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BIOSURFACTANT ASSISTED BIOREMEDIATION OF PETROLEUM AND POLYCYCLIC AROMATIC HYDROCARBONS IN AQUATIC AND SOIL MEDIA

Submitted By

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ABSTRACT

Title: **Biosurfactant Assisted Bioremediation of Petroleum and Polycyclic Aromatic Hydrocarbons in Aquatic and Soil Media**

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Petroleum hydrocarbons are organic pollutants of major concern due to their wide distribution, persistence, complex composition, and toxicity. They can bioaccumulate in food chains where they disrupt biochemical or physiological activities and can affect genetic integrity of many organisms, resulting in carcinogenesis, mutagenesis and impairment of reproductive capacity. Polycyclic aromatic hydrocarbons (PAHs) have been recognized as priority pollutants due to their carcinogenic, mutagenic and teratogenic properties. Bioremediation, which utilizes the metabolic versatility of microorganisms such as bacteria and fungi to degrade or detoxify hazardous wastes into harmless substances has been recognized as a sustainable, economic, environmentally friendly and versatile alternative for the remediation of many contaminated environments; however its effectiveness is limited by low bioavailability of nonaquous phase and soil-bound PAHs and petroleum hydrocarbons due to their low aqueous solubility, high hydrophobicity and strong sorption to soil. The purpose of this study was to investigate the PAHs and petroleum hydrocarbons bioavailability and subsequent biodegradation enhancement potential of biosurfactants.

Biosurfactants have steadily gained increased significance in environmental applications such as bioremediation dueto several advantages over surfactants of chemical origin, such as biodegradability, environmental compatibility, low toxicity, high selectivity and specific activity at extreme temperature, pH and salinity. A series of experiments was designed to investigate the bioavailability and subsequent biodegradation enhancement potential of the biosurfactants produced by the bacterial strains *Bacillus subtilis* CN2, *Ochrobactrum intermedium* CN3, *Paenibacillus dendritiformis* CN5 and *Bacillus cereus* SPL_4 in liquid

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culture and soil microcosms with PAH-enriched microbial consortium from chronically contaminated sites. The biosurfactants exhibited a high level of thermal stability, tolerance to extreme levels of salinity and a positive effect for increasing pH. They were identified after Fourier Transform Infrared (FT-IR) spectrometry, Thin Layer Chromatography (TLC) and Liquid Chromatography/Tandem Mass Spectrometry (LC–MS/MS) analyses. The biosurfactants physicochemical characterization displayed vast structural diversity and potent surface active properties of surface tension reduction and emulsion formation with a range of hydrocarbons. The lipopeptide biosurfactants produced by CN3 and CN2 enhanced degradations of used motor oil and petroleum sludge in liquid culture. In a shake flask pyrene degradation study, lipopeptide supplementations at 600 and 300 mg L^{-1} enhanced pyrene degradation to 83.5% and 67% respectively in 24 days compared to 16% degradation in its absence. However, degradation of pyrene was reduced to 57% as the lipopeptide supplementation was raised to 900 mg L^{-1}. This demonstrates that the biodegradation of pyrene was found to increase with an increase in the lipopeptide concentration up to a threshold level.

In a soil bioremedial study, microcosms supplemented with 0.2 and 0.6% (w/w) lipopeptide, 51.2% of 4-ring and 55% of 5- and 6-ring PAHs, 64.1% of 4-ring and 79% of 5- and 6-ring PAHs were removed respectively, compared to, 29% of 4-ring and 25.5% of 5- and 6-ring PAHs removal in the surfactant free control after 64 days of incubation. However, there was no statistically significant change in the degradation rates of low molecular weight PAHs in surfactant amended and surfactant free controls. The degradation of 5 and 6 ring PAHs was significantly enhanced ($p < 0.05$) in the higher surfactant dosage compared to the lower dosage. The results of this work demonstrated that the use of biosurfactants is a viable option to reduce clean-up time and for effective remediation of soil and aqueous media contaminated with polycyclic aromatic and petroleum hydrocarbons. The study demonstrated potential applications of microbial surfactants and provided an insight for further investigation of their large scale production for commercial applications.

Key words, PAH; biosurfactant; bioavailability; lipopeptide; bioremediation; desorption; solubilization; petroleum sludge.

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DECLARATION

I Fisseha Andualem Bezza, declare that the thesis which I hereby submit for a Doctor of Philosophy in Chemical Engineering at the University of Pretoria is my own work and has not been previously submitted by me for any degree at this or other institutions.

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Fisseha Andualem Bezza Date

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LIST OF ABBREVIATIONS AND ACRONYMS

ACN Acetonitrile

Ala Alanine

Asn Aspargine

ATR-FTIR Attenuated Total Reflectance Fourier Transform Infrared

ATSDR Agency for Toxic Substances and Disease Registry

BPDE Benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide

C:N:P Carbon, Nitrogen, phosphorus ratio.

CHN Carbon, Hydrogen and Nitrogen elemental analysis

CMC Critical Micelle Concentration

CMD Critical Micelle Dilution

CSH Cell Surface Hydrophobicity

Cys Cysteine

EDTA Ethylenediaminetetraacetic acid

FRTR Federal Remediation Technology Roundtable

FT-IR Fourier Transforms Infrared Spectroscopy.

Gly Glycine

HLB Hydrophile-Lipophile Balance

HMW High Molecular Weight PAHs

HOCs Hydrophobic Organic Compounds

HPLC High Performance Liquid Chromatography

IARC International Agency for Research on Cancer

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Ile Isoleucine

IPSC International Program on Chemical Safety

K_{oc} . Organic Carbon Normalized Partition Coefficient.

K_{ow}. Octanol-Water Partition Coefficient

LC-MS/MS Liquid chromatography tandem mass spectrometry

Leu Leucine

LMW Low Molecular Weight PAHs

LPS lipopolysachride

MFO Mixed function oxidase

MS Mass spectrum

MS/MS Tandem mass spectrum

MSM Mineral Salt Medium

NAPL None Aqueous Phase Liquid

OD Optical Density

PAH Polycyclic Aromatic Hydrocarbons.

PCBs Polychlorinated Biphenyls

PCP pentachlorophenol

POP Persistent Organic Pollutants.

PTFE Polytetrafluoroethene

RLs. Rhamnolpids

RT Retention Time

SACs Surface Active Compounds

SD Standard Deviation

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SOM Soil Organic Matter

TEFs Toxic Equivalency Factors

TEQ Toxic Equivalent

TLC Thin-Layer Chromatography

TOT Total Organic Carbon

TPH Total Petroleum Hydrocarbon

UPLC Ultra Performance Liquid Chromatography

WHO World Health Organisation

μ Specific Growth Rate

μ_{max} Maximum specific growth rate

$\mu_{D\,max}$ Maximum specific degradation rate

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Bezza, F. A. and Nkhalambayausi-Chirwa, E. M. (2015). Biosurfactant Assisted Bioremediation of polycyclic aromatic hydrocarbons (PAHs) in Liquid Culture System and Substrate Interactivity Factors. The Thirtieth International Conference on Solid Waste Technology and Management, Philadelphia, PA, USA.

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CHAPTER ONE

INTRODUCTION

1.1 Background

Advances in science and technology since the industrial revolution have increasingly enabled mankind to exploit natural resources. Excavating fossil fuels for energy and the advent of agricultural chemicals and pharmaceuticals have facilitated the improvement of the standard of living of millions of people in the world. Unfortunately, many of these inventions have a downside: chemicals needed for such improvements may have adverse health effects and impacts on the environment and humans (Valentín *et al.*, 2013). Relative to the pre-industrialization era, industrialization and intensive use of chemical substances such as petroleum hydrocarbons (e.g.,aliphatic, aromatic, polycyclic aromatic hydrocarbons (PAHs), BTEX (benzene, toluene, ethylbenzene, and xylenes), chlorinated hydrocarbons like polychlorinated biphenyls (PCBs), trichloroethylene (TCE), and perchloroethylene, nitroaromatic compounds, organophosphorus compounds, solvents, pesticides, and heavy metals are contributing to environmental pollution (Megharaj *et al.*, 2011). Some chemical compounds, such as organochlorides and nitroaromatic compounds, are purposefully synthesised, while the production and incineration of some other commodities, such as polyvinyl chloride (PVC) plastic, create undesired toxic by-products. After the chemical products have served their purpose, they often end up in the environment. The final destination of persistent contaminants is often the soil, or if they pass through a water treatment plant, either sewage sludge or sediment at the bottom of rivers, lakes or the sea, where they may accumulate, thereby rendering the environment hazardous to life (Valentín *et al.*, 2013).

PAHs and petroleum hydrocarbons are a major class of such hazardous and persistent organic pollutants released in to the environment, posing serious threat to terrestrial and aquatic ecosystems (Ite *et al.*, 2013). The term PAH generally refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983). PAHs are ubiquitous in the natural environment and are highly recalcitrant molecules due to their

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strong soil sorption capacity, molecular stability, and hydrophobicity (Ortega-Calvo *et al.*, 2013). Increased anthropogenic activities in the past 150 years have caused a significant increase of PAH concentrations in the natural environment (Cerniglia, 1992; Elliot, 2011).

Due to their high hydrophobicity and solid-water distribution ratio, PAHs in soil tend to interact with the non-aqueous phase and organic matter, and consequently become less available for further physicochemical or microbial removal (Johnsen *et al.*, 2005). PAHs are highly resistant to degradation as they are hydrophobic, have a high solid-water distribution ratio, and contain dense π-electron clouds that protect them from nucleophilic attack (Johnsen *et al*., 2005; Belcher, 2012). PAHs have received increased attention in recent years in environmental pollution studies because some of these compounds are highly carcinogenic or mutagenic (Hong *et al.,* 2016.). Eight PAHs typically considered as possible carcinogens are: benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene (B(a)P), dibenzo(a,h)anthracene, indeno(1,2,3-cd)pyrene and benzo(g,h,i) perylene. In particular, benzo(a)pyrene has been identified as being highly carcinogenic. The US Environmental Protection Agency (EPA) has promulgated 16 unsubstituted PAHs (EPA-PAH) as priority pollutants (Srogi, 2007).

Although PAHs originate from natural sources such as open burning, natural losses or seepage of petroleum or coal deposits, and volcanic activities, they are mainly derived from anthropogenic activities related to pyrolysis and incomplete combustion of organic matter (Abdel-Shafy and Mansour, 2015). Anthropogenic sources of PAH include burning of fossil fuel, coal tar, wood, garbage, refuse, used lubricating oil and oil filters, municipal solid waste incineration and petroleum spill and discharge (Haritash and Kaushik, 2009). PAHs are found in complex nonaqueous-phase liquids (NAPLs), such as creosote and coal tar, and soot-like materials, which are generally known as black carbon (Ortega-Calvo *et al.,* 2013). Creosote, with a PAH mass fraction of 85% (Mueller et al., 1989), is an obvious source. Coal tar creosote is the most common wood preservative in the United States. Various kinds of creosote are used for road paving, roofing, coking, and aluminum smelting (ATSDR, 1997). Petroleum is another significant source, it is a complex mixture containing thousands of hydrocarbons including PAHs. It is estimated that the annual global input of petroleum hydrocarbons to the environment is 1.7 – 8.8 million metric tons with anthropogenic sources responsible for the majority of it (Dimitriou-Christidis, 2005). Used lubricating oil contains several toxic components including up to 30% aromatic hydrocarbons,

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with as much as 22 ppm benzo(a)pyrene (Abioye, 2011). Upshall *et al.* (1992) reported that motor oil had a density of 0.828 g/ml and contained 14% aromatics and 65.4% aliphatics (by weight). In their study, the sum of 26 individual PAHs represented 0.17% of the oil, or 1.2% of the aromatic fraction.

Among the compounds of petroleum oil, the most representative low-molecular-weight molecules, such as straight, branched, cyclic alkanes and aromatic hydrocarbons, were shown to be readily degraded by many microorganisms (Zhang *et al.,* 2011). However, long-chain alkanes and polycyclic aromatic hydrocarbons (PAHs) were generally considered to be only slightly biodegradable due to their higher hydrophobicity (Kanaly and Harayama, 2000; Zhang *et al.,* 2011).

Although PAHs released into the environment could be removed through many physicochemical processes, including removal, alteration, volatilization, photo-oxidation, chemical oxidation, and adsorption. Such techniques involve excavation of contaminated soil and its incineration or containment become prohibitively expensive when amounts of contaminants are large, and in many cases, transfer the pollutant from one phase to another. On the other hand, bioremediation is recognized as an efficient, economic and versatile alternative with less input of chemicals and energy (Haritash and Kaushik, 2009).

Bioremediation is an option that offers the possibility to destroy the pollutants or transform them to innocuous substances (Niti *et al.,* 2013). Bioremediation is an ecologically sound and state-of-the-art technique that employs natural biological processes to completely eliminate toxic contaminants through biochemical transformation or mineralization. It may be any process that uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition (Chakraborty *et al.,* 2012; Mani and Kumar, 2014). As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and environmental soundness (Kumar *et al.,* 2011).

Biodegradation by natural populations of microorganisms represents one of the primary mechanisms by which petroleum and other hydrocarbon pollutants can be removed from the environment (Leahy and Colwell, 1990). The rate of biodegradation of hydrocarbons is dependent upon several physicochemical, as well as biological parameters. One of the important factors that

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limit biodegradation of hydrophobic pollutants in the environment is their limited availability to microorganisms. Due to their high hydrophobicity and solid-water distribution ratio, hydrocarbons in soil tend to interact with the non-aqueous phase and organic matter, and consequently become less available for further microbial degradation (Johnsen *et al.*, 2005).

Several approaches have been investigated to enhance bioavailability of contaminants in the environment. These include use of surfactants or cosolvents to enhance contaminant solubility and desorption as well as thermal treatment which is used to enhance desorption. To improve desorption efficiency of PAHs from soils as well as the mobility and bioavailability of PAHs in the aqueous phase, various solubility enhancement agents have been used, such as co-solvents and surfactants (Saichek and Reddy, 2004; Gómez *et al.*, 2010; Morillo *et al* ., 2014) but thermal and organic co-solvents can harm resident microbial population and arenot economically feasible.

Surfactant-enhanced bioremediation has been suggested as a promising technology for the remediation of hydrophobic organic compounds contaminated soil and groundwater (Zhang *et al.*, 2013). Surfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds (Singh *et al.*, 2007). At low concentrations, surfactants are soluble in water, and with increasing concentrations, they form micelle in solution. The concentration at which micelle begins to form is called the critical micelle concentration (CMC); after formation of micelles, solid PAHs may partition into the interior of the micelle, which increases the hydrocarbon's apparent solubility but some surfactants may increase the water solubility of hydrocarbon molecules below the CMC (Bordoloi and Konwar, 2009). Besides solubilization of hydrocarbons surfactants have also been reported to alter cell surface hydrophobicity of microorganisms, this interaction with microbial cell surface may have both stimulatory and inhibitory effects on the biodegradation of hydrocarbons based on the mechanism of hydrocarbon uptake for a specific microorganism (Bak *et al.*, 2015).

In recent years, much attention has been directed towards biosurfactants owing to their numerous advantages compared to chemical surfactants such as lower toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feed stocks (Safary

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et al., 2010; Chaprão *et al.,* 2015). Biosurfactants are structurally diverse and can have various chemical compositions mainly consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins depending on the producing microorganism, raw matter and process conditions (Makkar *et al.,* 2011)

1.2 Statement of the Problem

Widespread use, improper disposal, accidental spills and leaks of organic hydrocarbons like petroleum hydrocarbons, organic solvents, and creosote have resulted in long-term persistent sources of contamination of soil and groundwater, which becomes a major environmental issue because of their adverse effect on human health (Paria, 2008). Polycyclic aromatic hydrocarbons are recalcitrant compounds which present one of the most pressing problems for biotreatment of contaminated soils or sediments, these compounds comprise abroad class of persistent contaminants in soils and sediments including petroleum, tars and creosotes (NRC, 1997). Subsurface contamination by non-aqueous phase liquids (NAPLs) including coal tar and creosotes (complex hydrocarbon mixtures consisting of polycyclic aromatic hydrocarbons and other aromatic hydrocarbons), polychlorinated biphenyl (PCBs), and certain pesticides is a complex process and difficult to treat due to many reasons like the tendency of adsorption of contaminants onto the soil matrix, low water solubility and limited rate of mass transfer for remediation (Kavanaugh *et al.,* 2003; Paria, 2008). Bioremediation has shown promise as a potentially effective and low-cost treatment option, however, bioremediation of PAH and the other petroleum hydrocarbons in the environment is limited by low bioavailability to microbes because of their hydrophobicity, low aqueous solubility and their strong adsorption to the soil material (Makkar and Rockne, 2003). Microbial degradation of PAHs and other hydrophobic substrates is limited by the amounts dissolved in the water phase (Bosma *et al.,* 1997), with sorbed, crystalline, and non-aqueous phase liquids (NAPL)-dissolved PAHs being unavailable to PAH-degrading organisms. Bioavailability is considered a dynamic process, determined by the rate of substrate mass-transfer to microbial cells relative to their intrinsic catabolic activity (Bosma *et al.,* 1997; Johnsen *et al.,* 2005). PAH hydrophobicity results in these compounds being strongly sorbed onto soil particles, especially clays and soil organic matter and desorption of them from soil is considered to be a controlling factor in their biodegradation (Johnsen *et al.,* 2005; Wick *et al.,* 2011).

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1.3 Scope of the Study

In this study a series of biosurfacatant enhanced biodegradation experiments were conducted. This study is composed of three major parts:

- Isolation and characterization of PAH-degrading and biosurfactant producing bacterial strains and laboratory scale biosurfactant production, characterization and optimization.
- Conducting biosurfactant assisted mass transfer and biodegradation assays in liquid culture and soil microcosms.
- Develop an effective bioremediation strategy through *exogenous* biosurfactant addition.
- Stimulation of *in situ* biosurfactant production for enhanced decontamination of PAH and petroleum hydrocarbon contaminated soils and aqueous media by bioaugmentation of efficient hydrocarbon degraders enriched from chronically contaminated niche.

1.4 Research Hypothesis

In the current research, it is hypothesized that microbially produced biosurfactants will: (a) enhance pseudosolubilization, desorption and mobilization of PAHs and other petroluem hydrocarbons and (b) and subsequently enhance the biodegradation of the recalcitrant PAHs and petroleum hydrocarbons.

1.5 The Objective of the Present Research

1.5.1 General Objectives;

Evaluate the potential of biosurfactants produced by isolated strains in enhancing the bioavailability and subsequent biodegradation of the hydrophobic petroleum and polycyclic aromatic and petroluem hydrocarbons.

Develop an effective bioremediation strategy through *exogenous* biosurfactant addition and stimulation of *in situ* biosurfactant production for enhanced bioremediation of PAH contaminated soils and aqueous media by bioaugmentation of efficient hydrocarbon degraders enriched from chronically contaminated niche.

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1.5.2 Specific Objectives

- Isolate and characterize microbial strains that grow on coal tar creosote and model PAHs (Phenanthrene, Pyrene and Anthracene) and produce biosurfactants. Identification of these strains by analysis of the sequence of the gene encoding 16S rDNA.
- Laboratory scale biourfactant production and stimulation of over production through cosubstrate addition or growth limitation.
- Recovery and physicochemical characterization and stability study of the biosurfactants produced
- Perform biosurfactant enhanced desorption and solubilization mass trasfer studies of adsorped and crystalline PAHs.
- Perform laboratory microcosm tests to investigate the feasibility of using the biosurfactants produced from isolated microbial strains to enhance the bioremediation of petroleum sludge, used engine oil and PAH contaminated soil and water media.
- Determine the optimum concentration of the biosurfactants required for the bioremediation of PAHs in liquid culture contaminated soils. This includes the determination of appropriate microbial kinetic models to determine growth and substrate utilization parameters.
- Enrichment and 454-pyrosequencing characterization of efficient creosote PAH degraders from creosote contaminated soil.

1.6 Thesis Organization

This dissertation is a compilation of five manuscripts. The manuscripts (Chapters Four, Five, Six and Seven; Papers 1, 2, 3, 4 and 5) have been published in peer-reviewed international journals.

Chapter 1. The first chapter contains the introduction, the statement of the problem, and the objectives of this research, the scope of the research and the thesis organization or layout.

Chapter 2. The Literature Review, reviews previous work and developments in the area of biosurfactant assisted PAH and petroleum sludge bioremediation, including the type of microorganisms and biosurfactants used to treat PAH-contaminated sites, the attempts to improve bioavailability and biodegrdation of PAHs and optimization of biosurfactant production strategies for *in situ* and *ex situ* applications and an overview of the kinetic models as applied in the prediction of microbial growth and biodegradation of the PAHs.

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Chapter 3. Materials and Methods, it includes details of the chemicals and reagents used ,isolation and enrichment of microorganisms for biosurfactnat production and inoculation , the preparation of cell culture and soils used for the experiment, the experimental setup and procedures, the analytical methods and instruments used for sample quantification.

Chapters 4, 5, 6 and 7. Results and Discussion, present the experimental results, data analysis and discussion. Finally,

Chapter 8. Conclusions and Future Prospects; the conclusions from this work and recommendations for future studies are presented

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CHAPTER TWO

LITERATURE REVIEW

2.1 Background

Petroleum is the most important and predominant energy resource and raw material of chemical industry in modern society. However, spills, leaks, and other releases of petroleum often result in the contamination of soil and groundwater, especially when associated with accidental spills on a large scale (Xia *et al.,* 2014). Petroleum processing generates considerable amounts of a residue named oily sludge. One of the main issues faced by refineries and petrochemical industries is related to the safe disposal of this residue, since its destination and/or inappropriate treatment can cause serious impact to the environment and potential risk to human health (Cerqueira *et al.,* 2014). Oily sludges can bioaccumulate in food chains where they disrupt biochemical or physiological activities of many organisms, thus causing carcinogenesis of some organs, mutagenesis in the genetic material, impairment in reproductive capacity (Short and Heintz, 1997; Onwurah *et al.,* 2007). Oil sludge has been classified by the United States Environmental Protection Agency (US EPA) as a hazardous organic complex (US EPA 1994; Ubani *et al.,* 2013). The main use of oil is as an energy source in traffic, heating and electricity production, as approximately 34% of the global energy demand is met by oil products (Vicent *et al.,* 2013). Crude oil, generally referred to as petroleum, is a complex mixture of thousands of different chemical components, mainly hydrocarbons, which usually make up about 95 per cent of the crude oil. These hydrocarbons vary in toxicity and degradability, and range from very volatile, light materials like propane and benzene, to heavy compounds such as bitumens, asphaltenes, resins and waxes (http://oils.gpa.unep.org/facts/oil-what.htm). Crude oil is perhaps globally the most exploited non-renewable natural resource. Accordingly, substances deriving from crude oil are the most common polluters of the environment (Valentın *et al.,* 2013).

The petroleum hydrocarbons (PHCs) and other organic compounds in oily sludge can be generally classified into four fractions, including aliphatics, aromatics, polars or resins and asphaltenes (Hu *et al.,* 2013). The aliphatics and aromatic hydrocarbons usually account for up to 75% of PHCs in oily sludge and commonly oily sludge is composed of 40–52% alkanes,

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28–31% aromatics, 8–10% asphaltenes, and 7–22.4% resins by mass (Hu *et al.*, 2013). Crude oils contain 0.2 to 7% PAHs, with configurations ranging from two to six rings. Asphaltenes constituted 5% to 20% of crude oil with multiple PAH units containing 2–5 or more rings inside asphaltene molecules and some other heteroatom-containing (N, S and O) aromatic compounds (Xia *et al.*, 2014). Thus, alkanes and PAHs could be treated as typical and predominant fraction of crude oil (Xia *et al.*, 2014).

Among hydrocarbons, polycyclic aromatic hydrocarbons (PAHs) are a widespread class of environmental pollutants that are carcinogenic and mutagenic. They arise from the incomplete combustion of organic material, especially fossil fuels, from the discharge of petroleum and its products and from the post-depositional transformation of biogenic precursors (Demircioğlu, 2011). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants present in the vast majority of contaminated areas. They are recognized worldwide as priority pollutants in soils, sediments and waters because of their toxicity and carcinogenicity (Ortega-Calvo *et al.*, 2013). Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic compounds consisting of two or more combined benzene rings in linear, angular or cluster arrangements (Sims and Overcash, 1983). The PAHs most commonly encountered in the environment contain two (naphthalene) to seven (coronene) fused benzene rings, though PAHs with greater number of rings are also found (Singh and Ward, 2004). Like all hydrocarbons, PAHs contain only hydrogen and carbon. However, closely related compounds called heterocyclic aromatics, or polycyclic aromatic compounds, in which an atom of nitrogen, oxygen, or sulphur replaces one of the carbon atoms in a ring, are commonly found with PAHs from most sources (Jazestani, 2011).

PAHs contaminate the environment via many routes, including the burning of fossil fuels, the manufacture of gas and coal tar, wood processing, escaped automobile gasoline and the incineration of waste (Harvey, 1991; Peng *et al.*, 2008). Several industrial processes, such as coal coking; carbonization of coal and oil to produce manufactured gas, coal tars, carbon black, and pitch; catalytic cracking of petroleum feed stocks to produce refined petroleum products; manufacture of iron and steel; and aluminum smelting produce airborne particulates and solid wastes containing high concentrations of PAH (Neff *et al.*, 2005). Oil leakage from storage tank bottoms, oil-water separators, and drilling operations etc.has led to an increase in soil PAH concentration over the last few decades PAHs are also a major constituent of, Creosote (approximately 85% PAH by weight) and anthracene oil, which are commonly used pesticides for wood treatmen (Koul and Fulekar, 2013).

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Polycyclic aromatic hydrocarbons (PAHs) are one of the most important classes of environmental pollutants. Due to the persistent, toxic, mutagenic and carcinogenic characteristics of PAHs, 16 of them are on the US EPA list of priority pollutants (Liu *et al.*, 2009). PAHs are ubiquitously distributed in the environment, and have been detected in number of environmental samples, including air, water, soil, sediments, and also in foods, oils, and tars (Mahanty *et al.*, 2011). Point sources of PAH contamination are the most significant environmental concern. Though the areas contaminated are relatively small in size, the contaminant concentration at these sites is often high and associated with co-contaminants such as benzene, toluene, ethylene and xylene (BTEX) compounds, heavy metals and aliphatic hydrocarbons, which can hinder remediation efforts (Bamforth and Singleton, 2005).

PAHs originate from two main sources: these are natural (biogenic and geochemical) and anthropogenic. It is the anthropogenic source of PAHs that is the major cause of environmental pollution and hence the focus of many bioremediation programmes (Bamforth and Singleton, 2005). The widespread occurrence of PAHs is due to their production by virtually all types of combustion of organic materials. The anthropogenic sources of PAHs and their derivatives are diverse and include: incomplete burning of fuels, garbage, or other organic substances such as tobacco and plant material. Likewise, forest fires and volcanic eruptions can also contribute to the natural budget of the PAH inventory (Kim *et al.*, 2013).

PAHs are highly persistent in the environment due to their high hydrophobicity or low water solubility (Cerniglia, 1992). Generally, with an increase in number of fused rings in PAHs while solubility and volatility decreases, hydrophobicity of the compound increases (Wilson and Jones, 1993). There are more than 100 diverse PAH compounds, and most of them persist in the ecosystem for many years owing to their low aqueous solubility and their high adsorption to solid particles (Volkering *et al.*, 1992, 1993; Peng *et al.*, 2008).

2.1.1 Sources of PAHs

Anthropogenic sources include petrogenic and pyrolytic PAH contaminations. Pyrolytic sources include incomplete combustion of organic matter such as fossil fuels and biomass whereas the petrogenic PAHs are formed by petroleum products (Kafilzadeh, 2015). Crude and refined petroleum contain PAHs (i.e., petrogenic PAHs), are introduced to aquatic environments through accidental oil spills, discharge from routine tanker operations, municipal and urban runoff, and so on. The combustion of fossil fuels (coal and petroleum) and biomass also produces PAHs (i.e., pyrogenic PAHs), which are released into the environment in the

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form of exhaust and solid residues (Zakaria *et al.*, 2002). PAHs with four to six rings are generally formed through incomplete combustion of recent and fossil organic matter at high temperature during anthropogenic activities, such as burning of fossil-fuels, vehicular emissions, combustion processes of solid incineration plant and domestic heating, in addition during natural processes, such as forest and prairie fires (Bertrand *et al.*, 2015). These pyrogenic PAHs can be dispersed over long distances in the atmosphere and then deposited by wet and dry deposition (Cranwell and Koul, 1989). In contrast, significant sources of PAHs with two or three rings derived from petrogenic contamination are offshore petroleum hydrocarbon production and/or petroleum exportation (Bertrand *et al.*, 2015).

2.1.2 Occurrence of PAHs in the Environment

PAHs in the atmosphere

PAHs in the ambient air exist in vapor phase or adsorb into air borne particulate matter depending on the atmospheric conditions (ambient temperature, relative humidity, etc.), the nature (i.e., origin and properties) of the aerosol, and the properties of the individual PAH (Kim *et al.*, 2013). Low-molecular-weight PAHs occur in the atmosphere predominantly in the vapour phase, whereas high molecular weight PAHs (five or more rings) are largely bound to particles. Intermediate-molecular-weight PAHs (four rings) are partitioned between the vapour and particulate phases, depending on the atmospheric temperature (WHO, 2010). The majority of compounds, and especially those with four rings, are considered as semi-volatile and such compounds partition between the vapour and particle phases in the atmosphere. These compounds can deposit to surface water and soils where they have a long lifetime but subsequently re-evaporate to the atmosphere (Keyte *et al.*, 2013). In the atmosphere, PAHs may be subject to direct photolysis, although adsorption to particulates can retard this process. PAHs can also react with pollutants such as ozone, hydroxyl radicals, nitrogen dioxide and sulfur dioxide, yielding diones, nitro- and dinitro-PAHs, and sulfonic acids, whose toxicity may be more significant (WHO, 2010; Kim *et al.*, 2013). These indicate that PAHs are considered to be a significant hazardous substance to human health through breathing. In view of this health concern, monitoring the level of particle-bound PAHs in urban areas has become more important (Chetwittayachan *et al.*, 2002).

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PAHs in Soils

Among the different environments, soil is an important reservoir for PAHs, and gaseous and particulate PAHs are input to the soil by wet/dry atmospheric deposition at over short and long distances (Park *et al.,* 2001; Mahanty *et al.,* 2011). Accumulation of PAHs in soils may lead to further potential contamination of vegetables and food chains, and then cause direct or indirect exposure to humans (Srogi, 2007). PAHs deposited to terrestrial and aquatic biosystems are associated with particulate matter, soil sediment and other oily substances owing to their low aqueous solubility, elevated octanol–water and organic carbon coefficients as well and high melting and boiling points (WHO, 2010; Kim *et al.,* 2013). PAHs enter soil mainly via atmospheric deposition and other sources of PAHs pollution to soil are disposal of waste materials, creosote use, road runoffs and car tire shredding, accidental fuel spills and leakages as well as industrial wastewaters, sewage sludge and compost applied to agricultural land (Sims and Overcash, 1983; Maliszewska-Kordybach - 2005). As major reservoirs of PAHs soil is considered as a good indicator of environmental pollution and environmental risk for human exposure to PAHs (Kumar *et al.,* 2015). Soils can be contaminated with between 1 µg kg^{-1} and 300 g kg^{-1} PAHs, depending on the source of contamination (eg. old coal gasification sites have the higher levels stated) (Bamforth and Singleton, 2005).

PAHs in sediments

PAHs reach the hydrosphere mainly by dry and wet deposition and road runoff but additionally from industrial wastes containing PAHs and leaching from creosote-impregnated wood (WHO, 2003). Due to their low solubility in water and high affinity for particulate matter, PAHs are not usually found in water in high concentrations. The concentrations of total PAHs in water vary between 2.5 - 9.0 ng/L in tap water, 1.0 - 10 ng/L in groundwater, 2.7-7.3 ng/L rain water and about 10 - 830 ng/L in surface water. The European Union, EEC Directive 98/83/EC has set a limit of 0.1 ng/L for total PAHs in drinking water (Nkansah et al., 2012.)

2.1.3 Physicochemical Properties of PAHs.

The chemical properties, and hence the environmental fate, of a PAH molecule are dependent in part upon both molecular size, i.e., the number of aromatic rings, and molecule topology or the pattern of ring linkage. The chemical properties and, hence, the environmental fate of a PAH molecule are dependent in part upon both molecular size (the number of aromatic rings)

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and molecule topology, or the pattern of ring linkage. Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability (Kanaly and Harayama, 2000). In addition to increases in environmental persistence with increasing PAH molecule size, evidence suggests that, in some cases, PAH genotoxicity also increases with size, up to at least four or five fused benzene rings (Cerniglia, 1992). High molecular weight (HMW) PAHs are compounds with four or more fused benzene rings, whereas the low molecular weight (LMW) compounds consist of two to three fused benzene rings (Cerniglia, 1992). The high molecular weight (HMW) PAHs are less water-soluble, less volatile and more lipophilic than lower molecular weight (LMW) PAHs (Edokpayi *et al.*, 2016). The diversity of structures represented by the PAH group of compounds can be broadly categorized into alternant and non-alternant classes. Alternant PAHs contain only fused benzenoid rings (e.g. anthracene, phenanthrene and pyrene) whereas non-alternant PAHs contain four, five or six membered rings (e.g. fluorene and fluoranthene) (Harvey, 1997).

The physicochemical properties of selected PAHs are presented in Table 2.1 where it can be seen that volatility and aqueous solubility decrease with increasing molecular weight (Wilson and Jones, 1993). The octanol-water coefficient (K_{ow}) is a measure of hydrophobicity of organic chemicals. The decreasing solubility of PAHs and the increasing hydrophobic sorptive capacity (K_{ow}) as the molecular weight increases , coupled with decreasing volatilities (low Henry's Law constant) and the increasing general chemical stability mean that PAHs are environmentally persistent compounds that are strongly held to solids, both suspended particles and bottom sediment (Prabhukumar and Pagilla, 2010). As shown in Table 2.1 properties such as aqueous solubility and vapour pressure range in five and twelve orders of magnitude, respectively, moving from two to six benzene rings in the PAH-molecule. Thus, low molecular weight (LMW) PAHs are much more water soluble and volatile than their high molecular weight (HMW) relatives, while the HMW PAHs show higher hydrophobicity than the LMW compounds (Mackay *et al.*,1992; Lundstedt, 2003).

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Table 2.1: Properties of the16 US-EPA PAHs, adapted from Mackay *et al.*, 1992

	Ring number	Molecular weight	Aqueous Solubility(mg/L)	Log(K_{ow})	Vapour Pressure(Pa)
Naphthalene	1	128	31	3.37	1.1E+2
Acenaphthylene	2	152	16.1	4.00	0.9
Acenaphthene	2	154	3.8	3.92	0.3
Fluorene	3	166	1.9	4.18	0.09
Phananthrene	3	178	1.1	4.57	0.02
Anthracene	3	178	0.0045	4.54	0.001
Pyrene	4	202	0.13	5.18	0.0004
Fluoranthene	4	202	0.26	5.22	0.00123
Benzo[*a*]anthracene	4	228	0.011	5.91	2.80E-5
Chrysene	4	228	0.006	5.91	5.70E-7
Benzo[*b*]fluoranthene	5	252	0.0015	5.80	-
Benzo[*k*]fluoranthene	5	252	0.0008	6.00	5.20E-08
Benzo[a]pyrene	5	252	0.0038	5.91	7.00E-07
Dibenzo[*a,b*]anthracene	6	278	0.0006	6.75	3.70E-10
Indeno[*1,2,3-cd*]pyrene	6	276	0.00019	6.50	-
Benzo[*ghi]*perylene	6	276	0.00026	6.50	1.40E-8

PAH molecular stability and hydrophobicity are two primary factors which contribute to the persistence of HMW PAHs in the environment (Kanaly and Harayama, 2000). Thus, presence of dense clouds of π electrons on each side of aromatic rings restricts the PAHs to limited nucleophilic attack leading to biochemical stability (Johnsen *et al*., 2005).

2.1.4 Toxicity and Carcinogenicity

The PAHs benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k)fluoranthene, chrysene, benz(a,h)anthracene, and indeno(1,2,3-c,d)pyrene have been classified as proble human carcinogens based on sufficient evidence of carcinogenicity from studies in experimental animals (US EPA, 1984). Long-term studies of workers exposed to mixtures of PAHs and other workplace chemicals have shown an increased risk of skin, lung, bladder and gastrointestinal cancers (Johnson *et al.*, 2009).

PAHs are highly lipid soluble and thus readily absorbed from the gastrointestinal tract of mammals (Cerniglia, 1984). They are rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat. The PAHs require metabolic activation and conversion to display their genotoxic and carcinogenic properties, Pickering (1999). This happens as the PAHs are metabolized in higher organisms. PAHs do not accumulate in the same manner as some other lipophilic organic compounds, *e.g.* PCBs (Lundstedt, 2003).

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Instead, they are converted to more water-soluble forms, which facilitate their subsequent excretion from the organism. Subsequent metabolism renders them more water-soluble making them easier for the body to remove. However, PAHs can also be converted to more toxic or carcinogenic metabolites, reactive intermediates that may react with DNA to form adducts, preventing the gene involved from functioning normally). The enzyme system primarily responsible for PAH metabolism is the cytochrome P450 mixed function oxidase enzymes system, which converts the non-polar PAHs into polar hydroxy and epoxy derivatives (Hall *et al.*, 1989).

The resultant epoxides or phenols might get detoxified in a reaction to produce glucoronides, sulfates or glutathione conjugates. Some of the epoxides might metabolize into dihydrodiols, which in turn, could undergo conjugation to form soluble detoxification products or be oxidized to diol-epoxides. Many PAHs contain a 'bay-region' as well as 'K-region', both of which allow metabolic formation of bay- and K-region epoxides, which are highly reactive (Samanta *et al.*, 2002). Extensive and systematic studies on the tumorigenicity of individual PAH metabolites in animals have led to the conclusion that vicinal or so called bay-region diol epoxides are the ultimate mutagenic and carcinogenic species of alternant PAHs, although not necessarily the only ones (WHO, 2000). Benzo[*a*]pyrene (BaP), a highly carcinogenic polycyclic aromatic hydrocarbon (PAH) and one of the most extensively investigated of all chemical carcinogens (Scherer, 2014). The metabolism of BaP by the diol epoxide pathway is summarised in Figure 2.1. The initial step involves epoxide formation at the 7,8 or 9,10 position, catalysed by cytochromes P450 (Scherer, 2014). The principal route of further oxidative metabolism of the BaP-7,8-dihydrodiol is to the bay-region BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) (WHO, 2000). The bay region diol epoxide is considered a major ultimate carcinogen of BaP based on studies in cell culture and laboratory animals (WHO, 2000; Muñoz and Albores, 2011; Scherer, 2014).

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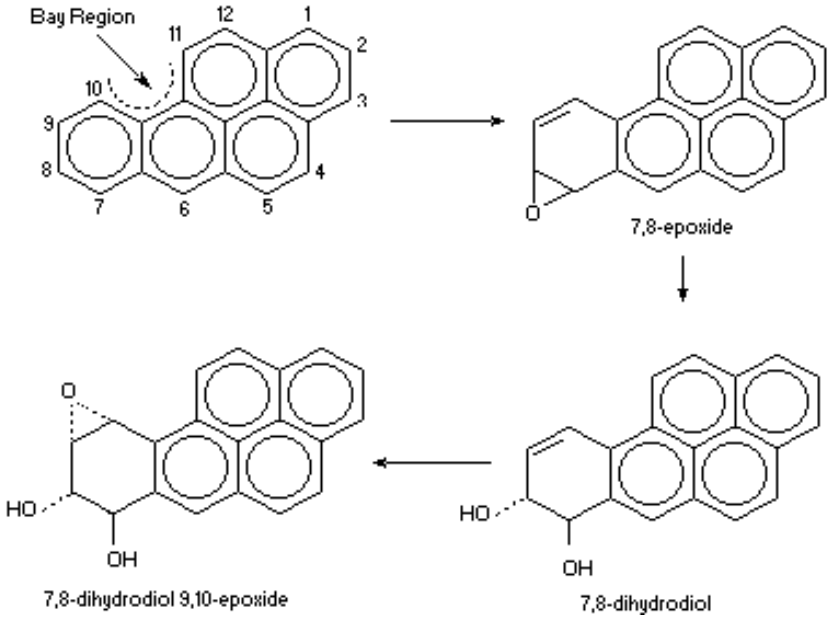

Figure 2.1 The bay region dihydrodiol epoxide route of metabolism of Benzo[a]Pyrene. (Adapted from IPCS (1998).

2.1.5 Environmental Fate of PAHs

Several distribution and transformation processes determine the fate of both individual PAH and mixtures (WHO, 1998). There are a variety of mechanisms by which PAHs are dissipated in the environment, including chemo-oxidation and photo oxidation; however microbial degradation is considered the primary route of degradation of PAHs in soils (Juhasz and Naidu, 2000). PAHs have differing half lives in environmental compartments (e.g. soil, air, water) depending on their structural susceptibility to chemical, physical or biological decomposition. PAHs released to the atmosphere are subject to short- and long-range transport and are removed by wet and dry deposition onto soil, water, and vegetation. In surface water, PAHs can volatilize photolyze, oxidize biodegrade, bind to suspended particles or sediments, or accumulate in aquatic organisms (with bioconcentration factors often in the 10-10,000 range) (ATSDR, 1995). The most important degradation process for PAHs in air and water is indirect photolysis under the influence of sensitizing radicals like OH, NO_3, and O_3. Under laboratory conditions, the reaction of the compounds with airborne hydroxyl radicals shows maximum half-lives between about 3 and 11 hours. For pure water, the photodegradation half-lives appear to be in the range of hours, whereas the half-lives increase drastically when sediment/water

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partitioning is taken into account (WHO, 2003). The adsorption of high-molecular-mass PAH on to carbonaceous particles in the environment should stabilize the reaction with OH radicals. The reaction of two- to four-ring PAH, which occur mainly in the vapour phase, with NO_3 leads to nitro-PAH, which are known mutagens (WHO, 1998).

2.2 Bioremediation of Polycyclic Aromatic and Petroleum hydrocarbons

Though contaminated sites and sites suspected of being contaminated may represent potential dangers, remediation and restoration of the sites represents a significant opportunity. Moreover restoring metropolitan contaminated sites reduces potential risks, supports economical land use and sustainable usage of natural resources (UIC, 2010).

Numerous remediation techniques have been developed and explored in response to the growing numbers of identified contaminated sites such as incineration, excavation, landfilling. These techniques are expensive, sometimes difficult to execute, inefficient, and often toxic pollutants are merely transported to another area for land filling or treated by a process that transfers them into another phase in the environment (Elliot *et al.*, 2010; Bustamante, 2012). Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. Bioremediation utilizes the metabolic versatility of microorganisms such as bacteria and fungi to degrade or detoxify hazardous wastes into harmless substances such as carbon dioxide, water and cell biomass. It is considered to be a safe, efficient, eco-friendly and economic means of removing pollutants from contaminated soil without simply enacting transfer to another medium (Vidali, 2001; Mohan *et al.*, 2006; Das and Chandran, 2010).

Microorganisms in general and bacteria in particular, harbor enormous metabolic diversity, allowing them to utilize the complex chemicals as energy sources. Further, their ability to undergo rapid genetic change also enhances their chance to acquire new metabolic potential for degradation of the recently introduced xenobiotic chemicals (Pandey *et al.*, 2009). Though biodegradation of wastes is a centuries-old technology, it is only in recent decades that serious attempts have been made to harness nature's biodegradative capabilities with the goal of large-scale technological applications for effective and affordable environmental restoration. This development has required a combination of basic laboratory research to identify and characterize promising biological processes, pilot-scale development and testing of new bioremediation technologies, their acceptance by regulators and the public, and, ultimately,

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field application of these processes to confirm that they are effective, safe, and predictable (Crawford and Crawford, 1996).

2.2.1 Microbial Degradation of PAHs

Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation, microbial degradation is the major degradation process (Haritash and Kaushik, 2009). Microorganisms play an important role in the degradation of PAHs in terrestrial and aquatic ecosystems, and microbial degradation is the main process in natural decontamination (Vinas *et al.*, 2005). The PAH-degrading microorganism could be algae, bacteria, and fungi. It involves the breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, H_2O, CO_2 (aerobic) or CH4 (anaerobic). There is a wide range of PAH-degrading bacteria that are ubiquitously distributed in the natural environment, such as in soils and sediments. Often PAH-contaminated soils and sediments host active populations of PAH degrading bacteria (Komukai-Nakamura *et al.*, 1996), and correlations have been observed that soils having a high total PAH content often contain more PAH degraders compared to soils with a lower total PAH content (Elliot *et al.*, 2010). Several PAH-degrading microorganisms, primarily bacteria, are capable to use the PAHs as a carbon and energy source, and may thus transform the contaminants into molecules that can enter the organisms' central metabolic pathways (Cerniglia 1992).

Biodegradation is defined as the biologically catalyzed reduction in complexity of chemical compounds (Alexander, 1994). It is based on two processes: growth and cometabolism.In the case of growth, organic pollutants are used as sole source of carbon and energy. This process results in a complete degradation (mineralization) of organic pollutants (Fritsche and Hofrichter, 2008).

Cometabolism is defined as the metabolism of an organic compound in the presence of a growth substrate which is used as the primary carbon and energy source. Cometabolism, the transformation of a substance without nutritional benefit in the presence of a growth substrate, is a common phenomenon of microbial activities. It is the basis of biotransformations (bioconversions) used in biotechnology to convert a substance to a chemically modified form. Microorganisms growing on a particular substrate gratuitously oxidize a second substrate (cosubstrate). The cosubstrate is not assimilated, but the product may be available as substrate for other organisms of a mixed culture (Fritsche and Hofrichter, 2008). In the case of a pure

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culture, co-metabolism is a dead-end transformation without benefit to the organism. In a mixed culture or in the environment, however, such an initial co-metabolic transformation may pave the way for subsequent attack by another organism (Johnsen *et al.*, 2005). Cometabolism, in particular, is an important interaction that transforms non-growth substrate PAH in the presence of growth substrate to enlarge the range and extent of PAH degradation (Zhong *et al.*, 2007a). Cometabolic degradation of recalcitrant high molecular weight PAHs with the degradation of readily utilized PAH had been reported in several studies (van Herwijnen *et al.*, 2003). However, cometabolism of recalcitrant PAHs is often concomitant with competitive inhibition on readily degradable PAHs, which possibly resulted from competition on energy or sharing a common enzyme system with a broad specificity for PAH degradation (Guha *et al.*, 1999; Zhong *et al.*, 2007a).

The extent and rate of biodegradation of PAHs depends on many factors including pH, temperature, oxygen, microbial population, degree of acclimation, accessibility of nutrients, chemical structure of the compound, cellular transport properties, and chemical partitioning in growth medium (Haritash and Kaushik, 2009). The most important hydrocarbon-degrading bacterial genera in soil environments include *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Burkholderia, Collimonas, Corynebacterium, Dietzia, Flavobacterium, Gordonia, Micrococcus, Mycobacterium, Nocardia, Nocardioides, Pseudomonas, Ralstonia, Rhodococcus, Sphingomonas, Variovorax* and other unculturable bacterial clones (Leahy and Colwell, 1990; Kanaly and Harayama, 2000; Robertson *et al.*, 2007; Chikere *et al.*, 2011).

Bacterial strains belonging to a few genera such as *Sphingomonas, Pseudomonas*, and *Mycobacterium* have been observed to dominate PAH degradation in soil (Bisht *et al.*, 2015). *Pseudomonas* has been the most extensively studied, owing to its ability to degrade so many different contaminants and its ubiquity in soils containing Petroleum Hydrocarbons (Dı´az, 2004; Robertson *et al.*, 2007). Members of the genus *Pseudomonas*, such as *Pseudomonas saccharophila* P15, isolated from creosote-contaminated soil, was shown to exhibit enhanced removal rates of fluoranthene, pyrene, benz[*a*]anthracene, chrysene and benzo[*a*]pyrene when salicylate was used as an inducer of PAH dioxygenase activity (Chen and Aitken, 1999; Kanaly and Harayama, 2010).

Hydrocarbon degrading fungi genera includes; *Allescheria, Aspergillus, Aureobasidium, Botrytis, Candida, Cephalosporium, Cladosporium Cunninghamella, Debaromyces, Fusarium*

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,Gonytrichum, Hansenula, Helminthosporium, Mucor, Oidiodendrum, Paecylomyces, Phialophora, Penicillium, Rhodosporidium, Rhodotorula, Saccharomyces, Saccharomycopisis, Scopulariopsis, Sporobolomyces, Torulopsis, Trichoderma Trichosporon (Floodgate, 1984). The presence of microorganisms with the appropriate metabolic capabilities is the most important requirement for hydrocarbon bioremediation. The communities which were exposed to hydrocarbons become adapted, exhibiting selective enrichment and genetic changes (Atlas, 1991; Adams *et al.,* 2015). The three interrelated means by which adaptation can occur are (1) induction and or depression of specific enzymes, (2) genetic changes which result in metabolic pathways and (3) selective enrichment of microbes able to transform the hydrocarbons (Leahy and Colwell, 1990; Chikere *et al.,* 2011).

While there are many organisms capable of degrading the low molecular weight (two-and 3 ring) PAHs, relatively few genera have been observed to degrade high molecular weight (> 3 ring) PAHs (Juhasz and Naidu, 2000). In the last 10 years many new bacteria that possess HMW PAH biodegradation capabilities for pyrene and fluoranthene have been isolated from different environments (Kanaly and Harayama, 2010). PAHs with molecular weight up to 202—including the high-molecular-weight (HMW) PAHs pyrene and fluoranthene can be degraded through growth-linked aerobic reactions (Bueno-Montes *et al.,* 2011). As yet no strains have been found to utilize PAHs with more than four rings (such as benzo(a)pyrene, benzo(k)fluoranthene, benzo(b)fluoranthene, dibenzo(a, h)anthracene etc.) as a sole carbon and energy source; but by cometabolic transformation (Elliot *et al.,* 2011).

A single species is capable of degrading alimited number of compounds but microbial consortium composed of different bacterial species possesses multilple metabolic capacities that increase the efficiency of the bioremediation process. In addition, a complex mixture of pollutants or organic compounds requires microbial communities to work together in order to efficiently degrade the pollutant, since a combination of genetic information among the group of organisms give the best potential for degradation (Jacques *et al.,* 2007; Tay *et al.,* 2015).

2.2.1.1 Bacterial Degradation of PAHs

Catabolism of hydrocarbons has long been considered as a strictly oxygen-dependent process. Common aerobic hydrocarbon-utilizing organisms are found among fungi and bacteria. In aerobic organisms, the initial attack of hydrocarbons always requires molecular oxygen as a co-substrate. The first enzymes in the metabolic pathways of alkanes are monooxygenases,

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while aromatic hydrocarbons are attacked by either monooxygenases or dioxygenases. These enzymes incorporate hydroxyl groups, derived from molecular oxygen, into the aliphatic chain or the aromatic ring (Heider et al., 1998).

The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form *cis*-dihydrodiols (Bamforth and Singleton, 2005). The first enzymatic process of aerobic PAH catabolism, the rate limiting step, is the cleavage of the aromatic ring. One important and well characterized mechanism for the aerobic bacterial metabolism of naphthalene, and in general for PAHs, is via the oxidative action of the naphthalene dioxygenase enzyme complex, which introduces molecular oxygen to the aromatic ring (Elliot *et al.*, 2010).

This enzyme complex specifically incorporates atoms of oxygen to the two carbon atoms that were previously sharing a bond, forming a cis-dihydrodiol (Fig. 2.2). This intermediate then undergoes re-aromatization by dehydrogenases to form dihydroxylated intermediates (catechol). These intermediates then undergo ortho or meta cleavage (fission) resulting in cis-muconic acid or 2-hydroxymuconic semialdehyde, respectively (Bamforth and Singleton, 2005; Elliot *et al.*, 2010). Ring cleavage results in the production of sucinic, fumaric, pyruvic, and acetic acids and aldehydes (Fig. 2.2), all of which are utilized by the microorganisms for the synthesis of cellular constituents and energy (Juhasz and Naidu, 2000). PAH degradation in anaerobic conditions has also been reported but the rate is much slower compared to that in aerobic conditions (Sun *et al.*, 2014).

2.2.1.2 Fungal Degradation of PAHs

Several studies have shown that diverse fungi are capable of PAH mineralization. There are two main types of fungal metabolism of PAHs; these are mediated by the non-ligninolytic and ligninolytic fungi (also known as the white-rot fungi), Bamforth and Singleton (2005). The nonligninolytic fungi uses the cytochrome P-450 monoxygenase system and the ligninolytic fungi uses the soluble extracellular enzymes of lignin catabolism, including lignin peroxidase (LiP), manganese peroxidase (MnP) and laccases (Peng *et al.*, 2008). These enzymes are nonspecific and oxidize a wide variety of organic compounds.

In nonligninolytic fungi, cytochrome P450 monooxygenase enzymes catalyze the oxidation of PAHs to arene oxides, which are initial products of PAH metabolism. While the resulting arene oxide intermediate is carcinogenic, it can undergo further metabolism by epoxide hydrolase to

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form a trans-dihydrodiol or undergo nonenzymatic rearrangement to produce a phenol which can form many conjugates (Fig. 2.2) and their toxicity thought to be low (Sutherland, 1992). In organisms that use the cytochrome P-450 system, the *trans*-dihydrodiol product cannot be used as an energy source (Pickard *et al.*, 1999; Pozdnyakova, 2012). However, *trans*-dihydrodiol are more soluble and potentially available for bacterial degradation (Al-Turki, 2009). The ligninolytic fungi, commonly known as white-rot fungi, cause white-rots of wood and have been screened for their PAH-degrading ability when grown under ligninolytic and nonligninolytic culture conditions (Verma *et al.*, 2007). Ligninolytic fungi find widespread use in bioremediation since they are able to completely mineralize PAHs to carbon dixode and water (Cerniglia, 1997).

The degradation of PAHs by ligninolytic fungi, including white-rot and litter-decomposing fungi, has been intensively studied. They produce extracellular enzymes with very low substrate specificity, making them suitable for degradation of lignin and different low- and higher-molecular-weight aromatic compounds (Haritash and Kaushik, 2009). Wood- and litter-decay fungi can mineralize PAHs with four and more condensed aromatic rings, in contrast to bacteria and soil fungi. They also can metabolize both individual PAHs and their complex mixtures, such as creosote (Pozdnyakova, 2012). There is significant interest surrounding the use of ligninolytic fungi to degrade PAHs, as they have low substrate specificity and are therefore able to degrade even the most recalcitrant of compounds. Also, the enzymes involved are extracellular, and are theoretically able to diffuse into the soil/sediment matrix and potentially oxidise PAHs with low bioavailability (Bamforth and Singleton, 2005).

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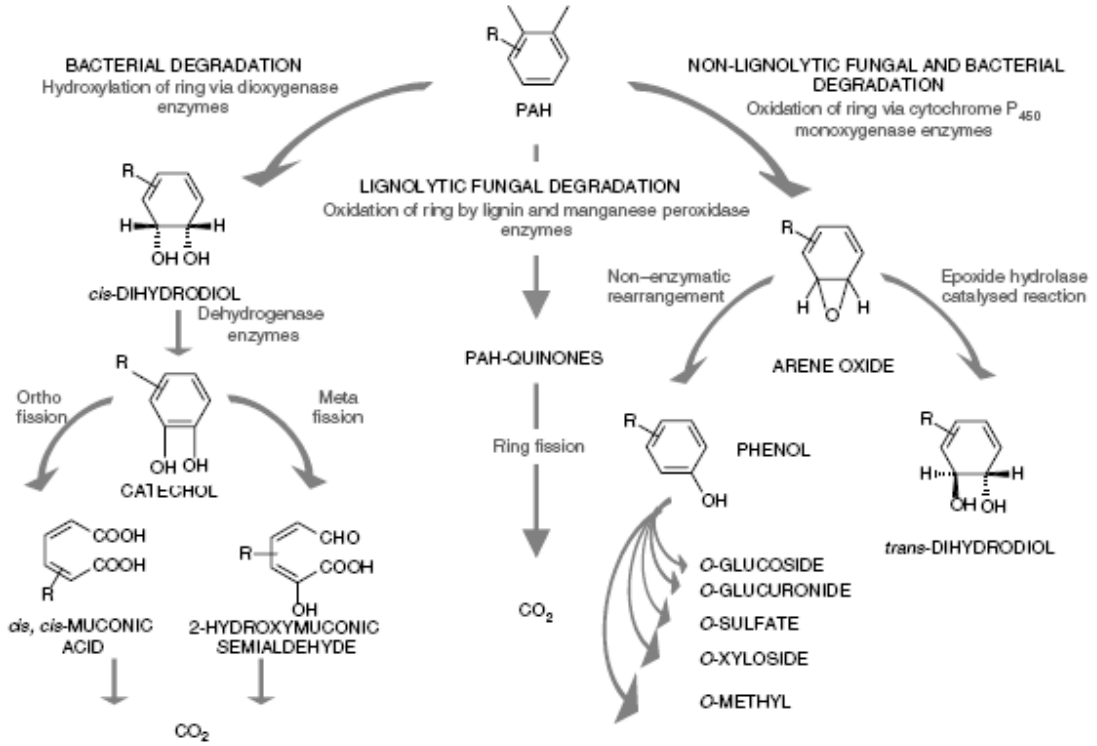

Figure 2.2: Pathways of microbial degradation of PAHs adapted from Cerneglia, 1992.

2.2.1.3 Anaerobic Degradation of Hydrocarbons

Although many PAHs are known to be biodegraded under aerobic conditions, most contaminated sediments are anoxic. Hence, anaerobic bacteria should play a more important role in biodegradation of PAHs in the natural environment (Li *et al.*, 2015b). At present aerobic processes are the most applied in the bioremediation of PAH contaminated soil but there is increasing experimental evidence of anaerobic PAH degradation in soil with alternative electron acceptors other than oxygen such as Iron Fe(III), nitrate and sulfate (Tomei and Daugulis, 2013). This approach is a promising alternative in soil bioremediation especially in subsurface and deep sediments and in cases of reduced soil porosity and air permeability (i.e., oil spills) that make oxygen transfer in the system difficult (Li *et al.*, 2010; Tomei and Daugulis, 2013). The anaerobic biodegradation of PAHs is a slow process, and its biochemical mechanism has not yet been elucidated (Haritash and Kaushik, 2009). Like their aerobic counterparts, anaerobic bacteria able to degrade xenobiotic compounds are diverse and present in various anaerobic habitats, including sediments, water-laden soils, feedlot wastes, sludge digesters, groundwater, and landfill sites (Zhang and Bennett., 2005). More recently, the

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number of pure cultures shown to metabolize various hydrocarbons with different electron acceptors have increased (Van Hamme *et al.*, 2003). This diverse set of bacteria, including members of the α-, β-, γ-, and δ-subclasses of the *proteobacteria*, form an excellent framework from which to elucidate the underlying biochemical and molecular mechanisms driving anaerobic hydrocarbon metabolism. While difficult, greater focus on isolating and characterizing the enzymes involved in anaerobic hydrocarbon metabolism is required (Van Hamme *et al.*, 2003). Several studies of anaerobic degradation of both low and high molecular weight PAHs have been demonstrated in soil and marine sediments with various electron acceptors (Liang *et al.*, 2014). Li *et al.* (2015b) reported that anaerobic biodegradation rates of PAHs, especially 4-ring PAHs were significantly enhanced by the inoculation of enriched PAH-degrading microbial consortium. The authors concluded that inoculation of the enriched PAH-degrading consortium supported higher microbial activity, leading to more rapid and complete biodegradation with the half-lives of the target PAHs shortened significantly.

2.2.2 Bioremediation Technologies

Bioremediation technologies can be broadly classified as *ex situ* or *in situ* (Kuppusamy *et al.*, 2016). *In situ* bioremediation is treatment of the contaminated material in place by implementation of microbial metabolic potential without the need for excavation of the contaminated sample, whereas *ex situ* bioremediation techniques are those treatments which involve the physical removal of the contaminated material for treatment (Farhadian *et al.*, 2007; Pandy *et al.*, 2009). *Ex situ* and *in situ* techniques each have specific benefits and costs.

2.2.2.1 In situ bioremediation

The prime benefit of *in situ* techniques is that contaminated soil or ground water need not be removed or transported. This approach can be less costly and less disruptive than ex situ treatments because no pumping or excavation is required. Moreover, exposure of site workers to hazardous contaminants during in situ treatment is minimal (Tabak *et al.*, 2005). The main advantage of in situ treatment is that it allows ground water, industrial sites, aquifers, subsurface soil and other inaccessible environmental niches to be treated without being brought to the surface (Farhadian *et al.*, 2007; Pandey *et al.*, 2009). *In situ* treatment is useful for contaminants that are widely dispersed in the environment, present in dilute concentrations, or otherwise inaccessible e.g., due to the presence of buildings or structures (Tabak *et al.*, 2005). *In situ* treatment, however, generally requires longer time periods, and there is less

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certainty about the uniformity of treatment because of the variability in aquifer characteristics and because the efficacy of the process is more difficult to verify (Vaněk *et al.*, 2010).

2.2.2.2 Ex situ bioremediation

Despite the high cost, *ex situ* treatment generally requires less time to achieve efficient contaminant cleanup, is easily monitored and achieves more uniformity (Kuppusamy *et al.*, 2016). *In situ* bioremediation may not be always suitable under situations like high concentrations of toxic pollutants causing microbial inhibition. Furthermore, due to the longer timescales involved the residual contaminant levels attainable might not always be low enough to meet legislation limits. Under such curcumstances, attention is focused on *ex situ* technologies, usually preferred when a safe and effective intervention is required i.e. in the presence of severe contamination by highly hazardous compounds (Angelucci and Tomei, 2016).

The other significant advantage with the application of *ex situ* bioremediation method is its independence from the environmental factors that could adversely affect the efficacy of the process. Additionally, since *ex situ* bioremediation is carried out in non-natural environments, the process can be manipulated easily by physico-chemical treatments of the target pollutant before and/or during the degradation (Kim *et al.*, 2005; Pandy *et al.*, 2009). It generally requires shorter time periods, and there is of more certainty about the uniformity of treatment because of the ability to monitor and continuously mix the groundwater. However, *ex situ* treatment requires pumping of groundwater and excavation of soil, leading to increased costs and engineering for equipmentand material handling.

Ex situ treatments are useful for the remediation of (i) sludges, soils or sediments polluted with high concentration of recalcitrant contaminants; (ii) clayish and stratified soils with low hydraulic conductivity and low permeability accompanied with high contents of organic matter and (iii) soils in regions and areas where environmental conditions are adverse to biological processes and (iv) contaminated sites that require a short remediation time because of regulatory or other pressures (Robles-González *et al.*, 2008). Generally the selection of each remdiation approach needs to be rationalized according to the targets and expected outcomes required

Ex situ bioremediation technologies can most easily be classified by the physical state of the medium to which they are typically applied (USEPA, 2006). Slurry Phase Bioreactors, Landfarming, Composting, and Biopiles are examples of *ex situ* treatment techniques. *Ex situ*

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treatment technology is further divided into slurry-phase bioremediation and solid-phase bioremediation (Pavel and Gavrilescu, 2008).

I Slurry-Phase Bioremediation (Bioreactors)

Slurry bioreactors are one of the most important types of *ex situ* technique. Treatment of soils and sediments in slurry bioreactors has become one of the best options for the bioremediation of soils polluted by recalcitrant pollutants under controlled environmental conditions (Robles-González, 2008). In this technique, contaminated solid or liquid material (water, sludge, sediment and soil) is processed through an engineered contamination structure (bioreactor—manufactured or engineered device that supports a biologically active environment) to achieve bioremediation (Kuppusamy *et al.*, 2016).

In slurry bioreactors contaminated soil is processed to separate stones and rubble and mixed with water or wastewater to give a slurry of predetermined consistency, with aqueous suspensions generally in the range of 10– 40% w/v, requiring an effective mixing device (mechanical or pneumatic). Electron acceptors and nutrients are added to the reactor, and parameters such as pH and temperature are controlled to optimize biological processes. Also, the reactor may be inoculated with specific organisms if a suitable population is not present. Targeted contaminants include petrochemicals, solvents, pesticides, wood preservatives, explosives, petroleum hydrocarbons, and other organic chemicals (Tomei and Daugulis, 2013; Juwarkar *et al.*, 2014).

The system can be operated under aerobic, anoxic and anaerobic conditions and in different feed modes: continuous, semicontinuous, and batch. Batch operation is the most commonly used mode offering better operability in the treatment of soils or sediments. As a modification of batch operation, the reactors are frequently operated as sequencing batch reactors with a work cycle consisting of feed phase (soil, water, and nutrient addition); reaction phase (the slurry is mixed until the desired decontamination degree is reached), settling, and extraction of the supernatant and of the decontaminated soil to be disposed of. Part of the slurry is left as inoculum for the next cycle. The biodegradation rate is rapid and remedial goals are achieved in few months. Also, bioslurry systems are technically versatile, simple and more effective than conventional bioremedial approaches. Since it is a closed system, it allows better control of temperature, pH, aeration, nutrient and surfactant additions, control of VOC emissions, monitoring of reactions, and microbial supplementations (Kuppusamy *et al.*, 2016).

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These characteristics, in turn, lead to several process advantages, (i) increased mass transfer rates and increased contact microorganisms/pollutant/nutrients; (ii) increased rates of pollutant biodegradation compared to *in situ* bioremediation or solid phase biotreatment: (iii) associated to (i) and (ii), significantly shorter treatment times can be achieved; (iv) possibility of using different electron acceptors. (v) control and optimization of several environmental parameters such as temperature, pH, etc.; (vi) effective use of biostimulation and bioaugmentaion; (vii) increased pollutant desorption and availability through the addition of surfactants and solvents (Robles-González *et al.*,2008).

II Solid-phase bioremediation

Solid-Phase bioremediation is an *ex situ* technology in which the contaminated soil is excavated and placed into piles. Bacterial growth is stimulated through a network of pipes that are distributed throughout the piles (Pavel and Gavrilescu, 2008). By pulling air through the pipes the necessary ventilation is provided for microbial respiration. Moisture is introduced by spraying the soil with water. Solid-phase systems require a large amount of space, and cleanups require more time to complete than with slurry-phase processes (USEPA, 2001). Some solid-phase treatment processes include land farming, soil biopiles, and composting.

Land farming

The term landfarming refers to the process where hydrocarbon contaminated soils are spread out in a layer of 0.3–1.0 m thick, nutrients are added and the soils are mixed periodically. During the process of landfarming, the total petroleum hydrocarbons, (TPH), may be lost through volatilization or biodegradation. Treatment systems for landfarms vary with climate, location, temperature and soil type. Enhanced bioremediation of contaminated soil typically involves the addition of nutrients and water, and periodic tilling or excavating to mix and aerate the soil (Paundy *et al.*, 2008)

The contaminants are degraded, transformed and immobilized by means of biotic and abiotic reactions. Sometimes, in cases of very shallow contamination, the top layer of the site may simply be tilled without requiring any excavation. Liners or other methods may be used to control leachate. This technology is designed primarily to treat soil contamination by fuels, PAHs, non-halogenated VOCs, SVOCs, pesticides, and herbicides. The process may be applied to halogenated organics, but is less effective, the more chlorinated or nitrated the compound, the more difficult it is to degrade (Dadrasnia *et al.*, 2013). Landfarming has several advantages.

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First, the equipment and energy consumption requirements are relatively modest (Filler *et al.*, 2006). The logistical requirements of landfarms are also comparatively small, and consequently landfarming is more cost-effective than many alternative approaches to the remediation of petroleum hydrocarbon contaminated soil (Filler *et al.*, 2006; Bolton 2012). However, managing air quality associated with volatilization of petroleum hydrocarbons from uncovered landfarms represents a limitation of this technique and often requires an emission control system to address reduced air quality (Camenzuli and Freidman, 2015; Environment Canada, 2013).

Other Factors that may limit the applicability and effectiveness of land farming include: A large amount of land is required, volatile contaminants must be pre-treated because they would volatilize into the atmosphere and cause air pollution, there is a possibility for contaminant movement from the treatment site to a previously undisturbed site (Khan *et al.*, 2004; Baciocchi *et al.*, 2012). The presence of metal ions may be toxic to microbes and may leach from the contaminated soil into the ground. It may not be effective for sites of high constituent concentrations of greater than 50,000 ppm TPH (USEPA, 1998). The prevention of groundwater and air pollution increases treatment cost, thereby reducing the main advantage of the technology. Both groundwater and air pollution have been taken into account in the ever more stringent environmental regulations and this has caused a reduction in recent landfarming application after its extensive use in the 1960s (Tomei and Daugulis, 2013).

Composting

Composting and mature compost have been successfully applied to the bioremediation of contaminated soils with PAHs, pesticides, petroleum and other pollutants by providing a degrading matrix, available nutrients, and large numbers of active microorganisms (Chen *et al*., 2015). Composting, defined as the controlled biological decomposition and stabilization of organic matter into a humus-like product called compost, is a common way of reclaiming organic wastes for agricultural use (Plaza *et al.*, 2009). Composting is a controlled biological process that treats organic contaminants using microorganisms under thermophilic conditions (40– 50 °C). In composting, soils are excavated and mixed with bulking agents and organic amendments, such as wood chips and vegetative wastes, to enhance the porosity of the mixture and support the development of a rich microbial population and elevated temperature which is characteristic of composting (USEPA, 2006; Lapsiya, 2011). Windrows and static piles are the two basic compost technologies used to treat contaminated soil. In the windrows composting

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process, the compost mixture is placed in long parallel rows called as windrows. The method draws oxygen into the pile by natural convection and mechanical turning. In static pile composting, the compost is placed over an aeration system comprised of blowers or vacuum pump and piping. Air is circulated forcibly or drawn through the pile to provide oxygen and regulate temperature (Lapsiya, 2011).

Suface irrigation often is used to maintain moisture content. Composting depends on the degradation of the organic materials, resulting in the thermogenesis and production of organic and inorganic compounds. The metabolically generated heat is trapped within the compost matrix, which leads to elevations in temperature, a characteristic of composting process (Semple *et al.,* 2001). With increases in the respiratory activity, there is an increase in temperature resulting in a decrease in mesophilic microbes and an increase in thermophiles and it is at these higher temperatures that most of the microbial decomposition and biomass formation takes place (Semple *et al.,* 2001). Organic amendments from compost are an important source of nutrients, which provide more available carbon sources for indigenous microbes. In addition, organic amendments from compost also directly increase the density of microbes that are responsible for the decomposition and biotransformation of pollutants in soils (Chen *et al.,* 2015.).

However, compost is a complex matrix, very rich in organic matter, especially in humic acids, which are known to heavily influence bioavailability of contaminants. For this reason, a careful assessment of the amendment effect on biodegradation of contaminants and on attainable end-points is required (Gandolfi *et al.,* 2010).

Soil Biopiles

Biopiling, also known as bioheaps, biocells or biomounds, is an *ex situ* bioremediation technology that has been extensively used for remediating a wide range of petrochemical contaminants in soils and sediments (Germaine *et al.,* 2015). Biopiles can be defined as an above ground engineered system, heaping contaminated soils into piles and stimulating aerobic microbial activity by providing oxygen, nutrients and/or microbial consortia to degrade petroleum compounds adsorbed to the soil (Khan *et al.,* 2004, Juwarkar *et al.,* 2014; Gomez and Sartaj, 2014).

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Biopile is one of the cost-effective remediation techniques that may be utilised to remediate petroleum hydrocarbon-contaminated soils. Biopiles are a hybrid of landfarming and composting in which excavated soils are mixed with soil amendments, placed on a treatment area and bioremediated using forced aeration, and it can be engineered or non-engineered. The basic biopile system includes a treatment bed, an aeration system, an irrigation/nutrient system and a leachate collection system; they are a refined version of landfarming that tends to control physical losses of the contaminants by leaching and volatilization (Ramadass *et al.*, 2015). Biopiles provide a favourable environment for indigenous aerobic micro-organisms. As a consequence of these optimum growth conditions, the enhanced microbial activity results in the degradation of the bioavailable organic pollutants (Gomez and Sartaj, 2013). The effectiveness of biopiling has been successfully demonstrated at laboratory and field scale for several different types of hydrocarbons (Coulon *et al.*, 2010; Gomez and Sartaj, 2014; Germaine *et al.*, 2015).

2.2.3. Factors Affecting Biodegradation Rate of PAHs

Microorganisms can degrade numerous of organic pollutants owing to their metabolic machinery and to their capacity to adapt to inhospitable environments. Thus, microorganisms are major players in site remediation (Joutey *et al.*, 2013). However, their efficiency depends on the existence of microbial consortia capable of degrading the pollutant, the bioavailability of the contaminant to microbial attack and certain environmental factors (soil type, temperature, soil pH, oxygen level of soil, moisture, electron acceptor agents, nutrient content of soil and other substances that may act as substrate cometabolites., PAH concentrations , toxicity, mobility, and contamination history of soil) contributing to microbial growth (Cerniglia, 1992; Alexander, 1999; Joutey *et al.*, 2013; Ukiwe *et al.*, 2013). Bioavailability of contaminants followed by contaminant mass transfer and subsequent metabolism are the factors believed to control the overall bioremediation efficiency in the soil matrix especially in regard to hydrophobic contaminants such as PAHs (Mohan *et al.*, 2006). The bioavailability of contaminants is influenced by many factors including physico-chemical properties of the PAHs (chemical structure, molecular weight, hydrophobicity), length of time the PAHs have been in the soil and soil properties, including soil texture, composition, cation exchange capacity, water content, pH and physical structure (Solomon *et al.*, 2013).

These factors affecting biodegradation rate of PAHs can be categorized in three domains (Vidali, 2001; Jazestani, 2011)

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1. PAH-related: physico-chemical properties of the PAHs, concentration, length of time the PAHs have been in the soil; and associated bioavailability of contaminants for the microbial population; toxic or inhibitory effects of the contaminants and their degradation products
2. Environment-related: sediment type, soil type, organic content, nutrient status, salinity, soil-to-water ratio, temperature, pH, redox potential; presence of oxygen or other electron acceptor
3. Bacteria-related: The existence of a microbial population capable of degrading the pollutants (types, population, distribution, and previous exposure). Microbial communities present in previously contaminated soil can metabolize PAHs at greater rates than soil microbial communities found in uncontaminated soils. An acclimation period is required for microbes after a contamination event before degradation processes commence. During this acclimation period, appropriate soil microbial communities are developed through growth and enzyme induction (Rathbone *et al.*, 1998).

2.2.4 Strategies to enhance bioremediation efficiency

Manipulations of the above environmental, bacteria and PAH related factors are the basis for bioremediation (Jazestani, 2011), with the subsequent goals of:

(1) Improving soil microbial habitat through fertilizer additions, tillage, liming, and/or

(2) Promote microbial functional groups capable of degrading PAHs.

(3) Increasing the bioavailability of the PAHs.

When naturally occurring metabolic processes are used to remediate pollutants without any additional alteration of site conditions, the process is called as intrinsic or natural attenuation. When working conditions at the site are engineered, i.e. designed to accelerate the bioremediation of contaminants, the process is referred to as engineered or enhanced bioremediation (Farhadian *et al.*, 2007). Enhanced bioremediation includes augmentation with various pollutant degrading organisms (bioaugmentation) or the provision of amendments to exploit the existing natural degradative capacity of the soil's indigenous microflora (biostimulation) (Megharaj *et al.*, 2011).

Natural or intrinsic bioremediation has been occurring since the beginning of life on earth, but the process is relatively slow, and it takes a long time to remove certan classes of insoluble and hard-to-degrade hydrocarbons from polluted sites. Therefore, in engineered bioremediation,

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attempts are made to speed-up the naturally occurring biodegradation by customizing local environmental conditions either by (1) bioaugmentation, in which microbes are added or by (2) biostimulation, i.e., providing/maintaining the favorable conditions for growth of the soil microorganisms (Yadav and Hassanizadeh, 2011).

2.2.4.1 Bioaugmentation

Bioaugmentation or seeding is the addition of highly concentrated and specialized populations (single strains or consortia) to the site contaminated with recalcitrant toxic compounds (Leahy and Colwell, 1990). This technique is best suited for sites that (i) do not have sufficient microbial cells or (ii) the native population does not possess the metabolic routes necessary to metabolize the compounds under concern (Tyagi *et al.,* 2011). Even when the appropriate catabolic microbes are present in the intrinsic microbial community at the contaminated site, the abundance and activity of the microorganisms may be too low for successful bioremediation. In such cases bioaugmentation of highly concentrated and specialized populations (single strains or consortia) capable to degrade the xenobiotic compounds of interest is used to enhance the degradative capacity of the microbial community and the transformation rate of the pollutants several folds (Nõlvak, 2012). Successful application is more difficult in a complex and dynamic matrix such as soil where many abiotic and biotic factors affect the survival and activity of the inoculated microorganisms. The abiotic factors include fluctuations in temperature, humidity level, pH, nutrient content and availability. Biotic factors include competition from indigenous bacteria, along with antagonistic interactions including antibiotic production by competing organisms, and predation by protozoa, bacteriophages and fungi (Tomei and Daugulis, 2013; Bisht *et al.,* 2015). The most commonly-used options for bioaugmentation covering the catabolic degradation route of the contaminant are; addition of a pre-adapted pure bacterial strain; addition of a pre-adapted consortium; introduction of genetically engineered bacteria; addition of biodegradation-relevant genes packaged in a vector to be transferred by conjugation into indigenous microorganisms (El Fantroussi and Agathos, 2005). Pre-exposure and subsequent re-exposure of a chemical pollutant enhances the metabolic potential of microorganisms (Reddy and Sethunathan, 1983). The phenomenon of retaining specific metabolic capacity after pre-exposure over long periods of time is referred to as ‘soil memory.’ The soil memory makes a contribution when the microorganisms are amended to a polluted site to hasten detoxification and/or degradation (Megharaj *et al.,* 2011).

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The relationship of the inoculated microorganisms with its new biotic and abiotic environments, in terms of survival, activity and migration, can be decisive in the outcome of any bioaugmentation strategy (El Fantroussi and Agathos, 2005; Pandey *et al*., 2009). Therefore, a comprehensive assessment of both abiotic and biotic environmental factors and their impacts on the bioaugmentation process are significant to confer the optimal efficiency to the process at the field site (Pandey *et al.*, 2009; Mrozik and Piotrowska-Seget, 2010). The best approach for selecting competent microbes should be based on the prior knowledge of the microbial communities inhabiting the target site (Thompson *et al.*, 2005).

It has been suggested that the best way to increase the survival of the inoculum is to base the selection of competent microbes on prior knowledge of the microbial communities inhabiting the target site (Hosokawa *et al.*, 2009), if this is not possible, candidate microbes should be chosen from the same ecological niche as the polluted area (El Fantroussi and Agathos, 2005). Indigenous microbes (pre-selected for bioaugmentation) are more likely to persist and propagate when reintroduced into the site, as compared to transient or alien strains to such habitat (Nõlvak, 2012). In cases like co-contamination with high metal concentrations and organic pollutants which would be inhibitory to the microrganisms, the use of multi-component systems such as a microbial consortium, which is a better representation of a real environment than models based on single-component systems, is the proposed strategy (Ledin, 2000). Co-inoculation of a consortium of bacteria, each with different catabolic degradation route, is often found to be more efficient for bioremediation process as it provides the metabolic diversity and robustness needed for field applications (Tyagi *et al.*, 2011).

2.2.4.2 Biostimulation

Petroleum hydrocarbons represent a large carbon source for the indegeneuous microorganisms in most hydrocarbon contaminated environments, whereas the presence of nitrogen and phosphorous is limited. Biostimulation refers to the addition of one or more rate-limiting nutrients to accelerate contaminant biodegradation rates (Nikolopoulou and Kalogerakis, 2009). It involves adjustments to the site by the addition of nutrients (nitrogen, phosphorus and trace minerals) while also making appropriate pH adjustments, soil moisture content, and aeration for the proliferation of indigenous microorganisms in order to provide bacterial communities with a favorable environment by which they can effectively degrade contaminants (Mohan *et al.*, 2006).

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Nutrient requirements of microorganisms are divided into three categories (macro, micro, and trace nutrients) which are based largely on the essential need and quantity required by the microorganism. For example, the macronutrients carbon, nitrogen, and phosphorus comprise approximately 50, 14, and 3% dry weight of a typical microbial cell, respectively (Liebeg and Cutright, 1999). Based on this criterion, the optimal C:N:P mole-ratio recommended for bioremediation applications is 100:10:1 (Cookson, 1995), ratio of 100:15:3 (Zitrides, 1983) and 120: 10: 1 by Alexander (1977). Wolicka *et al.* (2009) optimized the C:N:P ratio (at the level of 100:9:2, 100:10:1 or 250:10:3) before commencing *in situ* remediation of BTEX. On the other hand (Swannell *et al.,* 1996) suggested for oil spill remediation, around 1–5% N by weight of oil with a ratio of N:P between 5 and 10:1. These additions may be insufficient or inaccurate for polluted sites with different types of pollutants. Formulation of nutrient-treatment strategies and maintenance of control on the degradation rates and the outcomes of degradation need to be tailored to specific site/pollutant combinations (Megharaj *et al.*, 2011).

Since nitrogen and phosphorus supplies in soil systems are inadequate to support microbial growth and degradation of organic compounds, most bioremediation processes supply these two compounds. The availability of nitrogen and phosphorus usually limits the degradation of petroleum hydrocarbons in aqueous and terrestrial environments, but comparatively less work has been done to examine other nutrients that might affect biodegradation, but fortunately these are required only in small or trace amounts, which are generally supplied in a natural soil (Crawford and Crawford, 1996).

Numerous types of amendments such as inorganic fertilizers, wastewater sludge, sewage sludge compost, vermicompost, municipal solid waste compost, manure (Liu *et al.,* 2010) and biosolids, have been utilized to enhance the degradation of petroleum products in the subsurface of contaminated sites (Nõlvak, 2012). On the other hand, excessively high nitrogen levels can be toxic and can inhibit microbial activity; in an aquatic environment, excess nutrients can also cause algal blooms (Nikolopoulou and Kalogerakis, 2009). Therefore, nutrient injections must be thoroughly optimized according to the conditions of each treated sit (Nikolopoulou and Kalogerakis, 2009; Nõlvak, 2012). Addition of a carbon source as a nutrient in contaminated soil is known to enhance the rate of pollutant degradation by stimulating the growth of microorganisms responsible for biodegradation of the pollutant. It has been suggested that the addition of carbon in the form of pyruvate stimulates the microbial growth and enhances the rate of PAH degradation (Lee *et al.,* 2003).

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2.2.4.3 Increasing Bioavailability of PAHs

The rate at which hydrocarbon degrading microorganisms can convert chemicals depends on the rate of transfer to the cell (mass transfer) and the rate of uptake and metabolism (intrinsic activity) by the microorganisms (Bosma *et al.,* 1997; Semple *et al.,* 2003; Kutcherov, 2013). Bioavailability of a chemical is determined by the rate of mass transfer relative to the intrinsic activity of the microbial cells. Evidently, increased microbial conversion capacities do not lead to higher biotransformation rates when mass transfer is the limiting factor (Bosma *et al.*, 1997). The mass transfer of a contaminant determines microbial bioavailability. The term "bioavailability" refers to the fraction of contaminant in soil that can be utilized or transformed by living organisms (Semple *et al.,* 2003). It is evident that PAH bioavailability is limited by hydrophobicity and strong sorption/sequestration in micropores or organic matter, non-uniform spatial distribution of microorganisms and pollutants, and the retardation of substrate diffusion by the soil matrix (Mohan *et al.,* 2006). In particular, the higher is the hydrophobicity of the compound, the greater is its persistence in the environment, with deleterious, often irreversible effects on environmental health. The persistence of an organic contaminant is, however, not only due to its intrinsic molecular property, but it is much more a result of environmental microhabitats that affect both the mass transfer of the xenobiotic to microorganism and its degradative activity (Huesemann *et al.,* 2004; Andreoni and Gianfreda, 2007).

2.2.5 Factors That Affect PAH Bioavailability

Factors that affect PAH bioavailability can be categorized into three catagories.

- Physical and chemical properties of PAHs,
- Soil properties (soil organic matter, dissolved organic matter, moisture content, etc.) and
- Ageing of PAHs in soil

2.2.5.1 Effects of physicochemical properties of PAHs on bioavailability

Bioavailability is influenced by the molecular structure and size of PAHs. LMW PAHs are removed faster by physico-chemical and biological processes due to their higher solubility, volatility and the ability of many microorganisms to use them as sole carbon sources in comparison to the HMW PAHs (Solomon *et al.,* 2013). The bioavailability of a chemical is controlled by a number of physical-chemical processes such as sorption and desorption, diffusion, and dissolution (Bosma *et al.,* 1996). PAHs possess physical properties, such as low

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aqueous solubility and high solid–water distribution ratios, which stand against their ready bioavailability and microbial utilization (Johnsen *et al.,* 2005). The PAHs hydrophobicity increases nearly logarithmically with their size, which is the source of their strong tendency to sorb onto solid surfaces, such as clays and organic matter (Johnsen *et al.,* 2005; Ortega-Calvoa *et al.*, 2013).

Polycyclic aromatic hydrocarbon bioavailability is commonly equated with the amount of a given PAH that can be desorbed relatively rapidly from a solid-phase or nonaqueous compartment to the aqueous phase, where the compound can be accessed by indigenous PAH-degrading bacteria (Richardson and Aitken, 2011). Due to their hydrophobicity PAHs sorb onto the humic and fulvic acids in wet soils and on mineral surfaces, such as clay, in dry soils where aluminum and iron, via redox reactions, can form PAH radicals that then undergo polymerization (Garbarini and Lion, 1986; Chiou, 1999; Husain, 2008). Soil organic matter and other natural and anthropogenic domains such as residual coal tar, pitch, coke, soot, coal, and lampblack have been identified as important reservoirs for sorption of PAHs in soils and tend to exhibit significantly lower PAH availability than mineral particles (Richardson and Aitken, 2011).

2.2.5.2. Effects of soil or sediment properties and aging on PAHs bioavailability

The biodegradation potential of hydrocarbons is not only determined by their chemical composition, but also by the biological, physical and chemical characteristics of the soil environment (Betancur-Galvis *et al.,* 2006). The inherent difficulty with removing PAHs from soil is that they exhibit low aqueous solubilities coupled with a strong sorptive capacity to mineral surfaces (i.e. clays) and organic matter (i.e. humic and fulvic acids) in the soil matrix (Delle, 2001), and other soil constituents (Van Hamme *et al.,* 2003; Elliot *et al.,* 2011). The greater the surface area and cation exchange capacity, the greater the adsorption of PAH molecules. Soil moisture, temperature and pH all have an effect on the adsorption characteristics of PAHs (Providenti *et al.,* 1993; Elliot *et al.,* 2011) and this sorption capacity often renders PAH contaminants difficult to remove by thermal, physical, or chemical means. As a result of this sorption of PAHs to soil organic matter and sediment particle surfaces, their mobility and availability to microorganisms decrease and mass transfer to the aqueous phase becomes rate-limiting step (Atlas and Philip, 2005).

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Sorption of organic chemicals to soils and sediments often entails an initially rapid and reversible process followed by a period of slow sorption occurring over weeks, months, or perhaps years, and the slow sorption leads to a chemical fraction that then resists easy desorptivity and bioavailability (Hatzinger and Alexander, 1995). The PAH bioavailability is further complicated when PAHs interact with nonaqueous phase liquids (NAPL) and soil colloids, thus resulting less or completely unavailable for microorganisms (Efroymson and Alexander, 1995; Andreoni and Gianfreda, 2007).

An important process affecting the concentration of an organic compound in soil and consequently its availability is the "aging," through increasing contact time organic compounds become increasingly desorption-resistant, due to slow diffusion of organic compounds within solid organic matter components, the entrapment within nanopores in soil aggregates, and the formation of strong bonds between organic compounds and the soil (Li *et al.,* 2008). This process limits the release of PAHs into the bulk liquid phase, making them inaccessible to microorganisms, thus decreasing bioavaiability and biodegradation rates (Mohan *et al.,* 2006).

2. 3 Enhancing Bioavailability of PAHs Using Surfactants

One way to enhance the bioavability of PAHs is to apply surface-active agents (surfactants) as mobilizing agents (Makkar and Rockne, 2003). The biodegradation of soil-bound PAHs is, consequently, a two-step process that involves mobilization of PAHs from solid to aqueous phase and subsequent catabolism by microorganisms. The mass-transfer rate of PAHs into the aqueous phase was shown to be the rate limiting step in their biodegradation. It is, thus, very essential to enhance their mass transfer for a successful remediation process. To solve this problem, a surfactant enhanced bioremediation technique has been a research focus in recent years (Li and Chen, 2002). Surfactant-enhanced bioremediation has been considered to be a practical and effective remediation technique (Christofi and Ivshina, 2002; Paria, 2008; Xia *et al.,* 2014; Li *et al.,* 2015; Mao *et al.,* 2015).

Surfactants are able to improve the mass-transfer of hydrophobic pollutants from a solid or non-aqueous liquid phase into the aqueous phase by decreasing the interfacial tension and by accumulating the hydrophobic compounds in the micelles (Li and Chen, 2009). The addition of a surfactant to a contaminated soil can reduce the interfacial tension thus increasing the mass transfer and bioavailability of the contaminants (Mulligan *et al.,* 2001; Franzetti *et al.,* 2008; Bustamante *et al.,* 2012). Several studies have shown that various surfactants can enhance

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desorption (Zhu and Aitken, 2010; Bueno-Montes *et al.*, 2011; Congiu and Ortega-Calvo, 2014), solubilization (Pennell *et al.*, 1994; Zhao *et al.*, 2005; Hadibarata and Tachibana, 2010; Aryal and Liakopoulou-Kyriakides, 2013) and subsequent biodegradation of organic compounds.

Surfactant is an abbreviation for surface active agent, which literally means: a species, which is active at the interface. In other words, a surfactant is characterized by its tendency to adsorb at the surfaces and interfaces. The term interface denotes a boundary between any two immiscible phases while, the term surface indicates that one of the phases is a gas, generally air (Mehta *et al.*, 2010).

Surfactants are amphiphilic molecules consisting of a hydrophobic and hydrophilic domain. Due to the amphiphilic properties, surfactants often behave by partitioning at the interface of phases with different degrees of polarity and hydrogen bonding, such as between air and water, soil and water or oil and water boundaries. This unique property of surfactants lowers the surface tension at these interfaces and (dependent upon concentration) can act as emulsifying, foaming, wetting, solubilizing and dispersing agents (Bai *et al.*, 1997).

As surfactants are added to aqueous solution, they will tend to accumulate at fluid-fluid and fluid-solid interfaces and decrease the surface and interfacial tensions. Once a sufficient amount of surfactant has been added and approaches a certain concentration, known as the critical micelle concentration or CMC, the lipophilic moieties of the surfactant monomers associate with one another to form micelles consisting of a hydrophobic core surrounded by a hydrophilic mantle. The presence of micelles leads to an increase in the apparent solubility of HOCs; which is attributed to the incorporation of hydrophobic compounds within surfactant micelles this is also referred to as ‘solubilisation’ (West and Harwell, 1992; Pennell, 1993; Volkering *et al.*, 1997). The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached (Fig. 2.3).

At concentrations above the CMC, the surface tension remains nearly unchanged with increasing surfactant concentrations; additional quantities of surfactant in solution will promote the formation of more micelles. The formation of micelles leads to a significant increase in the apparent solubility of hydrophobic organic compounds. The effect of such a process is the enhancement of mobilization of organic compounds and of their dispersion in solution (Perfumo *et al.*, 2010).

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Further, micelle formation enables emulsification, wetting and spreading of nonaqueous phase liquids (Mulligan *et al.*, 2001; Attallah *et al.*, 2015). Physical properties of surfactant solutions, such as surface tension, detergency, osmotic pressure, electrical conductivity, etc., show an abrupt change in the neighborhood of the critical micelle concentration (CMC).

In the presence of a non-aqueous phase liquid (NAPL), surfactants concentrate at the liquid-liquid interface, reducing the interfacial tension. This may cause dispersion of NAPL droplets and stabilization of emulsions. A thermodynamically stable emulsion with a droplet diameter less than 1μm is called a micro-emulsion, while all other emulsions are called macro-emulsions (Volkering *et al.*, 1998).

There are two main types of emulsions: oil-in-water emulsions, in which oil droplets are dispersed in a continuous water phase, or water-in-oil emulsions, in which water droplets are dispersed in a continuous oil phase. When two immiscible liquids are in contact with each other, they will tend to maintain as small an interface as possible, emulsification involves the generation of a large total interfacial area for the miscibility of the liquids. In order to generate this large interfacial area, the interfacial tension must be lowered significantly, and the primary means by which the interfacial tension is lowered is through the addition of emulsifying agents, usually surfactants (James-Smith, 2006; Tadros, 2009).

Surfactants could significantly enhance the solubility of HOCs at a concentration greater than CMC and this characteristic made them usable as adhesives, flocculating, wetting and foaming agents, demulsifiers and penetrants based on their abilities to lower surface tensions, increase solubility, detergency power, wetting ability and foaming capacity (Mulligan and Gibbs, 1993).

The magnitude of the lowering of the surface or interfacial tension depends on the surfactant structure, concentration, and the physico-chemical conditions of the solution (e.g. pH, salt concentration, temperature, pressure, etc). When the critical micellar concentration, or CMC, is reached, many of the physical properties of the surfactant solution in water show an abrupt change. Some of these properties include the surface tension, osmotic pressure, electrical conductivity, and solubilisation (James-Smith, 2006).

The effectiveness of a surfactant is determined by its ability to lower the surface tension, which is a measure of the surface free energy per unit area required to bring a molecule from the bulk phase to the surface (Desai and Banat, 1997). A good surfactant can lower the surface tension

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of water from 72 to 35 mN m^{-1} and the interfacial tension (tension between non-polar and polar liquids) for water against n-hexadecane from 40 to 1 mN m^{-1} (Mulligan, 2005).

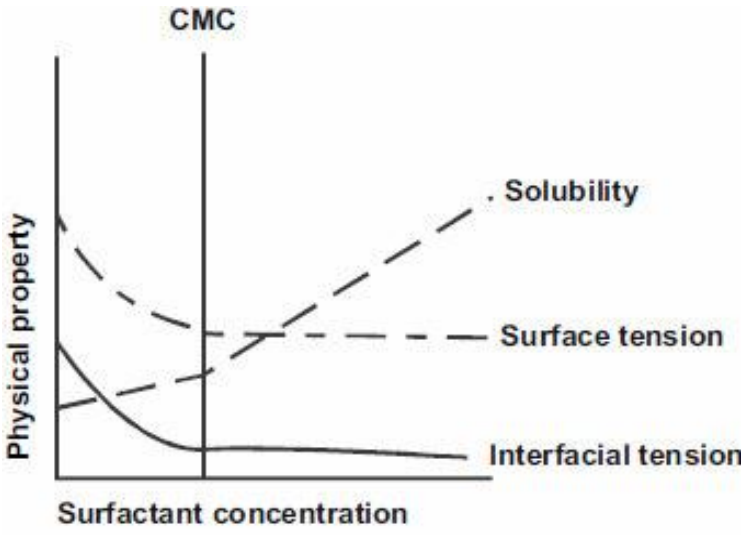

Figure 2.3 Relationship of surface tension, interfacial tension and the CMC with surfactant concentration (Mulligan, 2005).

The CMC is influenced by pH, temperature and ionic strength. Fig. 2.3 shows how other parameters vary as a function of surfactant concentration (Mulligan, 2005). Surfactants are classified according to the ionic charge residing in the polar part of the molecule. Hence anionic, cationic, nonionic and zwitterionic (combined presence of anionic and cationic charges) surfactants exist (Christofi and Ivshina, 2002)

2.4 Types of Surfactants

2.4.1 Synthetic Surfactants

The majority of work, by far, carried out on enhancing the solubility of organic hydrophobic contaminants in soils and other environments utilize synthetic surfactants. Synthetic surfactants have been shown to remove nonpolar compounds from surfaces but problems can be associated with their use, such as reduced availability of compounds sequestered into micelles, their toxicity, and ultimate resistance to biodegradation leading to increased pollution (Christofi and Ivshina, 2002).

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Studies with Synthetic surfactants have shown that surfactant additions approaching and above the surfactants critical micelle concentrations can inhibit PAH biodegradation via: (i) toxicity of surfactants dueo to surfactant induced permeabilization or lysis of bacterial cell membrane (Willumsen and Karlson, 1998; Avramova *et al.*, 2008), (ii) prevention of bacterial adhesion of some strains on hydrophobic substrates (Neu, 1996; Stelmack *et al.*, 1999), (iii) decreased bioavailability of PAH sequestered in surfactant micelles (Guha and Jaffé, 1996; Volkering *et al.*, 1995) and (iv) or preferential utilization of the surfactant over the target PAHs (Kim and Weber, 2003).

Although both synthetic surfactants and biosurfactants are used for the same general purposes, biosurfactants are distinguished from synthetic surfactants in that they are naturally produced by certain bacteria, yeasts and plants, whereas synthetic surfactants are chemically synthesised from different feedstocks, mainly petroleum.

As petroleum-based products they can be toxicants, recalcitrant to biodegradation, often making their production expensive and resulting in the production of toxic waste by-products (Mohee and Mudhoo, 2012; Chandra, 2015). Therefore, interest in microbial surfactants has been steadily increasing in recent years due to their diversity, environmentally friendly characteristics, lower toxicity, biodegradability, better environmental compatibility, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity (Rodrigues and Teixeira, 2010; Pereira *et al.*, 2013). Furthermore, these molecules can be tailor-made to suit different applications by changing the growth substrate or growth conditions (Rodrigues and Teixeira, 2010).

2.4.2 Biosurfactants

Biosurfactants are a structurally diverse group of surface active molecules synthesized by microorganisms. These molecules reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures, which makes them potential candidates for enhancing polycyclic aromatic hydrocarbons bioavailability to microorganisms and accordingly their biodegradation, oil recovering, and so on (Lotfabad *et al.*, 2009). The ability to biosynthesize biosurfactants is, often, coupled with the ability of these microorganisms to grow on immiscible carbon sources, such as hydrocarbons (Franzetti *et al.*, 2010).

Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity; higher biodegradability; better environmental compatibility, higher foaming, high selectivity

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and specific activity at extreme temperatures, pH, and salinity; and the ability to be synthesized from renewable feedstocks (Desai and Banat, 1997). Due to all these properties, they have steadily gained increased significance in industrial and environmental applications such as bioremediation, soil washing, enhanced oil recovery and other general oil processing and related industries (Fracchia *et al.,* 2012). Furthermore, potential commercial applications in several other industries including paint, cosmetics, textile, detergent, agrochemical, food and pharmaceutical industries begin to emerge (Banat *et al.,* 2000; Fracchia *et al.,* 2012).

Biosurfactants are mainly classified by their chemical structure and their microbial origin. They are made up of a hydrophilic moiety, comprising an acid, peptide cations, or anions, mono-, di- or polysaccharides and a hydrophobic moiety of unsaturated or saturated hydrocarbon chains or fatty acids. These structures confer a wide range of properties, including the ability to lower surface and interfacial tension of liquids and to form micelles and microemulsions between two different phases. These compounds can be roughly divided into two main classes (Neu, 1996): low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids, proteins and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins that are collectively called bioemulsans (Rosenberg and Ron, 1997) or bioemulsifiers (Smyth *et al.,* 2010; Banat *et al.,* 2010).

The terms ‘biosurfactants’ and ‘bioemulsifiers’ are often used interchangeably, however in general those that reduce surface and interfacial tension at gas-liquid-solid interfaces are called biosurfactants and those that mainly reduce the interfacial tension between immiscible liquids or at the solid-liquid interfaces leading to the formation of more stable emulsions are called bioemulsifiers or bioemulsans. The former group includes low molecular-weight compounds, such as lipopeptides, glycolipids, proteins, while the latter includes high molecular weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins (Banat *et al.,* 2010; Fracchia *et al.,* 2012). Biosurfactants have wide structural diversity, ranging from glycolipids, lipopeptides, and lipoproteins to fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants. This endows them with their unique properties, including better environmental compatibility, greater foaming properties, higher selectivity and biodegradability (Das *et al.,* 2008). These molecules can be tailor-made to suit different applications by changing the growth substrate or growth conditions (Banat *et al.,* 2000).

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Table 2.2 Main classes of biosurfactants and respective producer microorganisms adapted from Silva *et al.* (2014)

Class/Type of Biosurfactant	Microorganisms
Glycolipids	
Rhamnolipids	*Pseudomonas aeruginosa*
Sophorolipids	*Torulopsis bombicola*, *T. apícola*
Trehalolipids	*Rhodococcus, erythropolis, Mycobacterium* sp.
Lipopeptides	
Peptide-lipid	*Bacillus licheniformis*
Viscosin	*Pseudomonas fluorescens*
Serrawettin	*Serratia marcenscens*
Surfactin	*Bacillus subtilis*
Subtilisin	*Bacillus subtilis*
Gramicidin	*Bacillus brevis*
Polymyxin	*Bacillus polymyxia*
Fatty acids, neutral lipids and phospholipids	
Fatty acid	*Corynebacterium lepus*
Neutral lipids	*Nocardia erythropolis*
Phospholipids	*Thiobacillus thiooxidans*
Polymeric surfactants	
Emulsan	*Acinetobacter calcoaceticus*
Biodispersan	*Acinetobacter calcoaceticus*
Liposan	*Candida lipolytica*
Carbohydrate-lipid-protein	*Pseudomonas fluorescens*
Mannan-lipid-protein	*Candida tropicalis*
Particulate surfactant	
Vesicles	*Acinetobacter calcoaceticus*

Efficient surfactants have low CMCs, i.e. less surfactant is necessary to decrease the surface tension to a given level. Microbial surfactants are most effective and efficient at their CMC which can be 10–40 times lower than that of chemical surfactants, thus less surfactant is necessary to get a maximum decrease in surface tension (Fracchia *et al.*, 2012; Rodrigues, 2015). The biosurfactants and their surface activities are not generally affected by environmental conditions such as temperature and pH (Sharma, 2014). Abu-Ruwaida *et al.* (1991) reported that heat treatment (autoclaving at 120 °C for 15 min) of some biosurfactants caused no appreciable change in biosurfactant properties such as the lowering of surface tension or interfacial tension and the emulsification efficiency. A lipopeptide from *B. subtilis*

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LB5a was stable even after autoclaving (121 °C for 20 min) and after 6 months' storage at –18 °C. The surface activity has been found to be stable in the range of pH 5.0 to pH 11.0 and NaCl concentrations up to 20% (Nitschke and Pastore, 2006).

The best studied low molecular weight biosurfactants so far are glycolipids and lipopeptides (Franzetti *et al.*, 2010).

Glycolipids

Glycolipids constitute the biosurfactant category that is more common and studied due to its high production yield, as well as its high application potential (Franzetti *et al.*, 2010). Generally speaking, these compounds are formed by different types of mono, di-, tri- and tetra-saccharide carbohydrates in combination with hydrophobic fractions of one or more long chain fatty acids or hydroxyl fatty acids (Franzetti *et al.*, 2010; de Jesús Cortés-Sánchez *et al.*, 2013). The main glycolipids are rhamnolipids, sophorolipids, trehalolipids and mannosylerythritol lipids.

Lipopeptides

Lipopeptides are low molecular weight biosurfactants showing potent with excellent interfacial and biological activity (Franzetti *et al.*, 2010). Lipopeptides are mainly produced by *Bacillus* (such as *Bacillus subtilis, B. licheniformis and B. polymyxa*), *Pseudomonas, Streptomyces, Aspergillus, Serratia and Actinoplanes* (Liu *et al.*, 2015). Lipopeptides are composed of a fatty acid tail linked to a peptide moiety and correspond to agroup of isoforms that differ by the composition of the peptide moiety, the length of the fatty acid chain and the link between the two parts. Lipopeptides are classified as cyclic lipopeptides and linear ones based on their chemical structures (Liu *et al.*, 2015). Surfactin, iturin, fengycin and lichenysin are among the most documented lipopeptides. Also, viscosin, tensin, arthrofactin, pseudofactin and syringomycin mainly produced by *Pseudomonas* isolates are widely described in literature reviews. Generally, lipopeptide surfactants are characterized by their low critical micelle concentration(CMC) and diverse functional properties (such as emulsification /de-emulsification, dispersing, foaming, viscosity reducers, solubilizing and mobilizing agents and pore forming capacity) permitting their use in many domains (Mnif and Ghribi, 2015). Several isoforms can be produced by the same strain. For example, Xia *et al*. (2014) reported the coproduction of surfactin, iturin and fengycin by *Pseudomonas sp.* WJ6. Bacillus related lipopeptides and *Pseudomonas* related lipopeptides are the most studied (Mnif and Ghribi, 2015).

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2.5 Influence of biosurfactants on the bioavailability and subsequent degradation of hydrophobic organic compounds

Owing to the intracellular location of hydrocarbon-degrading enzymes three steps are needed for microbial metabolism of the hydrocarbons. First, bacteria need to have access to the target compounds, i.e. the compounds have to be dissolved in the aqueous phase or bacteria have to directly adhere to the hydrocarbons; second, hydrocarbons that are adsorbed on the surface of the cell are transported across the membrane into the interior of the microorganism; finally, these hydrocarbons are degraded in the presence of enzymes (Hua and Wang, 2014). Hydrocarbon metabolism is always restricted to water/hydrocarbon interfaces since the oxygenases involved in their catabolic pathways are never extracellular but always membrane bound enzymes (Franetti *et al.*, 2010a; Płociniczak *et al.*, 2011).

Production of biosurfactants by bacteria is considered an important microbial strategy that influences the bioavailability of hydrophobic chemicals by changing the surface properties of bacterial cell or by dissolving and emulsifying these non-hydrophilic hydrocarbons. The application of biosurfactants in the remediation of organic compounds, such as hydrocarbons, aims at increasing their bioavailability (biosurfactant-enhanced bioremediation) or mobilizing and removing the contaminants by pseudosolubilisation and emulsification in a washing treatment (Banat *et al.*, 2010).

Biosurfactants can enhance hydrocarbon bioavailability by two mechanisms. The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (Mulligan and Gibbs, 2004). Addition of biosurfactants can be expected to enhance hydrocarbon bioavailability by mobilization, solubilization or emulsification (Pacwa-Płociniczak *et al.*, 2011).

2.5.1 Mobilization and micellar solubilization,

The mobilization mechanism occurs at concentrations below the biosurfactant CMC. Mobilization can be divided into displacement and dispersion. Displacement is the release of hydrocarbon droplets from porous media owing to a reduction in interfacial tension. From a theoretical perspective, entrapped hydrocarbon will undergo displacement if the interfacial tension between the aqueous and hydrocarbon phase is reduced sufficiently to overcome the capillary forces that caused the formation of residual saturation (Bai *et al.*, 1997; Santos *et al.*, 2016). Dispersion is the process in which the hydrocarbon is dispersed into the aqueous phase

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as very small emulsions. Emulsions are generally not thermodynamically stable, however, owing to kinetic constraints, they may remain stable for significant time periods. Dispersion is related to both the interfacial tension and the surfactant concentration, and is different from displacement in that the displacement process is only related to the interfacial tension between aqueous and hydrocarbon phases and no emulsion forms (Bai *et al.*, 1997). In turn, above the biosurfactant CMC the solubilization process takes place, at these concentrations biosurfactant molecules associate to form micelles, which dramatically increase the solubility of the PAH. The process of incorporation of these molecules into a micelle is known as pseudosolubilization (Urum and Pekdemir, 2004). Supplementation of rhamnolipids above CMC enhanced the apparent aqueous solubility of hexadecane, favored biodegradation of hexadecane, octadecane, n-paraffins, creosotes and other hydrocarbon mixtures in soil and promoted bioremediation of petroleum sludge (Franzetti *et al.*, 2010).

2.5.2 Emulsification of non-aqueous phase liquid (NAPL) pollutant

Biosurfactants can decrease the interfacial tension between an aqueous and a non-aqueous phase. This may lead to the formation of micro-emulsions or, with energy input, to the formation of macro-emulsions. This results in an increase in the contact area, enabling improved mass transport of the pollutant to the aqueous phase and in mobilisation of sorbed liquid-phase pollutant; (Volkering *et al.*, 1998)

Extracellular biosurfactants and bioemulsifiers increase oil/water interfaces enhancing substrate mass transfer and allowing more microorganisms to contact the hydrocarbon substrates. Emulsifiers increase the hydrocarbon/water interfaces stabilizing oil droplets in the water/oil emulsion (Franzetti *et al.*, 2010). High-molecular-weight biosurfactants have a great potential in stabilizing emulsions between liquid hydrocarbons and water, thus increasing the surface area available for bacterial biodegradation (Banat *et al.*, 2010).

2.5.3 Biosurfactant induced cell surface hydrophobicity.

Surfactants can adsorb on to bacterial cells and change the cell surface hydrophobicity or increase the membrane permabilization (Kaczorek *et al.*, 2011). Cell surface hydrophobicity (CSH) is an important property of microorganism, which affects the efficiency of various bioprocesses, such as cell-to-cell interaction and adherence of bacteria to hydrophobic organic compounds. Thus, it is recognized as one of the crucial factors in determining microbial adhesion to bioremediation interfaces (Liu *et al.*, 2004; Liu *et al.*, 2014). Chen and Zhu (2005) found a more hydrophobic surface of *P. aeruginosa* cell after rhamnolipid-induced release of

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LPS from the cell surface and a subsequent enhancement of accumulation of hydrophobic hydrocarbons on the cell surface. Such effect may enhance the affinity of the degrading microbial cells to the hydrophobic organic contaminants (HOCs) and their subsequent metabolism. Further more since modifications of surface properties, caused by the surfactant, are significant at concentrations below the CMC, (Neu, 1996; Herman *et al.*, 1997; Zhong *et al.*, 2007; Rodrigues *et al.*, 2013), the approach makes extensive application of biosurfactants in this field economically possible. The release of lipopolysachride (LPS), an important hydrophilic component of the cell surface, by *Pseudomonas spp*. induced by sub-CMC levels of rhamnolipids allowed a more efficient uptake of hexadecane by rendering the cell surface more hydrophobic (Al-Tahhan *et al.*, 2000).

However, the mechanisms of uptake are complicated and may be strain and/or biosurfactant specific (Ward, 2010). The mechanisms of uptake of hexadecane by *Acinetobacter calcoaceticus* RAG1, *Rhodoccus erythropolis* ATCC 19558 and *R. erythropolis* BCG112 appeared to be different to that of *Pseudomonas aeruginosa* UG2.6. Rhamnolipid biosurfactant stimulated degradation of hexadecane by *P. aeruginosa* UG2, the rhamnolipid-producing organism, but did not promote biodegradation by other biosurfactant-producing strains, A. *calcoaceticus* RAG1, *R. erythropolis* ATCC 19558 or *R. erythropolis* BCG112 (Noordman and Janssen, 2002).

The microorganism's ability to place themselves in an ecological niche, in which they can multiply, is one of their survival strategies. Biosurfactants play a crucial role in this strategy, since they are responsible for the interaction of the microorganism with a specific surface. In the case of biosurfactants that are excreted, they can form a conditioning film on the interface, thereby stimulating certain microorganisms to attach to a surface while inhibiting the attachment of others (Rosenberg and Ron, 2005).

However, the sorption of surfactants to bacteria and to interfaces can either enhance or inhibit adhesion, depending on the nature of the surfaces and the surfactant itself (Banat *et al.*, 2010; Rodrigues *et al.*, 2013). Some strains have hydrophobic cell surfaces and that they increase or decrease their cell hydrophobicity by respectively exposing outwardly or inwardly the hydrophobic moieties of the cell-bound bisurfactants (Banat *et al.*, 2010). For example, the cell-surface hydrophobicity of *A. lwoffii Acinetobacter* RAG-1 strain that produces cell-bound biosurfactant showed a reduction of their hydrophobicity due to biosurfactant addition (Rosenberg *et al.*, 1983). Beal and Betts (2000) showed that the cell surface hydrophobicity

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increased by the biosurfactant strain more than a non-biosurfactant producing strain during growth on hexadecane. The rhamnolipids also increased the solubility of the hexadecane from 1.8 to 22.8 mg/L.

2.6 Biosurfactant Production

2.6.1 Biosynthesis

Two primary metabolic pathways, namely, hydrocarbon and carbohydrate, are involved in the synthesis of their hydrophobic and hydrophilic moieties, respectively. The pathways for the synthesis of these two groups of precursors are diverse and utilize specific sets of enzymes. In many cases, the first enzymes for the synthesis of these precursors are regulatory enzymes; therefore, in spite of the diversity, there are some common features of their synthesis and regulation (Desai and Banat, 1997). According to Syldatk and Wagner (1987), the following possibilities exist for the synthesis of different moieties of biosurfactants and their linkage:

(i) The hydrophilic and hydrophobic moieties are synthesized de novo by two independent pathways;
(ii) The hydrophilic moiety is synthesized de novo while the synthesis of the hydrophobic moiety is induced by substrate;
(iii) The hydrophobic moiety is synthesized de novo, while the synthesis of the hydrophilic moiety is substrate dependent; and
(iv) The synthesis of both the hydrophobic and hydrophilic moieties is substrate dependent.

The elucidation of these mechanisms for apaticular system is impotant for design of growth media and growth conditions for large scale production, as well as for the induction of biosynthetic pathways by addition of precursor molecules. The carbon source influences biosurfactant production either by induction or repression (Sineriz *et al.,* 2001)

Several studies have aimed to optimize the biosurfactant production process by changing the variables that influence the type and amount of biosurfactant produced by a microorganism Reis (2012). The production of microbial metabolites is governed by several factors: the nature of the carbon source; the concentrations of nitrogen and multivalent ions in the media; culture conditions like pH, temperature, agitation rate and oxygen availability; the nature of the

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selected microorganism; and, the adopted fermentation strategies (Nitschke *et al.*, 2005; Pornsunthorntawee *et al.*, 2010).

2.6.2 Kinetics of Fermentative Production

The fermentation process is the key factor which governs the overall economics of biosurfactant production as the raw materials account for about 10-30% of the overall cost of biosurfactant production. Also, the production patterns or kinetics of fermentative production by different species are different, which can be grouped into following types (Khire, 2010; Pornsunthorntawee *et al.*, 2010; Joshi and Desai, 2010)

1. Growth-associated: For growth-associated production, parallel relationships exist between growth, substrate utilization and biosurfactant production, as observed in rhamnolipid production by some *Pseudomonas* spp. Manresa *et al. (*1991) and lipopeptide biosurfactant C9-BS by *Bacillus subtilis* C9 (Kim *et al.*, 1997)

2. Growth limiting conditions: Production under growth-limiting conditions can be characterized by a sharp increase in the biosurfactant level as a result of a limitation of one or more medium nutrients (e.g., nitrogen or iron). This was observed in number of *Pseudomonas spp.*(Zhang and Miller, 1992) and *Bacillus spp.* (Davis *et al.*, 1999) ,when the culture reaches the stationary phase of growth due limitation of nitrogen and/or iron.

3. Resting cells: When using resting or immobilized cells, the microorganism is separated from the culture medium after cultivation under optimal growth conditions and the wet biomass is subsequently used for the biosurfactant production. For rhamnolipid production, the widely‐used fermentation strategies are production under growth‐limiting conditions and production by resting or immobilized cells (Nitschke *et al.*, 2005). Pilot plant production of rhamnolipid biosurfactant by resting cells of *Pseudomonas aeruginosa* resulted in the reduction of cost of product recovery as the growth and the product formation phases can be separated (Reiling *et al.*, 1986)

4. Precursor supplement: Addition of certain precursors in the fermentation medium causes both qualitative and quantitative increase in biosurfactant production. As reported for the addition of lipophilic compounds to the culture medium of *T. bombicola* (Cooper and Paddock,

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1984) resulted in increased biosurfactant yields. Therefore, process development and fermentations have to be optimized on a case by case basis.

2.6.3 Factors Affecting Biosurfactant Production

The type, quality and quantity of biosurfactant produced are influenced by the nature of the carbon source, the concentration of nitrogen, phosphorus, magnesium, ferric, and manganese ions in the culture medium and the culture conditions like pH, temperature, agitation rate and and oxygen availability; the nature of the selected microorganism; and, the adopted fermentation strategies (Guerra-Santose *et al.*, 1986; Desai and Banat, 1997; Nitschke *et al.*, 2005).

2.6.3.1 Nutritional Factors Affecting biosurfactant Production

Carbon Source

Biosurfactants are produced by a variety of microrganisms, either extracellularly or attached to parts of the cell, predominantly during their growth on water-immiscible substrates (Amaral *et al.*, 2010). Production can be growth associated. In this case, they can either use the emulsification of the substrate (extracellular) or facilitate the passage of the substrate through the membrane (cell membrane associated). Biosurfactants, however, are also produced from carbohydrates, which are very soluble (Mulligan *et al.*, 2005). Both water-soluble and water-insoluble carbon sources have been utilized for production of biosurfactant by *Pseudomonas sp* (Banat and Desai, 1997; Abdel-Mawgoud *et al.*, 2011). Biosurfactant product, however was inferior to that obtained with water-immiscible substrates such as n-alkanes and olive oil (Syldatk *et al.*, 1985).

Hydrophobic carbon sources such as vegetable oils are especially effective at promoting the production of rhamnolipids (RLs). Rhamnolipids are usually produced by *P. aeruginosa* also in media containing water-soluble carbon sources such as glucose, glycerol, mannitol, and ethanol as a carbon source, particularly, when the cells become limited for nitrogen (Guerra-Santos *et al.*, 1986; Mulligan and Gibbs, 1993; Sim *et al.*, 1997) indicating that they may serve other roles besides being involved in solubilizing hydrophobic substrates; nevertheless, the final surfactant concentration was lower than that obtained when *n*-alkanes and vegetable oils were the carbon sources (Pantazaki *et al.*, 2011). When *Arthrobacter paraffineus ATCC* 19558 was grown on D-glucose, supplementation with hexadecane in the medium during the

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stationary growth phase resulted in a significant increase in biosurfactant yield (Duvnjak *et al*., 1982). Other authors observed little biosurfactant production when cells were growing on a readily available carbon source; only when all the soluble carbon was consumed and when water-immiscible hydrocarbon was available was biosurfactant production triggered (Banat *et al.,* 1991; Desai and Banat, 1997). Sim *et al.* (1997) have tested mixture of vegetable oils (canola oil, soy bean and glucose), for rhamnolipid production by *P. aeruginosa* UW-1 and reported 10-12 fold increase in rhamnolipid production on vegetable oils in comparison to glucose.

Although it is also possible to produce biosurfactants in the presence of water soluble carbon sources, several studies show that often higher production yields are obtained when hydrophobic substrates are added (Amaral *et al.,* 2010). A number of works describe the importance of combining a water insoluble substrate with a carbohydrate in the culture medium. A very low yield was found when only either glucose or vegetable oil was used for the production of a biosurfactant by T. bombicola, but the yield increased to 70 g/L when both carbon sources were provided together (Cooper and Paddock, 1984).

Three mechanisms, namely, induction, repression, and nitrogen and multivalent ions, operate in the regulation of biosurfactant production (Banat and Desai, 1997). The carbon source influences biosurfactant synthesis by either induction or repression. Biosurfactant production can be induced by hydrocarbons or water-insoluble substrates, this effect, described by many authors, concerns many of the interfacially active compounds (Nitschke *et al.,* 2005). Rapid biosurfactant production was observed in a *Pseudomonas* strain during growth on glucose and oleic acid, when oleic acid was utilized upon the exhaustion of glucose (Banat *et al.,* 1991). The induction of sophorolipid synthesis by addition of long- chain fatty acids, hydrocarbons, or glycerides to the growth medium of *Torulopsis magnolia* (Tulloch *et al.,* 1962) and of glycolipid *in P. aeruginosa SB-30* by addition of alkanes (Chakrabarty, 1985) has been reported. Induction appears to be influenced not only by the lengths of fatty acids in the triglycerols, but also by the number of unsaturations (Lima *et al.,* 2003). On the contrary catabolic repression of rhamnolipid production was observed when applying glucose, acetate and tricarboxylic acids (Hauser and Karnovsky, 1957). When *P. aeruginosa* UG2 was grown on hydrophobic substrates such as corn oil, lard, and long chain alcohols, production was around 100-165 mg of rhamnolipid/g substrate, whereas when hydrophilic substrates such as

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glucose and succinic acid were used, only 12-36 mg/g substrate was obtained (Mata-Sandoval *et al.*, 1999).

Nitrogen, Minerals, and Iron Sources

Medium constituents other than carbon source also affect the production of biosurfactants (Ilori *et al.*, 2005). Nitrogen is important in the biosurfactant production medium because it is essential for microbial growth as protein and enzyme syntheses depend on it. Different nitrogen compounds have been used for the production of biosurfactants, such as urea, peptone, yeast extract, ammonium sulphate, ammonium nitrate, sodium nitrate, meat extract and malt extract, etc. Yeast extract is the most used nitrogen source for biosurfactant production, but its concentration depends on the microorganism and the culture medium (Amaral *et al.*, 2010).

Nitrogen can be an important key to the regulation of biosurfactant synthesis, and there is evidence that the nitrogen plays an important role in the production of surface-active compounds by microbes (Cameotra and Makkar, 1998). Among the inorganic salts tested, ammonium salts and urea were preferred nitrogen sources for biosurfactant production by *Arthrobacter paraffineus* (Duvnjak *et al*., 1983), whereas for overproduction of rhamnose lipids by *P. aeruginosa*, the highest yield are obtained if nitrate is used rather than ammonium as a nitrogen source (Wu *et al.*, 2008).

For the induction of Rhamnolipid (RL) formation in a biotechnological set-up, an appropriate limitation must be achieved. For this purpose, the limitation of nitrogen, phosphorus, or multivalent ions in combination with an excess carbon are employed (Abdel Mawgoud *et al.*, 2011). Abdel Mawgoud *et al.* (2011) reported that not only the type of carbon and nitrogen source but also the respective C/N ratios strongly influence total ramnolipid biosurfactant productivity. In fermentative processes, the C/N ratio affects the buildup of metabolites. High C/N ratios (i.e., low nitrogen levels) limit bacterial growth, favouring cell metabolism towards the production of metabolites.

In constrast, excessive nitrogen leads to the synthesis of cellular material and limits the buildup of products (Santos *et al.*, 2016). Guerra-Santos *et al.*(1986) reported that for *P. aeruginosa* DSM 2569 (37 °C, pH 6.5, glucose, nitrate) C/N ratios between 16/1 and 18/1 lead to the highest RL productivity while no RLs could be observed at C/N ratios lower than 11/1.

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Matsufuji *et al* (1997) reported that a high production of rhamnolipids was achieved when *Pseudomonas aeruginosa* IFO 3924 was cultivated under nitrogen-limiting conditions at a carbon to nitrogen (C/N) ratio of 18/1. Santa Anna *et al.* (2002) found that a C/N ratio of 60/1 caused the overproduction of rhamnolipids by *Pseudomonas aeruginosa* PA1.47. These results suggest that the effect of C/N ratio on the rhamnolipid production depends on the bacterial strains (Pornsunthorntawee *et al.*, 2010).

Many works have demonstrated that the limitation of multivalent ions and nitrogen is able to cause the overproduction of rhamnolipids. It was reported that the rhamnolipid production by *Pseudomonas aeruginosa* DSM2659 was promoted as the iron concentration in the culture media was reduced (Guerra-Santos *et al.*, 1984). Mulligan *et al.* (1989) found that an inorganic phosphate-limited medium provided the best yield of rhamnolipid production by *Pseudomonas aeruginosa* ATCC 9027. Abu-Ruwalda *et al.* (1991), observed nitrate to be the best source of nitrogen for biosurfactant production by *Pseudomonas* strain 44T1 and *Rhodococcus* strain ST-5 growing on olive oil and paraffin, respectively. The production started after 30 h of growth, when the culture reached nitrogen limitation, and continued to increase up to 58 h of fermentation. Production under growth-limiting conditions was observed in *Bacillus* spp. when the culture reaches the stationary phase of growth due to limitation of nitrogen (Davis *et al.*, 1999). Soberon-Chavez *et al*. (2005) reported that elevated C/N and C/P ratios promote rhamnolipids production, while high concentrations of divalent cations, especially iron, are inhibitory.

Iron limitation stimulates biosurfactant production in *P. fluorescens* (Persson *et al.*, 1990) and *P.aeruginosa* (Guerra-Santos *et al.*, 1986), whereas addition of iron and manganese salts stimulates biosurfactant production in both *B. subtilis* (Cooper *et al.*, 1981) and *Rhodococcus* sp. (Abu-Ruwaida *et al.*, 1991). The type of nitrogen source is crucial to cell growth and rhamnolipid production. It was found that sodium nitrate ($NaNO_3$) was the most efficient nitrogen source for the rhamnolipid production by *Pseudomonas aeruginosa* EM1 in terms of rhamnolipid yields; however, using urea and yeast extract, organic compounds, as nitrogen sources provided better cell growth (Wu *et al.*, 2008). In fact, it has been reported that the organic nitrogen source can promote cell growth, but it is unfavorable for the production of glycolipid biosurfactant (Pornsunthorntawee *et al.*, 2010).

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2.6.3.2 Environmental Factors

Environmental factors and growth conditions such as pH, temperature, agitation, and oxygen availability affect biosurfactant production through their effects on cellular growth or activity (Desai and Banat, 1997). Rhamnolipid production in Pseudomonas spp. was at its maximum at a pH range from 6 to 6.5 and decreased sharply above pH 7 (Guerra-Santos *et al.*, 1984).

While rhamnolipid formation with glycerol as the sole carbon source showed a wide optimum ranging from 27 °C up to 37 °C, production of rhamnolipids from *n*-alkanes had a sharp optimum at 37 °C (Syldatk *et al.*,1985).

Aeration and agitation rates are important factors that influence the production of biosurfactants, since they facilitate the oxygen transfer from the gas phase to the aqueous phase and it may also be linked to the physiological function of microbial emulsifiers. It has been suggested that the production of bioemulsifiers can enhance the solubilization of water-insoluble substrates and consequently facilitate nutrient transport to microorganisms. Therefore, higher shear stress may induce surfactant secretion as the contact of organic droplets dispersed in water with microorganisms becomes more difficult (Amaral *et al.,* 2010).

Wei *et al* (2005) reported that the rhamnolipid production by *Pseudomonas aeruginosa* J4 increased about 80% when the agitation rate was increased from 50 to 200 rpm. Further increasing the agitation rate decreased the transfer efficiency of oxygen gas into the liquid medium, leading to unsuitable conditions for the biosurfactant production

2.6.4 Recovery of Biosurfactants

Even if optimum production is obtained using optimal media and culture conditions the production process is still incomplete without an efficient and economical means for the recovery of the products. Thus, one important factor determimng the feasibility of a production process on a commercial scale is the availability of suitable and economic recovery and downstream procedures (Saharan *et al.,* 2011). Downstream processing costs account for nearly 60% of the total production costs, a significant proportion of the cost of production and the criteria that govern the selection of a specific recovery method include (Saharan *et al.,* 2011; Abdel Mawgoud *et al.,* 2011):

(1) The cost associated with the extraction method, which adds to the price of the final product

(2) The proposed purpose of the final product, which influences the level of purity required,

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(3) The adaptability of the method to a particular industrial fermentation process.

Biosurfactant recovery depends mainly on its ionic charge, water solubility, and location (intracellular, extracellular or cell bound) with respect to the cell surface. One of the simplest methods of recovery is by acid precipitation (De´ziel *et al.* 1999; Abdel-Mawgoud *et al.*, 2011).

At a pH of 2–3 rhamnolipids are protonated and become insoluble in water, therefore they precipitate. Yields of up to 98% have been reported for acid precipitation after cell removal and subsequent heat treatment in a patent. The precipitate can then be further processed by organic solvent extraction methods (Müller *et al.,* 2012). The used organic solvents are mostly ethyl acetate (Syldatk *et al.* 1985), chloroform (Mata-Sandoval *et al.* 1999), ethyl acetate or chloroform–methanol (2:1) are applied (Heyd *et al.*, 2008). In general, the extraction yield can be improved by an acidification of the sample prior to extraction, as biosurfactants are present in their protonated form and, hence, are less soluble in water. Extraction with chloroform-methanol, dichloromethane-methanol, butanol, ethyl acetate, pentane, hexane, acetic acid, ether, etc. constitutes the most commonly used method in biosurfactant downstream processing.

The most widely employed products are different ratios of chloroform and methanol, which facilitate the adjustment of the polarity of the extraction agent to the extractable target material. The disadvantages of using organic solvents for biosurfactant recovery include the large amount of solvent required and the increase in production costs due to the price of expensive solvents. (Santos *et al.,* 2016). In a recent development, continuous removal of biosurfactant during fermentation by different techniques has increased the cell density in the reactor and eliminated product inhibition, resulting in a severalfold net increase in biosurfactant yield (Desai and Banat, 1997). One of the successful techniques involved a continuous in situ removal of surfactin from fermentation broth by foam fractionation. In this technique, foam was collected and acidified to pH 2 with concentrated HCl and the precipitated surfactin was extracted in dichloromethane (Mulligan *et al.,* 1989).

2.6.5 Structural Characterization of Biosurfactants

For identification and chacterization of various elements of an unknown biosurfactant detailed structural analysis is necessary. This is achieved by mass spectrometry (MS), Infrared (IR) spectroscopy, and nuclear magnetic resonance (NMR) spectroscopy (Heyd *et al.,* 2008).

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Fourier transform infrared spectroscopy (FTIR) is most useful for identifying types of chemical bonds (functional groups), therefore it can be used to elucidate some unknown components of unknown given mixture. Also, one can use the unique collection of absorption bands to confirm the identity of a pure compound (Sasidharan *et al.,* 2010). Thin-layer chromatography (TLC) has also been extensively used for determining the composition of biosurfactants (Heyd *et al.,* 2008). For elucidation of the chemical structures of biosurfactant the crude extract is purified by solvent fractionation using silica gel column chromatography (Chiewpattanakul *et al.,* 2010). Several studies have been conducted on Spectroscopic Methods for Structural Analysis of of biosurfactants and other bioproducts (Heyd *et al.,* 2008; Kumirska *et al.,* 2010; Sharma *et al.,* 2015).

Sharma *et al.* (2014) evaluated the molecular composition of the crude biosurfactant by FTIR, TLC, ^{1}H NMR, ^{13}C NMR, and GC MS, and the results suggested that it consisted of several compounds such as octadecanoic acid as main lipid consisting of long aliphatic chain and polysaccharides. Structural characterization of biosurfactant-TT42 using TLC, Fourier transform infrared spectroscopy (FTIR), GC-MS, and matrix-assisted laser desorption ionization time of flight (MALDI-TOF) suggested that it was a mixture of lipopeptide species, all having a common hydrophilic cyclic heptapeptide head linked to hydrophobic tails of different lengths of 3β-OH-fatty acids (Lotfabad *et al.,* 2010). Suthar and Nerurkar (2016), conducted Structural characterization of extracted biosurfactant by thin-layer chromatography (TLC), which revealed the presence of two compounds corresponding to those of authentic mono- and di-rhamnolipid. The identity of two the structurally distinguished rhamnolipids was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Liquid chromatography/mass spectrometry (LC/MS) of extracted biosurfactant revealed up to seventeen different rhamnolipid congeners. Further quantification showed di-rhamnolipids as the major compound (77.2%), while monorhamnolipids comprising a smaller proportion (22.8%) of MR01 biosurfactant.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials and Methods

3.1.1 Chemicals

Acetonitrile, Ethyl acetate, Hexane and Acetone, all HPLC grade, were obtained from Merck, Germany, ultrapure water (Milli-Q water). Naphthalene (99%), Anthracene (99.5%), Phenanthrene (98%), Fluoranthene (98%), Pyrene (98%), were purchased from Sigma–Aldrich (USA). Stock Standard Solution of 16 PAHs (listed by the US Environmental Protection Agency; EPA);16 parent PAH compounds (naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3-cd]pyrene) each at 10µg/mL in methylene chloride, Chemicals used to prepare culture media, was obtained from Merk , Germany. Creosote was a gift from local wood treatment plant.

3.1.2 Stock Solutions

1. A stock solution of pyrene (5 mg mL^{-1}) was prepared by dissolving appropriate quantity of pyrene crystals in hexane. PAH stock solutions were stored at 4 °C in darkness.

2. The 16PAHs or individual PAHs Calibration standard solutions were prepared by suitable dilution of the PAH Standard Stock Solutions with acetonitrile.

3. Stock solutions of the biosurfactants were prepared at (10 mg mL^{-1}) from the extracted lipopeptides by dissolving the brownish lipopeptide recovered in Mineral Salt Medium (MSM) and heating for 10 minutes at 50 °C. Stocks were then stored at 4 °C.

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3.1.3 Mineral Salt Medium Composition

All media were prepared using distilled water; recipes are per litre of distilled water. The Media were sterilised via autoclaving at 121°C for 15 minutes. It was composed of 6.0 g/L $(NH_4)_2SO_4$, 0.4 g/L $MgSO_4$ x 7 H_2O, 0.4 g/L $CaCl_2$ x 2 H_2O, 7.59 g/L Na_2HPO_4 x 2 H_2O, 4.43 g/L KH_2PO_4, and 2 mL/Lof trace element solution.

The trace element solution consisted of; 20.1 g/L EDTA (disodium salt), 16.7 g/L $FeCl_3$ x 6 H_2O, 0.18 g/L $CoCl_2$ x 6 H_2O, 0.18 g/L $ZnSO_4$ x 7 H_2O, 0.16 g/L $CuSO_4$x 5 H_2O, and 0.10 g/L $MnSO_4$ x H_2O.

Nutrient Agar

This medium was prepared by adding 28 g of Nutrient Agar (composition agar, 15 g/Lmeat extract, 1 g/L, peptone, 5 g/L,sodium chloride, 5 g/L, yeast extract, 2 g/L) to one litre of distilled water

Nutrient broth

Medium prepared by adding 16g of broth (composition; meat extract, 1 g/L, peptone, 5 g/L,sodium chloride, 5 g/L, yeast extract, 2 g/L) to one litre of distilled water

3.2 Bacterial Strain

Bacterial strains / microbial consortia used in this work were enriched and /or selectively isolated from the contaminated environment, Environmental isolates or consortia were obtained from the sources using the methods contained in Section 3.3.1. Stocks of organisms were stored at −80 °C in MSM mixed with sterile glycerol at a final concentration of 30%.

3.3 Microbiological Methods

3.3.1 Enrichment and Isolation of PAH Degrading Bacteria

Soil samples were collected from a creosote wood treatment plant in Pretoria West, South Africa with chronic contamination of creosote and other hydrocarbon spills in pre-sterilized glass bottles and transported to the laboratory for isolation of bacteria. A microbial consortium was obtained via selective enrichment in 250 mL Erlenmeyer flasks. A 5 g contaminated soil

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sample was inoculated in to 100 mL of mineral salt medium (MSM) according to Trummler et al. (2003). Creosote 5% (v/v) was used as a sole carbon and energy source. The Enrichment was conducted at 30 °C and 120 rpm in a Labcon SPL-MP 15 Orbital Shaker (Labcon Laboratory Services, South Africa) and kept for about 7 days. After 7 days of incubation, 10 mL of enriched culture was transferred into another flask containing 100 mL fresh sterile MSM with Creosote 5% (v/v) and incubated. A total of five subsequent enrichments were carried out to selectively enrich PAH-utilizing microbial consortium. Isolation and purification of the bacteria were done via spread and streak plate techniques on Luria-Bertani (LB) agar. Aliquots from the culture dilutions (100 µL) were spread on the LB agar plates and incubated at 37 °C for 3 days. Pure cultures were stored at −80 °C in MSM mixed with sterile glycerol at a final concentration of 30%.

3.3.2 Screening of Biosurfactant-Producing Bacteria

One loop of each isolate is transferred to test tubes containing 5 ml sterile nutrient broth (NB) and shaken (150 rpm) at 30 °C for 24 h.

A 100-µl sample of each cell culture was transferred to 5 ml of MSM medium supplemented with 2% (v/v) of different carbon sources (glycerol) in a rotary shaker at 30 °C and 150 rpm for 24 h.

Preliminary Screening assays for biosurfactant-producing isolates is performed by using the qualitative drop-collapsing test and by oil spreading technique, of the culture supernatant after centrifugation at 8,500 rpm and 4 °C for 10 min

- Oil spreading technique- The oil spreading assay was developed by Morikawa *et al*. (2000). For this assay, 10 µl of crude oil is added to the surface of 40 ml of distilled water in a petri dish to form a thin oil layer. Then, 10 µl of culture or culture supernatant are gently placed on the centre of the oil layer. If biosurfactant is present in the supernatant, the oil is displaced and a clearing zone is formed. The diameter of this clearing zone on the oil surface correlates to surfactant activity, also called oil displacement activity. For pure biosurfactant a linear correlation between quantity of surfactant and clearing zone diameter is given.

- Drop collapse method- Drop collapse test was performed by following the procedure described by Jain *et al.* (1991) and modified by Bodour and Miller-Maier (1998). This assay

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relies on the destabilization of liquid droplets by surfactants. Therefore, drops of a cell suspension or of culture supernatant are placed on an oil coated, solid surface. If the liquid does not contain surfactants, the polar water molecules are repelled from the hydrophobic surface and the drops remain stable. If the liquid contains surfactants, the drops spread or even collapse because the force or interfacial tension between the liquid drop and the hydrophobic surface is reduced. 2µl of mineral oil was added to micro-titre plate and incubated at room temperature and then 5µl of culture was added to the surface of oil, bio-surfactant producing cultures gave flat drops with scoring system ranging from partial to complete spreading on the oil surface after inspecting it for one minute.

3.3.3 Characterization and Identification of Microorganisms

3.3.3.1 Identification of bacterial strain

After the screening for biosurfactant producing isolates bacterial cultures with the highest activity towards the biosurfactant production, were then taken for 16S rRNA gene sequencing identification in the university of Pretoria department of Microbiology. Genomic DNA was extracted from purified colonies according to the protocol described in the Wizard Genomic DNA purification kit(Promega Corporation, Madison, WI, USA). 16S rRNA genes were amplified by a reverse transcriptase–polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8–27; Primer pH to position 1541–1522 of the 16S gene under the following reaction conditions: 1 min at 94 °C, 30 cycles of 30 s at 94 °C, 1 min at 50 °C and 2 min at 72 °C, and a final extension step of 10 min at 72 °C). PCR fragments were then cloned into pGEM-Teasy (Promega) [Promega Wizard® Genomic DNA Purification Kit (Version 12/2010) (Tikilili and Chirwa, 2011). The 16S rRNA gene sequence of strains were aligned automatically to reference sequences of their respective genus obtained from the Gen Bank (http://www.ncbi.nlm.nih.gov/BLAST/), and a phylogenetic tree was constructed based on their neighbor-joining method using the software MEGA version 6.0 (Tamura *et al.*, 2013). Partially sequenced amplified 16S rDNA fragment was compared with other gene sequences in Gen Bank using (http://www.ncbi.nlm.nih.gov/BLAST/) and aligned with gene sequence of our isolates. The aligned sequences were used to construct a distance matrix, after the generation of 1000 bootstrap sets that was subsequently used to construct a phylogenetic tree using the neighbor-joining method MEGA version 6 software (Tamura *et al.*, 2013).

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All of the selected bacteria were maintained in a nutrient broth containing a glycerol solution at a concentration of 30% (v/v) and stored at - 80 °C before use.

3.3.3.2 High-Throughput 454 Pyrosequencing Analysis

3.3.3.2.1 DNA extraction, PCR amplification conditions and 454-pyrosequencing

DNA was isolated from the samples using a modified SK method according to Ságova-Mareˇcková *et al.* (2008). Three parallel extractions of soil material (0.5 g per extraction) were performed, and the resulting aliquots were pooled to form a single representative DNA sample. Samples were prepared for 454 pyrosequencing using two-step PCR (Sundberg *et al.*, 2013). Initial PCR Master mix was 1× Phusion HF buffer (with $MgCl_2$; Finnzymes Oy, Espoo, Finland), 0.2 mM of dNTP mixture, 0.5 U Phusion Hot Start DNA polymerase (Finnzymes), 0.5 µM of each primer, 1 µL of template, and sterile Milli-Q water to a final volume of 25 µL. The primers used for pyrosequencing were MPRK341F (5′CCTAYGGGRBGCASCAG-3′) and MPRK806R (5′GGACTACNNGGGTATCTAAT-3′). PCR reactions, bacteria and archaea 16S rRNA primers, and PCR thermal programs were carried out as previously described by Albers *et al*. (2015). Amplicons were purified (AmPure magnetic beads XP; Beckman Coulter, Inc.; Brea, CA) and quantified using a Qubit fluorometer (Invitrogen, Life technologies, Carlsbad, CA) before 454 pyrosequencing. A two region 454 sequencing run was performed on a 70 × 75 GS Pico Titer Plate by using a Genome Sequencer FLX pyrosequencing system according to the manufacturer's instructions (Roche, Mannheim, Germany). Sorting and trimming of sequences > 150 bp was done by the Pipeline Initial Process (http://rdp.cme.msu.edu) as previously described (Cole *et al.*, 2009).

3.3.3.2.2 Bioinformatic Analyses.

Sequence processing was performed in Mothur (Schloss *et al.*, 2009). In Mothur, poor quality sequences were set as sequences with a length less than 550 bases, contained ambiguous bases and homopolymers greater than 6 bases or did not have a barcode and a primer sequence. Multiple sequence alignments were performed using the program MAFFT, version 6.925 (Katoh *et al.*, 2009), with the E-INS-i strategy assuming multiple conserved regions and long gaps. After subsequent preclustering to an alignment of known 16S bacterial sequences, chimeras check with UCHIME (Edgar *et al.*, 2011), the sequences were aligned and clustered into operational taxonomic units (OTUs) using the furthest neighbour algorithm with 97%

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similarity threshold. Bacterial data were summarized at phylum, class, order, family, genus and species levels. The OTUs were taxonomically identified by the RDP-II Naïve Bayesian Classifier (Wang *et al.*, 2007) using an 80% confidence threshold.

3.4. Biosurfactant Production and Characterization

3.4.1 Culture Media for Biosurfactant Production

The organism was grown on the mineral salt medium (MSM) (Trummler *et al.*, 2003).

3.4.2 Biosurfactant Production, Recovery and Purification

The strain was stored at −80 °C in the MSM containing 30% (v/v) glycerol solution until use. To prepare pre-cultures, the strain was grown in 50 mL of the MSM supplemented with glycerol (4%, w/v) at 37 °C, pH 7 for 24 h in a rotary shaker Labcon SPL-MP 15 Orbital Shaker (Labcon Laboratory Services, South Africa) at 150 rpm. Afterwards, 5 mL of this pre-culture was transferred to a 1000 ml Erlenmeyer flask containing 500 ml of MSM supplemented with glycerol (4%, w/v) or 3% (v/v) sunflower oil and 0.01% (w/v) anthracene [for experiments with *Bacillus cereus* SPL-4 and for an other experiment with *Paenibacillus dendritiformis* CN5] and incubated for 72 and 120 h for glycerol and oil respectively at a shaker speed of 150 rpm. After growth for 72 h or 120 h for glycerol and oil correspondingly, cells were removed by centrifugation 10 min at 12,000 rpm at 4°C to obtain cell-free supernatants and crude biosurfactant was precipitated from the supernatant by adding 6 N HCl to pH of 2.0 and extracted with chloroform and methanol (2:1).

The solvent was evaporated in vacuum. The residue was dissolved in methanol and filtered through a 0.22 µm filter (Millipore). The crude extracts were further purified through a silica gel column, Silicagel (60–200; Merck KGa) column. Elution was carried out first with chloroform to remove pigment and impurities of hydrocarbons, then with a mixture of chloroform and methanol 80:20 v/v (100 ml), then 35:65 v/v (100 ml) collecting two fractions after discarding the first chloroform eluted fraction. The fractions eluted were collected and the solvents were evaporated in a rotary evaporator at 40 °C.

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3.4.3. Surface Active Properties

3.4.3.1 Surface Tension and Critical Micelle Concentration

The surface tension of cell free supernatant was measured using a Du Nouy tensiometer (KrüssTensiometer, K11 model - Germany), equipped with a 1.9 cm platinum ring, according to the De Nöuy ring method (Rodrigues *et al.*, 2006). Surface tension values represent the average of three independent measurements performed at room temperature (25 °C). The biosurfactant concentration is expressed in terms of critical micelle dilution (CMD), estimated by measuring the surface tension for varying dilutions (10- to 100-fold) of the sample. CMD is the dilution necessary to reach the CMC (critical micelle concentration), at which the surface tension starts to increase dramatically (Kim *et al.*, 1997).

When measuring the CMD the cell free broth were diluted 10 times CMD^{-1} or 100 times CMD^{-2} with Phosphate buffer solution (10 mM KH_2PO_4/K_2HPO_4 plus150 mM NaCl with pH adjusted to 7.0) and the surface tension was measured as described above(Pereira *et al.*, 2013)

3.4.3.2 Critical Micelle Concentration and Minimum Surface Tension Determination

Different concentrations of biosurfactant solution (0–5000 mg/L) were prepared by serial dilutions of stock solution in the Phosphate buffer solution (with pH adjusted to 7.0). The critical micelle concentration (CMC) was then determined from the break point of the surface tension versus logarithm of biosurfactant concentration curve. For the calibration of the instrument, the surface tension of distilled water was measured before each set of experiments Pornsunthorntawee *et al.*, 2008).

3.4.3.3 Emulsification Index (E_{24})

Emulsification index was estimated by the method according to Cooper and Goldenberg (1987). In this method 6 ml of cell free supernatant was added to 6 ml of various hydrocarbons (hexane, waste motor oil and cyclohexane) and then the mixtures were vortexed for 4 min and allowed to stand for 24 h. The emulsification index (E_{24}) was expressed as the percentage of the height of the emulsified layer to the height of the total liquid column. The stability of Emulsification index obtained was assessed over a 3 month period.

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3.4.4 Stability Studies

The stability studies were carried out with respect to the effect of temperature, pH, and salinity on the emulsification capacity of the biosurfactant. The analysis was done using the 24-hour cell-free culture broth obtained by centrifuging the culture sample at 12000 rpm for 20 minutes. To determine the thermal stability of the biosurfactant, cell-free broth was maintained at a constant temperature in the range 20–120 °C for 30 min and cooled at room temperature. To determine the effect of pH on the activity, the pH of the biosurfactant was adjusted at a value in the range 2.0–11.0. The effect of NaCl on the activity of the biosurfactant was investigated by adding NaCl in the range 5–20% (w/v). In each case, E_{24} values were performed with Hexane as described above (De Sousa and Bhosle, 2012).

3.4.5 Chemical Characterization of Biosurfactant

3.4.5.1 Fourier Transforms Infrared (FT-IR) Spectroscopy

FT-IR spectroscopy can be used to elucidate the chemical structures of some components in an unknown mixture by identifying the types of chemical bonds or the functional groups present in their chemical structures (Pornsunthorntawee *et al.*, 2008). Column purified biosurfactant was ground with KBr powder and was dispersed uniformly in a matrix of dry (paraffin) mar, compressed to form an almost transparent disk for Fourier transform infrared spectroscopy (FTIR) spectra measurement in the frequency range of 4,000–650 cm^{-1}. Chemical analysis of the components in the crude biosurfactant was characterized using FTIR spectrophotometer (Thermo-Nicolet, USA) equipped with OMNIC software for data analysis.

3.4.5.2 Thin-Layer Chromatography (TLC).

After hydrolysis of the column chromatographed biosurfactant with 6 M HCl at 110 °C for 24 h to cleave the chemical bonds between the moieties of fatty acids and amino acids. The aqueous fraction containing the amino acids was subjected to TLC analysis of silica gel 60 plates (F_{254}; Merck) with chloroform–methanol–H_2O (65:25:4, v/v/v) as the mobile phase. For the detection of peptides, the dry plates were sprayed with a solution of 0.25% (w/v) ninhydrin in acetone and kept at 115 ºC for 5 min (Xia *et al.*, 2014).

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3.4.5.3 Mass Spectrometric (MS) Analysis

The tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS) analysis was performed for the identification of column chromatographed, fractions. The samples were eluted first with chloroform to remove yellow pigment and impurities of hydrocarbons, then with a mixture of chloroform and methanol 80:20 v/v (100 ml), then 35:65 v/v (100 ml) collecting two fractions after discarding the first chloroform eluted fraction. The fractions eluted were collected and the solvents were evaporated in a rotary evaporator at 40 °C. LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Waters Synapt G2 mass spectrometer. Prior to analysis the MS was calibrated in both positive and negative ion resolution mode over the mass range of 100 - 2000 Da typically using sodium formate clusters. In this method, 5 µl of the extract was injected onto a Waters C_{18} BEH 1.7 µm (2.1 x 100 mm) column. The mobile phase consisted of A: Water (0.1% formic acid), B: Acetonitrile (0.1% formic acid) run with a 20-min gradient of (30 %, v/v Acetonitrile for 5 min; 30 –100 %, vol/vol Acetonitrile for 8 min and 100 %, v/v Acetonitrile for 2 min; 100 – 30%, v/v Acetonitrile for 1 min; 30 %, vol/vol Acetonitrile for 4 min) at a flow rate of 0.4 µl/min.The eluent flowed directly into the ESI source. The MS instrument was operated in MSE mode collecting both low energy (4 V) precursor and high energy (ramp: 20-40 V) product spectra. A 2 ng/µl solution of leucine enkephalin was used as the lockmass with a constant flow rate of 5 µl/min.

Mass spectra were attained in both positive and negative ion modes using the following parameters: scan time of 0.5 seconds; cone voltage of 30 V, the capillary voltage of 2.8 KV; source temperature 100 °C; desolvation temperature 300 °C; cone gas 100 L/h; desolavation gas 500 L/h.

3.5 Biosurfactant Assisted Mass Transfer Experiments

3.5.1 Pyrene Solubilization Experiment

The experiments of solubilization of pyrene (PYR) were done in Phosphate buffer solution (10 mM KH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) to achieve the complete dissolution of biosurfactant. For the study pyrene (2000 µl) was added from pyrene stock (5 mg mL^{-1}) solution in hexane to achieve a final concentration of 100 mg L^{-1} in a 250 mL Erlenmeyer flask. After complete evaporation of the solvent, 50 mL buffer solution and the

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required quantity of each surfactant was added from (10 mg mL^{-1}) stock solution so as to obtain a concentration of, 300 mg L^{-1}, 600 mg L^{-1} and 900 mg L^{-1} lipopeptide.
Sodium azide was added at a concentration of 300 mg L^{-1} so as to avoid microbial growth. Triplicate tests were conducted for each surfactant concentration. The biosurfactant produced by *Paenibacillus dendritiformis* CN5 (as described in Section 3.4.2) was used in this experiment. The flasks were sealed with Teflon-lined screw caps and incubated in a rotary shaker at 120 rpm and 32 °C for 48 h in dark. Afterwards, samples were transferred into 25-mL centrifuge tube to settle the non-solubilized solid pyrene (7000 rpm, for 10 min). The supernatant was extracted twice with equal volume of hexane (1:1, v/v). Immediately after addition of hexane, the flask was vortex-mixed for 4 min followed by centrifugation at 10,000 rpm for 10 min to separate the aqueous and organic phase. Five mL of the organic layer containing the extracted compound was drawn off with a pipette. The solvent was evaporated to dryness under a nitrogen stream and re-dissolved in equal volume (5 mL) of acetonitrile. The extract in acetonitrile (HPLC mobile phase) was passed through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter and HPLC analysed. The concentration of each PAH was calculated from 4-point standard calibration curves.

3.5.2 Phenanthrene and Pyrene Desorption Experiment

Soil was collected from a pristine supply and was sievedto < 2 mm size. Texture of the soil was 26% sand, 33% clay and 41% silt; the water holding capacity was 30% and the total organic carbon was 22 g/Kg. One-hundred grams of sterile dry soil were placed in a 1 L bottle and spiked with 80 mg of PHE and 80 mg of PYR dissolved in 100 mL of acetone to achieve soil contamination of 800 mg/kg of PHE and 800 mg/kg of PYR each. The soil was shaken vigorously for 5 min to promote homogeneous distribution of the PAHs in the soil. The amount of acetone added was sufficient to completely saturate the soil. The acetone in the mixture was allowed to evaporate for one week at 30 °C under a fume hood, and the contaminated soils were aged for 6 months at room temperature before the experiment starts to reach equilibrium.

A mass of 10 g of contaminated soil sample was weighed into each flask containing 50 mL of MSM 20% (w/v) with a different amount of lipopeptide. The lipopetide biosurfactant produced by Lbp5 strain was used for the desoption study. All aqueous solutions for soil tests contained 0.01 mol/L NaCl to maintain a constant ionic strength and 0.01% (w/w) of NaN_3 to inhibit microbial growth. The samples were shaken on a rotary shaker at 150 rpm in darkness at 32 °C. Triplicate samples were collected every 24 h by centrifugation for 10 min at 10,000 rpm.

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The biosurfactant produced by *Paenibacillus dendritiformis* CN5 (as described in Section 3.4.2) was used in this experiment.

The supernatant was drained off and the soil samples were airdried at room temperature. Five grams of the air-dried and homogenized soil sample were weighed directly in a flask where 30 mL of solvent hexane/acetone (1:1 v/v) were added and ultrasonicated twice (frequency 50-60 Hz, Bransonic 2200, Danbury, CT, USA) at 45 °C for 60 min (USEPA Method 3550C, 1998). The extracts were pooled and vacuum filtered (Whatman no.1 filter paper), the solvent was evaporated under a fume hood of dry nitrogen and the residual PAHs were recovered in 5 mL of acetonitrile (exchanged to mobile phase medium) and HPLC analysis was performed. The PAHs desorption percentage was computed from the difference of the initial and final concentrations ofthe soil.

3.6 Microbial Degradation Experiments of Petroleum Sludge, Motor Oil and PAHs

PAH degradation due to microbial activity was recognised as a greater decrease in the PAH concenttation in inoculated cultures compared to controls treated similarly. Cultures with sterilized and uninoculated PAH medium served as the controls. The media were sterilized by autoclaving (15 minutes at 121 °C/15 psi). PAHs and other hydrocarbons concentrations in liquid culture and soil microcosms were determined in triplicate by HPLC and GC (Sections 3.7.1 and 3.7.2) after extraction with Hexane. Microbial growth was established by an increase in microbial numbers as determined by the Standard Plate Count method (Section 3.7.3).

3.6.1 Shake Flask Biodegradation Experiments of Petroleum Sludge.

Shake flask biodegradation experiments were carried out in 500 mL Erlenmeyer flasks with 100 mL of mineral salt medium (Trummler *et al.,* 2003) and petroleum sludge (4.0%, v/v). Biodegradation experiment was conducted with three different sets in duplicates:

(1) Bacterial cells with petroleum sludge (4.0%, v/v);

(2) Bacterial cells with petroleum sludge (4.0%, v/v) and biosurfactant (50 mL cell free supernatant of 96 h culture); and

(3) An inoculated abiotic control.

The biosurfactant produced by *Ochrobactrum intermedium* CN3 (as described in Section 3.4.2) was used in this experiment. The biosurfactant solution was a cell-free broth obtained from the

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growth of strain CN3 isolate in nitrogen limited media for 96 h (Trummler *et al.*, 2003). Sterilized MSM containing 4% petroleum sludge (v/v) was inoculated with 1 mL inoculum *Ochrobactrum intermedium* CN3 strain (Each set of samples except the abiotic controls was inoculated with 1 mL aliquots of the PAH degraders from late-log precultures grown on 2% glycerol over night to achieve a final cell density of (10^7 CFU/mL) and the culture flasks were incubated for 21 days at 37 °C with 175 rpm of shaking. Samples were collected every 4 days and analyzed for cell growth based on CFU counts. Periodically, flasks were sacrificed in duplicate for analysis of hydrocarbons. Hydrocarbons were extracted by addition of n-hexane to the flasks and GC/MS analyzed.

Residual hydrocarbon was extracted using hexane after, 7, 14 and 21 days of growth and analyzed by GC. The biodegradation ofpetroleum hydrocarbon fractions specifically aliphatic and aromatics were analyzed after being fractionated as described by Mishra *et al.* (2001). After solvent evaporation, the residual TPH was fractionated into alkane, aromatic, asphaltene, and NSO (nitrogen, sulphur, and oxygen-containing compounds) fractions on a silica gel column.

For this purpose, samples were dissolved in n-Hexane and separated into soluble and insoluble fractions (asphaltene). The insoluble fraction (asphaltene) was filtered out on Whatman No. 1 paper and the soluble fraction was loaded on a silica gel column andeluted with different solvents. The alkane fraction was eluted with100 mL of hexane followed by the aromatic fraction, which waseluted with 100 mL of toluene. Finally, the NSO fraction was elutedwith methanol and chloroform 200 mL (1:1). The biodegradation of Predominant alkane and the aromatic fractions were analyzed in the current study using a Clarus 600 T GC/MS (Perkin–Elmer, Connecticut, USA) equipped with a Perkin–Elmer Elite—5MS capillary column (30 m×0.25 mm ID ×0.5 µm fixed phase) with helium as a carrier gas.

3.6.2 Biosurfactant-Enhanced Biodegradation of Motor Oil by Microbial Consortium.

The PAH-degrading microbial consortium that was enriched from creosote-contaminated soil sample (described in Section 3.3.1) was used in the biodegradation experiments. 16S rRNAgene sequence analysis revealed that the culturable isolates were predominantly composed of *Bacillus subtilis, Paenibacillus dendritiformis, Pseudomonas aeruginosa, Ochrobactrum intermedium.* This population was subcultured fortnightly on Trummler *et al.* (2003) MSM with 5% v/v used motor oil as a sole source of carbon and energy for 4 weeks. To obtain microbial inoculum, the consortium was precultured in MSM supplemented with

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1.0% (v/v) of motor oilin flasks on a rotary shaker (with shaking at 180 r.p.m) at 35 °C and pH 7 for 5 days. After 5 days, the culture broth was centrifuged andthe pellets were resuspended in MSM at OD_{600} of 0.8 to obtain ahighly concentrated microbial suspension for the experiments.

Shake flask biodegradation experiments were carried out in250 mL Erlenmeyer flasks with 150 mL of sterilized MSM (pH 7) supplemented with 3% (v/v) of used motor oil. Sterilized MSM was inoculated with 2% (v/v) microbial suspension and the culture flasks were incubated in an orbital shaker at 180 rpm and 35 °C for 3 weeks. Biodegradation experiments were conducted in three different sets using the above MSM. The biosurfactant produced by *Bacillus subtilis* CN2 (as described in Section 3.4.2) was used in this experiment.

(I) Bacterial cells (2%, v/v) + used motor oil (3%, v/v) + MSM;

(ii) Bacterial cells (2%, v/v) + used motor oil (3%, v/v) + biosurfactant

(50 mL cell free supernatant of 96 h culture, after diluting further with Phosphate buffer solution to obtain a concentration of 0.15% (w/v)) + MSM and

(iii) Uninoculated abiotic control; used motor oil (3%, v/v) + MSM.

The flasks were incubated in an orbital shaker at 180 rpm, and 35 °C for 3 weeks. All the experiments were carried out in duplicates and results were reported as mean ± Standard errors. Samples were withdrawn from each flask at a 4 day interval and aliquots of appropriate dilutions were plated (in triplicates) onto nutrient agar for total viable counts (TVC). Periodically, samples were extracted twice with hexane and analyzed on GC MS.

The Residual hydrocarbon was extracted from flasks using hexane after 1, 9, and 18 days of growth and analyzed by GC. The biodegradation of petroleum hydrocarbons and fractions (aliphatic, aromatic, resin and asphaltene) were analysed as described by Mishra *et al.* (2001). After solvent evaporation, the residual TPH was fractionated into alkane, aromatic, asphaltene, and NSO (nitrogen, sulfur, and oxygen-containing compounds) fractions on a silica gel column.

For this purpose, samples were dissolved in n-pentane and separated into soluble and insoluble fractions (asphaltene). The soluble fraction was loaded on a silica gel column and eluted with different solvents. The alkane fraction was eluted with 100 mL of hexane followed by the aromatic fraction, which was eluted with 100 mL of toluene. Finally, the NSO fraction was eluted with methanol and chloroform 200 mL (1:1). The aromatic fractions were analysed by

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gas chromatography (GC-FID). Degradation values from the uninoculated abiotic control were used to account for abiotic losses. All the experiments were carried out in duplicate and the mean values were used as results. For the quantification of the monitored hydrocarbons, standard calibration curves were constructed from dilutions of the oil sludge, and the calibration curves for each representative peak were used to calculate percentage degradations and expressed as percentages of respective controls.

3.6.3 Shake Flask Pyrene Biodegradation and Microbial Growth Kinetics Experiment

Pyrene biodegradation and microbial growth kinetics in the presence of different concentration of biosurfactant were studied under batch cultivation in a liquid culture medium. A stock solution of pyrene (5000 mg L^{-1}) was prepared by dissolving appropriate amounts of pyrene in hexane. An appropriate aliquot of the stock solution (200 µl) was added to sterile 250-mL Erlenmeyer flasks and left for a few hours in the fume hood to allow the solvent to evaporate. Mineral salt medium (100 mL) was added to each flask to achieve final pyrene concentration of 100 mg L^{-1}. The level of pyrene is generally lower in an aquatic environment (below several hundred ng L^{-1}) than that used in our experiments but it is unpredictably higher (up to several hundred µg L^{-1}) in some cases, e.g., oil spill accidents or coking wastewater (Luo *et al.*, 2014). We chose much higher concentration of pyrene in this study to see the biodegradation enhancing capacity of the surfactant for potential application to harsher environmental circumstances.

Four sets of runs consisting of 250-mL Erlenmeyer flasks containing 100 mL of liquid were operated in parallel to evaluate the biodegradation of pyrene in surfactant-free and surfactant amended systems at varying concentrations higher than the CMC. The flasks were supplemented with different concentrations of lipopeptide (no-surfactant control, 300 mg L^{-1}, 600 mg L^{-1} and 900 mg L^{-1}) from the stock solution of lipopeptide (10 mg/mL) prepared by dissolving in the MSM. The biosurfactant produced by *Paenibacillus dendritiformis* CN5 (as described in Section 3.4.2) was used in this experiment.

PAH degrading microbial consortium enriched from creosote contaminated soil using Creosote (described in Section 3.3.1 and Characterized as described in Section 3.3.3.2) was used for the biodegradation experiment. The bacterial consortium was incubated at 30 °C and 150 rpm in nutrient broth until reaching the late exponential phase. Cells were centrifuged at 5000 g for 10 min, washed twice and re-suspended in 0.85% NaCl to obtain a cell suspension with an optical

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density at 600 nm of 1.0, and the aliquots of this cell suspension (2 mL) were added to flasks containing pyrene in mineral salt medium as inoculum.

After inoculation the flasks were incubated in a rotary shaker (120 rpm) set at 30 °C. Uninoculated control flasks were also kept to account for abiotic loss of pyrene. Duplicate samples, were collected every 72 h for 24 d to measure residual pyrene concentration.

At specified time, residual pyrene was determined by extraction using the method reported by Ghosh *et al.* (2014) with minor modifications. Briefly, the entire content of a set of flasks (both control and experimental) in duplicate were extracted twice with hexane (extraction ratio 1:1). Immediately after addition of hexane, the flask was vortex-mixed for 4 min followed by centrifugation at 10,000 rpm for 10 min to separate the aqueous and organic phase. The upper aqueous phase was discarded by pipetting and the combined organic extract obtained from repeated extractions was passed through anhydrous sodium sulfate. Subsequently, hexane was evaporated to dryness and the pyrene extracted was dissolved in an equivalent volume of HPLC grade acetonitrile. The extract in acetonitrile was passed through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter and HPLC analysed. The concentration of each PAH was calculated from 4-point standard calibration curves. All the experiments were performed in duplicate and the error bars represent standard error.

3.6.4 Biodegradation Experiment of Polycyclic Aromatic Hydrocarbons (PAHs) in an aged Creosote Comtaminated Soil.

3.6.4.1 Soil microcosms

Laboratory-scale remediation of soil was performed as detailed below. Before incubation, all treatments were amended with inorganic sources of nitrogen (NH_4NO_3) and phosphorus (KH_2PO_4) to provide a final C:N:P molar concentration ratio equivalent to 100:10:1 (Cookson, 1995).

The treatments are comprised of,

(1) Soil that was autoclave sterilized (at 121°C for 30 minutes) as abiotic control to account for abiotic losses;

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(2) Microcosm amended with lipopeptide at a concentration of 0.2% (w/w), 2.2 times its critical micelle concentration (CMC) determined as 90.5 mg/L in distilled water (subsection 2.3.2.1);

(3) Microcosm amended with lipopeptide at a concentration of 0.6% (w/w), 6.7 times its critical micelle concentration (CMC). The biosurfactant was harvested from the supernatant of a cell culture of *Bacillus cereus* grown in a mineral medium with sun flower oil, as described earlier (subsection 2.3.1) and applied every 21 days;

(4) Control created using the same treatment protocol but with no added biosurfactant to the contaminated soil.

The biosurfactant produced by *Bacillus cereus* SPL-4 (as described in Section 3.4.2) was used in this experiment. The PAH-degrading microbial consortium (from bioremediated soil) used as inoculant in this study was previously enriched from a chronically PAH contaminated wood treatment plant soil, showing the capacity of degrading different groups of PAHs (Bezza and Chirwa, 2016). The microbial composition was characterized as described in section 3.3.3.2.

Each treatment except the abiotic control were inoculated with 5 mL microbial cell suspension (with OD_{600} = 1.0) and thoroughly mixed. For each treatment, two independent duplicates (200-mL capacity beakers) which were kept covered but unsealed, were prepared as microcosms, each containing 50 g of sieved (2 mm) soil.

All microcosms were supplemented with sterile water to approximately 65% of the soil's water-holding capacity and incubated at 37 °C in the dark for 64 days. Twice a week, the microcosm contents were mixed and the soil water content was restored by controlling the weight. Triplicate samples of 5 g of soil from each beaker were collected periodically for 64 d and analyzed by high performance liquid chromatography (HPLC) for PAH concentrations, and measurement of the microbial population.

3.6.4.2 Extraction and HPLC analysis of PAHs

The soil samples were extracted using the USEPA Method 3550B which was developed for extracting non-volatile and semi-volatile organic compounds from solids (U.S.EPA, 1996). The method involved air drying and homogenizing a 5 g soil sample and mixing with 30 mL of a solvent hexane: acetone (1:1, v/v) in flask, followed by sonication at 50 −60 Hz at 55 °C

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for 60 min (M1800 Ultrasonic bath, USA). The sample was transferred to centrifuge tubes and the soil particles were removed from the liquid by centrifugation at 2000 rpm for 10 min. The organic layer containing the extracted compounds was drawn off with a pipette. The extraction was performed twice before disposing the solids to achieve thoroughness of removal of PAHs from the soil. The final extract from each sample was vacuum-filtered to remove particles that might have been integrated into the supernatant during centrifugation.

The cleaned extract was evaporated to dryness under a nitrogen stream and re-dissolved in 5 mL of acetonitrile. The extract in acetonitrile (HPLC mobile phase) was passed through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter and HPLC analysed. The concentration of each PAH was calculated from 4-point standard calibration curves.

3.7 Analytical Methods

3.7.1 Determination of PAH Concentration by HPLC

PAHs were quantified using an external calibration method. Each PAH is identified by its retention time and absorption spectrum and quantified by its absorbance compared with four - point calibration curve for individual compounds prepared with the standards.

Retention time (RT) of the peaks of the 16 PAHs of the reference standard was used for analytes' peak identification. The chromatographic conditions applied were; 0–1 min, 70% acetonitrile (A) isocratic; 1–25 min, linear gradient 70% A–100% A; 25–35 min, 100% A isocratic; 35- 40 min, linear gradient 100%A – 70% A and finally, 40- 45 min 70% A isocratic back to the initial conditions and recondition the column. For HPLC analysis a Waters 2695 separation module equipped with photo diode array detector was used. The PAHs were separated with the Reverse phase a Waters PAH C_{18} column (4.6 mm×25 cm with 5 µm packing) at a column temperature of 25 °C, at 254 nm. In general, the precision of the retention time was between 0.04 and 0.36 minutes. The surrogate standards were also used for confirmation of the shift in the retention time. One working standard (16PAH) was injected at the beginning of daily measurement for the instrumental performance check i.e. Precision, repeatability, and random errors due to changes in the equipment conditions during the period of study.

The elution conditions applied were; 0–1 min, 70% acetonitrile, A isocratic; 1–25 min, linear gradient 70% A–100% A; 25–35 min, 100% A isocratic; 35- 40 min ,linear gradient 100%A – 70% A and finally, 40- 45 min 70%A isocratic back to the initial conditions and recondition

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the column. For HPLC analysis a Waters 2695 separation module equipped with photo diode array detector was used. The PAHs were separated with the Reverse phase a Waters PAH C_{18} column (4.6 mm×25 cm with 5 µm packing) at a column temperature of 25 °C, at 254 nm.

PAHs in solutions were analyzed by HPLC system with a slightly modified EPA Method 8310 (USEPA, 1986). For the HPLC analysis a Waters 2695 separation module equipped with photo diode array detector was used. The PAHs were separated with the Reverse phase a Waters PAH C_{18} column (4.6 mm×25 cm with 5 µm packing) at a column temperature of 25 °C, at 254 nm.

Each PAH is identified by its retention time and absorption spectrum and quantified by its absorbance compared with the external calibration curve prepared with the standards. The detection limit of the HPLC system was 0.01 mg/L. All tests were conducted in triplicate with uninoculated controls to monitor the abiotic/ volatilization losses and total recovery of contaminants. Quantitation was performed by external standard calibration with a five points calibration curves ranging from 0.1–100 mg/L

3.7.2 Gas Chromatographic (GC) Analysis of Aromatic and Aliphatic Fractions

The aromatic fractions in used motor oil and the aromatic and aliphatic fractions in petroleum sludge were analysed using a Clarus 600 T GC/MS (PerkinElmer, Connecticut, USA) equipped with a PerkinElmer Elite – 5MS capillary column (30 m × 0.25 mm ID × 0.5 µm fixed phase) with helium as a carrier gas. The oven temperature was kept initially at 60 °C for 5 min, followed by an increase to 300 °C at a rate of 15 C/min. Degradation of selected peaks was identified by searching for closest matches in the Wiley Mass Spectral Libraries. The MS was operated in the electron impact (EI) ionization mode to determine the appropriate masses for the selected ion monitoring ranged from 100 to 600 amu (atom to mass unit). All samples were performed in duplicates and the results were expressed as mean value.

3.7.3 Microbial Count

Standard plate count Method was used to quantify heterotrophic bacteria utiliziling the PAHs as a source of carbon. Serial dilutions were prepared in Ringer's solution and 100 µl of each dilution was spotted on Plate Count Agar (Oxoid, England) and incubated for 48 hours at 37 °C. Each assay was conducted in triplicate. Quantitative determinations were made on the basis of colony forming units (CFU). Results are reported as colony-forming units (CFUs)/mL. Plate count of bacterial soil population was performed by adding 1 g of soil sample to 9 mL of

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Ringer's solution. After appropriate serial dilutions, 100 µL of each dilution was spotted on Plate Count Agar and incubated for 48 hours at 37 °C. Results are reported as colony-forming units (CFUs)/g.

3.7.4. Cell growth determination

Biomassconcentration was measured by turbidimetry at 600 nm in a UV–Vis spectrophotometer (hekiosb), and the obtained-values were converted to grams of cell dry weight per liter using an experimental calibration curve (Biomass concentration (g L^{-1}) = 0.5415×Absorbance). Cells were harvested by centrifugation (10 min, 5000g) and suspended in distilled water for biomass determination. Excess pyrene suspended in the aqueous phase was found to have negligible effect on absorbance measurement as revealed through measurements in control flasks that were not inoculated with the bacterial culture.

3.7.5 Cell Surface Hydrophobicity (CSH) Test

Cell surface hydrophobicity was measured by bacterial adhesion to hydrocarbons (BATHs) According to slightly modified method described earlier (Zhong., 2007). Microorganisms grown on the hydrocarbons for a specified number of days were centrifuged at 12000 rpm for 10 mins. Then the precipitated cell pellets were washed twice with MSM. The cells were then resuspended in MSM and adjusted to an optical density of 1at 600 nm (OD_{600}). Afterwards, 2.0 ml of hexane was added to 8.0 ml of cell suspension and vortexed for 2 mins after 20 mins of phase separation the optical density of the aqueous phase was measured. Cell surface hydrophobicity was expressed by the ratio of the difference of the optical density (OD_{600}) of the initial cell suspension before the addition hexane (A_0) and the optical density (OD_{600}) of the aqueous phase after mixing (A) over the absorbance of the initial cell suspension. For each sample three independent determinations were made and the values are mean ± SD.

$$CSH\,(\%) = \frac{(A_0 - A)}{A_0} \times 100\%$$

3.7.6 Analysis for Total Organic Carbon (TOC) and Nitrogen

A Total Organic Carbon (TOC) Analyser (Model TOC-VWP, Shimadzu Corporation, Kyoto, Japan) was used to determine organic content of a washed eluent whereas a gravimetric method was used to determine total organic on the sample. The TOC analyzer was calibrated by dissolving different proportions of a 1000 mg/L potassium hydrogen phthalate stock solution

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in concentrations ranging from 0-5 mg/L in a 100 mL volumetric flask prior to analysing for totalcarbon. Perkin Elmer 2400 CHN/O Elemental Analyser was used to determine the soil samples percentage composition of Carbon and Nitrogen, which showed that the amount of Nitrogen in the soil sample was lower than the detection limit of the instrumnent.

3.8 Data Analysis

3.8.1 Modelling microbial growth and pyrene biodegradation profile assays

The logistics model was fitted to experimental data using Sigma Plot Software which seeks the values of the parameters that minimize the sum of the squared differences between the observed and predicted values of the dependent variable.

The growth rate according to Logistics model is given by a differential equation as follows (Fujikawa *et al.*, 2004)

$$\frac{dX}{dt} = \mu_{\max}(1 - \frac{X}{X_{\max}})X \qquad (3.1)$$

Where X is biomass concentration at time t and $\mu_{\max}$ is the the maximum specific growth rate, $X_{\max}$ is the maximum concentration (at the stationary phase), which is often called the carrying capacity ofthe environment. The logistic model contains the term, $1 - X / X_{\max}$, which suppresses the growth rate at a high population. When X is small during the lag phase, the value of this term is almost one, which does not affect the growth rate. As X increases to approach $X_{\max}$, the value approaches zero, thus making the growth rate almost zero during the stationary phase.

A growth curve described with this model is sigmoid on an ordinary Cartesian plane. At the beginning of incubation, when t = 0, the inoculums is considered as the initial concentration, i.e. $X = X_0$. Integrating Eqn (1) from t_0 to t gives the explicit form of biomass (X) as a function of time (t):

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$$X = \frac{X_{\max}}{1 + e^{[\ln(\frac{X_{\max}}{X_0} - 1) - \mu_{\max} t]}} \quad (3.2)$$

$$D = \frac{D_{\max}}{1 + e^{[\ln(\frac{D_{\max}}{D_0} - 1) - \mu_D t]}} \quad (3.3)$$

where X and D are the biomass ($g\ L^{-1}$) and the PAHs removal (%) at a specific moment of the culture time $t\ (d)$, X_O and D_O, are the initial biomass (g L^{-1}) and PAHs removal (%), $X_{\max}$ and $D_{\max}$ are the maximum biomass ($g\ L^{-1}$) and PAHs removal (%), $\mu_{\max}$ and $\mu_{D\max}$ represent the specific growth and specific degradation rates (d^{-1}) respectively.

The values of $\mu_{\max}$, $\mu_{D\max}$, X_O, $X_{\max}$, D_O, $D_{\max}$, were determined by minimizing the cumulative squared residuals between experimental and calculated values of the independent variables in equations in Equations (3.2 and 3.3) using Sigma Plot Software .

3.8.2 Modelling of Biodegradation Kinetics in soil microcosms

The data describing the degradation of the PAHs in the soil treatment were fit to the two-compartment first order decay model, which yielded the best fit overall (Congiu and Ortega-Calvo, 2014; Deary *et al.*, 2016)

$$\frac{S_t}{S_0} = F_{fast} e^{-k_{fast} t} + F_{slow} e^{-k_{slow} t} \quad (3.4)$$

Where S_t (mg kg^{-1}) is the PAHs content in the soil at time t (d) and S_0 (mg kg^{-1}) at the start of the experiment; F_{fast} and F_{slow} are the rapidly and slowly degrading fractions.

The values of F_{fast}, F_{slow}, k_{fast} (d^{-1}) and k_{slow} (d^{-1}) were determined by minimizing the cumulative squared residuals between experimental and calculated values of $\left(\frac{S_t}{S_0}\right)$ in Equation(3.4) using the software Microsoft Excel 2010 (SOLVER option).

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3.8.3. Statistical analysis

Data were analysed by unpaired two-tailed Student's t-tests or One-Way ANOVA, followed by Tukey's multiple comparison test using the statistical software SPSS version 21.0 for Windows (SPSS, Inc., Chicago, IL, USA). The differences between treatments were considered significant at $p < 0.05$.

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CHAPTER FOUR

APPLICATION OF BIOSURFACTANT FOR ENHANCING PETROLEUM SLUDGE AND USED MOTOR OIL BIOREMEDIATION

4.1 Application of Biosurfactant Produced by *Ochrobactrum Intermedium* CN3 for Enhancing Petroleum Sludge Bioremediation.

4.1.1 Introduction

The effective disposal of oily sludge wastes generated from petroleum industry during crude oil transportation, storage and refinery process is a worldwide problem (Zhang *et al.,* 2012). Improper disposal of oily sludge leads to environmental pollution, particularly soil contamination, and poses a serious threat to groundwater. Many of the constituents of oily sludge are carcinogenic and potent immuno-toxicants (Mishra *et al.,* 2001). The petroleum hydrocarbons (PHCs) and other organic compounds in oily sludge can be generally classified into four fractions, including aliphatics, aromatics, poles or resins and asphaltenes (Hu *et al.,* 2013). The aliphatics and aromatic hydrocarbons usually account for up to 75% of PHCs in oily sludge and commonly oily sludge is composed of 40–52% alkanes, 28–31% aromatics, 8–10% asphaltenes, and 7–22.4% resins by mass (Hu *et al.,* 2013).

Due to the existence of the high concentration of petroleum hydrocarbons (PHCs), oily sludge is considered to be hazardous to the environment and human health, thus requiring effective remediation (Zhang *et al.,* 2012). The remediation of contaminated sites can be achieved by physicochemical or biological methods. Conventional physicochemical methods can rapidly remove the majority of spilled oil but, in most cases, removal simply transfers contaminants from one environmental medium to another and can even produce toxic by-products. Moreover, crude oil cannot be completely cleaned up with physicochemical methods (Silva *et al.,* 2014). Bioremediation has gained increasing interest because it is, in contrast to physicochemical treatments, environment friendly, cost-effective, and efficient (Ismail *et al.,* 2013). However, bioremediation is limited by the low water solubility of the hydrophobic contaminants, limiting their availability to microorganisms (Ismail *et al.,* 2013).

A promising method that can improve bioremediation effectiveness of hydrocarbon contaminated environments is the use of biosurfactants that disperse the oil and accelerate its biodegradation (de Sousa and Bhosle, 2012). Production of biosurfactants by microorganisms

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is considered an important microbial strategy that influences the bioavailability of hydrophobic chemicals by changing the surface properties of bacterial cell or by dissolving and emulsifying these non-hydrophilic hydrocarbons (Xia *et al.*, 2014). Biosurfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic moieties that tend to interact with interfaces of various polarities and reduce the surface and interfacial tension of solutions leading to increased bioavailability by emulsifying and solubilization of hydrocarbons and subsequently rendering them available for microbial degradation (de Sousa and Bhosle, 2012; Xia *et al.*, 2014).

The aim of the present study is to isolate noble hydrocarbon degrading and biosurfactant producing strain and assess its feasibility in enhancing biodegradation of petroleum sludge in liquid culture. The strains were isolated from creosote contaminated soil that was obtained from wood treatment plant using creosote as a source of carbon and energy. *Ochrobactrum* stain CN3, which had the relatively higher degradation ability of petroleum sludge than others was selected and its biosurfactant production and biodegradation enhancement potentials were investigated. *Ochrobactrum* is an aerobic, Gram-negative *coccobacilli* belonging to the family *Brucellaceae* in the phylum *Proteobacteria* (Kumar *et al.*, 2014).

4.1.2 Screening and identification of biosurfactant-producing bacteria

A Total of 13 bacterial isolates were obtained from creosote contaminated soil, which are efficient degraders of Creosote. Biosurfactant producing isolates were screened using “Drop collapse” and “Oil Spread test”. Strain CN3 was one of these isolates that were found out to be efficient biosurfactant producers.

Based on the 16S rDNA gene sequences and using the Gen Bank BLAST tool, isolate CN3 was found to be closely related to members of the genus *Ochrobactrum*, and showed highest sequence similarity (100%) to *O. intermedium* (Fig. 4.1.1). The complete 16S rDNA sequence of the strain CN3 has been deposited in the GenBank database under the accession number of KP7939228. *O. intermedium* was also reported for its capability to degrade hydrocarbons and ability to degrade high molecular weight hydrocarbons, such as fluoranthene, pyrene, benz[a]anthracene, chrysene, benzo(k)fluoranthene and benzo(e)pyrene (Arulazhagan and Vasudevan, 2011).

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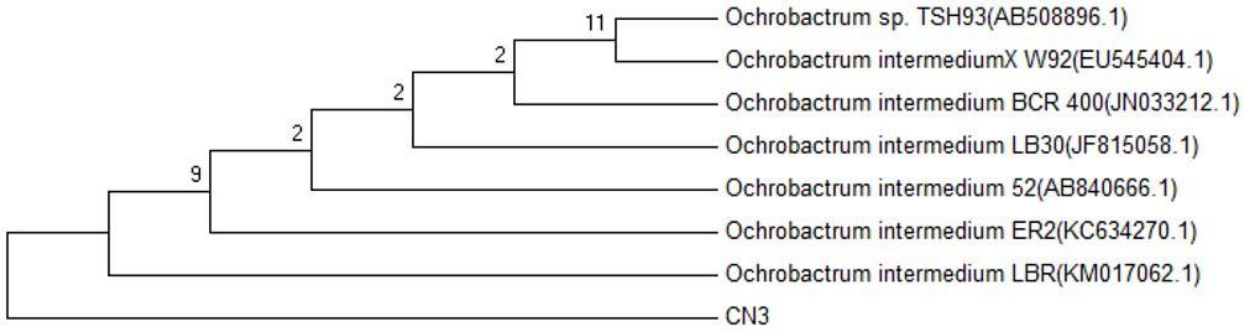

Figure 4.1.1 Phylogenetic relationship based on the 16S rDNA gene sequences between strain CN3 and species in the *Ochrobactrum* as determined by the neighbor-joining algorithm

4.1.3 Physical Properties of the Biosurfactant

4.1.3.1 Surface activity.

The surface tension of the whole broth dropped rapidly from around 71 mN m^{-1} to 36 ± 1 mN m^{-1} in the first 3 days of incubation. The biosurfactant concentration in the cell free broth was 25×CMD at the minimum surface tension. The biosurfactant has a critical micelle concentration (CMC) of 225 mg/L corresponding to minimum surface tension of 36 ± 1 mN m^{-1}. The cell free supernatant of *Ochrobactrum* CN3 was used to prepare emulsion of hexane, cyclo-Hexane and waste motor oil, which were found to remain stable up to 3 months.

4.1.3.2 Emulsification activity

The emulsification index of the cell free culture supernatant was found to be 86%, 79% and 65% with Cyclo-Hexane, hexane and waste motor oil respectively. The emulsions formed were stable for over 12 weeks. The ability of biosurfactants to emulsify hydrocarbon-water mixtures have been demonstrated to increase hydrocarbon degradation significantly and is thus potentially useful in oil spill management and enhanced oil recovery (Obayori *et al.*, 2009; Anyanwu *et al.*, 2011).

4.1.3.3 Stability studies

Biosurfactants are promising environmental molecules for bioremediation purposes; the conditions that affect the performance of a biosurfactant are usually salinity, pH and temperature. The stability of biosurfactants under extreme conditions is a pre-requisite for their

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potential applications in environmental, industrial and enhanced oil recovery applications in general (Rocha *et al.*, 2014). Therefore, the stability of biosurfactants at high temperature, salinities, and over a wide range of pH was studied.

4.1.3.3.1 Temperature stability

The applicability of the biosurfactant in several fields also depends on their stability at different pH, salinity, and temperature. The stability of the biosurfactant was tested over a wide range of temperature. The biosurfactant produced by *Bacillus subtilis* CN2 was shown to be thermo stable over arrange of temperatures (Fig. 4.1.2a). Heating of the supernatant to 125 °C for an hour caused no significant effect on the biosurfactant performances. This indicated that, emulsification activity of the lipopeptide was stable up to quite high temperatures, in contrast to the conventional synthetic surfactants such as SDS (Sodium Dodecyl Sulfate) , which exhibits a significant loss of emulsification activity above 70 °C as previously reported by several authors (Kim *et al.*, 1997). The stability of biosurfactants under extreme temperature conditions is a pre-requisite for their potential applications in environmental, industrial and enhanced oil recovery applications in general (Rocha *et al.*, 2014). Thus, our product is better than synthetic surfactants in terms of its thermostability and potential application under severe environmental conditions. The reason for its stability at high temperatures is due to the presence of their functional groups and are important properties of the biosurfactant produced probably caused by a better stability of fatty acids-surfactant micelles. Such extreme stability was reported by Abdel- Mawgoud *et al*. (2008) for the *P. aeruginosa* strain.

4.1.3.3.2 pH stability

The emulsification capacity of the CN3 biosurfactant for hexane demonstrated an increasing E_{24} value over the pH range of 5–12 and an appreciable decrease at pH < 5 (Fig. 4.1.2b). The reduction in emulsification activity at low pH scale (< 5) is due to the occurrence of precipitation, caused by the consequent insolubility of the biosurfactant produced by CN3 at these pH values (Abouseoud *et al.*, 2008; Rocha *et al.*, 2014).

Similar results were reported by Khopade *et al.* (2012). Increase in emulsion stability with increasing pH could be caused by a better stability of fatty acids-surfactant micelles in the presence of NaOH and the precipitation of secondary metabolites at higher pH values (Khopade *et al.*, 2012). Extreme pH values could possibly transform weak surface-active species into more active emulsifiers by increasing ionization (Abouseoud *et al.*, 2008).

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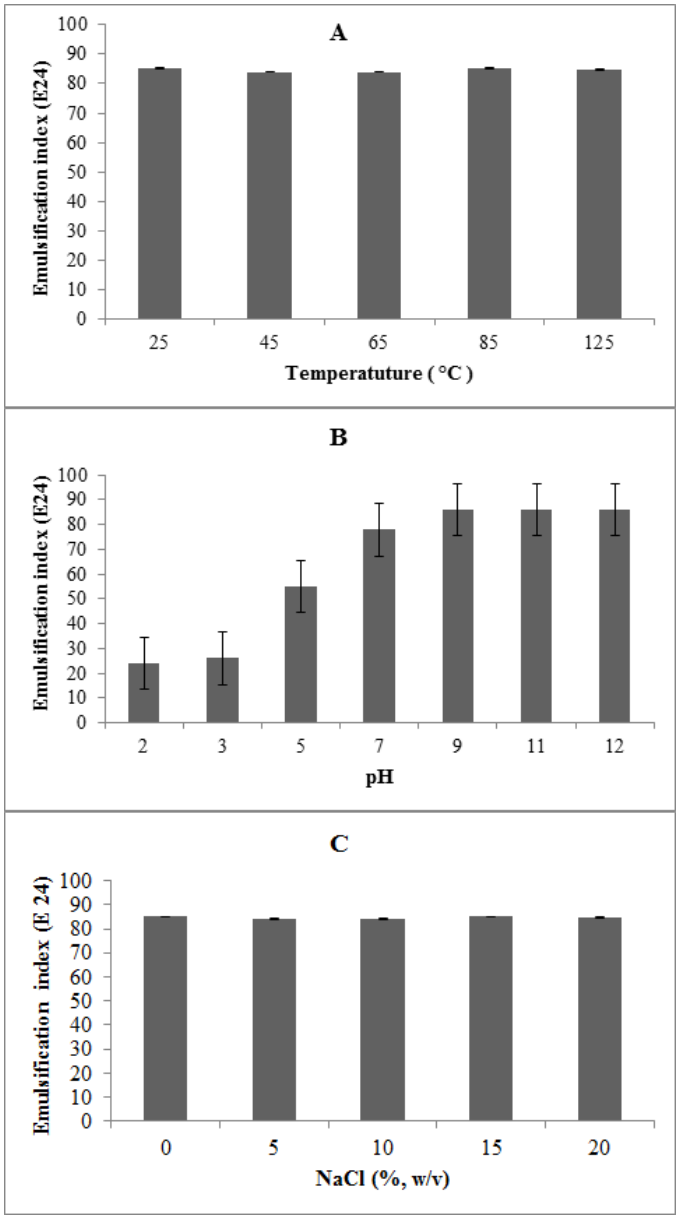

Figure 4.1.2 Effects of temperature (a), pH (b) and salinity (c) on the emulsification activity of biosurfactant produced by *Ochrobactrum intermedium* CN3. Data points represent the mean of three replicate flasks, while error bars represent standard errors.

4.1.3.3.3 Effect of salinity

The emulsification index (77%) of the produced biosurfactant was stable in the presence of NaCl in the concentration range of 2-20% w/v (Fig. 4.1.2C). Biosurfactants are more effective at a wide range of salinity values compared with synthetic surfactants (Silva *et al.*, 2014). This stability at high concentration of salt can be attributed to the presence of diverse functional

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groups in the microbially produced surfactants than the conventional chemical surfactants. The stability of the biosurfactant over a wide range of temperature, pH, and salt concentration suggests its potential application for enhanced oil recovery from oil reservoirs and various industrial activities. Tolerance of biosurfactants/ bioemulsifiers to NaCl as high as 25% (w/v) has been been reported earlier (De Sousa and Bhosle, 2012). In a similar study, Rhodococcus *sp*. TA6, isolated from oil-contaminated soil, produced a biosurfactant able to reduce the surface tension of the culture medium from 68 mN m^{-1} to 30 mN m^{-1} , maintaining its stability in solution with concentrations of up to 100 g L^{-1} NaCl (Shavandi *et al.*, 2011). Comparatively, the biosurfactants produced by the CN3 isolate demonstrated better activity and stability at higher concentrations of NaCl than previously reported studies (Anyanwu *et al.*, 2011; Khopade *et al.*, 2012).

Generally, the product exhibited a high level of thermal stability, a positive effect for increasing pH, and demonstrated a high level of tolerance of ionic strength, which shows clear perspectives for its use in extreme environmental conditionssuch as enhanced oil recovery and bioremediation of soil and marine environments (Abouseoud *et al.*, 2008; Anyanwu *et al.*, 2011).

4.1.4 Chemical characterization of the biosurfactant

The column puried biosurfactant was collected after solvent evaporation and used for FTIR, TLC and LC MS/MS characterization.

4.1.4.1 FTIR ATR chemical characterization

The FTIR spectrum of the column purified biosurfactant showed bands characteristic of peptides at 3,291.9 cm^{-1} resulting from N–H stretching mode (Fig. 4.1.3). The sharp peak around 1649.4 cm^{-1} (stretching mode of the CO–N bond) is due to amide group (Yilmaz et al., 2009; Ismail *et al.*, 2013). The presence of aliphatic chains was confirmed by the observation of peaks in the region 2850–2950 cm^{-1} due to the –C–H stretching mode of CH_3 and CH_2 groups in alkyl chains (Thaniyavarn *et al.*, 2003). The deformation vibrations from 1411 cm^{-1} to 1270 cm^{-1} reflect aliphatic chains (–CH_3, –CH_2–) of the fraction (Yilmaz *et al.*, 2009). This characteristically indicated the presence of fatty acid chain of lipopeptide biosurfactants.

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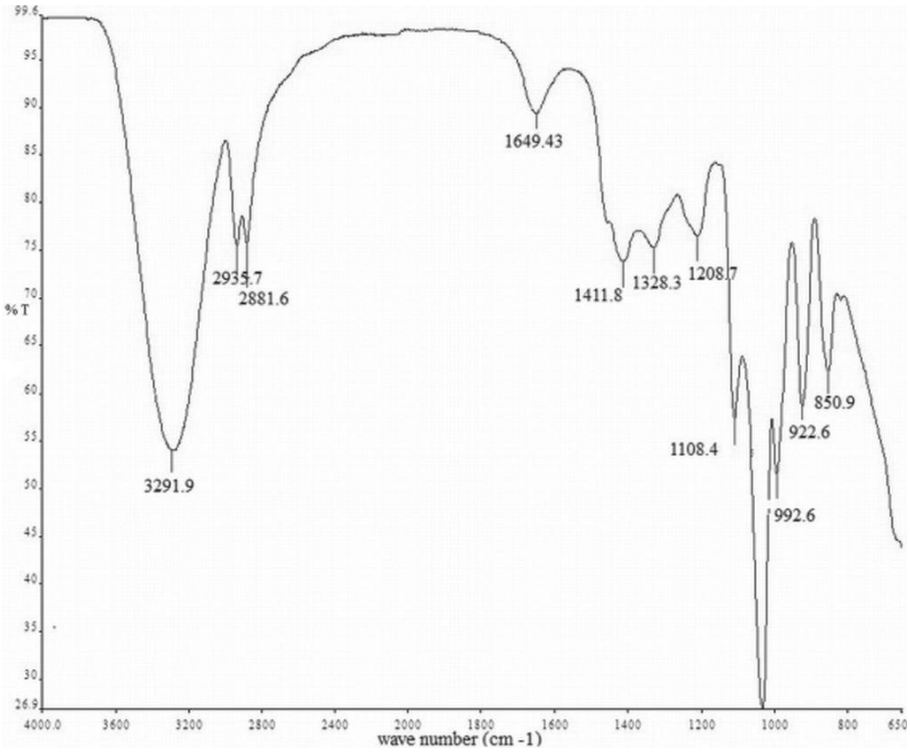

Figure 4.1.3 Fourier transform infrared (FT-IR) absorption spectrum of the biosurfactant produced by strain *Ochrobactrum intermedium* CN3.

4.1.4.2 TLC Analysis of Purified Biosurfactant

The fraction of the biosurfactant in the aqueous phase after acid hydrolysis indicated an R*f* value of 0.8 and showed positive reactions with ninhydrin on the TLC plate (Fig. 4.1.4), signifying that the biosurfactant consisted of peptide moieties and showing that it may be lipopeptide.

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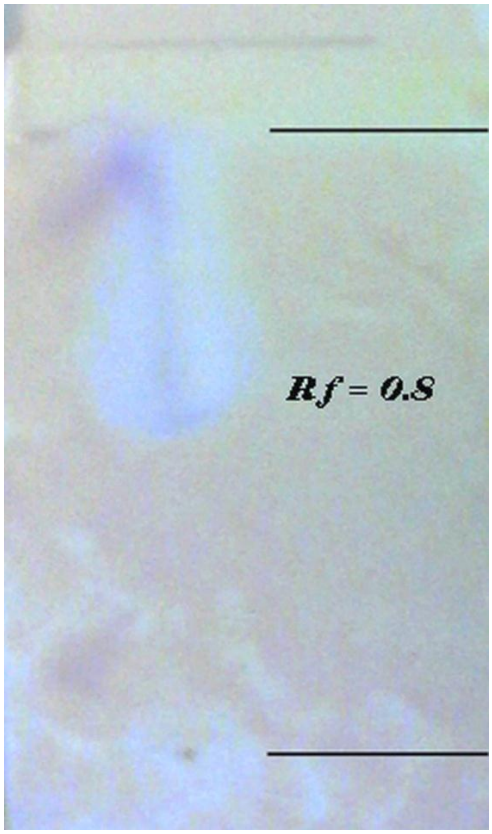

Figure 4.1.4 Thin-Layer Chromatography (TLC) of the biosurfactant obtained from *Ochrobactrum intermedium* CN3 after treatment with ninhydrin showed pink spots with R*f* value of 0.8

4.1.4.3 Mass Spectrometry

The molecular weight of the biosurfactant was determined by LC-MS/MS. In the MS/MS spectrum, 7 peaks were observed at a retention time of 4.139, 4.467, 4.475, 4.687, 9.546, 13.21 and 16 mins. The fatty acid or hydrophobic moiety of the biosurfactant eluted at 13.21 minutes, which is the predominant peak, was proposed to be triacontyl hexadecanoate fatty acid, $C_{56}H_{112}O_2$, molecular mass 816.86Da, With fragmentation at m/z 1350 (Fig. 4.1.5.1). The fragment ion at m/z 1350.50 corresponds to the loss of 444.2 from the $[M + H]^+$ 1794.7 ion. The base peak at m/z = 1350.5 is produced by a characteristic cleavage alpha to the carbon with the hydroxyl group and defines the position of the carbon with the hydroxyl group (Ryhage and Stenhagen, 1960).

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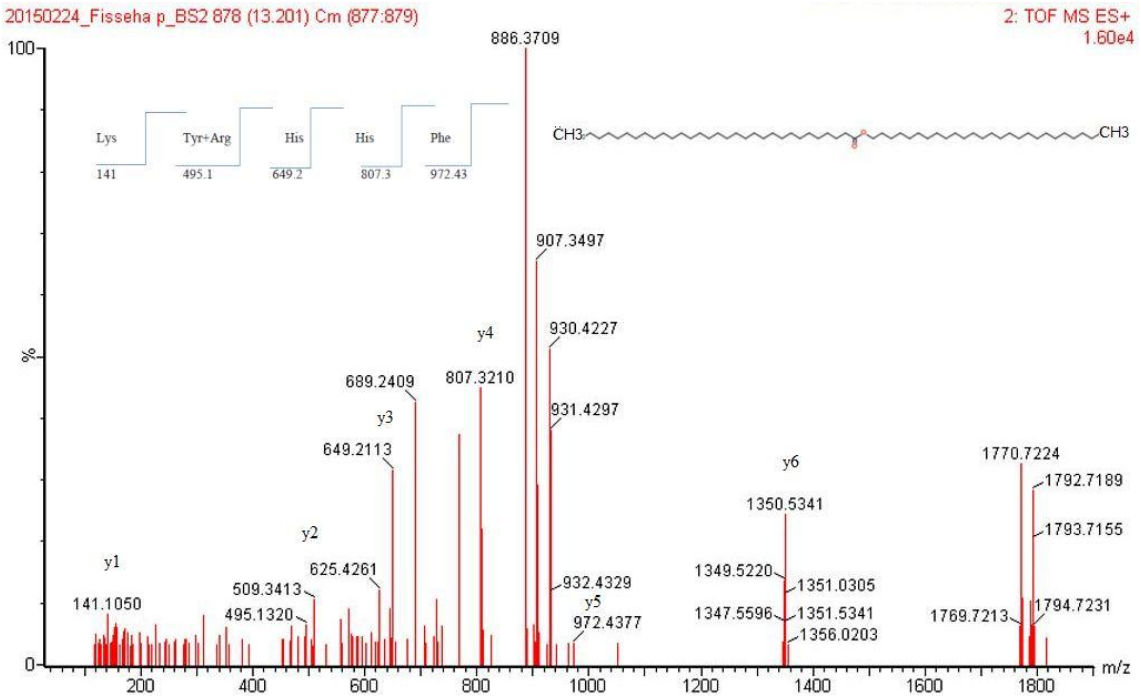

Figure 4.1.5.1 LC-MS/MS spectrum of $[M+H]^+$ precursor ion at m/z 1794.7, separated at retention time of 13.2 min, where *m/z* (x - axis) represents mass to charge ratio and % (y-axis) represents relative abundance of the fragment ions.

The product ions obtained for the precursor ion at m/z 1794.7 are shown in Figure 4.1.5.1. The y ion sequence was 1794.7 → 1350.50 (y6) → 972.4 (y5) → 807.5 (y4) → 649.2 (y3) → 495.1 (y2) → 141.1(y1), corresponding respectively to the losses of (fatty acid)-Phe(Phenylalanine)-His(Histidine)-His(Histidine)-Tyr(Tyrosine)-Arg(Arginine)-Lys(Lysine).

Peptide moiety was proposed to consist of a sequence of 6 amino acids. Fragments representing the calculated masses were derived from the stepwise cleavage (Fig 4.5.1.1) of the amino acids Phe (972.4), His (807.5), His (689.2), Tyr-Arg(495.1), Lys(141) following the method by (Symmank *et al.,* 2002). Thus, the lipopeptide surfactant produced by CN3 can be, composed possibly of 6 amino acids coupled to one molecule of Triacontylhexacosanoate. The molecular weight (MW) of the lipopeptide is determined as the sum of the peptide hydrophilic moiety and MW of the hydrophobic fatty acid moiety, $C_{31}H_{62}O_2$ which gives the MW of 1794.7 Da (Fig. 4.1.5.1). Other heavy molecular lipopeptide components were also identified with different retention times (Figures 4.1.5.2a, b, c and d).

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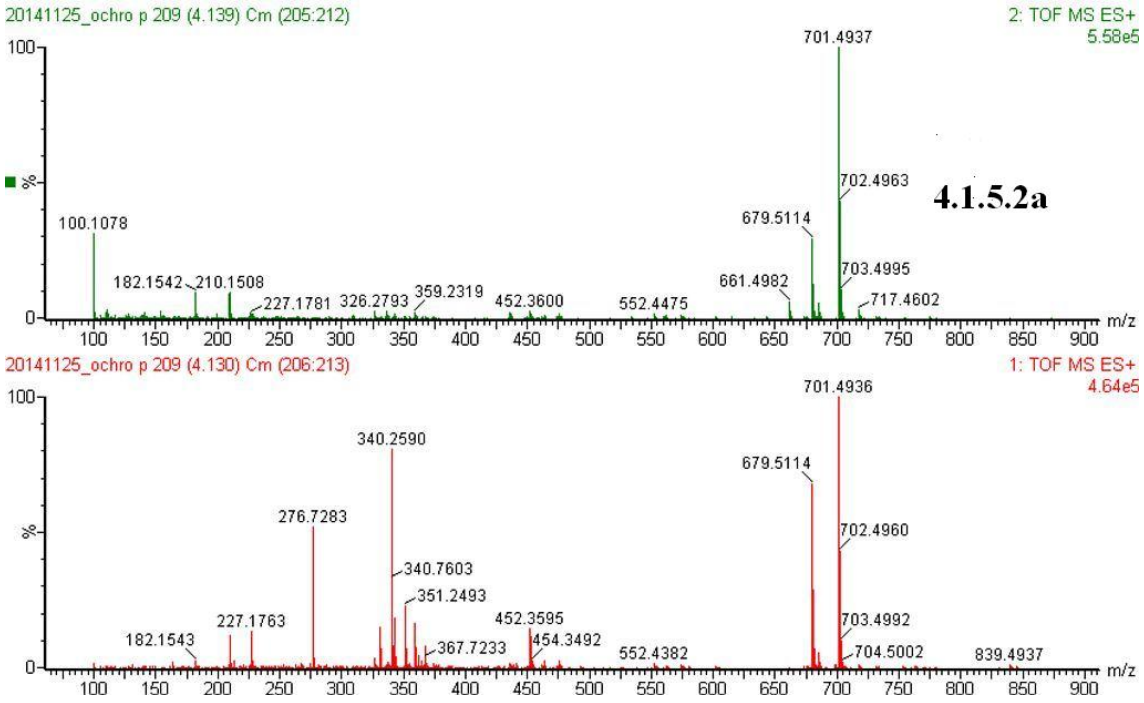
The produced biosurfactant is carbohydrate protein and lipid complexe having the composition of carbohydrate (18.5%), protein (17.7%) and lipid (63.8%) showing its glycolipopeptidal nature.



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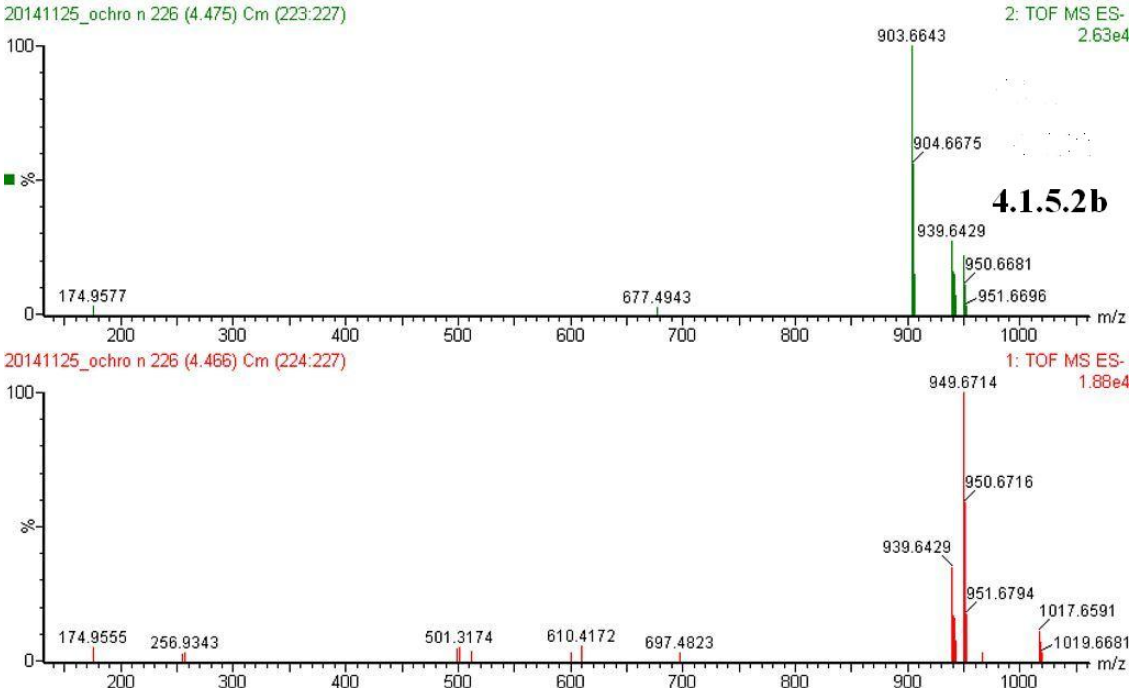

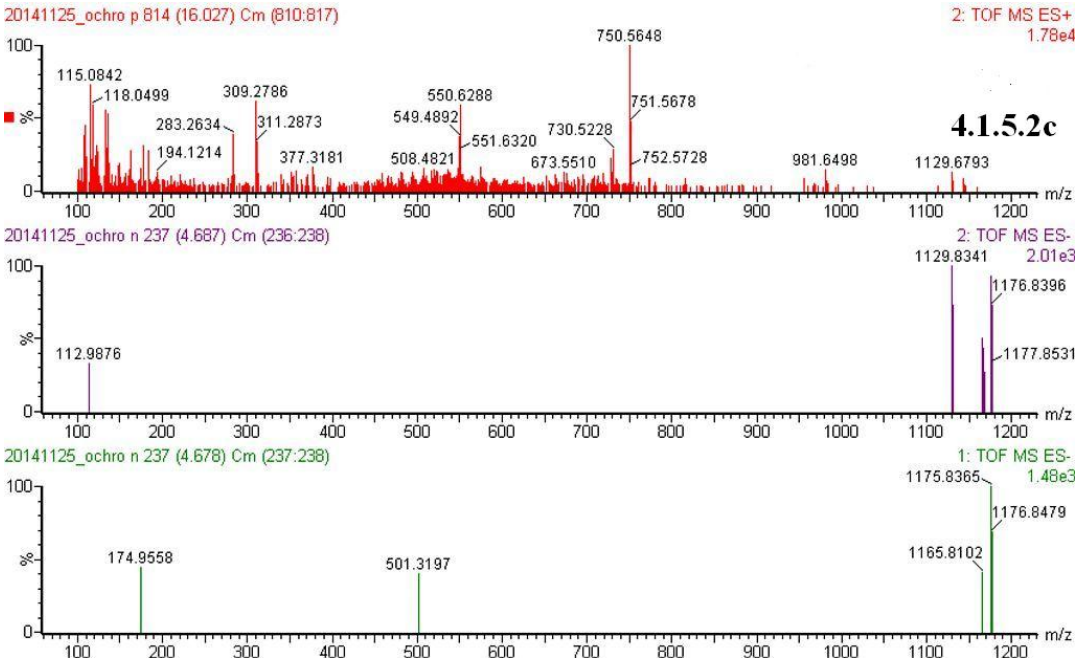

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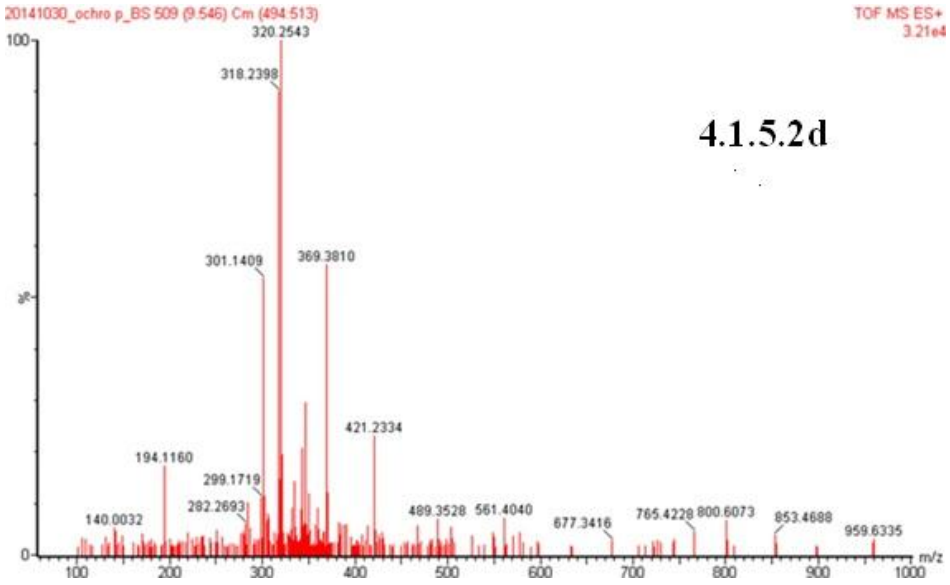

Figure 4.1.5.2 (a, b ,c and d) LC-MS/MS spectrum of $[M+H]^+$ precursor ions, separated at retention time of 4.13,4.475 ,4.678, 9.55 and 16 min.Where *m/z* (x - axis) represents mass to charge ratio and % (y-axis) represents relative abundance of the fragment ions.

The produced biosurfactant is carbohydrate protein and lipid complex having the composition of carbohydrate (18.5%), protein (17.7%) and lipid (63.8%) showing its glycolipopeptidal nature.

4.1.5 Biodegradation of Petroleum Sludge

Investigation of the effectiveness of each treatment on petroleum sludge biodegradation rate was estimated in terms of n-alkanes and PAHs reductions. The petroleum sludge sample was found to have alkanes ranging from C_8 to C_{18} and polycyclic aromatic hydrocarbons (Tables 4.1.1 and 4.1.2). Figures 6 and 7 show the depletion of the total petroleum hydrocarbons without and with biosurfactant supplementation respectively. In the abiotic control where the degraders are not supplemented with mineral salt medium and biosurfactant no significant degradation occurred. An enhanced reduction in the hydrocarbon content was observed in the 21 day treatment in the microcosms treated with the mineral salt medium and in the microcosm treated both with the mineral salt medium and the biosurfactant.

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Table 4.1.1 Monitored aliphatic hydrocarbon residual (%) after 7, 14 and 21 days of incubation in the biosurfactant supplemented and non-supplemented samples. Data are expressed as the mean ± standard deviation of two independent experiments performed in duplicate.

Time(d)	Compound	Biosurfactant+MSM +Bacterial Cell	MSM +Bacterial Cell
	Pentadecane		
0		100.0±0.5	100.0±0.8
7		58.0±2.5	78.6±4.3
14		23.4±1.7	55.7±1.3
21		17.4±1.2	37.8±5.6
	Hexadecane		
0		100.0±1.4	100.0±0.9
7		57.4±1.3	84.5±1.8
14		35.8±2.4	68.5±2.3
21		27.4±2.0	58.4±3.1
	Heptadecane		
0		100.0±2.1	100.0±0.6
7		73.2±3.1	89.4±1.6
14		62.5±2.5	82.2±2.3
21		28.3±1.8	61.3±3.1
	Octadecane		
0		100.0±1.3	100.0±2.1
7		73.4±1.5	87.3±0.8
14		62.6±2.3	84.5±2.3
21		36.1±0.9	61.4±1.3
	Nonadecane		
0		100.0±2.1	100.0±1.5
7		71.5±3.1	81.4±2.7
14		61.4±2.3	78.5±2.7
21		41.3±0.8	61.5±3.1

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Table 4.1.2 Monitored PAHs residual (%) after 7, 14 and 21 days of incubation in the biosurfactant supplemented and non-supplemented samples. Data are expressed as the mean ±standard deviation of two independent experiments performed in duplicate.

Time(d)	Compound	Biosurfactant+MSM +Bacterial Cell	MSM +Bacterial Cell
	Phenanthrene		
0		100.0±2.3	100.0±1.4
7		68.5±1.5	81.6±3.1
14		53.4±0.9	64.3±2.6
21		41.3±1.7	68.5±3.1
	Anthracene		
0		100.0±3.1	100.0±2.8
7		68.5±1.7	84.5±1.6
14		53.4±2.5	74.5±1.8
21		41.3±0.8	68.5±2.4
	Pyrene		
0		100.0±1.7	100.0±2.1
7		73.3±1.2	83.8±2.1
14		60.6±3.4	79.5±2.9
21		42.4±2.3	68.7±3.2

The degradation of the long chain n-alkanes and representative PAHs were monitored during the shake flask treatment. The degradation rates of the identified peaks of the *n*-alkanes (Nonadecane; Octadecane; Heptadecane; Hexadecane; Pentadecane and PAHs (Anthrecene, Phenanthrene and Pyrene) were monitored. *Ochrobactrum* showed an efficient degradation of the monitored n alkanes and PAHs.

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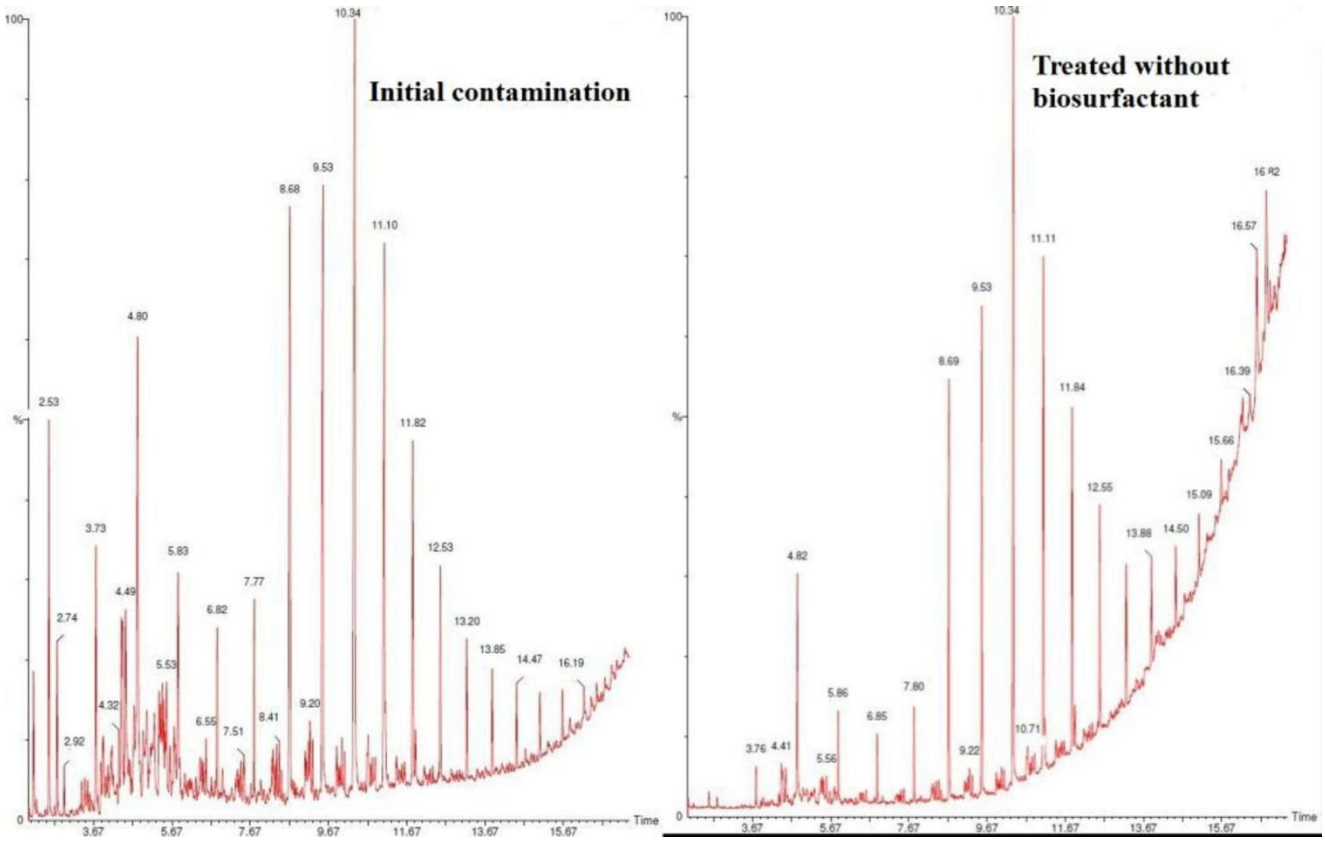

Figure 4.1.6 Chromatogram shows the degradation of the hydrocarbons by the isolate *Ochrobactrum intermedium* CN3 during the 21 day incubation, initial contamination (left) and the microcosm treated with no biosurfactant amendment (right).

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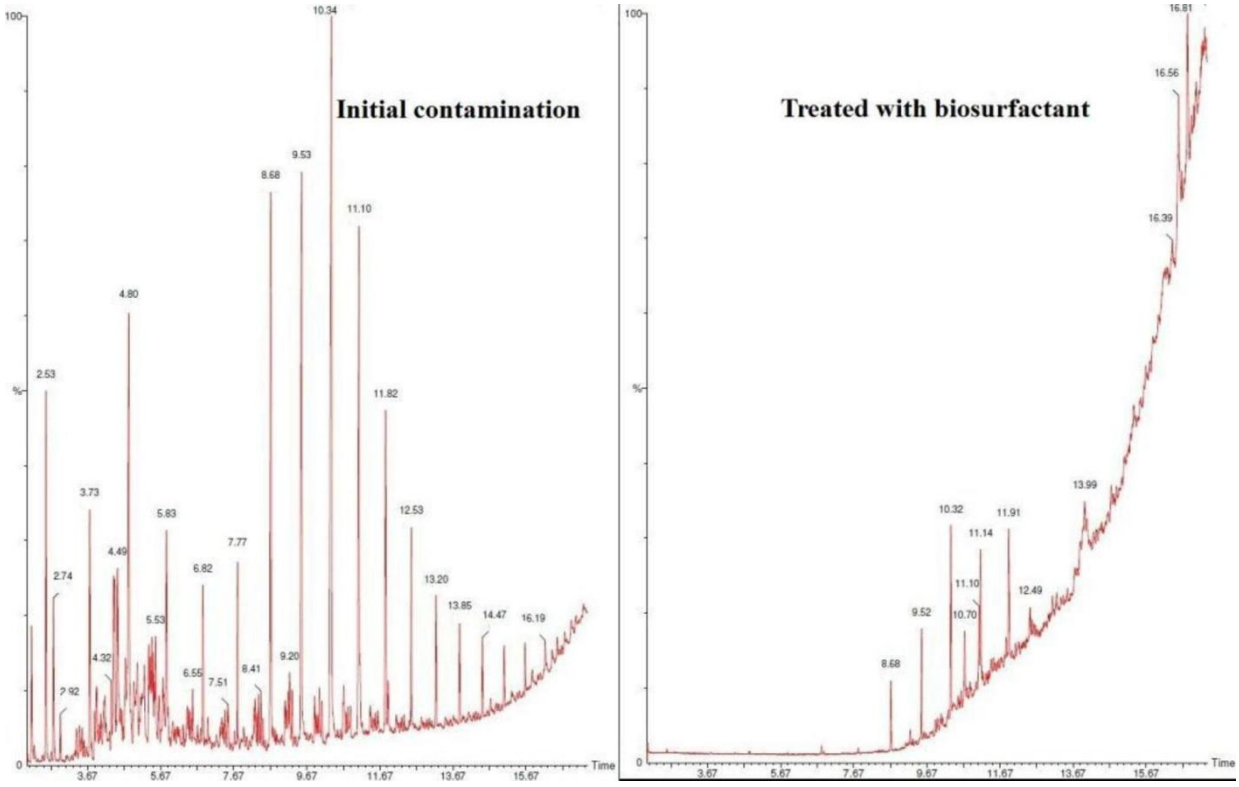

Figure 4.1.7 Chromatogram shows the degradation of the hydrocarbons by the isolate *Ochrobactrum intermedium* CN3 during the 21 day incubation, initial contamination (left) and the microcosm treated with biosurfactant amendment (right).

The short chain aliphatic hydrocarbons (C < 12 like Dodecane and Decane) were easily degraded during the first few days of incubation while the longer chain aliphatics with C > 12 and PAHs were resistant to biodegradation due to their low aqueous solubility. The biodegradation of the longer chain aliphatics monitored (Table 4.1.1 and 4.1.2) showed up to 64 % degradation in 21 days of incubation time. The biodegradability of the oil components generally decreases in the order of *n*- alkanes, branched-chain alkanes, branched alkenes, low-molecular-weight *n*-alkyl aromatics, monoaromatics, cyclic alkanes, PAHs, and asphaltenes (Tyagi *et al.*, 2011).

The degradation of the more hydrophobic longer chain hydrocarbons and PAHs degradation was highly enhanced in the presence of the biosurfactant (Fig. 4.1.7). As can be seen in Fig. 4.1.6 and Fig. 4.1.7 fastest eluting short chain alkanes were completely degraded in both the unamneded and amended microcosms in the first few days of incubation. However, the longer chain aliphatic and PAHs degradation was lower in the unamended microcosm while enhanced degradation was observed in the biosurfactant supplemented microcosms (Tables 4.1.1 and 4.1.2). The biodegradation of the PAHs monitored was up to 59, 77 and 58 % for Anthracene,

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Phenanthrene and Pyrene respectively, after 21 days of incubation (Table 4.1.2). The degradation of the PAHs was increased up to twofold in the presence of the biosurfactant indicating the enhancement provided by the biosurfactant. Sludge removal after 21 days of incubation was insignificant (< 3.2 ± 1.4 %) for the monitored components at the un-inoculated abiotic controls.

Biodegradation of aliphatic hydrocarbons by microbes can be enhanced by increasing their bioavailability through solubilization or emulsification or by adherence and uptake of oil directly from the oil–water interface. Extracellular biosurfactants or bioemulsifiers produced by microbes play an important role in enhancing the cellular uptake by pseudosolubilization, and increase the interfacial area by lowering the surface tension for mass transfer, where n-alkane droplets complex to biosurfactant and then complex gets dissociated within a hydrophobic compartment of the cell wall (Mishra and Singh, 2012). In a similar study Nikolopoulou et al. (Nikolopoulou *et al.*, 2013) reported that the specific degradation rate formedium to high chain *n*-alkanes C_{15}, C_{20}, C_{25} and C_{30} with inorganic nutrient addition and biosurfactant (rhamnolipids) addition in the presence of organic nutrients (uric acid and lecithin) was 3–3.5 times higher than in the control treatment. The biosurfactant in the current study enhanced the biodegradation of all the fractions of the hydrocarbons particularly those which are more hydrophobic longer chain *n*-alkanes and aromatic hydrocarbons.

Kang *et al.* (2010) observed effective biodegradation of crude oil in the soil, in their studies addition of sophorolipid in soil increased degradation of model compounds resulting in 80 % degradation of saturates and 72 % aromatics in 8 weeks. Their results indicated that sophorolipid may have potential for facilitating the bioremediation of sites contaminated with hydrocarbons having a limited water solubility and increasing the bioavailability of microbial consortia for biodegradation. Our observation in the current study is in accordance with other studies reported regarding biosurfactant enhanced biodegradations (Thavasi *et al.*, 2011; Moldes *et al.*, 2011; Xia *et al.*, 2014).

Production of biosurfactant by *Ochrobactrum intermedium* CN3 was observed from the high emulsification index (50±3%, with hexane) of the whole cell broth, surface tension reduction (35±2) of the growth medium and cell surface hydrophobicity (65±2%) observed. Dispersion/emulsification of crude oil in the culture medium suggests the presence of tensioactive agents. Production of biosurfactants is known amongst crude oil and hydrocarbon-degrading bacteria as this is the way by which hydrocarbonoclastic microbes overcome the

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hydrophobicity and low aqueous solubility of the hydrocarbon substrates (Ismail *et al.*, 2013). The emulsifying activity of culture supernatant of the un-amended microcosm was determined by measuring emulsification index as described by Cooper and Goldenberg (1987). The culture supernatant of *Ochrobactrum intermedium* CN3 was used to prepare emulsion of hexane and cyclohexane after 12 days of incubation. The culture supernatants showed emulsification index of between 74% and 85%, which were found to remain stable up to 3 weeks. However, the cell free supernatant didn't make an emulsification showing that the biosurfactant produced was cell bound and was not dispersed in the medium. High emulsification measured at day 12 of incubation showed stability, but the cell free supernatant didn't make an emulsification showing that the biosurfactant produced was cell bound and was not dispersed in the medium. Similar results were reported by (Franzetti *et al.*, 2008). The authors reported that when the *Gordonia sp.* BS29 was grown on n-hexadecane, the surface tension achieved in culture broth dropped to 30.0 mN m^{-1} whereas cell-free culture filtrates did not show a significant reduction in surface tension.

Often, biodegradation is stimulated by biosurfactants however inhibitory effects have also been reported (Chrzanowski *et al.*, 2012). A variety of factors and mechanisms have been proposed to explain the inhibition process some of these observations can be explained by one or more of the following effects: (a) toxicity of surfactants due to surfactant-induced permeabilisation or lysis of the bacterial cell membrane (b) Substrate toxicity resulting from an increase in bioavailability brought by surfactant solubilization (c) physical-chemical effects resulting in undesirable bacterial-cell/surfactant interactions, e.g., prevention of bacterial adhesion to the hydrophobic substrates, (d) the reduction of contaminant bioavailability due to its uptake into the surfactant micelle (e) surfactants' use as preferential growth substrates by degrading microorganisms (Avramova *et al.*, 2008).

Chrzanowski *et al.* (2012) observed that the addition of rhamnolipid Biosurfactant into samples containing either diesel or biodiesel fuel did not enhance the biodegradation efficiency of petroleum hydrocarbons. Rather the rhamnolipids were preferentially degraded by a soil-isolated consortium of hydrocarbon degraders. Biosurfactant rhamnolipidsat concentrations much less than their CMCs increased the cell surface hydrophobicity (CSH) of *Pseudomonas aeruginosa* due to the release of Liopoplysachrides (LPS) from the outer membrane and increased association of cells with hydrophobic organic contaminants (HOCs) resulting in an increased HOC degradation rate (Li and Zhu, 2012). On the other hand, Zhong *et al.* (2008)

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found that rhamnolipids in concentrations higher than their CMCs decreased the CSH of *P. aeruginosa*. Thus, in every bioremedial application of biosurfactants optimum level and compatibility studies need to be evaluated experimentaly prior to application.

Fast bacterial growth was observed during the first few days in both biosurfactant amended and unamend microcosms due to the easily degradable short chain aliphatic hydrocarbons (C ≤ 12 like Dodecane and Decane) which were easily degraded. However, bacterial count was lower in the unamended microcosm compared to the biosurfactant amended microcosm (Fig. 4.1.8). After day 10 of incubation following the depletion of easily degradable short chain aliphatics, enhanced biodegradation of long chain hydrocarbons and concomitant increase in bacterial count was observed in the biosurfactant amended microcosm.

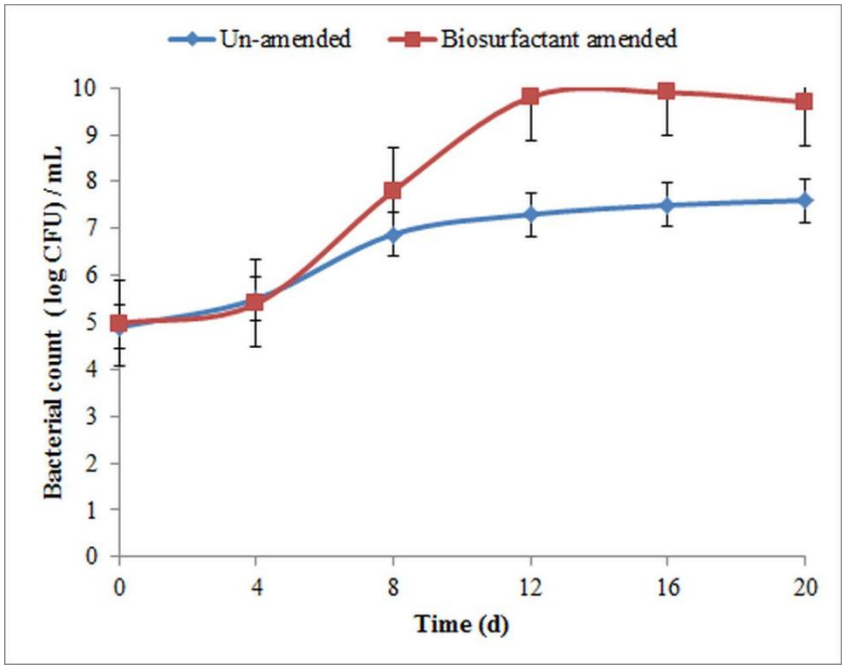

Figure 4.1.8 Bacterial count showing increase in bacterial growth in the biosurfactant amended and unamended microcosms. Error bars represent the standard errors of the mean.

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4.2 Production and Applications of Lipopeptide Biosurfactant for Bioremediation and Oil Recovery by *Bacillus Subtilis* CN2

4.2.1 Introduction

The release of contaminants, such as petroleum and its byproducts, into the environment is one of the main causes of global pollution and has become a focus of great concern both in industrialized and developing countries due to the broad environmental distribution in soil, groundwater, and air (Silva *et al.*, 2014). The presence of different types of automobiles and machinery has resulted in an increase in the use of lubricating oil. Spillage of used motor oils such as diesel or jet fuel contaminates our natural environment with hydrocarbons (Abioye *et al.*, 2012).

New motor oil contains a higher percentage of fresh and lighter (often more volatile and water soluble) hydrocarbons that would be more of a concern for acute toxicity to organisms. Used motor oil contains more metals and heavy polycyclic aromatic hydrocarbons (PAHs) that would contribute to chronic hazards including mutagenicity and carcinogenicity (Mandri *et al.*, 2007). PAHs represent the group of compounds in oil that has received the greatest attention due to their carcinogenic and mutagenic properties (Pampanin *et al.*, 2013). Thousand million gallons of waste engine oil is generated annually from mechanical workshops and discharged carelessly into the environment (Faboya, 1997; Adelowo *et al.*, 2006). According to USEPA (1996), only 1 L of used engine oil is enough to contaminate one million gallons of freshwater. Used engine oil also renders the environment unsightly and constitutes a potential threat to humans, animals, and vegetation (Abioye *et al.*, 2010).

Compared to physico-chemical methods, biological methods have gained increased acceptance in cleaning up the hydrocarbon contaminated sites because these are environmentally friendly, cost-effective, and efficient (Chandankere *et al.*, 2014). However, the general low bioavailability of hydrocarbons, which are highly recalcitrant molecules that can persist in the environment due to their hydrophobicity and low water solubility, is a hindrance to microbial degradation (Xia *et al.*, 2014). Release of biosurfactants is one of the strategies used by microorganisms to influence the uptake of PAHs and hydrophobic compounds in general (Johnsen *et al.*, 2005).

The aim of this study is to isolate potent hydrocarbon-degrading and biosurfactant-producing bacterial strain and to assess the abilities of the biosurfactant produced in assisting the

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biodegradation of representative PAHs in motor oil and assess its potential application for motor oil recovery from spiked sand. For the bioremediation purpose, potential hydrocarbon degrading microbial consortia isolated from creosote contaminated soil using coal tar creosote as a source of carbon and energy was used. *B. subtilis* CN2 was isolated as an efficient degrader and biosurfactant producer. The lipopeptide biosurfactant produced by the isolate was characterized and its efficacy in enhancing motor oil biodegradation in liquid culture was investigated.

4.2.2 Isolation and Screening of Bacterial Isolates for Biosurfactant Production

Among 13 bacterial isolates obtained, 7 were found to be potential biosurfactant producer when tested by drop-collapse and oil spread test for biosurfactant production. Bacterial isolate CN2 screened was found to possess maximum biosurfactant production ability. The positive result obtained from the above confirmatory assays confirmed the biosurfactant production by the strain. Based on the 16S rDNA gene sequences and using the Gen Bank BLAST tool, isolate CN2 (Figure 4.2.1) was found to be closely related to *Bacillus subtilis*, with a percentage of similarity of 100%. The complete 16S rDNA sequence of the strain CN2 was deposited in the GenBank database under the accession number KP793927.

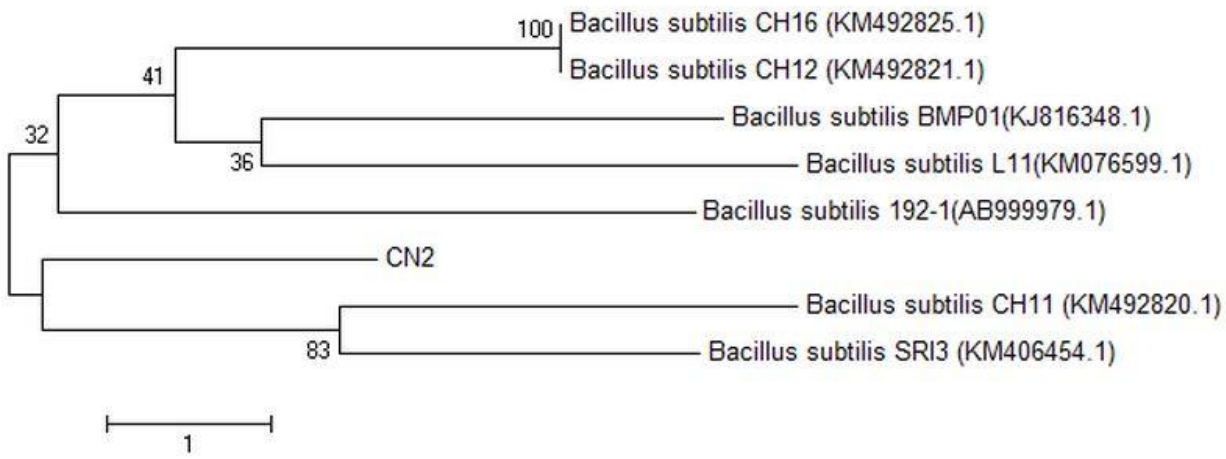

Figure 4.2.1 Phylogenetic tree based on 16S rRNA sequence, constructed by the neighbor-joining method, showing the position of strain CN2 among related members of the genus *Bacillus*. Reference strain organisms are included and sequence accession numbers are given in parentheses. Bootstrap values from 1000 replicates.

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4.2.3 Chemical Characterisation

The column purified biosurctant was collected after evaporation of the solvent for TLC , FTIR and LC MS/MS analysis

4.2.3.1 FTIR Characterisation

As presented in Figure 4.2.2, the FT-IR spectrum of the purified biosurfactant isolated from *B. subtilis* CN2 showed strongly absorbing bands at 3,286 cm^{-1} as a result of N–H stretching indicating the peptide groups. The presence of an aliphatic chain was indicated by the C–H modes from 2936 to 2882 cm^{-1} and 1412.6 –1328 cm^{-1}. The strong absorption band at 1,648.3 $cm^{-1}$is due to the amide band (C–O stretching in the peptide bond). The C–O stretching and C–O–C, ester groups were present at the 1,209 and 1,108 cm^{-1} ranges, respectively. The IR absorption pattern revealed the presence of peptide and carboxyl groups that indicated their lipopeptide nature (Sivapathasekaran *et al.*, 2009; Sriram *et al.*, 2011; Sousa *et al.*, 2014). Comparing with a standard sample of commercial surfactin from Sigma-Aldrich by Sousa *et al.* (2014), the presence of (Amide, peptide and aliphatic) functional groups was revealed, thus confirming the lipopeptidal nature of the biosurfactant.

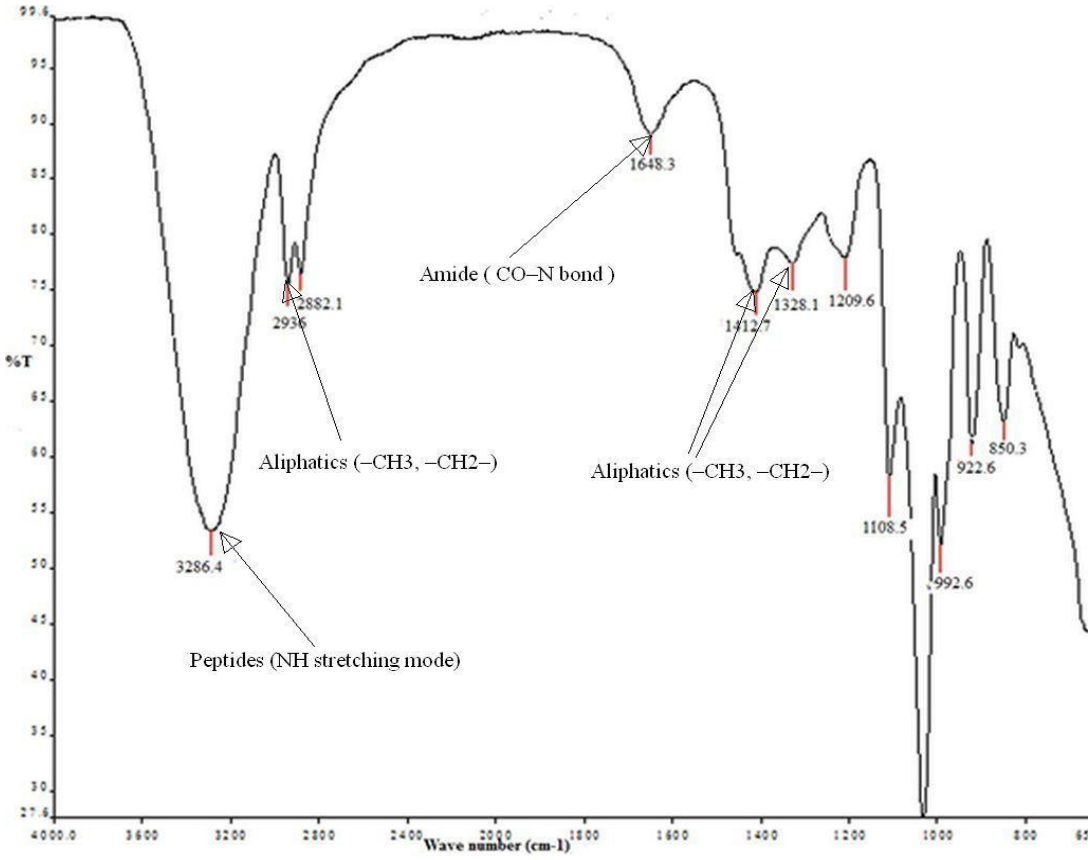

Figure 4.2.2 Fourier transform infrared spectra (FTIR) spectra of the biosurfactant produced by the *Bacillus subtilis* CN2

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4.2.3.2 Thin-Layer Chromatography (TLC) Analysis of Purified Biosurfactant

Thin-layer chromatography characterization revealed a pink spot with R_f value of 0.62 (Fig. 4.2.3) when sprayed with ninhydrin reagent, indicating the presence of amino acids. No spot was observed when sprayed with *p*-anisaldehyde confirming the absence of sugar moiety. The above result confirmed the lipopeptidal nature of the biosurfactant. Similar results were reported by Sriram *et al.* (2011).

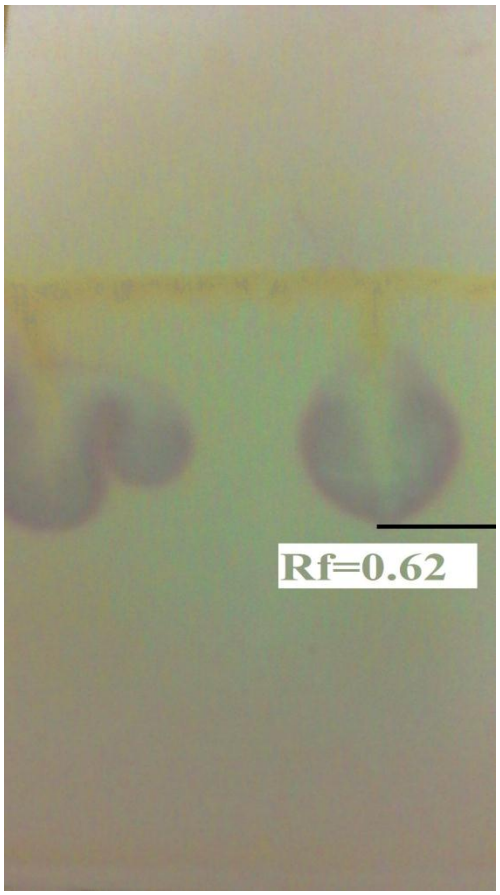

Figure 4.2.3 Thin-Layer Chromatography (TLC) of the biosurfactant obtained from *Bacillus subtilis* CN2 after treatment with ninhydrin 0.35 % (w/v, in acetone) revealed a pink spot with R*f* value of 0.62 indicating the presence of amino acids. Chromatograms were developed with chloroform: methanol: water (65:15:4 v/v)

4.2.3.3 Mass Spectrometry

As shown in LC–MS–MS spectral analysis of the purified fraction (Fig. 4.2.4**)**, a series of protonated ions were observed for the fraction of the purified sample eluting at 0.59 min, at

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m/z 1030, 1064, 1098, 1132, 1166, 1200, 1235, 1268, 1302, 1336, 1370, 1404, 1438 and 1472. These peaks differ by 34Da, suggesting a series of homologous molecules with different length of fatty acid chain. The difference of 17Da, 34 Da observed between peaks is due to NH_3 loss. These spectra are very similar to those reported by Ishikawa *et al.* (1988). A study on the amino acid sequence in iturin by Ishikawa *et al.* (1988) indicated that the difference between two peaks is 17. In a McLafferty rearrangement mechanism which is a reaction observed in mass spectrometry, the cleavage happens between alpha and beta atoms and two hydrogens are transferred to the alkoxy part including alpha atoms (Yang *et al.*, 2006). Lipopeptides can consist of short linear chains or cyclic structures of amino acids, linked to a fatty acid via ester or amide bonds or both. In a cyclic lipopeptide an ester or an amide is formed if a carbonyl group, laying in the C - terminal amino acid residue/residues, is bonded to an alcohol or an amine with a long chain and the alcohol or amine would have two or more gamma Hydrogen. As a result, for ester structure the OH_2 would be added to C-terminal part and its mass would be increased by 18.01; and to the amide structure the NH_3 would be added to C-terminal part and its mass would be increased by 17.01 (Yang *et al.*, 2006). Thus we can deduce that the biosurfactant produced by the strain *B. subtilis* CN2 is the cyclic lipopeptide iturin of molecular mass 1472Da.

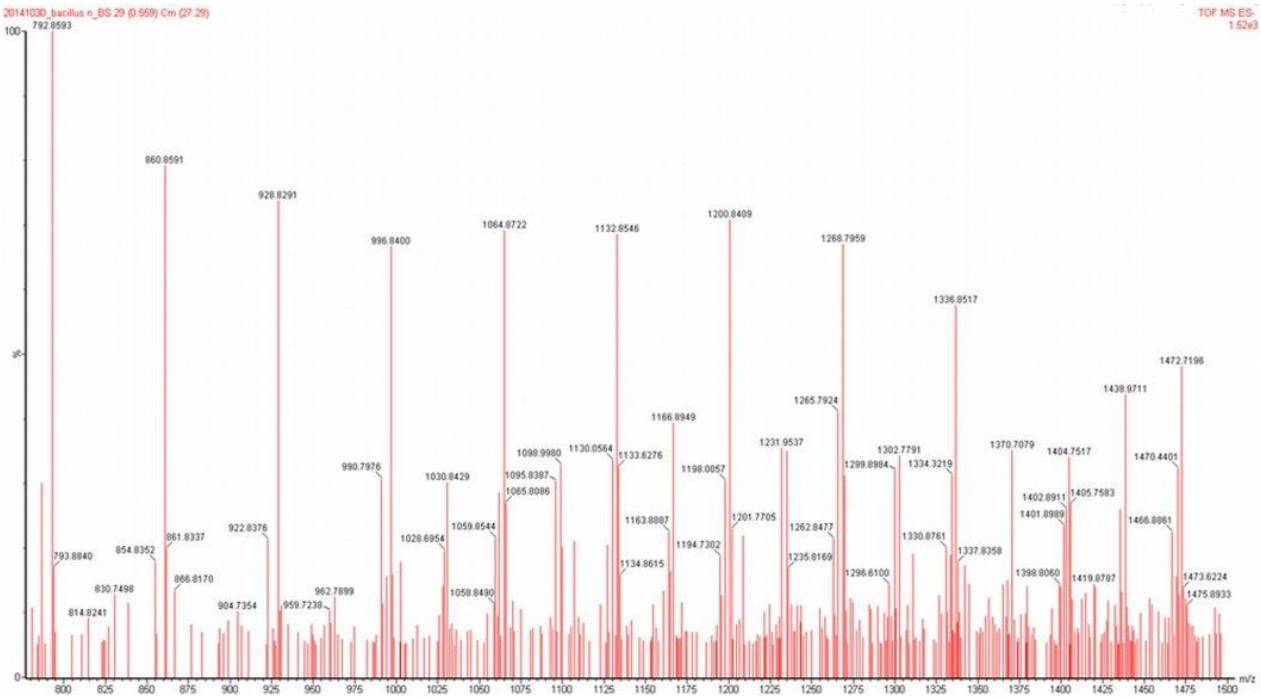

Figure 4.2.4 Tandem mass spectrometry (LC-MS/MS) Mass spectra of the fraction of the biosurfactants produced by *Bacillus subtilis* CN2 eluting at 0.569 min.

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4.2.4 Physical Properties of the Biosurfactant

4.2.4.1 Surface Activity

The isolate CN2 lowered the surface tension of culture broth from 72 to 32 mN m^{-1} after 72 h incubation. The biosurfactant concentration in terms of critical micelle dilution (CMD) in the cell free broth was 20×CMD at the minimum surface tension, indicating that the biosurfactant concentration in the cell free broth was 20 times its CMC. The emulsification capacity of the culture broth was not highest at this point and continued to increase with further growth and biosurfactant production. This reveals that the biosurfactant concentration became sufficient for micelle formation after 72 h of fermentation, beyond which constant surface tension is observed. The surface tension versus biosurfactant concentration plot was used to determine the CMC as the point of interception between the two lines that best fit the decline and the constant plateau of surface tension. The lipopeptide has a critical micelle concentration (CMC) of 185±10 mg L^{-1} corresponding to minimum surface tension of 31 ± 1 mN m^{-1} determined after column purification and serial dilution of the biosurfactant with Phosphate buffer solution (10 mM KH_2PO_4/K_2HPO_4 and 150 mMNaCl with pH adjusted to 7.0).

The CMC obtained in this study is comparable with the CMC reported in the literature for surfactin by Fox and Bala. (2000) who reported 0.15, 0.16 and 2.9 g L^{-1} depending on media used and 200 mg L^{-1} reported by Ismail *et al.* (2013). Thimon *et al.* (1992) reported that iturinic lipopeptides have close surface tension values, which are significantly higher than that of surfactin, the CMC values are quite variable from 9 µM for surfactin to 80, 160 and 170 µM for iturin C, bacillomycin D and bacillomycin L respectively which are iturinic compounds. The CMC values of iturine reported by Thimon et al are comparable to the CMC value of 127 µM of the iturin produced by CN2 strain in the current study. Hamley (2015) has reported critical micelle concentration of 25 – 40 ×10µM for iturin in water. Cooper *et al.* (1981) reported a CMC of 0.025 g L^{-1} for crystalline surfactin, some other values (13, 22, and 17 mg L^{-1}) have also been reported (Abdel-Mawgoud *et al.*, 2008).

An efficient biosurfactant can reduce the surface tension of water and air from 72 mN m^{-1} to less than 30 mN m^{-1} (Ismail *et al.*, 2013; Rodrigues, 2015). Accordingly, *B. subtillis* CN2 is a promising biosurfactant producer with a potential for use in environmental remediation and oil recovery strategies. Abdel-Mawgoud *et al.* (2008) demonstrated the high efficiency of a crude biosurfactant produced by *B. subtilis* BS5 based on the reduction of the surface tension of water from 70 to 36 mN m^{-1}. Vaz *et al*. (2012) and Ghojavand *et al.* (2008) reported surface

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tensions reductions to 29 mN m^{-1} and 27 mN m^{-1} respectively. For the *B. subtillis* CN2, the highest amount of biosurfactant produced was 7150 mg L^{-1} of culture medium obtained when using glycerol as the carbon source at concentration of 6% (w/v). Gudiña *et al.* (2015) reported about 1300 mg L^{-1} of biosurfactant production by *Bacillus subtilis* #573 strain using corn steep liquor (CSL) culture medium consisting of 10% (v/v) of CSL. Lin *et al.* (2008) and Rahman *et al.* (2006) reported highest iturin productions of 121.28 mg/L and 4000 mg/L respectively from *B. subtillis* strains.

4.2.4.2 Emulsification activity

In addition to surface activity, good emulsification property of a biosurfactant gives it additional environmental and industrial applications . The biosurfactant showed appreciable emulsification indices, with hexane (82±2%), cyclohexane (84±1%), and used motor oil (76±2.5%). The emulsions formed were stable for over 6 weeks. The results show that the biosurfactant could emulsify different hydrocarbons, which confirmed their applicability for hydrocarbon polluted media bioremediation through dispersing and enhancement of the availability of the recalcitrant hydrocarbons (Aparna *et al.,* 2012).

Ghojavand *et al.* (2008) studied the emulsification index of a biosurfactant synthesized by a member of the *B. subtilis group* (PTCC 1696) with several typical hydrocarbons, n-hexane, n-heptane, cyclohexane and n-nonane. The maximum emulsification activity of the biosurfactant was reported to be 64.4% with cyclohexane. For the isolate CN2, the highest emulsifying activity was obtained with glycerol and glucose while the use of hydrocarbons as the sole carbon source showed the least biosurfactant production and very low emulsificarion. This is in agreement with the results reported by other authors for different *B. subtilis isolates* (Joshi *et al.,* 2008; Pereira *et al.,* 2013). The CMC of the biosurfactant produced by the strain CN2 reported here is higher (i.e. less active) than previous studies. However, the emulsification index and yield of the biosurfactant is comparably higher when compared with *Bacillus subtillis* produced biosurfactants from soluble carbon sources such as glycerol and glucose (Ghojavand *et al.*, 2008; Vaz *et al.,* 2012; Rodrigues *et al.*, 2015).

4.2.4.3 Stability

4.2.4.3.1 Temperature stability

The applicability of the biosurfactant in several fields also depends on their stability at different pH, salinity, and temperature. The stability of the biosurfactant was tested over a wide range

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of temperature. The biosurfactant produced by *B. subtilis* CN2 was shown to be thermo stable. The emulsification activity and surface tension of the biosurfactant remained constant throughout the studied range of temperature (25-125 °C) with average surface tension value of 31±1 mNm^{-1} and average E_{24} value of 82±1%. Heating of the supernatant to 120 °C for half an hour caused no significant effect on the biosurfactant performances. Khopadea *et al.* (2012) reported that heating of the biosurfactant to 100 °C caused no significant effect on the biosurfactant performance. Heating at 125 °C for 30 min resulted in a slight increase in surface activity similar result was reported by Abdel-Mawgoud *et al*. (2008). Abdel-Mawgoud et al. suggested that this increase in activity may be attributed to the heat-dependent coagulation and precipitation of substances such as proteins contaminating the surfactin solution; which might have been coextracted with surfactin during extraction steps. The biosurfactant's stability at high temperature condition suggests the biosurfactant's potential for enhanced oil recovery and bioremediation of spills in thermophilic condition.The unique and distinct properties of biosurfactants when compared to their chemically synthesized counterparts are related to their surface activity, tolerance to pH, temperature and ionic strength, biodegradability and low toxicity (Chakrabarti, 2012; Vijayakumar and Saravanan, 2015). Especially since, industrial processes and microbial enhanced oil recovery involve exposure to extremes of temperature, pH and pressure, it is necessary to isolate novel microbial products that able to function under these conditions (Cameotra and Makkar, 2004).

4.2.4.3.2 pH stability

The biosurfactant demonstrated a stable emulsification and surface tension over the pH range of 5–12 and an appreciable decrease of E_{24} at pH < 5 and pH > 13 (Fig. 4.2.5). At pH 13, the value of emulsification activity (E_{24}) maintained was 92%. For pH values lower than 5, the samples become turbid, and showed reduction in emulsification activity and surface tension due to the initiation of precipitation of the biosurfactant which increases with decrease in pH. These results are in accordance with the ones reported by Abdel-Mawgoud *et al*. (2008), in which the lipopeptide surfactin was found to be soluble in aqueous solutions at pH values higher than 5.0. The authors demonstrated that the optimum solubility for surfactin is at pH 8.0–8.5. This may be attributed to the acidic nature of the lipopeptide. The molecule contains two carboxylic groups (glutamic acid residue and aspartic acid residue) that confer it its anionic nature (Vaz *et al.,* 2012). Similar results of increasing surface activity with increasing pH were

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reported by Khopade *et al*. (2012) and Abouseoud *et al.* (2008). Abouseoud *et al.* (2008) reported that pH increase from 5 to 12 decreased surface tension from 34 to 30 mNm^{-1}, and increased emulsion stability (15% increase of E_{24}). Increase in emulsion stability with increasing pH (Fig. 4.2.5) could be caused by a better stability of fatty acids-surfactant micelles in the presence of NaOH and the precipitation of secondary metabolites at higher pH values (Abouseoud *et al.*, 2008; Khopade *et al.*, 2012). Extreme pH values could possibly transform weak surface-active species into more active emulsifiers by increasing ionization (Shin *et al.*, 2004; Abouseoud *et al.*, 2008). The biosurfactant stability at high alkaline conditions also suggests the biosurfactant's potential for bioremediation of spills in marine environment of high salinity and alkaline condition.

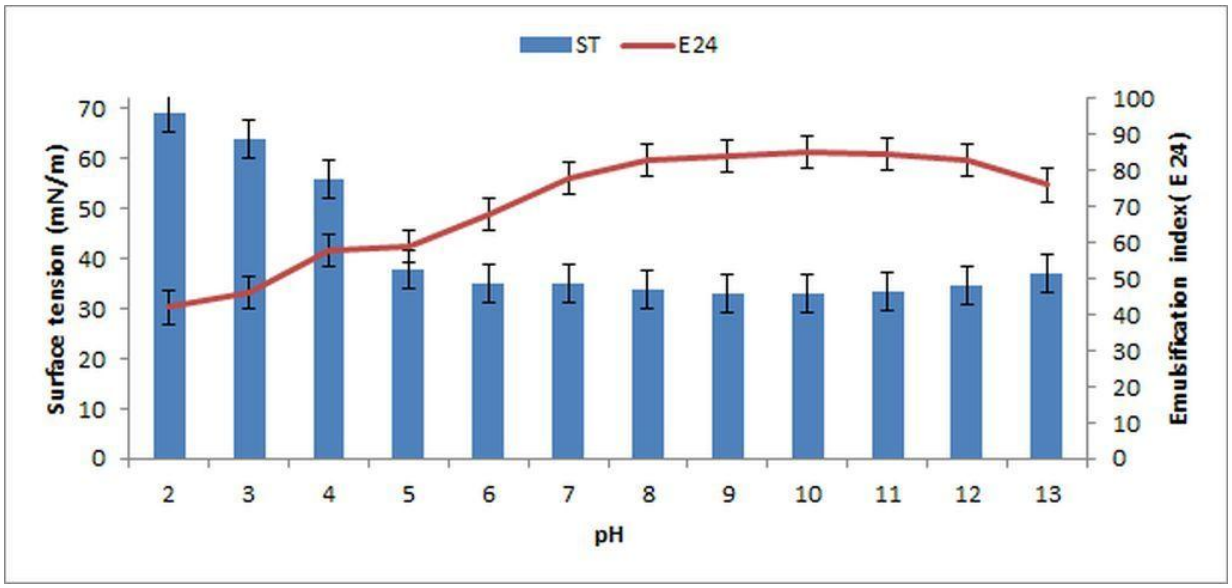

Figure 4.2.5 Influence of pH on the surface tension values (mN m^{-1}) and emulsifying index (%) of 72 hour cell-free broth of *Bacillus subtilis* CN2, obtained by centrifuging the culture at 12000 rpm for 20 minutes. The error bars represent standard errors of three independent experiments (n = 3).

4.2.4.3.3 Effect of salinity

The emulsification activity and surface tension of the biosurfactant remained constant throughout the studied range of salinity (5 -20%, w/v) with average surface tension value of 31±1 mN/m and average E_{24} value of 82±1%. Abouseoud *et al.* (2008) reported a similar effect

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on effectiveness upon the addition of 20 % sodium chloride, for a biosurfactant produced by *Pseudomonas fluorescens Migula* 1895-DSMZ. The similarity of stability results shows the effectiveness of microbially produced surfactants at extreme of salinity due to their unique structures which provide new properties that synthetic surfactants may lack. Previous reports showed that concentrations above 2% NaCl are enough to inactivate synthetic surfactants (Desai and Banat, 1997). Khopadea *et al*. (2012) reported that little changes were observed in increase in concentration of NaCl up to 9% (w/v) while at higher concentration of NaCl, the biosurfactant retained 80% of its activity. The biosurfactant produced by *B. subtilis* CN2 has stability at alkaline pH and high salinity; such a biosurfactant may be useful for bioremediation of spills in the marine environment because of its stability in alkaline condition and in the presence of salt (Khopadea *et al.*, 2012).

4.2.5 Biodegradation of Used Motor Oil

There was a significant increase in cell density in the culture broths in the 18 days of incubation, with an associated decrease in different components of the used engine oil, demonstrating that the consortium can utilize used motor oil as the sole carbon and energy source. High turbidity and enhanced bacterial growth were observed in the biosurfactant supplemented culture than the one without biosurfactant supplementation (Fig. 4.2.6). The total viable cell count increased (from 6.4 log (CFU) mL^{-1} at the day of incubation to to 9.99 log (CFU) mL^{-1}) on day 15 in the flask supplemented with the biosurfactant. In the microcosm with no biosurfactant supplementation the total viable cell count were considerably lower by up to two orders of magnitude (increase from 6.4 to 7.77 log (CFU) mL^{-1} compared to increase from 6.4 to 9.99 log (CFU) mL^{-1}) in both nutrients and biosurfactant supplemented flask.

Even though both microcosms containing used motor oil (3%, v/v) were supplied with nutrients from the mineral salt medium the greater bacterial growth in those microcosms supplemented with both nutrients and biosurfactant (compared with nutrients alone) suggests that by increasing hydrocarbon bioavailability the microbial growth was enhanced. The biosurfactant increased the surface area of the hydrophobic water-insoluble substrate and improved their bioavailability, thereby enhancing the growth of bacteria and the rate of bioremediation (Ron and Rosenberg, 2002). One biological strategy that can enhance contact between bacteria and water-insoluble hydrocarbons is emulsification of the hydrocarbon. Biosurfactants help to disperse the oil and increase the surface area for growth, unless the surface area of the

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hydrophobic substrate is increased exponential bacterial growth is not possible, and biomass will only increase arithmetically (McKew *et al.*, 2007).

Fresh motor oil usually contains very small amounts of polycyclic aromatic hydrocarbons (PAHs). On the other hand, used oil may contain minute quantities of additive, metals such as lead, zinc, barium and magnesium resulting from engine wear and higher percentages of alkylbenzenes, naphthalene, methyl naphthalene and higher polycyclic aromatic hydrocarbons (PAHs) as a result of pyrosynthesis (Lu and Kaplan, 2008; Obayori *et al.*, 2014). The degradation of representative PAHs and methylated derivatives was monitored during the shake flask treatment. The representative PAHs monitored were Naphthalene; Acenaphthene; Naphthalene 2-methyl-, Fluorene; Phenanthrene; Fluoranthene; Naphthalene, 1-phenyl-; Naphthalene, 2-phenyl- and 9-Allylanthracene. Biodegradation tests of the motor oil components in the laboratory scale microcosm experiment showed maximum degradation in the culture with biosurfactant amendment (Bacterial cells + used motor oil + biosurfactant + MSM) than the one without biosurfactant (Bacterial cells + used motor oil + MSM). Furthermore, there was no significant removal observed in the abiotic controls (less than 3.1 ±1.2%), confirming that the removal was due to biological activity.

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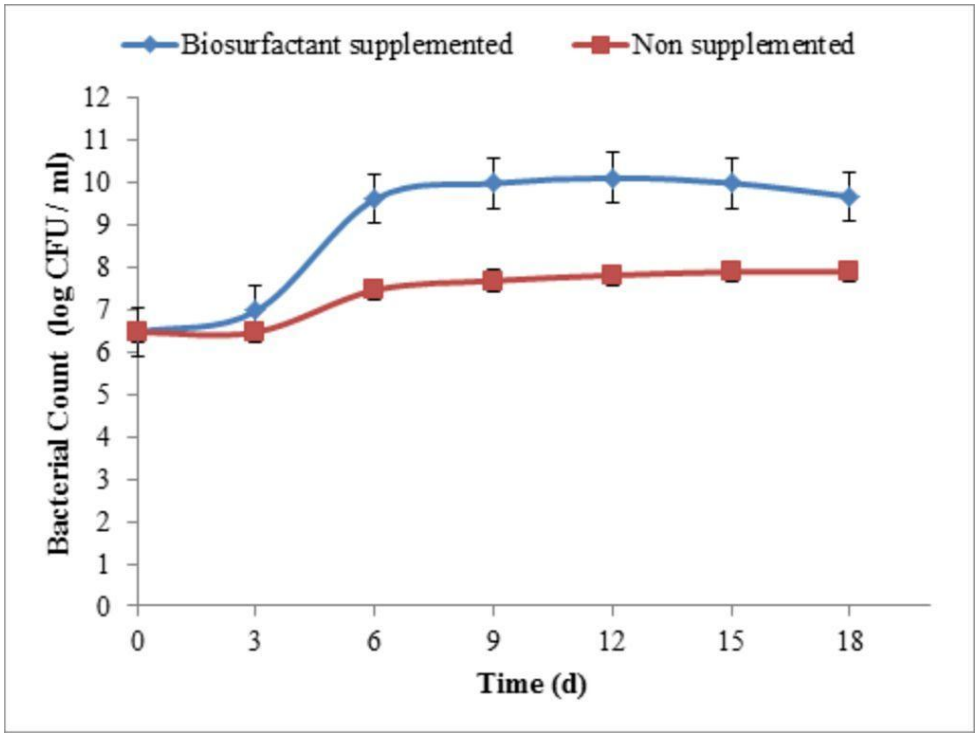

Figure 4.2.6 The growth kinetics of the consortium on MSM supplied with used motor oil (3% v/v) as the sole carbon source, with 0.15% (w/v) biosurfactant supplementation (*diamond*), with no biosurfactant supplementation (*square*). Data are expressed as the mean ± standard error of two independent experiments performed in duplicate. Other process parameters: pH, 7; temperature, 35 °C; agitation speed, 180 rpm.

The biosurfactant amendment more than doubled the degradation of the PAHs; mainly enhanced degradation of the more hydrophobic PAHs was observed (Figure 4.2.7A). At day 18 of incubation there was considerable disappearance of the peaks in the biosurfactant amended sample, whereas in the unamended microcosm persistence of the PAHs was observed. Mainly the more hydrophobic PAHs showed the least degradation in the microcosm without biosurfactant supplementation (Figure 4.2.7B). Enhanced bacterial growth was observed in the biosurfactant amended microcosm.

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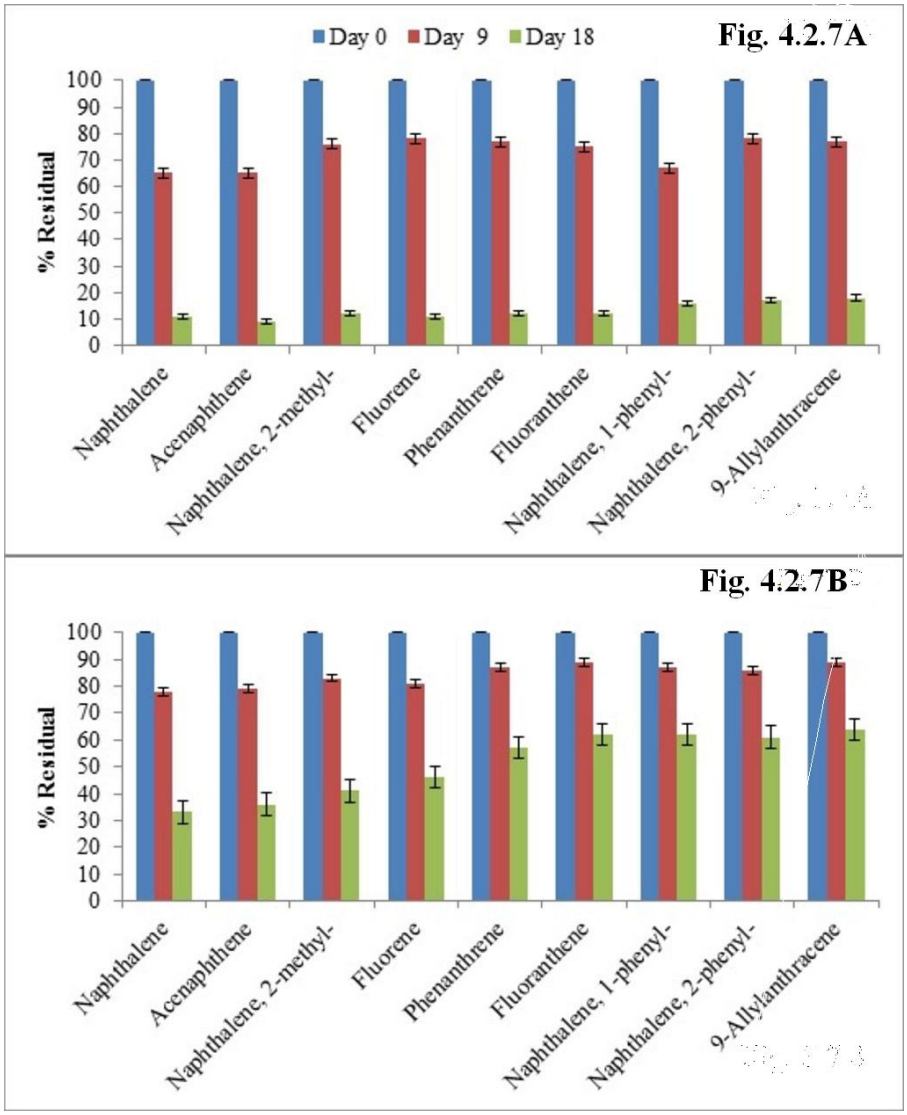

Figure 4.2.7 Percentage of residual monitored PAHs in used motor oil (3% v/v) amended flasks, as the sole source of carbon and energy, at day 0 (blue); day 9 (red) and day 18 (green): (A) With 0.15% (w/v) biosurfactant supplementation; (B) with no biosurfactant supplementation. Data are expressed as the mean ± standard error of two independent experiments performed in duplicate. Error bars denote standard errors of duplicate samples. Other process parameters: pH, 7.5; temperature, 35 °C; agitation speed, 180 rpm.

One of the main factors affecting the biodegradation efficiency of complex oily compounds is the low availability of contaminants for microbial attack. An alternative to expand bioavailability and contaminant metabolism is increasing substrate solubilisation by using biosurfactants (Cerqueira *et al.*, 2011). Biosurfactants can increase the aqueous solubilisation of poorly soluble compounds, including Polyaromatic hydrocarbons and enhance hydrocarbon

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bioremediation by two mechanisms (Pacwa-Płociniczak *et al.*, 2011). The first includes the increase of substrate bioavailability for microorganisms, while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells. By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds leading to increased mobility and bioavailability of hydrocarbons. The enhanced biodegradation levels obtained with biosurfactant demonstrated that that they represent the most efficient accelerators for hydrocarbon biodegradation through enhancing their bioavailability and facilitating their degradation by microorganisms (Noparat *et al.*, 2014).

Investigation of surface tension and emulsifying activity of the culture medium demonstrated that the microbial consortium were able to produce biosurfactants in mineral salt medium containing motor oil as the sole carbon source. The highest emulsification index (> 90 % with hexane) was observed in the biosurfacatnt supplemented microcosm and high surface tension reduction, lower than 35 mN m^{-1} was observed in both biosurfcatnt supplemented and non-supplemented microcosms. Most of the members of the consortium used in this study like, *Bacillus subtilis, Paenibacillus dendritiformis, Pseudomonas aeruginosa, Ochrobactrum intermedium* have been confirmed as potent biosurfactant producers in the screening tests conducted. Thus the additional initial biosurfactants added to the culture medium could be enough to facilitate the bacterial access to the crude oil followed with more biosurfactant production which resulted in the high emulsification index observed.

Thavasi *et al.* (2011) observed maximum crude oil degradation and biosurfactant production with *Pseudomonas aeruginosa* (89%, with 0.1% biosurfactant). Similar studies of biosurfactant enhanced degradation have been reported by many authors. Chandankere *et al.* (2014) reported that an effective biosurfactant-producer and hydrocarbon degrading bacterial strain, *Bacillus methylotrophicus* USTBa degraded 92% of crude oil in 2 weeks' time and produced biosurfactant during the course of hydrocarbon degradation that had the ability to decrease the surface tension of water from 72 to 28 mN m^{-1}. A biosurfactant from *L. delbrueckii* cultured with peanut oil cake as the carbon source was tested in biodegradation experiments with crude oil. While significant oil degradation occurred when the biosurfactant and fertilizers were added, the biosurfactant alone was also capable of promoting biodegradation to a large extent without added fertilizers (Thavasi *et al.*, 2011). Crude oil biodegradation with biosurfactnt from *Pseudomonas cepacia* CCT6659 cultivated with 2% soybean waste frying oil and 2% corn

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steep liquor as substrates showed 83% biodegradation activity in the first 10 days of the experiments when the biosurfactant and bacterial cells were used together (Silva *et al.*, 2014).

In addition to increasing bioavailability biosurfactant or biosurfactant producing bacteria reduce the toxicity level of heavy metals in the used motor and this supports the growth of other hydrocarbon degrading bacterial strains to supplement the process of oil degradation. The usefulness of biosurfactants for bioremediation of heavy metal contaminated media is mainly based on their ability to form complexes with metals. The anionic biosurfactants create complexes with metals in a nonionic form by ionic bonds (Pacwa-Płociniczak *et al.*, 2011).

4.2.6 Used Motor Oil Removal from Contaminated Sand

Petroleum hydrocarbon compounds bind to soil components and are difficult to remove due to their hydrophobicity. Biosurfactants can emulsify hydrocarbons enhancing their water solubility, decreasing surface tension and increasing the displacement of oil substances from soil particles (Batista *et al.*, 2010; Luna *et al.*, 2012). The results showed 84.6±7.1 and 15± 3% used motor oil recoveries from the contaminated sands in the cell free supernatant from *B. subtilis* CN2 and control (distilled water) respectively. This result is comparable with the ones reported by Liu *et al.* (2015) in which biosurfactant from *Bacillus subtilis* BS-37 recovered 96% of crude oil from oil-sand at the concentration of 300 mg L^{-1}, surfactin (LB) and surfactin (G) solution.

Joshi and Desai (2013) reported 30.22–34.19% additional oil recovery over the water flood residual oil saturation using crude biosurfactant obtained from *Bacillus* strains by sand pack column studies. Al-Wahaibi *et al.* (2014) reported 17–26% extra oil recovery using crude biosurfactant obtained from *B. Subtilis* B30. The current study showed that, the biosurfactatnt produced by *B. subtilis* CN2 has potential application in soil washing and enhanced microbial oil recovery.

4.3 SUMMARY

Based on the study it can be concluded that the isolate *Ochrobactrum intermedium* CN3 was able to grow on petroleum sludge as a source of carbon and energy and showed an enhanced degradation when supplemented with the biosurfactant it produced. Especially the effect of the biosurfactant was pronounced more on the biodegradation of the long chain hydrophobic aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) which are known for

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their recalcitrance and persistence in the environment due to their low solubility. The biosurfactant from *B. subtilis* CN2 strain increased the pseudosolubilization and emulsification of highly hydrophobic used motor oil sludge, thereby increasing the bioavailability and its subsequent degradation. The biosurfactant from the CN2 strain also showed an interesting potential application in soil washing or enhanced microbial oil recovery. Generally the findings demonstrated that thebiosurfactants can have remarkable potential for environmental application and enhanced oil recovery at harsh environmental conditions of extreme salinity, alkalinity and thermal conditions.

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CHAPTER FIVE

BIOSURFACTANT FROM *PAENIBACILLUS DENDRITIFORMIS* AND ITS APPLICATION IN ASSISTING PAH AND MOTOR OIL SLUDGE REMOVAL FROM CONTAMINATED SOIL AND SAND MEDIA

5.1 Introduction

Past industrial activities have left behind large areas contaminated with persistent organic pollutant and especially polycyclic aromatic hydrocarbons (PAHs). These compounds present a risk for human health and the environment, as they are mutagens, carcinogens and teratogens (International Agency for Research on Cancer, 2010; Barnier *et al.*, 2014). Bioremediation is an effective and economic technique for the cleanup of PAH contaminated sites and a wide variety of microorganisms were found to have the ability to degrade PAHs (Yu *et al.*, 2014). However bioremediation of PAHs in the environment is limited by their low bioavailability to microbes because of high hydrophobicity, low aqueous solubility and strong sorption to soil material, such as clays and organic matter (Malik *et al.*, 2011).

Thus, surfactant-enhanced remediation (SER) has been suggested as a promising technology for the remediation of hydrophobic organic compounds (HOCs) contaminated soils and groundwater (Mulligan *et al.*, 2001; Zhou and Zhu, 2007). Surfactants have been used for surfactant-enhanced bioremediation or other remedial technologies, such as soil washing, surfactant-enhanced phytoremediation, and surfactant-enhanced electrokinetic remediation (Mao *et al.*, 2015).

Surfactant enhanced desorption for HOCs is a key factor to the performance of SER technology in the remediation of contaminated soils and groundwater (Zhou and Zhu, 2007). Numerous studies have shown that biosurfactants are effective in washing soils contaminated with various hydrocarbon compounds (Urum *et al.*, 2005; Portet-Koltalo *et al.*, 2013; Singh and Cameotra, 2013; Mnif *et al.*, 2014). The addition of surfactant in PAH contaminated soil washing solutions act to reduce the surface and interfacial tension at the air–water and oil–water interface, thereby reducing the capillary force that holds the oil and soil, which may lead to the mobilizing and (or) the solubilizing of the oil (Urum *et al.*, 2005). Surfactants can greatly enhance the apparent solubility of an otherwise poorly soluble HOC by partitioning of the HOC

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into micelles, which occurs when the aqueous-phase concentration of the surfactant is above its critical micelle concentration (CMC). These micelles with hydrophilic surfaces and lipophilic cores can easily disperse the contaminants like nonaqueous phase liquids (NAPLs), and dramatically improve their solubility in water phase, thereby further promote desorption of contaminants from soil. The dissolved contaminants in aqueous phase have better mobility, being conducive to the subsequent removal of contaminants via either biotic routes e.g., plant uptake and microbial degradation or abiotic routes e.g., soil washing and subsequent separation, Mao *et al.* (2015).

Biosurfactants play a pivotal role in the biodegradation of hydrophobic aromatic compounds contained in petroleum and heavy oil (Banat *et al.*, 2010). Lipopeptides are a group of biosurfactants consisting of a hydrophobic fatty acid moiety and a hydrophilic peptide moiety in the molecule. Lipopeptides have low critical micelle concentration (CMC), stable emulsification properties, powerful surface activities and excellent foaming characteristics and show stable physical–chemical properties at different temperatures and pH levels (Xia *et al.*, 2014). Many types of biosurfactants have been synthesized from bacteria belonging to a wide variety of genera, such as *Pseudomonas*, *Bacillus*, *Acinetobacter*, and *Mycobacterium* (Banat *et al.*, 2010).

In this study, we report the production of lipopeptide biosurfactant by potent biosurfactant producing bacterium *Paenibacillus dendritiformis* CN5 strain isolated from creosote contaminated soil and investigate the efficacy of biosurfactant produced by the strain in enhancing desorption of PAHs from spiked soil and heavy oil recovery from contaminated sand. The experimental results can be used to investigate biosurfactant enhanced desorption of hydrophobic organic compounds (HOCs) and provide valuable information for the application of biosurfactant enhanced remediation technology for the restoration of organic contaminated soil and possible application of the biosurfactant for enhanced oil recovery

5. 2 Isolation of Bacterial Isolates for Biosurfactant Production

A Total of 13 bacterial isolates were obtained from creosote contaminated soil which are efficient degraders of Creosote. Strain CN5 was one of these isolates it was found out to be efficient biosurfactant producer. The 16S rRNA analysis revealed that strain CN5 was related to members of genus *Paenibacillus*, and showed highest sequence similarity (100%) to *Paenibacillus dendritiformis*. The 16S rRNA gene sequence of strain CN5 was aligned automatically to reference sequences of the genus *Paenibacillus dendritiformis* obtained from

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the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), and a phylogenetic tree was constructed (Fig. 5.1) based on the neighbor-joining method using the software MEGA version 6.0 (Tamura *et al.*, 2013). Members of the genus *Paenibacillus* have been reported in different studies to be present in soils contaminated with petroleum hydrocarbons and shown to degrade oil and PAHs (Ganesh and Lin, 2009; Gudiña *et al.*, 2015). Obuekwe *et al.* (2009) identified *Bacillus sp.* and *Paenibacillus sp*. as two of the most prominent crude-oil degraders in the Kuwait desert environment. The dominant isolation of these spore-forming gram-positive bacteria was predicated on their ability to survive the prevalent high soil temperature (40 - 50°C) and tailings from oil-recovery operations (Ganesh and Lin, 2009). Bacterial strains belonging to the *Paenibacillus* genus like *Paenibacillus sp.*, *Paenibacillus macerans* have been reported as efficient biosurfactant producers (Liang *et al.*, 2014; Gudiña *et al.*, 2015).

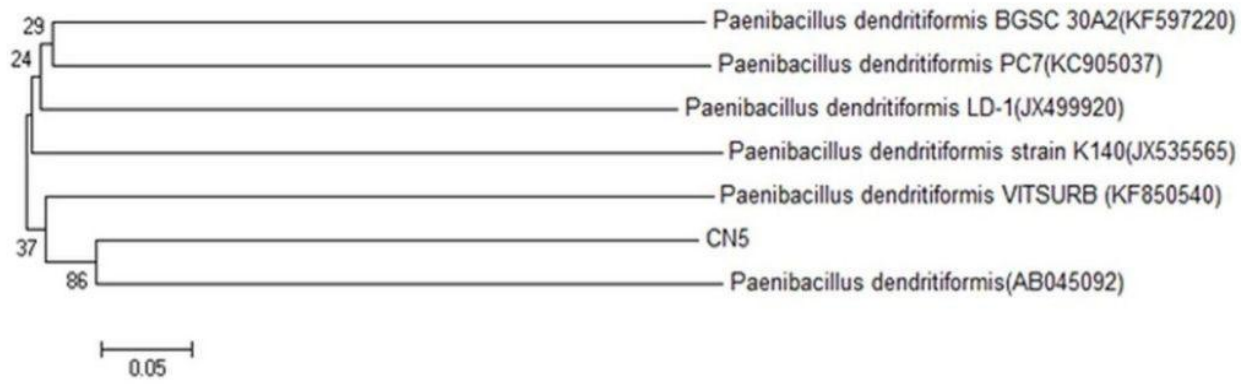

Figure 5.1 Phylogenetic relationship based on the 16S rDNA gene sequences between strain CN5 and species in the *Paenibacillus dendritiformis* as determined by the neighbour-joining algorithm. Numbers at nodes indicate levels of bootstrap support (%) based on a neighbour-joining analysis of 1000 resampled data sets. NCBI accession numbers are given in parentheses.

5.3 Physical Properties of the Biosurfactant

5.3.1 Surface activity

The surface tension of the whole broth dropped rapidly from around 71 mN m^{-1} to 34 ± 1 mN m^{-1}, in the first 3 days of incubation, the biosurfactant concentration is expressed in terms of critical micelle dilution (CMD), CMD is the dilution necessary to reach the CMC (critical

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micelle concentration), at which the surface tension starts to increase dramatically (Kim *et al.*, 1997). The biosurfactant concentration in the cell free broth was 18×CMD at the minimum surface tension. The lipopeptide has a critical micelle concentration (CMC) of 185 mgL^{-1} corresponding to minimum surface tension of 34.6 ± 1 mNm^{-1}. The ability to form and stabilize emulsions is an important parameter to evaluate the quality of a surface-active agent. The cell free supernatant of *Paenibacillus dendritiformis* CN5 was used to prepare emulsion of hexane, cyclo-Hexane and used motor oil, which were found to remain stable up to 2 months. The emulsification index of culture supernatant was found to be 74%, 82% and 72 % with hexane, cyclo-Hexane and used motor oil respectively.

5.3.2 Temperature salinity and pH stability of the biosurfactant

The surface tension and emulsification index of the culture supernatant was tested over a wide range of temperature. CN5 remained stable after incubation for an hour at 110 °C. The surface tension of the culture supernatant and emulsification activity were stable indicating high thermal stability. The surface tension and emulsification activity of culture supernatant also remained unaltered over a pH range of 6–10 (Fig. 5.2a). There was gradual increase in surface tension and decrease in emulsification activity at pH values lower than 5.5 and higher than 10. As the pH values lower than 4 the surface tension was increasing dramatically since the biosurfactant is not soluble at such acidic conditions, it precipitates. Several studies (Gudiña *et al.*, 2010; Al-Bahry *et al.*, 2013) reported stability of the biosurfactants in the range of 6 to 12 pH values, while for pH values lower than 6, surface tension starts increasing and the samples turbidity increases, due to partial precipitation of the biosurfactant. The effect of sodium chloride addition on the surface activity showed little changes in increased concentration of NaCl up to 15% (w/v). At higher concentration of NaCl (up to 20%), the biosurfactant retained 85% of the surface activity (Fig. 5.2b). The biosurfactant stability at high saline and alkaline conditions also suggests the biosurfactant's potential for bioremediation of spills in marine environment of high salinity and alkaline condition.

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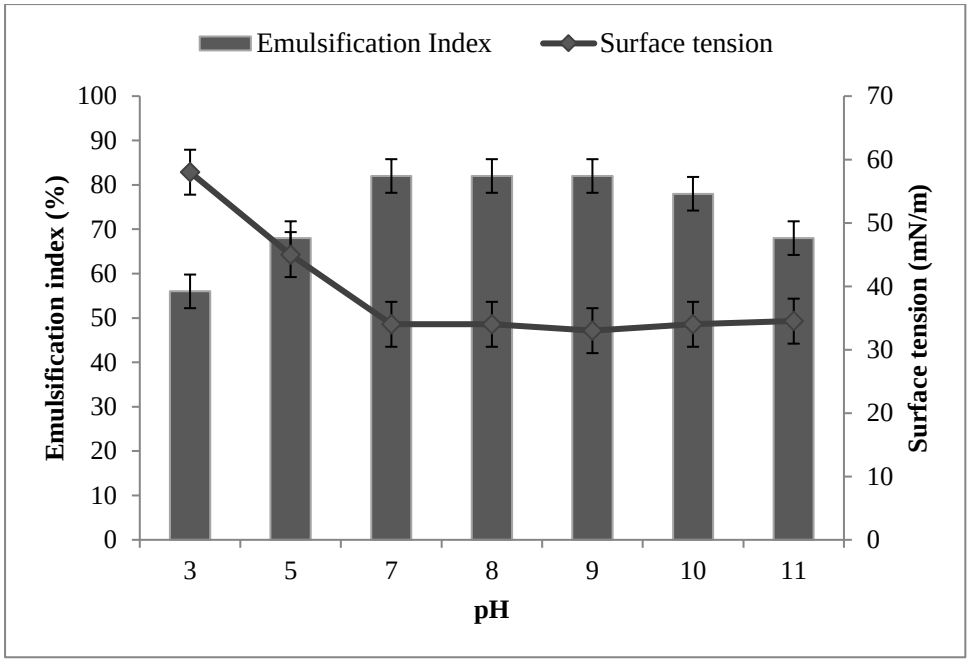

Figure 5.2a The surface tension and emulsification index values of the cell free broth produced by *Paenibacillus dendritiformis* CN5 over a wide range of pH values. The error bars represent standard errors of three independent experiments (n = 3).

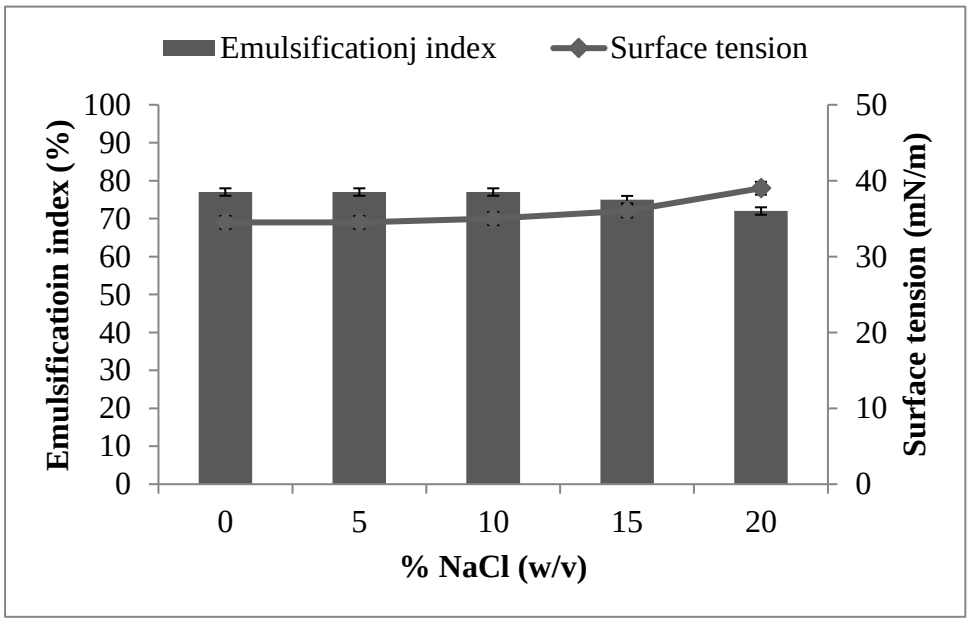

Figure 5.2b Effect of different salt (NaCl) concentration on surface tension and emulsification index values of the cell free broth produced by *Paenibacillus dendritiformis* CN5. The error bars represent standard errors of three independent experiments (n = 3).

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5.4 Chemical characterization of the biosurfactant

5.4.1 FTIR ATR Characterization

The FTIR is the most useful analysis for identifying types of chemical bonds (functional groups) present in the biologically active fraction of an unknown biosurfactant and thus determines its chemical nature. The FTIR spectrum of the column purified biosurfactant showed bands characteristic of peptides at 3,282.7 cm^{-1} resulting from N–H stretching mode (Fig. 5.3). The presence of aliphatic chains was confirmed by the observation of peaks in the region 2882–2935.8 cm^{-1} due to the –C–H stretching mode of CH_3 and CH_2 groups in alkyl chains (Thaniyavarn *et al.*, 2003). The deformation vibrations from 1412 cm^{-1} to 1210 cm^{-1} reflect aliphatic chains (–CH_3, –CH_2–) of the fraction. The sharp peak around 1648.41cm^{-1} (stretching mode of the CO–N bond) is due to amide group (Nasir and Besson, 2012). Absorbance in this region signifies the presence of peptide group in the molecule which indicates the presence of fatty acid chain in the biosurfactant. This type of FTIR spectra is characteristic of lipopeptides, e.g. other lipopeptide biosurfactants like lichenysin reported in literature have yielded similar IR absorption spectra (Yakimov *et al.*, 1995; Das *et al.*, 2008.).

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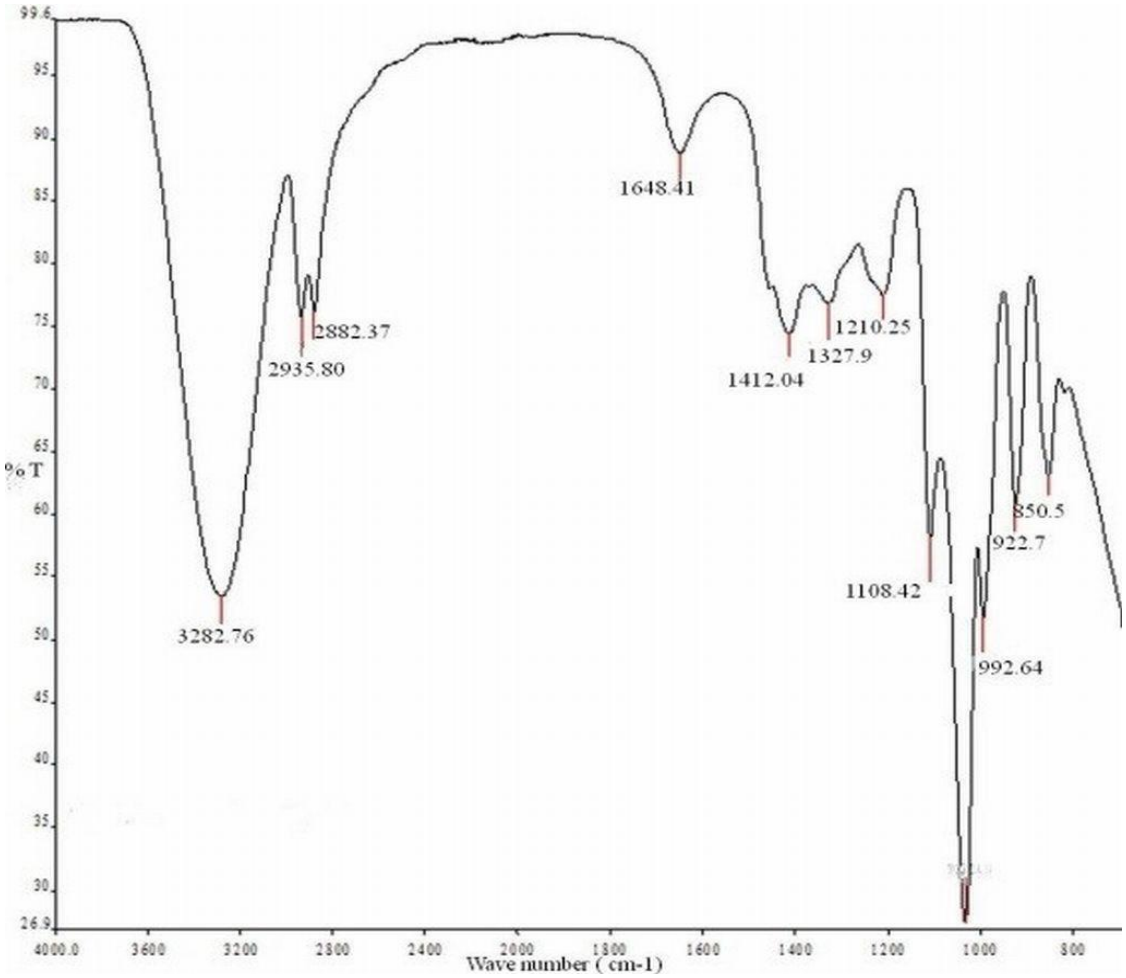

Figure 5.3. Fourier transform infrared (FTIR)-absorption spectrum of the biosurfactant produced by *Paenibacillus dendritiformis* CN5 Strain

5.4.2 TLC analysis of purified biosurfactant

The aqueous fraction of the biosurfactant after acid hydrolysis indicated an R*f* value of 0.7 and showed positive reactions with ninhydrin on the TLC plate (Fig. 5.4), signifying that the biosurfactant consisted of peptide moieties and showing that it may be lipopeptide.

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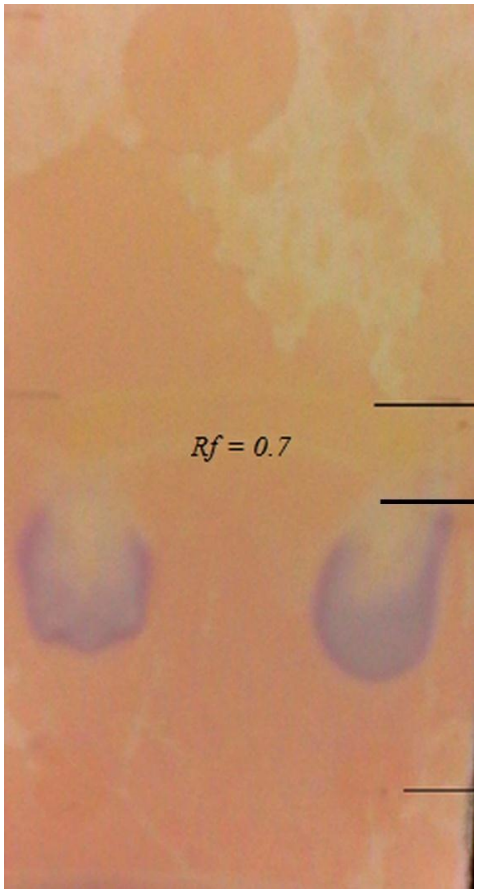

Figure 5.4. Thin-Layer Chromatography (TLC) analysis of the biosurfactant obtained from *Paenibacillus dendritiformis* CN3 after treatment with ninhydrin revealed a pink spot with R*f* value of 0.7.

5.4.3 Mass Spectrometry

A positive full scan LC-MS/MS chromatogram of the lipopeptide extract (eluted at 0.54) was elucidated using mass spectrometry technique. LC-MS/MS analysis are liable and simple method to elucidate the structure of lipopeptides. Lipopeptides always contain a peptide as backbone and a fatty acid chain residue. Tandem mass spectroscopy is well adapted to determine the peptide sequence by analyzing the *m/z* of the productions. However, the cleavage points of the peptide ring are crucial for interpreting the tandem mass spectrometry of lipopeptides (Ma and Hu, 2014). Each peptide fragment in a series differs from its neighbour by one amino acid. In principle, it is therefore possible to determine the amino-acid sequence by considering the mass difference between neighbouring peaks in a series, as is shown in Fig. 5.4 . The peaks obtained for different fragments in the MS/MS analysis at *m/z* 718, 588, 474, 344, 288, 198 and 125 display predicted peptide sequence of Cys-Gly-Ala-Gly-Ile-Asn-Leu.

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The peptide sequence is linked to an aliphatic chain of heavy molecular weight, fatty acid moiety, proposed to be $CH_3(CH_2)_{33}COOH$, to give the molecular mass of the lipopeptide *m/z* 1240 Da (Fig. 5.4).

The subsequent fragments with *m/z* 125, 198, 288, 344, 474, 588, 718, 832 and 968, 1098, 1166 and 1240 Da point to the following chain: lipophilic part + Leu -- Asn -- Ile -- Gly -- Ala -- Gly-- Cys. Thus, the lipopeptide surfactant produced by *Paenibacillus dendritiformis* CN5 can be, possibly composed of 7 amino acids coupled to one molecule of Pentatriacontanoic fatty acid ($CH_3(CH_2)_{33}COOH$) of molar mass 522 g/mol. The molecular weight (MW) of the lipopeptide is determined as the sum of the 7 peptide hydrophilic moiety and the MW of the hydrophobic fatty acid moiety, $C_{35}H_{70}O_2$, which gives the MW of the lipopeptide as 1240 Da (Fig. 5.5).

The current study reported production of novel lipopeptide of molecular weight (1240Da) by *Paenibacillus dendritiformis* CN5. Various lipopeptides of different molecular weight and shapes have been reported (Qian *et al.*, 2012; Guo *et al.*, 2012). Several bacterial species produce lipopeptides or peptidolipids, most of which have important biological functions. For example, many have surfactant, antibacterial or antifungal properties and have attracted interest from industry.

They can consist of short linear chains or cyclic structures of amino acids, linked to a fatty acid via ester or amide bonds or both (http://www.lipidhome.co.uk/lipids/complex/pc/index.htm). Cyclic lipopeptide was isolated from the *Paenibacillus* bacterial genus, consisting of a cyclic lipopeptide with a 15 fatty acid chain and 13 amino acid residues (Guo *et al.*, 2012). Cyclic lipopeptides from *Paenibacillus spp.* containing 15-guanidino-3-hydroxypentadecanoic acid as the fatty acid component linked to six-membered cyclic peptides was reported by Beatty, and Jensen (2002). Qian *et al.*, (2012) reported production of lipopeptide named *battacin* from *Paenibacillus tianmuensis*, with 8 amino acid residues. *Bacillus* or *Paenibacillus* species produce basic lipopeptides that consist of decapeptides (7-membered cyclic peptides attached to a linear peptide) linked to a fatty acid such as 6-methyl-octanoic or 6-methyl-heptanoic acids, and termed 'polymyxins' (http://www.lipidhome.co.uk/lipids/complex/pc/index.htm). Lipopeptides vary in their amino acid and/or fatty acid composition and all these variations in length and branching of the fatty acid chains and amino acid substitutions lead to remarkable lipopeptide diversity and activities (Sharma *et al.*, 2014).

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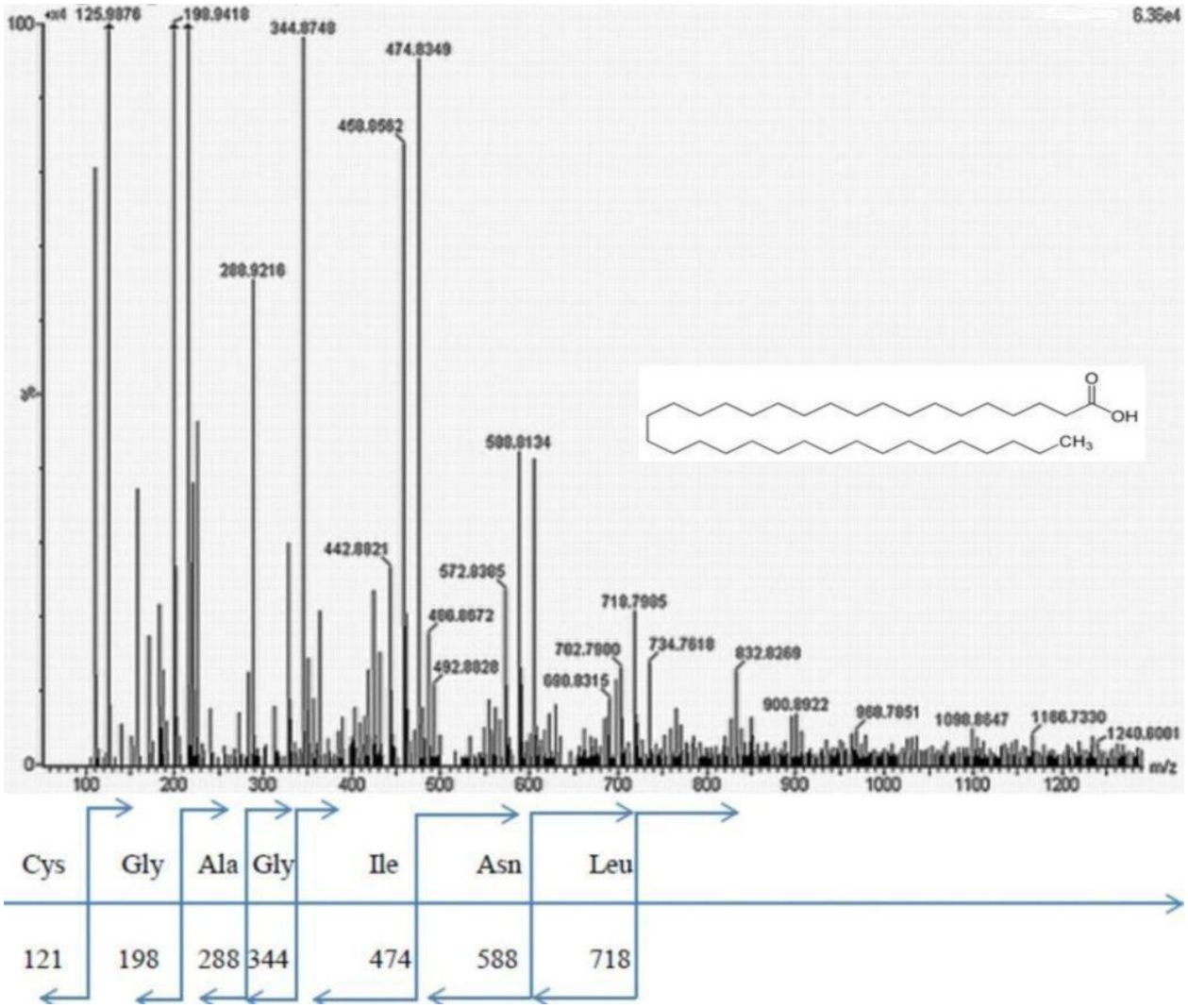

Figure 5.5. LC-MS-MS spectra of Lipopeptide eluted at a retention time of 0.54 min.

5.5 Batch Desorption Study

Figures 5.6 and 5.7 show the desorption rate of the PAHs phenanthrene, and pyrene respectively at an increasing concentration of the cell free supernatant used; 0% (distilled water), 15% , 50%, and 100% cell free broth. Phenanthrene a 3-ring PAH and Pyrene a 4-ring PAH were chosen to see the effect of the biosurfactant on the desorption rate of the PAHs with increasing hydrophobicity. The desorption extent of PAHs was positively correlated with the concentrations of biosurfactant, showing a proportional increase of desorption with the biosurfactant concentration increase. The cell free supernatant increased the desorption rates of the PAHs at a rate proportional to the concentration of the biosurfactant (Fig. 5.6 and Fig.

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5.7). When hydrophobic organic contaminants (HOC) like PAHs remediation of contaminated soil must be done, the first step of the process consists of promoting an increased desorption of PAHs by reducing interfacial tension between the water and the soil and by increasing the apparent solubility of the contaminant in the aqueous phase (Portet-Koltalo *et al.,* 2013). At concentrations above the CMC, hydrophobic pollutants can readily partition into the hydrophobic core at the centre of the micelle, thus increasing HOC aqueous concentration through micellar solubilisation and promoting the desorption of HOCs from soils into aqueous phases (Zhou and Zhu, 2007).

In addition, the desorption extent of PAHs was positively correlated with the concentrations of biosurfactant, showing a proportional increase of desorption with the biosurfactant concentration increase. On day 10 of incubation statistically significant ($p < 0.05$) desorption rates of Pyrene and Phenanthrene were observed in the 50 and 100% cell free broth culture where as the desorption rates were not significant ($p > 0.05$) at 15% cell free broth. On day 10 of incubation 83% of Pyrene was desorbed in the 100% cell free broth in comparison, 67% and 34% desorption rates were observed in the 50% and 15% cell free broth cultures respectively where as 25% desorption was observed in the demineralized water (control) microcosm. Likewise, 97.6%, 94%, 63.5% and 56% of Phenanthrene was desorbed at day 10 of incubation in the 100%, 50%, 15% and control respectively. According to our experimental design, biosurfactant concentrations in aqueous phase were 18CMC, 9CMC and 2.7CMC. Which resulted in increased desorption of PAHs with the increase in the biosurfactant aqueous concentration.

The lower desorption rate of Pyrene (Fig. 5.7), compared to Phenanthrene (Fig. 5.6) can be explained by the more hydrophobic nature of Pyrene, which can be reflected by the higher octanol/water partition coefficient (Log K_{ow}) of Pyrene as affinities for a specific sorbent are greater for more hydrophobic PAH (Cheng *et al.,* 2004). (Singh and Cameotra, 2013) reported that Lipopeptide biosurfactant, consisting of surfactin and fengycin obtained from *Bacillus subtilis* A21 removed a significant amount of petroleum hydrocarbon (64.5%) and metals from soils from industrial dumping site in a soil washing with 50CMC biosurfactant solution. Parameters like surfactant concentration, temperature, agitation condition and pH of the washing solution influenced the pollutant removing ability of the biosurfactant. Similar results of biosurfacant enhanced desorption of hydrocarbons were reported by (Lai *et al.,* 2009; Groboillot *et al.,* 2011; Portet-Koltalo *et al.,* 2013; Singh and Cameotra, 2013). Groboillot *et*

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al. (2011) reported that adding *amphisin* biosurfactant from *Pseudomonas fluorescens DSS73* displayed increased effectiveness in releasing polycyclic aromatic hydrocarbons (PAHs) strongly adsorbed to sediments when compared to a synthetic anionic surfactant.

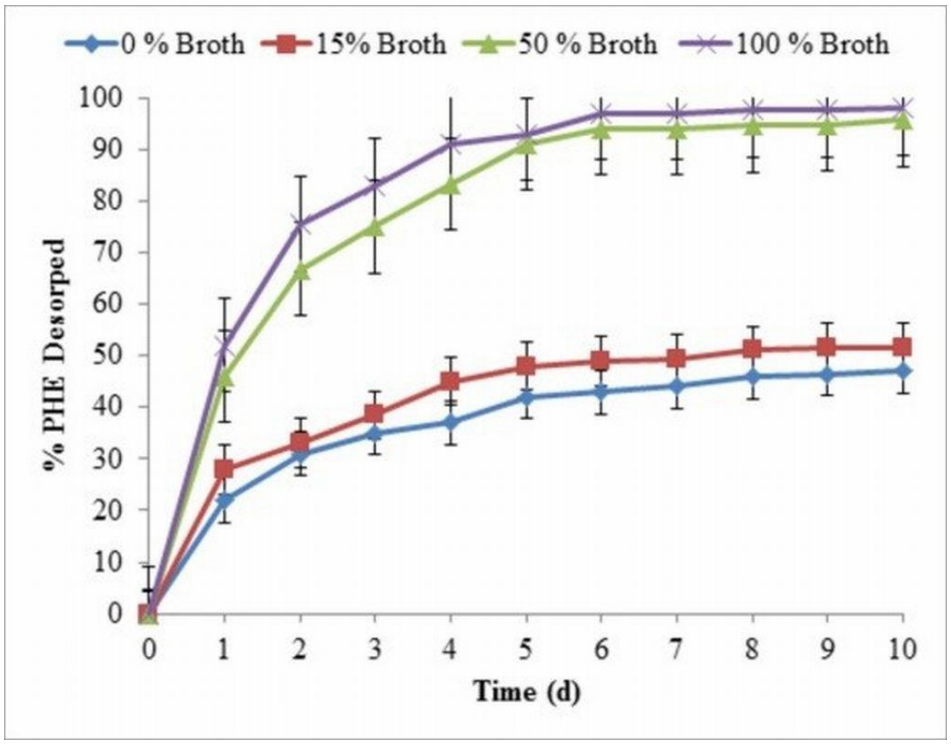

Figure 5.6 Percentage of Phenanthrene (PHE) desorbed at 15%, 50%, and 100% cell free broth and distilled water (control). Error bars represent the standard error of the mean for two duplicate experiments.

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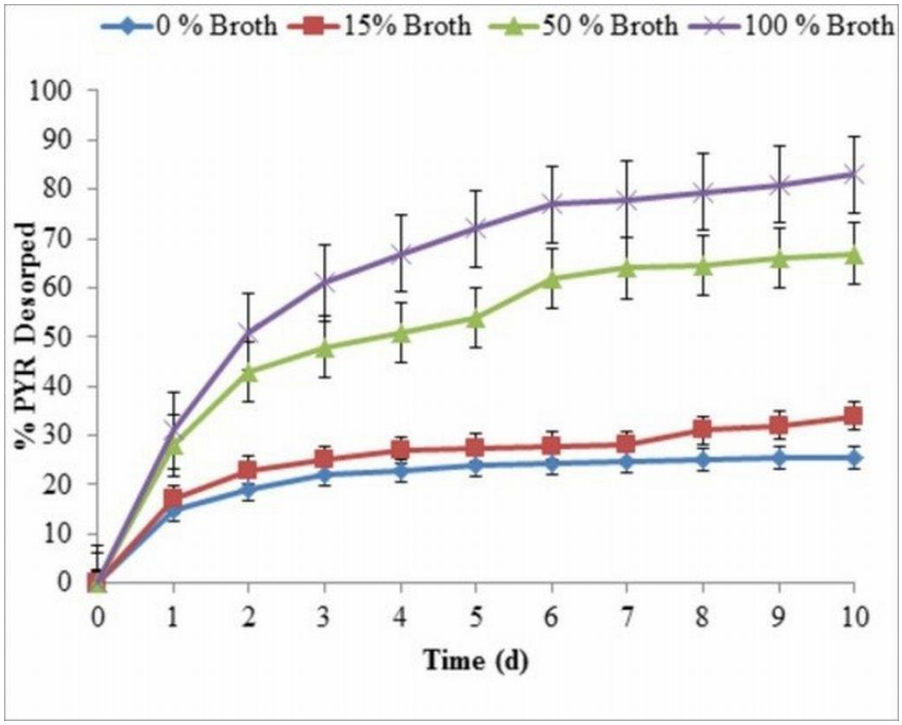

Figure 5.7 Percentage of Pyrene (PYR) desorbed at 15%, 50%, 100% cell free broth and distilled water (control). Error bars represent the standard error of the mean for two duplicate experiments.

5.6 PAH Desorption kinetics

Generally, solubilisation involves a series of processes: the diffusion of PAH molecules and surfactant micelles in the solution, and the desorption of micelles at the PAH/water interface. The micelle adsorption–desorption model proposed by Chan and Cussler (1976) can be used to describe the dynamic process of solubilisation. The desorption model may be represented by a pseudo-first-order kinetic equation (Long *et al.*, 2014).

$$-\frac{dC_t}{dt} = K_1 \cdot (C_e - C_t) \tag{5.1}$$

Where C_e and C_t are the concentrations of the PAH at equilibrium and time t (h), respectively; K_1 is the dissolution rate constant (h^{-1}) of pseudo-first-order kinetics. The linear expression derived from this equation is:

$$-C_t = C_e \cdot \left(1 - e^{-K_1 t}\right) \tag{5.2}$$

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The dissolution rate constant (K_1) and the equilibrium concentration of the PAH at time t (C_e) are determined by minimizing the cumulative squared residuals between experimental and calculated values of (C_t) in Equation 5.2 using the software Microsoft Excel 2010 (SOLVER option). Fitting the data to equation 5.2 gave sums of squared deviations in the range of 0.003-0.008, implying satisfactory fitness (Figures 5.8a, b, c and d). The values obtained for (C_e) and their rate constants (K_1) are presented in (Table 5.1 and Table 5.2) for different Lipopeptide concentrations.

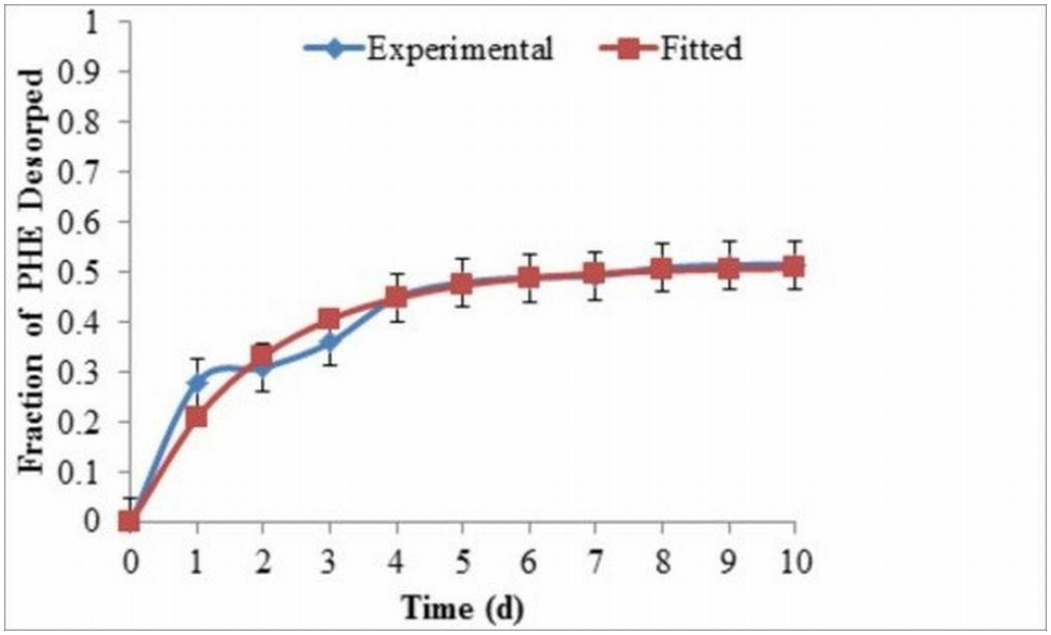

Figure 5.8a. Experimental and fitted results of PHE desorption with Lipopeptide amendment of 15% cell free broth. Error bars represent the standard error of the mean for two duplicate experiments

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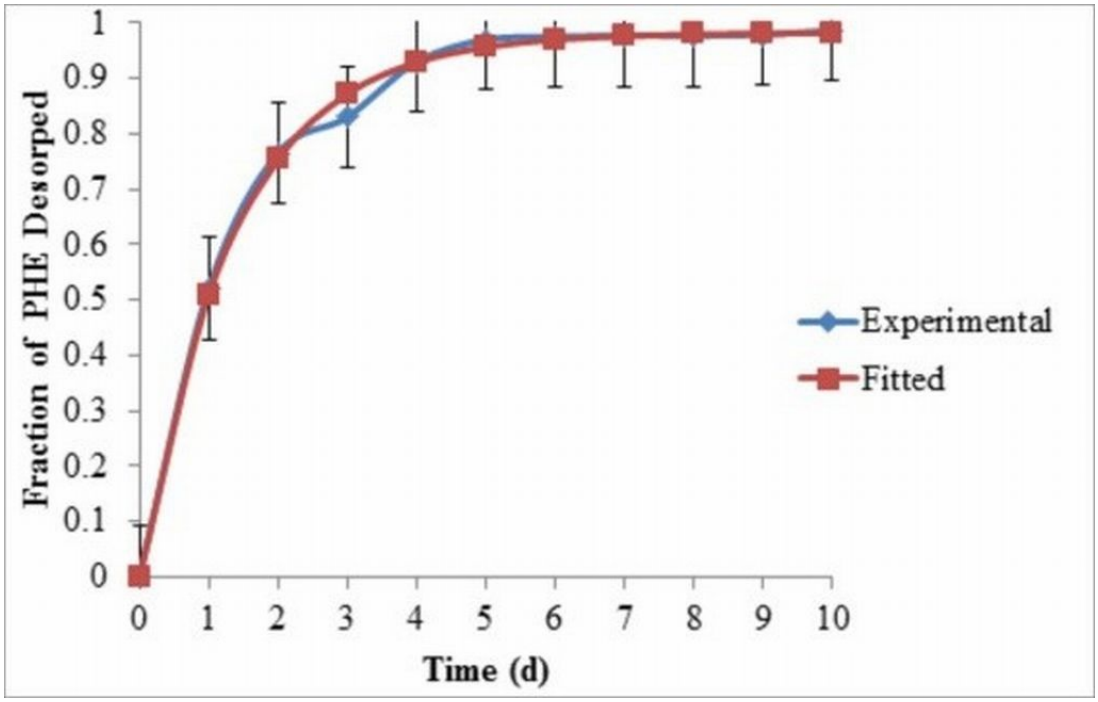

Figure 5.8b Experimental and fitted results of PHE desorption with lipopeptide amendment of 100% cell free broth. Error bars represent the standard error of the mean for two duplicate experiments

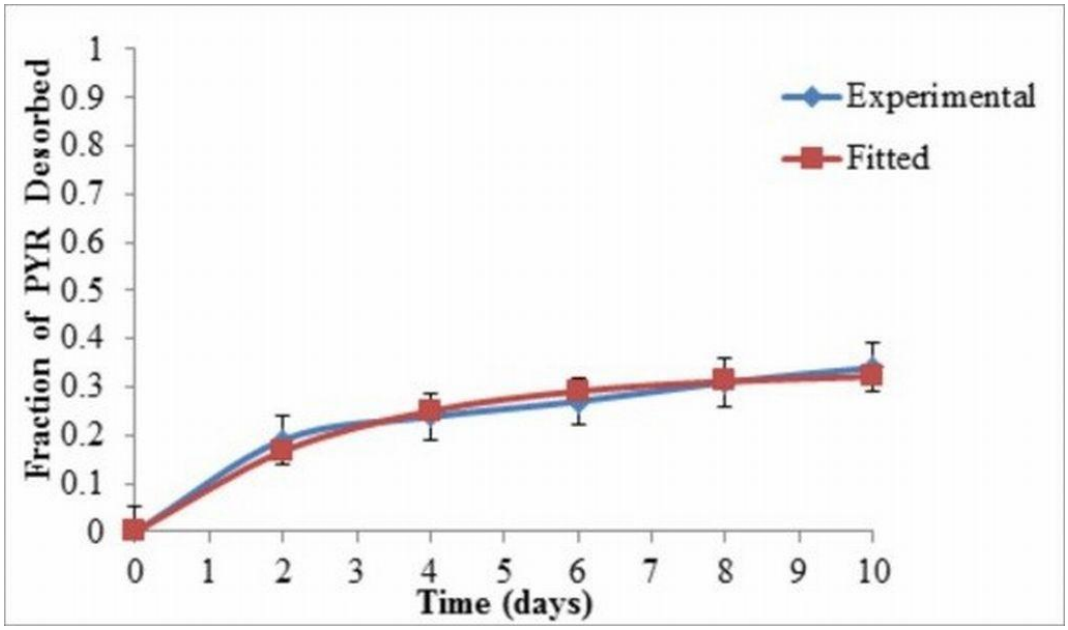

Figure 5.8c Experimental and fitted results of PYR desorption with Lipopeptide amendment of 15% cell free broth. Error bars represent the standard error of the mean for two duplicate experiments

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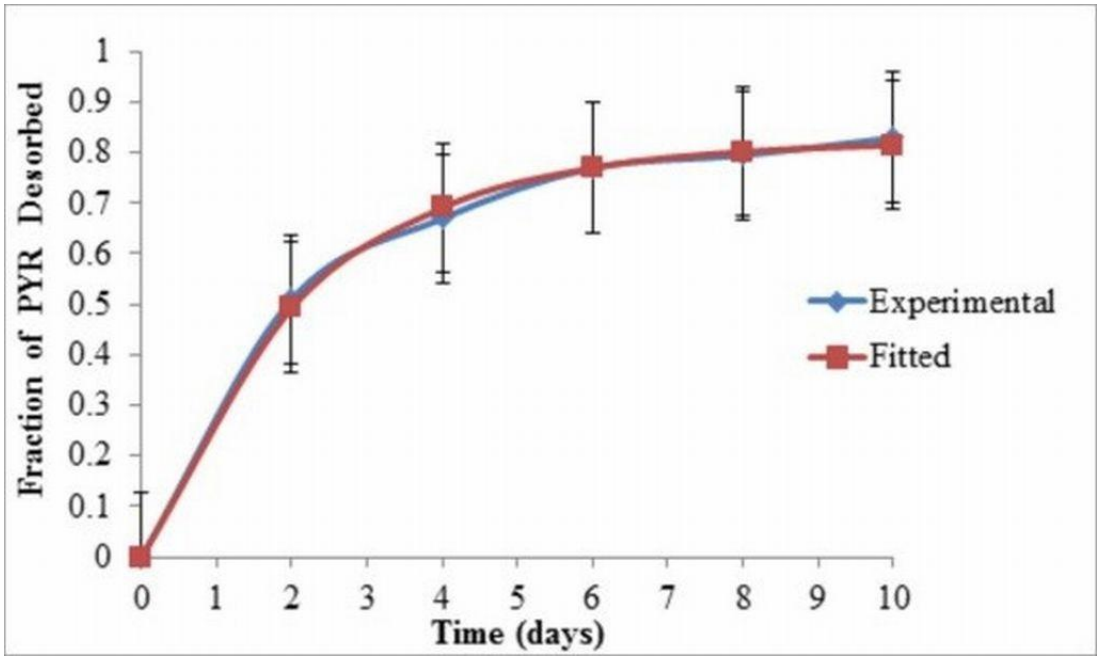

Figure 5.8d Experimental and fitted results of PYR desorption with Lipopeptide amendment of 100% cell free broth. Error bars represent the standard error of the mean for two duplicate experiments

Table 5.1 Best Fit Parameters of Phenanthrene desorption for the different Lipopeptide concentrations, results are mean ± SD of duplicate tests.

Lipopeptide conc. (% Broth)	C_e **(mg/L)**	$K_1(d^{-1})$
100%	0.989 ± 0.04	0.727 ± 0.03
50%	0.958 ± 0.12	0.686 ± 0.12
15%	0.679 ± 0.13	0.527 ± 0.03
0%	0.586 ± 0.18	0.330 ± 0.05

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Table 5.2. Best Fit Parameters of Pyrene desorption for the different Lipopeptide concentrations, results are mean ± SD of duplicate tests.

Lipopeptide conc. (% Broth)	C_e **(mg/L)**	$K_1(d^{-1})$
100%	0.824 ± 0.01	0.457± 0.06
50%	0.668 ± 0.13	0.425 ± 0.07
15%	0.331 ± 0.12	0.350 ± 0.09
0%	0.256 ± 0.09	0.313 ± 0.15

It can be noted that the dissolution rate (K_1) rate and equilibrium concentrations (C_e) of the PAHs increased up to two folds with increasing concentration of lipopeptide compared to the unamended microcosm. The desorption rate observed in the low lipopeptide concentration in the soil−water system wasnot statistically significant ($p > 0.05$) compared to the unamended microcosm. This is due to the high hydrophobicity of the PAHs and the fact that a portion of surfactant monomers in aqueous phase was lost as the result of surfactant sorption onto soil (Urum *et al.*, 2006; Zheng *et al.*, 2012). Various values of K_1 have been reported in litrature Long *et al.* (2014) reported values of K_1 an order of magnitude greater than the current study. The different values of the dissolution rate depend on a number of factors like contact time between PAH and sorbent (aging), soil organic matter and clay contents.

Biosurfactants with small CMC and high degree of sorption to soil can remove hydrocarbon pollutants, only if the washing solution has surfactant concentration much higher than CMC (Urum *et al.*, 2006; Singh and Cameotra, 2014). Te sorption of surfactants to soils represents a major constraint for their application in bioremediation, since it leads to a significantly high surfactant dose and promotes the sorption of PAHs back to the soil because of the modification of the soil surface and the increase in soil organic carbon content caused by the adsorbed surfactants (Congiu and Ortega-Calvo, 2014).

5.7 Application of the biosurfactant in oil removal from sand

The biosurfactant of the bacterial strain recovered 78 – 81% of heavy motor oil from the saturated sand at 30 °C in 24 hours in comparison to the distilled water control which desorbed

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11% of heavy motor oil. This would show the potential application of the biosurfactant for enhanced oil recovery and petroleum sludge washing. Results obtained by Abu-Ruwaida *et al.* (1991) for the cell-free broth containing a biosurfactant produced by *Rhodococcus* cells, showed the recovery of 86% of crude residual oil adsorbed in the sand, while distilled water removed about 65% of the oil. Petroleum hydrocarbon compounds bind to soil components and are difficult to remove due to their hydrophobicity.

Current oil recovery technologies depend on natural reservoir pressure and/or injection of gas or water into the reservoir to drive the oil to producing wells. However, these technologies mobilize only about 30–50% of the oil contained in the reservoir (Lundquist *et al.*, 2001; Fernandes *et al*., 2016). The low overall oil recovery is attributed to many factors, but the most important factors are the high viscosity of oil and the high interfacial tension between oil and injected water (Fernandes *et al*., 2016).

In recent years, a new set of oil recovery methods called enhanced oil recovery (EOR) techniques has been introduced. Enhanced Oil Recovery (EOR) refers to processes in which chemicals such as surfactants, emulsifiers, polymers, acids, alkali, dispersants, and solvents, are being used to lower interfacial tension, change the wettability, and control mobility in order to increase the amount of oil recovered from a previously water-flooded reservoir (ShamsiJazeyi *et al.*, 2014; Patel *et al*., 2015) . However, these methods carry with them their own inherent risks; in addition to the economic costs, the chemical pathways through which these products are generated often use toxic chemicals, such as ethylene oxide in the production of nonionic surfactants (Patel *et al*., 2015).

Considerable interest has been directed towards environmentally friendly and economically feasible microbiological techniques for enhancing oil recovery, known as microbial enhanced oil recovery (MEOR) (Patel *et al*., 2015). One of the main MEOR processes includes the use of microbial products, such as biosurfactants, to lower interfacial tension between brine and petroleum, and hence reduce the capillary forces that entrap oil in rock pores (Fernandes *et al*., 2016) . The use of biosurfactants, especially lipopeptides produced by *Bacillus species*, reduces the interfacial tension between oil and the aqueous phases required to mobilize residual hydrocarbons (McInerney *et al.*, 2005; Fernandes *et al*., 2016).

Our study showed high thermal, pH and saline stability of the biosurfactant over a wide range of temperature, pH and salinity. This demonstrates the potential application of the biosurfactant under harsh environmental conditions. Oil reservoir is one of the harshest environments, where

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temperatures can range from 20 to 90 °C, normal salinity to hypersalinity and pH over a wide range (Al-Bahry *et al.*, 2013). The biosurfactant produced by *Paenibacillus dendritiformis* CN5 will have an enormous potential under such harsh environmental conditions as it maintains its surface activity under a wide range of environmental factors. Many studies have similarly reported stability of biosurfactants over a wide range of temperature, pH and salinity (Gudina *et al.*, 2010; Al-Bahry *et al.*, 2013).

MEOR has several advantages compared to traditional EOR processes in that it does not consume large amounts of energy, as do thermal processes, nor does it depend on the price of crude oil, as do many chemical processes. The substances injected have low toxicity, high biodegradability and are effective for a wide range of pH and temperatures (Fernandes *et al*., 2016). MEOR can also be cost-effective, since these bioproducts can be generated from cheaper substrates and renewabale resources (Patel *et al*., 2015). Generally the versatility and efficiency of the lipopeptide biosurfactant demonstrated that this compound has a promising potential for microbially enhanced oil recovery and other industrial applications.

5.8 SUMMARY

The lipopeptide biosurfactant produced by *Paenibacillus dendritiformis* CN5 strain mobilized substantial amounts of residual oil from contaminated sand- and efficiently desorbed the hydrophobic PAHs. It was observed that increasing the concentration of biosurfactants appeared to enhance PAH removal from the contaminated soil. Generally, the lipopeptidal biosurfactant produced by *Paenibacillus dendritiformis* strain showed potential application in PAH contaminated soil washing, oil recovery and other environmental applications under harsh environmental conditions of extreme pH salinity and temperature.

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CHAPTER SIX

PYRENE BIODEGRADATION ENHANCEMENT POTENTIAL OF LIPOPEPTIDE BIOSURFACTANT PRODUCED BY *PAENIBACILLUS DENDRITIFORMIS* CN5 STRAIN

6.1 Introduction

Polcyclic aromatic hydrocarbons (PAHs) are a diverse class of organic compounds consisting of two or more fused aromatic rings. PAHs are ubiquitous in various environmental niches and have been of great concern due to their genotoxic, mutagenic and carcinogenic nature (Moscoso *et al.*, 2012; Ghosh *et al.*, 2014). PAHs are primarily formed during incomplete combustion of organic materials and are emitted into the environment mainly by anthropogenic activities such as coal combustion and refining processes as well as contamination associated with the transport and use of fossil fuels and derivatives (Ma *et al.*, 2013). While low-molecular-weight (LMW) PAHs (composed of two or three fused benzene rings) are readily degraded by bacteria, high-molecular-weight (HMW) PAHs consisting of four rings or more are recalcitrant to biodegradation and persist in the environment (John *et al.*, 2012). In general, an increase in the size of the molecule or the total number of aromatic rings and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability (Kanaly and Harayama, 2000).

Pyrene is a high molecular weight four-ring polycyclic aromatic hydrocarbon (PAH) that has low biodegradability and high persistence in the environment and has been listed as a priority pollutant by the United States Environmental Protection Agency (Teh and Hadibarata, 2014). We chose PYR as model HMW PAH as Pyrene has often been used as a model compound for high-MW PAH biodegradation because it is structurally similar to several carcinogenic PAHs (Peng *et al.*, 2008). Its quinone-based metabolites are mutagenic and more toxic than the parent compound and for these reasons, pyrene is a priority pollutant and considered as an indicator for monitoring PAH contaminated wastes (Saraswathy and Hallberg, 2005; Singh, 2006; Anastasi *et al.*, 2009).

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Bioremediation is an environmentally feasible and cost-effective approach for the cleanup of sites contaminated with polycyclic aromatic hydrocarbons (PAHs) but its effectiveness is limited by the low bioavailability of PAHs (Silva *et al.*, 2009; Zhao *et al.*, 2011).

It is generally believed that the use of surface-active compounds or surfactants may be a means in overcoming the low bioavailability problem (Zhang and Zhu, 2012). Surfactant-enhanced bioremediation has been suggested as a promising technology for the remediation of hydrophobic organic compounds contaminated soil and groundwater (Makkar and Rockne, 2003; Zhang and Zhu, 2012). Surfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds (Singh *et al.*, 2007). At low concentrations, surfactants are soluble in water, and with increasing concentrations, they form micelle in solution. The concentration at which micelle begins to form is called the critical micelle concentration (CMC); after micelles form, solid PAHs may partition into the interior of the micelle, which increases the hydrocarbon's apparent solubility but some surfactants may increase the water solubility of hydrocarbon molecules below the CMC (Chaprão *et al.*, 2015). Apart from the solubilization of hydrocarbons, surfactants can also change the surface properties of microbial cells, which lead to the attachment of hydrocarbon to bacteria and a consequent increase in hydrocarbon utilization (Sajna *et al.*, 2015).

In recent years, much attention has been directed towards biosurfactants owing to their greater advantages compared to chemical surfactants such as lower toxicity, higher biodegradability, better environmental capability, higher foaming, high selectivity, specific activity at extreme temperatures, pH and salinity, and the ability to be synthesized from renewable feed stocks (Chaprão *et al.*, 2015). Biosurfactants are structurally diverse and can have various chemical compositions mainly consisting of fatty acids, glycolipids, lipopeptides, lipopolysaccharides and lipoproteins depending on the producing microorganism, raw matter and process conditions (Makkar *et al.*, 2011)

Several studies have been conducted with addition of biosurfactants (Whang *et al.*, 2008; Sponza and Gok, 2010; Bak *et al.*, 2015; Sajna *et al.*, 2015) in bioremediation processes of hydrocarbon pollutions. While biodegradation is often stimulated by biosurfactants (Kang *et al.*, 2010; Bak *et al.*, 2015; Sajna *et al.*, 2015 Bezza and Chirwa, 2015a), no effect or even inhibitory effects have also been observed and may be caused by various reasons (Avramova *et al.*, 2008; Ławniczak *et al.*, 2013).

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The aim of the present study is to investigate the effect of the lipopeptide biosurfactant produced by gram positive *Paenibacillus dendritiformis* CN5 strain, previously isolated from wood treatment plant soil (Bezza and Chirwa, 2015c), on pyrene degradation by a microbial consortium predominantly composed of *Pseudomonas viridiflava* (49%) and *Pseudomonas nitroreducens* (32%) species. The biosurfactant produced by CN5 strain was purified; its surface activity was evaluated and the potential to aid the biodegradation of pyrene was studied. The optimum concentration that supported the maximum hydrocarbon degradation was evaluated.

6.2 Physicochemical Characterization of the Biosurfactant

6.2.1 Yield and surface activity of the crude biosurfactant

The biosurfactant showed potent surface active property, which reduced the surface tension of Phosphate buffer solution from 72.0 to 30 mN m^{-1}. The crude biosurfactant had a yield of 7.5 g L^{-1} and a Critical Micelle Concentration (CMC) value of 185 mg L^{-1} at the lowest surface tension displayed. We have previously reported biosurfacatant production by the strain using glycerol, hydrophilic substrate that reduced surface tension to 34 mN m^{-1}. Biosurfactants are produced by a variety of microbes, secreted either extracellularly or attached to parts of cells, predominantly during growth on water-immiscible (hydrophobic) substrates. However some microbial surfactants can be produced on water soluble substrates (Ciapina *et al.*, 2006). In the current study the strain produced more efficient biosurfactant when growing on hydrophobic substrates which reduced the surface tension of distilled water to 30 mN m^{-1} compared to the surface activity of the biosurfactant produced when growing on hydrophilic substrate. Several studies have reported production of biosurfactants by different strains when growing on both hydrophobic and hydrophilic substrates (Holden *et al.*, 2002). An efficient biosurfactant can reduce the surface tension of water and air from 72 to less than 40 mN m^{-1} (Ismail *et al.*, 2013). Accordingly, *Paenibacillus dendritiformis* CN5 is a promising biosurfactant producer with a potential for use in environmental remediation and oil recovery strategies. Sriram *et al*. (2011) reported that the CMC of the biosurfactant produced by *B. cereus* NK1 was 45 mg L^{-1} and occurred at the surface tension value of 36 mN m^{-1}. Extensive range of CMC values has been reported for various biosurfactants ranging from 9 mg L^{-1} to 140 mg L^{-1} (Sriram *et al.*, 2011).

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6.2.2 Chemical Characterization

6.2.2.1 Thin Layer Chromatography (TLC) analysis

The results of the TLC analysis showed pink spot after spraying with ninhydrin at R*f* value around 0.85 (Fig. 6.1), indicating the presence of aminoacids. This result demonstrates that the biosurfactant is lipopeptide with peptide hydrophilic moieties. Comparable results were reported by Sriram *et al*. (2011).

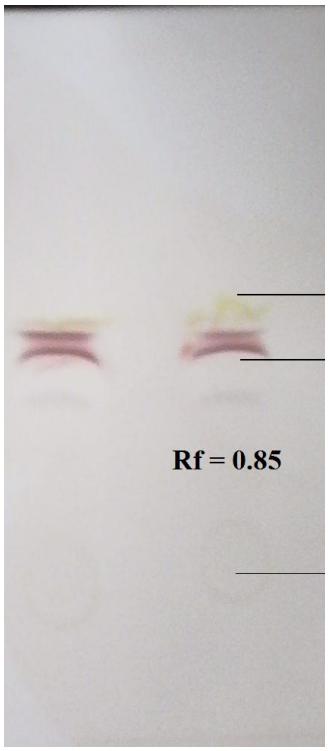

Figure 6.1 Thin layer chromatography analysis of lipopeptide produced by CN5 while growing on oil and anthracene. Developed with chloroform-methanol-water (65: 25: 4, v / v / v) and sprayed with ninhydrin (0.35%, v/v) showed a pink spot with R*f* (retention factor) value of 0.85.

6.2.2.2 Mass Spectrometry (LC MS/MS)

After column chromatography and partial hydrolysis, different lipopeptide classes were detected by LC MS/MS mass spectrometry (Fig. 6.2 A, B and Fig. 6.3). LC MS/MS mass spectral analysis revealed a cluster containing several molecules observed at m/z 1035.6,

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1057.6, (Fig. 6.2a), eluting at retention time of 7.27 min, m/z 1043.7, 1057.7, (Fig. 6.2b), at retention time of 15.957 min (proposed iturin) and m/z 1031, (Fig. 6.3), at retention time of 10.995 min (proposed surfactin) classes (Kim *et al.*, 2010; Pathak and Keharia, 2014). Ions at m/z 1043.7 and 1057.7 (Fig. 6.2b), are two homologues with a difference of 14 Da (-CH_2-), which have similar m/z values to the lipopeptide, iturin (Chen *et al.*, 2008).

The precursor ions at m/z 1035.6, 1057.6 with mass difference of 22 Da are assigned as sodium adducts of iturin homologue (Fig. 6.2A). Apart from the lipopeptide groups identified above, a number of ion peaks were detected by LC MS/MS. Ion clusters around m/z 1157–1323 eluting at retention time (10.51), 1083–1379 at retention time (11.5), (Fig. 6.4 A, B); 1299–1973 at retention time (10.42) and 1299–1973 at retention time (10.409), (Fig. 6.5A, B) indicate several higher mass lipopeptides present.

We have previously reported production of 7 amino acid lipopeptidal biosurfactant by the strain when growing on hydrophilic substrate (Bezza and Chirwa, 2015c). In this study apart from the lipopeptide groups identified above (Fig. 6.2A, B and Fig. 6.3), the detection of various fractions of lipopeptide, including higher mass molecular ions shown in (Fig. 6.4 A, B); 1299–1973 eluting at a retention time 10.42 min and; 1299–1973 at retention time 10.409 min (Fig. 6.5 A, B) indicate the presence of several higher mass lipopeptides.

The results demonstrate the diverse nature of lipopeptide biosurfactants produced by the strain. Among the different current classes of biosurfactants, lipopeptides, due to their structural novelty and versatility, represent one of the most important ones widely produced among *Bacillus* species (Fracchia *et al.*, 2012; Branquinho, 2014). Chemically, these compounds are constituted of 7– 10 amino acids, which are cyclized by a lactone ring to a β-hydroxy fatty acid with distinct chain lengths. Moreover, they are classified into different families, encompassing surfactins, iturins, fengycins, bacillomycins, mycosubtilin and kurstakins (Branquinho, 2014). The differences in the sequence of amino acids and carbon atoms in the fatty acids provide lipopeptides with diverse chemical structures and physiochemical properties (Liu *et al.*, 2015). Different bacterial strains may produce the same lipopeptide compound, and the same bacterial strains are found to produce lipopeptides belonging to different families as is the case of B. subtilis which can produce surfactin, iturin and fengycins simultaneously (Liu *et al.*, 2015).

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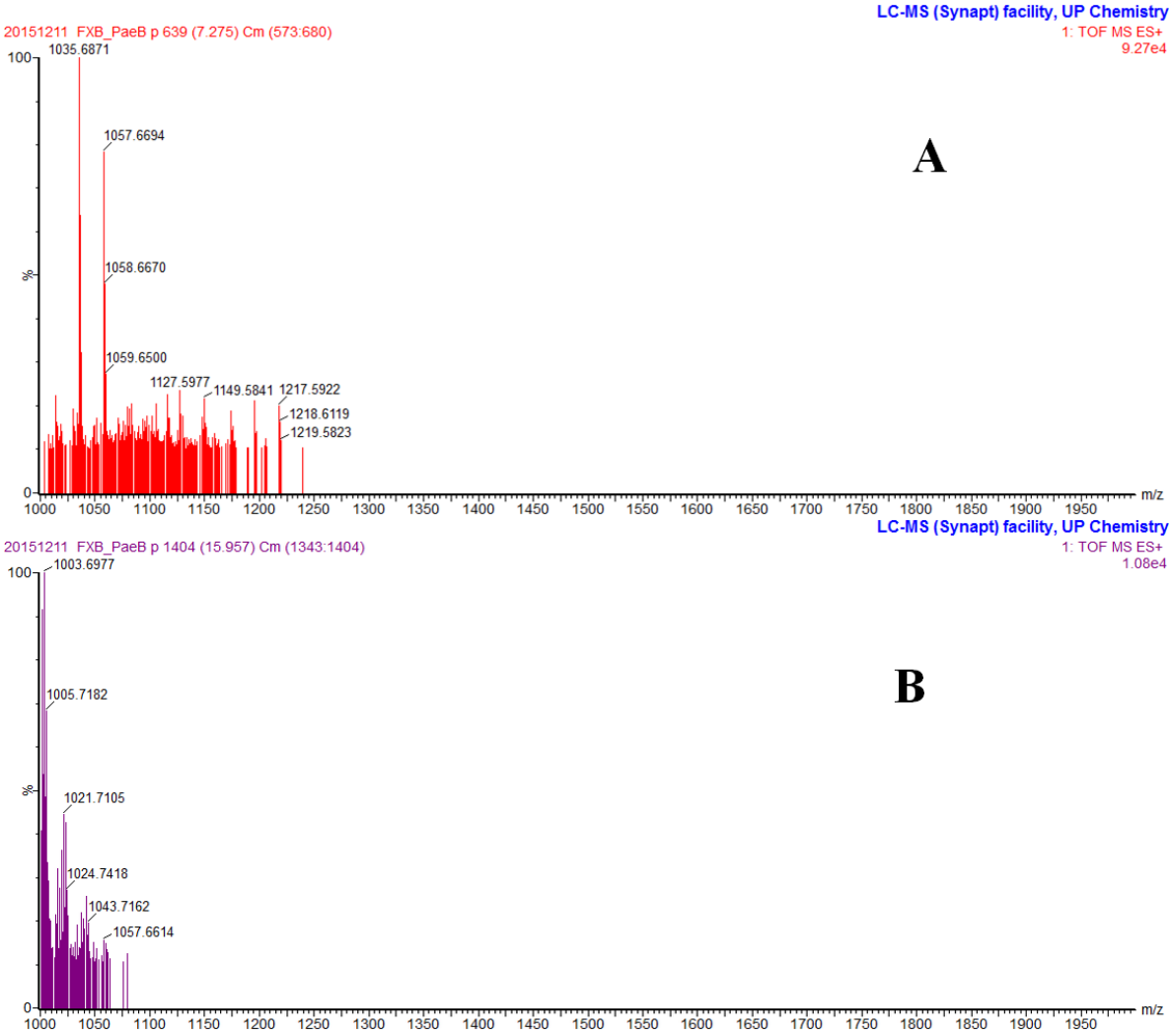

Figure 6.2 (A and B) Tandem mass spectrometry (LC–MS/MS) mass spectra of the fractions of the biosurfactant from *P. dendritiformis* eluting at retention time of 7.275 (A) and 15.957(B) respectively

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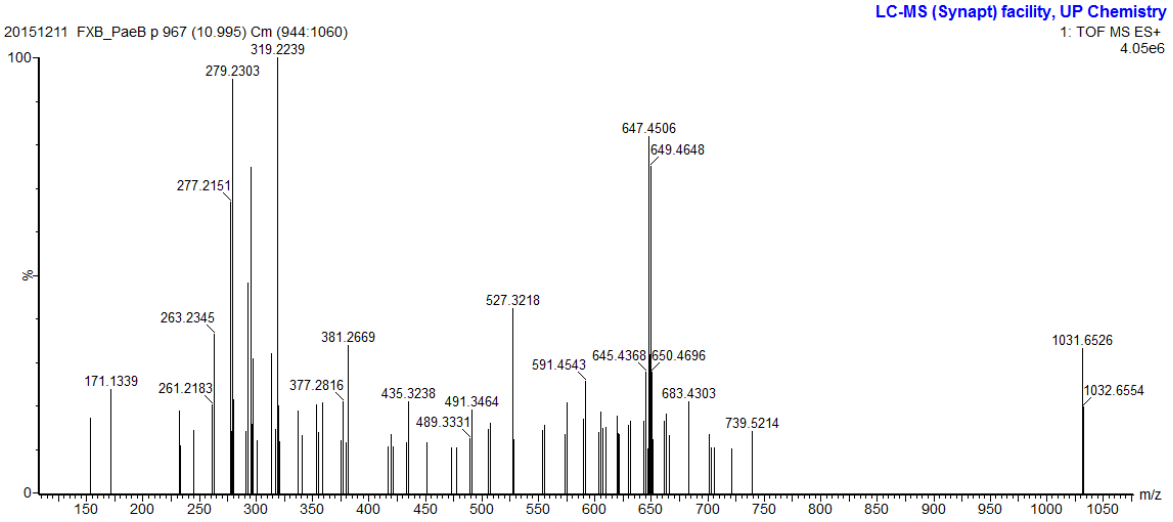

Figure 6.3 Liquid chromatography tandem mass spectrometry (LC–MS/MS) spectra of the fraction of the biosurfactant from *P. dendritiformis* eluting at retention time of 10.995 min

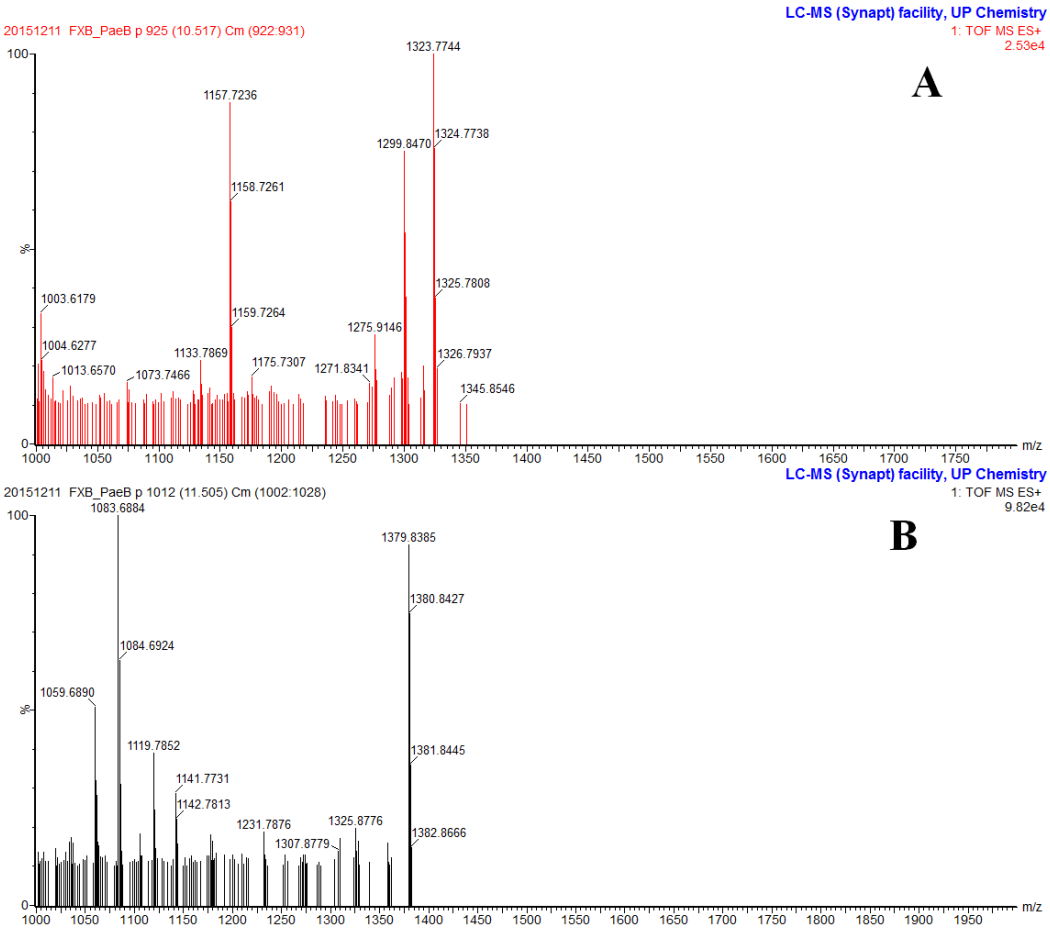

Figure 6.4 (A and B) Liquid chromatography tandem mass spectrometry (LC–MS/MS) spectraof the purified biosurfactant from P. dendritiformis eluting at retention time of 10.517 (a) and 11.505(b) respectively.

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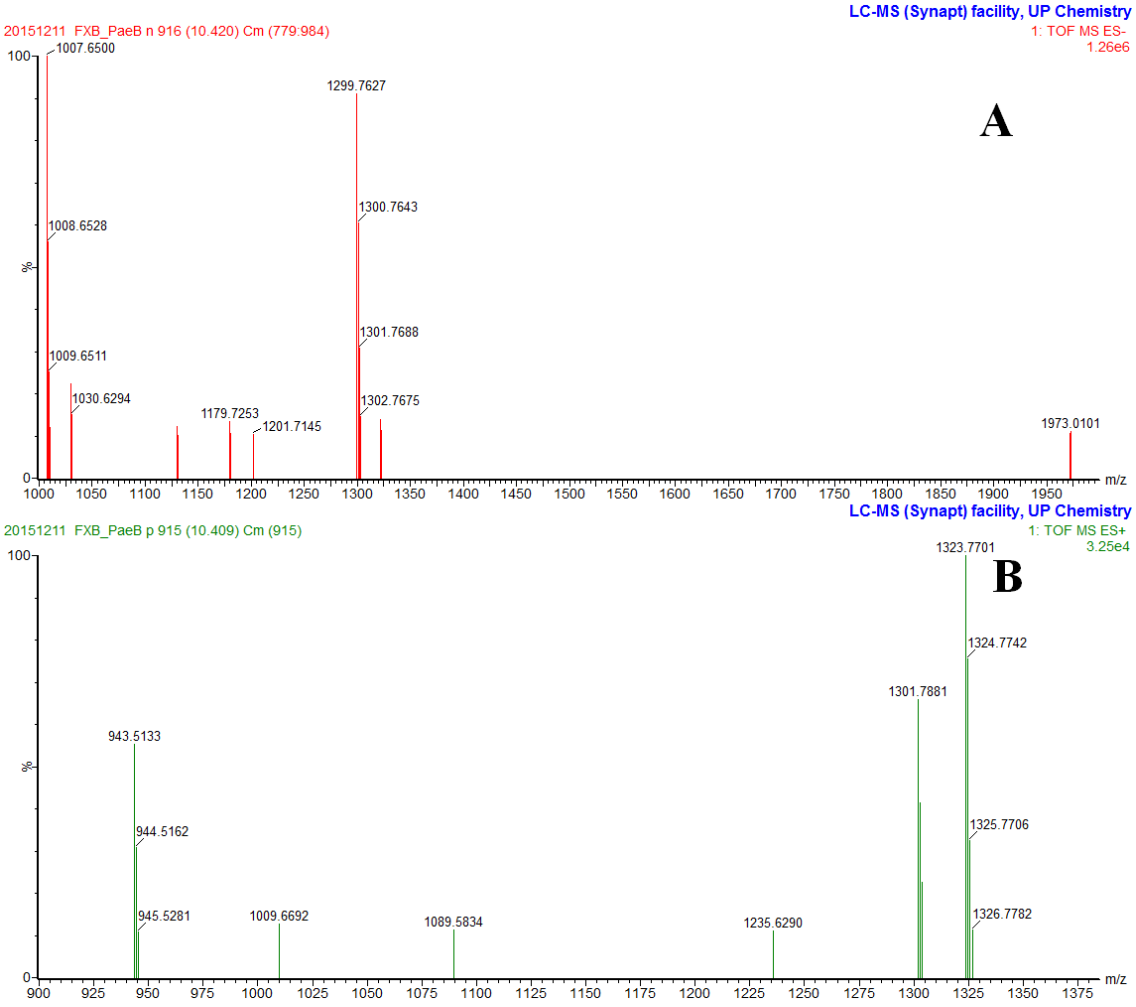

Figure 6.5 (A and B) Liquid chromatography tandem mass spectrometry (LC–MS/MS) spectra of the purified biosurfactant from P. dendritiformis eluting at retention time of 10.420 (a) and 10.409(b) respectively

6.3 Microbial Community Composition of the Inoculant

Using a similarity threshold of 97% to cluster sequences within the same operational taxonomic units (OTUs), a total of 320 OUTs were found from the creosote contaminated soil inoculum. *Proteobacteria* was the dominant bacterial phylum, representing 99% of the 16S rDNA reads from the 320 most abundant bacterial OTUs in the inoculum sample at 1% cut off. Most of the OTU in the sample (> 99 %) were affiliated to the *Gamma* division of the *Proteobacteria* (Fig. 6.6). The predominant OTUs, *Pseudomonas viridiflava* (49%) and *Pseudomonas nitroreducens* (32%) were associated with the genus *Pseudomona* (6.6). Strains affiliated with

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the *gammaproteobacteria* group are associated with members of the genera like *Pseudomonas, Stenotrophomonas*, which have been reported as being efficient hydrocarbon degraders (Mahjoubi et al., 2013). This microbial composition is in accordance with most studies showing the importance of the *Proteobacteria,* especially the *Gamma* division, in hydrocarbon-polluted soil or natural asphalts (Militon *et al.*, 2010)

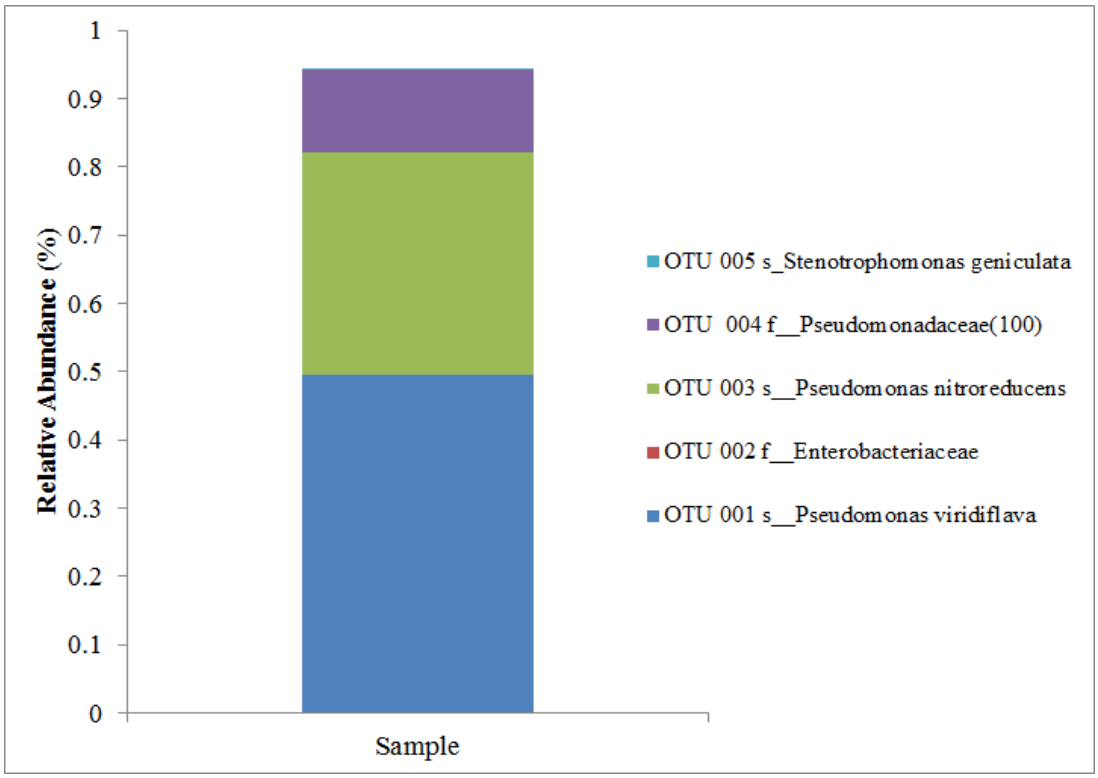

Figure 6.6 Relative abundances of different classes in the dominant *Proteobacteria* Phylum. The relative abundance is presented in terms of percentage in total effective bacterial sequences in the sample. OTU s and OTU f represent Operational Taxonomic Units at species and family levels.

6.4 Solubilization Assay of Pyrene

The effect of the biosurfactant on the solubility of PAH (pyrene) was determined in the presence of lipopeptide at concentrations of 300, 600 and 900 mg L^{-1}. Data obtained are presented in Fig. 6.7. Above the CMC, the biosurfactant was effective in enhancing pyrene solubility and a relationship between solubilized pyrene and biosurfactant concentration up to about (900 mg L^{-1}) can be observed. The solubility of PAHs in the buffer medium increased linearly up to 21.3 mg L^{-1} at 900 mg L^{-1} addition of lipopeptide isolated from CN5 strain. The

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solubilization of Pyrene was increased up to 157.8 times its solubility in water (0.135 mg L^{-1}, 1 atm, 25 °C) at 900 mg L^{-1} lipopeptide amendment. A linear relationship between hydrophobic compound solubility and surfactant concentration beyond CMC has been previously demonstrated for commercial surfactants and biosurfactants (Abouseoud *et al.*, 2010).

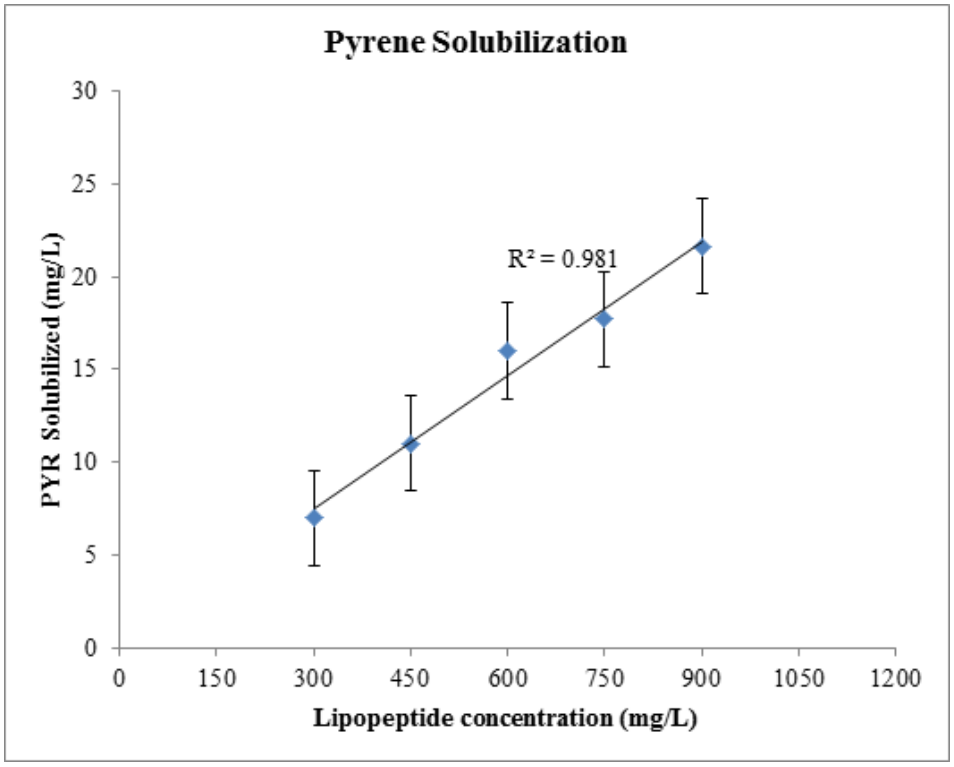

Figure 6.7 Variation of pyrene solubilization with increasing concentration of lipopeptide after 48 h of shaking at 120 rpm and 32 °C in darkness.

6.5 Microbial Growth and Pyrene Biodegradation Kinetics

65.1 Growth Kinetics

Efficiency in the degradation of pyrene was determined by measuring the cell growth and hydrocarbon utilization. Cell growth is indicative of hydrocarbon degradation as complete mineralization results in the formation of biomass, CO_2 and H_2O. The growth of microbial consortium on pyrene at different concentration of lipopeptide ranging from 300 to 900 mg L^{-1} and surfactant free control is illustrated in Fig. 6.8. The microbial consortium showed substantially increased biomass growth in the lipopeptide supplemented treatments compared to the surfactant free control ($p < 0.05$), but there was no significant change in growth rates between amended treatments ($p > 0.05$). The results suggest that the lipopeptide stimulated bacterial growth up to the level of 600 mg L^{-1}, while at 900 mg L^{-1} the lipopeptide showed

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comparative reduction in the growth of the microbial consortium indicating the toxicity of biosurfactant at higher concentrations. Since microbial biomass is sometimes used as a valuable tool for studying dynamics of pollutant biodegradation, modelling the experimental growth and degradation parameters can be relevant to achieve a better knowledge on the bioremediation process. The logistic model was used to fit the experimental biomass and biodegradation data, which was previously described for other biodegradation processes (Moscoso *et al.*, 2012; Shao *et al.*, 2015). The model described the experimental data well with R^2 values varying between 0.97 and 0.99 (Fig. 6.8).

$$X = \frac{X_{\max}}{1 + e^{[\ln(\frac{X_{\max}}{X_0} - 1) - \mu_{\max} t]}} \tag{6.1}$$

$$D = \frac{D_{\max}}{1 + e^{[\ln(\frac{D_{\max}}{D_0} - 1) - \mu_D t]}} \tag{6.2}$$

where X and D are the biomass ($g\ L^{-1}$) and the PAHs removal (%) at a specific moment of the culture time $t\ (d)$, X_O and D_O, are the initial biomass (g L^{-1}) and PAHs removal (%), $X_{\max}$ and $D_{\max}$ are the maximum biomass ($g\ L^{-1}$) and PAHs removal (%), $\mu_{\max}$ and $\mu_{D\max}$ represent the specific growth and specific degradation rates (d^{-1}) respectively.

The values of $\mu_{\max}$, $\mu_{D\max}$, X_O, $X_{\max}$, D_O, $D_{\max}$,were determined by minimizing the cumulative squared residuals between experimental and calculated values of the independent variables in equations in Equations (6.1 and 6.2) using Sigma Plot Software .

The best-fit model parameters (Equations (6.1) and (6.2)) are presented in Table 6.1, and the resulting isotherm is plotted as the solid line in Fig. 6.8 and 6.9. The biomass parameters, displayed quite higher levels of the maximum specific growth rate ($\mu_{\max}$) and carrying capacity ($X_{\max}$) values in the lipopeptide amended treatments compared to surfactant free control.

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The μ_{max} values were substantially higher in the 600 mg L^{-1} amended treatment (0.27 d^{-1}) than the surfactant free control (0.18 d^{-1}) and other dosages. The maximum specific growth rate level values are comparable to those reported by Obayori *et al.* (2008) when studied pyrene biodegradation by a Pseudomonas species isolated from polluted tropical soils (0.024 h^{-1}). The specific growth rate on pyrene for some other reported pyrene degraders are 0.014 and 0.013 h^{-1} for two *Pseudomonas sp.* K-12 and B-24 respectively (Thibault *et al.*, 1996) and 0.03–0.085 h^{-1} for *P. aeruginosa* RS1 over the concentration range 25–500 mg L^{-1} (Ghosh *et al.*, 2014).

Likewise, X_{max} values displayed highest microbial growth at 600 mg L^{-1} lipopeptide amendment (4.90 g L^{-1}) and, lowest at surfactant free control (1.12 g L^{-1}) in the 24 day incubation period, which indicated significantly enhanced ($p < 0.05$) microbial growth at the optimal amount of lipopeptide compared to surfactant free control.

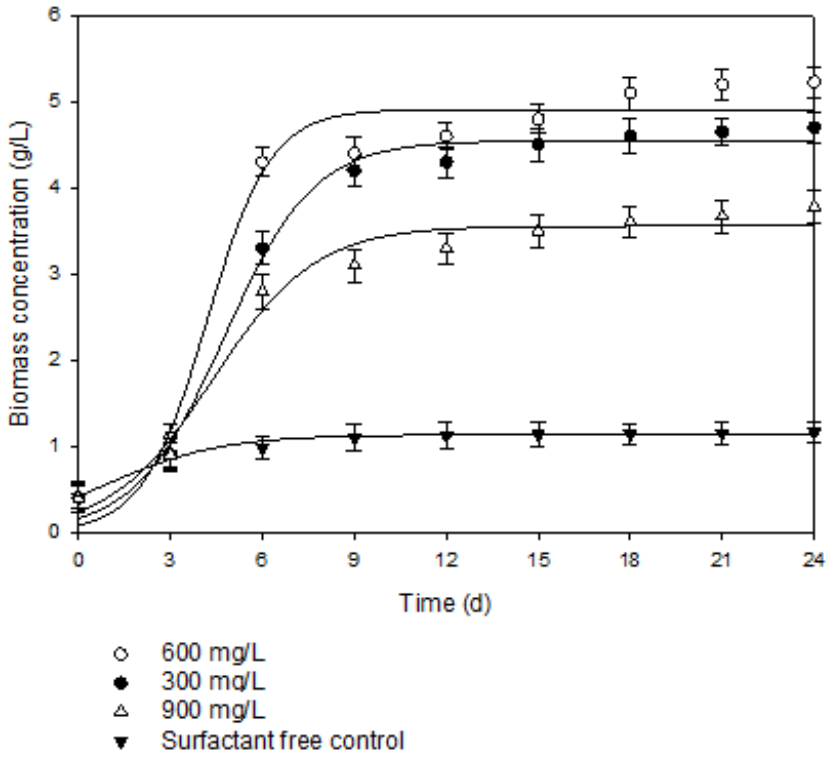

Figure 6.8 Growth profiles of the microbial consortium on pyrene over a period of 24 days with and without lipopeptide supplementation at different concentrations in liquid culture**.** The

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curves are the best fit to a logistic model (Eq. (6.1)) using the parameters listed in Table 6.1. Data represent the mean ± standard deviation of triplicate samples.

6.5.2 Biodegradation Kinetics

The pyrene depletion profile at the end of the 24-day incubation period for each treatment schedule using the microbial consortium is shown in Figure 6.9. Pyrene degradation percentages under the various treatment conditions ranged between 83 and 17% for biosurfactant supplemented and non-supplemented cultures, respectively. For all control assays, no abiotic losses were detected during incubations, thus removal of pyrene can be attributed only to microbial metabolism. For the three sets of lipopeptide concentrations evaluated, there was significant ($p < 0.05$) pyrene degradation in the treatments supplemented with 300 and 600 mg L^{-1} lipopeptide amendments, but the degradation at 900 mg L^{-1} was not statistically significant compared to the non-amended treatment. For lipopeptide concentrations tested, pyrene removal was highest at 600 mg L^{-1} lipopeptide concentration (83%) followed by 300 mg L^{-1} (68%). In comparison, when lipopeptide concentration was increased to 900 mg L^{-1}, the extent of pyrene degradation was reduced (56%). These results indicate that at higher concentration of biosurfactant the microbial consortium could have stressed from increased concentration of pseudosolubilized pyrene or the biosurfactant itself which may interfere with the cellular membrane of the bacteria above the threshold level. The apparent solubility (pseudosolubility) of otherwise slightly soluble hydrophobic organic compounds like pyrene can be dramatically enhanced in solutions of surfactants at concentrations greater than the CMC. The hydrophobic core of each micelle can accommodate a certain amount of hydrophobic organic compound (pyrene) as a solubilizate. Solubilization of hydrophobic substances commences at the CMC and in general is a linear function of surfactant concentration over a wide range of surfactant concentrations greater than the CMC (Edwards, *et al.*, 1991).

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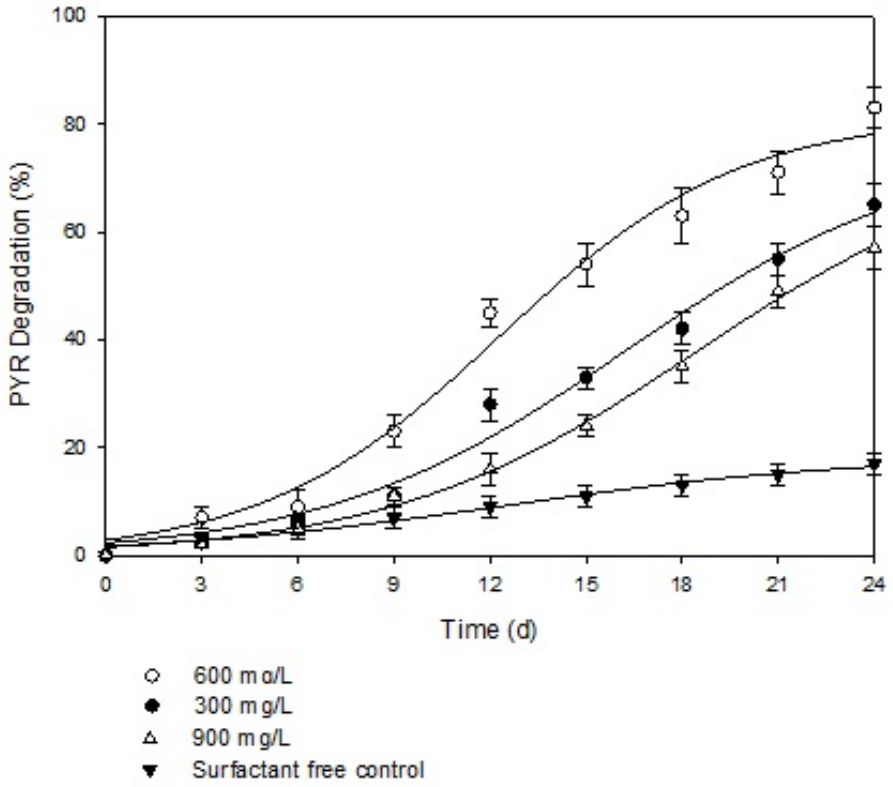

Figure 6.9 Percentage degradation of pyrene in liquid culture over a period of 24 days with and without lipopeptide supplementations. The curves are the best fit to a logistic model (Eq. (6.2)) using the parameters listed in Table 6.1. Data represent the mean ± standard deviation of triplicate samples.

The maximum specific removal rate ($\mu_{D\max}$) of PYR and the coefficients of determination that were obtained by fitting to the kinetic model are presented in Table 6.1. The $\mu_{D\max}$ values were considerably higher in the 600 mg L^{-1} amended treatment (0.97 d^{-1}) than the surfactant free control (0.54 d^{-1}) and other dosages. These values are comparable to the values reported by Shao *et al.* (2015), 0.97 vs. 0.95 d^{-1} and much higher than those reported for bacterial culture of *B. pumilus, Dyella ginsengisol* and *Mycobacterium barasi* (Chang *et al.*, 2008) for PYR, 0.97 vs. 0.36 d^{-1}.

Moreover, $D_{\max}$ values displayed highest degradation of PYR at 600 mg L^{-1} lipopeptide amendment (81.5%) and, lowest at surfactant free control (18.5%) in the 24-day incubation period, which indicated significantly enhanced degradation of PYR at the optimal amount of lipopeptide.

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Table 6.1 Best-fit parameters of logistic model for biomass growth and pyrene degradation with lipopeptide supplementation at different concentrations.

Lipopeptide concentration	***Biomass Parameters***				***PYR biodegradation Parameters***			
	$\mu_{max}(d^{-1})$	$X_{max}(g/L)$	$X_0(g/L)$	R^2	$\mu_{D\,max}(d^{-1})$	$D_0(\%)$	$D_{max}(\%)$	R^2
0	0.18	1.12	0.42	0.97	0.54	1.75	18.5	0.98
300 mg/L	0.21	4.54	1.65	0.98	0.69	2.32	75.6	0.97
600 mg/L	0.27	4.90	0.68	0.99	0.97	2.89	81.52	0.99
900 mg/L	0.2	3.56	0.24	0.98	0.60	1.5	75.3	0.97

Biosurfactants can enhance hydrocarbon bioremediation by two mechanisms (Pacwa-Plociniczak *et al.*, 2011). The first includes the increase of substrate bioavailability for microorganisms (emulsified or solubilized hydrocarbons) while the other involves interaction with the cell surface which increases the hydrophobicity of the surface allowing hydrophobic substrates to associate more easily with bacterial cells (direct contact of cells with hydrocarbon droplets). Usually both mechanisms take place but the dominance of one or the other depends on the strain (Cameotra and Singh, 2009). Enhanced solubilisation of PAHs is a key factor for the application of biosurfactant enhanced bioremediation technology and is always used as an index in evaluating the capabilities of surfactants in removing PAHs from contaminated soils. Thus increased solubility by pseudo-solubilization of PAHs in biosurfactant micelles increases PAHs bioavailability and their subsequent degradation.

Generally, the study showed that the Lipopeptide increased solubility (Fig. 6.7) of the PAHs and enhanced their degradation at optimum amounts of supplementation, but failed to enhance the degradation process at higher concentrations of Lipopeptide supplementation (900 mg L^{-1}). Biosurfactants may exhibit inhibitory effects and be detrimental to degrading microbial organisms at higher concentrations as they are antimicrobial products of microbial origin (Sachdev and Cameotra, 2013). Microbial surfactants like iturin, fengycin and lichenysin are recognized by their pore forming abilities and membrane permeabilizing properties (Inès and Dhouha, 2015). Surfactant molecules at a high concentration may form mixed micelles with membrane lipids, which may solubilize cell membranes and lead to necrosis and lysis of cells (Li and Chen, 2009). Another likely cause of a reduced rate of bioremediation in the presence of higher concentration of biosurfactant is due to increased toxicity of the hydrophobic contaminant due to its increased solubility (Obayori *et al.*, 2008; Silva *et al.*, 2014). Similar

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observations were reported by Sajna *et al.* (2015) whereby maximum degradation of hydrocarbons by *P. putida* was observed at 2.5 mg L^{-1} supplementation of biosurfactant and the authors suggested that the concentration of biosurfactants to be added should be less than 11.07 mg L^{-1}, as higher concentrations would negatively affect the growth of the organism and its hydrocarbon biodegradation efficiency.

Several studies have been conducted with addition of biosurfactants (Whang *et al.*, 2008; Sponza and Gok, 2010; Bak *et al.*, 2015; Sajna *et al.*, 2015) in bioremediation processes of hydrocarbon pollutions. While biodegradation is often stimulated by biosurfactants (Kang *et al.*, 2010; Bak *et al.*, 2015; Sajna *et al.*, 2015 Bezza and Chirwa, 2015a), no effect or even inhibitory effects have also been observed and may be caused by various reasons (Avramova *et al.*, 2008; Ławniczak *et al.*, 2013).

Some of the reasons have been attributed to surfactants' use as preferential growth substrates by degrading microorganisms (Kim and Weber, 2003) or to their toxicity (Whang *et al.*, 2008; Avramova *et al.*, 2008; Li and Chen, 2009; Chakraborty *et al.*, 2010). Substrate toxicity resulting from an increase in bioavailability brought by surfactant solubilization (Bramwell and Laha, 2000) or the reduction of contaminant bioavailability due to its uptake into the surfactant micelle contributes also for the non-efficiency of the surfactants used (Doong and Lei, 2003; Zeng *et al.*, 2011).

Contrasting effects of the same surfactant on the biodegradation of PAHs by different bacteria were reported (Wong *et al.*, 2004; Zhao *et al.*, 2011). This indicates that the effects of surfactants on the biodegradation are dependent on the interactions not only between surfactants and PAHs, but also between surfactants and PAH degrading microorganisms, e.g., the utilization of surfactants as growth substrates in preference to PAH compounds or the toxicity of surfactants to microorganisms (Wong *et al.*, 2004; Haritash and Kaushik, 2009; Zhao *et al.*, 2011). The degree to which inhibitory and stimulatory effects of surfactants on biodegradation of hydrocarbons depends on the characteristics and dosages of surfactants (Zhao *et al.*, 2011; Zhang *et al.*, 2013; Liu *et al.*, 2016). Thus it is important to have a better understanding of the interactions between either surfactants and PAHs or surfactants and bacterial cells (Zhang *et al.*, 2013; Liu *et al.*, 2016).

Moldes *et al.* (2011) carried out studies focused on assessing the influence of biosurfactants from *Lactobacillus pentosus* on the biodegradation efficiency of octane in soil by

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autochthonous microflora. After 15 days, the biodegradation efficiency reached 59 % and 63 % for soil contaminated with 700 and 70,000 mg/kg of octane in the presence of biosurfactants, while in their absence the removal rate was at 1 % and 24 %, accordingly. The authors suggested that mobilization of octane molecules and subsequent increase in their bioavailability was the main cause of the observed differences. Supplementation of rhamnolipids at 15 mg L^{-1} concentration improved the efficiency of polyaromatic hydrocarbons (PAH) removal and soluble COD reduction to 90% and 99%, respectively indicating its potential to treat the waste water abundant in polyaromatic hydrocarbons (Sponza and Gok, 2010).

Zeng *et al.* (2011) studied the effect of monorhamnolipids on the degradation of hexadecane by Candida tropicalis, where the CMC of rhamnolipids was determined to be 38.0 mg L^{-1} and enhanced biodegradation was observed with the supplementation of 19.0 mg L^{-1} of rhamnolipids. Solubility of petroleum hydrocarbons was improved remarkably by the addition of biosurfactants such as rhamnolipids and surfactin above their CMC. However, at higher concentration, biosurfactants may have an inhibitory effect on the bioremediation as they exhibit antimicrobial activity (Whang *et al.*, 2008).

While the microbial toxicity of surfactants is a possible cause of bioremediation inhibition, many surfactants are not toxic to microorganisms at concentrations near their CMC values (Silva *et al*., 2014). Most of the biosurfactants exhibit toxicity at higher concentration, which affects the cell surface properties or metabolic pathways of microbes, leading to the decline in biomass required for degrading the hydrocarbon, and thus hampers the bioremediation (Sajna *et al.*, 2015). The surfactant molecules at a high concentration above CMC may form mixed micelles with membrane lipids, which may solubilize cell membranes. This will lead to the necrosis and lysis of cells (Li and Chen, 2009). Another possible cause of a reduced rate of bioremediation in the presence of (bio) surfactant is due to increased toxicity of the hydrophobic contaminant due to its increased (pseudo)solubility. Biosurfactants increase the apparent aqueous solubility of hydrophobic substrates (Silva *et al.*, 2014). On the other hand, some biosurfactants or pseudosolubilized contaminants may exhibit selective toxicity toward specific pure cultures but may have a limited inhibitory impact in a remediation system involving a diverse indigenous microbial population (Singh *et al.*, 2007).

Other possible explanations, for inhibited degradation at higher surfactant concentrations, is that increasing the surfactant concentration formed surfactant hemi-micelles on the bacterial cell surface and that subsequent hydrophilic moiety of surfactants extended to the aqueous

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phase result in relatively lower cell surface hydrophobicity of bacteria (Li *et al.*, 2015). As mentioned previously, the results could be due to the reduced bioavailability of pyrene in the micellar phase at a higher surfactant concentration (Li and Chen, 2009).

Surfactants' detrimental effect on bioremediation due to their inherent toxicity to microorganisms and due to enhancement in bioavailability of toxic components was observed to depend on the specific bacteria involved (Allen *et al.*, 1999), which means that the specific interactions between bacteria and surfactant also play an important role (Li and Chen, 2009). Thus, choice of appropriate surfactant type and dose based on the target pollutants and the microorganisms involved is essential. Surfactant-aided biodegradation may also be influenced by the inherent ability of the hydrocarbon-degrading microorganisms to utilize a specific hydrocarbon group type (aliphatic or aromatic) and the inherent mode of uptake of hydrocarbons by the microorganisms (Mohanty and Mukherji, 2012).

6.6 SUMMARY

Generally, our study demonstrated that the lipopeptide increased solubility of the PAH and enhanced its subsequent degradation in aqueous system at optimal amount of supplementation (600 mg L^{-1}), but was less effective to enhance the degradation process at higher concentration of lipopeptide supplemented (900 mg L^{-1}). The lipopeptide stimulated bacterial growth up to the level of 600 mg L^{-1}, while at 900 mg L^{-1} it showed comparative reduction in the growth of the microbial consortium. The positive effect of the lipopeptide biosurfactant on the hydrocarbon biodegradation and microbial growth have been invariably attributed to the increased solubility and dissolution of hydrocarbons or enhanced mass transport in the presence of the surfactant.

The results suggested that at high concentration of biosurfactant the strain could have stressed from increased concentration of pseudosolubilized pyrene or the biosurfactant itself which may interfere with the cellular membrane of the bacteria above the threshold level. Among several factors the degree to which inhibitory and stimulatory effects of surfactants on biodegradation of hydrocarbons depends on the characteristics and dosages of surfactants. Accordingly, to exploit successful bioremediation potential of biosurfactants optimal threshold level of the biosurfactant need to be determined experimentally prior to wide scale application. The results of this study could be of particular importance for bioremediation of hydrocarbon contaminated

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environments, mainly contaminated with HMW PAHs from industrial and petroleum processing areas that need immediate restoration for public use.

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CHAPTER SEVEN

THE ROLE OF LIPOPEPTIDE BIOSURFACTANT ON MICROBIAL REMEDIATION OF AGED POLYCYCLIC AROMATIC HYDROCARBON (PAHS)-CONTAMINATED SOIL

7.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of organic compounds that consist of two or more fused benzene rings and/or pentacyclic molecules that are arranged in various structural configurations (Bamforth and Singleton, 2005). PAHs are apriority group of environmental contaminants that pose a threat to human health and ecological security owing to their toxic, carcinogenic, and mutagenic properties (Haritash and Kaushik, 2009). They are highly hydrophobic and have a strong sorption tendency with soil and solid sediments present in water bodies. In particular, natural organic matter and other geochemical components, such as black carbon and clay minerals, are responsible for this strong sorption, which results in recalcitrance, especially in the case of aged contaminants (Congiu and Ortega-Calvo, 2014). PAHs are ubiquitous pollutants and are generated mainly from anthropogenic activities such as the burning of fossil fuels, the use of wood preservatives such as creosote and the generation of wastes from coal gasification plants and other industrial activities (Bezza and Chirwa, 2015a).

A typical source of PAH contamination in soil is coal-tar creosote, which was commonly used to preserve and waterproof crossties and power line poles. Coal tar creosote is a dark, oily liquid formed by fractional distillation of crude coal tars in 200 – 400 °C. It consists of a complex mixture of several hundred chemicals, of which only 20% are present in amounts greater than 1%. The composition of creosote varies depending on the origin of the coal and the distillation process, but six major compound classes can be sorted out: i.e. aromatic hydrocarbons, tar acids/phenolics, nitrogen-, sulphur- or oxygen containing heterocycles and aromatic amines (Simpanen *et al.*, 2016). The main compound class of creosote is the PAHs, which can make up approximately 85% of creosote composition (Mueller *et al.*, 1989).

Conventional remediation technologies including land removal and incineration or land-filling are expensive, and in many cases transfer the pollutant from one phase to another. On the other

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hand, bioremediation is gaining wider approval as a feasible treatment technology to transform the compounds to less hazardous/nonhazardous forms with less input of chemicals, energy, and time (Kulik *et al.*, 2006; Haritash and Kaushik., 2009). However, the rate and extent of degradation of PAHs are often restricted due to their poor aqueous solubility, high hydrophobicity and strong sorption to soil or sediment matrix (Seo *et al.*, 2009; Ghosh and Mukherji, 2016). One of the effective ways to increase the bioavailability of hydrophobic pollutants in soil is using surfactants to enhance the desorption and solubilization of the hydrocarbons, thereby facilitating their assimilation by microorganisms (Silva and Sarubbo, 2015; Congiu *et al.*, 2015).

Chemical and biosurfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility and mobility of hydrophobic or insoluble organic compounds (Singh *et al.*, 2007). Surfactants can increase the rate of PAH desorption from soil through two mechanisms: micellar solubilization and direct modification of the contaminant matrix. Micellar solubilization involves partitioning of PAHs into surfactant micelles at aqueous-phase surfactant concentrations above the critical micelle concentration (CMC), increasing the rate of desorption by maximizing the concentration gradient between the sorbent and aqueous phase (Adrion *et al.*, 2016).

The extensive sorption of surfactants to soil, however, leads to a substantially higher surfactant dose to reach the CMC in a soil/water system than in the absence of soil (Zhu and Aitken, 2010). On the other hand, surfactants have been shown to increase desorption of PAHs from contaminated field soil at doses corresponding to aqueous-phase surfactant concentrations less than the CMC in the soil/water system through modification of contaminant matrix (Frutos *et al.*, 2011; Adrion *et al.*, 2016). In addition, at sub CMC levels, surfactant monomers accumulate at the soil-contaminant and soil-water interfaces and increase the contact angle between the soil and the contaminant (i.e. change the wettability of the system), thereby promoting the separation of the contaminant from the soil particles (Deshpande *et al.*,1999). Apart from interactions with the pollutants and contaminant matrix biosurfactants may induce changes in the properties of cellular membranes, resulting in increased microbial adherence, which is considered to be relevant in terms of biodegradation efficiency (Ławniczak *et al.*, 2013).

Several researchers have reported enhancement in biodegradation of PAHs in presence of biosurfactants (Whang *et al.*, 2009; Sponza and Gok, 2011; Lin *et al.*, 2015; Bezza and Chirwa,

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2016). However, sometimes no beneficial effect and detrimental effect have been observed (Shin *et al.*, 2005; Whang *et al.*, 2008; Adrion *et al.*, 2016). Possible reasons for inhibition in biosurfactant-mediated degradation include surfactant toxicity to microorganisms due to permeabilization of cell membrane (Avramova *et al.*, 2008), accumulation of inhibitory products of incomplete metabolism due to the substantial increase of substrate availability by solubilization (Zhu and Aitken, 2010) and decreased bioavailability of micelle solubilized PAHs or preferential utilization of the surfactant over the target PAHs (Kim and Weber, 2003; Ghosh and Mukherji, 2016). In recent years, the interest in biosurfactants has been remarkably increasing due to many advantages compared with chemical surfactants, including lower toxicity, superior biodegradability and environmental-friendliness, vast structural diversity and specific activity at extreme conditions (temperature, pH, salinity) (Cao *et al.*, 2009; Xia *et al.*, 2014).

Biosurfactants are structurally diverse groups produced by several types of microorganisms such as bacteria, yeasts and fungi as membrane components or secondary metabolites (Cao et al., 2009). Among the many classes of biosurfactants, lipopeptides represent a class of microbial surfactant with remarkable surface properties and biological activities, such as surplus crude oil recovery, such as surplus crude oil recovery, efficient removal of petroleum hydrocarbons and heavy metals from contaminated soils and so on (Cao *et al.*, 2009; Xia *et al.*, 2014).

Previous studies have reported application of lipopetidal and rhamnolipid biosurfactants for bioremediation of polycyclic aromatic hydrocarbons in liquid culture and soil slurries (Sponza and Gok, 2011; Lin *et al.*, 2015; Bezza and Chirwa, 2015b). Nevertheless applications of lipopetidal biosurfactants for remediation of PAH contaminated soil in solid phase has rarely been reported.

The objective of this study was to investigate the impact of lipopeptidal biosurfactant, produced by *Bacillus cereus* SPL-4 on biodegradation of low and high molecular weight PAHs by microbial consortium from previously bioremediated soil.

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7.2 Biosurfactant Producing Strain Screening and Identification.

The strain SPL-4, which produces an effective biosurfactant, was isolated from Creosote contaminated wood treatment plant soil and screened for biosurfactant production capability using the Drop Collapse and Oil Spreading test methods as described in (subsection **3.3.2)**. The drop collapse and oil spreading tests showed positive result and the bacterial isolate SPL-4 screened was found to possess considerable biosurfactant production ability. Based on the 16S rDNA gene sequences and using the Gen Bank BLAST tool, isolate SPL-4 was found to be closely related to *Bacillus cereus*, with a percentage of similarity of 100%. The 16S rRNA gene sequence of the SPL-4 strain was aligned automatically to reference sequences of the genus *Bacillus* obtained from the Gen Bank (http://www.ncbi.nlm.nih.gov/BLAST/), and a phylogenetic tree was constructed (Fig. 7.1) Based on the neighbor-joining method using the software MEGA version 6.0 (Tamura *et al.*, 2013). The complete 16S rDNA sequence of the strain SPL-4 was deposited in the Gen Bank database under the accession number KU900609. Biosurfactant Production by the strain, recovery and purification were conducted as described in subsection 3.4.2.

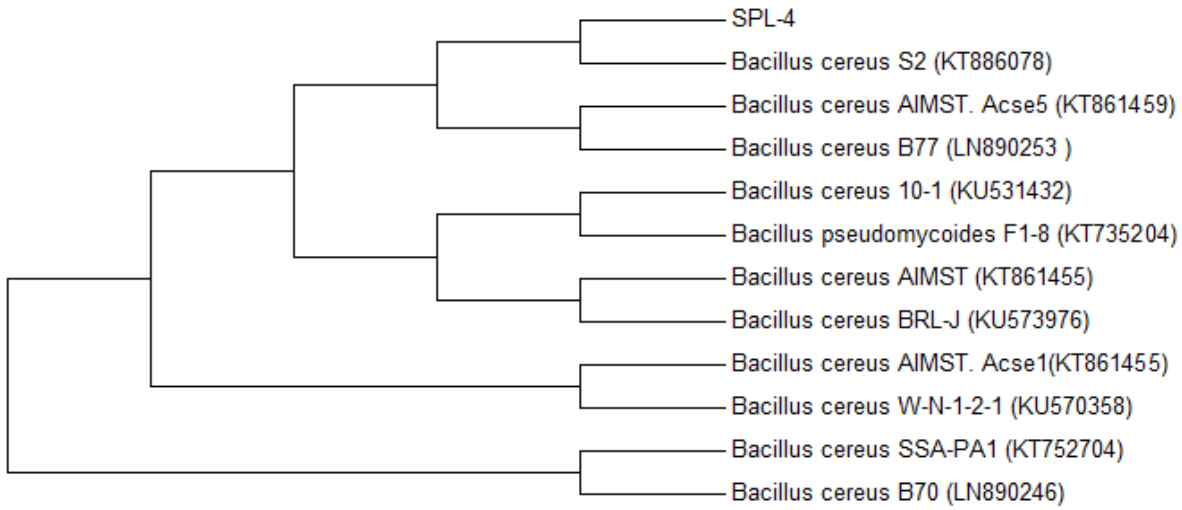

Figure 7.1 Phylogenetic tree based on 16S rRNA sequence, constructed by the neighbor-joining method, showing the position of strain SPL-4 among related members of the genus *Bacillus*. Reference strain organisms are included and sequence accession numbers are given in parentheses. Bootstrap values from 1000 replicates.

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7.3 Yield and Physicochemical Characterization of the Biosurfactant

7.3.1 Yield and surface activity of the crude biosurfactant

The biosurfactant showed potent surface active property, which reduced the surface tension of Phosphate buffer solution from 72.0 to 29.5 mNm^{-1} . An efficient biosurfactant can reduce the surface tension of water and air from 72 mNm^{-1} to less than 40 mNm^{-1} (Ismail *et al.*, 2013). Accordingly, *B. cereus* SPL-4 is a promising biosurfactant producer with a potential for use in environmental remediation and oil recovery strategies. The crude biosurfactant had a yield of 8.2 g L^{-1} and a Critical Micelle Concentration (CMC) value of 90.5 mg L^{-1} at the lowest surface tension displayed. The CMC obtained in this study is comparable with the CMC value of 200 mg L^{-1} of crude biosurfactant reported by Ismail et al. (2013) that reduced the surface tension of water from 68 to 42 mNm^{-1}. Sriram *et al.* (2011) reported that the CMC of the biosurfactant produced by *B. cereus* NK1 was 45 mg L^{-1} and occurred at the surface tension value of 36 mNm^{-1}. Extensive range of CMC values has been reported for various biosurfactants ranging from 9 mg L^{-1} to 140 mg L^{-1} (Sriram *et al.*, 2011).

7.3.2 Chemical charcaterization of the biosurfactant

7.3.2.1 Thin layer chromatography (TLC) analysis of purified biosurfactant

Thin-layer chromatography characterization of the column purified biosurfactant revealed two pink spots with R*f* values of 0.42 and 0.94 (Fig. 7.2), when sprayed with ninhydrin reagent, indicating the presence of amino acids. The above result confirmed the lipopeptidal nature of the biosurfactant. Similar results were reported by Sriram *et al. (*2011).

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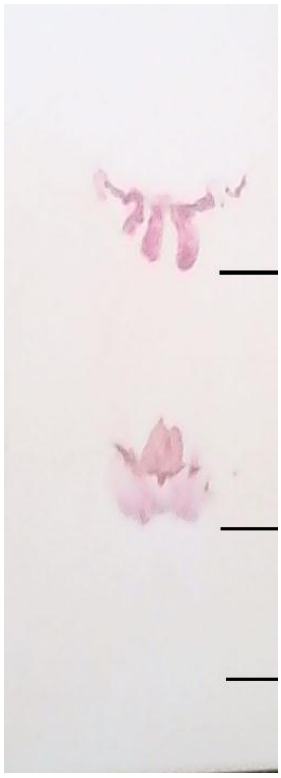

Figure 7.2 Thin-layer chromatography (TLC) of the biosurfactant obtained from Bacillus cereus SPL-4 after treatment with ninhydrin 0.35% (w/v, in acetone) revealed pink spots with R*f* values of 0.42 and 0.94 indicating the presence of amino acids. Chromatograms were developed with chloroform: methanol: water (65:15:4 v/v).

7.3.2.2 Mass spectrometry

LC–MS/MS spectral analysis of the purified sample (Fig. 7.3, A and B**)** revealed two groups of molecular ions in the m/z range 1000–1200 and 1400–1869 eluting at retention times of 8.87 and 12.7 mins. The groups of peaks could be attributed to the isoform ensembles of surfactins,iturins, and fengycins, which represent the well-known biosurfactant families by *B. subtilis* strains. The former represents a mixture of surfactins and iturins, whereas the latter may be assigned as fengycins. The fengycin family could be readily distinguished by its distinctly higher masses in the range of m/z 1421–1566 (Pathak *et al.*, 2012). The molecular ion observed at m/z 1,869 (Fig. 7.3A), represents a fraction of lipopeptide that cannot be assigned in any of the three classes and the molecular ion at 1065.7 (Fig. 7.3B), represent iturin class (Mandal *et al.*, 2013). Several other mass ions produced by the strain include m/z 953 and 1356 (Fig. 7.4). Moreover, simultaneous co-production of two distinct classes of lipopeptide by the SPL-4 strain is observed from the two spots on the TLC analysis.

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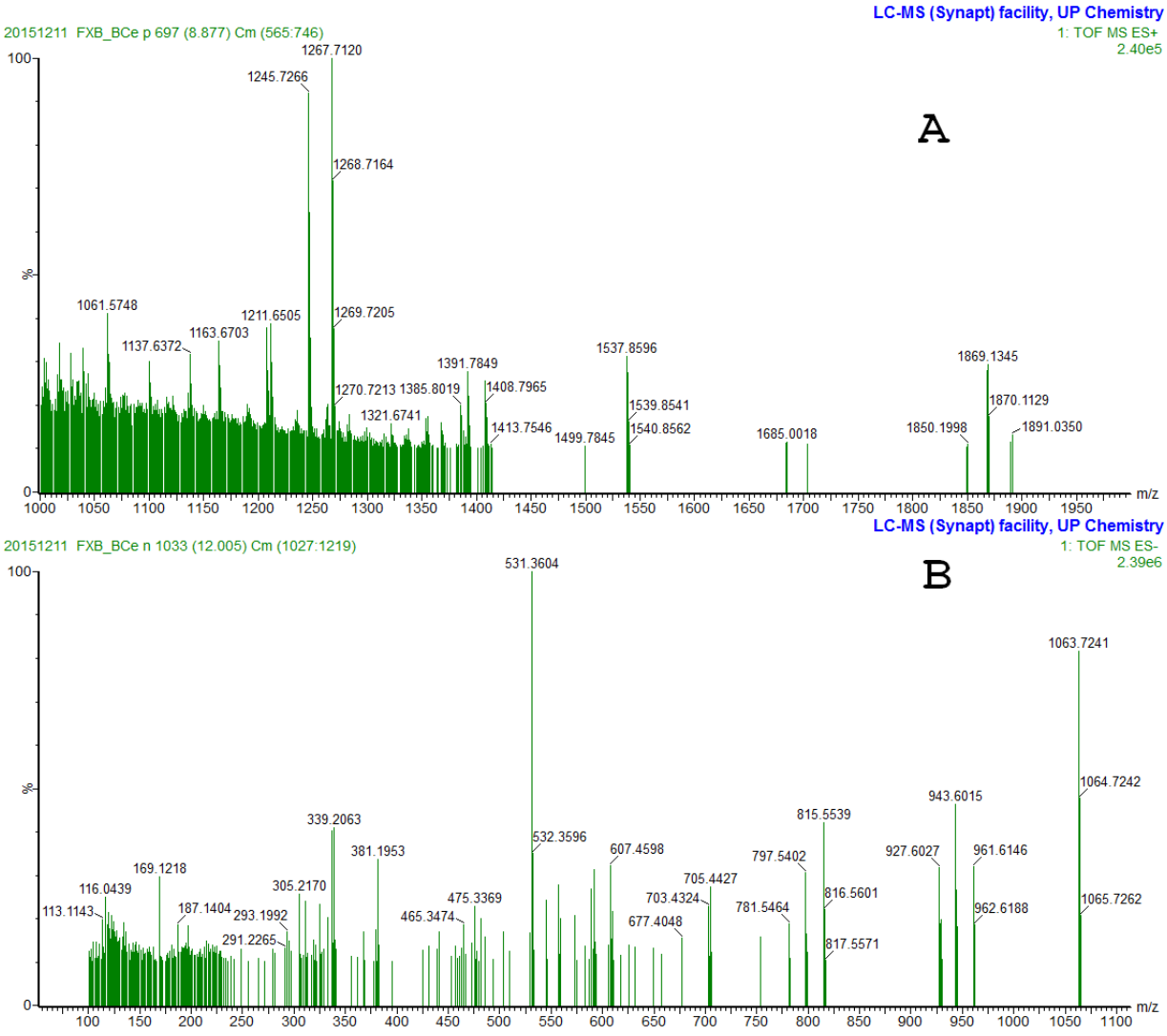

Figure 7.3 A and B Liquid chromatography tandem mass spectrometry (LC–MS/MS) spectraof the fractions of the biosurfactant produced by *Bacillus cereus* SPL-4 eluting at 8.87 min (A) and 12.0 min (B) retention times.

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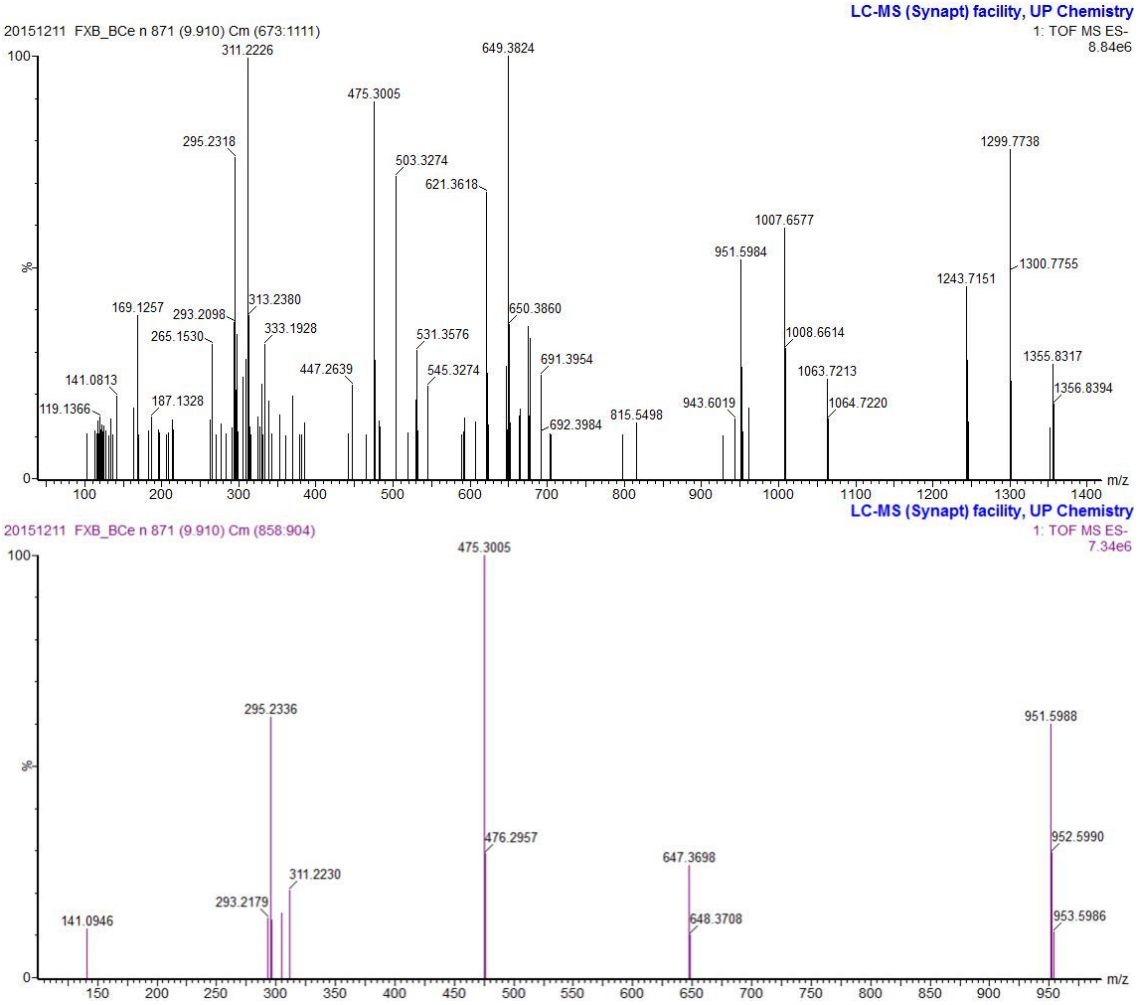

Figure 7.4 (A and B) Liquid chromatography tandem mass spectrometry (LC–MS/MS) spectraof the fractions of the biosurfactant produced by *Bacillus cereus* SPL-4 eluting at 9.910 min retention time.

7.4 Biodegradation of PAHs

Total PAH (the sum of 13 compounds) concentration in the wood treatment plant soil was 6745.5 mg kg^{-1} (Tables 7.1 and 7.2). Two and three-ring PAHs dominated (58.3%) in the plant soil, 4-ring PAHs covered 32.2% while 5 and 6-ring PAHs represented 9.5% of the total PAH concentration. The proportion of low-molecular-weight (LMW) PAHs in the wood treatment plant soil in this study was similar to those reported by Hu and Aitken (2012) (> 50% for 3-ring and 30% for 4-ring PAHs). The biodegradation of the 13 PAHs in soil phase was monitored for a period of 64 days; Tables 7.1 and 7.2 present the initial and final concentrations of PAHs in the soil after different treatments were carried out. The autoclave sterilized abiotic controls didn't show significant losses (< 5%). All treatments showed a significant reduction

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in low molecular weight PAHs concentration following 64 days of incubation ($p < 0.05$) while high molecular weight PAHs were significantly reduced in surfactant treated microcosms (Fig. 7.5).

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Table 7.1 13 EPA-PAHs concentration (mg kg^{-1}dry soil) of aged contaminated soil before and throughout the 64-day incubation period [a] , with no surfactant amendment, Naphthalene (NAP), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene(FLR), pyrene (PYR), benzo[a]anthracene (BaA), chrysene(CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenz[a,h]anthracene (D(a,h)A) , benzo [g,h,i] perylene (B(g,h,i)P).

PAHs[b]	Day 0[b]	Day 8[b]	Day 16[b]	Day 24[b]	Day 32[b]	Day 40[b]	Day 48[b]	Day 56[b]	Day 64[b]
NAP	2787.4± 75	2552.5±62	1913.2±57	1447.8±72	1230.2±95	1054.3±63	879.9±21	264.5±6	124.6±11
FLU	368.2±39	266.5±11	248.9±17	236.7±13	227.8±23	221.9±27	216.2±17	201.4±9	171.7±19
PHE	593.4±11	505.7±15	476.4±19	354.4±14	343.4±7	332.1±5	319.6±11	240.6±15	145.2±12
ANT	185.1±05	141.1±05	126.5±4	115.3±6	109.1±7	108.6±3	103.2±4	102.5±13	91.4±9
Total 2 and 3 ring	**3934.1**	**3465.8**	**2765**	**2154.2**	**1910.5**	**1716.9**	**1518.9**	**809**	**532.9**
FLR	863.9±87	813.9±61	772.4±73	763.6±85	755.6±91	751.4±79	749.5±81	724.5±56	632.7±34
PYR	784.5±31	703.5±45	675.9±23	667.6±21	655.4±40	641.7±33	634.9±41	619.9±39	518.1±56
B[a]A	288.4±09	266.4±11	258.4±8	251.0±12	248.6±11	244.8±7	242.6±6	238.9±13	233.5±19
CHR	234.4±11	175.5±13	171.2±16	167.7±11	166.4±19	165.2±24	164.5±13	161.4±18	158.6±9
Total 4 ring	**2171.2**	**2009.3**	**1877.9**	**1822.9**	**1862**	**1803.1**	**1791.5**	**1744.7**	**1542.9**
B[b]F	154.6±06	147.7±03	143.1±3	139.5±3	138.1±3	136.7±3	134.6±3	132.2±13	129.4±11
B[k]F	121.3±09	113.4±07	111.7±8	109.9±4	107.5±6	105.2±8	104.4±7	80.8±7	71.6±8
B[a]P	124.6±12	113.6±11	109.2±8	107.2±4	104.4±3	101.5±7	99.5±21	97.2±9	94.2±9
D[a,h]A	108.4±03	88.3±04	82.5±11	79.3±7	78.3±9	77.2±8	76.9±7	76.3±8	70.1±5
B[g,h,i]P	131.3±07	121.1±07	119.7±17	118.2±12	117.5±17	116.9±21	115.7±10	113.0±23	111.8±16
Total 5 and 6 ring	**640.2**	**584.1**	**566.2**	**554.1**	**545.8**	**537.5**	**531.1**	**499.5**	**477.1**

[a] Mean values±S.D. for duplicates; [b] No Surfactant amendment.

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Table 7.2 13 EPA-PAHs concentration (mg kg^{-1}dry soil) of aged contaminated soil before and throughout 64-day incubation [a], at 0.2%(w/w) Lipopeptide and 0.6%(w/w) Lipopeptide ; Naphthalene (NAP), fluorene (FLU), phenanthrene (PHE), anthracene (ANT), fluoranthene(FLR), pyrene (PYR), benzo[a]anthracene (BaA), chrysene(CHR), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenz[a,h]anthracene (D(a,h)A), benzo [g,h,i]perylene (B(g,h,i)P)

PAHs [c]	Day 0[c]	Day 8[c]	Day 16[c]	Day 24[c]	Day 32[c]	Day 40[c]	Day 48[c]	Day 56[c]	Day 64[c]
NAP	2787.4± 75	1683.8±81	1384.1±78	1112.7±92	1090.9±87	816.9±45	550.3±31	221.1±13	--------
FLU	368.2±39	286.5±31	268.7±23	252.5±21	243.7±18	232.3±12	211.6±17	180.6±15	154.1±11
PHE	593.4±11	445±21	425±17	405.2±35	365.2±41	331.4±19	318.8±23	269.9±16	150.1±9
ANT	185.1±05	143.7±13	131.2±10	125.1±9	119.8±	114.6±13	110.5±10	82.7±9	76.8±7
Total 2 and 3 ring	**3934.1**	**2559**	**2209**	**1895.5**	**1819.6**	**1495.2**	**1191.2**	**754.3**	**381**
FLR	863.9±13	681.1±97	646.5±41	634.4±44	628.8±32	614.6±27	609.7±34	589.8±23	384.8±20
PYR	784.5±21	674.4±53	578.1±23	566.5±31	545.8±11	527.2±21	521.4±27	505.7±19	443.4±23
B(a)A	288.4±09	221.2±12	209.1±11	201.5±21	186.2±13	179.1±19	167.1±12	162.7±9	158.6±8
CHR	234.4±11	168.6±9	157.0±9	154.5±13	144.5±10	123.2±11	119.5±11	98.5±6	74.1±3
Total 4 ring	**2171.2**	**1745.3**	**1590.6**	**1556.9**	**1505.3**	**1444.1**	**1417.7**	**1356.7**	**1060.9**
B(b)F	154.6±06	106.5±13	103.4±12	95.2±8	93.1±9	91.5±7	88.7±6	86.1±7	83.2±5
B(k)F	121.3±09	67.0±8	58.4±8	52.7±3	48.5±4	46.9±4	43.1±3	41.1±3	37.9±2
B(a)P	124.6±12	83.9±9	81.1±11	76.4±6	74.3±7	64.7±6	57.5±6	56.5±7	54.9±4
D(a,h)A	108.4±03	69.2±7	60.8±9	55.7±5	48.6±5	44.2±3	48.7±3	42.4±5	36.1±5
B(g,h,i)P	131.3±07	91.6±11	86.2±10	84.4±7	82.4±8	78.7±6	77.8±4	76.3±5	75.7±7
Total 5 and 6 ring	**640.2**	**418.2**	**389.9**	**364.4**	**346.9**	**326**	**315.8**	**302.4**	**287.8**
PAHs[d]	**Day 0[d]**	**Day 8[d]**	**Day 16[d]**	**Day 24[d]**	**Day 32[d]**	**Day 40[d]**	**Day 48[d]**	**Day 56[d]**	**Day 64[d]**
NAP	2787.4± 75	1920.5±43	1720.7±67	1443.3±34	1399.8±42	1318.9±55	820.5±11	246.8±9	-------
FLU	368.2±39	221.4±12	215.4±11	211.2±10	207.1±12	197.4±13	195.5±21	181.6±9	91.4±6
PHE	593.4±11	349.9±21	311.7±21	283.3±13	251.9±11	231.4±14	205.7±12	182.8±16	96.3±9
ANT	185.1±05	124.4±9	119.8±11	117.3±12	111.7±11	109±8	103.7±9	95.7±6	78.1±6
Total 2 and 3 ring	**3934.1**	**2616.2**	**2367.6**	**2055.1**	**1970.5**	**1856.7**	**1325.4**	**706.9**	**265.8**
FLR	863.9±13	558.7±21	531.6±23	471.3±13	462.9±17	429.8±11	401.3±13	371.1±13	300.2±12
PYR	784.5±21	612.5±45	517.1±31	416.4±11	381.4±9	359.7±7	357.5±17	331.3±21	301.9±21
B(a)A	288.4±09	224.9±14	204.9±10	171.8±9	158.2±21	141.1±8	138.8±11	136.2±9	134.6±13
CHR	234.4±11	116.8±9	110.6±9	67.8±7	65.8±8	62.9±5	59.5±8	53.5±7	42.9±5
Total 4 ring	**2171.2**	**1512.9**	**1364.2**	**1127.3**	**1068.3**	**993.5**	**957.1**	**892.1**	**779.6**
B(b)F	154.6±06	77.6±8	73.7±8	68.5±5	64.8±9	60.7±6	57.8±9	48.9±4	37.0±3
B(k)F	121.3±09	84.7±11	34.4±4	25.7±2	22.3±3	21.1±3	19.7±3	17.7±2	16.3±2
B(a)P	124.6±12	57.5±5	46.5±6	37.8±2	35.2±4	34.5±5	32.1±5	29.2±2	28.1±3
D(a,h)A	108.4±03	51.3±6	33.3±4	22.2±3	21.2±3	20.5±3	18.2±3	17.2±3	16.9±3
B(g,h,i)P	131.3±07	72.7 ±7	53.4±7	46.5±4	42.9±4	39.2±2	37.2±4	36.8±4	35.2±4
Total 5 and 6 ring	**640.2**	**343.8**	**241.3**	**200.7**	**186.4**	**176**	**165**	**149.8**	**133.5**

[a]Mean values ± S.D. for duplicates; [c] 0.2% (w/w) Lipopeptide; [d] 0.6% (w/w) Lipopeptide; ----- represents below instrument detection level.

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7.4.1 Soil treatment withou biosurfactant (lipopeptide) amendment

The 2 and 3-ring PAH content had decreased considerably over the period of 64 days. Overall 13.5% of 2 and 3-ring, 74.8% of the 4-ring and 75% of the 5 and 6 ring PAHs remained in the soil after the treatment (Fig. 7.5a). The treatment showed a significant reduction in low molecular weight PAHs concentration while there was no significant reduction of the high molecular weight PAHs (Fig. 7.5a).

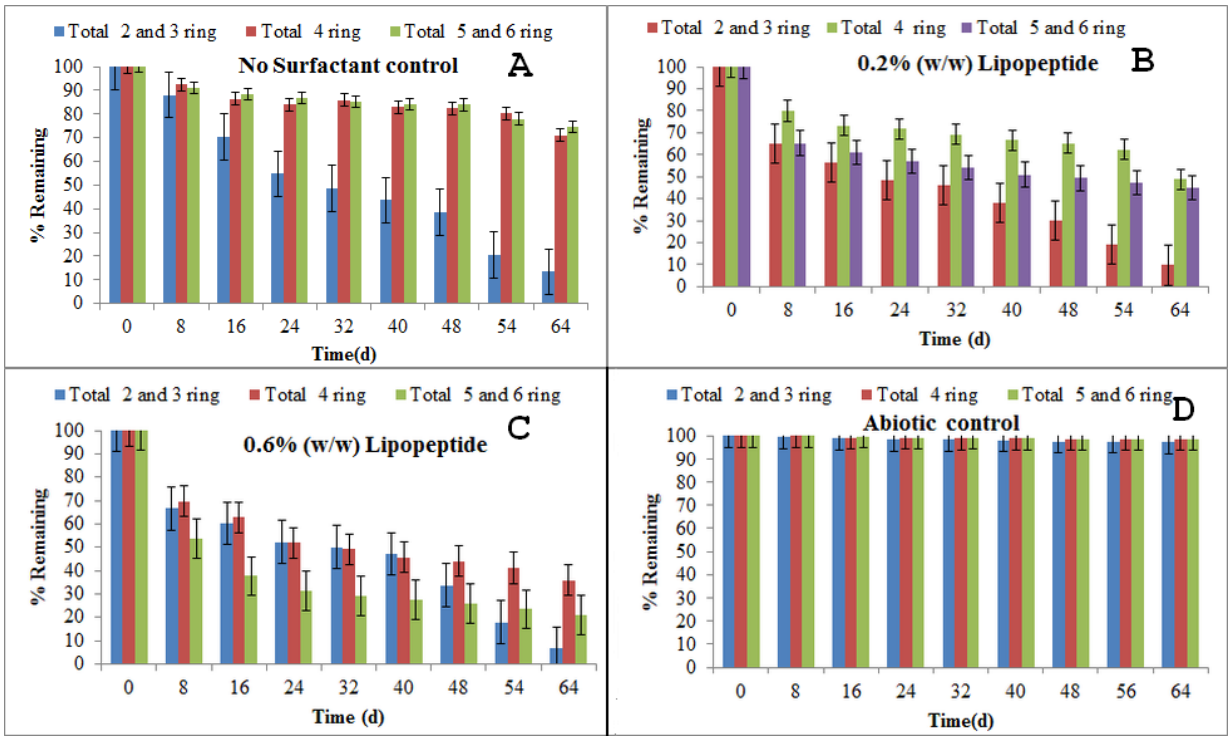

Figure 7.5 Percentage of remaining PAHs according to their ring size in the different treatments during the 64 day incubation period; no surfactant control(A), 0.2% (w/w) lipopeptide amended (B), 0.6%(w/w) lipopeptide amended (C), sterilized abiotic control (D). All data are expressed as means ± SD of duplicate experiments.

7.4.2 Soil treatment with biosurfactant (lipopeptide) amendment

Statistically significant reduction ($p < 0.5$) of HMW PAHs was observed in both the 0.2% and 0.6% lipopeptide amended treatments compared to the surfactant free control (Fig. 7.5a, b and c). The lower molecular weight (2 and 3-ring PAHs) content of the soil was reduced to 9.7% while the 4, 5 and 6-ring PAHs content was reduced to 48.9 and 44.9% respectively after treatment with 0.2% (w/w) lipopeptide (Fig. 7.5b). Particular PAH group concentration in the

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soil after an incubation period of 64 days indicated enhanced degradation of the 4 ring PAHs (26% increment) and 5 and 6 ring PAHs (26% increment) compared to the treatment without surfactant (Fig. 7.5b). Residual PAH concentrations were significantly reduced ($p < 0.05$) in the 0.6% (w/w) lipopeptide amended microcosm compared to the 0.2% (w/w) amendment. Acceleration of PAH degradation was most pronounced for the 5 and 6-ring PAHs in both amendments (Fig. 7.5b, c). Biodegradation of 4 ring PAHs was enhanced twofold whereas the 5 and 6 ring PAHs was enhanced threefold in the 0.6% (w/w) lipopeptide amended microcosm compared to the treatment without surfactant (Fig. 7.5 a, c).

The total PAH concentration in soil with 0.2% and 0.6% (w/w) lipopeptide amended treatments declined from (6745.5± 121) to (1729.7± 115) and (1178.9 ± 205) mg kg^{-1} respectively compared to 2552.9 ± 245 mg kg^{-1} decline in no surfactant control after 64 days of incubation. In treatments where lipopeptide was added the extent of PAH degradation was greatly enhanced (Fig. 7.5b,c) with residual concentrations significantly lower ($p < 0.05$) than in the surfactant free control. On the other hand, there was no significant difference in the removal of 2 and 3 ring PAHs ($p > 0.05$), which were most abundant and easily degraded in all the treatments.

The supplementation of the surfactant had no statistically significant effect on the biodegradation of phenanthrene, fluorene and naphthalene in any of the dosages ($p > 0.05$). Thus the percentages of naphthalene fluorene and phenanthrene removed were not significantly different for biosurfactant amended and unamended microcosms (Tables 7.1 and 7.2). A positive effect of surfactant addition was observed on the dissipation of anthracene compared to other three ring PAHs, as it showed the lowest degradation with no-surfactant control. The absence of significant effect on the removal of the low molecular weight PAHs other than anthracene in the presence of the surfactant may be attributed to the higher aqueous solubility of naphthalene (31 mg L^{-1}), fluorene (1.9 mg L^{-1}) and phenanthrene (1.1 mg L^{-1}) compared to anthracene (0.045 mg L^{-1}), what caused less bioavailability restrictions to biodegradation of naphthalene, fluorene and phenanthrene.

All the 4-ring PAHs exhibited significantly high removal rates compared to the surfactant free controls ($p < 0.05$). Chrysene which has the highest hydrophobicity and lowest solubility (Table 2.1) showed the highest dissipation rate (82%) compared to the other 4 ring PAHs pyrene (62%), benzo[a]anthracene (53.2%) and fluoranthene (65%) in the 0.6% w/w lipopeptide supplemented microcosms (Table 7.2). In contrast to the 3 ring PAHs the effect of the

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surfactant supplementation was pronounced more in the 4 ring PAHs dissipation rates. All the four ring PAHs especially benzo[a]anthracene showed recalcitrance and least degradation in surfactant free controls compared to surfactant supplemented treatments due to their high hydrophobicity and electrochemical stability which in turn resulted in decreased bioavailability and high sorption tendency to soil organic matter and clay particles. Within the 4 ring PAH group chrysene and benzo[a]anthracene have the highest hydrophobicity and lowest solubility (Table 2.1) due to their angular pattern of ring linkage (Kanaly and Harayama, 2000). The hydrophobicity and recalcitrance of a PAH molecule depends on the number of aromatic rings and on the pattern of ring linkage (Kanaly and Harayama, 2000). PAHs with linear structure are more unstable than their angular counter parts, increased size and angularity of PAH structure increases its hydrophobicity and electrochemical stability, which in turn affect their vapour pressure, solubility and adsorption characteristics (Mukherji and Ghosh, 2012). Despite lowest solubility and high hydrophobicity the highest degradation rate (82%) of chrysene observed in the 0.6% w/w lipopeptide supplemented treatments compared to the other 4 ring PAHs can be attributed to other factors besides bioavailability limitations. Of these factors the presence of low-molecular weight PAHs may facilitate overall degradation of a complex mixture by inducing enzymatic systems that cometabolize the high-molecular-weight, recalcitrant structures (Zylstra *et al.,* 1994; McLellan *et al.,* 2002). Previous studies of PAH degradation by microbial cultures presented evidence that there are interactions between PAHs in mixtures that may result in inhibition of degradation as well as synergy (Bouchez *et al*., 1995; Molina *et al*., 1999; McLellan *et al.,* 2002). The factors affecting these interactions are complex and may include hastening by cross induction of required degradative enzymes (Molina *et al*., 1999), co-metabolism (Bouchez *et al*., 1995) and preferential utilization of high-affinity substrates besides bioavailability limitations. Park *et al.* (1990) reported similar observation whereby higher-molecular-weight PAHs were more resistant to biotransformation when present as pure compounds in soil than when present in complex waste mixtures in soil, whereas lower-molecular-weight PAHs were transformed more rapidly as pure compounds.

The 5 and 6 ring PAHs showed significant degradation rates in surfactant amended controls compared to surfactant free controls ($p < 0.05$). Benzo[a]pyrene which is highly hydrophobic and least degradable 5 ring PAH showed 56.4% and 75.4% degradation rates at 0.2% w/w and 0.6% w/w lipopeptide supplementations compared to 24% in the surfactant free control (Tables 7.1 and 7.2). Benzo[*b*]fluoranthene, benzo[*k*]fluoranthene and dibenz[*a*,*h*]anthracene also showed statistically significant degradations in the surfactant amended microcosms ($p < 0.05$,

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Tables 7.1 and 7.2) compared to surfactant free controls. The 6 ring PAH, benzo[*g*,*h*,*i*]perylene displayed 71.4 % dissipation when amended with 0.6%, w/w compared to 15% degradation in the unamneded and 42% in the 0.2% w/w amended treatments. There was significantly high removal rate of all 5 and 6 ring PAHs in the 0.6% w/w lipopeptide supplemented treatment ($p < 0.05$) compared to 0.2% w/w supplemented treatments. While both the dosages of lipopeptide supplementation displayed significant removal rates of the 4 ring PAHs compared to surfactant free controls ($p < 0.05$), there was no significant change in the removal rates of the PAHs at the two dosages of lipopeptide supplementation ($p > 0.05$).

In general, both the surfactant dosages enhanced the removal of 4-ring PAHs, although the higher dosage enhanced the removal of the 5 and 6 ring PAHs significantly (Fig. 7.5c).

It is generally accepted that low level of bioavailability is one of the most important factors involved in the slow biodegradation of hydrophobic organic compounds in soil. They are easily adsorbed to clay or humus fractions in the soil, and pass very slowly to the aqueous phase, where they are metabolized by microorganisms. It has previously been demonstrated that biosurfactants accelerate the degradation kinetics of PAH and PAH bioavailability in both aqueous phase and the solid phases (Congiu and Ortega-Calvo, 2014; Bezza and Chirwa, 2016).

Though both lipopeptide dosages enhanced degradation of the HMW PAHs compared to the surfactant free controls, increasing the lipopeptide added from 0.2% to 0.6% w/w displayed significantly higher enhancement in the degradation of the HMW PAHs ($p < 0.05$), Tables 7.1 and 7.2. Reduced degradation of the HMW PAHs in the 0.2% (w/w) lipopeptide amended treatment compared to the 0.6% (w/w) treatment can be attributed to sorption of the lipopeptide on soil and organic matter in soil. Surfactants can increase the rate of PAH desorption and subsequent degradation from a soil through micellar solubilisation, direct modification of the contaminant matrix and surfactant induced modification of cell surface (Zhang *et al.*, 2013). The extensive sorption of surfactants to soil, however, leads to a substantially higher surfactant dose to reach the CMC in a soil/water system than in the absence of soil (Zhu and Aitken, 2010).

In contrast to solubilisation, modification of the contaminant matrix can occur at concentrations above and below the CMC. Surfactants have been shown to increase desorption of PAHs from contaminated field soil at doses corresponding to aqueous-phase surfactant concentrations less

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than the CMC in the soil/water system (Zhu and Aitken, 2010; Alden *et al.*, 2016). Suggested effects of surfactants on the contaminant matrix include increased PAH diffusivity and increased soil interfacial surface area caused by wetting and dispersion of non-polar matrices (Alden *et al.*, 2016). Various authors observed that surfactants that associate with none-aqueous phase liquids (NAPLs) in contact with soil influence their wetting behaviour and correspondingly help release the NAPLs from the soil, even when the aqueous-phase surfactant concentration is below the CMC (Dong *et al.*, 2004; Zhu and Aitken, 2010). Alternatively, biosurfactants may also induce changes in the properties of cellular membranes, resulting in increased microbial adherence to the hydrophobic pollutants (Zhang *et al.*, 2013).

The surfactant at the 0.2% (w/w) may have enhanced the rate of PAH biodegradation by increasing the interaction of bacteria with PAH-containing soil compartments. This could occur through increased soil interfacial surface area onto which bacteria may adhere or through modification of cell- or soil-surface properties to favour adhesion. Surfactants can alter cell surface hydrophobicity in ways that can either promote or inhibit bacterial adhesion. Several previous studies reported enhanced biodegradation of PAHs and other hydrophobic contaminants in the presence of externally added biosurfactants (Whang *et al.*, 2009; Sponza and Gok 2011; Congiu and Ortega-Calvo, 2014; Bak *et al.*, 2015; Bezza and Chirwa, 2016). Though the application of biosurfactants in bioremediation has been reported highly beneficial in many studies, inhibitory effects have also been observed (Shin *et al.*, 2005; Whang *et al.*, 2008).

Whang *et al.* (2008) demonstrated that surfactin at 40 mg/L stimulated diesel biodegradation as well as biomass growth by an enriched diesel-degrading bacterial consortium in batch diesel/water cultures. However, higher concentrations of surfactin inhibited degradation of petroleum hydrocarbons in the diesel/water systems prompting the authors to conclude that the possible inhibitory effects of surfactin on bioremediation needs careful determination. Besides their efficient surface active properties lipopetidal biosurfactants like viscosin, and surfactin, are concomitantly potent antibiotics (Raaijmakers *et al.*, 2010) and would have detrimental effect on the degrading microorganism beyond threshold level that needs to be determined in advance. It is important to take in to consideration that a biosurfactant affects bacteria differently, depending on their individual uptake strategies of hydrophobic compounds (Bak *et al.*, 2015). Some studies have revealed that bacterial adhesion to the PAHs was prevented by addition of surfactants and their degradation was inhibited (Abbasnezhad *et al.*, 2011). Adrion

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et al. (2016) suggested that the limited PAH removal in the presence of the rhamnolipid used in their study was due to inhibited bacterial adhesion to PAH-containing soil domains.

Microbial degradation studies have shown that it is much more difficult to remove high molecular weight (HMW) PAHs with four or more fused aromatic rings than it is to remove low molecular weight PAHs since HMW PAHs are thermodynamically stable and hydrophobic with low aqueous solubility and high sorption to soil particles (Kim *et al.*, 2007). Extensive well-documented biodegradation studies of PAHs in field-contaminated soils show that generally the rate of PAH biodegradation tends to decrease with increasing ring number (Haritash and Kaushik, 2009). Similar to our previous study (Bezza and Chirwa, 2016) the results from the biodegradability test in this study confirm the decreasing degradability of the compounds with increasing ring number in the no surfactant control, on the other hand the biosurfactant amended treatments showed significantly enhanced degradation of 5 and 6-ring high molecular weight PAHs compared to the lower molecular weight 4-ring PAHs.

The rate of degradation of 5 and 6-ring PAHs was significantly higher than four ring PAHs in the 0.6% (w/w) surfactant amended treatment ($p < 0.05$). However in terms of mass removal, fluoranthene was the most degradable, in HMW PAHs, with a decrease from 863.9 to 300.2 mg kg^{-1} by day 64, a mass decrease of 563.7 mg kg^{-1}, much higher than the mass decrease of 94.5 mg kg^{-1} in benzo (*g, h, i*) perylene a 6-ring PAH which showed comparably higher percentage removal than fluoranthene (Table 7.1). The 5–6 rings achieved up to 79.9% removal but the mass degraded was lesser compared to 4 ring PAHs. This shows the potent metabolic potential of the microbial consortium which was previously sourced from creosote contaminated soil to degrade different group of PAHs concomitantly. The degradation of the PAHs in aged soils appears to be controlled by mass transfer rather than the biodegradation rate as previously reported (Li *et al.*, 2008).

The processes by which organic compounds become increasingly desorption-resistant in soil results from sequestration, which originated from the slow diffusion of organic compounds within solid organic matter components, the entrapment with in nanopores in soil aggregates, and the formation of strong bonds between organic compounds and the soil (Hatzinger, and Alexander, 1995). Once this bioavailability limitation of the PAHs was overcome, the microbial consortium showed concomitant degradation of all PAH groups. This result was consistent with previous findings in which preferential degradation of the high-molecular-weight PAHs (with 5- and 6-rings) were reported (Li *et al.*, 2008; Guo *et al.*, 2016). These

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results were especially important for high molecular weight PAHs and could be a real advantage for remediation of aged, contaminated soils.

Enhanced degradation of HMW PAHs might be due to cometabolization by the enzymes induced through LMW PAHs or some metabolites such as salicylate in the degradation process (Chen and Aitken, 1999). Wang *et al.* (2010) reported significant degradation of HMW PAHs involving benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, and benzo[k]fluoranthene, which are generally recalcitrant to microbial attack, following sequential utilization, after the initial degradation of 3-ring and some 4-ring PAHs.

7.5 Biodegradation Kinetics

In our study, 64 d degradation kinetics curves were modeled for 4, 5 and 6-ring PAH groups at no surfactant and two different lipopeptide supplementation dosages, using the two-compartment first order decay model (Congiu and Ortega-Calvo, 2014; Deary *et al.,* 2016). The data describing the degradation of the PAHs were fit to the model (Equation 7.1), which generated the best fit overall (Congiu and Ortega-Calvo, 2014; Deary *et al.,* 2016)

$$\frac{S_t}{S_0} = F_{fast} e^{-k_{fast} t} + F_{slow} e^{-k_{slow} t} \qquad (7.1)$$

Where S_t (mg kg^{-1}) is the PAHs content in the soil at time t (d) and S_0 (mg kg^{-1}) at the start of the experiment; F_{fast} and F_{slow} are the rapidly and slowly degrading fractions.

The values of F_{fast}, F_{slow}, k_{fast} (d^{-1}) and k_{slow} (d^{-1}) were determined by minimizing the cumulative squared residuals between experimental and calculated values of $\left(\frac{S_t}{S_0}\right)$ in Equation (7.1) using the software Microsoft Excel 2010 (SOLVER option).

For all the PAHs, the experimental results fitted with the two-compartment model satisfactorily (Fig. 7.6(A) and (B)). Fitting the data to Equation (7.1) gave sums of squared deviations ranging from 0.0018 to 0.00033, indicating satisfactory fitness. The parameter estimates for the two-compartment model for the PAH groups are given in Table 7.3. The dissipation rate

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constants for the two-compartment model followed the progression of k_{fast} >> k_{slow} and were generally in the order of 10^{-1} and 10^{-3} d^{-1}, respectively.

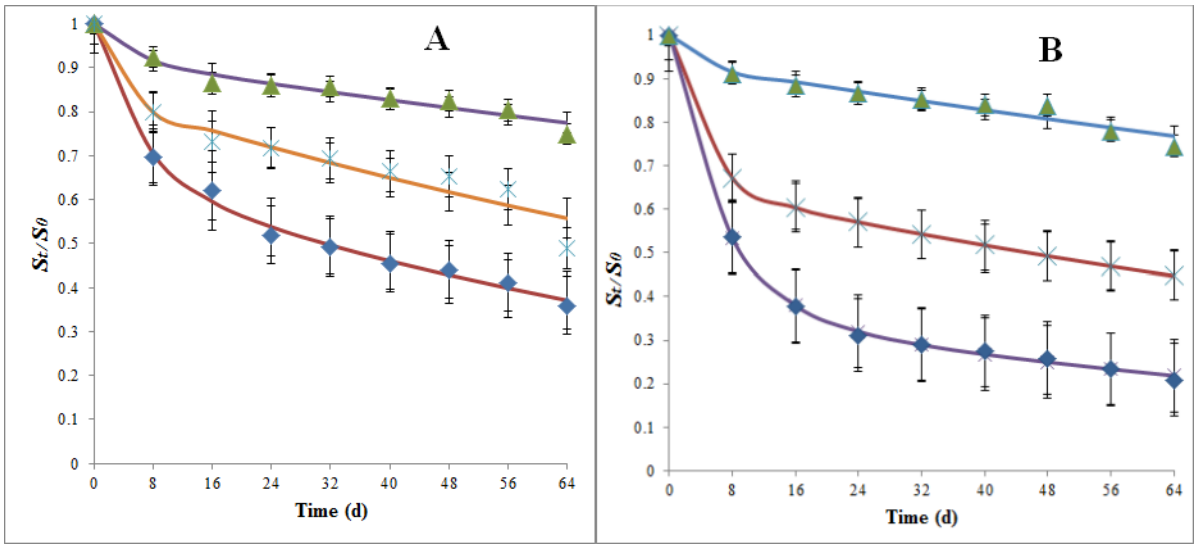

Figure 7.6 Two-compartment model fits to 4-ring PAHs (A) and 5 and 6-ring PAHs (B) biodegradation kinetics data with no surfactant (▲) and in the presence of 0.2% (w/w) Lipopetide (×) and 0.6% (w/w) Lipopeptide (◆). Data points are mean values from three independent experiments. Error bars represent standard errors.

Table 7.3 Best-fit parameters of the two-compartment model for the different lipopeptide supplementations for 4-ring, 5 and 6-ring PAH groups.

PAH (Ring roups)	Lipopeptide % (w/w)	F_{fast}(%)	$k_{fast}(d^{-1})$	F_{slow}(%)	$k_{slow}(d^{-1})$
Total 4- Ring	**0.0**	07.80±0.2	0.205	92.20±1.0	0.002
	0.2	16.10±0.3	0.534	83.90±0.3	0.006
	0.6	34.00±0.6	0.164	66.00±1.1	0.009
Total 5 and 6 Ring	**0.0**	06.20±0.8	0.967	93.80±0.5	0.003
	0.2	34.20±0.5	0.256	65.80±1.0	0.006
	0.6	63.10±0.4	0.152	36.90±0.4	0.008

The calculation of the maximum rates of degradation (k_{fast}) and F_{fast} values with equation 7.1 (Table 7.3) verified that, the biosurfactant enhanced the degradations significantly. These two parameters described well the changes in degradation caused by the biosurfactant amendment.

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Fast degradation fractions, F_{fast}, were increased in the biosurfactant amended treatments, which led to progressively higher rates of degradations compared to the surfactant-free controls. The total 5 and 6-ring PAHs fast degradation fraction was 6% in the surfactant free control compared to 34.2 and 63% in the 0.2% (w/w) and 0.6%(w/w) lipopeptide amended treatments respectively (Table 7.3). Specific representative 4-ring, 5 and 6-ring PAHs experimental results fitted adequately with the two-compartment model and exhibited increasing fast degradation fractions, F_{fast}, in the biosurfactant amended treatments (Fig. 7.7).

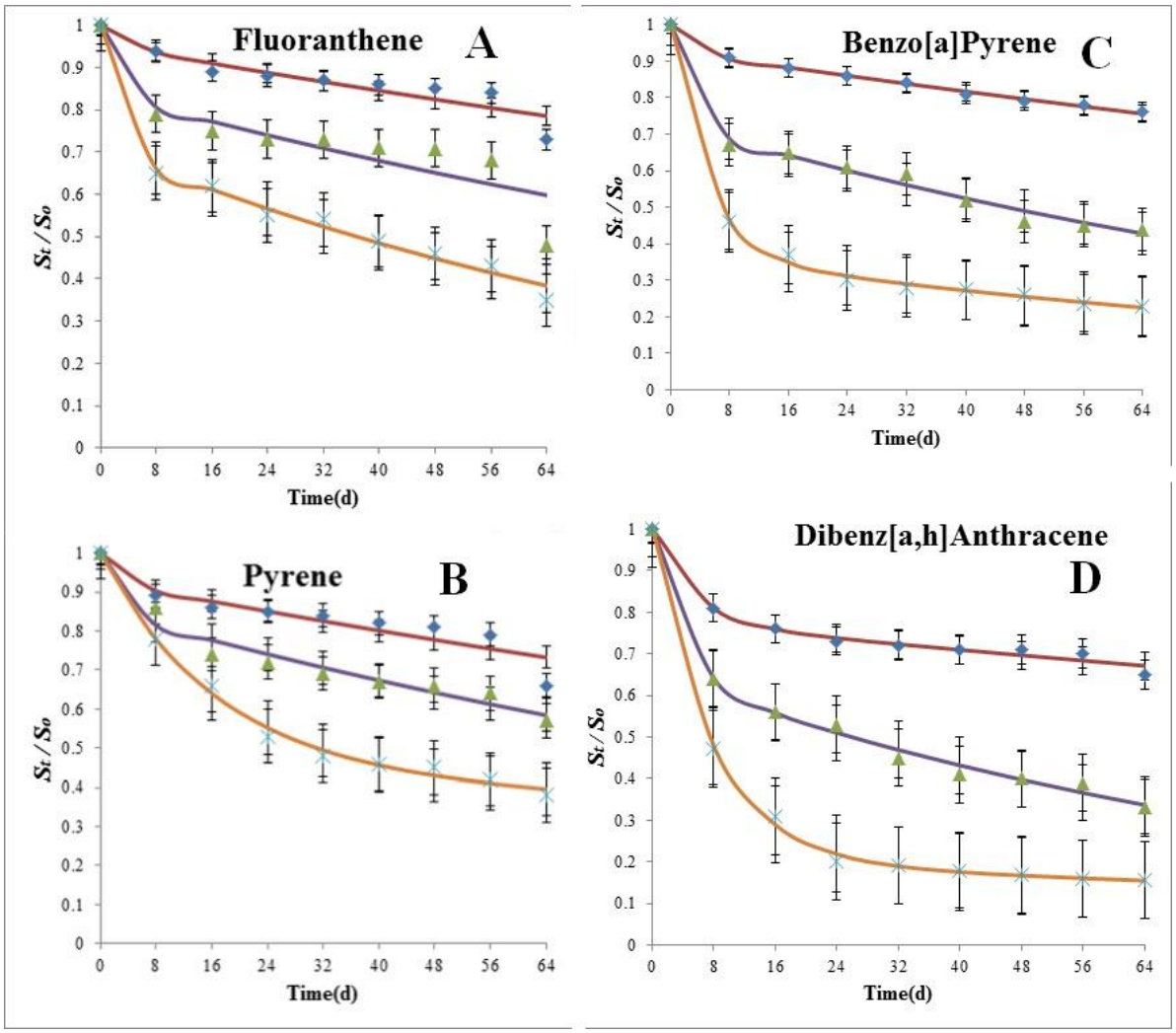

Figure 7.7 Two-compartment model fits to representative 4-ring PAHs (A,B) and 5-ring PAHs (C,D) biodegradation kinetics data with no surfactant (♦) and in the presence of 0.2% (w/w) Lipopeptide (▲) and 0.6% (w/w) Lipopeptide (×). Data points are mean values of two independent experiments performed in duplicates. Error bars represent standard errors.

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These results are comparable with values reported in other studies for PAH removals from aged soils and sediments (Congiu and Ortega-Calvo, 2014; Jho *et al.*, 2014). The values of k_{slow} were two to three orders of magnitude lower than the k_{fast} values for all PAH groups in different samples (Table 7.3), which is consistent with other studies that apply the two-compartment biodegradation model (Jho *et al.*, 2014; Congiu and Ortega-Calvo, 2014; Deary *et al.*, 2016). These results could be considered to well validate the biphasic behaviour of organic compounds degradation and to confirm the supposition of the model. Many studies have reported that PAH degradation in soil over extended periods can be described by biphasic kinetics, comprising an initial rapid phase over several days in which the PAHs are degraded by soil microorganisms followed by a slower phase over hundreds of days that reflects sequestering of the PAHs by soil organic matter during the soil ageing process (Deary *et al.*, 2016).

7.6 Microbial Community Composition

Using a similarity threshold of 97% to cluster sequences within the same operational taxonomic units (OTUs), a total of 53 OTUs were obtained in the sample. The overall distribution of the main prokaryotic groups (phyla or classes) showed dominance of sequences within, *Gammaproteobacteria, Alphaproteobacteria, Bacilli, Beta protobacteria and, Acidobacteria*. *Proteobacteria* was the dominant bacterial phylum, representing 88.8% of the 16S rDNA reads from the 53 most abundant bacterial OTUs and *Firmicutes* (2.22%), *Actinobacteria* (1.6%), *Acidobacteria* (1.6%) were observed in the sample at 1% cut off.

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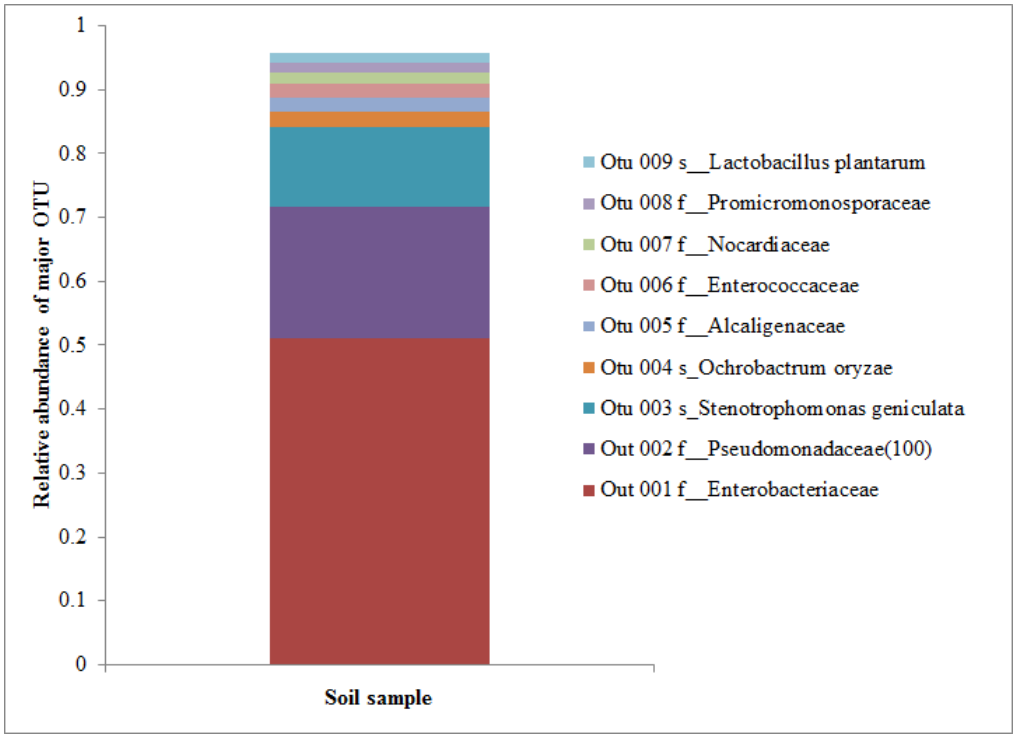

Figure 7.8 Relative abundance of Predominant Operational taxonomic units (OTUs) found in the soil sample at family (f) and species (s) levels.

Proteobacteria have been identified in many studies as the predominant phylum in soil samples playing an integral role in nutrient cycling (Militon *et al.,* 2010). The *Proteobacteria* encompass enormous morphological, physiological and metabolic diversity, and are of great importance to global carbon, nitrogen and sulphur cycles (Militon *et al.*, 2010). Bacteria in the phyla *Proteobacteria, Actinobacteria, Firmicutes, Bacteroidetes and Chlamydiae* have been reported as hydrocarbon degraders (Prince *et al.*, 2010). The current study showed that besides the above mentioned phyla *Verrucomicrobia; Chloroflexi; Planctomycetes*; WPS-2; *Chloroflexi; Armatimonadetes; Gemmatimonadetes*; WPS-2; TM7 bacterial phyla were observed in the creosote contaminated soil. Our results are in agreement with most studies showing the importance of the *Proteobacteria,* especially the Gamma division, in hydrocarbon-polluted soil microbial communities or natural asphalts (Militon *et al.,* 2010). Most of the predominant OTUs belong to the families *Enterobacteriaceae,* and *Pseudomonadaceae* at the family level while *stenotrophomonas geniculate* and *Ochrobactrum oryzae* were found to be the most abundant species (Fig. 7.8). The community was dominated by members of the *Enterobacteriaceae family* (~50%), representative genera in this family like *Klebsiella* and *Serratia* were reported as efficient biosurfactant producers and capable of hydrocarbon

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degradation (Saimmai *et al.*, 2012). *Pseudomonas spp* which are representative members of the *Pseudomonadaceae* family are able to endure and metabolize contaminants that are considered very toxic to other bacteria. Several studies have proved that *Pseudomonas sp*. can utilize a vast range of contaminants either naturally present or xenobiotic (Palleroni *et al.*, 2010).

7.7 Microbial Growth Pattern

To determine the growth of PAH degrading microbial population, microbial count was undertaken over the 64 day period.

The results showed the population of hydrocarbon degraders increased from three to five orders of magnitude in all the treatments (Fig. 7.9). The PAH-degrading bacteria showed a significant increase in the number of colony forming units (CFU) in soils amended with the biosurfactant treatment compared to the control after day 16 of incubation. Addition of surfactant to the soil increased the number of microorganisms present after day 16 by up to two orders of magnitude (increase from 8.8 to 11.7 log (CFU) g^{-1} compared to increase from 8.8 to 9.1 log (CFU) g^{-1} soil in no surfactant control). However there was no significant change in microbial growth during the first 16 days of incubation which may be due to the abundance of easily degradable low molecular weight PAHs in all treatments. The comparably high microbial growth in all treatments suggests that soil microbial communities possessed a sufficiently high PAH degradation capacity.

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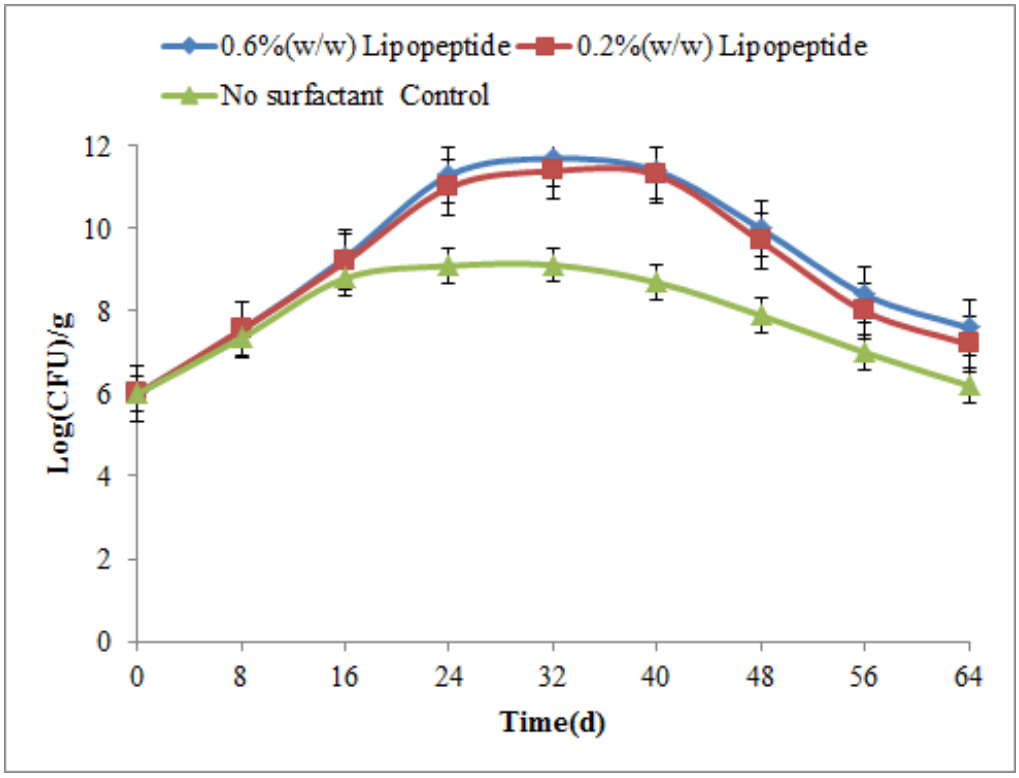

Figure 7.9 The growth kinetics of microbial consortium on the creosote PAHs as the sole carbon source: with 0.2% (w/w) biosurfactant supplementation (*square*), 0.6% (w/w) biosurfactant supplementation (*rhombus*) with no biosurfactant supplementation (*triangle*). Data are expressed as the mean ± standard error of two independent experiments performed in duplicate.

7.8 SUMMARY

Generally, it can be concluded that bioremediation approach for creosote PAH-contaminated soil encompassing biosurfactant supplementation demonstrated more efficient and effective remediation potential. The biosurfactant enhanced the bioavailability and hence the biodegradation of the aged PAHs significantly. Although high-molecular-weight (HMW) PAHs generally persist longer in soils than low-molecular-weight (LMW) PAHs and other hydrocarbons, HMW PAHs were removed significantly from the contaminated soil in the surfactant amended microcosms compared to the surfactant free controls. The degradation of the HMW PAHs in the aged soil appears to be controlled by mass transfer rather than the metabolic potential of the microorganisms. The processes by which organic compounds become increasingly non-bioavailable in soil results from sequestration, which originated from the slow diffusion of organic compounds within solid organic matter components, the

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entrapment with in nanopores in soil aggregates, and the formation of strong bonds between organic compounds and the soil. Once this bioavailability limitation of the PAHs was overcome through biosurfactant supplementation, the microbial consortium showed concomitant degradation of all PAH groups. These results were especially important for high molecular weight PAHs and could be a real advantage for remediation of aged, contaminated soils. Thus the present work shows the potential use of the lipopetidal biosurfactant produced for the bioremediation of creosote and other petroleum hydrocarbon polluted environmental media.

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CHAPTER EIGHT

CONCLUSIONS AND FUTURE PROSPECTS

8.1 Conclusions

This research was undertaken to investigate the potential application of biosurfactants to enhance bioavailability and subsequent biodegradation of the persistent petroleum and polycyclic aromatic hydrocarbons. To accomplish these aims a set of five experiments was conducted. Bioremediation uses microorganisms, fungi, green plants or their enzymes to return the natural environment altered by contaminants to its original condition. As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and environmental soundness. One of the important factors that limit biodegradation of hydrophobic pollutants in the environment is their limited availability to microorganisms. Due to their high hydrophobicity and solid-water distribution ratio, hydrocarbons in soil tend to interact with the non-aqueous phase and organic matter, and consequently become less available for further microbial degradation.

In this sense, the objective of this work was to evaluate the role of biosurfactants produced by various strains in enhancing the degradation of these persistent hydrophobic contaminants in liquid culture and soil media.

Accordingly, efficient PAH degrading and biosurfactant producing strains were isolated from creosote contaminated wood treatment plant soils and characterized as (*Bacillus subtilis* CN2, *Ochrobactrum intermedium* CN3, *Paenibacillus dendritiformis* CN5, *Bacillus cereus* SPL-4).

The biosurfactants produced by the strains exhibited a high level of thermal stability, tolerance to extreme levels of salinity and a positive effect for increasing pH. The biosurfactants were identified after Fourier Transform Infrared (FT-IR) spectrometry, Thin Layer Chromatography (TLC), liquid chromatography/tandem mass spectrometry (LC–MS/MS) analyses. The biosurfactants physicochemical characterization displayed vast structural diversity (Chapters Four, Five, Six and Seven) and potent surface active properties of surface tension reduction and emulsion formation with a range of hydrocarbons. All the studies showed the potential

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abilities of the biosurfactants to reduce surface tensions and increase the bioavailability by emulsification, solubilization and desorption of contaminants.

The ability of the isolate *Ochrobactrum intermedium* CN3 to degrade petroleum sludge and the effect of its biosurfactant addition on biodegradation enhancement potential was tested in liquid culture experiments. The isolate degraded up to 40% of the hydrophobic long chain aliphatic and polycyclic aromatic hydrocarbons in liquid cultures with 4% (v/v) crude oil sludge while the biosurfactant amended microcosm showed up to 70% degradation of the most hydrophobic components of the petroleum sludge in 3 weeks. This suggests the potential application of the isolate and its biosurfactant in bioremediation of contaminated environment and microbial enhanced oil recovery (Chapter 4, Paper 1).

Lipopeptide biosurfactant produced by the isolate *Bacillus subtilis* CN2 more than doubled the degradation rate of the PAHs in used motor oil sludge in liquid culture (3%, v/v) compared to its absence. The lipopeptide mainly enhanced degradation of the more hydrophobic PAHs in the used motor oil sludge which is predominantly composed of PAHs (Chapter Four, Paper 2).

Mass transfer enhancement potential of the lipopeptide biosurfactant produced by *Paenibacillus dendritiformis* CN5 was studied (Chapter Five, Paper 3). The biosurfactant desorbed more than 96% of phenanthrene and 83% of pyrene from the contaminated soil in 5 days and recovered 81% of heavy used motor oil sludge from the spiked sands in 24 h. This suggests the potential application of the biosurfactant for the removal of PAHs and motor oil sludge from contaminated media and its potential application for enhanced oil recovery,

The lipopeptide biosurfactant produced by CN5 strain showed a superior surface activity when it was grown on oil and anthracene (Chapter Six, Paper 4) than when grown on glycerol (Chapter Five). The lipopeptide at 600 and 300 mg L^{-1} enhanced pyrene degradation to 83.5% and 67% respectively in 24 days compared to 16% degradation in its absence. However, degradation of pyrene was reduced to 57% as the lipopeptide supplementation was raised to 900 mg L^{-1}. This demonstrates that the biodegradation of pyrene was found to increase with an increase in the lipopeptide concentration up to a threshold level. These results suggest that at higher concentration of biosurfactant the microbial consortium could have stressed from increased concentration of pseudosolubilized pyrene or the biosurfactant itself which may interfere with the cellular membrane of the bacteria above the threshold level.

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The impact of lipopeptidal biosurfactant, produced by *Bacillus cereus* SPL-4 on biodegradation of low and high molecular weight PAHs in creosote contaminated soil by microbial consortium from previously bioremediated soil was investigated (Chapter Seven, Paper 5). In the microcosms supplemented with 0.2 and 0.6% (w/w) lipopeptide, 51.2% of 4-ring and 55% of 5- and 6-ring PAHs, 64.1% of 4-ring and 79% of 5- and 6-ring PAHs were removed respectively, compared to, 29% of 4-ring and 25.5% of 5- and 6-ring PAHs removal in the surfactant free control after 64 days of incubation. However, there was no statistically significant change in the degradation rates of LMW PAHs in surfactant amended and surfactant free controls. The degradation of 5 and 6 ring PAHs was significantly enhanced ($p < 0.05$) in the higher surfactant dosage compared to the lower dosage. The results suggest that biosurfactant assisted degradation by microbial consortium may be a promising practical bioremediation strategy for aged PAH-contaminated soils.

Based on the results of this work, the hypothesis has been confirmed, indicating that the use of biosurfactants in bioremediation of polycyclic aromatic and petroleum hydrocarbons is a viable option to reduce clean-up time and for effective remediation of soil and aqueous media.

8.2 Future Prospects

Given pervasive nature and large scale anthropogenic production of the persistent polycyclic aromatic and petroleum hydrocarbon products, feasible remedial action through biosurfactant supplementation is a viable option for their immediate and timely remediation owing to their carcinogenicity and other health concerns. Further researches are required to scale up the small scale studies to large scale practical real world applications with various heterogeneities and conduct biosurfactant production at industrial scale accordingly. To facilitate field applications of this biosurfactant enhanced bioremediation technology, a large-scale test is anticipated to incorporate heterogeneities in geological/hydrological characteristics and in microbial and hydrocarbon distributions of real world contaminated sites. However, the first challenge in the production and use of biosurfactants is the cost issue when it comes to large scale production, but it can be offset when an immediate restoration of environmental media is mandatory by regulatory bodies or health concerns for instance. Hazardous waste contaminated sites like closed landfills, abandoned gas stations, old manufacturing facilities, and former dry cleaning facilities predominantly in metropolitan areas pose a potential health risk. The bioremediation and restoration of contaminated sites thus would be a requisite to protect public health and

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contribute to the creation of more vibrant communities. Especially due to scarcity of land in metropolitan areas bioremediation and reuse of municipal lands would be viable option that would require remediation of hazardous wastes to a level acceptable to regulatory bodies.

The major concern for large scale production and use of biosurfactants is related to the yield of biosurfactants produced, the substrates needed, and the downstream processing required. Eventually, however, biosurfactants will need to be produced in sufficient quantity and at an attractive price to compete with chemical surfactants, before they will become a major replacement for the surfactants currently used (Marchant and Banat, 2012). Thus researches need to be done on possibility of *in situ* biosurfactant production through nutrient supplementation, growth limitation and optimization of culture conditions for enhanced production.

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