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Biodegradation of polycyclic aromatic hydrocarbon contaminants in a mixed culture bioreactor

by

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DECLARATION

I, Olusola S. Amodu, declare that the contents of this thesis represent my own unaided work, and that the thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Olusola S. Amodu	*February 30, 2015*
Signed	**Date**

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common and recalcitrant environmental contaminants – known for their potential toxicity, mutagenicity, and carcinogenicity to humans. Biosurfactant application can enhance the biodegradation of PAHs. The main object of this work was to explore the novelty of biosurfactant produced by the isolated strains of *Bacillus* sp and *Pseudomonas aeruginosa* grown exclusively on *Beta vulgaris*, and the modification of the zeolites nanoparticles by the biosurfactant, for enhanced biodegradation of PAHs in soil. Novel biosurfactant-producing strains were isolated from hydrocarbon-contaminated environments, while several agrowaste were screened as primary carbon sources for the expression of biosurfactants, which were quantified using various standardized methods. The biosurfactants produced achieved considerable emulsification activity for hydrocarbons. Different culture conditions – agrowaste concentration, pH, and temperature were optimized for enhanced biosurfactant production by *Bacillus licheniformis* using the statistical modelling of response surface methodology (RSM); by numerical optimisation techniques, the optimum conditions were found to be pH of 6.72, an agrowaste concentration of 4% (w/v), and a temperature of 44.5 °C. Furthermore, the kinetics of cell growth, substrate utilization, and biosurfactant production by *Bacillus licheniformis* STK 01 grown on *Beta vulgaris* was investigated, as well as the effects of using polyurethane foam as a biocarrier. The highest biosurfactant production was 5.8 ± 0.5 g L^{-1} when *Beta vulgaris* waste was used without the biocarrier, while biosurfactant production increased up to 9.78 ± 1.02 g L^{-1} in a mineral salt medium (MS) without the biocarrier. Although the addition of polyurethane foam enhanced cell proliferation considerably when MS substrate was used, it repressed biosurfactant production. The biosurfactant produced from *Beta vulgaris* and MS lowered the surface tension of broths to 30 and 23.5 mN m^{-1} respectively. Moreover, from kinetic data analysis, cell growth, *B. vulgaris* utilization, and biosurfactant production were best fitted to the logistic model and modified Monod equation, respectively, with the maximum cell growth rate of 0.026 h^{-1}, cell yield of 0.617, Monod constant being 0.418 g L^{-1}, and biosurfactant production rate 0.140 h^{-1}

The effect of biosurfactant supplementation on the biodegradation of PAHs with 4, 5, and 6 benzene rings, by *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03, was evaluated in soil for a period of 60 days at the optimized conditions. The isolates were able to degrade the studied contaminants in mono- and co-cultures soil, with *B. licheniformis* STK01 cultures achieving the highest degradation rates. Biosurfactant supplementation significantly enhanced the degradation of benzo(*ghi*)perylene – a 6-ring benzene HMW PAH. The highest degradation rates

achieved in biosurfactant-supplemented cultures were: 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene, 86.17% for benzo(a)pyrene, and 60.90% for benzo(*ghi*)perylene. Degradation kinetic data were suitably described by first-order kinetics, with the rate constants showing that phenanthrene degradation was the fastest for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}), while the degradation rates for others followed in the order of their increasing molecular weight.

The biosurfactant produced by *Bacillus licheniformis* grown on *B. vulgaris* was used to modify the surface of zeolites, synthesised by direct fusion of sodium hydroxide, coal fly ash and magnetite nanoparticles. The micrographs of the synthesized zeolites and the resultant magnetic composite showed pore structures of nanocubes. The acidity and functional group analyses signified asymmetric and symmetric stretching vibrations of O–H and internal tetrahedron vibrations of Si-O and Al-O. The modification of the surface of zeolites with biosurfactant increased the BET surface area by more than 120% in comparison with the unmodified zeolites. In this study, biosurfactant-modified zeolite was found to enhance the degradation of PAHs in soil after the 30-day experiment, in comparison with the soil culture without amendment, achieving the highest degradation levels of 88.55% for phenanthrene, 74.45% for pyrene, 63.33% for benz(a)anthracene, 67.66% for benzo(a)pyrene, and 56.37% for benzo(*ghi*)perylene. Analysis of rate kinetics did not show many discrepancies between the amended soil and soil without amendment.

This study shows that exclusive utilization of agrowaste as microbial substrates for biosurfactant production is promising for enhanced biodegradation of PAHs, particularly the high molecular weights, both in *in-situ* and *ex-situ* operations. The modification of zeolite surface with biosurfactant offers an environmentally benign option to enhance the affinity of zeolites for hydrophobic hydrocarbon contaminants.

Keywords: Agrowaste; *Bacillus licheniformis*; Bioconversion; Biodegradation; Biosurfactant; Kinetics; Modelling; Magnetic nanoparticles; Polycyclic Aromatic Hydrocarbon; Solid State Fermentation

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To my lovely wife – Bridget

and

my little angels –

Yemi, Tolu, and Bomi

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Abbreviations	Definition
A	Cross-sectional area
A_{OD}	Oil displacement activity
BaA	Benzo*(a)*anthracene
BaP	Benzo*(a)*pyrene
BghiP	Benzo*(a)*perylene
BET	Brunauer–Emmet–Teller
BJH	Barrett–Joyner–Halenda
BMZ	Biosurfactant modified zeolites
BMMZ	Biosurfactant modified magnetic zeolites
CCD	Composite experimental design
CFU	Colony-forming unit
CMC	Critical micelle concentration
C_e	Equilibrium concentration (mg L^{-1})
D	Diffusion coefficient
DCM	Dichloromethane
DNS	Dinitrosalicyclic acid
DOM	Dissolved organic matter
EDS	Energy dispersive X-ray spectrometer
E_{24}	Emulsification index
EPA	Environmental Protection Agency
FA	Fly ash
FTIR	Fourier transform infrared
GC-FID	Gas chromatography-flame ionization detector
HDTMAB	Hexadecyltrimethyl ammonium bromide
HEX	Hexane
HLB	Hydrophilic-lipophilic balance
HMW	High molecular weight
HOCs	Hydrophobic organic compounds
HPLC	High-performance liquid chromatography
IEA	International Energy Agency
k	Kinetic rate constant
K	Adsorption equilibrium constant
K_1	Hill-de Boer equilibrium constant (L mg^{-1})

K_2	Energetgy of interaction between adsorbed molecules (kJ mol^{-1})
K_S	Monod saturation constant
LM	Logistic model
LMW	Low molecular weight
Log K_{ow}	Octanol – water partition coefficient
LLP	Logistic incorporated Leudeking–Piret model
LLPM	Logistic incorporated Leudeking–Piret modified model
LP	Leudeking–Piret model
LPM	Leudeking–Piret modified model
m	Cell maintenance parameter
MLLPM	Modified logistic incorporated Leudeking–Piret modified model
n	Freundlich constant
P	Product concentration (g L^{-1})
PCR	Polymerase chain reaction
Phe	Phenanthrene
P_m	Maximum biosurfactant production (g L^{-1})
P_o	Initial biosurfactant concentration (g L^{-1})
PXRD	Powder X-ray diffraction
P_r	Specific production rate (h^{-1})
Py	Pyrene
q_e	Equilibrium adsorption capacity (mg g^{-1})
q_m	Maximum equilibrium adsorption capacity (mg g^{-1})
ΔQ	Variation of adsorption energy
r	Cell growth inhibitory effect
rDNA	Ribosomal deoxyribonucleic acid
RSM	Response surface methodology
r_p	Volumetric production rate of biosurfactant (g L^{-1} h^{-1})
r_x	Cell growth rate (g L^{-1} h^{-1})
r_s	Substrate utilization rate (g L^{-1} h^{-1})
R^2	Coefficient of determination
R	Universal gas constant (KJ mol^{-1} K^{-1})
S	Limiting substrate concentration (g)
SDBS	Sodium dodecyl benzene sulfonate
SEM	Scanning electron microscopy
SPE	Solid phase extraction
SSE	Sum of square error
t	Time (h)
T	Temperature
TGA	Thermogravimetric analysis

TOM	Total organic matter
USEIA	US Energy Information Administration
X	Cell concentration (g L^{-1})
X_m	Maximum biomass concentration (g L^{-1})
$Y_{p/s}$	Product yield coefficient (g biosurfactant/ g substrate)
$Y_{x/s}$	Biomass yield coefficient (g biomass/g substrate)
μ	Specific growth rate (h^{-1})
μ_m	Maximum cell growth rate
μ_o	Initial specific growth rate (h^{-1})
Å	Angstrom
°C	Degree Celsius
°K	Degree Kelvin
eV	Electronvolt
h	Hours
min	Minutes
kg	Kilogram
L	Litre
mL	Millilitre
μL	Microlitre
rpm	Revolutions per minute
β	Redlich-Peterson constant
θ	Surface coverage (q_e/q_m)
ε	Adsorption energy potential
λ	Growth-dependent parameter
γ	Non-Growth-dependent parameter

Chapter 1

INTRODUCTION

1.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are one of the most common and recalcitrant environmental contaminants. These organic pollutants, which consist of clustered benzene rings, are released into the environment mostly through anthropogenic sources, and particularly because of incomplete combustion of fossil fuels. They are potentially toxic, mutagenic and carcinogenic to human health; this is the primary reason that some of them have been classified as priority pollutants by the Environmental Protection Agency (USEPA, 1999). PAHs have long been identified in soil, water, sediments, and air (Grimmer, 1983, Johnsen *et al.*, 2005). Recently they have been found in vegetative plant parts and charcoal-grilled meat (Farhadian et al., 2011, Wick et al., 2011). They are hydrophobic and, as a result, soil particles and organic matter constitute their major repository when released into the environment. However, through geochemical processes such as precipitation, leaching, and erosion, they are mobilized into water bodies. About 85% of the world's energy is derived from combustion of fossil fuels (USEIA, 2013) which suggests that PAH pollution is ubiquitous. Hence, the removal of PAHs from contaminated media has attracted possibly more attention in recent years than any other known contaminants.

Several remediation methods have been used over the years, particularly, chemical methods and/or physico-chemical methods such as photochemical treatment, oxidation, chlorination, membrane filtration, ozonation, biodegradation, and adsorption (Bohn *et al.*, 1985, Pierzynski, 2000,

Zheng *et al.*, 2007, Bernal-Martínez *et al.*, 2007, Ferrarese *et al.*, 2008). The success of a bioremediation study carried out in 1991 on the Valdez sea port in the Gulf of Alaska (Pritchard and Costa, 1991, Bragg *et al.*, 1994), sparked tremendous research interest in the potential of the microbial community for the bioremediation of PAHs. Consequently, robust biological systems have been designed and deployed for PAHs degradation, focusing on the isolation of PAH-degrading microorganisms to the application of engineered microbial cultures for use in biostimulation, bioaugmentation, co-metabolism, and adsorption, among others (Mills and Frankenberger, 1994, Straube et al., 2003, Mancera-Lopez et al., 2008, Arbabi et al., 2009, Wick et al., 2011).

According to Amodu et al. (2013), most earlier studies focused attention on the low molecular weight (LMW) fractions such as naphthalene and phenanthrene, while there were limited studies on the bioremediation of high molecular weight (HMW) PAHs such as benz(a)anthracene, benzo(a)pyrene and benzo(ghi)perylene. This is due to the relatively high hydrophobicity and low bioavailability of the HMW PAHs. However, these fractions are the major PAH priority pollutants found in contaminated media (Juhasz and Naidu, 2000, Wu et al., 2010). Currently, identification of more prolific and biologically evolved microbial species has led to the development of various techniques being explored to enhance the bioavailability and biodegradation of HMW PAHs (Husain, 2008, Chaudhary et al., 2011, Moscoso et al., 2012, Kunihiro et al., 2013, Jorfi et al., 2013, Mishra and Singh, 2014, Wang et al., 2014, Lu et al., 2014). Nonetheless, the preponderance of these studies was on PAHs degradation in aqueous phase, as a secondary remediation process after extraction from solid matrices such as soil, using washing as an extraction method prior to biodegradation due to the sequestration of these contaminants in soil. An approach that can achieve high degradation of PAHs in soil without a pre-treatment step is less intrusive to the environment and more suitable for *in-situ* bioremediation.

Moreover, the low bioavailability, including mass transfer limitations of PAHs, has continued to be a major challenge to their biodegradation. For this reason, the use of biosurfactant has been explored in recent years as one of the emerging approaches to enhance PAHs' desorption and transfer from their repositories, so that they are available in aqueous phase for microbial degradation. Naturally, PAH-degrading bacteria are able to access hydrophobic substrates in the environment through: 1) the synthesis of biosurfactants, and 2) their ability to directly attach to the hydrophobic substrate by modifying their cell membrane hydrophobicity (Das and Mukherjee, 2007a). Biosurfactant-enhanced bioavailability often occurs via two mechanisms: 1) pre-micellar lowering of the surface tension, thereby enhancing the mobilization of the contaminants from particulate matrices resulting in increased sorption mass transfer, and 2) micellar solubilization. Furthermore, the effectiveness of crude biosurfactant in environmental studies makes its use promising (Mukherjee et al., 2008, Mutalik et al., 2008, Makkar et al., 2011). This implies that purification-related costs can be circumvented in such applications of the bioproduct.

Another challenge is the economic viability of the biodegradation technologies being developed, which is accentuated by the low yield of the requisite metabolites and the cost of precursors and microbial substrates. To circumvent this challenge, the optimization of microbial culture conditions for enhanced biosurfactant production is well reported (Ilori et al., 2005, Rodrigues et al., 2006c, Joshi et al., 2008, Najafi et al., 2010, Zhu et al., 2013). This can be either by direct quantification of biosurfactant produced or determining relative quantities such as the critical micelle dilution (CMC). Such optimization studies make it possible to be able to assess influential parameters that ultimately lead to peak process performance. In contaminants' biodegradation, the optimized parameters can be used to regulate the microbial activity so that the target contaminant is degraded.

Recent researchers have identified a plethora of renewable and low-cost agro/agro-industrial wastes as substrates (Das and Mukherjee, 2007b, Makkar et al., 2011, Zhu et al., 2013). However, most often these substrates are supplemented with refined compounds to make up for certain essential nutrients required for microbial growth and synthesis of bioproducts that enhance pollutant availability including biodegradation. This is not yet considered a holistic approach for environmental studies, and requires a new approach as proposed in this study. The exclusive application of agrowaste that needs no supplementation with refined substrate for biosurfactant production could ensure the sustainability of this approach for the enhanced biodegradation of PAHs. Another less invasive approach for enhanced solubility and biodegradation of recalcitrant PAHs is by microbial co-cultivation and co-metabolism, with a lowered surface tension aqueous phase (Moscoso et al., 2012).

Furthermore, a number of nanocomposite adsorbents have been used in recent times such as graphene oxide and mesoporous organosilica for the immobilization of hydrophobic organic compounds in their pores, thereby increasing their solubility in aqueous phase. In order to increase the affinity of the adsorbents for hydrophobic compounds, they have been modified with chemical surfactants (Dong *et al.*, 2010, Fungaro *et al.*, 2011, Lin *et al.*, 2011, Schick *et al.*, 2011, Vidal *et al.*, 2013). The chemical surfactant often used for zeolites' modification is hexadecyltrimethyl ammonium bromide (HDTMAB). However, the modification of the adsorbents with biosurfactant could make the approach environmentally benign and sustainable.

1.2 Research questions

From the challenges raised above, the following research questions are pertinent:

- Are there lignocellulosic substrate(s), including precursors, suitable for use for microbial growth and synthesis of biosurfactant, so that the renewable substrates will not need supplementation with refined nutrients?

- Are there suitable mathematical models to suitably model the microbial growth rate, the consumption rate of the renewable resources, and biosurfactant production?

- How effective is the biosurfactant produced from the renewable resources in enhancing the biodegradation of HMW PAHs in soil?

- What is the effect of microbial co-cultivation and co-metabolism on the biodegradation of PAHs? That is, how can the mixed culture be exploited with a view to improving the production of suitable products for the biodegradation of PAHs on a large scale?

- How can a magnetic adsorbent and the biosurfactant modified nanocomposite be explored for immobilization of PAHs, thereby enhancing the bioavailability and biodegradation of the contaminants?

1.3 General objective

Based on these challenges and the previous research efforts, the objective of this research was to isolate a prolific biosurfactant-producing and PAH-degrading microorganism that will be able to utilize organic waste exclusively, and optimize the cultural conditions for high yield of the requisite biological products which can be used to enhance the bioavailability and subsequent biodegradation of PAHs.

1.4 Specific objectives

1. To isolate biosurfactant-producing and PAH-degrading bacterial species that will be able to utilize agrowaste as a sole substrate for growth and synthesis of surface-active agents (biosurfactant), for use to enhance the bioavailability and biodegradation of PAHs.

2. To identify a suitable agrowaste substrate for biosurfactant production and to investigate the effectiveness of the biosurfactant produced for the emulsification of hydrocarbon compounds.

3. To optimize culture conditions for the high yield of biosurfactant using the response surface methodology and to model microbial growth, substrate utilization, and biosurfactant production with suitable mathematical models.

4. To investigate the effects of microbial co-culture, co-metabolism, and biosurfactant supplementation on the biodegradation of PAHs in soil. This will include determining PAHs' degradation kinetics.

5. To synthesize a magnetic zeolite and modify it with a biosurfactant synthesized from agrowaste and apply the nanocomposite to further enhance the biodegradation of PAHs in soil samples.

PREFACE TO THE THESIS

The research work presented in this thesis was conducted at the Chemical Engineering Department; Bioresource Engineering Research Laboratory, Department of Biotechnology; and Instrumentation and Analytical Laboratory, Department of Chemistry – all on the Cape Town campus of the Cape Peninsula University of Technology, South Africa.

The thesis comprises nine chapters.

Chapter 1 gives a brief introduction to the thesis, the objectives of the study, and provides a preface to the thesis.

Chapter 2 contains an overview of the literature on the environmental fate of PAHs, and the role of biosurfactant in their bioavailability and biodegradation. It further highlights some of the challenges of PAHs removal from contaminated samples and some of the techniques in use to tackle these challenges, with emphasis on the use of biological systems.

Chapter 3 highlights the materials, methods and procedures used for experimentation and results analyses.

Chapter 4 discusses the isolation of biosurfactant-producing microorganisms, their DNA extraction and PCR amplification of 16s rDNA, and the identification of suitable agrowaste that can be used by the microbial isolates as a growth substrate and for the synthesis of biosurfactant, without supplementing the waste with any refined carbon source or nutrient element. It further discusses the different standardized methods used for biosurfactant screening and characterization, and finally examines the capability of the synthesized biosurfactant to emulsify various hydrocarbon compounds.

Chapter 5 focuses on the optimization of biosurfactant production by *Bacillus licheniformis* STK 01 while using *Beta vulgaris* waste as an exclusive carbon and nutrient element source – the microbial strain and agrowaste are both identified in Chapter 3. The chapter presents the central composite

design of experiments, statistical analysis and modelling, graphical representation of the response surface, and the numerical optimization option of the response surface methodology using Design-Expert® software.

Chapter 6 contains the kinetic modelling of cellular growth, substrate utilization, and biosurfactant production from *B. vulgaris* by *Bacillus licheniformis* STK 0. It also presents the quantification of biosurfactant by using high-performance liquid chromatography (HPLC), and the determination of critical micelle concentration (CMC). The models used in this chapter are the logistic model, modified Monod model, modified logistic model, Leudeking–Piret model, and logistic incorporated Leudeking–Piret model.

Chapter 7 explicates the application of the synthesized biosurfactant to enhance the biodegradation of PAHs in soil samples. It explains PAHs extraction from soil samples using ultrasonication, sample clean-up with solid-phase extraction column, and analytes quantification using gas chromatography equipped with a flame ionization detector (GC-FID). It further presents the kinetics of biodegradation of PAHs, considering the effects of co-metabolism and bacterial co-culture in addition to biosurfactant application.

Chapter 8 presents the modification of zeolite nanoparticles with the synthesized biosurfactant and the application of the resultant composite to enhance the biodegradation of PAHs. In this chapter, the synthesis and characterization of the magnetic zeolite used is also presented. It further examines the biodegradation kinetics of PAHs as the adsorbent provided a base support for microbial cells and contaminant immobilization.

Chapter 9 contains the summary of the work and general concluding remarks.

Chapter 2

PAHS' ENVIRONMENTAL FATE, BIOAVAILABILITY AND BIODEGRADATION: THE ROLE OF BIOSURFACTANT

2.1 Introduction

Hydrocarbons are compounds made of hydrogen and carbon atoms. Based on the arrangement and/or geometry of the carbon atoms, they can be classified as aliphatic and cyclo-hydrocarbons. Similarly, they can be classified as saturated and unsaturated hydrocarbons, depending on the saturation of carbon-carbon bonds. The saturated cyclic hydrocarbons are called cycloalkanes, and they are more reactive than their open chain counterparts. The most profuse of unsaturated cyclic hydrocarbon homologues are the aromatics that have a special stability and properties because of a closed loop of electrons, of which benzene is the most common. A benzene ring contains six carbon atoms, each carbon atom joined to a hydrogen bond (C_6H_6). Generally, a benzene ring is considered to be the backbone of the family of aromatic compounds. Aromatic compounds containing several rings are known as polycyclic aromatic hydrocarbons (PAHs), or polyarenes, or polynuclear aromatic hydrocarbons (PAHs). These compounds are of most concern, owing to their toxicity and the tendency to become recalcitrant in the environment (Wrenn and Venosa, 1996).

Until the advent of automobile as a major mode of transportation, petroleum was of little use except as a lubricant and replacement for whale oil in oil lamps; consequently, hydrocarbons in

the environment did not pose much of a challenge (Laws, 2000). However, as years passed by, hydrocarbon products such as kerosene, lubricant oil, diesel, and gasoline became major industrial products and subsequently began to enter into the environment in larger quantities. Now, more than 30 PAH compounds and several thousands of PAH derivatives have been identified which have mutagenic and carcinogenic effects, making them the largest class of chemical carcinogens identified to date (Bjorseth, 1985).

PAHs can be classified, based on the number of fused benzene rings, as low molecular weight (LMW) and high molecular weight (HMW) compounds. The rings can however be arranged linearly, angularly, or in a clustered form (Sims and Overcash, 1983, Grimmer, 1983, Harvey, 1998, Dabestani and Ivanov, 1999). The LMW are those with two to three clustered rings, while those with four or more are referred to as HMW. Based on the ring structure, PAHs are also classified as alternant and non-alternant. Alternant PAHs such as anthracene, phenanthrene, and chrysene are derived from benzene by fusion of additional six-membered benzene rings, and contain fewer than eight rings (Harvey, 1998). Non-alternant PAHs may contain rings with fewer than six carbon atoms in addition to six membered rings – examples are acenaphthylene, fluoranthene, and Indeno(1,2,3,-cd)pyrene.

These PAHs are hydrophobic; thus their solubility in water as well as in organic solvents varies depending on the molecular weight, structural orientation, type of solvent, and octanol-water partition coefficient. Generally, as the number of benzene rings in a PAH compound increases, solubility decreases (Wild and Jones, 1995, Wick et al., 2011). However, an exception to this rule has been observed based on the symmetry, planarity, and the presence of substituents in the PAH structures. For instance, solubility has been found to increase in linearly fused PAH as the number of rings increases, but has not been observed in angularly fused PAHs (Harvey, 1998). Planar PAHs are less reactive (i.e. less soluble) and biologically less toxic (Dabestani and Ivanov, 1999). Substituted PAHs are those in which a functional group in the compound has been replaced with another functional group. For example, in a methyl-substituted PAH, one of the functional groups has been replaced by a univalent compound with the general formula $-CH_3$. As the compounds deviate from planarity, they tend to be more soluble in organic solvents. Most by-products of PAH degradation tend to be more polar and have higher solubility in the environment than the parent compounds (Wick *et al.*, 2011).

PAHs are formed and released into the environment through natural and anthropogenic sources. Natural sources include volcanoes and forest fires, while anthropogenic sources include majorly fossil fuel combustion, wood burning, and municipal and industrial waste incineration. These organic contaminants tend to adsorb onto soil and sediment, owing to their hydrophobicity, thus making them recalcitrant in the environment. PAHs are potentially toxic, mutagenic and carcinogenic to humans. Consequent upon these health risks, 16 of them have been identified as priority

pollutants by the Environmental Protection Agency (USEPA) of the United States of America (USEPA, 1999). These 16 EPA polycyclic aromatic hydrocarbons are listed in Table 2.1.

Table 2.1: The 16-EPA PAHs and their physical properties

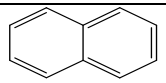
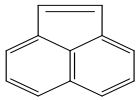
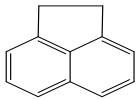
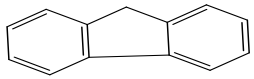
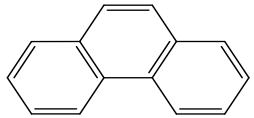
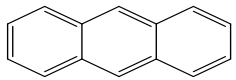
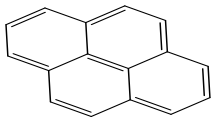
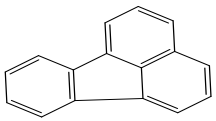
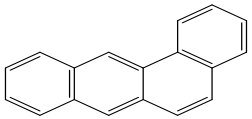
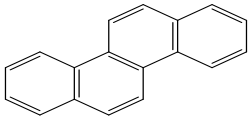
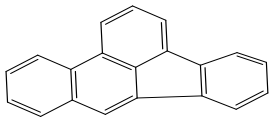
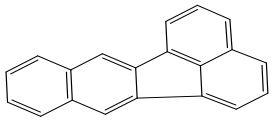
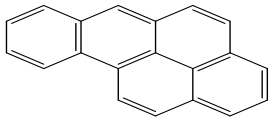
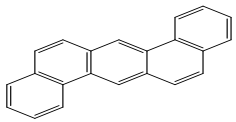
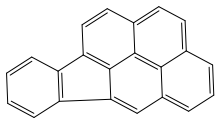
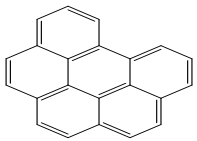
PAH Name	Number of rings	Molecular formula	MW (g $mole^{-1}$)	Mt Point °C	BPt, °C	Solubility in water (mg L^{-1})	Vapour Pressure (Pa)	Log K_{ow}
Naphthalene		$C_{10}H_8$	128	80.2	218.0	31	$1.0*10^{2}$	3.37
Acenaphthylene		$C_{12}H_8$	152	91.8	280	16.1	$9.0*10^{-1}$	4.00
Acenaphthalene		$C_{12}H_{10}$	154	96	278	3.8	$3.0*10^{-1}$	3.92
Fluorene		$C_{13}H_{10}$	166	116	295	1.9	$9.0*10^{-2}$	4.18
Phenanthrene		$C_{14}H_{10}$	178	100	339	1.1	$2.10*10^{-2}$	4.57
Anthracene		$C_{14}H_{10}$	178	217	340	0.045	$1.0*10^{-3}$	4.54
Pyrene		$C_{16}H_{10}$	202	150.4	393	0.13	$6.0*10^{-4}$	5.18
Fluoranthene		$C_{16}H_{10}$	202	108.8	383	0.26	$1.2*10^{-3}$	5.22

Table 2.1 cont'd

Benz(a)anthracene *		$C_{18}H_{12}$	228	158	400	0.011	$2.8*10^{-5}$	5.91
Chrysene *		$C_{18}H_{12}$	228	253.8	431.0	0.006	$5.7*10^{-7}$	5.91
Benzo(b)fluoranthene *		$C_{20}H_{12}$	252	168	N/A	0.0015	-	5.80
Benzo(k)fluoranthene *		$C_{20}H_{12}$	252	217	N/A	0.0008	$5.2*10^{-8}$	6.00
Benzo(a)pyrene *		$C_{20}H_{12}$	252	179	496	0.0038	$7.0*10^{-7}$	5.91
Dibenz(a,h)anthracene *		$C_{22}H_{14}$	278	262	524	0.0006	$3.7*10^{-10}$	6.75
Indeno(1,2,3,-cd)pyrene *		$C_{22}H_{12}$	276	163	536	0.00019	-	6.50
Benzo(ghi)perylene *		$C_{22}H_{12}$	276	278	500	0.00026	$1.4*10^{-8}$	6.50

The US EPA has classified PAHs (*) as possible human carcinogens. Kow is octanol-water partition coefficient.

Several methods have been used for the removal of PAHs from contaminated samples such as: extraction (Silva *et al.*, 2005, Yap *et al.*, 2012, Song *et al.*, 2012, Lau *et al.*, 2014), adsorption/biosorption (Chang *et al.*, 2004, Vidal *et al.*, 2011, Kaya *et al.*, 2013), co-metabolism (Reda, 2009, Moscoso *et al.*, 2012), and biosurfactant application**.** Some of these remediation methods have found field applications, while a good number of others still remain as microcosms or at pilot stage. Overall, environmental remediation of PAH contamination proceeds either by biological process, or physical or chemical techniques, or a combination of either two of the three, with each technique having its advantages and disadvantages. The use of microorganisms and/or biological products to decontaminate or transform pollutants from the environment to less harmful intermediates or innocuous products, is considered an environmentally benign approach for the clean-up of PAH contamination. Hence, this chapter will highlight some of these methods, but with emphasis on the biological systems.

The economic viability of bioremediation technology and the low bioavailability and mass transfer limitations of PAHs from several matrices into the aqueous phase for microbial degradation, still constitute a major challenge. Research efforts have focused on developing novel techniques to circumvent these challenges, such as biophysical processes (e.g. bioextraction, biosorption and biodegradation) and physicochemical processes. The potential application of biologically produced active surface agents, known as biosurfactants, to enhance the bioavailability of PAHs, has been extensively researched (Das and Mukherjee, 2007b, Makkar *et al.*, 2011, Zhu *et al.*, 2013). Such surface-active agents can be synthesized from cheap renewable resources such as agricultural by-products and/ or agro-industrial wastes.

Researchers have identified a number of suitable agrowaste substrates for this purpose (Sousa *et al.*, 2012, Sobrinho *et al.*, 2013). Some of the identified agrowaste do not contain all the required nutrients or do not contain the nutrients in the required proportion for microbial growth and for the synthesis of the requisite metabolites, and therefore need to be supplemented with refined nutrients. This approach is deemed feasible on a large scale owing to possible cost reduction and process sustainability, especially for *ex-situ* bioremediation. However, one of the concerns, also investigated, is the comparative effectiveness of biosurfactants produced from agrowaste and those produced from refined glucose. Factors affecting microbial growth and biosurfactant yield have also been studied and optimized to enhance the effectiveness of the system in the environmental bioremediation of PAHs. Bioremediation methods are considered to be relatively inexpensive and less invasive compared with several physicochemical remediation processes (Acevedo *et al.*, 2010, Bautista *et al.*, 2010, Acevedo *et al.*, 2011). Recently, some novel adsorbents have been synthesized for the adsorption of hydrophobic organic contaminants as well as the synergistic effects of adsorbent modification with surfactants

(Fungaro *et al.*, 2011, Schick *et al.*, 2011, Kaya *et al.*, 2013, Vidal *et al.*, 2013). In general, the use of microorganisms for degradation seems to be the only non-intrusive technology for the ultimate clean-up of contaminated environments; several other methods only translocate the contaminants from one medium to another (Wick *et al.*, 2011).

In addition to the concerns raised above, this review chapter describes the environmental behaviour of PAHs, and how it affects bioavailability. It discusses some of the recent approaches to enhance bioavailability. It also examines the effectiveness of biosurfactants produced from renewable resources for the bioremediation of PAHs, with a view towards system sustainability as well as minimizing the cost associated with the biological process. The chapter further discusses the effects of bioavailability on the bioremediation of PAHs and relevant kinetic models that can be used to predict microbial cell growth, substrate utilization, and biosurfactant production.

2.2 Sources and environmental fate of PAHs

2.2.1 Sources of PAHs

PAH compounds are formed and released into the environment through both natural and anthropogenic sources. Natural sources include volcanoes, forest fires, and biogenecity. Anthropogenic (or petrogenic and pyrolitic) sources include combustion of fossil fuels, wood burning, municipal and industrial waste incineration, asphalt roads, roofing tars, coal tar, coke, crude oil, creosote, discharges from industrial plants and waste water treatment plants, hazardous waste sites, coal gasification sites, smoke houses, aluminium production plants, atmospheric contamination of leafy plants, and charbroiled meat (Wick *et al.*, 2011).

PAH compounds have been reported in air, water, soil, food containing plant material, and thus can enter humans through any of these media (Björseth *et al.*, 1979, Cerniglia, 1992, Gao and Zhu, 2004), as depicted in Fig. 2.1. The main sources of PAHs in water bodies are atmospheric particulate matter deposition, runoff of polluted ground water sources, industrial effluent discharge, municipal wastewater discharge, and oil spills (Dabestani and Ivanov, 1999, Latimer and Zheng, 2003). Owing to the hydrophobicity of PAHs, the contaminants tend to adsorb to particulate matter, and are thus found in low concentrations in water bodies. PAH concentration measured in water bodies ranges from less than 1 to 800 µg L^{-1} (Chen *et al.*, 2004, Surif, 2013). Meanwhile, concentration ranges of µg/kg to g/kg can accumulate in solid media such as soil, sediments, or dredging, depending on the proximity of the area to the PAH sources such as creosote production sites, wood preserving sites, petrochemical sites,

gas manufacturing plant sites, and oil spilled sites (Juhasz and Naidu, 2000, Latimer and Zheng, 2003, Chen *et al.*, 2004). From atmospheric deposition and accumulations in soil, PAHs can be absorbed by plant roots. This varies from one geographical location to another, as the contaminant concentration is expected to be more in industrialized regions. Hence, PAHs can be contacted by humans through inhalation of contaminated air particulates, consumption of contaminated leafy vegetables, fats, oils, grilled and smoked meats, and ingestion of contaminated water (Menzie *et al.*, 1992).

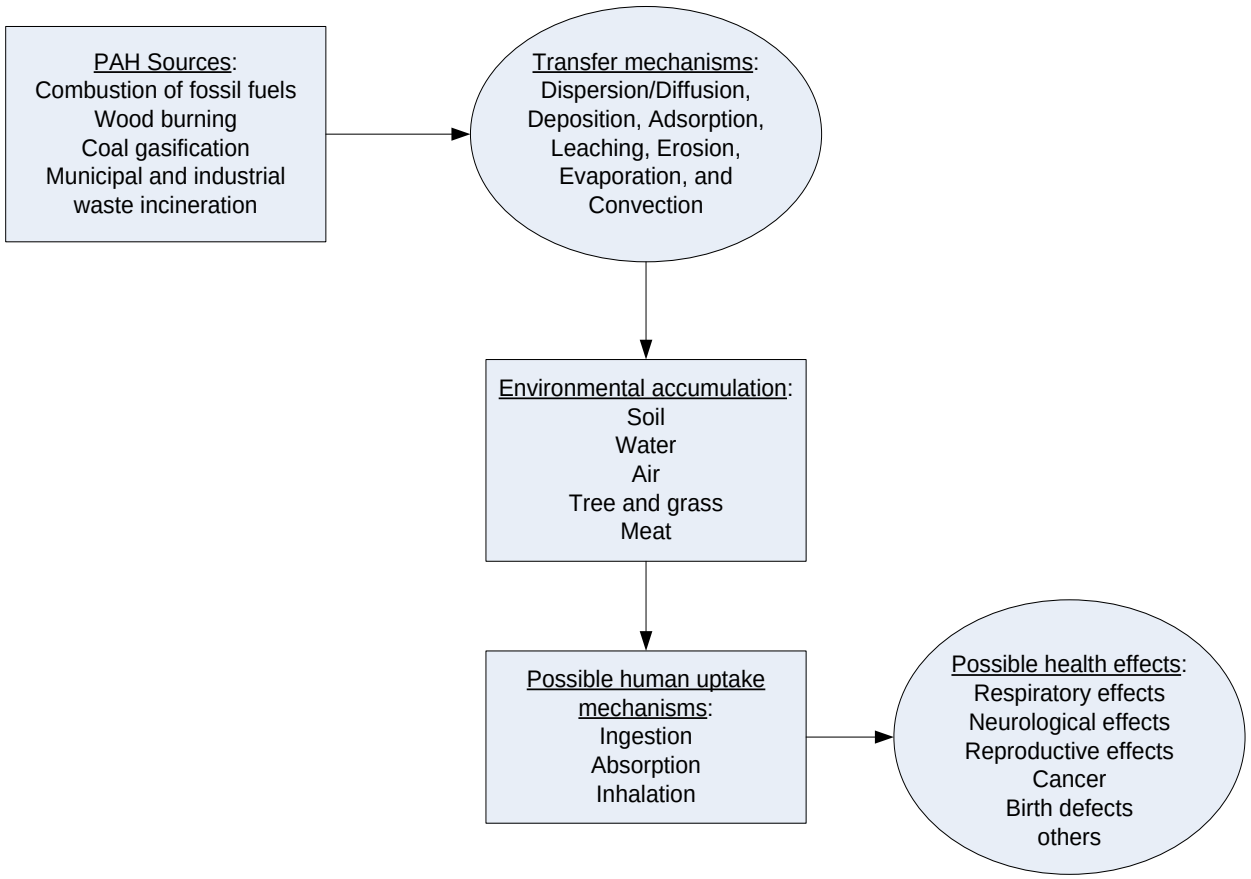


Figure 2.1: Environmental fate of PAH contaminants and possible health effects

PAHs' environmental behaviour depends on their physicochemical properties. They are semi-volatile substances at atmospheric conditions and frequently occur both in the vapour phase as well as particulate matter, depending on the vapour pressure of each PAH (Wingfors *et al.*, 2001, Basheer *et al.*, 2003). As the molecular weight of PAHs increases, hydrophobicity/lipophilicity increases, water solubility

decreases, vapour pressure decreases, and the compounds become sequestered within the soil matrix, making them non-bioavailable for degradation. Because of their hydrophobicity, they tend to partition into soil particles, and in water as non-aqueous phase liquid (NAPL) or as dissolved contaminants (Harvey, 1998). Soil contamination is therefore the commonest form of contamination, and because these compounds are recalcitrant pollutants, their levels may remain high enough to pose a threat (not only to the environment) long after their release. Therefore, soil contamination constitutes a major environmental challenge.

2.2.2 Environmental fate

When PAH compounds are released into the environment, there can be degradation, accumulation, or translocation. In every case, at least one or two of these processes usually occurs. These processes are controlled by a number of factors that include soil type (mineral and organic matter content), variation in soil depth and seasonal changes, and physicochemical properties of the contaminant (e.g. aqueous solubility, polarity, hydrophobicity, and molecular structure).

The major pathway for the removal of PAHs in soil is by microbial metabolism. However, the translocation of the contaminant can take place through the following mechanisms: leaching, volatilization, precipitation and sorption. The physical and chemical properties of the particular PAH compound being degraded, as well as environmental factors such as soil temperature, moisture content, pH, and oxygen concentration, can affect the level and rate of the degradation process (Manilal and Alexander, 1991, Weissenfels *et al.*, 1992).

Similarly, the concentration, together with the impact of PAHs, may depend on the proximity of the source of generation. Hence, PAH accumulations in urban soil are reportedly higher than in remote soils, ranging from 0.5 – 50 mg kg^{-1}, with high concentration observed in densely populated areas (Wild and Jones, 1995, Morillo *et al.*, 2007, Jiang *et al.*, 2009). In addition, significantly higher concentrations are found in industrial sites. For example, a total PAH concentration of 5863 mg kg^{-1} was reported at a creosote site, 18704 mg kg^{-1} at a wood preserving site, 821 mg kg^{-1} at a petrochemical processing site, and 451 mg kg^{-1} at a gas manufacturing plant site (Juhasz and Naidu, 2000). Studies on PAH concentrations' variation in different soil textures showed this order: coarse sand > fine > silt > clay, while biodegradation rate followed the reverse order (Amellal *et al.*, 2001, Owabor and Ogunbor, 2010). This is because the finer particle size fractions have more water, nutrients, and soil organic matter retaining capacity than the large particle size fractions with little internal surface for PAH adsorption.

Furthermore, the seasonal variation in the concentration of 15 PAHs was reported by Hong et al. (2007), and was highest in autumn and lowest in summer, for pyrene and benzo(a)pyrene.

2.2.3 Accumulation, sorption, and sequestration

An understanding of the mechanisms and dynamics of PAHs accumulation and sequestration in the environment is important for a successful bioremediation process, especially for freshly contaminated sites, most often with oil spills. Two stages of kinetics are generally observed when PAHs are released into the environment (Xing and Pignatello, 1997). A portion of the contaminant gets adsorbed quickly onto the soil surface and mesopores, whereas the remaining fraction is sorbed through the soil surface into the intrapores more slowly over weeks, months or even years – a phenomenon that is often referred to as ageing. Ageing of hydrophobic organic compounds (HOCs) in the environment is largely influenced by the soil organic matter, inorganic constituents of the soil, soil texture (with particular reference to pore size), concentration and physicochemical properties of the contaminants, along with microbial activity (Hatzinger and Alexander, 1995, Guthrie and Pfaender, 1998, Nam and Alexander, 1998).

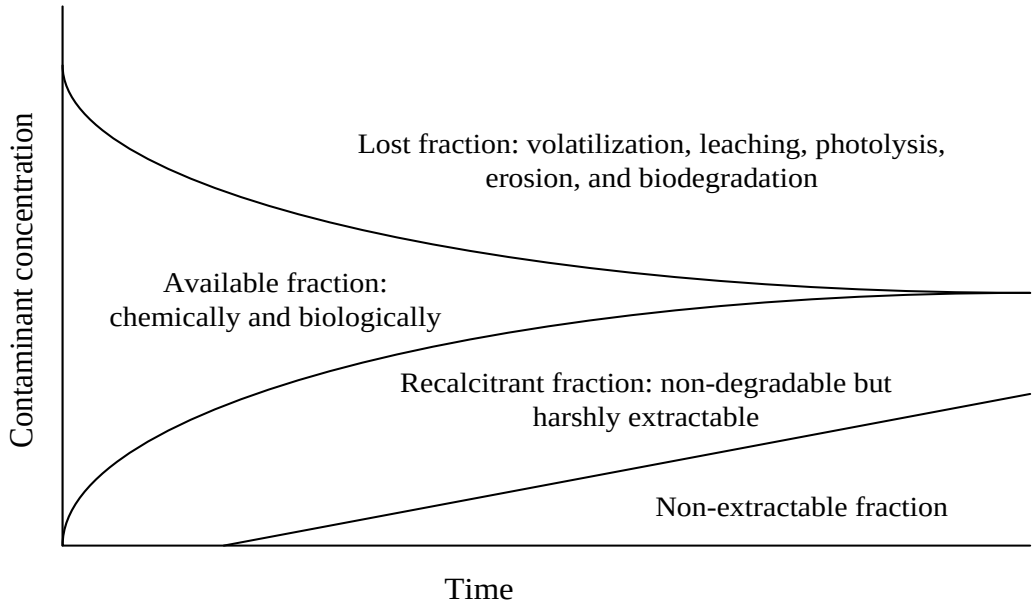


Figure 2.2: The influence of contact time on the extractability and bioavailability of a contaminant (Semple *et al.*, 2003)

An increased contact time between the contaminants and soil increases the level of sorption and sequestration of the contaminants within the soil matrices, making them unavailable for degradation (Fig. 2.2). In cases of fresh contamination, a large fraction of the contaminant is available for autochthonous microorganisms; in this case, a bacteria metabolic process has been identified as the most robust and rapid way of decontaminating such an environment. Sequestration of the contaminants slows down microbial activities as the process becomes substrate limited, making the contaminants recalcitrant (Wick *et al.*, 2011). At this stage, the microorganisms begin to synthesize metabolites such as biosurfactants to enhance solubilization and diffusion of the contaminants, in order to increase their bioavailability. Microorganisms undergo this process to survive in the environment where the only carbon source is not readily available. Hence, low bioavailability has been identified as the major challenge to environmental remediation of PAHs. However, several methods have been reported to enhance the bioavailability of PAHs. Some of these methods are discussed briefly in this chapter.

2.2.4 Transfer mechanisms of PAHs in the environment

2.2.4.1 Volatilization

Volatilization describes the loss of organic contaminants such as PAHs from soil surface or surfaces of liquid media. The rate and extent of the volatilization is influenced by the physicochemical properties of the contaminants as well as weather conditions such as vapour pressure, temperature, and air flux. Henry's law (Eq. 2.1) has been used to predict the volatilization of various organic chemicals as well as PAH compounds (Achman *et al.*, 1993, Alaee *et al.*, 1996).

$$H = (C_a / C_w)RT, \tag{2.1}$$

where C_a is the concentration of PAHs in air (mol m^{-3}), C_w is the concentration of PAHs in water (mol m^{-3}), R is the universal gas constant (8.314 Pa m^3 mol^{-1} K^{-1}), T is absolute temperature (K), and H is the Henry's law constant (Pa m^3 mol^{-1}). The rate of volatilization of individual PAH compounds may also differ based on the variations in their vapour pressure, molecular weight and structure, as well as

solubility. Generally, the understanding of the rate of volatilization may be expedient for assessing PAHs' accumulation and/or sequestration in the environment.

2.2.4.2 Leaching

The translocation of PAHs either laterally or downward through soil stratification is usually very low because of their high hydrophobicity. This phenomenon is dependent on water content, soil texture and organic matter content, seasonal variation, and anthropogenic activities such as land farming and irrigation (Petruzzelli *et al.*, 2002, Ran *et al.*, 2007). The leaching of soluble PAHs can be described by Darcy's law (Eq. 2.2).

$$q = -kA(dh/dl), \qquad 2.2$$

where q is the flow rate (volume/time), A is the cross-sectional area, k is the hydraulic conductivity of the soil/sediment, while dh/dl is the change in head per unit distance of contaminant movement (hydraulic gradient). Darcy's law is often considered valid for slow and viscous liquids, of which subsurface water flow and the percolation of soluble contaminants through soil pores, are suitable representations. This understanding might be helpful in assessing the possibility and level of hydrophobic contaminant in subsurface water. For instance, Enell *et al.* (2004) investigated the leaching of PAHs through soil and reported that about 0.3% of the total PAHs were leached over a period of three months under saturated conditions. Some other researchers have reported on the leaching of PAHs (Brandt and De Groot, 2001, Kim and Osako, 2003, Legret *et al.*, 2005). In addition to the parameters mentioned above, upon which leaching of pollutants depends, contaminant solubility and partitioning coefficients are important factors for evaluating the risk of pollutant leaching.

2.2.4.3 Adsorption

In environmental samples, PAHs are transported and get adsorbed in the soil micropores where they become inaccessible to microorganisms. The contaminants thus become sequestered and settled within the pores, making them persistent. The interaction between pollutants and the soil constituents may significantly change the migration behaviour of the pollutants and the extent of sequestration. The finer the soil texture, the longer it takes for the contaminants to settle within the pores, and once

settled, they are difficult to desorb – an example is adsorption in clay. Adsorption also describes the uptake of PAHs by plant roots or animal and human ingestion. This is one of the fastest ways of PAHs' translocation in the environment. The rate of adsorption, in this case, can be influenced by factors such as exposure time, cell membrane transport, susceptibility, and plant species. Several adsorption models have been used to describe PAHs' adsorption from aqueous phase – the most common are the Langmuir, Freundlich, Tempkin, Dubinin–Radushkevich, and Redlich–Peterson models (Dowaidar *et al.*, 2007, Long *et al.*, 2008, Yang *et al.*, 2013).

2.4.2.4 Erosion

Transfer of PAHs by water transport and wind is one of the major mechanisms for the transfer of the contaminant in the environment. This mechanism is influenced by soil aggregation, wind speed, and rainfall. Erosion contributes significantly to the accumulation of PAHs in water bodies, which is often the repository of most environmental pollutants.

2.4.2.5 Diffusion, dispersion, and convection

The bioavailability of PAHs in aquifers is largely dependent on the chemical and physicochemical properties of the PAHs and that of the solvent that determines the presence and concentration of PAHs in the solution, and that subsequently control the environmental distribution. The transport of PAHs to get adsorbed onto and into soil particles, as well as desorption from the soil surface and intrapores to become available for microbial degradation in aqueous media, is influenced by diffusion, dispersion, and convection. All contaminant species in solution are subject to these mechanisms, whether in the same solution or in contact with a stagnant solution. In cases where a polluted site is in contact with stagnant water, the only possibility for transport of the contaminants will be diffusion through the aqueous phase, in which case, the mean pathway (*X*) is given as (Eq. 2.3):

$$X = \sqrt{(2Dt)}, \tag{2.3}$$

where *D* is the diffusion coefficient in (m^2 $year^{-1}$) and *t* is time (year). Typically the diffusion coefficient *D* will be higher in pure water than in porous media, where the effect of constriction and tortuosity can affect the value of D.

2.3 PAHs removal from contaminated samples

2.3.1 Extraction techniques

In most cases extraction studies are conducted with the aim of mobilizing the hydrophobic organic compounds (HOCs) into a suitable organic phase and concentrated for quantification. The quantity of extractable contaminant is used as a representation of what can be available for microbial degradation. Since it is difficult to ascertain the level of contamination of a particular contaminant, such as PAHs, in the environment, exhaustive extraction methods have been adopted to extract as much as possible from various matrices. Researchers have argued that the non-extractable fraction may not be available as to pose health risks to the environment (Semple *et al.*, 2003). Extraction studies are often limited to quantification as a mimicry of bioavailability which, in effect, amount to mere relocation of the contaminants, such as from soil into aqueous phase or from water into another liquid. These contaminants often find their way back into the environment if not reduced to innocuous end-product(s); this is cyclical (Fig. 2.3). However, a combination of extraction method with biological treatment, whereby extraction is used as a pre-treatment step, can enhance the efficiency of the bioremediation.

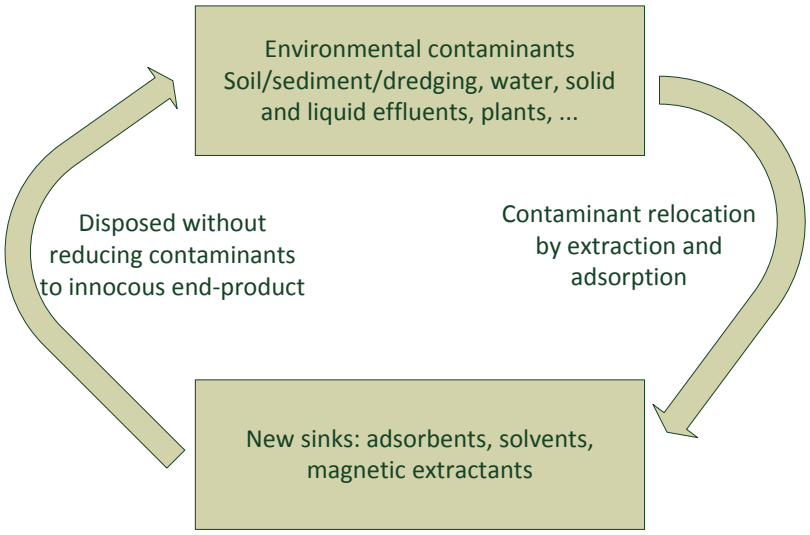


Figure 2.3: Cycle of contaminant translocation by adsorption and extraction

Some of the extraction methods in use are Soxhlet extraction, ultrasonication, supercritical and subcritical fluid extraction, ionic liquid extraction, etc. The limitations of these methods are summarized in Table 2.2. For further study on the extraction techniques, analytical chemistry textbooks on extraction can be consulted. Review articles written by Poole and Poole (2010) and Welton (1999) are also recommended for further reading.

Table 2.2: Various extraction methods for PAHs – advantages and limitations

Extraction methods	Technology involved and advantages	Limitations	References
Ultrasonic agitation/ Sonication	Uses acoustic energy of ultrasonic wave.	Not suitable for LMW PAHs.	(Green and Akgerman, 1996, Saldana et al., 2005)
Soxhlet extraction	Uses principles of evaporation and condensation; often the desired fraction has limited solubility in the extracting solvent while the impurity is insoluble in that solvent.	Process is labour intensive, time consuming, and uses large volume of solvent.	(Luque de Castro and Garcıa-Ayuso, 1998)
Supercritical and subcritical fluid extraction	Uses environmentally benign fluids above their critical conditions to extract the desired components. Suitable for extracting VOCs.	Often difficult to optimize.	(Anitescu and Tavlarides, 2006).
Microwave assisted extraction	Uses heat and microwave radiation energy. Advantages include reduction in solvent usage and time, and enhancement of the extraction of non-polar samples.	Solvent needs to be physically removed from the sample matrix upon completion of the extraction prior to further analysis.	(Letellier et al., 1999; Wang et al., 2007)
Ionic liquid extraction	Uses salts which are liquids at ambient temperatures, with unique properties of non-volatility, non-flammability, and excellent chemical and thermal stability.	Ionic liquids often contaminate the extracted analytes. This results in distorted chromatograms when gas chromatography is used for the determination step.	(Liu et al., 2003, Poole and Poole, 2010)

VOCs – volatile organic compounds

2.3.2 Application of semipermeable membrane devices to predict bioavailability

Semipermeable membrane devices (SPMDs) are lay-flat tubes, made of low-density polyethylene membrane, and contain a thin film of a high-molecular weight lipid tube filled with

isooctane, ion liquid or triolein containing cellulose acetate. Triolein, a major non-polar lipid found in aquatic organisms, is often used based on certain advantages it possesses such as: easy availability as a high purity synthetic product, low melting point, and large capacity to dissolve non-polar compounds. In addition, the SPMD sampler is easy to use, less expensive, more reproducible than live biota samplers, and only bioavailable compounds are sampled. The application of SPMDs used to mimic the absorption of compounds through cell membranes was introduced by Huckins *et al.* (1993), and has been successfully used in the aquatic environment (Verweij *et al.*, 2004, Gourlay *et al.*, 2005). Their deployment in soil environment to predict the bioavailability of PAHs has also attracted remarkable research interest (Gourlay *et al.*, 2005, Ahn *et al.*, 2005, Tao *et al.*, 2008)

2.3.3 Factors affecting PAHS extraction in soil: moisture content and other soil characteristics

A number of factors can affect the extraction of PAHs from environmental samples, and these include: temperature, extraction solvent and volume, extraction time, extraction method, soil texture, and dissolved organic matter. According to Tao *et al.* (2006) in their study on the effects of soil organic matter on the extractability of different PAHs compounds from seven soil samples, it was reported that the recovery of individual PAHs as well as the sum of the four PAH compounds studied was correlated with the DOM but not correlated with the total organic matter (TOM). This means that the DOMs enhance the extraction of PAHs while TOM retains them in soil. The results of the study actually supported some of the properties of PAH compounds as discussed earlier in this chapter; PAH compounds are hydrophobic and thus partitioned toward solid particles in the environment.

Various reports on the effects of temperature and pressure on the recovery efficiency of PAHs showed stochastic results. In a study on PAHs' extraction from three environmental samples (marine sediment, diesel soot, and air particulate matter), an increase in temperature from 80 to 200 °C led to an increase in the recovery of PAHs for all the three samples (Yang *et al.*, 1995). An increase in extraction temperature from 40 to 200 °C was also found to increase the recovery of PAHs from spiked soil as well as from natural contaminated marine sediment and railroad bed soil (Langenfeld *et al.*, 1995). These authors had earlier observed that temperature was more important than pressure for achieving high extraction efficiencies when the interaction between pollutant molecules and sample matrices are strong, noting that increasing temperature from 50 to 200 °C increased PAHs' recovery from soil, while being independent of pressure increase. On the other hand, temperature increase did not influence PAHs' recovery from air particulate matters (Langenfeld *et al.*, 1993). Moreover, Latawiec and Reid (2010) studied the extraction of different PAH compounds and contended that there were no significant differences between extractions when temperature was increased to 200 °C. Similarly, in an

extraction study of different PAH compounds and their alkyl – and nitro – derivatives, it was observed that the compounds exhibited different threshold temperatures; highest recovery efficiency for some compounds occurred when the extraction was carried out at 100 °C, while for others, the temperature was raised to 200 °C (Schantz *et al.*, 2012).

The suitability of various extraction solvents (mostly organic solvents), extraction time and cycles, and extraction methods, has been reported for PAHs' recovery from environmental samples, as discussed previously. The application of surfactants for PAHs' recovery is discussed in detail in a subsequent section of this chapter. But, in general, it appears that PAH extraction efficiency from soil and particulate matrix is largely controlled by the physicochemical properties of the matrix, contaminant ageing in the matrix, and how the contaminant is incorporated into the matrix.

2.4 PAHs degradation and bioremediation techniques

The ultimate goal of degradation is the complete mineralization of PAHs to innocuous end products, that is, CO_2, water, microbial carbon, and other inorganic compounds, while paying attention to the environmental impact of the bioremediation exercise. Unfortunately, degradation of PAHs may result in the accumulation of metabolites (mainly ketones, quinones, and dicarboxylic acid anhydrides) that can be more toxic and/or more soluble than the parent compound (Lundstedt, 2003). In the field study of PAHs, a brief spike in leachate toxicity was observed owing to the accumulation of more soluble metabolites. However, after remediation was complete, the final toxicity was negligible compared with the original compounds, because the metabolites tended to be less stable and more soluble, making them more available to microbial degradation. Fluoranthene degradation has equally been found to produce more soluble and potentially leachable metabolites (Vessigaud *et al.*, 2007). The rate and extent of PAHs' biodegradation in the environment are controlled by many factors such as the physicochemical properties of the contaminants, depth into soil/to groundwater, soil texture, environmental distribution and concentration, pH and alkalinity, organic matter, nutrients, soil gas O_2 and CO_2, soil moisture, and microbial populations.

2.4.1 Chemical degradation techniques

Several chemical degradation techniques have been investigated for PAH degradation. The common method is chemical oxidation, which uses ozone and Fenton reagents – where oxidants (ozone, hydrogen peroxide, hypochlorites, chlorine and chlorine dioxide) are injected into the contaminated soil

to convert PAHs to more stable and less mobile forms (Valderrama *et al.*, 2009). Ultraviolet radiation can also be combined with ozonation to enhance the degradation of PAHs (Ledakowicz *et al.*, 2001). Other methods include electrokinetic degradation (often integrated as an assist in biological oxidation), photochemical degradation, and thermal decomposition (Harvey, 1998, Pierzynski, 2000). Combination of oxidation and bioremediation, whereby oxidation was used as a pre-treatment to microbial degradation, has been reported (Kulik *et al.*, 2006).

Chemical oxidation procedures have the advantages of better process control and relative insensitivity to external environmental disturbances, compared with biodegradation approach (Rivas, 2006). Nonetheless, there are several drawbacks to oxidation of PAHs, and these have raised considerable concerns about its continued applications. These drawbacks include: oxidant introduction negatively impacting subsurface soils; decreased soil permeability by colloid formation; release of previously sorbed metals to groundwater resources; and toxic by-product production, including heat and gas production.

2.4.2 Biological degradation and bioremediation techniques

Bioremediation or biological remediation is the use of biological systems such as bacteria, fungi, and enzymes to remove pollutants from the environment. Bacteria, being the most profuse in the environment, assume a dominant role in soil and in the marine ecosystems. For passive approaches, the existing microorganisms naturally attenuate the pollutants but in actively engineered bioremediation, the addition of specific enzymes, microorganisms, and nutrients may be required. Such remediation methods have the potential to be less expensive, less invasive or more environmentally friendly than many chemical or physical remediation options (Baker and Herson, 1994, Seah *et al.*, 2001, Ahuja *et al.*, 2004, Acevedo *et al.*, 2010, Acevedo *et al.*, 2011). The rate of PAHs' biodegradation in the environment is determined by the autochthonous microbial populations of hydrocarbon-degrading microorganisms and the physiological capabilities of the populations, in addition to other various abiotic factors that may influence the growth of the hydrocarbon degraders. Moreover, the composition of the microbial community and its adaptive response to the presence of the contaminant affects PAHs' biodegradation.

Several gram-negative bacterial and some gram-positive species have been reported for their ability to degrade numerous PAHs. These genera include *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., and *Acinetobacter* sp. (Chen *et al.*, 1999, Boonchan *et al.*, 2000, Dandie *et al.*, 2004, Mishra and Singh, 2014, Ghosh *et al.*, 2014). Many of these species have recorded high metabolization rates, especially for low molecular weight (LMW) PAHs, while a few have been able to demonstrate similar abilities for the HMW PAHs, with more than three benzene rings. However, fungi have also shown the ability to

transform and mineralize organic contaminants owing to their enzymatic and mycelium system which enable them to access the pollutants in the soil (Pointing, 2001).

Moreover, a number of studies have often focused on PAHs' degradation in the aqueous phase, as a secondary remediation process after extraction from solid matrices such as soil, using washing as an extraction method prior to degradation due to the sequestration of these contaminants in soil. As a result of the small intraparticle pores in soil grains, gram-negative bacteria appear to be better degraders of PAHs in soil owing to their thin cellular membrane which may assist in higher PAH mass transfer across the cellular membrane, thereby facilitating sorption subsequent to intracellular degradation (Ma *et al.*, 2013). Furthermore, earlier studies had reported that most indigenous bacteria may be physically precluded from some intraparticle soil grain pores because of the mean diameter of the pores that the immobilised bacteria require to penetrate to access bound pollutants (Alexander, 1977, Lawrence *et al.*, 1979b). This perhaps elucidates limitations associated with the bioremediation of contaminated soil with a high fraction of clay and silt. Low bioavailability and mass transfer limitations are the challenges to PAHs' bioremediation processes, particularly in soil. These limitations are often influenced by the contaminants' molecular structure, weight, weathering, ageing, and the soil physicochemical characteristics.

These challenges, in addition to the drawbacks of conventional chemical methods for the remediation of environmental contaminants, have necessitated the design of more robust biological systems and optimization for the degradation of PAHs and other pollutants. The quest includes the use of bioaugmentation, biostimulation, bacterial co-culture, co-metabolism, and genetically modified microbial strains, including isolation and identification of more prolific microorganisms. Most common remediation techniques are listed in Table 2.3.

2.4.3 Factors that may limit biodegradation and bioremediation of PAHs

In environmental bioremediation, it is important to know what contaminants and co-contaminants are present that may affect the microbial degradation of the target compounds. In certain instances, co-contaminants can become competitive to the target contaminant, thereby inhibiting the degradation of the target compounds. On the other hand, co-contaminant/co-metabolism can enhance microbial cell proliferation, and thus enhance the degradation of the target contaminant. In a biodegradation study of PAHs (BaP and Phe), the presence of Phe was found to inhibit the degradation of BaP – a more hydrophobic compound (Wang *et al.*, 2014). In such a scenario, optimization of culture parameters may be necessary to control the microbial metabolism so that the target compound will be degraded.

Table 2.3: Some conventional bioremediation technologies and their limitations

Methods	Technologies involved	Typical equipment types	Limitations/challenges
Land farming	A combination of biostimulation, bioaugmentation, and/or surfactants, combined with tilling and mixing the soil/sediment to improve distribution of the contaminants and oxygen supply.	Containment pad, leachate collection and management system, tilling equipment, leachate collection and treatment system.	Maintaining oxygen diffusion throughout the contaminated soil is often a challenge. Possibility of groundwater contamination with leachates except carried out on water-impervious platform.
Biopile/ Biocell	Involves excavating and treating soil in a pile or in a layer. Controls consist of impermeable barriers designed to prevent contaminant migration, and pipes through the soil for aeration and nutrient distribution.	Soil pile support pad or container, aeration pipes and blowers, off-gas treatment equipment, leachate collection and treatment system, nutrient feed and chemical stabilizer system.	Treatment of large contaminated area is a challenge. It's an intrusive approach on the eco-system.
Composting	Contaminated material is mixed with an organic substrate (i.e. straw, wood chips or bark), supplemented with inorganic nutrients and placed in a pile.	Mixing equipment for organic additives, organic additives and bulking agents, soil pile support pad and aeration pipes and blowers for windrow turning machine or composting reactor, off-gas treatment equipment.	Optimization of aeration, temperature, moisture and pH, C: N ratio is necessary for successful operation during composting.
Slurry-Phase Bioreactors (soil and sediment)	Contaminated sediment/soil is put into bioreactors in small batches, mixed with nutrients and microbial cultures and aerated.	Soil/sediment mixing equipment, bioreactor with aeration components, clarifier, off-gas treatment system, nutrient feed and chemical stabilizer system.	Often difficult and not economical for large quantities of sediment.
Bioventing	An *in situ* remediation approach by aerating indigenous microorganisms using extraction or injection wells.	Air injection wells, blowers, soil-gas monitoring points, off-gas treatment equipment (if required).	Low soil permeability (particularly in clay and silts). Vapour build up in basements can affect air injection wells. Low soil moisture and off-gases at the soil surface may limit operation.
Phyto-remediation	Uses plants to reclaim contaminated areas through increasing microbial activity in the rhizosphere while breaking down the contaminant.	An on-site strategy which does not necessarily need any containment.	A very lengthy process, usually considered a secondary treatment method. It's often cumbersome to identify plants with appropriate root morphological properties.

Soil texture is another factor that often influences the biodegradation of hydrophobic organic contaminants. An understanding of contaminant distribution in the soil may help in developing strategies for delivery and assessing the effectiveness of remedial reagents. Mass transfer limitation tends to occur in less permeable soils such as silts and clays that slow the migration of contaminants and often contain more contaminants than the more permeable strata. A high content of silt and clay in the soil can preclude the delivery of mobilization agents, such as biosurfactant, and even bacteria species from accessing the contaminants (Alexander, 1977, Lawrence *et al.*, 1979a).

Low concentrations of essential nutrients can be limiting to microbial degradation, slowing the degradation process and extending the time required for treatment. In some soils, nutrient is not present in required concentrations and proportions, and as such can limit or stop the degradation process. Petroleum oil-contaminated sites are usually rich in carbon but can be deficient in mineral nutrient or elements required to support microbial growth (Congress, 1991, Prince, 1997)**.** Marine and other ecosystems are often deficient in mineral nutrients because autotrophic organisms consume them in competition with the oil-degrading species. A typical example is the clean-up of the oil tanker Exxon Valdez oil spill of 1989 in Prince William Sound and the Gulf of Alaska (Pritchard and Costa, 1991, Bragg *et al.*, 1994) – a study that has since sparked further research interest in the potential of microbial communities in the biodegradation of hydrocarbon contaminants. However, it may be difficult to quantify the carbon content of environmental samples to determine nutrient deficient conditions, but information on the primary nutrient elements (N, P, K), is essential (Rentz *et al.*, 2005).

One of the most significant limitations to biodegradation of PAHs or other HOCs is low bioavailability. A necessary condition for microbial degradation of contaminants is the availability of the contaminants and, perhaps, the contact time of the microorganism with the contaminant. Usually, PAHs in the environment exhibit three regimes in terms of their availability: a fraction is readily available for microbial degradation, a slowly bioavailable fraction, and an unavailable fraction, as shown in Fig. 2.2. The subject of PAHs availability is discussed further in Section 2.6.

The physicochemical properties of contaminants could also be a limiting factor to microbial degradation of PAHs. The phytoremediation of PAH compounds (naphthalene and phenanthrene) has validated the chemotaxis of PAH-degrading rhizosphere bacteria – *Pseudomonas alcaligenes*, *P. stutzeri*, and *P. putida* – to plant root exudates (Ortega-Calvo *et al.*, 2003). But the same bacteria were repelled by anthracene and pyrene, demonstrating how the physicochemical properties of contaminants can affect biodegradation. The attraction of competent bacteria to the root zone may improve bioavailability and increase PAH degradation in the rhizosphere.

In summary, these limiting factors are highlighted below (Alexander, 2000, Goetz and Brenner, 2002, Straube *et al.*, 2003, Das and Chandran, 2010):

- Nutrient limitations.
- Non-optimal abiotic conditions of temperature, pH, salt, oxygen concentration and toxins.
- Lack of a prolific microbial community that can degrade PAH compounds or low microbial biomass.
- Limited PAH bioavailability.
- Physicochemical characteristics of PAH compounds.
- Exhaustion of a co-metabolic growth substrate.
- Predation of contaminant-degrading bacteria by opportunistic predators.
- Energetic limitations and build-up of toxic intermediates.

The manipulation of the above limitations is the basis for bioremediation technologies. Some of the technologies are briefly discussed in Section 2.4.4.

2.4.4 Techniques to enhance biodegradation and bioremediation: Biostimulation and bioaugmentation

Biostimulation is the addition of nutrients or carbon sources to the microbial culture containing the pollutants in order to stimulate or enhance the activities of the microorganism. The presence of nitrogen and phosphorous is limited in most contaminated sites, especially sites contaminated with hydrocarbon products. Thus, the addition of rate-limiting nutrients can accelerate the rate and extent of bioremediation, which is referred to as biostimulation. Addition of nutrient-rich organic matter obtained from domestic sewage treatment, inorganic fertilizers (rich in N and P), and commercial fertilizer, and biosolids rich in N and P, to enhance microbial degradation of hydrocarbon oil pollutants, is well reported (Mills and Frankenberger, 1994, Sarkar *et al.*, 2005). Supplementation of PAH-contaminated soil with glucose, sodium succinate and starch was shown to enhance bioremediation significantly. Besides nutrient supplementation, the availability of alternative carbon sources, as observed in the case of the Exxon Valdez oil spill bioremediation, can influence the mineralization potential of the degrading microorganisms.

Similarly, in certain operations, autochthonous microorganisms may need to be augmented with 'customized' or pre-adapted microbial strains to achieve the desired level of bioremediation. Microbial species normally used in this technology are genetically engineered bacterial strains or consortia that can adapt to the contaminated environment and as well as consolidation of the activities of the

indigenous microbial populations. Enhanced degradation of PAHs following bioaugmentation is well reported (Reda, 2009, Li *et al.*, 2009, Alisi *et al.*, 2009). An 85% removal efficiency of Phe from PAH-contaminated soil by a mixed cultures of *Pseudomonas fluorescene*, *Serratia liquifaciens*, *Bacillus* and *Micrococcus* strains was reported by Arbabi *et al.* (2009). The influence of bioaugmentation with *Pseudomonas* sp, *Pseudomonas aeruginosa* and consortium on PAH-contaminated soil was also reported elsewhere, whereby a biodegradation efficiency of 87.8%, 85.5%, and 92.8% respectively, were reported for Phe (Nasseri *et al.*, 2010).

The combination of biostimulation and bioaugmentation techniques has been found to be equally effective in enhancing bioremediation, and even more in some studies than using either of the two. A microcosm and pan studies to evaluate the effect of biostimulation with slow release of nitrogen fertilizer and bioaugmentation with biosurfactant-producing *Pseudomonas aeruginosa* strain 64 on a soil with 11300 mg kg^{-1} total PAH concentrations showed that the two techniques reduced PAH levels seven times more than the control after 16 months (Straube *et al.*, 2003). The synergistic effects of biostimulation and bioaugmentation were observed with three fungi strains of *Rhizopus* sp, *Penicillium funiculosum* and *Aspergillus sydowii* isolated from a PAH aged soil (Mancera-Lopez *et al.*, 2008). It was reported that the fungi were able to remove, respectively, 36%, 30%, and 17% more PAHs in comparison with biostimulation alone. Different techniques are used at a field scale for biostimulation and bioaugmentation, depending on the volume of material being remediated.

2.5 PAHs' adsorption onto nano-composite adsorbents

As mentioned earlier in this chapter, several physical and chemical methods have been used for PAH removal from water and wastewater such as photochemical treatment, oxidation, chlorination, membrane filtration, ozonation, biodegradation, and adsorption (Bohn *et al.*, 1985, Pierzynski, 2000, Zheng *et al.*, 2007, Bernal-Martínez *et al.*, 2007, Ferrarese *et al.*, 2008). Adsorption is a traditionally known method of migrating materials from one medium to another by immobilizing the material onto an appropriate support. Adsorption has shown high proficiency for organic compounds, as well as PAHs' removal from water and wastewater (Chang *et al.*, 2004, Ali *et al.*, 2012, Kaya *et al.*, 2013).

Consequently, a number of high-efficiency adsorbents have been developed, from inexpensive natural and synthetic materials for use in recent times, which include carbon nanocomposite materials (such as graphene oxide), mesoporous organosilica, organic and inorganic modified zeolites, and activated carbon (Vidal *et al.*, 2011, Zhao *et al.*, 2011, Bruna *et al.*, 2012, Nkansah *et al.*, 2012, Zhang *et al.*, 2013). Static adsorption study of three polycyclic aromatic hydrocarbons (PAHs) – naphthalene,

acenaphthene, and fluorene, from aqueous solutions onto hypercrosslinked polymeric adsorbent, showed that the molecular size of adsorbates has a distinct influence on the adsorption capacity of polymeric adsorbent for the PAHs; the larger the adsorbate molecular size, the lower the adsorption equilibrium capacity (Long *et al.*, 2008).

2.5.1 Activated carbon/activated sludge

Activated carbons are produced from heat beneficiation of carbonaceous materials. This treatment confers on the materials sponge-like characteristics, creating within and on the surface of the materials a network of pores for adsorption. Activated carbons are the most widely used adsorbents and they have been found suitable for absorbing an array of contaminants, including the PAH compounds. The adsorption of PAHs in a domestic wastewater treatment plant was investigated in a one-year period using quartz sand, kaolinite, and natural clay as inorganic adsorbents and activated sludge as an organic adsorbent for adsorbing naphthalene, phenanthrene, and pyrene.

It has been widely recognized that adsorption may play the main role in PAHs' removal in most wastewater treatment plants owing to the high affinity of PAHs for particulate matter (Lei *et al.*, 2007). Their different molecular structures strongly affect their aqueous solubility, octanol/water partition coefficient (Kow), and consequently their equilibrium partitioning coefficients for adsorption onto various particle surfaces. The tendency of increasing adsorption of PAHs onto solids with increasing fused rings has also been noticed in other studies (Mastral *et al.*, 2002, Liu *et al.*, 2011). The coupling of solid-phase extraction (SPE) using activated carbon with a monolithic column–high-performance liquid chromatography (MC–HPLC) method was developed for the high-efficiency enrichment and rapid determination of 16 polycyclic aromatic hydrocarbons (PAHs) in water (Ma *et al.*, 2011). Although activated carbon is predominantly used in adsorption studies (Ania *et al.*, 2008, Luna *et al.*, 2011), it has some drawbacks that can limit its application, such as ease of desorption of highly volatile adsorbates, and ineffectiveness in the removal of oil, grease, and organic contaminants (Chang *et al.*, 2004, Kaya *et al.*, 2013).

2.5.2 Zeolite application as adsorbents: its' synthesis from fly ash

Zeolites are crystalline hydrated aluminosilicate minerals which are characterized by a framework of tetrahedral molecules of SiO_4 and AlO_4, joined together by shared oxygen atoms

(Mumpton, 1977). The peculiar properties of zeolites enhance their applications in the removal of various cations and organics from contaminated water and wastewater (Kazemian and Mallah, 2006, Wang and Peng, 2010). Zeolites, in contrast to activated carbon, exhibit certain advantages such as excellent ion exchange capacities, catalytic properties, easy regeneration without losing their adsorption capacities, high selectivity, etc. (Khalid *et al.*, 2004, Mahabadi *et al.*, 2007).

Zeolites and many low-cost adsorbents have been synthesized from agricultural and industrial wastes. The volarization of these inexpensive and renewable biomasses, including other waste materials, offers additional sources of carbon for adsorbents' synthesis. Some of these wastes include bamboo dust, sewage char and tyres, coconut shell and husk, groundnut shell, oil palm shell and fibre, wheat bran, rice husk and straw, fly ash, etc. (Yamashita *et al.*, 2001, Tan *et al.*, 2008a, Tan *et al.*, 2008b, Rafatullah *et al.*, 2010, Yang *et al.*, 2011).

Fly ash is an inorganic residue of coal-fired power plants. Its characteristics vary tremendously and are dependent on its origin (Janoš *et al.*, 2003, Wang *et al.*, 2005, Rafatullah *et al.*, 2010). The chemical constituents generally comprise silica, alumina, magnetite, carbon, and traces of unburned residue (Ahmaruzzaman, 2010) with an approximate surface area of 15.6 m^2 g^{-1} (Wang *et al.*, 2005). According to the report of the International Energy Agency (IEA, 2013) and the US Energy Information Administration (USEIA, 2013), coal was adjudged the second primary source of energy in the world next to oil, with a consumption growth rate of 60 % recorded from 2000 to 2012, and accounting for more than 30% of the world's total energy mix. Obviously, large quantities of fly ash are produced annually. Hence, studies into the chemical constituents of these wastes have led to their profitable applications, among others for adsorption and zeolites synthesis (Sun *et al.*, 2010, Fungaro *et al.*, 2011, Ali *et al.*, 2012, Fungaro and Magdalena, 2014). Although some researchers have reported the efficiency of fly ash in the removal of textile dyes, phenolic compounds, phosphate, and heavy metals (Ahmaruzzaman, 2009, Chatterjee *et al.*, 2010, Koukouzas *et al.*, 2010), limitations such as lack of molecular selectivity and low binding capacity and kinetics to target molecules need to be overcome. The pyrolysis and modification of fly ash helps to improve the adsorptive capacity of this waste material.

In recent times, more efficient adsorbents such as zeolites and magnetic nanoparticles have been produced with high selectivity towards target molecules (Zhao *et al.*, 2011, Nkansah *et al.*, 2012, Zhang *et al.*, 2013). Zeolites synthesized from fly ash have demonstrated their affinity for the adsorption of organic compounds from water and wastewaters (Janoš *et al.*, 2003). The natural adsorption ability of fly ash makes it suitable for a host of adsorbents synthesis. A synergy of fly ash and magnetite has been used as a support for molecularly imprinted polymers, which was found to be very effective for the

selective adsorption of nonylphenol (Pan *et al.*, 2013). In addition, the magnetic composite form exhibited a high binding capacity and fast mass transfer.

Magnetically imprinted nanoparticles for separation of hydrocarbons/hydrocarbon derivatives or oils from water and minerals have been reported (Alfadul, 2007, Yamaura and Fungaro, 2013). Zeolites synthesized from fly ash may demonstrate similar adsorption affinity for HOCs like PAHs, owing to the magnetite composition of fly ash. Moreover, parameters such as Si/Al ratio, cation type, number and location mostly affect adsorption, and are thus often manipulated by chemical treatments to improve the efficiency of zeolites. Furthermore, the hydrophobicity of PAHs has remained a major challenge in most studies focusing on their treatment, thus several methods have been employed to enhance their solubility in aqueous media, which include adsorption by using zeolites.

2.5.3 Zeolite modification by surfactants

Since zeolites are hydrophilic and thus do not have large adsorption capacities for hydrophobic organic compounds, surfactant modification by simple ion-exchange mechanisms has been applied to significantly enhance their capability for the removal of hydrocarbon contaminants like PAHs from water and wastewater (Kaya *et al.*, 2013). Surfactant modification of zeolites is influenced by hydrophobic effects and cation exchange. The positive moieties of cationic surfactants readily exchange with the replaceable cations on the external surface of zeolite (Fig. 2.4), forming surfactant monolayers (Simpson and Bowman, 2009). At a surfactant loading above the critical micelle concentration, surfactants' molecules aggregate on the zeolites' surface to form bilayers, in which case, the upper layer is bound to the lower through hydrophobic bonding between the negative moieties of surfactants in both layers (Guan *et al.*, 2010, Lin *et al.*, 2011).

The modification process allows the exchangeable cations on the zeolites' surface to be replaced with cations from the surfactant molecule – usually the quaternary ammonium compounds, with cationic head groups and a hydrocarbon long chain molecule as the surfactant tail (Shen, 2001, Gök *et al.*, 2008, Park *et al.*, 2011, Kaya *et al.*, 2013). Thus, the zeolite surface is modified, which allows the adsorbent to retain organic compounds (Vidal *et al.*, 2013). The chemical surfactant that is commonly used for zeolites' modification is hexadecyltrimethyl ammonium bromide (HDTMAB). An array of contaminants such as anions, cations, and organics – phenol, 4-chlorophenol, Orange II, bisphenol A, and sodium dodecyl benzene sulfonate (SDBS), have been reportedly removed by surfactant-modified zeolites (SMZ) from aqueous solution (Dong *et al.*, 2010, Fungaro *et al.*, 2011, Lin *et al.*, 2011, Schick *et al.*, 2011, Vidal *et al.*, 2013). Furthermore, zeolites modified by hexadecyltrimethylammonium were

effective for the removal of inorganic cations, inorganic anions and neutral organic materials (Tan, 2002). The surfactant modified zeolite (SMZ) was found to be stable in high ionic strength and in a wide range of pH, and also maintained more than 90% of its adsorptive capacity after regeneration. The environmental implication of using chemical surfactant for zeolite modification could be a concern. Therefore, modification using biosurfactant is deemed suitable.

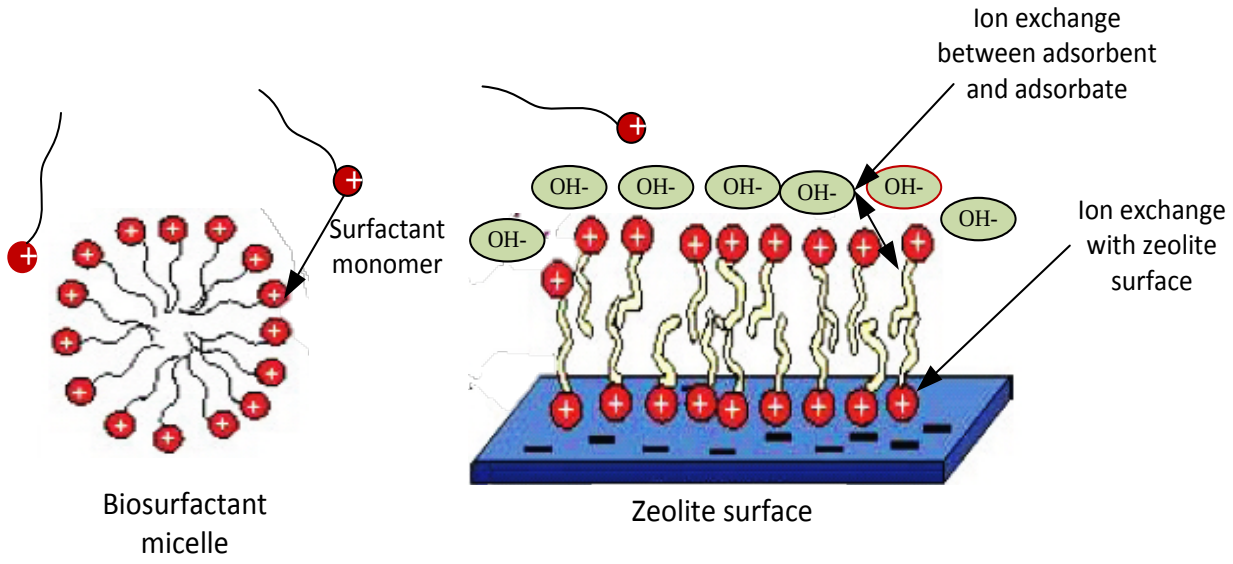


Figure 2.4: Surface modification of a zeolite by biosurfactant (Ranck *et al.*, 2005)

2.6 Bioavailability – a major factor affecting PAHs' biodegradation[1]

2.6.1 PAHs: various views and definition of bioavailability

Polycyclic aromatic hydrocarbon mobilization, degradation and sequestration in the environment are contingent upon the bioavailability of the PAHs. Researchers and/or scientists differ in their opinions of what the exact definition of bioavailability should be (Stokes *et al.*, 2005). The following definitions are listed in a technical report published by the European Centre for Ecotoxicology and

[1] ***Published in Chapter 8 of Environmental Biotechnology- New Approaches and Prospective application INTECH Open Access Publisher. URL – http://www.intechopen.com/books/environmental-biotechnology-new-approavhes-and prospective applications.***

Toxicology of Chemicals (ECETOC, 2002): (i) "The ability of a substance to interact with the biosystem of an organism" (Van Leeuwen, 1995); (ii) "the portion of the total quantity or concentration of a chemical in the environment or a portion of it that is potentially available for biological action" (Van Leeuwen, 1995); (iii) "the amount/percentage of a compound that is actually taken up by an organism as the outcome of a dynamic equilibrium of organism-bound sorption processes, and soil particle-related exchange processes, all in relation to a dynamic set of environmental conditions" (Herrchen, 1997). The NRC report also noted numerous definitions: "Bioavailability may represent the fraction of a chemical accessible to an organism for absorption, the rate at which a substance is absorbed into a living system, or a measure of the potential to cause a toxic effect". Often, environmental scientists consider bioavailability to represent the accessibility of a soil-bound chemical for assimilation and possible toxicity (Alexander, 2000), while toxicologists consider bioavailability as the fraction of chemical absorbed and able to reach systemic circulation in an organism. Another view of bioavailability is represented by a chemical's crossing a cell membrane, entering a cell, and becoming available at a site of biological activity. Others might think of bioavailability more specifically in terms of contaminant binding to or release from a solid phase.

Obviously, the various definitions given to the term bioavailability by scientists in various disciplines are capable of causing semantic confusion and thus garner more attention than proffer solutions. These definitions were compiled in this chapter to present a simple and workable definition of the term 'bioavailability' as it is important to estimate; the extent of contaminants desorption from the sorbed phase, the non-desorbable residue as against the minimum level required in the environment for such contaminants, and thus assessing the overall success of the bioremediation exercise. Considering these several opinions about bioavailability, two words are common to almost all: 'uptake' or 'absorbed' and 'available'.

Based on this observation, bioavailability can be defined as the amount of available contaminants in the environment that can be absorbed by microorganisms and/or biological products. Other clauses such as the fraction of contaminants taken up or absorbed, the fraction of contaminant that is potentially available, the mobilization or transportation of contaminants from the sorbed phase, etc., which are often included in the definition of bioavailability and thus cause confusion, are intrinsic factors or features of bioavailability.

The major limitation to PAHs' biodegradation and bioremediation is the low bioavailability of the contaminant. Hence, studies on bioavailability are crucial to link the amount of PAHs taken up by microorganism with the actual amounts that are available to cause adverse effects in the environment.

Many factors have been known to affect PAH bioavailability, which are (Stokes *et al.*, 2005, Harmsen, 2007, Khan *et al.*, 2011):

- Physical and chemical properties of PAH s (low molecular weight and high molecular weight).
- Soil properties (soil organic matter, dissolved organic matter, moisture content, etc.).
- Ageing PAHs in soil and receptor microorganism.

2.6.2 Effects of physical and chemical properties of PAHs on bioavailability

Bioavailability is influenced by the molecular structure and size of PAHs. LMW PAHs are removed faster by physicochemical and biological processes owing to their higher solubility and volatility including the ability of many microorganisms to use them as sole carbon sources in comparison with the HMW PAHs (Alexander, 1999). Bioavailability changes with time and weathering (Uyttebroek *et al.*, 2007). Ageing is a central term concerning availability and refers to the process of organic compounds in soil becoming less susceptible to degradation, extractability and other related processes in a time- dependent manner (Semple *et al.*, 2003, Bergknut, 2006). Ageing increases sorption propensity of soil contaminants, making them more recalcitrant to diffusion and mobility, which consequently lead to low bioavailability. Both the physicochemical properties of the contaminant and the soil characteristics influence ageing, which may include several steps and processes such as chemical oxidation reactions incorporating them into natural organic matter (Burgos *et al.*, 1996, Bosma *et al.*, 1996), slow diffusion into very small pores and absorption into organic matter, or entrapment due to the formation of semi-rigid films around non-aqueous-phase liquids (NAPL) with a high resistance towards NAPL-water mass transfer (Luthy *et al.*, 1997).

The effect of molecular mass on the bioavailability of phenanthrene and pyrene was illustrated by a study conducted on four soils by Ling *et al.* (2010). It was shown that the non-desorbing residual concentrations of pyrene, which have a higher molecular weight and adsorption tendency, showed greater retention in soil than phenanthrene, in all the four tested soils, after the 16 weeks of ageing. The available residual concentrations of phenanthrene and pyrene generally decreased with ageing time, and the PAHs were more readily available at the start of the incubation, but their availabilities decreased rapidly with increasing the soil-PAH contact time. Generally, the degradation efficiency of the available PAHs in soil is higher for PAHs with low molecular weight.

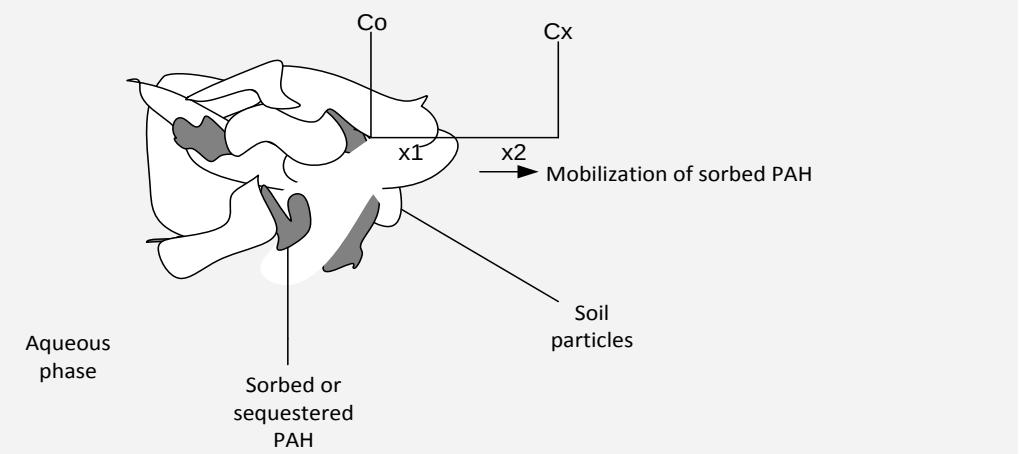
2.6.3 Effects of soil or sediment and dredging properties on PAHs bioavailability

Soil properties such as organic matter content, soil texture, soil depth, particle size, pH, porosity, intrinsic permeability, moisture-holding capacity, and cation-exchange capacity influence PAH bioavailability. Soil properties can vary greatly from one region to another. They can even vary within the same region spatially and with depth (McNally *et al.*, 2007).

Microorganisms have a range of tolerance to these factors, which affects their growth and activities. Soil structure such as aggregation has been found to decrease PAH availability through physical sequestration of PAHs on the interior of aggregates (Wu and Gschwend, 1986). For instance, Nam *et al.* (1998) established that PAH bioavailability to phenanthrene degraders declined with time in soils with more than two percent soil organic matter. Hundal *et al.* (2001) reported on the retention of large amounts of phenanthrene by smectite clays. A report has shown the positive impact of soil organic matter on PAHs' distribution in soils (Yang *et al.*, 2010b). It was observed that when the soil organic matter was increased from 0.2 to 7.1%, the average non-bioavailable amount of acenaphthene, anthracene, fluoranthene, and pyrene was almost tripled from 436.9 to 1205.8 ng/g.

2.6.4 Effects of mass transfer on bioavailability and bioremediation of PAHs

The overall PAHs biodegradation concept can be conceptually divided into the following steps: desorption to the aqueous phase (Fig. 2.5); mass transfer to biologically accessible regions; and biological uptake and transformation (Reid *et al.*, 2000, Semple *et al.*, 2003). These steps occur sequentially, so that the overall bioremediation rate can be limited and controlled by any of these steps.



X1 is the diffusion distance from interior to the surface while x2 is the diffusion distance from the surface to microbial site. Co-Cx is the concentration gradient.

Figure 2.5. Diffusion of entrapped PAH out of soil micropores into aqueous phase to become available to microorganism

The impact of desorption rate on overall bioremediation is expected to be greatest in the case where biodegradation rates are higher relative to desorption rates. This occurs when the active microorganisms are capable of high biodegradation rates and either porous media have high capacity for solute, or porous media have large diffusion distances (Zhang *et al.*, 1998, Johnsen *et al.*, 2005). Based on this, it is important to examine how the physical morphology of surface and subsurface soils can impact the biodegradation of sorbed organic chemicals. Naturally occurring particles contain pores of different sizes, many of which are smaller than the sizes of microorganisms. For example, analysis of one of the coarser sand sizes from the Borden aquifer in Ontario, Canada, indicated that roughly 50% of the intraparticle pore volume resides in pores that are less than 0.1 µm in diameter (Ball *et al.*, 1990).

Pores with diameter larger than 1 µm comprised 12% of the total pore space, and only about 5% of the pore volume was attributed to pores larger than 2 µm. Considering that most indigenous bacteria are 0.5 to 1.0 µm in diameter (Alexander, 1977), bacteria will be physically excluded from most of the intraparticle pores of these grains. The mean diameter of intraparticle pores occupied by bacteria has been estimated to be typically larger than 2 µm (Lawrence *et al.*, 1979a), and this is likely to be larger than intraparticle pore spaces of many natural sorbent solids. However, for those pores accessible to bacteria, slow mass transfer of contaminants from the pore interior as well as from the pore surface can limit the extent of microbial growth and consequently bioremediation.

The slow intraparticle mass transfer caused by the interactions between soil constituents and organic contaminants was investigated using synthetic aggregates and porous granules of Teflon® (Harms and Zehnder, 1995). Considering the influence of soil aggregate size, it was suggested that the available surface area of the aggregates would be a major factor in determining the rate of biodegradation. Amellal *et al.* (2001) investigated the effects of soil aggregate size fractions on distribution and degradation of a mixture of eight PAH compounds. The distribution of PAHs and of the degrading bacteria was determined in the bulk soil as well as in four sized aggregate fractions corresponding to sand, coarse silt, fine silt and clay. Bacterial communities of PAH degraders were present in a higher density in the aggregates corresponding to sand and least to those corresponding to clay. Chemical analysis shows that the remaining PAHs (low and high molecular weight) were much more concentrated in the fine soil fractions (fine silt and clay) and were present at a very low content in the larger aggregate size fractions. The nano-pore-sized clay and silt sand obviously precluded the penetration of PAH compounds and the bacterial cells. Other factors that may affect contaminant bioavailability are the level of contamination and availability of suitable microbial communities.

2.7 PAHs bioavailability enhancement

2.7.1 Biosurfactants and the mechanism of enhancing PAHs bioavailability

Biosurfactants are surface-active agents produced by microorganisms. All biosurfactants consist of two parts, a polar (hydrophilic) moiety and a non-polar (hydrophobic) group (Fig. 2.6). A hydrophilic group consists of mono-, oligo- or polysaccharides, peptides or proteins and a hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols (Lang, 2002).

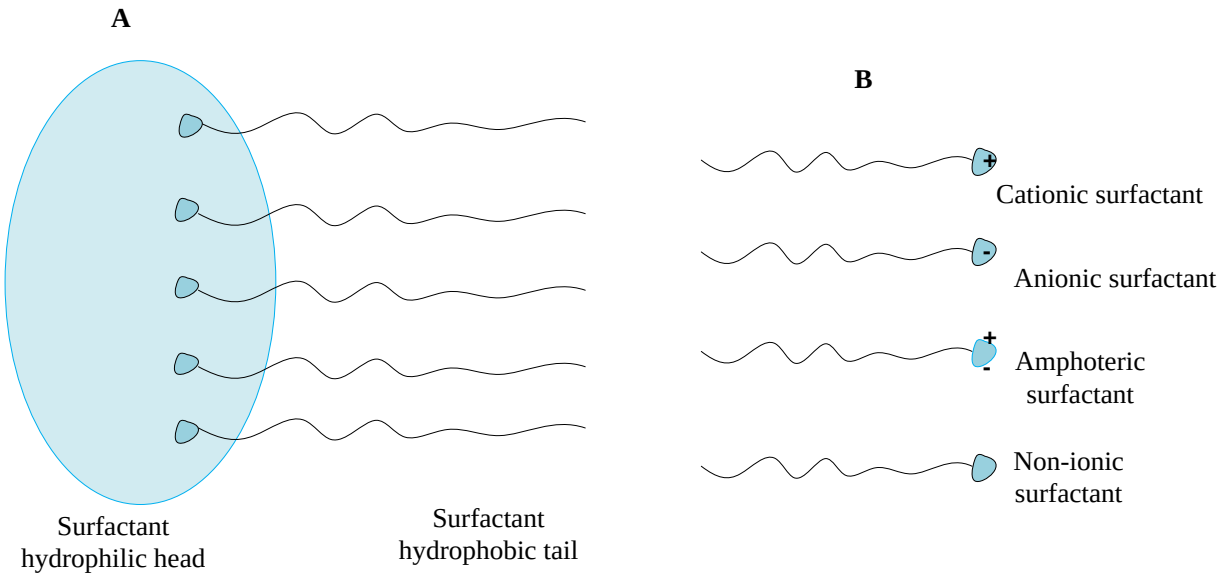


Figure 2.6: Surfactant moieties (A) and surfactant classification based on charges (B)

Owing to their amphiphilic structure, biosurfactants show a wide range of properties, including the lowering of surface and interfacial tension of liquids, the ability to form micelles and micro-emulsions between two different phases, and the ability to increase the surface area of hydrophobic water-insoluble substances, and thus increase the water bioavailability of such substances. The advantages of biosurfactant over their chemically synthesized equivalents include (Kosaric, 1992, Kosaric, 2001, Rahman *et al.*, 2002, Das and Mukherjee, 2007a):

- Environmental friendliness
- They are biodegradable, less toxic and non-hazardous

- Better foaming properties and higher selectivity
- They are active at extreme temperatures, pH and salinity
- They can be produced from wastes and from various by-products

The fact that biosurfactant can be synthesized from many inexpensive renewable resources makes its application for enhancing HOCs' bioavailability promising. Another advantage is the concomitant effects of utilizing waste substrates, thereby reducing their environmental pollution. Biosurfactants increase the bioavailability of PAHs, resulting in enhanced growth of the degrading microorganism and the biodegradation of the contaminants (Kuyukina *et al.*, 2005, Das and Mukherjee, 2007a, Kang *et al.*, 2010). The structure of surfactin – an example of polypeptides biosurfactant – is shown in Fig. 2.7.

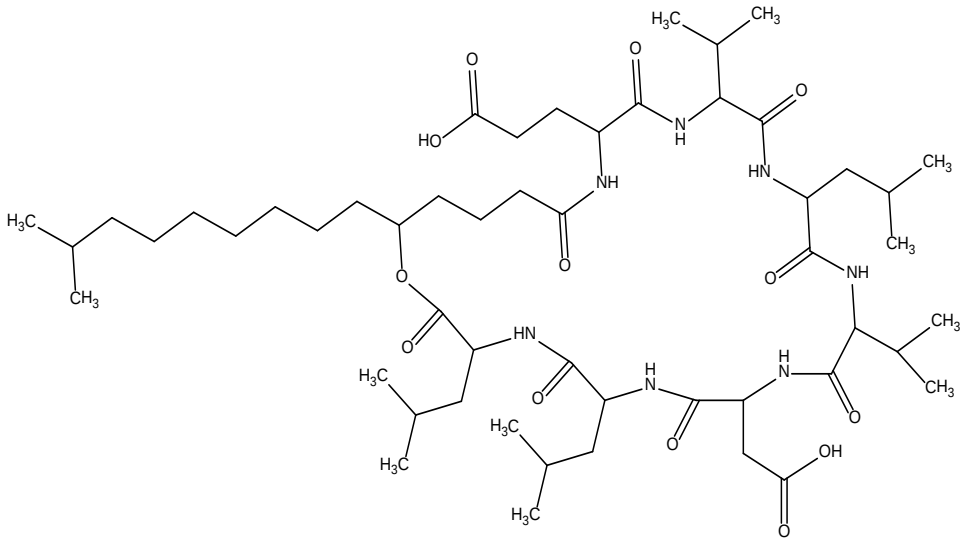


Figure 2.7: Chemical structure of surfactin

An important feature of the physicochemical properties of surfactants is their hydrophilic-lipophilic balance (HLB) (Tiehm, 1994, González *et al.*, 2011). The HLB value indicates whether a surfactant will produce a water-in-oil or oil-in-water emulsion. Emulsifiers with lower HLB values of 3 to 6 are lipophilic and promote water-in-oil emulsification, while emulsifiers with higher HLB values between 10 and 18 are more hydrophilic and promote oil-in-water emulsion formation (Nilanjana and Preethy, 2010). A classification based on HLB values has been used to evaluate the suitability of different

surfactants for various applications. For example, it has been reported that successful surfactants are those with the ability to promote desorption of contaminants from contaminated soils and are normally those with HLB values above 10 (Volkering *et al.*, 1997).

Another feature is the critical micelle concentration (CMC) which is the concentration above which the formation of micelles is thermodynamically favoured (Haigh, 1996). 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. Owing to the reduction of the interfacial force, contact of biosurfactants with a soil-oil system increases the contact angle and reduces the capillary force holding the oil and soil together. Above the biosurfactant CMC, the solubilization process takes place. At these concentrations, biosurfactant molecules aggregate to form micelles, which dramatically increase the solubility of the oil. The hydrophobic parts of the biosurfactant molecules interconnect inside the micelle while the hydrophilic ends are then exposed to the aqueous phase on the exterior. Consequently, the interior of a micelle creates an environment compatible for hydrophobic organic molecules. The process of incorporation of these molecules into a micelle is known as solubilization (Urum and Pekdemir, 2004).

The formation of micelles leads to a significant increase in the apparent solubility of hydrophobic organic compounds, even above their water solubility limit, as these compounds can partition into the central core of a micelle. The effects of such a process are the reduction of surface and interfacial tension, enhancement of mobilization and mass transfer of contaminants from soil particles into the aqueous phase, and consequently the bioavailability of the hydrophobic contaminants for microbial attack (Perfumo *et al.*, 2010).

2.7.2 Application of biosurfactants for enhancing PAHs' bioavailability and biodegradation

Only limited numbers of microorganisms are capable of degrading HMW PAHs. Hence, as discussed previously, this limitation is due to low bioavailability of the contaminants, which is a result of their hydrophobicity and strong adsorptive capacity in particulate matters (Volkering *et al.*, 1995, Harayama, 1997). Several studies have reported on the ability of biosurfactant to enhance aqueous concentration of HOCs. For example, an emulsifying agent produced by *P. aeruginosa* UG2 increased the solubility of hexachlorobiphenyl added to soil slurries, and resulted in a 31% recovery of the compound in the aqueous phase (Berg *et al.*, 1990). A rhamnolipid from bacteria, in combination with the oleophilic fertilizer *Inipol EAp*-22, was found to increase the degradation rate of hexadecane, benzene, toluene, *o*- and *p*-Cresol and naphthalene both in aqueous phase and in soil bioreactors (Griffin *et al.*, 1995).

Churchill et al. (1995) also reported increased rates of biodegradation of aliphatic and aromatic hydrocarbons by pure bacterial cultures. Table 2.4 enumerates the environmental applications of the various classes of biosurfactant.

The efficiency of biosurfactants in the remediation of soil contaminated by phenanthrene and polychlorinated biphenyls (PCBs) was also reported (Miller, 1995). In an investigation of the capacity of PAH-utilizing bacteria to produce biosurfactants using naphthalene and phenanthrene, Deziel *et al.* (1996) quantified biosurfactant production that was responsible for an increase in the aqueous concentration of naphthalene. This indicates a potential role for biosurfactants in increasing the solubility of such compounds. Similarly, Zhang *et al.* (1997) determined the effect of two biosurfactants on the dissolution and bioavailability of phenanthrene and reported increases in both solubility and the degradation rate of phenanthrene. Kanga *et al.* (1997) applied glycolipid biosurfactants produced by *Rhodococcus sp*. H13A and a synthetic surfactant (Tween® 80) for enhanced substrate solubility. Using naphthalene and methyl-substituted derivatives in crude oil as representative of the PAH content, they observed that both surfactants lowered surface tension in solutions from 72 to 30 mN m^{-1}. The biosurfactants were efficient in increasing the solubility of the hydrocarbons, particularly the substituted derivative. In a laboratory column study, Noordman *et al.* (1998) applied biosurfactants for the enhanced removal of phenanthrene from phenanthrene-contaminated soil eluting with an electrolyte solution containing rhamnolipid. The enhanced removal of phenanthrene occurred mainly by micellar solubilization.

Microbially produced biosurfactants were studied to enhance crude oil desorption and mobilization in model soil column systems (Kuyukina *et al.*, 2005). The results showed that the ability of biosurfactants from *Rhodococcus ruber* to remove the oil from the soil core was 1.4 to 2.3 times greater than that of a synthetic surfactant of suitable properties, Tween® 60. The biosurfactant was less adsorbed to soil components than synthetic surfactant, thus rapidly penetrating through the soil column and effectively removing 65–82% of the crude oil. Chemical analysis showed that the crude oil removed by the biosurfactant contained a lower proportion of high-molecular-weight paraffins and asphaltenes. The capability of biosurfactants and biosurfactant-producing bacterial strains to enhance organic contaminants' availability and biodegradation rates was reported by several authors (Deziel *et al.*, 1996, Rahman *et al.*, 2003, Inakollu *et al.*, 2004). The role of sophorolipid in biodegradation of aliphatic and aromatic hydrocarbons and Iranian light crude oil under laboratory conditions was investigated by Kang *et al.* (2010). It was observed that the addition of the biosurfactant to soil increased biodegradation of tested hydrocarbons, with the rate of degradation ranging from 85% to 97% of the total hydrocarbons.

Table 2.4: Biosurfactant-producing organisms: classification and application in environmental biotechnology (Pacwa-Płociniczak *et al.*, 2011)

Biosurfactant		Microorganism	Applications in Environmental Biotechnology	References
Group	Class			
Glycolipids	Rhamnolipids	*Pseudomonas aeruginosa, Pseudomonas* sp.	Enhancement of the degradation, dispersion, emulsification of different classes of hydrocarbons and vegetable oils; removal of metals from soil.	(Sifour *et al.*, 2007, Whang *et al.*, 2008)
	Trehalolipids	*Mycobacterium tuberculosis, Rhodococcus erythropolis, Arthrobacter* sp.*, Nocardia* sp.*, Corynebacterium* sp.	Enhancement of the bioavailability of hydrocarbons.	(Franzetti *et al.*, 2010)
	Sophorolipids	*Torulopsis bombicola, Torulopsis petrophilum, Torulopsis apicola*	Recovery of hydrocarbons from dregs and muds; removal of heavy metals from sediments; enhancement of oil recovery.	(Baviere *et al.*, 1994, Pesce, 2002, Whang *et al.*, 2008)
Fatty acids, phospho-lipids and neutral lipids	Corynomycolic acid	*Corynebacterium lepus*	Enhancement of bitumen recovery.	(Gerson and Zajic, 1978)
	Spiculisporic acid	*Penicillium spiculisporum*	Removal of metal ions from aqueous solution; dispersion action for hydrophilic pigments; preparation of emulsion-type organogels; microencapsulation;	(Hong *et al.*, 1998)
	Phosphati-dylethanolamine	*Acinetobacter* sp.*, Rhodococcus erythropolis*	increasing the tolerance of bacteria to heavy metals.	(Appanna *et al.*, 1995)
Lipopeptides	Surfactin	*Bacillus subtilis*	Enhancement of the biodegradation of hydrocarbons and chlorinated pesticides; removal of heavy metals from a contaminated soil, sediment and water; increasing the effectiveness of phytoextraction;	(Awashti *et al.*, 1999)
	Lichenysin	*Bacillus licheniformis*	enhancement of oil recovery.	(Thomas *et al.*, 1993)
Polymeric biosurfact-ants	Emulsan	*Acinetobacter calcoaceticus RAG-1*	Stabilization of the hydrocarbon-in-water emulsions.	(Zosim *et al.*, 1982)
	Alasan	*Acinetobacter radioresistens KA-53*		(Toren *et al.*, 2001)
	Biodispersan	*Acinetobacter calcoaceticus A2*	Dispersion of limestone in water.	(Rosenberg *et al.*, 1988)
	Liposan	*Candida lipolytica*	Stabilization of hydrocarbon-in-water emulsions.	(Cirigliano and Carman, 1985)
	Mannoprotein	*Saccharomyces cerevisiae*		(Cameron *et al.*, 1988)

Their results indicated that sophorolipid may have the potential for facilitating the bioremediation of sites contaminated with hydrocarbons having limited water solubility and increasing the bioavailability of microbial consortia for biodegradation. The solubility and utilization of pyrene as a sole carbon source by the biosurfactant-producing bacterial strains, *Bacillus subtilis* DM-04, *Pseudomonas aeruginosa mucoid* (M) and *nonmucoid* (NM), isolated from a petroleum-contaminated soil were also reported (Das and Mukherjee, 2007a). The biosurfactants produced by the bacteria under the study were capable of enhancing the solubility of pyrene in aqueous media and can influence the cell surface hydrophobicity of the biosurfactant-producing strains that results in a higher uptake of pyrene.

2.7.3 Biosurfactant production from renewable resources

The bioconversion of waste materials is considered to be of importance for the development of sustainable biotechnology processes in the near future because of its favourable economics, low capital and energy cost, reduction in environmental pollution, and relative ease of operation (Makkar *et al.*, 2011). Producing usable products from agrowaste and agro industrial waste is therefore a feasible and favourable option (Makkar and Cameotra, 2002, Moldes *et al.*, 2007). Modern society produces high quantities of waste materials through activities related to industries such as those in the forestry, agriculture and municipal areas of operation (Martins *et al.*, 2006, Montoneri *et al.*, 2009).

The use of alternative substrates such as agro-based industrial wastes is one of the attractive strategies for the production of economical biosurfactants to enhance bioremediation of environmental hydrophobic contaminants. It has been suggested that successful approaches to more economical production technologies of biosurfactants will be a collaborative approach involving process development and sustainable raw material supplies. According to Marchant and Banat (2010), emphasis should be on the cost-effective management of downstream processing. These inexpensive agro-industrial waste substrates include olive oil mill effluent, plant oil extracts and waste, distillery and whey wastes, potato process effluent and cassava wastewater (Makkar *et al.*, 2011). These waste materials are some examples of food industry by-products or waste that can be used as feedstock for biosurfactant production. Vegetable oils are lipid molecules that comprise saturated or unsaturated fatty acids with a 16- to 18-carbon atom chain.

Table 2.5: Yield and lowest surface tension reduction achieved for biosurfactant produced from agro/agro industrial waste and mineral salt

Microorganisms	Isolation environment	Primary carbon source used	BS yield (g L^{-1})	ST reduction (mN m^{-1})	References
Sphingobacterium sp. 6.2S	Volcanic soil	Mineral salts	9.6	22.0	(Burgos-Diaz *et al.*, 2011)
B. subtilis FE-2	n/r	Wheat bran	n/r	24.0	(Veenanadig *et al.*, 2000)
Candida sphaerica UCP0995	n/r	Ground nut oil residue + corn steep liquor	9.0	25.0	(Luna *et al.*, 2012)
B. subtilis ATCC 21332	n/r	Cassava wastewater	2.2	25.9	(Nitschke and Pastore, 2004)
B. subtilis LB5	Wax apple orchard		3.0	26.6	
Candida sphaerica UCP0995	Mangrove sediment	Soybean oil residue + corn steep liquor	6.36	26.0	(Sobrinho *et al.*, 2013)
Pseudomonas aeruginosa AT110	Food oil-contaminated site	soybean oil refinery wastes	9.5	26.8	(Abalos *et al.*, 2001)
B. subtilis LAMI009	Wastewater treatment (chlorination) tank	Glycerol from biodiesel synthesis	0.27	27.0	(Sousa *et al.*, 2012)
P. aeruginosa strain BS2	Oily sludge	Distillery and whey wastes + glucose	0.97	27.0	(Dubey and Juwarkar, 2001)
B. licheniformis TT42	Tuva-Timba hot water spring	Mineral salts	n/r	28.0	(Nerurkar, 2012)
B. subtilis strain JA-1	Oil reservoir	Glucose	0.32	28.3	(Wang *et al.*, 2011)
P. aeruginosa J4	Petrochemical wastewater	Luria-Bertani (LB) medium	n/r	30.0	(Whang *et al.*, 2008)

n/r – Not reported

Studies involving the application of a variety of vegetable oils for biosurfactant production from canola, corn, sunflower, safflower, olive, rapeseed, grape seed, palm, coconut, fish and soybean oil have been reported. The world production of oils and fats is about 2.5 to 3 million tons, 75% of which are derived from plants and oil seeds (Dumont and Narine, 2007). The high content of fats, oils and other nutrients in these wastes makes them interesting and cheap raw materials for industries involved in useful secondary metabolite production. Furthermore, from an economical point of view, nutrient- rich agricultural residues can be employed for producing useful biological products such as biosurfactants. These materials are among the most abundant organic carbons available on earth (Kukhar, 2009) and they are the major components of different waste streams from various industries.

In recent times, research studies have focused on the application of agro-industrial wastes or by-products for the production of biosurfactants and their use in crude form or the direct use of surfactant-producing strains for bioremediation processes. This is due to the high cost of biosurfactant purification and the greater stability and sustainability provided by these biosurfactant-producing strains in bioremediation processes. The production and properties of a biosurfactant, synthesized by *Bacillus subtilis* LB5a strain, using cassava wastewater as a substrate, was investigated. The microorganism was able to grow and to produce a surfactant on cassava waste, reducing the surface tension of the medium to 26.6 mN m^{-1} and giving a crude surfactant concentration of 3.0 g L^{-1} after 48 h (Nitschke and Pastore, 2006). The biosurfactant obtained was capable of forming stable emulsions with various hydrocarbons. Panesar *et al.* (2011) investigated the suitability of molasses, the sugar industry by-product, for biosurfactant production using *Pseudomonas aeruginosa* strain ATCC 2297**.** An attempt was also made to replace the costly nitrogen sources with agro-industrial by-products to formulate a low-cost medium for biosurfactant production. The strain was found to display maximum emulsification activity on a molasses medium after 120 h of incubation period under optimized conditions. Biosurfactant production by a strain of *Pseudomonas aeruginosa* using palm oil as a sole carbon source was investigated (Thaniyavarn *et al.*, 2006). The *P. aeruginosa* strain gave emulsification index results of 100% when diesel was used as an oil phase and was able to reduce surface tension of three tested inorganic media to approximately 33 mN m^{-1}. The versatility of a bacterial strain isolated from a hydrocarbon-based source at a palm oil mill has also been reported (Wan Nawawi *et al.*, 2010). The strain showed a high bacterial growth on sludge palm oil with a surface tension of 36.2 mN m^{-1} and was therefore proposed for biosurfactant production by liquid state fermentation.

2.8 Kinetics of biosurfactant production, cell growth, and substrate utilization: a tool to predict microbial operations during PAHs' degradation

2.8.1 Biomass growth kinetics

2.8.1.1 Monod equation

The growth rate of microbial biomass during fermentation is often found to be directly proportional to the concentration of the viable cells. This fundamental first-order growth model, which was proposed by Malthus (1798), has been used to characterize the exponential growth dynamics of microbial cells. This relationship is mathematically expressed as:

$$dX/dt \propto X$$

Hence,

$$\frac{dX}{dt} = r_x = \mu, \tag{2.4}$$

where *dx/dt* is the growth rate (g L^{-1} h^{-1}); *X* is the cell concentration (g L^{-1}); μ is the specific growth rate (h^{-1}); while *t* is the time (h). The relationship between cell growth rate and substrate concentration was established by the following Monod equation (Monod, 1949):

$$\mu = \frac{\mu_m S}{K_S + S}, \tag{2.5}$$

where μ_m is the maximum specific growth rate (h^{-1}); K_S is the saturation constant, which represents substrate concentration (when $\mu = ½\ \mu_m$); and *S* is the limiting substrate concentration.

2.8.1.2 Logistic model (LM)

To obtain a mathematical representation of cell proliferation of *B. licheniformis* on *Beta vulgaris*, a number of biomass growth models were investigated. A mechanistic model, proposed by Verhulst (1838) as an improvement to the Malthusian exponential model, often referred to as a logistic model, was used. The model embedded several assumptions; however, it is found to be valid – during the early stages of cell growth, for large populations, for homogeneous biological populations, and for a fairly constant growth rate. The model is given as:

$$\frac{dX}{dt} = \mu_o X\left(1 - \frac{X}{X_m}\right) \tag{2.6}$$

where μ_o represents the initial specific growth rate (h^{-1}); X_m is the maximum biomass concentration (g L^{-1}); and X is the biomass concentration (g L^{-1}).

On integration, Eq. 2.6 yields:

$$X(t) = \frac{X_o X_m \ exp(\mu_0 \, t)}{X_m - X_o \ + \ X_o \ exp(\mu_o \, t)} \tag{2.7}$$

which can be rearranged to give:

$$\ln\left(\frac{X}{X_m - X}\right) + ln\left(\frac{X_m}{X_o} - 1\right) = \mu_o t \tag{2.8}$$

An appropriate plot of Eq. 2.8 will give a straight line that will lead to the determination of μ_o.

2.8.1.3 Modified logistic model (LM)

To extend the validity of the logistic model beyond the early stages of cell growth, mainly the exponential phase, the model was modified by incorporating a biomass growth-dependent inhibitory effect '*r*' which accounts for the growth variation from the exponential correlation (Mulchandani *et al.*, 1988).

$$\frac{dX}{dt} = \mu_o X\left[1 - \left(\frac{X}{X_m}\right)^r\right], \qquad \text{for } r > 0 \tag{2.9}$$

However, as *r* approaches zero, *dx/dt* also tends to zero and there will be complete inhibition of cell growth. On the other hand, the equation reduces to the logistic model, when *r* equals 1. When *r* ranges between 0 and 1, a higher extent of growth inhibition is observed. But when *r* >1, the growth lies between exponential and logistic forms.

Eq. 2.9 can be rearranged to give;

$$\frac{dX}{X\ ({X_m}^r - X^r)} = \frac{\mu_m}{{X_m}^r}\ dt \tag{2.10}$$

while integration by partial fraction, with the initial conditions $X = X_o$ at $t = t_o$, gives:

$$X(\mathrm{t}) = \frac{{X_m}^r\ exp(\mu_o rt)}{1 - \left(\frac{X_o}{X_m}\right)^r\ [(1 - exp(\mu_o rt)]^{-1}} \tag{2.11}$$

2.8.2 Biosurfactant production kinetics

2.8.2.1 Logistic form model (LM)

An analogous of the logistic growth model, proposed by Mercier *et al.* (1992) for the prediction of lactic acid synthesis from fermented corn, which has also been used to model the kinetics of biosurfactant production (Lotfabad *et al.*, 2009), was used in this study to model biosurfactant synthesis from *B. vulgaris*. The model was developed in similar fashion to the microbial growth model, as presented in Eq. 2.12 below:

$$\frac{dP}{dt} = P_r P\left(1 - \frac{P}{P_m}\right) \tag{2.12}$$

$$P_r = {r_p}/{P_o}$$

Integration of Eq. 2.12 gives an expression for biosurfactant production (P), similar to Eq. 2.7 above for determining cell biomass:

$$P = \frac{P_o P_m\ exp(P_r\ t)}{P_m - P_o\ +\ P_o\ exp(P_r\ t)} \tag{2.13}$$

where P is the biosurfactant concentration (g L^{-1}); P_m is the maximum biosurfactant production (g L^{-1}); r_p is the initial volumetric production rate (g L^{-1} h^{-1}); while P_o is the initial biosurfactant concentration (g L^{-}

[1]). Rearranging Eq. 2.13 gives Eq. 2.14, from which a linear plot can be obtained to determine the value of P_O

$$\ln(\frac{P}{P_m - P}) + ln\left(\frac{P_m}{P_o} - 1\right) = P_r t \tag{2.14}$$

2.8.2.2 Leudeking–Piret model (LP)

When the biological product is cell-mass dependent or depends on some constituent of cells, then the rate of formation of product directly relates to the rate of growth. Luedeking and Piret (1959) considered this relationship of cell growth to product formation, and thus proposed a model to study the kinetics of lactic acid fermentation, which can also be used in this case to predict biosurfactant production. It assumes that the microbial product formation depends on the instantaneous biomass concentration and the growth rate, as represented in Eq. 2.15 below:

$$\frac{dP}{dt} = a\frac{dX}{dt} + bX \tag{2.15}$$

where *a* and *b* are coefficients for growth and non-growth product formation, respectively.

2.8.2.3 Logistic incorporated Leudeking–Piret model (LLP)

The Leudeking–Piret model was modified by incorporating the logistic model (Weiss and Ollis, 1980), substituting Eq. 2.6 for *dt/dx* and Eq. 2.7 for *X*, in Eq. 2.15, and rearrange to obtain:

$$\frac{dP}{dt} = a + \frac{b}{\mu_o\left(1 - \frac{X}{X_m}\right)} \tag{2.16}$$

By integrating Eq. 2.16, with the initial conditions of $X = X_o$ and $P = P_o$, at $t = 0$, and substituting for *X* from Eq. 2.7, gives:

$$P(t) = P_o + aX_o\left[\frac{exp(\mu_o t)}{1-\left(\frac{X_o}{X_m}\right)(1-exp(\mu_o t))}-1\right]+b\frac{X_m}{\mu_o}ln\left[1-\frac{X_o}{X_m(1-exp(\mu_o t))}\right] \quad (2.17)$$

2.8.3 Substrate utilization kinetics

2.8.3.1 Modified Monod model

The rate of substrate utilization by a bacterium in a batch system is related to the biomass growth rate (Robinson and Tiedje, 1983), as shown in Eq. 2.18 below:

$$-\frac{ds}{dt} = -r_S = \frac{1}{Y_{X/S}}\frac{dX}{dt} \quad (2.18)$$

where *ds/dt* is the rate of substrate utilization (g $L^{-1}h^{-1}$), and $Y_{X/S}$ is the yield coefficient (g biomass/g substrate), which relates cell concentration to substrate utilization as:

$$X = Y(S_O - S) + X_O \quad (2.19)$$

Substituting Eqs 2.4, 2.5, and 2.19 into Eq. 2.18 gives:

$$\frac{ds}{dt} = -\left(\frac{\mu_{mS}}{K_S+S}\right)[Y(S_O - S) + X_O]/Y \quad (2.20)$$

which can be integrated to give:

$$C_1 \ln\left\{\frac{[Y(S_O-S)+X_O]}{X_O}\right\} - C_2 \ln\left(S/S_O\right) = \mu_m t \quad (2.21)$$

where $C_1 = (K_S Y + S_O Y + X_O)/(YS_O + X_O)$ and $C_2 = K_S Y/(YS_O + X_O)$

Equation 2.21, which often gives the familiar elongated *S*-shape or sigmoidal curve for substrate depletion, cannot be solved explicitly for *S*. However, by numerical approximation, *S* can be evaluated as a function of *t*.

2.8.3.2 Leudeking–Piret modified model (LPM)

Additionally, in biological fermentation, the specific rate of substrate consumption can be represented by incorporating both product synthesis rate and the maintenance of cells (*m*) into Eq. 2.18. The three processes are mathematically related as:

$$-\frac{ds}{dt} = Y_{S/P}\frac{dP}{dt} + Y_{S/X}\frac{dX}{dt} + mX \tag{2.22}$$

Substitute Eq. 2.15 into Eq. 2.22 to obtain:

$$-\frac{ds}{dt} = (Y_{S/P}\, a + Y_{S/X})\frac{dX}{dt} + (Y_{S/P}\, b + m)X \tag{2.23}$$

2.8.3.3 Logistic incorporated Leudeking–Piret modified model (LLPM)

Rearrange Eq. 2.23 and substitute Eq. 2.6 for *dt/dx* to obtain:

$$-\frac{ds}{dX} = (Y_{S/P}\, a + Y_{S/X}) + \frac{(Y_{S/P}\, b + m)}{\mu_o\left(1 - \frac{X}{X_m}\right)} \tag{2.24}$$

Integrating Eq. 2.24 above with the initial conditions $X = X_o$ and $S = S_o$, at $t = 0$ gives:

$$-(S_t - S_o) = (Y_{S/P}\, a + Y_{S/X})(X_t - X_o) + \frac{(Y_{S/P}\, b + m)X_m}{\mu_o}\left[ln\left(1 - \frac{X_t}{X}\right) - ln\left(1 - \frac{X_o}{X_m}\right)\right]$$

By substituting Eq. 2.7 and rearranging, we have:

$$S_t = S_O - \lambda X_o \left[\frac{exp(\mu_o t)}{1 - \left(\frac{X_o}{X_m}\right)(1 - exp(\mu_o t))} - 1 \right] - \frac{\gamma X_m}{\mu_o}[1 - \frac{X_o}{X_m}(1 - exp(\mu_o t))] \quad (2.25)$$

where $\lambda = Y_{s/p}a + Y_{S/X}$ and $\gamma = Y_{S/P}\, b + m$

2.8.3.4 **Modified logistic incorporated Leudeking–Piret modified model (MLLPM)**

Rearrange Eq. 2.22 and substitute Eq. 2.9 for *dt/dx* to obtain:

$$\frac{ds}{dX} = (Y_{S/P}\, a + Y_{S/X}) + \frac{(Y_{S/P}\, b + m)}{\mu_o\, [(1 - \frac{X}{X_m})^r]} \quad (2.26)$$

Integrating Eq. 2.26 above and setting initial conditions of $X = X_o$ and $S = S_o$, at $t = 0$, we have:

$$S_t = S_o - \lambda \left[\left\{ \frac{{X_o}^r \exp(\mu_o t)}{1 - \left(\frac{X_o}{{X_m}^r}\right)\frac{1}{(1 - \exp(\mu_o t))}} \right\}^{\frac{1}{r}} - {X_o}^r \right] - \frac{\gamma {X_m}^r}{\mu_o} \ln \left[1 - \frac{X_o}{{X_m}^r}(1 - \exp(\mu_o t)) \right] \quad (5.24)$$

where λ and γ are the growth-dependent and non-growth dependent parameters.

2.9 Biodegradation kinetic models for PAHs remediation studies

2.9.1 First-order kinetics

Biodegradation rate of PAHs in the aqueous phase and the rate constant (*k*) can be determined using the reaction rate expression as follows:

$$-\frac{dC}{dt} = kC^n \tag{2.25}$$

C - is the concentration of PAH (mg L^{-1}), *t* the time (days), *k* the rate constant for chemical disappearance of PAH ($days^{-1}$) and *n* the reaction order, which is unity for first-order kinetics (Alexander, 1999, Kwon *et al.*, 2009). Based on the assumptions that only dissolved forms are available for biodegradation and that the biodegradation rate follows a first-order kinetics (Yang *et al.*, 2010a), the logarithm of the ratio of residual PAH concentration to its initial concentration (i.e., logarithm of *C/Co*) can be plotted as a function of time; hence the biodegradation rate, being the gradient of the plot, can be determined.

2.9.2 Michaelis–Menten kinetic model

The most commonly assumed relationship for the assessment of bioremediation is done using the biodegradation rate and the concentration *(C_S)* of the contaminants in the aqueous phase using the Michaelis–Menten kinetic model (Eq. 2.26) – an equivalent of cell growth model shown in Eq. 2.5:

$$V = \frac{V_{max}C_S}{K_m + C_S} \tag{2.26}$$

where *V* is the biodegradation rate, V_{max} the maximum biodegradation rate, K_m the Michaelis–Menten constant, and C_S the residual contaminant concentration. As depicted in Fig. 2.8, at a high C_S, *V* becomes independent of C_S and at a low C_S, the model is approximated to first-order kinetics, i.e., *V* is directly proportional to C_S. At all values of C_S, *V* is always proportional to the biocatalyst concentration available for the biodegradation process.

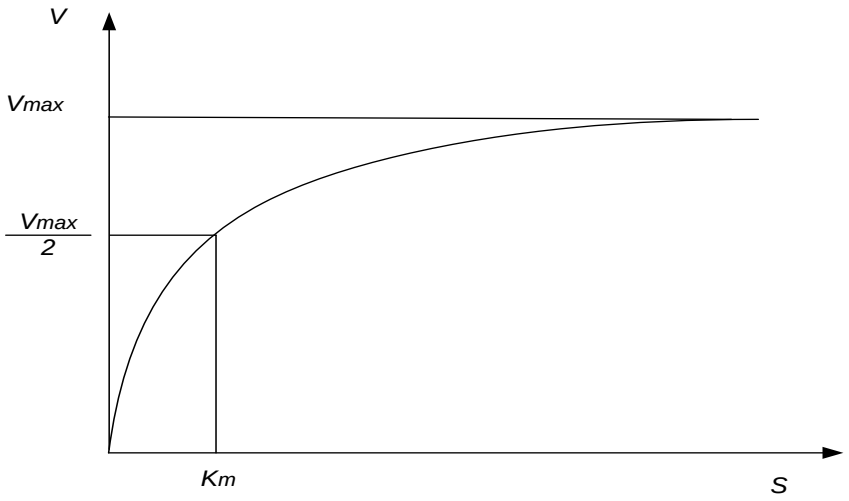


Figure 2.8: Michaelis–Menten kinetics rate profiles

At low PAH concentrations, as when the concentration of the contaminant is in the part per billion ranges usually encountered for underground water contaminations, insufficient energy and carbon source availability can become a limiting factor for microbial growth and maintenance. That is, a threshold may exist below which microbial biomass growth cannot be sustained. Such a minimum concentration for sustainable growth is defined as the concentration at which microbial growth is balanced by decay (Zhang *et al.*, 1998). Also at such a concentration, the biodegradation rate reverts to that described by first-order kinetics. However, the application of suitable microorganisms and/or their products can be used to enhance the concentration of PAHs in the aqueous phase through increased desorption rate and mobilization from the sorbed phase. At such an enhanced concentration, Michaelis–Menten rate kinetics becomes appreciably applicable and from its linearized plot (Fig. 2.9), the maximum PAH degradation rate can easily be determined.

The rate equations can also be simplified by making certain assumptions, particularly, the steady-state approximation, which assumes a negligible change in the concentration of the enzyme-substrate complex during the course of the reaction. Furthermore, the Michaelis–Menten reaction mechanism presupposes that catalysis is irreversible and that the enzyme is not subject to any product inhibition. This limits the suitability of using this model to predict biodegradation rates where chemical surfactants have been known to inhibit product formation (Zhao and Wong, 2009).

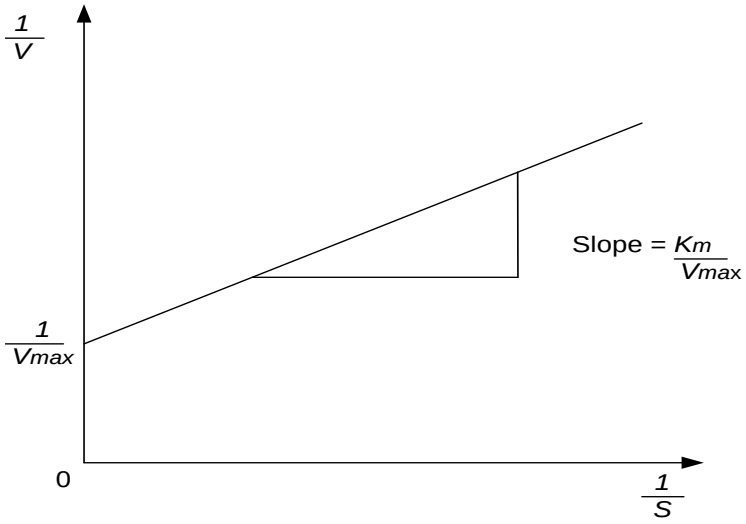


Figure 2.9: A plot of the linearized form of Michaelis–Menten rate equation

2.9.3 Freundlich adsorption isotherm

The Freundlich isotherm is an adsorption isotherm which relates the concentration of a solute on the surface of an adsorbent to the concentration of the solute in the liquid with which it is in contact. The isotherm is mathematically represented as:

$$Q = \frac{x}{m} = kC^{\frac{1}{n}} \tag{2.27}$$

for which the linearized form is:

$$\log Q = \log k + NlogC \qquad [N = \frac{1}{n}] \tag{2.28}$$

where *Q* is the sorbed amount of PAH (mg g^{-1} soil), *x* the mass of PAH (mg), *m* the mass of soil (g), *C* the equilibrium concentration of PAH in solution (mg L^{-1}), and *k* and *n* are constants for a given PAH and adsorbent, which can be either a solid or a liquid, at a particular temperature and thus define the isotherm's curvature. The amount of the sorbed PAH can be determined by plotting the isotherm's linearized form as a function of the equilibrium concentration of the PAH in solution (Fig. 2.10).

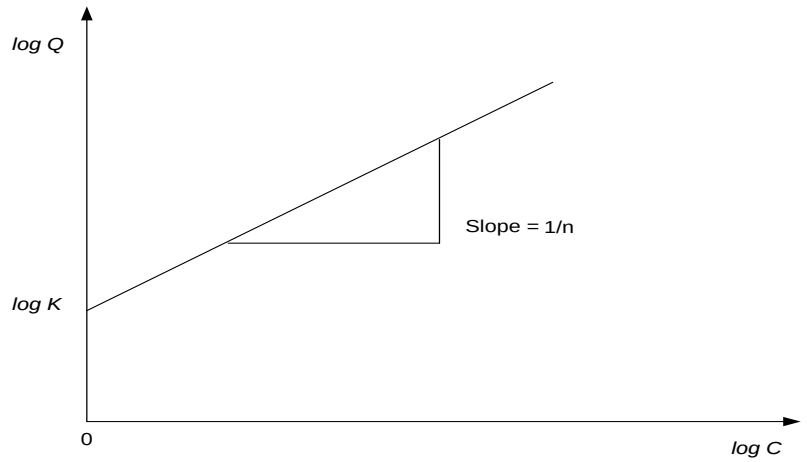


Figure 2.10: A plot of the linearized form of the Freundlich isotherm

The isotherm can also be represented in terms of sorbent pore pressure *P* as:

$$Q = kP^{\frac{1}{n}} \tag{2.8}$$

At a high pressure, the extent of adsorption is independent of pressure (i.e. $^{1}/n = 0$); at low pressure, it is dependent on pressure.

In a recent study of biodegradation of phenanthrene and pyrene in a slurry soil system, biosorption isotherms were found to fit well with the Freundlich isotherm. The Freundlich *n* values were approximated to unity, indicating that the biosorption was dominated by partitioning onto cell biomass and the soils' organic matter (Chen *et al.*, 2010, Chen and Ding, 2012). It also showed that the amount of PAH sorption by the microbial biomass was dependent on the concentration of the PAH present in the contaminated medium. The extent of adsorption varies directly with pressure until saturation pressure is reached. Beyond that point, the rate of adsorption reaches a maximum, even after applying higher pressure (Levenspiel, 1998), thus the Freundlich adsorption isotherm will not be suitable at a higher pressure. Although Langenfeld *et al.* (1993) have shown that pressure variation has no effect on the supercritical extraction efficiency of PAHs, the use of the Freundlich isotherm in biodegradation studies without considering the effects of pore pressure on sorption of PAHs can be problematic (Chen and Ding, 2012).

Table 2.6: Common adsorption isotherms that can be used to describe PAHs adsorption

Model	Mathematical representation
Langmuir	$q_e = \frac{Q_L\, K_L\, C_e}{1 + K_L\, C_e}$
Freundlich	$q_e = K_F {C_e}^{1/n}$
Elovich	$\frac{q_e}{q_m} = K_e C_e exp(-\frac{q_e}{q_m})$
Temkin	$\theta = \frac{RT}{\Delta Q}\, lnK_0 C_e$
BET	$q_e = \frac{Q_0\, B\, C_e}{(C_s - C_e)\left[1 + \frac{(B-1)C_e}{C_s}\right]}$
Kiselev	$K_1 C_e = \frac{\theta}{(1-\theta)(1+K_n\theta)}$
Redlich–Peterson	$q_e = K_F C_e/(1 + B{C^\beta}_e)$
Fowler–Guggenheim	$K_{FG} C_e = \frac{\theta}{1-\theta}\exp(2\theta w/RT)$
Langmuir–Freundlich	$q_e = \frac{q_{mLF}(K_{LF}C_e)^{mLF}}{1 + (K_{LF}C_e)^{mLF}}$
Fritz–Schlunder	$q_e = \frac{q_{mFS} K_{LF} C_e}{1 + q_m {C_e}^{mFS}}$
Sips model	$q_e = \frac{q_{mS} K_S {C_e}^{mS}}{1 + \beta_S {C_e}^{mS}}$
Hill–De Boer	$K_1 C_e = \frac{\theta}{1-\theta}\exp\left(\frac{\theta}{1-\theta} - \frac{K_2\theta}{RT}\right)$
Polanyi–Dubinin–Manes model	$\log q_e = \log Q_0 + a(\varepsilon/V_s)^b$ $\varepsilon = RT\ln(C_s/C_e)$

Symbols are defined in the glossary section.

Chapter 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganisms

Bacillus licheniformis STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 are biosurfactant-producing strains isolated from decaying wood, coal tar surfaces, and an oil spill site, respectively. They were maintained on slant agar at 4 °C and subculture every three weeks.

3.1.2 Chemical reagents

Phenanthrene (Phe), pyrene (Py), benz(a)anthracene (BaA), benzo*(a)*pyrene (BaP), and benzo(ghi)perylene [B(ghi)P] were all certified reference materials purchased from Sigma-Aldrich Corporation (Germany). Hexane (> 97%), dichloromethane (≥ 99.8%), anhydrous sodium thiosulfate (> 98%), cyclohexane (≥ 99.5%), and methanol (≥ 99.9%) were obtained from Sigma-Aldrich, while a C-18 Solid Phase Extraction (SPE) 6mL glass cartridge (0.5g solid phase) was purchased from SUPELCO (Bellefonte, PA, USA) and utilised for the clean-up and concentration of the PAHs under evaluation. Sodium hydroxide, anhydrous sodium aluminate,

3.1.3 Other materials

Pear, P (*Pyrus communis*), pineapple, PP (*Ananas comosus*), orange, OR (*Citrus sinensis*) and beetroot, B (*Beta vulgaris*) were obtained as waste from a fruit and vegetable processing facility close to the Cape Peninsula University of Technology, Cape Town campus. Spent brewer's yeast, BY (*Saccharomyces cerevisiae*) was obtained from a nearby brewery in Cape Town. Fly ash was obtained from a coal-fired plant in Gauteng, South Africa.

3.2 Methods and procedures

3.2.1 Isolation of microorganism, DNA extraction, and PCR amplification of 16S rDNA

The microorganisms isolated in this work were identified by morphological as well as molecular-16S ribosomal deoxyribonucleic acid (rDNA) sequencing analysis. Details on the isolation are presented in Appendix A.

Genomic DNA of the isolates was extracted using a Powersoil® DNA isolation kit (MOBIO Laboratories, San Diego, CA, USA). The total genomic DNA of the strains was extracted for PCR analysis using the method described by Boot *et al.* (1993) with slight modifications. The 16S rDNA gene was amplified by PCR using the following two universal primers: 1) Forward: 5'- AGA GTT TGA TCI TGG CTC AG -3' and 2) Reverse**:** 5'- ACG GIT ACC TTG TTA CGA CTT -3'. The PCR program was set for denaturation at 94 °C for 1 min, annealing at 46 °C for 1 min, and extension at 72 °C for 1 min, for a total of 30 cycles. The PCR products were analysed by electrophoresis at 100 mV for 40 min on a 1% agarose gel (Sigma-Aldrich, USA), using ethidium bromide (10 µg mL^{-1}) to ensure that fragments of the correct size were amplified. A 10-µL sample of the amplified product was added to 1 µL of the tracking dye, followed by loading onto the gel, which was visualized using a UV trans-illumination procedure (Wang *et al.*, 1996). The forward and reverse overlapping sequencing primers were used to sequence the entire length of the double-stranded DNA, which was then compared with known nucleotide sequences, listed in the NCBI Genbank database. The phylogenetic trees obtained by distance analysis based on the 16S rDNA gene, using the neighbour-joining option, are shown in Appendix A. The microbial cultures are always maintained on slant agar and subculture frequently, say every two to three weeks. However, for they can be preserved for a longer period on glycerol and in temperature around -50 to -80°C.

3.2.1.1 Inoculum preparation

An inoculum solution was prepared by transferring a loopful of the microbial cells from the slant agar into nutrient broth and incubated at 37 °C and 150 rpm for 24 h in an orbital shaking incubator. Serial dilutions were then prepared from the inoculum solution and cultured on nutrient agar at the same temperature of 37 °C for 24 h in a shaking incubator at 150 rpm. Isolated cells grew into colonies and were counted using a Quebec Darkfield Colony Counter (Reichert Scientific Instruments, USA). Typically, plates with fewer than 30 or more than 300 colonies were regarded as statistically unreliable (Benson, 2001). The number of colonies counted multiplied by the dilution factor gave 10^8 CFU mL^{-1}, which represented the inoculum concentration used.

3.2.2 Biosurfactant production and characterization

3.2.2.1 Screening of agro-waste: culture preparation and biosurfactant production

The agro-wastes screened were milled and oven-dried at 70 °C for 72 h and then pulverized into particles with diameters of less than 0.30 mm. Then, 250-mL Erlenmeyer flasks containing 100 mL of the culture media, i.e., 5% (w/v) of each of the agrowaste in distilled water, were prepared and autoclaved for 15 min at 110 °C. The cultures were allowed to cool to room temperature, inoculated with a 10% (v/v) inoculum of isolate cultures grown overnight subsequent to incubation at 37 °C and 180 rpm for 96 h. Each experiment was carried out in duplicate for the three isolates used, while uninoculated samples served as controls. After the four-day fermentation, suitable substrates were identified by assaying the activity of the broth supernatants (crude biosurfactant) using the following standard methods: drop-collapse, oil displacement, emulsification index, and surface tension determination. Figure 3.1 demonstrates the procedure followed to select an appropriate agrowaste for biosurfactant production.

Table 3.1. Nutritional compositions per 100g of agro-waste screened for biosurfactant production (USDA, 2011)

Nutritional composition	Agrowaste			
	Ananas comosus (Pineapple, PP)	*Beta vulgaris* (Beetroot, B)	*Citrus sinensis* (Orange, OR)	*Pyrus communis* (Pear, P)
Energy, kcal	50	43	47	58
Water, g	86	88	86.75	89.8
Protein, g	0.54	1.61	0.94	0.5
Carbohydrate, g	13.12	9.96	11.75	13.8
Total sugar, g	9.85	7.96	9.35	4.30
Ca ,mg	13	16	40	9
Fe, mg	0.29	0.8	0.10	0.17
Mg, mg	12	23	10	7
P, mg	8	64.6	14	11
K, mg	109	325	181	119
Na, mg	1.0	78	0	1.0
Zn, mg	o.12	0.35	0.07	0.1
Vitamin C, mg	47.8	8.1	53.2	6.1

3.2.2.2 Drop collapse test

Drop collapse tests were carried out according to the method described by Jain *et al.* (1991) and Bodour and Miller-Maier (1998). Mineral oil (4 µL) was added into the well regions of a 96-well micro-plate and allowed to equilibrate for 24 h, which was followed by the addition of 5 µL of the cell-free culture broth onto the oil-coated regions while the drop size was observed for 5 min with the aid of a magnifying glass. A result was considered positive for biosurfactant production when the oil drop diameter was at least 1 mm larger than that produced by deionized water (control). A 0.5% (v/v) Tween® 20 solution was used for comparison.

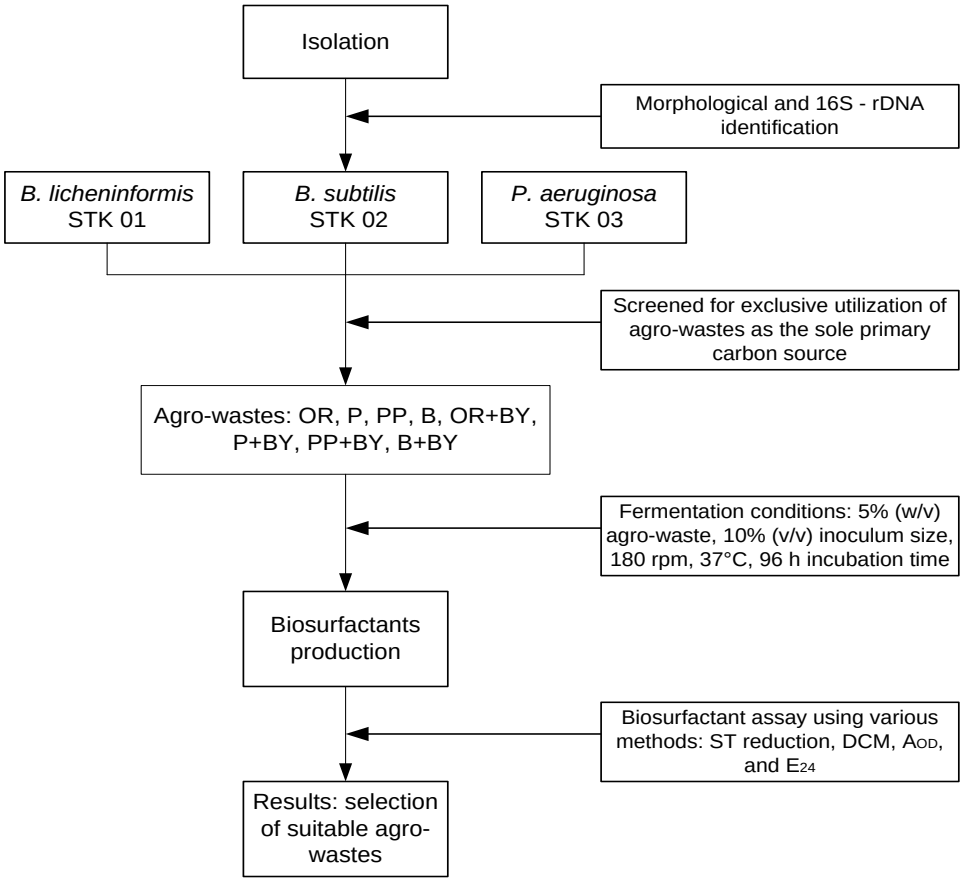


Figure 3.1: A flow chart showing the summary of experimental procedure for the selection of suitable agrowaste for biosurfactant production. 'OR' – *Citrus sinensis*, 'PP'- *Ananas comosus*, 'P' – *Pyrus communis*, 'B'- *Beta vulgaris*, 'BY'- *Saccharomyces cerevisiae*, 'ST'- Surface tension, 'DC'- Drop collapse, 'A_{OD}'- Oil displacement activity, 'E_{24}'- Emulsification index.

3.2.2.3 Oil displacement assay

Oil displacement assays were performed according to the method described by Morikawa *et al.* (2000); 40 mL of distilled water was added to a Pyrex Petri dish followed by the addition of 20 µL of mineral oil to the surface of the water. Thereafter, 10 µL of cell-free supernatant from the culture broth was added to the oil surface. The presence of a biosurfactant was indicated by a clear zone on the oil surface, while the diameter size of the cleared zone or displaced oil signified the biosurfactant activity. A negative control was maintained with distilled water (without biosurfactant), in which no oil displacement or clear zone was observed, while Tween® 20 (0.5% v/v) was used as a positive control. Oil displacement activity (A_{OD}) was determined as:

$$A_{OD} = \frac{\text{Diameter of cleared zone or displaced oil}}{\text{Diameter of oil surface}} \times 100 \quad (3.1)$$

3.2.2.4 Surface tension determination

The surface tension of the biosurfactant-containing culture broth was determined according to Podlogar *et al.* (2004). The surface tension of the cell-free supernatant was determined with a Krüss Processor Tensiometer (model K 100, Germany) at 25 ± 0.5 °C, using the Wilhelmy plate method (Gannon and Faber, 1978). Platinum was used as the plate material because it is chemically inert and easy to clean. The plate was flamed before and after each experimental run to avoid contamination. When the vertically suspended plate of the Tensiometer touches the surface of the biosurfactant, a force is generated which correlates with the surface tension and with the contact angle θ (Eq. 3.2), from which the surface tension can be determined.

$$\sigma = \frac{F}{L \cos \theta} \quad (3.2)$$

where σ is the surface tension (mN m^{-1}), F is the force (mN), and L is the wetted length (m).

3.2.2.5. Emulsification index

The emulsification index (E_{24}) was determined as reported by Cooper and Goldenberg (1987). Six hydrocarbons, *i.e.,* mineral oil, kerosene, diesel, lubricant motor oil, anthracene, and phenanthrene, were added to a cell-free supernatant containing the biosurfactant (6 mL hydrocarbon: 4 mL biosurfactant) in a test tube and homogenized by vortexing vigorously for 2 min. The mixtures were left to stand for 24 h, and the emulsion index (E_{24}) was calculated as indicated in Eq. 3.3. Tween® 20 (0.5% v/v) was used as the control.

$$E_{24} = \frac{\text{Total height of the emulsion}}{\text{Total height of aqueous phase + emulsion}} \times 100$$

(3.3)

3.2.2.6 Biosurfactant emulsion stability to pH and temperature variation

Emulsion stability at varied pH and temperature was assayed using the cell-free supernatant containing the biosurfactant. The pH of the biosurfactant was adjusted using 1 M HCl or 1 M NaOH in the range 2 to 12, after which the emulsification index of the samples was determined. Similarly, the effect of salinity on biosurfactant emulsification ability was investigated at varying concentrations of NaCl (4 to 10%, w/v). In both assays, the stability of the emulsion formed was assessed at different temperatures for 21 days.

3.2.2.7 Biosurfactant extraction and purification FTIR analysis

The pH of the cell-free supernatant containing crude biosurfactant was adjusted to 2.0 using 1 M HCl and keeping it at 5 ± 1 °C overnight, followed by centrifugation at 15,000 rpm and at 4 °C for 20 min to obtain a precipitate. The precipitate obtained was dissolved in 5 mL of distilled water and extracted using three cycles with an equal volume of a chloroform:methanol solution with a ratio of 2:1 (v/v). The organic layer was dialyzed and evaporated using a vacuum at ambient temperature. The dried extract obtained was used for functional group analysis using Fourier transform infrared spectroscopy (FTIR).

Biosurfactant samples were prepared for FTIR assays by milling the extracts with KBr subsequent to pressing with an 8,000-kg load (Specac Bench-Top Hydraulic Presses) for 20 min to form a thin wafer. IR spectra were monitored from 400 to 4000 wave numbers (cm^{-1}) using an FTIR spectrophotometer. Spectra showing the functional groups were used to study the composition of the biosurfactant. Absorption spectra were plotted using a built-in plotter, while the KBr disk was used as a background reference. Pure biosurfactant obtained from Sigma-Aldrich (98% pure surfactin) was used as a control.

3.2.2.8 Biosurfactant concentration and critical micelles concentration (CMC) determination

To determine the concentration of the biosurfactant produced, surfactin samples were filtered through Millipore membranes with pore size 0.22 µm and the concentration determined by a high-performance liquid chromatography (HPLC) – Shimadzu UFLC (Shimadzu Corporation, Kyoto, Japan) coupled with a model Shimadzu SPD-20A UV/Vis detector and a 20AHT auto sampler. A reverse phase C_{18} (RP) capillary column with a Techsphere 5 µm ODS column (250 x 4.6 mm Ea) was used for the analyte elution. For each assay, 10 µL of the filtrate was injected and eluted isocratically with a mobile

phase of water and acetonitrile (35:65 v/v) at a flow rate of 1.0 mL min^{-1} at 30 °C. The absorbance of the eluate was detected at 220 nm. Surfactin (> 98%) purchased from Sigma-Aldrich was used to generate the standard calibration curve (Appendix B) from which the concentration of the biosurfactant produced was assayed. The CMC was determined from the plot of surface tension against surfactant concentration.

3.2.3 Analytical methods and quantification

3.2.3.1 Total reducing sugar quantification

Total available sugar (TAS) in the culture medium was quantified using the dinitrosalicyclic (DNS) acid colorimetric method described by Miller (1959), with a slight modification. 1.5 mL of DNS reagent (containing, in w/v: 1% DNS; 0.2% phenol; 1% sodium hydroxide; and 0.05% sodium sulphite) was added to 1.5 mL aliquots of culture sample (10 times diluted) in a capped polypropylene test tube. The mixtures were heated for 15 min in a water bath at 90 °C and then cooled under running tap water adjusted to ambient temperature, before adding 0.5 mL of 40% potassium sodium tartrate. The absorbance of the mixture was measured in a UV spectrophotometer (Jenway, UK) at 575 nm with a slit width of 0.06 mm. A standard curve of absorbance against concentration (Appendix B) was prepared with a D(+) glucose solution across the range 0–1000 mg L^{-1} and used to estimate the sugar concentration in the culture.

3.2.3.2 Soil characterisation, PAH-spiked soil sample preparation, and culture conditions

Uncontaminated topsoil was obtained within the vicinity of the university (CPUT) and used in this study. The soil was characterized using an American Society for Testing and Material method (ASTM method DIN-4188) coupled with a United Soil Classification system. The soil contained 30% clay, 20% silt (half passing through no. 200 sieve), 20% fine and 30% coarse sand (half being retained on no. 4 to 200 sieve) – the soil was classified as a silt soil. It was collected and stored at 4 °C until use.

The degradation of PAHs in the soil was determined in the absence of indigenous microorganisms. A quantity of soil (200 g) was autoclaved twice at 121 °C and 1 bar for 30 min, within a 12 h interval. The soil was spiked with a mixture of 40 mg PAH per kg of soil, using each of the following PAHs: Phe, Py, BaA, BaP, and B(ghi)P. The spiking process was carried out as described by Brinch *et al.* (2002), with a minor modification; i.e., 20% of the soil was treated with the PAH mixture in acetonitrile.

After the volatilisation of the acetonitrile, the PAH-containing soil was mixed with 50% of the uncontaminated soil sample. After adequate mixing, the contaminated portion was then mixed with the remaining soil to ensure a uniform distribution of the PAHs in the soil. A mass (10 g) of the contaminated soil was weighed into 100 mL Erlenmeyer flasks (covered with foil on the exterior), with a glass weighing boat. The soil samples were inoculated with 8% (v/w) of overnight microbial cultures grown in nutrient broth, subsequent to incubation at 37 °C in a dark static incubator. Different cultures were studied, viz., the monocultures of each of the isolate (without supplementation with either biosurfactant or *B. vulgaris* waste); a culture of *B. licheniformis* supplemented with the crude biosurfactant produced from *B. vulgaris* (5%, v/w); a culture of *B. licheniformis* supplemented with dry milled *B. vulgaris* waste (5%, w/w); and a co-culture of the two *Bacillus* strains (without supplementation). The soil moisture content was maintained at 60% holding capacity as reported by Acevedo *et al.* (2011) by adding 5 mL of sterile water to each flask at 10-day intervals. Control experiments were prepared in the same manner without an inoculum, to account for PAHs' disappearance due to abiotic factors. Each experiment was carried out in duplicate. Samples were incubated for 60 days.

3.2.3.3 PAH extraction, clean up, and quantification

PAHs were extracted using an ultrasonication method. At the end of the 60-day experiment, samples were transferred from Erlenmeyer flasks into 100 mL amber bottles and extracted with 20 mL of hexane for 20 min at 25 °C in an ultrasonic bath. During the sonication, sample bottles were swirled intermittently to prevent the soil's settling at the bottom. This step was repeated twice for each sample while the supernatants were pooled into another bottle and centrifuged at 5,000 rpm for 10 min, in preparation for the clean-up process.

Clean-up procedure

An SPE column LC 18 cartridge was preconditioned with 30 mL of hexane (HEX) and dichloromethane (DCM), in this order: DCM – DCM/HEX (2:3, v/v) – DCM, with a volume of 10 mL being loaded at a time. Sodium thiosulfate (1.0 g) was added to the top of the silica gel in the cartridge prior to conditioning (EPAMethod610, 1984).The supernatant collected from the centrifugation above was passed through the conditioned SPE cartridge followed by the elution of the PAH analytes with HEX and DCM, in the same order as mentioned above, using 7.5 mL of the eluent each time. The eluant collected was dialyzed in a rotary evaporator; thereafter, the residue was reconstituted in DCM to 1 mL in an amber vial, followed by analysis with a gas chromatography-flame ionization detector (GC-FID).

The GC-FID analysis was performed using a 7890A Series GC-system (Agilent Technologies, CA, USA) equipped with a flame ionization detector, an Agilent capillary column USB499114H (20 m x 180 µm x 0.14 µm), and an auto sampler. The oven programme was 170 °C followed by ramping at 5 °C min^{-1} up to 300 °C with each ramping step being maintained for 3 min. Once a temperature of 300 °C was reached, the temperature was increased to 310 °C and held for 5 min. The total run time was 36 min, including 6 min post-run time to clean the column before subsequent injections. The carrier gas used was nitrogen, while a split-mode injection was used, with the injector temperature set to 250 °C. A calibration curve was plotted using external standards, with concentration in the range of 0.5 to 100 ppm (R^2 = 0.9996), which was used to quantify the concentration of each analyte. The recovery efficiency for phenanthrene, pyrene, and B(a)A was approximately about 90%, while that of B(a)P and B(ghi)P was 75% and 83%, respectively.

3.2.4 Magnetic zeolite synthesis, modification with biosurfactant, and characterization

The raw fly ash samples were first screened through a 212 µm sieve to eliminate the larger particles. A mixture of sodium hydroxide, fly ash, and the magnetite particles, in a predetermined ratio of 1:1.5: y (by weight), respectively, was milled and fused in an oven at a temperature of 550 °C for $1^1/_2$ h (Musyoka *et al.*, 2012, Mainganye *et al.*, 2013). The quantity of magnetite (y) in this composite varied from 0.1–0.75. The resultant fused magnetic zeolite (MZ) was then cooled to ambient temperature, milled further and dissolved in distilled water (1 g/5 mL water). The slurry obtained was stirred at 1500 rpm and at room temperature for 2 h. The resultant precipitate was filtered and washed repeatedly with distilled water to remove the remaining solids. The filtrate thus obtained was mixed with $NaAlO_2$(aq) in a ratio of 2.5:1 (v/v), stirred for 20 min and crystallized at 100 °C for 2–4 h. The purpose of the addition of the aluminate solution was to control the molar ratio for single-phase zeolite A synthesis. Twenty-five grams of the synthesized magnetic zeolite was mixed with 0.5 L of biosurfactant solution, and stirred for 7 h at 102 rpm at ambient temperature. The suspension formed was filtered and oven dried at 70 °C for 10 h, and then characterized.

3.2.4.1 Adsorbent characterization

BET surface area

For surface characterization of the synthesized zeolites, samples were degassed prior to analysis at 90 °C for 60 min, thereafter at 250 °C for 12 h, using a Micromeritics VacPrep® 061 Sample Degas System (Micromeritics, USA), while a 3Flex surface characterization analyzer (Micromeritics Instrument Corp., USA) was used. To determine the surface area, the temperature of the degassed samples was first reduced to that of liquid nitrogen. Then the absorbing gas (nitrogen) was admitted in incremental doses. The accumulated gas quantity adsorbed versus gas pressure data at one temperature were then plotted to generate an adsorption isotherm using the Barrett–Joyner–Halenda (BJH) method. The data obtained were treated in accordance with the Brunauer–Emmet–Teller (BET) gas adsorption method to calculate the specific surface areas for the sample in units of square meters per gram.

TGA analysis

Thermogravimetric analysis (TGA) was performed using a Mettler Toledo TGA-DSC 1 analyser. The experiment was carried out using nitrogen as a purge gas in the temperature range from ambient to 800 °C, with a heating rate of 10 °C/min and an inert gas flow rate of 70 mL/min.

SEM analysis

For the analysis of the morphology, samples were sprinkled on special glue mixed with carbon graphite and mounted on an aluminium stub, and analysed with an S200 scanning electron micrometer (SEM) equipped with an energy dispersive X-ray spectrometer (EDS) to determine the elemental composition of the samples.

PXRD and FTIR analysis

Mineralogical determination of the synthesized zeolites and fly ash was carried out by first reducing the sample particles into fine powder and then analysing them using a Bruker D8 Advance X-ray diffractometer (Bruker Corporation, Germany) equipped with a Co source and a Vantec position-sensitive detector. Powder X-ray diffraction (PXRD) patterns were collected within 2-theta range of 5 and 70° with a step size of 0.0062° and step time of 360 s under continuous rotation of the sample during the scan. The different functional groups and bonds present in the crystal samples were examined using a Fourier transform infrared (FTIR) spectrophotometer (PerkinElmer Ltd, UK). The

crystalized samples of zeolites and the powdered sample of FA were prepared as explained in Section 3.2.2, using the same hydraulic press, and analysed the same way

Chapter 4

EMULSIFICATION OF HYDROCARBONS BY BIOSURFACTANT: EXCLUSIVE USE OF AGROWASTE

4.1 Introduction[2]

The future commercialization of biosurfactants depends on research and development studies that can identify better, low-cost, renewable substrates to develop eco-friendly processes for the sustainable synthesis of suitable bioproducts. Compared with the traditional use of synthetic surface-active agents in soaps, laundry detergents, and personal care products, biosurfactants have applications in other fields such as polymerization, foods/beverages, cosmetics, pharmaceuticals, petroleum recovery, and environmental remediation (Banat *et al.*, 2000, Banat *et al.*, 2010). Biosurfactants are unique organic compounds synthesized biologically from natural or renewable raw materials. Because of their amphiphilic structure and distinctive functional groups, they possess desirable properties, such as wettability, micellization, surface tension lowering abilities, and formation of micro-emulsions between two different phases, which make them suitable for a variety of applications. For environmental bioremediation applications, these properties can enhance the bioavailability of hydrophobic contaminants, thereby increasing their biodegradation (Pacwa-Płociniczak *et al.*, 2011, Soberón-Chávez

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and Maier, 2011).Most environmental contaminants are hydrocarbon derivatives that are hydrophobic and recalcitrant, thus requiring surface-active agents to mobilize them from their repositories – usually sediments and soil – into the aqueous phase for microbial degradation. Consequently, surface tension and emulsification are two important properties used to evaluate and screen surfactants for their ability to enhance the bioavailability of hydrophobic contaminants. An effective surface-active agent should be able to lower the surface tension of the medium enough to create emulsions of two phases and thus enhance the solubility of the hydrophobic contaminants. Although a surface tension reduction below 35 mN m^{-1} has been benchmarked for effective biosurfactants (Fox and Bala, 2000, Nitschke and Pastore, 2006, Barros *et al.*, 2008), studies have shown some biosurfactants with a high capacity for emulsification of hydrophobic organic compounds whose medium surface tensions were above 35 mN m^{-1} (Lai *et al.*, 2009, Rocha *et al.*, 2009, Oliveira *et al.*, 2013). Likewise, emulsion stability is an important consideration in environmental application of biosurfactants. De-emulsification can occur because of acid stimulation and ionization of the constituents of interfacial films as a result of variations in soil temperature, pH, and salinity (Fortuny *et al.*, 2007).

Furthermore advances in the utilization of agro-waste/agro-industrial waste materials for the production of biosurfactants has increased, as more of these wastes are identified as appropriate carbon and nitrogen sources (Sousa *et al.*, 2012, Sobrinho *et al.*, 2013). Moreover, the increasing awareness of environmental regulations on pollution control has necessitated the use of eco-friendly feedstock and products. Despite these regulations, together with the relatively low availability and high cost of petroleum derivatives used for chemical surfactants' production, the possibility of replacing chemical surfactants with those produced biologically may not be realistic in the near future unless suitable biosurfactant-producing strains are identified. The quest also includes the availability of low-cost renewable resources such as solid agrowaste, agro-industrial waste, and effluent, which can be used exclusively (i.e., without augmentation with refined sugar or any source of trace elements) for biosurfactant production. This could be one of the options to circumvent the low yield and high cost associated with the full-scale commercialization of biosurfactant production. Utilization of agrowaste in this way will offer a concomitant advantage by reducing the pollution effects caused by these wastes and minimizing their disposal cost. In addition to these advantages, there is a plethora of suitable and easily accessible organic waste that can be used for biosurfactant production, thus improving the sustainability of such processes. Furthermore, the application of crude surfactants can be as effective as their refined counterparts for certain applications, especially for bioremediation of environmental contaminants (Nitschke and Pastore, 2006, Kang *et al.*, 2010, Amodu *et al.*, 2013).

Some agro-industrial wastes/agrowaste have been identified for biosurfactant production by certain microorganisms, depending on the nutritional composition required by specific microorganisms. They include olive oil mill effluent, biodiesel plant by-products, plant oil residue, distillery and whey waste, potato peels, and rice straw (Das and Mukherjee, 2007b, Makkar *et al.*, 2011, Sobrinho *et al.*, 2013, Zhu *et al.*, 2013). In these studies, the renewable resources are often supplemented with refined glucose and other compounds, such as nitrogen sources and trace elements required by the microorganisms. However, the exclusive application of agrowaste can offer considerable cost-effective and sustainable systems for the production of biosurfactants and easy adaptation for *in-situ* bioremediation of environmental contaminants. Microorganisms differ in their requirements for carbon sources, including quantities, as well as for other requisite micronutrients, for their metabolic activities. This makes it necessary to identify suitable agrowaste for each isolate that has shown a tendency for biosurfactant production on refined substrates.

4.2 Objectives

The objectives of the present study were:

- to isolate and identify biosurfactant-producing strains;
- to identify suitable agricultural solid waste for exclusive use by the isolated strains for biosurfactant production;
- to investigate the capacity of the produced biosurfactants to emulsify hydrocarbons; and
- to investigate the effects of pH and salinity on the stability of the emulsion formed.

4.3 Materials and method

Bacillus licheniformis STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 are biosurfactant-producing strains and were isolated as shown in Chapter 3. The strains were maintained on nutrient agar slants at 4 °C and sub-cultured every three weeks.

Several types of agrowaste, namely *Pyrus communis* (pear, P), *Ananas comosus* (pineapple, PP), *Citrus sinensis* (orange, OR), and *Beta vulgaris* (beetroot, B) were screened as primary substrates for biosurfactant production, as was the combination of each of the wastes with *Saccharomyces cerevisiae* (spent brewer's yeast, BY), as reported in Chapter 3 The nutritional compositions of these agrowaste are

listed in Table 3.1. The procedures for selecting an appropriate agrowaste for biosurfactant production, extraction, activity, and stability assays are in Chapter 3.

4.4 Results and discussion

4.4.1 Microbial identification

The morphological identification showed that two of the isolates were gram-positive and spore-forming *Bacilli* sp. with reddish-pink rod-shaped colonies, while the third was a gram-negative non-spore-forming strain, identified as *Pseudomonas* sp. The DNA sequence revealed that the gram-positive biosurfactant-producing isolates were closely related to *Bacillus licheniformis* strains ZML-1 (96%), SCCB-37 (96%), and 1-FTM8 (96%) and *Bacillus subtilis* strains ZBSF-1 (98%) and SML-2 (98%). The isolates were thus identified as *Bacillus licheniformis* and *Bacillus subtilis*, respectively. *Bacillus licheniformis* was designated strain STK 01, while the *B. subtilis* strain was STK 02. Similarly, the gram-negative strain isolated belongs to the clad of *Pseudomonas aeruginosa*, sharing the highest similarity with strains AMBAS7 (97%) and SK9 (97%). Hence, it was identified as *Pseudomonas aeruginosa* and designated strain STK 03. It has been suggested that a bacterial strain can be regarded as novel when the genomic similarity to its closest neighbour is less than or equal to 97% (Stackebrandt and Goebel, 1994). This correlation was, however, revised in 2005, recommending a nucleotide sequence similarity value of 98.5%, based on the DNA-DNA hybridization data used for delineating species (Stackebrandt and Ebers, 2006, Stackebrant, 2011). With regard to this recommendation, the bacterial strains isolated in this study were regarded as novel strains. A phylogenetic tree was constructed by distance analysis based on the 16S rDNA gene, using a neighbour-joining option (as presented in Appendix A).

4.4.2 Identification of suitable agrowaste substrates for biosurfactant production

The isolated strains *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 have shown biosurfactant-producing properties on refined substrates, reducing the surface tension of broth to 28.5, 30.2, and 32.0 mN m^{-1}, respectively. Thus, to identify appropriate renewable substrates that can be used by the microorganisms exclusively for growth and for biosurfactant production, several agrowaste/products were screened. The results obtained from the screening methods showed that the three bacterial isolates were able to utilize *Beta vulgaris* waste. The explanation for this observation could be adduced based on the nutritional composition of *B. vulgaris* (Table 3.1). In addition to the sugar

content of the agrowaste, which serves as a carbon and nitrogen source, *B. vulgaris* is richer in essential macronutrients required for microbial cell structure and metabolism, compared with other agrowaste screened. For the drop collapse test (Table 4.1), distilled water and Tween® 20 were used as negative and positive controls, respectively.

Table 4.1. Drop collapse assay for the screening of various agrowaste for biosurfactant production

Agrowaste	Biosurfactant activity from various agrowaste		
	B. licheniformis STK 01	*B. subtilis* STK 02	*P. aeruginosa* STK 03
Citrus sinensis, OR	+	NA	+++
Ananas comosus, PP	++	++	NA
Beta vulgaris, B	+++	+++	++
Pyrus communis, P	+	NA	NA
OR + BY	+	+	++
PP + BY	++	++	NA
B +BY	+++	+++	++
P + BY	NA	NA	NA

'NA' – no drop collapse, '+++'- complete collapse within 2 min, '++'- collapse after 2 min, '+' – collapse after 4 min of biosurfactant addition, 'BY' - *Saccharomyces cerevisiae*. Controls: distilled water: 'NA', Tween® 20: '+++'

The effectiveness of the biosurfactant produced was displayed by a rapid and complete collapse of its droplets on oil surfaces, as observed for *B. vulgaris* cultures, and also when it was supplemented with *Saccharomyces cerevisiae*. However, some of the agrowaste used were found to be unsuitable for biosurfactant production by the microorganisms, as the droplets of their supernatants maintained an oval shape on oil surfaces, similar to the experiments in which water droplets were used. For instance, the broth obtained after 96 h of fermentation of *Pyrus communis* with the three microorganisms did not seem to demonstrate any surface-active properties. Meanwhile, *Citrus sinensis* was shown to be a suitable nutrient source only for *P. aeruginosa* among the three microorganisms. This microbial selectivity of agrowaste substrate has been reported (Singh *et al.*, 2007, Kumar *et al.*, 2011).

Similar results and trends were observed for the surface tension studies. Considerable surface tension reduction was achieved by the crude biosurfactant produced from *B. licheniformis* and *B. subtilis* on *Beta vulgaris*. These strains reduced the surface tension of broth to 30.2 and 32.98 mN m^{-1}, respectively. Nonetheless, *P. aeruginosa* seemed to thrive more on *C. sinensis*, giving a surface tension reduction of 29.06 mN m^{-1} (Table 4.1). Comparable surface tension reductions have been reported for some agrowaste/agro-industrial wastes, such as oil-refining extracts and waste, distillery and whey waste, potato peels, cassava wastewater, and rice straw (Makkar *et al.*, 2011, Amodu *et al.*, 2013).

Table 4.2: Surface tension determination of biosurfactants produced from various agrowastes

Agrowaste	Surface tension (mN m^{-1})			
	B. licheniformis	*B. subtilis*	*P. aeruginosa*	Control
Citrus sinensis, OR	39.64 ± 0.01	40.15 ± 0.02	29.06 ± 0.04	43.05 ± 0.01
Ananas comosus, PP	37.66 ± 0.02	38.91 ± 0.01	39.86 ± 0.01	45.07 ± 0.01
Beta vulgaris, B	30.20 ± 0.03	32.98 ± 0.05	30.37 ± 0.01	45.30 ± 0.02
Pyrus communis, P	46.81 ± 0.01	47.68 ± 0.02	45.05 ± 0.04	47.42 ± 0.02
OR + BY	43.05 ± 0.02	43.20 ± 0.01	35.04 ± 0.03	45.50 ± 0.03
PP + BY	41.62 ± 0.01	42.04 ± 0.01	41.52 ± 0.01	47.20 ± 0.02
B + BY	31.53 ± 0.01	44.09 ± 0.02	41.08 ± 0.01	41.35 ± 0.01
P + BY	48.00 ± 0.03	48.25 ± 0.01	46.80 ± 0.02	53.8 ± 0.01

'BY': *Saccharomyces cerevisiae*

Additionally, *S. cerevisiae*, even though a good protein source, did not enhance biosurfactant production when used to supplement agrowaste in this study.

Biosurfactants produced from the various agrowaste were further screened using the oil displacement method (Fig. 4.1), which showed the spreading and wettability effects of the produced biosurfactants. These are essential properties required for surface-active agents used for industrial cleaning, bioremediation of hydrophobic contaminants, and oil recovery from reservoirs (Banat *et al.*, 2010). Again, the results obtained were similar to those observed for the surface tension and drop collapse methods. A 95% oil displacement was produced by biosurfactants from *B. subtilis* and *P.*

aeruginosa; both microorganisms grown on *Beta vulgaris* waste, as compared with 85% observed for 0.5% Tween® 20 used as the control. The assessment also showed that none of the microorganisms could use *P. communis* waste as a nutrient source for biosurfactant production.

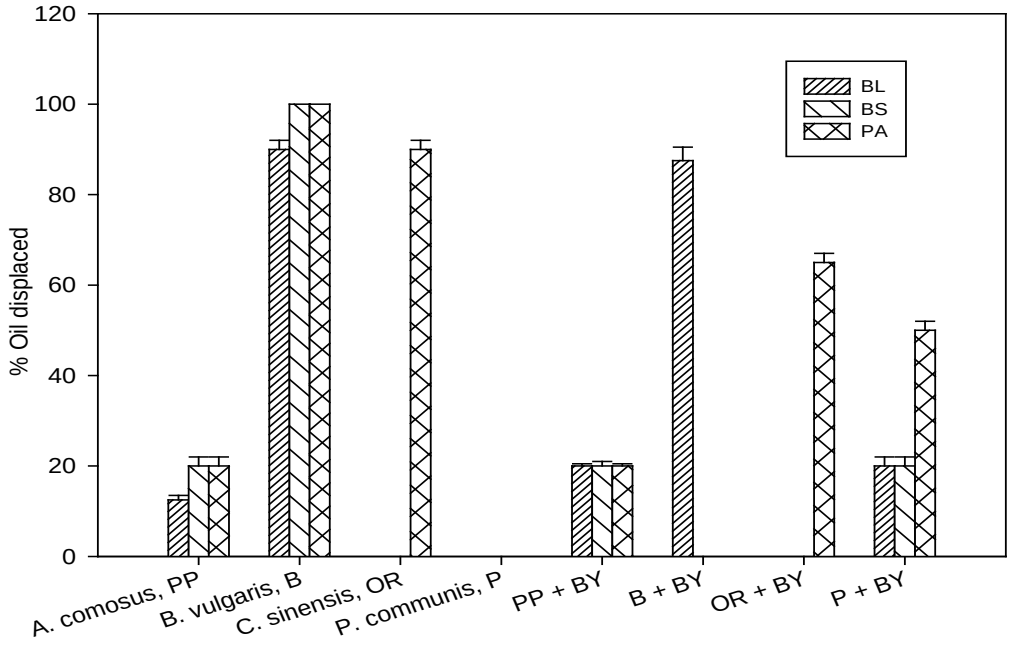


Figure 4.1: Oil displacement activity of biosurfactants produced exclusively from solid agrowaste. 'BY'- *Saccharomyces cerevisiae*, 'BL'- *Bacillus licheniformis* STK 01, 'BS'- *Bacillus subtilis* STK 02, 'PA'- *Pseudomonas aeruginosa* STK 03; Controls: Distilled water and 0.5% Tween® 20. Tween® 20 gave 85% oil displacement.

Microorganisms differ in their nutrient requirements, compositions, and fermentation conditions, which could influence their metabolic activities (Coulon *et al.*, 2005). Hence, in prospecting for suitable renewable substrates for microbial growth and biosurfactant production, it is expedient to perform a screening test for surface activity on the fermented broth rather than screening based on nutritional and chemical compositions. This study showed the possibility of effective biosurfactant production from solid agrowaste without supplementation with refined nutrient sources.

4.4.3 Biosurfactant activity assay

Furthermore, the activity of biosurfactants produced by the microorganisms was assayed against different hydrocarbon compounds by the emulsification method (Figs. 4.2 and 4.3). The ability of biosurfactants to create emulsions of hydrocarbon compounds, and thereby increase their bioavailability, is often used as a basis for determining their effectiveness in environmental bioremediation of hydrophobic contaminants. Emulsions are formed when a liquid phase is dispersed as microscopic droplets in another liquid phase. The biosurfactant produced showed a high hydrocarbon emulsification index, particularly for heavy hydrocarbons (Fig. 4.2). The highest emulsification values recorded for biosurfactants produced by *B. licheniformis* STK 01 were 49, 65.5, and 95% for phenanthrene, anthracene, and lubricant oil, respectively. The biosurfactant from *P. aeruginosa* STK 03 gave a 66.7% emulsification index for phenanthrene, while a 70% index was recorded for kerosene. Similarly, the biosurfactant produced by *B. subtilis* STK 02 showed a 90% emulsification index for lubricant oil, but failed to emulsify phenanthrene and anthracene. The results obtained in this study were similar to those reported by Sumiardi *et al.* (2012), whereby the highest emulsification index of 93.7% was achieved for a hydrocarbon compound by a bacterial consortium. Emulsification indices in the range of 69 to 71% were also reported for diesel and kerosene by a biosurfactant expressed by *Agrobacterium* spp. QS-6 (Lai *et al.*, 2009). Oil emulsification using biosurfactants can be influenced by some thermodynamic and rheological properties of the system, including aqueous phase composition (salinity and pH), organic phase composition, emulsion-stabilizing nature of the biosurfactants, the presence of fine particulates, and temperature (Kosaric, 1992). This result shows the stability of these isolates for biosurfactant production, particularly for applications in environmental bioremediation of heavy hydrophobic contaminants, whereby the bioprocesses used are supported by cheap and easily accessible agrowaste substrates.

The surface activity of biosurfactants produced by *B. licheniformis* STK 01 and *P. aeruginosa* STK 03, grown on the same agrowaste, was compared in this study. The two microorganisms produced biosurfactants that both lowered the surface tension of the fermented broth to 30 mN m^{-1}. However, Fig. 4.2 shows a disparity in their emulsification activity. The biosurfactant produced by *B. licheniformis* STK 01 exhibited a better emulsification tendency for all the hydrocarbons used, except for kerosene. This shows that surface tension reduction only may not be appropriate to assess the emulsification capacity of biosurfactants.

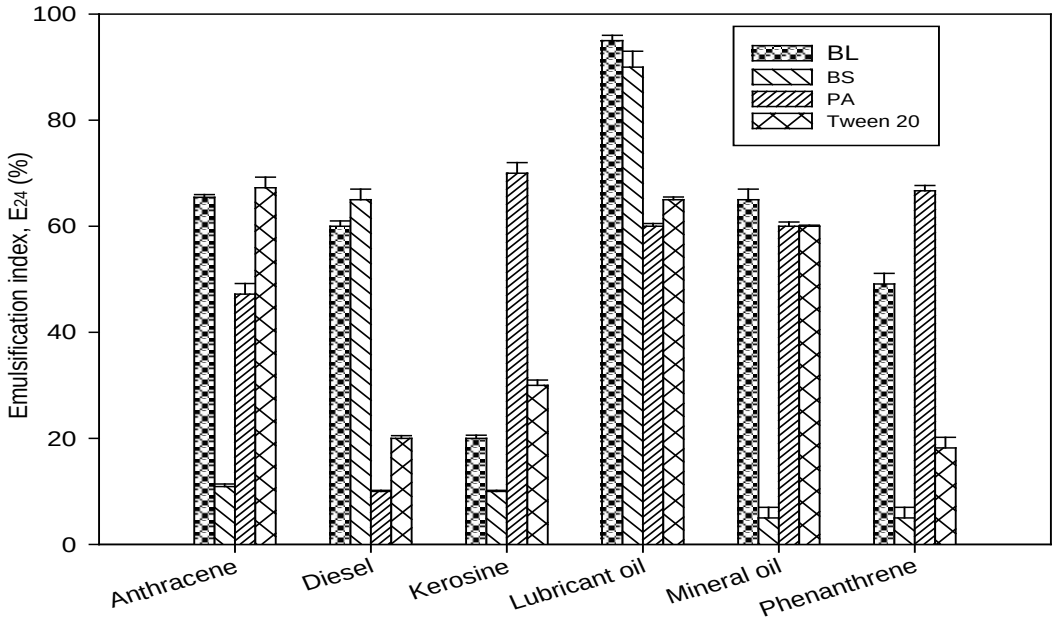


Figure 4.2: Emulsification activity of biosurfactants produced by *Bacillus licheniformis* STK 01(BL), *Bacillus subtilis* STK 02(BS), and *Pseudomonas aeruginosa* STK 03(PA) exclusively from *Beta vulgaris*. 0.5% Tween® 20 was used as a positive control while uncultured broth of *B. vulgaris* was used as a negative control which showed 20 and 10% E24 for lubricant oil and mineral oil respectively, and zero for the other hydrocarbons.

Although the two microorganisms were grown on the same solid agrowaste, they had different metabolic pathways and thus produced biosurfactants with different functional groups. This in turn affects the formation and stability of the hydrocarbon emulsion. A similar scenario was reported (Lai *et al.*, 2009, Rocha *et al.*, 2009, Oliveira *et al.*, 2013), where it was hypothesized that the different metabolic activities of biosurfactant-producing microorganisms affect the chemical structure and functional groups of the biosurfactant produced, and thus the emulsification index.

In Figure 4.3, a comparison was made between the emulsification activities of biosurfactants produced from two different agrowastes – *A comosus* and *B. vulgaris* – but by the same microorganism. The biosurfactant produced by *B. subtilis* STK 02 from *A. comosus* waste lowered the surface tension of broth to 38.91 mN m^{-1}, while that which was produced from *Beta vulgaris* reduced the surface tension to 32.98 mN m^{-1}, as shown in Table 4.3; the latter demonstrated a higher emulsification tendency, as expected. The results obtained showed that emulsification activity of biosurfactants produced by the same microorganisms, for particular hydrocarbons, is proportional to the extent to which they can lower the media surface tension, irrespective of the nutrient sources used. This is thus important when

assessing the emulsification tendency of biosurfactants based on their capacity to lower surface/interfacial tension.

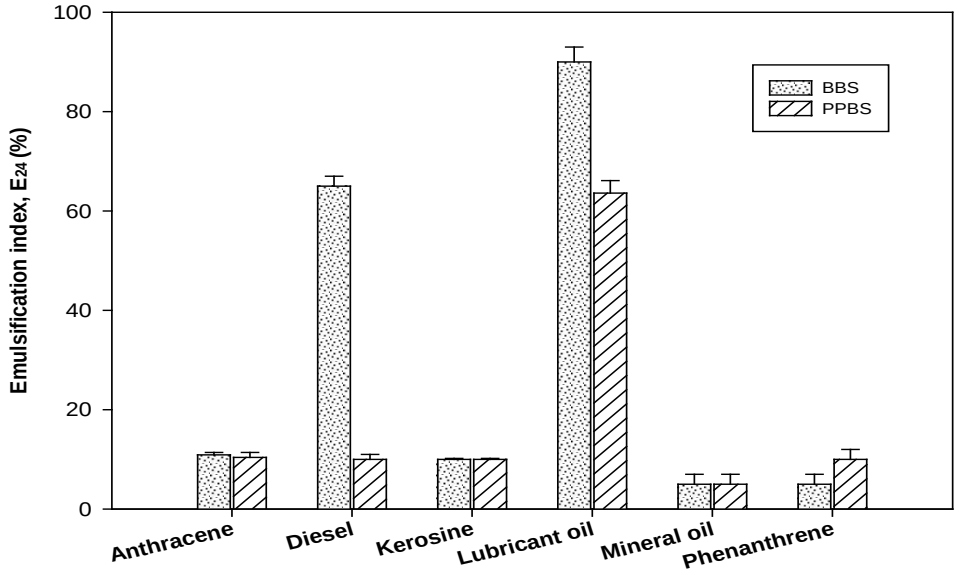


Figure 4.3: Comparison of the emulsification index of biosurfactants produced by *Bacillus subtilis* STK 02 grown exclusively on *B. vulgaris* (BBS) and *A. comosus* (PPBS)

4.4.4 Emulsion stability

Biosurfactant emulsion stability under varying conditions is of great importance, as it can impair their applicability. De-emulsification may not be desirable in most environmental applications, whether oil-in-water or water-in-oil emulsion is being considered. The former is encountered during mobilization of hydrophobic contaminants from their sinks, usually sediments or soil matrices, to become available for microbial degradation. The stability of the emulsion formed by the biosurfactant produced by *B. licheniformis* STK 01 while using *Beta vulgaris* waste was investigated at various pH levels, salinities, and temperatures (Fig. 4.4).

The variation in hydrocarbon emulsification with respect to pH shown in Fig. 4.4a indicates how much effect the environmental pH can have on the continuous bioavailability of hydrocarbon contaminants. The emulsification index (E_{24}) rose gradually to a maximum at a pH between 5 and 8, with optimum at 6 to 7, and thereafter showed exponential de-emulsification. In the same vein, the highest E_{24} was observed at a salt concentration range of 6 to 7% w/v (Fig. 4.4b). The hydrocarbon

representatives used were lubricant oil and *n*-hexane, for pH and salinity studies, respectively. This made it possible to test the stability of the emulsion formed with light as well as heavy hydrocarbons. The changes in the pH of biosurfactant solutions can affect the physicochemical properties of the hydrocarbon and the formation and rigidity of the interfacial films, thus causing the emulsions formed to coalesce. Emulsion stability can be severely upset due to acid stimulation and ionization of interfacial films' constituents (Fortuny *et al.*, 2007). Salinity can also have an important effect on hydrocarbon emulsification and stability owing to ionization caused by the interaction of ions present in the salt solution with the asphaltenes-resins, aromatic, and saturated hydrocarbons.

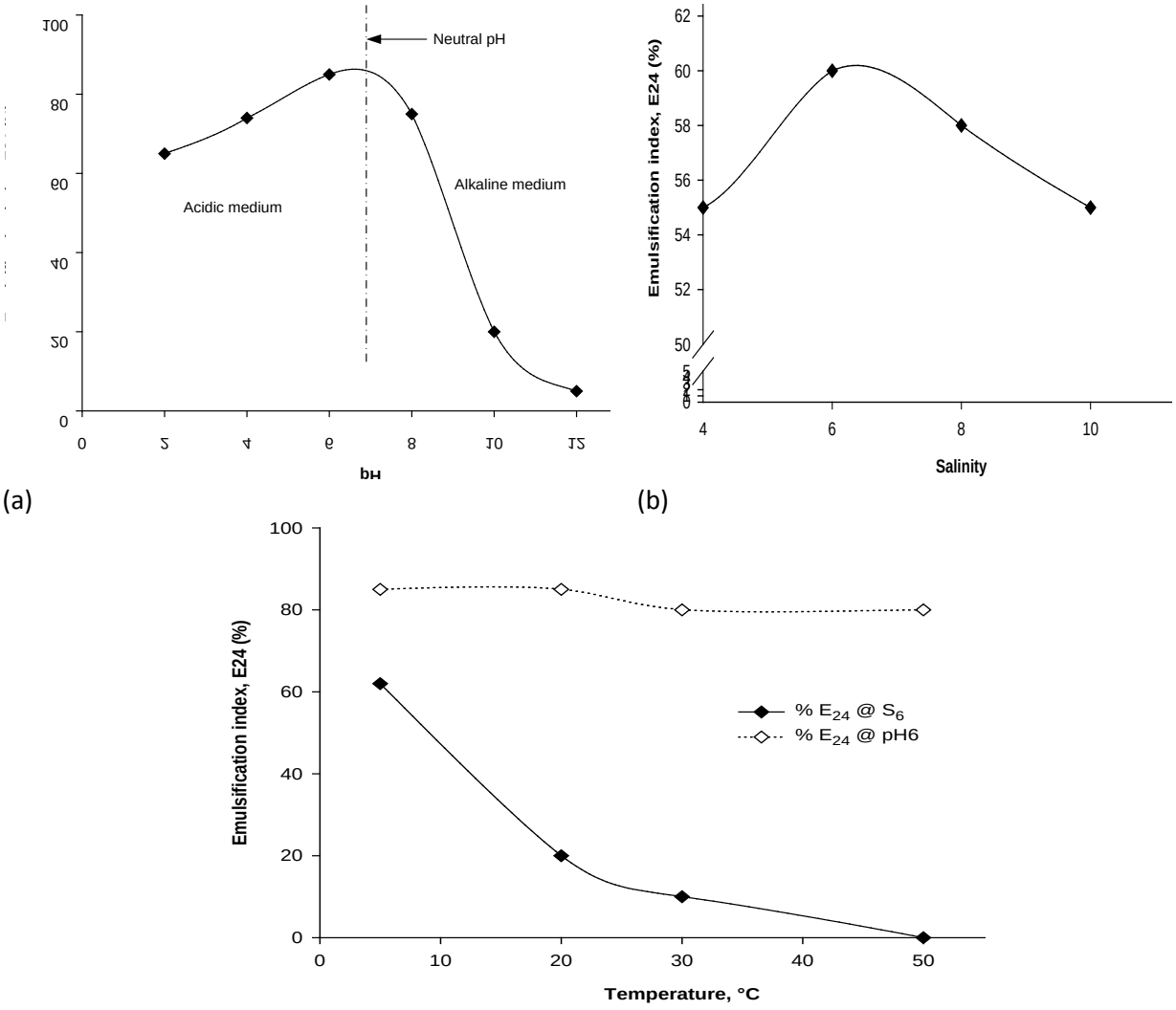


Figure 4.4: (a) Hydrocarbon emulsification as a function of pH – demonstrated with lubricant oil; (b) effect of salinity on emulsification – demonstrated with *n*-hexane; and (c) thermal stability of emulsion formed at 6 %(w/v) salt concentration (% E_{24}@S6) and at pH 6 (% E_{24}@pH6)

According to the USDA Natural Resources Conservation Service (USDA, 2001) and Department of Natural Resources and Mines of Queensland (QNRM, 2006), most soils have pH values between 3.5 and 10. Typically, this pH range changes to between 5 and 7 during rainfall, whereas in the dry season the range is from 6 to 9. It has been reported that most soil microbes thrive in a slightly acidic pH range (6 to 7) because of the high bioavailability of nutrients in that pH range (Sylvia *et al.*, 2005, Das *et al.*, 2007). Seasonal variations in soil moisture, temperature, and plant growth usually cause changes in soil pH and salinity as well as microbial activities, such as, in this case, biosurfactant synthesis and continuous emulsification of environmental contaminants.

Figure 4.4c shows the variability and stability of emulsions at different temperatures. The emulsions formed at normal pH (i.e., pH 6) and salinity (i.e., 6% w/v) were subjected to various temperatures. It was observed that temperature plays a major role in emulsion stabilization. Emulsion stability decreased at higher temperatures by affecting the physical properties of oil, water, interfacial films, and surfactant solubility in the oil and water phases. For the salinity stability test, which was demonstrated by *n*-hexane emulsification, the E_{24} value decreased significantly and approached zero as the temperature increased. This was due to the high volatility of *n*-hexane; the interfacial films around the n-hexane droplets coalesced, leading to the de-emulsification of hexane. In fact, about 33% of the hexane evaporated at 50 °C. Lubricant oil emulsion, on the other hand, was relatively stable owing to the low volatility of the oil, but could also decrease significantly if the temperature was increased further. According to the USDA (2001), typical soil temperature ranges from 20 to 50 °C throughout the year. This suggests one of the reasons for seasonal variation in the accumulation and bioavailability of environmental contaminants and their biodegradation (Nedwell, 1999, Coulon *et al.*, 2005). The effect of salinity on emulsion stability may not be severe, except in sites that are prone to erosion, leachate sinks, or areas that are erosion product repositories, such as sediments. Such environments have higher saline content, but typical soils generally have a neutral salinity (USDA, 2001, QNRM, 2006).

4.4.5 FTIR analysis of biosurfactant produced by *B. licheniformis* STK 01 grown on *Beta vulgaris*

The biosurfactant produced by *B. licheniformis* STK 01 grown on *Beta vulgaris* was subjected to further characterization by FTIR, having demonstrated highest surface tension reduction and emulsification for the hydrocarbons used in this study. The IR spectrum of the biosurfactant showed strong absorption bands, elucidating the presence of peptide components at 3368 cm^{-1} for the biosurfactant produced, compared with 3309 cm^{-1} for commercial surfactin, which emanated from the bond-stretching of NH.

Figures 4.5 and 4.6 show the translucent disks and the chromatograms of the biosurfactant produced, respectively.

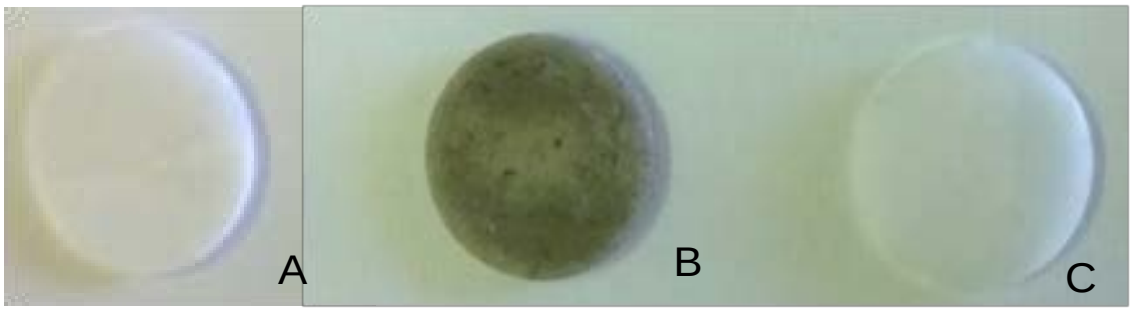


Figure 4.5: Pictures of thin wafer used for FTIR analysis: A – potassium bromide (KBr) pellet used as blank; B – combination of biosurfactant extract obtained from *Beta vulgaris* with KBr; C – standard surfactin from Sigma-Aldrich with KBr

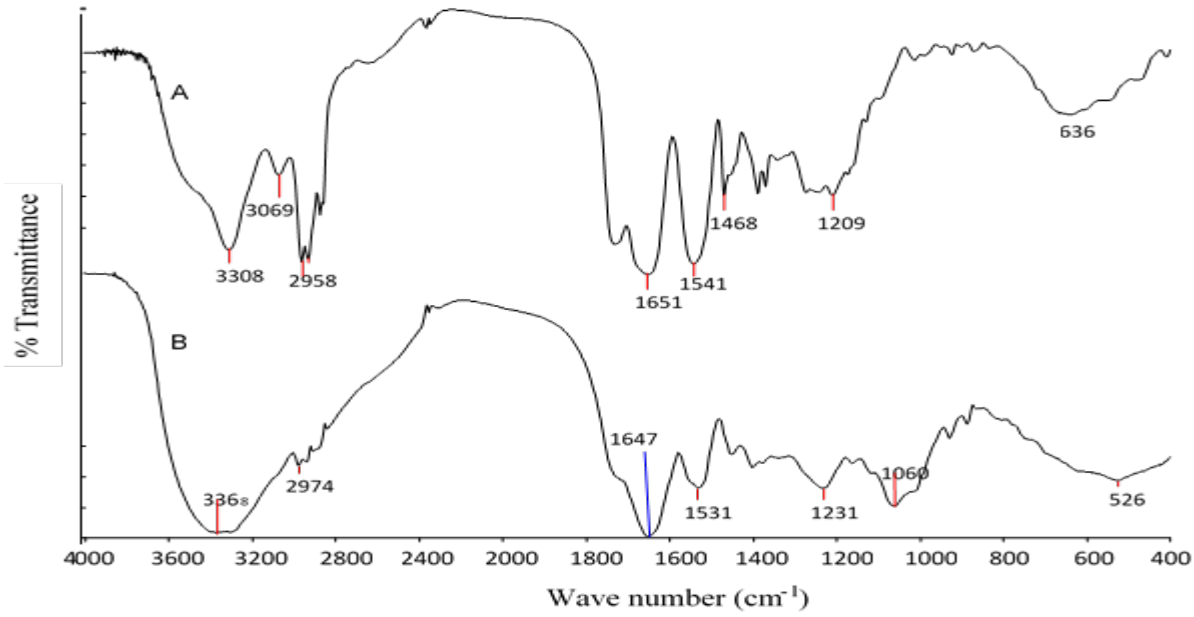


Figure 4.6: FTIR of commercial surfactin (A) and crude biosurfactant (B) produced by *Bacillus licheniformis* STK 01, grown exclusively on *Beta vulgaris*

The presence of CO and CN bonds in the two samples is signified by wave numbers 1651 to 1531 cm^{-1}. Also, the presence of an aliphatic group was observed at 3000 to 2850 cm^{-1} for CH_2 and CH_3, indicating that the biosurfactant produced is a lipopeptide. A carbonyl moiety at 1731 cm^{-1} was observed in the commercial surfactin, but was not conspicuous in the produced biosurfactant. Similar results have been reported in previous studies (Das and Mukherjee, 2007b, Oliveira *et al.*, 2013). The commercial surfactin used (98% pure) was obtained from Sigma-Aldrich.

4.5 Summary

This study revealed that exclusive utilization of solid agrowaste for microbial growth and effective biosurfactant production is feasible and has promising application with a view to enhancing the bioavailability and bioremediation of recalcitrant environmental contaminants. Among the agrowastes screened for biosurfactant production in this study, *Beta vulgaris* proved to be the most suitable substrate; the biosurfactants produced by the three bacterial isolates – *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 – were able to lower the surface tension of the culture medium to 30.0, 32.98, and 30.37 mN m^{-1}, respectively. These surface tension reductions exemplified the suitability of using microbial isolates supported exclusively on agrowaste for biosurfactant production.

The FTIR analysis of the biosurfactant produced by *B. licheniformis* STK 01, which demonstrated highest surface tension reduction and emulsification tendency for the hydrocarbons used in this study, indicated that the biosurfactant produced might be a lipopeptide. The emulsification of heavy hydrocarbons and environmental contaminants by the produced biosurfactants suggests the potential application of the isolates in utilizing cheap agrowaste for biosurfactant production, as well as their application for bioremediation of hydrophobic contaminants in the environment. Investigation of the emulsion formation and stability showed that the highest emulsification occurred at a pH range of 6 to 7 and 6 to 7 % w/v salt concentration. These have been reported to be the ranges of these parameters in typical environmental soils (USDA, 2001). This further suggests the suitability of the agrowaste with the isolated microorganisms for continuous bioavailability of environmental contaminants for *in-situ* bioremediation. The study also showed how severe temperature variation can reduce emulsion stability, particularly for hydrocarbons with relatively high volatility, thus explaining one of the reasons for seasonal variation in the accumulation, bioavailability, and biodegradation of hydrocarbon contaminants in the environment.

The next chapter focuses on the optimization of culture parameters for the optimum yield of biosurfactant by *B. licheniformis* STK 01, while the microorganism is grown on *B. vulgaris*, which was identified as the most suitable agrowaste in this chapter

Chapter 5

OPTIMIZATION OF BIOSURFACTANT PRODUCTION BY *B. LICHENIFORMIS* STK 01 GROWN EXCLUSIVELY ON BETA VULGARIS WASTE USING RESPONSE SURFACE METHODOLOGY

5.1 Introduction[3]

The application of crude biosurfactants has been reported for environmental remediation processes and enhanced oil recovery from storage tanks and capillaries (Mukherjee *et al.*, 2008, Mutalik *et al.*, 2008, Amodu *et al.*, 2013), implying that purification-related costs can be circumvented in such applications of the bioproduct. Furthermore, the process can become more economically viable when appropriate renewable resources are identified that will serve as exclusive sources of nutrients for microbial growth and synthesis of the requisite metabolites. Considering all cost-determining variables in biosurfactant production, the choice of suitable, low-cost raw materials can account for 10 to 30% of the overall cost (Cameotra and Makkar, 1998, Makkar and Cameotra, 2002). Therefore, the utilization of agrowaste, as well as suitable microorganisms under optimized conditions, can significantly increase biosurfactant yield, thereby enhancing the economic viability of the large-scale production of crude biosurfactants for the bioremediation of environmental contaminants.

[3] ***Published as an articles in BioResources 9 (3), 5045 - 5065***

Surface tension reductions in the range of 27 to 35 mN/m have been reported for the biosurfactants produced, as well as emulsification activity in the range of 20 to 75% for various hydrocarbon compounds (Fox and Bala, 2000, Nitschke and Pastore, 2006, Barros *et al.*, 2008, Oliveira *et al.*, 2013). Although the biosurfactants produced from these renewable substrates, often supplemented with refined nutrients, have been shown to be effective, identification of renewable substrates that can be used exclusively is expedient for the sustainability of the process. Yet, exclusive utilization of renewable resources, particularly solid wastes, for biosurfactant production, is rarely reported.

In addition, optimization of nutritional as well as cultivation parameters for enhanced biosurfactant production, either by direct quantification of biosurfactant produced or relative quantities such as the critical micelle dilution, is well reported in the literature (Ilori *et al.*, 2005, Rodrigues *et al.*, 2006c, Joshi *et al.*, 2008, Najafi *et al.*, 2010, Zhu *et al.*, 2013). These parameters include pH, temperature, agitation, inoculum size, cultivation time, oxygen availability, substrate concentration, and composition. Using design of experiment (DoE), the most influential parameters can be obtained or specified heuristically in order to reduce the number of variables. Interestingly, the response surface methodology (RSM) used in this study offers a statistical design of experiments to assess influential parameters that ultimately lead to peak process performance and the discovery of optimum conditions at a minimal cost. The use of a suitable substrate, in this case agrowaste, is inherently dependent on the amount of free and usable sugars, including trace elements, available for microbial growth and the expression of the biosurfactants. This study will most likely present the first report on the exclusive utilization of solid agrowaste as substrate for biosurfactant production.

5.2 Objective

The focus of this chapter is the optimization of culture conditions (temperature, pH, and substrate concentration) to enhance the surface activity of the biosurfactant produced by *B. licheniformis* grown exclusively on *B. vulgaris*. The production of an effective biosurfactant from appropriate agrowaste, such as those that do not require supplementation with refined substrates, could ensure the sustainable and economical production of biosurfactant, thus finding an application in the bioremediation of environments polluted with hydrocarbon contaminants.

5.3 Material and methods

Bacillus licheniformis STK 01 was maintained on nutrient agar slants at 4 °C and sub-cultured every three weeks as mentioned earlier. Inoculum preparation procedure is as shown in Chapter 3. 250 mL Erlenmeyer flasks containing 100 mL of the culture media were inoculated with 8% (v/v) of the 10^8 CFU mL^{-1}, and incubated in an orbital shaking incubator at 150 rpm at various temperatures, and at concentrations specified for *B. vulgaris* waste in Table 5.1, for a maximum of 240 h. Samples were taken after 120 and 240 h to assay the surface activity of the biosurfactants produced. An uninoculated culture of *B. vulgaris* served as the control. Surface tension and emulsification were determined as reported in Chapter 3.

5.3.1 Response surface methodology (RSM): central composite design experiments

RSM offers a statistical design of experiments to assess influential parameters that ultimately lead to peak process performance and the discovery of optimum conditions at a minimal cost. Central composite experimental design (CCD) was applied in this study for evaluating three variables, substrate concentration, pH, and temperature, allowing a minimum number of experimental runs for determining the optimum fermentation conditions for maximizing biosurfactant production and thus the surface activity of the broth. The ranges of the variables were specified based on optimum values reported for most bacteria, considering the lowest and highest values possible (Guerra-Santos *et al.*, 1984, Powalla *et al.*, 1989, Abushady *et al.*, 2005). The experimental design was generated using Design-Expert® software version 6.0.8 (Stat-Ease Inc., USA). Each variable was analysed at five levels coded as −α, −1, 0, +1, and +α representing core factorial, centre, and axial points. A set of 20 runs was carried out consisting of the following: a 2^k complete factorial design (where k = 3, i.e., the number of the test variables), six axial points representing two outlier points on the axis of each variable at a distance of + α from the high level (+1) and − α from the low level (−1), which equals $2^{k/4}$ (i.e., 1.68 for k = 3) and six centre points (level 0). Each sample was inoculated with 8% (v/v) of 10^8 CFU mL^{-1}, while the uninoculated broth served as a control at various specified temperatures. The pH of the samples was adjusted accordingly with the addition of 1 M NaOH or 1 M HCl. The results presented were three replicate measurements from two flasks.

5.3.2 Statistical analysis and modelling

Suitable statistical models were chosen to model the interactions among the different experimental variables and their effect on surface tension reduction by the biosurfactants produced, based on the Sequential Model Sum of Squares and a Lack-of-Fit Test. The response, measured after 120 and 240-h incubation periods, was modelled with an overall mean and a response surface quadratic model, respectively.

The results obtained after 240 h of incubation gave both the highest surface tension reduction and better statistical fitness, and were therefore subjected to further analysis by Analysis of Variance (ANOVA) to assess the significance of each variable on the surface activity of the biosurfactants produced. An empirical model that could relate the response measured to the independent variables was obtained using multiple regression analysis. The response (*Y*), after 240 h of the fermentation system, can be represented by the following quadratic model:

$$Y = \alpha_o + \sum_{i=1}^{n} \alpha_i X_i + \sum_{i=1}^{n} \alpha_{ii} X^2{}_i + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \alpha_{ij} X_i X_j + \varepsilon \quad (5.1)$$

where X_1, X_2, X_3, …, X_n are the independent coded variables, α_0 is the offset term, and α_i, α_{ii}, and α_{ij} account for the linear, squared, and interaction effects, respectively, and ε is the random error.

However, a model reduction may be expedient if there are many redundant model terms, excluding those required to support hierarchy such as α_1, α_2, and α_3. Statistical properties of the model were further analysed with the normal probability and the externally studentized plots to validate the normality of the residuals and the influential terms.

Table 5.1. The various media components included in CCD experiments and their corresponding high, medium, and low concentration levels

Variables	Code	Units	High levels (+1)	Medium levels (0)	Low levels (−1)
Substrates Conc.	*A*	% (w/v)	8.00	6.00	4.00
pH	*B*	-	8.00	7.25	6.50
Temperature	*C*	°C	42.00	37.00	32.00

Run	*A*	*B*	*C*	Surface Tension (mN/m)		
				120 h	240 h (Experimental value)	240 h (Predicted value)
1	0	0	0	38.60	49.80	49.95
2	0	0	0	38.60	49.80	49.95
3	+1	−1	−1	42.58	42.70	39.54
4	0	0	0	38.60	49.80	49.95
5	+1	+1	+1	41.40	33.16	35.42
6	0	0	0	38.60	49.80	49.95
7	+1	−1	+1	35.30	34.00	33.17
8	0	0	+ α	33.82	31.62	28.62
9	0	0	−α	37.46	37.43	35.14
10	+ α	0	0	40.50	41.57	41.99
11	−α	0	0	40.27	45.20	39.44
12	0	+ α	0	47.27	48.90	40.16
13	0	− α	0	33.90	32.00	35.45
14	−1	−1	−1	34.32	33.48	34.00
15	0	0	0	38.60	49.80	49.95
16	−1	−1	+1	33.48	31.00	30.75
17	−1	+1	+1	32.11	30.00	36.91
18	+1	+1	−1	35.14	34.98	38.96
19	−1	+1	−1	34.26	33.73	38.31
20	0	0	0	38.60	49.80	49.95

Table 5.2. Central composite experimental design for three variables and the corresponding responses

A, *B*, and *C* represent the coded level of variables; α represents the axial point with a coded level of 1.68

5.4 Results and discussion

5.4.1 Central composite experimental design

To determine the optimum components of culture medium to maximize effective biosurfactant production, three operational parameters were studied for their individual as well as their interactive effects, using RSM (Table 5.1). A total of 20 experimental runs were carried out as generated by the Design-Expert® software. The results presented in Table 5.2 showed stochastic variations in the responses measured, suggesting the effects of the various culture components on microbial activities. There was considerable surface tension reduction of broth to 30, 31, 31.62, and 32 mN m^{-1}, the lowest observed for runs 17, 16, 8, and 13, respectively. This corresponds to a reduction of 20 mN m^{-1} relative to the control (uninoculated broth). Comparing the responses measured after 120 h and 240 h of fermentation, the surface tension values were lower for some runs for the fermentation period of 240 h, which was expected, since the solid residues of the substrate were not removed, and consequently the bound sugars were released continuously to sustain microbial growth and biosurfactant production.

However, for some runs, the surface tension values measured for the 240 h fermentation were higher than those measured after 120 h. This scenario, where the surface activity of biosurfactant decreases with time owing to variations of the system parameters, has been observed previously (Das and Mukherjee, 2007b, Oliveira *et al.*, 2013). It is akin to enzyme deactivation after long use or subjection to unfavourable conditions. Biosurfactants are produced extracellularly and the process has been reported to be growth dependent (Lin *et al.*, 1998, Sahoo *et al.*, 2011). Meanwhile, the surface activities are dependent on thermodynamic properties such as temperature and biosurfactant concentration. Deactivation of biosurfactants by stationary-phase cultures was observed for *B. licheniformis* KGL 11 grown in a mineral salt medium supplemented with refined glucose (Lin *et al.*, 1998). This could be a result of changes in metabolic activities as the biosurfactant production process goes through different stages of microbial growth. It can be explained that during cell lysis, which occurs owing to prolonged incubation and probably after the CMC was reached, the concentration of biosurfactants increased considerably, forming more micelles. Chapter 6 discusses the kinetics of biosurfactant production from *B. vulgaris* by this novel strain i.e., *B. licheniformis*, where this scenario is further explicated.

A closer look at the results presented in Table 5.2 shows that the highest surface tension reduction (Runs 17, 16, and 8) occurred at temperatures of 42 and 45.4 °C and substrate concentrations of 4 and 6% (w/v), suggesting that the isolated strain might be an extreme mesophile. Conversely, the supernatants produced from central points, at the extreme high pH, and at the extreme low substrate

concentration (Runs 1, 11, and 12), did not show any biosurfactant activity. The interactive effects of the studied variables are better depicted with graphical representation of the response surface model discussed in Section 5.3.3.

Because the production of biosurfactant is growth dependent, often at the exponential growth phase (Lin *et al.*, 1998, Reis *et al.*, 2004, Sahoo *et al.*, 2011, Oliveira *et al.*, 2013), it can be deduced that the optimum effective biosurfactant production corresponds to the highest surfactant activity at the broth-air interface, just before the onset of CMC. This in turn corresponds to the highest surface tension reduction. The reduction in the surface tension of the culture broth to 30 mN m^{-1} showed the potency of the isolated strain in using unconventional substrates for biosurfactant production. Some researchers have tried to benchmark an effective biosurfactant by its ability to lower the surface tension of water below 35 mN m^{-1} (Mulligan, 2005, Costa *et al.*, 2006, Gudiña *et al.*, 2010). Several studies have demonstrated surface tension reduction similar to the results obtained in this study. Queiroga *et al.* (2003), investigating the ability of 13 microbial strains to produce biosurfactant using glycerol and glucose as carbon sources, observed a surface tension reduction of the medium to 30 mN m^{-1}. Recently, Oliveira *et al.* (2013) showed a surface tension reduction of the fermented broth medium to 30 mN m^{-1} by the biosurfactant produced by a *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice supplemented with $(NH_4)_2SO_4$ as a nitrogen source.

Furthermore, Barros *et al.* (2008) reported that the biosurfactant produced by *Bacillus subtilis* LB5, grown in cassava wastewater, reduced the surface tension of water from 72.31 to 27.01 mN m^{-1}. Nitschke and Pastore (2006), working with the same strain of *Bacillus subtilis* grown in cassava wastewater, reported a surface tension reduction of the medium to 26.6 mN m^{-1} by the biosurfactant produced. However, it may be inexpedient to compare surface tension reduction values as stated here, bearing in mind that the onus should be on producing effective biosurfactants, not only in their surface activities, but also in terms of cost and sustainability. The media containing the biosurfactants, the extent of purification involved, as well as the substrates used, whether refined sugars or renewable resources with or without supplementation, should form the basis of comparison. The agrowaste used as a substrate in this study proved to be effective, giving results comparable to those obtained for refined substrates (Joshi *et al.*, 2008, Wang *et al.*, 2011, Sousa *et al.*, 2012).

5.4.2 Statistical model analysis and validation

The statistical model summary based on the Sequential Model Sum of Squares and Lack-of-Fit Test elucidated the fitness of mean and quadratic models for the responses measured after 120 and 240

h fermentation periods, respectively. Moreover, the data obtained for the 240 h fermentation were optimized, having given the highest surface tension reduction and better statistical model fitness. Using ANOVA to assess the significance of each variable in the model, an empirical quadratic model was obtained from Eq. 5.1 that could relate the surface tension of the biosurfactant measured to the independent variables.

Table 5.3: ANOVA for response surface quadratic model

Model Coeff.	Coeff. Estimate	DF	Standard Error	95% CI Low	95% CI High	F Value	Prob > F	Significance
α_0	49.95	1	1.97	45.51	54.39	4.47	0.0043	**
α_1	0.77	1	1.32	−2.18	3.72	0.34	0.5731	NS
α_2	1.40	1	1.32	−1.55	4.35	1.12	0.3150	NS
α_3	−1.94	1	1.32	−4.89	1.01	2.15	0.1732	NS
α_{11}	−3.26	1	1.29	−6.13	−0.39	6.40	0.0299	*
α_{22}	−4.30	1	1.29	−7.17	−1.43	11.13	0.0075	**
α_{33}	−6.39	1	1.29	−9.26	−3.52	24.63	0.0006	***
α_{12}	−0.98	1	1.73	−4.83	2.88	0.32	0.5847	NS
α_{13}	−0.54	1	1.73	−4.39	3.31	0.097	0.7617	NS
α_{23}	+0.70	1	1.73	−3.15	4.56	0.17	0.6925	NS

(***): significant at level 99.99%; (**); significant at level > 99%; (*); significant at level 95%; NS = Not significant; CL = Confidence level; DF = Degree of freedom; Values of "Prob > F" less than 0.05 indicate model terms are significant, while values greater than 0.1 indicate the model terms are not significant.

The predicted response (*Y*) for the fermentation system was as follows:

$$Y = 49.95 + 0.77A + 1.40B - 1.94C - 3.26A^2 - 4.30B^2$$

$$-6.39C^2 - 0.98AB - 0.54AC + 0.70BC \quad (5.3)$$

where *A*, *B*, and *C* are the coded values for substrate concentration, pH, and temperature, respectively. Statistical analysis performed to determine and quantify the interactive effects of the coefficient in predicting the surface tension reduction of the biosurfactant showed a stochastic variation (Eq. 5.2). The interaction coefficients were estimated by taking the average of the two confidence levels (Table 5.3). By considering coefficients with significant effects, Eq. 5.3 can be reduced to the following:

$$Y = 50 - 2.71A^2 - 3.91B^2 - 6.72C^2 \quad (5.4)$$

Statistically, a model reduction may be appropriate for improving the model if there are more redundant model terms than the significant ones, excluding those required to support hierarchy. A model reduction was observed to enhance the fitness of the experimental data. The ANOVA of the quadratic regression model for the surface activity of the biosurfactant showed that the model was significant at the 99.8% level (Table 5.3), implying that the total variance in the response could be explained using this model. The Model F-value of 6.54 also enhances the significance of the model. There was only a 0.2% chance that a model F-value this large could occur because of noise. Adequate precision measures the signal-to-noise ratio, and a ratio greater than 4 is desirable. Thus, the adequate precision ratio of 7.802 observed in this study indicated an adequate signal, further suggesting that this model can be applied to navigate the design space. The coefficient of variation value (CV% = 11.8) equally underscored the precision and reliability of the model.

Table 5.4: ANOVA for surface tension reduction by biosurfactant in CCD

Source of variation	Sum of squares	DF	Mean sq	F-value	Significance
Regression	961.19	9	106.80	4.47	**
Residual	239.02	10	23.90		
Lack of fit	239.02	5	47.80	0.000	***
Pure error	0.000	5	0.000		
Total	1200.00	19			

Std. Dev. = 4.89; C.V = 12%; R^2 = 0.8008; Adj R^2 = 0.6216; Pred R^2 = 0.5208; DF = Degree of freedom

The calculated value of the coefficient of determination (R^2 = 0.8044) implies that at least 80% of the variability in the actual and predicted values can be explained by the model. The non-significance of the F-value of the Lack-of-Fit Test (Table 5.4) also showed the strength of the model for the experimental data. The diagnostic details of the model, using studentized residual (Fig. 5.1), indicated normality in the error term, further justifying the fitness of the model.

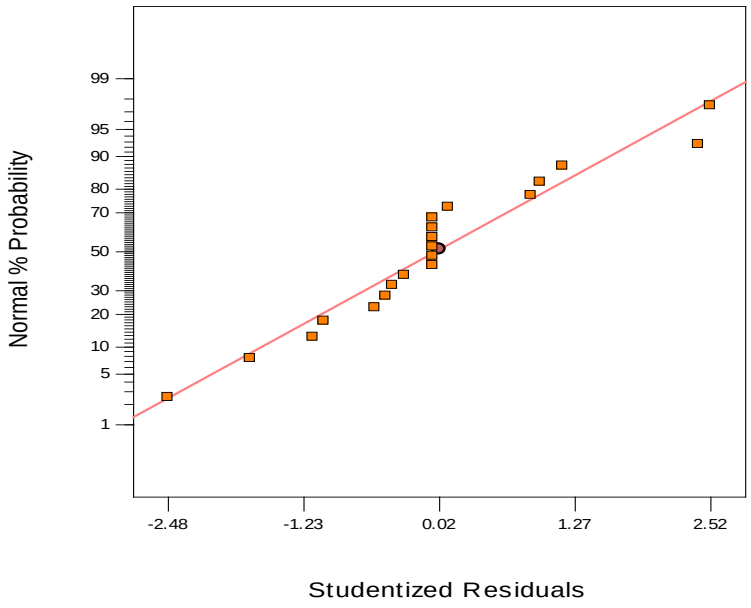


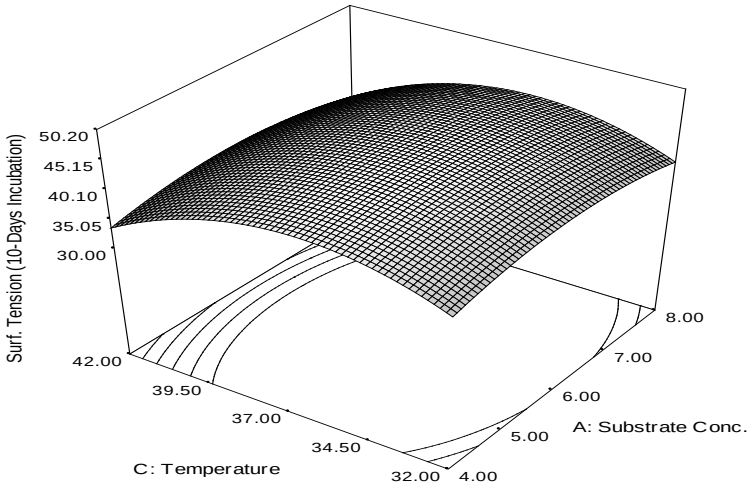
Figure 5.1: Normal probability plot of the residuals

A high degree of correlation was observed between the experimental and predicted values that showed the accuracy and applicability of the model for predicting biosurfactant production. One unique aspect of this study is that it is possible to predict the optimum at which the biosurfactant produced will be most active in lowering the surface tension.

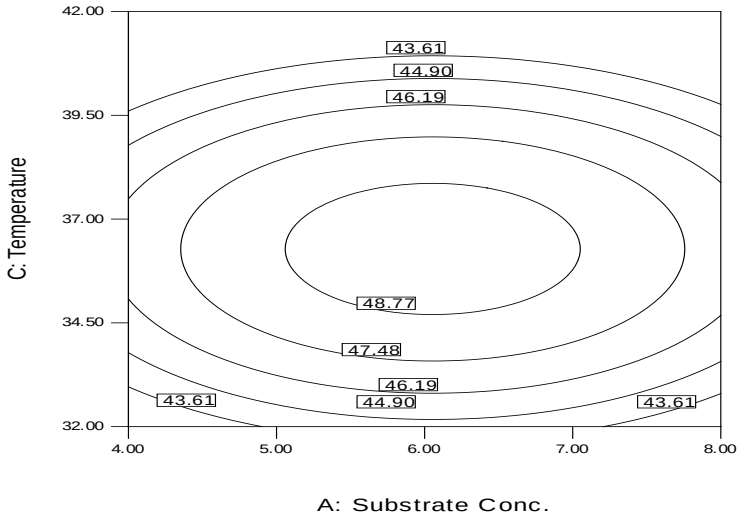
5.4.3 Graphical representation of the response surface model

The interactive effects of the independent variables on the system's response were investigated by plotting three-dimensional curves of the response against any two of the variables while keeping the third constant (Fig. 5.2). Such response surface plots allow for easy interpretation of experimental results and the prediction of optimal conditions. The 3-D and contour plots can be used to determine

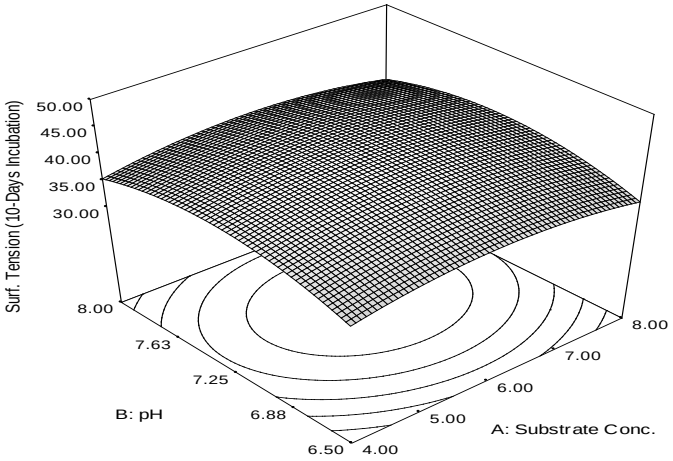
the level of interaction between the independent variables. An elliptical contour shape shows a perfect interaction between the two independent variable plots while a circular contour shows a non-interactive effect on the system response (Khuri and Cornell, 1996, Montgomery, 2008). The response contour plots showed ellipses for all the variable pairs plotted in Fig. 5.2, with Fig. 5.2b and Fig. 5.2f showing complete interactions.



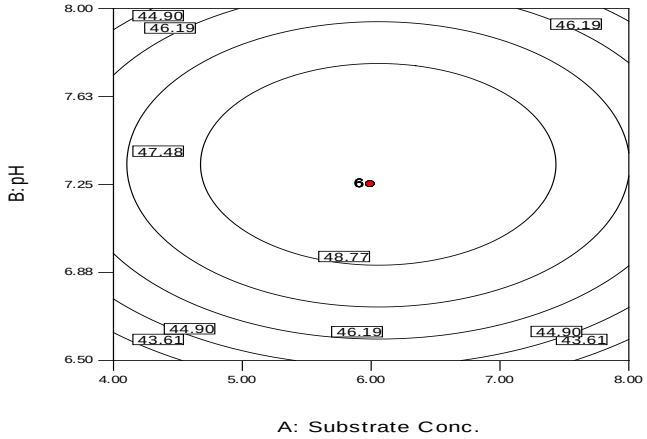
(a)



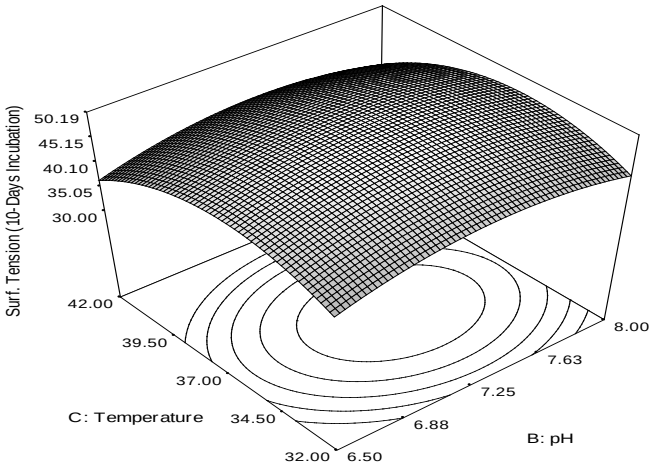
(b)


Surf. Tension (10-Days Incubation)
50.00
45.00
40.00
35.00
30.00
8.00
7.63
7.25
6.88
6.50
B: pH
4.00
5.00
6.00
7.00
8.00
A: Substrate Conc.

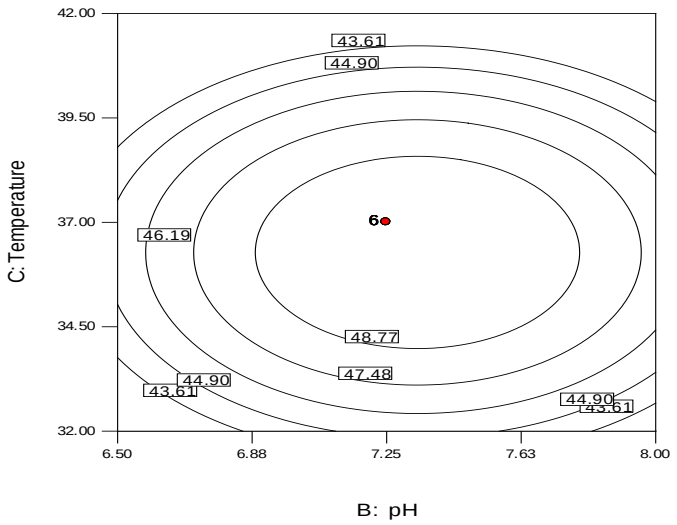
(c)


8.00
7.63
7.25
6.88
6.50
B: pH
44.90
46.19
46.19
47.48
48.77
44.90
43.61
46.19
44.90
43.61
4.00
5.00
6.00
7.00
8.00
A: Substrate Conc.

(d)


Surf. Tension (10-Days Incubation)
50.19
45.15
40.10
35.05
30.00
42.00
39.50
37.00
34.50
32.00
C: Temperature
6.50
6.88
7.25
7.63
8.00
B: pH

(e)



(f)

Figure 5.2**:** 3-D plots a, c, and e and contour plots b, d, and f show the interactive effects of the independent variables on the effectiveness of the biosurfactant produced

It was observed from the responses measured that substrate concentration and temperature played more significant roles as seen in the experimental runs at temperatures 42 and 45 °C and at substrate concentrations of 2 and 4% (w/v). In Fig. 5.2d, the contour plot was not perfectly elliptical, indicating fewer interactions between the independent variables. The composition and quantity of the substrate have been identified as the most important factors affecting the production of biosurfactant (Joshi *et al.*, 2007, Das *et al.*, 2009). The results of this study supported that observation.

5.4.4 Process optimization

The optimization of the response in this study was carried out by the numerical option of the Design-Expert® software, in which the input factors were combined to achieve peak process performance. In the numerical optimization process, the desired goal for each process variable and response is selected. The weight or importance can give more or less emphasis on an individual goal relative to the others.

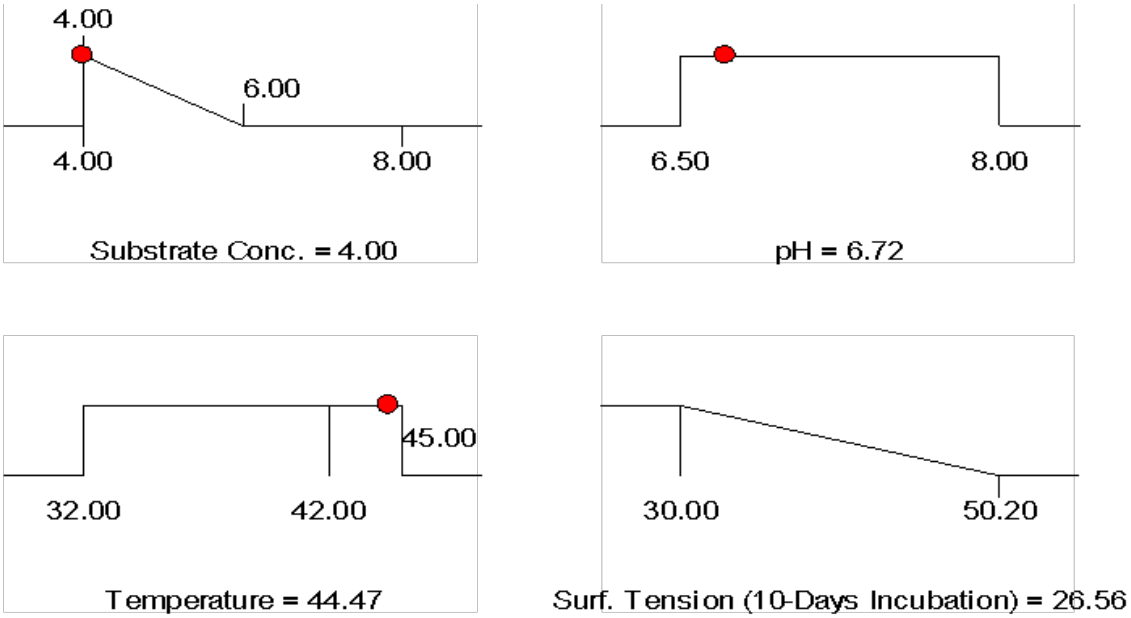


Figure 5.3: Desirability ramp for the numerical optimization of three independent variables: substrate concentration, pH, and temperature

The input variables can be set to maximize, minimize, target, within range, or none, while the response is often set to minimum or maximum. In this analysis, substrate concentration was set at target; pH and temperature were set within range. The response was set at minimum, since the desirable optimum is the combination of the independent variables that will give the maximum reduction in surface tension. Design-Expert® software searches for and lists solutions to match the set criteria from the most to the least desirable – desirability ranges from zero (i.e., at least one goal was unachievable) to one (i.e., all goals were easily met). Fig. 5.3 shows the desirability ramp generated from 10 solutions via numerical optimization. The optimum point with the maximum desirability function was selected. Hence, the optimum condition for the maximum surface tension reduction of 26.56 mN m^{-1} was found to be at a pH of 6.72, a substrate concentration of 4% (w/v), and a temperature of 44.47 °C. The experiment conducted at these optimum conditions with the *B. licheniformis* STK 01 isolated, produced a biosurfactant which lowered the surface tension of broth to 30 mN m^{-1} when the organism was grown on *B. vulgaris*, and 23.5 mN m^{-1}, when grown on refined substrate. So far, this is the lowest ever reported for a biosurfactant, other than a study by Burgos-Diaz *et al.* (2011), where a surface

tension reduction to 22.0 mN m^{-1} was reported, in which case, the authors suspected the activity of a consortium.

5.5 Summary

The surface tension reduction of culture broth to 30 mN m^{-1} reported in this study suggests the novelty of the microbial isolate in its ability to utilize solid agrowaste for growth and biosurfactant production without supplementation with refined nutrients, yielding results comparable to those reported for refined substrates. No work has thus far been reported on the utilization of *B. vulgaris*, and most likely this study is the first to report the exclusive utilization of solid agrowaste for biosurfactant production. The analysis of response measured from the CCD using response surface plots identified substrate concentration and temperature as the most significant factors affecting biosurfactant production. By numerical optimization, the optimum conditions were found to be a pH of 6.72, a substrate concentration of 4% (w/v), and a temperature of 44.47 °C, under which a surface tension reduction to 26.56 mN m^{-1} was predicted. The experiment conducted to validate the optimum conditions specified by the RSM in this study showed remarkable results.

The biosurfactant produced on *B. vulgaris* within 96 h lowered the surface tension of the broth to 30 mN m^{-1}, while that which was produced on glucose, at the same optimum conditions, lowered the surface tension to 23.5 mN m^{-1} – this is one of the greatest surface tension reductions ever reported for a biosurfactant. The biosurfactant produced showed a high emulsification tendency for hydrocarbon, giving a 70% emulsification index for paraffin oil, which suggests its suitability for use as a bioemulsifier. The biosurfactants' ability to lower surface and/or interfacial tension, thereby enhancing the emulsification of hydrophobic compounds rather than the quantity produced, is often the measure of their effectiveness.

Thus, more attention should be focused on optimization of the surface activity of biosurfactants. Moreover, the cutting edge in this area of research appears to be the production of effective biosurfactants at a reasonable cost; the exclusive application of renewable substrates could also ensure the sustainability of the process, particularly for the enhanced bioremediation of environmental contaminants. This study has provided a basis for further investigation on the kinetics of biosurfactant production from *B. vulgaris* and possible large-scale fermentation for *B. licheninformis* STK 01 lichenysin production.

Since the optimum conditions for a high yield of biosurfactant have been obtained, it is therefore necessary to model the proliferation of *B. licheniformis* while being grown on *B. vulgaris*

substrate, and to be able to predict the consumption of the substrate and biosurfactant production using suitable mathematical models. This is discussed in Chapter 6.

Chapter 6

KINETIC MODELLING OF CELLULAR GROWTH, SUBSTRATE UTILIZATION, AND BIOSURFACTANT PRODUCTION FROM *BETA VULGARIS-BACILLUS LICHENIFORMIS* CULTURES

6.1 Introduction[4]

Typically, microbial culture preparation from agrowaste, mostly in microcosm studies, is such that the requisite nutrients are extracted from the agrowaste while the solid residue is discarded (Lin *et al.*, 1998, Reis *et al.*, 2004, Rodrigues *et al.*, 2006b, Sahoo *et al.*, 2011, Oliveira *et al.*, 2013). With the extracted nutrient readily bioavailable in aqueous phase, the substrate consumption kinetics often follow a decay curve pattern, which has been described by some existing models (Babu *et al.*, 1996, Rodrigues *et al.*, 2006a, Oliveira *et al.*, 2013). However, these kinetic studies may not represent cases often encountered in bioremediation of environmental contaminants, where most biological metabolic processes are nutrient limiting owing to the low bioavailability of the primary carbon source.

However, it has been shown in the previous chapters that such solid precipitates could sustain the microbial metabolism during biosurfactant production. An example is in composting of organic

[4] ***Submitted for publication in Biochemical Engineering Journal BEJ –D-15-00170***

waste for enhanced soil remediation (Wick *et al.*, 2011). In this case, the total sugar concentration of the culture medium, at any particular time, is the sum of the available sugar in the aqueous phase and the residual bound sugar, until the total nutrient is exhausted.

Although several studies have been reported on biosurfactant production, limited information exists on the kinetic modelling (Babu *et al.*, 1996), and it appears no model has been used to describe this scenario.

Moreover, the suitability of the existing models, such as the Monod equation and logistic models, that are often used to explain substrate kinetics with defined initial concentration, is not certain. Nonetheless, the kinetics of substrate utilization and biosurfactant synthesis, for such a system, is necessary for bioreactor design and control.

6.2 Objectives

The objectives of this chapter were: 1) to investigate the kinetics of cell growth, substrate utilization, and biosurfactant production, at the optimized conditions, while the microorganism is grown on *B. vulgaris* waste and in a mineral salt medium; and 2) to investigate the possibility of using polyurethane foam as a biocarrier for enhanced biosurfactant production. The view was to provide an understanding of the kinetics and to determine the kinetic parameters suited to the condition described above by fitting the kinetic data to available models, viz., Monod equation, logistic models, Leudeking–Piret model, and the logistic incorporated Leudeking–Piret model.

6.3 Materials and methods

The agrowaste (*Beta vulgaris*) culture was prepared as discussed in Chapter 3. Polyurethane biocarrier was milled, washed thrice with formalin (4%, v/v) and rinsed thoroughly with sterile water, then oven dried for 2 h at 65 °C. The sterilized biocarrier (1.6%, w/v) was added to the culture media – *B. vulgaris* (5%, w/v) and mineral salt (MS), containing 50 mL of culture media in 100 mL Erlenmeyer flasks. The cultures containing *B. vulgaris* were autoclaved at a temperature of 110 °C and 15 bars for 15 min, while the MS cultures were autoclaved under the same conditions but at 121 °C. The autoclaved cultures were allowed to cool to room temperature, then inoculated with 8% (v/v) of the overnight-grown culture of *B. licheniformis*, and incubated at 150 rpm and 44.5 °C for 192 h. The pH of the culture media was adjusted to 7 prior to autoclaving. The MS medium used was composed of, in gram per litre: *Lab-Lemco* powder 1.0; yeast extract 2.0; peptone 5.0; and sodium chloride 5.0 at pH 7.4 ± 0.2 at 25°C

(Oxoid, UK). Samples were taken periodically, while using destructive sampling mode, and assayed as described in Chapter 3. Each experiment was carried out in triplicates while uninoculated cultures served as control. The total reducing sugar content of *B. vulgaris* was quantified as described in Chapter 3, as well as the determination of biosurfactant concentration using a high-performance liquid chromatography (HPLC).

6.4 Kinetic models

6.4.1 Biomass growth kinetics

Cell proliferation of *B. licheniformis* on *Beta vulgaris* was mathematically represented by the logistic model which was proposed by Verhulst (1838), which is an improved Malthusian exponential model (Eq. 2.6).

$$\frac{dX}{dt} = \mu_o X\left(1 - \frac{X}{X_m}\right) \tag{2.6}$$

where μ_o represents the initial specific growth rate (h^{-1}); X_m is the maximum biomass concentration (g L^{-1}); and X is the biomass concentration (g L^{-1}). Recall Eq. 2.7:

$$X(t) = \frac{X_o X_m \ exp(\mu_0 \, t)}{X_m - X_o \ + \ X_o \, exp(\mu_o \, t)} \tag{2.7}$$

To extend the validity of the logistic model, a biomass growth-dependent inhibitory effect 'r', which accounts for the growth variation from the exponential correlation, was incorporated (Mulchandani *et al.*, 1988) as given in Eq. 2.11:

$$X_t = \frac{{X_m}^r \, exp(\mu_o r t)}{1 - \left(\frac{X_o}{X_m}\right)^r \, [(1 - exp(\mu_o r t)]^{-1}}$$

(2.11)

Complete inhibition of cell growth often occurs as *r* approaches zero, while the equation reduces to the logistic model when *r* equals to 1. But when *r* >1, the growth lies between exponential and logistic forms.

6.4.2 Biosurfactant production kinetics

An analogy of the logistic model for biomass growth, proposed by Mercier *et al.* (1992) was used in this study to model biosurfactant synthesis from *B. vulgaris* (recall Eq. 2.13):

$$P(t) = \frac{P_o P_m \ exp(P_r\, t)}{P_m - P_o \ + \ P_o \, exp(P_r\, t)} \tag{2.13}$$

where *P* is the biosurfactant concentration (g L^{-1}); P_m is the maximum biosurfactant production (g L^{-1}); r_p is the initial volumetric production rate (g L^{-1} h^{-1}); while P_o is the initial biosurfactant concentration (g L^{-1}).

A logistic incorporated Luedeking and Piret (1959) model, which relates cell growth to product formation, was also used in this study to predict biosurfactant production (Eq. 2.17):

$$P(t) = \ P_o + aX_o \left[\frac{exp(\mu_o t)}{1 - \left(\frac{X_o}{X_m}\right)(1 - exp(\mu_o t))} - 1 \right] + b\frac{X_m}{\mu_o} ln\left[1 - \ \frac{X_o}{X_m(1 - exp(\mu_o t))}\right] \tag{2.17}$$

where *a* and *b* are growth and non-growth product formation coefficients respectively.

6.4.3 Substrate utilization kinetics

Substrate consumption was modelled by the modified Monod, Leudeking–Piret, and modified Leudeking–Piret models as presented in Eqs 2.21, 2.25, and 2.27, respectively.

$$\frac{K_S Y + S_O Y + X_O}{Y S_O + X_O} \ln\left\{\frac{[Y(S_O - S_t) + X_O]}{X_O}\right\} - \frac{K_S Y}{(Y S_0 + X_O)} \ln\left({}^{S_t}/_{S_O}\right) = \mu_m t \quad (2.21)$$

$$S_t = S_O - \lambda X_o \left[\frac{exp(\mu_o t)}{1 - \left(\frac{X_o}{X_m}\right)(1 - exp(\mu_o t))} - 1\right] - \frac{\gamma X_m}{\mu_o}\left[1 - \frac{X_o}{X_m}(1 - exp(\mu_o t))\right] \quad (2.25)$$

$$S_t = S_o - \lambda\left[\left\{\frac{{X_o}^r \exp(\mu_o t)}{1 - \left(\frac{X_o}{{X_m}^r}\right)\frac{1}{(1 - \exp(\mu_o t))}}\right\}^{\frac{1}{r}} - {X_o}^r\right] - \frac{\gamma {X_m}^r}{\mu_o} \ln\left[1 - \frac{X_o}{{X_m}^r}(1 - \exp(\mu_o t))\right] \quad (2.27)$$

where $\lambda = Y_{s/p} a + Y_{S/X}$ and $\gamma = Y_{S/P}\, b + m$ (m is the cell maintenance factor); $Y_{x/s}$ is the yield coefficient (g biomass/g substrate); *Ks* is the saturation constant; S_o is the initial substrate concentration; while S_t is the limiting substrate concentration. Equation 2.21, which often gives the familiar sigmoidal curve for substrate depletion, cannot be solved explicitly for S_t. However, by numerical approximation, *S* was evaluated as a function of *t*.

6.5 Results and discussion

6.5.1 Cell proliferation and effect of biocarrier

The kinetics of cell growth, substrate utilization, and biosurfactant production was reported in this study, while the microorganism was grown exclusively on *B. vulgaris*, and in an MS medium. Figure 6.1 shows the growth curve of *B. licheniformis* STK 01 grown exclusively on *B. vulgaris*, and in MS. The trend of Fig. 6.1 is typical of a microbial growth pattern, showing lag, exponential, stationary, and death phases (Monod, 1949, Reis *et al.*, 2004). Microbial growth peaked after 24 h of lag phase and increased steadily until the peak of the exponential phase was reached in 72 h for all the experimental conditions (i.e., using *B. vulgaris* and MS, with and without biocarrier). Although the extent of lag phase may depend on several factors (such as culture conditions, type of substrate used, and the form in which the

sugar is present, the microorganism, and cell concentrations), the biocarrier appears to enhance the onset of the exponential phase (Fig. 6.1).

The use of polyurethane foam as a biocarrier is well reported for its ability to enhance microbial growth through creation of bio-balls and biofilm stabilization (Shim and Kawamoto, 2002, Lu *et al.*, 2010, Chu and Wang, 2011). However, the effect was not so conspicuous in this study, except with *B. vulgaris*, where the growth rate almost doubled for culture supported on the carrier, than in the MS medium. The maximum cell concentration was, on average, 2 x 10^{10} CFU mL^{-1}, while it went up to 3.6 x 10^{10} CFU mL^{-1} for the *B. vulgaris* culture supported on the carrier, showing the effect of biocarriers in cell proliferation (Fig. 6.1). Some other biocarriers as well as nutrient supplements have been reported to enhance cell growth (Fox and Bala, 2000, Reis *et al.*, 2004, Lu *et al.*, 2010, Chu and Wang, 2011). In biological systems, biocarriers are often used for cell immobilization, to provide supports for biofilm formation and stability, which subsequently lead to enhanced cell growth and metabolite formation. The optimum cell concentration was maintained up to 96 h, after which there was a death phase. The cell biomass yield in grams per grams of *B. vulgaris* substrate consumption ($Y_{X/S}$) was found to be 0.1012.

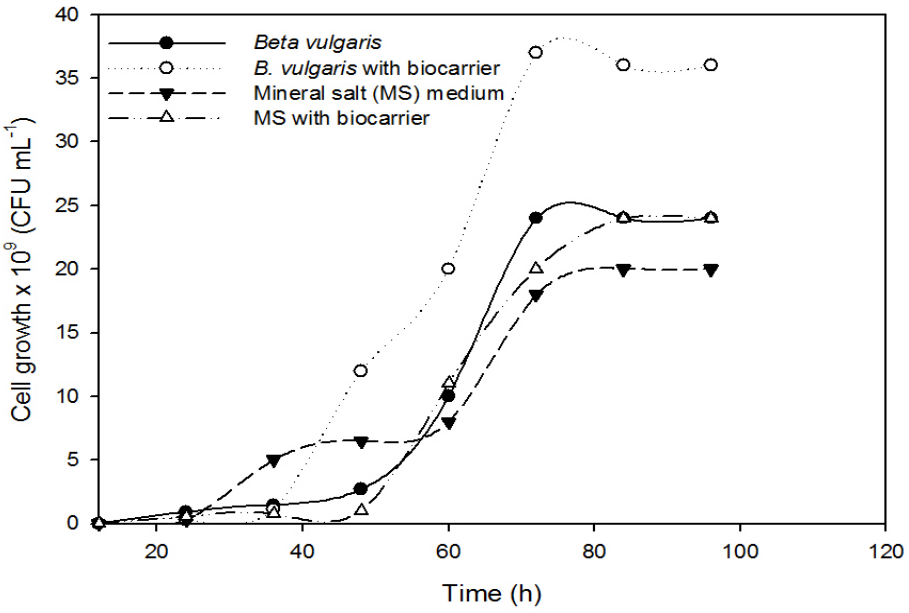


Figure 6.1: Biomass growth of *B. licheniformis* STK 01 on *B. vulgaris* and in mineral salt medium and the effects of polyurethane biocarrier

6.5.2 Biosurfactant production, surface tension reduction and critical micelles concentration (CMC)

In Figure 6.2, the onset of the exponential growth phase corresponds to the production of biosurfactant which increased gradually until equilibrium was reached after 40 h of fermentation with the *B. vulgaris* culture, while for the MS medium, it became equilibrated after 140 h for the culture with biocarrier, and after 160 h for MS without the carrier. With the agrowaste substrate – *B. vulgaris*, the highest biosurfactant production was 5.8 ± 0.5 g L^{-1} and 6.2 ± 0.04 g L^{-1} for the culture without and with the biocarrier, respectively. Similarly, for the MS medium, biosurfactant production rose to 9.78 ± 1.02 g L^{-1} and 8.04 ± 0.28 g L^{-1}, without and with the biocarrier, respectively. Such high yield of biosurfactant has been reported previously (Burgos-Diaz *et al.*, 2011, Luna *et al.*, 2013, Rufino *et al.*, 2014). Biosurfactant production is considered to be growth dependent, reaching the peak often at the mid-exponential phase or at the onset of the stationary growth phase (Lin *et al.*, 1998, Reis *et al.*, 2004, Sahoo *et al.*, 2011), as similarly observed in this study. Although the addition of polyurethane foam increased cell proliferation, it had no significant effect on biosurfactant production as shown by the errors associated with the mean values of triplicate analysis of biosurfactant concentration reported above. This study further showed its repressive effect in the case of the MS medium (Fig. 6.3) as reported by Reis *et al.* (2004), where the addition of carriers and a carbon source stimulated cell proliferation to the detriment of the required bioproduct.

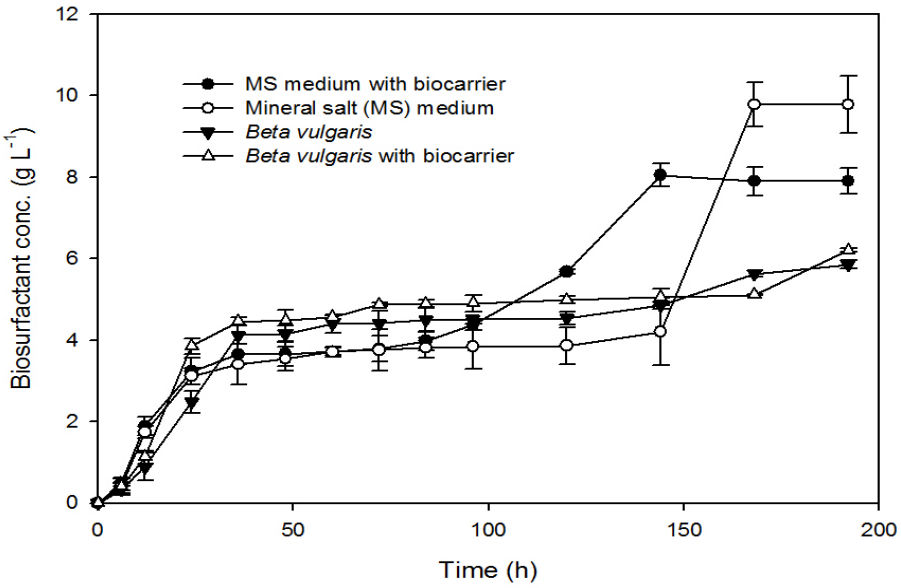


Figure 6.2: Biosurfactant production by *B. licheniformis* STK 01 grown on *B. vulgaris* and in a mineral salt medium

Given a microbial substrate in excess of carbon, microorganisms begin to accumulate biosurfactants when certain nutrients, particularly nitrogen, become limiting (Fox and Bala, 2000). Although the limitation of other requisite elements such as phosphorus, iron, calcium, and magnesium can induce such metabolite accumulation, the maximum biosurfactant production observed in this study might be due to nitrogen depletion that consequently increased the carbon-nitrogen ratio, and thus enhanced the rate of biosurfactant production. Furthermore, the induction of cell lysis can also contribute to increasing the quantity of biosurfactant that was released into the broth. After the equilibrium biosurfactant production, it was observed that a considerable amount of residual sugar was still remaining, showing that the fermentation process was not carbon limiting. In a similar study of biosurfactant production from whey fermentation (Rodrigues *et al.*, 2006a), a high sugar content was left at the end of the fermentation; the authors suggested that the *Lactobacillus* strain used metabolized medium nutrients other than the pentose sugar present in the whey.

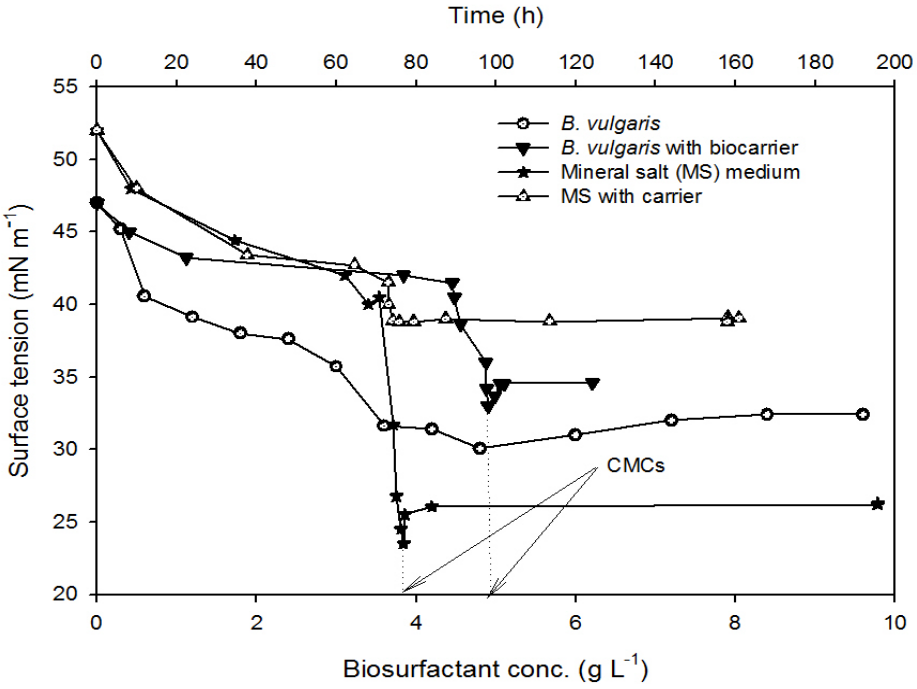


Figure 6.3: Surface activity and the critical micelle concentration (CMC) of biosurfactant produced by *B. licheniformis* STK 01 while grown on *B. vulgaris* and in mineral salt medium

The activity of the biosurfactants produced was evidenced in the surface tension of the culture broth. The biosurfactant produced lowered the surface tension of the *B. vulgaris* broth from 47 to below 30 mN m^{-1} after 72 h incubation, which is comparable with values reported when refined substrates or

agrowaste supplemented with refined substrates were used (Nitschke and Pastore, 2006, Makkar *et al.*, 2011, Oliveira *et al.*, 2013). Additionally, the biosurfactant produced when the microorganism was grown in the MS medium gave a further reduction of the broth surface tension to 23.5 mN m^{-1} (Fig. 6.3). This is the highest surface tension reduction reported, to the best of the author's knowledge, for a *Bacillus licheniformis* (Table 6.1). Although a reduction to 22.0 mN m^{-1} was reported by Burgos-Diaz *et al.* (2011), it was attributed to the activity of the microbial consortium. Table 6.1 shows some of the lowest surface tension values that have been reported to date for microbially produced surface-active agents.

Table 6.1: Some of the lowest surface tension to date reported for biosurfactants

Microorganisms	Isolation environment	Primary carbon source used	BS yield (g L^{-1})	ST reduction (mN m^{-1})	References
B. licheniformis STK 01	Rotten wood	Mineral salts	9.78	23.5	This study
Sphingobacterium sp. *6.2S*	Volcanic Soil	Mineral salts	9.6	22.0	(Burgos-Diaz *et al.*, 2011)
B. subtilis FE-2	n/r	Wheat bran	n/r	24.0	(Veenanadig *et al.*, 2000)
Candida sphaerica UCP0995	n/r	Ground nut oil residue + corn steep liquor	9.0	25.0	(Luna *et al.*, 2013)
B. Subtilis ATCC 21332	n/r	Cassava wastewater	2.2	25.9	(Nitschke and Pastore, 2004)
B. subtilis LB5	Wax apple orchard		3.0	26.6	
Candida sphaerica UCP0995	Mangrove sediment	Soybean oil residue + corn steep liquor	6.36	26.0	(Sobrinho *et al.*, 2013)
B. subtilis LAMI009	Wastewater treatment tank	Glycerol from biodiesel synthesis	0.27	27.0	(Sousa *et al.*, 2012)
B. licheniformis TT42	Tuva-Timba hot water spring	Mineral salts	n/r	28.0	(Nerurkar, 2012)
B. subtilis strain JA-1	Oil reservoir	Glucose	0.32	28.3	(Wang *et al.*, 2011)

BS – biosurfactant; *ST* – surface tension; n/r – not reported

The critical micelle concentration (CMC) is another important characteristic that is often used to assess the effectiveness of surfactants. It is the saturation concentration of surfactant that is required to achieve the highest surface tension reduction; any additional surfactant beyond this concentration may not result in reducing surface tension, but aggregate in the bulk of the solution to form a polymeric molecule known as the micelle. Nonetheless, beyond the CMC, the presence of micelle can lead to increased solubility. As shown in Fig. 6.3, biosurfactant concentration became equilibrated at 3.82 g L^{-1} and 4.49 g L^{-1}, respectively, when MS and *B. vulgaris* waste were used. The CMCs were attained at the onset of the stationary growth phase, i.e., after 72 h. Such high CMC values have been reported previously. Liu *et al.* (2010) reported a CMC value of 2.96 g L^{-1} for a biosurfactant synthesized by *Bacillus subtilis* CCTCC AB93108, while a value as high as 14 g L^{-1} has been reported by Rodrigues *et al.* (2006d) for a crude biosurfactant produced by *Lactococcus lactis* 53. However, a low CMC is usually preferred, which implies that a low concentration of surfactant can be used to achieve the highest surface activity.

6.5.3 Dynamics of substrate utilization in batch culture

With the bound sugar continually released into the aqueous phase for easy accessibility to the microorganism as the reaction progresses, the kinetic data shown in Fig. 6.4 was observed. Conventionally, the agrowaste is heated or autoclaved to release the sugar content into the aqueous phase while the residue is discarded after filtration (Lin *et al.*, 1998, Rodrigues *et al.*, 2006b, Oliveira *et al.*, 2013). In this study, both the sugar released into the aqueous phase after autoclaving and the solid residue were used for the microbial culture.

Fresh *B. vulgaris* in distilled water contains about 5.5 g of sugar per 100 g of *B. vulgaris*, and this value can increase to about 8.8 g per 100 g of *B. vulgaris*, or even more, if boiled for about 45 min (USDA, 2011). This suggests that by using only the filtrate for microbial fermentation, while the residue is being discarded, close to half of the total sugar content could be wasted. Based on the above information on the total reducible sugar content of *B. vulgaris*, sugar concentration in the experimental flask was determined to be 3.52 g L^{-1}, while the initial measured glucose concentration was 2.705 g L^{-1}.

The available sugar concentration continued to fluctuate with time as more residual sugar was being released into the aqueous phase, reaching peaks of 3.33 g L^{-1} and 3.52 g L^{-1}, after 96 h and 36 h fermentation period, for *B. vulgaris* culture without the biocarrier (*BR*) and *B. vulgaris* with the biocarrier (BR_C) respectively, before the death phase, as the substrate became exhausted. At every given time during the fermentation, the total sugar concentration is the sum of substrate consumed, the available substrate in the aqueous phase, and the residual sugar in the *B. vulgaris* residue.

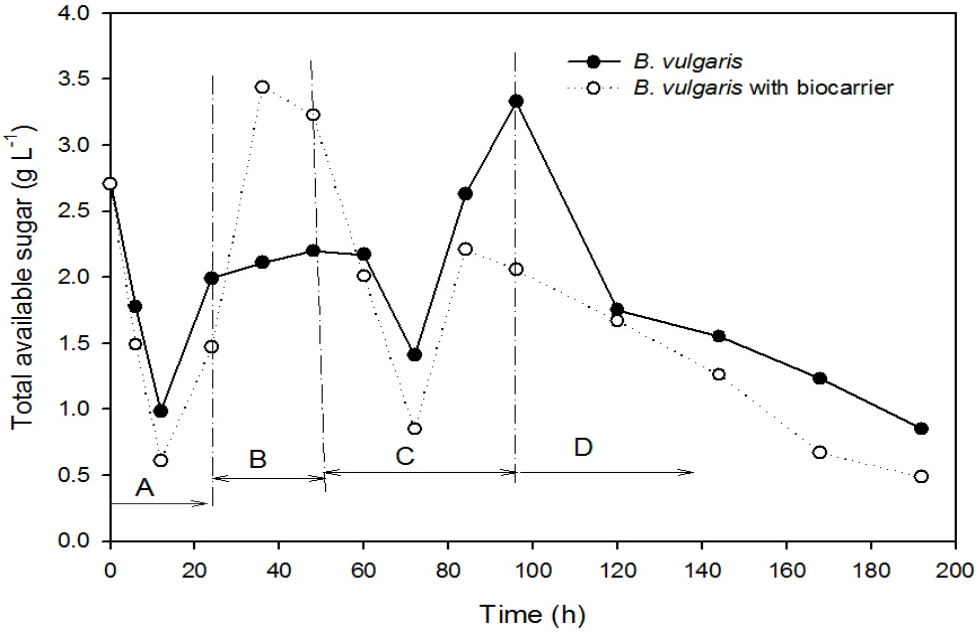


Figure 6.4: Substrate utilization kinetics by *B. licheniformis* STK 01 showing the corresponding cell growth phases – Lag phase: B - Pre-exponential phase; C - Exponential and stationary phase; D - Death phase

It was observed that the two overlapping lowest substrate availability after 72 h correspond to the maximum cell concentration, biosurfactant production, and the surface tension reduction. This showed a correlation between substrate consumption, cell growth, and product formation. The yield coefficient, $Y_{P/S}$, was found to be 0.459 g of biosurfactant per g of *B. vulgaris* consumed. In view of the continuous applications of solid agrowaste as substrates in biosurfactant production and in field studies of biodegradation of hydrocarbon contaminant, the *B. vulgaris* was used in this study to sustain microbial growth and biosurfactant formation.

6.5.4 Kinetic models

In this study, several models were examined for their fit for the experimental data, using the Solver routine in Microsoft Excel™ to minimize the sum of square errors (SSE) between the measured and predicted variables. The intention was to determine kinetic parameters of the model that best describe the experimental data based on the SSE and R^2 values. Figures 6.5, 6.6, and 6.7 show the fit of

relevant models that best described the microbial growth, biosurfactant production, and substrate utilization, respectively. The analysis of the microbial growth data showed that the classical logistic model (LM) can be used to describe the growth of *B. licheniformis* on *B. vulgaris* waste with good accuracy. The logistic model (LM) and its modified form (Eq. 2.11) were found to be valid in describing the different phases of growth, with the coefficient of correlation (R^2) being 0.9903 and 0.8511, respectively (Table 6.2). The models LM and MLM gave the maximum cell concentration of 24.99 and 22.86 x 10^9 CFU mL^{-1}, respectively, which are comparable with the experimental value of 24 x 10^9 CFU mL^{-1}. However, the models could not describe the death phase. This observation had actually been emphasized as one of the limitations of the logistic model; it is only found to be valid during the early and the exponential phases of cellular growth (Mulchandani *et al.*, 1988). In bioreactor design, death phase parameters are not often considered. Furthermore, the growth inhibitory effect (*r*) found to be 0.235, showed a high extent of inhibition, which occurred between the onset of the exponential growth phase.

Similarly, the LM for product formation gave a good description of the biosurfactant production (R^2 = 0.9978) as well as the logistic incorporated Leudeking–Piret model (R^2 = 0.9855). The maximum production of biosurfactant (P_{max}) and volumetric production rate (r_p) were found to be 4.785 g L^{-1} and 0.02965 g $L^{-1}h^{-1}$, respectively, while the specific production rate P_r was found to be 0.1402 h^{-1}. The growth-dependent parameter '*a*' and the non-growth dependent parameter '*b*' were found to be 0.00217 and 0.000422, respectively. The P_{max} obtained from model simulation (4.785 g L^{-1}) was comparable with the experimental value of 4.855 g L^{-1}.

Neglecting osmotic pressure and substrate diffusion effects as well as gradient substrate release of the residual sugar, the substrate utilization kinetic pattern shown in Fig. 6.4 was adjusted to the familiar sigmoidal curve, and was adequately modelled by the logistic incorporated Leudeking–Piret modified model (R^2 = 0.9823), the modified logistic incorporated Leudeking–Piret model (R^2 = 0.9807), and the modified Monod model (R^2 = 0.9934), with the best fit shown in Fig. 6.7. The yield coefficients $Y_{X/S}$ and $Y_{P/S}$ were determined to be 0.142 g cell biomass/g substrate consumed and 0.108 g biosurfactant/g substrate consumed, respectively. The cell maintenance parameter '*m*' was, however, determined to be 0.0057, signifying that the energy derived from *Beta vulgaris* metabolism used for cellular maintenance was insignificant. The initial substrate concentration obtained from the models was 3.557 g L^{-1}, which is also comparable with the experimental value of 3.52 g L^{-1}. In addition, from the modified Monod equation, the cell biomass yield on substrate consumption was found to be 0.6167, while the maximum growth rate μ_m and the Monod constant K_S were determined to be 0.0261 h^{-1} and 0.4178 g L^{-1} respectively.

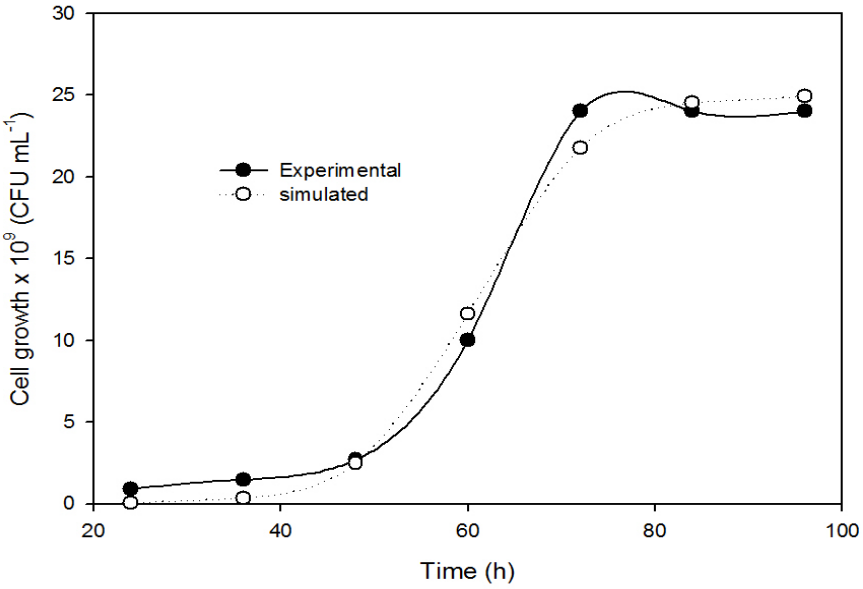


Figure 6.5: Logistic model of *B. licheniformis* STK 01 growth on *B. vulgaris* waste as the only nutrient source

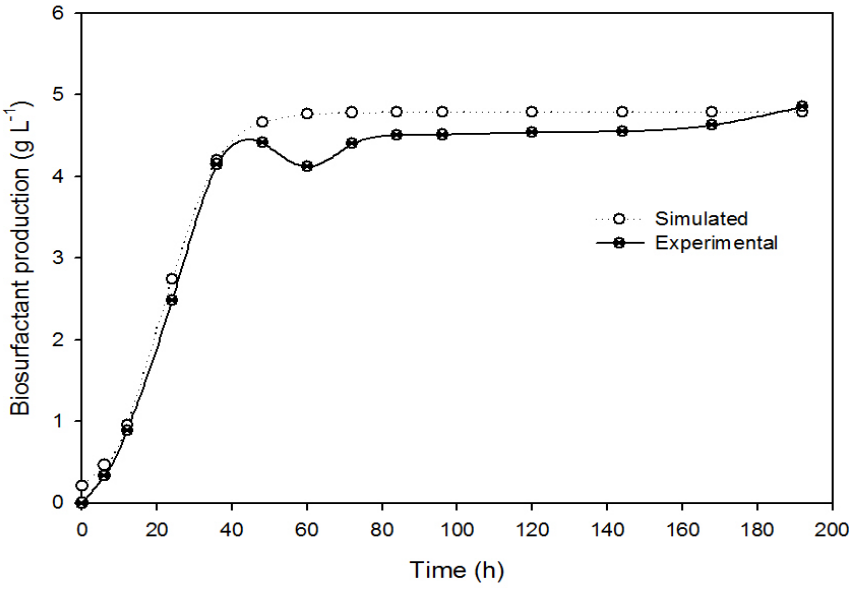


Figure 6.6: Logistic model for biosurfactant production by *B. licheniformis* STK 01 grown exclusively on *B. vulgaris*

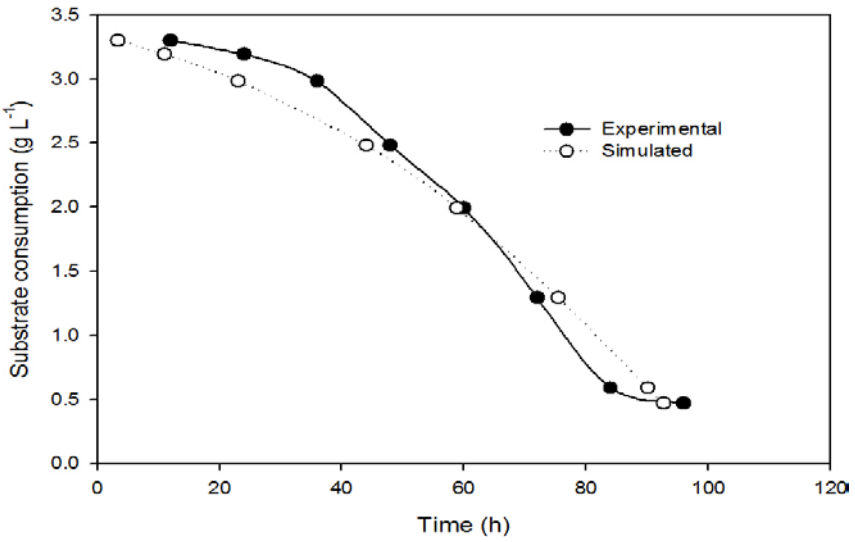


Figure 6.7: Modified Monod model describing *B. vulgaris* substrate utilization by *B. licheniformis* STK 01 for biosurfactant synthesis

Table 6.2: Estimated kinetic model parameters for biomass growth, biosurfactant production, and substrate utilization by *B. licheniformis* STK 01 for biosurfactant production

Symbols	Cell growth models		Product formation models		Substrate utilization models		
	LM	MLM	LM	LLPM	MM	LLPM	MLLPM
X_O (g L^{-1})	0.74E-3	0.711	-	0.335	0.868	0.78	0.78
X_M (g L^{-1})	24.993	22.857	-	23.683	-	23.007	23.007
μ_O (h^{-1})	0.1713	0.108	-	0.365	-	0.03778	0.03778
μ_m (h^{-1})	-	-	-	-	0.0261	-	-
K_S (g L^{-1})	-	-	-	-	0.4178	-	-
R	-	0.235	-	-	-	-	1.786
P_O (g L^{-1})	-	-	0.2115	-	-	-	-
P_M (g L^{-1})	-	-	4.785	-	-	-	-
P_r (h^{-1})	-	-	0.1402	-	-	-	-
So (g L^{-1})	-	-	-	-	3.4232	3.557	3.555
$Y_{X/S}$	-	-	-	-	0.6167	0.142	0.142
$Y_{P/S}$	-	-	-	-	-	0.108	0.108
A	-	-	-	0.002176	-	0.0015	0.0015
B	-	-	-	0.000422	-	0.0005	0.0005
M	-	-	-	-	-	0.0057	0.0057
R^2	0.9903	0.8511	0.9978	0.9855	0.9934	0.9823	0.9807
SSE	9.98E-05	760.55	4.362	2.112	0.0015	1.405	-3.701

LM-Logistic model; MLM-Modified logistic model; M-Monod equation; LP-Leudeking–Piret model; LLPM-Logistic incorporated Leudeking–Piret modified model; MLLPM-Modified logistic incorporated Leudeking–Piret model; MM-Modified Monod equation; SSE-Sum of square error

6.6 Summary

In this chapter, the kinetics of cell growth, substrate utilization, and biosurfactant production by *Bacillus licheniformis* STK 01 grown on *Beta vulgaris* and in mineral salts was investigated, as well as the effects of using polyurethane foam as a biocarrier. The cell growth kinetics illustrated the conventional growth phases, with maximum growth rate being achieved after 72 h fermentation for both media used. With the agrowaste substrate, *B. vulgaris*, the highest biosurfactant production was 5.8 ± 0.5 g L^{-1} and 6.2 ± 0.04 g L^{-1} for culture without and with the biocarrier, respectively. Similarly, for the MS medium, biosurfactant production increased to 9.78 ± 1.02 g L^{-1} and 8.04 ± 0.28 g L^{-1} without and with the biocarrier, respectively.

Biosurfactant production was shown to be growth dependent, peaking at the end of the exponential phase. The addition of polyurethane foam showed a repressive effect on biosurfactant production when the MS medium was used, although it enhanced cell proliferation considerably. The biosurfactant produced lowered the surface tension of the *B. vulgaris* broth from 47 to less than 30 mN m^{-1} after 72 h incubation. Likewise, the biosurfactant produced when the microorganism was grown in MS gave a surface tension reduction of the broth to 23.5 mN m^{-1}, representing one of the highest surface tension reductions reported for a biosurfactant. This study revealed a correlation between substrate consumption, cell growth, and product formation.

The logistic model (LM) gave a good description of the experimental data up to the end of the stationary phase of cell growth (R^2 = 0.9903). Similarly, biosurfactant production was modelled by the logistic model (R^2 = 0.9978) as well as by the logistic incorporated Leudeking–Piret model (R^2 = 0.9855), while substrate utilization was best explained mathematically by the modified Monod equation (R^2 = 0.9934).

As discussed in the previous chapters, the isolated microorganisms were able to use *B. vulgaris* as a primary substrate for biosurfactant production. The culture conditions for the maximum biomass growth and biosurfactant production have been optimized, followed by obtaining suitable models to be able to predict the cell growth and the synthesis of biosurfactant. Therefore, it is imperative to assess the applicability of the isolated strains with the identified agrowaste (*Beta vulgaris*) and the biosurfactant produced to enhance the bioavailability and thus, biodegradation of PAHs in soil samples. As reported in Chapter 3, the microorganisms assessed were isolated from environments saturated with heavy hydrocarbon compounds – coal tar, lignocellulosic waste, and an oil-spill site. This suggested that these organisms can adapt to harsh environmental conditions and also can degrade heavy hydrocarbon contaminants. This is the focus of the next chapter

Chapter 7

KINETICS OF BIODEGRADATION OF HMW PAHS IN SOIL: EFFECTS OF CO-METABOLISM, BACTERIAL CO-CULTURE, AND BIOSURFACTANT

7.1 Introduction [5]

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous and recalcitrant contaminants, released into the environment through natural and anthropogenic sources. These sources are mainly biogenic, petrogenic and pyrolitic (Harvey, 1998, Wick *et al.*, 2011). Owing to their hydrophobicity, they tend to fuse to non- and porous particulate matter, making soil and sediment a suitable repository. Several PAHs have been identified as potential human mutagens and carcinogens (Grimmer, 1983, USEPA, 1999). Chemical and biological methods have been used to remediate PAH contaminated matrices, with the bioremediation approach being deemed suitable because it is environmentally benign and less invasive. Of the sixteen PAHs classified as priority pollutants by the United States Environmental Protection Agency (USEPA, 1999), eight were identified as potential human carcinogens. These eight PAHs belong to the high molecular weight (HMW) class, a group associated with a higher tendency to bioaccumulate in environmental matrices. Recent research studies have focused on the degradation of these HMW PAHs by identifying more prolific and biologically evolved microbial species, and exploring various techniques to enhance the PAHs' bioavailability and subsequent biodegradation (Zhou and

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Sheng, 2006, Husain, 2008, Chaudhary *et al.*, 2011, Moscoso *et al.*, 2012, Kunihiro *et al.*, 2013, Jorfi *et al.*, 2013, Mishra and Singh, 2014, Wang *et al.*, 2014, Lu *et al.*, 2014).

Although several bacterial strains have shown their ability to degrade low molecular weight (LMW) PAHs, a few have been able to demonstrate an ability to biodegrade HMW PAHs, with more than three benzene rings. Many gram-negative bacterial and some gram-positive species have been reported for their ability to degrade numerous PAHs. These species include *Bacillus* sp., *Pseudomonas* sp., *Rhodococcus* sp., and *Acinetobacter* sp. (Boonchan *et al.*, 2000, Dandie *et al.*, 2004, Mishra and Singh, 2014, Ghosh *et al.*, 2014), with degradation rates achieved ranging from 28 to 85%, predominantly for 4-benzene ring PAHs such as pyrene and benzo(a)anthracene. Moreover, a number of these studies often focused on PAH degradation in the aqueous phase, as a secondary remediation process after extraction from solid matrices such as soil, using washing as an extraction method prior to degradation due to the sequestration of these contaminants in soil. As a result of the small intraparticle pores in soil grains, gram-negative bacteria appear to be better degraders of PAHs in soil, owing to their thin cellular membrane which may assist in higher PAH mass transfer across the cellular membrane, thereby facilitating sorption subsequent to intracellular degradation (Ma *et al.*, 2013). Moreover, earlier studies had reported that most indigenous bacteria may be physically precluded from some intraparticle soil grain pores because of the mean diameter of the pores that the immobilised bacteria require to penetrate to access bound pollutants (Alexander, 1977, Lawrence *et al.*, 1979b). This perhaps elucidates limitations associated with the bioremediation of contaminated soil with a high fraction of clay and silt. Low bioavailability and mass transfer limitations are the challenges to PAH bioremediation processes, particularly in soil. These limitations are often influenced by the contaminant's molecular structure, weight, weathering and the soil physicochemical characteristics. Several methods have been adopted to circumvent these challenges, such as extraction (Silva *et al.*, 2005, Yap *et al.*, 2012, Song *et al.*, 2012, Lau *et al.*, 2014), adsorption/biosorption (Chang *et al.*, 2004, Vidal *et al.*, 2011, Kaya *et al.*, 2013), co-metabolism (Reda, 2009, Moscoso *et al.*, 2012), and biosurfactant application. Rather than utilizing soil washing, an approach that is less intrusive and harmful to the environment is often preferred.

The application of biosurfactants to increase the bioavailability of pollutants in the environment seems to be a suitable method, considering that the process is benign. In addition, the availability of an array of suitable wastes for biosurfactant synthesis provides an avenue to mitigate the cost associated with their production. Several studies have reported the enhancement of PAH bioavailability and subsequent biodegradation in the presence of biosurfactants, with a rhamnolipid emulsan produced by *Pseudomonas fluorescens* observed to enhance the biodegradation of pyrene from 91 to 98% within 10 days (Husain, 2008). Similarly, the addition of biosurfactant synthesized by a *Pseudomonas aeruginosa*

SP4 to a soil artificially contaminated with pyrene, was found to enhance pyrene degradation from 59.8 to 84.6% (Jorfi *et al.*, 2013). Naturally, PAH-degrading bacteria are able to access hydrophobic substrates in the environment through: 1) the synthesis of biosurfactants, and 2) their ability to attach directly to the hydrophobic substrate by modifying their cell membrane hydrophobicity (Harvey, 1998, Wick *et al.*, 2011). Biosurfactant-enhanced bioavailability often occurs via two mechanisms: 1) pre-micellar lowering of the surface tension, thereby enhancing the mobilization of the contaminants from particulate matrices resulting in increased sorption mass transfer, and 2) micellar solubilization (Amodu *et al.*, 2013).

Another less invasive approach for enhanced biodegradation of recalcitrant PAHs is by microbial co-cultivation using liquefied substrate with a lower surface tension. The biodegradation of phenanthrene, pyrene, and benzo(a)anthracene by a bacterial consortium of *Staphylococcus warneri* and *Bacillus pumilus* increased from a maximum of 85% for mono-septic cultures, obtained for each PAH, to biodegradation rates greater than 90% when co-cultivated cultures were used in a bioreactor system containing a mixture of PAHs, with a lowered surface tension aqueous phase (Moscoso *et al.*, 2012). Although significant degradation rates were achieved in these studies for some HMW PAHs, particularly those with four-benzene rings, the degradation of five- and six-membered benzene ring PAHs such as benzo(a)pyrene and benzo(ghi)perylene, is minimally reported.

As reported in Chapter 3, novel bacterial strains were isolated – *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 – that had shown abilities for biosurfactant syntheses, with significant hydrocarbon emulsification indices and surface tension reduction under various environmental conditions. The preliminary investigations on LMW PAH degradation using these isolates showed that these microbial strains are potential degraders of a mixture of PAHs, including the HMWs.

7.2 Objectives

The objectives of this chapter were:

- to investigate the capability of the isolated strains mentioned above to degrade a mixture of PAHs in soil;
- to investigate the effect of biosurfactant produced from *Beta vulgaris* waste and the synergy of microbial co-culture cultivation on PAH degradation; and
- to study the biodegradation kinetics of PAHs assuming a first-order degradation kinetic model.

7.3 Materials and methods

The microorganisms used (*Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03) were maintained on nutrient agar slants at 4 °C, and subsequently subcultured every three weeks. The procedures for soil characterization and samples preparation, with PAH extraction and clean-up, are presented in Chapter 3. The soil used consist of 30% clay, 20% silt, 20% fine, and 30% coarse. The soil was artificially spiked with a PAH mixture comprising Phenanthrene (Phe), pyrene (Py), benz(a)anthracene (BaA), benzo*(a)*pyrene (BaP), and benzo(ghi)perylene (BghiP).

A mass (10 g) of the PAH-contaminated soil was weighed into 100 mL Erlenmeyer flasks (covered with foil on the exterior), with a glass weighing boat. The soil samples were inoculated with 8% (v/w) of overnight microbial cultures grown in nutrient broth, subsequent to incubation at 37 °C in a dark static incubator, as explained in Chapter 3.

Different cultures were studied, viz., the monocultures of each of the isolate (without supplementation with either biosurfactant or *B. vulgaris* waste); a culture of *B. licheniformis* supplemented with the crude biosurfactant produced from *B. vulgaris* (5%, v/w); a culture of *B. licheniformis* supplemented with dry milled *B. vulgaris* waste (5%, w/w); and a co-culture of the two *Bacillus* strains (without supplementation). The soil moisture content was maintained at 60% holding capacity as reported by Acevedo *et al.* (2011) by adding 5 mL of sterile water to each flask at 10-day intervals. Control experiments were prepared in the same manner without an inoculum, to account for PAHs' disappearance due to abiotic factors. Each experiment was carried out in duplicate. Samples were incubated for 60 days, initially without periodic assessment of degradation efficiency.

Samples were extracted, cleaned-up, and analysed with a gas chromatography-flame ionization detector (GC-FID) as described in Chapter 3. The recovery efficiency for phenanthrene, pyrene, and B(a)A was approximately about 90%, while that of B(a)P and B(ghi)P was 75 and 83%, respectively.

To study the kinetics of PAH biodegradation, the experimental procedures were repeated by spiking the soil with 50 mg, each of Phe, Py and BaA, and with 25 mg each of BaP and BghiP. The spiking procedure is discussed in Chapter 3. A mass (50 g) of the contaminated soil was transferred into 250 mL Erlenmeyer flasks covered in foil and incubated at 43 ± 2 °C in a dark shaking incubator at 180 rpm for 60 days. Samples were analysed periodically, and before each sample was taken, the flasks were swirled thoroughly to ensure homogeneity. Two control experiments were used, one for each culture. All experiments were carried out in triplicate for this set of experiments. Analytes extraction, clean up, and

analysis using GC were done as described in Chapter 3. The rate constant (*k*) was determined using first-order decay rate (Eq. 2.25)

7.4 Results and discussion

7.4.1 PAH biodegradation

The biodegradation of phenanthrene, pyrene, benz(a)anthracene, benzo(a)pyrene, and benzo(ghi)perylene by *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, *Pseudomonas aeruginosa* STK 03, is shown in Table 7.1. The cultures containing *B. licheniformis* were supplemented with biosurfactant and with a solid agrowaste (*Beta vulgaris*) extract to investigate the effect of co-metabolic substrate utilization on PAH biodegradation.

Table 7.1: PAH degradation: a) by mono-septic cultures of *Bacillus licheniformis, Bacillus subtilis* and *Pseudomonas aeruginosa*; b) in co-cultures of *Bacillus licheniformis and Bacillus subtilis* including *Beta vulgaris* and biosurfactant supplementation

Mono-septic cultures

PAH	*B. licheniformis*			*B. subtilis*			*P. aeruginosa*		
	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$
Phe	38.20	3.28	91.43	34.03	5.16	84.83	34.21	5.79	83.97
Py	38.71	8.38	78.35	28.56	7.44	73.96	35.61	10.99	69.15
B(a)A	35.55	8.86	75.07	35.11	13.27	62.21	34.11	12.59	63.09
B(a)P	36.96	11.59	68.63	33.60	15.16	54.90	20.82	5.12	75.40
B(ghi)P	32.44	15.34	52.73	32.52	19.35	40.50	26.59	11.06	58.42

Co- and augmented cultures

PAH	*B. licheniformis & B. subtilis*			*B. licheniformis & B. vulgaris*			*B. licheniformis &* biosurfactant		
	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$	C_i	C_f	$\%R_{bd}$
Phe	34.561	3.34	90.34	37.18	3.69	90.07	38.84	1.21	96.88
Py	36.741	8.99	75.54	35.50	5.39	84.82	35.11	4.01	88.58
B(a)A	35.200	9.74	72.34	38.51	6.47	83.03	34.46	8.16	76.31
B(a)P	36.005	10.01	72.20	32.74	10.72	67.27	35.71	8.82	75.29
B(ghi)P	35.602	13.97	60.76	37.43	15.58	58.36	33.87	13.24	60.90

– Phenanthrene, Py – Pyrene, BaA – Benz(a)anthracene, BaP – Benzo(a)pyrene, B(ghi)P – Benzo(ghi)perylene, %R*bd*- percentage biodegradation; Ci/f – initial and final concentration (mg L-1).

The biosurfactant used was produced by a *B. licheniformis* strain used in this study using *Beta vulgaris* waste extract. The level of degradation in this study decreased with an increase in the molecular weight of the contaminants for all the cultures studied, with an exception observed for the culture containing *P. aeruginosa*, whereby the degradation of the five-benzene ring PAH (BaP) was higher than that for PAHs with four-benzene rings (BaA and Py).

For all the experiments, the level of degradation ranged from 73.97 to 96.88% for phenanthrene, 69.15 to 88.58% for pyrene, 62.21 to 83.30% for BaA, 54.90 to 75.40% for BaP, and from 40.50 to 60.90% for BghiP. The recalcitrance of PAHs to microbial degradation often increases with increasing molecular weight, as observed in this study, and as reported by others (Chaudhary *et al.*, 2011, Lors *et al.*, 2012). This phenomenon is expected to occur in PAH degradation because as the molecular weight increases, the tendency for the compound to sequester in soil matrix and become non-bioavailable increases. However, other researchers have also reported certain cases whereby HMW PAHs were degraded more than the LMW PAHs. Zhang *et al.* (2009), for instance, reported a 97.7% degradation for pyrene and 82.1% for anthracene. Similarly, Acevedo *et al.* (2011), in a study of the biodegradation of some HMW PAHs, reported a degradation level of 60% for pyrene and 75% for BaP. Further research work may be required in this area to explicate the effects of the structural symmetry of pollutants on their biodegradation.

In comparing the degradation levels in monoseptic cultures, it was observed that *B. licheniformis* achieved higher degradation levels than the other two isolates, for all the PAHs, except in the case of BghiP, where *P. aeruginosa* performed better in degrading the 6-membered ring. Generally, the microbial isolates were all found to demonstrate an elevated ability for the degradation of HMW PAHs. Few bacteria species have demonstrated a similar proficiency in a soil environment. In a study of BaP degradation by Mishra and Singh (2014), *Pseudomonas aeruginosa* PSA5 and *Rhodococcus* sp. NJ2 were found to degrade 88 and 47% of the contaminant, respectively, during a 25-day incubation period in a mineral salt medium.

Considering the biosurfactant-supplemented *B. licheniformis* cultures and the mono-culture without biosurfactant supplementation, it was observed that the degradation of Py, BaP, and BghiP increased from 78.35 to 88.58%, 68.63 to 75.29%, and from 52.73 to 60.90%, respectively. Several other studies have reported biosurfactant-enhanced degradation of PAHs in soil. For example, Husain (2008) observed that a rhamnolipid biosurfactant (emulsan) produced by *Pseudomonas fluorescens* enhanced the degradation of pyrene from 91 to 98% after 10 days of bioremediation studies. Similarly, the addition of biosurfactant synthesized by *Pseudomonas aeruginosa* SP4 to a soil artificially contaminated with pyrene, was found to enhance degradation from 59.8 to 84.6% (Jorfi *et al.*, 2013).

Table 7.2: Kinetics of PAHs degradation by *B. licheniformis* STK 01 with and without biosurfactant supplementation

Microorganism		% Degradation								
		Day 3	**Day 8**	**Day 15**	**Day 21**	**Day 28**	**Day 35**	**Day 42**	**Day 50**	**Day 60**
Bacillus licheniformis STK 01	Phe	7.10	17.17	54.68	66.48	81.86	84.60	93.36	96.81	97.44
	Py	6.88	4.36	34.47	53.08	55.14	63.36	70.93	78.12	89.12
	BaA	7.45	8.25	51.84	56.05	66.40	67.54	73.23	74.02	76.03
	BaP	5.82	6.85	26.05	32.47	52.54	57.33	70.34	82.36	83.05
	BghiP	3.68	9.50	16.49	22.72	28.87	34.54	39.87	45.44	51.58
Bacillus licheniformis STK 01 supplemented with biosurfactant	Phe	1.55	14.23	30.89	46.30	67.46	83.69	96.78	100	100
	Py	4.65	7.90	18.38	25.91	41.43	76.18	82.99	91.82	95.32
	BaA	9.85	12.24	29.86	56.58	65.81	70.76	78.80	81.90	82.71
	BaP	6.79	7.53	21.32	38.49	40.04	43.65	66.47	85.39	86.17
	BghiP	1.28	7.45	20.40	27.60	34.86	41.33	47.26	57.63	61.37

Phe – Phenanthrene, Py – Pyrene, BaA – Benz(a)anthracene, BaP – Benzo(a)pyrene

Furthermore, the synergistic effects of *B. licheniformis* and *B. subtilis* co-culture, as well as the supplementation with *B. vulgaris* on PAH degradation was observed in this study, particularly for cultures containing B(ghi)P. *Beta vulgaris* supplementation here could serve as a co-metabolic substrate – an approach that has been reported for enhanced biodegradation of PAHs (Reda, 2009, Moscoso *et al.*, 2012). The presence of phenanthrene or other LMW PAHs can also serve as competitive substrates. However, in such a situation, optimization of culture parameters may be required to control the microbial metabolic activity to avoid cell proliferation at the expense of degrading the target contaminants. In a study of BaP degradation by *Lasiodiplodia theobromae* that lasted for 35 days, the presence of Phe in the culture was found to inhibit BaP degradation, as phenanthrene, being a LMW PAH, is easier to metabolize (Wang *et al.*, 2014). The microorganism may however adjust to the nutrient-limiting conditions and thus metabolize BaP, if the experiment had lasted longer. Usually in a culture medium, the tendency is for the microorganisms to first metabolize a readily accessible substrate, and under the deficiency of certain nutrient elements, like nitrogen, the organisms then shift

their metabolic pathways by producing surface active agents extracellularly to solubilize the non-readily available substrate.

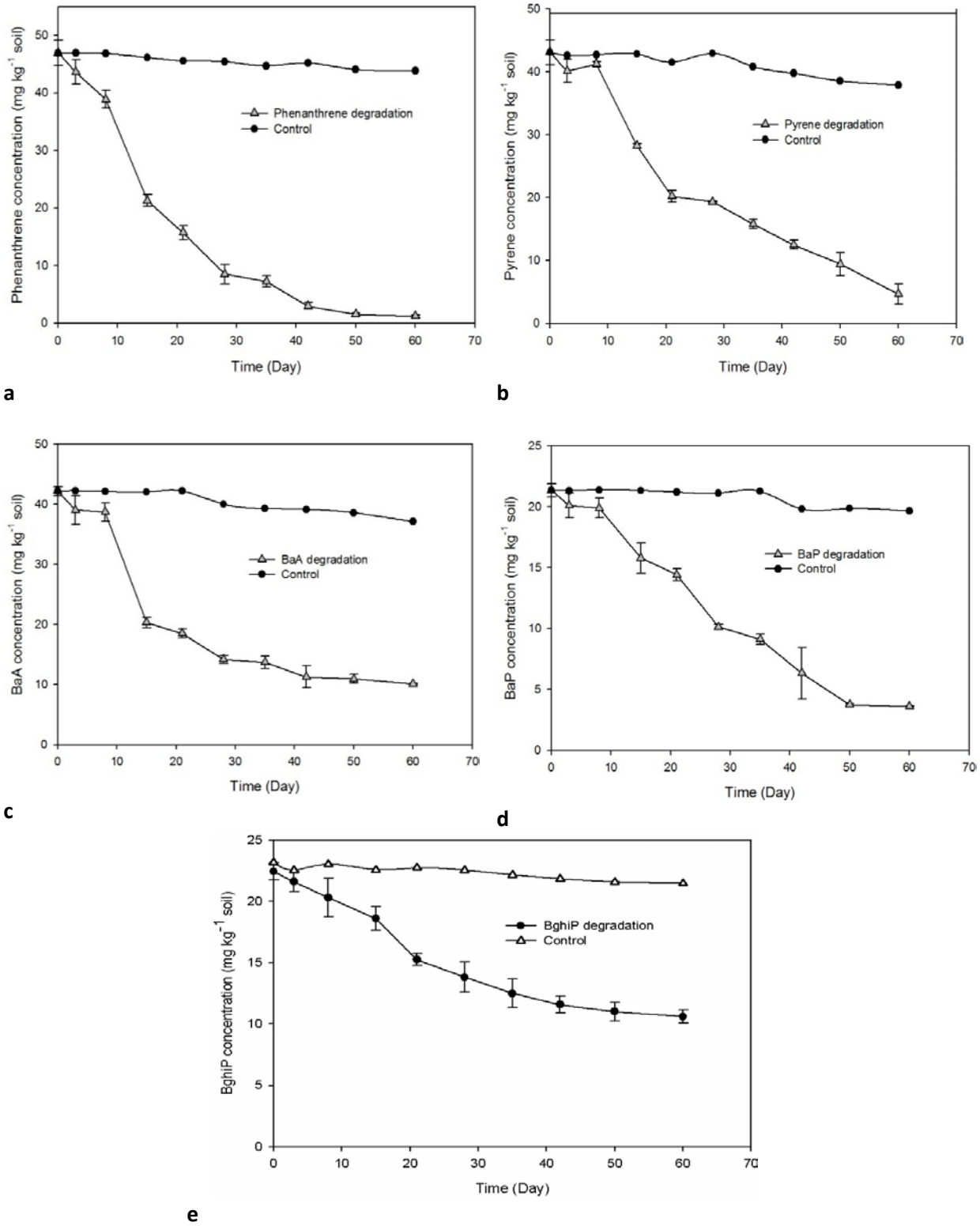
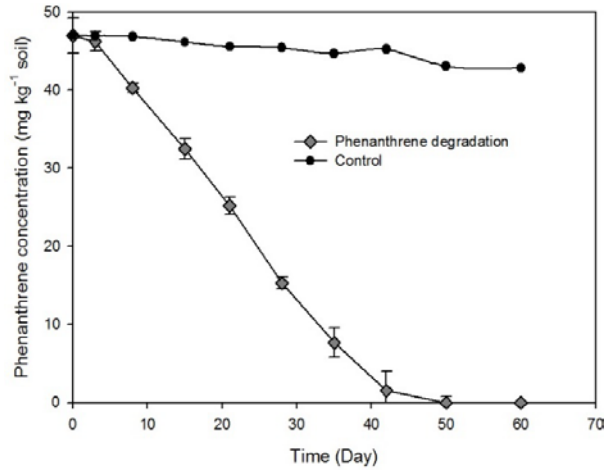
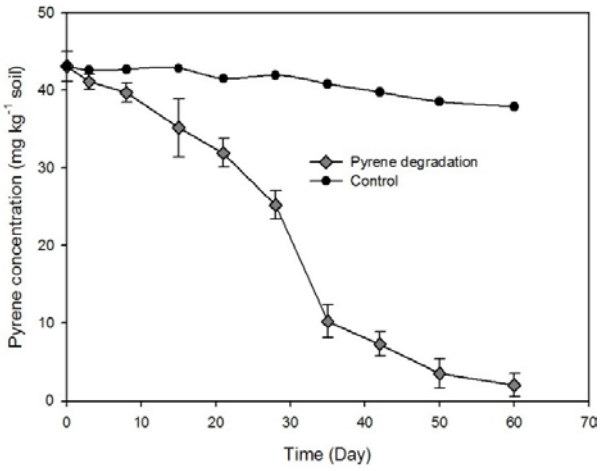


Figure 7.1: Biodegradation profile for (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene, (d) benzo(a)pyrene, and (e) benzo(ghi)perylene by *Bacillus licheniformis* STK 01. Error bars represent the standard deviation of three replicate determinations



a



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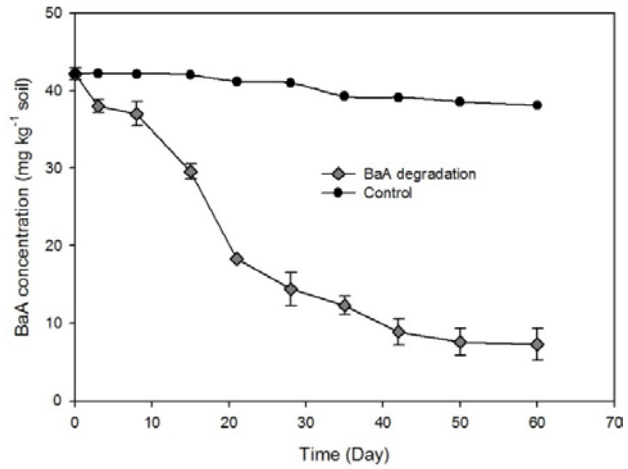

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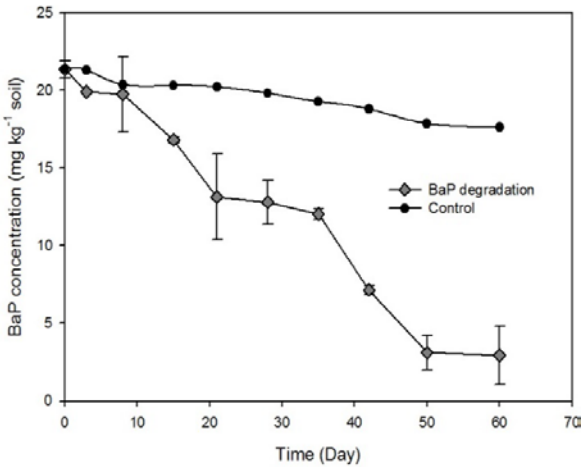

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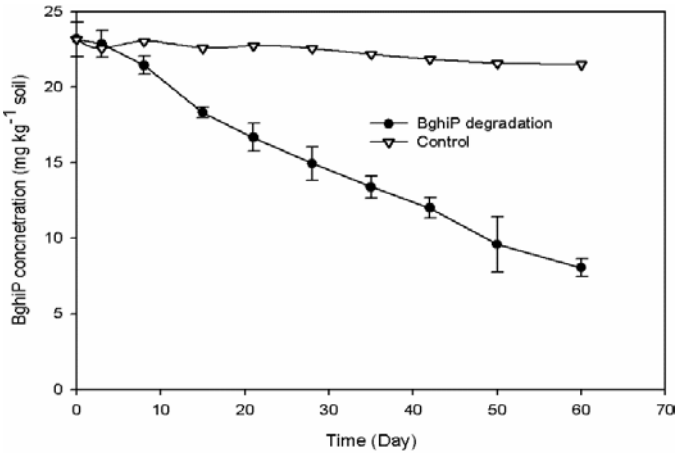


e

Figure 7.2: Biodegradation profile for (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene, (d) benzo(a)pyrene, and (e) benzo(ghi)perylene by *Bacillus licheniformis* STK 01 with addition of biosurfactant. Error bars represent the standard deviation of three replicate determinations.

7.4.2 Kinetic study of PAH degradation

As observed in the degradation results shown in Table 7.1, *B. licheniformis* STK 01, as well as its supplementation with biosurfactant, demonstrated higher degradation levels for most of the PAH compounds studied than the other cultures.

From the degradation kinetic profiles of the contaminants (Figs. 7.1 and 7.2), a brief lag phase was noticeable up to day 7, prior to a decrease in concentration of the PAHs in the soil, which was not observed for phenanthrene. Moreover, the degradation profiles showed that most of the PAHs were degraded between day 7 and 40. For example, about 70% of Phe was degraded within the first 21 days (Fig. 7.1a), while the degradation of BaA and BaP reached an equilibrium after 50 days (Fig. 7.1 and 7.2). As the concentration of the contaminants decreased, sorption into soil intrapores increased, making the remaining fractions less bioavailable, thus reducing degradation. This phenomenon is common in PAH-degradation studies, an observation particularly associated with HMW PAHs – a phenomenon which was observed in this study for B(a)A and BaP. Furthermore, BaP degradation (Fig 7.2d) seemed to indicate two different phases of degradation – a seemingly stationary phase followed by a first-order decrease in concentration between day 10 to 20, and between day 35 and 50. This may be attributed to the biosurfactant supplementation which seemed to increase the bioavailability of the contaminant from the soil matrix in a discrete form.

The degradation rates determined for Phe, Py, BaA, BaP, and BghiP after the 60-day experiment were 97.44%, 89.12%, 76.03%, 83.06%, and 51.58% respectively, for cultures without biosurfactant supplementation. Furthermore, the addition of biosurfactant slightly enhanced the degradation levels to 100%, 95.32%, 82.71%, 86.17%, and 61.37% for Phe, Py, BaA, BaP, and BghiP, respectively. A comparison of the inoculated cultures with the control experiments revealed that PAHs' disappearance due to abiotic factors was negligible. Previous studies on the kinetics of PAH degradation in soil have reported results comparable with some of the results presented in this study. A study by Acevedo *et al.* (2011) on the kinetics of PAH degradation in soil for 60 days reported that most PAH compounds studied were degraded within 14 to 35 days, while 60% and 75% degradation was achieved for Py and BaP, respectively. Lors *et al.* (2012) investigated the degradation kinetics of the 16 PAHs in soil for 200 days and observed that the highest degradation occurred in the first two months for most of the PAHs, with the LMWs being degraded within 7 to 34 days. In the same study, an average of 90% degradation was established for most of the 16 PAHs studied, while 85% and 35% were recorded for the 4- and 5- ring PAHs.

Analyses of rate kinetics showed that phenanthrene degradation was the fastest, both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}), compared with the rate constants determined for Py, BaA, and BaP (Fig. 6.3 and Table 6.3). The rate constant values obtained in the culture with biosurfactant supplementation were in the order: $k_{phe} > k_{py} > k_{BaA} > k_{BaP} > k_{BghiP}$, while for the culture without biosurfactant addition the order observed was: $k_{phe} > k_{py} > k_{BaA} = k_{BaP} > k_{BghiP}$

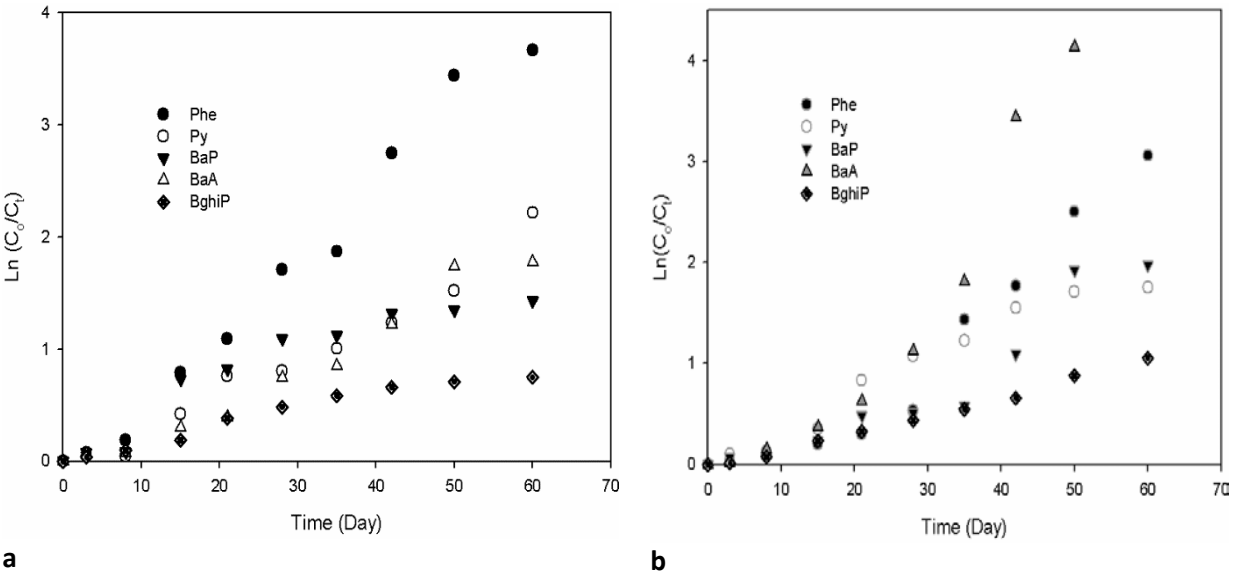


Figure 7.3: Linearized plot of first-order degradation kinetic model for (a) *B. licheniformis* STK 01 and (b) *B. licheniformis* STK 01 + biosurfactant

Table 7.3: PAH degradation rate constant and regression determining coefficients

PAH compounds	*B. licheniformis* STK 01		*B. licheniformis* STK 01 supplemented with biosurfactant	
	k (day^{-1})	R^2	k (day^{-1})	R^2
Phenanthrene	0.0620	0.9759	0.0664	0.8382
Pyrene	0.0332	0.9602	0.0432	0.9208
Benz(a)anthracene	0.0290	0.8724	0.0292	0.8647
Benzo(a)pyrene	0.0291	0.9496	0.0272	0.8387
Benzo(ghi)perylene	0.0140	0.9502	0.0167	0.9878

7.5 Summary

The bacterial isolates – *Bacillus licheniformis* STK 01, *Bacillus subtilis* STK 02, and *Pseudomonas aeruginosa* STK 03 – used in this study were shown to be novel in degrading HMW PAHs, particularly, benzo(ghi)perylene – a 6-benzene ring PAH compound whose substantial degradation is rarely reported. A high degradation capability was observed for all the cultural set ups, with the highest being 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene, 86.17% for benzo(a)pyrene, and 60.90% for benzo(ghi)perylene. The results obtained showed that both gram-positive and gram-negative bacteria are effective degraders of PAHs. Biosurfactant supplementation was found to significantly enhance the degradation of all the PAHs studied, especially the HMWs. On the other hand, culture supplementation with *Beta vulgaris* as a co-metabolic substrate as well as co-culture of *B. licheniformis* STK 01 and *B. subtilis* STK 02, only enhanced the degradation of B(ghi)P. Co-metabolism increased the degradation of B(ghi)P from 52.73 to 58.36%, while co-culture raised the degradation level from 52.73 to 60.76%. First-order reaction rate kinetics was found to fit well the kinetic data, and analyses of the rate constant showed that phenanthrene degradation was the fastest both for cultures without biosurfactant ($k = 0.0620\ day^{-1}$) and with biosurfactant ($k = 0.0664\ day^{-1}$), compared with the rate constant determined for pyrene, benz(a)anthracene, and benzo(a)pyrene. From this study, the effects of structural symmetry of PAHs on biodegradation as well as the effects of micellar core solubilization and pre-micellar surface activity on the kinetics of PAH biodegradation are recommended for further investigation. Overall, the isolated microorganisms were shown to be able to degrade HMW PAHs.

Although efficient biodegradation of HMW PAHs was achieved, the biodegradation rate could be higher than what was observed in this chapter. This became the focus for the next chapter. Magnetic nanoparticles were synthesized and modified with biosurfactant produced from *B. vulgaris*, and applied to enhance the biodegradation rates of PAHs.

Chapter 8

SYNTHESIS, CHARACTERIZATION, AND APPLICATION OF BIOSURFACTANT-MODIFIED NANOPARTICLES TOWARDS ENHANCED BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

8.1 Introduction

Several physical and chemical methods have been used for PAH removal from water and wastewater such as photochemical treatment, oxidation, chlorination, membrane filtration, ozonation, biodegradation, and adsorption (Bohn *et al.*, 1985, Pierzynski, 2000, Zheng *et al.*, 2007, Bernal-Martínez *et al.*, 2007, Ferrarese *et al.*, 2008). Adsorption, being a traditionally known method of migrating materials from one medium into another, is extensively reported for the adsorption of organic compound removal from water and wastewater (Chang *et al.*, 2004, Ali *et al.*, 2012). Consequent upon the high removal efficiency achievable, adsorption has been used for PAH removal from wastewater (Kaya *et al.*, 2013).

A number of highly efficient adsorbents have been developed for use in recent times which include: carbon nanocomposite materials (such as graphene oxide), mesoporous organosilica, and modified zeolites (with organic and inorganic materials) (Vidal *et al.*, 2011, Zhao *et al.*, 2011, Bruna *et al.*, 2012, Nkansah *et al.*, 2012, Zhang *et al.*, 2013). Owing to some drawbacks associated with the use of

activated carbon, such as ease of desorption of highly volatile adsorbates, and ineffectiveness in the removal of oil, grease, and organic contaminants, zeolites have received more attention recently, particularly for the removal of oily contaminants (Chang *et al.*, 2004, Wang and Peng, 2010, Kaya *et al.*, 2013). In addition, zeolites exhibit certain advantages which include excellent ion exchange capacities and high selectivity, catalytic properties, and easy regeneration without losing their adsorption capacities (Khalid *et al.*, 2004, Mahabadi *et al.*, 2007). Recently, fly ash has been recognized as a good precursor for the synthesis of zeolites with magnetic properties (Sun *et al.*, 2010, Fungaro *et al.*, 2011, Ali *et al.*, 2012, Fungaro and Magdalena, 2014). The peculiar properties of zeolites enhance their applications in the removal of various cations and organics from contaminated water and wastewater, including soil.

But since zeolites are hydrophilic and do not have large adsorption capacities for hydrophobic organic compounds, their applications for contaminants like PAHs may be a challenge. Nonetheless, adsorption-dependent parameters such as Si/Al ratio, and cation type, number and location, are often manipulated to improve the adsorption efficiency of zeolites for hydrophobic compounds. One such treatment method is the use of surfactant modification by ion-exchange mechanisms (Kaya *et al.*, 2013). Surfactant modification of zeolites is influenced by hydrophobic effects and cation exchange, whereby the positive moieties of cationic surfactants are readily exchanged with the replaceable cations on the external surface of the zeolites, forming surfactant layers (Simpson and Bowman, 2009). The modification process allows for the exchangeable cations on zeolites surface to be replaced with cations from the surfactant molecule – usually the quaternary ammonium compounds with cationic head groups and a hydrocarbon long-chain molecule as the surfactant tail, are used (Shen, 2001, Gök *et al.*, 2008, Park *et al.*, 2011, Kaya *et al.*, 2013). Thus, the zeolite surface becomes hydrophobic, which allows the adsorbent to retain organic compounds (Vidal *et al.*, 2013). The chemical surfactant that is commonly in use for zeolite modification is hexadecyltrimethyl ammonium bromide (HDTMAB).

Although an array of contaminants such as anions, cations, and organics – phenol, 4-chlorophenol, Orange II, bisphenol A, and sodium dodecyl benzene sulfonate (SDBS) have been reportedly removed by surfactant-modified zeolites (SMZ) from aqueous solution (Dong *et al.*, 2010, Fungaro *et al.*, 2011, Lin *et al.*, 2011, Schick *et al.*, 2011, Vidal *et al.*, 2013), the environmental friendliness of the nanocomposite, especially for bioremediation of environmental contaminants, still raises concerns.

8.2 Objectives

The objectives of this section were: 1) synthesis magnetic zeolites and modification of the synthesised zeolite with biosurfactant produced by *Bacillus licheniformis* STK 01 on *Beta vulgaris* waste; 2) to characterise the zeolite produced as well as the modified nanocomposite using the SEM equipped with an energy dispersive spectrometer (EDS), TGA, FTIR, XRD, and BET surface area analyser; 3) to investigate the ability of the zeolite and the modified nanoparticles to enhance the biodegradation rates of PAHs. In addition, the toxicity of the synthesised adsorbents was investigated.

8.3 Materials and method

Magnetic zeolite preparation, modification, and characterization methods are presented in Chapter 3. The soil used was composed of 40% clay, 20% silt, and 40% fine sand. A portion (25 g) of the contaminated soil was weighed into 100 mL Erlenmeyer flask, with a glass weighing bow. Two cultures were prepared: one with the contaminated soil amended with biosurfactant (2 g of BS added), while the other contained only the contaminated soil samples. The samples were inoculated with 8% (v/w) of an overnight grown culture of *B. licheniformis* STK 01 and incubated at 44 ± 1 °C in a dark shaking incubator at 180 rpm for 30 days. The flask with soil amendment was shaken in an incubator shaker for 72 h (an equilibrium adsorption time determined for naphthalene adsorption unto the biosurfactant modified zeolite [BMMZ]) prior to inoculation. The soil water content was maintained at 60% of the water-holding capacity as reported by Acevedo *et al.* (2011). Both cultural set ups were carried out in triplicate. Control experiments without inoculum were set up in the same way, one for each set up, to account for PAHs' disappearance due to abiotic factors. Samples were taken periodically, cleaned up and analysed, as explained in Chapter 3, over a period of 30 days.

8.4 Results and discussion

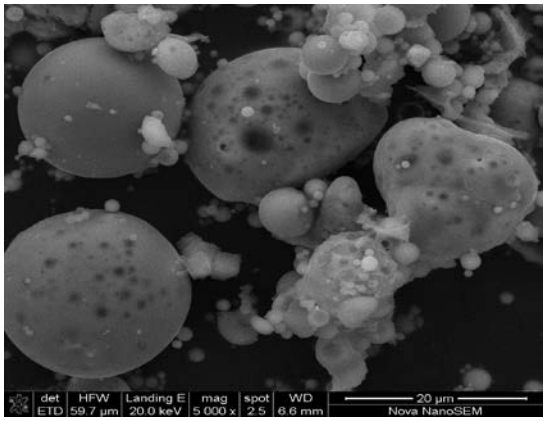
8.4.1 Nano-composite characterization

Magnetic zeolite was synthesized, in a batch system, by direct fusion of coal fly ash (FA), sodium hydroxide (NaOH), and magnetite nanoparticles (Fe_2O_3), in a ratio of 1:1.5: y, respectively; y represents 0.1, 0.2, 0.3, 0.5, 0.75. Zeolite (Z), without the addition of magnetite particles, was also synthesized from the fly ash. The optimized ratio of FA and NaOH combination has been reported earlier (Musyoka *et al.*, 2012, Mainganye *et al.*, 2013). Since the magnetite ratio can influence the affinity of the nano-composite for the hydrocarbon contaminants, the quantity used was varied. The resultant magnetic

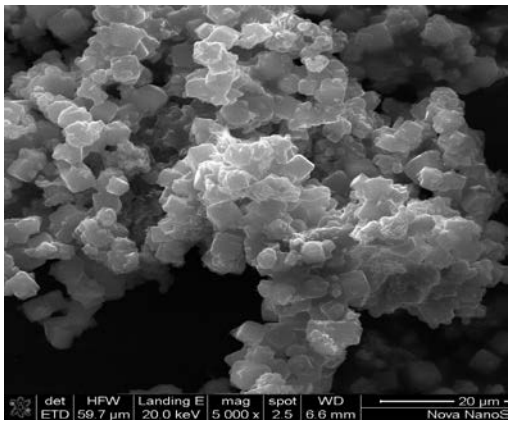
zeolites were designated MZ1, MZ2, MZ3, MZ4, and MZ5, while the respective biosurfactant modified products were represented as BMMZ1, BMMZ2, BMMZ3, BMMZ4, and BMMZ5. The combination of the precursor that gave the highest adsorption was found to be 1: 1.5: 0.3 (in grams), for FA, NaOH, and Fe_2O_3 respectively, based on the preliminary experiments on naphthalene adsorption (results not shown). Hence, MZ3 and BMMZ3 were shown to be better adsorbents and as such, they were characterized.

The morphologies and crystallographic examination of the synthesized adsorbents using scanning electron microscopy (SEM), at 5000 x magnification, showed distinct nanocubes for the zeolites (Z) and BMMZ, while that of MZ showed a fibre-like shape of amorphous materials on a 20 µm scale (Fig. 8.1). The surface of the FA showed the presence of occluded OH^- condensate and amorphous carbon. The mineralogical composition of the zeolites and for FA as by the XRD pattern obtained, revealed that the samples contained some amorphous materials. The peaks observed are similar to those often reported for zeolites synthesized from fly ash as containing basically quartz, magnetite, hematite, and mullite (Fig. 8.2). The most predominant peak, which occurred at 2Θ° of 30, is often due to the presence of quartzite mineral (Williams and Roberts, 2009, Musyoka *et al.*, 2012).

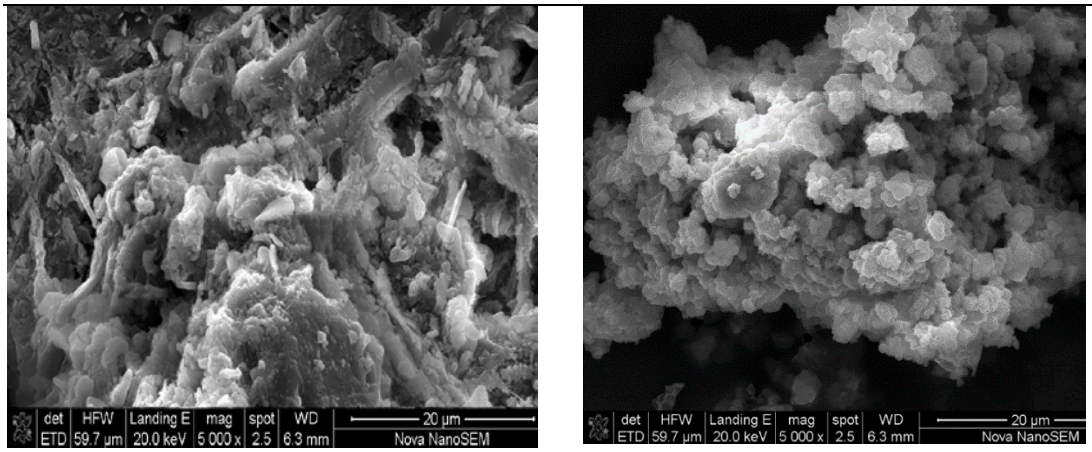
In addition, elemental distribution of the samples was assessed with an energy dispersive X-ray spectroscopy (EDS). As shown in Table 8.1, the increase in the Fe content of the MZ may obviously be due to the presence of $Fe^{2+}/Fe^{3+}$of the magnetite particles. Furthermore, according to the International Zeolite Association (IZA) and the International Mineralogical Association (IMA), zeolites with an Si/Al ratio of 1 – 1.5, in their framework, are classified as zeolite X (Alberti *et al.*, 1997, Tavolaro and Drioli, 1999, Rivera-Garza *et al.*, 2000). Thus, a Si/Al ratio of 1.2, obtained from the EDS analysis, showed that the synthesized zeolite, is zeolite X, with pore sizes from 0.45 – 0.80 nm.



FA



Z



MZ **BMMZ**

Figure 8.1: Scanning electron micrographs of fly ash (FA), zeolite (Z), magnetic zeolite (MZ), and biosurfactant modified magnetic zeolite (BMMZ). The surface morphology of the samples was examined using scanning electron microscopy (SEM), and the corresponding micrographs were obtained, at 5000 x magnification.

Table 8.1: Energy dispersive spectrometer analysis of fly ash (FA), zeolite (Z), magnetic zeolite (MZ), and biosurfactant-modified magnetic zeolite (BMMZ)

Chemical element	FA	Z	MZ	BMMZ
C	34.40	22.55	20.68	-
O	46.63	46.38	46.19	45.48
Na	-	9.97	23.83	14.13
Al	4.44	8.78	3.19	11.74
Si	19.04	10.44	3.09	16.07
Ca	2.25	1.29	0.22	1.50
Fe	-	0.59	2.80	9.69
K	-	-	-	1.38

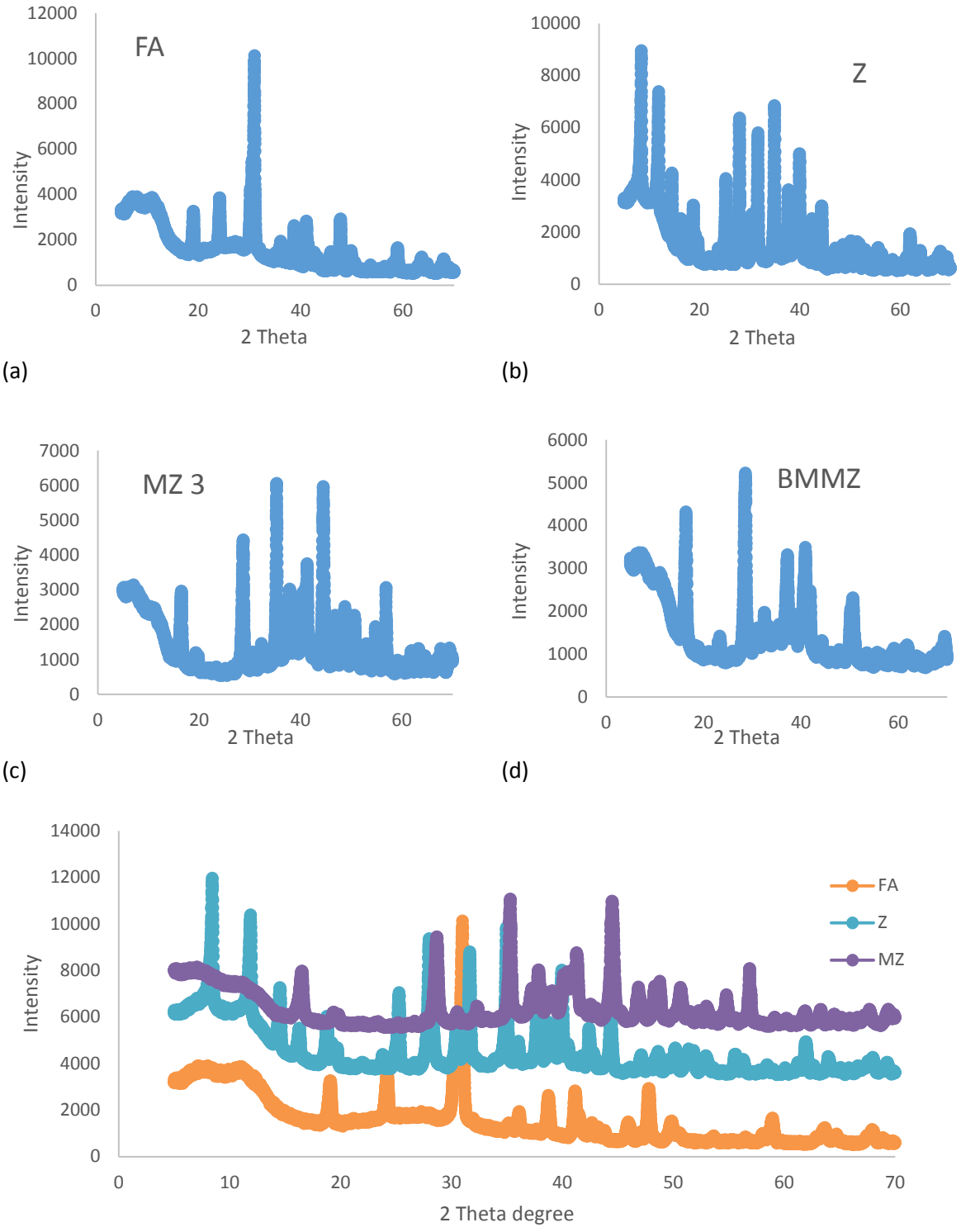


Figure 8.2: XRD spectra of a) fly ash (FA), b) zeolite X (Z), c) magnetic zeolite (MZ), d) biosurfactant-modified zeolite (BMMZ)

The FTIR spectra of the zeolites and the magnetic zeolite, represented in Fig. 8.3, showed highest bands from 3457 to 3415 cm^{-1}, which signified asymmetric and symmetric stretching vibrations of O–H. Bands between 453 and 465 cm^{-1} represented internal tetrahedron vibrations of Si-O and Al-O, 1450, and 866 cm^{-1} denoted zeolite formation, while band at wavelength 1449 cm^{-1} is an O-H bending mode. The shifting of Si-O or Al-O band was signified at 1096 cm^{-1}, with stretching vibrations to lower frequencies 984 cm^{-1} as shown for Z and $BMMZ_3$. In addition, the weak bands at 1646 and 1651 cm^{-1} are attributed to the bending mode of H_2O molecules. The shifting of Si-O or Al-O at 1096 cm^{-1} appears to be more conspicuous in FA.

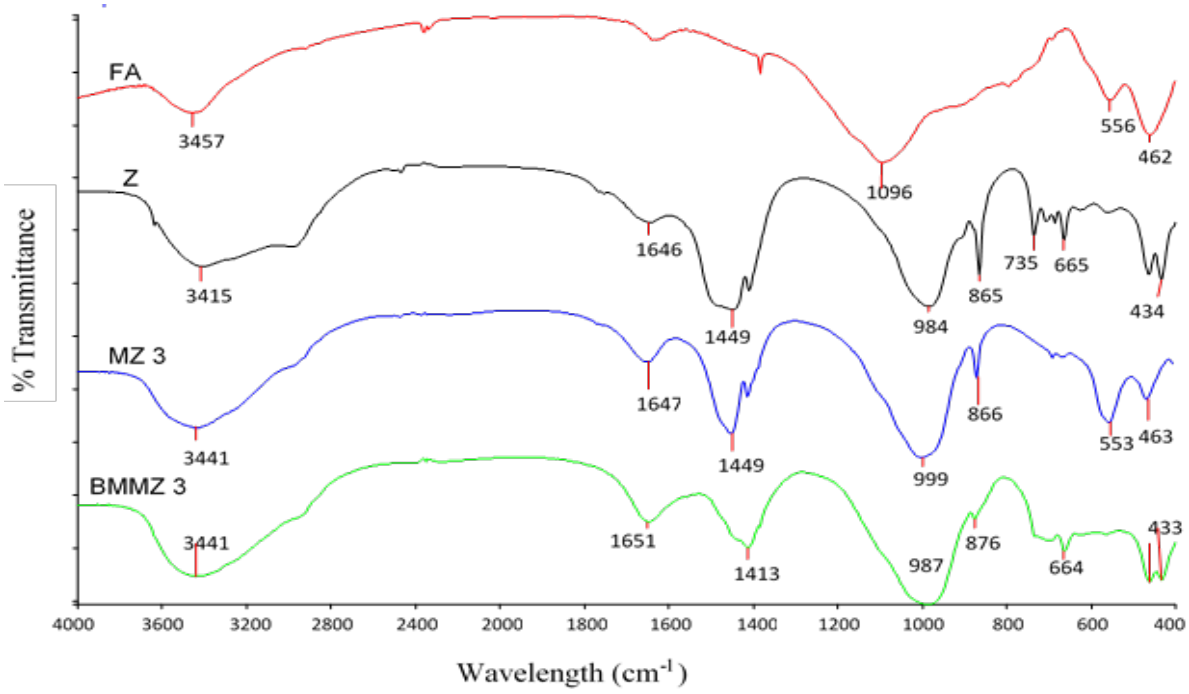


Figure 8.3: FTIR spectra of fly ash (FA) and the synthesized zeolites – zeolite X (Z), magnetic zeolite (MZ), and biosurfactant-modified zeolite (BMMZ)

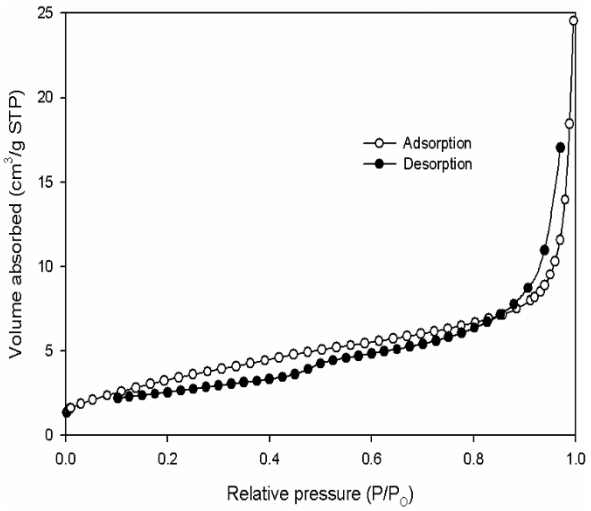
8.4.2 N_2 adsorption and TGA analysis

Typical N_2 adsorption/desorption isotherms for the synthesized zeolite, MZ, and BMMZ are shown in Fig. 8.4. The isotherms show a type-4H hysteresis loop as characterized by the IUPAC, which is

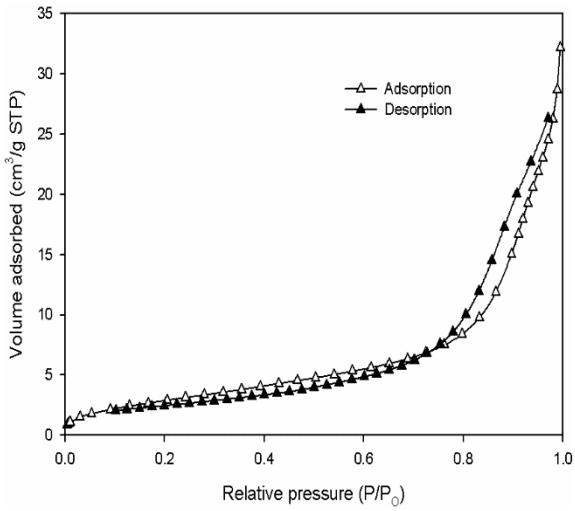
often associated with slit-shaped pores – the type that is mainly obtained with activated carbons (Lippens and De Boer, 1965, He *et al.*, 1998). This hysteresis loop resulted from capillary condensation in the mesopores. And considering that this type of loop does not peak at *P/Po*, it was difficult to establish the limiting boundary of the desorption curve. The adsorption of N_2 unto the adsorbents was generally slow as shown in Fig. 8.4; for Z, about 70% of the gas was adsorbed at P/P_O between 0.9 and 1.0. Similarly, for MZ3, about 78% of the adsorbent pores were covered at P/P_O between 0.8 and 1.0, while for the BMMZ only about 30% of the sorption capacity was used up to P/P_O of 0.8. The slow rate of adsorption observed was due to the unexpectedly low BET surface area determined for these nanoparticles (Fig. 8.4 and Table 8.2). However, it is remarkable to note that the modification of zeolite with biosurfactant increased the surface area by more than 120%. Furthermore, N_2 adsorption increased with respect to increased surface area, with BMMZ recording the highest adsorption of 75.21 cm^3g^{-1} at standard temperature and pressure. In observing that the two branches of a loop cannot satisfy the requirement of thermodynamic reversibility, this means that some distinctive metastable states exist in the process of adsorption and desorption of the adsorbate.

In order to further understand the adsorption capacity of the synthesized zeolites, a t-plot was generated using equation: $t = [13.99 / (0.034 - \log (P/P_O))]^{0.5}$, proposed by Harkins-Jura, to determine the micropore volume (V_{mic}) and mesopore volume (V_{mes}) as well as the external surface area (Fig. 8.5). The V_{mic} and V_{mes} were obtained from the intercepts of the curves with the y-axis, with the thickness range: 3.5 Å to 6 Å. Moreover, the BET and Langmuir surface areas were determined for the zeolite materials by measuring the amount of N_2 adsorbed at different relative pressures (Fig. 8.6).

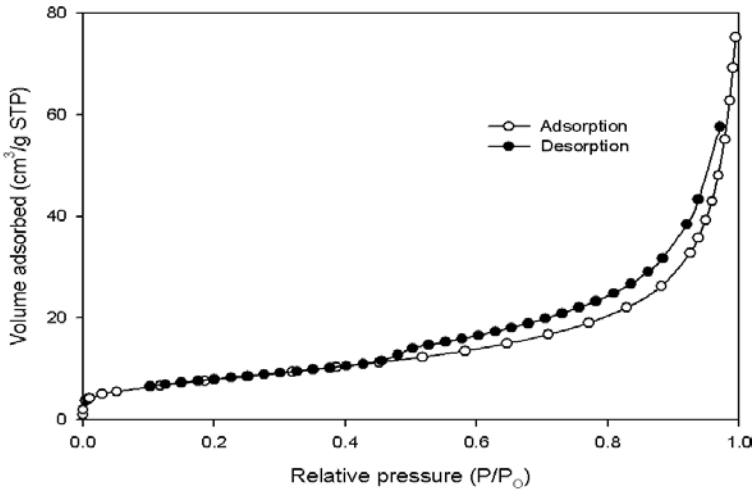
The thermogravimetric analysis (TGA) was performed to assess the thermal stability of the materials (Fig. 8.7). The mass change observed as temperature changed from 22 to 795 °C, was due to the evaporation of volatiles such as water. The weight loss accounts for about 20% of the original weight for both Z and BMMZ tested. The weight loss for both samples occurred in two stages – the evaporation of the more volatile fractions, which is often ascribed to the evaporation of the OH^- condensate, was observed from 22 to 200°C, followed by the evaporation of the less volatile fractions, usually occluded hydrocarbon compounds. Usually, the evaporation of strongly adsorbed fractions within the intrapores of the zeolites occurs at a relatively higher temperature. A similar trend had been reported earlier (He *et al.*, 1998).



Z



MZ3



BMMZ3

Figure 8.4: Nitrogen adsorption isotherms of zeolite (Z), magnetic zeolite (MZ), and biosurfactant-modified magnetic zeolite (BMMZ)

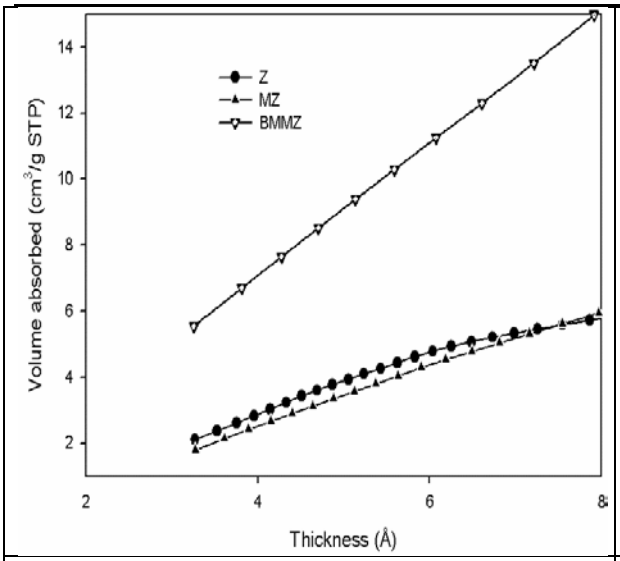


Figure 8.5: The t-plots for zeolite (Z), magnetic zeolite (MZ), and biosurfactant-modified magnetic zeolite (BMMZ)

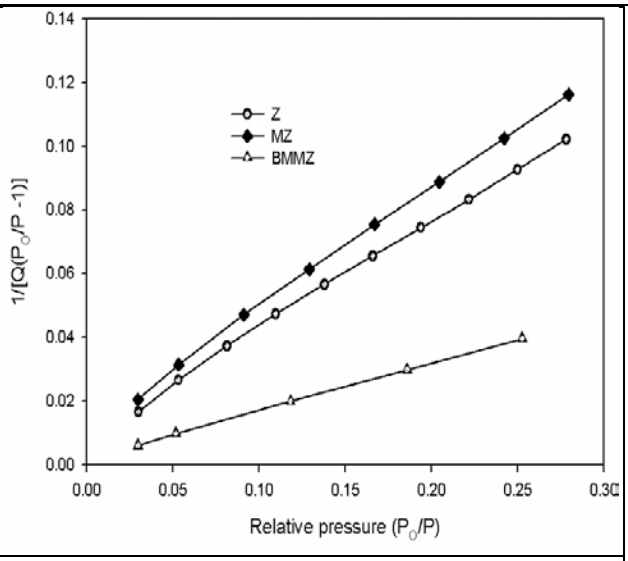


Figure 8.6: BET surface area plots for zeolite (Z), magnetic zeolite (MZ), and biosurfactant-modified magnetic zeolite (BMMZ)

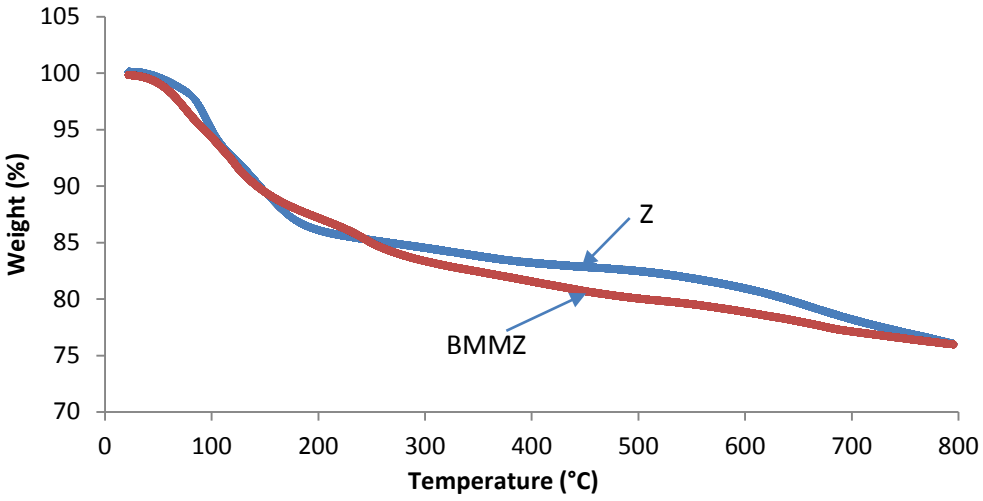


Figure 8.7: Thermogravimetric analysis (TGA) for zeolite (Z) and biosurfactant-modified magnetic zeolite (BMMZ)

Table 8.2: BET analysis of fly ash (FA), zeolite (Z), magnetic zeolite (MZ), and biosurfactant-modified magnetic zeolite (BMMZ)

Parameters	FA	Z	MZ	BMMZ
S_{BET} ($m^2 g^{-1}$)	6.05	12.56	11.16	28.68
S_L ($m^2 g^{-1}$)	27.00	46.78	48.73	125.52
S_{EXT} ($m^2 g^{-1}$)	7.44	14.92	14.47	31.35
Average pore diameter (Å)	58.19	121.17	178.70	162.51
V_{mic} ($cm^3 g^{-1}$)	0.0007	0.0015	0.0018	0.0015

S_{BET} – BET surface area, S_L - Langmuir surface area, V_{mic} – micropore volume, S_{EXT} – external surface area

8.4.3 Application of BMMZ as an adsorbent for enhanced PAHs biodegradation

The biodegradation of phenanthrene, pyrene, benz(a)anthracene, benzo(a)pyrene, and benzo(ghi)perylene by *Bacillus licheniformis* STK 01 is shown in Table 8.3. Higher degradations were achieved in the culture containing soil amended with biosurfactant-modified magnetic zeolite. The biosurfactant used was produced by *B. licheniformis* strain used in this study using a *Beta vulgaris* waste extract, which was identified as a novel substrate for microbial growth and biosurfactant synthesis in Chapter 4. The level of degradation in this study decreased with an increase in the molecular weight of the contaminants, for all the cultures studied with an exception observed for the soil-amended culture where the degradation of BaP (a 5-benzene ring PAH) was higher than that of BaA (a 4-benzene ring).

Table 8.3: Degradation of PAHs by *Bacillus licheniformis* STK 01 in soil without amendment and in soil amended by biosurfactant-modified magnetic nanoparticles

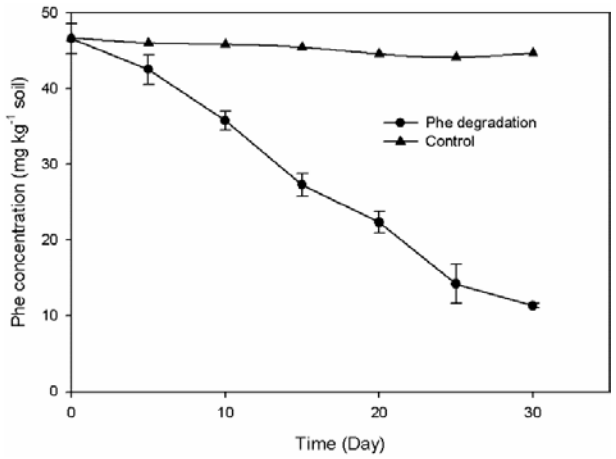
PAHs	% Degradation					
	Day 5	**Day 10**	**Day 15**	**Day 20**	**Day 25**	**Day 30**
	Soil without amendment					
Phe	7.58	21.99	39.91	49.87	67.79	74.69
Py	8.29	18.99	24.38	51.46	57.48	66.25
BaA	4.17	17.19	35.51	41.90	56.81	59.29
BaP	0.73	5.74	21.10	32.23	39.61	54.58
BghiP	0.99	6.61	18.80	36.46	44.98	46.42
	Soil amended with biosurfactant-modified magnetic nanoparticles					
Phe	6.21	10.41	21.18	36.68	61.21	88.55
Py	3.41	9.83	13.84	30.16	52.36	74.45
BaA	2.31	11.27	26.60	48.96	58.73	63.33
BaP	4.05	14.57	28.63	43.33	54.01	67.66
BghiP	2.87	9.75	23.64	40.84	51.06	56.37

Phe – Phenanthrene, Py – Pyrene, BaA – Benz(a)anthracene, BaP – Benzo(a)pyrene, BghiP – Benzo(ghi)perylene

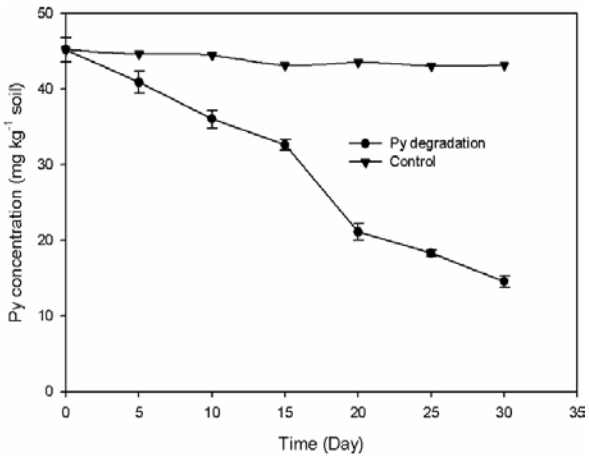
For all the experiments, the level of degradation ranged from 74.69 to 88.55% for Phe, 66.25 to 74.45% for Py, 59.29 to 63.33% for BaA, 54.58 to 67.66% for BaP, and from 46.42 to 56.37% for BghiP. The recalcitrance of PAHs to microbial degradation often increases with increasing molecular weight, as observed in this study, and as reported by others (Chaudhary *et al.*, 2011, Lors *et al.*, 2012). This phenomenon is expected to occur in PAH degradation because as the molecular weight increases, the tendency for the compound to sequester in the soil matrix and become non-bioavailable increases. However, other researchers have also reported certain cases whereby HMW PAHs were degraded more than the LMW PAHs. Zhang *et al.* (2009), for instance, reported a 97.7% degradation for pyrene and 82.1% for anthracene. Similarly, Acevedo *et al.* (2011), in a study of the biodegradation of some HMW PAHs, reported a degradation level of 60% for pyrene and 75% for BaP. Further research work may be required in this area to explicate the effects of structural symmetry of pollutant on their biodegradation.

In comparison, the levels of degradation in the culture with soil amended with biosurfactant-modified zeolites increased by 18.55% for Phe, 12.38% for Py, 6.81% for BaA, 23.96% for BaP, and 21.43% for BghiP. Generally, the higher degradation levels obtained for the soil amended with biosurfactant-modified magnetic zeolite were due to increased contact time created by the nanoparticles, thereby increasing the bioavailability of the contaminants. Few bacteria species have demonstrated a similar proficiency in a soil environment within the period of 30-day degradation reported in this study. In a similar study, the biodegradation of BaP was reported by Mishra and Singh (2014), whereby *Pseudomonas aeruginosa* PSA5 and *Rhodococcus* sp. NJ2 were found to degrade 88 and 47% of the contaminant, respectively, during a 25-day incubation period in a mineral salt medium.

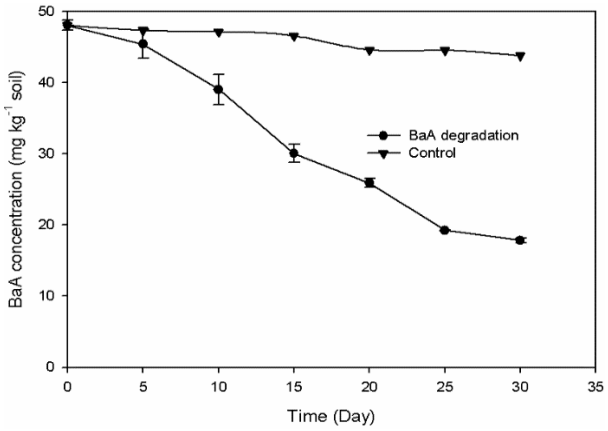
From the degradation kinetic profiles of the contaminants (Figs. 8.8 and 8.9), a brief lag phase was noticeable up to day 5, prior to a decrease in concentration of the PAHs in the soil, compared with the 7-day lag phase observed in the previous study (Chapter 7). Moreover, the degradation profiles showed that most of the PAHs were degraded between days 5 and 25, the profiles showing that the levels of degradation could still increase significantly if the experiment had lasted longer. When comparing the levels of degradation obtained in this study with those recorded after day 35 in Chapter 7 (Fig. 7.2), it was observed that the BMMZ enhanced the levels of degradation for Phe and BaP. In general, as the concentration of the contaminants decreased, sorption into soil intrapores increased, making the residual fractions less bioavailable and thus reducing degradation. This phenomenon is common in PAH degradation studies, particularly for the HMW PAHs.



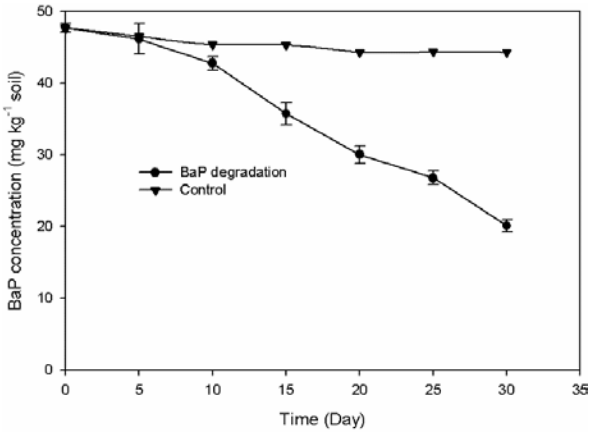
(a)



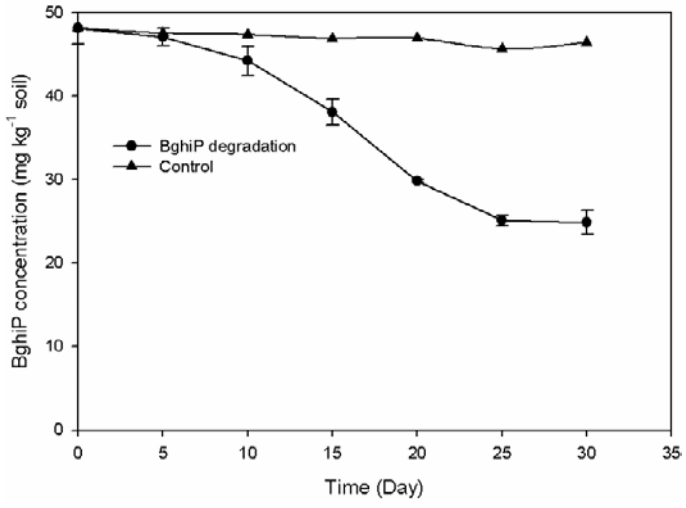
(b)



(c)



(d)



(e)

Figure 8.8: Biodegradation profile for (a) phenanthrene, (b) pyrene, (c) benz(a)anthracene, (d) benzo(a)pyrene, and (e) benzo(ghi)perylene in soil by *Bacillus licheniformis* STK 01. Error bars represent the standard deviation of three replicate determinations

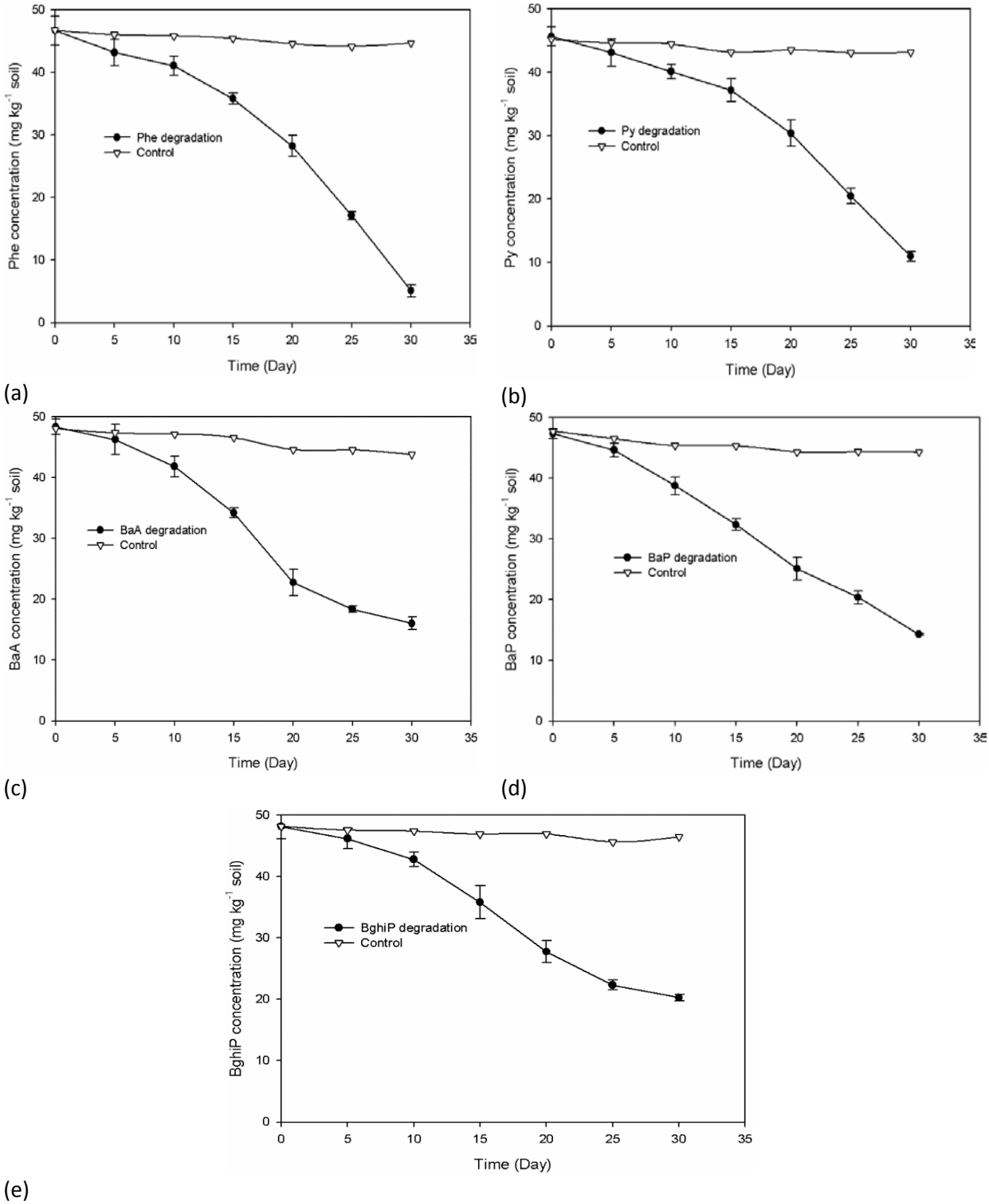
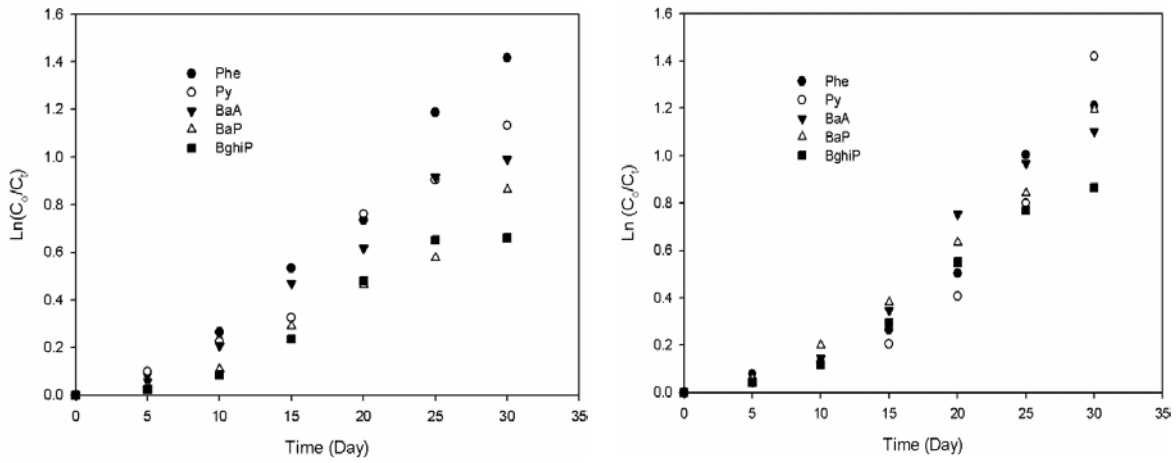


Figure 8.9: Biodegradation profile for a) phenanthrene, b) pyrene, c) benz(a)anthracene, d) benzo(a)pyrene, and e) benzo(ghi)perylene in soil amended by the addition of biosurfactant-modified magnetic nanoparticles by *Bacillus licheniformis* STK 01. Error bars represent the standard deviation of three replicate determinations

The degradation rates determined for Phe, Py, BaA, BaP, and BghiP after the 30-day experiment, in comparison with soil without amendment, increased from 74.69 to 88.55%, 66.25 to 74.45%, 59.29 to 63.33%, 54.58 to 67.66%, and from 46.42 to 56.37%, for Phe, Py, BaA, BaP, and B(ghi)P, respectively. A comparison of the inoculated cultures with the control experiments revealed that PAH disappearance due to abiotic factors was negligible. Previous studies on the kinetics of PAH degradation in soil have reported results comparable with those obtained in this study. A study by Acevedo *et al.* (2011) on the kinetics of PAH degradation in soil for 60 days reported that most PAH compounds studied were degraded within 14 to 35 days, while 60% and 75% degradation were achieved for Py and BaP, respectively. Lors *et al*. (2012) investigated the degradation kinetics of the 16 PAHs in soil for 200 days and observed that the highest degradation occurred in the first two months for most of the PAHs, with the LMWs being degraded within 7 to 34 days. In the same study, an average of 90% degradation was noted in most of the 16 PAHs studied, while 85% and 35% were recorded for the 4- and 5- ring PAHs.

Analyses of rate kinetics did not show much discrepancies between the amended soil and soil without amendment (Table 8.4). Phenanthrene degradation was a bit faster in the soil culture without amendment ($k = 0.0431\ day^{-1}$) than in the amended soil ($k = 0.0339\ day^{-1}$). In the same vein, the degradation of BaP was faster in the amended soil culture ($0.0341\ day^{-1}$) than in that without amendment ($0.0242\ day^{-1}$). But generally, the rate of degradation proceeded in the order of increasing molecular weight for soil culture without amendment; $k_{phe} > k_{py} > k_{BaA} > k_{BaP} > k_{BghiP}$.



(a) (b)

Figure 8.10: Linearized plot of first-order biodegradation kinetic model for PAHs in a) soil without amendment and b) soil amended with biosurfactant-modified magnetic nanoparticles

Table 8.4: PAH degradation rate constant and regression determining coefficients

PAH compounds	Soil without amendment		Soil amended with biosurfactant-modified magnetic zeolites	
	k (day $^{-1}$)	R^2	k (day $^{-1}$)	R^2
Phenanthrene	0.0431	0.9440	0.0339	0.8506
Pyrene	0.0349	0.9373	0.0332	0.7561
Benz(a)anthracene	0.0327	0.9612	0.0348	0.9158
Benzo(a)pyrene	0.0242	0.9161	0.0341	0.9296
Benzo(ghi)perylene	0.0221	0.9070	0.0272	0.9261

8.5 Summary

In this study, zeolites and magnetic zeolites were synthesized by fusion of coal fly ash (FA), sodium hydroxide (NaOH), and magnetite (Fe_2O_3) nanoparticles, and subsequently modified with a biosurfactant. The optimum ratio of the Fe_2O_3 in this composite was found to have significant effects on the adsorptive capacity of the synthesized adsorbents. The combination of the precursor that gave the highest adsorption was found to be 1: 1.5: 0.3 (in grams), for FA, NaOH, and Fe_2O_3 respectively. The elemental composition analysis of the synthesized particles showed that zeolite X was produced based on the Si/Al classification by the International Zeolite Association. The micrographs of the zeolites revealed zeolite X and the biosurfactant-modified zeolites as nanocubes, while the magnetic zeolites possess a rod-like shape with a high quantity of amorphous materials. The acidity and functional group analyses signified asymmetric and symmetric stretching vibrations of O–H and internal tetrahedron vibrations of Si-O and Al-O. The BET surface areas determined for these nanoparticles were unexpected, the highest being 28.68 m^2 g^{-1}. However, it is remarkable to note that the modification of zeolite with biosurfactant increased the surface area of the zeolite by more than 120%. The thermogravimetric analysis (TGA) showed about 20% loss in weight of the zeolites, over a temperature range of 22 to 795 °C, which occurred in two stages – the evaporation of the more volatile fractions (often the OH^- condensate), followed by the evaporation of the strongly adsorbed and less volatile fractions (usually occluded hydrocarbon compounds).

The degradation study on PAHs validates the applicability of biosurfactant-modified zeolites to enhance degradation. The enhanced biodegradation was due to the enhanced bioavailability of the

contaminants in the pores of the nanoparticles and the ability of the particles to act as biofilms or a support to immobilize the microbial cells as well as the contaminants.

Scientific advancement from this study are:

- The application of a consortium of prolific biosurfactant-producing microorganisms, with mutant genes to adapt to HMW organic compounds in the environment and operating under optimized culture conditions, is more promising for degradation of HMW organic contaminants such as BghiP.

- Exclusive application of agrowaste as the primary source of carbon and nutrient element as microbial substrate, without supplementation with chemical substances or refined nutrients, is effective for microbial growth and synthesis of biosurfactant. This could increase the economic viability of biosurfactant production and PAH bioremediation operations.

- Magnetic adsorbents and their modification with surface-active agents are increasingly recognized as effective adsorbents for hydrophobic contaminants. Hence modification with environmentally benign surface-active agents (biosurfactants), rather than with chemical surfactant, is necessary to sustain the novel technology as society becomes more stringent in respect of environmental legislation.

Consequent upon these advancements, the degradation of major hydrophobic organic contaminants, as reported for HMW PAHs in this study, can be achieved and sustained. These advancements are further highlighted in the summary and concluding remarks presented in the next chapter.

Chapter 9

SUMMARY AND CONCLUDING REMARKS

Polycyclic aromatic hydrocarbons (PAHs) are the world's largest class of carcinogens known to date, not only because of their ability to cause gene mutation and cancer, but also owing to their persistency in the environment. They are particularly recalcitrant owing to their molecular weight, and hydrophobic nature, and thus accumulate in various matrices in the environment. The sources of these contaminants are natural as well as anthropogenic. Natural sources include volcanoes and forest fires, while major anthropogenic sources include incomplete combustion of fossil fuel, wood burning, municipal and industrial waste incineration. Owing to the hydrophobic nature of PAHs, soil and sediments are often their repositories in the environment – a focus of this study therefore was to employ a degradation strategy suited to implementation on a large scale.

Several remediation methods have been used over the years, particularly chemical methods and/or physicochemical methods. The success of the bioremediation of the oil tanker Exxon Valdez oil spill of 1989 in Prince William Sound and the Gulf of Alaska, carried out in 1991, sparked unprecedented research interest in the potential of the microbial community in the bioremediation of PAHs. Consequently, robust biological systems have been designed and deployed for PAH degradation, starting

with the isolation of PAH-degrading microorganisms to engineered microbial strains and cultures – which include biostimulation, bioaugmentation, bioreactors application, enzymatic bioremediation, phytoremediation, and land farming, among others.

However, the low bioavailability and mass transfer limitations of PAHs have continued to be a major challenge to their biodegradation. For this reason, the use of biosurfactant has been deployed in recent years to enhance PAHs' desorption and transfer from their sinks, and thus make them available in aqueous phase for microbial degradation. Another challenge is the economic viability of biodegradation technology, which is accentuated by the low yield of the requisite metabolites and the cost of precursors and microbial substrates. Recent researchers have identified a plethora of renewable and low-cost agro/agro-industrial wastes as substrates to facilitate the biodegradation of environmental pollutants. But frequently these substrates are supplemented with refined and/or processed chemical compounds, such as phosphate fertilizer and refined glucose to compensate for certain requisite nutrient elements required for microbial metabolism. This is not considered a holistic approach in the use of renewable waste/biomass as microbial substrate. Based on these challenges and the previous research efforts, the objective of this research work was to: isolate prolific biosurfactant-producing and PAH-degrading microorganisms that would be able to utilize agrowaste exclusively, optimize the cultural conditions for high yield of the requisite metabolites, and apply the isolate for PAH degradation.

To achieve the stated objectives, the study began with bioprospecting, which led to the isolation of *B*. *licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03, from lignocellulosic materials, coal tar, and an oil-spill site (Chapter 3). Several agrowaste were screened as substrate for microbial growth and biosurfactant production and eventually, *Beta vulgaris* was identified as the most suitable substrate – containing high sugar content and appropriate nutrient elements. The biosurfactants produced by the three bacterial isolates – *B. licheniformis* STK 01, *B. subtilis* STK 02, and *P. aeruginosa* STK 03 – were able to lower the surface tension of the culture medium to 30.0, 32.98, and 30.37 mN m^{-1}, respectively. High emulsification indices were equally achieved for some heavy hydrocarbons with the synthesized biosurfactant. These surface tension (ST) reductions and the emulsification indices exemplified the suitability of using the biosurfactant to enhance the bioavailability of PAHs; this thus suggests the potential capability of the isolate to degrade the hydrophobic compounds. Furthermore, the analysis of the biosurfactant produced showed a lipopeptide type – with the ability to induce OH-group into the clustered benzene rings of PAHs in order to enhance their solubility.

In addition, by the numerical optimization option of the surface response methodology (RSM), the optimum conditions for biosurfactant synthesized by *B. licheniformis* STK 01 were found to be a pH of 6.72, a substrate concentration of 4% (w/v), and a temperature of 44.47 °C, under which an ST

reduction to 26.56 mN m^{-1} was predicted (Chapter 4). The experiment conducted to validate the optimum conditions specified by the RSM showed remarkable results: the biosurfactant produced on *B. vulgaris* within 96 h lowered the ST of the broth to 30 mN m^{-1}, while that which was produced on glucose, at the same optimum conditions, lowered the ST to 23.5 mN m^{-1}. The latter represents one of the highest ST reductions ever reported for a biosurfactant.

Moreover, the kinetics of cell growth, substrate utilization, and biosurfactant production by *B. licheniformis* STK 01 showed that the maximum growth rate of the bacterium was achieved after 72 h fermentation, when grown on *B. vulgaris* as well as in a mineral salt (MS) medium. In this study, the effects of using polyurethane foam as a biocarrier were also investigated (Chapter 5). With the agrowaste substrate – *B. vulgaris*, the highest biosurfactant production was 5.8 ± 0.5 g L^{-1} and 6.2 ± 0.04 g L^{-1} for culture without and with the biocarrier, respectively. Similarly, for the MS medium, biosurfactant production increased to 9.78 ± 1.02 g L^{-1} and 8.04 ± 0.28 g L^{-1} without and with the biocarrier, respectively. The biosurfactant yield recorded in this study is remarkable compared with that of previous studies.

Generally, biosurfactant production was shown to be growth dependent, reaching the maximum at the end of the exponential phase. The addition of polyurethane foam did enhance cell proliferation considerably, but repressed biosurfactant production when the MS medium was used. The growth kinetic data were best fitted to the logistic model, while biosurfactant production was modelled by both the logistic model (R^2 = 0.9978) and the logistic incorporated Leudeking–Piret model. The maximum cell growth rate (μ_m) of 0.026 h^{-1}, cell yield ($Y_{S/X}$) of 0.617, and production rate (P_r), of 0.140 h^{-1} were determined. Similarly, substrate utilization was best explained mathematically by the modified Monod equation, with the Monod constant (K_S) being 0.418 g L^{-1}. The study revealed a correlation between substrate consumption, cell growth, and product formation.

At the optimized conditions stated above, the microbial isolates proved to be novel degraders of high molecular weights (HMW) PAHs, particularly, benzo(*ghi)*perylene – a 6-benzene ring PAH compound whose substantial degradation is rarely reported. A high degradation capability was observed for all the cultural set-up, with highest being 100% for phenanthrene, 95.32% for pyrene, 82.71% for benz(a)anthracene, 86.17% for benzo(a)pyrene, and 60.90% for benzo(*ghi)*perylene (Chapter 6). Biosurfactant supplementation was found to significantly enhance the degradation of all the PAHs studied. On the other hand, culture supplementation with *Beta vulgaris* as a co-metabolic substrate as well as co-culture of *B. licheniformis* STK 01 and *B. subtilis* STK 02, only enhanced the degradation of B(*ghi*)P. The degradation rate kinetic data fitted well to the first-order reaction rate model, with

phenanthrene degradation being the fastest both for cultures without biosurfactant (k = 0.0620 day^{-1}) and with biosurfactant (k = 0.0664 day^{-1}), compared with the other PAH compounds.

Furthermore, the biosurfactant produced from *B. vulgaris* was used to modify the surface of a magnetic zeolite synthesized from the fusion of coal fly ash (FA), sodium hydroxide (NaOH), and magnetite (Fe_2O_3) nanoparticles, and applied towards enhancing PAH biodegradation. The resultant nano-composite was characterized with an X-ray diffractometer (XRD), a scanning electron micrometer (SEM) equipped with energy dispersive spectrometer (EDS), a thermogravimetric analyzer (TGA), and an FTIR machine. The BET surface area of the magnetic nanoparticles was also determined (Chapter 8). In comparison with soil sample without amendment, the degradation rates recorded for soil sample with biosurfactant-modified zeolite amendment, increased from 74.69 to 88.55%, 66.25 to 74.45%, 59.29 to 63.33%, 54.58 to 67.66%, and from 46.42 to 56.37%, for Phe, Py, BaA, BaP, and B(*ghi*)P, respectively. This result validates the applicability of biosurfactant-modified zeolites to enhance bioavailability and thus biodegradation of PAHs, by acting as support for biofilm formation and immobilization of the microbial cells as well as the contaminants. The PAHs adsorbed into the intrapores of the magnetic adsorbent can be accessed directly by the microbial cells, unlike the soil particles (particularly clay) where some gram-positive bacterial cells are often excluded.

In conclusion, this study has provided a basis for further investigation on the possibility of a large-scale production of biosurfactant from *B. vulgaris* or other suitable agro/agro-industrial waste, without supplementation with chemical compounds or refined glucose source. Since the ability of biosurfactants to lower surface and/or interfacial tension is often the measure of their effectiveness, more attention should be focused on optimization of the surface activity of biosurfactants, rather than the quantity produced. Moreover, the cutting edge in this area of research appears to be the production of effective biosurfactants at a reasonable cost; the exclusive application of renewable substrates could also ensure the sustainability of the process, particularly for the enhanced bioremediation of environmental contaminants.

The synergy of bacterial co-culture and co-metabolism for enhanced biodegradation of PAHs was demonstrated in this study. The modification of the zeolite surface with biosurfactant and the application of the resultant nanocomposite towards enhancing the biodegradation of PAHs are reported, for the first time, in this study. The results obtained showed that the synthesised nano-composite is promising for absorbing and enhancing the bioavailability and biodegradation of hydrophobic hydrocarbon contaminants.

However, certain challenges arise from this work. The degradation of PAHs is expected to decrease with increasing molecular weight, structural complexity, and number of condensed benzene rings. But as observed in this study, and by other authors, the degradation levels of some PAHs with a higher number of benzene rings were slightly higher than those with fewer rings. Hence, the effects of structural symmetry of PAHs on biodegradation as well as the effects of micellar core solubilization and pre-micellar surface activity on the kinetics of PAH biodegradation are recommended for further investigations. One of the advantages of the nano-composite, which was not investigated in this study, is regeneration and reusability without significant loss of active sites. Hence, further research on the magnetic nano-composite will be to investigate its recoverability from contaminated soil particles.

Chapter 10

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Chapter 11

APPENDICES

Appendix A

The microbial isolation and PCR amplification were discussed in Chapter 3. The gene sequencing was done by a genomics company (Inqaba Biotec) in Pretoria, South Africa. The amplified 16s-rDNA gene and the gene sequences for the three strains used were presented in appendix A including phylogenetic trees. The calibration curves for the quantification of total reducing sugar for HPL quantification of biosurfactant were presented.

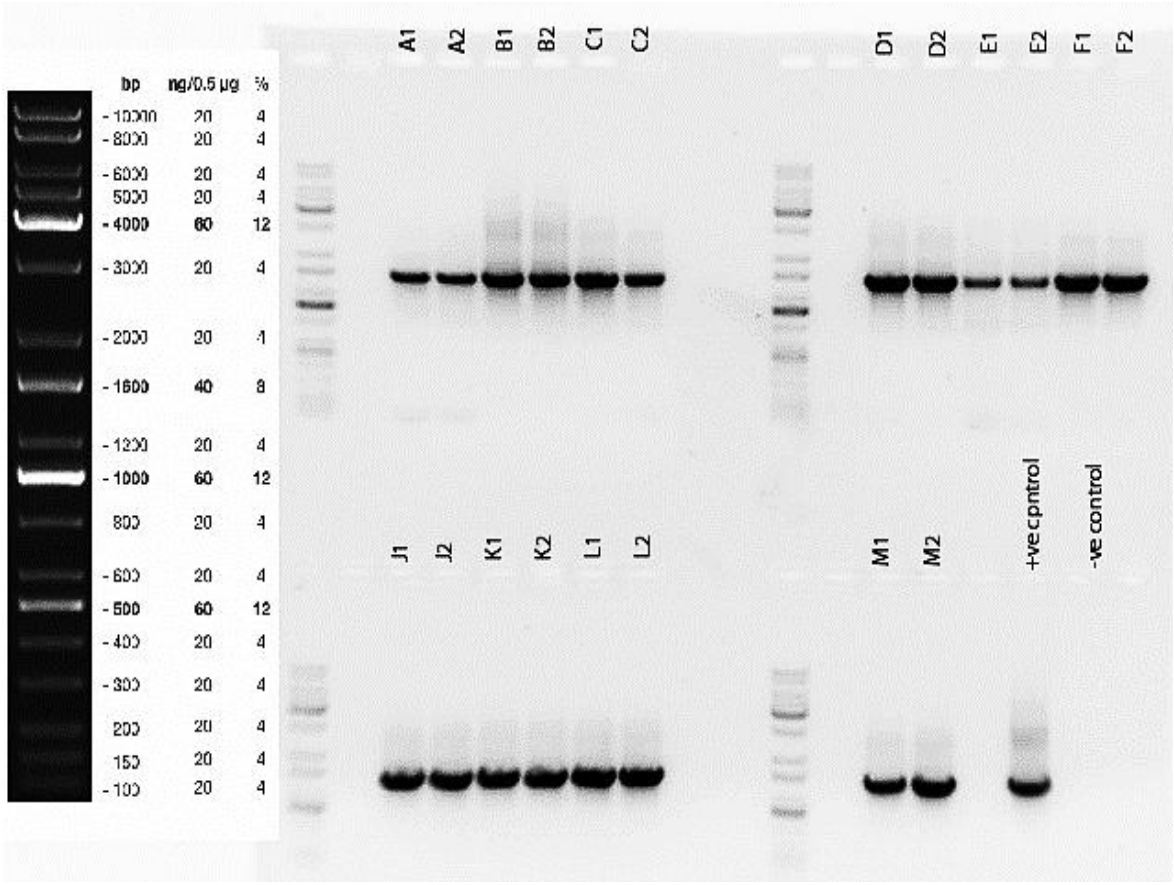


Figure A1: 16s rDNA gene amplification of isolated strains

The primers used for amplification are the forward (F) and reverse (R) primers.

16s-F1: 5'- AGA GTT TGA TCI TGG CTC AG -3'

16s-R5: 5'- ACG GIT ACC TTG TTA CGA CTT -3'

Of all the 10 microbial strains isolated, the sequences below are shown for the three strains that demonstrated highest biosurfactant production from the exclusive use of agrowaste substrate and highest hydrocarbon emulsification, which were also used for the degradation studies

>Bacillus licheniformis [strain STK 01]

AGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCT
GTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTGAACC
GCATGGTTCAATTATAAAAGGTGGCTTTTAGCTACCACTTACAGATGGACCCGCGGC
GCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGA
GTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTC
GAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG
TCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAG
CGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTC
TGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGG
TAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGC
TGCAGCAAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAA
CGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTCCC

>Bacillus subtilis [strain STK 02]

GCTACCATGCAGTCGTAACAAGCAGACCCGGACTTGCTCCCTGATGTTAGCGGCGGA
CGGGTGAGTAACACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACC
GGGGCTAATACCGGATGGTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTC
GGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGA
AAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTG
TTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTAACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCG
TTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGA
AAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGACGCTGAGGAGCGAAAG
CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTG
CTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC
GCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAA

>Pseudomonas aeruginosa [strain STK 03]

GCTCCTGGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTG
GGGGATAACGTCCGGAAACGGGCGCTAATACCGCATACGTCCTGAGGGAGAAAGT
GGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGG
TGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGT
CACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATA
TTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTC
GGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTT
TTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT
ACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGT
TCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTA
CTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG
TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACA
CTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC
CGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCA

The above sequences were registered on the NCBI (Registration number – 1794534). The accession numbers generated for the strains are shown in Table A1.

Table A1: GenBank accession numbers generated for the isolated strains

Isolated bacterial strains	GenBank accession numbers
Bacillus licheniformis STK 01	KR011152
Bacillus subtilis STK 02	KR011153
Pseudomonas aeruginosa	KR011154

Brief information on the Phylogenetic tree construction

The sequence results were assessed with Chromas Lite software while the consensus sequence was built for each of the strains using the BioEdit software. The consensus sequences were blasted on the NCBI (an online GenBank) in order to identify the level of similarity of the isolated strains with those that have been registered by other researchers. Existing sequences online can be assessed at http://www.ncbi.nlm.nih.gov or from the EMBL data base at http://srs.ebi.ac.uk. Gen similarity less than 97% may be considered for an evolutionary strain, as mentioned in Chapter 4. The collated sequences from the NCBI were aligned using MAFFT version 6. This activity is easier performed online at http://mafft.cbrc.jp/aligment/server or http://www.genome.jp/tools/mafft.

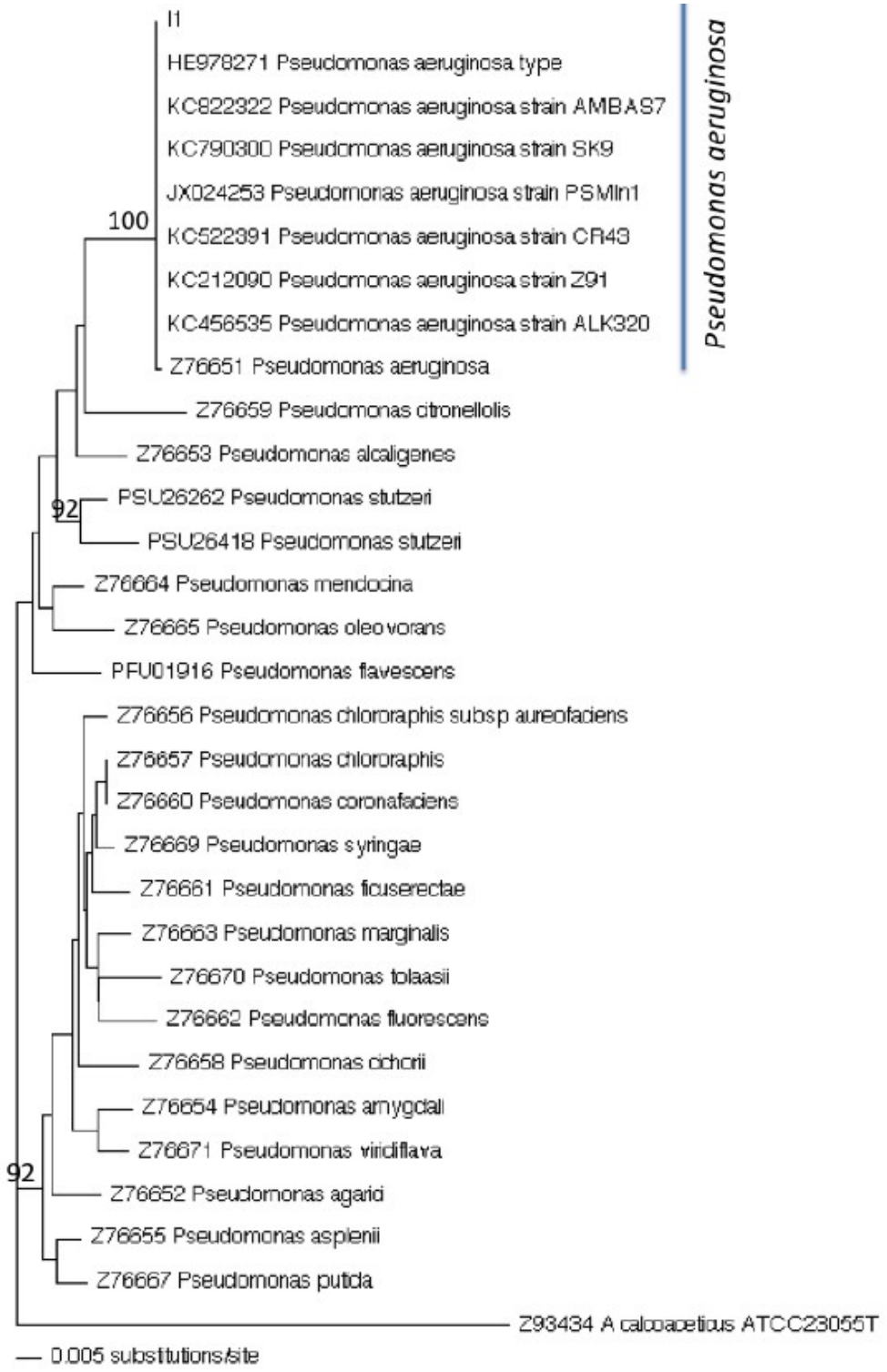


Figure A2: Phylogenetic tree for the P. aeruginosa obtained by distance analysis based on the 16S rDNA gene, using neighbour-joining option. "Type" indicates a type strain.

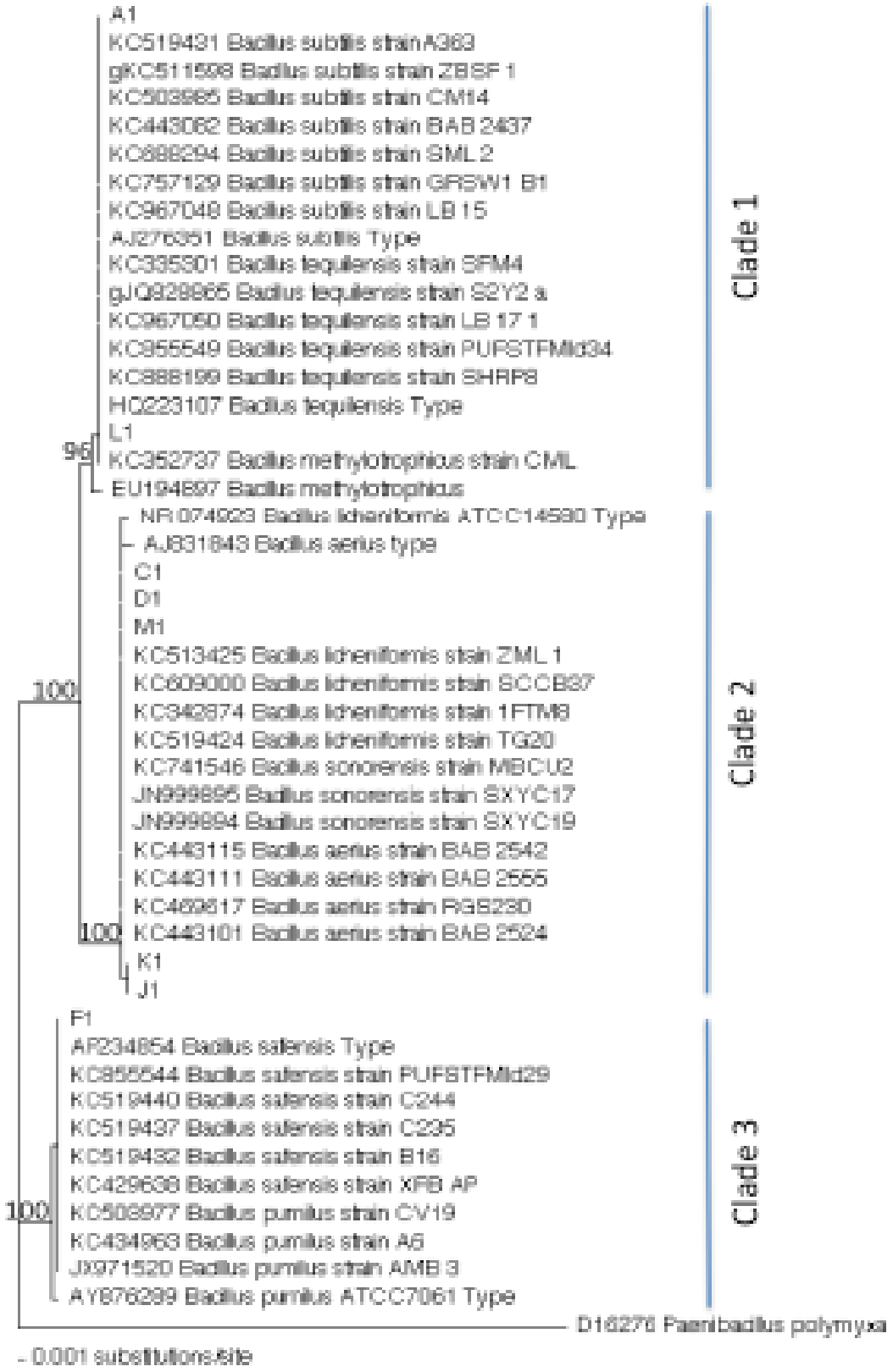


Figure A3: Phylogenetic tree for the Bacillus strains obtained by distance analysis based on the 16S rDNA gene, using neighbour-joining option. "Type" indicates a type strain.

Figure A4: a) Beta vulgaris, b) B. vulgaris inoculated culture, c) biosurfactant extraction, and d) zeolite modification with biosurfactant

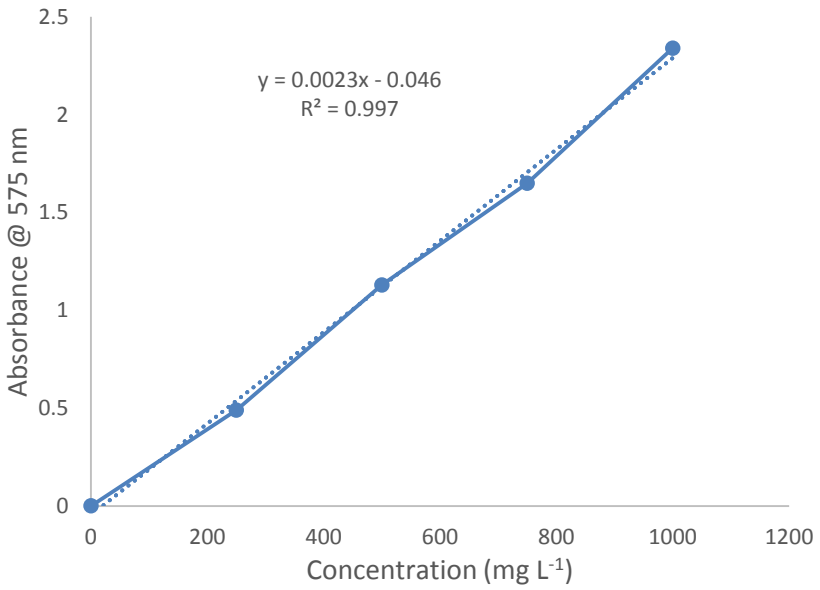


Figure A5: Calibration curve for the quantification of total reducing sugar

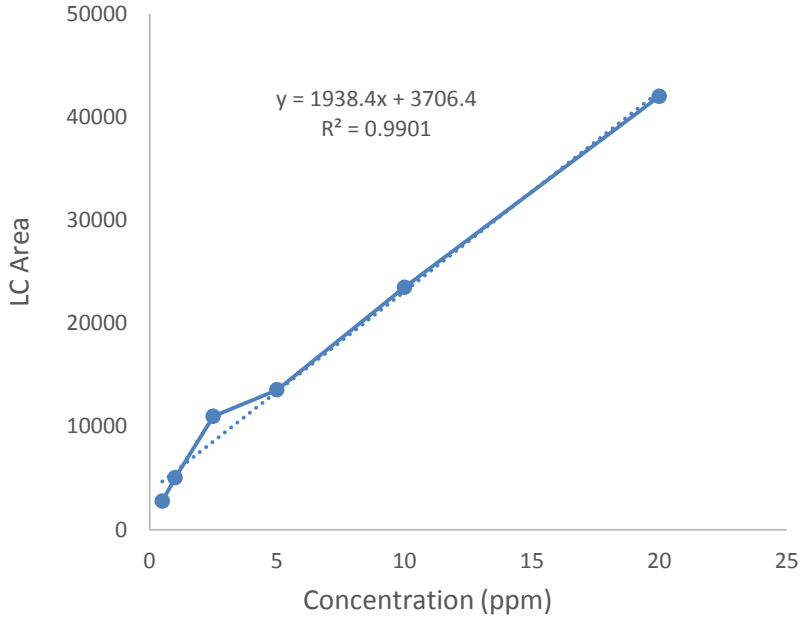
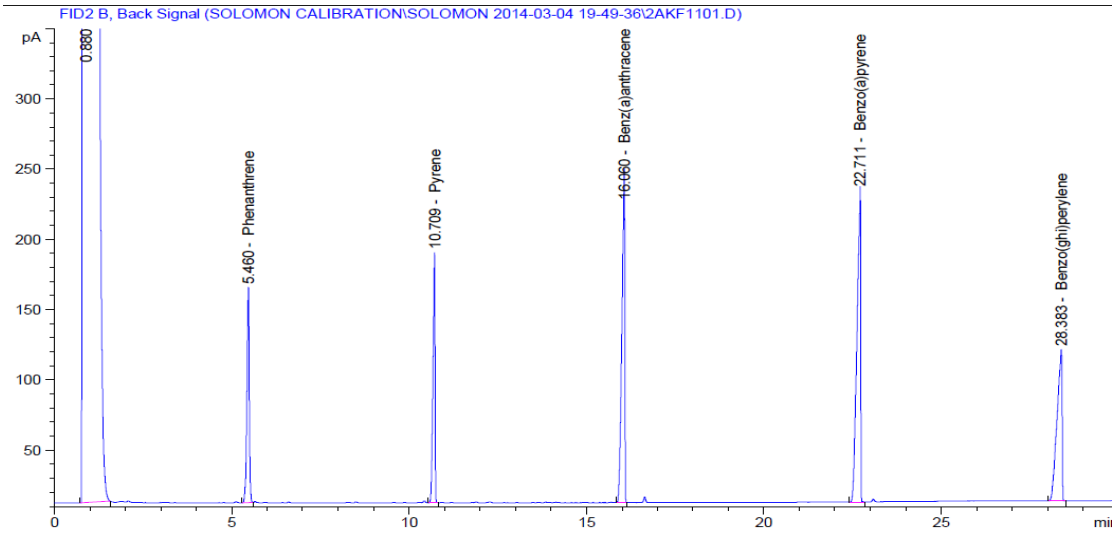


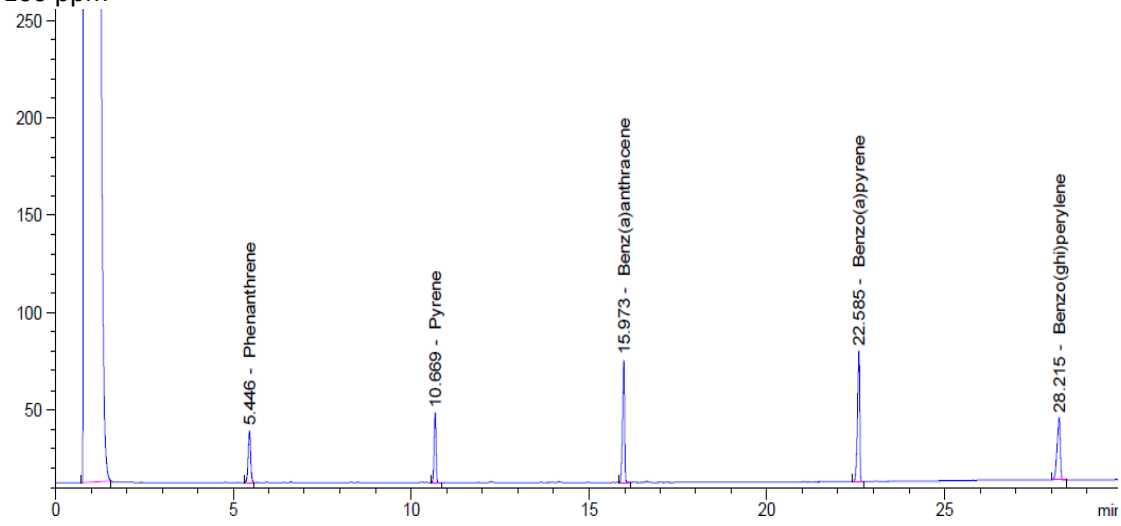
Figure A6: HPLC calibration curve for the determination of biosurfactant

Appendix B

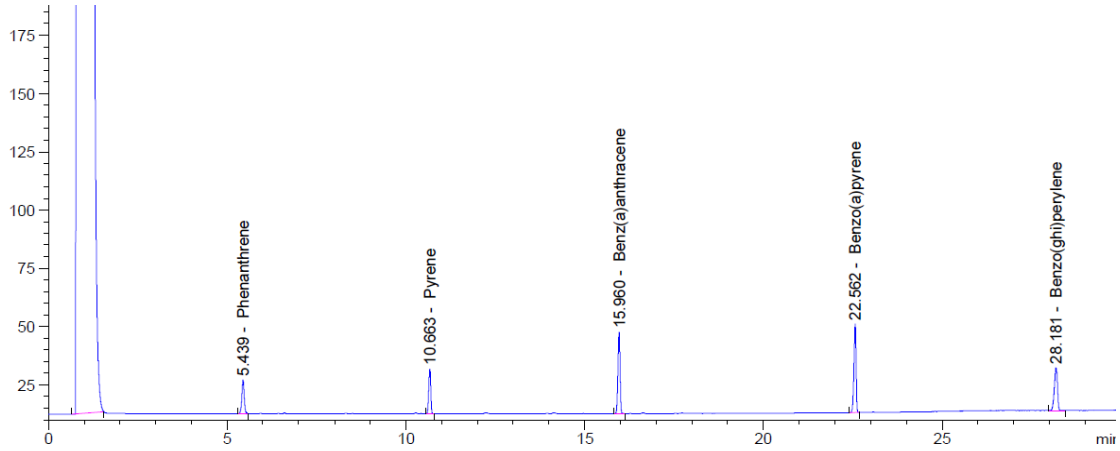
Some of the chromatograms for GC-FID calibration with PAHs including those of the analytes are presented in appendix B.



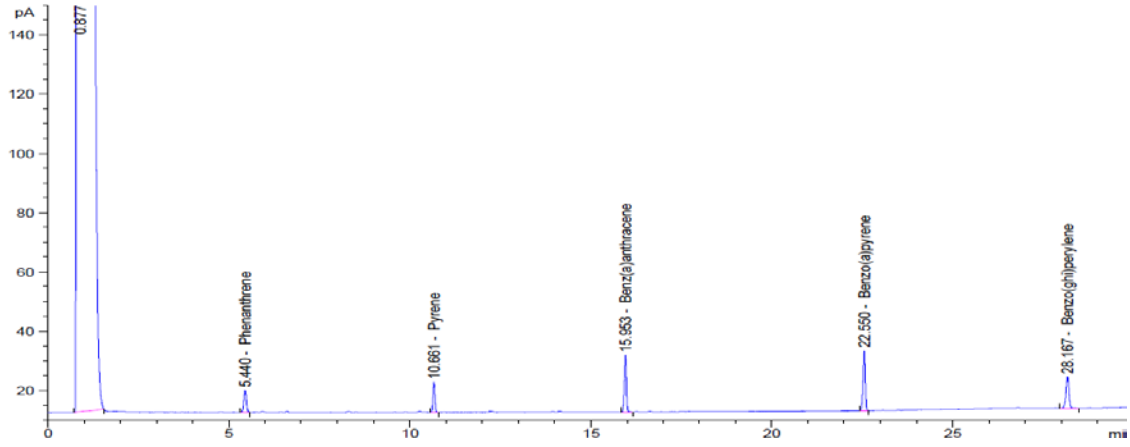
100 ppm



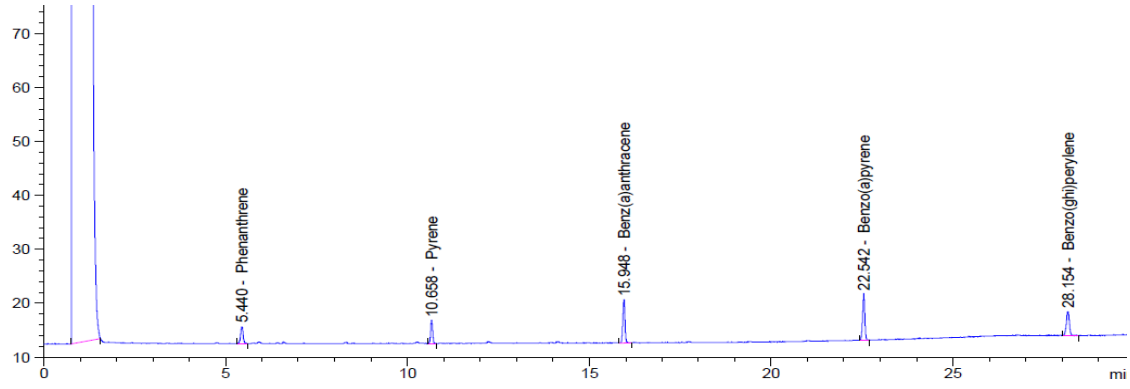
20 ppm



10 ppm

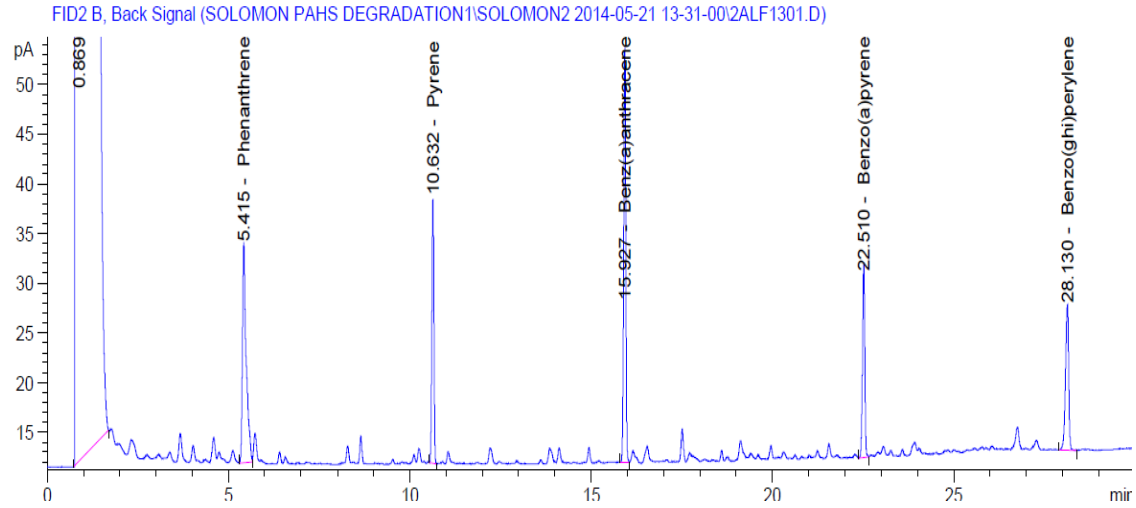


5 ppm

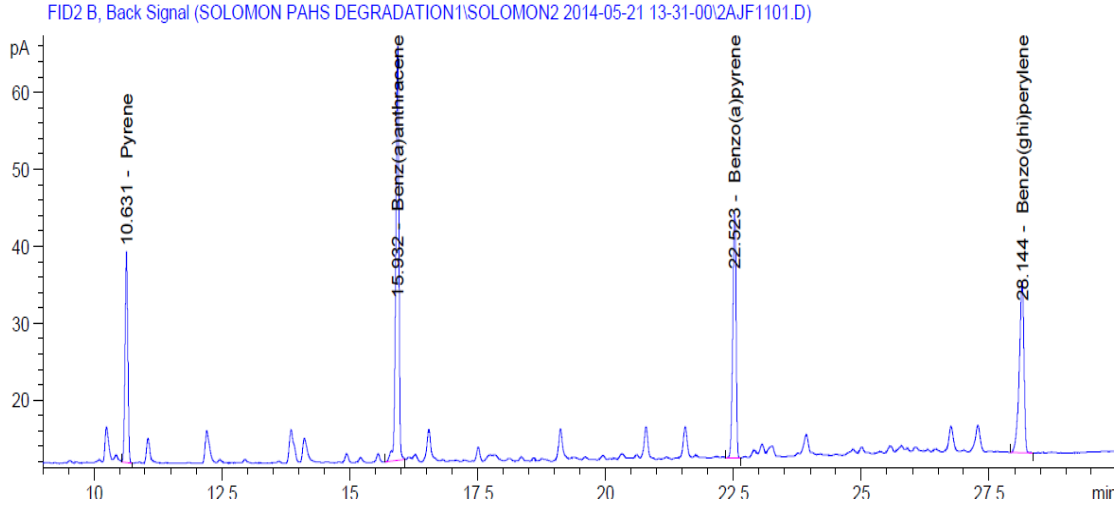


2 ppm

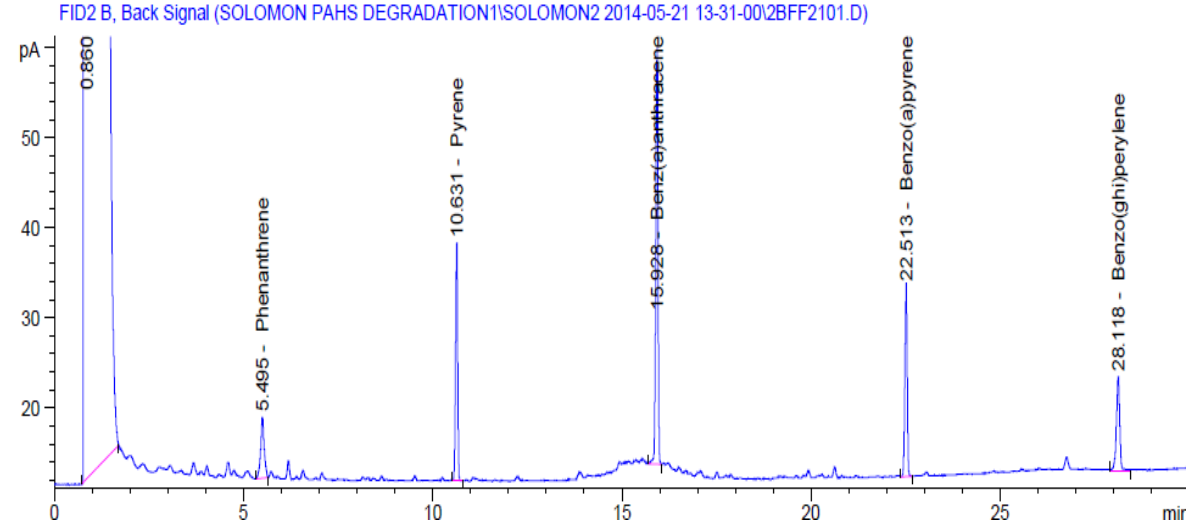
Figure B1: Some of the chromatograms for standard PAHs used for GC calibration


FID2 B, Back Signal (SOLOMON PAHS DEGRADATION1\SOLOMON2 2014-05-21 13-31-00\2ALF1301.D)
pA
0.869
5.415 - Phenanthrene
10.632 - Pyrene
15.927 - Benz(a)anthracene
22.510 - Benzo(a)pyrene
28.130 - Benzo(ghi)perylene
mir

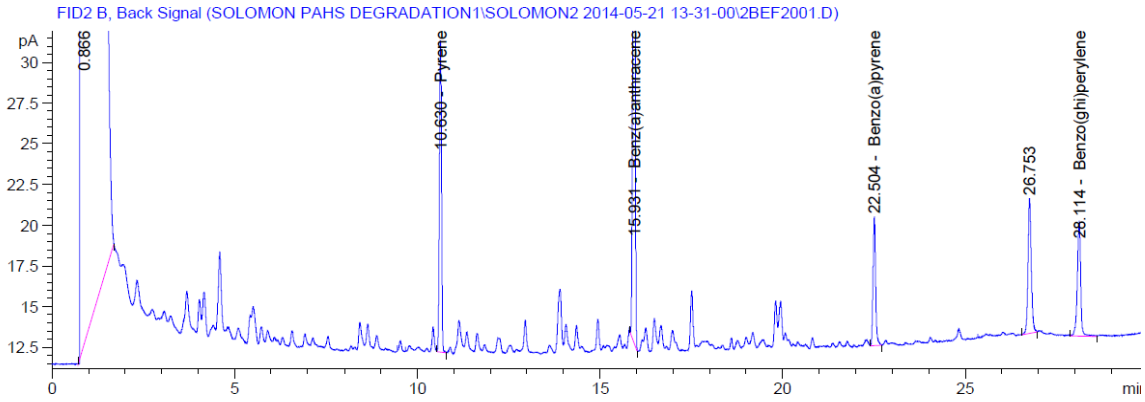
PA


FID2 B, Back Signal (SOLOMON PAHS DEGRADATION1\SOLOMON2 2014-05-21 13-31-00\2AJF1101.D)
pA
10.631 - Pyrene
15.932 - Benz(a)anthracene
22.523 - Benzo(a)pyrene
28.144 - Benzo(ghi)perylene
mir

BL

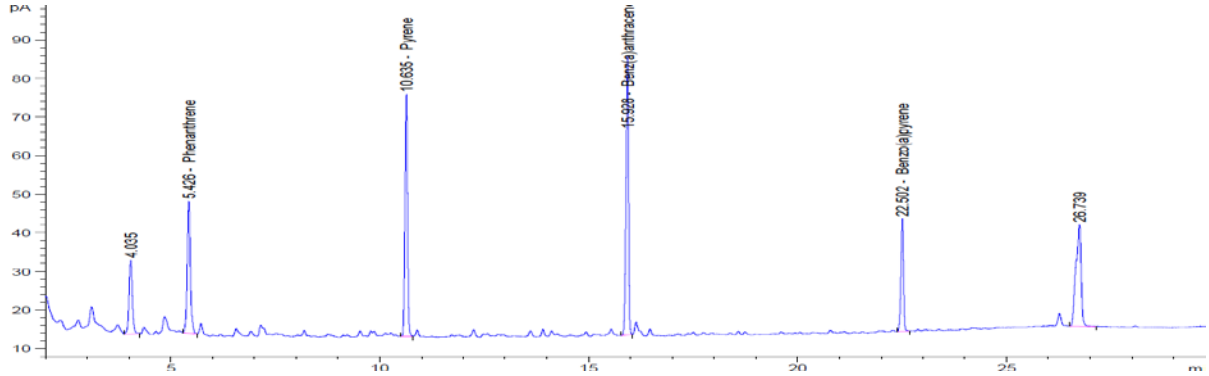

FID2 B, Back Signal (SOLOMON PAHS DEGRADATION1\SOLOMON2 2014-05-21 13-31-00\2BFF2101.D)
pA
0.860
5.495 - Phenanthrene
10.631 - Pyrene
15.928 - Benz(a)anthracene
22.513 - Benzo(a)pyrene
28.118 - Benzo(ghi)perylene
mir

BL+BS

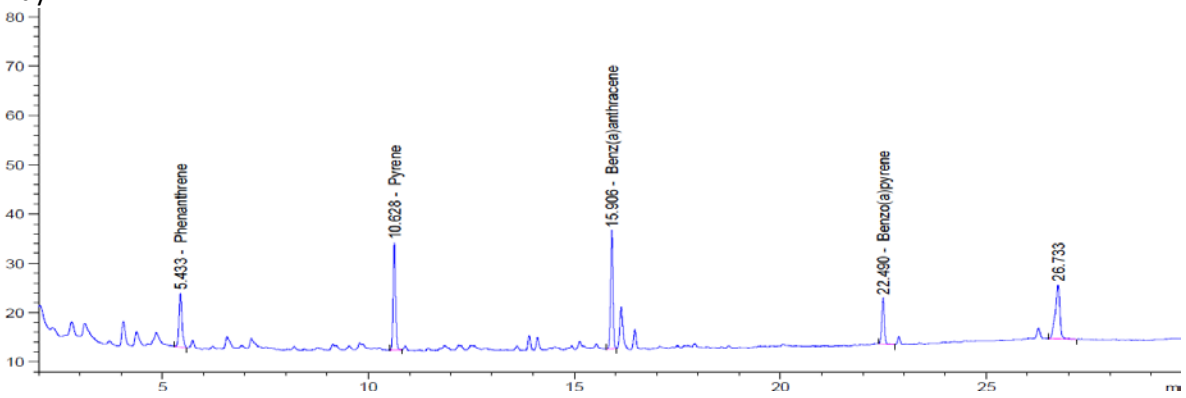


BL + Biosurfactant

Figure B2: Some of the chromatograms recorded on GC-FID for PAHs' analytes after 60 days biodegradation with mono- and mixed- cultures: BL – *Bacillus licheniformis* STK 01; BS – *Bacillus subtilis* STK 02; PA – *Pseudomonas aeruginosa* STK 03



Day 42



Day 50

Figure B3: Some of the chromatograms for PAH biodegradation kinetics by *Bacillus licheniformis* STK 01 supplemented with biosurfactant.

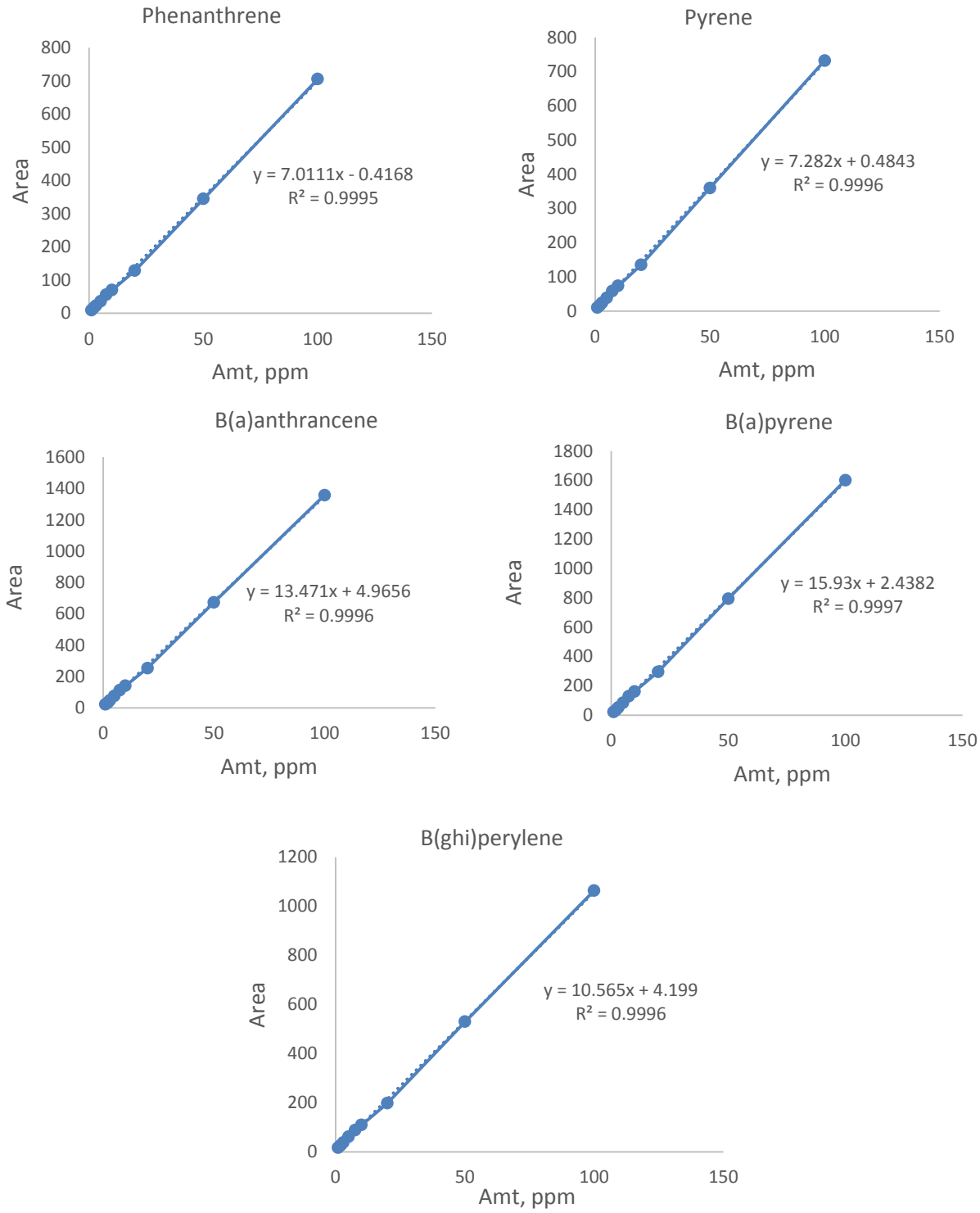


Figure B4: GC-FID calibration curves

Appendix C

Detailed kinetic data for PAHs' biodegradation are presented in appendix C.

Table C1: PAHs biodegradation kinetic data obtained for degradation in soil amended with biosurfactant (Chapter 7)

Microorganism	PAHs		Day 0	Day 3	Day 8	Day 15	Day 21	Day 28	Day 35	Day 42	Day 50	Day 60
Bacillus licheniformis STK 01	Phe	Conc., ppm	46.952 ± 2.27	44.619 ± 2.11	38.890 ± 1.54	21.280 ± 1.02	15.736 ± 1.26	8.517 ± 1.73	7.229 ± 0.96	3.000 ± 0.53	1.500 ± 0.21	1.200 ± 0.20
		% Degrad	0.00	7.10	17.17	54.68	66.48	81.86	84.60	93.36	96.81	97.44
	Py	Conc., ppm	43.097 ± 1.92	40.132 ± 1.87	41.218 ± 0.42	28.243 ± 0.37	20.218 ± 0.91	19.332 ± 0.08	15.791 ± 0.74	12.527 ± 0.68	9.430 ± 1.85	4.689 ± 1.60
		% Degrad	0.00	6.88	4.36	34.47	53.08	55.14	63.36	70.93	78.12	89.12
	BaA	Conc., ppm	42.172 ± 0.72	39.032 ± 2.43	38.6915 ± 1.50	20.311 ± 0.88	18.536 ± 0.77	14.170 ± 0.64	13.691 ± 1.03	11.290 ± 1.81	10.956 ± 0.75	10.107 ± 0.11
		% Degrad	0.00	7.45	8.25	51.84	56.05	66.40	67.54	73.23	74.02	76.03
	BaP	Conc., ppm	21.354 ± 0.55	20.111 ± 1.01	19.891 ± 0.81	15.791 ± 1.25	14.420 ± 0.51	10.134 ± 0.22	9.111 ± 0.41	6.333 ± 2.14	3.766 ± 0.07	3.620 ± 0.02
		% Degrad	0.00	5.82	6.85	26.05	32.47	52.54	57.33	70.34	82.36	83.05
	BghiP	Conc., ppm	22.440 ± 0.72	21.615 ± 0.82	20.308 ± 1.57	18.609 ± 1.97	17.266 ± 0.48	15.822 ± 1.23	14.499 ± 1.17	13.286 ± 1.69	12.023 ± 1.75	10.612 ± 0.55
		% Degrad	0.00	3.68	9.50	16.49	22.72	28.87	34.54	39.87	45.44	51.58
Bacillus licheniformis STK 01 + Biosurfactant	Phe	Conc., ppm	46.952 ± 2.27	46.225 ± 1.22	40.273 ± 0.54	32.449 ± 1.31	25.213 ± 1.11	15.279 ± 0.75	7.657 ± 1.88	1.511 ± 2.50	0.7935 ± 0.75	0.000
		% Degrad	0.00	1.55	14.23	30.89	46.30	67.46	83.69	96.78	98.31	100
	Py	Conc., ppm	43.097 ± 1.92	41.093 ± 0.97	39.691 ± 1.24	35.175 ± 3.71	31.931 ± 1.82	25.243 ± 1.86	10.266 ± 2.11	7.333 ± 1.57	3.527 ± 1.91	2.018 ± 1.54
		% Degrad	0.00	4.65	7.90	18.38	25.91	41.43	76.18	82.99	91.82	95.32

Table C1 - Biodegradation kinetic data (Chapter 7) cont'd

	BaA	Conc., ppm	42.172 ± 0.72	38.017 ± 0.82	37.012 ± 1.57	29.579 ± 0.97	18.311 ± 0.08	14.420 ± 2.13	12.333 ± 1.17	8.942 ± 1.69	7.632 ± 1.75	7.290 ± 2.05
		% Degrad	0.00	9.85	12.24	29.86	56.58	65.81	70.76	78.80	81.90	82.71
	BaP	Conc., ppm	21.354 ± 0.55	19.903 ± 0.12	19.747 ± 2.44	16.801 ± 0.18	13.134 ± 2.76	12.804 ± 1.41	12.034 ± 0.35	7.159 ± 0.28	3.119 ± 1.10	2.954 ± 1.88
		% Degrad	0.00	6.79	7.53	21.32	38.49	40.04	43.65	66.47	85.39	86.17
	BghiP	Conc., ppm	23.170 ± 1.12	22.873 ± 0.89	21.443 ± 0.62	18.322 ± 0.37	16.680 ± 0.91	14.949 ± 2.10	13.398 ± 0.74	12.008 ± 0.68	9.594 ± 1.85	8.691 ± 1.6
		% Degrad	0.00	1.28	7.45	20.40	27.60	34.86	41.33	47.25	57.63	61.37
Control	Phe	Conc., ppm	46.952	46.921	46.815	46.122	45.556	45.402	44.674	45.192	44.024	43.812
	Py		43.097	42.571	42.670	42.825	41.501	42.927	40.811	39.751	38.517	37.884
	BaA		42.172	42.210	42.110	42.005	42.177	42.001	40.252	40.112	40.553	40.107
	BaP		21.354	21.300	21.363	21.314	21.201	21.110	21.270	19.805	19.851	19.633
	BghiP		23.170	23.190	23.080	22.590	22.750	22.550	22.322	22.251	22.191	22.050

Table C2: PAHs biodegradation kinetic data obtained for degradation in oil amended with magnetic nanoparticles (Chapter 8)

PAHs		Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
		Soil without amendment						
Phe	Conc., ppm	46.587 ± 2.01	42.527 ± 1.94	35.742 ± 1.22	27.295 ± 1.51	22.338 ± 1.41	14.207 ± 2.56	11.307 ± 0.281
	% Degrad	0.00	7.58	21.99	39.91	49.87	67.79	74.69
Py	Conc., ppm	45.192 ± 1.58	40.917 ± 1.45	36.021 ± 1.17	32.612 ± 0.72	21.116 ± 1.05	18.291 ± 0.44	14.550 ± 0.74
	% Degrad	0.00	8.29	18.99	24.38	51.46	57.48	66.25
BaA	Conc., ppm	48.036 ± 0.72	45.373 ± 2.03	39.011 ± 2.15	30.032 ± 1.28	25.901 ± 0.58	19.223 ± 0.09	17.812 ± 0.37
	% Degrad	0.00	4.17	17.19	35.51	41.90	56.81	59.29
BaP	Conc., ppm	47.690 ± 0.61	46.172 ± 2.09	42.761 ± 0.95	35.751 ± 1.55	30.03 ± 1.23	26.771 ± 0.94	20.108 ± 0.81
	% Degrad	0.00	0.73	5.74	21.10	32.23	39.61	54.58
BghiP	Conc., ppm	48.144 ± 1.91	47.053 ± 1.07	44.230 ± 1.74	38.061 ± 1.52	29.810 ± 0.17	25.093 ± 0.61	24.874 ± 1.42
		0.00	0.99	6.61	18.80	36.46	44.98	46.42
		Soil amended with biosurfactant modified magnetic nanoparticles						
Phe	Conc., ppm	46.684 ± 2.31	43.158 ± 2.10	41.045 ± 1.51	35.801 ± 0.90	28.213 ± 1.71	17.112 ± 0.67	5.114 ± 1.01
	% Degrad	0.00	6.21	10.41	21.18	36.68	61.21	88.55
Py	Conc., ppm	45.622 ± 1.52	43.093 ± 2.15	40.094 ± 1.12	37.158 ± 1.79	30.381 ± 2.08	20.495 ± 1.20	11.015 ± 0.75
	% Degrad	0.00	3.41	9.83	13.84	30.16	52.36	74.45
BaA	Conc., ppm	48.306 ± 1.27	46.226 ± 2.50	41.801 ± 1.68	34.181 ± 0.82	22.752 ± 2.17	18.371 ± 0.55	16.045 ± 1.03
	% Degrad	0.00	2.31	11.27	26.60	48.96	58.73	63.33
BaP	Conc., ppm	47.312 ± 0.78	44.628 ± 1.12	38.755 ± 1.47	32.339 ± 0.96	25.109 ± 1.84	20.388 ± 1.09	14.320 ± 0.18
	% Degrad	0.00	4.05	14.57	28.63	43.33	54.01	67.66
BghiP	Conc., ppm	48.110 ± 2.01	46.158 ± 1.67	42.745 ± 1.16	35.793 ± 2.68	27.751 ± 1.78	22.319 ± 0.82	20.253 ± 0.49
	% Degrad	0.00	2.87	9.75	23.64	40.84	51.06	56.37
Phe	Conc., ppm	46.687	46.017	45.815	45.422	44.556	44.112	44.674
Py		45.192	44.615	44.467	43.125	43.501	43.020	43.112
BaA		48.036	47.321	47.110	46.565	44.577	44.511	43.752
BaP		47.690	46.512	45.363	45.314	44.311	44.330	44.275
BghiP		48.144	47.523	47.362	46.872	46.912	45.609	46.422