BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF BACTERIAL BIOSURFACTANT

A thesis submitted in partial fulfillment of the requirements for award of the degree of

Doctor of Philosophy

By

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December, 2013

Dedication

I dedicate this thesis to my ever loving parents and brother

Abstract

Accident soil contamination by petroleum hydrocarbons can be remediated by physical, chemical, or biological techniques. Among the available remediation techniques, use of microorganisms is an emerging environmental–friendly technology[1, 2].

Biosurfactants of bacterial origin hold distinct advantages over synthetic ones including biodegradability and biocompatibility, multifunctional characteristics, stable activity under extreme environmental conditions, and thus can be more efficient in remediation of petroleum hydrocarbon contaminated soil[2, 3]. Rhamnolipids produced by *Pseudomonas aeruginosa* have been one of the most widely studied biosurfactants[3]. In spite of numerous advantages over the synthetic chemical surfactants, biosurfactants are still unable to contend with the synthetic surfactants. This could be due to their high production cost in relation to the inefficient bioprocessing techniques and poor strain productivity.

In the backdrop of above mentioned information, the present investigation has been focused on (i) screening and taxonomic identification of efficient biosurfactant producing bacteria (ii) optimization of culture conditions, (iii) characterization of isolated biosurfactants, (iv) investigation on the biological activity of biosurfactants and (v) investigation on the possible industrial applications of both the biosurfactants and their producing bacteria.

Four potential bacterial strains have been selected from a total of 52 bacterial strains possessing the ability to produce biosurfactant and utilize hydrocarbons, particularly the purified petroleum hydrocarbons. Biochemical and molecular characterization revealed that all four isolates were closely related strains of *Pseudomonas aeruginosa*. Blood agar assay, CTAB agar test and orcinol assay clearly suggested the glycolipidic nature of biosurfactant, produced by the bacterial strains. The strains were subjected to growth in mineral salt medium (MSM) supplemented with various carbon sources including aliphatic, aromatic and polyaromatic components of crude petroleum. The bacterial strains could degrade nearly all the

tested aliphatic hydrocarbons, but their growth on aromatic and polyaromatic hydrocarbons was not good. The bacterial strains could produce higher yield of biosurfactants with the use of n-hexadecane as sole source of carbon (2.83-4.57 $g.l^{-1}$). The agro industrial wastes like waste residual glycerol and residual kitchen oil were found to be promising alternate cheap carbon sources for the production of rhamnolipid (1.52-3.90 $g.l^{-1}$ and 0.44-2.26 $g.l^{-1}$ respectively).

The critical micelle concentration (CMC) values of the biosurfactants produced by the bacterial strains were in the range of 45-105 $mg.l^{-1}$. The interfacial tension against diesel decreased from 29 mNm^{-1} to a range of 1.5-3.4 mNm^{-1} in the use of biosurfactants produced by the bacterial strains. Biosurfactant at normal and CMD^{-1} (critical micelle concentration) were stable and showed optimum surface activity between the pH 5.0-8.0, temperature 25-75°C, salinity up to 4% and in the presence of various metal ions except Al^{+3}. The cell free culture supernatant of the bacterial strains produced stable foam with F_{24}% in the range of 50.4-65.5% and was relatively stable upto 24 h. The emulsification activity (E_{24}%) of the cell free culture supernatant of the bacterial strains against diesel was quite stable in the pH range of 5.0-8.0, temperature 25-75°C and up to 3% salinity. The bacterial strains exhibited wide variability in surface hydrophobicity that increased with the complexity of the carbon sources.

Purification and characterization of biosurfactants using TLC, FTIR and LC-MS indicated the presence of different congeners of rhamnolipid molecules. Moreover, the occurrence of rhamnolipid molecules was found to be quite variable. Differential scanning calorimetric (DSC) and thermo-gravimetric analysis (TGA) indicated a comprehensive thermally stable structure of the biosurfactants.

Reduction in the viscosity of crude oil following the treatment from 48.7 to 34.6 Pa.sec indicated the ability of bacterial strains to alter the physicochemical properties of the crude oil by degrading the fractions of crude oil. The solubilization assays of anthracene, phenanthrene and naphthalene clearly showed that solubilization of polyaromatic hydrocarbons (PAHs) depends on the concentration of biosurfactants. Bacterial strains OBP1 and OBP4 appeared to be the best degraders of crude oil. Out

of the eleven different bacterial consortia consortium I and II were efficient in degrading 78.6-80.4% of aliphatic, 42.4-42.7% of aromatic and 19.2-21.6% of nitrogen sulphur oxygen containing compounds (NSO compounds) in 30 days of culture which was much higher as compared to individual strains.

The maximum recovery of crude oil from the petro-sludge or contaminated soil was obtained with use of the bacterial biosurfactants at their respective CMCs. About 9.3-11.4% of residual crude oil was recovered from the saturated sand pack column using cell free culture supernatant of the bacterial strains containing biosurfactant as compared to the control.

The degradation of biosurfactants by *P. aeruginosa* strain (MTCC8165) was comparatively less than as compared to *Bacillus circulans* strain (MTCC8167) which preferred that the biosurfactants can't be easily degraded by its source species. The biosurfactants showed appreciable antibacterial activity against a wide variety of bacteria and was more pronounced towards Gram-positive. Rhamnolipids exhibited excellent chemo-attractant property. Biosurfactants above CMC showed phytotoxicity on germinating rice and mung seeds; but showed no larval mortality of *Aedes albopictus* at almost all the recommended concentrations. The treatment of the mouse fibroblast cell line with the biosurfactants exhibited no cytotoxicity within the CMC and also indicated no acute dermal toxicity as confirmed by the haematological tests. Dermal toxicity test has been done to assess the allergic or immunological effect of rhamnolipid on the skin (dermal tissue) of animal model system (rabbit) through haematological assays.

The biosurfactant of OBP1 was more efficient in the synthesis of silver and iron oxide nanoparticles and provided significant stabilization towards nanoparticles and protected silver nanoparticles from exposure to salt (NaCl). Further both nanoparticles exhibited significant antibacterial activity. The nucleophilic addition of 6-amino-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione and 6[(dimethylamino) methyleneamino]-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione with aldehyde (aromatic, aliphatic and heterocyclic) and biosurfactants of OBP1 was achieved in

water at room temperature with higher yield of products as against their counterparts without the use of biosurfactants. Degradation studies of synthetic hyperbranched epoxy/OMMT clay nanocomposites based polymer in the presence of bacterial strain OBP1 clearly indicated the efficiency of the bacterial strain in the biodegradation of synthetic polymers.

The physico-chemical properties of the biosurfactant and the efficiency of the bacterial strains in utilizing the petroleum hydrocarbons clearly indicate their potential application in bioremediation and industrial processes. Further, the present study opens up a possibility to study the effect of rhamnolipids on flora and fauna present at the site of application to ensure their remediation efficiency without causing harm to the on-site living systems.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis **"Biochemical and Molecular Characterization of Bacterial Biosurfactant"**, submitted to **Department of Molecular Biology and Biotechnology, Tezpur University**, Tezpur, Assam in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology and it has not been previously considered for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition from any University, Institute or other organizations.

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All helps received from various sources have been duly acknowledged.

No part of this thesis has been submitted elsewhere for award of any degree.

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Acknowledgements

It gives me immense pleasure and joy to express my heartfelt gratitude to my supervisor, Prof. Bolin Kumar Konwar, Professor Department of Molecular Biology and Biotechnology, Tezpur University, Napaam, and presently Vice chancellor, Nagaland University (Central) HQ: Lumami, Dist. Zunheboto, Nagaland, who has guided me throughout the entire work. Without his constant inspiration, encouragement and constructive criticism, the present investigation would not have been possible.

I would like to express my gratitude to my Doctoral committee members, Prof. K. K. Baruah, Professor Department of Environmental Science, Tezpur University and Prof. T. K. Maji, Professor Department of Chemical Sciences, Tezpur University, for their kind advices and guidance during the tenure of my research work.

My sincere thanks to Prof. A. K. Buragohain, Prof. A. K. Mukherjee, Prof. S. Baruah (HOD), Dr. S. K. Ray, Dr. M. Mandal, Dr. A. M. Ramteke, Dr. T. Medhi, Dr. R. Doley, Dr. E. Kalita, Dr. S. P. G. Ponnam, Dr. R. Mukhopadhyay, Dr. S. Saha, Dr. A. N. Jha, Dr. L. Bora, Dr. N. D. Namsa, Dr. Satish, Dr. S. Dasgupta and Dr. J. P. Saikia for their help and support.

I would like to express my sincere thanks to Dr. A.K. Thakur, Department of Chemical Sciences, Tezpur University

for his kind advice and constant encouragement during the tenure of my research period.

Special thanks to my senior Dr. Subrata Das for his friendly advices and constant encouragement.

It is a great pleasure to offer my sincere thanks to all staff members of the Department, specifically Dr. Kalyan K. Hazarika, Dr. Naba K. Bordoloi, Mr. Pranab Mudoi, Mr. Samar Kakati, Mr. Deepak Deka and Mr. Bania for their support during the entire course of investigation.

I would like to acknowledge CDRI Lucknow, NCCS Pune, IIT Guwahati, IIT Kharagpur, Assam Agriculture University Jorhat, DRL Tezpur, CSIR-NEIST Jorhat, CIF NEHU Shillong, Sophisticated Instrument Facilities and infrastructure received from the Department of Chemical Sciences and other Departments of Tezpur University.

I am also grateful to Dr. Ranjan Dutta Kalita, Dr. Sudhir Kr. Roy, Vishwajyoti Baruah, S. Tiken Singh, Jai Prakash Yadav and Abhijeet Dutta for their support.

Special thanks to Yasir Bashir for his constant support and encouragement throughout my Ph.D tenure.

I thank Pinkee Phukon, Mayur Mausum Phukan, Anggana Ray, Krishna Gogoi, Kalpana Sagar, Salam Pradeep Singh, Shyamalima Gogoi, Pranjal Pratim Das and Bhaskarjyoti Gogoi for their help.

I also offer thanks to Dr. (Mrs.) Juri Konwar, Chandrika and Chandrim Konwar for their support throughout the work.

I offer my sincere thanks to the Basin Manger and all officials and personnel of ONGC, Cinnamara, Jorhat, Assam for their support and assistance.

The financial assistance in the form of a Senior Research Fellowship received from the Oil and Natural Gas Corporation sponsored project entitled "Bioremediation of crude oil contaminated soil" (PI: Prof. B. K. Konwar) is duly acknowledged.

The reason for my being and the reason for making me what I am are my parents. I thank them for everything. They faced all the hardships to see me succeed in every endeavor of mine. My brother, Manoj Bharali, has always given me the hope and the support whenever I needed and I sincerely thank him for all his support.

I thank all those people who helped me in many other ways yet whose name couldn't be mentioned.

At last, I thank Almighty God for keeping me safe and happy.

Bharali

Date 30·12·2013 (Pranjal Bharali)

CONTENTS

List of Tables

List of Figures

List of Abbreviations

ABS	Absorbance
A.U.	Absorbance unit
BLAST	Basic local alignment search tool
BS	Biosurfactant
CMC	Critical micelle concentration
CMD	Critical micelle dilution
CFU	Cell forming unit
CTAB	Cetyl trimethylammonium bromide
DNA	Deoxyribonucleic acid
DB	Dry biomass
EOR	Enhanced oil recovery
E_{24}%	Emulsification index
EI	Electron bombardment ionization
F_{24}%	Foaming index
FTIR	Fourier transform infrared spectroscopy
g	gram
GC	Gas chromatography
h	hour
l	litre
LC	Liquid chromatography
LB	Luria Bertani
L	Levo rotatory
MEOR	Microbial enhanced oil recovery
MSM	Mineral salt medium
MS	Mass spectroscopy
mg	milligram
$mN.m^{-1}$	Milli Newton per meter
min	minutes
n	Normal

NaCl	Sodium chloride
nm	Nanometer
O.D.	Optical density
PBS	Phosphate buffer saline
rmp	Revolution per minute
r-RNA	Ribosomal ribonucleic acid
RL	Rhamnolipid
RE	Rhamnose equivalent
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TPH	Total petroleum hydrocarbon
TLC	Thin layer chromatography
TEM	Transmission electron microscope
ST	Surface tension
SEM	Scanning electron microscope
SNP	Silver nano particles
UV/Vi	Ultra violet/Visible
v/v	Volume/volume
viz.	Namely
w/v	Weight/volume
XRD	X-ray
µg	Microgram
µl	Microlitre

Chapter I
Introduction

1.1 Surfactant

Surfactants are surface active agents, with broad range properties including the lowering of surface and interfacial tensions of liquids[1, 2]. These surface active compounds are a group of commonly used chemicals in everyday life. Characteristically, these are organic amphipathic compounds containing the hydrophilic (polar) moiety usually referred to as the 'head', and the nonpolar hydrophobic moiety known as the 'tail'. Because of their amphiphilic nature, surfactant molecules accumulate at the interfaces such as solid-liquid, liquid-liquid or vapour-liquid. The hydrophobic portion concentrates at the surface with a strong attraction to surrounding solution while the hydrophilic portion is oriented towards the solution with weak to non-attraction forces. At interface, these molecules reduce the free energy of the system by reducing the forces of repulsion between unlike phases or surfaces and allow the two phases to mix more easily. Surfactant molecules have the ability to lower the surface tension, increase solubility, detergency power, wettability and foaming capacity[3]. The unique surface active properties of surfactants have been exploited in various areas such as detergents, emulsification, adhesion, coatings, wetting, foaming, soil and water remediation, paints, chromatographic separations, medicine, agriculture, cosmetics, personal care and almost every sector of modern industry[4]. Surfactants are also the key ingredients found in detergents, shampoos, toothpaste, oil additives, and a number of other consumer products[5].

1.2 *Chemical nature of surfactant*

Surfactants are classified with varying chemical structures according to their ionic charge residing in the polar part of the molecule. Hence anionic, cationic, non-ionic and zwitterionic (combined presence of anionic and cationic charges) surfactants exist[1, 5]. The hydrophobic moiety is usually a hydrocarbon chain of varying length in different surfactants[1, 6]. Surfactants are mainly synthesized from chemical based

materials such as petroleum derived hydrocarbons, lignosulfonates or triglycerides[6]. Majority of the synthetic surfactants includes linear alkyl benzenesulphonates, alcohol sulphates, alcohol ether sulphates, alcohol glyceryl ether sulphonates, α-olefin sulphonates, alcohol ethoxylates and alkyl phenol ethoxylates[7]. Surfactants are potentially useful in every industry dealing with multiphase systems. For example, Sodium dodecyl sulphate (SDS, $C_{12}H_{25}SO_4^-Na^+$) a widely used anionic surfactant. It contains a straight chain aliphatic hydrocarbon with a sulphate group[1].

1.3 Actions of surfactant

Surfactants enhance the aqueous solubility of non-aqueous phase liquids (NAPLs) by reducing their surface/interfacial tension at air-water and water-oil interfaces[5]. The effectiveness of a surfactant is determined by its ability to lower the surface tension, which is a measure of the surface free energy per unit area or the work required to bring a molecule from the bulk phase to the surface[8]. Another distinguishing character of surfactants is their ability to self-assemble in solution into dynamic aggregates called micelles. As the interfacial tension is reduced at the air-liquid interface and the aqueous surfactant concentration increased, the monomers aggregate to form micelles. The minimum concentration of the surfactant at which micelles first initiate to form is referred to as critical micelle concentration (CMC)[9]. The concentration corresponds to the point where the surfactant molecules reduce the maximum surface tension of the system. The CMC of a surfactant is influenced by pH, temperature and ionic strength[6]. The physical properties used to characterize surfactants that depend on CMC include emulsion formation, oil solubilization, foaming and detergency, interfacial and surface tensions. Generally the dispersions of the complex fluids form and stabilize by absorbing surfactants onto air-liquid interface, known as foams. These dispersed fluids contain small bubbles with large surface areas, which can be stabilized using surfactants. Formation of heavy foams signifies the better detergency character which indicates the effectiveness of the surfactant in separating oily material from a particular medium[5].

The dynamics of the surfactant adsorption is of vast significance for practical applications such as foaming, emulsifying or coating processes, where bubbles or drops are rapidly generated and need to be stabilized. The dynamics of surfactant adsorption depends on its diffusion coefficient. As the interface is created, the adsorption of the surfactant is limited by the diffusion at the interface. In certain cases, there is an existence of energy barrier for the adsorption or desorption of the surfactants at the interface, then the adsorption dynamics is known as 'kinetically limited'. Such energy barrier is due to the steric or electrostatic repulsions between the surfactant molecules. The surface rheology of surfactant layers, including their elasticity and viscosity plays a very vital role in foam or emulsion stability.

The immiscible liquids such as oil and water when mixed, one liquid is dispersed into the other, and small droplets form emulsion. An emulsion is defined as a "heterogenous system, consisting of at least one immisicible liquid dispersed in another in the form of droplets, whose diameters, in general, exceed 0.1 mm[1, 2]. The behavior of the emulsion is related to the equilibrium phase behavior of the oil/water/surfactant system from which it is made[5]. Formation of such small droplets provides a large amount of interfacial surface area and hence greater interfacial free energy in the system. In normal condition, the droplets may rapidly coalesce and two separate phases will form to minimize the interfacial area of the system[9]. With the addition of surfactants, emulsion formed may stabilize by reducing the interfacial tension and decreasing the rate of coalescence[8]. Usually there are two types of emulsion, i.e. water-in-oil (w/o) or oil-in-water (o/w). The term hydrophilic-lipophilic balance (HLB) is used to classify which type of emulsion the emulsifier will favour.

1.4 Surfactants market

There are surfactants used in detergents and cleaners (54%); as auxiliaries for textiles, leather and paper (13%); in chemical processes (10%); in cosmetics and pharmaceuticals (10%); in the food industry (3%); in agriculture (2%) and in others (8%)[10]. The world production of surfactants is estimated at 15 M ton per year, of which about half are soaps. The other surfactants produced on large scale are linear

alkyl benzene sulfonates (1700 k ton per year), lignin sulfonates (600 k ton per year), fatty alcohol ethoxylates (700 k tons per year), alkyl phenol ethoxylates (500 k ton per year) [11]. The total production of the surfactant has exceeded around 10 million tons in 2007 for their increasing demands in various industries such as polymers, lubricants and solvents[12, 13]. The market is expected to grow over US $41 billion in 2018 with an average annual growth of 4.5% [14, 15, 16]. Such increase in growth rate is related to the world demand in detergents since this sector uses over 50% of surfactant production[17]. Out of the total production of surfactants, about 54% are consumed as household or laundry detergents and only 32% destined for industrial use[18, 19, 20].

1.5 Disadvantages of synthetic surfactants

As a part of the global effort to reduce hazardous wastes, greener processes with reduced waste products are being progressively integrated with modern developments[21]. Since all the conventional surfactants commercially available today are synthetic and of petroleum origin, they not only cost high but also pose potential threats to the environment due to their recalcitrant and persistent nature[22, 23]. There is a concern on the possible toxic effect of the synthetic surfactants on aquatic organisms, especially if they are used in near shore waters[24]. Besides these, the effects of synthetic surfactants on biostimulation of indigenous microorganisms in enhancing the removal of organic pollutants yielded inconsistent results. Such decrease in the rate of biodegradation of organic pollutants especially at higher concentrations of surfactant could be due to the interaction of surfactant with the lipid membrane[22]. It is a well known fact that surfactant molecules at higher concentration affect enzymes and other cellular proteins necessary for basic functions of the microorganisms[25]. Most anionic and non-ionic surfactants are nontoxic, having LD_{50} comparable to sodium chloride. Prolonged exposure of skin to surfactants can cause chaffing because surfactants disrupt the lipid coating that protects skin and cells[11]. Moreover, most of synthetic surfactants during the manufacturing process and the byproducts cause serious environmental hazards[26]. With the increased environmental awareness among consumers and new stringent legislations, environmental compatibility of surfactants has become an important factor in their application for various uses[27, 28]. As a result,

with advances in biotechnology, attention has been paid to the alternative environmental friendly processes for the production of different types of surfactants from microorganisms[29]. Hence, the use of biosurfactants in place of chemical surfactants can minimize the threats caused by the synthetic surfactant[22].

1.6 Green surfactant or bio-surfactant

In ancient times some natural surfactants such as soap (fatty acid salt), lecithin (phospholipid) and saponins (glycolipid) were extracted from plants or animals and widely used in households and industry[30]. Natural surfactants are usually present in lesser quantity in their natural sources and the cost involvement in their extraction procedure exceeds the cost of chemical synthesis[30]. Therefore, the investigation for an alternative source of natural surfactant is significant.

Investigation of the literature indicates that the capability to produce natural surfactant, well-known as biosurfactants is prevalent among the bacterial and archeal domains[31]. Biosurfactants are heterogeneous groups of surface-active microbial surfactants produced by a wide variety of microorganisms such as bacteria, actinomycetes, fungi, yeast etc[32]. These surface active molecules have been reported as being produced on the microbial cell surfaces and excreted extracellulaly[33]. Basically they are amphipathic molecules that comprise a hydrophilic portion, which might consist of monosaccharides, oligosaccharides, or polysaccharides, amino acids or peptides, or carboxylate or phosphate groups and a hydrophobic portion, which is composed of saturated or unsaturated (hydroxy) fatty acids or fatty alcohols. These molecules partitioned at the interface between fluid phases with different degree of polarity and hydrogen bonding such as in oil/water or air/water interfaces[34]. Such accumulation of biosurfactant molecules at the interfaces ultimately reduces the surface and interfacial tension of the system. They impart better wetting, spreading, foaming, detergency and emulsifying traits, rendering them most versatile process chemicals[35]. This quallities make them more competitive and suitable to various application needs [36, 37, 38].

1.7 Role of biosurfactant in producing microbes

Biosurfactant possess diverse properties and physiological functions such as increase in the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation[39]. However, it is impossible to make any generalization or identify one or more common roles to all microbial surfactants[40]. Most of the bacteria isolated from sites having history of contaminations by hydrocarbons and derivatives are Gram negative, and it might be a characteristic that contributes to the survival of the populations in such harsh environments[41, 42]. Most of them produce biosurfactants, amphiphilic molecules of diverse chemical nature, which improve the ability of microbial cells to utilize hydrophobic compounds as growth substrates[7, 43]. Biosurfactants are either produced on microbial cell surfaces or excreted extracellularly[7, 44, 45]. The capacity of bacteria to produce biosurfactant specifically with antimicrobial property could be a survival strategy allowing them to flourish ahead of other organisms in the competitive environments[46]. The mode of action of biosurfactants is the modification of the cell surface hydrophobicity[47] and/or in promoting emulsification and/or solubilization of substrates[48].

1.8 Types of biosurfactants

The initial classification of the biosurfactant was on the molecular weights, chemical properties and cellular localizations. The low molecular weight biosurfactants such as glycolipids, lipopeptides, flavolipids, corynomycolic acids and phospholipids lowers the surface and interfacial tensions at the air/water interfaces. The high molecular weight biosurfactants are called bioemulsans, such as emulsan, alasan, liposan, polysaccharides and protein complexes. These biosurfactants are efficient emulsifiers at low concentrations and exhibit considerable substrate specificity in stabilizing oil-in-water emulsions[19, 22]. However, general classification based on parent chemical structure and classified as glycolipids, lipopeptides, lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polymeric lipids[49]. Therefore, it can be expected to have diverse properties and physiological functions of

biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation[39].

1.9 Biosurfactants with special reference to rhamnolipids

Rhamnolipids are known to be produced by the different species of *Pseudomonas* bacteria, especially by *Pseudomonas aeruginosa*, a leading commercial biosurfactant which is suitable for considerable applications[50, 51]. Rhamnolipids, the glycolipid-type biosurfactants produced by *Pseudomonas aeruginosa* in the late log and stationary phases of growth[27] are among the most effective biosurfactants and have been applied in various industries and bioremediations[27, 52, 53]. The production of rhamnolipids is known to be crucial for *Pseudomonas aeruginosa* to survive and thrive under specific conditions[54]. Rhamnolipids basically are glycolipid in nature, composed of a hydrophilic head formed by one or two rhamnose molecules, known respectively as mono-rhamnolipid and di-rhamnolipid, and a hydrophobic tail which contains up to three molecules of hydroxyl fatty acids of varying chain length from 8 to 14 of which β-hydroxydecanoic acid is predominant[35, 55, 56]. Thus, distribution of rhamnolipid congeners always exists. Although, there are several types of rhamnolipid species reported in the literature, all of them possess similar chemical structure and have an average molecular weight of 577 [52, 57, 58]. The crude biosurfactants extracted from the liquid culture of *Pseudomonas* strains were found to reduce the surface-tension of water from 72 to 30 mNm^{-1} [57] with a critical micelle concentration of 5-200 $mg.l^{-1}$ and exhibited an emulsification index of above 70% [37, 53, 55, 56, 59]. With respect to their production, they show higher yield as compared to other known biosurfactants. The bacterial genus *Pseudomonas* has been highlighted for its ability to use diverse hydrophobic or hydrophilic substrates such as hydrocarbons, vegetable oils, carbohydrates, or even wastes from the food industry as carbon source to produce rhamnolipid-type biosurfactants[13, 58, 60]. Another advantage of rhamnolipids over the other bio-surfactants is the ease at which they can be isolated from the culture as they are extracellularly produced[13, 52, 61]. Being of microbial origin, rhamnolipids with high biodegradability possess an additional advantage over the synthetic surfactants in soil

washing and bioremediation processes[23, 61]. Rhamnolipids reveals the potential to improve the microbial degradation of chlorinated hydrocarbons, PAHs, heavy metals and petroleum hydrocarbons from contaminated soil and water[3]. They are not only efficient surfactants but also exhibit excellent antimicrobial activity against several other microorganisms[51, 57, 62, 63] and disrupt host defenses during infections[64]. Rhamnolipids have been investigated in several applications which include bacteriocide[65], fungicide[66], wound healing[67] and others[51]. They also assist in cell surface motility[68] and influence the architecture of biofilms, especially in the formation and maintenance of fluid channels within the exo-polymeric matrix after bacteria adhere irreversibly on a substratum[69]. The production of rhamnolipid is known to be regulated by quorum sensing mechanism and is depends on various environmental and nutritional factors which include pH, temperature, phosphates and iron content, as well as the nature of the carbon source[54].

1.10 Advantages of biosurfacatnts over synthetic surfactants

The most attractive aspects of biosurfactant use are their biodegradability and ecological acceptance[60]. Biosurfactants could retain their surface active properties even under extreme conditions of temperature, pH, salinity and metal salts[32, 40, 70]. Due to the low irritancy and compatibility with human skin[71] they are constantly used in the sector of cosmetics and pharmaceutical industries[51]. Because of these properties, biosurfactants have a broad range of potential applications, including in detergent, pharmaceuticals, agriculture, cosmetics, food, cleanser, paint and petroleum based industries[29, 35]. Other main advantages of biosurfactants includes bioavailability, activity under diverse conditions, ecological acceptability, low toxicity, their capacity to be modified by biotechnological techniques and their capability of increasing the bioavailability of poorly soluble organic compounds, such as polyaromatic hydrocarbons (PAH)[72]. From an environmental standpoint, biosurfactants have promising applications in various fields including bioremediation of contaminated environments, tertiary oil recovery such as MEOR (microbial enhanced oil recovery), flotation process, detergent formulations etc. [60, 73]. Biosurfactants could also be easily produced from renewable resources through microbial fermentation[22]. In the recent

years, biosurfactants have been gaining much attention in the field of nanobiotechnology because of their unique chemical composition[74, 75]. Various aspects of biosurfactants, such as their biomedical and therapeutic properties as well as natural roles have been recently reviewed[63, 76].

1.11 Application of biosurfactants

Biosurfactants are beginning to attain a status of potential effective substance in various fields[10]. Various applications of biosurfactants have been extensively reviewed[35]. At production level, along with the utilization of cheap renewable substrates and organic wastes, the cost of the biosurfactants has become competitive with that of the cost of the synthetic chemical surfactants.

1.11.1 For the production of specific compounds

Biosurfactants are being considered as an alternative to the high value synthetic chemical whose use may have toxic environmental impacts[26]. The pyrenacylester of rhamnolipids are reported to be synthesized for its use in monitoring the polarity and fluidity of solid surfaces and also used in determining the impact of coatings on the surface properties[77]. Rhamnolipid from *P. aeruginosa* is a superior source of rhamnose as it is excreted in late log and stationary phases of growth[26]. Rhamnolipids have been a source of stereo specific L-rhamnose, which is used in the production of high quality flavoring compounds and as starting material for the synthesis of some organic compounds[78].

1.11.2 In laundry and other sector

Rhamnolipid were applied in the formulation of laundry detergents and examined their effectiveness in removing sunflower oil, chocolate, and albumen stains from cotton fabrics[79]. Some other commercial applications of biosurfactants are in the pulp and paper, the paint, textiles and ceramics industries[26].

1.11.3 In medicine

Currently, biosurfactants are being investigated and exploited for medical purposes[63, 80]. Biosurfactants have a range of therapeutic applications; such as rhamnolipids produced by *P. aeruginosa*, lipopeptides produced by *B. subtilis* and *B. licheniformis* as biocidic agents[26, 63]. The possible applications of biosurfactants as emulsifying agent for transporting drugs to the site of infection, for supplementing pulmonary surfactant and as adjuvant for vaccines were assessed[81]. The surfactin produced by *Lactobacillus acidophilus* RC 14 has been investigated for its possible application as anti-adhesive biological coatings for catheter materials[82]. They are also known to have the potential use as major antimycoplasmic, antiviral, anti-tumor agent, inhibitor of fibrin clot formation, immunomodulatory, hypocholesterolemic, anti-adhesive and most recently as dispersants for nanoparticles[63, 83].

1.11.4 In cosmetic industry

The cosmetic and health care industries use large amounts of biosurfactants in several different formulations. Products like insect repellents, antacids, acne pads, anti-dandruff products, contact lens solutions, deodorants, nail care products, anti-wrinkle and anti-ageing products and toothpastes require surfactants that have high surface and emulsifying activities. These characteristics of surfactants play a vital role in maintaining the texture consistency of these products[27, 51, 84]. Biosurfactants also acquires the position in the market of personal care products due to its low toxicity, excellent moisturizing properties and skin compatibility[85]. Currently, there are patents for the use of rhamnolipids to make liposomes and emulsions[51].

1.11.5 In agriculture

Biosurfactants have been evaluated for their potential role in controlling various plant pathogens. The proposed mechanism for their action is that it intercalates into and disrupts the plasma membrane[26]. The rhamnolipids isolated from *P. aeruginosa* was efficient in biological control of zoosporic plant pathogens at very low concentrations and reported to be successful in controlling the disease in a

hydrophonic recirculating cultural system[86]. Surfactin and a similar lipopeptide, iturin A, produced by *Bacillus subtilis* RB14 were reported to suppress the damping off disease of tomato seedlings caused by *Rhizoctonia solani*[87]. Rhamnolipids and other biosurfactants were reported to have zoosporicidal activity against species of *Pythium*, *Phytophthora*, and *Plasmophora* at concentrations ranging from 5 to 30 μg ml^{-1} [26]. Rhamnolipid at a rate of 1% emulsion was successfully used for the treatment of *Nicotiana glutinosa* leaves infected with tobacco mosaic virus and for the control of potato virus X disease[88].

1.11.6 Metallurgy processes

Biosurfactants are used in the dispersion of inorganic minerals in mining and various manufacturing processes. Rosenberg *et al.*[89] reported the production of an anionic polysaccharide called as biodispersan by *Acinetobacter calcoaceticus* A2 that prevents the flocculation and effect dispersion of limestone 10% in water. The use of biosurfactant isolated from *Nocardia amarae* was reported to be used for the removal and recovery of non-ionic organics from aqueous solutions[90]. Polman *et al.*[91] reported partial solubilization of North Dakota Beulah Zap lignite coal with the use of crude biosurfactant isolated from *Candida bombicola.* Surfactin, rhamnolipids and sophorolipid were used in batch washing experiments to remove heavy metals from sediments[83]. Other commercial applications of biosurfactants involve the processing of uranium ore and mechanical dewatering of peat[26, 40]. The foaming ability of biosurfactants isolated from *Pseudomonas aeruginosa* was investigated for its possible application in coal and mineral flotation as a frother and co-frother[92].

1.11.7 In food industry

Biosurfactants are routinely used in the food industry as emulsifiers in the processing of raw materials. Other applications of biosurfactants are in bakery and meat products where they influence the rheological characteristics of flour or to emulsify the partially broken fat tissue[93]. Lecithin and its derivatives are currently used as emulsifiers in the food industry worldwide[94]. Biosurfactants produced by thermophilic dairy *Streptococci sp.* used for fouling control of heat-exchanger plates

in pasteurizers as they hindered the colonization of *Staphylococcus thermophilus* responsible for fouling[95]. There could be a possible application of biosurfactants in immunonutrition[96]. Diacylmannosylerythritol, a glycolipid type biosurfactant produced by *Candida antarctica* as an effective anti-agglomeration agent in the slurry system[97]. Such type of biosurfactants exhibited a remarkable effect on the slurry, attaining a high ice-packing factor (35%) for 8 h at a biosurfactant concentration of 10 $mg.l^{-1}$.

1.11.8 Microbial enhanced oil recovery

Biosurfactants have been shown in many cases to have emulsification properties equivalent to that of the industrially available emulsifying agents and the most desirable character is their biodegradable nature. There is a possible use of biosurfactants in mobilizing heavy crude oil, transporting petroleum in pipelines, viscosity control, managing oil spills, oil-pollution control, cleaning oil sludge from oil storage facilities, soil/sand bioremediation and microbial enhanced oil recovery (MEOR)[98]. MEOR is a less expensive process as compared to CEOR because microorganisms can synthesize useful products by fermenting low-cost substrates or raw materials. Furthermore, microbial products are biodegradable and have low toxicity[99, 100, 101, 102]. Single microorganism or consortium could be used to degrade heavy oil fractions, as a result the oil viscosity decreases and it becomes more fluid, lighter and more valuable[103]. Biosurfactants were produced by culturing the necessary microbes in the basal salt medium containing 2% w/v glucose and oleic acid together as carbon source and was used as a substitute for the chemical surfactants in a test carried out on an oil storage tank belonging to Kuwait Oil Company[98]. Clark *et al.*[104] estimated that about 27% of oil reservoirs in USA are amenable to microbial growth and MEOR on the basis of computational survey.

1.11.9 Environmental applications

Biosurfactants have been extensively studied and applied directly in the presence and absence of microorganisms for bioremediation of organic pollutants and heavy metals on the laboratory scale[3, 54]. Biosurfactants are used in various industrial

and environmental applications which frequently involve exposure to extreme environmental conditions. As a result, researchers have focussed on isolating and screening strains that are able to produce biosurfactant under extreme environments, especially for MEOR and bioremediation purposes[105]. The effectiveness of biosurfactant in separating crude oil was equivalent to those of synthetic surfactant and much higher than that of natural plant derived surfactant-saponin[106] and synthetic Tween 60[107]. In the case of removing hexadecane from the contaminated sand, biosurfactant was found to be much more efficient than SDS and Tween 80[108]. The addition of biosurfactant produced by *Candida antarctica* to the fermentation process of n-undecane improved degradation rate of petroleum hydrocarbons, while application of synthetic surfactant Tween 40 and Span 80 didn't show any improvement[109]. There are a number of microorganisms such as *Pseudomonas aeruginosa* known to degrade hydrocarbons by using as carbon sources[110] and produce biosurfactants[111]. Biosurfactants are very effective in enhancing oil biodegradation either by enhancing the uptake of hydrocarbon or by specific adhesion/desorption mechanisms[112]. In the recent time, focus is given to the possible application of biosurfactant in its attempt to recover residual oil from oil sludge and in enhanced biodegradation of oil sludge process[113, 114]. Biosurfactants form complexes preferably with toxic heavy metal cations which include Cd^{2+}, Pb^{2+}, Zn^{2+}, Ar^{2+} etc[1, 115] than with other non-toxic metals such as Ca^{2+} and Mg^{2+} for which they have much lesser affinity[39]. Due to the anionic nature of rhamnolipids, they are able to take out metal ions from the soil such as arsenic, cadmium, copper, lanthanum, lead and zinc due to their complexation ability[1, 115]. The order of rhamnolipid stability constant for the complexation with metals, tested at pH 6.9 was in the order Al^{2+}> Cu^{2+}> Pb^{2+}> Cd^{2+}> Zn^{2+}> Fe^{3+}> Hg^{2+}> Ca^{2+}> Co^{2+}> Ni^{2+}>Mn^{2+}> Mg^{2+}>K^{2+} [116].

1.11.10 In nanotechnology

Considering the requirement of greener bioprocesses and novel enhancers for the synthesis using microbial processes, biosurfactants; and/or biosurfactant producing microbes are emerging as an alternate source for nanomaterial synthesis /functionalization and its subsequent applications[117, 118]. These nanomaterial-

biomolecule multifunctional systems could be used to mimic the behavior of biomolecules in cells and therefore could be helpful in explaining the mechanisms of complex biological processes with several potential applications[119]. Recent developments show that biosurfactants are multifunctional smart molecules which would become a part of diverse biotechnological applications including biocontrol, drug delivery vehicle, and bioremediation. Biosurfactant mediated nanomaterial synthesis and/or stabilization is a recent development in the field of nanotechnology. Biosurfactants especially rhamnolipids have been used as green capping agents for nanoparticle synthesis. At present, focus on biosurfactant-mediated processes is steeply increasing due to their potential implication in the synthesis of various metal nanoparticles such as NiO, ZnS, Ag, Au etc[120, 121, 122, 123].

1.12 Limiting factors for commercialization of biosurfactant

Attention towards biosurfactants has been gradually increasing in recent years due to the possibility of their production through fermentation technology and their potential applications in specific areas such as environmental protection. In spite of numerous advantages over the synthetic chemical surfactants, biosurfactants are still unable to compete with the chemically synthesized surfactants in the surfactant market. This could be due to their high production costs in relation to the inefficient bioprocessing techniques, poor strain productivity and the need to use costly substrates[17, 71]. This has led to concerted efforts during the last decade, focussed on minimizing production costs in order to facilitate wider commercial use. The success of biosurfactant production depends on the development of cheaper processes and the use of low cost raw materials, which account for 10–30% of the overall cost[124]. Four factors need to be focussed to reduce cost of production of biosurfactants: the microbes, process, microbial growth substrate or process feed stock and the process byproducts[22, 26, 125].

1.13 Petroleum contamination in Assam

The Indian petroleum industry is one of the six core industries of the country and contributes over 15% to GDP. India is the 6th largest consumer of oil in the world

and the ninth largest crude oil importer. Oil continued to remain the top item in the country's import list during 2012-13. In India, particularly the state Assam has a huge reserve of oil and natural gas. It has 1.3 billion tonnes of proven crude oil. Assam is the first state in the country where for first time oil well was dug mechanically at Digboi in Tinsukia district in the year 1889. The state has the oldest refinery in the country established in the year 1901 which started to produce 500 barrels of crude oil per day and established to refine the crude oil in Digboi itself. Subsequently, various petroleum industries were set up to exploit the crude oil and natural gas of Assam which includes Oil India Limited (OIL), Oil and Natural Gas Corporation (ONGC), Indian Oil Corporation Limited (IOCL) consisting of two major refineries Guwahati refinery and Bongaigaon refinery, Assam Gas Company Limited (AGCL) and Numaligarh Refinery Limited (NRL).

Industrial wastes from petroleum-based industries are identified as one of the major sources of pollution. The oil enriched geographical regions of Assam are very fertile and under the traditional agricultural practices since time immemorial. The people of the state mostly depend on agriculture and the major crops of the region are rice and tea. Exploration activities in the oil fields often cause spillages of crude oil from the oil wells into the nearby forests, tea cultivations and agricultural fields. Such problems are rising with the increase in the scale of oil exploration activities. The incidences of crude oil contamination are more often in Upper Assam area resulting in the destruction of soil quality that consequently damages crop cultivations. Moreover, there have been instances where pipelines transporting crude oil from the site of drilling to the refinery are damaged and large quantities of oil discharged into open fields. Apart from exploration activities, refining and transportation of crude and refined products do contribute towards pollution of soil and water affecting agriculture, aquaculture and human health[126]. Therefore, the problem of crude oil pollution in Upper Assam needs effective remediation solutions for the security and the sustenance of crop cultivations and biodiversity of the region.

Soil which is accidentally contaminated with petroleum hydrocarbons can be remediated by physical, chemical or biological methods. Among all, *in situ*

bioremediation is considered to be environmentally friendly because it restores the soil structure, requires less energy input, and involves the complete destruction or immobilization of the contaminations rather than their transfer from one environmental compartment to another which mainly occur in physical or chemical treatment processes[127]. Although most of the hydrocarbons are biodegradable, but the rate of biodegradation in the environment is limited due to their hydrophobicity or less accessibility to microbes and low aqueous solubility[7]. One of the approaches to enhance biodegradation of crude oil contamination is the use of biosurfactant[128], which could increase the solubility of hydrophobic substrates/oils in aqueous medium to enhance the bioavailability of the hydrophobic substrates leading to higher oil degradation.

The treatment of crude oil contaminated soil and environments through the indigenous biosurfactant producing bacteria having the capacity to degrade the petroleum hydrocarbons seems to be advantageous[1]. As most of the bacteria dwelling in the crude oil contaminated environment have the capacity to utilize the components of crude oil as the source of carbon and energy for their growth and tend to secrete biosurfactants which in turn help in the degradation of crude oil components[7, 43, 129]. Moreover, the production of biosurfactant at the site of treatment with the producer bacteria doesn't require rigorous testing like that of the chemical surfactants because of their environmentally compatible nature[52]. Therefore, the application of biosurfactants in bioremediation might be more acceptable from the social point of view. Thus there is a need for increased production of biosurfactants and their characterization.

1.14 Objectives of the investigation

In the backdrop of all above mentioned information, the present investigation has been undertaken with the following objectives:

1. Screening of biosurfactant producing bacteria from various environmental and petrochemical waste samples.
2. Biochemical and molecular characterization of efficient biosurfactant producing bacterial strains.
3. Optimization of culture conditions of the efficient bacterial strains for the optimum level production of biosurfactants.
4. Physical and chemical characterization of biosurfactants produced by the efficient bacterial strains.
5. Investigation on the biological activity of the biosurfactants produced by the potential strains.
6. Investigation on the possible industrial applications of the selected bacterial strains and their isolated biosurfactants.

Chapter II
Review of Literature

2.1 Biosurfactants

Virtually all surfactants are chemically synthesized chiefly from petroleum hydrocarbons. Nevertheless, in the recent years, biosurfactants have been receiving much more attention due their diversity, selectivity, environmentally friendly nature, performance under extreme conditions, possibility of large-scale production, and potential applications in environmental protection[10]. Further, increasing environmental concern had also led to consider the biological surfactants in various applications, especially in the bioremediation related technologies.

2.2 Classification of biosurfactants

Most commonly, surfactants are generally categorized according to the type of the polar group present. On the basis of chemical composition of the polar head group, they are classified as nonionic, anionic, cationic and zwitterionic[10]. Rosenberg and Ron[43] suggested that biosurfactants could be divided into low molecular weight molecules and high molecular weight polymers. The lower molecular weight biosurfactants which includes glycolipids, lipopeptides, flavolipids, corynomycolic acids and phospholipids, efficiently lower the surface and interfacial tensions at the air/water interfaces. The high molecular weight polymers also known as bioemulsans which includes emulsan, alasan, liposan, polysaccharides and protein complexes, are highly efficient emulsifiers that work at low concentrations, exhibit considerable substrate specificity and are more effective in stabilizing oil-in-water emulsions[19]. However, general classification of biosurfactant is based on the parent chemical structure and their surface properties and is represented in the following groups.

2.2.1 Glycolipids

They contain carbohydrates groups that are acylated with aliphatic acids or hydroxyaliphatic acids. The connection is by means of either an ether or ester group.

2.2.1.1 Trehalose lipids: Microbial trehalolipid, a glycolipid type biosurfactant produced by most of the sp. of *Mycobacterium*, *Corynebacterium* and *Nocardia*. Trehalolipid consists of disaccharide trehalose linked to C-6 and C-6' to mycolic acid. Mycolic acids are the long chain, α-branched and β-hydroxy fatty acids. Trehalolipids from diverse organisms differ in the size and structure of mycolic acid, the number of carbon atoms present and the degree of unsaturation[130]. *Rhodococcus erythropolis* and *Arthrobacter* sp. were reported to produce trehalolipid that reduces the surface tension and interfacial tension of the culture broth[131].

2.2.1.2 Rhamnolipid: Jarvis and Johnson[132], reported the production of rhamnolipid from *Pseudomonas aeruginosa*, an important biosurfactant with tremendous applications both in industrial and environmental sector. Rhamnolipid usually contains one or two molecules of rhamnose which are connected to one or two molecules of β-hydroxydecanoic acid. The -OH group of one of the acids is involved in glycosidic linkage with the reducing end of the rhamnose disaccharide, the -OH group of the second acid is occupied in ester formation[133]. L-Rhamnosyl-Lrhamnosyl- β-hydroxydecanoyl-β-hydroxydecanoate and L-rhamnosyl- β- hydroxydecanoyl-β-hydroxydecanoate, referred to as rhamnolipids 1 and 2 respectively, are principal glycolipids synthesized by *P. aeruginosa*[134]. The pure rhamnolipid lowered the interfacial tension against n-hexadecane to about 1 mNm^{-1} and had a CMC of 10±30 $mg.l^{-1}$, depending on the pH and salt conditions[135].

2.2.1.3 Sophorolipids: Sophorolipids are mainly produced by yeast such as *Torulopsis bombicola*[136, 137]. These molecules mainly composed of a dimeric carbohydrate sophorose attached to a long-chain hydroxyl fatty acid by a glycosidic linkage. Generally, they are found as a mixture of free acid form and macro-lactones. These biosurfactants are a combination of at least six to nine varied hydrophobic sophorolipids. Hu and Ju[138] reported the uses of lactone form of the sophorolipid in various applications. The sophorose lipids lower surface and interfacial tensions, although they are not effective emulsifying agents[136]. The pure lactonic sophorose lipid (10 $mg.l^{-1}$) lowered the interfacial tension between n-hexadecane and water from

40 mNm^{-1} to about 5 mNm^{-1}, relatively independently of pH (6±9), salt concentration and temperature (20±90 °C)[43].

Marine *Alcaligenes* sp. produces glucose-lipid containing biosurfactant, where the lipophilic component consisting of four β-hydroxydecanoic acids linked together by ester bonds is coupled glycosidically with C-1 of glucose[139]. *Alcanivorax borkumensis*, a marine bacterium was also reported to produces an anionic glucose lipid type biosurfactant consisting of a tetrameric oxyacyl side chain with N-terminal esterified with glycine[140]. Schulz *et al.*[141] reported a marine *Arthrobacter* sp. SI1 that produced *disaccharide* trehalose (trehalose tetraester) and trehalose dicorynomycolates (trehalose diester) when grown on mihagol-S and ethanol separately as carbon sources. The minimal interfacial tensions (between aqueous salt solutions and n-hexadecane) achieved with corynomycolic acids, trehalose monocorynomycolates, and trehalose dicorynomycolates were 6, 16 and 17 mNm^{-1} respectively[43]. Suzuki *et al.*[142] identified trehalose dimycolates, a type of glycolipid, present in the emulsion layer of culture broths of *Arthrobacter paraffineus* during their growth on hydrocarbon substrates. Wagner and co-workers have carried out extensive studies of trehalose dimycolates produced by *Rhodococcus erythropolis* with special reference to their interfacial activities and possible application in enhanced oil recovery[143]. Mannosylerythritol lipids, extracellular microbial surfactants, have several interesting biological properties such as they inhibit growth of human promyelocytic leukemia cell lines and induce monocytic differentiation[144].

2.2.2 Lipopeptides and lipoproteins

2.2.2.1 Surfactin: *Bacillus subtilis* produces a cyclic lipopeptide called surfactin or subtilisin[145, 146]. These groups of biosurfactant contain a lipid linked to a polypeptide chain. *Bacillus subtilis* produces cyclic lipopeptide type biosurfactant known as surfactin that has various potential applications. It is composed of a seven amino-acid ring structure joined to a fatty acid chain by means of lactone linkage. It reduces the surface tension from 72 to 27.9 mNm^{-1} at a concentration as low as 0.005% and shows a minimum interfacial tension against hexadecane upto 1 mNm^{-1} [145].

2.2.2.2 Iturin: Iturin A was isolated from a *Bacillus subtilis* strain taken from the soil in Iturin (Zaire)[147]. The Iturin groups of compounds are cyclic lipo-heptapeptides which contain a β- amino fatty acid in its side chain and reported to have potent antifungal agents which can be used as biopesticides for plants protection[148]. These molecules have remarkable efficacy against a broad variety of clinically important pathogenic fungi and yeast strains. However, their application in medicine is limited because of possible toxicity[149].

2.2.2.3 Fengycin: Fengycin, a type of lipopeptide biosurfactant consists of lipodecapeptide with β-hydroxy fatty acid in its side chain[148]. This group of compounds comprises of C15 to C17 variants, which have characteristic Ala-Val dimorphy at position 6 of the peptide ring[150].

2.2.2.4 Lichenysin: *Bacillus licheniformis* produces several type biosurfacants such as lichenysin that act synergistically and exhibit stability at extreme temperature, pH and salinity. These molecules are similar in their structural and physico-chemical properties to surfactin[151]. Biosurfactants produced by *B. licheniformis* are able to reduce the surface tension of water to 27mNm^{-1} and the interfacial tension between water and n-hexadecane to 0.36 mNm^{-1}.

Several lipopeptide including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins) shows both the antibiotics as well as the potent surface-active properties[43]. The polymixins are a group of closely related lipopeptide antibiotics produced by *Bacillus polymyxa*, *Bacillus brevis* and other related bacilli[152]. Polymyxin B is composed of deca-peptide in which amino acids 3±10 form a cyclic octapeptide. A branched-chain fatty acid is connected to the terminal 2, 4-diaminobutyric acid (Dab)[43].

The synthesis of one or more peptide antibiotic during the early stages of sporulation is specific characteristics common to the selected members of the genus Bacillus[153]. *Bacillus brevis* produces the cyclosymmetric decapeptide antibiotic known as gramicidin S. In solution, gramicidin S exists in the form of a rigid ring with the two positively charged ornithine side-chains constrained to one side of the ring and the

side-chains of the remaining hydrophobic residues oriented toward the opposite side of the ring[154]. *Pseudomonas* strains produce viscosin, a peptidolipid biosurfactant that lowers surface tension to 27 mNm^{-1} [155].

2.2.3 Fatty acids, phospholipids, and neutral lipids

Various bacteria and yeasts secrete large amounts of fatty acids and phospholipid surfactants during their growth on n-alkanes[156]. The hydrophilic and lipophilic balance (HLB) is directly proportional to the length of the hydrocarbon chain in their structures. These surfactants are able to produce optically clear microemlsions of alkanes in water. Kappeli and Finnerty[157] reported that *Acinetobacter* sp.. When grown on n-alkane produces phosphatidylethanolamine-rich vesicles that form optically clear microemulsions of alkanes in water and lowers the interfacial tension between hexadecane and water to less than 1 mNm^{-1} with a critical micelle concentration (CMC) of 30 $mg.l^{-1}$ [131]. *Myroides* sp. SM1 was found to produce bile acids; cholic acid, deoxycholic acid and their glycine conjugate when cultivated in Marine broth[158]. *Thiobacillus thiooxidans* produces a measurable amount of phospholipids and has a role in the wetting of element sulfur[159]. Miyazima *et al.*[160] reported the production of phospholipids by the fungus *Aspergillus* sp. that grown on hydrocarbons. The extracellular free fatty acids are in the range of C_{12} to C_{14} and the complex fatty acid contains hydroxyl groups and alkyl branches[131]. Such fatty acids are produced by various microorganisms during their growth on alkanes and exhibit the properties of surfactants. *Arthrobacter* AK-19[161] and *Pseudomonas aeruginosa* 44T1[162] have been shown to accumulate up to 40-80% w/w lipid when cultivated on hexadecane and olive oil, respectively.

2.2.4 Polymeric biosurfactants

These are high molecular weight biopolymers that exhibit useful properties such as high viscosity, tensile strength and resistance to shear and are known to have a variety of industrial applications[163]. The best-studied polymeric biosurfactants are emulsan, biodispersan, liposan and other polysaccharide–protein complexes[164].

2.2.4.1 Emulsan: *Acinetobacter calcoaceticus* RAG-1 produce an extracellular potent polyanionic amphipathics heteropolysaccharide bioemulsifier[165]. The heteropolysaccharide backbone contains repeating trisaccharide of N-acetyl-D-galactosamine, N-acetyl-galactosamine uronic acid, and an unidentified N-acetyl amino sugar[166]. On the basis of dry weight, 10-15% of fatty acids are shown to be linked to the polysaccharide through O-ester linkages[167]. Emulsan does not appreciably reduce the surface tension but it is an effective emulsifying agent for hydrocarbons in water[168], even at a concentration as low as 0.001 to 0.01%.

2.2.4.2 Biodispersan: *Acinetobacter calcoaceticus* A2 was reported to produce an extracellular, non-dialyzable dispersing agent called biodispersion[89]. It is an anionic heteropolysaccharide having an average molecular weight of 51, 400 and four reducing sugars, namely glucosamine, 6-methyl aminohexose, galactosamine uronic acid and an unidentified amino sugar[169].

2.2.4.3 Liposan: Liposan is an extracellular water-soluble emulsifier produced by *Candida lipolytica* and composed mainly of 83% carbohydrate and 17% protein[156]. Husain *et al.*[170] reported a polymeric type biosurfactant produced by *Pseudomonas nautica* which consists of proteins, carbohydrates and lipid at the ratio of 35:63:2, respectively. Zinjarde and Pant[171] also reported that *Yarrowia lipolytica*, a tropical marine strain produces an emulsifier (lipid-carbohydrate-lipid) complex associated with the cell wall in the earlier stages of growth but displayed the extracellular emulsifier activity towards the stationary phase during their growth on alkanes or crude oil.

2.2.4.4 Other polysaccharide protein complexes: The surface active properties of *Acinetobacter calcoaceticus* BD4 is due to the production of heteropolysaccharide-containinng capsules[172]. These capsules are composed of repeating units of heptasaccharide and are released in the medium during the growth on hydrocarbons. Sar and Rosenberg[173], polysaccharides alone showed no emulsification activity, but polysaccharides released with protein during the growth of a parent strain on ethanol or by a mutant strain BD-413 showed potent emulsification activity. Cameron *et al.*[174]

reported the production of mannoprotein emulsifier from *Saccharomyces cerevisiae* that emulsifies many oils, alkanes and organic solvents and the emulsions were reported to be stable at extreme temperature, pH, and salt concentrations. Kappeli *et al.*[175] had isolated a mannan-fatty acid complex from alkane-grown *Candida tropicalis* that stabilized hexadecane in water emulsions. *Shizonella malanogramma* and *Ustilago maydis* were reported to produce a biosurfactant that was characterized as erythritol- and mannose-containing lipid[176]. Cameotra and Singh[177] had isolated, purified and characterized an emulsifying and solubilizing factor from hexadecane-grown *Pseudomonas* sp.. Desai *et al.*[178] reported the production of bioemulsifier which is composed of trehalose (50% carbohydrate) and lipid-*o*-dialkyl monoglycerides (10% lipid and 19.6% protein) by *Pseudomonas fluorescens* during growth on gasoline. *Bacillus subtilis* FE-2 had reported to produce a glycolipopeptide that was capable of emulsifying water-immiscible organophosphorous pesticides[179].

2.2.5 Particulate biosurfactants

Extracellular membrane vesicles partition hydrocarbons to from a microemulsion, which plays a very important role in alkane uptake by microbial cells[164]. *Acinetobacter* sp.. HO1-N was reported to secrete extracellular vesicles having a diameter of 20–50 nm and a buoyant density of 1.158 cubic $g.cm^{-1}$. Such vesicles are mainly consists of protein, phospholipids and lipopolysaccharide[157]. The cellular lipid content of *Pseudomonas nautical* reported to be increased in eicosane-grown cells up to 3.2 fold, compared with acetate-grown cells. Husain *et al.*[170] reported that phospholipids, mainly the phosphatidyl-ethanolamines and phosphatidylglycerides, were accumulated in eicosanes-grown cells of *Pseudomonas nautical*. For *Sphingomonas* sp., the cell surface of bacteria of this strain was covered with extracellular vesicles when grown on polyaromatic hydrocarbons. Nevertheless, the surfaces were smooth when cells were grown on a hydrophilic substrate such as acetate[164].

2.3 Natural roles of biosurfactant

When taking the account of biosurfactant's role in microbial physiology, it is important to highlight their production by the various groups of microorganisms, also possessing diverse chemical structures and surface properties. Thus, it is rational to believe that diverse groups of biosurfactants have different natural roles which are specific to the physiology and ecology of the producing microorganisms[40]. Therefore, it will not be correct to draw any generalization for assigning common functions to all microbial surfactants.

2.3.1 Increasing the surface area of hydrophobic water-insoluble substrates

During the growth of microorganisms certain growth stimulating compounds are produced which tend to emulsify the hydrophobic substrates extending the interfacial area between the microorganisms and the substrate which further facilitates mass transfer of the substrate to the surface of microorganisms[30]. Work of Zhang and Miller[47] confirmed the effect of biosurfactants on hydrocarbon biodegradation with the increase of microbial accessibility to insoluble substrates and thus enhance their biodegradation. Chang *et al.*[180] reported that biosurfactants increase the apparent solubility of hydrophobic organic compounds at concentrations above the critical micelle concentration (CMC), which enhance their availability for microbial uptake. Whang *et al.*[181] showed that two different types of biosurfactants such as rhamnolipid and surfactin produced by several sp. of *P. aeruginosa* and *B. subtilis* respectively, increases the solubility and bioavailability of a petrochemical mixture and also stimulate indigenous microorganisms for enhanced biodegradation of diesel contaminated soil. Biosurfactant-negative mutants of *P.aeruginosa* KY-4025[182] and *P.aeruginosa* PG-201[183] 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 microorganism in the respective hydrocarbon. Franzetti *et al.*[19] and Franzetti *et al.*[184] reported that the rate of biodegradation is dependent on the chemicophysical properties of the biosurfactants and not by the effects on microbial metabolism. Reid *et al.*[185] and Stokes *et al.*[186] in their review

highlighted that biodegradation assays depends on soil slurries and solubilization of target contaminants, which gives an estimation of bioaccessibility rather than bioavailability. Burgos-Díaz *et al.*[187] reported that exogenously added biosurfactant can increase the apparent water solubility of organic compounds and alter its bioavailability by mediating interactions between the hydrophobicity of the cell surface and the substrate surface.

2.3.2 Increasing the bioavailability of hydrophobic water-insoluble substrates

The interaction of microorganisms with hydrophobic organic chemicals and the role of biosurfactants in their bioavailability have been reviewed extensively by Salihu *et al.*[22], Van Hamme *et al.*[38] and Ron and Rosenberg[40]. Volkering *et al.*[188] reviewed the probable modes of how microorganisms interact with hydrophobic organic compounds and suggested that the microorganisms may access the hydrophobic substrate via direct contact or by contact with pseudosolubilized substrate in surfactant micelles or emulsion droplets. In the case of direct contact, the hydrophobicity of both the cell surface and the substrate surface will determine the interaction and biosurfactants may play a role in mediating such interactions[38]. Rosenberg[189] found that emulsan, an extracellular polymeric heteropolysaccharide capsule, is used by *Acinetobacter calcoaceticus* to facilitate detachment from crude oil droplets exhausted of substrate. Once the utilizable substrates have been consumed the emulsan coat is shed off and changing the hydrophobic oil surface to a hydrophilic one. In *Acinetobacter radioresistens* KA53 the bioemulsifiers, alasan, was found to increase the solubility of PAHs by 6 to 27-fold[169]. For pseudosolubilization, addition of exogenous biosurfactant or surfactant can enhance a noticeable aqueous solubility of organic compounds and modify the bioavailability[190, 191]. Miller and Bartha[192] showed that micelles or other aggregates are formed that partition hydrophobic substrates and may enhance biodegradation by allowing for closer cell–substrate interactions, or may fuse directly with microbial membranes resulting in direct substrate delivery. Rhamnolipid has been found to remove LPS in a dose-dependent manner from *P.aeruginosa* resulting in increased cell surface hydrophobicity and enhanced uptake of hydrophobic substrates[193]. Some hydrocarbon-degrading microbes

respond to these non soluble carbon sources by producing surface-active compounds, as well as by changing cell surface properties such as cell surface hydrophobicity[47, 194, 195].

2.3.3 Binding of the heavy metals

Since many contaminated sites are also co-contaminated with metals, biosurfactants have also been explored for metal chelation[38]. Mulligan *et al.*[3] have recently evaluated remediation technologies for metal-contaminated soils. Rhamnolipids are able to remove metals, ions and forms stable complexes with metals in the following order: Al^{3+}> Cu^{2+}> Pb^{2+}> Cd^{2+}> Zn^{2+}> Fe^{3+}> Hg^{2+}> Ca^{2+}> Co^{2+}> Ni^{2+}> Mn^{2+} > Mg^{2+}> K^{+} [115, 196, 197]. Tan *et al.*[197] studied the effect of monorhamnolipid produced by *P. aeruginosa* ATCC 9027 on the formation of metal complexes and reported that surfactant–metal interactions are rapid and stable. The mechanism of reduced toxicity was apparently via rhamnolipid complexation of cadmium as well as by rhamnolipid induced lipopolysaccharide removal from the cell surface[38]. Mulligan *et al.*[198] have been reported to use surfactin from *Bacillus subtilis* to treat soil and sediments contaminated with Zn, Cu, Cd, oil and grease. Mulligan and Yong[199] used biosurfactants from *Bacillus subtilis* ATCC 21332, *P. aeruginosa* ATCC 9027 and *Torulopsis bombicola* ATCC 22214 to examine the removal of metals from oil-contaminated soil. Surfactin, rhamnolipids and sophorolipids produced by the microorganisms were extracted using methods described in Mulligan *et al.*[3, 198] and Mulligan and Gibbs[6]. Sandrin *et al.*[200] reported that exogenously added rhamnolipid reduces cadmium toxicity for *Burkholderia* sp. growing on either naphthalene or glucose as sole carbon source. In sorption, metal-ligand complexation, complexation with soil constituents and cation exchange processes are involved[201] affecting access of the metal to the microorganisms.

2.3.4 Pathogenesis

Biosurfactant noticeably influence the physiological behaviour of microbes such as their role in plant and animal pathogenesis and the same have been extensively reviewed by Cameotra and Makkar[36] and Peypoux *et al.*[202]. Biosurfactant are regarded

to function as a "dispersing agent" in pathogenic microorganisms effecting plants or animals as a "wetting agent" for the surface of host cell[30]. *Pseudomonas syringae* produces two necrosis-inducing lipopeptide toxins, syringopeptin and syringomycin, types of pore forming cytotoxins that form ion channels permeable to divalent cations during plant pathogenesis[203]. Plant pathogenic bacterium, *Pseudomonas fluorescens* 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[204]. *Serratia marcescens*, an opportunistic pathogenic bacterium produces serrawettin a type of nonionic cyclic depsipeptide that helps in wetting of the host cell surface and dispersion of the bacteria[205]. Several sp. of *P. aeruginosa* are reported to be pathogenic and causes serious infections in immunocompromised patients and individuals suffering from cystic fibrosis (CF)[206]. *P. aeruginosa* has been reported to produces a heat-stable extracellular glycolipid called hemolysin that has hemolytic activity[207]. The di-rhamnolipid type biosurfactant from *Burkholderia pseudomallei* is similarly hemolytic for erythrocytes of various sp. and also cytotoxic at high concentrations for non-phagocytic and phagocytic cell lines[208]. Zulianello *et al.*[209] showed shown that *P. aeruginosa* requires the production of rhamnolipids to invade respiratory epithelia reconstituted with primary human respiratory cells.

2.3.5 Antimicrobial activity

With the interest in developing novel antimicrobials for therapeutic and agricultural applications, a number of biosurfactants with antibiotic properties have been described. A *Pseudomonas* sp. derived from marine alga produces eight types of Massetolides A–H, novel cyclic depsipeptides. These eight Massetolides A–H were and was found to exhibit *in vitro* antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium-intracellulare*[210]. Sotirova *et al.*[211] showed that rhamnolipids from *Pseudomonas* sp. PS-17 interact with *P. aeruginosa* causing a reduction in LPS content, changing the outer membrane proteins and had a direct impact on bacterial cell surface morphology. The antibacterial property of biosurfactant and their application in the field of medicine is extensively reviewed by

Rodrigues *et al.*[63]. Kim *et al.*[212] studied the effects of rhamnolipid B on a range of plant pathogenic fungi including *Phytophthora capsici* and *Colletotrichum orbiculare* and observed to cause zoospore lysis, inhibition of zoospore and spore germination, and hyphal growth inhibition. Andersen *et al.*[213] reported to isolate a *Pseudomonas* sp. DSS73 strain from the rhizoplane of sugar beet seedlings that showed antagonism towards the root-pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani*. Yoo *et al.*[66] investigated rhamnolipids as alternative antifungal agents against typical plant pathogenic oomycetes, including *Phytophthora* sp. and *Pythium* sp. Viscosinamide, a new antibiotic isolated from *Pseudomonas fluorescens*, with biosurfactant properties and was found to have antifungal properties[214]. Bechard *et al.*[215] isolated an antimicrobial lipopeptide from a strain of *B. subtilis* and demonstrated a broad spectrum of activity against Gram-negative bacteria, lesser activity against Gram-positive organisms and was active against one of the two fungi assayed. Grangemard *et al.*[216] reported the chelating properties of lichenysin, a cyclic lipopeptide produced by *Bacillus licheniformis*, which might explain the membrane disrupting effect of lipopeptides. Carrillo *et al.*[217] studied the molecular mechanism of antibiotic and other important biological actions of surfactin produced by *Bacillus subtilis*. Nielsen and Sorensen[218], found three cyclic lipopeptides (viscosinamide, tensin, amphisin) produced by *P. fluorescens* in the rhizosphere of germinating sugar beet seeds and considered that such lipopeptides confer a competitive advantage to the organism during colonization. Vatsa *et al.*[219] reviewed the zoosporicidal activity of rhamnolipids against various fungal phytopathogens. In the literature properties of rhamnolipids against the algae *Heterosigma akashiwo*, viruses, amoeba like *Dictyostelium discoideum* and mycoplasma have also been reported but don't have significant effect on yeasts[219].

2.3.6 Effecting attachment of microorganism to surfaces

One of the most essential survival strategies of microorganisms is their ability to establish themselves in an ecological niche where they can propagate. In such strategy the key element is the structure of cell-surface, responsible for the attachment of the microbes to the proper surface[43]. Neu[220] has reviewed how surfactants can affect

the interaction between bacteria and interfaces. Zhang and Miller[47] observed that cell surface hydrophobicity of *P.aeruginosa* was greatly increased by the presence of cell-bound rhamnolipid. In case of *Acinetobacter* strains the cell-surface hydrophobicity was reduced in the presence of its cell-bound emulsifier[89]. Such observations suggest that microorganisms can use their biosurfactants to regulate their cell-surface properties in order to attach or detach from surfaces according to the need. Rosenberg[189] found that emulsan, an extracellular polymeric heteropolysaccharide capsule, is used by *Acinetobacter calcoaceticus* to facilitate detachment from crude oil droplets with the exhaustion of carbon source.

2.3.7 Biosurfactant production and quorum sensing

Being a virulence factor, the production of bioemulsifier produced by the pathogens, it has been suggested, initiates when the cell density is high enough to cause a localized attack on the host[63]. Ron and Rosenberg [40] reported that bacteria growing at the oil-water interface starts producing emulsifier when the cellular density become higher, resulting in the increase in the surface area of oil droplets and this allows more bacteria to attach on the extended surface area of the oil drops. On the other hand, when these usable fractions of the hydrocarbon present in the oil are consumed, the production of the emulsifier allows the bacteria to get detached from the "used" droplets and find a new one.

2.3.8 Role of biosurfactant in biofilms

Rhamnolipid production appears as a regulator for determining cell-surface hydrophobicity and modification of adhesive interactions, especially when there are changes in nutritional conditions[68, 220]. Rhamnolipids are reported to be involved in biofilm development. Exogenous rhamnolipids induce a release of lipopolysaccharides (LPS) and consequently enhance the cell surface hydrophobicity, which might favor primary adhesion of planktonic cells[47, 194]. Alasan, an exocellular polymeric emulsifier produced by *Acinetobacter radioresistens* was KA53 reported to bind on the surface of *Sphingomonas paucimobilis* EPA505 and *Acinetobacter calcoaceticus* RAG-1 and modify their surface properties. Moreover, when the alasan-producing *Acinetobacter*

radioresistens KA53 was co-culture with *Acinetobacter calcoaceticus* RAG-1, alasan was released from the producing strain and bound to the recipient RAG-1 cells[221]. Such horizontal transfer of bioemulsifier between the bacterial sp. has considerable implications in natural microbial communities, co-aggregation and biofilm formation. Rhamnolipids assist the surface-associated migration of bacteria in the biofilm and therefore the initial microcolony formation and differentiation of the biofilm structure takes places[222].

2.4 Genetic regulation of biosurfactant synthesis

The regulation of biosurfactant production has been investigated at the molecular level for the glycolipids of *P.aeruginosa* and a few lipopeptides of *Bacillus sp.* Peypoux *et al.*[202] had shown that biosurfactant production in these bacteria was induced by molecular signal involved in quorum sensing[223]. However, whether quorum sensing is the environmental clue to biosurfactant production in general is still not known. Burger *et al.*[224] proposed that rhamnolipid synthesis proceeds by two sequential glycosyl transfer reactions, each catalyzed by a different rhamnosyltransferase. Ochsner and Reiser[225] and Ochsner *et al.*[226] made significant contribution to the genetics of rhamnolipid biosynthesis. Mono-rhamnolipid (rhamnolipid 1) synthesis is catalyzed by the enzyme rhamnosyltransferase 1, encoded by the *rhlAB* and is present in a single operon. The second rhamnosyltransferase 2 responsible for the synthesis of di-rhamnolipid (rhamnolipid 2), encoded by *rhlC*, had been characterized and its expression had been shown to be co-coordinately regulated with *rhlAB* by the same quorum sensing system[227]. The *rhlR* and *rhlI* genes are arranged sequentially and regulate *rhlAB* genes expression. *RhlI* protein forms N-acylhomoserine lactones, which act as autoinducers and influence *RhlR* regulator protein. Induction of *rhlAB* depends on quorum-sensing transcription activator *RhlR* complexes with the autoinducer N-butyryl-homoserine lactone (C4-HSL).

The biosynthesis of surfactin is catalyzed non-ribosomally by a large multienzyme peptide synthetase complex called the surfactin synthetase, consisting of three protein subunits-SrfA, ComA (earlier known as SrfB) and SrfC[202]. The peptide

synthetase required for the amino acid moiety of surfactin is encoded by four open reading frames (ORFs) in the *srfA* operon namely *SrfAA*, *SrfAB*, *SrfAC* and *SrfAD* or *SrfA-TE*. This operon also contains *comS* gene lying within and out-of-frame with the *srfB*. The other three ORFs are absolutely essential as compared to *SrfAD* for the biosynthesis of surfactin. The gene *sfp* encodes for phosphopantetheinyl transferase and is absolutely essential for the activation of surfactin synthetase by posttranslational modification. When the cell density is high, ComX, a signal peptide after being modified by the gene product of comQ, accumulates in the growth medium[228]. Quorum sensing controls *srfA* expression by ComX. The histidine protein kinase ComP donates a phosphate to the response regulator ComA and interacts with ComX, which in turn activates the transcription of the *srf* operon[223].

Other types of biosurfactants whose molecular genetics have been decoded in the recent years include arthrofactin, iturin, lichenysin, mannosylerythritol lipids (MEL) and emulsan. Arthrofactin is a cyclic lipopeptide-type biosurfactant produced by *Pseudomonas* sp.. MIS38. Three genes designated as *arfA*, *arfB*, and *arfC* form the arthrofactin synthetase gene cluster and encodes for ArfA, ArfB & ArfC proteins respectively, which assemble to form a unique structure for catalyzing the biosynthetic reactions[223]. Lichenysin is another type of lipopeptide synthesized non-ribosomally by a multienzyme peptide synthetase complex. The *lic* operon of *B. licheniformis* is 26.6 kb long and consists of genes *licA* (three modules), *licB* (three modules) and *licC* (one module). The domain structures of these seven modules resemble that of surfactin synthetases SrfA-C[223]. Iturin A is a type of lipopeptide biosurfactant produced by *B. subtilis* RB14, composed of four open reading frames (ORFs) contains *ituD*, *ituB*, *ituC* and *ituA* genes which encodes for putative malonyl coenzyme A transacylase, peptide synthetase consisting of four amino acid adenylation domains and peptide synthetase respectively while the fourth gene *ituA* encodes for ItuA, having three functional domains homologous to β-ketoacyl synthetase, amino transferase and amino acid adenylation[223]. *Ustilago maydis* produces two kinds of glycolipid type biosurfactants, mannosylerythritol lipid (MEL) referred to as ustilipids[229] and ustilagic acid that are cellobiose lipids. *emt1* and *cyp1* are the two genes involved in the synthesis of MEL

and ustilagic acid, respectively. It is assumed that Cyp1 protein is associated with the terminal and/or sub-terminal hydroxylation of an unusual fatty acid present in cellobiose lipids[223]. During the stationary phase of growth, *Acinetobacter lwoffii* RAG-1 secrete a potent bioemulsifier on the cell surface known as emulsan[230]. A 27kb gene cluster termed wee encodes the genes *wza*, *wzb*, *wzc*, *wzx* and *wzy* required for the biosynthesis of emulsan[231]. It was later established that Wzc and Wzb encodes a protein tyrosine kinase and protein tyrosine phosphatase, respectively[232] .

2.5 Biosynthetic pathways of biosurfactant synthesis

Biosurfactants are synthesized by two primary metabolic pathways viz hydrocarbon and carbohydrate pathways[7, 163]. Metabolic pathways involved in the synthesis of the precursors of hydrophilic and hydrophobic domains of biosurfactants are diverse and utilize definite set of enzymes. Syldatk and Wanger[233] suggested some of the possible features for the biosynthesis of biosurfactants and their regulation which includes (i) *de novo* synthesis of hydrophilic and hydrophobic moieties by two independent pathways followed by their linkage to form a complete biosurfactant molecule, (ii) *de novo* synthesis of the hydrophilic moiety and the substrate-dependent synthesis of the hydrophobic moiety and its linkages, and (iii) *de novo* synthesis of the hydrophobic moiety and the substrate-dependent synthesis of the hydrophilic moiety followed by its linkage. The biosynthesis of both hydrophobic and hydrophilic moieties depends on the type of substrate used for the production of biosurfactant[163].

The biosynthesis of surfactin by *Bacillus subtilis* has been extensively studied by Kluge *et al.* [234] and reviewed by Desai and Desai[163]. The formation of surfactin occurs non-ribosomally and two different mechanisms are involved in the activation of amino acid. Nakano *et al.*[235] reported that two components of surfactin synthesizing enzyme complex of *B subtilis* are homologous to tyrocidine synthase I and gramicidin S synthase. In addition, the biochemical studies confirmed the occurrence of surfactin synthesis via a thio-template mechanism[236]. Enzymatic synthesis of surfactin requires ATP, Mg^{2+}, precursors and sucrose. The fatty acid component of surfactin is incorporated only as an acetyl-CoA derivative and L-isomer of amino acids are

incorporated in the peptide chain[236]. The enzymes involved also catalyze the ATP-Pi-exchange reactions which are mediated by the amino acid components of surfactin. This pattern was consistent with a peptide-synthesizing system that activates its substrate simultaneously as aminoacyl phosphates[234]. In case of surface active compound herbicollin A, both the lipid and peptide domain have been found to be directly synthesized from carbohydrates. Addition of amino acids or fatty acids in the growth medium affected the yield but not the structure of the surfactant[163]. Research investigations have shown that in Gramicidin-S a type of surface active antibiotic, lipopeptide, is synthesized non-ribosomally by a multienzyme complex with the involvement of pantetheine cofactor by a thio-template mechanism[237].

Regarding the biosynthesis of glycolipids, the pathway of the sugar-lipid biosurfactant formation depends on the microorganism producing it. An example of glycolipid synthesis is the biosynthesis of anionic rhamnolipids by *Pseudomonas* sp.. Rhamnolipid synthesis using enzymology and different radioactively labeled precursors and the proposed biosynthetic pathway has been studied extensively by Hauser and Karnovsky[238] and reviewed by Banat *et al.*[35]. Syldatk *et al.*[239] reported that the composition of biosurfactant produced by *Pseudomonas* sp. is affected by the type of carbon substrate used and the cultivation conditions but the hydrocarbon substrate having different chain length has no effect on the chain length of the fatty acid moiety in glycolipids. Almost the similar results were observed during the production of bioemulsifier by *Acinetobacter sp.* H01-N using alkane as the substrate[240]. Suzuki *et al.*[241] reported the influence of substrate on the sugar moiety of the glycolipid synthesized by *Arthrobacter paraffineus*. The non-ionic trehalose lipid is formed when *A. paraffineus*is grown on n-alkanes, but fructose lipids are produced when fructose is used as the sole carbon source[30].

Other examples of *de novo* synthesis of biosurfactant are cellobiose lipid by *Ustilago zeae*[242] and sophorolipid by *Torulopsis bombicola*[30] from different hydrophobic substrates. During the synthesis of trehalose mono-and dicorynomycolates in *Rhodococcus erythropolis*, the sugar moiety of the surfactant is *de novo* synthesized and the chain length of the lipid moiety is dependent on

hydrocarbon substrate used in the medium[243]. A similar pathway has been found to be working in *Rhodococcus erythropolis* for the synthesis of trehalosetetraesters[233], in *Candida sp.* for the synthesis of mannosylerythritol lipids[244], and in *Nocardia erythropolis*[245] for extracellular glycolipid synthesis.

2.6 Regulation of Biosurfactant Synthesis

The chemical composition, level of production and surface properties of the biosurfactant depend not only on the producer strain but also on various factors such as nature and concentration of macro and micro nutrients, culture conditions including pH, temperature, agitation and dilution rate[6, 29].

The carbon source is the most important factor influencing the biosurfactant synthesis either by induction or by repression[124]. The commonly used carbon sources include carbohydrates, hydrocarbons and vegetable oils. It has been concluded from a number of studies that different carbon sources can influence the composition of biosurfactant formation[22, 246]. *Actinobacter calcoaceticus* and *Arthobacter paraffineus*[247] fail to produce surface-active compounds when grown on organic acids and D-glucose as carbon source, respectively. Previous reports indicate that the addition of water-immiscible substrates result in the induction of biosurfactant production. Tulloch *et al.*[248] have found the induction of sophorolipid synthesis by the addition of long chain fatty acids, hydrocarbons or glycerides to the growth medium of *Torulopsis magnoliae*[249]. *Arthrobacter* produces 75% extracellular biosurfactant when grown on acetate or ethanol but it is totally extracellular when grown on hydrocarbon[4]. Hauser and Karnovsky[238] have demonstrated a severe decrease in the synthesis of rhamnolipid on addition of glucose, acetate, and tricarboxylic acids during the growth on glycerol. A similar observation was reported for the synthesis of liposan in *Candida lipolytica*[156]. On the other hand, surfactin produced by *Bacillus subtilis* is usually observed with glucose as the carbon source and is inhibited by the addition of hydrocarbons in the medium[250].

Nitrogen is another important factor that plays an important part in the regulation of biosurfactant synthesis. It may also contribute to pH control[251]. Duvnjak

et al.[247] found that urea led to a satisfactory biosurfactant production. Moreover, nitrogen limitation also changed the composition of the biosurfactant production[163, 239]. Among the inorganic salts tested, ammonium salts and urea were preferred for biosurfactant production by *Arthobacter paraffineus*, whereas nitrate supported maximum biosurfactant production in *P. aeruginosa*[7]. Yeast extract was found to be required for glycolipid production by *Torulopsis bombicola*, but was very poor for *P. aeruginosa*[251]. Supplementation of ammonia in the nitrate-containing medium delays the production of biosurfactant in *Corynebacterium*[163]. Several investigators observed rhamnolipid in the fermentation broth of *P. aeruginosa* with the exhaustion of nitrogen and beginning of the stationary phase of growth[206]. Nitrogen limitation not only causes over production of biosurfactants but also changes the composition of biosurfactants produced[163, 239]. According to Hommel *et al.*[137] it is the absolute quantity of nitrogen and not its relative concentration that is important to give an optimum biomass yield while the concentration of hydrophobic carbon source determines the conversion of carbon available to the biosurfactant.

Phosphate limitation also influences the metabolism of biosurfactant[251]. Clarke *et al.*[252] observed enhanced production of rhamnolipid by *P. aeruginosa* ATCC 9027 under phosphate limited conditions. The change in activity of several intracellular enzymes dependent on phosphate levels indicated a shift in biosurfactant metabolism[4]. Iron limitation is reported to stimulate the production of biosurfactants in *P. fluorescens*[253]. However, the production of surfactin by *B. subtilis* is reported to be stimulated by the addition of iron and manganese salts to the medium[250]. The limitation of multivalent cations also causes overproduction of biosurfactants[124, 254]. Higher yield of rhamnolipid could be achieved in *P. aeruginosa* DSM 2659 by limiting the concentration of Mg^{2+}, Ca^{2+}, K^{+}, Na^{+} and trace salts[163].

Environmental factors such as temperature, pH, agitation and oxygen availability also affect the production of biosurfactant production through their effect on cellular growth or activity[22, 124, 129, 246]. Temperature may cause alteration in the composition of the biosurfactant produced by *Pseudomonas* sp. DSM-2874[239]. Banat[255] reported a thermophilic *Bacillus* sp. which could grow and produced

biosurfactant at temperatures above 40 °C. In *Torulopsis bambicola* the pH of the medium plays an important role in sophorolipid production[256]. Powalla *et al.*[257] reported that penta and disaccharide lipid production by *Nocardia corynbacteroides* was unaffected in the pH range of 6.5 to 8.0. Mulligan and Gibbs[4] reported that increase in agitation speed caused shear effect which reduced the production of biosurfactant by *Nocardia erythropolis*. Conversely, production of biosurfactant by yeast increased when the agitation and aeration rates increased[7]. The role of abiotic factors on the production of rhamnolipid by *P.aeruginosa* was extensively reviewed by various authors[50, 258, 259].

2.7 Screening for biosurfactant producing microorganisms

Numerous methods have been tried for the high-throughput screening. These procedures are reliable and significantly accelerate the screening process towards high biosurfactant producer strains[260]. The Du-Nouy-Ring assay using a tensiometer is most widely applied for screening of biosurfactant producing microbes[59]. This method was based on measuring the force required to detach a ring or loop of wire from an interface or surface. Jain *et al.* [261] developed rapid drop-collapsing test, a simple method of detecting biosurfactant production. In this technique, a drop of a cell suspension is placed on an oil-coated surface and the drops containing biosurfactant collapse within few second whereas non-surfactant containing drops remain stable. Persson and Molin[262] described a similar assay using a glass surface instead of the oil coated surface. Vaux and Cottingham[263] developed and patented a method called microplate assay. This assay was based on the change in the optical distortion which was caused by surfactants in an aqueous solution. Maczek *et al.* [264] developed a qualitative technique suitable for high-throughput screening, known as penetration assay. This assay was based on the contact of two insoluble phases which leads to a change in color. Another method of detection of biosurfactant production is oil spreading technique. This technique measures the diameter of clear zones caused when a drop of biosurfactant-containing solution is placed on an oil-water surface[265]. Another most widely used assay which was based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg[266]. Emulsification capacity

was expressed as emulsification index (E_{24}), where E_{24} is the emulsification percentage obtained by vigorously shaking of culture supernatant with kerosene. This method is most suitable for emulsifying biosurfactants. Rosenberg *et al.* [267] developed the bacterial adhesion to hydrocarbons (BATH) method, a simple photometrical assay for measuring the hydrophobicity of bacteria. Cell surface hydrophobicity is an important character associated with the adherence of bacterial cell to various liquid hydrocarbons[268]. A simple replica plate assay for the identification and isolation of hydrophobic microbes was developed by Rosenberg[269]. The basis of this assay is the adherence of bacterial strains to hydrophobic polystyrene surface which correlates to cell surface hydrophobicity. Siegmund and Wagner[270] developed a semi-quantitative CTAB agar plate method for the detection of extracellular glycolipids or specifically for anionic type biosurfactants. The interaction between the anionic biosurfactant secreted by the microbes with the cationic surfactant CTAB (cetyltrimethyl ammonium bromide) and methylene blue results in the formation of insoluble ion pair. The resulting productive colonies are surrounded by dark blue halos[271]. Another method of detecting biosurfactant producing microbes is by their ability to cause haemolysis of RBC on solid media plate and was developed by Mulligan *et al.* [272]. Blood agar lysis has been used to quantify surfactin[273] and rhamnolipids[207]. Schenk *et al.* [274] developed a high-performance liquid chromatographic method of detection of rhamnolipid produced by *Pseudomonas aeruginosa*. A similar method of detection of biosurfactant production in the cell-free fermentative broth of *Bacillus subtilis* ATCC 21332 was proposed by Lin *et al.* [275]. Among all the known techniques reported till date, drop collapse assay, microplate assay and penetration assay are considered as high-throughput screening (HTS) methods because these techniques are rapid and reliable[271].

2.8 Production of biosurfactant from cheaper and available carbon sources

The success of commercial level biosurfactant production depends on the development of cheaper processes and the use of low-cost raw materials, accounting for 50% of the final product cost[124]. Agro-industrial wastes are obtained at low cost from the respective processing industries and are as potent as low-cost substrates for

industrial level biosurfactant production. Potato process effluents generated from potato processing industries were reported to be used for the production of biosurfactant by *B. subtilis*[276]. Cassava wastewater generated during the preparation of cassava flour is a potential substrate for the production of surfactin and rhamnolipid by *B. subtilis*[277] and *P. aeruginosa*[278] respectively. George and Jayachandran[279] analyzed the rhamnolipid biosurfactants produced through submerged fermentation using orange fruit peelings as sole carbon source. Maria *et al.* [280] reported the utilization of cashew apple juice supplemented with peptone and nutritive broth for the cultivation of *P. aeruginosa* to obtain biosurfactants. Patel and Desai[281] reported the use of molasses and corn-steep liquor as the primary carbon and nitrogen source for the production of rhamnolipid using *P. aeruginosa* GS3. Dubey and Juwarkar[282] studied the production of biosurfactant using industrial waste from distillery using *P. aeruginosa* BS2. Kitchen waste oils generated from domestic uses, vegetable oil refineries or the soap industries have been reported to be suitable for the production of biosurfactant through microbial fermentation[41, 283]. Lima *et al.* [284] reported the use of residual waste of soybean oils for the production of biosurfactant by submerged fermentation in stirred tank reactors using *P. aeruginosa* PACL. Soap stock, an industrial waste by-product has been used to produce emulsan, bio-dispersan and rhamnolipid by *Acinetobacter calcoaceticus* A2[89] and *P. aeruginosa* LBI[283] respectively through batch fermentation. Anastasia *et al.* [285] reported the use of sunflower seed oil and oleic acid for the production of rhamnolipids by *Thermus thermophilus* HB8. Palm oil was reported to be used for the simultaneous production of polyhydroxyalkanoates and rhamnolipids by *P. aeruginosa*[286]. Hazra *et al.* [287] reported the utilization of de-oiled cakes of mahua (*Madhuca indica*), karanja (*Pongamia pinnata*), jatropha (*Jatropha curcus*) and neem (*Azadiracta indica*) for the production of rhamnolipid using *P. aeruginosa* AB4. Pratap *et al.* [288] reported the use of non-edible traditional oils such as neem oil, jatropha oil and karanja oil for the production of rhamnolipid using *P.aeruginosa* ATCC 10145. *P. aeruginosa* 47T2 was reported to produce rhamnolipid when grown on olive oil waste water or in waste frying oils obtained from olive/sunflower (50:50; v/v)[84, 289, 290]. Daniel *et al.* [291] used dairy wastes as carbon substrates and achieved production of high concentrations of

sophorolipids using two-stage cultivation process for the *yeast Cryptococcus curvatus* ATCC 20509. Deshpande and Daniels[292] used animal fat for the production of sophorolipids biosurfactant using the yeast, *Candida bombicola.*

2.9 Industrial application of biosurfactants

2.9.1 In petroleum industry

Over the recent years, many studies have shown the capability of biosurfactants and biosurfactant-producing bacterial strains to enhance availability and biodegradation rates of organic contaminants[47, 293, 294]. Research investigations conducted by Zhang and Miller[295] confirmed that biosurfactant effects the degradation of hydrocarbon by increasing microbial accessibility to insoluble substrates and thus enhance their biodegradation. Glycolipid biosurfactants have also been shown to enhance the hydrocarbon removal (from 80 to 90–95%) from soil; furthermore, the biosurfactant was reported to increase hydrocarbon mineralization by two-fold and shorten the adaptation time of microbial populations to fewer hours[4]. Holakoo and Mulligan[296] reported the usefulness of biosurfactants for oil spills remediation and for dispersing oil slicks into fine droplets and converting mousse oil into oil-in-water emulsion. Barkay *et al.* [297] used the bioemulsifier alasan produced by *Acinetobacter radioresistens* KA53 to enhance PAH solubility, and degradation results showed 6.6, 25.7 and 19.8-fold increases in the solubilities of phenanthrene, fluoranthene and pyrene respectively. Similarly, the solubilization of PAH has been reported with the rhamnolipids produced by *Pseudomonas aeruginosa* and other pseudomonads[83, 298]. Balachandran *et al.* [299] reported the degradation of petroleum and polyaromatic hydrocarbons and metabolism of naphthalene by *Streptomyces* sp. isolated from oil contaminated soil. Mata-Sandoval *et al.*[300] reported that biodegradation of chlorinated and polychlorinated biphenyl hydrocarbons can be enhanced by addition of glycolipids to the medium. Several sp. of *P. aeruginosa* and *B. subtilis* produce rhamnolipid and surfactin respectively; these two biosurfactants have been shown by Whang *et al.*[181] to increase solubility and bioavailability of a petrochemical mixture and also stimulate indigenous microorganisms for enhanced biodegradation of diesel

contaminated soil. Bordoloi and Konwar[298] reported that pyrene was solubilized more by the biosurfactant of *P.aeruginosa* (MTCC 7815) and *P.aeruginosa* (MTCC 7812); phenanthrene by *P.aeruginosa* (MTCC 8165); fluorene by *P.aeruginosa* (MTCC 7812) and *P.aeruginosa* (MTCC 8163); crude oil by the biosurfactant of *P.aeruginosa* (MTCC 8165). Addition of rhamnolipids above their critical micellar concentration (CMC) enhanced the apparent aqueous solubility of hexadecane, enhanced biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and promoted bioremediation of petroleum sludges[2, 193, 195, 294]. Das and Mukherjee[301] reported that inoculation of the contaminated soil with *P. aeruginosa* M and NM consortium and *B. subtilis* strain along with their respective biosurfactant reduced the TPH levels from 84 to 21 and 39 $g \cdot kg^{-1}$ of soil, respectively within 120 days of treatment. *Gordonia* sp. BS29 growing on aliphatic hydrocarbons as sole carbon source has been found to produce bioemulsan, which effectively degrades crude oil, PAHs and other recalcitrant branched hydrocarbons from the contaminated soils[184]. Reddy *et al.* [302] reported 93.92% degradation of phenanthrene by a biosurfactant producing *Brevibacterium* sp. PDM-3 strain and also reported the ability of the bacterial strain to degrade other polyaromatic hydrocarbons such as anthracene and fluorene.

2.9.2 In microbial surfactant enhanced oil recovery (MEOR)

Biosurfatant MEOR represents one of the most promising methods to recover a substantial proportion of the residual oil from mature oil fields[101]. The efficiency of MEOR has been proven in field studies in the U.S., Czech Republic, Romania, Hungary, Poland, and Holland, with significant increase in oil recovery observed in all cases[7, 133]. Jinfeng *et al.*[103] (2005) conducted microbial enhanced water-flooding experiment in a Guan 69 Unit in Dagang Oilfield in China by injection of a mixture of *Arthrobacter* sp. (A02), *Pseudomonas* sp. (P15) and *Bacillus* sp. (B24) strain suspension and the nutrient solution through injection wells in an ongoing water flood reservoir where the temperature reached 73°C. They observed that the oil production steadily increased after microbial water-flooding. In recent years, physical stimulation test such as sand pack column experiment has been served as an excellent laboratory

instrument to investigate and understand the mechanism and performance of biosurfactant flooding in enhanced oil recovery[32, 98, 303]. Bordoloi and Konwar[2] treated crude oil saturated sand pack column with cell free culture broths containing biosurfactant of four different *P. aeruginosa* strains (MTCC 7815, MTCC 7814, MTCC 7812 and MTCC 8165) and were reported to release about 15% more crude oil at 90°C than at room temperature and 10% more than at 70°C under laboratory conditions. Pornsunthorntawee *et al.* [304] compared the oil recovery from the sand-packed column saturated with motor oil using the biosurfactants produced by *B. subtilis* PT2 and *P. aeruginosa* SP4 with three synthetic surfactants. The results showed that the biosurfactants produced by *B. subtilis* PT2 and *P. aeruginosa* SP4 were more efficient in oil recovery, removing about 62% and 57% respectively, of the tested oil while synthetic surfactants were able to release approximately 53–55%. Suthar *et al.*[100] compared the oil recovery upon application of bioemulsifier and biosurfactant to a sand pack column designed to stimulate an oil reservoir. They reported that crude bioemulsifier produced by *B. licheniformis* K125 gave better oil recovery than the biosurfactant by *B. mojavensis* JF2 and *B. licheniformis* TT42. Oil recovery experiments in physical simulation showed 7.2-14.3% recovery of residual oil after water flooding when the biosurfactant of three strains of *P. aeruginosa*, *B. subtilis* and *R. erythropolis* was added[32]. Gudiña *et al.*[102] studied the efficiency of biosurfactant produced by the strains of *B. subtilis*, isolated from the crude oil samples of Brazilian Oil field and suggested their usefulness for MEOR applications due to their unique properties including thermo- and salt-tolerance; stable surface activity and hydrocarbon degradation.

2.9.3 In technologies for cleaning up

Soil washing technology is characterized by chemico-physical properties of the biosurfactant and not by their effect on metabolic activities or changes in cell-surface properties of bacteria[101]. However, the processes may enhance the bioavailability for bioremediation. Such cleanup process is highly desirable as it is economically rewarding and environmentally friendly[305]. Abu-Ruwaida *et al.*[303] showed that cell-free broth of *Rhodococcus sp.* containing biosurfactant removed about 86% of

adsorbed crude oil from the contaminated sand. Urum *et al.*[106] investigated the efficiency of different surfactant solutions in removing crude oil from contaminated soil using a soil washing process. They demonstrated higher crude oil elimination by synthetic surfactant-sodium dodecyl sulfate (SDS) and rhamnolipid biosurfactants (46% and 44% respectively) than natural surfactant saponins (27%). Franzetti *et al.*[19] showed that the BS29 bioemulsans from *Gordonia sp.* are promising washing agents for remediation of hydrocarbon-contaminated soils. The mean of the crude oil removal for bioemulsans was 33%. The BS29 bioemulsans were also able to remove metals (Cu, Cd, Pb, Zn, Ni) but their potential in the process was lower than rhamnolipids. Costa *et al.*[306] reported that increasing the rhamnolipid concentration produced by *P. aeruginosa* L2-1 on cassava wastewater enhanced the removal of crude oil from the contaminated sand from 69% at CMC to 84% at CMC + 5% (w/w). Aparna *et al.*[23] reported that the *Pseudomonas* sp. 2B biosurfactant solution at 0.01% and 0.05% biosurfactant concentrations was capable to remove 89% and 92% of the oil absorbed in the sand respectively while the distilled water and sodium dodecyl sulphate (SDS) removed 48% and 63% of the contaminated oil respectively.

Thimon *et al.*[307] reported that glutamate residues of surfactin can bind metals such as Mg, Mn, Ca, Ba, Li and rubidium. Soil washing with 0.25% surfactin removed 70% of the Cu and 22% of the Zn[198]. Using micellar-enhanced ultrafiltration, 85–100% removal of cadmium, copper and zinc by surfactin from contaminated water was achieved[198]. Rhamnolipids have been reported to be used for the removal of heavy metals such as Ni and Cd from soils due to their anionic nature, with efficiencies of 80–100% in the lab and 20–80% in the field samples[308].

Fermentative broth of the bacterial strain (Pet 1006) 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[309]. Joseph and Joseph[113] separated the residual oil from the petroleum sludge generated from the crude oil refinery by directly inoculating the strains of *Bacillus sp.* and by addition of the cell free culture supernatant of the bacteria. The removal efficiency of the bacterial

strains was in the range between 91.67 to 97.46%. Helmy *et al.*[114] reported the application of biosurfactant produced by *Azotobacter vinelandii* AV01 for enhanced oil recovery from the oil sludge and recovered upto 15% of oil from the sludge.

2.9.4 In the agriculture sector

Rhamnolipids have a direct biocide action on various plant disease causing bacteria and fungi[219]. They are reported to increase the susceptibility of certain Gram-positive bacteria to specific antibiotics[310]. Nielsen *et al.*[218] demonstrated that viscosinamide, a new cyclic depsipeptide (CLP) produced by *P. fluorescens* DR54 exhibited strong biosurfactant properties and some had antibiotic properties towards root-pathogenic microfungi. Andersen *et al.*[311] isolated a cyclic lipopeptide amphisin from *Pseudomonas sp.* DSS73 from the rhizoplane of sugar beet seedlings that exhibited antagonism towards the root-pathogenic microfungi *Pythium ultimum* and *Rhizoctonia solani*. Rhamnolipids were reported to exert high zoosporicidal activity, probably through zoospore lysis, against various zoosporic phytopathogens, including sp. from the *Pythium, Phytophthora*, and *Plasmopara* genera[86]. Interestingly, fluorescent *Pseudomonads* are effective in the biological control of plant pathogens. Furthermore antiviral, algicidal, mycoplasmicidal, and antiamoebal properties of rhamnolipids have also been reported[312, 313]. The efficacy of rhamnolipid has also been demonstrated in the near commercial, hydroponics, recirculating cultural system[86]. Apart from antimicrobial properties, surface active compounds are used in agricultural sector for hydrophilization of heavy soil which results in soil improvement. Patel and Gopinathan[314] reported that glycolipopeptide produced by *Bacillus* strains were able to form a stable emulsion in the presence of the organophosphorous pesticide fenthion and helps in spontaneous distribution in water. Banat *et al.*[35] reported the biodegradation of around 40 % of chlorinated pesticide α- and β endosulfan by the biosurfactant produced by *B. subtilis* MTCC2423.

2.9.5 In medicines and therapeutics

Rhamnolipids have permeabilizing effects on Gram-positive and Gram-negative human bacterial strains reinforcing their potential in biomedicine[315].

Lipopeptides such as pumilacidin and surfactin have been reported to act as antiviral agents[316]. The loss of membrane integrity as opposed to other vital physiological processes makes surfactin and other lipopolypeptides potentially important as the next generation of antibiotics[36]. Gan *et al.*[317] reported that both *Lactobacillus fermentum* RC-14 and its secreted biosurfactant significantly inhibited *Staphylococcus aureus* infection and bacterial adherence to surgical implants. In current biofilm preventive strategies various research investigations suggested the use of biosurfactants as anti-adhesion with antimicrobial biological coatings for catheter materials or other medical surfaces[36, 63]. Sophorolipids are reported to have activity against human immunodeficiency virus[318]. Similarly, rhamnolipid and its complex with alginate, both produced by a *Pseudomonas sp.* strain showed significant antiviral activity against herpes simplex virus types 1 and 2 [319]. Research investigation of Seydlová and Svobodová[320], Park and Kim[321] and Cao *et al.*[322] indicated that surfactin has potent immunosuppressive capabilities which suggested important therapeutic implications for transplantation and autoimmune diseases including allergy, arthritis and diabetes. Clinical trials using rhamnolipids for the treatment of psoriasis, lichen planus, neurodermatitis and human burn wound healing have confirmed excellent ameliorative effects of rhamnolipids when compared to conventional therapy using corticosteroids[67, 323]. Rhamnolipids also display differential effects on human keratinocyte and fibroblast cultures[208].

2.9.6 In the food industry

In the food industry biosurfactants provide multiple functions and act as emulsifying/foaming agents, stabilizers, antioxidant agents, and anti-adhesives[324]. The addition of polymeric surfactants forms very stable emulsions which improves the texture and creaminess of low-fat dairy products such as soft cheese and ice creams[43]. Shepherd *et al.*[325] reported the successful use of extracellular carbohydrate-rich compound from *Candida utilis* as emulsifying agent in salad dressing formulations. Biosurfactants have been reported to control the agglomeration of fat globules, stabilize aerated systems, improve texture and shelf-life of starch-containing products, modify rheological properties of wheat dough and improve consistency and texture of

fat-based products[326]. In bakery and ice cream formulations biosurfactants act by controlling consistency, retarding staling and solubilizing flavor oils; they are also utilized as fat stabilizer and anti-spattering agents during cooking of oil and fats[327]. Nitschkea and Costa[324] suggested the use of rhamnolipids to improve properties of butter cream, croissants and frozen confectionery products. Iyer *et al.*[328] reported the isolation of a bioemulsifier from a marine strain of *Enterobacter cloac*ae and described it as a potential viscosity enhancing agent because it imparts high-quality viscosity at acidic pH, allowing its use in food products containing citric or ascorbic acid. Biosurfactants have been reported to be used in the pre-conditioning of material surfaces (stainless steel, polystyrene and poly tetrafluoroethylene) found in food-processing environments to prevent the adhesion of food-borne pathogens to such solid surfaces[329, 330].

2.9.7 In nanotechnology

Biosurfactant mediated nanomaterial synthesis and/or stabilization is a recent development in the field of nanotechnology. The biosurfactant mediated process and microbial synthesis of nanoparticles are now emerging as clean, nontoxic and environmentally acceptable "green chemistry" procedures[118, 331, 332]. The focus on the biosurfactant-mediated processes is steeply increasing due to their potential implication on the synthesis of silver nanoparticles[74, 121]. Palanisamy[333] reported the synthesis of stable NiO nano-rods by a water-in-oil micro emulsion technique using rhamnolipid biosurfactant. Literature related to NiO nano-rod synthesis using rhamnolipids as biosurfactants revealed that particle morphology can be tuned by altering the pH[74]. Recently biosurfactants have been shown to be promising candidates for the "green" stabilizing agent of nanoparticles. Mulligan *et al.*[83] reported the use of rhamnolipid biosurfactants as dispersants for nanoparticles. Biswas and Raichur[334] evaluated the efficiency of rhamnolipids for the synthesis and stabilization of nano zirconia particles. Reddy *et al.*[121] reported the use of surfactin as an environmentally friendly stabilizing agent in the synthesized silver nanoparticles. Reddy *et al.*[331] synthesized, for the first time, surfactin-mediated gold nanoparticles, opening the way to a new and fascinating application of biosurfactants in the biomedical field. Kiran *et*

al.[332] reported the application of brevifactin, a novel lipopeptide biosurfactant produced by the marine actinobacterium *Brevibacterium casei* MSA19 for the synthesis and stabilization of silver nanoparticles. The use of gold nanoparticles, in particular, is currently undergoing a dramatic expansion in the field of drug and gene delivery, targeted therapy and imaging[335]. Hazra *et al.* [336] reported the biomimetic fabrication of biocompatible and biodegradable core–shell polystyrene/biosurfactant bionanocomposites for protein drug release.

Chapter III
Materials and Methods

3.1 Materials

3.1.1 Collection of environmental samples

Crude oil contaminated soil and water samples as well as petroleum sludge samples were collected from Borhula Oil Fields of Assam and Assam-Arkan basin, ONGC, Jorhat, Assam, India. Soil samples were collected from just below the top surface layer (~3 cm) and soil samples of sub-surface were collected from a depth of 8 cm. Soil moisture level was maintained with the addition of 0.85% (w/v) saline water at regular intervals. In addition, sludge samples were also collected from Lakuwa Oil Fields of ONGC, Sibsagar, Assam, India. All collected samples were stored at room temperature for subsequent use.

3.1.2 Collection of crude oil samples

Crude oil samples were supplied by INBIGS, ONGC, Jorhat collecting from oil fields such as Nambar reserve, Lakuwa and Geleky. Collection of crude oil samples was done directly from the well-head outlet in sterile reagent bottles.

3.1.3 Other materials

The constituents of mineral salt medium (MSM), bacteriological media and media for biochemical tests were purchased from Hi-Media Laboratories Pvt. Ltd., India, Merck Biosciences, BDH and Ranbaxy. Hydrocarbons used in the study were purchased from Merck, Germany. Carbon sources such as de-oiled cake of mustard, sesame seed oil and castor seed oil were purchased from local stores in Tezpur. Nahor seed oil, Jatropha seed oil, Pongamia seed oil and waste raw glycerol were collected from the Department of Energy, Tezpur University, Assam. Waste residual kitchen oil was collected from the Nilachal Men's hostel, Tezpur University, Assam. Sugarcane bagasse and waste residual molasses were collected from a local sugarcane processing

industry at Tezpur. All other reagents of analytical grade were purchased from Sigma (USA) and Merck (Germany).

3.1.4 Use of waste renewable carbon sources for biosurfactant production

The waste glycerol produced during the conversion of Jatropa seed oil to biodiesel was collected from Department of Energy, Tezpur University, Assam. The pH of the waste was adjusted to 7.0 by adding 5N NaOH. The glycerol fraction from the neutralized waste product was separated using a glass separating funnel and dried at 50°C in an oven to remove the residual moisture.

3.2 Cultivation of microorganisms

3.2.1 Isolation of microorganisms by enrichment culture

The bacterial strains were isolated from the crude oil contaminated soil and water as well as petroleum sludge samples using enrichment culture technique. A sample weighing 5.0 g was added to 100 ml of mineral salt medium (MSM) formulated according to Bordoloi and Konwar[2] in a 500 ml Erlenmeyer flask supplemented with 1% (v/v) of membrane filtered (0.22 μm) n-hexadecane as the sole source of carbon and incubated at 37°C on an orbital incubator shaker for 7 days at 180 rpm. After 10 cycles of enrichment, 1ml of the saturated culture was diluted 10^4 times and an aliquot of 100 μl was spread on MSM agar plates having 0.1% (v/v) n-hexadecane as the carbon source. The culture plates were incubated at 37°C for 48 h. Morphologically different bacterial colonies thus obtained were further purified on the mineral salt agar medium (MSAM) plates with or without 0.1% (v/v) n-hexadecane to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated and isolates showing pronounced growth on n-hexadecane were selected and preserved for further characterization[337, 338].

3.2.2 Isolation of microorganisms by direct culture technique

Crude oil contaminated soil and water and petroleum sludge samples were serially diluted with sterile saline water (0.9% NaCl, w/v) using the standard dilution

technique. The total viable bacterial populations were determined by spread-plating each sample after appropriate dilution (10^{-5}–10^{-6} fold) onto nutrient agar. The morphologically different bacterial colonies thus obtained were further purified on nutrient agar. Bacterial isolates were further grown on Bushnell-Hass medium to ensure their hydrocarbon utilizing capacity. The medium was supplemented with 1% (v/v) n-hexadecane and was used for the screening of potential hydrocarbon degrading and biosurfactant producing bacterial isolates[339].

3.2.3 Maintenance and preservation of isolated microorganisms

The bacterial pure cultures were preserved at 4°C in both nutrient agar plates and slants. Cultures were sub-cultured at an interval of 30 days. For long time storage, frozen stock cultures were prepared in 15 % (v/v) glycerol and stored at −70°C.

3.3 Screening of biosurfactant producing bacteria

3.3.1 Screening for surface activity

The broth culture of each bacterial isolate was centrifuged at 8000 rpm at 4 °C in a Remi 22R centrifuge for 15 min after 96 h of culture. The culture supernatant was collected and its surface tension was measured using a digitalized tensiometer (Krűss Tensiometre K9 ET/25). Before using the platinum ring, it was thoroughly washed three times with distilled water followed by acetone and then allowed to dry at room temperature[59].

3.3.2 Drop collapse test

Screening for biosurfactant production was done using the qualitative drop-collapse test described by Bodour and Maier[59]. For the experiment, 2 µl of crude oil was dropped at the centre of each well on a 96-well micro-titer plate lid and allowed to stand for 24 h for equilibration. An aliquot of 5 µl of culture medium of 48 h duration, before and after centrifugation (10, 000 rpm for 15 min at 4°C), was dropped on the oil-coated wells and then the drop size was observed after 1 min with the help of a magnifying glass. If the drop diameter was found to be at least 1 mm larger than the

one produced by the de-ionized water drop; the result was considered to be positive for the biosurfactant production.

3.3.3 Oil-displacement test

The oil displacement test was done by adding 50 ml of distilled water to a Petri dish with a diameter of 45 cm. An aliquot of 500 µl of crude oil was dropped onto the surface of the water; this was followed by the addition of 100 µl of cell free culture supernatant of the bacterial culture, onto the surface of the oil. The diameter of the clear zone was then measured. Each experiment was repeated thrice to determine an averaged value of the diameter of the appeared clear zone[340].

3.4 Characterization of biosurfactant producing bacteria

3.4.1 Growth characteristics of selected bacterial strains

The pure culture of each bacterial isolate was prepared by using Luria Bertani broth and incubated overnight in an orbital incubator shaker at 37°C with 180 rpm. An aliquot of 100 µl of the above fresh culture broth containing 1×10^{8} ml^{-1} microbes (as calculated from McFarland turbidity method) was inoculated to the 250 ml Erlenmeyer flask containing 100 ml of MSM. All the cultures were supplemented with 1%.(v/v) of n-hexadecane and incubated at 37°C with 180 rpm on an orbital incubator shaker. The growth of the bacterial isolates was monitored by determining the cell forming unit (cfu. ml^{-1}) at a time interval of 12 h for 180 hours.

3.4.2 Biomass determination

Biomass of the bacterial isolates was determined by centrifuging the culture broths at 8,000 rpm for 15 min at 4°C followed by washing twice with phosphate buffered saline (Appendix I). The biomass was dried overnight at 45°C and weighed. In the case of aliphatic hydrocarbons (pentane, n-hexane, heptane, iso-octane, dodecane, tridecane, n-hexadecane, octadecane, eicosane, triacontane and liquid paraffin) and petroleum products (phenol, benzene, toluene, xylene, kerosene, diesel, lubricating oil and crude oil), 1% (v/v) of each carbon source was added to the culture

separately and in the case of polycyclic aromatic hydrocarbons (pyrene, anthracene, naphthalene, fluorene and phenathrene), the culture medium was supplemented with 50 µg of each type of carbon source [298].

3.4.3 Taxonomic identification

3.4.3.1 Biochemical and morphological tests

The taxonomic identification of the selected bacterial isolates was carried out using the standard morphological, physiological and biochemical tests as described by Cappuccino and Sherman[341]. The bacterial isolates were taxonomically identified up to genus level with the help of standard procedures described in Bergey's Manual of Systematic Bacteriology[342].

3.4.3.2 16S rRNA gene sequencing and phylogenetic analysis

The 16s rRNA gene sequences of the selected bacterial isolates were sequenced at National Centre for cell science (NCCS), Pune University, Pune, India. The BLAST search was performed for the 16s rRNA gene sequences of the selected bacterial isolates at the NCBI database using nucleotide BLAST. Subsequently, the 16s rRNA gene sequences of various bacterial strains showing maximum similarity from the BLAST result were taken for phylogenetic analysis using Mega 5.1 version and nucleotide frequency count analysis using CLC Main work bench software.

3.5 Detection and quantification of biosurfactant

3.5.1 Cetyl trimethyl ammonium bromide (CTAB) agar test

The bacterial strains were spread over the mineral salt medium containing CTAB (0.2 $g.l^{-1}$), methylene blue (5 $mg.l^{-1}$), agar (16 $g.l^{-1}$) as solidifying agent and 0.1% (v/v) n-hexadecane as sole source of carbon. Biosurfactant production could be detected by the formation of dark blue halos around the bacterial colonies[270].

3.5.2 Blood hemolysis agar test

Hemolytic activity was determined on nutrient blood agar media plates containing mammalian blood (goat blood). With the help of a sterile cork borer a single well of 6.0 mm diameter was made at the center of blood agar plate. An aliquot of 100 µl of cell free culture supernatant was inoculated into the well and incubated at 37°C for 48 h. Hemolytic activity indicates complete lysis of red blood cells surrounding the well containing bacterial culture supernatant. The diameter of the clear zone depends on the concentration of the biosurfactant produced by the bacteria[270, 272].

3.5.3 Orcinol assay

An aliquot of 0.5 ml of cell free culture supernatant was extracted twice with 1 ml of diethyl ether. The ether fraction was collected, allowed to dry under fume hood and dissolved in 0.5 ml H_2O. Sample was further diluted to 10^{-1} dilution in 0.19% orcinol solution prepared in 53% (v/v) of concentrated H_2SO_4. The sample was then placed in boiling water for 30 min, cooled at room temperature for 15 min, and the absorbance at 421 nm was measured. Glycolipid concentration was calculated from a standard curve prepared with L-rhamnose and expressed as rhamnolipid values by multiplying rhamnose values with a coefficient of 3.4 obtained from the correlation of pure rhamnolipid/rhamnose[343].

3.6 Isolation and purification of biosurfactant

3.6.1 Isolation of biosurfactant

The culture supernatant was first centrifuged at 8,000 rpm for 20 min at 4 °C to remove the bacterial cells. The cell free culture supernatant was then acidified to pH 2 with 6 N HCl and allowed to stand overnight at 4°C to precipitate the biosurfactant. The precipitate was harvested by centrifugation at 12,000 rpm for 15 min at 4°C. The recovered precipitate was extracted thrice with ethyl acetate at room temperature. The organic phase was collected in a round-bottom flask and connected to a rotary evaporator (Eyela, CCAS-1110, Rikakikai Co. Ltd., Tokyo) to remove the solvent.

The process yielded a viscous honey-colored residue. The residue was then washed twice with n-hexane to remove any residual n-hexadecane. Finally, the yellowish product was dissolved in ethyl acetate, filtered, and concentrated using a rotary evaporator[53, 344]. It was weighted and expressed as $g.l^{-1}$.

3.6.2 Purification of biosurfactant

Purification of the isolated crude biosurfactants was carried out using thin layer chromatography (TLC) with slight modifications. Briefly, aqueous slurry of silica gel 60 was prepared and was used for making preparative thin layer chromatography (TLC) glass plates (20×20 cm). 2 g of crude biosurfactant was dissolved in 4 ml chloroform and loaded onto the activated TLC plates with the help of glass capillary. The purification was carried out using chloroform: methanol: H_2O (65:15:2, v/v/v) as mobile phase. After the competitions of the separation process the plates were exposed to the iodine vapour to make visible the separated fraction. The separated fractions were then collected by scrapping out the separated fractions from the preparative plates. Finally the separated compound was recovered from the silica gel by washing the collected TLC fractions with chloroform and methanol (2:1, v/v) through a glass column. During the purification steps analytical TLC (using chloroform: methanol: H_2O, 65:15:2 v/v/v as mobile phase) and the reduction in the surface tension of water by the separated TLC fraction was carried out to check the purity and surface activity of the isolated fractions.

3.7 Biochemical characterization of biosurfactant

3.7.1 Quantification of total protein content

The total protein content in the isolated biosurfactant samples was estimated by standard Folin-Lowry method[35] using bovine serum albumin (BSA) as standard. The total protein content of unknown samples was calculated from the standard curve obtained by plotting optical density (OD_{660}) versus concentration of BSA (1 $mg.ml^{-1}$).

3.7.2 Estimation of total carbohydrate content

The total carbohydrate content in the isolated biosurfactant samples was quantified by standard phenol-sulphuric acid method[346] using D-glucose as standard. The carbohydrate content of the unknown samples was calculated from the standard curve obtained by plotting optical density (OD_{490}) versus concentration of D-glucose ($0.1\ mg.ml^{-1}$).

3.7.3 Estimation of total lipid content

The total lipid content in the isolated biosurfactant samples was estimated gravimetrically using standard protocol of Folch *et al.*[347]. Briefly, 50µg of isolated biosurfactant was homogenized with a chloroform-methanol mixture (2:1, v/v). The crude extract was then mixed thoroughly with 1.0 ml of water and was allowed to separate into two distinct phases. The upper aqueous phase was removed with the help of a micropipette. Finally, the lower organic phase was collected, dried and the total lipid content was determined gravimetrically.

3.8 Physical characterization of biosurfactant

3.8.1 Determination of reduction in surface and interfacial tension

An aliquot of 5 ml culture broth was collected at a regular interval of 12 h for a period of 168 h to determine the reduction in surface tension. The culture broth of each bacterial strain was centrifuged at 8,000 rpm for 15 min at 4°C. The surface tension of the cell free culture supernatant was measured by using a digitalized tensiometer (Krüss Tensiometre K9 ET/25) at 25±1°C. Before using the platinum ring, it was thoroughly washed three times with distilled water followed by acetone and then allowed to dry at room temperature[59]. The interfacial tension was measured against diesel. Each experiment was repeated thrice for taking the average value.

3.8.2 Critical micelle concentration (CMC) and critical micelle dilution (CMD)

Critical micelle concentration (CMC) of the isolated biosurfactant samples was determined by measuring the surface tension of the aqueous biosurfactant solutions at

different dilution concentrations upto the constant value of surface tension. Further, the surface tension of aqueous biosurfactant samples was determined at their critical micelle dilutions of 10 and 100 times i.e., CMD^{-1} and CMD^{-2}, respectively. For the calibration of the instrument the surface tension of the pure water was measured before each set of experiment. The platinum ring was washed thrice with distilled water followed by acetone and then allowed to dry at room temperature. Each measurement was repeated thrice and average value was taken[59, 348].

3.8.3 Foaming index

An aliquot of 20 ml of the biosurfactant solution ($1g.l^{-1}$) was transferred to a glass measuring cylinder of 50 ml volume and compressed N_2 gas was passed through the solution at a flow rate of 0.5 $l.min^{-1}$ for 2 min[92]. The foaming index of the biosurfactant was calculated after 24 h by using the following equation:

$$\text{Foaming index } (F_{24}\%) = \left(\frac{\text{Height of the foam}}{\text{Total height of the liquid+foam}}\right) \times 100$$

3.8.4 Emulsification activity

The emulsification index was measured using the standard method as described by Cooper and Goldenberg[266]. Different hydrocarbons were used for testing the emulsification efficiency. 3 ml of the test hydrocarbon was added to 2 ml of cell free culture supernatant in a glass test tube and homogenized in a vortex at high speed for 2 min. The resulting mixture was kept at room temperature for 24 h and emulsification index (E_{24}) was calculated by the following formula:

$$\text{Emulsification index } (E_{24}\%) = \frac{\text{Height of the emulsion layer}}{\text{total height of the mixture}} \times 100$$

3.8.5 Determination of biosurfactant stability

Stability studies were carried out using cell free culture supernatant obtained after centrifuging the bacterial cultures at 8,000 rpm for 15 min at 4°C. Aliquots of 10 ml cell free culture supernatant were subjected to 4°C, 25°C, 37°C, 50°C, 75 °C, 100°C

for 60 and 121°C for 30 min and cooled to room temperature after which the surface tension of the culture supernatant was measured at normal concentration, at CMD^{-1} and CMD^{-2} and emulsification index against diesel was measured. To study the pH stability of the biosurfactant, the pH of the culture supernatant was adjusted to different pH values (2–11) and the surface tension of the treated culture supernatant was measured at normal concentration, at CMD^{-1} and CMD^{-2} and emulsification index against diesel was measured. Similarly, the effect of NaCl concentration (1–5 %) (w/v) on the surface tension of the culture supernatant at normal concentration, CMD^{-1} and $CMD^{-2}$and emulsification activity was determined. To investigate the effect of metal ions on the surface activity, the culture supernatants of the bacterial strains were mixed with different metal ions of K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Al^{3+} (20,000 $mg.l^{-1}$) separately. Changes in the surface tension of the culture supernatant at normal concentration, CMD^{-1} and CMD^{-2} for each case were measured. In this study, the synthetic surfactant sodium dodecyl sulphate (SDS) was used as a standard. The assays were carried out in triplicates.

3.8.6 Determination of cell surface hydrophobicity or BATH assay

The cell surface hydrophobicity of the bacterial cells was measured by using the standard procedure of Rosenberg *et al.*[267]. Briefly, bacterial strains were grown on n-hexadecane (2% v/v) and glucose (2% w/v) separately in mineral salt medium (MSM). Bacterial cells were harvested from culture medium by centrifugation at 8,000 rpm for 15 min at 4°C and washed twice in PUM buffer (Appendix I) and suspended in the same buffer to give an optical density of approximately 0.5–0.6 at 600 nm. The cell suspension (2.0 ml) with 0.5 ml of test hydrocarbon was vortexed in a test tube vigorously for 3 min and allowed to settle down for 15 min at room temperature. The bottom aqueous phase was carefully removed and the optical density was measured at 600 nm in a spectrophotometer. The cell surface hydrophobicity was expressed as the percentage of adherence to hydrocarbon and calculated by the following formula:

$$\left(1-\frac{\text{Optical density of the aqueous phase}}{\text{Optical density of the initial cell suspension}}\right)\times 100$$

3.9 Optimization of culture conditions for biosurfactant production

3.9.1 Effect of different carbon sources on biosurfactant production

Different carbon sources on the basis of their increasing complexity and hydrophobicity, including glucose, glycerol, vegetable oil (soyabean oil) and petroleum hydrocarbons (n-hexadecane, octadecane, diesel and crude oil) were screened to determine their effectiveness in biosurfactant production. After the completion of the fermentation, various parameters such as reduction in the surface tension, yield of biosurfactant and dry biomass were assessed.

Concentrations of the efficient carbon source 1.0, 1.5, 2.0, 2.5 and 3 % (w/v) were used to determine the optimum level of biosurfactant production by the bacterial strains. After the completion of the fermentation process, various parameters such as reduction in the surface tension, yield of biosurfactant and dry biomass were assessed.

3.9.2 Role of nitrogen sources on biosurfactant production

Different organic and inorganic nitrogen sources were tested for their role on biosurfactant production which included beef extract, yeast extract, peptone, ammonium dihydrogen orthophosphate ($NH_4H_2PO_4$), urea (H_2NCONH_2), ammonium chloride (NH_4Cl), potassium nitrate (KNO_3), ammonium nitrate (NH_4NO_3) and ammonium sulphate $(NH_4)_2SO_4$. The nitrogen sources were used in different concentrations and combinations to determine their effect on the production of biosurfactant. After the completion of fermentation, various parameters such as reduction in the surface tension, yield of biosurfactant and dry biomass were determined.

3.9.3 Effect of macro and micro-nutrients on biosurfactant production

Various concentrations of macronutrients like Na and K were added in the culture medium in the form of KH_2PO_4 and Na_2HPO_4 to determine their influence on the production of biosurfactant. Similarly, various concentrations of micronutrients were prepared and added in the culture media. The micronutrient solution included

$FeSO_4 \cdot 7H_2O$ (1000 $\mu g.l^{-1}$), $CuSO_4 \cdot 5H_2O$ (50$\mu g.l^{-1}$), H_3BO_3 (10 $\mu g.l^{-1}$); $MnSO_4$ (10 $\mu g.l^{-1}$), $ZnSO_4 \cdot 7H_2O$ (70 $\mu g.l^{-1}$) and MoO_3 (10 $\mu g.l^{-1}$) (Appendix I). A control experiment was run simultaneously without supplying any of the micronutrients. With the completion of the fermentation process, various parameters such as reduction in the surface tension, yield of biosurfactant and dry biomass were determined.

3.9.4 Effect of temperature on biosurfactant production

To determine the effect of temperature on biosurfactant production, bacterial cultures were incubated at a temperature range from 30 to 42 °C with shaking at 180 rpm. On completion of fermentation process, various parameters such as reduction in the surface tension, yield of biosurfactant and dry biomass were determined.

3.9.5 Effect of pH on biosurfactant production

To determine the effect of pH on the production of biosurfactants by the bacterial strains, the pH of the media were adjusted between pH 6.5-7.2 and the production of biosurfactants was observed with the assessment of parameters like reduction in the surface tension, yield of biosurfactant and dry biomass were determined.

3.9.6 Effect of agitation (shaking) on biosurfactant production

Erlenmeyer flasks of 250 ml volume containing 100 ml of mineral salt medium inoculated with the bacterial strains were used for the production of biosurfactant. The conical flasks were incubated in an orbital incubator shaker with the agitation rate set at 100, 120, 150, 180, 200 and 220 rpm to determine the impact of agitation on biosurfactant production. A flask without any agitation was maintained and considered to be at 0 rpm for mixing as the control. After the completion of the fermentation, parameters like reduction in the surface tension, yield of biosurfactant and dry biomass were determined.

3.9.7 Screening of low cost renewable carbon substrate

Different carbon substrates such as agro-industrial wastes, non-edible vegetable oils, kitchen wastes and petroleum refinery wastes were screened for their role in the production of biosurfactant. Agro-industrial wastes containing de-oiled mustard seed cakes, waste raw glycerol of biodiesel, waste residual molasses and sugarcane bagasse. Jatropa seed oil (*Jatropha curcas*), Nahor seed oil (*Mesua ferrea*), Castor seed oil (*Ricinus communis*), Pongamia seed oil (*Pongamia glabra*) and Sesame seed oil (*Sesamum indicum*) were selected as non-edible vegetable oils for the experiment. Waste residual kitchen oil and oily sludge produced by the petroleum refinery were also used for the purpose. With the completion of the fermentation process, parameters like reduction in surface tension, yield of biosurfactant and dry biomass were determined for each carbon substrate.

3.10 Chemical characterization of biosurfactant

3.10.1 Thin layer chromatography (TLC)

The isolated biosurfactant samples were dissolved in chloroform to result a concentration of $0.3 g.l^{-1}$. An aliquot of 100 µl of the biosurfactant solution was applied on 20×20 cm TLC plates (TLC Silica gel 60 F254, Merck, India). The plates were developed in a solvent system consisting of chloroform: methanol: acetic acid (65:10:2, v/v/v). For detecting the carbohydrate components in the separated fraction, the TLC plates were sprayed with anthrone reagent prepared in concentrated H_2SO_4. For the detection of lipid components, the TLC plates were exposed to the iodine fume. Presence of protein or amino acid components in the separated fractions was determined by spraying the TLC plates with ninhydrin solution[279, 349].

3.10.2 Fourier transform infrared spectroscopy (FTIR)

For elucidating the chemical bonds or the functional groups present, the isolated biosurfactant samples were subjected to FTIR analysis. The lyophilized biosurfactant sample of 1.0 mg was ground with 1.0 g of KBr and pressed with 7,500 kg for 30 s to result in a translucent KBr pellet. The IR spectra of the samples were

recorded on a Nicolos Impact I 410, FTIR system, USA, with a spectral resolution and wave number accuracy of 4 and 0.01 cm^{-1} respectively and 32 scans with correlation for atmospheric CO_2[350].

3.10.3 Liquid chromatography and mass spectroscopy (LC-MS)

Rhamnolipid mixtures were separated from the isolated biosurfactant samples and identified by LC-MS. Biosurfactants isolated from the bacterial strains OBP1 and OBP2 were characterized using a UPLC-ESI-MS (Waters) while the biosurfactants from the bacterial strains OBP3 and OBP4 were characterized using LC-MS-MS (Agilent 6520). Samples were prepared by diluting with methanol at a concentration of 10 mg l^{-1}; 100 µl of the same was injected into a C8 WP-300 (5 µm) 150×4.6 mm column. The LC flow rate was 1.0 $ml.min^{-1}$. For the mobile phase an acetonitrile-water gradient was used; starting with 30% of acetonitrile for 4 min, followed by 30-100% acetonitrile for 40 min then standby for 5 min and return to the initial condition. MS was performed with a single quadrupole mass spectrometer, equipped with a pneumatically assisted electrospray (ES) source and negative ion mode was used. The capillary was held at a potential of −3.5 KV and extraction voltage at −75 V. Full scan data were obtained by scanning from m/z 100 to 750 in the centroid mode using scan duration of 2.0 s and an inter-scan time of 0.2 s[84].

3.10.4 Thermogravimetric analysis (TGA)

The thermal stability and decomposition profile of the biosurfactant samples were determined by using a thermogravimetric analyzer (Shimadzu TGA-50, Japan) operated at a heating rate of $10°C.min^{-1}$ under nitrogen flow rate of 30 $ml.min^{-1}$. The biosurfactant samples were heated from 30 to 600°C

3.10.5 Differential scanning calorimetry (DSC)

Differential Scanning Calorimetry (DSC) was used to assess the thermal properties of biosurfactant samples. 10 mg of dried biosurfactant was weighed into the aluminum pans and sealed hermitically prior to analysis. Sample was then heated at 10 $°C.min^{-1}$ under a dry nitrogen purge (50 $ml.min^{-1}$) from 30°C–400°C. Analysis of data

for obtaining the onset peak and melting temperature as well as enthalpy was carried out using the universal V3.5 BTA instrument software. The melting temperature (T_m) was determined from the differential scanning calorimetry (DSC) endotherms.

3.11 Application of bacterial strains and biosurfactants in bioremediation

3.11.1 Reduction of viscosity by the biosurfactant

The culture broths of the bacterial strains after 4 weeks of incubation with crude oil were extracted thrice with the equal volume of dichloromethane, dried over anhydrous sodium sulphate, filtered and concentrated in vaccum. Viscosity measurement was performed on Ostwald viscometers, which allows the determination of viscosity of the control/treated crude oil. All determinations were carried out at 25 °C using a concentration of 1 $mg.ml^{-1}$ of the extracted crude oil dissolved in hexane.

3.11.2 Solubilization of polyaromatic hydrocarbon (PAH) by biosurfactants

The solubilization assay was carried out as described by Barkay *et al.*[297]. Three polyaromatic hydrocarbons (PAH) anthracene, phenanthrene and naphthalene were selected. Stock solution of all the three PAHs (6 $mg.ml^{-1}$) were prepared in hexane. From the stock, 1µl was distributed in glass test tubes so as to achieve 0.6 µg PAH in each. The test tubes were kept open in a fume hood to remove the solvent. This was followed by addition of 3.0 ml of assay buffer (20 mM Tris-HCl, pH 7.0) and 1.0 ml of biosurfactant solution in the increasing concentrations (100-800 $\mu g.l^{-1}$) were added to the above test tubes. Test tubes were capped and incubated overnight at 30°Cin an orbital incubator shaker at 150 rpm in dark. The solutions in the test tubes were filtered through membrane filter (pore size 1.2 µm) and 2.0 ml of the filtrate was extracted with equal volume of hexane. The resulting mixture of filtrate and hexadecane was centrifuged at 10,000 rpm for 10 min to separate the aqueous and hexane phase. PAHs in the hexane extracts were measured spectrophotometrically at 250, 253, and 273nm[297]. Test tubes containing the assay buffer with PAHs but without biosurfactant served as the positive control while test tubes with assay buffer and

biosurfactant, but without PAHs were used as blank. All experiments were performed in triplicates.

3.11.3 Degradation of petroleum products

3.11.3.1 Assay on residual petroleum

Verification experiment of hydrocarbon degradation was carried out on the residual petroleum products by using combined solvent extraction and column chromatographic techniques as described by Queiroga *et al.*[351]. The residual crude oil were separated from the culture broth with the help of glass separating funnel and were recovered by washing twice with 30 ml of hexane, dried over anhydrous sodium sulfate, filtered and concentrated in vacuum. Residual petroleum product 100 mg was fractionated on a chromatographic column (1.2 mm×30 cm) packed with 6.0 g silica gel (60-80 mesh size) and saturated with hexane for overnight period. The aliphatic hydrocarbons were eluted with 30 ml of hexane, aromatic hydrocarbons with 30 ml of hexane-diethyl ether mixture solution (9:1, v/v) and resins and asphaltenes with 30 ml of chloroform-methanol-water mixture solution (21:8.4:0.6, v/v/v). All the three extracts were dried at room temperature over anhydrous sodium sulphate and concentrated in vacuum. The residual hydrocarbon components were determined by measuring the weight of the dry extract. The pattern of biodegradation and abiotic loss were evaluated by comparing their weights against the control samples as well as the fresh weight of petroleum products.

3.11.3.2 Gas chromatographic (GC) analysis of degraded petroleum products

The saturated hydrocarbons were analyzed using a PerkinElmer gas chromatograph-mass spectrometer (GC-MS; model CP-3800 gas chromatograph and Saturn 2200 mass spectrometer, Varian Technologies Japan, Inc.) equipped with a capillary column (TC-1, length 30 m, ID 0.25 mm, film thickness 0.1 lm) obtained from GL Science. For crude oil analysis, column temperature was first held at 50 °C for 5 min, and then raised to 280°C. All analyses were carried out with the split ratio

of 20:1. Helium was used as the carrier gas with a flow rate of 0.8 ml.min^{-1}. Injector temperature was set at 250 °C.

3.11.3 Release of oil from sand pack column

The suitability of the isolated biosurfactant in microbial enhanced oil recovery (MEOR) was evaluated using the sand pack technique as described by Suthar *et al.*[100]. Glass column of 20 mm × 25 mm × 85 mm dimensions with a sieve (100 µm pore size) was packed with 150 g of acid washed sand of 140µm particle size. The column was then saturated with 50 ml of crude oil supplied by ONGC, Assam, and India having a density of 0.86 g.cc^{-1} at 15°C. A volume of 50 ml aqueous biosurfactant solution was pumped into the column and the amount of oil released was measured. Similarly, cell free culture supernatant of the bacterial strain was also used the recovery process. To determine the influence of temperature on the recovery process, the experiment was carried out at room temperature, 50, 70 and 90°C. Sand pack column saturated with crude oil without the addition of aqueous biosurfactant was kept as control. The mean and standard deviation of triplicates for each treatment were calculated.

3.11.4 Soil washing experiment

Dried acid washed sand was mixed with crude oil (10%, w/w) and left at room temperature for 12 days. The crude oil contaminated sand samples weighing 20 g was transferred to each of 250 ml Erlenmeyer flasks containing 100 ml of aqueous biosurfactant solution at various concentrations (0.001, 0.005, 0.007, 0.01 and 0.1 % w/v) and kept at 150 rpm for 24 h at room temperature. Similarly, the cell free culture supernatants of the bacterial strains were used in place of biosurfactant solution. The contaminated sand samples were separated, dried and washed twice with dichloromethane. The solvent part was removed and the residual oil was determined gravimetrically. The percentage of oil removed was calculated using the equation:

$$\text{Crude oil removed (\%)} = \frac{(Oi\text{-}Or)}{Oi} \times 100\%$$

Where, Oi is the initial oil in the sand sample (g) before washing and Or is the oil remaining in the sand sample (g) after washing[306]. The mean and standard deviation of triplicates for each treatment were calculated.

3.11.5 Separation of residual crude oil from petroleum sludge

The petroleum sludge was mixed with acid washed sterile sand to achieve the sludge concentration between 1-9% (w/w). The sludge samples weighing 20 g of the five concentrations were transferred to 250 ml Erlenmeyer flasks containing 100 ml of aqueous biosurfactant solution (0.001, 0.01 and 0.1 % w/v) separately and kept at constant shaking (100-180 rpm) between 3-18 days at room temperature. After the treatment, the culture flasks were allowed to settle for few hours and the treated sludge samples were recovered. The total petroleum hydrocarbon (TPH) of the sludge sample after treatment was estimated and expressed as residual TPH. Flask receiving sludge sample with only water was kept as control[113].

3.12 Biological activity of biosurfactants

3.12.1 Effect on seed germinations

The phytotoxicity of the isolated biosurfactant samples was evaluated in static test by seed germination and root elongation as described by Tiquia *et al.*[353]. The isolated biosurfactant samples were dissolved in water at three different concentrations i.e. below CMC, at CMC and above CMC. The bioassay was determined in 50 ml glass Petri dish containing Whattman No.1 filter paper. Mung bean (*Vigna radiate*) as dicotyledonous and rice (*Oryza sativa*) as monocotyledonous plant were selected for the bioassay. The seeds were pre-treated with 0.1% (w/v) $HgCl_2$ solution and 10 seeds were transferred into each Petri dish which was inoculated with 5 ml of the test biosurfactant solution at 25±2°C. After 3-7 days of incubation in dark, the germination of seed, root elongation (≥5mm) and germination index (GI) were determined as follows:

$$\text{Relative seed germination (\%)} = \left(\frac{\text{numbers of seed germinate in the extract}}{\text{number seeds germinate in the control}}\right) \times 100$$

$$\text{Relative root length (\%)} = \left(\frac{\text{mean root length in the extract}}{\text{mean root length in the extract}}\right) \times 100$$

$$\text{Growth Index (GI) (\%)} = \left(\frac{(\%\text{ of seed germination}) \times (\%\text{ of root growth})}{100\%}\right)$$

Petri dishes containing the seeds and water were kept as controls. The mean and standard deviation of triplicate samples from each concentration were calculated.

3.12.2 For insect larvicidal activity

The mosquito larvicidal activity of the isolated biosurfactant samples was evaluated against the third instar larvae of *Aedes albopictus* using the standard protocol approved by World Health Organization (WHO)[354]. The larvae used in the bioassay were obtained from the mosquito rearing facility section of the Defense Research Laboratory, Tezpur, India. A total of 20 numbers of larvae per replicate were transferred to 250 ml glass beakers containing 200 ml of distilled water. The biosurfactant samples were dissolved in ethanol (≥ 99%, Merck) and added to the beakers at concentrations ranging from 100-1500 $mg.l^{-1}$. For each concentration three replicates were made. The beakers with larvae and water were kept as negative control. The same procedure was also used for determining the larvicidal property of cell free culture supernatant of the *P. aeruginosa* strains. The numbers of larvae killed were counted after 24 h, followed by counting the number of live larvae in each beaker. The mean and standard deviation of triplicates for each treatment were calculated.

3.12.3 For antimicrobial activity

The bacterial and fungal strains used in the research work were obtained from the Department of Molecular biology and Biotechnology, Tezpur, Assam, India and Department of Plant Pathology, Assam Agricultural University, Jorhat, Assam, India, respectively. The studied bacterial strains were *Escherichia coli* (MTCC 40), *Escherichia coli* (MG1655), *Bacillus subtilis* (MTCC 121), *Bacillus subtilis* (MTCC 441), *Staphylococcus aureus* (MTCC 737), *Staphylococcus aureus* (MTCC 3160),

Klebsiella pneumoniae (MTCC 618), *Pseudomonas aeruginosa* (MTCC 7815) and *Pseudomonas diminuta* (AUs).The fungal strains used in the investigation include *Candida albicans* (MTCC 227), *Fusarium oxysporium* (MTCC 284), *Aspergillus niger* (AUs), *Colletotrichum capaci* (AUs) and *Alternaria solani* (AUs). The antibacterial activity of biosurfactant was evaluated by well diffusion method[355]. Briefly, 200 µl of the log phase culture of the test microbes (10^7–10^8 cells as per McFarland standard) were seeded on the surface of the Mueller Hinton agar medium using a micropipette and spread over the medium uniformly using a sterile glass spreader. With the help of a sterile cork borer wells having 6 mm diameter each were made on Mueller Hinton agar (MHA) plates. The biosurfactant samples were dissolved in sterilized DMSO (10% v/v) and introduced into one of the wells. As the presence of 10% DMSO (v/v) had no detectable effect on bacterial growth, compounds at concentrations of 10 $mg.ml^{-1}$ were prepared in 10% DMSO (v/v). Streptomycin sulphate (1 $mg.ml^{-1}$) was taken as a positive control and 10% DMSO (v/v) as negative one. After the incubation of the plates at 37°C for overnight period, microbial growth was determined by measuring the diameter of inhibition zone using a transparent metric ruler. For antifungal investigation, the fungal strains ($0.5–2.5\times10^6.ml^{-1}$) were grown on Sabouraud dextrose agar (SDA). Biosurfactant solution was introduced into the wells in the similar manner as described for bacteria. After incubation for 36 h at 25°C, the growth was determined by measuring the diameter of inhibition zone. Nystatin ($1mg.ml^{-1}$) was used as positive while 10% DMSO (v/v) was kept as negative control.

The microbroth dilution method was performed to determine the minimum inhibitory concentration (MIC). The dissolved rhamnolipid solution was diluted to a series of tenfold in Luria Bertani (LB) broth, seeded in a 96-well culture plate, and then inoculated with a fresh bacterial inoculum. Inoculated microplates were incubated at 37 °C for 24 h. In case of fungi, the rhamnolipid solution was diluted in Sabouraud dextrose (SD) broth and the plates were incubated at 25°C for 24 h. Each biosurfactant concentration was tested in duplicates for each organism. Two wells containing suspension test organism with no drug (growth control) and 2 wells containing only

media (background control) were included in the microtitre plate. The viability of the treated cells was determined by MTT ·(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium) assay and the absorbance was measured at 570 nm and 405 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California) for bacterial and fungal strains respectively. The MIC was determined as the lowest concentration of biosurfactant required to inhibit the growth of each organism. The mean and standard deviation of triplicates for each treatment were calculated.

3.12.4 Determination of chemo-attractant property of biosurfactant

The chemotaxis property of the biosurfactant samples was examined by using Chemical Gradient Motility Agar (CGMA) method as described by Garg and Kanitkar[356]. Motility agar medium (MAM) containing 0.7% agar was used for the assay (Appendix I). After the solidification of the medium, three long rectangular wells were made. The well present on the right and left side were loaded with rhamnolipid (0.5%, w/v) and streptomycin (1mg.ml^{-1}), respectively and kept undisturbed for 60 min at room temperature. The well present at the center was loaded with 100 µl of the fresh overnight grown test bacterial culture and incubated at 37 °C for 24 h. 5% (w/v) glucose was used as a positive control because it has an excellent chemo-attractant property. The biosurfactant (rhamnolipid) used in the present investigation was isolated from *P. aeruginosa* OBP1 because this strain showed the highest antimicrobial property among the four selected bacterial strains of *P. aeruginosa*.

3.12.5 Assessment of cell cytotoxicity of the isolated biosurfactants

Primary mouse connective tissue cell line (L929) was obtained from the National Centre for Cell Science (Pune, India). The cells were maintained in Dulbecco's minimum essential medium (DMEM) containing 2 mM.l^{-1} glutamine, 1.5 g.l^{-1} sodium bicarbonate ($NaHCO_3$), 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum and 1% antibiotic antimycotic solution (1000 U.ml^{-1} penicillin G, 10 mg.ml^{-1} streptomycin sulfate, 5

$mg.ml^{-1}$ gentamicin and 25 $\mu g.ml^{-1}$ amphotericin B). Cells were maintained at 37°C in a saturated-humidity atmosphere containing air 95% /5% CO_2.

A simple, non-radioactive and colorimetric MTT (3-[4, 5-dimethylthia-zole-2-yl]-2, 5-diphenyl tetrazolium bromide) dye conversion assay was used[357] to quantitatively measure the cell toxicity, For MTT assay viability studies, mouse fibroblast cell line L929 was cultured at a density of 1×10^4 cells per well in a 100 µl volume of cell culture medium (DMEM supplemented with 10% fetal bovine serum) in a 96-well cell culture plate. After 24 h, cultured cells were treated with a series of different biosurfactant concentrations (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 $\mu g.ml^{-1}$) of OBP1, OBP2, OBP3 and OBP4 dispersed in 100 µl per well DMEM without serum and phenol red, and incubated further for 4 hours with MTT dye. After the incubation, an aliquot of 100 µl of dimethylsulfoxide (DMSO) was added to each well to dissolve blue formazan precipitate, and absorbance was measured at 570 nm using a microtitre plate reader (Bio-Rad Model 680; Hercules, California). The cell viability was expressed as a percentage of the control by the following equation:

$$\text{Viability } \% = \frac{N_t}{N_c} \times 100$$

Where, N_t is the absorbance of the compound treated cells and N_c is the absorbance of the untreated cells. All experiments were performed in quadruplets.

3.12.6 Acute dermal toxicity test

The primary skin irritation potential of isolated biosurfactants was studied on rabbits. The isolated biosurfactant samples were dissolved in ethanol (≥99%, Merck) at a concentration of $1mg.ml^{-1}$and were evenly applied to the shaved skin of the rabbits under a patch. After the treatment of 24 h, the patch was removed and the compound applied site was wiped off with distilled water to remove the residual test substance. Treated skin sites of the animals were examined for erythema, edema and eschar at 72 h after the application of the test compound. The blood samples were also collected from the test animals at 72 h to determine the biochemical changes in the

blood after the application of the test compound[358, 359]. All experiments were performed in quadruplets.

3.12.7 Biodegradation of biosurfactant

The biodegradation assay of the isolated biosurfactants was carried out using the procedure of by Zeng *et al.*[360]. The experiment was carried out in 250 ml Erlenmeyer flasks containing 100 ml of culture media consisting of NH_4Cl (1.0 g), K_2HPO_4 (1.0 g), KH_2PO_4 (1.0 g), $MgSO_4.7H_2O$ (0.05 g), $CaCl_2$ (0.02 g), $FeSO_4.7H_2O$ (250 μg), glucose (1.0 g) and biosurfactant sample (1.0 g). The pH of the culture medium was adjusted to 7.2 and sterilized by autoclaving at 121°C for 15 min. Gram-negative bacteria *Pseudomonas aeruginosa* (MTCC 8165) and Gram-positive bacteria *Bacillus circulans* (MTCC8167) were selected for the biodegradation assay. Pure cultures of the bacterial strains were prepared using Luria Bertani (LB) broth and incubated overnight in an orbital incubator shaker at 37 °C with 180 rpm. An aliquot of 100 μl of overnight grown culture broth containing 1×10^8 ml^{-1} cells (as calculated from McFarland turbidity method) was inoculated to the 250 ml volume Erlenmeyer flask containing 100 ml of mineral salt medium (MSM). The degradation experiment was carried out at 37°C in an orbital incubator shaker at 180 rpm. For analytical use, 2 ml of the culture broth was removed from culture flask at time intervals of 24 h. After centrifuged at 8,000 rpm for 15 min, the cell free culture supernatant was separated and used for the determination of rhamnolipid concentration by using orcinol as described by Chandrasekaran and Bemiller[343].

The top surface soil was collected from the Departmental garden and distributed equally in several earthen pots. Rhamnolipid solution was added into the earthen pots and the ratio of soil and rhamnolipid was adjusted to 800:1 (w:w). Water was added to earthen pots and the water content was adjusted to about 60%. The earthen pots were kept open air in a shed. The water content in the earthen pots was determined at an interval of 24 h and was maintained between 50-60% with fresh sprinkling. For analytical use, the soil sample was mixed up with water at the ratio of 1:20 and incubated on an orbital incubator shaker at 100 rpm for 30 min until the

suspension was formed. The residual suspension was centrifuged at 8,000 rpm for 15 min to recover the supernatant and used for the determination of rhamnolipid content using the standard procedure as described above. Each experiment was repeated thrice to determine the mean and standard deviation.

3.13 Application of biosurfactant in nanotechnology

3.13.1a Biosurfactant assisted synthesis of iron oxide nanocrystals (IONRLs)

IONRLs were prepared using the protocol as described by Konwarh *et al.*[361] with slight modification. Briefly, 0.1 M $FeCl_2 \cdot 4H_2O$ (≥99.0%, BDH) solution in 50:50 (v/v) Millipore water : methanol containing 2% (w/v) biosurfactant of bacterial strain OBP1 was subjected to sonication for 3 min with 60% amplitude and 0.5 cycles instead of constant magnetic stirring, as reported previously. 1.0 M NaOH (Merck) solution was added drop wise to the above mixture with constant stirring at room temperature till pH 8.4 was attained. Addition of 3% (v/v) H_2O_2 (Ranbaxy) to the resultant dark green suspension yielded a black dispersion that was attracted by a permanent magnet. The mixture was then subjected to sonication at the same parameters mentioned previously. After separating the BIONPs by centrifugal decantation, they were washed with distilled water followed by washing with acetone and re-dispersion in water

3.13.1b Characterization of IONRLs

Water-dispersed iron oxide nanoparticles were casted on carbon-coated copper grids and their morphology was observed using a JEOL, JEM 2100 transmission electron microscope (TEM) at an operating voltage of 200 KV.

3.13.2a Biosurfactant assisted synthesis of silver nanoparticles (SNP)

The rhamnolipid (RL) produced by *P. aeruginosa* OBP1 was dispersed in distilled water at its CMC concentration (45.0 $mg.l^{-1}$). The SNP was synthesized in the RL dispersed distilled water following the method as described by Phukon *et al.*[362]. Briefly, 30 ml of RL (45.0 $mg.l^{-1}$) solution was taken in a 250 ml Erlenmeyer flask

and sodium borohydride ($NaBH_4$) was dissolved into the solution to get a concentration of 0.002 M. The Erlenmeyer flask was placed into an ice bath and was allowed to cool for 20 min. The assembly was stirred gently using a magnetic stirrer. Now, 10 ml 0.001 M $AgNO_3$ solution was added drop wise, about 1 drop per second, until the whole amount was used up. After the addition of the $AgNO_3$, the solution turned light yellow in colour and the silver nanoparticles (SNP) were synthesized in the RL solution.

3.13.2b Characterization of SNP/SNP-RL

The morphology and size of the particles were investigated by scanning electron microscopy (SEM) using model no. JSM-6390LV of JEOL, Japan. The samples were directly observed under SEM without platinum or gold coating. Transmission electron microscopy (TEM) analysis was done using a 200 KV system of JEOL JEM 2100, Japan.

3.13.3c Possible protection of silver nanoparticles against salt using rhamnolipid

The SNPRL colloid was subjected to UV–visible scan (Thermo Scientific, UV-10 model) from 300 to 700 nm at 0, 10, 16, 22, 27, 32 and 33 days interval from the day of synthesis. After finding the SNPRL colloid samples to be stable for more than 1 month, they were exposed to 0, 2, 20, 60 mg $NaCl.ml^{-1}$ of the colloid. A freshly prepared SNP negative control was also put in contact with 2 mg NaCl $ml^{-1}$of the colloid. All spectra were recorded with a gap of 5 min between NaCl addition and spectra recording[122].

3.13.3d Determination of antibacterial property of SNP/SNP-RL and ION/ION-RL

The agar well diffusion method was used for the determination of antibacterial activities of the IONRL, SNPRL, ION, SNP and RL. The rhamnolipid (RL) used in the present investigation was isolated from *P. aeruginosa* OBP1 strain. The tested bacterial strains, *Staphylococcus aureus* (MTCC 3160), *Escherichia coli* (MTCC 40), *Pseudomonas aeruginosa* (MTCC 8163) and *Bacillus subtilis* (MTCC 441) were

grown on Mueller-Hinton agar plate. Wells were made with the help of sterile cork borer and 100 µl of SNPRL, SNP and RL along with positive control streptomycin ($1mg.ml^{-1}$) were added. The plates were incubated at 37°C for 24 h and the observed zones of inhibition were measured using transparent metric ruler. The mean and standard deviation of triplicates data from each sample were calculated.

3.14 Industrial applications of biosurfactants and producing bacteria

3.14.1 Application of biosurfactant in synthesis of bis-uracil derivatives

The rhamnolipid (RL) produced by *P. aeruginosa* OBP1 was dispersed in distilled water at its CMC (45 $mg.l^{-1}$). 6-[(dimethylamino) -methylene]1,3-aminouracil (210 mg, 1 mM) was added to the 8 ml of the above biosurfactant solution in a 100 ml round-bottomed flask and the mixture was stirred at room temperature until all the 6-[(dimethylamino)methylene]1,3-aminouracil got dissolved. To the above mixture 15% (w/v) of p-toluenesulfonic acid (PTSA) was added to enhance the organic chemical reaction. Further, benzaldehyde (108 mg, 1 mmol) was added drop wise to the above mixture solution with constant stirring at room temperature and the product was formed within 5-10 min. The product was separated from the reaction mixture through TLC, dissolved in distilled ethanol (≥98%) and warmed. The ethanol solution of the product was then filtered, allowed to cool and evaporated at room temperature to obtain the square shaped yellow shining transparent crystal product. The crystals were collected, dried and stored in glass vials for further characterization. The same procedure was followed for the synthesis of other derivatives of bis-uracil. For comparison, the same experiment was conducted in water with the addition of biosurfactant. Reaction schemes are given in Appendix I.

3.14.2 Use of biosurfactant producing bacteria in the degradation of modified hyperbranched epoxy/OMMT clay nanocomposites

Mineral salt medium (MSM) was used for assessing the biodegradation of modified hyperbranched epoxy/OMMT clay nanocomposites as described by Dutta *et al.*[363]. Vegetable oil based branched polyester modified hyperbranched epoxy

nanocomposites were prepared with organically modified montmorillonite clay (OMMT) at different clay loadings (1, 2.5 and 5 wt % with respect to the total amount of resin) by the Department of Chemical Sciences, Tezpur University, Tezpur, Assam. The prepared nanocomposite films were introduced into the sterilized mineral salt medium (MSM) under the laminar air hood. The biosurfactant producing bacterial strain *P. aeruginosa* (OBP1) was selected for the biodegradation assay. The nanocomposite films were the only sole carbon source in the culture medium required for the growth of the bacterial strain. An aliquot of 100 µl of overnight grown culture both containing 1×10^8 ml^{-1} cells (as calculated from McFarland turbidity method) was inoculated to the 250 ml volume Erlenmeyer flask containing 100 ml of mineral salt medium (MSM). The culture flasks were kept at 37°C with 180 rpm on an orbital incubator shaker for 5 weeks. The growth of the bacterial strain was monitored by measuring the optical density at 630 nm at a time interval of 7 days. Culture media without any polymer film was kept as control.

3.15 Statistical analysis

Data collected represent the mean of at least three replications and the error bars indicate the standard deviations. The analyses were carried out using Microsoft Excel Software, version 2000 (Microsoft Corporation).

Chapter IV
Results

4.1 Isolation of potential biosurfactant producing bacteria capable of utilizing hydrocarbon from the environmental samples

The environmental samples consist of crude oil-contaminated soils of oil fields, petroleum sludge and waste residual crude oil dumping sites from ONGC, Jorhat, Assam; petroleum sludge from the oil fields of ONGC, Sibsagar, Assam; contaminated soil samples from the different oil depots of Tezpur, Assam. The bacterial strains were isolated from the above mentioned environmental samples using both the enrichment and direct plate culture techniques. A total of 52 culturable isolates were obtained based on distinctly different colony morphology as shown in Table 4.1.

Table 4.1. Morphological characters of bacterial isolates obtained from crude oil-contaminated samples

Sl. No	Bacterial isolates	Size	Pigment	Form	Margin	Elevation
1	JB08S11	Large	Green	Irregular	Lobate	Flat
2	JB08S12	Large	White	Circular	Entire	Flat
3	JB08S13	Large	Yellow	Circular	Undulate	Flat
4	JB08S14	Moderate	White	Circular	Entire	Raised
5	JB08S15	Moderate	White	Irregular	Lobate	Raised
6	JB08S16	Small	White	Circular	Entire	Raised
7	JB08S17	Small	Light yellow	Circular	Entire	Raised
8	JB08S18	Small	White	Circular	Entire	Flat
9	JB08S21	Large	Light yellow	Irregular	Lobate	Flat
10	JB08S22	Large	White	Circular	Undulate	Flat
11	JB08S23	Small	White	Circular	Entire	Raised
12	JB08S24	Small	White	Irregular	Lobate	Flat
13	JB08S25	Pin point	White	Circular	Entire	Raised
14	JB08S31	Large	Yellow	Circular	Entire	Convex
15	JB08S32	Large	White	Circular	Entire	Raised
16	JB08S33	Large	Green	Circular	Undulate	Flat

17	JB08S34	Large	Yellow	Irregular	Lobate	Flat
18	JB08PS35	Moderate	White	Irregular	Lobate	Raised
19	JB08PS36	Moderate	White	Irregular	Lobate	Raised
20	JB08PS37	Moderate	White	Circular	Entire	Raised
21	JB08PS38	Moderate	Green	Circular	Serrate	Raised
22	JB08PS39	Pin point	White	Circular	Entire	Flat
23	JB08PS41	Moderate	White	Circular	Undulate	Flat
24	JB08PS42	Pin point	White	Circular	Entire	Raised
25	JB10OD11	Large	Light yellow	Irregular	Lobate	Flat
26	JB10OD12	Large	White	Rhizoid	Filamentous	Flat
27	JB10OD13	Moderate	Yellow	Irregular	Lobate	Raised
28	JB10OD14	Moderate	White	Irregular	Entire	Raised
29	JB10OD15	Small	White	Circular	Serrate	Convex
30	JB10OD21	Large	White	Circular	Entire	Flat
31	JB10OD22	Large	Yellow	Irregular	Lobate	Raised
32	JB10OD23	Moderate	White	Circular	Entire	Raised
33	JB10OD24	Moderate	Yellow	Circular	Entire	Convex
34	JB10OD25	Pin point	White	Circular	Lobate	Raised
35	S10PSS11	Large	Light yellow	Rhizoid	Filamentous	Flat
36	S10PSS12	Large	White	Circular	Entire	Convex
37	S10PSS13	Moderate	White	Circular	Entire	Convex
38	S10PSS14	Moderate	Yellow	Circular	Undulate	Flat
39	S10PSS15	Moderate	White	Circular	Entire	Raised
40	S10PSS16	Small	White	Circular	Serrate	Flat
41	S10PSS17	Small	Yellow	Circular	Entire	Raised
42	S10PSS21	Large	White	Circular	Serrate	Raised
43	S10PSS22	Large	White	Irregular	Lobate	Flat
44	S10PSS23	Moderate	White	Circular	Entire	Convex
45	S10PSS24	Small	Green	Circular	Entire	Flat
46	S10PSS25	Pin point	Green	Circular	Entire	Flat
47	T11PS11	Moderate	White	Circular	Entire	Flat
48	T11PS12	Moderate	White	Circular	Serrate	Flat
49	T11PS21	Large	Green	Circular	Entire	Flat
50	T11PS22	Small	Yellow	Circular	Entire	Raised
51	T11PS23	Small	Yellow	Circular	Entire	Raised
52	T11PS24	Pin point	White	Circular	Entire	Raised

The bacterial isolates were further re-cultured to obtain the pure colonies of each individual strain and maintained in nutrient plates and also in stab agar cultures at 4°C. The bacterial isolates were sub-cultured at an interval of 30 days in nutrient broth agar. For long term maintenance, the bacterial isolates were preserved in 15% (w/v) glycerol and stored at −70°C.

After the initial screening at 37°C, all 52 bacterial strains were re-cultured in 100 ml mineral salt medium supplemented with 1% (v/v) of n-hexadecane as the sole source of carbon and incubated at 37°C for 7 days at a constant rpm of 150. After the sixth cycle of subcultures, the bacterial isolates were cultured on Bushnell-Hass medium supplemented with 1% (v/v) n-hexadecane to establish their hydrocarbon utilizing ability. The bacterial isolates were grown for 96 h at 37°C with 150 rpm to determine the total dry biomass yield and the same are presented in Table 4.2.

Table 4.2 Biomass yields in Bushnell-Hass medium supplemented with 1% (v/v) n-hexadecane after 96 h

S.No	Bacterial isolates	Biomass ($g.l^{-1}$)
1	JB08S11	2.32±0.75
2	JB08S12	0.45±0.32
3	JB08S13	1.87±0.43
4	JB08S14	4.78±0.21
5	JB08S15	4.23± 0.65
6	JB08S16	2.15±0.31
7	JB08S17	4.54±0.65
8	JB08S18	0.20±0.55
9	JB08S21	4.93±0.83
10	JB08S22	2.8±0.32
11	JB08S23	4.46±0.27
12	JB08S24	2.83±0.11
13	JB08S25	5.02±0.45
14	JB08S31	4.93±0.83
15	JB08S32	2.20±0.29
16	JB08S33	4.75±0.43
17	JB08S34	0.32±1.2
18	JB08PS35	5.10±0.82

19	JB08PS36	0.71±0.37
20	JB08PS37	1.65±0.44
21	JB08PS38	4.77±0.62
22	JB08PS39	2.53±0.83
23	JB08PS41	0.16±0.97
24	JB08PS42	4.92±1.1
25	JB10OD11	5.08±0.41
26	JB10OD12	0.67±0.67
27	JB10OD13	2.83±0.93
28	JB10OD14	4.86±0.83
29	JB10OD15	4.25±0.53
30	JB10OD21	3.52±0.77
31	JB10OD22	5.02±0.84
32	JB10OD23	3.94±0.53
33	JB10OD24	2.17±0.91
34	JB10OD25	4.94±0.63
35	S10PSS11	5.05±0.82
36	S10PSS12	3.32±0.32
37	S10PSS13	4.93±0.54
38	S10PSS14	4.05±0.82
39	S10PSS15	4.88±0.43
40	S10PSS16	3.62±0.81
41	S10PSS17	5.09±0.80
42	S10PSS21	3.58±0.31
43	S10PSS22	4.95±0.52
44	S10PSS23	0.07±1.1
45	S10PSS24	0.27±0.62
46	S10PSS25	5.02±0.41
47	T11PS11	4.75±0.53
48	T11PS12	1.88±0.41
49	T11PS21	3.91±0.74
50	T11PS22	5.10±0.18
51	T11PS23	4.90±0.42
52	T11PS24	2.96±0.62

Results represent mean ± S.D of three individual experiments

A total of 23 isolates JB08S14, JB08S17, JB08S21, JB08S23, JB08S25, JB08S31, JB08S33, JB08PS35, JB08PS38, JB08PS42, JB10OD11, JB10OD14,

JB10OD22, JB10OD25, S10PSS11, S10PSS13, S10PSS15, S10PSS17, S10PSS22, S10PSS25, T11PS11, T11PS22 and T11PS23 possessed better growth in Bushnell-Hass medium supplemented with 1% (v/v) n-hexadecane on the basis of increased dry biomass yield. The isolates were further screened for their ability to produce biosurfactant by culturing on n-hexadecane supplemented media.

To evaluate biosurfactant production, the bacterial isolates were grown in MSM supplemented with 1% (v/v) n-hexadecane and incubated at 37°C and 150 rpm for 8 days. After 96 h of incubation, the reduction in the surface tension of the culture medium by the individual bacterial isolates was determined and is presented in Table 4.3.

Table 4.3 Surface activity exhibited by bacterial isolates grown in mineral salt medium (MSM) supplemented with 1% (v/v) n-hexadecane after 96 h

S.No	Bacterial isolates	Reduction in surface tension (mNm^{-1})	Drop collapse test	Oil displacement test (cm^2)
1	JB08S14	43.8±0.42	+	12.3±0.23
2	JB08S17	32.6±0.85	+++	35.6±0.34
3	JB08S21	38.2±0.24	+++	33.4±0.42
4	JB08S23	36.0±0.42	+++	35.3±0.15
5	JB08S25	47.8±0.62	+	11.6±0.32
6	JB08S31	39.5±0.29	++	28.5±0.26
7	JB08S33	38.9±0.73	+++	30.7±0.44
8	JB08PS35	52.3±0.44	-	-
9	JB08PS38	39.2±0.82	+++	29.8±0.21
10	JB08PS42	38.7±0.48	+++	30.2±0.34
11	JB10OD11	43.6±0.57	+	26.8±0.40
12	JB10OD14	55.8±0.23	-	-
13	JB10OD22	46.3±0.55	+	23.9±0.27
14	JB10OD25	34.3±0.65	+++	36.±0.33
15	S10PSS11	39.1±0.63	+++	29.9±0.28
16	S10PSS13	44.7±1.1	+	19.8±0.32
17	S10PSS15	56.2±0.45	-	-
18	S10PSS17	39.8±0.28	+++	31.0±0.41
19	S10PSS22	40.4±0.73	++	26.8±0.11
20	S10PSS25	51.1±0.11	-	-

21	T11PS11	46.8±0.45	+	21.7±0.23
22	T11PS22	38.6±0.23	+++	35.7±0.34
23	T11PS23	47.9±0.97	+	16.6±0.27

Results represent mean ± S.D of three individual experiments. Abbreviation used: − = negative, + = positive, ++ = significant, +++ = Excellent

Out of 23 bacterial strains, 17 strains which include JB08S17, JB08S21, JB08S23, JB08S31, JB08S33, JB08PS38, JB08PS42, JB10OD11, JB10OD22, JB10OD25, S10PSS11, S10PSS13, S10PSS17, S10PSS22, T11PS11, T11PS22 and T11PS23 exhibited the reduction in the surface tension of the culture medium from 67.5±0.93 mNm^{-1} to a minimum of 32.6±0.55 mNm^{-1}. The flask containing the culture medium alone was taken as control and it exhibited the maintenance of the same surface tension value of 67.8±1.3 after 96 h. Out of 23 bacterial isolates, 19 showed positive value in the drop collapse test. In the case of drop-collapse test, if the cell free culture supernatant contains biosurfactant, the droplets of the culture broth on the oil-coated wells will collapse but no change occurs in the shape of the droplets if the broth is without biosurfactant. Similarly, in oil displacement test, the addition of cell free culture supernatant of 19 bacterial isolates caused oil to spread and formed a wide clear zone on the oil-water surface confirming the presence of biosurfactant.

The objective of the present investigation was to identify bacteria capable of producing biosurfactant and could utilize the petroleum hydrocarbons efficiently. Out of 23 bacterial isolates, only 17 could exhibit both properties. On further experimentation, only 4 isolates viz. JB08S17, JB08S21, JB08S23 and JB10OD25 were found to be the potential users of hydrocarbons and producers of biosurfactants as determined by their biomass yield and surface properties exhibited while growing on n-hexadecane. Therefore, these four bacterial isolates were selected for taxonomic identification and further studies. The bacterial isolates JB08S17, JB08S21, JB08S23 and JB10OD25 were further designated as OBP1, OBP2, OBP3 and OBP4, respectively for the sake of convenience.

4.2 Characterization of potential biosurfactant producing bacterial isolates

4.2.1 Morphological and physiological characterization

The biochemical, morphological and physiological characterization of the bacterial isolates was carried out following the procedures described by Cappuccino and Sherman[341] and data thus obtained are presented in Table 4.4.

Table 4.4 Biochemical characterization of bacterial isolates

Biochemical test	Bacterial isolate			
	OBP1	OBP2	OBP3	OBP4
1. Gram staining	Negative	Negative	Negative	Negative
2. Shape of the cell	Straight rod	Straight rod	Straight rod	Straight rod
3. Capsule staining	Positive	Positive	Positive	Positive
4. Endospore staining	Negative	Negative	Negative	Negative
5. Motility test	Positive	Positive	Positive	Positive
6. Acid production from				
a. Glucose	Positive	Positive	Positive	Positive
b. Fructose	Positive	Positive	Positive	Positive
c. Xylose	Positive	Positive	Positive	Positive
d. Maltose	Negative	Negative	Negative	Negative
e. Lactose	Negative	Negative	Negative	Negative
f. Manitol	Negative	Negative	Negative	Negative
g. Salicin	Negative	Negative	Negative	Negative
h. Sucrose	Negative	Negative	Negative	Negative
i. Oxidase test	Positive	Positive	Positive	Positive
7.Catalase test	Positive	Positive	Positive	Positive
8. H_2S production	Negative	Negative	Negative	Negative
9. Gelatin hydrolysis test	Positive	Positive	Positive	Positive
10. Starch hydrolysis	Negative	Negative	Negative	Negative
11. Methyl red test	Negative	Negative	Negative	Negative
12. Voges-Proskauer test	Negative	Negative	Negative	Negative
13. Indole test	Negative	Negative	Negative	Negative
14. Citrate utilization test	Positive	Positive	Positive	Positive
15. Nitrate reduction test	Positive	Positive	Positive	Positive
16. Production of fluorescence	Positive	Positive	Positive	Positive
17. Production of pyocyanin	Negative	Negative	Negative	Negative

The colony morphology of the bacterial isolates is shown in Figure 4.1. The characterization details revealed four selected bacterial isolates belonging to the genus *Pseudomonas*.

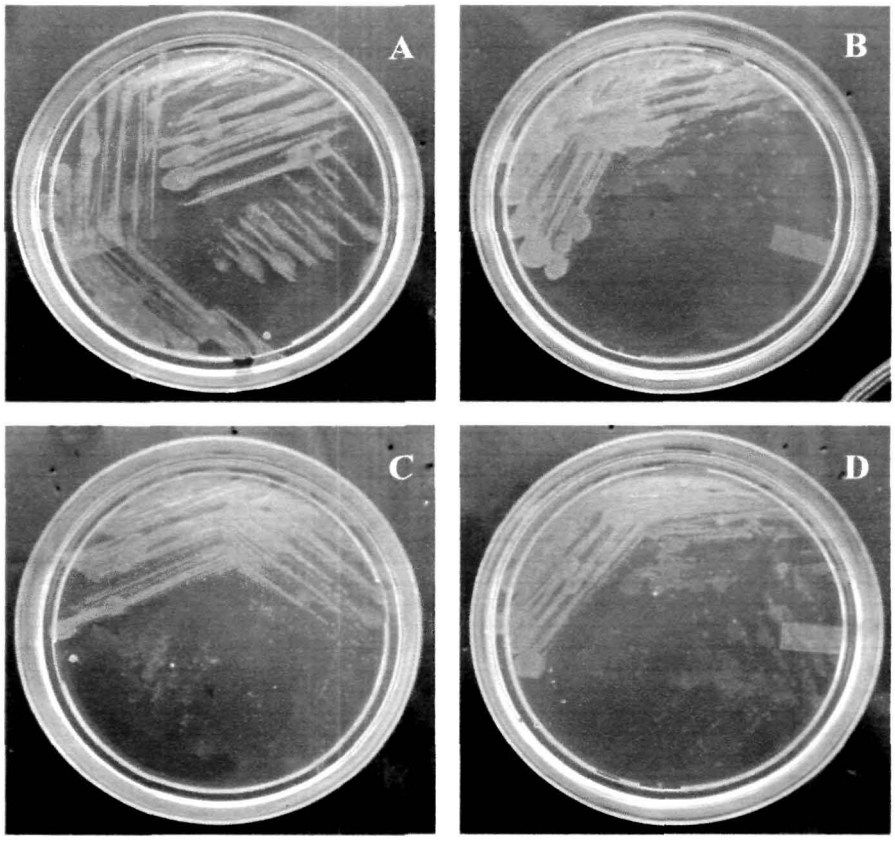
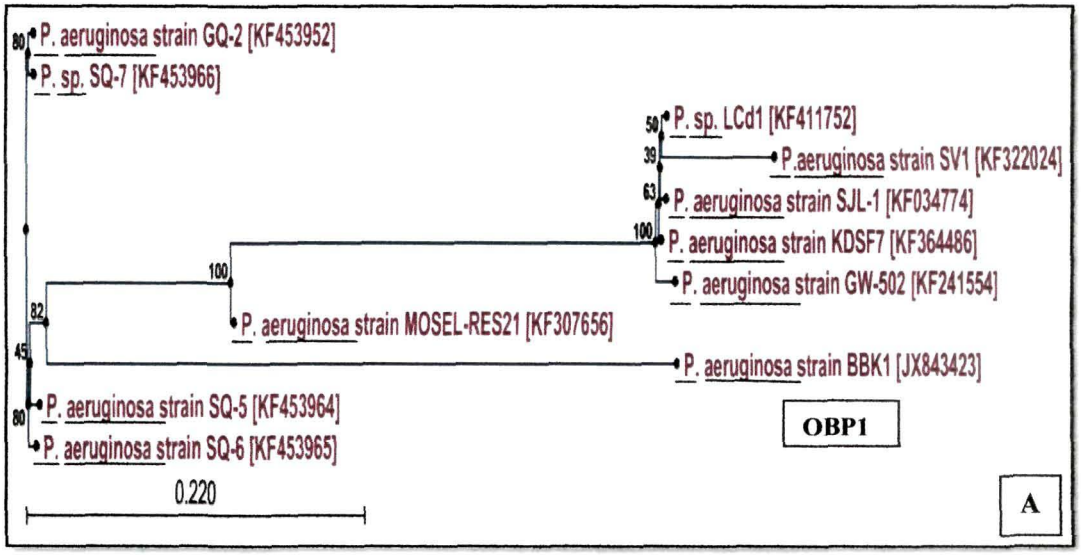


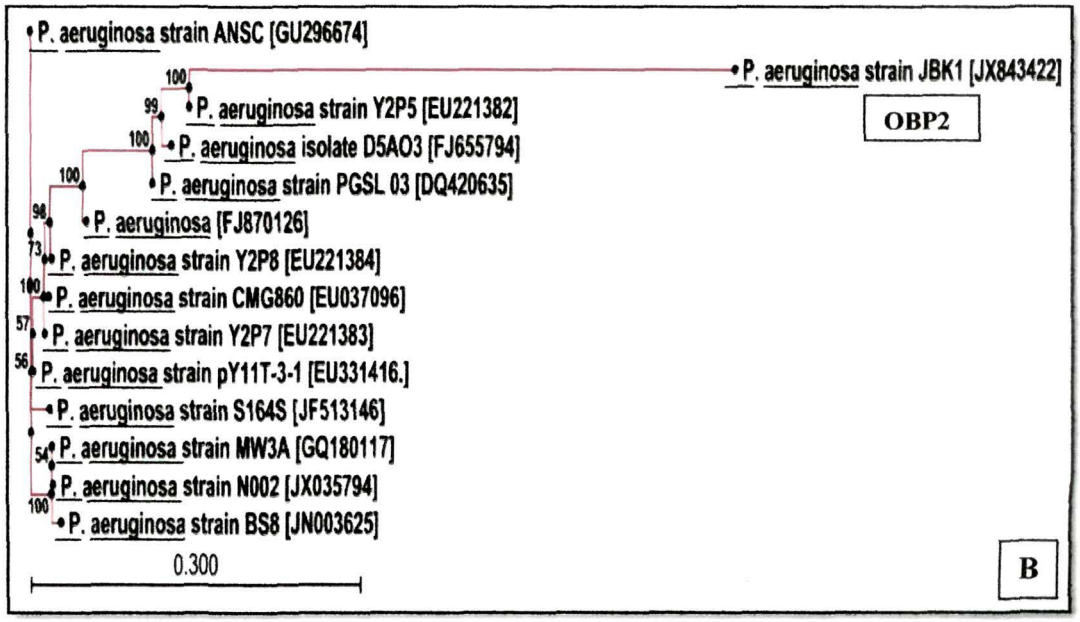
Figure 4.1. Colony morphology of pure cultures on nutrient agar (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3 and (D) *P. aeruginosa* OBP4

4.2.2 Molecular characterization of the bacterial isolates

The selected bacterial isolates were subjected to partial sequencing of their 16S rRNA genes. On the basis of partial 16S rRNA gene sequencing as well as with the use of NCBI GenBank BLAST tool, these four bacterial isolates OBP1, OBP2, OBP3 and OBP4 were found to be closely related to the genus *Pseudomonas aeruginosa* with percent similarity of 99% in all the strains. NCBI BLAST result of these bacterial isolates showed maximum similarity with 34 different strains of *Pseudomonas*

aeruginosa in respect of their score, query coverage, E-value and maximum identity. The partial 16S rRNA gene sequences of the bacterial strains OBP1, OBP2, OBP3 and OBP4 were deposited in the GenBank database under the accession numbers 1568190, 1568199, 1568206 and 156820, respectively. Further, putative phylogenetic trees for the bacterial isolates were constructed using Neighbour-Joining method and are shown in Figure 4.2.





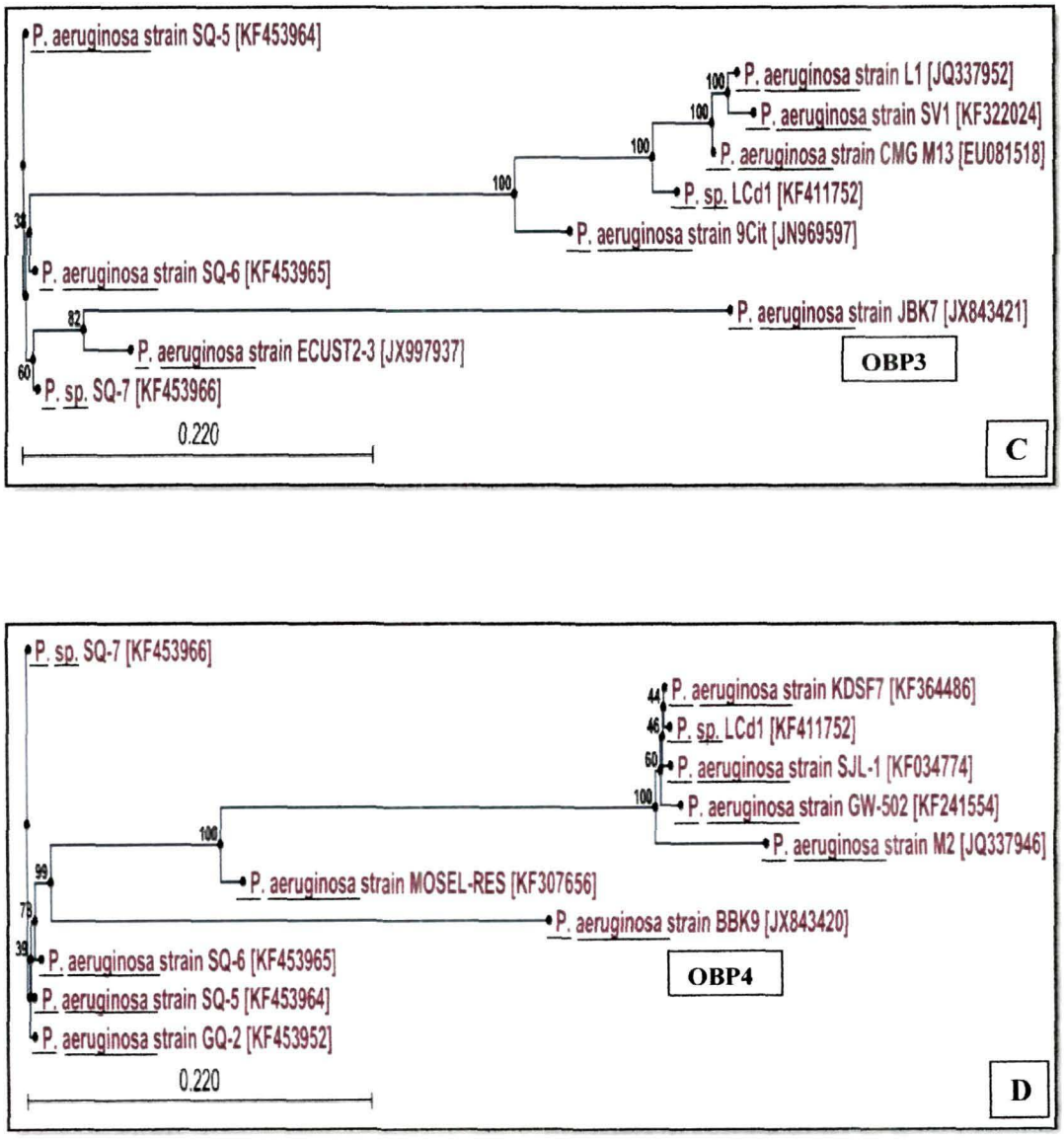


Figure 4.2. Phylogenetic tree generated using Neighbour-Joining method showing the similarity of selected strains with other 16S rRNA gene sequences of *P. aeruginosa*. Bootstrap values are expressed as percentages of 1000 replications. Bar, 0.01 substitutions per nucleotide position. (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3 and (D) *P. aeruginosa* OBP4

The nucleotide frequency count analyses were done for the bacterial isolates using CLC main work bench software. The nucleotide frequency counts showed that

ATGC, C+G and A+ T compositions of bacterial strains OBP1, OBP2, OBP3 and OBP4 have almost similar frequency (99%) distribution as compared to other reported strains of *Pseudomonas aeruginosa*.

4.3 Ability of the bacterial strains to utilize different hydrocarbons

The bacterial strains were able to grow in MSM supplemented with different hydrocarbons as the sole source of carbon and energy. The growth performance of the bacterial strains on the hydrocarbon sources has been presented in Table 4.5. The bacterial strains exhibited better growth on n-hexadecane, octadecane, tridecane, dodecane, diesel and crude oil supplemented media. Less or no growth was observed in the case of kerosene, lubricating and paraffin, pentane, hexane, heptane, iso-octane, eicosane and triacontane.

Table 4.5 Ability of the bacterial strains to utilize different components of crude petroleum

Carbon sources	**Properties**	***P. aeruginosa* strains**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
Pentane	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.3±0.21	67.5±0.42	68.8±0.43	67.9±0.32
Hexane	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	67.9±0.32	68.5±0.61	68.1±0.45	67.7±0.32
Heptane	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.4±0.24	67.8±0.57	67.6±0.31	68.3±0.36
iso-Octane	DB ($g.l^{-1}$)	-	-	-	
	ST (mNm^{-1})	67.9±0.36	68.2±0.51	67.7±0.48	67.9±0.52
Dodecane	DB ($g.l^{-1}$)	3.28±0.65	3.31±0.34	2.55±0.53	2.37±0.65
	ST (mNm^{-1})	36.8±0.21	43.4±0.63	41.6±0.27	38.9±0.52
Triadecane	DB ($g.l^{-1}$)	3.37±0.64	3.48±0.45	3.61±0.44	3.54±0.32
	ST (mNm^{-1})	36.0±0.24	42.5±0.57	39.7±0.31	37.5±0.36
n-Hexadecane	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
Octadecane	DB ($g.l^{-1}$)	4.83±0.34	3.82±0.56	4.12±0.74	4.24±0.37
	ST (mNm^{-1})	31.9±0.85	39.6±0.24	38.4±0.42	36.8±0.65
Eicosane	DB ($g.l^{-1}$)	-	-	-	-

	ST (mNm^{-1})	68.6±0.18	68.4±0.61	67.9±0.28	68.2±0.20
Triacontane	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	67.7±0.49	68.3±0.61	68.5±0.28	67.9±0.19
Paraffin	DB ($g.l^{-1}$)	1.38±0.22	1.13±0.46	1.52±0.27	1.47±0.51
	ST (mNm^{-1})	45.3±0.29	47.2±0.27	44.5±0.25	45.6±0.55
Phenol	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.3±0.23	68.6±0.23	67.8±0.27	67.4±0.82
Benzene	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.3±0.18	68.6±0.23	67.9±0.41	68.4±0.21
Toluene	DB ($g.l^{-1}$)	0.36±0.13	-	0.78±0.52	1.04±0.22
	ST (mNm^{-1})	54.7±0.39	68.7±0.41	52.2±0.17	52.5±0.48
Xylene	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.2±0.16	68.5±0.42	68.7±0.19	68.5±0.15
Naphthalene	DB ($g.l^{-1}$)	-	-	0.83±0.66	-
	ST (mNm^{-1})	68.7±0.69	68.2±0.36	53.5±0.35	68.7±0.92
Anthracene	DB ($g.l^{-1}$)	0.45 ±0.37	-	0.66±0.27	0.93±0.41
	ST (mNm^{-1})	54.9±0.83	67.7±0.23	54.2±0.44	53.6±0.58
Phenanthrene	DB ($g.l^{-1}$)	0.61±0.34	-	1.05±0.29	1.18±0.73
	ST (mNm^{-1})	54.2±0.59	68.6±0.15	53.8±0.43	53.2±0.46
Pyrene	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	67.8±0.91	68.7±0.20	67.6±0.82	68.7±0.73
Fluorene	DB ($g.l^{-1}$)	-	-	-	-
	ST (mNm^{-1})	68.8±0.83	67.9±0.93	68.6±0.43	67.9±0.42
Diesel	DB ($g.l^{-1}$)	4.54±0.93	3.97±0.53	4.91±0.40	5.04±0.62
	ST (mNm^{-1})	32.0±0.42	37.5±0.28	36.2±0.69	34.3±1.0
Kerosene	DB ($g.l^{-1}$)	2.73±0.76	2.30±0.56	2.54±3.2	2.78±0.74
	ST (mNm^{-1})	39.6±0.62	42.2±0.29	40.5±0.83	39.2±0.44
Lubricating oil	DB ($g.l^{-1}$)	2.31±0.56	1.67±0.21	2.39±0.82	2.24±0.29
	ST (mNm^{-1})	41.5±0.37	43.8±0.18	40.7±0.64	41.4±1.0
Crude oil	DB ($g.l^{-1}$)	3.71±0.44	3.27±0.62	3.86±0.82	4.07±0.53
	ST (mNm^{-1})	32.7±0.66	40.4±0.25	39.5±0.41	37.7±0.16

Results represent mean ± S.D of three individual experiments

In the case of aromatic hydrocarbons like toluene the bacterial strains OBP4, OBP3, and OBP1 showed slight growth but no growth on benzene, phenol and xylene supplemented media. The bacterial strains OBP4, OBP3, and OBP1exhibited minimum growth on PAHs like phenanthrene and anthracene but no growth on pyrene and fluorene. Among the four bacteria, only OBP3 exhibited growth on naphthalene

supplemented medium. Among the tested hydrocarbons, the bacterial strains showed good performance in n-hexadecane supplemented MSM. The capability of the bacterial strains to utilize a particular hydrocarbon as the sole source of carbon and energy was different from each other as revealed by their biomass yield and efficiency to reduce the surface tension.

4.4 Detection and quantification of biosurfactant

To detect and quantify the surface active glycolipids three independent experiments were performed with blood agar assay, CTAB agar test and orcinol assay. The cell free culture supernatants of the selected bacterial strains were analyzed for their hemolytic activity on blood agar plates at 37°C overnight. The bacterial strains exhibited distinct zone of hemolysis in blood-agar plates containing 2% (v/v) goat blood and the same is shown in Figure 4.3.

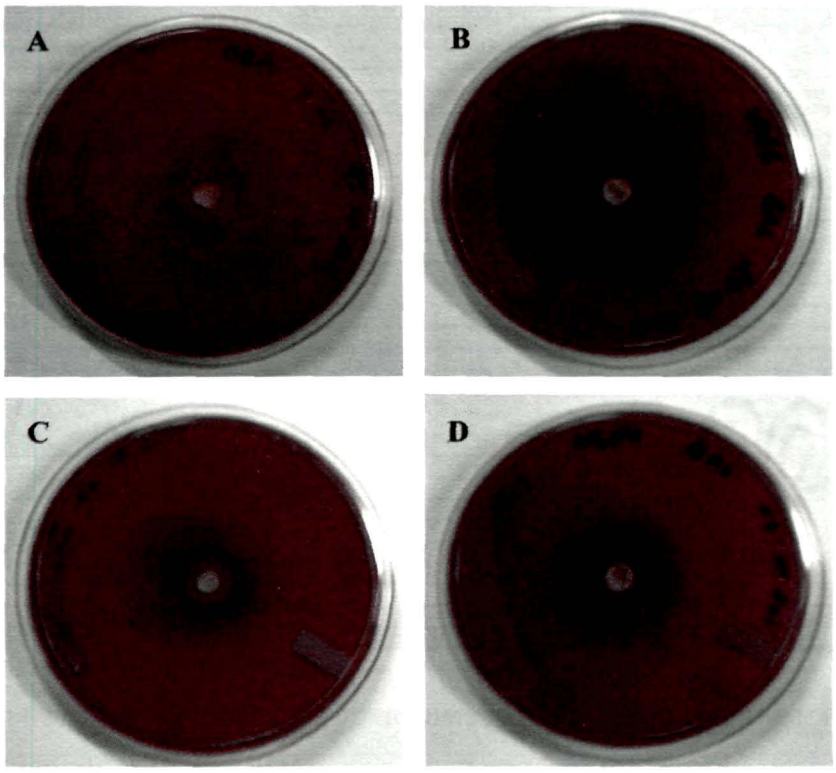


Figure 4.3. Haemolysis on blood agar medium by cell free culture supernatant of bacterial strains. (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3 and (D) *P. aeruginosa* OBP4

The hemolysis assay confirmed the production of biosurfactant and the same was considered as the preliminary criterion for the production of biosurfactant.

The cell density of the individual bacterial strains was reduced by serial dilution and then they were spread over the MSM agar plates supplemented with CTAB ($0.2g.l^{-1}$), methylene blue dye ($5\ mg.l^{-1}$) and n-hexadecane (0.1%, v/v) and incubated at 37°C for 48 h. All four bacterial strains could grow on CTAB agar plates forming blue halos around the colonies. The appearance of blue halos around the colonies on the blue agar plates confirmed the production of extracellular anionic biosurfactants by the strains and the same are shown in Figure 4.4.

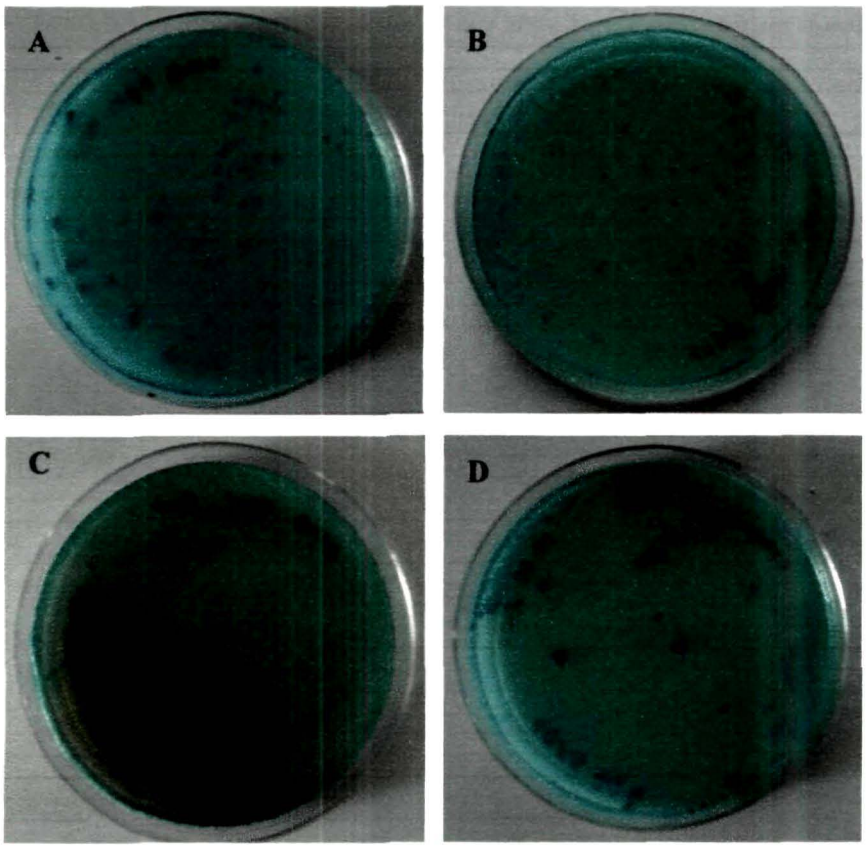


Figure 4.4. CTAB agar assay for the detection of glycolipid biosurfactant secreted by the bacterial colonies. (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3 and (D) *P. aeruginosa* OBP4

The concentration of biosurfactant present in the culture medium of the bacterial strains was determined using the orcinol assay and the production was in the range of 8.8-12.3 $g.l^{-1}$. The same is presented in Fig 4.5.

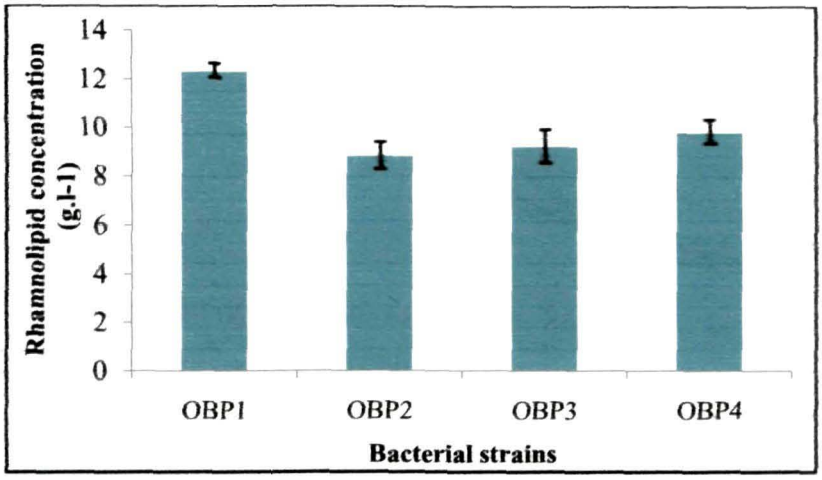


Figure 4.5. Biosurfactant concentration ($g.l^{-1}$) produced by *P. aeruginosa* strains in mineral salt medium supplemented with 2% n-hexadecane. Results represent the mean of three independent experiments ± standard deviation

4.5 Optimization of culture conditions for biosurfactant production

4.5.1 Growth characteristics and biosurfactant production

The bacterial strains OBP1, OBP2, OBP3 and OBP4 could grow on MSM supplemented with n-hexadecane causing reduction of surface tension of the culture medium from 68.5 mNm^{-1} to 31.1, 37.6, 35.5 and 33.2 mNm^{-1}, respectively between 84-96 h of incubation. Growth curve of the bacterial strains revealed the maximum biomass productions between 120-144 h of incubation and the same is presented in Figure 4.6. The production of biosurfactant started after 36-48 of incubation, the highest production was achieved towards the early stationary phase between 108-120 h. The biomass and biosurfactant production were in the range of 4.73-5.10 $g.l^{-1}$ and 2.83-4.57 $g.l^{-1}$, respectively. The Scanning Electron Micrographs of the bacterial strains grown on n-hexadecane supplemented medium are shown in Figure 4.7.

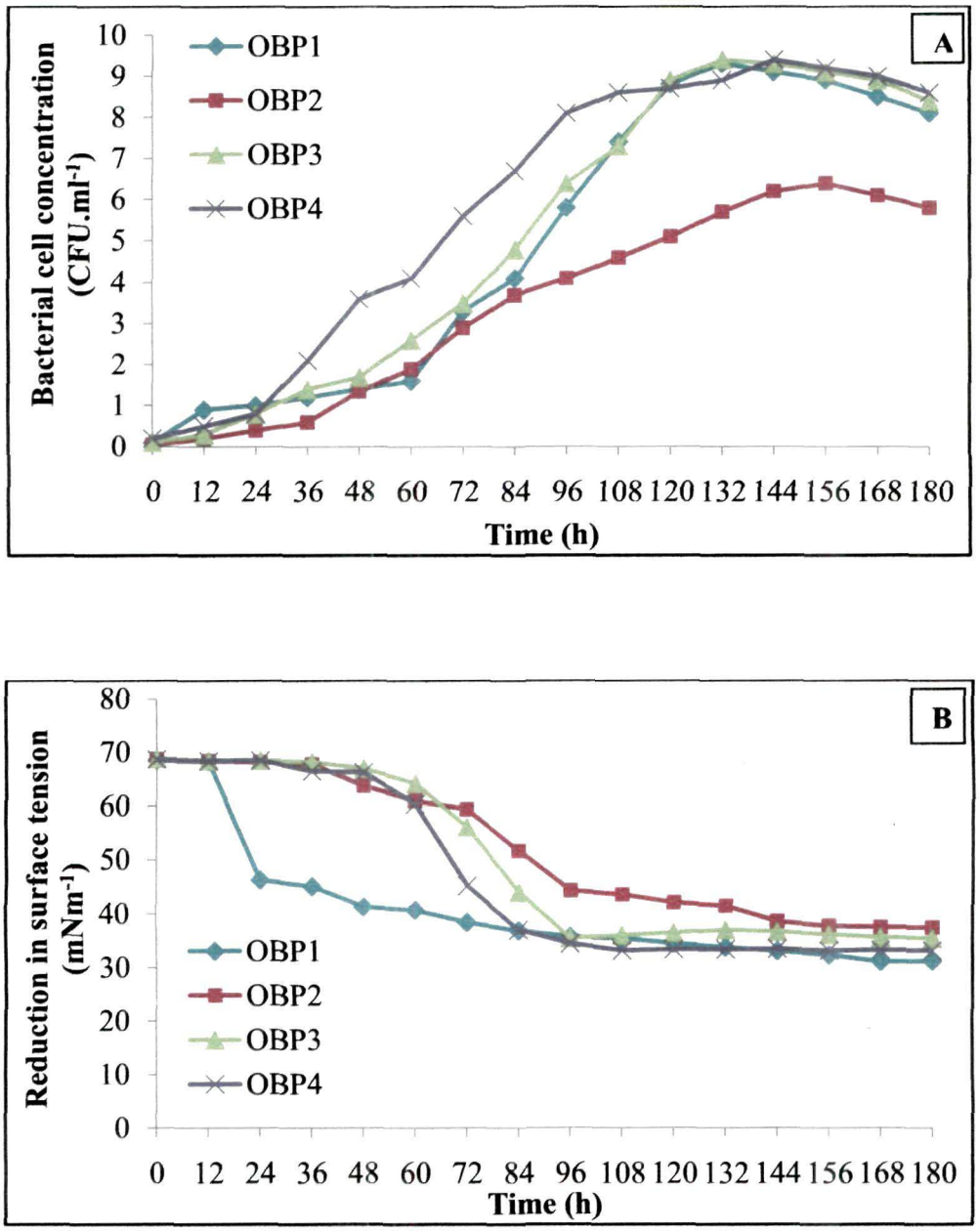


Figure 4.6. Time profile of (A) growth and (B) reduction in the surface tension of the culture broth by the *P. aeruginosa* strains in mineral salt medium supplemented with 2% n-hexadecane. Results represent the mean of three independent experiments

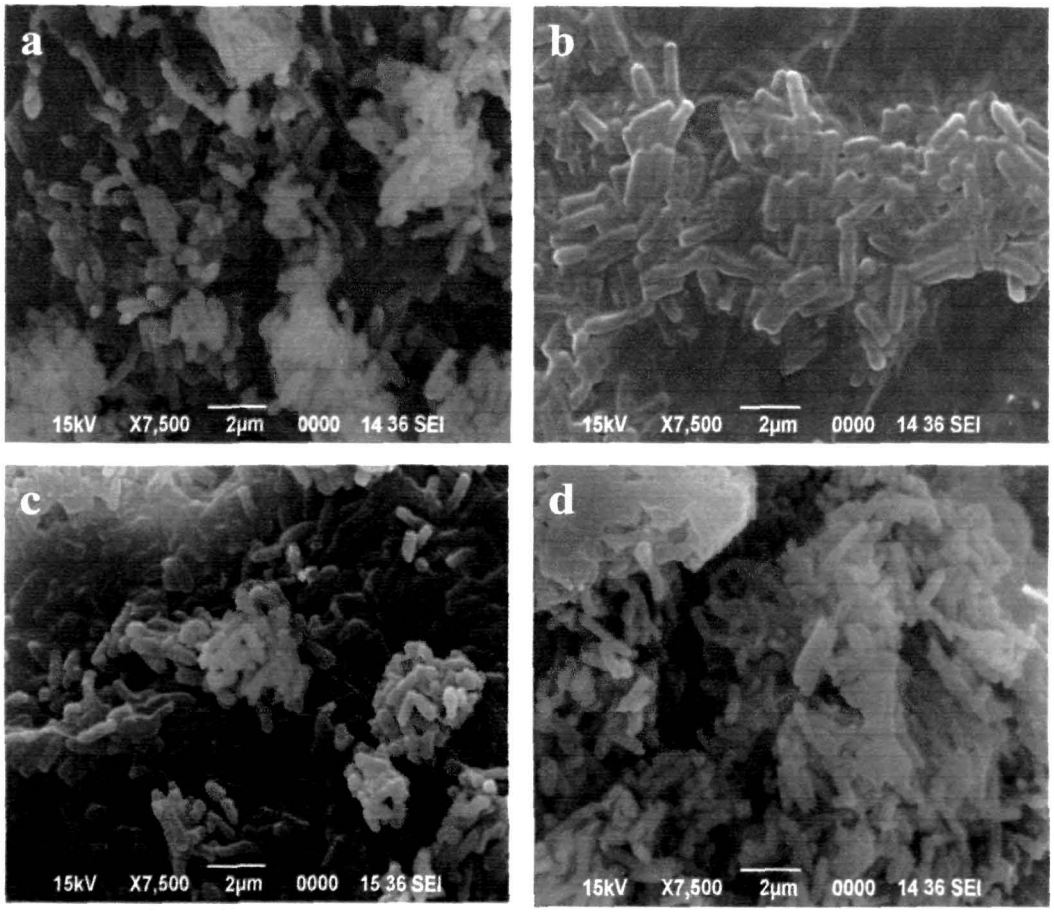


Figure 4.7. Scanning electron micrograph of *P. aeruginosa* strains showing growth on n-hexadecane. (a) *P. aeruginosa* OBP1, (b) *P. aeruginosa* OBP2, (c) *P. aeruginosa* OBP3 and (d) *P. aeruginosa* OBP4

4.5.2 Effect of different carbon sources on biosurfactant production

A total of seven carbon sources viz. glucose, glycerol, n-hexadecane, octadecane, soyabean oil, diesel and crude petroleum were assessed for their effectiveness in producing biosurfactants. The data are presented in Table 4.6. All four bacterial strains could grow on mineral salt medium supplemented with the substrates. The carbon sources allowed good bacterial growth whereas the production of biosurfactant was quite different. Among the substrates, n-hexadecane was found to be most suitable for the production of biosurfactant by all four bacterial strains followed by diesel.

Table 4.6 Influence of different concentrations of n-hexadecane on growth and biosurfactant production of *P. aeruginosa* strains

Carbon sources	**Biomass ($g.l^{-1}$)**	**Maximum reduction in surface tension (mNm^{-1})**	**Yield of crude biosurfactant ($g.l^{-1}$)**
Glucose			
P. aeruginosa OBP1	5.04±0.45	50.7±0.23	0.12±0.42
P. aeruginosa OBP2	5.06±0.23	51.4±0.16	0.09±0.12
P. aeruginosa OBP3	4.91±0.63	50.6±0.41	0.10±0.38
P. aeruginosa OBP4	4.96±0.28	51.8±0.36	0.07±0.18
Glycerol			
P. aeruginosa OBP1	4.96±0.21	49.2±0.27	0.30±0.35
P. aeruginosa OBP2	5.12±0.40	46.6±0.38	0.39±0.52
P. aeruginosa OBP3	5.03±0.29	49.5±0.53	0.26±0.11
P. aeruginosa OBP4	5.08±0.57	48.6±0.45	0.33±0.50
n-hexadecane			
P. aeruginosa OBP1	4.87±0.63	31.1±0.88	4.57±0.53
P. aeruginosa OBP2	5.03±0.37	37.6±0.51	2.86±0.28
P. aeruginosa OBP3	4.73±0.72	35.5±0.38	2.83±0.43
P. aeruginosa OBP4	5.10±0.21	33.2±0.79	3.17±0.37
Octadecane			
P. aeruginosa OBP1	4.83±0.34	31.9±0.85	4.21±0.34
P. aeruginosa OBP2	3.82±0.56	39.6±0.24	2.23±0.52
P. aeruginosa OBP3	4.12±0.74	38.4±0.42	2.37±0.50
P. aeruginosa OBP4	4.24±0.37	36.8±0.65	2.58±0.44
Diesel			
P. aeruginosa OBP1	4.54±0.93	32.0±0.42	3.04±0.60
P. aeruginosa OBP2	3.97±0.53	37.5±0.28	2.47±0.38
P. aeruginosa OBP3	4.91±0.40	36.2±0.68	2.64±0.87
P. aeruginosa OBP4	5.04±0.62	34.3±1.0	2.96±0.92
Crude oil			
P. aeruginosa OBP1	3.71±0.44	32.7±0.66	2.53±0.47
P. aeruginosa OBP2	3.27±0.62	40.4±0.25	1.48±0.52
P. aeruginosa OBP3	3.86±0.82	39.5±0.41	2.20±0.73
P. aeruginosa OBP4	4.07±0.53	37.7±0.16	2.46±0.28
Soyabean oil			
P. aeruginosa OBP1	2.51±0.56	38.3±0.61	1.71±0.22
P. aeruginosa OBP2	2.05±0.29	42.8±0.52	1.24±0.45

P. aeruginosa OBP3	1.13±0.37	45.6±0.37	1.05±0.58
P aeruginosa OBP4	1.05±0.35	46.5±0.29	0.97±0.33

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

The substrates octadecane and crude petroleum could also significantly increase the biosurfactant production by all four bacterial strains. The strains showed significant difference in their growth and surface properties when grown on the selected vegetable oil. However, OBP1 exhibited better performance in terms of bacterial biomass and biosurfactant production in soyabean oil. The substrates glucose and glycerol were proficient in terms of biomass production, but not so in biosurfactant production.

4.5.3 Concentration effect of n-hexadecane on biosurfactant production

Biosurfactant production by the bacterial strains initially increased with the increasing concentration of n-hexadecane until it reached the maximum value and then leveled off. Moreover, the growth of the bacterial strains significantly reduced as the concentration of n-hexadecane exceeded more than 2.5% (v/v) resulting in insignificant production of biosurfactant in the culture medium. The same are presented in Table 4.7.

Table 4.7 Influence of different concentrations of n-hexadecane on growth and biosurfactant production of *P. aeruginosa* strains

Percentage of n-hexadecane (v/v)	**Properties**	***P. aeruginosa* strain**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
1.0	DB ($g.l^{-1}$)	4.47±0.71	4.53±0.67	4.58±0.64	4.62±0.93
	ST (mNm^{-1})	32.6±0.85	38.2±0.82	36.0±0.42	34.3±0.65
	BS ($g.l^{-1}$)	3.96±1.00	2.48±0.31	2.76±0.97	2.92±0.49
1.5	DB ($g.l^{-1}$)	4.65±0.52	4.94±0.36	4.73±0.72	4.89±0.56
	ST (mNm^{-1})	32.0±0.95	37.9±0.49	35.5±0.38	33.9±0.74
	BS ($g.l^{-1}$)	4.43±0.74	2.70±0.32	2.83±0.43	3.03±0.52
2.0	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.66±0.52	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	36.2±0.86	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.72±0.73	3.17±0.37

2.5	DB ($g.l^{-1}$)	4.70±0.48	4.89±0.62	4.28±0.66	4.91±0.52
	ST (mNm^{-1})	32.8±0.62	38.0±0.47	38.2±0.29	33.8±0.80
	BS ($g.l^{-1}$)	4.41±0.71	2.77±0.79	2.18±0.57	3.04±0.39
3.0	DB ($g.l^{-1}$)	3.20±0.65	4.08±0.36	3.73±0.82	3.88±0.75
	ST (mNm^{-1})	36.2±0.48	39.9±0.84	40.8±0.37	38.6±0.62
	BS ($g.l^{-1}$)	3.84±0.92	2.02±0.55	1.93±0.77	2.53±0.45

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant.

The population of colony forming unit (cfu) of OBP3 bacterial strain got reduced with increase in the concentration of n-hexadecane above 1.5%, whereas the same happened in the case of other three bacterial strains at a higher concentration (2.0 %). A maximum $cfu.ml^{-1}$ of OBP1, OBP2 and OBP4 was detected by the plate count technique in the culture medium supplemented with 2.0 % n-hexadecane whereas OBP3 possessed a maximum $cfu.ml^{-1}$ in 1.5% n-hexadecane. As shown in Table 4.8, the number of $cfu.ml^{-1}$ of the bacterial strains OBP1, OBP2 and OBP4 increased from 5.5×10^7 to 7.5×10^{11} with the increase in the concentration of n-hexadecane from 1.0 to 2.0%; however, a similar change from 4.8×10^8 to 5.4×10^9 $cfu.ml^{-1}$ in the case of OBP3 occurred from 1.0 to 1.5% n-hexadecane. The increase in the concentration of n-hexadecane above 2.0% showed sharp reduction in the $cfu.ml^{-1}$ of the bacterial strains and the same in the case of OBP3 at above 1.5%.

Table 4.8 Influence of different concentration of n-hexadecane on the colony forming unit (CFU) of *P. aeruginosa* strains

Bacterial strain	**Concentration of n-hexadecane**				
	1.0%	1.5%	2.0%	2.5%	3.0%
P.aeruginosa OBP1	3.9×10^8	4.7×10^8	5.9×10^9	4.2×10^8	2.3×10^5
P.aeruginosa OBP2	5.5×10^7	6.1×10^8	6.6×10^9	5.8×10^7	3.5×10^5
P aeruginosa OBP3	4.8×10^8	5.4×10^9	3.9×10^8	2.6×10^5	1.1×10^3
P aeruginosa OBP4	4.5×10^8	6.8×10^9	7.5×10^{11}	6.1×10^8	3.8×10^6

Results represent mean of three individual experiments. NB: CFU, colony forming unit

4.5.4 Effect of nitrogen sources on biosurfactant production

Nitrogen source plays a crucial role in the production of biosurfactants by bacteria. Bacterial strains exhibited poor growth and surface activities in the nitrogen-deficient media. Both organic and inorganic nitrogen sources influenced the growth and biosurfactant production in the bacterial strains. Data are presented in Table 4.9.

Table 4.9 Influence of various nitrogen sources on growth and biosurfactant production of *P. aeruginosa* strains

Different nitrogen sources	Properties	*P. aeruginosa* strain			
		OBP1	OBP2	OBP3	OBP4
Nitrogen Free	DB ($g.l^{-1}$)	0.51±0.18	0.24±0.10	0.27±0.21	0.33±0.14
	ST (mNm^{-1})	56.4±0.23	58.8±0.15	58.7±0.27	59.7±0.07
	BS ($g.l^{-1}$)	0.10±0.27	0.05±0.21	0.07±0.31	0.07±0.22
NH_4Cl	DB ($g.l^{-1}$)	3.17±0.29	2.86±0.78	3.27±0.51	2.97±0.80
	ST (mNm^{-1})	40.4±0.71	42.5±1.00	42.0±0.24	42.5±0.67
	BS ($g.l^{-1}$)	2.05±0.45	1.04±0.67	1.0±0.22	1.28±0.49
$(NH_4)_2SO_4$	DB ($g.l^{-1}$)	3.78±0.51	4.18±0.56	3.96±0.74	4.23±0.37
	ST (mNm^{-1})	36.7±0.72	40.9±0.24	39.8±0.42	38.6±0.65
	BS ($g.l^{-1}$)	3.14±0.66	1.13±0.30	1.07±0.71	2.38±0.43
NH_4NO_3	DB ($g.l^{-1}$)	2.76±0.56	3.17±0.70	3.44±0.65	3.28±0.97
	ST (mNm^{-1})	39.2±0.73	42.6±0.93	41.0±0.39	41.4±0.71
	BS ($g.l^{-1}$)	2.03±0.41	1.10±0.72	1.06±0.86	1.68±0.64
$NH_4H_2PO_4$	DB ($g.l^{-1}$)	3.57±0.42	3.38±0.43	3.71±0.51	3.62±0.50
	ST (mNm^{-1})	38.2±0.36	42.2±0.72	40.8±0.88	40.6±0.62
	BS ($g.l^{-1}$)	2.34±0.67	1.02±0.56	1.10±0.90	1.86±0.47
KNO_3	DB ($g.l^{-1}$)	2.94±0.56	2.13±0.67	3.07±0.79	2.26±0.87
	ST (mNm^{-1})	42.4±0.35	44.5±0.56	43.6±0.81	43.8±0.56
	BS ($g.l^{-1}$)	1.06±0.77	0.83±0.38	0.78±0.95	1.04±0.33
H_2NCONH_4	DB ($g.l^{-1}$)	2.34±0.72	2.48±0.46	2.27±0.67	2.61±0.25
	ST (mNm^{-1})	37.3±0.41	41.7±0.29	40.5±0.51	39.3±0.65
	BS ($g.l^{-1}$)	2.32±0.54	1.12±0.52	1.15±0.83	2.19±0.43
Yeast extract	DB ($g.l^{-1}$)	2.63±0.43	2.77±0.38	2.58±0.62	3.12±0.93
	ST (mNm^{-1})	37.8±0.20	40.9±0.73	41.7±1.02	38.8±0.52
	BS ($g.l^{-1}$)	2.15±0.76	1.10±0.55	1.06±0.83	2.07±0.73
	DB ($g.l^{-1}$)	2.42±0.36	2.43±0.81	2.27±0.52	2.86±0.29

Beef extract	ST (mNm^{-1})	39.6±0.72	42.3±0.95	40.5±0.63	41.5±0.33
	BS ($g.l^{-1}$)	1.87±0.29	1.11±0.21	1.16±0.47	1.14±0.71
Peptone	DB ($g.l^{-1}$)	2.58±0.81	2.82±0.43	2.65±0.59	3.04±0.11
	ST (mNm^{-1})	38.4±0.33	42.8±0.62	42.4±0.28	40.2±0.52
	BS ($g.l^{-1}$)	2.08±0.27	1.07±0.83	1.10±0.51	1.95±0.76

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Among the inorganic sources, $(NH_4)_2SO_4$ was found to be the best for growth and biosurfactant production as compared to the other sources. Among the organic nitrogen sources, urea was found to be efficient against yeast extract, beef extract and peptone promoting growth and biosurfactant production in all the bacterial strains.

4.5.5 Effect of concentration and combinations of nitrogen sources on biosurfactant production

The type and concentration of inorganic nitrogen sources effected the production of biosurfactants. The highest production was obtained with $(NH_4)_2SO_4$ at a concentration of 2.0 $g.l^{-1}$ in all the bacterial strains except for OBP 2 which exhibited optimal biosurfactant production at 1.0 $g.l^{-1}$. Similarly, urea at the concentration of 2.0 $g.l^{-1}$ was proved to be the best organic nitrogen source. Further, the combination of both $(NH_4)_2SO_4$ and urea at a concentration of 2.0 $g.l^{-1}$ each was found to be efficient for the growth and biosurfactant production. Data thus obtained are presented in Table 4.10a, b and c, respectively.

Table 4.10a. Influence of different concentration of $(NH_4)_2SO_4$ on growth and biosurfactant production of *P. aeruginosa* strains

Concentration of $(NH_4)_2SO_4$ ($g.l^{-1}$)	**Properties**	***P. aeruginosa* strain**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
1.0	DB ($g.l^{-1}$)	3.53±0.47	3.89±0.65	3.78±0.25	4.08±0.63
	ST (mNm^{-1})	37.7±0.36	37.7±0.69	41.3±0.52	39.8±0.81
	BS ($g.l^{-1}$)	2.97±0.38	1.00±0.30	0.87±0.46	2.19±0.45
2.0	DB ($g.l^{-1}$)	3.78±0.51	4.18±0.56	3.96±0.74	4.23±0.37
	ST (mNm^{-1})	36.7±0.72	40.9±0.24	39.8±0.42	38.6±0.65
	BS ($g.l^{-1}$)	3.14±0.66	1.13±0.30	1.07±0.71	2.38±0.43

3.0	DB ($g.l^{-1}$)	3.64±0.58	3.96±0.52	3.83±0.68	4.14±0.35
	ST (mNm^{-1})	37.0±0.36	38.5±0.40	40.6±0.50	39.4±0.30
	BS ($g.l^{-1}$)	3.03±0.73	1.06±0.28	0.96±0.83	2.23±0.44

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Table 4.10b. Influence of different concentration of urea (H_2NCONH_4) on the growth and biosurfactant production of *P. aeruginosa* strains

Percentage of H_2NCONH_4 ($g.l^{-1}$)	**Properties**	***P. aeruginosa* strain**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
1.0	DB ($g.l^{-1}$)	2.22±0.64	2.31±0.38	2.13±0.56	2.43±0.54
	ST (mNm^{-1})	38.9±0.28	42.5±0.62	41.8±0.72	41.6±0.28
	BS ($g.l^{-1}$)	2.18±0.54	1.04±0.58	1.04±0.71	1.97±0.83
2.0	DB ($g.l^{-1}$)	2.34±0.72	2.48±0.46	2.27±0.67	2.61±0.25
	ST (mNm^{-1})	37.3±0.41	41.7±0.29	40.5±0.51	39.3±0.65
	BS ($g.l^{-1}$)	2.32±0.54	1.12±0.52	1.15±0.83	2.19±0.43
3.0	DB ($g.l^{-1}$)	2.28±0.45	2.40±0.84	2.20±0.82	2.55±0.80
	ST (mNm^{-1})	37.9±0.38	42.2±0.71	41.3±0.39	40.4±0.42
	BS ($g.l^{-1}$)	2.27±0.23	1.07±0.56	1.09±0.84	2.07±0.74

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Table 4.10c. Combined effect of two different nitrogen sources on the growth and biosurfactant production of *P. aeruginosa* strains

Bacterial strain	**MSM + H_2NCONH_4 (2.0 $g.l^{-1}$) + $NH_4(SO_4)_2$ (2.0 $g.l^{-1}$)**		
	DB ($g.l^{-1}$)	ST (mNm^{-1})	BS ($g.l^{-1}$)
P. aeruginosa OBP1	4.87±0.63	31.1±0.88	4.57±0.53
P. aeruginosa OBP2*	5.03±0.37	37.6±0.51	2.86±0.28
P. aeruginosa OBP3	4.73±0.72	35.5±0.38	2.83±0.43
P. aeruginosa OBP4	5.10±0.21	33.2±0.79	3.17±0.37

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant.* In case of OBP2, the concentration of $NH_4(SO_4)_2$ was 1.0 $g.l^{-1}$

4.5.6 Effect of macro and micro-nutrients on biosurfactant production

Metal ions are known to play a crucial role in growth and production of biosurfactants as they participate in various metabolic pathways in the form of cofactors of many enzymes. The influence of macronutrients such as Na_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ are presented in Table 4.11-4.14.

Table 4.11. Influence of magnesium sulphate ($MgSO_4.7H_2O$) on growth and biosurfactant production of *P. aeruginosa* strains

Concentration of $MgSO_4.7H_2O$ ($g.l^{-1}$)	**Properties**	***P. aeruginosa* strains**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
0.0	DB ($g.l^{-1}$)	4.23±0.73	4.34±0.38	4.21±0.59	4.41±0.83
	ST (mNm^{-1})	38.6±0.35	43.4±0.76	42.6±0.38	45.0±0.20
	BS ($g.l^{-1}$)	3.95±0.82	2.16±0.13	2.30±0.61	2.55±0.47
0.1	DB ($g.l^{-1}$)	4.59±0.72	4.84±0.37	4.50±0.76	4.87±0.43
	ST (mNm^{-1})	32.5±0.41	38.4±0.30	36.2±0.73	34.1±0.17
	BS ($g.l^{-1}$)	4.39±0.62	2.65±0.76	2.71±0.38	2.94±0.52
0.2	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.83±0.43	3.17±0.37
0.3	DB ($g.l^{-1}$)	4.79±0.57	4.93±0.25	4.67±0.46	4.96±0.60
	ST (mNm^{-1})	31.7±0.50	38.1±0.74	35.8±0.90	33.7±0.35
	BS ($g.l^{-1}$)	4.48±0.94	2.82±0.67	2.78±0.22	3.11±0.71

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Table 4.12. Influence of calcium chloride ($CaCl_2 \cdot 2H_2O$) on growth and biosurfactant production of *P. aeruginosa* strains

Concentration of $CaCl_2 \cdot 2H_2O$ ($mg.l^{-1}$)	**Properties**	***P. aeruginosa* strain**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
0	DB ($g.l^{-1}$)	4.34±0.71	4.56±0.22	4.28±0.53	4.60±0.36
	ST (mNm^{-1})	37.2±0.94	42.7±0.12	41.5±0.29	44.3±0.50

	BS ($g.l^{-1}$)	4.04±0.42	2.20±0.52	2.33±0.73	2.39±0.71
25	DB ($g.l^{-1}$)	4.67±0.49	4.93±0.81	4.57±0.72	4.95±0.64
	ST (mNm^{-1})	31.8±0.32	38.2±0.73	35.9±0.91	34.0±0.37
	BS ($g.l^{-1}$)	4.40±0.63	2.68±0.30	2.74±0.54	3.01±0.52
50	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.83±0.43	3.17±0.37
75	DB ($g.l^{-1}$)	4.77±0.33	4.97±0.83	4.70±0.54	5.04±0.70
	ST (mNm^{-1})	32.0±0.51	38.4±0.65	36.0±0.50	33.8±0.22
	BS ($g.l^{-1}$)	4.51±0.39	2.80±0.73	2.77±0.61	3.09±0.45

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Table 4.13. Influence of potassium dihydrogen phosphate (KH_2PO_4) on growth and biosurfactant production of *P. aeruginosa* strains

Concentration of KH_2PO_4 ($g.l^{-1}$)	**Properties**	***P. aeruginosa* strains**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
0.0	DB ($g.l^{-1}$)	3.87±0.27	4.04±0.55	3.73±0.26	4.14±0.42
	ST (mNm^{-1})	38.7±0.41	43.6±0.82	42.5±1.03	41.0±0.57
	BS ($g.l^{-1}$)	2.78±0.18	1.34±0.54	1.41±0.35	1.56±0.26
0.875	DB ($g.l^{-1}$)	4.52±0.56	4.79±0.98	4.36±0.42	4.81±0.70
	ST (mNm^{-1})	32.9±0.74	38.8±0.14	36.7±0.30	34.2±0.54
	BS ($g.l^{-1}$)	4.32±0.22	2.61±0.63	2.70±0.58	2.89±0.31
1.75	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.83±0.43	3.17±0.37
3.5	DB ($g.l^{-1}$)	4.76±0.47	4.95±0.73	4.65±0.38	5.01±0.52
	ST (mNm^{-1})	31.8±0.75	38.2±0.59	36.0±0.27	33.9±0.44
	BS ($g.l^{-1}$)	4.47±0.38	2.78±0.96	2.75±0.53	3.07±0.90

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Table 4.14. Influence of disodium hydrogen phosphate (Na_2HPO_4) on growth and biosurfactant production of *P. aeruginosa* strains

Concentration of Na_2HPO_4 ($g.l^{-1}$)	Properties	*P. aeruginosa* strains			
		OBP1	OBP2	OBP3	OBP4
0.0	DB ($g.l^{-1}$)	3.28±0.63	3.54±0.82	2.96±0.37	3.66±0.41
	ST (mNm^{-1})	39.5±0.19	44.6±0.41	43.3±0.58	42.0±0.62
	BS ($g.l^{-1}$)	2.08±0.37	1.05±0.66	1.17±0.29	1.26±0.45
1.81	DB ($g.l^{-1}$)	4.48±0.71	4.72±0.37	4.29±0.96	4.75±0.65
	ST (mNm^{-1})	33.7±0.49	39.0±0.80	37.1±0.63	34.9±0.23
	BS ($g.l^{-1}$)	4.22±0.52	2.49±0.56	2.65±0.48	2.83±0.74
3.61	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.83±0.43	3.17±0.37
7.22	DB ($g.l^{-1}$)	4.73±0.84	4.90±0.55	4.60±0.39	4.87±0.21
	ST (mNm^{-1})	32.2±1.02	38.5±0.94	36.2±0.67	34.7±0.83
	BS ($g.l^{-1}$)	4.40±0.76	2.76±0.38	2.69±0.25	3.00±0.57

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

The concentrations of Na_2HPO_4, KH_2PO_4, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ at 3.6 $g.l^{-1}$, 1.75 $g.l^{-1}$, 0.2 $g.l^{-1}$, 1.0 $mg.l^{-1}$ and 50.0 $mg.l^{-1}$, respectively were found to be optimum for the production of biosurfactant by the bacterial strains.

The use of negative control having no micronutrients in the culture media and application of 100 $\mu l.l^{-1}$ of each of the stock solution of $CuSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 5H_2O$, H_3BO_3, $ZnSO_4 \cdot 7H_2O$ and MnO_3 in 1l of MSM was found to be effective in growth and biosurfactant production of *P. aeruginosa* strains and the data are presented in Table 4.15.

Table 4.15. Influence of trace elements on growth and biosurfactant production of *P. aeruginosa* strains

Micronutrients * ($\mu l.l^{-1}$)	Properties	*P. aeruginosa* strains			
		OBP1	OBP2	OBP3	OBP4
0	DB ($g.l^{-1}$)	4.54±0.83	4.67±0.52	4.38±0.77	4.66±0.19
	ST (mNm^{-1})	35.8±0.20	41.0±0.49	39.5±0.41	38.7±0.62

	BS ($g.l^{-1}$)	4.24±0.62	2.38±0.37	2.44±0.83	2.81±0.70
50	DB ($g.l^{-1}$)	4.79±0.62	4.94±0.27	4.67±0.71	5.02±0.58
	ST (mNm^{-1})	31.7±0.80	38.2±0.15	36.0±0.98	33.8±0.37
	BS ($g.l^{-1}$)	4.50±0.31	2.80±0.66	2.78±0.43	3.11±0.65
100	DB ($g.l^{-1}$)	4.87±0.63	5.03±0.37	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	37.6±0.51	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.86±0.28	2.83±0.43	3.17±0.37
200	DB ($g.l^{-1}$)	4.83±0.73	5.02±0.50	4.70±0.27	5.07±0.83
	ST (mNm^{-1})	31.6±0.40	38.0±0.97	35.8±0.63	33.7±0.40
	BS ($g.l^{-1}$)	4.54±1.02	2.83±0.58	2.78±0.75	3.13±0.81

* Stock solution of the micronutrients solution was prepared as described in the Appendix I. Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

4.5.7 Effect of temperature on biosurfactant production

The bacterial strains could grow and produce biosurfactant in almost all temperatures applied and data thus obtained are presented in Table 4.16.

Table 4.16. Influence of temperature (°C) on growth and biosurfactant production of *P. aeruginosa* strains

Temperature (°C)	**Properties**	***P. aeruginosa* strains**			
		OBP1	**OBP2**	**OBP3**	**OBP4**
35	DB ($g.l^{-1}$)	4.45±0.56	4.52±0.62	4.33±0.55	4.92±0.18
	ST (mNm^{-1})	32.8±0.24	38.8±0.76	36.3±0.39	35.0±0.98
	BS ($g.l^{-1}$)	4.11±0.32	2.33±0.51	2.51±0.52	3.04±0.36
37	DB ($g.l^{-1}$)	4.87±0.63	4.95±0.38	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	38.1±0.41	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.82±0.82	2.83±0.43	3.17±0.37
40	DB ($g.l^{-1}$)	4.85±0.64	5.03±0.37	4.68±0.82	5.05±0.28
	ST (mNm^{-1})	31.1±0.28	37.6±0.51	36.1±0.45	34.3±0.80
	BS ($g.l^{-1}$)	4.50±0.83	2.86±0.28	2.78±0.71	3.07±0.31
42	DB ($g.l^{-1}$)	1.08±0.77	2.65±0.80	1.02±0.29	1.10±0.40
	ST (mNm^{-1})	49.8±0.41	40.4±0.62	50.7±1.04	49.2±0.12
	BS ($g.l^{-1}$)	0.95±0.94	1.28±0.27	0.78±0.83	1.03±0.56

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

Biosurfactant production increased with an increase in temperature from 30–37°C, remained nearly constant at 37–40°C and then decreased when temperature was increased further to above 40°C. The optimal temperature for growth and biosurfactant production was found to be 37°C in the case of bacterial strains OBP1, OBP3 and OBP4, but the strain OBP2 exhibited maximum growth and production of biosurfactant at 40°C.

4.5.9 Effect of pH on biosurfactant production

The influence of the initial pH of the culture medium on the production of biosurfactant by the bacterial strains was determined and data is presented in Table 4.17.

Table 4.17. Influence of pH on growth and biosurfactant production

pH	Properties	*P. aeruginosa* strains			
		OBP1	OBP2	OBP3	OBP4
6.5	DB ($g.l^{-1}$)	4.66±0.83	4.50±0.28	4.57±0.35	4.92±0.38
	ST (mNm^{-1})	31.8±0.39	39.8±0.62	36.4±0.82	34.7±0.20
	BS ($g.l^{-1}$)	4.43±0.27	2.24±0.70	2.70±0.80	3.03±0.25
6.8	DB ($g.l^{-1}$)	4.87±0.63	4.91±0.56	4.73±0.72	5.10±0.21
	ST (mNm^{-1})	31.1±0.88	38.5±0.24	35.5±0.38	33.2±0.79
	BS ($g.l^{-1}$)	4.57±0.53	2.60±0.40	2.83±0.43	3.17±0.37
7.0	DB ($g.l^{-1}$)	4.80±0.54	5.03±0.37	4.68±0.38	5.01±0.35
	ST (mNm^{-1})	32.4±0.71	37.6±0.51	36.0±0.75	33.8±0.22
	BS ($g.l^{-1}$)	4.50±0.19	2.86±0.28	2.79±0.49	3.06±0.57
7.2	DB ($g.l^{-1}$)	4.48±0.42	4.58±0.72	4.25±0.33	4.55±0.81
	ST (mNm^{-1})	35.7±0.86	38.4±0.25	39.2±0.71	36.2±0.72
	BS ($g.l^{-1}$)	3.94±0.55	2.77±0.80	2.31±0.29	2.85±0.40

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

The bacterial strains could grow in all the tested pH values; however growth and biosurfactant production got reduced at higher acidic and alkaline pH levels. Growth and production of biosurfactants were better at slightly acidic to neutral pH values. The optimal pH was found to be 6.8 except for OBP2 which exhibited optimal growth and biosurfactant production at a pH of 7.0

4.5.9 Effect of shaking on biosurfactant production

The influence of shaking on the production of biosurfactant under the different agitation rates ranging from 100-220 rpm is presented in the Table 4.18.

Table 4.18. Influence of agitation (rpm) on growth and biosurfactant production of *P. aeruginosa* strains

Agitation (rpm)	Properties	*P. aeruginosa* strains			
		OBP1	OBP2	OBP3	OBP4
0	DB ($g.l^{-1}$)	2.63±0.73	2.94±0.83	2.56±0.15	2.19±0.59
	ST (mNm^{-1})	40.7±0.63	44.7±0.49	43.6±0.62	44.3±0.35
	BS ($g.l^{-1}$)	0.94±0.37	0.73±0.56	0.65±0.39	0.76±0.27
100	DB ($g.l^{-1}$)	4.16±0.52	4.25±0.35	4.27±0.58	4.05±0.43
	ST (mNm^{-1})	39.8±0.66	42.7±0.48	40.8±0.31	41.6±0.52
	BS ($g.l^{-1}$)	1.21±0.42	0.83±0.30	0.98±0.92	0.90±0.61
120	DB ($g.l^{-1}$)	4.22±0.73	4.48±0.41	4.34±0.89	4.37±0.26
	ST (mNm^{-1})	34.9±0.52	39.2±0.20	38.5±0.42	39.2±0.47
	BS ($g.l^{-1}$)	4.23±0.35	2.47±0.28	2.43±0.56	2.47±0.39
150	DB ($g.l^{-1}$)	4.65±0.39	4.94±0.72	4.51±0.53	4.76±0.80
	ST (mNm^{-1})	32.7±0.92	38.8±0.38	36.8±0.71	37.5±0.45
	BS ($g.l^{-1}$)	4.39±0.70	2.73±0.47	2.69±0.95	2.88±0.37
180	DB ($g.l^{-1}$)	4.87±0.34	5.03±0.56	4.73±0.74	4.90±0.35
	ST (mNm^{-1})	31.1±0.85	37.6±0.24	35.5±0.42	34.8±0.82
	BS ($g.l^{-1}$)	4.57±0.85	2.86±0.30	2.83±0.71	3.04±0.39
200	DB ($g.l^{-1}$)	4.71±0.61	4.87±0.99	4.58±0.37	5.10±0.37
	ST (mNm^{-1})	31.8±0.38	38.4±0.52	36.7±0.61	33.2±0.65
	BS ($g.l^{-1}$)	4.45±0.22	2.70±0.49	2.65±0.30	3.17±0.43
220	DB ($g.l^{-1}$)	3.93±0.47	4.04±0.29	3.86±0.39	4.82±0.48
	ST (mNm^{-1})	33.0±0.31	39.3±0.42	38.5±0.15	35.7±1.22
	BS ($g.l^{-1}$)	3.68±0.55	2.32±0.71	2.24±0.44	2.90±0.81

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

With increase in shaking from 100 to 180 rpm, biosurfactant production increased sharply with higher cell growth. However, increasing of shaking above 180 rpm caused heavy foaming and reduced the level of biosurfactant production. The bacterial strains showed higher growth and biosurfactant production at the optimum

shaking speed of 180 rpm except for OBP4 which exhibited optimal growth and biosurfactant production at 200 rpm.

4.5.10 Effect of low cost carbon substrate in biosurfactant production

The bacterial strains were able to utilize vegetable oils. However, in terms of bacterial biomass and biosurfactant production, bacterial strain OBP1 was found to be the best. Hence, for the further studies the strain OBP1 was selected for screening the different inedible vegetable oils as carbon substrate for the production of biosurfactant. Data obtained from the experiment are presented in Table 4.19.

Table 4.19. Influence of various inedible vegetable oil as carbon source on biosurfactant production of *P. aeruginosa* OBP1

Vegetable oil	DB ($g.l^{-1}$)	ST (mNm^{-1})	BS ($g.l^{-1}$)
Jatropha curcas	1.88±0.6	40.6±1.2	1.02±0.9
Mesua ferrea	3.84±0.3	36.4±0.4	2.34±0.3
Ricinus communis	3.47±0.8	38.3±0.8	2.01±0.6
Sesamum indicum	4.42±0.5	37.1±0.2	2.57±0.7
Pongamia glabra	2.56±0.3	39.8±0.8	1.76±0.5

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant.

The inedible vegetable oils such as sesame seed oil followed by nahor seed oil proved to be promising for the production of biosurfactants and Table 4.19 shows the same. Among the other tested substrates, waste glycerol followed by petroleum refinery sludge and waste residual kitchen oil were found to be suitable for the production of biosurfactant and the data thus obtained are presented in Table 4.20

Table 4.20. Influence of various low cost carbon substrates on biosurfactant production of *P. aeruginosa* strains

Carbon sources	Properties	*P. aeruginosa* strains			
		OBP1	OBP2	OBP3	OBP4
Waste glycerol of biodiesel	DB ($g.l^{-1}$)	3.28±0.76	2.75±0.51	2.63±0.28	3.40±0.62
	ST (mNm^{-1})	37.6±0.40	33.7±0.80	39.5±0.63	37.0±0.38

	BS ($g.l^{-1}$)	1.85±0.56	3.90±0.42	1.52±0.51	2.24±0.18
De-oiled mustard seed cakes	DB ($g.l^{-1}$)	2.54±0.80	1.16±0.37	1.03±0.90	0.95±0.74
	ST (mNm^{-1})	39.8±0.32	44.5±0.59	49.5±0.72	49.3±0.58
	BS ($g.l^{-1}$)	1.48±0.64	0.47±0.83	0.27±0.25	0.28±0.95
Waste residual molasses	DB ($g.l^{-1}$)	5.07±0.55	5.02±0.83	4.97±0.56	5.04±0.51
	ST (mNm^{-1})	50.4±0.37	50.6±0.59	51.2±0.75	51.7±0.43
	BS ($g.l^{-1}$)	0.14±0.45	0.10±0.35	0.18±0.62	0.16±0.59
Sugarcane bagasse	DB ($g.l^{-1}$)	2.46±0.57	2.52±0.70	2.48±0.45	2.41±0.52
	ST (mNm^{-1})	51.8±0.38	52.4±0.47	52.7±0.80	51.5±0.35
	BS ($g.l^{-1}$)	0.13±0.40	0.12±0.59	0.07±0.19	0.18±0.22
Waste residual kitchen oil	DB ($g.l^{-1}$)	2.98±0.55	1.48±0.38	1.28±0.50	1.07±0.41
	ST (mNm^{-1})	37.3±0.36	40.3±0.20	45.6±0.71	42.7±0.35
	BS ($g.l^{-1}$)	2.26±0.70	0.91±0.56	0.44±0.43	0.68±0.70
Petroleum refinery sludge	DB ($g.l^{-1}$)	3.10±0.25	3.27±0.81	4.02±0.60	3.22±0.95
	ST (mNm^{-1})	37.0±0.32	39.8±0.70	37.6±0.83	36.5±0.52
	BS ($g.l^{-1}$)	1.96±0.60	1.03±0.46	1.85±0.79	2.33±0.23

Results represent mean ± S.D of three individual experiments. NB: DB, dry biomass; ST, surface tension; BS, yield of biosurfactant

4.6 Physical characterization of biosurfactant

4.6.1 Reduction in surface, interfacial tension (IFT) and critical micelle concentration (CMC)

The biosurfactants produced by *P. aeruginosa* strains were able to reduce the surface tension of the culture medium. Data are presented in Table 4.21. Surface tension of the culture medium as acted by the inoculated bacterial strains was drastically reduced from 68.5 to about 31.1mNm^{-1}.

Table 4.21. Properties of biosurfactant produced by *P. aeruginosa* strains

Bacterial strains	BS ($g.l^{-1}$)	ST (mNm^{-1})	IFT[a] (mNm^{-1})	CMC ($mg.l^{-1}$)
P. aeruginosa OBP1	4.57±0.65	31.1±0.88	1.5±0.65	45±0.86
P. aeruginosa OBP2	2.86±0.79	37.6±0.51	3.4±0.48	105±0.34
P. aeruginosa OBP3	2.83±0.63	35.5±0.38	2.8±0.93	90±0.58
P. aeruginosa OBP4	3.17±0.37	33.2±0.79	2.2±0.28	65±0.94

Results represent mean ± S.D of three individual experiments. NB: BS, yield of biosurfactant; ST, surface tension; IFT[a], interfacial tension against diesel; CMC, critical micelle concentration

Reductions in the IFT of diesel containing culture supernatant of four bacterial strains as compared to the control culture medium without any bacteria are presented in Table 4.21. The minimum IFT of the culture supernatant containing the biosurfactant of OBP1, OBP2, OBP3 and OBP4 was 1.5, 3.4, 2.8 and 2.2 mNm^{-1}, respectively.

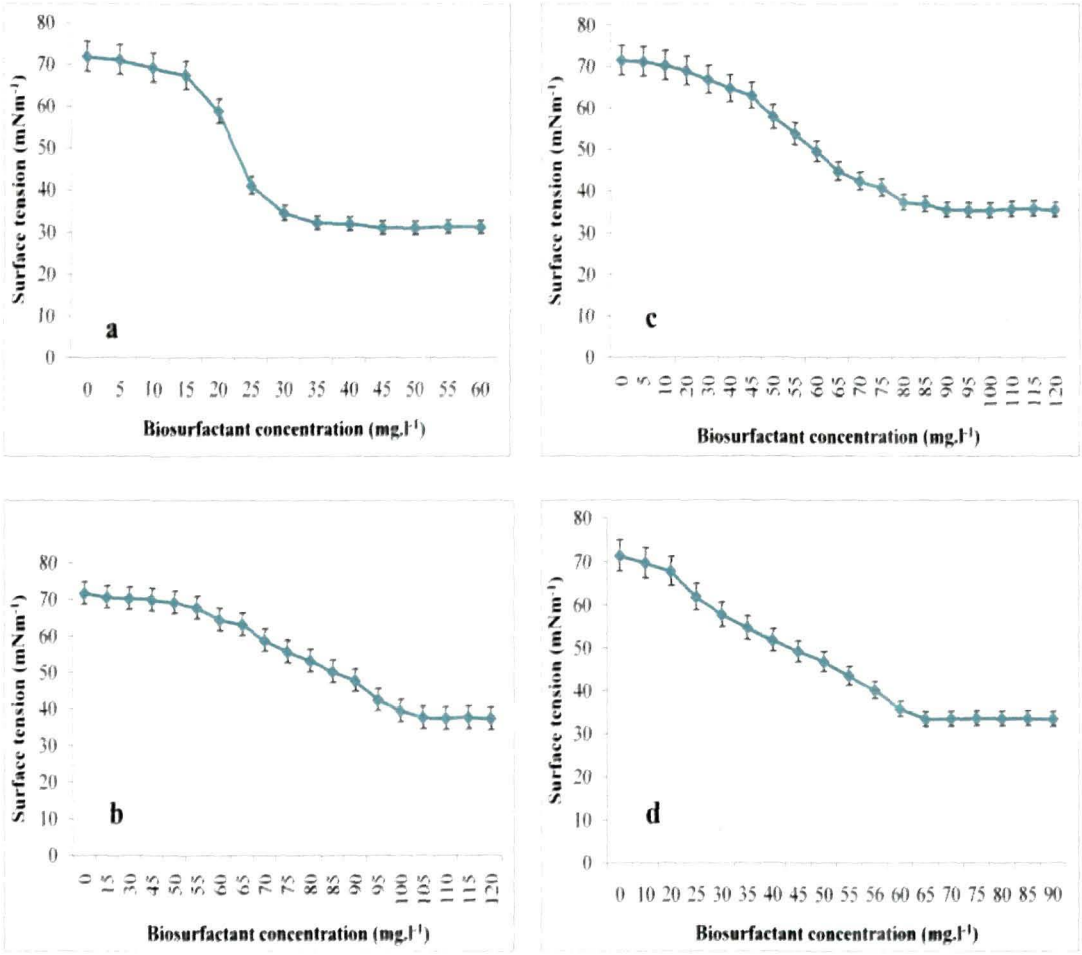


Figure 4.8. Determination of critical micelle concentration (CMC) of the biosurfactants produced by *P. aeruginosa* strains. Results represent the mean of three independent experiments ± standard deviation. (a) *P. aeruginosa* OBP1, (b) *P. aeruginosa* OBP2, (c) *P. aeruginosa* OBP3 and (d) *P. aeruginosa* OBP4

The CMC values were determined by diluting the isolated biosurfactant solution of the bacterial strains to several times in sterile distilled water. The values were found to be in the range of 45-105 $mg.l^{-1}$ and are presented in Table 4.21 and Figure 4.8. The surface tension values at the CMC for the biosurfactant of OBP1, OBP2, OBP3 and OBP4 were in the order of 31.1, 37.6, 35.5 and 33.2 mNm^{-1}, respectively. After the attainment of the CMC no further reduction in the surface tension was observed.

4.6.2 Influence of temperature, pH, salinity and metal ion concentration on the surface activity of isolated biosurfactant

The cell-free culture supernatant containing the biosurfactant showed almost stable surface activity over a wide range of pH values. The effects of pH on biosurfactants were determined at normal concentration, CMD^{-1} (10 times dilution) and CMD^{-2} (100 times dilution). Data are presented in Table 4.22.

Table 4.22. Influence of pH on the surface activity of biosurfactant produced by *P. aeruginosa* strains in 2% n-hexadecane supplemented medium at normal and critical micelle dilutions (CMD^{-1} and CMD^{-2})

Bacterial strains	pH	Surface tension (mN/m)		
		Cell-free culture supernatant	CMD^{-1}	CMD^{-2}
P. aeruginosa OBP1	2	34.8±1.00	35.6±0.32	48.2±0.46
	5	31.8±0.42	32.0±0.56	42.5±0.49
	7	31.1±0.37	31.8±0.47	41.9±0.55
	8	31.2±0.22	31.9±0.28	42.8±0.42
	11	32.6±0.28	33.8±0.14	45.6±0.65
P. aeruginosa OBP2	2	41.5±0.58	48.9±0.78	63.7±0.31
	5	37.8±0.35	41.0±0.23	58.3±0.46
	7	37.6±0.11	40.7±0.83	57.6±0.29
	8	38.1±0.34	41.5±0.69	58.8±0.36
	11	39.7±0.53	43.2±0.45	61.0±0.53
P. aeruginosa OBP3	2	40.4±0.36	46.3±0.93	63.5±0.29
	5	35.7±0.31	38.7±0.67	56.8±0.49
	7	35.4±0.19	38.2±0.30	55.6±1.00

	8	36.5±0.83	39.5±0.47	57.4±0.19
	11	38.7±0.62	42.5±0.61	60.8±0.38
P. aeruginosa OBP4	2	38.5±0.43	43.3±0.60	57.1±0.51
	5	33.7±0.76	39.8±0.71	50.3±0.94
	7	33.1±0.93	39.5±0.35	48.7±0.54
	8	33.6±0.51	39.7±0.29	51.4±0.66
	11	35.8±0.23	41.4±0.72	54.9±0.39

The data shown here are mean values of triplicates

Biosurfactants at normal and CMD^{-1} concentrations showed no significant difference in their surface activity at all the tested pH levels. However, the concentration at CMD^{-2} exhibited reduction in the surface activity due to the lower concentration which leads to increase in surface tension. The activity of the biosurfactants produced by the bacterial strains was found to be optimum between the pH of 5-8. Extreme pH below 5 and above 8 caused increased surface tension.

The cell free culture supernatant of the bacterial strains retained the surface activity even after 60 min of incubation at temperatures ranging from 4-100°C. The cell free culture supernatants remained effective even after autoclaving at 121°C for 30 min. Concentrations of the supernatant at CMD^{-1} and CMD^{-2} on exposure to temperatures 4-100°C for 60 min exhibited almost stable surface tension. Data obtained are presented in Table 4.23.

Table 4.23. Influence of temperature on the surface activity of biosurfactant produced by *P. aeruginosa* strains in 2% n-hexadecane supplemented medium at normal and critical micelle dilutions (CMD^{-1} and CMD^{-2})

Bacterial strains	**Exposure to 60 min at the temperature (°C)**	**Surface tension (mN/m)**		
		Cell-free culture supernatant	**CMD^{-1}**	**CMD^{-2}**
P. aeruginosa OBP1	4	32.3±0.23	33.5±0.39	42.7±0.92
	25	31.2±0.62	31.3±0.19	41.9±0.36
	37	31.1±0.56	31.5±0.17	41.6±1.20
	50	31.5±0.72	31.8±0.39	41.9±0.39
	75	31.8±0.18	32.3±0.40	42.4±0.41
	100	32.0±1.23	36.3±0.94	47.2±0.55

	121 (for 30 min)	32.3±0.81	35.9±0.40	47.7±0.56
P. aeruginosa OBP2	4	38.3±0.41	43.0±0.25	58.1±0.29
	25	37.5±0.38	40.3±0.48	57.6±0.38
	37	37.6±0.28	40.8±0.39	57.5±0.65
	50	37.8±0.37	41.1±0.52	57.8±0.75
	75	38.0±0.51	41.6±0.41	58.2±0.28
	100	39.4±0.20	43.9±0.40	59.3±0.47
	121 (for 30 min)	39.2±0.51	44.2±0.35	59.1±0.76
P. aeruginosa OBP3	4	36.8±0.39	39.6±0.77	56.8±0.87
	25	35.5±0.38	38.4±0.93	55.3±0.54
	37	35.3±0.40	38.3±0.38	55.5±0.39
	50	35.3±0.19	38.7±0.84	55.9±0.82
	75	35.9±0.36	39.4±0.62	56.4±0.39
	100	38.3±0.72	41.5±0.65	59.2±0.35
	121 (for 30 min)	38.2±0.10	41.8±0.94	59.6±0.62
P. aeruginosa OBP4	4	34.7±0.39	40.6±0.54	49.5±0.82
	25	33.2±0.91	39.8±0.77	48.7±0.22
	37	33.2±0.32	39.7±0.83	48.8±0.91
	50	33.5±0.17	39.7±0.39	49.2±0.30
	75	33.7±0.63	40.2±0.98	49.7±0.45
	100	34.0±0.72	43.8±0.74	51.4±0.76
	121 (for 30 min)	34.6±0.83	44.1±0.59	51.2±0.98

The data shown here are mean values of triplicates

At CMD^{-2}, the cell free culture supernatant exhibited comparatively lesser surface activity than CMD^{-1} due to lowering of surfactant concentration. The cell free culture supernatants at CMD^{-1} and CMD^{-2} remained effective similar to that of normal concentration even after autoclaving at 121°C for 30 min.

The biosurfactant retained its surface activity by reducing the surface tension upto a concentration of 4% NaCl and the effect of salinity on biosurfactants was determined at normal, CMD^{-1} and CMD^{-2} concentrations. Results thus obtained are shown in Table 4.24.

Table 4.24. Influence of salinity on the surface activity of biosurfactant produced by *P. aeruginosa* strains in 2% n-hexadecane supplemented medium at normal and critical micelle dilutions (CMD^{-1} and CMD^{-2})

Bacterial strains	NaCl Concentration (g %)	Surface tension (mN/m)		
		Cell-free culture supernatant	CMD^{-1}	CMD^{-2}
P. aeruginosa OBP1	0	31.2±0.87	31.4±0.56	41.7±0.92
	2	31.4±0.36	31.3±0.20	41.3±0.39
	3	31.7±0.90	31.8±0.54	42.2±0.49
	4	34.5±0.13	39.3±0.73	48.2±0.28
	5	40.4±0.64	42.6±0.29	53.7±0.36
P. aeruginosa OBP2	0	37.4±0.43	40.6±0.48	57.7±0.26
	2	37.6±0.26	40.9±0.71	57.5±0.40
	3	37.9±0.39	41.0±0.39	57.8±0.40
	4	40.5±0.48	43.7±0.51	59.6±1.00
	5	46.6±0.65	50.3±0.30	61.9±0.49
P. aeruginosa OBP3	0	35.4±0.28	38.3±0.83	55.5±0.72
	2	35.4±0.76	38.5±0.62	55.6±0.49
	3	35.7±0.39	39.9±0.96	55.6±0.49
	4	37.3±0.49	41.0±0.70	58.3±0.02
	5	43.1±0.65	48.8±0.52	59.8±0.39
P. aeruginosa OBP4	0	33.1±0.38	39.7±0.30	48.6±0.56
	2	33.3±0.29	39.6±0.73	48.6±0.75
	3	33.6±0.48	39.7±0.40	48.9±0.95
	4	36.0±0.20	42.5±0.67	50.4±0.34
	5	39.6±0.29	48.2±0.93	53.7±0.94

The data shown here are mean values of triplicates

The reduction in surface tension of the cell free culture supernatant was almost similar to that of CMD^{-1} referring to the intact efficiency of biosurfactant at CMD^{-1}. At higher dilution of CMD^{-2} the cell free culture supernatant exhibited significant activity indicating their efficiency even at lower concentrations. However, the level of surface activity was comparatively lower than that of normal and CMD^{-1}. The cell-free culture supernatant of the bacterial strains were treated with different metal ions like K^+, Ca^{2+}, Mg^{2+}, Fe^{2+} and Al^{3+} (2% w/v). The culture supernatant at CMD^{-1} and CMD^{-2} on

exposure to the different metal ions exhibited almost stable surface tensions. Data obtained are presented in Table 4.25.

Table 4.25. Influence of metal ions on the surface activity of biosurfactant produced by *P. aeruginosa* strains in 2% n-hexadecane supplemented medium at normal and critical micelle dilutions (CMD^{-1} and CMD^{-2})

Bacterial strains	**Exposure to metal ions (2% w/v)**	**Surface tension (mN/m)**		
		Cell-free culture supernatant	**CMD^{-1}**	**CMD^{-2}**
P. aeruginosa OBP1	K^+	31.2±0.40	37.4±0.75	45.7±0.60
	Ca^{2+},	31.1±0.58	36.3±0.37	45.6±0.45
	Mg^{2+},	31.3±0.67	36.5±0.53	45.7±0.60
	Fe^{2+}	31.2±0.22	36.3±0.63	45.5±0.45
	Al^{3+}	34.7±0.36	38.9±0.52	48.8±0.69
P. aeruginosa OBP2	K^+	37.6±0.54	43.2±0.56	58.2±0.45
	Ca^{2+},	37.5±0.65	42.4±0.23	57.5±0.23
	Mg^{2+},	37.4±0.83	42.7±0.75	57.4±0.34
	Fe^{2+}	37.7±0.31	43.4±0.32	57.8±0.62
	Al^{3+}	39.3±0.53	46.5±0.69	60.7±0.42
P. aeruginosa OBP3	K^+	35.7±0.67	41.5±0.98	52.5±0.51
	Ca^{2+},	35.5±0.34	40.5±0.54	51.6±0.11
	Mg^{2+},	35.3±0.37	40.5±0.29	51.5±0.27
	Fe^{2+}	35.2±0.83	40.6±0.78	51.7±0.39
	Al^{3+}	37.7±0.56	43.2±0.43	53.8±0.83
P. aeruginosa OBP4	K^+	33.5±0.29	39.7±0.93	47.0±0.36
	Ca^{2+},	33.2±0.65	39.4±0.53	46.9±0.73
	Mg^{2+},	33.3±0.56	39.6±0.77	46.7±0.29
	Fe^{2+}	33.4±0.93	39.5±0.49	46.9±0.60
	Al^{3+}	35.8±0.37	41.6±0.61	49.2±0.35

The data shown here are mean values of triplicates

In the case of trivalent metal (Al^{3+}) there was a significant reduction in the surface activities of biosurfactants at all concentrations.

4.6.3 Emulsification activity (E_{24}%)

The emulsification activity of the biosurfactants present in the culture supernatant of the bacterial strains was assessed against the different hydrocarbons and is presented in Figure4.9.

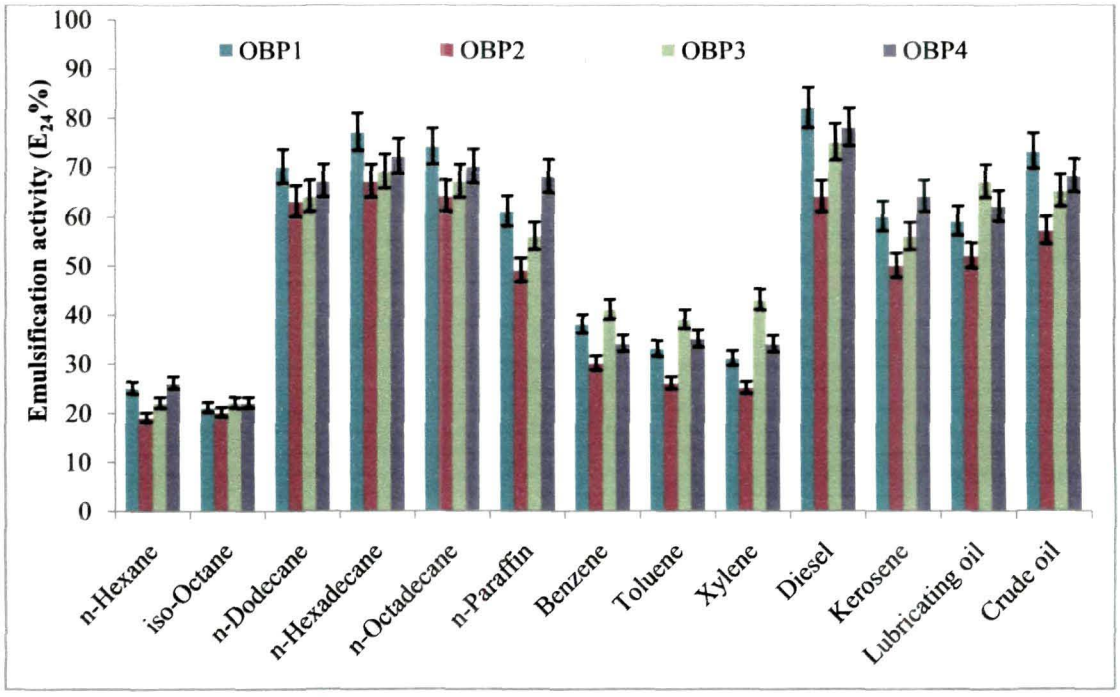
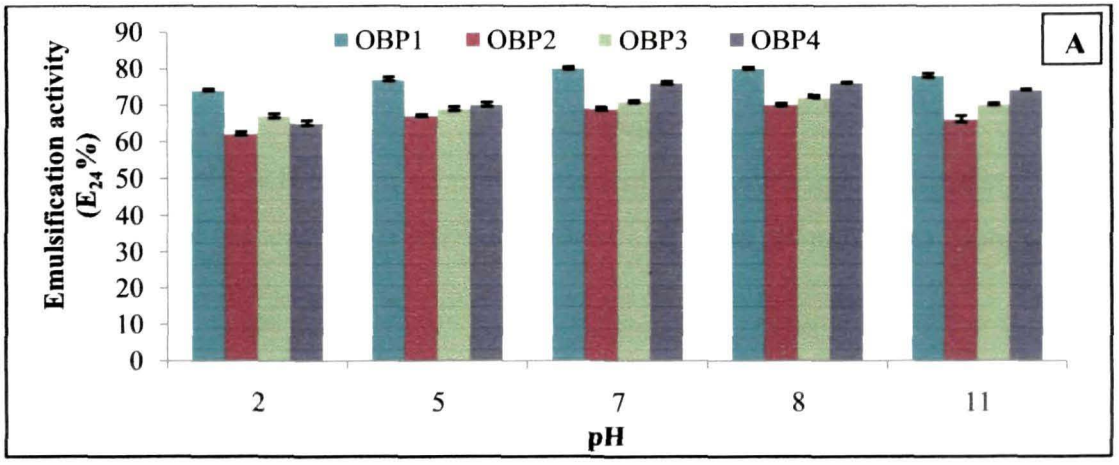


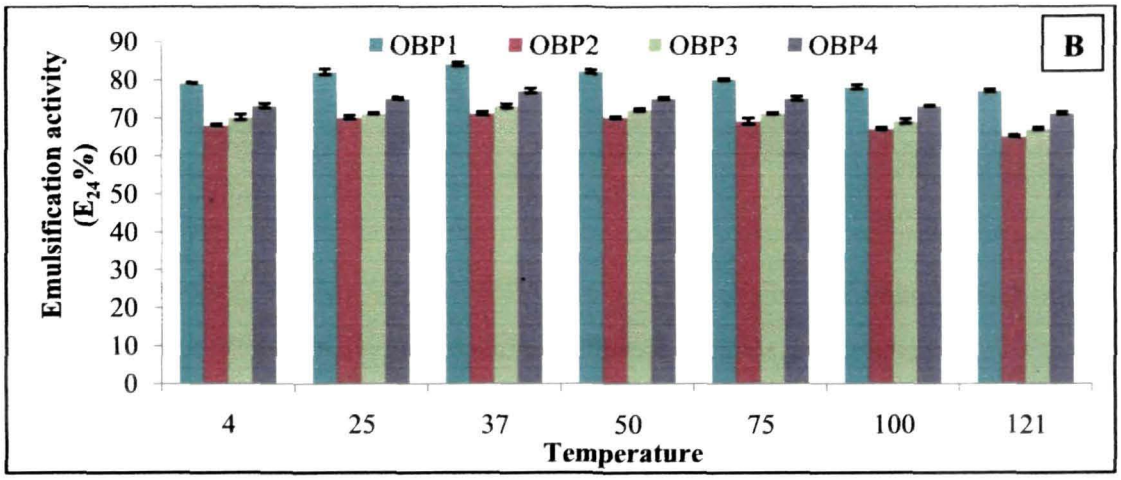
Figure 4.9. Emulsification indices (E_{24}%) exhibited by the culture supernatants of *P. aeruginosa* strains with various hydrophobic substrates. Results represent the mean of three independent experiments ± standard deviation

The bacterial strains showed wide difference in the emulsification activity against the test hydrocarbons. However, the cell free culture supernatant of the bacterial strains exhibited appreciable emulsification indices against diesel, n-hexadecane, n-octadecane, crude oil, n-dodecane, lubricating oil, n-paraffin and kerosene were in the range of 64-82%, 67-77%, 64-74%, 57-73%, 63-70%, 52-67%, 49-68%, and 50-64% respectively. Retention of emulsions even after 30 days indicates the formation of a relatively stable emulsion. It was observed that the cell free culture supernatants of the bacterial strains couldn't emulsify the iso-octane upto a significant level.

4.6.4 Influence of temperature, pH, and salinity on the emulsification activity (E_{24}%)

The E_{24} of the cell free culture supernatant of the bacterial strains against diesel was quite stable at all pH levels; but the maximum activity was shown in the pH range of 5-8 (Figure 10a). E_{24} of the diesel supplemented cell free culture supernatant of the bacterial strains was quite stable at all temperatures from 4-100°C (Figure 10b). The E_{24} was also stable upto 30 days even when stored at 4°C. It is interesting to note that the biosurfactant retained its emulsifying activity even after heating at the autoclaving temperature of 121°C for 30 min, indicating the thermal stability of the cell-free culture supernatant of the bacterial strains. Further, The E_{24} against diesel also remained unchanged over the tested NaCl concentrations from 2-4% (Figure 10c).





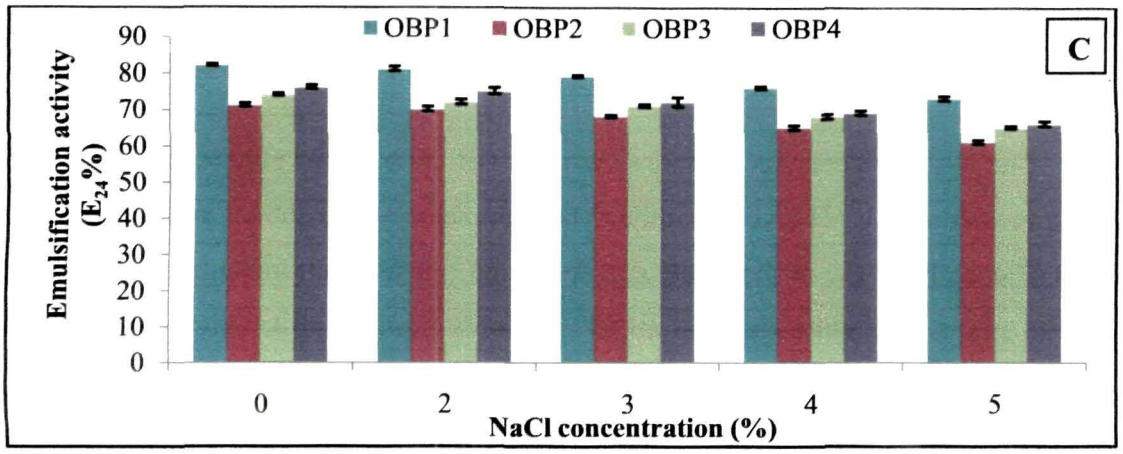


Figure 4.10. Effect of (A) pH, (B) temperature and (C) salinity (NaCl) on the emulsifying properties (E_{24}%) of culture supernatants of *P. aeruginosa* strains against diesel. Results represent the mean of three independent experiments ± standard deviation

4.6.5 Foaming index (F_{24}%)

The cell free culture supernatants of the bacterial strains produced stable foam with the foaming index (F_{24}%) in the range of 50.4-65.5%. F_{24}% is presented in Figure 4.11.

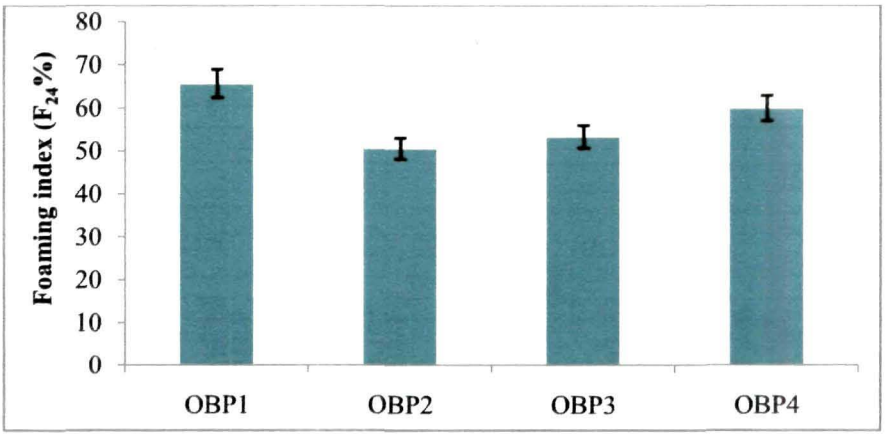


Figure 4.11. Foaming indices (F_{24}%) exhibited by the culture supernatant of *P. aeruginosa* strains. Results represent the mean of three independent experiments ± standard deviation

Foams produced by the cell free culture supernatants of all four bacterial strains remained relatively stable upto 24 h.

4.6.6 Cell surface hydrophobicity

Hydrophobicity of the bacterial cell surfaces growing on two different carbon sources such as glucose and n-hexadecane was determined and is presented in Figure 4.12. All the bacterial strains possessed wide extent of variability in their surface hydrophobicity against the tested hydrocarbons. The surface hydrophobicity of four strains of *P. aeruginosa* cells growing on n-hexadecane was observed to be much higher as compared to the bacterial strains growing on glucose containing medium. Further, it was observed that the cell surface hydrophobicity of the bacterial cells at the exponential growth phase was much lower than that of the bacterial cells at the stationary phase of growth.

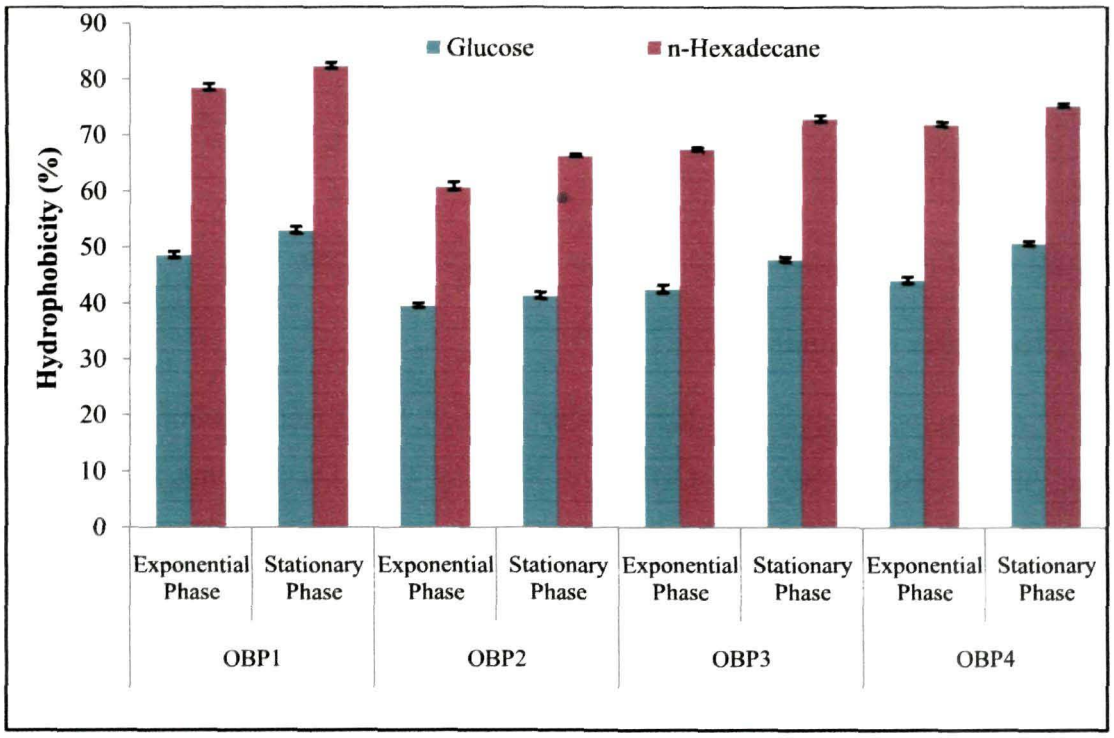


Figure 4.12. Hydrophobicity of bacterial strains at exponential and stationary phase of growth when cultivated in mineral salt medium supplemented with 2% n-hexadecane or glucose. Values are the mean of three independent experiments ± standard deviation

4.7 Chemical characterization of isolated biosurfactants

4.7.1 Biochemical characterization of isolated biosurfactants

Biochemical characterization of isolated crude biosurfactants from four bacterial strains is presented in Table 4.26. The isolated biosurfactants were glycolipid in nature.

Table 4.26. Biochemical characterization of biosurfactant produced by *P. aeruginosa* strains in mineral salt medium supplemented with 2% n-hexadecane

Biosurfactant sample	Carbohydrate content (%)	Lipid content (%)	Protein content (%)
OBP1	48.0±0.3	28.7±0.2	23.3±0.2
OBP2	51.7±0.1	28.3±0.6	20.0±0.1
OBP3	47.8±0.6	30.8±0.6	21.4±0.1
OBP4	49.3±0.2	29.0±0.5	21.7±0.4

Results represented mean ± S.D of three individual experiments

4.7.2 Thin layer chromatography (TLC)

Qualitative analysis of the partially purified biosurfactant samples isolated from OBP1, OBP2, OBP3 and OBP4 was done by TLC. On spraying with orcinol reagents, brown colored spots indicative of carbohydrate units were detected in silica plates. While exposing to the similar plates with iodine vapor, yellow spots indicative of lipids giving same R_f value as that of glycosyl units were observed on the same region and the same are presented in Figure 4.13. The biosurfactant of OBP1 showed the presence of four spots having R_f values: 0.26, 0.52 and 0.67. In the case of OBP2, only three spots with R_f values 0.33, 0.40 and 0.71 appeared. Three spots with almost similar R_f values 0.34, 0.55 and 0.73 were observed in the biosurfactant of OBP3 and OBP4.

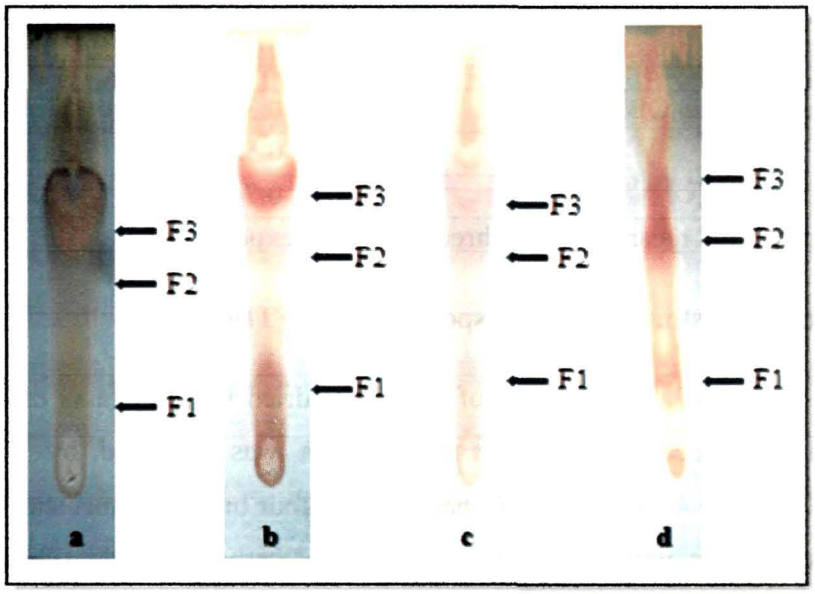


Figure 4.13. Thin-layer chromatogram of biosurfactants produced by *P. aeruginosa* strains when cultivated in mineral salt medium supplemented with n-hexadecane. (a) *P. aeruginosa* OBP1, (b) *P. aeruginosa* OBP2, (c) *P. aeruginosa* OBP3 and (d) *P. aeruginosa* OBP4. F1-F3 represents fractions that exhibit positive results of surface activity

For further purification of the isolated biosurfactants, preparative TLC plates were used to collect those fractions which exhibited the high surface activity in water and are shown in Table 4.27.

Table 4.27. TLC separation of partially purified biosurfactant produced by *P. aeruginosa* strains

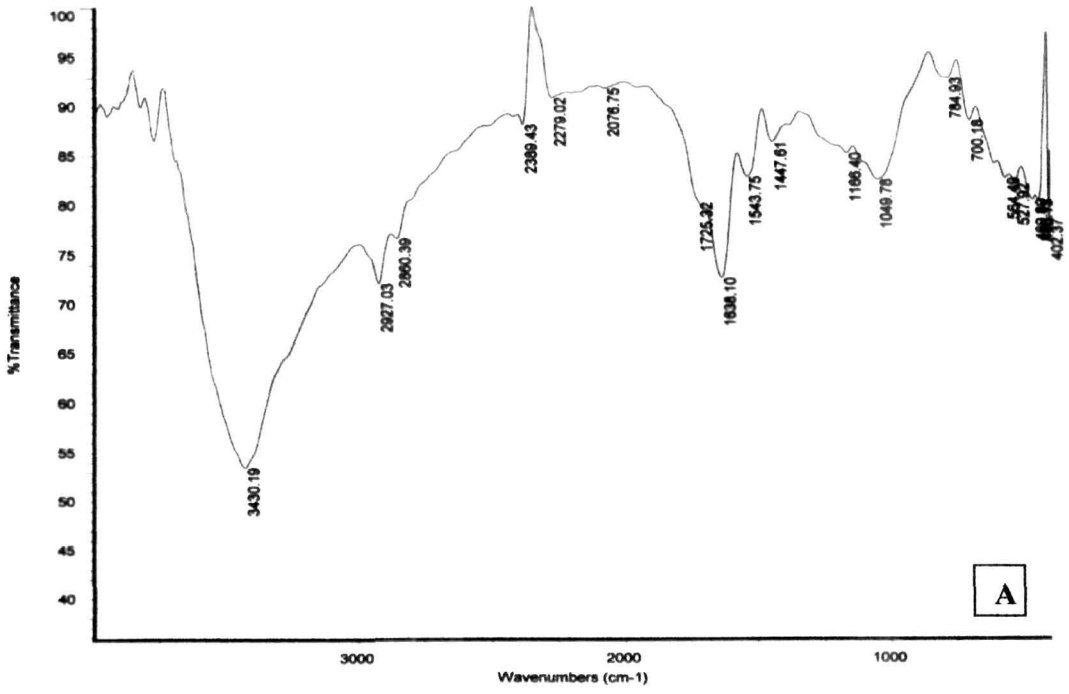
Bacterial strain	**TLC fractions**	**R_f values**	**Surface tension reduction (mNm^{-1})**
P. aeruginosa OBP1	S1	0.26	52.7±0.83
	S2	0.52	30.8±0.39
	S3	0.67	32.3±0.52
P. aeruginosa OBP2	S1	0.33	56.3±0.45
	S2	0.40	37.4±0.72
	S3	0.71	43.2±0.55
P. aeruginosa OBP3	S1	0.34	54.3±0.43

	S2	0.55	39.3±0.53
	S3	0.73	35.1±0.12
P. aeruginosa OBP4	S1	0.33	51.5±0.55
	S2	0.52	33.0±0.34
	S3	0.74	38.7±0.41

Results represented mean ± S.D of three individual experiments.

4.7.3 Fourier transforms infrared spectroscopy (FTIR)

The molecular composition of the freeze-dried biosurfactants of the bacterial strains on n-hexadecane supplemented medium was analyzed by FTIR and is presented in Figure 4.14. The FTIR spectra of all four biosurfactants showed different characteristic peaks with the presence of amino, carboxyl, hydroxyl and carbonyl groups. All four spectra showed the same essential adsorption bands; only the relative areas under the various absorption bands are slightly different.



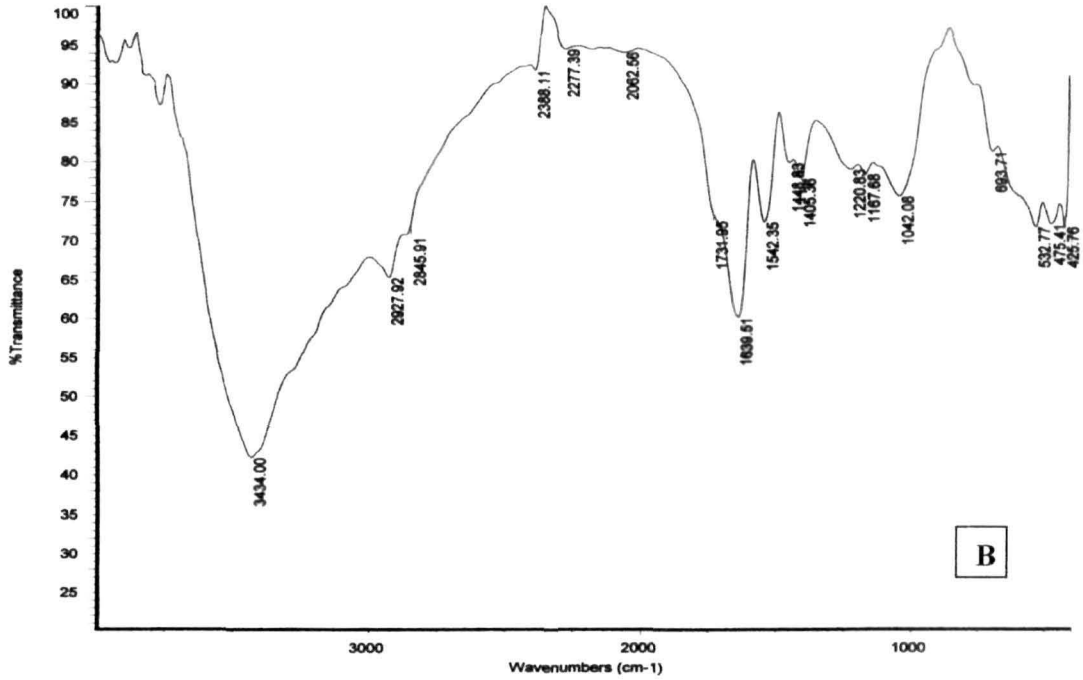
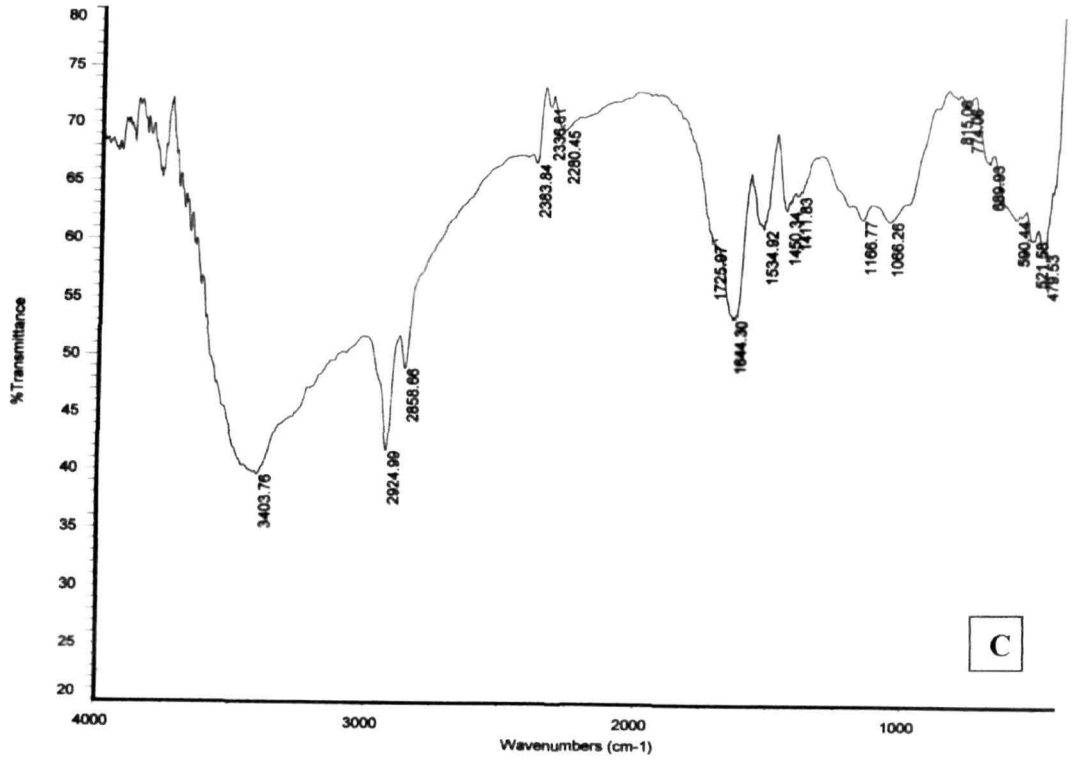


Figure 4.14a. The FTIR spectra of the biosurfactants produced by (A) *P. aeruginosa* OBP1 and (B) *P. aeruginosa* OBP2 in mineral salt medium supplemented with n-hexadecane.



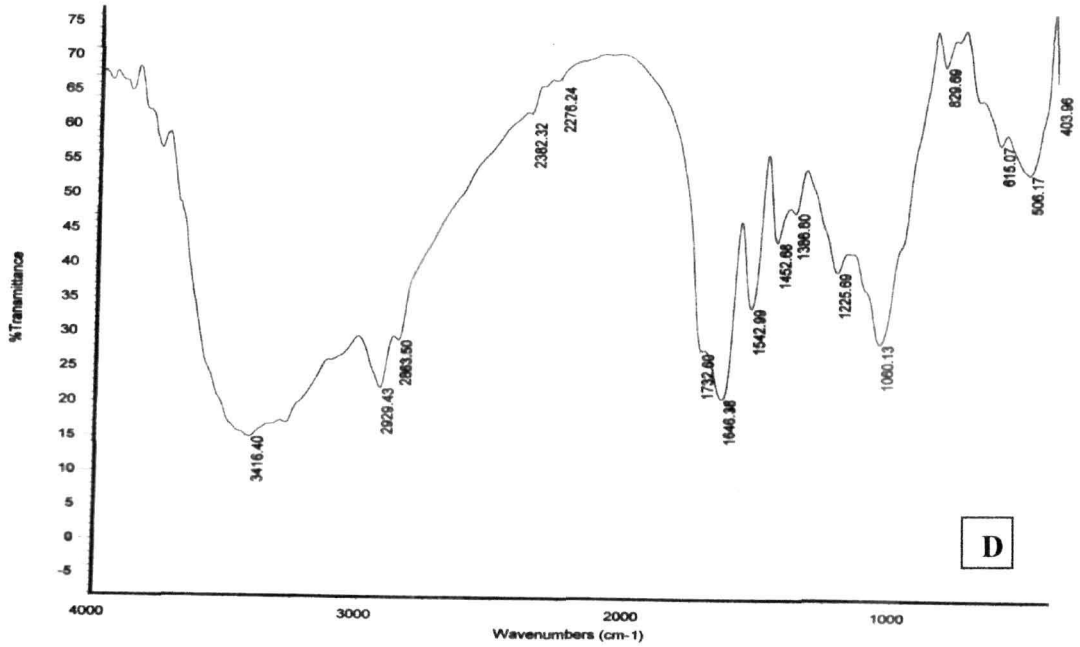


Figure 4.14b. The FTIR spectra of the biosurfactants produced by (C) *P. aeruginosa* OBP3 and (D) *P. aeruginosa* OBP4 in mineral salt medium supplemented with n-hexadecane.

FTIR spectrum of OBP1 biosurfactant (Figure 4.14a) possessed a characteristic band at 3430.19 cm^{-1} representing –OH groups. Bands at 2927.03 and 2860.39 cm^{-1} were caused by –CH stretching bands of $–CH_2$ and $–CH_3$ groups. Stretching bands at 1725.32 cm^{-1} showed the presence of carbonyl groups. Similarly, the FTIR spectrum of OBP2 biosurfactant (Figure 4.14a) exhibited the intense characteristic peak at 3434.00 cm^{-1} representing –OH groups. The intense stretching bands at 2927.92, 2845.91 and 1731.95 cm^{-1} corresponded to cm^{-1} $–CH_2$, $–CH_3$ and –C=O groups, respectively.

In the case of OBP3 biosurfactant (Figure 4.14b), the peaks at 3403.76 cm^{-1} indicated –OH groups. Other characteristic peaks at 2924.99, 2858.66 and 1725.97 cm^{-1} represented $–CH_2$, $–CH_3$ and –C=O stretching bands, respectively. The FTIR spectrum of OBP4 surfactant (Figure 4.14b) had an intense peak at a frequency of 3416.40 cm^{-1} referring to the presence of –OH groups. Peaks at 2929.43 and 2863.50 cm^{-1} represent stretching bands of $–CH_2$ and $–CH_3$ groups. Another characteristic peak at the frequency of 1732.60 cm^{-1} showed the presence of carbonyl stretching.

In most of the FTIR spectra possessed absorption at 1042.08-1066.26, 1534.92-1543.75 and 1638.10-1646.38 cm^{-1} representing –C=O amide I, –N/–C=O amide II and PII band: polysaccharide respectively which indicated the presence of protein and polysaccharide like substance in the isolated biosurfactants.

4.7.4 Liquid chromatography and mass spectroscopy (LC-MS)

The purified TLC fractions of OBP1 and OBP2 biosurfactant were further purified on a ultra pure liquid chromatography (UPLC) system to separate the rhamnolipid mixture and are presented in Figure 4.15a.

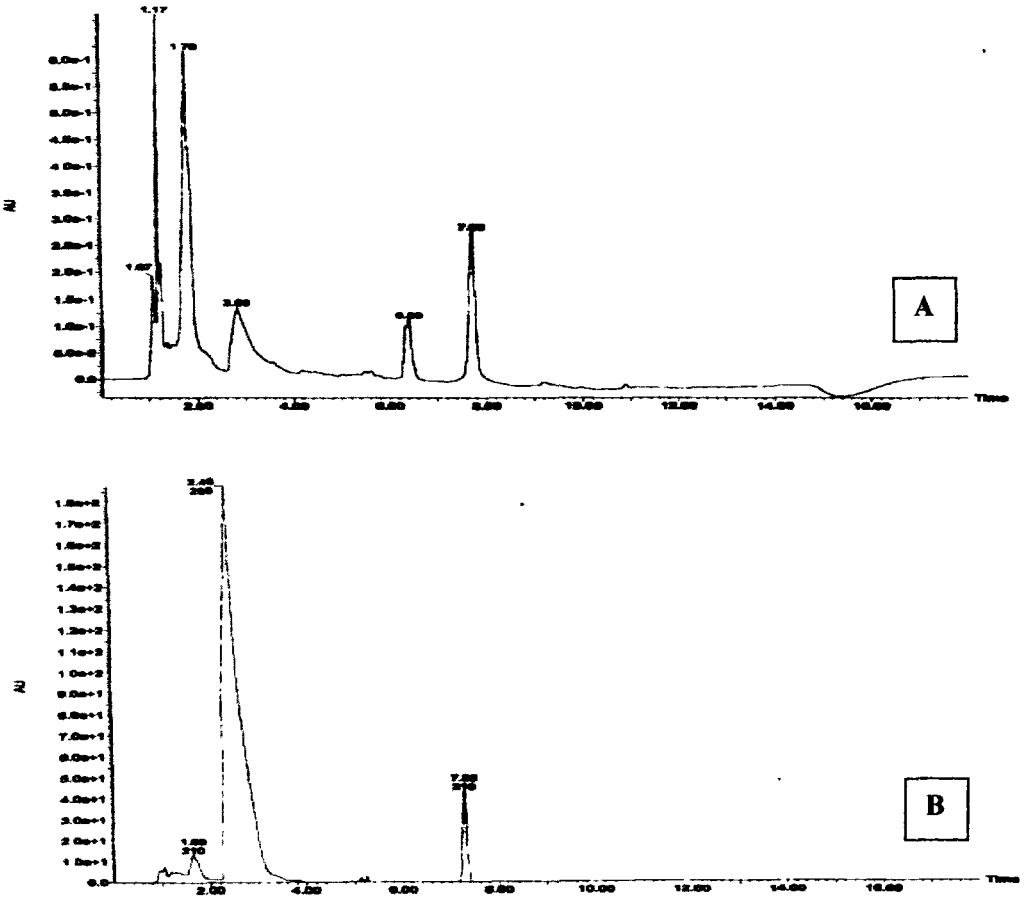


Figure 4.15a. LC profile of purified fraction of (A) *P. aeruginosa* OBP1 and (B) *P. aeruginosa* OBP2

In the case of OBP3 and OBP4, the TLC fraction showing the highest surface activity was purified and characterized on a liquid chromatography (LC) system and are shown in Figure 4.15b. The mass spectra of the purified rhamnolipid from the bacterial strain OBP3 and OBP4 showed the presence of rhamnolipid congers with multiple molecular ions (Appendix II) and the same are presented in Table 4.28.

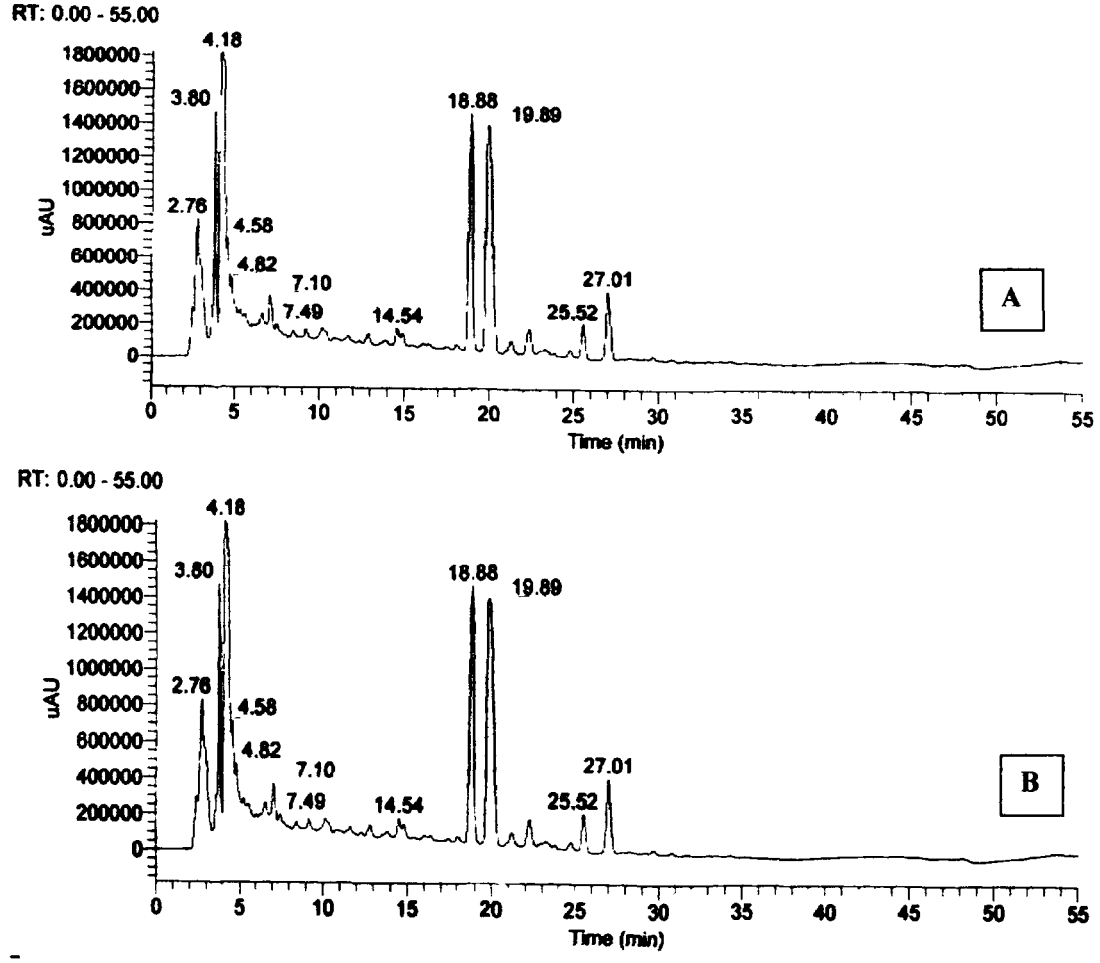


Figure 4.15b. UPLC profile of purified fraction of (A) *P. aeruginosa* OBP3 and (B) *P. aeruginosa* OBP4.

Table 4.28. Chemical composition of rhamnolipid mixture produced by *P. aeruginosa* strains as determined by mass spectroscopic analysis

Bacterial strains	Rhamnolipid congeners	Pseudomolecular ion (m/z)
	Rha-C_{10}	331
	Rha-$C_{12\,2}$	359

P. aeruginosa OBP1	Rha-C_8-C_{10}	479
	Rha-C_{10}-C_{10}	505
	Rha-C_{10}-$C_{12:1}$	528
	Rha-C_{12}-C_{10}	531
	Rha-C_{10}-C_{12}	531
	Rha- Rha-C_8-C_{10}	621
	Rha- Rha-C_{10}-C_{10}	648
	Rha- Rha-C_{10}-$C_{12:1}$	674
P. aeruginosa OBP2	Rha-$C_{8:2}$	302
	Rha-$C_{12:2}$	357
	Rha-C_{10}-C_{10}	501
	Rha-C_{10}-$C_{12:1}$	527
	Rha- Rha-C_{10}-C_{12}	531
	Rha- Rha-C_{12}-C_{10}	531
	Rha- Rha-C_{10}-C_{10}	648
	Rha- Rha-C_{10}-$C_{12:1}$	675
P. aeruginosa OBP3	Rha-$C_{8:2}$	302
	Rha-C_{10}	333
	Rha-C_8-C_{10}	477
	Rha- Rha-C_{10}	480
	Rha-C_{10}-C_{10}	502
	Rha-C_{10}-$C_{12:1}$	529
	Rha- Rha-C_{10}-C_{12}	531
	Rha- Rha-C_{12}-C_{10}	531
	Rha- Rha- C_8-C_{10}	622
P. aeruginosa OBP4	Rha-$C_{8:2}$	302
	Rha-C_{10}	332
	Rha-$C_{12:2}$	358
	Rha-C_8-C_{10}	477
	Rha- C_{10}-C_{10}	502
	Rha-C_{10}-C_{12}	532
	Rha- Rha-C_8-C_{10}	622
	Rha- Rha-C_{10}-C_{10}	648
	Rha- Rha-C_{10}-$C_{12:1}$	673
	Rha- Rha-C_{10}-C_{12}	679

4.7.5 Thermogravimetric analysis (TGA)

TGA analyses of the four isolated four biosurfactants were carried out (weight loss versus temperature) and are presented in Figure 4.16.

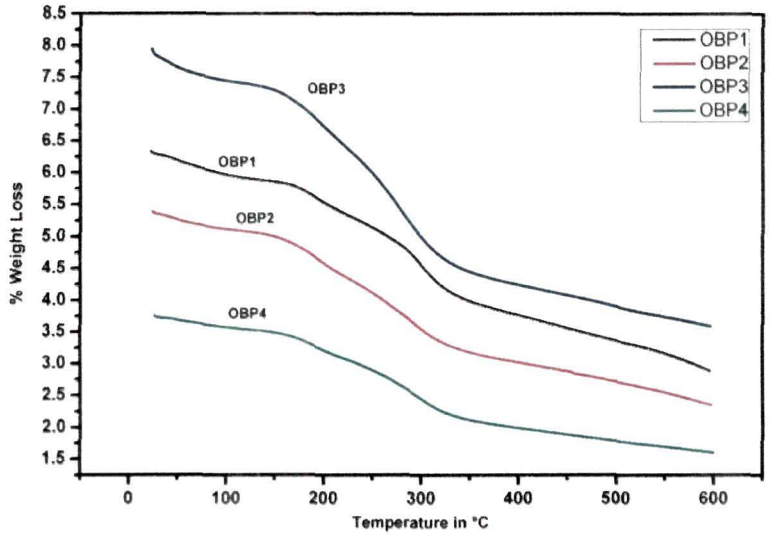


Figure 4.16. Thermogravimetric analysis (TGA) of dried biosurfactants produced by *P. aeruginosa* strains during growth in mineral salt medium supplemented with n-hexadecane.

Degradation study was done by heating the samples from 25 to 600°C. The thermogram of all the biosurfactant samples exhibited a two-step degradation pattern. The initial degradation of the biosurfactants of OBP1, OBP2, OBP3 and OBP4 occurred at around 156.6, 135.6, 135.6 and 145.3°C, respectively and its corresponding weight loss were in the order of 7.95, 6.26, 7.5 and 6.9 %, respectively. The second step degradation of the biosurfactant samples were observed at 284.4, 276.3, 257.0 and 269.9 °C, respectively and determined as degradation temperature (T_d). In the second step degradation, there were 24.1, 28.8, 26.1 and 26.9 % weight loss in the biosurfactant samples of OBP1, OBP2, OBP3 and OBP4, respectively. The retention of 43-44 % weight by the biosurfactant samples even after heating at 600°C reveals their thermo-stability.

4.7.6 Differential scanning calorimetry (DSC)

DSC thermograms of the four biosurfactants are presented in Figure 4.17. The thermogram of the biosurfactant samples revealed two different endothermic peaks. Biosurfactant of the bacterial strain OBP1 exhibited two distinct endothermic peaks at around 16 °C and 131°C. Also a third smaller endothermic peak was observed nearby

140 °C. The first peaks represent the enthalpy of dehydration and the second peak indicates the enthalpy of decomposition.

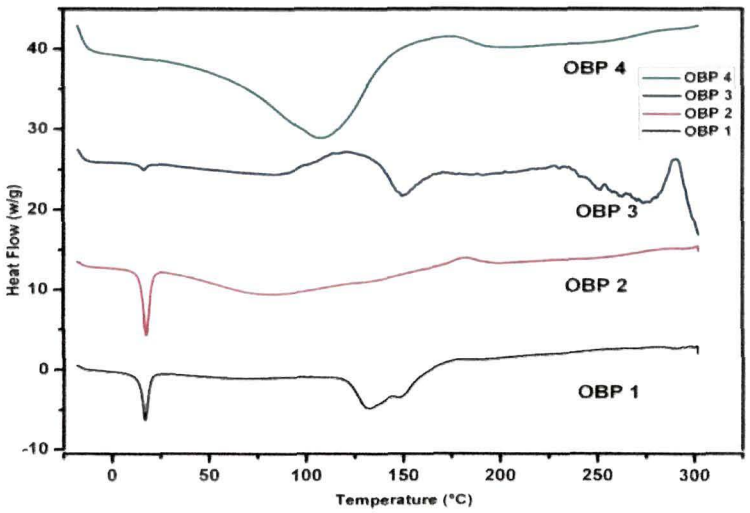


Figure 4.17. Differential scanning calorimetry (DSC) of dried biosurfactant produced by *P. aeruginosa* strains during growth in mineral salt medium supplemented with n-hexadecane

4.8 Application in the field of bioremediation

4.8.1 Reduction in the viscosity of crude oil

Treatment of crude petroleum with the bacterial strains showed noticeable reduction in the viscosity values as compared to the untreated control. The results are presented in Figure 4.18.

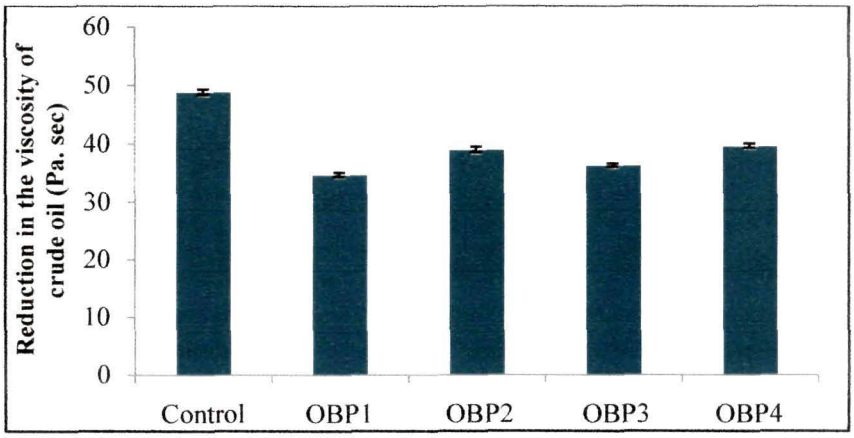


Figure 4.18. Reduction in the viscosity of crude oil after treatment with *P. aeruginosa* strains. Values are the mean of three independent experiments ± standard deviation.

However, bacterial strain OBP4 and OBP2 had a very clear impact on the viscosity of crude oil which significantly decreased from 48.7 Pa.sec before treatment to 34.6 Pa.sec after 30 days of the treatment.

4.8.2 Solubilization of polyaromatic hydrocarbon (PAH) by biosurfactant

The effect of biosurfactants on the solubility of PAHs such as phenanthrene, anthracene and naphthalene was determined in the presence of biosurfactant. As shown in Figure 4.19, biosurfactants possessed highly noticeable effect on the solubilization of the three tested PAHs.

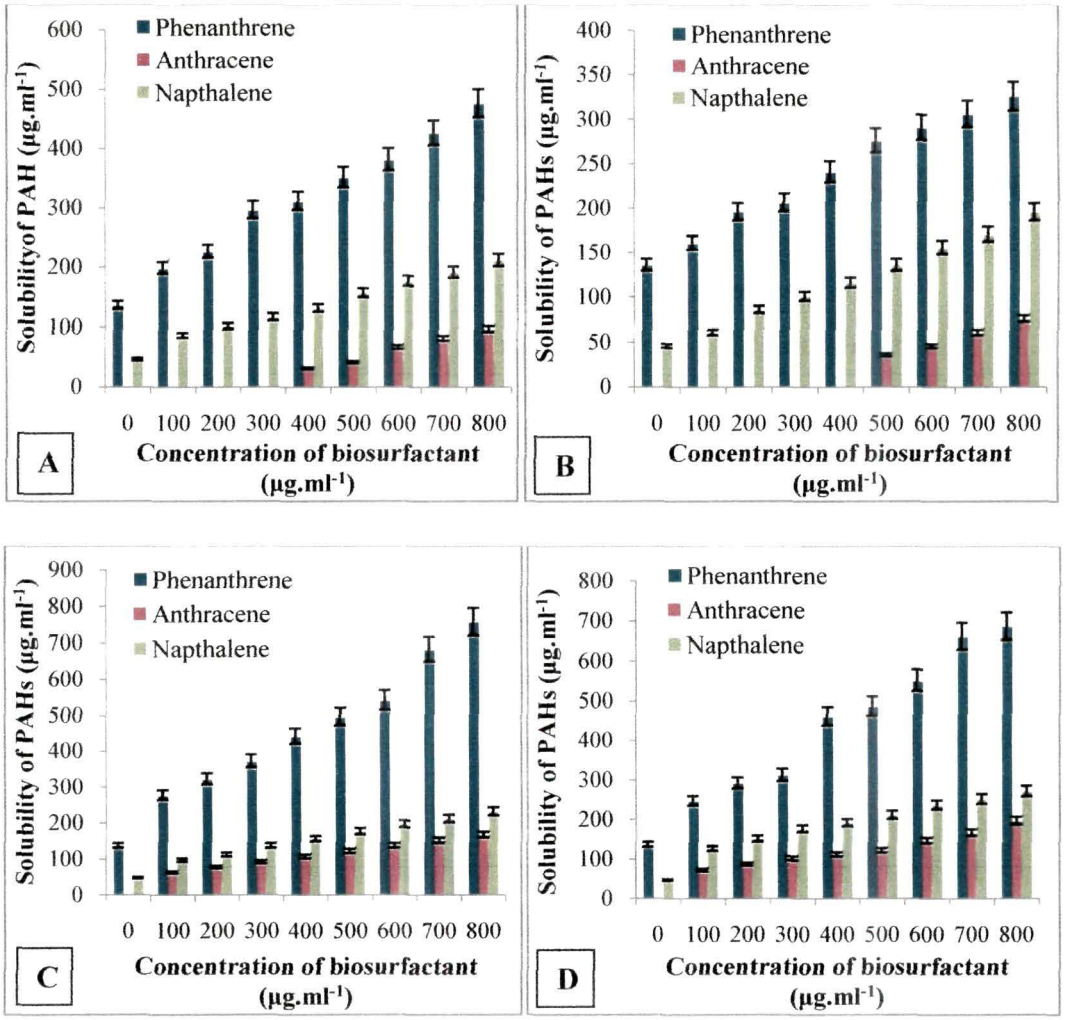


Figure 4.19. PAH solubilization assay showing the decrease in the available phenanthrene, anthracene and naphthalene concentration with

increasing concentration of biosurfactants produced by (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3, and (D) *P. aeruginosa* OBP4.. Values are the mean of three independent experiments ± standard deviation

The solubility of PAHs in water was found to be higher in the presence of biosurfactants as compared to the control having no biosurfactant. Biosurfactants either below or above its CMC were effective in solubilization of PAHs. Nevertheless, solubilization was much more pronounced when the concentration of biosurfactants produced by the bacterial strains were increased above their respective CMC values. The biosurfactants from all four bacterial strain exhibited appreciable solubilization of phenanthrene. The highest solubilization of phenanthrene was observed in OBP3 biosurfactant, followed by OBP4. In the case of anthracene, the biosurfactant from the bacterial strain OBP4 showed a significant level of solubilization as compared to the biosurfactant from the OBP3 and OBP1. Naphthalene was found to be solubilized significantly only by the biosurfactant from OBP4.

4.8.3 Biodegradation of crude oil by the bacterial strains

The bacterial strains were assessed for their ability to degrade crude oil components in the culture medium. The medium was supplemented with 2.0 ml (1.9 g) of crude oil and inoculated with the individual bacterial strains separately. After 30 days of incubation, the residual fractions of aliphatic, aromatic and NSO compounds were determined and are presented in Table 4.29.

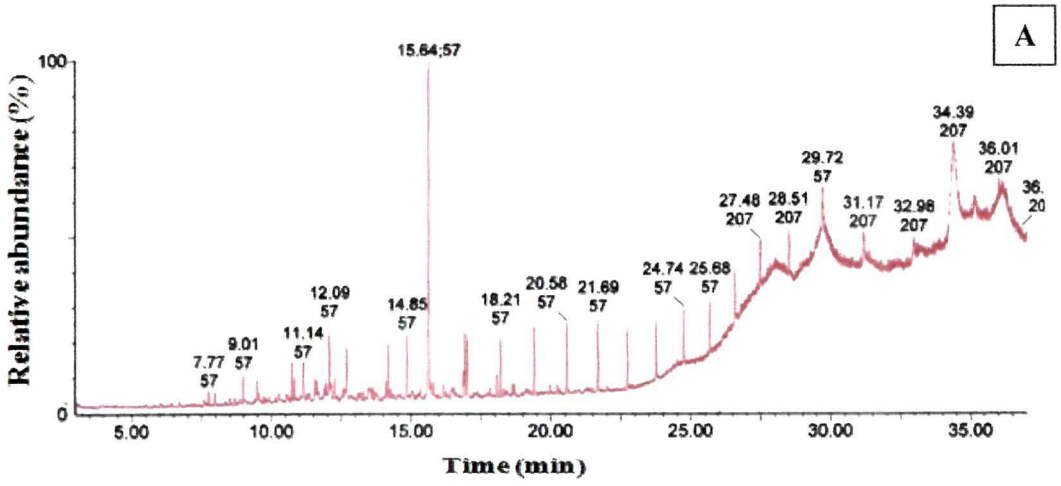
Table 4.29. Degradation of aliphatic, aromatic and NSO fractions of crude oil by *P. aeruginosa* strains after 30 days of treatment in liquid culture

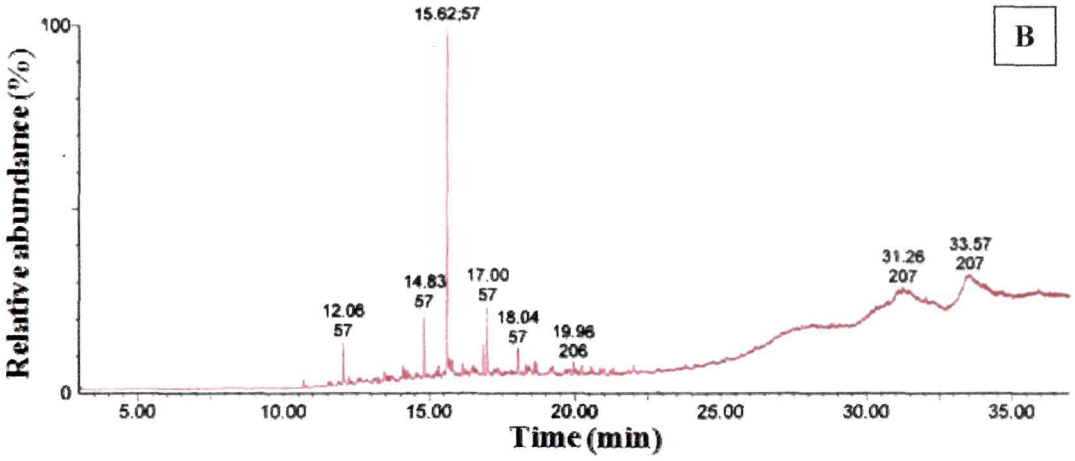
Bacterial strain	**Media supplemented with crude oil (g)***	**Aliphatic fraction degraded %**	**Aromatic fraction degraded %**	**NSO compounds degraded %**
Control	1.86	12.6±0.8	10.3±0.6	04.7±0.2
P. aeruginosa OBP1	1.86	72.8±0.2	25.2±0.6	11.5±0.5

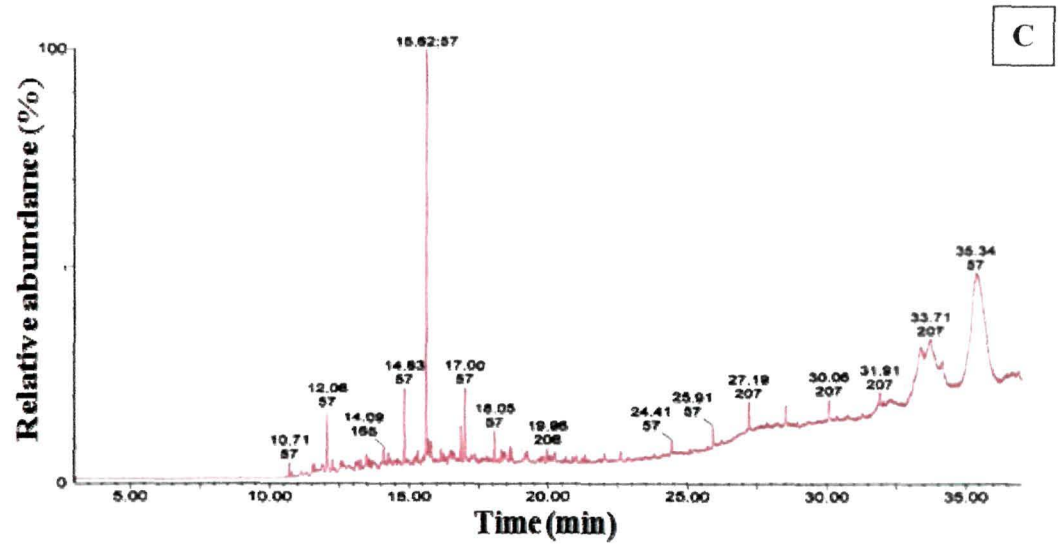
P. aeruginosa OBP2	1.86	67.5±0.7	12.3±0.9	07.3±0.1
P. aeruginosa OBP3	1.86	71.8±0.3	31.8±0.8	13.5±0.4
P. aeruginosa OBP4	1.86	73.0±0.3	30.3±0.3	14.7±0.7

*Determination on the basis of crude oil 2.0 ml (1.857 g). Results represented mean ± S.D of three individual experiments.

As shown in Table 4.29, the strain OBP4 followed by OBP1 exhibited high level degradation of aliphatic hydrocarbons with 73.0 and 72.8% respectively. On the other hand, the strain OBP3 and OBP2 possessed 71.8 and 67.5% degradation of aliphatic hydrocarbons, respectively. In the case of aromatic fraction, the bacterial strain OBP3 followed by OBP4, OBP1 and OBP2 showed better degradation of 31.8, 30.3, 25.2 and 12.3% respectively. The strain OBP4 exhibited the highest degradation of NSO compounds with 14.7% while OBP3, OBP1 and OBP2 degraded 13.5, 11.5 and 7.3%, respectively. The degradation of crude oil by the bacterial strains was further confirmed by gas chromatography (GC). The GC profiles of the saturated fractions of the crude oil after 30 days treatment with the bacterial strains along with the control were determined and are presented in Figure 4. 20.




B
Relative abundance (%)
100
0
15.62;57
12.06
57
14.83
57
17.00
57
18.04
57
19.96
206
31.26
207
33.57
207
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Time (min)


C
Relative abundance (%)
100
0
15.62;57
10.71
57
12.08
57
14.09
166
14.83
57
17.00
57
18.05
57
19.96
206
24.41
57
25.91
57
27.19
207
30.06
207
31.91
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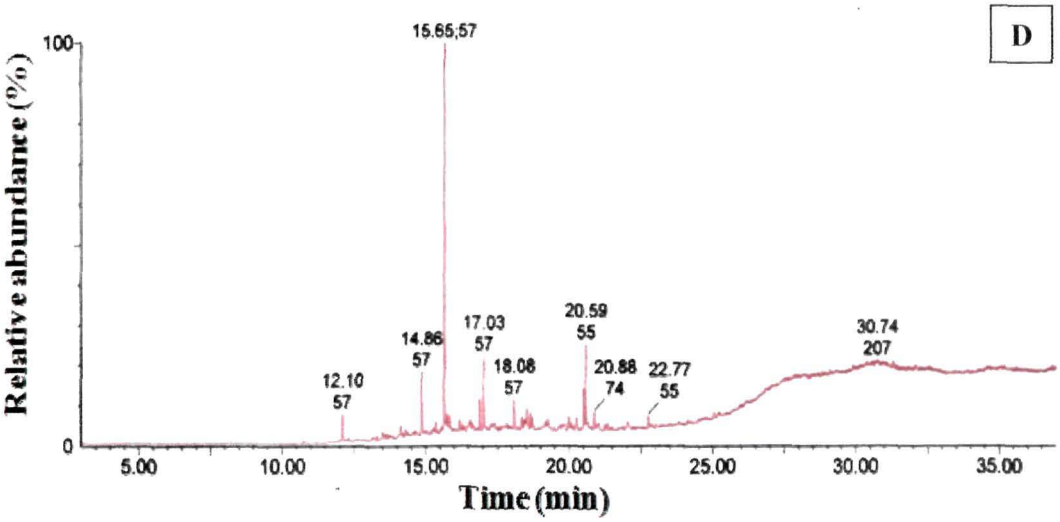
15.00
20.00
25.00
30.00
35.00
Time (min)


D
Relative abundance (%)
100
0
15.65;57
12.10
57
14.86
57
17.03
57
18.08
57
20.59
55
20.88
74
22.77
55
30.74
207
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Time (min)

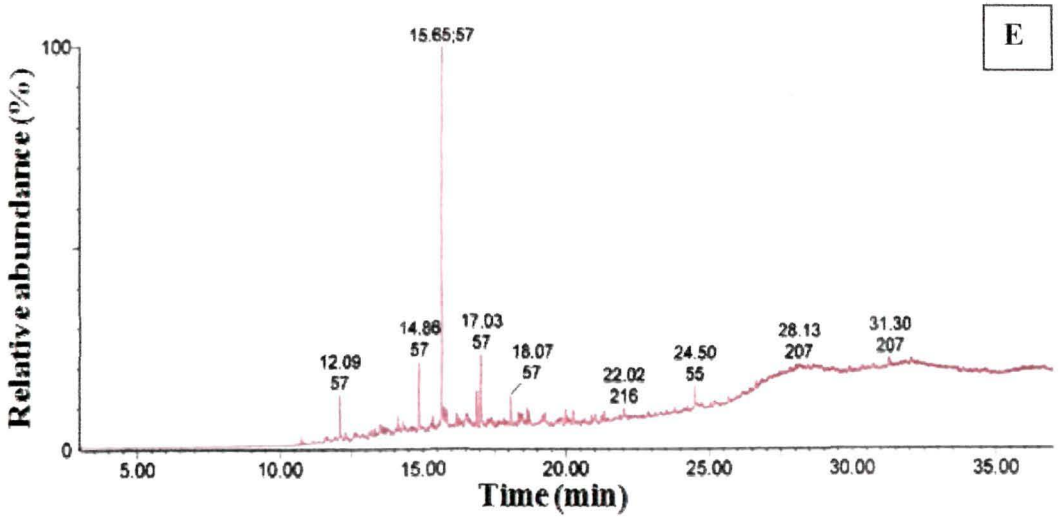


Figure 4.20. Gas chromatographic analysis of the saturate fraction of crude oil after treatment with bacterial strains. (A) Control without treatment, (B) *P. aeruginosa* OBP1, (C) *P. aeruginosa* OBP2, (D) *P. aeruginosa* OBP3, and (E) *P. aeruginosa* OBP4.

11 different combinations of bacterial strains were cultured in MSM supplemented with 2% (v/v) crude oil for a period of 96 h to determine their ability to grow on crude oil. The final dry biomass yield was determined and the data are presented in Table 4.30.

Table 4.30. Biomass of bacterial consortia in mineral salt medium supplemented with crude oil after 96 h of culture

S.No	Bacterial consortium	Dry biomass ($g.l^{-1}$)
1.	OBP1 + OBP2	1.45± 0.01
2.	OBP1 + OBP3	1.35± 0.04
3.	OBP1 + OBP4	0.85± 0.1
4.	OBP2 + OBP3	1.34± 0.2
5.	OBP2 + OBP4	0.71± 0.1
6.	OBP3 + OBP4	0.86± 0.1
7.	OBP1 + OBP3+ OBP4	2.72± 0.3
8.	OBP2 + OBP3+ OBP4	1.50± 0.4

9.	OBP1 + OBP2+ OBP4	1.90± 0.1
10.	OBP1 + OBP2+ OBP3	3.34± 0.1
11.	OBP1 + OBP2+ OBP3+ OBP4	3.33± 0.1

Results represented mean ± S.D of three individual experiments.

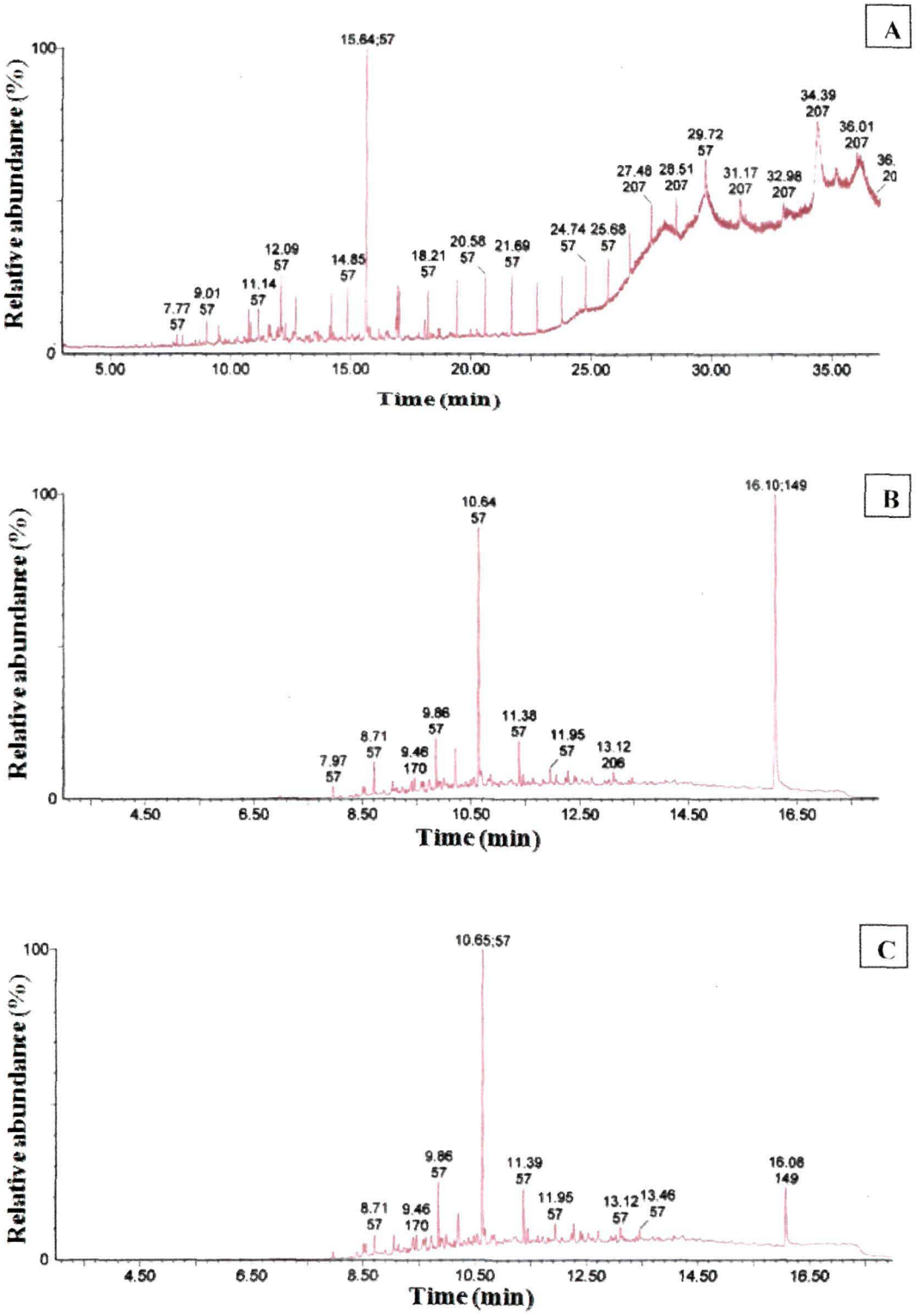
On the basis of growth performance as depicted by the dry biomass yield, bacterial consortia 7 and 11 designated as consortium I and consortium II were selected to assess their ability to degrade crude oil in MSM. The residual crude oil in the culture medium after 30 days of treatment by the two bacterial consortia separately was assayed and is presented in Table 4.31.

Table 4.31. Degradation of aliphatic, aromatic and NSO fractions of crude oil by bacterial consortia and in presence of biosurfactant after 30 days

Bacterial strain	**Culture media with crude oil (g)**	**Aliphatic fraction degraded (%)**	**Aromatic fraction degraded (%)**	**NSO compound degraded (%)**
Control	1.85	12.2±0.5	08.9±0.5	05.2±0.7
Consortium I	1.85	78.6±0.5	42.7±0.7	21.6±0.3
Consortium II	1.85	80.4±0.8	42.4±0.4	19.2±0.4
Consortium I + biosurfactant	1.85	80.7±0.3	43.8±0.5	22.5±0.7
Consortium II+ biosurfactant	1.85	81.6±0.7	42.6±0.2	20.7±0.5

*Determination on the basis of crude oil 2.0 ml (1.849 g). Results represented mean ± S.D of three individual experiments.

As shown in Table 4.31, the consortia was able to degrade 78.6–80.4% of aliphatic fractions, around 42.5% of aromatic fractions and 19.2-21.6% of NSO containing compounds of crude oil within 30 days. Similarly, the effect of biosurfactant on the degradation of crude oil by the bacterial consortia was estimated and the data are presented in Table 4.31. The GC profiles of the saturated fraction of crude oil in culture medium inoculated with consortium I, consortium II and in the presence of externally added biosurfactant after 30 days of incubation was determined and are presented in Figure 4.21.


A
Relative abundance (%)
100
0
15.64;57
34.39
207
36.01
207
29.72
57
27.48
207
28.51
207
31.17
207
32.98
207
36.
20
12.09
57
14.85
57
18.21
57
20.58
57
21.69
57
24.74
57
25.68
57
11.14
57
9.01
57
7.77
57
5.00
10.00
15.00
20.00
25.00
30.00
35.00
Time (min)
B
Relative abundance (%)
100
0
16.10;149
10.64
57
9.86
57
11.38
57
8.71
57
11.95
57
9.46
170
13.12
206
7.97
57
4.50
6.50
8.50
10.50
12.50
14.50
16.50
Time (min)
C
Relative abundance (%)
100
0
10.65;57
9.86
57
11.39
57
16.08
149
8.71
57
9.46
170
11.95
57
13.12
57
13.46
57
4.50
6.50
8.50
10.50
12.50
14.50
16.50
Time (min)

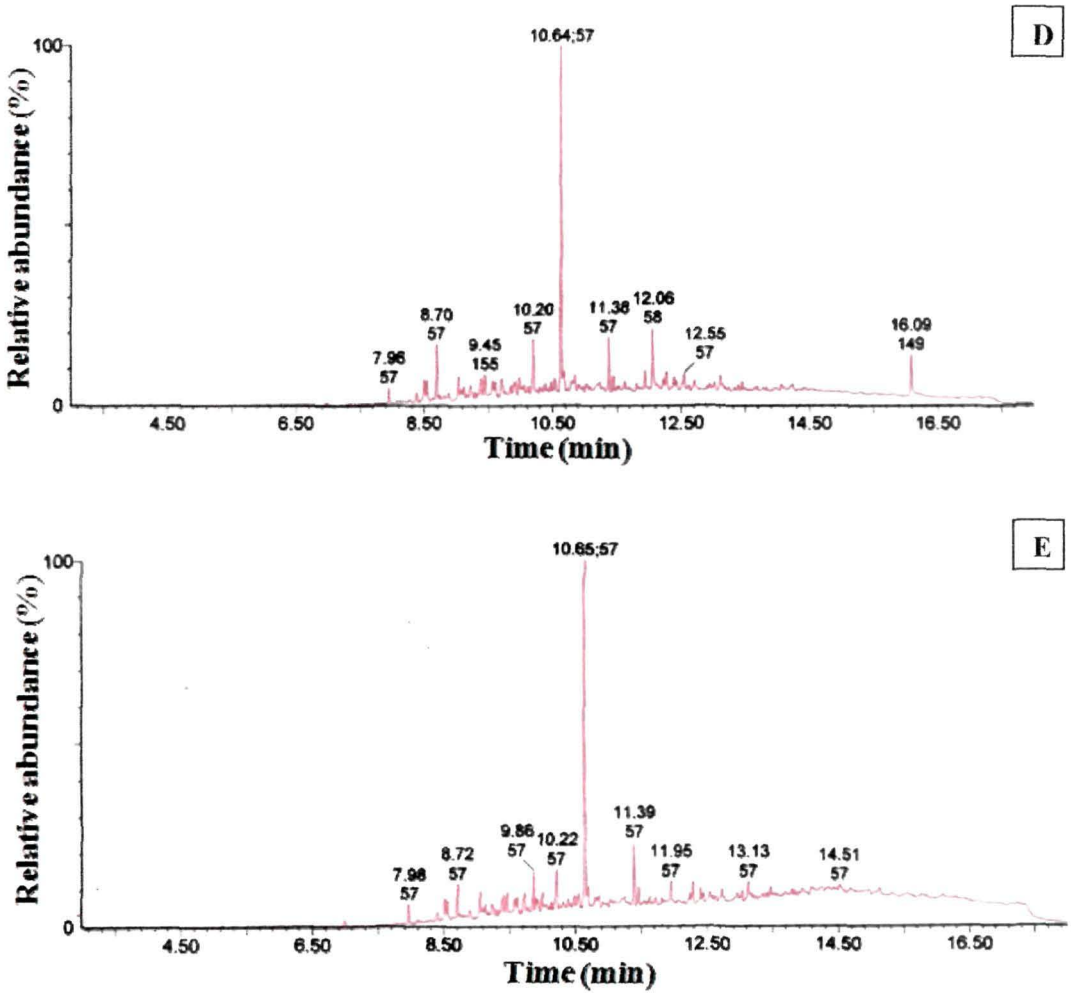


Figure 4.21. Gas chromatographic analysis of the saturate fraction of crude oil after treatment with (A) control (B) Consortium I, (C) Consortium II, (D) Consortium I+ biosurfactant, and (E) Consortium II+ biosurfactant

4.8.4 Separation of crude oil from contaminated sand using biosurfactants

The capability of the aqueous biosurfactant solutions to remove crude oil from the crude oil contaminated sand was investigated and the results are shown in Figure 4.22. The maximum crude oil removed by the biosurfactants isolated from the bacterial strains was attained within their CMC, showing a total removal upto a range of 54-61.7% and are presented in the Figure 4.22. However, increased concentration of biosurfactants beyond CMC did not enhance further removal of crude oil from the contaminated sand.

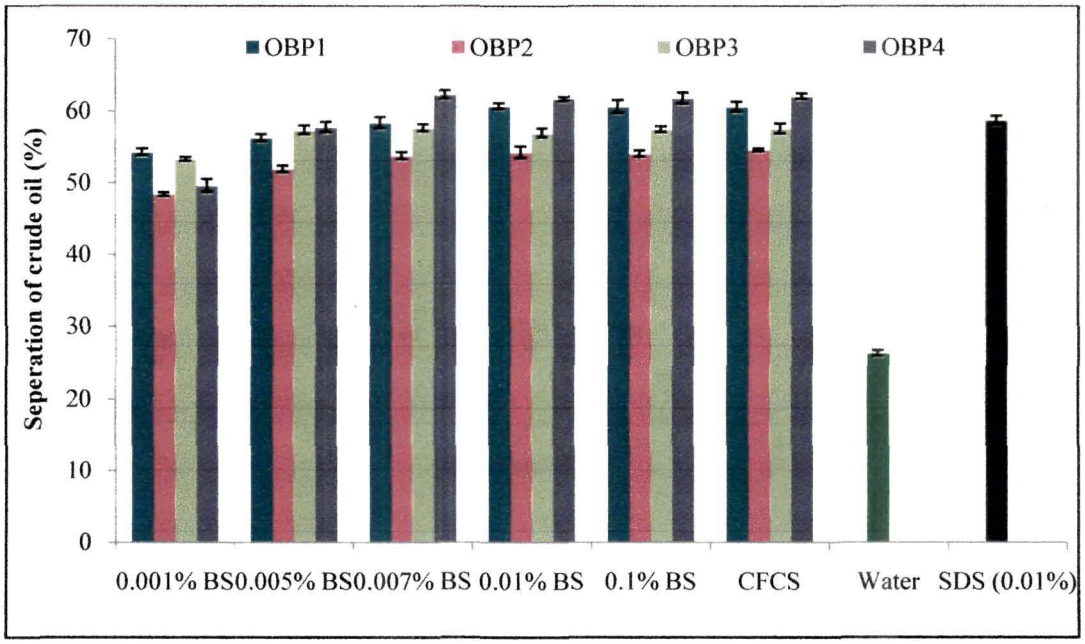


Figure 4.22. Removal of crude oil from the contaminated sand after washing with biosurfactant solution (BS) produced by *P. aeruginosa* strains. CFCS-Cell free culture supernatant, SDS-Sodium dodecyl sulphate. Values are the mean of three independent experiments ± standard deviation

The cell free culture supernatants were also found to be efficient in separating the crude oil from the contaminated sand and exhibited almost similar efficiency as compared to the purified biosurfactant solutions. The control with only distilled water was able to remove only 26.2% while SDS could able to separate 58.6% of crude oil from the contaminated sand.

4.8.5 Release of crude oil from the sand pack column by biosurfactants

The crude oil saturated sand pack columns were treated with individual cell free culture supernatant of the bacterial strains and incubated at room temperature, 50, 70 and 90°C to determine the release of crude oil from the column. The released crude oil was quantified and the data are shown in Figure 4.23. At room temperature the culture supernatant of the bacterial strains could recover 9.3-11.4% of crude oil from the saturated sand pack column, 6.4–8.7% at room temperature, 7.8-9.7% at 50°C, 8.4-10.5% at 70°C and 9.3-11.4% at 90°C.

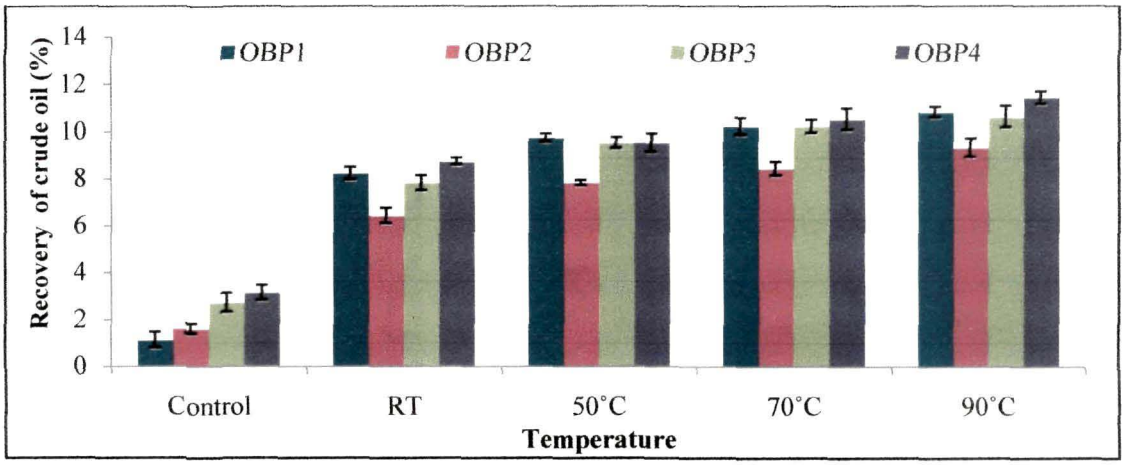
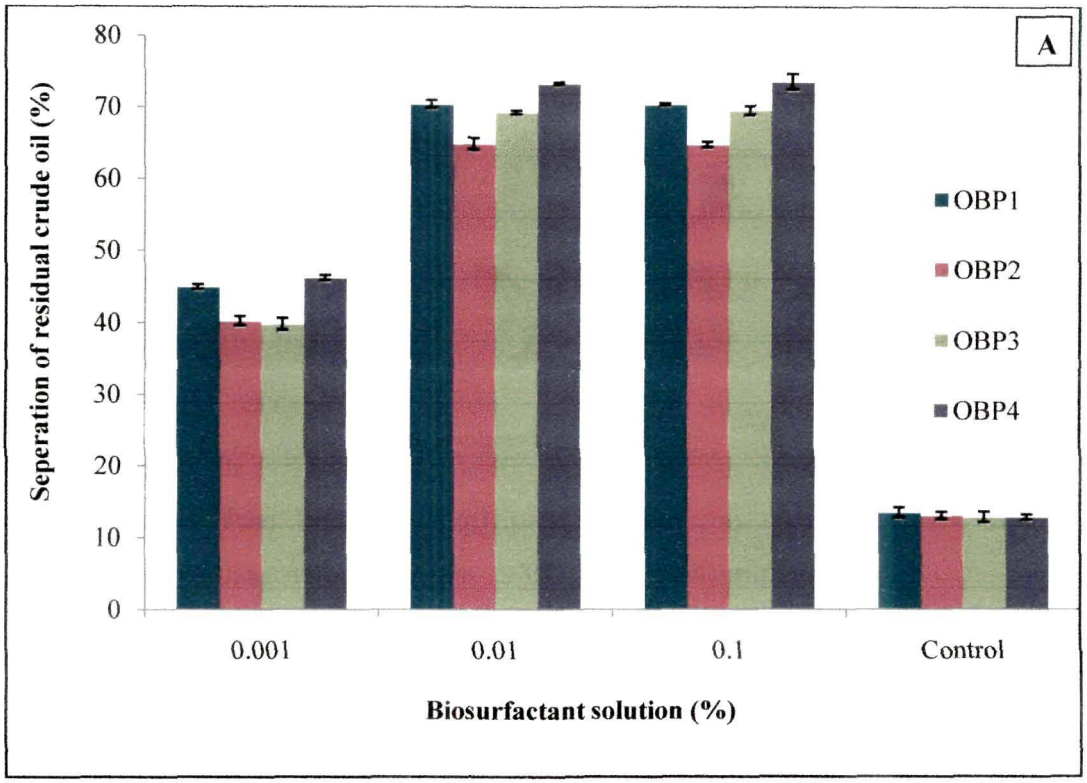


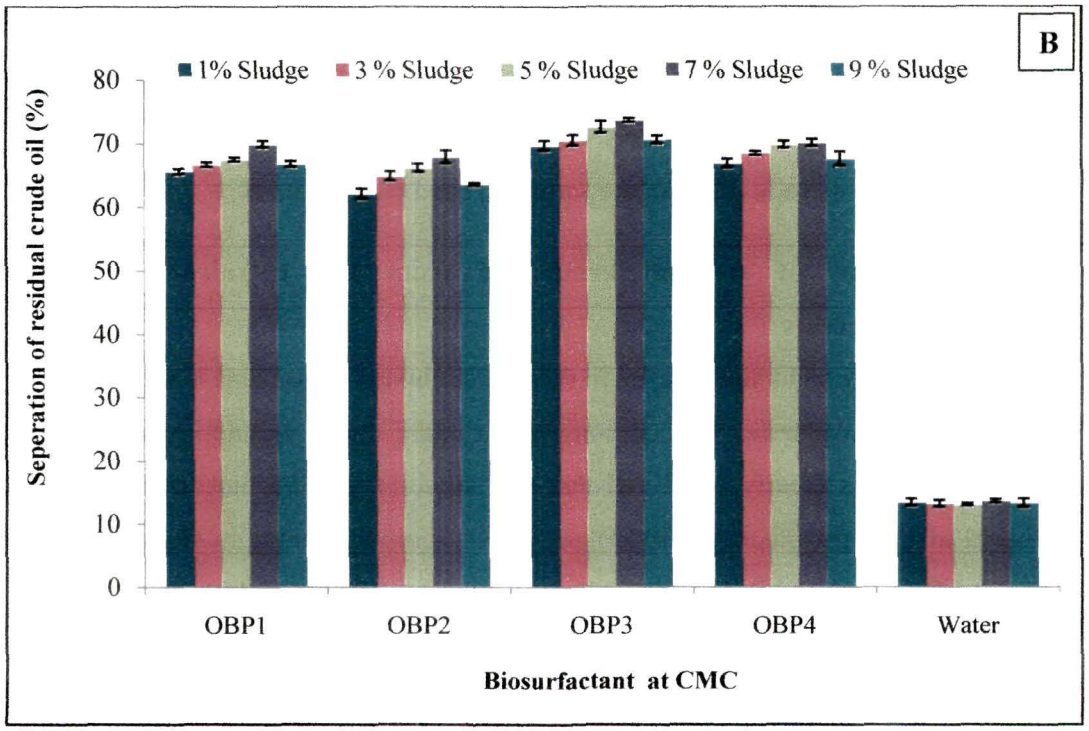
Figure 4.23. Recovery of crude oil (%) from the sand pack column at room temperature (RT), 50°C, 70°C, and 90°C after treatment with cell free culture broth of *P. aeruginosa* strains. Values are the mean of three independent experiments ± standard deviation.

The control with the sterilized culture medium could able to recover only 1.1–3.1% crude oil. The biosurfactants produced in the culture media by the bacterial strain while exposed to higher temperature between 70–90°C caused higher recovery of crude oil from the saturated sand pack column. This also proved the stability of biosurfactants in the recovery process being subjected to higher temperatures. Further, addition of fresh medium separately with the bacterial strains could not enhance the further recovery of crude oil from the saturated sand pack column.

4.8.6 Separation of residual crude oil from the petroleum sludge by biosurfactants

The sticky solid sludge used in the present investigation was blackish brown in color. The total petroleum hydrocarbon present in the sludge was approximately 785 ± 130 g/kg. Results clearly explained that the suitability of the biosurfactants for the removal of total petroleum hydrocarbon (TPH) from the petroleum sludge than those removed by water only. The biosurfactant solution of bacterial strain OBP1 could separate around 63.4-73.5% of residual crude oil from 7% petroleum sludge in 15 days of incubation and is presented in Figure 4.24.


A
Seperation of residual crude oil (%)
80
70
60
50
40
30
20
10
0
OBP1
OBP2
OBP3
OBP4
0.001
0.01
0.1
Control
Biosurfactant solution (%)


B
1% Sludge
3 % Sludge
5 % Sludge
7 % Sludge
9 % Sludge
Seperation of residual crude oil (%)
80
70
60
50
40
30
20
10
0
OBP1
OBP2
OBP3
OBP4
Water
Biosurfactant at CMC

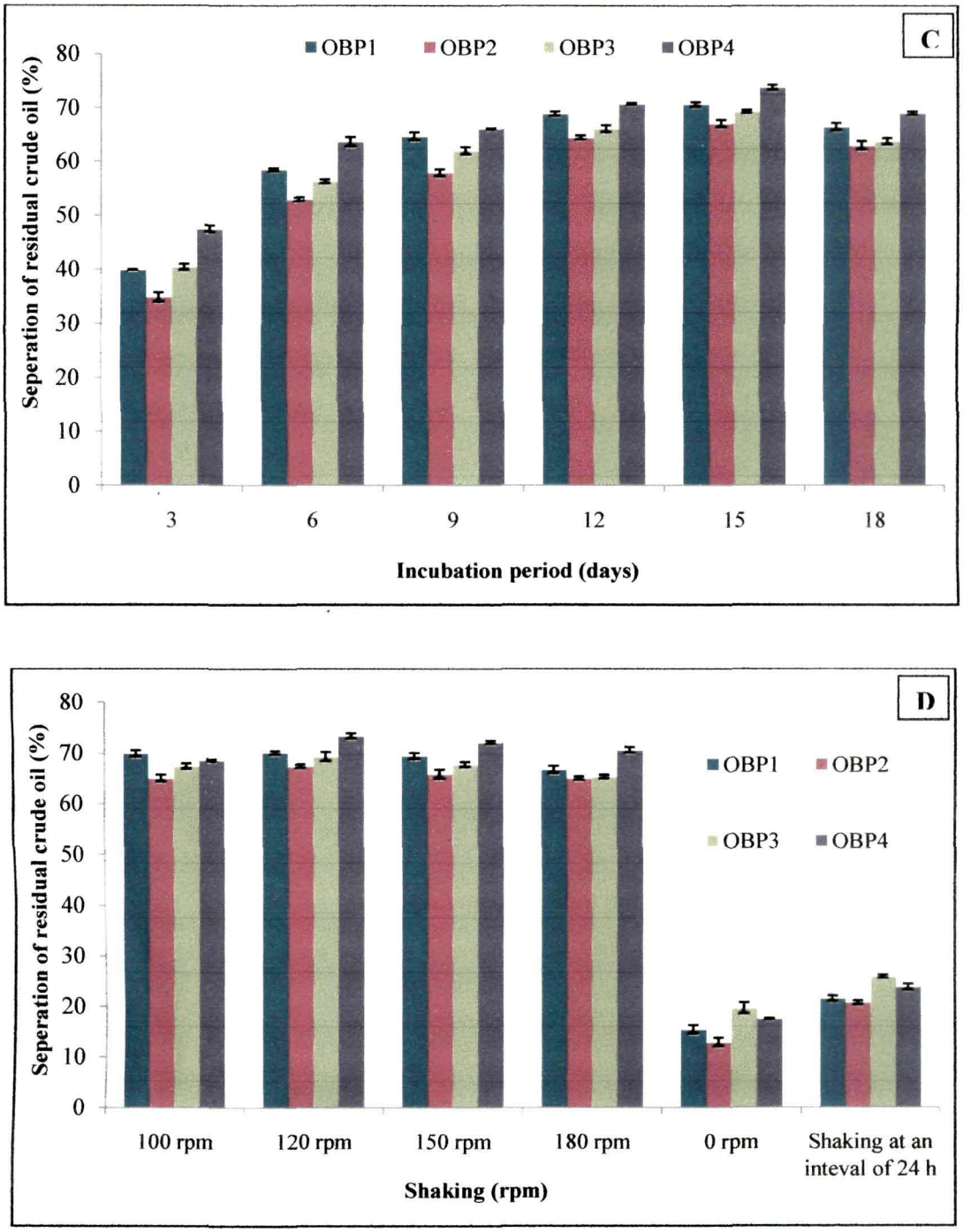


Figure 4.24. Effect of different parameters on the separation of residual crude oil from the petroleum sludge. (A) Biosurfactant concentration, (B) sludge concentration, (C) treatment period, and (D) shaking. Values are the mean of three independent experiments ± standard deviation.

The separation of the residual oil from the sludge gradually increased with increase in the biosurfactant concentration. The maximum recovery of crude oil by the biosurfactant solutions were achieved within their respective CMC values (p value < 0.05). In the case of concentrations above CMC, no enhancement in the release of oil from the sludge was observed (p value > 0.05) and is presented in Figure 4.24a. Similarly, cell free culture supernatants of the bacterial strains were also efficient in removing the residual crude oil from the sludge and were almost comparable to their respective biosurfactant solutions. Separation of oil was upto 7% (w/w) sludge concentration , but above this concentration there was no further increase in the release of the residual crude oil from the sludge (p value > 0.05), as they could not form homogenous slurry and the same is presented in Figure 4.24b.

The amount of residual crude oil released gradually increased along with the treatment time, but after 15 days of treatment there was no further increase (p value > 0.05) and the data are presented in Figure 4.24c. Much more residual oil was released when the flasks were continuously shaken as compared to occasional (p value < 0.05) and no shaking (p value < 0.05). The release of oil increased with the increase in the rpm value, however above 120 rpm there was a significant decrease in the separation of residual oil (p value > 0.05) and is presented in Figure 4.24d.

4.8.7 Degradation studies of bacterial biosurfactants

Biosurfactants isolated from the bacterial strains were significantly degraded by the bacterial strains *P. aeruginosa* (MTCC 8165) and *B. circulans* (MTCC8167) in liquid culture. The degradation patterns of biosurfactants are shown in Figure 4.25. In both liquid cultures of *P. aeruginosa* (MTCC 8165) and *B. circulans* (MTCC8167) no inhibitory effect of the tested biosurfactants were observed. The bacterial strains showed normal growth behaviour and are presented in the Figure 4.25. Both strains could utilize all four types of biosurfactants efficiently as revealed by the decrease in the biosurfactant concentration and increase in biomass content in the culture medium with the increase in the incubation period. However, the rate of degradation in all four types of biosurfactants was more in the case of *B. circulans* (MTCC8167) as compared to *P. aeruginosa* (MTCC 8165).

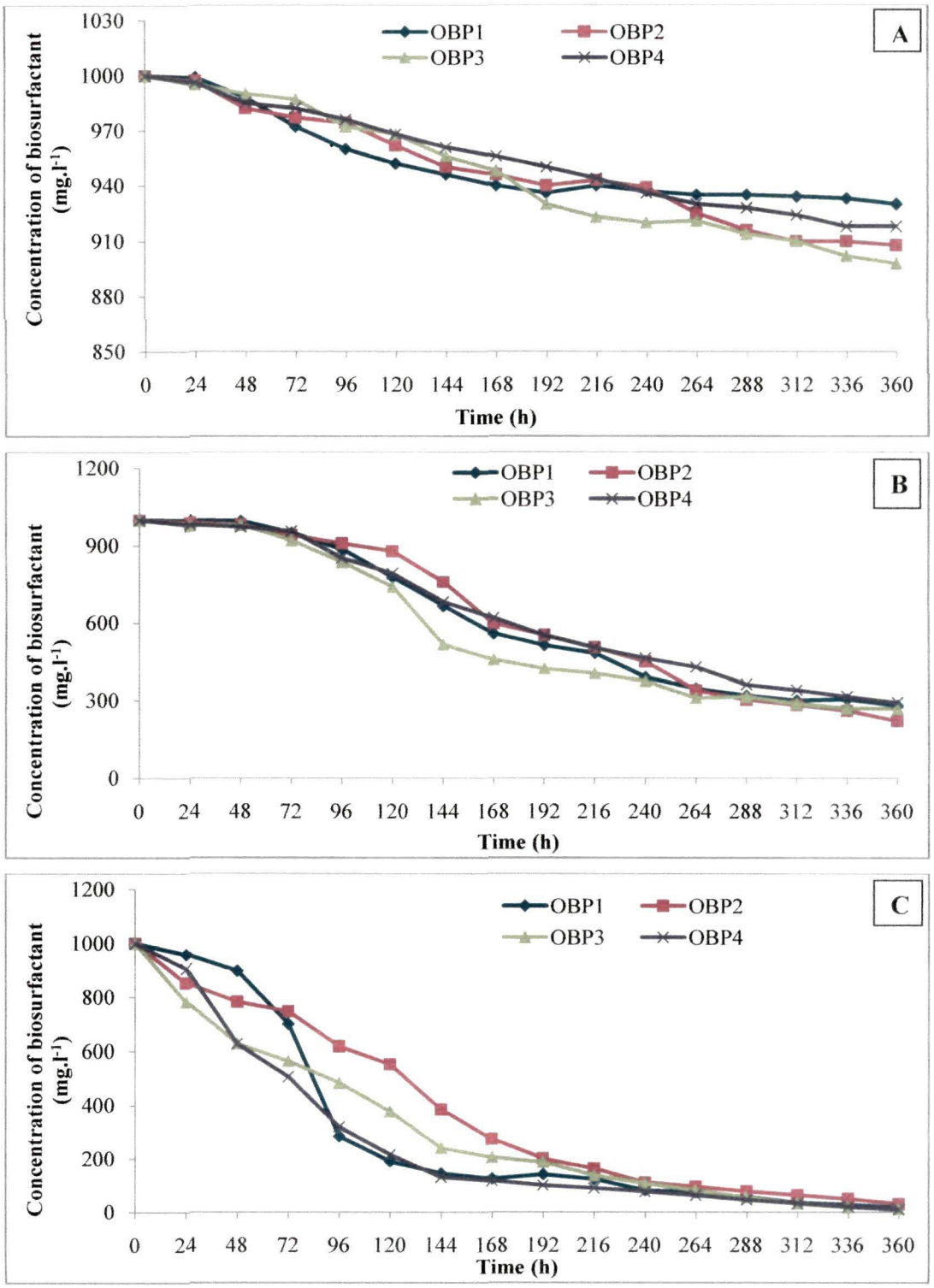


Figure 4.25. Degradation of biosurfactants produced by the selected bacterial strains by (A) *P. aeruginosa* (MTCC 8165), (B) *B. circulans* (MTCC8167), and

(C) in garden soil. Values are the mean of three independent experiments ± standard deviation

4.9 Biological activity of isolated biosurfactant

4.9.1 Effect of biosurfactant on the seed germination and growth

The seed germination tests were performed with the addition of biosurfactant at various concentrations to evaluate their toxicity towards the tested plants and the results are presented in Figure 4.26.

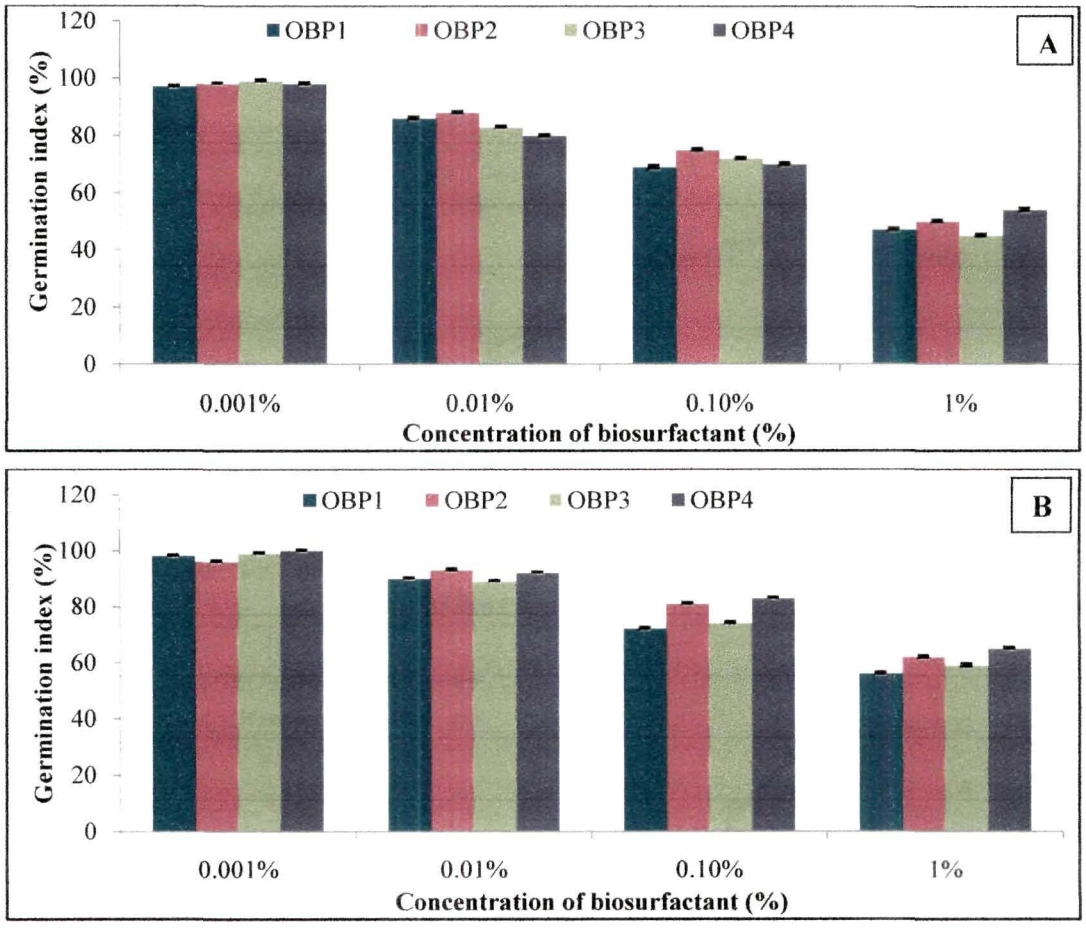


Figure 4.26. Effect of biosurfactants produced by *P. aeruginosa* strains on the germination index of (A) rice and (B) mung bean. Values are the mean of three independent experiments ± standard deviation

The biosurfactant solutions were tested at below CMC, at CMC and above CMC in order to determine the effect of biosurfactant concentrations on seed germination. The biosurfactant solutions at lower concentration did not exhibit any

inhibitory effects on the seed germination and root elongation of mung bean and rice. Normal development of leaves and elongation of primary roots with root hairs were observed in both plants. The GI of mung bean (96-100%) was higher up to CMC concentrations of the biosurfactants as compared to rice (97-99%). Increased concentrations of biosurfactants above CMC values caused reduction in GI values to 55-65% and 54-45% in mung bean and rice, respectively.

4.9.2 Larvicidal activity of biosurfactants

The isolated biosurfactants from all four strains showed no mortality of mosquito larvae at the recommended concentrations fixed by WHO. However, all four biosurfactants exhibited almost negligible mortality (%) towards the 3rd instar *Aedes albopictus* larvae at a dose of 100-1000 mg.l^{-1} and the results are presented in Table. 4.32.

Table 4.32. Mosquito larvicidal potency of biosurfactants produced by *P. aeruginosa* strains

Biosurfactant (mg.l^{-1})	**Mortality (%) of mosquito larvae after 24 h**			
	OBP1	**OBP2**	**OBP3**	**OBP4**
Control	-	-	-	-
100	-	-	-	-
300	-	-	-	-
500	-	-	-	-
700	-	-	-	-
1000	-	-	10±2.0	-
1500	10±2.5	10±1.5	15±2.0	10±1.5

Results represented mean ± standard deviation of three independent experiments.

Mortality of mosquito larvae at much higher concentrations above the one recommended by the WHO indicated the ineffectiveness of the isolated biosurfactants.

4.9.3 Antimicrobial activity of biosurfactants

Biosurfactant produced by the bacterial strains OBP1 exhibited the highest antibacterial activity against the tested microorganisms followed by OBP3, OBP2 and OBP4. The biosurfactant showed high activity against *Escherichia coli* (MG1655) and *Klebsiella pneumoniae* (MTCC 618), but less against *Pseudomonas aeruginosa* (MTCC 7815), *Bacillus subtilis* (MTCC 441), *Pseudomonas diminuta* (AUs) and *Staphylococcus aureus* (MTCC3160).

Table 4.33. Antimicrobial properties of biosurfactants produced by *P. aeruginosa* strains

Microorganisms	**Zone of inhibition (mm)**			
	Purified biosurfactants from			
	OBP1	**OBP2**	**OBP3**	**OBP4**
Escherichia coli (MTCC40)	16±0.2	15±0.4	16±0.3	14±0.3
Escherichia coli (MG1655)	18±0.3	1±0.8	18±0.9	16±0.4
Bacillus subtilis (MTCC 441)	14±0.4	10±0.3	12±0.2	10±0.1
Bacillus subtilis (MTCC 121)	12±0.1	12±0.7	14±0.4	12±0.4
Staphylococcus aureus (MTCC 737)	14±0.3	14±0.2	14±0.5	12±0.5
Staphylococcus aureus (MTCC 3160)	12±0.3	12±0.8	10±0.2	10±0.3
Klebsilla pneumoniae (MTCC 618)	14±0.4	16±0.9	14±0.8	14±0.8
Pseudomonas aeruginosa (MTCC 7815)	10±0.7	08±0.2	08±0.3	08±0.4
Pseudomonas diminuta (AUs)	12±0.3	12±0.7	10±0.7	10±0.4
Candida ablicans (MTCC 227)	09±0.7	07±0.2	08±0.6	07±0.7
Fusarium oxysporium(MTCC 284)	10±0.2	12±0.5	08±0.7	07±1.2
Aspergillus niger (AUs)	10±0.8	08±1.0	07±0.2	-
Colleototricum capaci (AUs)	10±0.1	08±0.3	-	-
Alternaria solani (AUs)	10±0.3	07±0.8	-	-

Results represented mean ± standard deviation of three independent experiments.

The biosurfactant displayed antifungal activity with higher MIC values against tested fungal strains compared to bacterial strains.

Table 4.34. Antimicrobial potency of biosurfactants produced by the *P. aeruginosa* strains

Microorganisms	Minimum inhibitory concentration ($mg.ml^{-1}$)			
	Purified biosurfactants from			
	OBP1	OBP2	OBP3	OBP4
Escherichia coli (MTCC40)	0.5±0.3	0.5±0.5	0.5±0.1	0.5±0.1
Escherichia coli (MG1655)	0.25±0.8	1.25±0.2	0.25±0.6	0.5±0.5

Bacillus subtilis (MTCC 441)	0.5±0.2	2.0±0.6	1.0±0.2	2.0 ±0.2
Bacillus subtilis (MTCC 121)	1.0±0.2	1.0±0.2	0.5±0.6	1.0±0.7
Staphylococcus aureus (MTCC 737)	0.5±0.5	0.5±0.6	0.5±0.4	1.0±0.5
Staphylococcus aureus (MTCC 3160)	0.5±0.1	1.0±0.3	2.0±0.1	2.0±0.2
Klebsiella pneumoniae (MTCC 618)	0.25±0.3	0.5±0.6	0.5±0.3	0.5±0.8
Pseudomonas aeruginosa (MTCC 7815)	2.0±0.7	4.0±0.5	4.0±0.9	4.0±0.3
Pseudomonas diminuta (AUs)	1.0±0.3	1.0±0.1	2.0±0.3	2.0±0.8
Candida ablicans (MTCC 227)	2.0±0.1	4.0±0.6	4.0±0.6	4.0±0.3
Fusarium oxysporium(MTCC 284)	2.0±0.5	1.0±0.7	4.0±0.2	4.0±0.2
Aspergillus niger (AUs)	2.0±0.2	4.0±0.7	-	-
Colleototricum capaci (AUs)	2.0±0.4	4.0±0.2	-	-
Alternaria solani (AUs)	2.0±0.2	2.0±0.4	-	-

Results represented mean ± standard deviation of three independent experiments.

4.9.4 Chemo-attractant property of biosurfactant

The biosurfactant produced by the bacterial strain OBP1 was found to be rhamnolipid in nature (described in the section 3.6.2 and 4.7.4) and this biosurfactant at a concentration of 0.5% (w/v) was used to determine its chemo-attractant property on *Staphylococcus aureus* (MTCC 3160) and *Klebsiella pneumoniae* (MTCC 618). Glucose 5% (w/v) was the positive control. The cultures were incubated for 24h. Both bacterial strains *Staphylococcus aureus* (MTCC 3160) and *Klebsiella pneumoniae* (MTCC 618) exhibited growth almost similar to that of the control (Glucose, 5% w/v). However, the bacterial strain failed to show any growth towards streptomycin (1.0 $mg.ml^{-1}$) containing wells and the same are shown in Figure 4.27. The tested bacterial strains showed positive chemotaxis response towards the rhamnolipid biosurfactants similar to that of glucose but negative chemotaxis response towards the streptomycin.

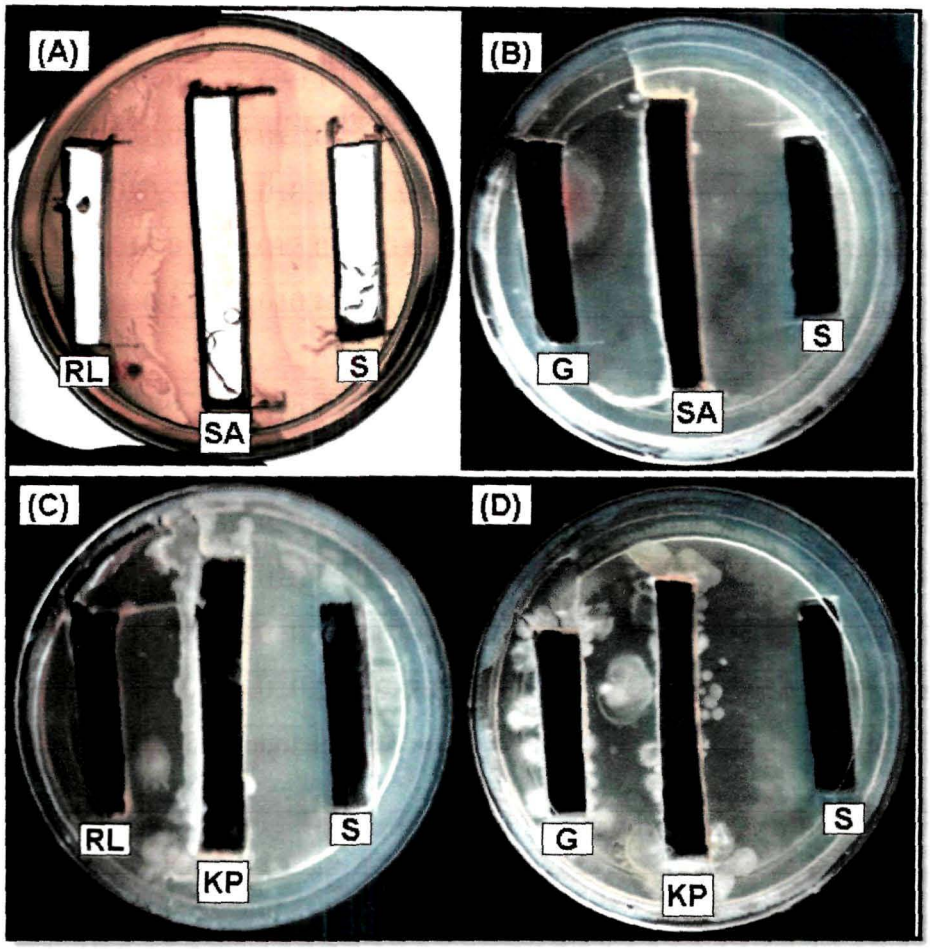


Figure 4.27. Chemotaxis activity of (A) *Staphylococcus aureus* (SA) with RL and streptomycin (S); (B) *Staphylococcus aureus* (SA) with glucose (G) and streptomycin (S); (C) *Klebsiella pneumoniae* (KP) with RL and streptomycin (S); and (D) *Klebsiella pneumoniae* (KP) with glucose (G) and streptomycin (S)

4.9.5 Cell cytotoxicity of isolated biosurfactant

The biosurfactants of the bacterial strains did not exhibit any inhibitory effect on the primary mouse fibroblast cell line (L929). Data are presented in Figure 4.28. Biosurfactant of OBP1, OBP2, OBP3 and OBP4 upto a concentration of 100 $\mu g.ml^{-1}$ failed to prevent the growth of mouse fibroblast cells referring to lack of cell cytotoxicity at higher concentrations.

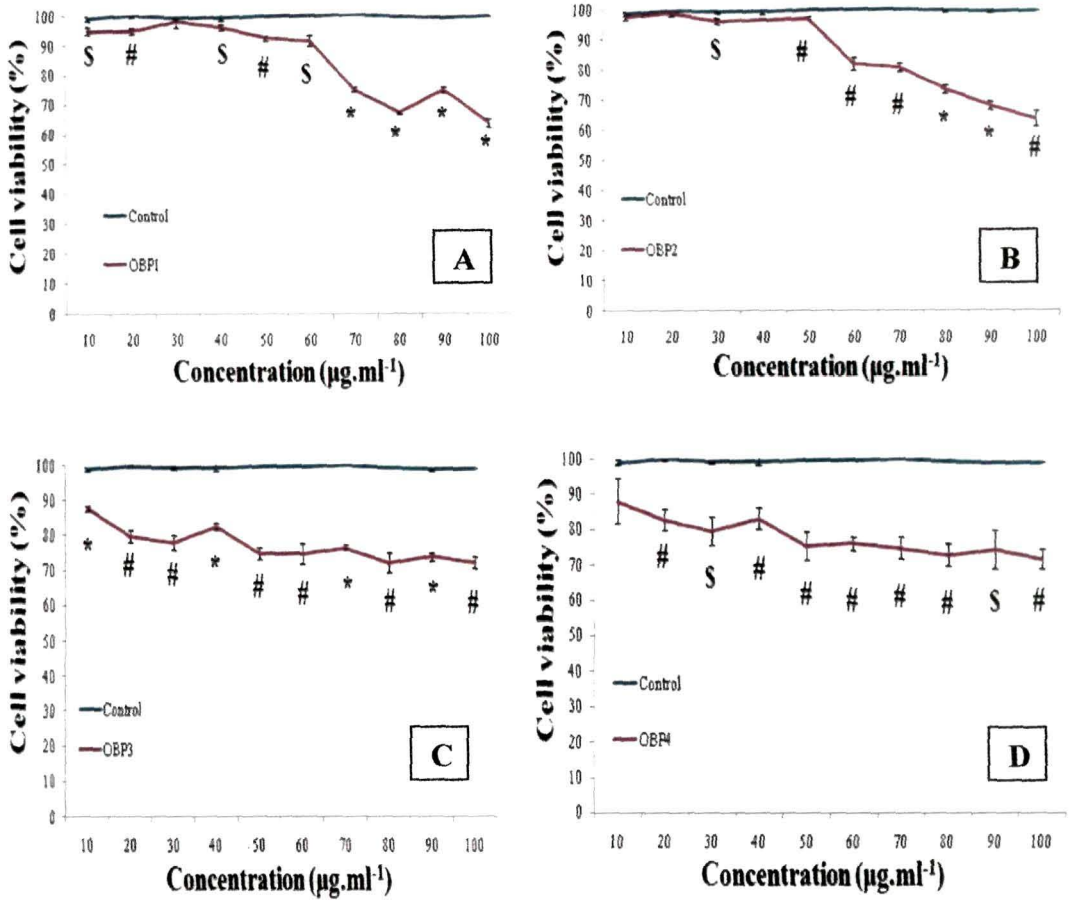


Figure 4.28. Effect of biosurfactants on the viability of mouse fibroblast cell line (L929) grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum. (A) *P. aeruginosa* OBP1, (B) *P. aeruginosa* OBP2, (C) *P. aeruginosa* OBP3, and (D) *P. aeruginosa* OBP4. Values are the mean of three independent experiments (mean ± SD). *, $p \leq 0.001$; #, $p \leq 0.01$; $, $p \leq 0.05$

4.9.6 The acute dermal irritation study with the isolated biosurfactants

The acute dermal irritation study showed no dermal reactions at 72 h such as erythema or edemas compared to their negative control after the application of biosurfactants on the shaved region of rabbits and are shown in Figure 4.29.

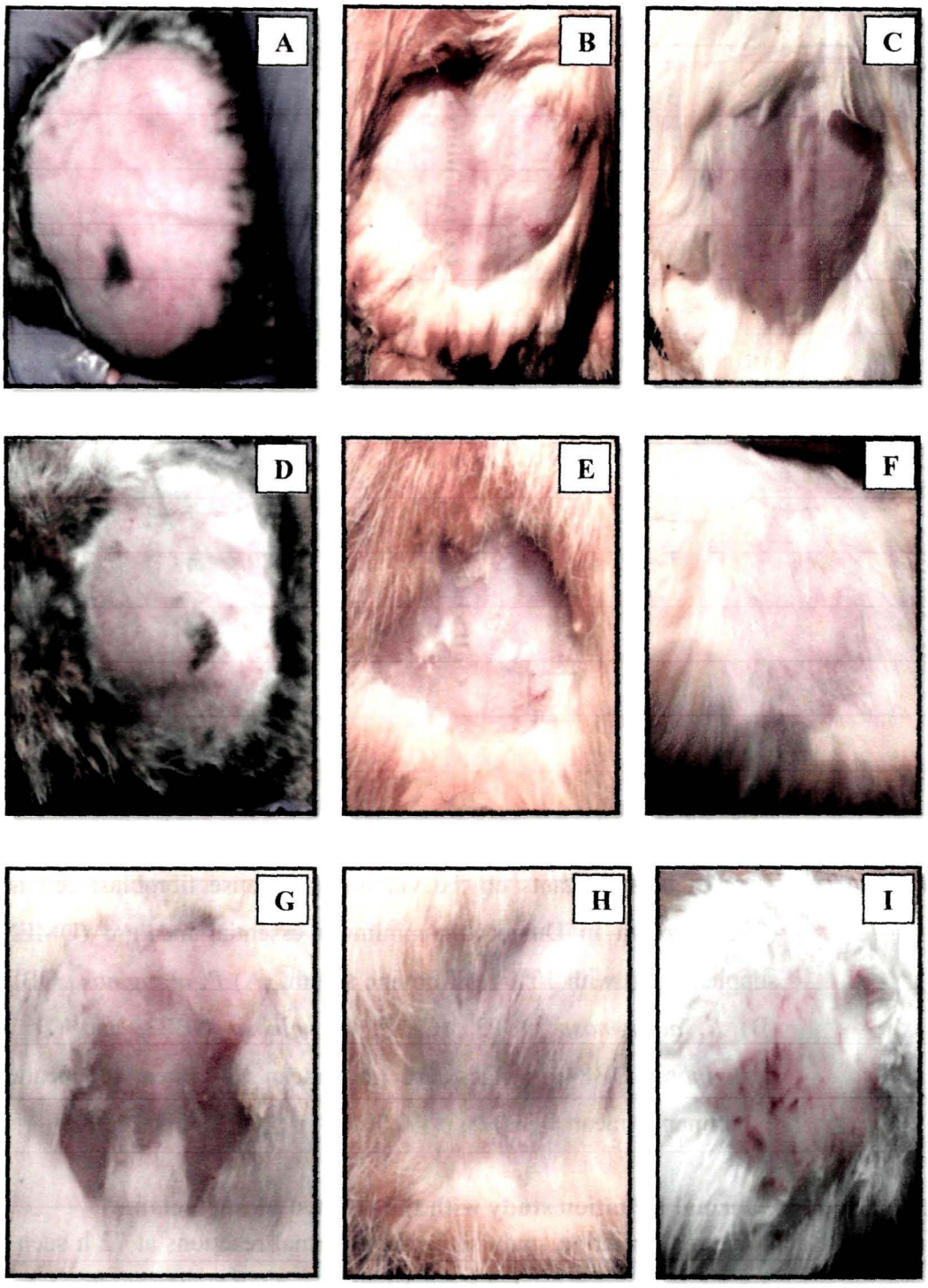


Figure 4.29. The acute dermal irritation study of transdermal patch in rabbits. (A) at 0 h and (D) after 72 h of treatment with biosurfactant from OBP1 strain; (B) at 0 h and (E) after 72 h of treatment with biosurfactant from OBP2

strain; (C) at 0 h and (F) after 72 h of treatment with biosurfactant from OBP3 strain; (G) at 0 h and (H) after 72 h of treatment with biosurfactant from OBP4 strain; (I) Control: after 72 h of treatment with 0.8% HCHO (v/v)

No significant reductions in the total body weight in the treated groups of rabbits were observed as compared to the control group. The concentration of biosurfactants above their CMC was non-toxic to the skin of rabbit. The acute dermal irritation study with the isolated biosurfactants also showed no adverse effect on the haematological parameters of the treated rabbits and is presented in Table 4.35.

Table 4.35. Results of haematological test after treatment with biosurfactants produced by *P. aeruginosa* strains

Components	Treatment 1 OBP1	Treatment 2 OBP2	Treatment 3 OBP3	Treatment 4 OBP4
WBC(K)	6.11±0.98	6.79±0.35	6.24±0.26	7.21±0.05
Neutrophil (%)	11.16±1.7	19.06±1.0	22.32±2.8	12.51±1.2
Lymphocyte (%)	68.72±6.2	81.25±2.2	61.06±0.4	71.25±0.2
Monocyte (%)	6.23±0.15	1.08±2.66	5.77±1.29	3.11±2.16
Eosinophil (%)	8.33±0.49	3.19±1.38	2.03±0.91	1.28±1.31
Basophil (%)	0.87±0.01	0.75±0.01	0.65±0.01	0.83±0.01
RBC(M)	10.02±0.1	7.98±0.15	7.62±0.76	5.36±0.81
Hb ($g.dl^{-1}$)	36.19±8.0	18.76±1.0	22.29±3.7	21.06±5.0
Hct (%)	34.07±1.0	42.21±3.2	49.39±0.2	32.31±1.2
MCV(fl)	76.33±3.9	51.79±2.5	70.86±2.0	41.62±2.1
MCH(pg)	16.02±0.5	13.07±1.0	14.96±1.3	13.37±2.2
MCHC ($g.dl^{-1}$)	37.18±3.9	41.07±0.5	29.10±2.4	42.17±0.2
Reticulocyte (%)	2.16±0.98	5.33±2.13	1.91±0.88	5.73±3.11
PLT(K)	1578±156.5	1708±118.3	1611±179.9	1428±104.1
PT(sec)	13.75±0.3	13.19±3.8	9.98±0.95	12.29±1.2
APTT(sec)	17.61±0.9	46.79±5.5	20.50±3.3	26.09±1.5

Components	Positive control	Negative control
WBC(K)	5.02±0.66	7.11±0.78
Neutrophil (%)	19.36±2.66	16.26±2.4
Lymphocyte (%)	51.69±1.90	52.69±0.9
Monocyte (%)	4.04±0.97	4.00±0.87
Eosinophil (%)	2.28±0.16	3.88±0.63
Basophil (%)	0.85±0.007	0.65±0.01
RBC(M)	8.36±0.66	5.96±0.78
Hb ($g.dl^{-1}$)	19.86±2.24	17.06±4.0
Hct (%)	49.66±4.0	42.16±2.1
MCV(fl)	49.36±3.2	48.96±3.6
MCH(pg)	11.55±5.2	10.35±5.9
MCHC ($g.dl^{-1}$)	44.98±4.5	41.98±0.5
Reticulocyte (%)	3.99±0.71	3.63±0.26
PLT(K)	1620±111.5	1360±107.3
PT(sec)	16.98±1.2	13.58±0.2
APTT(sec)	23.71±5.5	27.51±5.9

Mean ± S.D of three individual experiments

WBC: white blood cell, RBC: red blood cell, Hb: hemoglobin, Hct: hematocrit, MCV: mean corpuscular volume, MCH: mean corpuscular hemoglobin, MCHC: mean corpuscular hemoglobin concentration, PLT: platelet, PT: prothrombin time, APTT: activated partial thromboplastin time

4.10 Application of biosurfactant in nanotechnology

4.10.1 Biosurfactant assisted synthesis of iron oxide nanocrystals

The morphology of the magnetite crystals depends more on the ultrasonic irradiation than on the growth temperature. It was interesting to note that the randomly distributed biosurfactants could stabilize iron oxide nanorods and nanoplates in contrast to the spherical nanoparticles when PEG was used as the supporting matrix. The diameter of nanorods was distributed within a spectrum of (9.5-34) nm with

Plate No. 1

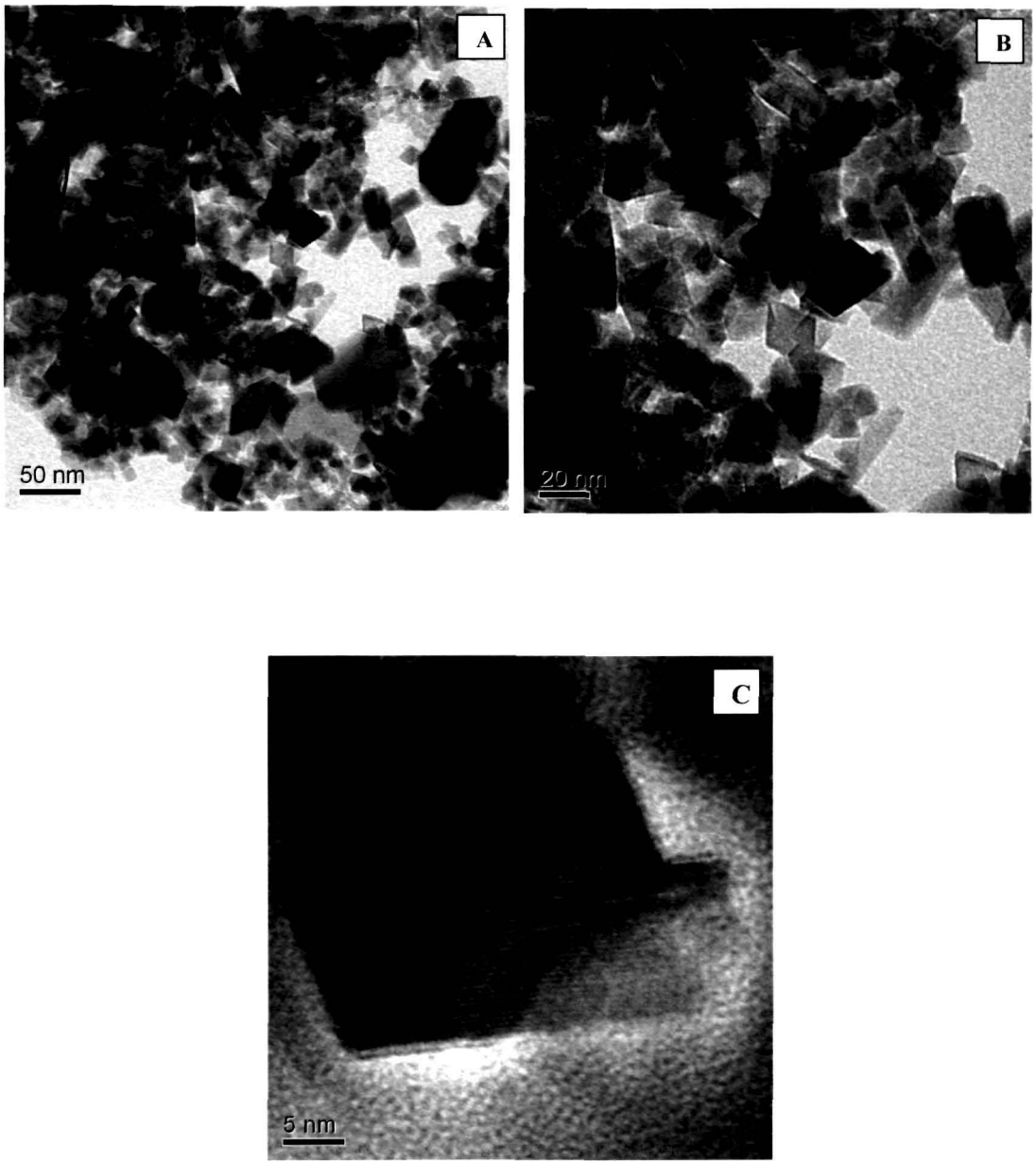


Figure 4.30. TEM micrograph of the biosurfactant assisted synthesis of iron oxide nanocrystals

Plate No. 2

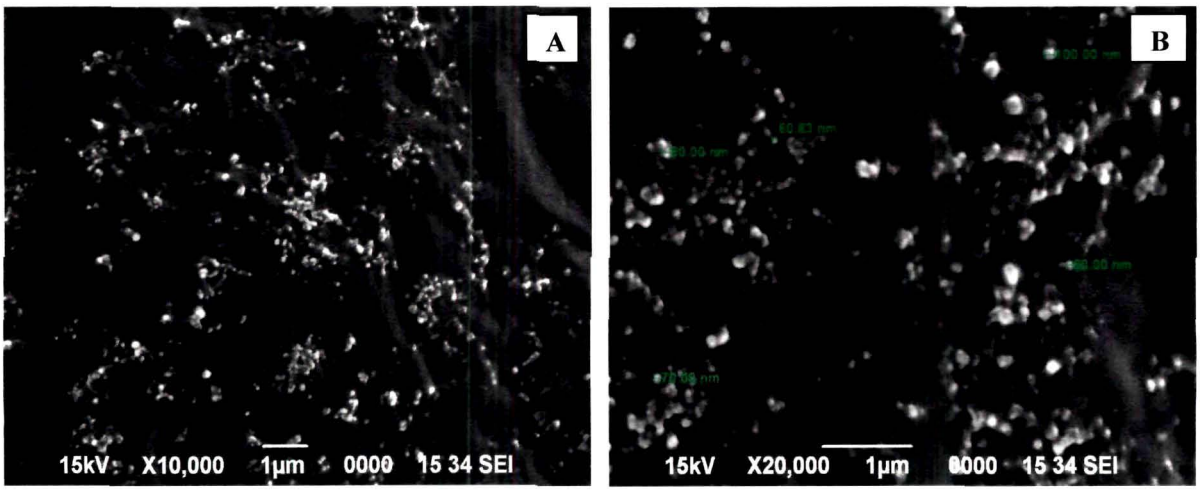


Figure 4.31. TEM micrograph of the biosurfactant assisted synthesis of iron oxide nanocrystals

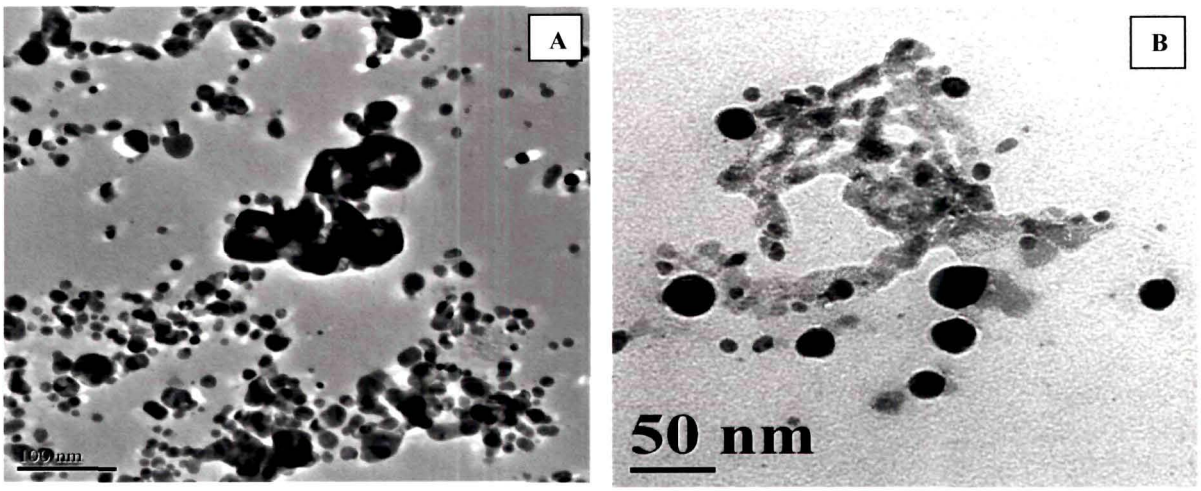


Figure 4.32. TEM micrograph of A) SNP and B) SNP/RL composites

average diameter of 23 nm. Nanorods were as long as 91 nm along their long axis. On the other hand, the nanodiscs appeared with strained geometry of 4-6 faces as shown in the **Figure 4.30.**

4.10.2 Biosurfactant assisted synthesis of silver nanoparticles

The synthesized colloidal composite (SNPRL) was analyzed using SEM. As seen in **Figure 4.31,** the particle size of SNPRL is distributed from 40-200 nm having majority of them within the range of 40-100 nm. The uniformity in the size may be attributed to the application of rhamnolipid. Detailed morphological study of the nanoparticles was performed using TEM and the same is presented in **Figure 4.32.**

The TEM micrographs represented naked and rhamnolipid coated SNPs. The rhamnolipid coated were almost spherical and well separated from each other with a particle size distribution of 20-40 nm. The micrograph also revealed uniform thickness coating (marked with arrow) of the rhamnolipid molecules on the silver nanoparticles. The small clusters of silver nanoparticles (particle size 20 nm) in rhamnolipid molecules might be due to drying of the colloidal sample during sample preparation on the carbon coated TEM-grid used for analysis.

4.10.3 Protection of silver nanoparticles by biosurfactant against salt treatment

As evident in Figure 4.33 there is consistency in the UV-Vis analysis of silver nanoparticles rhamnolipid (SNPRL) colloid during 10-33 days period as compared to the rapid fall during 0-10 days. The loss of intensity without broadening on day 10 as compared to day 0 suggested decrease in the number of SNP in the SNPRL colloid. During the period of study, the peak of the SNPRL shifted from 401 nm on day 0 to 404 nm on day 33rd.

In Figure 4.33, the magnification of the overlapping peaks from day 10 to 33 clearly showed changes in the peak position and intensity with respect to time. The intensity comparison of the SNPRL and RL colloids can be observed in Figure 4.34.

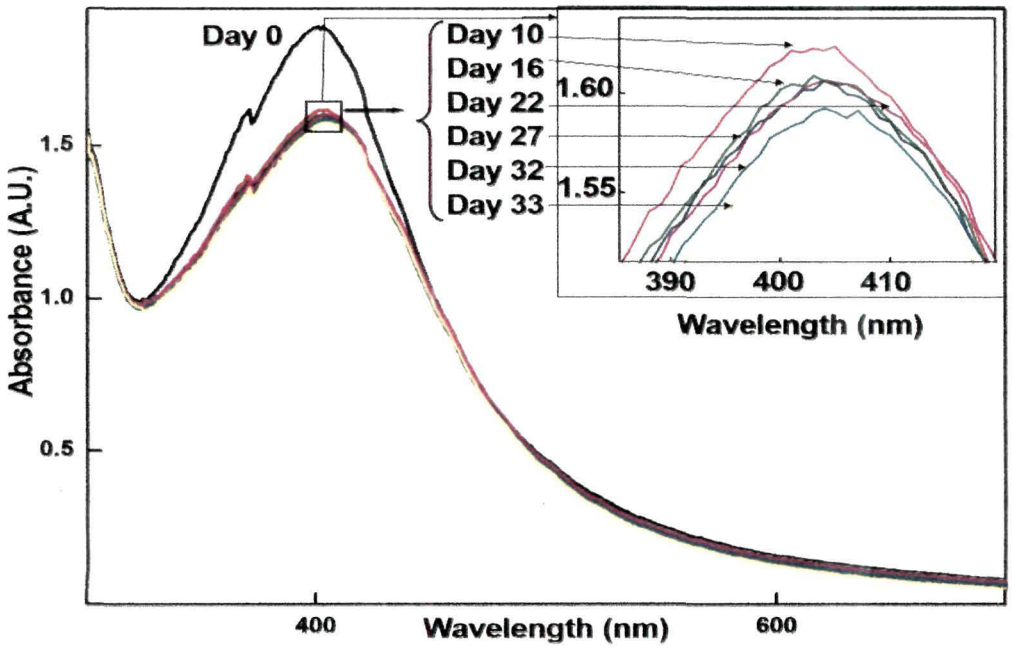


Figure 4.33. UV-Vis spectroscopic analysis of the silver nanoparticle colloid in rhamnolipid suspension with respect to time in days. The details on the absorption peaks during day 10 to 33 were shown in the right side corner

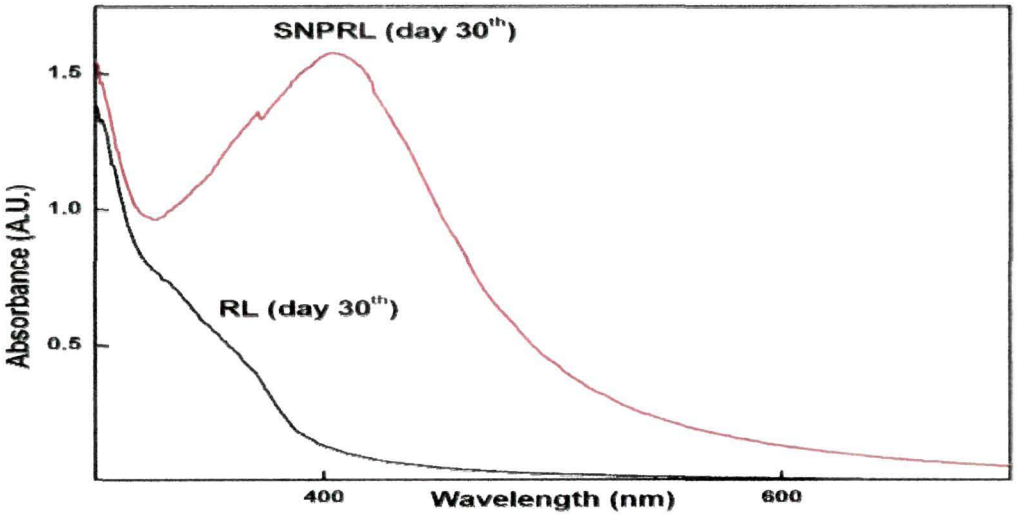


Figure 4.34. Graph showing the relative absorbance of the RL solution and SNPRL solution after synthesis on 30th day

As seen in Figure 4.35, the salt stability of the SNPRL was very high as compared to freshly prepared SNP colloid without rhamnolipid (RL). On application of NaCl to attain the final concentration of 2.0 $mg.ml^{-1}$ of the colloids, the SNPRL did not show any colour change.

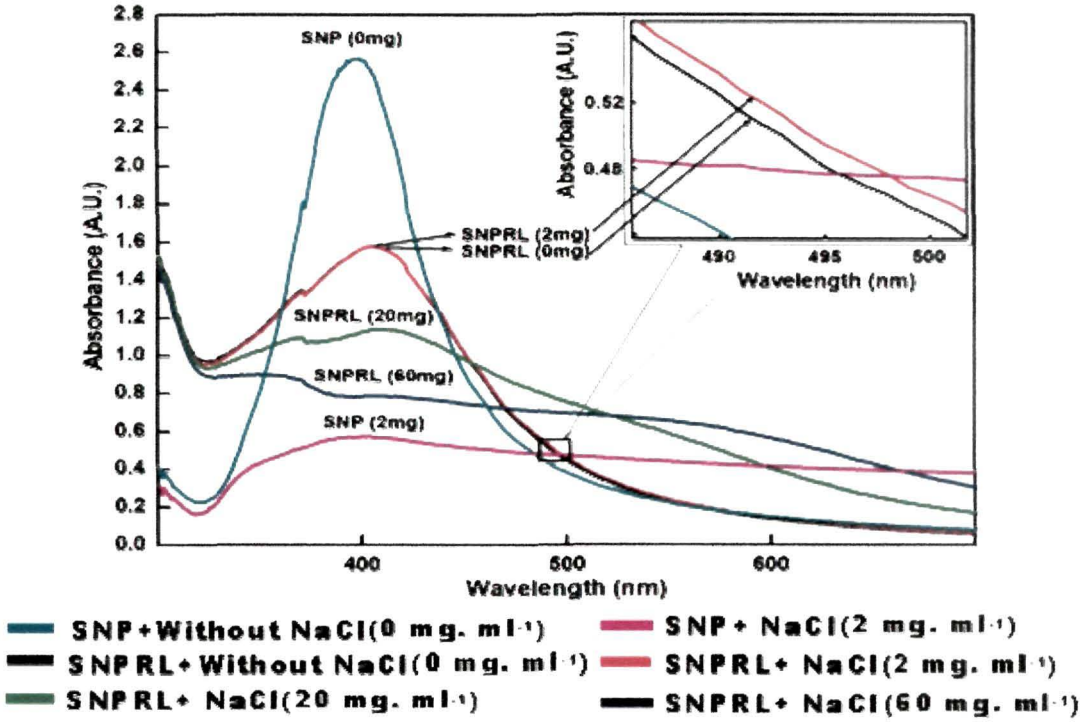


Figure 4.35. UV-Vis spectroscopic evidence of silver nano-particle rhamnolipid (SNPRL) stability during the treatment with NaCl (2 $mg.ml^{-1}$) and further degradation of SNPRL at an exposure of 60 $mg.ml^{-1}$ of NaCl. Freshly prepared silver nano-particles (SNP) was used as negative control.

When examined the same using UV-Vis spectrophotometer there was no change in the SNPRL (2 mg) as compared to blank SNPRL (0 mg), as shown in Figure 4.35. Broadening of the slight insignificant peak was observed in SNPRL near 490 nm suggesting a minor amount of SNP clumping. On the contrary, when the same concentration of NaCl was applied to the freshly prepared silver nanoparticles the intensity collapsed signifying that all the nanoparticles diminished (Figure 4.35) in SNP (0 mg) and SNP (2 mg). The SNPRL was found to be dominant in the protection of silver nanoparticles from NaCl as it took NaCl concentration of 60 $mg.ml^{-1}$ to

diminish all SNP in the colloid. The surface plasmon resonance property of the SNPRL was retained after addition of NaCl suggesting the presence of silver in the form of nanoparticles.

4.10.4 Antimicrobial activity of rhamnolipid nanocomposites of iron oxide nanocrystals (IONRL) and silver nano particles (SNPRL)

The antibacterial activity of SNP, ION, RL, SNPRL, and IONRL was depicted in Figure 4.36.

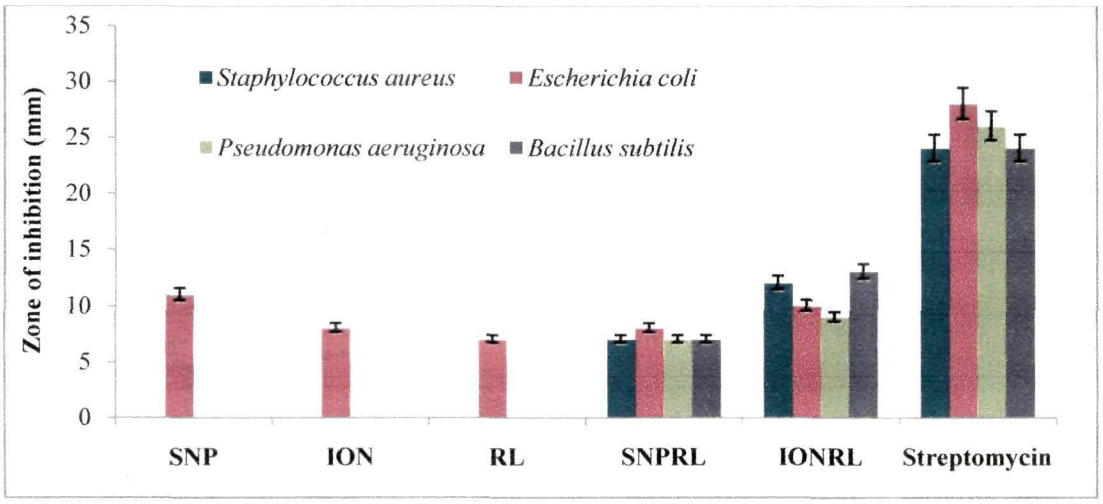


Figure 4.36. Antibacterial activity of (i) pristine SNP, ION and RL, (ii) SNPRL and IONRL nanocomposite, and (iii) streptomycin against different bacterial strains. Values are the mean of three independent experiments ± standard deviation

However, SNP, ION and rhamnolipid when applied individually did not possess lethal effect except in *E. coli*. Both SNPRL and IONRL possessed positive antibacterial activity against all four tested bacterial strains. The nanocomposites exhibited enhanced antibacterial activity as compared to that of pristine SNP, ION and rhamnolipid in the same concentrations.

4.11 Industrial applications of biosurfactant producing bacteria

4.11.1 Synthesis of bis-uracil derivatives in the presence of biosurfactant

The compound 6-[(dimethylamino) methyleneamino]-1, 3-dimethyl - pyrimidine-2, 4(1*H*, 3*H*)-dione (4) reacts with aldehydes (2) in water. But, the reaction did not proceed at all under this reaction condition. Using *p*-toluene sulphonic acid (*p*-

TSA) as catalyst or application of heat also did not support the reaction to proceed. Interestingly, when the reaction was carried out using the biosurfactant produced by *P. aeruginosa* OBP1 in water, the reaction took place, but produced very low yield. However, when *p*-TSA (15 mol %) was added as catalyst, the yield of the product was found to increase. In order to optimize the condition, the reaction between 6-[(dimethylamino) methyleneamino]-1, 3-dimethylpyrimidine-2, 4(1*H*, 3*H*)-dione (4) with benzalaldehyde (2a; R= $-C_6H_5$) was chosen as the model reaction. Results are elaborated in Table 4.36.

Table 4.36.Optimization of the reaction for the model reaction between 6-[(dimethylamino) methyleneamino]-1, 3-dimethyl -pyrimidine-2, 4(1*H*, 3*H*)-dione **[4]** and benzalaldehyde **[2a]**

Entry	**Reaction Condition**	**Time (h)**	**Yield [%][a] of (5a)**
1	Water, rt	48	No product found
2	Water, reflux	48	No product found
3	Water, reflux, 15 mol% *p*-TSA	12	Trace amount
4	CH_3CN, reflux	12	No product found
5	EtOH, reflux	12	No product found
6	MeOH, reflux	12	No product found
7	DMF, reflux	12	No product found
8	Toluene, reflux	12	No product found
9	$PhNO_2$, reflux	12	No product found
10	Water, SDS, [b] rt	12	56
11	Water, biosurfactant, rt	12	25
12	Water, biosurfactant, rt, 5 mol% *p*-TSA	12	40
13	Water, biosurfactant, rt, 10 mol% *p*-TSA	8	60
14	Water, biosurfactant, rt, 15 mol% *p*-TSA	4	75

[a] Yield [%] is referred to isolated yields and calculated from (mol of product)/(mol of initial substrate) ×100. [b] SDS=Sodium dodecyl sulphate

Accordingly, stirring of 1, 3-dimethyl-6-aminopyrimidine-2, 4(1H, 3H)-dione (1) and benzaldehyde (2a; R=Ph) together in the presence of biosurfactant solution at its CMC resulted in the formation of 5, 5'-phenylmethylenebis (1, 3-dimethyl-6-amino -pyrimidine-2, 4-dione) (3a) within 15 min in 99% yield (Scheme 2). The reaction was monitored by TLC. The reaction was clean providing only one product that is (3a).

To study the scope and limitation of the reaction, the above scheme was extended to other differently substituted aromatic, aliphatic and heterocyclic aldehydes and ketones (2a-r) under the same optimized reaction conditions. The results are summarized in Table 4.37 (a-r) along with comparison in the presence or absence of the biosurfactant.

Table 4.37. Synthesis of bisuracil derivatives (3) in the presence of biosurfactant (BS) vides Scheme1

Entry	Carbonyl Compounds (2)	Time (h)		Yield [%][a]	
		In presence of BS	In absence of BS[12]	In presence of BS	In absence of BS[12]
a	C_6H_5CHO	0.25	1	99	95
b	$C_6H_5CH=CHCHO$	0.91	4	95	93
c	*p*-$OMeC_6H_4CHO$	1.83	4	97	91
d	*p*-ClC_6H_4CHO	0.25	0.41	99	99
e	*p*-OHC_6H_4CHO	2	7	91	75
f	*o*-OHC_6H_4CHO	2	7	90	73
g	*p*-MeC_6H_4CHO	0.33	0.41	99	99
h	*p*-$NO_2C_6H_4CHO$	2.33	10	93	73
i	*m*-$NO_2C_6H_4CHO$	2.25	5	91	82
j	2-furaldehyde	0.33	1	99	99
k	Thiophene-2-carbaldehyde	0.75	6	99	87
l	Paraformaldehyde	0.41	1	93	91
m	CH_3CHO	0.75	3	94	92
n	$CH_3(CH_2)_2CHO$	0.83	3	95	93
o	$CH_3(CH_2)_3CHO$	0.83	3	95	90
p	$(CH_3)_2CO$	48	48	-[b]	-[b]
q	CH_3COPh	48	48	-[b]	-[b]
r	PhCOPh	48	48	-[b]	-[b]

[a]Yield (%) is referred to isolated yields and calculated from (mol of product)/(mol of initial substrate) ×100. [b] No product formation.

All the aldehydes (entries 2a-o, Table 4.37) reacted with equal ease within the short times, furnishing the products, bis-uracils (3a-o) possessed excellent yields (90-99%) without the formation of by-products. However, under the present condition,

also, the reaction with ketones (entries 2p-r, Table 4.37) failed. The yield of products increased and the reaction time reduced drastically along with reduction in the amount of water used from 30-35 ml but without biosurfactant to 8 ml.

In the next set of experiment, instead of 1, 3-dimethyl-6-aminopyrimidine-2, 4(1H, 3H)-dione (1), the reaction between 6-[(dimethylamino)-methyleneamino]-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione (4) and benzaldehyde (2a; R=$-C_6H_5$) was studied using the biosurfactant in water (Scheme 2).

Table 4.38. Synthesis of bisuracil derivatives (5) and (6) in the presence of biosurfactant vide Scheme 2

Entry	Carbonyl Compounds (2)	Time (h)	Yield of product (5) [%][a]	Yield of product (6) [%][a]
a	PhCHO	3.5	75	Trace
b	PhCH=CHCHO	10	25	Trace
c	*p*-OMePhCHO	3	trace	93
d	*p*-ClPhCHO	2	95	Nil
e	*o*-OHC_6H_4CHO	5	trace	75
f	*p*-OHPhCHO	5	trace	85
g	*p*-MePhCHO	4	15	87
h	*p*-NO_2PhCHO	4.5	96	Trace
i	*m*-NO_2PhCHO	4.5	60	37
j	2-furaldehyde	3	73	25
k	Thiophene-2-carbaldehyde	3.5	trace	93
l	Paraformaldehyde	10	trace	Nil
m	CH_3CHO	10	trace	Nil
n	$CH_3(CH_2)_2CHO$	10	trace	Nil
o	$CH_3(CH_2)_3CHO$	10	trace	Nil
p	$(CH_3)_2CO$	48	-[b]	-[b]
q	CH_3COPh	48	-[b]	-[b]
r	PhCOPh	48	-[b]	-[b]

[a]Yield [%] is referred to isolated yields and calculated from (mol of product)/(mol of initial substrate)×100. [b] No Product Formation.

Towards the completion of the reaction, a mixture of products, bisuracils (5) and (6) were obtained. The yield of the product(s) was also not satisfactory. In view of poor yield, 15 mol % of *p*-TSA was added to increase the yield of the products (Table

4.38). Remarkably, the reaction was very clean providing 5a (R=$-C_6H_5$) as the major product and trace amount of 6a.

The reaction was extended to other differently substituted aromatic, aliphatic, and heterocyclic aldehydes and ketones (2a-r) under the same optimized reaction condition. Out of two, one product was obtained always predominantly or exclusively. The results are summarized in Table 4.38 (entries a-r). All aldehydes (Table 4.38, entries 2a-o) reacted with equal ease within short duration, furnishing the products, 6-amino-6'-(dimethylamino)methyleneamino-1, 1', 3, 3'-tetramethyl-5, 5'-(phenylidene)-bis-[pyrimidine-2, 4(1H, 3H)-dione] derivatives (5a-k) and (6a-k) in good yields except (2b) and with no by-product formation. It was observed that the same reaction did not proceed in water in the absence of biosurfactant, thereby proving the necessity of biosurfactant. Similar to Scheme 1(entries p-r, Table 4.37), here also the reaction failed with ketones. However, with aliphatic aldehydes (Table 4.38, entries l-o), the reaction was not at all satisfactory.

To extend the scope and limitation of the reaction further, the reaction between 6-[(dimethylamino) methyleneamino]-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione (4) and dicarbaldehydes (7) (Scheme 4) was studied. The results are summarized in Table 4.39 (entries a and b). In the case of an aliphatic dicarbaldehyde, glutaraldehyde (Table 4.39, entry b) the bis-uracil adduct (8b) as major one (25%) was obtained, but in the case of p-benzenedicarbaldehyde (Table 4.39, entry a) bis-uracil adduct (8a) was obtained predominantly along with little amount of N-formylated bis-uracil derivatives (9). Un-reacted amount of 4 was recovered.

Table 4.39. Synthesis of bisuracil derivatives (8) and (9) in the presence of biosurfactant vide Scheme 4

Entry	**Dicarbonyl Compounds (7)**	**Time (h)**	**Yield of product (8) [%][a]**	**Yield of product (9) [%][a]**
a	*p*-$(CHO)C_6H_4(CHO)$	5	65	28
b	$CHO(CH_2)_5CHO$	10	25	Trace

[a]Yield [%] is referred to isolated yields and calculated from (mol of product)/(mol of initial substrate) ×100.

4.11.2 Degradation of complex synthetic polymers

The growth of *P. aeruginosa* strain OBP1 on various nanocomposite films such as hyper branched epoxy (HBE), modified hyperbranched epoxy (MHBE) and their clay nanocomposites along with the corresponding pristine polymeric films can be realized, as shown in Figure 4.37.

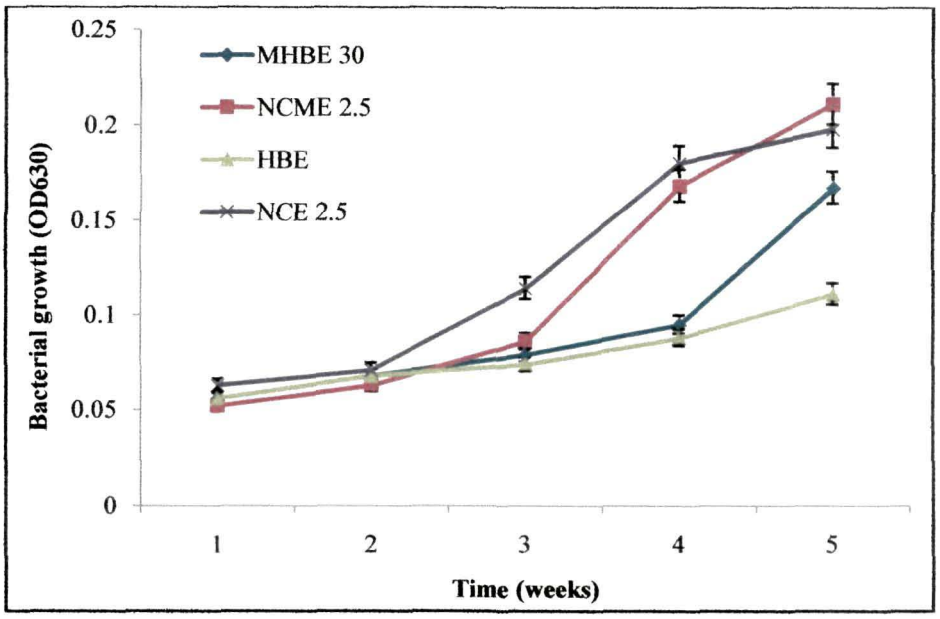


Figure 4.37. Growth curves of bacterial strain OBP1 on HBE, MHBE30, NCE2.5 and NCME2.5 their nanocomposites. Values are the mean of three independent experiments ± standard deviation

The growth of the bacterial strain on the tested polymers increased with the increase in exposure time to the bacterial strain and is presented in Figure 4.38. The growth rate in all the tested nanocomposites as well as on the pristine polymers was not significant up to two weeks of bacterial exposure. But after 2-3 weeks of treatment, the biodegradation rate increased sharply as could be realized from the bacterial population density determination by McFarland turbidity method. The biodegradation process caused severe damage to almost all the nanocomposites which could be observed from SEM micrographs of the recovered nanocomposite films following five weeks of bacterial exposure. The same are presented in Figure 4.38.

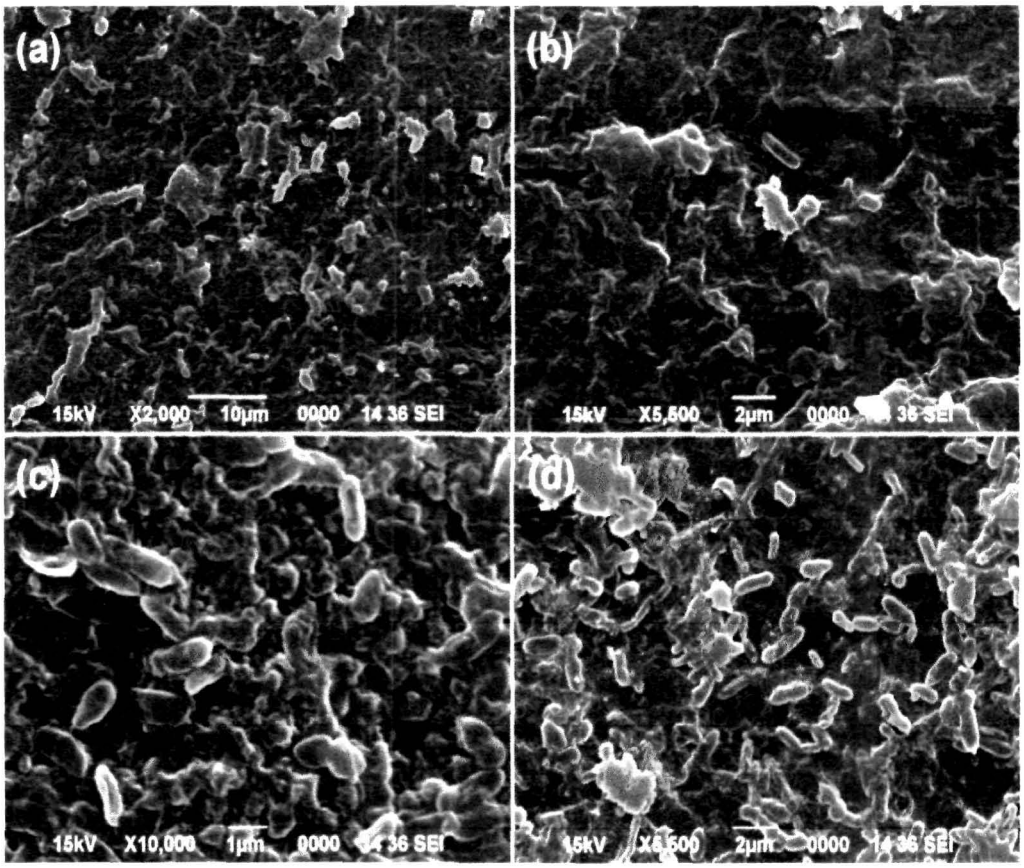


Figure 4.37. SEM micrographs of biodegraded nanocomposite films.(a) HBE, (b)NCE2.5, (c) NCME2.5, and (d) MHBE30

The same was further supported by the decrease in the weight of the nanocomposite films after biodegradation. However, the extent of biodegradation was found to be low in the case of pristine hyperbranched epoxy/clay nanocomposite in comparison to the modified hyperbranched epoxy nanocomposites, which could be seen clearly from the bacterial population as observed in SEM micrographs. Moreover, the rate of biodegradation was higher in the case of modified hyperbranched epoxy and its nanocomposites in comparison to the pristine HBE and its nanocomposite. The same are presented in Figure 4.38.

Chapter IV
Discussion

5.1 Isolation of potential biosurfactant producing bacteria capable of utilizing hydrocarbons from the environmental samples

A total of 52 bacterial strains with the ability to produce biosurfactant and utilize hydrocarbons, particularly the purified petroleum components such as n-hexadecane were isolated from hydrocarbon contaminated soil environments using the enrichment technique. Among them four bacterial strains were selected on the basis of their efficiency to utilize n-hexadecane and produce biosurfactants. Several reports suggested that the enrichment culture technique is efficient in isolating biosurfactant producing as well as hydrocarbon utilizing bacteria[364, 365]. Biochemical tests further confirmed that all four strains belonged to the species *P. aeruginosa*. Different dominant mesophilic bacteria such as *Pseudomonas putida*, *P. aeruginosa*, *P. saccharophila*, *Flavobacterium sp.*, *Burkholderia cepacia*, *Rhodococcus sp.*, *Stenotrophomonas sp.* and *Mycobacterium sp.* etc were reported to be isolated from the petroleum contaminated environments[133]. *Pseudomonas sp.* are commonly detected using both culture-dependent[366, 367] and culture-independent[368, 369] approaches and bacteria belonging to the species were isolated from the industrial waste-water samples and screened for growth on hydrocarbons and biosurfactant production[370, 371, 372]. The genus *Pseudomonas* is the most versatile group due to its inherent ability to utilize a diverse range of substrates as carbon source, particularly those found in petroleum[373, 374]. Superior performance of *P. aeruginosa* is due to the evolution of the alkane oxidation genes, which allow them to grow on alkanes as a sole carbon source[374]. Vila *et al.*[375] reported that the genes involved in the hydrocarbon degradation were present in the plasmids. Previous studies of Bordoloi and Konwar[2], Bora *et al.*[376], Saikia *et al.*[377] and Roy *et al.*[378] clearly supported the predominance of bacterial genus *Pseudomonas* in the North Eastern region of India especially in the petroleum contaminated environments of Assam and Assam-Arkan Basin, ONGC.

5.2 Characterization of potential biosurfactant producing bacteria

Majority of the bacterial strains isolated from crude oil contaminated environments were found to be Gram-negative. Previously it was reported that most of the bacteria isolated from such sites with a history of contaminations by hydrocarbons and its derivatives are Gram-negative and this could be a characteristic that contributes to survival of these populations in such harsh environments[41, 379]. Initial characterization of the potent bacterial isolates based on their morphological, physiological and biochemical properties revealed that all four isolates were closely related strains belonging to *Pseudomonas aeruginosa*. Finally, the genotypic analysis on the basis of partial 16S rRNA gene sequencing was undertaken to determine the precise taxonomic position of the strains. Alignment of 16S rRNA gene sequences of all four bacterial strains with sequences obtained by doing a BLAST NCBI[380] search revealed almost 99% similarity to *Pseudomonas aeruginosa*. Further, phylogenetic analysis of all four strains showed distinct variability in the phylogenetic relationship between the isolates. 16S rRNA sequencing as a basis of classification offers the distinct advantage of sensitivity, which is particularly important for the judicious identification of the closely related members of a particular species[381]. Moreover, the increasing availability and declining cost of high throughput sequencing operations makes the determination of nucleotide sequences for strain identification a viable option.

5.3 Ability of the bacterial strains to utilize different hydrocarbons

The selected bacterial strains exhibited better growth in the tested petroleum hydrocarbons. The overall preference of hydrocarbons was found to be in the order of n-hexadecane > octadecane > diesel > crude oil > tridecane > dodecane > kerosene > lubricating oil as evident from the increase in the bacterial biomass. The bacterial strains have the potentiality to degrade almost all the tested aliphatic hydrocarbons. The bacterial strain OBP1 followed by OBP3 and OBP4 exhibited the highest growth in dodecane, tridecane, hexadecane and octadecane-supplemented media as evident from the production of bacterial biomass. However, the bacterial isolate OBP3

exhibited almost similar biomass production in hexadecane and octadecane supplemented media but lower in tridecane and dodecane supplemented media. None of the strains were capable to utilize eicosane and triacontane. There are several reports on the degradation of short (C_8–C_{16}) and long chain (C_{44}) hydrocarbons[338, 352]. With the increase in the chain length of alkanes, the hydrophobicity of the molecule increases which in turn makes the molecules less soluble in water and reduces the bioavailability[289]. Several workers reported the capability to use aliphatic chains in the range of C_{12}–C_{24}, C_{12}–C_{34}, C_6–C_{28}, C_{12}–C_{32} and C_{12}–C_{28} by *P. aeruginosa* strains PAO1, RR1, (A1–A6), *P. fluorescens* CHA0 and *Pseudomonas sp.* PUP6[382, 383, 384].

Among the tested aromatic hydrocarbons such as benzene, toluene and xylene; the bacterial strains OBP4, OBP3 and OBP1 exhibited growth on toluene but not by the OBP2 strains. However, growth of the bacterial strains on aromatic hydrocarbons was not significant as compared to that on aliphatic hydrocarbons. The results are in consistent with the observation of Jones and Edington[385] who reported that only 0.5% of a large group of soil organisms could use aromatic hydrocarbons. In the case of polyaromatic hydrocarbons (PAHs), bacterial strains OBP4, OBP3 and OBP1 exhibited growth on anthracene and phenanthrene. The growth was significant in the case of OBP4. But no growth was observed in naphthalene except for OBP3. Cerniglia[386] reported that complete mineralization of high molecular weight PAHs could be achieved by only a limited number of microorganisms. Preference for toluene, anthracene and phenanthrene as the carbon source could be due to their isolation being from the environments, very frequently contaminated by aromatic and polyaromatic fractions of the crude oil. Bordoloi and Konwar[298] reported the isolation of several strains of *P. aeruginosa* from the petroleum contaminated sites of Assam, India capable of utilizing various PAHs such as phenanthrene, pyrene and fluorene as carbon substrates for their growth.

The bacterial strains exhibited better growth in diesel, crude oil and kerosene supplemented media as evident from the bacterial biomass. A greater portion of diesel, crude oil and kerosene contains aliphatic hydrocarbons and are reported to be more prone to microbial degradation[338, 352, 387].

5.4 Detection and quantification of biosurfactant

It is well known that microorganisms growing on hydrocarbons frequently produce biosurfactants with emulsification or surfactant activity[379]. Such microbial behavior has been considered as a biological strategy to facilitate the availability of hydrophobic substrates[7, 372]. In the present investigation, different assays based on biochemical and physical parameters were carried out with the objective to screen the potential hydrocarbon utilizing and biosurfactant producing bacterial strains from the petroleum contaminated environments. Among the physical parameter- based screening techniques, rapid drop collapse test is considered as a high throughput screening technique[260] which assists in the isolation of potent bacterial isolates. Jain *et al.*[261] suggested the drop collapse test to be a simple and sensitive technique used to detect and quantify the biosurfactant production. For direct detection of biosurfactant production by the bacterial isolates, two different independent techniques were performed which include hemolytic assay and CTAB agar test. Hemolysis assay is widely used for the screening of biosurfactant producing bacteria and could also be used for the quantification of glycolipid-type biosurfactant produced by bacteria[350]. Perfumo *et al.*[388] used blood agar plate technique to screen a novel thermophilic hydrocarbon degrading *Pseudomonas aeruginosa* AP02-1 producing rhamnolipid. CTAB agar test is another technique used for the detection of glycolipid-type biosurfactant production by the bacterial colonies in the culture plate directly[270]. Aparna *et al.*[23], Hazra *et al.*[287] and Perfumo *et al.*[388] reported screening of rhamnolipid producing thermophilic hydrocarbon-degrading *Pseudomonas aeruginosa* APO2, AB4 and 2B by CTAB agar blue plate method from the soil contaminated with petroleum products.

5.5 Growth characteristics and biosurfactant production

All four bacterial strains showed the requirement of similar nutrient and culture conditions which might be due to their isolation from the similar type of habitats that have the continuous exposure to the petroleum hydrocarbons. The growth curves of the bacterial strains in relation to duration of culture in mineral salt medium

supplemented with n-hexadecane as sole source of carbon, $(NH_4)_2SO_4$ and urea together as the nitrogen source showed nearly a parallel relationship between cell growth, reduction in the surface tension and biosurfactant production. Results clearly suggest a growth associated production of biosurfactants by the bacterial strains (Figure 4.6). Moreover, the preference for carbon substrates was quite similar among the strains. Though n-hexadecane was found to be the best carbon source for biosurfactant production by the bacterial strains but the pattern of growth behavior was relatively different from each other which might be due to the intrinsic variability for utilizing hydrocarbons. The initial reduction in the surface tension was about 45.1-66.3 mNm^{-1} after 36-48 h of inoculation during the early exponential phase of bacterial growth. Results indicate the initiation of biosurfactant production by the bacterial cells in the culture broth. The possible cause for the initiation of biosurfactant production in the early exponential phase could be due to availability of hydrocarbons as the source of carbon through pseudo-solubilization. After, 60-108 h of bacterial growth, the biosurfactant concentration increased rapidly, reaching its maximum after 120-132 h which was mainly towards the later stage of stationary phase of growth. Such behavior might be due to the release of cell bound biosurfactant into the culture broth[389]. The maximum surface activity in 156-168 h culture indicated the optimum level of biosurfactant production by the bacterial strains towards the stationary phase. The production of biosurfactant by the bacterial strains in n-hexadecane supplemented medium took place during the late exponential phase to early stationary phase of growth suggesting biosurfactant production as the secondary metabolite. Similar observations were reported by Makkar *et al.*[13], Lotfabad *et al.*[29], Wei *et al.*[52], and Abbasi *et al.*[92].

Various strains of *P. aeruginosa* were reported to show an over-production of rhamnolipid when cultures reached their stationary phase of growth[260, 281, 390, 391]. The depletion of nitrogen source in the culture medium mainly takes place during the stationary phase of cell growth[239, 252, 283, 392, 393]. Soberón-Chávez *et al.*[394] reported that N-limiting conditions do not favor rhamnolipid production, but production starts with the exhaustion of nitrogen in the culture medium[390]. Moreover, limitation of

multivalent ions such as Mg^{2+}, Ca^{2+}, K^{+}, Na^{+} and trace elements were reported to cause an enhancement in the rhamnolipid yield either in resting-cell cultures or during the stationary phase of growth[395]. The variation appeared in the pattern of rhamnolipid production was due to the use of different *Pseudomonas* strains, cultivation conditions which include pH and temperature, and media composition[396]. Hence there is a possibility that under the applied culture conditions, multiple limitations might occur having positive influence on rhamnolipid formation thus leading to an increase in the specific productivity per bacterial cell[396].

5.6 Effect of different carbon sources on biosurfactant production

The carbon source plays a crucial role in the production of rhamnolipid[27, 61]. In the present investigation, different carbon sources were used on the basis of their increasing complexity and hydrophobicity, which included glucose, glycerol, vegetable oils and petroleum hydrocarbons (n-hexadecane, octadecane, diesel and crude oil) to determine their effectiveness on rhamnolipid production (Table 4.6). The findings confirmed the ability of the bacterial strains in utilizing both the water-soluble and insoluble substrates for the production of biosurfactants corroborating the results of Perfumo *et al.*[388]. The hydrophobic substrates such as n-hexadecane, octadecane, diesel and crude oil could induce better biosurfactant production as compared to the vegetable oils and water miscible substrates. Biosurfactant production was reported to be induced by hydrocarbons or other water-insoluble substrates[397]. Another phenomenon is the catabolic repression of biosurfactant synthesis by glucose and other primary metabolites[133]. No surface-active agent could be isolated from the culture of *Arthrobacter paraffineus* when glucose was used as the carbon source instead of hexadecane[233]. Similarly, *P. aeruginosa* S7B1 was reported to produce a protein-like activator for n-alkane oxidation when grown on hydrocarbon supplemented medium but not on glucose, glycerol, or palmitic acid[398]. Hauser and Karnovsky[399] observed that rhamnolipid production by *P. aeruginosa* on glycerol was reduced sharply on adding glucose, acetate, succinate or citrate to the culture medium. According to Perfumo *et al.*[388], hydrocarbons are the excellent carbon sources for the production of rhamnolipid from *P. aeruginosa* strains.

The carbon source n-hexadecane was found to be the most efficient for the reduction of surface tension of the culture medium and achieving the highest biosurfactant production (Table 4.6). It was observed that the bacterial strain OBP1 also showed preference for the vegetable oil whereas OBP3 and OBP4 preferred mineral oils such as diesel and crude oil. However, the strain OBP2 preferred n-hexadecane. Such anomalous behavior of the bacterial strains clearly suggested that the carbon source preference for rhamnolipid production entirely depends on the bacterial strain. The effect of the nutrient medium and particularly the carbon source on the synthesis of rhamnolipids is still not well understood[388].

The bacterial strains were found to grow easily on mineral oils such as kerosene, diesel and crude oil. However, the pattern of biosurfactant production was different. The mineral oil, especially kerosene was much less efficient in rhamnolipid production by the bacterial strains. The bacterial strains possessed an efficient alkane utilizing ability. The involvement of *P. aeruginosa* in the production of rhamnolipid using water immiscible hydrocarbon like hexadecane as the carbon substrate was reported by Noordman and Janssen[193], Beal and Betts[195]and Tuleva *et al.*[350].

The tested vegetable oils: mustard oil, sunflower oil, soyabean oil, sesame seed oil, castor oil, nahor oil and jatropha oil, were less efficient in inducing rhamnolipid production but found to be better than glucose and glycerol. The strain OBP1 produced rhamnolipid efficiently when cultivated on the vegetable oil but production was less as compared to n-hexadecane. The probable reason for such behaviour might be due to the fact that most of *P. aeruginosa* species produce lipases which facilitate the assimilation of fatty acids present in the vegetable oil[27, 400]. The long chain fatty acids can either be further degraded via β-oxidation to support cell growth or might be transformed into the lipid precursor to promote biosynthesis of rhamnolipid[27, 52]. The production of rhamnolipid by the other three strains in vegetable oils was much lower.

The low production of rhamnolipid in water soluble substrates like glucose and glycerol could be reasonable as these substrates are quite soluble and hence more readily available to the bacterial cells. There is no requirement for the bacterial cells to

synthesize biosurfactant to improve their solubility or availability[52]. Further, catabolic repression of biosurfactant synthesis by glucose or primary metabolites is one of the important regulatory mechanisms found to be operative in the hydrocarbon utilizing microorganisms. The function of biosurfactant is related to the hydrocarbon availability or their uptake and therefore, they are synthesized predominantly by hydrocarbon degrading microorganisms[163]. The preference for carbon substrates among the studied bacterial strains of *P. aeruginosa* is quite different, and the complex hydrocarbons were found to be better than easily available carbon sources for the production of rhamnolipid. This clearly suggests that the preference for carbon sources for rhamnolipid production entirely depends on the bacterial strains.

5.7 Optimization of culture conditions for the production of biosurfactant

5.7.1 Effect of concentrations of n-hexadecane

The quality and quantity of biosurfactant produced by the bacterial strains were reported to be influenced by the nature of the carbon substrate[259, 400]. All four strains of *P. aeruginosa* were able to produce higher quantity of biosurfactants using n-hexadecane at the concentrations of **1.5-2.0%** with a maximum yield in the range of 2.83-4.57 $g.l^{-1}$. The culture medium of the bacterial strains attained a lower surface tension in the range of 31.1-37.6 mNm^{-1}. Chayabutra and Ju[401], Rocha *et al.*[402] and Tao *et al.*[403] discussed the utilization of hexadecane for the synthesis of rhamnolipid by various strains of *P. aeruginosa* which support the present findings.

All four bacterial strains exhibited better growth as was evident from the increased biomass density in the hexadecane-supplemented medium. Bacterial strains OBP1, OBP2 and OBP4 formed the highest $cfu.ml^{-1}$ in the range of 5.9×10^{9}-7.5×10^{11} on 2.0% n-hexadecane while OBP3 exhibited the maximum of 5.4×10^{9} $cfu.ml^{-1}$ on 1.5% n-hexadecane during incubation at 37°C with 180 rpm. The high concentration of n-hexadecane (>2.0%) reduced both the growth and the production of biosurfactants by all the four strains. Concentrations of n-hexadecane more than the optimum caused a drastic reduction in the bacterial population growth. The exact reason for such behavior is not well understood but may be related to the availability of dissolved oxygen. Wongsa *et al.*[352] stated that the inefficient oxygen supply in

shake-flask cultures might be responsible for the poor growth of *P. aeruginosa* WatG on petroleum refined products like kerosene and diesel, and reported it to be an oxygen-intensive metabolic process. Leahy *et al.*[404] reported that the effects of toxicity, enzyme inhibition, and oxygen limitation were minimized by using relatively low concentrations of hydrocarbons. According to Mehdi and Giti[388] the high concentration of crude oil reduced the growth rate of the different crude oil degrading bacterial strains of *Pseudomonas*, *Rhodococcus* and *Bacillus*.

5.7.2 Effect of nitrogen sources

Nitrogen being a vital component of proteins is required for the microbial growth and production of enzymes in the fermentation process[393]. The nitrogen source in the culture medium plays a significant role in the rhamnolipid production[33, 52] and contributes to pH control[22]. Several sources of nitrogen were reported to be used for the production of biosurfactants, such as urea, ammonium sulphate[2, 23], ammonium nitrate[52, 405, 406], sodium nitrate[29, 33, 53, 57, 349], yeast extract[33, 283, 350], meat and malt extract[300, 393]. The production of biosurfactants by the strains of *P. aeruginosa* was examined in the presence of various organic and inorganic nitrogen sources. NH_4NO_3, $NaNO_3$ and $(NH_4)_2SO_4$ enhanced the growth and production of biosurfactants; whereas KNO_3 lowered both growth and biosurfactant production (Table 4.9). The inorganic nitrogen sources such as NH_4NO_3 and $NaNO_3$ caused a similar pattern of growth of bacterial strains and biosurfactant production. $(NH_4)_2SO_4$ was found to be the best nitrogen source. Organic nitrogen source urea was found to be better than the yeast extract, leading to a high rhamnolipid yield (Table 4.9). In the case of urea and $(NH_4)_2SO_4$ each with 2.0 $g.l^{-1}$, the biosurfactants yield was 4.57, 2.86, 2.83 and 3.17 $g.l^{-1}$ in the case of OBP1, OBP2, OBP3 and OBP4, respectively which was much higher as compared to the individual nitrogen sources. Use of organic and inorganic nitrogen in combination was more effective in the production of biosurfactant[22]. Bordoloi and Konwar[2] obtained higher yield of rhamnolipid when $(NH_4)_2SO_4$ and urea were used as nitrogen sources. Mata-Sandoval *et al.*[300] reported increase in rhamnolipid production when $(NH_4)_2SO_4$ and trace metals were added throughout the fermentation process and the same supported our findings. Out of the defined media

like Luria broth, M9, modified mineral salt and basal salt media the modified mineral salt medium was found to be the best for the production of biosurfactant.

5.7.3 Effect of macro and micro-nutrients

On addition of Na_2HPO_4 and KH_2PO_4 at concentrations of 3.61 and 1.75 $g.l^{-1}$, respectively, the bacterial strains showed higher cell growth and maximum production of biosurfactants. The essential metal ions Mg^{2+}, Fe^{2+} and Ca^{2+} supplied in the form of $MgSO_4 \cdot 7H_2O$ ($g.l^{-1}$), $FeSO_4 \cdot 7H_2O$ ($g.l^{-1}$) and $CaCl_2 \cdot 2H_2O$ ($g.l^{-1}$), respectively, attributed to significant enhancement of cell growth and biosurfactant production. The best biosurfactant production was obtained with $MgSO_4 \cdot 7H_2O$ and $CaCl_2 \cdot 2H_2O$ at a concentration of 0.2 $g.l^{-1}$ and 50 $mg.l^{-1}$, respectively. Addition of trace elements like Zn^{2+}, Mn^{3+} and BO_3^{3+} in the medium significantly enhanced the production of biosurfactants. The concentration of metal ions plays a very important role in the production of biosurfactants as they form important cofactors of many enzymes[393]. Limitation of Ca^{2+}, Fe^{2+}, K^+, Mg^{2+}, Na^+ and other trace minerals towards the late exponential phase of growth were also reported to enhance the production of rhamnolipids[259, 396, 407, 408] Giani *et al.*[408] reported the highest rhamnolipid production from *P. aeruginosa* DSM 7107 and DSM 7108 in Ca^{2+}free media.

5.7.4 Effect of temperature

Temperature was reported to influence the production of biosurfactants[22, 393]. Temperature exhibited a noticeable influence on the production of rhamnolipids possibly due to its effect on the physiology of the bacterial strains. The optimum temperature (Tables 4.16) obtained in the present investigation was in the range of 37-40°C. Increase of temperature beyond the range caused a drastic reduction in the production of biosurfactant. Wei *et al.*[52] and Chen *et al.*[409] reported optimum activity of rhamnolipid production by *Pseudomonas aeruginosa* J4 between 30-37°C which decreased with further increase in the temperature. Temperatures between 28-40°C were reported for the production of rhamnolipid by various strains of *P. aeruginosa*[259, 393, 396]. Such variations in temperature clearly indicate the physiological variations among *P. aeruginosa* strains. Guerra-Santos *et al.*[410] observed that the temperature

range between 32–34°C resulted in higher rhamnolipid production by *P. aeruginosa* DSM 2659 strain. Subasioglu and Cansunar[411] obtained maximum rhamnolipid production by *P. aeruginosa* cultured in mannitol (20 $g.l^{-1}$) supplemented medium at 34.5°C and further increase in the temperature above 36°C caused a significant reduction in the production of biosurfactant. Silva *et al.*[56] determined the effect of two different temperatures, 28 and 37°C on the production of biosurfactant and observed no variations between both temperatures regarding the surface tension values and biosurfactant concentration.

5.7.5 Effect of pH

The pH of culture medium exhibited a clear influence on rhamnolipid production[412] which might be due to its effect on the cellular metabolism. The inoculated culture medium was found to be less turbid below pH 6.0 indicating less growth in the mineral salt medium and was further confirmed by the reduction in the bacterial biomass. However, the culture medium became turbid as a result of dense bacterial growth with the increase in pH of the medium from 6.5-7.2. Increase in the pH of the medium above 7.2 enhanced the bacterial growth but caused considerable reduction in the surface activity and the level of biosurfactant production. Similarly, decrease in the pH below 6.8 reduced the bacterial growth and effected biosurfactant production (Table 4.17). The optimal pH (6.8) obtained in the present investigation is in agreement with the pH values for rhamnolipid production by *P. aeruginosa* strains[52, 409]. Yateem *et al.*[413] reported the maximum production of biosurfactant at a neutral pH of 6.8 and further decreased as the pH of the medium moved towards alkalinity and the same has supported the present findings. Moreover, pH is known to have a profound impact on the behaviour of surface-active molecules[56]. Yateem *et al.*[413] reported that at lower pH (< 6.0), the rhamnolipid moiety, which has a pk_a of 5.6, remained at least 50% uncharged, but when the pH increased above 6.8, the rhamnolipid moiety became negatively charged and surface activity reached its maximum. Ishigaml *et al.*[414] also discussed the pH sensitivity of rhamnolipid biosurfactants produced by a species of *Pseudomonas*.

5.7.6 Effect of agitation

Oxygen is necessary for microbial metabolism[415]. Among the facultative microorganisms, *P. aeruginosa* are reported to be growing in the environments having low oxygen concentration[416]. However, the production of surface-active compounds by *P. aeruginosa* involves the stages of oxidation of the substrate[27]. Thus, agitation plays an important role on the production of biosurfactant by the bacterial strains promoting phase mixture and/at adequate oxygen transfer rate. Bailey and Ollis[417] reported that the agitation velocity of the culture medium was a determining factor in mixing both aqueous and hydrophobic phases as well as the mass transfer of oxygen into the bacterial cultures. The secondary function of agitation is to keep the microorganisms in suspension[418]. Moreover, agitation is a vital factor for the bacteria especially when the carbon sources are complex hydrocarbons. The utilization of n-alkanes in the shake-flask cultures are related with the availability of dissolved oxygen. Wongsa *et al.*[352] suggested that the utilization or degradation of hydrocarbons was an oxygen-intensive metabolic process. When the fermentation process was carried out at 180 rpm for 15 days, the bacterial strains produced the maximum biosurfactant. The production of rhamnolipid by the bacterial strains increased when the agitation rate was increased from 100 to 200 rpm (Table 4.18). However, the maximum production was achieved between 180-200 rpm (Table 4.18). Agitation speed above 200 rpm was reported to be unfavorable for the bacterial growth due to the shear damage even though it provided sufficient dissolved oxygen[415]. On the other hand, agitation at lower speed of 100-150 rpm caused stagnant regions in the fermentor due to improper mixing[419]. Moreover, the required agitation rate that favours the optimum production of biosurfactant varies from strain to strain suggesting differences in the oxygen consumption capacity for the metabolic processes. According to Cunha *et al.*[420] the increased agitation velocity caused a negative effect on the reduction in the surface tension by the produced biosurfactants during the cultivation of *Serratia sp.* SVGG16 on ethanol-blended gasoline and the optimum result was obtained with the lowest surface tension value of 34 mNm^{-1} at 100 rpm. Wei *et al.*[52] and Oliveira *et al.*[406] reported agitation affecting the mass transfer

efficiency of oxygen, components of the culture medium, and was considered to be crucial for the cell growth and biosurfactant formation by the aerobic bacterium *P. aeruginosa*.

5.8 Low cost carbon substrate for biosurfactant production

In the present investigation, agroindustrial wastes like residual glycerol and residual kitchen oil were found to be promising for the production of rhamnolipid as compared to other carbon substrates. It was estimated that 10 kg of glycerol wastes are produced for every 100 kg of biodiesel[421]. Moreover, due to the presence of impurities, further purification of the biodiesel-derived glycerol for its industrial applications was reported to be unprofitable. Non-edible vegetable oils such as sesame seed oil and nahor seed oil were found to be efficient. The variation in rhamnolipid production in the different vegetable oils might be associated with the composition of saturated and unsaturated fatty acids as well as the number of carbon atoms of the oils. Vegetable oils have been reported to be more efficient in rhamnolipid production by *P. aeruginosa* as compared to glucose, glycerol and hydrocarbons[27, 300]. They include palm seed oil, olive oil, sunflower oil, safflower oil, canola oil, soybean oil and corn oil[33, 61, 283, 344, 406]. However, based on cost and global food supply, the use of food-grade oils for producing rhamnolipid is not economically significant. Besides, the recent food storage crisis, limited land availability for crop cultivation and food industry with increasing food demand have persuaded the price of edible plant based oils to increase[422]. In this perspective, non-edible vegetable oils may be an alternate substrate for rhamnolipid production. Therefore, industrial wastes such as waste glycerol, petroleum refinery sludge, kitchen waste oil and non-edible oils such as nahor and sesame seed oil could be good carbon sources for the production of rhamnolipids.

5.9 Physical characterization of biosurfactant

5.9.1 Reduction in surface, interfacial tension (IFT) and critical micelle concentration (CMC)

The biosurfactant produced by the bacterial strains was able to reduce the surface tension of the culture medium significantly from 68.5 to 31.1 mNm^{-1}. However, the behavior of biosurfactant production by the bacterial strains was quite different (Figure 4.6). The effectiveness is measured by the minimum value to which the surface tension could be reduced, whereas efficiency is measured by the biosurfactant concentration required to produce a considerable reduction in the surface tension of water. The CMC values of the biosurfactants produced by the bacterial strains were in the range of 45-105 $mg.l^{-1}$ (Figure 4.8). On the basis of CMC, the biosurfactant of bacterial strain OBP1 could be considered as both efficient and effective while biosurfactant produced by OBP3 and OBP4 could be adjudged effective only. Efficient surfactants are known to have very low CMC values i.e. less surfactant is required to decrease surface tension[279] and exhibit some of the physical functions such as emulsification, solubilization and foaming even at a relatively low concentration[423]. Previously, a range of CMC values between 10-230 $mg.l^{-1}$ were reported for the rhamnolipids isolated from the different microbial sources[37]. The CMC values obtained in the present investigation differed within the strains as well as the other reported strains of *P. aeruginosa*. Such variation in the CMC values might be due to the differences in purity and composition of rhamnolipids[55, 56]. Further, due to the intrinsic variability of the rhamnolipids accumulated and the complexity of its composition, number and proportions of homologues, presence of unsaturated bonds, branching and length of the aliphatic chain of the rhamnolipid collectively affect the CMC and surface tension values between the rhamnolipids produced[56, 424]. The rhamnolipid homologues could also differ with the bacterium, medium and cultivation conditions[344]. The CMC values obtained in the present study were much lower than the chemical surfactants such as sodium dodecyl sulphate (SDS) having a CMC value of 2,100 $mg.l^{-1}$ [55].

It was reported that concentration of the surfactant below their CMC level reduces the surface and interfacial tension between air/water, oil/water and soil/water systems. Such reduction in the interfacial tension between crude oil and the soil particles, the capillary force that holds them together gets reduced resulting in their separation. Interfacial tension (IFT) is considered as an important factor in oil recovery because capillary number increases with decrease in the interfacial tension. Capillary number is determined by the ratio of viscous force to the capillary force[32]. The interfacial tension against diesel decreased from 29 to 2.3 mNm^{-1} by the bacterial strains. Similar interfacial tension values of 1.0 mNm^{-1} against kerosene was found in the case of *P. aeruginosa* 47T2 and 44T1 strain[425]; 1.3 mNm^{-1} in the case of *P. aeruginosa* LBI strain[426]; 2.0 mNm^{-1} against n-hexadecane in the case of *P. aeruginosa* UCP0992 strain[56] 1.85 mNm^{-1} against petroleum crude oil in the case of *P. aeruginosa* strain[32].

Critical micelle dilution (CMD) is an indirect means of measuring the surfactant production related to the range of the critical micelle concentration[406]. CMD is a very crucial factor for the oil recovery process. During the application of biosurfactants in MEOR technology, there is a higher chance of dilution of the introduced surfactant into the oil well due to the leakage of water from the water tables present nearby the oil reservoir. Therefore, it is very much important to determine the surface activity of the surfactant at different dilutions (Table 4.22-4.25) levels such as CMD^{-1} (1:10) and CMD^{-2} (1:100). Reduction in the surface tension at CMD^{-1} was almost similar to that of normal, whereas the CMD^{-2} caused a slight increase in the surface tension of the system due to the higher dilution.

5.9.2 Foaming index (F_{24}%) and its stability

The cell free culture supernatant containing the biosurfactant produced separately by each of four bacterial strains caused stable foam with F_{24}% in the range of 50.4-65.5%. The foam produced during the experiment was relatively stable upto 24 h. Such characteristics of biosurfactants indicate their possible application in coal and mineral froth-flotation as a frothing and co-frothing agent[92].

5.9.3 Emulsification activity (E_{24}%)

The emulsifying power is an important character of surfactants. Hence, emulsification index (E_{24}%) was determined for all four biosurfactants using cell free culture supernatant against the different hydrophobic substrates. The cell free culture supernatants showed appreciable emulsification indices (E_{24}%) with diesel, n-hexadecane, n-octadecane, crude oil, n-dodecane, lubricating oil, n-paraffin and kerosene (Figure 4.9). In addition to the surface and interfacial tensions, stabilization of an oil-water emulsion is usually used as an indicator of surface activity[427]. Further, the preference for hydrophobic substrates and the behavior of emulsification activity (E_{24}%) were quite different among *P. aeruginosa* four strains (Figure 4.9). Most of the microbial surfactants are substrate specific causing solubilization or emulsification of hydrocarbons at different rates[428]. The emulsifying activity (E_{24}%) was calculated using oil/water ratio 3:2, which indicates the constitution of the oil phase to be 60% of the total mixture volume. This signifies that the value of emulsifying activity (E_{24}%) greater than or equal to 60 is responsible for the complete emulsification of the oil phase[53]. Such conditions were observed in the case of cell free culture supernatant of the bacterial strains against diesel and n-hexadecane (Figure 4.9). The water-oil emulsions were found to be compact and remained stable at room temperature for more than one month suggesting possible application of the biosurfactants in the bioremediation process for enhancing the availability of the recalcitrant hydrocarbons. Moreover, the ability of the biosurfactants to emulsify specifically the crude oil products such as n-hexadecane, octadecane, kerosene, diesel and lubricating oil might facilitate their microbial assimilation which could be useful for the bioremediation of petroleum contaminated environments[27, 53]. Further, the ability of the biosurfactants to emulsify the vegetable oil also suggests their potential application in the pharmaceutical and cosmetic industries[56].

5.9.4 Influence of temperature, pH, salinity and metal ions on surface properties and emulsification activity (E_{24}%)

Environmental factors such as pH, temperature, salinity and metal ions are known to influence biosurfactant's activity and stability[23, 28, 32]. Biosurfactants produced by the bacterial strains at normal CMD^{-1} and CMD^{-2} concentrations were found to stable and showed optimum surface activity at pH 5-8 (Table 4.22). The emulsification activity (E_{24}%) of the cell free culture supernatant of the bacterial strains against diesel was quite stable at pH 5-8, though the optimum emulsifying activity was observed between pH 7-8 (Figure 4.10a). Considerable reduction in the stability as well as in the surface activity of biosurfactants was observed beyond pH 8. Such decrease in the surface activity might be due to the alteration of surfactant structures at the extreme pH conditions. Increase in pH from 5-8 caused an increase in the negative charge on the polar head of the rhamnolipid molecule (pK_a 5.6) enhancing its solubility in water. However, below pH 5, surface activity decreased due to the protonation of the rhamnolipid molecules affecting their precipitation[32]. Rhamnolipid molecules contain a single free carboxylic acid group at the β-hydroxyl fatty acid moiety and are responsible for the anionic nature of rhamnolipid[56]. According to Champion *et al.*[429] the increase of pH from 5.5-8.0 leads to increase in the negative charge on the polar head of rhamnolipid. A charge repulsion between the adjacent polar heads lead to the formation of larger head diameter and sequentially change the morphology of rhamnolipid molecules from bilayer sheets to vesicles and then to micelles. Biosurfactants produced by the different strains of *Pseudomonas aeruginosa* showed optimum surface activity with higher emulsification between the pH ranges of 4-12[23, 32, 53, 56].

Biosurfactants maintained their surface activity after exposure to temperatures from 4-100°C and showed appreciable thermostabilty at both normal and diluted conditions (CMD^{-1} and CMD^{-2}). The cell free culture supernatants also exhibited stable surface activity even after autoclaving at 121°C for 30 min (Table 4.23). Such thermal stability of biosurfactants indicates its utility in those industries where wet sterilization is of principal importance[56]. Extreme stability of biosurfactants was

reported by Abdel-Mawgoud *et al.*[53] for the *Pseudomonas aeruginosa* isolate Bs 20; Kiran *et al.*[21] for the marine bacterium *Brevibacterium aureum* MSA13 and Bharali *et al.*[430] for the bacterial strain of *Alcaligenes faecalis*. The present study clearly indicates the thermostability properties of the biosurfactants produced by four bacterial strains. Similar observation was reported by Desai and Banat[7]. The emulsification activity (E_{24}%) of the culture supernatant of the bacterial strains against diesel was quite stable at all the tested temperatures (Figure 4.10b). Emulsions were found to be stable upto one month at room temperature. It is interesting to note that biosurfactants retained about 53% of their original emulsifying properties even after autoclaving, indicating their thermal stability which broadens the scope of their application in MEOR.

The biosurfactants retained their surface activity up to the addition of 5% NaCl at both normal and CMD^{-1} conditions (Table 4.24). The culture supernatants retained significant surface activity at CMD^{-2} indicating their efficiency even at lower concentration. Emulsification activity (E_{24}%) of culture supernatants against diesel remained almost unchanged upto the addition of 3% NaCl (Figure 4.10c). Bognolo[431] reported that chemical surfactants usually get deactivated at around 2-3% of salt concentrations. Increase in the salt concentration beyond 4%, caused significant reduction in the emulsification activity (E_{24}%) of culture supernatants, indicating their ineffectiveness at such elevated salinity. When NaCl is added, the negative charge of carboxylic acid groups of rhamnolipid molecules is shielded by the Na^+ ions in the electrical double layer that leads to the formation of a close-packed monolayer. Thus, the formation of Na^+-rhamnolipid complex reduces surface tension values[56]. The stability of culture supernatant against higher pH and salinity suggests its applicability in bioremediation of marine environments and in industries where high salinities and pH prevail.

Biosurfactants showed appreciable surface activity in the presence of the different metal ions, but not in the case of Al^{3+} ions (Table 4.25). This could be due to the complex formation between the available rhamnolipid with Al^{3+}ions present in the cell free culture supernatant, as a result free rhamnolipid molecules in the solution gets

reduced. Consequently, the rhamnolipid molecules available at the interface are lesser which in turn reduces its surface activity. Rhamnolipids tends to form complexes with heavy metal cations more preferably than that of other non-toxic metal ions such as Ca^{2+}, Mg^{2+}, K^{+} etc., for which they possess much lesser affinity[1]. Similarly, Xia *et al.*[32] reported that the addition of trivalent ion (Al^{3+}) cause an increase in the surface activity of the biosurfactants produced by *Pseudomonas aeruginosa*. Cations of lowest to highest affinity for rhamnolipid biosurfactants were $K^{+} < Mg^{2+} < Mn^{2+} < Ni^{2+} < Co^{2+} < Ca^{2+} < Hg^{2+} < Fe^{2+} < Zn^{2+} < Cd^{2+} < Pb^{2+} < Cu^{2+} < Al^{3+}$ [6]. The anionic nature of the rhamnolipids enables them to absorb metals ions from the soil such as arsenic, cadmium, copper, lanthanum, lead and zinc due to their complexation ability[1, 115] and help in the remediation of toxic metal ions.

5.9.5 Cell surface hydrophobicity

The bacterial strains possessed wide variability in their surface hydrophobicity (Figure 4.12), and the same was found to increase with the complexity of carbon sources. In the case of n-hexadecane grown cells higher level of hydrophobicity and biosurfactant production were observed which indicated the bacterial strain's ability to have biosurfactant-mediated uptake of alkane. The cell surface hydrophobicity of bacteria in the exponential phase was much lower than that of the stationary phase which also proved higher levels of biosurfactant production in the late exponential phase of growth indicating the accumulation as secondary metabolite. Vasileva-Tonkova *et al.*[375] reported that cells in the early or mid-exponential growth phases were less hydrophobic than those in stationary phase which suggested that the production of biosurfactants contributed to cell surface hydrophobicity. Sotirova *et al.*[211] reported that the rhamnolipid at a concentration above CMC causes a reduction of the total cellular lipopolysaccharide (LPS) content of the bacterial cells of *P. aeruginosa* and concentration below CMC causes alteration in the composition of the outer membrane protein which contributes to the enhancement of surface hydrophobicity of the bacterial cells. Biosurfactants produced by the bacterial strains during growth on the immiscible carbon sources modify their cell surface physiology

and make them more hydrophobic which improve the adhesion of complex hydrocarbon to their surface or make it available to them[40, 47, 306].

5.10 Characterization of biosurfactants

5.10.1 Thin layer chromatography (TLC)

The TLC derived fractions showed positive reaction to sugars with orcinol reagents and lipids with iodine vapors, indicating the presence of both glycosyl units and lipid moieties on the same spots, but possessed negative reaction to amino groups with ninhydrin. The lower fraction with the average R_f value of 0.32 indicated the presence of di-rhamnolipids while other with an average R_f value of 0.71 for mono-rhamnolipid molecules. These observations were found to be consistent with the reports of Abdel-Mawgoud *et al.*[53], Monteiro *et al.*[55], Silva *et al.*[56], Abbasi *et al.* [92] and Lotfabad *et al.*[440]. The results of thin-layer chromatography (TLC) clearly confirmed the glycolipid nature of the biosurfactant produced by four bacterial strains. Moreover two independent assays comprising of CTAB agar test[270] and orcinol assay[343] along with TLC confirmed the production of rhamnolipid by the bacterial strains in the n-hexadecane supplemented medium.

5.10.2 Fourier transform infrared spectroscopy (FTIR)

The infrared spectroscopic analysis revealed the presence of glycolipid type compound in the isolated biosurfactant samples (Figure 4.14). The appearance of additional bands in the spectra might be the result from contamination of polypeptides and polysaccharides from cell debris during the extraction of biosurfactant from the culture supernatant[440]. Most of the known biosurfactants are glycolipids in nature[7], carbohydrates in combination with long-chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipids, trehalolipids and sorpholipids are among the best known glycolipids. *P. aeruginosa*[441], *R. erythropolis*[243], *Mycobacterium sp.*[146] and *Torulopsis bombicola*[256] were reported to produce biosurfactants that are glycolipid in nature. On the basis of biochemical, TLC and FTIR results, it was assumed that the biosurfactants produced by the bacterial strains had a common glycolipid type structure.

5.10.3 Liquid chromatography and mass spectroscopy (LC-MS)

The LC–MS analysis of the biosurfactant samples produced by *P. aeruginosa* strains confirmed the results of the TLC with peak values appearing at 171, 193, 205, 339 m/z indicated the presence of lipids and peak value at 163 for carbohydrate moiety[23, 389]. The m/z values obtained were consistent with the molecular structure of Rha-$C_{8:2}$, Rha-C_{10}, Rha-$C_{12:2}$, Rha-C_8-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12:1}$, Rha-Rha-C_8-C_{10}, Rha-Rha-C_{10}-C_{10}, Rha-Rha-C_{10}-$C_{12:1}$ and Rha-Rha-C_{12}-C_{10}. A total of twelve rhamnolipid homologs were identified from the biosurfactants produced by the bacterial strains which included both mono and di-rhamnolipids (Table 4.28). In the literature the number of rhamnolipid homologues reported varies from 4 to 28 [50, 434]. The predominant rhamnolipid components present in the biosurfactants of the bacterial strains were Rha-C_{10}, Rha-$C_{12:2}$, Rha-C_8-C_{10}, Rha-C_{10}-C_{10}, Rha-C_{10}-C_{12}, Rha-C_{10}-$C_{12:1}$, Rha-Rha-C_8-C_{10}, Rha-Rha-C_{10}-C_{10} and Rha- Rha-C_{10}-$C_{12:1}$. Results clearly showed the predominance of di-rhamnolipids over the mono-rhamnolipids and typically the main rhamnolipids were found to be Rha-C_{10}-C_{10}, Rha-Rha-C_8-C_{12} and Rha-Rha-C_{10}-C_{12}. The results of MS as described by Aparna *et al.*[23], Haba *et al.*[84], Deziel *et al.*[389], Yin *et al.*[423] and Lotfabad *et al.*[432] were found to be quite close to our findings with the higher abundance of dirhamnolipids over monorhamnolipids. The difference between the rhamnolipid composition and predominance of a particular type of congener in the present investigation was probably due to the factors like type of carbon substrate[389, 426], culture conditions[412], age of the culture[84] and the strains of *P. aeruginosa* used[389]. Moreover, the rhamnolipids congeners identified were also found to depend on the nature of the analytical methodology used[55]. Monteiro *et al.*[55], Benincasa *et al.*[62], Haba *et al.*[65, 84], Costa *et al.*[306], Déziel *et al.*[389], Costa *et al.*[412] and Lotfabad *et al.*[432] used HPLC/MS, ESI–MS and LC-MS; and reported the complete elucidation of rhamnolipid mixtures produced by the different strains of *P.aeruginosa*. However, no one used n-hexadecane as the sole source of carbon. Further, the variation in the culture composition and the differences in the strains could explain the differences between their results and the present work.

5.10.4 Thermogravimetric analysis (TGA)

For all four biosurfactants the weight loss corresponding to the temperature of 65-110°C was due to the loss of adsorbed moisture, consequently suggesting the samples being not completely anhydrous. Due to the presence of different types of moieties and linkages in the surfactant structures they display a two step degradation pattern as confirmed from the thermogram (Figure 4.16). The first step degradation started at 159°C and ended at 353°C resulting a weight loss of about 19% which might be due to the dehydration of the ester or the acid groups of the lipids. The same resulted in the generation of water or carbon dioxide as volatiles. The oxidative reactions are mainly responsible for the accelerated degradation. The second stage degradation might be due to the breaking of C-O linkages of the main structure. Finally, at high temperature there could be formations of heat stable structures as shown by the TGA thermogram. A higher amount of 37-43% residues were observed at 600°C that might be due to the formation of fused complex C-C linkages or char, which is highly thermostable in nature. Deprotonation of the glycosyl head at higher temperature consequently resulted in the depolymerization of rhamnolipid and subsequently lead to the generation of substantial amount of char. TGA clearly indicated the presence of di-rhamnolipid as the predominant form in the biosurfactant samples. Various factors such as interactions between the congeners of rhamnolipid molecules, appearance of steric hindrance as a result of branched aliphatic chains and acyclic structures collectively might attribute to the thermo-stability of the biosurfactants. Moreover, interaction and extensive H-bonding between the biosurfactant molecules were also evident from the FTIR studies.

5.10.5 Differential scanning calorimetry (DSC)

The DSC study was performed on the biosurfactants produced by the bacterial strains. All the samples except OBP4 exhibited two endothermic peaks at around 16°C and 131°C showing a lower enthalpy with lower temperature pre-transitions and higher enthalpy with higher temperature main transitions. First transition might be due to the formation of a crystalline phase by the aliphatic branching groups, which could

be inter or intra molecular. At low temperature there was a ceasation in the molecular motion of the molecules. The lower enthalpy transitions were mainly due to the chain unfolding of the lipids. The presence of flexible symmetric moieties and polar linkages in biosurfactant structures might form partially crystalline phases at lower temperatures which could be verified from the DSC thermogram (Figure 4.17). The second transition could be referred as the melting of those crystalline regions. Melting of crystalline phase at high temperature was due to the stability of the crystalline structure which might be because of polar nature of the moieties present in the biosurfactant structures. However, it was observed that the lower endothermic peak disappears in OBP4-biosurfactant suggesting higher concentration of dirhamnolipids. However, there are instances at which the heat capacity profile displays only one maximum. This is because of the fact that the DSC could describe the bulk behavior not single events which might not be detectable since they show only a small contribution to the overall melting [92]. In all the cases the increase in the dirhamnolipid concentrations resulted in broadening of the higher enthalpy transitions and shifting of the peak to lower values.

5.11 Application in the field of bioremediation

5.11.1 Reduction in the viscosity of crude oil

All four bacterial strains efficiently reduced the viscosity of the crude oil after 30 days of treatment under laboratory conditions. Results of the present investigation clearly indicated the ability of bacterial strains to degrade the heavy fractions of crude oil which in turn changed the physicochemical properties of the crude oil[443]. The use of microbes in degrading long chain alkanes might have several benefits such as minimizing paraffin precipitation or deposition problem along the production flow line, reduction of the viscosity of crude oil, increase in API gravity value and finally reduction of both pour point and paraffin content of crude oil[444]. Moreover, the biosurfactants produced during the bacterial growth on crude oil additionally reduces its viscosity by altering the surface and interfacial energy of the system, thereby increasing the mobility of crude oil in the pipelines. She *et al.*[445] used the

biosurfactant-producing indigenous *Bacillus* strains to degrade the higher fractions of crude oil. They also reported the enhancement in the flow characteristics of crude oil after the treatment in a petroleum reservoir of Daqing Oilfield. According to Gudiňa *et al.*[102] *Bacillus* strains were able to degrade large alkyl chains and reduce the viscosity of hydrocarbon mixtures. These reports supported our view of using the indigenous biosurfactant producing bacterial strains in reducing the viscosity of crude oil.

5.11.2 Solubilization of polyaromatic hydrocarbon (PAH) by the biosurfactant

Water solubility of some of the hydrophobic organic compounds HOCs could be increased with the addition of surfactant or biosurfactant[49]. The solubilization assays of anthracene, phenanthrene, and naphthalene clearly showed that with the increase in the concentration of biosurfactants upto a range of 45-120 $mg.l^{-1}$ could reduce the amount of available undissolved PAHs in the reaction mixture. Such behaviour of the biosurfactants is due to the fact that the concentration of biosurfactants above the CMC enhances the formation of micelle causing the undissolved organic components to dissolve within the micelle structure facilitating microbial uptake and bioremediation[431]. Das *et al.*[446] showed that the solubilization of anthracene increases with the increase in rhamnolipid concentration beyond 100 $mg.l^{-1}$. Yin *et al.*[431] reported the solubilization of phenanthrene at 50 $mg.l^{-1}$ of rhamnolipid produced by *P. aeruginosa* strain S6. Further, differences in the degree of solubilization by the biosurfactants might be due to differences in the physico-chemical characters of the tested PAHs and the types of rhamnolipid congers present in the biosurfactants[22, 53, 447, 448].

5.11.3 Biodegradation of crude oil by the bacteria strains

All four bacterial strains were found to be efficient in hydrocarbon degradation (Table 4.29). Bacterial strain OBP4 followed by OBP3 and OBP1 appeared to be the best degraders and worked best at neutral or near neutral pH. The genus *Pseudomonas* stands out as the most versatile group among the several genera of microorganisms that have the capability of hydrocarbon degradation and biosurfactant production[31, 133, 350, 392]. Das and Mukherjee[449] reported that strains of *Pseudomonas aeruginosa* were

much capable of degrading crude oil components as compared to the strains of *Bacillus subtilis*. Mehdi and Giti[338] showed that the strain of *Pseudomonas* was more efficient than the strains of *Bacillus* and *Rhodococcus* in degrading crude oil. The growth dynamics could either be due to the constitutive nature of hydrocarbon assimilation capability in the organism or reflected the adaptation of the strains as a result of previous exposure to exogenous hydrocarbons. This could be followed by simultaneous development of the capability to use the oil and/or its catabolic products as carbon and energy sources[387]. Gas chromatographic analyses of the treated crude oil with the bacterial strains showed a similar response of degradation, but different in details (Figure 4.20). Bioconversion of crude oil components leads to the enrichment of the lighter fractions of hydrocarbons having shorter retention time.

The bacterial strains were competent in degrading crude oil in the mineral salt medium and efficiently degraded n-alkanes in the range of C_9 to C_{18}. The high molecular weight n-alkanes could have been preferentially utilized as carbon and energy sources. Previous studies showed that alkane with C_{14}–C_{20} carbon atoms permits abundant growth for most of the bacteria[299, 440]. However, the bacterial strains were not much efficient in utilizing aromatic and polyaromatic compounds. The predominance of mineralization of aliphatic over aromatic hydrocarbons with greater rate of degradation by the bacterial community was reported by several authors[368, 387, 441]. The pure cultures of the individual bacterial strains possess limited preferred substrates and thereby are assisting less significantly in utilizing the complex hydrocarbon mixtures present in the crude oil[442]. Lal and Khanna[368] reported that degradation of crude oil by microbes habitually occurs with the utilization of alkanes or light aromatic fractions, while the higher molecular weight aromatics, resins and asphaltenes are considered as recalcitrant. Adebusoye *et al.*[387] reported that the individual organisms such as *Corynebacterium spp.*, *Acinetobacter lwoffi* and *Pseudomonas aeruginosa* could metabolize only a limited range of hydrocarbon substrates. Biodegradation studies conducted by Sharma and Pant[443] showed that 50% of the aliphatic fractions of the crude oil of Assam (India) were degraded by the isolates of *Rhodococcus*. Several studies pointed out that the extent of oil and total

petroleum hydrocarbon biodegradation are closely linked to the type of oil and its molecular composition[299]. The present study showed that the presence of crude oil in the culture medium had no inhibitory effect on biosurfactant production by the bacterial strains and might assist in the biodegradation of high molecular weight n-alkanes (C_{12}-C_{18}).

5.11.4 Biodegradation of crude oil by bacterial consortium

Individual microorganisms could metabolize only a limited range of hydrocarbon substrates; hence the assemblages of mixed populations with overall broad enzymatic capacities are required to bring the rate and extent of petroleum biodegradation further[444]. Out of the eleven different combinations tried, the combination 7 and 11, named as consortium I and II exhibited the highest dry biomass production of 2.73 ± 0.3 and 3.33 ± 0.1 $g.l^{-1}$, respectively in the MSM supplemented with crude oil within 96 h. Both the consortia comprising of all four different strains of *P. aeruginosa* except OBP2 in consortium I were found to efficiently degrade the crude oil under the shaking condition. Individually the bacterial strains were capable to utilize the different fractions of crude oil (Table 4.30), especially the aliphatic fractions in the range of 67.5-73.0%. However, the present investigation clearly revealed that the consortium I and II were efficient in degrading 78.6-80.4% of aliphatic, 42.4-42.7% of aromatic and 19.2-21.6% of NSO compounds in 30 days of culture (Table 4.31). Degradation of the aromatic fraction upto 42.4-42.7% could not be attained in the case of any single bacterium (Table 4.30). Such potency of consortia in degrading aromatic fraction supports the co-metabolism behaviour between the bacterial strains. The metabolic intermediates produced by one bacterial strain could be utilized by the other members of the consortium as the substrate for their growth and biosurfactant production[3]. Ghazali *et al.*[444] reported that biodegradation of complex hydrocarbons usually required the cooperation of more than a single species. This is particularly true in the case of pollutants that are made up of many different compounds such as crude oil or petroleum. Addition of biosurfactant (45 $mg.l^{-1}$) produced by the bacterial strain OBP1 to both of the consortia leads to an enhancement in the degradation of crude oil indicating the effectiveness of

biosurfactant in the biodegradation process (Table 4.31). Further, the GC analysis of the crude oil confirmed the enhancement in the degradation process (Figure 4.21). Research conducted by Itoh and Suzuki[182], Rahman *et al.*[249] and Zhang *et al.*[289] reported that the addition of the rhamnolipid produced by *Pseudomonas aeruginosa* enhances hydrocarbon degradation by the same organism.

Gas chromatographic profile of the saturated fraction of the crude oil inoculated with Consortia I and II exhibited much reduced noise level as compared to the non-inoculated medium (Figure 4.21). Presence of certain distinct unreduced peaks indicates the accumulation of bacterial by-products which are not degraded further by the members of the consortia. The results clearly established that both consortia along with the biosurfactant were efficient in degrading the different components of crude oil. In designing a consortium, solubility and accessibility of the hydrophobic compounds available in the crude oil are the two key aspects. Since only 0.02% of crude oil is soluble in water, hence there is a need for emulsification of the crude oil in the medium[445].

5.11.5 Separation of crude oil from contaminated sand by the biosurfactant

The aqueous biosurfactant solution of the bacterial strains could efficiently separate the crude oil from the contaminated sand which clearly indicated their capability in the soil washing. The maximum removal of crude oil by the biosurfactants was achieved within their CMC, but by increasing the concentration beyond the CMC the removal of crude oil from the contaminated sand could not be enhanced. With reduction in the interfacial tension between crude oil and the sand particles, the capillary force that holds them together in the sand-oil mixture gets reduced. Such reductions in the tension further increase the contact angle between the oil and soil particles and change the wettability of the system. This ultimately results in the mobilization of the crude oil from the sand-oil mixture to the aqueous solution. Such effect is directly related to the biosurfactant concentration in the solution until it reaches the CMC, the concentration at which the surfactant molecules start to form micelles and show the lowest tensional force[7, 80]. Differences in the separation

behavior of the biosurfactants produced by the bacterial strains in the washing experiment suggests that the biosurfactant mediated removal of oil from the sand particles is also dependent on the physico-chemical properties of the biosurfactant and combined behavior of surfactant/crude oil/sand systems[5]. Efficient washing off of crude oil from the contaminated sand could be achieved even with synthetic surfactant SDS. It is now well established that synthetic surfactants are more recalcitrant than the petroleum hydrocarbons and potentially toxic to the environment[446, 447]. Hence, use of biosurfactant seems to be more advantageous.

5.11.6 Release of crude oil from the sand pack column by the biosurfactant

The available residual oil in the sand pack column was mobilized during the passage of the cell free culture broth containing biosurfactant and began to exude with the effluent. Nearly, 6.4-11.4% of residual crude oil was recovered from the saturated sand pack column using cell free culture supernatant of the bacterial strains as compared to the control. Results clearly indicated mobilization of crude oil by biosurfactant in the sand packed column. Parameters like interfacial tension are important in the recovery of crude oil as the capillary number is dependent on the ratio of viscous to capillary forces[32]. Capillary forces arise due to the interfacial tension between oil and water phases, which oppose the externally applied viscous forces and responsible for large quantities of oil that are left behind after water flooding[100]. With the introduction of biosurfactants into the column system, it starts to decrease the interfacial tension at oil/brine interface which in turn increases the capillary number of the system. Further, increase in capillary number lowers the residual oil saturation in the column and increases the recovery process. Suthar *et al.* [100] reported reduction in the interfacial tension directs the mobilization of irregular oil lump to form available oil banks in between the reservoir rocks.

Further, exposure of all four types of biosurfactants to high temperature between 70-90 °C increased the release of the residual crude oil by 8.4-10.5% and 9.3-11.4%, respectively from the column. When temperature was raised between 50-90°C, there was a reduction in the inherent viscosity of the crude oil. This might be because

of the reduction in the compaction between the molecules of crude oil which makes the crude oil more mobile as compared to the room temperature. With increase in the temperature, the air which was previously trapped in between the vacant spaces of the sand particles of the column starts to move out and provides more space for the crude oil to flow. Further, reduction in the capillary forces with the involvement of biosurfactant enhances the release of the crude oil from the column. Such behaviour in the release of crude oil from the column with the enhancement of temperature in the present investigation was in agreement with the previous reports of Bordoloi and Konwar[2]. The results also indicated the retention of surface properties of the biosurfactants even after exposure to higher temperatures. Such thermo-stable property of biosurfactants is very crucial for their application in MEOR where the biosurfactants have to withstand the prevailing extreme temperatures inside the oil reservoirs.

5.11.7 Separation of residual crude oil from the petroleum sludge by the biosurfactant

The biosurfactant of four bacterial strains were capable of separating residual crude oil (63.4-73.5%) from the petroleum sludge (Table 4.24) having upto 7% (w/w) of the sludge concentration, but above this concentration there was no further increase in the release of oil. In fact it was reported that the preparation of homogeneous slurry could be a critical factor in the treatment of sludge which could limit the process[448]. The release of residual oil from the sludge gradually increases with the increase in the duration of treatment (upto 15 days) with continuous shaking (120 rpm). The disturbance caused by the continuous shaking further separated the loosely bound oil droplets from the soil particles due to the reduction of interfacial tension. With the increase in the biosurfactant concentration, there was increase in the removal of residual crude oil from the sludge. Such result was due to the reduction in the surface tension and interfacial tension which lead to a gradual decrease in the capillary force that hold soil and oil. This further enhanced the contact angle between soil and oil, resulting in a change in the wettability of the system. The interfacial tension of the system decreased gradually until it reached its CMC after which it remained constant.

The same mechanism described in the case of soil washing experiment is probably responsible for the mobilization of residual crude oil. The physico-chemical properties of biosurfactant and combined behavior of surfactant/crude oil/soil systems probably cause the effect[5]. A successful attempt in using biosurfactant to recover oil from the sludge was also reported by Banat *et al.*[309]. Joseph and Joseph[113] separated the residual oil from the petroleum sludge generated from the crude oil refinery by directly inoculating the strains of *Bacillus spp.* and with the addition of the cell free culture supernatant of the bacteria. Helmy *et al.*[114] reported the application of the biosurfactant produced by *Azotobacter vinelandii* AV01 for enhanced oil recovery from the oil sludge and recovered upto 15% of oil from the sludge. SDS is also effective but hazardous to the environmental components[446, 447]. Hence, the use of biosurfactant seems to be more advantageous over the chemical surfactants because of their biodegradability, less toxicity and effectiveness at extreme temperatures, pH and salinity[35, 129, 428, 430]

5.12 Biodegradation of biosurfactant

Biodegradation of biosurfactants produced by four *P. aeruginosa* strains confirmed their biodegradability nature when co-cultured with glucose in the culture medium. Several reports demonstrated co-degradation or sole utilization as a source of carbon and energy by various bacterial monocultures[360, 449]. Moreover, the degradation behaviour of biosurfactant depends on the type of bacterial species involved. In the present investigation, the bacterial strains used for the degradation study are potential degraders of complex hydrocarbons isolated from the petroleum hydrocarbon contaminated soil[2]. Chrzanowski *et al.*[450] suggested that rhamnolipids might be preferentially degraded by a consortium of hydrocarbon degraders due to structural similarities between lipid moieties of rhamnolipids and fatty acid moieties present in the biodiesel. In the present study, the degradation of biosurfactant produced by *P. aeruginosa* strain (MTCC8165) was comparatively lower than that of *Bacillus circulans* strain (MTCC8167), which signifies that biosurfactants can't be easily degraded by its source species. Similar observation was reported by Zeng *et al.*[449]. Providenti *et al.*[451] investigated the degradation behaviour of rhamnolipid and reported

that it could be easily utilized by bacterial consortia in sandy loam, silt loam and creosote contaminated soil, but immune to *P. aeruginosa* when it was present as the sole source of carbon. Zeng *et al.*[449] reported that rhamnolipid was degraded efficiently in compost without creating any disturbance to the microbial community present in the composting matrix, indicating its potential compatibility in environmental applications. Recent studies clearly demonstrated that the application of synthetic surfactants in bioremediation process influences the dynamics of microbial community and the hydrocarbon degradation rates[446, 447]. According to Chrzanowski *et al.*[450] biodegradation of rhamnolipids did not favor the growth of any specific consortium member which confirmed that the employed biosurfactant did not interfere with the microbial equilibrium during diesel/biodiesel biodegradation.

5.13 Biological activity of the isolated biosurfactant

5.13.1 Effect of biosurfactant on seed germination and growth

At low concentrations, the biosurfactants did not show phytotoxicity whereas at the increased concentration of biosurfactants beyond their CMC caused decrease in the GI of both seeds. Tiquia *et al.*[353] reported that GI 80% could be an indicator for the absence of phytotoxicity. The increased concentrations of the biosurfactant solutions above the CMC caused reduction in the GI clearly indicating the inhibitory effect on seed germination and root elongation. The reduction in GI of mung bean was moderate to 55-65% but it was prominent in rice to 45-54%. The progressive decrease in the GI with the increasing concentration of biosurfactants might be due to the alternations in the permeability of the cellular membrane induced by biosurfactants[452]. According to Millioli *et al.*[453] the sole presence of rhamnolipids may influence the GI of lettuce (*Lactuca sativa*) and the GI dropped linearly with the increasing concentration of rhamnolipids. Silva *et al.*[56] reported that cabbage (*Brassica oleracea*) tolerated the presence of rhamnolipids without showing any significant reduction in the GI upto the CMC of the tested biosurfactant which was 700 $mg.l^{-1}$. Marecik *et al.*[452] reported the appearance of notable changes in the GI value of alfalfa (*Medicago sativa*), mustard (*Sinapis alba*) and sorghum (*Sorghum saccharatum*) at two different

concentrations of rhamnolipid, the first at CMC and the second far above it. They also reported the presence of inhibitory effect of rhamnolipids on the germination of alfalfa and mustard and most prominent for monocotyledonous plant like sorghum while cuckoo flower species (*Cardamine pratensis*) remained unaffected suggesting that the phytotoxicity of rhamnolipids may be plant specific.

5.13.2 Larvicidal activity against mosquito

In the present study, biosurfactants produced by *P. aeruginosa* strains did not show any larvicidal potency against *Aedes albopictus* at normal concentrations as recommended by World Health Organization (WHO). The same was observed at much higher concentrations. This clearly indicated that the tested biosurfactants were not lethal to the mosquito larvae. Several studies reported that only certain cellular proteins secreted by *P. aeruginosa* have virulence affect on various insects. Mostakim *et al.*[454] reported the larvicidal activity of *P. aeruginosa* against the 3rd instar larvae of *Bactrocera oleae*, the most serious pest in olive cultivation. Among the known biosurfactants, the cyclic lipopeptide surfactin, produced by *B. subtilis* subsp. *subtilis* was reported to exhibit potential larvicidal and mosquitocidal activities[358]. Since the biosurfactants from *P. aeruginosa* strains possessed negligible mosquito larvicidal potency as compared to many other currently available preparations, the application of such biosurfactants in contaminated environments during the remediation process might not cause any undesirable effects to the beneficial insects or their larvae.

5.13.3 Antimicrobial activity

The MIC values were much less for Gram-positive bacteria, indicating their effectiveness at low concentrations except for few bacteria such as *Staphylococcus aureus*. However, the MIC values were much higher for Gram-negative bacteria because of the surface proteins and lipopolysaccharides (LPS) which are the two main constituents of the cell wall. The LPS either acts as a barrier or provides protection to the inner sensitive membrane and cell wall from the toxic compounds[455, 456]. The present study suggested that the rhamnolipid molecules having both hydrophobic and hydrophilic groups could insert their fatty acid components into the cell membrane

that cause considerable alteration in the ultra structure of the cell such as ability of the cell to interiorize plasma membrane. Alternately, it might also be possible that insertion of the shorter acyl tails of the rhamnolipid into the cell membrane causes disruption between cytoskeleton elements and the plasma membrane, allowing the membrane to lift away from the cytoplasmic constituents[7, 42]. Gram negative bacteria are known to have intrinsic resistance against a variety of antibiotics due to the transenvelope multidrug resistance (MDR) pump[474]. Therefore, treatment of such MDR strains with rhamnolipids seems to be more advantageous as it interacts only with the bacterial cell surface[475]. Several workers reported enhancement of the cytoplasmic membrane permeability by rhamnolipids with the consequential alteration leading to cell damage[315, 476].

5.13.4 Chemo-attractant property

Concentration gradient motility agar (CGMA) assay showed positive chemo-attractant property of rhamnolipids at their CMC towards *Staphylococcus aureus* (MTCC 3160) and *Klebsiella pneumoniae* (MTCC 618) confirming chemo-attractant property. Such chemo-attractant property of the test compounds enhances its antibacterial property towards the targeted pathogen[476, 477]. It has been suggested that when the microbes come in contact with rhamnolipid molecules, they might cause disruption of the cell surface of bacteria[476] by being integrated in between the phospholipids of plasma membrane. Rhamnolipids can also enhance permeability of the cytoplasmic membrane with the consequential alteration leading to cell damage[315]. Such unique property of rhamnolipids suggests their application in increasing the efficacy of several drug molecules such as nisin. According to Magalhaes *et al.*[310] the combinations of nisin, an antimicrobial peptide produced by *Lactococcus lactis* and rhamnolipids have a strong synergistic effect on the cytoplasmic membrane of *Listeria monocytogenes*, a serious food born pathogen.

5.13.5 Cell cytotoxicity

The biosurfactants of *P. aeruginosa* strains were found to be cytotoxic to growing mouse fibroblast cells (L929) at concentrations above 100 $\mu g.ml^{-1}$, probably

by directly perturbing cell membranes[478]. The haemolytic and cytotoxic activities of the rhamnolipid are due to the detergent like properties and cell membranes are possibly perturbed by the introduction of fatty acid chains into the organized lipid layers of cells. Häuler *et al.*[208] reported the time and dose dependent cytotoxicity against non-phagocytic HeLa and phagocytic HL60 cells by the rhamnolipid of *Burkholderia pseudomallei*. Lotfabada *et al.*[440] observed no inhibitory effect of the purified rhamnolipid fractions RL-a and RL-b isolated from *P. aeruginosa* MR01 on the normal Vero cell line at concentrations up to 50 $\mu g.ml^{-1}$. Rhamnolipid, especially the dirhamnolipid (50 $\mu g.ml^{-1}$) in the presence of serum, favors the keratinocyte differentiation and inhibits the proliferation of fibroblasts thus helping in the tissue repair[67]. These features broaden the application of rhamnolipids in the new advanced field of medicine for wound healing. Several others reported the non-toxic and anti-proliferative properties of rhamnolipids[440] suggesting that the exposure of biosurfactants to the human skin during the field operation process of bioremediated sites doesn't cause any hazard.

5.13.6 Acute dermal irritation study

Acute dermal irritation study of the transdermal patch in rabbits showed no dermal responses such as erythema or edema as compared to the negative control against the application of biosurfactants. The total body weight in the transdermal patch-treated groups of rabbits did not differ significantly from the control group. Loss of body weight is an important indicator of gross toxicity. Severe toxicity or interference with absorption of nutrients is reflected by the reduced body weight[359]. Hence, it could be concluded that the application of rhamnolipid biosurfactants have neither potency to produce severe tissue destruction nor does it seem to interfere with the absorption of nutrients. Further, the study showed that the patch-treated group did not show any hematological and biochemical changes confirming their non-toxicity to the mammalian skin. Maier and Soberon-Chavez[27] reported that rhamnolipid produced by *P. aeruginosa* have extremely low irritancy and even anti-irritating effects, as well as compatibility with human skin. Clinical trials for the treatment of psoriasis, lichen planus, neurodermatitis and human burn wound healing confirmed excellent

ameliorative effect of rhamnolipids as compared to the conventional therapy using corticosteroids[67, 323]. Rhamnolipids also exhibited differential effects on human keratinocyte and fibroblast cultures[67]. The innovative application of biosurfactants has appeared with the suggestion that biosurfactants may aid wound healing[461], hence opening up new avenues for incorporating biosurfactants into a wide range of skin care products in place of chemical surfactants and this could lead to healing of minor skin lesions[461].

15.14 Application of biosurfactants in nanotechnology

5.14.1 Biosurfactant assisted synthesis of iron oxide nanocrystals and silver nanoparticles

The biosurfactants are emerging as a green surfactant alternate to their chemically synthesized counterparts for the synthesis and stabilization of nanoparticles. Rhamnolipid biosurfactants produced by the bacterial strain OBP1 exhibited stabilization of silver and iron oxide nanoparticles. Such behaviour of biosurfactants is due to the solubilization or incorporation of the reactant species and/or synthesized particles into the micellar phase and adsorption of nanoparticles on the surface of the biosurfactant micelles[462]. It is possible that the positive charge on Ag^{+}/Fe^{2+} leads to the formation of the ion pair with the negative head of rhamnolipid micelles which concentrate the Ag^{+}/Fe^{2+} within the small volume through the electrostatic interactions into the reaction sites[462]. Kiran *et al.*[332] suggested that the presence of biosurfactant in the colloidal solution of nanoparticles would act as the stabilizing agent that prevents the formation of aggregates, and favours the production and stability of nanoparticles under the experimental conditions. Silver nanoparticles (SNP) synthesized in the rhamnolipid colloid were found to be stable for more than one month. The SNP in rhamnolipid (RL) colloid (SNPRL) was not affected by the addition of external NaCl upto 60 $mg.ml^{-1}$ and this also prevented the destruction of silver nanoparticles. The inference has suggested that the RL shall undergo vesicle formation to prevent silver nanoparticles' exposure to NaCl[122]. Xie *et al.*[117] also reported the use of rhamnolipid biosurfactant as stabilizing agent of silver

nanoparticles. Biswas and Raichur[334] used the rhamnolipid biosurfactant to evaluate its effect on the synthesis and stabilization of nano zirconia particles.

5.14.2 Antibacterial property of ION-RL and SNP-RL nanocomposite

Iron oxide nanocrystal-rhamnolipid (ION-RL) and silver nanoparticle-rhamnolipid (SNP-RL) nanocomposites were found to be effective against Gram positive and Gram negative bacteria suggesting their broad spectrum antibacterial properties. Different strains belonging to the same species of bacteria exhibited different susceptibility towards the biosurfactant, ION-RL and SNP-RL. The excellent antibacterial efficacy of the surfactant-adsorbed nanoparticles could be envisaged as a complex interplay of the following factors:

1. Better accessibility and as such greater activity of the surface adsorbed biosurfactant moieties. The immobilization of bioactive molecules onto nanomaterials displays enhanced activity, stability and reusability[122, 361].
2. Triangular nanoplates are much more competent than spherical counterparts[361]. The evolution of hierarchical structure with more of strained facets or planes in the iron oxide nanostructure could be ascribed to the antimicrobial action. The differential action against Gram positive and Gram negative bacteria may be credited to the varied cell wall architecture and the surface moieties that interact differentially with the rhamnolipid and the biosurfactant assisted nanocomposites of iron oxide and silver nanoparticles.

5.15 Industrial applications of biosurfactant producing bacteria

5.15.1 Synthesis of bis-uracil derivatives in presence of biosurfactant

A highly efficient and environmentally benign nucleophilic addition of 6-amino-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione and 6 [(dimethylamino) methyleneamino]-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione with aldehydes (aromatic, aliphatic and heterocyclic) using biosurfactant isolated from OBP1 achieved in water at the room temperature with higher yield of products as compared

to their counterparts without the application of biosurfactants. There could be two factors responsible for increased yield of products; first, the equilibrium of the nucleophilic reaction is shifted far to the right in the presence of the biosurfactant. Gradual addition of surfactant into the reaction mixture to an extent of CMC led to the formation of hydrophobic pockets within the bulk water solvent due to hydrophobic interior of micelle, which paves the way for bringing the reactants to close proximity facilitating product formation. The biosurfactant usually interferes with the existing solubility of the solvation layer, which led to the other probable interactions and chemical events that might be responsible for the release of binding energy. The released binding energy in turn helps in expediting the reaction towards completion in the presence of catalysts. Secondly, from the plausible reaction mechanism, the nucleophilic 5^{th} position of 4 attacks of the carbon centre belonging to the aldehyde, followed by the elimination of water molecule, a second molecule of uracil derivative then attacks via its nucleophilic 5^{th} position affording the product. Dehydration has successfully been achieved in water due to the hydrophobic nature of the biosurfactant interior[463]. The biosurfactant has been recovered and recycled for several times and used repeatedly.

5.15.2 Degradation of complex synthetic polymers

P. aeruginosa was reported to be a versatile bacterial strain capable of utilizing various types of carbon sources ranging from simple glucose to complex petroleum based hydrocarbons[373, 374]. From biodegradation studies of synthetic modified hyperbranched epoxy/OMMT clay nanocomposites based polymers, it is clear that *P. aeruginosa* strain OBP1 is capable of utilizing the synthesized polymers as is evident by an increase in bacterial population density. Further, the rate of increase in the bacterial cell density increases with the treatment time. This was particularly observed in the case of modified hyperbranched epoxy nanocomposites which might be due to the catalytic role of clay in the hydrolysis of the ester groups present in the modified systems[363]. The presence of terminal hydroxyl groups in the clay layers can cause heterogeneous hydrolysis after absorbing water in the presence of microbes. This process is known to require some time for the initiation which might be the reason for

Chapter V
Conclusion

Conclusions drawn from the present investigation are:

1. A total of 52 bacterial isolates were isolated from the various crude petroleum contaminated sites of Assam. Out of them 17 isolates exhibited significant reduction in the surface tension of the culture media and showed positive results in drop collapse and oil displacement tests. Finally, 4 isolates namely OBP1, OBP2, OBP3 and OBP4 were found to be potential in the utilization of hydrocarbons and production of biosurfactant.

2. The potential four bacterial isolates belonged to the genus *Pseudomonas*. The partial sequencing of 16S rRNA gene and NCBI GenBank BLAST search proved these four bacterial isolates to be closely related to *Pseudomonas aeruginosa*.

3. The partial 16S rRNA gene sequences of *Pseudomonas aeruginosa* strains OBP1, OBP2, OBP3 and OBP4 are deposited in the GenBank database under accession number 1568190, 1568199, 1568206 and 156820, respectively. The nucleotide frequency count showed ATGC, C+G and A+ T compositions of the bacterial strains to have almost similar frequency (~99%) distribution as compared to the other reported strains of *Pseudomonas aeruginosa*.

4. The bacterial strains could utilize a wide spectrum of hydrocarbons as the sole source of carbon and energy. All four bacterial strains exhibited preference for high molecular weight aliphatic hydrocarbons as compared to aromatic and polyaromatic hydrocarbons (PAHs).

5. Haemolytic and CTAB agar assay confirmed the biosurfactant producing ability of the bacterial strains and also provided a criterion for the production of rhamnolipid. These four *Pseudomonas aeruginosa* strains possessed presumptive production of extracellular anionic biosurfactants.

6. Among the carbon substrates used for the production of biosurfactants, n-hexadecane was found to be the most suitable for the bacterial strains. Diesel was also found to be an efficient carbon source as well in terms of reduction in the surface tension. The level of biosurfactant production in diesel-supplemented medium was almost similar to that of n-hexadecane.

7. Optimization of media components including carbon, nitrogen, macro-micro nutrients, and culture conditions including temperature, pH and agitation (rpm) leads to the enhancement of biosurfactant production in all four bacterial strains.

8. Among the low cost and renewable carbon substrates screened for the production of biosurfactant by these four bacterial strains, waste glycerol followed by kitchen waste oil (*Sesamum indicum*) and Nahor seed oil (*Mesua ferrea*) were found to be suitable.

9. Physical parameters like reduction in surface tension (ST), interfacial tension (IFT), critical micelle concentration (CMC), emulsification activity (E_{24}%) and foaming index (F_{24}%) of the biosurfactants isolated from the bacterial strains clearly confirmed their effective surface-active properties.

10. Biosurfactants produced by the bacterial strains exhibited excellent surface properties and remained stable while exposed to extreme conditions like high temperature, pH, salinity and metal ion concentration. The stability of biosurfactants in higher dilutions (CMD^{-1} and CMD^{-2}) further confirmed their intact surface properties.

11. Chemical characterization of isolated biosurfactants with TLC, FTIR and MS confirmed their glycolipidic nature. Further, mass spectroscopic studies confirmed the production of rhamnolipids by the bacterial strains; however di-rhamnolipids were found to be predominant over mono-rhamnolipids.

12. The isolated biosurfactants were efficient within their CMCs in solubilizing PAHs and thereby could remove the crude oil from the contaminated sand. This effected recovery of residual crude oil from the petroleum sludge. The same was true in the

case of crude oil recovery from the crude oil saturated sand pack column. Further, the increase in the release of crude oil at higher temperature of 70-90°C confirmed thermo-stability of biosurfactant in crude oil separation and recovery processes.

13. All four bacterial strains could grow in crude oil supplemented media utilizing them as the sole source of carbon. The efficiency of each bacterial strain in degrading the test hydrocarbons within 30 days was established by liquid chromatography followed by gravimetric analysis. Further, GC analysis of the saturated fractions of the hydrocarbons confirmed the degradation ability of each individual bacterial strain.

14. Bacterial Consortia I, comprising of OBP1, OBP3, OBP4 and Consortia II, comprising of OBP1, OBP2, OBP3 and OBP4 were found to be more effective in biodegradation of crude oil. Further, addition of biosurfactant to the Consortia enhanced the biodegradation process.

15. Biosurfactant solutions below the CMC were found to be non-toxic on germinating seeds of rice and mung bean, but at concentrations above the CMC exhibited inhibitory effect on seed germination and root elongation which was more pronounced in the case of rice.

16. Biosurfactant of these four bacterial strains showed no larval mortality of the insect *Aedes albopictus* at almost all the recommended concentrations of World Health Organization. However, at higher concentrations of 1000-1500 $mg.l^{-1}$ could kill only about 3% of the larvae.

17. Biosurfactant of the bacterial strains OBP1 exhibited the highest antibiotic activity against the test microorganisms. Further, this biosurfactant exhibited an excellent chemotactic response towards *Staphylococcus aureus* (MTCC 3160) and *Klebsiella pneumoniae* (MTCC 618) strains.

18. Biosurfactants did not exhibit inhibitory effect on the mouse fibroblast cell line L929 upto 100 $\mu g.ml^{-1}$. Biosurfactants having the concentrations above CMCs

revealed non-toxicity to the skin of rabbit, and showed no adverse effect on the haematological parameters of the treated rabbits.

19. Two different types of nanoparticles such as iron oxide nanocrystal (IONRL) and silver nanoparticles (SNPRL) were synthesized in the presence of biosurfactant of the bacterial strain OBP1 and characterized. The SNPRL was found to stable up to 31 days and protected the silver nanoparticles from NaCl up to a concentration of 60 $mg.ml^{-1}$.

20. Both IONRL and SNPRL nanocomposites exhibited considerable antibacterial properties against a wide range of bacterial strains.

21. Biosurfactant of OBP1 was found to be highly efficient in nucleophilic addition reactions of 6-amino-1, 3-dimethyl pyrimidine-2, 4 (1H, 3H)-dione and 6 [(dimethyl amino) methylene amino]-1, 3-dimethylpyrimidine-2, 4(1H, 3H)-dione with aldehydes in water at room temperature to give higher yield of products.

22. The bacterial strain OBP1 exhibited growth on various nanocomposite films such as hyper branched epoxy (HBE), modified hyperbranched epoxy (MHBE) and their clay nanocomposites along with the corresponding pristine polymeric films and could utilize vegetable oil based polymer films as carbon substrate. Further, gravimetric analysis and SEM micrographs confirmed the degradation of the tested polymer.

Future research

1. Molecular interaction studies of rhamnolipid congeners with biological macromolecules (molecular docking and molecular dynamics studies/approach) to understand the behaviour of rhamnolipid molecules in a biological system.

   Through bioinformatics tools such as molecular dynamic studies could be exploited for studying the molecular interaction of rhamnolipid congeners with different biological macromolecules particularly with membrane proteins. Such a study will help to elucidate the behaviour of rhamnolipid molecules within the biological system.

2. Study on the preparation of biocompatible rhamnolipid-nanocomposites and their possible application in biomedical fields especially as a drug carrier.

   Most of the nano-materials can't be directly used as carrier molecules for drug delivery. To make them effective for drugs there shall be encapsulation with a compatible agent (bio-coating) such as rhamnolipid for loading the medicinal compounds on it. This has to be worked out for specific medicinal compounds like cancer medicine for targeted drug delivery.

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Appendix I

1. Mineral salt medium (MSM)

Table 1. Composition of mineral salt medium ($g.l^{-1}$)

Sl. no	Chemical	Per litre	Sl. no	Chemical	Per litre
1.	Urea	2.0 g	7.	$FeSO_4.7H_2O$	1.0 mg
2.	$(NH_4)_2SO_4$	2.0 g	8.	$CuSO_4.5H_2O$	50 µg
3.	Na_2HPO_4	3.61 g	9.	$MnSO_4.5H_2O$	10 µg
4.	KH_2PO_4	1.75 g	10.	H_3BO_3	10 µg
5.	$MgSO_4.7H_2O$	0.2 g	11.	$ZnSO_4.7H_2O$	70 µg
6.	$CaCl_2.2H_2O$	50 mg	12	MnO_3	10 µg

pH of the medium was adjusted to 7.0.

2. Chemical gradient motility agar (CGMA)

Table 2. Composition of chemical gradient motility agar ($g.l^{-1}$)

Sl. no	Chemical	Composition ($g.l^{-1}$)
1.	Peptone	5.0
2.	Beef extract	3.0
3.	NaCl	5.0
4.	Agar	10

pH of the medium was adjusted to 6.9

3. Phosphate buffered saline (PBS)

For the preparation of potassium phosphate buffer, first 1 M stock solutions of K_2HPO_4 and KH_2PO_4 at the concentrations of 174.18 $g.l^{-1}$ and 136.09 $g.l^{-1}$ respectively in distilled water were prepared. By mixing prescribed volumes of 1 M K_2HPO_4 and 1 M KH_2PO_4 the required pH containing 0.1M buffer was prepared.

Table 3. Composition of 0.1M potassium phosphate buffer

pH	Volume of 1M K_2HPO_4 (ml)	Volume of 1M KH_2PO_4 (ml)
6.8	49.7	50.3
7.0	61.5	38.5

4. Phosphate urea magnesium sulphate (PUM) Buffer

Table 4. Composition of PUM buffer

Sl. no	Chemical	Composition ($g.l^{-1}$)
1.	$K_2HPO_4.4H_2O$	22 2 g
2.	$KH_2PO_4.4H_2O$	7.26 g
3.	Urea	1.8 g
4.	$MgSO_4.7H_2O$	0.2 g

pH of the medium was adjusted to 7.2.

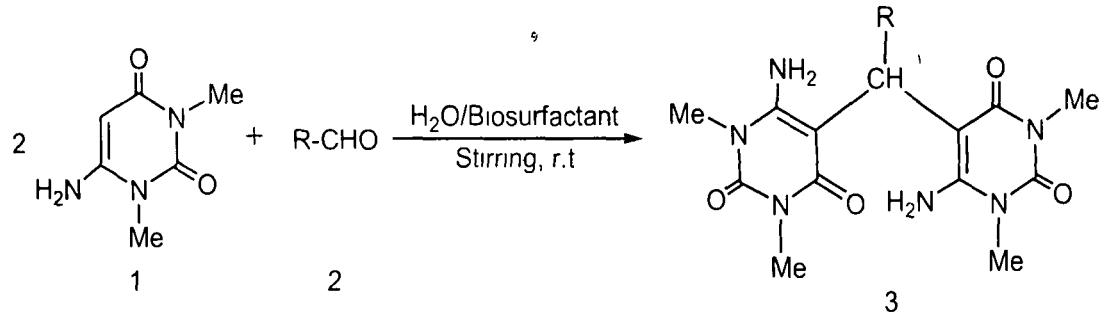
5. Micro and trace element stock solution

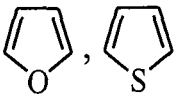
Table 5. Concentration of stock solutions

Sl. no	Chemical	Stock solution	Volume used per litre
1.	$FeSO_4.7H_2O$	2.5 g. 250 ml^{-1}	100 µl
2.	$CuSO_4.5H_2O$	50 mg. 100 ml^{-1}	100 µl
3.	$MnSO_4.5H_2O$	9.2 mg. 100 ml^{-1}	100 µl
4.	H_3BO_3	10 mg. 100 ml^{-1}	100 µl
5.	$ZnSO_4.7H_2O$	70 mg. 100 ml^{-1}	100 µl
6.	MnO_3	10 mg. 100 ml^{-1}	100 µl

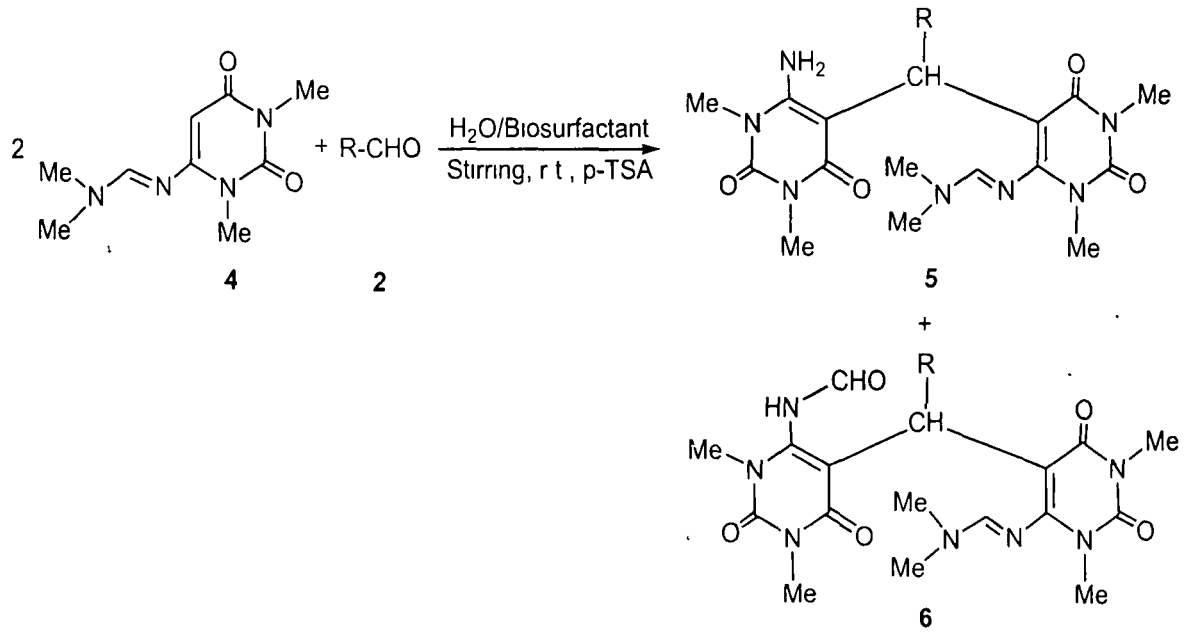
6. Reaction schemes for the synthesis of bisuracil derivatives

Scheme 1. Synthesis of bisuracil derivatives (3) in the presence of biosurfactant

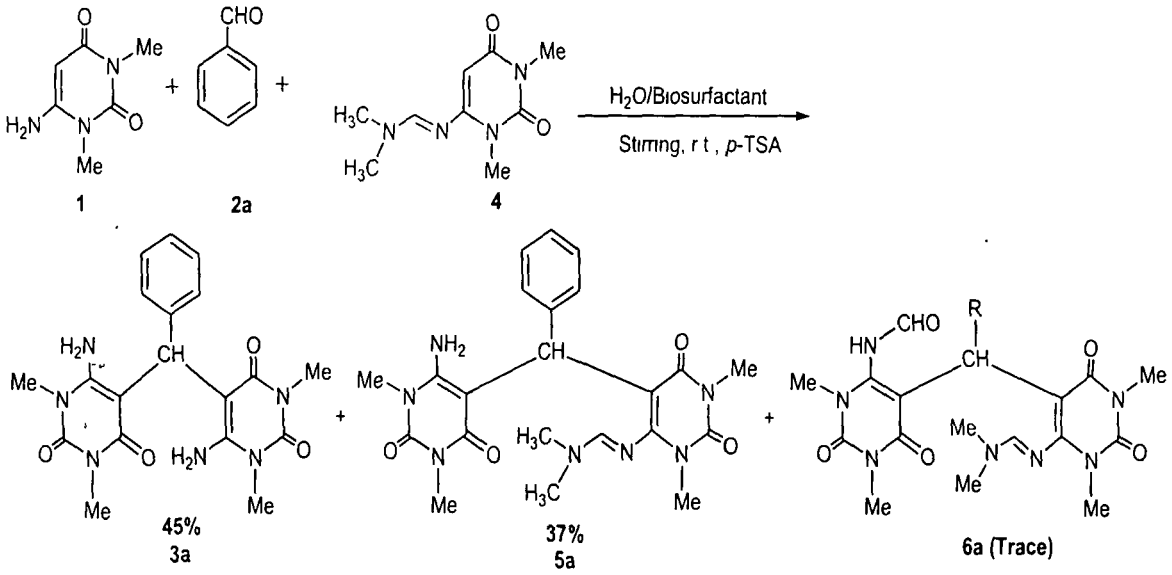


R= Ph, CH=CH-Ph, *p*-OCH_3Ph, *p*-ClPh, *p*-OHPh,*o*-OHPh, *p*-CH_3Ph, *p*-NO_2Ph, *m*-NO_2Ph, H, CH_3, CH_3-$(CH_2)_3$, CH_3-$(CH_2)_2$, 

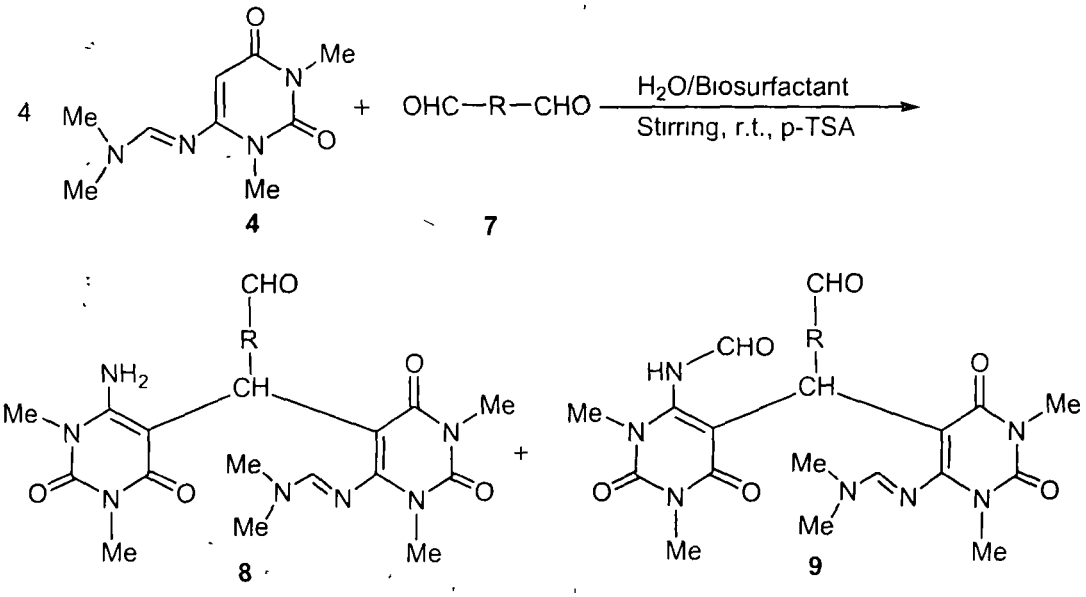
Scheme 2. Synthesis of bisuracil derivatives (5) & (6) in the presence of biosurfactant



Scheme 3. Competitive reaction between 1 and 4 with 2a



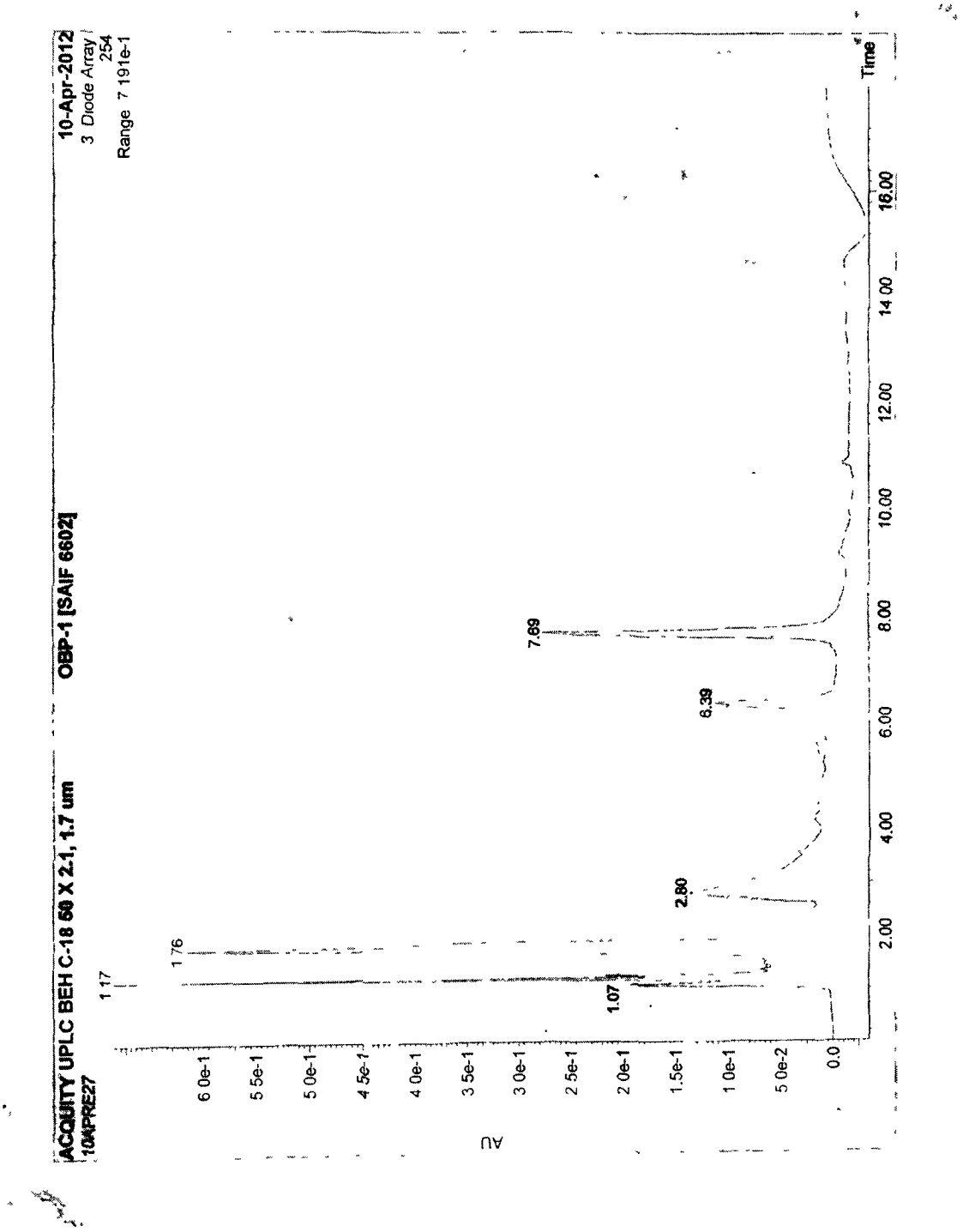
Scheme 4. Synthesis of bisuracil derivatives (8) and (9) in the presence of biosurfactant

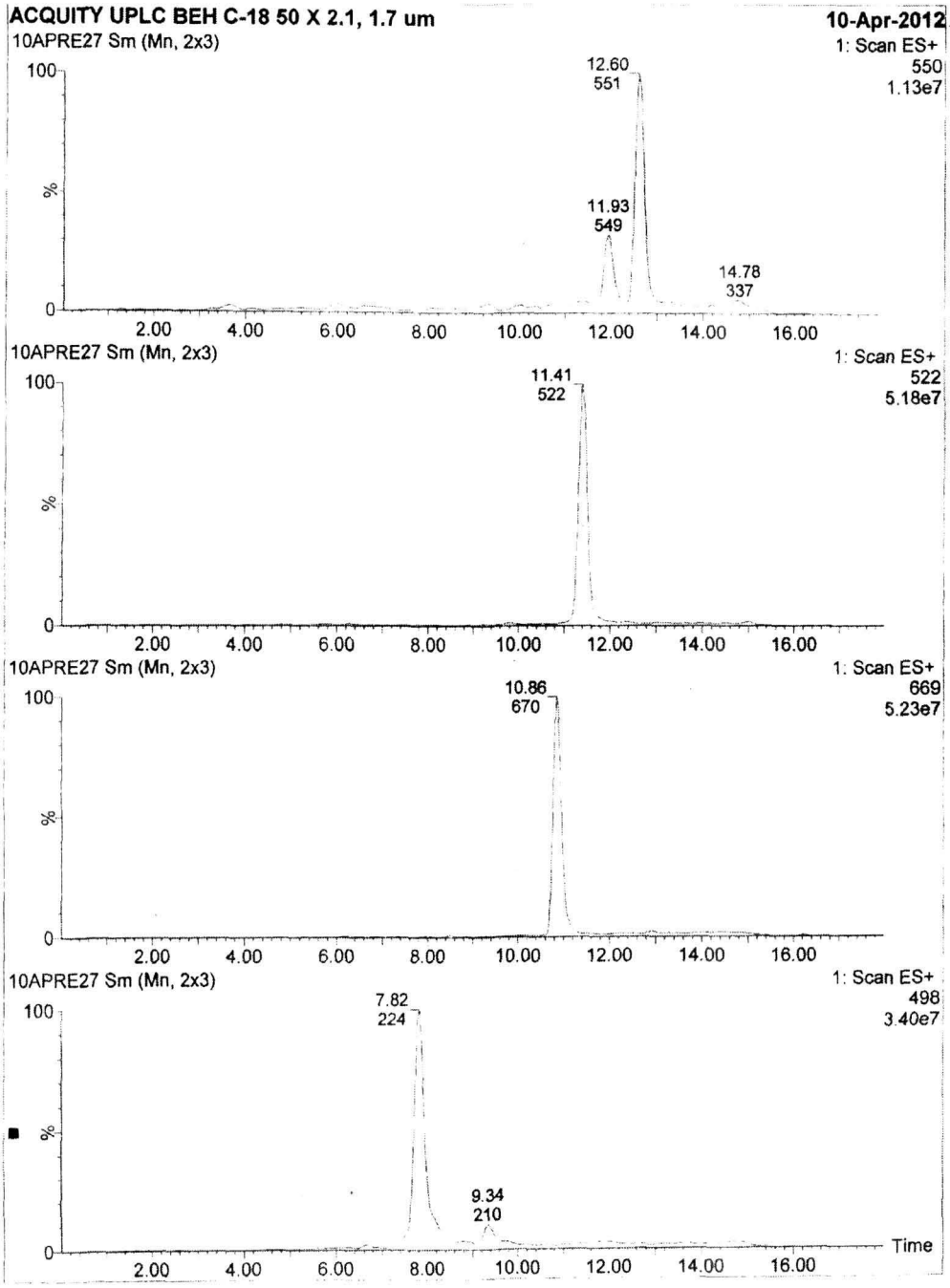


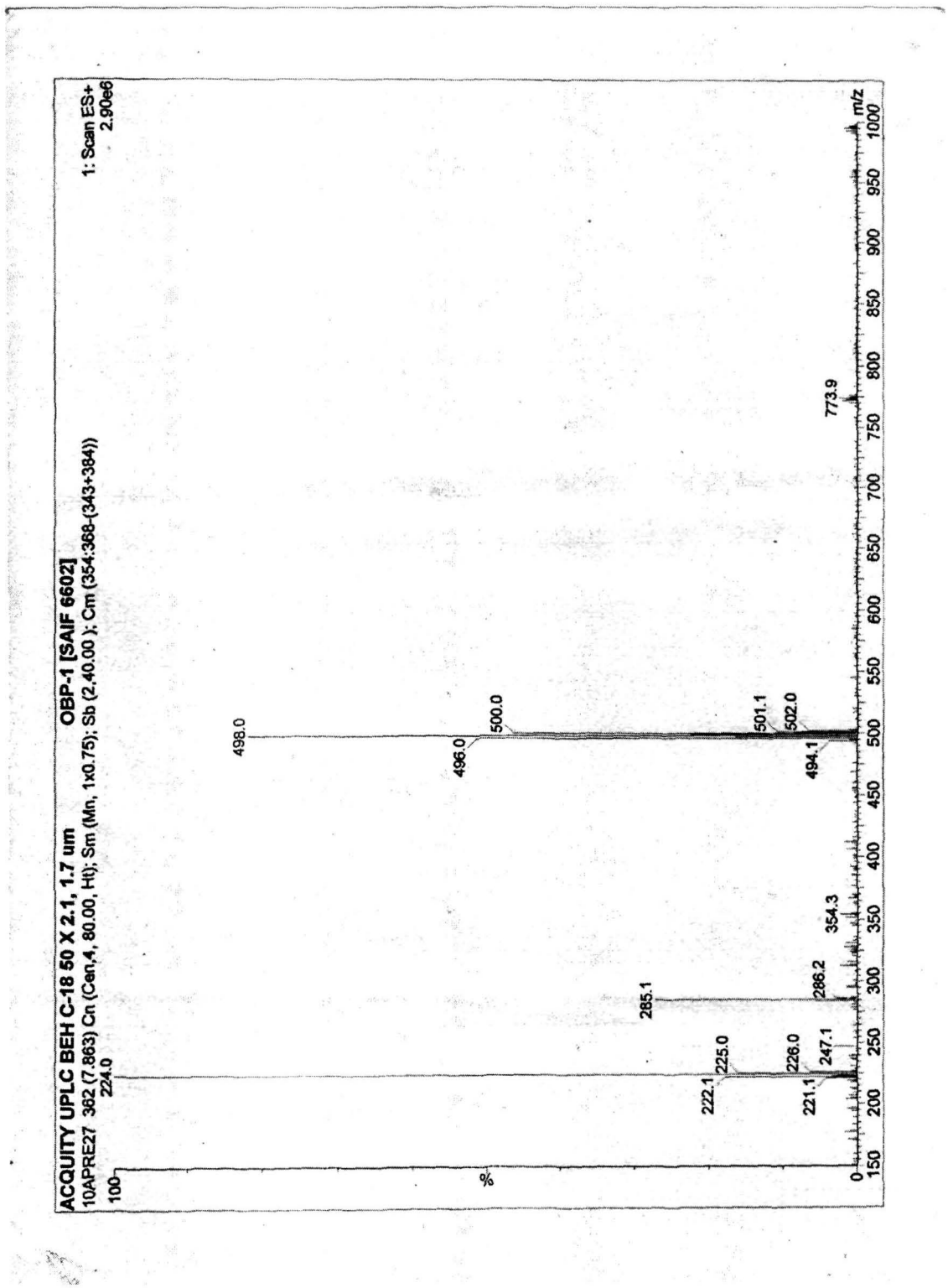
Appendix II

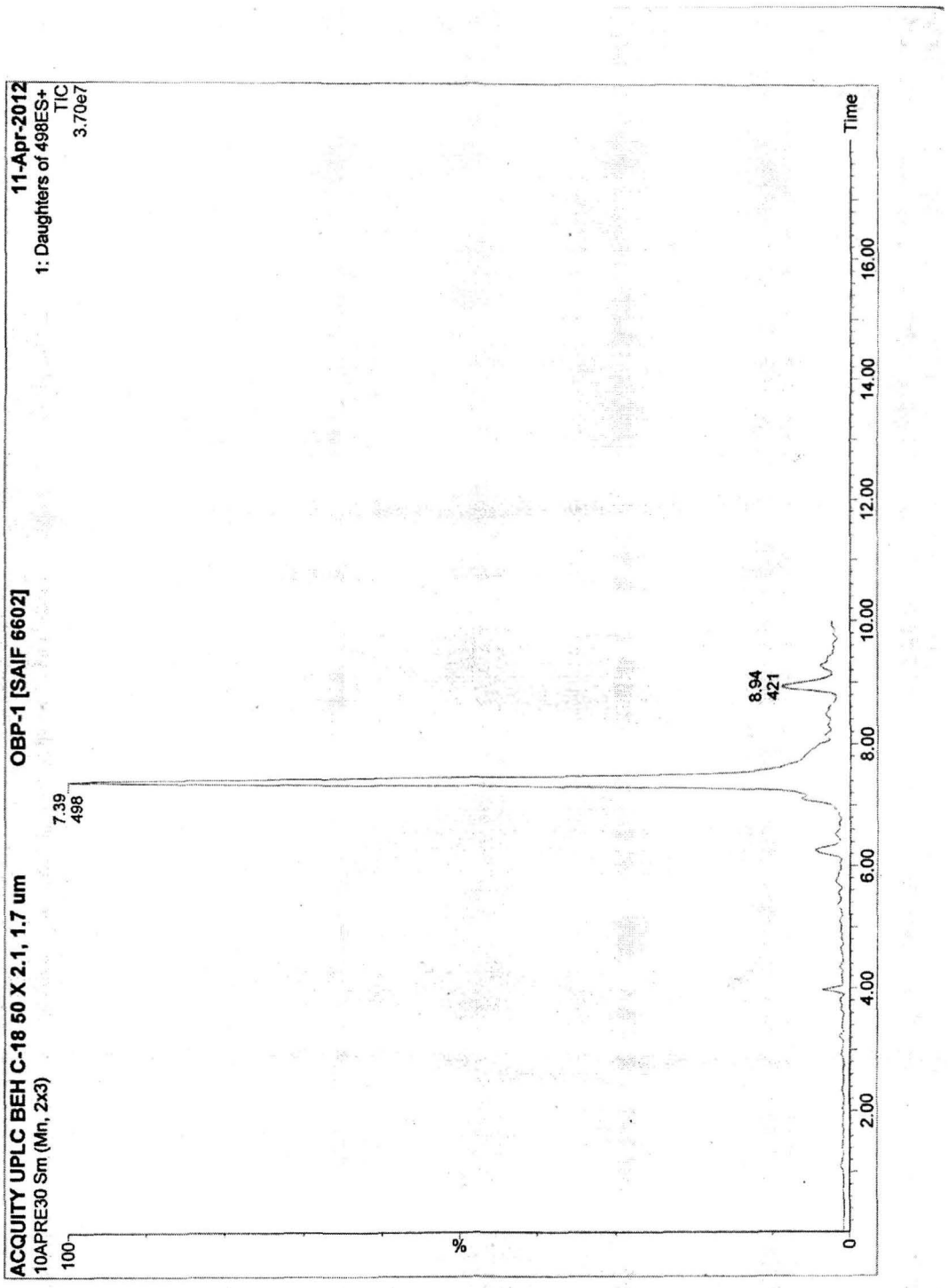
Mass spectra of the biosurfactant samples

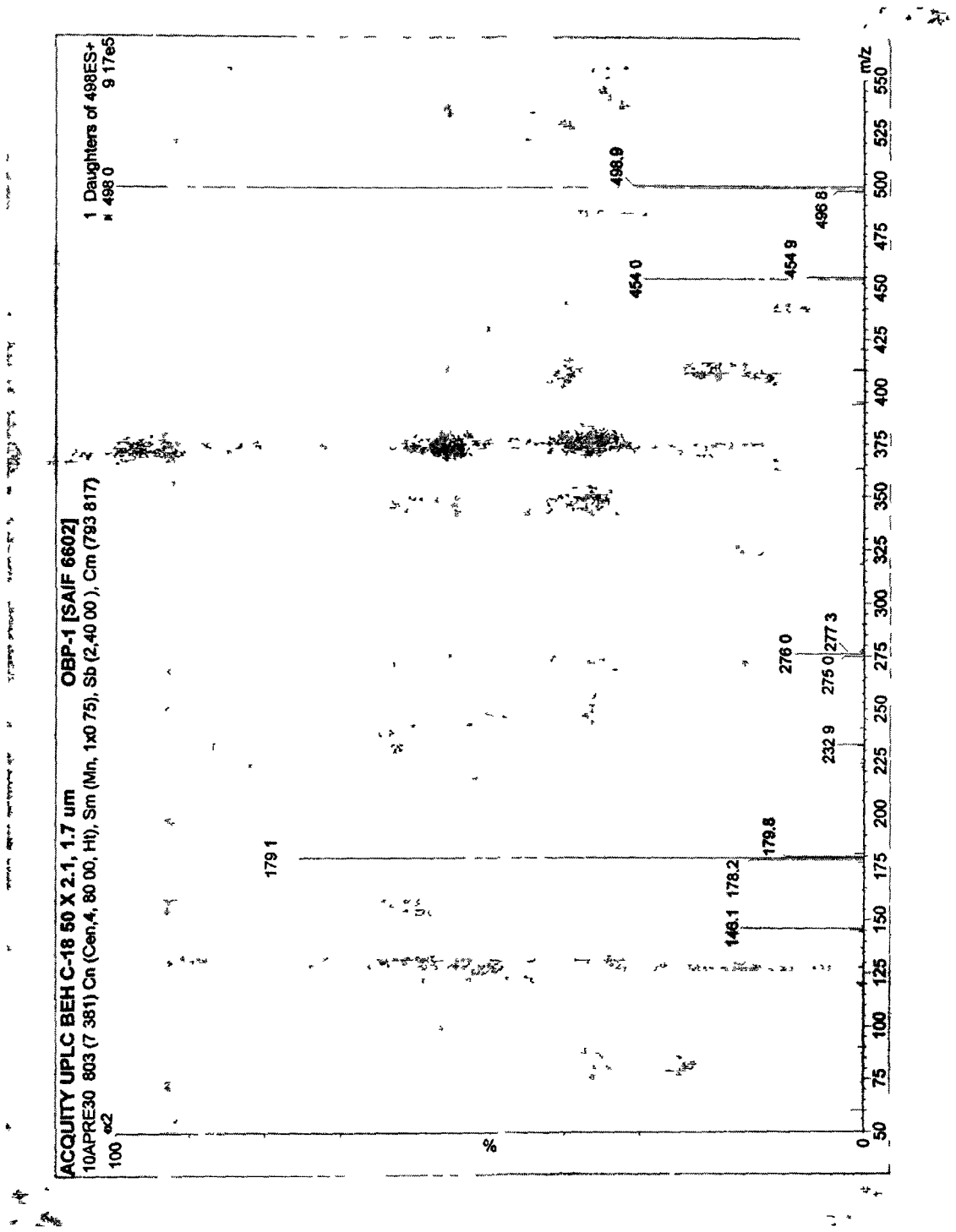
1. UP-LC-MS spectra of Bacterial strain OBP1

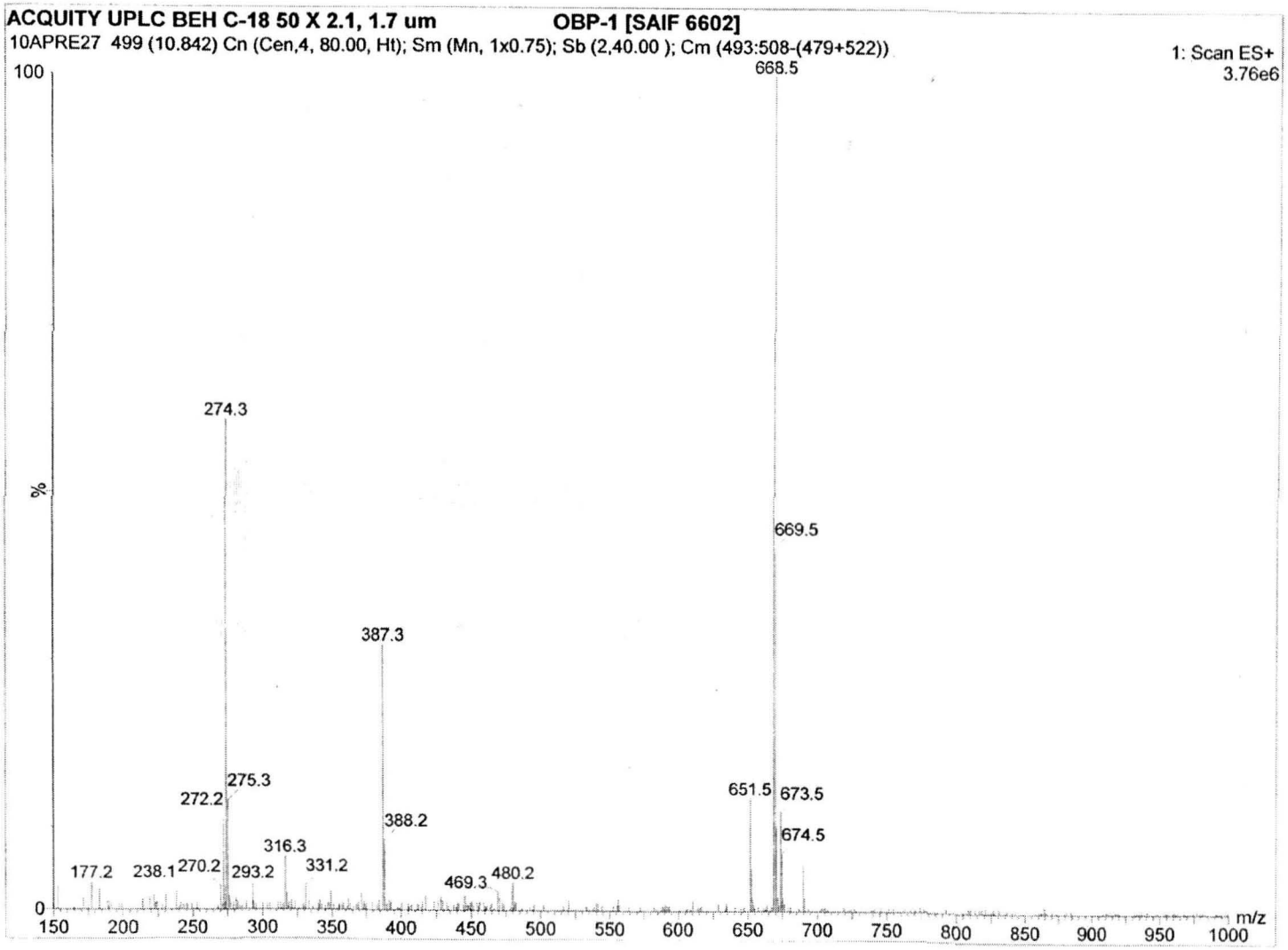


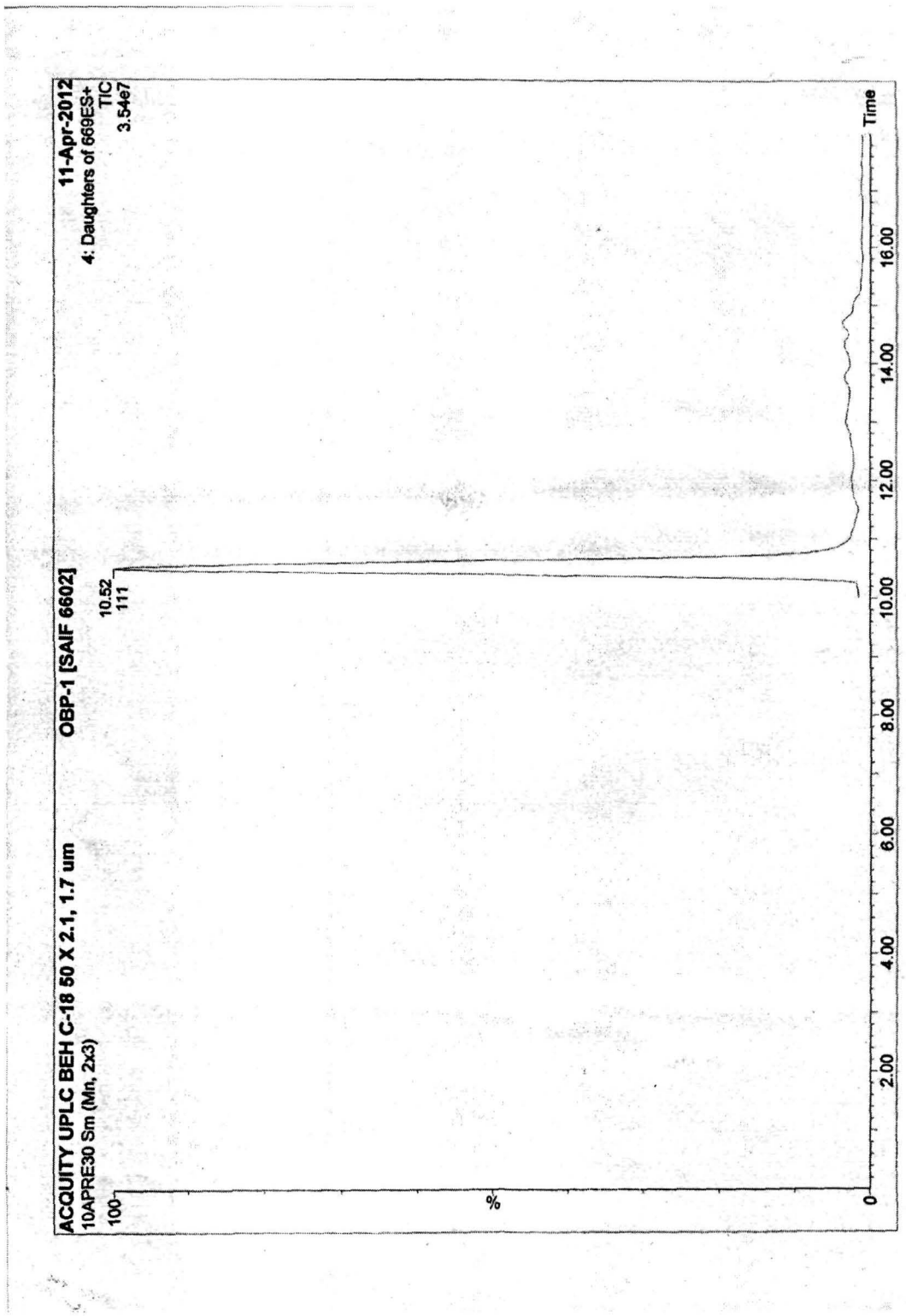

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10-Apr-2012
10APRE27 Sm (Mn, 2x3)
1: Scan ES+
550
1.13e7
12.60
551
11.93
549
14.78
337
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
10APRE27 Sm (Mn, 2x3)
1: Scan ES+
522
5.18e7
11.41
522
10APRE27 Sm (Mn, 2x3)
1: Scan ES+
669
5.23e7
10.86
670
10APRE27 Sm (Mn, 2x3)
1: Scan ES+
498
3.40e7
7.82
224
9.34
210
Time

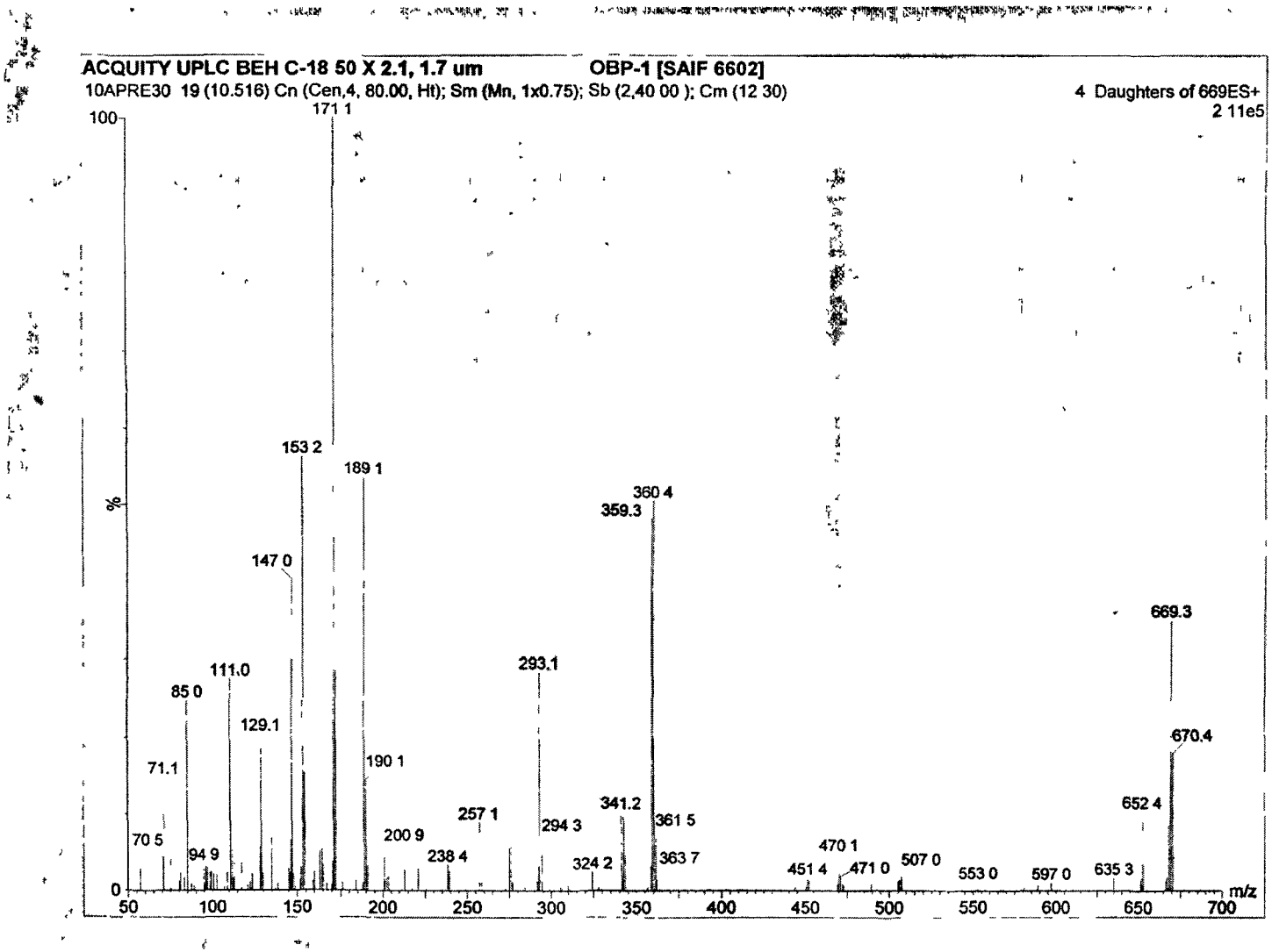

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE27 362 (7.863) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (354:368-(343+384))
1: Scan ES+
2.90e6
100
%
0
224.0
498.0
496.0
500.0
501.1
502.0
494.1
773.9
285.1
222.1
225.0
226.0
221.1
247.1
286.2
354.3
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
m/z

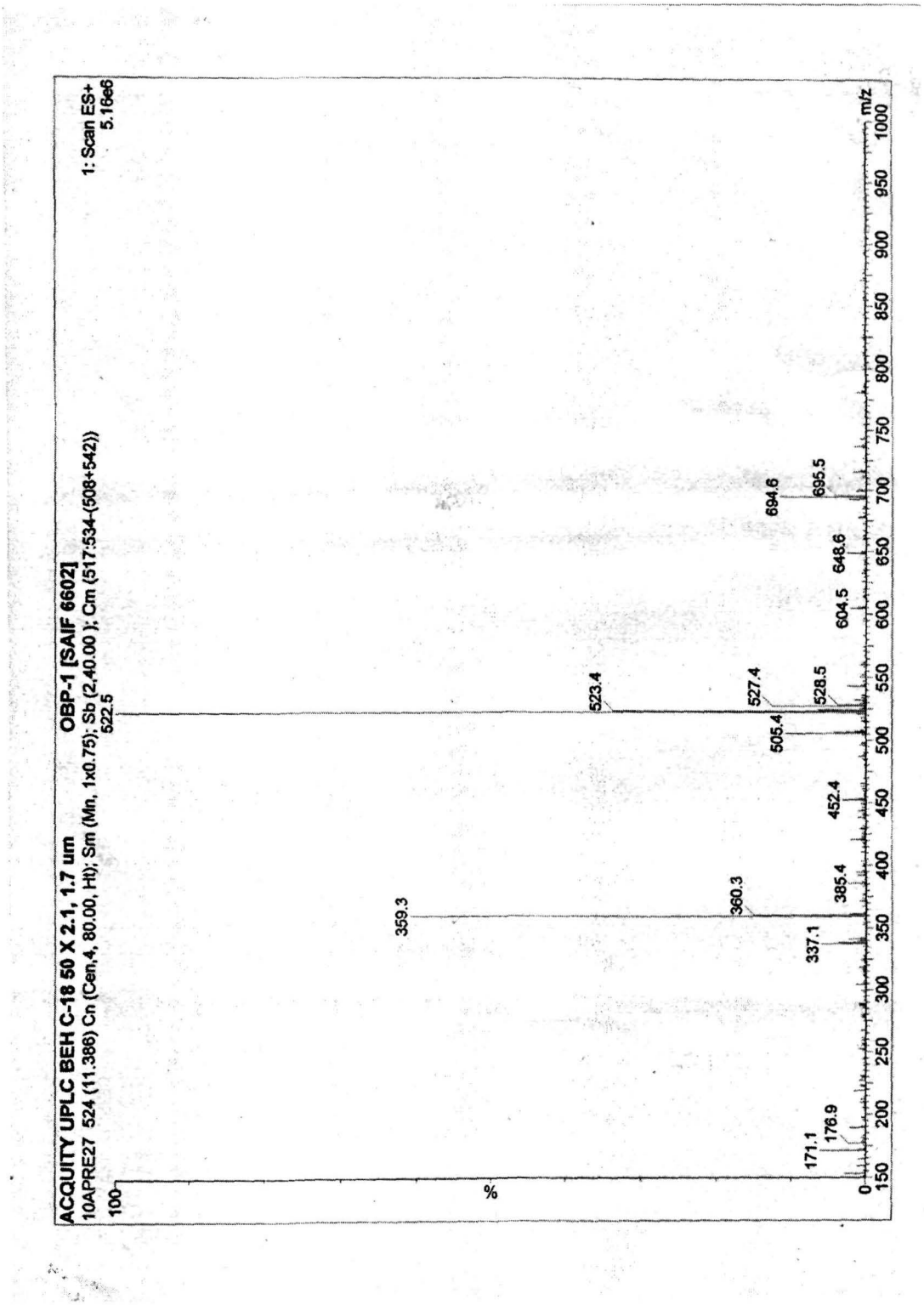

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE30 Sm (Mn, 2x3)
OBP-1 [SAIF 6602]
11-Apr-2012
1: Daughters of 498ES+
TIC
3.70e7
7.39
498
8.94
421
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time

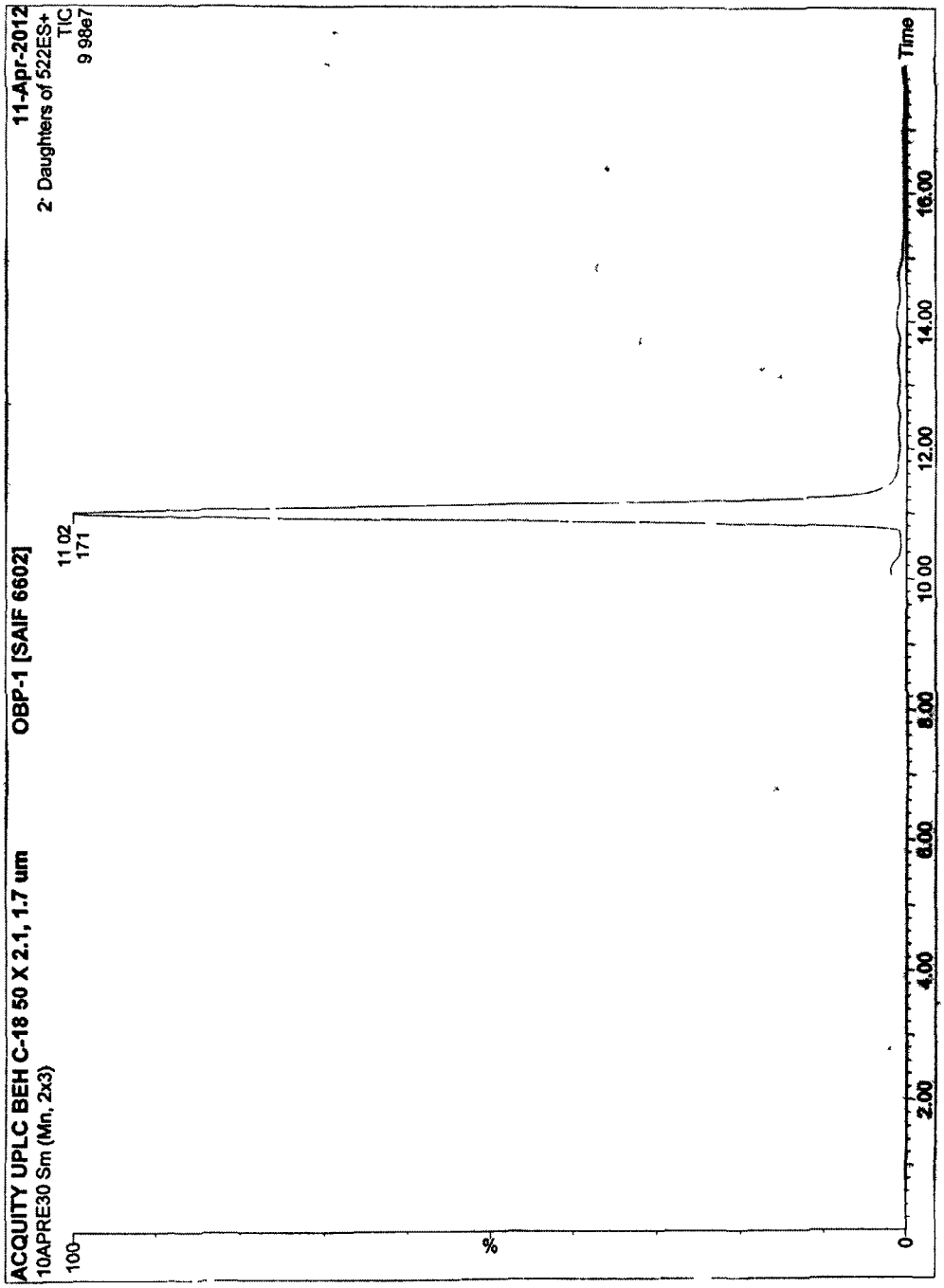

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE30 803 (7 381) Cn (Cen,4, 80 00, Ht), Sm (Mn, 1x0 75), Sb (2,40 00), Cm (793 817)
OBP-1 [SAIF 6602]
1 Daughters of 498ES+
9 17e5
498 0
100
%
0
m/z
50
75
100
125
150
175
200
225
250
275
300
325
350
375
400
425
450
475
500
525
550
179 1
146.1
178.2
179.8
232 9
275 0
276 0
277 3
454 0
454 9
496 8
498.9

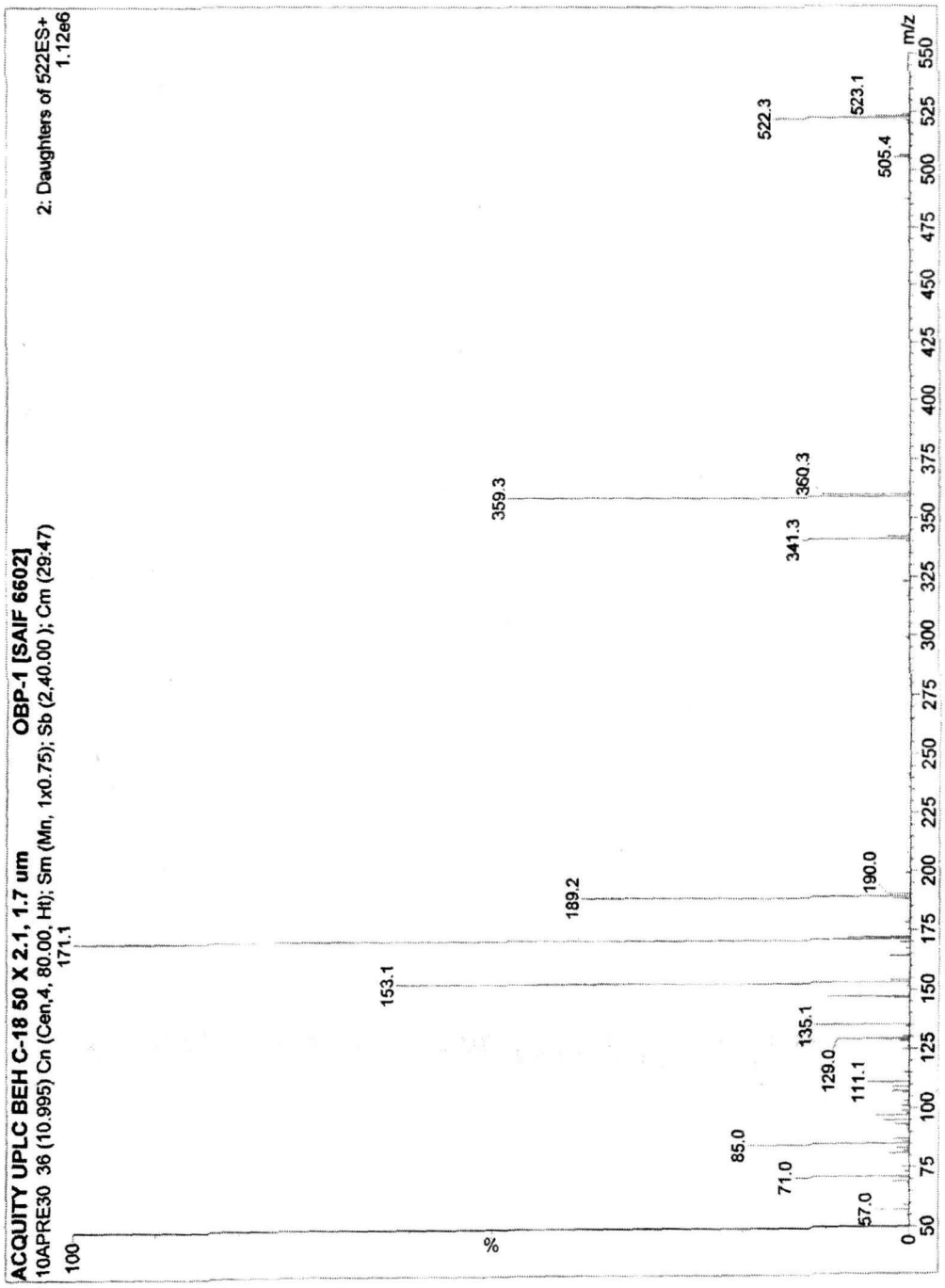

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE27 499 (10.842) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (493:508-(479+522))
1: Scan ES+
3.76e6
100
%
0
668.5
669.5
274.3
387.3
275.3
272.2
388.2
316.3
651.5
673.5
674.5
177.2
238.1
270.2
293.2
331.2
469.3
480.2
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

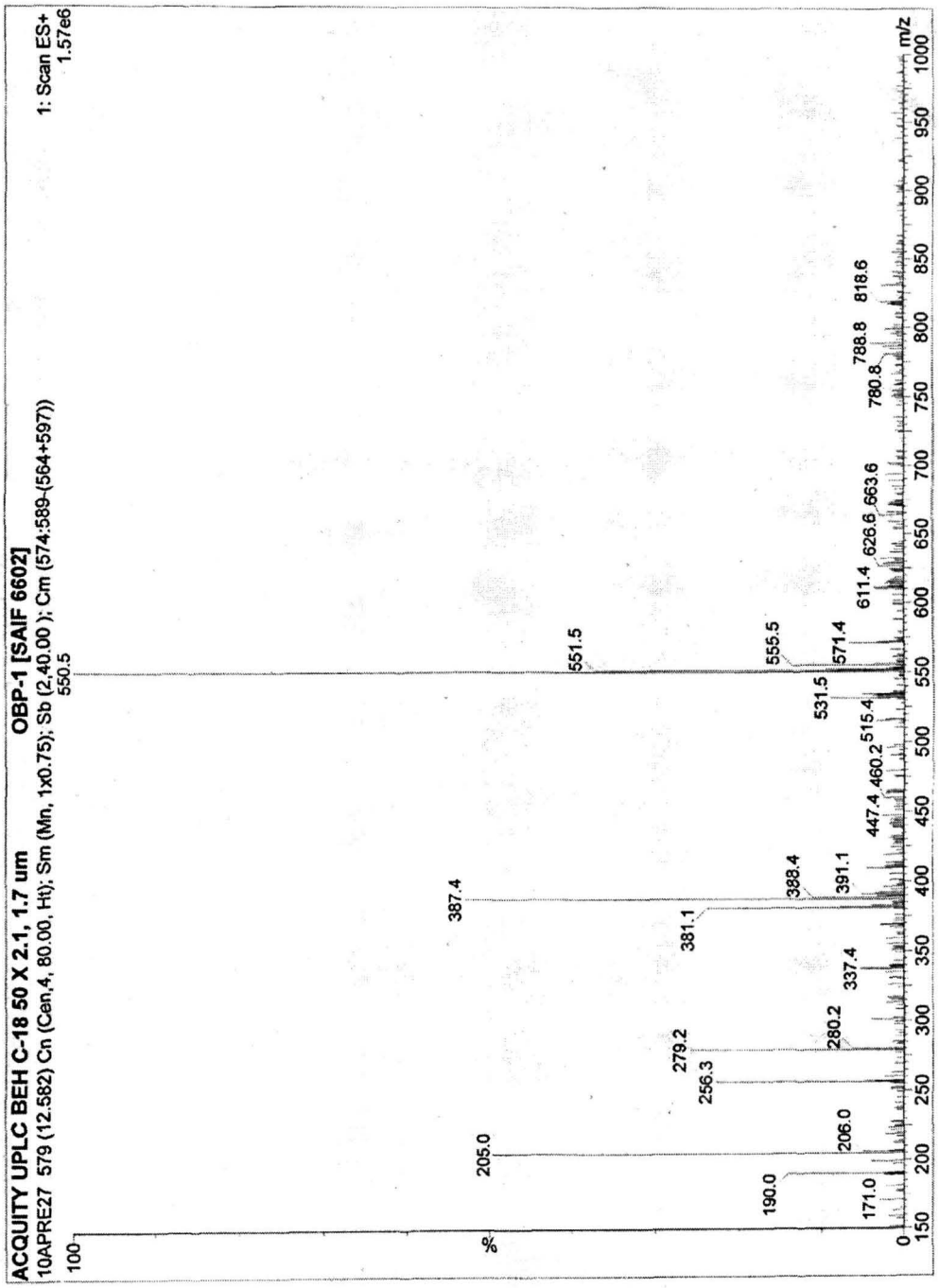

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE30 Sm (Mn, 2x3)
OBP-1 [SAIF 6602]
11-Apr-2012
4: Daughters of 669ES+
TIC
3.54e7
10.52
111
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time

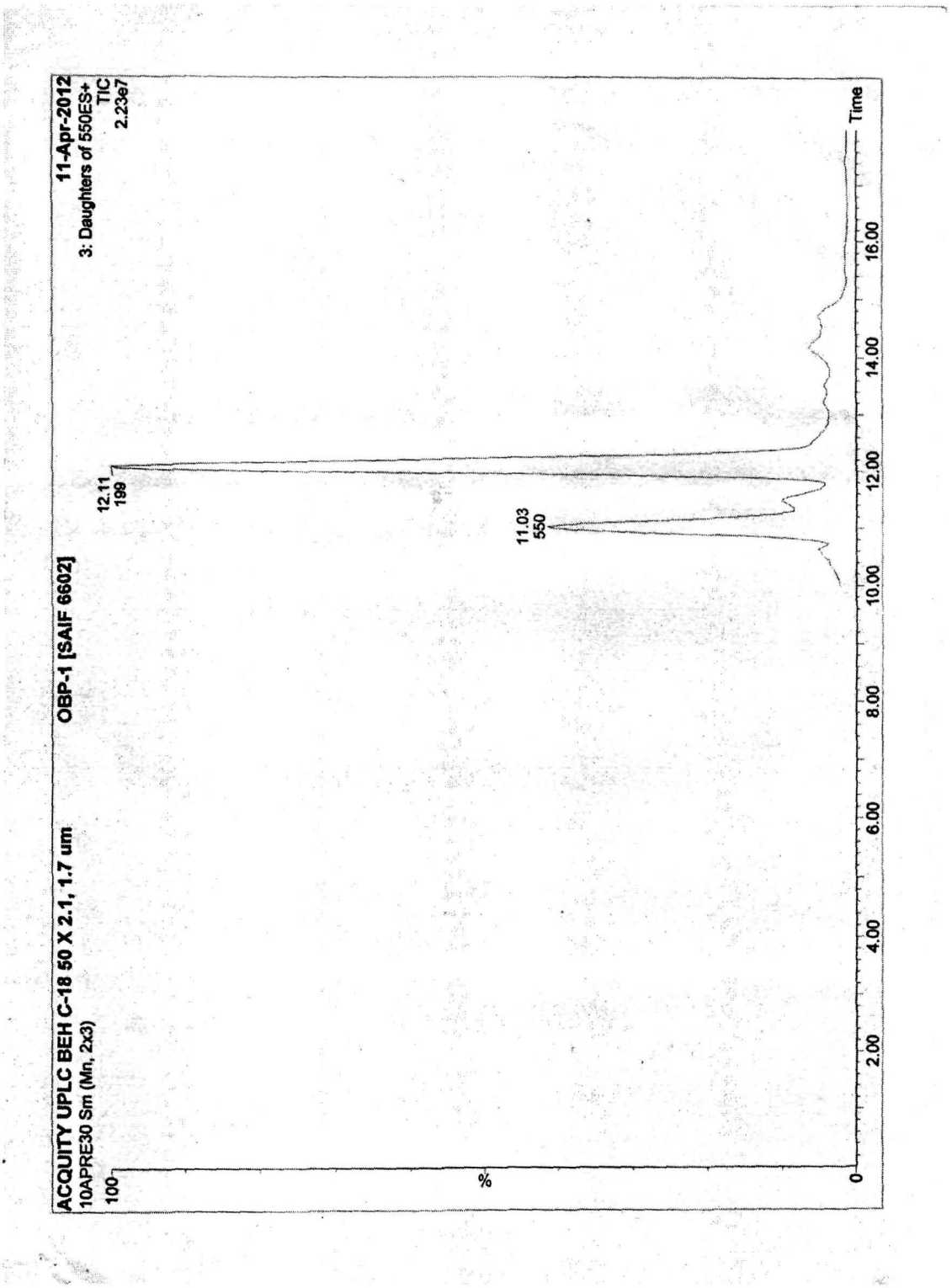

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE30 19 (10.516) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40 00); Cm (12 30)
4 Daughters of 669ES+
2 11e5
100
%
0
171 1
153 2
189 1
360 4
359.3
147 0
669.3
293.1
111.0
85 0
129.1
670.4
71.1
190 1
341.2
257 1
652 4
294 3
361 5
200 9
70 5
94 9
238 4
324 2
363 7
470 1
451 4
471 0
507 0
553 0
597 0
635 3
m/z
50
100
150
200
250
300
350
400
450
500
550
600
650
700

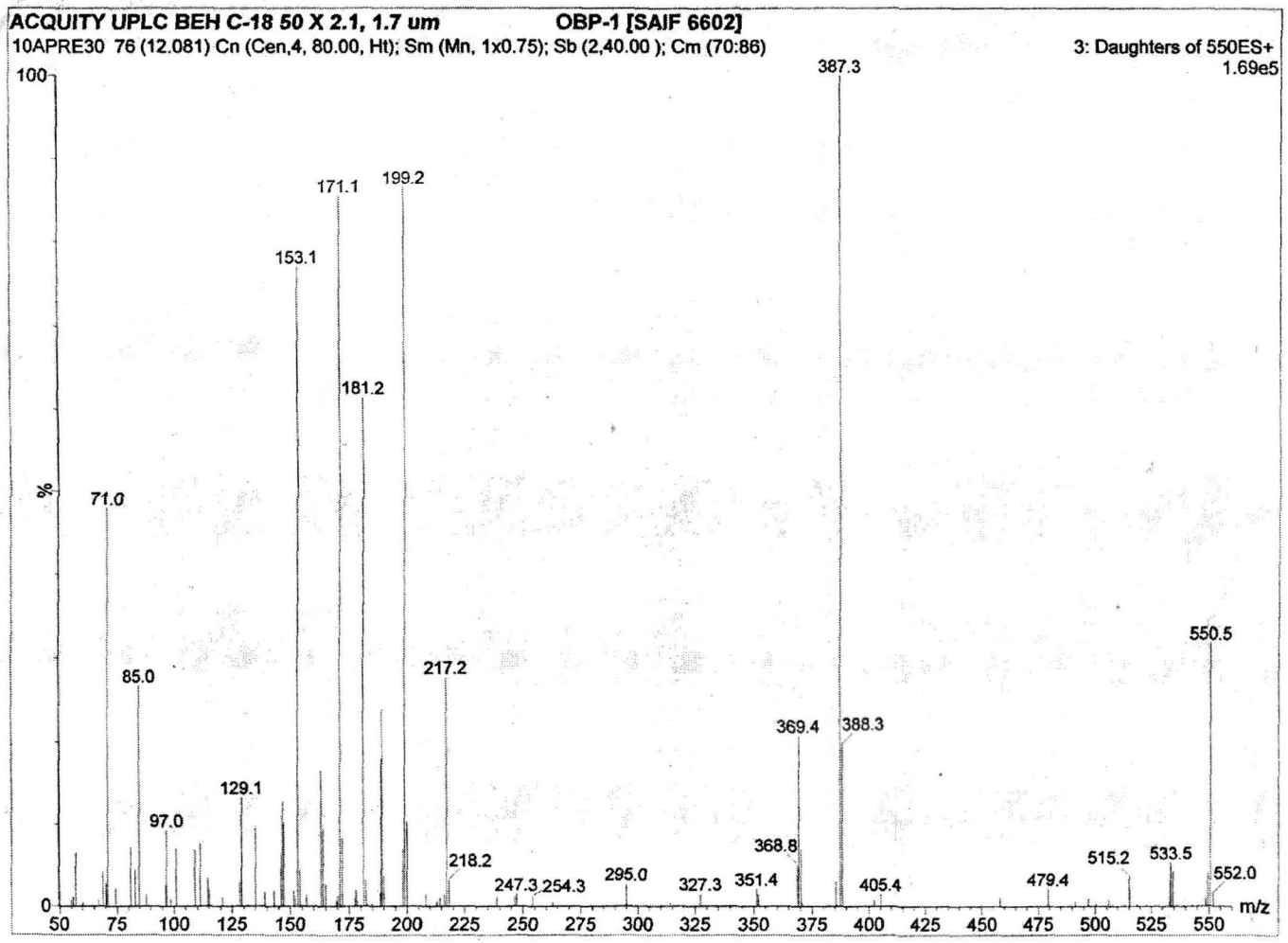

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE27 524 (11.386) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (517:534-(508+542))
OBP-1 [SAIF 6602]
1: Scan ES+
5.16e6
100
%
0
522.5
523.4
359.3
360.3
527.4
505.4
528.5
604.5
648.6
694.6
695.5
337.1
385.4
452.4
171.1
176.9
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z


ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE30 Sm (Mn, 2x3)
OBP-1 [SAIF 6602]
11-Apr-2012
2: Daughters of 522ES+
TIC
9.98e7
11.02
171
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time

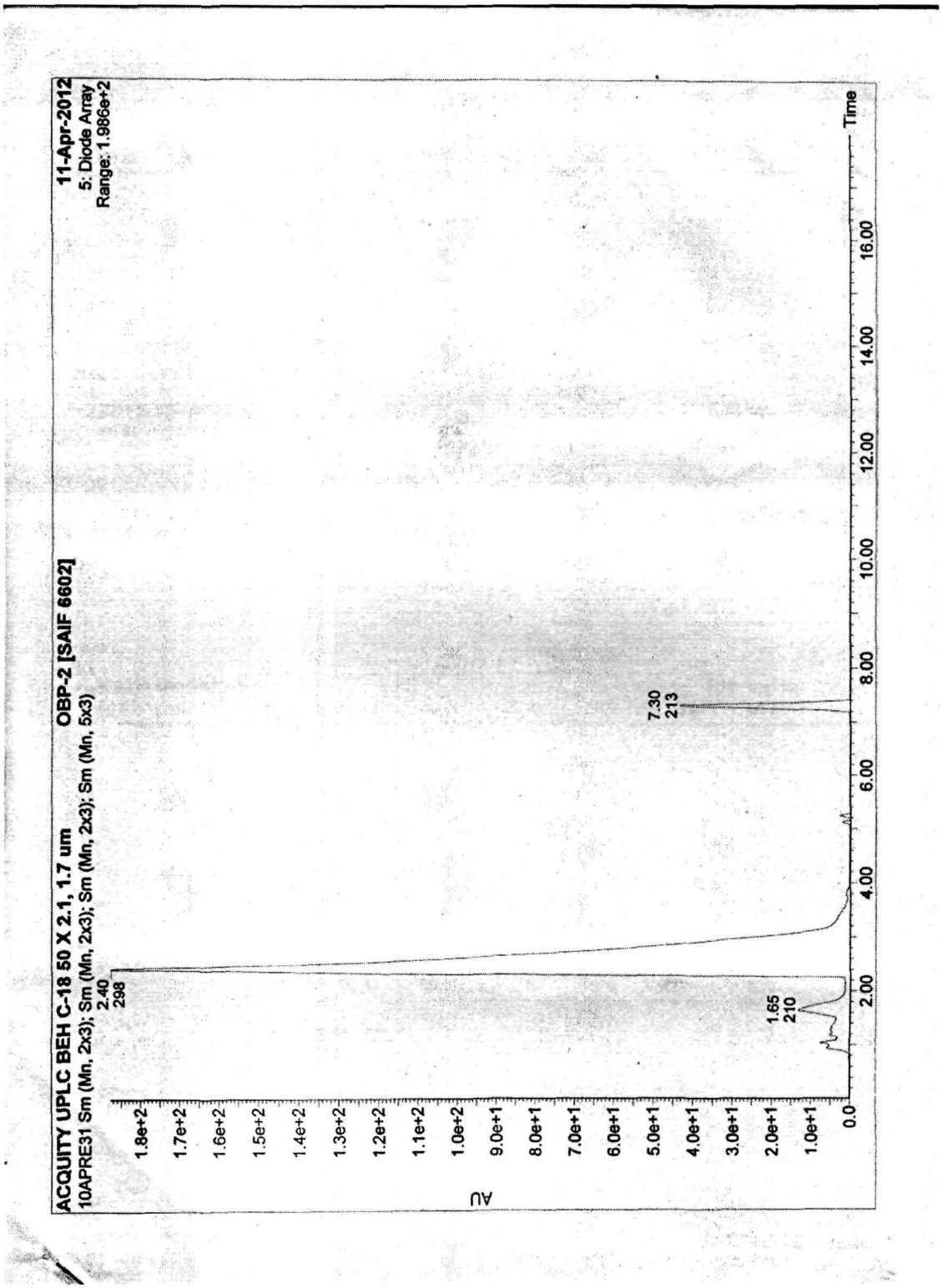

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE30 36 (10.995) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (29:47)
2: Daughters of 522ES+
1.12e6
100
%
0
171.1
153.1
189.2
359.3
341.3
360.3
190.0
522.3
523.1
505.4
85.0
71.0
57.0
135.1
129.0
111.1
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550
m/z

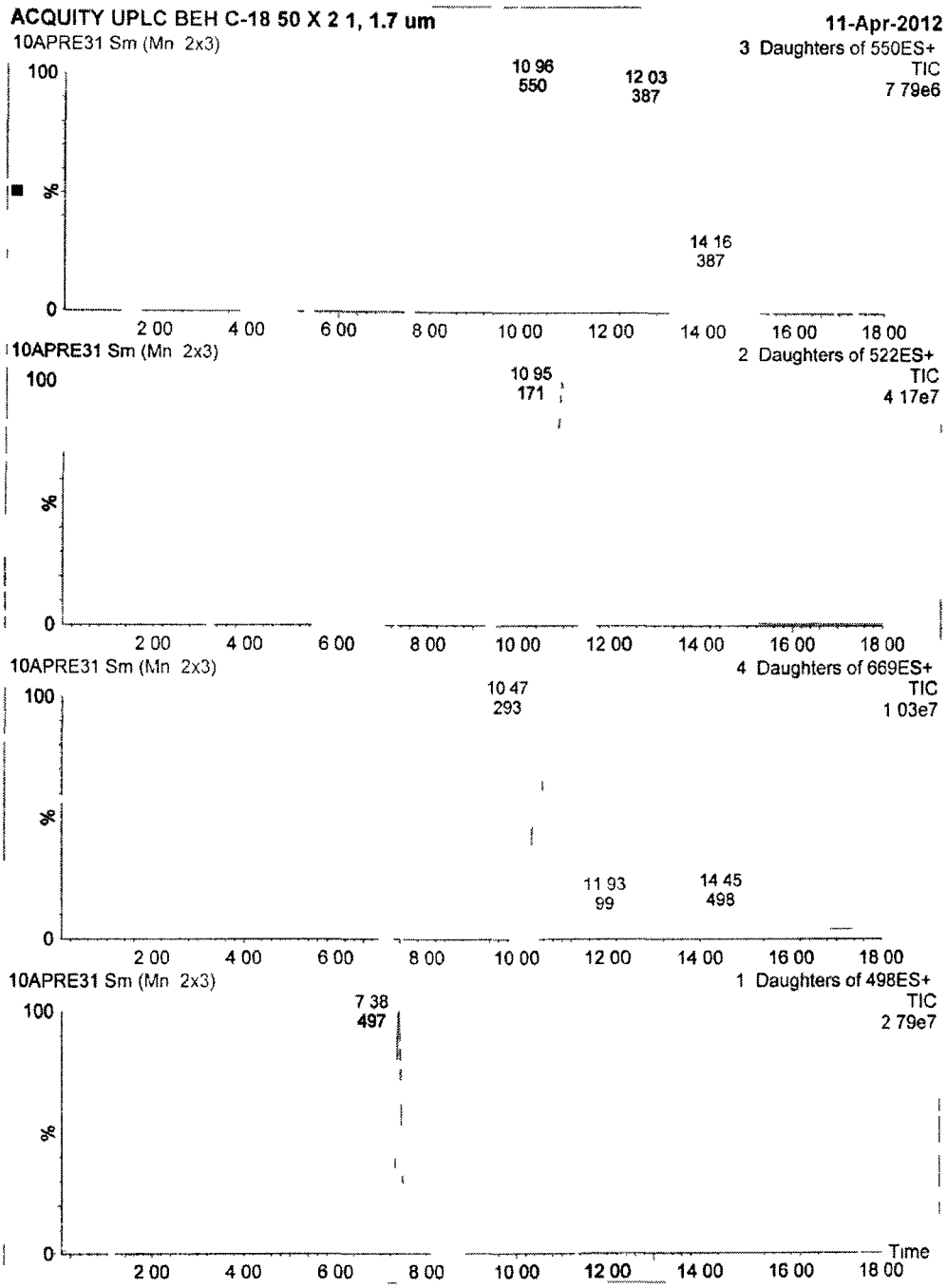

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE27 579 (12.582) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (574:589-(564+597))
1: Scan ES+
1.57e6
100
%
0
205.0
387.4
550.5
551.5
555.5
571.4
190.0
206.0
256.3
279.2
280.2
337.4
381.1
388.4
391.1
447.4
460.2
515.4
531.5
611.4
626.6
663.6
780.8
788.8
818.6
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
m/z

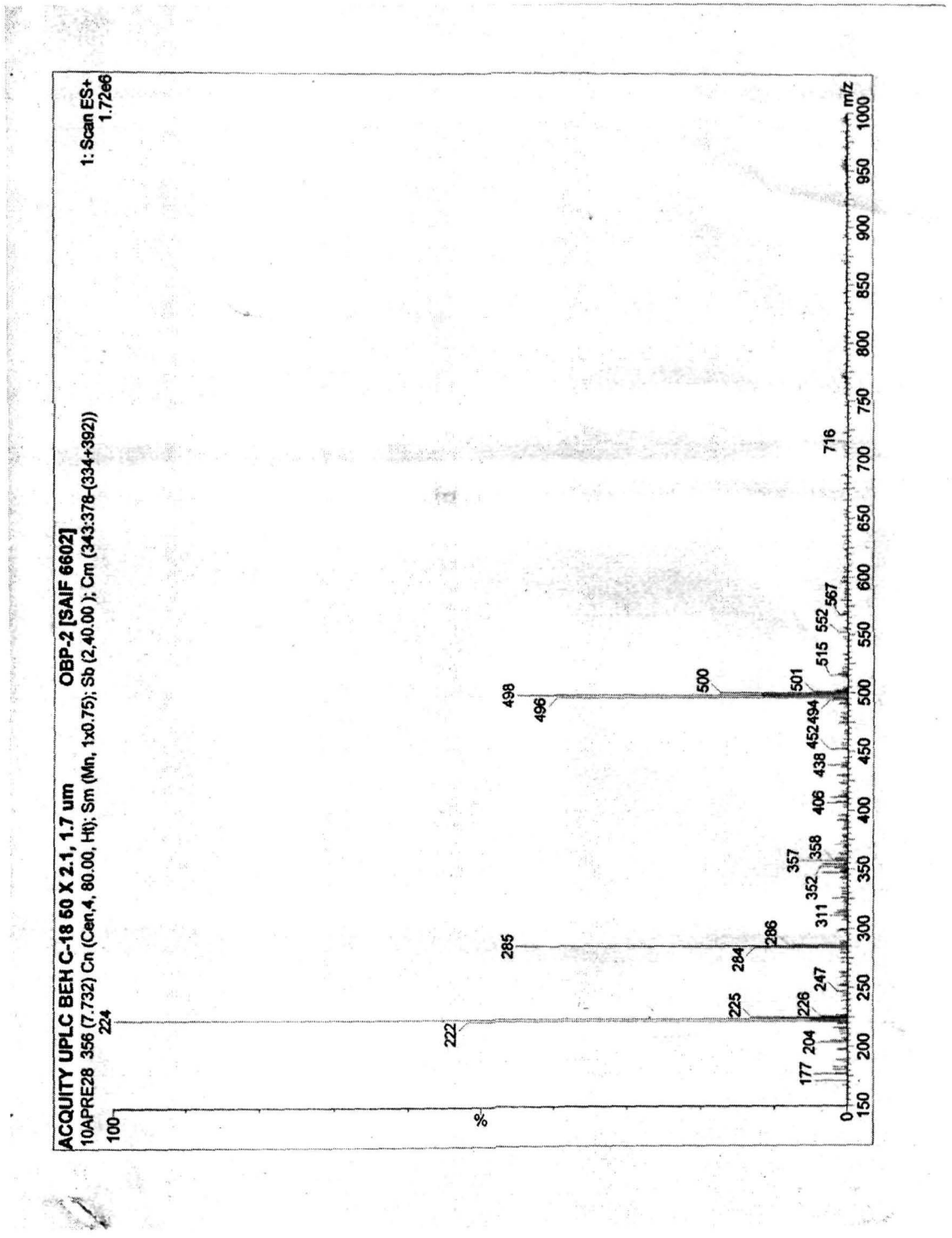

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE30 Sm (Mn, 2x3)
OBP-1 [SAIF 6602]
11-Apr-2012
3: Daughters of 550ES+
TIC
2.23e7
12.11
199
11.03
550
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
Time

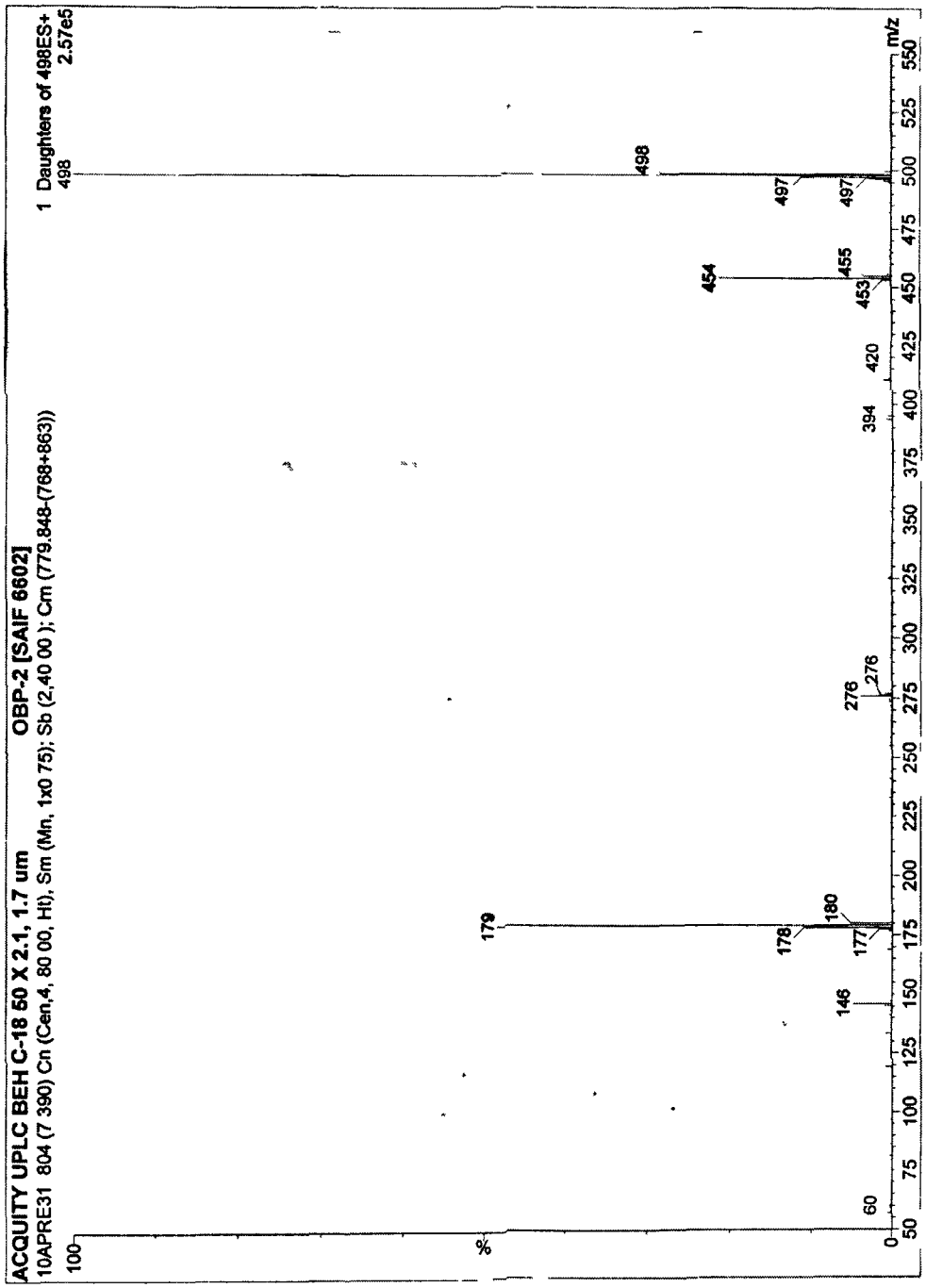

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-1 [SAIF 6602]
10APRE30 76 (12.081) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (70:86)
3: Daughters of 550ES+
1.69e5
%
m/z
387.3
171.1
199.2
153.1
181.2
71.0
85.0
217.2
550.5
369.4
388.3
129.1
97.0
368.8
218.2
515.2
533.5
552.0
247.3
254.3
295.0
327.3
351.4
405.4
479.4

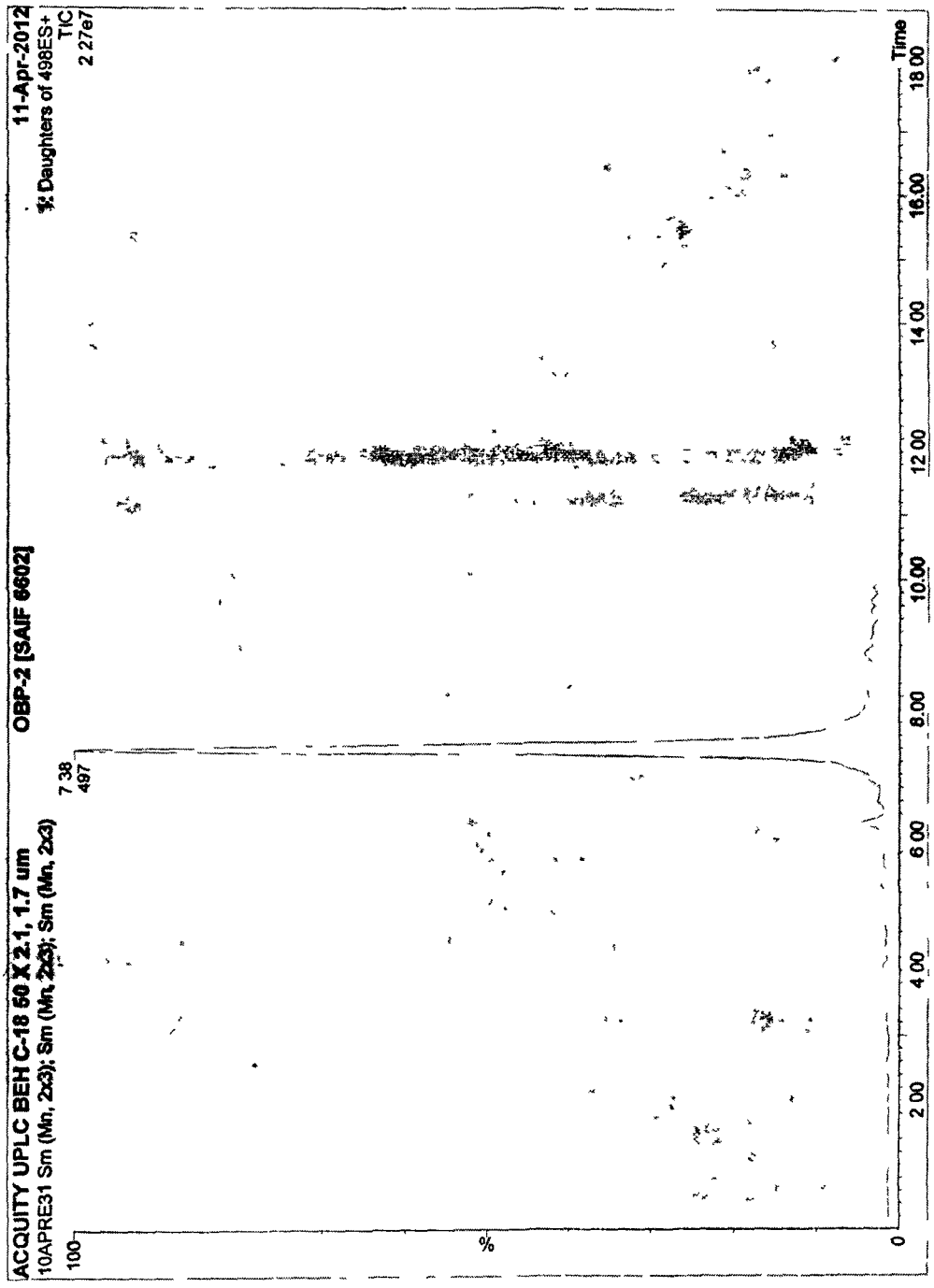
2. UP-LC-MS spectra of Bacterial strain OBP2

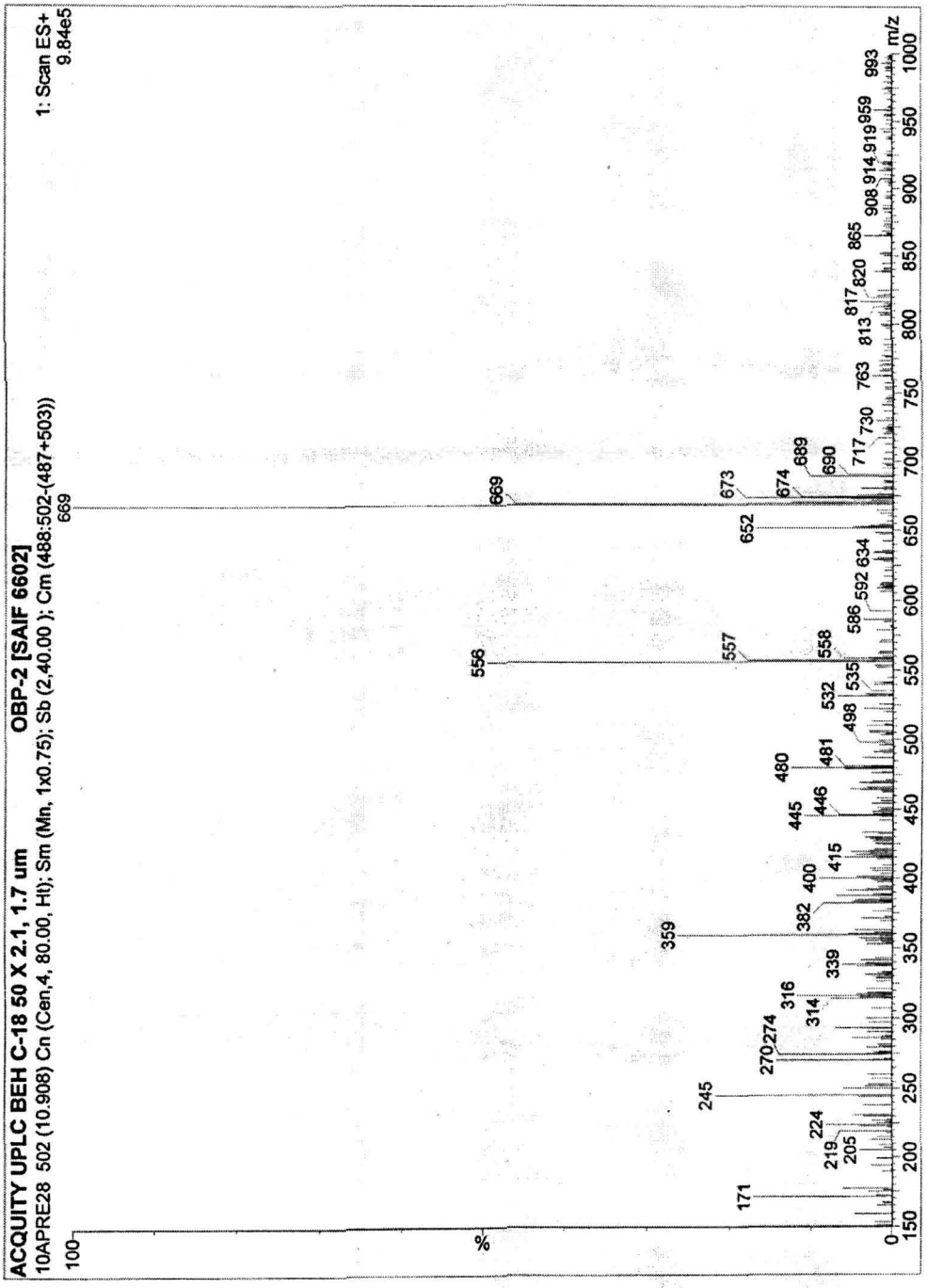


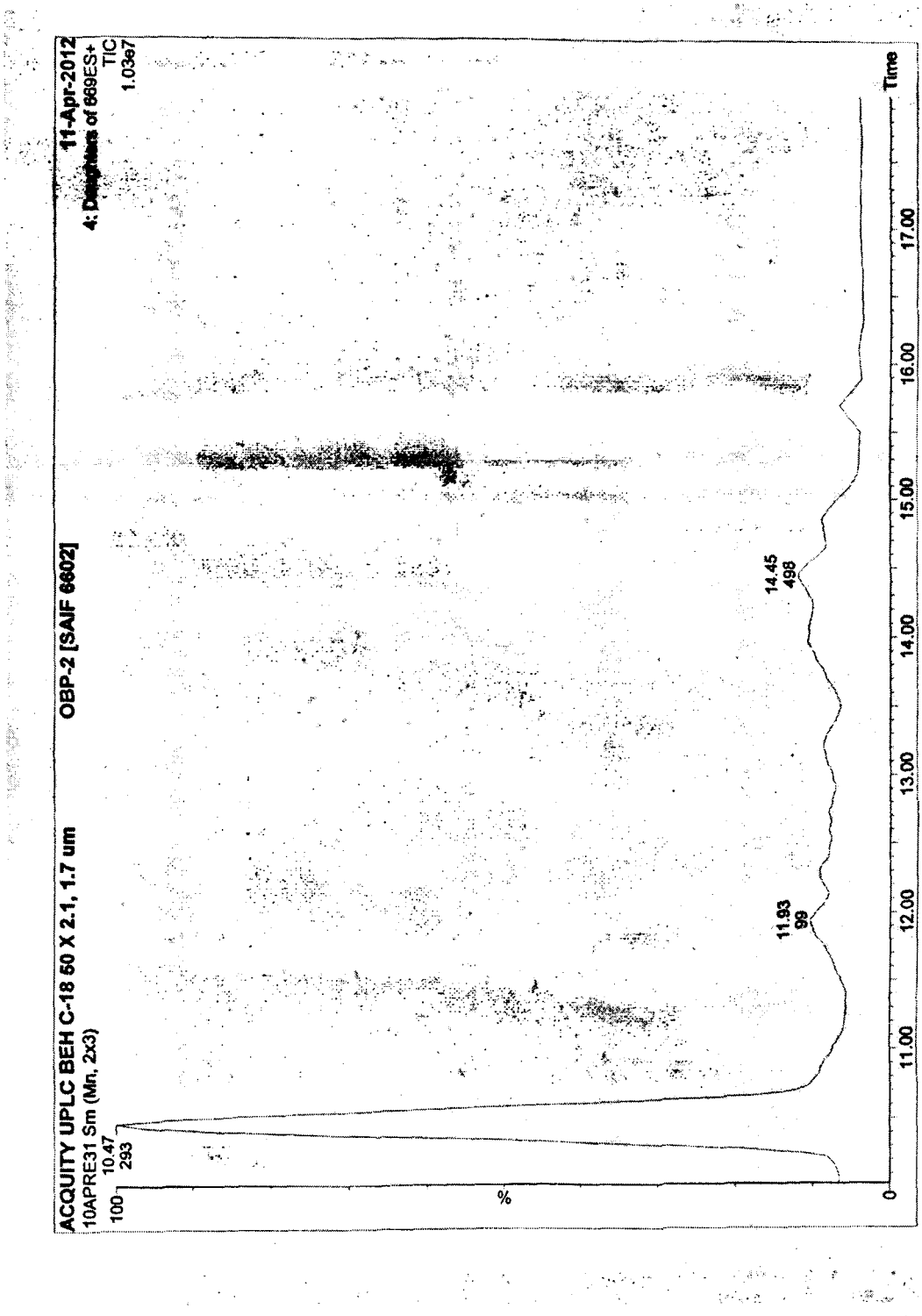

ACQUITY UPLC BEH C-18 50 X 2 1, 1.7 um
11-Apr-2012
10APRE31 Sm (Mn 2x3)
3 Daughters of 550ES+
TIC
7 79e6
10 96
550
12 03
387
14 16
387
100
%
0
2 00
4 00
6 00
8 00
10 00
12 00
14 00
16 00
18 00
10APRE31 Sm (Mn 2x3)
2 Daughters of 522ES+
TIC
4 17e7
10 95
171
10APRE31 Sm (Mn 2x3)
4 Daughters of 669ES+
TIC
1 03e7
10 47
293
11 93
99
14 45
498
10APRE31 Sm (Mn 2x3)
1 Daughters of 498ES+
TIC
2 79e7
7 38
497
Time

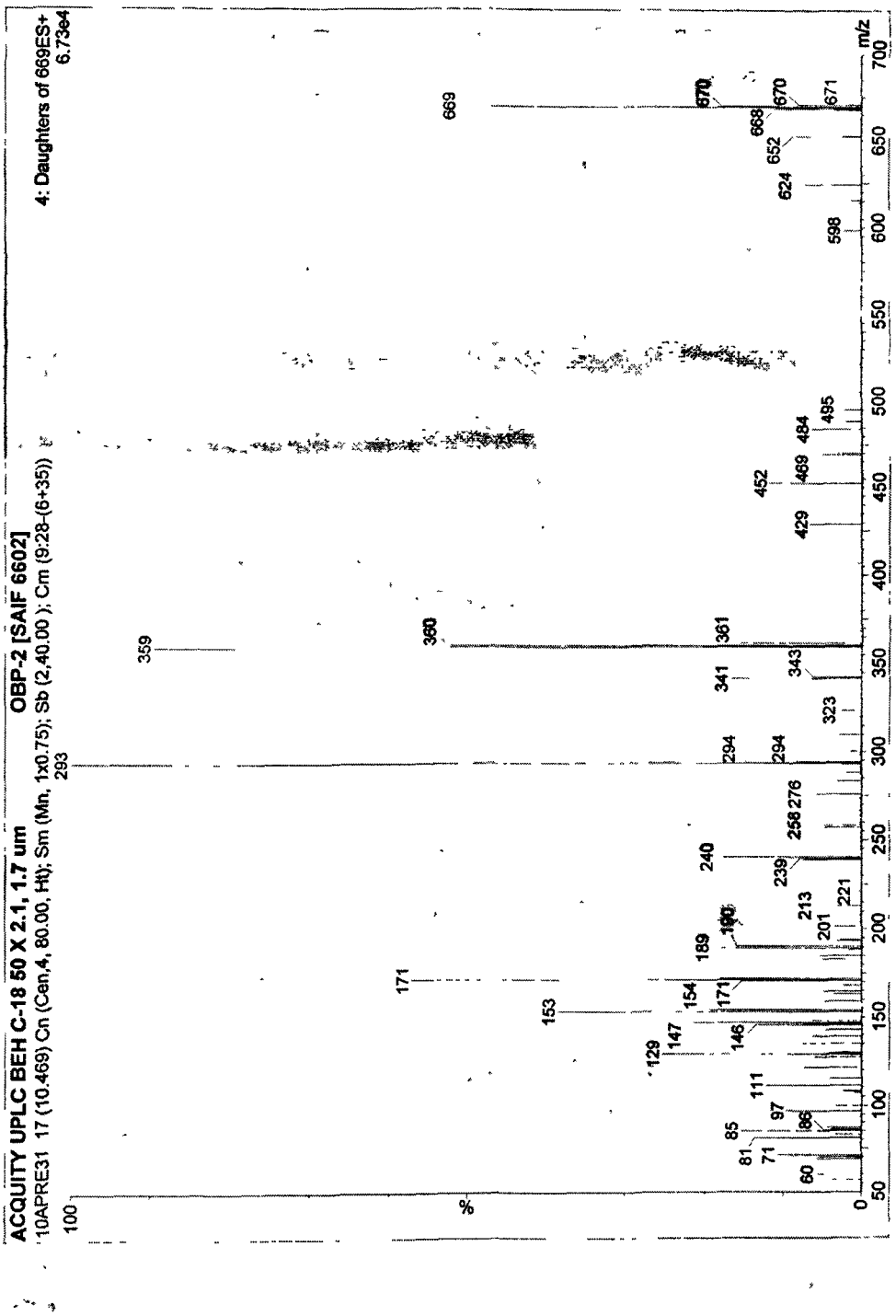

ACQUITY UPLC BEH C-18 60 X 2.1, 1.7 um
OBP-2 [SAIF 6602]
10APRE28 356 (7.732) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (343:378-(334+392))
1: Scan ES+
1.72e6
100
%
0
150 200 250 300 350 400 450 500 550 600 650 700 750 800 850 900 950 1000
m/z
177 204 222 224 225 226 247 284 285 286 311 352 357 358 406 438 452 494 496 498 500 501 515 552 567 716

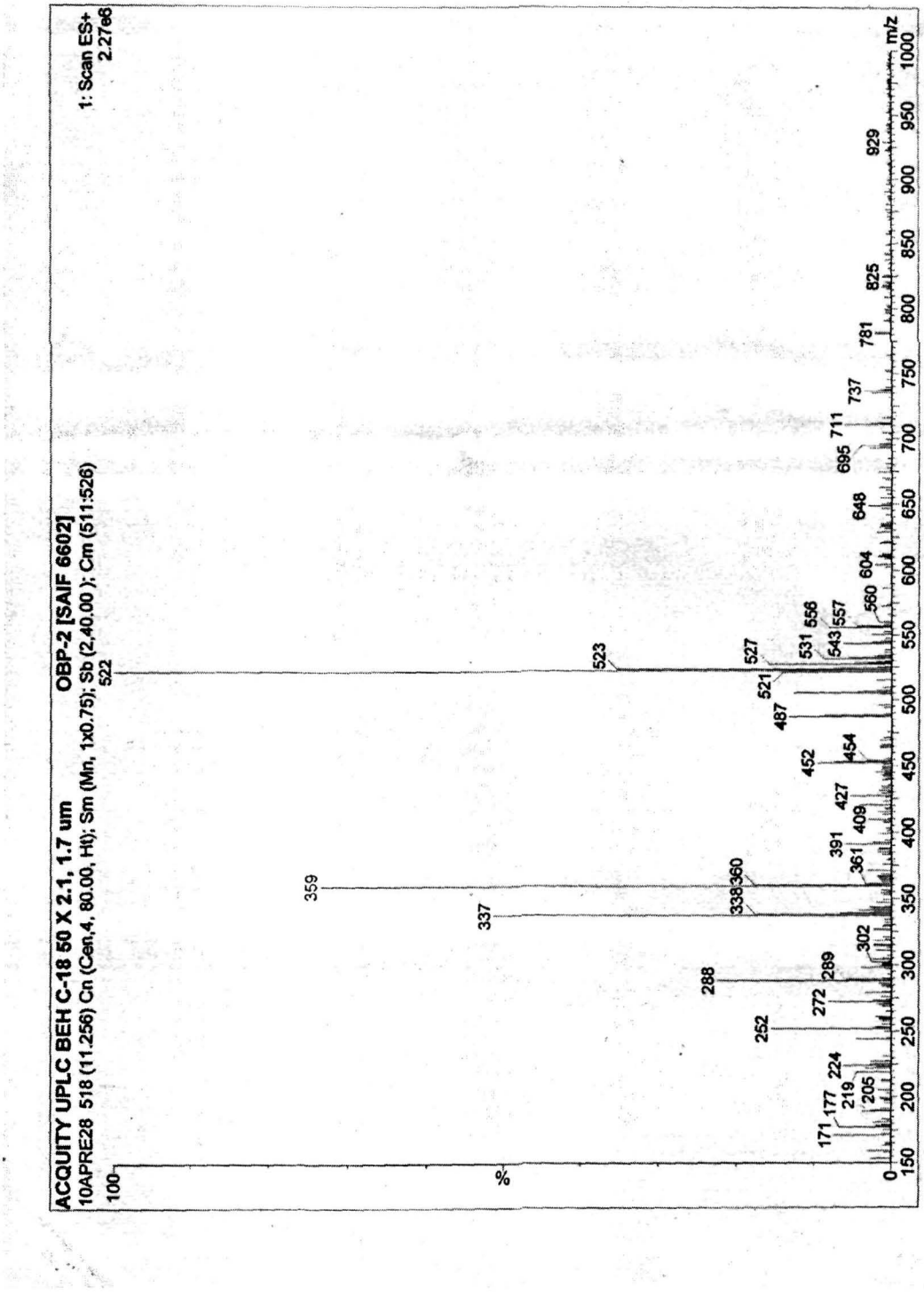

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um OBP-2 [SAIF 6602]
10APRE31 804 (7 390) Cn (Cen,4, 80 00, Ht), Sm (Mn, 1x0 75); Sb (2,40 00); Cm (779.848-(768+863))
1 Daughters of 498ES+ 2.57e5
498
100
%
0
60
50 75 100 125 150 175 200 225 250 275 300 325 350 375 394 420 425 450 475 500 525 550 m/z
498
497
497
454
455
453
276
276
179
180
178
177
146

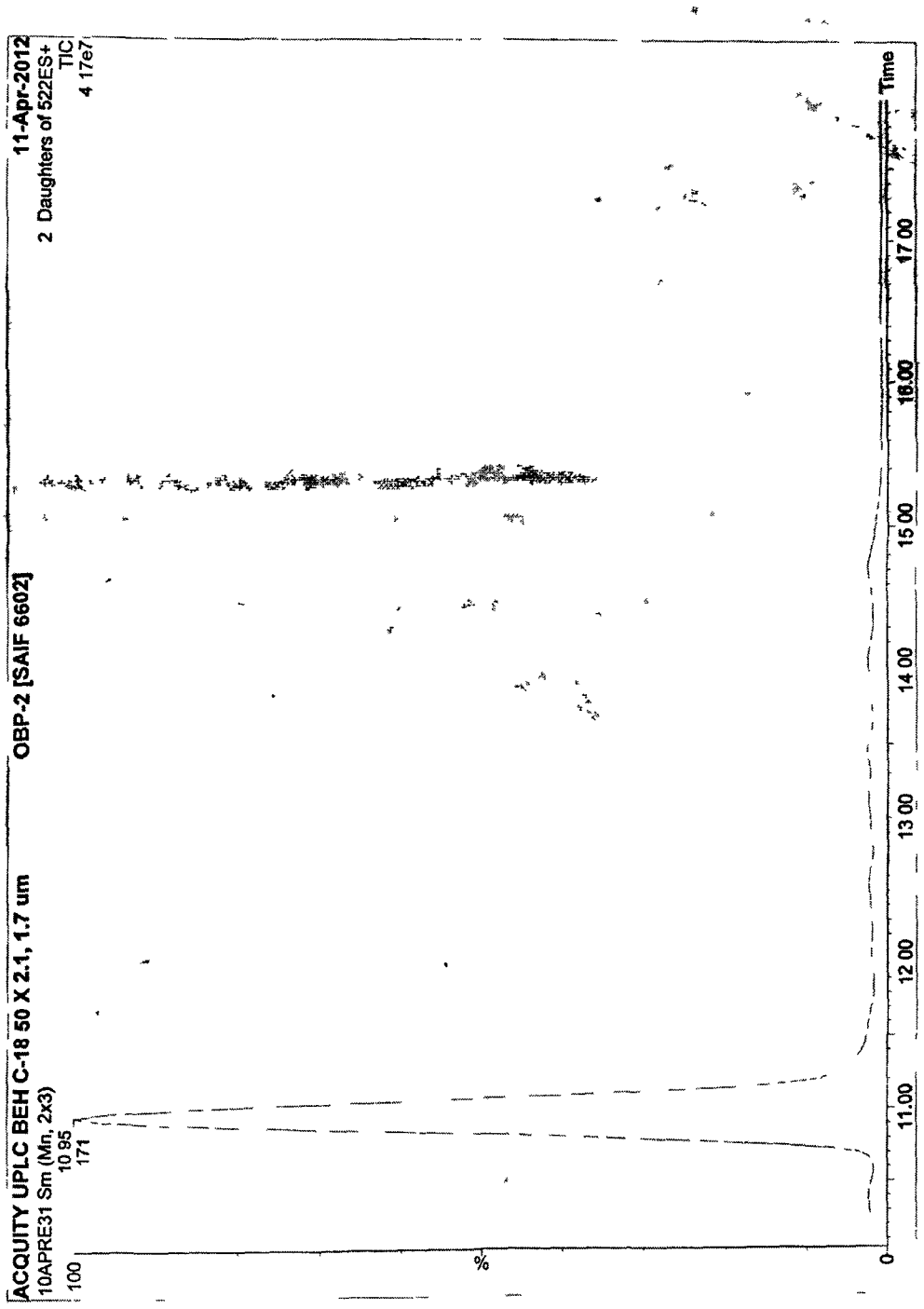

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE31 Sm (Mn, 2x3); Sm (Mn, 2x3); Sm (Mn, 2x3)
OBP-2 [SAIF 6602]
11-Apr-2012
Daughters of 498ES+
TIC
2.27e7
7.38
497
100
%
0
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
Time

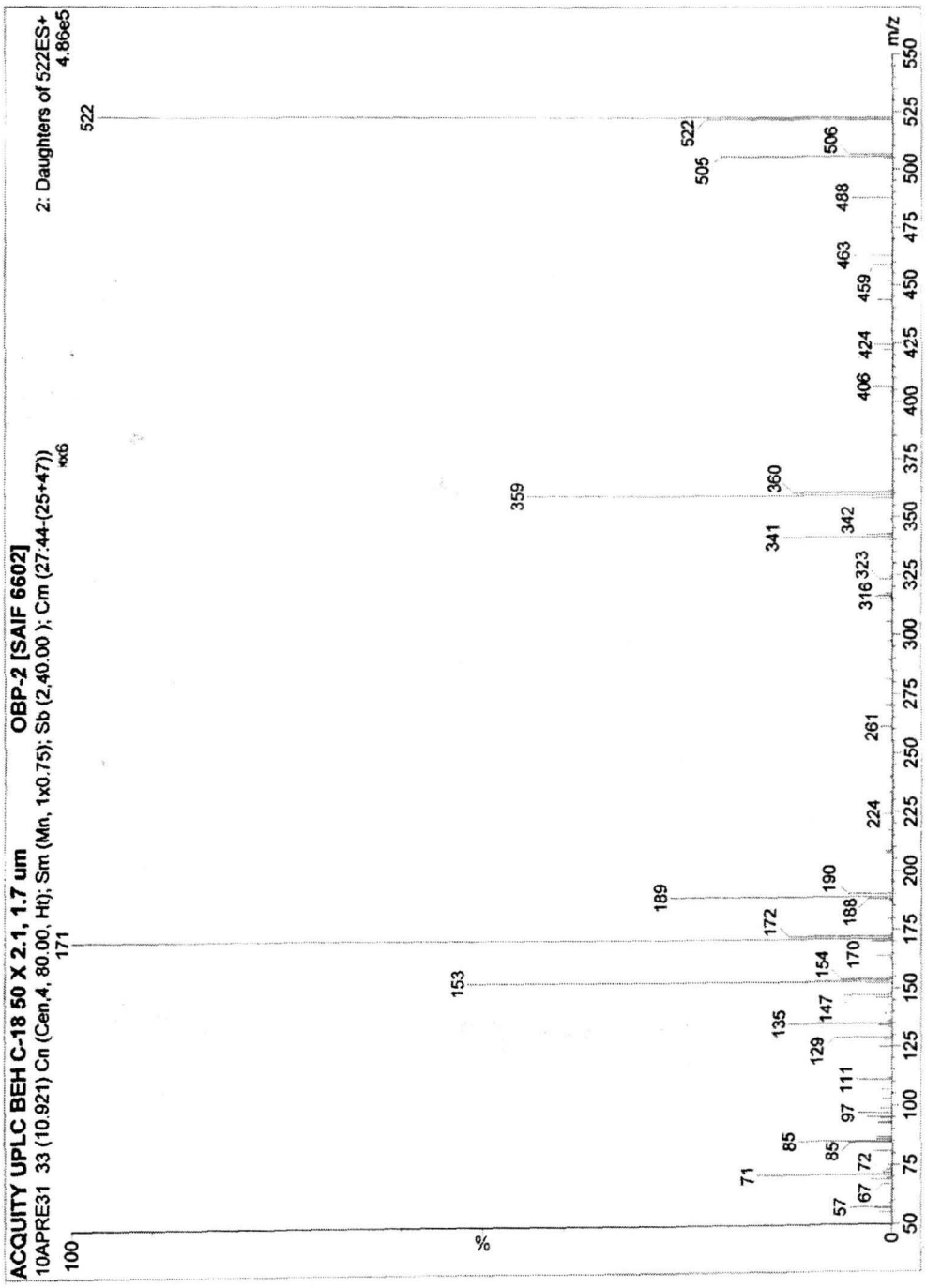

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-2 [SAIF 6602]
10APRE28 502 (10.908) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2.40,00); Cm (488:502-(487+503))
1: Scan ES+
9.84e5
100
%
0
m/z
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000
171
205
219
224
245
270
274
314
316
339
359
382
400
415
445
446
480
481
498
532
535
556
557
558
586
592
634
652
669
673
674
689
690
717
730
763
813
817
820
865
908
914
919
959
993

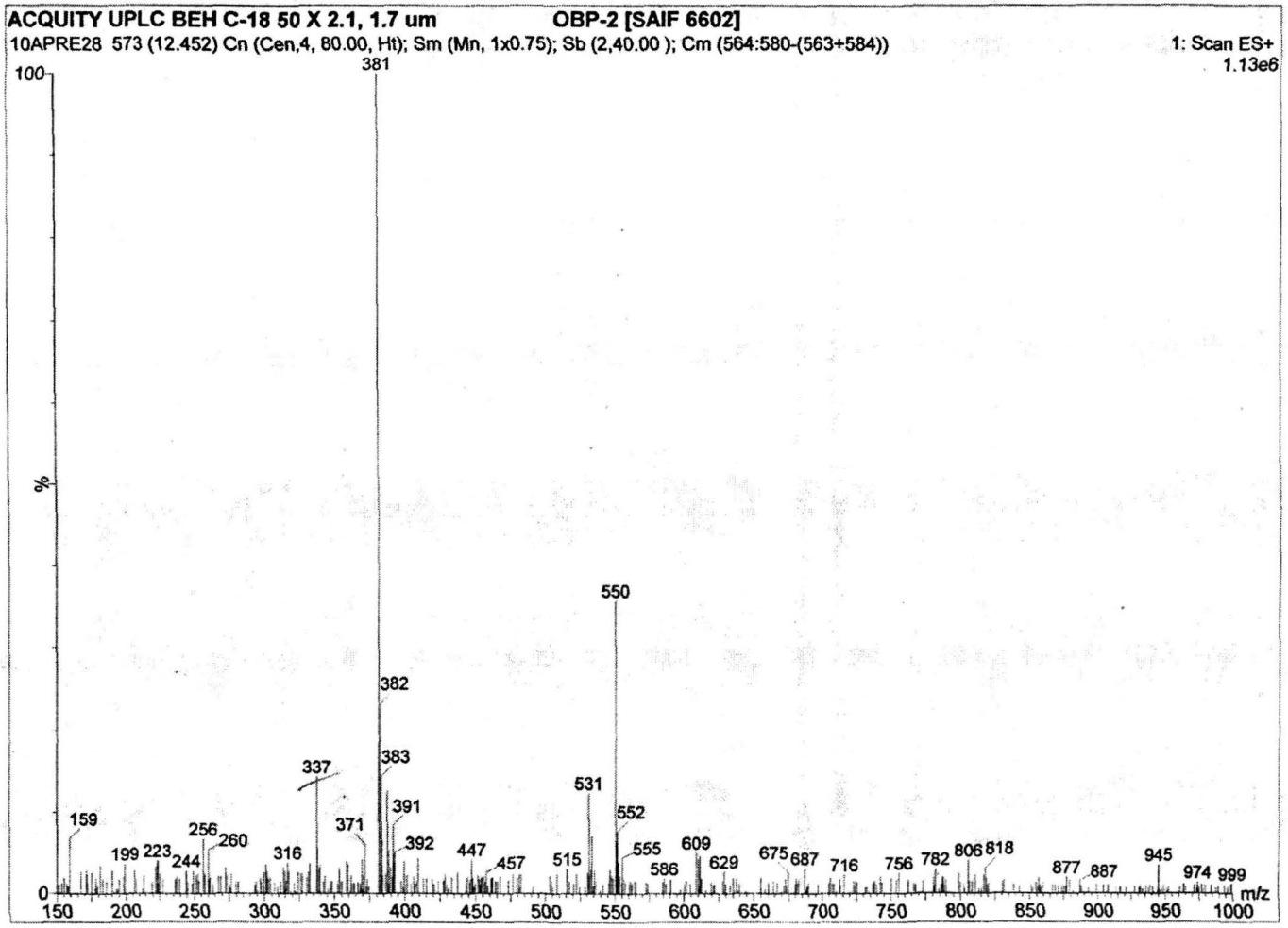

ACQUITY UPLC BEH C-18 60 X 2.1, 1.7 um
10APRE31 Sm (Mn, 2x3)
OBP-2 [SAIF 6602]
11-Apr-2012
4: Daughters of 669ES+
TIC
1.03e7
10.47
293
11.93
99
14.45
498
100
%
0
Time
11.00
12.00
13.00
14.00
15.00
16.00
17.00

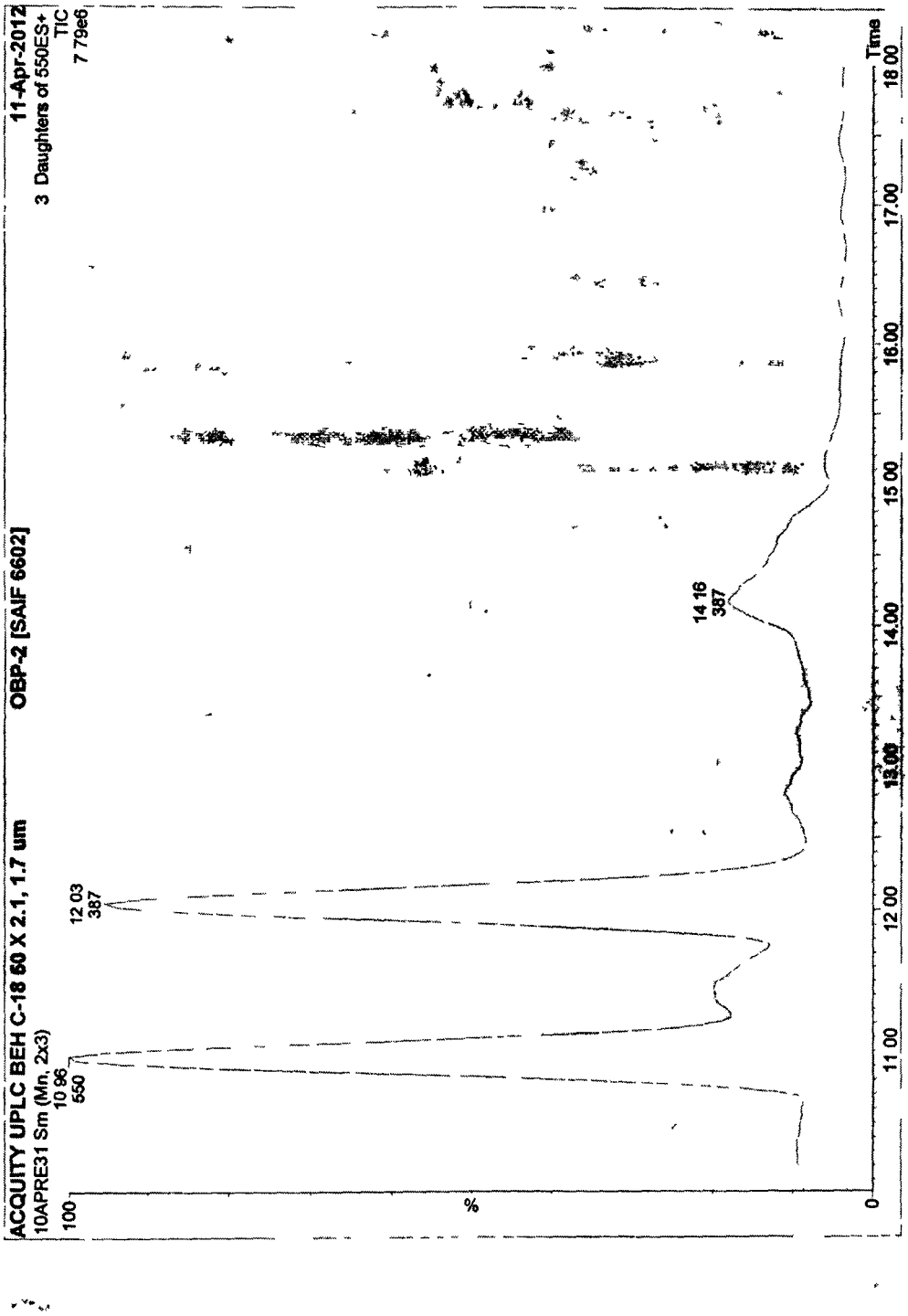

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE31 17 (10.469) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (9:28-(6+35))
OBP-2 [SAIF 6602]
4: Daughters of 669ES+
6.73e4
%
m/z

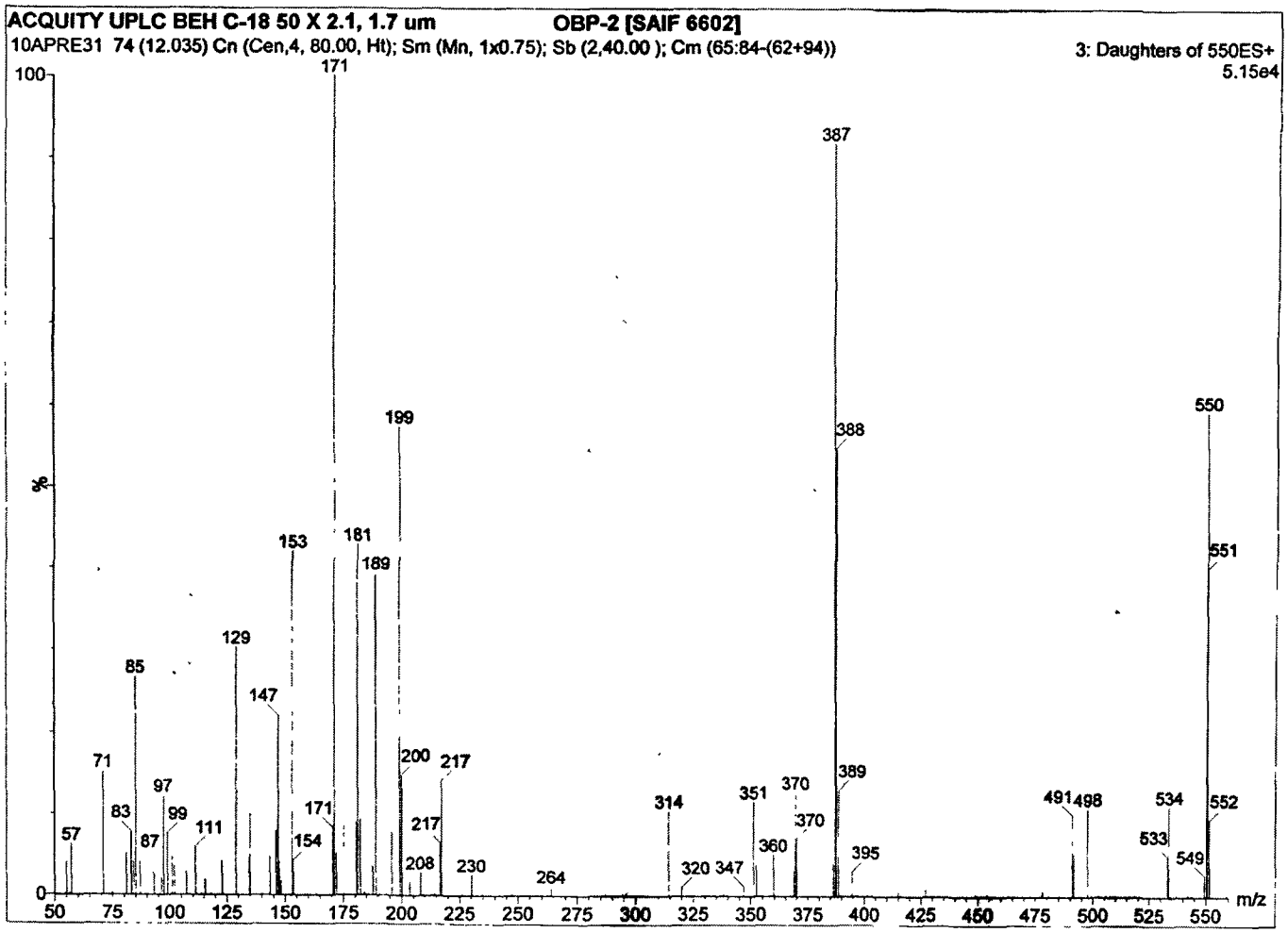

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE28 518 (11.256) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (511:526)
OBP-2 [SAIF 6602]
1: Scan ES+
2.27e6
%
m/z


ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
10APRE31 Sm (Mn, 2x3)
OBP-2 [SAIF 6602]
11-Apr-2012
2 Daughters of 522ES+
TIC
4 17e7
10.95
171
100
%
0
Time
11.00
12.00
13.00
14.00
15.00
16.00
17.00


ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-2 [SAIF 6602]
10APRE31 33 (10.921) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (27:44-(25+47))
2: Daughters of 522ES+
4.86e5
100
%
0
m/z
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550
57 67 72 85 85 97 111 129 135 147 153 154 170 171 172 188 189 190 224 261 316 323 341 342 359 360 406 424 459 463 488 505 506 522 522

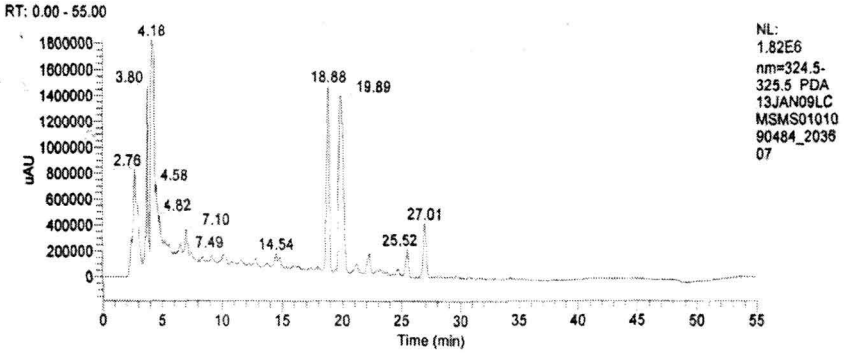

ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-2 [SAIF 6602]
10APRE28 573 (12.452) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (564:580-(563+584))
1: Scan ES+
1.13e6
100
%
0
381
550
382
383
337
531
391
552
159
371
256
260
555
609
199
223
244
316
392
447
457
515
586
629
675
687
716
756
782
806
818
877
887
945
974
999
m/z
150
200
250
300
350
400
450
500
550
600
650
700
750
800
850
900
950
1000

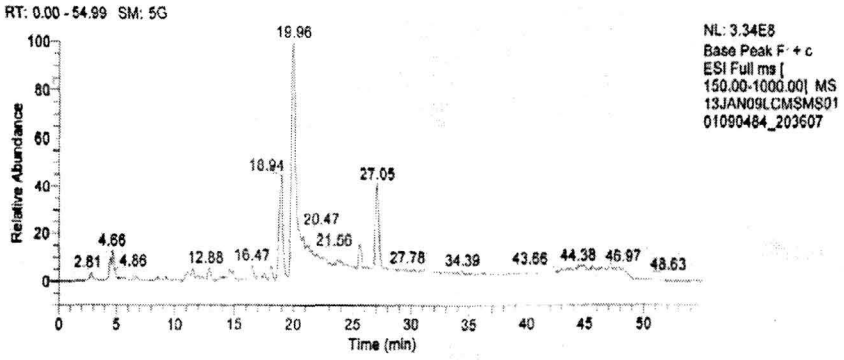

ACQUITY UPLC BEH C-18 60 X 2.1, 1.7 um
10APRE31 Sm (Mn, 2x3)
OBP-2 [SAIF 6602]
11-Apr-2012
3 Daughters of 550ES+
TIC
7 79e6
10 96
550
12 03
387
14 16
387
100
%
0
11 00
12 00
13 00
14 00
15 00
16 00
17 00
18 00
Time


ACQUITY UPLC BEH C-18 50 X 2.1, 1.7 um
OBP-2 [SAIF 6602]
10APRE31 74 (12.035) Cn (Cen,4, 80.00, Ht); Sm (Mn, 1x0.75); Sb (2,40.00); Cm (65:84-(62+94))
3: Daughters of 550ES+
5.15e4
100
%
0
m/z
50 75 100 125 150 175 200 225 250 275 300 325 350 375 400 425 450 475 500 525 550
171
387
199
550
388
153
181
189
551
129
85
147
71
200
217
97
389
370
351
314
491
498
534
552
83
99
111
171
217
370
57
87
154
360
533
549
208
230
264
320
347
395

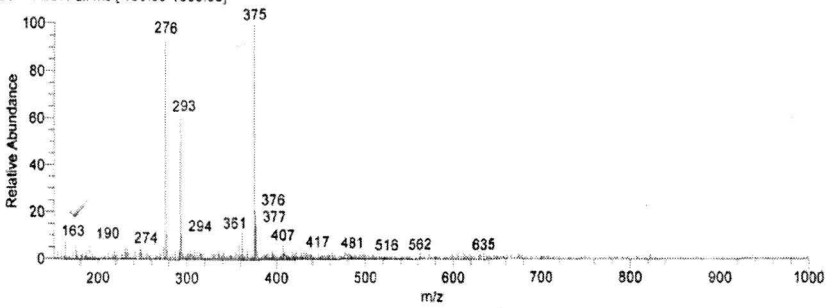
3. LC-MS spectra of Bacterial strain OBP3

```
13JAN09LCMSMS0101090484_203607
Type: Unknown ID: OBP-03 Row: 1
Sample Name:
Comments:                SAIF7023
:
:
DDPLS1:
:                        C:\DATA2013\SAIF7023\13JAN09LCMSMS01010904
                         84_203607.oad
DDRLS1:
Instrument Method:       C:\Xcalibur\methods\SAIF7023A.meth
Processing Method:
Vial:                    D:18
```

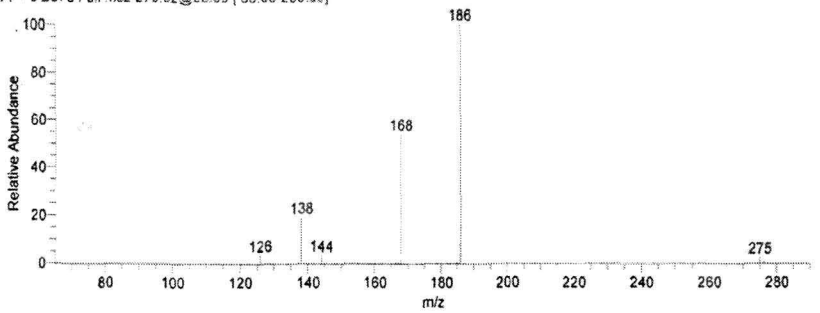




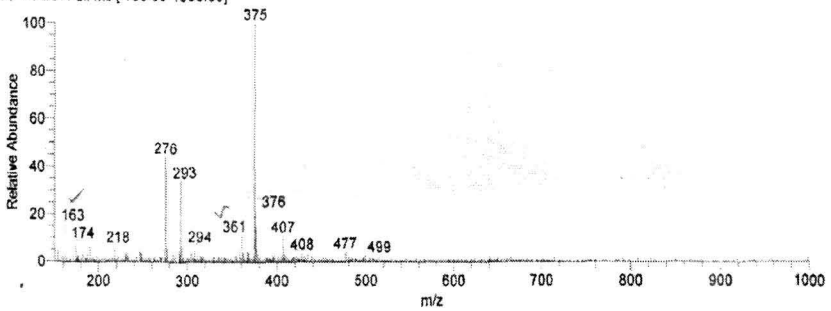
13JAN09LCMSMS0101090484_203607 #155-167 RT: 2.72-2.85 AV: 4 SB: 137 0.00-1.67 , 40.37-42.02 NL: 8.87E6
F: + c ESI Full ms [150.00-1000.00]



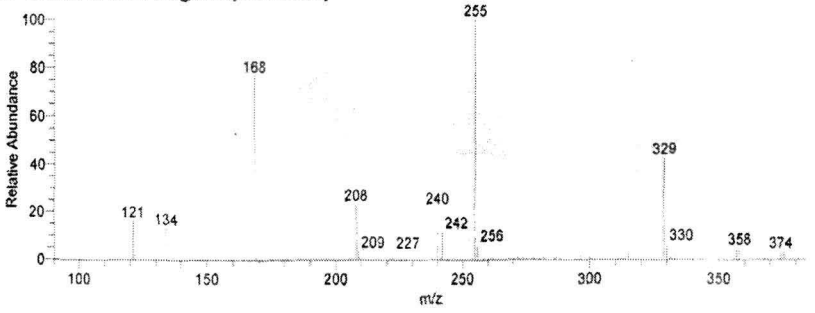
13JAN09LCMSMS0101090484_203607 #153-169 RT: 2.69-2.78 AV: 3 NL: 2.96E5
F: + c ESI d Full ms2 276.02@35.00 [85.00-290.00]



13JAN09LCMSMS0101090484_203607 #161-172 RT: 2.81-2.93 AV: 4 SB: 137 0.00-1.67 , 40.37-42.02 NL: 9.93E6
F: + c ESI Full ms [150.00-1000.00]

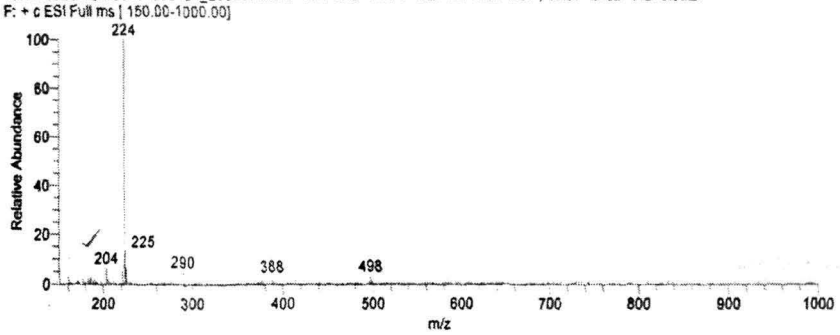


13JAN09LCMSMS0101090484_203607 #170-180 RT: 3.00-3.05 AV: 2 NL: 5.13E5
F: + c ESI d Full ms2 374.99@35.00 [90.00-385.00]

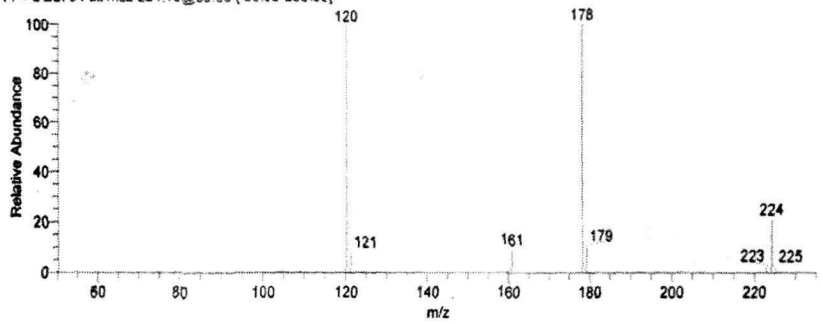


2

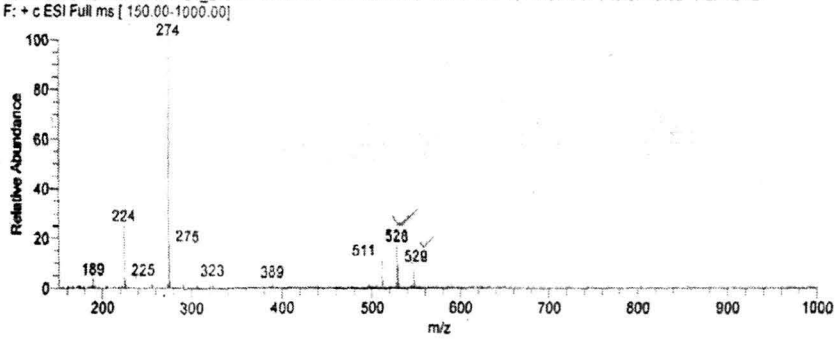
13JAN09LCMSMSD101090484_203607 #258 RT: 4.42 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 3.02E7
F: + c ESI Full ms [150.00-1000.00]



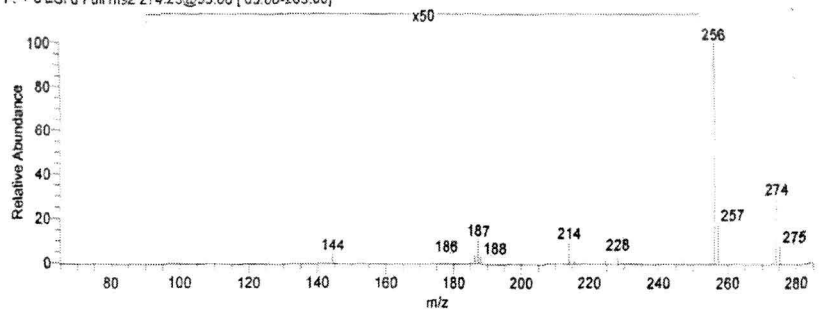
13JAN09LCMSMS0101090484_203607 #252-268 RT: 4.36-4.56 AV: 6 NL: 7.10E6
F: + c ESI d Full ms2 224.10@35.00 [50.00-235.00]



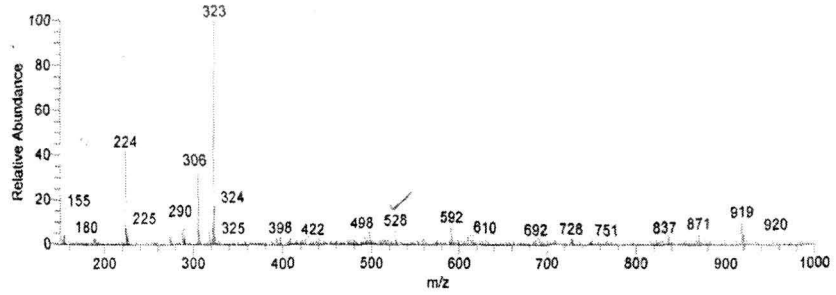
13JAN09LCMSMS0101090484_203607 #272-279 RT: 4.62-4.70 AV: 3 SB: 137 0.00-1.67 , 40.37-42.02 NL: 4.27E7
F: + c ESI Full ms [150.00-1000.00]



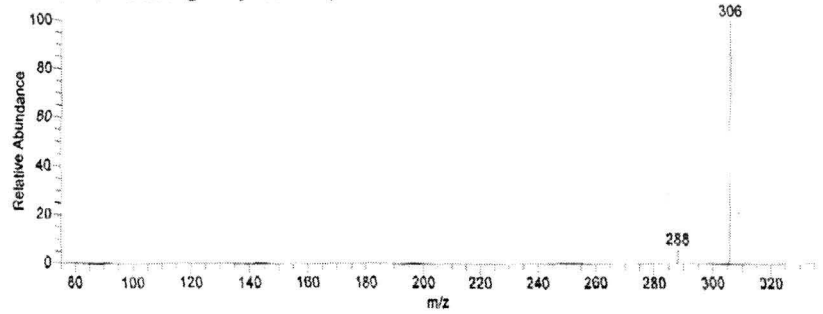
13JAN09LCMSMS0101090484_203607 #271-278 RT: 4.60-4.68 AV: 3 NL: 2.68E7
F: + c ESI d Full ms2 274.23@35.00 [65.00-285.00]



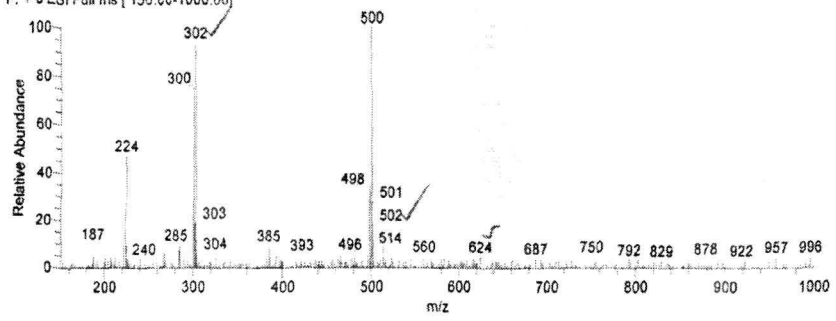
13JAN09LCMSMS0101090484_203607 #291 RT: 4.86 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.69E7
F: + c ESI Full ms [150.00-1000.00]



13JAN09LCMSMS0101090484_203607 #283-297 RT: 4.80-4.92 AV: 4 NL: 2.08E7
F: + c ESI d Full ms2 322.96@35.00 [75.00-335.00]

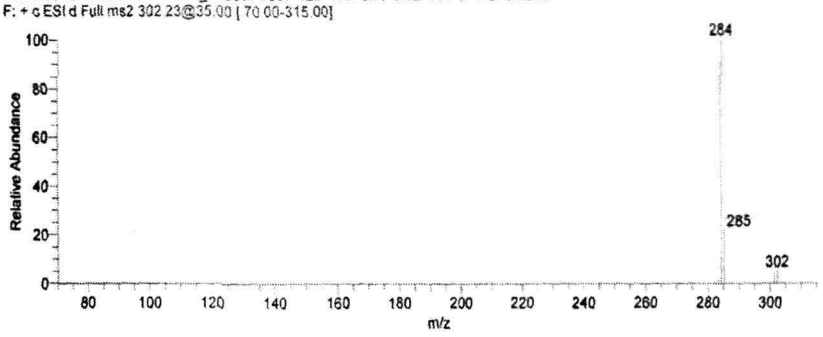


13JAN09LCMSMS0101090484_203607 #402 RT: 6.71 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 6.38E6
F: + c ESI Full ms [150.00-1000.00]

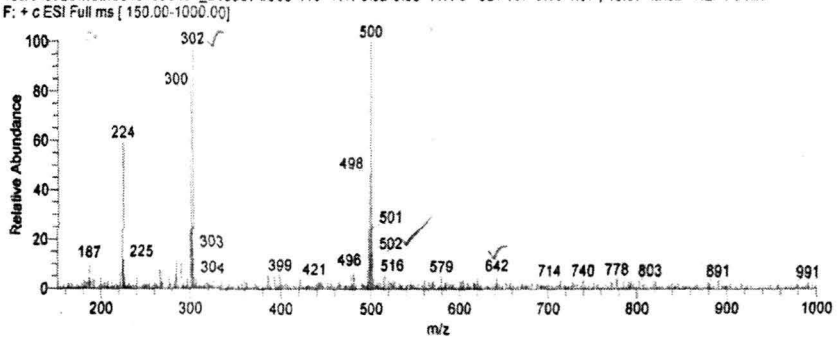


4

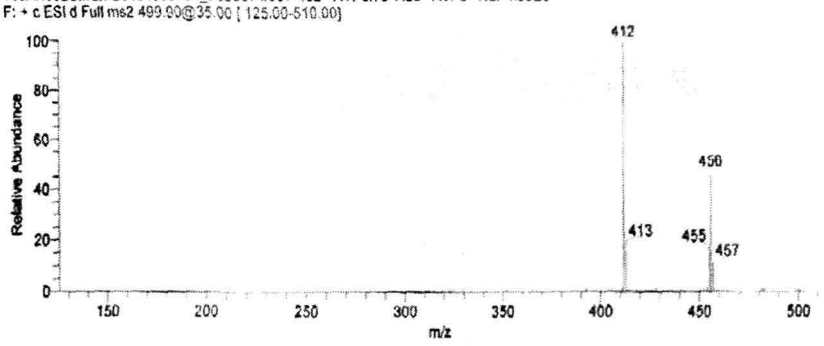
13JAN09LCMSMS0101090484_203607 #397-422 RT: 6.77-6.82 AV: 2 NL: 3.62E6
F: + c ESI d Full ms2 302.23@35.00 [70.00-315.00]



13JAN09LCMSMS0101090484_203607 #395-413 RT: 6.62-6.85 AV: 6 SB: 137 0.00-1.67 , 40.37-42.02 NL: 4.54E6
F: + c ESI Full ms [150.00-1000.00]

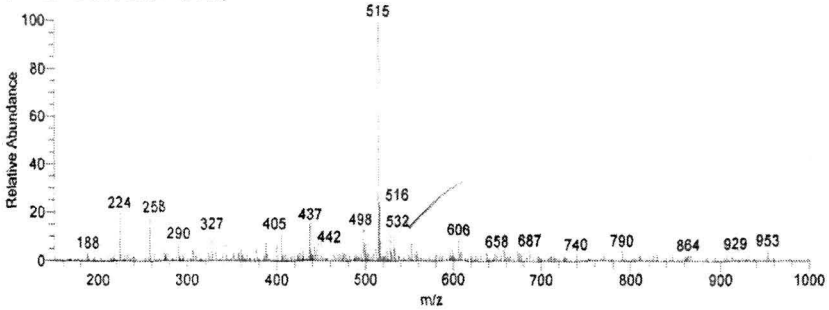


13JAN09LCMSMS0101090484_203607 #397-432 RT: 6.73-7.05 AV: 6 NL: 4.08E6
F: + c ESI d Full ms2 499.00@35.00 [125.00-510.00]

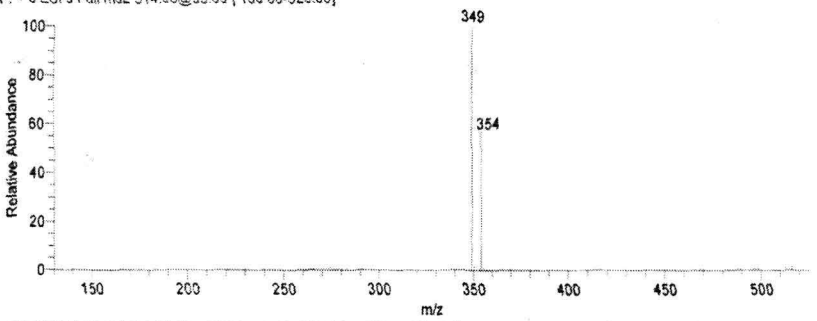


5

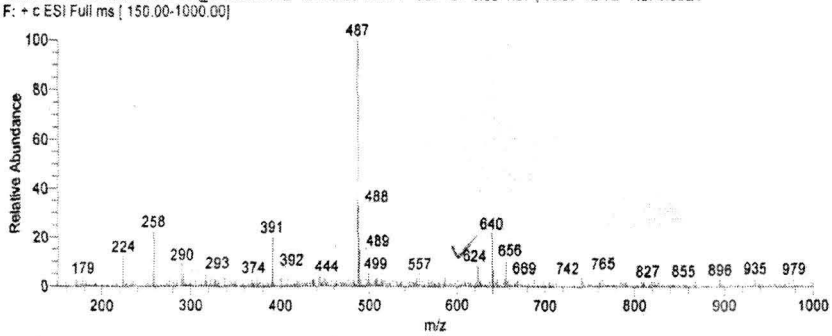
13JAN09LCMSMS0101090484_203607 #496 RT: 8.47 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 5.56E6
F: + c ESI Full ms [150.00-1000.00]



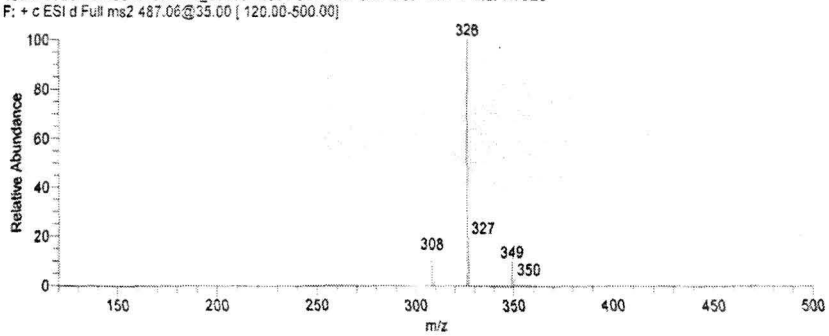
13JAN09LCMSMS0101090484_203607 #490-506 RT: 8.44-8.62 AV: 5 NL: 2.91E6
F: + c ESI d Full ms2 514.98@35.00 [130.00-525.00]



13JAN09LCMSMS0101090484_203607 #542 RT: 9.24 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 6.39E6
F: + c ESI Full ms [150.00-1000.00]

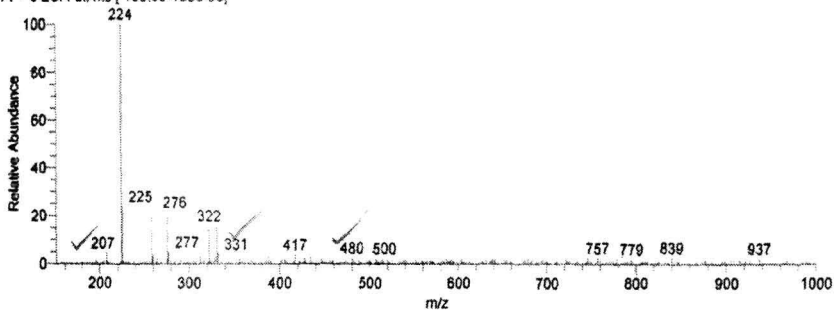


13JAN09LCMSMS0101090484_203607 #531-561 RT: 9.21-9.35 AV: 4 NL: 7.75E6
F: + c ESI d Full ms2 487.06@35.00 [120.00-500.00]

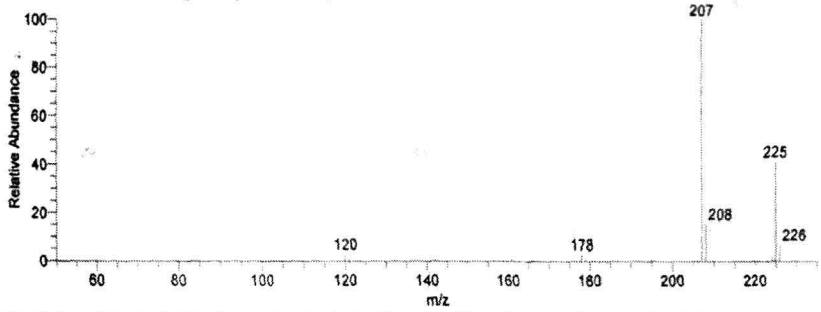


6

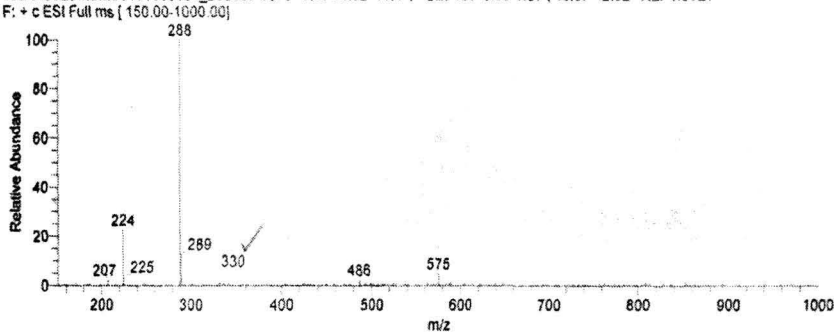
13JAN09LCMSMS0101090484_203607 #648 RT: 11.08 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.11E7
F: + c ESI Full ms [150.00-1000.00]



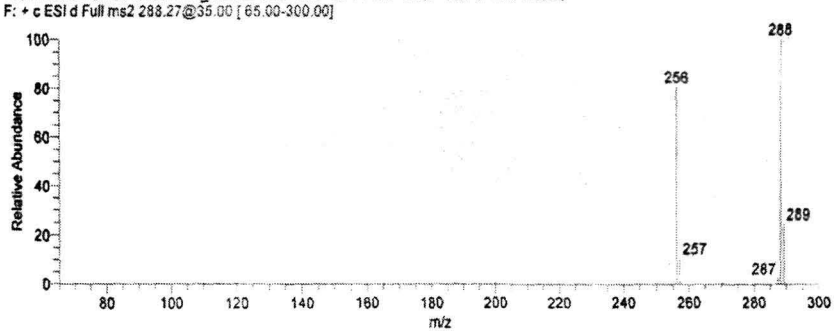
13JAN09LCMSMS0101090484_203607 #631-654 RT: 10.84-11.15 AV: 8 NL: 6.22E6
F: + c ESI d Full ms2 224.10@35.00 [50.00-235.00]



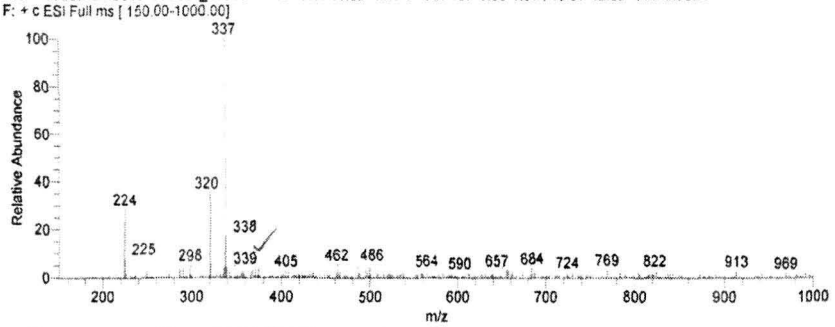
13JAN09LCMSMS0101090484_203607 #678 RT: 11.52 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.91E7
F: + c ESI Full ms [150.00-1000.00]



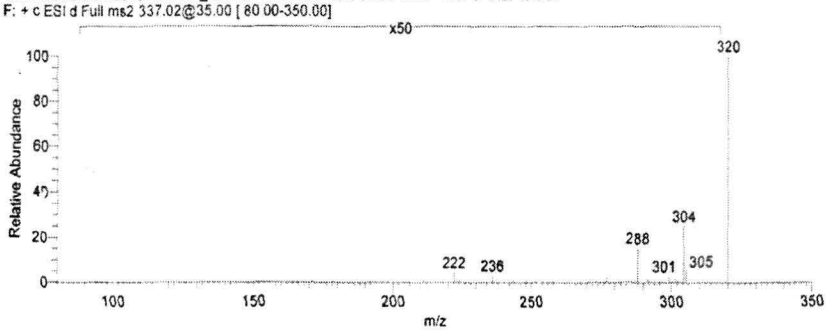
13JAN09LCMSMS0101090484_203607 #662-690 RT: 11.37-11.87 AV: 8 NL: 7.38E6
F: + c ESI d Full ms2 288.27@35.00 [65.00-300.00]



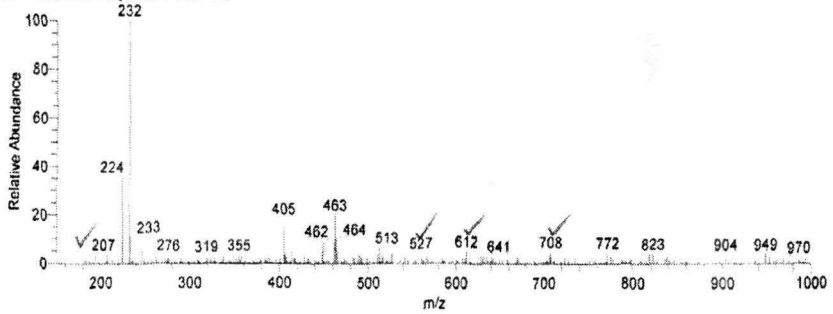
13JAN09LCMSMS0101090484_203607 #705 RT: 11.92 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 8.93E6
F: + c ESI Full ms [150.00-1000.00]



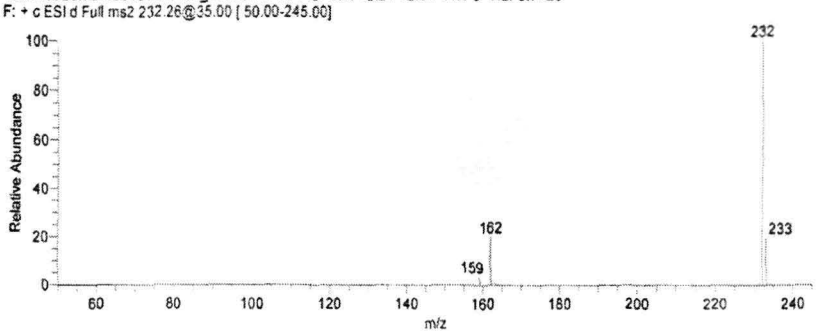
13JAN09LCMSMS0101090484_203607 #672-728 RT: 11.85-12.07 AV: 6 NL: 1.12E7
F: + c ESI d Full ms2 337.02@35.00 [80.00-350.00]



13JAN09LCMSMS0101090484_203607 #729 RT: 12.28 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 6.86E6
F: + c ESI Full ms [150.00-1000.00]

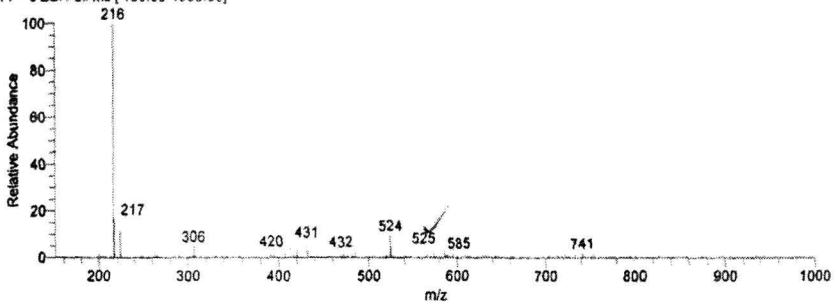


13JAN09LCMSMS0101090484_203607 #715-742 RT: 12.21-12.44 AV: 6 NL: 3.74E6
F: + c ESI d Full ms2 232.26@35.00 [50.00-245.00]

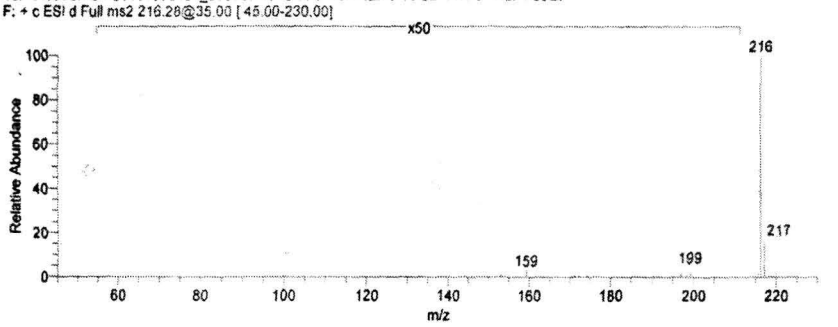


8

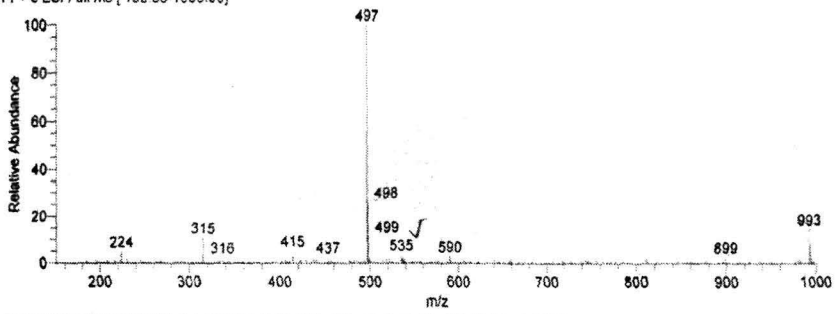
13JAN09LCMSMS0101090484_203607 #762 RT: 12.84 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.82E7
F: + c ESI Full ms [150.00-1000.00]



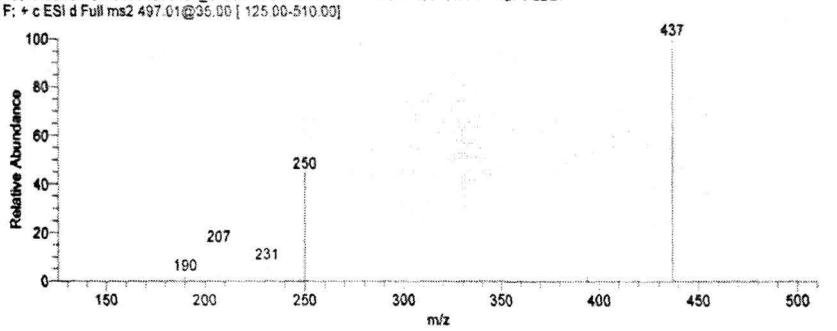
13JAN09LCMSMS0101090484_203607 #743-776 RT: 12.73-13.02 AV: 8 NL: 1.36E7
F: + c ESI d Full ms2 216.28@35.00 [45.00-230.00]



13JAN09LCMSMS0101090484_203607 #863 RT: 14.54 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.65E7
F: + c ESI Full ms [150.00-1000.00]

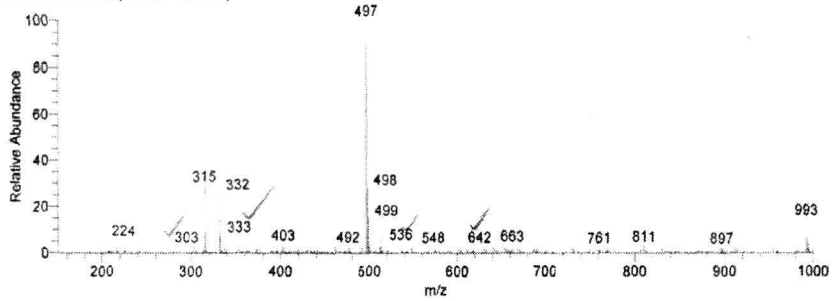


13JAN09LCMSMS0101090484_203607 #851-875 RT: 14.43-14.68 AV: 7 NL: 1.22E7
F: + c ESI d Full ms2 497.01@35.00 [125.00-510.00]

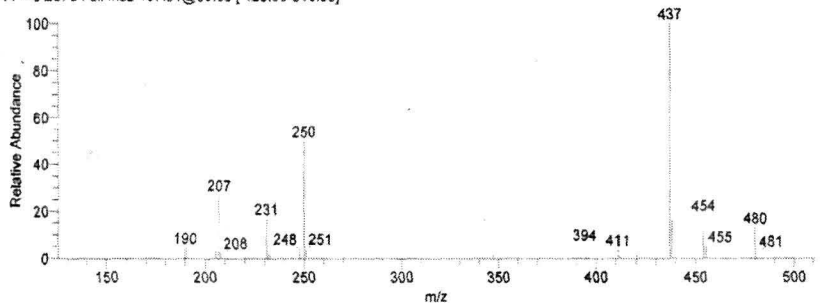


9

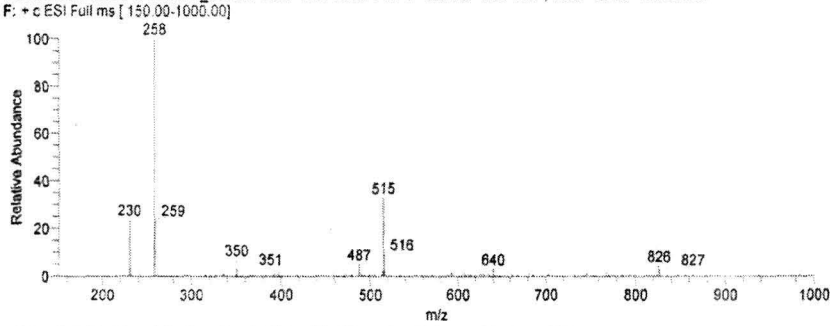
13JAN09LCMSMS0101090484_203607 #887 RT: 14.87 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.42E7
F: + c ESI Full ms [150.00-1000.00]



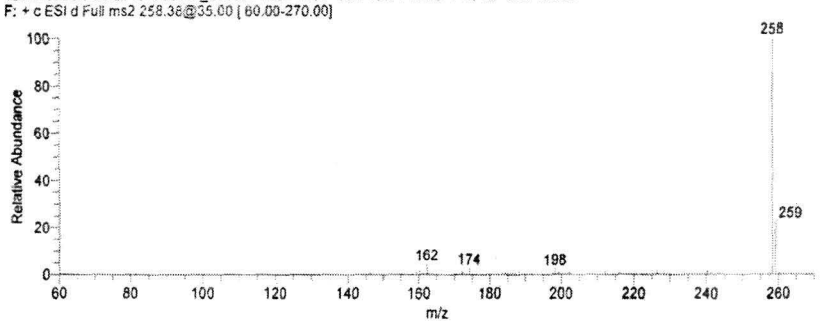
13JAN09LCMSMS0101090484_203607 #878-893 RT: 14.77-14.93 AV: 5 NL: 8.89E6
F: + c ESI d Full ms2 497.01@35.00 [125.00-510.00]

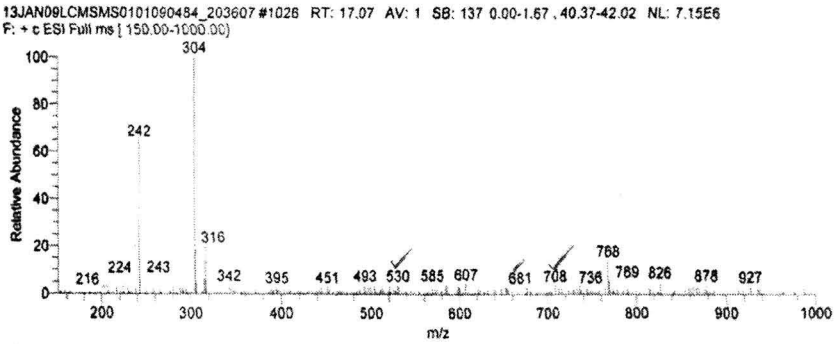


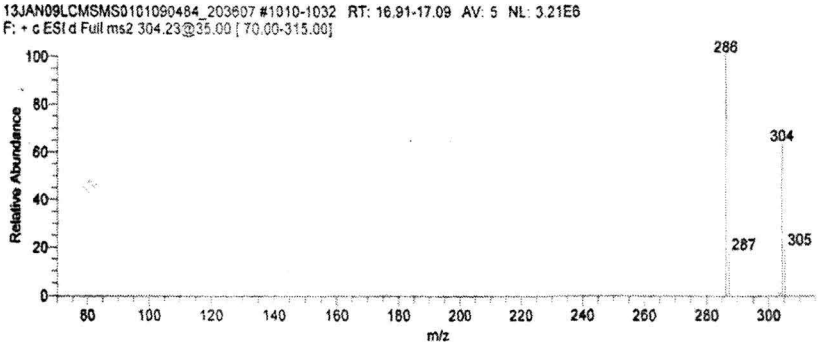
13JAN09LCMSMS0101090484_203607 #984 RT: 16.43 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 2.07E7
F: + c ESI Full ms [150.00-1000.00]

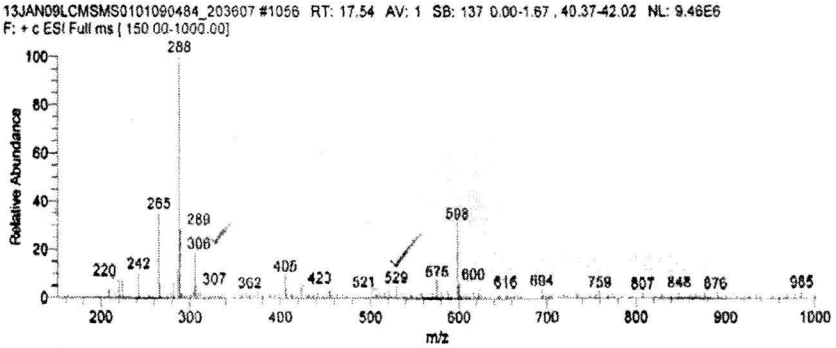


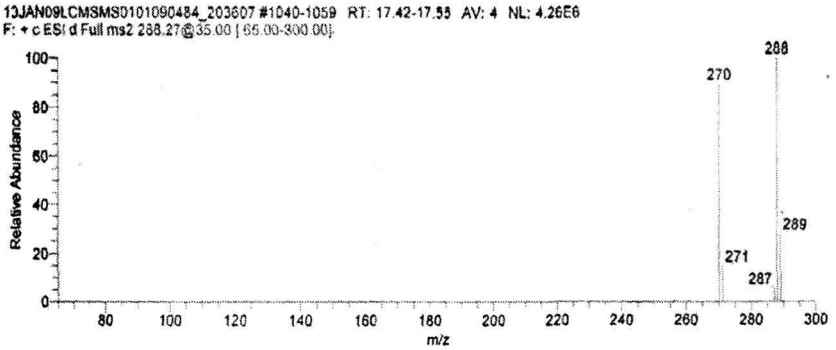
13JAN09LCMSMS0101090484_203607 #962-1003 RT: 16.24-16.70 AV: 12 NL: 1.19E7
F: + c ESI d Full ms2 258.36@35.00 [60.00-270.00]




13JAN09LCMSMS0101090484_203607 #1026 RT: 17.07 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 7.15E6
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
304
242
316
216
224
243
342
395
451
493
530
585
607
681
708
736
768
789
826
878
927

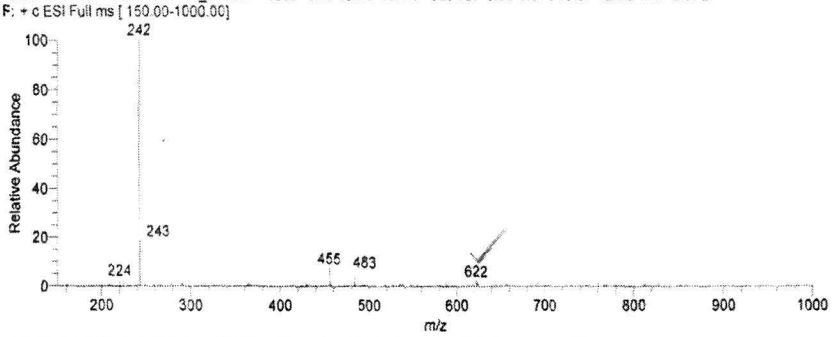

13JAN09LCMSMS0101090484_203607 #1010-1032 RT: 16.91-17.09 AV: 5 NL: 3.21E6
F: + c ESI d Full ms2 304.23@35.00 [70.00-315.00]
Relative Abundance
m/z
286
304
287
305


13JAN09LCMSMS0101090484_203607 #1056 RT: 17.54 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 9.46E6
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
288
265
289
306
598
220
242
307
362
405
423
521
529
575
600
616
694
759
807
848
876
965

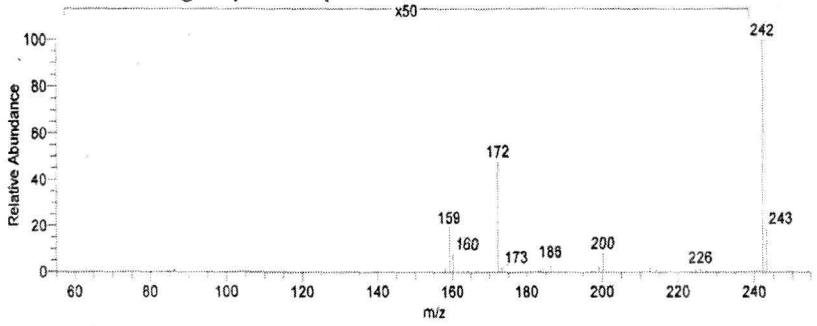

13JAN09LCMSMS0101090484_203607 #1040-1059 RT: 17.42-17.55 AV: 4 NL: 4.26E6
F: + c ESI d Full ms2 288.27@35.00 [65.00-300.00]
Relative Abundance
m/z
288
270
289
271
287

11

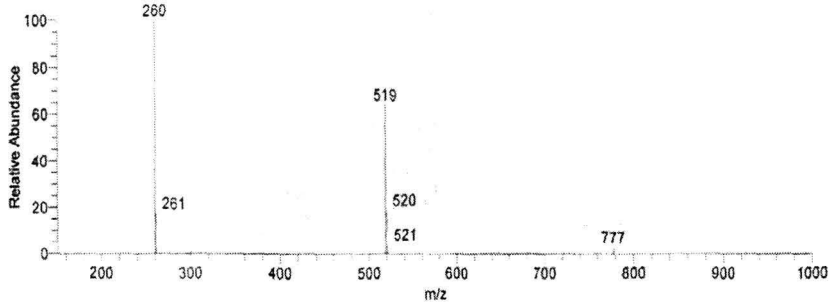
13JAN09LCMSMS0101090484_203607 #1092 RT: 18.11 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 2.03E7
F: + c ESI Full ms [150.00-1000.00]



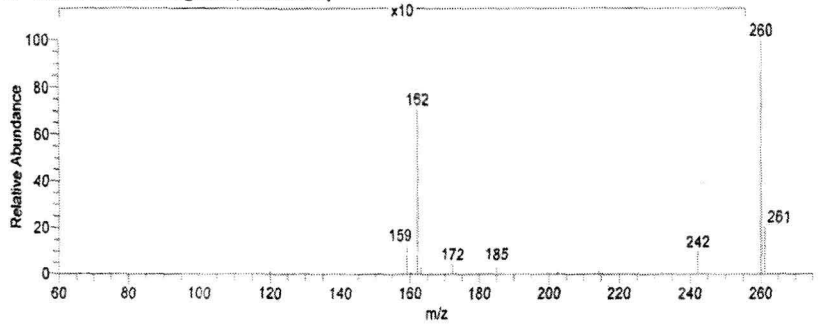
13JAN09LCMSMS0101090484_203607 #1075-1105 RT: 17.86-18.29 AV: 11 NL: 1.35E7
F: + c ESI d Full ms2 242.26@35.00 [55.00-255.00]



13JAN09LCMSMS0101090484_203607 #1121-1170 RT: 18.57-19.16 AV: 17 SB: 137 0.00-1.67 , 40.37-42.02 NL: 8.28E7
F: + c ESI Full ms [150.00-1000.00]

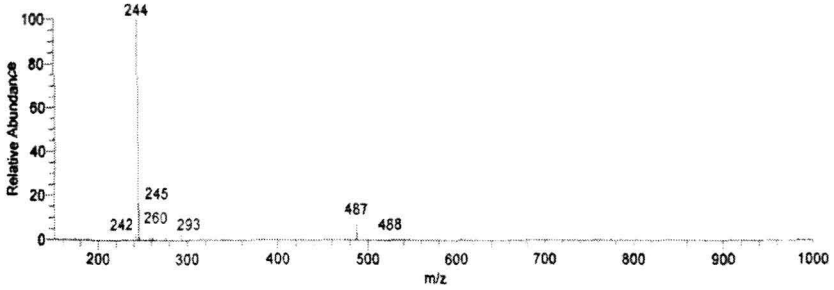


13JAN09LCMSMS0101090484_203607 #1113-1173 RT: 18.54-19.22 AV: 19 NL: 7.84E7
F: + c ESI d Full ms2 260.26@35.00 [60.00-275.00]

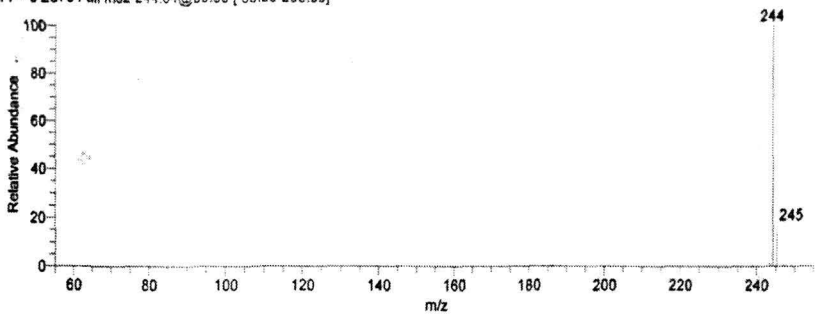


12

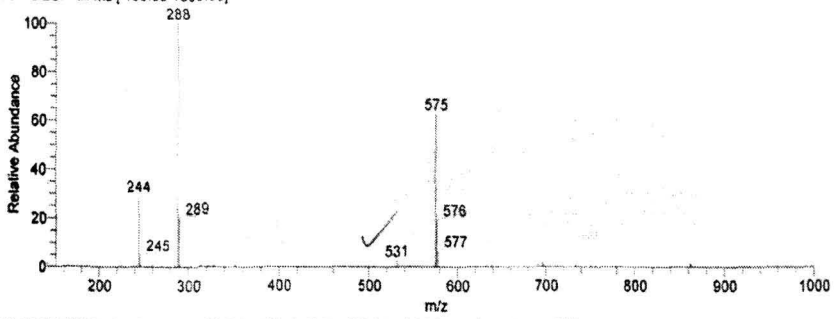
13JAN09LCMSMS0101090484_203607 #1247 RT: 20.17 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.41E8
F: + c ESI Full ms [150.00-1000.00]



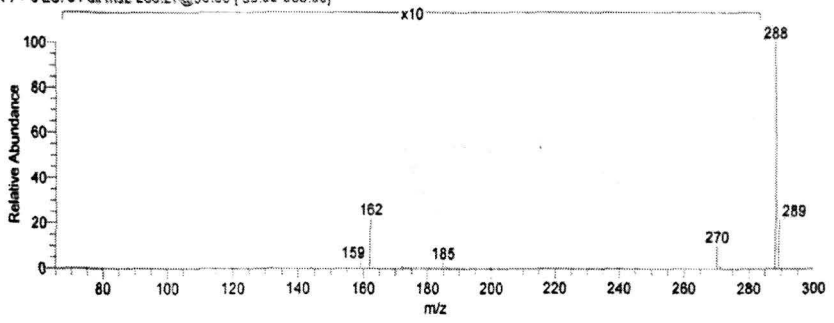
13JAN09LCMSMS0101090484_203607 #1196-1263 RT: 19.65-20.37 AV: 21 SB: 39 0.00-1.67 , 40.37-42.02 NL: 1.99E8
F: + c ESI d Full ms2 244.31@35.00 [55.00-255.00]

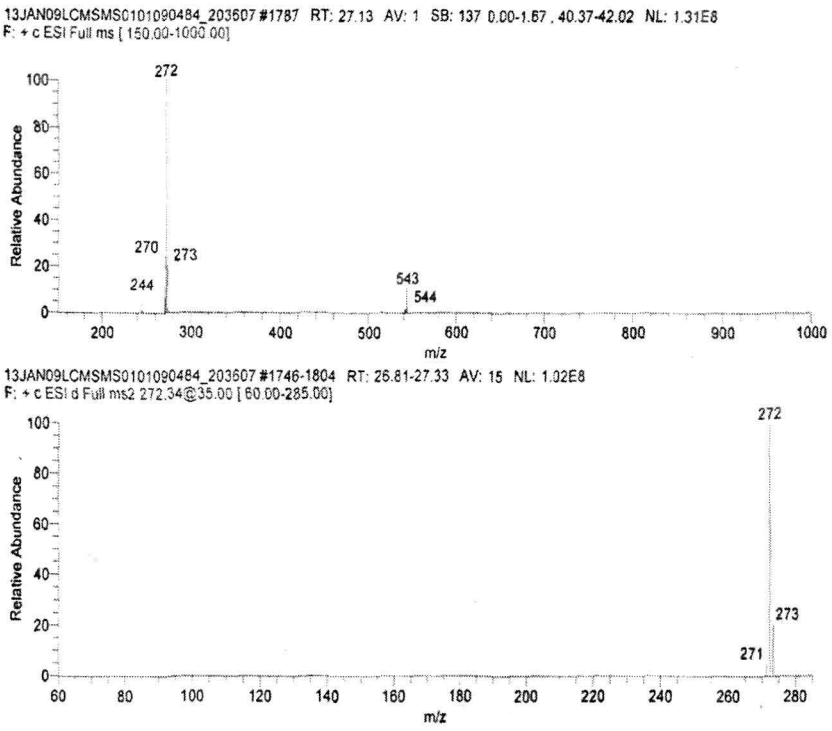


13JAN09LCMSMS0101090484_203607 #1661 RT: 25.50 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 4.47E7
F: + c ESI Full ms [150.00-1000.00]



13JAN09LCMSMS0101090484_203607 #1643-1692 RT: 25.40-25.81 AV: 12 NL: 4.59E7
F: + c ESI d Full ms2 288.27@35.00 [55.00-300.00]




13JAN09LCMSMS0101090484_203607 #1787 RT: 27.13 AV: 1 SB: 137 0.00-1.67 , 40.37-42.02 NL: 1.31E8
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
272
270
273
244
543
544
13JAN09LCMSMS0101090484_203607 #1746-1804 RT: 26.81-27.33 AV: 15 NL: 1.02E8
F: + c ESI d Full ms2 272.34@35.00 [60.00-285.00]
Relative Abundance
m/z
272
273
271

4. LC-MS spectra of Bacterial strain OBP4

```
13JAN09LCMSMS0301090486_203702
Type: Unknown ID: OBP-04 Row: 1
Sample Name:
Comments:                SAIF7023
:
:
DDPLS1:
:                        C:\DATA2013\SAIF7023\13JAN09LCMSMS03010904
                         86_203702.oad
DDRLS1:
Instrument Method:       C:\Xcalibur\methods\SAIF7023A.meth
Processing Method:
Vial:                    D:19
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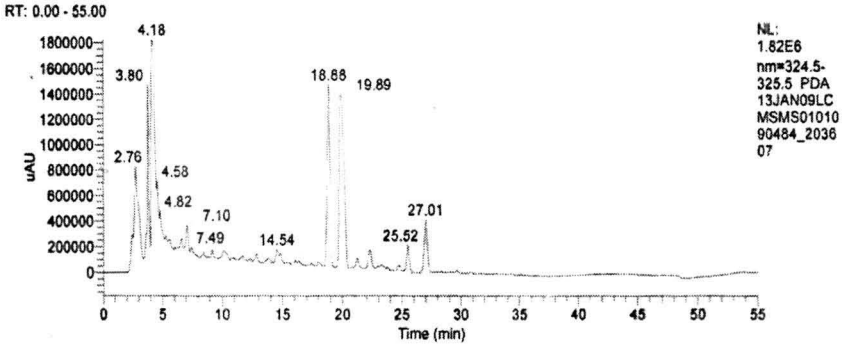
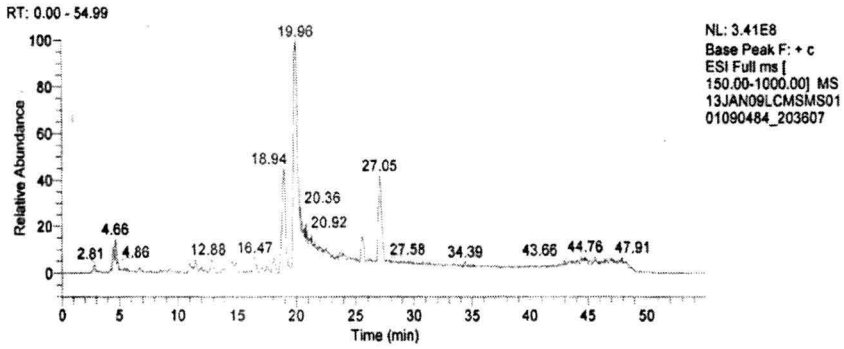
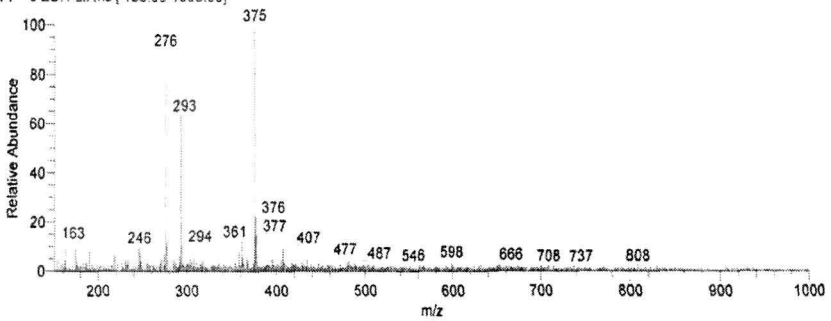
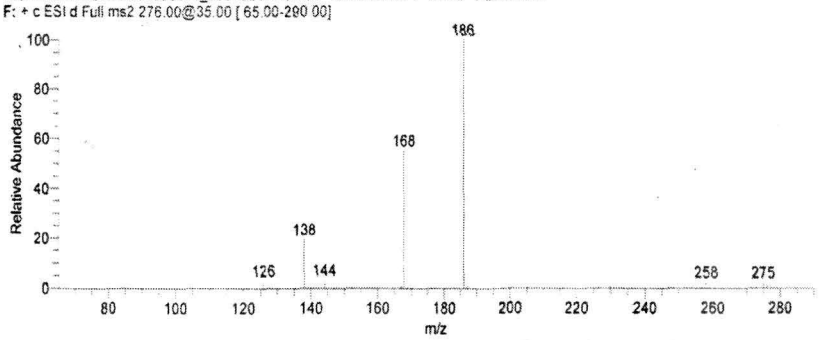




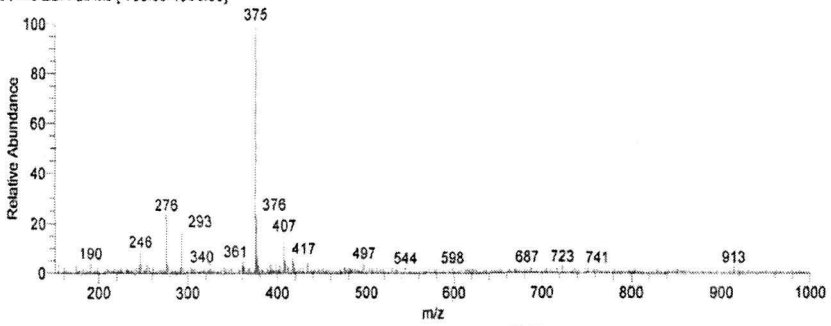
13JAN09LCMSMS0101090484_203607 #152-177 RT: 2.67-2.98 AV: 8 SB: 331 0.00-1.75 , 49.83-54.31 NL: 6.48E6
F: + c ESI Full ms [150.00-1000.00]



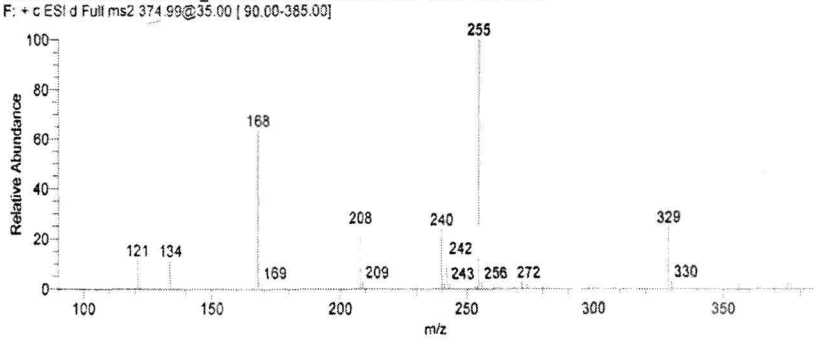
13JAN09LCMSMS0301090486_203702 #126-192 RT: 2.66-2.71 AV: 2 NL: 3.35E6
F: + c ESI d Full ms2 276.00@35.00 [65.00-290.00]



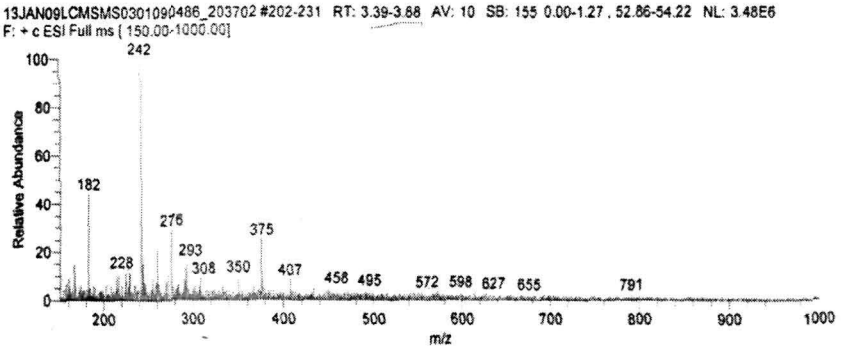
13JAN09LCMSMS0301090486_203702 #168 RT: 2.81 AV: 1 SB: 155 0.00-1.27 , 52.86-54.22 NL: 2.08E7
F: + c ESI Full ms [150.00-1000.00]

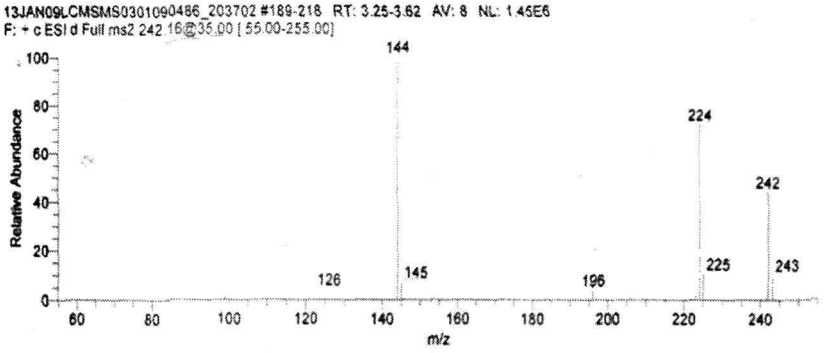


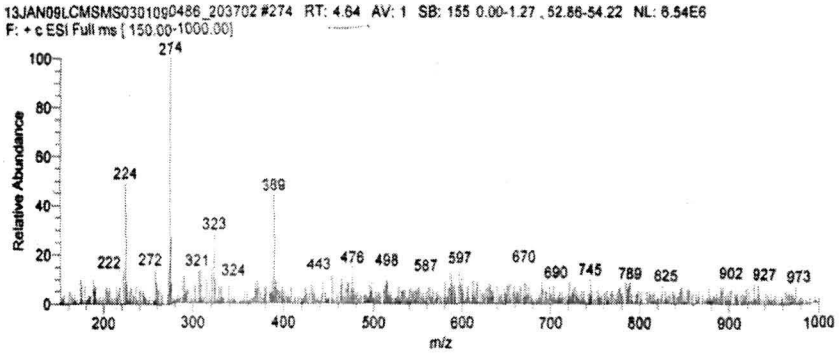
13JAN09LCMSMS0301090486_203702 #140-206 RT: 2.91-3.00 AV: 2 NL: 1.72E6
F: + c ESI d Full ms2 374.99@35.00 [90.00-385.00]

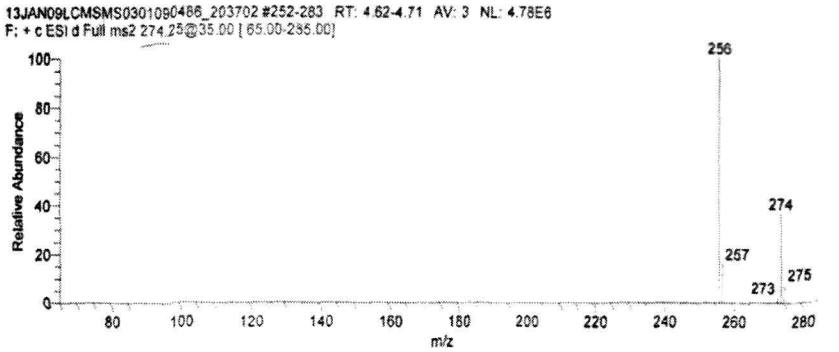


2


13JAN09LCMSMS0301090486_203702 #202-231 RT: 3.39-3.88 AV: 10 SB: 155 0.00-1.27 , 52.86-54.22 NL: 3.48E6
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
242
182
276
375
293
228
308
350
407
458
495
572
598
627
655
791

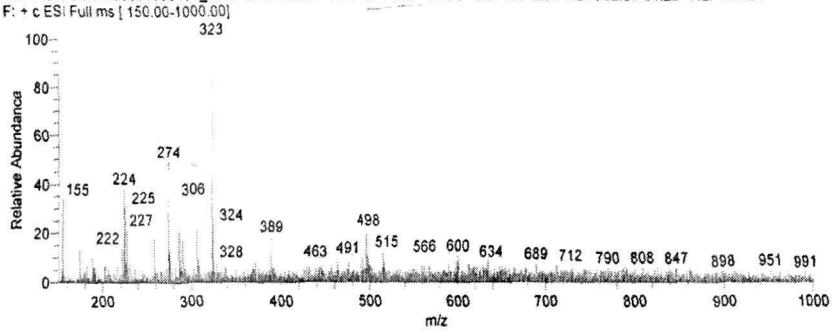

13JAN09LCMSMS0301090486_203702 #189-218 RT: 3.25-3.62 AV: 8 NL: 1.45E6
F: + c ESI d Full ms2 242.16@35.00 [55.00-255.00]
Relative Abundance
m/z
144
224
242
126
145
196
225
243


13JAN09LCMSMS0301090486_203702 #274 RT: 4.64 AV: 1 SB: 155 0.00-1.27 , 52.86-54.22 NL: 6.54E6
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
274
224
389
323
222
272
321
324
443
476
498
587
597
670
690
745
789
825
902
927
973

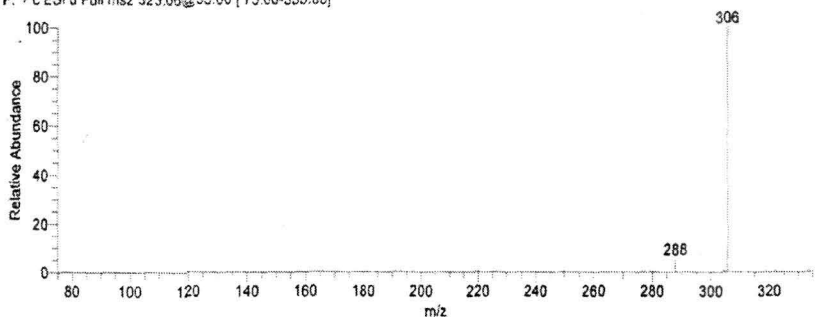

13JAN09LCMSMS0301090486_203702 #252-283 RT: 4.62-4.71 AV: 3 NL: 4.78E6
F: + c ESI d Full ms2 274.25@35.00 [65.00-285.00]
Relative Abundance
m/z
256
274
257
273
275

3

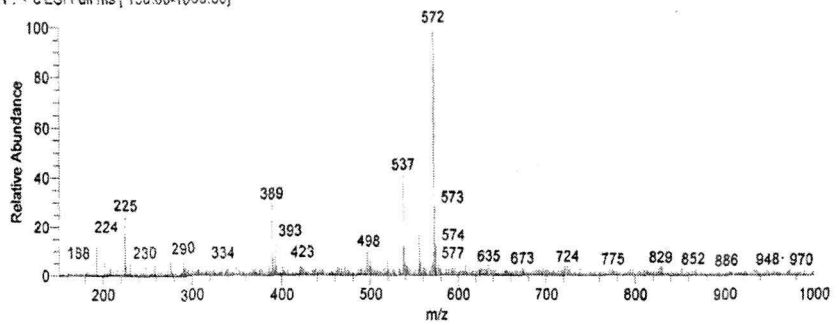
13JAN09LCMSMS0301090486_203702 #274-298 RT: 4.64-5.04 AV: 9 SB: 155 0.00-1.27 , 52.86-54.22 NL: 4.52E6
F: + c ESI Full ms [150.00-1000.00]



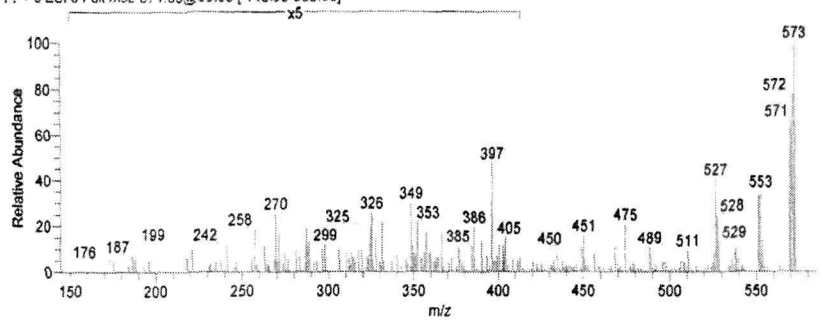
13JAN09LCMSMS0301090486_203702 #122-402 RT: 4.75-4.89 AV: 4 NL: 8.07E6
F: + c ESI d Full ms2 323.06@35.00 [75.00-335.00]



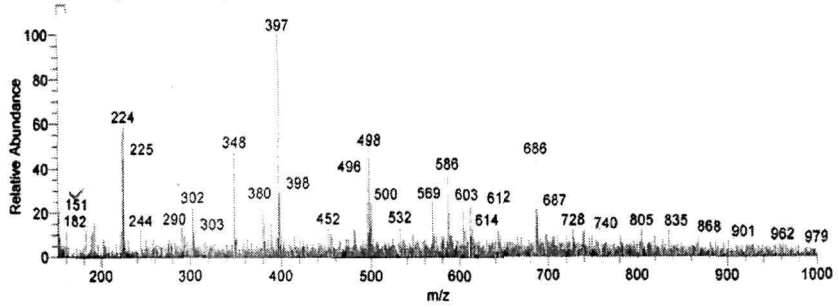
13JAN09LCMSMS0301090486_203702 #319-327 RT: 5.36-5.50 AV: 3 SB: 155 0.00-1.27 , 52.86-54.22 NL: 9.48E6
F: + c ESI Full ms [150.00-1000.00]



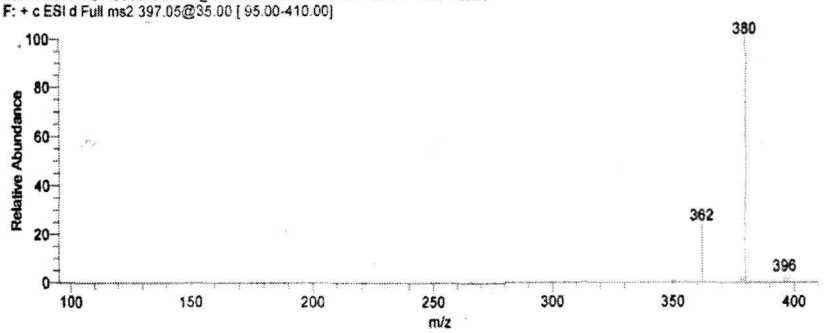
13JAN09LCMSMS0301090486_203702 #309-331 RT: 5.37-5.52 AV: 3 NL: 4.36E4
F: + c ESI d Full ms2 571.63@35.00 [145.00-585.00]



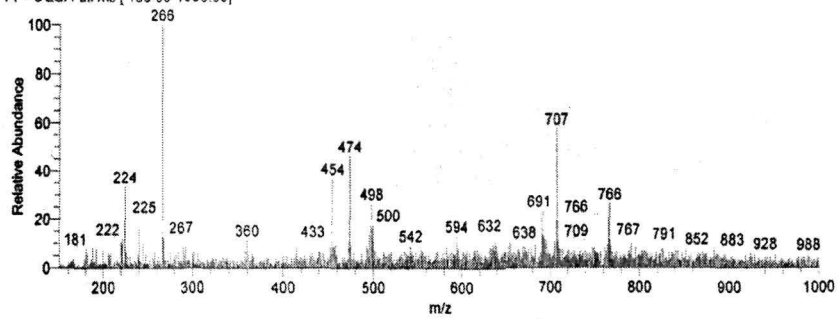
13JAN09LCMSMS0301090486_203702 #347-369 RT: 6.00-6.34 AV: 7 SB: 155 0.00-1.27 , 52.86-54.22 NL: 2.39E6
F: + c ESI Full ms [150.00-1000.00]



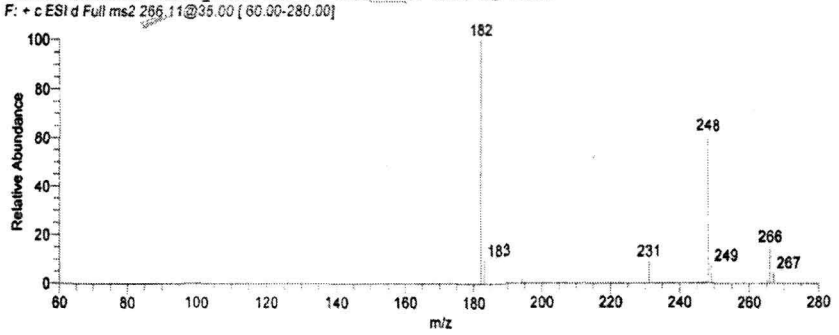
13JAN09LCMSMS0301090486_203702 #359 RT: 6.21 AV: 1 NL: 1.25E6
F: + c ESI d Full ms2 397.05@35.00 [95.00-410.00]



13JAN09LCMSMS0301090486_203702 #383-405 RT: 6.62-6.99 AV: 8 SB: 155 0.00-1.27 , 52.86-54.22 NL: 3.04E6
F: + c ESI Full ms [150.00-1000.00]

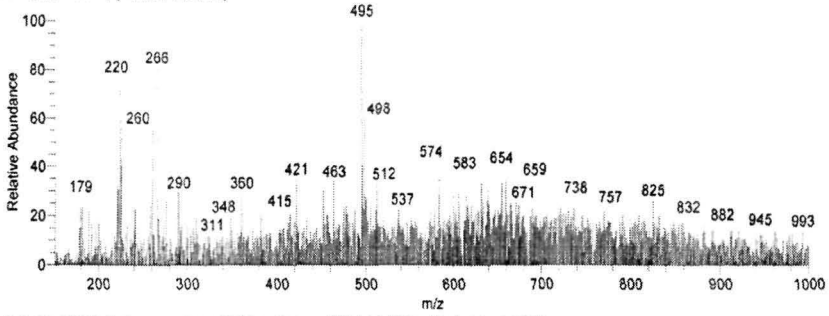


13JAN09LCMSMS0301090486_203702 #381-417 RT: 6.86-7.01 AV: 4 NL: 1.75E6
F: + c ESI d Full ms2 266.11@35.00 [60.00-280.00]

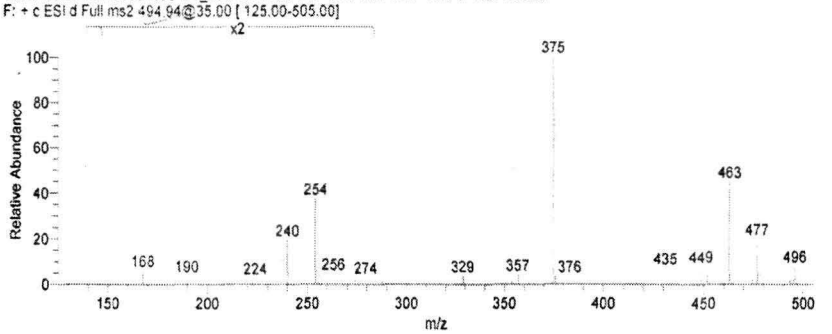


5

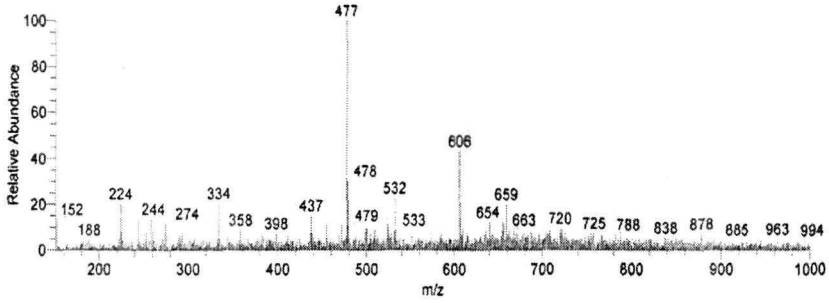
13JAN09LCMSMS0301090486_203702 #401-428 RT: 6.93-7.47 AV: 10 SB: 155 0.00-1.27 , 52.86-54.22 NL: 1.05E6
F: + c ESI Full ms [150.00-1000.00]



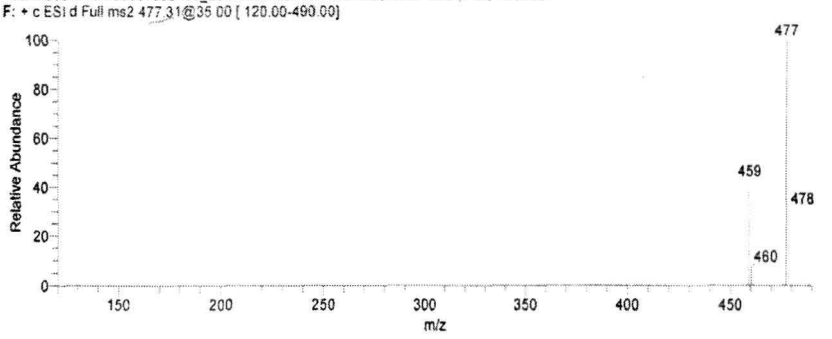
13JAN09LCMSMS0301090486_203702 #402-444 RT: 7.27-7.37 AV: 3 NL: 1.50E6
F: + c ESI d Full ms2 494.94@35.00 [125.00-505.00]



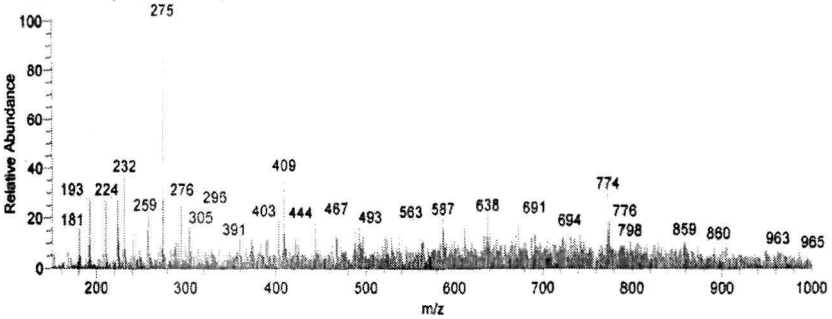
13JAN09LCMSMS0301090486_203702 #472-486 RT: 8.26-8.47 AV: 6 SB: 155 0.00-1.27 , 52.86-54.22 NL: 3.00E6
F: + c ESI Full ms [150.00-1000.00]



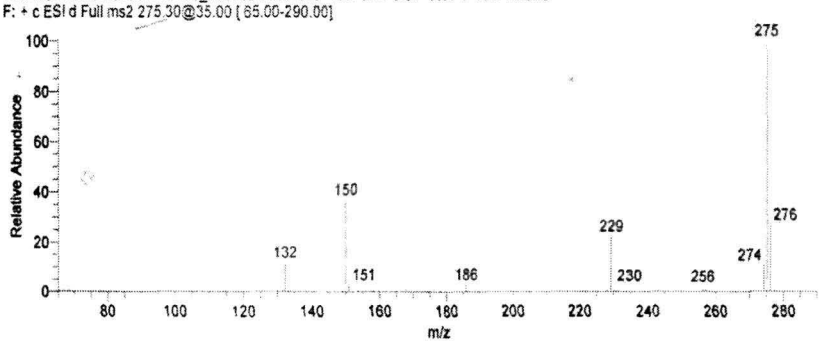
13JAN09LCMSMS0301090486_203702 #470-480 RT: 8.30-8.39 AV: 3 NL: 4.57E6
F: + c ESI d Full ms2 477.31@35.00 [120.00-490.00]



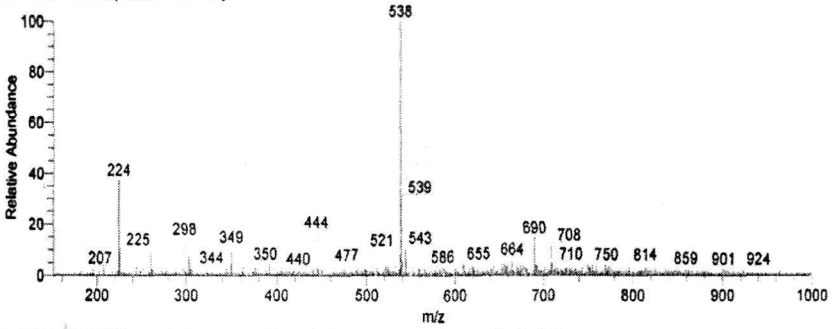
13JAN09LCMSMS0301090486_203702 #533-548 RT: 9.40-9.69 AV: 8 SB: 155 0.00-1.27 , 52.86-54.22 NL: 1.56E6
F: + c ESI Full ms [150.00-1000.00]



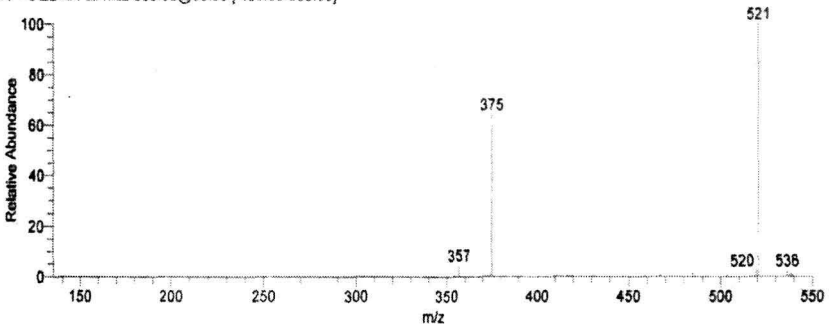
13JAN09LCMSMS0301090486_203702 #533-548 RT: 9.44-9.61 AV: 4 NL: 1.06E6
F: + c ESI d Full ms2 275.30@35.00 [65.00-290.00]



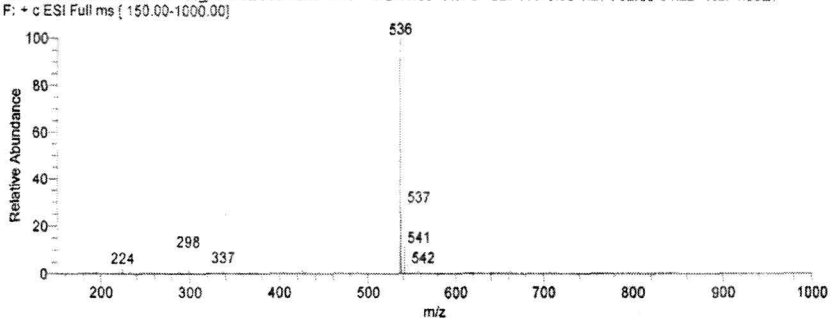
13JAN09LCMSMS0301090486_203702 #624-635 RT: 11.11-11.26 AV: 4 SB: 155 0.00-1.27 , 52.86-54.22 NL: 7.43E6
F: + c ESI Full ms [150.00-1000.00]



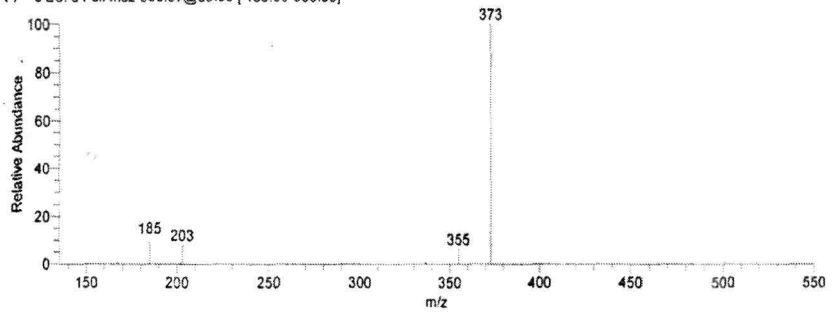
13JAN09LCMSMS0301090486_203702 #623-636 RT: 11.13-11.27 AV: 4 NL: 3.13E6
F: + c ESI d Full ms2 538.02@35.00 [135.00-550.00]



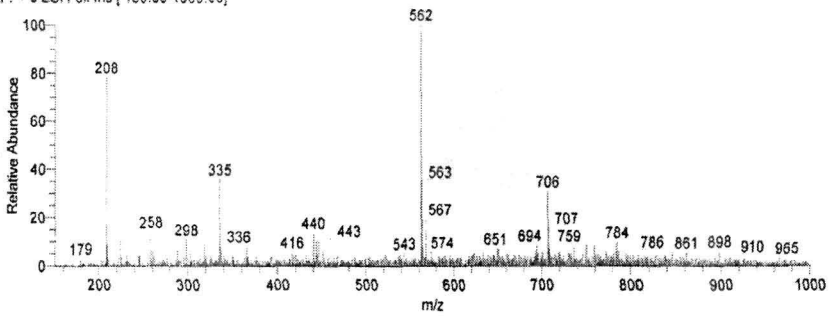
13JAN09LCMSMS0301090486_203702 #659-674 RT: 11.72-11.88 AV: 5 SB: 155 0.00-1.27 , 52.86-54.22 NL: 4.06E7
F: + c ESI Full ms [150.00-1000.00]



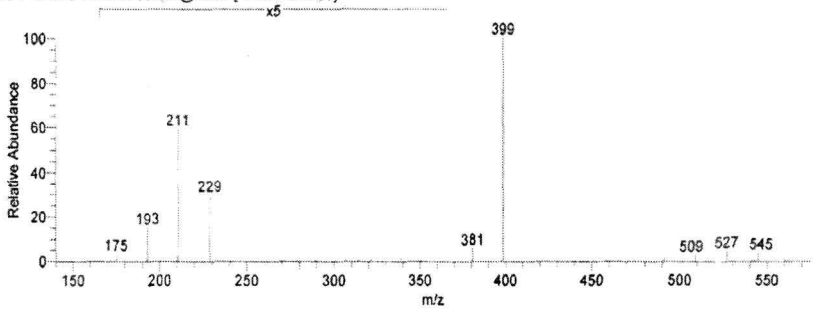
13JAN09LCMSMS0301090486_203702 #657-676 RT: 11.69-11.93 AV: 7 NL: 2.66E7
F: + c ESI d Full ms2 536.07@35.00 [135.00-550.00]



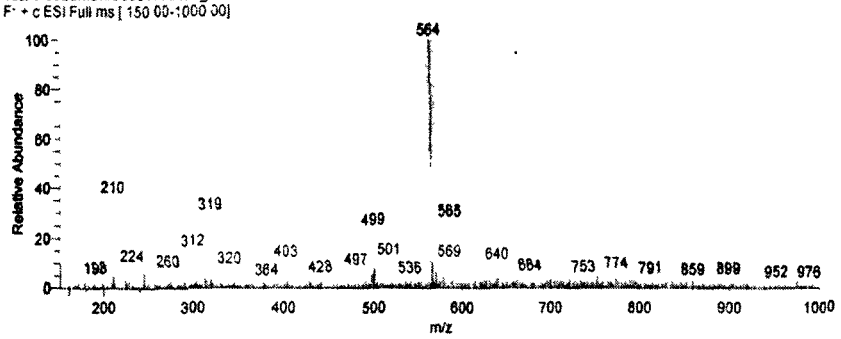
13JAN09LCMSMS0301090486_203702 #776-791 RT: 13.75-14.00 AV: 6 SB: 155 0.00-1.27 , 52.86-54.22 NL: 3.25E6
F: + c ESI Full ms [150.00-1000.00]



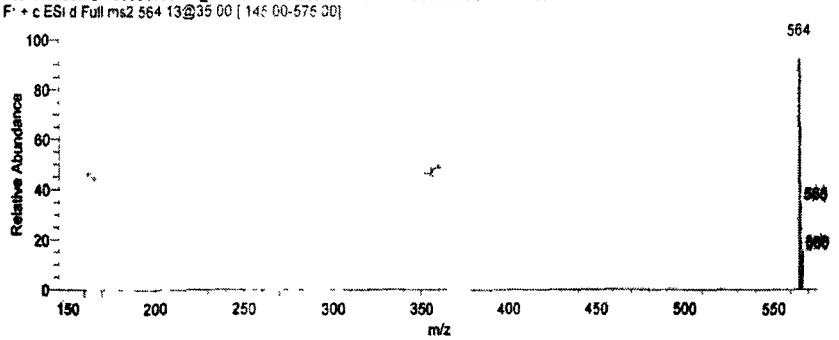
13JAN09LCMSMS0301090486_203702 #779-800 RT: 13.88-13.97 AV: 3 NL: 4.42E6
F: + c ESI d Full ms2 562.08@35.00 [140.00-575.00]



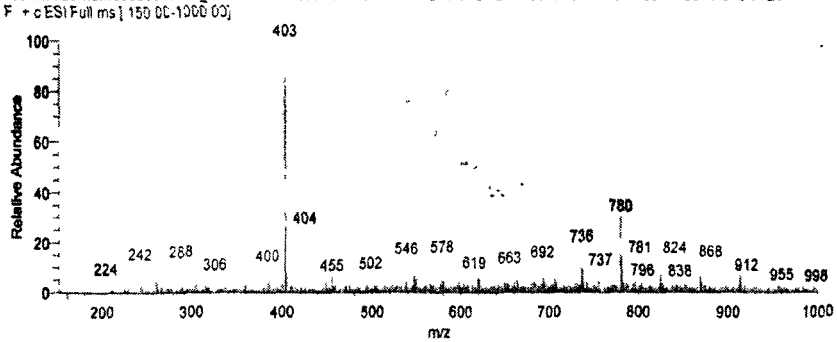
13JAN09LCMSMS0301090486_203702 #837-848 RT 14 80-14 95 AV 4 SB 155 0 00-1 27 52 86-54 22 NL 4 60E6
F: + c ESI Full ms [150 00-1000 00]



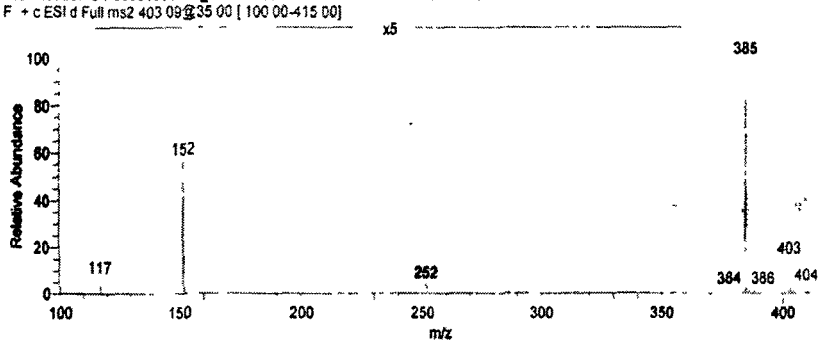
13JAN09LCMSMS0301090486_203702 #933-956 RT 16 47-16.77 AV: 7 NL 1 20E7
F: + c ESI d Full ms2 564 13@35 00 [148 00-575 00]



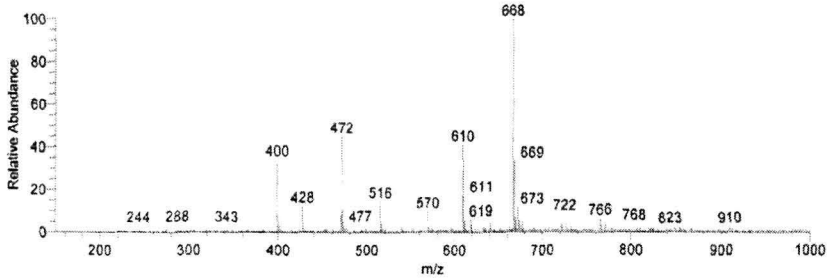
13JAN09LCMSMS0301090486_203702 #984-997 RT 17 24-17 43 AV 5 SB 155 0 00-1 27 , 52 86-54 22 NL 3 81E6
F + c ESI Full ms [150 00-1000 00]



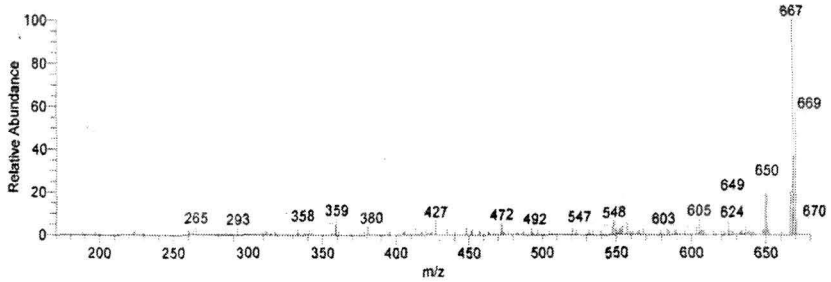
13JAN09LCMSMS0301090486_203702 #982-994 RT 17.20-17 40 AV 5 NL: 2 92E6
F + c ESI d Full ms2 403 09@35 00 [100 00-415 00]



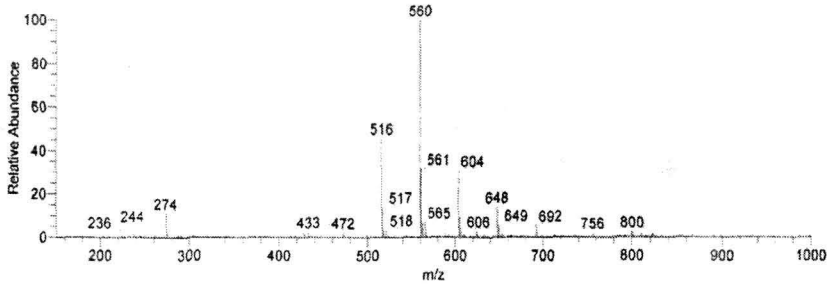
13JAN09LCMSMS0301090486_203702 #1072-1084 RT: 18.73-18.92 AV: 4 SB: 155 0.00-1.27 , 52.86-54.22 NL: 8.66E6
F: + c ESI Full ms [150.00-1000.00]



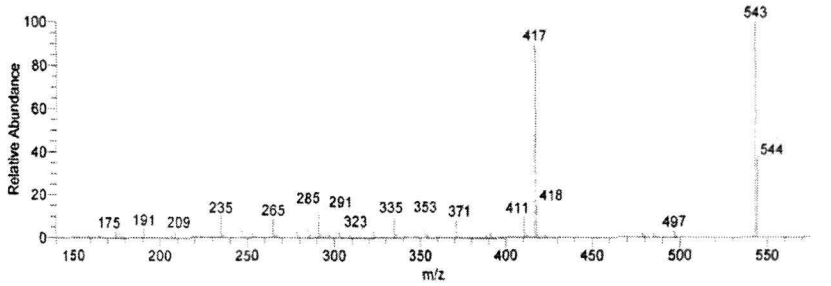
13JAN09LCMSMS0301090486_203702 #1034-1139 RT: 18.79-18.94 AV: 3 NL: 3.66E4
F: + c ESI d Full ms2 668.00@35.00 [170.00-680.00]



13JAN09LCMSMS0301090486_203702 #1118-1138 RT: 19.45-19.70 AV: 7 SB: 155 0.00-1.27 , 52.86-54.22 NL: 1.58E7
F: + c ESI Full ms [150.00-1000.00]

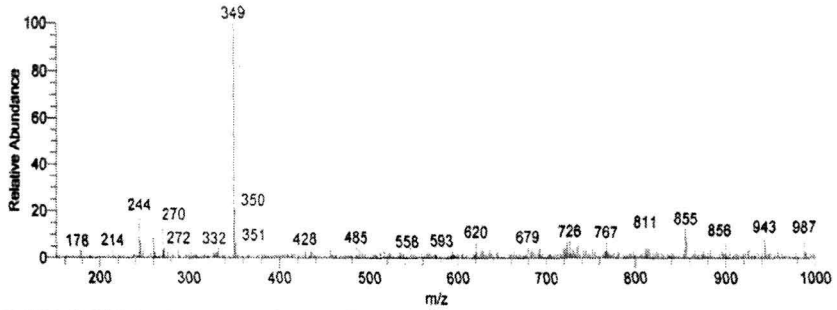


13JAN09LCMSMS0301090486_203702 #1086-1192 RT: 19.51-19.80 AV: 8 NL: 6.58E6
F: + c ESI d Full ms2 560.29@35.00 [140.00-575.00]

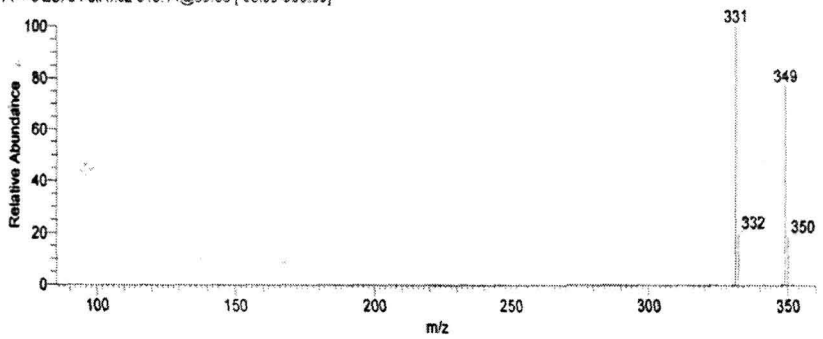


10

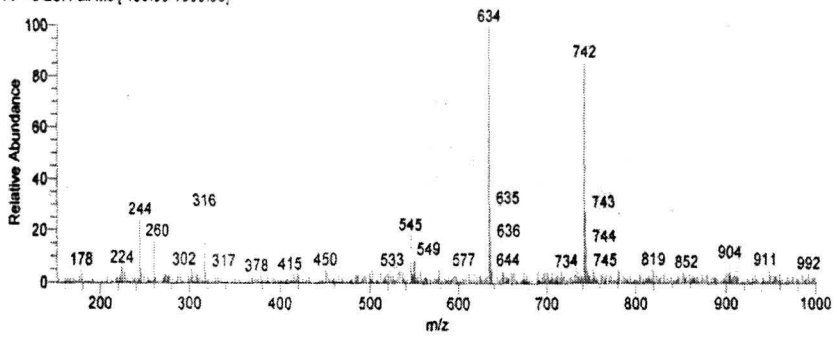
13JAN09LCMSMS0301090486_203702 #1389 RT: 23.78 AV: 1 SB: 155 0.00-1.27 , 52.86-54.22 NL: 5.60E6
F: + c ESI Full ms [150.00-1000.00]



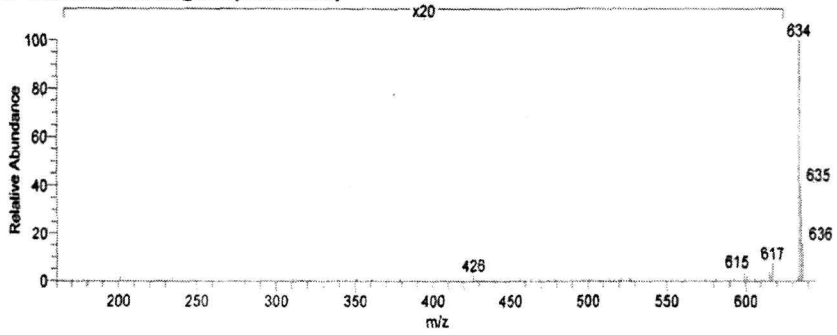
13JAN09LCMSMS0301090486_203702 #1350-1405 RT: 23.70-24.02 AV: 8 NL: 4.44E6
F: + c ESI d Full ms2 349.14@35.00 [85.00-360.00]



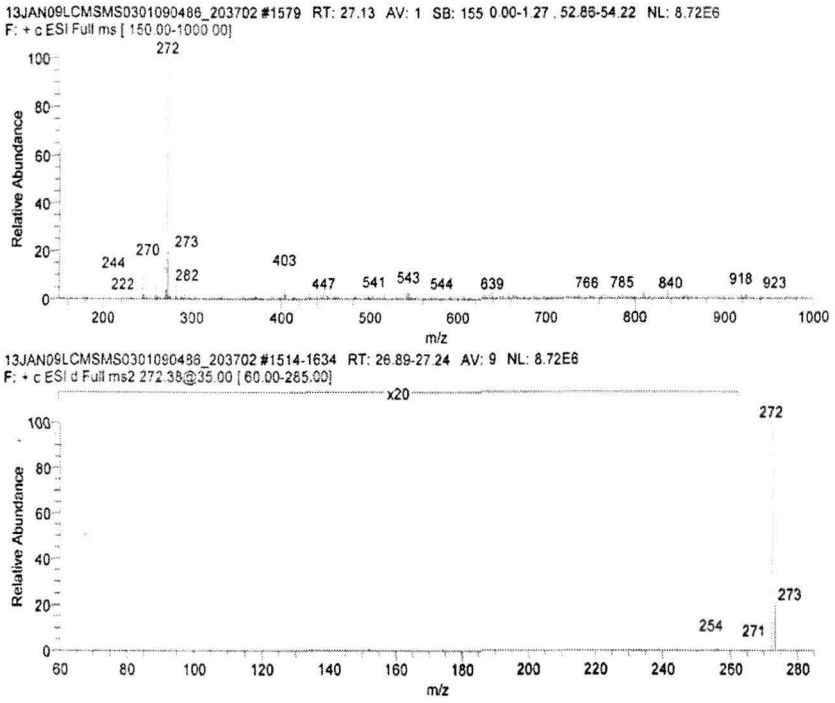
13JAN09LCMSMS0301090486_203702 #1515 RT: 26.01 AV: 1 SB: 155 0.00-1.27 , 52.86-54.22 NL: 4.00E6
F: + c ESI Full ms [150.00-1000.00]



13JAN09LCMSMS0301090486_203702 #1468-1572 RT: 25.91-26.34 AV: 4 NL: 4.09E6
F: + c ESI d Full ms2 634.42@35.00 [160.00-645.00]



11


13JAN09LCMSMS0301090486_203702 #1579 RT: 27.13 AV: 1 SB: 155 0.00-1.27 , 52.86-54.22 NL: 8.72E6
F: + c ESI Full ms [150.00-1000.00]
Relative Abundance
m/z
272
273
270
244
222
282
403
447
541
543
544
639
766
785
840
918
923
13JAN09LCMSMS0301090486_203702 #1514-1634 RT: 26.89-27.24 AV: 9 NL: 8.72E6
F: + c ESI d Full ms2 272.38@35.00 [60.00-285.00]
x20
Relative Abundance
m/z
272
273
254
271

List of publication

Journal publications (peer reviewed national/international journals)

1. Bharali, P., & Konwar, B.K. Production and Physico-chemical Characterization of a Biosurfactant Produced by *Pseudomonas aeruginosa* OBP1 Isolated from Petroleum Sludge, Appl. Biochem. Biotechnol. **164**, 1444--1460, 2011.
2. Bharali, P., Das, S., Konwar, B.K., & Thakur, A.J. Crude biosurfactant from thermophilic *Alcaligenes faecalis*: Feasibility in petro-spill bioremediation, Inter. Biodeter. Biodegr. **65**, 682--690, 2011.
3. Roy, B., Bharali, P., Konwar, B.K., & Karak, N. Silver-embedded modified hyperbranched epoxy/clay nanocomposites as antibacterial materials, Bioresour.Technol. **127**, 175--180, 2013.
4. Bharali, P., Saikia, J.P., Ray, A., & Konwar, B.K. Rhamnolipid (RL) from *Pseudomonas aeruginosa* OBP1: A novel chemotaxis and antibacterial agent, Colloids Surf. B Biointerfaces, **103**, 502–509, 2013.
5. Saikia, J.P., Bharali, P., & Konwar, B.K. Possible protection of silver nanoparticles against salt by using rhamnolipid, Colloids Surf. B Biointerfaces, **104**, 330--332, 2013.
6. Singh, S,P., Bharali, P., & Konwar B.K. Optimization of Nutrient Requirements and Culture Conditions for the Production of Rhamnolipid from *Pseudomonas aeruginosa* (MTCC 7815) using Mesua ferrea Seed Oil, Indian.J. Microbiol. **53**, 467--476, 2013.
7. Bharali, P., Saikia, J.P., Paul, S., & Konwar, B.K. Colloidal silver nanoparticles/rhamnolipid (SNPRL) composite as novel chemotactic antibacterial agent, Inter. J. Biol.Macromol. **61**, 238--242, 2013.
8. Das, S., Kalita, S.J., Bharali, P., Konwar, B.K., Das, B., & Thakur, A.J. Organic Reactions in "Green Surfactant": An Avenue to Bisuracil Derivative, ACS Sustainable Chem. Eng., DOI: 10.1021/sc4002774, Publication Date (Web): September 5, 2013.

9. Ray, A., Bharali, P., & Konwar, B.K. Mode of Antibacterial Activity of Eclalbasaponin Isolated from *Eclipta alba*, Appl. Biochem. Biotechnol. DOI 10.1007/s12010-013-0452-3, 08 September, 2013
10. Mudoi, P., Bharali, P., Konwar, B. K. Study on the Effect of pH, Temperature and Aeration on the Cellular Growth and Xanthan Production by *Xanthomonas campestris* Using Waste Residual Molasses, J. Bioproces. Biotechniq. 2013, 3:3. DOI.org/10.4172/2155-9821.1000135
11. Bora, C., Bharali, P., Baglari, S., Dolui, S.K., & Konwar, B.K. Strong and conductive reduced graphene oxide/polyester resin composite films with improved mechanical strength, thermal stability and its antibacterial activity, Composites Sci. Technol. **87**, 1--7, 2013.
12. Pramanik, S., Bharali, P., Konwar, B.K., & Karak, N. Antimicrobial hyperbranched poly (ester amide)/polyaniline nanofiber modified montmorillonite nanocomposites, Mater. Sci. Eng.C, Available online 31 October 2013, DOI i.org/10.1016/j.msec.2013.10.021.

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1. Bharali, P., & Konwar, B.K. Role of biosurfactant in reducing surface tension of crude oil and its biodegradation, 96th Indian Science Congress, NEHU, Shillong, January 3rd -7th , 2009.
2. Bharali, P., & Konwar, B.K. Isolation of indigenous biosurfactant producing *Pseudomonas aeruginosa* strains from the oil drilling sites of Assam, International Conference on Climate Change and Bioresource Technology (ICCCB-2010), Bharathidasan University, Department of Biotechnology, Tiruchirappalli, Tamil Nadu, February 9th -12th, 2010.
3. Bharali, P., & Konwar, B.K. Application of rhamnolipid in separation of residual crude oil from petroleum sludge under laboratory conditions, National seminar on Medicinal plant and microbe diversity and their pharmaceuticals, Tezpur University, Department of Biotechnology & Molecular Biology, Tezpur, December 19th-21st, 2010.

4. Bharali, P., & Konwar, B.K. Isolation and production of biosurfactant from *Alcaligene faecalis* isolated from the oil drilling sites of Assam, International Seminar on Bioresources and Human Sustenance, Cotton College & Zoological society of Assam (ZSA), Department of Zoology, Guwahati, October 20th-22nd, 2011.
5. Bharali, P., & Konwar, B.K. Utilization of biodiesel derived waste glycerol as an economically cheaper substrate for the production the of biosurfactant by indigenous *Pseudomonas aeruginosa* JBK1, Tezpur University, Department of Biotechnology & Molecular Biology, Tezpur, December 19th-21st, 2010.