PRODUCTION AND USE OF BIOSURFACTANTS IN BIOREMEDIATION OF HEAVY METAL POLLUTED SOIL

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ABBREVIATIONS

Symbol	Name
As	Arsenic
Cd	Cadmium
CO_2	Carbon dioxide
CEC	Cation exchange capacity
Cr	Chromium
Cu	Copper
CMC	Critical micelle concentration
$cubicgcm^{-1}$	Cubic gram per centimetre
^oC	Degree centigrade
DNA	Deoxyribonucleic acid
FTIR	Fourier Transform Infra Red
EC	Electrical conductivity
GCMS	Gas Chromatography and Mass Spectroscopy
$g\ cm^{-3}$	Gram per centimetre cube
gL^{-1}	Grams per litre
Pb	Lead

LPS	Lipopolysaccaride
MEL	Mannosylerythritol lipids
Hg	Mercury
μScm^{-1}	microSiemens per centimeter
meq	Milliequivalents
$mg\,kg^{-1}$	Milligram per kilogram
nm	Nanometre
NESREA	National Environmental Standards and Regulations Enforcement Agency
NARICT	National Research Institute for Chemical Technology
Ni	Nickel
N	Nitrogen
ppm	Parts per million
%	Percentage
P	Phosphorus
PAHs	Poly aromatic hydrocarbons
K	Potassium

NaCl	Sodium chloride
TPH	Total petroleum hydrocarbon
USEPA	United State Environmental Protection Agency
WHO	World Health Organisation

ABSTRACT

Bacteria were isolated from waste lubricating oil polluted soil and screened for potential to produce biosurfactants using oil spreading or displacement, oil drop collapse, emulsification, and blood haemolysis methods. Of the 45 isolates, 20 isolates (44.44 %) were positive for oil spreading, 22 isolates (48.89%) had haemolytic activity (β and α), 5 isolates (11.11%) were able to collapse oil droplet within 30seconds and 7(15.56%) were good emulsifiers. Based on these capabilities and consistency in activity, five of the isolates emerged for biosurfactant production. They were identified as *Pseudomonas aeruginosa* MPE40, *Micrococcus kristinea* MPE12, *Acinetobacter iwoffi* MPE25, *Bacillus firmus* MPE30, and *Pseudomonas paucimobilis* MPE17. Strains MPE40, MPE30, MPE25, MPE17 and MPE12 generated 1.7g/100ml, 1.6g/100ml, 1.6g/100ml, 1.0g/100ml, and 0.8g/100ml of biosurfactant after 7days respectively in the presence of 1% (v/v) diesel as sole source of carbon and energy. Biochemical, physical, chemical characterization as well as identification of the functional groups of the biosurfactants were done using TLC, GC-MS and FTIR. Chemical analyses of the biosurfactants indicated that they were glycolipids, composed majorly of Palmitic Hexadecanoic, Octadecanoic acid, methyl esters and hydroxylated fatty acids linked to a decanoic acid. Fourier transform infrared analysis showed that the surfactants consisted of carboxyl, hydroxyl, amino and sugar derivative groups. Specifically, strains MPE40, MPE30, MPE25, MPE17 and MPE12 produced the biosurfactants designated Rhamnolipid Bios-40, Firmnolipid Bios-30, Sulfoglycolipid Bios-25, Glycolipopeptide Bios-17, Disulfoglycolipid Bios-12 respectively. The potential of the biosurfactants for heavy metal removal in soil was also studied and the results revealed percentage removal in the range of 68.05%−95.5% for lead (Pb) and 75.7%−91.0% for chromium (Cr). It was further observed that pH of the heavy metal contaminated soil amended with biosurfactants was low (5.24-6.71 for Pb and 4.57-6.21 Cr. contaminated soil) as compared to that of original unpolluted soil (6.78) this indicates high removal of metals from the contaminated soil and effective remediation of metals with the biosurfactants. The metal adsorption by rhamnolipids Bios-40 of *Pseudomonas aeruginosa* was found to be more effective both to lead and chromium, although others had a comparative satisfactory removal. Thus, the results suggest that the biosurfactant have the potential to be used as an alternative remediation tool for treatment of soil contaminated with heavy metals.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background to the Study

The tremendous growth of industries worldwide in the last few decades and the associated anthropogenic activities has often resulted in environmental pollution. Heavy metals such as Chromium (Cr), Lead (Pb), Mercury (Hg) and Arsenic (As) are prominent components of industrial effluents which are discharged into the environment and consequently pollute the ecosystem. The presence of these heavy metals in the environment has been a subject of great concern due to their toxicity, non-biodegradable nature and the long biological half-lives for their elimination from biological tissues (Ishita, Asha, Juwarkar, and Tejo, 2011).

Heavy metals are dangerous group of soil pollutants and exposure to these heavy metals either through ingestion, inhalation, or skin contact is usually chronic due to food chain transfer and their routine penetration into ground and natural water sources, leading to organ damage (like liver damage), cancer and in extreme cases death (Abdurrahim, Abdulrhman, Ali, Mokhtar and Bassam, 2012).

Pollution of soils may come about because of the accumulation of heavy metals and metalloids through outflows from the quickly expanding industries and industrial activities, mine tailings, transfer of waste exceptionally stacked with metals, leaded fuel and paints, application of fertilizers, composit, sewage slop, pesticides, wastewater watering system, deposits from combustion of coal, petrochemicals spillage, and statements from the air (Zhang, Liu and Wang, 2010).

Soils are the major sinks for heavy metals discharged into the ecosystem by anthropogenic activities, most metals don't experience microbial or chemical degradation and their total concentration in soils continues for quite a while after their release into the environment which is not so for organic contaminants which are oxidized to carbondioxide (CO_2) by microbial activity (Adriano, 2003; Kirpichtchikova, Manceau, Spadini, Panfili, Marcus, and Jacquet, 2006). Therefore adequate restoration of soil ecosystems contaminated by heavy metals is required and the process by which these heavy metals are removed from soil using biological means is called bioremediation. In this study biosurfactants which are products from microorganisms were used in the remediation of heavy metal from soil.

Biosurfactants are surface-active agents that are produced extracellularly by microorganisms such as bacteria, yeasts and fungi (Mulligan, 2005). Examples include *Pseudomonas aeruginosa* which produces rhamnolipids, *Bacillus subtilis* which produces a lipopeptide called surfactin and *Candida* (formerly *Torulopsis*) *bombicola* one of the few yeasts to produce high yield of sophorolipids from vegetable oils (Mulligan, 2005). Biosurfactant have several advantageous properties in contrast with chemical surfactants, these includes, no or lesser toxicity, highly biodegradable, better environmentally compatible and non hazardous, higher foaming properties, there activities are highly selective and specific even at extreme temperatures, pH and salinility and also they have the ability to be synthesized from renewable feed-stock (Thenmozhi, Sornalaksmi, Praveenkumar, and Nagasathya, 2011). Most known biosurfactants are glycolipids. Among the glycolipids, the best known are rhamnolipids, trehalolipids, and sophorolipids (Desai and Banat, 1997). Rhamnolipids are said to possess anionic properties and because of these anionic nature, they are capable of

removing heavy metals and ions such as copper, arsenic, lanthanum, zinc and lead from soil due to their complex ability (Herman, Artiola, and Miller, 1995).

1.2 Statement of Problem

The pollution caused by heavy metals has been a subject of concern especially after the Zamfara State (Nigeria) lead poisoning in 2010 that claimed the lives of so many people especially children. The method used in mitigating this problem was through excavation of the soil to landfill and covering the original polluted soil surface with clean soils (Andrew, Dan, David, Bret, Andrea, and Jack, 2010). This method of mitigation (landfill) is limited due to lack of available land and landfill sites. In addition heavy metals are non-biodegradable and because of this they tend to accumulate in living organisms causing serious health effects, including reduction of growth and development, cancer, nervous system and organs damage, and in extreme cases, death. These have prompted the search for alternative ways to mitigate this problem and one of these ways is the use of biosurfactants produced from soil bacteria.

1.3 Justification

One of the most important environmental problems throughout the world today is the contamination of soil by heavy metals as a result of increase in industries and industrialized processes (Nouri, Mahvi, Babaei, and Ahmadpour, 2006; Doumett, Lamperi, Checchini, Azzarello, Mugnai, and Del-Bubba, 2008). In developing countries like Nigeria, increase in activities like vehicle repairs, vulcanizing, welding, auto-electrical works, battery charging and motor transportation have introduced heavy metals into the air and subsequently deposited into nearby soils (Mabogunje, 1990; Adefolalu, 2009). It has been reported that heavy metals have the ability to accumulate and cause toxicity in biological systems and they can remain almost indefinitely in the

soil environment, although availability can change considerably depending on their chemical speciation in the soil. (Gray, Mclaren, and Roberts, 2003; D'amore, Al-abed, Scheckel, and Ryan, 2005; Wuana, Okieimen, and Imborvungu, 2010).

Therefore adequate protection and restoration of the soil ecosystems is required and these can be achieve through remediation of soils that are contaminated with heavy metals (Nouri *et al.*, 2008; Nwachukwu, Feng, and Alinnor, 2010;), but the remediation techniques commonly used include: *ex-situ* (excavation) or *in-situ* (on-site) soil washing/leaching/ flushing with chemical agents, chemical immobilization/stabilization method to reduce the solubility of heavy metals by adding some non-toxic materials into the soils, electrokinetics (electromigration), covering the original polluted soil surface with clean soils, and dilution method (mixing polluted soils with surface and subsurface clean soils to reduce the concentration of heavy metals) (Fawzy, 2008; Nouri, Khorasani, Lorestani, Karami, Hassani, and Yousefi, 2009; Kord, Mataji, and Babaie, 2010; Wuana *et al.*, 2010); all these methods have been considered to be destructive, not ecofriendly, expensive and do not provide permanent or lasting solution to the problems. There is therefore, the need for sustainable alternatives that make use of inexpensive, environmentally friendly materials in the detoxification and removal of heavy metals from soil. Hence this study used biosurfactants to bind metals and removes them from soil.

1.4 Aim

The aim of this study was to produce biosurfactants and use them to reclaim heavy metal polluted soil.

1.5 Objectives

The objectives of this study were:

i. To isolate and identify biosurfactant producing bacterial from soil

ii. To screen the bacterial isolates for ability to produce biosurfactants

iii. To extract and identify the biosurfactants.

iv. To remediate heavy metal contaminated soil using the biosurfactants

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Definition and Classification of Biosurfactants

Biosurfactants are amphiphilic compounds which are secreted extracellularly or produced on living surfaces mainly on surfaces of microorganisms or as part of their cell membrane and it contains both hydrophilic and hydrophobic moieties which reduce the surface and interfacial tension of the surface and interface respectively (Luna, Rufino, Sarubbo, Campos-Takaki, 2013).

Unlike the chemically produced surfactants that are classified according to the type of the polar group present and their dissociation pattern in water; biosurfactants are classified by their microbial origin, mode of action, chemical composition, molecular weight, and physico-chemical properties. Taking into account their molecular weight they are partitioned into low-molecular mass biosurfactants which incorporate glycolipids, phospholipids, and lipopeptides and high-molecular mass biosurfactants/bioemulsifiers include the particulate surfactants, amphipathic polysaccharides, lipopolysaccharides, proteins, lipoproteins and other complex mixtures of these biopolymers (Rosenberg and Ron, 1999; Magdalena, Grażyna, Zofia, and Swaranjit, 2011).

Biosurfactants with low-molecular-mass are efficient in reducing surface and interfacial tensions, whereas the biosurfactants with high-molecular-mass are more effective and efficient at stabilizing oil-in-water emulsions (that is, they act as emulsion-stabilizing agents) (Rosenberg and Ron, 1999; Calvo, Manzanera, Silva-castro, Uad, and Gonzalez-lopez, 2009). They do these by accumulating at the interface between two immiscible liquids or between a fluid (liquid) and a solid thereby lowering surface

(liquid-air) and interfacial (liquid-liquid) tension, by reducing the repulsive forces between two non similar phases thus allowing these two phases to mix and interact more easily (Figure 2.1). Majority of these biosurfactants are ionic, neutral or anionic and the hydrophobic moieties are usually based on long-chain fatty acids or fatty acid derivatives, whereas the hydrophilic moieties can be a phosphate, amino acid, carbohydrate, or cyclic peptide (Nitschke and Coast, 2007). The major groups of biosurfactants and microorganisms that produced them are presented in table 2.1.

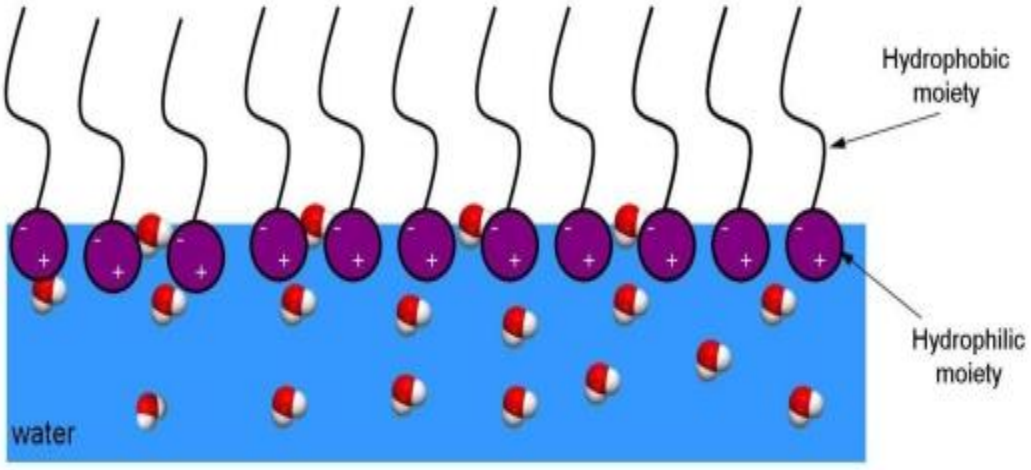


Figure 2.1: Accumulation of biosurfactants at the interface between liquid and air (Soberón and Maier, 2011).

Active biosurfactants can lower the surface tension of water from 72 mNm^{-1} to 30 mNm^{-1} and the interfacial force between water and n-hexadecane from 40 mNm^{-1} to 1 mNm^{-1}, these activities of biosurfactants depend largely on its concentration in solution when the critical micelle concentration (CMC) is obtained, when these concentrations obtained by the biosurfactant in the solution exceeds the CMC, the biosurfactant molecules begins to associate and form micelles, bilayers and vesicles (Figure 2.2), which quickens the reduction of the surface and interfacial tension thereby increasing the solubility and bioavailability of organic and inorganic compounds (pollutants) (Whang, Liu, Ma, and Cheng, 2008).

Table 2.1: Major classes of biosurfactants and microorganisms involved

Surfactant class	Microorganism
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Trehalose lipids	*Arthobacter* sp., *Rhodococcus erythropolis*
Sophorolipids	*Candida bombicola, C. apicola*
Mannosylerythritol lipids	*C. antartica*
Lipopeptides	
Lichenysin	*Bacillus licheniformis*
Viscosin	*P. fluorescens*
Surfactin/iturin/fengycin	*B. subtilis*
Serrawettin	*Serratia marcescens*
Surface-active antibiotics	
Gramicidin	*Brevibacterium brevis*
Polymixin	*B. polymyxa*
Antibiotic TA	*Myxococcus Xanthus*
Fatty acids or neutral lipids	
corynomicolic acids	*Corynebacterium insidibasseosum*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Alasan	*A. radioresistens*
Liposan	*C. lipolytica*
Lipomanan	*C. tropicalis*
Particulate biosurfactants	*A. calcoaceticus*
	Cyanobacteria

Source: (Karanth, Deo, and Veenanadig, 1999; Chakrabarti and Sneha, 2012)

The efficiency of surfactants is commonly measured using the CMC and an efficient biosurfactants usually have low CMC, this means that, litle quantity of biosurfactant is required to decrease the surface tension. Formation of Micelle has a very significant role in microemulsion formation (these microemulsions are stable and clear liquid mixtures of oil domains and water separated by monolayer or aggregates of biosurfactants) (Nguyen, Youssef, McInerney, and Sabatini, 2008).

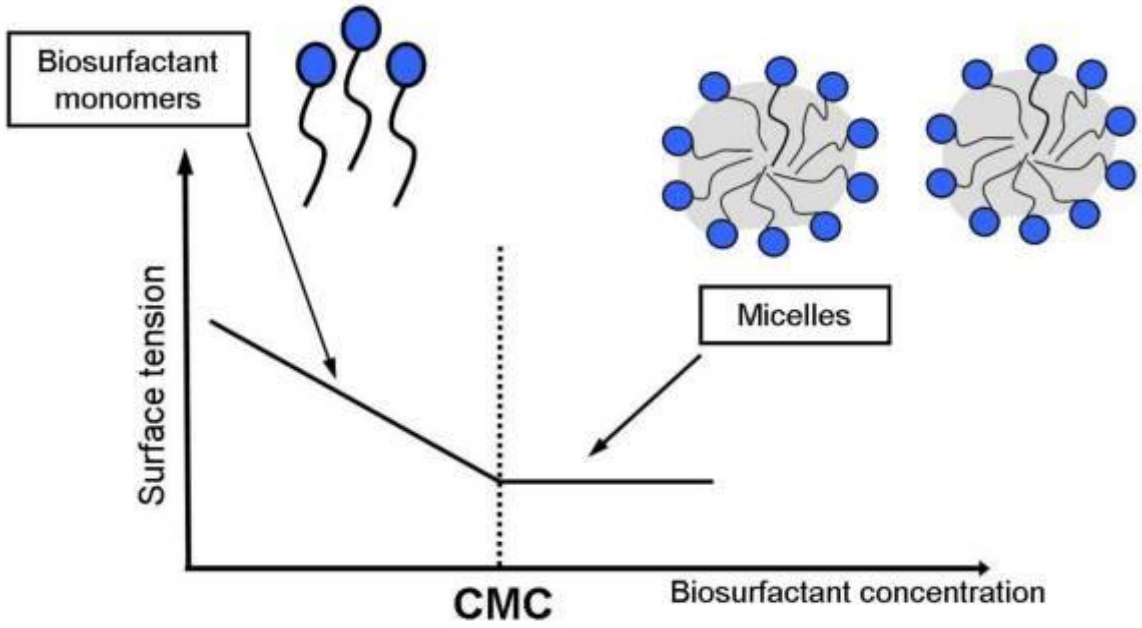


Figure 2.2: The relationship between formation of micelles, biosurfactant concentration and surface tension (Whang *et al.*, 2008).

CMC: critical micelle concentration

2.1.1 Glycolipids

Glycolipids are long-chain aliphatic acids or hydroxyaliphatic acids that are attached to carbohydrates which are in connection with an either an ether or ester group. Most of the biosurfactants are glycolipids, among the glycolipids, the best known are rhamnolipids, sophorolipids and trehalolipids (Banat, Franzetti, Gandolfi, Bestetti, and Marchant, 2010; Abdurrahim *et al*., 2012).

(i). Rhamnolipids: These are type of glycolipids in which one or two molecules of rhamnose are connected to one or two molecules of β-hydroxydecanoic acid; one of the -OH group of the acids is involved in glycosidic linkage with the reducing end attached to the rhamnose disaccharide while the second -OH group of the acid is occupied in ester formation (Karanth *et al.*, 1999). The production of rhamnose which contains glycolipid was first studied in *Pseudomonas aeruginosa* by Jarvis and Johnson (1949). L-Rhamnosyl-L-rhamnosyl-β- hydroxydecanoyl-β-hydroxydecanoate (Figure 2.3) and L-rhamnosyl- β-hydroxydecanoyl-β-hydrtocydecanoate, referred to as rhamnolipids 1 and 2 respectively, are principal glycolipids synthesized by *P. aeruginosa* (Edward and Hayashi, 1965).

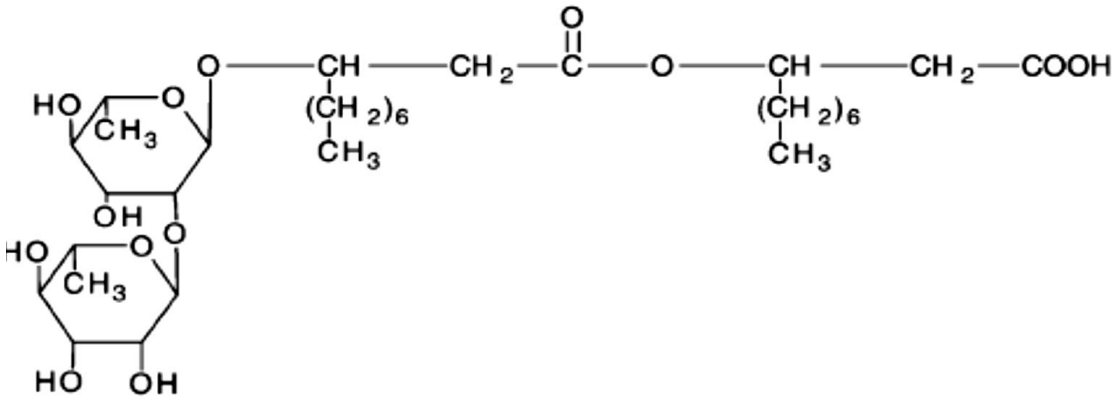


Figure 2.3: Structure of rhamnolipid (Edward and Hayashi, 1965; Chakrabarti and Sneha, 2012)

(ii).Trehalolipids: Various structural types of microbial trehalolipid biosurfactants have been reported (Figure 2.4). Disaccharide trehalose is linked at C-6 and C-6 to mycolic acid having long chain of α-branched and β-hydroxy fatty acids (Franzetti, Gandolfi, Bestetti, Smyth, and Banat. 2010). Trehalolipids produced by various organisms varies in structure of mycolic acid; size, degree of unsaturation and the number of carbon atoms present (Asselineau and Asselineau, 1978). Trehalose lipids obtained from and

Arthrobacter sp and *Rhodococcus erythropolis* reduced the surface tension and interfacial tension in culture broth (Kretschmer, Bock, and Wagner, 1992).

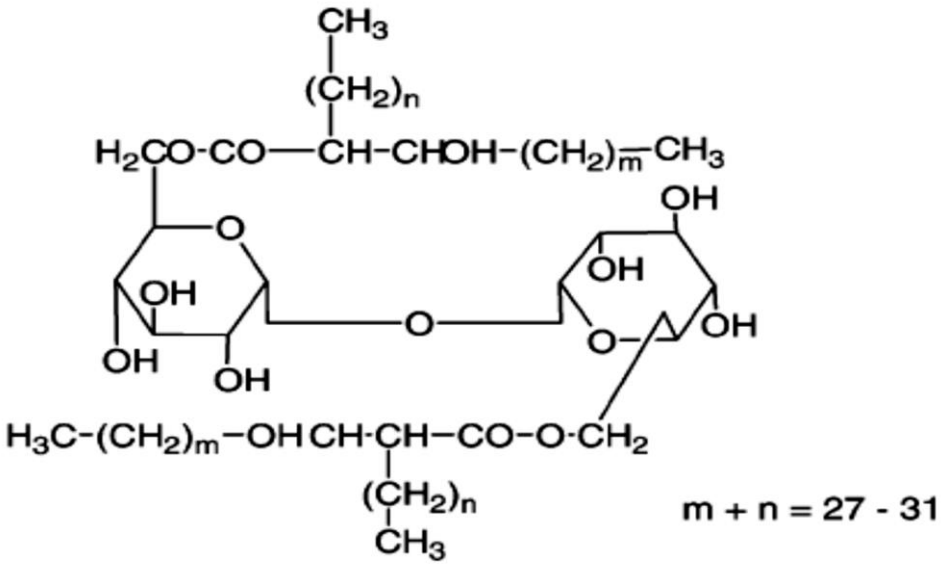


Figure 2.4: Structure of trehalose lipids (Franzetti *et al.*, 2010; Chakrabarti and Sneha, 2012)

(iii). **Sophorolipids:** These glycolipids consists of a dimeric carbohydrate sophorose attached to a long-chain hydroxyl fatty acid by a glycosidic linkage, consisting of mixture of free acid form and macrolactones with combinations of at least six to nine varied hydrophobic sophorolipids (Figure 2.5) and are majorly synthesized by yeast such as *Torulopsis bombicola, T. petrophilum* and *T. apicola* (Hu and Ju, 2001).

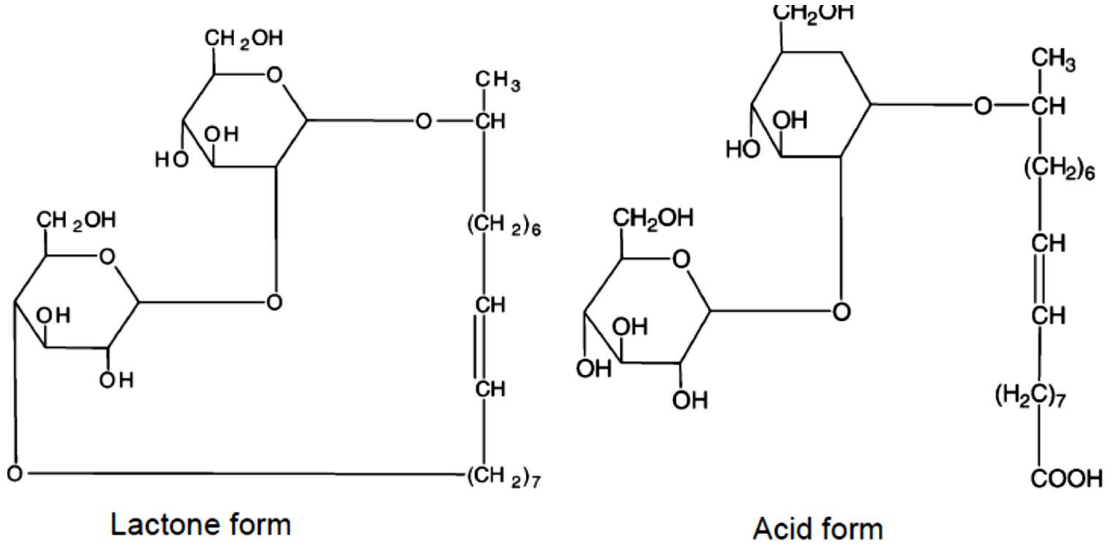


Figure 2.5: Structure of lactonized aAnd free-acid forms of sophorolipids (Hu and Ju, 2001).

2.1.2 Lipopeptides and lipoproteins

Lipopeptides and lipoproteins biosurfactants contain a lipid linked to a polypeptide chain with a lactone linkage. Majority of them are in form of cyclic lipopetides, including decapeptide antibiotics (gramicidins) and lipopeptide antibiotics (polymyxins). Surfactin synthesized by *Bacillus subtilis* which is made up of seven amino-acid ring structure joined to a fatty-acid chain *is* one of the commonly produced (Figure 2.6) (Pornsunthorntawee, Arttaweeporn, Paisanjit, Somboonthanate, and Chavadej, 2008).

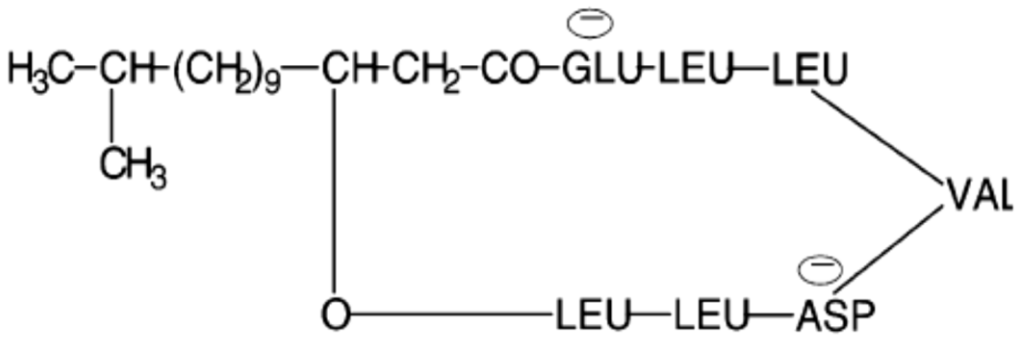


Figure 2.6: Structure of surfactin (Pornsunthorntawee *et al.*, 2008)

2.1.3 Lichenysin

Lichenysin is a type of biosurfactant that act synergistically and exhibit great temperature tolerance, salt and pH stability. It is commonly synthesized by *Bacillus licheniformis,* although their structural and physio-chemical properties are similar to that of surfactin (Madslien, Ronning, Lindback, Hassel, Anderson, and Granum, 2013). *Bacillus licheniformis* can produce biosurfactants that are capable of reducing the surface tension of water and the interfacial tension between water and n-hexadecane from $72mNm^{-1}$ to $27mNm^{-1}$ and $0.36mNm^{-1}$ respectively (Madslien *et al*., 2013).

2.1.4 Fatty acids, phospholipids and neutral lipids

Fatty acids and phospholipid biosurfactants are synthesize by yeasts and many bacteria especially during their growth on n-alkanes with the lipophilic and hydrophilic balance (LHB) directly proportional to the length of the hydrocarbon chain in their structures (Cirigliano and Carman, 1985). For instance phosphatidylethanolamine (Figure 2.7) synthesized by *Acinetobacter* sp forms optically clear microemulsions of alkanes in water while that synthesized by *R. erythropolis* lowers the interfacial tension between hexadecane and water to less than 1mN/m and a critical micelle concentration (CMC) of 30 mg/l (Kretschmer *et al.,* 1992).

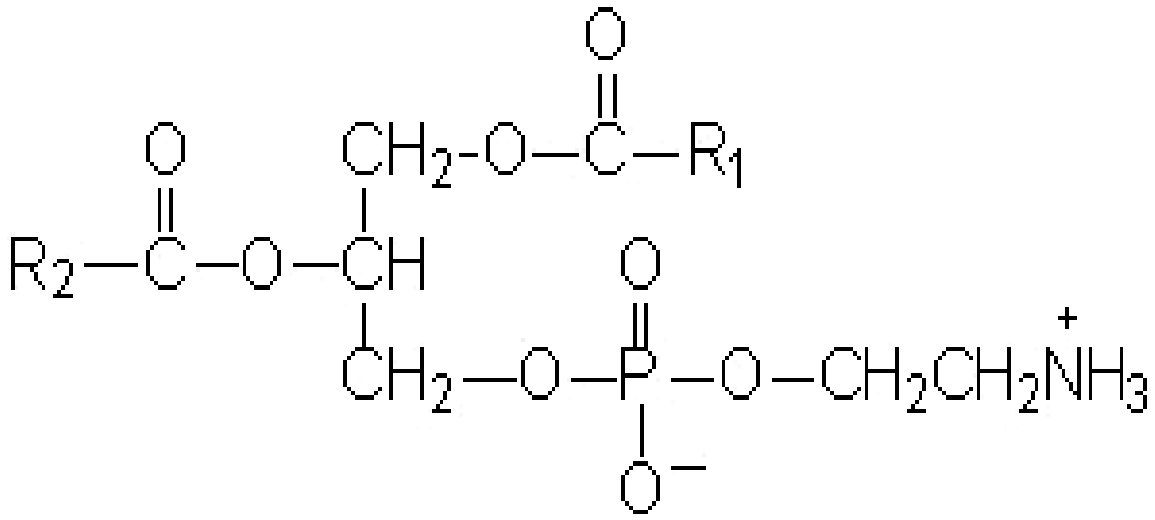


Figure 2.7: Structure of phosphatidylethanolamine, a biosurfactant produced by *Acinetobacter* sp. R1 and R2 (Desai and Banat, 1997)

2.1.5 Polymeric biosurfactants

The best-studied polymeric biosurfactants are alasan, liposan, emulsan and some other polysaccharide–protein complexes. Emulsan (Figure 2.8) is an effective emulsifying agent for hydrocarbons in water even at a concentration as low as 0.001 to 0.01%. Liposan is an extracellular water-soluble emulsifier synthesized by *Candida lipolytica* and is composed of 83% carbohydrate and 17% protein (Cirigliano and Carman, 1984).

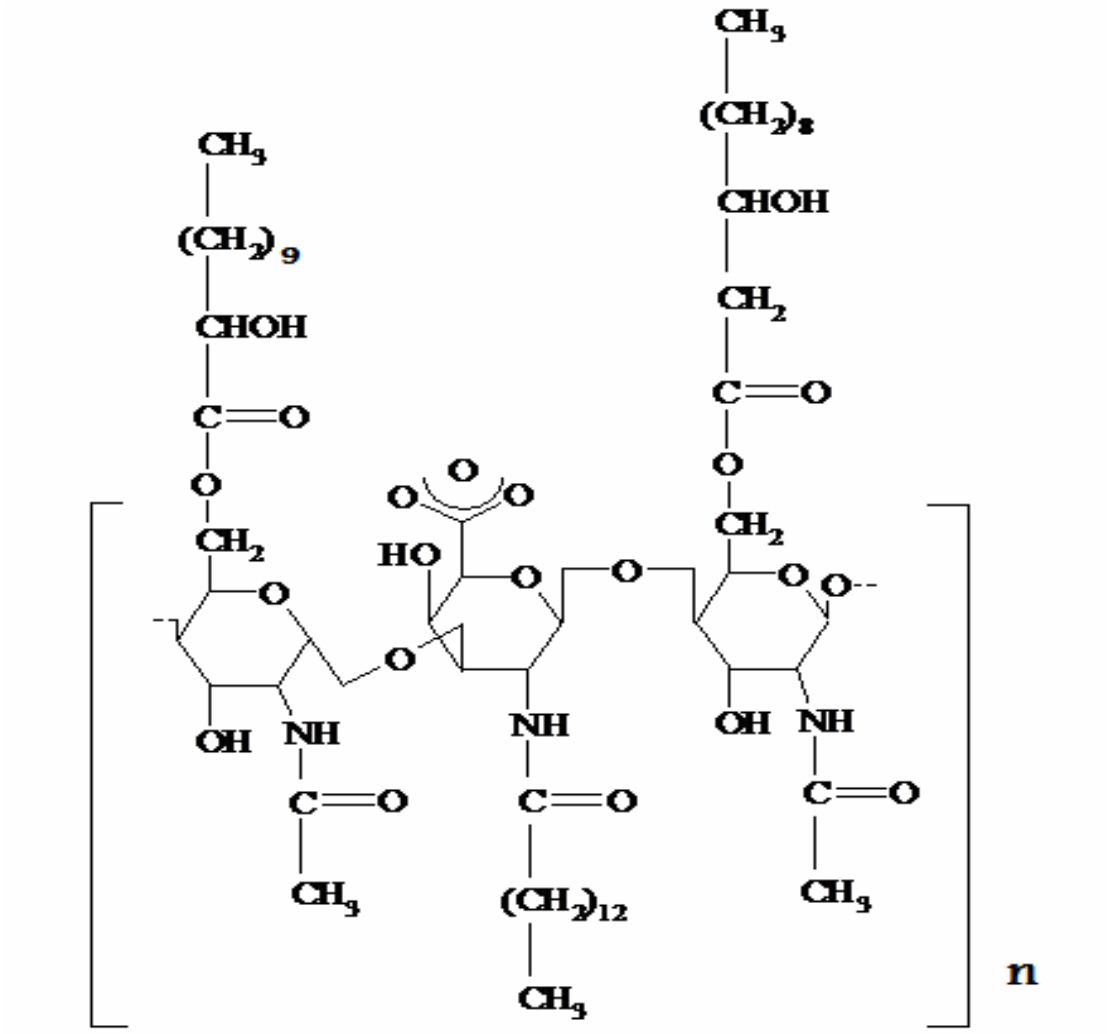


Figure 2.8: Structure of emulsan, produced by *Acinenetobacter calcoaceticus* (Chamanronkh, Mazaheri, Noohi, Yahyai. 2008).

2.1.6 Particulate biosurfactants

These are type of biosurfactants that contain extracellular membrane vesicles that partition hydrocarbons to from a microemulsion, which plays essential role in alkane uptake by microbial cells (Mukherijee, Das, and Sen, 2006). These vesicles especially those produced by *Acinetobacter* sp. have diameter range of 20–50nm and a buoyant density of 1.158cubicgcm^{-1} and they consists majorly of glycolipids, with trace amount of protein, phospholipids and lipopolysaccharide (Kappeli and Finnerty, 1979; Mukherijee *et al.*, 2006).

2.2 Properties of Biosurfactants

Biosurfactants are of increasing interest for commercial use because of the continually increasing spectrum of available substances. The major distinctive features of biosurfactants and a brief description of each property are given below:

(i) Surface and interface activity: A good surfactant can lower surface tension of water from 72 to 35mNm^{-1} and the interfacial tension of water/ hexadecane from 40 to 1mN/m (Mulligan, 2005). For instance; surfactin produced from *B. subtilis* reduced the surface tension of water to 25mNm^{-1} and interfacial tension of water/hexadecane to less than 1 mN/m (Cooper, MacDonald, Duff, and Kosaric, 1981). Rhamnolipids from *P. aeruginosa* decreased the surface tension of water to 26mNm^{-1} and the interfacial tension of water/hexadecane to less than 1mNm^{-1} (Hisatsuka, Nakahara, Sano, and Yamada, 1971) while sophorolipids from *T. bombicola* reduced the surface tension of water to 33mNm^{-1} and the interfacial tension to 5mNm^{-1} (Cooper and Cavalero, 2003). In general, biosurfactants are more efficient and effective than chemical surfactants even at a low concentration (Desai and Banat, 1997, Mulligan *et al.*, 2014).

(ii). Temperature, pH and ionic strength tolerance: Many biosurfactants and their surface activities are not affected by environmental conditions such as temperature and pH. For example, a biosurfactant (lichenysin) produced from *Bacillus licheniformis* remained stable (unaffected) even when the temperature was rised to 50°C, pH of 4.5–9.0, 50gl^{-1} of NaCl and Calcium concentration of 25gl^{-1} (McInerney, Javaheri, and Nagle, 1990; Madslien *et al.*, 2013) also a lipopeptide obtained from *B. subtilis* remained stable even after autoclaving at 121°C for 20 minutes and after 6 months at –

18°C; the surface activity did not change from pH 5 to 11 and NaCl concentrations up to 20% (Nitschke and Pastore, 1990).

(iii). Biodegradability: microbial-produced surfactants are easily degraded unlike the synthetic surfactants (Mohan, Nakhla, and Yanful, 2006) and environmentally friendly for use in bioremediation (Mulligan, 2005) and dispersion of oil spills.

(iv). Low toxicity: Reports about the toxicity of biosurfactants are rarely available, this is because they are usually seen as low or non-toxic products and therefore are appropriate for pharmaceutical, food and cosmetic uses. An investigator reported that a Corexit which is a synthetic surfactant displayed a lathel concentration that is up to 50% of the test species (LC50) against *Photobacterium phosphoreum* which was ten times lower than that desplyed by rhamnolipids (biosurfactant), signifying the larger toxicity of the chemically derived surfactant (Chakrabarti and Sneha, 2012). Comparing the toxicity of four synthetic surfactants, six biosurfactants and two commercial dispersants, it was reported that most of the biosurfactants degraded faster than the synthetic surfactants and dispersants, except for a synthetic sucrose-stearate that have structure homology that is similar to those of glycolipids, degraded more rapidly than the biogenic glycolipids (Poremba, Gunkel, Lang, and Wagner. 1991). It was also reported that biosurfactants showed higher effective concentration (EC50) with 50% decrease of test population values than synthetic dispersants (Poremba *et al.,* 1991).

The mutagenic and toxicity effect of a chemically derived surfactant was compared with biosurfactant from *P. aeruginosa.* The result reviewed that the chemical surfactant had higher level of mutagenic and toxicity effect whereas the biosurfactant was considered to be slightly non-toxic and non mutagenic (Flasz, Rocha, Mosquera, and Sajo, 1998).

(v) Chemical diversity: The diversity of chemical composition of biological surfactants provides a broad selection of surface-active agents with attributes related to unique applications.

2.3 Applications of Biosurfactants

Biosurfactants are ecologically safe and can be applied in wastewater treatment and bioremediation of soil. Some of the potential applications of biosurfactants in pollution and environmental control are microbial enhanced oil recovery, hydrocarbon degradation in the soil environment, in aquatic environment and hexa-chloro cyclohexane degradation, and removal of heavy metal from contaminated soil (Singh, Hamme and Ward, 2007).

2.3.1 Biosurfactants and metal remediation

Soil environment polluted with overwhelming metals is generally extremely dangerous for human and other living organisms in the environment even at low concentrations. On the other hand, there are numerous methods used to clean up soils polluted with heavy metals and the remediation of these soils incorporates tried and true systems, for example, excavation, and transfer of polluted soil to landfill destinations or biological methods (Asci, Nurbas, and Acikel, 2010). Natural systems are methodologies that utilization plants (phytoremedation) or microorganisms (bioremediation) to remediate metals from soil.

Heavy metals are not biodegradable yet they might be transformed from one chemical form to another which changes their toxicity and mobility. Heavy metals could be impacted by microorganisms in many ways some of which are: conversion response through redox process, alkylation, accumulation by metabolism-independent (passive), metabolism-dependent (active) uptake, by affecting pH or by producing substances which change mobility of the metals (Chakrabarti and Sneha, 2012).

Two techniques, for example, Soil washing and Soil flushing are utilized in the remediation of metal contaminated soil. The soil washing procedure is an ex situ technique that include the removal of the polluted soil from the site and put into the glass flask and washed with biosurfactant solution while the soil flushing is an in-situ innovations that makes utilization of drain channels and trenches for gathering and applying the biosurfactant solution to soil at site (Singh and Cameotra, 2004). Interestingly, biosurfactants might be utilized for metal remediation from soil by applying the biosurfactants to a little a piece of contaminated soil put in a bond blender and after that a complex called biosurfactant-metal complex is formed, these complex can be flushed out of the soil and soil deposited back to its original position and the biosurfactant metal complex will then be treated to precipitate out biosurfactant, leaving the metal behind. The formed bond that inbetween the positively and negatively charged metal and surfactant respectively is so strong that metal-surfactant complex can be removed from the soil matrix by flushing water through the soil. This method also can be used with more pumping activities in the removal of contaminations from deeper subsurfaces (Magdalena *et al*., 2011).

2.3.1.1 Mechanism of removal of metals by biosurfactants

The use of microbially synthesized surfactants do not have any questionable advantages because bacterial strains are able to produce surface active compounds do not need to have survival ability in heavy metal-contaminated soil.

The effectiveness of biosurfactants for bioremediation of heavy metal contaminated soil is principally dependent on their own ability form complexes and bind with metals. These biosurfactants (especially the anionic type) in a non ionic form by ionic bonds create complexes with metals; these ionic bonds are generally more stronger compared to the metal's bonds with the soil and result to the formation of metal-biosurfactant complexes which are desorbed from the matrix of soil into the soil solution because of reduction of the interfacial force. The cationic biosurfactants make use of ion exchange mechanism to replace negatively charged metal ions (by competition) for some surfaces that are negatively charged (Magdalena *et al*., 2011). Biosurfactant micelles can also be use to remove metal ions from soil surfaces and this is achieved where the polar heads of the micelles bind with the metals and mobilize it in water (Figure 2.9) (Mulligan and Gibbs, 2004; Asci *et al.*, 2008).

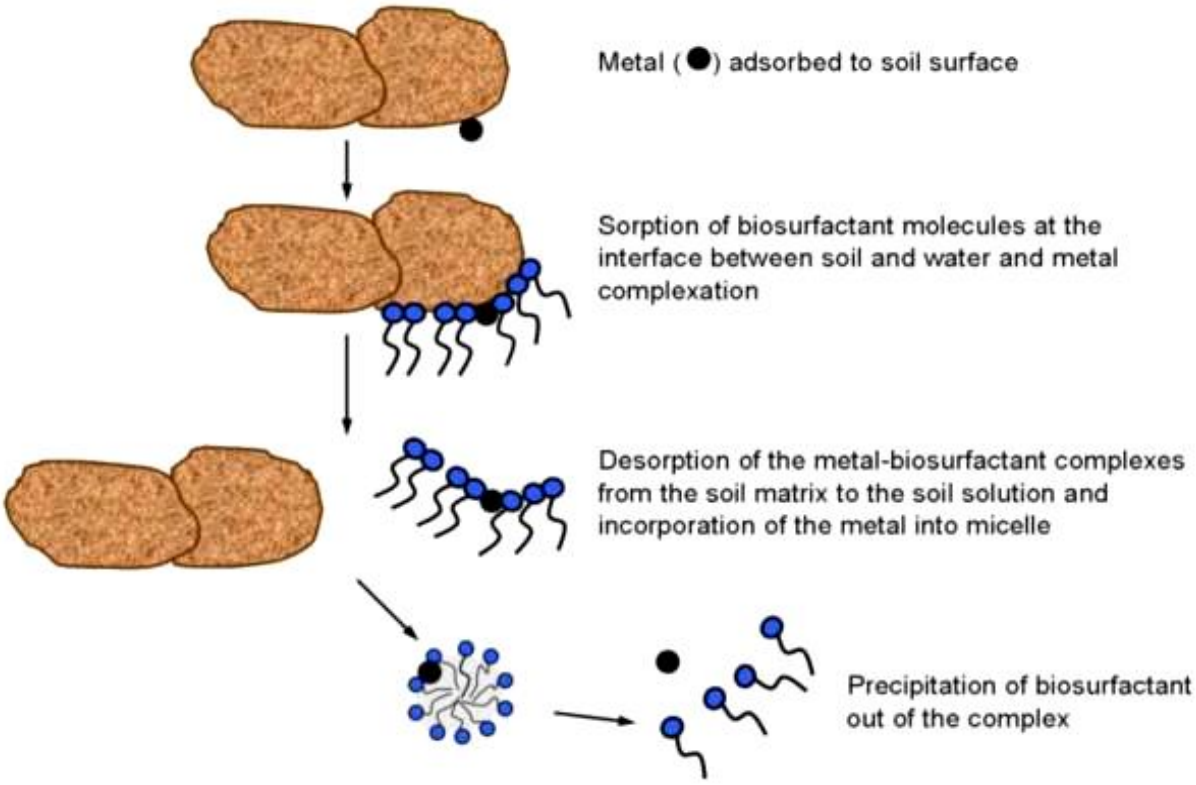


Figure 2.9: Mechanism of biosurfactant activity in metal-contaminated soil (Mulligan and Gibbs, 2004; Asci *et al*., 2008; Magdalena *et al*., 2011).

2.3.1.2 Applications of the process

Biosurfactants have been found useful in bioremediation of metal-contaminated soils, this fact was established by Juwarkar, Dubey, Nair, and Singh (2008) who tested the potential of biosurfactants (di-rhamnolipid) produced by *Pseudomonas aeruginosa* BS2 in the decontamination and mobilization of soil contaminated with heavy metal. In order to study the feasibility of di-rhamnolipid to remove lead, cadmium, copper, and chromium, from soil, 0.1% di-rhamnolipid biosurfactant solution was introduced into a glass column filled with soil spiked with heavy metal, after washing the soil with the biosurfactant solution they observed that the di-rhamnolipid was able to remove heavy metal selectively from the soil in the order of Cd = Cr > Pb = Cu > Ni. Similarly, Wang

and Mulligan (2004) assessd this feasibility by using foam from rhamnolipid to take out Cd and Ni from a sandy soil, the results revealed that the use of the foam had an effect on the movement of the biosurfactant in the porous medium, thereby increased the contact of the biosurfactant with the metals and allowed percentage removal of 68.1% and 73.2% of Ni and Cd, respectively, as compared to 61.7% of Cd and 51% of Ni, removal by the rhamnolipid solution alone.

The ability of biosurfactant produced by bacteria from marine environment to remove heavy metals from solutions was also investigated by Das, Mukherjee, and Sen (2009); the study revealed that tested anionic biosurfactant was able to bind the metal ions and the percentage removal of Pb and Cd metals varied with the different concentrations of metals and biosurfactants and they included that the capability of these biosurfactants to chelate toxic heavy metals and form an insoluble precipitate might be useful in treatment of soil and wastewater containing heavy metal.

The remediation of heavy metals from sediments might be enhanced by use of solution containing inorganic compound and biosurfactants. For example, the reports of Dahrazma and Mulligan (2007) revealed a high rate removal nickel and copper from sediments with addition of 1% NaOH to the solution of rhamnolipid. Numerous of these metals exist mostly in the organic fraction in the environment but with the addition of OH^- to the sediment it desolved this fraction, making available more metals for removal by a rhamnolipid biosurfactant.

Also the potential of biosurfactants was investigated for mobilization of arsenic from mine tailings; the experimental results showed significantly that the addition of

rhamnolipid enhanced mobilization of As from the mine tailings. The mobilization increased with biosurfactant concentration and relatively became stable when the rhamnolipid concentration was above $100mg^{-L}$.

It was reported that heavy metals removal linearly increased with increasing concentration of surfactant below the CMC and remained relatively constant above the CMC. The CMC of the biosurfactant used by Wang and Mulligan (2009) was around $30mg^{-L}$. In this experiment the high rhamnolipid concentration needed, might as a result from the dilution, binding effects of the mine tailing particles as well as sorption of the biosurfactant to the mine tailings. The biosurfactant influenced the As mobilization by lowering the interfacial force between the mine tailings and As compound through aqueous complexes and micelles formation and by improving the wettability of the mine tailings. The results obtained from the study, indicated that biosurfactants have potential to be used in the remediation of As-contaminated mine tailings and they can also be effectively used to remove As from soils.

Besides the mobilization of heavy metal with biosurfactant, they can be associated with other procedures involve in heavy metal remediation; for instance, entrapping of trivalent chromium within micelles which offers microbial threshold and some amount of resistance toward high concentration of Cr (III) (Magdalena *et al*., 2011).

The feasibility of biosurfactant from marine isolate (Bacillus sp. MTCC 5514) was investigated by Gnanamani, Kavitha, Radhakrishnan, Rajakumar, Sekaran, and Mandal (2010) the remediation process was carried out in two phases, in the first phase, an extracellular chromium reductase was used to reduce Cr (VI) (the toxic state) to Cr (III) (less toxic), which was then entrapped by the biosurfactants preventing the bacterial cells from being exposed directly to chromium (III) in the second phase (that is, in the

second phase the bacteria was used); during these processes the bacterial cells developed tolerance and resistance toward hexavalent and excessive trivalent chromium concentrations and thereby remained active throughout the period investigated. Also a biosurfactant, BS29 produced by *Gordonia* sp. was also able to remove metals (Cu, Cd, Pb, Zn, Ni) from soil, but their potential in the process was lower than rhamnolipids (Franzetti *et al*., 2010).

2.3.2 Biosurfactants in co-contaminated sites remediation

The United States Environmental Protection Agency (USEPA) estimated about 40% sites that are co-contaminated with organics and metals such as arsenic, lead, and cadmium and the presence of these toxic metals in most cases causes inhibition of organic compound biodegradation. A number of achievable techniques that may reduce bioavailability of metal and/or improve microbial tolerance to metals consist of using metal-resistant bacteria, addition of montmorillonite, clay courts minerals—kaolinite, chelating agents (EDTA), phosphate, calcium carbonate, and biosurfactants (Sandrin and Maier, 2003; Magdalena *et al*., 2011) but the biosurfactants produced by microorganisms are more promising in influencing biodegradation of organic compound in the presence of metals and their application in in-situ co-contaminated sites bioremediation seems to be more economical and environmentally compatible than using metal chelators or modified clay complexes (Sheng, He, Wang, Ye, and Jiang, 2008).

Sandrin, Chech, and also Maier (2000) demonstrated that heavy metal-complexing rhamnolipids lessened metal toxicity allowing increased degradation of organic compounds by *Burkholderia* sp. under laboratory conditions (Magdalena *et al.*, 2011).

The researcher recommended that rhamnolipid lowered the metal toxicity to help survival of microbial consortia within the co-contaminated soil through combination of metal complexation and modification of the surface properties of the bacterial cells, which was achieved through the synthesis of lipopolysaccharide (LPS), leading to increased bioremediation effect (Magdalena *et el.,* 2011). Maslin and also Maier (2000) analyzed the effect connected with rhamnolipids that is generated by several *Pseudomonas aeruginosa* strains around the phenanthrene degradation through native populations within two soils co-contaminated along with phenanthrene and also cadmium. The analysis demonstrated that the rhamnolipids had ability to complex cationic metals, in so doing improving the bioavailability of phenanthrene. The biodegradation connected with phenanthrene was improved in response to application of rhamnolipid from 7.5% to 35% in one of the soil, and also from 10% to 58% from the second soil.

2.3.3 Potential food applications

Biosurfactants might be investigated for various food-processing application, some are:

(i) Food-formulation ingredients

Biosurfactants, besides ability to diminish surface and interfacial tension, they can accordingly be use in encouraging the establishment and stabilization of emulsions, the surfactants have different functions in food. For instance, to control the aggregation of fat globules, stabilization of circulated air through frameworks, improvement of texture and shelflife of products like starch, modification of rheological properties of wheat dough and improvement of constancy and texture of fat-based products (Kachholz and Schlingmann, 1987). In bread shop and dessert definitions, biosurfactants act by

controlling the consistency, abating staling and solubilising the flavor oils; they are alagents throughout cooking of fats and oil, enhance the stability of dough, volume, and comservation of bakery products (Van and Vanzeveren, 2004).

A study conducted by Van and Vanzeveren (2004) revealed that rhamnolipids improved the properties of butter cream and frozen confectionery products. The investigators also reported that L-Rhamnose had substantial potential as a forerunner for flavouring and it is already used industrially as a precursor of high-quality flavour components like furaneol.

(ii) Anti adhesive agents

Biosurfactants have antiadhesive properties that prevent the adherancee of bacterial cells (biofilm) to surfaces. Biofilms are group of bacteria that have formed a colony on a surface. The biofilm consists not only of bacteria, but it also involves all the extracellular material produced at the surface and any trapped material within the matrix formed. Microbial biofilms which can be contained in the foodstuff industry surfaces are usually probable options for toxic contamination in which may lead to food spoilage and transmission of diseases (Hood and Zottola, 1995). The involvement of biosurfactants in microbial adhesion and detachment from surfaces has been investigated. Biosurfactant from *Streptococcus thermophilus* has been used to manage fouling in heat-exchanger plates in pasteurizers. It does this by slowing down the colonization of other thermophilics utilized as fat stabilizers and antispattering strains of *Streptococcus* that are responsible for fouling. The treatment of stainless steel surfaces with a biosurfactant obtained from *Pseudomonas fluorescens* suppresses the growth and the attachment of *Listeria monocytogenes* (Catherine, Thierry, and Marie, 2008).

2.3.4 Applications of biosurfactants in pharmaceutics and medicine

(i) Therapeutic and biomedical applications and antimicrobial activity

Several biosurfactants have exhibited antimicrobial activity against various bacteria, algae, fungi, and viruses, for instance, lipopeptide iturin from *B. subtilis* showed strong antifungal activity, 8mM of surfactin inactivated an enveloped virus such as herpes and retrovirus (Vollenbroich, Özel, Vater, Kamp, and Pauli, 1997); rhamnolipids repressed the growth of harmful bloom algae, *Heterosigma akashivo* and *Protocentrum dentatum* at a varying concentrations from 0.4mgl^{-1} to 10.0 mgl^{-1}. A rhamnolipid at a concentration of 32 mgml^{-1} exhibited inhibitory activity against *Escherichia coli*, *Micrococcus luteus* and *Alcaligenes faecalis* and at 16mgml^{-1} inhibited *Serratia arcescens and Mycobacterium phlei* while at 8mg/ml it inactivated *Staphylococcus epidermidis*, it also possess an excellent antifungal properties against *Aspergillus niger* (16 mgml^{-1}), *Chaetonium globosum*, *Enicillium crysogenum*, *Aureobasidium pullulans* (32mgml^{-1}) and the phytopathogenic *Botrytis cinerea* and *Rhizoctonia solani* (18 mgml^{-1}) (Abalos, Pinazo, Infante, Casals, Garcýa, and Manresa, 2001). Similarly it was reported that a glycolipid surfactant from *Candida antartica* (mannosylerythritolcations lipid) (MEL) had antimicrobial activity specifically against Gram-positive bacteria (Kitamoto, Yanagishita, Shinbo, Nakane, and wagner, 1993) and also exerted growth inhibition and differentiation-inducing activities against human leukemia cell lines by directly affecting intracellular signal transduction through phosphate cascade system (Tahzibi, Kamal, and Mahaheri, 2004; Tabatabaee, Mazaheri, Noohi, and Sajadianva, 2005; Thaniyavarn, Chongchin, Wanitsuksombut, and Thaniyavarn, 2006; Tachaoei, Leelapornpisid, Antiarwarn, and Lumyong, 2007).

(ii) Anti adhesive agents

Biosurfactants have been found to inhibit the adhesion of pathogenic organisms to solid surfaces and infection sites; Rodrigues, Mei, Teixeira, and oliveira (2004) reported that pre-coated of vinyl urethral catheters rubbed by surfactin solution before used decreased to a large extent the amount of biofilm formed by *Salmonella typhimurium*, *Salmonella enteric*, *E.coli* and *Proteus mirabilis*. The investigators also recorded decreased numbers of microbe on prostheses and induced decrease in the airflow resistance that occurred on voice prostheses after biofilm formation. Similarly the pre-treatment of silicone rubber with surfactant produced by *S. thermophilus* inhibited 85% adhesion of *Candida albicans* while surfactants produced from *Lactobacillus fermentum* and *L. acidophilus* adsorbed on glass and prevented 77% adherence by uropathogenic cells of *Enterococcus faecalis* as well as prevented infections of *Staphylococcus aureus* and its adherance to surgical implants (Gan, Kim, Reid, Cadieux, and Howard, 2002).

(iii) Immunological adjuvants

Lipopeptides have been identified to contain potent non toxic and non-pyrogenic immunological adjuvants. Rodrigues *et al.* (2006) reported that the mixture of these lipopeptides with conventional antigens enhanced humoral immune response.

2.3.5 Anticancer activity

The biological activities of seven microbial extracellular glycolipids, in conjuction with mannosylerythritol lipids-A, mannosylerythritol lipids-B, rhamnolipid, polyol lipid, sophorose lipid, etc. have been studied. All these glycolipids, except for rhamnolipid, were able to induce cell differentiation instead of cell proliferation in the human promyelocytic leukaemia cell line HL60. STL and MEL noticeably increased common differentiation characteristics in monocytes and granulocytes respectively.

Exposure of B16 cells to MEL lead to the condensation of chromatin, DNA fragmentation and sub-G1 arrest (the sequence of events in apoptosis). This is the first evidence that growth retards, apoptosis and differentiation of the mouse malignant melanoma cells can be induced by glycolipids (Zhao, 1999). In addition, exposure of PC12 cells to MEL enhanced the activity of acetylcholine esterase and interrupted the cell cycle at the G1 phase, with resulting outcome of neurites and partial cellular differentiation (Wakamatsu *et al*., 2001).

2.3.6 Anti-human immunodeficiency virus and sperm immobilizing activity

The increased incidence of Human Immunodeficiency Virus (HIV)/AIDS in women aged 15–49 years has identified the urgent need for a female-controlled, effective and safe vaginal topical microbicide. To overcome this challenge, sophorolipid synthesized by *C.bombicola* and its structural analogues have been studied for their spermicidal, anti-HIV and cytotoxic activities (Shah, Doncel, Seyom, Eaton, and Gross, 2005). The sophorolipid diacetate ethyl ester derivative is the most potent spermicidal and virucidal agent of the series of sophorolipids studied. Nevertheless, it also induced sufficient vaginal cell toxicity to raise concerns about its applicability for long-term microbicidal contraception.

2.3.7 Agents for respiratory failure

A deficiency of pulmonary surfactant which is a phospholipid protein complex is responsible for the failure of respiration in prematurely born infants. Isolation of the genes for protein molecules of this surfactant and cloning in bacteria has made possible its fermentative production for medical applications (Gautam and Tyagi, 2005).

2.3.8 Role of Biosurfactants in Biodegradation Processes

A better technique that can effectively enhance the remediation of hydrocarbon contaminated environments is the use of biosurfactants. They influence hydrocarbon degradation in two ways. The first includes; increase the substrate availability for microbial utilization, while the second process involves cell surface interaction which elevates the hydrophobicity of the surface permitting the hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004). These reduction of surface and interfacial tensions by biosurfactants results in the increase of surface areas of insoluble compounds leading to increased bioavailability and mobility of hydrocarbons. Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilisation or emulsification (Figure 2.10) (Déziel, Paquette, Villemur, Lepine, and Biasaillon, 1996; Nievas, Commendatore, Estevas, and Bucalá, 2008).

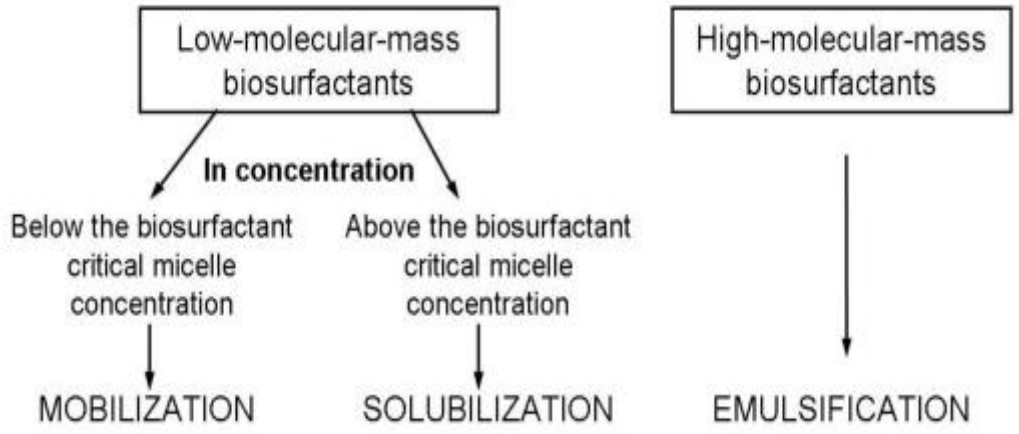


Figure 2.10: Mechanisms of hydrocarbon removal by biosurfactants depending on their molecular mass and concentration (Urum and Pekdemir, 2004).

Mobilization mechanism of biosurfactant in hydrocarbon removal usually takes place at concentrations below the biosurfactant CMC, at such concentrations; biosurfactants reduce the surface and interfacial tension between air/water and soil/water systems. Due to the reduction of the interfacial force, contact of biosurfactants with soil/oil system increases the contact angle and reduces the capillary force holding oil and soil together. In turn, above the biosurfactant CMC the solubilisation (that is, incorporation of these molecules into a micelle) process takes place. At these concentrations biosurfactant molecules associate to form micelles, which dramatically increase the solubility of oil, the hydrophobic ends of the biosurfactant molecules then connect together inside the micelle creating an environment compatible for hydrophobic organic molecules while the hydrophilic ends becomes exposed to the aqueous phase on the exterior (Urum and Pekdemir, 2004).

2.3.9 Soil washing technology

Soil washing technology (an ex-situ method performed in reactors or tank where environmental conditions can be manipulated and controlled without restrictions) is characterized by physicochemical properties of the biosurfactant and not by their effect on metabolic activities or changes in cell-surface properties of bacteria (Banat *et al*., 2010). However, the processes may enhance the bioavailability for bioremediation. Aqueous solutions of biosurfactants can also be used to release compounds characterized by low solubility from soil and other media in process called washing. Urum, Grigson, Pekdemir, and McMenamy (2006) investigated the efficiency of different surfactant solutions in removing crude oil from contaminated soil using a soil washing process; the investigators concluded that higher crude oil elimination was obtained by synthetic surfactant-sodium dodecyl sulphate (SDS) and rhamnolipid biosurfactants (46% and 44%, respectively) than natural surfactants—saponins (27%).

Lai *et al.* (2009) studied the ability of removing total petroleum hydrocarbon (TPH) from soil by two biosurfactants (rhamnolipid and surfactin) and two synthetic surfactants (Tween 80 and Triton X-100). The TPH removal efficiency was examined for low TPH-contaminated (LTC) and high TPH-contaminated (HTC) soils (containing 3000 and 9000 mg·kg−1 dry soil of TPH, respectively). They observed that addition of 0.2 mass% of rhamnolipid, surfactin, Triton X-100 and Tween 80 to LTC soil resulted in a TPH removal of 23%, 14%, 6% and 4%, respectively, while for HTC soil a significantly higher TPH removal efficiency of 63%, 62%, 40% and 35%, respectively, was observed. These results indicated that biosurfactants, rhamnolipid and surfactin showed superior performance on TPH removal, compared to synthetic surfactants.

The bioremediation of soils contaminated by aliphatic and aromatic hydrocarbons (microcosm bioremediation experiment), PAHs, and heavy metals (batch experiment) was evaluated by Franzetti *et al*.(2010) using surface-active compounds (extracellular bioemulsan and cell-bound biosurfactant) produced by *Gordonia* sp. The results showed that the bioemulsans produced by *Gordonia* sp. strain BS29 were able to enhance the biodegradation of recalcitrant branched hydrocarbons and achieved 33% removal of metal.

2.3.10 Microbial enhanced oil recovery (MEOR)

(i) Mechanism of MEOR

Biosurfactants can also be involved in microbial enhanced oil recovery (MEOR). MEOR methods are used to recover oil remaining in reservoirs after primary (mechanical) and secondary (physical) recovery procedures (Magdalena *et al.*, 2011). It is an important tertiary process where microorganisms or their metabolites, including biosurfactants, biopolymers, biomass, acids, solvents, gases and also enzymes, are used

to increase recovery of oil from depleted reservoirs. Application of biosurfactants in enhanced oil recovery is one of the most promising advanced methods to recover a significant proportion of residual oil. The remaining oil is often located in regions of the reservoir that are difficult to access and the oil is trapped in the pores by capillary pressure by biosurfactants causing reduction in the interfacial tension between oil/water and oil/rock which in turn reduces the capillary forces preventing oil from moving through rock pores (Figure 2.11) (Suthar, Hingurao, Desai and Nerurkar, 2008).

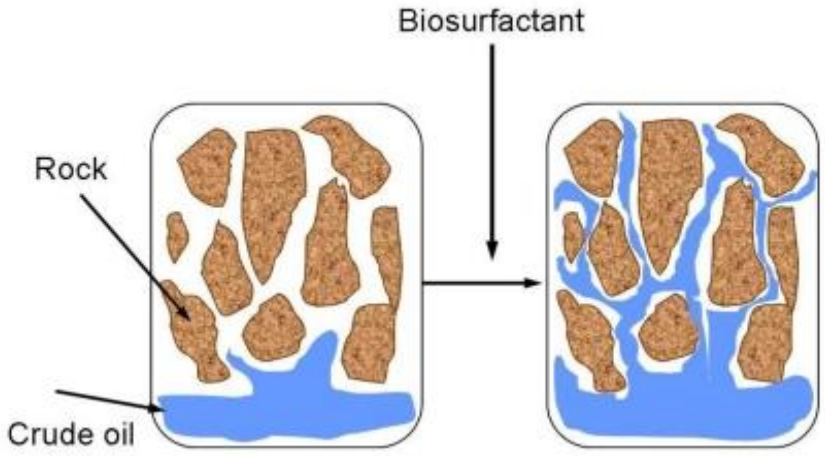


Figure 2.11: Mechanism of enhanced oil recovery by biosurfactants (Suthar *et al*., 2008)

(ii) Applications of MEOR

Laboratory studies on MEOR were investigated by utilizing core substrates and columns containing the desired substrate, usually sand, this substrate was used to demonstrate the usefulness of biosurfactants in recovery of oil from reservoirs. For this purpose, a glass column was packed with dry sand, then the column was saturated with

crude oil and aqueous solution of biosurfactant was poured into the column. The potential of biosurfactants in MEOR was estimated by measuring the amount of oil released from the column after pouring the aqueous solution of biosurfactant in the column. The experiment was carried out in room temperature, 70 and 90 °C to evaluate the influence of temperature on biosurfactant-induced oil recovery (Magdalena, *et al*., 2011).

Biosurfactants can also be used to extract hydrocarbon compounds from oil shales in order to utilize it as a substitute for petroleum energy fuel. In studies conducted by Haddadin *et al.* (2009) biosurfactants produced by *Rhodococcus erythropolis* and *Rhodococcus ruber* were successfully used for desorption of the hydrocarbons from El-Lajjun oil shale.

2.4 Heavy Metals

Industrial and agricultural activities have intensified as a result of global population growth and technological advancement, which has lead to a considerable increase in the concentration of heavy metals in the environment. Inorganic pollutants (like heavy metals) can be mineralized completely to non-dengerious form like the organic pollutant and their toxicity is intrinsic to their atomic structure (Bonaventura and Johnson, 1997). Pollution caused by heavy metals in ecosystems has been recognized as a severe environmental concern, due to their tendency to accumulate in plant and animal tissues, long shelf life and non-biodegradability (Otitoloju, Ajikobi and Egonmwan, 2009).

2.4.1 Sources of heavy metals contamination in soils

Heavy metals are present naturally in the environment from the formation procedures in weathering of parent materials at degrees refered as trace and non toxic (<1000 mg kg−1). Because of the disruption of geochemical cycle of metals by man, almost all the soil both in urban and rural environment may perhaps pile up a number of these metals to a level that is above the original natural values high enough to become hazardous to human, animals, plants and ecosystems. (D'Amore *et al.,* 2005). The heavy metals essentially become contaminants in the soil environments because (i) their rates of generation via man-made cycles are more rapid relative to natural ones, (ii) they become transferred from mines to random environmental locations where higher potentials of direct exposure occur, (iii) the concentrations of the metals in discarded products are relatively high compared to those in the receiving environment, and (iv) the chemical form (species) in which a metal is found in the receiving environmental system may render it more bioavailable (D'Amore *et al*.,2005). Some of the sources of heavy metal contamination of soil are:

(i) Fertilizers: fertilizrs are added frequently to soil during intensive farming system inorder to add nutrients (N, P, and K) for growth of crop. The mixtures used as a medium to supply these elements usually have trace amount of heavy meatal (e.g. Cd and Pb) as impurities, which significantly increase after continuous application of fertilizer (Jones and Jarvis, 1981).

(ii) Pesticides: Some pesticides commonly used in horticure and agriculture in the past contained high concentrations of metals. For instance, 10% of the chemicals approved for use as fungicides and insecticides were based on compounds which contain Cu, Zn Pb, Hg, or Mn. Examples of such pesticides are copper-containing fungicidal sprays such as Bordeaux mixture (copper sulphate) and copper oxychloride.

Lead arsenate was used in fruit orchards for many years to control some parasitic insects. Arsenic-containing compounds were also used to control cattle ticks and pests in banana; timbers have been preserved with formulations of Cu, Cr, and As (CCA), and there is now evidence where concentrations of soil by these elements have greatly exceeds background concentrations. Such contamination has the capacity to cause problems, especially if the sites are restructured for other agricultural or non-agricultural purposes (McLaughlin *et al*., 2000).

(iii) Biosolids and Manures: Biosolids (sewage sludge) are primarily organic solid products, generated from wastewater. Heavy metals mostly present in biosolids are Pb, Ni, Cd, Cr, Cu, and Zn, and the metal concentrations are influenced by the kind of procedure applied during the treatment, the intensity and type of industrial activity. The application of numerous biosolids (e.g., livestock manures, composts, and municipal sewage sludge) to land inadvertently leads to the accumulation of heavy metals in the soil (McLaren, Clucas, and Taylor, 2005). Under certain conditions, applications of biosolids added metals to soils and these metal can be leached downwards through the soil profile and contaminate groundwater (McLaren *et al.*, 2004).

(iv) Wastewater: The application of municipal and industrial wastewater and related effluents to land is a common practice in many parts of the world (Reed, Crites, and Middlebrooks, 1995). Worldwide, it is approximated that 20 trillion hectares of arable land are generally irrigated with waste water. In many Asian and African cities scientific studies suggest that agriculture dependant on waste water irrigation makes up 50 percentage of the plant supply to towns (Bjuhr, 2007). Farmers generally are not interested in the environmental hazards emanating from this, but rather they are mainly enthusiastic about maximizing their yield and profits. Although the heavy metal levels

in waste water effluents are comparatively low but long-term irrigation of land with such can eventually result in accumulation of heavy metal in the soil.

(v) Metal mining and milling processes and commercial wastes: Exploration and milling connected with heavy metal ores by industrial sectors|companies have left many countries the legacy of wide distribution of metal contaminants in soil. During mining and tailings, weightier and larger debris resolved to the bottom of the flotation cell which is later directly discharged by normal depressions, which include onsite wetland causing increasesd concentrations (DeVolder, Darkish, Hesterberg and Pandya, 2003). Extensive lead (Pb), zinc (Zn) ore mining and smelting have resulted in contamination of soil that poses risk to human and ecological health (Basta and Gradwohl, 1998). Industries such as textile, tanning, petrochemicals from accidental oil spills or utilization of petroleum-based products, pesticides, and pharmaceutical facilities can generate high amount of metal (Basta and Gradwohl, 1998).

(vi) Air-borne sources: Airborne sources for metals consist of stack or duct emissions connected with air flow, propane, or vapor streams, and fugitive emissions for example airborne dirt and dust from storage space places or waste piles. Metals from air borne sources are likely to be launched as particulates within the gas flow. Many metals for example, As, Cd, and Pb can volatilize in the course of high-temperature processing. These metals will convert to oxides and condense as fine particulates unless a reduced atmosphere is maintained (Smith, Means, Chen, Alleman and Chapman, 1995). Stack emissions can be distributed over a wide area by natural air currents until dry and/or wet precipitation mechanisms remove them from the gas stream. Fugitive emissions are often distributed over a much smaller area because emissions are made near the ground. In general, contaminant concentrations are lower in fugitive emissions compared to stack emissions but the type and concentration of metals emitted from both

sources will depend on site-specific conditions; all solid particles in smoke from fires and in other emissions from factory chimneys are eventually deposited on land or sea.

Most forms of fossil fuels contain some heavy metals and this is, therefore, a form of contamination which has been continuing on a large scale since the industrial revolution began. For example, very high concentration of Cd, Pb, and Zn has been found in plants and soils adjacent to smelting works (Smith et al., 1995). Another major source of soil contamination is the aerial emission of Pb from the combustion of petrol containing tetraethyl lead; this substantially contributes to the content of Pb in soils in urban areas and in those adjacent to major roads. Zinc and Cd may also be added to soils adjacent to roads, the sources being tyres, and lubricant oils (USEPA, 1998).

2.4.2 Heavy metals and their effects

The most common heavy metals found at contaminated sites, in the order of abundance are Pb, Cr, As, Zn, Cd, Cu, and Hg (USEPA, 1998). The fate and transport of a heavy metal in soil depends significantly on the chemical form and speciation of the metal. Once these heavy metals are in the soil, they are adsorbed by fast initial reactions (minutes, hours), followed by slow adsorption reactions (days, years) and are, therefore, redistributed into different chemical forms with varying bioavailability, mobility, and toxicity (Shiowatana,*et al.*, 2001; Buekers, 2007). This distribution is believed to be controlled by reactions of heavy metals in soils such as (i) precipitation and dissolution of mineral, (ii) desorption, ion exchange, and adsorption, (iii) aqueous complexation, (v) biological mobilization and immobilization, and (v) plant uptake (Levy, Barbarick, Siemer, and Sommers, 1992). Some heavy metals are discussed below:

(i) Lead

Lead (Pb) is a metal belonging to Group IV and Period 6 of the Periodic Table with atomic number 82, atomic mass 207.2, density 11.4 $g\,cm^{-3}$, melting point 327.4°C, and boiling point 1725°C. It is a naturally occurring, bluish-gray metal usually found as a mineral combined with other elements, such as sulphur (i.e. PbS, $PbSO_4$), or oxygen ($PbCO_3$), and ranges from 10 to 30 $mg\,kg^{-1}$ in the earth's crust (Raymond and Felix, 2011). Lead is not an essential element; it performs no known essential function in the human body. About half, of the Pb used, goes for the manufacture of Pb storage batteries, ammunition, solders, plumbing, cable covers, bearings, pigments, and caulking (Raymond and Felix, 2011).

Lead can exist in food, water, air, and soil and can enter human body through dermal contact, inhalation, or ingestion. The health effects of lead poisoning are both acute and chronic, and are particularly severe in children (Landrigan, Schechter, Lipton, Fahs, and Schwartz, 2002; Woolf, Goldman, and Bellinger, 2007; Kaul and Mukerjee, 2009; Andrew *et al.*, 2010). These adverse impacts can include neurological damage, nerve disorders, reduced intellegient quotient (IQ), muscle and joint pain, anaemia, loss of memory and concentration, infertility, increased blood pressure, and chronic headaches. Due to the small size of this metal, even small amounts of it in human body can be associated with long-term neurological and cognitive defects. When pregnant women are exposed to lead, it can result in damage to the foetus and eventually birth defects. At high concentrations, lead poisoning can cause seizures and death (National Safety Council, NSC, 2009).

The worst and most recent heavy metals incidence in the Nigerian record was that of Zamfara lead poisoning that claimed the lives of over 500 children within seven months in 2010; where illegal miners from seven villages of Bukkuyum and Gummi local governments of the State brought rocks containing gold ore into the villages for small scale mining operations (Médecins Sans Frontières MSF, 2010); Blacksmith Institute, 2010); Galadima *et al*., 2011). Jamiu *et al*. (2013) reported an increased rate of Pb in Abeokuta to a level far above permissible level. High concentration of lead was detected in blood of 87 children in Kaduna State (Galadima and Garba, 2012). Other incidence of Pb include catastrophe in Dakar, Senegal, where between November 2007 and March 2008, 18 children died from acute lead poisoning due to lead dust and soil exposure from ULAB recycling; Haina in the Dominican Republic, where at least 28% of children required immediate treatment for lead exposure, and 5% had blood-lead levels that put them at risk for neurological damage (Andrew *et al.*, 2010).

(ii) Chromium

Chromium (Cr) is a first-row -block transition metal of Group VIB in the Periodic Table with atomic number of 24, atomic mass 52, density 7.19 gcm^{-3}, melting point 1875°C, and boiling point 2665°C. It is one of the less common elements and does not occur naturally in elemental form, but only in form of compounds (Smith *et al.*, 1995). They occur in two major forms, Cr (III) and (VI). Cr (VI) is the more toxic and mobile form commonly found at contaminated sites, but it can be reduced to Cr (III) by soil microorganisms while Cr (III) mobility can be decreased by adsorption to clays and oxide minerals (Chrostowski, Durda, and Edelmann, 1991). Cr. enters the air, water, and soil through natural processes and human activities such as chemical, steel, leather and textile manufacturing, electroplating and tanning and coal combustion.

The effect of Cr varies; for organisms it alters their genetic makeup and causes cancer; in soil it can cause soil acidification and influences the uptake of available nutrients in soil by plant root and eventually plant may die; In water it damages the gills of fish; in animal the inhalation or ingestion of Cr containing food or water can lower their ability to fight disease, cause tumour formation, infertility and birth defects (www.lenntech.com; Blacksmith Institute, 2010).

High incidence rate of chromium were detected in Kano (Nigeria) in 2012 and this was attributed to the high indiscriminate discharge of tannery effluent and this led to the closure of four tannery companies by National Environmental Standard and Regulations Enforcement Agency (NESREA) (Mustapha, 2012). Although there has been no report about the outbreak of chromium but research has revealed that the concentration of chromium in some part of Zaria (Nigeria) (Garba *et al*., 2010) and Enugu (Nigeria) is now on the increase, high above the permissive level (Ibeto and Okoye, 2010).

(iii) Arsenic

Arsenic (As) is a metalloid in Group VA and Period 4 of the Periodic Table that occurs in a wide variety of minerals, mainly as As_2O_3, and can be recovered from processing of ores containing mostly Cu, Pb, Zn, Ag and Au. It is also present in ashes from coal combustion. Arsenic has the following properties: atomic number 33, atomic mass 75, density 5.72 $g\,cm^{-3}$, melting point 817°C, and boiling point 613°C, and exhibits fairly complex chemistry and can be present in several oxidation states (Smith *et al*., 1995). In aerobic environments, As (V) is dominant, usually in the form of arsenate (AsO_4^{3-}) in various protonation states: H_3AsO_4, $H_2AsO_4^-$, $HAsO_4^{2-}$, and AsO_4^{3-}. Arsenate and

other anionic forms of arsenic behave as chelates and can precipitate when metal cations are present. Biotransformation (via methylation) of arsenic creates methylated derivatives of arsine, such as dimethyl arsine $HAs(CH_3)_2$ and trimethylarsine $As(CH_3)_3$ which are highly volatile. Since arsenic is often present in anionic form, it does not form complexes with simple anions such as Cl^- and SO_4^{2-}. Many As compounds adsorb strongly to soils and are therefore transported only over short distances in groundwater and surface water. Arsenic is associated with skin damage, increased risk of cancer, and problems with circulatory system (Raymond, and Felix, 2011).

Arsenic compound cause both short-term and long term effects in individuals, plants, animals and organisms; these includes: irritation of the stomach, decrease production of red and white blood cells, cancer development(both skin lung liver and lymphatic cancer), infertility and miscarriages and DNA damage. In plant inhibit photosynthesis and growth (Department of Earth Science and Engineering, 2012).

(iv) Cadmium

Cadmium is located at the end of the second row of transition elements with atomic number 48, atomic weight 112.4, density $8.65\ g\,cm^{-3}$, melting point 320.9°C, and boiling point 765°C. Cd together with Hg and Pb, Cd is one of the big three heavy metal poisons and is not known for any essential biological function. Cadmium is directly below Zn in the Periodic table and has a chemical similarity to that of Zn, an essential micronutrient for plants and animals; this can accounts to some extent for Cd's toxicity; because Zn being an essential trace element, its substitution by Cd may cause the malfunctioning of metabolic processes (Campbell, 2006). The most significant use of Cd is in Ni/Cd batteries, as rechargeable or secondary power source exhibiting high output, long life, low maintenance, and high tolerance to physical and electrical stress.

Cadmium is also present as an impurity in several products, including phosphate fertilizers, detergents and refined petroleum products. In addition, acid rain and the resulting acidification of soils and surface waters have increased the geochemical mobility of Cd, and as a result its surface-water concentrations tend to increase as lake water pH decreases (Campbell, 2006).

Cadmium is very biopersistent but has few toxicological properties and, once absorbed by an organism, remains resident for many years (Weggler, McLaughlin, Graham, and Robin, 2004). Cadmium in the body is known to affect several enzymes. It is believed that the renal damage that results in proteinuria is the result of Cd adversely affecting enzymes responsible for reabsorption of proteins in kidney tubules. The activity of arylsulfatase, lipoamide dehydrogenase, alcohol dehydrogenase, and delta-aminolevulinic acid synthetase are decreased by Cadmium whereas it increases the activity of pyruvate decarboxylase, pyruvate dehydrogenase, and delta-aminolevulinic acid dehydratase (Manahan, 2003).

The most stunning as well as advertised happening associated with cadmium poisoning lead by diet consumption associated with cadmium by people in the Jintsu River Valley, near Fuchu, Japan. The victims were afflicted by itai itai disease, which means ouch, ouch in Japanese. The symptoms are the result of painful osteomalacia (bone disease) combined with kidney malfunction. Cadmium poisoning in the Jintsu River Valley ended up being attributed to irrigated grain polluted by upstream mine producing Pb, Zn, and Cd. The major threat to human health is chronic accumulation in the kidneys leading to kidney dysfunction. The main routes by which Cd enters the body are through food intake and tobacco smoking (Raymond and Felix, 2011).

(v) Mercury

Mercury belongs to same group of the periodic table with Zn and Cd. It is the only liquid metal at standard temperature and pressure (stp). It has atomic number 80, atomic weight 200.6, density 13.6 g cm^{-3}, melting point −13.6°C, and boiling point 357°C and is usually recovered as a by-product of ore processing (chemical rubber company (CRC) Handbook, 2011). Major source of Hg contamination include release from combustion coal, mining industry, the manufacturing of fungicides, releases from manometers at pressure-measuring stations along gas/oil pipelines, thermometers, thermostats and spills from broken materials containing Hg and dental amalgam for making fillings for teeth (ATSDR, 2004). After release to the environment, Hg usually exists in mercuric (Hg^{2+}), mercurous (Hg_2^{2+}), elemental (Hg^o), or alkylated form (methyl/ethyl mercury). Under anaerobic conditions, both organic and inorganic forms of Hg may be converted to alkylated forms by microbial activity, such as by sulfur-reducing bacteria. Mercury targets the brain and kidneys (Roberts, 1999; ATSDR, 2004; Raymond, and Felix, 2011).

Symptoms of acute exposure are cough, sore throat, shortness of breath, abdominal pain, nausea, vomiting, diarrhea, headache, weakness, visual disturbances, tachycardia, hypertension, and a metallic taste in the mouth.Chronic exposure to mercury may result in permanent damage to the central nervous system (Ewan, 1996) and kidneys. Mercury can also cross the placenta from mother to fetus (levels in the fetus are often double those in the mother) and accumulate, resulting in mental retardation, brain damage, cerebral palsy, blindness, seizures, and inability to speak. Symptoms of chronic exposure in adults and children could include tremors, anxiety, forgetfulness, emotional instability, insomnia, fatigue, weakness, anorexia, cognitive and motor dysfunction, and kidney damage (www.lenntech.com).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Collection of Samples

(i) Waste lubricating oil contaminated soil

Waste lubricating oil polluted soil samples were collected from different points in an automobile workshop beside Living Faith Church (few kilometres away from Federal University of Technology, Bosso Campus), Minna, Niger state, Nigeria into sterile sample bottles using a soil auger and were transported to the laboratory for the isolation of bacteria.

(ii) Uncontaminated soil

Uncontaminated soil was collected from a farmland near the botanical garden of the Federal University of Technology, Minna, Bosso Campus, Nigeria, into sterile sample bottles using a soil auger and was transported to the laboratory for heavy metal bioremediation study.

(iii) Metal compounds

The metal compounds, lead [$Pb(NO_3)_2$] and chromium [$Cr(NO_3)_3$] with 99% purity were obtained from the Department of Chemistry, Federal University of Technology, Minna, Nigeria.

(iv) Hydrocarbons

The crude oil used was Bonny light crude (BLC) obtained from Port Harcourt refinery, Alesa Eleme, Rivers State, Nigeria. Diesel and kerosene was obtained from Garma Petroleum Ltd, Bosso Road, Minna, Niger State, Nigeria.

3.2 Media Used

R2B broth, R2A agar, Blood agar, Muller Hinton broth (MHB), Motility agar, Simon citrate agar, Nutrient agar, Nutrient broth, starch agar, mineral salt medium (MSM) were used. The composition and preparation of the media are presented in Appendix A.

3.3 Isolation of Bacteria

The isolation of bacteria was done using the method of Anandaraj and Thivakaran (2010) and Erum *et al.* (2012)**.** Five grams of the oil polluted soil sample was inoculated in fifty millilitres of R2B broth and incubated at 37ºC for 24 hours. After incubation, the medium was serially diluted from 10^{-1} to 10^{-6} in sterile water. From the dilutions (10^{-1} to 10^{-6}), 1ml was transferred to sterile Petri dishes and over that 20ml of R2A agar was poured. Then the plates were incubated at 25ºC for 48 hours along with controls (which had sterile water and sterile R2A agar uninoculated) and this was done in triplicates. After incubation, morphologically different colonies were selected and subcultured repeatedly to obtain pure cultures. The pure cultures obtained were stored in nutrient agar (NA) slants and kept under refrigerated conditions (4ºC) for further screening.

3.4 Characterization and Identification of Biosurfactant Producing Bacteria

The potential biosurfactant producing bacteria were characterized based on their gram stain reaction and biochemical tests. Some of the biochemical tests are described below (following the methods of Fawole and Ose, 1988; Oyeleke and Manga, 2008). The isolates were identified by comparing their characteristics with those of known taxa using Bergey's Manual of Determinative Bacteriology (George, Julia and Timothy, 2004).

(i) Gram staining

A wire loop was flamed to red hot and allowed to cool; this sterile loop was used to pick culture from a discrete colony. A smear of the discrete colony was made on a clean slide which was allowed to air dry before it was fixed by passing it gently over a flame. The fixed smear was flooded with crystal violet for 60seconds after which the stain was drained off and washed over a running tap; then it was flooded with lugol's iodine for 60seconds and washed gently using tap water. This was flooded with 95% alcohol to decolorize for 30 seconds and rinsed with water. The slide was counter stained using safranin for 30 seconds, after which the slide was washed gently with water and was left to air-dry. This was viewed under oil immersion objective lens of the microscope. Gram-positive bacteria appeared purple/blue while gram-negative appeared red/pink.

(ii) Catalase test

Two drops of 3% hydrogen peroxide (H_2O_2) was placed on each end of a clean grease free slide and labelled A and B. With the help of a clean glass rod the test organism was transferred to drop A and was observed immediately for gas bubbling (effervescence) while drop B served as control. Result was recorded as negative or positive based on the evolution of gas or bubbles formed.

(iii) Indole test

Test organisms were grown in 5ml of 1% peptone water at 37^{o}C for 48 hours. This was followed by the addition of 0.5ml of Kovac's reagent (prepared by dissolving 5g P-dimethylamino-benzaldehyde in 75ml amyl alcohol and 25ml concentrated HCl, Appendix B) and shaken gently. Appearance of red colour ring at the reagent layer was recorded as positive result while absence of colored ring indicated negative result.

(iv) Oxidase test

Three drops of a freshly prepared oxidase reagent (tetraethyl-p-phenylenediamine dihydrochloride) was placed on a piece of filter paper placed on a clean Petri dish, and a sterile wooden stick was used to collect the test organism and smear it on the filter paper. The appearance of a blue-purple colour within ten seconds was recorded as positive result while the absence of blue-purple colour after fifteen seconds was recorded as negative.

(v) Citrate test

Twenty four point two eight grams (24.28g) of Simmons citrate agar was weighed and dissolved into 100ml of distilled water by heating. Then it was dispensed into test tubes. The citrate agar in the test tubes was sterilized by autoclaving at 121°C for 15minutes, and then it was placed in an angular position for it to gel into slant. The test organisms were streak inoculated into the citrate agar slants and incubated at 37^{o}C for 4days. Colour change from green to blue indicated a positive result, while no colour change (green colour retained) indicated a negative result.

(vi) Carbohydrate utilization test (acid and gas production from carbohydrate)

One hundred millilitres (100ml) of peptone water was prepared with the addition of two grams (2g) of the test sugar (sucrose, fructose, D-mannitol, lactose, D-glucose, sorbitol, arabinose, D-mannose) and 0.08gram of phenol red was incorporated as indicator. Five millilitres (5ml) of the mixture was dispensed into test tubes and sterilized by autoclaving at 121^{o}C for 15minutes along with an inverted Durham's tube inside the medium and was allowed to cool. Thereafter, the test organisms were inoculated into the sterile medium, a control was set up without the inoculation of the test organism.

This was incubated at 37ºC for 24- 48 hours after which the medium was observed for colour change from red to orange (yellow) indicating acid production while a void in the Durham's tube indicated formation of gas.

(vii) Triple Sugar Iron Agar test (TSI) (Hydrogen sulphide production)

Triple sugar iron agar slant was prepared and the test isolates were aseptically inoculated (using inoculating wire loop) into the agar by stabbing the agar to the bottom and streaking the surface of the slant. This was incubated along with uninoculated duplicate tubes as controls at 37ºC for three days, it was examined daily and the results were recorded with black precipitate indicating hydrogen-sulphide production, the extent of yellow coloration to indicated sucrose, lactose and glucose fermentation while the presence of cracks in the butt pushed from the bottom indicated gas formation.

(viii) Motility test

(a) Stab culture techniques

Motility medium was prepared in a test tube using 10g of peptone, 5g of agar agar, and 5g of sodium chloride (NaCl) per liter. This was sterilized by autoclaving at 121ºC for 15minutes. The bacterial isolates were inoculated into the sterile motility medium by stabbing with a sterile needle to a depth of about 2 cm just about the centre of the medium. The tubes were then incubated at 37ºC for 18-24 hours. Organisms that grew only along the line of stab as compared to the control was recorded as non-motile whereas those that grew along the line of stab and diffused into the medium away from the line of stabbing causing turbidity (rendering the medium not clear or opaque) was recorded as motile.

(ix) Hanging drop techniques

A clean grease free cavity slide was placed on the bench with the cavity uppermost, then a clean cover slip was held between two fingers and a drop of molten vaseline was carefully placed on the four edge of the cover slip. Then a drop of 18 hours old bacterial suspension was placed gently at the centre of the cover slip to which vaseline was applied; this was quickly and carefully inverted to the cavity slide, making the drop of the bacterial suspension on the cover slip to suspend in the centre of the slide. The slide along with the cover slip was examined for motility, first by using low power objective (x10) to focus the edge of the drop after which it was carefully turned to high power objective (x40) with reduced illumination. Organisms whose movement was directional, different from zigzag movement was recorded as positive (motile)

(x) Urease production

Bacterial isolates were inoculated in urea agar slants in bijou bottles and were incubated at 37^{o}C for 24 hours. Bright pink (or red colour) indicated a positive reaction while a negative reaction was indicated by the absence of coloration (i.e. the colour remains pale yellow).

(xi) Methyl red (MR) and Voges Proskauer (VP) test

The bacterial isolates were inoculated into test tubes containing 2ml of sterile glucose phosphate peptone water labelled A and B and incubated at 37^{o}C for 48 hours. To test tube A, four drops of methyl red reagent was added using a Pasteur's pipette; this was mixed by shaking gently and was observed for immediate colour change. Positive and negative M-R inference was indicated by bright red rings on the surface of the medium and yellow colour respectively. To tube B, one millilitre of 40% potassium hydroxide (KOH) and 3ml of 5% alcoholic alpha-naphtol was added and shaken properly. This

was allowed to stand for 3 minutes. A pink colour formation within 2-3minutes was recorded as positive V-P reaction while no colour change (i.e. remains black) indicated negative reaction.

(xii) Spore staining

Smears of the bacterial isolates were flooded with malachite green. The slides were brought to steaming for 3 minutes (by placing the stained slide over boiling water) and rinsed with tap water. Safranine solution was applied and was left for 30 seconds, afterwhich it was washed, dried and examined under the oil immersion objective. The spores stained green and the remainder of the cell were light red.

(xiii) Hydrolysis of macromolecules

(a) Starch hydrolysis

Starch agar was prepared by adding 2g of soluble starch into 100ml of nutrient agar. This was sterilized by autoclaving at 121^{o}C for 15 minutes, and then poured into sterile Petri dishes and allowed to solidify at room temperature. The bacterial isolates were streak inoculated onto the surface of the starch agar and incubated at 37^{o}C for 48 hours. After incubation, the plates (both inoculated and uninoculated) were flooded with Gram's iodine and were observed for holo zones around the isolates. The uninoculated plates remained blue-black (negative) while the inoculated plates with zone of clearing around the colonies of the streaked isolates were recorded as positive.

(b) Protein hydrolysis

The test organisms were streaked on sterile milk agar and incubated along with uninoculated milk agar plates at 37^{o}C for six days. This was observed daily after 24

hours for clear halo zone around the test isolates which indicated positive result (i.e. organism utilizes the protein) while absence of halos indicated negative result.

3.5 Screening of Isolates for Biosurfactant Production

The isolates were screened for ability to produce biosurfactants using the following methods:

3.5.1 Haemolytic activity test

Blood agar was prepared following the method used by Ijah and Olarinoye (2012); after which the isolates were streaked on the blood agar and the plates incubated at 28ºC for 48 hours. The plates were examined visually for zone of clearance (haemolysis) around the colonies. Isolates that had ability to lyse red blood cells and form a clear zone around colonies were noted as biosurfactant producers and recorded as positive (+) while those that could not form halo zones were recorded as non-biosurfactant producers (negative). Complete and incomplete haemolysis was designated as β (beta) and α (alpha) haemolytic activity respectively (Misawa, Hirayama, Itoh, Takahashi, 1995 and Erum *et al*., 2012).

3.5.2 Drop collapse test

Drop Collapse Assay developed by Jain, Collins-Thompson, Lee, and Trevors, (1991) was adopted. Two microlitres (2µl) of the cell free supernatant obtained after the centrifugation of eighteen hours old broth culture at 6000rpm for 30minutes using IEC FL 40R Centrifuge, USA, were placed on an oil coated solid surface and the shape of the drop was noted after 1 minute. The culture supernatant that collapsed the oil drop was indicated as positive showing the presence of biosurfactant and the culture

supernatant which failed to collapse the oil drop and gave rounded drops which appeared like air bubble was indicated as negative showing absence of biosurfactant.

3.5.3. Oil spreading or oil displacement techniques

Oil displacement method according to Jaysree *et al.* (2011) was used to determine the diameter of the clear zone, which occurred after adding surfactant-containing solution on an oil-water interphase. In this test, 25ml of distilled water was added to a Petri dish which was 90mm in diameter and 100µl of crude oil was added to the water surface followed by the addition of 20µl of cell free culture supernatant obtained after the centrifugation of eighteen hours old broth culture at 6000rpm for 30minutes. The diameter of the oil as displaced by the cell free supernatant and the clear zone formed were visualized under visible light and this was measured after 30seconds.

3.5.4 Emulsification capacity (E24)

Emulsification capacities of the isolates were tested using the method of Cooper and Goldenberg (1987). Two millilitres (2 ml) of kerosene and 2 ml cell free supernatant obtained after the centrifugation of eighteen hours broth culture at 6000rpm for 30 minutes using IEC FL 40R Centrifuge, USA, was added into a test tube; the mixture was homogenized by vortexing at high speed for two minutes using Stuart auto votex mixer (AE-11D, Great Britain). The homogenized mixture was allowed to stand for 24 hours undisturbed. After 24 hours, the height of the stable emulsion layer and the total height of the mixture were measured by using a meter rule; the values obtained were used to calculate the emulsification index (E_{24}), thus:

$$E_{24} = \frac{\text{height of emulsion layer}}{\text{total height of aqueous layer}} \times 100$$

3.6 Biosurfactant Production

The potential biosurfactant producing bacterial isolates were inoculated into a sterile Muller Hinton broth and incubated at 37^{o}C for 12 hours, then one millilitre of the 12 hours old culture was transferred into 100ml of freshly prepared mineral salt medium of Jacobucci *et al*.(2001) containing one millilitre of diesel oil. The medium was then incubated at 25^{o}C for 7 days with shaking at 300 oscillations per minute using flask shaker (Stuart SFI, ST15 OSA, United Kingdom) (Figure 3.1).

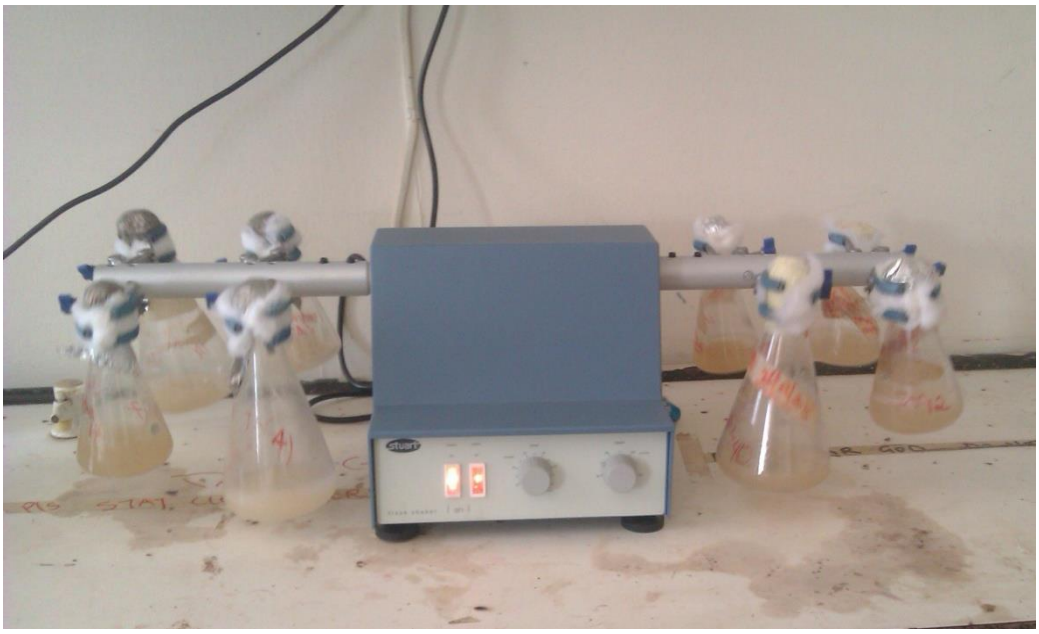


Figure 3.1 Production processes for biosurfactant

3.6.1 Biosurfactant extraction

Extraction of biosurfactant was done using acid precipitation method according to Ibrahim *et al*. (2013). In this method, the bacterial isolates were removed after 7days of incubation by centrifugation at 6000rpm, using a centrifuge (model IEC FL 40R, USA) at 4^{o}C for 30 minutes. The cell free culture supernatant was acidified with 1M of freshly prepared sulphuric acid (H_2SO_4) to obtain a pH of 2.0. The acidified cell free

supernatants were then used for the extraction of the biosurfactant. To every 100ml of the acidified cell free supernatant, 100ml of mixture of chloroform: methanol in the ratio of 2:1 (v/v) was added. The mixture was allowed to react for 30seconds, after which it was shaken vigorously until two phase separation was obtained. The upper layer containing majorly the reagents was decanted and the lower layer containing the biosurfactant was concentrated using a rotary evaporator (Model RE300, England), where most of the solvent evaporated and the left over sediment was poured into a test tube and centrifuged at 600rpm for 20 minutes. A whitish colour sediment was obtained as the biosurfactant.

3.6.2 Determination of dry weight of biosurfactants

The initial weight of sterile Petri plate was taken, and then the extracted biosurfactant was poured into the plates. This was placed in the hot air oven at 100^{o}C for 30 minutes. After drying, the plates and contents were reweighed. The weight of biosurfactant produced was determined using the formula = (Weight of the plate after drying -weight of the empty plate)**.**

3.6.3 Characterization of biosurfactants

(i) **Thin layer chromatography**

Preliminary characterization of the biosurfactant was done by Thin Layer Chromatography (TLC). Commercially prepared Silica gel (F254) plates (G60, Merck, Darmstadt, Germany) were activated at temperature of 160^{o}C using the hot air oven for one hour. Then ten different points of location were made at a distance of 2cm away from the base of the TLC plate; these points were labelled according to the code of the biosurfactants using pencil (that is, two points for one sample). 10µl of each test sample

(biosurfactants) were placed on each specific labelled point using capillary tubes and was allowed to dry for 10 minutes. Then it was placed inside a TLC tank flooded with an organic solvent (chloroform-methanol-water) at 70:10:0.5 (v/v/v) and covered with the lid. The test sample and solvent travelled along the TLC plates and after 30 minutes the movement stopped. The plates were removed and allowed to air dry for 10 minutes and were viewed under a UV light using mineral light lamp (model UVGL-15, England) and the separated spots were marked (circled). Then the spots were sprayed with colour developing reagents (Ninhydrin solution and Anthrone); all the developed spots retained a yellow colour of anthrone reagent indicating positive reaction for glycolipids according to Anandaraj and Thivakaran (2010).

The retention factor was determined using the expression: distance travelled by the test sample divided by the distance travelled by the solvent

$$RF = \frac{\text{distance travelled by sample}}{\text{distance travelled by solvent}}$$

(ii) Gas chromatography and mass spectroscopy (GC-MS)

GCMS analysis of the biosurfactant was carried out according to the method used by National Research Institute for Chemical Technology, NARICT (2013). The Crude biosurfactant (1mg) was mixed with 5% HCl- chloroform: methanol reagent (1ml). After the reaction was quenched with 1ml of distilled water, the sample was extracted with n-hexane and injected into GCMS (Model QP2010 PLUS, Shimadzu, Japan) equipped with a RTX-5MS (30m×0.2mm) capillary column and mass selective detector (AOC-20i) set to scan from m/z 40 to m/z 800 at scan rate of 1.2 scans per second. The oven temperature was initially programmed at 80^{o}C for 3 minutes and then increased at

the rate of 10°C per minute to 280°C. The carrier gas was Helium at a flow rate of 1.58ml min^{-1} and a split ratio of 50:1.0.

(iii) Fourier transform infra red (FTIR) analysis

In order to determine the functional groups in the biosurfactants, FTIR analysis was carried out according to the method used by NARICT (2013). One milligram of the extracted biosurfactant was ground with 100 mg of potassium bromide (KBr) and pressed with a silver coated hand presser at 7500 kg for 30 seconds to obtain translucent pellets. The pellet obtained was inserted into fourier transform infrared spectrophotometer (FTIR-8400S, Shimadzu, Japan) where the infrared spectra were recorded within the range of 4500-500cm^{-1} wave number. All measurements consisted of 500 scans, and KBr pellet was used as background reference.

3.7 Bioremediation Studies

The bioremediation studies were done following the method of Charoon and Siripun (2010) with little modification.

3.7.1 Preparation of metal stock solution

Each metal salt (Lead trioxonitrate $(PbNO_3)_2$ and chromium nitrate $Cr(NO_3)_2$) was weighed according to their molecular weight (that is, 1.599g and 4.55g respectively). This was dissolved in 1000ml of distilled water to make a standard stock solution of 1000ppm, it was shaken for 15 minutes and then left to stand for 24 hours to obtain complete dissolution. Then 10ml of the 1000ppm was withdrawn into a 100ml volumetric flask and distilled water was added to make up to 100ml mark to make the concentration 100ppm.

3.7.2 Pollution of soil with heavy metals

The uncontaminated soil sample was crushed in a mortar with pestle and was passed through 2.0 mm sieve to remove debris. It was air dried for 48 hours at room temperature; then it was contaminated artificially in the laboratory using the prepared metal salts solution. One kilogram of the soil sample was weighed into 1000ml capacity flasks and then 500ml of 100ppm metal salt solution was added into the flask containing the soil; this was shaken vigorously using an orbital shaker (Model HY-B11, USA) at 9000rpm for six hours to ensure maximum homogenous mixing, after that the mixture was allowed to stand for 14 days with intermittent shaking. After 14 days the supernatant was discarded and the contaminated soil was oven dried at 120^{o}C for three hours to achieve sterilization and metal-soil binding. Then some physicochemical properties (such as pH, cation exchange capacity (CEC), electrical conductivity (EC), permeability) of the soil were determined to confirm the presence of metal ions. Hundred gram (100g) each from the polluted soil was placed into eight different sterile 250ml flasks (labelled mpe12, mpe17, mpe25, mpe30, mpe40, A, B, C) for bioremediation (Charoon and Siripun, 2010).

3.7.3 Bioremediation of heavy metal polluted soil with biosurfactants

To each of the flask containing the polluted soil, 10mg/ml of the produced biosurfactants was inoculated into the flask according to their specified label and these were incubated at 28^{o}C ±2^{o}C for 4 weeks in triplicates. Control experiments consisted of the polluted soil alone without biosurfactant (A), polluted soil with distilled water alone (B) and polluted soil with mixture of chloroform and methanol (C).

The pH of the soil and atomic absorption spectroscopic (AAS) method was used in monitoring the removal of heavy metal by the biosurfactants (Charoon and Siripun, 2010).

(i) pH measurement

The pH of the heavy metal polluted soil treated with biosurfactants was determined by suspending 2g of the treated soil sample in 20ml of distilled water in a 200ml capacity beaker, swirled and allowed to stand for 10 minutes. The pH meter (pH meter 3015, Jenway, U. K) was standardized with buffer of pH 4 and 7. The pH of the soil samples was determined by inserting the pH electrode into the solution and the pH value was recorded when readings on the pH meter were at a stable state. The determination was carried out every two weeks (14days interval) for a total duration of 28 days.

(ii) AAS measurement

Determination of residual heavy metals removal in the soil amended with biosurfactants was done by removing one gram of the treated soil into a 100ml beaker with 20ml of acid mixture containing 100ml of concentrated Perchloric acid ($HClO_4$) and 300ml of Nitric (HNO_3) acid in the ratio of 1:3. This mixture was heated at high temperature for 30minutes to 1 hour using a heating mantle (thermostat hotplate, Gallenhamp, USA) until the soil was left only with whitish silicates. Then the digest was allowed to cool for 10minutes, after which it was filtered into a 50ml volumetric flask using Whatman No.1 filter paper. The filtrate was diluted (that is, water level was made up to the mark), after which the diluted filtrate was used for the heavy metal determination, using atomic absorption spectrophotometer (Shimadzu, wAArd80, Japan,) with a slit no.0.2nm loaded with air/acetylene in the ratio 60:85 as the fuel gas.

3.8 Data Analysis

The results were statistically analyzed and differences between and within groups were examined using one-way analysis of variance (ANOVA) of the Duncan descriptive test via the statistical package (SPSS) version 20. Statistical differences were set at $p<0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Potential of Bacterial Isolates for Biosurfactant Production

Forty five bacterial isolates were obtained from waste lubricating oil contaminated soil and tested for their potential to produce biosurfactants using four different methods.

4.1.1 Haemolytic activity on blood agar

Out of the 45 isolates screened for potential to produce biosurfactants only ten (22.22%) produced transparent cleared zones ($\beta-$ haemolysis, Appendix E_a) while twelve (26.67%) showed partial haemolysis with greenish appearance($\propto-$haemolysis, Appendix E_b). Twenty three isolates (51.11%) showed no haemolysis (Appendix E_c). The results of the haemolytic activity are shown in Table 4.1.

Since biosurfactants are known to cause lysis of red blood cells, it was considered that the organisms that lysed the red blood cells by showing transparent ring around the colonies in this study were potential biosurfactant producers. This correlates with the studies of Rashedi *et al.* (2005); Plaza *et al.* (2006); Anandaraj and Thivakaran (2010); Ijah and Olarinoye (2012); Ibrahim *et al.* (2013) and Parthasarathi *et al.* (2014) who used blood haemolysis test for screening biosurfactant producing organisms. However the method is considered as a preliminary screening method and should be supported by other techniques as recommended by Mulligan, Cooper and Neufeld (1984) and Youssef, Duncan, and Nagle (2004).

Table 4.1: Haemolytic activity of bacterial isolates on blood agar

Isolates code	Beta (**β**) heamolysis	Alpha(∝)heamolysis	No-heamolysis
Control A	-	-	+
Control B	-	-	+
MPE 1		+	
MPE 2		+	
MPE 3	-	-	+
MPE 4	-	+	
MPE 5	-	+	
MPE 6	-	+	
MPE7	-	+	
MPE 8	+	-	
MPE 9	-	-	+
MPE 10	+	-	
MPE 11	-	-	+
MPE 12	+	-	
MPE 13	+	-	
MPE 14	-	+	
MPE 15	-	-	+
MPE 16	-	+	
MPE 17	-	+	
MPE 18	-	+	
MPE 19	-	-	+
MPE 20	+	-	
MPE 21	-	-	+
MPE 22	-	-	+
MPE 23	-	-	+
MPE 24	-	-	
MPE 25	+	-	
MPE 26	-	-	+
MPE 27	-	-	+
MPE 28	-	-	+
MPE 29	-	-	+
MPE 30	+	-	
MPE 31	-	+	
MPE 32	-	-	
MPE 33	-	+	
MPE 34	-	-	+
MPE 35	-	-	+
MPE 36	-	-	+
MPE 37	-	-	+
MPE 38	-	-	+
MPE 39	+	-	
MPE 40	+	-	
MPE 41	-	-	+
MPE 42	-	-	+
MPE 43	-	-	+
MPE 44	-	-	+
MPE 45	+	-	
Total	10(22.22%)	12(26.67%)	23(51.11%)

Key: +: positive; - : negative

4.1.2 Oil spreading or displacement potential of the bacterial isolates

The forty five bacterial isolates were also screened for their potential to displace and spread crude oil. Of this number, 20 isolates (44.44%) were able to displace the oil (Plate I). The diameter of oil spread ranged from 1.0cm to 6.5cm within 3 to 70 seconds (Table 4.1). It was observed that *Pseudomonas aeruginosa* MPE 40 and *Bacillus firmus* MPE 30 had the highest diameter of displacement of 6.5cm within 5 seconds, meaning that they had strong ability to displace crude oil. Since the larger the diameter the higher the surfactant activity according to Rodrigues, Teixeira, van der, Mei and Oliveira, (2006); Erum *et al.* (2012) and Ibrahim *et al.* (2013). The results showed that MPE 40 and MPE 30 are potent biosurfactant producers. Plaza *et al.* (1986); Youssef *et al.* (2004) and Plaza *et al*. (2006) demonstrated that the oil spreading technique is a reliable method to detect biosurfactant production by diverse microorganisms. The assay was also applied for screening by Huy, Jin and Amada (1999); Nasr *et al.* (2009); Anandaraj and Thivakaran (2010) and Parthasarathi *et al.* (2014).

4.1.3 Emulsification capacity of the isolates

The emulsification capacities of the isolates were tested and it was observed that all the organisms screened had different emulsifying capacities which ranged from 11.0% to 70.20% (Table 4.3). From the result, six (12.5%) of the isolates (MPE 3, 5, 45, 37, 32 and 39) had very low emulsification index of 21.53%, 19.77%, 18.45%, 17.07 %,13.8%, and 11.00% respectively, 21 isolates (46.67%) had emulsification index ranged from 40% to 49% considered to be moderate, while seven (15.56%) (MPE 12, 16, 17, 25, 28, 30, and 40) showed percentage emulsification (50, 56.63, 58.80, 64.57, 66.20 and 70.20% respectively) that is significantly high (Table 4.3).

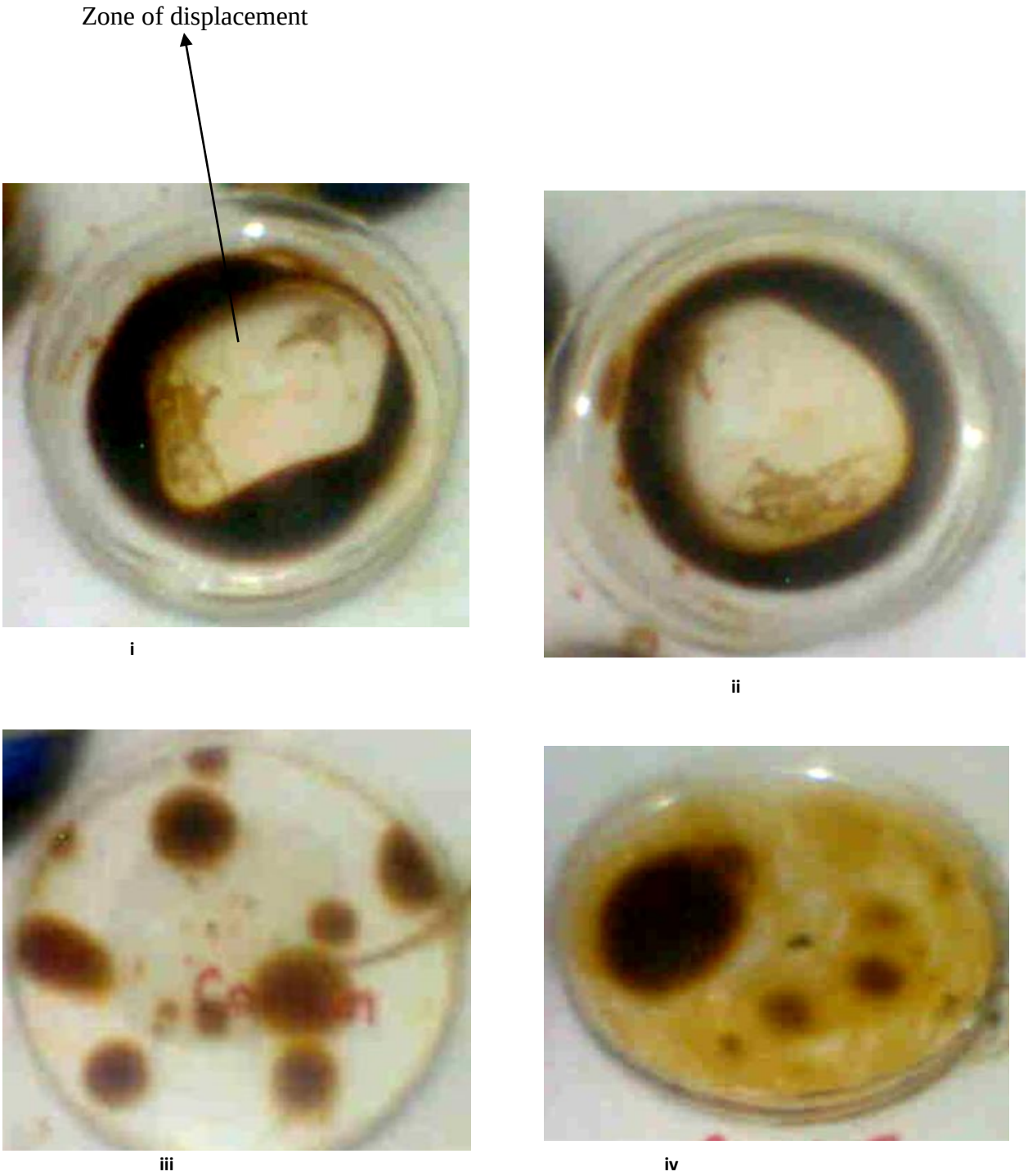


Plate I: Oil displacement caused by bacterial isolates

i& ii = positive displacement

iii & iv = negative displacement (controls)

Table 4.2: Oil spread/displacement caused by bacterial isolates

Isolates codes	Diameter(cm)	Time(s)	Interpretation
MPE40	6.5	3	Positive
MPE30	6.5	4.50	
MPE12	6.0	12	
MPE17	6.0	8.11	
MPE33	6.0	45	
MPE28	5.7	50	
MPE25	5.0	4	
MPE13	5.0	23	
MPE24	4.8	8	
MPE31	4.5	10	
MPE41	5.5	70	Negative
MPE10	5.0	60	
MPE8	4.0	60	
MPE19	3.4	12	
MPE26	3.0	20	
MPE27	2.5	25	
MPE20	2.0	8	
MPE4	2.0	33	
MPE1	1.0	60	
MPE6	2.5	30	
MPE5	-	-	
MPE7	-	-	
MPE9	-	-	
MPE11	-	-	
MPE2	-	-	
MPE3	-	-	
MPE15	-	-	
MPE16	-	-	
MPE18	-	-	
MPE21	-	-	
MPE22	-	-	
MPE23	-	-	
MPE29	-	-	
MPE32	-	-	
MPE34	-	-	
MPE35	-	-	
MPE36	-	-	
MPE37	-	-	
MPE38	-	-	
MPE39	-	-	
MPE42	-	-	
MPE43	-	-	
MPE44	-	-	
MPE45	-	-	
Control A	-	-	
Control B	-	-	

Key: A=Distilled water; Negative= diameter less than 4.5cm and occurs after 30seconds
B: Sterile broth; Positive: diameter from 4.5cm and above and occurs within 30 seconds , cm = centimetre, s = seconds

Note: The results are arranged in ascending order of displacement with time.

Table 4.3: Emulsification capacity of the isolates

Isolates code	E_{24} (%) ± Se	Isolates code	E_{24} (%)± Se
Control A	0.00 ± 0.00^{a}	MPE23	48.90 ± 0.55^{j}
MPE1	48.90 ± 3.0^{j}	MPE24	43.40 ± 0.50^{h}
MPE2	38.97 ± 0.55^{h}	MPE25	64.57 ± 4.37^{l}
MPE3	21.53 ± 3.56^{e}	MPE26	38.90 ± 0.25^{h}
MPE4	42.10 ± 0.90^{h}	MPE27	39.33 ± 0.34^{h}
MPE5	19.77 ± 3.09^{e}	MPE28	49.43 ± 0.57^{j}
MPE6	48.37 ± 3.63^{j}	MPE29	38.93 ± 0.56^{h}
MPE7	46.30 ± 0.50^{i}	MPE30	66.20 ± 0.25^{l}
MPE8	47.10 ± 0.60^{j}	MPE31	39.27 ± 0.73^{h}
MPE9	46.57 ± 1.09^{i}	MPE32	13.80 ± 0.00^{ab}
MPE10	47.77 ± 0.54^{j}	MPE33	47.00 ± 1.24^{j}
MPE11	38.97 ± 0.55^{h}	MPE34	44.20 ± 0.12^{h}
MPE12	50.00 ± 1.04^{j}	MPE35	24.03 ± 0.29^{e}
MPE13	47.70 ± 0.55^{j}	MPE36	30.10 ± 0.38^{g}
MPE14	43.71 ± 0.81^{h}	MPE37	17.07 ± 02.14^{c}
MPE15	43.41 ± 0.51^{h}	MPE38	47.50 ± 2.50^{j}
MPE16	56.63 ± 1.39^{jk}	MPE39	11.00 ± 0.29^{a}
MPE17	58.80 ± 0.64^{k}	MPE40	70.20 ± 0.65^{l}
MPE18	24.57 ± 2.18^{e}	MPE41	43.73 ± 1.73^{h}
MPE19	29.80 ± 15.05^{g}	MPE42	43.00 ± 0.058^{h}
MPE20	28.40 ± 14.25^{f}	MPE43	40.63 ± 0.15^{h}
MPE21	45.37 ± 0.67^{i}	MPE44	38.50 ± 0.31^{h}
MPE22	42.97 ± 5.33^{h}	MPE45	18.45 ± 0.45^{d}

Key: $\%E_{24}$= Percentage emulsification index; Control A= distilled water; ± Se=Standard error; the attached letters signifies significant difference ($p<0.05$). Numbers bearing same letters within rows are not significantly different.

It was observed that the level of growth and emulsification capacity varied with the isolates probably due to the varying ability of the isolates to produce extracellular emulsifying agents or due to release of hydrocarbon degradative enzymes during

growth. This result correlate with those of Kokub *et al*. (1989); Rocha *et al.* (1992) and Parthasarathi *et al.* (2014) who assessed potent biosurfactant producers and observed that most cases of variation in emulsification index and stability depend mostly on the rate of production of extracellular emulsifying agents during the breakdown of hydrocarbons. Krepsky *et al*. (2007) added that emulsification index can vary with bacterial growth phase, interactions and hydrophobic compound tested.

Similarly, Rahman, Rahman, Lakshmanaperumalsamy, Marchant and Banat (2003) and Mital, Jadhav, Kalme, Tamboli, and Govindwar (2011) reported that good and effective biosurfactant producers were not substrate specific but depended largely on the concentration or quantity of the surface active agent in the solution. In order words, potent producers, irrespective of the carbon substrate can still give significant $\%E_{24,}$ although the value may vary from one carbon source to another; for instance, *Pseudomonas desmolyticum* NCIM 2112 had E_{24} of 77, 70.33, 59.33 and 56.66% when grown on diesel, groundnut oil, toluene and corn oil respectively. Therefore the low $\%E_{24}$ demonstrated by MPE 3, 5, 45, 37, 32 and 39 may not be attributed to the carbon source; this suggests that they may be poor or non-biosurfactant producers.

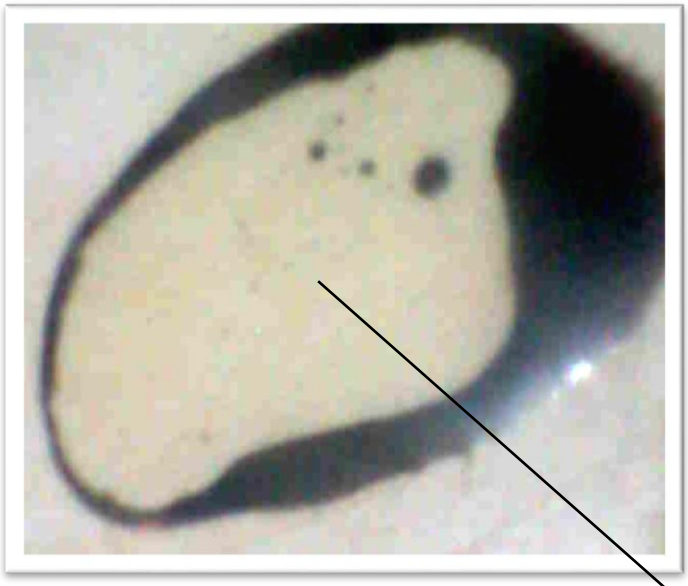
Several other researchers such as Neu and Poralla (1990); Makkar and Cameotra (1997); Willumsen and Karlson (1997); Christova, Tuleva, Lalchev, and Jordanov (2004); Plaza *et al*. (2004); Chen, Baker, and Darton (2007); Bosch *et al.* (2008); Jaysree *et al*. (2011); Preethy and Nilanjana (2011); Erum *et al*. (2012); Nalini, Parthasarathi, and Thandapani (2013) had also used this method in the isolation of potent biosurfactant producers, adding that the higher the emulsification index the more potent the biosurfactant. Therefore in this study isolates with E_{24} above 50% were considered potent producers of biosurfactants. Thus MPE 12, 16,17,25,30 and 40 (which are *M. kristinae, P. paucimobilis, A. iwoffii, B. firmus and P.aeruginosa*

respectively) were adjudged potent producers of biosurfactant (Table 4.3) representing only 15.56%. However, MPE 30 and 40 were not significantly different ($p>0.05$) in their emulsification ability (Appendix C).

4.1.4 Drop collapse potential of the bacterial isolates

Out of the forty five isolates screened only twelve (26.67%) showed positive result (that is, dispersed the oil around the liquid droplet). Out of the twelve only six (41.67%) were able to collapse the oil droplet (that is, caused it to spread out and appeared flat on the solid surface, Plate II) on which the cell free supernatants was placed within one minute. The results of the oil drop collapse caused by the bacterial isolates are shown in Table 4.4.

Viramontes-Ramos *et al*.(2010) investigated the potential of 324 microbial isolates from hydrocarbon contaminated soil on oil drop collapse for biosurfactant production and found that only seventeen (5.25%) were positive, and out of the seventeen only eleven (64.71%) were able to collapse oil drop within one minute. Similar results was obtained by Saravana and Vijayakumar (2012) and Tarango, Moorillon, Casarrubias, Chavira and Borunda (2012) who screened 243 and 802 isolates from oil contaminated soil samples and only 10 (4.12%) and 40(4.99%) respectively gave positive result. These differences in results may be attributed to the physiological characteristics of the organisms as well as their genetic and molecular composition.



i

ii

Cleared zone of collased drop

iii

iv

I,ii, & iii = positive result; iv = negative result

Plate II: Oil drop collapse caused by bacterial isolates

Table 4.4: Oil drop collapse caused by bacterial isolates

Isolates codes	Reaction	Isolates codes	Reaction
MPE1	-	MPE25	++
MPE2	-	MPE26	+
MPE3	-	MPE27	+
MPE4	++	MPE28	-
MPE5	-	MPE29	-
MPE6	-	MPE30	++
MPE7	-	MPE31	-
MPE8	-	MPE32	-
MPE9	-	MPE33	-
MPE10	-	MPE34	-
MPE11	-	MPE35	-
MPE12	++	MPE36	-
MPE13	-	MPE37	+
MPE14	-	MPE38	-
MPE15	-	MPE39	-
MPE16	-	MPE40	++
MPE17	++	MPE41	Nd
MPE18	-	MPE42	-
MPE19	+	MPE43	-
MPE20	+	MPE44	-
MPE21	-	MPE45	-
MPE22	-	Control A	-
MPE23	-	Control B	-
MPE24	+		

Key:+=positive only; ++= positive and collapsed; −: negative; Nd= not determined

It was observed that the number of isolates that responded positively to oil drop collapse were usually few as compared to other screening method; this may be attributed to the fact that the method is highly sensitive as it requires only an aliquot amount of cell free supernatant to cause destabilization of the liquid droplet resulting from the reduction in

the force or interfacial tension between the liquid drop and oil surface. Several researchers (Jain *et al.,* 1991; Satpute *et al.,* 2008; Thavasi, Jayalakshmi and Banat, 2010; Tarango *et al.,* 2012; Ibrahim *et al.,* 2013; Pereira *et al.,* 2013) have proved this method to be highly sensitive, very effective, and reliable in identification of potent biosurfactant producer. Based on this therefore, isolate MPE 4, 12, 17, 25, 30 and 40 (identified as *M. kristinae, P. paucimobilis, A. iwoffii, B. firmus and P.aeruginosa* respectively) used in this study were considered potent producers of biosurfactants.

4. 2 Characterization and identification of Biosurfactant Producing bacteria

Based on the consistent positive reactions on all four biosurfactant screening tests carried out, the isolates, MPE12, 17, 25, 30, and 40 emerged as the most effective biosurfactant producers among the 45 isolates screened. Therefore, only the five isolates were characterized and identified as *Pseudomonas aeruginosa* MPE40, *Bacillus firmus* MPE30, *Acinetobacter iwoffii* MPE25, *Pseudomonas paucimobilis* MPE17, and *Micrococcus kristinae* MPE12 (Table 4.5).

4.3 Production of Biosurfactants

4.3.1 Extraction of the biosurfactants

Acid precipitation method was used in the extraction of the biosurfactant which appeared as whitish precipitate (Plate III).

Table 4:5 Morphological and biochemical characteristics of biosurfactant producing bacteria

Test	MPE40	MPE30	MPE25	MPE17	MPE12
Gram reaction	−	+	−	−	+
Shape	Rod	Rod	Rod	Rod	Cocci
Motility	+	+	−	+	−
Catalase	+	+	+	+	+
Coagulase	−	−	+	−	+
Oxidase	+	−	−	+	+
Citrate	+	−	−	−	−
Nitrate rdn	+	+	−	+	−
Indole	−	−	−	−	−
Urease	−	−	−	−	−
H_2S	+	−	−	−	+
Gas	+	+	−	+	−
MR	−	−	−	+	−
VP	−	−	−	−	+
Spore	−	+	−	−	−
Starch hyd.	+	+	−	+	−
Protien util.	+	+	+	+	Nd
Sucrose	+	+	−	_	+
Glucose	+	−	+	+	+
Lactose	+	−	−	Nd	−
D-Mannitol	+	−	−	−	Nd
D-mannose	Nd	−	−	Nd	+
Fructose	+	−	+	−	+
Maltose	−	−	−	−	+
Galactose	Nd	_	−	+	+
Sorbitol	+	−	+	−	−
Identification	*Pseudomonas Aeruginosa*	*Bacillus Firmus*	*Acinectobacter iwoffii*	*Pseudomonas paucimobilis*	*Micrococcus kristinae*

Key: H_2S=Hydrogen sulphide, rdn=reduction, += positive, −=negative, Nd= Not determined, MR= Methyl Red, VP= Voges Proskauer, hyd.=hydrolysis, util= utilization

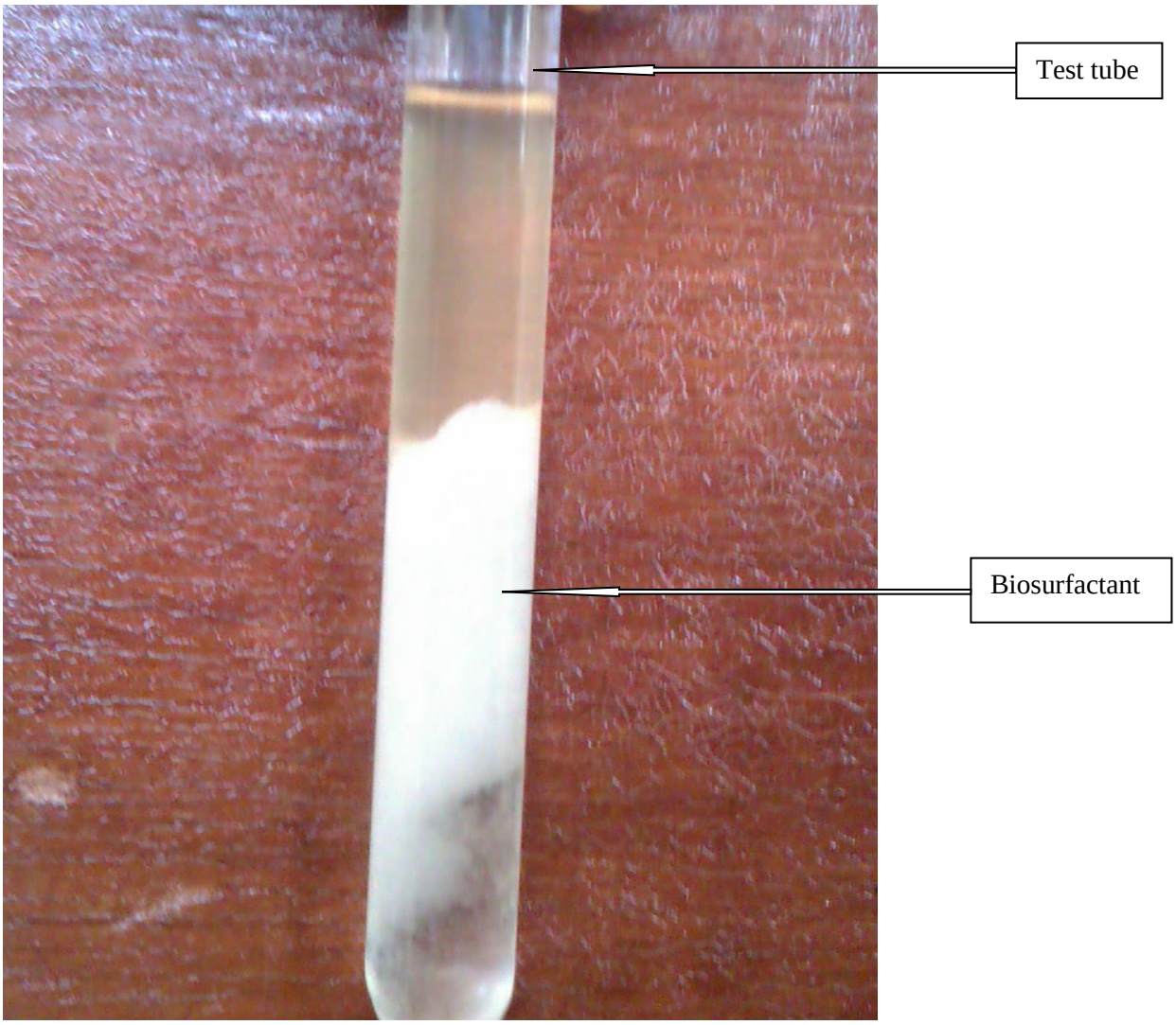


Plate III: Extracted biosurfactant

4.3.2 Dry weight of the biosurfactants

The quantity of biosurfactant produced (Table 4.6) by *P. aeruginosa* MPE40 (1.7g/100ml) was more than that of *P. paucimobilis* MPE17 (1.0g/100ml); this shows that organisms of the same genus, though members of different species have varying ability and capacities in their rate of production, this concurs with the reports of Cooper and Goldenberg (1987). However the yields obtained in this present study is about 5 times higher than (0.45g/l) obtained from *P. aeruginosa* by Ishita *et al.* (2011). This

difference may be attributed to different carbon sources used in the medium and the duration of incubation.

Table 4.6: Quantity of the biosurfactants produced by bacterial isolates

Coded Isolates	Quantity(g/100ml) of biosurfactants
Micrococcus kristinae MPE12	0.8
Pseudomonas paucimobilis MPE17	1.0
Acinectobacter iwoffii MPE25	1.6
Bacillus firmus MPE30	1.6
Pseudomonas aeruginosa MPE40	1.7

Similarly in Table 4.6 *A. iwoffi* MPE25 and *B. firmus* MPE30 had the same amount of biosurfactant (1.6g/100ml), even though they belong to different genera and species while *M .kristinae* MPE12 had the lowest yield of 0.8g/100ml. This implies that the biosurfactant production capacity of organisms could be due to the nature and genetic makeup of the organisms. The amount of biosurfactant produced from *M. kristinae* MPE12 (0.8g/100ml) correlates with those obtained from *M. kristinae* (8.0g/l) by Ibrahim *et al*. (2013) while those from *B. firmus* MPE30 (1.6g/100ml) were 4.5times more than that observed in *B. firmus* (7.15 g/ml) by Ibrahim *et al*. (2013). Also 1.78 and 1.69g/500ml of biosurfactant (emulsan) was obtained from *Acinetobacter calcoaceticus* PAY-4 and IL-1 when grown on crude oil respectively as reported by Chamanrokh *et al.* (2008) which was lower than that obtained in this study.

The study revealed that the strains used are efficient biosurfactant producers with lowest biosurfactant concentration of 0.8g/100ml which is equivalent to 8.0g/l. However, it is quite an appreciable quantity compared to that observed for rhodofactin by Peng *et al.*

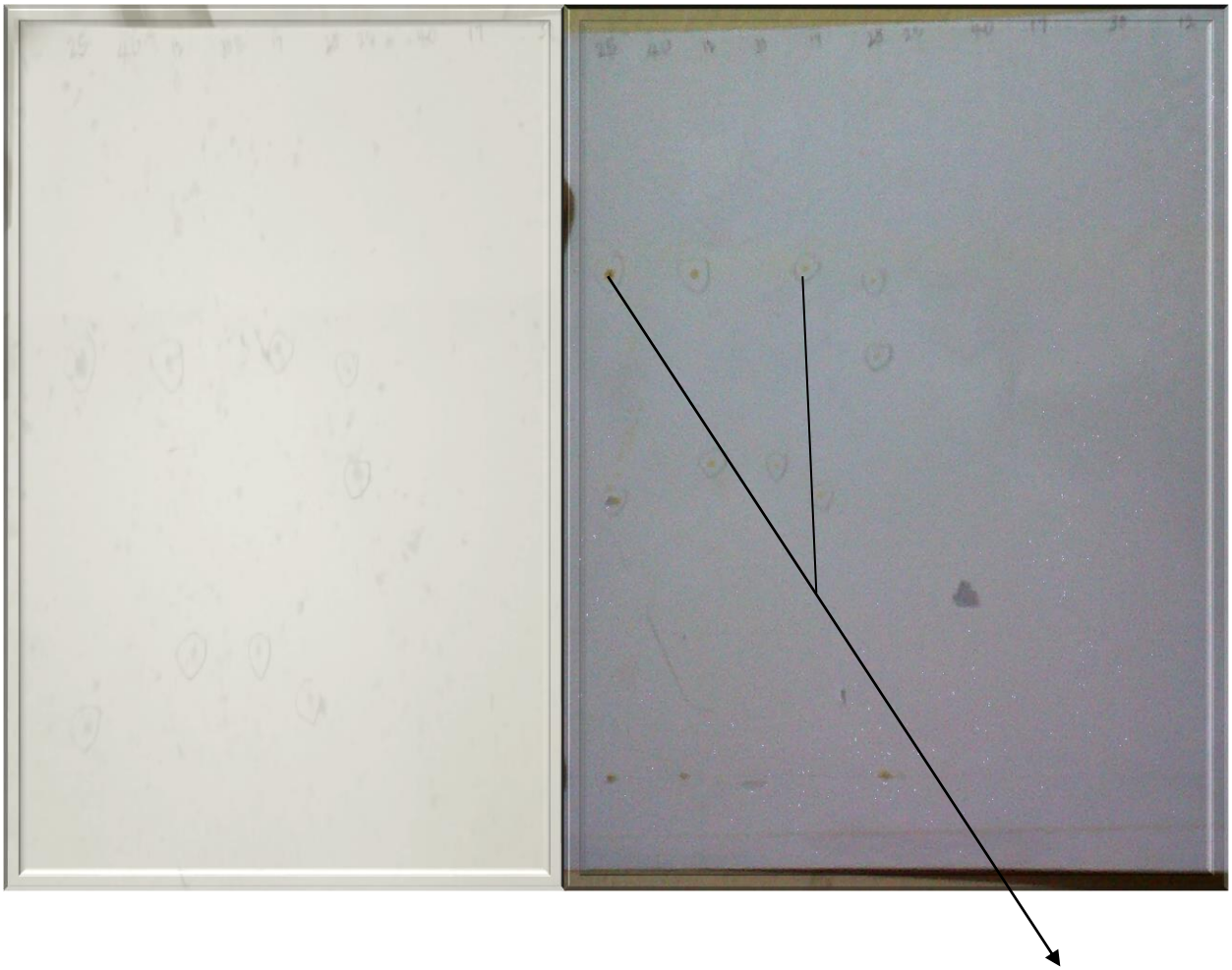
(2008) in which only 3.4 g/l of the biosurfactant was obtained after optimization. Nevertheless, differences may be attributed to the temperature of the environment; the composition and pH of the medium as well as the substrate used as carbon source; since, the quantity and type of biosurfactant produced vary on the substrate type (Ibrahim *et al*., 2013). The present study used diesel oil as carbon source while the study by Peng *et al.* (2008) made use of hexadecane.

4.4 Characterization of Biosurfactants

4.4.1 Thin layer chromatography

Thin layer chromatographic analysis

Thin layer chromatographic analysis of biosurfactant produced, showed yellow spots on spraying with Anthrone reagent (Plate IV) meaning that the biosurfactant belong to the class of glycolipids. From the result obtained, it was observed that biosurfactant Bios-12 produced by *M. kristinae* MPE12 had only one spot (Rf 0.56) while the other four (Bios-17, 25, 30 and 40) had two spots each (lower and upper) with varying Rf values (Table 4.7). The detection of yellow colour spots confirmed the presence of lipids (glycolipid group) in the biosurfactants, but Rf values varied with each sample probably due to variation in peptide linkage with varying length to fatty acids.



Yellow spots (glycolipids)

Plate IV: TLC plates showing the developed spots

Table 4.7: TLC showing the Rf value of various samples

Biosurfactant Code	No. of spots	Rf value	
		Lower	Upper
Bios-12	1	0.56	0.00
Bios-17	2	0.73	0.86
Bios-25	2	0.44	0.81
Bios-30	2	0.50	0.84

Bios-40	2	0.56	0.91

Rf= Retention factor

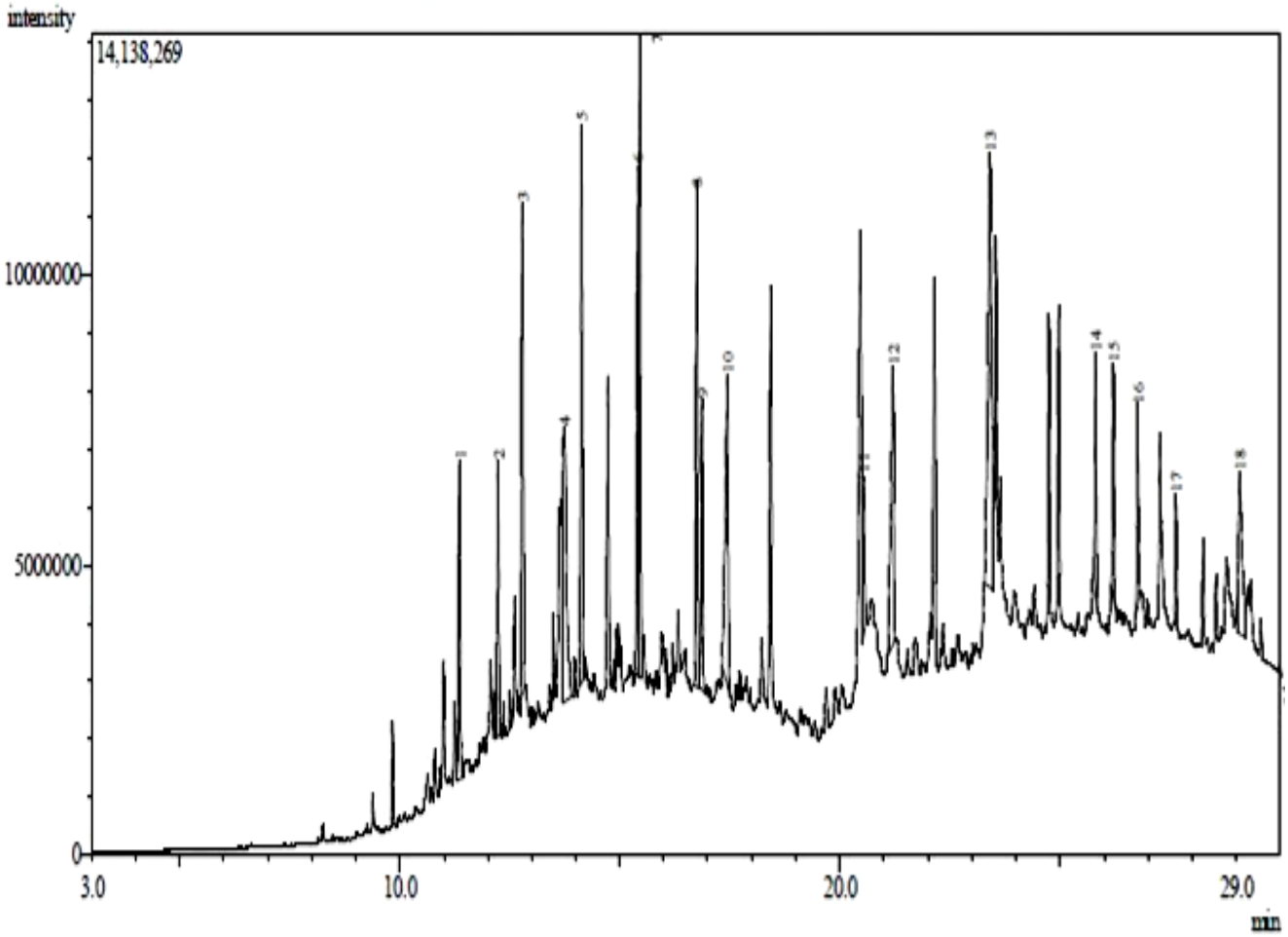
The Rf value of the biosurfactant, Bios-25 produced by *A. iwofii* were (0.44 and 0.81) in this study compared favourably with the glycolipids from *Renibacterium salmoninarium* 27BN (Rf =0.42 and 0.83) studied by Nelly *et al*. (2013).

The Rf of the biosurfactants, Bios-17 (Rf 0.73 and 0.86) and Bios-40 (Rf 0.56 and 0.91) produced by *Pseudomonas* species (strain MPE17 and MPE40 respectively) correspond to those of rhamnolipids; this was confirmed when compared with biosurfactant studied by various researchers. For instance, rhamnolipid produced by *P. aeruginosa* GL1 was characterized by Arino *et al.* (1996) who reported that the Rf values of different spots detected, corresponded to R1 0.72 (Rha-C10C10,), R2 0.40 (Rha-C10), R3 0.32 (Rha-Rha-C10C10) and R4 0.13 (Rha-Rha- C10).

The production of biosurfactant from *P. aeruginosa* MTCC2297 cultivated in orange fruit peelings was studied by George and Jayachandran (2009) and the detected spots had Rf values of 0.19 (dirhamnolipids), 0.36 (monorhamnolipids), 0.59 and 0.71, 0.82 and 0.98 (various rhamnolipid forms). Similarly, mixture of two rhamnolipids with Rf value of 0.72 and 0.45 was produced by Haba *et al*. (2000); Devendra *et al*. (2011) reported the production of glycolipid surfactant from *Serratia marcescens* having Rf of 0.72. Also, Vyas and Dave (2011) reported the production of glycolipid from *Norcardia otitidiscariarum* MTCC6471 with Rf of 0.72. It is therefore obvious that the result of this study compares favourably with established fact about the Rf of glycolipids.

4.4.2 Gas Chromatography and Mass Spectroscopy (GCMS)

The chromatograms for biosurfactants produced are shown in Figures 4.1−4.5. The GC-MS revealed the presence of unknown compounds especially the fatty acyl components



in the biosurfactants, each peak represents a compound and they are clearly explained in Tables 4.8-4.12.

Note: Peaks bearing the numbers 1, 2, 3, to 18 are peaks representing the major fatty acyl components in the Bios-40. Other peaks not numbered are the carbonhydrate moieties of the biosurfactants not identified.

Figure 4.1 Chromatogram for biosurfactant Bios-40 from *Pseudomonas aeruginosa* MPE40

From the chromatogram (Figure 4.1), peak number, 1, 3, and 4 represents fatty acids such as palmitic (n-C_{17}), stearic (n-C_{19}), and oleic acid (n-C_{19}), (all saturated), the fatty acid moiety of peak no. 9, 11, 12 and 16 consists of saturated hydroxyl fatty acids of n-C_{35}, n-C_{19}, n-C_{22}, and iso-C_{17} carbon lengths respectively (Table 4.8).

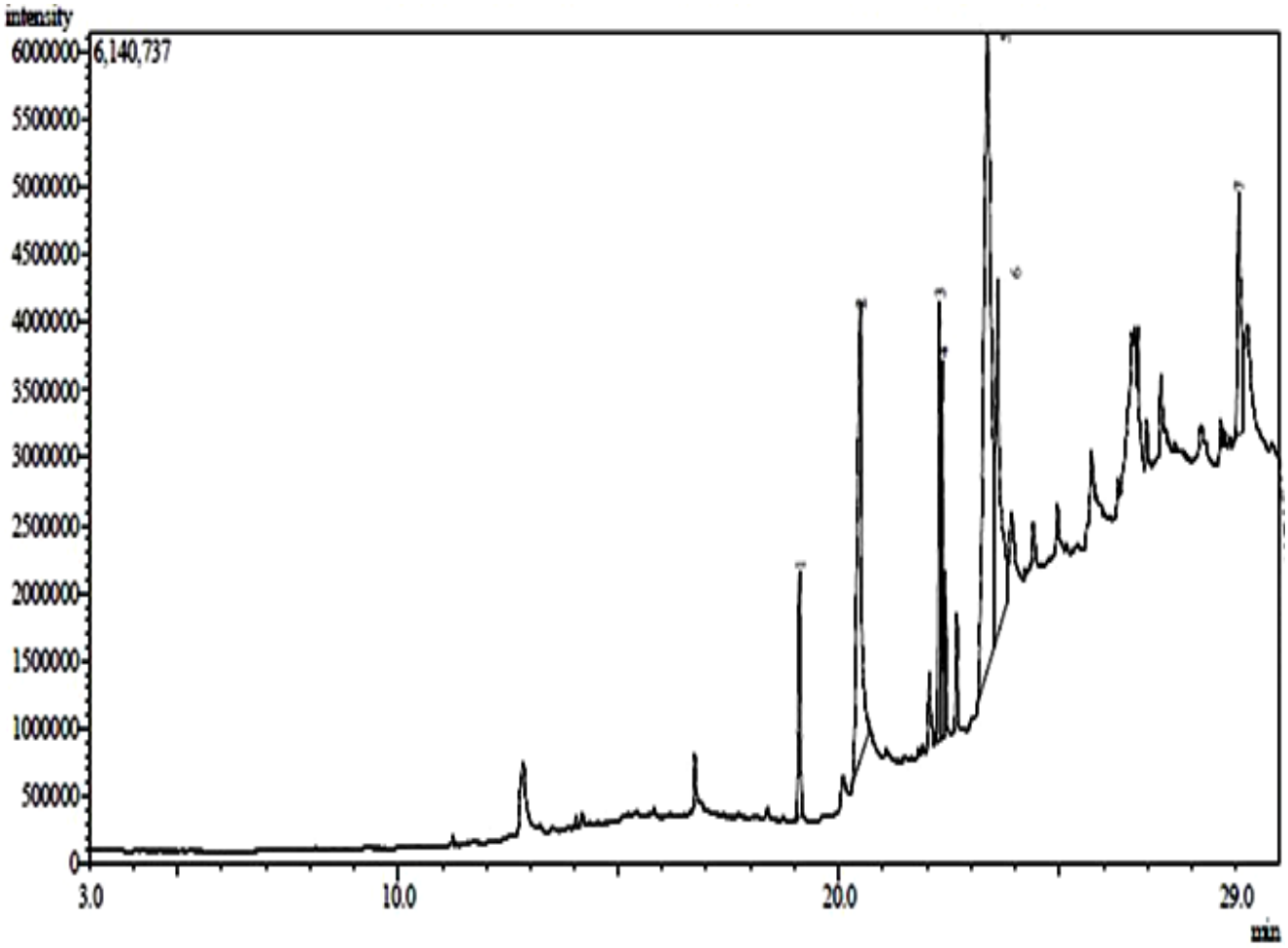
Table 4.8: Compounds present in the biosurfactant Bios-40

Peak No.	Retention time (min.)	%Height	Compound name	Molecular formula (Mw)
1	11.355	5.28	Palmitic acid	$C_{17}H_{34}O_2$ (270)
2	12.231	4.57	1-(+)-ascorbic acid 2,6-dihexadecanoate	$C_{38}H_{68}O_8$ (652)
3	12.788	8.16	Oleic acid	$C_{19}H_{36}O_2$ (296)
4	13.737	4.57	Stearic acid	$C_{19}H_{38}O_2$ (298)
5	14.133	9.26	Octadec-9-enoic acid	$C_{18}H_{34}O_2$ (282)
6	15.409	8.46	Octadecanoic acid$$ Stearic acid	$C_{18}H_{36}O_2$ (284)
7	15.457	10.67	1[[[(2-aminoethoxy) hydroxyphosphinyl] methyl]-1,2-ethanediyl ester	$C_{34}H_{74}NO_8P$ (691)
8	16.755	8.38	Eicosanoic acid	$C_{21}H_{42}O_2$ (326)
9	16.869	4.83	2-hydroxy-1,3-propanediylester	$C_{35}H_{68}O_5$ (568)
10	17.441	5.16	Heneicosane	$C_{21}H_{44}$ (296)
11	20.542	2.61	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$ (330)
12	23.677	9.12	octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	$C_{22}H_{44}O$ (372)
14	25.809	4.01	E, E, Z-1, 3 ,12-Nanodecatriene-5,14-diol	$C_{19}H_{34}O_2$ (294)
15	26.219	3.91	9,12-octadecadienoic (Z,Z)-2,3-dihydroxypropyl ester	$C_{21}H_{38}O_4$ (354)
16	26.762	3.43	(R)-14-methyl-8-hexadecyn-1-ol	$C_{17}H_{32}O$ (252)
18	29.084	2.70	Propyleneglycol monoleate	$C_{21}H_{40}O_3$ (340)

Min=Minutes, Mw=Molecular weight; Note: How each compound was analysed and fragmented to obtain the specific fatty acyl groups in the compound is shown in Appendix G.

Biosurfactants are amphiphilic compounds with hydrophilic and hydrophobic moieties (Rosenberg and Ron, 1999; Urai *et al*., 2007; Magdalena *et al.,* 2011; Luna *et al.,* 2013). The presence of various chains of fatty acids as revealed by the GC-MS analysis has confirmed the hydrophobic part of the biosurfactant. Bios-40 is suspected to be a rhamnolipid because of the presence of hydroxyl group attached to long chain fatty acid while the presence of decanoic acid confirms it a glycolipid (Table 4.8).

Production of rhamnose-containing glycolipids was first described in *P. aeruginosa* by Jarvis and Johnson (1949) the investigators reported that rhamnolipids contain either two rhamnose attached to hydroxyl decanoic acid or one rhamnose connected to identical fatty acid. These have been confirmed by several other researchers such as Lin (1996); Mulligan (2005); Catherine (2009); Saravanan and Vijayakumar (2012) and Gomathy and Senthilkumar (2013). Studies have shown that *P. aeruginosa* can produce 28 different homologues of rhamnolipids (De'ziel *et al.,* 1999; De'ziel *et al.,* 2000). Based on these established facts, it can be deduced that Bios-40 is a rhamnolipid consisting of two 2-hydroxyl groups attached to octadecanoic and octadecadienoic acid (Table 4.8).



Note: Peaks bearing the numbers 1, 2, 3, 4, 5, 6, & 7 are peaks representing the major fatty acyl components in the Bios-17. Other peaks not numbered are the carbonhydrate moieties of the biosurfactants not identified and other compounds present.

Figure 4.2 Chromatogram for biosurfactant Bios-17 from *Pseudomonas paucimobilis* MPE17

The chromatogram for biosurfactant Bios-17 (Figure 4.2), the peak number, 1, 3, and 4 represents fatty acids such palmitic, linoleic, and oleic acid, with the linoleic acid esterified to long chain 9, 12-octadecadienoic acid (Z, Z), 5 and 7 consist of octadec-9-enoic acid and propyleneglycol monoleate respectively which are unsaturated fatty acid of 18 and 21 carbon length (Table 4.9).

Table 4.9: Compounds present in the biosurfactant Bios-17

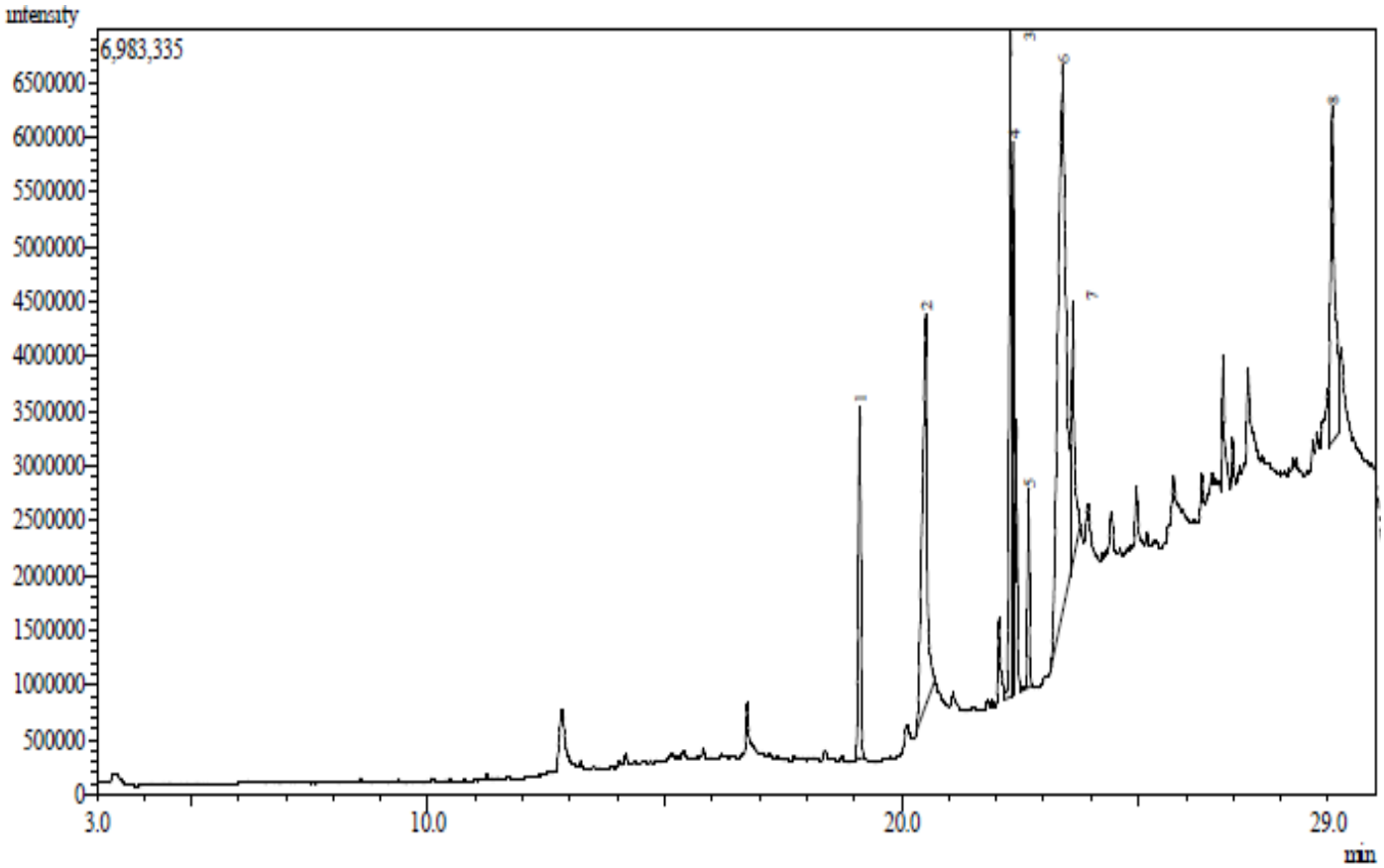
Peak No.	Retention time (min.)	%Height	Compound name	Molecular formula (Mw)
1	19.107	8.92	Palmitic acid	$C_{17}H_{34}O_2$ (270)
2	20.489	16.61	1-(+)-Ascorbic 2,6-dihexa decanoate	$C_{38}H_{68}O_8$ (652)
3	22.284	15.97	9,12-octadecadienoic acid(Z,Z)-methy ester$$Linoleic acid	$C_{19}H_{34}O_2$ (294)
4	22.340	13.72	Oleic acid	$C_{19}H_{36}O_2$ (296)
5	23.372	23.14	Octadec-9-enoic acid	$C_{18}H_{34}O_2$ (282)
6	23.600	12.86	Octadecanoic acid $$ stearic acid	$C_{18}H_{36}O_2$ (284)
7	29.071	8.78	Propyleneglycol monoleate	$C_{21}H_{40}O_3$ (340)

Min=Minutes, Mw=Molecular weight; %=percentage. Note: How each compound was analysed and fragmented to obtain the specific fatty acyl groups in the compound is shown in Appendix G.

Comparing the compounds present in the biosurfactant Bios-17 produced by *P. paucimobilis* MPE17 and Bios-40 from *P. aeruginoas* MPE40 as revealed from the chromatographic analysis, it was observed that most of the compounds in Bios-40 was present in Bios-17, but Bio-17 differs from Bios-40 in the absence of hydroxyl- groups while Bios-40 lack linoleic acid. This implies that the biosurfactants (Bios-17 and -40) are different even though they are of the same group of glycolipids; these differences may be attributed to the different in species, strain, number of carbon atom and degree of saturation as well as the fatty acids constituents. This result is similar to that obtained by Mital *et al*. (2011); the investigators reported variations in the composition of fatty

acid chains of the biosurfactant produced from *Pseudomonas aeruginosa* Pd2112 when compared to those produced by *P. chlororaphils.*

Furthermore, it has been reported that Pseudomonads are the best studied and well known biosurfactant producers, among which *P. aeruginosa, P. putida, P. fluorescens, P. nautical P. rubescens, P. chlororaphis* and *Burkhoderia pseudomallei* produced glycolipid biosurfactant with different compound composition (Husain *et al.,* 1997; Haussler *et al.*,1998; De'ziel *et al.,* 1999; De'ziel *et al.,* 2000; Gunther *et al.,* 2005; Kumar *et al.,* 2008; Perfumo *et al.,* 2006; Surekha *et al.,* 2010; Perfumo *et al.,* 2010 and Tarango *et al.,* 2012).



Note: Peaks bear the numbers 1, 2, 3, 4, 5, 6, 7, & 8 are peaks representing the major fatty acyl component in the Bios-30. Other peaks not numbered are the carbonhydrate moieties of the biosurfactants not identified.

Figure 4.3: Chromatogram for biosurfactant Bios-30 from *Bacillus firmus* MPE30

Table 4.10: Compound preset in the biosurfactant Bios-30

Peak No.	Retention time (min)	%Height	Compound name	Molecular formula (Mw)
1	19.117	10.68	Hexadecanoic acid, methyl ester $$Palmitic acid	$C_{17}H_{34}O_2$ (270)
2	20.514	11.84	Tetradecanoic acid Myristic acid	$C_{14}H_{32}O_2$ (228)
3	22.302	20.16	Linoleic acid	$C_{19}H_{34}O_2$ (294)
4	22.356	16.76	9-Octadecenoic acid	$C_{19}H_{36}O_2$ (296)
5	22.682	6.01	Methyl ester $$Stearic acid	$C_{19}H_{38}O_2$ (298)
6	23.399	16.55	Octadec-9-enoic acid	$C_{18}H_{34}O_2$ (282)
7	23.617	7.84	Octadecanoic acid $$ Stearic acid	$C_{18}H_36O_2$(284)
8	29.093	10.17	9-octadecenoic acid (Z)-,2,3-dihydroxypropyl ester$$ olein	$C_{21}H_{40}O_4$ (354)

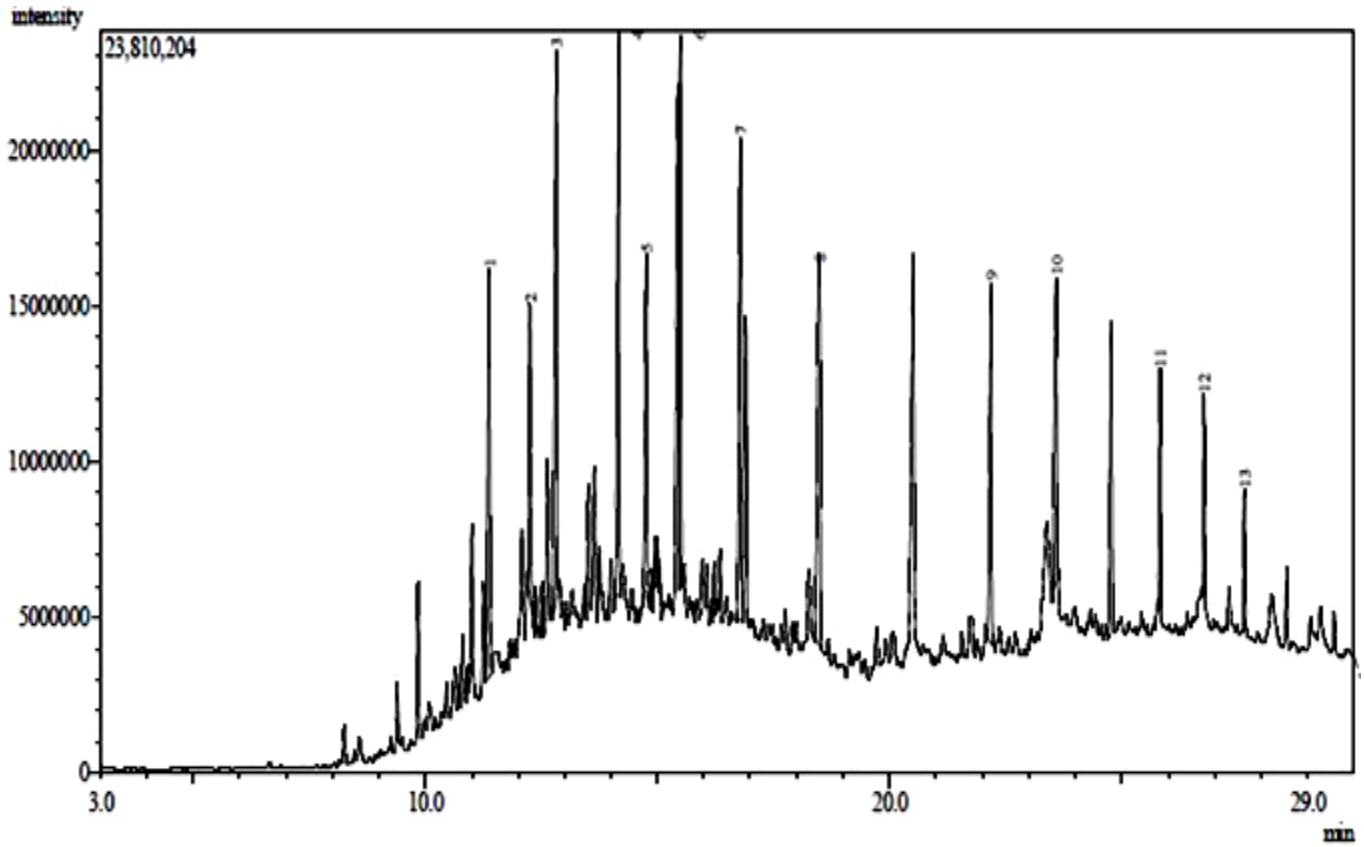
Min= minutes; mw=molecular weight; %=percentage. Note: How each compound was analysed and fragmented to obtain the specific fatty acyl groups in the compound is showed in Appendix G.

The chromatogram for Bios-30 (Figure 4.3) revealed 8 major peaks whose compounds are presented in Table 4.10, in addition to those compounds are, Heptadecanoic acid and 16 methyl-,methyl ester $$ Methyl isostearate $$ Methyl 16-methylheptadecanoate ($C_{19}H_{38}O_2$) which are highly branched chain fatty acid usually represented with the prefix "iso" and "anteiso" attached to a carbon atom(for example, iso-C_{19} and anteiso

C_{19}). The biosurfactant are predominated with fatty acyl compound of n-C_{19} (Octadecanoic acids).

The membrane lipids of *Bacillus firmus* RAB32 as classified by Clejan, Krulwich, Mondrus, and Seto-Young, (1996) consist of n-$C_{14:0}$, iso-$C_{14:0}$, iso-$C_{17:0}$, iso-$C_{12:0}$, n-$C_{18:1}$, n-$C_{18:2}$, n-$C_{18:0}$, anteiso-$C_{17:1}$, iso-$C_{16:0}$, n-$C_{16:0}$, n-$C_{16:1}$, anteiso-$C_{15:0}$ and iso-$C_{15:0}$ fatty acid. Most of the fatty acyl components as identified by Clejan *et al.* (1996) were present in the biosurfactant Bios-30 with the exception of iso-$C_{16:0,}$ iso-$C_{15:0}$ and anteiso-$C_{15:0.}$ On the other hand Bios-30 contains n-C_{19} and n-C_{21} which are not present in the membrane lipids of *B. firmus* RAB32.

These variations in composition and branched chain of the fatty acids could possibly be attributed to the culture strains, the constituent of the medium (*B. firmus* RAB32 were supplemented with phosphorus in order to produce phospholipids, this led to the production of phosphoglycolipid), the carbon source (the present study used diesel while the investigators used D, L-malate), and conditions for growth. However, all the carbon atoms were linked to decanoic acid same with those in this study confirming Bios-30 as a glycolipid.



Note: Peaks bearing the numbers 1, 2, 3, 4, to 13 are peaks representing the major fatty acyl component in the Bios-25. Other peaks not numbered are the carbonhydrate moieties of the biosurfactants not identified and other compound that are present but not included in the table.

Figure 4.4: Chromatogram for biosurfactant Bios-25 from *Acinetobacter iwoffi* MPE25

Thirteen major peaks were revealed in the chromatographic analysis of the biosurfactant Bios-25 produced by *A.iwoffii*. Each peak represents a major compound present in Bios-25 (Figure 4.4). The compounds present in Bios-25 are different from those of Bios-40, 17 and 30, except for Octadecanoic and Hexadecanoic acid, meaning that, they are not same biosurfactant, but the presence of decanoic acid confirms it a glycolipid (Table 4.11). However, compounds in peaks 2, 5 and 8 compare favourably with those cited in the literatures.

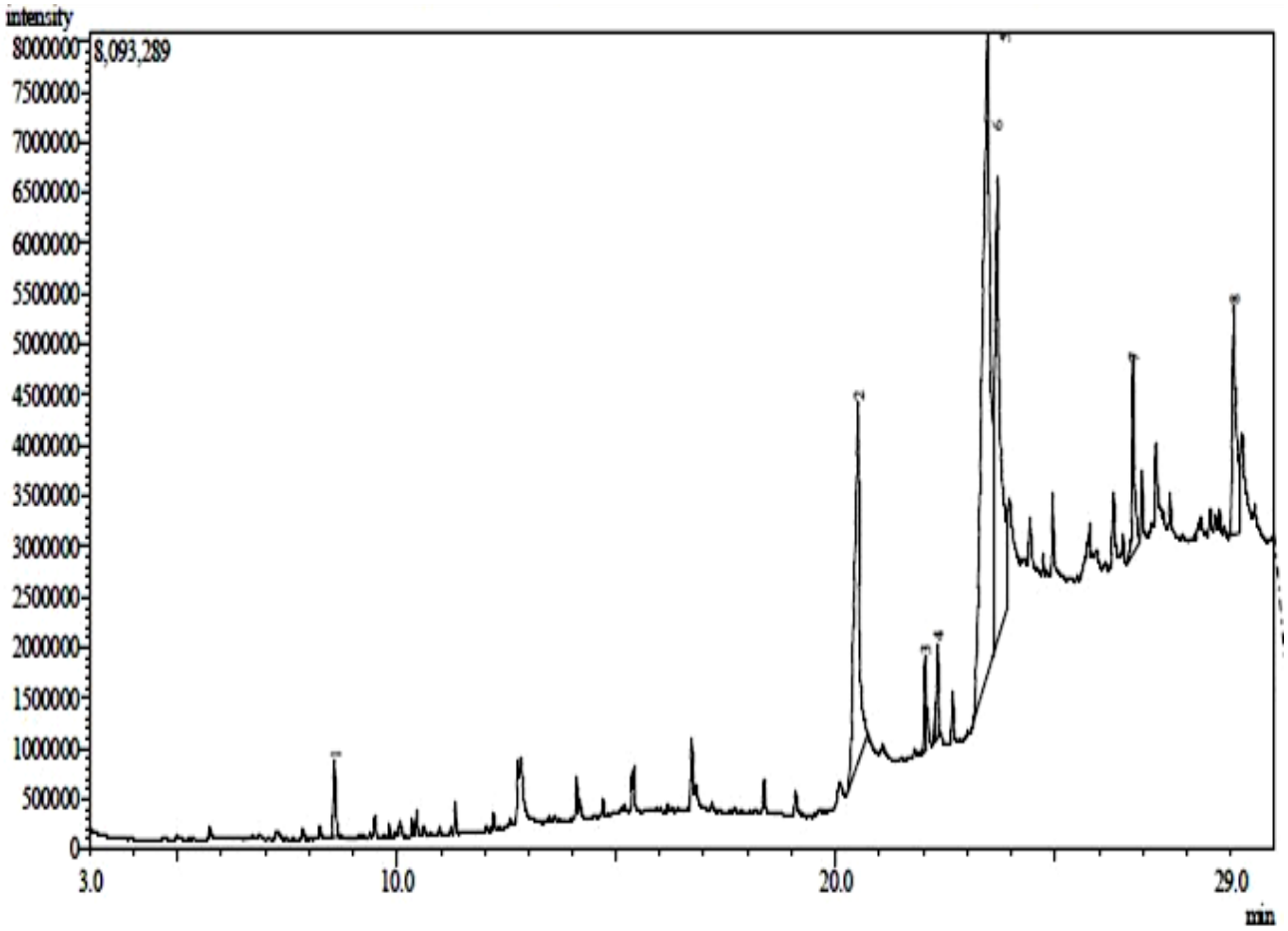
Table 4.11: Compounds present in the biosurfactant Bios-25

Peak No.	Retention time (min)	%Height	Compound name	Molecular formula(Mw)
1	11.372	8.44	Tetradecanoic acid	$C_{14}H_{30}O_4$(198)
2	12.254	6.06	4,6-dimethyl-$$ 4,6-Dimethyldodecanoic acid	$C_{14}H_{30}O_2$(198)
3	12.816	11.40	n-pentadecanoic acid	$CH_3(CH2)_{13}CH_3$ (212)
4	14.165	11.71	Hexadecanoic acid	$C_{16}H_{34}O_2$(226)
5	14.758	7.06	Sulphurous acid, dodecyl 2-ethylhexyl ester	$C_{21}H_{44}O_3S$ (376)
6	15.497	11.45	2,6,10,14-tetramethylpentadecanoate	$C_{19}H_{40}O_5$ (268)
7	16.795	10	Octadecanoic acid	$C_{18}H_{38}$ (254)
8	18.485	8.07	2,3-dihydroxydodecanoic acid	
9	22.183	7.46	Heneicosane	$C_{21}H_{44}$ (296)
10	23.580	6.28	Docosane	$C_{22}H_{46}$ (310)
11	25.829	5.02	Tetracosanoic acid	$C_{24}H_{50}O_2$(338)
12	26.775	4.23	Tetratriacontane	$C_{34}H_{70}$ (478)
13	27.644	2.82	Triacontane	$C_{30}H_{62}$ (422)

Min= minutes, Mw=Molecular weight, %=percentage

The composition of the glycolipid and phospholipids from *Acinectobacter* MO 56 and *Acinectobacter* HO1-N respectively composed of glucose and a mixture of 3-(3'-hydroxytetradecanoyloxy) decanoate and 3-(3'-hydroxyhexadecanoyloxy) decanoate (Rosenberg *et al.,* 1979; Satpute *et al.,* 2010; Kapadia and Yagnik, 2013). Recently, as cited by U.S patent, an *Acinectobacter iwoffii* RAG-1 was identified to possess aminopolysaccharide backbone composed of sugar units of (D-galactosamine,D-galactosamine uronic acid and 2,4-diamino,6-deoxy glucose) and mainly hydroxylated fatty acids (β-Hydroxy butyrate and β-Hydroxydodecanoic acid) linked to polymeric backbone in both ester and amide linkage. Despite the number of polymeric biosurfactants discovered, they still remain totally or partially uncharacterized due to their size and structural complexity (Satpute *et al.,* 2010; Kapadia and Yagnik, 2013).

From the chromatographic analysis of the Biosurfactant Bios-12, eight major peaks were revealed (Figure 4.5) representing major fatty acyl compounds present in Bios-12. Peaks 3, 5, and 6 consisted of fatty acyl compounds with eighteen carbons (n-C_{18}) (Table 4.12). Additional compounds (delta (sup9)-cis-oleic acid (ð^{9}-cis-n-C_{18}), Pentadecanoic acid (n-C_{15}) and 1, 2-Tetradecandiol) to those listed in Table 4.12 were also identified from the m/z scans, associated with peak 5, 6 and 7 respectively. Wieser, Denner, Kampfer, Schumann, Tindall and Steiner, (2002) as cited by Lin and Harichund (2011) reported that fatty acids abundantly present in *Micrococcus* species are anteiso-C_{15} and iso-$C_{15.}$



Note: Peaks bearing the numbers 1, 2, 3, 4, 5, 6, 7, 8 are peaks representing the major fatty acyl components in the Bios-12. Other peaks not numbered are the carbonhydrate moieties of the biosurfactants not identified and other possible compounds present but not listed in the Table 4.12

Figure 4.5 Chromatogram for biosurfactant Bios-12 from *Micrococcus kristinae* MPE12

Table 4.12: Compounds present in the biosurfactant Bios-12

Peak No.	Retention time(min)	%Height	Compound name	Molecular formula(Mw)
1	8.582	3.54	Azulene $$bicyclo[5.3.0]decapentaene	$C_{10}H_8$(128)
2	20.517	16.66	n-Hexadecanoic acid (n-C_{16}), palmitic acid Tridecanoic acid	$C_{16}H_{32}O_2$ (256)
3	22.043	4.38	13-hexyloxacyclotridec-10-en-2-one	$C_{18}H_{32}O_2$ (280)
4	22.329	4.37	9-Octadecenoic(Z)-methyl ester $$ oleic acid	$C_{19}H_{36}O_2$ (296)
5	23.465	29.77	Octadec-9-enoic acid	$C_{18}H_{34}O_2$ (282)
6	23.697	21.49	Stearic acid	$C_{18}H_{36}O_2$ (284)
7	26.785	9.17	15-hydroxypentadecanoic acid	$C_{15}H_{30}O_3$ (258)
8	29.073	10.62	Oleoyl chloride$$oleic acid chloride	$C_{18}H_{33}ClO$ (300)

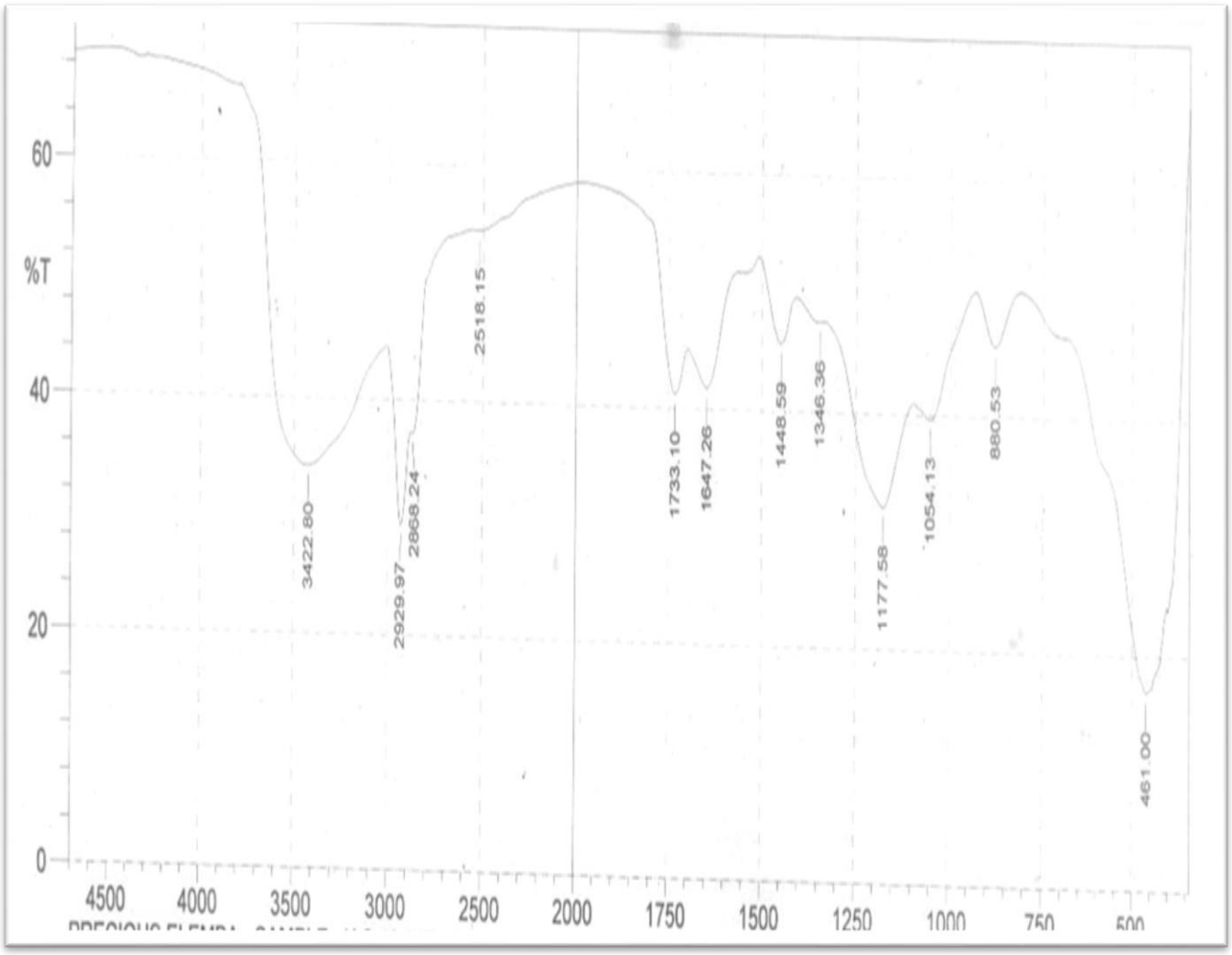
Min= minutes; Mw= molecular weight

The results of the study conducted by Pakkiri, Wolucka, Lubert, and Waechter (2004) revealed that Tridecanoic acid (C_{13}), Tetradecanoic acid (C_{14}), 13-Methyltetradecanoic acid (iso-C_{15}), 12-Methyltetradecanoic acid (anteiso-C_{15}), Hexadecanoic acid (C_{16}-br), Hexadecenoic acid ($C_{16:1}$), Hexadecenoic acid (C_{16}), and Heptadecanoic acid (C_{17}-br) are major fatty acids present in lipid produced by *Micrococcus luteus*. All these compounds are present in the biosurfactant Bios-12 with slight difference in the positioning of the methyl group and saturation.

Bios-12 produced from *Micrococcus kristinae* MPE12 has two unsaturated, one branched (iso) with the methyl group attached to position 9 of the carbon chain and oleic acid attached to a chloride (n-C_{18}-Cl) (Table 4.12 peak no 4 and 5) while that reported by the investigators had two fatty acids to which bromine is attached (Hexadecanoic acid (C_{16}-br) and Heptadecanoic acid (C_{17}-br)), two branched and one saturated fatty acid. The difference in saturation and branching may be as a result of the culture strains used. However, the entire fatty acyl component are attached to a decanoic acid this confirms Bios-12, a glycolipid.

4.5.3 Fourier Transform Infra Red (FTIR) Analysis of Biosurfactants

The spectra for biosurfactants produced from the FTIR analysis are shown in Figures 4.6−4.10. The results revealed peaks of different shapes (broad, asymmetric, weak), each represents specific functional groups that are present on the molecular chain in the biosurfactants studied.



The various numbers attached to each peak represents the wavelength (cm) associated with each functional group identified

Figure 4.6: Infrared spectra for biosurfactant Bios-40 from *Pseudomonas aeruginosa* MPE40

From the infra red (IR) analysis (Figure 4.6), the spectral at region 461cm-1 represents a lactone ring, the broad band at 3422.80cm^{-1} shows the hydroxyl group (O-H) stretching from carboxylic acids (COOH), the sharp pointed band at 2929.97cm^{-1} and the attached 2868.24cm^{-1} represent an sp2 CH group comprising -CH_3,-CH_2 and -CH groups of alkane, Carbonyl (C=O) band stretching from the esters group was found at 1733.1cm^{-1}, 1054.13 and 1448.59cm^{-1} represents asymmetric stretch of C-O-C from ester (Table 4.13).

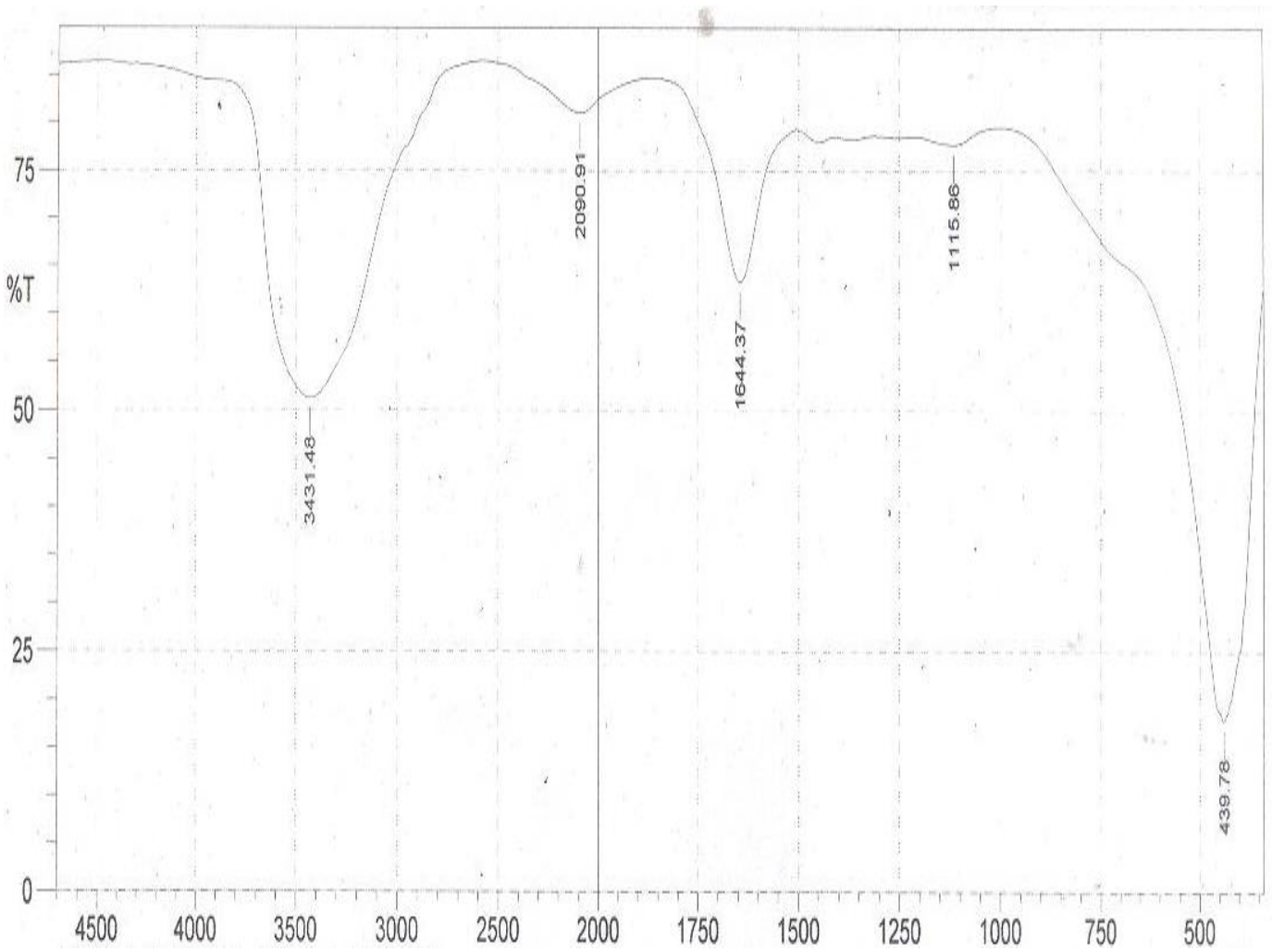
Table 4.13: Infra red interpretation for the biosurfactant Bios-40 produced by *Pseudomonas aeruginosa* MPE40

Peak	Wave No.(cm^{-1})	Functional groups	Peak	Wave No.(cm^{-1})	Functional groups
1	461	Lactone Ring	7	1647.26	C=C stretch
2	880.53	C−H bending	8	1733.1*	C=O, ester
3	1054.13*	C−O−C asymmetric	9	2518.15	O−H Alcohol
4	1177.58*	C−O	10	2868.24*	-C-H_2 alkane
5	1346.36	C−N	11	2929.97	Sp^2 C-H alkane
6	1448.59*	C-O-C	12	3422.8*	O-H, COOH

*peaks that characterized rhamnolipid/glycolipid

The above information from the respective wave numbers confirmed the glycolipid nature of biosurfactants (Table 4.13). This result is in agreement with the report of Thenmozhi *et al*. (2011) that characterized a rhamnolipid from *Pseudomonas aeruginosa* PDKT-2. Similar results were reported by Tuleva *et al*. (2002) and Saravanan and Vijayakumar (2012) with variations in peak region of 1104 cm^{-1} for C–O–C stretching and at 1351 cm^{-1} for CH_2 group from carboxyl ester. The difference in wave numbers as represented by various peaks may be due to the strain or the culture

conditions used which may have resulted in variation in the composition of fatty acid chains of rhamnolipids or different position of the functional groups present in the compounds. From the TLC, GC-MS and FTIR analysis and the kind of organism that produced this biosurfactant (Bios-40), it is confirmed that the biosurfactant is a rhamnolipid, and belong to glycolipid group, made up of aliphatic acid and ester; therefore this study designates the biosurfactant as rhamnolipid Bios-40.



The various numbers attached to peaks are the wavelength (cm) corresponding to each functional group

Figure 4.7 Infrared spectra for biosurfactant Bios-17 from *Pseudomonas paucimobilis* MPE17

The infra red (IR) spectrum of biosurfactant Bios-17 (Figure 4.7) revealed five major functional groups (Table 4.17). The broad N-H(amine) band stretching from peptides, free O-H stretch from alcohol and hydrogen bonded (H-bonded) OH stretch within the vibration spectra of 3431.48cm^{-1}, the vibration at 1644.37cm^{-1} indicates the lactone carbonyl adsorption (C=O) from aliphatic and –C=C stretch from aromatic, the sharp and longest peak at 439.78cm^{-1} confirms the presence of -C-Cl in a 6-membered ring in the sample, and at 1115.86 is the skeletal vibration stretch of C-C and C-O.

Table 4.14: Infra red Interpretation for biosurfactant Bios-17 produced by *Pseudomonas paucimobilis* MPE17

No. of Peak	Wave No.(cm^{-1})	Functional groups	No. of Peak	Wave No.(cm^{-1})	Functional groups
1	439.78	6 member Ring in and out of plane	3	1644.37*	C=C , N-H and C=O
2	1115.86	C-C, C-O	4	2090.91*	C=N (stretch, aliphatic nitriles (w)
3	1644.37*	C=C , N-H and C=O	5	3431.48	Free OH, H-bonded OH stretch, N-H stretch(1 amine)

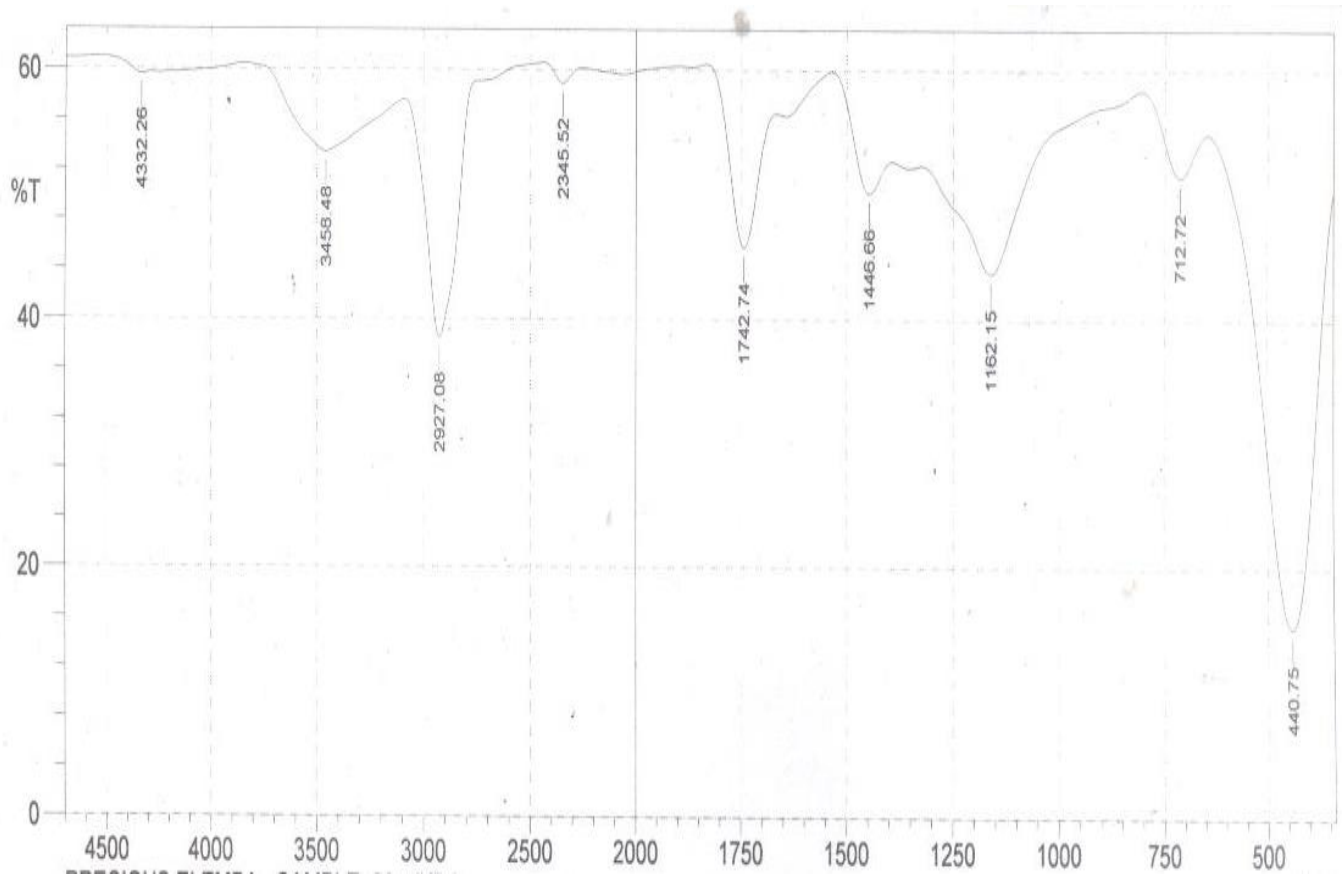
*nature of lipopeptide biosurfactants

The presence of repeated peptide moiety (N-H) and aliphatic nitriles (C-N) from the respective wave numbers (Table 4.14) confirmed the lipopeptide nature of biosurfactant as it has been previously described in literatures (Arima *et al.,* 1968; Nitschke *et al.,* 2005; Thavasi *et al.*, 2010; Mital *et al.*, 2011; Nalini *et al*., 2013). This result agrees with those of Thavasi *et al.* (2010). Similar results were obtained by Joshi *et al.* (2008); Faria *et al.* (2011) and Jorge *et al.*(2013) with slight difference in the wave numbers 3305cm^{-1}, 1643cm^{-1} and 1543 cm^{-1} representing the same functional groups (N-H-stretching, CO–N bond, and N–H bond combined with C–N respectively). Nalini *et al.* (2013) reported the presence of strong adsorption bands of peptide, at 3287cm^{-1} resulted from the stretching mode of N-H, stretching mode of the C=O bond and the deformation

mode (combined C-N stretch mode) of the N-H bond due to lactone carbonyl adsorption at 1652cm^{-1}. The variations in the wave numbers having the same functional group as compared to results of Joshi *et al.* (2008); Faria *et al.* (2011); Jorge *et al*. (2013) and Nalini *et al*. (2013) may be due to the positioning of the carbon atom to which the functional group is being attached.

From the TLC, GCMS and FTIR analysis of this biosurfactant Bios-17 it is identified as glycolipopetide and it is designated as glycolipopeptide Bios-17. Glycolipopeptide biosurfactant identified in this present study is similar to the results obtained from non marine *Corynebacterium* isolates by Zajic *et al.* (1977) and Cooper *et al.* (1979), *Corynebacterium* spp. by Akit, Cooper, Mannien and Zajic (1981) and *Corynebacterium kutscheri by* Thavasi *et al*. (2010).

The IR spectrum of Bios-30 (Figure 4.8) revealed nine major functional groups (Table 4.15) in the biosurfactant of which broad band at the wave number 3458.52cm^{-1} indicated the presence of O-H bonds stretching from the carboxylic acids (COOH).
The sharp C-H bond was observed at 2927.48cm^{-1}, while the ester carbonyl group was at 1742.74cm^{-1}. C-H bond of CH_2 group was observed at 1446.66cm^{-1} stretching from aliphatic and the N-H bond represented by the wave number 712.72 cm-^{1}.



The various numbers attached to peaks are the wavelength (cm) corresponding to each functional group

Figure 4.8: Infrared spectra for biosurfactant Bios-30 from *Bacillus firmus* MPE30

Table 4.15: Infra red Interpretation for the biosurfactant Bios-30 produced by *Bacillus firmus* MPE30

Peak	Wave No.(cm^{-1})	Functional groups	Peak	Wave No.(cm^{-1})	Functional groups
1	440.75	Ring in and out of plane bending	5	1742.74*	C=O ester
2	712.72	C-C skeletal vibration	6	2345.52	C≡N, aliphatic nitrile
3	1162.15*	C-O stretch(v)	7	2927.48*	C-H
4	1446.66*	C-H, binding aliphatic	8	3458.48*	O-H stretch, ROH,
5	1742.74*	C=O ester	9	4332.26	Aromatic and aliphatic C-H stretch

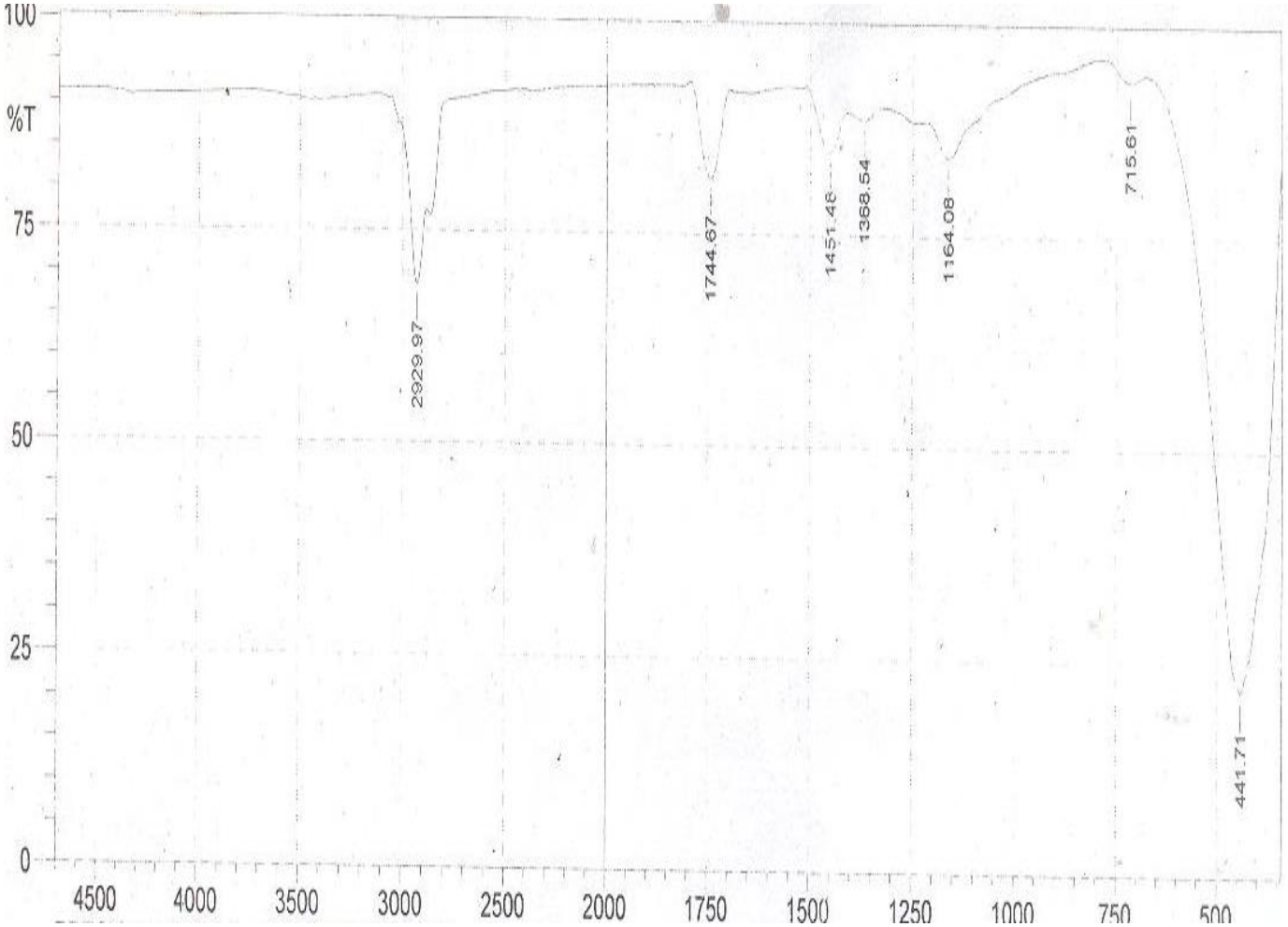
*major glycolipid peaks

These bands confirmed the glycolipid nature of the biosurfactant; although studies (Nakano and Zuber (1989), Nitschke and Pastore (2004), Nitschke *et al.* (2005), Mital *et al*. (2011); Nalini *et al*. (2013) have shown that *Bacillus* species produce majorly lipopeptide but *Bacillus firmus* MPE30 used in this study produced a glycolipid.

In addition, the GC-MS analysis (Figure 4.3 and Table 4.10) of the biosurfactant also confirmed the above results with peaks observed to consist of lipids and carbohydrate moieties without peptides. Although we are not aware of any report on the production and characterization of biosurfactants from *B. firmus*, but biosurfactant produced by *B. megaterium and Streptococcus thermophilus* A was classified as a glycolipid with carbohydrate and lipid combination. The FTIR analysis of the biosurfactant revealed that, the most important bands were located at 2929cm^{-1} (for the CH aliphatic stretching), 1700cm^{-1} (for the C=O ester bond), and 3342cm^{-1} (for O–H bonds) confirming the presence of glycolipid moieties (Rodrigues *et al*., 2006; Thavasi *et al*., 2010).

The IR spectrum of Bios-25 (Figure 4.9) revealed seven major functional groups (Table 4.15) in the biosurfactant of which the band at the wave number 1744.67cm^{-1} indicated the presence of carbonyl (C=O) absorption band stretching from ester indicating the presence of carboxyl group. The sp^2 C−H stretch from aliphatic was observed at 2929.97cm^{-1} and a weak asymmetric stretching peak at 1368.54cm^{-1} indicating C-O-C stretching of the rhamnose; these band characteristics confirm the glycolipid nature of the biosurfactant (Tabatabaee *et al.,* 2005; Chamanrokh *et al.,* 2008). Furthermore, a weak symmetric peak at 1164.08cm^{-1} representing sulfonyl (S=O) stretching indicated the presence of sulfate was observed. Eman (2012) reported the presence of S=O

stretching from sulfate in a biosurfactant (bioflocculant) at the absorption peak of 1248.68 cm^{-1}.



The various numbers attached to peaks are the wavelength (cm) corresponding to each functional group

Figure 4.9: Infrared spectra for biosurfactant Bios-25 from *Acinetobacter iwoffii* MPE25

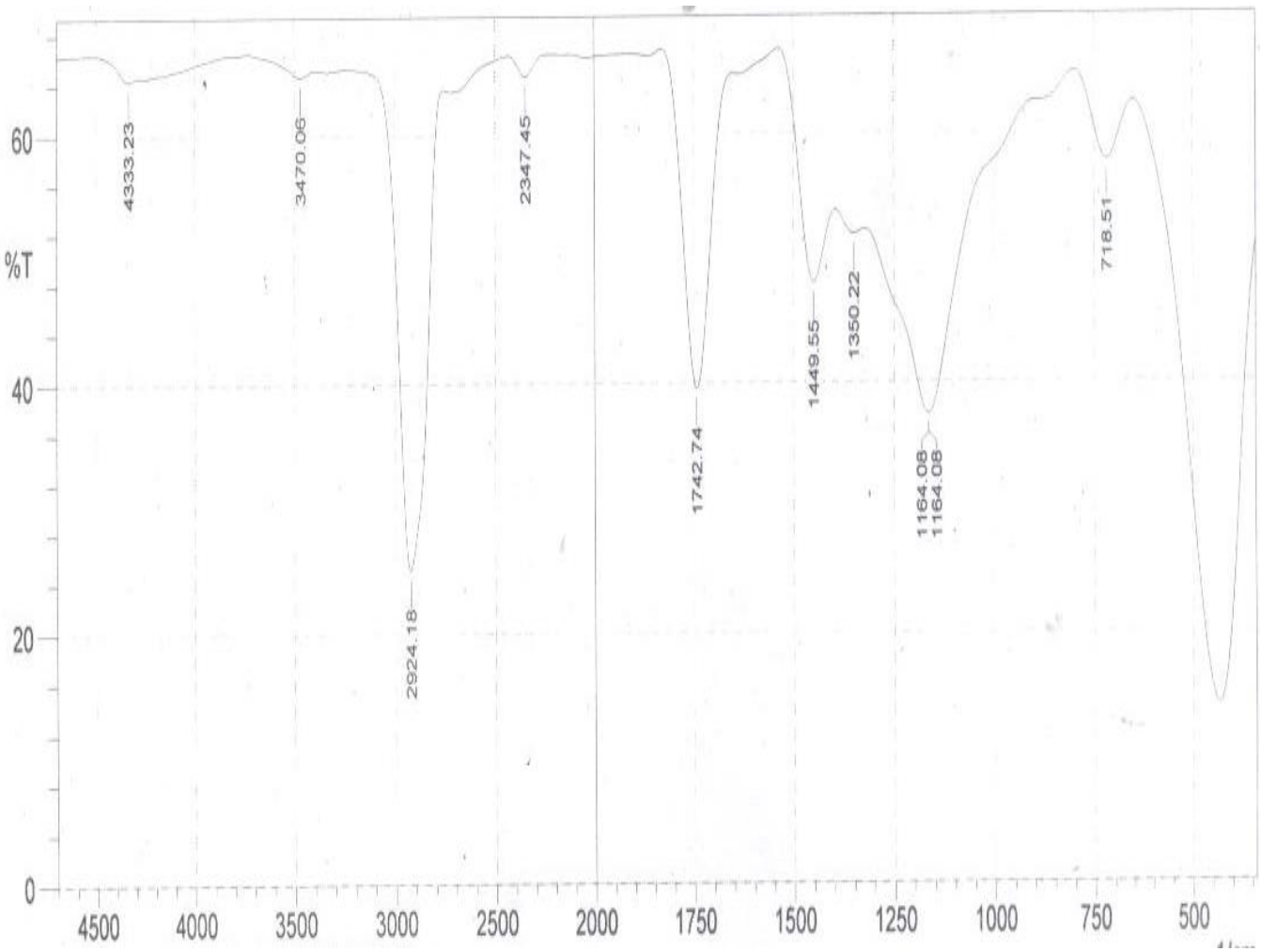
Table 4:16: Infra red interpretation for the biosurfactant Bios-25 from *Acinetobacter iwoffii* MPE25

No. of Peak	Wave No.(cm^{-1})	Functional groups
1	441.71	Stretches from C≡C−H (s)
2	715.61	C=C-H
3	1164.08	S=O from sulfonyl
4	1368.54*	C-O-C asymmetric stretch (vs)
5	1451.48*	C−H bending from aliphatic
6	1744.67*	C =O stretch(v)
7	2929.97*	Sp^2 C-H stretch from aliphatic

*peaks that characterized glycolipid

The GC-MS analysis (Figure 4.4, Table 4.11) revealed a fatty acid component with Sulphurous acid attached to dodecyl 2-ethylhexyl ester (that is a Sulphurous ester) which is not related to any biosurfactant known so far. The FTIR spectra confirmed the glycolipid nature and the S=O functional group proves that sulfonate is present in the compound. Following the naming scheme of Sanghoo, Kang, Kim, and Jung (2002); Mohammad, Hausmann, Lepine, Muller, and Deziel (2011) and other well studied and fully characterized biosurfactants, this biosurfactant is a sulfoglycolipid and should be designated as sulfoglycolipid Bios-25. Sanghoo *et al*. (2002) designated a biosurfactant phosphoglycolipid as a result of the unique phosphorus ester linked to a fatty acid chain revealed by GCMS and NMR spectrum and a phosphonyl band of P-O-C stretching observed at 780cm^{-1} in the FTIR spectra.

The IR spectrum of the biosurfactant Bios-12 (Figure 4.10), revealed ten functional groups (Table 4.15) in the biosurfactant of which rocky band at the wave number 4333.23cm^{-1} indicated the aromatic and aliphatic C-H stretch. The week O-H bond stretching from carboxylic acid (COOH) was observed at 3470.06cm^{-1}, the weak asymmetric stretch of C-O-C at 1350.22cm^{-1} and anhydride ester bond of C=O represented by sharp symmetric peak was observed at 2924.18cm^{-1} (Figure 4.10).



The various numbers attached to peaks are the wavelength (cm) corresponding to each functional group

Figure 4.10: Infrared spectra for biosurfactant Bios-12 produced by *Micrococcus kristinae* MPE12

Table 4.17: Infra red intrepretation for the biosurfactant Bios-12 from *Micrococcus kristinae*

Peak	Wave No.(cm^{-1})	Functional groups	Peak	Wave No.(cm^{-1})	Functional groups
1	718.51	C≡C-H,C-H bending	6	1742.74*	C=O (anhydride,ester)
2	1164.08	S=O stretch	7	2347.45	C≡N aliphatic stretch
3	1164.08	S=O stretch	8	2924.18*	C-H
4	1350.22*	C-O-C, a symmentric and C-H aliphatic bending	9	3470.06*	O-H,COOH dimmers
5	1449.55*	C-H aliphatic bending; C=C aromatic stretch	10	4333.23	Aromatic and aliphatic C-H stretch

*peaks that characterized glycolipid/rhamnolipids

These bands are characteristics of glycolipids and correspond with the studies of Pornsunthorntawee *et al*. (2008); Bondarenko *et al*. (2010) and Mital *et al.* (2011). However, the peak at 1164.08cm^{-1} has a double stretch which represent a disulfonyl (S=O) group indicating the presence of sulfates. Eman (2012) reported the presence of S=O stretching present in a biosurfactant (bioflocculant) at the absorption peak of 1248.68 cm^{-1}.

Micrococcus species (both the mutant and wild strain) have been reported to have surface active molecules consisting of phospholipid, glycolipid and neutral lipid (de Bony, Gilleron, Welby, Laneelle, and Tocanne, 1989; Pakkiri, Wolucka, Lubert, Waechter 2004; Leroy and Charles, 2005). In addition, a glycolipid biosurfactant has

being produced from *Micrococcus luteus* BN56 while growning on n-hexadecane (Tulevaa *et al.,* 2009).

Leroy and Charles (2005) reported a phospholipid with a phosphorus content (PG and PI) from *Micrococcus luteus* and its mutant strain mms1 and mms2, but in this present study with *Micrococcus kristinae* MPE12 a glycolipid consisting of two sulfonyl (S=O) group as revealed from the FTIR analysis was produced, and it was designated as disulfoglycolipid Bios-12 following the naming scheme of Sanghoo *et al*. (2002) where a biosurfactant was designated as phosphoglycolipid as a result of a the presence of phosphonyl band of P-O-C stretching.

4.5 Bioremediation of Heavy Metal Contaminated Soil

4.5.1 Physicochemical properties of the soil

The physical and chemical (physicochemical) properties of both the metal polluted and the unpolluted soil was determined and the results obtained are shown in Table 4.18.

Table 4.18: Physicochemical properties of unpolluted and heavy metal polluted soils

Soil Sample	pH	Organic Matter (%)	Permeability	Porosity	CEC (meq $100g^{-1}$)	EC(μScm^{-1})	Moisture (%)	Particle size (%)			Metal conc. (ppm)	
								Sand	Silt	Clay	Pb	Cr
Unpolluted soil	6.78	8.09	1.08×10^{-3}	22.62	25.4	120	6.18	54.9	17.14	21.8	5.0	0.6
Soil + Pb	5.24	2.18	1.02×10^{-3}	17.07	19.3	288	7.20	54.9	17.14	21.8	105.0	Nil
Soil+ Cr	4.57	2.67	1.04×10^{-3}	16.62	20.5	348	7.21	54.9	17.14	21.8	Nil	100.6

Key: Conc: Concentration, ppm: part s per million; Pb: Lead; Cr: Chromium; CEC: cation exchange capacity; EC: electrical conductivity; meq: milliequivalents; and μScm^{-1}: microSiemens per centimeter

The pH, CEC, porosity, permeability value, and organic matter content of the heavy metal polluted soil were lower than the unpolluted soil while EC and moisture content of the polluted soil were higher than the unpolluted soil (Table 4.18). These changes could be attributed to the presence of metallic elements present in the soil, showing that the metals (lead and chromium) actually bounded to the soil.

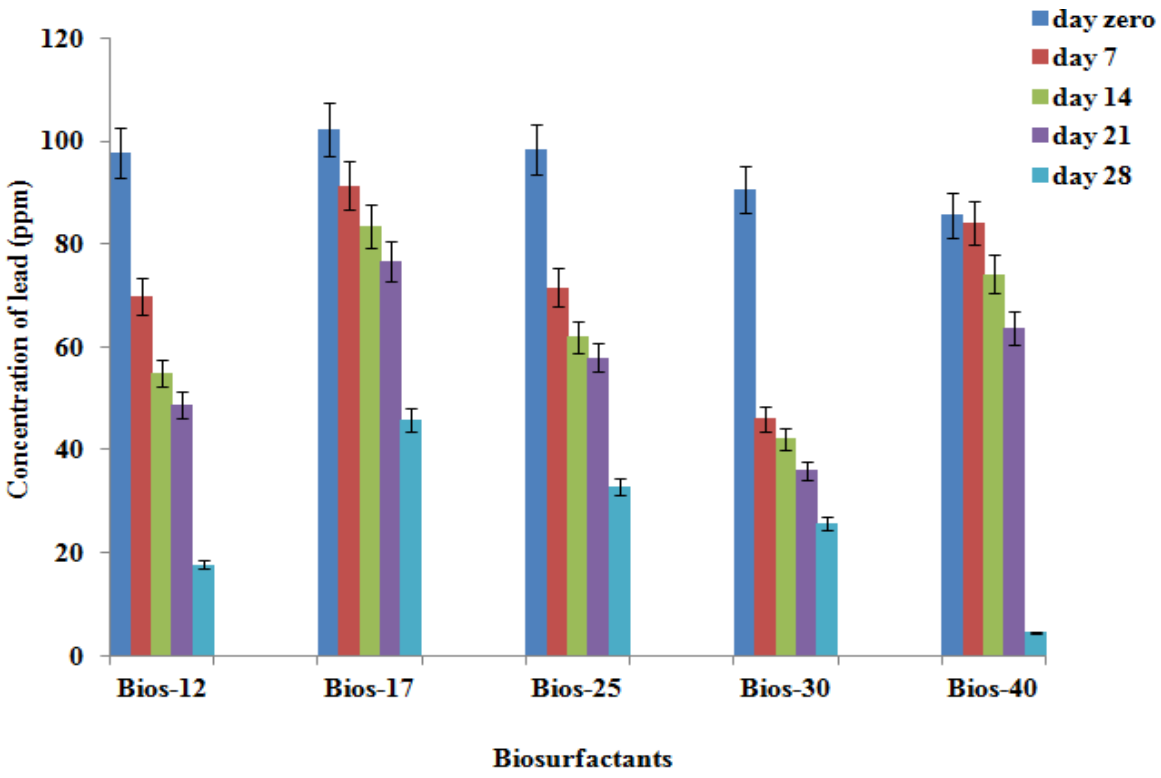
The CEC and porosity of the heavy metal polluted soil decreased as compared to the unpolluted soil.The changes in soil porosity and CEC may be due to the competitive metal sorption in soil and concentration of the heavy metals used for contamination (Juwarkar *et al.,* 2007; Wuana *et al*., 2010) because the CEC itself is an indicator of ion availability in soil (Larson and Pierce, 1994). Schoenholtz, Miegroet and Burger (2000) observed reduction in soil organic matter resulting from adsorption of metallic pollutant to soil surface.

Furthermore, the concentration of lead and chromium in the polluted soil was, higher than the original (unpolluted) soil, indicating that most of the metal ions were successfully introduced into the soil. The particle size analysis revealed textural fraction of sand (54.9%), silt (17.14%) and clay (21.8%) thus classifying the original unpolluted soil as loamy (Liu, Varonsson, Bergstrom, and Sharply, (2012) and they remained unchanged even after pollution, this implies that the presence of heavy metals in soil does not affect the texture of soil.

Parthasarathi and Sivakumaar (2011) recorded no change in particle size distribution, changes in pH value and CEC when a soil was spiked with nickel, cadmium and copper. Similar results were obtained by Liang, Chen, Song, Han, and Liang, (2011). The investigators reported that the presence of charged ions on soil surface resulted in the decrease in cation exchange capacity and reduced pH value. Also, Other researchers such as Rahatgaonkar and Mahore (2008); Zhan *et al.* (2009); Damodaran, Suresh, and Mohan (2011) reported changes in soil physicochemical properties due to metal contamination, as obtained from this present study.

4.5.2 Heavy metal removal from soil using biosurfactants

The feasibility of heavy metal removal from contaminated soil by biosurfactants produced in this study was monitored within a period of twenty eight days and the results obtained are presented in Figures 4.11- 4.12.



Bios-12=(disulfoglycolipid Bios-12), Bios-30=(firmnolipid Bios-30), Bios-25=(sulfoglycolipid Bios-25), and Bios-17(glycolipopeptide Bios-17); Bios-40=(rhamnolipid Bio-40). ppm=parts per million.

Figure 4.11: Effect of biosurfactants on lead polluted soil

Results illustrated in Figure 4.11 shows a progressive decrease with time (days), in the concentration of lead (Pb) in the soil, meaning that, as time (days) increased, the concentration of the heavy metal in the soil decreased gradually with effectiveness of the biosurfactant in the order of Bios-40>12>30>25>17.

The concentration of lead in the soil (105.60ppm) was reduced to 18.37ppm, 46.45ppm, 33.55ppm, 25.70ppm and 4.71ppm by biosurfactants Bios-12, Bios-17, Bios-25, Bios-30, and Bios-40 respecctively after 28days (Figure 4.11). Although, it was generally observed that the biosurfactant Bios-30 was faster in action than the other biosurfactants, though it did not achieve the best result (highest rate of metal removal) after 28days while the lowest reduction rate was observed in Bios-17. These variations in the rate of reduction by the biosurfactants could be attributed to the rate of biosurfactant adsorption to metal which may be influenced by the different molecular composition, size and length of carbon chain of the biosurfactants.

The results (Figure 4.11) also revealed that the biosurfactants Bios-40 (rhamnolipid Bios-40), Bios-12(disulfoglycolipid Bios-12), Bios-30(glycolipid Bios-30), Bios-25 (sulfoglycolipid Bios-25), and Bios-17(glycolipopeptide Bios-17) achieved percentage lead removal in the order of 95.5%> 82% >75.52%> 68.05%>55.76% from soil as compared to 0.24% and 3.07% removal with distilled water and solvent (controls) respectively; the results revealed a significant decrease in lead concentration at $p<0.05$ (Appendix D).

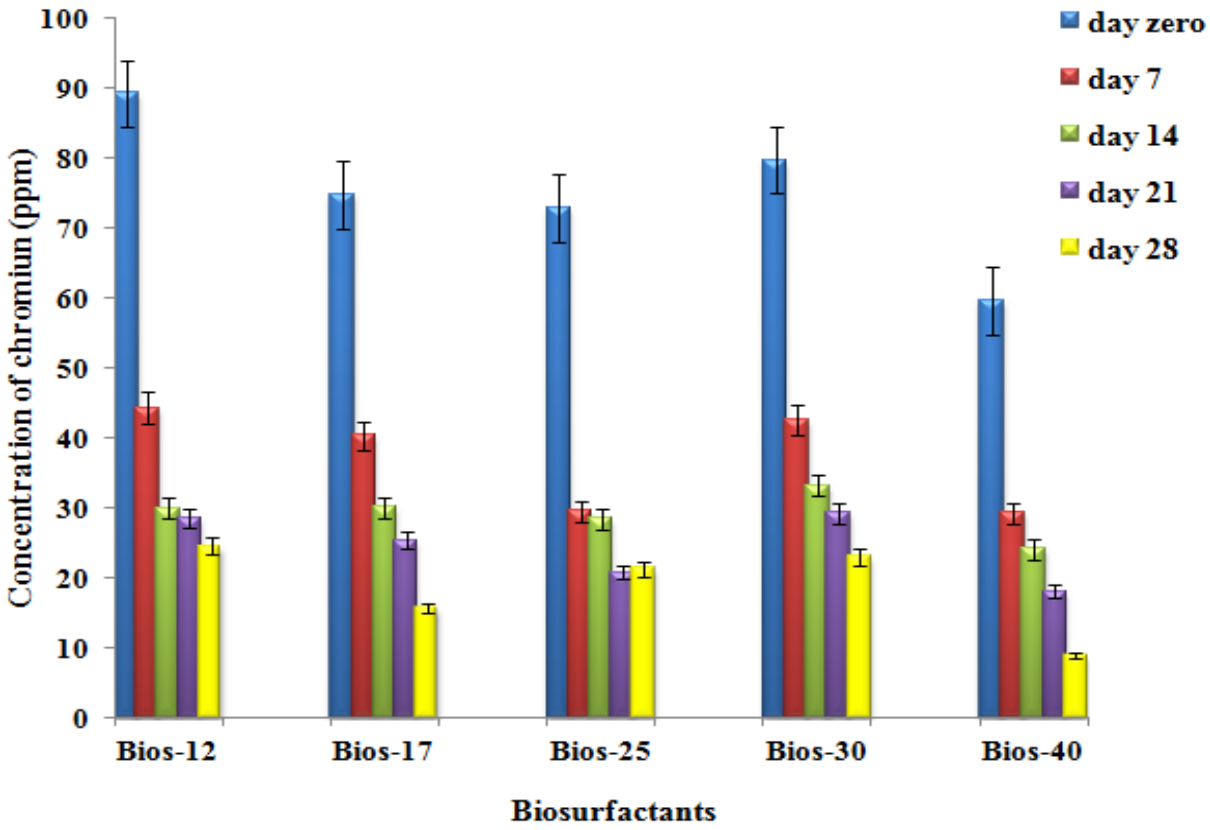
The percentage removal by the biosurfactant Bios-40 (identified as rhamnolipid Bios-40) in this study was as high as 95.5% compared to 15.3%, 88%, and 62% achieved by a rhamnolipid biosurfactant used by Neilson *et al.* (2002) and Daharazma and Mulligan (2007), Juwarker *et al.* (2007), and Abdurrahim *et al*. (2012) respectively in the treatment of lead contaminated soil. These differences in results could be attributed to variations in concentration (10mg/ml) of biosurfactant used as compared to 80ppm used

by Abdurrahim *et al*. (2012) and 5ppm used by Neilson *et al.* (2002). Also 98.3% removal of Pb from aqueous solution was achieved by a polysaccharide produced by *B. firmus* as reported by Salehizadeh *et al.* (2003) which is higher than 75.5% removal obtained by biosurfactant (Bios-30) produced by *B. firmus* MPE30 in this study; the reason being that the mobility of heavy metal in aqueous solution is different from that in soil.

In addition, the biosurfactant Bios-17 produced from *P. paucimobilis* MPE17 had the lowest lead removal of 55.76% as compared to the maximum removal of 95.5% by Bios-40 from *P. aeruginosa* showing that biosurfactant activity varies from species to species irrespective of the fact that they are of same genera. This was proved when biosurfactant from *Pseudomonas* sp.CH8 showed percentage efficiency removal of lead up to 90%, as compared to 50%, 45% and 25% removal of lead by biosurfactants of *Pseudomonas* CH2,CH6 and CH12 respectively (Lin and Harichund, 2011).

So far, we are not aware of any report on the use of biosurfactant produced from *Micrococcus* species in remediation of heavy metal. However, *Micrococcus* sp. BRM7 with ability to produce biosurfactant had being successfully used to adsorb 17% and 34% of strontium within an incubation time of 2 and 4days respectively as compared to its non biosurfactant producing counterpart (*Micrococcus* sp. ARMT8) that died off after 24 hours (Issam *et al.,* 2012). The researchers added that the use of the surfactant alone may be effective in remediation of heavy metal other than strontium (Young *et al.,* 2010; Issam *et al.,* 2012) and this has been proved in this present study that obtained 82% removal of lead from lead contaminated soil using biosurfactant designated as

disulfoglycolipid Bios-12. Hence, it was deduced that biosurfactants used in this study demonstrated metal sorption capacity with varying levels of efficiency.



Bios-12=(disulfoglycolipid Bios-12), Bios-30=(firmnolipid Bios-30), Bios-25=(sulfoglycolipid Bios-25), and Bios-17(glycolipopeptide Bios-17); Bios-40=(rhamnolipid Bio-40). ppm=parts per million

Figure 4.12 Effect of biosurfactants on chromium (Cr.) polluted soil

A significant reduction in the concentration of metal (Cr) was observed after the addition of biosurfactant as compared to control.

The concentration of chromium in the soil (100.60ppm) was reduced to 24.48ppm, 15.70ppm, 21.35ppm, 23.03ppm and 9.02ppm by Bios-12, Bios-17, Bios-25, Bios-30, and Bios-40 respecctively after 28days (Figure 4.12).

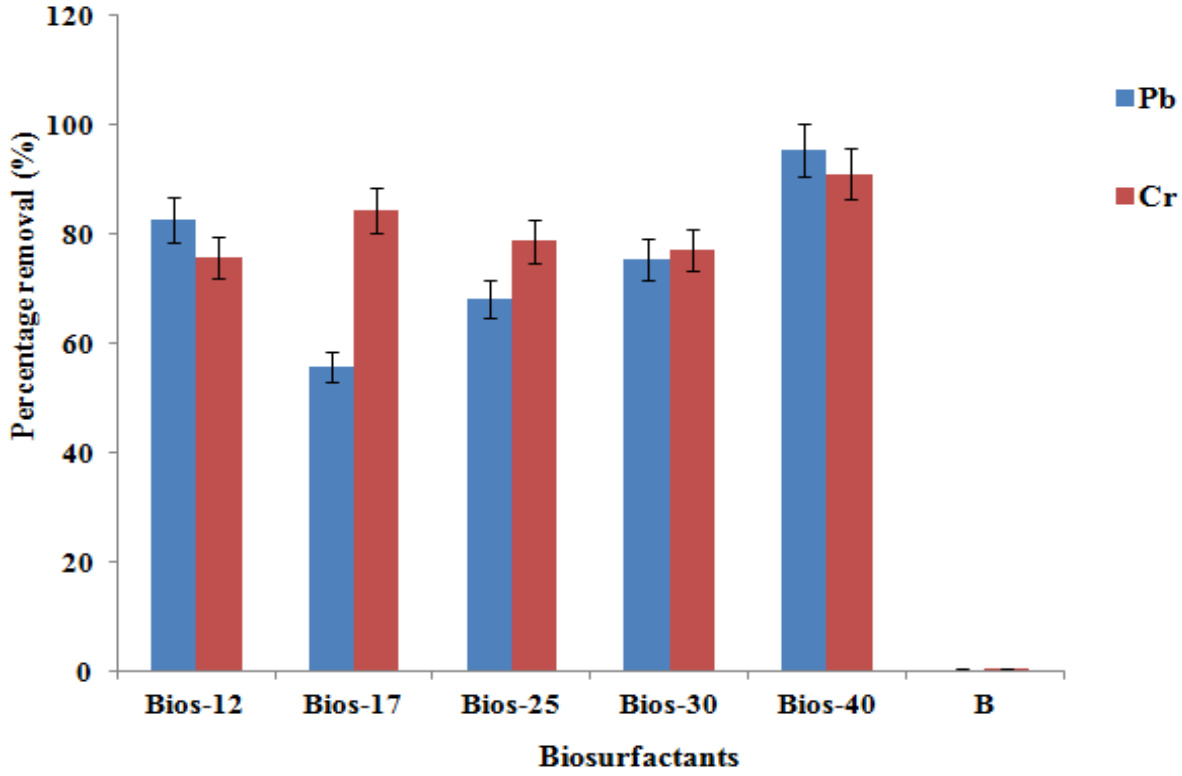
It was generally observed that as time (days) progressed the concentration of chromium in the soil decreased, meaning that, the percentage removal of heavy metal by the biosurfactants increased. However the trend of reduction of chromium concentration differed from one biosurfactant to another. The rate of reduction by Bios-40 was faster than all the other biosurfactants and it also achieved the best result (highest rate of metal removal) after 28 days. This variation in the rate of metal removal by the biosurfactants could be attributed to the size of the biosurfactant, the degree of saturation, the substituent, length of carbon chain, as well as their adsorption capacity and different sorptive interactions exhibited by the biosurfactants to the soil system (Asci *et al*., 2008).

The results (Figure 4.15), revealed that the biosurfactants, Bios-40, Bios-17, Bios-25, Bios-30, and Bios-12 achieved an effective chromium removal in the order of 91.03% >84.4% >78.8% >77.1% >75.7% as compared to 0.4% and 2.3% removal with distilled water and solvent (controls) respectively after 28days. Thus Bios-40 had the maximum removal efficiency of 91.03% which was closely followed by Bios-17 (84.4%) > Bios-25 (78.8%) >Bios-30(77.1%) and Bios-12 had the minimum removal efficiency of 75.7%. The results were significantly difference at $p<0.05$ (Appendix D).

The biosurfactant produced by *P. aeruginosa* MPE40 (rhamnolipid Bios-40) *and P. paucimobilis* MPE17 (glycolipopeptide Bios-17) in this study were more effective with percentage removal of 91.03%, and 84.4% respectively after 28days. These results agree with the report of Massara *et al*. (2007). Similarly Singh, Juwarkar, Mudhoo, and Dubey (2012) reported 92% removal of chromium from a soil spiked with 100mg/kg heavy metal within an interval of 24 days by rhamnolipid. A batch and column experimental setup by Lidi *et al*. (2012) was used to evaluate the efficiency of

biosurfactants (saponin and sorphorolipid) in removal and recovery of heavy metals from sludge and they observed 73.2% removal of lead, 64.2% of nickel, and 56.1% of chromium whereas 89.7%, 91.1%, and 99.1% of Pb, Ni and Cr. respectively were recovered after two weeks.

The adsorption efficiency for chromium achieved by biosurfactant (Firmnolipid Bios-30) from *Bacillus firmus* MPE30 in this study was 77.11% which is lower than 90% and 85% achieved by biosurfactant from *Bacillus subtilis* WD90 in nickel and cadmium respectively(Kaewchai and Prasertsan, 2002). Percentage removal obtained by sulfoglycolipid Bios-25 and disulfoglycolipid Bios-12 from *Acinectobacter iwoffii* MPE25 and *Micrococcus kristinae* MPE12 in this study was 75.67 and 78.78% respectively. Although there has been no report to the best of our knowledge on the use of biosurfactant from these species in metal remediation; it has been cited by researchers that *Acinectobacter* RAG-1 can bind up to 240µg uranium per emulsion or emulsan [(UO_2^{2+})/mg emulsion or emulsan] (Zosim, Gutnick and Rosenberg, 1983; Niu, Xu and Wang, 1993; Miller, 1995; Abdurrahim *et al*., 2012) which has been confirmed in ths study.



Bios-12=(disulfoglycolipid Bios-12), Bios-30=(firmnolipid Bios-30), Bios-25=(sulfoglycolipid Bios-25), and Bios-17(glycolipopeptide Bios-17); Bios-40=(rhamnolipid Bio-40); ppm=parts per million; B=control soil

Figure 4.13: Comparison between the percentage removal of lead and chromium by biosurfactant

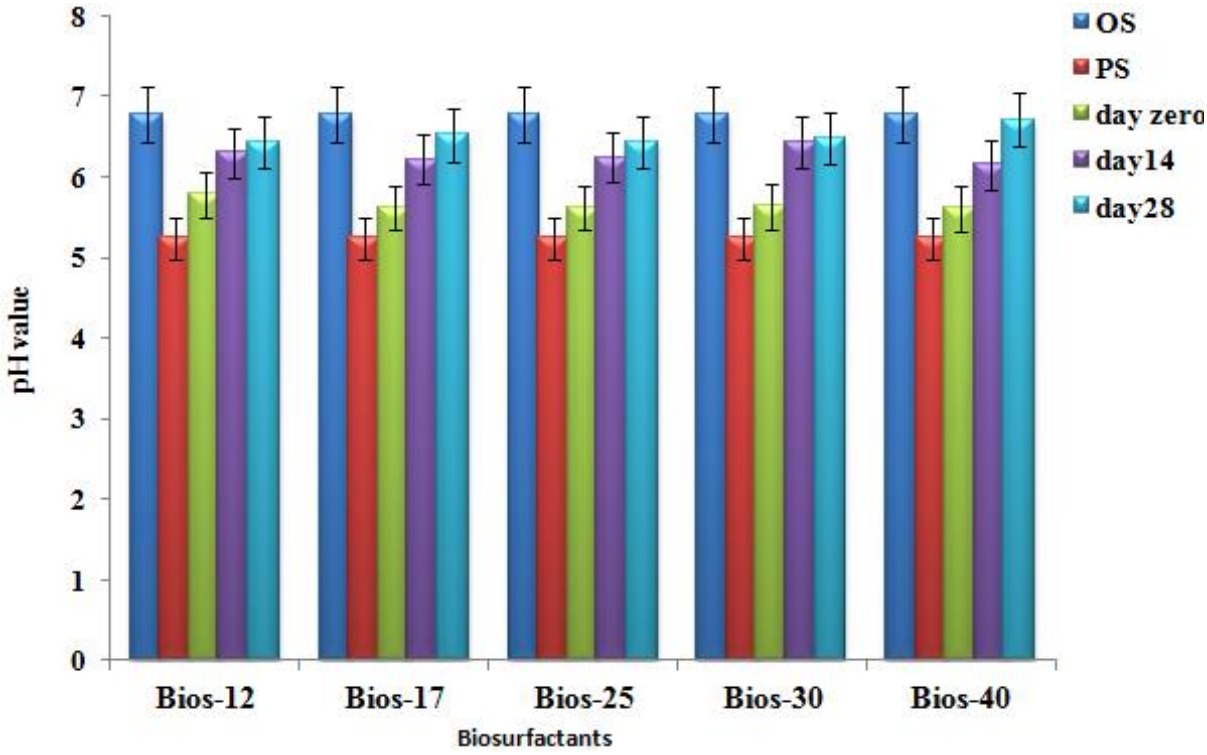
The biosurfactants exhibited different removal efficiencies for both lead and chromium (Figure 4.13). Only Bios-40 and Bios-12 had higher percentage removal for lead at 95.5% and 82.50% respectively while the other three biosurfactants (in the order Bios-17 >25 >30) were more effective on chromium at 84.39%, 78.78% and 77.11% respectively. Comparing the effectiveness and trend of reduction in all the five biosurfactants on the metals, it was deduced that the biosurfactants were more effective on chromium than lead (Cr >Pb) and this is possibly due to its (Pb) strong association and adsorption with soil (Wuana *et al.*, 2012) which contrbutes to its high toxicity to the environment.

Differences in Cr and Pb removal indicate that biosurfactants facilitates mobilization of metals selectively, and their adsorption is dependent on the characteristics of the metal due to the specificity of biosurfactant for each metal in the soil (Lidi *et al.,* 2012). Ochoa-Loza *et al*. (2009) had also reported preferential complexion of metals in the range of Cd, Pb and Hg by biosurfactant. Similarly Lin and Harichund (2011) added that differences in affinity of metals for biosurfactants are due to charge density, attractive interaction, and types of conformation formed by these polymers with the adsorbed ions.

All the five biosurfactants produced in this study demonstrated metal sorption potential with varying levels of efficiency (Figures 4.11, 4.12 and 4.13). Several other researchers (Kaewchai and Prasertsan, 2002; Wang and Mulligan, 2004; Das and Santra, 2007; Noghabi *et al.*, 2007; Lin and Harichund, 2011) have reported varied efficiencies by biosurfactants in metal removal.

4.5.3: pH of heavy metal polluted soil treated with biosurfactant

The pH of metal polluted soil remediated with biosurfactants was monitored and the results obtained are presented in Figures 4.14 and 4.15 for lead and chromium respectively.



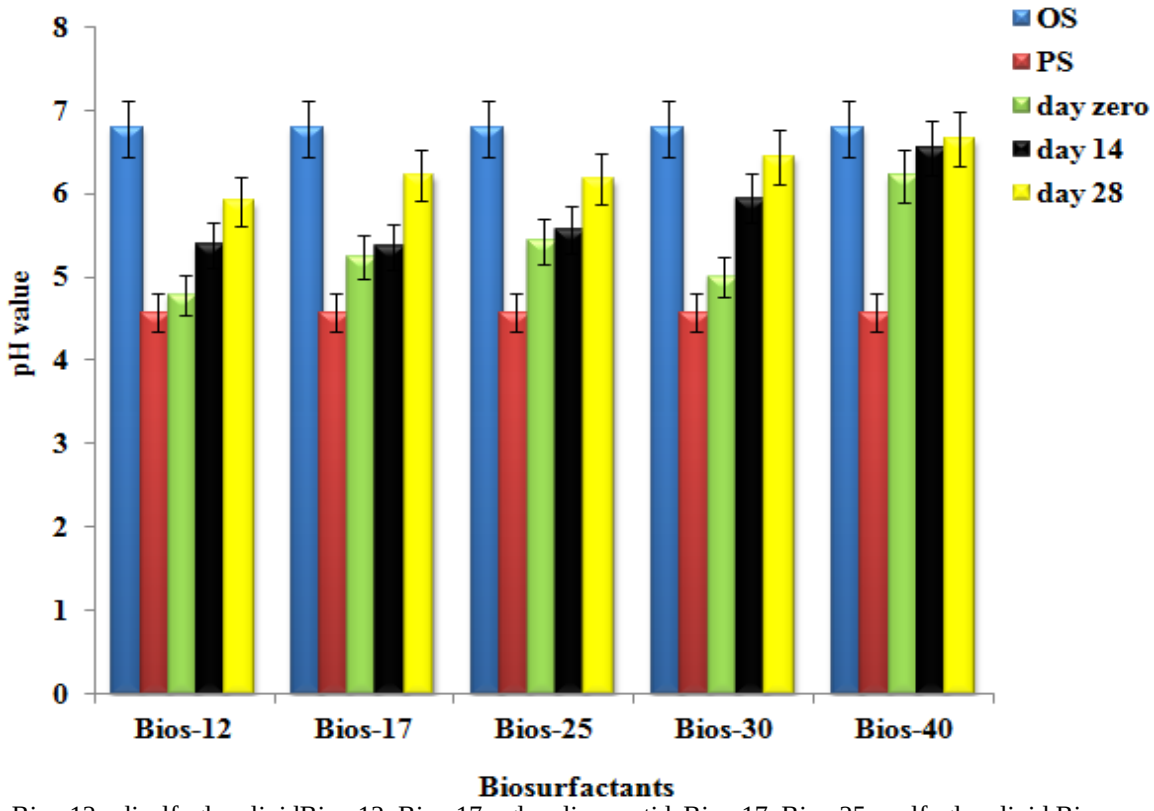
Bios-12= disulfoglycolipidBios-12, Bios-17= glycolipopeptideBios-17, Bios-25= sulfoglycolipid Bios-25, Bios-30= Firmnolipid Bios-30, and Bios-40= Rhamnolipid Bios-40**;** OS=original soil sample before pollution; PS= polluted soil sample with lead

Figure 4.14: pH of lead polluted soil treated with biosurfactants

From the result as illustrated in Figure 4.14 it was observed that the pH of the unpolluted soil was higher (6.78) than the lead polluted soil (5.24). However the pH of the polluted soil increased gradually from day zero to 28days.

As the concentration of the metal decreased in the soil (Figure 4.11), the pH of the soil increased progressively (Figure 4.14), with a range of 5.24−6.43 for Bios-12, 5.24−6.52 (Bios-17), 5.24−6.44 (Bios-25), 5.24−6.44 (Bios-30), and 5.24−6.71 (Bio-40). When the pH of the remediated soil was compared with the pH (6.78) of the original soil (before pollution with Pb) and the soil polluted with Pb (5.24), there were significant differences ($p<0.05$).

However there was no significant difference ($p>0.05$) between the treated soil and the original soil sample after 28days, meaning that the soil was almost returning to its original state after 28days of inoculation with the biosurfactants signifying that the biosurfactants were effective in the metal removal. Sanjee, Asha, Ackmez, and Kirti. (2012) reported an increase in pH value (6.60-6.78) of a heavy metal contaminated soil when treated with di-rhamnolipid as an evidence of surfactant effectiveness in remediation of metal.



Bios-12= disulfoglycolipidBios-12, Bios-17= glycolipopeptideBios-17, Bios-25= sulfoglycolipid Bios-25, Bios-30= Firmnolipid Bios-30, and Bios-40= Rhamnolipid Bios-40; OS=original soil sample before pollution; PS= polluted soil sample with chromium

Figure 4.15: pH of chromium polluted soil treated with biosurfactants.

From the result as illustrated in Figure 4.15, it was observed that the pH of the unpolluted soil was higher (6.78) than the chromium polluted soil (4.57).

However the pH of the polluted soil increased gradually from day zero to 28days. It was also observed that the pH decreased from 6.78 to 4.57 as Cr. was added to the soil and thereafter increased with the addition of biosurfactants.

The effect of the biosurfactants on pH (Figures 4.14 and 4.15) was noticed that as the metal concentration decreased as the day progresses the pH of the soil increases. This is in agreement with Lidi *et al*. (2012) who recorded that the removal efficiency of heavy metals by biosurfactants generally ascended with decreasing concentration of metal and increasing pH value. Singh *et al.* (2012) also recorded increased in pH value from 6.60 to 6.78 after remediation period.

Significant correlation coefficient with pH and metal concentration was observed to be ($r=0.922$, $P< 0.05$) and ($r=0.903$, $P<0.05$) for lead and chromium respectively. This strong correlation value demonstrates the strenghth of association between pH and metal concentration in soil. This correlation can be explained by considering the fact that pH is the main soil characteristic that influences the CEC of the soil which in turn shows the association and adsorption status of metal to soil (de Matos *et al.*, 1996; de Matos *et al.*, 2001).

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

Five bacterial species identified as *Bacillus firmus* MPE30, *Pseudomonas aeruginosa* MPE40, *Pseudomonas paucimobilis* MPE17, *Micrococcus kristinae* MPE12 and *Acinectobacter iwoffii* MPE25 isolated from hydrocarbon contaminated soil were identified as potent producers of biosurfactants (1.0−1.7g/100ml) with good emulsification activity (58.8−70.2%). Based on TLC, GCMS and FTIR analyses the biosurfactants were identified as firmnolipids, rhamnolipid, glycolipopeptide, disulfoglycolipid and sulfoglycolipid respectively composed of various fatty acyl groups of palmitic, oleic, Octadecanoic and Hexadecanoic acids, hydroxylated fatty acids (2,3-dihydroxydodecyl, 2-hydroxy-1-(hydroxymethyl) ethyl ester), branched chain (iso) and methyl esters (anteiso) fatty acids as well as OH, COOH, C-O-C, C=O (from the esters) and sp^2-CH_2, C≡C and N-H as the major and minor functional groups respectively.

The feasibility of metal removal by the biosurfactants was also studied by monitoring the pH of the soil and use of AAS analysis. The results revealed that the biosurfatants were effective and achieved significant percentage removal of 75-91.03% and 55-95.5% for chromium and lead respectively. Moreso, the soil pH was restored to near neutrality and slightly acidic for lead and chromium respectively. From the study conducted so far, it is suggested that the biosurfactants can be used as an effective agent for bioremediation of lead and chromium polluted soil as well as mopp up oil spill since they are good emulsifiers.

5.2 Recommendations

It is recommended that:

i. The biosurfactant producing bacteria and their biosurfactants are recommended for bioremediation of polluted soil.
ii. Further research should be carried out on the structural characterization, purification of the various compounds and gene regulation of biosurfactant production.
iii. The ability of the biosurfactants to remove heavy metals from contaminated soil in the field should be investigated.
iv. The optimal conditions for production of the biosurfactants should be determined.
v. The identified biosurfactant producing bacteria should be genetically engineered or modified in order to obtain higher yield of the biosurfactants.
vi. The biosurfactants produced should be tried on other applications such as enhanced oil recovery and bioremediation of oil spills since they are good emulsifiers.

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APPENDIX A

MEDIA USED

(i) R2B Broth

Dipotassium phosphate (K_2HPO_4)	0.3g
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	0.5g
Soluble starch	0.5g
Glucose	0.5g
Sodium pyruvate	0.3g
Yeast extracts	0.5g
Peptone	0.25g
Peptic digest of animal tissue	0.25g
Casein acid hydrolysate	0.5g
Distilled water	1liter
pH	7.1±0.2

These were weighed into a flask, and 1000ml of distilled water was added to dissolve the salts, which was then sterilized by autoclaving at 121^oC for 15minutes

For 100ml quantity: 0.36g of the composition was weighed and dissolved in 100ml

(ii) R2A agar

Dipotassium phosphate (K_2HPO_4)	0.3g
Magnesium sulphate heptadydrate ($MgSO_4.7H_2O$)	0.05g
Soluble starch	0.5g
Dextrose	0.5g
Sodium pyruvate	0.3g
Peptone	0.25g
Casamino acid	0.5g

Agar agar (No.4 Oxoid)	15g
Distilled water	1liter
pH	7.2±0.2

These were weighed into a flask, and 1000ml of distilled water was added, it was then heated for 3 minutes using burner to dissolve the salt, after which, it was sterilized by autoclaving at 121^{o}C for 15minutes

For 100ml quantity: 1.88g of all the composition was weighed and dissolved in 100ml after which, it was sterilized by autoclaving at 121^{o}C for 15minutes

(iii) Nutrient agar (NA)

lab-lemco powder	1.0g
Yeast extract	2.0g
Peptone	5.0g
Sodium chloride (NaCl)	5.0g
Agar (No.3 Oxoid)	15g

This was prepared according to the manufacture's direction (28g makes 1liter)

For every 200ml of NA that was prepared 5.6g of NA was weighed and dissolved in 200ml of distilled water, it was then sterilized by autoclaving at 121^{o}C for 15mnutes.

(iv) Muller Hinton broth (MHB) (Oxoid, CM0405)

Beef dehydrated infusion	300g
Casein hydrolysate	17.5g
Starch	1.5g
pH	7.3±0.1 at 25^{o}C

This was prepared according to the manufacture's direction. 21.0g was weighed into a flask and then dissolved with a1000ml distilled water. It was sterilized by autoclaving at 121^{o}C for 15mnutes.

(v) Blood agar

Blood agar was composed of human blood and nutrient agar (see composition of nutrient agar).

Nutrient agar base that was sterilized by autoclaving at 121ºC at 15lbs pressure for 15 minutes after which it was allowed to cool to 45ºC before pouring the 5% human blood and allowed to solidify.

(vi) Nutrient broth (Oxoid)

Peptic digests of animal tissue	5.0g
Sodium chloride (NaCl)	5.0g
Beef extract	1.5g
Yeast extract	1.5g
Distilled water	1litre
pH	7.4±0.2 at 25 ºC

13g of NB was weighed, dissolved in 1liter, and then sterilized by autoclaving at 121ºC for 15mnutes.

(vii) M-R and V-P Media (Glucose-phosphate medium)

Peptone	5g
K_2HPO_4	5g
Glucose	5g
Distilled	100ml
pH	7.5

The components (15g) were introduced into 100ml of distilled water in a conical flask, and then 2ml of the mixture was dispensed into test tubes and was sterilized by autoclaving at 121ºC for 15minutes.

(viii) Sugar Fermentation Test Broth

Phenol red indicator	0.04g
Peptone water (merck)	2g
Distilled water	100ml

The phenol red was dissolved along with the peptone water in 100ml of distilled and was sterilized along with inverted Durham's tubes inside the mixture, at 121^{o}C for 15minute. Then 10ml of filter sterilized sugars (glucose, D-manitol, sucrose, sorbitol etc dissolved in sterile water and heated for 5minutes over a hot burner) were added.

(ix) Motility Test Media

Peptone water (merck)	2.0g
Sodium chloride	1.5g
Agar agar (oxoid)	2.5g
Distilled water	100ml
pH	7.1±0.2

The composition was weighed and dissolved in 100ml of distilled water; it was dispensed into test tube (5ml) before it was sterilized by autoclaving at 121^{oC} for 15minutes.

(x) Simmons Citrate Agar (biomark™)

Ammonium dihydrogen phosphate	1 g
Dipotassium phosphate	1 g
Sodium chloride	5 g
Sodium citrate	2g
Magnesium sulfate	0.2g
Agar	15g

Bromothymol blue.	0.08g
Distilled water	1000 ml
pH	6.9±0.2

(xi) Starch Agar

Beef extract	1.5g
Soluble starch	5g
Agar	12g
Distilled water	500ml
pH	7.3±0.2

This was weighed into a flask containing distilled water and dissolved by heating over a Bunsen burner for 10minutes, before it was sterilized by autoclaving at 121^{oC} for 15minutes.

(xii) Urea agar base

Yeast extract	0.1 g
Monopotassium phosphate	0.091 g
Disodium phosphate	0.095 g
Urea	20.0 g
Phenol red	0.01 g
Distilled water (sterile)	1000ml
pH	6.9±0.2

All the composition was weghed and dissolved in distilled water and then sterilized by autoclaving at 121^{oC} for 15minutes.

(xiii) Mineral salt medium MSM (Jacobucci *et al.*, 2001)

$MgSO_4$	5g
$NaNO_3$	3g
$KH2PO_4$	1g

Yeast extract	1g
Peptone	0.3g
Distilled water	100ml
pH	7.4±0.2

APPENDIX B

REAGENTS USED

(i) Acid Ferric Chloride

$FeCl_3.6H_2O$	12g
Conc. HCl	2.5ml
Distilled H_2O	100ml

(ii) Benedict Solution

Sodium citrate	17.3g
$NaCO_3$ anhydride	10g
$CuSO_4.5H_2O$	1.73g
Distilled H_2O	100ml

(iii) Ehrlich's Reagents

P-dimetylaminobenzaldehyde	1g
Absolute Ethanol	95ml
Conc. HCl	20ml

(iv) Kovac Reagent

P-dimetylaminobenzaldehyde	5g
Amy alcohol(95%)	75ml
Conc. HCl	25ml

(v) Crystal violet

Crystal violet powder	0.5g
Distilled water	100ml

(vi) Safranine

Safranin powder	2.5g
95% ethyl alcohol	100ml
Distilled water	900ml

(vii) Methyl red solution

Methyl red	0.04g
Absolute ethanol	40ml
Distilled water	100ml

(viii) Alpha (α)-naphthol solution

5% α-naphthol in absolute ethanol

(ix) Nitrite test reagents

Solution A

0.8% Sulphanilic acid

5N-acetic acid

Solution B

0.6% dimethyl-∝-naphthylamine

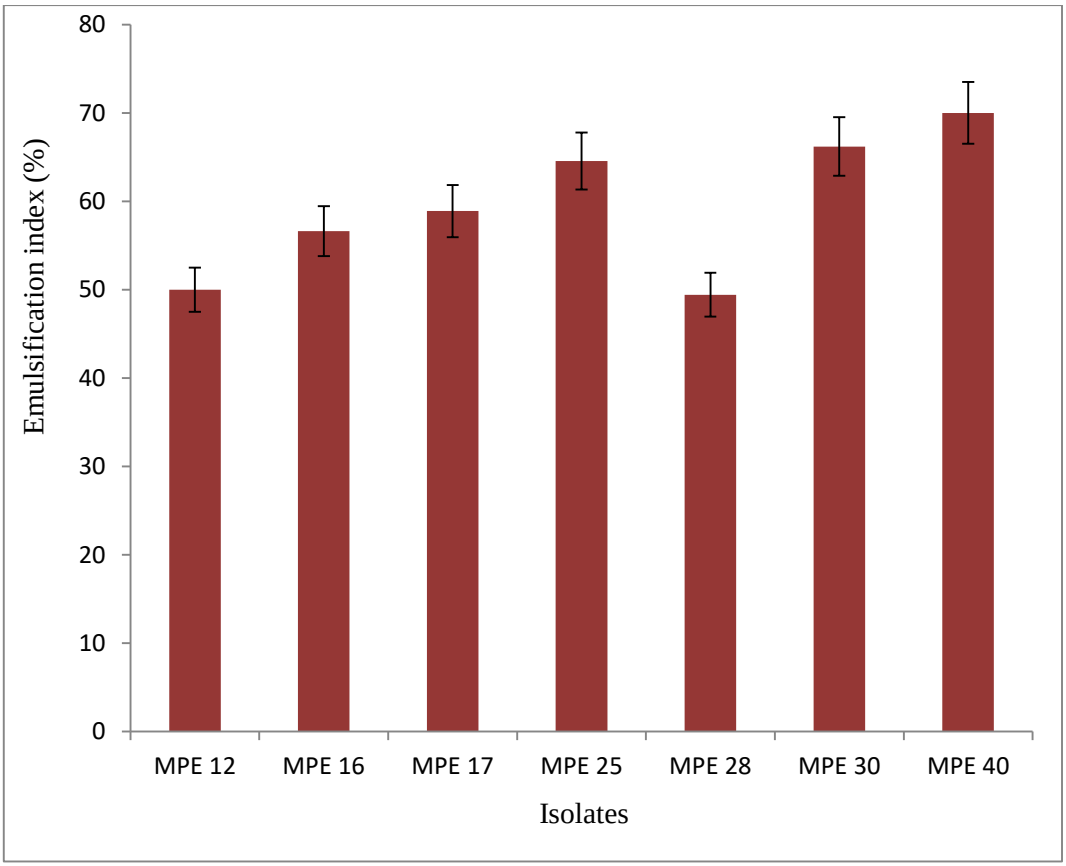
5N-acetic acid

1% Zinc dust

(x) Oxidase reagent: 1% tetrametyhl-p-phenylenediamine aqueous solution

APPENDIX C

Appendix C: Emulsification index (E_{24}) of selected potent producer of biosurfactant



APPENDIX D

Efficacy of Biosurfactant on Metal Removal

Appendix D: Lead polluted soil treated with biosurfactants

Time (Days)	Biosurfactants(mg/ml)					Controls		
	Biompe12	Biompe17	Biompe25	Biompe30	Biompe40	A	B	C
O	97.67±0.02^{c}	102.17±0.09^{e}	98.42±0.28^{d}	90.51±0.04^{b}	87.59±0.01^{a}	105.00±0.06^{f}	104.99±0.07^{f}	104.99±0.12^{f}
7	69.92±0.17^{b}	91.77±0.22^{d}	71.63±0.69^{b}	46.02±5.20^{a}	81.36±4.10^{c}	104.98±0.36^{e}	104.88±0.61^{e}	102.28±0.34^{e}
14	56.82±2.10^{b}	85.65±3.10^{e}	61.33±1.20^{c}	42.98±0.65^{a}	74.07±0.70^{d}	104.92±0.07^{f}	104.26±0.26^{f}	101.85±0.34^{f}
21	49.06±0.17^{b}	77.04±0.17^{e}	57.98±0.58^{c}	36.01±0.10^{a}	65.00±1.20^{d}	104.85±0.15^{f}	104.18±0.13^{f}	101.78±0.13^{d}
28	18.37±0.55^{b}	46.45±0.44^{e}	33.55±0.36^{d}	25.70±0.92^{c}	4.710±0.14^{a}	104.78±0.64^{g}	104.61±0.16^{g}	101.74±0.13^{f}
%rv	82.50	55.76	68.05	75.52	95.50	0.24	0.37	3.07

The results are presented as mean ± standard error of 3 measurements.

Key: %rv (ŋ) = percentage removal; these was calculated based on the initial metal content (control) in the soil sample using the equation: (ŋ) =CM-CMF/CM ×100

Where; CM is initial concentration of heavy metals (control, i.e. without treatment) and CMF is the final concentration of heavy metal

(after treatment with biosurfactants)

Controls:

A= contaminated soil with heavy metal (pb) alone

B= Pb contaminated soil treated with distilled water

C= Pb contaminated soil treated with reagents

Appendix D2: Chromium polluted soil treated with biosurfactants

Time (Days)	Biosurfactants(mg/ml)					Controls		
	Biompe12	Biompe17	Biompe25	Biompe30	Biompe40	A	B	C
O	89.19±0.76^{d}	73.94±0.48^{b}	72.83±3.29^{b}	79.69±0.34^{c}	59.66±0.68^{a}	100.60±0.01^{e}	100.54±0.46^{e}	99.63±0.23^{e}
7	44.28±0.42^{c}	43.34±0.48^{b}	29.54±0.25^{a}	42.47±2.33^{bc}	29.27±0.46^{a}	100.54±0.04^{d}	100.20±0.20^{d}	99.29±0.35^{d}
14	29.94±0.64^{b}	30.06±0.94^{b}	28.50±0.09^{b}	33.16±0.71^{c}	24.08±1.39^{a}	100.44±0.02^{d}	100.20±0.20^{d}	99.23±0.29^{d}
21	28.57±0.88^{d}	25.38±0.31^{c}	20.82±0.06^{b}	29.26±0.87^{d}	18.07±0.85^{a}	100.35±0.03^{e}	100.20±0.20^{e}	98.73±0.69^{e}
28135r5	24.48±0.05^{d}	15.70±0.54^{b}	21.35±0.47^{c}	23.032±1.2^{cd}	9.02±1.10^{a}	100.36±0.06^{f}	100.20±0.20^{f}	98.30±0.35^{e}
%rv	75.64	84.39	78.78	77.11	91.034	0.24	0.40	2.29

The results are presented as mean ± standard error of 3 measurements.

Key: %rv (ŋ) = percentage removal; these was calculated based on the initial metal content (control) in the soil sample using the equation: (ŋ) =CM-CMF/CM ×100

Where; CM is initial concentration of heavy metals (control, i.e. without treatment) and CMF is the final concentration of heavy metal

(after treatment with biosurfactants)

Controls:

A= contaminated soil with heavy metal (Cr) alone

B= Cr. contaminated soil treated with distilled water

C= Cr. contaminated soil treated with reagents

Appendix D3: The pH of lead contaminated soil before and after treatment with biosurfactant

Biosurfactant/time(days)	Before treatment		After treatment		
	OSS	SSPP	O	14	28
Bios-12	6.78	5.24	5.78±0.51	6.23±0.12	6.43±0.51
Bios-17	6.78	5.24	5.62±0.70	6.22±0.04	6.52±0.70
Bios-25	6.78	5.24	5.62±0.70	6.24±0.65	6.44±0.70
Bios-30	6.78	5.24	5.64±0.12	6.44±0.12	6.49±0.12
Bios-40	6.78	5.24	5.61±0.25	6.15±0.02	6.71±0.25
A	6.78	5.24	5.23±0.05	5.23±0.01	5.23±0.05
B	6.78	5.24	5.22±0.11	5.21±0.9	5.22±0.11
C	6.78	5.24	5.20±0.12	5.20±0.12	5.20±0.12

Key: OSS=original soil sample: SSPP= soil sample polluted with lead before treatment with biosurfactants.

A= contaminated soil with heavy metal (pb) alone; B= Pb contaminated soil treated with distilled water; C= Pb contaminated soil treated with reagents

Appendix D4: The pH of chromium contaminated soil before and after treatment with biosurfactant

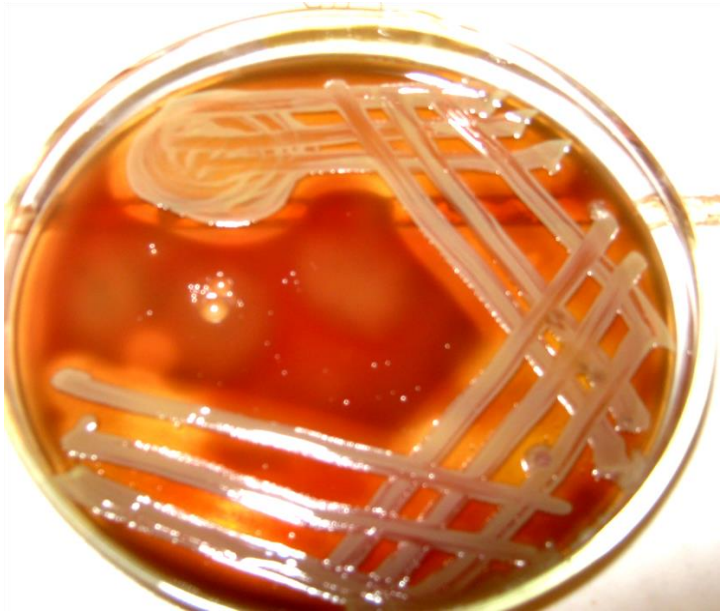
Biosurfactant/time(days)	Before treatment		After treatment		
	OSS	SSPP	O	14	28
Bios-12	6.78	4.57	4.78±0.51	5.38±0.12	5.90±0.51
Bios-17	6.78	4.57	5.23±0.70	5.34±0.04	6.22±0.70
Bios-25	6.78	4.57	5.42±0.70	5.57±0.65	6.17±0.70
Bio-30	6.78	4.57	5.00±0.12	5.94±0.12	6.44±0.12
Bios-40	6.78	4.57	6.21±0.25	6.55±0.02	6.21±0.25
A	6.78	4.57	4.57±0.05	4.57±0.01	4.57±0.05
B	6.78	4.57	4.57±0.11	4.59±0.90	5.20±0.11
C	6.78	4.57	4.57±0.12	4.94±0.12	5.24±0.12

Key: OSS=original soil sample: SSPP= soil sample polluted with Cr. before treatment with biosurfactants.

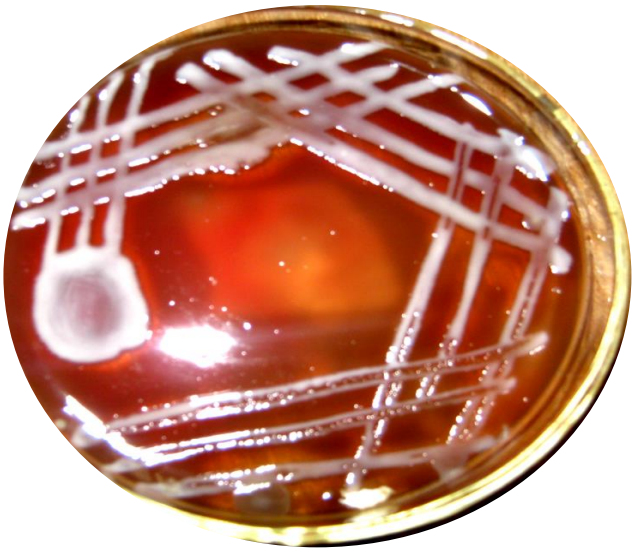
A= contaminated soil with heavy metal (Cr) alone;B= Cr. contaminated soil treated with distilled water; C= Cr. contaminated soil treated with reagents

APPENDIX E

Appendix E: Blood haemolysis due to bacterial growth



a: β-Haemolysis



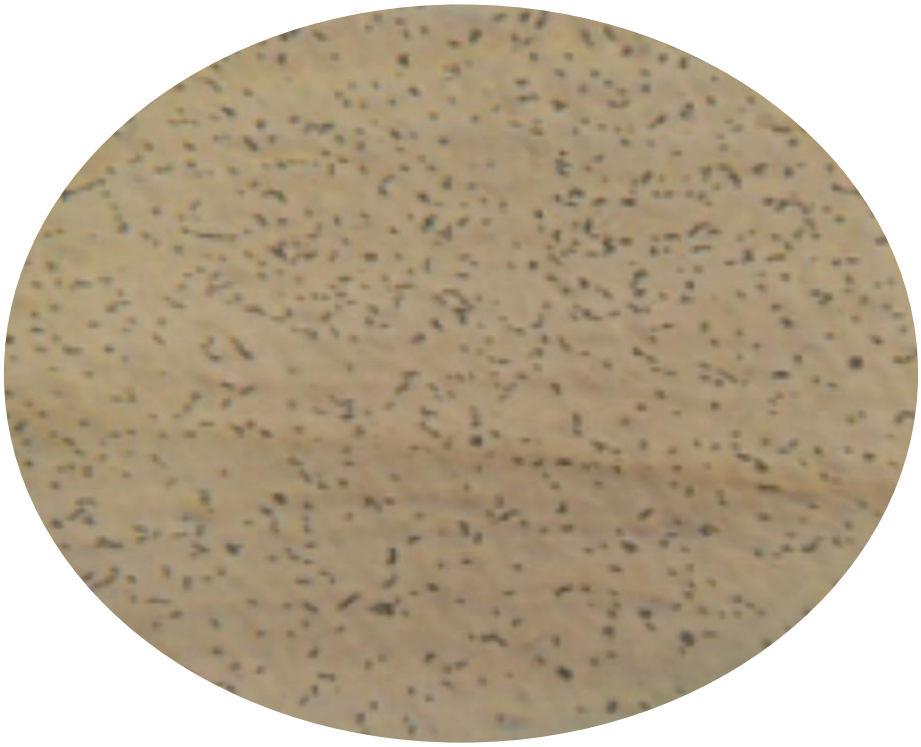
c: No-Haemolysis

b: α-Haemolysis

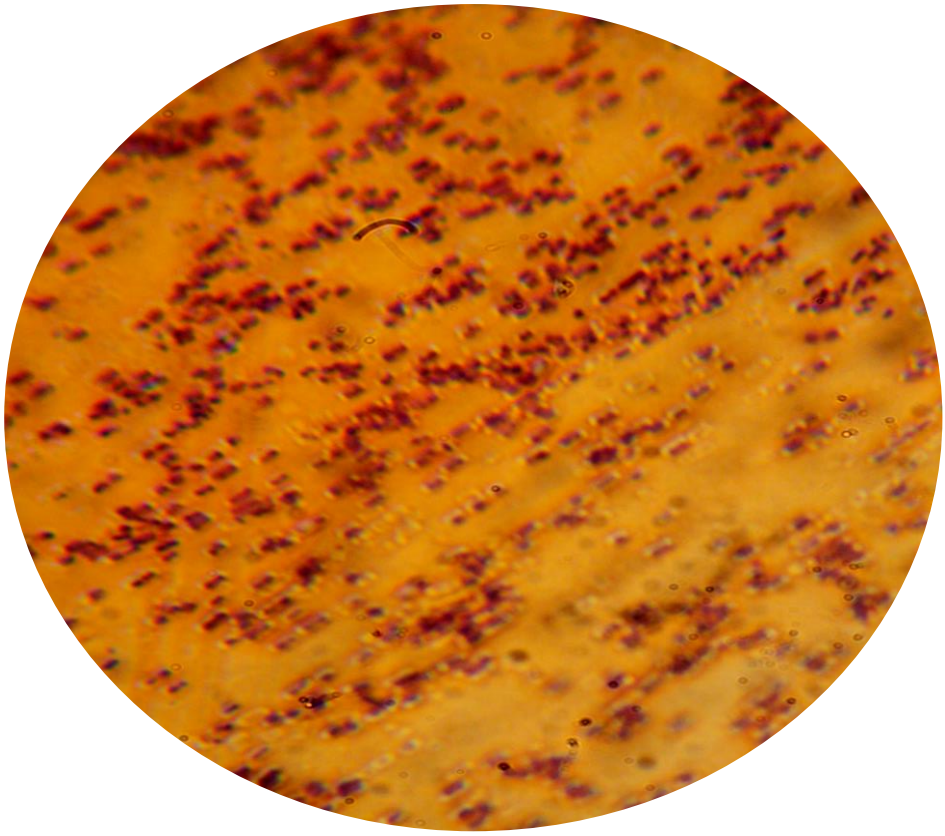
APPENDIX F

MICROSCOPIC VIEW OF THE TEST ORGANISMS

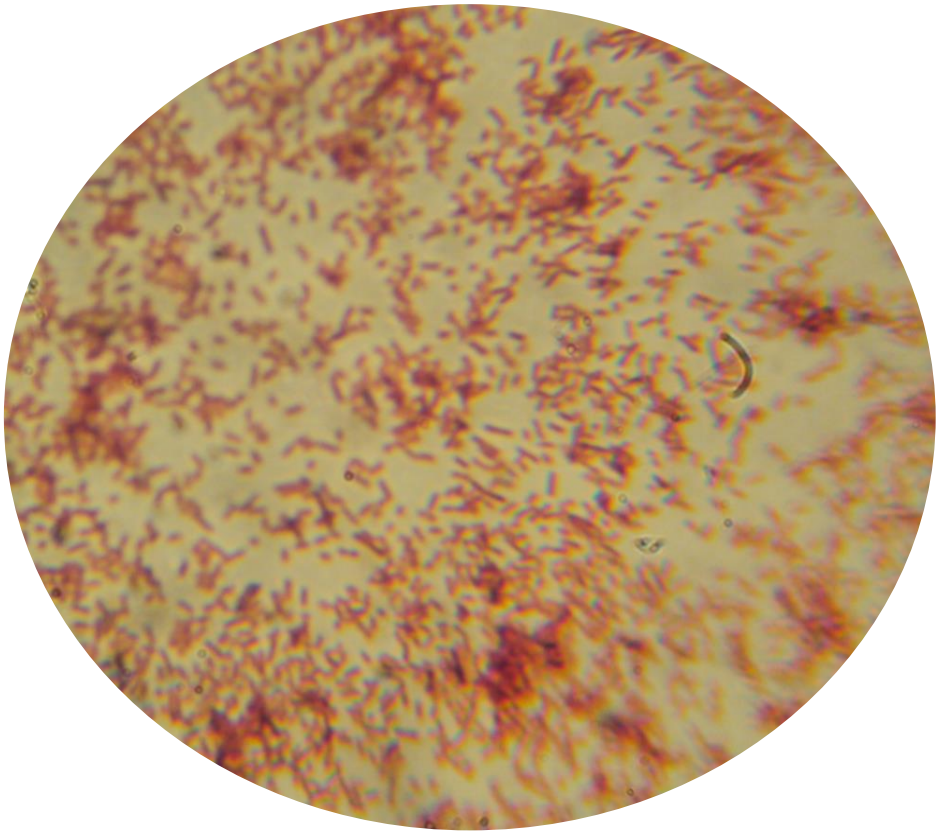
Appendix F1: Microscopic view of *Micrococcus kristinea* MPE12



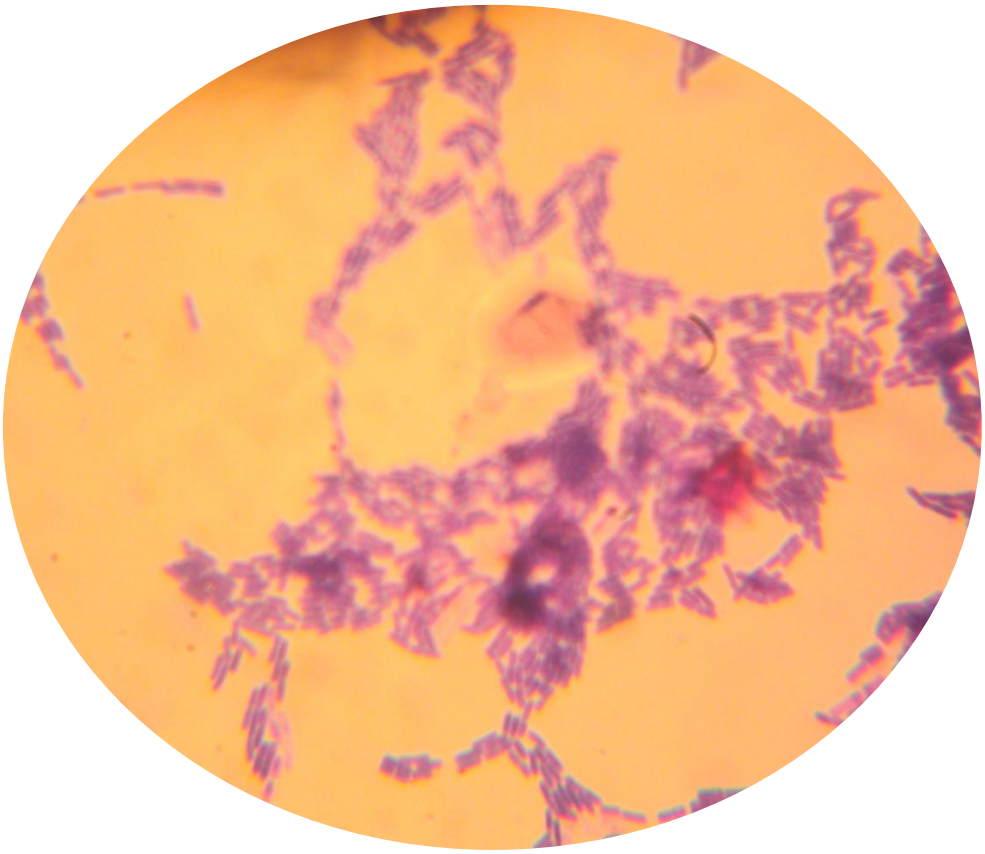
Appendix F2: Microscopic view of *pseudomonas paucimobilis* MPE17



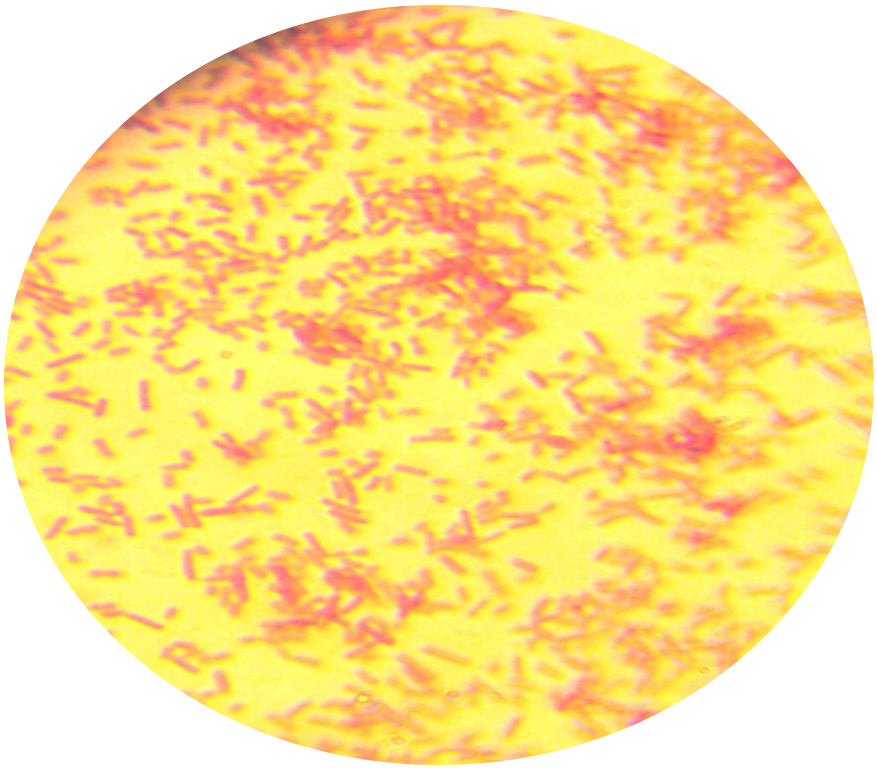
Appendix F3: Microscopic view of *Acinetobacter iwoffii* MPE25



Appendix F4: Microscopic view of *Bacillus firmus* MPE30



Appendix F5: Microscopic view of *Pseudomonas aeruginosa* MPE40

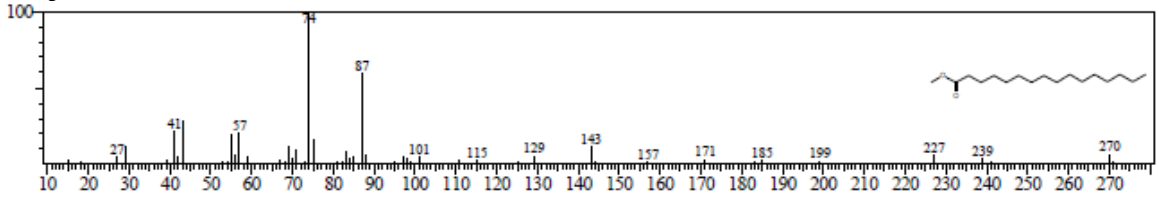


Appendix G

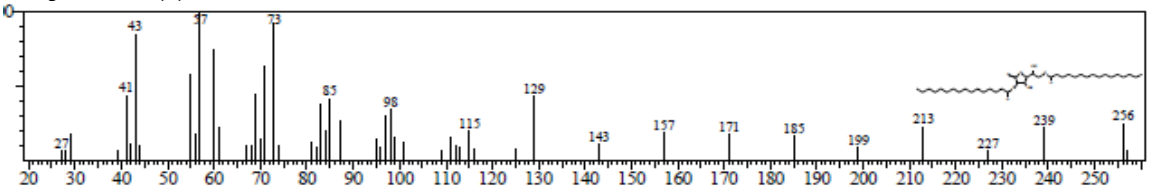
SPECTRUM CAMPARISON

Appendix G: Mass spectrum of the peaks in the GC profile identified

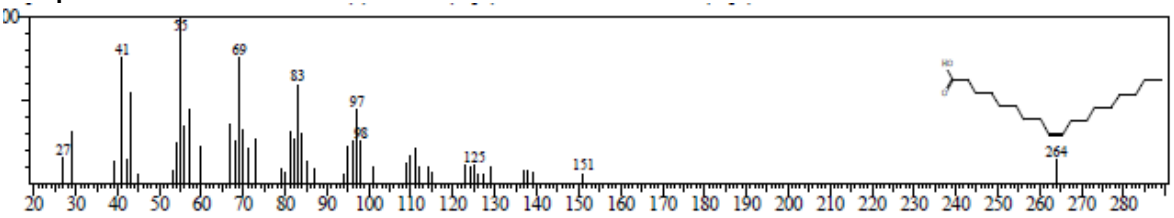
CompName: Palmitic acid



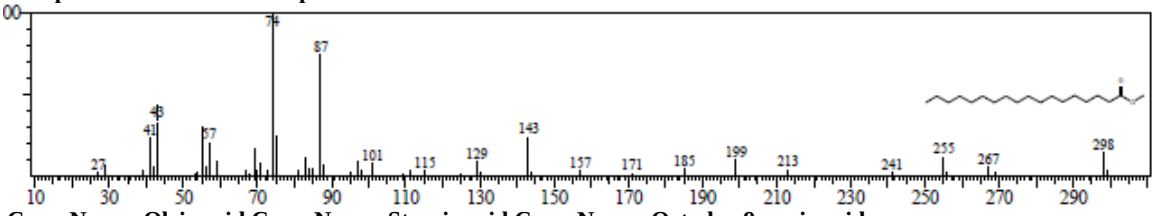
CompName: 1-(+)-ascorbic acid 2,6-dihexadecanoate



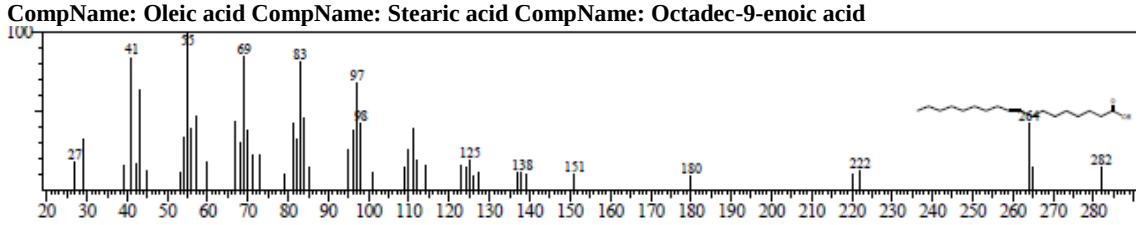
CompName: Oleic acid



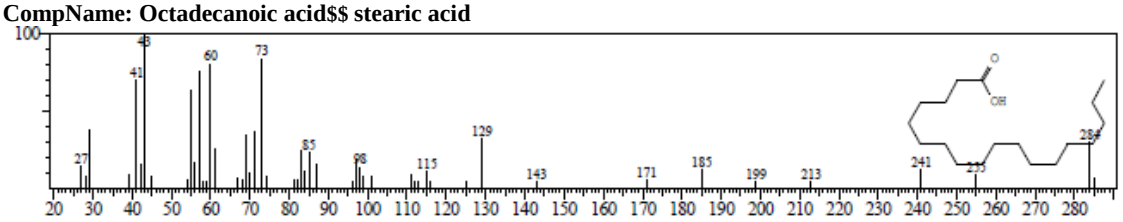
CompName: Oleic acid CompName: Stearic acid



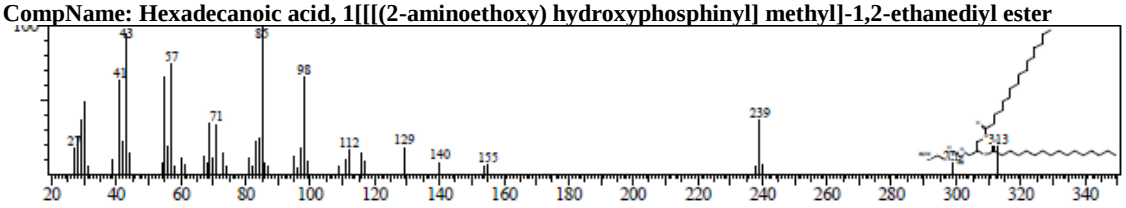
CompName: Oleic acid CompName: Stearic acid CompName: Octadec-9-enoic acid



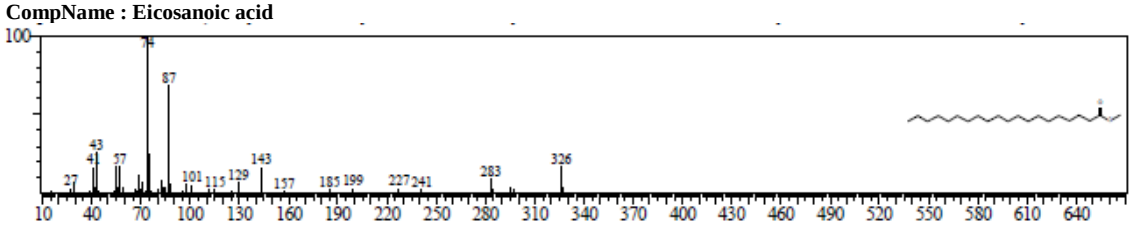
CompName: Octadecanoic acid$$ stearic acid



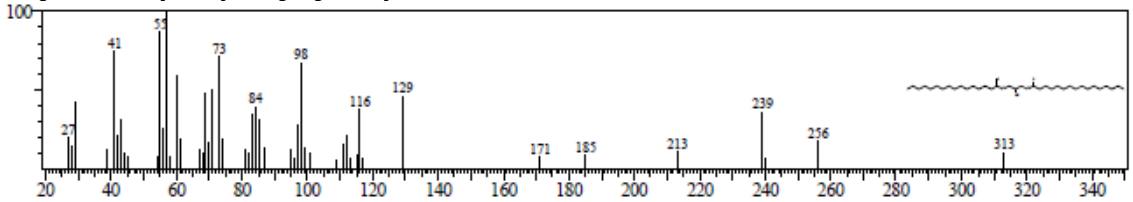
CompName: Hexadecanoic acid, 1[[[(2-aminoethoxy) hydroxyphosphinyl] methyl]-1,2-ethanediyl ester



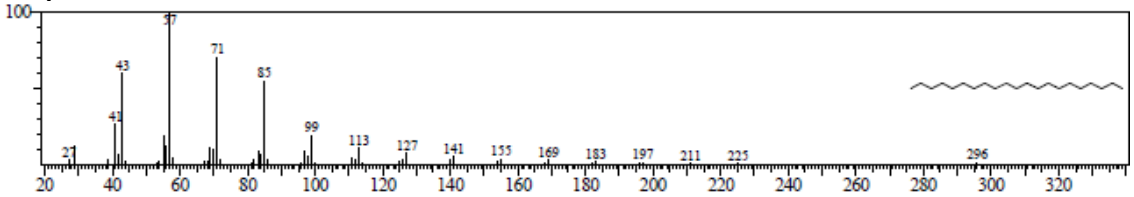
CompName : Eicosanoic acid



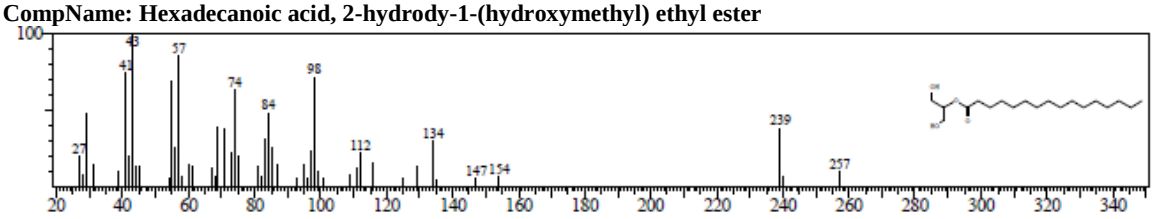
CompName: 2-hydroxy-1,3-propanediyl ester



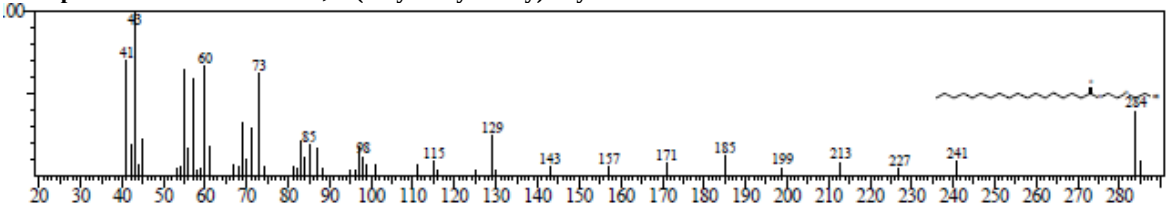
CompName:Heneicosane



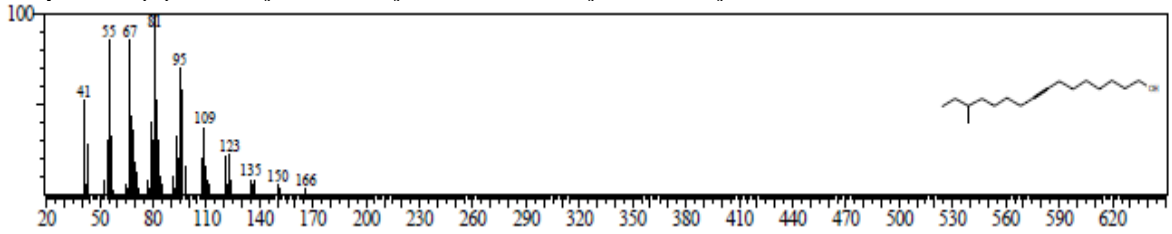
CompName: Hexadecanoic acid, 2-hydrody-1-(hydroxymethyl) ethyl ester



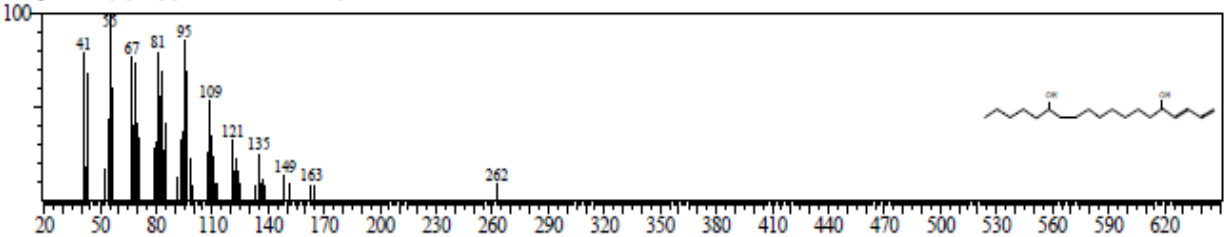
CompName:octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester



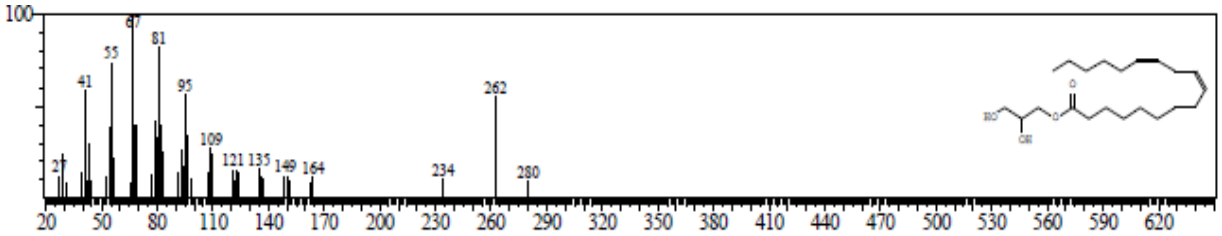
Comp Name: (R)-14-methyl-8-hexadecyn-1-ol $$ 14-methyl-8-hexadecyn-1-ol



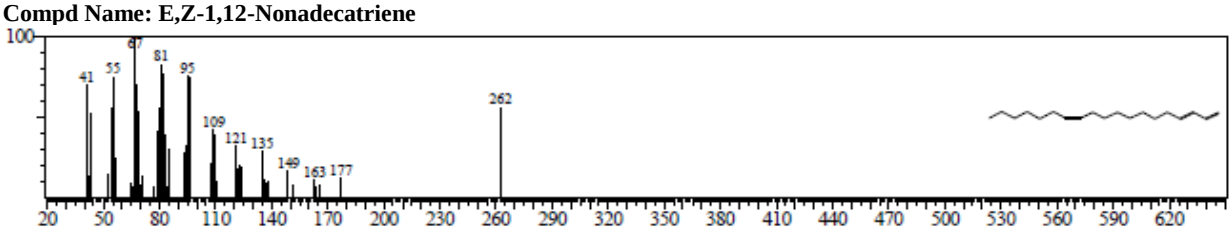
Comp Name: E, E, Z-1, 3 ,12-Nanodecatriene-5,14-diol



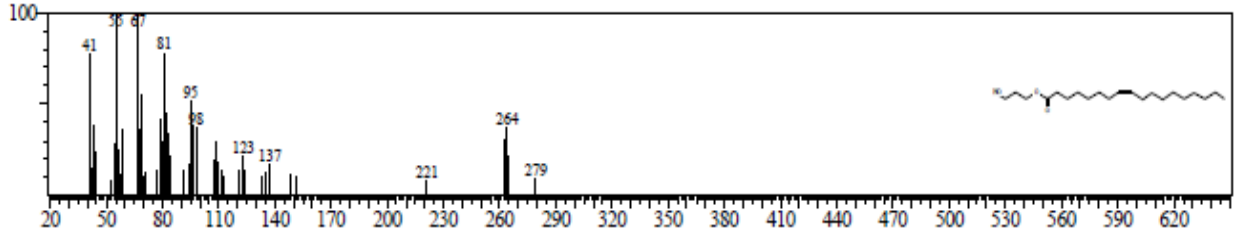
Compd Name: 9, 12-octadecadienoic (Z,Z)-2,3-dihydroxypropyl eater,$$ linolein



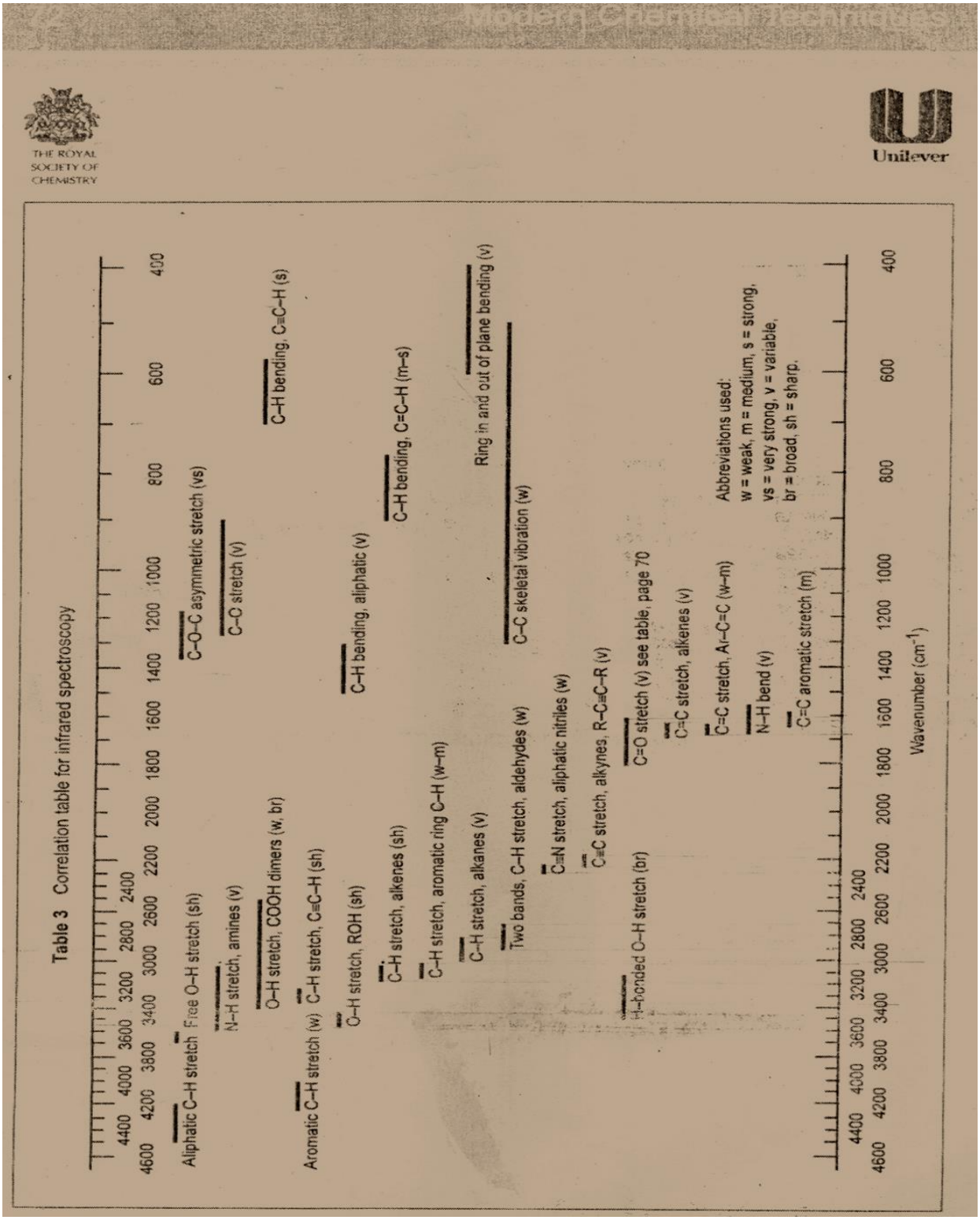
Compd Name: E,Z-1,12-Nonadecatriene



Comp Name: Propyleneglycol monoleate



Appendix H: Standard Correlation Table



APPENDIX I

Appendix I: Designation of the biosurfactants produced

Microbial origin	Group of biosurfactant	Name assigned
Pseudomonas aeruginosae MPE40	Glycolipid	RhamnolipidBios-40
Pseudomonas paucimobilis MPE17	Glycolipid/Lipopeptide	Glycolipopeptide Bios-17
Bacillus firmus MPE30	Glycolipid	FirmnolipidBios-30
Acinectobacter iwoffii MPE25	Glycolipid	Sulfoglycolipid Bios-25
Micrococcus kristinae MPE12	Glycolipid	DisulfoglycolipidBios-12

APPENDIX J

Appendix J_1: Correlation between concentration and pH for lead

Correlations

Lead		ConcentrationO	pHO	concentration14	pH14	concentration28	pH28
ConcentrationO	Pearson Correlation	1	.213	.372	.550	.824	-.062
	Sig. (2-tailed)		.731	.538	.337	.086	.921
	N	5	5	5	5	5	5
pHO	Pearson Correlation	.213	1	-.364	-.379	-.217	.088
	Sig. (2-tailed)	.731		.547	.529	.726	.888
	N	5	5	5	5	5	5
Concentration14	Pearson Correlation	.372	-.364	1	.056	.262	-.741
	Sig. (2-tailed)	.538	.547		.929	.670	.152
	N	5	5	5	5	5	5
pH14	Pearson Correlation	.550	-.379	.056	1	.922*	.558
	Sig. (2-tailed)	.337	.529	.929		.026	.328
	N	5	5	5	5	5	5
Concentration28	Pearson Correlation	.824	-.217	.262	.922*	1	.295
	Sig. (2-tailed)	.086	.726	.670	.026		.629
	N	5	5	5	5	5	5
pH28	Pearson Correlation	-.062	.088	-.741	.558	.295	1
	Sig. (2-tailed)	.921	.888	.152	.328	.629	
	N	5	5	5	5	5	5

*. Correlation is significant at the 0.05 level (2-tailed). Key: Numbers O, 14, 28 are (Time) days

Appendix J_2: Correlation between concentration and pH for chromium

Correlations

Chromium		concO	pHO	conc14	pH14	conc28	pH28
concentrationO	Pearson Correlation	1	.816	.760	.468	.903*	-.850
	Sig. (2-tailed)		.092	.136	.427	.036	.068
	N	5	5	5	5	5	5
pH0	Pearson Correlation	.816	1	.274	.019	.603	-.512
	Sig. (2-tailed)	.092		.656	.976	.282	.378
	N	5	5	5	5	5	5
Concentration14	Pearson Correlation	.760	.274	1	.857	.799	-.736
	Sig. (2-tailed)	.136	.656		.063	.105	.157
	N	5	5	5	5	5	5
pH14	Pearson Correlation	.468	.019	.857	1	.642	-.447
	Sig. (2-tailed)	.427	.976	.063		.243	.451
	N	5	5	5	5	5	5
Concentration28	Pearson Correlation	.903*	.603	.799	.642	1	-.931*
	Sig. (2-tailed)	.036	.282	.105	.243		.021
	N	5	5	5	5	5	5
pH28	Pearson Correlation	-.850	-.512	-.736	-.447	-.931*	1
	Sig. (2-tailed)	.068	.378	.157	.451	.021	
	N	5	5	5	5	5	5

*. Correlation is significant at the 0.05 level (2-tailed).

Key: Numbers O, 14, 28 are (Time) days; Conc= concentration