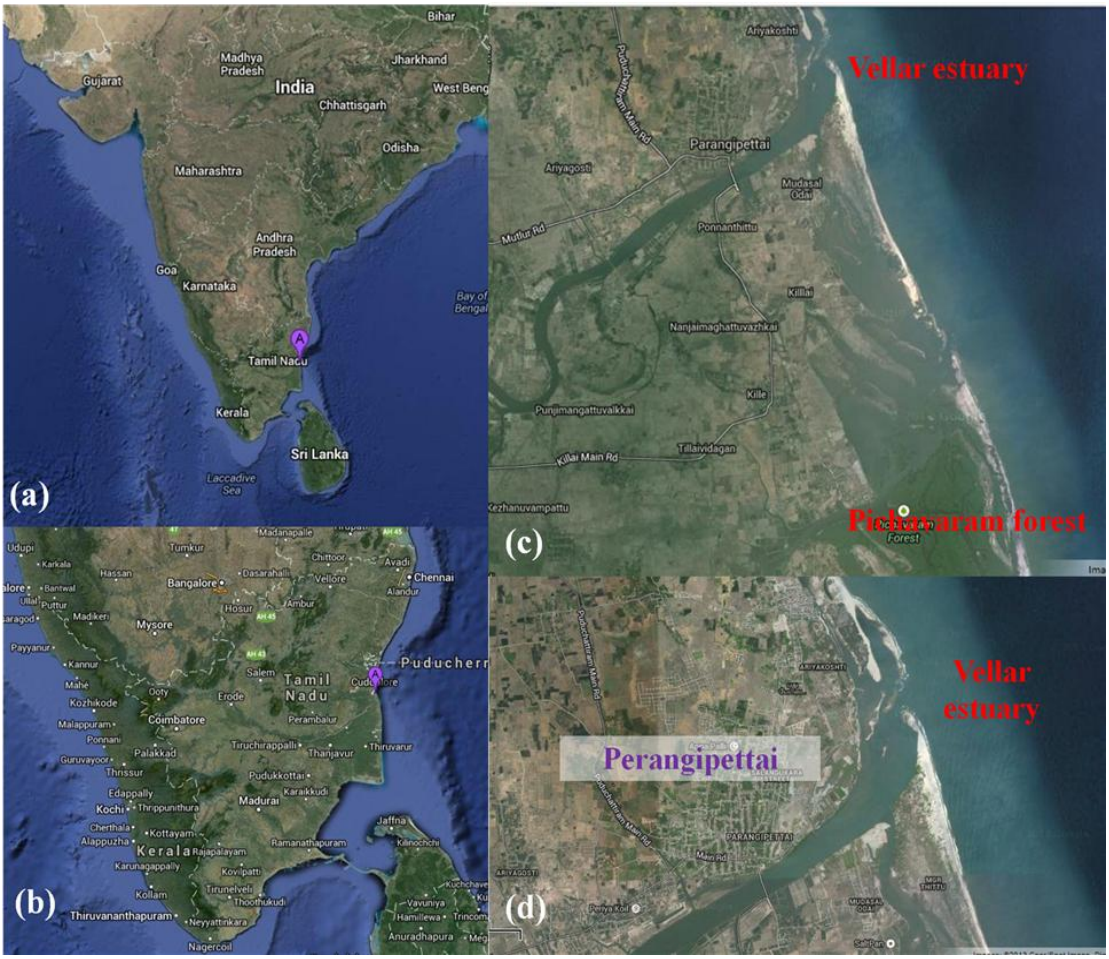


Figure 2.10. Vellar estuary at Perangipettai. Location of (a) Perangipettai in India (b) and in Tamil nadu . (c) Vellar estuary and Pichavaram national mangrove forest system and (d) and Perangipettai at the Vellar estuary .

2B.2. Materials and Methods

Isolate selected for further studies *S. silvestris* AM1 was maintained on Zobell marine medium (Appendix). ZM medium was also used for bioemulsifier production.

2B.2.1. 16S rDNA analysis:

The 16S rDNA of the bacterium was amplified with eubacterial universal primers (27f, 5'-GAGAGTTTGATCCTGGCTCAG-3'; 1107r, 5'-GCTCGTTGCGGGACTTAACC-3' and 1541r, 5'- AAGGAGGTGATCCAGCCGC-3') and aligned to get a 1503bp sequence which was submitted to NCBI GenBank (GenBank Accession number GU226320). The strain was submitted to Microbial culture collection centre, NCCS, Pune India (Accession No MCC 2096). 16S rDNA sequences of 49 submissions belonging to *Solibacillus* sp. were downloaded from

NCBI GenBank and analysed for evolutionary relatedness using MEGA 5.1. For studying evolution of *S. silvestris* AM1 and other strains of *Solibacillus* sp. isolated from similar intertidal zones of Indian coast (isolates V2 and B8 with GenBank accession Nos. JQ582448 and JQ582451 respectively) analysis was done with type strain *S. silvestris* HR3-23 and outgroup *Lacobacillus casei* (T) (GenBank accession No. D16551) using MEGA 5.1. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site

2B.2.2. DNA-DNA hybridization:

DNA-DNA hybridization was carried out between standard strain *S. silvestris* HR3-23 (MCC2084^T) and *S. silvestris* AM1 to evaluate the species similarity. DNA was prepared as described by Marmur, (1961) with additional modifications, DNA-DNA hybridization was done by spectrophotometry (Agilent, Cary 300 UV-Vis, with a thermostatted 6×6 multicell block Peltier and *in-situ* temperature probe) method as described by Ley, *et al.* (1970) under the consideration of the modification described by Huss, *et al.* (1983).

2B.2.3.Scanning electron microscopy:

Solibacillus silvestris strain AM1 was grown on Luria Bertani medium for 24 hours. Cells were pelleted at 10,000RPM and washed with Phosphate buffered saline, PBS (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4; pH 7.2 $\pm$0.2). The culture was fixed in 2.5% (v/v) glutaraldehyde and dehydrated in a series of increasing ethanol concentrations i.e., 10, 25, 50, 75 and 100% for 10 min each (modified Rheims *et al*. 1999). After sputter coating with silver (99%), the cells were observed with a JSM-5610LV Scanning electron microscope.

2B.2.4.Fatty acid methyl ester (FAME) analysis:

Extraction of lipids from *S. silvestris* strain AM1 grown on ZM medium was done by using method described by Lewis, *et al*, (2000). The AOAC (Association of Official Analytical Chemists, 1995) method was followed to esterify the lipid extract. Fatty acids were separated by using a Shimadzu QP2010 quadrupole Gas Chromatography Mass Spectrometer (GC-MS) instrument equipped with a Carbowax (30 m x 0.25 mm ID; 0.25-µm film thickness) capillary column (Cromlab S.A.). Helium was used as the carrier gas. Injector and detector temperatures were set at

250°C. Injection was performed in split mode (1:15). The column temperature was programmed initially at 50°C for 2 min and then to increase at a rate of 10°C per min to a final temperature of 230°C. The esters were separated at constant pressure (23.1 kPa) and peaks were identified by comparing the mass spectra with the mass spectral data base.

2B.2.5.Phenotypic strain characterization using Biolog microbial ID system:

S. silvestris strain AM1 was tested for carbon substrate utilization in Microlog-2.0 using GP2 plates and kept in incubation for 72Hr at 35°C. The list of 95 carbon substrates used is given in Table 2.3.

2B.2.6.Antibiotic sensitivity:

S. silvestris AM1 was checked for antibiotic sensitivity with respect to antibiotic discs (HiMedia laboratories Pvt. Ltd., India). The antibiotics tested include Kanamycin (30 µg), Ampicillin (25 µg), Tetracycline (30 µg), Gentamycin (50 µg), Chloramphenicol (30 µg), Streptomycin (25 µg), Rifampicin (30 µg) kept on a lawn of bacterium grown on Mueller Hinton agar medium (HiMedia, India).

2B.2.7. Biochemical characterization of *S. silvestris* AM1:

The biochemical characters of *S. silvestris* AM1 was checked with different biochemical tests and characterized according to Sneath, *et al.* (1986). The biochemical tests done were Indole, Methyl red, Voges–Proskauer, Citrate utilization, Triple sugar Iron test,presence of Lysine decarboxylase, Gelatine hydrolase, Nitrate reductase, Cellulase, Pectinase, Amylase, Urease, Protease, Oxidase and Oxidation-fermentation tests for Xylose, Lactose, Glucose, Sucrose, Mannitol and Maltose (MacFaddin, 2000).

Table 2.3. Substrates tested for utilization in Microlog-2.0 using GP2 plates.

Sl.No	Substrate	Sl.No	Substrate	Sl.No	Substrate
1	α-cyclodextrin	33	β-Methyl-D-Galactoside	65	Pyruvatic acid methyl ester
2	β-cyclodextrin	34	3-Methyl Glucose	66	Succinic acid-Mono methyl ester
3	Dextrin	35	α-Methyl-D-Glucoside	67	Propionic acid
4	Glycogen	36	β-Methyl-D-Glucoside	68	Pyruvic acid
5	Inulin	37	α-Methyl-D-Mannoside	69	Succinamic acid
6	Mannan	38	Palatinose	70	Succinic acid
7	Tween 40	39	D-Psicose	71	N-Acetyl-L-Glutamic acid
8	Tween 80	40	D-Raffinose	72	L-Alaninamide
9	N-Acetyl-D-Glucosamine	41	L-Rhamnose	73	D-Alanine
10	N-Acetyl-β-D-Mannosamine	42	D-Ribose	74	L-Alanine
11	Amyggdalin	43	Salicin	75	L-Alanyl glycine
12	L-Arabinose	44	Sedoheptulosan	76	L-Aspargine
13	D-Arabitol	45	D-Sorbitol	77	L-Glutamic acid
14	Arbutin	46	Stachyose	78	Glycil-L-Glutamic acid
15	D-Cellobiose	47	Sucrose	79	L-Pyroglutamic acid
16	D-Fructose	48	D-Tagatose	80	L-serine
17	L-Fucose	49	D-Trehalose	81	Putrescine
18	D-Galactose	50	Turanose	82	2,3-Butanediol
19	D-Galacturonic acid	51	Xylitol	83	Glycerol
20	Gentiobiose	52	D-Xylose	84	Adenosine
21	D-Gluconic acid	53	Acetic acid	85	2'-Hydroxy Adenosine
22	α-D-Glucose	54	α-Hydroxybutyric acid	86	Inosine
23	m-Inositol	55	β-Hydroxybutyric acid	87	Thymidine
24	α-D-Lactose	56	γ-Hydroxybutyric acid	88	Uridine
25	Lactulose	57	p-Hydroxy-Phenylacetic acid	89	Adenosine-5'-Monophosphate
26	Maltose	58	α-Ketoglutaric acid	90	Thymidine-5'-Monophosphate
27	Maltotriose	59	α-Ketovelaric acid	91	Uridine-5'-Monophosphate
28	D-Mannitol	60	Lactamide	92	D-Fructose-6-Monophosphate
29	D-Mannose	61	D-Lacticacid-Methyl ester	93	α-D-Glucose-1-phosphate
30	D-Melezitose	62	L-Lactic acid	94	D-Glucose-6-phosphate
31	D-Melibiose	63	D-Malic acid	95	D-L-α-Glycerol Phosphate
32	α-Methyl-D-Galactoside	64	L-Malic acid		

2B.3. Results and Discussion

S. silvestris AM1 is a novel bioemulsifier producing strain. In the present studies its polyphasic characterization is presented

2B.3.1. 16S rDNA analysis:

The genera *Solibacillus* has been reported with only one species, *S. silvestris* and many 16S rDNA sequences of it are available in GenBank. After alignment with Clustal W, uncorrected distances and pairwise deletion options were selected to generate a conservative estimate of divergence among the selected sequences. The evolutionary distances were computed using the p-distance method (Nei & Kumar, 2000) and are in the units of the number of base differences per site. All positions containing gaps and missing data in the aligned sequences were eliminated. The bootstrap consensus tree constructed, inferred from 5000 replicates and percentages of replicates were considered for trees as shown in Figure 2.11. Various algorithms were applied (neighbor joining, maximum parsimony and maximum likelihood with MEGA 5.1) to simulate the evolution of sequences and compared them in MEGA 5.1 to construct phylogenetic trees.

The analysis involved 49 nucleotide sequences. All ambiguous positions were removed for each sequence pair. There were a total of 857 positions in the final dataset. Branches corresponding to partitions reproduced in less than 20% bootstrap replicates were collapsed. When analyzed for evolutionary relationships, all the three intertidal zone strains of *Solibacillus* sp. namely AM1, V2 and B8 clustered differently with respect to the type strain HR3-23. In each of the method used, the sequences formed minimum of five clusters with biggest cluster bearing the type strain (*S. silvestris* HR3-23) and sequenced strain of *Solibacillus* sp. (StLB046).

The type strain HR3-23 showed p-distance values of zero with all the methods. *Solibacillus* strains namely V2 and B8 gave same values. When tested for Neighbor joining, Maximum evolution, Maximum likelihood and UPGMA methods, *Solibacillus silvestris* strains AM1 and V2/B8 gave the values 0.01087 and 0.0076, 0.01087 and 0.0076, 0.0083 and 0.0169 and 0.00923 and 0.00923 respectively while *Lactobacillus casei*, gave the p-distance values of 0.07458, 0.07458, 0.0787 and 0.07993 respectively. From 49 16S rDNA sequences analysed, there were total of 857 positions in the final dataset aligned using Clustal W. The nucleotide frequencies

were 26.34% (A), 20.82% (T/U), 31.16% (C), and 21.68% (G). The transition/transversion rate ratios were k1 = 1.65 (purines) and k2 = 1.958 (pyrimidines). The overall transition/transversion bias was found to be R = 0.916, where R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]. Transition/transversion (ti/tv) bias is a known property for evolution of DNA sequences. Estimation of ti/tv helps in understanding the patterns of DNA sequence evolution and reliable estimation of sequence distances and also for phylogeny reconstructions. Thus the comparison of closely related sequences can yield accurate estimates of patterns of substitutions with respect to comparison of divergent sequences (Yang & Yoder, 1999). Although the ti/tv rate for purines was lesser than pyrimidines, the overall ti/tv bias was almost 1.

The analysis given here shows the distinct branching of some of the strains like DFM76b, DWM125a, DEM132, StLB306 and R-26228 in different method of trees constructed. Thus many of the strains included in GenBank may be considered for a new species other than *S. silvestris* and further studies are needed.

2B.3.2. DNA-DNA hybridization:

Genomic relatedness/similarity between the strains *S. silvestris* HR3-23 and *S. silvestris* sp.AM1 was found to be >70 % (80 ±3%) and thus they belong to the same species according to the recommendations of the *ad hoc* committee (Wayne, *et al.*, 1987) for the species definition of bacteria.

2B.3.3. Scanning electron microscopy of *S. silvestris* AM1:

The rod shaped bacterium can easily be distinguished in the electron micrograph (Figure 2.12). The length of the bacterium was calculated to be 2.9µM ($\pm$0.2µM) and breadth to be 1.25µM ($\pm$0.2µM).

2B.3.4. FAME analysis of *S. silvestris* AM1:

Kämpfer, *et al.* (2003) recommended certain phenotypic and chemotaxonomic characteristics of type strain of *Bacillus* (i.e. *Bacillus subtilis*) as the 'core characteristics' for an isolate to be classified in genus *Bacillus*. *S. silvestris* differed from *B. subtilis* in cell-wall type, pattern of polar lipids and fatty acids and hence was separated from genus *Bacillus*. The iso fatty acids (iso- $C_{16:1}$ and iso-$C_{15:0}$) are predominant fatty acids in *S. silvestris* while members of the genus *Bacillus*, including

B. subtilis contain iso and antiso $C_{15:0}$ as their major fatty acids (Krishnamurthi, *et al.*, 2009).

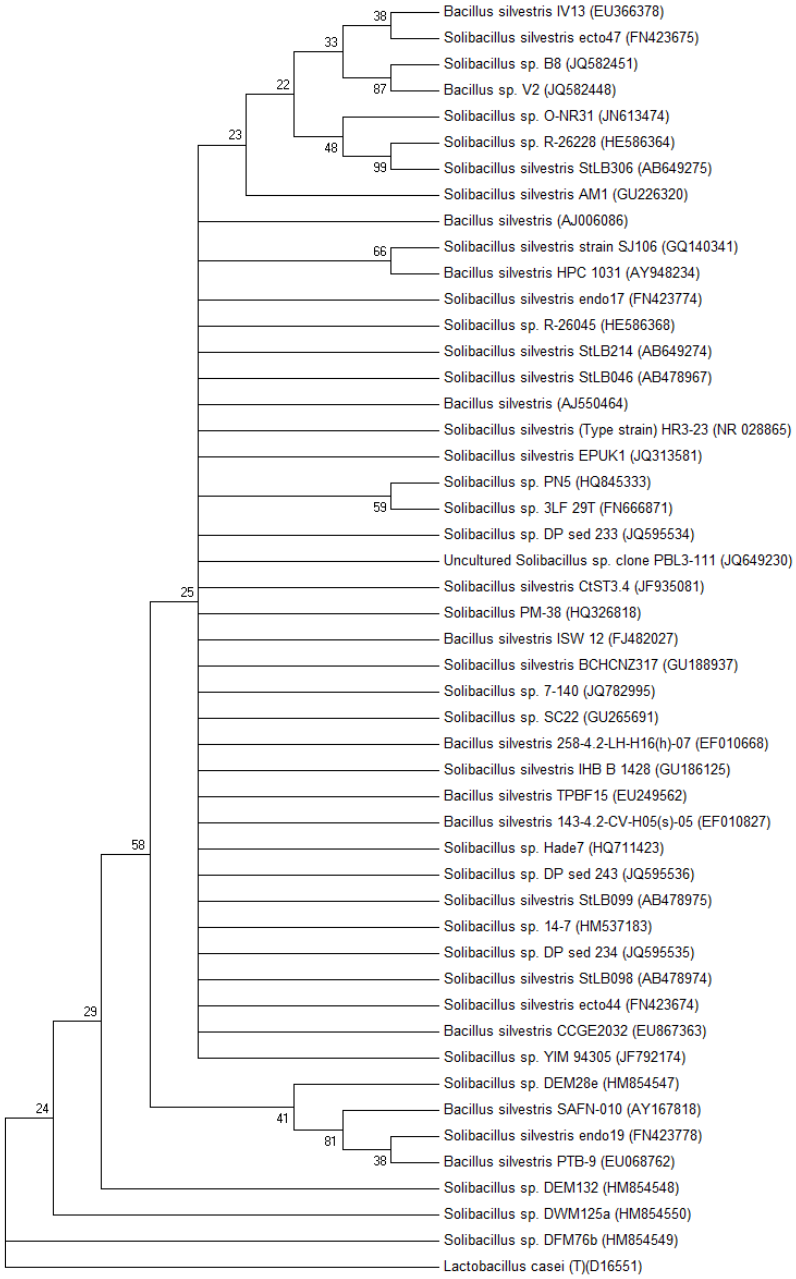


Figure 2.11. Analysis of evolutionary history of 49 sequences of *Solibacillus* spp. downloaded from GenBank inferred using the Maximum Parsimony method. The bootstrap consensus tree inferred from 5000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 20% bootstrap replicates are collapsed. All positions containing gaps and missing data were eliminated. There were a total of 121

positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura, *et al.*, 2011).

The fatty acid profile of *S. silvestris* strain AM1 demonstrated the the presence of Iso-$C_{15:0}$ (33.62%), Iso-$C_{18:0}$ (19.62%), Iso-$C_{16:1}$ n-9 (13.1%), Iso-$C_{16:0}$ (12.34%), Iso-$C_{17:0}$ (7.23%), Iso-$C_{14:0}$ (6.29%), Iso-$C_{16:1}$ n-7 (4.54%). Rheims, *et al.* (1999) and Krishnamurthi, *et al.* (2009) had reported the presence of Iso-$C_{16:1}$ is one of the peculiar cell wall characteristic of *Solibacillus silvestris.* The characters of *S. silvestris* AM1 are peculiar and its surface properties seem to change with medium used for culture.

2B.3.5. Phenotypic strain characterization using Biolog microbial ID system:

Unlike the type strain *Solibacillus silvestris* HR3-23^T, *S. silvestris AM1* can grow in salt concentrations of upto 7% NaCl, protease and amylase positive, no utilization of glucose (α-D-glucose, D-gluconic acid, α-D-glucose-1-phosphate, D-glucose-6-phosphate, 3-methyl glucose, α-methyl-D-glucoside, β-methyl-D-glucoside), fructose (D-fructose, D-fructose-6-monophosphate) and sucrose as sole carbon source. It showed marked utilization of acetic acid and pyruvic acid while D-galactose and D-tagatose were the only two tested hexoses shown to be utilized by the bacterium. Interestingly *S.silvestris*strain AM1 showed utilization of β-hydroxybutyric acid, γ-hydroxybutyric acid, p-hydroxy-phenylacetic acid and 2,3-butanediol.

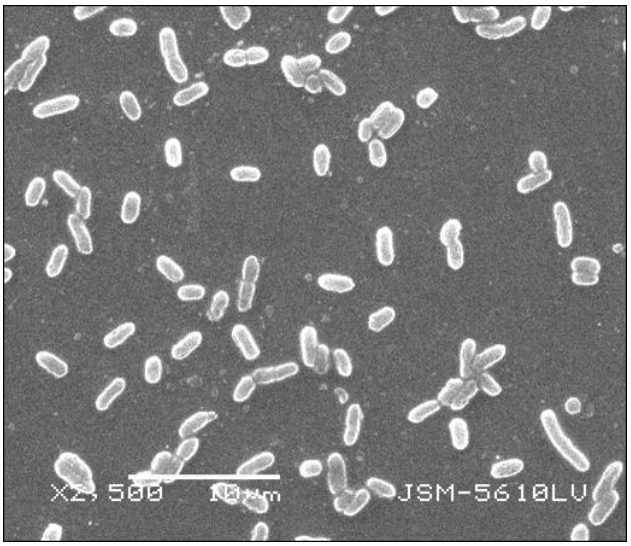


Figure 2.12. Scanning electron micrograph of *S. silvestris AM1*

Table 2.4. Biochemical characterization of *S. silvestris* AM1 and its results

Characterization	Results
Gram reaction	Positive thick rods
Sporulation	Spherical centrally positioned spores
Motility	Tumbling motility
Size	Length 2.9 (±0.2µM);breadth 1.25µM (±0.2µM)
Aerobic/anaerobic	Aerobic
Growth in NaCl	Upto 7%
Indole production	Negative
Methyl red	Negative
Voges–Proskauer	Negative
Citrate utilization	Negative
Triple sugar Iron	Alkaline / No change
Lysine decarboxylase	Negative
Gelatine hydrolase	Negative
Nitrate reductase	Negative
Cellulase	Negative
Pectinase	Negative
Amylase	Negative
Urease	Negative
Protease	Positive
Oxidase	Weakly positive
Oxidation fermentation tests	
Xylose	Oxidation / fermentation
Lactose	Non-reactive
Glucose	Non-reactive
Sucrose	Non-reactive
Mannitol	Non-reactive
Maltose	Non-reactive

S. silvestris strain AM1 differed from reported type strain *B. silvestris* HR3-23^T (Rheims, *et al.*, 1999) in gelatin hydrolase and extracellular protease production with weak positive reactions and 0-7% salt tolerance a little more than reported(0-5%) and showed resistance to Streptomycin. The salt tolerance of *S. silvestris* strain AM1 can be attributed to its micro-adaptation to marine environment. This can also be the reason for its dependency on Zobell marine medium for production of bioemulsifier. The non-utilization of Glucose and related sugars but utilization of acetic acid and pyruvic acid shows that *S. silvestris* strain AM1 uses alternative pathways for energy production than related to glucose.

2B.3.6. Antibiotic sensitivity:

S. silvestris AM1 showed resistance to Streptomycin which persisted even after the concentration was increased to 250µg.While it was sensitive to other antibiotics like Kanamycin, Ampicillin, Tetracycline, Gentamycin, Chloramphenicol and Rifampicin.

2B.3.7. Biochemical characterization of *S. silvestris* AM1:

Utilization of some of the substrate in Biolog test results were also repeated in biochemical tests and it was again confirmed that the bacterium could not utilize glucose, lactose,mannitol, sucrose, maltosethus giving negative results for tests. The biochemical tests results are given in table 2.4.

The novel bioemulsifier producing strain isolated from Vellar estuary, Parangipettai, India was found to produce a bioemulsifier that could emulsify various hydrocarbons. The bacterium also showed quorum quenching and biofilm forming ability with marginal decrease in surface tension. 16S rDNA, FAME analysis and DNA-DNA hybridization analysis confirms the bacterium to be a unique strain of *Solibacillus silvestris* with apparent inability to utilize common carbohydrates. With so much special characteristics, and properties, it was of great interest to further characterize and study the bioemulsifier. Further studies in this regard are given in succeeding chapters.

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Chapter 3

Factors influencing the production of bioemulsifier by *S. silvestris* AM1

कर्पूरधूलिरचितालवालः कस्तूरिकापंकनिमग्ननालः|
गंगाजलैः सिक्तसमूलवालः स्वीयं गुणं मुञ्चति किं पलाण्डुः||

Meaning:
If an onion plant is grown in camphor bed, musk is used as a soil for it, or it is watered with Ganga-jala (the holiest of the waters), will it give up its characteristic pungent odour? The central idea is that a person's basic character remains the same, no matter what efforts you take to change him.

Chapter 3:

Factors influencing the production of bioemulsifier by *S. silvestris* AM1

3.1. Introduction

Bioemulsifiers are extracellular polymers that are released into surrounding medium during the growth of the producing strain producing it (Amiriyan, *et al.*, 2004). Bioemulsifier producing microorganisms can be divided into three categories: There have been many reports about bioemulsifier producers with alkanes as carbon source, water soluble substrates as carbon sources and those with alkanes and water soluble substrates as carbon sources as listed by Amaral, *et al.* (2008).

For bioemulsifier production, some isolates needed induction as was seen in the case of *A. calcoaceticus* RAG-1 for production of Emulsan. The inducer needed here was ethanol. *Bacillus* strains FE-1 and FE-2, reported for bioemulsifier production (Patel & Gopinathan, 1986) were grown in the presence of fenthion as the sole carbon source while *B. stearothermophilus* VR-8, a hotspring isolate (Gurjar etal., 1995) could produce bioemulsifier in presence of 4% crude oil as an inducer. Many isolates from solid waste oil samples (Toledo, *et al.*, 2006) and *Bacillus* sp. DHT from oil contaminated soil (Kumar, *et al.*, 2007) could produce bioemulsifiers by utilizing the hydrocarbon carbon source. *Bacillus* species reported from hydrocarbon (Sathe, *et al.*, 2012) and oil contaminated soils (Klawech, *et al.*, 2013) utilized soybean oil and waste lubricating oil respectively as the carbon sources for production of respective bioemulsifiers. Many *Bacillus* species isolates were also reported for production of bioemulsifier in water soluble substrates like the two strains of *Bacillus,* IAF 343 and IAF 346 (Cooper and Goldenberg, 1987). *Bacillus* sp.CP912 and *B. licheniformis* K125 were reported to be glucose induced bioemulsifiers (Yun & Park, 2000); Suthar et al., 2008). *B. amyloliquifaciens* LP03 reported for antifungal bioemulsifier (Lee, *et al.*, 2007) was grown in casein peptone as the carbon source while *B. licheniformis* ACO1 isolated from petroleum reservoirs produced bioemulsifier by using yeast extract as the sole carbon source without any hydrocarbon inducer. Mulligan & Cooper (1985) have reported the salient features of the bioemulsifiers and biosurfactants produced from unusual carbon sources.

Traditionally microbiologists have used factorial designs for optimizing the production of industrially important metabolites. Those microorganisms whose growth responses not tested can be calculated by modeling the behavior of microorganisms with respect to the main controlling environmental (including nutritional) factors. Changes in formulation of media necessitate repetition of the challenge test and develop a systematic database of the microbial response with respect to the factors tested. The responses can be predicted by using many factorials and set of conditions. "Predictive microbiology" can be considered as an applicative research where the microbial to different factors responses from past observations are used to predict the reproduction of mathematical representations of similar responses. The term "Quantitative microbial ecology" has been applied as an alternative to predictive microbiology to study the microbial responses to environmental (including nutritional) factors and summarized into equations or mathematical models (Gibson, *et al.*, 1988, Kalathenos Panayotis, *et al.*, 1995, Fakruddin, *et al.*, 2011). Studies of all the environmental factors including the nutritional, temperature and salinity for production of biosurfactants and bioemulsifiers are scanty. Generalization of production with respect to nutritional factors needs studies with respect to other environmental factors including temperature, salinity, etc. (Ilori, *et al.*, 2005).

Solibacillus silvestris AM1, selected for further studies was checked for different conditions and factors influencing its bioemulsifier. The purpose of the studies undertaken here was not the optimization of production medium but rather to understand the effect of nutrients on bioemulsifier AM1 and whether these environmental factors would greatly affect the bioemulsifier production and how does it affect the interactions between different bacteria present in the niche.

3.2. Materials and Methods

Production of bioemulsifier by *S. silvestris* AM1 was checked in reported media as given in Appendix and compared to isolation and production medium i.e. Zobell marine medium (Appendix). The reported media used for production of bioemulsifiers were taken from Pfiffner, *et al.* (1986), Cooper & Goldenberg (1987), Gurjar, *et al.* (1995), Yun & Park (2003), Suthar, *et al.* (2008).

3.2.1. Emulsification index (%EI):

Emulsification index (%EI) was calculated as given in chapter 2, section 2A.2.5.

3.2.2. Effect of pH, temperature and NaCl concentration on bioemulsifier production:

The ability of *S. silvestris* sp. AM1 to produce bioemulsifier at different pH and temperature was tested by culturing 2% of the bacterium in ZM broth adjusted to different pH (6.0, 6.5, 7.0, 7.5, 8.0) and incubated at different temperature (30, 35, 40, 45, and 50°C) for 48h and then assaying for its %EI. Production of bioemulsifier in presence of higher NaCl concentrations was tested by inoculating 2% culture in ZM medium added with NaCl to give final concentrations of 2, 3, 4, 5, 6 and 7%. All the experiments were conducted in triplicates. The effect of NaCl on growth of the *S.silvestris* AM1 was also studied.

3.2.3. Inoculum size and age:

Overnight grown bacterial culture (when the bacterium is in stationary phase) was pelleted and washed with phosphate buffered saline (PBS) and adjusted to 0.6 OD at 600nm and 1, 2 and 3% inocula were individually added into sterile ZM broth (in triplicates) and incubated for 48h at 35°C. Similarly, in another batch, inoculum was added individually into sterile ZM medium and incubated at 35°C. After 6, 12, 18, 24 and 48h of incubation, aliquotes were removed, cells pelleted and adjusted to 0.6 O.D. (using PBS) and 1% was inoculated into 50ml sterile ZM medium in triplicates. After incubation, in both batches the culture was checked for O.D. and centrifuged for 10,000rpm for 10min. The cell-free supernatant was checked for %EI.

3.2.4. Growth and bioemulsifier production by *S. silvestris* AM1 in low concentrations of galactose:

The medium used in the studies here was supplemented with galactose as sole carbon source instead of peptone and yeast extract in ZM medium (section 2B.3.5). Growth and production of cell-bound and cell-free bioemulsifier was checked in different concentrations of galactose (1, 5, 10, 15 mg/ml) in ZM salt solution.

3.2.5. Statistical studies for medium component analysis:

Effect of components of Zobell-Marine (ZM 2116) medium was checked for their influence on the bioemulsifier production. The results were analyzed with ANOVA and t-test using GraphPad Prism 5 software.

3.2.6. One factor at a time (OFAT):

Six macronutrients out of 16 total components of the ZM medium were studied by single factorial design. One independent macronutrient was taken with all the other components at their fixed level. Inoculum of 0.6 O.D was inoculated and the medium containing organism was incubated for 48hrs at 35°C. After 48hrs the medium was centrifuged at 10,000rpm for 15 min. The supernatant was further analyzed for emulsifying ability (Table 3.1).

Table 3.1. Concentration of the components (variables) used in OFAT and Plackett Burman (PB) studies for the production of bioemulsifier by *S. silvestris* AM1.

Sl. No.	Components	ZM medium	OFAT (g%)			PB (g%)	
			Low	Medium	High	High (+)	Low (-)
1	Peptone	0.5	0.25	0.5	0.75	3	0.3
2	Yeast extract	0.1	0.05	0.1	0.15	1	0.1
3	NaCl	1.95	0.97	1.95	2.9	2.5	1.5
4	$MgCl_2$	0.88	0.44	0.88	1.32	1	0.25
5	Na_2SO_4	0.33	0.16	0.33	0.48	0.5	0.4
6	$CaCl_2$	0.18	0.09	0.18	0.27	0.4	0.2
7	Fe-citrate	0.01	-	0.01	-	0.01	0
8	Na_2HCO_3	0.016	-	0.016	-	0.016	0
9	KCl	0.055	-	0.055	-	0.2	0.05
10	KBr	0.008	-	0.008	-	0.04	0.008
11	NaF	0.00024	-	0.00024	-	0.0024	0
12	$SrCl_2$	0.0034	-	0.0034	-	0.0034	0
13	H_3BO_3	0.0022	-	0.0022	-	0.0022	0
14	Na_2SiO_3	0.0004	-	0.0004	-	0.004	0
15	$(NH_4)_2NO_3$	0.00016	-	0.00016	-	0.00016	0
16	Na_2HPO_4	0.0008	-	0.0008	-	0.0008	0

3.2.7. Plackett Burman design:

To evaluate the relative significance of the components of the ZM medium, the Plackett-Burman experimental design (Plackett & Burman, 1946) was used. After

OFAT studies, each variable were tested at 2 levels, a higher (+) and a lower (-). The 16 medium components were used as independent variables and three dummy variables were set to estimate the experimental error (Table 3.2). The rows in Table 3.2 show experimental trials and each column represents different variables. The medium components and the design of experiment are as given in table 3.2 The results were obtained in triplicates.

The effects of each variable was determined with the following equation

$$E_{xi} = (\Sigma M_{i+} - \Sigma M_{i-})/N$$

where, Exi is the concentration effect of the tested variable, Mi+ and Mi- are the emulsification indices from the trials where the variable (Xi) measured was present at high and low concentrations respectively and N is the number of trials (Yu, *et al.*, 1997, Srinandan, *et al.*, 2010)**.** Since the objective of this study is to evaluate the relative effect of each variable, a significance level of less then 0.2 is acceptable (Stowe & Mayer, 1966, Yu, *et al.*, 1997). Significance of the triplicate values of each trial was calculated using Prism 5.0 software. The results were analyzed with student's *t*-test using GraphPad Prism 5 software

3.2.8. Response surface methodology:

Response surface methodology using Box-Behnken design was used to estimate the nutritional requirements and interactions between them of *S.silvestris* AM1 for production of bioemulsifier using central composite design. The medium components used in the design were A: peptone (3, 1.65 and 0.3g%),B: yeast extract (0.1, 0.55 and 1g%), C: $MgCl_2$ (0.25, 0.625 and1g%) and D: KCl (0.05, 0.125and 0.2g%). The behavior of the system was explained by,

$$Y = \beta_o + \Sigma\beta ixi + \Sigma\beta ijxixj + \Sigma\beta iixi^2$$

where, Y is the predicted response, β_o is offset term, βi is a linear offset, βii is squared offset and βij is interaction effect. xi dimensionless coded value of Xi. This equation was further solved by using the software Design-Expert (version 8.0, Stat-Ease, Minneapolis, MN, USA) and the responses of the dependent variables were estimated.

Table 3.2. Plackett-Burman design matrix for 16 medium components of Zobell marine medium

	Variables																		
Trials	**X1**	**X2**	**X3**	**X4**	**X5**	**X6**	**X7**	**X8**	**X9**	**X 10**	**X 11**	**X 12**	**X 13**	**X 14**	**X 15**	**X 16**	**D1**	**D2**	**D3**
1	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-
2	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+
3	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+
4	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-
5	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-	-
6	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-	-
7	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+	-
8	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-	+
9	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+	-
10	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-	+
11	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+	-
12	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+	+
13	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+	+
14	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+	+
15	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-	+
16	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-	-
17	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+	-
18	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+	+
19	+	-	-	+	+	+	+	-	+	-	+	-	-	-	-	+	+	-	+
20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

3.2.9. Critical Micellar dilution (CMD) in ZM and AM1 media:

The minimum concentration of biosurfactant corresponding to the dilution at which micelles begin to form is referred to as its critical micellar concentration (CMC), which can also be expressed in terms of CMD (Makkar & Cameotra, 1997). Minimum biosurfactant/bioemulsifier required for effective micelle formation expressed as CMD is a measure of bioemulsifier concentration and its production. By diluting the crude bioemulsifier produced in ZM and AM1 medium (newly obtained combination of ZM medium components after statistical design analysis) with distilled water, the %EI is measured. The dilution at which, %EI starts to fall abruptly is the CMD, where it is proportional to the amount of biosurfactant/bioemulsifier present in the sample (Makkar & Cameotra, 1997).

3.2.10. Growth and bioemulsifier producing ability of *S. silvestris* AM1 in oligotrophic or nutrition deficient media:

To check the bioemulsifier production by *S. silvestris* AM1 in marine oligotrophic conditions, its BE production (cell-bound and cell-free) was tested in lower concentrations of ZM medium. The bacterium was inoculated in synthetic marine salt (ZM salts) solution containing different concentrations of protein (0.5, 1.0, 1.5, 2.0, 2.5, 5.0, 7.5, 10.0, 12.5 mg/ml Peptone). It was also checked for growth and bioemulsifier production in $1/10^{th}$ and $1/100^{th}$ diluted ZM medium and mixture of influencing factors from it (termed as AM1 medium referring to *S.silvestris* AM1 producing bioemulsifier). Zobell Marine medium was further diluted to $1/10^{th}$, $3/10^{th}$, $5/10^{th}$, $7/10^{th}$, $9/10^{th}$ and $1/100^{th}$ of its original constitution.

3.2.11. Time-course bioemulsifier production:

50ml of Zobell Marine (ZM) medium and AM1 medium were inoculated individually with 0.6 O.D. corresponding to 6.3×10^7 cfu of *S. silvestris* AM1 cells. The system was kept for incubation at 30°C. Every hour, 2ml aliquots were taken out and centrifuged at 10000 RPM for 10min. The cell pellet was given two washes with phosphate buffered saline (PBS; 140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4 at pH 7.3) and 2ml cell suspension was made in PBS. Centrifugation of supernatant was done and cell suspensions were checked for %EI (E_{24}) for cell bound bioemulsifier production while the cell free bioemulsifier was estimated in the supernatant.

Time course of bioemulsifier production by *S. silvestris* AM1 in oligotrophic conditions of peptone was checked by growing it in presence of 0.5 mg/ml Peptone in ZM salt solution at a sampling interval of two hours from the point of inoculation. The cell bound and cell free bioemulsifier was estimated as above.

3.2.12. Growth and production of bioemulsifier AM1 in xenobiotics:

S. silvestris AM1 was isolated from Vellar estuary which is constantly polluted by the four irrigation channels. There have also been numerous reports of hydrocarbon and pesticide contamination at vellar estuary (Rajasegar, *et al.*, 2002, Sarkar, *et al.*, 2008). The bioemulsifier producers from this site should therefore possess high tolerance and resistance to hydrocarbons and pesticides. Growth and production of bioemulsifier by *S. silvestris* AM1 in presence of these xenobiotics namely hydrocarbons and pesticides was explored.

3.2.12.1. Effects of hydrocarbons on growth and bioemulsifier production of *S. silvestris* AM1:

The hydrocarbons used in this study include polyaromatic naphthalene, trichlorobenzene, benzene and catechol. The concentration range for each hydrocarbon was chosen on the basis of their water solubility. While benzene (33.3% v/v) and trichlorobenzene (5% v/v) were prepared in methanol as stock solutions, naphthalene stock was prepared as 5mg/ml in water. Concentration of hydrocarbons used in this study is given in table 3.3. Catechol is an intermediate compound during the degradation pathway for aromatic hydrocarbons like BTX (benzene, toluene and xylene). The concentration of catechol used was 250µg/ml. The bacterium was grown in presence of Catechol for 48 hours, at 35°C in shaking condition (180 rpm). After incubation, growth was checked at 600nm and % EI was measured.

1% of 0.6 O.D. *S. silvestris* AM1 was inoculated into ZM medium tubes with hydrocarbons in the concentration given and incubated at 35°C with shaking for 48h. 25 µg/ml of Streptomycin was incorporated in the medium to avoid bacterial contamination. After incubation, O.D. was checked at 600nm and %EI of the culture broth was checked as given previously. Growth in presence of aromatic compounds was checked by meta-cleavage Dioxygenase plate assay (Tuah, *et al.*, 2009). For further confirmation, basic silver-mirror test was performed.

3.2.12.2. Effects of pesticides on growth and bioemulsifier production of *S. silvestris* AM1:

Major pesticides used in Indian fields were taken for studying their effects on bioemulsifier production and growth of *S. silvestris* AM1. Pesticides used in this study include methomyl, cypermethrin and an organophosphorous pesticide acephate with stock solutions of concentrations 10mg/ml (w/v). All the pesticides selected were water soluble and are considered to be major environmental hazards. While cypermethrin is a liquid, it was added directly into the experimental systems, acephate and methomyl were used after filtration through 0.22µm filter. Concentration of pesticides used with their recommended field concentration (RFC) (Singh et al., 2009) is given in table 3.3. Thus the study includes concentrations of pesticides above their RFC.

Table 3.3. Concentration of xenobiotics used to check their effect on growth and bioemulsifier production by *S. silvestris* AM1

Xenobiotics	Concentration (mg/ml)					RFC * (Singh et al., 2009)
	1	2	3	4	5	
Hydrocarbons						
Benzene	0.8	1.6	2.4	3.2	4.0	0
TCB *	72.5	145	217.5	290	362.5	0
Naphthalene §	10	20	30	40	50	0
Pesticides						
Acephate §	50	100	150	200	250	50
Methomyl §	100	200	300	400	500	60
Cypermethrin	0.5	1.0	1.5	2.0	2.5	65

RFC, Recommended field concentration (µg/ml); *= $\times 10^{-6}$mg/ml; §= $\times 10^{-3}$mg/ml

3.2.13. Statistical analysis:

All experiments were conducted in triplicate and analyzed withANOVA and t-test using GraphPad Prism 5 software.

3.3. Results and Discussion

Zobell marine medium, formulated in early 20th century is the only medium available for studying marine heterotrophic bacteria without the recurrent need for sea water. Bioemulsifier production was checked in ZM and different reported media and the results show that the production of bioemulsifier was highest i.e. 61.8% in ZM

medium as given in figure 3.1. Other media reported by Pfiffner, *et al.* (1986), Cooper & Goldenberg (1987), Gurjar, *et al.* (1995), Yun & Park (2003), Suthar, *et al.* (2008)when checked for bioemulsifier production by *S.silvestris* AM1 gave less than 50% EI as compared to that in ZM medium (Figure 3.1).The media of Pfiffner et al., and Cooper-Goldenberg used glucose as carbon source which is not utilized by *S. silvestris* AM1, while the media of Gurjar et al., Yun and Park and Suthar et al., have peptones or yeast extract which support bioemulsifier production as can be seen further in this chapter. Better %EI in the last three media of the series established the requirement of protein source for the production of bioemulsifier by *S.silvestris* AM1.

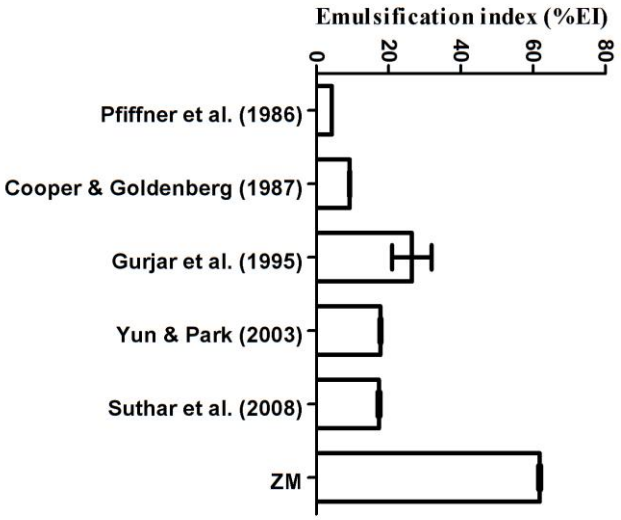

Figure 3.1. Bioemulsifier production by *S. silvestris* AM1 in reported production media.

3.3.1. Physical factors affecting the bioemulsifier AM1 production:

In Vellar estuary, physical factors like temperature, pH and salinity were observed by many reports and showed distinct seasonal variations (Sundaramanickam, *et al.*, 2008).

3.3.1.1. Effect of pH, temperature and NaCl concentration on bioemulsifier production:

Optimum condition for bioemulsifier production by *S. silvestris* AM1 was found to be pH 7.4 and a temperature of 35°C (Figure 3.1 and 3.2). As the inherent property of the components of ZM medium was changed (precipitated) beyond the pH range 6.0 to 8.0, this range was selected to test its effect on production. The

bioemulsifier production is not affected significantly with minor changes in pH selected (Figure 3.1).

The bioemulsifier production was also not affected between temperature range of 30- 37°C, but its production reduced drastically above 40°C. Bioemulsifier production is directly correlated to the growth of the bacteria producing it and therefore indirectly to the physical factors like temperature tested here (Figure 3.2). The optimum growth was at pH 6.0 and 30°C.

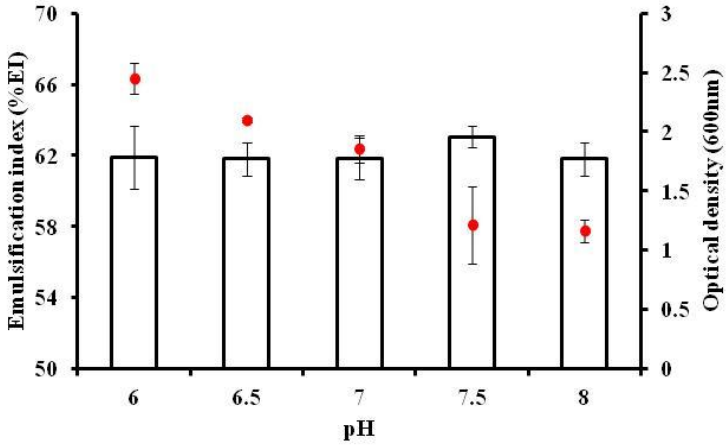

Figure 3.1. Effect of pH on bioemulsifier (bar) production by *S. silvestris* AM1 and its growth in O.D. (dot-marker) in ZM medium

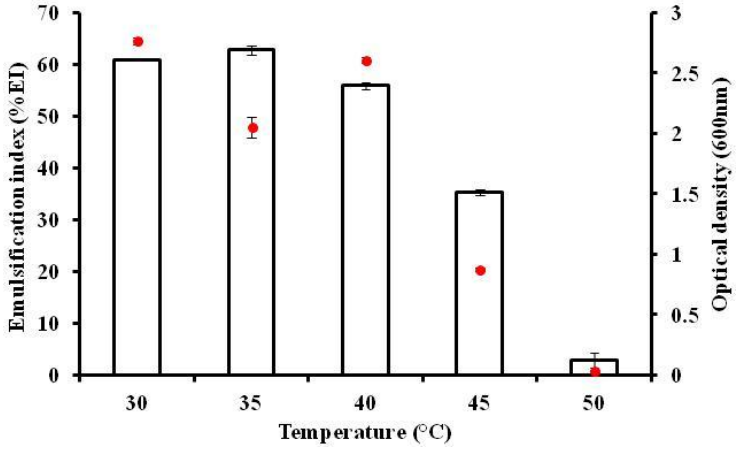

Figure 3.2. Effect of Temperature on bioemulsifier (bar) production by *S. silvestris* AM1 and its growth in O.D. (dot-marker) in ZM medium

The NaCl concentrations taken were above the range of ZM medium with 7% being the highest for growth (Figure 3.3). The growth of *S. silvestris* AM1 in different pH ranges tested here does not change as rapidly as in case of extreme temperatures

and NaCl concentrations. At higher temperatures (>45°C) and higher NaCl concentrations (7%), the growth of the bacterium decreased drastically.

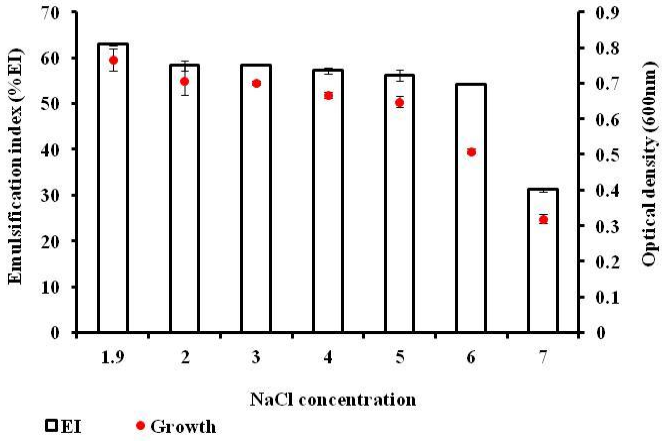

Figure 3.3. Effect of NaCl concentration on bioemulsifier (bar) production by *S. silvestris* AM1 and its growth in O.D. (dot-marker) in ZM medium

3.3.1.2. Inoculum size and age:

Inoculum age and inoculum size are the two factors usually known to affect the production of microbial surfactants like bioemulsifiers. The size and age of the bacteria for production of a specific compound are important factors in deciding final yield of the product. When tested for effects of inoculum size, it was seen that percentage inoculums gave highest %EI (60.8%). Increasing the percentage of inocula upto 2 and 3% did not further increase the %EI or the growth. Therefore 1% inoculums was selected. This may be due to exhaustion of nutrients (Figure 3.4).

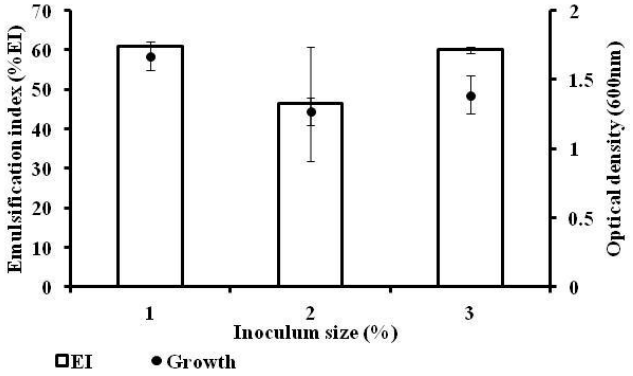

Figure 3.4. Effect of inoculum size on bioemulsifier production by *S. silvestris* AM1 (bar = %EI; dot-marker= growth in O.D.)

Inoculum age shows that the bioemulsifier activity is highest when the culture is after 6h old (60.8%EI), when the bacterium is in its log phase of growth (Figure

3.5). Similar results were obtained when the culture inoculum was 18h old giving 60.3%EI, and showed higher consistency and greater viscosity than 6h inoculum even when the culture inoculum was in stationary phase of growth. Inoculum age did not have any marked effect on emulsification as can be seen in figure 3.4. Hence 18h inoculums gave maximum EI while it gave least growth.

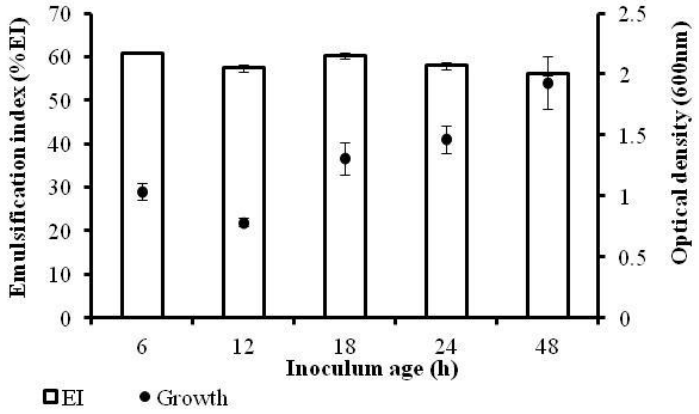

Figure 3.5. Effect of inoculum age on bioemulsifier production by *S. silvestris* AM1 (bar = %EI; dot-marker= growth in O.D.)

3.3.2. Nutritional factors affecting the bioemulsifier AM1 production:

Addition of carbon sources to ZM medium did not induce bioemulsifier production as can be seen in figure 3.6. *S.silvestris* AM1 has a limited ability as discussed in previous chapter to utilize the carbohydrates. Here the inability of glucose, sucrose and other sugars in enhancing the bioemulsifier production was also observed (Figure 3.6). As galactose was one of the few sugars utilized by *S. silvestris* AM1 as can be seen from the Biolog data (chapter 2. Section 2B.3.5), it was checked for increasing the bioemulsifier production. Presence of galactose marginally increased emulsification process. As given in Figure3.7, galactose, used as a sole carbon source, the growth of *S. silvestris* AM1 with increase in galactose concentration, but resulted in small increase of 2 - 5.3% EI in bioemulsifier production.

3.3.2.1. OFAT studies:

S. silvestris AM1, exhibited better production of bioemulsifier in ZM medium than in other reported production media (i.e., there was less or negligible bioemulsifier in reported production media) like Pfiffner, *et al.* (1986), Cooper & Goldenberg (1987), Gurjar, *et al.* (1995), Yun & Park (2003), Suthar, *et al.* (2008).

This suggests that proteinaceous medium is important criteria for bioemulsifier production by *S. silvestris* AM1.

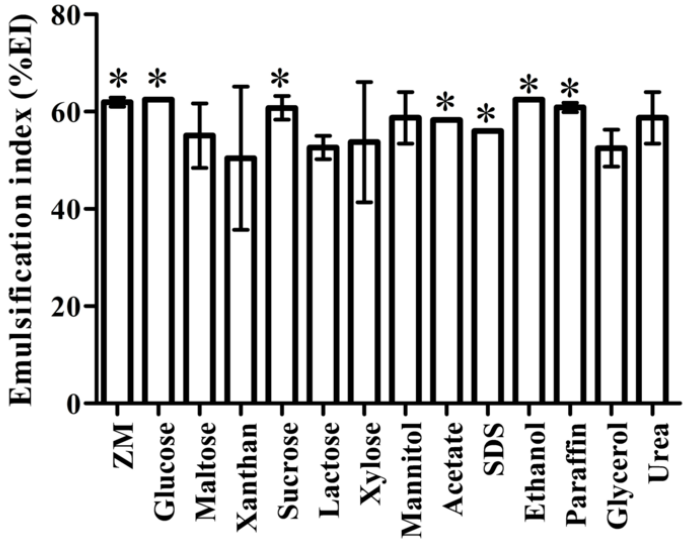

Figure 3.6. Emulsification index (%EI) of *S. silvestris* AM1 grown in ZM medium and its amendments with given sources (*=p<0.001*)

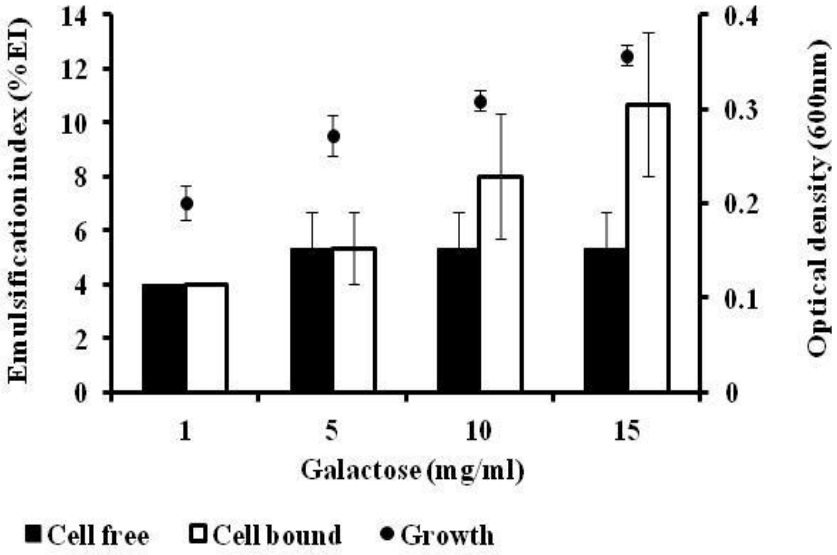

Figure 3.7. Growth and bioemulsifier production by *S. silvestris* AM1 in ZM medium with galactose as sole carbon source . (bar = %EI; dot-marker= growth in O.D. and n=3; P-values: *=p < 0.01)

As reported in the literature, the one factor at a time (OFAT) approach is time consuming but can be used for identifying critical components of the medium under study (Sivapathasekaran, *et al.*, 2010, Srinandan, *et al.*, 2010). The bioemulsifier

produced in the range of peptone and $MgCl_2$ used in OFAT studies was not significantly increased (p-value = 0.4 and 0.9 respectively). Only 57-59% EI was produced in presence of the two compounds. Changes in the concentration of yeast extract affected the production of bioemulsifier significantly (p-value < 0.02) giving emulsification between 54-64% EI. NaCl, Na_2SO_4 and $CaCl_2$ showed varied effect on the production of bioemulsifier with the EI range between 49-59%. Thus, according to OFAT studies, the ranges of peptone and $MgCl_2$ concentrations taken have little significance on bioemulsifier production while yeast extract was found to be a significant factor.

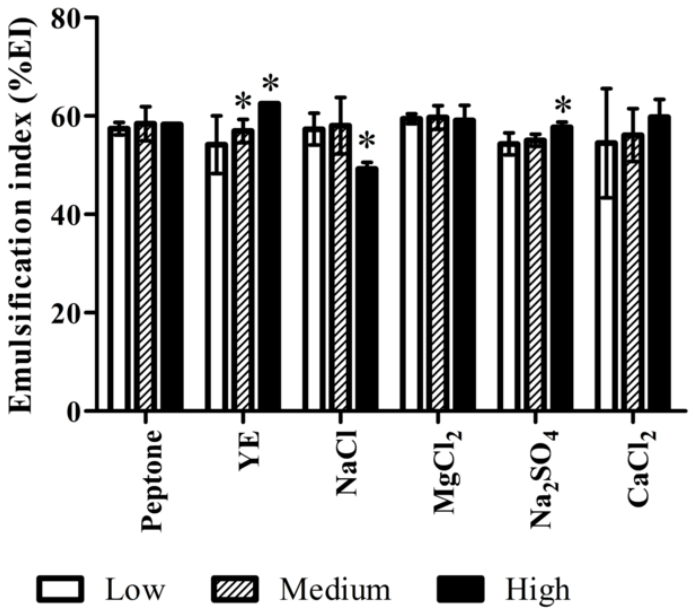

Figure 3.8. Effect of changing one factor at a time (OFAT) of the macronutrients from ZM medium on emulsification of *S. silvestris* AM1. (p-value, *<0.001).

3.3.2.2. Plackett-Burman design:

Plackett-Burman being a widely used statistical design for screening of many factors, significance of different components of ZM on bioemulsifier production was examined by the same statistical design. The bioemulsifier production quantified as emulsification index (%EI) from the cell-free supernatant of the 16 media trials of Plackett-Burman design are given in table3.4. As shown in Figure 3.9, trial 9 showed highest EI of 68.9% and trial 5 showed 66.3% EI. Trials 1, 11, 12, 13, 15, 16 and 18 gave 60-64% EI, which was comparable to 62.5% EI obtained in ZM medium. Trial 4 gave lowest EI of 9.5%. Trials 3, 4 and 8 gave below 20%EI.

Effects (Exi) of the components of ZM medium were calculated as given before (Figure3.10). A positive (Exi) value of the variable is considered to be positively influencing in bioemulsifier production by *S. silvestris* AM1 in higher ranges of the concentrations taken, and a negative result shows its positive influence in lower concentrations. Peptone and yeast extract influenced significantly on the production of bioemulsifier ($p<0.05$) while $MgCl_2$ and KCl influence with lower significance ($p<0.2$). As shown in Figure 3.10 peptone, yeast extract and KCl showed positive Exi values, where as $MgCl_2$ showed negative Exi value showing its positive and negative influence in enhancing the production of bioemulsifier by *S. silvestris* respectively (PB design in table 3.2).

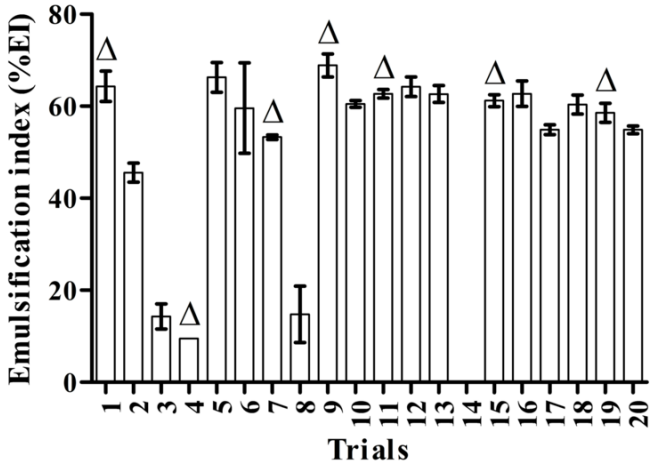

Figure 3.9. The emulsification activity of S. *silvestris* AM1 in 20 trial media of Plackett-Burman design (p-value, Δ<0.001)

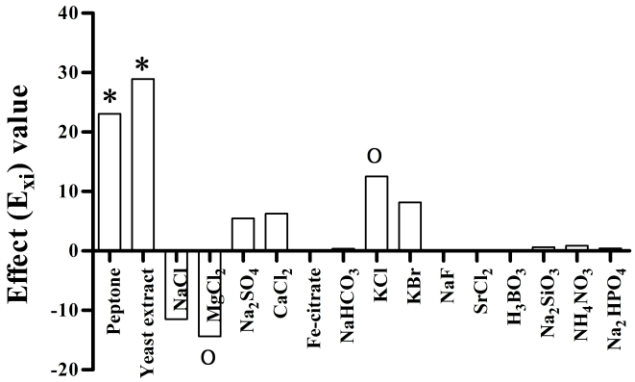

Figure 3.10. Effect of each media component of ZM medium on bioemulsifier AM1 production (p-value, *<0.05, O<0.2).

Different media components were evaluated statistically using Plackett Burman design for their significance, peptone and yeast extract showed positive effect and KCl and $MgCl_2$ exhausted negative effect on emulsifier production (Figure 3.10). Although it is known that less than 5% variation in the confidence of the matrix is sufficient, the bioemulsifier studies with matrix analysis of Plackett-burman design is robust and it shows that the probability of observations arising by more than 20% of the confidence is rarely due to chance. Hence $MgCl_2$ and KCl, both giving significance (p-value<0.2) were selected for further studies. Using Plackett Burman design, the medium components giving highest emulsification (%EI = 68% in trial 9) was achieved where yeast extract is a significant factor which was also seen in OFAT studies.

Table 3.4. Results of Plackett-Burman design for evaluating the components of ZM medium

Components	High (+) in g%	Low (-) in g%	E(xi)	t(Xi)	p-value	1-pvalue	Confidence
Peptone	3	0.3	23.09	2.82	0.04	0.96	96.29
Yeast extract	1	0.1	28.91	3.53	0.02	0.98	98.33
NaCl	2.5	1.5	11.48	1.40	0.22	0.78	78.03
$MgCl_2$	1	0.25	14.37	1.75	0.14	0.86	86.03
$Na2SO_4$	0.5	0.4	5.47	0.67	0.53	0.47	46.65
$CaCl_2$	0.4	0.2	6.26	0.76	0.48	0.52	52.08
Fe-citrate	0.01	0	0.08	0.08	0.94	0.06	5.55
$Na2HCO_3$	0.016	0	0.39	0.39	0.74	0.26	26.40
KCl	0.2	0.05	12.55	1.53	0.19	0.81	81.40
KBr	0.04	0.008	8.17	1.00	0.36	0.64	63.60
NaF	0.0024	0	0.17	0.17	0.88	0.12	12.18
$SrCl_2$	0.0034	0	0.00	0.00	1.00	0.00	0.01
$H3BO_3$	0.0022	0	0.18	0.18	0.88	0.12	12.49
Na_2SiO_3	0.004	0	0.61	0.61	0.61	0.39	39.44
NH_4NO_3	0.00016	0	0.85	0.85	0.48	0.52	51.53
Na_2HPO_4	0.0008	0	0.44	0.44	0.71	0.29	29.45

3.3.2.3. Response surface methodology:

Response surface methodology is used to estimate the main effects of individual variables, also to optimize the response. The four components of ZM medium shortlisted for their significant positive influence in production of bioemulsifier by *S. silvestris* AM1 were analyzed using Box-Behnken design in Design Expert 8.0 software. These components were studied for their interactions and

use of this model for higher production of bioemulsifier. After the analysis, the response yielded a linear model as there was an insignificant interaction seen among the components for bioemulsifier production. Peptone and yeast extract were found to be most significant components influencing the production with p-values of <0.0001 and 0.0044 respectively giving the hot zone as given in Figure 3.11. Since F-value of model was found to be 10.22, there is only a 0.01% chance that a 'model F-value' this large could occur due to noise. The lack of fit value for the model was not significant (p=0.7494). Thus, there is a 74.94% chance that a lack of fit F-value of 0.68 could occur due to noise or pure error. Thus the model and its terms were found to be significant.

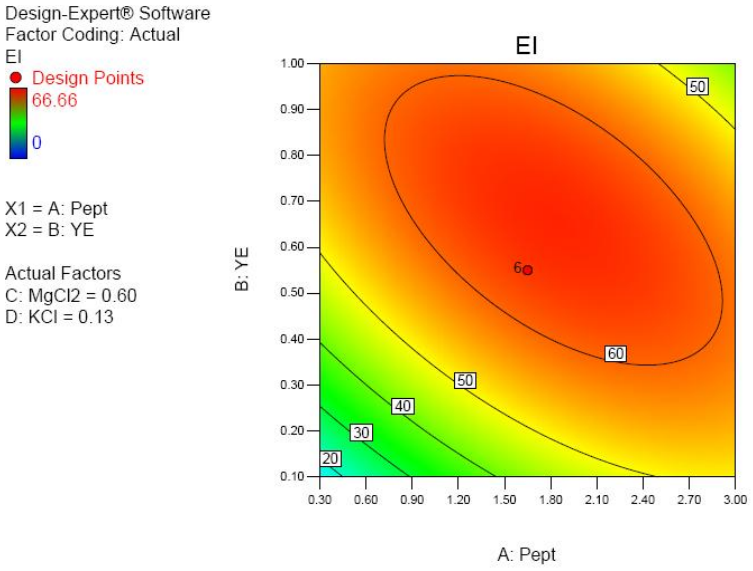

Figure 3.11. Contour graph of analysis of emulsification index by StatEase software

Similar to Plackett-Burman design, peptone and yeast extract were found to be the most significant factors among the four components shortlisted from Zobell marine medium. Box-Behnken (BB) design helped to identify the significant components of the medium and find their optimum concentrations corresponding to selected responses.

3.3.2.4. Validation of the factors:

The factors studied, physical (temperature, pH, inoculums age and inoculums size) and nutritional (the nutritional factors from ZM medium) influencing the production of bioemulsifier were thus checked by response surface methodology. By

incorporating the statistically optimzed conditions and the selected factors (Peptone, 3g%; Yeast extract, 1g%; MgCl2, 1g% and KCl, 0.2g%), making up an optimized medium, 10% increase in production of bioemulsifier by *S. silvestris* AM1 was obtained.

3.3.2.5. Critical micellar dilution (CMD):

The modified ZM medium, after PB design and BB methology had four significant components making up the new AM1 medium. The AM1 medium was checked for bioemulsifier production by *S.silvestris* AM1. The CMD obtained in AM1 and ZM media were compared. With bioemulsifier produced having little effect, both the media had the same CMD, but showed difference in emulsification activity (Figure 3.13). *S. silvestris* AM1 produced 68% EI with respect to ZM medium's 62% EI, an increase of almost 9.7% increase of emulsification index (3.12). The emulsifying activity was obtained even after 1000 times dilution. The bioemulsifier production in ZM medium was markedly less than in the new medium as the emulsification activity is not seen in ZM medium after 1000times dilution.

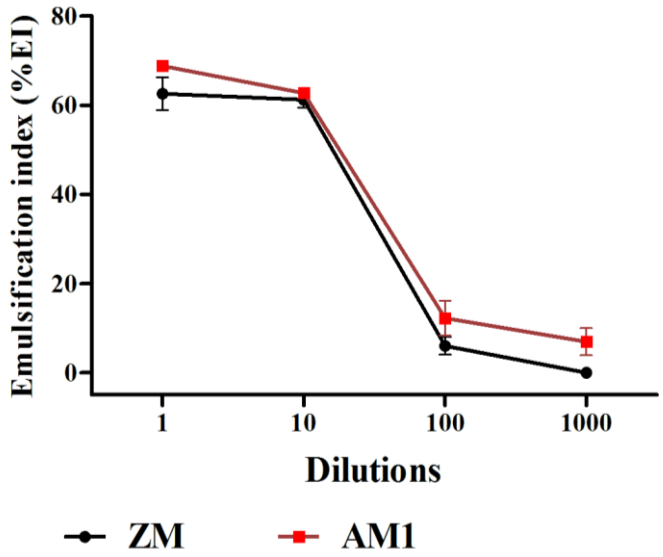

Figure 3.12. Critical micellar dilution (CMD) of bioemulsifier produced in ZM medium and optimized AM1 medium.

As remarked earlier, in absence of an assay method, the emulsification activity on dilution is a measure of the bioemulsifier production.

3.3.2.6. Effect of oligotrophic nutrient conditions on bioemulsifier production by S. silvestris AM1

S. silvestris was checked for its ability to produce bioemulsifier in extremely diluted medium and low concentrations of ZM medium and in turn peptone. In 1/10th and 1/100th diluted media, *S. silvestris* AM1 produces very low concentration of both cell bound (cb) and cell free (cf) bioemulsifier as seen in Figure 3.13 . Its growth also proportionately decreased with medium dilution.Maximum %EI of 60% was obtained in 7/10 dilution, then 5/10 and subsequently 3/10 of ZM medium. The 3/10 dilution also gave of nearly 50%EI.

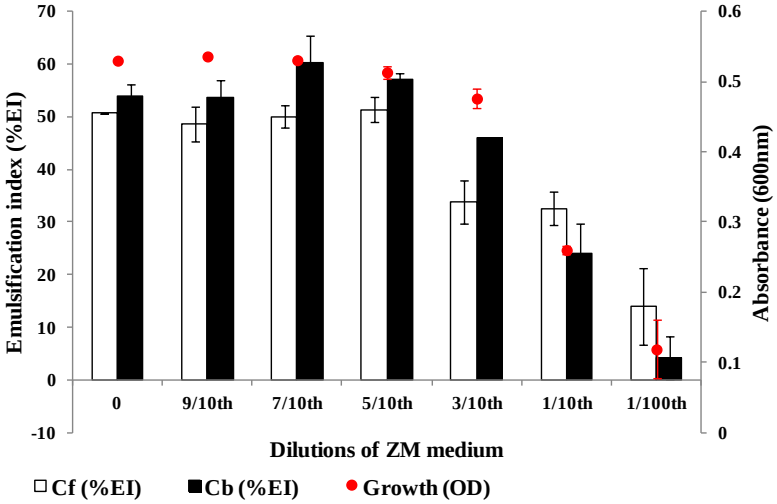

Figure 3.13. Effect of dilution of ZM medium on bioemulsifier production and growth of *S. silvestris* AM1 (bars Cb = cellbound %EI and Cf= cell free %EI dot-marker= growth in O.D.)

When oligotrophic condition of AM1 medium was checked it was found that the production of bioemulsifier by *S. silvestris* AM1 in AM1 medium is higher than in ZM medium, also the loss in activity has not as rapid in AM1 medium as seen in ZM medium at 1/100 dilution (Figure 3.13 and 3.14). In case of ZM medium, both cell bound and cell free emulsifier activity decreased. Also with dilution of AM1 medium the growth of the bacterium is not affected adversely (Figure 3.14). This may be due to the very high concentration of peptone initially in the medium itself. Thus at 1/100th dilution, the AM1 medium supplemented growth and bioemulsifier production better than the 1/100th diluted ZM medium.

Peptone at 0.5mg/ml as the sole carbon source in ZM salt solution gave slight emulsification. 7.5mg/ml peptone was found to be optimal for maximum cell bound

as well as cell free bioemulsifier production. The cell bound bioemulsifier production increased above 1mg/ml peptone concentration in the medium while the cell free bioemulsifier was released at about 1.5 mg/ml and above and beyond 2.5 mg/ml i.e., from 5mg/ml to 12.5 mg/ml it attained a plateau.

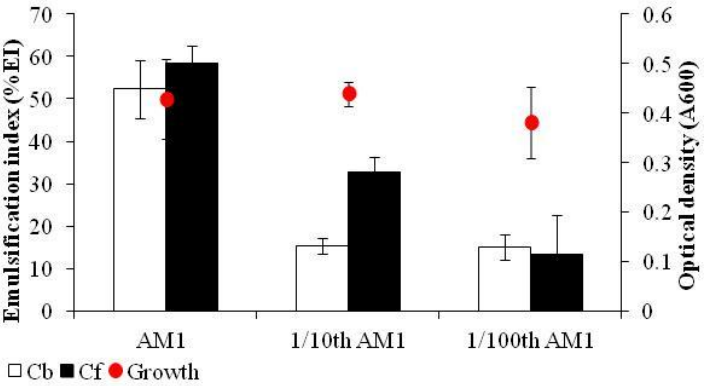

Figure 3.14. Effect of dilution of AM1 medium on bioemulsifier production and growth of *S. silvestris* AM1 (bars Cb = cellbound %EI and Cf= cell free %EI dot-marker= growth in O.D.)

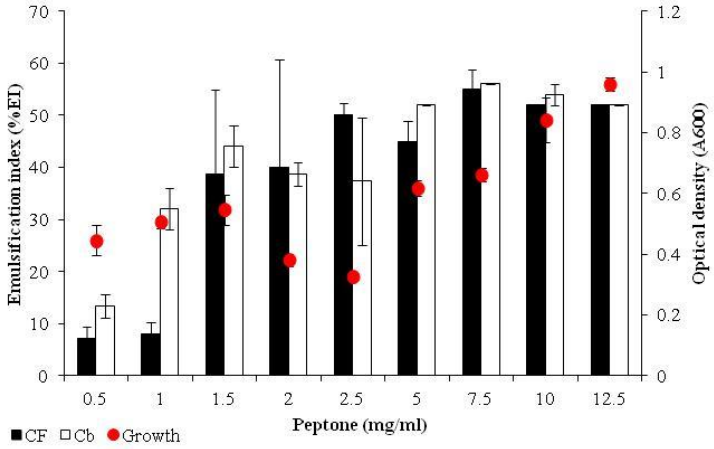

Figure 3.15. Effect of different concentration of peptone supplemented in ZM salt solution on bioemulsifier production and growth of *S. silvestris* AM1. (bars Cb = cellbound %EI and Cf= cell free %EI dot-marker= growth in O.D.)

3.3.3. Time course production of bioemulsifier AM1 :

S. silvestris AM1 reached its stationary phase of growth in 12h in ZM medium (figure 3.16). Here the cell-bound emulsification of *S. silvestris* AM1 started to increase after 4h. But the cell-free bioemulsifier release started only at about 12h. The

production of bioemulsifier in ZM medium was not growth associated and seemed to be a secondary metabolite of *S. silvestris* AM1. The bioemulsifier production reached both cell free and cell bound maximum of 62.5% at 22-24h.

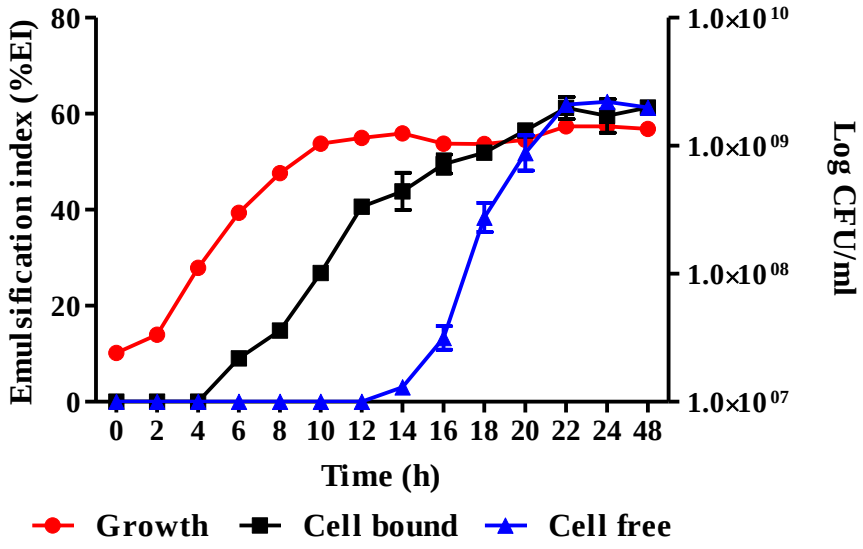

Figure 3.16. Time course bioemulsifier production by *S. silvestris* AM1 in Zobell marine medium

In AM1 medium the both cell bound and cell free bioemulsifier shot up at 8h coinciding with and in mid -logarithmic growth phase (Figure 3.17). The production of bioemulsifier was growth associated here. Maximum bioemulsifier emulsification of 64% was obtained at 22 -24h of growth as in ZM medium. However the main difference in the bioemulsifier production in ZM and AM1 media was manifested at 48h where in the latter %EI attained was 68% which was 62% in ZM medium.

In ZM medium the bioemulsifier production was growth associated and released later which seemed to be well in the stationary phase of the culture. While in AM1 medium the bioemulsifier production is cell bound and was released simultaneously as it is produced not associated with growth profile of the organism. Thus, in presence of production enhancing medium components, the cell-bound bioemulsifier expression is delayed by 4h, but, cell-free bioemulsifier release wass preponed by 6h.

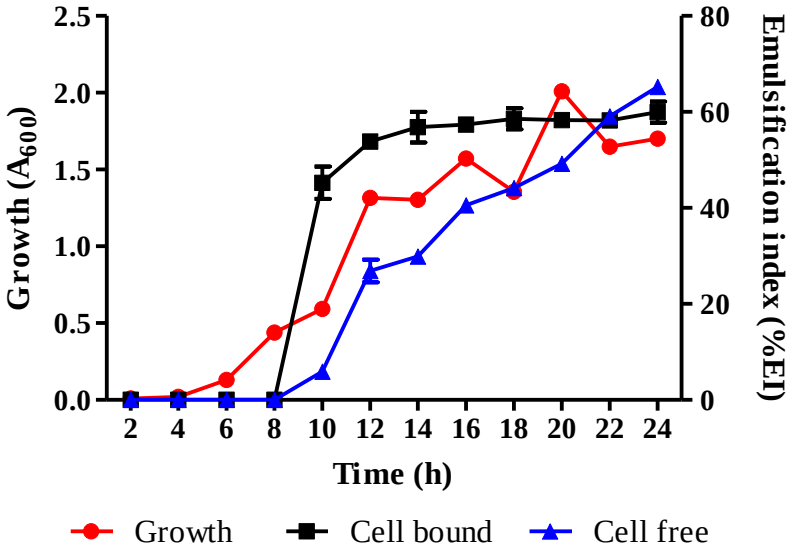

Figure 3.17. Growth (absorbance at 600nm) and emulsification (cell bound and cell free %EI) with respect to time is shown in AM1 medium.

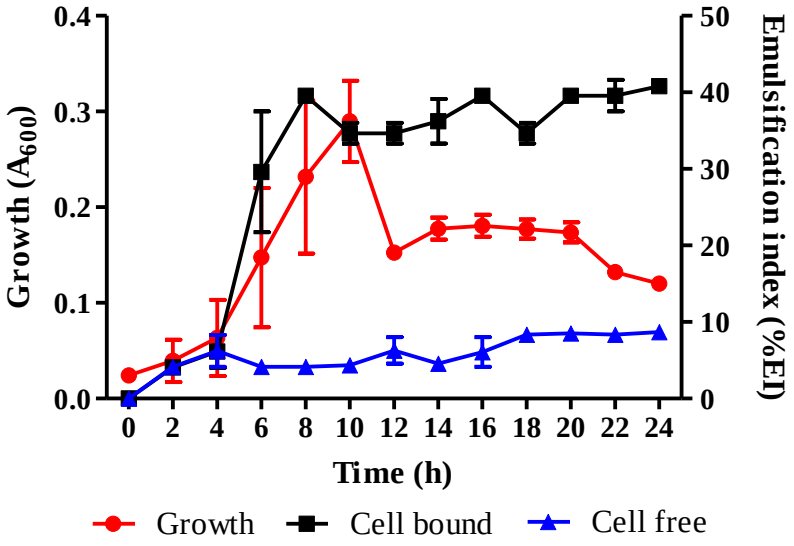

Figure 3.18. Time course production of bioemulsifier by *S. silvestris* AM1 in low peptone supplemented ZM salt solution.

In comparison to growth in ZM medium or AM1 medium, the growth of *S. silvestris* AM1 was significantly low ($p < 0.0001$) in low peptone conditions. After 12^{th} h of growth, the OD drops drastically probably due to exhaustion of the nutrients. The cell bound emulsification shot up at 4h and was growth associated. It was maximum of about 40% at about 8h after which remained more or less constant upto

24h.Highest cell free %EI observed in low peptone conditions was very low of 13.3%. The cell free emulsification was however comparable with AM1 and ZM medium even after 24h in low peptone medium.

Rajasegar, *et al.* (2002) reported the rapid increase of shrimp farms (42 till 1995, covering an area of 150ha.) along the vellar estuary. The feed pellet given to the shrimps have become the major source of eutrophication in the estuary. This release effluent from the shrimp farms into the backwaters, estuaries and mangroves affected the biological communities and has become a major concern for environmentalists (Rajasegar, *et al.*, 2002, Sundaramanickam, *et al.*, 2008). This may explain the presence of peptone dependent bioemulsifier production by *S. silvestris* AM1 as it was isolated from Vellar estuary.

Shoham, *et al.* (1983) reported Emulsan accumulates on the cell-surface of *A. calcoaceticus* RAG-1 as a mini-capsule during logarithmic phase of growth and like many other extracellular polysaccharides, it is produced after the cells reach stationary phase, the results correlate with bioemulsifier production in *S. silvestris* AM1. The time difference between the expression of cell-bound bioemulsifier and the activity of bioemulsifier in the supernatant can be attributed to possible saturation of the surface of the bacterium with bioemulsifier. As the lesser significant components of ZM medium were eliminated as in AM1 medium, the bioemulsifier release by the bacterium is changed effectively. Release of cell-free bioemulsifier is decreased by 8h (Figure 3.15), while the lag phase of the bacterium is increased by 2h.

In natural conditions, due to competition for nutrients, bacteria produce more cell-bound bioemulsifier than cell free. The cell free emulsification shown was at the basal level, but for the emulsification activity to be visible, the concentration of bioemulsifier should exceed the CMC level.

3.3.4. Growth and production of bioemulsifiers in xenobiotics:

Petroleum hydrocarbons and Polychlorinated compounds are widespread pollutants in the environment and possess known or suspected toxic, mutagenic and carcinogenic properties. The biodegradation of these compounds has been studied intensively and many bacterial strains have been isolated for their ability to degrade and use them as sources of carbon and energy (Luz, *et al.*, 2004). Vellar estuary, the original habitat of *S. silvestris* AM1 received effluents from different anthropological

sources. The four irrigation channels that drain pesticides and hydrocarbons into the Vellar estuary, contaminate the area and microbes isolated from this area are tolerant to these xenobiotics (Rajasegar, *et al.*, 2002). The organic carbon, total nitrogen and phosphorus content also varied with respect to the textural type of the sediments and the prevailing season (Rajasegar, *et al.*, 2002). Significant increase in hexachlorocyclohexane and other pesticide levels is reported in Vellar estuary during the wet season corresponding to the time of increased agricultural use of pesticides (Sarkar, *et al.*, 2008), thus these were studied.

3.3.4.1. Effects of hydrocarbons on growth and bioemulsifier production of S. silvestris AM1:

Due to their toxic, mutagenic and carcinogenic properties, the fate of Polycyclic aromatic hydrocarbons (PAHs) is of great concern and a major portion of which is carried out by the microorganisms in nature. naphthalene is a low molecular weight PAH and a large number of strains are known to metabolize or co-metabolize it (Bastiaens, *et al.*, 2000).

As shown in figure 3.19, for *S.silvestris* AM1 growth and bioemulsifier production do not get altered at higher concentration of naphthalene such as 50µg/ml. Beyond 30µg/ml concentration, the bioemulsifier production started gradual decrease and it can be seen that naphthalene concentration used here is not toxic for the organism as growth remained unaffected. Bioavailability of PAHs to microorganism is mainly limited by its low water solubility in environment and they tend to absorb on few organic matter (Bastiaens *et al*. 2000). Bioemulsifier are known to uptake such carbon sources with high affinity due to its amphipathic nature (Satpute, *et al.*, 2010). Thus bioemulsifier produced by *S.silvestris* AM1 possess the potential of naphthalene sorption present in environment.

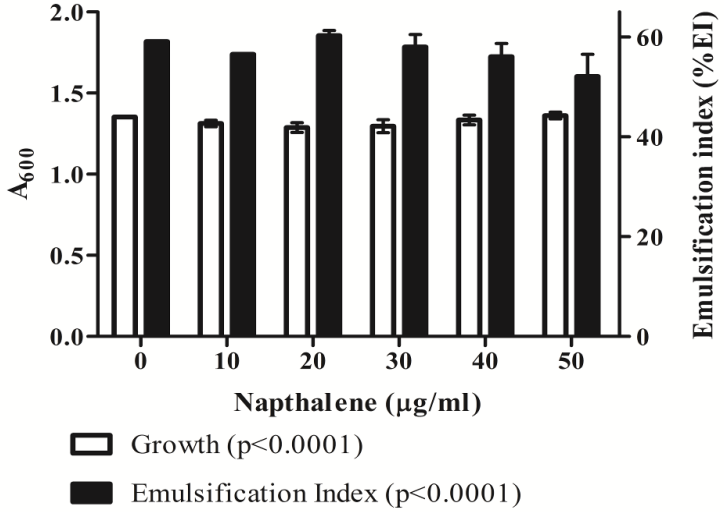

Figure 3.19. Growth and %EI of *S.silvestris* AM1 in presence of naphthalene.

Various organisms like *P.putida*, *Sphingomonas* sp., and *Mycobacterium* sp. are known to degrade such low-molecular weight PAHs in the environment. It was observed that they can grow on such carbon sources mainly due to their hydrophobic cell surface properties and presence of biosurfactant (in this case, a bioemulsifier) helps in increasing hydrophobicity and hence availability of less water soluble molecules to the organism (Guo, *et al.*, 2010). Aerobic microbial dehalogenation of chloroaromatics is generally observed in environment. *Pseudomonas* and *Nocardia* spp. performs hydrolytic dechlorination and utilizes such components (Rehm & Reed, 2009).

Figure 3.20, shows that at lower concentration of toxic trichlorobenzene (TCB) such as 72.5ng/ml, growth of *S.silvestris* AM1 doesn't get inhibited when compared with the growth in absence of TCB. But as the concentration of TCB increases in medium it gradually affects the growth. Bioemulsifier production was completely inhibited in presence of TCB. Even at lowest concentration of TCB no bioemulsifier production was observed. Generally halogenated xenobiotics, environmental pollutant are known to be degraded by anaerobic bacteria. Thus *S.silvestris* AM1 may not be able to perform such hydrolytic reaction to tolerate presence of TCB in the environment.

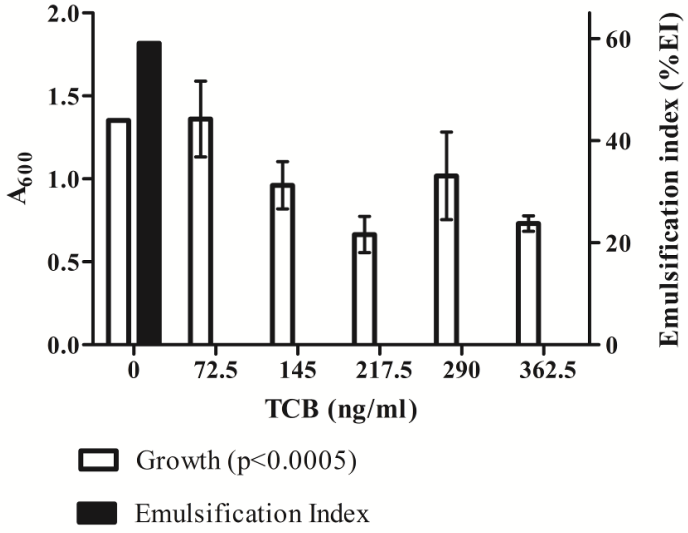

Figure 3.20. Growth and %EI of *S. silvestris* AM1 grown in presence of trichlorobenzene.

Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA) 2005 ranks benzene third in its priority list of Hazardous Organic Substances (Da Silva & Alvarez, 2007).

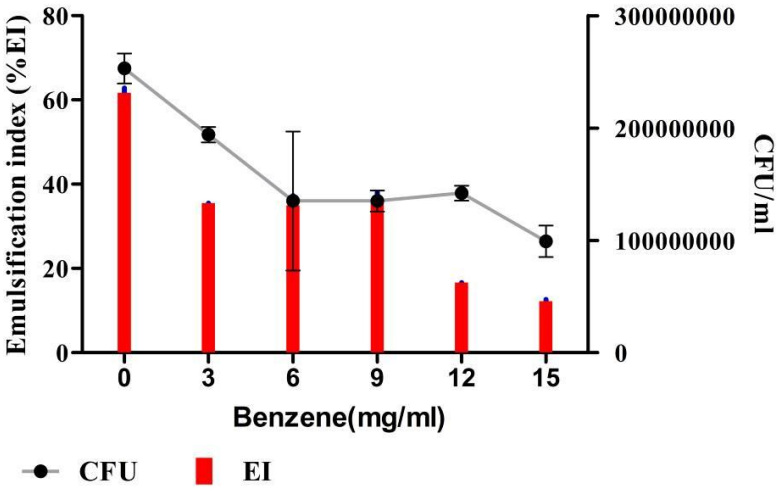

Figure 3.21. Growth and %EI of *S.silvestris* AM1 in presence of benzene.

Growth of *S.silvestris* AM1 decreases with the increased concentration of benzene and EI was also reduced along with growth (Figure 3.21). The predominant degraders of organo-pollutants in the oxic zone of contaminated areas are chemo-organotrophic species able to use a huge number of natural and xenobiotic compounds as carbon sources and electron donors for the generation of energy. Various Gram-

negative spp. like *Acinetobacter* spp., *Pseudomonas* spp. and Gram- positive organism like *Arthrobacter* spp. and *Bacillus* spp. are known to degrade long chain alkanes by oxygenation and ring- cleavage of xenobiotics (Rehm & Reed, 2009, Liu, *et al.*, 2010). *Bacillus* spp., *Pseudomonas* spp., and *Acinetobacter* are known to degrade benzene efficiently. Benzene degradation generally follows BTX degradation pathway, in which catechol is an important intermediate. Organisms like *Pseudomonas putida* and *Rhodococcus* spp. can grow in presence of such high concentrations (Liu, *et al.*, 2010).

Meta-Cleavage Dioxygenase assay was performed according to Tuah *et al* (2009) and no colour change was observed on ZM agar plate suggesting the catechol degradation was via ortho-cleavage pathways. From Silver-mirror test it was confirmed that acetaldehyde in the supernatant of culture grown in presence of catechol for 48h. Thus it may be possible that *S.silvestris* AM1 might degrade catechol via meta-cleavage pathways. Further confirmation is required to confirm the ability of *S.silvestris* AM1 to degrade catechol and it mechanism.

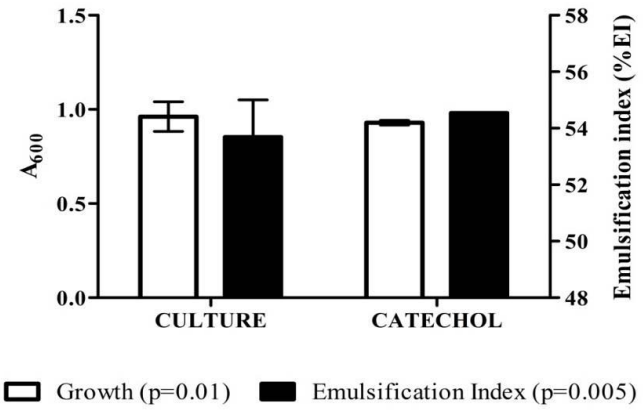

Figure 3.22. Growth and %EI of *S. silvestris* AM1 in presence of catechol.

Figure 3.22 shows catechol does not affect growth as well as emulsification of *S.silvestris* AM1. Thus results show that high concentration of benzene does inhibit growth of *S.silvestris* AM1. Catechol is one of the mono aromatic compound and important intermediate of BTX degradation pathway. Co-metabolism in natural environment is one of the important aspects for the survival of microorganisms. Thus tolerance to catechol also suggests that if *S.silvestris* AM1 does not utilize benzene itself but can grow on the component present due to degradation of benzene by other surrounding organisms present in niche.

3.3.4.2. Effects of pesticides on growth and bioemulsifier production of S. silvestris AM1:

Excessive use of pesticides in agriculture has led to several problems in environment. Biological degradation of such compound is best alternative to reduce the toxicity produced by pesticides in fields as well as marine coast. Several reports suggest that many organism like *Paenibacillus sp*.D1, marine *Cyanobacterium P.valderianum* shows growth in presence of various pesticides (Singh, *et al.*, 2009).

Widely used water soluble pesticides in field like organophosphate insecticide- acephate, carbamate insecticide-methomyl and pyrethroid insecticide-cypermethrin were taken into consideration for this study. These show toxicity to large number of marine organism and mammals. Concentration of pesticide for the study was decided on the basis of its Required Field Concentration (RFC) and its water solubility as given in table 3.3 (Singh *et al.*2009).

Acephate is a member of organophosphate group of insecticides and currently used in industrialized and developing countries. It is also known as acetylcholine esterase inhibitor and thus display neurotoxicity in mammals and insects (Singh *et al.*2009).

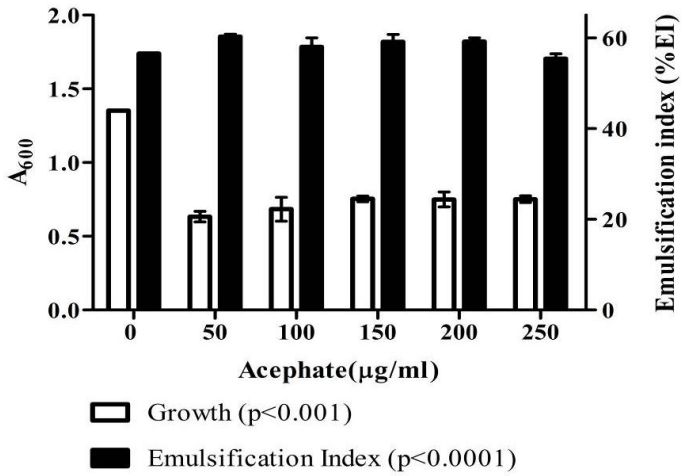

Figure 3.23. Growth and %EI of *S.silvestris* AM1 in presence of Acephate.

S.silvestris AM1 can tolerate higher concentration of acephate present in its environment (Figure 3.23) and comparatively less growth as compared to control but production of extracellular bioemulsifier was not altered. Although acephate does not affect production of bioemulsifier it may be toxic to some extent to *S.silvestris* AM1. Singh, *et al.* (2009), reported that growth and production of chitinase from

Paenibacillus sp. D1 is not affected by acephate significantly. Concentration of acephate used for study with *S.silvestris* AM1 was taken higher than its Required Field Concentration (RFC). Thus at high concentration of 250 µg/ml, production of bioemulsifier suggest that acephate might be working as a stimulator for production of bioemulsifier. In such way it may enhance bioavailability of acephate in the niche environment.

Methomyl is a carbamate group of insecticide which works as acetylcholine esterase inhibitor. *S.silvestris* AM1 showed reduction in growth in presence of methomyl (Figure 3.24). While comparing it with control, even at the lower concentration of methomyl used (100µg/ml), drastic change in growth of the organism was be observed. Production of bioemulsifier was also seen to be getting reduced as the concentration of methomyl increases. Concentration range used for this study that is 100-500 µg/ml is higher than RFC. Methomyl is known as toxic for various terrestrial and marine organisms. Study with *Paenibacillus sp.*D1 shows that, it does not reduce growth significantly of it (Singh, *et al.*, 2009). As shown in table 3.33, the RFC for methomyl is 60 µg/ml and the results here indicate the higher release of bioemulsifier in presence of a toxic compound (above its minimum toxicity level)shows the ability of *S. silvestris* AM1 to tolerate the higher toxic compounds by release of bioemulsifier AM1.

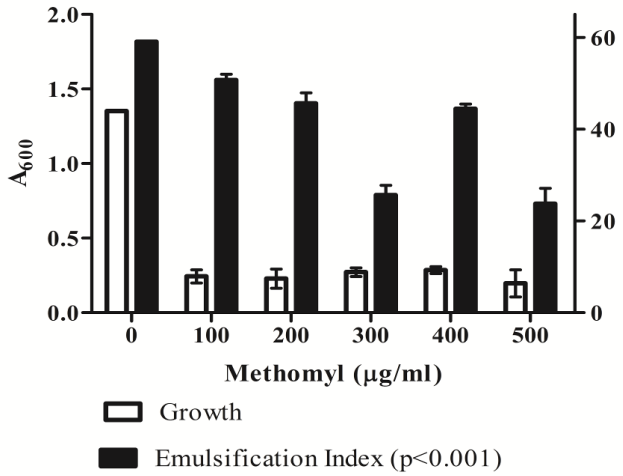

Figure 3.24. Growth and %EI of *S.silvestris* AM1 in presence of methomyl.

Cypermethrin is a member of pyrethroid insecticide group and functions as sodium channel modulater and is known for neurotoxicity. This insecticide is also known to affect nitrification and nodulating bacteria (Singh *et al.*2009).

Growth of *S. silvestris* AM1 is affected positively in presence of cypermethrin with increasing concentration (Figure 3.25). But this growth was relatively less while comparing it with control. No bioemulsifier production could be observed in presence of cypermethrin. Thus cypermethrin shows no toxicity towards *S.silvestris* AM1 but inhibits production of bioemulsifier completely.Study with other organism like *Paenibacillus sp*. D1 was observed in presence of cypermethrin, it shows complete inhibition of growth (Singh *et al.*2009).

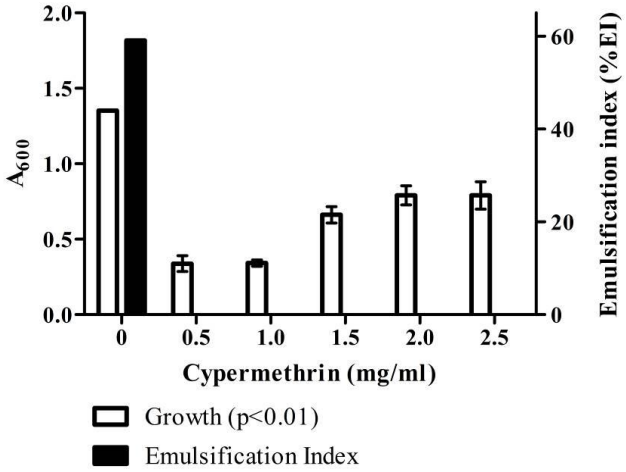

Figure 3.25. Growth and %EI of *S.silvestris* AM1 in presence of cypermethrin.

Efficient bioemulsifier production by *S. silvestris* AM1 was observed after 48h in ZM medium at 35°C and a pH of 7.5. The bioemulsifier production is initially cell bound which is exhibited at 6h and is released into the environment at 16h.

Thus from these studies, it can be asserted that the bioemulsifier production in *S. silvestris* AM1 is significantly enhanced and positively controlled by the presence of proteins in the medium. As discussed in previous chapter (Chapter 2), *S. silvestris* AM1 lacks the ability to utilize many major carbohydrates (section 2B.3.5) and is steadily evolving away from the type strain and members of the *Solibacillus* genera. Rajasegar, *et al.*, (2002), reported that Vellar estuary is riddled with rapidly growing shrimp and other aquaculture industries and feed pellet released by these ponds lead to periodic eutrophication. The data suggests that *S. silvestris* AM1, isolated from this area has evolved a mechanism to sustain on the protein rich area with ability to produce bioemulsifier even in oligotrophic conditions for proteins. The bacterium has evolved unique resistance to xenobiotics like hydrocarbons and pesticides and retaining its ability to produce bioemulsifier in many of their presence.

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Chapter 4

Biochemical and physical characterization of bioemulsifier AM1 and its emulsions

स्वभावसुंदरं वस्तु न संस्कारमपेक्षते।
मुक्तारत्नस्य शाणाश्मघर्षणं नोपयुज्यते॥
- दृष्टांत कलिका ४९

Meaning:
Simple and beautiful object does not need processing.
A freed pearl doesn not need to be polished again.
-Drushtanta kalika (49)

Chapter 4

Biochemical and physical characterization of bioemulsifier AM1 and its emulsions

4A. Biochemical characterization of bioemulsifier AM1

4A.1. Introduction

As introduced in the chapter 1, emulsifiers are a subclass of surfactants that stabilize dispersions of one liquid in another like oil-in-water (oil-in-water emulsions). They are high molecular weight polymers that bind tightly to surfaces. Microbial emulsifiers or bioemulsifiers are found to have wide spectrum of activity which is reflection of their complex chemistry as well as the diversity of bacterial genera producing them (Osterreicher-Ravid, *et al.*, 2001, Ron & Rosenberg, 2001, Abdel-Mawgoud, *et al.*, 2010, Satpute, *et al.*, 2010a, Satpute, *et al.*, 2010b).

Elucidation of the structure and chemical composition of a biomolecule is essential, as these characteristics dictate its function and interactions. Bioemulsifiers with various chemical compositions from eubacteria (including actinomycetes), archaea, algae, cyanobacteria, fungi and yeasts have previously been reported. Polysaccharides and their derivatives have been reported as bioemulsifiers in *Acinetobacter* (Kaplan & Rosenberg, 1982), *Klebsiella* (Bryan, *et al.*, 1986), *Candida* species (Cirigliano & Carman, 1984), *Pseudomonas* (Bonilla, *et al.*, 2005) *and Sphingomonas* species (Ashtaputre & Shah, 1995). Many marine bacteria isolated from Indian coast with emulsification activity have been reported previously and detailed analyses of these bacteria were also discussed in many reviews (Iyer, *et al.*, 2005, Kokare, *et al.*, 2007, Kumar, *et al.*, 2007, Nerurkar, *et al.*, 2009, Satpute, *et al.*, 2010b). Many of these bioemulsifiers reported were found to be derivatives of polysaccharides (Iyer, *et al.*, 2005, Kokare, *et al.*, 2007, Kumar, *et al.*, 2007). Carbohydrates conjugated with proteins or other hydrophobic moieties, such as lipids and fatty acids, that are capable of emulsification have been reported in *Bacillus* sp.(Gurjar, *et al.*, 2008, Suthar, *et al.*, 2009) and fungi such as *Trichosporon* sp.(de Souza Monteiro, *et al.*, 2012). Carbohydrates, lipids and proteins in different combinations from *Geobacillus* sp.(Zheng, *et al.*, 2011) and *Penicillium* sp.(Luna-Velasco, *et al.*, 2007) have been studied for their emulsification properties. Many

other emulsifying polymers, such as lipo-peptides, are found in *Bacillus* (Liu, *et al.*, 2010) and *Streptococcus* sp.(Jenkinson, 1992).

The bioemulsifiers reported from *Bacillus* strains FE-1 and FE-2, both isolated from fenthion contaminated soil produced a high molecular weight, lysozyme sensitive and thermostable glycolipopeptide bioemulsifier and another consisting of carbohydrate, lipid, and peptide respectively (Patel & Gopinathan, 1986). Cooper and Goldenberg (1987) isolated *Bacillus* strains IAF 343 and IAF 346 where the former produced a neutral lipid bioemulsifier, latter produced a polysaccharide bioemulsifier. A proteinaceous bioemulsifier with minor carbohydrate and lipid content was reported from *Bacillus stearothermophilus* VR-8 (Gurjar et al., 1995) and a *Bacillus* sp. CP912 was reported for homopolysaccharide bioemulsifier (Yun and Park 2000).

Bacillus amyloliquifaciens LP03 was produced antifungal lipopeptide surface active agent with high emulsification activity (Lee, *et al.*, 2007). In two separate reports, *Bacillus licheniformis* strains ACO1 and K125 were shown to be producing polysaccharide rich (Dastgheib, *et al.*, 2008) and polysaccharide-protein-lipid (Suthar, *et al.*, 2008) bioemulsifiers respectively. Kumar et al. (Kumar, *et al.*, 2007) reported a lipoprotein bioemulsifier from *Bacillus* sp. DHT resistant to high range of salinity and temperatures. Many reports where bioemulsifier producing *Bacillus* strain isolates were from contaminated soils (Toledo, *et al.*, 2008, Sathe, *et al.*, 2012, Klawech, *et al.*, 2013) and marine waters are available (Pavitran, *et al.*, 2006).

A few reports of proteinaceous bioemulsifiers exist those studied include a multi-protein complex bioemulsifier from *Methylobacterium* sp. (Joe, *et al.*, 2013) and *Methanobacterium thermoautotrophicum* (Trebbau de Acevedo & McInerney, 1996). Moreover, the studies on glycoprotein bioemulsifiers are rare, and the only reports are from *Pseudoalteromonas* sp.(Gutierrez, *et al.*, 2008) and *Antarctobacter* sp.(Gutiérrez, *et al.*, 2007).

In the present chapter in section A, the results of purification and physicochemical characterization of the bioemulsifier produced by the selected novel isolate *Solibacillus silvestris* AM1 are discussed. Additionally, a comparison of the emulsion formed by bioemulsifier AM1 with Emulsan from *Acinetobacter calcoaceticus* RAG-1 with varied solvents was characterized with respect to its stability. Further, in the second section B, the characters of the emulsion produced by bioemulsifier AM1 was studied.

Interesting observations regarding bioemulsifier AM1 showing amyloid nature aggregating into fibrils and noted in this work. Amyloid fibres are ordered protein aggregates generally associated with many neurodegenerative diseases like Alzheimer's, Parkinson's and various Prion diseases. The first of these bacterial functional amyloids reported, Curli fibres of *Escherichia coli* strains had a basic subunit of CsgA components while next to be discovered, thin aggressive fimbriae (Tafi) of *Salmonella* strains also showed similar subunits (Olsén, *et al.*, 1989, Collinson, *et al.*, 1991). Similar functional bacterial amyloids were reported from *Bacillus subtilis* made up of Tas A fibrils present as a mixture with exopolysaccharides released which stabilize the bacterium's biofilm (Soreghan, *et al.*, 1994, Gebbink, *et al.*, 2005, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). Other species of *Bacillus* genera also were reported subsequently for amyloid like proteins for varied cellular functions (Bowen, *et al.*, 2002, Wijman, *et al.*, 2007, Jordal, *et al.*, 2009).

As their production is reported from different microorganisms like, *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroides* and also in fungi with relatively no relation to each other at the amino acid level, there seems to be a preferential selection of amyloid curve during the evolution for various functions. Although list of bacteria capable of producing functional amyloids is increasing rapidly, there exists only few reports with respect to their function in environment (Romero, *et al.*, 2010, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). Among the few reported bacterial amyloids, many still need to be further studied in depth. Biophysical properties and ecological details of amyloids other than from *E. coli* and *Pseudomonads* and few other bacteria are still lacking (Nielsen, *et al.*, 2011). Here, an attempt to study the amyloid property of *S. silvestris* bioemulsifier AM1 was also studied.

4A.2. Materials and Methods

4A.2.1. Microorganisms, media and growth conditions:

(i) *Solibacillus silvestris* AM1 produced bioemulsifier in ZM medium and with peptone as the major carbon source as discussed in chapter 3. The strain was submitted to Microbial culture Collection Centre, NCCS, Pune, India (Accession No MCC 2096).

(ii) *Acinetobacter calcoaceticus* RAG-1 (MTCC 2409, ATCC 31012) was procured from Microbial Type Culture Collection (MTCC), Chandigarh, India. *A. calcoaceticus* RAG-1 was maintained on Luria agar. The production of Emulsan was conducted according to medium reported by Goldman, *et al.* (1982), in Erlenmeyer flasks at 30°C under shaking conditions for 72h.

4A.2.2. Emulsification index (%EI):

The emulsification index (%EI or $E_{24,}$ E_{48} and E_{72}) of the emulsion was calculated using methods discussed in Chapter 2, section 2A.2.5 (Cooper & Goldenberg, 1987).

4A.2.3. Purification:

Emulsion was prepared using culture medium containing crude bioemulsifier as described above. After 24 h, the emulsion was separated by centrifuging several times at 10000 rpm to remove oil. The major aqueous portion was filtered through a membrane filter with pore size of 0.2-µm and dialyzed (10 kDa cut-off) for 24 h using distilled water with a change of medium at every 6 h; then, the sample was passed through an ultrafiltration (UF) cassette (Amicon; OMEGA filters, Millipore Inc., USA) with a cutoff of 100 kDa. The retained material was washed 2-3 times with phosphate buffer (100 mM KH_2PO_4 and 100mM K_2HPO_4; pH 7.2 ±0.2) and collected in 5 ml buffer. This solution was then applied to a 50-cm gel filtration column with an inner diameter of 1 cm and a bed volume of 23.56 cm^3 filled with G200 (Sephadex, SIGMA chemical company, USA) using phosphate buffer, pH 7.2 ±0.2 as the mobile phase and a flow rate of 0.4 ml/min. After each purification step, the %EI was determined. After gel filtration chromatography (GFC), the active fractions producing emulsifier were pooled and lyophilized. The purified bioemulsifier AM1 was stored at 4°C for further studies.

4A.2.4. Biochemical characterization of Bioemulsfier AM1

4A.2.4.1. Carbohydrate and protein estimation:

Carbohydrate and protein content in the purified bioemulsifier were quantified using the methods of Dubois, *et al.* (1956) and Lowry, *et al.* (1951) respectively.

4A.2.4.2. Proteinase K treatment:

The bioemulsifier was treated with Proteinase K (10 mg/ml; Bangalore GeNei, Bengaluru, India) at 37°C for 30 min; afterwards, the %EI was determined as described above.

4A.2.4.3. Thin layer chromatography:

Bioemulsifier AM1 was examined for the presence of carbohydrates and lipid moieties using TLC. For this, it was then hydrolyzed for an hour with 1N HCl and run on silica gel 60 F_{254}plates (MERCK, Germany) in a chloroform–methanol–acetic acid–water system with ratios of 25:15:4:2 (Cooper & Goldenberg, 1987). α-Naphthol solution (1:1 of 10 mg α-Naphthol in 100 ml methanol and 5% H_2SO_4) was used to detect carbohydrates. For detection of lipids the bioemulsifier AM1 was run in the same solvent system and detected with iodine vapours (Suthar*, et al.*, 2008).

4A.2.4.4. High Performance Thin Layer Chromatography (HPTLC):

For HPTLC, purified bioemulsifier AM1 was hydrolyzed as described above and lyophilized. Methanolic solutions of hydrolyzed bioemulsifier (50 mg/ml) and standard sugars (glucose, galactose and ribose at concentrations of 1 mg/ml and fructose, rhamnose and xylose at concentrations of 2 mg/ml) were individually applied as 8-mm-wide bands (10 µl each) on the silica plates using an automatic TLC sample application device (Linomat V, CAMAG, Switzerland) with a constant flow of nitrogen gas. The same solvent system as the TLC was used, and HPTLC was conducted in a glass twin trough chamber (CAMAG, Switzerland). Plates were developed with an α-naphthol-H_2SO_4 reagent followed by heating at 110°C for 10 min and then scanning at 540 nm using scanner 3 (CAMAG, Switzerland), with integrated winCATS4 software.

4A.2.4.5. Glycoprotein staining:

The bioemulsifier was analyzed by native PAGE to study its glycoprotein nature. Samples were prepared by dissolving lyophilized bioemulsifier AM1 in 1% SDS (sodium dodecyl sulfate) with 0.01 M dithiothreitol (DTT). Equal volumes of 2 mM sodium phosphate buffer (pH 7.1) in 8 M urea were added to the sample to obtain the final SDS concentration of 0.5%. The sample (10 µl) was loaded onto a 12% native PAGE gel. Glycoprotein staining was performed as described by Segrest & Jackson (1972). Following overnight fixing with periodic acid-Schiff (PAS) fixative

(40:5:50 methanol: glacial acetic acid: water), the gel was treated with 0.7% periodic acid solution (in 5% acetic acid) for 2-3 h and then with 0.2% sodium metabisulfite solution (in 5% acetic acid) for 2-3 h. Subsequently, the gel was stained with Schiff's reagent for 12-18 h at room temperature and destained with PAS destaining solution (5:7.5:87.5 methanol : glacial acetic acid : water).

4A.2.4.6. LC/MS-MS:

The single largest band of untreated purified multimeric bioemulsifier with a molecular weight (MW) greater than 200 kDa was removed from the native PAGE gel and sent to the Centre for Genomic Applications (TCGA), New Delhi, India. The 30 kDa band of bioemulsifier subunit was removed from the SDS PAGE gel and sent to Centre for Cellular and Molecular Platforms (C-CAMP), Bengaluru, India for LC/MS-MS and Mascot analysis. This analysis was performed twice. The LC/MS-MS data received from all three samples were analyzed using Mascot.

4A.2.5. Physical characterization of Bioemulsifier AM1

4A.2.5.1. Fourier transform infrared (FTIR) analysis:

Bioemulsifier AM1 (1 mg) dissolved in deionized water was used to prepare a KBr pellet. FTIR spectra were recorded on a Shimadzu 8400S spectrophotometer. The spectra were obtained over the frequency range of 4000-500 cm^{-1} after 4 scans. Deionized water was used as a background control.

4A.2.5.2. Polarizing microscope:

The purified bioemulsifier AM1 (mg/ml) was resuspended in phosphate buffer (pH 7.3) spread into a smear on the clean grease-free glass slide and stained with 50µM congo red at room temperature for 1 min, washed with the buffer and distilled water in succession. The samples were then studied with Zeis Photomicroscope equipped with LEICA DM 2500P with 500X magnification (King, *et al.*, 1997).

4A.2.5.3. Circular Dichroism (CD) spectrum analysis:

A suspension of purified bioemulsifier AM1 (1mg/ml) was made in deionized water. CD spectra were recorded in the range of 195-250 nm wavelength on Jason J-108 (Japan) CD spectrophotometer at room temperature (26°C).

4A.2.5.4. SDS- PAGE analysis of denaturant treated purified bioemulsifier AM1:

Various denaturing substances (1-2% w/v), such as urea and dithiothreitol (DTT), and surfactants, such as SDS, Tween20, and Triton X100, were mixed with the purified bioemulsifier individually and incubated for 2 min with and without boiling treatment. The treated bioemulsifier was resolved using 12% SDS-PAGE, detected with silver staining and analyzed with AlphaEaseFC-v4.0 (AlphaInnotech, USA) software.

4A.2.5.5. Transmission electron microscope observations (TEM):

Purified bioemulsifier (500 µg) was applied to a carbon-coated grid for 1 min, stained with 1% uranyl acetate for 30 sec and subjected to transmission electron microscopy using JEOL JEM 2100 at Central Salt & Marine Chemicals Research Institute, Bhavnagar, Gujarat, India.

4A.2.5.6. Temperature, pH and salt stability of bioemulsifier:

Purified bioemulsifier AM1 (0.5 mg/ml) was examined for thermal stability by incubating in a boiling water bath (100°C) for 0.5, 1, 2, 3, 4 and 5 h. The same amount of bioemulsifier was dissolved in salt solutions of 1-5 M NaCl to examine its salt tolerance. Its pH stability was studied by preparing 0.5 mg/ml purified bioemulsifier AM1 solutions in standard buffers within the 3-9 pH range. The following buffers were used: pH 3-4 citrate buffer, pH 5-6 acetate buffer, pH 7 phosphate buffer and pH 8 and 9 Tris-Cl buffers. Bioemulsifier activity was examined by measuring emulsification, as indicated by %EI, before and after treatment in the conditions described. All tests were performed in triplicate and analyzed with one sample t-test using GraphPad Prism 5 software.

4A.2.5.7. Critical micellar dilution (CMD) measurement:

The minimum concentration of biosurfactant/bioemulsifier corresponding to the dilution at which micelles begin to form is referred to as its critical micellar concentration (CMC), which can also be expressed in terms of CMD (Makkar & Cameotra, 1997). The dilution of biosurfactant and bioemulsifier at which the %EI starts falling abruptly is the critical micellar dilution (CMD). In the absence of a direct assay test, the CMD can be used as a measure of the amount of bioemulsifier present in the original sample (Makkar & Cameotra, 1997) . The CMD can be estimated by diluting the cell-free supernatant with distilled water and measuring the %EI at

various dilutions (performed in triplicate). The previously reported bioemulsifier, Emulsan from *A. calcoaceticus* RAG-1, was used for comparison.

4A.2.6. Congo red-plate assay:

The method to detect the presence of amyloid proteins as shown by Romero, *et al.* (2010) was modified to observe the amyloid nature of the extracellular bioemulsifier produced by the colonies. Media used for the test were Bushnell Haas Medium (amended with 1% acetate as carbon source) and Zobell Marine medium both supplemented with congo red (25mg/l).

4A.2.7. Statistical analysis:

All experiments were conducted in triplicate and analyzed with ANOVA and t-test using GraphPad Prism 5 software.

4A.3. Results and Discussion

The results of biochemical and physical characterization of the bioemulsifier AM1 carried out to find out its chemical nature and other associated functional properties are discussed here.

4A.3.1. Charactrization of Bioemulsifier:

Production of a bioemulsifier by *S. silvestris* AM1 was most effective in ZM medium, and less or no bioemulsifier was produced in other reported production media (Pfiffner, *et al.*, 1986, Cooper & Goldenberg, 1987, Gurjar, *et al.*, 1995, Yun & Park, 2003, Suthar, *et al.*, 2008). The presence of protein in the medium was essential (Figure 3.1) for bioemulsifier AM1 production, as was also observed in the case of *B. stearothermophilus* VR-8 (Gurjar, *et al.*, 1995). A notable feature is that the *S. silvestris* AM1 produces a potent bioemulsifier in hydrocarbon-less medium, and no inducer is required. *A. calcoaceticus* RAG-1 produces Emulsan, which has been extensively studied and has been noted for requirement of ethanol for production of bioemulsifier (Patil & Chopade, 2001, Amiriyan, *et al.*, 2004). Crude bioemulsifier AM1, crude Emulsan, and 0.1% Xanthan were found to give 62.5%, 60% and 94% emulsification indices, respectively.

4A.3.2. Purification:

The culture supernatant containing bioemulsifier AM1 was dialyzed to remove residual salts from the medium. It was then concentrated using ultrafiltration cassettes

with 100 kDa cutoff. Following gel filtration chromatography (GFC) of the concentrate, the emulsification activity was found in 7-9th fractions of the flow through of a 30 ml gel column and was enhanced by 49.6-fold (Table 4.1). Gel filtration beads appeared to exclude the bioemulsifier because the emulsification activity was repeatedly observed in the flow through indicating that bioemulsifier AM1 was larger than 200 kDa.

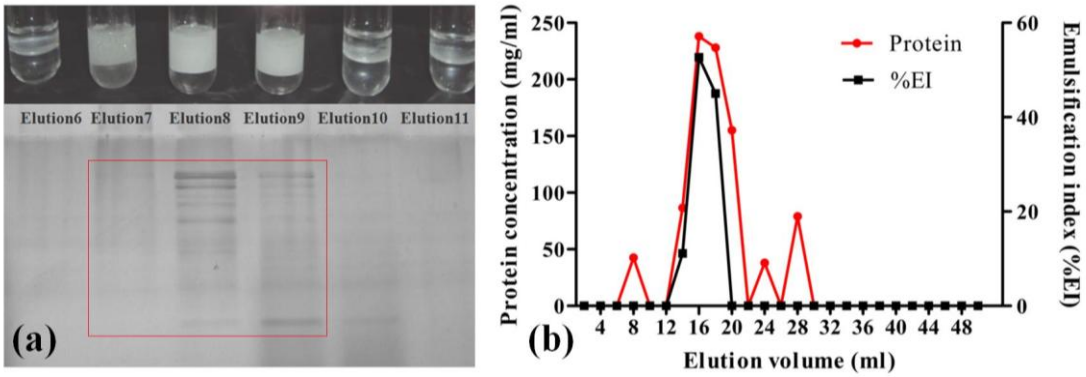

Figure 4.1. Gel filtration chromatography. (a) Emulsification index (%EI) and SDS PAGE of the eluted fractions obtained in gel filtration. **(b)** Elution profile of total proteins and emulsification activity (%EI) obtained from gel filtration chromatography G200 (Sephadex).

Table 4.1. Purification of bioemulsifier AM1

Purification stages	Total protein (mg/ml)	EI (ml^{-1})	Sp.Activity (EI/mg)	Fold purification
Crude	8.73 (±1.14)	61.8 (±0.69)	7.15 (± 0.86)	1
Dialysate	8.04 (± 0.53)	60.99 (±0.12)	7.60 (±0.51)	1.07 (±0.05)
Ultra filtration (UF)	3.50 (±0.34)	57.2 (±0.68)	16.4 (±1.8)	2.3 (± 0.02)
Gel filtration (GFC)	0.15 (±0.03)	52.38(± 0.25)	344.8 (±83.74)	49.6 (±17.79)

Values in parenthesis = Standard deviation (SD)

The elution profile of GFC is given in Figure 4.1 (a) and (b). Bioemulsifier from each of the active fractions was resolved into multiple bands using SDS PAGE (Figure 4.1a and b), and these bands could not be eliminated even after additional ultrafiltration and gel filtration steps. Surprisingly, an additional band of proteins of

less than 100 kDa were always visible in SDS PAGE, even after repeatedly recycling the filtrate through 200 kDa gel columns (Figure 4.2). Therefore, it can be inferred that the bioemulsifier eluted from the gel-filtration column must be a multimer that dissociated into smaller subunits during electrophoresis, appearing as less than 100 kDa bands (Figure 4.2).

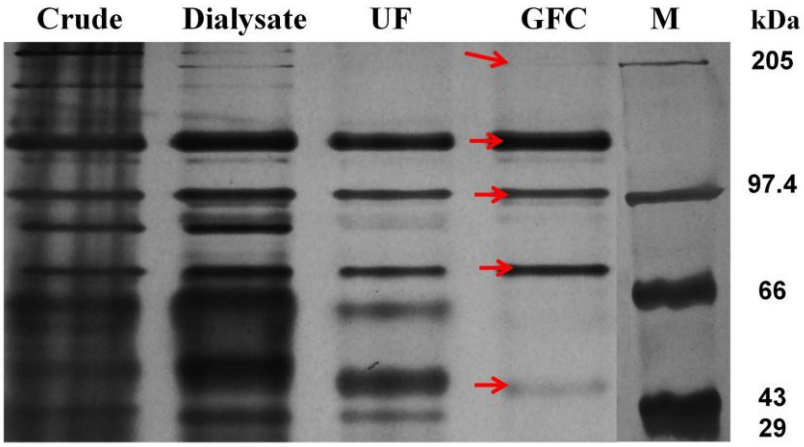

Figure 4.2. SDS-PAGE analysis of purification steps of bioemulsifier AM1: Lane 1- crude supernatant , Lane 2- dialysate, Lane -3 ultrafiltrate (UF), Lane 4- active fraction from gel-filtration chromatography (GFC) and Lane 5- molecular markers in SDS- PAGE (10%).

4A.3.3. Biochemical characterization of bioemulsifier AM1:

4A.3.3.1. Chemical analysis of bioemulsifier AM1:

The total carbohydrate content of purified bioemulsifier AM1 showed 36.5 µg of sugars per mg of protein. The carbohydrate component in this proteinaceous bioemulsifier is 3.6%, which is much less compared to the glycoprotein bioemulsifier reported by Gutiérrez et al. that contains 15.4% carbohydrate (Gutiérrez*, et al.*, 2007b). Thus, the bioemulsifier from *Solibacillus silvestris* AM1 is proteinaceous with a minor carbohydrate component.

4A.3.3.2. Proteinase K treatment:

Purified bioemulsifier AM1 lost its activity after Proteinase K treatment (Figure 4.3). Loss of bioemulsifier activity after enzymatic treatment indicates that it contains an active protein component.

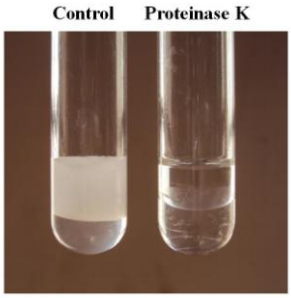

Figure 4.3. Emulsification activity of bioemulsifier AM1 after Proteinase K treatment

4A.3.3.3. Thin Layer Chromatography and HPTLC:

The purified bioemulsifier AM1 does not contain lipid, as no spot was observed in thin layer chromatography (Figure 4.4a). The solvent system described by Cooper and Goldenberg for TLC and HPTLC separated all the standard reported sugars effectively (Cooper & Goldenberg, 1987). The spots for crude bioemulsifier were observed near fructose on TLC but differed with respect to RF (Figure 4.4b). In figure 4.5 HPTLC densitogram of bioemulsifier AM1 along with standard sugars is shown in Figure 4.5. The software used for this analysis, winCATS, predicted the presence of galactose and xylose or ribose in the bioemulsifier AM1 with high probability.

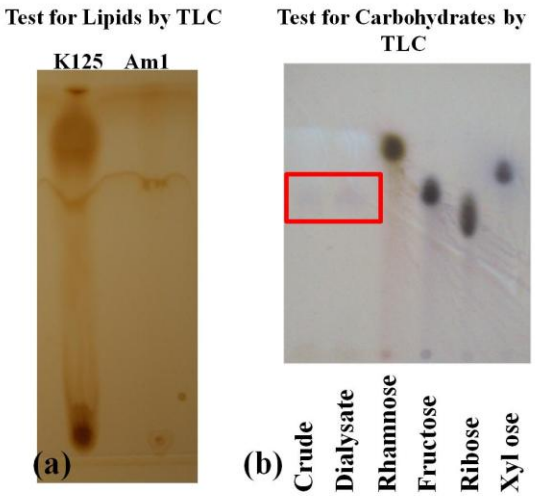

Figure 4.4. TLC of bioemulsifier from *S. silvestris* AM1 for (a) lipid, run with standard bioemulsifier from *B. licheniformis* K125 and (b) carbohydrate standards and bioemulsifier AM1. The red box indicated the spots for crude bioemulsifier

As seen in figure 4.5 the peak I of bioemulsifier AM1 coincides with galactose and peak II with ribose or xylose. Because ribose is a C3 epimer of xylose, these two sugars could not be distinguished using HPTLC. Similar protein-sugar conjugates are reported to be responsible for the stability of many extracellular bacterial proteins, such as flagellin (Schirm, *et al.*, 2004, Taguchi, *et al.*, 2010).

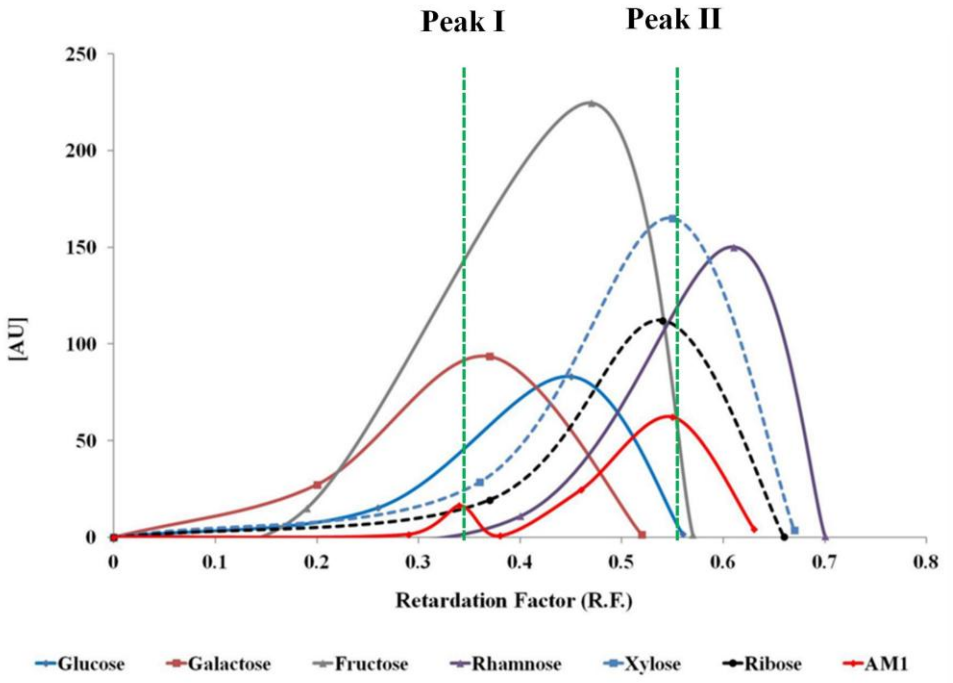

Figure 4.5. High performance thin layer chromatography (HPTLC) densitogram of bioemulsifier AM1 and standard sugars at 540nm. Peak I and Peak II represent the two peaks for sugars represented by bioemulsifier AM1.

4A.3.3.4. Carbohydrate analysis of the bioemulsifier AM1:

To determine the nature of the carbohydrate component of the glycoprotein bioemulsifier, native PAGE and further HPTLC was performed. A pink band detected by PAS staining was observed, corresponding to bioemulsifier subunits above the 100 kDa position in native PAGE, as shown in Figure 4.6.

Solibacillus sp. has not been reported to produce any extracellular proteinaceous bioactive molecule to date. Our work presents the first report of the production of bioemulsifier from *Solibacillus* sp.

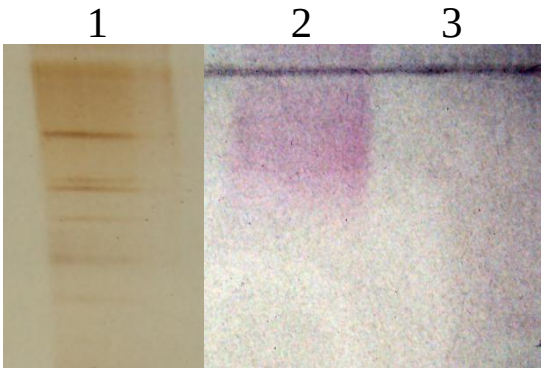

Figure 4.6. Native PAGE(12%) of active fraction GFC. Lane 1- silver staining , Lane 2- Periodic acid-Schiff's stain (PAS) staining for glycoprotein and Lane 3- control lane.

4A.3.3.5. LC/MS-MS:

Mascot analysis (Figure 4.7) of the LC/MS-MS spectrum of the single largest (200 kDa) band of purified bioemulsifier AM1 from Native PAGE provided the highest peptide score, matching two peptides: VIGPVVDVEFPR and FTQAGSEVSTLLGR of the β-subunit of F0F1 ATP synthase from *Corynebacterium diphtheriae* NCTC 13129 (NP_939413), as shown in Table 4.2a. Similarly, LC/MS-MS followed by Mascot analysis of a single band of 30 kDa provided a high peptide score, matching with three flagellin hag peptides (AGDDAAGLAISEK, INRAGDDAAGLAISEK and LGAYQNR) from *Bacillus halodurans* C-125 (FLA_BACHD). This analysis was performed twice to confirm the results (Table 4.2b).

As described by Hirose *et al.,* the fragment of flagellin AGDDAAGLAISEK is contained within the sequence INRAGDDAAGLAISEK (Hirose, *et al.,* 2000). These sequences show 94.44% homology with the flagellin sequence from *Solibacillus silvestris* StLB046, present in NCBI database with a few variations in the amino acids (Table 4.2b). According to Sakamoto, *et al.* (1992), the amino acid sequences of *B. subtilis* flagellin shows a pattern of similarities in the N- and C-terminal regions and dissimilarity in the central regions. *B. halodurans* C-125 flagellin, with which the peptide match for bioemulsifier AM1 was obtained, has a similar variable central region as observed in *B. subtilis* flagellin (Sakamoto, *et al.,* 1992).

Table 4.2. Comparison of amino acid sequence of peptides provided by LC-MS/MS and MASCOT analysis of the 200kDa (a) and 30kDa (b) proteins comprising bioemulsifier AM1.

	Analysed Peptide	Source	Organism	% Peptide similarity
(a)	VIGPVVDVEFPR FTQAGSEVSTLLGR	This study	*Solibacillus silvestris* AM1	100%
	VIGPVVDVEFPR FTQAGSEVSTLLGR	NCBI (NP_939413)	F0F1 ATP synthase subunit beta *Corynebacterium diphtheriae* NCTC 13129	100%
	V**M**GPVVDV**R**F**Q**S FTQAGSEVS**A**LLGR	NCBI (NP_244621)	F0F1 ATP synthase subunit beta *Bacillus halodurans* C-125	84.61%
	V**M**GPVVDV**K**F**AN** FTQAGSEVS**A**LLGR	NCBI (YP_006463939)	F0F1 ATP synthase subunit beta *Solibacillus silvestris* StLB046	80.77%
(b)	AGDDAAGLAISEK INRAGDDAAGLAISEK LGAYQNR	This study	*Solibacillus silvestris* AM1	100%
	AGDDAAGLAISEK INRAGDDAAGLAISEK LGA**M**QNR	NCBI (NP_244483)	Flagellin *Bacillus halodurans* C-125	97.22%
	AGDDAAGLAISEK **V**NRAGDDAAGLAISEK LGA**V**QNR	NCBI (YP_006463850)	Flagellin *Solibacillus silvestris* StLB046	94.44%
	AGDDAAGLAISEK INRAGDDAAGLAISEK LGAYQNR	NCBI (BAH80325)	Flagellin *Bacillus* sp. Kps3	100%

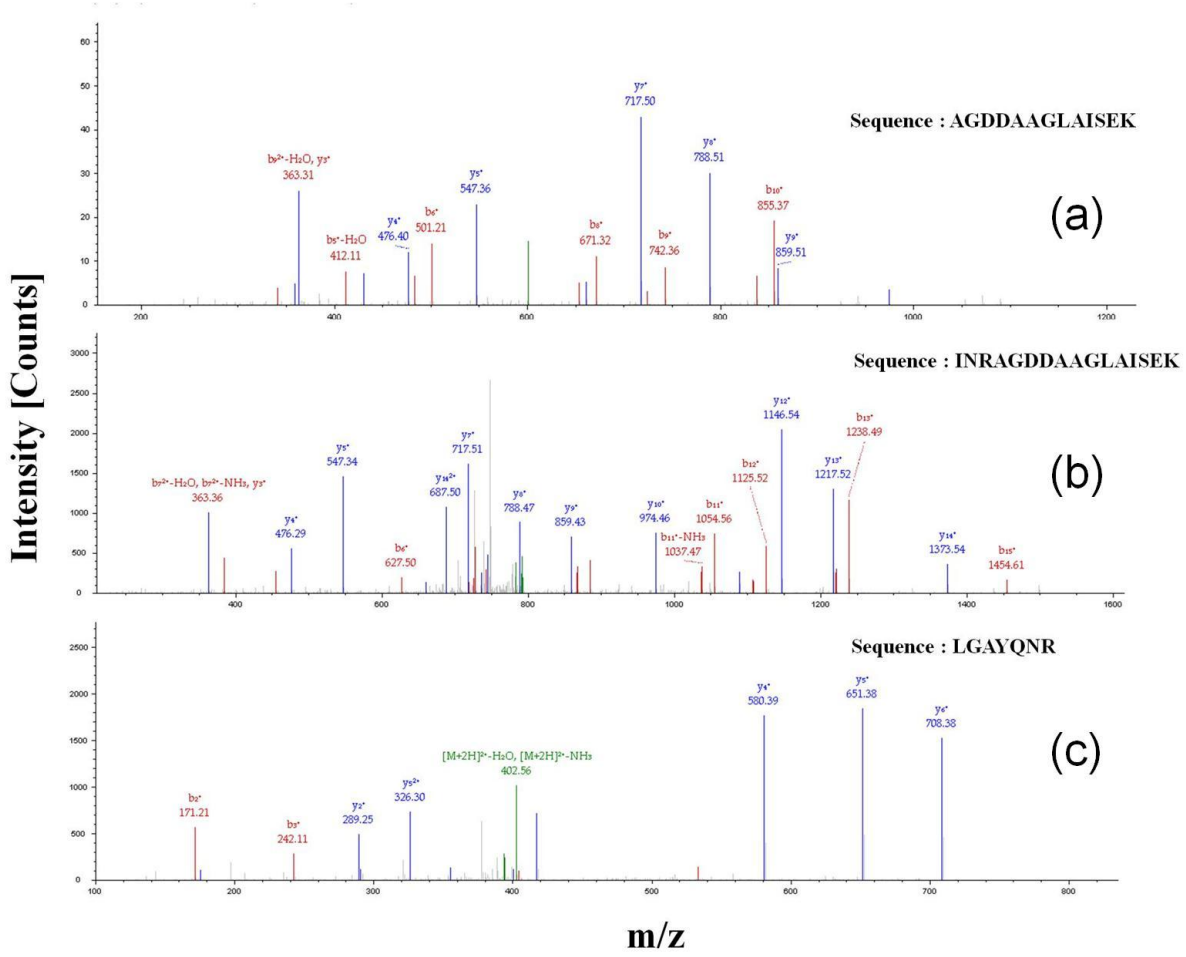

Figure 4.7. LC/MS-MS spectra of three peptides sequences (a, b & c) obtained from purified bioemulsifier AM1

Similar observations were reported for other sequenced flagellins. While conserved regions of the flagellins play an important structural role by forming hairpin loop structures, the central variable region forms the outer surface of the flagellin filament (Homma, *et al.*, 1987). Reports suggest that flagellins produced by bacteria inhabiting extreme environments are stabilized by their central regions. Albertini, *et al.* (1991), studied the entire complex of genes involved in flagellation and motility and reported an open reading frame (ORF) that translated into a protein with almost 30% identity to the β-subunit of *E. coli* ATP synthase. Ours is the first study reporting the similarity of a bioemulsifier to flagellin.

4A.3.4. Physical characterization of bioemulsifier AM1:

Functions of amyloids in bacteria are still not well described but seem to include fimbriae and other cell appendages for adhesion and biofilm formation, cell envelope components, spore coating, formation of large extracellular structures, amyloids acting as cytotoxins and probably several others, as yet unknown. Amyloids assembly is known to be depicted by proteinaceous surface structures of bacteria (Neilson et al. 2011), therefore bioemulsifier AM1 was subjected to various analysis performed for amyloids.

4A.3.4.1. Fourier transform infrared (FTIR) analysis:

FTIR spectroscopy was used to study the chemical structure of purified bioemulsifier AM1. Figure 4.8a represents the spectrum of purified bioemulsifier AM1 at 500-4,000 wavenumbers (cm^{-1}). Peak assignment was performed as per Jagmohan (2001), Suthar, *et al.* (2008). The FTIR spectrum in Figure 4.8a depicts –OH hydrogen bond stretching of 3400 cm^{-1} (a), –CH stretching of 2930 cm^{-1} (b), NH-CO stretching of 1690 cm^{-1}(c), -OH deformation bending of 1274 cm^{-1} (d) and C-O-C ester stretching of 1066 cm^{-1}(e).

In Figure 4.8b, FTIR spectrum of bioemulsifier AM1 from the amide I region of the spectrum (1600-1700 cm^{-1}) shows the peptide carbonyl stretching frequency, which is sensitive to the local conformation,(Jagmohan, 2001) and additionally, carbohydrate ester bond stretching was observed. This implies towards the glycoprotein nature of bioemulsifier AM1.

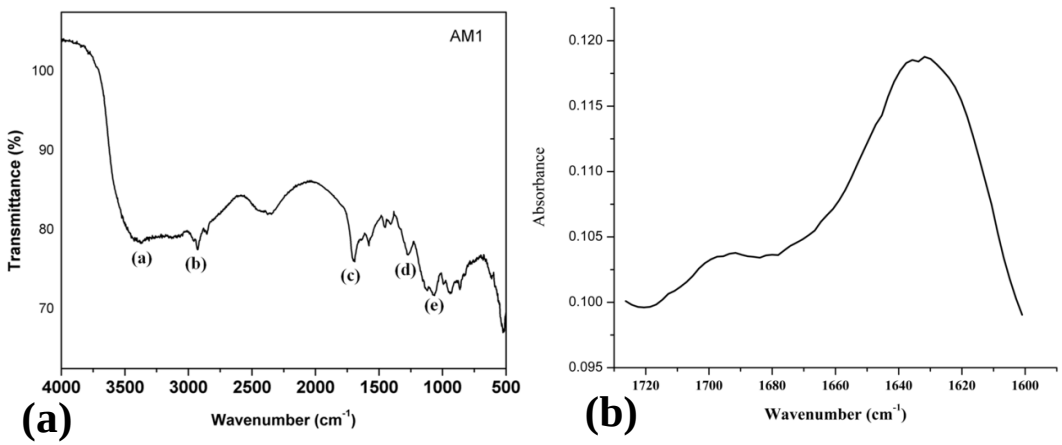

Figure 4.8. Fourier Transform Infrared (FTIR) Spectroscopy of the bioemulsifier AM1 depicted in (a) transmittance (%) for a range of 500-4000 cm^{-1} and (b) absorbance of amide I region for the region of 1600-1800 cm^{-1}.

Amyloid fibres are known to possess a a quaternary structure of characteristic cross-β sheets with β strands oriented perpendicular to fibril axis. Amphipathicity is also a common property seen in amyloids with some reported to be able to reduce surface tension(Soreghan, *et al.*, 1994, Gebbink, *et al.*, 2005, Wang & Chapman, 2008, Nielsen, *et al.*, 2011, Blanco, *et al.*, 2012). Although there have been reports of benign amyloid proteins, recent studies have revealed a new class of amyloid proteins extensively employed by microorganisms termed as "functional amyloids". Presence of characteristic amyloid-like *β* sheet structure of the bioemulsifier AM1 was supported by FTIR spectroscopy results. The major wavenumber was found to be around 1631 cm^{-1} and a minor peak of 1693 cm^{-1} was also seen which were suggested to be indicative for antiparallel *β* strands of an amyloid *β* sheet (Nilsson, 2004, Cerf, *et al.*, 2009, Zanetti Polzi, *et al.*, 2011).

4A.3.4.2. UV Cicular Dichroism (CD) spectrum analysis:

FTIR and CD spectra are usually used for secondary structure analysis of proteins. The far UV CD spectrum of purified bioemulsifier AM1 as depicted in figure 4.9, shows absorption minimum near 220-225nm. As per Jordal et al., (2009), this is a typical amyloid spectral property indicating a *β* sheet secondary structure in agreement with the expectations for the cross *β* amyloid fibrils. Therefore bioemulsifier AM1 can be positively said to have amyloid like structural property. These *β*-type spectral features are known to be predominantly associated with

filamentous structures and especially reported in amyloid proteins (Benditt, *et al.*, 1971, King, *et al.*, 1997, Fowler, *et al.*, 2005, Harada & Kuroda, 2010).

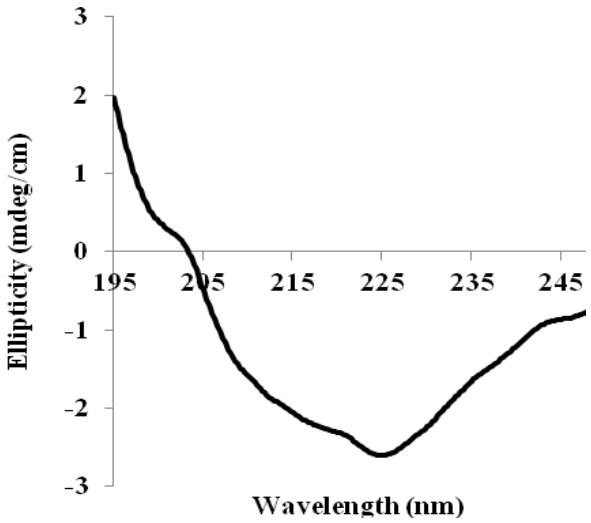

Figure 4.9. Circular Dichroism (CD) spectrum of the bioemulsifier AM1

4A.3.4.3. SDS-PAGE analysis of denaturant treated bioemulsifier AM1:

A multimeric protein, upon surfactant treatment, should dissociate into monomeric subunits, as was observed with bioemulsifier AM1. To further study this dissociation, bioemulsifier AM1 was treated with various denaturants. Only SDS treatment at high concentration was found to dissociate bioemulsifier AM1 into its individual subunits. At 2% SDS concentration, AM1 dissociated completely, as was shown by the appearance of a single band of approximately 30 kDa molecular weight (Figure 4.10).

High SDS concentration dissociated the multimers into individual subunits of 30 kDa, which was not observed with other denaturants, such as urea, dithiothreitol (DTT), Triton X100, Tween 20 and even low concentration of SDS (1%) with or without boiling (Figure 4.11). SDS disrupted the tertiary structure of the bioemulsifier and resolved the multimer into its monomeric subunits that were localized in a single band of 30 kDa. Upon boiling and 1% SDS treatment, the bioemulsifier separated into five distinct bands after SDS-PAGE: 28, 57, 83, 114 and 136 kDa in approximately 30 kDa increments. From these results, we can predict that protein bioemulsifier AM1 is a homopolymer composed of 30 kDa monomeric subunits. On the contrary, the disappearance of the bands observed in presence of complexing agents like

glutaraldehyde which can bind closely related proteins indicated strongly towards the multimeric nature that too a homopolymer of the protein (homo-multimer).

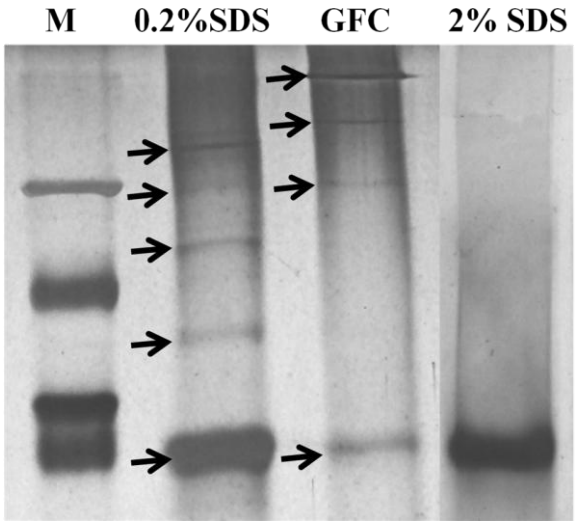

Figure 4.10. SDS-PAGE (10%) analysis of purified bioemulsifier AM1. Lane 1- Higher range protein marker (M) , Lane 2- 0.2% SDS treated active fraction of Gel filtration chromatography (GFC), Lane -3 active fraction of GFC, Lane 4- 2% SDS treated active fraction of GFC.

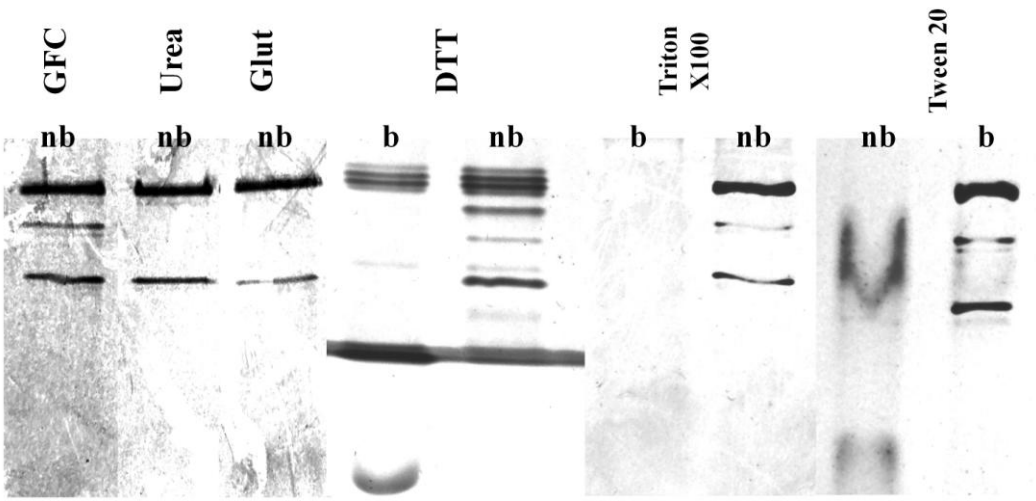

Figure 4.11. SDS-PAGE (10%) analysis of denaturant treated purified bioemulsifier AM1 (after treatment with urea, glutaraldehyde and also dithiothreitol (DTT), Triton X100 and Tween 20 with boiling (b) and without boiling (nb) in 10% SDS-PAGE)

4A.3.4.4. Transmission electron Microscopy (TEM) and Polarizing microscopy:

The glycoprotein bioemulsifier AM1 was checked for its aggregation properties. The electron microscopic structure of the bioemulsifier aggregate was found to be filamentous with ≈5nm width as seen in bacterial functional amyloids (Figures 4.12a and b). Two or more filaments could laterally associate to give a wider width than that seen in typical amyloid filaments.

After TEM, variety of techniques were used to confirm the amyloid nature of bioemulsifier AM1. Congo-red birefringence is considered to be one of the standard assays for amyloids (Wolman & Bubis, 1965). Bioemulsifier AM1 stained red with Congo-red dye and exhibited typical amyloid-like greenish-yellow to greenish-blue colour in polarized light microscope (Figure 4.13).

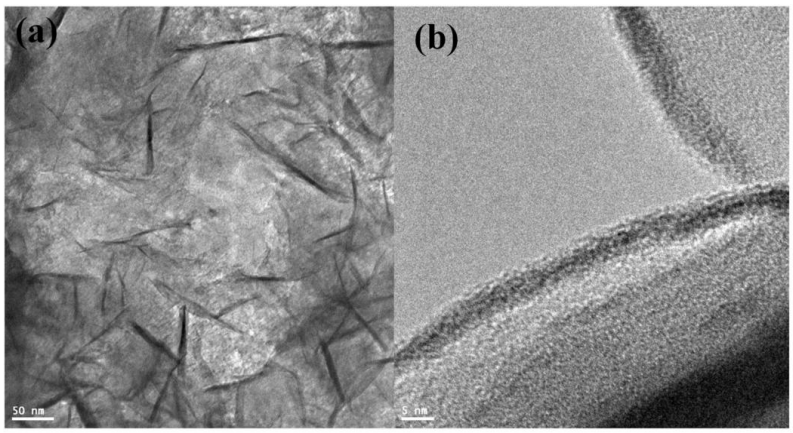

Figure 4.12. Transmission electron micrograph of purified bioemulsifier AM1 (a, bar = 50 nm; b, bar = 5 nm).

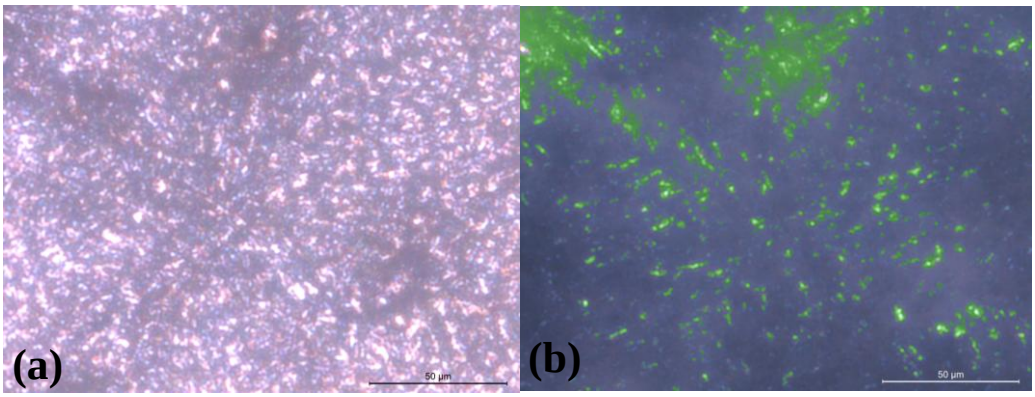

Figure 4.13. Polarizing microscope images of bioemulsifier AM1 stained with congo red. (a) Light microscopy and (b) polarization microscopy (bar = 50µm)

A total score of 4 must be achieved by satisfying a combination of the following criteria: β sheet secondary structure (score=2), congo red binding (score=2), denaturant or protease resistance (score=1), ThT or ThS binding (score=2), gel formation (score=1) and protofibril intermediate (score=1). Using these standard criteria and scoring technique set by Nilsson, (2004) for identification of amyloid fibrils, the bioemulsifier AM1 gets a score of 5 that is the first three criteria. With minimum score for 4 required to be achieved by the protein in question for catagorization into amyloid proteins, bioemulsifier AM1 can be considered as a glycoprotein amyloid with emulsification properties.

4A.3.4.5. Congored-plate assay

Bacteria producing amyloids on their surface are known to be taking up the congo-red stain when grown in its presence (Romero, *et al.*, 2010). As discussed, the bioemulsifier produced by *S. silvestris* AM1 has amyloid properties. *S. silvestris* AM1 is known to be producing cell bound and cell free bioemulsifier AM1 in protein rich medium like ZM medium and negligible or no bioemulsifier in medium containing no protein-carbon source. Figure 4.14a does not show the typical red coloration of the *S. silvestris* AM1 colonies when it produces bioemulsifier. When such media were amended with congo-red, the colonies producing bioemulsifier AM1 in ZM agar medium exhibited typical red colour (figure 4.14b), demonstrating the presence of amyloid bioemulsifier AM1 on their surface. Thus indicating amyloid like properties associated with the cell bound bioemulsifier.

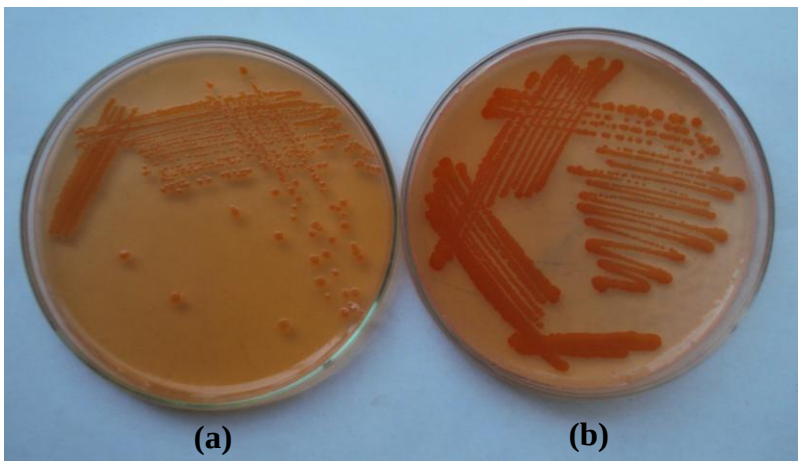

Figure 4.14. *S.silvestris* AM1 grown in (a) Bushnell Haas (amended with 1% acetate) medium and (b)Zobell Marine agar medium

4A.3.4.6. Temperature, pH and salt stability of bioemulsifier AM1:

As evident in Figure 4.15a, bioemulsifier AM1 is highly thermostable, retaining 33.81% E_{24} even after 5 h of treatment in boiling water (100°C). However, the emulsion formed was not stable after 24 h. The E_{24} remained between 55.1 and 40.5% after treatment with pH values ranging from 4 to 9 (Figure 4.15b). The emulsification activity was negligible below pH 4. The E_{24} of the bioemulsifier dropped from 47 to 30% after 2 M NaCl treatment but remained constant up to 5M NaCl treatment (Figure 4.15c). Nevertheless, the E_{24} of the bioemulsifier never dropped below its half-life in any of the conditions tested (other than pH 3).

The results of the experiments described here and in the previous section (SDS PAGE analysis) show that the bioemulsifier resists extreme temperatures, NaCl concentration, pH, and dissociation by detergents. Multimeric association of the emulsifier could be due to either hydrophobic interactions or to ionic, hydrogen or covalent bonds. Because the bioemulsifier AM1 multimer could not be disrupted completely by detergents, it may not be dependent on hydrophobic interactions. Additionally, it was previously observed that boiling in the presence of detergents results in the dissociation of the multimeric complex; therefore, we can conclude that hydrophobic interactions are only partially responsible for multimer stability, which was also reported by Law & Levine (1977). Stability in high salt concentrations and at high pH values indicates that inter-subunit interactions may not to be due to ionic or hydrogen bonds. Amyloid fibre formation and stability are reported to be also dependent on hydrophobic interactions (Marshall, *et al.*, 2011).

As described by Dimmitt & Simon (1971), purified flagellins are relatively stable compared to those obtained by shearing flagellar filaments. The variable region of flagellin from *B. halodurans* C-125 is hypothesized to give stability to the protein in the host's alkaline environment (Sakamoto, *et al.*, 1992). There exists a high probability that the resilience to NaCl concentration and pH, as well as to temperature (thermostability) is properties imparted to a bioemulsifier because of its primary structure bearing similarity to flagellin. The best match of the flagellin-like peptide of bioemulsifier AM1 with *B. halodurans* C-125 and *Bacillus* sp. Kps3 that are grown in extreme environments also supports this observation (Table 2.2).

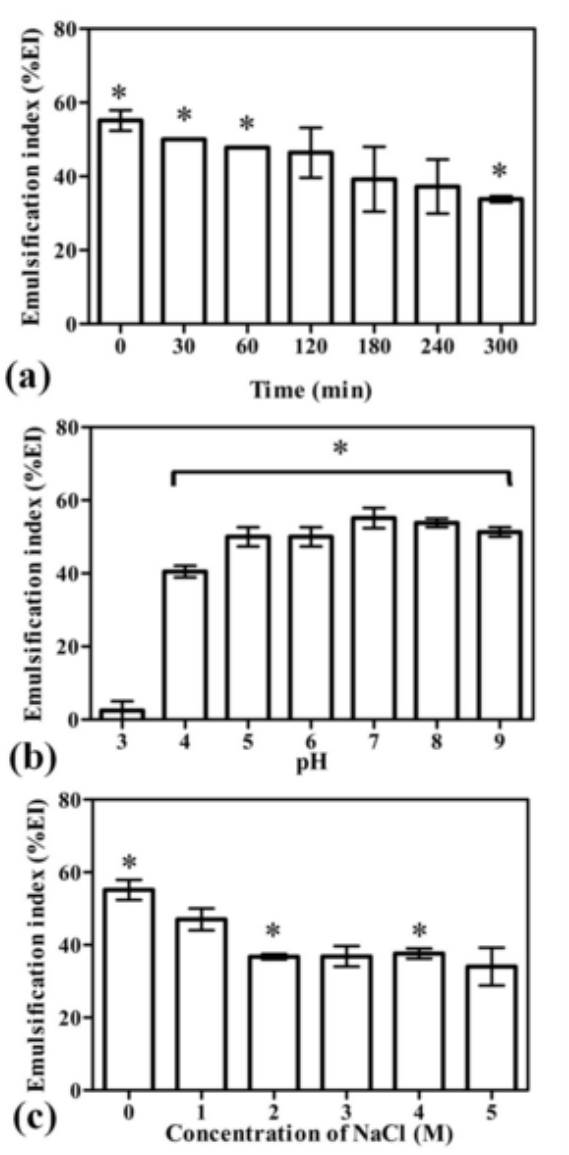

Figure 4.15. Stability of the bioemulsifier in (a) boiling-water-bath (b)pH; (c)NaCl concentration (*=p<0.001).

4A.3.4.7. Critical micellar dilution (CMD):

If the concentration of bioemulsifier produced by a bacterium is above its CMC, an increase in its concentration cannot be detected. Consequently, two cultures with very different concentrations of bioemulsifier may display the same activity. This problem can be overcome by serially diluting until a sharp decrease in emulsification is observed, in this case by measuring critical micellar dilution (CMD) (Walter, *et al.*, 2010).

As shown in Figure 4.16, the CMD^{-1} of bioemulsifier AM1 was twenty times diluted as compared to Emulsan which was diluted 6.66 times, i.e., a three times

higher dilution is required to reduce the activity to zero comparing to Emulsan. According to Oliveira, *et al.* (2006) and Makkar & Cameotra (1997), CMD is an indirect means of measuring the surfactant production relative to the CMC range. Thus, it can be inferred from Figure 4.16 that *S. silvestris* AM1 has significantly higher (P = <0.001) bioemulsifier production than *A. calcoaceticus* RAG-1.

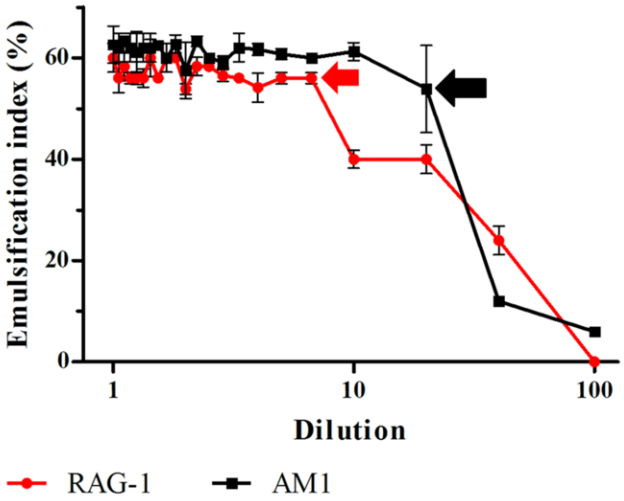

Figure 4.16. Critical Micellar Dilution (CMD) of bioemulsifier produced by *S. silvestris* AM1.

4B. Characterizatiion of emulsion

4B.1. Introduction

Dispersion, usually temporary mixture of two immiscible phases and if it lasts long, it is said to be a suspension. Thus the difference between dispersion and suspension is indistinct but it is known that more uniform the specific gravities of the two phases, and smaller the particle size of the dispersed phase, the dispersion lasts long. When the particle size is very small (colloidal), the system approaches the appearance of a solution. When there is a strong attraction between dispersing medium and the particles, the system is called an emulsoid. When this emulsoid (or Lyo-philic or showing affinity) requires a stabilizing (emulsifying) agent for greater/lesser stability, the colloid is called an emulsion (Harkins, 1947, Bennett, *et al.*, 1968).

Surface active compounds, when added into water, are adsorbed at the surface resulting in decreased surface tension. When the liquids are shaken, a large area free of surfactant is created. The molecules from the bulk solution, the air-water or oil-water interfaces adsorb and cover the equilibrium monolayers and prevent bubbles and droplets from coalescence, thus forming emulsions and foams. Depending on the laboratory method used, the interfaces are stretched rapidly and ruptured in different flow conditions and forming emulsions. Hence different types of flow used lead to different rupture mechanisms. Surface rheology plays role in the formation of emulsion once the threshold for rupture is crossed in a well defined flow. The energy used during emulsification process is directly proportional to elasticity and affect emulsion stability. Thus the importance of emulsification is a direct result of high surface area to volume relationship. An emulsion can be defined as a dispersion of two mutually immiscible liquids usually containing an emulsifying agent (surface active agents) (Harkins, 1947, Bennett, *et al.*, 1968, Langevin, 2000).

Emulsions have been historically considered to be dispersion of oil and water. Hence emulsions are also classified into oil-in-water (O/W), water-in-oil (W/O) and dual emulsions. An oil-in-water (O/W) emulsion has oil as the internal phase and water as the external phase and can be easily diluted by adding more water at the cost of stability. A water-in-oil (W/O) emulsion consists of dispersion of oil external phase with water internal phase and cannot be diluted by water. By process of inversion,

simultaneous switching of internal and external phases occurs as a result of mechanical or chemical action. Thus the relative concentration of the phases drive the change and an emulsifying agent fairly soluble in both the phases can help in formation of these dual emulsions (Harkins, 1947, Bennett, *et al.*, 1968).

The particle size of an emulsion is one of its most important characteristics. It is the function of nature of the phases in mixture, their quantity, also type of the emulsifying agent and the processing method used. For an emulsion droplet, the most thermodynamically stable energy configuration in an emulsion is spherical as it minimizes the surface area thus reducing the surface energy of the particle. This same minimizing surface energy tries to stabilize the configuration of emulsion as a whole which is as two distinct phases with lowest thermodynamic energy levels. Hence the type and concentration of emulsifier should provide sufficient thermodynamic energy to maintain the particulate dispersion of the internal phase. Increasing the concentration of emulsifier in the emulsion will result in smaller particle size (Bennett et al., 1968).

Rheology can be defined as the study of the deformation and flow of matter. In rheological terms, the tendency of a fluid to flow is called fluidity and the measure of its resistance to flow is called viscosity. Viscosity can also be defined as the force required to compel two parallel liquid surfaces of unit area separated by a distance of one differential unit to slide past each other with a constant unit of velocity. Thus viscosity is the factor of proportionality between rate of shear and shearing stress. When a fluid's flow is independent of the amount of shear, the fluid is termed as a Newtonian fluid while when a fluid has a viscosity as a function of shear and time of its application, it is called as a non-Newtonian fluid. A non-Newtonian fluid can be further classified into, plastic, pseudoplastic, thixotropic, dilatants and rheopectic. The shear stress of a plastic fluid should first exceed a minimum threshold value before it flows as seen in some synthetic resins. Pseudoplastic fluid viscosity decreases proportionally to the increase in shear rate irreversibly. The apparent viscosity of a thixotropic substance decreases reversibly with time to particular minimum value with constant increase in shear. Dilutant fluid's apparent viscocity is known to increase instantaneously with increase in shear rate as seen in starch in water while in rheopectic fluid, the viscosity increases to a maximum value at any constant rate of shear. The application of the emulsion industrially is dependent upon the emulsion's

rheological properties. Emulsion characteristics are important for its applications hence the characterization of emulsion formed by bioemulsifier AM1 was done (Bennett et al., 1968).

4B.2. Materials and Methods

4B.2.1. Comparative relative emulsion stability (%ES) in different solvents:

The emulsification activity of bioemulsifier AM1 was compared with Emulsan in 14 different solvents and was examined for up to seven days. Xanthan (0.1%) was used as a positive control. The following solvents were used: hexane (H), heptane (Hp), decane (D) and hexadecane (Hd), benzene (B), toluene (T), xylene (X) and trichlorobenzene (TCB), paraffin oil (Po), cottonseed oil (Co), groundnut oil (Go), silicone oil (So), white oil (Wo) and kerosene (K). Each test was performed in triplicate.

Relative emulsion stability (%ES) was calculated using the following formulae (Das, *et al.*, 1998, Kebbouche-Gana, *et al.*, 2009):

$$\%\ \textbf{Emulsion volume}\ (\%\textbf{EV}) = \frac{\text{Emulsion height (mm)} \times \text{Cross} - \text{section area (mm}^2\text{)}}{\text{Total volume of mixture (mm}^3\text{)}} \times 100$$

$$\%\ \textbf{Emulsion stability}\ (\%\textbf{ES}) = \frac{\%\text{EV at time } t \times \text{h}}{\%\text{EV at time 0h}} \times 100$$

4B.2.2. Bright-field microscopy of emulsion droplets:

Microscopic examination of emulsions formed with the previously listed fourteen solvents was performed using a bright-field Olympus microscope, model CX41, at 100X magnification. The images were analyzed using ProgRes CapturePro 2.7 imaging software (JENOPTIK optical systems, USA).

4B.2.3. Particle size analysis and Shear rate of the emulsion droplets:

Emulsions were prepared by homogenizing 0.5 mg/ml purified bioemulsifier with paraffin oil and TCB (1:1). Particle size analysis of the emulsion droplets was performed using Sympatec HELOS –BF particle size analyzer. Rheological properties of the emulsion, such as shear stress and shear viscosity, were measured by a Brookfield LV DV-III rheometer using CPE52.

4B.3. Results and Discussion

In this section, results of the characterization of emulsion produced by bioemulsifier AM1 are presented.

4B.3.1. Characterization of the emulsion formed by bioemulsifier AM1:

The relative emulsion stability (%ES) of bioemulsifier AM1 was found to be 89.7, 84.8, 81.7 and 79.32% with TCB, decane, groundnut oil and xylene, respectively, and these values were greater than those given by Emulsan (Figure 4.18). As shown, the best emulsion stabilization by bioemulsifier AM1 was in TCB, while the most negligible was in hexadecane. In hexane, heptane, toluene, silicone oil and paraffin oil, %ES values between 47.48 and 61.30% were found. In benzene, cotton seed oil, kerosene and industrial white oil, the relative %ES range was between 24.51 and 35.26%. The maximum relative emulsion stability of 107.7% was found to be Emulsan in silicone oil (Figure 4.18). Bioemulsifier AM1 showed stability in all types of solvent, whether aliphatic, aromatic or in oils. However, Emulsan showed better stability with oils and very low stability in aliphatic and aromatic solvents. The differences between all values of solvents used for bioemulsifiers AM1 and RAG-1 has a 0.37% chance of random occurrence in this experiment (p-value = 0.0037).

Bright-field microscopic studies of the emulsions formed by bioemulsifier AM1 corresponding to E_{24} and E_{168} in the solvents studied, showed a marked increase in droplet size, except in the case of TCB where there was a decrease in droplet size and in the case of hexadecane where no emulsion was formed (Figure 4.19). Droplet size variation was least in the emulsion with decane. Figure 4.19 shows benzene losing most of its smaller emulsion droplets after 168 h, while in trichlorobenzene, bigger droplets became less common after 24 h. Among oils, the emulsion droplet size increased in kerosene and white oil (Figure 4.20). The density of droplets increased in the emulsion formed at 168 h for stable emulsions, such as those formed in TCB and decane.

A bioemulsifier capable of producing a good and stable emulsion with all types of hydrophobic solvents (aliphatics, aromatics and oils) would be a versatile process material in industries. The results herein achieved are in accordance with the previous report that describes the inability of *A. calcoaceticus* RAG-1 to effectively emulsify low-molecular weight benzene derivatives, aromatic compounds containing

more than one ring, branched chain aliphatics from pentane to octadecane and unstable emulsions formed with kerosene and gasoline (Zuckerberg, *et al.*, 1979).

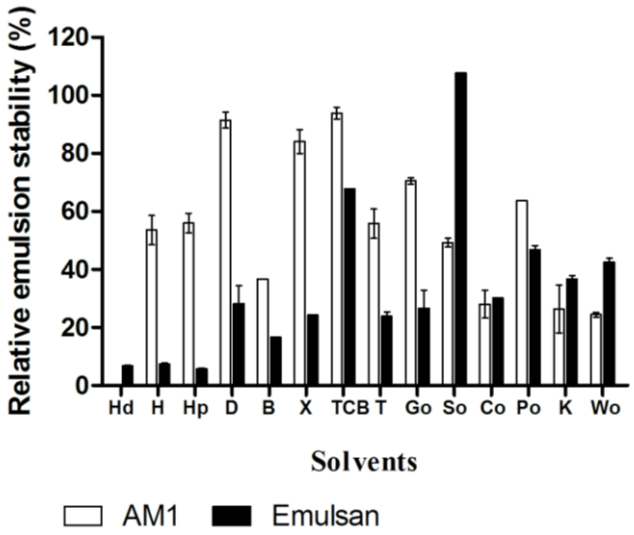

Figure 4.18. Comparative relative emulsion stability (ES%) of bioemulsifier from AM1 and Emulsan in 14 different solvents with respect to the standard emulsifier Xanthan. Hexane (H), heptane (Hp), decane (D) and hexadecane (Hd), benzene (B), toluene (T), xylene (X) and trichlorobenzene (TCB), paraffin oil (Po), cottonseed oil (Co), groundnut oil (Go), silicone oil (So), white oil (Wo) and kerosene (K)

Many reported organisms, such as *Planococcus maitriensis* Anita I (Kumar, *et al.*, 2007), *Antarctobacter* sp. TG22 (Gutiérrez, *et al.*, 2007) and *Penicillium citrinum* (De Morais, *et al.*, 2006), displayed good emulsification with oils. The yeasts *Torulopsis petrophilum* (Cooper & Paddock, 1983) and *Saccharomyces cerevisiae* (Cameron, *et al.*, 1988) were reported to produce compounds that emulsify in aliphatic and aromatic compounds. However, reports of microorganisms producing stable emulsions in all the three are rare. Thus, the ability of bioemulsifier AM1 to emulsify aliphatics, aromatics and oils is a significant property, and its efficiency can be increased further by optimizing the medium components and by yield enhancement.

4B.3.2. Particle size analysis and shear rate of emulsion droplets:

The particle size distribution patterns of emulsion droplets in paraffin and TCB are shown in Figure 4.21. A particle size shift was observed in TCB, while no

shift was observed in paraffin oil. Particle size of the emulsion with TCB was increased at 24 h (Sauter Mean Diameter, SMD= 127.69 μm) as compared to at 168 h (SMD= 97.47 μm), coinciding with a shift from a bimodal distribution at 24 h to a unimodal distribution after 168 h. Conversely, particle size in the emulsion formed using bioemulsifier AM1 and paraffin light oil (Po) was relatively small at 24 h (SMD= 49.18 μm) and increased thereafter, showing larger particles in emulsion at 168 h (SMD= 68.25 μm).

In an emulsion, smaller droplets are considered to be more rigid than larger droplets because they are deformed into polyhedral shapes. The formation of two distinct phases by coalescence of these larger droplets leads to a greater decrease in free energy than is achieved by mere aggregation, as observed in particle size shifts in these experiments (Figure 4.21). When two droplets come together, either by Brownian collision, shear-induced collision or gravitational force, the interaction of the two droplets (inter-particle forces) and the viscous force from the fluid drainage increase the contact area between the two particles, thus increasing the area of interface. This rapid stretching of the interface increases the local interfacial tension and opposes stretching (Goodwin, 2009).

Differences in static values and dynamic surface tension resist expansion and force the movement of the stabilizing agent (bioemulsifier) into the interaction zone, resulting in the Marangoni effect (drainage of fluids is opposed by fluids drawn into the thin interfacial film by viscous drag). Stability is sustained if the emulsifier is strongly adsorbed at the interface when the droplets come together. In a polydispersed emulsion, larger particles will have lesser solubility than smaller particles and tend to grow at the expense of the latter, which tend to dissolve and lead to Oswald ripening (Shaw, 2003). TCB, having higher density than water, forms a layer at the bottom of the test-tube. Thus, the emulsion formed with it is always in a compressed form because of the constant presence of an aqueous phase over it, leading to a size shift during the incubation and stabilization of the emulsion by smaller droplet packing and separation of the larger droplets from the emulsion into the immiscible phase.

As described by Bennett *et al.* (1968), the viscosity of an emulsion is a factor of proportionality between the rate of shear and shearing stress. The gradual reduction in viscosity (η) of both emulsions formed in paraffin and TCB shows their non-Newtonian nature and shear thinning effect for shear rate values used in this study.

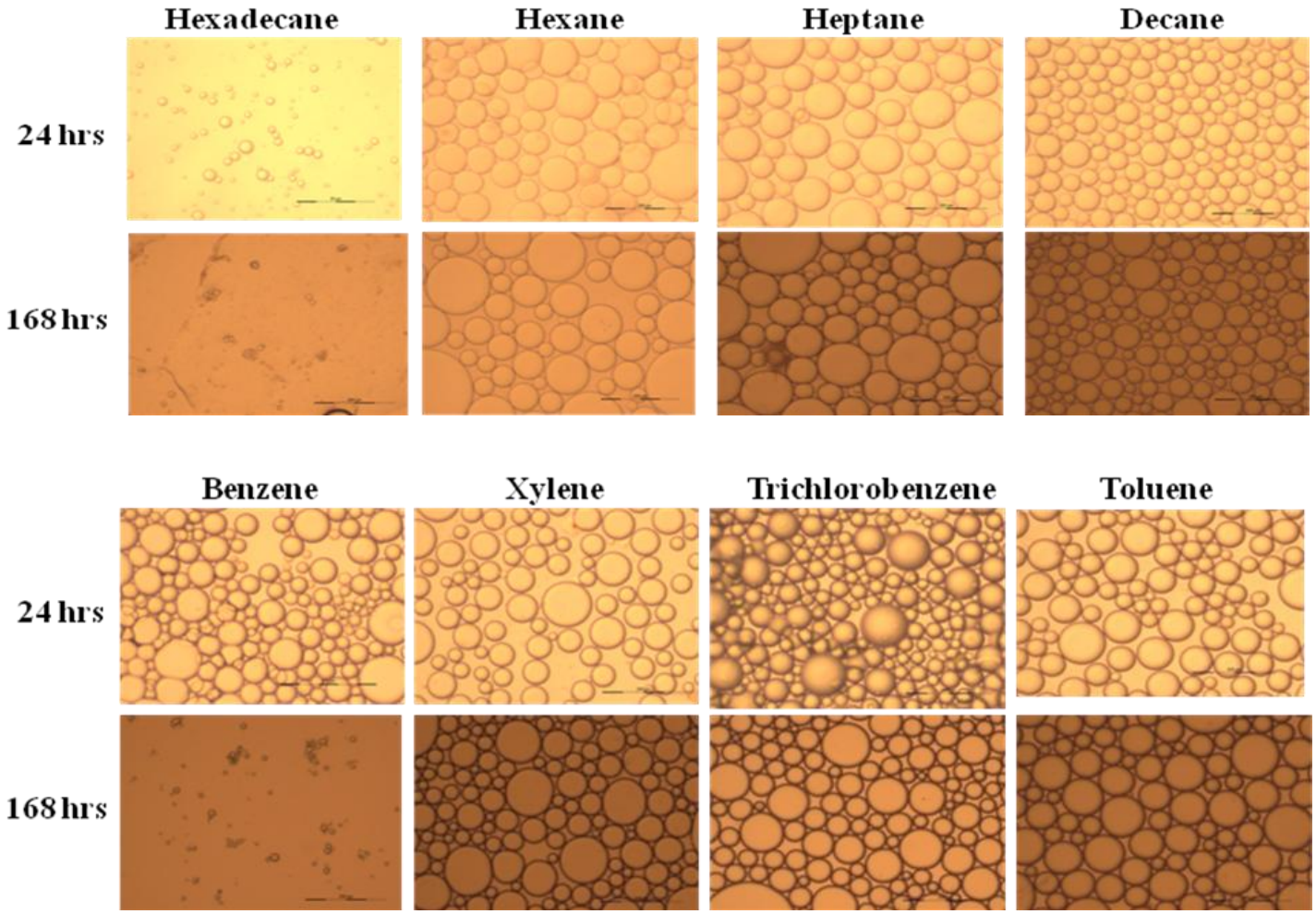

Figure 4.19. Brightfield micrographs of the emulsion of *S.silvestris* AM1 with aliphatic (first row) and aromatic (second row) solvents at 24h & 168h

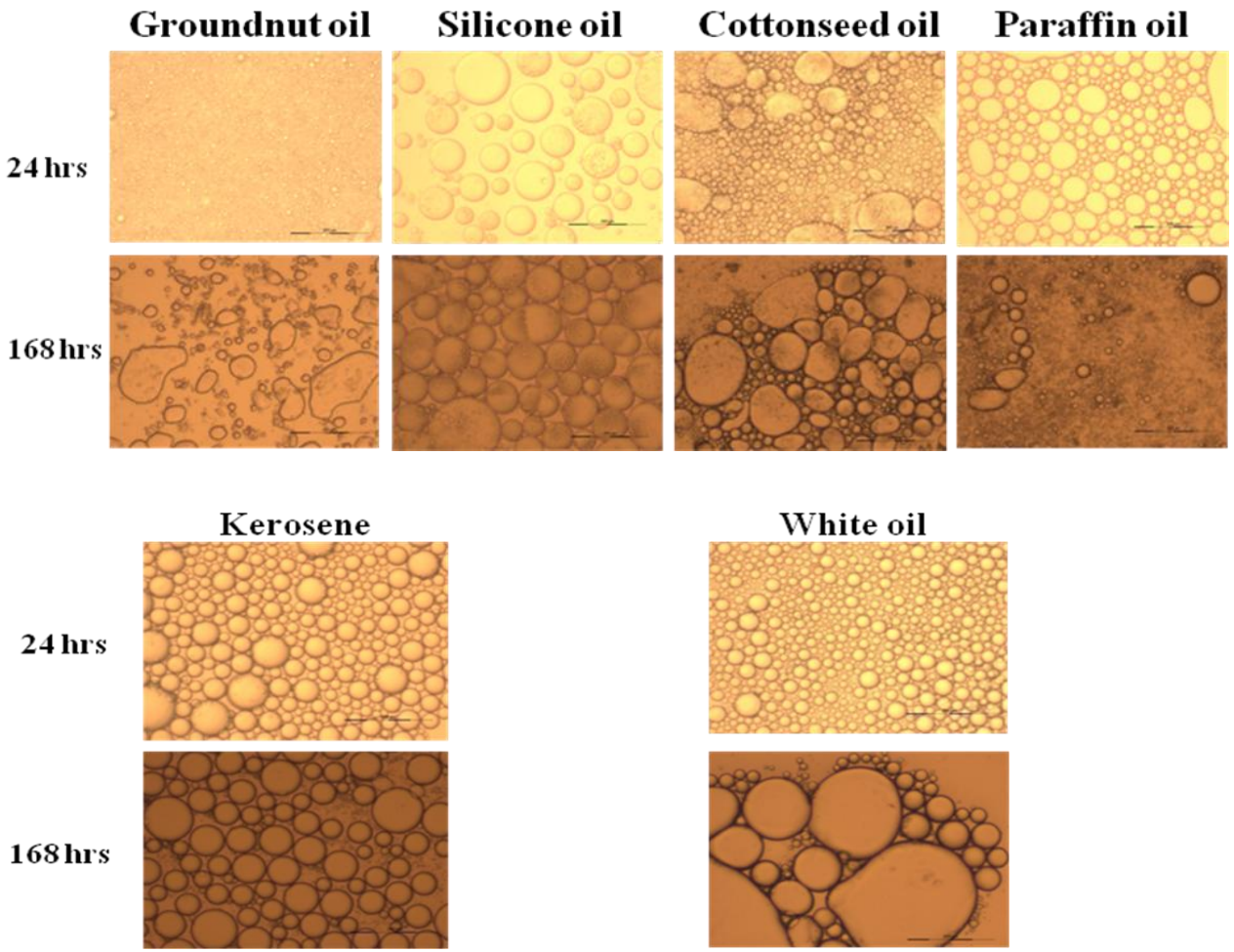

Figure 4.20. Brightfield micrographs of emulsions of *S.silvestris* AM1 with different oils as solvents at 24h & 168h

The loss of emulsion viscosity and the shear thinning phenomenon exhibited by the emulsion formed using the two solvent systems can be attributed to the proteinaceous bioemulsifier AM1 and hydrocarbon system. Pseudoplastic non-Newtonian emulsions, as observed in paints, show an instant decrease in viscosity with an increase in shear rate, as is observed in the rheogram of bioemulsifier AM1 depicted in Figure 4.22. This result means that the emulsion formed by bioemulsifier AM1 is non-adhesive, viscous and stable to temperature and stress over extended time periods, which is a distinct advantage in cosmetic, nutritive and pharmaceutical formulations.

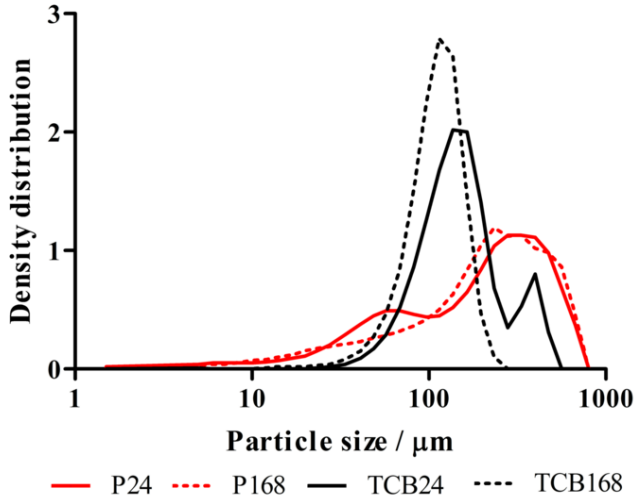

Figure 4.21. Particle size analysis of emulsion droplets formed using paraffin light oil and trichlorobenzene after 24 h and 168 h against bioemulsifier AM1.

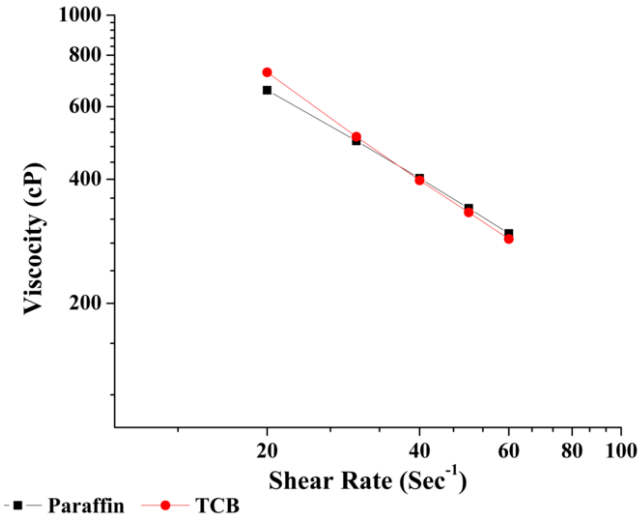

Figure 4.22. Analysis of shear rate and viscosity change of the emulsion produced by bioemulsifier AM1 with paraffin and TCB.

A novel estuarine bacterial strain, *Solibacillus silvestris* AM1, was found to produce an extracellular, multimeric glycoprotein bioemulsifier, termed AM1, with a MW of 200 kDa and containing 30 kDa monomeric subunits. The bioemulsifier contained 3.6% of the minor carbohydrate components galactose and ribose/xylose. LC/MS-MS of the 30 kDa subunit revealed its homology with a flagellin-like protein arranged in the form of fibers, as shown by a transmission electron micrographs. This is the first report of a flagellin-like protein that exhibits bioemulsifier activity being produced from a member of the *Solibacillus* genus. Bioemulsifier AM1 has a high emulsification index of 62.5% with 10^{-2} critical micellar dilution. It was found to be thermostable and active in the pH 5-9 and 0-5 M NaCl ranges. Moreover, AM1 formed stable emulsions with a broad range of solvents, including aliphatics, aromatic hydrocarbons and oils, performing better than the well-known bioemulsifier Emulsan. Emulsions formed with trichlorobenzene and paraffin oil have a pseudoplastic non-Newtonian rheological property, as observed by particle size and shear stress analysis. AM1, an eco-friendly bioemulsifier, formed stable emulsions in varied physical conditions, and these attributes may prove to be advantageous in cosmetic, pharmaceutical and environmental applications.

An interesting observation in this chapter is regarding the amyloid nature of bioemulsifier AM1. Picking the cue from TEM observations of fibre-like bioemulsifier conformation, secondary structure analysis was done by FTIR, CD and Congo red birefringence in polarizing microscopy, where the antiparallel β strands observed established the amyloid nature of bioemulsifier AM1. The amyloid nature of the cell bound bioemulsifier was also shown in Congo red plate assay, where the cell surface amyloid biomolecules are stained red. According to Neilson *et al*. (2011), the functions of bacterial amyloids are not yet clear, but the list of bacteria producing them is expanding. Since bioemulsifier AM1 production was triggered only in certain nutritional conditions, it can be said that it is produced only when its role is needed by the bacterium, envisaged as in adhesion and thereby biofilm formation as observed in chapter 5.

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Chapter 5

Ecophysiological studies of *S.silvestris* AM1 and its bioemulsifier

लोके प्रकृतिभिन्नेऽस्मिन् न कश्चित् कस्यचित् प्रियः।
कार्यकारणसंबद्धं वालुकामुष्टिवज्जगत्॥
- सौंदरानंद १५-३५

Meaning:
In this world where everyone is different, nobody is in love with each other. Requirement of each other makes people to be together like sand particles held in hand (temporary).

-Soundarananda kavya (15-35)

Chapter 5

Ecophysiological studies of *S.silvestris* AM1 and its bioemulsifier

Ecophysiological studies analyze the responses of organisms to the environment and the analysis of physiological and interaction mechanisms involved from microorganism to a similar microbial group grading to community and ecosystem (Pardo, 2005). Interaction of microorganisms in a settled niche depends on their ability to produce substances such as biosurfactants, organic acids or peroxides, quorum quenching strategies etc. (Sadowska, *et al.*, 2010). In order to understand the ecophysiology of *S. silvestris* AM1 the studies in this chapter involve elucidation of

(A) Natural role of bioemulsifier in *S. silvestris* AM1

(B) Interaction of *S. silvestris* AM1 bioemulsifier with other bacteria from foreign and shared habitats.

5A. Natural role of bioemulsifier in *S. silvestris* AM1

According to Ron and Rosenberg (2001) bioemusifiers have definite functions/roles in the microbes that produce them. The various roles they play for microorganisms are (i) Cell-cell interaction; (ii) Biofilm formation; (iii) Bioavailibilty of water insoluble substrates; (iv) Quorum sensing and (v) Virulence factors in bacterial pathogenesis etc. (Ron & Rosenberg, 2001). The emphasis of this study is on the following roles:

(i) Role in cell-cell interaction:

Biosurfactant/bioemulsifier produced by microbes either remain localized on cell surface or released extracellularly in the environment. When biosurfactants/bioemulsifiers remain bound to cell surface, it controls the adherence property to various surfaces (Neu, 1996, Satpute, *et al.*, 2010). Adhesion is a complex process involving hydrophobicity and specific ligand-receptor mechanisms. Adherence of bacterial cells is usually related to cell surface characteristics. Auto-aggregation has been correlated with adhesion, which is known to be a prerequisite for colonization and biofilm formation. Cell aggregation is a multistep process of motility, collision and adhesion and involves recruitment of planktonic cells from the

surrounding medium (co-aggregation) as a result of cell–cell interactions mediated by physical factors (Collado, *et al.*, 2006, Iliuta & Larachi, 2006).

It had been reported that in *Serratia marcescens*, serratamolide (a surface active agent) is bound to its cell surface via its hydrophobic end (Rosenberg, *et al.*, 1983). Thus the cell surface hydrophobicity is reduced by bound bioemulsifier. Also such microorganisms can readily bind to hydrophilic surfaces via the hydrophilic portion of the bioemulsifier. Opposite has been reported in case of cell surfaces of *Micrococcus* and *Corynebacterium*, in which the biosurfactant is oriented via its hydrophilic end attached to cells surface, thus reducing cell surface hydrophilicity. These microorganisms adhere readily to hydrophobic surfaces (Dyar, 1948).

(ii) Role in biofilm formation:

It has been reported in various studies that bioemulsifier/biosurfactant assists in biofilm formation of that particular microorganism producing them. Thus adhesion property of bioemulsifier plays an important role in initiation of biofilm (Satpute, *et al.*, 2010). Osterreicher-Ravid, *et al.* (2001) had reported the phenomena of horizontal transfer of bioemulsifier from one bacterial species to another. Hence it shows that the bioemulsifier/biosurfactant of a particular microorganism can bring about biofilm formation of a non-biofilm forming microorganism by adhering to the surfaces of such cells or it may adhere to the surface on which biofilm has to be formed and allow biofilm formation of non-biofilm forming microorganism.

(iii) Role in hydrocarbon solubilisation:

One of the main reasons for the prolonged persistence of hydrophobic hydrocarbons in contaminated environments is their low water solubility, which increases their sorption to soil particles and limits their availability to biodegrading microorganisms. Thus, approaches to enhancing biodegradation often attempt to increase the apparent solubility of hydrophobic hydrocarbons by treatments such as addition of synthetic surfactants or biosurfactants (Barkay, *et al.*, 1999).

Hydrocarbons become incorporated within the hydrophobic core of micelles and this effectively enhances their dispersion into the aqueous phase and hence their bioavailability for uptake by cells. This process has been largely studied with alkanes as model substrates and is referred to as ‘‘micelle solubilisation’’ or ‘‘pseudo-solubilisation’’ (Marchant & Banat, 2012).

Ecophysiological strategy employed by the bacteria inhabiting the intertidal niche and hitherto neglected aspect was studied here. With this perspective ecophysiological studies regarding elucidation of natural role of the bioemulsifier in *S. silvestris* AM1 inhabiting this habitat were initiated. The work carried out was divided into

I) Studies on surface properties of *S. silvestris* AM1 and its mutants and

II) Surface interaction studies between substratum and *S. silvestris* AM1

5AI. Studies on surface properties of *S. silvestris* AM1 and its mutants

5AI.1. Introduction

Although bioemulsifiers are known for influencing the surfaces of bacteria producing it, only few reports actually have studied this facet of bioemulsifiers in detail. In this section, of *S.silvestris* AM1 has been studied in detail with respect to its adhesion to hydrocarbons, cell-cell aggregation, substratum adhesion and biofilm formation.

5AI.2. Materials and methods

5AI.2.1. Microbial adhesion to hydrocarboms (MATH) test:

Microbial adhesion to hydrocarbon (MATH) test was performed according to van der Mei, *et al.* (1997) to understand cell adhesion properties. Four aromatic and four aliphatic Hydrocarbons with which MATH Test was performed are given in table 5.1.

For performing MATH Test culture was grown in ZM broth for 48 h at 35 °C and 180rpm. Grown culture was collected by centrifugation at 10,000 rpm for 10 minutes, washed and resuspended in sterile Phosphate buffered saline (PBS) (Appendix) and O.D. at 600nm was checked and adjusted between 0.4-0.6 with sterile PBS. 3 ml bacterial suspension was taken and 150 µl of hydrocarbon was added in respective test tube. System was vortexed for 10s and kept for 10 min at static condition to allow microbial adhesion to particular hydrocarbon to take place. After incubation absorbance was checked at 600nm of aqueous phase taking care that, immiscible hydrophobic phase was not involved in measurement. After obtaining result of absorbance, % Hydrophobicity was calculated with the equation:

$$\%\text{Hydrophobicity} = 1 - \left(\frac{\text{Final O.D.}}{\text{Initial O.D.}}\right) \times 100$$

Table 5.1. Hydrocarbons used in MATH test

Aromatic Hydrocarbons	Aliphatic Hydrocarbons
Benzene	Hexane
Trichlorobenzene	Heptane
Xylene	Hexadecane
Toluene	Decane

5AI.2.2. Cell aggregation assay:

The cell aggregation assay was performed according to Kos *et al*. (2003). *S.silvestris* AM1 culture was grown in 50 ml ZM medium in presence of hydrocarbon like benzene (3.2 mg/ml) and pesticide like acephate (50µg/ml) for 24 hours at 35°C on shaking condition (180 rpm). Overnight grown culture was centrifuged at 10,000 rpm for 15 min and finally washed resuspended in sterile PBS. Absorbance of this system was adjusted at 0.4-0.6 with sterile PBS at 600 nm. Flask was kept undisturbed and in sterile condition during the experiment and at the interval of 30 minutes O.D. was checked for all 3 experimental systems prepared in triplicates i.e., culture grown in ZM broth, culture grown in ZM broth with benzene, culture grown in ZM broth with acephate. This experiment was carried out for continuous 5 hours and Auto-aggregation % was calculated.

$$\text{Auto aggregation (\%)} = \frac{OD_{initial} - OD_{final}}{OD_{initial}} \times 100$$

Here $O.D._{initial}$ was the absorbance of t=0 and $O.D._{final}$ was absorbance checked at particular time interval.

5AI.2.3. Biofilm formation assay:

Biofilm formation was studied according to Srinandan, *et al.* (2010). *S. silvestris* AM1 was observed as a good biofilm former organism on polystyrene based surface comparing to glass surface. *S. silvestris* AM1 was grown in presence and absence of additional bioemulsifier in microtitre plates. The media used were Luria

Bertani medium and ZM medium and the biofilm assay was carried out as given in Chapter 2, section 2A.2.9.

Biofilm formation was analyzed in non-production medium i.e., ZM salt solution amended with 1% acetate and production medium i.e., ZM medium. Biofilm in production medium was treated with 100µl of Proteinase K (1mg/ml in Phosphate buffer of pH 7.3) at 37°C for 2h (Chaignon, *et al.*, 2007).

For time course experiment of biofilm formation, *S. silvestris* AM1 was grown in ZM medium in microtitre plates, incubated for 48h and the cells in the supernatant (planktonic) were washed with PBS and plated onto ZM plates for cfu was enumerated at every 2h interval. Biofilm assay was carried out as described in Chapter2, section 2A.2.9.

Biofilm formation in presence of xenobiotic was studied by inoculating 2 % of *S. silvestris* AM1 inoculum into ZM medium in 24 well microtitre plate along with xenobiotics (hydrocarbons and pesticides) with concentrations given in table 5.2. This system was incubated at 35° C in static condition for 48h. After incubation, biofilm assay was carried out as given in Chapter2, section 2A.2.9.

Table 5.2 Concentrations of hydrocarbons used for biofilm studies

Sr.No.	Component	Concentration used
1	Benzene	1.6 (mg/ml)
2	Trichlorobenzene	72.5(ng/ml)
3	Acephate	100 (µg/ml)
4	Methomyl	200(µg/ml)
5	Cypermethrin	0.5(mg/ml)
6	Catechol	250 (µg/ml)

5AI.2.4. Mutagenesis:

5AI.2.4.1. UV mutagenesis:

The overnight grown wildtype culture, *S. Silvestris* AM1 was washed once with PBS and OD_{600} was set to 0.2-0.4, 10ml of this suspension was then exposed to UV doses of 0.002, 0.004, 0.006 and 0.008 J in UV crosslinker (Wilber-Louromat, France). The 10% survival dose (D_{10}) was taken and the exposed cells were then

diluted to 10^{-7} and spread on the LB agar plates containing streptomycin, in dark. The plates were incubated at 35ºC for 24-48h. The mutants obtained were analyzed for emulsification activity

5AI.2.4.2. Transposon mutagenesis:

Bacillus subtilis strains containing Tn917 were obtained from Bacillus Genetic Stock Centre (BGSC), Columbus, Ohio, USA. Plasmids from these strains used for mutagenesis were plasmids pTV 32 (pE194Ts-*rep*, Tn*917- erm cat*) and pTV32ts (pE194 ts *ori*; Tn*917-lacZ erm cat;* thr-5 trpC2). Plasmids (50ng/µl) were extracted according to Sambrooke et al., (Sambrook & Russell, 2001). The transposon carried erythromycin resistance gene while the plasmid encoded for chloramphenicol resistance gene (Figure 5.1)

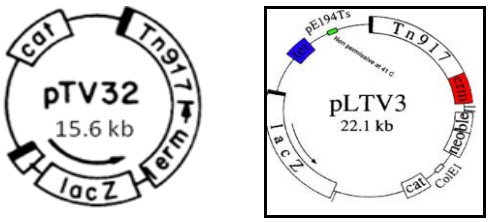


Figure 5.1. Temperature sensitive plasmids used for transposon mutagenesis.

The plasmids pTV32 and pLTV32ts were transformed in wildtype *S. silvestris* AM1 by electroporation. The bacterium was grown in electroporation growth medium (Appendix) overnight. 1.5ml of overnight grown culture (OD-0.8) was cooled on ice bath, centrifuged at 7000rpm for 5min at 4ºC. The pellet was washed four times in ice cold electroporation medium (Appendix). Cells were resuspended in 1/40th of initial volume and competent cells were stored at -80ºC.

The competent cells (60µl) were electroporated with plasmid (50ng/µl) at 2100 volts, 5ms. The electroporated cells were grown in outgrowth medium (Appendix) for 1-2 h at 30ºC and then spread on LB and ZM agar plates containing Erythromycin + Streptomycin and Erythromycin + Chloramphenicol + Streptomycin combinations of antibiotic (all 25 µg/ml concentration), incubated at 40ºC (nonpermissive temperature for plasmid). The mutants obtained were gridded on ZM agar plates containing appropriate antibiotic.

The transposon presence was confirmed by using Tn917 specific primers (3990f, 5`-GTTACACCTAGCGAAGCAGAAAT- 3`; 4501r, 5`-

ATGAGTAGCTTCCCTTGTATTAGT-3') and PCR conditions reported by Puopolo *et al*. (2007).

PCR conditions:An initial denaturation of 95°C for 5 min and subsequent steps for 30 cycles of denaturation at 95°C for 30s; annealing at 48ºC for 30s; elongation at 72°C for 30s; and final extension of 72°C for 10 min (Puopolo, *et al.*, 2007).

5AI.2.4.3. Mutant analysis:

Mutants obtained were analysed with amplified 16S rDNA restriction analysis (ARDRA) using universal eubacterial primers as given in chapter 2, 2A.2.7 and restriction enzymes *Alu*I, *Msp*I, *Hha*I (MBI, Fermentas, USA). Mutants were checked for their emulsification activities as given in chapter2, section 2A.2.5 and the cell bound activity was checked as given in chapter3, section 3.2.11. The putative mutants with no bioemulsifier activity (%EI) were selected for further studies.

5AI.2.4.4. Microbial adhesion to hydrocarbons (MATH) test of the S.silvestris AM1 mutants:

Hydrophobicity of the *S.silvestris* AM1 mutants was studied in triplicates as given in section 5AI.2.1.

5AI.2.4.5. Biofilm assay S.silvestris AM1 mutants:

24h old mutants and wildtype grown in ZM broth was added to sterile 24 well microtitre plate and checked for biofilm formation with sterile uninnoculated media as control. The plate was incubated for 48h at 35 ºC. After 48h, the biofilm assay was carried out as described in section 5AI.2.3.

5AI.2.5. Statistical analysis:

All experiments were conducted in triplicate and analyzed t-test using GraphPad Prism 5 software.

5AI.3. Results and Discussion

The function/role of bioemulsifier in *S.silvestris* AM1 was evaluated by performing MATH test, cell aggregation and biofilm formation of the wildtype and bioemulsifier non-producing UV and transposon mutants. Results obtained are compared and discussed here.

5AI.3.1. Microbial Adhesion to Hydrocarbon (MATH) of *S. silvestris* AM1 with different hydrocarbons:

Increase in % hydrophobicity suggests that culture can adhere to that hydrocarbon or cell surface properties show interaction with particularly the hydrocarbon and production of bioemulsifier may play a role in it. Biosurfactants/ Bioemulsifiers produce micelle around hydrocarbons thereby increasing the availability of such hydrocarbon. Aggregation assays and Microbial Adhesion to Hydrocarbon (MATH) test in reports have demonstrated significant differences in cell surface properties among various tested strains (Collado, *et al.*, 2006). Figure 5.2 shows MATH test carried out for *S.silvestris* AM1 in presence of various hydrocarbons.

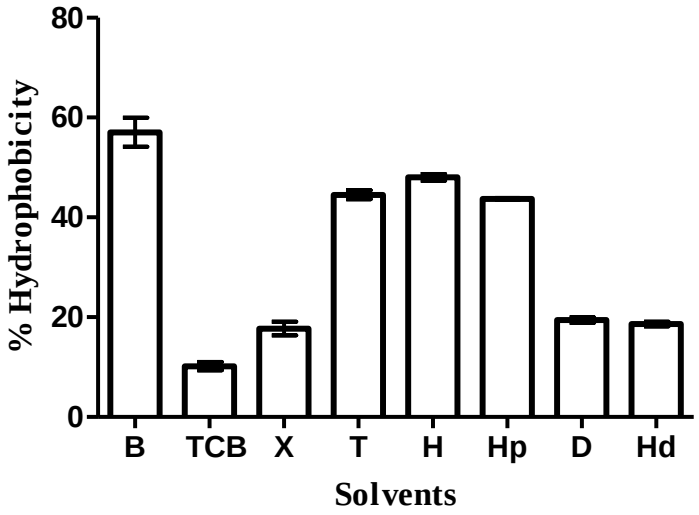


Figure 5.2 MATH Test of *S.silvestris* AM1 with various hydrocarbons. The hydrocarbons used: B- benzene, TCB- tricholobenzene, X-xylene, T- toluene, H-hexane, Hp- heptane, D- decane and Hd- hexadecane

The % hydrophobicity of *S. silvestris* AM1 was highest at about 57% with benzene as shown in Figure 5.2. % hydrophobicity of the cell surface of *S. silvestris* AM1 increased in presence of compounds like hexane, heptane and benzene, where as it relatively decreased in presence of compounds like xylene, hexadecane, decane and trichlorobenzene.

5AI.3.2. Cell aggregation assay of *S. silvestris* AM1:

Cell aggregation is a multistep process of motility, collision and adhesion and involves recruitment of planktonic cells from the surrounding medium (co-aggregation) as a result of cell-cell interactions mediated by physical factors like cell-surface properties (Iliuta & Larachi, 2006).

Cell/auto aggregation assay was performed with *S. silvestris* AM1 grown in ZM medium in presence of hydrocarbon like benzene and pesticide like acephate. The results were obtained at interval of 30 minutes with *S.silvestris* AM1 grown in presence of different conditions. As given in figure 5.3, aggregation ability of *S.silvestris* AM1 changed with change in medium condition. Culture showed relative increase in auto-aggregation % in presence of benzene and acephate as compared to unsupplemented medium. The autoaggregation in presence of benzene also showed increasing trend however at 90 and 270 minutes, there is a dip in autoaggregation for some unknown reasons not understood from this experiment. In all the cases auto-aggregation % increased with time, suggesting that cell-cell interaction increase with time and this cell surface property plays an important role in adhesion. Change in various parameters like pH, temperature, cell surface hydrophobicity cause change in auto-aggregation of a culture (Kos, *et al.*, 2003). Thus above results suggest that cell surface property may get altered or the surface hydrophobicity changed when the organism grew in presence of benzene or acephate and therefore showed increase in auto-agggregation.

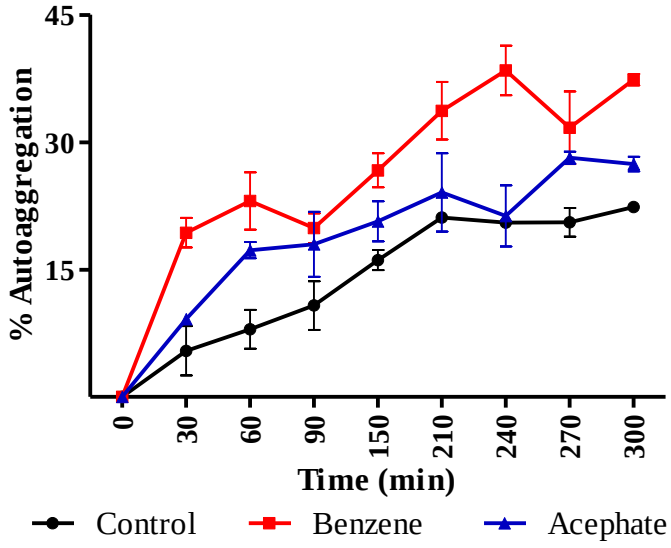


Figure 5.3: Cell aggregation assay of *S.silvestris* AM1.

5AI.3.3. Biofilm formation:

5AI.3.3.1. Influence of bioemulsifier addition on biofilm formation of S. silvestris AM1:

Biofilm development is affected by surface and interface properties, nutrient availability, and composition of microbial community, hydrodynamics, interspecies interaction, and cellular transport. The accumulation of biofilm is the net result of various sequential processes such as microbial attachment, adhesion, growth, aggregation of cells into microcolonies and cells and aggregates. Cell surface hydrophobicity also plays an important role in detachment from solid surface. Thus cell exist in both, planktonic and sessile forms during survival (Iliuta & Larachi, 2006).

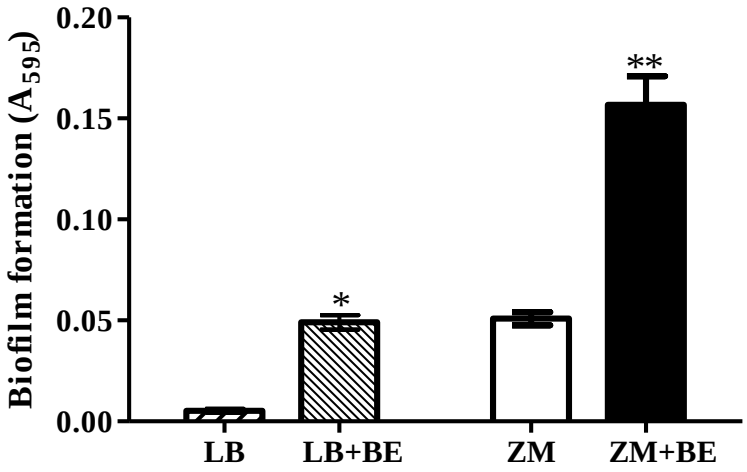


Figure 5.4. Biofilm formation of *S. silvestris* AM1 grown in Luria broth (L) and Zobell Marine broth (ZM) in presence and absence of bioemulsifier (BE). (p value : *<0.01, **<0.05)

Biofilm formation of *S. silvestris* AM1 was enhanced in presence of externally supplied bioemulsifier in case of LB as well as ZM medium (Figure 5.4). A significant increase in biofilm formation in presence of bioemulsifier indicated its role in biofilm formation. Biofilm formation was notably less in non production medium.

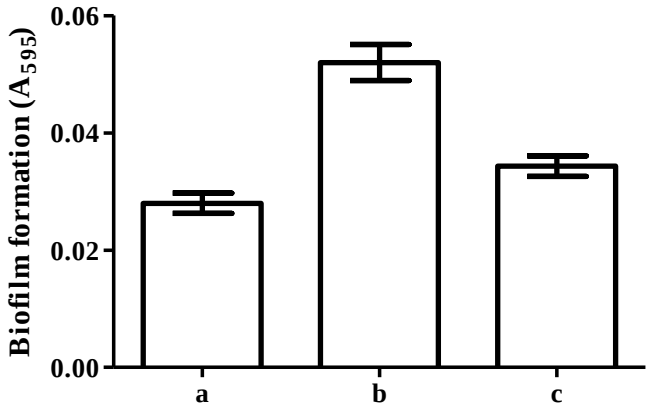


Figure 5.5. Biofilm formation of *S. silvestris* AM1. (a) non-production medium, (b) production medium and (c) Proteinase K treatment in production medium

In proteinase K treatment the biofilm formed was less as compared to the bioemulsifier amended condition pointing towards the possible role of bioemulsifier AM1 in biofilm formation (Figure 5.5).

5AI.3.3.2. Influence of xenobiotics on biofilm formation of S. silvestris AM1:

Figure 5.6 shows biofilm formation of *S.silvestris* AM1 in presence of various hydrocarbons as well as pesticides. Result of biofilm assay showed that *S.silvestris* AM1 forms biofilm in ZM medium. Biofilm formation was observed in presence of hydrocarbon like benzene, catechol and trichlorobenzene and pesticides like acephate, methomyl and cypermethrin. The biofilm formation in presence of benzene and methomyl was slightly affected while in presence of cypermethrin biofilm formation was least. Acephate and catechol enhanced the biofilm formation compared to control (unsupplemented medium).

In chapter 3 it was seen that *S. silvestris* AM1 can form bioemulsifier in presence of xenobiotics, the additional ability to form biofilm in presence of xenobiotics can help degrading bacteria in the habitat where bacteria producing bioemulsifiers are present.

5AI.3.3.3. Time course of biofilm production:

The biofilm formation by *S.silvestris* AM1 increased after 5h of incubation (Figure 5.7) which corresponded to the time of bioemulsifier production as discussed

in chapter 3, section 3.3.3. The increase in biofilm formation decreased in time corresponding to increase in planktonic bacteria in the well.

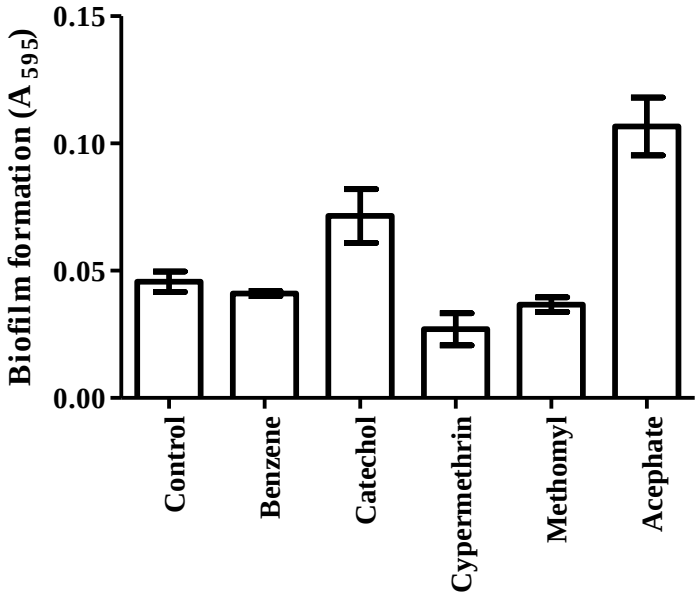


Figure 5.6. Biofilm formation of *S. silvestris* AM1 in presence of xenobiotics

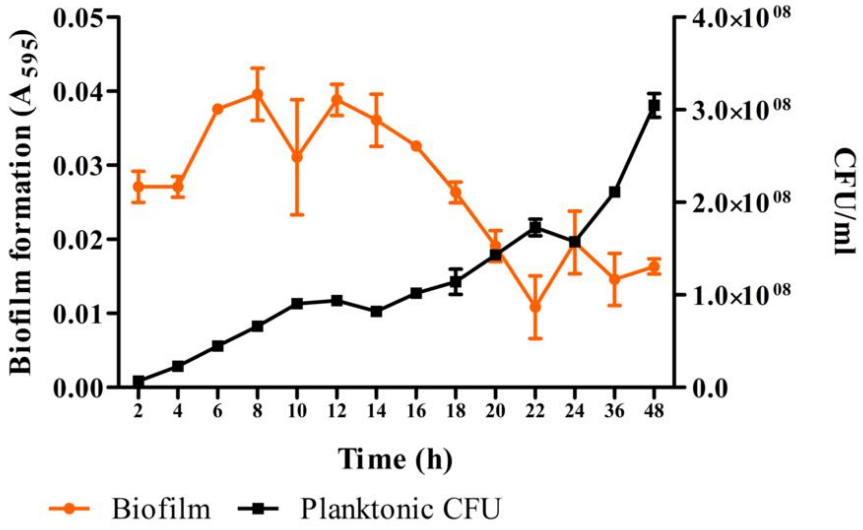


Figure 5.7. Time course of biofilm formation and accompanying planktonic growth by *S. silvestris* AM1

5AI.3.4. Mutant analysis:

The UV mutagenesis generated 177 mutants. From these many, only two mutants- ULA 29 and ULC16 showed complete loss of bioemulsifier production when checked on basis of their E_{24} index (Figure 5.8b)

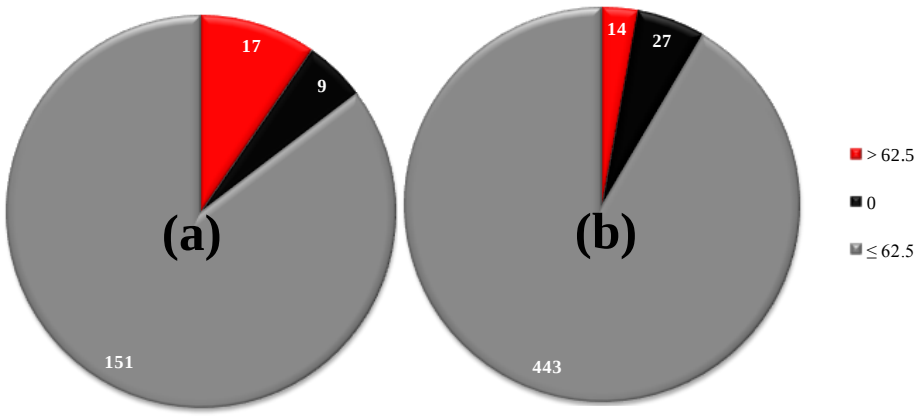


Figure 5.8. Bioemulsifier production production by mutants of *S. silvestis* AM1. (a) Tn917 mutants and (b) UV mutants.

In transposon mutagenesis after electroporation, the transformed cells were grown at non-permissive temperature i.e. 40ºC, where the plasmid gets degraded and transposon Tn917 on plasmid may get mobilized on to the chromosome. This transposition is reported to be stable as there is no more generation of specific transposase to mobilize the transposon further. This transposon specific transposase gene was on the plasmid which on degradation got destroyed. Also, other characters conferred by plasmid get destroyed (chloramphenicol resistance; cat^s).

So the mutants obtained should be having the transposon conferred antibiotic resistance – erythromycin (erm^r); and should be able to grow at non permissive temperature. This was checked by their growth on ZM agar plates containing Erythromycin and Streptomycin. The mutants did not grow on ZM plates with chloramphenicol, thus proving degradation of plasmid and successful transposition.

The mutants derived from transformation with both plasmids pTV32 and pLTV32ts were checked for their emulsification activity which is depected in figure 5.8a. Two isolates from tranposon mutagenesis, TLB 46 and TLA 17 were selected for amplification of Tn917. The 500bp amplicon of Tn917 specific primer from TLA17 and TLB46 confirmed the Tn mutants (Figure 5.9). Further, ARDRA was performed for the transformants and the restriction pattern for mutants as well as wild type was similar indicating that mutants were indeed *S. silvestris* AM1 (Figure 5.10). Thus the null mutants or the mutants showing loss of emulsifying activity were

proven to be originated from the same wildtype. Most of the UV mutants and the selected mutant ULA29 reverted to wild type.

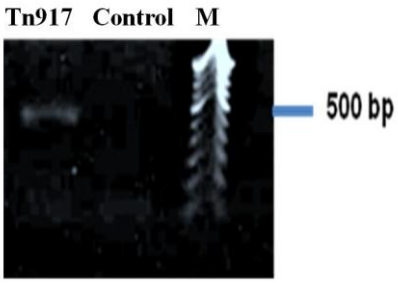


Figure 5.9. Amplification of Tn917 in transposant TLA17 using specific primers. The marker (M) used is 100 bp ladder.

5AI.3.5. Cell surface hydrophobicity of mutants:

When checked for hydrophobicity, the null mutants showed very high values in comparison to the wildtype in all the six hydrocarbons tested. Only the results of mutant ULA 29 showed similarity to wildtype that too with respect to aliphatic hydrocarbons (Figure 5.11).

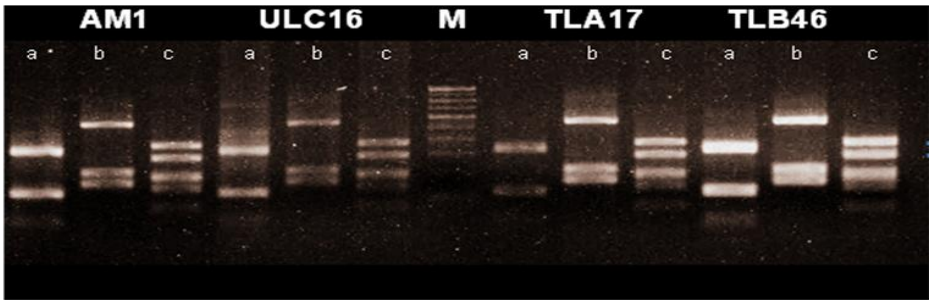


Figure 5.10. ARDRA pattern of UV mutants (ULC 16) and transposants (TLA 17 and TLB 46) of *S. silvestris* AM1 (a =*Alu*I,b =*Msp*I and c =*Hha*I).

Cell surface hydrophobicity is the result of the hydrophobic behaviour of cell surfaces which is manifested by anionic or negative charges on the surface. These contribute to the ability to attach stably to the surfaces. Many surfactants and bioemulsifiers enhance these interactions with the surfaces as well as between surrounding cells contributing to biofilm formation. These interactions are not only due to hydrophobic interactions of the hydrophobic part of the molecule but also to the hydrophilic reactions of the hydrophilic part (Neu *et al.*,1996).

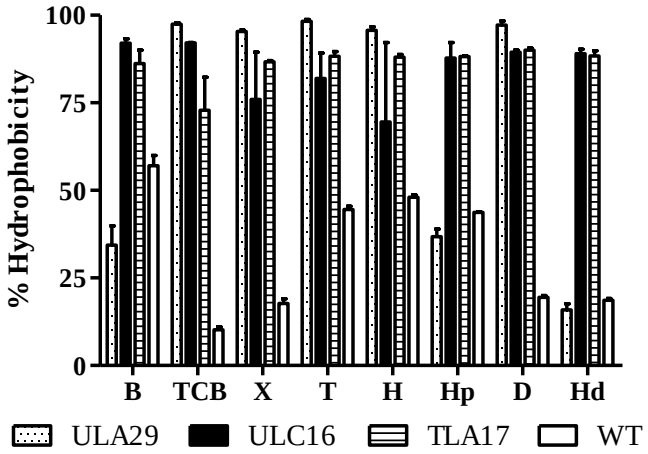


Figure 5.11. Cell surface hydrophobicity in wildtype *S.silvestris* AM1 and mutants. The hydrocarbons used: B- benzene, TCB- tricholobenzene, X-xylene, T-toluene, H- hexane, Hp- heptanes, D- decane and Hd- hexadecane.

The masking effect contributed by the bioemulsifier of *S. silvestris* AM1 on the cell surface hydrophobicity may contribute to the reversible attachment to surfaces. This property is advantageous for easy detachments in case of advancement of predator, starvation/ nutrient depletion or adverse environmental condition. Such a phenomenon is seen in many other oganisms producing surface active compounds. The detachment happens by release or excretion of such compounds from surface (Shunmugaperumal, 2010).

This point is strongly emphasized by MATH test done for null mutants. It was seen that cell surface hydrophobicity in mutants was more than that in wildtype (figure. 5.11). Moreover this finding also shows the behaviour of bioemulsifier of *S. silvestrs* AM1 in changing the surface hydrophobicity. The cell surface of wildtype and mutants being hydrophobic, shows better adherence to hydrocarbons. So, a mutant cell adheres to hydrocarbon in concentration that could be detrimental to cell. However, the presence of bioemulsifier masks this property in wildtype cells thus reducing % hydrophobicity. This could mean that in a way presence of bioemulsifier increases the tolerance level of bacterium towards hydrocarbons. The wildtype shows % hydrophobicity below 60 for almost all hydrocarbons; mutants show higher than 60 % for almost all except in case of TLA17 for hexane (Hx) and heptane (Hp).

5AI.3.6. Biofilm formation by mutants:

As seen from biofilm assay with mutants as well as wildtype using partial hydrophobic surface- polysterene, the formation of biofilm was better in case null mutants, except for TLB 46 (Figure 5.12). The increase in cell surface hydrophobicity contributes to irreversible attachment to the hydrophobic surfaces increasing the extent of biofilm formation. Such an attachment cannot be detached easily and so may be disadvantageous in adverse environment and shows failure to attach smooth surfaces with rapid proliferation of colony. It cannot form cell aggregates or multispecies biofilm and so needs hydrophobic counterpart or adapter like biosurfactant produced by other cells in vicinity. This further suggests the role of bioemulsifier in environment in appropriate dettachment, stable multispecies biofilm and further proliferation of biofilms.

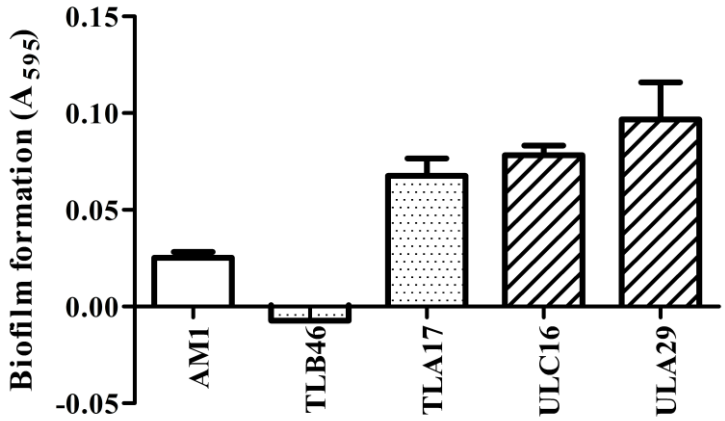


Figure 5.12. Biofilm formation of wild type *S. silvestris* AM1 and its mutants

Thus from this section, it can be concluded that the bioemulsifier AM1 produced on the cell surface of *S. silvestris* AM1 changed the surface characteristics of the bacterium. Benzene and acephate brought about increase in auto aggregation due to the change in cell surface hydrophobicity. The mutant analysis shows that hydrophobicity increased in absence of the bioemulsifier which facilitated the biofilm formation by irreversible attachment. Increased hydophobicity of mutants as compared to wildtype improved adhesion to hydrocarbons in high concentration at the cost of viability. Hence it can be said that the presence of bioemulsifier may be masking the cell surface of the wildtype giving tolerance to hydrocarbons.

5AII. Surface interaction studies between substratum and *S. silvestris* AM1

5AII.1. Introduction

According to Neu (1996), biofilm formation is a 'surface phenomenon' as it involves the interaction of two surfaces, one from bacteria and another is the surface on which biofilm has to be formed. As the surface property changes, the ability of adhesion of microbial cells to such surfaces also changes making bacterial adhesion a complicated mechanism (Neu, 1996). Research on bacterial adhesion and its significance is a large field covering marine science, soil and plant ecology, food industry and biomedical field. Although tremendous work has been done in last decade on this aspect of bacterial ecophysiology, a lot of questions still remain unanswered (Neu, 1996, An & Friedman, 1998).

Surfaces may be classified into two types: hydrophobic and hydrophilic surfaces. Hydrophobic surfaces offer attachment of microbial cells with hydrophobic surfaces and hydrophilic surfaces offer attachment of microbial cells with hydrophilic surfaces. Thus there has to be absolute compatibility of both the surfaces involved in biofilm formation. Biofilm formation in laboratory is usually checked in microtitre plates which have a polystyrene surface. The surface properties can be amended by treating these plates with various reagents. Few of the treatments are described in brief:

A. Sulfonation: When polystyrene surfaces are treated with sulphuric acid, sulfonate group is added to polystyrene. This process is termed as sulfonation of polystyrene (Figure 5.13). Usually polystyrene expresses a small amount of hydrophobicity. However due to addition of sulfonate group the polystyrene shows maximum amount of hydrophilicity, thus changing the native properties of polystyrene.

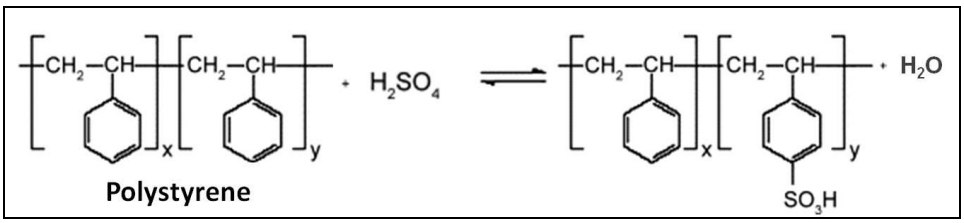


Figure 5.13. Sulfonation of polystyrene surfaces

B. Silanization: The treatment of polystyrene surfaces with various silanes is depicted in figure 5.14. Polystyrene readily gets charged with –OH in presence of

water. Due to silanization the hydrophobic property of polystyrene is enhanced. Increased hydrophobicity will allow only hydrophobic microbial surfaces to adhere to it.

The surface free energies and their components between two interacting surfaces are extremely important since not only do they dictate the strength of the interaction, but also control processes like the stability of aqueous colloidal suspensions, the dynamics of molecular-self assembly, wetting, spreading, adhesion and de-adhesion. Interfacial surface tensions between the two phases control these interactions.

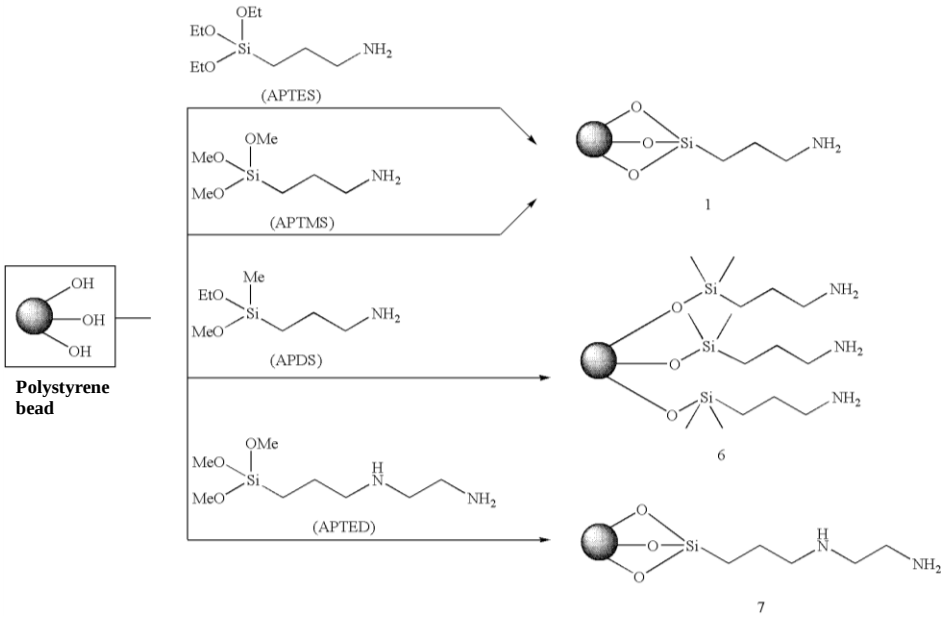


Figure 5.14. Silanization of polystyrene surfaces. This figure depicts silanization of hydroxyl group on polystyrene bead surfaces by various silanes.

Thus for studying this, understanding the surface properties specifically surface free energy components of the solids is necessary (Good, *et al.*, 1992, Yildirim, 2001). Wetting experiments have been standard approach for determining the surface free energies of the solids and the interfacial free energies between the interacting surfaces. Contact angle method, which is a measure of the surface hydrophobicity has been widely used to characterize the surface properties of the solids (Absolom, *et al.*, 1983, Pringle & Fletcher, 1983, Busscher, *et al.*, 1984, Van Oss, 1989, Good, *et al.*, 1992, Yildirim, 2001, Palmer, *et al.*, 2007, Busscher, *et al.*, 2010, Hori & Matsumoto, 2010).

A biofilm is a layer-like aggregation of microorganisms attached to a solid surface. Previous study with *S.silvestris* AM1 showed that it cannot form biofilm on a glass surface but it showed formation of biofilm on microtiter plate that is on polystyrene surface. In this section an attempt to understand the properties of bacterial cell surface and that of substratum of attachment and their interplay leading to adhesion as in biofilm formation has been done.

5AII.2. Materials and Methods

5AII.2.1. Sulfonation of microtitre plates:

To a sterile microtitre plate 100µl of 98% sulfuric acid was added and treated with sulfuric acid (H_2SO_4) for 30 minutes. 2-3 washings were given with 500µl of distilled water to remove excess of sulfuric acid and were exposed to UV treatment for 15 minutes (Kuo & Hoch, 1996).

5AII.2.2. Silanization of microtitre plates:

To a sterile microtitre plate 200 µl of 10% APTMS i.e (Amino)-Propyl-trimethoxy silane was added and kept for 10 minutes. 2-3 washings were given with 500 µl of distilled water and exposed to the UV treatment for 15 minutes (Protocol modified from (Camesano, *et al.*, 2000).

5AII.2.3. Contact angle measurements and calculation of Interfacial interaction energy:

The contact angle measurements were done for treated (silanized and sulfonated) and untreated surfaces of polystyrene and *S. silvestris* AM1 grown on sterile membrane filter on bioemulsifier production (ZM medium) and non-production (ZM salt solution amended with 1% acetate) media. The wettability analysis of the surfaces was done using tensiometer (Data physics, Germany) and contact angle measurements were studied using Dynamic contact angle measurement devices and analysis was done by SCAT software (Dataphysics, Germany) at Central Salt and Marine Chemical Research Institute (CSMCRI), Bhavnagar, Gujarat, India and calculation of interfacial interaction energy was done as given below.

The total interfacial interaction energy or the free energy of interaction (ΔG^{Tot}) was studied for interaction between non-treated, sulfonated and silanized polystyrene microtitre plate cut bases with bacterial lawns grown on membrane filters

(0.4µm) with bioemulsifier production and non-production medium bases. The calculation of total interfacial interaction energy between two surfaces (ΔG_{PwB}^{Tot}) namely, polystyrene (*P*) and bacteria (*B*) immersed in polar liquid, water (*W*) was done as per Fowkes (1964), Van Oss, *et al.* (1988a), Van Oss, *et al.* (1988b), Van Oss (1989)).

Using these approaches, the surface free energy of a phase *i* can be represented as,

$$\gamma_i = \gamma_i^{LW} + \gamma_i^{AB} \quad (1)$$

Thus polar and apolar surface tension components can be considered as additive. Where γ_i^{LW} and γ_i^{AB} refer to apolar (Lifshitz-van der Waals interaction energy) and polar (acid-base) components of surface free energy respectively. Here, Lifshitz-van der Waals (LW) interactions include the dispersion (London-forces of Hamaker's approach), induction (Debye's dipole-induced dipole interactions) and orientation (Keesom's dipole-dipole interactions). The polar interactions between Lewis acids (electron acceptor) and bases (electron donor) on the surface are generally considered intermolecular interactions.

The changes in free energy associated with the solid-liquid interactions (ΔG_{SL}) is given by the following Dupre's equation,

$$\Delta G_{SL} = \gamma_{SL} - \gamma_S - \gamma_L \quad (2)$$

Where interfacial tension between the solid and liquid is γ_{SL}, γ_S is the surface tension of solid and γ_L is the surface tension of liquid.

Lifshitz-van der Waals forces are universal and always available at the surface. It needs to be stressed here that, apolar interactions are additive while acid-base interactions are not, essentially due to their asymmetric properties.

According to Van Oss-Chaudhury-Good (OCG) approach, the surface free energy change upon two interacting surfaces (Solid-liquid) is given by,

$$\Delta G_{SL} = -2\sqrt{\gamma_S^{LW}}\gamma_L^{LW} - 2\sqrt{\gamma_S^{+}}\gamma_L^{-} - 2\sqrt{\gamma_S^{-}}\gamma_L^{+} \quad (3)$$

Where electron donor (γ^-) and electron acceptor (γ^+) interactions of the two phases (solid,S and liquid,L) are taken into consideration. The equation 3 allows us to determine the interfacial surface tensions of two interacting surfaces (solid and

liquid). This equation (equation 3) also has 4 unknowns for the calculation of γ_{SL}; the surface free energy components of solid, i.e., γ_S, γ_S^{LW}, γ_S^+ and γ_S^-. The surface free energy components of the liquids are generally available in literature.

Thus substituting equations 3 in equation 2 for ΔG_{SL}, we get,

$$\gamma_{SL} = \gamma_S + \gamma_L - 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right) \quad (4)$$

Work of adhesion (W_{ad}) or Gibbs free energy of interaction can be related to the interfacial energies through Young's equation,

$$\gamma_L \, Cos\theta = \gamma_S - \gamma_{SL} \quad (5)$$

From combining equations 2 and 5 for solving γ_{SL},

$$-\Delta G_{SL} = \gamma_L(1 + Cos\theta) = W_{ad} \quad (6)$$

Substituting equation 6 in equation 3,

$$\gamma_L(1 + Cos\theta) = 2\left(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^+\gamma_L^-} + \sqrt{\gamma_S^-\gamma_L^+}\right) \quad (7)$$

Using equation 7, solid surface can be characterized in terms of its surface free energy components, i.e., γ_S^{LW}, γ_S^+ and γ_S^-. These values can be determined by taking contact angle values of three different liquids of known properties (in terms of γ_L^-, γ_L^+ and γ_L^{LW}) on the solid surface of interest. Using three different equations with the three unknowns which can be further solved to obtain the values of γ_S^{LW}, γ_S^+ and γ_S^-. If one of the three liquids used in the study is apolar, equation 7 becomes,

$$(1 + Cos\theta)\gamma_L = 2\sqrt{\gamma_S^{LW}\gamma_L^{LW}} \quad (8)$$

Because γ_L^-, γ_L^+ are zero. Thus, using contact angle values of a single liquid with known γ_L^{LW} and γ_L, γ_S^{LW} can be solved. With the predetermined values of γ_S^{LW}, values of γ_S^+ and γ_S^- can be determined by solving two simultaneous equations from equation 7.

The interaction energy (ΔG_{PwB}) of surfaces namely bacterial surface (*B*) and polystyrene surface (*P*) both immersed in water, equation 2 can be re-written as,

$$\Delta G_{PwB} = \gamma_{PB} - \gamma_{Pw} - \gamma_{Bw} \quad (9)$$

Further, apolar (Lifshitz-van der Waals interaction energy) and polar (acid-base) components of the total interfacial interaction energy can be studied by using the following equations of attraction

$$\Delta G_{PwB}^{LW} = \gamma_P^{LW} + \gamma_B^{LW} - 2\sqrt{\gamma_P^{LW}\gamma_B^{LW}} \quad (10)$$

$$\Delta G_{PwB}^{AB} = 2\left(\sqrt{\gamma_P^+} - \sqrt{\gamma_B^+}\right)\left(\sqrt{\gamma_P^-} - \sqrt{\gamma_B^-}\right) \quad (11)$$

As mentioned earlier LW and AB components are additive to give the total interfacial interaction energy.

$$\Delta G_{PwB}^{Tot} = \Delta G_{PwB}^{LW} + \Delta G_{PwB}^{AB} = \gamma_{PB} - \gamma_{Pw} - \gamma_{Bw} \quad (10)$$

The values here can be negative, zero or positive. When negative, corresponds to attractive interaction energy and when positive, the energy connotates the repulsion between the two surfaces immersed in a polar liquid.

5AII.2.4. Influence of surface properties of substratum on biofilm formation:

S. silvestris AM1 was grown on different surfaces of polystyrene microtitre plates and in ZM medium (bioemulsifier production medium) and ZM salt solution with 1% acetate as the carbon source (bioemulsifier non-production medium). The biofilm plate was prepared as described above and incubated at 30°C for 48h. Biofilm assay was carried out as described in Chapter2, section 2A.2.9. Time bound biofilm formation by *S. silvestris* AM1 was also checked.

5AII.2.5. Time course microbial adhesion to hydrocarbons (MATH) test of *S. silvestris* AM1 in presence of benzene:

Hydrophobicity changes of the *S.silvestris* AM1 culture was studied on the hourly basis in triplicates as given in chapter 5AI, section 2.1with hourly reading. The hydrocarbon used here was benzene as MATH test results of *S.silvestris* AM1 showed highest surface hydrophobicity in it.

5AII.2.6. Time course cell aggregation assay of *S. silvestris* AM1:

Cell aggregation test was carried out as given in chapter 5AI, section 2.2 with hourly reading to study its time course.

5AII.2.7. Atomic Force Microscopy (AFM):

The AFM imaging of the cell surface of *S. silvestris* AM1 was performed using Ntegra Aura microscope (NT-MDT Co., Russia) and image analysis was done by Nova software (Novascan Technologies, Inc., USA).

5AII.3. Results and Discussion

The results of surface interaction studies of *S. silvestris* AM1 with self and abiotic surfaces are discussed in this section.

5AII.3.1. Surface interplay-biofilm: Influence of surface properties of substratum on biofilm formation

Biofilm formation is a surface phenomenon. Nature of the surface of adhering microorganism and the surface of the interface play important role in biofilm formation. For efficient biofilm formation there has to be absolute compatibility between these two surfaces. Two major parameters of surfaces are important in biofilm formation: hydrophobicity and hydrophilicity of the surfaces.

Hence the property of polystyrene surface (microtitre plate well) was changed to study the effect of surfaces on bacterial adhesion and biofilm formation. On the basis of degree of hydrophobicity, the relationship between the different surfaces used in this study is:

Silanized > Untreated > Sulfonated

In other words it can be said that hydrophobicity of silanized surfaces is maximum as compared to untreated surfaces and sulfonated surfaces. Or it can also be said that sulfonated surfaces are highly hydrophilic as compared to untreated surfaces and silanized surfaces.

The overall biofilm formation by *S. silvestris* AM1 in medium conducive for bioemulsifier production was higher than in non-production medium. In presence of bioemulsifier AM1, the biofilm formation in sulfonated polystyrene surface was least while best on silanized polystyrene surfaces. In non-production medium, untreated polystyrene showed least biofilm formation while silanized surface encouraged highest biofilm formation (Figure 5.15).

5AII.3.2. Surface interplay-thermodynamics: Contact angle measurements and calculation of Interfacial interaction energy:

As seen in figure 5.16a among surfaces, untreated polystyrene showed lowest and silanized polystyrene showed highest contact angle (θ) measurements. Bacterial surfaces showed marginal decrease contact angle (θ) in presence of bioemulsifier AM1.

Using this θ value, when interfacial free energy of interaction between *S. silvestris* AM1 and polystyrene surfaces was calculated, interaction energy was higher in all the three cases, untreated, silanised and sufonated in bioemulsifier production medium (Figure 5.16b). Intrestingly the bacterial cells with or without bioemulsifier showed better self-interaction than with that of surfaces. Theoretically the interaction between *S.silvestris* AM1 with and without cell surface bioemulsifier exhibited intermediate values of interfacial interaction energy.

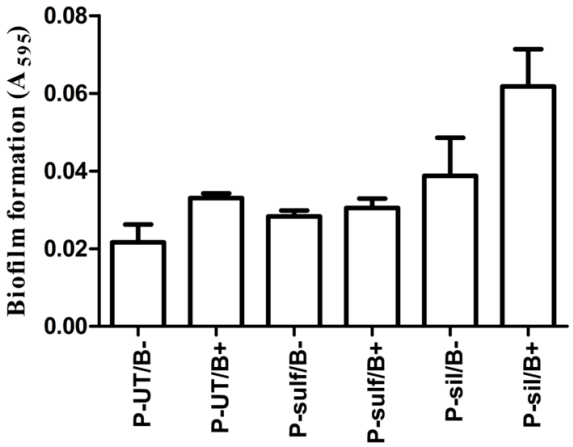


Figure 5.15. Biofilm formation by *S.silvestris* AM1. On untreated (P-UT), sulfonated (P-sulf) and silanized (P-sil) surfaces of polystyrene in bioemulsifier production (B^+) and non-production (B^-) medium.

A correlation between biofilm and free energy of interaction was analyzed (Figure 5.17 is formed by combining Figure 5.15 and Figure 5.16b) where in, it clearly shows the correlation between the biofilm formation of *S. silvestris* AM1 on different surfaces and their corresponding interfacial interaction energies. This successfully validated the theoretical calculations of interfacial interaction energy. This means that the reduction in interfacial interaction energy concomitantly facilitated more adhesion leading to biofilm formation.

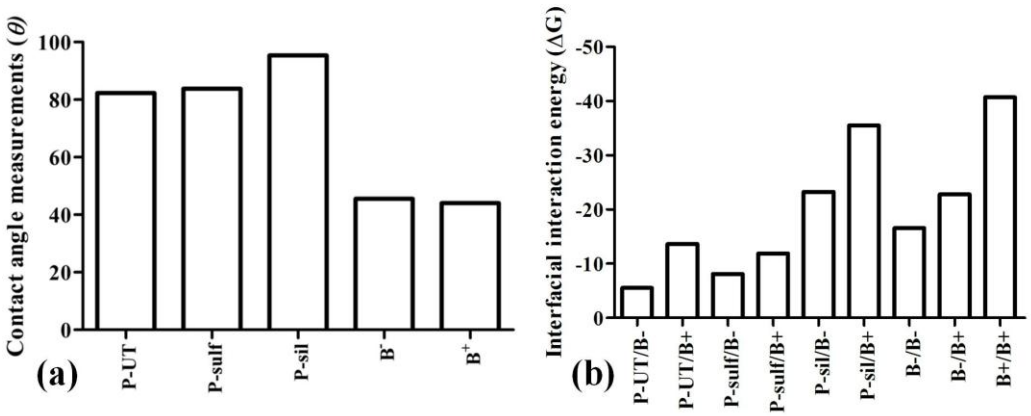


Figure 5.16. Contact angle (θ) measurements and calculated results of interfacial interaction energy. (a) θ values of *S. silvestris* AM1 in production (B+) and non production (B-) media on untreated (P-UT), sulfonated (P-sulf) and silanized (P-sil) surfaces of polystyrene, (b)calculated interfacial interaction energy from (a).

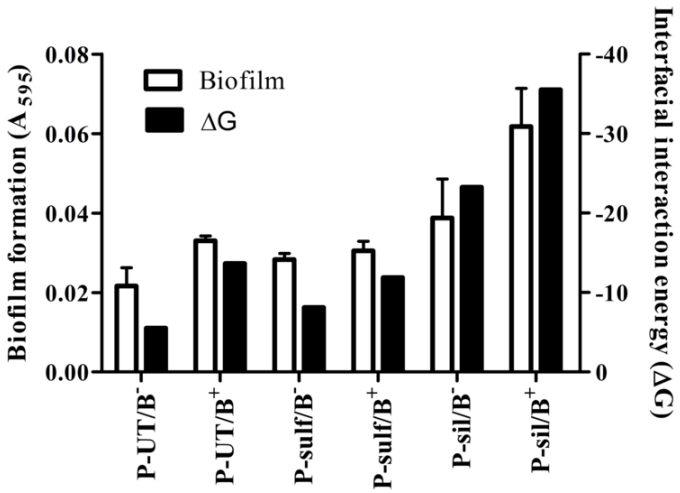


Figure 5.17. Merged figures 5.15 and Figure 5.16b for comparison of biofilm formation of *S. silvestris* AM1 on different hydrophobic surfaces and interfacial interaction energy in bioemulsifier production (B^+) and non production medium (B^-).

5AII.3.3. Time course of Microbial adhesion to hydrocarbons (MATH) test of *S. silvestris* AM1 in presence of benzene:

As seen in Figure 5.18, the cell surface hydrophobicity increases initially (1-4h) and starts to drop corresponding to the production of cell bound bioemulsifier (6h). The drop in hydrophobicity before the actual rise in emulsification activity can be possibly because of non-attainment of CMC for cell bound bioemulsifier. The cell

bound bioemulsifier activity can be detected only after a minimum threshold of their concentration is breached (CMC). The results also show that the decrease in % hydrophobicity attains a plateau. Thus the results here indicate that there is a possibility of certain hydrophobic surface portions of the cell surface being capped or masked by bioemulsifier AM1.

Bioemulsifiers have been long hypothesized to change the cell surface hydrophobicity owing to their nature of amphipathicity. (Palmer, *et al.*, 2007) have listed reports of enhancement and inhibition of cell adhesion to surfaces with respect to hydrophobicity. There have been similar extensive reports on change of cell surface hydrophobicity in *Serratia marcescens*, *Alcanivorax borkumensis*, *Pseudomonas aeruginosa* and *Acinetobacter calcoaceticus* and many more also with respect to utilization of recalcitrant carbon sources (Rosenberg, *et al.*, 1983, Zhang & Miller, 1994, Neu, 1996, Pruthi & Cameotra, 1997, Yakimov, *et al.*, 1998).

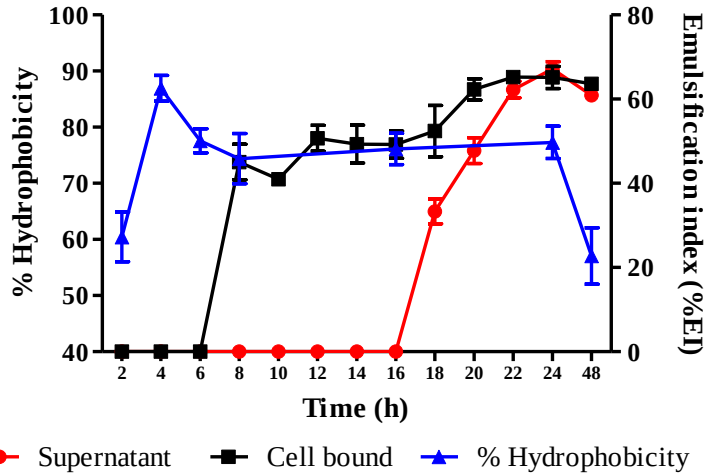


Figure 5.18. Time course of cell surface hydrophobicity of *S. silvestris* AM1 with respect to cell bound and cell free bioemulsifier production in ZM medium.

5AII.3.4. Time course cell aggregation assay of *S. silvestris* AM1:

Cell aggregation is a measure of cell-cell interaction and cell surface hydrophobicity in a population of cells. For *S. silvestris* AM1, initial cell to cell aggregation in *S. silvestris* AM1 rises significantly ($p<0.05$) after the time of bioemulsifier production. This result also confirms the results of thermodynamic studies shown in Figure 5.19.

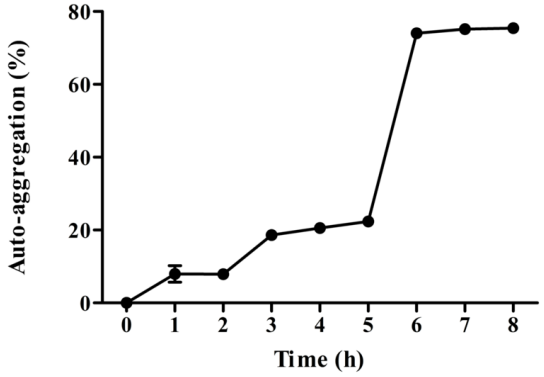


Figure 5.19. Time course of cell-cell aggregation in *S. silvestris* AM1.

5AII.3.5. Atomic force microscopy (AFM)

For many years, electron microscopy was thought to be the only technique to study the cell surface properties. Now, AFM is an established tool for characterizing the biological materials. It requires very little preparations and gives the detailed images of bacterial cell surface (Del Sol, 2007). Cell surface topology that accopanies the cell-bound stage of the bioemulsifier in *S. silvestris* AM1 was studied by AFM. This same technique was used by Del Sol et al., (2007) to study the cell surface changes that accompany the complex life cycle of *Streptomyces coelicolor*. AFM image of of *S. silvestris* AM1 show the tufts of bioemulsifier-like fibrils on the surface of the bacterium (Figure 5.20). Here it suggests that the bioemulsifier is distributed as islands on the surface of the bacterium.

AFM imaging of the cell surface topology of *S. silvestris* AM1 further helped to visualize the tuft like appearance of bioemolecules possibly the bioemulsifier AM1 in isolated islands on it which was also correlated with cell surface hydrophobicity studies where it was observed that some of the portions of the cell surface could possibly capped or masked by the bioemulsifier AM1.

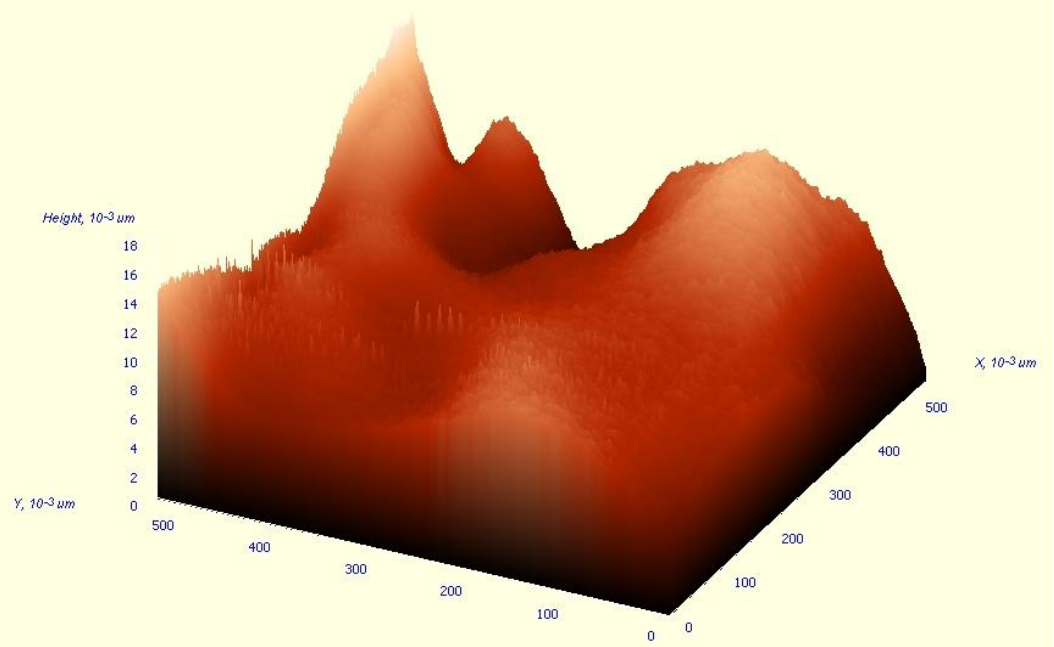


Figure 5.20. Atomic force microscopy of the cell surface of *S. silvestris* AM1

This section's results suggest that the hydrophobicity change resulting due to the production of bioemulsifier enhanced the bacterium's interaction with different surfaces as shown by the free energy studies. The distribution of bioemulsifier on the surface of *S. silvestris* AM1 seems to be restricted to few islands as visualized by AFM imaging beyond which, the bacterium starts to release it into environment changing the surface properties of substratum and increasing the wetability of the surface.

From the above studies, the natural role of bioemulsifier in *S. silvestris* AM1 is envisaged as follows: It changes the cell surface hydrophobicity and acts as a protectant against the hydrocarbon toxicity. Additionally, it aids in cell aggregation and adhesion to substratum and consequently helps in biofilm formation. In addition to emulsification activity, the bioemulsifier AM1 also possesses biodispersant and hydrocarbon solubilization properties as will be discussed in next section.

5B. Interaction of *S. silvestris* AM1 bioemulsifier with other bacteria

5B.1.Introduction

Bacteria produce a variety of surface active agents such as biosurfactants and bioemulsifiers, which are amphiphilic in nature ideally suited to interact with interfaces. Bioemulsifier producing microorganisms are reported to get enriched in contaminated area due to their ability to emulsify the hydrocarbons as carbon source and increasing its bioavailability (Baldi, *et al.*, 1999, Pavitran, *et al.*, 2006, Calvo, *et al.*, 2008, Das, *et al.*, 2009, Dusane, *et al.*, 2011).

Benzene, toluene and xylene (BTX) are an important family of organopollutants and are of great environmental concern throughout the world due to their greater migration into aquatic systems, toxicity and volatility. Microbial co-operation is essential for efficient hydrocarbon bioremediation in limited hydrocarbon availability. In microcosm experiments, it has been seen that assemblage of microorganisms in presence of different enzymes and enhancers (like fertilizers) can utilize hydrocarbons more efficiently (Mukherjee & Bordoloi, 2012, Choi, *et al.*, 2013, Zhang, *et al.*, 2013).

For study of ecophysiological potential of bioemulsifier AM1 in influencing other microorganisms, microcosm setup was used. This set up is such that it mimics environmental conditions of the habitat and the microbes involved. This model can be used to study the effects of commensalism between selected two groups of bacteria. For this bioemulsifier producing bacteria *S. silvestris* AM1 or bioemulsifier AM1 was co-inoculated in a microcosm setup with BTX degrading bacteria *Rheinheimera* sp. isolated from the same environmental sample. To understand the intertidal zone habitat and interaction of these two specific groups of bacteria, studies were carried out as given in this section. These studies should provide some information about the microbial interactions in this niche. Additionally studies were also done to check the effects of bioemulsifier AM1 on biofilm formation and degradation capability of other bacteria.

5B.2.Materials and Methods

5B.2.1. Microorganisms:

Following bacterial cultures were used for the studies.

(i) Biofilm forming *Staphylococcus aureus* ATCC 6538 and *Paracoccus* sp. W1b.

(ii) *Bacillus* sp. AV8 (GenBank accession No. J966102) isolated from the gut of a Nereid (*Nereis chilkaensis*), capable of degrading many xenobiotics, obtained from Central Institute for Fisheries Education, Mumbai, India.

(iii) BTX degrading bacterial isolates from same sediment sample from which the bioemulsifier producer *S. silvestris* AM1 was isolated.

5B.2.2. Crude oil dispersion:

For oil dispersion studies, a mixture of sterile ZM medium in 250ml Erlenmeyer flasks with 10% cell free supernatant as described earlier, 1% sodium azide (to avoid microbial contamination) and 2% crude oil was incubated for 72h at room temperature (30°C) in shaking condition at 180rpm. Then after further vigorous shaking, 5ml sample was transferred immediately to 150×14mm test tubes and allowed to stand for 2 min. 2ml of aliquot was carefully removed from the middle of the liquid column and its optical density was checked at 600 nm. Control experiment was setup with only medium in place of cell-free supernatant (Reisfeld, *et al.*, 1972).

5A.2.3. Solubilization assay:

The solubilization of hydrocarbons namely hexane and benzene was checked by using crude bioemulsifier AM1. Solubilization studies were carried out as described by Barkay, *et al.* (1999). The hydrocarbons namely benzene, toluene, xylene and hexane (concentration of 30 mg/ml) were mixed in Bushnell Haas medium (Appendix) and screened for their respective λ_{max}. For pilot assay 100µl of crude bioemulsifier was added and for further experiments 50 and 100 µg/ml of partially purified (purification as described in previous chapter 4 without gel filtration chromatography) bioemulsifier was added. Total volume of system was set at 5 ml, tubes were kept on shaker for 1-2h at 30°C and O.D was measured at λmax of the hydrocarbon in the medium with appropriate controls.

5B.2.4. Effect of bioemulsifier AM1 on degraders from foreign habitat:

To check the effects of bioemulsifier AM1 on degraders from different habitats, Nereid (*Nereis chilkaensis*) gut isolate *Bacillus* sp. AV8 (GenBank accession No. J966102) was used.

For hydrocarbon utilization assay the *Bacillus* sp.AV8 was grown in Luria broth overnight at 30°C. 1% of 0.6 O.D. inoculum was added into sterile Bushnell Haas medium (BHM, Appendix) containing hydrocarbons as the sole carbon source (5 mg/ml of benzene, toluene, xylene, hexane, decane, hexadecane, heptane and 1 mg/ml of trichlorobenzene) individually. To this 10 µg/ml (1 mg/ml is the stock) of partially purified bioemulsifier was added and system was incubated at 30°C for 72h. After incubation, absorbance was checked at 600nm with respective controls.

5B.2.5. Effect of bioemulsifier AM1 on degraders from shared habitat:

5B.2.5.1. Isolation and characterization of degraders from Vellar estuary sample:

0.5g of the soil sample from Vellar estuary was added into BHM amended with 1% hydrocarbons viz. benzene, toluene, crude oil and crude oil + benzene as sole carbon source and incubated for 10 days at 30°C. After 10 days of incubation, the growth was transferred to $1/10^{th}$ ZM agar plates. The colonies showing different morphologies were re-streaked into a fresh $1/10^{th}$ ZMA plates (Figure 5.20).

5B.2.5.2. Screening of Vellar estuary isolates:

Isolates obtained were checked for utilization of crude oil (1%) as the sole carbon source in BHM. Growth of these isolates was determined by turbidity at 600 nm. Isolates forming a film of growth on the crude oil were considered biofilm formers.

Inhibition of growth of *S. silvestris* AM1 was done by standard well agar diffusion method in Luria agar. For this assay, 100µl of 0.6 OD of *S. silvestris* AM1 was spread on the surface of $1/10^{th}$ ZM agar. 6mm diameter borer was used to make wells in the agar plate. 50µl of isolate inoculums was added in each well. Plates were incubated at 30˚C and inhibition zones were observed after 24h. Emulsification index (%EI) and test for surface tension reduction were checked as discussed in chapter2 (sections 2A.2.5 and 2A.2.6 respectively).Isolates incapable of growing in presence of crude oil carbon source, inhibiting the growth of *S. silvestris* AM1, showing

emulsification activity (%EI) and surface tension activity were eliminated from further studies.

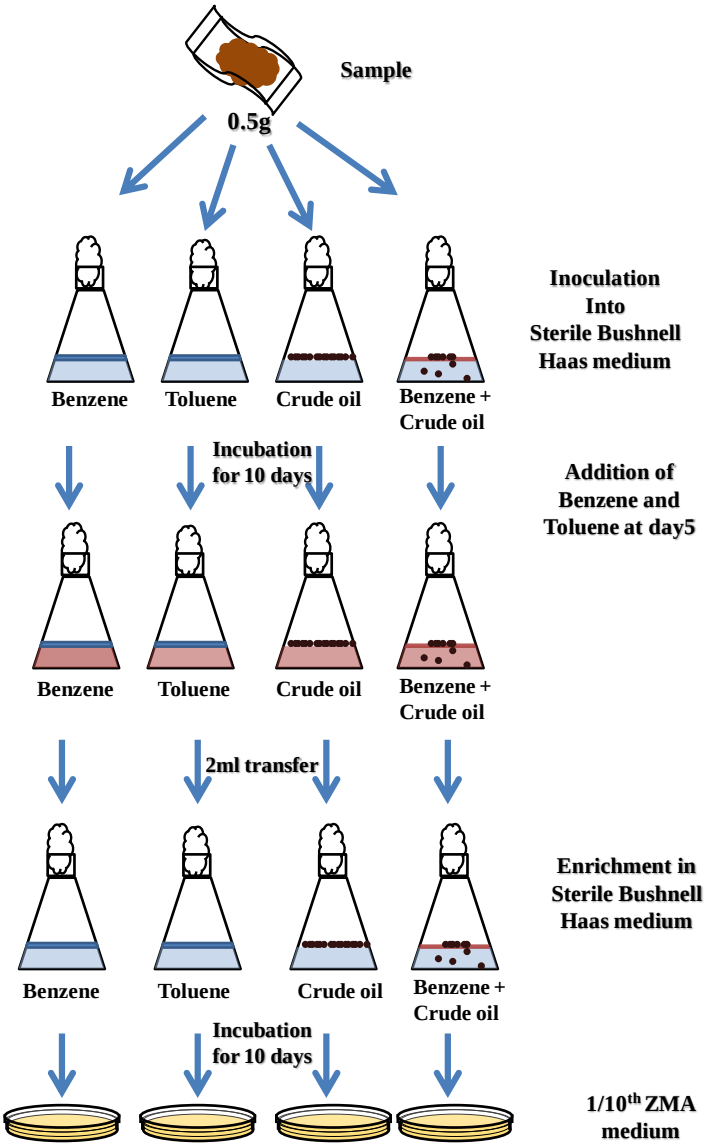


Figure 5.20. Strategy used for isolation of degraders from Vellar estuary sediment sample.

5B.2.5.3. PCR and Amplification of 16S rRNA gene of 23 isolates:

The 16S rRNA gene of the isolates was amplified using universal eubacterial primers discussed in Chapter 2, section 2A.2.7.

5B.2.5.4. Amplified rDNA Restriction Analysis:

Amplicons obtained were digested by the enzymes, *Alu*I, *Msp*I, *Hha*I and subjected to electrophoresis as discussed in Chapter 2, section 2A.2.7. The restriction profiles were analysed using AlphaEaseFC 4.0 (Alpha Innotech, USA) and NTSYS programmes and a consensus tree was plotted.

Eight isolates 4 forming biofilm 4 not forming biofilm but giving distinct OTUs were sent for commercial (outsourced) 16S rDNA sequencing (Xcelris labs, Ahmedabad, India). The sequences obtained were analysed with NCBI-BLAST. The sequence of the nearest match of each of the isolate was used for making a phylogenetic tree using MEGA 5 software and the chimera analyses of sequence was done with Pintail version 1.0, and were further submitted to NCBI-GenBank.

5B.2.5.6. Utilization of benzene, toluene and xylene (BTX) by selected isolates:

Isolates CO1 and isolates CO6 were selected for further screening as former could from biofilm while the latter could not. Isolates CO1 and CO6 were inoculated into $1/10^{th}$ ZM broth with 0, 10, 15, 30, 45 and 60 mg/ml of the three hydrocarbons, benzene, toluene and xylene combined. Culture blanks were setup for each concentration of hydrocarbon. This setup was incubated at 30˚C for 72h. The growth of the bacteria with BTX as the carbon source was analyzed by OD at 600nm.

5B.2.5.7. Microcosm studies:

Short-term microcosm studies were setup in specialized flasks (Figure 5.21). The flasks were made up of having inlet for hydrocarbons, outlets for air and GC sampling. The hydrocarbon inlet keeps the hydrocarbons trapped in the tube and makes it available for the period of incubation. The air outlet is kept open for 1min after the addition of the hydrocarbon to ensure the saturation of the inner environment of the flask with the hydrocarbon vapor.

The microcosm experiment was set up by mixing the sediment sample (from the intertidal zone sample used to isolate bioemulsifier producer and the degrader) with thoroughly washed and autoclaved (5 times) sand and $1/10^{th}$ ZM medium at 6.4 pH (native pH of the sediment). Different experimental microcosms were prepared in triplicate as given in table 5.3. The systems setup contained 100µg/ml of partially purified bioemulsifier, 30mg/ml BTX (10mg/ml of each hydrocarbon), 100µl bioemulsifier producing bacteria culture (0.6 OD_{600}), and 100µl BTX degrading bacterial degrader cultures (0.4 OD_{600}).

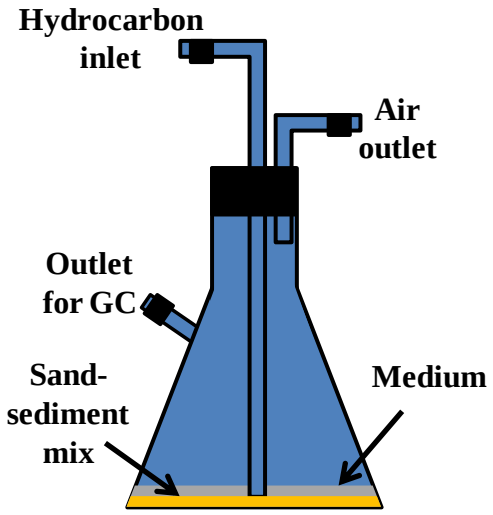


Figure 5.21. Schematic diagram of special flasks used for microcosm studies.

Table 5.3. Microcosm experimental setup

Experiment No.	System
1.	Sediment+ Sand+ Medium mixture (M)
2.	M+ Hydrocarbon (HC)
3.	M+ Bioemulsifier producing bacteria (P)
4.	M+ BTX degrading bacteria (D)
5.	M+ P+ HC
6.	M+ D+ HC
7.	M+ Purified bioemulsifier AM1(BE) +HC
8.	M+ D+ BE
9.	M+ D+ BE+ HC
10.	M+ D+ P+ HC

Aliquots from microcosm flasks were analyzed using gas chromatography (7890 GC system, Agilent Technologies, USA) equipped with a flame ionization detector (FID) and Restek 624 column of 30M length. The temperature of the GC oven was programmed to increase from 36°C (held for 20 min) to 240°C (4.6/min) at a rise of 10°C/min with FID detector at 200°C. Injector temperature was 140°C with injection volume of 1ml at a flow rate of 35cm/sec. Growth as cfu/ml of degrading bacteria (Ampicillin resistant) and bioemulsifier producing bacteria (Streptomycin resistant) was estimated on antibiotic selective media plates. The slurry of the

microcosm mainly containing medium and bacterial growth was centrifuged and supernatant was checked for emulsification and checked for protein release.

5B.2.6. Effect of bioemulsifier AM1 on biofilm forming bacteria:

24h old cultures *Paracoccus sp.* W1b, *Staphylococcus aureus* ATCC 6538, grown in Luria Bertani broth were pelleted and resuspended in PBS with a final O.D. of 0.6. The inocula were added into MM2 medium (as described by Srinandan et al., 2010) and Tryptic Soy Glucose broth respectively in sterile 24 well microtitre plates with and without the presence of 100 µg/ml (1 mg/ml is the stock) of partially purified bioemulsifier. The effect of bioemulsifier was assessed by washing a pre formed biofilm in presence of bioemulsifier AM1 with PBS for 2-3 times and treating with 100µl of Proteinase K (1mg/ml in Phosphate buffer of pH 7.3) at 37°C for 2h (Chaignon, *et al.*, 2007). Biofilm assay was carried out as describes in chapter 5, section 5AI.2.3.

5B.2.7. Nucleotide sequence accession numbers:

GenBank accession numbers of the hydrocarbon degrading bacteria isolated were *Bacillus cohnii* B2 (KF156796), *Bacillus methanolicus* B4 (KF156797), *Bacillus cereus* Co1 (KF156798), *Rheinheimera* sp. Co4 (KF156799), *Rheinheimera* sp.Co6 (KF156800), *Bacillus firmus* CoB1 (KF156801), *Bacillus aquimaris* CoB4 (KF156802) and *Bacillus thuringiensis* T3 (KF156803) (Table 5.5).

5B.2.8. Statistical analysis:

All experiments were conducted in triplicate and analyzed with t-test using GraphPad Prism 5 software.

5B.3. Results and Discussion

In addition to emulsification activity that the bioemulsifier AM1 possesses, its crude oil dispersion and hydrocarbon solubilization ability were also checked to evaluate its role in the environment. In this section, the interactions between the significant ecophysiological groups of bacteria were the focus of study. For this, hydrocarbon degrading bacteria from the same intertidal niche as that of *S. silvestris* AM1 were isolated, screened for BTX degrading ability to select a special strain to be used in the microcosm studies.

5B.3.1. Crude-oil dispersion:

The results with crude bioemulsifier of *S. Silvestris* AM1, shows that the bioemulsifier dispersed the crude oil efficiently with respect to the control within a time period of 72h (Figure 5.22 and 5.23). The crudeoil attached to the walls was also significantly reduced with repect to the test flask (Figure 5.22).

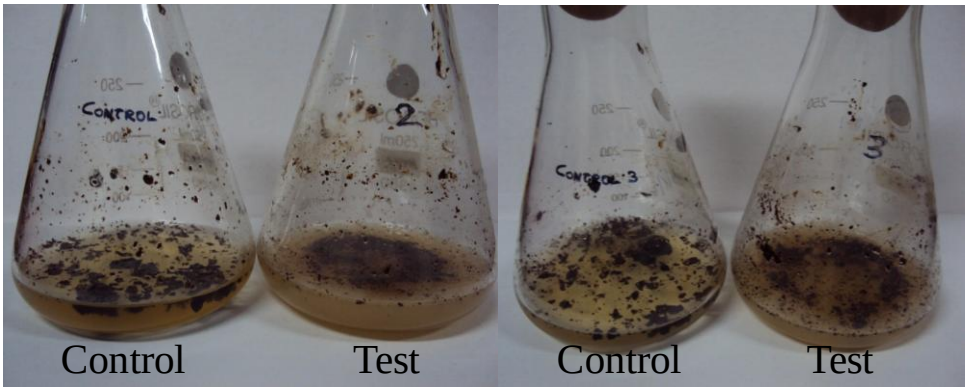


Figure 5.22. Crude oil dispersion assay of bioemulsifier AM1

5B.3.2. Hydrocarbon solubilization studies:

The water immiscible hydrocarbons coalesce generally in aqueous environment and will be distributed at the air-water interface due to their low density than water. As discussed in chapter 1, bioemulsifiers are known to increase the bioavailability of water immiscible carbon sources by solubilizing these compounds. While studying bioemulsifier from *Acinetobacter radioresistens* KA53, Barkay et al., (1999) reported an enhanced solubilization of polyaromatic hydrocarbons (PAHs).

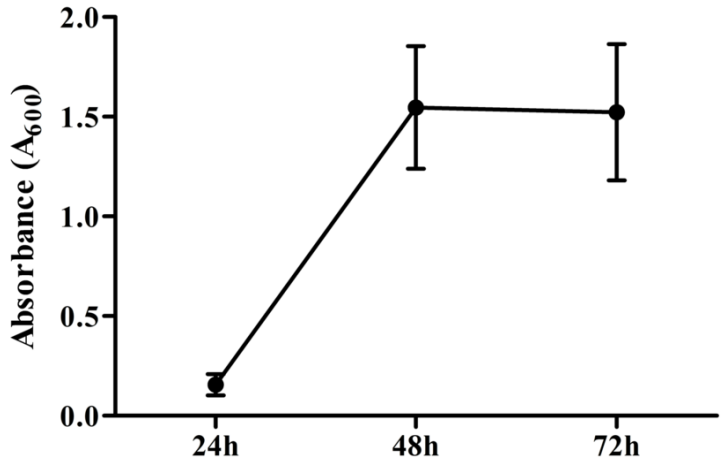


Figure 5.23. Crude oil dispersion assay of crude bioemulsifier AM1. Loss of transmittance of light in the test flask containing bioemulsifier with respect to 100% transmittance in control flasks where bioemulsifier was absent.

5B.3.2.1. Solubilization of aliphatic and aromatic hydrocarbons by crude bioemulsifier AM1:

The effect of crude bioemulsifier AM1 on the apparent aqueous solubility of benzene and hexane (both 5mg/ml) was determined. The solubilization was checked at the respective λ_{max} of the hydrocarbons. In aqueous environment, the solubilization of benzene and hexane is negligible as shown in Figure 5.24. The negative value for benzene may be because of clearing of the medium due to precipitation by benzene. Notably, the presence of crude bioemulsifier increases the solubilization of both the hydrocarbons hexane and benzene significantly.

5B.3.2.2 Solubilization of hydrocarbons by partially purified bioemulsifier AM1:

When 50mg partially purified bioemulsifier AM1 was checked for its ability to solubilize the hydrocarbons BTX, the hydrocarbons retained by the medium in presence of bioemulsifier was similar to that of crude bioemulsifier (Figure 5.25). The concentration of bioemulsifier was directly proportional to the amount of solubilization taking place. Toluene has higher ability to be retained in the medium than the other two components of BTX, thus giving higher readings even in blank while the high volatility of xylene makes it absent from the medium in absence of bioemulsifier. A point to mention here is that the solubilization assay of Barkay et al., (1999) is time bound and in environment as the time increases, the amount of hydrocarbons solubilized also increases.

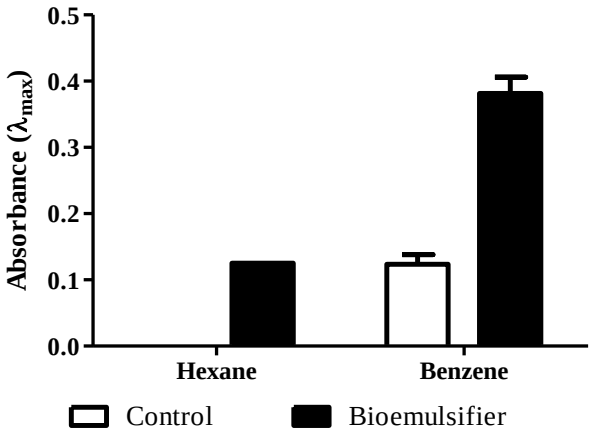


Figure 5.24. Solubilization of hexane (black bars) and benzene (white bars) in presence and absence of crude bioemulsifier AM1. (λ_{max} for hexane=202.5 nm and λ_{max} for benzene=280 nm).

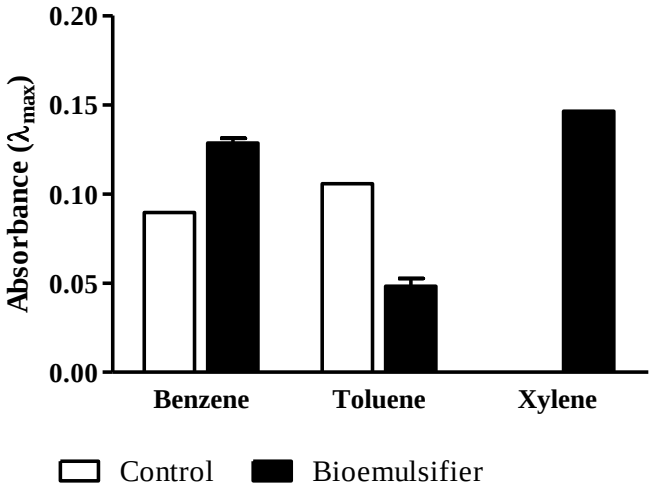


Figure 5.25. Solubilization of benzene, toluene and xylene (all 30mg/ml) in presence of 50 µg/ml of partially purified bioemulsifier AM1

Thus from these studies, it can be seen that bioemulsifier AM1 increases the solubility of hydrocarbons like BTX. The concentration of bioemulsifier Alasan (produced by *A. radioresistens* KA53) reported by Barkay *et al*, 1999 was 500µg/ml and of polyaromatic hydrocarbon used was 60µg/ml. In a similar report by (Toren, *et al.*, 2002), they used 40 µg/ml of Alasan and about 100 µg/ml hydrocarbons.

However, in present study solubilization of BTX was shown by using 30mg/ml of hydrocarbons and 100 µg/ml of bioemulsifier AM1. Effective solubilization even after using such a high concentration of hydrocarbons indicates the high efficiency of bioemulsifier AM1 in solubilization as compared to Alasan. Also from these studies, it can be proposed that bioemulsifier AM1 may have probable role in increasing the bioavailabilty of hydrocarbons in aqueous environment for degradation by microorganisms, suggesting bioremediation potential of bioemulsifier AM1.

5B.3.3. Effect of bioemulsifier AM1 on growth of hydrocarbon degrading bacteria from foreign habitat:

Bacillus sp. AV8 was isolated from Nereis (Marine tubeworm) gut. This bacterium can degrade wide variety of hydrocarbons such as benz(k)fluoranthene, benz(k)pyrene, chrysene, dibenz(a,h)anthracene, fluorine, naphthalene, phenanthrene, pyrene and 2,4,6-trichlorophenol. The hydrocarbons used for this study were selected from both aliphatic (hexane, decane, hexadecane and heptane) and aromatic (benzene, toluene, xylene and trichlorobenzene) group of hydrocarbons. *Bacillus* sp. AV8 exhibited almost equal amount of growth using hexane and hexadecane as sole source of carbon in the absence and presence of bioemulsifier AM1 (5B.6a) while its growth was enhanced in presence of bioemulsifier AM1 with benzene and trichlorobenzene as sole source of carbon (Figure 5.26).

Growth of *Bacillus* sp.AV8 in presence of decane, heptane, toluene and xylene as sole source of carbon showed negative results indicating toxicity or starvation (Figure 5.26c). It can be observed that *Bacillus* sp. AV8 shows higher growth using the specified hydrocarbons as sole source of carbon in the absence of bioemulsifier AM1 as compared to in the presence of bioemulsifier (Figure 5.26). Thus it can be indirectly concluded that either bioemulsifier AM1 was not solubilizing those hydrocarbons adequately to enhance the growth of degrading bacteria, or the degrading bacteria was unable to take-up the solubilised hydrocarbons. Since bioavailability of these two hydrocarbons is scarce in the aqueous environment, *Bacillus* sp. AV8 shows either same growth profile in presence and in absence of bioemulsifier AM1 or shows negative growth due to unavailability of solubilized hydrocarbons because of its surface-non compatibility with the hydrocarbon-emulsifier micelle complex.

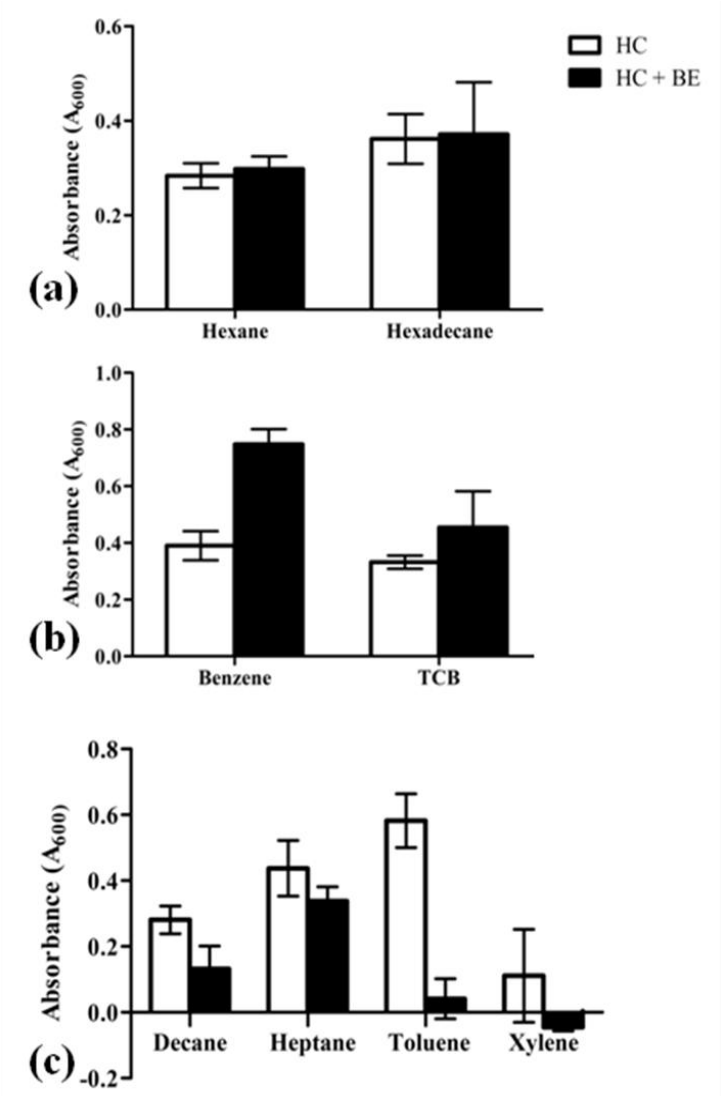


Figure 5.26. Effect of bioemulsifier AM1 on growth of *Bacillus* sp. AV8 from foreign habitat. Hydrocarbon concentration= 5mg/ml.(a) no effects, (b) enhancement and (c) inhibition of growth in presence of bioemulsifier AM1

In order to get effective solubilization, there should be compatibility between bioemulsifier and hydrocarbons and also between bioemulsifier and the surface of the degrader. If there is incompatibility between any of the two factors involved in solubilization then there is reduced amount of solubilisation and growth. The factor involved in development of this incompatibility can be attributed to amount of hydrophobicity possessed by the bioemulsifier. Thus, in the presence of bioemulsifier AM1 due to its incompatibility with the given hydrocarbons the bioavailability of these hydrocarbons was not increased.

5B.3.3.1. Effect of bioemulsifier AM1 on growth of hydrocarbon degrading bacteria from shared habitat:

5B.3.3.1.1. Isolation and characterization of hydrocarbon degrading bacteria from Vellar estuary sample:

S. silvestris AM1 was isolated from estuarine sediment sample of Vellar estuary, Parngipettai, Tamil Nadu, India. The estuary is constantly polluted by four channels that contaminate the estuary. This soil sample was used to isolate hydrocarbon degrading bacteria present in the same environment of *S. silvestris* AM1. Ecophysiological significance of bioemulsifier AM1 with respect to other organisms namely hydrocarbon degrading bacteria in the shared habitat of bioemulsifier producing bacteria *S.silvestris* AM1 was studied here.

As the isolation process involved hydrocarbons (benzene, toluene, crude oil and crude oil plus benzene) as the sole source of carbon, the isolates obtained were presumed to be hydrocarbon utilizers. The list of isolates obtained on the basis of sole carbon sources used and hydrocarbons used are given in table 5.4:

Table 5.4. List of isolates along with sole carbon source used to in their isolation procedure.

Benzene	Toluene	Crude Oil	Crude Oil + Benzene
B1	T1	Co1	CoB1
B2	T2	Co2	CoB2
B3	T3	Co3	CoB3
B4	T4	Co4	CoB4
B5	T5	Co5	CoB5
B6		Co6	
		Co7	

The above 23 isolates were then screened for their growth using combination of benzene+toluene and crude oil as source of carbon (Figure 5.27). As the isolation strategy included selective isolation of degraders, all isolates showed growth in presence of crude oil and benzene + toluene. Isolates B1, B2, B3, B4, Co1, Co4, CoB2, CoB3, CoB4 and T1 showed good growth in presence of crude oil as sole carbon source and Isolates B1, B4, B5, B6, Co3, Co4, Co6, CoB1, CoB3, CoB4 and T2 showed good growth in presence of benzene and toluene as source of carbon (Figure 5.27). It was observed that isolates Co1, CoB1, CoB4, CoB5, T2 and T3

showed biofilm formation in crude oil while rest showed dispersion of crude oil (Figure 5.28)..

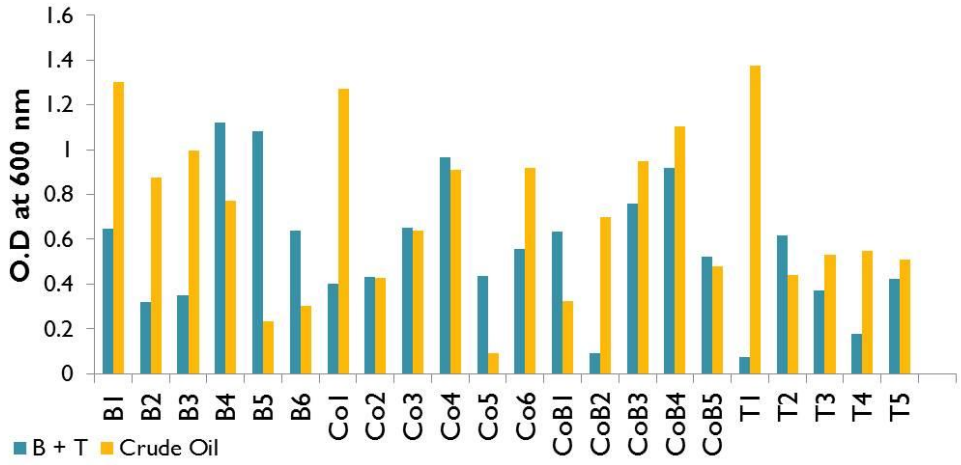


Figure 5.27. Hydrocarbon degrading isolates screened for growth in crude oil and benzene+toluene (B+T) as only carbon sources.

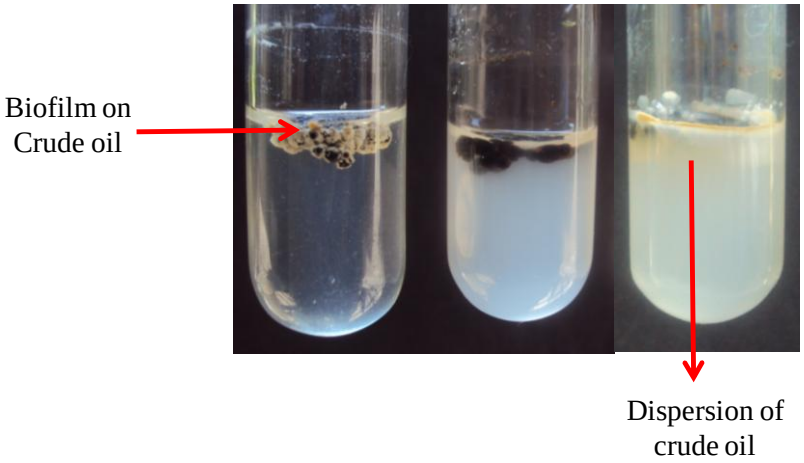


Figure 5.28. Biofilm formation and dispersion of crude oil by the hydrocarbon degrading isolates.

Further the 23 isolates were screened for their surface tension reducing ability, emulsification activity and also for their inhibitory activity against *S. silvestris* AM1 (Figure 5.29). From figure 5.29 it can be seen that isolates Co3, CoB1, CoB2 and CoB3 decreased surface tension of the growth medium ($1/10^{th}$ ZM medium) while isolates B3, Co3, Co5 and Co7 showed bioemulsifier production. Isolates CoB3, CoB4, T1 and T2 inhibited growth of *S. silvestris* AM1**.**

Isolate	Inhibition	Biofilm
Medium		
B1	No	No
B2	No	No
B3	No	No
B4	No	No
B5	No	No
B6	No	No
CO1	No	Yes
CO2	No	No
CO3	No	No
CO4	No	No
CO5	No	No
CO6	No	No
CO7	No	No
COB1	No	Yes
COB2	Yes	No
COB3	Yes	No
COB4	No	Yes
COB5	Yes	Yes
T1	Yes	No
T2	No	Yes
T3	No	Yes
T4	No	No
T5	No	No

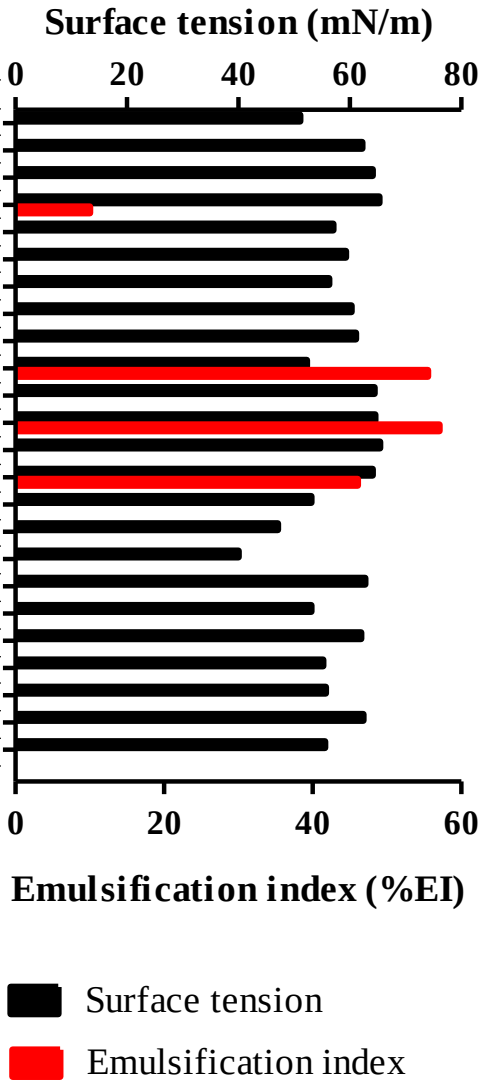


Figure 5.29. Biofilm formation, surface tension reduction, emulsification and inhibition against *S. silvestris* AM1 of the 23 hydrocarbon degrading isolates.

As shown in Figure 5.27, isolates B1-B4, Co1, Co4, Co6, Co7, CoB3, CoB4 and T1 showed good growth in presence of hydrocarbons as the sole carbon source in Bushnell Haas medium. Isolates B1, B2, B4 and Co4 showed pigmentation and precipitation in presence of hydrocarbons. Isolates B3, Co3, Co5 and Co7 produced bioemulsifier and showed %EI of 10, 55.5, 57.1 and 46.1 respectively. Isolate CoB2 and CoB3 showed BS production (47.1 and 40mN/m respectively). Isolates Co1, CoB4 and CoB showed biofilm formation while isolates CoB2, CoB3, CoB5 and T1 exhibited antagonism to *S.silvestris* AM1. Thus isolates showing emulsification, surface tension reduction and antagonism towards bioemulsifier producer were not taken for further studies.

All the 23 isolates were highly oligotrophic showing no growth in medium rich with nutrients. Out of 23 isolates, 5 isolates could not be subcultured further. 16S rRNA gene sequence analysis of the 18 isolates and ARDRA was performed by using *Alu* I, *Hha* I and *Msp* I. Upon ARDRA analysis , the hydrocarbon degrading isolates gave thirteen distinct OTUs (Figure 5.30).

Dendrogram constructed from ARDRA profile of the 18 isolates were used as criteria for excluding certain isolates from further studies. Since the aim of this study is to determine the role played by bioemulsifier AM1 in assisting hydrocarbon degrading bacteria isolated from the shared environment of *S. silvestris* AM1, the isolates which showed the ability to reduce surface tension, exhibited emulsification, inhibited growth of *S. silvestris* AM1 were not selected for further studies. The isolates B1, B3, B5, B6, Co2, Co3, Co5, Co7, CoB2, CoB3, CoB5, T1, T2, T4 and T5 were the ones which were excluded from further studies.

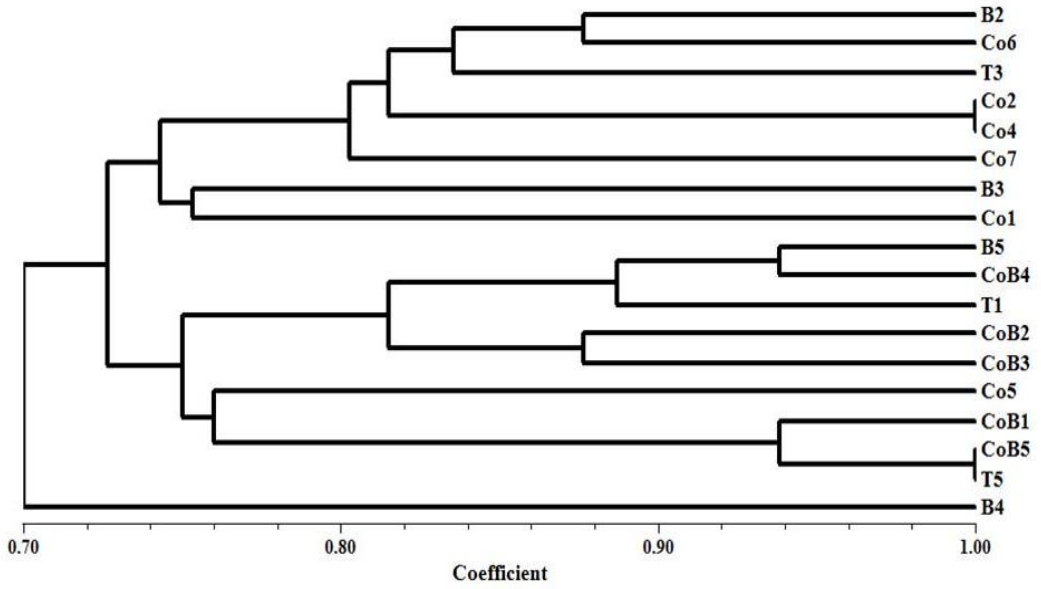


Figure 5.30. Dendrogram based on ARDRA profile of the 23 hydrocarbon degrading isolates

After ARDRA analysis, four biofilm forming isolates (Co1, CoB1, CoB4, T3) capable and four biofilm non forming isolates (B2, B4, Co4, Co6) were selected for 16S rDNA sequencing. The results of the sequencing are given in table 5.5.

The isolate Co6 along with isolate Co4 were the only Gram negative bacteria isolated from estuarine sample. Isolate Co6 showed high identity with *Rheinheimera aquimaris* SW-353 (NR_044068) and therefore considered as genus *Rheinheimera*

(Table 5.5). Out of the 4 biofilm forming isolates, Co1 was selected and out of the 4 non-biofilm forming isolates Co6 was selected for further studies. When tested for growth in utilizing BTX, Co6 showed better growth than Co1 (Figure 5.31).

Table 5.5. Identification of selected eight hydrocarbon degrading isolates based on 16S rRNA gene sequencing.

Isolate	Closest match	Identity %	Coverage %	GenBank Accession number
B2	*Bacillus cohnii* DSM 6307 (NR_026138)	99%	98%	*Bacillus cohnii* B2 (KF156796)
B4	*Bacillus methanolicus* NCIMB 12524 (AB112729)	100%	85%	*Bacillus methanolicus* B4 (KF156797)
Co1	*Bacillus cereus* ATCC 14579 (NR_074540)	99%	97%	*Bacillus cereus* Co1 (KF156798)
Co4	*Rheinheimera nanhaiensis* E407-8 (FJ169968)	72%	83%	*Rheinheimera* sp. Co4 (KF156799)
Co6	*Rheinheimera aquimaris* SW-353 (NR_044068)	83%	87%	*Rheinheimera* sp. Co6 (KF156800)
CoB1	*Bacillus firmus* clone B563 (DQ290000)	98%	99%	*Bacillus firmus* CoB1 (KF156801)
CoB4	*Bacillus aquimaris* BVC63 (JQ407795)	91%	92%	*Bacillus aquimaris* CoB4 (KF156802)
T3	*Bacillus thuringiensis* WS2617 (Z84585)	100%	99%	*Bacillus thuringiensis* T3 (KF156803)

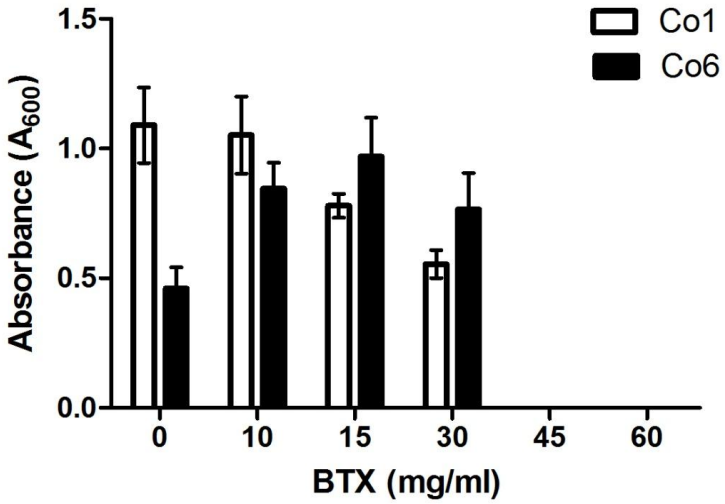


Figure 5.31. Growth of hydrocarbon degrading isolates Co1 and Co6 on BTX.

5B.3.3.1.2. Microcosm studies:

When consortia are used for microcosm studies, it is difficult to characterize the effect of enhancement of degradation with respect to a single bacterium. Therefore the microcosm interaction studies were performed with individual bioemulsifier producing *S. silvestris* AM1 and non-bioemulsifier producing, hydrocarbon degrading isolate *Rheinheimera* spp. Co6 isolates. Figure 5.32 depicts the microcosm experiment.

Figure 5.32. Microcosm setup to study the interaction between bioemulsifier producing *S. silvestris* AM1 and its bioemulsifier with hydrocarbon degrading *Rheinheimera* sp.Co6

The gas chromatography results of each microcosm experimental system are given in the figure 5.33. As per figure 5.33g, considering the residual hydrocarbon concentration in control (M+HC) as 100%, the residual hydrocarbon concentration reduced by 45.3% in benzene, 51.6% in toluene and 82.8% in xylene when *Rheinheimera* sp.Co6 was inoculated in the microcosm(M+HC+D). Similarly these figures were 50.9%, 47% and 72.2% for benzene, toluene and xylene respectively when *S. silvestris* AM1 was inoculated (M+HC+P). The residual hydrocarbon concentration did not show much change in above two cases. These values were similar to the microcosm (M+HC+D+P) where both the degrader and the producer were co-inoculated, where the residual hydrocarbon concentration was 55%, 63.5% and 64.9% for benzene, toluene and xylene respectively. So, the presence of both *Rheinheimera* sp.Co6 and *S.silvestris* AM1 could utilize 9 – 35ppm of hydrocarbons supplemented individually as well as when co-inoculated. However, interestingly as

was hypothesized the supplementation of purified bioemulsifier AM1 in the microcosm along with the degrader *Rheinheimera* sp.Co6 alone significantly (**=$p<0.01$) reduced the residual hydrocarbons to 23.6%, 33.3% and 36.5% for benzene, toluene and xylene respectively. Generally, the hydrocarbon utilization was 23-82%. This degradation of BTX in presence of both the degrader and bioemulsifier producer may be due to many factors from competition to cooperation.

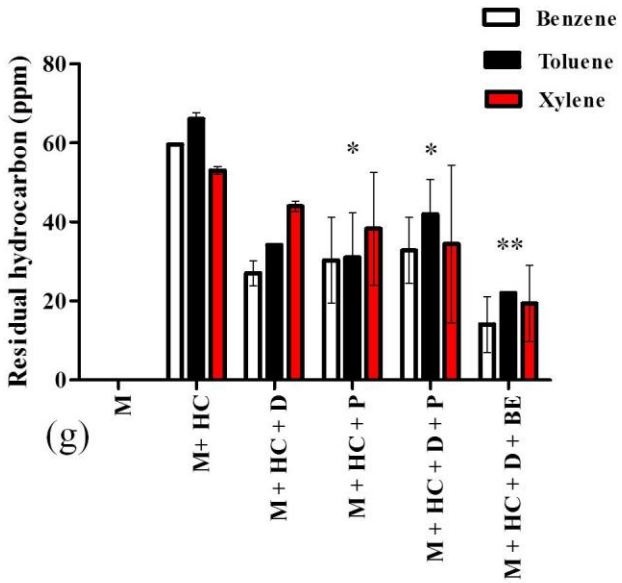
In order to demonstrate the growth of the bacteria under study in the microcosms; based on antibiotic resistance pattern of *S. silvestris* AM1 (streptomycin resistance) and *Rheinheimera* sp. Co6 (ampicillin resistance) selective agar plates were prepared to estimate their growth in terms of CFU/ml (Table 5.6). As observed, the growth was stably maintained in all the microcosms inoculated and it was reiterated that the organisms did not have any negative effects on each others growth.

To check the emulsification activity in the residual slurries of the microcosms, the %EI was checked. As expected, the %EI of *S. silvestris* AM1 in microcosm (M+P) was highest. Presence of bioemulsifier in the microcosm (M+BE, M+HC+BE, M+BE+D and M+HC+BE+D) gave 0 - 34%EI (Figure 5.34).

Mukherjee & Bordoloi (2012) had reported the essential presence of minimum amount of phosphate and nitrate sources in the microcosms. Here, $1/10^{th}$ ZM medium was used since *S. silvestris* AM1 could produce bioemulsifier only in ZM medium. A member of ecohpysiological group of aerobic, mesophilic, heterotrophic, sporulating, bacteria, *S. silvestris* AM1 showed brisk growth in absence of the hydrocarbon. The residual hydrocarbon remaining after its growth in presence of BTX showed that hydrocarbons were utilized by bioemulsifier producer also. The significantly higher degradation (lower retention of residual hydrocarbons) of BTX by the producer (*S. silvestris* AM1) can be expected as the bioemulsifier producers are known to possess higher utilization capability owing to their products.

Experiment with microcosms

Microcosm	Hydrocarbon	Area	Area%	Result (ppm)
Medium control	Benzene	63177	1.1002	Not detected
	Toluene	Not detected	Not detected	Not detected
	Xylene	Not detected	Not detected	Not detected
(b) M+HC	Benzene	2647848	24.2973	60.15
	Toluene	1810957	16.6178	66.89
	Xylene	435723	3.9983	54.74
(c) M+HC+D	Benzene	949127	13.6261	21.48
	Toluene	917127	13.1667	33.75
	Xylene	333333	4.7855	41.46
(d) M+HC+P	Benzene	2188579	20.6957	49.23
	Toluene	1379635	13.0462	50.56
	Xylene	506040	4.7852	63.01
(e) M+HC+P+D	Benzene	2092780	20.0595	47.29
	Toluene	1558294	14.9364	57.26
	Xylene	552870	5.2993	69.15



Experiment	System
M	Sediment+ Sand+ Medium mixture
M+HC	M+ Hydrocarbon (HC)
M+HC+D	M+ HC+BTX degrading bacteria (D)
M+HC+P	M+ HC+Bioemulsifier producing bacteria (P)
M+HC+P+D	M+HC+P+D
M+HC+BE+D	M+ HC+Purified bioemulsifier AM1(BE) +D

Figure 5.33. Residual hydrocarbon analysis of the microcosms by Gas Chromatography. GC data of residual hydrocarbons in terms of area, area% and ppm of hydrocarbons in microcosms a- f; (g) Graphical representation of the residual hydrocarbons(ppm) in microcosms a- f. (P value: *<0.05; **<0.01).

Table : 5.6. Growth of bacteria in different microcosms

Microcosms	Bacteria inoculated	CFU/ml
M+P	*S. silvestis* AM1	$15x10^3$
M+D	*Rheinheimera* sp.Co6	$9\ x10^2$
M+HC+P	*S. silvestis* AM1	$2x\ 10^3$
M+HC+D	*Rheinheimera* sp.Co6	$45x10^3$
M+D+BE	*Rheinheimera* sp.Co6	$1x10^2$
M+HC+D+BE	*Rheinheimera* sp.Co6	$60x\ 10^3$
M+HC+D+P	*Rheinheimera* sp.Co6	$2x10^3$
	S. silvestis AM1	$7x10^3$

D- *Rheinheimera* sp. Co6 and P- *S. silvestis* AM1

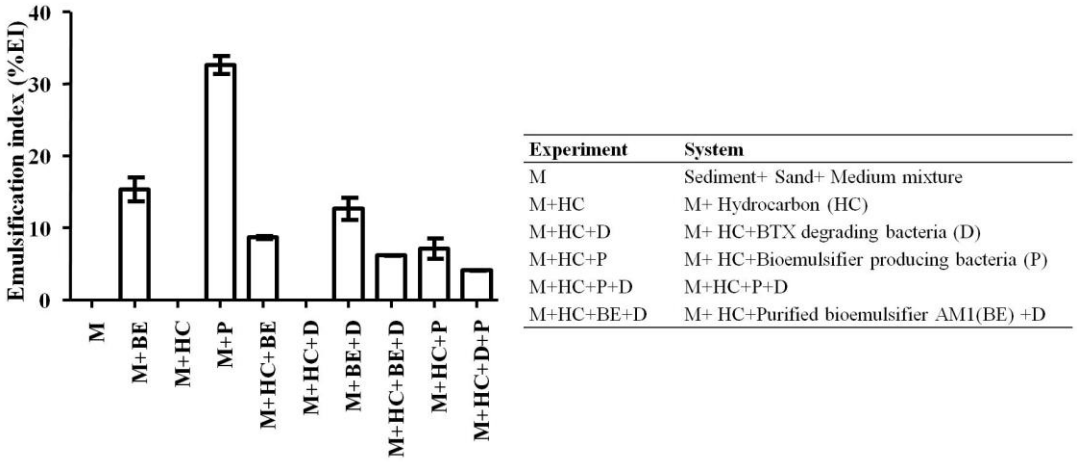


Figure 5.34. Emulsification activity in residual slurry of microcosms. M-Medium, BE- bioemulsifier, HC- hydrocarbon, D- *Rheinheimera* sp. Co6, P- *S.silvestris* AM1

From the concentration of BTX used in the present study almost 60-70% degradation was seen with the help of a single degrader in presence of the bioemulsifier. It can be concluded from microcosm studies that the ecophysiological group of bacteria under study can efficiently interact and influence other group of microorganisms in this intertidal zone environment. Enhancement of degradation by the degraders has been reported previously by other reports also (Mukherjee & Bordoloi, 2012). But influence and direct impact of presence of this ecophysiological group of bacteria can be significant in the natural environments where even the

bioemulsifier producing bacterium is benefited in presence of the degrader for its growth in higher toxic environments.

Lüttge & Scarano (2004) reported that ecophysiological performance varied largely at intra specific level in time and space, often (but not always) at inter-species level, ecophysiological performance was related to species dominance in the community. As seen in the present study, the presence of xenobiotics may select the bacteria capable of degrading and not completely wiping out the other bacteria which are shielded by the degrader's presence and constantly supporting the growth of the degraders. The bioemulsifier produced by these bacteria can enhance the bioremediation process in their habitat as seen in microcosm experiments.

5B.3.4. Effect of bioemulsifier AM1 on biofilm formation of other bacteria:

S. aureus ATCC 6538, was checked for its ability to form biofilm in presence of bioemulsifier AM1 in its optimized medium for biofilm formation (Tryptic soy Glucose broth, TSGB). Figure 5.35 shows that addition of bioemulsifier AM1 enhances biofilm formation significantly. *Paracoccus sp.* W1b displays maximum biofilm formation in presence of MM 2 medium (Srinandan et al., 2010). Figure 5.35 shows that in the absence of AM1 bioemulsifier, *Paracoccus sp.* W1b exhibited more biofilm formation than in the presence of bioemulsifier AM1. The conditioning film produced by the bioemulsifier AM1 or the orientation of the bioemulsifier on the surface of *Paracoccus* sp. W1b may be resulting or affecting the attachment of *Paracoccus sp.* W1b to the surface and hence decreasing its biofilm. Both the isolates showed almost revertion of biofilms after treatment with Proteinase K.

S. aureus produces maximum biofilm in presence of glucose in the Tryptic soy broth (TSB). However the results of this study clearly indicate that in the presence of AM1 bioemulsifier the biofilm formation of *S. aureus* ATCC 6538 is almost twice its maximum value. This suggests that the AM1 bioemulsifier facilitates the increased attachment of the *S. aureus* ATCC 6538 to the surface as a result of which there is enhanced biofilm formation. Also, pre-existing AM1 bioemulsifier in environment must be forming conditioning film over surfaces. When compatible microorganisms would come in the vicinity of such 'conditioned' surfaces there attachment to the surfaces would be enhanced by the bioemulsifier. Here the bioemulsifier would act as anchoring agents which will facilitate the building of a

stable biofilm. Also it can be concluded that when non-compatible microorganisms would come in the vicinity of such 'conditioned' surfaces, their attachment would be vetoed to the surfaces thus preventing biofilm formation. Addition of proteinase K decreased the biofilm of *S. aureus* ATCC6538 while in case of *Paracoccus* sp.W1b, the proteinase K treatment did not affect the biofilm formed.

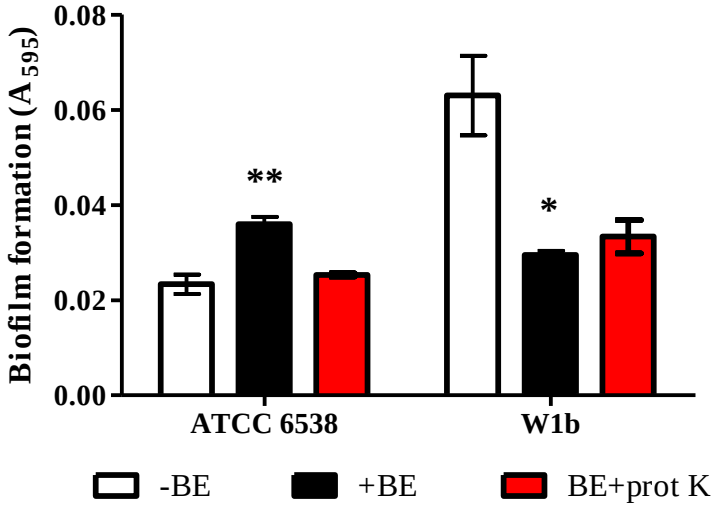


Figure 5.35. Biofilm formation of *Staphylococcus aureus* ATCC 6538 and *Paracoccus* sp. W1b in presence of bioemulsifier AM1 (BE). (**= P<0.005; P<0.05).

Thus, the results from microcosm experiments suggests that the bioemulsifier AM1 has positive influence on degradation of hydrocarbons by hydrocarbon degrading bacteria of the same habitat as the bioemulsifier producer, *S. silvestris* AM1. The bioemulsifier AM1 also affected the biofilm forming bacteria by enhacing or decreasing their biofilms.

In ecophysiological terms, the interaction involved two groups of bioemulsifier producing and hydrocarbon degrading bacteria inhabiting same niche of the environment in this case, intertidal zone of Vellar estuary. The interaction mechanism involved the bioemulsifier which helps in degradation of hydrocarbons by bacteria from the same habitat as *S. silvestris* AM1 as the degradation potential of the degrader is increased in presence of bioemulsifier. Significantly the biofilm formation in certain bacteria show differential responses towards the presence of bioemulsifier AM1 in their environment.

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APPENDIX

Appendix

Media

- **Zobell Marine (ZM-2216; HiMedia, India) medium (in g%, wt/vol):**
  Peptic digest of animal tissue, 0.5; Yeast extract, 0.1; Ferric citrate, 0.01; NaCl, 1.945; $MgCl_2$, 0.88; Na_2SO_4, 0.324; $CaCl_2$, 0.18; KCl, 0.055; Na_2CO_3, 0.016; KBr, 0.008; $SrCl_2$, 0.0034; H_3BO_3, 0.0022; Na_2SiO_3, 0.0004; NaF, 0.00024; $(NH_4)_2NO_3$, 0.00016; Na_2HPO_4, 0.0008 and pH 7.6±0.2 in 100 ml Distilled water

- **Synthetic marine salt solution (in g%, wt/vol):**
  NaCl, 1.945; $MgCl_2$, 0.88; Na_2SO_4, 0.324; $CaCl_2$, 0.18; KCl, 0.055; Na_2CO_3, 0.016; KBr, 0.008; $SrCl_2$, 0.0034; H_3BO_3, 0.0022; Na_2SiO_3, 0.0004; NaF, 0.00024; $(NH_4)_2NO_3$, 0.00016; Na_2HPO_4, 0.0008 and pH 7.0 ± 0.2 in 100 ml Distilled water.

- **Nutrient broth (in g%, wt/vol):**

  Beef extract, 0.3; Peptone, 0.5 and NaCl, 0.5

- **Luria Bertani (HiMedia, India) medium (in g%, wt/vol):**
  Casein enzymic hydrolysate, 1; Yeast extract, 0.5; NaCl, 0.5 and pH 7.0±0.2 in 100 ml Distilled water.

- **Phosphate Buffer Saline i.e.PBS**
  137 mM NaCl , 2.7 mM KCl ,10mM Na_2HPO_4 , 2 mM KH_2PO_4 at pH 7.3

- **Bushnell Haas Medium (BHM):**
  $MgCl_2$, 0.02g%; $CaCl_2$, 0.002g%; KH_2PO_4, 0.1g%; K_2HPO_4, 0.1g%; NH_4NO_2, 0.1g%; $FeCl_3$, 0.005g%; pH 7.0 (± 0.2).

- **Production media (all wt/vol or g%):**

  - Pfiffner, *et al.* (1986) in g%, wt/vol: Medium E containing NaCl, 5.0; (NH4)2SO4, 0.1; MgSO4, 0.05 and sucrose, 1.0; in 100 mM phosphate buffer (pH 7.0) supplemented with NaNO3, 0.1 and yeast extract, 0.05.

- Cooper & Goldenberg (1987) in g%, wt/vol: KH_2PO_4, 0.3; Na_2HPO_4 0.6; $(NH_4)_2SO_4$ 0.1; sucrose 1.0; tryptic soy, 0.4; yeast extract, 0.01; and trace amounts of sodium EDTA, $FeSO_4$, $CaCO_3$, $MgSO_4$, and $MnSO_4$.

- Gurjar, *et al.* (1995) in g%, wt/vol: Peptone, 1; Beef extract, 1g; NaCl, 0.5 and crude oil 2 with 7.0 pH.

- Yun & Park (2003) in g%, wt/vol: Glucose, 2; Bacto-peptone 0.3; $MgSO_4$· 7H2O, 0.05; KH2PO4, 0.03 and K2HPO4, 0.07.

- K Jenny's medium (Suthar *et al*., 2008) in g%, wt/vol: Glucose, 1g; NaNO3, 0.28; KCl, 0.05; MgSO4 0.02; EDTA, 0.02; CaCl2·2H2O, 0.003; 84% H3PO3, 0.2ml; ZnSO4·7H2O, 0.08mg%; MgSO4·7H2O 0.02; H3BO3 6µg%; NaMoO4, 2µg%; CuSO4·5H2O, 0.01mg%; CoCl2·6H2O, 0.01mg%; F2SO4·7H2O, 0.03mg%; NaCl, 3.5 and pH 7.0.

- **Emulsan production medium reported by Goldman *et al.* (1982):** Constituents in g%, wt/vol of K_2HP0_4 .$3H_20$, 2.22; KH_2PO_4, 0.726; $MgSO_4$.$7H_20$, 0.02; $(NH_4)_2SO_4$, 0.4

Electroporation

- Growth medium: Luria broth + 0.5M Sorbitol
- Washing solution: 0.5M Sorbitol + 0.5M Mannitol + 10% Glycerol
- Electroporation solution: 0.5M Sorbitol + 0.5M Mannitol + 10% Glycerol
- Outgrowth medium: Luria broth+ 0.5M Sorbitol + 0.38M Mannitol

SUMMARY

मोहं ऋणद्धि विमलीकुरुते च बुद्धिं,
सूते च संस्कृत पदव्यवहारशक्तिं।
शास्त्रांतराभ्यसन योग्यतया युनक्ति
तर्कश्रमो न तनुते किमिहोपकारं॥
-विश्वगुणादर्श चंपू ५५६

Meaning:
The study of Tarka-shastra (science of dialectics, logic, reasoning and art of debate for analyzes of subjects) removes delusions, cleans the mind and helps us to talk in the words of civilized. Makes us eligible to study other subjects and hence it helps us the most.

SUMMARY

Chapter 2:

Isolation and screening of bioemulsifier producing *Bacillus* sp. and characterization of the selected strain

2A. Isolation and screening of bioemulsifier producing *Bacillus* sp.

- For the isolation of ecophysiological group of Gram positive sporulating, bioemulsifier/biosurfactant producing, biofilm forming, quorum quenching bacteria from intertidal zone of Indian coast 16 water and sediment samples were collected from 12 sampling sites of 6 coastal states of India.
- 227 isolates were screened to get 54 isolates giving emulsification and 40 isolates exhibiting haemolysis. From these, 14 isolates were selected on the basis of emulsification index (%EI) above 40%.
- Isolates AM1, CAM11, T12, B8, V5, V6, Gh13 M12 and V2 showed effective emulsification of 43-62.5% EI while reduction of surface tension by isolates Vh13, Gh13, CAM11 and V6 was highest between 30-23. Also isolates CAM11,V2, AM1, Vh5, V6, CAo3 and Gh13 showed quorum quenching activity and isolates V2, CAo3, V6, T12, CAM11 and Vh13 showed biofilm formation.
- After clustering the fourteen isolates on the basis of ARDRA, twelve isolates were subjected to 16S rRNA gene sequencing and identified as *Bacillus* , *Lysinibacillus Solibacillus* and *Sporosarciona* spp. and their sequences were deposited in NCBI GenBank.
- Though all the twelve formed biofilm and possessed quorum quenching ability, biofilm forming ability was best in V2 and quorum quenching ability was best in CAM11.
- This is the first time, bioemulsifier production by *Solibacillus, Sporosarcina, Lysinibacillus, B.thuringiensis* and *B.flexus* with emulsification activity is reported.

- *Bacillus thuringiensis* strain Gh13, *Solibacillus silvestris* strain AM1 and *Sporosarcina soli* strain M12 were checked with four aliphatic, four aromatic hydrocarbons and six oils for emulsification. Isolate AM1 and isolate Gh13 showed maximum emulsification of 68.11% and 56.7% respectively in trichlorobenzene, and isolate M12 gave maximum of 52.3% in heptane.

2B. Characterization of the selected strain of *Bacillus* sp.

- *S. silvestris* AM1 was selected for further studies since it gave maximum % EI. Phenotypic characterization based on biochemical tests of isolate AM1 supported 16S rDNA sequencing identification as *S. silvestris*.

- Results of Biolog test of *S. silvestris* AM1 showed that it could utilize compounds like acetic acid, pyruvic acid and hydroxybutyric acid, etc. But it could not utilize glucose and its derivatives (α-D-glucose, D-gluconic acid, α-D-glucose-1-phosphate, D-glucose-6-phosphate, 3-methyl glucose, α-methyl-D-glucoside, β-methyl-D-glucoside), fructose (D-fructose, D-fructose-6-monophosphate) and sucrose as sole carbon source.

- After DNA-DNA hybridization, type strain *S. silvestris* HR3-23 (MCC2084^T) and *S. silvestris* AM1 were found to be 80% similar. FAME analysis of the two strains also showed high similarity, thus confirming the identity of *S. silvestris* AM1.

- The genus *Solibacillus* has only one species listed, *S. silvestris*. When 49 sequences of 16S rRNA gene of *Solibacillus silvestris* was downloaded from GenBank and was analyzed for evolutionary relationships, all the three intertidal zone strains of *Solibacillus* sp. isolated in this study namely AM1, V2 and B8 clustered differently with respect to the type strain HR3-23. These three strains also showed significant transition/transversion (ti/tv) bias, a known property for evolution of DNA sequences with respect to the type strain *S. silvestris* HR3-23.

- The analysis done here also shows the distinct branching of some of the strains like DFM76b, DWM125a, DEM132, StLB306 and R-26228 in different method of tree construction. Thus many of the strains included in GenBank

may be considered for a new species other than *S. silvestris* however, further studies are needed to conclude this.

Chapter 3:

Factors influencing the production of bioemulsifier by *S. silvestris* AM1

- Optimum condition for bioemulsifier production by *S. silvestris* AM1 was found to be pH 7.4 at a temperature of 35°C. The bioemulsifier production was also not affected between pH range of 6-8 and temperature range of 30-37°C with resistance of upto 7%NaCl concentration.
- The bioemulsifier production by *S. silvestris* AM1 was very less or negligible in other production media reported in literature.
- The bioemulsifier production was optimum at 1% inoculum of 12-18h old culture.
- In OFAT studies peptone and yeast extract were found to be most significant components influencing the bioemulsifier production with p-values of <0.0001 and <0.0044 respectively.
- After Plackett Burman design experiments, Peptone, Yeast extract, $MgCl_2$ and KCl were found to be affecting bioemulsifier production in *S. silvestris* AM1.
- Using Box-Behnken design in Response surface methodology, effects of significant components (individual variables) from placket burman design were estimated for their effects. After the analysis, the response yielded a linear model as there was no interaction seen among the components for bioemulsifier production. Peptone and yeast extract were found to be most significant components influencing the production with p-values of <0.0001 and 0.0044 respectively. Thus the model and its terms were found to be significant.
- The bioemulsifier production of *S. silvestris* AM1 showed 62.5% emulsification index with activity retained after 100 times dilution.

- When the significant factors of ZM medium responsible for bioemulsifier production by *S. silvestris* AM1 were checked, 68% EI and activity retention till 1000 times dilution were observed.
- Cell bound and cell free bioemulsifier was produced by *S. silvestris* AM1 even at 100 times dilution of ZM medium.
- Bioemulsifier production by *S. silvestris* AM1 was observed even at low peptone i.e., 0.5mg/ml concentration.
- In time course study *S. silvestris* AM1 showed cell bound bioemulsification activity after 6^{th} hour of incubation and cell free emulsification activity was observed after 16^{th} hour of incubation. The bioemulsifier AM1 production is growth associated.
- *S. silvestris* AM1produced relatively less cell free bioemulsifier compared to cell bound bioemulsifier in low peptone medium.
- Presence of xenobiotics including hydrocarbons like naphthalene, benzene, catechol, pesticides like acephate, methomyl and cypermethrin does not affect the production of bioemulsifier by *S. silvestris* AM1.

Chapter 4

Biochemical and physical characterization of bioemulsifier AM1 and its emulsions

4A. Biochemical characterization of Bioemulsfier AM1

- The bioemulsifier was purified by gel filtration chromatography with G200 column after dialysis and ultrafiltration.
- During purification, the bioemulsifier eluted from the flow-through fractions, indicating the size of the bioemulsifier may be above 200kDa.
- The bioemulsifier lost its full activity by treatment with Proteinase K, indicating its protein nature.

- The bioemulsifier AM1 was found to be constituting of 36.5µg of carbohydrate moiety per mg of protein.
- No lipid content in the bioemulsifier could be seen through TLC.
- The glycoprotein nature of the bioemulsifier was indicated in native PAGE by glycoprotein staining.
- In FTIR, the bioemulsifier gave distinct peaks at 1695 and 1632 cm^{-1} the peptide carbonyl bond stretching frequency and carbohydrate ester bond stretching at 1066 cm^{-1} confirming its glycoprotein nature.
- The carbohydrate moiety, as seen in HPTLC was found to be consisting of galactose and xylose or ribose.
- LC/MS-MS of the bioemulsifier AM1 provided in 5 peptide sequences. Two of them were found matching with the β-subunit of F0F1 ATP synthase, while three matched with flagellin hag peptides.
- Reports of flagellin proteins from *Bacillus subtilis* giving match in peptide analysis by LC/MS-MS with β-subunit of F0F1 ATP synthase also exists. Thus it can be said that bioemulsifier from *S. silvestris* AM1 is a flagellin-like glycoprotein.
- In FTIR a major peak found around 1631 cm^{-1} and a minor peak of 1693 cm^{-1} suggested antiparallel β strand structure like that of an amyloid β sheet.
- In CD spectra, there was clear marked drop at 225nm of UV wavelength which is typical amyloid spectral property indicating a secondary β sheet structure.
- During SDS PAGE analysis, it was seen that bioemulsifier AM1 always gave multiple bands, even after purification and the highest band was above 200kDa in size.
- The bioemulsifier AM1 was found to be resistant to moderate (0.1-1%) treatment with denaturants such as urea, dithiothreitol (DTT), Triton X100, Tween 20 and SDS.

- On 2% SDS treatment the bioemulsifier resolved into its monomeric units of 30kDa which was also corresponding to the size of flagellin protein.
- The bioemulsifier was found to be resistant to boiling temperature upto 5h, pH range of 4-9 and also for 1-5M NaCl concentration.
- Bioemulsifier, when checked on TEM, appeared as fibrillar in nature with 5nm width.
- The bioemulsifier stained with Congo red and when observed in polarizing microscope, the bioemulsifier emitted green-yellow-blue color due to Congo red birefringence.
- On Congo red containing bioemulsifier production medium *S. silvestris* AM1 gave red colored colonies due to amyloid nature of the bioemulsifier produced extracellularly as opposed to pale colonies on bioemulsifier non-production medium.
- FTIR, CD spectrum, Polarization microscopy, resistance to surfactants, temperature and pH, fibrillar nature under TEM and red colonies on Congo red medium established that *S. silvestris* AM1 bioemulsifier is amyloid in nature.
- In comparison to Emulsan of *Acinetobacter calcoaceticus* RAG-1, *S. silvestris* AM1 was found to be producing higher amount of bioemulsifier and broad emulsification spectrum of hydrocarbons and oils.

4B. Characterization of emulsion

- The relative emulsion stability (%ES) of bioemulsifier AM1 was found to be 89.7, 84.8, 81.7 and 79.32% with TCB, decane, groundnut oil and xylene, respectively which is higher than Emulsan.
- Trichlorobanzene (TCB) showed highest emulsion stability and the stability was due to the shift in particle size of the emulsion droplets towards smaller size as observed in brightfield microscopy. While the loss of stability in

paraffin oil can be attributed to Oswald's ripening, where the droplets gradually increased in size.

- The gradual reduction in viscosity of both emulsions formed in paraffin and TCB shows their non-Newtonian nature and shear thinning effect under shear rate values used.

Chapter 5

Ecophysiological studies of *S. silvestris* AM1 and its bioemulsifier

5A. Natural role of bioemulsifier in S. silvestris AM1

5AI. Studies on surface properties of *S. silvestris* AM1 and its mutants

- In MATH test, *S.silvestris* AM1 showed increased cell surface hydrophobicity in presence of compounds like hexane, heptane and benzene, whereas decreased hydrophobicity in presence of compounds like xylene, decane, trichlorobenzene and hexadecane.

- Surface hydrophobicity changed when *S. silvestris* AM1 grew in presence of benzene or acephate and therefore showed increase in auto-agggregation.

- Biofilm formation of *S. silvestris* AM1 was enhanced in presence of externally supplied bioemulsifier in case of LB as well as ZM medium.

- In bioemulsifier production medium *S. silvestris* AM1 biofilm formation increases and on proteinase K treatment the loss of biofilm corresponds similar to the biofilm formed in absence of bioemulsifier.

- *S. silvestris* AM1 produced higher biofilm in presence of catechol and acephate .

- The biofilm formation by *S. silvestris* AM1 increased corresponding to the time of cell bound bioemulsifier production observed earlier.

- Transposon (Tn917) and UV mutagenesis approach generated 177 and 484 mutants, out of which 9 and 27 mutants respectively exhibited 0%EI or null mutation for emulsification.

- The null mutants of *S. silvestris* AM1 exhibited higher cell surface hydrophobicity than the wild type in each of the hydrocarbon tested. They also exhibited higher biofilm formation than the wild-type.

5AII. Surface interaction studies between substratum and *S. silvestris* AM1

- Polystyrene was made differentially hydrophobic in the increasing hydrophobicity order of Silanized > Untreated > Sulfonated.
- The biofilm formation by *S. silvesttris* AM1 was highest on silanized polystyrene when compared to untreated and sulfonated in bioemulsifier production as well as non production medium.
- Untreated polystyrene showed lowest and silanized polystyrene showed highest contact angle (θ) measurements. Bacterial surfaces showed marginal decrease contact angle (θ) in presence of bioemulsifier AM1.
- Using θ values, when interfacial free energy of interaction between *S. silvestris* AM1 and polystyrene surfaces was calculated, it was higher in all the three cases, untreated, silanized and sulfonated in bioemulsifier production medium. The bacterial cells with or without bioemulsifier showed higher self-interaction than with that of surfaces.
- A correlation between the biofilm formation of *S. silvestris* AM1 on different surfaces and their corresponding interfacial interaction energies was observed, indicating that the reduction in interfacial interaction energy increased adhesion and therefore biofilm formation.
- *S. silvestris* AM1 cell surface hydrophobicity increased initially and was found to be maximum at fourth hour of growth, after which it gradually decreased as the production of cell bound bioemulsifier increased.
- *S. silvestris* AM1 exhibited lower cell-cell autoaggregation initially which increased significantly after sixth hour.

- AFM imaging of the cell surface topology of *S. silvestris* AM1 further helped to visualize the tuft like appearance of bioemolecules possibly the bioemulsifier AM1 in isolated islands.

5B. Interaction of *S. silvestris* AM1 bioemulsifier with other bacteria

- Bioemulsifier AM1 could disperse crude oil effectively
- Both crude as well as purified bioemulsifier AM1 could solubilize aliphatic and aromatic hydrocarbons tested (benzene, toluene and xylene).
- When checked with hydrocarbon utilizing bacteria from foreign habitats (*Bacillus* sp. AV8), bioemulsifier AM1's presence could not elicit the enhanced utilization of hydrocarbons except marginally in benzene and trichlorobenzene.
- 23 hydrocarbon degrading isolates were obtained from sediment sample of Vellar estuary with benzene, toluene, crude oil and crude oil and benzene as carbon source.
- After ARDRA, four isolates capable of forming biofilm and four non-biofilm formers were selected and subjected to 16S rRNA gene sequencing.
- The isolates were identified as *B. cohnii*, *B. methanolicus, B. cereus, B. firmus, B. aquimaris, B.thuringiensis* and *Rheinheimera* spp.
- *B. cereus,* a biofilm forming isolate and *Rheinheimera* sp. Co6, a biofilm non-forming isolates were screened for their BTX utilization and *Rheinheimera* sp. Co6 was found to be tolerant and able to utilize 15mg/ml and more of BTX mixture.
- *Rheinheimera* sp. Co6 was selected for microcosm studies with bioemulsifier producer, *S. silvestris* AM1.
- In microcosm experiments, it was evident that the not just the presence of bioemulsifier producer *S. silvestris* AM1, but also the presence of bioemulsifier itself increased the utilization and degradation of BTX by *Rheinheimera* sp. Co6.

- The microcosm experiments suggest that the bioemulsifier AM1 has positive influence on degradation of hydrocarbons by hydrocarbon degrading bacteria *Rheinheimera* sp. Co6.
- In presence of externally supplied bioemulsifier AM1, the biofilm formation of *S. aureus* ATCC 6538 is enhanced significantly while that of *Paracoccus sp.* W1b is reduced.

CONCLUSION

शुश्रूषा श्रवणं चैव ग्रहणं धारणं तथा |
ऊहापोहोर्थ विज्ञाम्प;ानं तत्वज्ञाम्प;ानं च धीगुणाः ||

Meaning:
Willing to listen, to actually listen, to understand what we listen, to be able to remember what we have listened, to be able to deduce some conclusions and put forth arguments, to be able to formalise and conclusively put forth the thought, knowledge of the around and Philosophy - these are the eight facets of 'buddhi'.

CONCLUSION

Major studies on bioemulsifier till now have always been with respect to their applications in bioremediation and industries. Genus *Acinetobacter* has been studied in detail for their ability to produce bioemulsifier and their functions. The diversity of bioemulsifiers produced by different kingdom of life shows the importance of bioemulsifier like molecules. In different microorganisms, bioemulsifiers have been studied for influencing adhesion and biofilm formation. Due to their direct interaction with the host and other microorganisms in the environment, bioemulsifiers are known to be and having ecophysiological potential to influence the microorganism in settling in a niche.

Since last few years, the order *Bacillales* which include Gram positive aerobic sporulating rods have been rearranged to form many families with respect to their divergence for genus *Bacillus.* These bacteria include maximum number of strains reported for industrially and environmentally important metabolites, but their bioemulsifiers were never studied in detail to assert their role in influencing the ecophysiology of the bacteria involved. In the context of untapped resources and functionality, only 1% of the total marine bacteria have been studied.

There is paucity in studies on various roles or functions of bioemulsifier in bacteria barring *Acinetobacter* that produces Emulsan. With this perspective the present studies on ecophysiological potential of bioemulsifier from *Bacillus* spp. have been undertaken.

Enrichment and isolation approach and specially selected intertidal zone sampling sites from all the western and one eastern coastal states of India provided bioemulsifier producing isolates belonging to *Bacillus* spp. Emulsifiying bacteria were commonly found in the sampling sites selected, Diverse bioemulsifier producing *Bacillus* strains inhabit the intertidal niche. Among those selected were species belonging to *Bacillus*, *Lysinibacillus*, *Sporosarcina* and *Solibacillus* genera. Presently only one species has been reported in genus *Solibacillus.* According to the transition/transversion analysis of *Solibacillus* sequences cited in literature preliminary observation was that many of the strains included in GenBank may be

considered for a new species other than *S. silvestris*, however further studies are needed. A novel bioemulsifier producing strain specially selected for its high bioemulsifier production was identified as *Solibacillus silvestris* AM1 which showed high degree of match to *Solibacillus* genus (Type HR3-23 strain) in DNA-DNA hybridization and FAME analysis, but showed slight variation when compared to transition transversion ratios in 16S rRNA gene analysis and phenotypic characters.

Isolated from Vellar estuary, Parangipettai, India, *S. silvestris* AM1 was found to produce a bioemulsifier that could emulsify various hydrocarbons and oils. It lacked the ability to utilize many common carbohydrates, while presence of proteins in its nutrient medium was significant in bioemulsifier production and even dilution of nutrients supported the production of bioemulsifier. It did not produce the bioemulsifier in any other reported bioemulsifier production media. The periodic eutrophication of Vellar estuary due to shrimp farm wastes and agricultural runoffs has compelled micro-adaptation strategies in *S. silvestris* AM1. The results here suggests that *S. silvestris* AM1, isolated from this area has evolved mechanisms to sustain on the protein rich area with ability to produce bioemulsifier even in oligotrophic conditions of proteins. These strategies also include its ability to produce bioemulsifier and form biofilm in presence of xenobiotics. *S. silvestris* AM1 produced bioemulsifier in cell bound state which was subsequently released into the environment.

The bioemulsifier Am1 produced by *S. silvestris* AM1 was found to be a multimeric glycoprotein of more than 200kDa in size with 30kDa monomeric subunits. The 3.6% carbohydrate entity in the bioemulsifier consisted of galactose and xylose/ribose. Mascot analysis of peptide sequences obtained from bioemulsifier protein sequencing demonstrated its relation to bacterial flagellin protein. It exhibited salt and thermostability and activity in broad pH range. It also possessed resistance to moderate levels of surfactants and sensitivity to proteinase K.

Among a handful of reports of protein bioemulsifier in literature exists and here first time a *Solibacillus* strain has been found to produce a protein bioemulsifier.

Its resistance to physicochemical factors was a significant property which pointed to its structural features. The bioemulsifier showed some interesting results when checked for FTIR, CD spectrum and TEM. The bioemulsifier aggregates when seen in TEM, appeared like fibres and exhibited presence of antiparallel β strands in FTIR, CD spectra and polarizing microscopy, which are characteristics of amyloid nature of proteins. According to Neilson *et al.* (2011), these bacterial functional amyloids are produced by a diverse group of bacteria, but only few have been purified and studied for their function. Thus bioemulsifier AM1 clearly exhibited amyloid assembly characteristics.

Bioemulsifier AM1 showed a broad range of emulsification activity in aromatic, aliphatic hydrocarbons and also in oils. Highest stability of the emulsions was observed in trichlorobenzene where the particle size decreased with time, stabilizing the emulsion. These emulsions formed by bioemulsifier AM1 in presence of tricholobenzene and paraffin oil exhibited pseudoplastic non-Newtonian rheological property, as observed by particle size and shear stress analysis. The bioemulsifier AM1 in addition to its emulsification activity was also found to be able to function as a biodispersant and biosolubilizer of hydrocarbons and crude oil.

Bioemulsifiers although reported from many microorganisms have been underestimated for their influence on bacterial ecophysiology. It was seen that amendment of medium with bioemulsifier increased the biofilm formation in *S. silvestris* AM1 and *S. aureus* ATCC 6538 which was lost by treatment with proteinase K, while biofilm formation was severely affected in *Paracoccus* sp.W1b. The biofilm formation in *S. silvestris* AM1 increased corresponding to the time of bioemulsifier production. Null mutants for bioemulsifier production obtained from transposon mutagenesis showed higher cell surface hydrophobicity and biofilm formation when compared to the wild type. Studies with interfacial interaction energy and subsequent biofilm formation on different surfaces indicated the importance of cell surface bioemulsifier and its influence in bacterial adhesion and biofilm formation. Cell surface hydrophobicity showed marked decreased and cell-cell aggregation increased during the production of cell bound bioemulsifier. These results clearly suggest the influence of cell bound bioemulsifier on bacterial cell surface topology and determine

cell adhesion to some extent. These results show that pre-existing AM1 bioemulsifier in environment will form conditioning film over surfaces (animate and inanimate), and when a compatible microorganisms would come in the vicinity of such 'conditioned' surfaces there attachment to the surfaces would be enhanced by the bioemulsifier. Here the bioemulsifier would act as anchoring agents which will facilitate the building of a stable biofilm. Also it can be concluded that when non-compatible microorganisms would come in the vicinity of such 'conditioned' surfaces, their attachment would be vetoed to the surfaces thus preventing biofilm formation.

For studying ecophysiological potential of a microorganism and the compound it is producing, it is necessary to understand its interaction with the microorganisms that share their niche. Bacteria isolated from same habitat of *S. silvestris* AM1 were capable of utilizing BTX and one of their representative *Rheinheimera* sp.Co6 was selected for microcosm studies. Microcosm studies clearly show that when bioemulsifier AM1 was present, the biodegradation of BTX increased significantly. The utilization of BTX was almost similar by the hydrocarbon degrading ,the bioemulsifier producer and the two together. The growth of both the added isolates was not hampered in presence of the each other which was possible due to competition in a microenvironment as in a microcosm. The bioemulsifier production by *S. silvestris* AM1 was also not hampered in the microcosm and it was also seen in the microcosm that the bioemulsifier was produced in the presence of degrader in the same microcosm. Thus, the results from microcosm experiments suggests that the bioemulsifier AM1 has positive influence on degradation of hydrocarbons by hydrocarbon degrading bacteria co-inhabiting the same habitat as the bioemulsifier producer, *S. silvestris* AM1. In case of *Bacillus* sp. AV8, it was seen that the bioemulsifier could not enhance its degrading capabilities. The organism probably lacks the surface compatibility for bioemulsifier AM1.

From the studies undertaken in present work, the natural role of bioemulsifier in *S. silvestris* AM1 is envisaged as follows: It changes the cell surface hydrophobicity and acts as a protectant against the hydrocarbon toxicity. It aids in cell aggregation and adhesion to substratum and consequently helps in biofilm formation.

In addition to emulsification activity, the bioemulsifier AM1 also possesses biodispersant and hydrocarbon solubilization properties and therefore it helps other compatible hydrocarbon degrading bacteria.

Thus from this study, it can be seen that a flagellin-like glycoprotein bioemulsifier with amyloid characteristics produced by an intertidal zone isolate, *S. silvestris* AM1 can influence the host by changing its surface characteristics and increasing its interfacial interaction energy. The bioemulsifier could also influence other bacteria in adhesion and degradation positively or negatively depending on their surface compatibility to the bioemulsifier and ability to recognize it on an abiotic surface.

LIST OF PUBLICATIONS & POSTERS PRESENTED

LIST OF PUBLICATIONS

- **"Physicochemical characterization of a thermostable glycoprotein bioemulsifier from *Solibacillus silvestris* AM1"**
  A.R. Markande, S.R. Acharya, A.S. Nerurkar
  Process Biochemistry, In Press
  Available online 1 September 2013

- **"Characterization of ecophysiological group of bioemulsifier producing bacteria isolated from intertidal zone of Indian coast"**
  Communicated

- ***"*Biochemical diversity of microbial bioemulsifiers and their roles in the natural environment*"***
  Invited article in Book entitled *"Current Issues in Microbiology"* by IGNOU (Accepted)

LIST OF POSTERS PRESENTED

- **"Isolation and characterization of bioemulsifier producing *Bacillus* sp."**
  Presented at 50th annual conference of Association of Microbiologists of India at NCL, Pune; from 15-18 Dec 2009
  Awarded Best poster in Environmental microbiology section

- **"Bioemulsifier from *Solibacillus silvestris* sp. AM1"**
  Presented at the 79th annual meeting of Society of Biological Chemists (India), IISc, Bangalore; from 13-15 Dec 2010

- **"Bioemulsifier from *Solibacillus silvestris* AM1 and its ecological significance".**
  "Presented at 52nd Annual Conference of Association of Microbiologists of India held at Chandigarh from November 3-6, 2011

- **"Characterization of novel bioemulsifier producing isolate, *Solibacillus silvestris* AM1 mutants"**
  "Presented at 52nd Annual Conference of Association of Microbiologists of India held at Chandigarh from November 3-6, 2011

- **"Effects of bioemulsifier from *Solibacillus silvestris* AM1".**
  Presented at 53rd Annual Conference of Association of Microbiologists of India (AMI); International conference on "Microbial world: Recent innovations and future trends"; held at KIIT University, Bhubaneswar, Odisha from November 22-25, 2012

- **"Studying the role of bioemulsifier in influencing cell surface properties of *Solibacillus silvestris* AM1".**
  Presented at "The 5th Congress of European Microbiologists (FEMS 2013)" at Leipzig, Germany, from July 21-25, 2013