ISOLATION AND CHARACTERIZATION OF AMPHIPHILIC FUNCTIONAL BIOPOLYMERS

A Thesis

submitted in the partial fulfillment of the requirement for the award of the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Under the guidance of:

Dr. MOUSHUMI GHOSH
Associate Professor

Submitted by:

ARUN KUMAR
Roll No. 301101005

DEPARTMENT OF BIOTECHNOLOGY & ENVIRONMENTAL SCIENCES

THAPAR UNIVERSITY, PATIALA (PUNJAB)-147004

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in this thesis entitled "**Isolation and characterization of amphiphilic functional biopolymers**" in partial fulfilment of the requirement for the award of the degree of Masters of science in Biotechnology, Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is an authentic record of my work during the period of six months from January, 2013 to July 2013, under the guidance of Dr. Moushumi Ghosh, Associate Professor, Thapar University, Patiala. I have not submitted the matter embodied in this thesis for the award of any other degree or diploma.

Arun Kumar
15/7/13

ARUN KUMAR

(301101005)

CERTIFICATE

This is to certify that the thesis entitled "**Isolation and characterization of amphiphilic functional biopolymers**" submitted by Arun Kumar in partial fulfilment of the requirement for the award of Degree of Masters of Science in Biotechnology to Thapar University, Patiala, is a record of student's own work carried out by him. The report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Dr. Moushumi Ghosh
Associate Professor, Supervisor
DBTES, TU
Patiala

Dr. M.S. Reddy
Head
DBTES, TU
Patiala

Dr. S.K. Mohapatra
Dean
Academic Affairs,
Thapar University
Patiala

ABSTRACT

Amphiphilic compounds are produced extracellularly by microorganisms on cell surfaces or excreted extracellularly. They contain hydrophilic and hydrophobic moieties that produce surface and interfacial tension between molecules at the surface and interface respectively. These biopolymers are promising natural surfactants and emulsifiers that offer several advantages, such as lower toxicity, biodegradability and ecological acceptability over the chemically synthesized surfactants/emulsifiers. So, the present study focused on isolation of amphiphilic biopolymer producing bacteria from environmental samples and assessing the potential of these isolates by various screening methods.

In this study, from a total of 156 samples collected from diverse environmental sources, 23 bacterial isolates were screened for amphiphilic biopolymer producing ability using a variety of tests like drop collapse test, BATH (Bacterial adhesion to hydrocarbon) assay, hemolytic activity assay, CTAB Agar plate method, surface tension measurement and emulsification activity. Five bacterial strains with higher emulsification activity (45% to 64%) were finally selected. Further studies on amphiphilic compounds production by these isolates revealed no significant effect ($p < 0.05$) of temperature and pH on the emulsification activity against different hydrocarbons (diesel, xylene and petrol). High biopolymer production (250 - 405 mgL^{-1}) from all the five strains was achieved in mineral salt medium with glucose as sole carbon source at 60 hrs. Characterization of amphiphilic biopolymers elaborated by the isolates revealed a chemical composition of proteins (6-12 %) and sugars (1-5 %). Emulsification activity of the biopolymers varied against different hydrocarbons tested. The results of the study of these amphiphilic biopolymers have possible application in bioremediation of environment contaminated with various hydrocarbons and other industrial applications.

Keywords: Amphiphilic biopolymer, bioremediation, emulsification, hydrocarbons, hydrophilic, hydrophobic.

LIST OF ABBREVIATIONS

AB	Amphiphilic biopolymers
BATH	Bacterial adhesion to hydrocarbons
BE	Bioemulsifiers
BS	Biosurfactants
BSA	Bovine serum albumin
CTAB	Cetyl trimethylammonium bromide
CPC	Cetylpyridinium chloride
FTIR	Fourier transform infrared spectroscopy
IFT	Interfacial tension
MELs	Mannosylerythritol lipids
MSM	Mineral salt medium
NB	Nutrient broth
OD	Optical density
O/W	Oil in water
SAC	Surface active compound
SEM	Scanning electron microscope
SFT	Surface tension
SIM	Sulfide indole motility medium
SDS	Sodium dodecyl sulfate
TCA	Tri-chloroacetic acid
UV	Ultra-violet
W/O	Water in oil

LIST OF SYMBOLS

°C	Degree(s) Celsius
g	Gram
mg/mL	Milligram per millilitre
mg/L	Milligram per litre
µg/mL	Microgram per millilitre
µl	Microlitre
µM	Micromolar
min	Minute
M	Molarity
mm	Millimetre
N	Normality
Na	Sodium
nm	Nanometer
%	Percentage
rpm	Revolutions per minute
sec	Second
spp.	Species (plural)
w/v	Weight by volume
U	Unit
V	Volt
wt	Weight

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INTRODUCTION

1. INTRODUCTION

Biopolymers are a diverse group of organic compounds that are produced by a wide variety of microorganisms, extracellularly or on cell surfaces. The microbial compounds containing hydrophilic (mono-, oligo- or polysaccharides, peptides) and hydrophobic (saturated, unsaturated and hydroxylated fatty acids or fatty alcohols) moieties are known as amphiphilic biopolymers. They exhibit many pronounced surface and emulsifying activities. These biopolymers are classified in two categories i.e. low-molecular weight and high molecular-weight molecules. Compounds that reduce surface and interfacial tension at gas-liquid-solid interfaces are called biosurfactants and those that mainly reduce the interfacial tension between immiscible liquids or at the solid-liquid interfaces leading to the formation of more stable emulsions are called bioemulsifiers or bioemulsans. Biosurfactants are low-molecular-weight compounds, such as proteins, lipopeptides, glycolipids, while the bioemulsifiers are high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins. Among the different classes of amphiphilic biopolymers, rhamnolipid and surfactin are the best studied biosurfactants (Ellaiah *et al.*,2002; Carrillo *et al.*, 2003; Rodrigues *et al.*, 2010).

The attributes such as low toxicity, high biodegradability, low irritancy and compatibility with human skin make amphiphilic biopolymers promising natural surfactants with several advantages over chemically synthesized surfactants. Amphiphilic biopolymers have gained importance in the field of cosmetics, oil recovery, environment bioremediation, metal treatment and processing, paper producing and paint, food processing and pharmaceutical industries. Moreover microorganisms which produce biosurfactants help to amplify the bioavailability of hydrocarbons by enhancing the contact between pollutant and the microorganisms in the presence of amphiphilic biopolymer.

Although, biosurfactants exhibit such important advantages, they are yet to find extensive industrial use; this is their because of relatively high production cost and lower

yield. One possible strategy to address this problem is to characterize microorganisms capable of high polymer production. In the present study, an attempt was made to isolate amphiphilic biopolymer producing bacteria from environmental samples and assessing the potential of these isolates by various standard methods. Further, the obtained biopolymers with potential emulsification activity were physico-chemically and morphologically characterized.

SCOPE OF THE STUDY

Amphiphilic biopolymers are surface active compounds (SAC) which have the enormous market demand in the industries. This demand is currently met by numerous synthetic mainly petroleum based chemical surfactants which are mostly toxic in nature. The growing awareness towards the use of renewable based products and "green products" have stimulated the development of alternatives to these chemical surfactants. Microbial biopolymer with amphiphilic nature may acts as potential alternative as they possess the advantage of being biodegradable and non-toxic, which encourages its use in bioremediation strategies. However though various biopolymers have been reported till date but still the limitations related to their production cost and yield persists. Therefore, there is a constant need of a systematic study in isolating efficient amphiphilic biopolymer producing microbes from diverse environmental niches. The characterization of such biopolymers is anticipated to play a significant role in designing unique, sustainable bioremediation approaches.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 Amphiphilic biopolymers (AB)

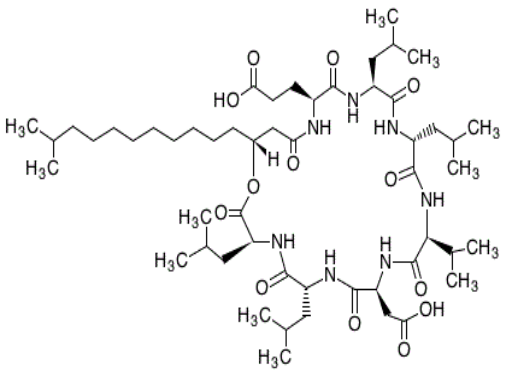
Many microorganisms can produce variety of amphiphilic compounds, with both hydrophilic and hydrophobic moieties present within a molecule, which allow them to exhibit surface activities at interfaces. Amphiphilic compounds that reduce surface and interfacial tension at gas-liquid-solid interfaces are called biosurfactants. These compounds mainly reduce the interfacial tension between immiscible liquids or at the solid-liquid interfaces leading to the formation of more stable emulsions are called bioemulsifiers or bioemulsans. Biosurfactants are low-molecular-weight compounds, such as proteins, lipopeptides, glycolipids, while the bioemulsifiers are high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins (Thavasi *et al.*, 2011). Hydrophobic portion of the amphiphilic biopolymer protrudes towards hydrophobic phase or oil and hydrophilic phase oriented towards solution or hydrophilic phase or water.

Their diverse functional properties namely, emulsification, wetting, foaming, cleansing, phase separation, surface activity and reduction in viscosity of heavy liquids such as crude oil, make them suitable for utilization for many industrial and domestic application purposes (Gautam and Tiagi, 2006; Franzetti *et al*., 2008). Biosurfactants have several advantages over synthetic surfactants such as, their biodegradability, environmental compatibility, low toxicity, high selectivity and specific activity at extreme temperatures, pH and salinity (Banat, 1993). Amphiphilic biopolymers have significant role in industrial and environmental applications, such as bioremediation and enhanced oil recovery. Besides they have potential commercial applications in various industries such as paint, cosmetics, textile, detergent, agrochemical, food and pharmaceutical industries (Perfumo *et al*., 2006; Satpute *et al.*, 2010).

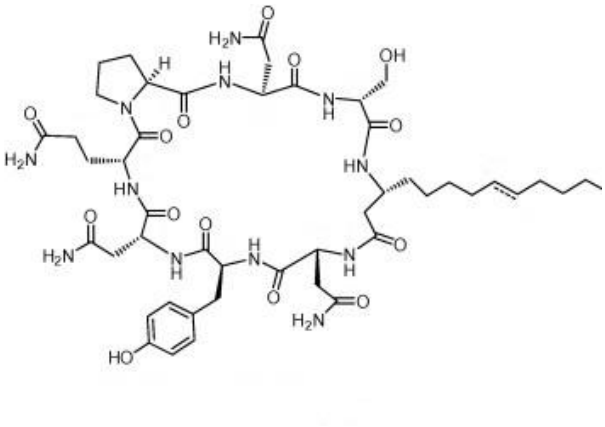
2.2 Classification of microbial amphiphillic biopolymers

2.2.1 *Low molecular weight amphiphilic biopolymers*

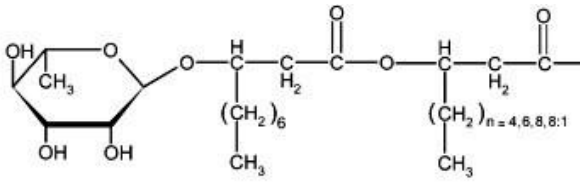
Low-molecular-weight amphiphilic biopolymeric compounds are lipopeptides and glycolipids have been studied extensively. Lipopeptides are mainly produced by members of the *Bacillus* sp. In fact, a cyclic lipopeptide, surfactin produced by *Bacillus subtilis* is considered the most active biosurfactant discovered so far (Ron and Rosenberg, 2001). Another lipopeptide, lichenysin, was extracted from the supernatant of *Bacillus licheniformis* culture (Rismani *et al.*, 2006). Glycolipids are also classified under the category of low molecular weight amphiphilic biopolymers. Glycolipids are generally mono or disaccharides compounds acylated with long chain fatty acids or hydroxyl fatty acids. Rhamnolipids, mannosylerythritol lipids (MELs), sophorolipids (Figure 1 (e) and 1 (f)) and trehalolipids are the best-studied structural subclasses of glycolipids. Rhamnolipids (Figure 1(c) and 1 (d)) are produced mainly by *Pseudomonas aeruginosa*, that are composed of one or two rhamnose sugar units linked to one or two β-hydroxyfatty acid chains (Perfumo *et al.*, 2006; Raza *et al.*, 2009). These molecules display high surface activities and many potential applications in the biomedical field due to their antibacterial, antifungal, antiviral, anti-adhesive reported properties.



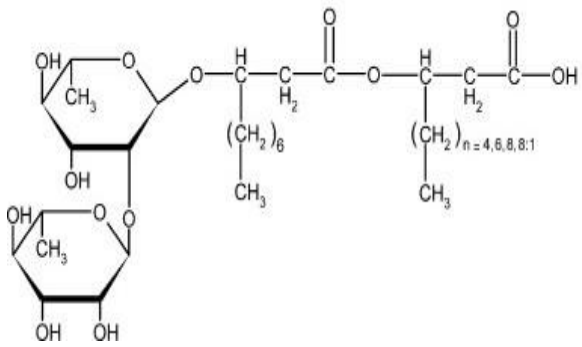
a) Surfactin



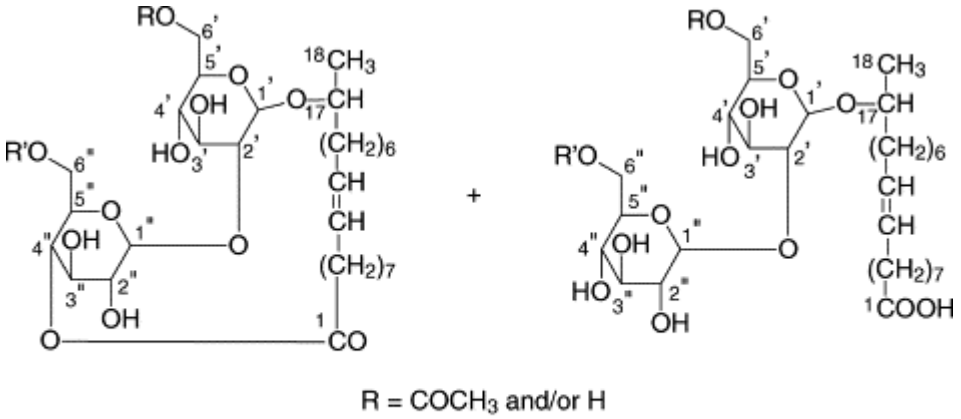
b) Iturin A



c) Mono-rhamnolipid



d) Di-rhamnolipid



Lactonic form (Major)

e) Lactonic sophorolipids

Acid form (Minor)

f) Acidic sophorolipids

Figure 1. Chemical structure of low molecular weight amphiphilic biopolymers

2.2.2 *High molecular weight amphiphilic biopolymers*

Emulsan (Figure 2) is the most commonly studied high molecular weight (1000 kDa) amphiphilic biopolymer (Chamanrokh *et al*., 2003). It is a kind of lipopolysaccharide isolated from *Acinetobacter calcoacetius* RAG-1 ATCC 31012. RAG-1 emulsan is formed of anionic heteropolysaccharide and protein. Due to the presence of fatty acids it exhibits surface activity and about 15% of the emulsan dry weight is due to fatty acids. These fatty acids are attached to the polysaccharide backbone via O-ester and N-acyl linkages. Alasan is another high molecular weight (1000 kDa) biosurfactant or amphiphilic biopolymer, a complex of an anionic polysaccharide and a protein isolated from *Acinetobacter radioresistens*. Toren *et al.,* (2001) reported that alasan is produced by *Acinetobacter radioresistens* KA53 and purified polysaccharide (apo-alasan) has very low emulsification activity.

These high molecular weight biopolymers possess effective emulsifying activity, but they do not reduce the surface tension as much as biosurfactants and are called as bioemulsifiers. Many bioemulsifiers show effective emulsification activity even at high temperatures, including the protein complex from *Methanobacterium thermoautotrophium* and the protein-polysaccharide-lipid complex of *Bacillus stearothermophilus* ATCC 12980. Liposan is an extracellular emulsifier produced by *Candida lipolytica* and mannanprotein is reported to be produced by *Saccharomyces cerevisiae*. Bioemulsifiers have promising applications in food, cosmetic, and petroleum industries (Cameotra and Makkar, 2004).

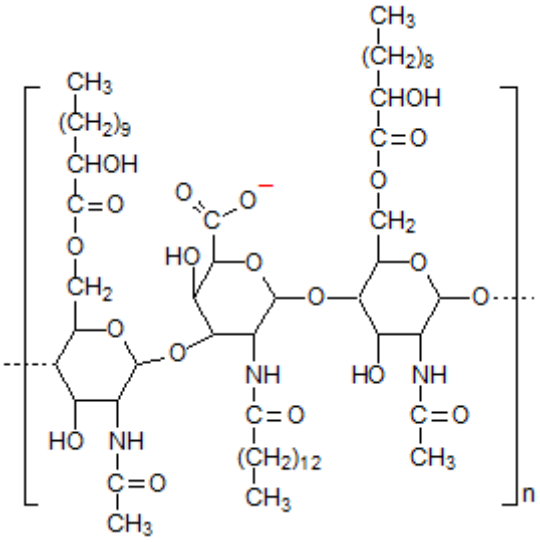


Figure 2. Chemical structure of Emulsan

Table 1. Amphiphilic biopolymers and their applications

Amphiphilic Biopolymer		Microorganism	Application
Group	**Class**		
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa*, *Pseudomonas* sp.	Enhancement of the degradation and dispersion of different classes of hydrocarbons Emulsification of hydrocarbons and vegetable oils Removal of metals from soil Antimicrobial activity against *Mycobacterium tuberculosis*
	Sophorolipids	*Torulopsis bombicola*, *Torulopsis petrophilum*, *Torulopsis apicola*	Recovery of hydrocarbons from dregs and muds Removal of heavy metals from sediments Enhancement of oil recovery
	Trehalolipids	*Mycobacterium tuberculosis*, *Rhodococcus erythropolis*, *Arthrobacter* sp., *Nocardia* sp., *Corynebacterium* sp.	Enhancement of the bioavailability of hydrocarbons antiviral activity against HSV and influenza virus
	Lichenysin	*Bacillus licheniformis*	Enhancement of oil recovery Antibacterial activity

Lipo-peptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides Removal of heavy metals from a contaminated soil Sediment and water; increasing the effectiveness of phytoextraction
Fatty acids, phospho-lipids and neutral lipids	Phosphatidyl-ethanolamine	*Acinetobacter* sp., *Rhodococcus erythropolis*	Increasing the tolerance of bacteria to heavy metals
	Coryno-mycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery
	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions from aqueous solution Dispersion action for hydrophilic pigments Preparation of new Emulsion-type organogels Superfine microcapsules (vesicles or liposomes), Heavy metal sequestrants
Polymeric Structures	Emulsan	*Acinetobacter calcoaceticus* RAG-1	
	Alasan	*Acinetobacter radioresistens* KA-53	Stabilization of the hydrocarbon-in-water emulsions
	Liposan	*Candida lipolytica*	
	Mannoprotein	*Saccharomyces cerevisiae*	
	Biodispersan	*Acinetobacter calcoaceticus* A2	Dispersion of limestone in water

2.3 Functional properties

It has been suggested that location and size of the hydrophilic and hydrophobic functional groups is crucial in determining the property of a AB, which consequently determines the practical application of AB in various industrial applications related to detergents, oil recovery, cosmetics, food, pharmaceutics, agriculture and mining (Desai and Banat, 1997).

2.3.1 *Surface activity*

SFT (Surface tension) and IFT (Interfacial tension) are important properties of surfactant (Figure 3A and 3B). These strong intermolecular attractive forces build the tension on the surface, i.e. called as SFT (Figure 3A). SFT of the distilled water is 72 mN/m and SFT value gets reduced on the addition of biosurfactant. *Bacillus sp*. produces biosurfactant namely surfactin that is found to be most effective biosurfactant reducing water SFT from 72 to 27 dynes/cm (Thavasi *et al.*, 2011).

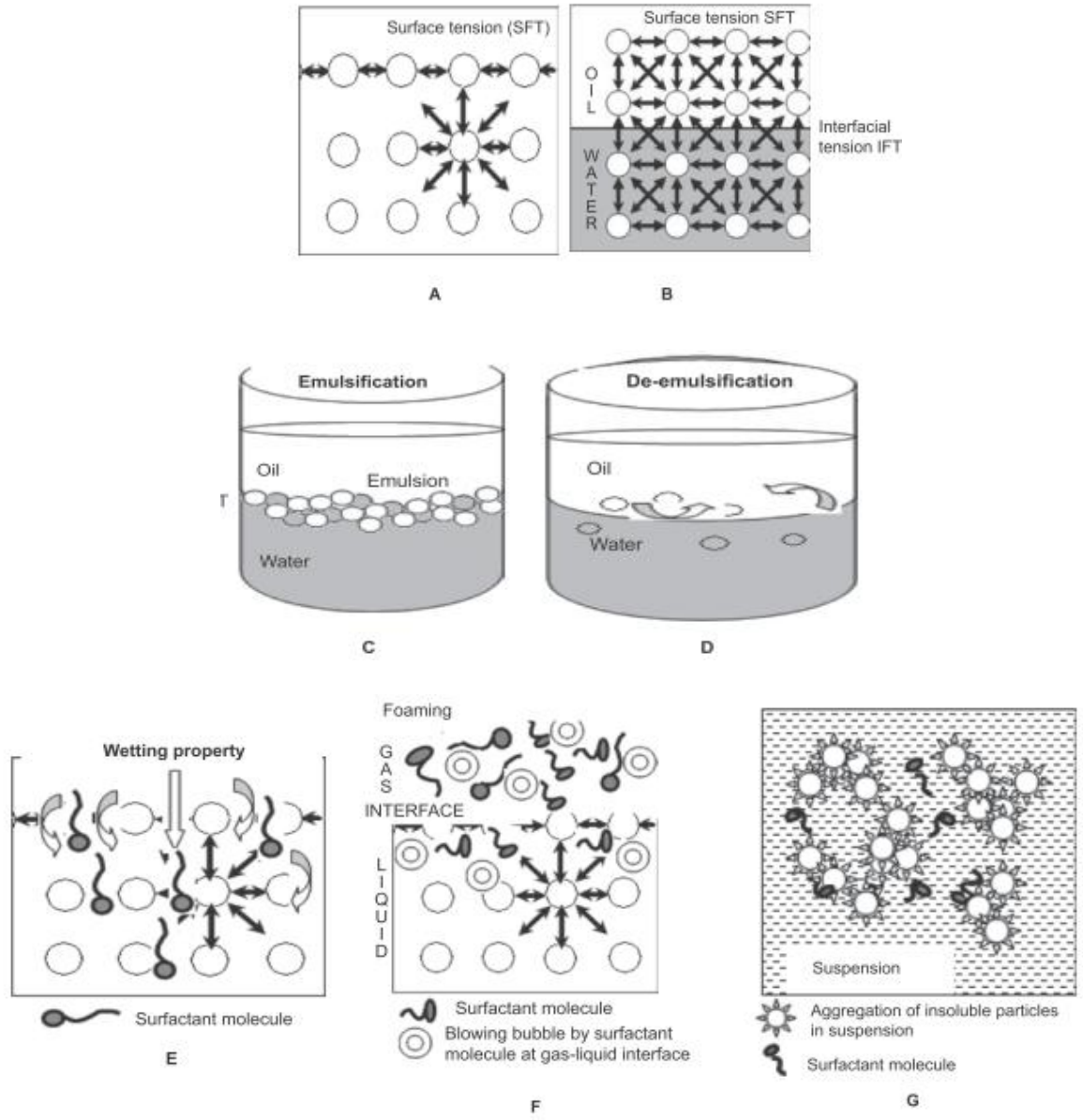


Figure 3. Different functional properties of amphiphilic biopolymers; (A) Surface tension; (B) Surface and interfacial tension (C) Emulsification (D) De-emulsification (E) Wetting (F) Foaming and (G) Adsorption.

2.3.2 *Emulsification*

Dispersion of one liquid into another leading to the mixing of two immiscible liquids is called emulsification (Figure 3 C) and the liquid formed by emulsification is called emulsion. The structure of emulsifier has two characters in the molecules, one is adaptable to

water (hydrophilic group), and the other is estranged to water (hydrophobic group). It represents a micellular solubilization; however, the resultant solubilized particles are much bigger. Emulsan is the extracellular form of a polyanionic, cell associated heteropolysaccharide produced by the oil-degrading bacterium *Acinetobacter calcoaceticus* RAG-1 (Rosenberg *et al.,* 1984). Emulsan has potential applications in the petroleum industry.

2.3.3 *De-emulsification*

This process breaks emulsions through the disruption of stable surface between the bulk phase and the internal phase (Figure 3 D) which is important in oil production processes, where natural emulsifying agents hinders the production processes. De-emulsion is achieved by disturbing the thermodynamic conditions at the interface. Equipment used in petroleum industry mainly suffers from the corrosion therefore, before downstream oil processing, de-emulsification action is needed. Different industries such as mining, food, nuclear fuel reprocessing, cosmetics and pharmaceuticals are dependent on this property (Kosaric *et al.*, 1987). De-emulsification capabilities of mixed bacterial population were usually tested by using a kerosene-water and petroleum-oil emulsion system, where up to 96% of de-emulsification was achieved. Mixed culture products exhibit high de-emulsifying activity as compared with the most effective pure culture (Nadarajah *et al.,* 2002).

2.3.4 *Wetting*

It is the spreading and penetrating power of a compound that lowers the SFT, when added to a liquid. It reduces attractive forces of similar molecules and increases the attraction towards unlike surfaces. Amphiphilic biopolymers act as wetting agents by getting into the pores and fissures rather than bridging them with the SFT (Figure 3 E). Sophorolipids extracted from *Candida apicola* have the wetting activity and it is used in cosmetic industry (Bhadoriya *et al.,* 2013).

2.3.5 *Foaming*

Surfactants get concentrated at a gas-liquid interface leading to the formation of bubbles through the liquid and on the interface resulting in foam formation (Figure 3 F). Bubbling techniques help studying the foaming properties of surfactin, sodium dodecyl sulphate (SDS), and bovine serum albumin (BSA). Surfactin exhibit excellent foaming properties when compared to sodium dodecyl sulphate (SDS) (Dubey *et al.*, 2005).

2.3.6 *Adsorption*

This functional property helps the surfactant molecules to get adsorb on hydrophobic substrates (Figure 3 G). Recovery of rhamnolipid BS JBR215 by this technique has been reported (Wei *et al.*, 2005). Adsorption property of a surfactant is an important factor to enhance oil recovery. Adsorption facilitates strong interactions of surface active molecules with the rock than with the oil and hence can increase the recovery of oil from rocks (Curbelo *et al*., 2007).

2.3.7 *Dispersion*

Dispersion property of surfactant keeps insoluble particles in suspension by preventing insoluble particles to form aggregates with each other. This property also leads to desorption of hydrophobic molecules from rock surfaces enhancing mobility and recovery and has application in oilfield chemistry. A psychrophilic yeast *Candida antarctica* has been shown to produce a glycolipids mannosylerythritol lipids that act as emulsifying agent and dispersant and currently used in skin smoothing and anti-wrinkle products (Bhadoriya *et al.*, 2013).

2.3.8 *Detergency*

Washing and cleansing activity is associated with detergency and amphiphilic biopolymers act as detergents. Glycolipids are used as components of detergent formulations. Low-

foaming sophorolipids from *Candida bombicola* appear suitable due to their high detergency ability, low cytotoxicity and high biodegradability and general environmentally acceptable properties (Hirata *et al.*, 2009).

2.3.9 *Flocculation*

It is a process, where the emulsion droplets stick together to form a cluster that can be broken up by mechanical action restoring the emulsion to its original form, *Cyanobacterium phormidium produce* emulcyan that acts as flocculating as well as emulsifying agent (Assadi *et al.*, 2010).

2.3.10 *Phase separation*

Once the surfactant is added to immiscible liquids, it leads to the formation of emulsion. However, after some period, the emulsion droplet of like molecules begins to assemble and come together leading to the separation of the two phases. This process is called phase separation.

2.3.11 *Viscosity reduction*

Due to high viscosity of crude oil, it resists to flow and becomes very difficult for transport. Heavy crude oil (high density) contributes significant contents of nitrogen, oxygen, sulphur compounds and heavy metal contaminants. Such viscosity of heavy oils is reduced by using surfactants to increase mobility and ease of transportation.

2.3.12 *Solubilization*

Surfactants enhance solubilization of insoluble material. At high concentration of surfactant, micellar structures are formed. Insoluble molecules are encapsulated into a micellar structure and brought into solution at higher level. This property is important to form water-insoluble substances in aqueous solutions, or water-soluble substances in organic solvents. BS/BE are more effective than the synthetic surfactants to solubilize complex compound mixture to an aqueous solution. Perfumo *et al*., (2006) reported on the roles of BS

and BE in accessing hydrophobic substrates while Wong *et al.,* (2004) also reported similar observation on the effects of synthetic surfactants and BS Corrosion inhibition. Corrosion inhibitors are material that protects against the wearing away of appliance surfaces.

2.4 Screening methods for detection of amphiphilic biopolymer producers

Various methods have been reported till date used to screen, detect or evaluate potential amphiphilic biopolymer (biosurfactant and bioemulsifier) producing microorganisms, each has its own advantages and limitations as discussed below:

2.4.1 *Agar plate overlaid with hydrocarbons*

Pure isolates are streaked on oil coated agar plates and incubated for one week at desired temperature. Colonies surrounded by an emulsified halo are detected as biosurfactant producers (Morikawa *et al.,* 2000). This is the efficient method where observation of emulsified halo around the culture is the direct indication of amphiphilic biopolymer producing microorganisms.

2.4.2 *Aximetric drop shape analysis (ADSA)*

The drop shape analysis is another optical method for the determination of the surface tension. For screening purposes it was first applied by Vander *et al.,* (1991). The underlying principle is that the shape of a liquid droplet depends greatly on the liquid surface tension. Droplets of liquids with a low surface tension are more apt to deviate from a perfectly spherical shape than droplets of liquids with a high surface tension. The surface tension of the suspension can then be calculated from the droplet profiles with a solution scheme developed by Rotenberg *et al.,* (1984). As shown by Vander *et al.,* (1991) the drop shape analysis can be used to monitor bacterial biosurfactant production.

2.4.3 *Cell surface hydrophobicity technique*

There is a direct correlation between cell surface hydrophobicity and amphiphilic biopolymer production. Depending upon the hydrocarbon uptake behaviour, microorganisms may have high or low surface hydrophobicity. Generally, those microbes which can take hydrocarbon by direct uptake mode, shows high surface hydrophobicity. Cell bound BS production is also associated with hydrocarbon uptake (Franzetti *et al.,* 2008). On the other hand, Bouchez *et al.* (1999) demonstrated that microbes show low surface hydrophobicity when BS/BE are released extracellularly, where hydrocarbon uptake is mediated through the BS. Hydrophobic interaction chromatography, salt aggregation test, bacterial adherence and adhesion (Rosenberg *et al.*, 1984) by replica plating technique helps to identify BS producers.

2.4.4 *Blue agar plate method*

Blue agar or CTAB plate agar method was developed by Siegmund and Wagner, (1991). This technique was specially developed for detection of glycolipids such as rhamnolipids by *Pseudomonas* sp. It can be applied for detection of similar type of BS from other gram negative isolates. This method has been used in many screenings (Ellaiah *et al.,* 2002; Thavasi *et al.*, 2011). Anionic BS forms insoluble ion pair with the cationic CTAB-MB and formation of dark blue halo around the culture is considered as positive for BS production. It is an excellent technique that has been used generally for detection of glycolipids BS.

2.4.5 *Hemolytic activity*

Amphiphilic biopolymers have ability to lyse the erythrocytes. It is a qualitative screening test for detection of amphiphilic biopolymer producers. This technique was developed by Mulligan *et al.*, (1984). Solid media such as Luria agar (LA), nutrient agar (NA), supplemented with 5% fresh whole blood are used (Carrillo *et al*., 2003; Banat, 1993). Blood agar is a complex medium hence it is very difficult to test the BS productivity of a

culture at different culture conditions directly on the agar (Youssef *et al.,* 2004). Hemolytic activity however has been considered an unreliable criterion for the detection of BS activity (Satpute *et al.,* 2010).

2.4.6 *Modified drop collapse method*

Jain *et al.,* (1991) developed the drop collapse assay. This assay relies on the destabilization of liquid droplets by surfactants. The stability of drops is dependent on biosurfactant concentration and correlates with surface and interfacial tension.

Persson and Molin (1987) described a similar assay using a glass surface instead of the oil coated surface. Furthermore, Bodour *et al.,* (1998) showed that for pure surfactant, this assay can even be quantitative by measuring the drop size with a micrometer. An important distinction of this assay is that it can be transferred to an automated screening in microplates, as it has been reported by Maczek *et al.,* (2007). They stained the culture supernatant to enhance the visual effect. The drop collapse assay is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample (Plaza *et al.,* 2006). In addition, it can be performed in microplates (Maczek *et al.,* 2007). This assay has been applied several times for screening purposes (Bodour *et al.,* (2003), Youssef *et al.,* (2004)). But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces.

2.4.7 *Oil spread method*

The oil spreading assay was developed by Morikawa *et al*., (2000). The oil spreading method is rapid and easy to carry out, requires no specialized equipment and just a small volume of sample. It can be applied when the activity and quantity of biosurfactant is low. Youssef *et al*., (2004) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms.

2.4.8 *Emulsification index (EI)*

Another popular assay based on the emulsification capacity of biosurfactants was developed by Cooper and Goldenberg, (1987). The emulsion index E_{24} is calculated as the ratio of the height of the emulsion layer and the total height of liquid. E_{24} correlates to the surfactant concentration. Evaluating the emulsification capacity is a simple screening method suitable for a first screening of biosurfactant producing microbes. It is applied in many screenings, (Nadarajah *et al.,* 2002; Toren *et al.,* 2001 and Fracchia *et al.,* 2012), whereas the kerosene can be replaced with other hydrophobic compounds, e.g., hexadecane. But surface activity and emulsification capacity do not always correlate (Vander *et al.,* 1993). Consequently, this method gives just an indication on the presence of amphiphilic biopolymers.

2.4.9 *Hydrophobic Interaction Chromatography (HIC)*

This is a method which allows the simultaneous isolation and screening of amphiphilic biopolymer producing microbes (Fracchia *et al.,* 2010). They used hydrophobic interaction chromatography (HIC) for this purpose. HIC is a chromatographic procedure based on hydrophobic interaction between the non-polar groups on a hydrophobic chromatographic resin and the non-polar regions of a particle. HIC is very convenient because screening and isolation of potential strains can be combined in one step. Fracchia *et al.,* (2012) demonstrated that HIC is a reliable screening method for biosurfactant production. The technique is also valid for comparative analysis of the hydrophobic properties of microorganisms.

2.4.10 *Tensiometeric measurement of surface tension*

The direct measurement of the interfacial or surface activity of the culture supernatant is the most straight forward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes (Vander *et al.,*1991).

The Du Nouy Ring method is based on measuring the force required to detach a ring or loop of wire from an interface or surface (Fracchia *et al*., 2012). Instead of a ring, a platinum plate, a so called Wilhelmy plate, can be applied in the same manner (Tuleva *et al*., 2005). The Du Nouy Ring assay is widely applied for screening of biosurfactant producing microbes (Cooper *et al*., 1987; Bodour *et al.,* 1998 and Toren *et al*., 2001).

The advantage of this method is the accuracy and the ease of use, however, it requires specialized equipment. A disadvantage is that measurements of different samples cannot be performed simultaneously. Other limitations of this assay include the volume of sample required for analysis, usually some milliliters and the restricted range of concentrations that can be analyzed without dilution (Bodour *et al*., 1998).

2.5 Recovery and purification of biosurfactants/bioemulsifiers

Recovery and purification of biotechnological products in downstream processing costs usually account for approximately 60% of the total production costs which makes commercial production of BS and BE quite expensive. Methods to reduce costs through the use of inexpensive and renewable substrates are therefore necessary (Desai and Banat, 1997; Banat *et al.,* 2000; Makkar and Cameotra, 1997). However, a great deal of monetary input is required in the purification processes (Rodrigues *et al.,* 2010). During all these process the risk of contamination with undesired compounds from fermentation procedures always exist. Ionic charge (chromatography), solubility (water/organic solvents) and location (intracellular, extracellular, cell bound) ultimately determines the purification procedure for AB to be extracted. Generally, purification and precipitation of high molecular weight BS is carried out using ammonium sulphate, followed by dialysis to remove any small molecules. Other methods also involved the use of tri-chloroacetic acid (TCA), acetone precipitation (Rosenberg *et al*., 1984; Patil and Chopade, 2001) ethanol and chloroform/methanol.

2.6 Biological activities of amphiphilic biopolymers

Amphiphilic biopolymers have been shown to have important roles in biomedical application. Some of the most powerful molecules (eg. surfactin, mannosylerythritol lipids (MELs) and trehalose lipids) are known to have anti-inflammatory, anti-tumour, immunosuppressive and immunomodulating functions, in addition to other properties such as self-assembling, human cells stimulation and differentiation, interaction with stratum corneum lipids, cell-to-cell signaling and hemolytic activity.

With most pathogenic microorganisms gaining antibiotic resistance, it is the promising to search new antimicrobial drugs against many pathogens. According to Donadio *et al.*, (2002) microbial metabolites have been recognized as a major source of compounds with ingenious structures and potent biological activities. Among these, biosurfactants have been reported to be suitable alternatives to synthetic medicines and antimicrobial agents and may therefore be used as effective and safe therapeutic agents (Cameotra and Makkar, 2004).

Lipopeptide is kind of low molecular weight amphiphilic biopolymer have the most potent antimicrobial activity (Deleu *et al*., 2008). Surfactin, is the first isolated biosurfactant, through hydrophobic interactions, it can penetrate into the membrane thus influencing the ordering of the hydrocarbon chains and thus varying the membrane thickness (Carrillo *et al*., 2003). Such membrane disruptions are a nonspecific mode of action and are advantageous for action on different cell membranes of both Gram-positive and Gram-negative bacteria. It has been suggested that such action by surfactin type peptides on membrane integrity rather than other vital cellular processes may perhaps constitute the next generation of antibiotics (Rodrigues *et al.*, 2010).

Naruse *et al*., (1990) reported the antiviral effects of surfactin and its analogues. More effective inactivation of enveloped viruses (retroviruses and herpes viruses), were observed

as compared to non-enveloped viruses, showing that inhibitory action links may be due to physico-chemical interactions with the virus envelope (Vollenbroich *et al.,* 1997). Antiviral activity of some lipopeptides therefore may take place as a result of the viral lipid envelope and capsid disintegration due to ion channels formation, with consequent loss of the viral proteins involved in virus adsorption and/or penetration (Seydlova and Svobodova, 2008). In vitro experiments showed that both surfactin and fengycin produced by *Bacillus subtilis* fmbj were able to inactivate cell-free virus stocks of porcine parvovirus, pseudorabies virus, new castle disease virus and bursal disease virus and could effectively inhibit infections and replication of these viruses (Huang *et al*., 2006).

They have antifungal activities, although their action against human pathogenic fungi has been rarely described (Abalos *et al*., 2001; Chung *et al.,* 2000). It has been demonstrated that glycolipids, such as cellobiose lipids and rhamnolipids and cyclic lipopeptides including surfactin, iturin and fengycin can all have varying degrees of antimicrobial activities (Cameotra *et al.,* 2004).

Some reports have shown interesting anti-mycoplasma effects for surfactins. In biomedical research mycoplasma contamination is a frequently occurring serious limitation, particularly when it affects the irreplaceable cell lines which ultimately ends up destroyed. Earlier studies showed that surfactin treatment of mammalian cells that had been contaminated with mycoplasmas permitted specific inactivation of mycoplasmas without significantly damaging effects on cell metabolism in the culture (Vollenbroich *et al.,* 1997). Kumar *et al*., (2007) reported that surfactin was used to eliminate mycoplasma from an extensively infected irreplaceable hybridoma cell line. There were apparent indications of limited elimination, suggesting the possible use of surfactin in achieving total decontamination. However, it was observed that surfactin was toxic to the infected hybridoma cells plated at various cell densities and exposure times, therefore it was suggested

that preliminary tests should be carried out to determine the cytotoxicity of surfactin before use in decontamination. Another study confirmed surfactin potential to eliminate mycoplasma cells independently of the target cell, which is a significant advantage over the mode of action of conventional antibiotics (Fassi *et al*., 2007). This study also indicated that surfactin is able to exhibits a synergistic effect in combination with enrofloxacin, and resulted in mycoplasma-killing activity of about two orders of magnitude greater than when the molecules were used separately.

2.7 Biotechnological commercial applications of amphiphilic biopolymers

Biosurfactants and bioemulsifiers are mainly related to the oil industry, enhanced oil recovery and bioremediation technologies. However, currently due to their broad-range of functional properties amphiphilic biopolymers found are being increasingly used in various industries like detergents and soaps, petroleum, textile, agriculture, cosmetic, medicine and food (Satpute *et al.*, 2010).

OBJECTIVES

The present work aims to contribute to isolation of potential amphiphilic functional biopolymer producing bacteria from environmental samples.

This study therefore emphasized on

- Isolation and screening of amphiphilic biopolymer producing bacteria
- Characterization and evaluation of potential amphiphilic biopolymers

MATERIALS AND METHODS

3.1 Isolation of amphiphilic biopolymer (AB) producing bacterial strains

3.1.1 *Sample collection*

For the isolation of AB producing microorganisms 156 samples (64 soil, 40 contaminated water and 52 sludge samples) were collected from three industrial units and three environmental sites (Table 2). The soil samples were collected in sterile zip locked specimen carrier bag (Himedia, (Mumbai)) and sludge samples were collected in sterile screw capped containers. All the samples were transported in ice to the lab and analyzed within 8 hrs.

Table 2. Various industrial units or environmental sites

Sr. No.	Industrial units/Environmental sites
1.	Patiala Distillery Manufacturing Ltd.
2.	Sunrise Chemicals, Patiala
3.	Federal Mogul Goetze (India), Patiala
4.	Bhakhra Canal, Patiala
5.	River (I) near Patiala
6.	River (II) near Patiala

3.1.2 *Chemicals and media*

All the chemicals and reagents used were of highest analytical grade available and were purchased from Sigma, MO, USA (unless otherwise specified). Standard media components were purchased from Sigma Aldrich (USA) and Himedia (Mumbai).

In order to isolate the amphiphilic biopolymer producing bacteria nutrient broth, agar medium and mineral salt medium were used. The composition of mineral salt medium (grams per litre of distilled water); peptone (5.0), diammonium sulphate (2.0), yeast extract (1.0), $CaCl_2.2H_2O$ (0.7), NaCl (0.1), $MgSO_4$ (0.2), K_2HPO_4 (1.0), Dextrose (1.0), pH 7.0 ± 0.2.

3.2 Screening of AB producing bacterial strains

Biopolymer producing bacteria were isolated from sludge samples collected from different environmental sites and industrial units located across Patiala region in India. Isolates were obtained by serial dilution plating on nutrient agar on mineral salt medium (MSM) agar and plates were incubated at 37°C for 24 hrs. The cell free culture broth or bacterial strains were used for the screening of AB producing bacteria. All the screening experiments were performed in triplicates (until otherwise mentioned) and the mean values are reported. All the cultures were grown at 37°C for 24 hrs routinely and then stored at 4°C for further work.

3.2.1 *CTAB agar plate method*

The CTAB agar plate method is a semi quantitative assay for the detection of extracellular glycolipids or other anionic surfactants. It was developed by Siegmund and Wagner in1991. The microbes of interest are cultivated on a light blue mineral salts agar plate containing the cationic surfactant cetyltrimethylammonium bromide and the basic dye methylene blue. If anionic surfactants are secreted by the microbes growing on the plate, they form a dark blue, insoluble ion pair with cetyltrimethylammonium bromide and methylene blue. Thus, productive colonies are surrounded by dark blue halos.

3.2.2 *Hemolytic activity*

Hemolytic assay was performed in 5% (v/v) human blood (Himedia, Mumbai) agar plates. Bacterial culture grown in mineral salt medium was streak-inoculated on to blood agar plates and incubated for 48 hrs at 37ºC. The plates were visually inspected for clear zone (hemolysis) around the colony. The clear zone is a qualitative method used as an indicator of biosurfactant production (Mulligan *et al.,* 1984; Rodrigues *et al.,* 2010). Standard cultures were used as controls.

3.2.3 *BATH (Bacterial adhesion to hydrocarbon) assay or cell surface hydrophobicity test*

Cell hydrophobicity was measured by bacterial adherence to hydrocarbons according to a method described by Rosenberg *et al*., (1984). The cell pellets were collected, washed twice and suspended in a buffer salt solution (K_2HPO_4 16.9 gL^{-1} and KH_2PO_4 7.3 gL^{-1}) and diluted using the same buffer solution to an optical density (OD) of ~ 0.5 at 610 nm. To the cell suspension (2 ml) in test tubes 100 µl of diesel oil was added and vortex-shaken for 3 min. After shaking, crude oil and aqueous phases were allowed to separate for 1 h. Optical density of the aqueous phase was then measured at 610 nm in a spectrophotometer (UV-1800, Shimadzu). From the absorbance (OD) values, percentage of cells attached to crude oil was calculated using the following formula:

$$\% \text{ of bacterial cell adherence} = (1-(OD_A / OD_B)) \times 100$$

Where: OD_A was the absorbance of the cell suspension after addition of oil
OD_B is the absorbance of cell suspension (set as 0.5)

3.2.4 *Drop collapse test*

Drop collapse test was performed as described by Bodour and Maier (1998). Two microlitres of diesel oil was added to the 96 well microtitre plates. The plates were equilibrated for 1 hr at 37 ºC and five microlitres of culture supernatant was added to the surface of the oil. The shape of drop on oil surface was observed after 1 min. The culture supernatant that make the oil drop collapsed was indicated as positive result and that drops remain beaded were scored as negative. SDS (Sodium dodecyl sulphate) was used as positive control.

3.2.5 *Surface tension measurement*

For the screening of the biopolymer to be amphiphilic nature, surface tension was determined by ring method (Thavasi *et al*., 2011). Du Nouy ring tensiometer was used at room temperature (25± 2°C) at end of the incubation time that was 60 hrs. These

measurements were made on cell free broth after centrifugation. Distilled water was taken as control.

3.2.6 *Emulsification stability (E_{24}) test*

Emulsification stability or emulsification index was determined as per the method described by Thavasi *et al.*, (2011). E_{24} of samples was determined by adding 2 ml of oil or hydrocarbon (Petrol and Diesel (Dhillon petrol pump, Bhadson road, Patiala), Xylene (AR, Ranbaxy), vegetable oil (Easy Day market, Bhadson road, Patiala) kerosene (Depo, Tripuri, Patiala)) to the same amount of cell free broth and vortex for 2 min and leaving to stand for 24 hours. The E_{24} index is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm).

$$E_{24} = (\text{Height of emulsified layer} / \text{total height}) * 100$$

3.3 Optimization studies

A) Optimization of Temperature

Stability studies were performed using the cell-free culture broth obtained by centrifugation at 10000 rpm for 10 min when the cultivation was maintained after 60 h. Two millilitres of the culture free of cells was maintained at 60ºC, 80ºC, 100ºC and 120ºC for 15 min and then kept at room temperature. At last E_{24} was measured with different hydrocarbons (petrol, diesel and xylene).

B) Optimization of pH

To study the effect of varying pH on the stability and emulsification activity of cell free culture of the cell-free culture, 100 mL of culture free of cells was taken from the late stationary phase (60 hrs) and was adjusted to different pH values (2, 4, 6 and 8) with 1N NaOH or 1N HCl solution. At last E_{24} was measured with different hydrocarbons (petrol, diesel and xylene) at room temperature.

3.4 Cell growth, extraction and purification of amphiphilic biopolymer

The growth kinetics of the amphiphilic biopolymer producing strains were studied in the mineral salt medium in batch culture. The emulsification activity and yield of the obtained biopolymers was recorded simultaneously.

On the basis of emulsification index, supernatant of 60 hrs old culture was used for the extraction of AB. Precipitation of AB from the supernatant was done with double the volume of chilled acetone for 24 hrs. In order to recover the biopolymer, the mixture was centrifuged at 10000 rpm for 10 min at 4ºC and the pellet obtained was purified by CPC (cetylpyridinium chloride) treatment. Further purification was done by addition of equal volume of NaCl (1N) and double the volume absolute ethanol and centrifuged at 10000 rpm for 20 min at 4ºC. Pellet was dissolved in water, frozen at -20ºC and lyophilized. The characterization of five biopolymers (powdered form) was performed through biochemical analysis and FTIR spectroscopy.

3.5 Compositional analysis of ABs

3.5.1 *Determination of total proteins* (Lowry *et al.,* 1951)

Folin-Lowry method was used to determine the total protein in the amphiphilic biopolymer. Bovine serum albumin (BSA) was used as standard in different concentrations (0.1 - 0.5 $mgmL^{-1}$) and biopolymer stock was 1 $mgmL^{-1}$. 50 mL of reagent A and 50 mL of (reagent B) were mixed to make the complex-forming reagent. 1 mL of freshly mixed complex-forming reagent was added to 0.2 mL of the sample or standard. The solution was left undisturbed for 10 min at room temperature. The absorbance was measured at 750 nm. The amount of total protein present in the sample was calculated from the standard curve prepared by using pure BSA as standard.

3.5.2 ***Determination of total sugars*** (Dubois *et al.*, 1956)

The total sugar or carbohydrate content of amphiphilic biopolymer was determined by phenol sulphuric acid method; 200 µl of phenol reagent (5 % v/v in water) was added to standards as well as samples. After the addition of phenol reagent 1.0 mL of concentrated sulphuric acid was rapidly added to the surface of the solution without touching the sides of the test tube. The tubes were left undisturbed for 10 min at room temperature. After incubation the tubes were shaken vigorously and absorbance was measured after 30 min at 490 nm. The total sugar content present in the sample was calculated from standard curve (Glucose, 0-1 $mgmL^{-1}$).

3.5.3 ***Determination of hexosamine***

To determine the hexosamine content in amphiphilic biopolymer Elson-Morgan method (1934) was followed; 250 µl of standards (0-1 $mgmL^{-1}$, galactosamine) and samples were added to 50 µl of reagent A (Annexure I). Then each mixture was heated to 100ºC for 3 min. After cooling rapidly to room temperature 1.5 mL of reagent B (Annexure I) was added, washing down any condensate formed. The mixture was then incubated 37ºC for 20 min, after cooling to room temperature, the absorbance was measured at 585 nm. The hexosamine content present in the sample was calculated from standard curve (Galactosamine, 0-1 $mgmL^{-1}$).

3.5.4 ***Determination of uronic Acid***

The uronic acid content of amphiphilic biopolymer was determined by method described by Haug and Larsen, (1962). 250 µl of sample or standards (0-1 $mgmL^{-1}$, D-glucuronic acid) and controls were cooled in an ice bath. 1.5 mL of ice cold reagent A (Annexure I) was carefully added with mixing and cooling in ice bath. The mixture was rapidly cooled in the ice-bath. 50 µl of reagent B (Annexure I) was added and mixed well.

Reheating was done at 100ºC for 15 min. The mixture was cooled rapidly to room temperature and the absorbance was measured at 525 nm. The uronic acid content present in the sample was calculated from standard curve (D-glucuronic acid, 0-1 $mgmL^{-1}$).

3.5.5 ***Determination of pyruvic acid***

The pyruvic acid content of amphiphilic biopolymer was determined by method described by Friedman and Haugen, (1943). For the determination of the pyruvic acid content of the amphiphilic biopolymer samples (5 mg dissolved in 5mL distilled water) were firstly treated with perchloric acid (50%) for deproteinization and were kept at 30ºC for 30 min. Then 1 mL of DNP reagent (Annexure I) was shaken to the extract. Further, 4 mL of water and 10 mL 2.2 N NaOH were added. The tubes were shaken and absorbance was measured at 416 nm. A standard curve was made by using pyruvic acid (0-3 $mgmL^{-1}$).

3.6 Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore can be used to elucidate some components of an unknown mixture. One milligram of freeze-dried partially purified biosurfactant was ground with 100mg of KBr and pressed with 7500 kg for 30 seconds to obtain translucent pellets. Infrared absorption spectra were recorded on a FTIR system (Spectrum RX-IFTIR, Perkin-Elmer) with a spectral resolution and wave number accuracy of 4 and 0.01cm -1, respectively. All measurements consisted of 500 scans, and a KBr pellet was used as background reference.

3.7 Characterization of potential biopolymer producing bacteria

3.7.1 *Hydrogen Sulfide Test*

Sulfide indole motility medium (SIM) agar tubes were inoculated with 24-48 hrs grown cultures with stab inoculation and incubated at 37ºC for 24 hrs. The strains that were produce black coloration along the line of the stab inoculation were positive for hydrogen sulfide test. Uninoculated tube was taken as negative control. Standard cultures were used as controls.

3.7.2 *Urease Test*

Urea broth was inoculated with 24 hrs grown cultures by mean of loop inoculation and incubated at 37ºC for 24 hrs. On the addition of phenol red indicator the strain that produces pink color was positive and no change in the color of the broth was negative for urease test.

3.7.3 *Methyl Red Test*

Using sterile technique, MR-VP broth was inoculated with 24 hrs grown cultures and incubated at 37ºC for 24-48 hrs. After incubation few drops of methyl red indicator was added to the culture. Red color indicates the MR positive strain.

3.7.4 *Voges-Proskauer Test*

Using sterile technique, MR-VP broth was inoculated with 24 hrs grown cultures and incubated at 37ºC for 24-48 hrs. The reagent used in this test, Barrett's reagent, consists of a mixture of alcoholic α-naphthol and 40% potassium hydroxide solution. Development of a deep rose color in the culture 15 min following the addition of Barrett's reagent is indicative of the presence of acetylmethylcarbinol and represent a positive result. The absence of rose coloration is a negative result.

3.7.5 *Citrate Utilization Test*

Using sterile technique, cultures were inoculated by streak inoculation on the Simmon Citrate agar slants and incubated at 37ºC for 24-48 hrs. Citrate positive cultures were

identified by the presence of growth on the surface of the slant along with blue color of the agar slants. There is no growth in case of citrate negative cultures and color of the media remains green.

3.7.6 *Indole Production Test*

SIM (Sulfide indole motility medium) agar tubes were inoculated with 24-48 hrs grown cultures with stab inoculation and incubated at 37ºC for 24 hrs. Cultures producing a red layer on the addition of Kovac's reagent are indole positive. Kovac's reagent is composed of *p*-dimethylaminobenzaldehyde, butanol and hydrochloric acid.

3.7.7 *Starch Hydrolysis Test*

Starch agar plates were made for inoculation. The petriplates was divided into small sections. Then plate inoculated by spot inoculation method and incubated at 37ºC for 24-48 hrs. After incubation the plate was flooded with Gram's iodine solution, allowed the Gram's iodine for 30 sec and then pours off the excess. Then the cultures were observed for the presence and absence of the blue-black color surrounding the growth of each colony.

3.7.8 *Catalase Test*

Trypticase soy agar plates were made for inoculation. Each petriplate was divided into four sections. Plates were inoculated by spot inoculation method and incubated at 37ºC for 24-48 hrs. 3-4 drops of hydrogen peroxide (3%) were allowed to flow over each colony. Cultures were observed for the presence or absence of bubble formation.

RESULTS AND DISCUSSIONS

4. RESULTS AND DISCUSSION

4.1 Isolation of AB producing bacterial strains

A total of 156 samples (collected from industrial sites) were collected from various regions of Patiala, Punjab, INDIA (Table 2). Preliminary screening was done on the basis of mucoid nature of colonies and 71 bacterial isolates were screened. Further all the bacterial isolates were screened for gram character and 23 were found to be gram negative. In the present study all the 23 bacterial strains were grown in minimal mineral salt media with glucose as sole carbon source, and further the AB producing strains were screened through various screening methods.

4.2 Screening of bacterial isolates for AB producing ability

The isolates were named on the basis of the source they were collected from. Table 3 illustrated that out of 23 isolates, 11 strains (PD2, SC1, R(I)2, R(II)1, R(II)2, FMG4, FMG5, AK 21 and SC4) were found to be positive for the CTAB agar plate method showing blue zone around the well. SDS (Sodium dodecyl sulfate) was used as control in CTAB agar plate method. This method was used to detect glycolipids or anionic amphiphilic biopolymers (Siegmund & Wagner, 1991). The CTAB agar assay is a specific for anionic biosurfactants and its major limitation is that CTAB is harmful and may inhibit the growth of some microbes. So, another screening method opted for screening was hemolytic assay.

Hemolysis is widely used to screen amphiphilic biopolymer producing bacteria and in some cases, have been shown to be invaluable (Banat, 1993; Youssef *et al.*, 2004). Results of present study on hemolytic activity of 23 bacterial strains revealed that 11 strains (PD1, PD3, SC1, SC2, R(II)1, R(II)2, BC1, BC2, FMG6, FMG8 and SC3) showed hemolysis and other bacterial strains were found to be partially hemolytic or non hemolytic in nature (Table 3). Various authors recommended blood agar lysis as a preliminary screening method for AB production (Mulligan *et al.* 1984; Youssef *et al.,* 2004).

BATH (Bacterial Adhesion to Hydrocarbons) assay conducted in this study with 23 isolates revealed that SC3 showed highest adhesion to diesel oil that is 42.03 %, and R(I)2 showed 40.5% adhesion to hydrocarbon (Table 3). Other isolates showed less adhesion or no adhesion to the hydrocarbon. This assay is meant for estimation of cell hydrophobicity. Cell adherence with hydrophobic compounds like crude oil is considered as an indirect method to screen bacteria for amphiphilic biopolymer production because the cells attach themselves with oil droplets by producing surface active amphiphilic biopolymers (Rosenberg *et al*., 1980).

Drop collapse assay has been reported by several authors (Vander *et al.,* 1991; Youssef *et al*., 2004; Thavasi *et al*., 2011) for screening of AB (low molecular weight). Result of drop collapse test (performed with petrol and diesel oil as hydrocarbons) revealed that SC2, R(II)1, R(II)2, FMG1, BC2, BC3, FMG5, FMG7, FMG8 and SC3 were positive for drop collapse test. Table 3 revealed the results of drop collapse method against all the 23 bacterial strains. But it displays a relative low sensitivity since a significant concentration of surface active compounds must be present in order to cause a collapse of the aqueous drops on the oil or glass surfaces. Surface tension measurement of cell fee culture broth revealed that out of 23 strains, all strains showed reduction in surface tension in the range of 48-64 mN/m. The highest surface tension reduction was observed in namely R(II)2 and FMG4. The two strains R(II)2 and FMG4 showed reduction in surface tension to 47.9 mN/m and 48.7 mN/m, respectively.

The direct measurement of the interfacial or surface activity of the culture supernatant is the most straight forward screening method and very appropriate for a preliminary screening of biosurfactant producing microbes. This gives a strong indication on biosurfactant production. There was a direct correlation found between drop collapse method and surface tension assays. Strains highly active in any one of these methods was active in

the other method. Similar correlation was reported by Bodour and Miller-Maier, (1998). As the result of these screening methods, five bacterial strains (R(I)1, FMG2, FMG3, FMG8 and SC3) were screened for further study.

Table 3. Screening of AB producing bacterial strains

Sr. No.	Isolate	Drop collapse assay[a]	Bath assay[b]	CTAB assay[c]	Hemolysis assay[d]
1.	PD1	-	-	-	+
2.	PD2	-	-	+	-
3.	PD3	-	-	-	+
4.	SC1	-	++	+	++
5.	SC2	++	+ +	-	+
6.	R(I)1	-	-	-	-
7.	R(I)2	-	++ +	++	-
8.	R(I)3	-	-	-	-
9.	R(II)1	++	-	+	+
10.	R(II)2	++	-	++	+
11.	FMG1	++	-	-	-
12.	FMG2	-	+	-	-
13.	FMG3	-	+ +	-	-
14.	FMG4	-	-	+	-
15.	BC1	-	+	-	++
16.	BC2	++	-	-	+
17.	BC3	++	-	-	-
18.	FMG5	++	-	+	-
19.	FMG6	-	+ +	-	++
20.	FMG7	++	+	-	-
21.	FMG8	++	+	+	+
22.	SC3	++	+++	-	++
23.	SC4	-	+	++	-
24.	SDS (+control)	+++		+	
25.	DW (- control)	-		-	

Drop collapse assay[a]: '+++' flat drop after 1 min, '++' less flat drop after 1 min, '+' little flat as compared to –ve control

BATH assay[b]: '+++' cell adhesion > 40 %, '++' cell adhesion > 10 % and '+' cell adhesion > 0.2

CTAB assay[c]: '++' blue zones with > 0.6 cm, '+'blue zones with 0.6 cm

Hemolytic assay[d]: '++' hemolytic, '+' partially haemolytic, '-' non-hemolytic

In this study, emulsification stability or emulsification index was checked with different kinds of hydrocarbons or oils (e.g. petrol, diesel, xylene, mustard oil and kerosene). Results observed revealed that most of the cell free broths of the isolates showed positive emulsification with petrol, diesel, and xylene but there were no isolate showing emulsification with mustard oil and kerosene (Table 4). Results revealed that out of 23 bacterial isolates, only five bacterial strains showed emulsification activity against three hydrocarbons (diesel, petrol and xylene), and all the other 18 isolates showed very low or negligible emulsification activity. Emulsification index of R(I)1 with petrol, diesel, xylene was 50, 13.6 and 54.5 respectively; E_{24} of FMG8 with petrol, diesel, xylene was 45, 9 and 45 respectively. Emulsification assay is an indirect method to screen amphiphilic biopolymer production (Youssef *et al.*, 2004). It was assumed that if a cell free culture broth used for this assay, contains amphiphilic biopolymer, it will emulsify the hydrocarbons present in the test solution.

Table 4. Emulsification Index of amphiphilic biopolymer producing bacterial isolates towards different hydrocarbons

Sr. No.	Isolate	Petrol	Diesel	Xylene	Mustard oil	Kerosene
1.	PD1	-	-	-	-	-
2.	PD2	+ (10)	-	-	-	-
3.	PD3	-	-	-	-	-
4.	BC1	+ (10)	+ (9)	++ (21.7)	-	-
5.	BC2	-	-	-	-	-
6.	BC3	-	-	++ (58.3)	-	-
7.	R(I)1	++ (50)	+ (13.6)	++ (54.5)	-	-
8.	R(I)2	-	-	-	-	-
9.	R(I)3	-	-	-	-	-
10.	R(II)1	-	-	-	-	-
11.	R(II)2	-	-	-	-	-
12.	FMG1	-	-	-	-	-
13.	FMG2	++ (40)	-	-	-	-
14.	FMG3	-	-	++ (63.6)	-	-
15.	FMG4	++ (31.5)	+ (4.5)	+ (8.6)	-	-
16.	FMG5	++ (40)	+++(63.6)	-	-	-
17.	FMG6	+ (11)	-	+ (8.6)	-	-
18.	FMG7	-	+ (8.6)	+ (10)	-	-
19.	FMG8	++ (45)	+ (9)	+++ (45)		
20.	SC1	-	-	-	-	-
21.	SC2	-	-	-	-	-
22.	SC3	+ (10.5)	-	+ (9)	-	-
23.	SC4	-	-	-	-	-
24.	SDS (+ control)	+++	+++	+++	+++	+++
25.	Water (- control)	-	-	-	-	-

Values are in percentage

Microbial ABs are complex molecules, comprising of different structures that include lipopeptides, glycolipids, polysaccharide protein complexes, fatty acids and phospholipids. The commercial importance of AB is evidenced from the increasing trends in their production and the number of industrial applications (Ron and Rosenberg, 2001; Cameotra *et al.,* 2004). Their environmental uses are related principally to the bioremediation of petroleum hydrocarbons in groundwater and soil and in the degradation of hazardous

compounds. In the oil industry, they are used in microbial-enhanced oil recovery, in the cleaning of contaminated vessels and to facilitate transportation of heavy crude oil by pipeline (Perfumo *et al.*, 2006). So, on the basis of above mentioned screening methods, five potential bacterial strains were selected for maximum AB producing ability.

4.3 Growth kinetics and amphiphilic biopolymer production

In order to establish the amphiphilic biopolymer production kinetics by all the five strains, they were cultured on mineral salt medium. The emulsification activity as well as biopolymer synthesis (yield) was measured at various growth phases during the growth in batch culture.

Table 5. Biopolymer yield (mg/L) by a) R(I)1, b) FMG2, c) FMG3, d) FMG8 and e) SC3

Time (hrs)	R(I)1	FMG2	FMG3	FMG8	SC3
0	0	0	0	0	0
4	0	0	0	0	0
8	0	0	0	0	0
12	3.1±0.1	3.4±0.1	7.5±0.1	4±0.1	4.8±0.1
16	4.6±0.1	5.1±0.1	9.1±0.1	8.1±0.1	9.6±0.2
20	10±0.2	11.5±0.2	14.3±0.2	14.1±0.2	12±0.2
24	32±0.2	26±0.3	29±0.4	24±0.2	32±0.2
28	40.1±0.4	35.2±0.2	32.6±0.3	31.8±0.4	36.1±0.4
32	52.5±0.5	49.5±0.5	44.5±0.5	41.9±0.5	41.6±0.5
36	70.3±0.7	54.2±0. ±0.5	58.5±0.5	59.2±0.5	55.5±0.5
40	110.6±0.6	81.2±0.6	82.5±0.6	90.1±0.6	104.1±0.6
44	170.6±0.8	101.1±0.6	109.4±0.6	120.4±0.8	131.4±0.8
48	228.2±0.9	120.1±0.8	140.5±0.8	165.7±0.8	161.3±0.8
52	295.9±1	171.5±0.8	190.9±0.8	192±0.8	199.5±0.8
56	371±1	203.8±0.8	211.1±0.8	220.3±0.8	215.5±0.8
60	404.8±1	239.1±0.8	254.6±0.8	263.2±1	259.4±0.8
64	403.1±1	234.2±0.9	249.1±0.9	261±1	252.9±1
68	395.5±1	231.4±0.9	248.8±1	260.7±1	251.9±1
72	368.7±1	219.5±1	233.2±1	246.2±1	240.3±1

± represents standard deviation from triplicate data

As shown in the Table 5 and Figure 4, the biopolymer production was observed to increase during the stationary phase and its yield showed decline after 60 hours.

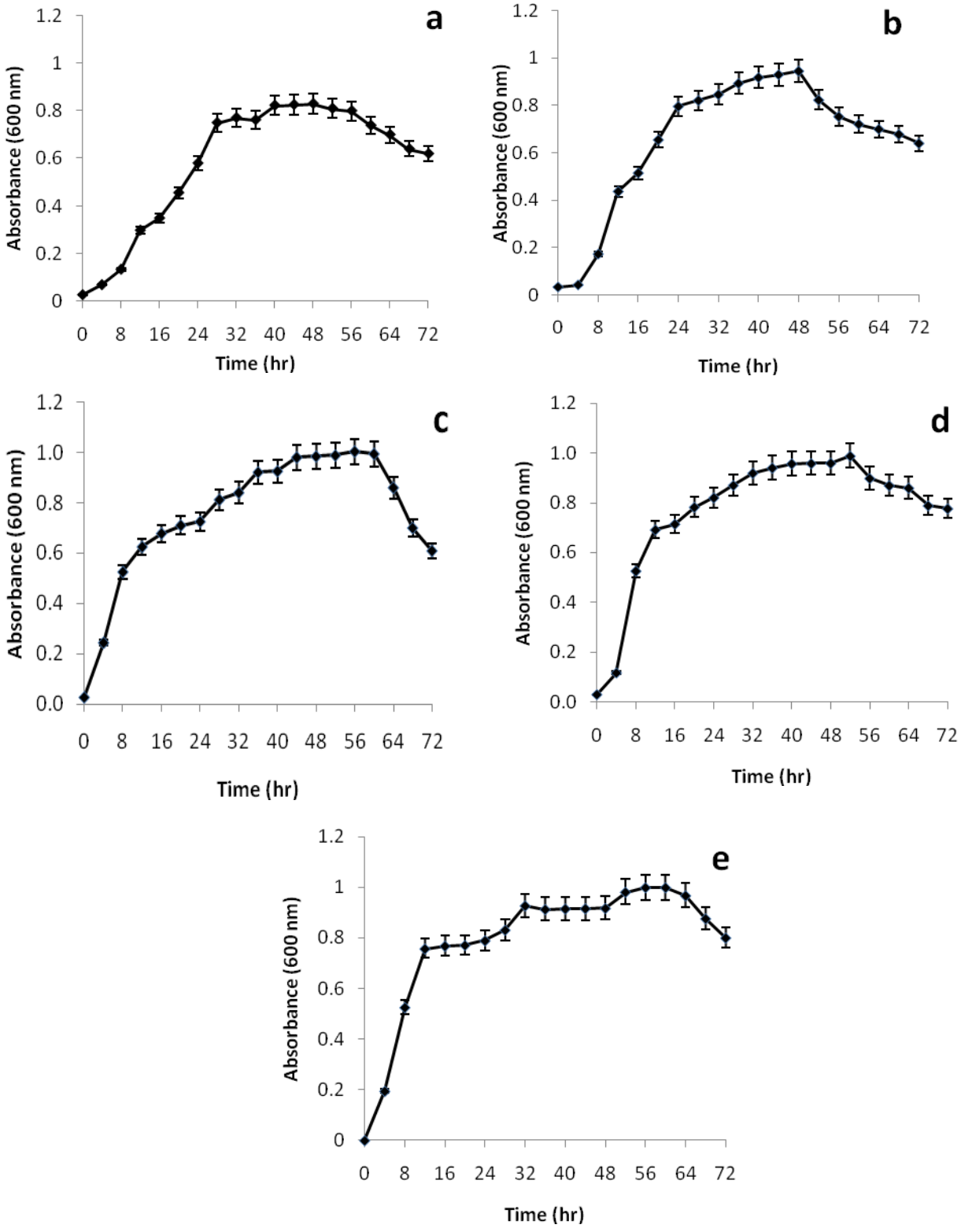


Figure 4. Growth kinetics of biopolymer producing strains a) R(I)1, b) FMG2, c) FMG3, d) FMG8 and e) SC3. Error bar indicate standard deviation from mean on the basis of three replicate experiments.

Initially with increase in the production of amphiphilic biopolymer, the emulsification rate also increased. Simultaneously results of emulsification activity with hydrocarbons (petrol, diesel and xylene) at the interval of 12 hour were depicted in Table 6. Results revealed that all the five strains were observed with lower emulsification activity for first 24 hours against the selected hydrocarbons. A significant increase ($p < 0.05$) in the activity was observed till 60 hours of bacterial growth and further decline in the activity was observed. Specific growth rates of the bacterial isolates (R(I)1, FMG2, FMG3, FMG8 and SC3) were 0.173, 0.198, 0.182, 0.277 and 0.289 respectively.

Table 6. Emulsification index of biopolymer producing bacterial strains with time towards different hydrocarbons

Cell Free Broth	Oil or hydrocarbon	12 hrs	24 hrs	36 hrs	48 hrs	60 hrs	72 hrs
R(I)1	Petrol	-	5.5±0.1	5.3±0.1	5.0±0.1	45.5±0.1	15±0.1
	Diesel	-	-	-	9±0.1	4.3±0.1	4.5±0.1
	Xylene	4.3±0.1	4.3±0.1	4.3±0.1	4.5±0.1	8.7±0.1	9.5±0.1
FMG2	Petrol	-	43.5±0.1	20±0.1	56.5±0.6	14.3±0.1	18.2±0.1
	Diesel	9±0.1	13.04±0.1	14.3±0.1	18.2±0.1	9.1±0.1	9.1±0.1
	Xylene	-	59.1±0.6	18.2±0.1	54.5±0.5	52.4±0.7	59.1±0.1
FMG3	Petrol	-	-	19.04±0.2	5±0.1	11.1±0.1	14.3±0.1
	Diesel	-	-	9.5±0.1	4.7±0.1	19.04±0.2	9.5±0.1
	Xylene	-	14.2±0.1	14.3±0.1	4.5±0.1	61.9±0.9	59.1±0.1
FMG8	Petrol	5.5±0.1	33±0.3	50±0.5	21.0±0.4	47.3±0.5	30±0.1
	Diesel	4.3±0.1	18.1±0.2	9.5±0.1	17.3±0.2	52.2±0.5	-
	Xylene	-	17.3±0.2	54.1±0.6	54.3±0.6	65.2±0.6	56.5±0.6
SC3	Petrol	23.5±0.2	-	-	36.8±0.4	50±0.1	50±0.6
	Diesel	8.7±0.1	-	9.5±0.1	8.7±0.1	27.3±0.1	9.5±0.1
	Xylene	26.1±0.2	-	-	27.3±0.4	45.2±0.5	40.0±0.5

± represents standard deviation from triplicate data

As the five bacterial strains (R(I)1, FMG2, FMG3, FMG8 and SC3) showed higher emulsification activity, they have been further used for amphiphilic biopolymer production. Glucose was only used as carbon substrate in this study for AB production. In earlier studies

by Youssef *et al*., (2004) maximum biopolymer production of 90 mg/L by *B. subtilis* by using glucose as carbon source. Cooper & Goldenberg, (1987) reported sucrose as carbon source for maximum biopolymer production by *B. cereus*. Following the culturing of bacteria for 60 hours and extraction the solid yield was 404.8 mgL^{-1} (R(I)1), 239.1 mgL^{-1} (FMG2), 254.6 mgL^{-1} (FMG3), 263.2 mgL^{-1} (FMG8) and 259.4 mgL^{-1} (SC3). The amphiphilic biopolymers obtained following lyophilization had white appearance and hygroscopic nature.

4.4 Optimization of physical conditions emulsifying activity of functional AB

4.4.1 *Effect of temperature on emulsification activity*

The effect of temperature on emulsification activity of all the five potential strains is illustrated in Table 7. The temperature range was from 60ºC to 120ºC for the optimization study. The results obtained were promising as even after the 15 min exposure to the higher temperature (100ºC and 120ºC) FMG8 and SC3 strains showed good emulsification activity in comparison to at 60ºC and 80ºC with petrol, diesel and xylene. R(I)1, FMG2 and FMG3 showed no emulsification at 120ºC, whereas emulsification was observed against all the three hydrocarbons at 100ºC, 80ºC and 60ºC.

Table 7. Effect of temperature on emulsification activity of biopolymer produced by five bacterial strains

Isolates	Temperature	Petrol	Diesel	Xylene
R(I)1	60ºC	-	4.5±0.1	-
	80ºC	11.1±0.1	14.3±0.1	14.3±0.1
	100ºC	52.6±0.6	22.8±0.2	9.1±0.1
	120ºC	-	4.8±0.1	-
FMG2	60ºC	5.3±0.1	4.8±0.1	4.8±0.1
	80ºC	10.0±0.1	9.5±0.1	14.3±0.2
	100ºC	52.6±0.7	22.8±0.1	9.1±0.1
	120ºC	-	-	-
FMG3	60ºC	10±0.1	9±0.1	8.7±0.1
	80ºC	11.8±0.1	5.0±0.1	10±0.1
	100ºC	52.6±0.6	45.5±0.6	45.5±0.6
	120ºC	-	-	-
FMG8	60ºC	16.6±0.2	14.3±0.2	14.3±0.2
	80ºC	22.2±0.2	23.8±0.2	14.3±0.2
	100ºC	31.2±0.2	23.8±0.2	14.3±0.2
	120ºC	15±0.2	21.7±0.2	25±0.2
SC3	60ºC	26.3±0.2	10±0.2	23.8±0.2
	80ºC	47.4±0.6	31.8±0.5	54.5±0.7
	100ºC	47.6±0.5	28.6±0.2	57.2±0.7
	120ºC	52.6±0.6	47.8±0.4	56.5±0.7

± represents standard deviation from triplicate data

4.4.2 *Effect of pH on emulsification activity*

The pH range from 2 to 8 was used for the optimization studies. As shown in Table 8 the change in pH had no effect on the emulsification activity of the bacterial strains. Cell free broths of the isolates showed good emulsification activity with petrol as compared to diesel and xylene.

Table 8. Effect of pH on emulsification activity of biopolymer produced by five bacterial strains

Isolates	pH	Petrol	Diesel	Xylene
R(I)1	2	38.9±0.3	4.7±0.1	9.1±0.1
	4	38.9±0.3	9.5±0.1	9.5±0.1
	6	11.1±0.1	9.5±0.1	9.5±0.1
	8	5.3±0.1	-	-
FMG2	2	16.7±0.2	4.5±0.1	4.5±0.1
	4	35.3±0.3	14.3±0.2	21.2±0.2
	6	16.7±0.2	-	4.8±0.1
	8	30.0±0.3	-	4.8±0.1
FMG3	2	47.4±0.5	4.8±0.1	36.4±0.3
	4	47.4±0.5	9.5±0.1	52.4±0.7
	6	44.4±0.5	20.0±0.2	33.3±0.3
	8	5.9±0.1	4.8±0.1	9.5±0.1
FMG8	2	5.9±0.1	18.2±0.3	-
	4	22.2±0.2	9.1±0.1	19.0±0.2
	6	-	9.1±0.1	4.8±0.1
	8	-	9.1±0.1	4.8±0.1
SC3	2	5.6±0.1	23.8±0.2	4.8±0.1
	4	10.0±0.1	9.5±0.1	13.6±0.2
	6	11.1±0.1	19.0±0.2	14.3±0.2
	8	5.3±0.1	9.1±0.1	4.3±0.1

± represents standard deviation from triplicate data

An important property of surfactants is their stability over a wide range of temperature, time, and pH (Rufino *et al*., 2007, Xing *et al*., 2009). AB extracted from *Bacillus subtilis* CCTCC AB93108 reported by Zhi-feng *et al.*, (2010) stabilized emulsions of several aromatic and aliphatic hydrocarbons, such as benzene, xylene, n-pentane, n-nonane, gasoline and diesel oil. This showed both high emulsification activity and stability over a wide range of temperature (40−100℃) for long duration. Our results were in accordance with the earlier studies on the effect of pH and temperature on the emulsification activity (Zhi-feng *et al.*, 2010).

4.5 Compositional Characterization

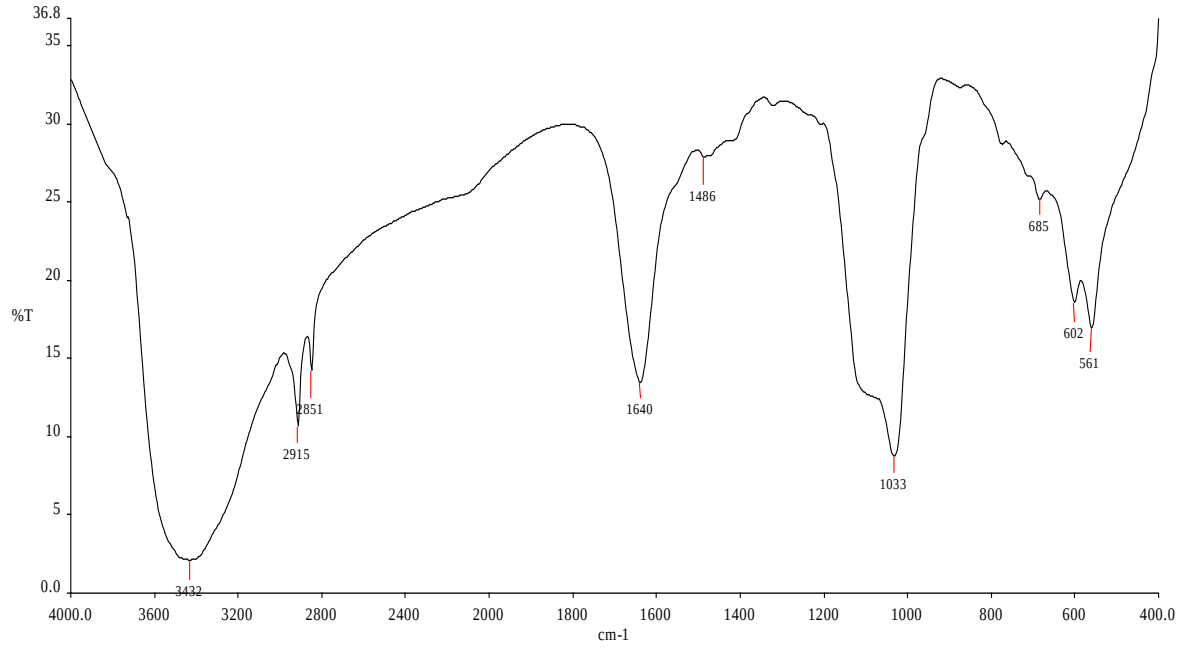
Table 9. Compositional characterization of biopolymers

Biopolymer	R(I)1	FMG2	FMG3	FMG8	SC3
Total Sugar	3.6 %	3.6 %	4.8 %	5.1 %	1.0 %
Total protein	12.0 %	8.0 %	7.0 %	6.0 %	8.0 %
Uronic acid	11.7 %	12.4 %	9.3 %	34.3 %	31.4 %
Pyruvic acid	0.8 %	0.9 %	1.2 %	1.0 %	1.0 %
Hexosamine	1.4 %	2.2 %	1.9 %	2.5 %	2.8 %

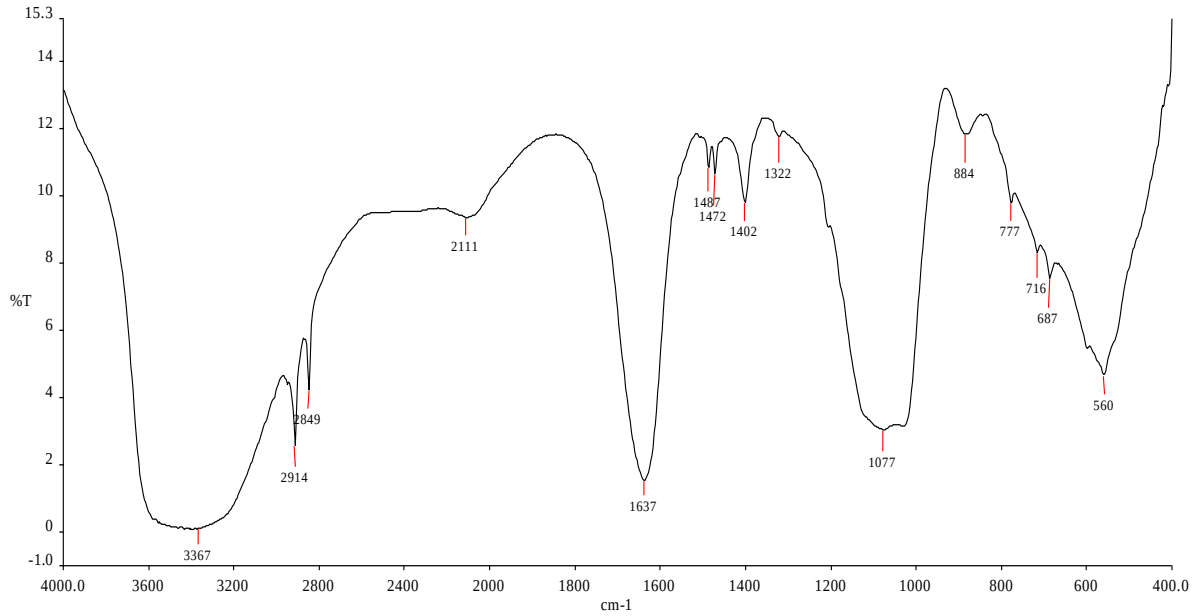
Table 9 showed the composition (%) of the five amphiphilic biopolymers. All the five amphiphilic biopolymers are the mixture of carbohydrate, protein, uronic acid, pyruvic acid.

4.7 Functional group analysis: IR

(a)



(b)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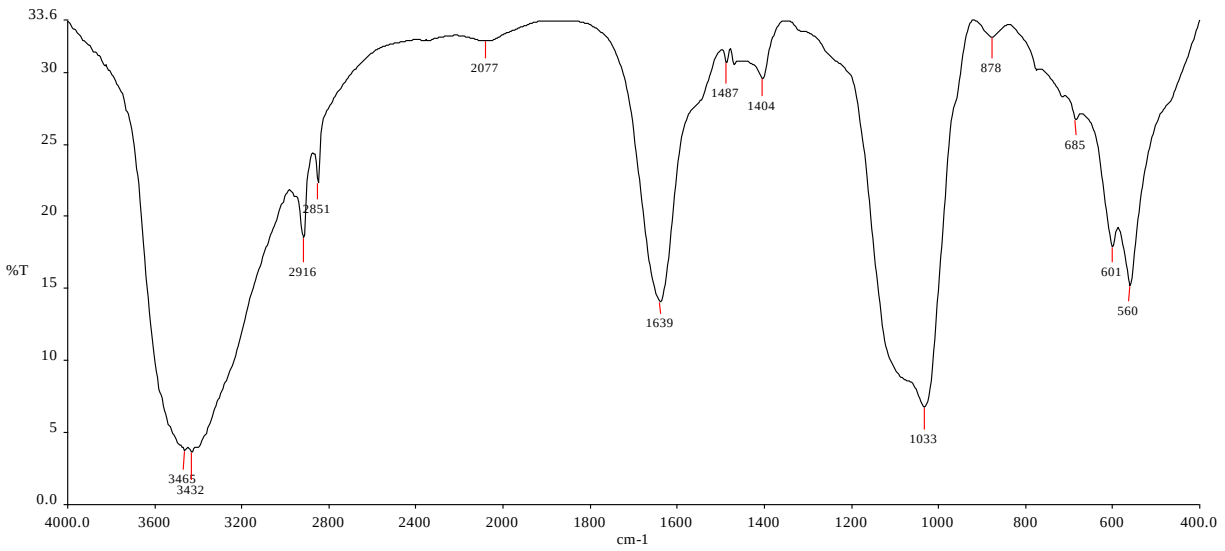
(c)

(d)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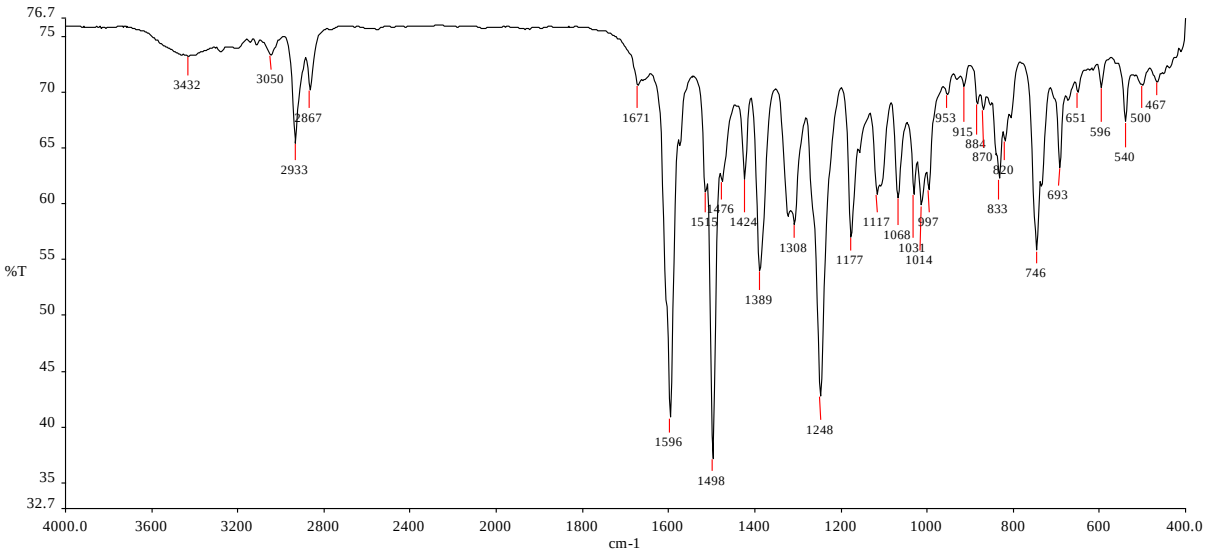

(e)

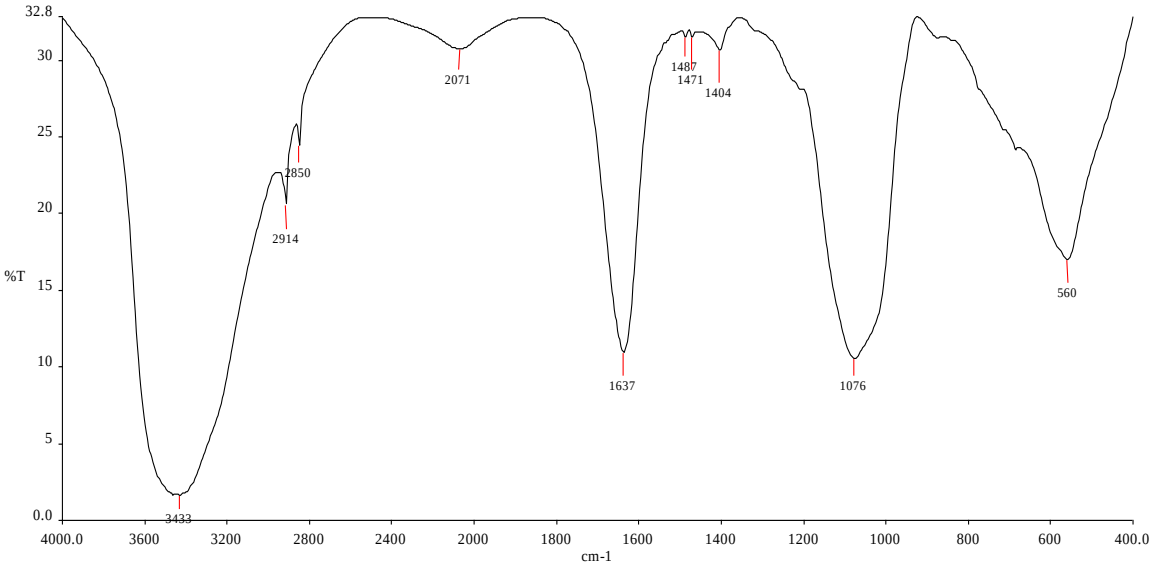

Figure 5. FTIR spectra of amphiphilic biopolymers produced by a) R(I)1, b) FMG2, c) FMG3, d) FMG8 and e) SC3

Figure 5 (a) depicts the FTIR spectral analysis of AB (R(I)1, which inferred that wave number 3432 cm^{-1} indicated the presence of N-H/C-H bonds of protein. CH_2 /C-H asymmetric vibrations were found at 2915 and 2851cm^{-1} which confirmed the (C-H) presence

of alkanes. 1640 and 1486cm^{-1} bands showed that the biopolymer had C=O stretching. Wave number 1033 cm^{-1} indicated the presence of C-O stretching. Bands in the region 685, 602 and 561 cm^{-1} showed the presence of C-H/C-C=O deformation. In the case of FMG2 biopolymer, FTIR spectral analysis (Figure 5 (b)) inferred that wave number 3367 cm^{-1} indicated the presence of amine groups. 2914, 2849, 2111, 1487, 1472 and 1402 cm^{-1} confirmed the presence of alkanes. The wave number 1637 cm^{-1} indicated the presence of peptide groups. The wave number 1322 cm^{-1} showed the presence of CH_3 deformation and 1077 cm^{-1} indicated the presence of C-O bond. C-H in-plane deformation showed by wave number 884 cm^{-1} and C-H/C-C=O deformation was found in the region having wave numbers 777, 716, 687 and 560 cm^{-1}.

The wave number 3465 and 3432 cm^{-1} indicated the presence of amine groups and 2916, 2851, 2077, 1487 and 1404 cm^{-1} confirmed the presence of alkanes was depicted in FTIR spectrum of FMG3 biopolymer shown in Figure 5 (c). The wave number 1639 cm^{-1} indicated the presence of peptide groups and peaks at 1033 cm^{-1} indicated the presence of C-O bond. C-H in-plane deformation showed by wave number 878 cm^{-1} and C-H/C-C=O deformation was shown by the bands 685, 601 and 560 cm^{-1}.

In FTIR spectral analysis of FMG8 biopolymer (Figure 5 (d)) the wave number 3432 cm^{-1} indicated the presence of N-H/C-H bonds of protein. CH_2 /C-H asymmetric vibrations were found at 3050, 2933 and 2867 cm^{-1} which confirmed the (C-H) presence of alkanes. The wave numbers 1671, 1596, 1515, 1498, 1476, 1424 and 1389 cm^{-1} were indicated the presence of C=O functional group in FMG8 biopolymer. 1308, 1248, 1177, 1117cm^{-1} revealed the abundance of C-N functional group. Wave numbers in the region 1068 - 953 cm^{-1} indicated the presence of carboxyl group. Bands were laid in the range 915-467 cm^{-1} shown presence of C-H group.

FTIR spectral analysis of AB (SC3) showed that wave number 3433 cm^{-1} indicated the presence of N-H/C-H bonds of protein. CH_2/C-H asymmetric vibrations were found at 2914, 2071 and 2850 cm^{-1} which confirmed the (C-H) presence of alkanes. The wave number 1637 cm^{-1} indicated the presence of peptide groups. 1487, 1471 and 1404 cm^{-1} bands were due to the presence of stretching of C=O bond. The wave number 1076 cm-1 indicated the presence of C-O bond (Figure 5 (e)). The band at 560 cm-1spectrum are due to C-H in plane deformation.

Overall, the infra-red spectral analysis for all the five amphiphilic biopolymers showed that these polymers are the mixtures of carbohydrates, lipids and proteins.

4.8 Morphological and biochemical characterization

Morphological and biochemical characteristics of five bacterial strains were analysed using keys Bergey's manual of determinative bacteriology. These biochemical tests included indole test, methyl red test, Voges-Proskauer test, citrate utilization test, starch hydrolysis, lipase activity and catalase test. Morphologically the bacterial colonies appeared to be cream or white colored circular, mucoid, gram negative and motility was observed in FMG8. Out of these five isolates four were found to be catalase positive.

Table no. 1 Characterization of isolated bacterial strains

	R(I)1	FMG2	FMG3	FMG8	SC3
Gram stain	-	-	-	-	-
MR	+	+	+	-	+
VP	-	-	-	+	-
Starch Hydrolysis	-	+	+	-	+
Lipase Activity	-	-	-	-	-
Hydrogen Sulfide Test	+	-	-	-	-
Citrate Utilization Test	-	-	-	+	+
Indole Production Test	+	+	-	-	-
Urease Test	-	-	-	-	-
Catalase Test	-	+	+	+	+

From the biochemical identification of the bacterial isolates, R(I)1 identified as the member of pseudomonadaceae family and other isolates (FMG2, FMG3, FMG8 and SC3) belongs to enterobacteriaceae family.

AB (biosurfactants & bioemulsifiers) are widely used in hydrocarbon bioremediation field since they can enhance the growth on hydrophobic surface and can increase the nutrient uptake of hydrophobic substrates thereby overcoming the poor availability of hydrocarbon contaminants to microorganisms (Perfumo *et al.,* 2006; Nguyen *et al.,* 2010). Synthetic surfactants currently used are not environmental friendly (toxic). Thus hazards caused by synthetic surfactants have drawn much attention to microbial biosurfactants (Kiran *et al.,* 2010).

In conclusion, the present study resulted in isolation of amphiphilic polymer producing bacteria with promising emulsification activity. These strains represent a valuable source for new compounds with surface-active properties, and potential application in various industries.

CONCLUSION

CONCLUSIONS

Sludge and soil samples from various industrial units and environmental sites were screened for the isolation of the amphiphilic biopolymer producing bacteria. Five potential amphiphilic polymer producing bacterial strains were selected with high emulsification activity. Studies revealed that highest biopolymer yield yield (404.8 mgL^{-1} (R(I)1), 239.1 mgL^{-1} (FMG2), 254.6 mgL^{-1} (FMG3), 263.2 mgL^{-1} (FMG8) and 259.4 mgL^{-1} (SC3)) and emulsification activity (45.5 % (R(I)1), 56.5 % (FMG2), 61.9 % (FMG3), 65.2 % (FMG8) and 50 % (SC3) was at 60 hrs of growth. Biochemical analysis and FTIR spectra of five amphiphilic biopolymers indicated proteins, polysaccharides, acids (pyruvic acid & uronic acids) and amino sugars as predominant structural constituents.

Robustness of amphiphilic biopolymers was evident from stability studies where temperature (60°C, 80°C, 100°C and 120°C) and pH (2, 4, 6 and 8) does not affect the emulsification activity with different hydrocarbons. The thermal stability of the AB increases its scope of application in a broader perspective including at conditions where high temperatures prevail as in microbial enhanced oil recovery. Considering the current social and technological development, utilization of amphiphilic biopolymers, which are environmentally friendly and highly functional materials, should be strongly encouraged. It may be concluded that these five bacterial strains were capable of producing amphiphilic biopolymers that would help in degrading hydrocarbon pollutants to the environment and other in industrial applications. Overall a potential scope of further studies and possible feasibility of new commercial applications are implicated from these findings. Further studies however are mandatory (complete structure, effect of environmental variables etc.) for achieving the above.

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ANNEXURE

ANNEXURE I

1. Mineral Salt Medium

Composition	**gL^{-1}**
Peptone	5.0
Yeast extract	1.0
Diammonium sulphate	0.5
$CaCl_2.2H_2O$	0.7
NaCl	0.1
$MgSO_4.7\ H_2O$	0.2
K_2HPO_4	1.0
Dextrose	1.0
Distilled water	1.0 L
pH (7 ± 0.2)	

2. Blood Agar

Sheep blood agar	37
Blood	5-10% (v/v)
Distilled water	1.0 L
pH (7.4 ± 0.2)	

3. CTAB Agar

CTAB	0.2 mg/mL
Methylene blue	5 µg/mL
Agar	1.5 %
Distilled water	100 mL
pH (7.4 ± 0.2)	

4. SIM Agar

Peptone	30.0
Beef extract	3.0
Ferrous ammonium sulphate	0.2
Sodium thiosulfate	0.025
Agar	3.0
Distilled water	100 mL
pH (7.3 ± 0.2)	

5. Starch Agar

Peptone	5.0
Beef extract	3.0
Starch	2.0
Agar	15.0
Distilled water	1000 mL
pH (7.2 ± 0.1)	

6. Simmons Citrate Agar

Ammonium di-hydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium chloride	5.0
Sodium citrate	2.0
Magnesium sulfate	0.2
Agar	15.0
Bromothymol blue	0.08
Distilled water	1000 mL
(pH 6.6 ± 0.2)	

7. Trypticase soy agar

Trypticase	15.0
Phytane	5.0
Sodium chloride	5.0
Agar	15.0
Distilled water	1000 mL
(pH 7.3± 0.2**)**	

8. Folin-Lowry Method

Reagent A (Alkaline solution 50 mL)	
Na_2CO_3	2.0
NaOH	0.2
Distilled water	50 mL
Reagent B (50 mL)	
$CuSO_4$	0.25
Na-K-tartarate	0.5
Distilled water	50 mL

9. ElsonMorgsn method (Hexosamine)

Reagent A (100 mL)	
Dipotassium tetraborate tetrahydrate	6.1
Reagent B	
4-N,N dimethyl-p-aminobenzaldehde	1 g
Glacial Acetic acid	50 mL
HCl(11.5 N)	1.5 mL
Standard solution (galactosamine)	0-1 $mgmL^{-1}$

10. Craazole Assay (Uronic Acid)

Reagent A (100 mL)	
Sodium tetraborate decahydrate	0.9 g
Distiiled water	10 mL
Concentrated H_2SO_4 (ice cold)(98%)	90 mL

Reagent B (100 mL)	
Carbazole	100 mg
Absolute Ethanol	100 mL
Standard solution (glucuronic acid)	0-1 $mgmL^{-1}$

11. Friedman method (Pyruvic Acid)

Perchloric acid	50%
DNP reagent (2, 4-dinitrophenylhydrazine)	500 µM
HCl (2.0 N)	100 mL
Sodium hydroxide	2.2 N
Standard solution	0-3 $mgmL^{-1}$

12. Total sugars

Phenol	5%
Sulphuric acid (concentrated)	
Sugar standards (glucose, galactose, xylose, maltose, mannose)	1 $mgmL^{-1}$

ANNEXURE II

Figure A. Standard curve of carbohydrate assay. Relationship between carbohydrates (as mg glucose) and absorbance using phenol-sulfuric acid assay. R^2=0.9051

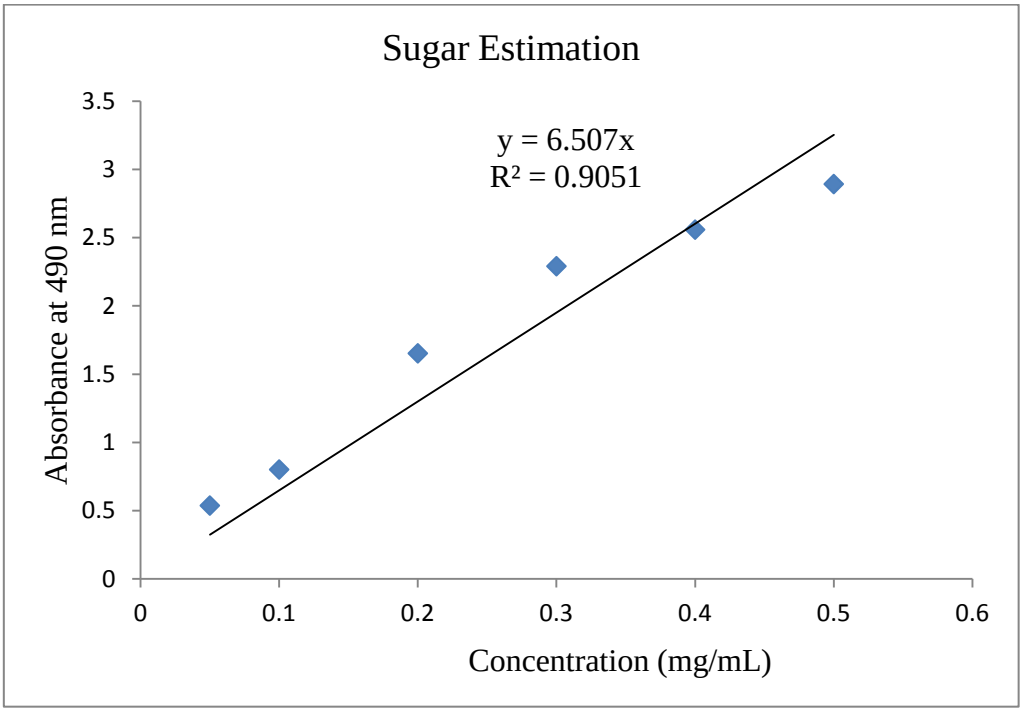

Figure B. Standard curve of protein assay. Relationship between protein (as mg BSA) and absorbance using Folin-Lowry assay. R^2=0.9763

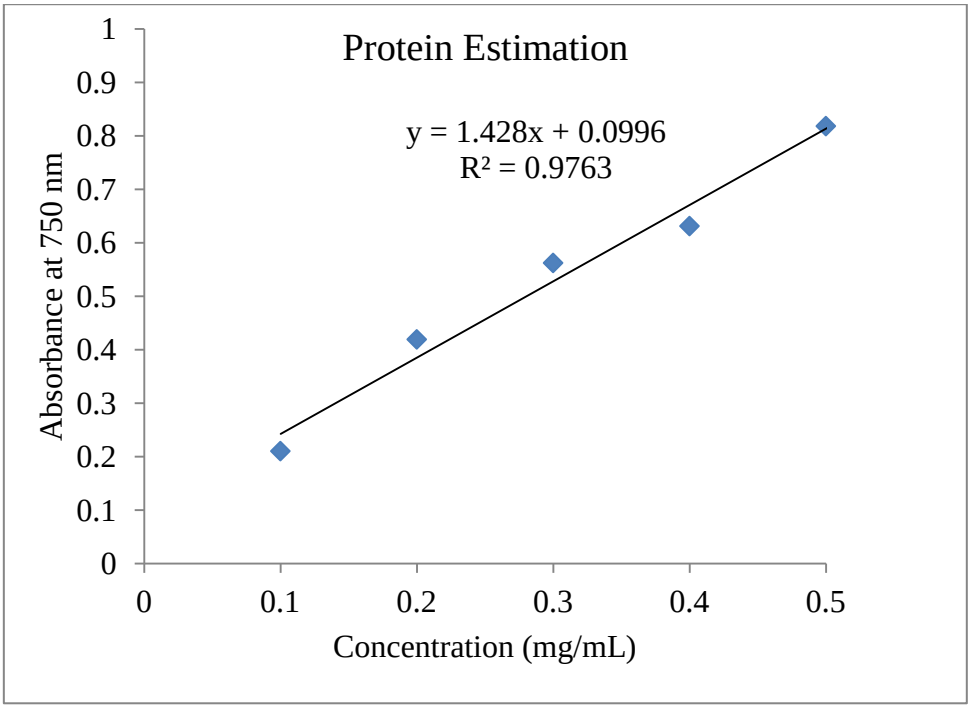

Figure C. Standard curve of uronic acid assay. Relationship between uronic acid (as mg glucuronic acid) and absorbance using uronic acid assay. R^2=0.9587

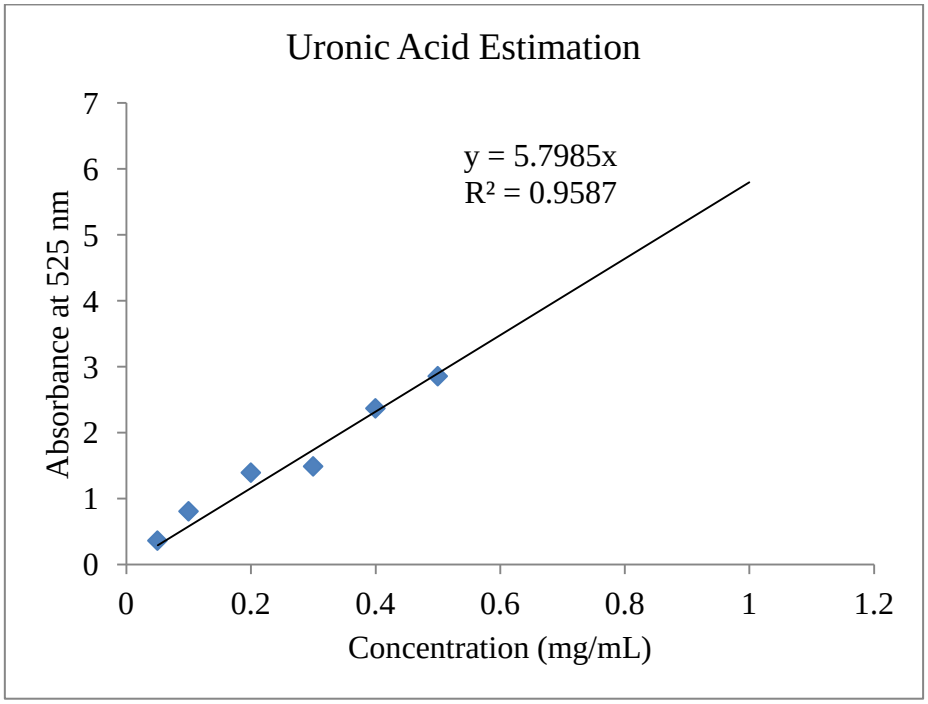

Figure D. Standard curve of pyruvic acid assay. Relationship between pyruvic acid (as µg) and absorbance using Friedman method. R^2=0.9602

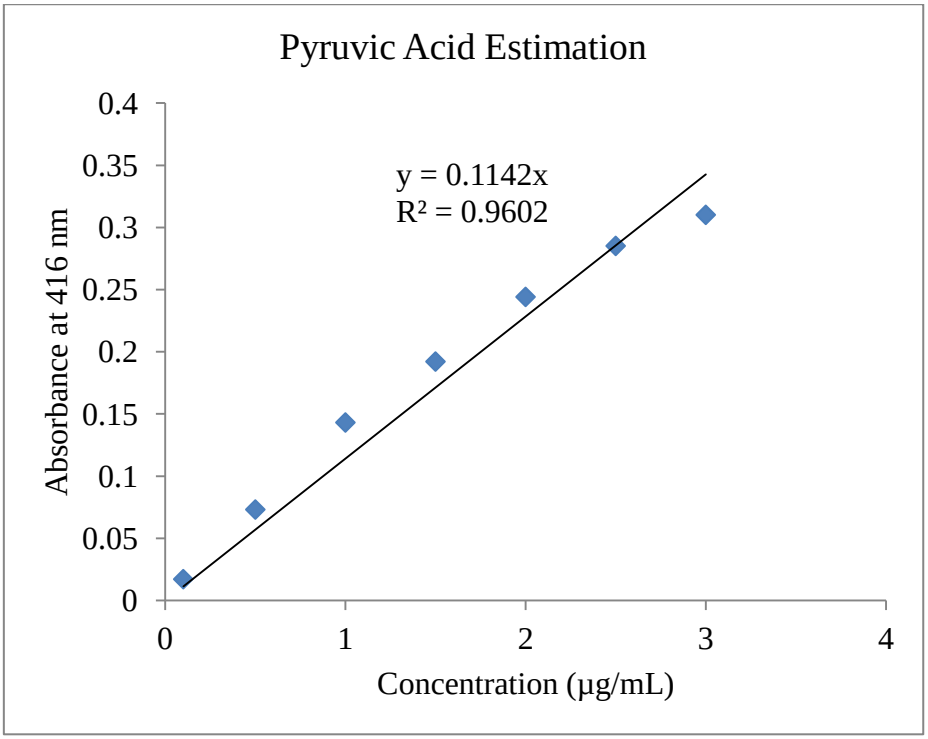

Figure E. Standard curve of hexosamine assay. Relationship between hexosamine (as mg galactosamine) and absorbance using Elson Morgan method. R^2=0.9589