BIOSURFACTANT ENHANCED BIOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBON CONTAMINATED ENVIRONMENTAL MEDIA

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ABSTRACT

Title: Biosurfactant Enhanced Bioremediation of Polycyclic Aromatic Hydrocarbon Contaminated Environmental Media

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Polycyclic aromatic hydrocarbons (PAHs) are a diverse class of toxicants that are ubiquitously and persistently present in the environment. These compounds present a risk for human health and the environment, as they are mutagens, carcinogens and teratogens. Bioremediation has shown promise as a potentially effective and low-cost treatment option, but concerns about the slow process rate and bioavailability limitations have hampered more widespread use of this technology. In the fundamental work of this thesis a series of experiments was designed utilizing the biosurfactant produced by *Pseudomonas aeruginosa* LBP5, LBP9 and CB1. Specifically, these experiments were designed to determine if the presence of various levels of partially purified biosurfactants produced by the isolates, would affect the degradation of a range of PAHs. The biodegradation and biotransformation of PAHs were studied in three bioremedial systems: soil slurry, liquid culture experiments with enriched consortium on PAHs from petroleum contaminated sites and Bioslurry reactor study with autochthonous consortium. Biosurfactant-producing and polycyclic aromatic hydrocarbon degrading microorganisms were isolated from petroleum-contaminated crane service station soil and creosote contaminated wood treatment plant soils in Pretoria area. Bacterial isolates LBP9 and LBP5 isolated from crane service station soil and isolates CB1, CN2, CN3, CN5 isolated from creosote contaminated soil were found to be the most efficient biosurfactant producing strains.

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The biosurfactant produced by the strains LBP9, LBP5 and CB1 were extracted and characterized by attenuated total reflectance Fourier transform infrared spectroscopy (ATR FTIR) and Thin layer chromatography (TLC).

Evaluation of the ability of the LBP9 biosurfactant for applications in enhancing biodegradation of mixed polycyclic aromatic hydrocarbons (PAHs) with a consortium of bacteria indicated that the biosurfactant was able to enhance the removal of significant amount of PAHs from the liquid culture medium at different concentrations. In this study at 400 mg/L amendment of lipopeptide the solubility of Phenanthrene, Fluoranthene and Pyrene was increased to 19.4, 33 and 45.4 times their aqueous solubility, respectively, and the extent of substrate utilization rate of the PAHs was enhanced up to 3 fold in the sole substrate microcosms.

A second goal of these experiments was to discern the efficacy of exogenous lipopeptide application and stimulation of *in situ* biosurfactant production through biostimulation / nutrient amendments in the removing of polycyclic aromatic hydrocarbons (PAH) from creosote PAH contaminated soil. This work also suggests that it may be more practical to stimulate indigenous biosurfactant production within a soil than to add pre-purified compound. In general, the results presented in the studies show the potential of biosurfactants in assisting the bioremediation of polycyclic aromatic hydrocarbon contaminated environmental media in a reasonable timeframe.

Key words: Bioremediation; PAH; Biosurfactant; Bioavailability; Lipopeptide; exogenous addition; *in situ* production.

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DECLARATION

I Bezza Fisseha Andualem, declare that the thesis which I hereby submit for a Masters of Engineering in Chemical Engineering degree 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.

Bezza Fisseha Andualem   Date

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LIST OF ABBREVIATIONS AND ACRONYMS

ACN Acetonitrile

ATSDR Agency for Toxic Substances and Disease Registry

B[a]P Benzo[a]pyrene

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

CHN Carbon, Hydrogen and Nitrogen elemental analysis

CSH Cell Surface Hydrophobicity

EDTA Ethylene diamine tetraacetic acid

FLU Fluoranthene

FT-IR Fourier Transforms Infrared Spectroscopy.

HMW High Molecular Weight PAHs

HOCs Hydrophobic Organic Compounds

HPLC High Performance Liquid Chromatography

IARC International Agency for Research on Cancer

K_{oc} . Organic Carbon Normalized Partition Coefficient.

K_{ow}. Octanol-Water Partition Coefficient

LMW Low Molecular Weight PAHs

LPS lipopolysachride

MFO Mixed function oxidase

NAPL None Aqueous Phase Liquid

OD Optical Density

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PAH Polycyclic Aromatic Hydrocarbons.

PCBs Polychlorinated Biphenyls

PCP pentachlorophenol

PHE Phenanthrene

POP Persistent Organic Pollutants.

PTFE Polytetrafluoroethene

PYR Pyrene

RT Retention Time

SD Standard Deviation

SOM Soil Organic Matter

TOT Total Organic Carbon

TPH Total Petroleum Hydrocarbon

MSM Mineral Salt Medium

TLC Thin layer chromatography

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LIST OF PAPERS AND CONFERENECE PRESENTATIONS

CONFERENCE PRESENTATIONS

Bezza, F. A. and Nkhalambayausi-Chirwa, E. M. (2014). Intervention Strategy To Alleviate Bioavailability Limitations Of Polycyclic Aromatic Hydrocarbon Contaminated Soil And Water Media Bioremediations. WISA 2014 Biennial Conference & Exhibition, 2014, South Africa

Bezza, F. A. and Nkhalambayausi-Chirwa, E. M. (2015). Desorption Kinetics of Polycyclic Aromatic Hydrocarbons (PAHs) From Contaminated Soil and the Effect of Biosurfactant Supplementation on the Rapidly Desorbing Fractions. The Thirtieth International Conference on Solid Waste Technology and Management, Philadelphia, PA, USA.

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CHAPTER 1

INTRODUCTION

1.1 Background

Advances in science and technology since the industrial revolution have increasingly enabled humans to exploit natural resources. However, the development and improvement of society through industrialization and urbanization comes with the cost of consistent deterioration and degradation of the natural environment through generation of toxic and hazardous pollutants. Polycyclic aromatic compounds (PAHs) are a major class of such hazardous and persistent organic pollutants, posing serious threat to terrestrial and aquatic ecosystems (Mahanty *et al.,* 2011). Thus, ever-growing demands for energy, security, food, health care and consumables have placed unprecedented pressure on our ecosystem and accentuated the need for sustainable management of the environment (Jazestani, 2011).

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 hydrophobic compounds and their persistence in the environment is chiefly due to their low water solubility (Cerniglia, 1992). Generally, PAH solubility decreases and hydrophobicity increases with an increase in number of fused benzene rings. In addition, volatility decreases with an increasing number of fused rings (Wilson and Jones, 1993).

Occurrence of PAHs in the environment is due to both natural and anthropogenic processes (Cerniglia, 1984). However, most PAHs in the environment possessing potential hazard to human health are anthropogenically produced. Natural sources are forest and rangeland fires, oil seeps, and volcanic eruptions. 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). 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 microbial degradation (Johnsen *et al.,* 2005). This persistence coupled with their ubiquitous, toxic, mutagenic, and carcinogenic

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properties (Cerniglia, 1992; Pothuluri and Cerniglia, 1998) makes PAHs pollutants of great environmental concern (Cerniglia, 1992). Concern was so great that in the late 1970s the United States Environmental Protection Agency (USEPA) listed 16 PAHs as Priority Pollutants.

Although PAH may undergo adsorption, volatilization, photolysis, and chemical degradation, microbial degradation is the major degradation process. The remediation and reclamation of land contaminated with hazardous materials has received increasing attention internationally in recent years, with enhanced awareness of the potential adverse effects on human health and the environment (Othman *et al.,* 2011). The hazards associated with the PAHs can be overcome by the use of conventional methods which involve removal, alteration, or isolation of the pollutant. Such techniques involve excavation of contaminated soil and its incineration or containment. These technologies are expensive, and in many cases transfer the pollutant from one phase to another. On the other hand, bioremediation is the tool to transform the compounds to less hazardous/non-hazardous forms with less input of chemicals, energy, and time and (Haritash and Kaushik, 2009).

Bioremediation has shown a promise as a potentially effective and low-cost treatment option, but concerns about the slow process rate and bioavailability limitations have hampered more widespread use of this technology. The success of any bioremediation technology depends on a number of factors including site characteristics, environmental factors (e.g., temperature, pH, electron acceptor, nutrients), the nature of the contamination, whether appropriate biodegradative metabolic potential present, and the bioavailability of the contaminants to degrading microorganisms within the site (Maier, 2000).

The term ‘bioavailability’ refers to the fraction of a chemical in a soil that can be taken up or transformed by living organisms (Semple *et al.,* 2004). There are several constraints that can limit the bioavailability of organic compounds in the environment. These are low aqueous solubility, sorption, and micropore exclusion (Maier, 2000). Bioavailability has been identified as a major limitation to the widespread use of many biological technologies and determining (the) factors governing the availability of pollutants for bioremediation and devising ways to increase their availability for destruction are identified as high priority areas of research (Maier,2000).

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Attempts to improve the biodegradation of PAHs in soil by increasing their bioavailability include the use of surfactants, solvents or solubility enhancers (Reid *et al.,* 2004; Yu, 2006). It is postulated that cyclodextrins may enhance the mass transfer of pollutants from soil surfaces to microorganisms, thereby promoting more rapid biodegradation of pollutants in soil (Reid *et al.,* 2004; Villaverde *et al.,* 2013). On the contrary, a study conducted by Stroud et al.(2009) showed that the introduction of hydropropyl-ß-cyclodextrin (HP-β-CD), a well-known PAH solubility enhancer, significantly increased the solubilization of PAHs although it did not improve the biodegradation rate of PAHs, HP-β-CD interfered with the microbial mineralisation of the hydrocarbons which resulted in lower mineralisation extents. Furthermore the solubilization effect of cosolvents is usually not significant for volume-fraction concentrations under 10% (Wang and Brusseau, 1993). The other option to enhance the bioavailability of PAHs is to add surfactants directly to soil *in situ* or *ex situ* in bioreactors. Both chemically and biologically derived surfactants (biosurfactants) have the potential to increase the bioavailability of PAHs via mechanisms such as emulsification of NAPLs, enhancement of the apparent solubility of the PAHs or mobilization of PAHs adsorbed to the soil (Volkering *et al.,* 1995). Surfactants have been found to enhance the degradation rates of individual PAHs in pure and mixed cultures (Luning Prak and Pritchard, 2002). 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). These compounds are usually toxic to the environment and non-biodegradable. They may bio-accumulate and their production, processes and by-products can be environmentally hazardous (Banat *et al.,* 2000). Biosurfactants have numerous advantages over their chemical counterparts such as low toxicity; low CMC, biodegradability, ecological acceptability, high selectivity, and effectiveness at extreme temperatures, pH, and salinity can be produced from several inexpensive waste substrates, thereby decreasing their production (Pacwa-Plociniczak *et al.,* 2011).

1.2 Statement of the problem

Polycyclic aromatic hydrocarbons are recalcitrant compounds which present one of the most pressing problems for biotreatment of contaminated soils or sediments, these compounds comprise a broad class of persistent contaminants in soils and sediments including petroleum, tars and

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creosotes (NRC, 1997). Bioremediation has shown promise as a potentially effective and low-cost treatment option, however, bioremediation of PAH in the environment is limited by low bioavailability to microbes because of the hydrophobicity and low aqueous solubility of PAHs and their strong adsorption to the soil material (Makkar and Rockne, 2003). Strong sorption to the solid phase has generally accounted for the inability of soil microorganisms to metabolize a significant number of PAHs in the environment (Weissenfels *et al.,* 1992). 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).

1.3 Research Hypothesis

In the current research, it is hypothesized that microbial produced biosurfcatants will: (a) enhance pseudosolubilization, desorption and mobilization of PAHs and (b) and subsequently enhance the biodegradation of the recalcitrant PAHs.

1.4 The objective of the present research

1.4.1 General Objectives;

To investigate the efficacy of biosurfactants in abating the bioavailability limitations of the recalcitrant polycyclic aromatic hydrocarbons for their effective bioremediation and to develop integrated biostimulation and bioaugmentation strategies to stimulate *in situ* production of biosurfactant and assist effective removal of these hazardous pollutants.

1.4.2 Specific objectives

- Isolate and characterize microbial strains that grow on model PAHs (naphthalene, phenanthrene, fluoranthene, and pyrene) and produce biosurfactants. Identification of these strains by analysis of the sequence of the gene encoding 16S rDNA.
- Produce biosurfactants in lab, characterize, and optimize their production.
- Perform laboratory microcosm tests to investigate the feasibility of using the biosurfactants produced from isolated microbial strains to enhance the bioremediation of PAH contaminated soil and water media.

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- ➢ To determine the optimum concentration of the the biosurfactant required to biodegrade PAHs in contaminated soils. Includes the determination of appropriate microbial kinetic models to determine growth and substrate utilization parameters.
- ➢ Develop a more effective bioremediation strategy to stimulate *in situ* biosurfactant production for the enhanced decontamination of PAH contaminated soils and water media by the indigenous population.

1.5 Scope of the study

In this study three 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, soil bioslurry microcosms.
- Develop a more effective and feasible bioremediation strategy through stimulation of *in situ* biosurfactant production through nutrient amendment and manipulation of controlling parameters.

1.6 Research Methodology

In the first experiment selective enrichment and isolation of PAH degraders and screening of biosufactant producer strains was conducted from petroleum contaminated soil on PAH (Naphthalene). Biosurfactant was produced from the selectively isolated strain and biodegradation experiments were undertaken in liquid culture and soil slurry microcosms, the microbial inoculum used in this experiment was the efficient PAH degrader and biosurfactant producer *pseudomonas aeruginosa* LBP9 monoculture.

In the second experiment microbial consortium enriched on the mixed PAHs was used in the multisubstrate biosurfactant assisted biodegradation experiment, since any one bacterial strain degrades only a limited number of PAH components, in order to achieve wide and extensive PAH biodegradation a consortium with many strains will be needed (Viñas *et al.,* 2002). This experiment investigated the biosurfactant-enhanced biodegradation of the mixed PAHs

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(Fluoranthene, Pyrene and Phenanthrene) in liquid culture media using microbial consortium from automobile garage soil in Pretoria area enriched on mixed PAHs (Naphthalene, Phenantheren and Anthracene). Enrichment cultures were established in Mineral Salt Medium containing a mixture of three PAHs (Naphthalene Phenanthrene, Anthracene) at a final concentration of 250 mg/L of each. The consortium was developed by weekly transfers in fresh medium for 5 subsequent transfers.

The third experiment was an integrated bioremediation strategy where by biosurfactant assisted bioremediation of heavily creosote-contaminated soil was conducted in bioslurry reactors using indigenous microbial consortium enriched on creosote and reintroduced in to the bioslurry reactors. Combined biostimulation and environmental specific bioaugmentation strategy were conducted to stimulate *in situ* biosurfactant production by the indigenous bacteria and enhance bioremediation efficiency. Efficacy of *in situ* biosurfactant production and removal of the monitored PAHs were evaluated in the biostimulated and biosurfactant amended microcosms.

1.7 Thesis Organization

This thesis consists of five chapters.

Chapter 1. The first chapter contains the introduction, the statement of the problem, 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 bioremediation, including the type of microorganisms and biosurfactnts 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.

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.

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

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

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CHAPTER 2

Literature Review

2.1 Background

Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic hydrocarbon compounds made up of two or more fused benzene rings, arranged in linear, angular or clustered structures (Sims and Overcash, 1983; Reda, 2009). PAHs are ubiquitous, and have been detected in animal and plant tissues, sediments, soils, air, surface water, drinking water, industrial effluents, ambient river water, well water, and groundwater (Eisler, 1989). 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).

Polycyclic aromatic hydrocarbons (PAHs) belong to the group of persistent organic pollutants (POPs). These are organic contaminants that are resistant to degradation, can remain in the environment for long periods, and have the potential to cause adverse environmental effects. PAHs have generated considerable interest, not only because of their wide distribution in the environment, but also their carcinogenic and mutagenic potential. PAHs have also been reported to have reproductive, developmental, hemato-, cardio-, neuro-, and immuno-toxicities in humans and laboratory animals (ATSDR, 1995). There are several hundred PAHs, 16 of which have been included in the U.S. EPA's list of 188 hazardous priority pollutants (U.S.EPA, 1990).

2.1.1 Sources and distribution of PAH in the Environment

PAHs originate from natural and anthropogenic sources. Natural sources of PAHs include forest fires, natural petroleum seeps, and post depositional transformation of biogenic precursors (Young and Cerniglia, 1995). There are two types of anthropogenic source of PAHs, that is, petrogenic and pyrogenic sources (Zakaria *et al.,* 2002; Wang *et al.,* 1999). Pyrogenic (pyrolytic) PAHs are generated through incomplete combustion of organic matter (e.g. coal, petroleum, wood). Pyrogenic sources include industrial operations and power plants using fossil fuels, smelting,

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waste incinerators, exhaust from vehicles powered by gasoline or diesel fuel, and forest fires, coal tars, produced by the high temperature treatment of coal (Wang *et al.,*1999). Crude oil and petroleum products contain PAHs and form another important primary source of PAHs (petrogenic source). They are introduced to the environments through accidental oil spills, discharge from routine tanker operations, municipal and urban runoff, intentional dumping of such materials as creosote, coal tar and petroleum products and so on (Kannan *et al.,* 2005; Zakaria *et al.,* 2002). Creosotes and coal tar, which are by-products of coking, contain significant quantities of PAHs e.g. creosote contains up to 85% PAHs (Cerniglia, 1984).

2.1.2 Occurrences of PAHs in the Environment

PAHs are ubiquitously distributed in the environment, and have been detected in a number of environmental samples, including air, water, soil, sediments, and also in foods and oils (Mahanty *et al.,* 2011). As noted previously, the main source of PAHs in the environment is of anthropogenic origin, namely the combustion of fossil fuels such as petroleum and coal (Bamforth and Singleton, 2005; Mahanty *et al.,* 2011). The concentration of PAHs in the contaminated environment thus depends on its proximity to the production source, level of urbanization and industrial activity, and mode of transport (Kanaly and Harayama, 2000). Gaseous and particle-bound PAHs can be transported over long distances, allowing their deposition in sediments, soils, and plants. Anthropogenic combustion of fossil fuels and long range atmospheric transport of PAHs has contributed to the dispersal of PAHs throughout the environment (Juhasz and Naidu, 2000). Important sources of PAHs in surface waters include deposition of airborne PAHs, urban storm water runoff, runoff of polluted ground sources and pollution of rivers and lakes by industrial effluents, municipal wastewater discharge, and oil spills (Wick *et al*., 2011). Since PAHs have low solubility and tend to adsorb to particulate matter, PAHs accumulate in fine grain sediments, partitioning to organic carbon-coated particles. As such, sediments may be considered as a reservoir for PAH accumulation (Juhasz and Naidu, 2000).

PAHs in soils

Among the different environments, soil is an important reservoir for PAHs (Ockenden *et al.,* 2003), and gaseous and particulate PAHs are input to the soil by wet/dry atmospheric deposition over short and long distances (Park *et al.,* 2001). Other sources include sludge disposal from public

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sewage treatment plants, automotive exhaust, tire and asphalt wear, irrigation with coke oven effluent, leachate from bituminous coal storage sites, and use of compost-based fertilizers. Levels of PAHs in soils have increased in the past 100-150 years because of growing industrial activities (ATSDR, 1995). Polycyclic aromatic hydrocarbon concentrations in urban industrial soils can be 10-100 times higher than in remote soils (Wild and Jones, 1995). Soil samples from of industrial sites, coal power and a coal tar distillation sites are usually reported to have concentration of PAH up to 3000 mg/kg of dry soil (Lors *et al.,* 2012).

2.1.3 Physicochemical Properties of PAHs

All PAHs are solids ranging from colorless to pale yellow to white (Mackay *et al.,* 1992). Polycyclic aromatic hydrocarbons are classified as low molecular weight (LMW) if they have two or three fused rings or high molecular weight (HMW) if they have four or more fused rings. LMW PAHs are degraded and volatilized more rapidly than HMW PAHs (Harvey, 1997). 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

Generally, an increase in the size and angularity of a PAH molecule results in a concomitant increase in hydrophobicity and electrochemical stability, and due to their lipophilic nature, PAH have potential for bio-magnification through tropic transfers (Harvey, 1997; Kanaly and Harayama, 2000).

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

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. Metabolism of PAHs occurs via the cytochrome P450-mediated mixed

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function oxidase system with oxidation or hydroxylation as the first step (Stegeman *et al.,* 2001). Due to their lipophilic nature, PAHs have a high potential for biomagnification through trophic transfers (Kanaly and Harayama, 2000). 16 PAHs are classified as priority pollutants by the U.S. Environmental Protection Agency, and B*a*P is included as 1 of 12 target compounds or groups defined in the Environmental Protection Agency's new strategy for controlling persistent, bioaccumulative, and toxic pollutants (Renner, 1999). Many PAHs were the first compounds known to be associated with carcinogenesis (Lee and Grant 1981).

The most common mechanism of carcinogenesis induced by PAHs is DNA damage through the formation of adducts. Alternatively, in the presence of reactive oxidative species, DNA damage can also result (ATSDR, 1995). Several PAHs such as benzo[a]pyrene (B[a]P), benzo[a]anthracene , benzo[b]fluoranthene, benzo[k]fuoranthene, chrysene, and indeno[1,2,3-c,d]pyrene, have produced carcinogenic, mutagenic, and genotoxic effects in animal experiments (Chen and Liao, 2006, Johnson *et al.*, 2009). 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). Biological effects of PAHs have also been associated with metabolic activation and elevated level of DNA adducts leading to mutation and transformation such as PAH-DNA adducts in persons who smoke or are exposed to PAH in the workplace and ambient air (Chen and Liao, 2006).

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). 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.

There are three main pathways for activation of PAHs; the formation of a PAH radical cation in a metabolic oxidation process involving cytochrome P450 peroxidase, the formation of PAH-o-

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quinones by dihydrodiol dehydrogenase-catalyzed oxidation and finally the creation of dihydrodiol epoxides, catalyzed by cytochrome P450 enzymes (Muñoz and Albores, 2011)

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. Carcinogenicity has been demonstrated by some of these epoxides (Goldman *et al.,* 2001).The most common mechanism of metabolic activation of PAHs, such as benzo[a]pyrene (B[a]P), is via the formation of bay-region dihydrodiol epoxides e.g.benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), via CYP450 and epoxide hydrolase (EH) (Muñoz and Albores, 2011).

2.1.5 Fate of PAHs in the Environment

There are a variety of mechanisms by which PAHs are degraded 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. PAHs can volatilize photolyze, oxidize biodegrade, bind to suspended particles or sediments, or accumulate in aquatic organisms (ATSDR, 1995).

2.1.5.1 Biological degradation

Biological degradation appears to be the main process responsible for the removal of PAHs in soil (Wilson and Jones, 1993). Microbial degradation of organic contaminants can occur naturally, supported by available electron donors, electron acceptors and nutrients, or through human intervention using enhanced or engineered bioremediation technologies (Scow and Hicks, 2005). Bioremediation and biotransformation harness the naturally occurring, microbial metabolic versatility such as bacteria and fungi, to transform the PAHs to other organic compounds or to inorganic end products such as carbon dioxide and water (Cerniglia, 1984). The latter process has

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been referred to as mineralization. Some 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 1984; 1992).

2.2 Bioremediation

A variety of technologies are currently available to treat soil contaminated with hazardous materials, including excavation and containment in secured landfills, vapour extraction, stabilization and solidification, soil flushing, soil washing, solvent extraction, thermal desorption, vitrification and incineration (US Environmental Protection Agency, 1988). Many of these technologies, however, are either costly or do not result in complete destruction of contamination. On the other hand, biological treatment `bioremediation' appears to be among the most promising methods for dealing with a wide range of organic contaminants, particularly petroleum hydrocarbons (Balba *et al.,* 1998).

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 (Mohan *et al.,* 2006).

Some 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 1984; 1992). In other instances PAHs are transformed by microorganisms growing on other carbon and energy sources and in these cases the products of the PAH oxidation reactions are not further assimilated by the microorganism. This process, called co-metabolism does not result in growth of the microorganism, and sometime the co - substrate, i.e. the PAH, is only transformed into another compound without any apparent benefit for the organism. Transformation can lead to complete detoxification, breakdown of products, which may be further attacked by other microbial groups, or in some cases, to more toxic metabolites (Khadrani *et al* 1999).

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2.2.1 Microbial Metabolism of PAH

2.2.1.1 General Features of PAH Degradation

There has been increasing interest in the bioremediation of terrestrial and aquatic PAH-contaminated environments. PAHs are degraded by photo-oxidation and chemical oxidation, but biological transformation is probably the prevailing route of PAH loss (Juhasz and Naidu, 2000). The recalcitrance of PAHs to microbial degradation increases directly with the molecular weight and the octanol: water partition coefficient (log K_{ow}) (Cerniglia, 1992).

A wide array of bacterial, fungal and algal species have been found to degrade PAH compounds, with bacteria constituting the most important group of degraders (Cerniglia *et al.,* 1992; Kastner *et al*., 1994). Fungi on the other hand mainly biotransform PAHs, to metabolites which can then be acted upon by other organisms (Cerniglia, 1992). Microorganisms have been found to degrade PAHs either via metabolism or cometabolism. Co-metabolism is especially important for the degradation of mixtures of PAHs. Although anaerobic metabolism is also well documented, most attention has been paid to the aerobic metabolism of PAHs and the common metabolic pathways for this, their rates of degradation and the enzymatic and genetic regulation involved are quite well understood (Samanta *et al.,* 2002).

Bacteria such as *Pseudomonas, Mycobacterium, Rhodococcus, Flavobacterium, Acinetobacter, Arthrobacter, Bacillus* and *Nocardia* are considered the primary degraders of polycyclic aromatic hydrocarbons (PAHs) in soil (Kanaly and Harayama, 2000; Chaillan *et al.,* 2004; Robertson *et al.,* 2007). *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).

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). Currently, there is only limited information regarding the bacterial biodegradation of five- and six-ring PAHs in environmental samples and pure or mixed cultures. High-molecular-weight PAHs, however, are more recalcitrant in the environment and resist both chemical and microbial degradation (Kanaly and Harayama, 2000; Cerneglia and Sutherland, 2001)

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2.2.1.2 Bacterial Degradation of PAHs

Oxidation of aromatic ring is the principal mechanism involved during PAH biodegradation (Cerniglia, 1992). The first step in the microbial degradation of PAHs is the action of dioxygenase, (Fig. 2.1) which incorporates atoms of oxygen at two carbon atoms of a benzene ring of a PAH resulting in the formation of cis-dihydrodiol (Juhasz and Naidu, 2000). The initial ring oxi-dation is usually the rate-limiting step in the biodegradation reaction of PAHs (Cerniglia, 1992). *cis*-Dihydrodiols are re-aromatised through *a* *cis*-dihy-drodiol dehydrogenase to yield a dihydroxylated de-rivative (Cerniglia, 1984). Further oxidation of the *cis*-dihydrodiols leads to the formation of catechols (Gibson and Subramanian, 1984; Juhasz and Naidu, 2000). Catechol is a key intermediate from which ring cleavage can occur. Catechol can be oxidised via two pathways. The *ortho* pathway involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield cis, cis-muconic acid. On the other hand, the *meta* pathway involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group (Juhasz and Naidu, 2000). Ring cleavage results in the production of sucinic, fumaric, pyruvic, and acetic acids and aldehydes (Fig. 2.1), 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 (Chang *et al.,* 2002; Rothermich *et al.,* 2002; Sun *et al.,* 2014).

2.2.1.3 Fungal Degradation of PAHs

Diverse group of ligninolytic and nonligninolytic fungi have the ability to oxidize PAHs. In contrast to bacteria, nonligninolytic fungi and prokaryotic algae (cyanobacteria) metabolize PAHs in pathways that are generally similar to those used by mammalian enzyme systems (Cerniglia, 1992).

The enzymes of lignin degradation (lignin peroxidase) are extracellular and have a broad substrate specificity, which makes them attractive candidates for environmental clean-up (Cerniglia and Sutherland, 2001).White rot fungi which can biodegrade or initiate the biodegradation of a wide range of pollutants by means of their extracellular, nonspecific and non-stereoselective enzyme system have received considerable attention in this respect (Cerniglia and Sutherland, 2001).

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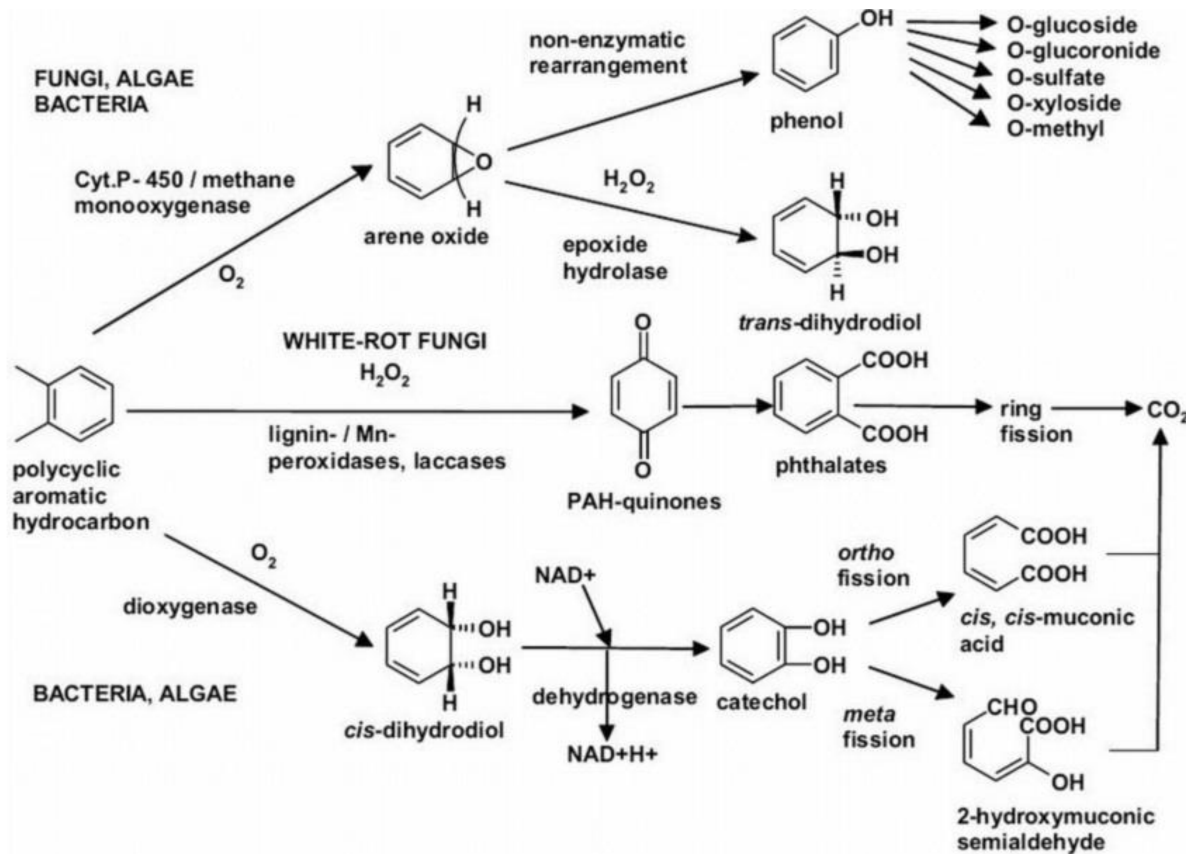

Figure 2.1: Pathways of microbial degradation of PAHs adapted from Cerneglia 1992.

Most degradative mechanisms reported are cometabolic, where an alternate carbon source is utilized for energy and growth, while the PAH is transformed as a consequence of this growth (Márquez-Rocha *et al.,* 2000). Enzymes from both fungal and mammalian systems oxidize PAHs to arene oxides by the cytochrome P-450 enzyme system by incorporating one atom of the oxygen molecule into the PAH to form an arene oxide and the other atom into water. Then (Fig. 2.1) the oxides can isomerize to yield phenols or undergo enzymatic hydration to yield trans-dihydrodiols (Gibson and Subramanian, 1984). Some ligninolytic fungi can further metabolize PAH quinones by cleaving the aromatic rings, with subsequent breakdown of the PAH to carbon dioxide (Cerniglia and Sutherland, 2001)

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2.2.2 Bioremediation technologies

Bioremediation technologies can be broadly classified as *ex situ* or *in situ* (Talley and Sleeper 1997). *Ex situ* technologies are those treatment modalities which involve the physical removal of the contaminated soil to another area (possibly within the site) for treatment. Bioreactors, land farming, composting, and some forms of solid-phase treatment are all examples of *ex situ* treatment techniques. In contrast, in situ techniques involve treatment of the contaminated material in place (Juwarkar *et al.,* 2010). *In situ* remediation techniques involve leaving the soil in its original place and bringing the biological mechanisms to the soil. *In situ* treatment of contaminated soils has the advantage of reducing cost by eliminating the need to transport the soil in which the pollutants reside, and some environmental impacts may be reduced. However, *in situ* processes may be limited by the ability to control or manipulate the physical and chemical environment in place.

2.2.2.1 *Ex-Situ* bioremediation

This method of treatment has the advantage of more control over parameters such as moisture content, temperature, and nutrient content. Treatment can be carried out more quickly once actual decontamination has begun, but the quantities of contaminated soil usually present at the sites make *ex-situ* treatment cost prohibitive, due to the costs associated not only with the remediation processes, but also with the excavation and transportation of the soil (koning *et al.,* 2000). *Ex-situ* treatment technology is further divided into slurry-phase bioremediation and solid-phase bioremediation (Pavel and Gavrilescu, 2008).

Although some contaminated sites can be controlled more easily and maintained with *ex-situ* settings, others are more effective with *in situ* techniques (Talley and Sleeper, 1997). For this reason, a decision on the selected alternative has to be made based on a variety of technical and economic factors involved in each project.

I Slurry-phase Bioremediation (Bioreactor)

Slurry-Phase bioremediation, also known as bioreactors, is a controlled treatment that involves the excavation of the contaminated soil, mixing it with water and placing it in a bioreactor (Pavel and Gavrilescu, 2008). The soil is treated in aqueous suspension, typically 10 to 30% w/v and that mechanical or pneumatic mixing is provided.

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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 (O_2, SO_4^{-2}, CO_2, NO_3^-); (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 (EPA, 2001). Some solid-phase treatment processes include land farming, soil biopiles, and composting.

Land farming: Land farming, also known as land treatment, is an *ex situ* bioremediation technique that involves the excavation of the contaminated soil and spreading it on a thin surface. Biodegradation of pollutants is stimulated aerobically by tilling or plowing the soil. It mainly focuses on stimulating the indigenous microorganisms in the soil by providing nutrients, water and oxygen (Pavel and Gavrilescu, 2008). Additional amendments or bulking agents sometimes are added to speed up the remediation process by increasing aeration, by addition of co-substrates and/or nutrients to stimulate microbial metabolism, or lime to adjust pH, or bacterial inoculations for seeding (Gomez, 2014). Although landfarming has a number of advantages, including minimal operation costs and maintenance requirements and the ability to be used to treat large quantities of contaminated sludge, there are also a number of drawbacks and limitations associated with the process (Prichard *et al.,* 2006). In general, the deliberate contamination of large tracts of land with oily sludge containing recalcitrant compounds is not environmentally acceptable (Koch, 2011).

Soil Biopiles

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This biotreatment includes the preparation of bioreactors in the form of piles or cells, allowing for the control of several factors like moisture, heat, nutrients, microbial consortia, oxygen and pH to enhance the biodegradation process. The soil is amended and piled over a piping system, which enables the supply of forced or passive air into the soil matrix, and leachate collection system for its recycle into the pile (US EPA, 2012; Gomez, 2014). Soil biopiles, is a biodegradation technique used for the remediation of excavated soil contaminated with petroleum contents. While tilling or plowing aerates land farms, biopiles are aerated by forcing air to move by injection through perforated piping placed throughout the pile (EPA, 2003; Pavel and Gavrilescu, 2008). The soil is usually mixed with a bulking agent (straws) to improve aeration and therefore enhance the growth of the microbial population. It can be engineered to be potentially effective for any combination of site conditions and/or techniques, and most of the influential parameters can be controlled (US EPA, 2012).

2.2.2.2 *In-Situ* Bioremediation

In situ processes treat soils and ground water in place, without removal. The Potential benefits of *in situ* bioremediation are its noninvasive character and its supposed cost-effectiveness. Although some sites may be more easily controlled and maintained with ex situ configurations, others are more effective with in situ treatment (Talley and Sleeper, 1997). However, *in situ* processes may be limited by the ability to control or manipulate the physical and chemical environment in place. As off-site treatments tend to be expensive, the *in situ* bioremediation processes, such as monitored natural attenuation, biostimulation, bioaugmentation and rhizoremediation, have increasingly become an attractive way to rehabilitate contaminated sites, especially those polluted by organic contaminants (Ayoub *et al.,* 2010). The competent microbial community and the whole bioremediation process of xenobiotics is influenced by a multitude of environmental parameters such as temperature, the availability of oxygen (or an alternative electron acceptor), the type and concentration of nutrients, salinity, pressure, water activity, pH and process-inhibiting co-contaminants on the site as well as the chemical composition, physical state, concentration, availability and toxicity of the target contaminant. If any of these factors is suboptimal or absent at the field site, the success rate of applied bioremediation may decrease (Nõlvak, 2012). The provision of the amount of O_2 needed to biologically degrade hydrocarbons is one of the major problems of *in situ* clean up using biological methods. The oxygen may be introduced as air, liquid

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oxygen, or (in) organic peroxides (Cuypers, 2001). One method to address this problem is bioventing, forced aeration of the soil matrix to supply O_2 for biodegradation (Lee *et al.,* 2006). Recognized limitations are physico-chemical constraints (bioavailability, desorption kinetics), geological constraints (permeability, vertical and horizontal conductivity, water depth), and treatment times. Moreover, the systems may be very heterogeneous and difficult to monitor. As a consequence, effective and complete treatment is difficult to ascertain (Cuypers, 2001).

2.2.3 Factors Affecting Biodegradation Rate of PAHs

Several factors influence the rate of PAHs biodegradation and their degree of persistence (Cerniglia 1984; Alexander, 1999) among which are the existence of a 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 1984, 1992; 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.

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

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

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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).

2.2.3.1 The Effect of Environmental Factors on PAHs Bioremediation

Many studies have investigated the efficacy of bioremediation on a bench scale and under ideal laboratory conditions, such as neutral pH and mesophilic temperatures. However, it is apparent that environmental factors that vary from site to site (such as soil pH, nutrient availability) and the bioavailability of the contaminant can influence the process of bioremediation by inhibiting growth of the pollutant-degrading microorganisms. Generally speaking, temperature, acidity/alkalinity (pH), oxygen, nutrients, light irradiation, and bioavailability are considered the most common relevant factors affecting PAHs bioremediation (Bamforth and Singleton, 2005).

2.2.3.2 Bioavailability factors

The efficiency of the biological treatment of soils containing hydrophobic organic contaminants is highly limited by contaminant bioavailability. The term ‘‘bioavailability’’ of soil pollutants refers to the fraction of a chemical that can be taken up or transformed by living organisms (Semple *et al.,* 2003). Polycyclic aromatic hydrocarbons (PAHs), interact with organic matter and their different fractions through mechanisms such as physical and chemical adsorption, affecting their mobility and bioavailability through the soil (Šašek *et al.,* 2003).

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.

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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). Allowing a pollutant to degrade via intrinsic bioremediation (where the target pollutant is biodegraded without intervention) is usually a slow process. Consequently, enhanced bioremediation, involving either biostimulation or bioaugmentation is increasingly being investigated (Juhasz and Naidu, 2000).

2.2.4.1 Bioaugmentation

Bioaugmentation is bioremediation strategy, which improves the biodegradative capacities of contaminated sites by introduction of single or consortia of microorganisms with desired metabolic capabilities (Mrozik and Piotrowska-Seget, 2010). 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 fold (Mohan *et al.,* 2006). 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). The process of bioaugmentation may use degrader organisms, or addition of biosurfactant-producing bacteria. (El Fantroussi and Agathos, 2005). In some cases, it may be possible to stimulate *in situ* production of biosurfactants through the addition of nontoxic organic materials or culture of plants that enhance the growth and activity of biosurfactant-producing bacteria (Nõlvak, 2012).

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).

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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).

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2.2.4.2 Biostimulation

Biostimulation is one important strategy which has been used extensively for enhancing the bioremediation of PAHs in soil (Mohan *et al.,* 2006). 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). Nutrients (i.e. nitrogen, phosphorus) often become limiting factors, especially when the contaminant functions as a C source (i.e. petroleum products) (Nõlvak, 2012). 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). If the ratio of organic C: N: P is higher than about 300:15:1, supplemental nitrogen and or phosphorous should be added. However, overloading of nitrogen (e.g. C: N = 1.8:1) can impair biodegradation, possibly due to ammonia toxicity (Zhou and Crawford, 1995). Numerous types of amendments such as inorganic fertilizers, waste water sludge, sewage sludge compost, municipal solid waste compost, and manure and biosolids have been utilized to enhance the degradation of petroleum products in the subsurface of contaminated sites (Nõlvak, 2012).

2.2.4.3 Increasing bioavailability of PAHs.

It is evident that PAH bioavailability is limited by low solubility 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).

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2.2.5 The Factors that affect PAH bioavailability

Factors that affect PAH bioavailability can be categorized into three catagories (Harmsen, 2007).

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

2.2.6.1. Effects of physical and chemical properties of PAHs on bioavailability

The aqueous solubility, and as a consequence, the bioavailability of PAHs decrease almost logarithmically with increasing molecular mass (Mohan *et al.,* 2006). PAHs possess physical properties, such as low aqueous solubility and high solid–water distribution ratios, which stand against their ready bioavailability and microbial utilization (Johsen *et al.,* 2005). The high partitioning to the solid phase and low solubility of these compounds results in low bioavailability to the organism(s) which is the most important factor responsible for slow biodegradation of the PAHs (Makkar and Rockne, 2003).

2.2.6.2. Effects of soil or sediment properties 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 interactions are considered to be the major rate-limiting factors in field scale applications. PAHs may be strongly adsorbed onto soil particles, especially clays and desorption of PAHs from soil is considered to be a controlling factor in their biodegradation. Low proportions of clay and silt in soil have been correlated to higher PAH bioavailability (Mohan *et al.,* 2006). PAHs can undergo rapid sorption to mineral surfaces (i.e. clays) and organic matter (i.e. humic and fulvic acids) in the soil matrix (Delle, 2001).

2.2.6.3 Effects of PAH residence time (aging) on PAHs bioavailability in soil.

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 partitioning, adsorption, chemisorption, and

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covalent binding between PAHs and SOM are believed to be responsible for the decline in their degradation over time (Mohan *et al*, 2006). 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)

2.2.5 Enhancing bioavailability of PAHs using surfactants

Surface-active compounds may be used to increase the bioavailability of otherwise poorly accessible carbon sources, thus helping to overcome the diffusion-related mass transfer limitations (Singh *et al.,* 2007).The addition of a surfactant to a contaminated soil can reduce the interfacial tension thus increasing the mass transfer of the contaminants (Franzetti *et al.,* 2008). Several researchers have shown that various surfactants can enhance desorption (Xu *et al.,* 2006), solubilisation (Prak and Pritchard, 2002) and biodegradation of organic compounds (Thavasi *et al.,* 2011a; Xia *et al.,* 2014).

2.2.5.1 Characteristics and properties of surfactants

Surface active agents, or surfactants, are chemical compounds that have the potential to alter the properties of fluid interfaces. The surfactant molecule is typically composed of a strongly hydrophilic (water loving) group, or moiety, and a strongly hydrophobic (water fearing) moiety (West and Harwell, 1992). The entire surfactant monomer is often referred to as amphiphilic because of its dual nature. The surface activity of surfactants derives from their amphiphilic structure moiety (West and Harwell, 1992). The hydrophilic group of the surfactant monomer provides most surfactants with a high solubility in water; the hydrophobic group of the monomer, however, prefers to reside in a hydrophobic phase. These competing effects result in the accumulation of surfactant monomers at the interfaces, with the hydrophobic tail group embedded in the None-aqueous phase and the hydrophilic head group oriented toward the water phase (volkering *et al.,* 1997). The structural features of amphiphiles confer them the ability to concentrate and alter the conditions at interfaces, such as the ability to lower surface and interfacial tension of liquids and increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or insoluble organic compounds (Franzetti *et al.,* 2010a; Xia *et al.,* 2014).

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

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to the surface (Desai and Banat 1997). For example, a good surfactant can lower the surface tension of water from 72 to 35 mN/m and the interfacial tension (tension between non-polar and polar liquids) for water against n-hexadecane from 40 to 1 mN/m (Mulligan, 2005). The surface tension correlates with the concentration of the surface-active compound until the critical micelle concentration (CMC) is reached (Fig. 2.3).

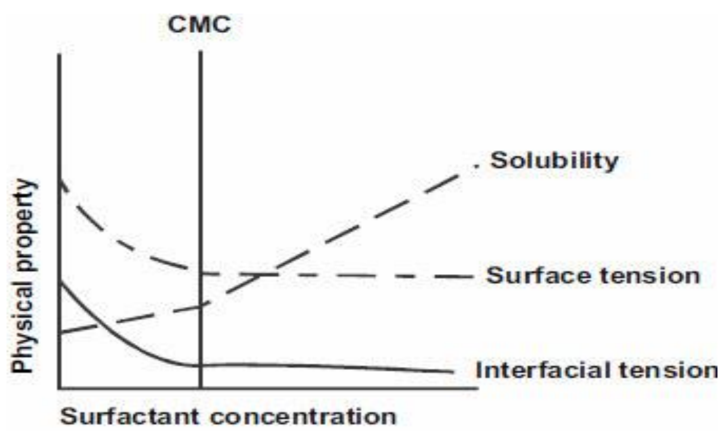

Figure 2.3: Relationship of surface tension, interfacial tension and the CMC with surfactant concentration (Mulligan, 2005).

2.2.5.2 Critical micelle concentration (CMC);

Fundamental property of surfactants is the ability to form micelles which is responsible for the excellent detergency and dispersing properties of these compounds (Franzetti *et al*, 2010). As surfactants are added to aqueous solution, they will tend to accumulate at fluid-fluid and fluid-solid interfaces. Once a sufficient amount of surfactant has been added to aqueous solution, however, aggregations of surfactant monomers referred to as micelles will form. As shown in Figure 2.3, the threshold concentration at which micelles begin to form is termed the Critical Micelle Concentration (CMC). The presence of micelles leads to an increase in the apparent solubility of HOCs; this is also referred to as 'solubilisation' (Volkering *et al.,* 1997, West and Harwell, 1992). Micelles are capable of dissolving hydrophobic contaminants in their hydrophobic core, which results in an increased apparent aqueous solubility of the pollutants (Edwards *et al.,* 1991; Prak and Pritchard, 2002).

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At concentrations above the CMC, 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).

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)

2.3 Types of surfactants

2.3.1 Synthetic Surfactants

Synthetic surfactants have been shown to remove nonpolar compounds from surfaces but problems can be associated with their use (Christofi and Ivshina, 2002) such as reduced availability of compounds sequestered into micelles, their toxicity, and ultimate resistance to biodegradation leading to increased pollution (Mulligan *et al*., 2001). Micellariazation of contaminants by synthetic surfactants has been shown to inhibit biodegradation at concentrations at or above their CMC as it may prevent microbial access to the contaminants (Willumsen *et al.,* 1998). Indeed, many synthetic surfactants are known to exert an inhibitory effect on PAH-degrading microorganisms (Tsomides *et al.,* 1995).This may be a function of the toxicity of the surfactant to degrading microorganisms through increased permeabilization and disruption of cell membranes (Willumsen *et al.,* 1998); toxicity of increased soluble fractions of the hydrophobic toxicant; prevention of microbial contact with the toxicant (Neu,1996); unavailability of micelle trapped toxicant; or competitive substrate utilization (Tiehm, 1994; Christofi and Ivshina, 2002). These compounds are usually toxic to the environment and non-biodegradable. They may bio-accumulate and their production, processes and by-products can be environmentally hazardous (Banat *et al.,* 2000). Therefore, with current advances in biotechnology, attentions have been paid to the alternative environmental friendly processes for production of different types of biosurfactants from microorganisms (Lotfabad *et al.,* 2009).

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2.3.2 Biosurfactants

Biosurfactants are a range of structurally diverse molecules produced by different microorganisms. Their hydrophilic moiety is mainly comprised of an acid, peptide cations, or anions, mono-, di- or polysaccharides while their hydrophobic moiety can be an unsaturated or saturated hydrocarbon chains or fatty acids (Banat *et al.,* 2010). They are mainly classified by their chemical structure and their microbial origin and molecular mass. Most are either anionic or neutral, while only a few with amine groups are cationic. 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.,* 2010a).

Biosurfactants (Microbial surface-active compounds), have numerous advantages over their chemical counterparts ,they offer several advantages over synthetic surfactants ;low toxicity, low CMC, biodegradability, ecological acceptability, high selectivity, and are effective at extreme temperatures, pH, and salinity (Płociniczak *et al.,* 2011). They have a 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). Synthetic surfactants are readily available and of (relatively) low cost, so are extensively utilized in remediation processes of contaminated water or soil (Bustamante *et al.,* 2012). On the other hand, the low yields and high costs that can be incurred in the production of biosurfactants (Mukherjee *et al.,* 2006; Banat *et al.,* 2010) have restricted their use.

2.3.3 Microorganisms and Characteristics of Biosurfactants Produced/ Classification

These compounds can be roughly divided into two main classes (Neu 1996); low-molecular-weight compounds called biosurfactants, such as lipopeptides, glycolipids and high-molecular-weight polymers of polysaccharides, lipopolysaccharides proteins or lipoproteins that are collectively called bioemulsans (Rosenberg and Ron1997) or bioemulsifiers (Smyth *et al.* 2010). The former group includes molecules which efficiently lower surface and interfacial tension, while the latter is composed of amphiphilic and polyphilic polymers which are more effective in

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stabilizing oil-in-water emulsions but do not lower the surface tension as much (Franetti *et al.*, 2010a). The low-molecular-weight biosurfactants are generally glycolipids, such as rhamnolipids, trehalose lipids, sophorolipids and fructose lipids, or lipopeptides, such as surfactin. The high molecular-weight bioemulsifiers are amphiphilic or polyphilic polysaccharides, proteins, lipopolysaccharides and lipoproteins (Perfumo *et al.,* 2010).

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

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2.3.4 Influence of biosurfactants on the bioavailability of hydrophobic organic compounds

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).Thus, microbial growth on hydrocarbons can be limited by the interfacial surfaces leading to a linear growth rather than exponential one. 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). Such properties play an important role in various fields like bioremediation, biodegradation, oil recovery, food, pharmaceutics, and many other applications in different industrial sectors (Banat *et al.,* 2010; Banat *et al.,* 2014). Addition of biosurfactants can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (Pacwa-Płociniczak *et al.,* 2011).

The mobilization mechanism occurs at concentrations below the biosurfactant CMC. At such concentrations, biosurfactants reduce the surface and interfacial tension between air/water and soil/water systems (West and Harwell, 1992). Due to the reduction of the interfacial force, contact of biosurfactants with soil/oil system increases the contact angle and reduces the capillary force holding oil and soil together. In turn, above the biosurfactant CMC the 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).

2.3.5 Bioremediation mechanisms

2.3.5.1 Emulsification

For bacteria growing on hydrocarbons, the growth rate can be limited by the interfacial surface area between water and oil. When the surface area becomes limiting, biomass increases

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arithmetically rather than exponentially (Shreve *et al.,* 1995). 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.,* 2010a). 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 (Franzetti *et al.,* 2010b).

2.3.5.2 Micellization

Surfactant-enhanced remediation (SER) is suggested as a promising technology that has recently been extensively exploited for the remediation of organic contaminated soil and water (groundwater or surface water) (West and Harwell, 1992; Paria, 2008). Surfactants are present as dispersed molecules (monomers) at low concentrations, while above their critical micelle concentration (CMC) the monomers aggregate in solution to form micelles consisting of a hydrophobic core and a hydrophilic shell. The formation of micelles can partition hydrocarbon into the hydrophobic micellar core with increased apparent aqueous solubility (Volkering *et al.,* 1998). 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.,* 2010a).

2.3.5.3 Regulation adhesion/de-adhesion of microorganisms to and from hydrocarbons

A proposed role of biosurfactants in hydrocarbon uptake is the regulation of cell attachment to hydrophobic and hydrophilic surfaces by exposing different parts of cell bound biosurfactants, thus changing cell-surface hydrophobicity (Franzetti *et al.,* 2010a). This natural role can be exploited by adding (bio) surfactants to increase the hydrophobicity of degrading microorganisms and to allow cells' easier access to hydrophobic substrates (Shreve *et al.,* 1995). 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). Further investigation of the mechanism of interaction of rhamnolipid with the *Pseudomonas* cell surface has revealed that

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the rhamnolipids cause a loss of LPS (Al-Tahhan *et al.,* 2000). The loss of LPS results in an increases in the relative hydrophobicity of the cell. Researches suggest that this mechanism may be more important for *in situ* bioremediation of hydrocarbons (Maslin and Maier, 2000; Maier and Soberon-Chavez, 2000).

2.3.6 Screening of Bio surfactant Producing Microorganisms

The principal aim in screening for new biosurfactants is finding new structures with strong interfacial activity, low critical micelle concentration (CMC), high emulsion capacity, good solubility and activity in a broad pH-range. A complete strategy for screening of new biosurfactants or producing strains consists of three steps: sampling, isolation of strains and investigation of strains (Walter *et al.,* 2010).

Isolation

For the screening of biosurfactant producing microbes, enrichment cultures utilizing hydrophobic compounds as the sole carbon source are applied. This is an indirect screening method as the growth on hydrophobic compounds indicates the production of biosurfactants, but not always correlates with this trait (Walter *et al.,* 2010). Sampling of contaminated sites combined with direct isolation or enrichment culture is an approved strategy for discovering new biosurfactant producing strains (Walter *et al.,* 2010).

Screening Methods

The methods for a general screening of biosurfactant producing strains are based on the physical effects of surfactants. The screening methods can give qualitative and/or quantitative results. For a first screening of isolates, qualitative methods are generally sufficient. The following methods are rapid and easy to carry out.

Drop collapse test

In the drop collapse method, 2 µl of mineral oil was added to each well of a 96-well micro titer plate lid. The lid was equilibrated for 1 h at room temperature, and then 5 µl of the culture was

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added to the surface of oil (Bodour and Miller-Maier, 1998). The shape of the drop on the surface of oil was inspected after 1 min. If the drop remained beaded; the result was scored as negative. If the drop collapsed, the result was scored as positive. Cultures were tested in triplicate. Distilled water and MSM without inoculation were used as negative controls

Oil Spread test

For the oil spreading technique, 50 ml of distilled water was added to a large petri dish (25 cm diameter) followed by the addition of 20 µL of crude oil to the surface of the water. Ten microliters of culture were then added to the surface of oil (Morikawa *et al.,* 2000). The diameter of the clear zone on the oil surface was measured and related to the concentration of biosurfactant. Cultures were tested in triplicate. Distilled water and MSM without inoculation were used as negative controls

2.4 Biosurfactant Production

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

2.4.1 Kinetics of Fermentative Production

Depending upon the nature of the biosurfactant and the producing microrganisms, the following patterns of biosurfactant production by fermentation are possible: (a) growth-associated production, (b) production under growth limiting conditions, (c) production by resting/nongrowing cells, and (d) production with the precursor augmentation. In the case of growth-associated biosurfactant production, there exists a parallel relationship between the substrate utilization, growth and biosurfactant production (Desai and Banat, 1997; Rodrigues et al., 2006). In the growth-associated case, there is a direct link between biosurfactant production and cell multiplication, and

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production stops when cells cease multiplying. Production under growth-limiting conditions occurs only when growth is limited by one or more nutrients (e.g., nitrogen or iron). In the case of production by resting cells, microbes use the carbon source to produce biosurfactants without multiplying. Production with precursor addition describes the stimulation of biosurfactant production by adding surfactant precursors in the medium.

2.4.2 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, phosphor, magnesium, ferric, and manganese ions in the culture medium and the culture condition was well as pH, temperature, agitation and dilution rate in continues culture (Guerra-Santose *et al.,* 1986; Desai and Banat, 1997; Nitschke *et al.,* 2005).

2.4.2.1 Nutritional Factors Affecting biosurfactant Production

Carbon Source

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 RLs. When *Arthrobacter paraffineus ATCC* 19558 was grown on D-glucose, supplementation with hexadecane in the medium during the 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; Al-Araji *et al*., 2007).

Three mechanisms, namely, induction, repression, and nitrogen and multivalent ions, operate in the regulation of biosurfactant production (Banat and Desai 1997). 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

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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. On the contrary catabolic repression of rhamnolipid production was observed when applying glucose, acetate and tricarboxylic acids (Hauser and Karnovsky 1957).

Nitrogen, Minerals, and Iron Sources

Medium constituents other than carbon source also affect the production of biosurfactants. 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). (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

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 RL productivity. Sobez-Chávez *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). Guerra-

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Santos et al.(86) 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 .

2.4.2.2 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).
Syldatk et al. (1985) showed that resting cells of *Pseudomonas sp.*DSM 2874 produced up to 15 g/L of different rhamnolipids in simple phosphate buffer or a sodium chloride solution with optimal pH value in the range 6 – 7.2. While rhamnolipid formation with glycerol as the sole C-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.

2.4.3 Recovery of biosurfactants

Downstream processing can represent a significant proportion of the final cost of production of biosurfactants and the criteria that govern the selection of a specific recovery method include (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,
(3) The adaptability of the method to a particular industrial fermentation process.

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 biosurfactants are protonated and become insoluble in water, thus precipitate. Yields of up to 98% were reported for acid precipitation after cell removal and subsequent heat treatment (Mixich *et al.* 1997). This aims to obtain aqueous culture medium-free crude extract. However, at this pH other molecules also precipitate. Therefore precipitation is generally followed by organic solvent extraction methods. 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

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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.

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CHAPTER 3

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 10 μg/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. Stock solutions of individual PAHs (phenanthrene, fluoranthene and pyrene) were prepared in acetone for liquid culture or soil experiments at (1 mg/mL). PAHs for mixed substrate experiments (phenanthrene, fluoranthene and pyrene, were prepared at 4 mg/mL each). PAH stock solutions were stored at 4 °C in darkness.
2. The 16 PAHs or individual PAHs Calibration standard solutions were prepared by suitable dilution of the PAH Standard Stock Solutions with acetonitrile.
3. Standard working solutions was prepared by diluting 0.5 mL of a 10 μg/mL PAHs standard stock solution to 10 mLwith acetonitrile or acetone.
4. Biosurfactant Stock Solutions. Stock solutions of the biosurfactants (Lipopeptides as identified later), were prepared (2 mg/mL) from the extracted lipopeptides by dissolving the brownish

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lipopeptide recovered in Mineral Salt Medium (MSM) and heating for 10 minutes at 50°C. Stocks were then stored at 4°C.

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/L 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

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 4°C.

3.3 Microbiological Methods

3.3.1 Enrichment and isolation of PAH Degrading Bacteria

Samples of contaminated soil were collected from petroleum contaminated crane service station soil that has been contaminated by spilt oil for decades around Pretoria. The enrichment and

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isolation of the PAH-degrading microbial consortium was performed by using Naphthalene as the sole carbon and energy source. The Naphthalene was dissolved in acetone and added to the MSM 12 hrs before the inoculation to get the acetone evaporated not to harm the consortium

Initially, the bacterial consortium was enriched by adding 5g of soil sample to 150 mL of mineral salt medium (MSM) in a 250-mL Erlenmeyer flask containing 250 mg/L Naphthalene added from the stock solution. The flask was shaken in an orbital shaker (150 rpm) at 30°C for 5 days for microbial enrichment.

After 5 days, an aliquot of 10 ml enriched culture was inoculated into another 250-ml conical flask containing 10 mL MSM with 250 mg/L PAH (Naphthalene) for the first enrichment. Five consecutive enrichments were carried out under the same condition to enrich a PAH-degrading microbial consortium. The bacterial colonies were isolated by streaking the enriched consortium on nutrient agar plates containing naphthalene powdered grains as a carbon and energy source provided in a vapour form put on the lid. Morphologically distinct colonies were re-isolated by transfer onto naphthalene containing agar plates at least three times to obtain pure cultures. Pure cultures were stored at −80°C in MSM mixed with sterile glycerol at a final concentration of 30%.

3.3.2 Screening and identification of biosurfactant-producing bacteria

3.3.2.1 Screening for biosurfactant producing isolates

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

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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.

- 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). It depends on principle that a drop of liquid containing a bio-surfactant will collapse completely over oil surface. 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.

3.3.2.2 Characterization of the microorganisms

After the screening for biosurfactant producing isolates 5 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. 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 -70°C before use.

3.4. Biosurfactant Production

3.4.1 Culture Media for biosurfactant production

The organism was grown on the mineral salt medium (MSM) (Trummler *et al.,* 2003) with some modification, changing the nitrogen source to NH_4NO_3 with equivalent molar mass of nitrogen.

3.4.2 Methods of biosurfactant production

3.4.2.1 Preparation of Culture

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

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to a 1000 ml Erlenmeyer flask containing 500 ml of MSM supplemented with glycerol (4%, w/v) and incubated for 72h at a shaker speed of 150 rpm. After growth for 72h (lowest surface tension was recorded at 72h), 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. The acid precipitate was recovered by centrifugation (12,000 rpm for 15 min at 4°C) and was further 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 mm filter (Millipore).

3.4.2.2 Preparation of Biosurfactant

For the induction of biosurfactant overproduction in the experimental setup an appropriate limitation must be achieved. For this purpose the limitation of nitrogen (nitrogen free set up) is conducted. Two-step process was developed according to (Trummler *et al.,* 2003) for optimized production of biosurfactant using resting *Pseudomonas aeruginosa* cells. The actual rhamnolipid production was preceded by a culture for cell material production. This step was performed in shaking flasks with growth medium containing the carbon source.

The biosurfactant production step was performed in another 2L flask with the production medium under nitrogen free (no nitrogen source) growth limited production medium. Two culture media produced one for growth of the organisms, and the other one nitrogen free media for the growth limited overproduction of biosurfactant.

Growth medium was composed of 3.63 g/L $(NH_4)\ NO_3$; 0.4 g/L MgSO4 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/L of 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. Growth limited (Nitrogen-free) medium has the same composition as the growth medium, except that no $(NH4)_2SO_4$ was added. All media were adjusted to pH 7.

3.4.2.2.1 Culture of *Pseudomonas aeruginosa* for cell mass production

A preculture was set up with 20 ml of growth medium supplemented with 2% (v/v) carbon source, Glycerol. The media was inoculated with 1 ml of *Pseudomonas aeruginosa* and glycerol and

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incubated for 18 h at 30 °C, 120 rpm. Shaking flasks, containing a total of 1.5 L of growth medium with 10% (w/v) carbon source, were inoculated with a total of 15 ml of the preculture and shaken for 72h at 30°C, 120 rpm. Cultures were adjusted to pH 6.5. Cell material was eventually harvested by centrifugation (10000 rpm, 10 min). To be used for the subsequent production by resting or immobilized cells production strategy. The free-cell culture medium was checked for surface activity and oil displacement test.

3.4.2.2.1 Production of biosurfactant by *Pseudomonas aeruginosa* in shaking flasks

The desired cell mass was suspended in a suitable volume of production medium. Final concentration of wet biomass did not exceed 5% (w/v). Glycerol and hexane carbon sources were added to the cell suspension, and incubated at 37°C, 120 rpm; and checked for optimum production.

Periodically aseptic removal of samples to monitor surface tension is carried out. The initial surface tension of the medium was 72 mN/m. The surface tension of the medium started to decrease, at the third day the surface tension of the reached ~ 35 mN/m. The biosurfcatnt was harvested on the fifth day.

3.4.3. Surface Tension and Emulsification Index (E_{24}) Measurements

3.4.3.1 Surface tension and Critical micelle concentration (CMC) measurements

The surface tension of the culture broth supernatant and biosurfactant solution was measured according to the Ring method as previously described (Rodrigues et al., 2006). Using a Du Nouy tensiometer (KrüssTensiometer, K11 model - Germany), equipped with a 1.9 cm platinum ring. Surface tension values represent the average of three independent measurements performed at room temperature (25°C). For the calibration of the instrument, the surface tension of the pure water was measured before each set of experiments.

Biosurfactant concentration, expressed in terms of critical micelle dilution (CMD), was estimated by measuring the surface tension for varying dilutions of the sample. When measuring the CMD the cell free broth were diluted 10 times CMD^{-1} or 100 times CMD^{-2} with Phosphate buffer solution (10 mMKH_2PO_4/K_2HPO_4 and 150 mM NaCl with pH adjusted to 7.0) and the surface tension of each sample was measured as described above. The surface tension versus biosurfactant

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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 critical micelle concentration (CMC) is determined after 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).

3.4.3.2 Measurement of Emulsification Index (E_{24})

To determine the emulsification index a mixture of 4 ml supernatant and 4 mL of hexane was vortexed for 5 minutes and the height of emulsion layer was measured after 24 h. The emulsification index (E_{24}) was calculated by dividing the measured height of the emulsion layer by the total height of the mixture and multiplying by 100 (Cooper and Goldenberg, 1987).

3.4.4. Biosurfactant Recovery

Cells were removed from the culture broth by centrifugation at 10000 rpm, for 15 min. After the removal of the bacterial cells by the centrifugation, to remove residuals it was filtered with Millipore membrane filter. The clear sterile supernatant served as the source of the crude biosurfactant. The biosurfactant was recovered from the cell free culture supernatant by cold acetone precipitation or acid precipitation alternatively.

1. Cold acetone precipitation. Three volumes of chilled acetone was added and allowed to stand for 10 h at 4°C. The precipitate was collected by centrifugation and Acetone was evaporated and biosurfactant was collected and weighted (Pruthi and Cameotra, 2003). About 20 g of the crude biosurfactant was extracted per liter of culture medium.

2. Acid Precipitation. After growth for 72h (lowest surface tension was recorded at 72 h), 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. The acid precipitate was recovered by centrifugation (12,000 rpm for 15 min at 4°C) and was further 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 mm filter (Millipore). The crude extracts were further purified through a silica gel column, Silica gel (60–200; Merck KGa) column

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3.4.5. Chemical Characterization of Biosurfactant

3.4.5.1 Fourier transforms infrared 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 4,000–400 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 ℃ 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 on 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 °C for 5 min (Xia *et al.,* 2014).

3.5 Biosurfactant Assisted Mass Transfer Experiments

3.5.1 Phenanthrene Fluoranthene and Pyrene solubilisation Experiments

The aqueous solubility experiment of PAHs was determined in the presence and the absence of the biosurfactant. For each experiment, 20 mg of Phenanthrene, Fluoranthene and Pyrene each was dissolved in acetone and added to 100 mL flasks. The solvent was allowed to evaporate overnight, leaving a thin coating of the PAHs covering the bottom of the flasks.

100 mL of MSM with different conc. of lipopetide (0, 100,150, 200, 400,800, 1600 mg/L) was added to the flasks from the lipopetide stock solution. The flask was placed in a gyratory shaker (120 rpm) maintained at 30 °C. After 24 hs, and 48 hrs triplicate 25-mL samples were removed from each flask using a pipette. This liquid was filtered through a 0.22 µm polytetrafluoroethene

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(PTFE) syringe filters to remove undissolved crystals , extracted with hexane , evaporated and the residue is resuspended with equal volume of Acetonitrile (exchanged with mobile phase medium) and filtered into 2 mL amber vials. The amount of phenanthrene in the solution was determined by high-performance liquid chromatography (HPLC).

3.5.2 Batch Desorption Study

Soil was collected from a pristine supply and was sieved to < 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) 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. The supernatant was drained off and the soil samples were air dried 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 of the soil.

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3.6 Microbial Degradation Experiments of 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). PAH concenttation in liquid and soil cultures were determined in triplicate by HPLC (Section 2.8) after extraction with Hexane / acetone. Microbial growth was established by an increase in microbial numbers as determined by the Standard Plate Count method (Section 2.4.9).

3.6.1 Biosurfactant-enhanced biodegradation of phenanthrene by *Pseudomonas aeruginosa* LBP9 isolate.

3.6.1.1 Biodegradation of Phenanthrene in liquid culture system.

For the Phenanthrene degradation experiments, which were conducted in triplicate, 10 mL of phenanthrene from the stock solution (described in section 3.1.2) was added to 100 mL flasks. The solvent was allowed to evaporate overnight, leaving a thin coating of phenanthrene covering the bottom of the flasks. MSM (50 mL) were added in each flask to achieve a final PAH concentrations of 200 mg/L. Each set of samples was inoculated with 1 mL aliquots of the phenanthrene degraders from late-log precultures to achieve a final cell density of (10^7 CFU mL^{-1}) approximately optical density (OD) of 2 and different concentrations of Lipopeptide. Lipopeptide biosurfactant produced by the isolate *Psuedomonas aeruginosa* LBP9 was used in the biodegradation experiments.The flasks were sealed with Aluminium foil caps and incubated at 30 °C on a rotary shaker at 150 rpm. Periodically, triplicate flasks were sacrificed to determine the amount of Phenanthrene remaining using the following procedure. Biosurfactant produced by the isolate was used in Creosote contaminated soil biodegradation experiment. The flasks were then extracted with 100 mL of hexane twice, centrifuged at 6500 rpm for 6 mins to remove emulsifications during solvent extraction. The solvent was pooled and evaporated. The residual Phenanthrene was dissolved in equal volume 50 mL of acetonitrile (exchanged to mobile phase medium), and the samples were HPLC analysed. The amount of Phenanthrene in the resuspended solvent was quantified by reversed phase high-performance liquid chromatography using a linear gradient elution condition

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(section 3.8.2a). All tests were conducted in triplicate with uninoculated controls to monitor the volatilization losses and total recovery of contaminants.

3.6.1.2 Biodegradation of Phenanthrene in Bioslurry System

Degradation trials in soil slurries were conducted in 100-mL Erlenmeyer flasks which were capped with aluminium foil. 5gm of 800 mg/kg Phenanthrene spiked soils were added to the flasks along with 50 mL of liquid media at 10% (w/v), with addition of different concentration of biosurfactant (0,400 mg/L, 700 mg/L). Each set of samples was inoculated with 1 mL aliquots of the PAH degraders from late-log precultures to achieve a final cell density of (10^7 CFU/mL) approximately optical density (OD) of 2 and different concentrations of Lipopetide. On days 2, 4, 6, 8, 10, 12 and 14 samples were collected from the flasks and Phenanthrene concentration in the aqueous and soil media was determined. To determine the amount of Phenanthrene in solution the aqueous phase was separated from the soil by centrifugation at 5000 rpm for 20 min. 10 mL of the supernatant was sampled, extracted with hexane and the hexane was evaporated, the residue is resuspended in acetonitrile and filtered through 0.22 µm syringe filter. Phenanthrene in solution was analyzed by HPLC Waters 2695 C_{18} reverse phase column (250 × 4.6 mm, 5 µm). To determine the Phenanthrene in the soil, the soil was air dried and extracted in ultrasonic bath (USEPA methods 3550C). Five grams of soil extracted with 30 mL of solvent hexane; acetone (1; 1) trice and pooled. Acetone; hexane (1;1) was added to the sample which was vortexed for 2 min and Phenanthrene extracted for 1 h in a Sonic Bath. Samples were centrifuged for 10 min at 10,000 rpm, the hexane was evaporated, and the residue is resuspended in acetonitrile and filtered through 0.22 µm syringe filter. Phenanthrene in solutions was analysed by HPLC system with a slightly modified EPA Method 8310 (EPA 1986).

3.6.2 Biosurfactant-assisted mixed PAHs degradation by PAH-degrading microbial consortium.

The soil used for enrichment of PAH- degrading microbial communities was collected from automobil garage and car service station at Pretoria, South Africa. The soil had been exposed to oil spills for several years. A total of five subsequent enrichments were carried out to enrich PAH degrading Bacterial consortium to be used for the liquid culture and soil experiments (according to Section 3.3.1).

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The PAH mixture of Naphthalene (Naph), Phenanthrene (Phe) and Anthracene (Ant) was used as a sole source of carbon and energy. For the substrate utilization experiments in ternary, binary and sole substrate studies, which were conducted in triplicate, 1 mL (4 mg/mL stock solution) of phenanthrene, fluoranthen and pyrene was added to 100 mL flasks from their respective stock solutions (described in section 3.1.2). The solvent was allowed to evaporate overnight, leaving a thin coating of the mixed PAHs covering the bottom of the flasks. MSM (40 mL) were added in each flask to achieve a final PAH concentrations of 300 mg/L (100mg/L each). For the sole and binary substrates as well the respective PAH was added the same way to give a final concentration of 300 mg/L from their stock solutions. Then each set of samples was inoculated with 1 mL aliquots of the PAH degraders from late-log precultures to achieve a final cell density of (10^7 CFU mL^{-1}) approximate optical density (OD) of 2 and different concentrations of Lipopeptides and Sodium azide -killed abiotic controls were supplied with the same combination of the PAHs.

The flasks were sealed with Aluminum foil caps and incubated at 30 °C on a rotary shaker at 120rpm. Periodically, triplicate flasks were sacrificed to determine the amount of PAHs remaining using the following procedure. The entire flasks were extracted with 40 mL of hexane twice, centrifuged at 6500 rpm for 6 minutes to remove emulsifications during solvent extraction. The solvent was pooled and evaporated. The residual PAHs was dissolved in equal volume 40 mL of acetonitrile (exchanged to mobile phase medium) , and the samples were stored at -20 °C until HPLC analysis was performed. The biodegradation of phenanthrene was computed in two ways; direct measurement of phenanthrene loss and bacterial cell counts to evaluate cell growth using the Standard Plate Count method.

3.6.3 Biosurfactant Enhanced Bioremediation of PAHs in Creosote Contaminated Soil.

Soil for this study was obtained from a previous wood treatment plant in the Gauteng region South Africa. It has been persistently contaminated with industrial creosote for about 16 years. Soil samples were transported to the laboratory in new plastic containers, where they were air dried at room temperature, passed through a 2 mm sieve, and kept at 4°C prior to analysis. *Pseudomonas aeruginosa* CB1 strain was selected based on Drop Collapse test as efficient biosurfactant producer and the biosurfactant produced by the isolate was used in Creosote contaminated soil biodegradation experiment.

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3.6.3.1. Enrichment of Indigenous Consortium from the Creosote Contaminated Soil

A microbial consortium was obtained via selective enrichment in 250 mL Erlenmeyer flasks. 5 gm contaminated soil sample was inoculated into to 250 mL of mineral salt medium (MSM) and was supplied with 10 mL of Creosote. Creosote 5 % (v/v) was used as a sole source of carbon and energy. Enrichment was conducted at 30°C and 120 rpm rotary shaker and kept for about 7days. After 1 week 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 creosote PAH degrading Bacterial consortium (according to Section 3.3.1.)

Due to the creosote contamination at the site, high levels of PAHs, PCBs and other petrochemical organic contaminants were detected in the soil. Since the soil had been contaminated for decades, organisms in the soil are expected to be acclimated to PAHs. The well adapted indigenous microbial community were further enriched on creosote as a sole source of carbon and energy with the mineral salt medium and reintroduced for the biodegradation experiment.

3.6.3.2. Soil characterization

The soil sample was collected from the top surface layer (15 cm) at a wood impregnation plant in the outskirts of Pretoria (Gauteng, South Africa).The soils were characterized according to pH, Total Organic Carbon and soil texture. PH measured using slurries consisting of soil mixed with distilled water.

Soil texture.

Soils were fractionated into three sizes, i.e., sand (53–2000 µm), silt (2–53 µm), and clay (< 2 µm) by using a combination of wet sieving and centrifugation (Stemmer *et al.,* 1998). The soil texture was 36% silt, 30% sand and 34 % clay.

Soil sample analysis

To determine the PAHs in the soil, 5 g samples were extracted using the USEPA Method 3550C which was developed for extracting non-volatile and semi-volatile organic compounds from solids (U.S.EPA, 1996) as described in section 3.6.1.2 and HPLC analysed. Most of the common PAHs were detected in the soil sample from the site at levels shown in Table 7.1. Notably, naphthalene

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(Nap), acenaphthene (Ace), fluorene (Flr), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flu) and pyrene (Pyr) were among the most abundant PAHs detected in the soil. After the total organic carbon (TOC) and Nitrogen Analysis, section 3.7.4, the approximate organic composition in the soil was 210 g/kg TOC of which 3.062 g/kg (1.5%) was PAHs, the concentration of each showed (Table 7.1). But contains very low amounts of N and, the microcosms were supplemented with NH_4NO_3 and KH_2PO_4, to bring C: N: P ratio to 100: 10: 1.

3.6.3.3. Bio-slurry Reactor Operation

Degradation studies were conducted in five bio-slurry reactors using 400 g of soil suspended in 1000 mL distilled water. 2 L Erlenmeyer flasks were used as bioreactors with continuous mixing using overhead mechanical mixers. Reactor 1 was supplemented with 3 g kg^{-1} crude biosurfactant (no added nutrients), Reactor 2 was supplemented with 3 g kg^{-1} crude biosurfactant and biostimulated with 11.5 g NH_4NO_3 and 1.5 g KH_2PO_4, Reactor 3 was biostimulated with 11.5 g NH_4NO_3 and 1.5 g KH_2PO_4 (no biosurfactant), Reactor 4 was the un-amended biotic control, and Reactor 5 was the sterilized abiotic control. Reactor 2 and 3 were supplemented with the nutrients twice at the beginning of the experiment and at the third week to obtain a C: N: P ratio of 100:10:1 in the soil (Cookson, 1995). All reactors were installed in a water bath at constant temperature of 37±1°C. The sterile abiotic control (Reactor 5) was prepared by autoclaving the soil slurry at 121°C (2.0 bar) for 15 min. 10 mL aliquots of the PAH degraders from late-log precultures was re-inoculated into the four bio-slurry reactors apart from Reactor 5 on day 2 of incubation to achieve a final cell density of (10^7 CFU/mL). All reactors were vigorously mixed using overhead mechanical mixers. Water lost in the reactors via evaporation was replaced daily to keep the working volume constant. The make-up was not used to adjust for volume loss due to drawing of samples. Because the biosurfactants are readily biodegradable, the microcosms received a fresh addition of surfactant (3g/kg soil) every three weeks for the 45 days' time.

Samples (25-mL aliquots) of the bio-slurry contents were drawn at predetermined intervals and centrifuged at 6000 rpm for 10 min. The harvested soil was air-dried after which 5 g was subjected to ultrasonic extraction followed by High Performance Liquid Chromatograph (HPLC) analysis of PAHs in the extracts. The samples were extracted using the USEPA Method 3550C 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 5g soil sample and mixing with 30

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mL of a solvent hexane: acetone (1:1 v/v) in flask, followed by sonication at 50-60 Hz at 55°C 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 2,000 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 injected into an HPLC through a 0.22µm polytetrafluoroethylene (PTFE) filter syringe. 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 (16 PAH) was injected at the beginning of daily measurement for the

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instrumental performance check i.e. Precision, repeatability, and random errors due to changes in the equipment conditions during the period of study.

Two chromatographic conditions were applied for the Phenanthrene, Pyrene, and Fluoranthene analysis in (Experiments 1 and 2). The amount of PAHs in the resuspended solvent was quantified by reversed phase high-performance liquid chromatography (HPLC) using a linear gradient acetonitrile & ultra-pure-water mobile phase over 30 min at a flow rate of 1 mL/min.

The elution conditions applied were; 0–1 min, 70% acetonitrile(ACN) isocratic; 1–10 min, linear gradient 70% ACN–100% ACN; 10–20 min, 100% ACN isocratic; 20- 25 min ,linear gradient 100%ACN– 70% ACN and finally, 25- 30 min 70%ACN isocratic back to the initial conditions and recondition the column (for the first two experiments).

3.7.1.2 For the 16 PAH Analysis (sub section 3.6.3)

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 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 C18 column (4.6 mm×25 cm with 5 µm packing) at a column temperature of 25°C, at 254 nm (for the 3rd experiment).

PAHs in solutions were analyzed by HPLC system with a slightly modified EPA Method 8310 (EPA 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 C18 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

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3.7.2 Microbial Count

Standard plate count Method was used to quantify heterotrophic bacteria utiliziliing 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.

3.7.3 Cell Surface Hydrophobicity Test

Cell surface hydrophobicity was measured by bacterial adhesion to hydrocarbons (BATHs) According to slightly modified method described by Zhong et al. (2007a). Microbial consortia grown on the mixed PAHs for 8 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 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.

$$\text{Hydrophobicity (\%)} = \left(\frac{A_O - A}{A_O}\right) \times 100 \quad (3.1)$$

3.7.4 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 in concentrations ranging from 0-5 mg/L in a 100 mL volumetric flask prior to analysing for total carbon. Perkin Elmer 2400 CHN/O Elemental Analyser was used to determine the soil samples

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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 of Desorption Kinetics

A two-compartment first-order rate constant model was used to fit the desorption data (Cornelissen *et al.,* 1998, Poot *et al.,*. 2014)

$$S_t / S_0 = F_{rap} e^{-k_{rap} t} + F_{slow} e^{-k_{slow} t} \quad \text{(3.2)}$$

Where S_t (mg kg^{-1}) is the PAHs content in the soil at time t (h) and S_0 (mg kg^{-1}) at the start of the experiment; F_{rap} and F_{slow} are the rapidly and slowly desorbing fractions ; k_{rap} and k_{slow} (h^{-1}) are the rate constants of rapid and slow desorption compartments, assuming that $k_{slow} << k_{rap}$. It is assumed that the two defined fractions covered the entire amount of PAHs S_t (no other compartment), which leads to:

$$F_{rap} + F_{slow} = 1 \quad \text{(3.3)}$$

Values of F_{rap}, F_{slow}, k_{rap}, *and* k_{slow} were determined by minimizing the cumulative squared residuals between experimental and calculated values of (S_t/S_0) in Equation 3.2 using the software Microsoft Excell 2010 (SOLVER option).

3.8.2. Modelling the biodegradation kinetics

Sole substrate biodegradation kinetics modelling

The Monod equation is commonly used to model the kinetics of biodegradation of organic compounds and associated biomass growth, the Monod model, is shown as Eq. (3 and 4) (Monod, 1949). The differential equations for time dependence of concentration of a growth-limiting substrate and biomass growth are:

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$$\frac{dC}{dt} = -q_{\max} \frac{C}{K_S + C} X \quad (3.4)$$

$$\frac{dX}{dt} = Yq_{\max} \frac{C}{K_S + C} X - bX \quad (3.5)$$

Where variables are concentration of the PAH substrate C (mg/L), biomass concentration X (mg protein /L), and time t (d). The parameters are the maximum substrate utilization rate per unit biomass, $q_{\max}$ (mg substrate/mg protein/h), the half-saturation coefficient, K_s (mg/L), the yield coefficient, Y (mg protein/mg substrate), and the endogenous decay rate, b (h^{-1}).

Due to very low growth cells (*X*) is assumed constant and *C* is the only variable. The parameters are $q_{\max}$ and K_s.

Single substrate degradation experiments can be used to estimate the kinetic parameters $q_{\max}$ and K_s for each substrate. The Monod model was fitted to the sole substrate experimental data to yield biokinetic parameters q_{max} and K_s. The data set is comprised of the measured variable, C measured over the independent variable, *t* (days, hours). The kinetic parameters, $q_{\max}$ and K_s, determined from single component degradation processes are used in multisubstrate utilization rate models in which more than one growth limiting substrate is present.

Multisubstrate model with competitive inhibition.

The multisubstrate model with competitive inhibition represents the case, in which multiple substrates are available to the microorganisms, but the substrates compete for the limited number of enzymes present during competitive inhibition, substrates compete for binding sites in order to be metabolized by the bacterial population. For a simple inhibition system, when the inhibitor is present as an alternate substrate, the multisubstrate model demonstrated by (Guha *et al.,* 1999; Knightes and Peters, 2006) is used (Equation 3.6).

$$\frac{dC}{dt} = q_{\max,i} \frac{C_i}{K_{s,i,} + \sum_{j=1}^{n} \frac{K_{s,i}}{K_{s,j}} C_j} X \quad (3.6)$$

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$q_{\max,i}$ is the maximum substrate utilization rate per unit biomass for substrate i (mg substrate/mg protein/d) and $K_{s,i}$ is the half-saturation coefficient for substrate i (mg/L)., C_i is the concentration of substrate i, (mg/L) , $K_{s,i}$ is the Half-saturation constant for substrate i, and $K_{s,j}$ is the half saturation constant for each substrate j.

The model uses the parameters derived from the sole substrate case (Guha *et al.,* 1999; Knightes, 2000; Knightes and Peters, 2006). It requires that the compounds be utilized through a common pathway (Segel, 1975).

Equation 3.6 can be extended for any number of components provided the compounds exhibit competitive inhibition kinetics. Thus, in Equation 3.6, $K_{s,j}$ represented a constant, while $K_{s,i}$ and $q_{\max,i}$ are fitting parameters. There are two parameters in this equation, $q_{\max}$ and K_s. In order to estimate kinetic parameters for single substrate experiments, nonlinear curve fitting and parameter estimation was performed with AQUASIM software package (Reichert, 1998). After the experimental data of PAHs degradation was fitted, the two parameters, $q_{\max}$ and K_s were estimated.

Parameter estimation

AQUASIM contains a dynamic equation Solver, which is capable of performing parameter estimation to find the best fit of the model output to the experimental data. The parameter estimation procedure minimizes deviations between measured (experimental) values and values predicted by the specified model. The parameters are estimated by minimizing the sum of the squares of the weighted deviations, given as

$$\chi^2 = \sum_{i=1}^{n} \left(\frac{f_{meas,i} - f_i(p)}{\sigma_{meas,i}} \right)^2 \quad (3.7)$$

Where $f_{meas,i}$ is the ith measured value, $f_i(p)$ is the calculated value from the model using parameter values p and $\sigma_{meas,i}$ is the estimated standard deviation of $f_{meas,i}$. During the fitting of the model to the experimental data, the kinetic parameters, $q_{\max}$ and K_s, were adjusted by AQUASIM until Chi square (χ^2) reached a minimum value. The weighted error between

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experimental and model data (χ^2) can be used as a measure of the goodness of fit between experimental and predicted data.

A correlation coefficient (R^2) is determined using excel software to evaluate the adequacy of fit along with the Chi square (χ^2) analysis and visual judgment.

3.8.3. Statistical analysis

The mean and standard deviation of three replicates were calculated. A significance level of ($p < 0.05$) was used throughout the study. All statistical analyses were performed using the software called Statistical Package for Social Sciences (SPSS version 22.0, SPSS Inc., USA).

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CHAPTER 4

BIOSURFACTANT CHARACTERIZATION AND ITS MASS TRANSFER ENHANCEMENT STUDIES

4.1 Microbiological Methods

4.1.1 Screening and identification of biosurfactant-producing bacteria

A total of 11 morphologically different colonies were isolated, from petroleum contaminated crane service station soil and 5 of them were found to be efficient biosurfactant producers as checked by "drop collapse" and "oil spreading" screening methods. Strains LBP9 and LBP5 were selected as powerful biosurfactant producing bacteria. The 16S rRNA analysis revealed that strain LBP9 and LBP5 were related to members of genus *Pseudomonas*, and showed highest sequence similarity (100%) to *Pseudomonas aeruginosa*.

The 16S rRNA gene sequence of strains LBP9 and LBP5 was aligned automatically to reference sequences of the genus *Pseudomonas aeruginosa* obtained from the GenBank (http://www.ncbi.nlm.nih.gov/BLAST/), and a phylogenetic tree was constructed (Fig. 4.1) based on the neighbor-joining method using the software MEGA version 6.0 (Tamura et al., 2013). Strains LBP9 and LBP5 cultures were preserved in the MSM broth with 30% (w/v) glycerol at −80 °C.

4.1.2 Biosurfactant properties

4.1.2.1 Surface Activity of the Biosurfactants

The surface tension of the whole broth dropped rapidly from around 71 mN/m to 34 ± 1 mN/m, in the first 3 days of incubation. The surface tension of the culture supernatants was lowered to ±35 mN/m after 3 to 6 days. The amounts of biosurfactants produced were therefore sufficient to reach the lowest possible surface tension, the critical micelle concentration. The biosurfactant concentration is expressed in terms of critical micelle dilution (CMD), CMD is the dilution

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necessary to reach the CMC (critical micelle concentration), at which the surface tension starts to increase dramatically (Kim *et al.,* 1997). The biosurfactant concentration in the cell free broth was 30×CMD at the minimum surface tension. The biosurfactants produced by the strains LBP9 and LBP5 have comparably equal critical micelle concentrations (CMC) of 150±10 mg/L corresponding to minimum surface tensions of 32 ± 1 mN/m and 35 ±2 mN/m respectively.

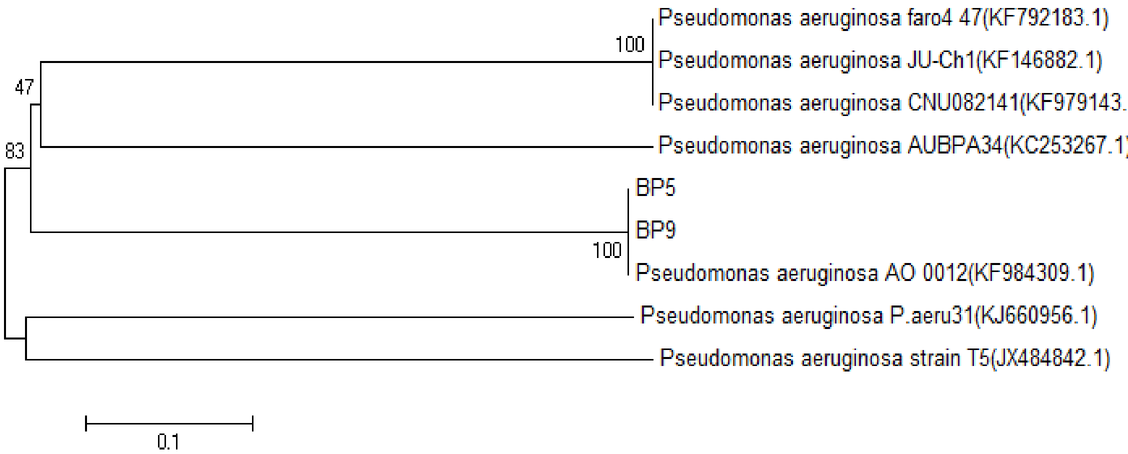

Figure 4.1: Phylogenetic relationship based on the 16S rDNA gene sequences between strains LBP5, LBP9 and species in the *Pseudomonas aeruginosa* as determined by the neighbour-joining algorithm.

4.1.2.2 The emulsification index (E_{24}) of the crude biosurfactant

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 *Pseudomonas aeruginosa* LBP5 and LBP9 strains were used to prepare emulsions of hexane, cyclo-Hexane and used motor oil, which were found to remain stable up to 2 months. The emulsification indices of the culture supernatants were found out to be 65±2 %, 78±1 % and 76±1 % for LBP5 and 72±3% , 81±2% and 78±2% for LBP9 strains with Hexane, cyclo-Hexane and toluene respectively.

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4.1.3 Chemical characterization of the biosurfactant

4.1.3.1 FTIR ATR Characterization

About 20 g of crude biosurfactant was produced per liter of culture medium. The FTIR spectrum of the column purified biosurfactant showed bands characteristic of peptides at 3,235.48 cm^{-1} resulting from N–H stretching mode (Fig. 4.2). The sharp peak around 1642.17cm^{-1} (stretching mode of the CO–N bond) is due to amide group (Nasir and Besson, 2012). The deformation vibrations from 1458.02 cm^{-1} to 1210 cm^{-1} reflect aliphatic chains (–CH_3, –CH_2–) of the fraction. This characteristically indicated the presence of fatty acid chain of lipopeptide biosurfactants. Sharp peaks in the range of 1350 – 1000 cm-1 (C-N stretching mode) indicated the presence of amine groups which show that peptide containing moieties were present in the compound.

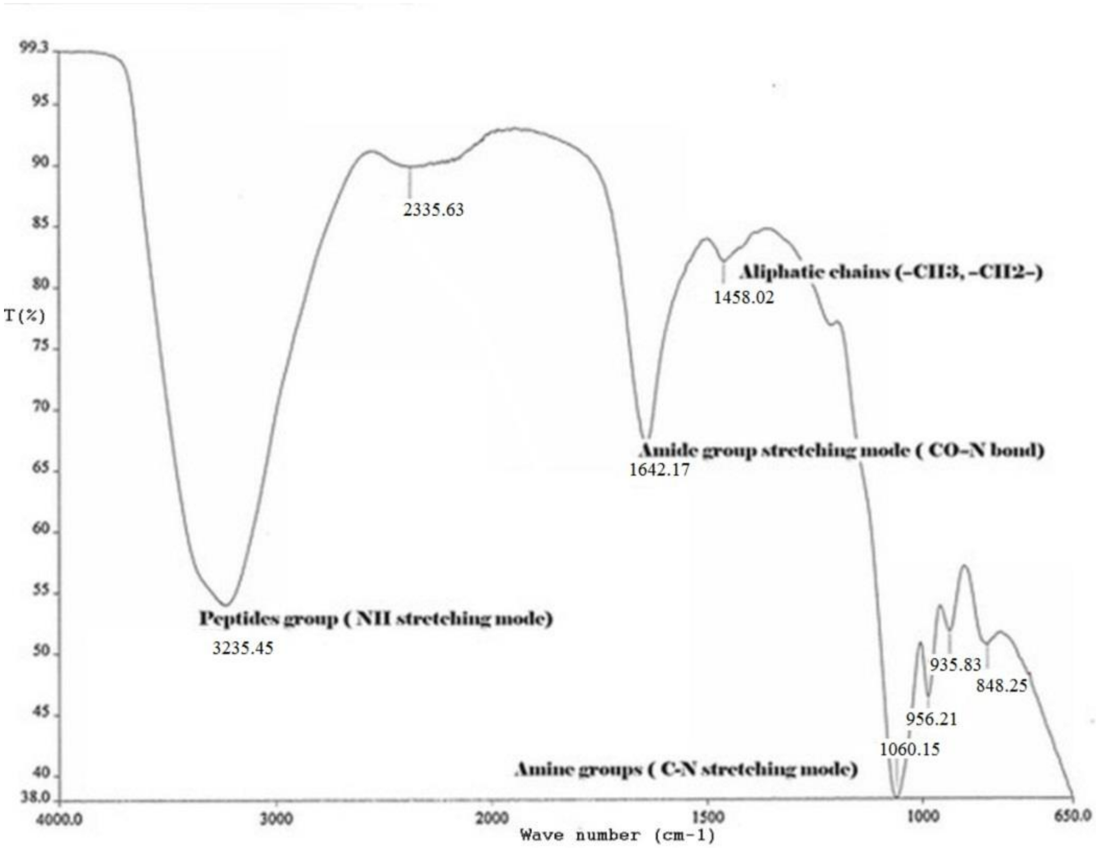

Figure 4.2: Fourier transform infrared (FTIR)-absorption spectrum of the biosurfactant produced by *Pseudomonas aeruginosa LBP9* Strain

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The results show that the product contained aliphatic and conjugated carbony (amide) that are the essential parts of lipopeptide (Hou et al., 2014). The FTIR spectra of the column purified biosurfactants from both the strains LBP5 and LBP9 showed the same functional groups, Figure 4.2 portrays the FTIR spectra of the biosurfactant produced by LBP9 strain.

4.1.3.2 TLC analysis of purified biosurfactant

The aqueous fraction of the biosurfactant after acid hydrolysis indicated an R*f* value of 0.62 and showed positive reactions with ninhydrin on the TLC plate (Fig. 4.3), signifying that the biosurfactant consisted of peptide moieties and showing that it may be Lipopeptide.

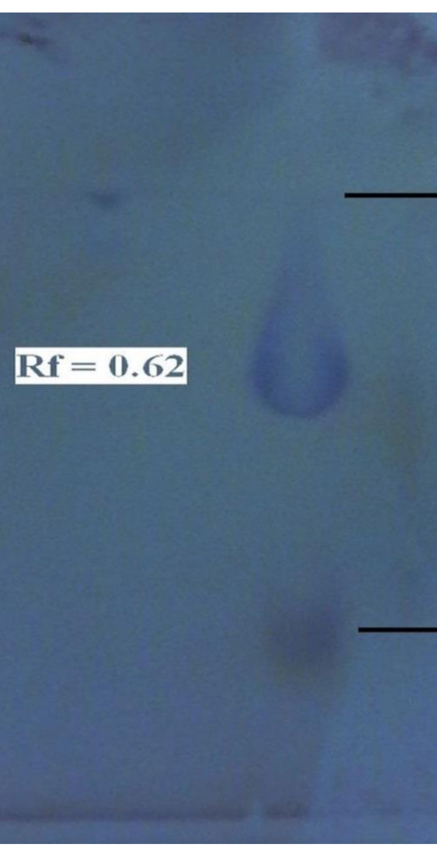

Figure 4.3: Thin-Layer Chromatography (TLC) of the biosurfactant obtained from *Pseudomonas aeruginosa* LBP9, after treatment with ninhydrin revealed pink spot with R*f* value of 0.62. TLC was carried out on silica gel plates using chloroform–methanol–H_2O (65:25:4, v/v/v) as mobile phase.

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4.2 Biosurfactant assisted Mass transfer studies

4.2.1 Phenanthrene and Pyrene solubilisation assay

The effect of lipopeptide on the apparent aqueous solubility of Phenanthrene, Fluoranthene and Pyrene was determined in the presence of increasing concentration of lipopeptide (0 to 1600 mg/L). The concentration of solubilized PAHs increased linearly with increasing concentration of lipopeptide (r^2 = 0.92, 0.94 and 0.91 for Phenanthrene, Fluoranthene and Pyrene respectively). At 400 mg/L of Lipopeptide supplementation, the solubilized PHE concentration was 30.3 mg/L, 19 fold higher than its aqueous solubility at room temperature (1.78 mg/L), while the amounts of fluoranthene and pyrene solubilized were 33 and 45 fold their aqueous solubilities respectively at the same concentration of Lipopeptide. It can be noted, (Fig. 4.4) that the effect of the biosurfactant was more pronounced on the less soluble PAHs (PYR > FLU > PHE).

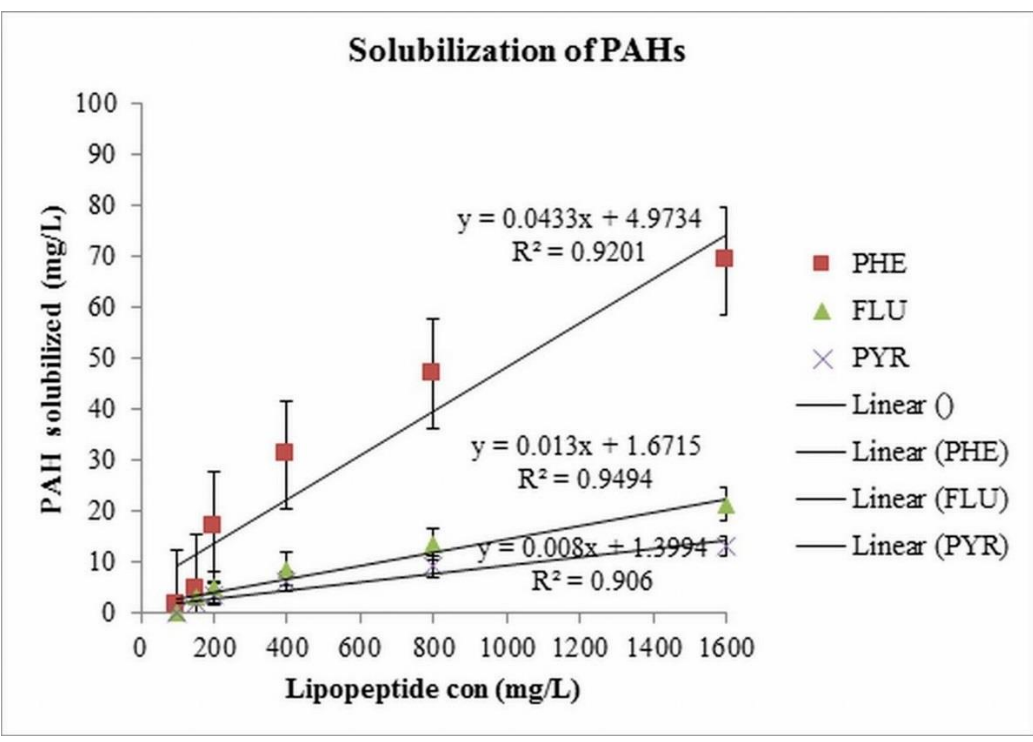

Figure 4.4: Effect of biosurfactant concentration on the dissolution of Phenanthrene (PHE), Fluoranthene (FLU) and Pyrene (PYR) (Symbols are experimental data, and lines are data fits).

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This is in line with the results reported by Cheng et al. (2004) who reported a linear relationship between the concentrations of surfactants at concentrations above their respective CMC and solubilization of PAHs. Previous studies have also shown that solubilization of hydrophobic substances commences at the CMC and in general the amount solubilized is a linear function of surfactant concentration above this concentration (Edwards *et al.,* 1994). Generally, surfactants can increase the solubility of PAHs by partitioning them into the hydrophobic cores of surfactant micelles above the critical micelle concentration (CMC) (Edwards *et al.,* 1991; Zhang *et al.,* 2010) and then enhancing the desorption of PAHs from solid into aqueous phase improve the bioavailability of PAHs for microbial remediation .

4.2.2 Batch Desorption Study of Phenanthrene and Pyrene

Desorption kinetics

The amount of PAHs desorbed from the soil increased as the concentration of biosurfactant in the equilibration solution and contact time increased (Fig. 4.5 and 4.6). Biosurfactant concentration is usually a critical factor for the removal of hydrophobic organic compounds (HOC) from soil. In the soil samples equilibrated with solution containing Lipopeptide at 700 mg/L, 71 % of the sorbed PHE and 48% of PYR were released after 5days of equilibration from the contaminated soil of 800 mg/kg contamination level. This rapid desorption phase was followed by a second phase characterized by a slower rate, which remained constant until the end of the experiment. 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 soil into aqueous phase (Cheng *et al.,* 2004).

Low desorption was observed when no or relatively low concentration of lipopeptide was present in the soil− water system due to the high octanol−water partition coefficient of PHE and PYR (log K_{ow} of 4.57 and 5.18 respectively) and the fact that a portion of surfactant monomers in aqueous phase was lost as the result of surfactant sorption onto soil (Greenberg *et al.,* 2005; Zhang *et al.,* 2010). Consequently much higher chemical or biosurfactant concentrations are required to promote pseudosolubilisation of hydrophobic contaminants present in soil compared to

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requirements for solubilisation in aqueous media alone. In fact examples involving chemical surfactants show that the surfactant concentration required for soil biotreatment may have to be increased by an order of magnitude compared to the amount of surfactant required for biotreatment in an aqueous system (Sarubbo *et al.,* 2012). The amount of surfactant required to desorb HOCs in soil/sediment-water systems which is considerably greater than the CMC in water, is described as critical desorption concentration (CDC), above which desorption process was sharply accelerated with increasing surfactant concentration (Cheng *et al.,* 2004; Greenberg *et al.,*2005).

In the 700 mg/L and 400 mg/L supplemented systems PYR and PHE desorbed remarkably during the rapid desorption stage while there was no significant desorption achieved at 150 mg/L suggesting that the concentration is too low to promote pseudo solubilisation of the PAHs. The results in this study are similar to previous studies (Urum *et al.,* 2003; Kuyukina *et al.,* 2005; Schwab *et al.,* 2007) who reported that increasing the concentration of biosurfactant could enhance the removal of PAHs and total petroleum hydrocarbons from contaminated soil. On the contrary, the toxic effect of some biosurfactants need to be considered when the biosurfactant is used to facilitate biodegradation of PAH pollutants with the indigenous microbial population in the soil as excessive biosurfactant addition would adversely affect the microorganisms (Barnier *et al.,* 2014). However, for the purpose of washing hydrocarbon -contaminated soil, removing HOC pollutants, for oil recovery or further *ex-situ* treatment, the amount of biosurfactant used could be much higher (Urum *et al.,* 2003; Schwab *et al.,* 2007).

The extent and rate of sorption and desorption correlate to the organic matter content and texture of the soil and the hydrophobicity of the PAHs. The lower desorption rate of PYR (48%) compared to PHE (71%) can be explained by the more hydrophobic nature of PYR, which can be reflected by the higher octanol / water partition coefficient (Log K_{OW}) of PYR. Similar results of greater affinities for a specific sorbent for more hydrophobic PAH have been reported by (Brinck *et al.,* 1999; Richardson *et al.,* 2011).

The shape of the desorption kinetic curves obtained were similar to those observed by previous authors using other extraction techniques either with model sorbents (Cornelissen *et al.*,1998) or sediments (Cornelissen *et al.*,1997; Congiu *et al.*,2014).

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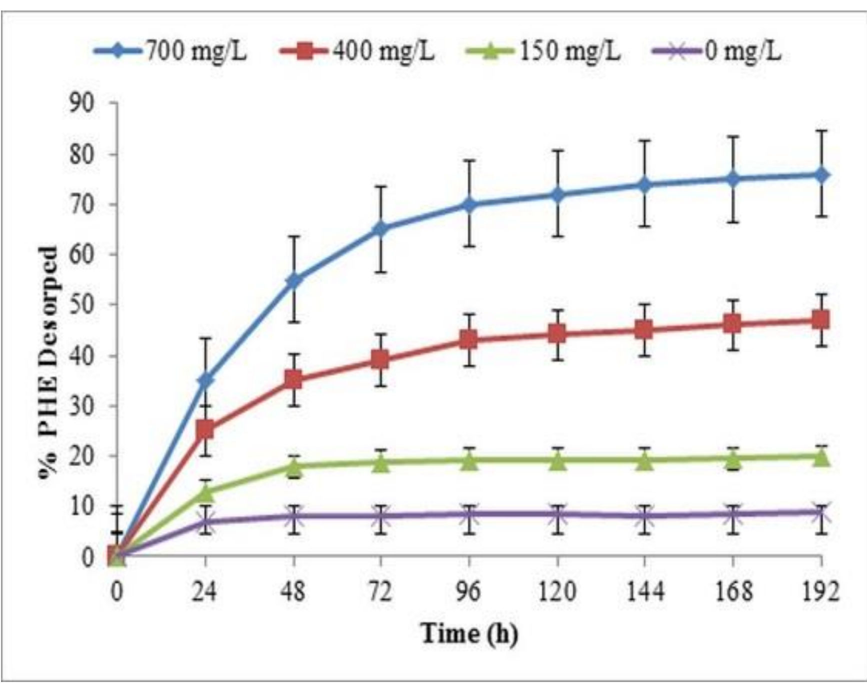

Figure 4.5: Percentage of Phenanthrene (PHE) desorbed with different concentrations of Lipopeptide. Values are the mean ± standard deviation (n = 3).

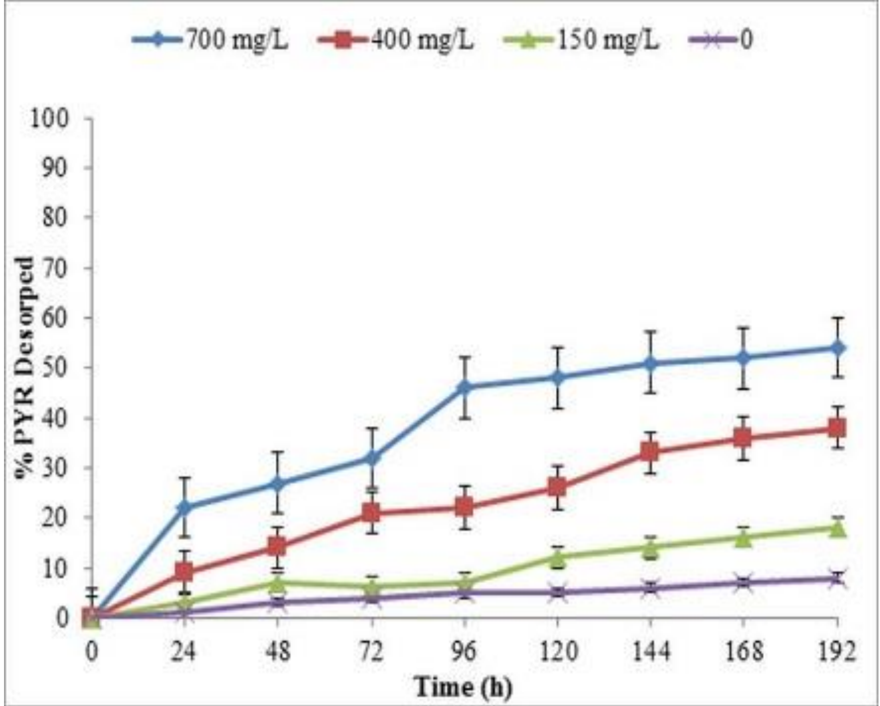

Figure 4.6: Percentage of Pyrene (PYR) desorbed with different concentrations of Lipopeptide. Values are the mean ± standard deviation (n = 3).

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All desorption kinetics curves do include the initial rapid desorption followed by the slow desorption rates (Fig. 2 and 3). The amount desorbed increased with the increased biosurfactant supplementation, as reported in previous studies (Brinck *et al.,* 1997; Sverdrup *et al.,* 2002).

The amount of the PAHs desorbed from the soil increased as the concentration of surfactant in the equilibration solution and contact time increased. In soils equilibrated with solution containing Lipopetide at 700 mg/L, 71 % of the sorbed PHE and 48% of PYR were released after 5 days of equilibration from the artificially contaminated soils of 800 mg/kg contamination level each (Figs 4.5 and 4.6). A rapid release of the sorbed fraction was observed for the first 5 day time followed by the slow release afterwards

Desorption Kinetics Modelling

The mathematical fitting of 192 h desorption kinetics enabled us to determine a rapidly desorbing fraction, which is generally considered to be the bioavailable fraction (Cornelissen *et al.,* 1998; Schwab *et al.,* 2007). Kinetic curves are modelled for each PAH at each Lipopeptide supplementation dosage, using the two-compartment model. For all PAHs, the experimental results fitted with the two compartment model satisfactorily (Fig. 4.7). Fitting the data to the two compartment model (**Equations, 3.2** in the **Data Analysis subsection 3.8**) gave sums of squared deviations ranging from 0.00761-0.0013, implying satisfactory fitness. The values obtained for the rapidly and slowly desorbing fractions (F_{rap}, F_{slow}) and their rate constants (k_{rap}, k_{slow}) are presented in Table 4.1.

Table 4.1: Best-fit parameters of the two-compartment model for the different lipopeptide supplementations for each PAH.

PAH	Lipopeptide (mg/L)	F_{rap} (%)	$k_{rap}(h^{-1})$	F_{slow} (%)	k_{slow} (h^{-1})
PHE	700	72.8±1.2	0.028	0.00025	27.2±1.3
	400	39.0±0.9	0.027	0.00074	61±0.7
	150	18.2±1.1	0.031	0.00028	81.8±2.1
PYR	700	51.2±1.3	0.012	0.00025	48.8±1.4
	400	24.5±1.2	0.015	0.00075	75.5±1.2
	150	6.4±0.9	0.087	0.00064	93.6±1.7

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As expected, desorption rate constants for the two-compartment model followed the progression of k_{rap}>k_{slow} and were generally in the order of 10^{-2} (h^{-1}) and $10^{-4}(h^{-1})$ respectively. Such findings are in accordance with values reported in other studies for PAH-spiked soils and sediments (Van Noort *et al.,* 2003; Barnier *et al.,* 2014). The data for k_{rap} in Table 4.1 suggest a slight decrease on increasing molecular weight of PAHs.

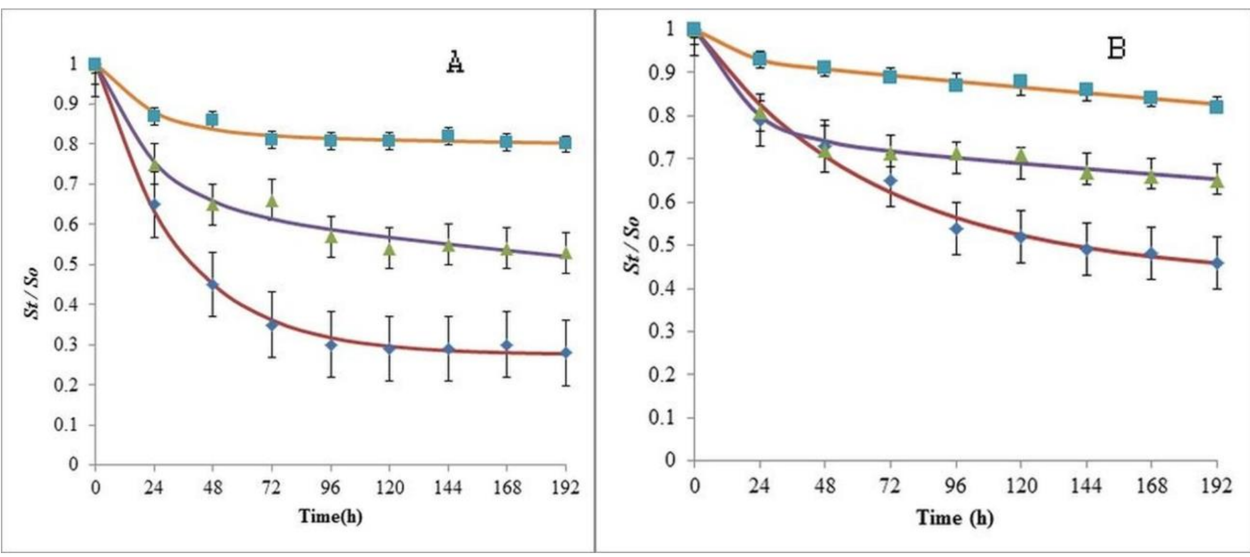

Figure 4.7: Two-compartment model fits to PHE (A) and PYR (B) desorption kinetics data in the presence of 150 mg/L (■), 400 mg/L (▲) and 700 mg/L (◆) of Lipopeptide. Data points are mean values from three independent experiments. Error bars represent standard errors

The 8-day desorption percentage of PHE in the three samples of different lipopeptide supplementation levels (Figure 2) ranged from 72.8% to 18.1%. The same trend for a greater extent of desorption taking place in the 700 mg/L amended sample than in the 400 and 150 mg/L amended ones. When the two-compartment model was used to analyse the data (Table 4.1), the rapid/ slow desorption fractions were 72.8/27.2%, 39/61% and 18.2/81.8% for the 700, 400 and 150 mg/L supplementations, respectively. A less rapid fraction was observed for the 150 and 400 mg/L amendments as compared to the 700 mg/L amendment. The values of k_{slow} were two to three orders of magnitude lower than the k_{rap} values for all PAHs in different samples, which is consistent with other studies that apply the two-compartment desorption model (Richardson and Aitken,2011;

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Zheng *et al.,*2012). These results could be considered to well validate the biphasic behaviour of organic compounds desorption and to confirm the supposition of the model. In addition, the extractability of the PAHs in the studied soils decreased generally with increasing molecular weight of the contaminating compound. The rapidly desorbing fraction decreased with the increasing hydrophobicity of PAHs and a positive relationship was found between the F_{slow} and the hydrophobicity of PAHs. A similar lipophilicity trend has also been observed for chlorobenzenes, other PAHs and polychlorinated biphenyls (Lai *et al.,* 2009). This behaviour is the result of the increase in the hydrophobicity of PAHs as their molecular weight increases (four-ring PAHs have octanol/water partition coefficients (log K_{ow}) in the range of 5.20 – 5.80, in comparison to 3.94 – 4.60 for three-ring PAHs. This increased hydrophobicity indicates a greater tendency to remain adsorbed to organic matter in the soil (Sanchez-Trujillo *et al.,* 2013).

It is assumed that the quantity of contaminant extracted by a non-exhaustive extraction technique or Tenax beads gives a measurement of the available pollutant pool (Barnier *et al.,* 2014). The rapidly desorbing (bioavailable) fraction has been used successfully to predict the extent of PAH degradation in field-contaminated sediments (Richardson and Aitken, 2011). In this study, the addition of increasing concentrations of lipopetide helped to increase the PAH desorption and expand the rapidly desorbing fraction. Accordingly, from the increasing rapidly desorbing fraction, which is the microbially degradable component of PAH contamination, we can predict an increase in the achievable bioremediation performance.

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CHAPTER 5

BIOSURFACTANT-ENHANCED BIODEGRADATION OF PHENANTHRENE BY PSEUDOMONAS AERUGINOSA LBP9 ISOLATE

5.1 Biodegradation of Phenanthrene by LBP9 isolate in Liquid Culture.

Phenanthrene (PHE) was selected as a model PAH to investigate the influence of the Lipopeptide produced by *Pseudomonas aeruginosa* LBP9 on the bioavailability and biodegradation of PAHs. Aqueous phase biodegradation of Phenanthrene in the presence and absence of Lipopeptide can be summarized as shown in (Fig. 5.1). There was 15% degradation at the Lipopeptide unamended control; 20% ; 55% and 6% biodegradation rates at 100 mg/L , 400 mg/L and 700 mg/L Lipopeptide amendments respectively during the first 3days of incubation. Lower degradation of phenanthrene (6%) was observed in the 700 mg/L Lipopeptide amended flask than in the the unamended control (15%) during the first three days of incubation. At the sixth day there was 92% degradation in the 400 mg/L lipopetide amended culture where as there was only 27% degradation in the unamended control (Fig. 5.1). Degradation followed first order kinetics in all four treatments with an initial lag of approximately 2 days, followed thereafter by rapid rates of degradation that were higher for the 400 mg/L and 300 mg/L Lipopeptide-amended treatments compared to the unamended control and 700 mg/L treated microcosms. Cultures with 400 mg/L biosurfactant addition expressed significantly more biodegradtion of PHE than in the absence of biosurfactants ($p < 0.05$). While the biodegradtion rates at the 100 mg/L and 700 mg/L Lipopeptide amendments were not significant ($p > 0.05$), compared to the culture with absence of Lipopeptide. Results with Lipopeptide thus suggest that optimum amount of Lipopeptide supply may enhance degradation of Phenanthrene.

Biosurfactant production was shown as a key characteristic of alkane-degrading bacteria, which serves to augment alkane bioavailability and thus degradation rate (Olivera *et al.,* 2009; Tecon and van der, 2010). A similar effect has been proposed for the bacterial degradation of polycyclic aromatic hydrocarbons (PAHs) (Johnsen *et al.,* 2005). The inhibitory effect due to the Lipopeptide addition of 700 mg/L suggests that the increased Lipopeptide could have been toxic to the

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phenanthrene degrading culture or alternatively stress caused by the solubilized phenanthrene by the increased amount of Lipopeptide could have caused toxicity to the bacteria and inhibited their degradation activity, or the Lipopeptide e was serving as a preferred carbon source. Similar results were reported by Whang et al. (2008) during studies focused on biosurfactant-mediated biodegradation of diesel contaminated water and soil carried out by autochthonic soil microorganisms during batch diesel/water experiments and biopile tests. The authors observed that even though diesel solubilization was slightly higher for surfactin, especially above the CMC value, the presence of this surfactant may limit the biodegradation rate at concentrations above 40 mg/L (with a complete inhibition at 400 mg/L). The external addition of biosurfactants, however, was shown to increase the solubilization of PAHs from non-aqueous phase liquids and solid particles (Garcia-Junco *et al.,* 2003). Nevertheless, an augmentation of PAH solubilization is not necessarily associated with an equivalent increase of its bioavailability to microorganisms (Tecon and van der Meer, 2010).

The nature of biosurfactant effects on PAH degradation rate are complex and is debated for more than decades (Volkering *et al.,* 1997). The effect of biosurfactants on the microorganisms depends on factors, such as: biosurfactant concentration, environmental and cultural conditions, and characteristic and properties of microorganisms as cellular ultra-structure (Van Hamme *et al.*, 2006). In fact, inhibitory effects may be dueto (c) preferential degradation of the surfactant, slowing the pollutant degradation (Tiehm, 1994), (v) decreased microbial mobility, and (vi) lowered bioavailability by inhibiting bacterial attachment, dispersing soil colloids causing clogging of pores, or interfering with the natural interactions of microbes with the pollutant.

Other studies showed ehnhanced degradation with different *Pseudomonas aeruginosa* strains; study with the astrain, *Pseudomonas marginalis*, indicated that the biosurfactant produced by the strain solubilized polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene and enhanced biodegradation (Burd and Ward, 1996).

Other research by Garcia-Junco *et al.* (2001) indicated that addition of rhamnolipids led to attachment to the phenanthrene that enhanced bioavailability and hence degradation of the contaminant by *Pseudomonas aeruginosa.*

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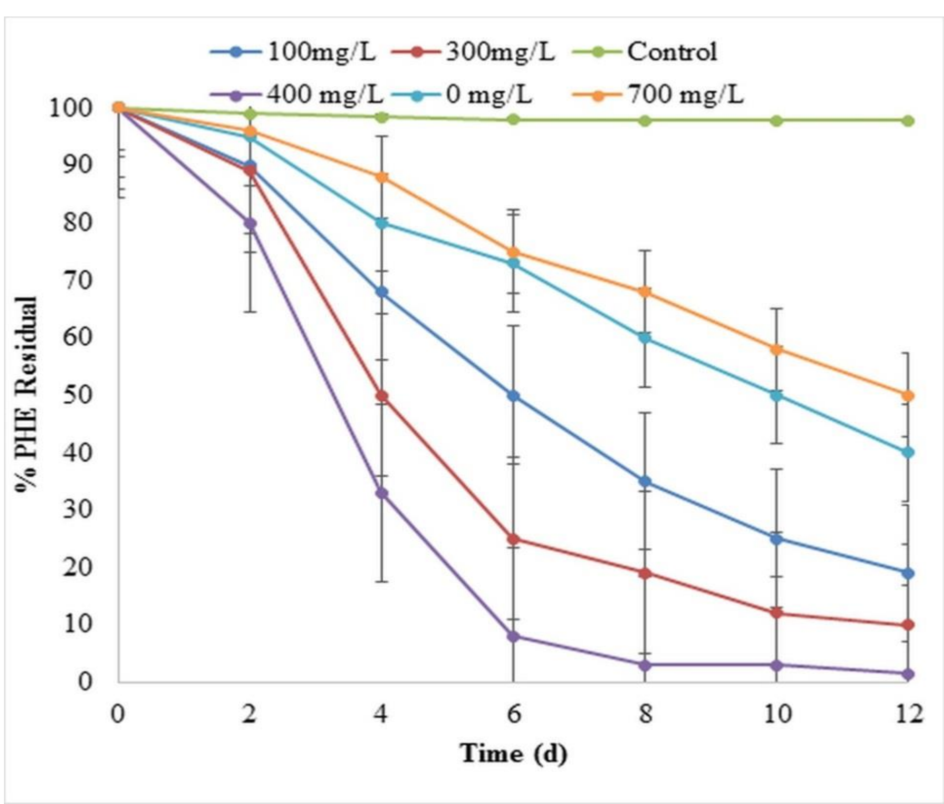

Figure 5.1: Aqueous phase biodegradation of Phenanthrene with different concentration of Lipopeptide by *Pseudomonas aeruginosa* LBP9 isolate

5.2 Bioslurry phase degradation of phenanthrene by LBP9 isolate

The amount of Phenanthrene desorbed from the soil increased as the concentration of surfactant in the solution increased (Fig. 5.2). In the soil equilibrated with solution containing Lipopeptide at 700 mg/L, more than 71% of the sorbed Phenanthrene was released after 5 days of equilibration from the artificially contaminated soil (800 mg/kg contamination level). The aqueous phase concentration rapidly increased during the initial fast desorption of the first 1 to 4 days, as the rate of desorption is greater than the rate of biodegradation bacterial metabolism is the rate limiting factor. Whereas for the unamended control the aqueous phase concentration of phenanthrene is very low. Because of the low desorption rate of the adsorbed substrate, there is low bioavailablity of phenanthrene to the degraders, and low degradation rate as a result, so desorption was the rate limiting factor.

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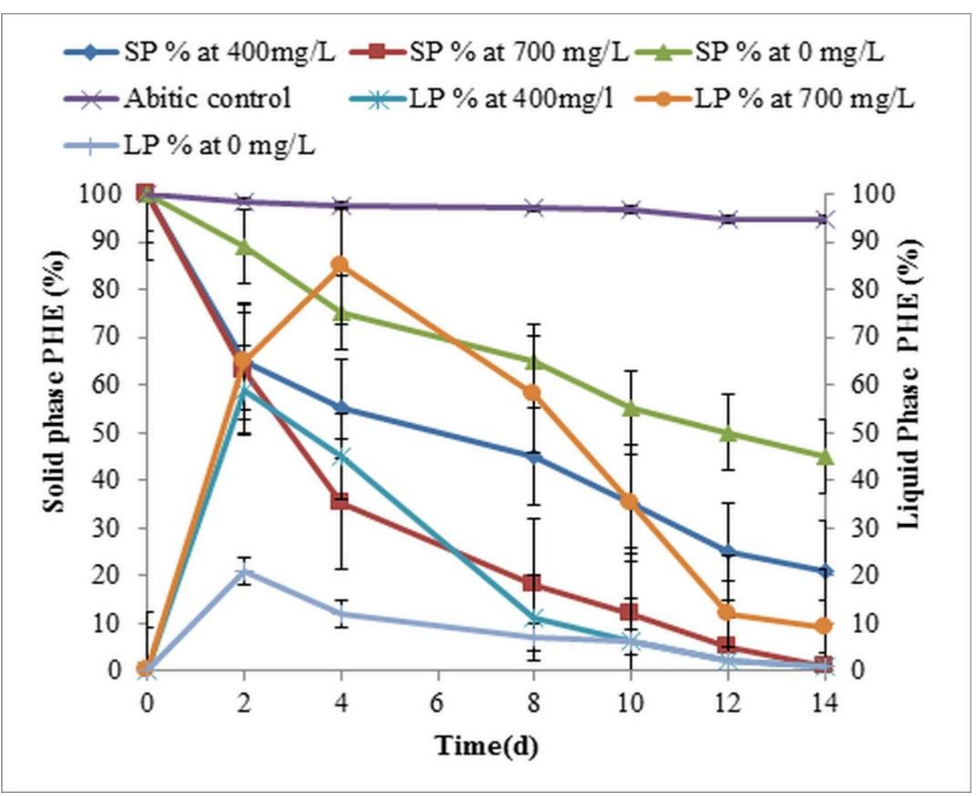

Figure 5.2: Percentage residual of phenanthrene in the solid phase and aqueous phase. SP represents the solid phase and LP represents the liquid phase percentages of Phenanthrene.

The data (Figure 5.2) shows, under the test conditions used in the experiments, that the rate of degradation of phenanthrene is significantly slower than the rate of desorption for the compound for the first days of fast desorption. But later on phenanthrene consumption by the isolates exceeded the phenanthrene desorption rate. Whereas the amendment of 700 mg/L resulted in inhibited degradation relative to the others at the kick-off but 4 days later rapid degradation commenced, probably due to longer lag phase for the bacteria to adapt to the increased concentration of solubilised contaminant which may cause toxicity beyond threshhold limit. The data (Fig. 5.2) shows, under the test conditions used in the experiments, that the rate of degradation of phenanthrene is significantly slower than the rate of desorption for the first days of fast desorption. The rate of degradation controls environmental fate during this period of fast desorption, since desorption appears to be more rapid than degradation. In this phase, bacterial growth was controlled only by metabolic activity, and not by bioavailability, with a corresponding exponential growth in biomass.

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Exponential growth ceased when phenanthrene consumption by the isolates exceeded the phenanthrene desorption rate. However to improve bioavailability, the phenanthrene degrading and biosurfactant producing bacteria *Pseudomonas aeruginosa* that we used for the biodegradtion test had developed strategies such as biosurfactant production. As observed in the experiment, the loss of phenanthrene, bacterial growth observed in the Standard Bacterial Count and the very high emulsification observed during hexane extraction of phenanthrene confirm the *in situ* production of biosurfactant.

This is in consistence with the studies by (Grosser *et al.,* 2000; Uyttebroek *et al.,* 2006) which showed that although it was assumed that the desorption rate of an organic contaminant into the aqueous phase is the rate-limiting step for microbial degradation of sorbed organic contaminants, the studies showed that the microbial utilization rate of a sorbed substrate can be higher than expected from its abiotic desorption rate.To improve bioavailability, PAH-degrading bacteria have developed strategies such as substrate source attachment and high specific substrate affinity toward PAHs to increase the diffusive flux to the cells and production of biosurfactants, to increase the apparent dissolved concentration of PAHs (Johnsen *et al.,* 2005).

Another important aspect to be considered is the sorption of biosurfactants onto soil, a condition that can cause natural surfactant losses, which in turn reduce the performance of the solubilization of hydrophobic contaminants (Zhou and Zhu, 2007), so the critical micelle concentration to reach solubilisation is much higher than the aqueous concentration. Degradation took place in all the microcosm experiments even though the rates and extents of degradation vary. The percentage of Phenanathrene degraded is relative to the amount of Lipopeptide supplied up to the optimum amount, for example the 400 mg/L Lipopeptide amended microcosm showed high desorption and the highest rate of degradation. Whereas the amendment of 700 mg/L resulted in inhibited degradation relative to the others at the outset but 6 days later rapid degradation was observed. This longer lag phase may be due to toxicity and increased substrate concentration following enhanced solubilization that took the bacteria longer time to adapt. Noticeably, the addition of surfactants to contaminated systems can enhance the desorption and mobility of hydrophobic contaminants, but the increased aqueous contaminant concentrations do not necessarily result in concomitant enhancement of biodegradation (Stelmack *et al.,* 1999).

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Microbial growth of up to three orders of magnitude occurred in the micrcosms from 2.88 log (CFU /mL) at the beginning of the experiment to 4.7 log CFU/mL and to 7.7 log CFU/mL in the zero Lipopeptide supplemented and 400 mg/L supplemented microcosms respectively (Fig. 5.3).

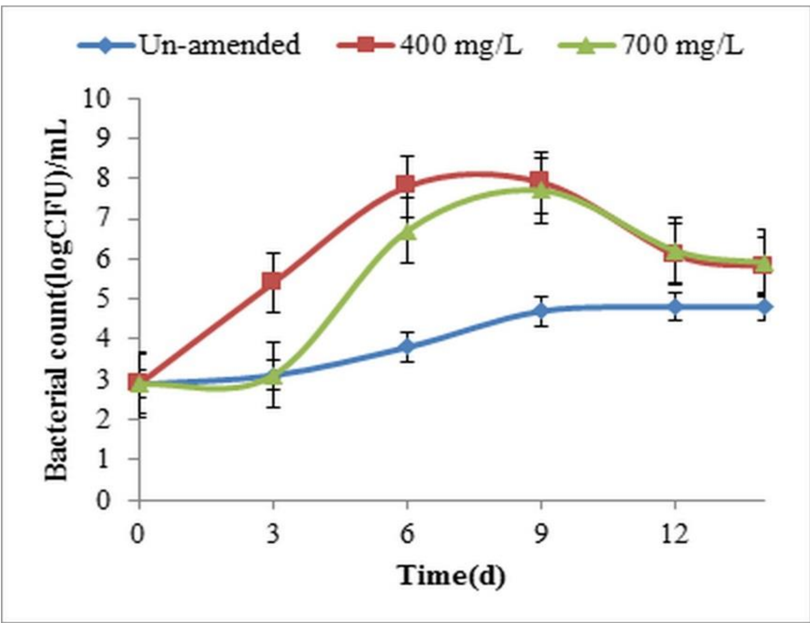

Figure 5.3: Bacterial count showing increase in bacterial growth in the biosurfactant amended and unamended microcosms.

For the Lipopeptide un-amended microcosm as well since the inoculant *Pseudomonas aeruginosa* species is a potent biosurfactant producer, it was able to produce its own biosurfactant and desorp the substrate as is normally expected even ethough the extent and rate is less than the biosurfactant amended ones. This study also demonstrated that bioaugmentation with surfactant-producing bacteria could enhance *in situ* bioremediation of soils contaminated with polycyclic aromatic hydrocarbons (PAHs).

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CHAPTER 6

BIOSURFACTANT-ASSISTED MIXED PAHS DEGRADATION BY PAH-DEGRADING MICROBIAL CONSORTIUM

6.1 Microbial Composition

The predominant microbial cultures were isolated and characterized by their morphological and biochemical characteristics. Studies of morphological and 16S rRNA identification of the dominant bacterial isolates revealed that the cultures were predominantly composed of *Serratia Marcescens, Klebsiellla Oxytoca, Pseudomonas aeruginosa, Bacillus cereus, Bacillus subtillis* as identified by the nucleotide 16S rRNA gene sequencing. Partially sequenced amplified 16S rDNA fragments were 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 1,000 bootstrap sets that was subsequently used to construct a phylogenetic tree (Fig. 6.1) using the neighbour-joining method MEGA version 6 software (Tamura *et al.,* 2013).

6.2 Biosurfactant-enhanced biodegradation of ternary mixture of PAHs in liquid culture

The lipopetide biosurfactant produced by LBP9 was used in this biodegradation study; the lipopetide has a critical micelle concentration (CMC) of 150 mg/L. During the biodegradation of the mixed PAHs consisting of Phenanthrene, Fluoranthene and Pyrene, differing degradation rates and extent were observed for each PAH at different concentrations of Lipopeptide. The data indicate that optimum degradation of PHE were observed on the 200 mg/L and 400 mg/L amended microcosms whereas the 800 mg/L and 1600 mg/L amended microcosms showed inhibited degradation rates (Fig. 6.2).

Increased bioavailability does not necessarily support enhanced biodegradation of the PAHs. On day 24 of incubation better degradation was observed as well in the inhibited microcosms after an extended lag phase, nevertheless the degradation was not statistically significant ($p > 0.05$).

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Serratia marcescens strain HS-L5(JF718272.1)
Serratia marcescens strain CM96(KF782799.1)
61
Serratia marcescens strain Z6002(KC212065.1)
97
Serratia sp. WN2(KP993209.1)
ML2
Serratia nematodiphila strain faro7 41A(KF792276.1)
95
ML3
Enterobacter cloacae strain M3207d (FR821640.1)
Klebsiella oxytoca strain M.pstv.35.3(KM108559.1)
99
Klebsiella sp. SW81(HM584796.1)
Klebsiella oxytoca strain ss-11(GU993916.1)
Klebsiella oxytoca strain SB2908(FR821668.1)
ML5
Pseudomonas aeruginosa strain 503(KF039909.1)
Pseudomonas aeruginosa strain cifa chp14(KC895885.1)
100
Pseudomonas aeruginosa strain ALK317(KC456532.1)
Pseudomonas aeruginosa strain ALK316(KC456531.1)
Bacillus cereus strain MAL 3A(KM251856.1)
Bacillus cereus strain AVSCE-4(KM236236.1)
84
Bacillus cereus strain MAL 3E(KM251858.1)
Bacillus cereus strain MAL 5A(KM251862.1)
ML1
100
ML4
Bacillus subtilis strain BCH1074(KM983009.1)
Bacillus subtilis subsp.(LC040931.1)
99
Bacillus subtilis strain 2xWM-ARB26(KJ961510.1)
Bacillus subtilis PPB11(KM092527.1)
0.05

Figure 6.1: Phylogenetic relationships on the basis of 16S rRNA gene sequence data of the predominant bacteria isolated from automobil garage and car service station soil and some reference strains, using neighbor-joining method, Based on 1000 bootstrap resamplings. The accession numbers are given in parentheses.

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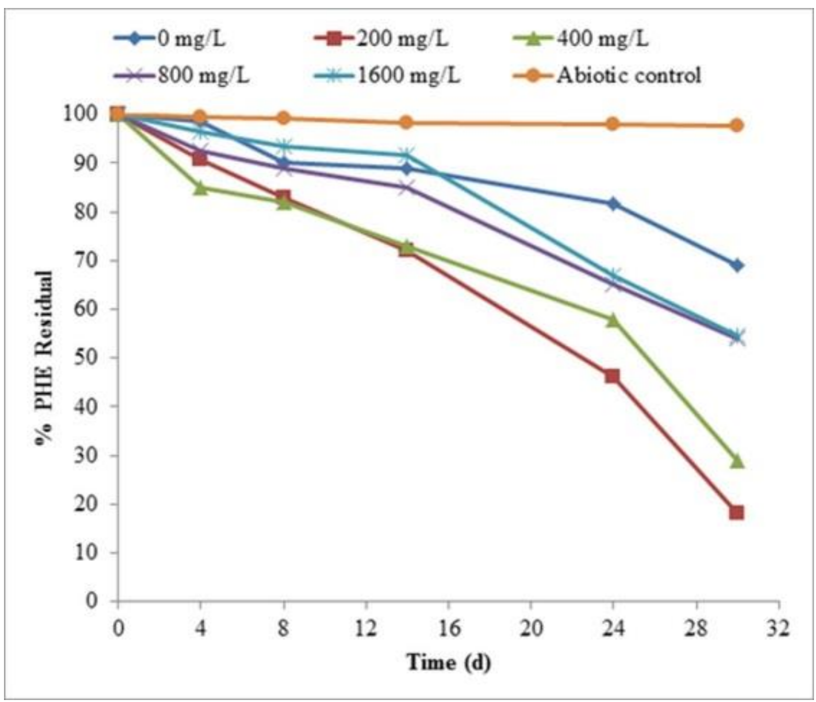

Figure 6.2: Percentage of Phenanthrene residual left at different concentration of Lipopeptide Values are the mean ± standard deviation (n = 3). Error bars represent standard errors.

At day 30 the maximum degradation rates of 82% and 71% were observed at 200 mg/L and 400 mg/L amendments while only 31% took place in the unamended control (in comparison to the abiotic control). The concentrations of Phenanthrene in the abiotic controls dropped to 98 % at day 4 and were not having significant losses afterwards.

Comparable results were obtained for Fluoranthene (Fig. 6.3) where optimal degradation happened at 200 mg/L and 400 mg/L Lipopeptide amendments and at higher concentrations of 800 mg/L and 1600 mg/L the rates were inhibited.

At day 30 the maximum degradation of 69% and 57% were observed at 200 mg/L and 400 mg/L amendments while only 21.8% took place in the unamended one (in comparison to the abiotic control). The losses of Fluoranthene in the abiotic controls were less than 1% throughout the experiment.

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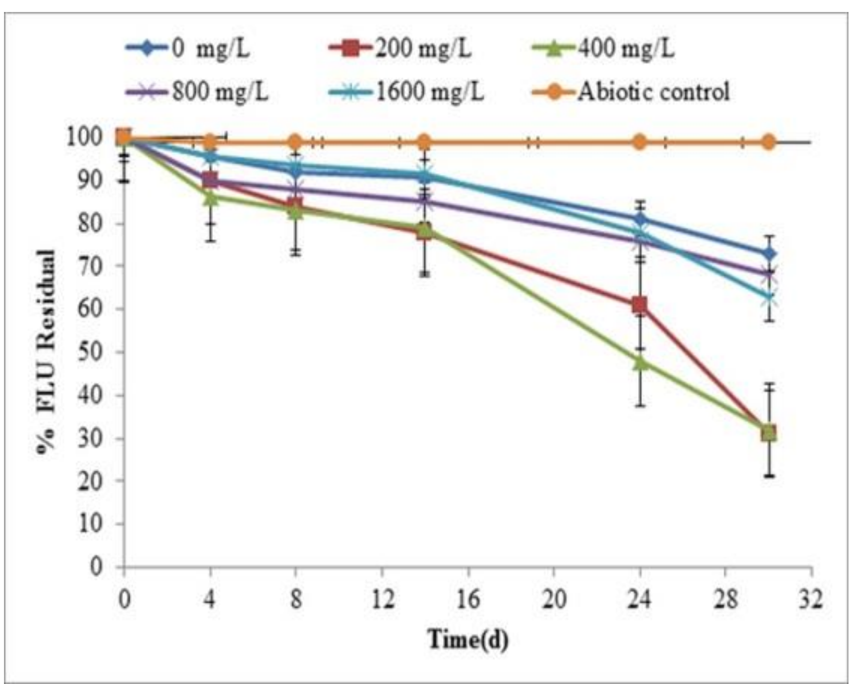

Figure 6.3: The percentage of Fluoranthene residual left at different concentration of Lipopeptide Values are the mean ± standard deviation (n = 3). Error bars represent standard errors.

For Pyrene (Fig. 6.4), the degradation was lesser when compared to Phenanthrene and Fluoranthene. Similar results of optimum degradation were observed at 200 and 400 mg/L, amendments. At day 30 the maximum degradations of 43% and 33% were observed at 200 mg/L and 400 mg/L amendments while only 12.2% degradation took place in the unamended one (in comparison to the abiotic control). The losses of Pyrene in the abiotic control were less than 1% throughout the experiment

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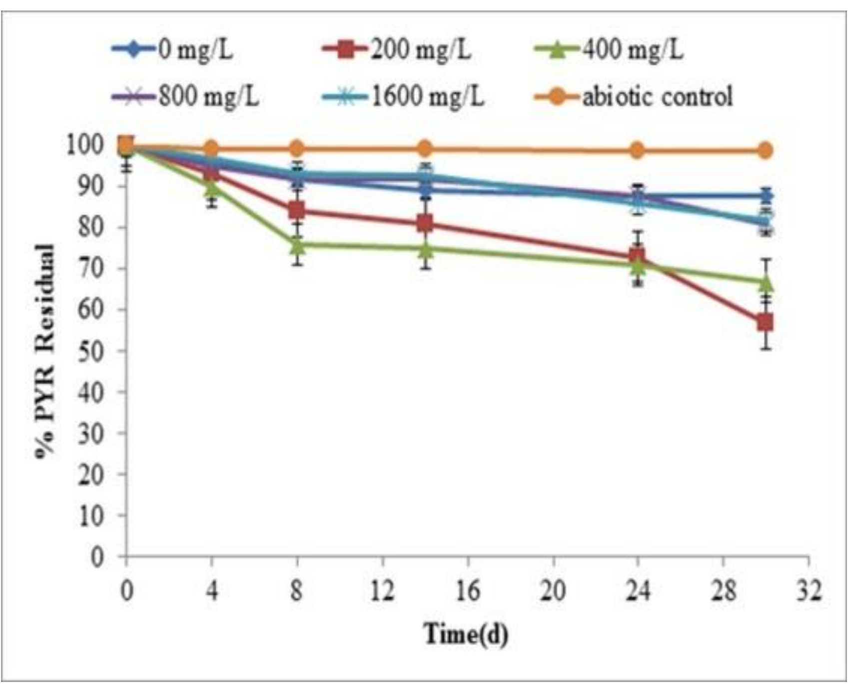

Figure 6.4: Percentage of Pyrene residual left at different concentration of Lipopeptide, Values are the mean ± standard deviation (n = 3).

The consortium showed 24.3% and 52% degradations of Fluoranthene at the 800 mg/L and 400 mg/L amendments respectively on day 24 (in comparison to the abiotic control). The extent of degradation of Phenanthrene was 42% at 400 mg/L, and 35% at the 800 mg/L amendments. Pyrene being more hydrophobic and more resistant to microbial biodegradation was 29% degraded at 400 mg/L amendments on day 24 of incubation, while its degradation was only 12% at 800 mg/L amendment.

6. 3 Biosurfactant-enhanced biodegradation of sole and dual substrate PAHs

On a parallel experiment under the same conditions but with Phenanthrene, Fluoranthene and Pyrene alone as sole substrate biosurfactant assisted biodegradation tests were conducted. Stimulated and inhibited degradation rates were observed at different amounts of Lipopeptide amendments. From the multisubstrate experiment it can be noted that that the optimum amount of Lipopeptide for effective performance of the consortium in all the microcosms were 200 mg/L and 400 mg/L Lipopeptide amendments. In the sole substrate studies the amendments at 0, 400 mg/L and 800 mg/L were studied.

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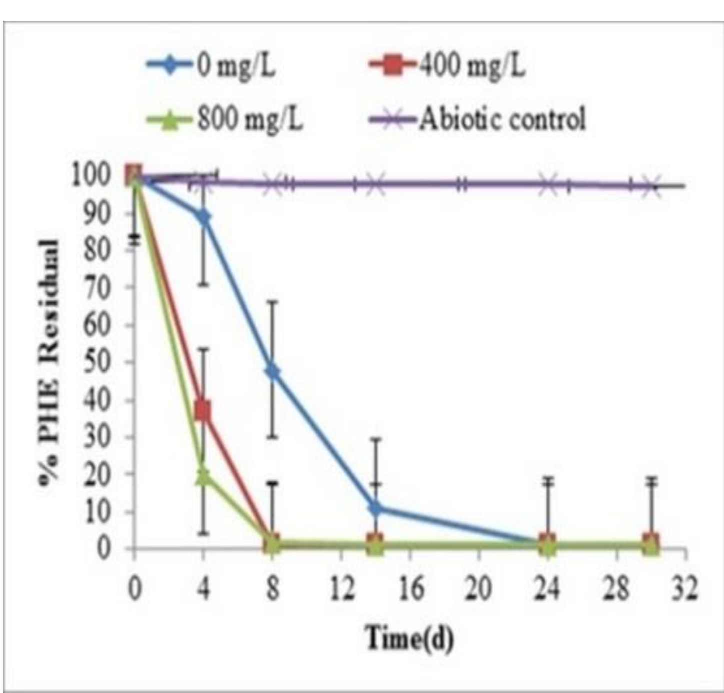

Figure 6.5a: Percentage of Phenanthrene residual when alone, Values are the mean ± standard deviation (n = 3). Error bars represent standard errors.

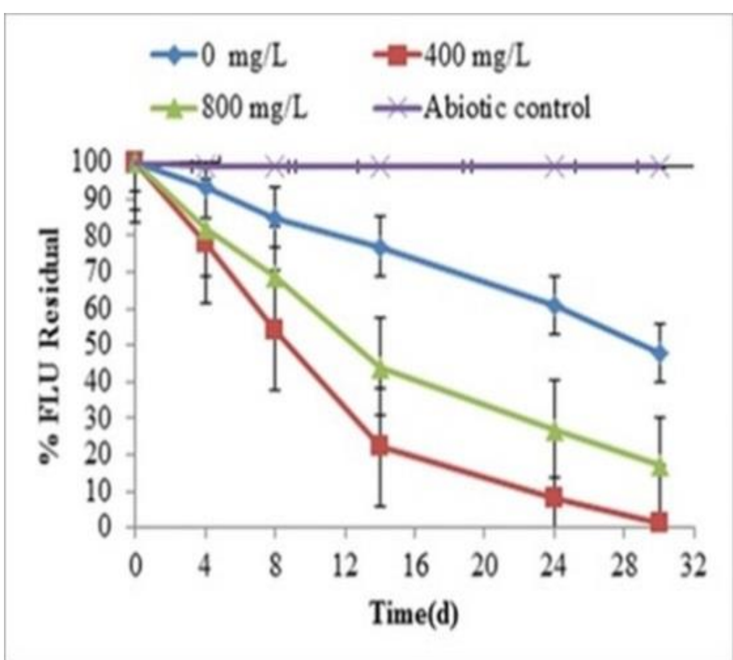

Figure 6. 5b: Percentage of Fluoranthene residual when alone, Values are the mean ± standard deviation (n = 3). Error bars represent standard errors.

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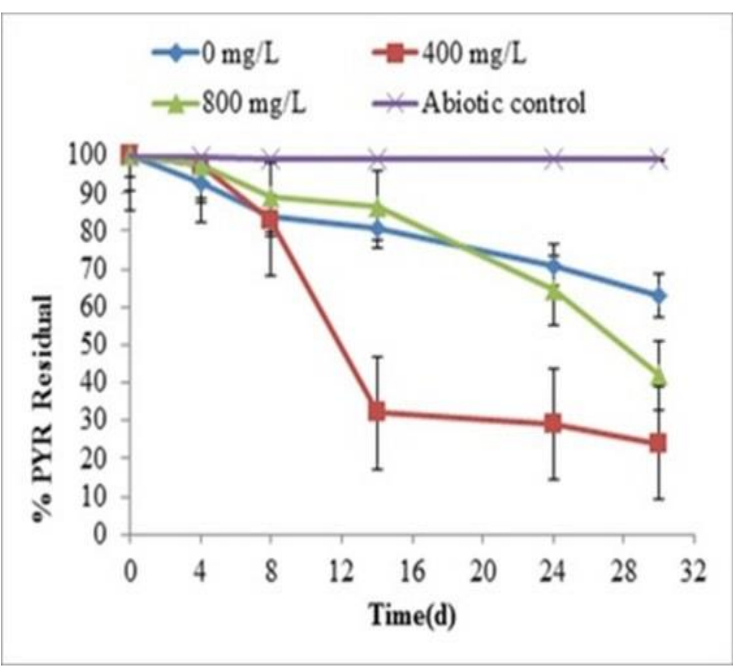

Figure 6.5c: Percentage of Pyrene residual when alone, Values are the mean ± standard deviation (n = 3). Error bars represent standard errors.

The consortium degraded phenanthrene fastest (Fig. 6.5a), then Fluoranthene (Fig. 6.5b) and Pyrene at last (Fig. 6.5c). Decrease in degradation extent was observed as recalcitrant nature of the PAHs increase with decreasing solubility and increasing molecular weight.

The consortium displayed inhibited biodegradation of Pyrene (Fig. 6.5c) in the Lipopeptide amended microcosms during the first 4 and 8 day experiments in all the microcosms. After day 14 of incubation an enhanced degradation of Pyrene was observed in the 400 mg/L Lipopeptide amended microcosm as it degraded 68% of the Pyrene while there was only 19% degradation in the unamended control and 13.4% in the 800 mg/L amended microcosm. At day 30, 76% of the Pyrene was degraded in the 400 mg/L amended microcosms while the unamended control and 800 mg/L amended microcosms showed 37% and 58% degradation respectively in relation to the abiotic control. Statistically significant biodegradation rates ($p < 0.05$) were observed in the sole substrate experiments at 400 mg/L amendments whereas the degradation at 800 mg/L showed inhibition and were not statistically significant($p > 0.05$) according to student two tailed - test in comparison to the unamended controls.

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In the above experiments the biosurfactant enhanced the biodegradation of the PAHs in both single and mixed PAHs by up to three folds at optimum level of concentration.

The optimum amount of Lipopeptide for statistically significant ($p < 0.05$) degradation performance of the consortium were observed to be the 200 mg/L and 400 mg/L amendments .On the multisubstrate experiments 78% and 71% of Phenanthren was degraded on day 30 at 200 mg/L and 400 mg/L Lipopeptide amendments respectively while 31% degradation was observed on the unamended control (Fig. 6.2). Fluoranthene depletion was 69% and 57% at 200 mg/L and 400 mg/L amendments respectively during which only 21.8 % degradation was observed on the unamended microcosm at day 30 (Fig. 6.3). On the other hand 43 % and 33 % degradation of Pyrene, the least soluble most recalcitrant PAH, was observed at 200 mg/L and 400 mg/L Lipopeptide amendments whereas only 12.2% degradation was observed on the unamended control on day 30 of incubation (Fig. 6.4).

When in the dual substrate with Phenanthrene the degradation rate of Pyrene was inhibited as only 37% was degraded on day 30 of incubation at 400 mg/L amendment of Lipopeptide and the unamended control showed only 16% degradation (Fig. 6.6a). In the dual substrate microcosm with Pyrene, the Phenanthrene biodegradation rate was 82% at day 8 and more than 95% was degraded at day 30 with 400 mg/L amendment (Fig. 6.6b). It can be seen that the degradation rate of Phenanthrene was not that much inhibited unlike the multi substrate experiment but still it can be observed that the rate was less than the sole substrate rate (Fig. 6.5a).

Microbial count results indicate that viable cell counts (log CFU/mL) increased from 5.1 to 7.42, 8.42 and 7.9 at day 30 with 0, 400 mg/L and 800 mg/L lipopeptide amendments respectively in the mixed substrate microcosm. This shows that the highest bacterial count is happening at the optimum lipopeptide concentration at which substrate consumption rate is higher. At 800 mg/L Lipopeptide concentration a slower growth in bacterial count was observed at day 30, which increased from 5.1 to 6.9 in the case of PYR, and from 5.1 to 6.5 in the mixed substrate microcosm, whereas at 400 mg/L amendment a faster bacterial growth was observed in both samples (Fig. 6.7). The highest bacterial count was observed for PHE which corresponds to its easiness and low recalcitrance to bacterial degradation which supported faster microbial growth from day 4 to 12 then bacterial count started declining following substrate depletion.

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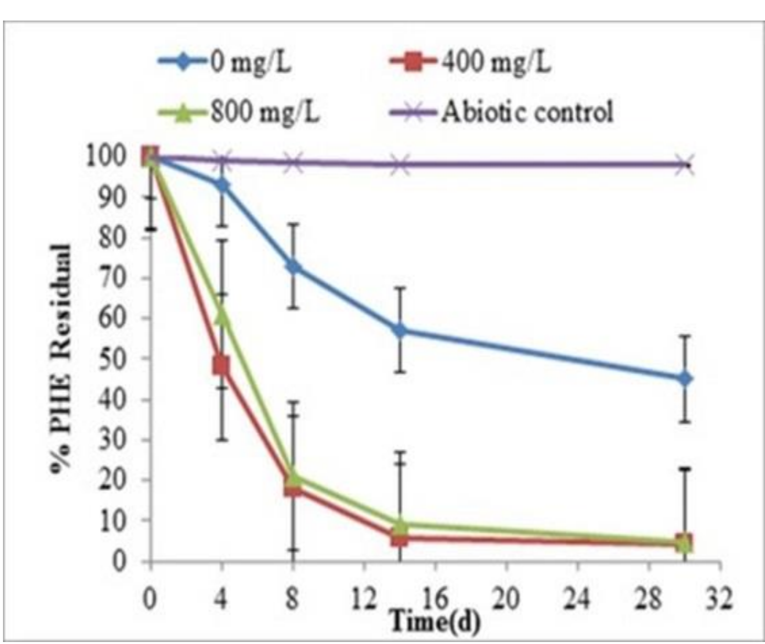

Figure 6.6a: Percentage of Phenanthrene residual in the binary substrate with Pyrene.Values are the mean ± standard deviation (n = 3), error bars represent standard errors.

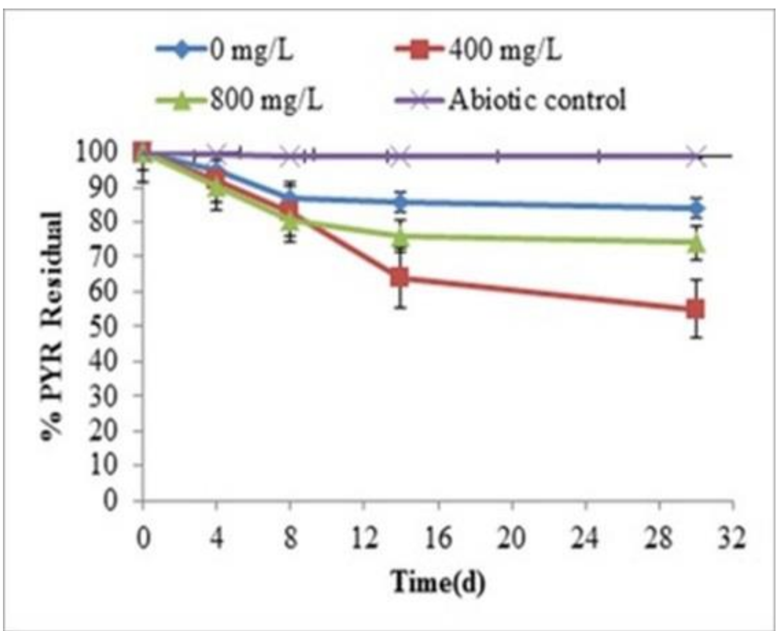

Figure 6.6b: Percentage of Pyrene residual in the binary substrate with Phenanthrene .Values are the mean ± standard deviation (n = 3), error bars represent standard errors.

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Figure 6.7: Viable Bacterial Counts in sole substrate PAHs, PHE (A), PYR (B) and in the mixed substrate PAHs of PHE, FLU, and PYR (C) under different Lipopeptide amendments.

In this study, the cell surface hydrophobicity percentage increased with the increase in Lipopeptide amendment from approximately 22% to 28% ,35% and 45% for the 200 mg/L, 400 mg/L, 800mg/L and 1600mg/L amended microcosms respectively, compared to approximately 11% in the unamended control (Fig. 6.8)

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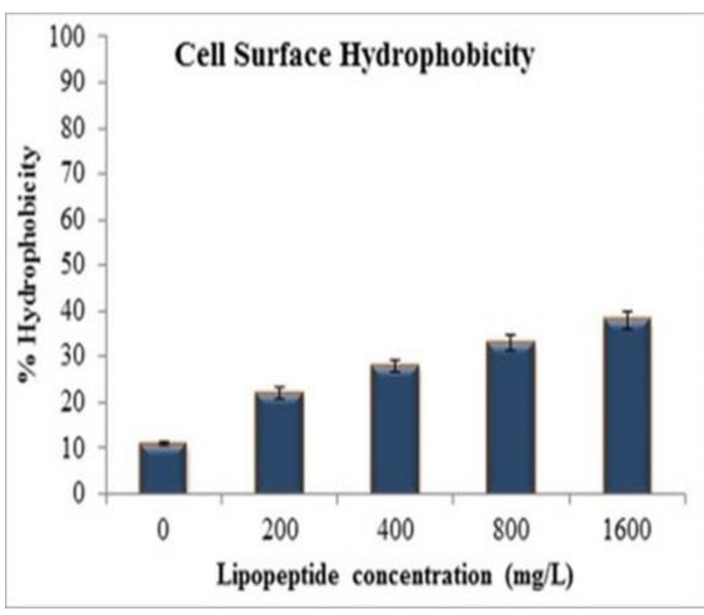

Figure 6.8: Cell Surface Hydrophobicity (CSH) of the microbial consortia in the ternary PAH microcosm in the presence of different concentration Lipopeptide. The values given are mean ± SD of three independent experiments taken at day 8 of incubation.

This work was initiated to study the role of biosurfactants on the biodegradation of mixed and sole substrate PAHs by microbial consortium. The biosurfactant used in our experiments are among the most studied surfactants in bioremediation/ biodegradation of PAHs (Bordoloi and Konwar 2009; Xia et al., 2014). It was shown that the lipopeptide biosurfactant at optimum amounts has a stimulating effect on the dynamics of the PAH degradation. Similar observations of biosurfactant enhanced bioremediation of polycyclic aromatic hydrocarbons and crude oil were reported by (Thavasi *et al.,* 2011a; Sponza and Gok, 2012; Xia *et al.,* 2014).

In the experiments the degradation rate at Lipopeptide concentrations of 800 mg/L and 1600 mg/L was not statistically significant ($p > 0.05$). 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

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surfactant micelle (e) surfactants' use as preferential growth substrates by degrading microorganisms (Avramova *et al.,* 2008). Surfactant toxicity includes cellular toxicity from interaction of surfactant molecules with cell membranes or cell membrane bound proteins, inhibition of enzymes of the catabolic pathway either by association with the enzyme or with the substrate, the accumulation of toxic intermediates due to incomplete metabolism incurred from substrate–surfactant interactions (Sponza and Gok, 2012). A similar observation was reported by Whang et al. (2008) during studies focused on biosurfactant-mediated biodegradation of diesel contaminated water and soil carried out by autochthonic soil microorganisms during batch diesel/water experiments and biopile tests. The authors observed that even though diesel solubilization was slightly higher for surfactin, especially above the CMC value, the presence of this surfactant may limit the biodegradation rate at concentrations above 40 mg/L (with a complete inhibition at 400 mg/L).

Biosurfactants can enhance hydrocarbon bioremediation by two mechanisms (Stoimenova *et al.,* 2009; 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. 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. Overall study showed that the Lipopeptide increased solubility (Fig. 4.2) of the PAHs and cell surface hydrophobicity (Fig. 6.8) of the microbial consortium and enhanced their degradation at optimum amounts of supplementation but failed to enhance the degradation process at higher concentrations of Lipopeptide supplementations. Bacterial CSH has been reported to be one of the most important parameters regulating the interactions among cell, hydrophobic substrates and various solid surfaces (Chakraborty and Mukherji, 2010). In general, microorganisms with high CSH adhere to or adsorb hydrocarbons ardently. The effects of lipopeptide on bacterial CSH studied (Fig. 6.8) revealed the interactions between surfactant and bacterial cell surface. The surfactants at concentrations range of 0 – 1600 mg/L promoted CSH,

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but addition of surfactant at 800 mg/L and 1600 mg/L (6.4 and 12.8 CMC) may have increased PAH bioavailability to a level toxic for the consortium, thus decreasing the growth and consequently the degradation of PAHs or there were inhibitory effects due to the toxicity of the high concentration of biosurfactant itself that inhibited the growth and PAH biodegradation activity of the consortium (Ward, 2010). Observation of toxicity of increased biosurfactant concentration on the bacteria was reported by (Vasileva-Tonkova *et al.,* 2011; Sotirova *et al.,* 2009). Vasileva-Tonkova et al. (2011) reported complete inhibition of the growth of *B. subtilis* 168 at higher concentrations of biosurfactant. Earlier reports suggest that both nonionic surfactants and biosurfactant have an ability to modify the cell surface (Zhang *et al.,* 2013). Cell surface hydrophobicity is influenced by surfactant type and concentration along with the physiological status of the cell (Zhong *et al.,* 2008). Sotirova et al., (2009) reported that rhamnolipids modify the cell hydrophobicity by reduction of lipopolysaccharides (above CMC). The modification of CSH by the addition of surfactants occurred due to removal of lipopolysaccharide (LPS) (Li and Zhu, 2012; Zhang *et al.,*2013) or the adsorption/Partition of surfactants (Zhong *et al.,* 2007a).The lipopolysaccharides are important to gram-negative bacterial cells since they help to provide a permeability barrier to hydrophobic substances and protect the sensitive inner membrane and the cell wall from the effect of hydrocarbons and other toxic compounds (Vasileva-Tonkova *et al.,*2011). So the removal of Lipopolysahride increases cell permeability to the highly bioavailable contaminant concentration above a threshold level whereby toxic effects of the contaminant are exhibited (Makkar and Rockne, 2003; Ward, 2010).

The increased solubility of the mixed PAHs enhanced the degradation at optimum amount of Lipopeptide, however when the PAHs were present simultaneously inhibitory effects were observed showing substrate interactivity effect as another factor that determines their degradation despite the increased bioavailability of the PAHs. Phenanthrene degradation appears inhibited by the presence of Fluoranthene and Pyrene. The degradation of Phenanthrene in the ternary microcosm was reduced, despite its increased bioavailability, when it is in the mixed PAHs. In the experiment under the same conditions with phenanthrene alone as single substrate (Fig. 6.4a) it took 4 days for its 63% degradation at 400 mg/L amendment, while it took 24 days to attain 42% degradation in the ternary substrate experiment under the same condition (Fig. 6.1), this clearly shows that the degradation of the easily degradable phenanthrene was inhibited in the mixed PAHs experiment. These results were consistent with the several previous reports (Zhong *et al.,* 2010;

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Lu *et al.,* 2014). When Phenanthrene was present, the degradation of Pyrene was inhibited in the binary mixture of PAHs. This result is in line with the results obtained by previous studies (McNally *et al.,* 1999), in which the degradation of Pyrene by pure culture of *Pseudomonas putida strain KBM-1* was competitively inhibited by the presence of Phenanthrene. This observation contradicted the result reported from related research by Lu et al. (2014) where Pyrene degradation was reported to have been enhanced by presence of Phenanthrene.

PAH biodegradation by microbial culture is liable to positive and negative effects of substrates and their conversion products (Baboshin and Golovleva, 2011). Reasons for retarded PAH biodegradation rates include substrate competitive inhibition (Stringfellow and Aitken 1995) and inhibition by formation of toxic intermediates (Casellas *et al.,* 1998) and hastened by cross induction of required degradative enzymes (Molina *et al.,* 1999), co-metabolism (Bouchez *et al.,* 1995), and the increase of biomass concentration during the growth (Guha *et al.,* 1999). Similarly Guha et al. (1999) observed substrate interactions in binary and ternary mixtures of naphthalene, phenanthrene and pyrene using a PAH-degrading consortium. In a similar study Zhong et al.(2007b) reported that removal of phenanthrene by a *Sphingomonas sp.* was either incomplete in presence of Fluoranthene or delayed in presence of Anthracene or Fluorene—indicating interactive behaviour among the PAHs on the rate and extent of their biodegradations in mixture. Stringfellow and Aitken (1995) studied biodegradation kinetics of Phenanthrene in binary mixtures, containing the compound with naphthalene, 1-methylnaphthalene, 2-methylnaphthaleneor fluorene, and observed competitive inhibition towards Phenanthrene degradation, suggesting that these PAHs probably share a common enzyme system. Similar observations were reported by (Ma *et al.,* 2013) that the same or similar enzymes may be responsible for the degradation of mixed PAHs. Competition for the active site(s) of the enzyme would occur when multiple substrates are simultaneously present (Mahanty *et al.,* 2010; Ma *et al.,* 2013). Competitive metabolism is a common phenomenon among PAH-degrading organisms, and this is based on the observation of competitive interaction in physiologically diverse microorganisms (Stringfellow and Aitken, 1995).

6.4 Modelling of biodegradation kinetics.

Kinetic analysis is a key factor for understanding biodegradation process, bioremediation effectiveness. Monod model was chosen because it incorporates both the microbial growth rate

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and the degradation of available substrates and additionally this model can be extended to sole- and multi-substrate systems, assuming common enzyme systems (Guha *et al.,* 1999).The Monod model establishes the relationship between the growth rates (μ), concentration of a single growth substrate (C), and relates growth with substrate utilization q (Monod 1949). The parameters used in the Monod model are the maximum specific growth rate $\mu_{\max}$ or maximum substrate utilization rate (q_{max}) and the affinity coefficient (K_s).

6.4.1 Sole-substrate model.

The simplest means of describing the biodegradation rate is the sole-substrate biodegradation model, based on Monod kinetics, as mentioned in the **Data Analysis Subsection** 3.8.2, is used.

$$\frac{dC}{dt} = -q_{\max} \frac{C}{K_S + C} X \quad \ldots\ldots \quad (6.1)$$

$$\frac{dX}{dt} = Yq_{\max} \frac{C}{K_S + C} X - bX \quad \ldots\ldots \quad (6.2)$$

Where $q_{max,i}$ is the maximum substrate utilization rate per unit biomass for substrate i (mg substrate/mg protein/h) and $K_{s,i}$ is the half-saturation coefficient for substrate i (mg/L). The parameters used in the Monod model are the maximum substrate utilization rate (q_{max}) and the Half-saturation constant or affinity coefficient (K_S). The affinity coefficient or the half velocity constant represents the concentration at which the substrate utilization rate is equal to half of the maximum substrate utilization rate (Monod 1949).

6.4.2 Multisubstrate model with competitive inhibition.

In most cases, aqueous-phase PAH mixture conversion can be satisfactorily described by a multisubstrate model, according to which all mixture components compete for the same common active site in accordance with the principles of Michaelis kinetics (Guha *et al.,* 1999; Lotfabad and Gray 2002; Knightes and Peters 2006; Dimitriou-Christidis and Autenrieth, 2007; Baboshin and Golovleva, 2011). Several models have been proposed to predict the type of interaction in such complex substrate systems. Most models have been tested with only two substrates, and their applicability to larger mixtures has been assumed without validation (Mohanty *et al.,* 2013). However, a few models have been proposed and tested for multicomponent mixtures with more than 2 components. Some examples are growth of a mixed culture on benzene, toluene,

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ethylbenzene, and *o*- and *p*-xylene (BTEX compounds) (Bielefeldt and Stense, 1999), and the biodegradation of three polynuclear aromatic hydrocarbons (Guha *et al.,* 1999). (Guha *et al.,* 1999) proposed a multisubstrate Monod kinetic model for determining substrate interactions between PAHs. (Guha *et al*., 1999) studied substrate interactions in binary and ternary mixtures of NPH, PHEN, and PYR using a PAH-degrading consortium. That work demonstrated the importance of substrate interactions in simple mixtures and demonstrated the feasibility of using predictive multisubstrate modeling, laying the groundwork for its use in more complex Systems (Knightes and Peters, 2006). The multisubstrate model with competitive inhibition represents the case in which multiple substrates are available to the microorganisms but the substrates compete for the limited number of enzymes present. For a simple inhibition system, when the enzyme binds with the inhibitor and the given substrate, the Michaelis–Menten kinetics may be represented as

$$\frac{dC}{dt} = q_{\max,i} \frac{C_i}{K_{s,i}(1+\frac{C_i}{K_I}) + C_i} X \quad \text{(6.3)}$$

Equation 6.3 is valid for a non-reactive inhibitor (Stryer 1995).

On the other hand when the inhibitor is present as an alternate substrate, the multisubstarate model demonstrated by (Guha *et al.,* 1999; Knightes and Peters, 2006) is used (**Equation 6.4**).

$$\frac{dC}{dt} = q_{\max,i} \frac{C_i}{K_{s,i,} + \sum_{j=1}^{n} \frac{K_{s,i}}{K_{s,j}} C_j} X \quad \text{(6.4)}$$

$q_{max,i}$ is the maximum substrate utilization rate per unit biomass for substrate i (mg substrate/mg protein/h) and is $K_{S,i}$ the half-saturation coefficient for substrate i (mg/L)., C_i is the concentration of substrate i, (mg/L) , is $K_{S,i}$ the Half-saturation constant for substrate i, and $K_{S,j}$ is the half saturation constant for each substrate j. The model uses the parameters derived from the sole substrate case (Knightes and Peters, 2000). It requires that the compounds be utilized through a common pathway (Segel, 1975). Equation 6.4 can be extended for any number of components provided the compounds exhibit competitive inhibition kinetics.Thus, in Equation 4, $K_{S,j}$ represented a constant, while $K_{S,i}$, $q_{\max,i}$ are fitting parameters.

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Parameter estimation

The parameters obtained from the sole substrate experiments formed a basis for modelling multicomponent systems (Knightes and Peters, 2006). No new parameters are estimated for the multisubstrate experiments.

In determining the parameters the Monod kinetic model was fitted in to the experimental data, the parameters $K_{S,i}$ and $q_{\max,i}$ are estimated and the deviation between the model and experimental results was compared after consecutive iteration up until the fitting of the experimental data to the model shows minimum deviation using AQUASIM software package (Reichert, 1998).The values of the parameters are determined as shown in Table 6.1.

Table 6.1 Monod kinetic parameters ($q_{\max}$ and K_S) obtained from the sole-substrate biodegradation experiments at different concentrations of Lipopeptide (0, 400 mg/L and 800 mg/L). The corresponding p-Values read from Table of Critical Chi Square values are < 0.05, which gives alevel of confidence of 95%.

Parameters at different Lipopeptide concentration		PAHs		
Lipopeptide conc.	Parameters and goodness of fit(χ^2)	PHE	FLU	PYR
0 mg L^{-1}	K_S (mg/L)	34.00	79.76	100
	q_{max} (mg mg $^{-1}$ d^{-1})	11.40	3.39	2.78
	χ^2	12.6	14.5	16.6
400 mg L^{-1}	K_S (mg/L)	20.00	52.00	100
	q_{max} (mg mg $^{-1}$ d^{-1})	24.03	10.4	8.80
	χ^2	11.6	13.4	17.6
800 mg L^{-1}	K_S (mg/L)	33.23	100	100
	q_{max} (mg mg $^{-1}$ d^{-1})	28.07	8.73	1.79
	χ^2	12.4	15.7	16.7

As can be seen from Tables 6.1, the results of parameter estimation performed by AQUASIM, along with the minimum Chi square values obtained show the high level goodness of fit. Entering

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the Chi square distribution table with 2 degrees of freedom (df = 2) and reading the values of $\chi 2$ we find the alpha level of significance. The corresponding probability is less than 0.050 probability levels, which shows 95% level of confidence.

From the Monod substrate utilization rate parameters for the sole substrate utilizations (Table 6.1) Phenanthrene utilization rate q_{max} increased from 11.4 to 24.3 $mg\,mg^{-1}d^{-1}$ with the addition of 400 mg/L of Lipopeptide and to 28.07 $mg\,mg^{-1}d^{-1}$ with 800 mg/L amendment. FLU utilization rate increased from 3.39 $mg\,mg^{-1}d^{-1}$ to 10.4 $mg\,mg^{-1}d^{-1}$ with 400 mg/L amendment of Lipopeptide while with 800 mg/L amendment it showed lower utilization rate of 8.73 $mg\,mg^{-1}d^{-1}$. PYR utilization rate increased from 2.78 $mg\,mg^{-1}d^{-1}$ with no amendment to 8.8 $mg\,mg^{-1}d^{-1}$ with an amendment of 400 mg/L of Lipopeptide and the utilization rate was reduced to 1.79 $mg\,mg^{-1}d^{-1}$ at 800 mg/L Lipopeptide amendments (Table 6.1).

The substrate utilization rate enhancement effect of the biosurfactant was observed on the more hydrophobic PAHs FLU and PYR. The substrate utilization rate increased three fold (from 3.39 to 10.4 $mg\,mg^{-1}d^{-1}$ and from 2.78 to 8.8 $mg\,mg^{-1}d^{-1}$) for FLU and PYR respectively at 400 mg/L amendment. While the the rate was slowed at 800 mg/L amendment to 8.73 and 1.79 $mg\,mg^{-1}d^{-1}$ for FLU and PYR respectively.

Very fast utilization rate of Phenanthrene was observed (from 11.4 $mg\,mg^{-1}d^{-1}$ to 24.03 $mg\,mg^{-1}d^{-1}$ and 28.07 $mg^{-1}d^{-1}$) at both 400 mg/L and 800 mg/L amendments respectively. Phenanthrene degradation was stimulated at 800 mg/L amendment as well unlike the inhibited Pyrene and Fluoranthene degradations at the same Lipopeptide dosage. This may be due to the adaptation of the consortium as it was enriched on Phenanthrene, Anthracene and Naphthalene mixtures. Moreover rapid substrate utilization rate of the lowest molecular weight Phenanthrene was observed with the utilization rate decreasing as the molecular weight and hydrophobicity increases.

After the experimental data were fitted in to mulstisubstrate model (Equation 6.4) and parameters were determined using AQUASIM software (Reichert, 1998) parameter estimation technique. The values of the maximum substrate utilization rate (q_{max}) at 400 mg/L amendment in the multisubstrate were determined as 5.6 $mg\,mg^{-1}d^{-1}$ for PHE, 5.51 $mg\,mg^{-1}d^{-1}$ FLU and 5.43

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$\mathrm{mg\ mg^{-1}d^{-1}}$ for PYR. This shows a decrease from the sole substrate utilization rates of 24.03, 10.4 and 8.8 $\mathrm{mg\ mg^{-1}d^{-1}}$ for Phenanthrene, Fluoranthene and Pyrene respectively (Table 6.2).

Table 6.2: Estimated kinetic parameters of multi substrate experiments after fitting of the experimental results, at 400 mg/L amendment.

Parameters	Phenanthrene	Fluoranthene	Pyrene
q_{max} (mg mg $^{-1}$d^{-1})	5.6±0.80	5.51±0.12	5.43±0.3
K_S (mg/L)	16.7±2.10	9.2±1.6	11±1.0
$\chi 2$	176	132	106

The sole substrate and multisubstrate with competitive inhibition model predicted degradation rates of Phenanthrene, Fluoranthene and Pyrene at 400 mg/L Lipopeptide amendment are portrayed in (Fig. 6.9) and (Fig. 6.10) respectively. Generally the model predicted kinetic parameters well, although much better fitting was obtained when sole substrate data were fitted. In the multisubstrate cases, the fitting was slightly deviated as observed from the higher Chi square values (Table 6.2), however the correlation coefficient value is 96% at 95% level of confidence, Figs. 6.10. The results demonstrate that the multisubstrate model is able to predict the inhibitory effect of substrates to each other in the PAH mixtures.

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Figure 6.9: Comparison of sole substrate experimental observations (dotted lines) for Phenanthrene, Fluoranthene and Pyrene to sole-substrate (solid line) models.

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Phenanthrene in the multisubstrate

C[mg/l]

Time[d]

Fluoranthene in the multisubstrate

C [mg/l]

Time[d]

Pyrene in the multisubstrate

C [mg/l]

Time[d]

Figure 6.10: Comparison of multisubstrate experimental observations (dashed lines) for Phenanthrene, Fluoranthene and Pyrene to multisubstrate with competitive inhibition (solid line) models

6.4.3 Parameter sensitivity analysis

Sensitivity analysis was performed to evaluate the uniqueness of parameter estimates and the relative importance of parameters over the range of substrate concentrations (Robinson and Characklis, 1984). The sensitivity functions of a PHE, FLU and PYR concentration with respect to the model parameters i. e., $q_{\max}$ and K_S were analyzed. The plots shown in Fig. 6.11 show that

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the sensitivity functions of concentration of the PAHs(C) with respect to $q_{\max}$ and K_S are approximately linearly dependent. The dependence of C on the other two parameters $q_{\max}$ and K_S leads to a similar shape of the changes in C, just with a different sign and magnitude. This means that a change in C caused by a change in one parameter can be compensated by an appropriate change in the other parameter.

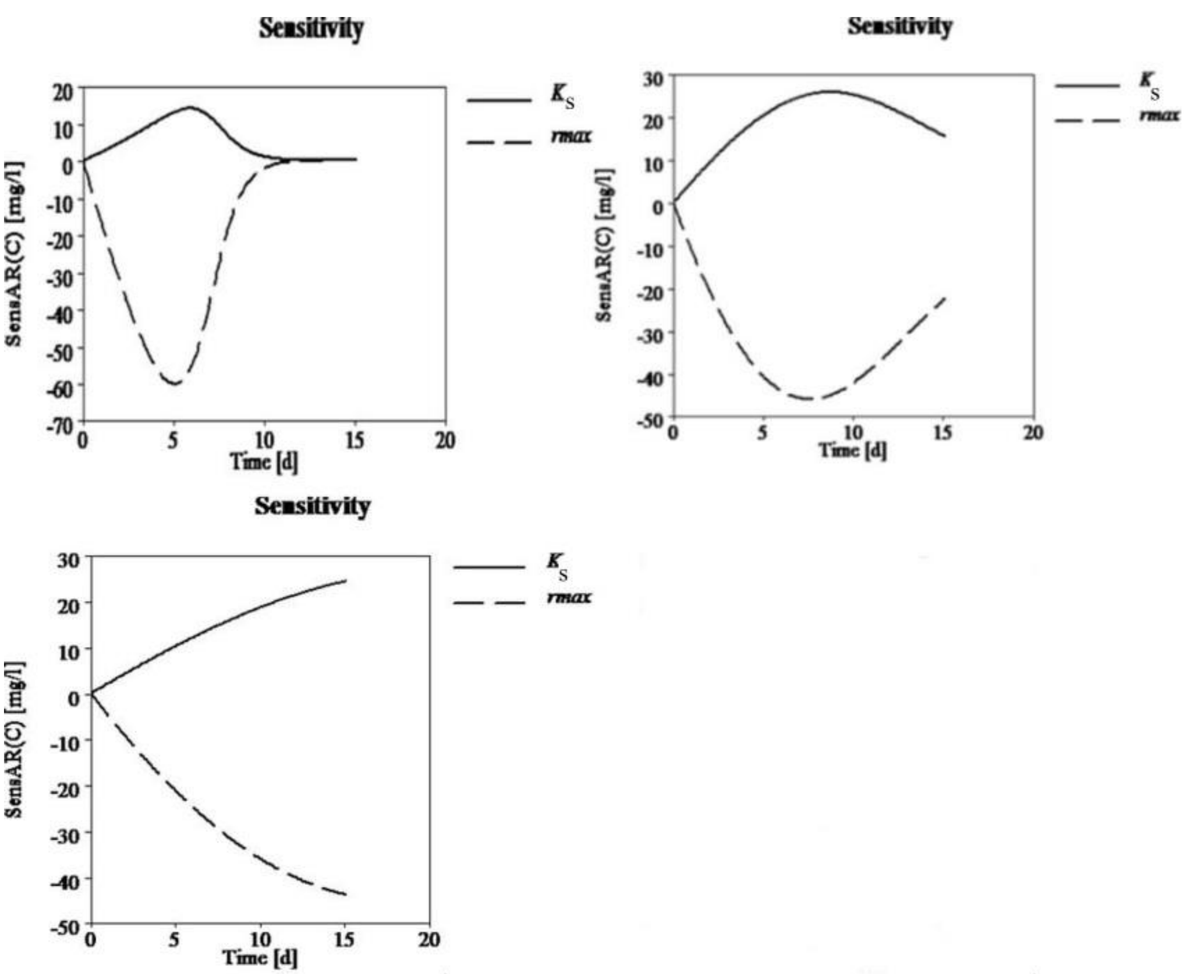

Figure 6.11: Sensitivity functions of PHE, FLU and PYR degradations by the microbial consortium at 400 mg/L Lipopeptide supplementation with respect to $q_{\max}$ and K_S

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6.5 Summary

It is generally accepted that limited bioavailability is the most important factor involved in the slow degradation of PAHs. The present study demonstrated that the biosurfactant enhanced the bioavailability and hence the degradation of the PAHs. Biosurfactants at optimum amounts enhanced the degradation of the pollutants even though the degradation in the multisubstrate microcosms depended on the interaction of the substrates as well. The success of biosurfactants in enhancing bioremediation depends on several factors including the biosurfactant type and its dose to be applied at the contaminated site, the hydrocarbon degrading microorganisms' response to solubilized hydrocarbons, the interaction of the hydrocarbon degraders with the biosurfactants and surfactant toxicity.

Biosurfactant dose determination plays a key role in the success of the bioremediation process. In the current study it can be noted that at the optimum amounts of biosurfactant the biodegradation rates were enhanced up to threefold while it inhibited the degradations of the PAHs beyond the optimum dose. The administration of high dose has a harmful effect on hydrocarbon degraders due to the toxicity of the biosurfactant itself to the degrading microorganisms or due to the solubilized PAHs toxicity, which are toxic to the degraders above a threshold level that would cause the membrane disruption of the microorganisms. Despite biosurfactant enhanced bioavailability of the PAHs Microbial degradation was affected by interaction effects of the multiple substrates present in the microcosm. When present in mixtures, PAHs have the capacity to influence the rate and extent of biodegradation of other components of the mixture. In some cases, these interactions may be positive, resulting in an increase in biodegradation of one or more components while in other cases negative effects have been reported. In the present study competitive inhibition was observed in the multisubstrate microcosm which resulted in inhibited substrate utilization rate of each PAH in the multisubstrate system.

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CHAPTER 7

BIOSURFACTANT ENHANCED BIOREMEDIATION OF AGED PAHs IN CREOSOTE CONTAMINATED SOIL

7.1 Microbial Culture

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 CB1 was one of these isolates that were found out to be efficient biosurfactant producers. The 16S rRNA analysis revealed that strain CB1 was related to members of genus *Pseudomonas*, and showed highest sequence similarity (100%) to *Pseudomonas aeruginosa.* Partially sequenced amplified 16S rDNA fragments were 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 1,000 bootstrap sets that was subsequently used to construct a phylogenetic tree using the neighbour-joining method MEGA version 6 software (Tamura *et al.,* 2013). The complete 16S rDNA sequences of the strains CB1, CB2, CB3, CB4, CN1, CN2, CN3 and CN5 have been deposited in the GenBank database under the accession numbers KP793922, KP793923, KP793924, KP793925, KP793926, KP793927, KP7939228, and KP793929 respectively. All the strains were preserved in the MSM broth with 30% (w/v) glycerol at −80 °C and used in subsequent biodegradation experiment.

Based on the 16S rRNA Gene Sequencing characterization, the strains isolated from the Creosote contaminated soil contained efficient PAH degrading and biosurfactant producing species like *Bacillus stratosphericus, Bacillus subtilis, and Bacillus megaterium, Paenibacillus dendritiformis, Pseudomonas aeruginosa, Ochrobactrum intermedium* (Figure 7.1).

These are only few members of the consortium which were identified as efficient biosurfactant producers. The strains isolated from the Creosote contaminated soil contained efficient PAH degrading and biosurfactant producing species like Bacilli – i.e., *Bacillus stratosphericus, Bacillus subtilis,* and *Bacillus megateriumi*, *Ochrobactrum sp* and *Pseudomonas aeruginosa*

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KJ856901 Bacillus subtilis subsp. subtilis strain K-5
KJ862218 Bacillus subtilis strain 5B
KJ733888 Bacillus subtilis strain Fse36
KJ607888 Bacillus subtilis subsp. subtilis strain RC1
KJ437491 Bacillus subtilis strain VIT-KADJK
84
KJ437478 Bacillus subtilis strain VIT-AAJ
KF181164 Bacillus subtilis strain JK17
CN2
CN1
CB3
63
LK939130 Bacillus subtilisstrain Y1
CB2
JQ647879 Bacillus subtilis strain SCSAAB0014
59
JQ818392 Bacillus stratosphericus strain NIOAD10
JX035948 Bacillus subtilis strain JDG32
Bacillus megaterium strain B-27
CB4
HF584842 Bacillus megaterium BD17-B11
HF584869 Bacillus megaterium BD18-E14
KJ534461 Bacillus megaterium strain RC55
JQ916899 Paenibacillus sp. LPUANSHUL
100
CN5
93
JN642249 Paenibacillus sp. DF9SA
JQ420900 Paenibacillus dendritiformis strain K125
JX393011 Ochrobactrum tritici strain W49-1
HQ235650 Ochrobactrum sp. SRDTH12
96
CN3
KJ831424 Ochrobactrum sp. 906B6 12AESBL
KJ831479 Ochrobactrum sp. ESBL496B15 13 3A
CB1
JF732908 Pseudomonas aeruginosa strain 211A
JQ792037 Pseudomonas aeruginosa strain 4nm-Pk-2011
JX008025 Pseudomonas aeruginosa strain HHP01
KJ999614 Pseudomonas aeruginosa strain
JQ312064 Paenibacillus alvei strain AIMST Ehe32
0.5

Figure 7.1: Phylogenetic tree diagram showing the identity of biosurfactant producing and PAH degrading species identified as most closely associated with *Pseudomonas aeruginosa, Bacillus ,* *Paenibacillus* and *Ochrobactrum sp.*

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7.2 Biosurfactant activity and characterization

Before going into detail investigation of the biosurfactant and culture performance, it was necessary to determine the nature of the crude biosurfactant produced by the isolate *Pseudomonas aeruginosa* CB1. The FTIR spectrum of the column purified biosurfactant showed bands characteristic of peptides at 3,217 cm^{-1} resulting from N–H stretching mode (Fig. 7.2). The absorption at wave numbers of 1458 cm^{-1}, reflect aliphatic chains ($–CH_3$, $–CH_2–$) of the fraction. The sharp peak around 1642 cm^{-1} (stretching mode of the CO–N bond) is due to amide group (Nasir and Besson, 2012). Additional reverse peaks in the wave number range 1100-1040 cm^{-1} indicated the presence of amine groups. The nature of compound that best fits this description is the lipopeptide. The Lipopeptidal structure of biosurfactant produced by *Pseudomonas aeruginosa* in this study showed an identical FTIR spectrum as the one produced by *Bacillus subtillis* (Joshi *et al.,* 2008). Thavasi et al. (2011b) also arrived at a similar conclusion alluding to the lipopeptide structure of the biosurfactant produced by *Pseudomonas aeruginosa* species.

In the TLC analysis, treatment with ninhydrin reagent showed positive reactions and revealed pink spot at R*f* value of 0.74 (Fig. 7.3). Signifying that the biosurfactant consisted of peptide moieties and showing the lipopeptidal nature of the biosurfactant. The biosurfactant produced by the strain *Pseudomonas aeruginosa* CB1 has a critical micelle concentrations (CMC) of 175±10 mg/L corresponding to minimum surface tension of 32± 1 mN/m.

7.3 Bioavailability and Biodegradability of PAHs

The concentration of 15 PAHs in creosote was monitored against purchased standards using the HPLC. Chromatographic peaks in the sample are matched with peaks and their retention times of known standards of the PAHs. Short- to intermediate-term degradability of PAHs in the solid phase of the soil slurry was determined after 25 days of incubation (Table 7.1). The long-term performance of the culture was evaluated after incubation for 45 days (Table 7.2).

The data in Table 7.1 and 7.2 show that degradability of the PAHs decreased with increasing ring-number. The decrease in degradation of PAHs with increasing ring number was also confirmed earlier by Tikilili and Chirwa (2011). The highest degradation efficiency was observed for Phenanthrene (88% removal) in the biosurfactant only amended reactor (Reactor 1). The pattern

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of degradation of PAHs was studied by evaluating the degradation rate of phenanthrene in the two phases (solid phase and aqueous phase) against time (Figure 4). Phenanthrene (3-ring) was chosen as the model compound since it is in the intermediate range of rings and its degradation could show a realistic pattern of the degradation of the PAHs.

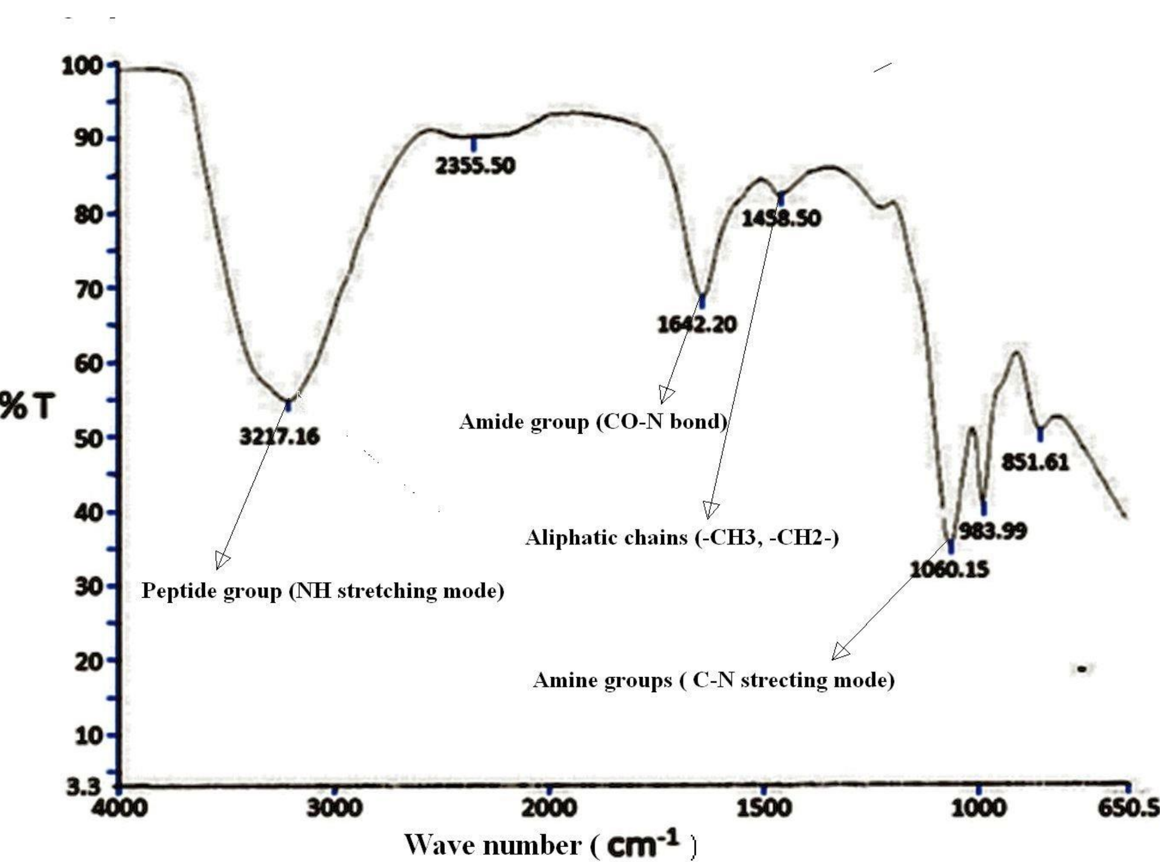

Figure 7.2: Fourier transform infrared absorption spectra of crude biosurfactant produced by a pure colony of *Pseudomonas aeruginosa* CB1.

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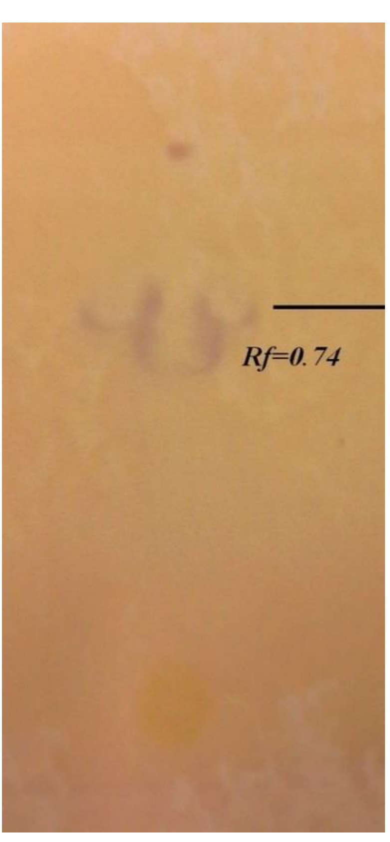

Figure 7.3: Thin-Layer Chromatography (TLC) of the biosurfactant obtained from *Pseudomonas aeruginosa* CB1, after treatment with ninhydrin revealed pink spot with R*f* value of 0.74. TLC was carried out on silica gel plates using chloroform–methanol–H_2O (65:25:4, v/v/v) as mobile phase.

The presence of the biosurfactant resulted in the increase of the aqueous phase concentration of the PAH, which resulted in the increased bioavailability and its fast subsequent degradation. Nevertheless *ex situ* supplementation of biosurfactant beyond optimum level could result in toxicity levels above the tolerance of the organisms. The toxicity may be dueto the biosurfactant overdose or increased stress caused by the solubilized PAHs. Shin et al. (2006) reported that rhamnolipid inhibited degradation of phenanthrene by a two-species consortium of *Sphingomonas* and *Paenibacillus sp.,* even though in pure culture the rhamnolipid inhibited only *Sphingomonas sp*. The authors suggested that the increased stress caused by the solubilized phenanthrene or the increased toxicity of rhamnolipid in the presence of solubilized phenanthrene could have resulted in the inhibitory effect in the case of *Paenibacillus sp*.

Observation of toxicity of increased biosurfactant concentration on the bacteria was reported by (Vasileva-Tonkova *et al.,* 2011). The authors reported complete inhibition of the growth of *B.*

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subtilis 168 at higher concentrations of biosurfactant. Biosurfactant induced toxicity can be caused dueto overdosage beyond threshold level which causes disruption of cellular membranes by interaction with lipid component and reactions of surfactant molecules with proteins that is essential to the functioning of the cell (Hames *et al.,* 2014). It is important therefore for the dissolution process to be optimized and experimentally determined to avoid toxicity and just fast enough to avoid starvation of cells since the PAHs in this system were the only carbon and energy sources.

Samples from the reactors were assayed for biosurfactant production utilizing Emulsification index (E_{24}) measurement using the method described by Cooper and Goldenberg (1987). The emulsification index of the sample was determined at day 18 and was found out to be 90% and 96%, with Hexane in the biostimulated and instantaneous biosurfactant and fertilizer stimulated reactors respectively. This continuous *in situ* biosurfactant production is due to growth limitation following nitrogen depletion and reaching stationary growth phase which is in line with previous observations reported (Hwang and Cutright, 2002). However in the second round supplementation of the nutrients on day 25 the emulsification observed was dissipated. The observed dissipation may be attributed to preferential microbial consumption of the biosurfactant produced indicating the necessity of nutrient limitation for *in situ* biosurfactant production.

Amendment of the bioslurry reactor with nutrients (11.5 g/L NH_4NO_3 and 1.5 g/L KH_2PO_4) and biosurfactant (Reactor 2) showed relative inhibition at the beginning as indicated by the decrease in the degradability of the compounds (51% naphthalene and 81% phenanthrene at day 25) as opposed to removal in the reactor with biosurfactant amendment but without nutrient amendment (Reactor 1: 74% naphthalene and 88% phenanthrene removed). The other 3 and 4 ring PAHs removal was also comparatively inhibited in Reactor 2 as opposed to Reactor 1 during the first 25 days of incubation. These results suggest that addition of nutrients in Reactor 2 may have resulted in preferential utilization of the biosurfactant amended as carbon source. Among the reactors inoculated with the microbial culture, the one that was not supplemented with the biosurfactant or nutrients in day 2 (Reactor 4) performed poorest. Only 30% naphthalene and 59% phenanthrene was degraded in this reactor. This reactor validated that the soil contaminated

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Table 7.1: Biodegradation of PAHs in creosote contaminated soil after incubation of the soil for 25 days; the results represent the mean ± standard deviation of the three replicates.

Compound	Initial Conc. mg kg^{-1}	Final Concentration in the different bioslurry reactors (mg kg^{-1})							
		R1 [a]	Removal %	R2	Removal %	R3	Removal %	R4	Removal %
Nap	425±16	112±10.6	74±0.7	210±15	51±0.3	162±5.6	62±0.2	296±21	30±0.2
Ace	272±11	41±3.9	85±0.9	46±2.8	83±0.4	60±12	78±3.2	163±17	40±0.5
Flou	383±25	55±5.7	86±1.2	107±15	72±1.7	116±19	70±2.2	211±31	45±1.2
Phe	233±3.6	27±0.56	88±0.1	44±3.3	81±0.5	44±07	81±2.1	95±9.4	59±0.6
Ant	131±7.8	42±7.2	68±2.2	49±7.2	63±1.6	61.5±4.2	53±0.4	81±7.7	38±0.5
Flr	321±19	58±8.4	82±2.0	141±10	56±0.5	160±7.8	50±0.3	305±51	5±0.2
Pyr	703±35	127±9.4	82±0.7	202±21	71±0.9	197±17	72±0.7	303±21	57±0.4
BaA	145±17	30±5.3	79±3.6	56±13	61±4.1	57±7.8	61±2.1	82±09	43±1.1
Chr	62±7.8	12±2.4	81±3.4	17±2.8	73±3.1	16±1.4	74±1.7	39±5.6	37±1.3
BbF	57±7.4	39±4.2	32±0.9	43±06	25±0.9	45±05	21±0.5	37±8.3	35±2.4
BkF	152±11	38±7.6	75±3.4	45±5.4	70±1.4	59±08	61±1.4	61±11	60±2.3
BaP	24±3.2	18±3.2	25±1.2	20±3.5	17±0.8	22±4.6	8±0.4	23±4.2	04±0.2
DahA	56±07	20±4.9	64±4.8	17±3.7	70±4.4	24±03	57±1.8	27±7	52± 4.3
BPer	97±11	15±3.1	85±4.7	16±2.6	84±3.3	31±5.4	68±3.1	66±6	32±0.7
IcdP	3.6±0.5	-- [b]	--	--	--	--	--	--	--
Total (mg kg^{-1})	**3064.6±14**	**635±5.9**	**79±2.5**	**1013±9.4**	**67±2.1**	**1055±8.8**	**66±1.7**	**1789±18.8**	**42±1.5**

[a] Reactor labels, R1 = reactor charged with biosurfactant and cells added on day 2, R2 = reactor charged with biosurfactant and nutrients NH_4NO_3 and KH_2PO_4 at time zero and cells on day 2, R3 = reactor charged with nutrients only and cells added on day 2, and R4 = Control reactor with cells added on day 2 (biotic control).

[b] '--' concentration was below instrument detection limit.

Naph	Naphthalene	Phe	Phenanthrene	Pyr	Pyrene	BbF	Benzo[b]fluoranthene	DahA	Dibenz[a,h]anthracene
Ace	Acenaphthene	Ant	Anthracene	BaA	Benz[a]anthracene	BkF	Benzo[k]fluoranthene	BPer	Benzo[ghi]perylene
Flou	Fluorene	Flr	Fluoranthene	Chr	Chrysene	BaP	Benzo[a]pyrene	IcdP	indeno(1,2,3-cd)pyrene

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Table 7.2: Biodegradation of PAHs in creosote contaminated soil after incubation of the soil for 45 days; the results represent the mean ± standard deviation of the three replicates.

Compound	Initial Conc.	Final Concentration in the different bioslurry reactors (mg kg^{-1})							
	mg kg^{-1}	R1 [a]	Removal %	R2	Removal %	R3	Removal %	R4	Removal %
Nap	425±16	34±7.4	92±0.2	102±4.7	76±0.3	125±9.1	71±0.5	256±17	40±0.4
Ace	272±11	32±3.1	88±0.9	32±2.6	88±0.7	46±1.7	83±0.3	74±6.1	73±0.3
Flou	383±25	64±6.7	83±1.3	34±4.1	91±1.7	52±3.6	86±0.8	187±11	51±0.7
Phe	233±3.6	5.1±0.7	98±1.8	31±3.2	87±0.9	19.5±2.4	92±1.4	50.7±4.5	78±0.1
Ant	131±7.8	29±3.3	78±1.3	39±4.2	70±2.0	39.5±1.6	70±1.1	76±3.5	42±0.6
Flr	321±19	31±4.1	90±1.9	42±2.6	87±0.7	37.1±2.7	88±0.9	136±11	58±0.6
Pyr	703±35	82±2.6	88±0.4	94±5.1	87±0.5	106±7.6	85±0.7	258±9.7	63±0.4
BaA	145±17	32±1.6	78±1.3	51±3.2	65±1.1	53.3±2.4	63± 1.0	93±7.3	36±0.7
Chr	62±7.8	6.9±0.8	89±1.4	8.4±0.9	86±2.3	10.7±1.1	83±2.2	13±3.2	79±2.4
BbF	57±7.4	12±0.6	79±1.5	7.8±02	86±7.1	10.5±0.8	82±2.1	21.7±2.5	62±2.5
BkF	152±11	33±2.8	78±0.9	27±5.2	82±3.5	51.5±3.7	66±0.7	45.5±6.2	70±0.9
BaP	24±3.2	16±1.1	33±0.8	15±2.6	38±1.8	20.5±2.1	15±0.5	22±2.7	08±2.1
DahA	56±07	20±2.4	64±1.9	15±1.2	73±1.6	31.4±3.1	44±1.1	25.1±2.9	55±1.8
BPer	97±11	17±3.4	82±4.3	8.1±1.6	92±4.8	45±2.6	54±1.9	56±3.7	42±2.2
IcdP	3.6±0.5	-- [b]	--	--	--	--	--	--	--
Total (mg kg^{-1})	**3064.6±14**	**414±3.4**	**86.5±1.6**	**506.3±3.3**	**83±2.7**	**648±3.8**	**79±1.2**	**1317±7.5**	**57±1.3**

[a] Reactor labels, R1 = reactor charged with biosurfactant and cells added on day 2, R2 = reactor charged with biosurfactant and nutrients NH_4NO_3 and KH_2PO_4 at time zero and cells on day 2, R3 = reactor charged with nutrients only and cells added on day 2, and R4 = Control reactor with cells added on day 2 (biotic control)
[b] '--' concentration was below instrument detection

Naph	Naphthalene	Phe	Phenanthrene	Pyr	Pyrene	BbF	Benzo[b]fluoranthene	DahA	Dibenz[a,h]anthracene
Ace	Acenaphthene	Ant	Anthracene	BaA	Benz[a]anthracene	BkF	Benzo[k]fluoranthene	BPer	Benzo[ghi]perylene
Flou	Fluorene	Flr	Fluoranthene	Chr	Chrysene	BaP	Benzo[a]pyrene	IcdP	indeno(1,2,3-cd)pyrene

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by the wood mill (the bio-slurry soil source) is lacking the nutrients and the low bioavailability of the PAHs is responsible for the observed poor performance even if it has been inoculated with the same consortium. In actual application, PAH degrading organisms for the clean-up of the site have to be sourced from the sources which had been acclimated through long term exposure. A creosote exposed microbial culture as applied in this study could serve the purpose. No removal of PAHs from the solid phase was observed in the sterilized abiotic control (Reactor 5) showing that abiotic losses were insignificant.

The bioslurry reactors incubated over a longer period of 45 days showed further degradation of PAHs in all reactors with live cultures. It was observed that the impact of additional nutrients in Reactor 2, which caused the batch to remain behind in performance during the first 25 days as shown by the 68.3% and 66.2%, 2–3 ring and 4 ring PAHs removal as opposed to 80.7% and 81.6% 2–3 ring and 4 ring PAHs removal in Reactor 1, was not significant after a long incubation time as Reactor 2 caught up with Reacror 1 (Fig.7.4). This confirms that addition of nutrient aids in the *in situ* biosurfactant production following nutrient depletion and subsequent growth limitation. Besides biosurfactant application and nutrient biostilmulation enhanced degradations in the reactors can be attributed to the synergistic effects of elevated temperature and slurring process. Raising the temperature increases desorption, which makes more organic material available for microbial degradation. Trably and Patureau (2006) investigated the ability of indigenous aerobic microorganisms to degrade low and high molecular mass PAHs in sewage sludge fed in continuous bioreactors at temperature increased from 35°C to 45°C and 55°C where biodegradation of the high molecular mass PAHs was enhanced from 50% to 80%.

7.4 Effect of Ring Number on Degradation

Several researchers have previously reported that PAH compounds with higher ring numbers are more difficult to degrade than low ring number compounds such as naphthalene and acenaphthene. The low degradability of high ring number compounds has been attributed, partly, to low bioavailability due to very low solubility limits (Xu and Obbard, 2004) and partly due to the high metabolic energy required in multiple ring openings which involves more complex metabolic pathways (Kim *et al.,* 2007). The results from the biodegradability test in this study confirm the decreasing degradability of the compounds with increasing ring number.

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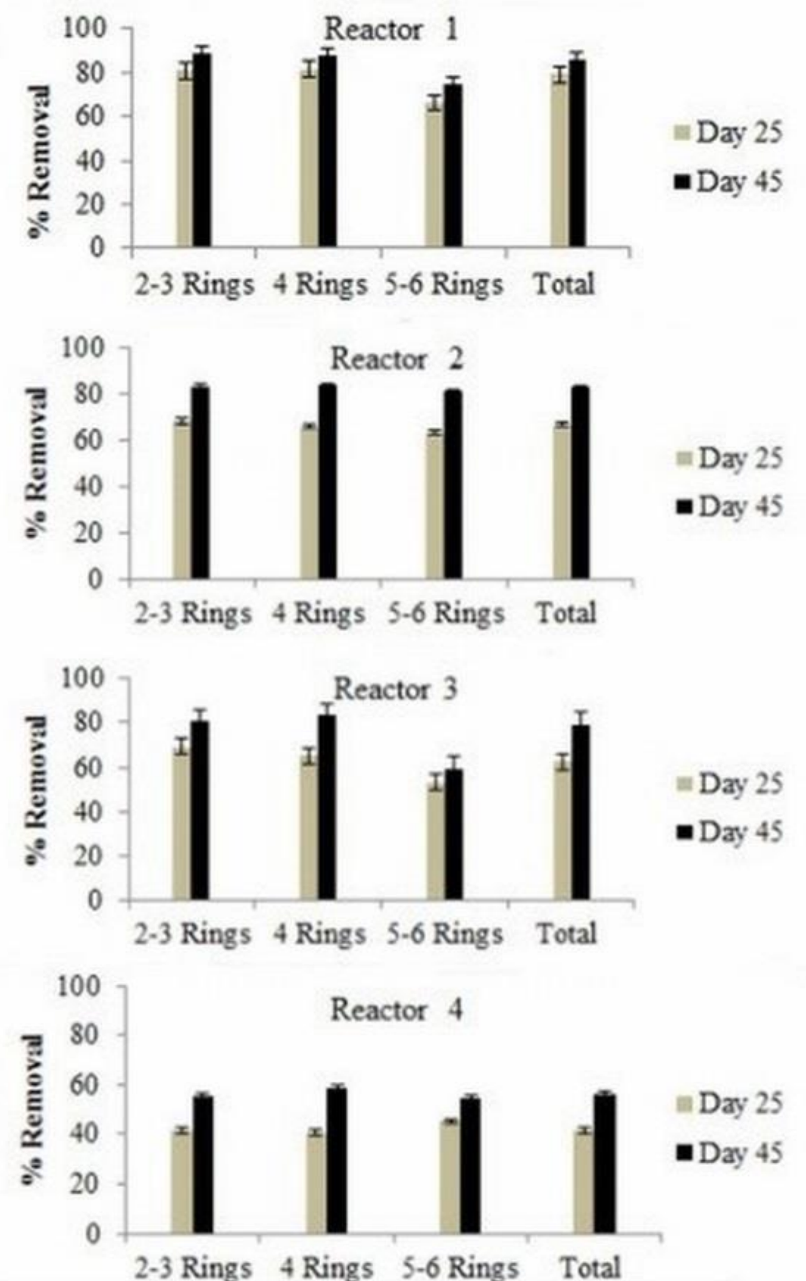

Figure 7.4: Percentage removal of the PAHs according to their ring size in the different reactors at day 25 (Orange) and day 45 (Black). The results represent the mean from three independent experiments ± standard deviations. Error bars represent the standard deviation of the mean of the three replicates

The most highly biodegradable compound at day 25 was Phenanthrene which was 88% degraded followed by flourene, another three-ring compound at 86% degradation. In terms of mass removed based on the amount degraded, Pyrene was the most degradable with a decrease from 703 to 127 mg kg^{-1} by day 25, a mass decrease of 576 mg kg^{-1}, much higher than the

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mass decrease of 328 mg kg^{-1} in flourene (Table 7.1). The 5-6 rings achieved up to 81.5 % removal but the mass conversion was very low. All compounds were similarly affected by the addition of nutrients as shown by the decreased efficiencies between the Reactor 1 (biosurfactant only) and Reactor 2 (biosurfactant + nutrients). Degradation increased further after further incubation to 45 days. The inhibitive effect of additional nutrients was less pronounced after 45 days which might either be due to culture acclimation to harsher conditions or decrease in the toxic compounds with time resulting in the Reactor 2 performance catching up with Reactor 1.

The biotic control (R4) had very low performance compared to the reactors supplemented with biosurfactant, nutrients, biosurfactant and nutrients at day 2. The loss from the abiotic control (R5) is insignificant (not shown) since losses due to photodegradation, volatilization and other abiotic loses were minimal.

7.5 PAH biodegradation kinetics

Liquid phase biodegradation experimnet was conducted to evaluate the impact of the biosurfactant on the biodegradation of Model PAH (Pyrene, supplemented to achieve a final concentration of 100 mg/L), according to the method 3.6.1.1.

Pyrene biodegradation kinetic parameters were determined by assuming Monod biodegradation kinetics:

$$\frac{dC}{dt} = -q_{\max} \frac{C}{K_S + C} X \quad (7.1)$$

Where the variables are substrate concentration C (mg/L), biomass concentration, X (mg protein/L), and time t(d). The parameters are $q_{\max}$, the biomass-normalized substrate utilization rate (mg substrate/mg protein/h) and K_S , the half-saturation coefficient for the substrate (mg/L).

The experimental data are fitted in to the Monod model (Equation 7.1) and the substrate utilization rates (q_{max}) and half saturation constant were determined using AQUASIM software package parameter estimation technique (Reichert, 1998). After the Monod model (equation 3) is directly fitted to the set of experimental data (Fig. 7.5), substrate utilization rates (q_{max}) were determined to be; 4.2; 16.1 and 19.4 mg/mg/d respectively for unamended one, 400 mg/L and

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700 mg/L amended microcosms. This shows more than 4 fold increase in the substrate utilization rate of the 700 mg/L Lipopeptide amended reactor compared to the unamended one.

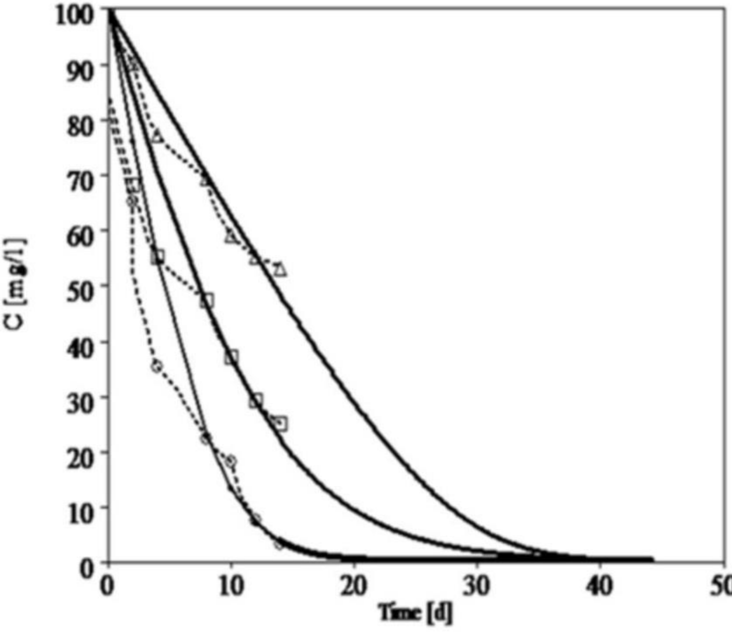

Figure 7.5: Model fits for Pyrene biodegradation in the microcosms supplemented with 700 mg/L Lipopeptide (*square*), 400 mg/L Lipopeptide (*rhombus*) and 0 mg/L (*triangle*) and their experimental data (dotted lines).

7.6 Significance of Results

The results highlight the importance of dissolution rate on biodegradability of persistent compounds in the creosote contaminated soil media. In the current study, PAH contamination of soil was observed coming from creosote contamination of the wood treatment plant. The results suggest that the desorption and subsequent biodegradation of the persistent PAHs from the creosote contaminated soil was enhanced through biosurfactant supplementation alone and simultaneous biosurfactant and nutrient supplementation. Biostimulation by addition of N and P, a strategy that has often been reported by various studies (McKew et al., 2007) proved an effective approach for enhanced PAH degradation. However bioavailability of the PAHs can also be a limiting factor owing to their hydrophobic nature and low water solubility. As can be observed in the nutrient-only amended treatment (Reactor 3) degradation of PAHs at day 45 increased to levels comparable to reactors amended with both nutrients and biosurfactant (Reactor 2) , from the emulsion observed it can be evident that the PAH degrading community produced their own surfactants and subsequently hydrocarbon bioavailability was enhanced. Thus *in situ* biosurfactant production through nutrient-only biostimulated treatment

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(introduction of additional nutrients of 11.5 g/L NH_4NO_3 and 1.5 g/L KH_2PO_4) resulted in considerably significant degradation and removal of the creosote PAHs as well. Just the right amount of biosurfactant was required to kick start the culture by release of enough carbon source into solution, otherwise the culture at day zero could struggle since there will not be enough bioavailable carbon source in the system.

The technique suggested in this study could prove useful in the bioremediation of PAH contaminated soil and sediments through supplying oxygen and nutrients. The evaluated process was aerobic thus it supported an array of aerobic bacteria including *Bacillus subtillis* and *Pseudomonas aeruginosa*. The limitation of the process could be in the production of the initial biosurfactant which was complicated and time consuming which requires either *ex situ* biosurfactant application or *in situ* biosurfactant production through supplementation of nutrients. Fortunately, once this biosurfactant production process has started, the rest of the process will be self-regenerating and self-sustaining, after the culture has established itself.

7.7 Summary

The results show that the biosurfactant applied twice over the course of the incubation assisted the remediation by increasing the bioavailability of the PAHs and enhanced the degradation potential of the culture. With this system, enough biosurfactant could be added to allow the cultures to grow to an effective population followed by *in situ* production to achieve the long-term stability and enhanced bioremediation of a contaminated environment. The experiments in this study showed that *in situ* biosurfactant production can be stimulated through growth limitation following nutrient depletion. The biosurfactant produced *in situ* helped desorb and emulsify the hydrophobic contaminants and increase their bioavailability as confirmed from the stable emulsions formed. *In situ* biosurfactant production for bioremediation purposes is economically feasible and has multi-faceted advantages as it can use the contaminant as source of carbon for biosurfactant production.

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CHAPTER 8

CONCLUSIONS AND RECOMMENDANTIONS

Bioremediation with monoculture and consortia inoculation together with optimum amount of biosurfactant amendment enhanced PAH bioavailability and subsequent dissipation and assisted PAH-degrading microbial activities in different bioremedial studies. Due to their biodegradability and low toxicity, biosurfactants such as Lipopeptides are very promising for use in bioremediation technologies. In addition, there is the potential for *in situ* production, a distinct advantage over synthetic surfactants. Biosurfactants produced from *Pseudomonas aeruginosa strain* LBP5 and LBP9 were effective in enhancing the solubilization as well as desorption of the crystalline and sorped PAHs, and increasing the rate of mass transfer to the aqueous phase. It can be concluded that the biosurfactant can have a potential applications in environmental remediation through alleviation of mass transfer limitations and enhancing subsequent biodegradation. Biosurfactants may also have inhibitory effect on the biodegradation of PAHs since their effects will be dose- and species dependent. Thus effects of biosurfactants on microbial community and optimum dosage should be experimentally determined in advance before a wide scale application. Besides considerable acceleration of degradations, biosurfactants are natural, biocompatible compounds and do not introduce any element that alters natural habitat. One problem with commercial applications of biosurfactants is that they are made in low concentrations, which make product recovery difficult and expensive. A major solution would possibly be use of alternative substrates and medium optimization approaches to reduce cost and optimize biosurfactant production. Better fermentation conditions (medium optimizations) and cheaper substrates are needed to reduce costs and expand the applications of biosurfactants.

The multisubstrate and sole substrate microcosm studies demonstrated that in the liquid culture experiments the biosurfactant enhanced the bioavailability and hence the biodegradation of the PAHs in the sole substrate experiment, on the other hand inhibition was observed in the liquid culture multisubsrate experiments. Besides substrate bioavailability it was observed that substrate interactions affect the biodegradation kinetics in PAH contaminated liquid cultures. When in mixtures, PAHs can influence the rate and extent of biodegradation of other PAH components positively or negatively. Reasons for retarded PAH biodegradation rates include

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substrate competitive inhibition and inhibition by formation of toxic intermediates. Further studies are recommended on the possibilities to accelerate multisubstrate biodegradation efficiencies through different approaches that would hasten biodegradation by cross induction of required degradative enzymes, co-metabolism and the increase of biomass concentration during the growth.

From the study either *ex situ* or *in situ* biosurfactant supply approaches (stimulating microorganisms in the soil to produce these compounds) can be considered efficient. Though the *ex situ* method also showed efficient performance, it is expensive due to capital involved surfactant production, purification and introduction into media. For bioremediation applications, in-soil biosurfactant production may have advantages compared with adding exogenously produced biosurfactants. In-soil production can reduce costs since the bacteria use the pollutants as carbon and energy sources. *In situ* biosurfactant production may enhance compatibility between biosurfactants and microorganisms to ensure biodegradation of solubilised or desorbed contaminants. Moreover production of biosurfactant by number of biosurfactant producing species in the community will have potential synergistic role of multiple biosurfactants.

The results of the creosote contaminated soil bioremediation study indicated that nutrient-limited conditions prevent microbial consumption of the biosurfactants produced or exogenously added. The long-term stability of the Lipopeptides of the biosurfactant amended microcosm of the study was a result of the nitrogen limitation which favoured enhanced degradation of high molecular weight PAH which degrade after the degradation of lighter molecular weight PAHs. In light of this result the potential benefit of the biosurfactants in assisting bioremediation is a promising bioremediation strategy for aged PAH-contaminated soil. Elucidation of the potential applicability of this strategy will require further studies involving different soil types and contaminants under field conditions. Further studies are suggested to investigate the potential sustainable exogenous application and *in situ* production of biosurfactants for the bioremediation and restoration of contaminated environmental media with in a reasonable timeframe.

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