ISOLATION, CHARACTERIZATION AND SOME INDUSTRIAL APPLICATION OF BIOSURFACTANTS PRODUCED BY *Bacillus subtilis* AND *Pseudomonas aeruginosa* STRAINS

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
Doctor of Philosophy

BY
KISHORE DAS, M.Sc.
Registration no. 049 of 2004

June, 2005

School of Science and Technology

ONGC-Centre for Petroleum Biotechnology
Department of Molecular Biology and Biotechnology
Tezpur University

Dedicated to my beloved parents

Sri. Kuladhar Das

&

Smt. Joymati Das

TEZPUR UNIVERSITY

Dr. A.K. Mukherjee, *M Sc.Ph D.*
Reader

DEPARTMENT OF MOLECULAR BIOLOGY AND BIOTECHNOLOGY
Tezpur-784028, Assam, India

CERTIFICATE BY THE SUPERVISOR

This is to certify that Mr. Kishore Das, M.Sc. has work out the thesis entitled "Isolation, characterization and some industrial application of biosurfactants produced by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains" under my supervision. He has fulfilled the requirements of the regulations relating to the nature and prescribed period of research at the Tezpur University. The Thesis embodied accounts for his own findings and has not been submitted previously anywhere for any degree whatsoever by either him or anyone else.

Date: 27 June, 2005

A.K. Mukherjee
(A. K. Mukherjee)

Ph: 03712-267007-9 Ext. 5405 (O); Fax: 03712-267006 (O); **E-mail:** akm@tezu.ernet.in

Acknowledgements

It gives me immense pleasure and joy to express my heartfelt gratitude to my supervisor, Dr. Ashis Kumar Mukherjee, Reader, Department of Molecular Biology and Biotechnology, Tezpur University, who has guided me throughout this work. Without his constant inspiration, encouragement and constructive criticism, the present investigation would not have been possible.

I would like to express my sincere thanks to Prof. P.C. Deka, Vice-chancellor, Tezpur University and Chairman, ONGC-Centre for Petroleum Biotechnology, T.U. and Prof. B.K. Konwar, Head, ONGC-Centre for Petroleum Biotechnology and Department of Molecular Biology and Biotechnology, Tezpur University, for their kind advise and constant encouragement during the tenure of my research period.

I express my deep sense of gratitude to Prof. Late Anil K. Lala, Indian Institute Technology, Mumbai, India, Dr. P. Deepalakshmi, Indian Institute of Science, Bangalore and Dr. S. Mayil Raj, Institute of Microbial Technology, Chandigarh, India for their kind help in carrying out few important analyses. I am also indebted to Dr. P. Dutta and Mr. C.M. Sharma of Indian Council of Medical Research (ICMR), Dibrugarh, India for their help in taxonomic identification of mosquito larvae. My sincere thanks goes to Dr. Tapan Chakrabarty, Institute of Microbial Technology, Chandigarh, India for critically reviewing part of my research work and for his valuable suggestions. The help, I received from Ms. Abira Mukherjee, Tezpur in statistical analyses of my data is worth mentioning.

It is a great pleasure for me to offer sincere thanks to all the faculty and staff members of MBBT department and specially to my colleague Mr. Robin Doley, for their friendly advice and constant encouragement. I am also grateful to Mr. Bhaben Tanti, Naba Bordoloi, Ms. Sangeeta Nath, Ms. Deepmoni Deka, Mr. J. Buragohain, Mr. D. Choudhary, Mr. R. Kandali, Mr. K. R. Barman and others for their kind help and suggestions. I express my sincerely gratefulness to the Dept. of Chemical Sciences, Tezpur University for helping me in carrying out the FT-IR analyses.

The help and encouragement received from the ONGC personnel are highly solicited. My sincere thanks goes to Mr. S. K. Majumdar, ONGC Jorhat, Dr. D. Ray and Ms. J. Thomas, KDMIPE, ONGC, Dehradun for their timely

encouragement. The help and encouragement received from Mr. Jasbir Singh, ONGC, Jorhat and Mr. Raghu Nandan, ONGC, Sibsagar is worth mentioning. No word could express my deep thanks for Mr. Y.N. Trivedi, Mr. U. Unniyal, Mrs. D. Rana, Mr. P. Goswami, Mr. Hazarika and Mr. A. Baruah, Institute of Biotechnology and Geotectonic studies (INBIGS), ONGC, Jorhat, for their valuable suggestion and inspiration to pursue the present work. Moreover the cooperation received from the ONGC personnel in Sibsagar and Jorhat is worth mentioning without their help the sample collection and field visit would have never been possible.

I am grateful to the Basin Manger, ONGC, Jorhat and Head, INBIGS, Jorhat for their kind permission to publish the research papers in scientific journals. I am also thankful to D. P. Sahashrabude, Basin Manager, Assam & Assam-Arakan, ONGC, Jorhat for according the kind permission to submit the Ph.D. thesis to the Tezpur University.

Last but not the least, I would like to express my deep sense of gratitude to all of my family members for their enthusiastic inspiration to me throughout my academic career.

The financial assistance received as JRF and SRF from the Oil and Natural Gas Corporation Ltd. (ONGC) in pursuing the present work is highly acknowledged.

Kishor Das

Date: 27/6/2005
Place: Tezpur

(Kishore Das)

Declaration

I hereby declare that due to the lack of proper facility at Tezpur University, following experiments/sample analyses were carried out at other institutes. Further, I declare that no part of this has been reproduced elsewhere for award of any other degree.

1. GC analyses of FAME of the bacterial strains was done by Dr. S. Mayil Raj at Institute of Microbial Technology, Chandigarh, India.

2. MALDI-TOF mass spectra analyses of biosurfactants was done by Prof. Anil. K Lala Indian Institute of Technology, Mumbai and by Dr. P. Deepalakshmi, Indian Institute of Science, Bangalore

Kishore Das

Date: 27/6/2005
Place: Tezpur

(Kishore Das)

List of Abbreviations

ALP= Alkaline phosphatase

A.U = Absorbance unit

B. = *Bacillus*

BSA = Bovine serum albumin

CFU= Colony forming unit

CLPs= Cyclic lipopeptides

CMC = Critical micelle concentration

CMD= Critical micelle dilution

CSH = Cell surface hydrophobicity

DNA = De-oxy ribose Nucleic Acid

E.I= Emulsification index

FAME= Fatty acid methyl ester

g = Gram

GC = Gas chromatography

h= Hour

ISR = Inter spacer region

l= Litre

lbs = Pound

LD_{50} = Lethal dose for 50% larval death

LD_{100} = Lethal dose for 100% larval death

M = Mucoid

MALDI-TOF -MS = Matrix Assisted Laser Desorption / Ionisation Time of Flight Mass Spectrophotometry

MEOR = Microbial enhanced oil recovery

mg= Milligram

min = Minute

mN/m = Milli newton per metre

N = Normal

NM= Non- mucoid

nm = Namometer

O.D = Optical density

P = *Pseudomonas*

PAHs= Poly aromatic hydrocarbons

PBS = Phosphate buffer saline

PCR-RFLP= Polymerize chain reaction- restriction fragment length polymorphism

pM= Pico-mole

PPGP = Platelet poor goat plasma

RBC = Red blood corpuscles

RP-HPLC = Reverse Phase High Performance Liquid Chromatography

rpm = Revolution per minute

S.D= Standard deviation

S.I= Similarity index

ST= Surface tension

SDS = Sodium dodecyl sulphate

SGOT= Serum glutamic oxaloacetic transaminase

SGPT= Serum glutamic pyruvic transaminase

TFA = Trifluroacetic acid

TPH = Total petroleum hydrocarbon

UV/Vis = Ultra violet/Visible

CONTENTS

CHAPTER I

INTRODUCTION

1.1 An introduction to biosurfactants

In 1960, there was rapid development of chemical industry and this has led to the development of a wide variety of petroleum-based chemical surfactants. However, natural surfactants produced from animal or plant materials, such as soap, lecithin and saponin have long been consumed for domestic and industrial use even before the advent of chemical surfactants (Kitamoto et al., 2002).

With increasing environmental awareness and emphasis on a sustainable society in harmony with the global environment, during the recent years, natural surfactants (Holmberg, 2001; Makkar and Camoetra, 2002) are getting much more attention. Among the natural surfactants, ones of microbial origin are especially classified into biosurfactants. These biosurfactants are defined as "structurally diverse groups of surface-active molecules synthesized by microorganisms". These molecules reduce surface tension in both aqueous solutions and hydrocarbon mixtures. These properties create micro-emulsion in which micelle formation occurs where hydrocarbons can be solubilized in water, or water in hydrocarbon (Banat, 1995). Surfactant are amphipathic molecules with both hydrophilic and hydrophobic moieties that partition preferentially at the interface between fluid phases with different degrees of polarity and hydrogen bonding such as oil/ water or air/ water interfaces. In addition to lowering the interfacial tension, the surfactant can also dominate the interfacial rheological behavior and mass transfer. These properties render surfactants capable of reducing surface and interfacial tension. Such characteristics make surfactant an excellent detergency, emulsifier, foaming and dispersing agents.

The interface between a liquid and a gas, or an immiscible liquid or an insoluble solid has special physical characteristics. At the interface, the intermolecular forces in the bulk material are unbalanced, leaving excess free energy, known as the surface energy in the exposed layer of molecules. For liquids, this is measured as a macroscopic property known as the surface tension. Amphipathic molecules and particles tend to accumulate at the interfaces and affect the surface tension between two immiscible liquids or surface energies at solid-liquid interface.

The surface tension of water is approximately 72 mN/m at room temperature and it results primarily from the effects of hydrogen bonding (Desai and Banat, 1997). Depending upon the concentration and surfactant types, many surfactants can reduce surface tension of water from 72 mN/m to approximately 30 ± 5 mN/m. This is achieved due to surfactant being amphiphilic molecules it act as a bridge between the two materials meeting at the interface. Biosurfactant activities can also be determined by measuring the changes in surface and interfacial tensions, stabilization or destabilization of emulsions and hydrophilic-lipophilic balance (HLB). Surface tension at the air/ water and oil/water interfaces can easily be measured with a tensiometer using plate or de Nuoy Ring method. A good biosurfactant producer was defined as one being able to reduce the surface tension of the growth medium by ≥ 20mN/m compared with distilled water. The definition of this threshold level was adopted from the literature where a culture is considered promising if it reduces the surface tension of a liquid medium to 40mN/m or less (Cooper, 1986).

Biosurfactants are synthesized by a wide variety of microorganisms. A search of the literature indicates that the ability to produce biosurfactants is widespread in the bacterial and archaeal domains as shown in Fig.1.1 (Maier, 2003). Some of the bacterial genera reported to produce surfactants include *Pseudomonas* (Maier and Sobero´n-Cha´vez, 2000), *Rhodococcus* (Bryant, 1990), *Mycobacterium* (Wong et al., 1979), *Nocardia* (Ioneda et al., 1970), *Flavobacterium* (Bodour et al., 2003), *Corynebacterium* (Cooper et al., 1982), *Clostridium* (Cooper et al., 1980), *Acinetobacter* (Ka¨ppeli and Finnerty, 1979), *Thiobacillus* (Beebe and Umbreit, 1971), *Bacillus* (Yakimov et al., 1995), *Serratia* (Matsuyama et al., 1985), *Arthrobacter* (Morikawa et al., 1993), and *Alcanivorax* (Yakimov et al., 1998). Although the ability to produce biosurfactants is widespread, the type of biosurfactant produced is genus and sometimes even species specific. For example, *Pseudomonas aeruginosa* produces rhamnolipid, a glycolipid as major biosurfactant, whereas *Pseudomonas fluorescens* produces viscosin, a lipopeptide biosurfactant.

Biosurfactant-producing organisms have been isolated from a wide diversity of environments including soil, seawater, marine sediments, and oil fields (deep subsurface environments). A study of polyaromatic hydrocarbon (PAH)-degrading isolates obtained from contaminated soil sites showed that 67% of these isolates are capable of producing biosurfactants (Willumsen and Karlson, 1997).

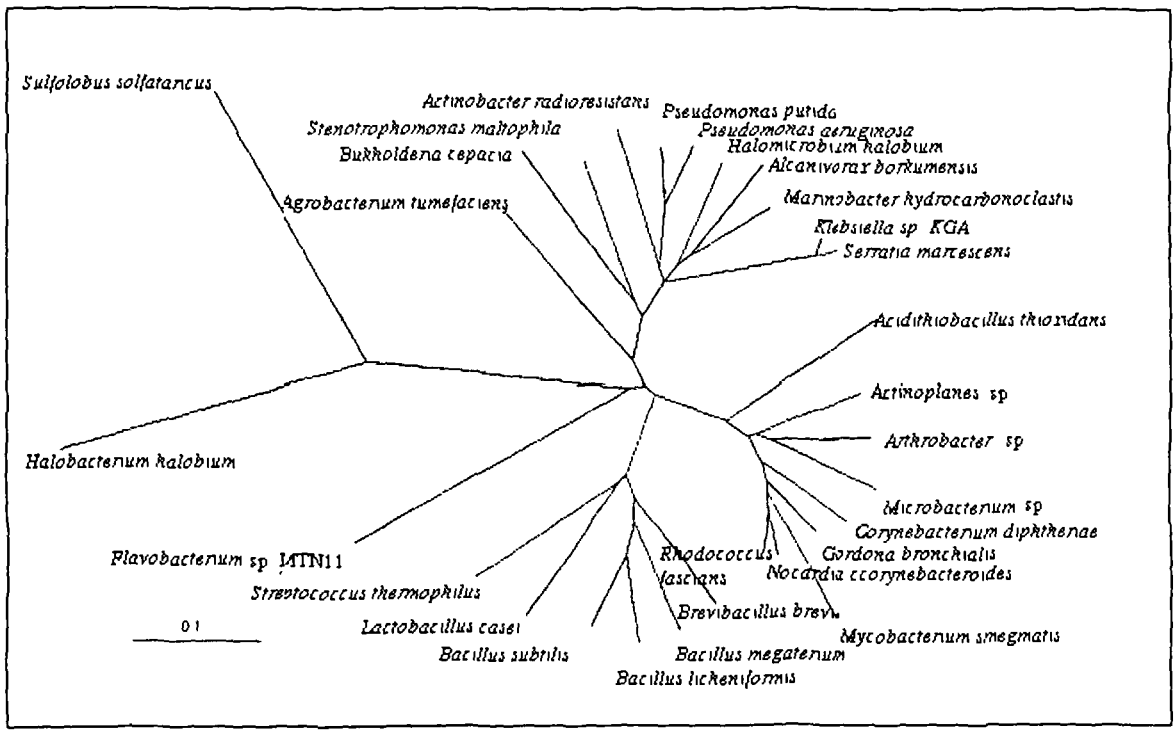

Fig.1.1. A phylogenetic tree based on the 16 S rDNA sequences from genera representing microorganisms that produce biosurfactants (Bodour and Maier, 2003).

1.2 Advantage of biosurfactants over synthetic surfactants

Chemically synthesized surfactants have been used in the oil industry to aid the clean up of oil spills, as well as to enhance oil recovery from oil reservoirs. These synthetic surfactants are not biodegradable and can be toxic to the environment. Biosurfactants, however have been shown in many cases to have equivalent emulsification properties and are biodegradable (Banat, 1995). The biosurfactants are considered to be superior to chemical surfactants on the basis of following observations/ properties (Kitamoto et al., 2002)-

(i) Possessing of one or more functional groups and chiral centers,

(ii) Higher biodegradability and lower toxicity,

(iii) Lower critical micelle concentration and higher surface activity,

(iv) Gradual adsorption and continuing activity,

(v) Superior ability to form molecular assembly and liquid crystal,

(vi) Possession of biological activity (antimicrobial and antitumor actions, etc).

Thus, there is an increasing interest in the possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, managing oil spills, oil-pollution control, cleaning oil sludge from oil storage facilities, soil/sand bioremediation and microbially enhanced oil recovery (MEOR) (Banat, 1995) and development of safe and effective biopesticides and antibiotics. Due to low anti-irritating effects and compatibility with skin, biosurfactants are preferred to synthetic surfactants in cosmetic, pharmaceuticals, food and health care industries (Makkar and Cameotra, 2002; Maier, 2003)

1.3 Major classes of biosurfactants

Biosurfactant are generally categorized by their chemical composition as well as by their microbial origin, whereas unlike chemically synthesized surfactant, which are classified according to the nature of their polar grouping. According to the microbial origin, biosurfactants are grouped into bacterial or fungal biosurfactant. From the point of chemical composition of biosurfactant, the major classes include glycolipid, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants. Some of the properties of the biosurfactants with the producing microbes are displayed in table 1. A detail classification of biosurfactants is presented in Review of Literature section (page no. 11)

1.3.1 Major classes of biosurfactants produced by *Bacillus* and *Pseudomonas* strains

B. subtilis are known to produce cyclic lipopeptides (CLPs) including surfactins, iturins, fengycins, and lichenysins, as the major classes of biosurfactants (Deleu et al., 1999; Vater et al.,2002). Among the various CLPs isoforms, surfactins are known to be the most efficient surfactants (Cooper et al., 1980; Peypoux, 1999), whereas iturin groups of CLPs are best known for their excellent antimicrobial properties (Ohno et al., 1996; Singh and Cameotra, 2004).

Production of rhamnose-containing glycolipid biosurfactants was first described in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949). Rhamnolipids, one of the best-studied glycolipids, consist of one or two molecules or rhamnose and are linked to one or

two molecules of β- hydroxydecanoic acid (Jarvis and Johnson, 1949; Sim et al., 1997). Rhamnolipids produced by *P. aeruginosa* strain were reported to exhibit antimicrobial properties (Haba et al., 2003) and algicidal activities (Wang et al., 2005). Various soil fluorescent *Pseudomonas* sp. are also known to produce cyclic lipopeptides with antibiotic and biosurfactant properties (Nielsen et al., 2002). Cyclic lipopeptides from fluorescent *Pseudomonas* sp. are also reported to have zoosporicidal activities against multiple oomycete pathogens, including *Pythium* species and *Phytophthora infestans* (de Souza et al., 2003). However these are the reports on biosurfactants produced by *Pseudomonas* sp. growing under mesophilic conditions.

1.4 Natural roles of biosurfactants to their producing microbes

When considering the natural roles of biosurfactants, it is important to emphasize that they are produced by a wide variety of diverse microorganisms and have very different chemical structures and surface properties. It is therefore reasonable to assume that different groups of biosurfactants have different natural roles in the growth of the producing microorganisms. One group of biosurfactants would have an advantage in a specific ecological niche, whereas another group of emulsifiers would be more appropriate for a different niche (Ron and Rosenberg, 2001). This diversity makes it difficult to generalize about the natural role of biosurfactants. The following natural roles of biosurfactants have been suggested (Ron and Rosenberg, 2001).

(i) Increasing the surface area of hydrophobic water-insoluble substrates.
(ii) Increasing the bioavailability of hydrophobic water-insoluble substrates.
(iii) Binding of heavy metals.
(iv) Pathogenesis.
(v) Antimicrobial activity.
(vi) Regulating the attachment-detachment of microorganisms to and from surfaces.
(vii) Emulsifier production and quorum sensing.
(viii) Role of emulsifiers in biofilms formation.

Table1.1. Microbial source and properties of important types of microbial surfactants

Class of biosurfactant		Organisms	Surface tension (mN/m)	Interfacial tension (mN/m)	CMC
Glycolipid	Rhamnolipids	*Psuedomonas aeruginosa*	29	0.25	0.1-10
		Pseudomonas sp.	25-30	1	
	Trehalolipids	*Rhodococus erythropolis*	32-36	14-17	4
		Nocardia erythropolis	30	3.5	20
		Mycobacterium sp.	38	15	0.3
	Sophorolipids	*Torulopsis bombicola*	33	1.8	
		Torulopsis apicola	30	0.9	
		Torulopsis petrophilium			
	Cellobiolipids	*Ustilage zeae*			
		Ustilage maydis			
Lipopeptides & lipoproteins	Peptide-lipid	*Bacillus licheniformis*	27	0.1-0.3	12-20
	Serrawettin	*Serratia marcesscens*	28-33		
	Viscosin	*Pseudomonas fluorescens*	26.5		150
	Surfactin	*Bacillus subtilis*	27-32	1	23-160
	Subtilin	*Bacillus subtilis*			
	Gramicidins	*Bacillus brevis*			
	Polymyxins	*Bacillus polymyxa*			

Table 1.1. Continued.

Biosurfactant		Organisms	Surface tension (mN/m)	Interfacial tension (mN/m)	CMC
Fatty acids, neutral lipids, and phospholipid	Fatty acid	*Corynebacterium lepus*	30	2	150
	Neutral lipid	*Nocardia erythropolis*	32	3	-
	Phospoholipids	*Thiobacillus thiooxidans*			
Polymeric surfactant	Emulsan	*Acinetobacter calcoaceticus*			
	Biodispersan	*Acinetobacter calcoaceticus*			
	Mannan-lipid protein	*Candida tropicalis*			
	Liposan	*Candida lipolytica*			
	Carbohydrate-protein-lipid	*Pseudomonas fluorescens, Debaryomyces polymorphis*	27		10
	Protein PA	*Pseudomonas aeruginosa*			
Particulate biosurfactant	Vesicles and fimbriae	*Acinetobacter calcoaceticus*			
	Whole cells	Variety of bacteria			

Table cited from Desai, J.D and Banat, I.M. (1997) Microbiol. Mol. Bio. Rev. 61, 47-64

1.5 Industrial application of biosurfactants

Currently, the surfactant industry, which exceeds $9 billion per year, markets predominantly synthetic surfactants (Makkar and Cameotra, 2002; Maier, 2003). However, some biosurfactants, e.g., rhamnolipid, sophorolipid, and spiculosporic acid, can be produced commercially at levels nearing and even exceeding 100 g/liter (Ishigami et al., 2000; Maier and Sobero´n-Cha´vez, 2000; Rau et al., 1996). At this level, the cost of producing biosurfactants becomes competitive with the cost of producing synthetic surfactants. As the production cost becomes competitive and as the commercial availability of biosurfactants increases, the commercial use of biosurfactants can be expected to grow tremendously in the coming decade.

The use of biosurfactants has been reviewed extensively by Bodour and Maier (2002). Potential applications of biosurfactants in the field of medicine include use as antibiotics for protection of cell cultures in medical research (Vollenbroich et al., 1997a), as antivirals (Kracht et al., 1999; Vollenbroich et al., 1997b), as antiinflammatory agents (Kim et al., 1998), in the prevention of biofilm formation (Busscher et al., 1997), and as antitumor agents (Isoda et al., 1997). In addition to these clinical applications, a number of industrial applications for biosurfactants have been investigated. Suggested applications are quite varied. One interesting example is their use to enhance the heterogeneous enzymatic hydrolysis of cellulose and steam-exploded wood (Helle et al., 1993).

Biosurfactants and chemically modified biosurfactants are being investigated for use in the field of high-value products, like, the use of biosurfactants for monitoring the micro-polarity and micro- fluidity of surfaces (Ishigami and Suzuki, 1997). A second example is the production of stereospecific L-rhamnose. Rhamnolipids have been suggested as an economical source of L-rhamnose, which is used commercially in the production of high-quality flavor compounds and as a starting material for synthesis of some organic compounds (Linhardt et al., 1989). Surfactants and emulsifiers are routinely used in the food industry. Mannoprotein emulsifier produced by *Saccharomyces cerevisiae* could be used as a mayonnaise additive (Torabizadeh et al., 1996). This emulsifier is found in the cell wall and can be obtained cheaply and in high yield [80–100 g/kg (g/g wet cells)]. In addition it is stable from pH 3 to 11, and the by-products can be used as animal feed supplements.

The cosmetic and health care industries use large amounts of surfactants for a wide variety of products, including insect repellents, antacids, acne pads, antidandruff products, contact lens solutions, hair color and care products, deodorants, nail care products, lipstick, eye-shadow, mascara, toothpaste, denture cleaners, antiperspirants, lubricated condoms, baby products, foot care products, antiseptics, shaving and depilatory products, and moisturizers (Kleckner and Kosaric, 1993). There are several reports on the efficacy of biosurfactants in biological control. Rhamnolipids have been shown to have activity against zoosporic plant pathogens at very low concentrations and were successful at control in a near-commercial hydroponic recirculating cultural system (Stanghellini and Miller, 1997). Similarly, surfactin and a similar lipopeptide, iturin A, produced by *Bacillus subtilis* RB14, have been identified as playing a role in the suppression of damping off disease of tomato seedlings caused by *Rhizoctonia solani* (Asaka and Shoda, 1996).

Biosurfactants have long been suggested for use as additives for microbially enhanced oil recovery (MEOR), bioremediation of crude oil contaminated environment (Mulligan, 2005), for the cleaning of oily sludge from storage tanks (Banat, 1995) and metal and organic contamination (Maslin and Maier, 2000; Sandrin et al., 2000; Maier, 2003). For these applications isolates from extreme environments (temperature, salinity, or pH) are of special interest (Gurjar et al., 1995; Trebbau de Acevedo and McInerney, 1996). More recently it has been suggested that biosurfactants may aid in preventing fouling of industrial surfaces by preventing biofilm formation (Busscher et al., 1996).

In the field of environment protection, biosurfactants have been explored for their bioremediation potential, and show promise for application to sites impacted by both organic and metal contaminants. Specific applications include enhancing contaminant biodegradation in sites that are contaminated with organics alone (Bregnard et al., 1998; Herman et al., 1997; Jain et al., 1992; Oberbremer et al., 1990) or that are co-contaminated with metals and organics (Maslin and Maier, 2000; Sandrin et al., 2000). Biosurfactants have also shown potential for use as additives to aid in cleaning or flushing organic (Van Dyke et al., 1993; Bai et al., 1998; Ivshina et al., 1998) or metal contaminants out of tanks or soils (Zosim et al., 1983; Torrens et al., 1998; Mulligan et al., 1999; Ochoa-Loza et al., 2001) or crude petroleum contaminated land and water ecosystem (Banat, 1995; Mishra et al., 2001; Ron and Rosenberg, 2002)

1.6 Aim and objectives of the present study

The proposed work has the following objectives:

a. Screening of microorganisms from various environmental samples for the production of biosurfactants under thermophilic growth condition of bacteria.

b. Taxonomic identification of hyper producing microbial strains.

c. Optimization of culture conditions of potential microbes for maximum biosurfactant production.

d. Isolation (Downstream processing) of biosurfactant(s) from potential microbes and biochemical characterization of biosurfactants.

e. To investigate the possible industrial applications of the promising microbes and isolated biosurfactants.

CHAPTER II

REVIEW OF LITERATURE

2.1 Biosurfactants: A general consideration

Biosurfactants are a heterogeneous group of surface-active molecules produce by microorganisms. The term biosurfactant has been used very loosely and refers to any usable and isolate-able compound obtained from microorganisms that has some influence on interface (Desai and Desai, 1993; Desai and Banat, 1997). Both mesophilic and thermophilic microorganisms are known to produce biosurfactants. Mesophilic microorganisms are those having the optimum growth temperature ranging from 25 °C to 40 °C (Pelczar, Jr. et al., 1993; Tortora et al., 2001). Thermophilic microorganisms have growth optimum temperature starting from 42 °C and above (Sharp and Munster, 1986; Weigel and Ljungdaht, 1986; Alcamo, 1997).

Worldwide surfactant market was calculated around $ 9.4 billion per annum (Shaw, 1994; Desai and Banat, 1997; Makkar and Cameotra, 2002) and their demand was expected to be increased at a rate of 35 % toward the end of 20^{th} century (Greek, 1991; Desai and Banat, 1997). Almost all surfactants currently in use are chemically synthesized from petroleum hydrocarbons. Chemical surfactants are classified into (I) Anionic (ii) Cationic (iii) Nonionic (iv) Zwitterionics (Rosen, 1988).

2.2 Types of biosurfactants

As describe earlier biosurfactant are generally categorized by their chemical composition and their microbial origin. Accordingly biosurfactant are divided into five broad groups viz., glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants and particulate surfactants (Desai and Banat, 1997; Maier, 2003).

2.2.1 Glycolipids

Glycolipids are the most commonly isolated and studied biosurfactants. They are carbohydrates in combination with long chain aliphatic acids or hydroxy aliphatic acids. The

best examples of glycolipids studied from the point of view of surfactant characterization and properties are (i) trehalose lipids, (ii) rhamnolipids and (iii) sophorolipids (iv) Diglycosyl diglycerides, (v) Mannosylerythritol lipids.

Other types of glycolipids have been reported in the literature such as cellobiose lipid (Spoeckner et al., 1999), glucose lipid (Ishigami et al., 1994), glycoglycerolipid (Nakata, 2000), sugar-based bioemulsifiers (Kim et al., 1996, 2000; Van Hoogmoed et al., 2000), and many different hexose lipids (Cairns et al., 1982; Ha et al., 1991; Fiebig et al., 1997; Golyshin et al., 1999;).

2.2.1.1 Trehalose lipids

Several structural types of microbial trehalose lipid biosurfactants have been reported (Lang and Wagner, 1987; Li et al., 1984). Disaccharide trehalose linked at C-6 and C-6' to mycolic acids (Fig. 2.1) is associated with most species of *Mycobacterium*, *Nocardia* and *Corynebacterium.* Mycolic acids are long-chain, ∝- branched -β- hydroxy fatty acids. Trehalolipids from different organisms differ in the size and structure of mycolic acid, the number of carbon atoms and the degree of unsaturation (Asselineau and Asselineau, 1978; Cooper et al 1989; Lang and Wagner 1987; Syldatk and Wagner, 1987). Trehalose dimycolate produced by *Rhodococcus erthropolic* has been extensively studied (Kretschmer et al., 1982; Rapp et al., 1979). *R erythropolis* and *Arthrobacter sp.* produce trehalose lipids, which lower the surface and interfacial tensions in the culture broth to 25 to 40 mN/m and 1- 5 mN/m respectively. Philp et al. (2002) reported the production of trehalose lipids from alkanotrophic *Rhodococcus ruber* on gaseous alkanes propane and butane.

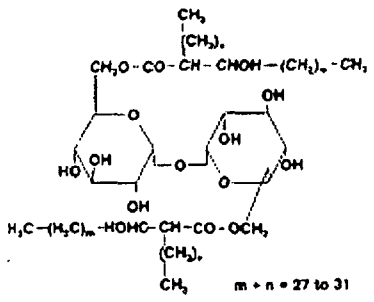

Fig. 2.1. Structure of trehalose lipids.

2.2.1.2 Rhamnolipids: a major class of biosurfactants produced by *Pseudomonas* sp.

Certain species of *Pseudomonas* are known to produce large amounts of glycolipids containing one or two molecules of rhamnose linked to one or two molecules of β-hydroxydecanoic acid (Jarvis and Johnson, 1949; Edward and Hayashi, 1965; Itoh et al., 1971; Chayabutra and Ju 2001; Haba et al. 2003; Benincasa et al. 2004). Jarvis and Johnson (1949) first reported the production of rhamnose containing glycolipids in *Pseudomonas aeruginosa*. Edward and Hayashi (1965) have reported formation of glycolipid, type R-1 containing two rhamnose and two β hydroxydecanoic units (Fig. 2.2) by *Pseudomonas aeruginosa*. Hisatsuka et al., (1971) measured the surface active properties of the R-1 lipid. A second kind of rhamnolipid (R-2) containing one rhamnose unit (Fig. 2.2) was reported by Itoh et al., (1971). Rhamnolipds from *Pseudomonas sp.* have been demonstrated to lower the interfacial tension against n- hexadecane to 1 mN/m and the surface tension to 25-30 mN/m (Geuerra- Santos et al 1986; Lang and Wagner 1987; Parra et al 1989). Rhamnolipid biosurfactant can emulsify alkanes and stimulate the growth of *Pseudomonas aeruginosa* in hexadecane (Hisatsuka et al 1971)

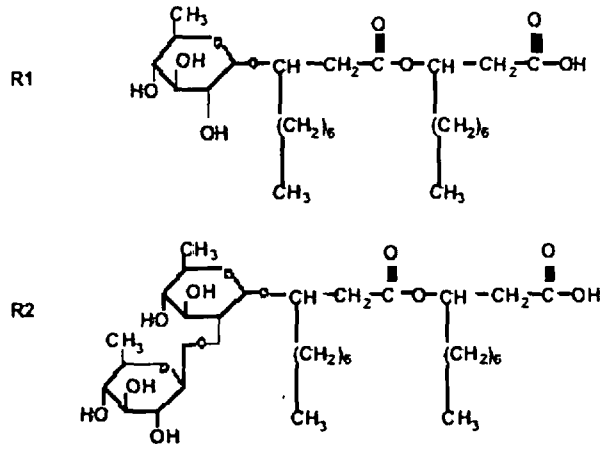

Fig2.2. Structure of R1 and R2 rhamnolipids.

2.2.1.3 Sophorolipids

Sophorolipids consist of a dimeric carbohydrate sophorose linked to a long chain hydroxy fatty acid (Fig. 2.3) and are mainly produced by yeast such as *Torulopsis bombicola* (Cooper and Paddock, 1983) and *T. apicola* (Tullock et al., 1967). These biosurfactants are a mixture of at least six to nine different hydrophobic sophorosides. Hommel et al.

(1987,1994), have reported the production of a mixture of water-soluble sophorolipids from yeast. Culter and Light, (1979) showed *Candida bogoriensis* produces glycolipids in which sophorose is linked to docosanoic acid diacetate. *T. petrophilum* produces sophorolipids on water insoluble substrates such as alkanes and vegetable oil (Cooper and Paddock 1983, Rau et al., 2001). *T. petrophilum* produces sophorolipids on water insoluble substrates such as alkanes and vegetable oil. These surface active compounds are chemically identical to those produced by *T. bombicola*, but cannot emulsify alkanes or vegetable oils (Cooper and Paddock 1983). These findings contradict the conventional belief that microbial emulsifier and surfactants are produced to facilitate the uptake of water-insoluble substrates. Although sophorolipids can lower surface and interfacial tension, they are not effective emulsifying agents (Cooper and Paddock, 1984). A protein containing alkane emulsifying agent was formed when *T petrophilum* was grown on a glucose- yeast extract medium (Cooper and Paddock, 1983).

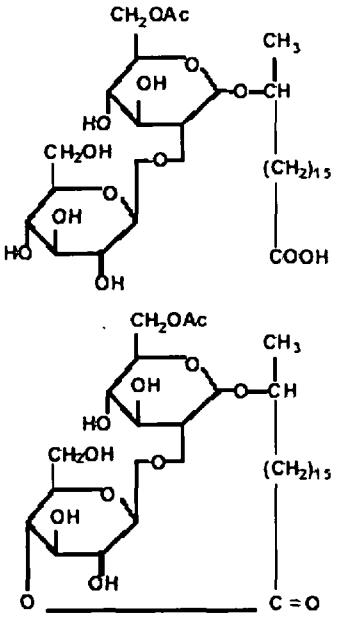

Fig. 2.3. Structure of sophorolipid.

2.2.1.4 Diglycosyl diglycerides

Diglycosyl diglycerides are glycolipids in nature and present in the cell membrane of a wide variety of bacteria (Shaw 1970,1974). The surfactant properties of this type of surfactant have not been tested. The structure of this class of molecule contains a polar, water-soluble

head and two lipophilic alkyl tails (Brundish et al., 1967). Wiken and Knox (1970) reported micelle formation by glycosyldiglycerides isolated from *Lactobacillus fermenti.*

2.2.1.5 Mannosylerythritol lipids

Mannosylerythritol lipids are glycolipids that contains mannosylerythritol as a sugar moiety (Fig. 2.4) and are mainly synthesised by yeast like *Candida* sp. B-7 (Kawashima et al., 1983), *Candida antarctica* T-34 (Kitamoto et al., 1990, 1992, 1993), *Candida* sp. SY 16 (Kim et al., 1999). The hydrophobic moiety, fatty acids, of the biosurfactant was determined to be hexanoic, dodecanoic, tetradecanoic and tetradecenoic (Kim et al., 1999). Kitamoto et al., (1990, 1993) reported that mannosylerythritol lipid-A and mannosylerythritol lipid-B were confirmed to be diacetyl dialkanoyl mannosylerythritol and monoacetyl dialkanoyl mannosylerythritol. This glycolipid biosurfactant lowered the surface tension of water to 29 dyne/cm at critical micelle concentration of 10 mg/l and the minimum interfacial tension was 0.1 dyne/cm against kerosene (Kim et al., 1999).

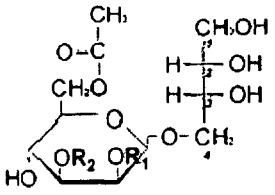

Fig. 2.4. Structure of mannosylerythritol lipids.

2.2.2 Amino acid containing surfactants

Decapeptide antibiotics (Gramicidins) and lipopeptide antibiotic (Polymyxins) produced by *Bacillus brevis* (Marahiel et al., 1977) and *B. polymyxa* (Suzuki et al., 1965) respectively possess remarkable surface-active properties.

Certain microorganisms produce biosurfactants which are chemically peptide containing lipid and exhibit excellent surfactant activity. These include ornithine-containing lipids from *P. rubescens* (Wilkinson, 1972) and *T. thiooxydans* (Knoche and Shively, 1972), cerilipin, and ornithine and taurine containing lipids from *Agrobacterrium tumefacians* IFO 3058 (Tahara et al., 1976) and *Streptomyces sioyaensis* (Kawanami et al., 1968).

Fluorescent *Pseudomonas* sp. are know to produce biosurfactants that contained peptide and lipid called cyclic lipopeptides that are reported to have antimicrobial and zoosporicidal activities (Nielsen et al., 2002; de Souza et al., 2003). Viscosinamide, tensin and apmhisin are the major CLPs produced by *P. fluorescens* (Henriksen et al., 2000; Nielsen et al., 1999, 2002; Sørensen et al., 2001). All these CLPs from *P. fluorescens* are reported to have antagonistic activities against the important plant-pathogenic microfungi *Pyuthium ultimum* and *Rhizoctonia solani* (Nielsen et al., 1999, 2002; Nielsen and Sørensen, 2003).

2.2.2.1 Lipopeptides

2.2.2.1.1 Surfactin

Surfactin, a cyclic lipopeptide (Fig. 2.5) is one of the most effective biosurfactants known so far, which was first reported in *B. subtilis* ATCC-21332 (Arima et al., 1968). In a concentration as low as 0.005 % (w/v), this surfactant can lower the surface tension from 72 to 27.9 mN/m (Arima et al., 1968). Its is named surfactin because of its exceptional surfactant activity (Peypoux1999). The ability of surfactin to lyse red blood cells has led to the discovery of development of a quick method for the screening of biosurfactant producing microbes (Mulligan et al., 1984). Surfactant produced by *B. subtilis* QMB exhibits similar structure and physiochemical properties to surfactin (Bernheimer and Avigad, 1970). The surfactin group of compounds are cyclic lipoheptapeptides which contain a β- hydroxy fatty acid in its side chain (Vater et al., 2002). Recent studies indicate that surfactin shows potent antiviral and antimycoplasma activities. This group of compounds also serves as antiviral, antitumoral agents as well as inhibitors of enzymes (Vollenbroich et al., 1997 a, b). Although, such properties of surfactins qualify them for potential applications in medicine or biotechnology, they have not been exploited extensively till date.

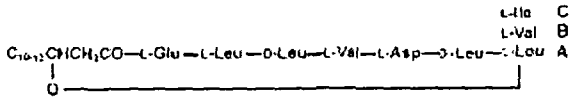

Fig. 2.5. Structure of surfactin.

2.2.2.1.2 Iturin

Iturin A, the first compound of the iturin group (Fig. 2.6) and its best known member, was isolated from a *Bacillus subtilis* strain taken from the soil in Ituri (Zaire) during the year 1957 (Delcambe et al., 1957). The subsequent isolation from other strains of *Bacillus subtilis* of five other lipopeptides such as iturin A_L, mycosubtilin, bacillomycin L, D, F and L_C (or bacillopeptin), all having a common pattern of chemical constitution, led to the adoption of the generic name of "iturins" for this group of lipopeptides (Peypoux et al., 1978, 1980, 1984,1985,1986; Kajimura, 1995). The iturin group of compounds are cyclic lipoheptapeptides which contain a β- amino fatty acid in its side chain (Vater et al., 2002). Lipopeptids belonging to the iturin family are potent antifungal agents which can be used as biopesticides for plant protection (Vater et al., 2002). Iturins are interesting in their remarkable efficacy against a broad variety of clinically important pathogenic yeast and fungi. However, their use in clinical trials is limited to local treatments, because of a possible toxicity (Bonmatin et al., 2003).

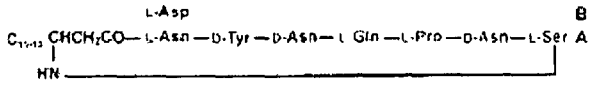

Fig. 2.6. Structure of iturin.

2.2.2.1.3 Fengycin

Fengycin is a lipodecapeptide with β- hydroxy fatty acid in its side chain (Fig. 2.7) (Vater et al., 2002). The fengycin group of compounds comprise of C_{15} to C_{17} variants which have a characteristic Ala-Val dimorphy at position 6 of the peptide ring (Nishikori et al., 1986;Vanittanakom et al., 1986; Schneider et al., 1999; Vater, 2002).

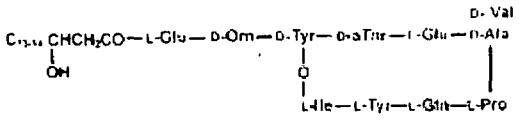

Fig. 2.7. Structure of fengycin.

2.2.2.1.4 Lichenysin

Lichenysin is also a lipopeptide surfactant produced by *B. licheniformis* JF2 (Javaheri et al., 1985; McInerney et al., 1990), which exhibit similar structure and physiochemical properties to that of surfactin. *B. licheniformis* also produce several other surface active agents which act synergistically and exhibit excellent temperature, pH and salt stability (McInerney et al 1990). BL-86, a surfactant produced by *B. licheniformis* 86, lowered surface tension of water to 27 dynes/ cm and interfacial tension between water and n- hexadecane to 0.36 dynes/ cm (Horowitz et al., 1990). This lipopeptide surfactant is stable over a wide range of pH, temperature and NaCl concentration and promotes dispersion of colloidal 3- silicon carbide and aluminum nitride slurries much more efficiently then commercial agents (Horowitz and Currie, 1990). BL-86 is found to be a mixture of lipopeptides with major components ranging in size from 979 to 1091 daltons with varying increments of 14 daltons. These are seven amino acids per molecule in which, lipid portion is composed to 8-9 methylene groups and a mixture of linear and branched tails (Horowitz and Griffin, 1991).

2.2.3 Phospholipids & fatty acid

2.2.3.1 Phospholipid

Certain hydrocarbon degrading bacteria and yeast produce appreciable amounts of phospholipids when grown on *n*-alkanes (Cirigliano and Carman, 1985, Cooper et al., 1978). This type of biosurfactants is able to produce optically clear microemulsions of alkane in water. Phospholipids produce by *Thiobacillus thioxidan* help in wetting of elemental sulfur (Beeba and Umbreit, 1971). *Aspergillus* sp. produces appreciable amount of phospholipids when grown on hydrocarbons (Miyazima et al., 1985). In *Acinetobacter* sp. strain HO1-N, phosphatidylethanolamine (Fig. 2.8) rich vesicles are produced which form optical clear microemulsions of alkanes in water (Kappeli and Finnerty, 1979).

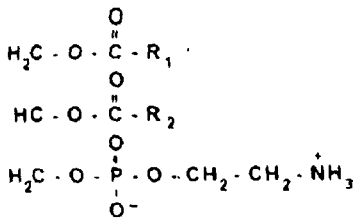

Fig. 2.8. Structure of phosphatidylethanolamine, a potent biosurfactant produced by *Acinetobacter* sp. R_1 and R_2 are hydrocarbon chains of fatty acids.

2.2.3.2 Fatty acid

Certain hydrocarbon degrading microbes produce extracellular free fatty acids when grown on alkanes and exhibit good surfactant activity. The important candidates are saturated fatty acids in the range of C_{12} to C_{14} and complex fatty acids containing hydroxyl groups and alkyl branches (Mac Donald et al., 1981; Kretschmer et al., 1982), *Arthobacter* strain AK-19 (Wayman et al., 1984) and *P. aeruginosa* 44Ti (Robert et al., 1989) accumulate up to 40-80% (w/w) of such lipids when cultivated on hexadecane and olive oil respectively. *R. erythropolis* produces phosphatidylethanolamine having CMC value of 30 mg/litre and lowers the interfacial tension between water and hexadecane to less than 1 mN/m (Kretschmer et al., 1982)

2.2.4 Polymeric biosurfactants

Polymeric biosurfactants are high molecular weight biopolymers, which exhibit properties like high viscosity, tensile strength and resistance to shear. For these properties, polymeric biosurfactants have a variety of industrial applications.

2.2.4.1 Emulsan

Acinetobacter calcoaceticus RAG-1 produces a potent extracellular polymeric bioemulsifier called emulsan (Rosenberg et al., 1979). Emulsan has been characterized as a polyanionic amphipathic heteropolysaccharide, the heteropolysaccharide backbone consisting of repeating unit of trisaccharide of N- acetyl-D-galactosamine, N-acetylgalactosamine uronic

acid and an unidentified N- acetylamino sugar (Fig. 2.9) (Zukerberg et al., 1979). Fatty acids constitute about 10%-15% of the dry weight are linked covalently to the polysaccharide through O-ester linkages (Belsky et al., 1979; Gutnik and Shabtai, 1987; Zuberberg et al., 1979). Emulsan is a very effective emulsifying agent for hydrocarbon in water even at a concentration as low as 0.001% (w/v) to 0.01% (w/v). It is one of the most powerful emulsion stabilizer known to-date and resists inversion even at water-to-oil ration of 1:4 (Belsky et al 1979; Gutnik and Shabtai, 1987; Zuberberg et al., 1979). On long standing, emulsion separates into two layers. The upper cream layer, which is known as emulsanosol, contains approximately 70 to 75% oil. Emulsanosols remain stable for months and have the ability to withstand enormous shear without any inversion (Zosim et al., 1982). Shoham et al., 1983 has isolated the enzyme responsible for the depolymerization of emulsion, which depolymerizes by transelimination.

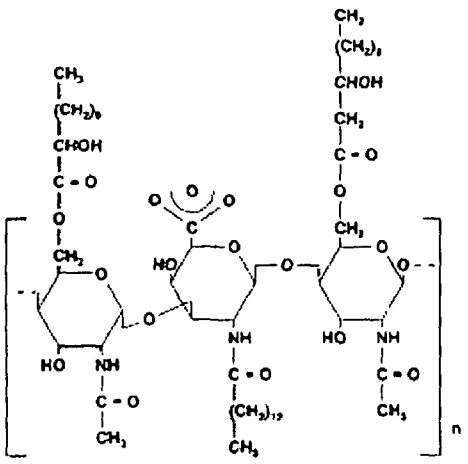

Fig. 2.9. Structure of emulsan, produced by *Acinetobacter calcoaceticus*.

2.2.4.2 Biodispersan

A. calcoaceticus A-2 produces an extracellular, nondialyzable dispersing called biodispersan (Rosenberg et al, 1988). The surface active component of biodispersan is an anionic heteropolysaccharide, with an average molecular weight of 51,400 and contains four reducing sugars namely glucosamine, 6- methylaminohexose, galactosamine uronic acids, and an unidentified amino sugar (Rosenberg et al., 1988)

2.2.4.3 Alasan

Alasan is an anionic alanine- containing heteropolysaccharide protein biosurfactant produced by *Acinetobacter radioresistens* KA-53. It was first described and isolated by Navonvenezia et al., (1995). It is found to be 2.5 to 3 time more active after heating at 100°C under neutral or alkaline condition. Alasan produced by *Acinetobacter radioresistens* KA-53 was reported to solubilise and degrade polyaromatic hydrocarbons (Barkay et al., 1999)

2.2.4.4 Liposan

Candida lipolytica produce an extracellular water soluble emulsifier called Liposan (Cirigliano and Carman, 1984; Kappeli and Fiechter, 1977), which is composed of 83% (w/v) carbohydrate and 17% (w/v) protein (Carigliano and Carman, 1984). The carbohydrate portion is a heteropolysaccharide consisting of glucose, galactose, galactosamine, and galacturonic acid. A potent bioemulsifier with carbohydrate as a major component produced by a gram negative bacterium is reported by Palejwala and Desai in 1989. It has been demonstrated by Sar and Rosenberg (1983) that polysaccharide has no emulsification activity alone but become a potent emulsifier when combined with some proteins released during the growth of *Acinetobacter calcoaceticus* on ethanol.

2.2.4.5 Emulsifying biopolymer from fungus

The production of large amounts of mannoprotein by *Saccharomyces cerevisiae* exhibiting excellent emulsifier activity toward several oils, alkanes, and organic solvents had been reported by Cameron et al., (1988). The purified emulsifier contains 44% mannose and 17% protein. A mannan- fatty acid complex from alkane grown *Candida tropicalis* was isolated by Kappeli et al., (1978, 1984). This complex stabilizes hexadecane- in- water emulsion. *Schizonella malanogramma* and *Ustilago maydis* produce biosurfactant that has been characterized as erythritol- and mannose-containing lipid (Fautz et al., 1986).

2.2.4.6 Emulsifying protein (PA) from *Pseudomonas aeruginosa*

An emulsifying protein (PA) along with rhamnolipid from *P. aeruginosa* was isolated by Hisatsuka et al., (1972,1977). It has a molecular weight of 14,300 and contains 147 amino

acids, of which 51 are serine and threonine (Hisatsuka et al., 1977). Production of peptidoglycolipd that bears 52 amino acids, 11 fatty acids, and a sugar unit by *P. aeruginosa* P-20 has been reported by Koronelli et al., (1983). An emulsifying and solubilizing factor containing protein and carbohydrate was isolated from *Pseudomonas* sp. grown on hexadecane (Beeba and Umbreit, 1971; Miyazima et al., 1985). The production of bioemulsifier, composed of 50% carbohydrate, 19.6 % protein and 10% lipid by *P. fluorescens* was reported by Desai et al., (1988).

2.2.5 Particulate biosurfactant

Some examples of particulate biosurfactant, which are extracellular membranes vesicles of microbial cells, that help in emulsification of hydrocarbon are given below.

2.2.5.1 Extracellular membrane vesicles of *Acinetobacter* sp.

Alkane uptake by microbial cells is also mediated by extracellular membrane vesicles that partition hydrocarbons to form a microemulsion.

Accumulation of extracellular membrane vesicles having 20-50 mm diameter and a bouyant density of 1.158 g/cm^2 has been reported in *Acinetobacter* sp. HO1-N cells (Kappeli and Finnerty, 1979). The purified vesicles are composed of protein, phospholipid and lipopolysaccharide. The vesicles have phospholipid content five times higher and a polysaccharide content 360 fold higher than that observed in the outer membrane of the same organism.

2.2.5.2 Microbial cells with high cell surface hydrophobicities

Surface active compound in most hydrocarbon- degrading and pathogenic bacteria is attributed to several cell surface components which include structures such as M protein and lipoteichoic acid in the case of group A streptococci, protein A in *Staphylococcus aureus*, layer A in *Aeromonas salmonicida*, prodigiosin in *Serratia* sp., Gramicidins in *Bacillus brevis* spores, and thin fimbriae in *A. calcoaceticus* RAG-1 (Fattom and Shilo, 1985; Rosenberg, 1986; Desai, 1987; Lang and Wagner, 1987).

2.3 Screening and detection of biosurfactant producing potential microorganisms

Quick, reliable methods for screening biosurfactant producing microbes had largely attributed for the recent advances in the field of microbial surfactants.

Axisymmetric drop shape analysis (ADSA) by profile was developed by Van der Vegt et al. (1991) for the assessment of potential biosurfactant- producing bacteria. In this technique, drops of culture broth are placed on a fluoro-ethylene-propylene surface and the profile of the droplet is determined with a counter monitor. Surface tensions are calculated from the droplet profiles by ADSA. Biosurfactant producing bacterial strains only show reduction in surface tension. The ability of the anionic surfactants to react with the cationic indicator to form a colored complex forms the basis of calorimetric estimation of biosurfactant (Shulga et al., 1993).

Another simple method of detecting biosurfactant production is a rapid drop- collapsing test. In this rapid method a drop of a cell suspension is placed on an oil- coated surface, and drops containing biosurfactants collapse whereas non-surfactant containing drops remain stable (Jain et al., 1991; Bodour and Miller-Maier, 1998). This is an easy method particularly when large number of samples are concerned (Bodour et al., 2003), but it has not been correlated to surface tension reduction to confirm its reliability.

Another method of detecting biosurfactant producing microbes is by their ability to haemolysis RBC on solid media plate. The hemolytic activity of biosurfactant was first discovered when Bernheimer and Avigad (1970) reported that the biosurfactant produced by *Bacillus subtilis*, surfactin, lysed red blood cells. Most types of biosurfactants have the ability to haemolysis red blood corpuscles (Mulligan et al., 1984; Banat, 1993; Carrillo et al., 1996; Yonebayashi et al., 2000). Blood agar lysis has been used to quantified surfactin (Moran et al., 2002) and rhanmolipids (Johnson and Boese-Marrazzo, 1980). In 1996 Carrillo et al., observed an association between hemolytic activity and surfactant production and recommended the use of blood agar lysis as primary screening method for biosurfactant production.

Oil spreading technique can also be utilize to detect biosurfactant production. This technique measures the diameter of clear zones caused when a drop of biosurfactant-

containing solution is placed on an oil-water surface: (Morikawa et al., 2000). These researchers used this method to compare the activity of both cyclic and linear forms of surfactin and arthrofactin. However, its ability to detect biosurfactant production in diverse organisms has not yet been tested. Youssef et al., (2004) compared three methods to detect biosurfactant production viz., drop collapse, oil spreading and blood agar for their ease of use and reliability in relation to the ability of the cultures to reduce surface tension. Matsuyama et al., (1991) described a direct thin- layer chromatographic technique for rapid characterization of biosurfactant-producing bacterial colonies. Colorimetric methods was developed by Siegmund and Wagner (1991) and Hansen et al., (1993) for screening of rhanmolipid producing and hydrocarbon-degrading bacteria respectively.

Another method is estimation of the emulsification (Van Dyke et al., 1993; Makkar and Cameotra, 1997,1998) and emulsification index after 24 hours value (E-24). E-24 is the emulsification percentage obtained by vigorously shaking culture broth samples with an equal volume of kerosene (Cooper and Goldenberg, 1987). This method is most suitable for emulsifying biosurfactant.

Cell surface hydrophobicity is an important aspect in bacterial cell adhesion to surface (vander Mei et al., 1987). Since hydrophobic surfaces are usually associated with molecules with low surface energy (Mozes and Rouxhet, 1987), Neu and Parolla (1990) used this property to screen microbes for biosurfactant production.

Biosurfactant production can also be detected by high-performance liquid chromatograph (Schenk et al., 1995; Lin et al., 1998). Schenk et al. (1995) developed a HPLC method for detection of rhamnolipid produced by *Pseudomonas aeruginosa*. A similar method of detection of biosurfactants production in the cell-free fermentative broth of *Bacillus subtilis* ATCC 21332 was proposed by Lin et al. (1998).

Most recently, Matrix-Assisted Laser Desorption/Ionization Mass spectrometry (MALDI-MS) has been used to screening the production of microbial biosurfactants and to characterize their molecular structure (Vater et al., 2002; Madonna and Voorhes, 2003)

2.4 Natural role of biosurfactants to their producing microbes

The exact physiological function of biosurfactants is still unknown, although there has been much speculation in the literature and it has been assumed that there may exist multiple functions for these unique and fascinating molecules. One suggested physiological role of biosurfactants is to facilitate growth of microorganisms on water-immiscible substrates by reducing the interfacial tension, and thus enhancing the bioavailability of the substrate. Other suggested functions include enhancement of adhesion of cells to insoluble substrates (Neu, 1996), antibiotic activity, and a possible role in pathogenesis of cystic fibrosis and burn wounds (Rumbaugh et al., 1999). It was also discovered that some biosurfactants strongly complex metals (Tan et al., 1994), suggesting a role for biosurfactants in the interaction of microorganisms with metals in their environment. Kitamoto et al., (2002) has proposed three major physiological role of biosurfactants, which includes (i) uptake of water- insoluble substrates (ii) support for infection to hosts and (iii) storage of carbon and energy sources. Some of the important natural roles of biosurfactants are highlighted below.

2.4.1 Increasing the surface area of hydrophobic water- insoluble substrates

Growth stimulating compounds are often accumulated in the culture medium, when microorganisms are cultured in water- insoluble substrates like n- alkanes or vegetable oils. These compounds emulsify the substrate, extending the interfacial area between the microorganisms and the substrate, thus facilitating mass transfer of the substrate to the surface of microorganisms (Kitamoto et al., 2002). Biosurfactant-negative mutants of *P. aeruginosa* KY-4025 (Itoh and Suzuki, 1972) and *P. aeruginosa* PG-201 (Koch et al., 1991) exhibited poor growth compared to the parent strains on n- paraffin and hexadecane, respectively and addition of rhamnolipid externally to the medium restored growth of the microorganisms in the respective hydrocarbon. However the addition of rhamnolipid has no growth stimulating effect with other genera of bacteria (Lang and Wullbrandt, 1999). For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil (Shreve et al., 1995). When the surface area become limiting, biomass increases arithmetically rather than exponentially. The evidence that emulsification is a natural process brought about by extracellular agents is indirect, and there are certain conceptual difficulties in understanding how emulsification can provide an evolutionary advantage for the producing microorganisms.

2.4.2 Increasing the bioavailability of hydrophobic water-insoluble substrates

One of the major reasons for the prolonged persistence of high-molecular hydrophobic compounds is their low water solubility, which limits their availability to biodegrading microorganisms. Biosurfactants can enhance growth on bound substrates by desorbing them from surfaces or by increasing their apparent water solubility (Deziel et al., 1996). Low molecular weight biosurfactants that have low critical micelle concentration (CMCs) increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities of micelles (Miller and Zhang, 1997). Much less is known about how polymeric biosurfactants increase the apparent solubilities of hydrophobic compounds.

2.4.3 Binding of heavy metals

A rhamnolipid biosurfactant has been shown to be capable of removing cadmium, lead and zinc from soil (Herman et al., 1997). Moreover, rhamnolipid eliminated cadmium toxicity when added to a 10-fold greater concentration than cadmium and reduced toxicity when added at an equimolar concentration (Sandrin et al., 2000). The mechanism by which rhamnolipid reduces metal toxicity may involve a combination of rhamnolipid interaction with the cell surface to alter cadmium uptake. 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* (Zosim et al., 1983).

2.4.4 Pathogenesis

Biosurfactant are considered to function as a "dispersing agent" in pathogenic microorganisms infecting plants or animals as well as a "wetting agent" for the surface of the host cells (Kitamoto et al., 2002). *Pseudomonas fluorescens*, a plant pathogenic bacterium, produces cyclic depsipeptides (viscosin), which reduce the surface tension on plant epidermis and thus accelerating wetting of the surface, dispersion of the bacteria and invasion and subsequent decay of the difficult-to-wet, waxy surface, etc (Hildebrand et al., 1998). Serrawettin, an nonionic cyclic depsipeptides produce by *Serratia marcescens*, an opportunistic pathogenic bacteria helps in wetting of the host cell surface and dispersion of the bacteria (Matsuyam and Nakgawa, 1996). Rhamnolipid is considered to be one of the virulence- associated exo-products of *P. aeruginosa*. Its production is also carried out and regulated in correlation with that of other virulence factors (Ron and Rosenberg, 2001).

2.4.5 Antimicrobial activity

Several lipopeptide surfactants are potent antibiotics (Marahiel et al., 1993; Yakimov et al., 1995; Tsuge et al., 1996). These include the cyclic lipopeptide surfactin or subtilysin; (Peypoux et al., 1999), iturin (Ohno et al., 1996; Singh and Cameotra, 2004) of *B. subtilis*, the extracellular hydrophobic peptide, streptofactin produced by *Streptomyces tendae* (Richter et al., 1998), the extracellular hydrophobic peptide, sterptofactin produced by *Streptomyces tendae* (Richter et al., 1998) and the cyclosymmetric decapeptide antibiotic gramicidin S of *B. brevis* (Krauss and Chan, 1983) and the polymyxins produced by *B. polymyxa* and related bacilli (Suzuki et al., 1969). It has been suggested that peptide antibiotics play a role in sporulation (Grossman, 1995).

2.4.6 Regulating the attachment- detachment of microorganisms to and from surfaces

When biosurfactant is excreted, it can form a conditioning film on an interface, thereby stimulating certain microorganisms to attach to the interface, while inhibiting the attachment of others (Neu, 1996). For example, the cell surface hydrophobicity of *P. aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid (Zhang and Miller, 1994), whereas the cell surface hydrophobicity of *Acinetobacter* strains was reduced by the presence of its cell- bound emulsifier (Rosenberg and Rosenberg, 1983). These findings suggest that microorganisms can use their biosurfactants to regulate their cell surface properties in order to attach or detach from surfaces according to need.

2.4.7 Emulsifier production and quorum sensing

For emulsifiers produced by pathogens, it has been suggested (Sulivan, 1998) that, being virulence factors, they are produced when the cell density is high enough to cause a localized attack on the host. It is easier to explain the need for bioemulsifiers in bacteria growing in hydrocarbons. As these bacteria are growing at the oil-water interface, the production of emulsifiers when the density is high will increase the surface area of the drops, allowing more bacteria to feed. Alternatively, when thee utilizable fraction of the hydrocarbon is consumed, as in the case of oil that consists of many types of hydrocarbons, the production of the emulsifiers allows the bacteria to detach from the "used" droplet and find a new one.

2.4.8 Role of bioemulsifiers in biofilms

Alasan, the exo-cellular polymeric emulsifier produced by *A. radioresistens* KA53, was shown to bind to the surface of *Sphingomonas paucimobilis* EPA505 and *A. calcoaceticus* RAG-1 and change their surface properties. The transfer could be shown after incubation of the recipient cells with the purified emulsifier. Moreover, when the alasan-producing *A. radioresistens* KA 53 was grown together with *A. calcoaceticus* RAG-1, alasan was released from the producing strain and became bound to the recipient RAG-1 cells (Osterreicher-Ravid et al., 2000). This horizontal transfer of bioemulsifiers from one bacterial species to another has significant implications in natural microbial communities, co-aggregation and bioflims (Davey et al., 2003).

2.5 Biosurfactant production under thermophilic condition

There are many reports of biosurfactant production by microbes in a mesophilic environment, but there are limited reports of biosurfactant production in thermophilic conditions. Thermophiles are heating loving microbes and can survive at a temperature ranging from 42-100°C. Philips and Perry, (1976) reported the ability of gram-negative bacteria to utilize hydrocarbon as the sole source of carbon and energy. A thermotolerant *Bacillus sp.* growing at 50°C on hydrocarbon containing medium was reported by Banat, (1993). *Streptococcus thermophilus* strains isolated from a pasteurization units in a dairy industry was reported to have biosurfactant producing ability (Busscher et al., 1994). *Bacillus licheniformis* JF2 isolated by Jenneman et al., (1983) from oil-field injection water have properties for potential use in *in situ* MEOR. The organisms could grow anaerobically and produce biosurfactant using glucose and $NaNO_3$ as respective carbon and nitrogen sources (Javaheri et al., 1985). *Bacillus licheniformis* JF2 could grown in a wide range of pH (4.6-9) and temperature and salinity 50°C and 10% respectively. The biosurfactant was not affected by typical parameters of many reservoirs like temperature, pH, salinity and calcium concentration (McInerney et al., 1990).

Yakimov et al., (1995) have reported the characterization of a new lipopeptide surfactant produce by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS 50. The organisms could produce biosurfactant when grown both aerobically and anaerobically at a temperature ranging from 35°C and 45°C and salt concentration optimal at 5%. The biosurfactant termed lichenysin A could reduced the surface tension of water from 72 mN/m

to 28 mN/m and have a CMC value of 12 mg/litre. Denger and Schink (1995) isolated biosurfactant producing two new strains of bacteria that produces the surface active compounds at salt concentration of around 8% and temperature 50°C using sugars as the carbon sources. Trebbau de Acevedo and Mc Inerney (1996) isolated bioemulsifiers from *Methanobacterium thermoautotrophicum* that form viscous emulsions but do not reduce the surface tension of water or interfacial tension between water and hexadecane. The emulsifier was active over a wide range of pH (5-10) and at salt concentration up to 200g/ liter. Makkar and Cameotra, (1997a, b, 1998) reported biosurfactant production by two strains *of B. subtilis* at 45°C. The organisms could produce biosurfactant over a wide range of pH and temperature ranging from 30°C to 45°C. The organisms could also grow and produce biosurfactant on molasses like cheaper carbon source. Recently it has been reported the biosurfactant production ability of *Pseudomonas* strains viz., *P. aeruginosa* M and NM and *Pseudomonas* DM-01 strain at 45°C (Das et al., 2003; Das and Mukherjee, 2005). The organisms could grow over a wide range of pH and could release about 60% and 80% crude oil and kerosene oil from oil saturated sand pack column indicating its potential in MEOR and oil sludge clean up.

2.6 Biosurfactant from non-conventional and cheaper carbon sources

The choice of inexpensive raw materials is an important factor that govern the production cost involved in the production of biosurfactant, as they account for 50% of the final product cost. Selection of waste substrate involves the problems of finding a waste with the right balance of carbohydrates and lipids to support optimal growth of the microorganisms, which may finally lead to the product formation (Makkar and Cameotra, 1999). Mulligan and Cooper (1985) used water collected during drying of fuel-grade peat for biosurfactant production by a *Bacillus subtilis* strain. Sheppard and Mulligan (1987) used peat hydrolysate for biosurfactant production. Rhamnolipid production by a *Pseudomonas* sp. was reported when the organism was grown on olive oil mill effluent (Mercede et al., 1993). Soap stick oil has been used for rhamnolipid production with *P. aeruginosa* D10 (Mercede and Manersa, 1994). Benincasa et al., (2002) reported biosurfactant (rhamnolipid) production by *Pseudomonas aeruginosa* LBI growing on soapstock and waste-water from sunflower oil production as the sole carbon source. Bednarski et al. (2004) reported glycolipid production by *Candida antarctica* ATCC 20509 and *Candida apicola* ATCC 96134 grown on oil refinery waste. Daniel et al., (1998) reported sophorolipids by *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509 from deproteinized whey

and rapeseed oil in a two stage fed batch process. *Bacillus subtilis* NB22 produced iturin and surfactin using wheat bran and okara (soybean curd residue) (Ohno et al., 1992,1996). Makkar and Cameotra (1997b) reported biosurfactant production by two *Bacillus* strains viz., *Bacillus subtilis* MTCC2423 and *Bacillus subtilis* MTCC 1427 using molasses (a by-product of sugar cane industry) as carbon source supplemented in mineral medium.

2.7 Industrial application of biosurfactants

2.7.1 Microbes and microbial surfactants in bioremediation

Oil-contamination soil is a common problem and its physical treatment methods or remediation techniques including excavation, incineration, landfarming and landfilling, can be difficult or economically not feasible (Banat 1995). One of the most economically feasible methods includes *in situ* bioremediation i.e., biodegradation of oil components present in the soil (Riser-Roberts, 1998; Ron and Rosenberg, 2002; Mulligan, 2005). Biodegradation is the partial simplification or complete destruction of the molecular structure of environmental pollutants by means of physiological reactions catalyzed by microorganisms (Alexander, 1981,1994; Atlas, 1992; Atlas and Cerniglia, 1995). Bioremediation is the intentional use of biodegradation process to eliminate environmental pollutants from sites where they have been released either intentionally or inadvertently. Bioremediation technologies use physiological potential of microorganisms, as documented most readily in laboratory assay, to eliminate or reduce the concentration of environmental pollutants in field sites to level that are acceptable to site owners and regulatory agencies that may be involved (Atlas, 1992; Shauver, 1993). Permeability of the microbial cell membrane might be adversely affected by the use of synthetic surfactant, which would interfere with the capacity of a microorganisms to biodegrade (Hunt et al., 1994). Microbial surfactants are generally much less toxic than chemical surfactants, at least as effective, and more readily biodegradable (Chakrabarty, 1995). Using microorganisms that produce their own biosurfactants capable of degrading pollutants can further lower treatment costs (Wilson and Jones, 1993)

Microbes have been long known to produce surface active compounds (biosurfactants) when grown on specific substrates (Zajic and Panchal, 1976) and majority of the oil degrading microorganisms secrete emulsifying agents (Reisfeld et al., 1972), which are produced throughout the growth periods. Naturally occurring biosurfactants seem to play a

very important role in degradation of petroleum hydrocarbons from polluted biosphere (Rambeloarisoa et al., 1984). Production of emulsifiers during active growth on hydrocarbons suggests that emulsifiers are involved in growth on hydrocarbons (Riser-Roberts, 1998).

There are two phases involved in the process of biodegradation (Oberbremer and Muller-Hurting, 1989). In the first phase, the most water-soluble compounds are degraded. After the interfacial tension is lowered by production of biosurfactants, the more resistant compounds can be degraded in the second phase. This is accomplished by microorganisms with high cell- surface hydrophobicity, which allows them to adhere to high-molecular-weight hydrocarbon (Rosenberg et al., 1992).

Fry et al., (1993b) has successfully remediated machine oil contaminated soil by using microbial inoculation and by biosurfactant treatment. Successful bioremediation of oil contaminated soil and groundwater from a US Arm engineering plant using naturally surfactants producing indigenous microorganisms was also reported by Fry et al., (1993 a). Harvey et al., (1990) tested the ability of biosurfactant producing *Pseudomonas aeruginosa* SB 30 strain to remove oil from Exxon Valdez Alaskan contaminated gravel in the laboratory. Bragg et al., (1994) reported the effectiveness of bioremediation activities on the Exxon Valdez Alaskan oil spill *in situ*. This was carried out by enriching the indigenous microflora using an oleophilic liquid fertilizer containing N and P. Bioremediation of crude oil contaminated desert soil in Kuwait both *in situ* and on situ was reported by Al- Awadhi et al. (1994).

2.7.2 Biosurfactant in oil clean up of storage tanks

Due to excellent emulsifying properties of biosurfactants, they are used as detergents in cleaning up hydrocarbon/crude oil storage tank. Banat et al., (1991) reported the ability of biosurfactants produced by a bacterial strain (Pet 1006) for cleaning up oil storage tanks and to recover hydrocarbons from emulsified sludge. 18-19 hrs fermentative broth containing biosurfactant produced by using 2% (w/v) glucose followed by 2% (v/v) oleic acid as carbon source in basal salt medium was used as a substitute for chemical surfactants in a test carried out on an oil storage tank belonging to Kuwait Oil Company, Kuwait (Banat et al., 1991). In a test for cleaning up of oil storage tank, about 91% crude oil could be recovered from the total sludge. The recovered crude oil was as par in quality to standard

Kuwaiti crude oil, which could be sold at a price that could cover the costs of the cleaning at approximately US $ 100 000 – 150 000 per storage tank. Such clean up process is highly desirable as it is economically rewarding and environmentally friendly (Lillienberg et al., 1992).

2.7.3 Microbial surfactants in Microbial Enhanced Oil Recovery (MEOR)

Approximately 30% of the oil present in a reservoir can be recovered using current enhanced oil recovery (EOR) technology (Singer and Finnerty, 1984). Poor oil recovery in existing producing wells may be due to low permeability or the high viscosity of the oil, which results in poor mobility. High interfacial tensions between the water and oil may also result in high capillary forces retaining the oil in the reservoir rock (Bubela, 1987). Due to failure of primary and secondary recovery technique to recover oil from reservoir, interests have evolved in tertiary recovery techniques (Morkes, 1993). MEOR is an important tertiary recovery technology that utilizes microorganisms and/ or their metabolic end products for recovery of residual oil from an oil reservoir.

The presence of different types of microorganisms with varying growth properties and metabolite production will have different effects on the reservoir environment (Banat, 1995). Thus it is necessary to consider all aspects of MEOR when trying to recover oil from reservoir by one mechanism such as the use of biosurfactants. There are several strategies involving the use of biosurfactant in MEOR (Shennan and Levi, 1987).

1. The first strategy involves injection of biosurfactant-producing microorganisms into a reservoir through the well, with subsequent *in-situ* propagation of microbes through the reservoir rock (Bubela, 1985)
2. The second strategy involves the injection of selected nutrients into a reservoir, to favour and encourage the growth of indigenous biosurfactant-producing microorganisms.
3. The third mechanism involves the production of biosurfactants in bioreactors *ex situ* and subsequent injection into the reservoir.

Laboratory studies on MEOR have typically utilized core samples and columns containing the desired substrate. The substances have been utilized to demonstrate the usefulness of biosurfactants in oil recovery from sand and limestone. Similarly, core samples have been

used to model the movement of microorganisms and nutrients through substrates to ascertain their usefulness after injection into oil reservoirs. Fermentative culture broth containing biosurfactant from *Rhodococcus* ST-5 (Abu- Ruwaida et al., 1991a,b) and the thermophilic *Bacillus* AB-2 (Banat 1993) could release 80% and 95% oil from sand-pack columns. Biosurfactant containing culture broth Pet 1006 could also release 95% residual oil from sand pack column (Banat, 1993). Biosurfactant produced by *Bacillus* sp C-14 used at concentration of 0.04 mg/ml could release oil from oily sand (Eliseev et al., 1991).

Field studies involving MEOR increases the production of oil by 250% using *Clostridium acetobutylicum* (Tanner et al., 1991). MEOR investigations in carbonate reservoirs showed an increase of 60-120% in oil production in Hungary (Hitzman, 1983) and 200% increase in Germany (Wagner, 1991). Oil production increased from 0.3 barrel/day to 1.6 barrel/day after injection of a commercial biosurfactant product (RAM Biochemicals Wel Prep 5) on three wells on the Burnett J lease, USA (Nelson and Launt, 1991).

2.7.4 Use of biosurfactants in food industries

Food surfactants may be broadly classified by the functions they serve. Accordingly they are grouped as solubilizers, emulsifiers, detergents, crystallization modifiers in both aqueous and non-aqueous systems, and as foaming, wetting, lubricating, and complexing agents. Table 2.1 gives the brief overview of the application of biosurfactants in various food items with the function, as cited by Kachholz and Schlingmann (1987). In the food industry, biosurfactants are used as emulsifiers in the processing of raw materials (Makkar and Cameotra, 2002). Other applications of surface-active compounds are in bakery and meat products where they influence the rheological characteristics of flour or the emulsification of partially broken fat tissue (Vater, 1986b). Lecithin and its derivatives are currently in use as emulsifiers in the food industry worldwide (Bloomberg, 1991). *Candida utilis* bioemulsifier has been used in salad dressing (Shepherd et al., 1995). Busscher et al. (1996) have used biosurfactants produced by thermophilic dairy Streptococci for fouling control of heat-exchanger plates in pasteurizers as they retard the colonization of *S. thermophilus* responsible for fouling.

Table 2.1 Functionality of Emulsifiers in different food items

Food item	Improved functionality through addition of emulsifier
Melted chocolate	Viscosity, yield point, crystallization
Fat icing Sugar icing fondants	Crystallization
Instant products	Wettability, dispensability, solubility
Fats	Antifreezing property
Frying margarine	Minimize spattering
Deep-frying fat	Minimize foaming

2.7.5 Use of biosurfactants in agricultural sectors

Surface active compounds like salts, polymeric fatty acids, or short- chained alkyl sulfonates are used in agricultural sector for hydrophilization of heavy soil. Good wettability and equal distribution are the precondition for loosening the soil. Hydrate formation between emulsifiers and water helps in soil improvement.

Some cationic surfactants are known to bind to the soil and influence its structure and water holding capacity. In addition to this, the influence at the same time directly affects the plants by stimulation of assimilation and photosynthesis, thereby increasing the growth and yield of the plant. Most of the pesticides are water insoluble and are marked in powder or in liquid concentrate form. Before spraying in field these are diluted with water and mixed with surface active compounds for spontaneous distribution of the water insoluble pesticides in the aqueous phase as well as an equal distribution on wetting of the treated areas.

Glycolipopeptide, produced by two *Bacillus strains* were able to form a stable emulsion in the presence of the pesticide fenthion (Patel and Gopinathan 1986). The compound had some activity against other liquid-immiscible organophosphorous pesticides, but not solid organophosphorus pesticides, or organochlorine pesticides or hydrocarbon. Biodegradation of the chlorinated pesticide α- and β endosulfan by biosurfactant was reported by Banat et al., (2000). They used biosurfactant produced by *B subtilis* MTCC2423 and reported around 40% biodegradation of the said pesticides.

2.7.6 Use of biosurfactants in pharmaceutical sectors

Biosurfactant have some therapeutic applications. Biosurfactants (Rhamnolipids) produced by *P. aeruginosa* (Itoh et al., 1971; Haba et al., 2003), lipopeptides produced by *B. subtilis* (Sandrin et al. 1990; Leenhouts et al., 1995; Vollenbroich et al, 1997 a, b) and *B. licheniformis* (Jenney et al., 1991; Fiechter, 1992; Yakimov et al., 1995) and mannosylerythritol lipids from *Candida antarctica* (Kitamoto et al, 1993) have been reported to have antimicrobial activities. Rhamnolipid biosurfactant produced by *Pseudomonas aeruginosa* was recently reported to have potential algicidal activity against some harmful algae (Wang et al., 2005). Surfactin was reported to have properties like hemolysis and inhibiting fibrin clot formation that indicates its potential use in the pharmaceutical sector (Bernheimer and Avigard 1970). It has also been reported that surfactin possess antitumor activity against Ehrlich's ascite carcinoma cells (Kameda et al., 1974), inhibiting cyclic adenosin 3′, 5′- monophosphate phosphodiesterase (Hosono and Suzuki, 1983) and having antifungal properties (Vater, 1986a). Iturin, a lipopeptide produced by *B. subtilis* was reported to have antifungal properties (Thimon et al., 1995). Pumilacidin, a surfactin analog was reported to have inhibitory effect against herpes simplex virus 1 (HSV-1), H^+, K^+-Atpase and gastic ulcer in vivo. Itokawa et al., (1994) reported the potential of surfactin against human immunodeficiency virus 1(HIV-1). Takizawa et al., (1995) reported significant stimulation of the proliferation of bone marrow cells from BALB/c female mice by lipopeptides produced by *S. amethystogenes*. Glycolipids from *C. antarctica* T-34 was reported to induce cell differentiation in the human promyelocytic leukemia cell line HL60. The reports on antibiotic effects (Neu et al., 1990) and inhibition of HIV virus growth in white blood corpuscles have opened up new arena in the potential application of this microbial surface active compounds in pharmaceuticals.

The involvement of biosurfactants in microbial adhesion and desorption has also been reported. *Streptococcus thermophilus* produce biosurfactant, which caused its own, desorption from glass, leaving a completely non-adhesive coating (Busscher et al., 1990). Release of biosurfactant by an oral *S. mitis* strain, which was responsible for a reduction in adhesion of *S. mutans* was reported by Pratt- Terpstar et al. (1989). Similarly, Velraeds-Martine et al. (1996) reported on the inhibition of adhesion of pathogenic enteric bacteria by biosurfactants produced by a *Lactobacillus* strain and later showed that the surface active compound caused an important, dose–related inhibition of the initial deposition rate of *Escherichia faecalis* and other bacteria adherent on both hydrophobic and hydrophilic substrates (Velraeds- Martine et al. 1997).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Collection of environmental samples

Soil and water samples contaminated with crude petroleum oil were collected from various locations of ONGC oil fields of Assam, India. Additionally, to it soil and water samples were also collected from "Garampani" -a hot spring in Golaghat district of Assam, India and from hotsprings of Himachal Pradesh, India. Soil samples used for bioremediation experiments were collected from crude petroleum contaminated sites of ONGC oil fields, Assam, India.

3.1.2 Other materials

Components of minimal (M9) salt media and media for biochemical tests were purchased from Hi-Media Laboratories Pvt. Ltd., India. Hydrocarbons used in the study were purchased from Merck- Schuchardt, Germany. Acetone and hexane were purchased from Merck India Ltd., India. Petrol, diesel and kerosene were procured from a local fuel filling station. Kits for testing the biochemical parameters of fish serum viz. serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP), triglycerides, total protein and total cholesterol were purchased from Monozyme India Limited, Mumbai, India. Kit for estimation of serum bilirubin content (Liquizone Bilirubin T & D) was procured from Ozone Biomedicals Pvt. Ltd, New Delhi, India. Molasses was purchased from known grocery stores in Tezpur. Mosquito larvae were collected from a water lodging area inside Tezpur University campus. Potato peels were collected from the kitchen waste of hostel mess, Tezpur University. Starter culture for alcohol production was obtained from local tribes of Assam, India. All other reagents of analytical grade were purchased from Sigma (USA). Primers for PCR-RFLP of *Bacillus* strains were procured from Sigma-Aldrich India Ltd. (Bangalore, India) and that for *Psuedomonas* strains were procured from Bioserve India Ltd. (Hydrabad, India).

3.1.3 Preparation of potato peels as carbon source

Potato peels collected from Tezpur University hostel canteen were washed several times with distilled water to remove the surface dirties followed by blenching them by immersing in hot water (75-80 °C) for 20 min. Peels were then dried at 45°C for 36 hours, grinded in a mixer grinder at room temperature (Remi-Auto-Mix-Blender, Remi Amupam Mixie, Mumbai, India) to form a paste. The paste was dried overnight at 45°C and sterilized at 120°C, 15 lbs. pressure and used subsequently as carbon source for microbes.

3.2 Isolation and preservation of biosurfactant producing microbes

3.2.1 Isolation of microbes by enrichment culture

One gm or one ml of each soil/ water sample was added to 100 ml of defined medium (M9) with 2% (v/v) n- hexadecane as a source of carbon and incubated at 45°C for 7 days with a shaking of 200 rpm. The composition (g / l) of the M9 medium (Atlas, 1993) was, 6.0 g Na_2HPO_4; 3.0 g KH_2PO_4; 1.0 g NH_4Cl; 0.5 g NaCl; 0.014 g $CaCl_2$; 0.245 g $MgSO_4.7H_2O$; 10 mg of thiamine-HCl and 1 ml of micro-nutrient stock (see section 3.3.6). After 7 days, 1 ml inoculum was transferred to the same (fresh) medium and incubation was carried out for another 7 days under the identical condition. The process was repeated three times and finally 1 ml inoculum from the third enrichment culture was appropriately serially diluted, and from which 100µl was plated on nutrient agar plates. Isolated pure colonies were picked up and preserved on glycerol stock at –80 °C.

3.2.2 Isolation of microbes directly from environmental sample (hot spring water)

Samples from hot springs were serially diluted with sterile Milli Q H_20, plated on nutrient agar plates followed by incubation of the samples at the temperature of their original habitats (43°C to 80 °C). Single colonies were picked up by conventional technique and preserved on glycerol stock at –80 °C.

3.2.3 Microorganisms from other sources

One of the biosurfactant producing bacterial strain was isolated from starter culture used for alcohol production by a local tribe of Assam, India. The starter culture was serially diluted in

sterile Phosphate buffered saline (PBS), and 100 µl of the appropriately diluted sample was plated in nutrient agar plates to get isolated pure colonies (Cappuccino and Sherman, 1999).

3.2.4 Routine maintenance and preservation of microorganisms

Pure culture of bacteria were preserved at 4°C in nutrient agar slants and transferred to fresh slants at an interval of one month. Isolates were also stored in 15% (v/v) glycerol in nutrient broth and kept at -80° C for long time storage.

3.3 Screening of biosurfactant producing thermophilic bacteria

3.3.1 Screening of thermophilic bacteria

Thermophilic bacteria were selected based on their capacity to grow at 45°C temperature. Briefly, isolated pure cultures were grown in duplicate for thrice at nutrient agar and mineral salt media agar plates and incubated at 45 C temperature for overnight. The pure colonies capable of growing in the plates were selected for further studies. These colonies were re-grown on broth culture at 45 C temperature to check their capability to grow at thermophilic condition.

3.3.2 Screening of biosurfactant producing thermophilic bacteria

In, the next steps, pure cultures of thermophilic bacteria were tested for their ability to produce biosurfactant. This was judge by their ability to reduce the surface tension of the growth medium (Maier, 2003; Mulligan, 2005).

3.4 Taxonomic identification of biosurfactant producing microbes

3.4.1 Biochemical and morphological tests

The hyper-producing, thermophilic bacteria were taxonomically identified by (a) standard biochemical test and (b) studying their morphological characteristics (Cappuccino and Sherman, 1999 and Bergey's Manual of Systematic Bacteriology, 1984,1986).

3.4.2 Fatty acid methyl ester (FAME) analysis

Strain typing based on their cellular fatty acids contents was done using Microbial Identification System (MIS) of Institute of Microbial Technology (IMTECH), Chandigarh, India. The MIS system determines the cellular fatty acid profile of the bacterial isolates by high resolution gas chromatography analysis. This profile is then compared by utilizing the MIDI software, which possess the database of cellular fatty acid profiles of known bacteria. A similarity index (SI) of 0.6 or higher indicated a very good match, whereas SI index lower than 0.25 indicated a poor match (Heyrman et al., 1999; Yuste et al., 2000).

3.4.3 PCR-Restriction Fragment Length Polymorphism

3.4.3.1 Isolation of chromosomal DNA

3.4.3.1.2 DNA extraction by alkaline lysis

Genomic DNA from *B. subtilis* strains (DM-03 and DM-04) was prepared as described by Ausubel et al., (1995). The cells were pelleted by spinning 4 ml of culture at 9,450 x g for 10 min in a Hettich Zentifugen, Germany and supernatant was decanted. Pellet was resuspended in 0.8 ml of solution I. To this resultant mixture 160 µl of lysozyme (10 mg/ml) was added and incubated at room temperature for 20 min. Subsequently 44.5 µl of 10% (w/v) SDS solution was added and re-incubated for 10 min at 50°C. There after 53.3 µl of Rnase A (10 mg/ml) was added and incubated at 37°C for 1.5 h. This was followed by addition of 45.3 µl of Na-EDTA (0.1 M, pH 8.0) and incubation at 50°C for 10 min. To remove the protein, 26.6 µl of proteinase K (5mg/ml stock) was added and incubated at 50°C for 16h. Equal volume of phenol (saturated with 0.1 M Tris HCl, pH 8.0) was added and mixed thoroughly. The mixture was centrifuging at 9,450 x g for 10 min, the upper (aqueous) phase was aspirated into sterile eppendorf and lower phase was discarded. 700 µl of (1:1) phenol and chloroform-isoamylalcohol (24:1) was added and mixed thoroughly. Following centrifuging at 9,450 x g for 10 min, the upper phase was transferred to a sterile microfuge tube, then equal volume of chloroform-isoamyalcohol (24:1) was added and spun at 9,450 x g for 10 min. The upper phase was transferred to a sterile microfuge tube and $1/10^{th}$ volume of salt (Na-acetate 3M, pH-7.0) was added. The DNA was precipitated by adding 2 volumes of ice-cold absolute ethanol in the above solution and the DNA pellet was recovered by centrifugation followed by removal of alcohol. DNA was resuspended in 10

mM Tris HCl-1mM EDTA buffer (pH 8.0) at a final concentration of 1 µg / ml and was stored at 4°C until it was further used.

3.4.3.1.2 Direct chromosomal DNA extraction

A direct method of DNA extraction from the *P. aeruginosa* strain was adopted following the protocol described by Barsotti et al., (2002). In brief, after removal the colonies from the surface of agar plate, the bacteria were suspended in a microfuge tube containing 300 µl of sterile MilliQ water and then vortexed for 20 sec. The DNA was extracted after cell lysis by immersing the tubes in boiling water bath for 10 min. The cell debris was pelleted by centrifugation at 16,000 x g for 10 sec (in a Hettich Zentifugen, Germany) and the supernatant containing DNA was removed and transferred to fresh microcentrifuge tube. The concentration of DNA was estimated spectrophotometry at 260 nm (Beckman DU 530^R spectrophotometer).

3.4.3.2 Synthesis of primers for ISR amplification

3.4.3.2.1 Synthesis of primers for ISR amplification of *B. subtilis* strains

All primers were designed by using Fast PCR TM primer analysis software, version 3.02.0130 ("PCR Team" Ruslan Kalendar). For PCR-restriction fragment length polymorphism (PCR-RFLP), oligonuclotide primer pairs 16S/p1 (5'-AGTCTGCAACTCGACTGCGTG-3') and 23S/p2 (5'-CAACCCCAAGAGGCAAGCCTC-3'), complementary to nucleotide sequence of 11128 to 11149 of 16S and 11988 to 12009 of the 23S rRNA gene (*B. subtilis* complete genome, Pubmed accession no. AL009126), were used to amplify approximately 0.88 kb of spacer region DNA in PCR system thermal cycler (GeneAmp* PCR system 9700, Applied Biosystems). For the specificity of the ISR amplification, the PCR products were used as templates in a second PCR (nested PCR) with primers 16S/p3 (5'-GGAAGGTGCGGCTGGATCACC-3') and 23S/p4 (5'-CCCGAAGCATATCGGTGTTCG-3'), which anneal to positions 11334 to 11355 of the 16S rRNA gene and position 11770 to 11791 of the 23S rRNA gene respectively. The nested PCR amplification includes the entire ISR and a part of the flanking rDNAs (ca. 28 bp of 16S rDNA and 83 bp of 23S rDNA).

3.4.3.2.2 Synthesis of primers for ISR amplification of *P. aeruginosa* strains

All primers were designed by using Fast PCR TM primer analysis software, version 3.02.0130 ("PCR Team" Ruslan Kalendar). PCR based RFLP of the two *Pseudomonas* strains, viz., *P. aeruginosa* M and NM strains oligonucleotide primers 16S/p5(5'-ATGGGAGTGGTTGCTCCAG-3') and 23S/p6(5'-GGATCAAAGTCTGTTTGCCGAC-3') were designed complementary to conserved regions annealing to positions 1406 to 1426 of the 16S rRNA gene to positions 84 to 104 of the 23S rRNA gene (*Pseudomonas aeruginosa* numbering; Genebank accession number PA0668.1) to amplify 0.8 kb of ISR in PCR system thermal cycler (GeneAmp*PCR system 9700, Applied Biosystems).The resultant PCR products contained the complete 16S-23S ribosomal inter spacer region (ISR) and parts of the flanking rDNA (ca. 130 bp of 16S rDNA and 104 bp of 23S rDNA). For the specificity of the ISR amplification, the PCR products were used as templates in a second PCR (nested PCR) with primers 16S/p7 (5'-GTAGCCGTAGGGGAACCTGC-3') and 23S/p8 (5'-GCTTTTCGCAGGCTACCACG-3'), which anneal to positions 1498 to 1518 of the 16S rRNA gene and position 76 to 56 of the 23S rRNA gene respectively. The nested PCR amplification includes the entire ISR and a part of the flanking rDNAs (ca. 38 bp of 16S rDNA and 76 bp of 23S rDNA).

3.4.3.3 PCR amplification and purification

All PCR reactions were carried out in a final volume of 25µl containing 2.5µl of 10x PCR buffer with $MgCl_2$, 2 µl of mixture containing each dNTP's at a concentration of 2.5 mM, each primer at a final concentration of 5.0 pM and 1 Unit of *Taq* DNA polymerase. The amplification reaction consists of 30-s denaturation step at 94°C, 30-s annealing step at 54°C (for the *Bacillus* strains) or 51°C (for the *Pseudomonas* strains) and a 2 min extension step at 72°C. After the completion 30 cycles of PCR, there was a final 5- min extension at 72°C. The PCR amplifications were performed using PCR system thermal cycler (GeneAmp* PCRsystem 9700, Applied Biosystems). PCR products were subsequently purified with a QIA-quick PCR purification kit (Qiagen) according to the manufacturer's instruction.

3.4.3.4 Restriction analysis

The purified PCR products (DNA) were digested with 10-15 units of *Mbo*I at 37 °C and the total digested products were separated in a 3% (w/v) agarose gel in Tris-borate-EDTA buffer. DNA fragments sizes were compared with standard DNA molecular weight markers (100 bp ladder) from Sigma-Aldrich. Gel images were documented with Geldoc-1000 (BioRad).

3.5 Measurement of surface activities of crude and purified biosurfactants

3.5.1 Surface / interfacial tension reduction measurement

Surface tension and critical micelle dilution (CMD^{-1}, CMD^{-2}) were determined using a Du-Nouy Tensiometer (Kruss 9KT Tensiometer, Kruss, Germany) at room temperature (25 C) using the ring correction mode of the instrument. The interfacial tension was measured by dipping the ring into the aqueous biosurfactant sample, layering an equal volume of *n*-hexadecane on the surface, and then measuring the force required to pull the ring through the aqueous –oil interface. All measurements were made with fresh interfaces (less than 5 min) at 25 ± 2 C (Neufield et al., 1980).

3.5.2 Critical Micelle Concentration (CMC)

Critical Micelle Concentration (CMC) was determined by the sudden break in the curve obtained by the plot of surface tension versus the plot of log of concentration of biosurfactant (Kim et al., 2000).

3.6 Optimization of culture conditions for optimum growth of and maximum biosurfactants production by microbes

3.6.1 Effect of different carbon sources on biosurfactant production

A total 16 different carbon sources were used to study their effect on biosurfactant production by the bacterial strains. Dextrose, molasses, starch, and potato peels, glycerol, hexadecane, diesel, petrol, kerosene, dodecane, liquid light paraffin, octane and octadecane were used at were used at a concentration of 2% (either w/v or v/v). The

aromatic hydrocarbons used in this study viz., pyrene, anthracene and phenethrene were dissolved in N-N-dinitroformamide solvent and used at a final concentration of 250 mg/l. After 48 hours (for the *B. subtilis* strains) or 96 hours (for the *P. aeruginosa* strains) of growth, the surface tension reduction, CMD values, yield of biosurfactant and dry biomass were recorded.

3.6.2 Effect of different concentrations of glycerol and glucose on biosurfactant production

Two best carbon sources, viz. glycerol and glucose were used at different concentrations ranging from 0.5% to 3% (w/v or v/v) to study their effect on biosurfactant production and growth of bacteria. After the incubation for desired time periods and temperatures, the surface tension reduction and CMD values of crude biosurfactant and dry bacterial biomass were recorded.

3.6.3 Effect of different nitrogen sources on biosurfactant production

The following ten inorganic and organic nitrogenous sources were tested for their effect on growth and biosurfactant production viz., beef extract, yeast extract, peptone, tryptone, ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$), urea (H_2NCONH_4), ammonium chloride (NH_4Cl), potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3) and ammonium sulphate {$(NH_4)_2SO_4$}. After 48 hours (for the *B. subtilis* strains) or 96 hours (for the *Pseudomonas sp.*) of bacterial growth, the surface tension reduction, CMD values, yield of biosurfactant and dry biomass were recorded.

3.6.4 Effect of different concentration of the nitrogen sources on biosurfactant production

Different concentrations of best nitrogen source for each bacteria (*B. subtilis* and *P. aeruginosa*), ranging from 0.05% to 0.4 % (w/v) were used to study the growth and biosurfactant production. After 48 hours (for the *Bacillus* strains) or 96 hours (for the *P. aeruginosa* strains) of growth, the surface tension reduction, CMD values and dry biomass were recorded.

3.6.5 Effect of cations and mineral salts on biosurfactant production

Graded amounts of metal ions, viz. Mg, Ca and mineral salts viz. KH_2PO_4, Na_2HPO_4 were added in the growth medium to see their influence on biosurfactant production. A control experiment was run in parallel without adding any cation. Following the incubation for desired time period and temperature, reduction in surface tension, CMD values and dry biomass (bacterial growth) were measured.

3.6.6 Effect of micro-nutrients on biosurfactant production

Various concentrations of trace elements (micronutrients) were prepared and added in various combinations to the growth media to check their effect on the enhancement of bacterial growth and biosurfactant production. The composition of micronutrient solution is (µg/l) $Fe_2SO_4.7H_2O$- 1000; $CuSO_4.5H_2O$- 50; H_3BO_3- 10; $MnSO_4$- 10; $ZnSO_4.7H_2O$- 70 and MOO_3- 10. A control experiment was run simultaneously without adding any micronutrient. After the required incubation time, the surface tension reduction, CMD values and dry biomass were recorded.

3.6.7 Effect of growth supplement (Thiamine-HCl) on biosurfactant production

Thiamine-HCl solution was prepared by dissolving 10 mg in a litre of deionized water (Atlas, 1993). Different concentrations of Thiamine-HCl (0.5ml/l to 1.5 ml/l) were added in the growth media to check their effect on biosurfactant production. A control was run without Thiamine-HCl. After incubation for the desired time period, the surface tension reduction, CMD values and dry biomass were recorded.

3.6.8 Effect of agitation (shaking) on biosurfactant production

In this experiment, 250 ml Erlenmeyer flask containing 100 ml mineral salt medium was used for growth and biosurfactant production studies. The flasks were placed in a incubator shaker and agitated at different revolution per minute (rpm) ranging from 100 rpm to 300 rpm to evaluate the impact of agitation on biosurfactant production. A flask without agitation (stationary) was also set and considered as control. After the desired incubation time period, the surface tension reduction, CMD values and dry biomass were recorded.

3.6.9 Effect of temperature and pH on biosurfactant production

For evaluation of the effect of pH on biosurfactant production and growth of bacteria, the pH of the growth media were adjusted from pH 3.0 to 12.0. In this different pH, growth of microbes and production of biosurfactant were observed. Similarly, for studying the effect of temperature, the microbes were incubated at 30°C - 55°C, with 200 rpm agitation. After the desired incubation period of bacteria, the surface tension reduction, CMD values, yield of biosurfactant and dry biomass were recorded.

3.6.10 Assessment of salt tolerance limit of isolated microbes for biosurfactant production

To check the salt tolerance limit of potential biosurfactant producing potential strains, the salinity (NaCl concentration) of the medium was adjusted from 0.5% (w/v) to 7.0% (w/v). Post incubation of bacteria at 45°C for the desired time periods, surface tension reduction, CMD values and bacterial growth were recorded.

3.6.11 Batch fermentation

For batch fermentation, cells were grown in 3-l working volume of culture (modified M9 media) in a Bioflow 110 Fermentor (New Brunchwick Scientific, USA) set at 45°C and pH 7.0 - 8.0 with an agitation of 200 rpm. When the cells reached the late log phase or stationary phase (48 hr for *Bacillus* strains and 96 hrs for *Pseudomonas* strains), they were harvested and biomass was determined as described in section 3.8. The cell-free clear supernatant was used for the surface activity assessment and purification of biosurfactants.

3.7 Isolation and purification of biosurfactants

3.7.1 Isolation

3.7.1.1 Isolation of biosurfactant from *B. subtilis* strains

Bacterial cells were removed from the medium by centrifugation at 7,650 x g for 25 min at 4 °C (using a Sorvall SS-34 rotor) and crude lipopeptide surfactants were isolated from the medium by the procedure of Vater et al. (2002). Briefly, surfactants were precipitated from

the cell free fermentative broth by adding 6 N HCl to obtain a final pH of 2.0. The acid precipitates were recovered by centrifugation at 7,650 x g for 15 min at 4°C (using a Sorvall SS-34 rotor) and were extracted with dichloromethane or methanol (lipopeptide fraction). When methanol was used as the solvent, the extract was neutralized immediately to avoid formation of methyl esters. The products were dried under vacuum, weighed and kept at – 20 °C until further use.

3.7.1.2 Isolation of biosurfactant from *P. aeruginosa* strains

Crude biosurfactants secreted by *Pseudomonas* strains were extracted from the cell-free culture broth post 96 h of growth. Extraction of biosurfactant was done by acetone precipitation followed by acidifying the broth to pH≈2.0 (Pruthi and Cameotra, 1995; Turkovskaya et al. 2001). The precipates were allowed to form overnight at 4°C. The product recovered by centrifugation at 7,650 x g for 15 min at 4°C (using a Sorvall SS-34 rotor) and dried under vacuum, weighed and kept at –20 °C until further use.

3.7.2 Purification of biosurfactants

3.7.2.1 Purification of biosurfactants from *B. subtilis* strains

Separation of lipopeptide isoforms from crude surfactants mixture of the *Bacillus* strains was carried out by high performance liquid chromatography (Waters) coupled with a reverse phase C_{18}-μ-Nova pak HPLC column (3.9 mm X 150 mm). Elution was carried out with an isocratic gradient of methanol: 10 mM potassium phosphate buffer, pH 6.0 (80:20) at a flow rate of 1-ml min^{-1}. Detection was monitored at 210 nm (Waters 2487 Dual λ Absorbance Detector, Milford, USA) and individual peak was screened for biosurfactant activity. The peak displaying highest surface tension reducing activity was pooled, dried and dissolved in 20% (v/v) acetonitrile containing 0.1% (v/v) trifluroacetic acid (TFA). The lipopeptides present in this peak were further separated by RP-HPLC on the same C_{18}-μ-Nova pak column. Elution was carried out with a linear gradient of 20 to 100% acetonitrile – 0.1 % (v/v) TFA (Vater et al., 2002).

3.7.2.2 Fractionation of biosurfactants from *P. aeruginosa* strains

Fractionation of biosurfactants isolated from the two *P. aeruginosa* strains was done on a Waters reverse phase C_{18}-μ-Nova pak (3.9 mm X 150 mm) HPLC column. Bound biosurfactants were eluted with a isocratic gradient of methanol (60%) and 10 mM K-P buffer, pH 6.0 (40%) at a flow rate of 1-ml min^{-1}. Detection was monitored at 210 nm (Waters 2487 Dual λ Absorbance Detector, Milford, USA) and individual peaks were collected manually. Peak showing maximum biosurfactant activity was lyophilized and stored at –20°C.

3.8 Dry biomass determination

Dry biomass, an indicator of bacterial growth was determined by the method of Makkar and Cameotra (1998). In case of aliphatic hydrocarbons (hexadecane, dodecane, diesel, kerosene, petrol, liquid light paraffin, octane and octadecane), the culture broth was treated with hexane to remove the residual hydrocarbons and in case of aromatic hydrocarbons (pyrene, phenethrene and anthracene), the culture medium was treated with ethyl acetate to remove the residual hydrocarbons. The culture broth was then centrifuged using a Sorvall SM-24 rotor at 12,365 x g for 25 min. Biomass obtained was dried at 100 °C and weighed.

3.9 Biochemical characterization of biosurfactants

3.9.1 Protein estimation

The protein content was estimated by Folin-Lowry method (Lowry et al., 1951) using BSA as a protein standard. The protein content of the unknown samples were calculated from the standard curve obtained by plotting optical density Vs concentration of BSA (1 mg/ml).

3.9.2 Estimation of carbohydrate content

Total carbohydrate was quantitated by Phenol-sulphuric acid method as described by Dubois et al. (1956) using D-glucose as standard. The optical density of the reaction mixture was measured at 490 nm against a reagent blank. The carbohydrate content of the

unknown samples were calculated from the standard curve obtained by plotting optical density Vs concentration of D-glucose (0.1 mg/ml).

3.9.3 Estimation of total lipid

The total lipid was estimated spectrophotometrically as described by Frings and Dunn, (1970). The concentrations of the unknown samples were calculated from the standard curve obtained by using phosphotidylcholine as lipid standard.

3.9.4 Assay of pH stability and thermostability of isolated biosurfactants

Graded concentrations of biosurfactants solutions were made (1 mg/ml to 10 mg/ ml) and the resulting solutions were heated at 100°C for different time periods (5 min to 30 min), cooled down to room temperature followed by measuring their surface tension and CMD values. Similarly, for assaying the pH stability, the biosurfactant aqueous solutions were adjusted to different pH values (ranging from pH 3 to 12) and surface tension and CMD values of the resulting solution were measured.

3.9.5 Emulsification assay and assessment of emulsion stability

3.9.5.1 Assessment of emulsification index of biosurfactants

Emulsification assay of biosurfactant was also done by the method described by Cooper and Goldenberg, (1987). Briefly, emulsification activity was measured by adding 6 ml of kerosene to 4 ml of aqueous biosurfactant solution and vortexing the mixture at high speed in SPINIX vortex, for 2 min. The resulting mixture was kept at room temperature (~25°C) for 24 h and emulsification index (E_{24}) was calculated as follows

E_{24}=Height of emulsion layer (cm) / Total height of the mixture (cm) X 100

3.9.5.2 Assessment of emulsion stability

Stability of the emulsion formed by the action of biosurfactant was assessed as described by Kim et al, (2000). Biosurfactant or SDS solution was dissolved at a final concentration of 0.2% (w/v) in PBS, pH 7.0, and 1 ml of hexadecane was added to make a final volume of 5

ml. Mixtures were vortexed at high speed for 1 min to form the emulsion. The emulsions were allowed to stand at room temperature for 10 min and then the absorbance at 540 nm was recorded using a Beckman DU 530^R spectrophotometer in every 5 min interval for 1 h. For control experiment, same volume of PBS buffer and hexadecane were used. For assessing the emulsion stability, the log of the absorbance was plotted against time. The slope (decay constant, K_d) of the line was calculated and expressed as emulsion stability.

3.9.6 Functional group determination by Infrared (IR) spectra analysis

The Infrared (IR) spectrum of the biosurfactants was recorded using KBr pellet in Nicolet Impact 410 FTIR spectrophotometer. Sample was prepared by dispersing the solid uniformly in a matrix of dry nujol (KBr) mull, compressed to form an almost transparent disc. The spectra showing the functional groups were used to study the composition of the biosurfactant. Absorption spectra were plotted using a built-in plotter. IR spectra were collected from 500-4000 wave numbers (cm^{-1}).

3.9.7 Mass determination by MALDI-TOF- MS analysis

Molecular mass of purified biosurfactant was determined by matrix-assisted laser desorption/ionization time of flight mass spectrophotometry (MALDI-TOF MS) using Ultraflex MALDI- TOF spectrophotometer (Bruker, Germany). Analysis was carried out in a α-ctabi-4-hydroxycinnamic acid (CHCA) matrix, suspended in 50% acetonitile and 0 1% TFA with 337 nm nitrogen laser shots. Spectra were obtained in positive ion mode and 25 kV reflector voltage was used. Identities of the biosurfactant isoforms were determined by comparison with known standards.

3.9.8 Light absorption spectra of crude biosurfactants

The crude biosurfactants from both the *B. subtilis* and *P. aeruginosa* strains, obtained after acid precipitation and solvent extraction was dissolved in 0.05M Na_2HCO_3 solution at concentration of 1 mg/ml and absorption at different wave lengths of light ranging from 190 nm to 510nm was measured to find the λ_{max} value.

3.10 Pharmacological characterization of biosurfactants

3.10.1 Interference in blood coagulation

Interference in blood coagulation (coagulant or anticoagulant effect) by crude/purified biosurfactant was assayed as described by Doley and Mukherjee (2003). Briefly, platelet poor plasma (PPP) from goat/healthy human donor was prepared by centrifuging (2,500 X g at a Remi Centrifuge) the citrated blood (1:9) twice for 15 min at 4 °C and used within 4 h of collection. To assay the recalcification time, specific amount of crude/purified biosurfactant (in a final volume of 30 µl) was added to 300 µl of PPP pre-incubated at 37 °C. The mixture was incubated for 2 min at 37 °C and 40 µl of 250 mM $CaCl_2$ was added to this reaction mixture. The clotting time of plasma was recorded with the help of stopwatch based on the first appearance of a fibrin clot. As a control, plasma aliquot was incubated with 30 µl of phosphate buffer saline (PBS) and coagulation time was determined identically.

3.10.2 Assay of hemolytic activity

Direct hemolytic activity was assayed as described by Doley and Mukherjee (2003). Briefly, graded concentrations of biosurfactant solutions were added to 5 % (v/v) of human erythrocyte suspension (in 100 mM phosphate buffer, pH 7.4) and final volume was adjusted to 3 ml. After incubating for 60 min at 37 °C, the reaction mixture was centrifuged and released hemoglobin was measured spectrophotometrically at 540 nm (U-2001 UV/Vis Spectrophotometer, Hitachi, Japan). For cent percent (100 %) hemolysis, RBC suspension was incubated with 0.1% (v/v) Triton X-100.

3.10.3 *In-vitro* tissue damage

For the assay of *in-vitro* tissue damaging activity, procedure of Doley and Mukherjee (2003) was followed. The percentage of hemoglobin released i.e. the *in-vitro* tissue damaging activity of crude /purified biosurfactant was calculated with respect to tissues incubated with 0.1% (v/v) Triton X-100 (100% activity).

3.11 Cell surface hydrophobicity

The surface hydrophobicity of the bacterial cells was measured by the method of Rosenberg et al. (1980). Briefly, cells were grown either on 2 % (v/v) hexadecane or 2% (w/v) glucose separately in basal salt medium for 20-22 h at 45°C. Then cells were harvested by centrifugation in a Sorvall SS-34 rotor at 7,000x g for 15 min, washed twice with phosphate buffer (50 mM, pH 7.5) and suspended in the same buffer in such a manner that the final absorbance was adjusted between 0.29- 0.31 at 600 nm. To 3.0 ml of washed cell suspension, various amounts of the test hydrocarbons (hexene, dodecane, hexadecane, xylene, benzene, hexane and toluene) were added. The mixtures were preincubation for 5 min at room temperature and then vortexed for 60 sec. The mixtures were allowed to stand at room temperature for 15 min followed by the removal of the aqueous phase with a Pasture pipette and the absorbance was recorded at 600 nm at U-2001 UV/Vis Spectrophotometer (Hitachi, Japan).

The cell surface hydrophobicity was expressed in terms of percent of cells transferred to the hydrocarbon phase, and calculated as shown below

$$\% \text{ Cell transferred to hydrocarbon phase} = 100 - \frac{\text{Absorbence at 600 nm post mixing}}{\text{Absorbence at 600 nm before mixing}} \times 100$$

3.12 Role of bacterial plasmid in biosurfactant production

3.12.1 Isolation of plasmid DNA

Plasmid DNA was isolated by the method of Sambrook et al., (1989). The cells were pelleted by from 15-20 ml of culture by centrifugation in a Sorvall SS-34 rotor at 5,050 x g for 10 min at 4°C. Pellets were resuspended in 1.5 ml of ice-cold solution I (see in Appendix) by vigorous vortexing. To this, 3 ml of freshly prepared solution II (see in Appendix) was added and mixed by inverting the tubes. Then 2.25 ml of ice-cold solution III (see in Appendix) was added and mixed by gently inverting the tubes followed by centrifugation at 11,950 x g for 10 min at 4 °C. The supernatant (containing plasmid DNA) was transferred to fresh tube and equal volume of Tris- saturated phenol: Chlorform-isoamyl alcohol (24:1) was added and mixed thoroughly by vortexing followed by

centrifugation in a Sorvall SS-34 rotor at 11,950 x g for 10 min. The supernatant was transferred to another fresh tube and 0.7 volume of isopropanol was added. Contents were mixed thoroughly and then centrifuged at 20,100 x g for 15 min at room temperature. The DNA pellet settled down at the bottom of the tube and pellet was washed twice with 1.5 ml of 70% ethanol. Plasmid DNA was recovered by centrifugation at 11,950 x g for 10 min followed by removal of the ethanol by drying in the air. Dried pellet was redissolved in 40 µl 1x TE (Tris EDTA) containing 120 µg/ml Rnase and resolved in 0.8% agarose gel and stained with ethidium bromide.

3.12.2 Curing of plasmid DNA

Plasmid curing was done by the method described by Hirota et al., (1960). The bacteria was grown in nutrient broth supplemented with acridine orange (final concentration ranging from 25 to 150 g mL^{-1}) and incubated overnight at 45°C. Culture tube with highest concentration of the dye and showing visible growth was selected and ten-fold diluted in fresh nutrient broth. About 100 µl of the diluted cultures were spread on to nutrient agar plates with the help of a sterile spreader. Mutants lacking the plasmid were isolated by plating in Ampicillin $^{+}$and Rifamycin $^{+}$ plates. Those mutants fail to grown in these antibiotics were further studied. The positive mutants were grown in the nutrient broth within overnight and on mineral salt medium within 48 hours followed by isolation of plasmid DNA by alkaline lysis method described in section 3.12.1. The positive mutant along with the wild type were grown in mineral salt medium and surface activities along with biosurfactant production and growth was recorded post 96 h of incubation at optimum growth temperature. Post 96 h of incubation, plasmid DNA was again isolated to reinforce the result of the experiment.

3.13 Some industrial application of biosurfactants produced by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains

3.13.1 Enhanced oil recovery: sand pack column test

The potential use of isolated biosurfactants in Microbial Enhanced Oil Recovery (MEOR) was evaluated by using the sand pack column technique as described by Abu Ruwaida et al., (1991). Glass columns (3 cm X 60 cm), packed with 100 g of acid washed sand were saturated either with 60 ml of kerosene or crude petroleum oil respectively. Release of oil

from sand by the action of biosurfactant was measured by applying aqueous solution of biosurfactant into the column and then measuring the amount of oil release. In control experiments, same volume of un-inoculated growth media (without biosurfactants) was applied in the column and the release of oil was measured.

3.13.2 Bioremediation of crude oil contaminated soil under laboratory condition

3.13.2.1 PAHs solubilization effect of biosurfactants

PAHs solubilization assay was done as described by Barkay et al., (1999). Briefly, 60 µg of PAHs (from 6mg/ml stock in acetone) were distributed into glass test tubes (10mm X 170 mm) and kept open inside an operating chemical fume hood to remove the solvent, followed by addition of 3 ml of assay buffer (20 mM Tris-HCl, pH 7.0) and 1.0 ml of biosurfactant solution (0.5 mg/ ml). Tubes were capped with plastic closures and incubated in a vertical position overnight at 30°C with shaking (200 rpm) in dark. Samples were filtered through 1.2 µm-pore-size-grade filters (Whatman) and 2.0 ml filtrate was removed in a clean tube to which 2.0 ml of hexane was added prior to extraction by vortexing for 2 min. This emulsion was centrifuged at 9,450 x g in a Hettich Zentrifuge, Germany for 10 min to separate the aqueous and hexane phases. PAHs in the hexane extracts were measured spectrophotometrically at 252, 250 and 273 mm for phenanthrene, anthracene and pyrene respectively. From a calibration curve of PAHs (in hexane) the concentration of each PAHs was determined. Assay buffer with biosurfactants but no PAHs was extracted with hexane in the same manner and served as blank. Control experiments were also run in parallel where no biosurfactant was added to PAHs before extraction with hexane.

3.13.2.2 Laboratory scale biodegradation experiment

The ability of the bacterial strains to remediate the crude oil contaminated soil sample was investigated by carrying out the biodegradation experiment for 120 days under laboratory condition (~25°C temperature). Ten kg of crude petroleum oil contaminated soil sample (level of contamination was detected as describe below) collected from ONGC field of North-East India, was layered in a rectangular tray of 30 cm X 45 cm X 10 cm (length X breadth X height) size. Prior to starting the biodegradation experiment the soil samples were treated with aqueous solutions (0.1% w/v) of biosurfactants obtained from the respective strains at a concentration of 100 ml biosurfactant solution/ kg of soil and then left

for 3 days at room temperature. The soil was then seeded with 1litre of mineral salt media containing $1x10^7$ CFU of either *B. subtilis* DM-04 strain or *P. aeruginosa* M and NM (1:1) strains. Each experiment was run in triplicate. The soil was thoroughly trilled at every two weeks interval. Glucose (100 mg/l) was supplemented at an interval of 30 days. To determine the total extent of biodegradation, the soil-phase TPH concentrations were analyzed after 120 days. A control experiment was also run in parallel where the soil was treated in the identical manner except that the mineral salts media (M9) was devoid of any microbes.

3.13.2.3 Chemical analysis of oily sludge

Total petroleum hydrocarbon (TPH) was extracted from 10 g of soil by sequentially extracted with 100 ml of hexane, methylene chloride, and chloroform (Mishra et al., 2001). All the three extracts were pooled and dried at room temperature by evaporation of the solvents under a gentle nitrogen stream in a fume hood. After evaporation of the solvent, the amount of residual TPH was determined gravimetrically (Mishra et al., 2001). Further fractionation of TPH into aromatic, aliphatic, asphaltene and NSO (nitrogen, sulfur and oxygen- containing compounds) was done on a silica gel column of 2cm X 30cm size (Walker et al., 1975). TPH extracts were dissolved in n- pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on the top of a silica gel G (60-120 mesh) column and eluted with solvents of different polarities. The alkane fraction was eluted with 100 ml each of hexane followed by eluting the aromatic fraction with 100 ml of toluene. The NSO fraction was eluted with 100 ml of methanol and chloroform (Mishra et al., 2001).

3.13.2.4 Analysis of alkane fraction by gas-chromatography

The alkane fraction was analyzed by gas chromatography (GC) using a FID detector (Varian Saturn 3800). The column was CPSil 8 low bleed (30 m X 0.25 mm X 0.25 μm) coupled with a CP-Sil 5 CB low bleed/MS (30 m X 0.25 mm X 0.25 μm) column with helium as a carrier gas. The column temperature was 80-240 °C for 30 min with 5 °C /min increment and hold at 240 °C for 30 min, the injector temperature was 240 °C and the transfer line temperature was 300 °C. Individual components present in the alkane fraction were determined by matching the retention time with authentic standards.

3.13.3 Biodegradation of pyrene

3.13.3.1 Media and microbial growth in presence of pyrene

The mineral salt medium used in this study was described previously. To measure the microbial growth at the expense of pyrene as sole source of carbon, this compound dissolved in N', N'-dimethylformamide was added to sterile mineral salts medium to give a final concentration of 250 mg/ litre (Boonchan et al., 2000). The flasks were shaken at 200 rpm and 37°C before inoculation for the removal of N', N'-dimethylformamide. If glucose was used as a second/co-carbon source, it was supplied to the culture medium at a final concentration of 100 mg / liter.

Replicate batch cultures were grown in 250-ml Erlenmeyer flasks containing 100 ml of M9 mineral salts medium supplemented with either pyrene or pyrene and glucose as carbon source(s). Incubation was performed at 45°C temperature and pH 7.0 for *P. aeruginosa* M and NM strains or 55 °C temperature and pH 8.0 for *B. subtilis* DM-04 strain with 200 rpm rotary shaking. Uninoculated flasks and flasks without pyrene were served as control. Pyrene and protein concentrations were measured after 48 h and 96 h of growth.

3.13.3.2 Determination of bacterial growth in presence of pyrene

Bacterial growth by utilizing pyrene was assessed by measuring the bacterial protein concentration, dry biomass and the residual pyrene remained in the culture medium (Vila et al., 2001). Bacterial dry biomass was determined after extracting the residual pyrene from the culture medium and then pelleting the cells (Makkar and Cameotra, 1998). The protein concentration was measured using the entire flask contents of duplicate cultures by a modification of the Lowry method (Daniels et al., 1994).

3.13.3.3 Extraction and quantification of residual pyrene by HPLC

The residual pyrene in the flasks was extracted with a mixture of chloroform and methanol (v/v=20:10 ml) as described by Zhang et al. (2004). One milliliter extracted samples were then filtered through a μm pore size filter and 20 μl of the filtrate was then analyzed for pyrene content by high-performance liquid chromatography (HPLC) on a Waters reverse-phase C_{18} Nova pak column (3.9 mm x 150 mm) by using isocratic elution with acetonitrile-

water (Pickard et al., 1999). Flow rate was adjusted to 1.0 ml/ min and elution of pyrene was monitored at 273 nm (Waters 2487 Dual λ Absorbance Detector, Milford, USA). The decrease in the amount of pyrene in experimental flasks was estimated by measuring the peak area of UV absorbance at 273 nm and by comparing with the peak area control flasks. Pyrene concentration was expressed as mean and standard deviations based on the results obtained with triplicate flasks.

3.13.3.4 Measurement of pyrene uptake by bacterial cells

Pyrene uptake by resting bacterial cells was measured by spectrophotometric rate assay as described by Stringfellows and Aitken (1995). Briefly, $1x10^7$ bacterial cells (final volume of 3.0 ml in 20 mM phosphate buffer containing 150 mM NaCl, pH 7.0) were placed in a 3.5 ml quartz cuvette in a U-2001 UV/Vis Spectrophotometer (Hitachi, Japan) and 60 μg of pyrene (in 10μl of acetone) was injected into the cuvette. A decrease in A_{273} was measured from 0 sec to 30 min post addition of pyrene. A control experiment was run in parallel where 10 μl of acetone (without pyrene) was added. Pyrene uptake was also measured in presence of a suspension of killed cells. From a standard curve of pyrene, decrease in pyrene content was calculated and results are expressed as μg of pyrene uptake by $1x10^7$ bacterial cells.

To study the bacterial uptake of biosurfactant solubilized pyrene, stock solution of pyrene (in acetone) was incubated with biosurfactants (0.5 mg/ml) from the respective bacterial strains for overnight and then biosurfactant solubilized pyrene was injected into the cuvette containing $1x10^7$ bacterial cells (final volume of 3ml). Pyrene uptake measurement was done in the same manner as described above.

3.13.3.5 Pyrene solubilization assay

Pyrene solubilization assay was done as described in section 3.13.2.1.

3.14.4 Antimicrobial activity of biosurfactants

E. coli and *Kluyvera cryocrescens* MTCC 3971 bacterial strains were grown in nutrient broth medium and yeast strains of *Saccharomyces cerevisiae* MTCC 3976, *Debaryomyces hansenii* MTCC 3977, *Pichia anamala* MTCC 3979 and *Candida glabrata* MTCC3981 were grown in YEPD (yeast extract peptone dextrose) broth medium till stationary phase at 37

°C. *Ralstonia solanacea* was grown on peptone sucrose medium. All the strains have been identified in Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Serial dilutions of the microbial cultures were made in sterile PBS, pH 7.0 and plated in nutrient agar and YEPD agar plates for bacterial and yeast strains respectively. Various concentrations of biosurfactant, dissolved in sterile 0.05M $NaHCO_3$ pH 8.6 were added to the respective medium and incubated for 24 h at 37 °C along with control (sterile 0.05M $NaHCO_3$ pH 8.6). The diameter of the clear zone around the point of application of biosurfactant, indicating the inhibition of bacterial growth, was measured.

3.13.5 Assessment of mosquito larvicidal potency of biosurfactants

3.13.5.1 Determination of larval potency (LC_{50} and LC_{100}) of biosurfactants

Assessment of the larvicidal potency of the crude CLPs against the early 3rd instar *Culex quinquefasciatus* larvae was based on standard methods for testing of larval susceptibility (World Health Organization, 1981) with the following modification. Briefly, 10 larvae were transferred on individual well of 6-welled Nunclon cell culture plate (Denmark) containing 10.0 ml of rearing tap water (pH was maintained to 7.0) and incubated in presence of graded amounts of CLPs dissolved in Milli Q water. The plates were incubated at room temperature (~23 °C) for 24-72 h, followed by counting the number of dead larvae in each well. A control experiment was run in parallel without adding CLPs. Control as well as treated larvae were observed from 24 h to 72h post application of CLPs and the lethal concentration 100% (LC_{100}) and 50% (LC_{50}) of the CLPs were calculated as described by Wang et al., (1999). For each concentration of biosurfactants, experiment was repeated thrice.

3.13.5.2 Influence of various physico-chemical factors on larvicidal efficacy

For assessing the heat or sunlight (UV) resistance of CLPs, the aqueous solutions (2 mg / ml) of CLPs were heated at 100 ° C for 10 min, 30 min and 60 min or exposed to sunlight for a duration of 6h and 8h respectively, and larvicidal efficacy (post 24 h LC_{50}) of heated/sunlight exposed CLPs was tested at each time interval. Effect of pH on the larvicidal efficacy of CLPs was assessed by maintaining the pH of the rearing water at 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 and then determining the post 24 h LC_{50} value of CLPs at each pH. There were three replicates for each experiment and a control was run in parallel.

3.13.5.3 Assessment of biosafety of CLPs to non-target organism

Indian major carp, *Labio rohita,* with a mean weight of 35.0 ± 2.0 g were used as a model aquatic organism for assessing the biosafety of *B. subtilis* lipopeptides to non-target species. The fish were obtained from a local fish farm, kept in glass aquarium containing 30 liter water (temperature 25 ± 2.0°C, pH 7.2-7.5) with constant aeration and were acclimatized for 4 weeks before starting the experiment. Fishes were divided into three groups (four fish / group)-A, B, and C, having three replicates for each group. All groups were fed the normal diet (Rao et al., 2004) at 3% of body weight. Group A fish served as control. Crude CLPs from *B. subtilis* DM-03 and DM-04 strains were mixed in the groups B and C water at a concentration of 120 mg/l and 300 mg/l respectively. The experiment was conducted for 4 weeks following which fish were sampled for weight gain and then sacrificed, blood was collected immediately, serum was separated and used for the assay of enzyme activity (SGPT, SGOT, ALP) and other biochemical parameters viz. total cholesterol, total protein, albumin, globulin, total billirubin and triglycerides by using commercial kits and following the instruction from manufacturers.

3.14 Statistical analysis

Statistical analysis was done by Student's "t" test (Daniel, 2000).

CHAPTER IV

ISOLATION AND TAXONOMIC IDENTIFICATION OF THE BIOSURFACTANTS PRODUCING POTENTIAL MICROBES

4.1 Results

4.1.1 Screening of microbes capable of producing biosurfactants at 45°C

From different environmental samples, 70 number culturable bacterial isolates were isolated and pure cultures of such strains were maintained. The environmental samples include crude oil contaminated soil and water samples from ONGC oil fields, refinery sludge from Guwahati Refinery, Assam, India, Hot water springs of Garampani, Assam and Manikaran, Himachal Pradesh; starter culture used for alcohol production by a local tribal group from North-eastern India.

The procedure of isolation, screening and subsequent selection of most potential strains, capable of producing significant amount of biosurfactants at thermophilic (>45°C) growth condition was based on the following two steps. In the first step isolation of microbes from different environmental samples was done either by enrichment culture or by direct plating and incubating the cultures at different isolation temperature (45°C and above) for 48 h. In the next step, the isolated microbes were grown in mineral-salts medium followed by assessing the surface tension of the cell free growth medium as shown in Table 4.1. The surface tension of the control (un-inoculated) medium was recorded as 65.0 ±1.0 (mean ± S.D). These steps were proved to be helpful in identifying the biosurfactants producing potential microbes.

Table 4.1. Isolation and screening of microorganisms for biosurfactants production. The bacteria were isolated from different environmental samples and tested for their ability to produce biosurfactants post 48 h or 96 h of growth at their isolation temperature. Surface tension of the growth (control) medium was 65.0 ±1.0 (mean ± S.D). Isolates shown in bold letter were considered as *biosurfactants producing potential strains based on the surface tension* reduction (< 32 mN/m). Value are mean ± S.D of triplicate determination.

S. No.	Isolates	Environmental sample	Isolation temperature (°C)	Surface tension of growth medium (mN/m)
1	PSg- 1	Petroleum Sludge	45	51.5 ± 0.5
2	PSg- 2			55.7 ± 0.9
3	PSg- 3			49.6 ± 1.0
4	PSg- 4			45.0 ± 0.6
5	**PSg- 5**			**30.2 ± 0.8**
6	PSg- 6			33.7 ± 1.0
7	PSg- 7			33.6 ± 1.0
8	PSg- 8			36.3 ± 1.0
9	**PSg- 9**			**30.8** ± 1.2
10	PSg- 10			32.9 ± 0.5
11	**PSg- 11**			**30.5** ± 0.8
12	**PS-1**	Petroleum contaminated soil samples	45	**28.0** ± 0.5
13	**PS-2**			**29.0** ± 0.6
14	**PS-3**			**27.5** ± 0.4
15	PS-4			37.5 ± 0.8
16	PS-5			40.6 ± 0.9
17	PS-6			46.8 ± 1.0
18	**PS-7**			**30.2** ± 0.7
19	PS-8			44.1 ± 0.8
20	**PS-9**			**29.6** ± 0.7
21	PS-10			50.0 ± 0.7
22	PS-11			52.0 ± 0.9
23	PS-12			45.0 ± 1.0
24	PS-13			55.0 ± 1.5
25	PS-14			45.1 ± 1.5
26	**PS-15**			**30.5** ± 0.7
27	PS-16			45.9 ± 1.0
28	PS-17			52.6 ± 1.0
29	PS-18			47.3 ± 1.0
30	PS-19			48.6 ± 0.8
31	PS-20			58.6 ± 0.7
32	PS-21			52.9 ± 0.6
33	PS-22			44.1 ± 0.6
34	PS-23			40.0 ± 0.7
35	PS-24			42.0 ± 0.6

Table 4.1 continued

S. No.	Isolates	Environmental sample	Isolation temperature (°C)	Surface tension (mN/m)
36	PS-25	Petroleum contaminated soil samples	60	46.6 ± 0.7
37	PS-26			54.1 ± 0.8
38	PS-27			50.1 ± 1.2
39	PS-28			42.8 ± 1.5
40	**PW-1**	Petroleum contaminated water samples	45	**30.5** ± 1.0
41	PW-2			55.0 ± 0.8
42	PW-3			40.5 ± 0.9
43	PW-4			40.2 ± 1.0
44	PW-5			47.0 ± 0.8
45	**PW-6**			**30.8** ± 1.2
46	**PW-7**			**28.8** ± 0.6
47	PW-8			37.3 ± 0.7
48	**PW-9**			**29.2** ± 0.8
49	**PW-10**			**29.1** ± 0.9
50	PW-11			42.8 ± 0.8
51	PW-12			45.0 ± 1.0
52	PW-13	Petroleum contaminated water samples	60	55.0 ± 1.5
53	PW-14			53.8 ± 0.5
54	PW-15			56.1 ± 0.9
55	PW-16			44.1 ± 1.1
56	HWS-1	Hot Water Springs (Assam)	45	49.1 ± 0.9
57	HWS-2			41.8 ± 0.8
58	HWS-3			40.1 ± 0.9
59	HWS-4			33.4 ± 0.6
60	HWS-5			34.2 ± 0.8
61	HWS-6			47.0 ± 1.0
62	HWS-7			43.5 ± 1.2
63	HWS-8			48.6 ± 1.5
64	HWS-9	Hot Water Springs (Himachal Pradesh)	80	62.6 ± 0.8
65	HWS-10			53.4 ± 0.3
66	HWS-11			59.0 ± 0.5
67	HWS-12			57.8 ± 0.4
68	HWS-13			61.2 ± 0.7
69	HWS-14			56.6 ± 1.0
70	**SC-1**	Starter culture cake	45	**32.0** ± 0.2

As shown in table 4.1 out of the seventy pure bacterial cultures, only fifteen strains viz., PSg-5, PSg-9, PSg-11, PS-1, PS-2, PS-3, PS-7, PS-9, PS-15, PW-1, PW-6, PW-7, PW-9, PW-10 and SC-1 reduced the surface tension of the growth medium from 65.5 mN/m to less than 30.0 ± 2.0 mN/m. These fifteen strains were considered as biosurfactants producing promising strains and characterized further.

In the next step, investigation was carried out to identify the biosurfactants producing most promising microbes among these 15 promising isolates. As shown in Table 4.2, out of these fifteen biosurfactants producing strain(s), only four strains were identified as hyperproducing strains, based on (i) surface tension reduction value of the growth medium and (ii) yield of biosurfactants. These four strains viz., **PS-1, PS-2, PS-3 and SC-1** could reduce the surface tension of the growth medium from 65 mN/m to 30 ± 2 mN/m and produced comparatively higher amount of biosurfactants (Table 4.2). It is worth mentioning that three of the potential isolates viz. PS-1, PS-2 and PS-3 were isolated from the same environmental sample. Therefore, these four microbes were selected for taxonomic identification and for further studies.

Table 4.2. Surface tension reduction and yield of biosurfactants from the potential microorganisms. Results represents mean ± S.D of three individual experiments.

Isolates	Surface tension reduction* (mN/m)	Dry biomass (g/l)	Yield of biosurfactants (g/l)
PSg-5	30.2 ± 0.8	1.2 ± 0.1	1.0 ± 0.05
PSg-9	30.8 ± 1.2	1.0 ± 0.1	1.2 ± 0.2
PSg-11	30.5 ± 0.8	0.8 ± 0.2	1.0 ± 0.3
PS-1	28.0 ± 0.5	2.0 ± 0.3	6.0 ± 0.4
PS-2	29.0 ± 0.6	1.9 ± 0.2	5.0 ± 0.3
PS-3	27.5 ± 0.4	1.9 ± 0.1	2.0 ± 0.5
PS-7	30.2 ± 0.7	0.6 ± 0.3	0.9 ± 0.07
PS-9	29.6 ± 0.7	0.7 ± 0.1	0.5 ± 0.08
PS-15	30.5 ± 0.7	0.8 ± 0.1	0.6 ± 0.1
PW-1	30.5 ± 1.0	0.5 ± 0.05	0.4 ± 0.1
PW-6	30.8 ± 1.2	0.3 ± 0.03	0.1 ± 0.05
PW-7	28.8 ± 0.6	0.8 ± 0.05	1.2 ± 0.05
PW-9	29.2 ± 0.8	1.0 ± 0.8	1.5 ± 0.05
PW-10	29.1 ± 0.9	0.8 ± 0.09	1.0 ± 0. 0.8
SC-1	32.0 ± 0.2	1.75 ± 0.1	3.5 ± 0.1

*The surface tension of the control medium was 65.0 mN/m.

4.1.2 Taxonomic identification of PS-1, PS-2, PS-3 and SC-1 strains

The taxonomic identification of the strains was done following the standards morphological, physiological and biochemical tests to reveal the genus of the microbes. This was followed by GC analysis of cellular FAME (fatty acid methyl ester) of the microbes for species identification and strain typing. The results of the biochemical and morphological tests of the potential bacterial isolates are shown below (Tables 4.3 to 4.6).

Table 4.3. Biochemical and morphological tests of PS-1. Experiments were repeated thrice to assure the reproducibility.

Morphology	
	Rod shaped with 1-2 μm in diameter, motile, Gram negative, border regular, surface raised, pigmentation in the colony and in mineral salt medium broth.
Growth	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at 8.5-9.0
Temperature	Range 30-55 °C, optimum at 45 °C
Catalase	Positive
Voges-Proskauer Test	Negative
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
L- Arabinose	Positive
D- Xylose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Negative
Lipid	Positive
Utilization of Citrate	Negative
Formation of Indole	Negative
Nitrate reduction	Positive
Litmus milk reaction	Peptonization

Table 4.4. Biochemical and morphological tests of PS-2. Experiments were repeated thrice to assure the reproducibility.

Morphology	Rod shaped with 1-2 μm in diameter, motile, Gram negative, border irregular, surface raised, dark green pigmentation in the colony and in mineral salt medium broth
Growth	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at 8.5-9.0
Temperature	Range 30-55 °C, optimum at 45 °C
Catalase	Positive
Voges-Proskauer Test	Negative
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
L- Arabinose	Positive
D- Xylose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Negative
Lipid	Positive
Utilization of Citrate	Negative
Formation of Indole	Negative
Nitrate reduction	Positive
Litmus milk reaction	Peptonization

Table 4.5. Biochemical and morphological tests of PS-3. Experiments were repeated thrice to assure the reproducibility.

Morphology	Rod shaped with 1-2 μm in diameter, motile, Gram positive, border irregular, surface raised
Growth	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at 8.5-9.0
Temperature	Range 30-60 °C, optimum at 55 °C
Catalase	Positive
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
L- Arabinose	Positive
D- Xylose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Positive
Utilization of Citrate	Positive
Formation of Indole	Negative
Nitrate reduction	Negative
Litmus milk reaction	Peptonization

Table 4.6. Biochemical and morphological tests of SC-1. Experiments were repeated thrice to assure the reproducibility.

Morphology	Rod shaped with 1-2 μm in diameter, motile, Gram positive, border irregular, surface raised
Growth	
Agar	Abundant, pale yellow
Broth	Good growth, with sediment
pH	4.0 –10.0, optimum at 8.5-9.0
Temperature	Range 30-60 °C, optimum at 50-55 °C
Catalase	Positive
Voges-Proskauer Test	Positive
Methyl Red Test	Negative
Acid from	
D- Glucose	Positive
L- Arabinose	Positive
D- Xylose	Positive
D- Mannitol	Positive
Gas from Glucose	Negative
Hydrolysis of	
Casein	Positive
Gelatin	Positive
Starch	Positive
Utilization of Citrate	Positive
Formation of Indole	Negative
Nitrate reduction	Negative
Litmus milk reaction	Peptonization

Bacterial morphology and the results of biochemical tests suggested that PS-1 and PS-2 belonged to the genus *Pseudomonas* whereas characteristic features of PS-3 and SC-1 suggested that they belonged to the genus *Bacillus* (Bergey's manual of Systematic Bacteriology, 1984, 1986). Moreover, ability of the *Pseudomonas* strains viz., PS-1 and PS-2 to grow on Pseudomonas Isolation Agar medium (Difco Laboratories, Detroit, USA) re-confirmed their genus as "*Pseudomonas*".

4.1.3 FAME analysis

The similarity index (SI) and species identification of these four bacterial strains are shown in Table 4.7. A major difference in the composition of cellular fatty acid was observed between both the *Pseudomonas* PS-1 and PS-2 species and on this basis, the former was identified as *Pseudomonas aeruginosa* mucoid (M) strain, whereas the latter strain was identified as *Pseudomonas aeruginosa* non-mucoid (NM) strain (Table 4.8). The *Bacillus subtilis* strains isolated from starter culture cake used for alcohol production and petroleum contaminated soil sample from ONGC oil field were designated as *B. subtilis* DM-03 and *B. subtilis* DM-04 respectively (Table 4.7). However, both the *P. aeruginosa* strains were isolated from a petroleum contaminated soil sample.

Table 4.7. Similarity index (S.I) of FAME analysis. S.I of cellular fatty acid methyl ester of the potential bacterial strains

Biosurfactants producing potential bacterial isolates	SI index	Matching strains	Microbe Designation
SC-1	0.55	*B. subtilis*	*B. subtilis* DM-03
PS-3	0.77	*B. subtilis*	*B. subtilis* DM-04
PS-1	0.91	*P. aeruginosa*	*P. aeruginosa* M
PS-2	0.88	*P. aeruginosa*	*P. aeruginosa* NM

Table 4.8. Composition of cellular fatty acid ester in *P. aeruginosa* mucoid (M) and non-mucoid (NM) strains. Determination was carried out by GC analysis the cellular fatty acid methyl esters of strains.

Name of the Fatty acid	% composition in *P. aeruginosa* M strain	% composition in *P. aeruginosa* NM strain
10:0 3OH (3-Hydroxy Decanoic acid)	6.11	4.18
12:0 (Dodecanoic acid)	6.86	9.36
12:0 2OH (2-Hydroxy Dodecanoic acid)	12.17	8.50
12:0 3OH (3-Hydroxy Dodecanoic acid)	10.56	7.46
16:0 (Hexadecanoic acid)	25.89	24.54
18:1 w7c (Delta 6–16-Methyl-heptadecanoic acid)	27.18	29.57
Summed feature 3 15:0 ISO (13-Methyl-tetradecanoic acid) 16:1 w7c (Delta6-14 Methylpentadecanoic acid)	11.22	13.84

4.1.4 PCR-Restriction fragment length polymorphism

4.14.1 PCR-RFLP of *B. subtilis* strains

For PCR-RFLP analysis of two *B. subtilis* strains, PCR amplification with primers 16S/ p1 and 23S/p2 by using the flanking terminal sequences of the 16S and 23S rRNA genes was performed with the isolated chromosomal DNA (Ausubel et al., 1995). PCR amplification with p1 and p2 primers of a segment of the ISR of 16S-23S rRNA of *B. subtilis* DM-03 and *B. subtilis* DM-04 yielded 0.8 kb and 0.77 kb products respectively (Fig. 4.1). Nested PCR amplification of these fragments with primers 16S/ p3 and 23S/ p4 resulted in a amplification of 0.5 and 0.48 kb for *B. subtilis* DM-03 and DM-04 strains respectively. (Fig.4.1).

Restriction digestions by *Mbo* 1 of the amplified 16S ISR (amplified with primer p1 and p2) of the two *Bacillus* strains resolved into different band patterns in 3% agarose gel (Fig.4.2).

4.4.2 PCR-RFLP of *P. aeruginosa* strains

PCR amplification of ISR of two *P. aeruginosa* strain with primers 16S/ p5 and 23S/p6 by using the flanking terminal sequences of the 16S and 23S rRNA genes was performed with chromosomal DNA (Barsotti et al., 2002).

PCR amplification with primers 16S/ p5 and 23S/p6 by using the flanking terminal sequences of the 16S and 23S genes yielded nearly identical band patterns of 0.7 kb, containing ISR and the flanking region. Nested PCR amplification of these fragments with primers 16S/ p7 and 23S/ p8 resulted in a similar profile of 0.5 kb amplification, containing the ISR and the flanking region (Fig. 4.3). *Mbo* 1 restriction digested PCR amplified products from both the *P. aeruginosa* M and NM strains were resolved into similar banding patterns in 3% agarose gel (Fig.4.4).

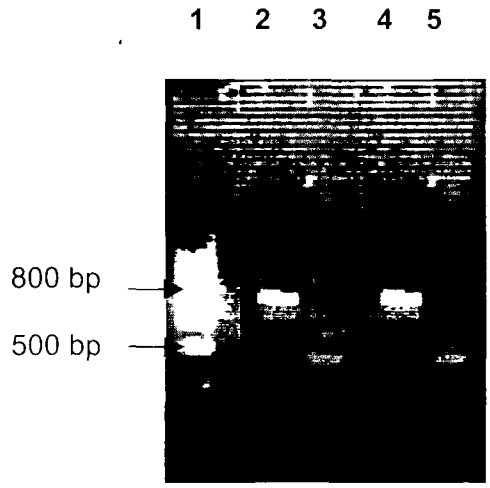

Fig. 4.1. PCR amplification of ISR of 16S-23S rRNA gene of *B. subtilis* strains

Lane1: Molecular weight marker in bp (100 bp ladder); Lane 2: amplified ISR of *Bacillus subtilis* DM-03 with primer (p1/p2); Lane3. amplified ISR of *B subtilis* DM-03 with nestled primer (p3/p4); Lane 4: amplified ISR of *B. subtilis* DM-04 with primer (p1/p2); Lane 5: amplified ISR of *B. subtilis* DM-04 with nestled primer (p3/p4).

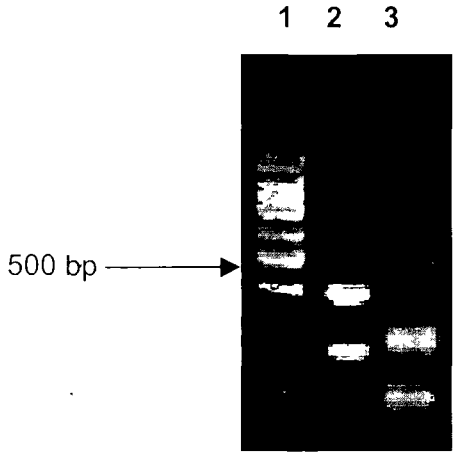

Fig. 4.2. Analysis of restriction digested PCR amplified ISR products of *B. subtilis* DM-03 and DM-04 strain.

Lane 1: 100 bp molecular weight marker; Lane 2: *Mbo* I digested PCR amplified ISR of 16S-23S rRNA of *B. subtilis* DM-03 strain; Lane 3: *Mbo* I digested PCR amplified ISR of 16S-23S rRNA of *B. subtilis* DM-04 strain.

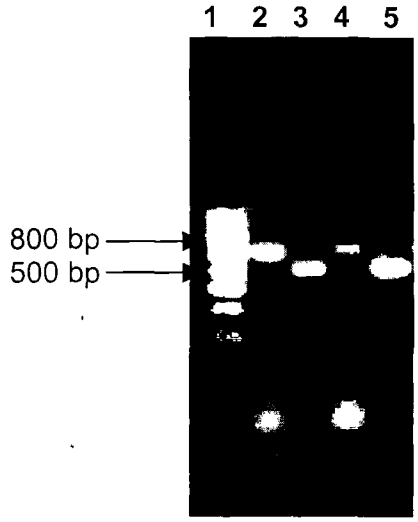

Fig. 4.3. PCR amplification of ISR of 16S-23S rRNA gene of *P. aeruginosa* strains

Lane1: Molecular weight marker in bp (100 bp ladder); Lane 2: amplified ISR of *P. aeruginosa* M strain with primer (p5/p6); Lane3: amplified ISR of *P. aeruginosa* strain with nestled primer (p7/p8) ; Lane 4: amplified ISR of *P. aeruginosa* NM strain with primer (p5/p6); Lane 5: amplified ISR of *P. aeruginosa* NM strain with nestled primer (p7/p8).

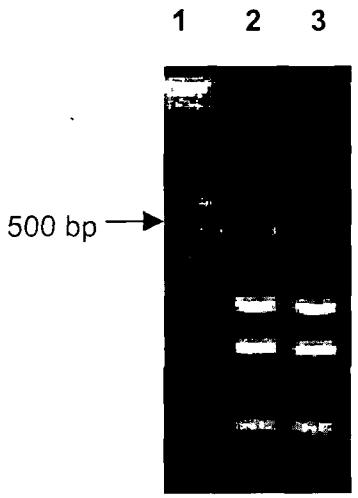

Fig. 4.4. Analysis of restriction digested PCR amplified ISR products of *P. aeruginosa* M and NM strain. Lane 1: 100 bp molecular weight marker; Lane 2: *Mbo* I digested PCR amplified ISR of 16S-23S rRNA of *P. aeruginosa* M strain; Lane 3: *Mbo* I digested PCR amplified ISR of 16S-23S rRNA of *P. aeruginosa* NM strain.

CHAPTER V

PRODUCTION, ISOLATION AND CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY *B. subtilis* STRAINS

5.1 Results

5.1.1 Optimization of culture conditions for maximum biosurfactants production

5.1.1.1 Growth characteristics and biosurfactants production

Maximum reduction in surface tension of growth medium with glucose as the carbon source was achieved in the late log phase (post 48h of microbial growth) when the surface tension was reduced from 60.5 mN/m to 29.1± 0.2 and 27.1± 0.3 mN/m by *B. subtilis* DM-03 and DM-04 strains respectively (Table 5.1). M9 media was found optimum for growth and biosurfactants production by both the *B. subtilis* strains. Optimization of M9 salts is described below. Two other media described by Goswami and Singh, (1991) and Banat, (1993) (composition given in section 3.3.6) were tried, however these were discarded due to poor growth of the microorganisms in these media. Bacterial growth rate pattern followed the similar trend. *B. subtilis* DM-03 strain achieved maximum growth at post 48h of inoculation, whereas highest biomass for *B. subtilis* DM-04 strain was recorded from 48 h to 72 h of growth in the presence of glucose as sole carbon source.

Table 5.1. Growth and surface tension reduction by *B. subtilis* strains at different time intervals. Data represents the mean ± S.D of four individual experiments.

Incubation time (h)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0	Surface tension (mN/m)	60.5± 0.2	60.5± 0.2
	Dry biomass (g/l)	0.0	0.0
24	Surface tension (mN/m)	35.6 ± 0.5	35.0 ± 0.3
	Dry biomass (g/l)	1.05 ± 0.05	1.0 ± 0.02
48	Surface tension (mN/m)	29.1± 0.3	27.1 ± 0.2
	Dry biomass (g/l)	2.0 ± 0.05	1.8 ± 0.05
72	Surface tension (mN/m)	30.0 ± 0.6	29.8 ± 0.6
	Dry biomass (g/l)	1.85 ± 0.1	1.85 ± 0.05
96	Surface tension (mN/m)	33.0 ± 0.6	32.0 ± 0. 4
	Dry biomass (g/l)	1.9 ± 0.05	1.8 ± 0.1
120	Surface tension (mN/m)	33.8 ± 0.8	33.0 ± 0.6
	Dry biomass (g/l)	1.8 ± 0.08	1.6 ± 0.05

5.1.1.2 Role of different carbon sources on growth and biosurfactants production

As shown in Table 5.2 and Table 5.3, the yield of biosurfactants from both the *Bacillus* strains was dependent on the choice of carbon source. The two *Bacillus* strains exhibited marked differences in their growth and surface activities (biosurfactants production) when grown on hydrocarbons. For example, in contrast to *B. subtilis* DM-04, *B. subtilis* DM-03 showed poor growth in all the tested hydrocarbons (Table 5.2 and Table 5.3). However, both the *B. subtilis* strains showed excellent growth and maximum biosurfactants production when glucose was used as the sole carbon source. Both the strains could utilize unconventional carbon sources viz., molasses and potato peels for growth and biosurfactants production. Glycerol proved to be another efficient carbon source in terms of surface tension reduction, but the yield of biosurfactants in glycerol was less as compared to glucose carbon source. *B. subtilis* DM-03 exhibited efficient surface tension reduction ability with appreciable yield of biosurfactants.

Table 5.2. Effect of different carbon sources on growth and biosurfactants production by *Bacillus subtilis* DM-03 and DM-04 strains post 48 hrs of inoculation. Results represents mean ± S.D of three individual experiments.

Carbon source	Reduction in surface tension of medium (Δ mN/m)*		Yield of crude biosurfactant (g/l)		Dry biomass (g/l)	
	DM-03	DM-04	DM-03	DM-04	DM-03	DM-04
Glycerol	37.5 ±0.2	35.6 ± 0.3^{c}	3.0 ± 0.3	1.5 ± 0.1^{c}	3.5 ± 0.4	2.5 ± 0.3^{a}
Glucose	30.7 ± 0.3	31.2 ± 0.4	3.5 ± 0.5	1.8 ± 0.3^{b}	2.0 ± 0.2	1.9 ± 0.2
Starch	37.0 ± 0.2	24.3 ± 0.4^{c}	2.8 ± 0.4	0.6 ± 0.1^{b}	2.1 ± 0.2	0.7 ± 0.1^{c}
Octane	11.2 ± 0.5	30.6 ± 0.3^{c}	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	0.7 ± 0.1
Dodecane	27.3 ± 0.5	35.7 ± 0.2^{c}	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
Hexadecane	20.0 ± 0.6	35.6 ±0.4^{c}	0.3 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	1.9 ± 0.2^{c}
Octadecane	19.0 ± 0.8	33.0 ± 0.3^{c}	0.3 ± 0.1	0.2 ± 0.1	0.4 ± 0.1	0.7 ± 0.1^{a}
Liquid light paraffin	20.3 ± 0.5	35.7 ± 0.3^{c}	0.3 ± 0.1	0.8 ± 0.2^{a}	0.4 ± 0.1	0.7 ± 0.1^{a}
Petrol	0.8 ± 0.4	14.7 ± 0.2^{c}	0.1 ± 0.1	0.7 ± 0.1^{b}	0.6 ± 0.1	0.7 ± 0.1
Diesel	1.0 ± 0.2	10.9 ± 0.1^{c}	0.2 ± 0.1	0.1 ± 0.0	0.5 ± 0.1	0.8 ± 0.1^{a}
Kerosene	2.6 ± 0.7	18.4 ± 0.3^{c}	0.1 ± 0.1	0.8 ± 0.1^{b}	0.5 ± 0.1	0.8 ± 0.1^{a}
Anthracene	9.3 ± 0.4	35.3 ± 0.5^{c}	0.2 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.6 ± 0.1$^{a, x}$
Pyrene	9.9 ± 0.6	37.4 ± 0.2^{c}	0.1 ± 0.1	0.2 ± 0.1^{a}	0.3 ± 0.1	1.0 ± 0.1$^{b, x}$
Phenanthrene	11.2 ± 0.3	36.7 ± 0.3^{c}	0.01 ± 0.0	0.1 ± 0.0^{c}	0.4 ± 0.1	0.6 ± 0.1$^{a, x}$

* Reduction in surface tension of medium by surface active compounds was measured with respect to respective uninoculated growth medium (control).

Significance of difference with respect to *B. subtilis* DM-03 strains ^{a} $p< 0.05$, ^{b} $p<0.01$, ^{c} $p< 0.001$.

Significance of difference with respect to utilization of hexadecane ^{x} $p< 0.001$

Table 5.3. Effect of different concentrations of glucose on biosurfactants production and growth of *B. subtilis* strains. *B. subtilis* strains were grown on different concentrations of glucose at 45°C temperature for 48 h, followed by assaying the surface tension reduction and measuring the bacterial growth. Values represents mean ± S.D of three individual experiments.

Percent of glucose (w/v)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0.5	Surface tension (mN/m)	34.2 ± 0.6	33.0 ± 0.5
	CMD^{-1} (mN/m)	48.2 ± 0.8	45.9 ± 0.6
	Dry biomass (g/l)	1.5 ± 0.5	1.4 ± 0.6
1.0	Surface tension (mN/m)	30.0 ± 0.6	29.8 ± 0.5
	CMD^{-1} (mN/m)	41.0 ± 0.4	40.5 ± 0.5
	Dry biomass (g/l)	1.88 ± 0.2	1.65 ± 0.3
2.0	Surface tension (mN/m)	29.3 ± 0.4	27.3 ± 0.5
	CMD^{-1}(mN/m)	38.0 ± 0.6	33.9 ± 0.8
	Dry biomass (g/l)	1.8 ± 0.2	1.7 ± 0.3
3.0	Surface tension (mN/m)	39.5 ± 0.5	34.8 ± 0.6
	CMD^{-1}(mN/m)	40.6 ± 0.4	36.5 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.5	1.8 ± 0.6

As shown in Table 5.3, glucose has a dose- dependent effect on the growth of and biosurfactants production by both the *B. subtilis* strains, but optimum growth or biosurfactants production was observed at 2% (w/v) glucose concentration.

5.1.1.3 Effect of different nitrogen sources on growth and biosurfactants production

Type of nitrogen source plays an important role in the growth of and biosurfactants production by both the *B. subtilis* strains. Both the strains exhibited poor growth and surface activity in nitrogen-depleted medium. Inorganic nitrogen sources were observed to be the best for biosurfactants production by *B. subtilis* DM-03 strains, whereas organic nitrogen sources proved to be optimum for *B. subtilis* DM-04 strain. For example, ammonium nitrate and tryptone proved to be the best sources of nitrogen for *Bacillus subtilis* DM-03 and DM-04 strains respectively (Fig. 5.1 & 5.2) for growth, good surface activities and high yield of biosurfactants.

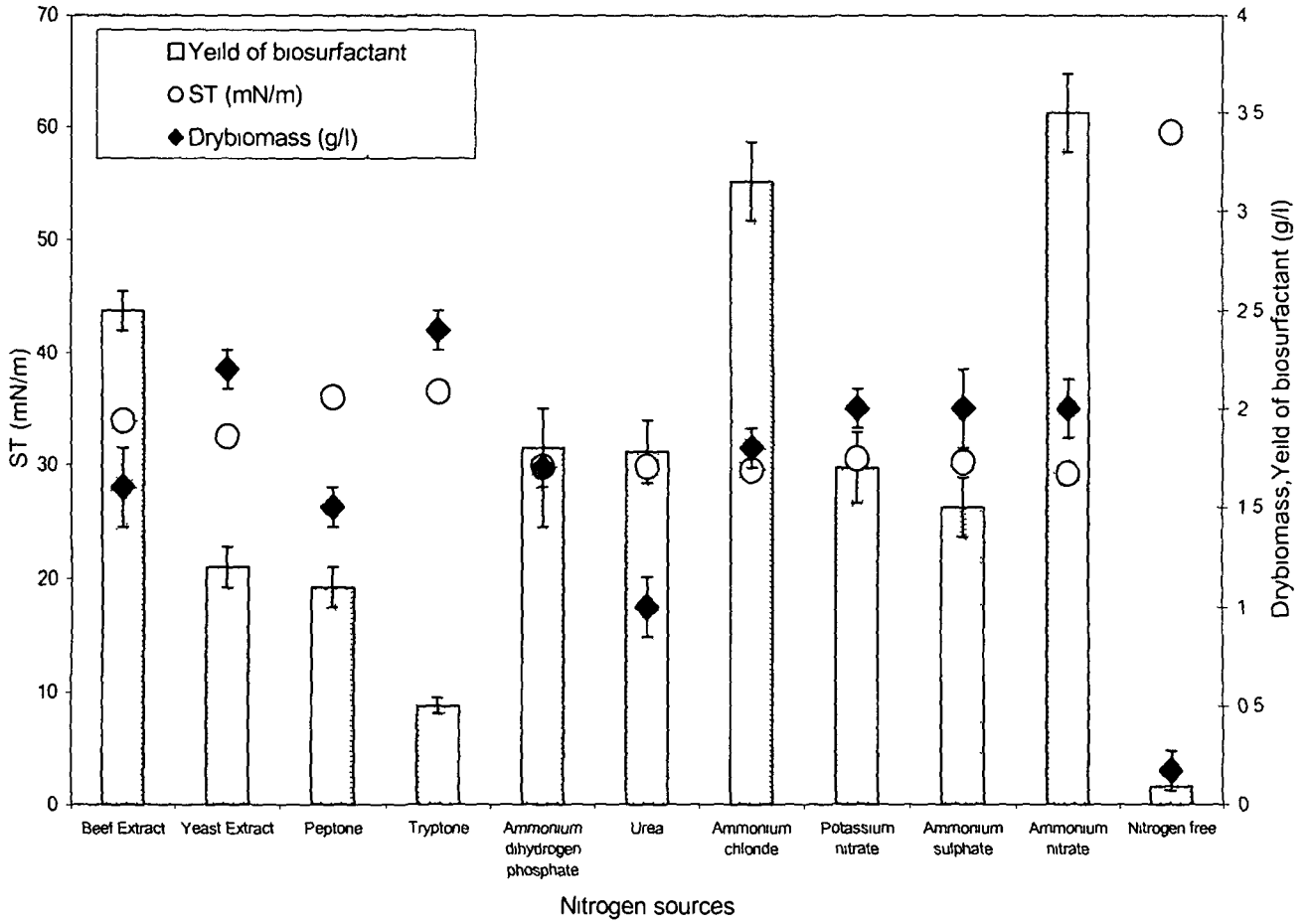

Fig. 5.1. Influence of various nitrogen sources on growth of and biosurfactants production by *B. subtilis* DM-03. Values are mean ± S.D of three determinations.

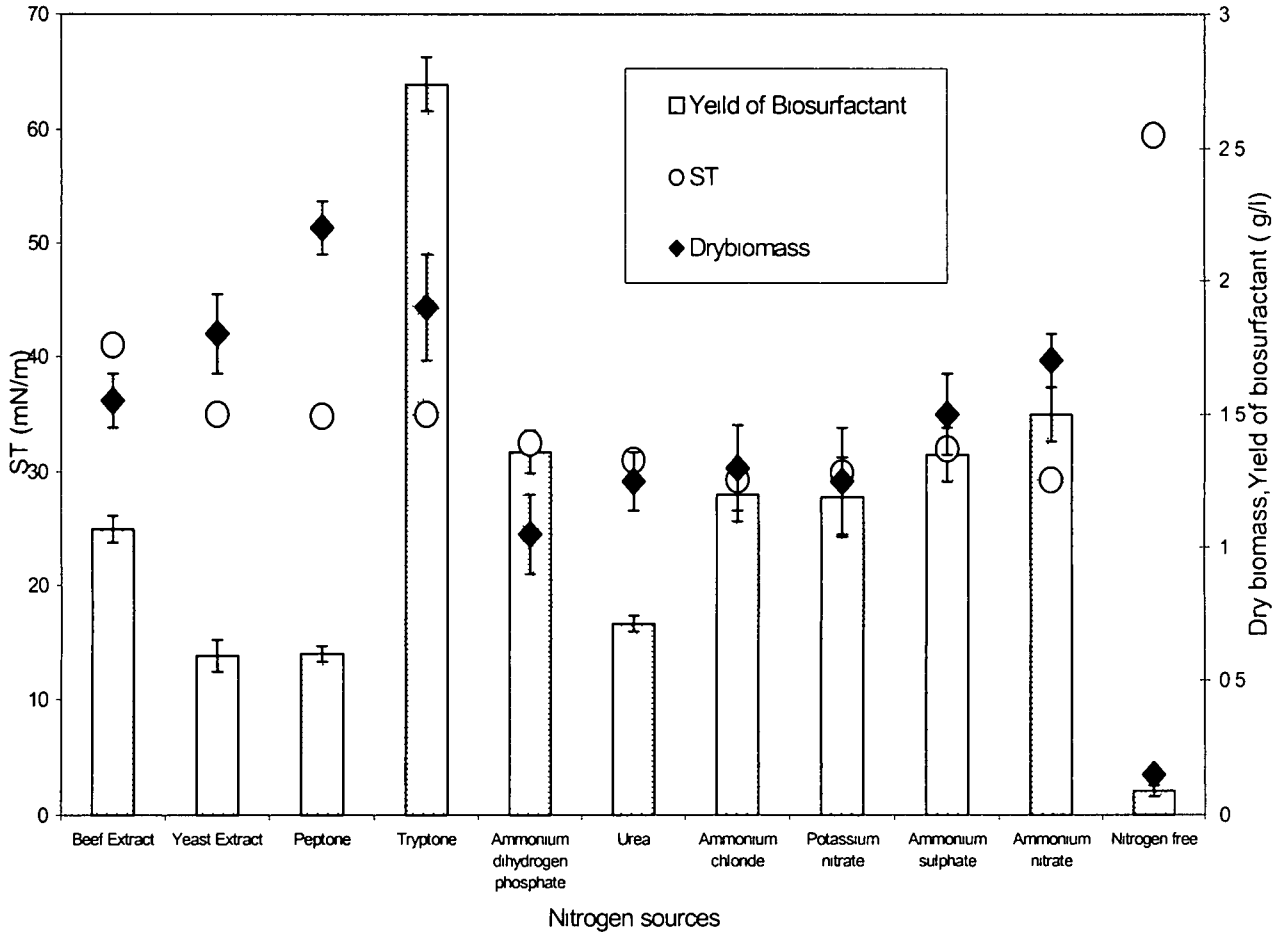

Fig. 5.2. Influence of various nitrogen sources on growth of and biosurfactants production by *B. subtilis* DM-04. Values are mean ± S D of three determinations

Table 5.4. Influence of different concentrations of nitrogenous source on growth and biosurfactants production. Results recorded post 48h of inoculation of *Bacillus* strains grown on glucose and either ammonium nitrate (for *B. subtilis* DM-03) or tryptone (for *B. subtilis* DM-04). Values represents mean ± S.D of three individual experiments.

Conc. of nitrogen source (g %)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0.05	Surface tension (mN/m)	35.5 ± 0.5	33.0 ± 0.4
	CMD^{-1} (mN/m)	47.0 ± 0.8	44.8 ± 0.6
	Dry biomass (g/l)	1.5 ± 0.2	1.4 ± 0.3
0.1	Surface tension (mN/m)	29.3 ± 0.4	27.5 ± 0.5
	CMD^{-1} (mN/m)	38.5 ± 0.5	34.0 ± 0.5
	Dry biomass (g/l)	1.95 ± 0.2	1.8 ± 0.3
0.2	Surface tension (mN/m)	33.2 ± 0.5	32.5 ± 0.4
	CMD^{-1} (mN/m)	39.0 ± 0.6	41.0 ± 0.5
	Dry biomass(g/l)	2.0 ± 0.7	1.95 ± 0.5
0.4	Surface tension (mN/m)	35.3 ± 0.4	33.0 ± 0.5
	CMD^{-1} (mN/m)	44.0 ± 0.6	44.6 ± 0.5
	Dry biomass(g/l)	1.8 ± 0.7	1.9 ± 0.5

Dose- dependent study showed that 0.1% ammonium nitrate and tryptone were optimal for growth of and biosurfactants production by *B. subtilis* DM-03 and *B. subtilis* DM-04 strains respectively (Table 5.4).

5.1.1.4. Influence of metal ions on biosurfactants production.

Metal ions have an important role on influencing the surface-active properties of the microorganisms. 0.246 g/l of $MgSO_4.7H_2O$; 0.14 g/l of $CaCl_2$; 3.0g/l of KH_2PO_4; 6.0 g/l of Na_2HPO_4 and 1.0 g/l of NaCl were found to be optimum for biosurfactants production by the *Bacillus* strains (Table 5.5-5.9)

Table 5.5. Influence of magnesium chloride on growth and biosurfactants production. Results recorded post 48 h of inoculation for *Bacillus* strains grown on glucose. Values represents mean ± S.D of three individual experiments.

Conc. of $MgCl_2.7H_20$ (g/l)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	43.5 ± 0.6	42.5 ± 0.5
	CMD^{-1} (mN/m)	62.3 ± 0.8	61.8 ± 0.6
	Dry biomass (g/l)	1.4 ± 0.3	1.25 ± 0.4
0.1	Surface tension (mN/m)	29.5 ± 0.5	29.3 ± 0.4
	CMD^{-1} (mN/m)	40.0 ± 0.8	30.8 ± 0.6
	Dry biomass (g/l)	1.75 ± 0.2	1.55 ± 0.3
0.25	Surface tension (mN/m)	29.3 ± 0.4	27.8 ± 0.5
	CMD^{-1} (mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.2	1.8 ± 0.3
0.3	Surface tension (mN/m)	35.5 ± 0.6	32.4 ± 0.5
	CMD^{-1} (mN/m)	48.8 ± 0.6	39.3 ± 0.8
	.Dry biomass (g/l)	1.89 ± 0.4	1.8 ± 0.3

Table 5.6. Influence of calcium chloride on growth and biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Values represents mean ± S.D of three individual experiments.

Concentration of $CaCl_2$ (g/l)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	43.5 ± 0.6	42.5 ± 0.5
	CMD^{-1} (mN/m)	62.3 ± 0.8	61.8 ± 0.6
	Dry biomass (g/l)	1.4 ± 0.3	1.25 ± 0.4
0.007	Surface tension (mN/m)	30.6 ± 0.5	31.3 ± 0.4
	CMD^{-1} (mN/m)	40.0 ± 0.5	37.0 ± 0.4
	Dry biomass (g/l)	1.8 ± 0.2	1.9 ± 0.3
0.014g/l	Surface tension (mN/m)	29.3 ± 0.4	27.3 ± 0.5
	CMD^{-1} (mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.2	1.8 ± 0.3
0.021	Surface tension (mN/m)	34.6 ± 0.5	32.8 ± 0.6
	CMD^{-1} (mN/m)	42.0 ± 0.6	37.5 ± 0.8
	Dry biomass (g/l)	1.8 ± 0.4	1.8 ± 0.3

Table 5.7. Influence of KH_2PO_4 on growth and biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Values represents mean ± S.D of three individual experiments.

Concentration of KH_2PO_4 (g/l)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	41.9 ± 0.6	41.5 ± 0.5
	CMD^{-1} (mN/m)	51.6 ± 0.8	50.0 ± 0.5
	Dry biomass (g/l)	1.75 ± 0.7	1.68 ± 0.2
1.5	Surface tension (mN/m)	37.5 ± 0.5	38.4 ± 0.6
	CMD^{-1} (mN/m)	42.3 ± 0.6	46.5 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.04	1.8 ± 0.09
3.0	Surface tension (mN/m)	29.3 ± 0.4	27.4 ± 0.5
	CMD^{-1} (mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	2.0 ± 0.15	1.9 ± 0.1
4.5	Surface tension (mN/m)	33.2 ± 0.4	35.5 ± 0.5
	CMD^{-1} (mN/m)	41.8 ± 0.6	50.5 ± 0.8
	Dry biomass (g/l)	1.95 ± 0.05	1.85 ± 0.05

Table 5.8. Influence of Na_2HPO_4 on growth and biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Values represents mean ± S.D of three individual experiments.

Concentration of Na_2HPO_4 (g/l)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	41.5 ± 0.5	42.9 ± 0.6
	CMD^{-1} (mN/m)	50.0 ± 0.5	53.6 ± 0.8
	Dry biomass (g/l)	1.8 ± 0.09	1.8 ± 0.08
3.0	Surface tension (mN/m)	37.5 ± 0.5	38.4 ± 0.6
	CMD^{-1} (mN/m)	42.3 ± 0.6	46.5 ± 0.5
	Dry biomass (g/l)	1.75 ± 0.07	1.68 ± 0.15
6.0	Surface tension (mN/m)	29.3 ± 0.4	27.1 ± 0.5
	CMD^{-1} (mN/m)	38.3 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	2.0 ± 0.09	1.9 ± 0.1
8.0	Surface tension (mN/m)	38.4 ± 0.6	37.5 ± 0.5
	CMD^{-1} (mN/m)	42.3 ± 0.6	46.5 ± 0.5
	Dry biomass (g/l)	1.85 ± 0.7	1.85 ± 0.2

Table5.9. Influence of NaCl on growth and biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Values represent the mean ± S.D of three individual experiments.

Concentration of NaCl (g/l)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	38.4 ± 0.6	37.5 ± 0.5
	CMD^{-1} (mN/m)	46.5 ± 0.5	42.3 ± 0.6
	Dry biomass (g/l)	1.75 ± 0.7	1.68 ± 0.2
0.25	Surface tension (mN/m)	33.3 ± 0.4	29.0 ± 0.5
	CMD^{-1} (mN/m)	40.8 ± 0.6	38.0 ± 0.8
	Dry biomass (g/l)	1.8 ± 0.2	1.7 ± 0.3
0.5	Surface tension (mN/m)	31.5 ± 0.4	28.0 ± 0.5
	CMD^{-1} (mN/m)	40.0 ± 0.5	34.0 ± 0.8
	Dry biomass (g/l)	1.8 ± 0.2	1.75 ± 0.3
1.0	Surface tension (mN/m)	29.3 ± 0.4	27.2 ± 0.4
	CMD^{-1} (mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.2	1.85 ± 0.3

5.1.1.5. Influence of growth supplement (Thiamine-HCl) on growth and biosurfactants production

The preparation of Thiamine-HCl solution has already been described in section 3.6.7. The control set (with no Thiamine-HCl solution) has comparatively less growth and poor surface activities, with all the bacterial strains under study. 1ml/l of the solution was found optimum for the *Bacillus* strains for optimum growth and surface activities (Table 5.10)

5.1.1.6 Influence of trace element solution on biosurfactants production and growth

Similarly trace elements also have a role in growth and biosurfactants production, as it is evident from the experiment with negative control (0ml/l). 1 ml/l was found optimum for all the bacterial strains for optimum growth and biosurfactants production (Table 5.11).

Table5.10. Influence of growth supplement (Thiamine-HCl) on biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Values represent the mean ± S.D of three individual experiments.

Concentration of Thiamine-HCl (ml/l) *	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0 (control)	Surface tension (mN/m)	41.5 ± 0.5	38.5 ± 0.6
	CMD^{-1} (mN/m)	50.0 ± 0.5	48.6 ± 0.8
	Dry biomass (g/l)	1.45 ± 0.4	1.2 ± 0.3
0.5	Surface tension (mN/m)	37.5 ± 0.5	38.4 ± 0.6
	CMD^{-1} (mN/m)	42.3 ± 0.6	46.5 ± 0.5
	Dry biomass (g/l)	1.75 ± 0.7	1.48 ± 0.2
1.0	Surface tension (mN/m)	29.3 ± 0.4	27.4 ± 0.5
	CMD^{-1}(mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.2	1.85 ± 0.3
1.5	Surface tension (mN/m)	33.2 ± 0.4	30.5 ± 0.5
	CMD^{-1} (mN/m)	40.8 ± 0.6	36.0 ± 0.8
	Dry biomass (g/l)	1.92 ± 0.2	1.9 ± 0.3

* Stock solution of the Thiamine-HCl was prepared as described in the section 3.6.5

Table 5.11. Influence of micronutrients on growth and biosurfactants production. Results recorded post 48h of inoculation for *Bacillus* strains grown on glucose. Data represents the mean ± S.D of three independent experiments.

Concentration of micronutrients (ml/l)*	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0	Surface tension (mN/m)	48.5 ± 0.6	47.5 ± 0.5
	CMD^{-1} (mN/m)	58.0 ± 0.6	55.8 ± 0.8
	Dry biomass (g/l)	1.5 ± 0.4	1.4 ± 0.3
1.0	Surface tension (mN/m)	29.3 ± 0.4	27.4 ± 0.5
	CMD^{-1} (mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass (g/l)	1.9 ± 0.2	1.85 ± 0.3
2.0	Surface tension (mN/m)	35.5 ± 0.5	37.4 ± 0.6
	CMD^{-1} (mN/m)	46.6 ± 0.6	48.4 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.7	1.7 ± 0.2
4.0	Surface tension (mN/m)	37.5 ± 0.5	38.4 ± 0.6
	CMD^{-1} (mN/m)	49.3 ± 0.6	50.5 ± 0.5
	Dry biomass (g/l)	1.75 ± 0.7	1.58 ± 0.2

* Stock solution of the micronutrient solution was prepared as described in the section 3.6.6

5.1.1.7 Influence of agitation on biosurfactants production and growth

Stationary culture or the negative control (agitated at 0 rpm) exhibited poor cell growth as well as poor surface activities. Agitation at 200 rpm was noted to be optimum agitation for both the *Bacillus* strains in the study (Table 5.12).

Table5.12. Influence of agitation on biosurfactants production. Results recorded post 48h of inoculation. Agitation was controlled by incubating the cultures at various revolutions per minute (rpm) shaking condition on an incubator shaker. Values represent the mean ± S.D of three independent experiments.

Agitation (rpm)	Properties	*B. subtilis* DM-03	*B. subtilis* DM-04
0	Surface tension (mN/m)	48.9 ± 0.5	49.5 ± 0.6
	CMD^{-1} (mN/m)	57.0 ± 0.5	58.8 ± 0.8
	Dry biomass (g/l)	1.3 ± 0.4	1.2 ± 0.3
100	Surface tension (mN/m)	35.0 ± 0.5	38.4 ± 0.6
	CMD^{-1} (mN/m)	40.3 ± 0.6	46.5 ± 0.5
	Dry biomass (g/l)	1.75 ± 0.7	1.68 ± 0.2
200	Surface tension (mN/m)	29.3 ± 0.4	27.4 ± 0.5
	CMD^{-1}(mN/m)	38.5 ± 0.6	34.0 ± 0.8
	Dry biomass(g/l)	1.9 ± 0.2	1.85 ± 0.3
300	Surface tension (mN/m)	32.5 ± 0.5	33.2 ± 0.4
	CMD^{-1} (mN/m)	40.0 ± 0.8	36.8 ± 0.6
	Dry biomass(g/l)	1.8 ± 0.2	1.75 ± 0.3

5.1.1.8 Influence of temperature and pH on growth and biosurfactants production

As shown on Tables 5.13 and 5.14, both the *Bacillus* strains could grow and produce biosurfactants in a wide range of pH (3.0-12.0) and temperature (30°C-55°C). However, these bacterial strains exhibited better growth and biosurfactants production in the alkaline or neutral range of pH as compared to the acidic pH. Growth and biosurfactants production were maximum at 45°C for *Bacillus subtilis* DM-03 and pH 8.0 (Table 5.13) whereas *Bacillus subtilis* DM-04 exhibited maximum growth and biosurfactants production at 55°C temperature pH 7.0 (Table 5. 14).

5.1.1.9 Effect of different concentrations of NaCl on biosurfactants production

As shown in Table 5.15, both the *Bacillus* strains could grow and produce biosurfactants at a salt (NaCl) concentration ranging from 0.5% (w/v) to 7.0% (w/v), although the growth rate was reduced with an increase in the salt concentration. Nevertheless, changes in the CMD values and yield of biosurfactants remained un-effected with an increase in the salt (NaCl) concentration. Comparative study showed that *B. subtilis* DM-04 exhibited better salt tolerance as compared to *B. subtilis* DM-03 strain (Table 5.15).

Table 5.13. Influence of different pH and temperature on the growth and surface activities of *B. subtilis* DM-03 strain. Results were recorded post 48h of incubation. Each results represents mean ± S.D of three independent experiments.

Temperature (° C)	pH	Surface tension reduction (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	Dry biomass (g/l)	Yield of biosurfactant (g/l)
30	3.0	50.0 ± 0.5	69.0 ± 1.0	71.5 ± 0.5	0.6 ± 0.05	0.8 ± 0.05
	4.0	48.0 ± 1.0	60.0 ± 1.0	68.6 ± 0.5	0.85 ± 0.07	1.45 ± 0.05
	6.0	42.0 ± 0.5	56.0 ± 0.4	65.5 ± 1.0	1.7 ± 0.05	2.0 ± 0.5
	7.0	30.5 ± 0.5	39.8 ± 0.2	47.0 ± 0.7	1.8 ± 0.08	3.15 ± 0.1
	8.0	30.0 ± 0.5	39.0 ± 0.4	46.0 ± 0.6	1.9 ± 0.09	3.38 ± 0.06
	10.0	34.0 ± 0.5	44.5 ± 0.5	50.3 ± 0.7	1.5 ± 0.05	2.8 ± 0.02
	12.0	31.5 ± 0.5	41.0 ± 1.0	48.0 ± 0.5	1.35 ± 0.05	2.15 ± 0.05
45	3.0	48.0 ± 1.0	68.0 ± 1.0	69.0 ± 1.0	0.65 ± 0.05	0.9 ± 0.06
	4.0	45.0 ± 1.0	59.0 ± 0.5	68.0 ± 0.8	0.78 ± 0.03	1.7 ± 0.03
	6.0	41.0 ± 0.5	55.0 ± 1.0	63.0 ± 0.5	1.75 ± 0.05	2.8 ± 0.09
	7.0	30.0 ± 0.5	36.0 ± 0.7	45.0 ± 0.7	1.9 ± 0.1	3.45 ± 0.1
	8.0	29.1 ± 0.2	35.0 ± 0.6	44.0 ±0.6	2.0 ± 0.2	3.5 ± 0.05
	10.0	30.0 ± 0.5	36.3 ± 0.7	44.5 ± 0.9	1.45 ± 0.05	3.0 ± 0.05
	12.0	30.5 ± 1.0	38.6 ± 0.4	55.5 ± 0.5	1.25 ± 0.050	2.5 ± 0.09
55	3.0	55.0 ± .5	70.5 ± 0.5	71.9 ± 1.0	0.65 ± 0.05	0.5 ± 0.05
	4.0	50.3 ± 0.7	68.0 ± 0.5	71.0 ± 0.5	0.7 ± 0.05	1.5 ± 0.05
	6.0	44.0 ± 0.5	58.0 ± 1.0	65.0 ± 0.5	1.5 ± 0.05	1.2 ± 0.09
	7.0	30.2 ± 0.8	39.0 ± 0.5	49.0 ± 0.5	1.7 ± 0.1	2.5 ± 0.1
	8.0	29.8 ± 0.2	36.0 ± 0.5	45.0 ±0.5	1.8 ± 0.09	3.0 ± 0.05
	10.0	31.5 ± 0.5	37.0 ± 0.6	50.0 ± 0.9	1.3 ± 0.05	2.4± 0.05
	12.0	32.5 ± 1.0	45.0 ± 0.5	58.5 ± 0.5	1.0 ± 0.05	2.0 ± 0.1

Table 5.14. Influence of different pH and temperature on growth and surface activities of *B. subtilis* DM-04 strain. Results recorded post 48 h of incubation. Each result represents mean ± S.D of three independent experiments.

Temperature (° C)	pH	Surface tension reduction (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	Dry biomass (g/l)	Yield of biosurfactant (g/l)
30	3.0	46.8 ± 0.2	68.0 ± 1.0	70.5 ± 0.5	0.45 ± 0.05	0.3 ± 0.05
	4.0	46.0 ± 0.5	60.0 ± 1.0	68.0 ± 0.8	0.8 ± 0.05	0.4 ± 0.05
	6.0	30.0 ± 0.5	39.0 ± 0.4	60.0 ± 1.0	1.5 ± 0.05	0.7 ± 0.5
	7.0	30.2 ± 0.5	39.5 ± 0.5	45.0 ± 0.7	1.6 ± 0.04	1.45 ± 0.1
	8.0	29.0 ± 0.5	38.0 ± 0.5	44.0 ± 0.5	1.8 ± 0.09	1.6 ± 0.04
	10.0	33.0 ± 0.5	44.5 ± 0.5	50.3 ± 0.7	1.5 ± 0.05	1.2 ± 0.02
	12.0	31.0 ± 0.5	45.0 ± 1.0	48.0 ± 0.5	1.3 ± 0.05	1.1 ± 0.09
45	3.0	48.0 ± 1.0	68.0 ± 1.0	69.0 ± 1.0	0.65 ± 0.05	0.3 ± 0.06
	4.0	45.0 ± 1.0	59.0 ± 0.5	68.0 ± 0.8	0.78 ± 0.03	0.8 ± 0.03
	6.0	41.0 ± 0.5	55.0 ± 1.0	63.0 ± 0.5	1.75 ± 0.05	0.9 ± 0.09
	7.0	27.2 ± 0.4	35.0 ± 0.7	45.0 ± 0.7	1.8 ± 0.1	1.5 ± 0.1
	8.0	29.1 ± 0.2	35.0 ± 0.6	44.0 ±0.6	1.85 ± 0.2	1.8 ± 0.05
	10.0	30.0 ± 0.5	36.3 ± 0.7	44.5 ± 0.9	1.45 ± 0.05	1.36 ± 0.05
	12.0	30.5 ± 1.0	38.6 ± 0.4	55.5 ± 0.5	1.25 ± 0.05	0.9 ± 0.09
55	3.0	49.0 ± .5	70.0 ± 0.5	70.5 ± 1.0	0.65 ± 0.05	0.3 ± 0.05
	4.0	50.3 ± 0.7	68.0 ± 0.5	71 0 ± 0.5	1.0 ± 0.03	1.2 ± 0.05
	6.0	44.0 ± 0.5	58.0 ± 1.0	65.0 ± 0.5	1.8 ± 0.05	1.5 ± 0.09
	7.0	30.2 ± 0.8	39.0 ± 0.5	49 0 ± 0.5	1.85 ± 0.1	4.73 ± 0.07
	8.0	27.3 ± 0.2	36.0 ± 0.5	48.0 ±0.5	1.9 ± 0.05	4.68± 0.05
	10.0	31.5 ± 0.5	37.0 ± 0.6	50.0 ± 0.9	1.5 ± 0.05	2.5 ± 0.1
	12.0	32.5 ± 1.0	45.0 ± 0.5	58.5 ± 0.5	1.3± 0.05	2.0 ± 0.1

Table 5.15. Growth and biosurfactants production under saline condition. Result recorded post 48 h of growth of the strains at 45° C temperature incubated with different concentrations of NaCl. Each value represents mean ± S.D of three independent experiments.

Strains	Concentra -tion of NaCl (g%)	Properties			
		Surface tension (mN/m)	CMD^{-1} (mN/m)	Dry weight (g/l)	Yield of bio-surfactants (g/l)
Bacillus subtilis DM-03	0.5	29.0 ± 0.2	40.0 ± 0.5	1.8 ± 0.05	3.0 ± 0.15
	1.0	28.9 ± 0.3	38.0 ± 0.7	1.8 ± 0.06	3.5 ± 0.1
	3.0	31.0 ± 0.4	39.0 ± 0.5	1.7 ± 0.02	2.8 ± 0.2
	4.0	31.5 ± 0.5	40.5 ± 0.5	1.5 ± -.04	2.7 ± 0.1
	5.0	32.5 ± 0.3	45.0 ± 0.3	1.2 ± 0.01	2.6 ± 0.08
	7.0	42.0 ± 0.1	55.0 ± 0.5	1.0 ± 0.02	2.5 ± 0.1
Bacillus subtilis DM-04	0.5	30.0 ± 0.4	36.3 ± 0.4	1.9 ± 0.02	1.2 ± 0.2
	1.0	27.2 ± 0.4	34.0 ± 0.8	1.9 ± 0.05	1.5 ± 0.2
	3.0	31.5 ± 0.3	37.2 ± 0.5	1.9 ± 0.04	1.0 ± 0.1
	4.0	33.0 ± 0.4	39.8 ± 0.6	1.65 ± 0.03	0.8 ± 0.15
	5.0	33.5 ± 0.5	40.1 ± 0.4	1.65 ± 0.02	0.8 ± 0.1
	7.0	40.5 ± 0.5	50.2 ± 0.5	1.5 ± 0.04	0.6± 0.1

5.1.2 Isolation of crude biosurfactants

The biosurfactant production by *B. subtilis* DM-03 and DM-04 strains was bacterial growth dependent, and the biosurfactant production failed to initiate before 24 hours of growth. As shown in Table 5.1, biosurfactant yield was directly proportional to the growth of bacteria (incubation time) until the bacterial growth entered the stationary phase. At this point, biosurfactant production was independent of bacterial growth rate. About 3.5 g and 1.8 g of crude biosurfactants were isolated by acid precipitation followed by solvent extraction from one litre cultures of *B. subtilis* DM-03 and DM-04 strains respectively when grown at 45°C temperature and with glucose as the sole carbon source. However, a much higher yield (4.68 g/l) of biosurfactant was obtained when *B. subtilis* DM-04 was grown at 55°C temperature (Table 5.14).

While separating biomass from culture broth, the surface activity of different fractions was tested in order to ensure that biosurfactant activity was associated with cell free supernatant. In case of water immiscible substrates the recovered residual hydrocarbons were tested for surface activity but negative result was confirmed by tensiometer. The clumping biomass, which was recovered by centrifugation or from the interface of aqueous and oil layers after solvent washing and drying, was resuspended in distilled water and surface tension was tested. The biomass failed to show any significant surface tension reduction ability (Table 5.16). The slight reduction in surface tension of water could be caused by bacterial cell surface itself. Since the biomass after centrifugation did not show any surface activity, this suggested that the biosurfactants production by the strains was completely extracellular. The aqueous phase, which was cell and oil free, showed both surface and interfacial activity and acidification of which resulted in the precipitation of biosurfactant.

5.1.3 Purification of biosurfactants

When crude biosurfactants, obtained by acid precipitation of the cell free supernatants from two *B. subtilis* strains (DM-03 and DM-04) were subjected to reverse-phase (RP) HPLC on a C_{18} column, they were resolved into eight (Bs03 M1 to Bs03 M8) {Fig. 5.3} and five (Bs04 M1 to Bs04 M5) {Fig.5.4} major peaks, respectively. When individual peaks were tested for surface active properties, peaks Bs03 M3 and Bs04 M2 with the retention time of 1.05 min and 1.01 min respectively, exhibited maximum biosurfactant activity (Table 5.21). These peaks were subjected to further purification on the same RP-HPLC column with a linear gradient of 20 to 100% acetonitrile –0.1 % (v/v) TFA; the peak Bs03 M3 was resolved into seven fractions (Fig. 5.5) whereas the peak Bs04 M2 was separated into five fractions (Fig. 5.6).

Table 5.16 Summary of surface activity of isolated crude biosurfactant from *B. subtilis* DM-03 and DM-04 strains. Data are from a typical experiment.

Fractions	Surface tension reduction (mN/m)
***B. subtilis* DM-03**	
Cell free extract	32.1
Biomass	60.0
HCl precipitated cell free extract	30.0
Dichloromethane extracted crude biosurfactant	29.1
***B. subtilis* DM-04**	
Cell free extract	29.1
Biomass	56.0
HCl precipitated cell free extract	28.0
Dichloromethane extracted crude biosurfactant	27.5

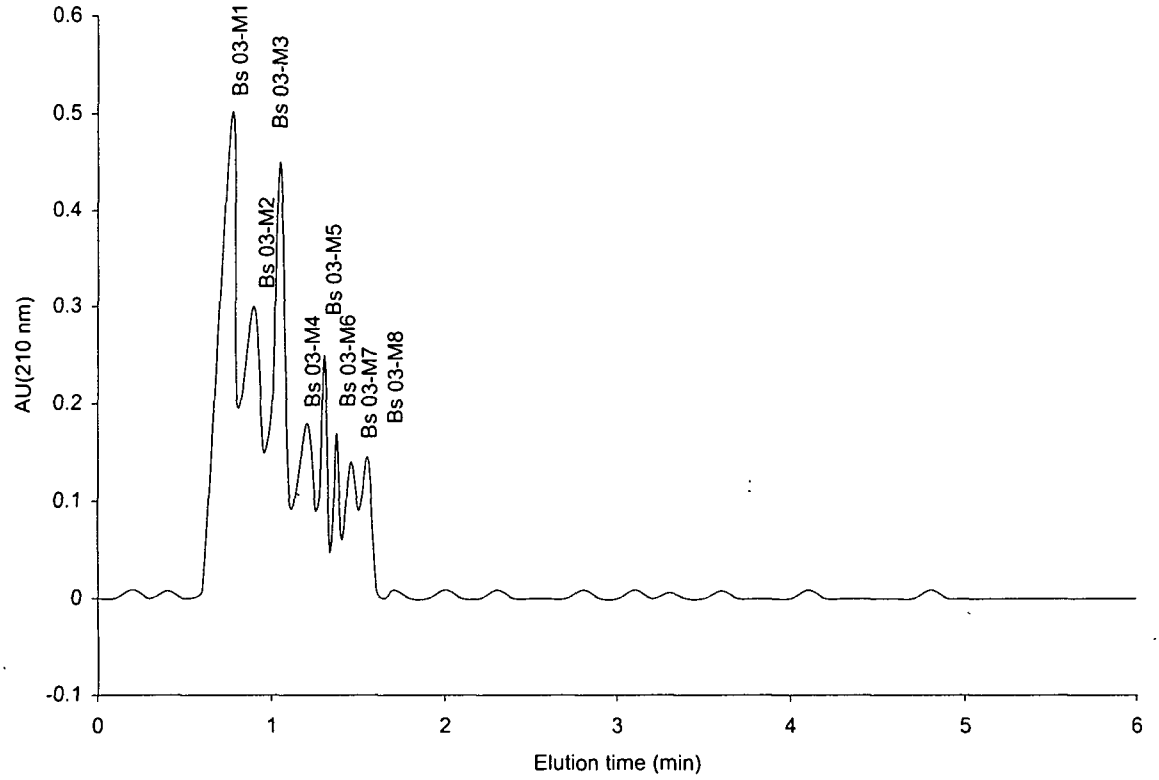

Fig.5.3. RP-HPLC of crude lipopeptide isolated from *B. subtilis* DM03 on a Waters $C_{18}\mu$ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate, pH 6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min and isocratic gradient with buffers A (20): buffer B (80%) and monitored at 210 nm.

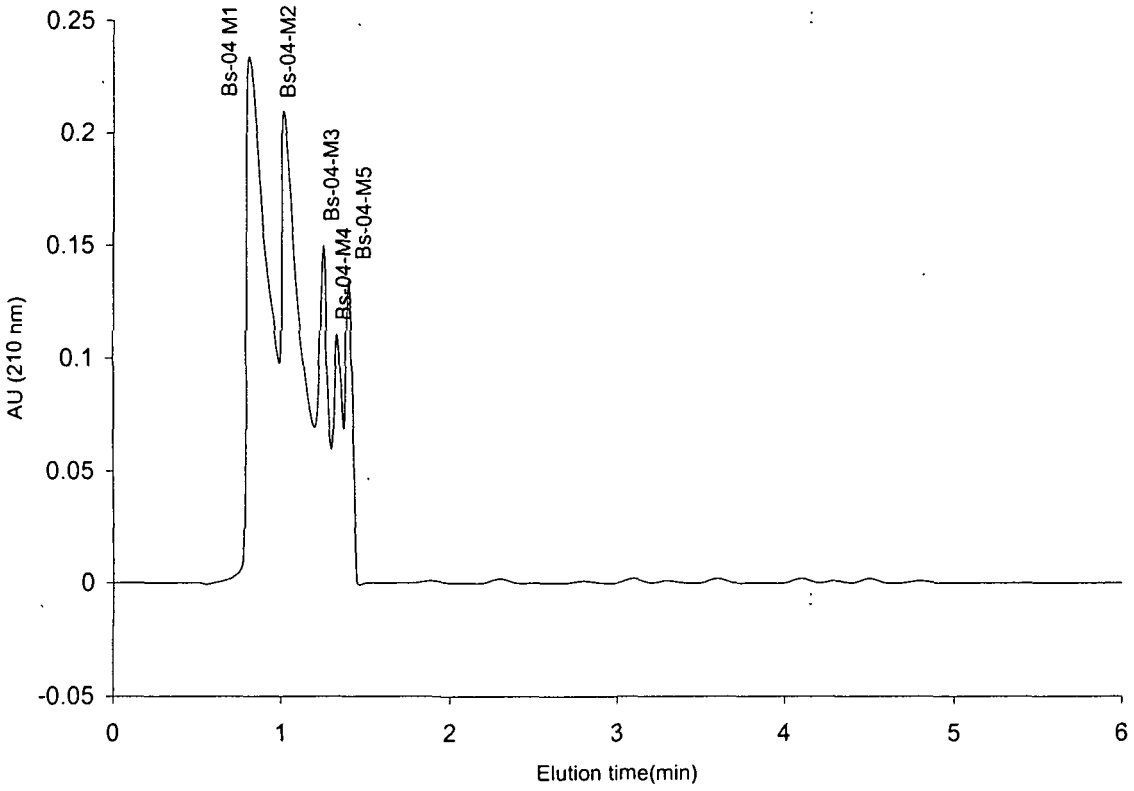

Fig.5.4. RP-HPLC of crude lipopeptide isolated from *B. subtilis* DM04 on a Waters C_{18}-μ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate, pH 6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min with buffer A (20): buffer B (80%) and monitored at 210 nm.

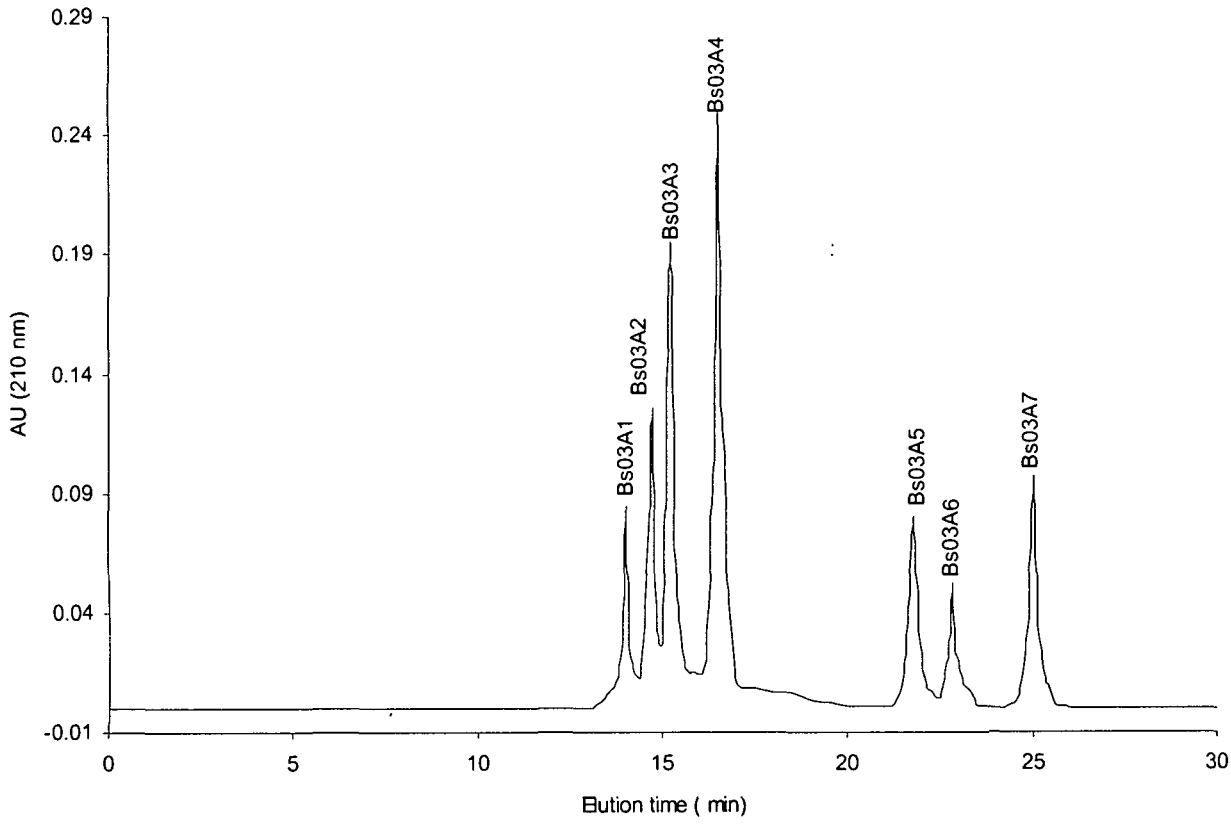

Fig.5.5. RP-HPLC of Bs03M3 on a Waters C_{18}-µ Nova pak RP-HPLC column. Buffer A was Milli Q H_20 containing 0.1% (v/v) TFA and buffer B was acetonitrile with 0.1% (v/v) TFA. Elution was performed at a linear gradient of 20-100% acetonitrole- 0.1% TFA having a flow rate of 1 ml/min and monitored at 210 nm.

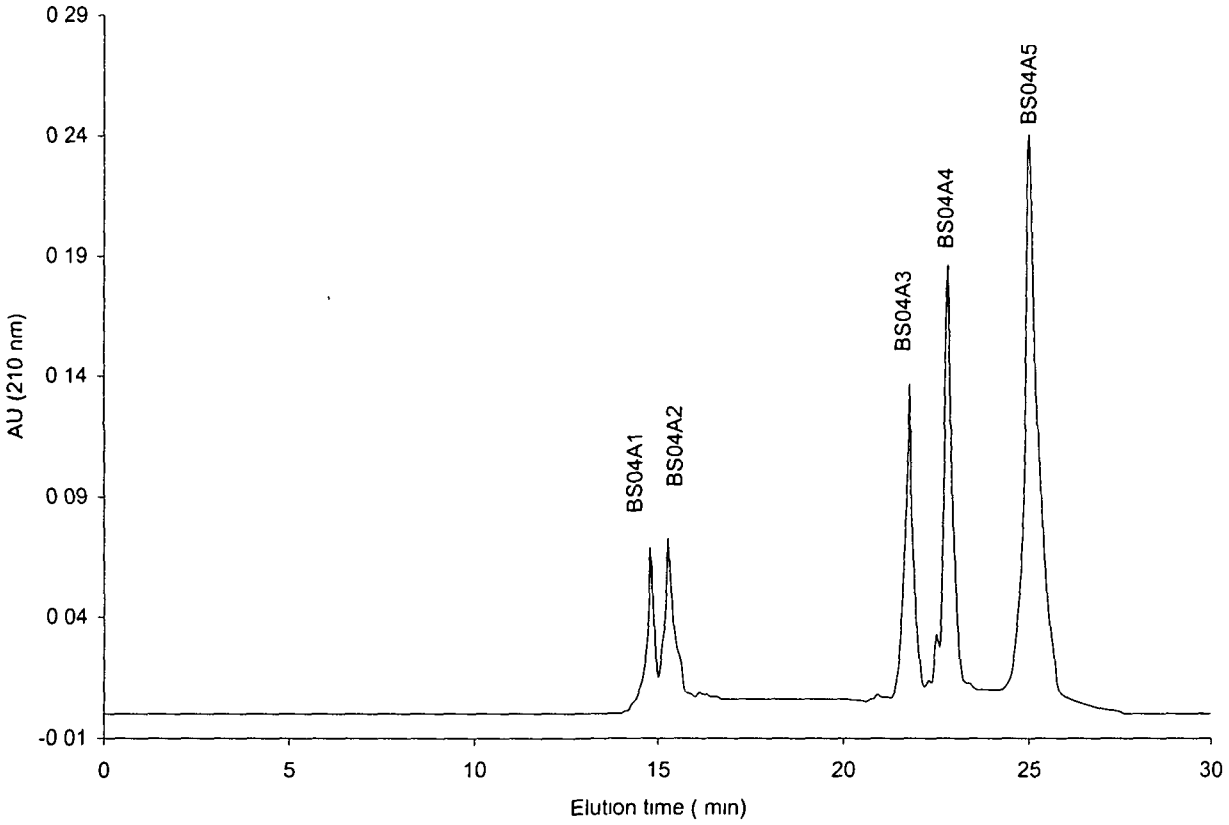

Fig.5.6. RP-HPLC of Bs04M2 on a Waters C_{18}-µ Nova pak RP-HPLC column. Buffer A was Milli Q H_20 containing 0.1% (v/v) TFA and buffer B was acetonitrile with 0.1% (v/v) TFA. Elution was performed at a linear gradient of 20-100% acetonitrole- 0.1% TFA having a flow rate of 1 ml/min and monitored at 210 nm.

5.1.4 Biochemical characterization of biosurfactants

5.1.4.1 Biochemical composition of biosurfactants

Biochemical composition of crude biosurfactants from *B. subtilis* DM-3 and DM-04 strains is displayed in Table. 5.17. It is quite evident that isolated biosurfactants from both the *B. subtilis* strains are lipopeptide in nature.

Table 5.17 Biochemical composition of biosurfactant from *B. subtilis* strains. Results represents mean ± S.D of three determinations.

Biosurfactants from	Protein content (%)	Lipid content (%)	Carbohydrate content (%)
B. subtilis DM-03	16.6 ± 0.05	21.9 ± 0.1	0
B. subtilis DM-04	18.4 ± 0.06	20.5 ± 0.05	0

5.1.4.2 Determination of critical micelle concentration (CMC) of crude biosurfactants

The CMC of biosurfactant from *B. subtilis* DM-03 and DM-04 was observed as 140 mg/l and 120 mg/l respectively (Fig.5.7) Above the CMC, no further reduction in surface tension was observed. The surface tension at the CMC for biosurfactants of *B. subtilis* DM-03 and DM-04 were 29.5 mN/m and 27.5 mN/m respectively.

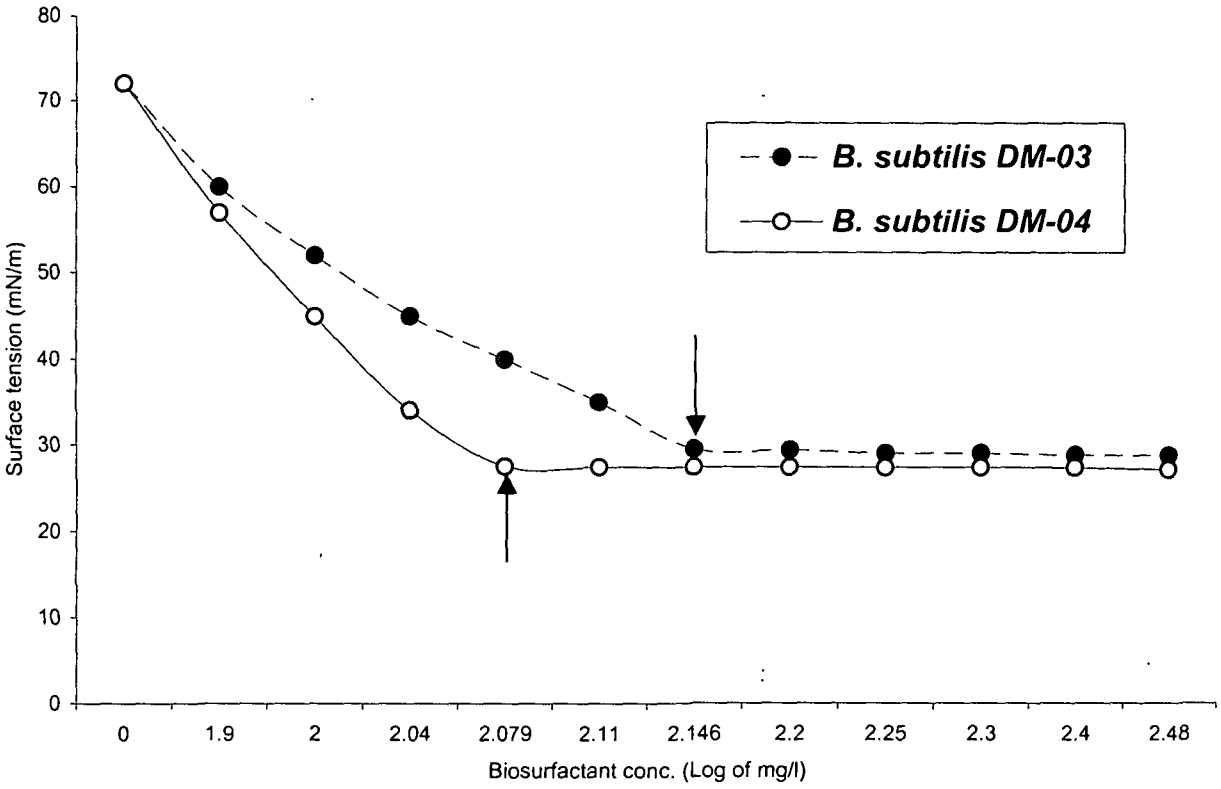

Fig.5.7. CMC of crude biosurfactants from *B. subtilis* DM-03 and DM-04 strains. The arrows indicate the CMC values. Results represent the mean of four individual experiments.

5.1.4.3 Heat and pH stability of biosurfactants

The crude biosurfactants from both the *Bacillus* strains retained their surface active properties even after heating them at 100°C for 60 min. For example, the lipopeptide fraction from either strain retained 93.6 – 96.3 % of original biosurfactant activity post heating at 100°C for 60 min (Table 5.18).

The crude biosurfactants from both the *B. subtilis* strains exhibited appreciable pH stability (Table 5.19). The biosurfactant activity was retained in a wide range of pH ranging from 3.0-11.0.

Table 5.18. Thermostability of biosurfactant from *B. subtilis* DM-03 and DM-04 strains. Aqueous solutions of biosurfactants were heated at 100°C for 60 min, cooled to room temperature followed by measurement of surface tension reduction. Results represent the mean ± SD of three individual experiment.

Bacterial strains	Surface tension reduction by biosurfactants		% Activity remaining post heating
	Before heating (mN/m)	Post heating (mN/m)	
B. subtilis DM-03	29.0 ± 0.2	30. ± 0.6	93.6 ± 0.4
B. subtilis DM-04	27.5 ± 0.1	28.5 ± 0.3	96.3 ± 0.2

Table 5.19 Assessment of pH stability of biosurfactant(s) from *B. subtilis* DM-03 and DM-04 strains. Results are from a typical experiment.

Source of crude biosurfactant	Surface active properties at pH range											
	pH 3.0		pH 5.0		pH 6.0		pH 7.0		9.0		11.0	
	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}
B. subtilis DM-03	32.4	38.0	31.0	36.0	30	34.5	29.0	34.0	29.5	35.0	31.3	36.0
B. subtilis DM-04	33.0	39.0	31.5	37.0	31.5	36.0	27.5	31.0	29.9	36.5	31.9	37.0

ST= surface tension. Surface tension of water is 72 mN/m

5.1.4.4 Emulsification property and emulsion stability

As shown in Table 5.20, the crude lipopeptide biosurfactants from *B. subtils* DM-03 and DM-04 strains displayed an emulsification index (E_{24}) of 60% and 70% respectively.

The emulsion formed by the crude biosurfactants secreted from *B. subtils* strains under study was found to be very stable as was evident from the calculation of the decay constant (K_d) described in the section 3.9.5.2. The stability of the emulsion formed by biosurfactants from *B. subtilis* strains was higher than the chemical surfactant SDS (Table 5.20 and Fig.5.8). The decay constant of the lipopeptide biosurfactant(s) from *B. subtilis* DM-03 and DM-04 was found to be –0.0135 and –0. 0159 respectively and that of SDS was found to be –0.0172 (Fig.5.8).

Table 5.20 E_{24} of biosurfactant from the bacterial strains. Calculation of E_{24} and K_d are described in the sections 3.9.5.1 and 3.9.5.2 respectively. Higher K_d value represents better emulsion stability as compared to lower K_d value. Results represent mean ± S.D of four individual experiments

Source of crude Biosurfactant /Chemical surfactant	Emulsification index (E_{24})	Emulsion stability (K_d)
Bacillus subtilis DM-03	60 ± 1.8	-0.0135
Bacillus subtilis DM-04	70 ± 2.1	-0.0135
SDS	ND	-0.0172

ND= not determined

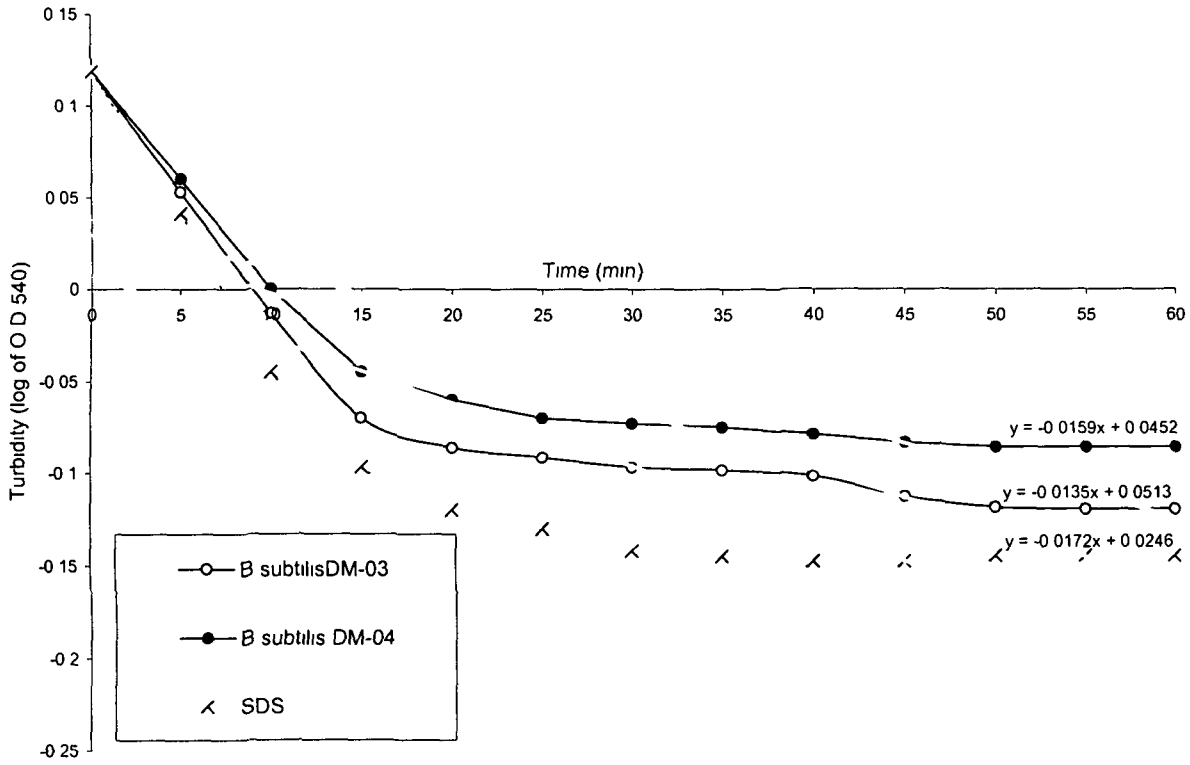

Fig.5.8. A comparison of emulsion stability of biosurfactants from *B. subtilis* DM-03, *B. subtilis* DM-04 and chemical surfactant SDS.

5.1.4.5 Light absorption spectra of crude biosurfactants

The λ_{max} for crude biosurfactants of the *Bacillus subtilis* DM-03 and DM-04 were noted at 219 nm and 207 nm respectively (Fig. 5.9 and Fig. 5.10).

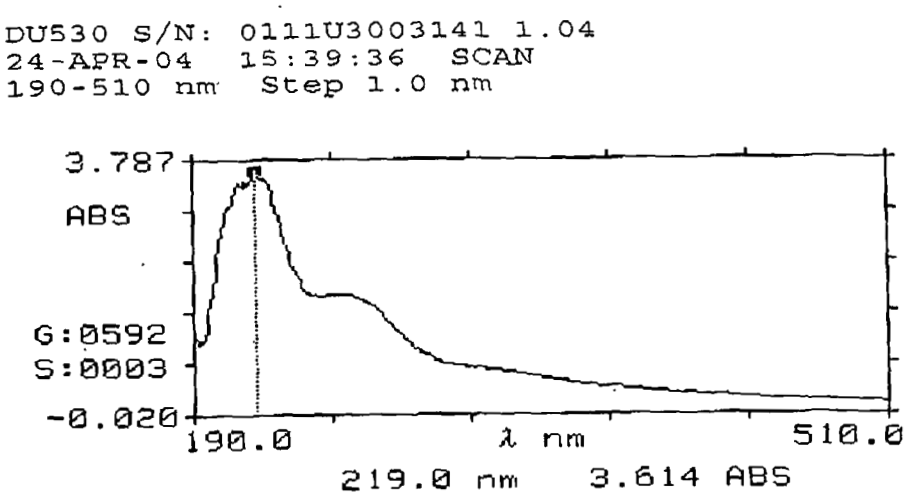

Fig.5.9. Light absorption characteristics of crude biosurfactants isolated from *B. subtilis* DM-03 strain.

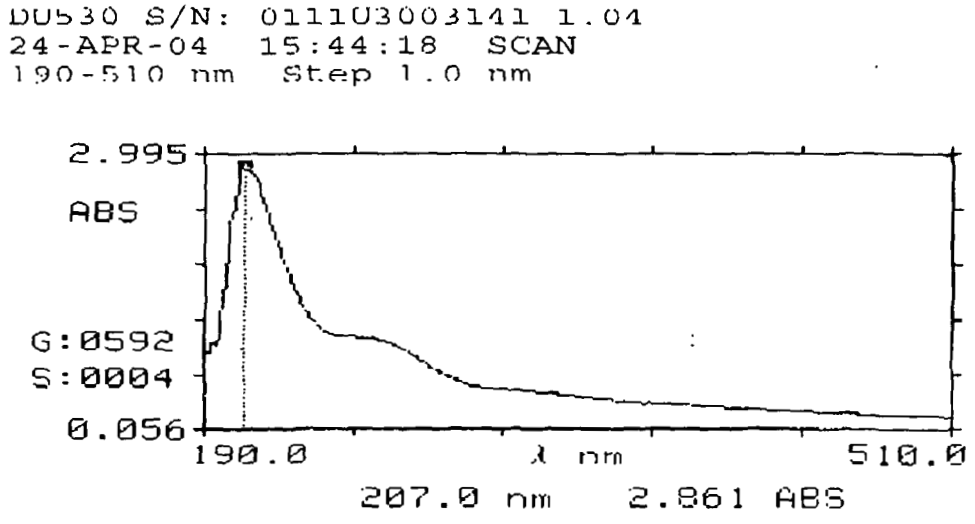

Fig. 5.10. Light absorption characteristics of crude biosurfactant isolated from *B. subtilis* DM-04 strain.

5.1.4.6 IR spectra of crude biosurfactants

IR spectra of the biosurfactant from both the *Bacillus* strains in nujol showed strong absorption bands at 1644 cm^{-1} as result of stretching mode of a CO-N and at 1542 cm^{-1} for the deformation mode of the NH bond combined with C-N stretching mode occurred The presence of an aliphatic chain was indicated by the C-H stretching modes at 2858-2926cm^{-1} and 1457-1400 cm^{-1} The band at 1736 cm^{-1} was due to lactone carbonyl absorption (Fig. 5.11 and Fig. 5.12). These result indicated that the biosurfactant contained aliphatic and carbonyl moieties.

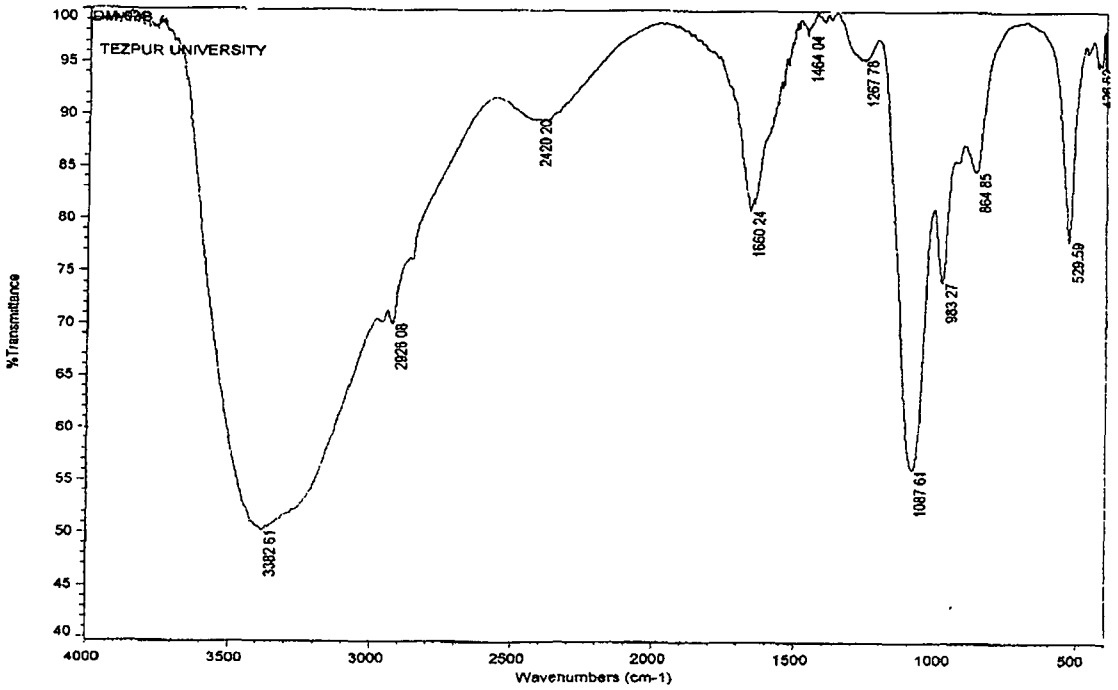

Fig.5.11. IR spectra of crude biosurfactants isolated from *B. subtilis* DM-03.

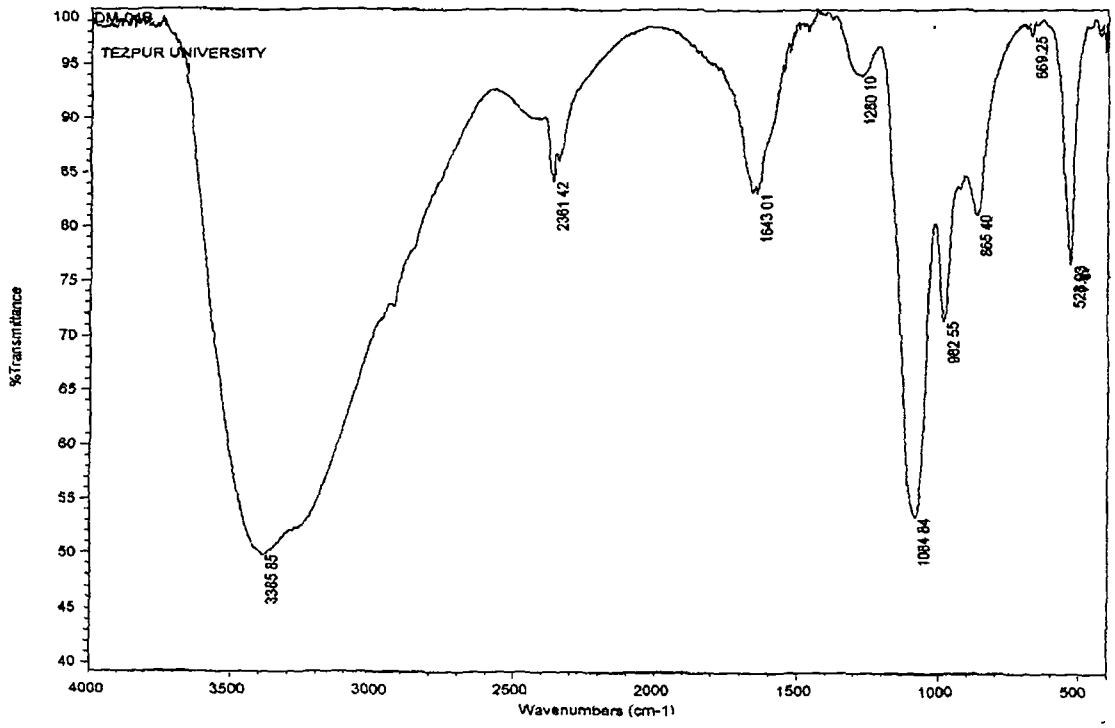

Fig.5.12. IR spectra of crude biosurfactants isolated from *B. subtilis* DM-04.

5.1.4.7 MALDI-TOF-MS characterization of HPLC purified biosurfactant fractions

The molecular mass and identities of each fraction of *B. subtilis* strains, as determined by MALDI-TOF mass spectra analysis of the RP-HPLC purified biosurfactant(s) along with their surface-active properties like surface tension reduction and CMC values are summarized in Table 5.21. Our results showed that iturin and surfactin isoforms represent the major lipopeptide biosurfactant produced by *B. subtilis* DM-03 and DM-04 strains respectively (Table 5.21). The mass spectra of these lipopeptides have peaks, which can be attributed to the protonated forms, as well as to the sodium and potassium adducts.

Table 5.21. Characterization of major biosurfactant fraction isolated from *Bacillus subtilis* DM-03 and DM-04 strains by acid precipitation followed by HPLC fractionation. Results are from a typical experiment.

Fraction	Final surface tension (mN/m) by100 µg of CLPs*	Amount recovered (mg)	Yield (%)	CMC (mg/l)	m/z ** (Da)	Assignment
***B. subtilis* DM-03**						
Cell free extract	32.3	130.0	100.0	-	-	-
Crude lipopeptide	29.1	92.3	71.0	140	-	-
Bs 03M3	29.0	2.7	2.0	132	-	-
Bs03A1	44.3	0.2	0.1	145	1170.6	C_{16} iturin $[M + K]^+$
Bs03A2	43.2	0.3	0.2	146	1122.6	C_{17} iturin $[M + K]^+$
Bs03A3	40.5	0.4	0.3	140	1136.6	C_{18} iturin $[M + K]^+$
Bs03A4	41.0	0.5	0.4	141	1112.5,1150.6	C_{19} iturin $[M+K]^+$,$[M + H]^+$
Bs03A5	28.2	0.2	0.1	89	1046.6	C_{13} surfactin $[M+K]^+$
Bs03A6	27.0	0.1	0.1	73	1060.5	C_{14} surfactin $[M + K]^+$
Bs03A7	25.0	0.2	0.2	75	1070.6	C_{15} surfactin $[M + K]^+$
***B. subtilis* DM-04**						
Cell free extract	29.1	123.6	100.0	-	-	-
Crude lipopeptide	27.1	80.9	65.6	120	-	-
Bs 04M2	26.9	2.7	2.2	112	-	-
Bs04A1	41.8	0.2	0.1	124	1122.6	C_{17} iturin $[M + K]^+$
Bs04A2	39.2	0.2	0.1	138	1136.6	C_{18} iturin $[M + K]^+$
Bs04A3	28.3	0.3	0.3	83	1046.6	C_{13} surfactin $[M + K]^+$
Bs04A4	27.1	0.5	0.4	72	1060.5	C_{14} surfactin $[M + K]^+$
Bs04A5	25.9	0.6	0.5	76	1058.6	C_{15} surfactin $[M + Na]^+$

*Surface tension of control is 72 mN/m.

** Determined by MALDI-TOF mass spectra analysis.

5.1.5 Pharmacological properties

5.1.5.1 Hemolytic activity

As depicted in Fig. 5.13, biosurfactant from both the *Bacillus* strains induced dose dependent direct lysis of washed human erythrocytes, but to a different extent. Two hundred µg of crude biosurfactants from *B. subtilis* DM-03 and DM-04 induced 3.5% and 2.9% hemolysis of the washed human erythrocytes respectively post 90 min of incubation at 37°C. The hemolytic effect of crude and purified lipopeptide biosurfactants from *B. subtilis* DM-03 and DM-04 are summarized in Table 5.22. Among the lipopeptide isoforms iturin induce comparatively higher hemolysis than those of surfactins group of lipopeptides.

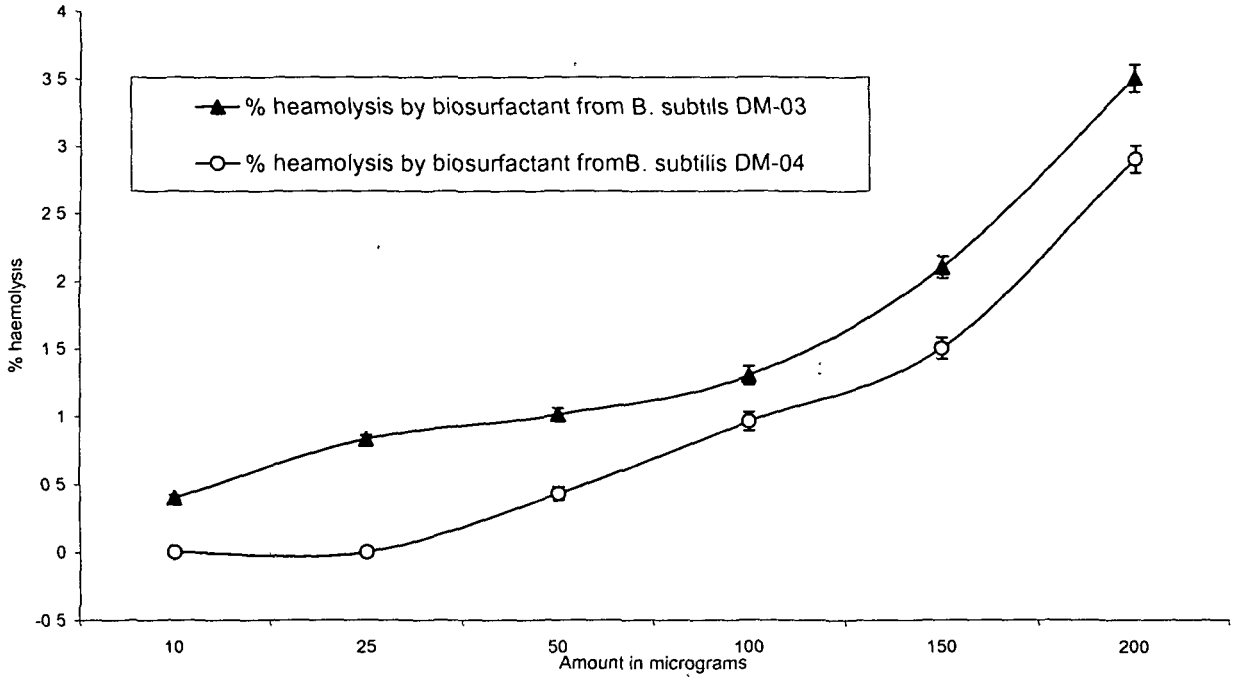

Fig.5.13. Dose dependent hemolysis of human erythrocytes by crude biosurfactants from *B. subtilis* DM-03 and DM-04 strains. Various concentrations of biosurfactants were incubated with 5% (v/v) washed human erythrocytes in a final of 3.0 ml at 37°C temperature for 60 min. Each value represents mean ± S.D of three independent experiments.

5.1.5.2 Interference in blood coagulation

Twenty µg of crude lipopeptide from *B. subtilis* DM-03 and DM-04 increased the Ca-clotting time of platelet poor goat plasma (PPGP) from 157±1 sec to 219±1 sec and 230 ±1 sec respectively revealing their anti-coagulant nature (Fig. 5.14). The anticoagulant nature of crude and individual lipopeptide fractions is shown in Table 5.22.

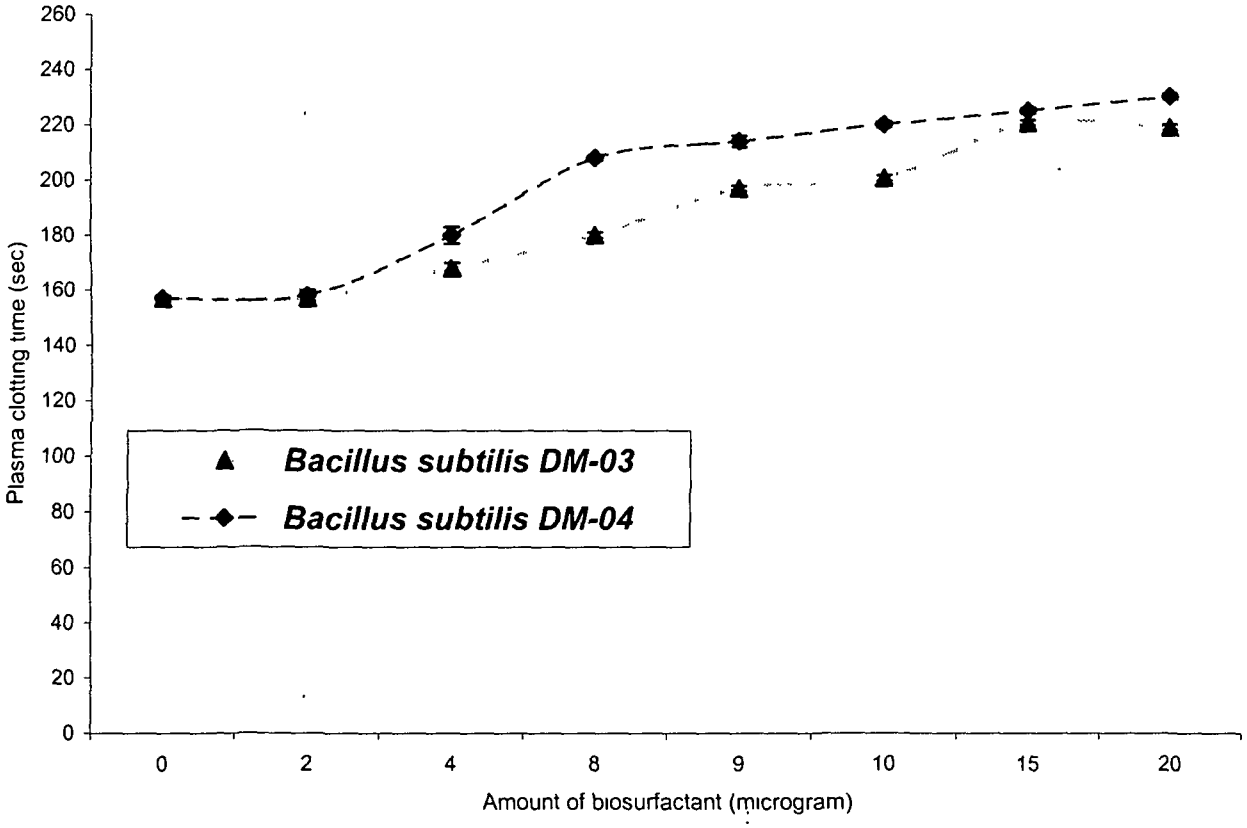

Fig.5.14. Dose dependent interference of *in-vitro* blood coagulation by biosurfactant from *B. subtilis* DM-03 and DM-04. Results represent the mean ± S.D of four individual experiments.

5.1.5.3 *In-vitro* tissue damaging activity

The bio-safety of the crude/purified biosurfactants was evaluated by assessing their ability to induce tissue damage in *in vitro* condition. Neither the crude lipopeptide nor their fractions from either *Bacillus* strains could show any appreciable liver tissue damaging activity as compared to chemical surfactant- Triton-X 100 (100% tissue damaging activity) and they were completely non-detrimental on the heart and lungs tissue even at a dose of 200µg/ ml demonstrating their bio-safety (Table 5.22).

Table 5.22. Pharmacological properties of crude/ purified lipopeptide biosurfactants from *B. subtilis* DM-03 and DM-04. Experiments were carried out as described in the material and methods section. Results represent mean ± S.D of three independent experiments.

Lipopeptide Fraction	Direct hemolysis*	Anticoagulant activity**	In vitro tissue damaging***		
			Liver	Heart	Lungs
***B. subtilis* DM-03**					
Crude lipopeptide	1.3 ±0.1	219.0 ±2	1.9 ±0.2	0.0	0.0
HPLC purified lipopeptides	1.02 ±0.2	213.0 ±1	0.9 ±0.1	0.0	0.0
C_{16} iturin $[M + K]^+$	0.30 ±0.1	178.0 ±1	0.0	0.0	0.0
C_{17} iturin $[M + K]^+$	0.28 ±0.1	178.0 ±2	0.0	0.0	0.0
C_{18} iturin $[M + K]^+$	0.33 ±0.2	210.0 ±1	0.0	0.0	0.0
C_{19} iturin $[M+K]^+$,$[M + H]^+$	0.38 ±0.1	213.0 ±1	0.0	0.0	0.0
C_{13} surfactin $[M+K]^+$	0.0	211.0 ±2	0.0	0.0	0.0
C_{14} surfactin $[M + K]^+$	0.0	218.0 ±1	0.0	0.0	0.0
C_{15} surfactin $[M + K]^+$	0.0	217.0 ±2	0.0	0.0	0.0
***B. subtilis* DM-04**					
Crude lipopeptide	0.96 ±0.1	230.0 ±1	1.0 ±0.1	0.0	0.0
HPLC prurified lipopeptides	0.73 ±0.1	227.0 ±2	0.6 ±0.1	0.0	0.0
C_{17} iturin $[M + K]^+$	0.31 ±0.2	183.0 ±3	0.0	0.0	0.0
C_{18} iturin $[M + K]^+$	0.30 ±0.1	179.0 ±1	0.0	0.0	0.0
C_{13} surfactin $[M + K]^+$	0.0	178.0 ±2	0.0	0.0	0.0
C_{14} surfactin $[M + K]^+$	0.0	211.0 ±2	0.0	0.0	0.0
C_{15} surfactin $[M + Na]^+$	0.01 ±0.01	216.0 ±2	0.0	0.0	0.0

* by 100 µg of crude/ purified lipopeptide biosurfactants .

** Plasma clotting time in sec by 20µg biosurfactant. Clotting time of normal plasma is 157±1 sec.

*** % hemoglobin release by 200 µg/ml of fraction

5.1.6 Cell surface hydrophobicity (CSH)

The cell surface hydrophobicity was evaluated by studying the ability of the cells to partition in the organic and aqueous phases, as described in section 3.11.

A comparison of CSH of the two *Bacillus* strains revealed that the cells of *B. subtilis* DM-04 were more hydrophobic in nature as compared to the cells from *B. subtilis* DM-03 in all the hydrocarbons used in this study (Fig.5.15). *B. subtilis* DM-03 cells could not be transferred to the aromatic hydrocarbons (benzene and toluene) and aliphatic hydrocarbon like hexane and only 12.74 % of these cells were transferred in dodecane. In contrast to this, cells from *B. subtilis* DM-04 exhibited much better hydrophobicity to the entire test hydrocarbons, except toluene (Fig.5.15).

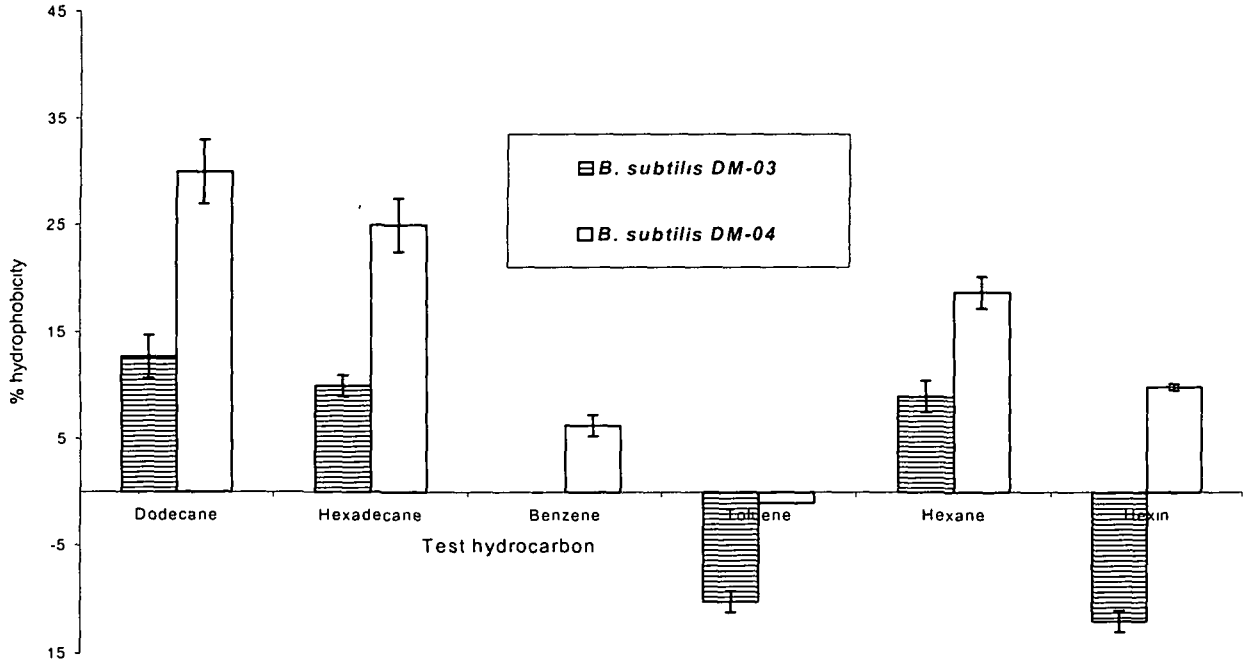

Fig. 5.15. Comparison of cell surface hydrophobicity of *B. subtilis* DM-03 and DM-04. Experiments were carried out as described in the text. Results represent the mean ± S.D of four individual experiments.

5.1.7 Effect of crude lipopeptide biosurfactant from a particular *B. subtilis* strain on enhancing the growth and substrate (hydrocarbon/ starch) utilization by another *B. subtilis* strain in the present study

As described earlier, both the *Bacillus* strains displayed maximum growth and biosurfactants production in the presence of dextrose and glycerol respectively. However, least growth as well as biosurfactant production by *B. subtilis* DM-03 was observed when hydrocarbons were used as sole carbon source(s). But the growth rate of DM-03 strain in hydrocarbons (eg. hexadecane and pyrene) was enhanced significantly ($p<0.001$) when crude lipopeptide isolated from *B. subtilis* DM-04 strain was added in the growth medium (Table 5.23). Similarly, *B. subtilis* DM-04 exhibited poor growth on starch, but excellent growth was observed in the same medium, when exogenously supplemented with crude lipopeptide isolated from *B. subtilis* DM-03 strain (Table 5.23).

Table 5.23. Effect of crude lipopeptide biosurfactant from a particular *B. subtilis* strain on the growth and substrate (hydrocarbon/ starch) utilization by another *B. subtilis* strain. Results represents mean ± S.D of three independent experiments.

Test organism (*B. subtilis* strain)	Carbon source	Exogenous added crude lipopeptides from	Dry biomass(g/l)	Surface tension (mN/m)	CMD^{-1} (mN/m)
DM-03	Hexadecane	None (control)	0.5 ± 0.05	46.5 ± 0.3	64.2 ± 0.4
DM-03	Hexadecane	*B. subtilis* DM04	1.9± 0.1 [a]	32.5 ± 0.2 [a]	38.5 ± 0.4 [a]
DM-03	Pyrene	None (control)	0.29 ± 0.08	57.5 ± 0.4	70.0 ± 0.4
DM-03	Pyrene	*B. subtilis* DM04	1.0 ± 0.05 [a]	34.2 ± 0.3 [a]	40.2 ± 0.4 [a]
DM-04	Starch	None (control)	0.7 ± 0.06	42.5 ± 0.3	52.0 ± 0.4
DM-04	Starch	*B. subtilis* DM03	3.0 ± 0.1 [a]	30.5 ± 0.1 [a]	38.0 ± 0.4 [a]

Surface tension of the control medium with hexadecane, pyrene and starch was 66.5 mN/m, 67.4 mN/m and 66.8 mN/m respectively. Statistical analysis: [a] $p<0.001$ with respect to control value.

5.1.8 Biosurfactants production by *B. subtilis* strains grown on non-conventional carbon source (potato peel)

5.1.8.1 Growth and biosurfactant(s) production by *B. subtilis* strains when grown on potato peels

It was observed that biosurfactant production by both the *Bacillus* strains using potato peel as sole carbon source was growth associated and maximum biosurfactant production was achieved post 72 hours of bacterial growth. A comparison of biosurfactant production showed that *B. subtilis* DM-03 (Fig.5.16) produced more biosurfactant then that of *B. subtilis* DM-04 using potato peels as source of carbon for growth (Fig.5.17).

5.1.8.2 Isolation and fractionation of biosurfactant(s) from *B. subtilis* strains grown on potato peels as carbon source

When subjected to RP-HPLC, the crude lipopeptide from *B. subtilis* DM-03 was resolved into five peaks (Fig. 5.18) where as the crude lipopeptide from *B. subtilis* DM-04 was fractionated into three peaks (Figure 5.19). Screening of the individual peak for surface tension reduction ability showed that the peaks eluted at a retention time of 1.05 min (Bs03pp3) and 1.01min (Bs04pp2) respectively for *B. subtilis* DM-03 and DM-04 retained the maximum surface active properties.

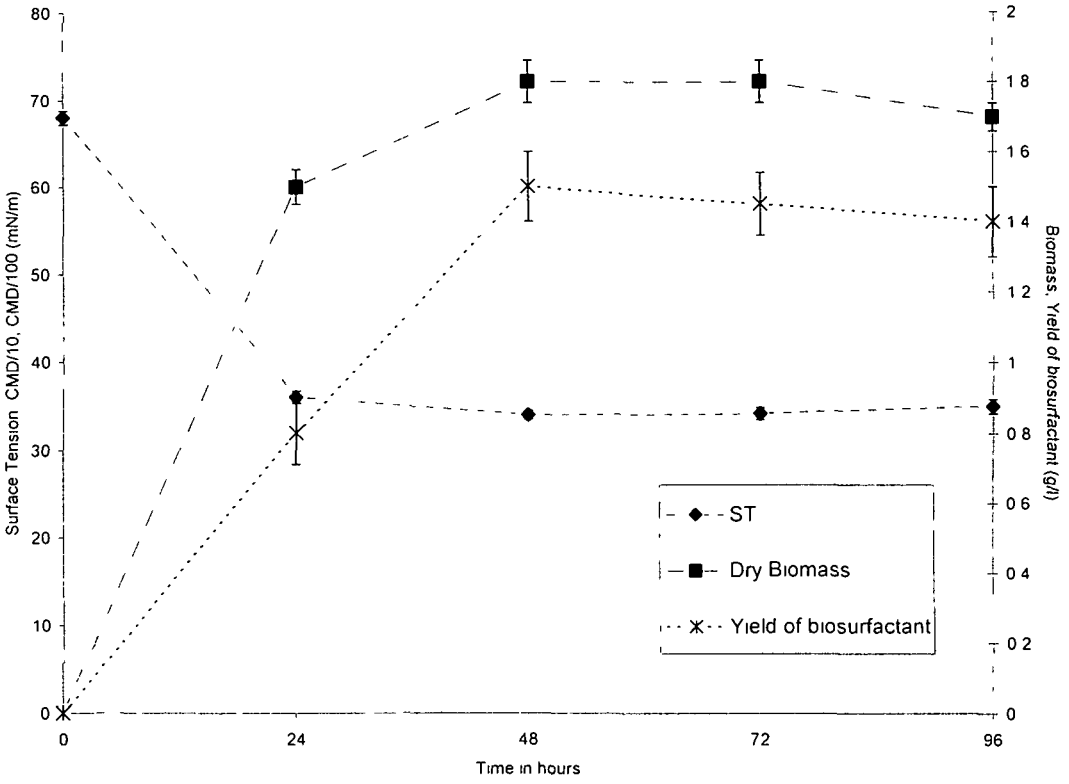

Fig. 5.16. Growth, biosurfactant production and surface activity of *B. subtilis* DM-03 when grown on mineral salt medium supplemented with 2% (w/v) dried potato peels. Results represents mean ± SD of three independent experiments.

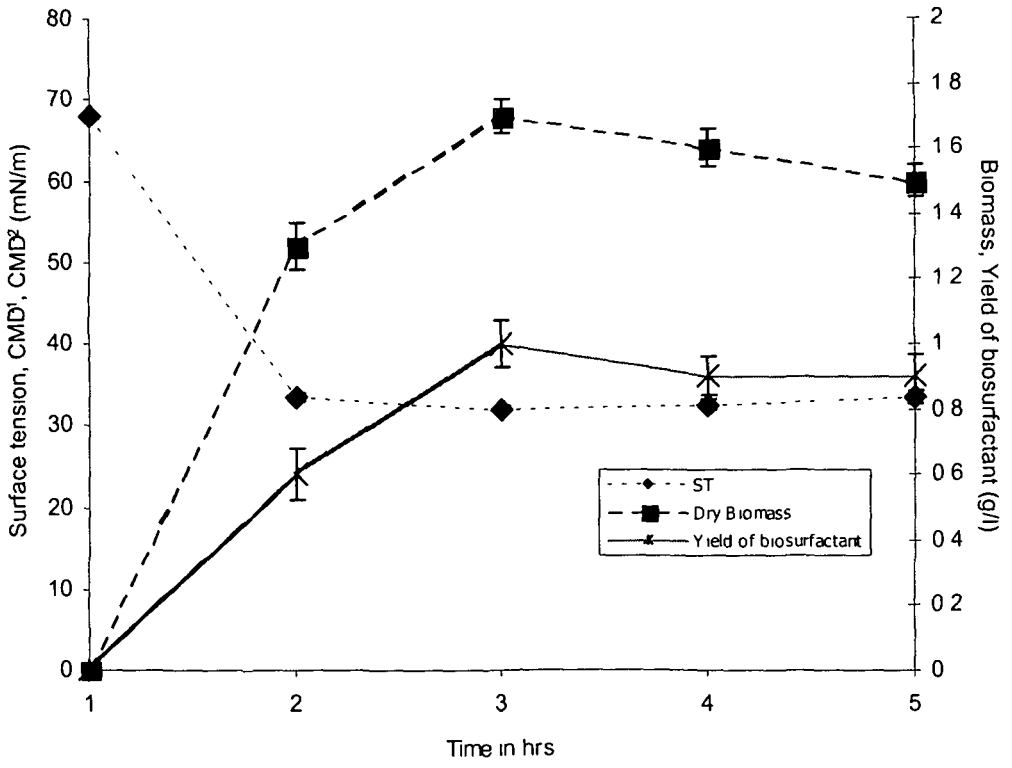

Fig. 5.17. Growth, biosurfactant production and surface activity of *B. subtilis* DM-04 strain when grown on mineral salt medium supplemented with 2% (w/v) dried potato peel. Results represents mean ± SD of three independent experiments.

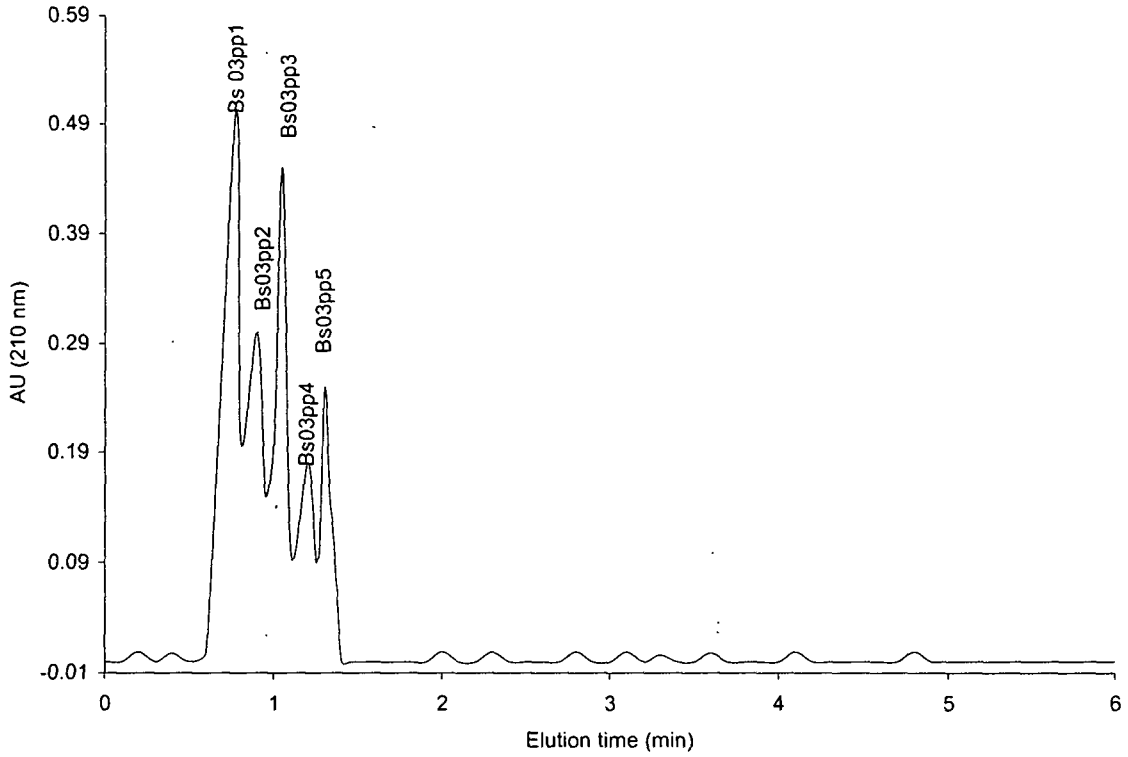

Fig. 5.18. RP-HPLC of crude lipopeptide (isolated from *B. subtilis* DM-03 grown on potato peel as carbon source) on a Waters C18µ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate buffer, pH 6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min. The elution was monitored at 210 nm. Experiment was carried out as described in the text.

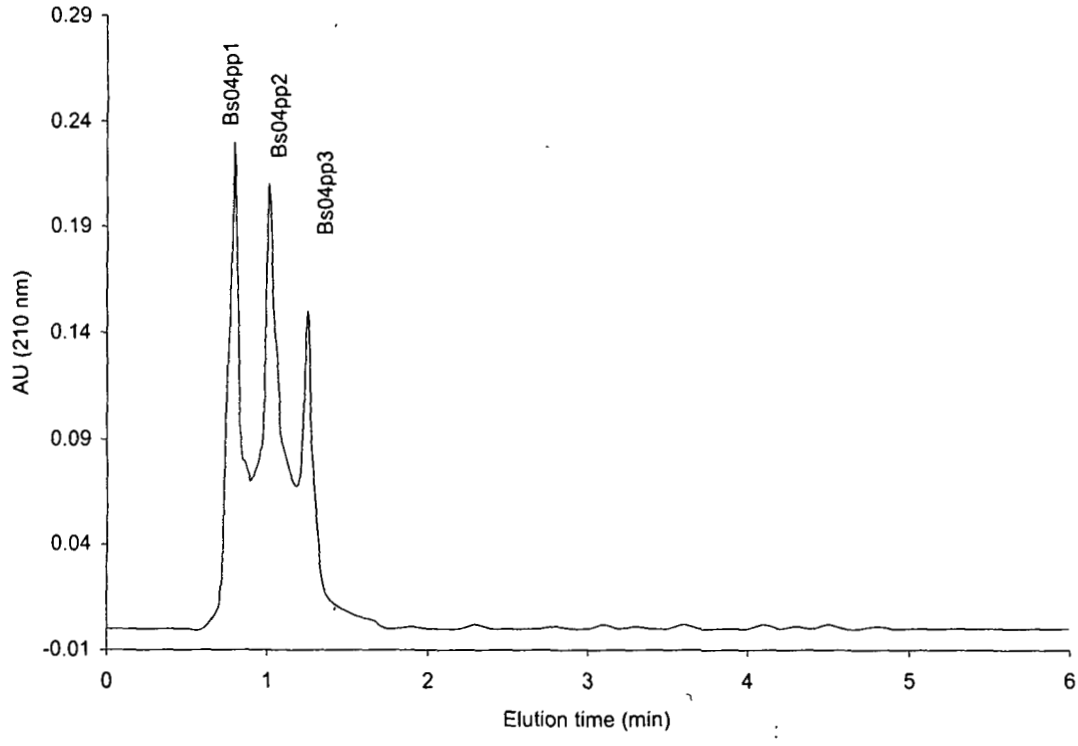

Fig. 5.19. RP-HPLC of crude lipopeptide (isolated from *B. subtilis* DM-04 grown on potato peel as carbon source on a Waters C18µ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate buffer, pH 6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min. The elution was monitored at 210 nm. Experiment was carried out as described in the text.

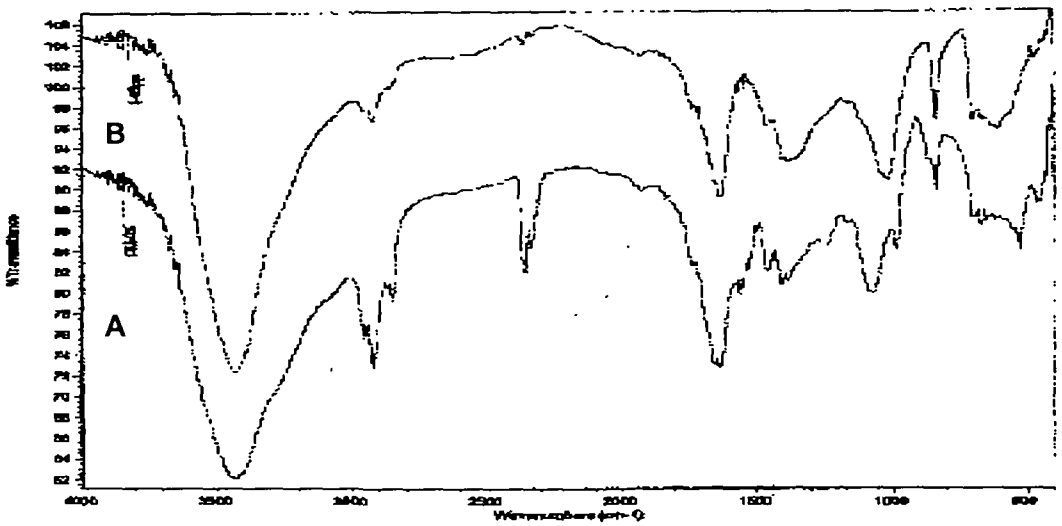

Fig. 5.20. A comparison of IR spectra of crude lipopeptide isolated from *B. subtilis* DM-03 grown on 2% (w/v) glucose and 2% (w/v) potato peels.

A: biosurfactants from *B. subtilis* DM-03 when grown on glucose.

B: biosurfactants from *B. subtilis* DM-03 when grown on potato peels.

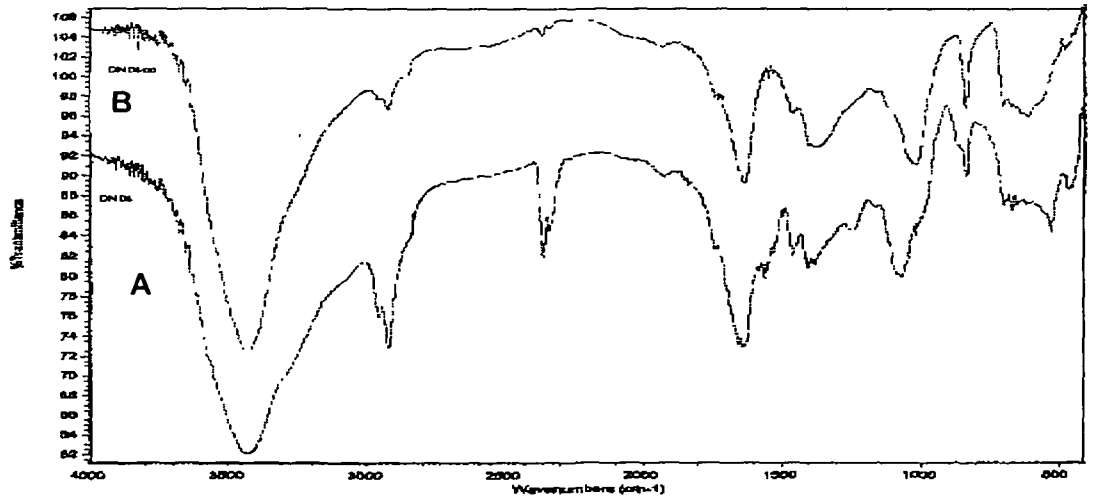

Fig 5.21. A comparison of IR spectra of crude lipopeptide isolated from *B. subtilis* DM-04 grown on 2% (w/v) glucose and 2% (w/v) potato peels.

A: biosurfactants from *B. subtilis* DM-04 when grown on glucose.

B: biosurfactants from *B. subtilis* DM-04 when grown on potato peels.

5.1.8.3 Characterization of biosurfactants from *B. subtilis* strains grew on potato peel as sole carbon source

Chemical analysis showed that *B. subtilis* biosurfactants were lipopeptide in nature with a protein and lipids contents of 15.56 % and 20.15 % respectively for *B. subtilis* DM-03 strain and 17.87 % and 18.20 % respectively for *B. subtilis* DM-04 strain.

Emulsification index E_{24} was measured as 55 % and 66 % respectively for *B. subtilis* DM-03 and DM-04 and could release 52 ± 1.0 % and 65 ± 1.5 % kerosene oil from sand pack column, respectively.

IR spectra of isolated crude biosurfactants from *B. subtilis* strains grown on potato peel and glucose did not exhibit any major difference. The intensity of the spectra around 2361 cm^{-1} and around 2900 cm^{-1} in the crude biosurfactants obtained when strains were grown in glucose were missing in isolated crude biosurfactants of either strains grown on potato peel. However other spectral peaks of biosurfactants were identical, irrespective of their culture in these two different carbon sources (Fig.5.20 and 5.21).

CHAPTER VI

PRODUCTION, ISOLATION AND CHARACTERIZATION OF BIOSURFACTANTS PRODUCED BY *P. aeruginosa* STRAINS

6.1 Results

6.1.1 Optimization of culture parameters for maximum yield of biosurfactants

6.1.1.1 Growth characteristics and biosurfactants production

Maximum reduction in surface tension was achieved post 96 hrs (stationary phase) of microbial growth. At this stage, surface tension of culture medium was reduced from 68.0 mN/m to 27.0 ± 0.3 mN/m and 29.0 ± 0.5 mN/m respectively by *P. aeruginosa* M and NM strains (Table 6.1). M9 media was found optimum for growth and biosurfactants production by both the *P. aeruginosa* strains. Optimization of M9 salts is described below. Two other media described by Goswami and Singh, (1991) and Banat, (1993) (composition given in Appendix) were tried, however these were discarded due to poor growth of the microorganisms in these media.

Table 6.1. Growth and biosurfactants production by *P. aeruginosa* strains at different time intervals. Data represents the mean ± S.D of four individual experiments.

Time (in h)	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0	Surface tension (mN/m)	68.0± 0.2	68.0± 0.2
	Dry biomass (g/l)	0.0	0.0
24	Surface tension (mN/m)	40.8 ± 0.8	41.5 ± 0.5
	Dry biomass (g/l)	1.0 ± 0.05	0.9 ± 0.08
48	Surface tension (mN/m)	35.0 ± 0.3	36.0± 0.2
	Dry biomass (g/l)	1.8 ± 0.09	1.7 ± 0.05
72	Surface tension (mN/m)	29.0 ± 0.2	33.0 ± 0.8
	Dry biomass (g/l)	2.4 ± 0.1	2.2 ± 0.08
96	Surface tension (mN/m)	27.0± 0.3	29.0 ± 0.5
	Dry biomass (g/l)	2.4 ± 0.1	2.2 ± 0.15
120	Surface tension (mN/m)	30.0 ± 0.8	33.0 ± 1.0
	Dry biomass (g/l)	1.9 ± 0.1	1.75 ± 0.15

6.1.1.2 Role of carbon sources on growth and biosurfactants production

As shown in Table 6.2, both the *P. aeruginosa* strains showed maximum growth and biosurfactants production when glycerol followed by glucose were supplemented as the carbon source. The production of biosurfactants by *P. aeruginosa* M strain was much higher than that of *P. aeruginosa* NM strain (6.4g/l Vs 5.0 g/l) under the same experimental condition (Table 6.2). Among the tested carbohydrates, starch served as the worst carbon source for bacterial growth and biosurfactants production. However, *Pseudomonas* strains under the study failed to produce appreciable amount of biosurfactants by utilizing unconventional carbon sources like molasses and potato peels (Table 6.2).

Table 6.2. Effect of carbon sources on biosurfactants production and growth of *P. aeruginosa* M and NM strains. Results recorded post 96 h of incubation. Data represents the mean ± S.D of three independent experiments.

Carbon source	Reduction in surface tension (Δ mN/m*)		Yield of crude biosurfactants (g/l)		Dry biomass (g/l)	
	P. M strain	*P.* NM strain	*P* . M strain	*P* . NM strain	*P* . M strain	*P* . NM strain
Glycerol	41.0 ± 0.3	39.0 ±0.2	6.49 ± 0.1	5.0 ± 0.15	2.4 ± 0.1	2.2 ± 0.2
Glucose	33.0 ± 0.2	32.0 ±0.4	3.68 ± 0.3	3.85 ± 0.25	2.9 ± 0.1	2.8 ± 0.2
Molasses	16.1 ± 0.3	14.7 ± 0.5	3.0 ± 0.1	2.27 ± 0.2	2.5 ± 0.3	2.35 ± 0.5
Potato peels	10.5 ± 0.5	0.9 ± 0.3	0.8 ± 0.05	0.7 ± 0.2	0.6 ± 0.1	0.5 ± 0.1
Starch	10.5 ± 0.4	10.0 ± 0.3	0.6 ± 0.06	0.4 ± 0.05	2.1 ± 0.1	2.0 ± 0.15
Octane	13.2 ± 0.2	13.2 ± 0.3	0.4 ± 0.05	0.4 ±0.1	1.25 ± 0.25	1.1 ± 0.1
Dodecane	33.2 ± 0.5	32.5 ± 0.2	0.55 ± 0.04	0.3 ± 0.07	1.2 ± 0.2	1.0 ± 0.1
Hexadecane	36.4 ± 0.3	36.5 ± 0.5	2.68 ± 0.1	2.08 ± 0.15	1.05 ± 0.05	1.0 ± 0.1
Octadecane	35.2 ± 0.5	34.2 ± 0.4	0.28 ± 0.08	0.3 ±0.05	1.05 ± 0.05	1.0 ± 0.1
Liquid light paraffin	16.8 ± 0.6	16.3 ± 0.2	0.6 ± 0.03	0.35 ± 0.05	1.09 ± 0.01	1.01 ± 0.01
Petrol	7.3 ± 0.5	6.7 ± 0.5	0.9 ±0.1	0.8 ± 0.1	0.81 ± 0.01	0.75 ± 0.05
Diesel	10.8 ± 0.7	10.5 ± 0.6	0.95 ± 0.05	0.88 ± 0.07	0.88 ± 0.02	0.8 ± 0.06
Kerosene	19.5 ± 0.5	19.1 ± 0.3	1.0 ± 0.1	0.9 ± 0.1	0.91 ± 0.01	0.86 ± 0.05
Anthracene	38.9 ± 0.6	28.9 ± 0.5	0.2 ± 0.05	0.6 ± 0.2	0.95 ± 0.05	0.9 ± 0.1
Pyrene	21.5 ± 0.3	22.4 ± 0.7	0.2 ± 0.05	0.24 ± 0.06	0.78 ± 0.02	0.73 ± 0.05
Phenanthrene	14.9 ± 0.7	15.5 ± 0.3	0.18 ± 0.02	0.17 ± 0.03	0.75 ± 0.05	0.7 ± 0.05

*It is the reduction in surface tension compared to respective growth medium (control) only.

Table 6.3. Effect of different concentrations of glycerol on growth and biosurfactants production by the *P. aeruginosa* strains. *P. aeruginosa* strains were grown at different concentrations of glycerol at 45°C temperature for 96 h, followed by assaying the surface tension reduction of growth medium and measuring the bacterial growth. Values represent the mean ± S.D of three individual experiments.

Percent of Glycerol (v/v)	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0.5	Surface tension (mN/m)	31.0 ± 0.5	31.8 ± 0.6
	CMD^{-1} (mN/m)	34.0 ± 0.5	35.8 ± 0.3
	Dry biomass (g/l)	1.7 ± 0.3	1.65 ± 0.3
1.0	Surface tension (mN/m)	28.0 ± 0.2	30.0 ± 0.5
	CMD^{-1} (mN/m)	31.6 ± 0.5	32.0 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.7 ± 0.5
2.0	Surface tension (mN/m)	27.3 ± 0.3	29.2 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.3	1.8 ± 0.3
3.0	Surface tension (mN/m)	32.0 ± 0.5	33.6 ± 0.6
	CMD^{-1} (mN/m)	34.9 ± 0.6	36.6 ± 0.3
	Dry biomass (g/l)	2.1 ± 0.3	2.0 ± 0.3

As shown in Table 6.3, glycerol has a dose- dependent effect on the growth of and biosurfactants production by *P. aeruginosa* strains, but optimum growth and biosurfactants production were observed at 2% (v/v) glycerol concentration.

6.1.1.3 Effect of various nitrogen sources on biosurfactants production

Type of nitrogen source has played an important role in the growth of and biosurfactants production by both the *P. aeruginosa* strains in the present study. Both the strains exhibited poor growth with least biosurfactants production in nitrogen-depleted medium (Fig. 6.1). Inorganic nitrogen sources were much better compared to organic nitrogen sources for optimum bacterial growth. Both the *P. aeruginosa* strains exhibited preference for ammonium chloride for growth and biosurfactants productions (Figs 6.1 & 6.2), but they showed comparatively less biosurfactants production when potassium nitrate was used as the nitrogenous source. Although, organic nitrogen sources like beef extract, yeast extract, peptone and tryptone stimulate the growth of the cells but interestingly, these nitrogen sources failed to induce significant biosurfactants production (Figs 6.1 & 6.2).

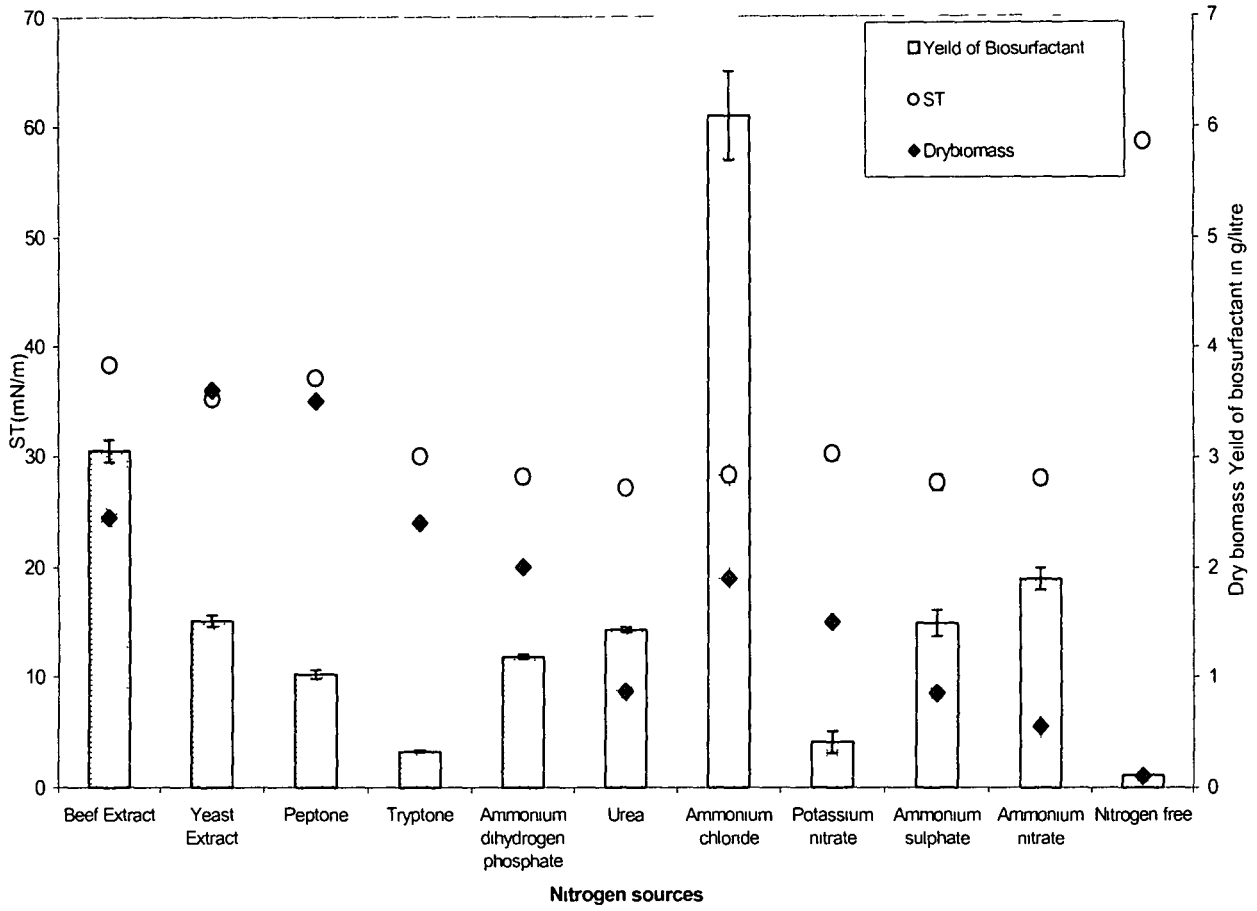

Fig. 6.1. Influence of various nitrogen sources on growth of *P. aeruginosa* M strain and biosurfactants production. Values are mean ± S D of three determinations

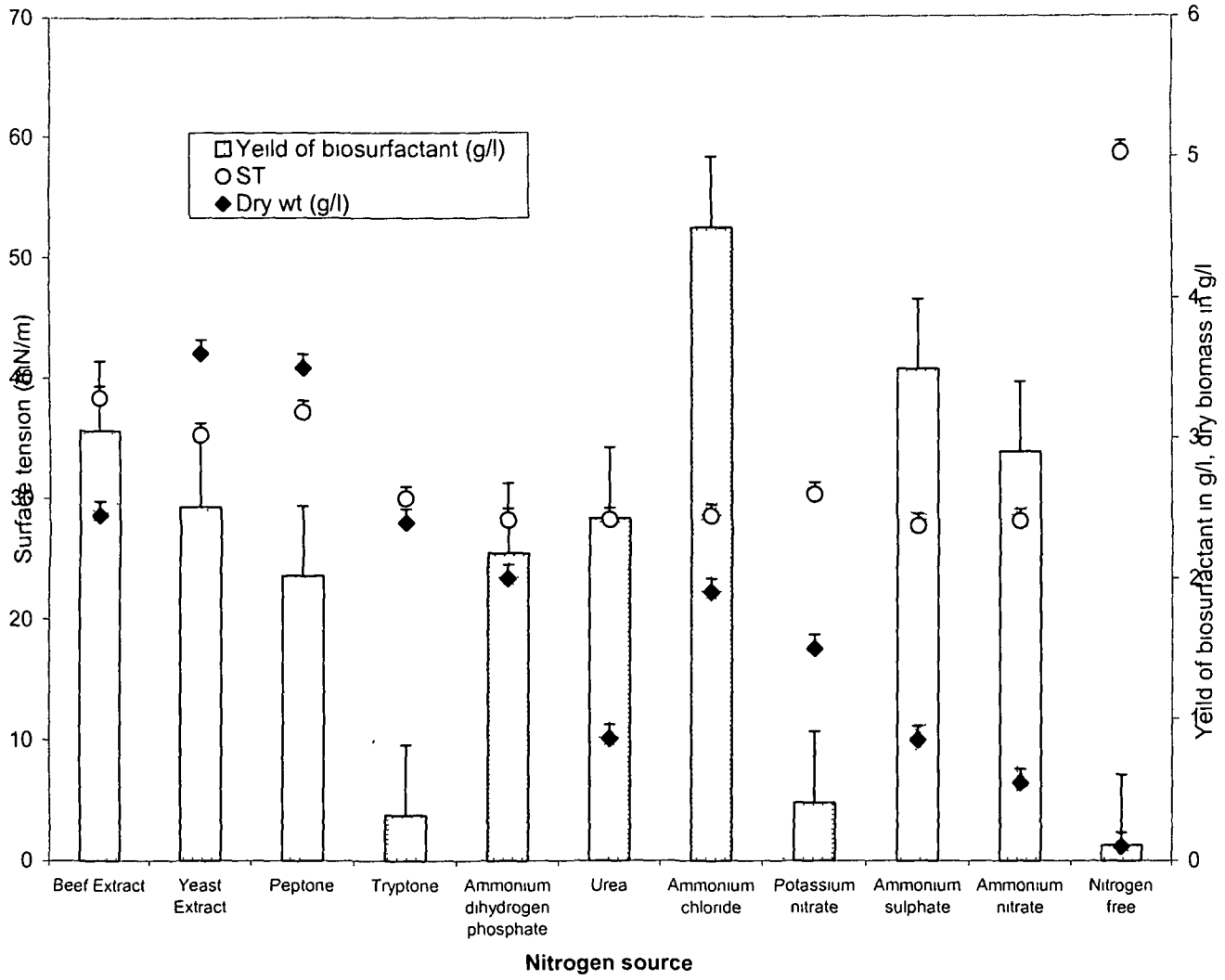

Fig. 6.2. Influence of various nitrogen sources on growth of *P. aeruginosa* NM strain and biosurfactants production. Values are mean ± S.D of three determinations.

Table6.4. Influence of different concentration of NH_4Cl source on growth and biosurfactants production by *Pseudomonas* strains. Results recorded post 96 h of growth of *Pseudomonas* strains on glycerol. Values represent mean ± S.D of three individual experiments.

Conc. of NH_4Cl (g %)	Properties	*P. aeruginosa* M strain	*P. aeruginosa* NM strain
0.05	Surface tension (mN/m)	33.0 ± 0.5	35.0 ± 0.5
	CMD^{-1} (mN/m)	35.0 ± 0.5	37.5 ± 0.3
	Dry biomass (g/l)	1.6 ± 0.3	1.5 ± 0.3
0.1	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.3	2.0 ± 0.3
0.2	Surface tension (mN/m)	31.8 ± 0.5	33.0 ± 0.5
	CMD^{-1} (mN/m)	36.0 ± 0.6	37.5 ± 0.5
	Dry biomass (g/l)	2.1 ± 0.4	1.85 ± 0.5
0.4	Surface tension (mN/m)	32.0 ± 0.3	34.5 ± 0.6
	CMD^{-1} (mN/m)	34.0 ± 0.6	36.6 ± 0.5
	Dry biomass (g/l)	2.0 ± 0.4	1.7 ± 0.5

Dose dependent study showed that 0.1% (g%) of ammonium chloride served as the best concentration for growth of and biosurfactants production by the *P. aeruginosa* strains (Table 6.4).

6.1.1.4 Influence of metal ions on biosurfactants production

Metal ions have an important role on influencing the surface-active properties of the microorganisms. 0.246 g/l of $MgSO_4.7H_2O$; 0.14 g/l of $CaCl_2$; 3.0g/l of KH_2PO_4, 6.0 g/l of Na_2HPO_4 and 0.5 g/l of NaCl were observed as the optimum concentration of metal ions for maximum yield of biosurfactants (Table6.5-6.9).

Table 6.5. Influence of magnesium chloride on growth and biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources Values represent mean ± S.D of three individual experiments.

Conc. of $MgCl_2.7H_20$ (g/l)	Properties	*P. aeruginosa* M strain	*P. aeruginosa* NM strain
0	Surface tension (mN/m)	38.5 ± 0.5	39.8 ± 0.4
	CMD^{-1} (mN/m)	44.0 ± 0.5	45.2 ±0.3
	Dry biomass (g/l)	1.6 ± 0.5	1.45 ± 0.4
0.1	Surface tension (mN/m)	27.8 ± 0.3	30.2 ± 0.5
	CMD^{-1} (mN/m)	32.5 ± 0.5	32.9 ± 0.3
	Dry biomass (g/l)	1.9 ±0.3	1.75 ± 0.3
0.25	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ±0.3
	Dry biomass (g/l)	2.1 ±0.3	2.0 ± 0.3
0.3	Surface tension (mN/m)	35.5 ± 0.5	35.8 ± 0.4
	CMD^{-1} (mN/m)	39.0 ± 0.5	40.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.5	1.82 ± 0.4

Table 6.6. Influence of calcium chloride on growth and biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of $CaCl_2$ (mg/l)	Properties	*P. aeruginosa* M strain	*P. aeruginosa* NM strain
0	Surface tension (mN/m)	38.5 ± 0.5	39.8 ± 0.4
	CMD^{-1} (mN/m)	44.0 ± 0.5	45.2 ±0.3
	Dry biomass (g/l)	1.6 ± 0.5	1.45 ± 0.4
7.0	Surface tension (mN/m)	28.5 ± 0.5	30.0 ± 0.5
	CMD^{-1} (mN/m)	33.0 ± 0.6	34.2 ±0.5
	Dry biomass (g/l)	2.0 ±0.3	1.8 ± 0.3
14.0	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ±0.3
	Dry biomass (g/l)	2.1 ±0.3	2.0 ± 0.3
21.0	Surface tension (mN/m)	34.5 ± 0.5	34.9 ± 0.4
	CMD^{-1} (mN/m)	39.3 ± 0.5	40.8 ±0.3
	Dry biomass (g/l)	1.9 ±0.5	1.75 ± 0.4

Table 6.7. Influence of KH_2PO_4 on growth and biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of KH_2PO_4 (g/l)	Properties	*P. aeruginosa* M strain	*P. aeruginosa* NM strain
0	Surface tension (mN/m)	36.5 ± 0.5	37.3 ± 0.4
	CMD^{-1} (mN/m)	40.1 ± 0.4	41.3 ±0.2
	Dry biomass (g/l)	1.8 ± 0.2	1.6 ± 0.5
1.5	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ±0.5
	Dry biomass (g/l)	1.9 ±0.15	1.75 ± 0.05
3.0	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ±0.3
	Dry biomass (g/l)	2.4 ±0.1	2.2 ± 0.2
4.5	Surface tension (mN/m)	29.3 ± 0.3	33.3 ± 0.6
	CMD^{-1} (mN/m)	34.0 ± 0.5	36.5 ±0.3
	Dry biomass (g/l)	2.3 ±0.08	2.1 ± 0.1

Table 6.8. Influence of Na_2HPO_4 on growth and biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of Na_2HPO_4 (g/l)	Properties	*P. aeruginosa* M strain	*P. aeruginosa* NM strain
0	Surface tension (mN/m)	36.5 ± 0.5	37.3 ± 0.4
	CMD^{-1} (mN/m)	40.1 ± 0.4	41.3 ± 0.2
	Dry biomass (g/l)	1.9 ± 0.05	1.75 ± 0.1
3.0	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.6 ± 0.05
6.0	Surface tension (mN/m)	27.1 ± 0.3	29.2 ± 0.5
	CMD^{-1} (mN/m)	30.7 ± 0.5	33.2 ± 0.3
	Dry biomass (g/l)	2.4 ± 0.1	2.2 ± 0.05
8.0	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ± 0.5
	Dry biomass (g/l)	2.1 ± 0.2	1.95 ± 0.5

Table 6.9. Influence of NaCl on growth and biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of NaCl (g/l)	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ±0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.6 ± 0.5
0.25	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	31.5 ±0.3
	Dry biomass (g/l)	2.0 ±0.3	1.8 ± 0.3
0.5	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ±0.3
	Dry biomass (g/l)	2.0 ±0.3	2.0 ± 0.3
1.0	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	31.5 ±0.3
	Dry biomass (g/l)	2.0 ±0.3	1.8 ± 0.3

6.1.1.5 Influence of growth supplement (Thiamine-HCl) on biosurfactants production

Bacteria grown on control medium (with no Thiamine-HCl solution) showed comparatively less growth and poor surface activities compared to *P. aeruginosa* M and NM strains supplied with Thiamine-HCl. 1ml/l of Thiamine-HCl was found to be optimum for the *P. aeruginosa* strains for maximum growth and biosurfactants production (Table 6.10)

6.1.1.6 Effect of trace elements solution on biosurfactants production

Trace elements played a significant role in growth of and biosurfactants production by bacteria under study as it was evident from the experiment with control (no trace element). 1 ml/l was observed as optimum for all the bacterial strains for maximum growth and biosurfactants production (Table 6.11).

Table 6.10. Influence of growth supplement (Thiamine-HCl) on biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of Thiamine- HCl (ml/l)*	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0.0	Surface tension (mN/m)	36.5 ± 0.5	37.3 ± 0.4
	CMD^{-1} (mN/m)	40.1 ± 0.4	41.3 ± 0.2
	Dry biomass (g/l)	1.5 ± 0.5	1.5 ± 0.4
0.5	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.7 ± 0.5
1.0	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.3	2.0 ± 0.3
1.5	Surface tension (mN/m)	27.9 ± 0.3	30.5 ± 0.5
	CMD^{-1} (mN/m)	32.0 ± 0.5	34.5 ± 0.3
	Dry biomass (g/l)	2.1 ± 0.3	2.0 ± 0.3

** Stock solution of Thiamine-HCl was prepared as described in the section 3.6.7.

Table6.11. Influence of trace elements on biosurfactants production. Results recorded post 96 h of growth of the strains in presence of glycerol and ammonium chloride as the respective carbon and nitrogen sources. Values represent mean ± S.D of three individual experiments.

Concentration of micronutrients (ml/l)*	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0.0	Surface tension (mN/m)	40.5 ± 0.5	42.3 ± 0.4
	CMD^{-1} (mN/m)	54.0 ± 0.5	55.5 ± 0.3
	Dry biomass (g/l)	1.5 ± 0.5	1.5 ± 0.4
1.0	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.3	2.0 ± 0.3
2.0	Surface tension (mN/m)	31.5 ± 0.5	32.5 ± 0.4
	CMD^{-1} (mN/m)	36.1 ± 0.6	37.5 ± 0.4
	Dry biomass (g/l)	1.9 ± 0.2	1.7 ± 0.5
4.0	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.6 ± 0.5

* Stock solution of micronutrients was prepared as described in the section 3.6.6.

6.1.1.7 Effect of agitation on biosurfactants production

Agitation of the culture flasks was very much important for the growth and biosurfactants production by microbes. Static culture, i.e., the negative control (agitation was 0rpm) had poor cell growth and exhibited poor surface activities. Agitation at 200 rpm was recorded as optimum condition for both the *Pseudomonas* strains in the study (Table 6.12).

Table6.12. Influence of agitation on biosurfactants production. Results recorded post 96 h of growth of the *Pseudomonas* strains. Agitation was achieved by incubating the cultures in an incubator and then shaking at various rpm. Values represent mean ± S.D of three individual experiments.

Agitation (rpm)	Properties	*P. aeruginosa* M	*P. aeruginosa* NM
0	Surface tension (mN/m)	48.5 ± 0.5	50.3 ± 0.4
	CMD^{-1} (mN/m)	58.0 ± 0.5	61.5 ± 0.3
	Dry biomass (g/l)	1.0 ± 0.5	1.5 ± 0.4
100	Surface tension (mN/m)	32.5 ± 0.5	34.0 ± 0.3
	CMD^{-1} (mN/m)	37.5 ± 0.6	39.5 ± 0.5
	Dry biomass (g/l)	1.8 ± 0.2	1.6 ± 0.5
200	Surface tension (mN/m)	27.3 ± 0.3	29.5 ± 0.5
	CMD^{-1} (mN/m)	31.0 ± 0.5	33.5 ± 0.3
	Dry biomass (g/l)	2.0 ± 0.3	2.0 ± 0.3
300	Surface tension (mN/m)	29.3 ± 0.3	33.3 ± 0.6
	CMD^{-1} (mN/m)	34.0 ± 0.5	36.5 ± 0.3
	Dry biomass (g/l)	1.9 ± 0.3	1.8 ± 0.3

6.1.1.8 Influence of pH and temperature on growth and biosurfactants production

As shown in Tables 6.13 and 6.14, both the *P. aeruginosa* strains could grow and produce biosurfactants in a wide range of pH (3.0-12.0) and temperature ranging from 30°C-50°C. Both the bacterial strains exhibited better growth and biosurfactants production in the alkaline pH as compared to the acidic pH (Table 6.13). Although, these strains have the capacity to grow and produce biosurfactants in a wide range of temperature, maximum growth and biosurfactants production were achieved when cultures were incubated at 45°C temperature. Increasing the temperature beyond 45°C, growth rate was declined with a concomitant reduction in biosurfactants production by both the *P. aeruginosa* strains.

Table 6.13. Influence of different pH and temperature on growth and surface activities of *P. aeruginosa* M strain. Results recorded post 96 h of bacterial growth. Results represent mean ± S.D of three independent experiments.

Temperature (°C)	pH	Surface tension reduction (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	Dry biomass (g/l)	Yield of biosurfactants (g/l)
30	3.0	54.5 ± 1.0	63.5 ± 0.5	68.3 ± 1.0	0.35 ± 0.08	1.0 ± 0.05
	4.0	53.6 ± 0.5	62.0 ± 0.4	68.0 ± 0.8	0.65 ± 0.05	1.2 ± 0.06
	6.0	47.7 ± 0.7	60.0 ± 0.6	65.0 ± 0.7	1.5 ± 0.05	1.6 ± 0.09
	7.0	27.5 ± 0.5	31.0 ± 1.0	45.0 ± 0.8	1.85 ± 0.1	3.77 ± 0.08
	8.0	28.0 ± 0.7	32.5 ± 0.9	46.0 ± 0.5	1.7 ± 0.05	3.52 ± 0.1
	10.0	29.0 ± 0.5	26.5 ± 0.5	37.3 ± 0.7	1.6 ± 0.07	3.45 ± 0.1
	12.0	54.5 ± 0.5	63.2 ± 0.8	65.5 ± 0.8	0.5 ± 0.04	1.8 ± 0.05
45	3.0	54.0 ± 0.8	62.0 ± 0.5	68.0 ± 0.5	0.4 ± 0.05	1.1 ± 0.04
	4.0	53.0 ± 0. 5	61.5 ± 0.5	67.3 ± 0.7	0.75 ± 0.06	2.5 ± 0.05
	6.0	47.0 ± 0.5	55.0 ± 1.0	63.0 ± 0.5	1.8 ± 0.08	3.5 ± 0.5
	7.0	27.0 ± 0.3	30.4 ± 0.2	40.0 ± 0.5	2.4 ± 0.2	6.49 ± 0.1
	8.0	28.0 ± 0.5	32.0 ± 0.5	44.0 ± 0.8	1.9 ± 0.05	3.98 ± 0.6
	10.0	30.0 ± 0.6	44.3 ± 0.7	44.5 ± 1.0	1.05 ± 0.04	2.31 ± 0.04
	12.0	48.5 ± 1.0	60.0 ± 1.0	62.0 ± 1.0	1.2 ± 0.05	2.0 ± 0.08
50	3.0	62.0 ± 1.0	69.5 ± 1.0	71.2 ± 1.0	0.3 ± 0.05	0.85 ± 0.05
	4.0	56.0 ± 0.8	68.0 ± 1.5	70.5 ± 0.8	0.45 ± 0.05	1.0± 0.05
	6.0	49.0 ± 0.7	63.5 ± 1.0	68.7 ± 0.7	1.1 ± 0.04	1.3 ± 0.1
	7.0	45.0 ± 0.5	62.0 ± 1.0	66.0 ± 1.0	1.2 ± 0.2	2.0 ± 0.1
	8.0	48.0 ± 0.9	63.0 ± 0.9	68.0 ± 1.5	1.0 ± 0.09	1.9± 0.1
	10.0	48.5 ± 1.0	63.5 ± 0.8	68.9 ± 1.0	0.9 ± 0.05	1.9 ± 0.05
	12.0	50.0 ± 1.5	64.0 ± 1.0	70.0 ± 1.0	0.6 ± 0.09	1.8 ± 0.05

Table 6.14. Influence of different pH and temperature on growth and surface activities of *P. aeruginosa* NM strain. Results recorded post 96 h incubation. Results represent mean ± S.D of three independent experiments.

Temperature (C)	pH	Surface tension reduction (mN/m)	CMD^{-1} (mN/m)	CMD^{-2} (mN/m)	Dry biomass (g/l)	Yield of biosurfactants (g/l)
30	3.0	59.7 ± 1.3	68.0 ± 0.8	68.5 ± 1.0	0.32 ± 0.08	0.9 ± 0.04
	4.0	60.9 ± 0.6	62.0 ± 0.4	68.1 ± 0.9	0.45 ± 0.05	1.0 ± 0.05
	6.0	49.0 ± 0.5	61.0 ± 0.5	67.0 ± 0.8	1.3 ± 0.05	1.5 ± 0.05
	7.0	30.5 ± 0.5	36.0 ± 0.5	50.0 ± 0.9	1.8 ± 0.09	4.0 ± 0.2
	8.0	31.5 ± 0.5	37.8 ± 0.4	56.0 ± 0.5	1.7 ± 0.05	3.6 ± 0.15
	10.0	31.6 ± 0.4	40.0 ± 0.5	60.0 ± 0.5	1.5 ± 0.05	2.9 ± 0.1
	12.0	54.5 ± 0.5	63.2 ± 0.8	65.5 ± 0.8	0.5 ± 0.04	0.8 ± 0.05
45	3.0	59.0 ± 0.8	67.7 ± 0.3	68.0 ± 0.5	0.42 ± 0.05	1.0 ± 0.05
	4.0	55.5 ± 0. 5	66.5 ± 0.5	67.5 ± 1.0	0.45 ± 0.05	2.0 ± 0.05
	6.0	46.0 ± 0.5	56.0 ± 1.0	65.0 ± 0.5	1.5 ± 0.05	2.5 ± 0.5
	7.0	29.0 ± 0.5	33.0 ± 0.4	43.0 ± 0.5	2.2 ± 0.15	5.0 ± 0.1
	8.0	29.5 ± 0.6	33.7 ± 0.3	44.0 ± 0.8	1.85 ± 0.05	4.0 ± 0.5
	10.0	31.4 ± 0.6	37.6 ± 0.4	50.5 ± 1.0	1.51 ± 0.09	3.5 ± 0.05
	12.0	48.5 ± 1.0	62.2 ± 1.8	69.0 ± 0.5	1.0 ± 0.05	1.0 ± 0.08
50	3.0	64.0 ± 0.5	69.5 ± 1.0	71.5 ± 0.5	0.25 ± 0.02	0.8 ± 0.05
	4.0	63.0 ± 0.5	69.0 ± 1.0	70.2 ± 0.3	0.40 ± 0.05	0.9± 0.05
	6.0	51.8 ± 0.4	65.5 ± 0.5	67.2 ± 0.8	0.63 ± 0.07	1.0 ± 0.05
	7.0	48.0 ± 0.5	62.5 ± 1.0	66.0 ± 0.5	0.9 ± 0.1	1.5 ± 0.05
	8.0	48.8 ± 0.4	64.0 ± 0.5	67.0 ± 0.5	0.8 ± 0.05	1.3± 0.1
	10.0	54.4 ± 0.6	66.0 ± 0.5	68.5 ± 1.0	0.85 ± 0.05	1.0 ± 0.05
	12.0	63.5 ± 1.0	69.2 ± 0.8	71.5 ± 1.0	0.55 ± 0.05	0.8 ± 0.06

6.1.1.9 Biosurfactants production under saline condition

As shown in Table 6.15, both the *Pseudomonas* strains possess the ability to produce biosurfactants under saline condition. The *Pseudomonas* strains could grow and produce biosurfactants at a salt (NaCl) concentration ranging from .05% to 5.0 % (w/v). However, the production of biosurfactants decreased dramatically when bacteria were grew at a NaCl concentration greater than 5.0 % (w/v)

Table 6.15. Growth of and biosurfactants production by *Pseudomonas* strains when grown on different saline (NaCl) concentrations. Results are from a typical experiment. Other experiments produced identical results.

Bacterial strains	Nacl (g%)	Properties			
		Surface tension of growth medium (mN/m)	CMD^{-1} (mN/m)	Dry weight (g/l)	Yield of biosurfactants (g/l)
P. aeruginosa M	0.5	28.0	33.0	2.0	6.0
	1	33.0	35.0	1.8	5.0
	3	40.0	50.5	1.7	4.8
	4	48.0	58.3	1.5	4.5
	5	50	62.8	1.0	3.0
	7	65.0	70.1	0.4	ND
P. aeruginosa NM	0.5	30.0	33.8	1.9	4.3
	1	30.5	35.5	1.8	4.0
	3	41.6	51.5	1.5	3.8
	4	49.0	60.0	1.4	3.5
	5	52.5	65.2	1.0	2.7
	7	66.9	71.0	0.3	ND

ND= could not be determined due to extremely low yield

6.1.2 Isolation of crude biosurfactants

The production of biosurfactants by *P. aeruginosa* strains was directly proportional to the growth of bacteria because biosurfactants production did not start before 24 hours of growth (Table 6.1). At stationary phase, biosurfactants production was independent of bacterial growth rate and maximum yield of biosurfactants occurred at this stage. About 6.49g and 5.0 g of crude biosurfactants were isolated by acetone-HCl precipitation from one litre stationary phase (96 h) culture of *P. aeruginosa* M and NM strains when grown at 45°C temperature with glycerol as the carbon source (Table 6.2).

While separating biomass from culture broth, the surface activity of different fractions was tested. In case of water immiscible substrate the recovered residual hydrocarbons were tested for surface activity, but negative result was confirmed by tensiometer. The clumping biomass which was recovered by centrifugation or from the interface of aqueous and oil layers after solvent washing and drying, it was resuspended in distilled water and surface activity of this water was tested, and the biomass also did not show any significant surface tension reduction ability. The slight reduction in surface tension of water could be caused by the bacterial cell surface itself (Table 6.16). Since, the biomass after centrifugation did not show any surface activity, suggesting that the biosurfactants production by the strains was completely extracellular. The aqueous phase, which was cell and oil free, showed both surface and interfacial activity and acidification of which resulted in the precipitation of biosurfactants.

Table 6.16 Summary of isolation of biosurfactants from *P. aeruginosa* M and NM strains. Data are from a typical experiment.

Fractions	Surface tension reduction (mN/m)	Yield of biosurfactants (mg)
***P. aeruginosa* M**		
Cell free extract	28.5	-
Biomass	53.0	-
Acetone added cell free supernatant	28.0	-
Acidified crude biosurfactants	28.0	80.0
HPLC fractions		
Pa M1	39.0	4.0
Pa M2	38.0	2.6
Pa M3	27.0	1.8
***P. aeruginosa* NM**		
Cell free extract	29.5	-
Biomass	55.0	-
Acetone added Cell free supernatant	29.0	-
Acidified crude biosurfactants	29.0	85.0
HPLC fractions		
Pa NM1	28.0	4.0
Pa NM2	35.0	2.4

6.1.3 Purification of biosurfactants

Fractionation of crude biosurfactants from *P. aeruginosa* M and NM strains by reverse-phase (RP)-HPLC on a C_{18} column resolved them into three and two peaks respectively (Figs.6.3 and 6.4). When individual peak was tested for surface active properties, peaks Pa M3 and Pa NM1 with retention time of 1.45 min and 0.96 min respectively, exhibited maximum biosurfactants activity (Table 6.16).

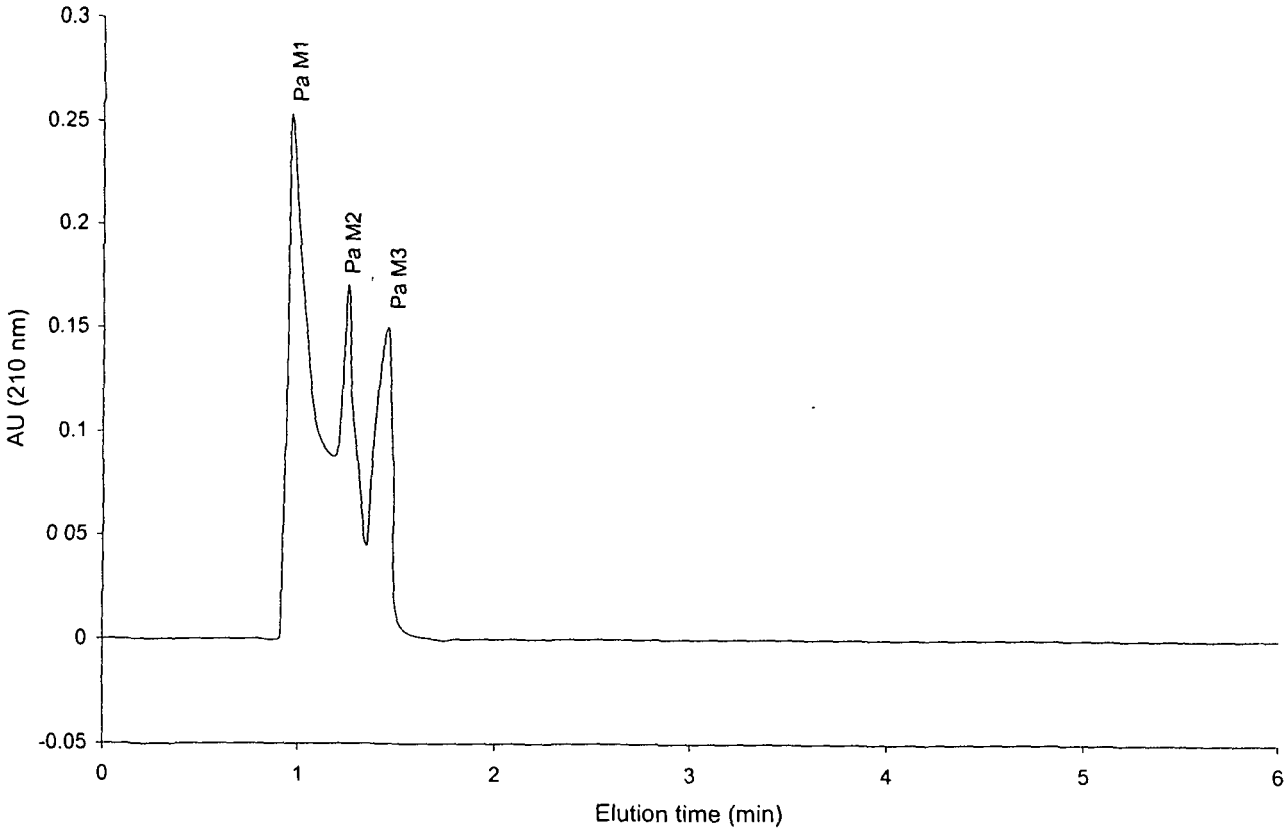

Fig. 6.3. RP-HPLC of crude biosurfactants from *P. aeruginosa* M strain on a Waters C_{18}- µ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate, pH6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min and monitored at 210 nm.

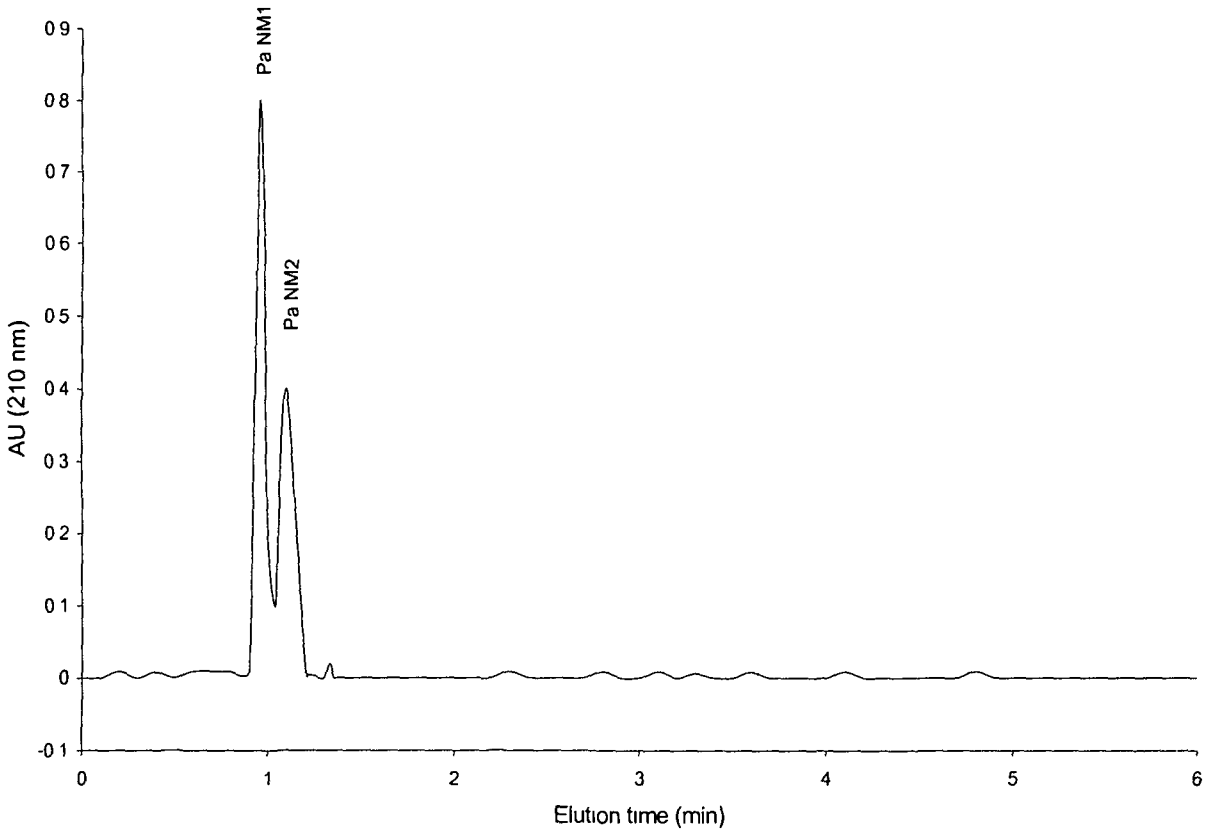

Fig.6.4. RP-HPLC of crude biosurfactants from *P. aeruginosa* NM strain on a Waters C_{18}- µ Nova pak RP-HPLC column. Buffer A was 10 mM potassium phosphate, pH6.0 and buffer B was methanol. Elution was performed at a flow rate of 1 ml/min and monitored at 210 nm.

6.1.4 Biochemical properties

6.1.4.1 Biochemical composition of biosurfactants

The biochemical composition of biosurfactants from *P. aeruginosa* strains is displayed in Table. 6.17. The carbohydrate, lipid and protein contents of biosurfactants vary depending upon the producing *Pseudomonas* strains.

Table 6.17. Biochemical composition of biosurfactants from the *P. aeruginosa* strains

Results represent mean ± S.D of four independent determinations.

Source of crude biosurfactants	Protein content (%)	Carbohydrate content (%)	Lipid content (%)
P. aeruginosa M strain	25.7 ± 0.2	41.0 ± 0.2	31.3 ± 0.1
P. aeruginosa NM strain	44.2 ± 0.1	30.0 ± 0.1	25.0 ± 0.1

6.1.4.2 Determination of critical micelle concentration of biosurfactants and chemical surfactant SDS

The CMC of biosurfactants from *P. aeruginosa* M and NM strains was found to be 80 mg/l and 110 mg/l respectively (Fig.6.5). Above the CMC, no further reduction in surface or interfacial tension was observed. The CMCs of the biosurfactants from both the *P. aeruginosa* strains were comparatively lower than that of the chemical surfactants SDS, the CMC value of which was detected as 2888.3 mg/l under identical experimental conditions (Fig.6.6). The surface tension of the crude biosurfactants from *P. aeruginosa* M and NM strains at CMC was recorded as 27.2 mN/m and 28.0 mN/m respectively and that of SDS was recorded as 33.5 mN/m.

At CMC, the crude biosurfactants from *P. aeruginosa* M and NM strains reduces the interfacial tension against hexadecane from 40 mN/m to 1.5 mN/m and 1.9 mN/ m respectively.

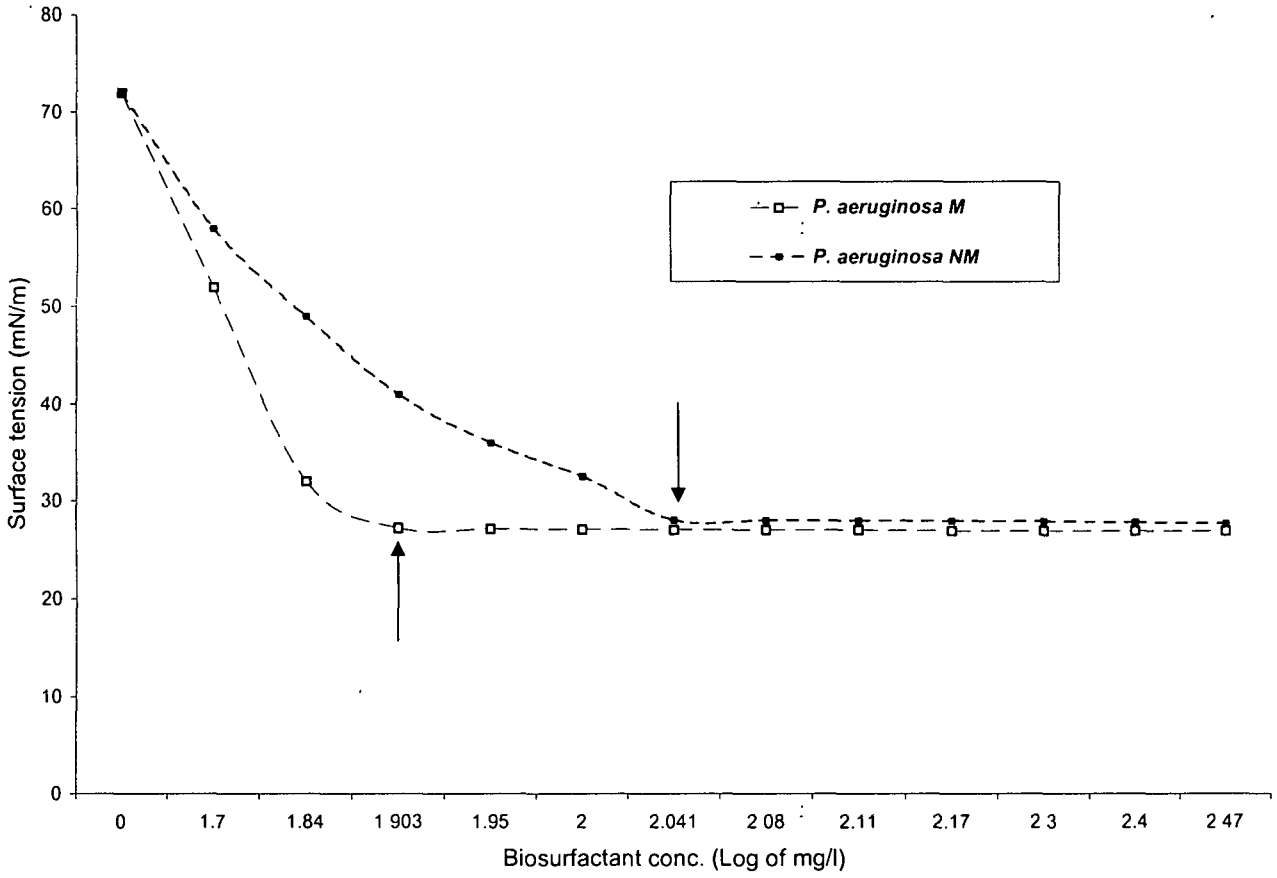

Fig.6.5. CMC of biosurfactants from *P. aeruginosa* M and NM. Results represent the mean of four individual experiments.

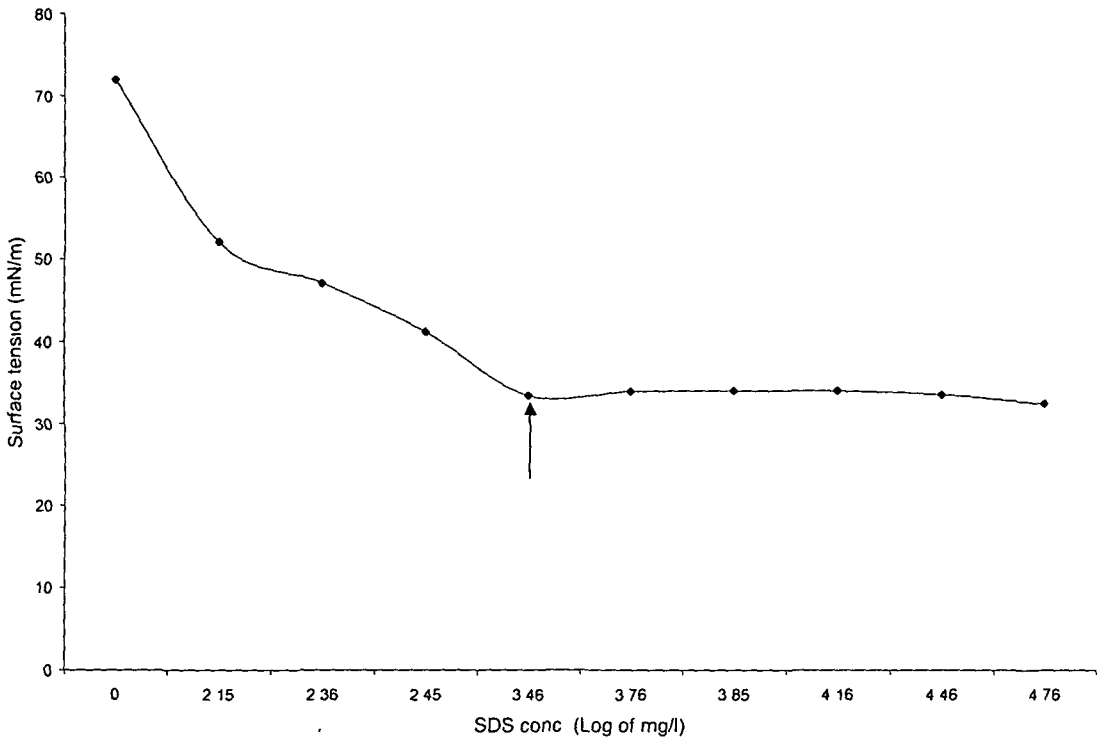

Fig.6.6. CMC of Sodium dodecyl sulphate (SDS). Results represent the mean of four independent determinations.

6.1.4.3 Heat and pH stability of biosurfactants

The crude biosurfactants from both the *P. aeruginosa* strains retained their surface active properties (95-96.6 % of original activity) post heating at 100°C for 60 min (Table 6.18). The biosurfactants activity was also retained in a wide range of pH ranging from 3.0- 11.0 (table 6.19).

Table 6.18. Thermostability of biosurfactants from *P. aeruginosa* M and NM strains. Results represent the mean ± S.D of four individual experiments.

Bacterial strains	Surface tension (mN/m)	Surface tension after heating (mN/m)	% activity remaining after heating
P. aeruginosa M	27.0 ± 0.5	28.0 ± 0.3	96.6
P. aeruginosa NM	28.0 ± 0.5	29.4 ± 0.5	95.0

Table 6.19. pH stability of biosurfactants from *P. aeruginosa* M and NM strains. Results are from a typical experiment.

Source of crude biosurfactants	Surface active properties at pH range											
	pH 3.0		pH 5.0		pH 6.0		pH 7.0		9.0		11.0	
	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}	ST	CMD^{-1}
P. aeruginosa M	33.5	36.0	30.0	33.5	28.5	32.0	27.5	31.0	29.0	33.0	32.0	34.5
P. aeruginosa NM	34.0	37.5	31.8	35.5	29.5	33.8	28.0	32.5	30.0	34.8	32.5	35.7

ST= Surface tension. Surface tension of water is 72 mN/m

6.1.4.4 Emulsification and emulsion stability

The emulsification index of biosurfactants from *P. aeruginosa* M and NM strains against kerosene was found to be 90% and 82% respectively (Table 6.20).

The emulsion formed by the crude biosurfactants from both the *Pseudomonas aeruginosa* strains under study were very stable as was evident from result of the decay constant (K_d). The biosurfactants from *P. aeruginosa* M was the most stable with a decay constant of –0.0031 followed by *P. aeruginosa* NM strain with K_d value of –0.0036 (Fig.6.7). The biosurfactants from the *P. aeruginosa* M and NM strains were 5.5 and 4.7 time respectively more stable than that of SDS, which had K_d value of –0.0172 (Fig.6.7).

Table 6.20. E_{24} of biosurfactants from *P. aeruginosa* strains. Results represent mean ± S.D of four individual experiments

Source of crude biosurfactants/ chemical surfactant	% Emulsification	Emulsion stability (K_d)
P. aeruginosa M	90 ± 1.0	-0.0031
P. aeruginosa NM	82 ± 2.0	-0.0036
SDS	ND	-0.0172

ND= not determined

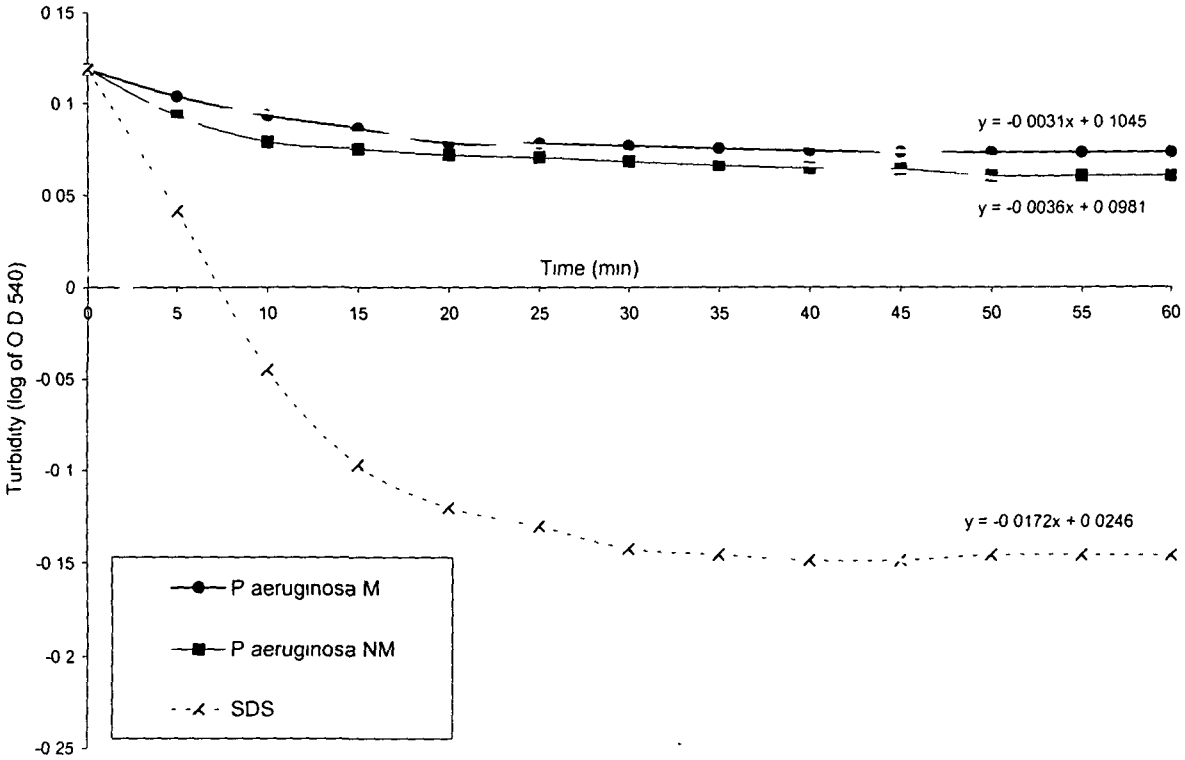

Fig.6.7. A comparison of emulsion stability of biosurfactants from *P. aeruginosa* M, NM strains and SDS. The equation of the line is given in the figure. The slope of the line is given in the equation of the line. Symbol used (♦): *P. aeruginosa* M (■); SDS (x)

6.1.4.5 Absorption maxima (λ_{max}) of crude biosurfactants

The λ_{max} for biosurfactants from *P. aeruginosa* M and NM strain was observed at 211 nm (Figure 6.8 and 6.9). Crude biosurfactants from either strain did not show absorption peak in any other wavelength of light.

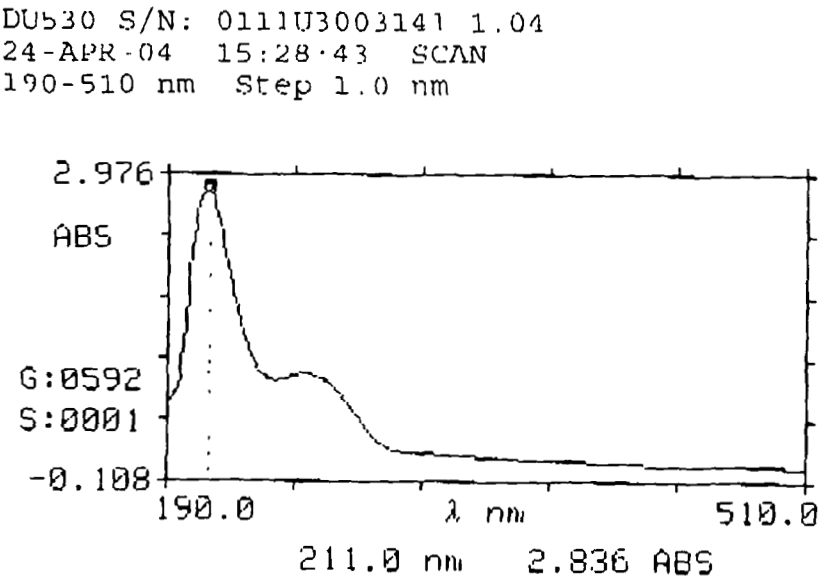

Fig.6.8. Absorption maxima (λ_{max}) of crude biosurfactants isolated from *P. aeruginosa* M strain

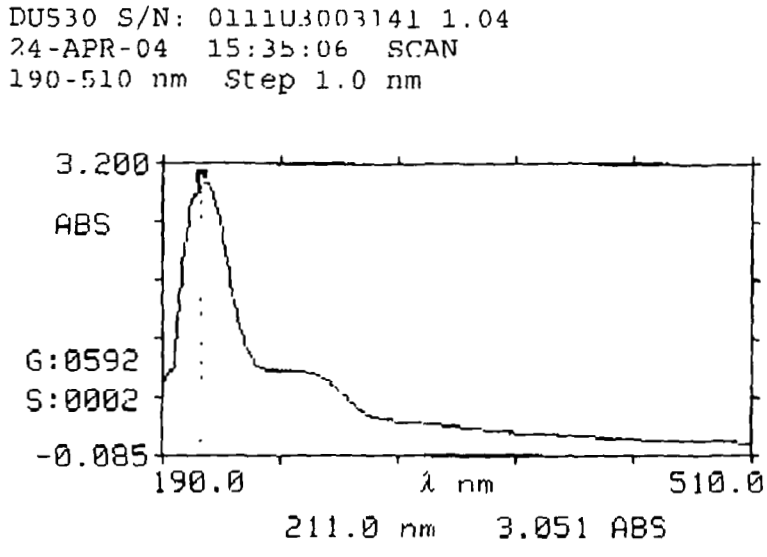

Fig.6.9. Absorption maxima (λ_{max}) of crude biosurfactants isolated from *P. aeruginosa* NM strain

6.1.4.6 IR spectra of biosurfactants

IR spectra of the biosurfactants from *P. aeruginosa* M and NM strains in nujol showed strong absorption bands at 1644 cm^{-1} as result of stretching mode of a CO-N and at 1542 cm^{-1} for the deformation mode of the NH bond combined with C-N stretching mode occurred. The presence of an aliphatic chain was indicated by the C-H stretching modes at 2858-2926cm^{-1} and 1457-1400 cm^{-1} The band at 1736 cm^{-1} was due to lactone carbonyl absorption (Fig. 6.10 and 6.11).

6.1.4.7 MALDI-T0F-MS characterization of RP-HPLC purification of biosurfactants fraction

The MALDI-TOF-MS of both the *Pseudomonas* RP-HPLC purified biosurfactants from both the *Pseudomonas aeruginosa* strains showed peaks at same m/z ratio, but HPLC purified biosurfactants from *P. aeruginosa* M strain showed two extra peaks at (m/z) 1788.3 and 1832.299 (Fig. 6.12). The mass spectra of these biosurfactants have peaks, which can be attributed to the protonated forms, as well as to the sodium and potassium adducts.

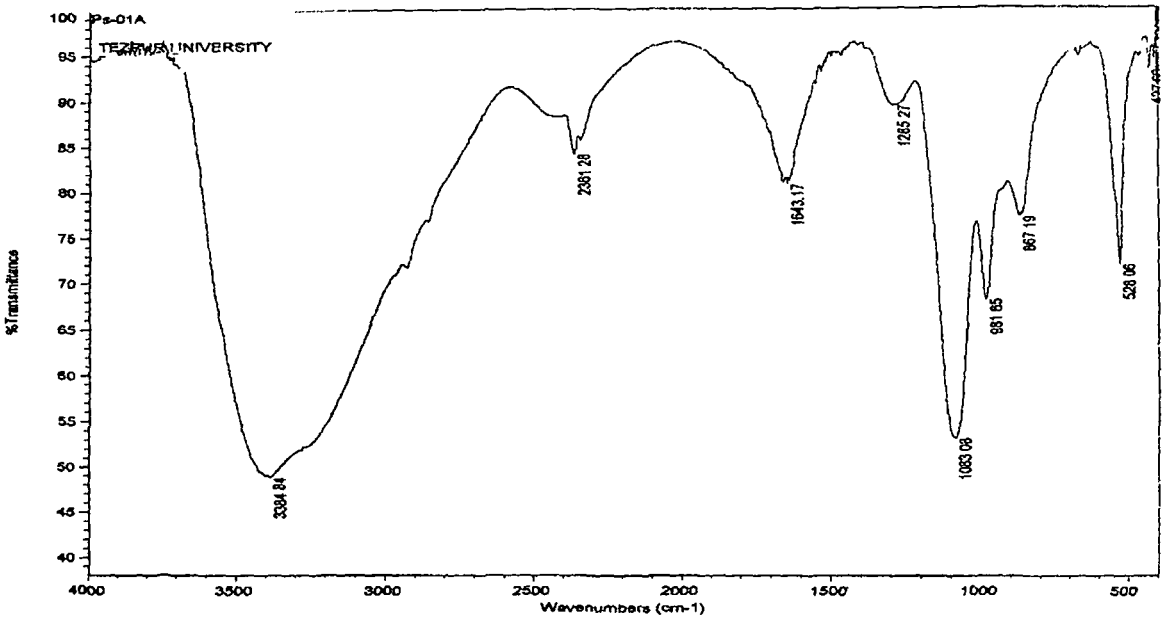

Fig.6.10. IR spectra of purified biosurfactants isolated from *P. aeruginosa* M strain.

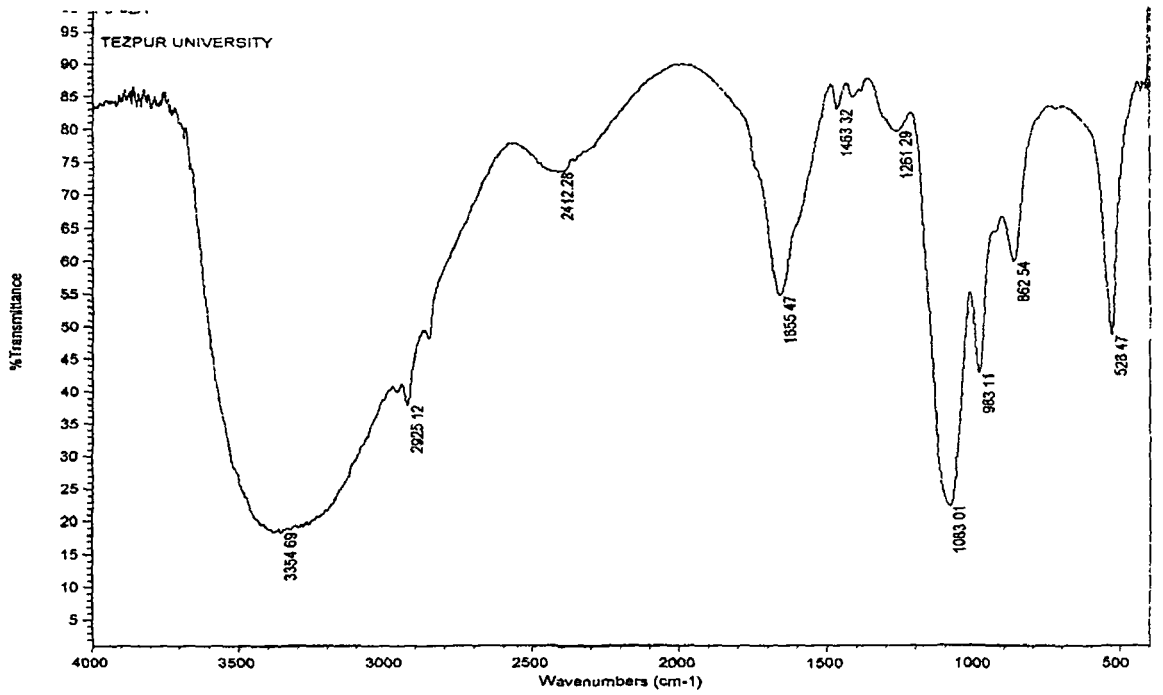

Fig.6.11. IR spectra of purified biosurfactants isolated from *P. aeruginosa* NM strain.

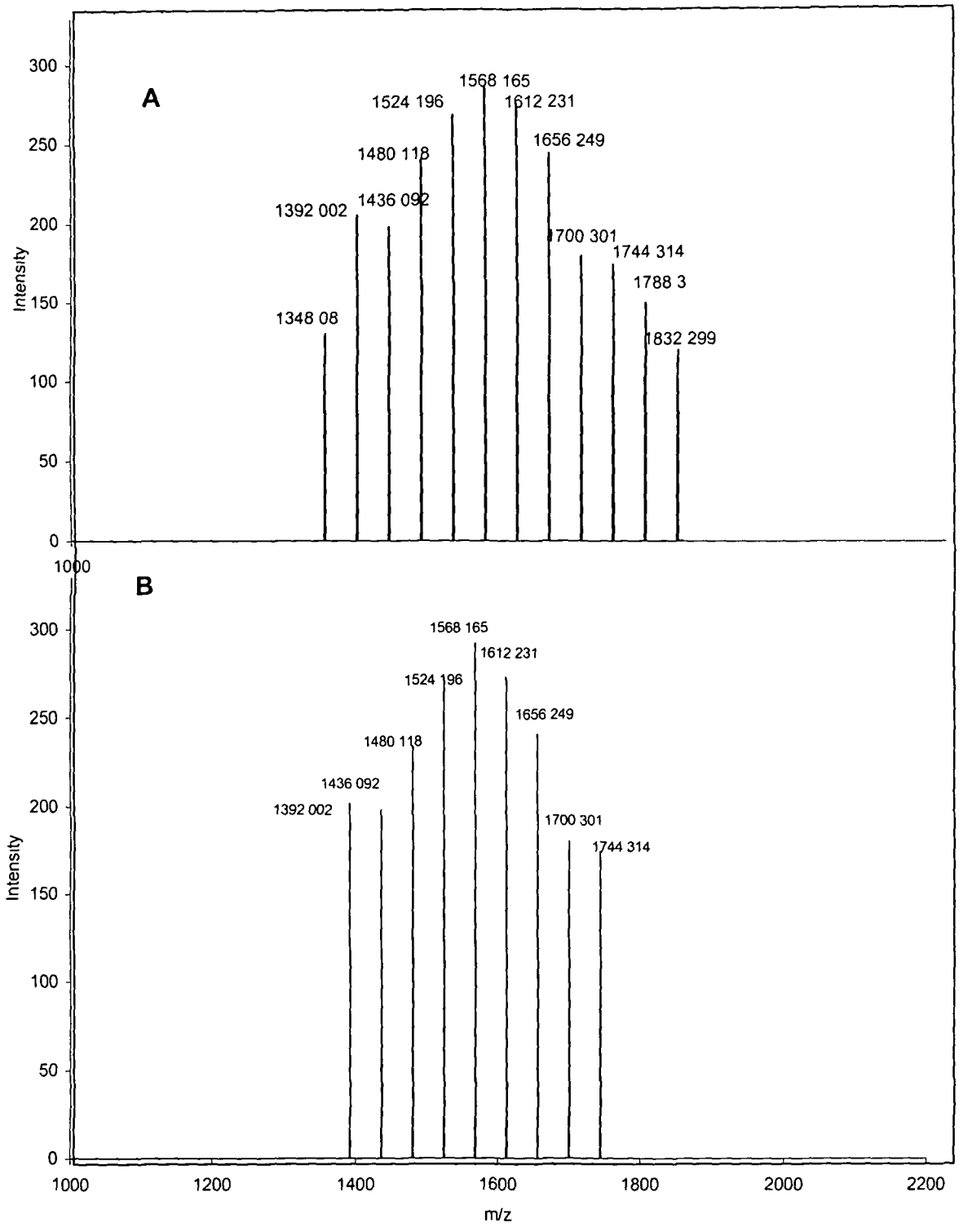

Fig. 6.12. MALDI-TOF mass spectra of the HPLC purified active peak.

(A) *P. aeruginosa* M strain (B) *P. aeruginosa* NM strain.

6.1.8 Pharmacological properties

6.1.8.1 Hemolytic activity

As shown in fig. 6.13, the biosurfactants from both the *P. aeruginosa* strains induced dose dependent direct lysis of washed human erythrocytes, but to a different extent. The hemolytic effect of two hundred microgram of crude biosurfactants from *P. aeruginosa* M strain was higher than that induced by identical dose of biosurfactants from *P. aeruginosa* NM strain (3.8 % Vs 2.8%).

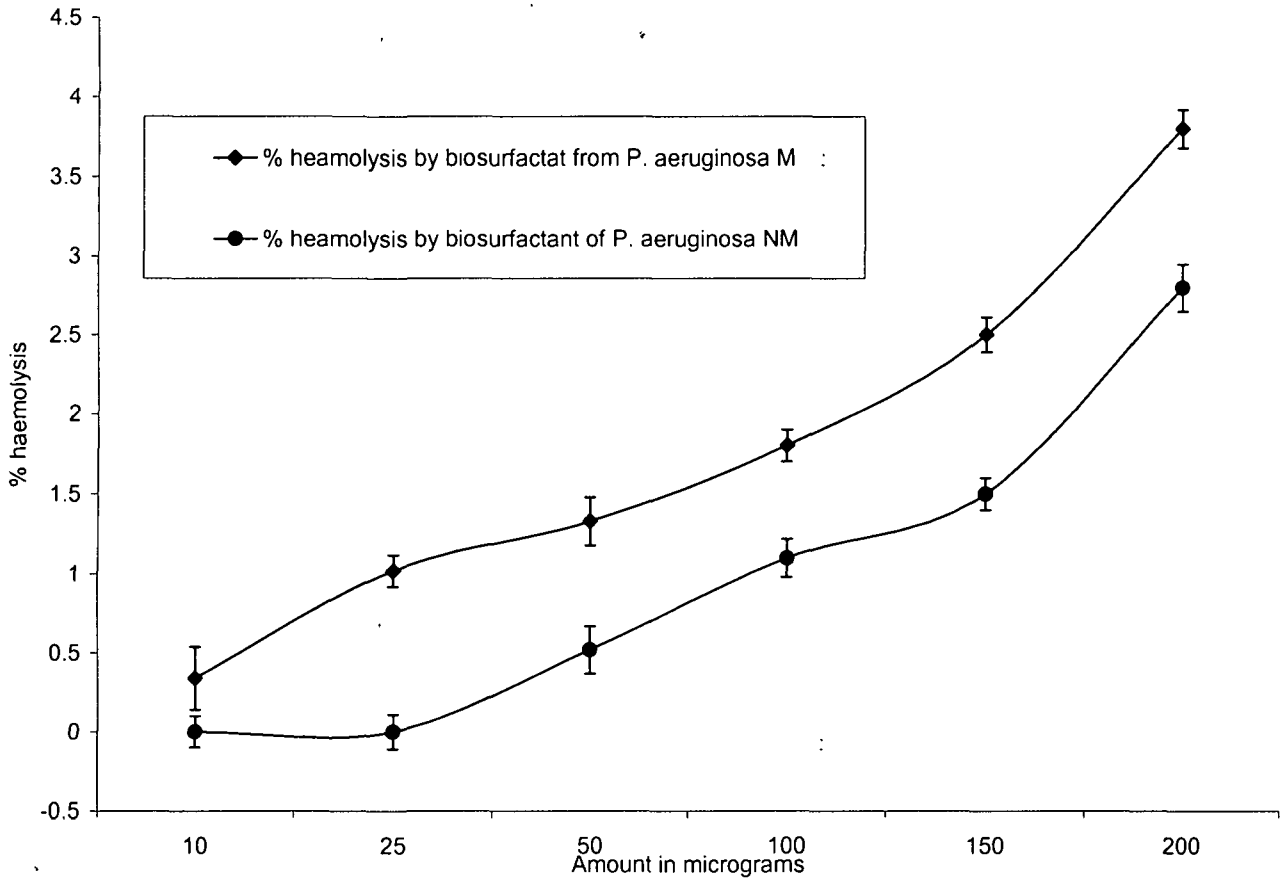

Fig.6.13. Hemolysis of human erythrocytes by the crude biosurfactants of *P. aeruginosa* M and NM strains. Various concentrations of biosurfactants were incubated with 5.0 % (v/v) washed human erythrocytes in final volume of 3.0 ml at 37°C temperature for 1 h. Each value represents mean ± S.D of three independent experiments

8.1.8.2 Interference in blood coagulation

In *in vitro* condition, crude biosurfactants from both the *P. aeruginosa* strains induced dose dependent coagulation of the platelet poor goat plasma (PPGP). For example, 20 µg biosurfactants from *P. aeruginosa* M and NM strains reduced the Ca-clotting time of PPGP from 157±1 sec to 100.4±1 sec and 99.1±1 sec respectively (Fig. 6.14).

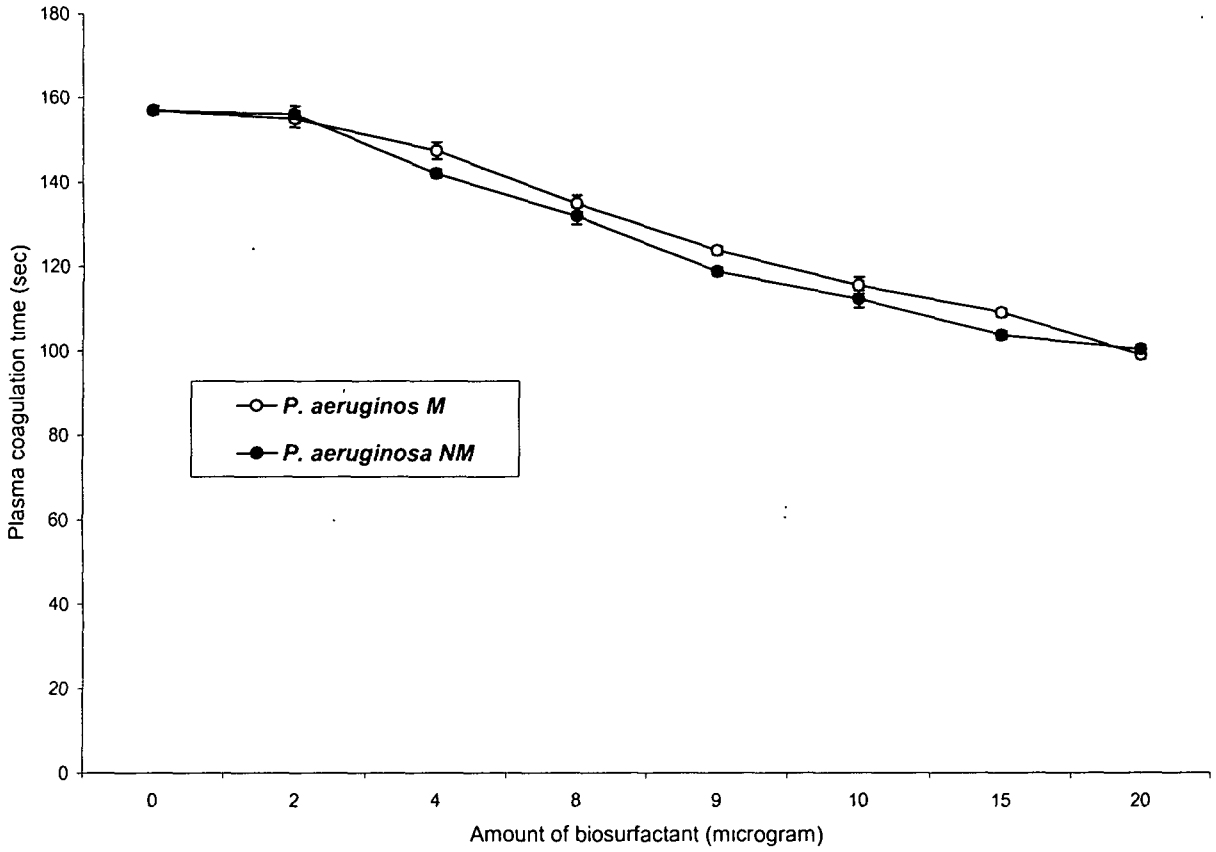

Fig.6.14. Dose dependent interference of *in-vitro* blood coagulation by biosurfactants from *P. aeruginosa* M and NM strains. Results represent the mean ± S.D of four individual experiments.

6.1.7.3 *In-vitro* tissue damaging activity

In *in vitro* condition, biosurfactants from *P. aeruginosa* M and NM stains failed to demonstrate any detrimental effect on the tested chicken tissues up to a concentration of 200µg/ ml (Table 6.21).

Table 6.21. *In-vitro* tissue damaging activity of crude biosurfactants. Results represent mean of three independent experiments. Cent percent hemolysis was induced by Triton X-100 and other values were compared with that.

S. No	Tissue	Source of biosurfactants	Final Concentration of biosurfactants (µg/ml)	% tissue damage
1	Chicken Liver	*P. aeruginosa* M	25	0.0
2			50	0.0
3			200	0.0
4		*P. aeruginosa* NM	25	0.0
5			50	0.0
6			200	0.0
7	Chicken Heart	*P. aeruginosa* M	25	0.0
8			50	0.0
9			200	0.0
10		*P. aeruginosa* NM	25	0.0
11			50	0.0
12			200	0.0
13	Chicken Lungs	*P. aeruginosa* M	25	0.0
14			50	0.0
15			200	0.0
16		*P. aeruginosa* NM	25	0.0
17			50	0.0
18			200	0.0
19	Chicken Kidneys	*P. aeruginosa* M	25	0.0
20			50	0.0
21			200	0.0
22		*P. aeruginosa* NM	25	0.0
23			50	0.0
24			200	0.0

6.1.9 Cell surface hydrophobicity (CSH)

The cells of *P. aeruginosa* stains were allowed to grow either in carbohydrate (2% w/v glucose) or in hydrocarbon (2% v/v hexadecane) medium and their ability to transfer the cells in the hydrocarbons was investigated. As shown in Figs. 6.15 and 6.16, the cells from both *P. aeruginosa* strains when grown on glucose were more efficiently transferred to different hydrocarbons but to a different extent. For example, there was comparatively poor transfer of cells in hexane, benzene and toluene as compared to transfer of cells in hexadecane and dodecane. This is an important finding because it may be useful for formulation of inoculum for bioremediation of hydrocarbons contaminated ecosystem.

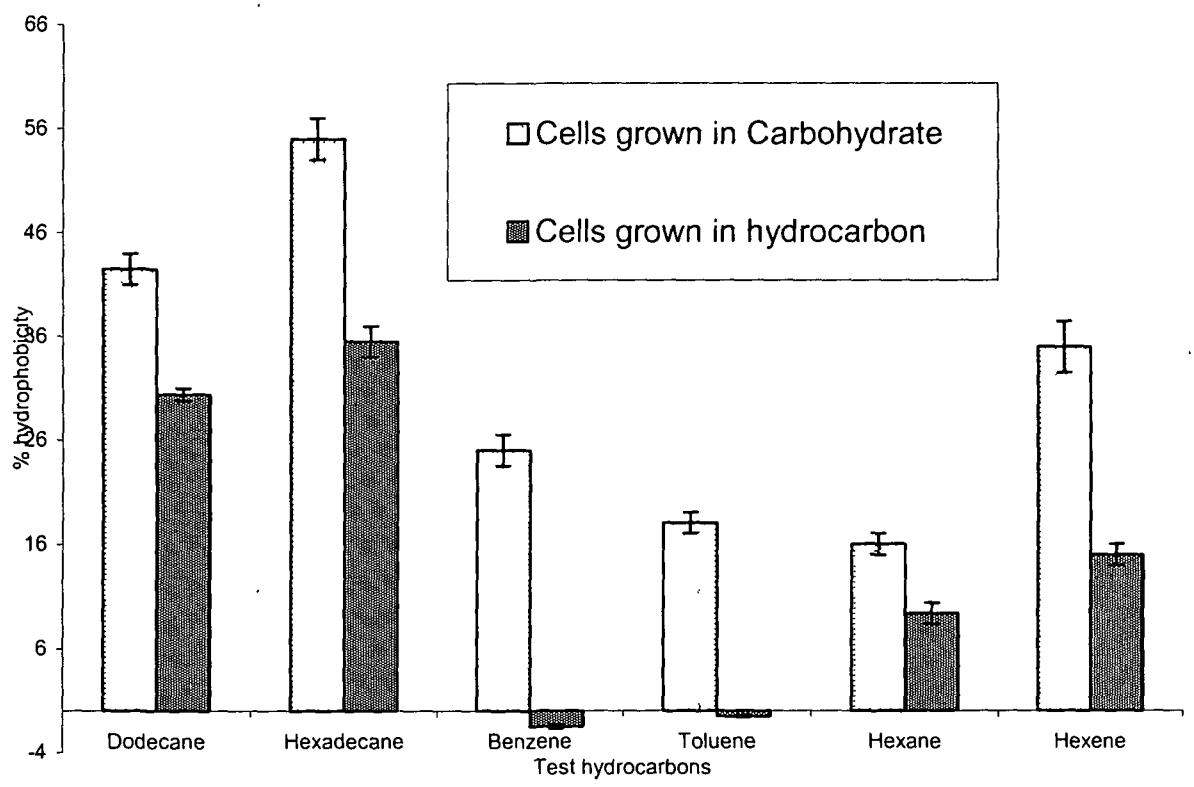

Fig. 6.15. Cell surface hydrophobicity (CSH) of *P. aeruginosa* M strain. The bacterial cells were grown either in glucose or in hexadecane and their cell surface hydrophobicity was assessed as described in the text. Results represent the mean ± S.D of four individual experiments.

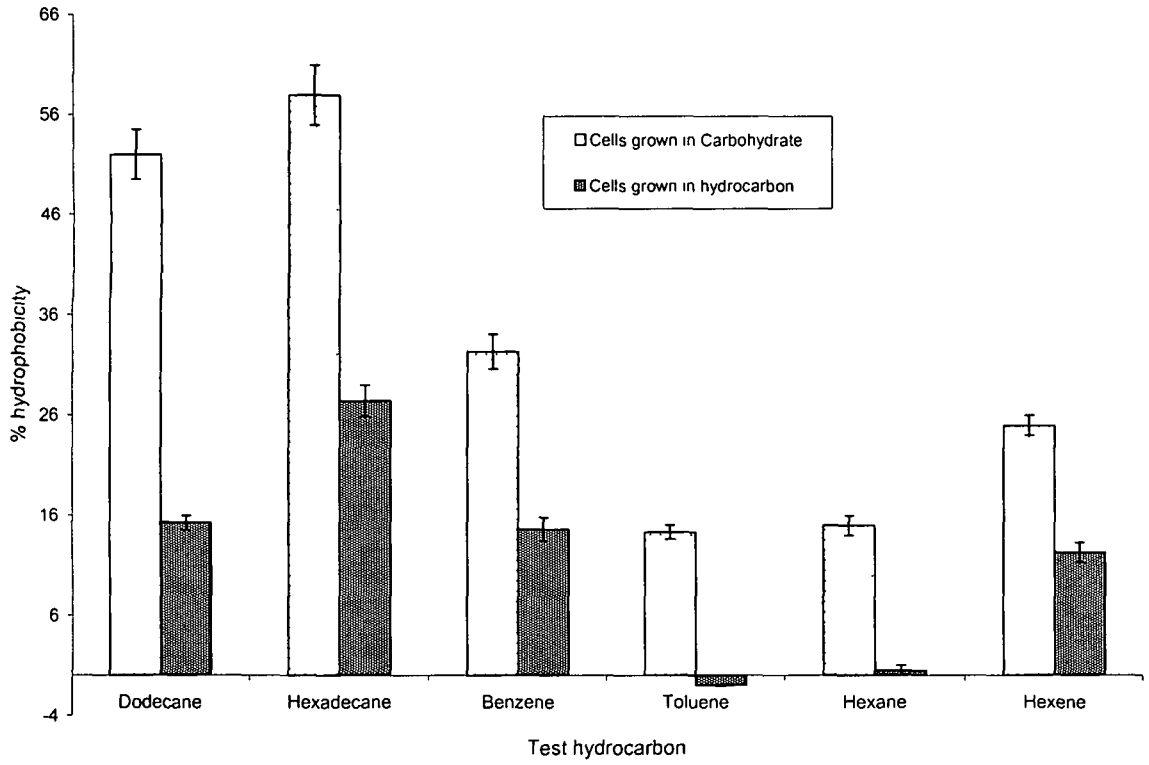

Fig. 6.16. Cell surface hydrophobicity (CSH) of *P. aeruginosa* M strain. The bacterial cells were grown either in glucose or in hexadecane and their cell surface hydrophobicity was assessed as described in the text. Results represent the mean ± S.D of four individual experiments.

6.1.10 Plasmid curing

Plasmid DNA of 1.9 kb was found only on *Pseudomonas aeruginosa* M strain {fig. 6.17 (A)} and not on *P. aeruginosa* NM strain. 125 μg/ml of acridine orange cured the plasmid DNA of *P. aeruginosa* M strain. We failed to isolate plasmid DNA from the cured mutants. Further, our study revealed that plasmid DNA has no role to play in biosurfactants production by *P. aeruginosa* M strain (Table 6.22). Plamid was also lacking in the mutated isolates after 96h of fermentation in the process of biosurfactants production {fig. 6.17 (B)}.

Table 6.22. A comparison of surface active properties of culture supernatant obtained post growth of wild and mutant strains of *P. aeruginosa* M. Results represent the mean ± S.D of three independent determinations.

Properties	Wild strain of *P. aeruginosa* M	Mutant strain of *P. aeruginosa* M
Surface tension (mN/m)	27.5 ± 0.2	27.7 ± 0.4
CMD^{-1}(mN/m)	32.3 ± 0.4	32.4 ± 0.3
Yield of biosurfactants (g/l)	6.0 ± 0.2	6.0 ± 0.3
Dry biomass(g/l)	2.1 ± 0.2	2.0 ± 0.1

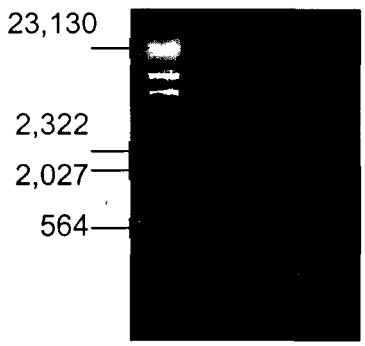

Figure 6.17(A)

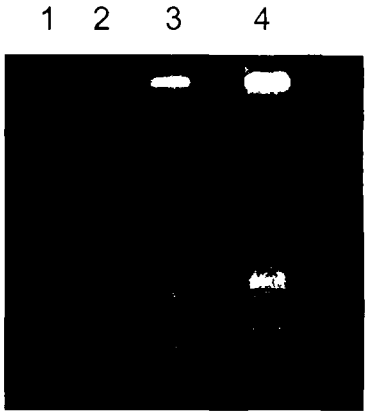

Figure 6.17(B)

Fig. 6.17 **(A) Plamid DNA of *P. aeruginosa* M strain**. Lane1 is the molecular weight marker (λ DNA-Hind III digest) and Lane 2 is the plasmid DNA from *P. aeruginosa* M strain.

(B) Plasmid isolated from 96 h culture of *P. aeruginosa* M wild and mutant strains. Both the wild and mutant strains were grown on modified M9 medium with glycerol (2% v/v) as sole carbon source. Lane 1, 2 & 3 shows the absence of plasmid DNA from the mutant strains. Lane 4 is the plasmid DNA as isolated from the wild (non-mutant strain) of *P. aeruginosa* M. Plasmid with isolated from the wild and mutated under identical condition.

CHAPTER VII

SOME INDUSTRIAL APPLICATION OF BIOSURFACTANTS ISOLATED FROM *B. subtilis* AND *P. aeruginosa* STRAINS

7.1 Results

7.1.1 Use of biosurfactants in releasing oil from saturated sand pack column

The crude biosurfactants from *B. subtilis* DM-03 and DM-04 could release 60% (Fig. 7.1) and 71.2% (Fig. 7.2) respectively of kerosene oil from saturated sand pack columns. On the other hand, same concentration of crude biosurfactants (150 mg/l) from *P. aeruginosa* M and NM strain could release 85% (Fig. 7.3) and 79% (Fig. 7.4) of kerosene oil from saturated sand pack column demonstrating that biosurfactants from *P. aeruginosa* strains were more efficient than biosurfactants from *B. subtilis* strains in releasing the oil from saturated sand pack columns.

Even after heating the aqueous solutions of biosurfactants obtained from *B. subtilis* or *P. aeruginosa* strains at 100°C for 60 min, there was no significant change in the ability of these biosurfactants to release oil from saturated sand pack column. The lipopeptides from *B. subtilis* DM-03 and DM-04 strains retained respectively 91.0% and 95.0% oil releasing activity post heating at 100°C for 60 min. On the other hand, 95.0% and 92.0% activity was retained post heating the biosurfactants from *P. aeruginosa* M and NM strain.

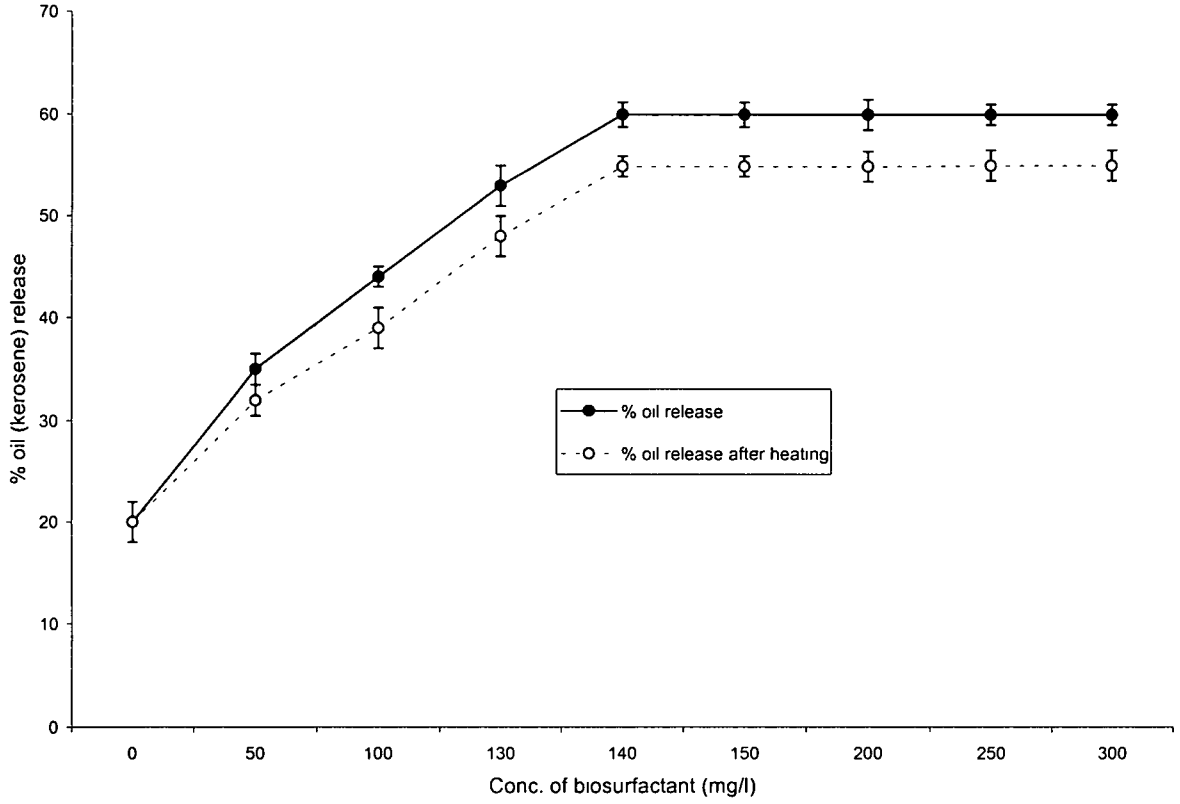

Fig. 7.1. Percentage of kerosene release from saturated sand pack column by graded amount of biosurfactants from *B. subtilis* DM-03 strain. The black line with black circle shows the percent of oil release by unheated biosurfactants, whereas the dotted line with white circle indicates the percentage oil release after heating the biosurfactants solutions at 100 °C for 60 min. Results represent the mean ± S.D of three independent determinations.

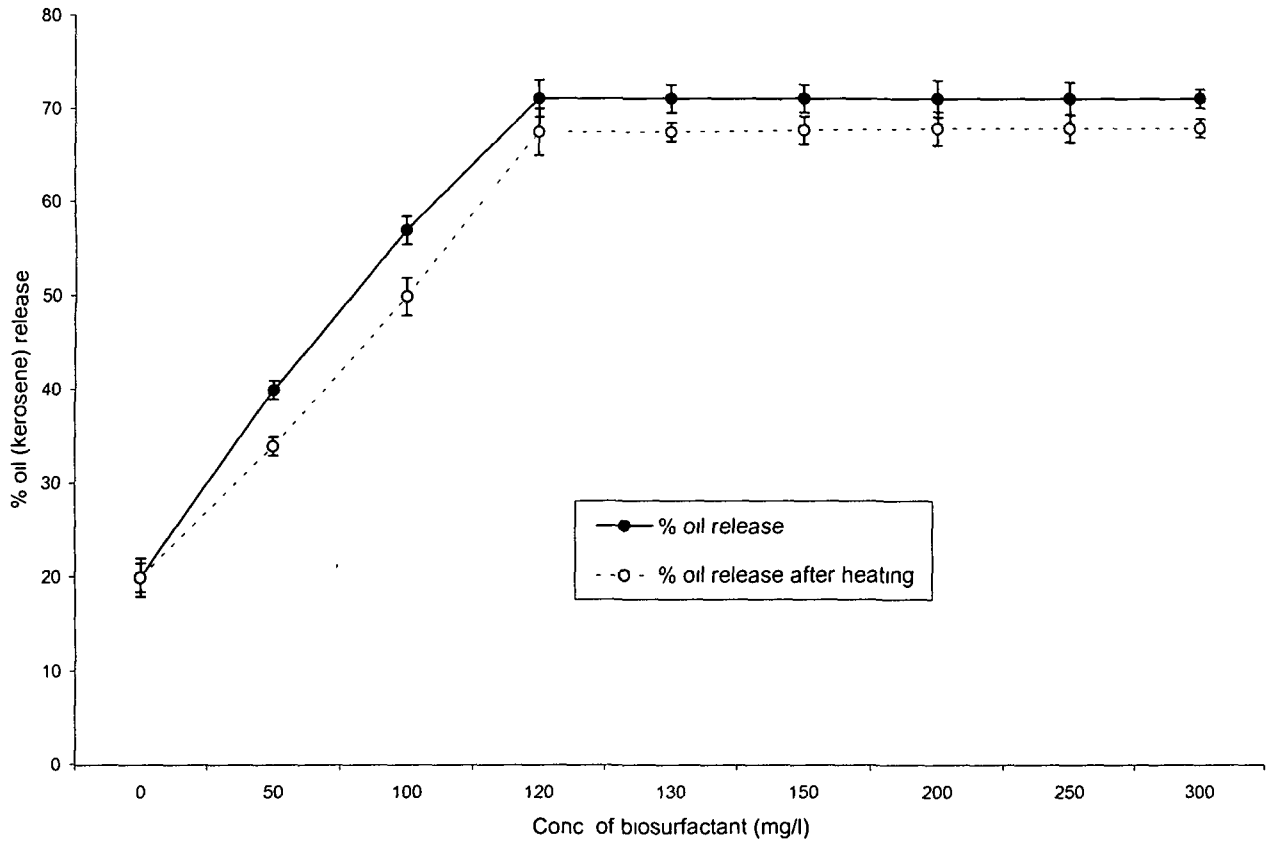

Fig.7.2. Percentage of kerosene release from saturated sand pack column by graded amount of biosurfactants from *B. subtilis* DM-04 strain. The black line with black circle shows the percent of oil release by unheated biosurfactants, whereas the dotted line with white circle indicates the percentage oil release after heating the biosurfactants solutions at 100 °C for 60 min. Results represent the mean ± S.D of three independent determinations.

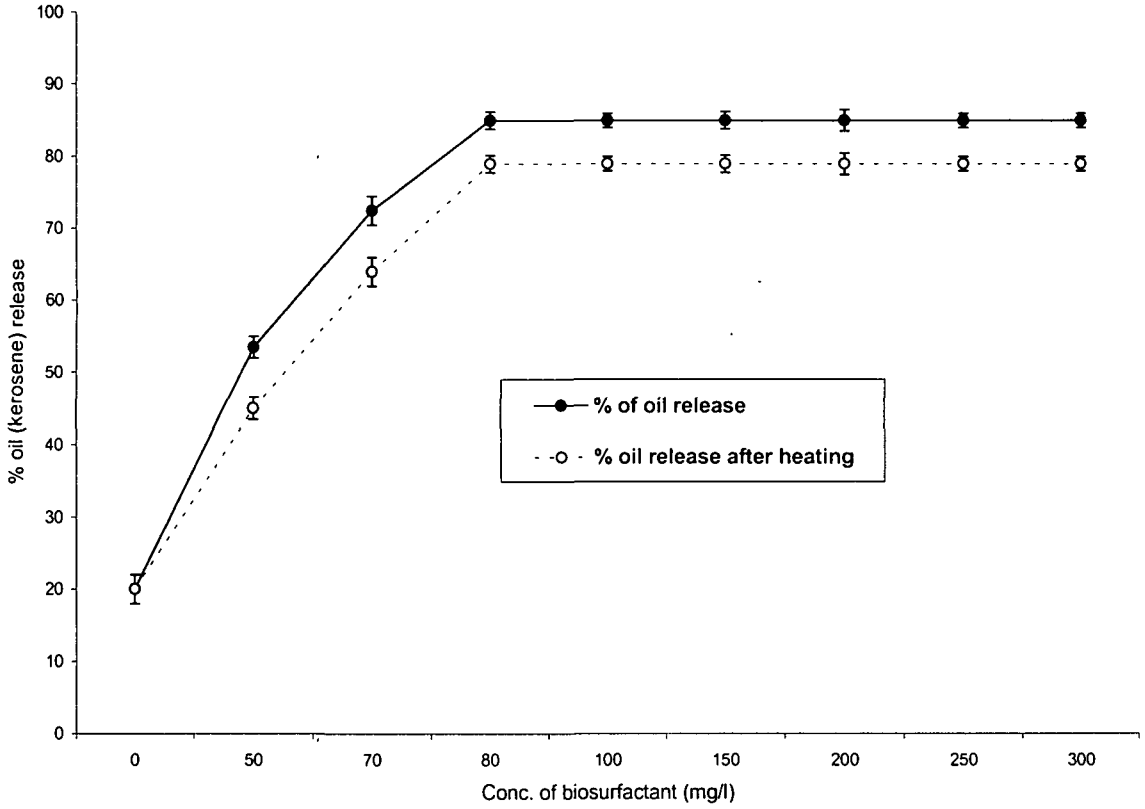

Fig.7.3. Percentage kerosene release from saturated sand pack column by graded amount of biosurfactants from *P. aeruginosa* M strain. The black line with black circle shows the percent of oil release by unheated biosurfactants, whereas the dotted line with white circle indicates the percentage oil release after heating the biosurfactants solutions at 100 °C for 60 min. Results represent the mean ± S.D of three independent determinations.

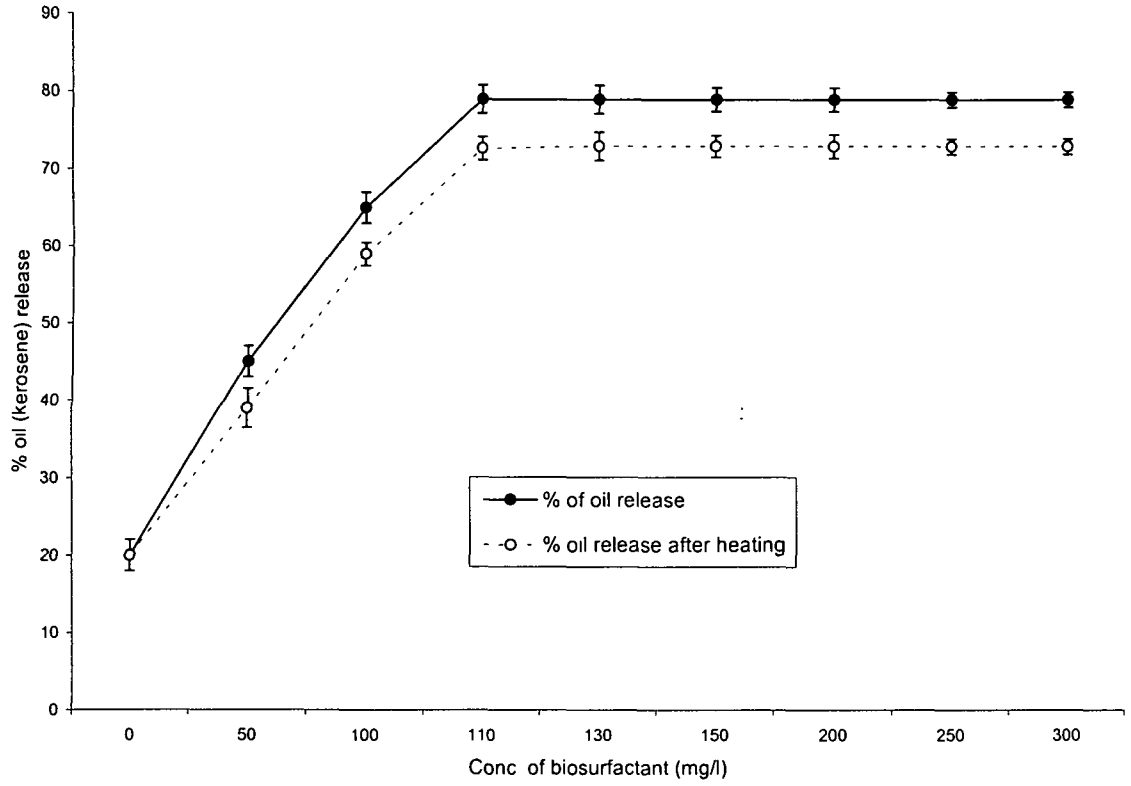

Fig.7.4. Percentage kerosene release from saturated sand pack column by graded amount of biosurfactants from *P. aeruginosa* NM strain. The black line with black circle shows the percent of oil release by unheated biosurfactants, whereas the dotted line with white circle indicates the percentage oil release after heating the biosurfactants solutions at 100 °C for 60 min. Results represent the mean ± S.D of three independent determinations.

7.1.2. Application of biosurfactants in bioremediation of soil samples (laboratory scale) contaminated with crude petroleum oil

7.1.2.1 PAH solubilization by biosurfactants

The effect of biosurfactants on the apparent aqueous solubility of PAH was determined by test-tube solubilization assay in the presence of graded amounts of biosurfactants (125 μg/ml to 500 μg/ml) obtained from bacterial strains used in this study. In general, the crude biosurfactants from all the three strains enhanced the apparent solubility of PAHs in a dose dependent manner (Table 7.1). However, solubilization of pyrene by biosurfactants (~ 5 time higher apparent solubility compared to control) was significantly higher when compared with the phenanthrene or anthracene solubilization effect of biosurfactants (2 to 3 times higher compared to control). Moreover, the crude biosurfactants produced by *P. aeruginosa* NM strain displayed higher pyrene solubilization effect ($p < 0.05$) compared to crude biosurfactants produced by *P. aeruginosa* M and *B. subtilis* DM-04 strains (Table7.1). On the other hand, crude biosurfactants from *B. subtilis* DM-04 strain at a dose of 500 μg/ml demonstrated significantly higher anthracene solubilization effect compared to the same property displayed by crude biosurfactants from other two *Pseudomonas* strains (Table 7.1).

7.1.2.2 Analysis of TPH content of soil before bioremediation

The soil sample selected for bioremediation experiment was loamy and dark brown in color. The pH of the soil was 6.8-7.0 before starting the bioremediation experiment (time zero) and did not change significantly at the end of experiment. This crude petroleum contaminated soil sample tested for bioremediation experiment contained alkanes (55.1%) as the largest constituent of the solvent extracted TPH, followed by the aromatic fraction (19.8), asphaltene fraction (15.6%) and 7% NSO (Fig.7.5).

Table 7.1. Dose dependent solubilization of PAHs by lipopeptides / biosurfactants isolated from *B. subtilis* DM-04, *P. aeruginosa* M and NM strains. Results represent mean ± S.D. of three independent experiments.

Source and concentration of crude biosurfactants (μg/ml)	Solubility of PAHs ((μg/ml)		
	Pyrene	Phenanthrene	Anthracene
***B. subtilis* DM-04**			
0	2.0 ± 0.3	0.5 ± 0.1	0.4 ± 0.1
125	5.2 ± 0.5	0.6 ± 0.1	0.6 ± 0.1
250	8.6 ± 0.3	0.7 ± 0.2	1.0 ± 0.3
500	10.2 ± 0.3	0.9 ± 0.1	1.4 ± 0.4
***P. aeruginosa* M**			
0	2.0 ± 0.3	0.5 ± 0.1	0.4 ± 0.1
125	5.0 ± 0.5	0.6 ± 0.2	0.5 ± 0.1
250	8.0 ± 0.5	0.8 ± 0.2	0.9 ± 0.3
500	9.9 ± 0.8	1.0 ± 0.3	1.1 ± 0.5
***P. aeruginosa* NM**			
0	2.0 ± 0.3	0.5 ± 0.3	0.4 ± 0.1
125	6.1 ± 0.3	0.7 ± 0.3	0.5 ± 0.3
250	8.3 ± 0.3	0.9 ± 0.3	0.9 ± 0.3
500	11.2 ± 0.3	1.1 ± 0.3	1.0 ± 0.3

Table 7.2. Biodegradation (%) of various fractions of crude petroleum oil contaminated soil sample. Experiment was done as described in the text. Each value represents mean ± S.D. from triplicate trays.

Microorganisms	Biodegradation (%) post 120 days of experiment			
	TPH	Alkane	Aromatic	NSO + Asphaltene
None (control)	5.1 ± 0.1	27.4 ± 5.0	19.2 ± 2.9	15.8 ± 3.1
B. subtilis DM-04	53.4 ± 2.1[a]	55.7 ± 5.4[a]	51.0 ± 5.2[a]	45.3 ± 4.2[a]
P. aeruginosa M and NM consortia	75.6 ± 4.2[a, b]	81.5 ± 7.3[a, b]	64.6 ± 6.0[a, b]	64.2 ± 5.8[a, b]

Significance of difference with respect to control experiment [a] $p < 0.001$
Significance of difference with respect to biodegradation by *B. subtilis* DM-04 [b] $p < 0.01$

7.1.2.3 Biodegradation of TPH and various fractions of crude petroleum oil

The results of the biodegradation experiment are shown in Table 7.2. The level of TPH contamination in soil was detected as 84.4 g/kg of soil before starting the bioremediation experiment (time zero), which decreased to 20.6 g/kg and 39.3 g/kg of soil at the end of experiment (after 120 days) due to biodegradation of TPH by *P. aeruginosa* M and NM consortia and *B. subtilis* DM-04 strain respectively. In untreated (control) soil, the TPH level was decreased to 80.1 g/kg of soil and after 120 days degradation of alkane, aromatic, NSO and asphaltene fractions was non-significant compared to biodegradation of same fractions by *B. subtilis* DM-04 and *Pseudomonas* consortia. However, all the microbes in the present study exhibited higher biodegradation of alkane fraction compared to aromatic or NSO fractions. Moreover, *Pseudomonas* consortia was shown to possess significantly higher potential ($p < 0.01$) for the biodegradation of TPH as well as various fractions of crude petroleum oil compared to *B. subtilis* DM-04 strain.

7.1.2.4 GC analysis of alkane fraction

Gas- chromatographic analyses of alkane fraction obtained from the petroleum contaminated soil before (0 days) and at the end (120 days) of bioremediation experiment have been shown in Fig. 7.6. GC analyses showed that level of alkanes in TPH was subsequently reduced after 120 days of bioremediation experiment with either *B. subtilis* DM-04 or *Pseudomonas* consortium.

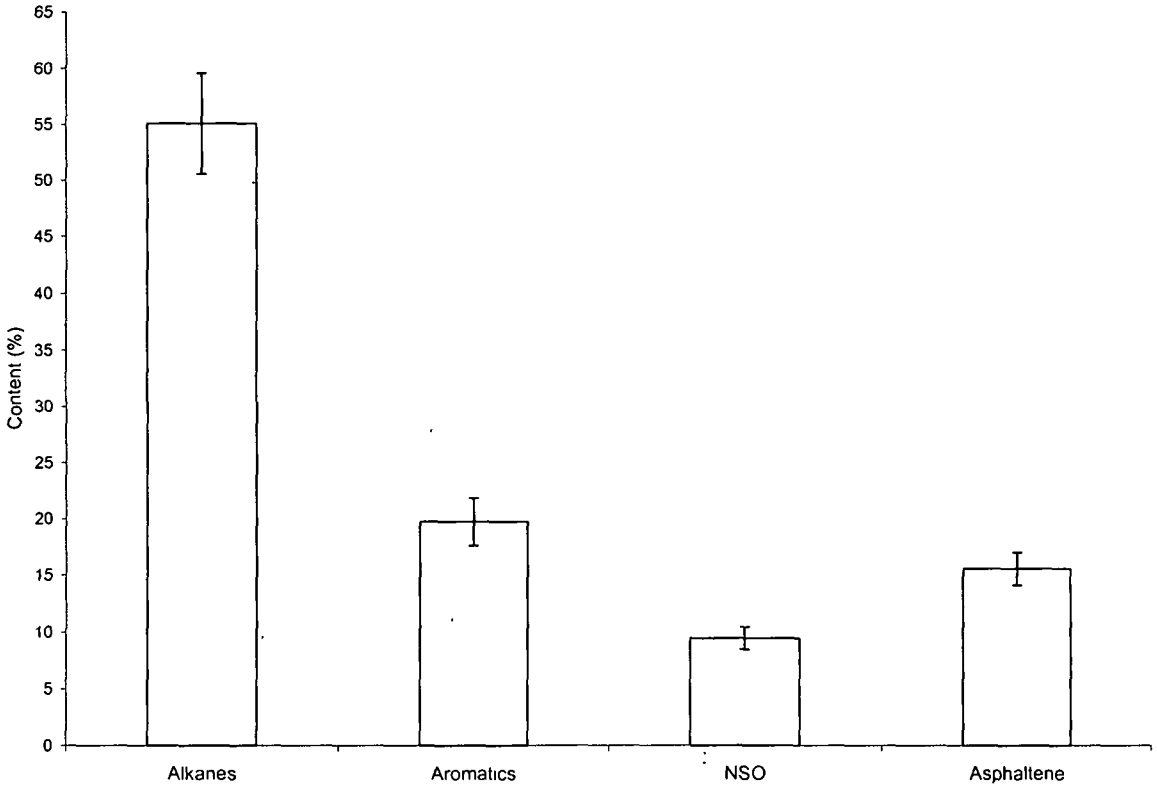

Fig. 7.5. The composition of total petroleum hydrocarbon (TPH) of the soil before starting the bioremediation experiment (time zero). Values represent mean ± S.D. of triplicate determinations

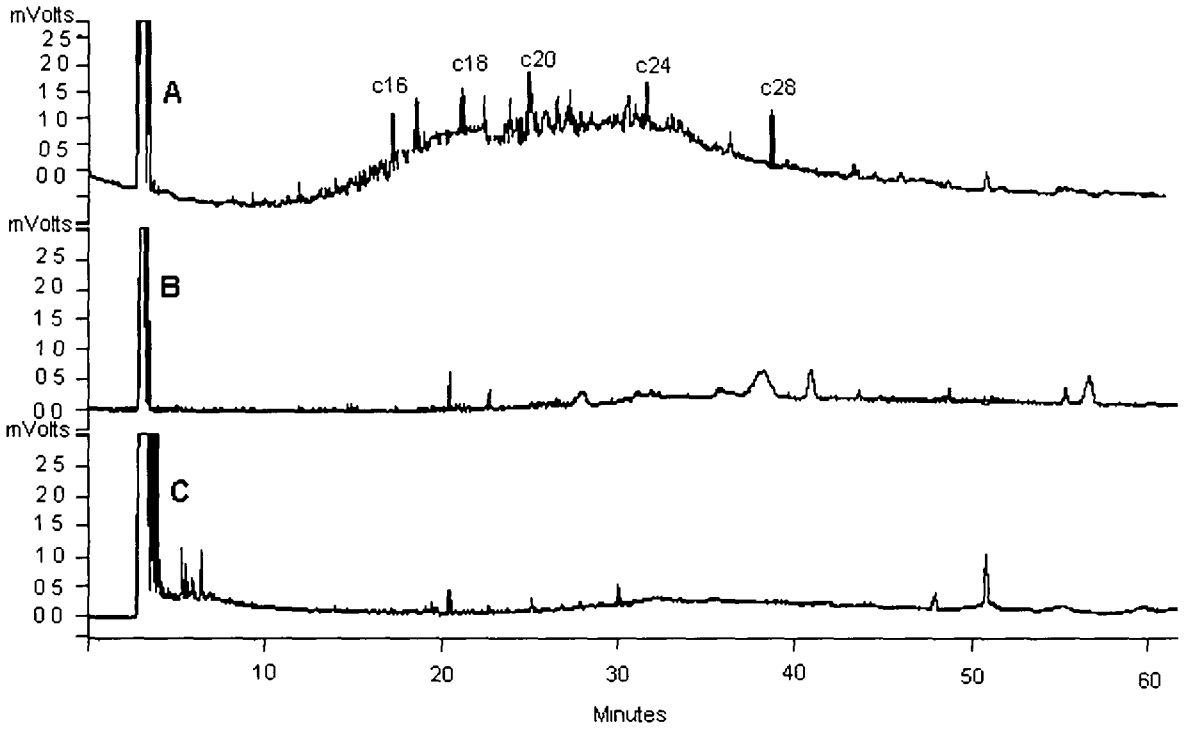

Fig.7.6. GC fingerprinting of alkane fraction of soil treated with *P. aeruginosa* M and NM strain and *B. subtilis* DM-04 strain.

(a) at 0 h and (b) after 120 days (end of bioremediation) with *B. subtilis* DM-04 strain

(c) after 120 days (end of bioremediation) with *P. aeruginosa* M and NM strains

7.1.3. Biodegradation of pyrene

7.1.3.1 Growth characteristics of microbes at the expense of pyrene:

All the microbes in the present study were able to utilize pyrene as a sole source of carbon and energy as was evident from the decrease in pyrene content from microbial culture containing pyrene- minimal salts medium (Fig. 7.7) with a concomitant increase in the absorbance of the culture medium at 600 nm and an increase in bacterial dry biomass and protein content with respect to time (Table 7.3). No significant microbial growth was observed up to 12 h post inoculation. However the growth rates of *B. subtilis* DM-04 strain in pyrene medium was significantly higher ($p<0.01$) compared to growth rate of *P. aeruginosa* M and NM strains. As shown in Table 7.3, addition of 100 mg/l of glucose in the growth medium resulted in a significant enhancement of pyrene utilization and growth of *B. subtilis* DM-04 as well as *P. aeruginosa* M and NM strains.

Table 7.3. Dry weight of the bacterial strains and yield of biosurfactants at different time period when grown on pyrene and pyrene + glucose. Experiments were performed as described in the text. Values represent the mean ± S.D of three individual experiments.

Bacterial strain	Carbon source		48 h		96 h	
	Pyrene	Glucose	Dry biomass (g/l)	Yield of biosurfactant (g/l)	Dry biomass (g/l)	Yield of biosurfactant (g/l)
B. subtilis DM-04	+	-	1.0 ± 0.1	0.2 ± 0.05	1.3 ± 0.1[a]	0.3 ± 0.05
	+	+	1.2 ± 0.1[c]	0.3 ± 0.05	1.6 ± 0.1[a,b,c]	0.6 ± 0.1[b]
P. aeruginosa M	+	-	0.7 ± 0.1	0.1 ± 0.01	0.8 ± 0.1	0.2 ± 0.1
	+	+	0.8 ± 0.1	0.2 ± 0.01	1.2 ± 0.1[b]	0.5 ± 0.1[c]
P. aeruginosa NM	+	-	0.6 ± 0.1	0.1 ± 0.01	0.7 ± 0.1	0.2 ± 0.7
	+	+	0.8 ± 0.1	0.2 ± 0.01	1.1 ± 0.1[a,b]	0.5 ± 0.1[b]

[a] Significance of difference with respect to growth after 48 h incubation $p< 0.05$

[b] Significance of difference with respect to growth in presence of pyrene alone $p<0.01$

[c] Significance of difference with respect to *P. aeruginosa* M and NM strain $p<0.01$

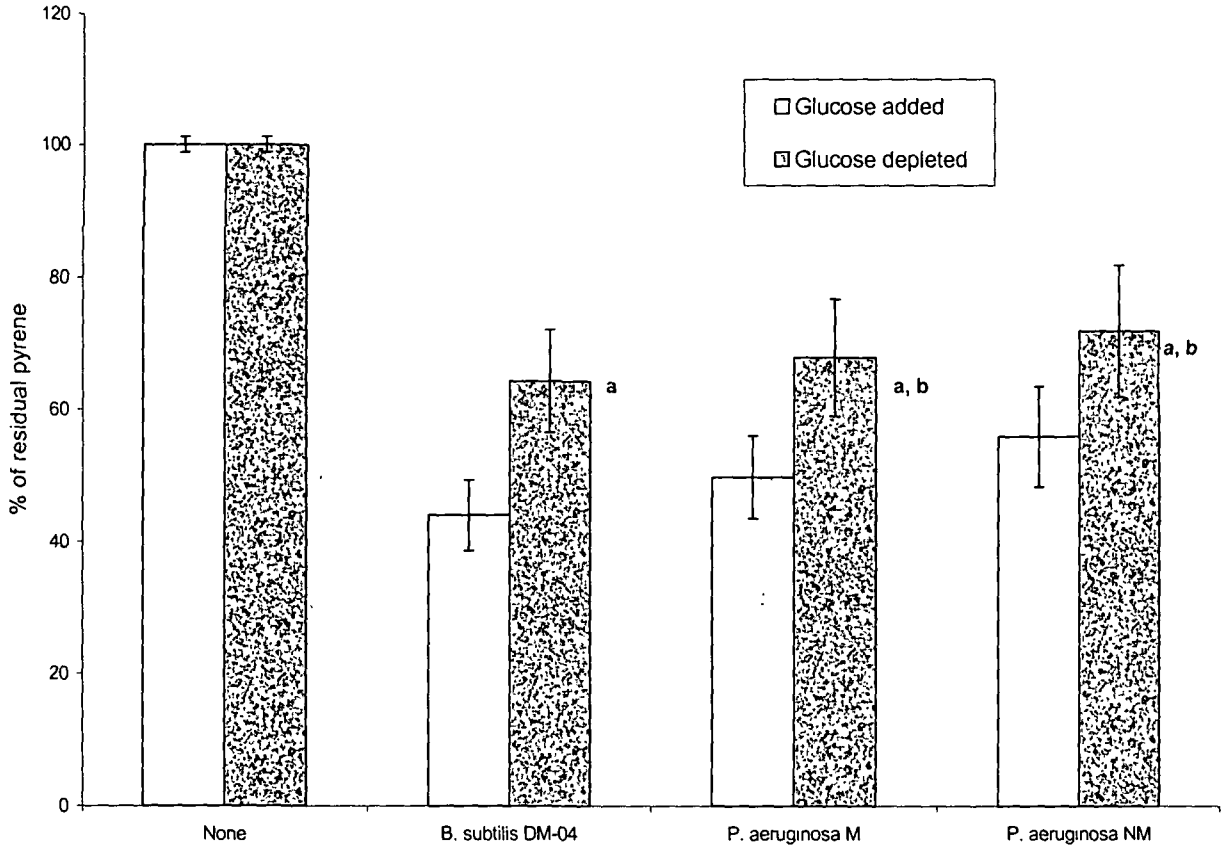

Fig.7.7. Residual pyrene in the culture supernatants of the 96 h bacterial growth. Pyrene extracted from the culture supernatant as describe in text. Percentage residual pyrene was calculated from the peak area of HPLC chromatogram. Peak area of the chromatogram from the uninoculated control flask was considered as cent percent (100%). Results represent mean ± S.D. of three independent experiments.

[a] Significance of difference with respect to pyrene utilization in presence of glucose $p< 0.01$

[b] Significance of difference with respect to pyrene utilization by *B. subtilis* DM-04 strain.

7.1.3.2 Biosurfactants production during microbial growth on pyrene

Observation of both surface-active properties and emulsifying activities supported that all the strains used in this study produced biosurfactants in pyrene-mineral salts medium (Table 7.3). The biosurfactants produced by *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains reduced the surface tension of growth medium from 67.4 ± 0.5 mN/m (mean ± S.D) to 30.0 ± 0.2 mN/m (mean ± S.D), 45.9 ± 0.3 mN/m (mean ± S.D) and 45.0 ± 0.7 mN/m (mean ± S.D) respectively. The secretion of biosurfactants started after 24 h of growth and continued up to growth at 96 h. However, the amounts of biosurfactants recovered from 96 h culture media of the microbes did not differ significantly.

7.1.3.3 Pyrene solubilization by biosurfactants

The effect of biosurfactants on the apparent aqueous solubility of pyrene was determined by test tube solubilization assay in the presence of biosurfactants (500 μg/ml) produced by respective bacterial strains. Solubility of pyrene in presence of biosurfactants from *B. subtilis* DM-04, *P. aeruginosa* M and NM strains was significantly higher then its solubility in absence of biosurfactants (Fig.7.8). Moreover, the crude biosurfactants secreted by *P. aeruginosa* NM strain showed higher pyrene solubilization effect ($p<0.05$) compared to pyrene solubilization by crude biosurfactants from *P. aeruginosa* M and *B. subtilis* DM-04 strains (Fig.7.8)

7.1.3.4 Role of biosurfactants in pyrene uptake

Table 7.4 shows that in all the three bacteria strains, pyrene uptake was enhanced significantly when it was added to bacterial cells after solubilization with biosurfactants obtained from the respective bacterial strains. Interestingly, *P. aeruginosa* NM strain did not demonstrate pyrene uptake up to 30 min incubation (Table 7.4) and 12 μg of pyrene uptake was observed 60 min post incubation with unsolubilized pyrene. Addition of biosurfactant solubilized pyrene resulted in a dramatic increase in pyrene uptake by this strain. Pyrene uptake did not occur in presence of a suspension of killed cells.

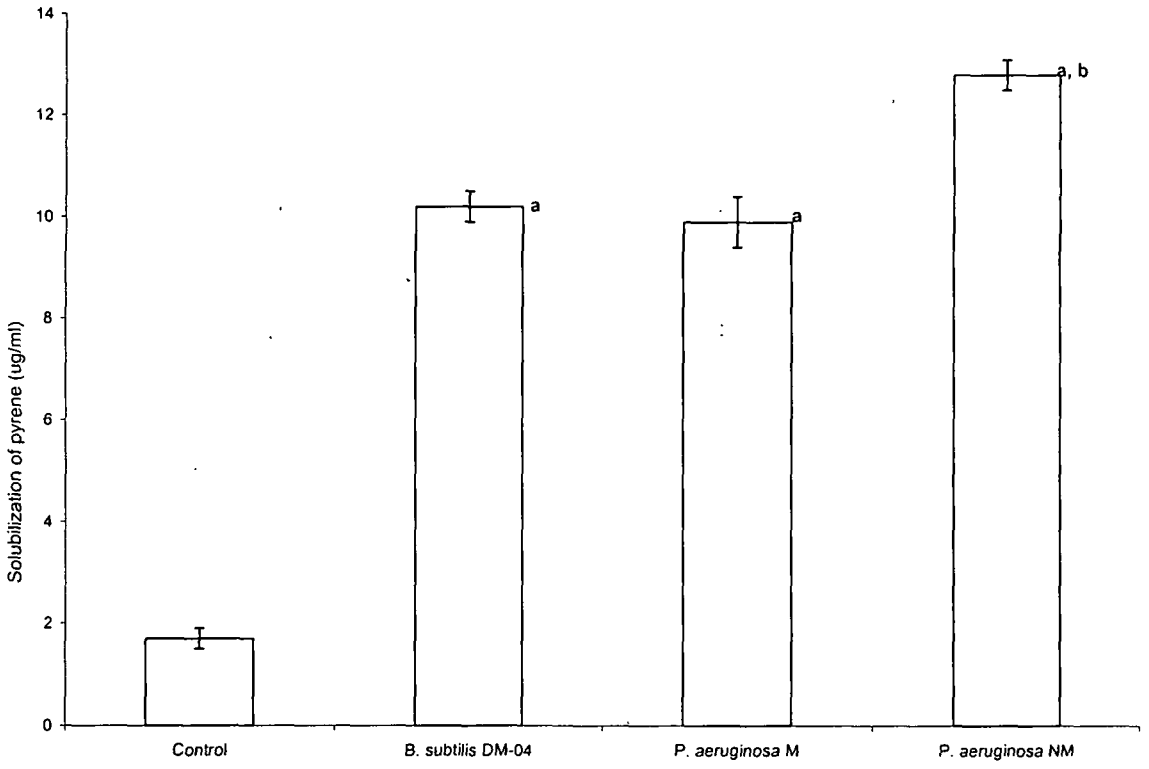

Fig.7.8 Solubilization of pyrene by the biosurfactants secreted by the bacterial strains.

Results represent mean ± S.D. of three independent experiments.

Significance of different with respect to pyrene solubilization in absence of biosurfactants (control) [a] $p < 0.001$

Significance of difference with respect to pyrene solubilization by biosurfactants from *B. subtilis* DM-04 and *P. aeruginosa* M strain [b] $p < 0.05$

Table 7.4 Effect of pyrene uptake by the bacterial cells. Experiments were performed as described in the text. Result represent the mean ± S.D of three individual experiments. "+" in condition implies the addition of microbial biosurfactants, whereas as "-" indicates no addition of microbial surfactant.

Strains	Conditions	Pyrene uptake (μg/1x10^7 bacterial cells)			
		0 min	5 min	10 min	30 min
B. subtilis DM-04	-	0	13.5 ± 1.9	14.7 ± 1.8	15.0 ± 2.0
	+	0	19.9 ± 2.1[a]	22.1 ± 2.0[b]	21.8 ± 2.9[b]
P. aeruginosa M	-	0	2.7 ± 1.1	2.8 ± 0.9	4.6 ± 1.1
	+	0	3.8 ± 1.2	6.1 ± 1.0[a]	8.4 ± 1.0[a]
P. aeruginosa NM	-	0	0	0	0
	+	0	2.0 ± 0.5[b]	2.9 ± 0.8[c]	4.1 ± 1.2[c]

Significance of difference with respect to pyrene uptake in the absence of biosurfactant [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$

7.1.4 Antimicrobial potency of biosurfactants

Crude lipopeptides from *B. subtilis* DM-03 showed significant antagonistic activity against *Kluyvera cryocrescens*, *Saccharomyces cerevisiae*, *Pichia anamala* and *Ralstonia solanaceae* but failed to inhibit *Candida glabrata* and *E. coli* strain up to 100 μg dose (Table 7.5). On the other hand, lipopeptides from *B. subtilis* DM-04 exhibited very weak antimicrobial activity as compared to lipopeptides from *B. subtilis* DM-03 strain (Table 7.6). For example, crude lipopepeptide from *B. subtilis* DM-03 arrested the growth of *Saccharomyces cerevisiae* and *Pichia anamala* at a dose of 25 μg, whereas crude lipopeptides from *B. subtilis* DM-04 exerted identical inhibition of these yeast strains only at a dose of 100 μg.

Biosurfactants from *P. aeruginosa* M strain exhibited wide range of antimicrobial activity as compared to biosurfactants from NM strain (Tables 7.7 and 7.8). Interestingly, the latter biosurfactants could arrest the growth of *K. cryocrescens strain* only and not the other tested microbes. Biosurfactants from either *Pseudomonas* strains bacteria under study could inhibit the growth of *Candida glabrata* MTCC3981 even at a dose of 100 μg.

However, the biosurfactants did not inhibit the growth of the respective producing microbes up to a concentration of 100 μg.

Table7.5. Antimicrobial potency of biosurfactants from *B. subtilis* DM-03 strain. + sign indicates inhibition of microbial growth whereas – sign indicates no influence on growth of the tested microorganism. (+): zone of inhibition around 5 mm around the application of biosurfactants. (+ +): zone of inhibition around 5 mm to 10 mm around the application of biosurfactants. (+ + +): zone of inhibition for more than 15 mm around the application of biosurfactants.

Microorganisms	Zone of inhibition			
	Lipopeptides from *B. subtilis* DM-03			
	25µg	50µg	75µg	100µg
Control	-	-	-	-
E coli	-	-	-	-
Kluyver cryocrescens	+	+ +	+ +	+ + +
Ralstonia solanaceae	+ +	+ + +	+ + +	+ + +
Saccharomyces cerevisiae	+	+ +	+ +	+ + +
Pichia anamala	-	+	+ +	+ +
Candida glabrata	-	-	-	-

Table 7.6. Antimicrobial potency of biosurfactants from *B. subtilis* DM-04 strain. + sign indicates inhibition of microbial growth whereas – sign indicates no influence on growth of the tested microorganism. (+): zone of inhibition around 5 mm around the application of biosurfactants. (+ +): zone of inhibition around 5 mm to 10 mm around the application of biosurfactants. (+ + +): zone of inhibition for more than 15 mm around the application of biosurfactants.

Microorganisms	Zone of inhibition			
	Lipopeptides from *B. subtilis* DM-04			
	25µg	50µg	75µg	100µg
Control	-	-	-	-
E coli	-	-	-	-
Kluyver cryocrescens	+	+	++	++
Ralstonia solanaceae	-	-	-	-
Saccharomyces cerevisiae	-	-	+	+
Pichia anamala	-	-	-	-
Candida glabrata	-	-	-	-

Table 7.7. Antimicrobial potency of biosurfactants from *P. aeruginosa* M strain. + sign indicates inhibition of microbial growth whereas – sign indicates no influence on growth of the tested microorganism. (+) : zone of inhibition around 5 mm around the application of biosurfactants. (+ +): zone of inhibition around 5 mm to 10 mm around the application of biosurfactants. (+ + +): zone of inhibition for more than 15 mm around the application of biosurfactants.

Microorganisms	Zone of inhibition			
	Biosurfactants from *P. aeruginosa* M			
	25µg	50µg	75µg	100µg
Control	-	-	-	-
E coli	+	+ +	+ +	+ + +
Kluyver cryocrescens	+	+ +	+ +	+ + +
Ralstonia solanaceae	+ +	+ + +	+ + +	+ + +
Saccharomyces cerevisiae	+	+ +	+ +	+ + +
Debanyomyces hansenii	+	+ +	+ +	+ + +
Pichia anamala	+	+ +	+ +	+ + +
Candida glabrata	-	-	-	-

Table 7.8. Antimicrobial activities of biosurfactants from *P. aeruginosa* NM strain. + sign indicates inhibition of microbial growth whereas – sign indicates no influence on growth of the tested microorganism. (+) : zone of inhibition around 5 mm around the application of biosurfactants. (+ +): zone of inhibition around 5 mm to 10 mm around the application of biosurfactants. (+ + +): zone of inhibition for more than 15 mm around the application of biosurfactants.

Microorganisms	Zone of inhibition			
	Biosurfactants from *P. aeruginosa* NM			
	25µg	50µg	75µg	100µg
Control	-	-	-	-
E coli	-	-	-	-
Kluyver cryocrescens	+	+	++	++
Ralstonia solanaceae	-	-	-	-
Saccharomyces cerevisiae	-	-	-	-
Debanyomyces hansenii	-	-	-	-
Pichia anamala	-	-	-	-
Candida glabrata	-	-	-	-

7.5. Assessment of mosquito larvicidal potency activity of biosurfactants

7.5.1 Larvicidal activity (LC_{50}, LC_{100}) of biosurfactants against *C. quinquefasciatus* larvae

As shown in Fig. 7.9, crude lipopeptides from both the *Bacillus* strains exhibited dose dependent larvicidal activity against 3rd instar *C. quinquefasciatus* larvae. This is the first evidence showing the larvicidal potency of the compounds from *B. subtilis* bacteria. However, the lipopeptides from *B. subtilis* DM-03 showed better larvicidal potency (post 24 h LC_{50} 120.0 ± 5.0 mg/l, mean ± S.D.) as compared to lipopeptides from *B. subtilis* DM-04 (post 24 h LC_{50} 300.0 ± 8.0 mg/l, mean ± S.D.). Cent percent mortality was observed only with the crude lipopeptides from *B. subtilis* DM-03 at a concentration of 300 mg/l and above post 48 h application. However 100% mortality of the larvae of could not be achieved with the lipopeptides from *B. subtilis* DM-04 strains up to a dose of 600 mg/l post 72 h application.

Biosurfactants from *P. aeruginosa* M could kill 40.0% of the 3rd instar *culex* larvae at a dose of 600 mg/l post 48 h of application (Table 7.9). On the other hand, identical dose of biosurfactants from *P. aeruginosa* NM could kill 30% of larval population post 72 hrs of application (Table 7.10).

7.5.2 Effect of physico-chemical and biotic factor on the larvicidal efficacy of lipopeptides from the *B. subtilis* strains.

A bacterial larvicidal preparation should have resistance against various detrimental physico-chemical and biotic factors such as temperature, water pH, sunlight (UV radiation) etc. But in the present study, the larvicidal potency (LC_{50}) of crude lipopeptides from *B. subtilis* DM-03 and DM-04 strains at 23 ° C was reduced to 4.2 and 4.5 folds respectively of their original activity at 35 °C (Table 7.11). Moreover, heating the crude lipopeptides at 100 °C up to 60 min did not significantly ($p > 0.05$) effect their larvicidal potency (Table 7.11), indicating the thermostable nature of *B. subtilis* CLPs. Our study showed that the crude lipopeptides from either *B. subtilis* strain was insensitive to UV/ sunlight exposure as sunlight irradiated and non-irradiated (control) lipopeptides were equally effective in killing the larvae (Table 7.11).

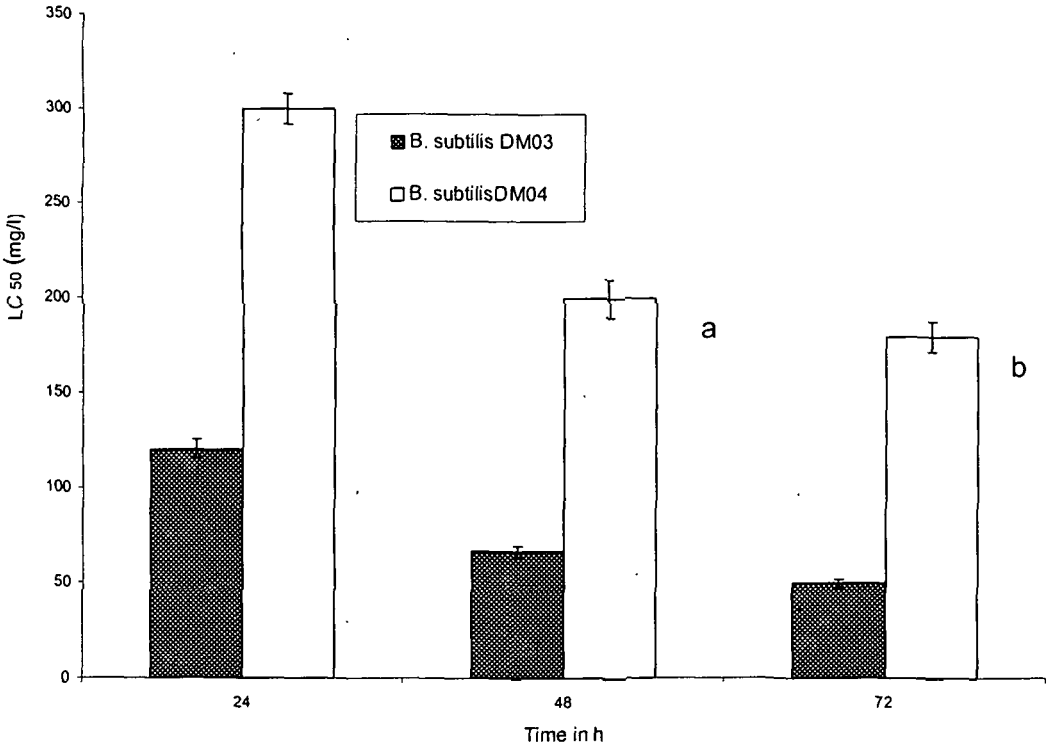

Fig 7.9. Lethality [LC $_{50}$ (mg/l)] of the crude lipopeptides from *B. subtilis* DM-03 and DM-04 against 3rd instar *Culex quinquefasciatus* larvae. Data recorded post 24 h, 48h and 72 h of lipopeptides treatment. Each Result represents the mean of three independent experiments. Significance of difference with respect to 24 h LC $_{50}$ value; ^{a} $p < 0.05$; ^{b} $p < 0.01$.

Table 7.9. Mosquito larvicidal potency of biosurfactants from *P. aeruginosa* M strain. Results represent mean ± S.D of three independent experiments.

S. No.	Biosurfactant concentration mg/l	% mortality in		
		24 hours	48 hours	72 hours
1	0 (Control)	0	0	0
2	25	0	0	0
3	50	0	0	0
4	66.6	0	0	0
5	120	0	0	0
6	180	0	0	0
7	200	0	0	5
8	300	0	10 ± 1	15 ± 1
9	400	10	15 ± 1.5	20 ± 2
10	500	15 ± 1	20 ± 1.5	25 ± 2
11	600	20 ± 2	40 ± 2	40 ± 2.5

Table 7.10. Mosquito larvicidal potency of biosurfactants from *P. aeruginosa* NM strain. Results represent mean ± S.D of three independent experiments.

S. No.	Biosurfactant concentration mg/l	% mortality in		
		24 hours	48 hours	72 hours
1	0 (Control)	0	0	0
2	25	0	0	0
3	50	0	0	0
4	66.6	0	0	0
5	120	0	0	0
6	180	0	0	0
7	200	0	0	0
8	300	0	0	0
9	400	0	0	0
10	500	0	16 ±1	16 ±1
11	600	16 ± 1.5	20 ± 2	30 ± 1.5

Table 7.11. Influence of physico-chemical factors on the larvicidal efficacy (LC_{50}) of crude CLPs from *B. subtilis* strains. Results recorded post 24 h application and each value represents the mean ± S.D of three individual experiments.

Physio-chemical parameters	LC_{50} (mg/l)	
	***B. subtilis* DM-03**	***B. subtilis* DM-04**
Incubation Temperature (°C)		
23	120 ± 5.0	300 ± 8.0
35	115 ± 5.1	286 ± 11.0
Heating period (min) at 100 (°C)		
Control (no heating)	120 ± 5.0	300 ± 8.0
10	122 ± 4.0	306 ± 5.0
30	124 ± 5.0	312 ± 7.0
60	130 ± 6.0	320 ± 10.0
pH of the water		
5.0	126 ± 4.5	320 ± 10.0
6.0	122 ± 4.0	310 ± 9.0
7.0	120 ± 5.0	300 ± 8.0
8.0	121 ± 3.9	305 ± 5.0
9.0	124 ± 3.5	308 ± 6.0
10.0	124 ± 4.0	314 ± 6.0
11.0	133 ± 6.0[a]	328 ± 11.0[a]
Exposure to sunlight (h)		
Control (No exposure)	120 ± 5.0	300 ± 8.0
6	125 ± 5.0	306 ± 9.0
8	129 ± 6.0	314 ± 10.0

Significantly different with respect to LC_{50} value at pH 7.0. [a] $p < 0.05$

7.5.3 Bio-safety of lipopeptide biosurfactants from *B. subtilis* strains to non-target aquatic organism.

The current ecotoxicological requirements for all new industrial chemicals are that acute toxicity tests must be carried out using fish (directive 79/831/EEC), and fish are still the backbone of the environmental safety testing. As shown in Table 7.12, crude CLPs from *B. subtilis* strains at a dose of post 24 h LC_{50} for mosquito larvae did not change the normal physiological as well as serum biochemical parameters of groups B and C fishes ($p > 0.05$) as compared to control (group A) fish, documenting their bio-safety to non-target aquatic organism.

Table 7.12. Effect of CLPs from *B. subtilis* DM-03 and DM-04 strains on the normal physiological and serum biochemical parameters of Indian major carp, *Labeo rohita*. Experiment was done as described in the text. Each value represents the mean ± S.D of three independent experiment. The results from group B and C fish do not differ significantly ($p<0.05$) from the results obtained from group A fish

Parameters	Group A	Group B	Group C
Physiological parameters			
Net weight gain per fish (g)	20.0 ± 2.0	18.0 ± 1.2	19.0 ± 1.0
Increase in length (cm)	2.0 ± 0.2	2.0 ± 0.1	1.9 ± 0.3
Physical appearance	Normal	Normal	Normal
Biochemical parameters of serum			
SGOT (IU/ L)	169.1 ± 6.0	173.9 ± 5.1	173.0 ± 7.0
SGPT (IU/ L)	214.1 ± 10.0	212.7 ± 11.0	213.5 ± 8.0
ALP (IU/ L)	1301.0 ± 40.0	1295.1 ± 50.0	1300.1 ± 40.0
Total Cholesterol (mg/dl)	88.0 ± 3.0	95.7 ± 4.0	95.5 ± 5.0
Triglyceride (mg/dl)	47.8 ± 1.0	50.1 ± 2.5	48.5 ± 1.5
Total protein (mg/dl)	5.1 ± 0.5	4.9 ± 0.2	4.9 ± 0.2
Albumin (mg/dl)	1.3 ± 0.1	1.4 ± 0.1	1.6 ± 0.2
Globulin (mg/dl)	2.9 ± 0.2	3.0 ± 0.5	3.1 ± 0.5
A/G ratio	0.45	0.47	0.52
Bilirubin (mg/dl)	4.13 ± 0.5	3.9 ± 0.3	4.4 ± 0.4

CHAPTER VIII

8.1 DISCUSSION

Phylogenetic analysis reveals that great diversity exists among biosurfactant producing microorganisms, suggesting that biosurfactant production is an important survival tool for the producing microbes and biosurfactant production appears to have evolved in an independent yet parallel fashion (Bodour et al., 2003). Cyclic lipopeptides including surfactin, iturin, fengycin, and lichenysin, are the major classes of biosurfactant produced by *Bacillus* species (Deleu et al, 1999;Vater et al, 2002). It has been shown that different strains of *B. subtilis* can produce significantly different classes of lipopeptides in a different combinations (Ahimou et al., 2000), however the exact contribution of theses molecules to their producing species, apart from influencing the cell surface hydrophobicity is not completely understood. In the present study, the significance of diverse CLP production by two *B. subtilis* strains isolated from extremely different habitats, one from petroleum contaminated soil sample and the other from the cake used for the alcohol production was explored. In these diverse environment, bacteria were dependent on the utilization of specific available substrate (e.g. hydrocarbon or starch) from the environment for their natural growth and survival.

A search of the literature indicates that the ability to produce biosurfactants is wide spread in the bacterial and archeal domains, and biosurfactants producing organisms have been isolated from a wide diversity of environments including soil, seawater, marine sediments and oil fields (Maier, 2003). The biosurfactants produced by *P. aeruginosa* at mesophilic growth conditions (30-37 °C) have been characterized as rhamnolipids (Guerra-Santos et al. 1984; Syldatk et al. 1985; Parra et al. 1989) and are found to be excellent emulsifiers of hydrocarbons. However, with the few exceptions, efficient biosurfactants production by *Pseudomonas sp.* at thermophilic growth condition (45 °C) has not been reported. In the present study, two *Pseudomonas aeruginosa* strains were isolated by enrichment culture from hydrocarbons contaminated soil samples and they produced significant amount of biosurfactants at 45°C temperature. Infact, this was the first report describing biosurfactants production by *P. aeruginosa* strains at 45°C temperature. Further, the biochemical properties and biological activities of these biosurfactants were characterized with an aim to explore their possible industrial applications.

8.1.1 The bacterial isolates

In this study, three out of the four potential bacterial strains (viz., *B. subtilis* DM-04, *P. aeruginosa* M and NM) were isolated from petroleum crude oil contaminated soil sample where as one strain (*B. subtilis* DM-03) was isolated from fermented food (Das et al., 2004). Among the environmental samples used for the isolation of microbes, 27.2%, 25% and 41.6% of the culturable microbes obtained from petroleum sludge, petroleum contaminated soil sample and petroleum contaminated water sample respective were efficient biosurfactants producers. *Pseudomonas* strains have often been isolated from soils contaminated by water-insoluble compounds such as petroleum products (Mac Elwee et al., 1990; Arino et al., 1996). In environments contaminated by unleaded gasoline, a recent study showed that they were particularly abundant since Ridgway et al. (1990) identified up to 86% of pseudomonads among 244 isolates. Exposure to hydrophobic pollutants in contaminated soils appeared to select biosurfactants producers, which could emulsify hydrophobic compounds (Francy et al., 1991; Ron and Rosenberg, 2001). Under these specific circumstances, *Pseudomonas* species appeared to exhibit the capacity to produce biosurfactants. However this property did not seem to be shared by other strains of this species which have been isolated from uncontaminated environment (Persson and Mollin, 1987). A study of polyaromatic hydrocarbons (PAHs) degrading isolates obtained from contaminated and soil sites showed that 67% of the isolates produced surfactants (Willumsen and Karlson, 1997). In a different study, twenty of the 21 soil samples (including uncontaminated, hydrocarbon-contaminated, metal contaminated and hydrocarbon-metal co-contaminated), were found to contain at least one biosurfactant producing isolate even using a very limited screening methology (Bodour et al., 2003). Hence, the association of biosurfactants production with microbial degradation of hydrocarbons is a well-established phenomenon. In fact, all the earlier reports on biosurfactant production came from the research works on hydrocarbon fermentation (Cooper and Zajic, 1980; Zajic and Steffens, 1984). So effort was directed to isolate the biosurfactants producing *P. aeruginosa* strains by enrichment culture on n-hexadecane as carbon source. The enrichment culture technique was extremely useful in selection and subsequent isolation of the hydrocarbons degrading potential bacteria. These bacteria were further characterized for biosurfactants production.

The soil borne *Bacillus* strain in the study, viz. *B. subtilis* DM-04 was isolated by crude petroleum contaminated soil sample and the food borne strain viz., *B. subtilis* DM-03 from fermented food without enrichment culture (by direct plating). Occurrence of *B. subtilis* strain

in soil has been reported earlier (Knox et al., 2000; Reva et al., 2004). However, strains of *Bacillus* and *Pseudomonas* at hydrocarbon contaminated sites have been determined to be biosurfactant producer (Jennings and Tanner, 2000). *B. subtilis* strains are always present in high numbers in fermented product (Sarkar et al., 1994; Nout et al., 1998; Roongsawang et al., 2000; Xia et al., 2005). This species has also been associated with incidents of food-borne disease related to unheated food such as ropy bread (Nout et al., 1998).

8.1.2 Fatty Acid Methyl Ester (FAME) profile analysis for taxonomic identification of isolated microbes

The analysis of fatty acid methyl ester (FAME) profiles by gas chromatography (GC) is a rapid and inexpensive technique that holds great promises in microbial identification. FAME analysis is based on the facts that there are a large number of different kinds of fatty acids in the cellular lipids of microorganisms and that different microorganisms have different combinations of these fatty acids. Because fatty acids can be readily volatized following methylation (that also prevent their oxidation during GC analysis), FAME profiles can easily be analyzed by gas chromatography following the standard protocol of Microbial Identification System. When FAME profiles study is coupled with principal component analysis, more accurate information can be obtained to identify similarities and differences in characters among microbial communities.

The analysis of whole cell FAME profiles has certain advantages over the conventional biochemical test used for strain identification (Buyer et al., 1996) like (i) Fatty acid analysis is more objective and less prone to human error. (ii) In contrast to biochemical tests, fatty acid composition is not influenced by plasmid loss or gain and is rarely influenced by organism mutation. (iii) Fatty acid analysis is based on species database (accounts for normal species variability) versus a series of yes/no answer (iv) Biochemical- based identification can take up to several days, compared to just several hours for fatty-acid-based identification.

Similarly, FAME analysis has some unique advantage over DNA based microbial identification which includes such as (i) the cost for fatty acid analysis is low as compared to microbes identification by DNA techniques (ii) technical proficiency required for DNA techniques is high (iii) fatty acid analysis can identify to the strain level versus the species level for most DNA based methods. (iv) DNA- based identification is very labor intensive (v)

DNA-based identification can take up to several day, compared to just hours for fatty-acid based identification.

Characterization of physiological and morphological properties and GC analysis of cellular FAME of two of the isolated bacterial strains viz., PS-3 and SC-1confirmed their identity as *Bacillus subtilis*. As discussed in section 8.1.3, based on the 16s-23S rRNA PCR-RFLP analysis, these were proved to be different strains, hence subsequently designated as *B. subtilis* DM-03 and *B. subtilis* DM-04 strains respectively.

Similarly, the physiological and morphological characterization along with GC analysis of cellular FAME of bacterial strains viz., PS-1 and PS-2 leads to their identification as *Pseudomonas aeruginosa.* A minor difference in the percentage composition of the cellular FAME was observed between these strains, and one of them was identified as *Pseudomonas aeruginosa* mucoid strain. This was named as *P aeruginosa* M where as the non-mucoid strain was designated as *P. aeruginosa* NM.

8.1.3 Strain differentiation by 16S-23S rRNA PCR-RFLP

The two strains of *B. subtilis* in this study viz., *B. subtilis* DM-03 and DM-04 were different at molecular level as was evident from the variation in the band patterns of the digested amplified ISR when resolved in 3% (w/v) agarose gel. However, the similar band patterns of the digested amplified ISR of both the *Pseudomonas* strains in 3% (w/v) agarose gel nullified the polymorphism in the ISR region of these strains, indicating that they probably belonged to the same phylogenetic group.

The sequencing of 16S rRNA alone cannot discriminate among closely related species because of the highly conserved nature of this region (Stackebrandt and Goebel, 1994). However, the ISR of 16S-23S was reported to possess much grater variability in the DNA sequence and proved to be much more useful for the differentiation of closely related bacterial strains (Jensen et al, 1993; Garcia- Martinez et al, 1999, 2001; Johnson et al., 2000; Shaver et al., 2002). Moreover, PCR- RFLP of the 16S-23S ISR was reported to be comparatively much cheaper and simpler technique than sequencing the whole ISR (Gurtler and Stanisich, 1996; Guasp et al., 2000; Kabadjov et al., 2002) and therefore advantage of this technique was exploited to differentiate the *B. subtilis* DM-03 and DM-04 and *P. aeruginosa* M and NM strains at molecular level.

8.1.4 Can the isolated *B subtilis* or *P. aeruginosa* strains be referred to as thermophilic bacteria?

Both the *B. subtilis* strains in the present study displayed optimum growth at 55°C temperature, demonstrating their thermophilic nature (Sharp and Munster 1986; Weigel and Ljungdht 1986). However maximum biosurfactants production was observed at 45 °C and 55 °C for *B. subtilis* DM-03 and DM-04 strains respectively, showing that optimum temperature for growth and biosurfactants production may not be same for a particular *B. subtilis* strain and hence it was concluded that temperature requirement was strain specific. Many of the previous reports supported this observation (Cooper et al., 1981; Makkar and Cameotra, 1997a; Kowall, 1998; Vater et al., 2002)

Both the *P. aeruginosa* strains in the present study displayed optimum growth and biosurfactants production at 45°C temperature, revealing moderate thermophilic nature of the strains (Sharp and Munser, 1986; Weigel and Ljungdht, 1986; Alcamo, 1997). However, due to poor growth of these strains at 50 °C, it would be better to refer them as thermotolerant, instead of stating them as thermophilic strains. Although there existed few reports on biosurfactants production by the *Bacillus* sp. under thermophilic condition (Banat 1993;Makkar and Cameotra 1997a,b, 1998); however not a single report existed showing the optimum temperature requirement for biosurfactants production by *Pseudomonas sp.* exceeds 45 °C. The optimum growth temperature for the *P. aeruginosa* M and NM strains was higher than that reported by Yuste et al., (2002). While studying rhamnolipid production by *Pseudomonas aeruginosa* 44T1 on glucose, Robert et al., (1991) noticed that the best temperature for the product formation was 37°C.

8.1.5 The choice of the nitrogen and carbon sources has major influence on the yield of biosurfactant production by the microbial community

The choice of the nitrogen and carbon source has a major influence on the yield and biosurfactant production by bacteria under study (Suzuki et al., 1974; Desai et al., 1994). The role of nitrogen in influencing biosurfactant production was quite evident in our study, because the microbes growing on nitrogen depleted medium showed poor surface activities (biosurfactant production) as well as least growth, which was in good agreement with the findings of Makkar and Cameotra (1997a, 1998). However, our observation as well as the

findings from other laboratories suggested that different strains of *B. subtilis* had different preferences for either organic or inorganic nitrogen for growth and biosurfactants production. For example, *B. subtilis* DM-03 preferred ammonium nitrate but *B. subtilis* DM-04 was grown well when medium was supplemented with tryptone; *B. subtilis* MTCC 1427 strain failed to utilize ammonium ions but exhibited a preference for nitrate ions (Makkar and Cameotra, 1998); *B. subtilis* MTCC 2423 was not able to utilize ammonium sulphate, but exhibited preference for nitrate ions (Makkar and Cameotra, 1997).

It has been well established that ammonium chloride served as the best source of nitrogen for growth and biosurfactants production for various *Pseudomonas* sp., like *Pseudomonas aeruginosa* 50.3 (Turkovskaya et al., 2001), *Pseudomonas aeruginosa* ATCC 10145 (Chayabutra and Ju, 2001). *Pseudomonas aeruginosa* PTCC 1637 (Tahzibi et al., 2004) etc. Present work also agreed with these findings and further, dose dependent study had proved that among the tested nitrogen sources, 0.1% (w/v) concentration of ammonium chloride served as the best nitrogen source maximum for growth of and biosurfactants production by *P. aeruginosa* M and NM strains. However, the observation that a further increase in the concentration of the ammonium chloride (>0.1 %) resulted in a decrease in the biosurfactants production may be related to the fact that an optimum C/N ratio was required by *P. aeruginosa* for maximum biosurfactants production. An increase in the nitrogen concentration resulted in a lower rhamnose production as well as higher interfacial tension values (Guerra-Santos et al., 1984). Syldatk et al. (1985) reported that nitrogen limitation in the medium caused an overproduction of rhamnolipids by *Pseudomonas* sp.

Haferberg et al., (1986) and Guerra Santos et al., (1984) reported that the majority of known biosurfactants are synthesized by microorganisms grown on water- immiscible hydrocarbons, but some have been produced on water soluble substrates such as glucose, glycerol and ethanol (Cooper et al., 1981; Palejwala and Desai, 1989). In the present study it was observed that *B. subtilis* strains preferred glucose (2% w/v) whereas, the *P. aeruginosa* strains had a preference for glycerol (2% v/v) for maximum growth and biosurfactants production, which reflected the difference in the choice of carbon source for growth and energy production by bacteria at the genus level.

The fact that glucose was the best carbon source for biosurfactants production by both the *Bacillus* strains in this study was in good agreement reports from many other laboratories (Nakano et al., 1988; Sandrin et al., 1990; Roongsawang et al., 2002; Vater et al., 2002).

Saccharose and fructose had also been mentioned as efficient carbon sources while the presence of glycerol severely decreased surfactin production. In contrast to other biosurfactants, surfactin biosynthesis did not follow stimulation by hexadecane (Cooper et al., 1981; Sandrin et al., 1990). Makkar and Cameotra (1997a, 1998, and 2001) described the ability of *Bacillus* strain in their study to use starch and sucrose as the preferred carbon sources for maximum growth and biosurfactants production. Utilization of starch as the carbon source for biosurfactants production was observed in case of *B. subtilis* DM-03 strain in the present study.

The *Pseudomonas* strains in present investigation exhibited maximum growth and biosurfactants production when the mineral salt medium (M9) was supplemented with glycerol. Glycerol was also used by some earlier investigators like Turkovskaya et al., (2001) for biosurfactants production. Although growth on glucose had resulted in the maximum decrease in the surface tension of the medium, but the emulsifying activity was only 40%, whereas glycerol was the best source for surfactant synthesis and exhibited a better emulsifying property of 60% (Turkovskaya et al., 2001). We have demonstrated that glucose was the second best carbon source for growth and biosurfactants production by both the *Pseudomonas* strains which is in good agreement with the findings of many other groups (Guerra- Santos et al., 1984; Reiling et al., 1986; Schenk et al., 1995; Turkovskaya et al., 2001).

8.1.6 Optimization of culture conditions is an important criteria for maximization of bacterial growth along with biosurfactants production.

Various key factors such as choice of carbon and nitrogen sources, growth temperature, growth period, NaCl and mineral-salts concentrations, agitation of culture medium etc, influenced the bacterial growth along with yield of biosurfactants. Therefore, it is utmost important to optimize the various culture conditions in an order to improve the biosurfactants yield or to get the best product.

M9 mineral salt medium was best for growth and biosurfactants production by both *the B. subtilis* and *P. aeruginosa* strains. The yield of biosurfactants (6.5g/l) from *P. aeruginosa* M strain in presence of glycerol as sole source of carbon was appreciably higher than the reported biosurfactants production by other *Pseudomonas* sp. (Jarvis and Johnson, 1949; Pruthi and Cameotra 1995). *Pseudomonas aeruginosa* LBI produced rhamnolipids during the

whole cell cycle. It began to accumulate in the medium soon after incubation and followed, in some cases, a diauxic patter (Benincasa et al, 2002. They had also reported the effect of aeration on rhamnolipid formation and observed that aeration rate (K_La) of 169.9 h^{-1} was optimum for rhamnolipid production by *Pseudomonas aeruginosa* LBI. A batch feed process often enhances the production yield (Linhardt et al., 1989). Benincasa et al. (2002) reported a product conversion yield of 70% and the production was 0.20 g/l per hour, which was higher than that reported by Reiling et al. (1986) while cultivating *P. aeruginosa* DSM 2659 on glucose under continuous culture conditions. $NaNO_3$ optimum for rhamnolipid production by *Pseudomonas aeruginosa* UW-1 (Sim et al, 1997), *Pseudomonas aeruginosa* 47T2 NCBIM 40044 (Haba et al., 2003), *Pseudomonas aeruginosa* LBI (Benincasa et al., 2002), *Pseudomonas aeruginosa* AT 10 (Abalos et al, 2002), *Pseudomonas aeruginosa* 47T2 NCBIM 40044 (Haba et al., 2003).

Further, It could be assumed that the ability of both the *Bacillus* strains to tolerate high salt (NaCl) concentration (5%, w/v) for growth and biosurfactants production makes them suitable candidates for their field application in saline environment (Bryant, 1987; Banat, 1995; Wilson and Bradley, 1997; Mulligan, 2005).

A biosurfactants yield of 15.6 g/l was reported by Sim et al., (1997), by cultivating *P. aeruginosa* ATCC 10145 on corn oil as the carbon source. *P. aeruginosa* UW-1 produced 24.3 g/l of biosurfactants using 6% (v/v) canola oil after 9 days of incubation at 30 °C (Sim et al., 1997).

As shown by many workers agitation of the Erlenmeyer flask has a major role in the bacterial growth and biosurfactants production (Syldatk and Wagner, 1987; Turkovskaya et al, 2001) Agitation helped in the mixing of mineral salts components of the medium. Intense aeration was crucial for the culture growth and biosurfactant synthesis. Besides supplying oxygen, aeration also helped in mixing the inoculum with the media composition. The agitation at 200 rpm was observed as optimum for all the four bacterial strains in the present study which was in a good agreement with the findings from many other laboratories (Makkar and Cameotra, 1997a,b, 1998; Sim et al, 1997). However, Turkovskaya et al, (2001) reported that agitation at 160 rpm was optimum for maximum biosurfactant production by *Pseudomonas aeruginosa* 50.3. An increase of agitation speed from 250 to 500 rpm caused a decrease in surfactant production by *Nocardia erythropolis* due to a shear rate effect on the growth kinetics of the microorganism (Syldatk and Wagner, 1987). We also observed a similar result.

Chayabutra and Ju, (2001) reported that *P. aeruginosa* ATCC 10145 could grow optimally at a hexadecane concentration of 8% (v/v). The findings of the optimum amount of NaCl requirement and pH, NH_4Cl for the optimum growth and biosurfactants production by the *Pseudomonas* strains in the present study was in good agreement with the findings of Chayabutra and Ju, (2001). Rhamnolipid production is influenced by the nutrients used in the culture media and also on the applied culture parameters. Guerra-Santos et al (1986) reported better yields of rhamnolipids, produce by *P. aeruginosa*, when the concentration of magnesium, calcium potassium, sodium salts and trace elements were minimized. Syldatk and Wagner (1987) reported a similar findings for a *Pseudomonas* sp. Guerra- Santos et al. (1986) also have found many operational problems such as foam formation and wall growth during continuous cultivation.

8.1.7 Correlation between diverse biosurfactants production and survival of producing bacteria in a particular habitat

Lipopeptide profile and bacterial cell surface hydrophobicity varies greatly within the strains. Although the ability of *Bacillus subtilis* to synthesize lipopeptides is independent of bacterial hydrophobicity, but the accumulation of extracellular lipopeptides in the culture medium induce changes in the cell surface hydrophobicity of the producing strain (Ahimou et al., 2000). The hydrophobic alterations suggested the contribution of lipopeptide molecules in adhering *B. subtilis* strains by hydrophobic interaction onto the surface of various hydrophobic substrates found in that habitat, for the easy uptake of the substrate(s), presumable by increasing the surface area of substrate and increasing their apparent solubility (Ahimou et al., 2000; Ron and Rosenberg, 2001). Therefore, it may be concluded that major CLP isoforms secreted by a specific *B. subtilis* strain, may help in the better utilization of specific hydrophobic substrate(s) (eg. starch in case of DM-03 where as hydrocarbons for DM-04 strain) available in the habitat of the lipopeptides producing strain. The following observations supported this hypothesis-

(a) *B. subtilis* DM-04 strain could utilize starch more efficiently ($p < 0.001$) only when growth medium was supplemented with crude CLPs secreted by *B. subtilis* DM-03 strain.

(b) Although *B. subtilis* DM-03 failed to utilize hexadecane or pyrene carbon source, but significant growth was achieved in the same hydrocarbon source(s) when incubated along with crude CLP isolated from *B. subtilis* DM-04 strain.

(c) The cell surface hydrophobicity of *B. subtilis* DM-03 strain was enhanced when incubated with lipopeptide from *B. subtilis* DM-04 strain, documenting that lipopeptides from the latter strain induce changes in the cell surface hydrophobicity of the former strain. This results in enhanced interaction of *B. subtilis* DM-03 with hydrocarbons and their subsequent utilization.

(d) Chemical analysis of the petroleum oil contaminated soil sample (from where DM-04 strain was isolated) revealed that it contains higher proportions of aliphatic hydrocarbons than the aromatic hydrocarbons and *B. subtilis* DM-04 was shown to be more efficient in utilization of aliphatic hydrocarbon viz. hexadecane as compared to aromatic hydrocarbons for growth and energy production. This finding reinforced that the type or amount(s) of the hydrophobic substrate(s) available in the parent habitat of the bacteria influences the biosurfactants production and subsequent enhance utilization of a specific group of substrate.

The surfactin molecules, owing to their larger molecular size and hence more space occupation, exhibit higher cell surface hydrophobicity than that of iturin molecules (Maget-Dana et al., 1992). This fact can be well correlated with findings from present study that *B. subtilis* DM-04 strain, producing higher amount of surfactin molecules exhibited greater cell surface hydrophobicity as compared to the *B. subtilis* DM-03 strain producing iturins as dominated forms of CLP.

Therefore in the present study, biosurfactants produced by two different strains of *B. subtilis*, isolated from extreme habitats were characterization, in an order to understand their natural role in the growth of the producing strain. The study supported the hypothesis that one group of biosurfactants would have an advantage in a specific ecological niche, whereas another group of emulsifiers would be more appropriate for a different niche (Ron and Rosenberg, 2001; Maier, 2003).

8.1.8 Surface activities (surface tension and CMC values) of biosurfactants are indicators of their efficiency

Both the *B. subtilis* strains were found to be efficient biosurfactant producers based on surface tension measurements of culture supernatants obtained from fermentation performed at various temperatures as well as pH. The biosurfactants produced by *B. subtilis* DM-04

exhibited better surface tension reducing ability in comparison to *B. subtilis* DM-04 strain. This observation supported that *B. subtilis* DM-04 strain produced higher amount of surface active compounds as compared to *B. subtilis* DM-03 strain. Production of higher amounts of surfactin by the former strain supported this observation. These surface tension reduction values were quite consistent with the reported values for the most efficient biosurfactants which had been isolated and studied so far (Cooper and Zajic, 1980; Cooper et al., 1981,1989; Desai and Banat, 1997).

The biosurfactants isolated from all the bacterial strains in the present study have lower CMC values as compared to the tested synthetic surfactant e.g. SDS. CMC is defined by the solubility of a surfactant within an aqueous phase and is commonly used to measure the efficiency of a surfactant (Desai and Banat, 1997). Biosurfactants posses low critical CMC values as compared to synthetic surfactants and thus have an advantage over chemical surfactants (Lin et al., 1998). Rhamnolipids (Steinbuchel, 1991) and lipopeptides are (Morikawa et al., 2000; Vater et al., 2002) the most efficient biosurfactants known till date. Their critical micelle concentrations (CMCs) in water can be as low as 10-20 mg/l, and the corresponding minimal surface tension (MST) ranges from 25 to 30 mN/m. Both properties compared very favorably to those of the synthetic surfactants, eg., CMC at 2023-2890 mg/l and MST at 37 mN/m for sodium dodecyl sulfate, and CMC at 590 mg/l and MST at 47 mN/m for alkylate dodecyl benzene (Lenz et al., 1992; Brandl et al.,1990; Margaert et al., 1992). The CMC values and the MST of biosurfactant from *Pseudomonas* strains in the present investigation are in close proximity with that reported by Haba et al. (2003), Syldatk et al. (1985), Mata-Sandoval et al. (1999). A mutant strain of *P. aeruginosa*, obtained by mutagenesis with N-Methyl-N'-nitro-N-nitrosoguanide designated as *P. aeruginosa* PTCC 1637 produced rhamnolipid biosurfactant having a CMC value of 9 mg/l (Tahzibi et al., 2004). The characteristic of rhamnolipids produced by the mutant strain was identical to that produced by the wild type bacteria. Moreover, biosurfactant from *P. aeruginosa* M strain was found to be excellent emulsifier and the stability of the emulsion was for a much higher period of time as compared to stability of emulsion reported by Turkovskaya et al. (2001). Biosurfactants from *Nocardia* was found to have 4.5 times more emulsion stability in comparison to the chemical surfactant SDS (Kim et al., 2000). In this study it was observed that emulsion stability of biosurfactants from *P. aeruginosa* M and NM stains is 5.4 times and 4.5 respectively more stable than SDS, as was evident from the calculation of decay constant (K_d).

8.1.9 MALDI-TOF MS analysis is a useful tool in determining the molecular mass of biosurfactants

It is well known that MALDI-TOF mass spectrometry is an innovative, highly efficient technique to characterize the molecular structures of the microbial biosurfactants or their secondary metabolites (Leenders et al., 1999; Vater et al., 2002; Bonmatin et al., 2003). The $[M+H]^+$ or $[M+Na]^+$ ions generated by this technique are very stable, thus leading to intense signals which are very useful for determining rapidly the homogeneity or heterogeneity of the samples by MS analysis (Bonmatin et al, 2003) including the molecular masses of samples with an accuracy of 0.01 to 0.02%. Therefore in this study, MALDI-TOF mass spectrometry was used as a tool to check the homogeneity of preparation and determining the molecular mass. By comparing the mass data obtained for individual fractions and by comparing the mass numbers reported for the lipopeptide complexes from other *B. subtilis* strains (Kakinuma et al., 1969; Kowall et al, 1998; Leenders et al, 1999; Peypoux et al, 1976, 1978, 1984; Schneider et al., 1999; Vater et al., 2002) the lipopeptide products of *B. subtilis* DM-03 could be identified as iturins (C_{16} –C_{19}) and surfactins (C_{13}-C_{15}) whereas the major lipopeptide isoforms of *B. subtilis* DM-04 were iturins (C_{17}-C_{18}) and surfactins (C_{13}-C_{15}). The isoforms of theses CLP were resolved on the basis of their hydrophobicities which in turn were determined mainly by the length of the fatty acid chains (Vater et al., 2002); therefore iturin isoforms were eluted first from the C18-μ Nova pack reverse phase HPLC column followed by surfactin isoforms. The *B. subtilis* DM-03 produced higher quantity of iturins as compared to surfactins (1.55 Vs 0.546 % of total lipopeptide) but the reverse was true for the *B. subtilis* DM-04 strain, where surfactin isoforms predominated over iturins. *B. subtilis* RB14, YB8 and ATCC6633 have been reported to co-produce surfactin and Iturin A, surfactin and plipastatin B1, and surfactin and mycosubtilin, respectively (Huang et al., 1993; Tsuge et al., 1996; Duitman et al., 1999). Therefore it seems that diverse CLP production by different strains of *B. subtilis* is rather a common ability and must confer some kind of competitive advantage to the producing microorganism in a particular environment (Maier, 2003). It appears that this ability has evolved across the bacterial domain in a parallel and non-convergent fashion with respect to biosynthesis and regulation (Maier, 2003)

In this study, the biosurfactants produced by both the *P. aeruginosa* strains were nearly identical demonstrating only minor difference exists in the biosurfactant isomers of these strains. In general, molecular mass of active biosurfactants from *P. aeruginosa* strains have been detected in the range from m/z 331 to 677 (Jarvis and Johnson, 1949; Chayabutra and

Ju, 2001; Haba et al., 2003; Benincasa et al., 2004), but very interestingly the m/z of most active biosurfactant isoforms secreted by *P. aeruginosa* M and NM strains were detected in the range from m/z 1348 to 1832.

8.1.10 Thermostability assay of biosurfactants: a helpful method in assessing the potency of biosurfactants before their industrial applications

Biochemical characterization of biosurfactants secreted either from *B. subtilis* DM-03 or DM-04 demonstrated their lipopepetide nature, whereas the secreted biosurfactants *P. aeruginosa* M and NM strains composed of mixture of carbohydrate, protein and lipid. High thermostable nature of these biosurfactants was evident when heating at 100 ° C for 60 min did not effect their tested biochemical or surface active properties. This was in good agreement with the earlier reports demonstrating thermostable nature of biosurfactants from other *B. subtilis* strains (Makkar and Cameotra, 1998) as well as from *Pseudomonas* strains (Johnson and Boese-Marrazzo, 1980; Turkovskaya et al., 2001). However, the crude lipopeptide from DM-04 strain preferred higher pH as compared to DM-03 strain for exerting maximum surface activity. This phenomena may be related to the presence of higher amount of surfactin isomers in the crude lipopeptide secreted by *B. subtilis* DM-04 strain because it is well established that surfactin molecules have a preference for higher pH (Morikawa et al., 2000).

8.1.11 Pharmacological characterization and biosafety assessment are important criteria before the field trial of biosurfactants

The iturin and not the surfactins isoforms, of both the *Bacillus* strains exhibited direct hemolytic activity on washed human erythrocytes, crude lipopeptide mixture from either strain showed higher hemolytic activity as compared to individual CLPs. However, because of the limited recovery of the HPLC purified iturins and surfactins molecules, the finding of Delue et al., (2003), who reported nearly 30% hemolysis by 10μM of surfactin, could not be confirmed. It seems that very low concentration of surfactins (0.05 μM in the present study) may not induce hemolysis and therefore hemolytic effect of these molecules are due to detergent like action rather than presence of any specific receptor(s) in the surface of RBC. Infact, Deleu at al., (2003) on the basis of computer stimulation modeling also suggested that hemolysis activity of surfactin results from the their detergent effect. The higher hemolytic activity of crude lipopeptide as compared to individual fractions was due to the synergistic

interaction between the surfactin and iturin molecules. The presence of surfactin, at a concentration at which alone, it is inactive, increases to a very large extent the haemolysis percent induced by iturin A (Maget-Dana et al., 1992).

The crude lipopeptide from *Bacillus subtilis* DM-03 and DM-04 exerted anticoagulant activity. Arima et al., (1968), first reported the anticoagulant property of surfactants from *Bacillus subtilis* strain. The concentration of surfactin needed to bring about 50% inhibition was about 30 µg/ml (Arima et al., 1968). The inhibition site of surfactin in Ca-clotting system is the polymerization step from fibrin monomer to fibrin polymer. The ability of surfactin to inhibit clot formation may be derived from the strong surface-active properties of surfactin (Arima et al., 1968).

The crude lipopeptide (100µg) from *Bacillus subtilis* DM-03 and DM-04 displayed *in vitro* liver tissue damaging activity, but lipopeptide biosurfactants from either source under the identical experimental condition, failed to show any detrimental effect on heart and lungs tissues showing a tissue specificity for these lipopeptides. Surfactant exerted its cellular effect by altering membrane integrity (Bernheimer and Avigad, 1970). The alteration in the membrane topography could be attributed to the ability of lipopeptide to interact with phospholipids, along with an interference with ion chelation systems (Peypoux et al, 1999). The cation membrane topography facilitated the lipopeptides to penetration into the membrane (Maget-Dana and Ptak, 1995). At a very low concentration the lipopeptide is miscible with phospholipids, the mechanism of interaction being assisted by conformational changes displayed by the peptide cycle when it collides with a lipid with which its has affinity. At high concentrations, the detergent effect was prominent and leaded to the membrane disruption (Carrillo et al., 2003). Carrillo et al., 2003 reported dose dependent surfactin induced vesicle-content leakage, finally leading to the destabilization of the membrane. The onset for membrane solubilization occurred at a surfactin / lipid ration of 0.92, which was termed as R_{sol} (Carrillo et al., 2003). It was also previously reported that in mixtures with dimyristoylphosphatidylcholine, surfactin displays fluid-phase immiscibility, suggesting the formation of surfactin-rich domains within the membrane (Grau et al., 1999). Sheppard et al., (1991) proposed the formation of ionic channels induce by surfactin in planar lipid bilayer membrane.

There was hardly any report available describing the pharmacological properties of biosurfactant produced by *P. aeruginosa*. The RBC hemolytic properties of biosurfactants

produced by both the *P. aeruginosa* strains could be exploited for rapid screening of the biosurfactant producing microbes in blood agar plates. Capability of rhamnolipids to cause hemolysis of erythrocytes was also reported earlier (Johnson and Boese-Marazzo, 1980; Tuleva et al., 2002). Since, the *P. aeruginosa* biosurfactants did not have any detrimental effects on tissues or living system, therefore the coagulant biosurfactants from both the *P. aeruginosa* strains can be exploited pharmaceutically for the development of topical (local) coagulant drugs to prevent the bleeding during minor surgical operations.

8.1.12 Plasmid DNA does not play any role in biosurfactant production by *P. aeruginosa* M strains.

Among the four bacterial strains, plasmid DNA was found on only on *P. aeruginosa* M strain. The present study revealed that *P. aeruginosa* M strain plasmid DNA had no role to play in biosurfactant production, since no significant change in the surface activity of culture supernatants as well as biosurfactant production by wild and mutants strains of *P. aeruginosa* M strain were observed. This finding reinforced the observations from other laboratories that major biosurfactant producing genes of *P. aeruginosa* are located disparately on the chromosome and not on plasmids (Maier, 2003).

8.1.13 Production of biosurfactants by utilizing non-conventional and cheaper carbon sources is cost effective and can further enhance the industrial application of biosurfactants

The economic of biosurfactants production is one of the factors, which determines its better acceptability for industrial applications and future prospect. Different ways should be explored to reduce the production costs through better yields of biosurfactants and product accumulation, economic engineering processes and use of cost-free or cost-credit feedstock for microbial growth and biosurfactant production (Makkar and Cameotra, 1997b). Currently microbial surfactants price ranges between 2 to 3 USD per 1 kg and are 20-30% more expensive than their synthetic equivalents. The choice of the medium components is very important since they constitute 50% of the total production costs. So, food industry by products or other wastes can be exploited from this point of view (Daniel and Otto, 1999; Haba et al., 2000). In recent year, researchers have targeted to use cheaper material for the production of biosurfactants.

Since *B. subtilis* secrete significant α- amylase enzymes in the culture supernatant (Das et al, 2004) and also produce lipopeptide biosurfactants, it was thought that biosurfactant production could be feasible from potato peels, a cheaper source of carbon. Present study vouch for the production of lipopeptide biosurfactants by thermophilic *Bacillus* strains using potato peels from kitchen wastes as the carbon source. There are numerous reports existed on biosurfactants production using industrial by products or waste. Mulligan and Cooper (1985) used water collected during drying of fuel-grade peat for biosurfactant production by a *Bacillus subtilis* strain. Sheppard and Mulligan (1987) used peat hydrolysate for biosurfactant production. Rhamnolipid production by a *Pseudomonas* sp. was reported when the organism was grown on olive oil mill effluent (Mercede et al., 1993), soap stick oil has (Mercede and Manersa, 1994), soapstock and waste-water from sunflower oil (Benincasa et al., 2002). Daniel et al., (1998) reported sophorolipids by *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509 from deproteinized whey and rapeseed oil in a two stage fed batch process. Bednarski et al., (2004) reported glycolipid production by *Candida antarctica* ATCC 20509 and *Candida apicola* ATCC 96134 grown on oil refinery waste. *B. subtilis* stains are known for utilization of waste substrates for biosurfactants production. For example, Ohno et al., (1996) reported the ability of *B. subtilis* NB22 strain to produce lipopeptide biosurfactant by utilizing soybean curd residue. Makkar and Cameotra (1997b) reported biosurfactant production by two *Bacillus* strain viz., *Bacillus subtilis* MTCC2423 and *Bacillus subtilis* MTCC 1427 using molasses (a by-product of sugar cane industry) as carbon source supplement in mineral medium, under thermophilic growth conditions. Hence, the utilization of potato peels in biosurfactants production by the *B. subtilis* strains could be expected to cut the production cost and subsequently commercialization of the products.

8.1.14 The isolated biosurfactants from *Pseudomonas* and *Bacillus* strains may be useful in MEOR application

The potential use of biosurfactant in Microbial Enhanced Oil Recovery (MEOR) was evaluated using the sand pack column technique. Among the four bacterial strains under study, biosurfactants from the *P. aeruginosa* were comparatively more efficient than *B. subtilis* strains in releasing appreciable amount of oil (kerosene) from sand pack column. The percent oil release from both the *P. aeruginosa* strains was in good agreement with the report by Pruthi and Cameotra (1997) using biosurfactant produced by a strain of *Serratia marcescens*. This recovery of oil from saturated sand pack column by either biosurfactant in the present study is much higher than that of the biosurfactants from *Bacillus subtilis* strains

as reported by Makkar and Cameotra, (1998), but lower than the biosurfactant from a thermophilic *Bacillus* sp. (Banat, 1993). The ability of both the stains to grown in high salt concentration, wide range of temperature (30-55°C) and pH (4-12) could be exploited for such industrial application, such as for *in situ* oil recovery from oil wells with moderate well temperature.

8.1.15 Application of biosurfactant producing bacteria and isolated biosurfactants in bioremediation of oil contaminated environmental samples: an eco-friendly strategy to degrade the hydrocarbon components

Oil pollution accidents are now a days become a common phenomenon and have caused ecological and social catastrophes (Shaw, 1992; Burger, 1993; Burns et al., 1993; Mishra et al., 2001). The ability of biosurfactants to emulsify hydrocarbon- water mixtures has been widely reported. These emulsification properties has also been demonstrated to enhance hydrocarbons degradation in the environment, hence making them potentially useful tools for oil spill pollution-control (Atlas and Bartha, 1992; Atlas, 1993b; Bertrand et al., 1994).

Many of the standard treatment processes used to decontaminate soil and groundwater have been limited in their application, are prohibitively expensive, or may be only partially effective (Nicholas, 1987). Problems associated with the clean up of leaking disposal sites and spills of toxic substances have demonstrated the need to develop remediation and waste reduction technologies that are effective, economical and rapidly deployable in a wide range of physical setting (Catallo and Partier, 1992).

Traditional methods of treating soil and groundwater contamination have relied upon removal or containment (Brown et al., 1986). Most of these treatment schemes are not completely effective and do not offer permanent solutions for containment or remediation. Some methods might even create additional uncontrolled hazardous waste. Therefore, this is the urgent demand by the society to greatly reduce the volume and toxicity of waste and development of safe, effective and economic alternatives for its disposal (Nicholas, 1987).

Both *in situ* and on-site treatment processes by involving the use of microorganisms to break down hazardous organic environmental contaminants, avoid the economic and technical disadvantages, as well as environmental risks, incurred by transport of hazardous wastes to alternative treatment facilities (Ahlert and Kosson, 1983; Lee and Ward, 1985).

An assessment and comparison of the capability of *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains was made to explore biodegradation efficiency of oily sludge, a hazardous hydrocarbon waste generated by the petroleum industry. Survival of the microorganisms in the soil after their seeding/ inoculation is a key deciding factor in the rate of biodegradation of hydrocarbons (Ramos et al., 1991). Since all the bacteria in the present study were isolated from a petroleum contaminated soil sample, therefore they survived and adopted the oil-contaminated soil environment very easily (Sugiura et al., 1997; Mishra et al., 2001; Rahman et al., 2003).

Natural soil bacteria present in this oily sludge may be in a dormant or slow-growing state, did not show appreciable biodegradation of hydrocarbons even when supplied with glucose or mineral salts media (control experiment). It has been reported that bioremediation is negligible, if the population of hydrocarbon-degrading microorganisms is less than 10^5 cfu/ g in soil (Forsyth et al., 1995; Mishra et al., 2001). These reports have supported our view that a specific group of bacteria or a bacterial consortium capable of degrading the petroleum hydrocarbons must be seeded in the soil at a population greater than $1x10^5$ cfu/ g in soil for the effective bioremediation of contaminated soil (Mishra et al., 2001). Further, it has been shown in the present study that supplying of glucose as co-carbon source along with pyrene (a major carbon source) enhanced the rate of degradation of pyrene by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains along with a concomitant increase in bacterial biomass. This leads to prompt us to supply glucose in the soil after a regular interval to increase the rate of biodegradation of TPH. However, the results are in contradiction to report of Chhatre et al., (1996) describing addition of nutrients in the soil was unlikely to have dramatic effect on the microbial degradation of crude oil.

In this study, *Pseudomonas* consortium was shown to possess significantly higher potential for the biodegradation of TPH as well as various fractions of crude petroleum oil compared to *B. subtilis* DM-04 strain. It is to be mentioned that *B. subtilis* DM-03 strain was not included in biodegradation study owing to its poor growth in hydrocarbons. An organic chemical may be subjected to non-enzymatic reactions brought about by microorganisms in the soil (Alexander, 1980); however, it is the enzymatic reactions that bring about the catebolism of organic compounds (Kanaly and Harayama, 2000). Key factors such as presence of a specific and or higher amount of inducible enzyme(s), substrate specificity of hydrocarbon degrading enzymes (Gibson et al., 1984; Kanaly and Harayama, 2000; Sharnagouda and Karegoudar, 2001) etc., may be responsible for higher metabolism of TPH by *Pseudomonas*

strains. Although the biochemical pathways for the biodegradation of various PAHs by microbes have been well established (Gibson et al., 1984), however the pathways for PAHs degradation in *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains are yet to be discovered. Apart from the biodegradative enzymes, presence of sphingolipids or other specific molecule in the outer membrane structure of bacteria, enabling them to adhere to a specific substrate, may also be responsible for higher bio-degradation of TPH or a specific fraction of petroleum hydrocarbon by a specific group of bacteria (Sugiura et al., 1997). Possibility of presence of these kinds of molecules in the outer membrane of *B. subtilis* DM-04 or *P. aeruginosa* strains should be investigated in near future.

It has been stated that biosurfactant production is an important survival tool for the producing microbes (Ahimou, 2000; Ron and Rosenberg, 2001; Maier, 2003) production of biosurfactant is related to the utilization of available hydrophobic substrate(s) by the producing microbes from their natural habitat, presumably by increasing the surface area of hydrophobic substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). This hypothesis supports our present observation, where biosurfactants from *B. subtilis* DM-04 or *P. aeruginosa* M and NM strains were shown to enhance the apparent solubility of PAHs in dose-dependent manner. Hence for efficient and complete biodegradation, solubilization of contaminating hydrocarbons with biosurfactants prior to bioremediation is advantageous (Rahman et al., 2003). To achieve this goal, the oil-sludge was treated with biosurfactants prior to inoculating the known *Bacillus* or *Pseudomonas* cultures with an aim to enhance the apparent solubility of hydrophobic hydrocarbons with a corresponding increase in their bioavailability for higher biodegradation by bacteria. Moreover, use of biosurfactant producing, hydrocarbon degrading, microorganisms for bioaugmentation to enhance hydrocarbon degradation offer the advantage of a continuous supply of no-toxic and biodegradable surfatant at low cost (Moran et al., 2000; Rahman et al., 2002).

GC/FID analyses and other study demonstrated that n-alkanes were preferentially degraded compared to PAHs by all the bacteria used in this study. The outer membrane permeability of bacteria may be one of the major factors to determine the biodegradability (Sugiura et al., 1997). Solubility may be another factor to influence biodegradability, it is being known that the solubility, and hence the accessibility to catabolic enzymes, of a hydrocarbon molecule decreases as the number of its carbon atom and cyclization increases (Sugiura et al., 1997).

In conclusion, biodegradation as a treatment alternative generally consists of optimizing various physical and chemical factors of soil including optimization of nutrient and biosurfactants concentrations to stimulate the growth of the organisms that will metabolize the particular contaminants present. Therefore it is necessary to establish the optimum environmental conditions, nutrient and biosurfactants application rates in the laboratory bench-scale studies prior to practical field application of microbes for effective bioremediation.

8.1.16 Comparative study of pyrene utilization by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains: Role of biosurfactants in enhancing the apparent solubility and metabolism of pyrene

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants occurring mostly as a result of fossil fuel combustion and as by-products of industrial activities. Since many of them are either known or suspected carcinogens and mutagens, exposure to PAHs may result in a significant health risk to human populations (White, 1986), and therefore their fate in nature is of great environmental and medical concern.

In this paper, we have presented the data demonstrating differences in utilization of pyrene as sole source of carbon and energy by tow non-actinomycetes groups of bacteria viz. *B. subtilis* DM-04 and *P. aeruginosa* N and NM strains, which were isolated from a petroleum contaminated soil sample. This difference in pyrene metabolism resulted in a significantly different growth of these bacteria. Further, we studied the role of biosurfactants produced by respective bacterial strains in enhancing the apparent solubility of pyrene that leads to a significant increase in pyrene metabolism by bacteria for growth and energy production.

There are many reports on bacterial degradation of pyrene, a high-molecular weight precondensed PAH, mainly by actinomycetes groups of bacteria such as *Mycobacterium* and *Rhodococcus* (Kanaly and Harayama, 2000; Vila et al., 2001). Besides, a variety of non-actinomyces bacteria such as *P. aeruginosa*, *P. pudita*, *Flavobacterium* sp. were reported to utilize pyrene, when supplemented with other forms of organic carbons (Trzesicka-Mlynarz et al., 1995). But subsequently it was demonstrated the possibility of utilization of pyrene or other PAHs as sole source of carbon and energy by *B. subtilis* DM-04 and *P. aeruginosa* strains owing to their ability to produce biosurfactants in the culture medium (Mueller et al., 1990; Deziel et al., 1996). In the present study, it was provided further evidence of utilization

of pyrene by *Bacillus* and *Pseudomonas* bacteria as sole source of carbon by demonstrating the correlation between increase in bacterial growth (dry biomass, protein concentration and O.D. of culture medium at 600 nm) and a concomitant decrease (degradation) in pyrene content from the culture medium with respect to time. However, there are also reports on soil *Pseudomonas* sp. capable of degrading PAHs, but fail to utilize them as sole source of carbon and energy (Foght et al., 1988).

The growth (dry biomass yield) of *B. subtilis* DM-04 and *P. aeruginosa* N and NM strains at the expense of pyrene as sole carbon source after 96 h of inoculation suggested an assimilation of about 35.6%, 32.0% and 28.0 % of pyrene carbon respectively, showing differences in metabolism and utilization of pyrene by these three bacterial strains, isolated from the same environmental sample. Guo et al., (2005) demonstrated that PAH- degrading bacteria isolated from mangrove sediments possess different potential to degrade PAHs, and degradation percentages were not related to levels of PAHs contaminated in mangrove sediments. Moreover, the result of the study was also in good agreement with the findings of many previous researchers, who observe that microbial growth on pyrene did not result in complete removal of the substrate (Mulder et al., 1998; Vila et al., 2001). Observed attenuation in pyrene degradation occurred presumable by biofilm formation on pyrene crystals and consequent prevention of pyrene dissolution, while previously accumulated intermediates would allow further cell growth (Vila et al., 2001.)

There may be several key factors such as presence of a specific and or higher amount of inducible enzyme(s), substrate specificity of PAH degrading enzymes (Sharanagouda and Karegoudar, 2001, Gibson et al., 1984), are responsible for higher metabolism of pyrene by *B. subtilis* DM-04 strain compared to *P. aeruginosa* M and NM strains. The biochemical pathways for the biodegradation of aromatic compounds by microbes have been well documented (Gibson et al., 1984), and initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of PAH to a dihydrodiol by a multi-component enzyme system (Kanaly and Harayama, 2000). The identification of metabolites accumulating during the growth of *Mycobacterium* sp. strain AP1 in pyrene suggested that this stain initiates its attack on pyrene by either monooxygenation or dioxygenation at its C-5, C-5 positions (Vila et al., 2001). However, the pathway(s) for pyrene degradation in *B. subtilis* DM-04 and *P. aeruginosa* M and NM strains are yet to be discovered.

Phylogenetic analysis revealed that great diversity exists among biosurfactant producing microbes suggesting that biosurfactant production is an important survival tool for the producing microbes and appears to have evolved in an independent yet parallel fashion (Bodour et al., 2003). It has been reported that biosurfactant producing bacteria are found in higher concentration in hydrocarbon contaminated soils (Bodour and Maier, 2003), however there are only few reports of microorganisms producing surface-active compounds while growing on PAHs (Deziel et al., 1996). In the present study, all the three bacteria were found to be efficient biosurfactant producers based on (i) surface tension measurement of culture supernatants obtained after 24 h of bacterial growth and (ii) by determining the yield of biosurfactants. The production of biosurfactant by all the three bacterial strains increased significantly when the medium was supplied with glucose (as a co-substrate) along with pyrene. Glucose, which is a versatile precursor for many biomolecules, acts as inducer and co-source of carbon, energy and reducing power for microbes that leads to substantial increase in bacterial biomass with a corresponding increase in biosurfactant production.

Previous study have documented that production of biosurfactant is related to the utilization of available hydrophobic substrates by the producing microbes from their natural habitat, presumably by increasing the surface area of substrates and increasing their apparent solubility (Ron and Rosenberg, 2001). Therefore, use of biosurfactants has been reported as a mechanism to enhance the bioavailibility of hydrophobic pollutants and PAHs for microbial degradation (Thiem, 1994; Guerin and Jones, 1988). It has been demonstrated that growth of *Mycobacterium* sp. strain LB 50 IT was directly related to substrate dissolution from crystals and to the uptake of substrate from the solution by microorganisms (Wick et al., 2001). Low molecular weight biosurfactant like lipopeptides that have low critical micelle concentrations (CMCs) increase the apparent solubility of hydrocarbons by incorporating them into the hydrophobic cavities off micelles (Miller and Zhang, 1997), whereas alasan, a high molecular weight bioemulsifier complex produced by *Acinetobacter radioresistens* KA 53 enhanced the aqueous solubility of PAHs by a physical interaction most likely of a hydrophobic nature and increases the biodegradation rate of PAHs (Barkay et al., 1999).

In this study, convincing data was presented showing that *B. subtilis* DM-04, *P. aeruginosa* M and NM biosurfactants at a concentration of 0.5 mg/ml subsequently enhance the apparent solubility of pyrene by factors 5 to 7 resulting in its higher uptake and metabolism by bacteria compared to non-solubilized pyrene. The difference in pyrene solubilization effect of biosurfactants from different bacterial strains in this study may be related to the chemical

nature as well as surface properties of biosurfactants. For example, major biosurfactants secreted by *B. subtilis* DM-04 were lipopeptide in nature containing higher amount of surfactins compared to iturins, whereas biosurfactants secreted by *P. aeruginosa* M and NM were found to be a complex mixture of lipopeptides and glycoproteins. Moreover, the significantly higher pyrene solubilization effect of biosurfactants from *P. aeruginosa* NM strain compared to *P. aeruginosa* M strain reinforces the hypothesis that a minor variation in biosurfactant isoforms between these two strains may result in a large variation of the emulsification property and specificity of biosurfactants. It may be concluded that higher pyrene solubilization effect of biosurfactants from *P. aeruginosa* NM strain dramatically enhanced the metabolism of pyrene, that sustained the growth of this bacteria in pyrene, otherwise it would not be able to grow on pyrene. Further studies to understand the microbial ecology of PAHs degrading communities and their application for the development of bioremediation strategies for PAHs are in progress.

8.1.17 Potential use of the isolated biosurfactants as antimicrobial agents

Several lipopeptide surfactants including the cyclic lipopeptides of *B. subtilis* are potent antibiotics (Ron and Rosenberg, 2001; Maier, 2003, Singh and Cameotra, 2004). Interestingly, the antibiotic potency and microbial specificity of the crude lipopeptides from *B. subtilis* DM-03 and DM-04 strains as well as between *P. aeruginosa* N and NM strains differed which may be due to the production of diverse biosurfactants isoforms. It is reasonable to assume that this antibiotic specificity of lipopeptides may have a natural role in enhancing the growth of the producing bacteria by inhibiting the other interfering microbes present in their original habitat. Differences in the antimicrobial potency of crude lipopeptide produced by *B. subtilis* DM-03 and DM-04 strains against *Saccharomyces cerevisiae* and *Pichia anamala* yeast strains, which were co-isolated along with *B. subtilis* DM-03 strain from fermented food, supported the hypothesis. These yeast strains, present in the natural habitat of the *B. subtilis* DM-03 strain, compete with the bacteria for the uptake of glucose formed by the hydrolysis of starch by the action of α-amylase secreted by *B. subtilis* strains. Therefore, to prevent these yeast strains from substrate (glucose) utilization and to favor the growth of producing bacteria, higher amounts of iturin isomers produced by *B. subtilis* DM-03 strain inhibits the yeast cells probably by disrupting the plasma membrane of yeast cells by formation of small vesicles and the aggregation of their intra membranous particles (Thimon et al., 1995).

Most work on biosurfactant applications has been limited to their use in pollution control (Banat, 1995) and enhancing the availability of various hydrophobic compounds for microbial degradation (Mulligan, 2005). In addition, biosurfactants might have other applications, particularly as antimicrobial agents. Antagonistic activity of *Pseudomonas* sp. biosurfactant was reported against pathogenic fungi *Pythium ultimum* and *Rhizoctonia solani* at a concentration of 100 µg (Nielsen et al., 2002). Rhamnolipids from *Pseudomonas aeruginosa* were reported to have antagonistic activity against a wide range of microorganisms (Jarvis and Johnson, 1949; Abalos et al., 2001; Haba et al., 2003; Benincasa et al., 2004), and bacteriostatic effect on *Mycobacterium tuberculosis*, a tuberculosis causing agent. Very recently, the algicidal potency of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa* against some harmful algae was demonstrated (Wang et al., 2005) In addition, rhamnolipids from *P. aeruginosa* have potential for biological control of zoosporic plant pathogens (Staghellini and Miller, 1997). The biosurfactants from *P. aeruginosa* M strains exhibited significant anti-microbial activity on all the tested microorganisms, except *Candida glabrata* and therefore their use as broad-spectrum antibiotics is highly promising. The current investigation provided the evidence that only a minor variation in the biosurfactant isoforms can cause a huge difference in their antimicrobial specificity. However, the antibiotic potency and specificity of the biosurfactants from these strains against the medically important microbes remains to be elucidated. Moreover, in order to develop a safe drug, it is essential to explore the pharmacological properties and toxicity of the biosurfactants. Neither of the biosurfactants in this study displayed any appreciable toxicity or pharmacological properties such as hemolysis, tissue damaging activity etc. implying their biosafety in humans.

8.1.18 Application of crude biosurfactants from *B. subtilis* DM-03 and DM-04 strains as potential mosquito larvicidal agent

Culex quinquefasciatus is the dominant vector of lymphatic filariasis, which infected some 120 million people worldwide and can also lead to genital damage and elephantiasis. More recently *Culex quinquefasciatus* is regarded as a potential vector of West Nile virus (Zinser et al., 2004). Although, lipopeptides particularly iturin are known to possess antimicrobial activities (Ohno et al, 1996; Cameotra and Makkar, 2004; Singh and Cameotra, 2004), but there has not been a single reports demonstration the anti-insecticidal (against mosquito larvae) potential of the lipopeptide.

Lipopeptide biosurfactants from *Bacillus subtilis* DM-03 exhibited better antimosquitocidal activity than *Bacillus subtilis* DM-04 in the tested 3rd instar *Culex quinquefasciatus* larvae. The difference in the larvicidal potency between the crude CLPs from *B. subtilis* DM-03 and DM-04 strains was due to presence of quantitatively as well as qualitatively different proportions of iturins and surfactins in the crude lipopeptides. The crude lipopeptides of *B. subtilis* DM-03 contained more iturin molecules whereas *B. subtilis* DM-04 strain contained higher amount of surfactins as revealed by MALDI-TOF mass spectral analyses in the present study. However, our results are in contradiction to the report of Assie et al. (2002), who had showed that surfactin molecules and not the iturins displayed a dose dependent insecticide activity. Therefore, the mosquito larvicidal activity of individual lipopeptides from *B. subtilis* and their exact mode of action remain to be elucidated. Mortality of the *culex* larvae may have occur due to the disruption of the cell membrane by detergent effect of the lipopeptides (Maget-Dana and Ptak 1995) with the formation of ionic channels on the membrane (Sheppard et al, 1991).

Several physico-chemical and biotic factors such as pH of water, temperature, sunlight, larval age etc. have been reported to influence the efficacy of bacterial formulation or toxins against the target mosquito larvae (Mulla, 1985; Becker et al, 1992; Mittal et al., 1995). For example, the efficacy of spherix (*B. sphaericus*) and bactoculicide (Bti) formulations against anopheline larvae were reduced to about 10 fold in laboratory bioassay at 21 ° C as compared to 31 ° C. But in the present study, larvicidal potencies (LC_{50}) of crude lipopeptides from *B. subtilis* DM-03 and DM-04 strains were reduced to 4.2 and 4.5 folds of their original activity respectively at 23 ° C as compared to 35°C. Moreover, heating the crude lipopeptide solutions at 100°C up to 60 min does not influence their larvicidal potency, showing extremely thermostable nature of these lipopeptides, this property makes them suitable for application in tropical countries.

It has been reported that protein toxins of Bti and *B. sphaericus* are highly sensitive to sunlight (UV radiation). For example, exposure to sunlight for 6 h reduces the biolarvicidal potency of Bti and Bs to about 50% and 75 % respectively of their original activity (Mittal, 2003). Sunlight irradiation brings about widespread destruction of indole residues of protein crystals from *B. thuringiensis* var *kurstaki* (Pozsgay et al., 1987). This study showed that the crude lipopeptides from either *B. subtilis* strain was insensitive to UV/ sunlight exposure as sunlight irradiated and non-irradiated (control) lipopeptides were equally effective in killing

the larvae. This clearly demonstrated the greater UV radiation stability of *B. subtilis* lipopeptides as compared to Bti and Bs toxins.

Previous studies have shown that stability, solubility and insecticidal activity of the crystal toxins of *B. thuringiensis* var *aizawai* and *B. thuringiensis* var *kurstaki* are effected by pH of the medium (Nishiitsusuji-Uwo et al., 1977; Gringorten et al., 1992). In the present study, there was significant ($p< 0.05$) decrease in the larvicidal potency (LC_{50}) of *B. subtilis* CLPs at pH 11.0, as compared to pH 7.0. The optimum range of pH at which *B. subtilis* CLPs exhibited maximum larvicidal efficacy was observed in between 5 to 10. The results of this study revealed that although the CLPs from *B. subtilis* strains possess lower mosquito larvicidal potency as compared to the currently available larvicidal preparations of the other bacteria, the higher stability of *B. subtilis* lipopeptides against heating, sunlight exposure and different pH render this bacterium to be considered as a potential candidate for mosquito larvicidal control.

In conclusion, the crude CLPs secreted by *B. subtilis* strain had shown larvicidal activity against *Culex* mosquito, can withstand many environmental stresses like extreme pH, sunlight/UV radiation etc., and they did not impart toxicity to the tested aquatic vertebrate *Labeo rohita* up to a concentration that induced mortality in the mosquito larvae. These properties can be exploited for the formulation of a safer, novel biopesticide for effective control of mosquito larvae. Further studies to assess the mosquitocidal activities of purified CLPs and their mode of action are in progress.

8.2 Conclusion

In the present study, biochemical and pharmacological properties of biosurfactants produced by two *B. subtilis* (DM-04 and DM-04) strains and two *P. aeruginosa* (M and NM) strains growing under thermophilic growth condition (45°C) were characterized. Further some of the potential industrial applications crude natural roles of the biosurfactants to their producing bacteria were also explored. Three out of the four bacteria, viz., *B. subtilis* DM-04, *P. aeruginosa* M and NM were isolated from petroleum oil contaminated soil samples whereas the remaining strain, viz. *B. subtilis* DM-03 was isolated from traditional fermented food.

The RFLP pattern of 16S-23S ISR of *B. subtilis* DM-03 and DM-04 strains showed clear difference as was evident from the different banding patterns in a 3% agarose gel.

Restriction digested amplified products of the 16S–23S ISR of *P. aeruginosa* mucoid (M) and non-mucoid (NM) strains were resolved into similar banding patterns in 3% agarose gel, documenting that these strains belong to same phylogenetic trait.

Both the *Bacillus* strains exhibited distinct preferences for the nitrogen source, pH and temperature optima for bacterial growth and biosurfactants production. For example, *B. subtilis* DM-03 had a preference for ammonium nitrate, whereas *B. subtilis* DM-04 had a preference for tryptone for maximum biosurfactants production. However, both the strains had a common preference for glucose as sole source of carbon for maximum biosurfactants production. The optimum pH for maximum growth of and biosurfactants production by *B. subtils* DM-03 and DM-04 strains was observed at pH 8.0 and pH 7.0 respectively. Comparative study showed that *B. subtilis* DM-04 had a better salt tolerance as compared to *B. subtilis* DM-03 strain.

Maximum growth of and biosurfactants production (6.5g/l Vs 5.0 g/l) by *P. aeruginosa* M and NM strains were observed at the stationary phase (96 h post inoculation) with (2.0% v/v) glycerol and (0.1% w/v) ammonium chloride as respective carbon and nitrogen sources and at an incubation temperature of 45°C and agitation set at 200 rpm.

In this study, least growth or biosurfactants production by *B. subtilis* DM-03 was observed when hydrocarbons were used as sole carbon sources. But the growth rate of DM-03 strain in hydrocarbons (eg. hexadecane and pyrene) was enhanced significantly ($p<0.001$) when crude lipopeptide isolated from *B. subtilis* DM-04 strain was added in the growth medium. Similarly, *B. subtilis* DM-04 exhibited poor growth on starch, but excellent growth was observed in the same medium, when exogenously supplemented with crude lipopeptide isolated from *B. subtilis* DM-03 strain.

Biochemical compositions of crude biosurfactants from *B. subtilis* DM-03 and DM-04 strains revealed the lipopeptide nature whereas, the secreted biosurfactants by the *P. aeruginosa* strains composed of mixture of carbohydrate, lipid and protein. The crude biosurfactants from both the *Bacillus* and *Pseudomonas* strains retained their surface-active properties in a wide range of pH as well as post heating at 100°C for 60 min.

MALDI-TOF mass spectra analyses showed that iturins and surfactins isoforms represented the major lipopeptide biosurfactants produced by *B. subtilis* DM-03 and DM-04 strains

respectively. The molecular mass of the biosurfactants present in these active peaks of *P. aeruginosa* M and NM strains were detected in the range from m/z 1348 to m/z 1832. The molecular mass of active biosurfactants from *P. aeruginosa* strains have been detected in the range from m/z 331 to 677, showing they were novel biosurfactants reported till date.

Pharmacological characterization of the crude biosurfactants from all the bacterial strains demonstrated that they induced dosed dependent lysis of washed human erythrocytes, but to different extent. Biosurfactants from both the *B. subtilis* strains demonstrated their anti-coagulant nature on platelet poor plasma, but biosurfactants from *P. aeruginosa* strains decreased the clotting time of platelet poor plasma. As the isolated crude biosurfactants from the *P. aeruginosa* strains failed to exhibit any detrimental effects on chicken heart, lungs, liver and kidney tissue, there is enough scope to develop topical (local) coagulant drugs to prevent the bleeding during minor surgical operations.

Present study shows that other industrial applications of biosurfactants and their producing microbes are highly promising. Laboratory scale study on bioremediation demonstrated the capacity of the *P. aeruginosa* strains and *B. subtilis* DM-04 strain to appreciable reduced the TPH of petroleum contaminated soil sample in a time period of 4 months. These strains were also shown to be efficient degraders of pyrene and *B. subtilis* DM-04 strain possessed significantly higher pyrene utilizing capacity compared to *P. aeruginosa* M and NM strains. Crude biosurfactants from all the bacterial strains exhibited antimicrobial activity against certain tested microorganisms, biosurfactants from *B. subtilis* DM-03 and *P. aeruginosa* M strains showed better anti-microbial potency compared to other two strains.

The lipopeptide biosurfactants from the *B. subtilis* strains exhibited dose dependent larvicidal activity against 3rd instar *C. quinquefasciatus* larvae and among the four strains, crude biosurfactants from *B. subtilis* DM-03 strain exhibited best larvicidal activity. Further, these lipopeptides were shown to be non-harmful to the aquatic non-target organism (*Labeo rohita*) and therefore, biosurfactants from *B. subtilis* DM-03 strain could be exploited for the development of eco-friendly biopesticides.

REFERENCES

Abalos, A., Pinazo, A., Infante, M.R., Casal, M., Garcia, F. and Menresa, A. 2001. Physicochemical and antimicrobial properties of new rhamnolipids produced by *Pseudomonas aeruginosa* AT10 from soyben oil refinery wastes. Langmuir 17, 1367-1371.

Abu- Ruwaida, A.S., Banat, I.M., Haditirto, S. and Kadri, M. 1991b. Nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodococcus* bacterium. World J. Microbiol. Biotechnol. 7, 53-61.

Abu- Ruwaida, A.S., Banat, I.M., Haditirto, S., Saleem, A. and Kadri, M. 1991a. Isolation of biosurfactant- producing bacteria-product characterization and evaluation. Acta Biotechnologica 11, 315-324.

Ahimou, F., Jacques, P. and Deleu, M. 2000. Surfactin and iturin A effects on *Bacillus subtilis* surface hydrophobicity. Enz. Microbiol. Technol. 27, 749-754.

Ahlert, R.C. and Kosson, D.S. 1983. *In situ* and on-site biodegradation of industrial landfill leachate. Report to Dept. of the Interior, Washington, D.C., on Contract No. 14-34-0001-1132. PB84-136787.

Al- Awadhi, N., Williamson, K.J. and Isok, J.D. 1994. Remediation of Kuwait's oil-contaminated soils. In Hydrocarbon Contaminated Soils and Groundwater, P.T. Kostecki and E.J. Calabrese (ed). Vol. 3. Lewis Publishers, Michigan, USA.

Alcamo, I.E. 1997. In: Fundamentals of Microbiology, 5th Edition. Pp 106, Addison Wesley Longman Inc., New York.

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. Science 211, 132-138.

Alexander, M. 1994. Biodegradation and Bioremediation. Academic Press, New York.

Arima, K., Kakinuma, A. and Tamura, G. 1968. Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: isolation, characterization and its inhibition of fibrin clot formation. Biochem. Biophys. Res. Commun. 31, 488-494.

Arino, S., Marchal, R. and Vandecasteele, J.P. 1996. Identification and production of a rhamnolipid biosurfactant by a *Pseudomonas* species. Appl. Microbiol. Biotechnol. 45, 162-168.

Asaka, O. and Shoda, M. 1996. Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. Appl. Environ. Microbiol. 62, 4081–4085.

Asselineau, C. and Asselineau, J. 1978. Trehalose containing glycolipids. Prog. Chem. Fats Lipids 16, 59-99.

Assie L.K., Deleu, M., Arnaud, L., Paquot, M., Thonart, P., Gaspar, Ch. and Haubruge, E. 2002. Insecticide activity of surfactins and iturins from a biopesticide *Bacillus subtilis* Cohn (S499 strain). Meded Rijksuniv Gent Fak Landbouwkd Toegep Biol Wet. 67, 647-655.

Atlas, R.M. 1992. Molecular methods for environmental monitoring and containment of genetically engineered microorganisms. Biodegradation 3, 137-146.

Atlas, R.M. 1993a. Handbook of microbiological media. L.C. Parks (eds). CRC Press, Florida, pp 529.

Atlas, R.M. 1993b. Bacteria and bioremediation of marine oil-spills. Oceanus 36, 71-81.

Atlas, R. M. and Bartha, R. 1992. Hydrocarbon biodegradation and oil-spill bioremediation. Adv. Microbiol. Eco. 12, 287-338.

Atlas, R. M. and Cerniglia, C.E. 1995. Bioremediation of petroleum pollutants. BioScience 45, 332-338.

Ausubel, F.M., Brent, R., Kinston, R.E., Moorse, D.D., Seidman, J.D., Smith, J.A. and Struhl, K. 1995. In: Short protocols in Molecular Biology. Pp. 2.12 - 2.14. Wiley, New York.

Bai, G., Brusseau, M. L. and Miller, R. M. 1998. Influence of cation type, ionic strength and pH on solubilization and mobilization of residual hydrocarbon by a biosurfactant. J. Contam. Hydrol. 30, 265–279.

Banat, I.M. 1993. The isolation of a thermophilic biosurfactant producing *Bacillus* sp. Biotech. Lett. 15, 591-594.

Banat, I.M. 1995. Biosurfactants production and possible uses in microbial enhanced oil recovery and oil pollution remediation: A review. Bioresour. Technol. 51, 1-12.

Banat, I.M., Makkar, R.S. and Cameotra, S.S. 2000. Potential commercial applications of microbiol surfactants. Appl. Microbiol. Biotechnol. 53, 495-508.

Banat, I.M., Samarah, N., Murad, M., Horne, R. and Banerjee, S. 1991. Biosurfactant production and use in oil tank clean-up. World J. Microbiol. Biotechnol. 7, 80-88.

Barkay, T., Navon-Venezia, S., Ron, E.Z. and Rosenberg, E. 1999. Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the bioemulsifier Alasan. Appl. Environ. Microbiol. 65, 2697-2702.

Barsotti, O., Decoret, D. and Renaud, F.N.R. 2002. Identification of *Streptococcus mitis* group species by RFLP of the PCR-amplified 16S-23S rDNA intergenic spacer. Res Microbiol. 153, 687-691.

Becker, N., Zgomba, M., Ludwig, M., Petric, D. and Rettich, F. 1992. Factors influencing the activity of *Bacillus thuringiensis* var *israelensis* treatments. J. Amer. Mosq. Contr. Assoc. 8, 286.

Bednarski, W., Adamczak, M., Tomasik, J. and Plaszczyk, M. 2004. Application of oil refinery waste in the biosynthesis of glycolipids by yeast. Bioresour. Technol. 95, 15-18.

Beebe, J.L. and Umbreit, W.W. 1971. Extracellular lipid of *Thiobacillus thiooxidans*. J. Bacteriol. 108, 612-615.

Belsky, I., Gutnick, D.L. and Rosenberg, E. 1979. Emulsifier of *Arthrobacter RAG-1*: determination of emulsifier bound fatty acids. FEBS Lett. 101, 175-178.

Benincasa M., Abalos A., Oliveria I. and Manresa, A. 2004. Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock. Antonie van Leeuwenhoek. 85, 1-8.

Benincasa, M., Contiero, J. Manresa, M.A. and Moraes, I.O. 2002. Rhamnolipid production by *Pseudomonas aeruginosa* LBI growing on soapstock as the sole carbon source. J. Food. Eng. 54, 283-288.

Bergey's manual of systematic bacteriology (1984) Krieg, N.R. and Holt, J.G. (ed.). Vol.1. Lippincott Williams & Wilkins, Baltimore, USA.

Bergey's manual of systematic bacteriology (1986) Sneath, P.H.A., Mair, N.S., Sharpe, M.E. and Holt, J.G. (ed.) Vol2. Lippincott Williams & Wilkins, Baltimore, USA

Bernheimer, A.W. and Avigad, L.S. 1970. Nature and properties of a cytological agent produced by *Bacillus subtilis*. J.Gen.Microbiol. 61, 361-369.

Bloomberg, G. 1991. Designing proteins as emulsifiers. Lebensmitteltechnologie 24, 130-131.

Bodour, A.A. and Miller-Maier, R.M. 1998. Application of a modified drop-collapse technique for surfactant quantitation and screening of biosurfactant-producing microorgams. J. Microbiol. Methods 32, 273-280.

Bodour, A.A., Drees, K.P. and Maier, R.M. 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Appl. Environ. Microbiol. 69, 3280-3287.

Bonmatin, J-M., Laprevote, O. and Peypoux, F. 2003. Diversity among microbial cyclic lipopeptides: Iturins and Surfactins. Activity- structure relationships to design new bioactive agents. Combinatorial Chemistry&HighThroughput Screening 6, 541-556.

Boonchan, S., Britz, M. and Stanley, G.A. 2000. Degradation and mineralization of high molecular weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Appl. Environ. Microbiol. 66, 1007-1019.

Bragg, J.R., Prince, R.C., Harner, E.J. and Atlas, R.M. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature 368, 413-418.

Brandl, H., Gross, R.A., Lenz, R.W. and Fuller, R.C. 1990. Plastics from bacteria and for bacteria: poly (β- hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. Adv. Biochem.Eng./Biotechnol. 41, 77-93.

Brandl, H., Gross, R.A., Lenz, R.W. and Fuller, R.C. 1990. Plastics from bacteria and for bacteria: poly (β- hydroxyalkanoates) as natural, biocompatible, and biodegradable polyesters. Adv. Biochem.Eng./Biotechnol. 41, 77-93.

Bregnard, T. P. A., Hohener, P. and Zeyer, J. 1998. Bioavailability and biodegradation of weathered diesel fuel in aquifer material under denitrifying conditions. Environ. Toxicol. Chem. 17, 1222–1229.

Brown, R.A., Loper, J.R. and McGarvey, D.C. 1986. In: In situ treatment of ground water: Issuess and answers. Proc. Hazar. Mat. Spills Conf. May 5-8, 1986, St. Louis, MO. 261-264.

Brundish, D.E., Shaw, N. and Baddiley, J. 1967. The structure and possible function of the glycolipid from *Staphylococcus lactis* I 3. Biochem. J. 105, 885-889.

Bryant, F. O. 1990. Improved method for the isolation of biosurfactant glycolipids from *rhodococcus* sp. strain H13A. Appl. Environ. Microbiol. 56, 1494–1496.

Bryant, R.S. 1987. Potential use of microorganisms in petroleum recovery technology. Proc. Okla. Acad.Sci. 67, 97-104.

Bubela, B. 1987. A comparison of strategies for enhanced oil recovery using *in situ* and *ex situ* produced biosurfactants. Surfactant Science Series. 25, 143-161.

Bubela, B. 1985. Effect of biological activity on the movement of fluids through porous rocks and sediments and its application to enhanced oil recovery. Geomicrobiol. J. 4, 313-327.

Burger, A.E. 1993. Estimating the mortality of seabirds following oil- spills – effects of spill volume. Mar. Poll. Bull. 26, 239-248.

Burns, K.A., Garrity, S.D. and Levings, S.C. 1993. How many years util mangrove ecosystems recover from catastrophic oil-spills. Mar. Pol. Bull. 26, 239-248.

Busscher, H. J., Bellon-Frontaine, M.N., Mozes, N., Van der Mei, M.C., Sjollerma, J. Cerf, O. and Rouxhet, P.G. 1990. Deposition of *Leuconostoc mesenteroides* and *Streptococcus thermophilus* to solid substrata in parallel plate flow chamber. Biofouling 2, 55-63

Busscher, H. J., van der Kuijl-Booij, M. and van der Mei, H. C. 1996. Biosurfactants from thermophilic dairy *Streptococci* and their potential role in the fouling control of heat exchanger plates. J. Ind. Microbiol. 16, 15–21.

Busscher, H. J., van Hoogmoed, C. G., Geertsema-Doornbusch, G. I., van der Kuijl-Booij, M. and van der Mei, H. C. 1997. *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. Appl. Environ. Microbiol. 63, 3810–3817.

Busscher, H.J., Bellon-Frontaine, M.N., Mozes, N., Van der Mei, H.C., Sjollerma, J., Cerf, O. and Rouxhet, P.G. 1990. Deposition of *Leuconostoc mesenteroides* and *Streptococcus thermophilus* to solid substrata in parallel plate flow chamber. Biofouling. 2, 55-63.

Busscher, H.J., Neu, T.R. and Van der Mei, H.C. 1994. Biosurfactant production by thermophilic dairy *Streptococci.* Appl. Microbiol. Biotechnol. 41, 4-7.

Buyer, J. S. and Kaufmanns, D. D. 1996. Microbial diversity in the rhizosphere of corn field grown under conventional and low input system. Appl. Soil Ecol. 5, 21-27.

Cairns, W. L., Cooper, D. G., Zajic, J. E., Wood, J. M. and Kosaric, N. 1982. Characterization of Nocardia amarae as a potent biological coalescing agent of wateroil emulsions. Appl. Environ. Microbiol. 43, 362–366.

Cameotra, S.S. and Makkar, R.S. 2004. Recent applications of biosurfactants as biological and immunological molecules. Curr. Option Microbiol. 7, 1-5.

Cameotra, S.S. and Singh, H.D. 1990. Purification and characterization of alkane solubilizing factor produced by *Pseudomonas* PG-1. J. Ferment. Bioeng. 69, 341-344.

Cameron, D.R., Cooper, D.G. and Neufeld, R.J. 1988. The mannoprotein of *Saccharomyces cerevisiae* is an effective bioemulsifier. Appl. Environ. Microbiol. 54, 1420-1425.

Cappuccino, J.G. and Sherman, N. 1999. Microbiology – A Laboratory Manual. Addison-Wesley Longman, Inc.

Carrillo, C., Teruel, J.A., Aranda, F.J. and Ortiz, A. 2003. Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim. Biophys. Acta. 1611, 91-97.

Carrillo, P.G., Mardaraz, C., Pitta-Alvarez, S.J. and Giulietti, A.M. 1996. Isolation and selection of biosurfctant-producing bacterial. World J. Microbiol. Biotechnol. 12, 82-84.

Catallo, W.J. and Portier, R.J. 1992. Use of indigenous and adapted microbial assemblages in the removal of organic chemicals from soils and sediments. Water Sci. Techno. 25, 229-237.

Chakrabarty, A.M. 1995. Microbial degradation of hazardous chemicals: evolutionary consideration and bioremediative developments. In Bioremediation: the Tokyo'94 Workshop. Org. Econ. Co-operation Devel. Paris. 71-80.

Chayabutra, C. and Ju, L-K 2001. Polydrooxyalkanoic acids and rhamnolipids are synthesized sequentially in hexadecane fermentation by *Pseudomonas aeruginosa* ATCC 10145. Biotechnol. Prog. 17, 419-423.

Chhatre, S., Purohit, H., Shanker, R. and Khanna, P. 1996. Bacterial consortia for crude oil spill remediation. Wat. Sci. Tech. 34, 187-193.

Cirigliano, M.C. and Carman, G.M. 1984. Isolation of a bioemulsifier *from Candida lipolytica*. Appl. Environ. Microbiol. 48, 747-750.

Cirigliano, M.C. and Carman, G.M. 1985. Purification and characterization of liposan, a bioemulsifier from *Candida lipolytica*. Appl. Environ. Microbiol. 50, 846-850.

Cooper, D.G. and Zajic, J.E. 1980. Surface active compounds from microorganisms. Adv. Appl. Microbiol. 26, 229-253.

Cooper, D.G., Akit, J. and Kosaric, N. 1982. Surface activity of the cells and extracellular lipids of *Corynebacterium fascians* CF15. J. Ferment. Technol. 60, 19–24.

Cooper, D.G., Zajic, J.E., Gerson, D.F. and Manninen, K.I. 1980. Isolation and identification of biosurfactants produced during anaerobic growth of *Clostridium pasteurianum*. J. Ferment. Technol. 58, 83–86.

Cooper, D.G. 1986. Biosurfactants. Microbiol. Sci. 3, 145-149.

Cooper, D.G., MacDonald, C.R., Duff, S.J.B. and Kosaric, N. 1981. Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. Appl. Environ. Microbiol. 42, 408-412.

Cooper, D.G., Zajic J.E. and Gerson, D.F. 1978. Production of surface active lipids by *Corynebacterium lepus*. Appl. Environ. Microbiol. 37, 4-10.

Cooper, D.G. and Goldenberg, B.G. 1987. Surface active agents from two *Bacillus* species. Appl. Environ. Microbiol. 53, 224-229.

Cooper, D.G. and Paddock, D.A. 1983. *Torulopsis petrophilum* and surface activity. Appl.Environ. Microbiol. 47, 1426-1429.

Cooper, D.G. and Paddock, D.A. 1984. Production of a biosurfactant from *Torulopsis bombicola*. Appl. Environ. Microbiol. 47, 173-176.

Cooper, D.G., Liss, S.N, Longay, R. and Zajic, J.E. 1989. Surface activities of *Mycobacterium* and *Pseudomonas*. J. Ferment. Technol. 59, 97-101.

Cutler, A.J. and Light, R. J. 1979. Regulation of hydroxydocosanoic and sophoroside production in *Candida bogoriensis* by the level of glucose and yeast extract in the growth medium. J. Biol. Chem. 254, 1944-1950.

Daniel, H.J. and Otto, R.T. 1999. Production of sophorolipids by whey. Biotechnol. Lett. 20, 1153-1156.

Daniel, H.J., Reuss, M. and Syldatk, C. 1998. Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage batch process using *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509. Biotechnol. Lett. 20, 1153-1156.

Daniel, W.W. 2000. In: Biostatistics: A foundation for analysis in the health sciences.7th ed. pp 166-167. John Wiley and Sons, Inc, New York,

Daniels, L., Handson, R.S. and Phillips, J.A. 1994. Chemicals analysis. Pp. 512. In: Gerhardt, A.P., Murray, R.G.E., Wood, W.A. and Krieg, N.R. (ed). Methods for general and molecular bacteriology. ASM Press, Washington, D.C.

Das, K. and Mukherjee, A.K. 2005. Characterization of biochemical properties and biological activities of biosurfactants produced by *Pseudomonas aeruginosa* mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. Appl Microbiol Biotechnol. (**In press**).

Das K., Doley, R. and Mukherjee, A.K. 2004. Purification and biochemical characterization of a thermostable, alkaliphilic, extracellular α-amylase from *Bacillus subtilis* DM-03, isolated from the traditional fermented food of India. Biotechnol. Appl. Biochem. 40, 291-298.

Das K., Mukherjee, A.K. and Konwar, B.K. 2003. Potential applications of biosrufactant, produced by thermophilic *Pseudomonas* sp. DM-02 strain, in microbial enhanced oil recovery (MEOR) and bioremediation. National Seminar on "Hydrocarbon Degrading Microbes" 22nd December, 2003, Tezpur University, India. Pp 12.

Davey, M.E., Caiazza, N.C. and Tootle, G.A.O. 2003. Rhamnolipid surfactant production affects bioflims architecture in *Pseudomonas aeruginosa* PA-01. J. Bacteriol. 185, 1027-1036

de Souza, J.T., de Boer, M., de Waard, P., van Beek, T.A. and Raaijmakers, J.M. 2003. Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl. Environ. Microbiol. 69, 7161-72.

Delcambe, L. and Devignat, R. 1957. Acad. R. Sci. Coloniales. 6, 1-78.

Deleu, M., Bouffioux, O., Razafindralambo, H., Paquot, M., Hbid, C., Thonart, P., Jacques, P. and Brasseur, R. 2003. Interaction of Surfactin with membrane: A computational approach.Langmuir. 19, 3377-3385.

Deleu, M., Razafindralambo, H., Popineau, Y., Jacques, P., Thonart, P. and Paquot, M. 1999. Interfacial and emulsifying properties of lipopeptides from *Bacillus subtilis*. Colloids Surf. A. 152, 3-10.

Denger, K. and Schink, B. 1995. New halo- and thermotolerant fermenting bacteria producing surface active compounds. Appl. Microbiol. Biotechnol. 44, 161-166.

Desai, A. J., Patel, K.M. and Desai, J.D. 1988. Emulsifier production by *Pseudomonas fluorescens* during the growth on hydrocarbon. Curr. Sci. 57, 500-501.

Desai, A.J., Patel, R.M. and Desai, J.D. 1994. Advances in production of biosurfactants and their commercial applications. J. Sci. Ind. Res. 53, 619-629.

Desai, J.D. 1987. Microbial surfactants: evalutation, types and future applications. J. Sci. Ind. Res. 46, 440-449.

Desai, J.D. and Desai, A.J. 1993. Production of biosurfactant. p 65-97. In:N. Kosaric (ed),. Biosurfactants: production, properties, and applications. Marcel Dekker, Inc. New York.

Desai, J.D. and Banat, I.M. 1997. Microbial production of surfactants and their commercial potential. Microbiol. Mol. Bio. Rev. 61, 47-64.

Deziel, E., Paquette, G., Villemur, R., Lepine, F. and Bisaillon, J-G., 1996. Biosurfactant production by a soil *Pseudomonas* strain growing on polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 62, 1908-1912.

Doley, R. and Mukherjee, A.K. 2003. Purification and characterization of an anticoagulent phospholipase A_2 from Indian monocled cobra (*Naja Kaouthia*) venom. Toxicon. 41, 81-91.

Dubois, M., Giles, K.A., Hamilton, J.K., Roberts, P.A. and Smith, F. 1956. Colorimetric method of determination of sugar and related substances. Anal.Chem. 28, 350-356.

Duitman, E.H., Hamoen, L.W., Rembold, M., Venema, G., Seitz, H., Saenger, W., Bernhard, F., Reinhardt, R., Schmidt, M., Ullrich, C., Stein, T., Leenders, F. and Vater, J. 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc. Natl. Acad. Sci. USA. 96, 13294-132.

Edward, J.R. and Hayashi, J.A. 1965. Structure of a rhamnolipid from *Pseudomonas aeruginosa*. Arch. Biochem. Biophys. 111, 415-421.

Eliseev, S.A., Vildanova- Martsishin, R., Shulga, A., Shabo, A. and Turovsky, A. 1991. Oil-washing bioemulsifier produced by *Bacillus* sp. Microbiol. J. 53, 61-66.

Fattom, A. and Shilo, M. 1985. Production of emulcyan by Phormidium J-1: its activity and function. FEMS Microbiol. Eco. 31, 3-9.

Fautz, B., Lang, S. and Wagner, F. 1986. Formation of cellobiose lipids by growing and resting cells of *Ustilago maydis*. Biotechnol. Lett. 8, 757-762.

Fiebig, R., Schulze, D., Chung, J.C. and Lee, S.T. 1997. Biodegradation of polychlorinated biphenyls (PCBs) in the presence of a bioemulsifier produced on sunflower oil. Biodegradation 8, 67–75.

Fiechter, A. 1992. Biosurfactants: moving towards industrial application. Trends Biotechnol. 10, 208-217.

Foght, J.M. and Westlake, D.W.S. 1988. Degradation of polycyclic aromatic hydrocarbons and aromatic heterocyctles of *Pseudomonas* species. Can. J. Microbiol. 34, 1135-1141.

Forsyth, J.V., Tsao, Y.M. and Bleam, R.D. 1995. Bioremediation: when bioaugmentation needed? In: Hinchee, R.E., Fredrickson, J. and Alleman, B.C. (eds) Bioaugmentation for site remediation. Columbus, O.H.: Battelle Press, 1-14.

Francy, D. S., Thomas J.M., Raymond, R.L. and Ward, C.H. 1991. Emulsification of hydrocarbons by subsurface bacteria. J. Ind. Microbiol. 8, 237-246.

Frings, C. S. and Dunn, R.T. 1970. A colorimetric method for determination of total lipids based on the sulfo-phospho-vanillin reaction. Am. J. Clin. Pathol. 53, 89-91.

Fry, I.J., Chakrabarty, A.M. and DeFrank, J.J. 1993a. *In situ* bioremediation of oil-contaminated soil and ground water of the Stafford army engine plant using natural surfactants. Proc. 1992 CRDEC Science Conf. on Chemical Defense.Edgewood, Maryland, USA, 362.

Fry, I.J., Checkai, R.T., Lynch, M., Rahrbaugh, D. and DeFrank, J.J. 1993b. Bioremediation of oil-contaminated soil by microbial inoculation or surfactant treatment. Proc.1993. CRDEC Science Conf. on Chemical Defense Edgewood, Maryland, USA.

Garcia- Martinez, J., Bescos, I., Rodriguez-Sala, J.J. and Rodriguez-Valera, F. 2001. RISSC: a novel database for ribosomal 16S-23S genes spacer regions. Nucleic Acids Res 29, 178-180.

Garcia- Martinez, J., Acinas, S.G., Anton, A. I. and Rodriguez-Valera, F. 1999. Use of the 16S-23S ribosomal spacer region in studies of prokaryotic diversity. J. Microbial. Methods. 36, 55-64.

Gibson, D.T. and Subramanian, V. 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. In: Gibson, D.T. (ed.) Microbial degradation of organic compounds. Marcel Dekker, Inc. New York, N.Y.

Golyshin, P. M., Fredrickson, H. L., Giuliano, L., Rothmel, R., Timmis, K. N. and Yakimov, M. M. 1999. Effect of novel biosurfactants on biodegradation of polychlorinated biphenyls by pure and mixed bacterial cultures. Microbiologica 22, 257–267.

Goswami, P. and Singh, H.D. 1991. Different modes of hydrocarbon uptake by two *Pseudomonas* species. Biotechnol. Bioeng. 37, 1-11.

Grau, A., Gomez Fernandez, J.C., Peypoux, F. and Ortiz, A. 1999. A study on the interactios of surfactin with phospholipid vesicles. Biochim.Biophysc Acta1418, 307-319.

Greek, B.F. 1991. Sales of detergents growing despite recession. Chem. Eng. News 69, 25-52.

Greek, B. F. 1990. Detergent industry ponders products for new decade. Chem. Eng. News 68, 37-38.

Grossman, A.D. 1995. Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. Annu. Rev. Acta 1418, 307-319.

Guasp, C., Moore, E. R. B., Lalucat, J. and Bennasar, A. 2000. Utility of internally transcribed 16S-23S rDNA spacer regions for definition of *Pseudomonas stutzeri* genomovars and other *Pseudomonas* species. Int. J. Sayst. Evol. Microbiol. 50, 1629-1639.

Guerin, W.F. and Jones, G.E. 1988. Mineralization of phenanthrene by a *Mycobacterium* sp. Appl. Environ. Microbiol. 54, 937-944.

Guerra-Santos, L.H., Kappeli, O. and Fiechter, A. 1984. *Pseudomonas aeruginosa* biosurfactant production in continuous culture with glucose as carbon source. Appl Environ Microbiol. 48, 301–305.

Guerra-Santos, L.H., Kappeli, O. and Fiechter, A. 1986. Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. Appl. Microbiol. Biotechnol. 24, 443-448.

Guo, C.L., Zhou, H.W., Wong, Y.S. and Tam, N.F.Y. 2005. Isolation of PAH-degrading bacteria from mangrove sediments and their biodegradation potential. Mar. Pol. Bul. (**In press**).

Gurjar, M., Khire, J. M. and Khan, M. I. 1995. Bioemulsifier production by *Bacillus stearothermophilus* VR-8 isolate. Lett. Appl. Microbiol. 21, 83–86.

Gutnik, D.L. and Shabtai, Y. 1987. Exopolysaccharide bioemulsifiers. Pp211-246. In: Kosaric, N, Cairns, W.L. and Gray, N.C.C.(ed.). Biosurfactants and biotechnology. Marcel Dekker, Inc. New York.

Ha, Y. W., Stack, R. J., Hespell, R. B., Gordon, S. H. and Bothast, R. J. 1991. Some chemical and physical properties of extracellular polysaccharides produced by *Butyrivibrio fibrisolvens* strains. Appl. Environ. Microbiol. 57, 2016–2020.

Haba, E., Espuny, M.J., Busquets, M. and Manresa, A. 2000. Screening and production of rhamnolipids by *Pseudomonas aeruginosa* 47T2 NCIB 40044 from waste frying oils. J. Appl Microbiol. 88, 379-87.

Haba E., Pinazo, A., Jauregui, O., Espuny, M. J., Infante M.R. and Manresa, A. 2003. Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044. Biotech. Bioeng. 81, 316-322.

Haferburg, D., Hommel, R., Claus, R. and Kleber, H.P. 1986. Extra-cellular microbial lipids as biosurfactants. Adv. Biochm. Eng. 33, 53-93.

Hansen, K.G., Desai, J.D. and Desai, A.J. 1993. A rapid and simple screening technique for potential crude oil degrading microorganisms. Biotechnol. Tech. 7, 745-748.

Harvey, S., Elashvili, I., Valdes, J.J., Kamely, D. and Chakrabarty, A.M. 1990. Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant. Bio/Tech., 8, 228-230.

Helle, S.S., Duff, S.J.B. and Cooper, D.G. 1993. Effect of surfactants on cellulose hydrolysis. Biotechnol. Bioeng. 42, 611-617.

Henriksen, A., Anthoni, U., Nielsen, T.H., Sørensen, J., Christophersen, C. and Gajhede, M. 2000. Cyclic lipoundecapeptide Tensin from *Pseudomonas fluorescens* strain 96.578. Acta Crtstallogr. C 56, 113-115.

Herman, D. C., Zhang, Y. and Miller, R. M. 1997. Rhamnolipid (biosurfactant) effects on cell aggregation and biodegradation of residual hexadecane under saturated flow conditions. Appl. Environ. Microbiol. 63, 3622–3627.

Hilderbrabrand, P.D., Braun, P.G., MaRae, K.B. and Lus, X. 1998. Role of the biosurfactant viscosin in broccoli head rot caused by a pectolytic strain of *Pseudomonas fluorescens*.Can. J. PlantPathol. 20, 296-303.

Hirota, V. 1960. The effect of acridine dyes on mating type factors in *E. coli.* Pro Natl Acad Sci USA, 46, 57-64.

Hisatsuka, K., Nakahara, T. Sano, N. and Yamada, K. 1971. Formation of rhamnolipid by *Pseudomonas aeruginosa*: its function in hydrocarbon fermentations. Agric. Biol. Chem. 35, 686-692.

Hisatsuka, K., Nakahara, T. and Yamada, K. 1972. Protein- like activator for n-alkane oxidation by *Pseudomonas aeruginosa* S_7B_1. Agric. Biol. Chem. 36, 1361-1369.

Hisatsuka, K., Nakahara, T., Minoda, Y. and Yamada, K. 1977. Formation of protein- like activator for n-alkane oxidation and its properties. Agric. Biol. Chem. 41, 445-450.

Hitzman, D.O. 1983. Petroleum microbiology and the history of its role in enhanced oil recovery. In Proc.1982 International Conf: Microbial Enhancement of Oil Recovery. Donaldson, E.C. and Clark, J.B. (eds). NTIS, Springfield, USA, 163-218.

Holmber, K. 2001. Natural surfactants. Cur. Opin. Colloid Interface Sci. 6, 148-159.

Hommel, R.K., Stuwer, O., Stuber, W., Haferburg, D. and Kleber, H.P. 1987. Production of water-soluble surface-active exolipids by *Torulopsis apicola*. Appl. Microbiol. Biotechnol. 26, 199-205.

Hommel, R.K., Weber, L., Weiss, A., Himmelreich, U., Rilke, O. and Kleber, H.P. 1994. Production of sophorose lipid by *Candida (Torulopisis) apicola* grown on glucose. J.Biotechnol. 33, 147-155.

Horowitz, S. and Currie, J.K. 1990. Novel dispersants of silicon carbide and aluminium nitrate. J. Dispersion Sci. Technol. 11, 637-659.

Horowitz, S. and Griffin, W.M. 1991. Structural analysis of *Bacillus licheniformis* 86. J. Ind. Microbiol. 7. 45-52.

Horowitz, S., Gilbert, J.N. and Griffin, W.M. 1990. Isolation and characterization of a surfactant produced by *Bacillus lichenifromis* 86. J. Ind. Microbiol. 6, 243-248.

Hosono, K. and Suzuki, H. 1983. Acylpeptides, the inhibitors of cyclic adenosine 3', 5' monophosphate phosphodiesterase III. Inhibition of cyclic AMP phosphodiesterase. J. Antibiot. 36, 679-683.

Huang, C-C., Ano, T. and Shoda, M. 1993. Nucleotide sequence and characteristics of the gene, *lpa*-14, responsible for biosynthesis of the lipopeptide antibiotics iturin A and surfactin from *Bacillus subtilis* RB14. J. Ferment. Bioeng. 76, 445-450.

Hunt, P.G., Robinson, K.G. and Ghosh, M.M. 1994. The role of biosurfactants in biotic degradation of hydrophobic organic compounds. In: Hydrocarbon Bioremediation. Hinchee, R.E., Alleman, B.C., Hoeppel, R.E., and Miller, R.N. (eds). Lewis Publishers, Boca Raton, FL.

Ioneda, T., Lederer, E. and Rozonis, J. 1970. Structure of trehalose diesters (cord factors) produced by *Nocardia asteroides* and *Nocardia rhodochrous*. Chem. Phys. Lipids. 4, 375–393.

Ishigami, Y. and Suzuki, S. 1997. Development of biochemicals—functionalization of biosurfactants and natural dyes. Prog. Org. Coatings. 31, 51–61.

Ishigami, Y., Gama, Y., Sano, Y., Lang, S. and Wagner, F. 1994. Interfacial and micellar behavior of glucose lipid. Biotechnol. Lett. 16, 593–598.

Ishigami, Y., Zhang, Y. and Ji, F. 2000. Spiculisporic acid. Functional development of biosurfactant. Chem. Today. 18, 32–34.

Isoda, H., Shinmoto, H., Kitamoto, D., Matsumura, M. and Nakahara, T. 1997. Differentiation of human promyelocytic leukemia cell line HL60 by microbial extracellular glycolipids. Lipids 32, 263–271.

Itoh, S. and Suzuki, T. 1972. Effect of rhamnolipids on growht of *Pseudomonas aeruginosa* grown on n-paraffin. Agric. Biol. Chem. 36. 2233-2235.

Itoh, S., Honda, H., Tomita, F. and Suzuki, T. 1971. Rhamnolipids produced by *Pseudomonas aeruginosa* grown on n-paraffin. J. Antibiot. 24, 855-859.

Itokawa, H., Miyashita, T., Morita, H., Takeya, K., Hirano, T., Homman, M. and Kitaro, O. 1994. Structural and conformational studies of [Ile7] and [Leu7] surfactins from *Bacillus subtilis*. Chem. Pharm. Bul. 42, 604-607.

Ivshina, I. B., Kuyukina, M. S., Philp, J. C. and Christofi, N. 1998. Oil desorption from mineral and organic materials using biosurfactant complexes produced by *Rhodococcus* species. World J. Microbiol. Biotechnol. 14, 711–717.

Jain, D. K., Lee, H. and Trevors, J. T. 1992. Effect of addition of *Pseudomonas aeruginosa* UG2 inocula or biosurfactants on biodegradation of selected hydrocarbons in soil. J. Ind. Microbiol. 10, 87–93.

Jain, D.K., Collins-Thompson, D.L., Lee, H. and Trevors, J. T. 1991. A drop-collapsing test for screening surfactant-producing microorganisms. J. Microbiol. Meth. 13, 271-279.

Jarvis, F.G. and Johnson, M.J. 1949. A glycolipid produced by *Pseudomonas aeruginosa*. J. Am. Chem. Soc. 71, 4124-4126.

Javaheri, M., Jenneman, G.E., McInnerney, M.J. and Knapp, R.M. 1985. Anaerobic production of a biosurfactant by *Bacillus licheniformis* JF-2. Appl. Environ. Microbiol. 50, 698-700.

Jenneman, G.E., McInerney, M.J., Knapp, R.M., Clark, J.B., Feero, J.M., Revus, D.E. and Menzie, D.E. 1983. A halotolerant biosurfactant producing *Bacillus* species potentially useful for enhanced oil recovery. Dev. Ind. Microbiol. 24, 485-492.

Jennings, E.M. and Tanner, R.S. 2000. Biosurfactant-producing bacteria found in contaminated and uncontaminated soils. Proceedings of the 2000 Conference on Hazardous Waste Research. 299-306.

Jenny, K., Kappeli, O. and Fiechter, A. 1991. Biosurfactants from *Bacillus licheniformis*, structural analysis and characterization. Appl. Microbiol. Biotechnol. 36, 5-13.

Jensen, M.A., Webster, J.A. and Straus, N. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl Environ. Microbiol. 59, 945-952.

Johnson, M.K. and Boese-Marrazzo, D. 1980. Production and properties of heat-stable extracellular hemolysin from *Pseudomonas aeruginnosa*. Infect. Immun. 29, 1028- 1033.

Johnson, Y.A., Nagpal, M., Krahmer, M.T., Fox, K.F. and Fox, A. 2000. Precise molecular weight determination of PCR products of the rRNA intergenic spacer region using electrospray quadrupole mass spectrmetry for differentiation of *B. subtilis* and *B. atrophaeus*, closely related species of bacilli. J. Microbiol. Methods. 40, 241-254.

Kabadjova, P., Dousset, X., Cam, V.L. and Pevost, H. 2002. Differentiation of closely related *Carnobacterium* food isolates based on 16S-23S ribosomal DNA inter spacer region polymorphism. Appl. Environ. Microbiol. 68, 5358-5366.

Kachholz, T. and Schlingmann, M. 1987. Possible food and agricultural application of microbial surfactants: an assessment. In: Kosaric N., Cairns W.L. and Grey, N.C.C (ed) Biosurfactant and Biotechnology, Vol 25, pp 194.Marcel Dekker, Inc., New York.

Kajimura, Y., Sugiyama, M. and Kaneda, M. 1995. Bacillopeptins, new cyclic lipopeptide antibiotics from Bacillus subtilis FR-2. J. Antibiot. (Tokyo). 48, 1095-103.

Kakinuma, A., Ouchida, A., Shima, T., Sugino, H., Isono, M., Tamura, G. and Arima K. 1969. Confirmation of the structure of surfactin by mass spectrometry. Agric. Biol. Chem. 33, 1669-1671.

Kameda, Y., Ouchira, S., Matsui Kkanatomo, S., Hase, T. and Atsusaka, T. 1974. Antitumor activity of *Bacillus natto* V. Isolation and characterization of surfactin in theculture medium of *Bacillus natto* KMD 2311. Chem. Pharm. Bull. 22, 938-944.

Kanaly, R.A. and Harayama, S. 2000. Biodegradation of high-molecular weight polycyclic aromatic hydrocarbons by bacteria. J. Bacteriol. 182, 2059-2067.

Kappeli, O. and Fiechter, A. 1977. Component from the cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis* with possible relation to hydrocarbon transport. J. Bacteriol. 131, 917-921.

Kappeli, O. and Finnerty, W.R. 1979. Partition of alkane by an extracellular vesicle derived from hexadecane- grown *Acinetobacter*. J. Bacteriol. 140, 707-712.

Kappeli, O., Walther, P., Muller, M. and Fiechter, A. 1984. Structure of cell surface of the yeast Candida tropicalis and its relation to hydrocarbon transport. Arch. Microbiol. 138, 279-282.

Kappeli, O., Muller, M. and Fiechter, A. 1978. Chemical and structural alterations at the cell surface of *Candida tropicalis*, induced by hydrocarbon substrate. J. Bacteriol. 133, 952-958.

Kawanami, J., Kimura, A. and Otsuka, H. 1968. Siolipin A: A new lipoamino acid ester isolated from *Streptomyces sioyaensis*.Biochim.Biophys.Acta. 151, 808-810.

Kim, K., Jung, S.U., Lee, K.K., Jung, J.-K., Park, J.K., Kim, K.K. and Lee, C.-H. 1998. Suppression of inflammatory responses by surfactin, a selective inhibitor of platelet cytosolic phospholipase A_2. Biochem. Pharmacol. 55, 975-985.

Kim, S.H., Lee, J.-D., Kim, B.-C. and Lee, T.-H. 1996. Purification and characterization of bioemulsifier produced by Acinetobacter sp. BE-254. J. Microbiol. Biotechnol. 6, 184–188.

Kim, H.S., Lim, E. J., Lee, S. O., Lee, J. D. and Lee, T.H. 2000. Purification and characterization of biosurfactants from *Nocardia* sp. L-417. Biotechnol. Appl. Biochem. 31, 249–253.

Kim, H.S., Yoon, B.D. Choung, D.H., Oh, H.M., Katsuragi, T. and Tani, Y. 1999. Characterization of a biosurfactant, mannosylerythritol lipid produced from *Candida* sp. SY16. Appl. Microbiol. Biotechnol. 52, 713-721.

Kitamota, D., Akiba, S., Hioki, C. and Tabuchi, T. 1990. Extracellular accumulation of mannosylerythritol lipids by a strain *Candida antarctica*. Agric. Biol. Chem. 54, 31-36.

Kitamota, D., Nakane, T., Nakao, N., Nakahara, T. and Tabuchi, T. 1992. Intracellular accumulatio of mannosylerythritol lipids as storage materials by *Candida antarctica*. Appl. Microbiol.Biotechnol. 36, 768-772.

Kitamoto, D, Isoda, H. and Nakahara, T. 2002. Funtions and potential applications of glycolipid biosurfactants- from energy-saving materials to gene delivery carriers. J. Biosci.Bioeng. 94, 187-200.

Kitamoto, D., Yanagishita, H., Shinbo, T., Nakane, T., Kamisawa, C. and Nakahara, T. 1993. Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by *Candida antarctica*. J.Biotechnol. 29, 91-96.

Kleckner, V. and Kosaric, N. 1993. Biosurfactants for cosmetics. In "Biosurfactants, Production, Properties, Applications" (N. Kosaric, Ed.), Surfactant Science Series, Vol. 48, pp. 373–389. Marcel Dekker, New York.

Knoche, H.W. and Shiveley, J.M. 1972. The structure of an ornithine- containing lipid from *Thiobacillus thiooxidans*. J. Biol. Chem. 247, 170-178.

Knox, O.G.G., Killham; K. and Leifert, C. 2000. Effect of increased nitrate availability on the control of pathogenic fungi by the soil bacterium *Bacillus subtilis*. Appl. Soil Eco. 15, 227-231.

Koch, A.K., Kappeli, O, Fiechter, A. and Reiser, J. 1991. Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol. 173, 4212-4219.

Koronelli, T.V., Komarova, T.I. and Denisov, Y.V. 1983. Chemical composition and role of peptidoglycolipid of *Pseudomonas aeruginosa*. Mikrobiologiya 52, 767-770.

Kowall, M., Vater, J., Kluge, B., Stein, T., Franke, P. and Ziessow, D. 1998. Separation and characterization of surfactin isoforms produced by *Bacillus subtilis* OKB 105. J. Colloid. Interface. Sci. 203, 1-8.

Kracht, M., Rokos, H., Ozel, M., Kowall, M., Pauli, G. and Vater, J. 1999. Antiviral and hemolytic activities of surfactin isoforms and their methyl ester derivatives. J. Antibiot. 52, 613–619.

Krauss, E.M. and Chan, S.I. 1983. Complexation and phase transfer of nucleotides by gramicidin S. Biochemistry 22, 4280-4285.

Kretschmer, A., Bock, H. and Wagner, F. 1982. Chemical and physical characterization of interfacial-active lipids from *Rhodococcus erthropolis* grown on n- alkane. Appl. Environ. Microbiol. 44, 864-870.

Lang, S. and Wagner, F. 1987. Structure and properties of biosurfactants.p.21-47. In. N. Kosaric, Cairns, W.L. and Gray, N.C.C (Ed.), Biosurfactants and biotechnology. Marcel Dekker, Inc, New York.

Lang, S. and Philp, J.C. 1998. Surface-active lipids in *Rhodococci.* Antonie van Leeuwenhoek. 74, 59-70.

Lang, S. and Wullbrandt, D. 1999. Rhamnose lipids-biosynthesis, microbial production and application potential. Appl. Microbiol. Biotechnol. 51, 22-32.

Lee, M.D. and Ward, C.H. 1985. Environmental and biological methods for the restorationof contaminated aquifers. Environ. Toxicol. Chem. 4, 743-750.

Leenders F., Stein, T.H., Kablitz, B., Franke, P. and Vater, J. 1999. Rapid typing of *Bacillus subtilis* strains by their secondary metabolities using matrix- assisted laser desorption / ionization mass spectrometry of intact cells. Rapid Commun Mass Spectrom 13, 943-949.

Leenhouts, J.M., Van der Winingard, P.W.J., De Kroon, A.I.P.M. and Der Kruijff, B. 1995. Anionic phospholipids can mediate membrane insertion of the anionic part of a bound peptide. FEBS Lett. 370, 361-369.

Li, Z.Y., Lang, S., Wagner, F., Witte, L. and Wray, V. 1984. Formation and identification of interfacial-active glycolipids from resting microbial cells of *Arthrobacter* sp. And potential use in tertiary oil recovery. Appl. Environ. Microbiol. 48, 610-617.

Lillienberg, L., Hogstedt, B. and Nilson, L. 1992. Health-effects of tank cleaners. Amer. Ind. Hygiene. Assoc. J. 53, 95-102.

Lin S.C., Chen Y.C. and Lin Y.M. 1998. General approach for the development of high-performance liquid chromatography methods for biosurfactant analysis and purification. J. Chromatography 825, 149-159.

Linhardt, R. J., Bakhit, R., Daniels, L., Mayerl, F. and Pickenhagen, W. 1989. Microbially produced rhamnolipid as a source of rhamnose. Biotechnol. Bioeng. 33, 365–368.

Lenz. R.W., Kim, Y.B. and Fuller, R.C. 1992. Production of unusual bacterial polyesters by *Pseudomonas oleovorans* through cometabolism. FEMS Micrbiol. Rev. 103, 207-214.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193.265-275.

MacDonald, C.R., Cooper, D.G. and Zajic, J.E. 1981. Surface-active lipids from *Nocardia erythropolis* grown on hydrocarbons. Appl. Environ. Microbiol. 41, 117-123.

MacElwee, C.G., Lee, H. and Trevors, J.T. 1990. Production of extracellular emulsifying agent by *Pseudomonas aeruginosa* UG1. J. Ind. Microbiol. 5, 25-32

Madonna, A.J. and Voorhees, K. T. 2003. Detection of cyclic lipopeptide biomarkers from *Bacillus* species using atmospheric pressure matrix-assisted laser desorption/ionization mass spectrometry. Anal. Chem. 75, 1628-1637.

Maget- Dana, R., Thimon, L., Peypoux, F. and Ptak, M., 1992. Surfactin/iturin A interactions may explain the synergistic effect of surfactin on the biological properties of iturin A. Biochime. 74, 1047-1051.

Maier, R.M. 2003. Biosurfactant: Evolution and diversity in bacteria. Adv. Appl. Microbiol. 52, 101-121.

Maier, R.M. and Soberon-Chavez, G. 2000. *Pseudomonas aeruginosa* rhamnolipids: biosynthesis and potential applications. Appl. Microbiol. Biotechnol. 54, 625-633.

Makkar, R.S. and Cameotra, S.S. 1997a. Biosurfactant production by a thermophilic *Bacillus subtilis* straim. J. Ind. Microbiol. Biotechnol.18, 37-42.

Makkar, R.S. and Cameotra, S.S. 1997b. Utilization of molasses for biosurfactant production by two *Bacillus* strains at thermophilic conditions.J.Am.Oil.Chem.Soc. 74, 887-889.

Makkar, R.S. and Cameotra, S.S. 1998. Production of biosurfactant at mesophilic and thermophilic conditions by a strain of *Bacillus subtis*. J. Ind. Microbiol. Biotechnol. 20, 48-52.

Makkar, R.S. and Cameotra, S.S. 1999. Biosurfactant production by microorganisms on unconventional carbon sources. J. Surfactant Detergents 2, 237-241.

Makkar, R.S. and Cameotra, S.S. 2001. Synthesis of enhanced biosurfactants by *Bacillus subtilis* MTCC 2423 at 45°C by foam fractionation. J. Surfactant Detergents 4, 355-357.

Makkar, R.S. and Cameotra, S.S. 2002. An update on the use of unconventional substrates for biosurfactant production and their new application. Appl. Microbiol. Biotechnol. 58, 428-434.

Marahiel, M., Denders, W., Krause, M. and Kleinkauf, H. 1977. Biological role of gramicidin S in spore functions. Studies on gramicidin-S negative mutants of *Bacillus brevis*. 9999. Eur. J. Biochem. 99, 49-52.

Marahiel, M., Nakano, M.M. and Zuber, P. 1993. Regulation of peptide antibiotic production in *Bacillus subtilis*. Mol. Microbiol. 7, 631-636.

Margaert, J., Anderson, C., Wouter, A, Swing, J. and Kersters, K. 1992. Biodegradation of polyhydrooxyalkanoates. FEMS Microbiol. Rev.103, 317-322.

Maslin, P. and Maier, R. M. 2000. Rhamnolipid-enhanced mineralization of phenanthrene in organic-metal co-contaminated soils. Biorem. J. 4, 295–308.

Mata-Sandoval J, Karns, J. and Torrents, A. 1999. High-performance liquid chromatography method for the characterization o rhamnolipids mixture produced by *Pseudomonas aeruginosa* UG2 on corn oil. J. Chromatogr. 864, 211-220.

Matsuyama, T. and Nakagawa, Y. 1996. Bacterial wetting agents working in colonization of bacteria on surface environments. Colloids Surf. 7, 207-214.

Matsuyama, T., Fujita, M. and Yano, I. 1985. Wetting agent produced by *Serratia marcescens*. FEMS Microbiol. Lett. 28, 125–129.

Matsuyama, T., Sogawa, M. and Yano, I. 1991. Direct colony thin-layer chromatography and rapid characterization of *Serratia marcescens* mutants defective in production of wetting agents. Appl. Environ. Microbiol. 53, 1186-1188.

McInerney, M.J., Javaheri, M. and Nagle, D.P. 1990. Properties of the biosurfactant produced by *Bacillus licheniformis* strain JF-2. J. Ind. Microbiol. 5, 95-102.

Mercede, M.E. and Manersa, M.A. 1994. The use of agroindustrial by-products for biosurfactant production. J. Am. Oil. Chem. Soc. 71, 61-68.

Mercede, M.E., Manersa, M.A., Robert, M., Epsuny, M.J., deAndres, C. and Guinea, J. 1993. Olive Oil Mill Effluent (OOME); New substrate for biosurfactant production, Bioresour.Technol. 43, 1-6.

Mercede, M.E. and Manersa, M.A. 1994. The use of agroindustrial by- products for biosrufactant production. J. Am. Oil Chem. Soc. 71, 61-64.

Miller, R.M. and Zhang, Y. 1997. Measurement of biosurfactant- enhanced solubilization and biodegradation of hydrocarbons Pp 59-66. In: Sheehan, D. (Eds), Methods in Biotechnology, vol.2. Humana Press, Totowa.

Mishra, S., Jyoti, J., Kuhad, R.C. and Lal, B. 2001. In Situ bioremdiation potential of an oily sludge- degrading bacterial consortia. Curr. Microbiol. 43, 328-335.

Mittal, P.K., Adak, T. and Sharma, V.P. 1995. Effect of water pH on the larvicidal activity of Spherix (*Bacillus sphaericus*) and Bactoculicide (*Bacillus thuringiensis* H-14) against mosquitoes. Natl Acad Sci Lett. 18, 189-191.

Miyazima, M., Iida, M. and Iizuka, H. 1985. Phosphoidlipid derived from hydrocarbons by fungi. J. Ferment. Technol. 63, 219-224.

Moran, A.C., Alejandra Martinez, M. and Sineriz, F. 2002. Quantification of surfactin in culture supernatant by hemolytic activity. Biotechnol. Lett. 24, 177-180.

Moran, A. C., Olivera, N., Commendatore, M., Esteves, J.L. and Sineriz, F. 2000. Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilis* O9. Biodegradation. 11, 65-71.

Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishe, Y. and Imanaka, T. 1993. A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. J. Bacteriol. 175, 6459–6466.

Morikawa, M., Hirata, Y. and Imanak, T. 2000. A study on the structure-function relationship of the lipopeptide biosurfactants. Biochim. Biophys. Acta. 1488, 211-218.

Morkes, J. 1993. Oil-spills-whose technology will clean up. R&D Mazagine 35, 54-56.

Mozes, N. and Rouxhet, P.G. 1987. Methods for measuring hydrophobicity of microorganisms. J. Microbiol. Methods 6, 99-112.

Mueller, J.G., Chapman, P.J., Blattman, B.O. and Pritchard, P.H. 1990. Isolation and characterization of a fluoranthene-utilizing strain of *Pseudomonas paucimobilis*. Appl. Envrion. Microbiol. 56, 1079-1086.

Mukherjee, A.K. and Das, K. 2005. Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. FEMS Microbiol. Eco. (**In press**)

Mulder, H, Breure, A.M, van Honschooten, D., Grotenhuis, J.T.C., van Andel, J.G. and Rulkens, W.H. 1998. Effect of biofilm formation by *Pseudomonas* 8909N on the bioavailability of solid naphthalene. Appl. Microbiol. Biotechnol. 50, 277-283.

Mulla, M. S. 1985. Field evaluation and efficacy of bacterial agents and their formulations against mosquito larvae. In: Laird, M., Miles, J.W. (Eds.), Integrated Mosquito Control Methodologies, vol. 2. Academic Press, London, 227–250.

Mulligan C.N. 2005. Environmental applications of biosurfactants. Environ Pollution. 133, 183-198.

Mulligan, C. N., Yong, R. N., Gibbs, B. F., James, S. and Bennett, H. P. J. 1999. Metal removal from contaminated soil and sediments by the biosurfactant surfactin. Environ. Sci. Technol. 33, 3812–3820.

Mulligan, C.N. and Cooper, D.G. 1985. Pressate from peat dewatering as a substrate for bacterial growth. Appl. Environ. Microbiol. 50.160-162.

Mulligan, C.N., Cooper, D.G. and Neufeld, R.J. 1984. Selection of microbes producing biosurfactants in media without hydrocarbons. J. Ferment. Technol. 62, 311-314.

Nakata, K. 2000. Two glycolipids increase in the bioremediation of halogenated aromatic compounds. J. Biosci. Bioeng. 89, 577–581.

Nakano, M. M., Marahiel, M.A. and Zuber, P. 1988. Identification of a genetic locus required for biosynthesis of the lipopeptide antibiotic surfactin in *Bacillus subtilis*. J. Bacteriol. 170, 5662-5668.

Navonvenezia, S., Zosim, Z., Gottieb, A., Legmann, R., Carmeli, S., Ron, E.Z. and Rosenberg, E. 1995. Alasan, a new bioemulsifier from *Acinetobacter radioresistens*. Appl. Environ. Microbiol. 61, 3240-3244.

Nelson, S.T. and Launt, P.D. 1991. Stripper well production increased with MEOR treatment. Oil and Gas J. 89, 114-118.

Neu, T.R. 1996. Significance of bacterial surface-active compounds in interaction of bacteria with interfaces. Microbiol. Rev. 60, 151-166.

Neu, T.R., Hartner, T. and Poralla, K. 1990. Surface active properties of viscosin, a peptidolipid antibiotic. Appl. Microbiol. Biotechnol. 32, 518-520.

Neu, T.R. and Paralla, K. 1990. Emulsifying agent from bacteria isolated during screening for cells with hydrophobic surfaces. Appl. Microbiol. Biotechnol. 32, 521-525.

Neufeld, R.J., Zajic, J.E. and Gerson, D.F. 1980. Growth characteristics and cell partitioning of *Acinetobacter* on hydrocarbon substrates. J. Ferment. Technol. 61, 315-321.

Nielsen, T.H. and Sørensen, D. 2003. Production of cyclic lipopeptides by Pseudomonas fluorescens strains in bulk soil and in the sugar beet rhizosphere. Appl. Environ. Microbiol. 69, 861-868.

Nielsen T.H., Christophersen, C., and Anthoni, U. and Sørensen, J. 1999. Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by *Pseudomonas fluorescens* DR54. J. Appl. Microbiol. 86, 80-90.

Nielsen T.H., Sørensen, D., Tobiasen, C., Andersen, J.B., Christophersen, C., Givskov, M. and Sørensen, J. 2002. Antibiotic and biosurfactant properties of cyclic lipopeptides produced by fluorescent *Pseudomonas* spp. from the sugar beet rhizophere. Appl Environ Microbiol. 68, 3416-3423.

Nishikori, T., Naganawa, H., Muraoka, Y., Aoyagi, T. and Umezawa, H. 1986. Plipastatins: new inhibitors of phopholipase A2, produced by *Bacillus cereus* BMG 302-fF67. II. Structure of fatty acid residue and amino acid sequence. J. Antibiot. (Tokyo) 39, 745-754.

Nout, M.J.R., Bakshi, D. and Sarkar, P.K. 1998. Microbiological safety of kinema, a fermented soya bean food. Food Control 9, 357-362.

Oberbremer, A. and Muelller-Hurting, R. 1989. Aerobic stepwise hydrocarbon degradation and formation of biosurfactants by and original soil population in a stirred reactor. Appl. Emicrobiol. Biotechnol. 31, 582-586.

Oberbremer, A., Mu¨ ller-Hurtig, R. and Wagner, F. 1990. Effect of the addition ofmicrobial surfactants on hydrocarbon degradation in a soil population in a stirred reactor. Appl. Microbiol. Biotechnol. 32, 485–489.

Ochoa-Loza, F. J., Artiola, J. F. and Maier, R. M. 2001. Stability constants for the complexation of various metals with a rhamnolipid biosurfactant. J. Environ. Qual. 30, 479–485.

Ohno, A., Takashi, A. and Shoda, M. 1996. Use of soybean curd residue, okara for the solid state substrate in the production of a lipopeptide antibiotic iturin A, by *Bacillus subtilis* NB22.Process Biochem. 31.801-806.

Ohno, A., Takashi, A. and Shoda, M. 1992. Production of antifungal peptide antibiotic iturin by *Bacillus subtilis* NB22 in solid state fermentation. J. Ferment. Bioeng. 75, 23-27.

Osterreicher- Ravid, D., Ron, E.Z. and Rosenberg, E. 2000. Horizontal transfer of an exopolymer complex from one bacterial species to another. Environ. Microbiol. 2, 366-372.

Palejwala, S. and Desai, J.D. 1989. Production of extracellular emulsifier by a Gram negative bacterium. Biotechnol. Lett.11. 115-118.

Panchal, C.J. and Zajic, J.E. 1978. Isolation of emulsifying agents from a species of *Corynebacterium*. Dev. Ind. Microbiol. 19, 569-576.

Parra, J.L., Guinea, J., Manresa, M.A., Robert, M.E., Mercade, M.E., Comelles, F. and Bosch, M.P. 1989. Chemical characterization and physiocolo-chemical behavior of biosurfactants. J. Am. Oil Chem. Soc. 66, 141-145.

Pattel, M.N. and Gopinathan, K.P. 1986. Lysozyme sensitive bioemulsifier for immiscible organophosphorous pesticides. Appl. Environ. Microbiol. 52, 1224-1226.

Pelczar-J.R., M.J., Chan, E.C.S. and Krieg, N.R. 1993. In: Microbiology.5th Edition. Pp. 108. Mc-Graw-Hill, Inc., New York.

Persson, A. and Molin, G. 1987. Capacity for biosurfactant production of environmental *Pseudomonas* and *Vibrionaceae* growing on carbohydrates. Appl. Microbiol. Biotechnol. 26, 439-442.

Peypoux, F., Besson, F., Michel, G., Lenzen, C., Dierickx, L. and Delcambe, L. 1980. Characterization of a new antibiotic of iturin group: bacillomycin D. J Antibiot (Tokyo). 33, 1146-1149.

Peypoux, F., Bonmatin, J.M. and Wallach, J. 1999. Recent trends in the biochemistry of surfactin. Appl.Microbiol.Biotechnol. 51, 553-563.

Peypoux, F., Guinand, M., Michel, G., Delcambe, L., Das, B.C. and Lederer, E. 1978. Structure of iturin A, a peptidolipid antibiotic from *Bacillus subtilis*. Biochemistry 17, 3992-3996.

Peypoux, F., Marion, D., Maget-Dana, R., Ptak, M., Das, B.C. and Michel, G. 1985. Structure of bacillomycin F, a new peptidolipid antibiotic of the iturin group. Eur. J. Biochem. 153, 335-340.

Peypoux, F., Michel, G. and Delcambe, L. 1976. Structure de la mycosubtiline, antibiotique isole de *Bacillus subtilis*. Eur. J. Biochem. 63. 391-398.

Peypoux, F., Pommier, M.-T., Das, B.C., Besson, F., Delcambe, L. and Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*, J. Antibiot. (Tokyo) 77, 1600-1604.

Peypoux, F., Pommier, M.-T., Das, B.C., Besson, F., Delcambe, L. and Michel, G. 1986. Revised structure of mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. J. Antibiot. (Tokyo) 39, 636-641.

Philips, W.E. and Perry, J.J. 1976. *Thermomisrobium fosteri* sp. nov. A hydrocarbon utilizing obligate thermophile. Int. J. Syst. Bacteriol. 26, 220-225.

Philp, J.C., Kuyukina, M.S., Ivshina, I.B., Dunbar, S.A., Christofi, N., Lang, S. and Wray, V. 2002. Alkanotripic *Rhodococcus ruber* as a biosurfactant producer. Appl. Microbiol. Biotechnol. 59, 318-324.

Pickard, M.A., Roman, R., Tinoco, R. and Vazquez-Duhalt, R. 1999. Polycyclic aromatic hydrocarbon metabolism by white rot fungi and oxidation by *Coriolopsis gallica* UAMH 8260 laccase. Appl. Environ.Microbiol. 65, 3805-3809.

Pratt-Terpstar, I.H., Weerkamp, A.H. and Busscher, H.J. 1989. Microbial factors in a thermodynamic approach of oral Streptococci adhesion to solid substrata. J. Colloid Interface.Sci. 129, 568-574.

Pruthi, V. and Cameotra, S.S. 1995. Rapid method for monitoring maximum biosurfactant produced by acetone precipitation. Biotechnol Techniques 9, 271-276.

Pruthi, V. and Cameotra, S.S. 1997. Production of a biosurfactant exhibiting excellent emulsification and surface active properties by *Serratia marcescens*. World J. Microbiol. Biotechnol. 13, 133-135

Rahman, K.S.M., Thahira-Rahman, J., Kourkoutas, Y., Petsas, I., Marchant, R. and Banat, I.M. 2003. Enhanced bioremediation of *n*- alkanes in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients. Bioresour. Technol. 90, 159-168.

Rahman, K.S.M., Thahira-Rahman, J., Lakshmanaperumalsamy, P. and Banat, I.M. 2002. Towards efficient crude oil degradation by mixed bacterial consortium. Bioresour. Technol. 85, 257-261.

Rambeloarisoa, E., Rontani, J.F., Giusti, G., Duvnjak, Z. and Bertrand, J.C. 1984. Degradation of crude oil by a mixed population of bacteria isolated from sea-surface foam. Mar. Biol. 83, 69-81.

Ramos, J.L., Duque, E. and Ramos-Gonzalez, M.I. 1991. Survival in soils of an herbicide-resistant *Pseudomonas putida* strain bearing a recombinant TOL plasmid. Appl. Environ. Microbiol. 57, 260-266.

Rao, Y.V., Romesh, M., Singh, A. and Chakrabarti, R. 2004. Potentiation of antibody production in Indian major carp *Labeo rohita*, rohu, by *Achyranthes aspera* as a herbal feed ingredient. Aquaculture. 238, 67-73.

Rapp, P., Bock, H., Wary, V. and Wagner, F. 1979. Formation, isolation and characterization of trehalose dimycolates from *Rhodococcus erthropolis* grown on n-alkanes. J. Gen. Microbiol.115, 491-503.

Rau, U., Hammen, S., Heckmann, R., Wray, V. and Lang, S. 2001. Sophorolipids: a source for novel compounds. Ind. Crops. Prod. 13, 85-92.

Rau, U., Manzke, C. and Wagner, F. 1996. Influence of substrate supply on the production of sophorose lipids by *Candida bombicola* ATCC 22214. Biotechnol. Lett. 18, 149–154.

Rautenbach, M., Swart, P. and van der Merwe, M.J. 2000. The interaction of analogues of the antimicrbial lipopeptide Iturin A_2 with alkali metal ions. Bioorganic.Med.Chem. 8, 2539-2548.

Reiling, H.E., Thanei- Wyss, U., Guerra-Santos, L.H., Hirt, R., Kappeli, O. and Fiechter A. 1986. Pilot plant prouduction of rhamnolipid biosurfactant by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. 51, 985-989.

Reisfeld, A., Rosenberg, E. and Gutnick, D. 1972. Microbial degradation of crude oil: factors affecting oil dispersion in sea water by mixed and pure cultures. Appl. Microbiol. 24, 363-368.

Reva, O.N., Dixelius, C., Meijer, J. and Priest, F.G. 2004. Taxonomic characterization and plant colonizing abilities of some bacteria related to *Bacillus amyloliquefaciens* and *Bacillus subtilis*. FEMS Microbiol. Eco. 48, 249-259.

Richter, M., Willey, M., Suessmuth, R., Jung, G. and Fieldler, H.P. 1998. Streptofactin, a novel biosurfactant with arial mycelium including activity from *Streptomyces tendae* Tue 901/8c. FEMS Microbiol. Lett. 163, 165-171.

Ridgway H.F., Safarik, J., Phipps, D., Carl, P. and Clark, D. 1990. Identification and catabolic activity of well-derived gasoline-degrading bacteria from a contaminated aquifer. Appl. Environ. Microbiol. 56, 3565-3575.

Riser- Roberts, E. 1998. In: Remediation of Petroleum Contaminated Soils. Biological, Physical, and Chemical Process. CRC Press LLC, Boca Raton, Florida. pp. 306.

Robert, M., Mercade, M.E., Bosch, M.P., Parra, J.L., Espuny, M.J., Manresa, M.A. and Guinea, J. 1989. Effect of the carbon source on biosurfactant production by *Pseudomonas aeruginosa* 44T. Biotechnol. Lett. 11, 871-874.

Ron, E.Z. and Rosenberg, E. 2001. Natural roles of biosurfactants. Environ. Microbiol. 3, 229-236.

Ron, E.Z., and Rosenberg, E. 2002. Biosurfactant and oil remediation. Curr. Opinon Biotechnol. 13, 249-252.

Rosenberg, E.G.A. and Rosenber, M. 1983. Inhibition of bacterial adherence to hydrocarbons and epithelial cells by emulsan. Infect. Immun. 39, 1024-1028.

Robert, M., Mercade´, M.E., Bosh, M.P., Parra, J.L., Espuny, M.J., Manresa, M.A. and Guinea, J. 1991. Optimizacio´n de la producio´n de biotensoactivos por *P. aeruginosa* 44 T1. Grasasy Aceites 42, 1-7.

Roongaswang, N., Thaniyavarn, J., Thaniyavarn, S., Kameyama, T., Haruki, M., Imanaka, T., Morikawa, M. and Kanaya, S. 2002. Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L., plipastatin, and surfactin. Extremophiles 6, 499-506.

Rosen, M.J. 1989 In: Surfactants and interfacial phenomena. 2nd Ed. Pp. 3-4. John Wiley & Sons, New York, USA.

Rosenberg, E. 1986. Microbial surfactants. Crit. Rev. Biotechnol. 3, 109-132.

Rosenberg, E., Legman, R., Kushmaro, A., Taube, R., Adler, E. and Ron, E.Z. 1992. Petroleum bioremediation- a multiphase problem. Biodegradation 3, 337-350.

Rosenberg, E., Rubinovitz, C., Legmann, R. and Ron, E.Z. 1988. Purification and chemical properties of *Acinetobacter calcoaceticus* A2 biodispersan. Appl.Environ. Microbiol. 54, 323-326.

Rosenberg, E., Zuckerberg, A., Rubinovitz, C. and Gutnick, D.L. 1979. Emulsifier *Arthrobacter* RAG-1: isolation and emulsifying properties. Appl. Environ.Microbiol. 37, 402-408.

Rosenberg, M., Gutnick, D. and Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. FEMS Microbiol. Lett. 9, 29-33.

Rumbaugh, K.P., Griswold, J.A., Iglewski, B.H. and Hamood, A.N. 1999. Contribution of quorum sensing to the virulence of Pseudomonas aeruginosa in burn wound infections. Infect. Immun. 67, 5854-5862.

Sambrook, J., Fritsch, E.F. and Maniates, T. 1989. In: Molecular Cloning 1. 2nd Edition. Nolan, C. (eds). Cold Spring Harbor Laboratory Press. New York.

Sandrin, T. R., Chech, A. M. and Maier, R. M. 2000. A rhamnolipid biosurfactant reduces cadmium toxicity during naphthalene biodegradation. Appl. Environ. Microbiol. 66, 4585–4588.

Sandrin, C., Peypous, F. and Michel, G. 1990. Coproduction of surfactin and iturin A lipopeptides with surfactant and antifungal properties by *Bacillus subtilis*. Biotechnol. Appl. Biochem.12, 370-375.

Sar, N. and Rosenberg, E. 1983. Emulsifier production by *Acinetobacter calcoaceticus* strains. Curr. Microbiol. 9, 309-314.

Sarkar, P.K., Tamang, J.P., Cook, P.E. and Owens, J.D. 1994. Kinema- a traditional soybean fermented food: proximate composition and microflora. Food Microbiol. 11, 47-55.

Schenk, T., Schuphan, I. and Schmidt, B. 1995. High- performance liquid chromatographic determination of rhamnolipids produced by *Pseudomonas aeruginosa*. J. Chromatography A. 693, 7-13.

Schneider, J., Taraz, K., Budzikiewicz, H., Jacques, P. and Thonart, P. 1999. The structure of two fengycins from *Bacillus subtilis* S499. Z. Naturforsch. 54, 859-865.

Sharanagouda, U. and Karegoudar, T.B. 2001. Degradation of 2- Methylnaphthalene by *Pseudomonas* sp. strain NGK 1. Curr. Microbiol. 43, 440-443.

Sharp, R.J. and Munster, B. 1986. Thermophiles. In: Herbert RA, Codd GA (eds). Microbes in extreme environments. Academic Press, London

Shauver, J.M. 1993. A regulator's perspective on in situ bioremediation. In *In situ* Bioremediation: When Does It Work? pp 99-103. National Academic Press. New York.

Shaver, Y.J., Nagpal, M.L., Rudner, R., Nakamura, L.K., Fox, K.F. and Fox, A. 2002. Restriction fragment length polymorphism of rRNA operons for discrimination and intergenic spacer sequences for cataloging of *Bacillus subtilis* sub-groups. J. Microbiol. Methods. 50, 215-223.

Shaw, A. 1970. Bacterial glycolipids. Bacteriol. Rev. 4, 365-377.

Shaw, A. 1974. Lipid composition as a guide to the classification of bacteria. Adv. Appl. Microbiol. 17, 63-108.

Shaw, D.G. 1992. The Exxon-valdez oil-spill-ecology and social consequences. Environ.Conserv.19, 253-258.

Shaw, A. 1994. Surfactants-94. Soap Cosmet. Chem. Specialisties 70, 24-34.

Shennan, J.L. and Levi, J.D. 1987. In situ microbial enhanced oil recovery. Pp 163-181.In: Biosurfactants and Biotechnology. N Kosaric, Cairns, W.L. and Gray, N.C.C. (eds). Marcel Dekker, New York.

Shepard, R., Rocky, J., Sutherland, I.W. and Roller, S. 1995. Novel bioemulsifier from microorganisms for use in foods. J. Biotechnol. 40, 207-217.

Sheppard, J.D., and Mulligan, C.N. 1987. The production of surfactin *by Bacillus subtilis* grown on pet hydrolysate.Appl.Microbiol.Biotechnol. 27, 110-116.

Sheppard, J.D., Jumarie, C., Cooper, D.G. and Laprade, R. 1991. Ionic channels induced by surfactin in planar lipid bilayer membranes. Biochim. Biophys. Acta 1064, 13-23.

Shoham, Y., Rosenberg, M. and Rosenberg, E. 1983. Bacterial degradation of Emulsan Appl. Environ. Microbiol. 46, 573-579.

Shreve, G.S., Inguva, S. and Gunnam, G. 1995. Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*. Mol. Mar. Biol. Biotechnol. 4, 331-337.

Shulga, A.N., Karpenko, E.V., Eliseev, S.A. and Turovsky, A. A. 1993. The method for determination of anionogenic bacterial surface-active peptidolipids. Microbiol. J. 55, 85-88.

Siegmund, I. and Wagner, F. 1991. New method for detecting rhamnolipids exerted by *Pseudomonas* species grown on mineral agar. Biotechnol. Tech. 5, 264-268.

Sim, L., Ward, O.P. and Li, Z-Y. 1997. Production and characterization of a biosurfactant isolated from *Pseudomonas aeruginosa* UW-1. J. Ind. Microbiol. Biotechnol. 19, 232-238.

Singer, M. E. Vogt. and Finnerty, W.R. 1984. Microbial metabolism of straight and branched alkanes. Pp 1-59.In: Petroleum Microbiology. R. Atlas (ed). Collier Mac Millan, New York.

Singh P. and Cameotra, S.S. 2004. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. 22, 142-146.

Sørensen, D., Nielsen, T.H., Christophersen, C., Sørensen, J. and Gajhede, M. 2001. Cyclic lipoundecapeptide amphisin from Pseudomonas sp. strain DSS73. Acta Crystallogr. C 57, 1123-1124.

Spoeckner, S., Wray, V., Nimtz, M. and Lang, S. 1999. Glycolipids of the smut fungus *Ustilago maydis* from cultivation on renewable resources. Appl. Microbiol. Biotechnol. 51, 33–39.

Stackebrandt, E. and Goebel, B. 1994. Taxonomic note. A place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. 44, 846-849.

Stanghellini, M. E. and Miller, R. M. 1997. Biosurfactants—Their identity and potential efficacy in the biological control of zoosporic plant pathogens. Plant Dis. 81, 4–11.

Steinbuchel, A. 1991. Recent advances in the knowledge of the metabolism of bacterial polyhydrooxyalkanoic acids and potential impacts on the production of biodgradable thermoplastics. Acta Biotechnol. 11, 419-427.

Stringfellow, W.T. and Aitken, M.D. 1995. Competitive metabolism of naphthalene, methylnaphthalenes and fluorene by phenanthrene-degrading *Pseudomonads*. Appl. Environ. Microbiol. 61, 357-362.

Sugiura, K., Ishihara, M., Shimauchi, T. and Harayama, S. 1997. Physiochemical properties and biodegradability of crude oil. Environ.Sci. Technol. 31, 45-51.

Sullivan, E. R. 1998. Molecular genetics of biosurfactant production. Curr. Opion. Biotechnol. 9, 263-369.

Suzuki, T., Tanaka, K., Matsubara, I. and Konishita, S. 1969. Trehalose lipid and α branched- β-hydroxy fatty acid formed by bacteria grown on n- alkanes. Agric. Biol. Chem. 33, 1619–1627.

Suzuki, T., Hayashi, K., Fujikawa, K. and Kieber, H.P. 1974. Sucrose lipids of *Arthrobacteria*, *Corynebacteria* and *Nocardia* grown on sucrose. Agric. Biol. Chem. 38, 557-563.

Suzuki, T., Hayashi, K., Fujikawa, K. and Tsukamoto, K. 1965. The chemical structure of polymyxin E. The identies of polymyxin E_1 with colistin A and polymyxin E_2 with colistin B. J. Biol. Chem. 57, 226-227.

Syldatk, C. and Wagner, F. 1987. Production of biosurfactants. p. 89-120. In: Kosaric, N., Cairns, W.L., and Gray, N.C.C (ed.), Biosurfactanst and biotechnology. Marcel Dekker, Inc., New York.

Syldatk, C., Lang, S. and Wagner, F.Z. 1985. Chemical and physical characterization of four interfacial- active rhamnolipids from *Pseudomonas* sp. DSM 2874 grown on n- alkanes. Z. Naturforsch 40C, 61-67.

Tahara, Y., Yamada, Y. and Kondo, K. 1976. A new lysin containing lipid isolated from *Agrobacterium tumefaciens*. Agric. Biol. Chem. 40, 1449-1450.

Tahzibi, A., Kamal, F. and Assadi, M.M. 2004. Improved production of rhamnolipids by a *Pseduomonas aeruginosa* mutant. Iranian Biomed. J. 8, 25-31.

Takizawa, M., Hida, T., Horiguchi, T., Hiramoto, A., Harada, S. and Tanida, S. 1995. Tan-1511 A, B and C, microbial lipopeptides with G-CSF and GM-CSF inducing activity. J. Antibiot. 48, 579-588.

Tan, H., Champion, J. T., Artiola, J. F., Brusseau, M. L. and Miller, R. M. 1994. Complexation of cadmium by a rhamnolipid biosurfactant. Environ. Sci. Technol. 28, 2402–2406.

Tanner, R.S., Udegbunam, E.O., McInerney, M.J. and Knapp, R.M. 1991. Microbially enhanced oil recovery from carbonate reservoirs. Geomicrobiol. J. 9, 169-195.

Tiehm, A. 1994. Degradation of polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. Appl. Environ. Microbiol. 60, 258-263.

Thimon, L., Peypoux, F., Wallach, J. and Michel, G. 1995. Effect of the lipopeptide antibiotic iturin A, on morphology and membrane ultrastructure of yeast cells. FEMS Microbiol. Lett. 128, 101-106.

Torabizadeh, H., Shojaosadati, S. A. and Tehrani, H. A. 1996. Preparation and characterization of bioemulsifier from *Saccharomyces cerevisiae* and its application in food products. Food Sci. Technol. 29, 734–737.

Torrens, J. L., Herman, D. C. and Miller-Maier, R. M. 1998. Biosurfactant (rhamnolipid) sorption and the impact on rhamnolipid-facilitated removal of cadmium from various soils. Environ. Sci. Technol. 32, 776–781.

Tortora, G.L., Funke, B.R. and Case, C.L. 2001. In: Microbiology-An Introduction. 7th Edition. Pp 158. Addison Wesley Longman, Inc. New York

Trebbau de Acevado, G. and McInerney, M.J. 1996. Emulsifying activity in thermophilic and extremely thermophilic microorganisms. J. Ind. Microbiol. 16, 1-7.

Trzesicka- Mlynarz, D. and Ward, O. P. 1995. Degradation of polycyclic aromatic hydrocarbon (PAHs) by mixed culture and its component pure culture, obtained from PAH-contaminated soil. Can. J. Microbiol. 41, 470-476.

Tsuge, K., Ano, T. and Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Arch. Microbiol. 165, 243-251.

Tuleva, B.K., Ivnov,G.R. and Christova, N.E. 2002. Biosurfactant production by a new *Pseudomonas putida* strain. Z. Naturforsch. 57, 356-360.

Tullock, P., Hill, A. and Spencer, J.F.T. 1967. A new type of marocyclic lactone from *Torulopsis apicola*. J. Chem. Soc. Chem. Commun. 584-586.

Turkovskaya, O.V, Dmitrieva, T.V and Muratova, A.Y. 2001. A biosurfactant-producing *Pseudomonas aeruginosa* strain. Appl. Biochem. Microbiol. 37, 71-75

Van der Vegt, W., Vander Mei, H.C., Noordmans, J. and Busscher, H.J. 1991. Assessment of bacterial biosurfactant production through axisymmetric drop shape analysis by profile. Appl. Microbiol. Biotech. 35, 766-70.

Van Dyke, M. I., Couture, P., Brauer, M., Lee, H. and Trevors, J. T. 1993. *Pseudomonas aeruginosa* UG2 rhamnolipid biosurfactants: Structural characterization and their use in removing hydrophobic compounds from soil. Can. J. Microbiol. 39, 1071–1078.

Van Dyke, M.I., Gulley, S.L., Lee, H. and Trevors, J.T. 1993. Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil. J. Ind. Microbiol. 11, 163-170.

Van Hoogmoed, C. G., van der Kuijl-Booij, M., van der Mei, H. C. and Busscher, H. J. 2000. Inhibition of *Streptococcus* mutans NS adhesion to glass with and without a salivary conditioning film by biosurfactant-releasing *Streptococcus mitis* strains. Appl. Environ. Microbiol. 66, 659–663.

Vander Mei, H.C., Weerkamp, A.H. and Busscher, H.J. 1987. A comparison of various methods to determine hydrophobic properties of streptococcal cell surfaces. J.Microbiol.Methods. 6, 277-287.

Vanittanakom, N., Loeffler, W., Koch, U. and Jung, G. 1986. Fengycin-a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29-3. J. Antibiot. (Tokyo) 39, 888-901.

Vater, J., Kablitz, B., Wilde, C., Franke, P., Mehta, N. and Cameotra, S.S. 2002. Matrix-Assisted Laser Desorption Ionization- Time of Flight Mass Spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. App. Environ. Microbiol. 68, 6210-6219.

Vater, P.J. 1986a. Lipopeptides, an attractive class of microbial surfactants. Prog. Colloid Polym. Sci. 72, 12-18.

Vater, P.J. 1986b. Lipopeptides in food application. Pp 419-446. In: Kosaric, N. (ed) Biosurfactant-production, properties and applications. Dekker, New York.

Velraeds-Martine, M.C., Vander Mei, H.C., Reid, G. and Busscher, H.J. 1996. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl. Environ. Microbiol. 62, 1958-1963.

Velraeds-Martine, M.C., Vander Mei, H.C., Reid, G. and Busscher, H.J. 1997. Inhibition of initial adhesion of uropathogenic *Enterococcus faecalis* to solid substrate by an absorbed biosurfactant layer from *Lactobacillus acidophilus*. Urology 49, 790-794.

Vila, J., Lopez, Z., Sabate, J., Minguillon, C., Solanas, A.M. and Grifoll, M. 2001. Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1: Actions of the isolate on two-and three-ring polycyclic aromatic hydrocarbons. Appl. Environ. Microbiol. 67, 5497-5505

Vollenbroich, D., O¨ zel, M., Vater, J., Kamp, R. M. and Pauli, G. 1997b. Mechanism of inactivation of enveloped viruses by the biosurfactant surfactin from *Bacillus subtilis*. Biologicals 25, 289–297.

Vollenbroich, D., Pauli, G., O¨ zel, M. and Vater, J. 1997a. Antimycoplasma properties and application in cell culture of surfactin, a lipopeptide antibiotic from *Bacillus subtilis*. Appl. Environ. Microbiol. 63, 44–49.

Walker, J.D., Colwell, R.R., Hamming, M.C. and Ford, H.T. 1975. Extraction of petroleum hydrocarbons from oil-contaminated sediments. Bull. Environ. Contam. Toxicol. 13, 245-248.

Wang, X., Gong, L., Liang, S., Han, X., Zhu, C. and Li, Y. 2005. Algicidal activity of rhamnolipid biosurfactants produced by *Pseudomonas aeruginosa*. Harmful Algae 4, 433-443.

Wang, X.H., Smith, R.; Fletcher, J.I., Wilson, H., Wood, C.J., Howden, M.E. and King, G.F. 1999. Structure-function studies of w-atracotoxin, a potent antagonist of insect voltage-gated calcium channels. Eur. J. Biochem. 264, 488-494.

Wagner, M. 1991. Microbial enhancement of oil recovery from carbonate reservoirs with complex formation characteristics. Dev. Petr. Sci. 31, 387-398.

Wayman, M., Jenkins, A.D. and Kormady, A.G. 1984. Biotechnology for oil and fat industry. J. Am. Oil Chem. Soc. 61, 129-131.

World Health Organization. 1981. Instruction for determining the susceptibility or resistance of mosquito larvae to insecticides. WHO/VBC/81. 807. World Health Organization, Geneva, Switzerland.

Wick, L.Y., Colangelo, T. and Harms, H. 2001. Kinetics of mass transfer-limited bacterial growth on solid PAHs. Environ. Sci. Technol. 35, 354-361.

Wicken, A.J. and Knox, K.W. 1970. Studies on the group F antigen of lactobacilli: isolation of a teichoic acid-lipid complex from *Lactobacillus fermenti* NCTC 6991. J. Gen. Microbiol. 60, 303-313.

Wiegel, J. and Ljungdahl, L.G. 1986. Importance of thermophilic bacterial in biotechnology. CRC Crit. Rev. Biotechnol. 3, 39-108.

Wilkinson, S.G. 1972. Composition and structure of the ornithine-containing lipid from *Pseudomonas rubescens*. Biochim. Biophys. Acta 270, 1-17.

Willumsen, P. A. and Karlson, U. 1997. Screening of bacteria, isolated from PAH-contaminated soils, for production of biosurfactants and bioemulsifiers. Biodegradation 7, 415–423.

Wilson, N.G. and Bradley, G. 1997. A study of a bacterial immobilization substratum for use in the bioremediation of crude oil in a saltwater system. J. Appl. Microbiol. 83, 524-530.

Wilson, S.C and Jones, K.C. 1993. Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAH)s: a review. Environ. Pollut. 81, 229-249.

Wong, M. Y. H., Steck, P. A. and Gray, G. R. 1979. The major mycolic acids of *Mycobacterium smegmatis*. J. Biol. Chem. 254, 5734–5740.

Xia, Y., Chen, W., Fu, X., Zhang, H., Yang, S. and Ding, X. 2005. Construction of an integrative food-grade expression system for *Bacillus subtilis*. Food Res. Int. 38, 251-256.

Yakimov, M. M., Golyshin, P. N., Lang, S., Morre, E. R. B., Abraham, W. R., Lunsdorf, H. and Timmis, K. N. 1998. *Alcanivorax borkumensis* a new hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol. 48, 339–348.

Yakimov, M.M., Timmis, K.N., Wray, V. and Fredrickson, H.L. 1995. Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS 50. Appl. Environ. Microbiol. 61, 1706-1713.

Yoneyayshi, H., Yoshida, S., Ono, K. and Enomoto, H. 2000. Screening of microorganisms for microbial enhanced oil recovery process. Sekiyu Gakkaishi. 43, 59-69.

Youssef, N.H., Duncan K.E., Nagle, D.P., Savage K.N., Knapp R.M. and McInerney, M.J. 2004. Comparison of methods to detect biosurfactant production by diverse microorganisms. J. Microbiol. Methods 6, 339-347.

Yuste L., Corbella, M.E., Turiegano, M.J., Karlson, U., Puyet, A. and Rojo, F. 2000. Characterization of bacterial strains able to grow on high molecular mass residues from crude oil processing. FEMS Microbiol. Eco. 32, 69-75.

Zajic, J.E., and Panchal, C.J. 1976. Bio-emulsifiers. Crit. Rev. Microbiol. 5, 39-66.

Zajic, J.E. and Panchal, C.J. 1996. {in 1997 vol}. Bio-emulsifiers. CRC Crit. Rev.Microbiol. 5, 39-66.

Zajic, J.E. and Seffens, W. 1984. Biosurfactants. CRC Crit. Rev. Microbiol. 5, 87-107.

Zhang, H., Kallimanis, A., Koukkou, A.I. and Drainas, C. 2004. Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. Appl. Microbiol. Biotechnol. 65, 124-131.

Zhang, Y. and Miller, R.M. 1994. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl. Environ.Microbiol. 60, 2101-2106.

Zosim, Z., Gutnick, D. and Rosenberg, E. 1983. Uranium binding by emulsan and emulsanosols. Biotechnol. Bioeng. 25, 1725–1735.

Zosin, Z., Gutnick, D.L. and Rosenberg, E. 1982. Properties of hydrocarbon-in-water emulsions stabilized by *Acinetobacter* RAG-1 emulsan. Biotechnol. Bioeng. 24, 281-292.

Zukerberg, A., Diver, A., Peeri, Z., Gutnick, D.L. and Rosenberg, E. 1979. Emulsifier of Arthrobacter RAG-1: chemical and physical properties. Appl. Environ. Microbiol. 37, 414-420.

Appendix

Mineral Salt media (Banat, 1993)

Composition (g/l) Na_2HPO_4, 2.2; KH_2PO_4, 1.4; $MgSO_4.7H_2O$, 0.6; $(NH_4)_2SO_4$, 3.0; Yeast extract, 1.0; NaCl, 0.05; $CaCl_2$, 0.02, $FeSO_4$, 0.1 and 1ml trace element solution.

Mineral Salt media (Goswami and Singh, 1991)

Composition (g/l) Urea, 2g; $(NH_4)_2SO_4$, 2g; Na_2HPO_4, 3.61; KH_2PO_4, 1.75; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.05; and 1 ml trace element solution.

Solution I

50 mM glucose
25 mM Tris-Cl (pH 8.0)
10 mM EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100 ml, autoclaved for 15 min at 10 lb/ sq. in. on liquid cycle and stored at 4°C.

Solution II

0.2 N NaOH (freshly diluted from 10 N stock)
1.0% SDS

Solution III

5 M potassium acetate	60 ml
glacial acetic acid	11.5 ml
H_2O	28.5 ml

The resulting solution is 3 M with respect to potassium and 5 M with respect to acetate.

LIST OF PUBLICATIONS

Research papers accepted for publication

1. **Das, K.** and Mukherjee, A. K. (2005) Characterization of biochemical properties and biological activities of biosurfactants produced by *Pseudomonas aeruginosa* mucoid and non-mucoid strains isolated from hydrocarbon-contaminated soil samples. ***Applied Microbiology and Biotechnology***, Springer Verlag GmbH, Germany (In press).

2. Mukherjee, A. K. and **Das, K.** (2005) Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. ***FEMS Microbiology Ecology***, Elsevier Publication, UK. (In press).

Revised manuscript communicated

1. **Das, K** and Mukherjee, A. K. (2005) Assessment of mosquito larvicidal potency of cyclic lipopeptides produced by *Bacillus subtilis* strains. **Acta Tropica**, Elsevier Publication, UK . (Manuscript communicated after minor revision).

Manuscript under preparation

1. **Das, K** and Mukherjee, A. K. (2005) A comparative study on the utilization of pyrene for growth and energy production by *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* M and NM strains isolated from a petroleum contaminated soil sample: Role of biosurfactants in enhancing the apparent solubility and metabolism of pyrene (under communication)

Research papers presented in National Seminar/ Symposium

1. **Das, K.,** Mukherjee, A. K. and Konwar, B.K. (2003). Potential application of biosurfactant produced by thermophilic *Pseudomonas* sp. DM-02 strain in Microbial Enhanced Oil Recovery (MEOR) and Bioremediation. *National Seminar on Hydrocarbon Degrading Microbes. 22nd December 2003.* Organized by Centre for Petroleum Biotechnology, Department *of Molecular Biology and Biotechnology, Tezpur University & sponsored by Oil and Natural Gas Corporation Limited at Tezpur University.*
2. **Das, K** and Mukherjee, A. K. (2004).Role of biosurfactant in the managent of oil pollution. *National Workshop on Regional Development: Case for North East India.* IIT, Guwahati.3-6 Feb, 2004. *Organized by Tezpur University, IIT Guwahati and C-MMACS, Bangalore, India.*

Corrigendum

S. No	Page No., paragraph/ section/line	Mistake	Corrigendum/ Should be read as
1	P11, 2nd paragraph, line 6	Rosen, 1988	Rosen, 1989
2	P13,42,59	"biosurfactants production" and "biosurfactants producing".	The term "biosurfactants-producing and biosurfactants producing" shuold be read as "biosurfactant- producing" and biosurfactant producing
3	P17,1st paragraph, line3	Delcambe et al., 1957	Delcambe and Devignat, 1957
4	P37, section 3.1, line3	Atlas, 1993	Atlas, 1993a
5	P38, section 3.3.1, line3	45 C	45 °C
6	P52, section 3.12.2 line1	Hirota et al., 1960	Hirota, 1960
7	P52, section 3.13.1 line2	Abu-Ruwaida et al, 1991	Abu-Ruwaida et al, 1991a
8	P53, section3.13.2.1 heading and line1	PAHs solubilization effect	PAH solubilization effect
9	P54, section 3.13.2.2, line 6	A control experiment was	A control was
10	P56, section 3.13.3.4, line 4	"60 µg of pyrene"	60 ng of pyrene
11	P 81, section 5.1.1.5 line 3	Final concentration of thiamine in the medium when 1 ml/L stock solution is used	10 µg/l
12	P142, section 6.1.4.7 line 1	MALDI-TOF-MS- RP HPLC purification of biosurfactant	MALDI-TOF-MS- RP HPLC purified biosurfactant
13	P 147, line 2	The term "Cent per cent"	Should be read as 100%
14	P 150, section 6.1.10	Term "mutant"	The word "mutant" should be read as "cured"
15	P 151, Figure 6.17(B)	The word "mutant"	The word "mutant" is deleted from the text.
16	P 152, line 2 & P 157, line 10	"On the other hand"	Should be read as "In addition to"
17	P158, Table 7.1	Legend of the table-solubility is expressed as µg/ml	Solubility should be read as ng/ml
18	P 161, Figure 7.5.	The Y axis label "Content (% TPH)"	Should be read as "Content (% TPH)"
19	P 164, Figure 7.7 Legend of the figure.	The designation "glucose depleted""	Should be read as "No glucose"
20	P167, Table 7.4	Legend of the table- pyrene uptake (µg/1 X 10^7 bacteria)	pyrene uptake (ng/1 X 10^7 bacteria)
21	P177, 1st paragraph, line 4	Roongsaway et al., 2000	Roongsaway et al , 2002

(Signature of student)

S. No	Page No., paragraph/ section/line	Mistake	Corrigendum/ Should be read as
22	P 177, section 8.1.2, line 5	The sentence "Because fatty acids.....during GC analysis".	The sentence is deleted from the text.
23	P 182, 3rd paragraph	The sentence "A biosurfactant yield.....at 30°C(Sim et al., 1997)".	The sentence is deleted from the text.
24	P 183, section 8.1.7, line 1	Could the author clarify the statement "Lipopeptide profile.....varies greatly within the strains"	Should be read as "Lipopeptide profile.....varies greatly between the strains"
25	P 190, line 10	The phrase "soap stick oil has.....sunflower oil.	The word "has" is being deleted from the text.
26	P 191, section 8.1.15, 2nd paragraph, line 6	Reference "Catallo and Partier,1992"	should be read as Catallo and Portier, 1992
27	P192, 2nd paragraph, line 3	"Control experiment"	Control
28	P195, 2nd paragraph, line 3	% assimilation of pyrene	Strains %assimilation of pyrene *B. subtilis* DM-04 = 47.7 % *P. aeruginosa* M = 32.4 % *P. aeruginosa* NM = 31.8 %
29	P195, 3rd paragraph, line 3 and 6	Gibson et al., 1984	Gibson and Subramanium, 1984
30	P196, last paragraph, line 1	"Convincing data was presented showing that---"	Should be read as "Data has been presented to show that"
31	Page 196, line 12	"Glucose acts as an inducer"	Should be read as "Glucose acts as an inducer of growth"
32	Page 199, line 13	The reference "Maget-Dana and Ptak,1995"	Should be read as "Maget-Dana et al.,1995
33	Page 199, 3rd paragraph, line 3	Abbreviations "Bs" and "Bti" have been used without defining.	Biolarvicides of bacterial origin from *Bacillus thuringiensis* ssp. *israelensis* and *Bacillus sphaericus* are know as "Bti" and "Bs" respectively.
34	Page 200, section 8.2, line 3	The sentence "Further some of the potential industrial applications crude natural roles of biosurfactants to the producing bacteria were also explore."	The sentence should be read as "Further, some of the potential industrial applications of biosurfactant were also explored."
35	P202, 3rd paragraph, line 2	Study on bioremediation	Study on biodegradation
36	P 214	Holmber,2001	Holmberg, 2001
37	P 236	Zosin et al., 1982	Zosim et al., 1982

(Signature of student)

Addendum:

Missing references from reference list

Gringorten, J. L., Milne, R. E., Fast, P. G., Sohi, S. S. and van Frankenhuyzen, K. 1992. Suppression of *Bacillus thuringiensis* δ-endotoxin activity by low alkaline pH. J. Invertebrate Pathology 60, 47-52.

Heyrman, J., Mergaert, J., Denys, R., Swings, J. 1999. The use of fatty acid methyl ester analysis (FAME) for the identification of heterotrophic bacteria present on three mural paintings showing severe damage by microorganisms. FEMS Microbiol. Letters 181, 55-62.

Maget-Dana,R and Ptak,M. 1995. Interactions of surfactin with membrane models. Biophys. J. 68, 1937-1943.

Nishiitsutsuji-Uwo, J., Ohsawa, A. and. Nishimura, M. S. 1977. Factors affecting the insecticidal activity of δ-endotoxin of *Bacillus thuringiensis*. J. Invertebrate Pathology 29, 162-169.

Pozsgay, M., Fast, P., Kaplan, H. and Carey, P. R. 1987. The effect of sunlight on the protein crystals from *Bacillus thuringiensis* var. *kurstaki* HD1 and NRD12· A Raman spectroscopic study. J. Invertebrate Pathology 50, 246-253.

White, K.L. 1986. An overview of immunotoxicology and carcinogenic polycyclic aromatic hydrocarbons. Environ. Carcinogen Rev. C4: 163-202.

Kishor Das

(Signature of student)

Addendum:

Figures

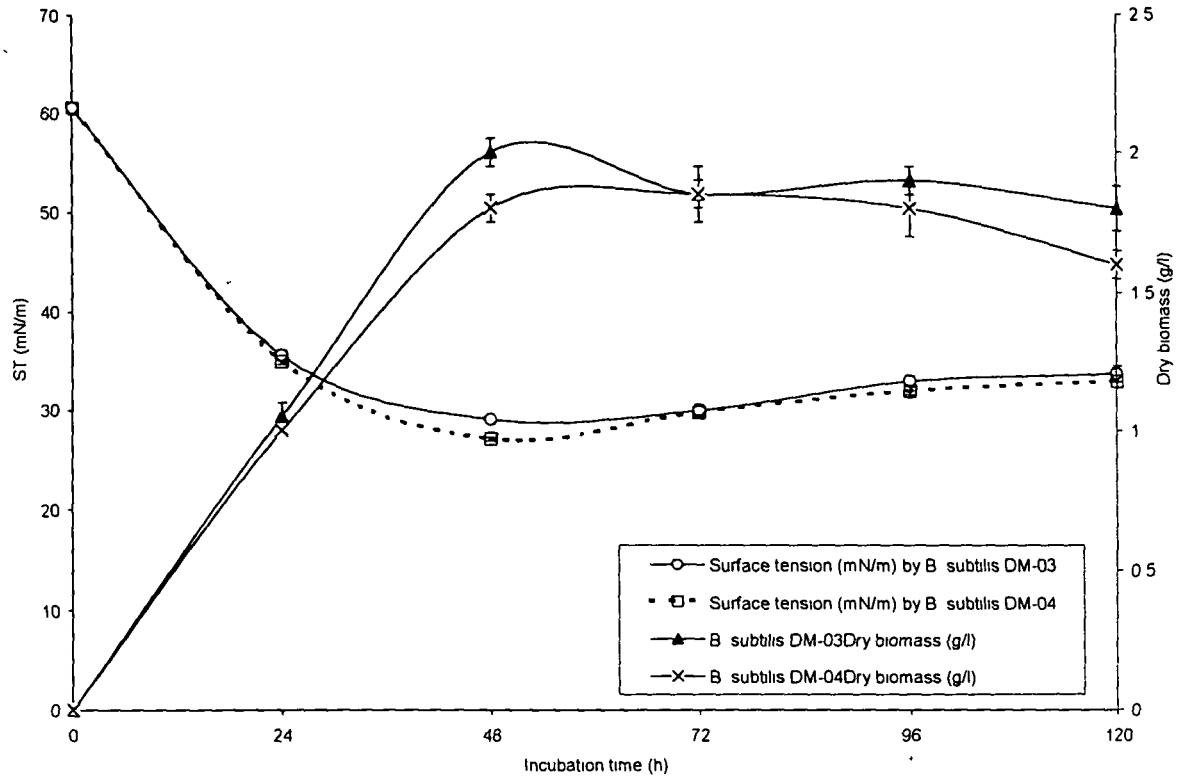

Figure 1. Growth and surface tension reduction by *B. subtilis* strains at different time intervals. Data represents the mean ± S.D. of four individual experiments.

(Signature of student)

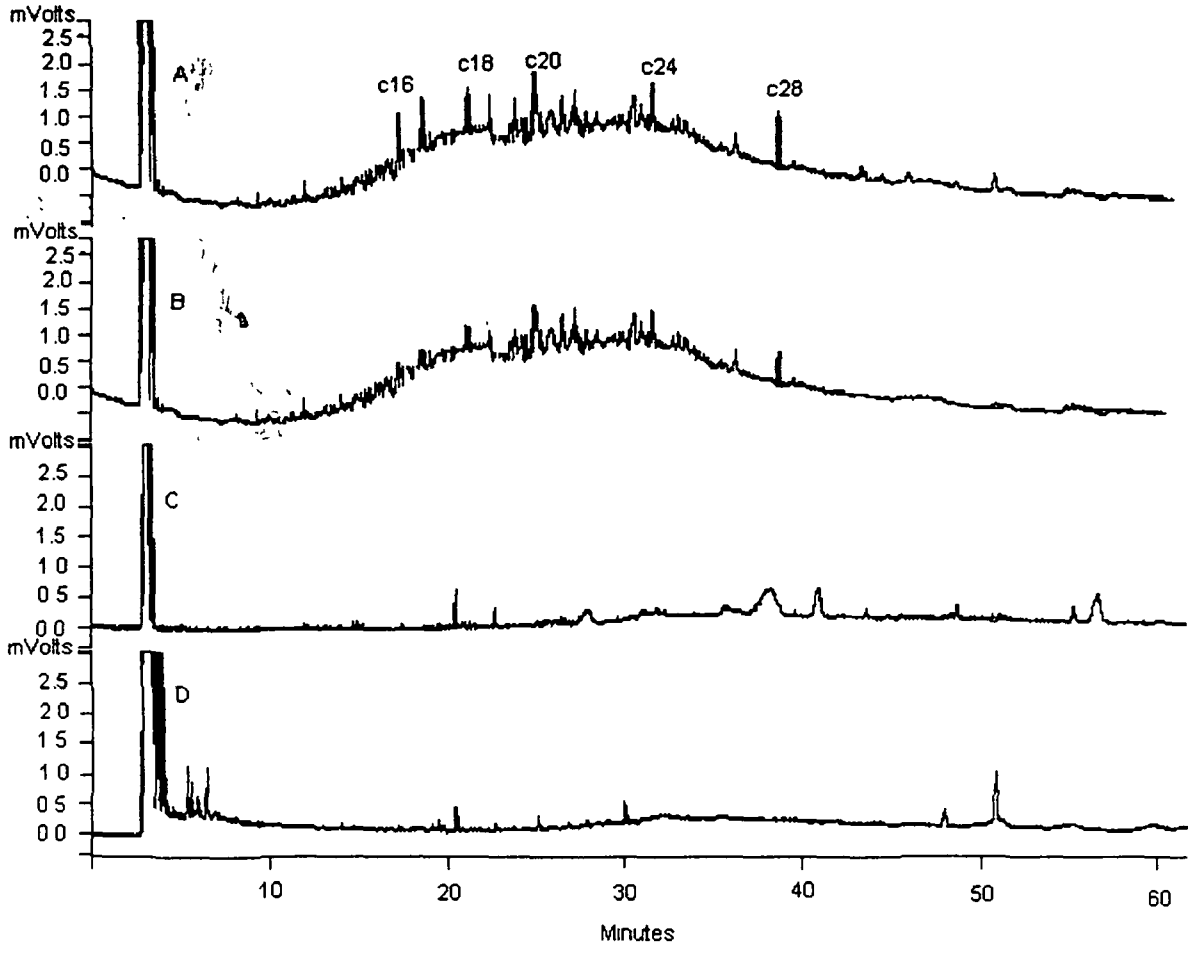

Figure 2. GC fingerprinting of alkane fraction of soil seeded with *P. aeruginosa* M and NM strain consortia and *B. subtilis* DM-04 strain

(a) at 0 d (beginning of bioremediation experiment)

(b) after 120 d without exogenously seed bacteria (control)

(c) after 120 d with *B. subtilis* DM-04 strain.

(d) after 120 d with *P. aeruginosa* M and NM strain

Kishor Das

(Signature of student)

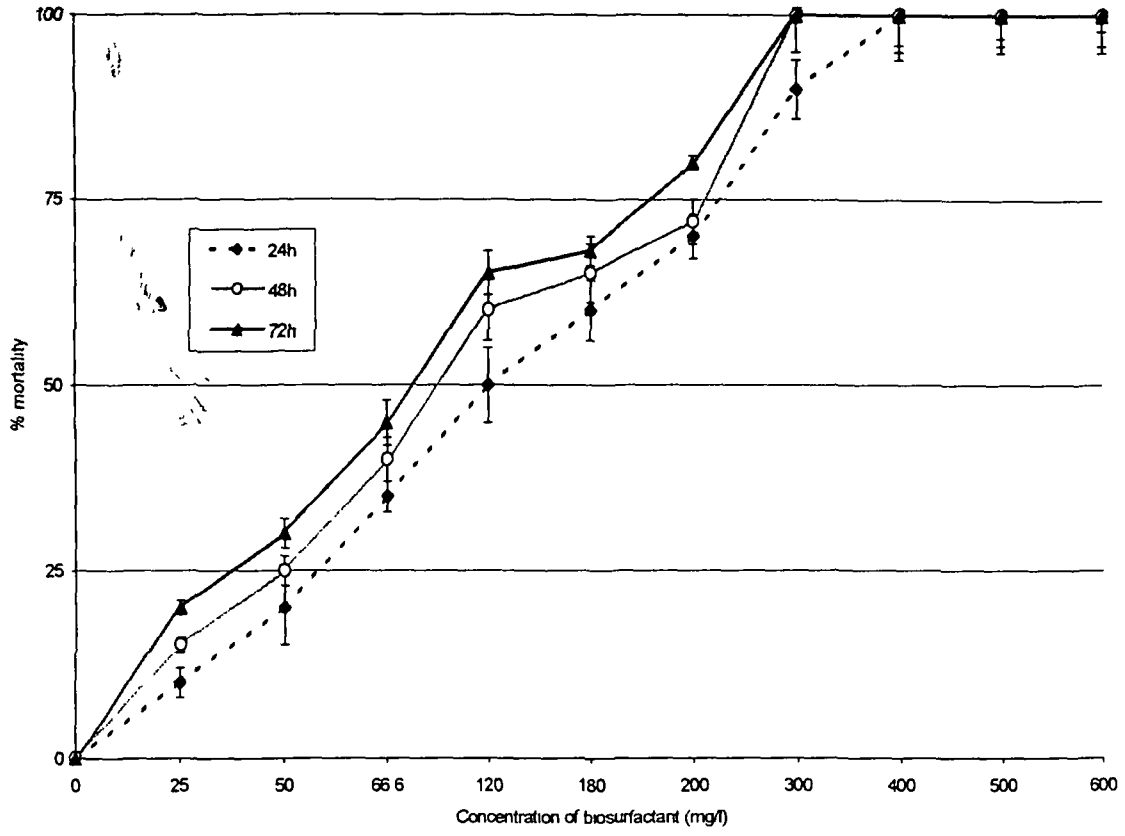

Figure 3. Dose dependent mosquito larvicidal potency of biosurfactant from *B. subtilis* DM-03 strain. Results represent mean ± S.D of three independent experiments.

(Signature of student)

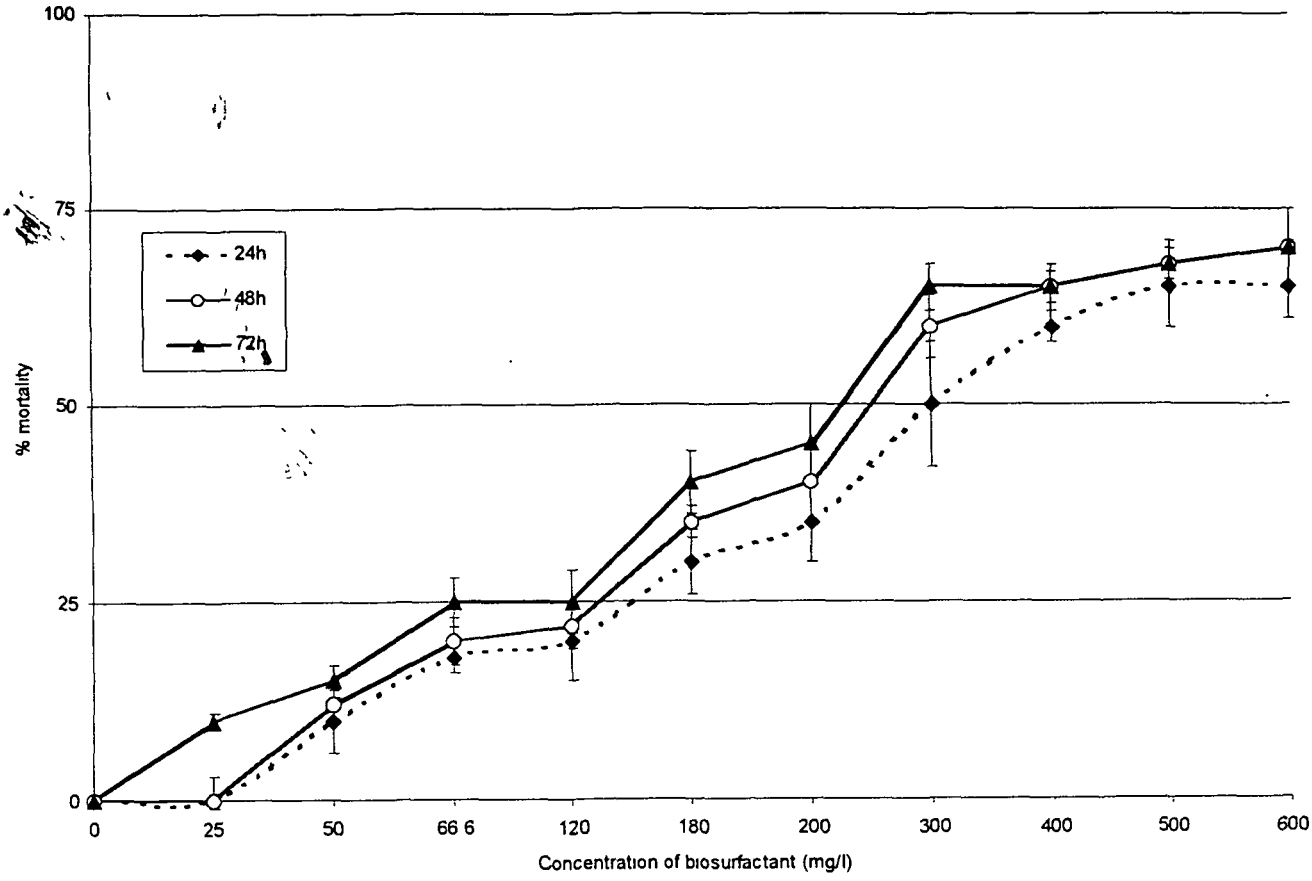

Figure 4. Dose dependent mosquito larvicidal potency of biosurfactant from *B. subtilis* DM-04 strain. Results represent mean ± S.D of three independent experiments.

(Signature of student)