STUDIES ON BIODEGRADATION OF PYRENE BY *MYCOBACTERIUM FREDERIKSBERGENSE*

A THESIS

submitted by

BISWANATH MAHANTY

for the award of the degree

of

DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOTECHNOLOGY

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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Dedicated
to
My Parents

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

DEPARTMENT OF BIOTECHNOLOGY

STATEMENT

I do hereby declare that the matter embodied in this thesis is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Kannan Pakshirajan and Dr. Veeranki Venkata Dasu.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

Date: 21st January, 2009. **Biswanath Mahanty**

INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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CERTIFICATE

It is certified that the work described in this thesis entitled "***Studies on biodegradation of pyrene by Mycobacterium frederiksbergense***" by Biswanath Mahanty for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Department of Biotechnology, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for a degree.

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ACKNOWLEDGEMENTS

I first wish to thank my loving parents, my siblings and my in-laws for being near to my heart and providing the support and encouragement.

It is with my deepest sense of appreciation that I express my heartiest acknowledgement to my research supervisors, Dr. K. Pakshirajan and Dr. V.V. Dasu, Department of Biotechnology, for their continuous care, support and encouragement throughout my research work. I must acknowledge the unconditional freedom to think, plan, execute and express, that I was given in every step of my research work, while keeping faith and confidence on my capabilities.

I am thankful to my doctoral committee members, Dr. P. Goswami, Dr. R. Swaminathan and Dr. P. Saha for their constructive criticism and precious suggestions. I owe my gratitude to the Department of Biotechnology, IIT Guwahati for providing me with the necessary facilities.

I would express thank to my friends Atul, Gopi, Ravi, Satish and Siva for their valuable suggestions; Achlesh, Kasturi, Kausik, Meher, Naresh, Pallab, Ravi, Sahu, Sanjay and Sukhwinder for providing me moral support whenever I needed.

How can I forget my "Jadavpur University", my friends over there? They are and will be always there in my soul. I must acknowledge my friends for their constant feeds.

Naturally, I need not acknowledge my wife Shampa, who is always there in my life. However, I have to thank her for joining with me in Guwahati, which involved delaying some of her own goals, for supporting me in every aspect of life, and for patiently hearing more about Pyrene and TPPBs than any 'normal' human being would ever want to.

Date: 21st January, 2009. **Biswanath Mahanty**

ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants produced via natural and anthropogenic sources, mainly generated during the incomplete combustion of solid and liquid fuels or derived from industrial activities. Due to high hydrophobicity and recalcitrant nature, they tend to contaminate soil and water and pose serious threat to receiving environment because of their mutagenicity and carcinogenicity. Of the various treatment methods for PAHs, biodegradation have been shown to be more successful, eco-friendly and cost effective one.

In this study, *Mycobacterium frederiksbergense*, preliminarily known to degrade pyrene, was investigated of its potential to degrade this model PAH compound, in single and mixed substrate condition, employing three different biodegradation systems. In the first slurry phase degradation system containing pyrene as the sole substrate, the *Mycobacterium* showed a lag of 48 h in pyrene degradation in batch shake flask for an initial concentration of 50 mg l^{-1}. Immediately following this lag period, pyrene was actively degraded with an observed rate of 19.86 mg l^{-1} d^{-1}. However, in fermenter, pyrene was degraded without any lag phase with an over all rate of 6 mg l^{-1} d^{-1}. To study biodegradation of pyrene in a ternary mixture along with naphthalene and anthracene in this slurry phase system, a 2^3 full factorial design of experiments was employed. Statistical analyses of the PAHs degradation rates by the culture performed in the form of analysis of variance (ANOVA) and student's t-test gave interpretation of main and interaction effects of the substrates.

ABSTRACT

In surfactant aided system for biodegradation of pyrene, initially five synthetic chemical surfactants viz. TritonX-100, Tween 80, Tween 20, sodium dodecyl sulphate (SDS) and cetyl trimethyl ammonium bromide (CTAB) were evaluated of their molar solubilisation ratio for pyrene, which inferred superiority of Tween 80 over others. Also, a biosurfactant produced by an indigenous microbial culture isolated from the soil of a gasoline filling station, was comparatively evaluated of its efficiency to solubilise pyrene. The partially purified biosurfactant also showed emulsification activity, stability at different temperature, pH towards a range of solvents. However, the culture failed to biodegrade pyrene. Tween 80 aided pyrene biodegradation in batch shake flask indicated a prolonged lag phase, which was however absent in fermenter experiments with a maximum degradation rate at 25 mg l^{-1} initial pyrene concentration. Results of Tween 80 aided mixed PAHs biodegradation studies with pyrene, naphthalene and anthracene were also analysed in terms of their main and interaction effects on their biodegradation.

To further evaluate the potential of the mycobacterium in biodegradation of pyrene, two liquid phase partitioning bioreactor (TPPB) system was developed. After an initial screening procedure, silicone oil was chosen as the non-aqueous phase liquid in the TPPB system. Further, results of hydrodynamic study in this TPPB system indicated the best operating conditions to be 1.5 vvm of aeration rate, 600 rpm agitation rate, with silicone oil fraction of 0.2. At this optimized sets of operating conditions in the TPPB system, biodegradation study carried out at different initial pyrene concentration in silicone oil (200-1000 mg l^{-1}) gave very high pyrene degradation rates as high as 270 mg l^{-1} d^{-1}. The pyrene degradation profiles at the various concentration levels were also fitted

to several kinetics models found in literature, and it was observed that three-half order kinetic model fitted the best compared to others.

From a simple cost-benefit analysis of the three biodegradation systems investigated in this work was performed considering the cost of inoculum preparation, media ingredients and its preparation (if any), power requirements while running the batch experiments in shake-flask or in bioreactor, the TPPB system proved to be the most effective.

However, to overcome certain limitations in the conventional TPPB system, pyrene in silicone oil was encapsulated into alginate-polyvinyl alcohol hydrogel for its biodegradation by the *Mycobacterium*. For this non-conventional TPPB system, five different bead types were prepared and individually characterized for their suitability in this study. The utility of the best pyrene encapsulated bead type in this non-conventional TPPB system was further confirmed by performing pyrene biodegradation experiments in shake flask.

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ABBREVIATIONS AND NOTATIONS

1-PY	1-pyrenol
AHR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
AOP	Advanced oxidation process
BaP	Benzo[a]pyrene
BES	Bis(ethyl hexyl) sebacate
BH	Bushnell Hass
BHI	Brain Heart infusion
CD	Cyclodextrin
CFMM	Carbon free minimal medium
CMC	Critical micelle concentration
CMCD	Carboxymethyl cyclodextrins
CTAB	Cetyltrimethyl ammonium bromide
CYP1A	Cytochrome P4501A
CYP450	Cytochrome P450
DCM	Dichloromethane
DF	Degree of freedom
DMSO	Dimethyl sulphoxide
DO	Dissolved oxygen concentration
DOE	Design of experiment
EROD	Ethoxyresorufin-o-deethylase
EVA	Ethylene-co-vinyl acetate
FF	Fixed wavelength fluorescence
HMW	High molecular weight
HPCD	Hydroxypropyl-β-cyclodextrin
HRP	Horseradish peroxidase
IPM	Isopropyl myristate

LMW	Low molecular weight
MnP	Manganese peroxidase
MS	Mean sum of squares
MSM	Minimal salt media
MSR	Molar solubilisation ratio
NAPL	Non-aqueous phase liquid
NB	Nutrient broth
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffer saline
PHWE	Pressurized hot water extraction
POP	Persistent organic pollutant
PQ	Pyrene quinone
PVA	Polyvinyl alcohol
SB	Styrene co-butadiene
SCE	Supercritical extraction
SCF	Super-critical fluid
SDS	Sodium n-dodecyl sulfate
SEM	Scanning electron microscopy
SF	Synchronous fluorescence
SS	Sum of squares
SVE	Soil vapor extraction
SVOC	Semi-volatile organic contaminant
SVTD	Soil venting-thermal desorption
TPAH	Total polycyclic aromatic hydrocarbon
TPPB	Two phase partitioning bioreactor
USEPA	United state environmental protection agency

C_f	Final PAH concentration (mg l^{-1})
C_i	Initial PAH concentration (mg l^{-1})
D_{OA}	Partition coefficient of oxygen between silicone oil and water
E	Enhancement factor of oxygen mass transfer in two-liquid phase system
k_1	First-order proportionality constant (h^{-1})
k_2	Second-order proportionality constant (h^{-2})
K_d	Decay constant for emulsion stability (d^{-1})
k_La	Oxygen mass transfer coefficient (h^{-1})
k_La_A	Volumetric oxygen mass transfer coefficients in aqueous phase (h^{-1})
k_La_O	Volumetric oxygen mass transfer coefficients in organic phase (h^{-1})
k_La_{TP}	Overall volumetric oxygen transfer coefficient (h^{-1})
K_{ow}	Octanol water partition coefficient
M^*	Equilibrium saturation concentrations of pyrene aqueous phase (mg l^{-1})
M_0	Initial amount of pyrene in the aqueous phase (mg l^{-1})
M_d	Residual pyrene content in the gelling medium after encapsulation (mg)
M_i	Initial pyrene content in the hydrogel mixture before encapsulation (mg)
M_t	Amount of pyrene released at time t
n	Release exponent for exponential release kinetics
P_g	Power requirement of the aerated bioreactor (Watt)
q	Degradation of PAHs (%)
R^2	Correlation coefficient of regression
S_{CMC}	Concentration of PAH at surfactant concentration of its CMC (M)
t	Time (s)
Vs	Superficial gas velocity (m s^{-1})
W_f	Final weight of hydrated beads after swelling experiments (mg)
W_i	Initial weight of beads before swelling experiments (mg)
X_{org}	Organic phase fraction of two liquid phase system
$\Delta\lambda$	Wavelength offset in synchronous fluorescence (nm)
$\Delta\lambda_{opt}$	Optimized wavelength offset in synchronous fluorescence (nm)

λ_{opt}^{max}	Optimized excitation wavelength for maximum SF intensity (nm)
$\lambda_{emission}$	Emission wavelength (nm)
$\lambda_{excitation}$	Excitation wavelength (nm)

CHAPTER 1

INTRODUCTION

1.1. Generalities

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous pollutants produced via natural and anthropogenic sources, generated mainly during the incomplete combustion of solid and liquid fuels or derived from industrial activities. PAHs cause contamination of soil and groundwater as they tend to adsorb and concentrate in the solid phase due to their high hydrophobicity and recalcitrance. They are harmful to environment and human health due to their mutagenicity, carcinogenicity and tendency to bioaccumulate in the food chain. This potential toxicity coupled with their persistency in soil, makes PAHs a class of problematic environmental contaminants (Cerniglia, 1992; Sutherland, 1992) of which about sixteen are listed in the US Environmental Protection Agency priority pollutant list (Kanaly and Harayama, 2000).

Carcinogenic and mutagenic properties of PAHs are known to vary with number of aromatic rings in their structure. The two-ringed naphthalene, three-ringed phenanthrene and anthracene are neither genotoxic nor carcinogenic; on the other hand, four-ringed as well as the five-ringed compounds benzanthracene and benzo[a]pyrene are both genotoxic and carcinogenic (Verschueren, 1983; Dibble *et al.*, 1990). Even though pyrene is not carcinogenic, it is still genotoxic and its quinone metabolites are mutagenic

and are more toxic than the parent compound. Pyrene has been used as an indicator of PAH pollution, and since its structure is found in the molecules of carcinogenic PAHs, it has been used as a model compound to study the biological degradation of PAHs (Ravelet *et al.*, 2000).

Various treatment methods have been demonstrated for treatment of PAH contaminated environments, which are either *in-situ* or *ex-situ* based on the site characteristics and technical feasibility of the treatment approaches. Important treatment technologies that have come up in the recent times, include but not limited to physical methods like, soil-vapor extraction, hot water extraction, super critical extraction and chemical methods like Fenton oxidation, ozonization. Some of these methods are briefed below:

Soil vapor extraction (SVE)

This technique consists in the application of vacuum to the soil matrix producing air flow in the soil, which due to free volatilization, desorption from the soil and dissolution from the aqueous phase transports the contaminants to extraction wells. Before reaching the atmosphere, the gas usually is treated to protect the air quality. This is one of the most widely used *in-situ* technology, and is considered simple, relatively cheap and very efficient in removing volatile and semi volatile organic compounds from the unsaturated soil. However, this method is found unsuitable for high molecular weight (HMW) PAHs because of their less volatility. Moreover, important limitation of this particular remediation technology is the difficulty in predicting the time required for remediating a contaminated site (Albergaria *et al.*, 2006).

Hot water extraction

Pressurized hot water extraction (PHWE) has been used largely in recent past for extracting hydrophobic PAHs from contaminated soil and sediments (Smith, 2002; Rivas *et al.*, 2008). In PHWE, the temperature of water is maintained in between 100°C and the critical temperature (T_c) at 374°C. As the temperature of water increases, its polarity decreases such that at 250°C the dielectric constant of water is equal to the dielectric constant of methanol under ambient conditions (Andersson *et al.*, 2002).

Supercritical extraction

Supercritical extraction employs supercritical fluids (SCFs) such as carbon dioxide (mixed with a co-solvent - methanol) or water for the extraction of volatile and persistent organic pollutants (POPs) including PAHs from environmental matrices (Anitescu and Tavlarides, 2006). In this technique, the SCF is chosen in such a way that the solvent possess a higher affinity for contaminants than the accompanying solid matrix together with enticing SCF properties, i.e. liquid-like density, low viscosity, high diffusivity and no surface tension, to extract contaminants at optimum temperature, pressure and flow rate conditions. SCE does not lead to destruction of contaminants, but extracted pollutants are highly concentrated, and in which form, they could, however, be subsequently destroyed more cost effectively (Anitescu and Tavlarides, 2006).

Fenton oxidation

In this technique, hydrogen peroxide (H_2O_2), a very strong non-selective liquid oxidizing agent with standard oxidation potential about 2.8 V, is used for the production

of hydroxyl radicals (·OH), which can react with PAHs for complete breakdown of the compounds. In Fenton's reagent, hydrogen peroxide is dosed together with a solution of a transition metal (typically iron) which dramatically increases the peroxide oxidative strength by enhancing the radical formation (Flotron *et al.*, 2005; Watts *et al.*, 2005). The typical Fenton's reaction is based on the hydrogen peroxide decomposition into hydroxyl radicals in the presence of ferrous iron. In modified Fenton's system, the radical formation is enhanced by the addition of chelating agents and/or by high peroxide concentrations where many complex reactions are involved and produces hydroxyl radicals and numerous other reacting species including hydroperoxide radicals ($HO_2^{\bullet}$), superoxide anions (O_2^{-}) and hydroperoxide anions (HO_2^{-}) (Watts *et al.*, 2005). As peroxide oxidation is exothermic, it can enhance desorption and dissolution of sorbed pollutants, making them more available for the oxidation treatment

Ozonation

Ozone is a highly reactive and powerful oxidant that has been used in the chemical industry as an oxidizing agent, and is also used extensively in treating water for drinking purpose (Rositono *et al.*, 2001). There has been considerable interest in using ozone to remediate contaminated soils containing non-volatile organic compounds that are not removed by conventional soil venting. Ozone can be used in the form of gas or liquid (Choi *et al.*, 2001), which after a short period of time reverts back to atmospheric oxygen leaving no toxic residues in the soil. Ozone has been reported to be useful for the transformation of PAHs in soil (Goi and Trapido, 2004), where the intermediates are more soluble in the aqueous phase (Kornmüller and Wiesmann, 2003).

The above techniques although seem promising for successful remediation of PAHs contaminated environment, major limitations of these techniques are:

- Most of these methods, particularly the physical ones result in a mere phase change of the PAHs from one environment to the other, rather than complete degradation/removal.
- Intermediates produced by these treatment methods are known to be more hazardous than the parent compounds.
- All these methods are nonselective often requires high energy and maintenance cost, and not economically viable in most cases.

Hence, as an alternative to the physico-chemical treatment methods, biological methods are emerging to be useful in remediation of PAH contaminated sites.

1.2. Biological Methods

Of the various methods tested to treat PAHs contaminated environment, biodegradation using microbes has been proved more successful and preferred in terms of cost and treatment efficiency (Tavlarides *et al.*, 2000). Various microorganisms capable of degrading PAHs have already been isolated of which non-actinomycetes species of *Bacillus, Pseudomonas, Rhodococcus, Beijerinckia, Flavobacterium, Brevibacterium, Cycloclasticus, Sphingomonas, Burkholderia* and *Mycobacterium* are most widely studied. These microorganisms can completely degrade or partially transform PAHs depending on whether they are present individually or as a consortium (Heitkamp and Cerniglia, 1989). However complete degradation of HMW PAHs can be achieved only by few potential microorganisms (Cerniglia, 1992). Various *Mycobacterium* strains have

largely been studied in recent years (Schneider *et al.*, 1996; Rehmann *et al.*, 2001; Moody *et al.*, 2002; Herwijnen *et al.*, 2003a; Moody *et al.*, 2004) due to their very high degradation capacity. *Mycobacterium* sp. is known to have a hydrophobic cell surface, which help them to grow even in hydrophobic environments as biofilm on sorbed solid PAHs. In addition, mycobacterium can utilize PAHs as sole carbon source and does not looses this capability even in presence of easily available substrates.

In biodegradation of PAHs, the major limiting factor is the reduced bioavailability of PAHs present in the contaminated environments to the degrading microbes. Moreover, in contaminated environment a large complex mixture of both LMW and HMW PAHs and their interaction plays a crucial role on the individual PAH mineralization rate and efficiency. PAHs, in general, do not promote the growth of microbes; on the other hand, if simple C-sources are present together with PAHs, microbial population may increase, but without any significant biodegradation of PAHs. Hence, by studying the degradation kinetics of PAHs, in single and mixed substrate conditions, knowledge about the kinetic parameters can be gathered which may be helpful to understand more complex systems. The effect of various substrates on the microbial degradation of PAHs has been the subject matter of a number of studies (Kelley and Cerniglia, 1991; McNally *et al.*, 1999; Johnsen *et al.*, 2005). However, these authors, almost totally neglected the facts that (i) microorganisms in real contaminated environment encounter systems mostly containing a mixture of substrates (PAHs) (ii) nature and extent of interaction among these substrates on each other degradation depends largely whether the substrates are present in aqueous solution or as suspension or in any non-aqueous phase liquid (NAPL). These neglected facts by other researchers were also considered for research in the present thesis work.

1.2.1. Surfactant aided biodegradation of PAHs

Having understood the fact that extremely low aqueous solubility and hence low bioavailability limits the potential of PAH biodegradation by microbes (Guha and Jaffe, 1996), attempts have been made in the recent past to overcome the bioavailability limitations majorly by the use of surfactants. However, use of surfactants have resulted contrasting effects ranging from enhancement to inhibition of PAH biodegradation. This is because surfactants used in the system may serve as a preferred substrate over the PAHs or it may sequester the PAHs in micelle phase making it practically non-bioavailable (Guha and Jaffe, 1996). Surfactants are also known to exhibit negative effects on biodegradation of PAHs either due to its own toxic effects (Tiehm, 1994) or due to the increased toxicity of PAHs at higher concentrations to the degrading microorganisms. Therefore, it appears that applicability of surfactants in biodegradation of PAHs can not be made too generalized, rather sufficient efforts should be made to understand the microbial system in the context of the PAHs under investigation.

1.2.2. Two liquid phase partitioning bioreactor system

Substrate delivery in the biological treatment of PAHs is a crucial and the most significant challenge, where addition of the substrate at high a concentration will most likely inhibit or even kill the organisms, and substrate addition at too low a rate will cause the cells to starve resulting in a sub-optimal process performance (Daugulis, 2001). The situation becomes more complicated when aqueous solubility of PAHs is extremely low and concentration is not precisely regulated with conventional feedback controls. In

such scenario, sustained and controlled delivery of PAHs to the degrading microbes become exceedingly important.

The most recent and promising technology for the bioremediation of PAHs seems to be use two-phase partitioning bioreactor (TPPB) system characterized by an immiscible organic phase loaded with a target substrate that partitions into the aqueous phase based on equilibrium considerations and real-time demand of the organisms (Daugulis, 2001), so that hydrophobic pollutants are delivered in the aqueous phase at sub-inhibitory levels to the microorganisms. TPPB system is a two-step process that involves initial partitioning of substrate from its highly soluble non-aqueous phase liquid (NAPL) to aqueous phase, followed by subsequent microbial metabolism. Hydrophobic nature of PAHs makes them ideal candidates for degradation in TPPB systems where high concentration and large surface areas can be achieved by dissolving PAHs in the dispersed organic phase (Daugulis and Janikowski, 2002; Marcoux *et al.*, 2000).

1.2.2.1. Critical design criteria for TPPB in biodegradation of PAHs

Selection of a suitable organic phase

The selection of organic phases is crucial particularly when dealing with a new contaminant and a new microbial strain; in addition to being non-volatile, inexpensive and readily available, the selected organic phase must be safe, stable, biocompatible and non-bioavailable. The last two properties of organic phase are most crucial and must be considered in the context of microorganism being employed in the TPPB system. The ability of microorganisms to grow and survive in an organic solvent is also related to the solvent polarity; logarithm of octanol-water partition coefficient (log P_{ow}) is currently

considered as one of the best measure of solvent biocompatibility, and solvents with high log P_{ow} values are generally regarded more biocompatible and safer than others with low log P_{ow} values. Being highly biocompatible, chosen solvent should also have a high solubility for the PAH with mass partition coefficient with a value as high as that is required for the aqueous phase concentration to remain at sub-inhibitory level. Another important design criterion is the increased surface area resulting from emulsion formation between the NAPL and the aqueous phase in the TPPB system. The increased surface area may be beneficial for PAH biodegradation, but if the emulsion is highly stable, it may create major problem in sampling of the two phases for analysis purpose.

Determination of optimum set of operating conditions

Microbial degradation rate of PAHs in TPPB largely relies upon the mass-transfer rate of substrate from the NAPL to the aqueous phase, which is mainly governed by the size of the interfacial area. The interfacial area in turn is a function of surface averaged droplet diameter and dispersed-phase volume fraction (phase ratio). Optimal phase ratio that will generate the highest interfacial area is influenced by the geometry of the bioreactor, such as length and diameter, the properties of the NAPL (density, viscosity etc.), and the agitation and aeration rates. Though increase in organic phase fraction is supposed to increase liquid-liquid interfacial area, it also increases the NAPL drop diameter, and for this reason optimal phase fraction in the system must be determined. Another interlinked parameter known to influence the performance of a TPPB system is the agitation rate. Increase in agitation intensity often results in decrease in the Sauter mean diameter and increase in the number of droplets. However, the distributions of drop

sizes, as well as its mean diameter are resultant of two simultaneous processes – break-up and coalescence of drops: high mixing rate produces smaller NAPL drops, but also multiply the probability of drop contact and coalescence. Furthermore, higher agitation rates may prevent contact between microorganisms and PAH-containing NAPL droplets, and the resulting higher shear stress could also be detrimental to the microorganisms. Quite obviously, in aerobic cultures, the oxygen transport is also linked to these set of operating conditions in a TPPB system and may get affected due to sub-optimal levels of the parameters.

Many studies have been reported regarding application of TPPB system for biodegradation of PAHs. However, little attention has been given towards proper step-wise selection of solvent and, more importantly, on arriving at the best set of operating conditions. In this present study, due attention has been given on solvent selection as well as on selecting the correct levels of operating parameters in relation to oxygen mass transfer in the TPPB system. Since most of the earlier studies have been limited to the use of the system in a more conventional way, potential of this system has not been fully realized. Hence, there is a need to develop and evaluate non-conventional TPPB methods for PAH biodegradation to keep the process cost at a minimum without compromising on the treatment efficiency.

1.3. Objective and Scope

Based on an extensive literature review on remediation of PAH contaminated environment, the present study focused on biodegradation of pyrene, both in single and mixed substrate conditions, by *Mycobacterium frederiksbergense.* In order to achieve this

objective, three different systems were investigated and evaluated of their potential. Following are the detailed investigations performed in the present research:

- Biodegradation of pyrene in slurry phase system by *M. frederiksbergense* under single and mixed PAHs conditions.
- Evaluation of different surfactants in enhancing pyrene bioavailability for biodegradation by the mycobacterium.
- Feasibility study to enhance the aqueous solubility of pyrene and its biodegradation using an indigenous mixed culture capable of producing biosurfactant.
- Evaluation of surfactant aided system in biodegradation of pyrene by *M. frederiksbergense* in single and mixed substrate conditions.
- Development and evaluation of a two liquid phase partitioning bioreactor system in biodegradation of pyrene by *M. frederiksbergense*.
- Cost-benefit analysis and comparison of the three systems investigated in the present research.
- Development and evaluation of a non conventional TPPB system for biodegradation of pyrene by *M. frederiksbergense*.

1.4. Organization of the Thesis

The presentation of the work has been divided into five chapters. The current **Chapter 1** presents a general introduction, objective and scope of the present work. While the literature that supports the present work is presented in **Chapter 2;** details of the materials and methods adopted in the present study are described in **Chapter 3**. **Chapter 3** essentially details the slurry phase, surfactant aided and TPPB systems for

biodegradation of pyrene by the mycobacterium. It also provides technical information about the analytical methods adopted in this present work. **Chapter 4** contains the results and discussions, where the results of pyrene degradation in slurry phase, surfactant aided system and TPPB system are presented and thoroughly discussed. This chapter addresses pyrene biodegradation, both in single and mixed substrate condition in all the systems. It also compares the efficiency of the three systems in terms of cost benefit analysis. Finally, the chapter emphasizes the development of a solvent encapsulated hydrogel system for biodegradation of pyrene. **Chapter 5** draws summary and appropriate conclusion based on the previous results and discussion; this chapter also provides some useful recommendations for researchers to carry out work in this field.

CHAPTER 2

LITERATURE REVIEW

2.1. Polycyclic Aromatic Hydrocarbons (PAHs) and the Environment

2.1.1. Physico-chemical properties of PAHs

The term PAH, in general, refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983). PAHs are highly persistent in the environment due to their high hydrophobicity or low water solubility (Cerniglia, 1992). Generally, with an increase in number of fused rings in PAHs while solubility and volatility decreases, hydrophobicity of the compound increases (Wilson and Jones, 1993).

Octanol water partition coefficient (K_{ow}) is used to represent the hydrophobicity of any compound, and compounds having high K_{ow} are known to absorb onto lipophilic substances. PAHs, according to the USEPA, are also defined as priority pollutants with K_{ow} values ranging from 3-6.5 and considered as the most lipophilic compounds. PAHs tend to adsorb in organic rich sediments, bioaccumulate and become less bioavailabile for degradation. Common properties and ring structures of some representative PAHs are presented in Table 2.1 and Figure 2.1, respectively.

Table 2.1: Physicochemical properties* of some important PAHs.

PAHs	No of rings	Melting point (°C)	Boiling point (°C)	water solubility (mg l^{-1})	log (K_{ow})
Naphthalene	2	80.2	218	32.0	3.37
Anthracene	3	216	340	0.07	4.45
Phenanthrene	3	101	340	1.29	4.46
Pyrene	4	149	360	0.14	5.32
Chrysene	4	255	488	0.002	5.61
Benzo[a]pyrene	5	179	496	0.0038	6.04
Indeno [1,2,3-c,d]pyrene	6	163	536	0.062	7.66

*Sims and Overcash, 1983.

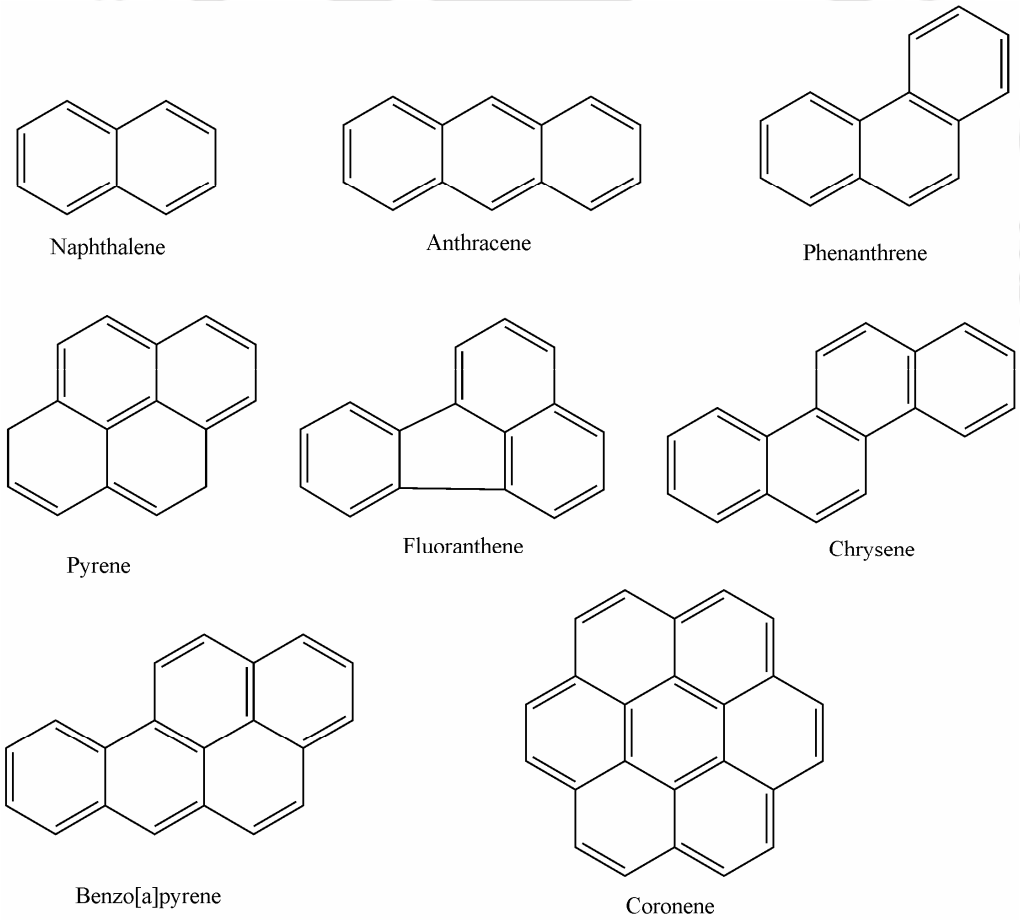


Figure 2.1: Chemical structure of some PAHs commonly encountered in the environment.

2.1.2. Sources and distribution of PAHs in the environment

PAHs originate from both natural and anthropogenic sources. Natural sources of PAHs include forest-fires, natural petroleum seeps and post-depositional transformation of biogenic precursors (Young and Cerniglia, 1995). Anthropogenic source of PAHs include combustion of fossil fuels (coal and petroleum) or wood (pyrolytic) and crude/refined petroleum through accidental oil spills, discharge from routine tanker operations (petrogenic) (Zakaria *et al.*, 2002; Kannan *et al.*, 2005). However, among these various natural and anthropogenic sources, emission from the latter is considered as the major source of these highly recalcitrant compounds in the environment. In recent literature, there are strong indications that the three PAHs naphthalene, phenanthrene and perylene are produced biologically in addition to the anthropogenic sources. Biological production of naphthalene has been concluded from its presence in *Magnolia* flowers (Azuma *et al.*, 1996) or flower scents of different *Annonaceae* species from the Amazon rain forest (Jürgens *et al.*, 2000). *Muscodor vitigenus,* an endophytic fungus growing in the Peruvian Amazon region have shown production of naphthalene (Daisy *et al.*, 2002). High naphthalene concentrations in *Coptotermes formosanus* termite nests of subtropical North America and nests of various termite genera from tropical Brazil suggest naphthalene synthesis by termites or associated microorganisms (Chen *et al.*, 1998; Wilcke *et al.*, 2003, 2004). There were early indications that phenanthrene can be produced biologically from alkyl-phenanthrene precursors in plant debris (Sims and Overcash, 1983). Perylene is known to be produced biologically in anaerobic environments in soils and sediments (Wilcke *et al.*, 2002)

PAHs are ubiquitously distributed in the environment, and have been detected in number of environmental samples, including air, water, soil, sediments and also in foods, oils and tars (Gocht *et al.*, 2001; Curtosi *et al.*, 2007; Shi *et al.*, 2005; Moret *et al.*, 2000; Guillen *et al.*, 2004; Ibáñez *et al.*, 2005). As noted earlier the main source of PAHs in the environment is of anthropogenic origin, namely combustion of fossil fuels, such as petroleum and coal (Wilcke, 2000; Bamforth and Singleton, 2005). Natural pyrogenic sources like vegetation fires (Vila-Escalé *et al.*, 2007; Olivella *et al.*, 2006) or volcanic eruptions (Stracquadanio *et al.*, 2003) are known to be additional sources of PAHs, but to a lesser extent. The concentration of PAHs in the contaminated environment thus depends on its proximity to the production source, level of urbanization and industrial activity and mode of transport (Kanaly and Harayama, 2000). Among the different environment, soil is an important reservoir for PAHs (Ockenden *et al.*, 2003), and both gaseous and particulate PAHs are input to the soil by wet/dry atmospheric deposition at over short and long distances (Park *et al.*, 2000), where these compounds accumulate mainly in the humus layer. PAH concentration in soil and sediments varies largely from few μg kg^{-1} to as high as mg kg^{-1} of soil. Table 2.2 presents some important findings from the literature on PAHs concentrations found in soils of different places. In a recent study by Wilcke (2007) on global patterns of PAHs in soil samples, collected from 12 different geographic regions, total PAH concentrations ranged between 4.8 and 186,000 μg kg^{-1}.

Table 2.2: Concentrations of PAHs in soil as reported in the literature.

Site	Total PAH (μg kg^{-1})	References
China	22-1,257	Hao *et al.*, 2007
Antarctica	41-8105	Aislabie, 1999
Western Europe	11 - 200	Atanassova and Brümmer, 2004
Hangzhou City, China	60-616	Chen *et al.*, 2004
Beijing, China	366-27,825	Tang *et al.*, 2005
Costa Rica	5	Daly *et al.*, 2007
India	3-28	Masih and Taneja, 2006
Hong Kong	ND* - 19,500	Chung *et al.*, 2007
Malaysia	110–330	Omar *et al.*, 2002
Japan	0.41–611	Yang *et al.*, 2002
South Korea	23-2,834	Nam *et al.*, 2003
Switzerland	50-619	Bucheli *et al.*, 2004
Nepal	184-10,279	Aichner *et al.*, 2007

* ND-not detected

2.1.3. Toxicity of PAHs

Many PAHs have toxic, mutagenic and/or carcinogenic properties, but numerous studies have indicated that one-, two- and three-ring compounds are acutely toxic (Sims and Overcash, 1983) and HMW PAHs are considered to be genotoxic (Lijinsky, 1991; Mersch-Sundermann *et al.*, 1992). Being highly lipid-soluble, these are readily absorbed from the gastro-intestinal tract of mammals (Cerniglia, 1984) and are rapidly distributed in a wide variety of tissues with a tendency for localization in body fat. PAH intermediates produced by incomplete degradation pose further potential risk for humans (Kazunga and Aitken, 2000).

The photo-oxidized PAHs are in many cases more toxic than the parent compounds (McConkey *et al.*, 1997). Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step (Stegeman *et al.*, 2001*)*.

A variety of PAHs taken up in the human body undergo metabolic activation. The initial step in the metabolism of PAHs involves the multifunctional P-450 enzyme system forming different epoxides, which are short-lived compounds and may rearrange spontaneously to phenols or undergo hydrolysis to dihydrodiols. These products may then be conjugated with glutathione, glucuronic acid or sulfuric acid for easy excretion. However, the dihydrodiols may also act as a substrate for cytochrome P-450 again to form new dihydrodiol epoxides, which are unfortunately poor substrates for further hydrolysis. These dihydrodiol epoxides may instead react with proteins, RNA and, most seriously, DNA, thus causing mutations and possibly cancer (Xue and Warshawsky, 2005).

PAHs are seldom encountered individually in the environment and many interactions occur within a mixture of PAHs whereby the potency of known genotoxic and carcinogenic PAHs can be enhanced (Kaiser, 1997). PAHs may also interact in the carcinogenic process, for example, promoting cellular proliferation (Delistraty, 1997). As a group, PAHs have shown varying ability to induce cancer; hence it is difficult to identify the structural features associated with their carcinogenic activity. However, for unsubstituted PAHs, it seems that a minimum of four benzene rings is required to exhibit carcinogenic activity (Pickering, 1999), but it still unproved that all PAHs with four benzene rings are carcinogenic. Some PAHs are very weak while others are strongly

carcinogenic, *e.g.* benzo[*a*]pyrene. Structure-activity relationships become even more complex when substitution of the molecular structure occurs; for example, although benz[*a*]anthracene is a fairly weak carcinogen, 7,12-dimethylbenz[*a*]anthracene is a very potent carcinogen. Furthermore, some environmental transformation products of PAHs may react directly with DNA causing mutations and possibly cancer without the need for metabolic activation (Moller *et al.*, 1985).

The toxicity of naphthalene has been reported in laboratory animals (Goldman *et al.*, 2001), and is shown to bind covalently with molecules in liver, kidney and lung tissues, thereby enhancing its toxicity from being a simple inhibitor of mitochondrial respiration (Falahatpisheh *et al.*, 2001). Acute naphthalene poisoning in humans can lead to haemolytic anaemia and nephrotoxicity, which is in addition to dermal and ophthalmologic changes in populations occupationally exposed to this compound. Phenanthrene, a three member ring PAH, is known to be a photosensitizer of human skin, a mild allergen and mutagenic to bacterial systems under specific conditions. It is a weak inducer of sister chromatid exchanges and a potent inhibitor of gap junctional intercellular communication (Weis *et al.*, 1998*)*. The toxicity of benzo[a]pyrene, benzo[a]anthracene, benzo(b)fluoranthene, benzo(k)fluranthene, dibenz(a,h)anthracene and indeno(1,2,3-c,d)pyrene are also studied and shown that they are carcinogenic (Liu *et al.*, 2001). Information on toxicity of other PAHs such as acenaphthene, fluranthene and flourene with respect to their toxicity to mammals is still unknown.

Pyrene has often been considered as a model compound for toxicity studies as its structure is often found in the molecules of other highly carcinogenic PAHs.

Brown *et al.* (2004) studied toxic effect of pyrene on survival, reproduction, ethoxyresorufin-*o*-deethylase (EROD) activity (to measure the catalytic activity of cytochrome P4501a) and catalase activity of earthworm *Lumbricus rubellus* in contact and soil tests. The authors observed that at higher pyrene concentrations there was a steady concentration related decrease in survival and calculated LC_{50} values of 6.8 mg l^{-1} for the contact test and 283 mg kg^{-1} in the soil test from the survival data. Cocoon production rate was significantly reduced compared to controls in the soil test at higher concentrations and was completely ceased at 640 mg kg^{-1}. Though, no EROD activity could be detected, catalase activity in the soil test was also significantly lower at the above concentration compared to all other treatments and the control.

Krasnov *et al.* (2005) studied toxic effect of pyrene on the transcriptomes of juvenile rainbow trout kidneys and livers by exposing the fish to sub-lethal doses for 4 d and measuring expression of 1273 genes using a cDNA microarray. The authors reported chemical toxicity in metallothionein and mitochondrial proteins of oxidative phosphorylation. Expression of mitochondrial and heat shock proteins were stimulated, whereas genes involved in humoral immune response and apoptosis were suppressed. Pyrene affected mainly genes implicated in the maintenance of the genetic apparatus, immune response, glycolysis and iron homeostasis.

Incardona *et al.* (2006) studied mechanism of developmental toxicity of pyrene in zebrafish, and they observed activation of the aryl hydrocarbon receptor (AHR) pathway by pyrene resulting in induction of cytochrome P4501A (CYP1A). The authors observed induced CYP1A expression throughout the vascular endothelium, including the majority of blood vessels in the head and trunk, and in developing hepatocytes, when exposed to

pyrene. Continuous exposure to pyrene (soon after fertilization) resulted in a syndrome of systemic toxicity in early larval stages. The visible signs of pyrene toxicity included dorsal curvature of the body axis, reduced peripheral circulation, anemia, pericardial edema that evolves into yolk sac edema, and cell death beginning in the brain and later involving the spinal cord. Pyrene-exposed larvae began to die during the fifth day when the liver of pyrene exposed larvae appeared opaque with irregular margins, appeared congested with enlarged vacuolated hepatocytes.

Petersen and Dahllöf (2007) studied potential toxicity of pyrene on natural algae from an arctic sediment from shallow-water marine bay. The authors observed direct toxicity of pyrene affecting the algal community reflected in decreased ^{14}C-incorporation. The decrease was most pronounced under light where a decrease to 34% compared to that of control (without pyrene) was observed. Ammonium, nitrate and silicate uptake by the algae and its total DNA content was significantly decreased in the presence of pyrene as a result of decreased growth leading to increased algal death.

Stabenau *et al.* (2008) studied effect of pyrene exposure in the leopard frog (*Rana pipiens*). After exposure for seven days in pyrene saturated water aquaria, the authors measured exercise duration, muscle contractile ability, glycogen levels and mitochondrial respiration, which revealed that pyrene exposure produced many adverse effects in leopard frogs including significant reductions in exercise performance, muscle contractile ability and alterations to muscle mitochondrial oxygen consumption.

2.1.4. Analysis of PAH in environmental samples

Currently available methods for quantitative estimation of PAHs are by HPLC, GC, LC-MS and GC-MS (Song *et al.*, 2002). Although well established, these methods are usually found tedious, time-consuming and cost intensive sample preparation or pretreatment consisting of an extraction followed by one or more purification steps, namely column chromatography, solid-phase extraction (Martinez *et al*., 2004).

From an analytical perspective, PAHs has shown to exhibit fluorescence properties, which allow their determination to be carried out even at very low concentrations. However, conventional fluorescence methods involving excitation/emission scan have limited practical applicability as most spectra obtained from complex mixtures cannot be often resolved satisfactorily without sample pretreatment due to overlaps in the broad spectral bands (Wise *et al.*, 1993). On the other hand, in synchronous fluorimetry (SF), by scanning both excitation and emission monochromators simultaneously while maintaining a constant wavelength interval ($\Delta\lambda$), simultaneous determination of PAHs in multi-component mixtures was possible with minimum or no pretreatment (Lloyd, 1971; Reynolds, 2003; Pulgarin and Bermejo, 2003). For identification of a particular PAH, the chosen value of $\Delta\lambda$ generally corresponds to maximum fluorescence intensity. The success of SF method in quantitative estimation of a target analyte relies on proper selection of excitation wavelength, wavelength offset, excitation and emission slit widths and concentration of the analyte.

Ferrer *et al.* (1998) used SF spectra of mixtures containing ten PAHs (anthracene, benz[a]anthracene, benzo[a]pyrene, chrysene, fluoranthene, fluorene, naphthalene,

perylene, phenanthrene and pyrene) for their determination by Partial Least Squares Regression (PLSR). Different procedures have been used for the pretreatment of the data in order to obtain better models, and the size of the calibration matrix has also been studied. The best models have been used for the determination of the above mentioned PAHs in spiked natural water samples at concentration levels between 4 and 20 μg l^{-1}.

Lage-Yusty *et al.* (2005) studied application of SF for determination of 13 PAHs from a 16 PAHs mixture, using second derivative SF at constant wavelength interval mode. The detection limits were found to be < 1 μg l^{-1}.

Hua *et al.* (2006) used SF method for quantifying benzo[a]pyrene in solvent extract from spiked soil. The authors validated the SF method against HPLC and found a good correlation between the two with a detection limit of 1.6 μg l^{-1} for the SF method. Moreover, the results demonstrated potential of SF method as a sensitive, accurate, rapid, simple, economic and efficient alternative to HPLC for detection and quantification of BaP in complex soil extracts.

Liu *et al.* (2006) studied SF spectra of pyrene in aqueous solution in concentration ranges of 1-50 μg l^{-1}. The authors observed that a wavelength difference ($\Delta\lambda$) of 38 nm between excitation and emission wavelengths was suitable for effective determination of pyrene and the peak was observed at excitation wavelength 335 nm. A linear relationship between SF intensity and concentration of pyrene in aqueous solution was thus established.

Xie *et al.* (2007) proposed SF with multiple standard additions (SFMSA) as a new method for determination of pyrene in mucus without any pretreatment. The authors

reported detection limit for pyrene as 0.47 µg l^{-1} with linear in the range up to 50 µg l^{-1}. The authors observed good agreement between SFMSA and GC/MS upon validation.

Tairova *et al.* (2009) evaluated the possibility of using the pyrene metabolite 1-hydroxypyrene as a biomarker of phenanthrene, anthracene, pyrene, benzo[a]pyrene and benzo[k]flouranthene exposure in the marine polychaete *Nereis diversicolor*, where concentrations of parent PAHs and 1-hydroxypyrene were measured after five days using HPLC with fluorescence detection (HPLC/F), SF and GC-MS. The SF measurements of 1-hydroxypyrene were validated by HPLC/F method where positive and significant correlation between these two was confirmed.

2.2. Remediation of PAHs Contaminated Environment

PAHs are characterized by their intrinsic hydrophobic nature and tendency to get adsorbed onto solid particulates, particularly in the organic fraction of the solids (Kohl *et al.*, 2005). Most influencing parameters affecting PAHs sorption onto soils are the solubility of the different PAHs and organic fraction content in soil. Other factors influencing their adsorption to the soil are temperature, salinity or the presence of dissolved organic matter (Huang *et al.*, 2003). Intimately related to the adsorption process is the sequestration phenomenon, where adsorbed hydrophobic substances show a declining availability from soil. The toxicity values of sequestered molecules are, obviously, quite different from those assigned to dissolved substances or weakly adsorbed molecules. Physically, this phenomenon consists in a migration of adsorbed substances into condensed organic matter and inaccessible micropores present in the soil (Brion and Pelletier, 2005). Various physical, chemical and biological methods either *ex-*

situ or in-situ for remediation of PAHs contaminated environments have been proposed (Venkata-Mohan *et al.*, 2006).

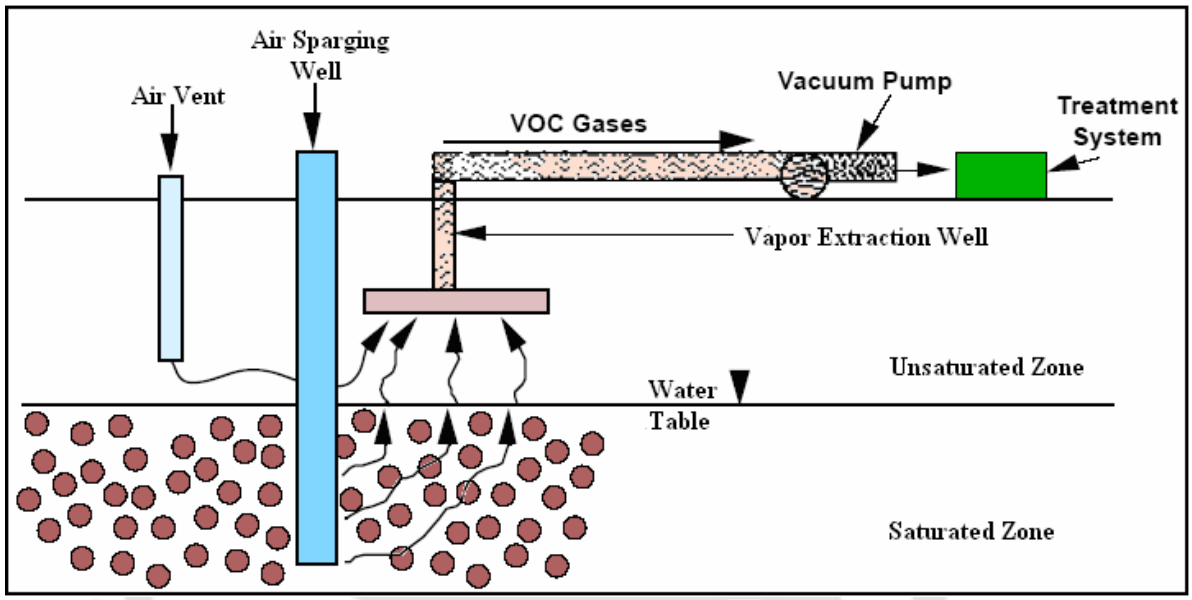
2.2.1. Physical methods

Of the various physical methods, incineration, soil vapor extractions (SVE), thermal de-sorption, soil washing/solvent extraction, disposal at hazardous land fills or deep well injection are quite well studied (Lagadec *et al.*, 2000). While each of these techniques has some advantages, there are serious concerns regarding their usage either from the consideration of the environment or economics or both. Incineration is the only destruction technology that completely degrades the toxic residues on soil, but is expensive. Similarly, solvent extraction requires expensive solvent regeneration, and thermal desorption produces air pollutants, which often necessitates secondary treatment of the off gases (Sahle-Demessie and Richardson, 2000). On the other hand, land filling and deep well injection methods are mere transfer of pollutants from one phase to the other and only postpones the PAH pollution problem to the future.

Soil vapor extraction (SVE) is one of the most widely used simple, efficient and cost effective *in-situ* technology used for removing volatile and semi volatile organic compounds from the contaminated soil, where vacuum is applied to the soil matrix that produces air flow in the soil, and, which due to free volatilization and desorption from the soil transports the contaminants to extraction wells (Figure 2.2). The off gas is usually treated before it reaches the atmosphere. However, this method is applicable for treating LMW PAHs and not ideally suited for HMW ones, mainly because of their less volatile nature. Moreover, important limitation of this remediation technology is the difficulty of

predicting the required time for cleaning up PAH contaminated environment (Albergaria *et al.*, 2006).

Figure 2.2: Schematic of soil vapor extraction system.



* Reproduced from USEPA technology fact sheet EPA 542-F-96-008

Harmon *et al.* (2001) conducted a feasibility study in support of a soil venting-thermal desorption (SVTD) process, which couples SVE with *in situ* heating, for remediating lampblack-impacted soil containing 11 PAHs totaling about 4100 mg kg^{-1} total PAH (TPAH). The results indicated that temperatures above 250°C were sufficient to mobilize most of the PAHs, where TPAH load in the soil was reduced to less than 100 mg kg^{-1} within 10 d.

Park *et al.* (2005) conducted laboratory and field pilot studies to evaluate the effectiveness of soil vapor extraction (SVE) system for the removal of semi-volatile organic contaminants (SVOCs) including PAHs from soils. They found that increased rate of air flow results in higher removal of contaminants, which, however, resulted in mass transfer limited volatilization at very high air flow rate. Field pilot study of a hot air

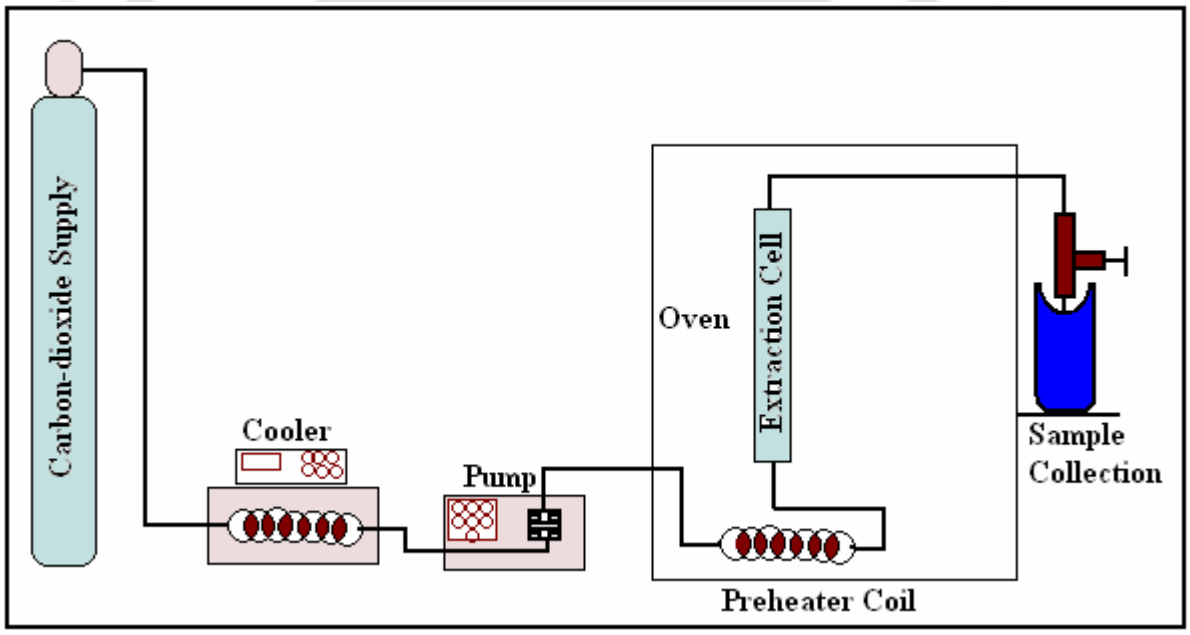
injection for the remediation of diesel-contaminated soil showed dramatic reduction of total TPAH concentrations (> 95%) within 30 d of test operation.

Hot water extraction or pressurized hot water extraction (PHWE) have been used largely in the recent past for extraction of hydrophobic PAHs from contaminated soil and sediments (Andersson *et al.*, 2002; Smith, 2002; Rivas *et al.*, 2008). In PHWE, the temperature of water is kept at 100°C and the critical temperature (T_c) at 374°C. Furthermore, the solvating properties of water are easily altered through changes in temperature and pressure. For example, at 250 °C, the dielectric constant of water is equal to the dielectric constant of methanol under ambient conditions (Andersson *et al.*, 2002), and, therefore, more polar contaminants can be extracted at lower temperatures; where as contaminants with low polarity could be extracted at higher temperatures, thus providing possibility of class selective extraction using this technique (Yang *et al.*, 1997). The major advantage of PHWE is because of the use of water, which is known to be cheaper and far more environmentally compatible than any known solvent. Dadkhah and Akgerman (2002) studied a small-scale batch extraction with/without *in situ* wet oxidation of PAHs in spiked and aged soils using subcritical water. They observed that removal of phenanthrene, anthracene, chrysene and benzo[a]pyrene from spiked soil in extraction-only experiments was from 79 to 99% depending on their molecular weight, which was however, in the range of 99.1% to 100% for the combined extraction and oxidation. In a more recent study by Dadkhah and Akgerman (2006), semi-continuous experiments with residence times of 1 and 2 h were performed using aged soil at 250°C and hydrogen peroxide as the oxidizing agent. In all combined extraction and oxidation

flow experiments, residual PAHs in the soil were undetectable in the liquid phase after the first 30 min of the experiments.

Supercritical extraction uses supercritical fluids (SCFs) such as carbon dioxide (mixed with co-solvent as methanol) or water for the extraction of volatile and persistent organic pollutants including PAHs from environmental matrices. Figure 2.3 represents a schematic of supercritical fluid extraction system. In addition to having higher affinity for contaminants than the accompanying solid matrix, the chosen SCF should have liquid-like density, low viscosity, high diffusivity and no surface tension for extracting the contaminants at optimum temperature, pressure and flow rate conditions.

Figure 2.3: Schematic of a super critical fluid extraction system.



Anitescu and Tavlarides (2006) recently reviewed the existing literature about SCE methods. It has been a general finding that SCE does not destroy contaminants rather extracted pollutants are highly concentrated that can be subsequently destroyed

more cost effectively. Librando *et al.* (2004) studied extraction efficiency of 11 PAHs including pyrene from spiked soil using CO_2 in the supercritical phase at 50-80°C, at a pressure of 230-600 bar, with three different organic solvents (methanol, *n*-hexane and toluene) added at 5% v/v. Using methanol as the co-solvent, an increase in the yield of recovered PAHs was observed, but higher temperature than 80°C caused a negative effect. The recovery yield for PAHs from the spiked soil sample was measured and found to be greater than 90%, and, in general, the species with a HMW showed better recovery yields than LMW compounds.

2.2.2. Chemical methods

Chemical oxidation using oxidants like ozone, hydrogen peroxide, permanganate and persulfate seems to be a promising technique for the remediation of environmental matrices contaminated with recalcitrant PAHs. In advanced oxidation processes (AOPs) various combinations of the chemical reactants generate highly reactive radicals capable of mineralizing even the most recalcitrant organic compounds. For the AOPs, the Fenton's reagent, activated persulfate and perozone are the most commonly used oxidants. For examples, Rivas (2006) and Kim and Choi (2002) considered gaseous ozone as an effective agent for the treatment of PAHs contaminated soils and sediments. Among the liquid oxidants, Fenton's reagent has been adopted by Bogan and Trbovic (2003), Bissey *et al.* (2006), Rivas (2006) for oxidation of PAHs in their studies.

Fenton's reagent

Hydrogen peroxide (H_2O_2) is a very strong non-selective liquid oxidizing agents that has widely been used in environmental applications. Though hydroxyl radicals ($^{\bullet}OH$) generated from hydrogen peroxide is capable of reacting with aromatic compounds, the reaction rate is quite slow. Therefore in Fenton's reagent, hydrogen peroxide is dosed together with a solution of a transition metal (mostly iron) for dramatically increasing the peroxide oxidative strength by enhancing the radical formation (Flotron *et al.*, 2005; Watts and Teel, 2005). The solubility of ferric ions generated from ferrous ions by hydroxyl radicals declines at higher pH (>3) due to precipitation of ferric ions as an oxyhydroxide complex (Bohn *et al.*, 1985), which could however be manipulated to occur at near-neutral pH by stabilizing the solubility of ferric ions with chelating agents. In modified Fenton's system, the radical formation is enhanced by the addition of chelating agents and/or by high peroxide concentrations where numerous reacting species in addition to hydroxyl radical are generated, including hydroperoxide radicals, superoxide anions and hydroperoxide anions (Watts and Teel, 2005). The classical Fenton's free radical mechanism in the absence of the target pollutants mainly involves the following sequence of reactions (Deng and Englehardt, 2006).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^- \quad \textbf{(2.1)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+}HO_2^{\bullet} + H^+ \quad \textbf{(2.2)}$$

$$^{\bullet}OH + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \quad \textbf{(2.3)}$$

$$^{\bullet}OH + Fe^{2+} \rightarrow Fe^{3+} + OH^- \quad \textbf{(2.4)}$$

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + O_2H^+ \quad \textbf{(2.5)}$$

$$Fe^{2+} + HO_2^{\bullet} + H^+ \rightarrow Fe^{3+} + H_2O_2 \quad \textbf{(2.6)}$$

$$2HO_2^{\bullet} \rightarrow H_2O_2 + O_2 \quad \textbf{(2.7)}$$

The generated hydroxyl radicals can attack the organic pollutants either by radical addition, hydrogen abstraction, electron transfer, or radical combination, where organic radicals (R˙), formed may rapidly and irreversibly react with O_2 generating intermediates, which may further continue to react with hydroxyl radicals and O_2 leading to further decomposition and even final mineralization to water and CO_2.

Nam *et al.* (2001) have observed Fenton's reagent to be very efficient in the destruction of a mixture of naphthalene, fluorene, phenanthrene, anthracene, pyrene, chrysene and benzo[a]pyrene in spiked soil. The degradation was more noticeable for pyrene (84.5%) and benzo[a]pyrene (96.7%). In soil from a contaminated site, the same treatment method destroyed more than 80% of 2- and 3-ring and 20-40% of 4- and 5-ring PAHs. However, use of modified Fenton's reagent (with catechol or gallic acid as chelator) resulted in a decline in overall performance relative to the unmodified Fenton's reagent.

Lindsey *et al.* (2003) showed carboxymethyl cyclodextrins (CMCD) as effective agent for degradation of pyrene by Fenton's degradation in the presence of soil organic matters, humic acid or hydroxyl radical scavenger. However, it was observed by the authors that with increase in the amounts of CMCD, the pyrene degradation fell, though the CMCD protected the hydroxyl radicals from scavenging action of Cl^-.

Jonsson *et al.* (2007) investigated as to how the chemical degradability of PAHs in soil samples is influenced by soil characteristics and by PAH physico-chemical properties in relatively mild, slurry-phase Fenton's reaction conditions. The authors observed LMW PAHs to be degraded to a greater extent (up to 89 and 59% with two and three rings, respectively) than highly hydrophobic HMW variants (0-38%). Anthracene,

benzo[*a*]pyrene and pyrene were found to be more susceptible to degradation compared to other structurally similar PAHs.

Lundstedt *et al.* (2006) studied effect of ethanol pre-treatment on efficiencies of Fenton's oxidation in remediation of a PAH-contaminated soil from a former gasworks site. Although the authors observed facilitated desorption and enhanced depletion of all PAHs in the soil, some PAHs, particularly anthracene, benzo[*a*]pyrene and perylene were more extensively depleted than others.

Ozonation

Ozone is a highly reactive and powerful oxidizing agent that has traditionally been used in the chemical industry and also in the treatment of drinking water (Rositono *et al.*, 2001). There has been considerable interest in using ozone to remediate contaminated soils which are otherwise non-responsive toward conventional soil venting. Moreover, ozone can be used in the form of gas or liquid (Choi *et al.*, 2001) which reverts back to atmospheric oxygen after a short period of treatment time leaving no residual contaminants in the soil. Goi and Trapido (2004) have reported the usefulness of ozone for the transformation of PAHs in contaminated soil where the intermediates generated were more soluble in the aqueous phase for easy biodegradation by microbes.

Kornmüller and Wiesmann (2003) studied ozonation kinetics of benzo[e]pyrene in oil/water-emulsions simulating the contaminated sites. Benzo[e]pyrene degradation rate constant for the ozonation process (1.02 min^{-1}) in oil/water-emulsions system was even higher when compared to ozonation process involving dissolved benzo[e]pyrene in

water. The results confirmed the applicability of ozone treatment in real contaminated site containing adsorbed PAHs in an inhomogeneous NAPL water mixture.

Bernal-Martínez *et al.* (2005) studied combined effect of anaerobic digestion with ozonation in removal of all 13 PAHs (including anthracene, pyrene, benzo[a]anthracene and chrysene) in an urban sludge. The authors observed improved PAH removal rate (61%) due to ozonation of anaerobically digested sludge, compared to untreated anaerobically digested sludge (50%). Moreover, PAH removal rate increased up to 81% when hydrogen peroxide was added during ozonation. However, higher doses of ozone treatment from the optimal level (1.5 g l^{-1}) did not improve PAH removal noticeably. In another recent study by the same group (Bernal-Martínez *et al.*, 2007), combining ozonation with anaerobic digestion increased biodegradability or bioavailability of each PAH (12 PAHs comprising HMW and LMW), and removals were well correlated to the PAH solubility.

O'Mahony *et al.* (2006) studied the use of ozone for the removal of phenanthrene from several different soils, both individually and in combination with microbial biodegradation. These authors observed negative impact of water content of the soil in the ozone treatment efficiency, and more than 50% and 85% removal in phenanthrene levels was achieved in air-dried soil and sandy soils, respectively, when treated with ozone for 6 h at 20 ppm. However, pre-ozonation did not enhance (or even some time retarded) subsequent removal of phenanthrene in the soils; the authors attributed this effect due to the possible release of toxic intermediates in this soil during ozonation.

2.2.3. Biological methods for PAHs remediation

Biological method of remediation refers to the application of biological systems or their products for degradation, mineralization or detoxification of target pollutants in a given environment. Biological treatment of PAHs has largely been reviewed by Cerniglia (1992), Shuttleworth and Cerniglia (1995), Samanta *et al.* (2002), Parales and Haddock (2004) and Johnsen *et al.* (2005). It has been observed that a catabolically diverse microbial community, consisting of bacteria, fungi and algae, along with some plants can metabolizes PAHs in the environment.

2.2.3.1. Plant assisted PAHs biodegradation

Though, not well explored, in phyto-remediation for cleanup of PAHs contaminated soil, plants are known to accumulate considerable amount of these contaminants in rhizosphere soil from the surroundings (Cunningham *et al.*, 1996).

Liste and Alexander (2000), while studying plat promoted degradation of pyrene in soil, observed that within approximately 8 weeks as much as 74% of the pyrene disappeared from vegetated soil compared to 40% or less from unplanted soil.

Lee *et al.* (2008) evaluated pyrene degradation ability of four native Korean plant species (*Panicum bisulcatum*, *Echinogalus crusgalli*, *Astragalus membranaceus* and *Aeschynomene indica*) in 80 d long green house experiments, where the authors have observed 77-94% of pyrene dissipation in planted soil in comparison to only 69% in unplanted control. In a similar but more recent study, Cheema *et al.* (*article in press*) conducted green house experiments, using tall fescue (*Festuca arundinacea*) for phyto-remediation of PAHs spiked soil, and reported about 91.7-97.8% of phenanthrene and

70.8-90.0% of pyrene degradation in the planted soils, which were 1.88-3.19% and 8.85-20.69% larger than those in corresponding unplanted control soils. The authors attributed the enhanced dissipation of pyrene or phenanthrene in planted soils to increased microbiological activity in the rhizosphere. However, the authors also reported that plant growth was negatively impacted at higher concentrations of the pyrene or phenanthrene.

It is quite clear from the above studies that, although phyto-remediation may be a viable option for bioremediation of PAH contaminated soil, the long time required for this technique associated with low treatment efficiency makes the approach not very much attractive. In a recent review by Gerhardt *et al.* (2009), the authors pointed out the inadequacy of current analytical techniques in detecting any decrease in PAHs concentration in contaminated soil even when the plants are actively facilitating the process. In addition the authors called for certain strategies to overcome toxicity of PAHs towards plants.

2.2.3.2. PAHs biodegradation by fungi and bacteria

A large number of fungi and bacteria have been isolated and studied of their potential to degrade PAHs (Müncnerová and Augustin, 1994; Juhasz and Naidu, 2000; Samanta *et al.*, 2002). And it has been proved that these microorganisms can utilize PAHs either as sole source of carbon or utilize along with other carbon sources by co-metabolism.

Fungal biodegradation of PAHs

Among the different fungal species, the ligninolytic fungi are known to degrade PAHs by virtue of their ability to naturally degrade the complex biopolymer - lignin (Kirk and Farrell, 1987), which is due to extracellular oxidative enzymes secreted by them (Bogan and Lamar, 1996; Pointing, 2001). White rot fungi possess a number of advantages over other bioremediation systems, for example, extracellular lignolytic system can degrade compounds that are not easily taken up by the fungi. Moreover, as the ligninolytic enzymes are produced in response to nutrient carbon nitrogen or sulphur starvation, the white rot fungi does not require acclimatization to PAHs (Barr and Aust, 1994). Table 2.3 presents a representative list of fungi that have been successfully used for bioremediation of PAHs.

In PAHs biodegradation study with *Pleurotus ostreatus* in creosote contaminated and artificially spiked soils, Eggen and Majcherczyk (1998) reported efficiencies of benzo[a]pyrene degradation of 28% and 40% respectively within a month, which improved only marginally upon further prolonged incubation. However, complete mineralization of benzo[a]pyrene due to white rot fungus in spiked soil was only 1.0%, which was even less at 0.1% in absence of the fungus.

Zheng and Obbard (2002) reported biodegradation of LMW and HMW PAHs by *Phanerochaete chrysosporium* in solid phase, soil slurry and surfactant solubilised systems. In the soil-slurry system, oxidation of phenanthrene, fluoranthene and pyrene was enhanced by up to 43% in the presence of the fungus as compared to in an un-inoculated control. However, oxidation of chrysene, benzo[a]pyrene and dibenz[ah]anthracene were very low in the same system. In contrast, in the surfactant

solubilised system, benzo[a]pyrene was efficiently oxidized to a residual of 0.35% of its initial concentration.

Table 2.3: Fungi capable of metabolizing phenanthrene, pyrene and benzo[a]pyrene.

PAH	Fungi	References
Phenanthrene	*Aspergillus niger*	Wang *et al*., 2008
	Bjerkandera adusta	Schützendübel *et al*., 1999
	Cunninghamella elegans	Lisowska *et al*., 2006
	Cyclothirium sp.	da Silva *et al*., 2004
	Cylindrocladium simplex	Lisowska and Dlugonski, 1999
	Phanerochaete chrysosporium	Zhang *et al*., 2008
	Pleurotus ostreatus	Byss *et al*., 2008
	Trametes versicolor	Han *et al*., 2004
Pyrene	*Aspergillus niger*	Wang *et al*., 2008
	Bjerkandera adusta	Valentín *et al*., 2007
	Cyclothirium sp.	da Silva *et al*., 2004
	Naematoloma frowardii	Sack *et al*., 1997
	Phanerochaete chrysosporium	Hammel *et al*.,1986
	Penicillium janthinellum	Saraswathy and Hallberg, 2002
	Pleurotus ostreatus	Bezalel *et al*.,1996
	Pleurotus sp.	Byss *et al*., 2008
Benzo[a]pyrene	*Cunninghamella elegans*	Cerniglia and Gibson, 1979
	Cladosporium sphaerospermum	Potin *et al*., 2006
	Cyclothirium sp.	da Silva *et al*., 2004
	Fusarium solani	Verdin *et al*., 2004
	Pleurotus ostreatus	Byss *et al*., 2008
	Trametes versicolor	Dodor *et al*., 2004

Valentín *et al.* (2006) studied biodegradation of chrysene, pyrene, fluoranthene and phenanthrene in mixture in forest and salt marsh soils by nine white rot fungal species. Among these nine species, *Bjerkandera adusta*, *Irpex lacteus* and *Lentinus tigrinus* were found highly efficient in degrading the PAHs in mixture at 67%, 55% and 53% respectively within 30 d. Moreover, in the PAHs mixture pyrene was degraded to the most extent (>80%) by these fungal strains.

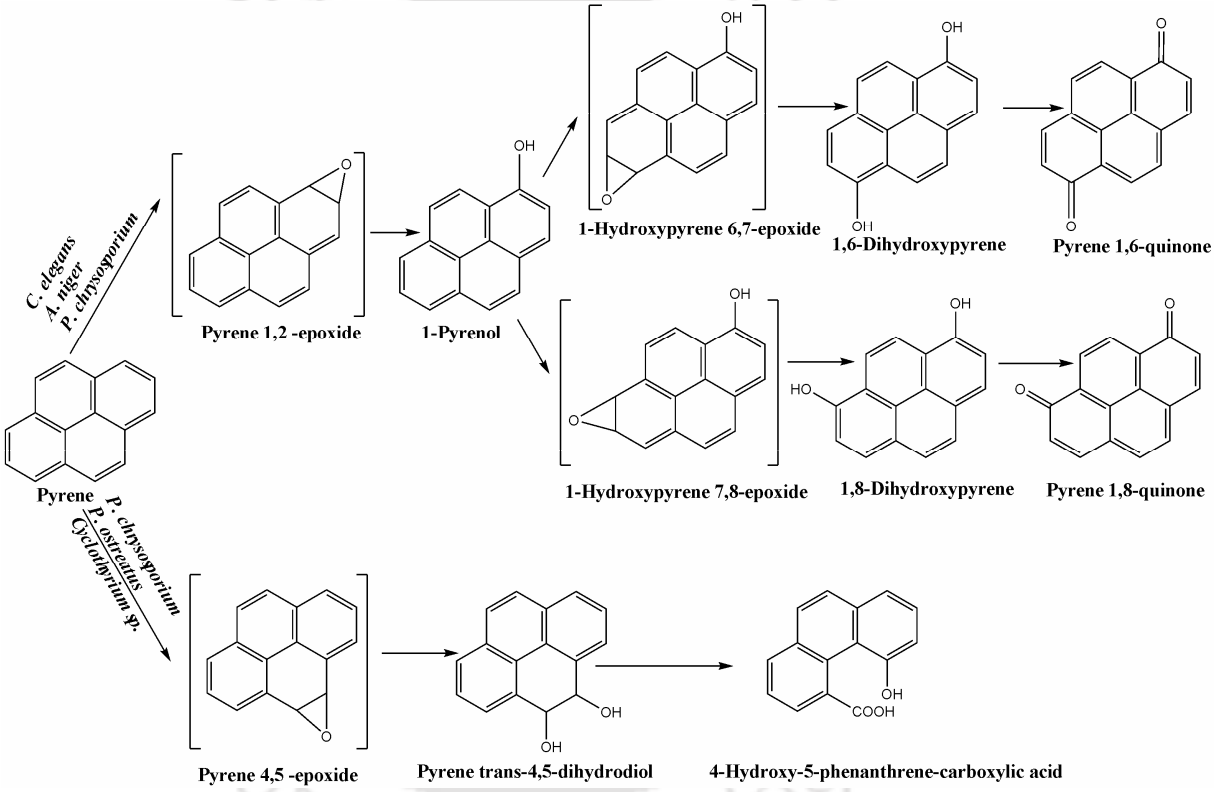
Baborová *et al.* (2006) reported purification of manganese peroxidase (MnP) from *Irpex lacteus*. *In vitro* degradation of pyrene and anthracene with the purified enzyme resulted in 92% and 100% removal of the two PAHs respectively, within 168 h for initial concentrations of 15 mg l^{-1} each in mixture.

Non-lignolytic fungi utilize cytochrome P450 (CYP450) enzyme complex to primarily detoxify PAHs by breaking them into less reactive intermediates, which could then be conjugated with soluble moieties for subsequent excretion from the body. Non-lignolytic fungi, such as *Cunninghamella elegans* and *Penicillium janthinellum* can metabolize a variety of PAHs to polar metabolites.

Cerniglia and Gibson (1979) studied biodegradation of benzo[a]pyrene by *C. elegans*, and observed about 18% conversion of benzo[a]pyrene over a period of 96 h; most of the metabolites formed in this study were of water soluble sulphate conjugated type – similar to metabolic pathway of higher organisms. In another study, Cerniglia *et al.* (1989) reported about 53% conversion of phenanthrene into a glucoside conjugate of 1-hydroxyphenanthrene within 72 h by *C. elegans*. Using *Penicillium janthinellum*, Launen *et al.* (1999) reported 100% conversion of pyrene into 1-pyrenol (1-PY) and pyrenequinones (PQ) under optimized growth conditions.

Fungal metabolism and detoxification of PAHs has been concisely reviewed by Müncnerová and Augustin (1994). Fungi metabolize PAHs to more water-soluble compounds, thereby facilitating their subsequent excretion for detoxification, similar to the transformation pathways found in humans and other mammals (Cerniglia, 1992). Figure 2.4 illustrates a typical fungal metabolic pathway for pyrene.

Figure 2.4: Fungal metabolism of pyrene*.



*Adopted after Cerniglia and Yang, 1984; Cerniglia and Sutherland, 2006.

The figure shows that in ligninolytic fungi the PAHs are transformed to quinones via aryl cation radicals (Cerniglia, 1997). White-rot fungi may then continue the degradation to carbon dioxide and water through ring cleavage; quinones appear to be dead-end products when complete mineralization is not possible. Non-ligninolytic fungi

oxidize PAHs via the cytochrome P-450 enzyme system to form phenols and trans-dihydrodiols, which can be conjugated and excreted from the organism.

Bacterial biodegradation of PAHs

Bacterial degradation of LMW PAHs, such as naphthalene and phenanthrene and HMW PAHs, such as pyrene has largely been investigated in the recent past. Table 2.4 is a representative list of such studies where PAHs have been shown to be degraded as the sole carbon source. Most of the isolated bacteria capable of mineralizing HMW PAHs like pyrene or fluoranthene belong to the genera *Mycobacterium*, *Rhodococcus*. PAHs with 5-ring (e.g. benzo[a]pyrene) has not been shown to be taken up as sole carbon source (Juhasz and Naidu, 2000), which could be attributed to the limited mass-transfer rates of HMW-PAHs to the bacterial cells for maintaining their basic metabolic requirements. It is also postulated that the low bioavailability of the PAH might have prevented the evolution of suitable enzymatic pathways in soil bacteria (Johnsen *et al.*, 2005). *Mycobacterium* sp. has been given particular attention in recent years for it's out standing capabilities to mineralize 3-, 4- ring PAHs (Wick *et al.*, 2003). *Mycobacterium frederiksbergense*, a novel PAH degrading microorganism, isolated from contaminated soil has shown good potential towards mineralization of pyrene, phenanthrene and fluoranthene as sole source of energy and are known to be excellent survivor of unfavorable conditions (Willumsen *et al.*, 2001). Compiled information regarding its characterization and genome level information available in public database is shown in Appendix.

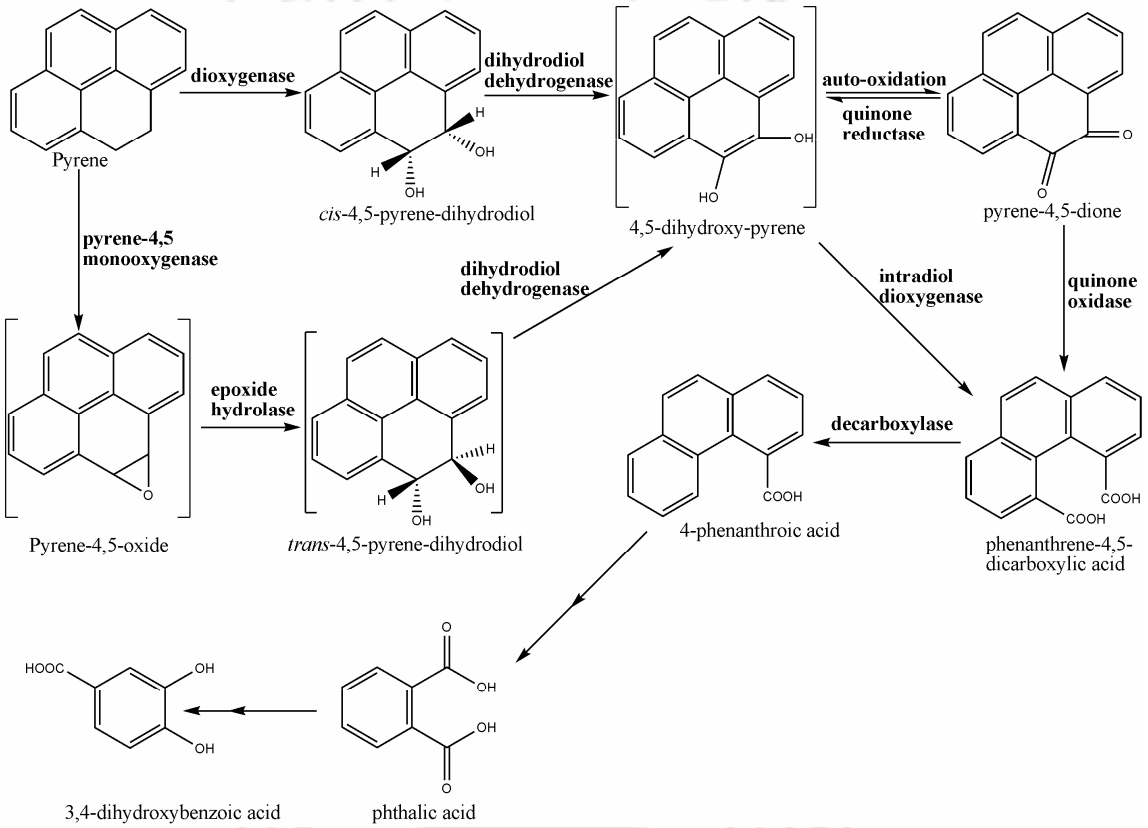
Table 2.4: Bacteria using naphthalene, anthracene and pyrene as sole carbon source.

PAH	Bacteria	References
Naphthalene	*Comamonas testosteroni*	Hoffman *et al.*, 2005
	Pseudomonas putida G7	Lee *et al.*, 2003
	Pseudomonas putida M8	Collina *et al.*, 2005
	Sphingomonas yanoikuyae B1	Kim and Zylstra, 1999
	Bacillus thermoleovorans	Annweiler *et al.*, 2000
	Rhodococcus sp. strain B4	Grund *et al.*, 1992
Anthracene	*Mycobacterium* sp. strain LB501T	Herwijnen *et al.*, 2003[a]
	Pseudomonas aeruginosa	Jacques *et al.*, 2005
	Paracoccus sp.	Zhang *et al.*, 2004
	Mycobacterium LP1	Pizzul *et al.*, 2007
	Pseudomonas aeruginosa	
	Alcaligenes eutrophus	Ilori and Amund, 2000
	Bacillus subtilis	
	Rhodococcus sp.	Dean-Ross *et al.*, 2001
Pyrene	*Mycobacterium strain AP1*	Vila *et al.*, 2001
	Mycobacterium strain CH1	Churchill *et al.*, 1999
	Mycobacterium strain KR2	Rehmann *et al.*, 1998
	Mycobacterium sp. *strain KMS*	Miller *et al.*, 2004
	Mycobacterium sp. *strain 6PY1*	Krivobok *et al.*, 2003
	Mycobacterium sp. *strain RJGII-135*	Grosser *et al.*, 1991
	Mycobacterium flavescens	Dean-Ross and Cerniglia, 1996
	Mycobacterium sp. *strain BB1*	Boldrin *et al.*, 1993
	Mycobacterium PYR-1	Heitkamp and Cerniglia, 1988
	Mycobacterium sp.	Jimenez and Bartha, 1996
	Stenotrophomonas maltophilia	Boonchan *et al.*, 2000
	Leclercia adecarboxylata	Sarma *et al.*, 2004
	Pseudomonas putida	Song, 2005

Most bacteria have been reported to mineralize PAHs under aerobic conditions in similar metabolic pathways (Figure 2.5), where the initial step of the metabolism of PAHs occurs via oxidation of the PAH to dihydrodiol by a multi-component enzyme system. These dihydroxylated intermediates may then be processed through either an ortho- or a meta- cleavage type of pathway leading to central intermediates such as protocatechuates and catechols, which are further converted to tricarboxylic acid cycle intermediates (Samanta et al, 2002). Presences of multiple alternative pathways have been proposed for some microorganism for degradation of some specific PAHs (Stingley et al, 2004[a]). *Mycobacterium vanbaalenii* PYR-1 seems to have numerous alternative degradation pathways and has been extensively studied (Heitkamp *et al.*, 1988; Khan *et al.*, 2001; Kim *et al.*, 2005; Stingley *et al.*, 2004[a,b]; Kim *et al.*, 2007), and is proposed to have both dioxygenase(s) and cytochrome P450 monooxygenase(s) to metabolize PAHs (Brezna *et al.*, 2006; Khan *et al.*, 2001). *M. vanbaalenii* can oxidize pyrene via two pathways, firstly by initial deoxygenation at the C-1 and C-2 positions and secondly dioxygenation initiated at the C-4 and C-5 positions generating pyrene *cis*-4,5-dihydrodiol. Re-aromatization of the dihydrodiol and subsequent ring cleavage dioxygenation leads to the formation of 4,5-dicarboxyphenanthrene, which is further decarboxylated to 4-phenanthroate. The subsequent intermediate *cis*-3,4-dihydroxyphenanthrene-4-carboxylate is produced by a second dioxygenation reaction; re-aromatization then forms 3,4-dihydroxyphenanthrene, which is further metabolized to 1-hydroxy-2-naphthoate. The next enzymatic reactions, including intradiol ring cleavage dioxygenation, result in the production of *o*-phthalate (Kim *et al.*, 2007). Incomplete metabolism of pyrene have been reported, where *cis-4,5-dihydro-4,5-dihydroxypyrene*

and *pyrene-4,5-dione* get accumulated in *Sphingomonas yanoikuyae* strain R1 and *Pseudomonas saccharophila* strain P15 (Kazunga and Aitken, 2000) which strongly inhibit degradation of phenanthrene and benzo[a]pyrene. Anaerobic degradation of PAHs has been suggested for a number of two- and three-ring PAHs, but not for PAHs with more than three rings (Meckenstock *et al.*, 2004).

Figure 2.5: Pyrene degradation pathway of *Mycobacterium* sp.



*Adopted from Liang *et al.*, 2006.

Also, anaerobic degradation of PAH has been demonstrated in several microcosm studies with nitrate, ferric iron, or sulfate as electron acceptors, and under methanogenic conditions particularly for naphthalene and phenanthrene. Degradation pathways study with naphthalene-degrading pure and enrichment cultures indicates 2-naphthoic acid as a central metabolite (Zhang *et al.*, 2000). Naphthalene is activated by addition of a C_1-unit

to generate 2-naphthoic acid. In the central 2-naphthoic acid degradation pathway the ring system is reduced prior to ring cleavage generating 5,6,7,8 tetrahydro-2-naphthoic acids (Meckenstock *et al.*, 2004).

2.2.3.3. Biodegradation of PAHs mixture

Degradation of individual PAHs is well established, but their degradation in complex mixtures has drawn interest only very recently. Degradation of PAHs in mixture may result in inhibition, cometabolism, augmentation or no effect at all.

Inhibition is the reduction in the rate and/or extent of degradation of one compound by the presence of another - the most common effect noted in the degradation of PAH mixtures. McNally *et al.* (1999) observed inhibited pyrene degradation in presence of phenanthrene in *Pseudomonas putida* strain. Tiehm and Fritzche (1995) observed the inhibition of pyrene degradation by fluorene in a pure culture of a *Mycobacterium* sp. grown on pyruvate. Herwijnen *et al.* (2003b) observed potential inhibiting effect of phenanthrene and fluoranthene on the degradation of fluorine by *Sphingomonas* sp. strain LB126 where the effect of phenanthrene was about 10 times stronger than the effect of fluoranthene. Yuan *et al.* (2000) investigated the potential biodegradation of PAHs by an aerobic mixed culture utilizing phenanthrene as its carbon source. The mixed culture was also capable of efficiently degrading pyrene and acenaphthene, but failed to degrade anthracene and fluorene. However, the aerobic culture showed increased degradation rates for fluorene and anthracene, and decreased degradation rates for acenaphthene, phenanthrene and pyrene when all the five PAHs were present in mixture. Inhibition to PAHs degradation in mixture condition suggests

that multiple PAHs are degraded using common enzymatic pathways leading to competition for the active sites of enzymes (Stringfellow and Aitken, 1995). Bouchez *et al.* (1995) suggested that changes in enzyme induction could cause inhibition, as one compound represses the synthesis of enzymes needed to degrade the other. Also, the aqueous solubility of individual compounds may influence the inhibition patterns observed. Several studies (Bouchez *et al.*, 1995; Stringfellow and Aitken, 1995; Tiehm and Fritzche, 1995) have reported that more soluble PAHs inhibit the degradation of the less soluble ones; however, no such correlation was observed by Ye *et al.* (1996). The use of mixed rather than pure cultures to degrade mixtures has been reported to be potential in mitigating the observed inhibition effects. Mixed cultures can display complementary degradative action, thus showing a greater tolerance for toxic products, where product intermediates can act as substrates for supporting the activity of other bacterial species.

Co-metabolism is the degradation of PAHs without generation of energy and carbon for the cell metabolism as a non-specific enzymatic reaction with a substrate competing with structurally similar primary substrate for the enzyme's active site. An example is the co-metabolism of benzo[a]pyrene by bacteria growing on pyrene (Boonchan *et al.*, 2000). Pure culture of *Pseudomonas* sp., unable to use fluoranthene as sole carbon source, can degrade the compound in presence of phenanthrene (Bouchez *et al.*, 1995). Cometabolism may be an important fate process when larger and often more recalcitrant PAHs are present in mixtures of more readily degraded, smaller PAHs. Somtrakoon *et al.* (2008) observed that pyrene or fluoranthene when supplied as the sole carbon source could not be degraded by *Burkholderia* sp. VUN10013. However, when added in a mixture along with phenanthrene, both pyrene and fluoranthene were degraded

without affecting the phenanthrene degradation. The amounts of pyrene and fluoranthene in liquid media at initial concentrations of 50 mg l^{-1} each decreased to 42.1% and 41.1%, respectively, after 21 days without any adverse effect on simultaneous phenanthrene degradation by this bacterial strain. Anthracene also stimulated the degradation of pyrene or fluoranthene by *Burkholderia* sp. VUN10013, but to a lesser extent than phenanthrene. The extent of anthracene degradation decreased in the presence of pyrene or fluoranthene. The presence of PAHs in a mixture produces interactive effects that can either increase or decrease the rate of utilization of individual PAHs. Dean-Ross *et al.* (2002) observed that *Mycobacterium flavescens* could utilize fluoranthene in the presence of pyrene, but utilization of pyrene was slower in the presence of fluoranthene than in its absence. Another strain, a *Rhodococcus* species, could utilize fluoranthene in the presence of anthracene; the presence of fluoranthene, however, slowed the rate of utilization of anthracene.

Augmentation describes cases in which degradation of one compound is enhanced by the presence of another one. Bouchez *et al.* (1995) found fluorene augmented degradation of phenanthrene by a pure culture of a *Rhodococcus* sp. Tiehm and Fritzche (1995) observed an increase in the degradation of pyrene in the presence of phenanthrene by a pure culture of a *Mycobacterium* sp. amended with surfactants. Recently, Lei *et al.* (2007) found comparable or higher efficiency in the removal of fluoranthene and pyrene in a mixture than under single substrate condition by microalgal species, suggesting a stimulatory effect between the two PAHs.

Mixtures of PAHs do not always produce any of these observable effects. For example, when present in high concentrations, the degradation of phenanthrene was not

affected by the presence of fluorene, fluoranthene or pyrene (Tiehm and Fritzche, 1995). At low PAH concentration in aqueous solution, inhibition was observed. In a study by Ye *et al.* (1996), the presence of PAHs, including benzo[a]anthracene, benzo[b]fluoranthene, chrysene, dibenzo[a,h]anthracene and fluoranthene, did not affect the degradation of benzo[a]pyrene by *Sphingomonas paucimobilis*. The authors suggested two explanations for these observations: (1) the compounds did not compete for the active sites of the enzyme responsible for benzo[a]pyrene degradation, (2) there are different enzymes responsible for degradation of the different PAHs.

Use of mixed culture or consortia for biodegradation of mixture of PAHs is likely to obviate the problems of observed inhibition by bacterial strains with complementary capacities, and facilitate complete mineralization of PAHs. Jacques *et al.* (2007) evaluated the capacity of a defined microbial consortium (six bacteria and a fungus) isolated from a PAHs contaminated site to degrade and mineralize different concentrations of anthracene, phenanthrene and pyrene in soil. They found that bacterial and fungal isolates from the consortium, when inoculated separately into the soil, were less effective in anthracene mineralization compared to the consortium, signifying synergistic promotion of PAHs mineralization by mixtures of the microbial consortium.

2.2.3.4. Biodegradation kinetics

Microbial growth and utilization of PAHs as substrates have been studied by many researchers and a variety of biodegradation kinetic models has been developed (Simkins and Alexander, 1985; Schmidt *et al.*, 1985), which allow prediction of

contaminant that remain at a certain time and calculation of time required to reduce a given load to certain concentration.

Caldini *et al.* (1995) studied biodegradation of chrysene as sole carbon source by *Pseudomonas fluorescens* strain. When chrysene was supplied as dissolved in water miscible solvent to the mineral media, it was rapidly degraded following first-order kinetics with maximum rate of 0.175 $\mu g\ l^{-1}\ d^{-1}$.

Poeton *et al.* (1999) investigated biodegradation kinetics of phenanthrene and fluoranthene for a PAH-degrading marine enrichment culture as a function of their dissolved concentrations in laboratory condition using radiolabeled PAHs. The authors observed that the degradation profiles for both phenanthrene and fluoranthene could be described by first order rate kinetics with rate coefficients in the range of 0.033-0.139 L $mg^{-1}\ d^{-1}$ for phenanthrene, and 0.132-0.162 L $mg^{-1}\ d^{-1}$ for fluoranthene.

Chang *et al.* (2002) studied anaerobic biodegradation of phenanthrene, pyrene, anthracene, fluorene and acenapthene with a consortia isolated from river sediments at optimal incubation conditions (pH 8.0 and 30°C). They observed faster rate of PAH degradation when presented as mixtures than individual. The authors observed that all the degradation data fitted well to first order kinetics, with half-lives of the five PAH lying in between 16 and 200 d.

Mollea *et al.* (2005) studied naphthalene biodegradation kinetics of two fungi *P. chrysosporium* and *T. harzianum* in soil microcosms, with different C/N ratio with maximal naphthalene concentration of 600 mg kg^{-1}. The authors observed saturation shaped degradation kinetics with *P. chrysosporium* when average depletion rates are

plotted against naphthalene concentration. In nitrogen-limited condition, the best-fitting kinetic model was found to be:

$$r_{Naphthalene} = \frac{K_1 \cdot C_{Naphthalene}}{K_2 + C_{Naphthalene}} \quad \textbf{(2.8)}$$

where, $r_{Naphthalene}$ is kinetic rate and $C_{Naphthalene}$ is naphthalene concentration in microcosm study and K_1 and K_2 are constant terms in the model.

Thiele-Bruhn and Brümmer (2005) carried out bioremediation experiments with PAH contaminated soil using outdoor pot trials for 168 weeks. The authors observed largest degradation for acenaphthene (88%) and smallest for anthracene (22%), where kinetics were characterized by a first initial phase of fast degradation followed by a subsequent diminished rate of degradation for individual PAHs. The kinetic data was found to be best fitted by single and two coupled first order exponential equations (Equation 2.9 and 2.10, respectively), from an initially chosen set of seven differential rate equations.

$$C_t = C_0 \cdot e^{-k \cdot t} \quad \textbf{(2.9)}$$

$$C_t = C_{0,1} \cdot e^{-k_1 \cdot t} + C_{0,2} \cdot e^{-k_2 \cdot t} \quad \textbf{(2.10)}$$

where, C_0 and C_t are concentration of the PAHs at initial and at time‘t’, respectively; k, k_1 and k_2 are the rate constant(s) in first order and coupled first order equations.

Rončević *et al.* (2005) studied bioremediation of PAHs on a laboratory scale, inoculated with adopted bacterial population. The authors reported that modified first order rate kinetics (Equation 2.11) was more reliable in describing the biodegradation profiles of HMW PAHs in their study. However, degradation of pyrene or chrysene were found to follow simple linear function.

$$C_t = C_0 \cdot e^{-k \cdot t^{1/2}} \quad \textbf{(2.11)}$$

Recently Xia *et al.* (2006) reported biodegradation of chrysene, benzo[a]pyrene and benzo[g,h,i]perylene with phenanthrene as a co-metabolic substrate in natural waters from the Yellow River. Biodegradation kinetics of the PAHs was studied by fitting to a biodegradation kinetics model for organic compounds not supporting growth. They found that integrated form of the model (Equation 2.12), proposed by Schmidt *et al.* (1985), was more suitable than others.

$$S = S_0 \cdot e^{-(K_1/r)[\exp(rt)-1]} \quad \textbf{(2.12)}$$

Where, K_1 is the biodegradation rate constant and r is the maximum specific growth rate of microorganism; S_0 and S are concentration of PAH at initial and at time't' respectively.

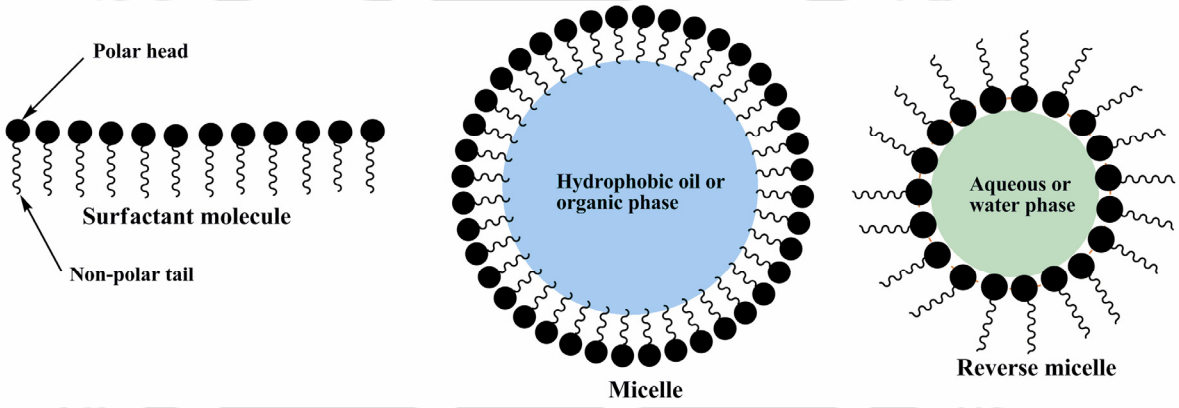
Recently, Chen *et al.* (2008) investigated biodegradation of phenanthrene in contaminated sediment slurry by *Sphingomonas* sp. isolated from surface mangrove sediment. The authors observed that phenanthrene biodegradation profiles were best described by the first order rate model, with a rate constant of 0.1185 h^{-1} under the optimal conditions of temperature, salinity and C/N ratio.

2.3. Surfactant Aided Biodegradation of PAHs

Surfactants are synthetic or biogenic amphiphilic organic compounds containing both hydrophobic tail and hydrophilic head, and are soluble in both organic solvents and water. They reduce the surface tension of water or interfacial tension between oil and water by adsorbing at the liquid-gas interface or liquid-liquid interface, respectively. Surfactants can also aggregate in the bulk solution as vesicles and micelles in a concentration dependent manner, which begin to form above a certain concentration

known as the critical micelle concentration or CMC. When micelles form in water, their tails form a core that can encapsulate solvent/oil droplets, and their polar heads form an outer shell that maintains favorable contact with water. However, when surfactants assemble in oil, the aggregate is referred to as a reverse micelle, where the heads are in the core and the tails maintain favorable contact with oil. Figure 2.6 schematically represents the formation of micelle and reverse micelle (Tanford, 1980).

Figure 2.6: Schematic representation of micelle and reverse micelle formation by surfactants.



2.3.1. Chemical surfactants

Surfactants have frequently been used to increase the rates of desorption of hydrophobic compounds from soil and subsequent solubilization into aqueous micelles affecting the PAH accessibility to microorganisms (Edwards *et al.*, 1991). Surfactants are also known to exhibit negative effects on biodegradation of PAHs, due either to the surfactant toxicity or to the increased toxicity of PAHs at enhanced concentrations (due to surfactants) to microorganisms. They may also compete for PAHs as alternate carbon source, and hence negatively affect the PAH biodegradation. Majority of the studies

where surfactants have been used for biodegradation of PAHs focuses on non-ionic surfactants, due to their minimal toxicity compared to other types of surfactants. Properties of commonly used surfactants in biodegradation are shown in Table 2.5.

Table 2.5: Properties of commonly used synthetic surfactants in PAHs biodegradation.

Surfactants	Molecular Weight	CMC (M) $\times 10^{-5}$	Molar solubilization ratio*		
			Pyrene	Phenanthrene	Naphthalene
SDS	288	800	0.010	0.0243	0.073
Triton X-100	625	17	0.029	0.095	0.18
Brij 30	363	5.5	-	0.086	0.23
Brij 35	1198	9.2	0.038	0.124	0.27
Tween 20	1228	3.5-4.7	0.067	0.14	-
Tween 80	1310	1.2	0.086	0.165	0.38

* *Source*: Zhu and Feng, 2003; Prak and Pritchard, 2002; Zhao *et al.*, 2005.

Volkering *et al.* (1995) observed that presence of the synthetic nonionic surfactants Triton X-100, Tergitol NPX, Brij 35 and Igepal CA-720 increased apparent solubility of PAHs in batch biodegradation studies without any toxic effects up to surfactant concentrations of 10 g l^{-1}. However, it was observed that the substrate present in the micellar phase was not readily available for degradation by the microorganisms.

Doong and Lei (2003) reported enhanced bioavailability of naphthalene, phenanthrene and pyrene by non-ionic surfactants in the order of Brij 30, Triton X-100, Tween 80 and Brij 35, which correlated well with the extent of polyoxyethylene chain of the surfactants. However, Brij 35 and Tween 80 inhibited the growth of *Pseudomonas putida*, and Triton X-100 and Brij 30 were utilized as the sole carbon and energy sources at concentrations above CMC values. Conclusively, the addition of surfactants decreased the mineralization rate of pyrene.

Zhu and Feng (2003) studied solubility enhancement of naphthalene, acenaphthylene, anthracene, phenanthrene and pyrene by micellar solutions of single and mixed anionic-nonionic surfactants (Triton X-100, Triton X-305, Brij 35 and SDS). Through this study, it was shown that PAHs are solubilised synergistically in mixed anionic-nonionic surfactant solutions, particularly at low surfactant concentrations, and the synergistic action of the mixed surfactants followed the order SDS-TX 305 > SDS-Brij 35 > SDS-TX100.

Yang *et al.* (2004) in their study to quantify the bioavailability of surfactants-solubilised phenanthrene to a mixed phenanthrene-degrading culture, reported that the concentrations of Tween 20 above CMC could increase the solubility of phenanthrene and at these concentration, the surfactant was neither toxic to the phenanthrene degrading bacteria, nor inhibited biodegradation of phenanthrene. Phenanthrene solubilised in the micelles of Tween 20 in liquid media was therefore bioavailable and degradable by the mixed culture.

Kim and Weber (2005) examined the effect of nonionic surfactant (Tween 80) on the bioavailability of PAHs, and they observed that the mixed consortium partially and preferentially utilized readily available portions of the surfactant as carbon sources (16-18% of the initial surfactant dose) resulting in destabilization of dispersed-phase micelles along with significant decrease in molar solubilization ratio (MSR) and micelle-water partition coefficient values.

Sartoros *et al.* (2005) studied biotransformation and mineralization of a mixture of anthracene and pyrene by an enrichment culture in presence of a non-ionic surfactant

Tergitol NP-10, at 10°C and 25°C. Though, the addition of surfactant at 25°C increased the overall mineralization of anthracene and pyrene, it had a negative impact at 10°C.

Zhao *et al.* (2005) investigated the effects of mixed anionic–nonionic surfactants: SDS mixed with Tween 80, Triton X-100 and Brij-35, respectively, on the solubility enhancement and biodegradation of phenanthrene in its aqueous phase. The authors observed that CMC of mixed surfactants were much lower than that of SDS when presented individually. Given the molar fraction of nonionic surfactant and the total surfactant concentrations, SDS-Tween 80 mixture enhanced the solubility of phenanthrene most significantly among the three mixed systems; SDS-Brij35 exhibited the largest extent of synergistic solubilization. More importantly, phenanthrene in aqueous solution containing a mixture of surfactants was readily degraded with no inhibitory effects on the microorganism.

Yu *et al.* (2007) observed similar observation of anionic-nonionic mixed surfactants (SDS mixed with Triton X-100) on desorption and biodegradation of phenanthrene in soil-water system. In this study, mixed surfactants with relatively smaller ratio were found to promote phenanthrene biodegradation, but the biodegradation was inhibited at larger ratio of SDS in the mixed solutions, probably due to preferential utilization of SDS by phenanthrene degraders.

Jin *et al.* (2007) investigated effects of concentration, polar or ionic head group and structure of surfactants on the biodegradation of PAHs in aqueous phase, as well as their effects on bacterial activity. The authors noted the toxicity ranking of studied surfactants as: non-ionic surfactants < anionic surfactants < cationic surfactants. For the same head group and similar molecular structure, toxicity was observed to decrease as the

chain length increased. Also, it was noted that surfactant addition was not beneficial to the removal of phenanthrene, presumably due to the preferential utilization of surfactants at low levels and due to the high toxicity of the surfactants at elevated levels.

Recently, Dar *et al.* (2007) reported mixed micelle formation of binary and ternary cationic-nonionic surfactant mixtures and studied solubilization behavior towards PAHs. Cationic surfactants exhibited lesser solubilization capacity than nonionic; however, increase in hydrophobicity of head groups of cationic surfactants by incorporating ethyl or benzyl groups enhanced their solubilization capacity. Cationic-nonionic binary combinations showed better solubilization capacity than pure cationic, nonionic or cationic-cationic mixtures. Equimolar cationic-cationic-nonionic ternary surfactant systems showed lower solubilization efficiency than their binary cationic-nonionic counterparts, but higher than cationic-cationic ones.

Using *Pseudomonas alcaligenes*, Hickey *et al.* (2007) observed enhanced degradation of fluoranthene in presence of Tween 80 utilizing the synthetic surfactant as growth substrates.

Cyclodextrins (CD) are a well-known family of oligomers of glucose produced by the reaction of certain bacterial enzymes on starch having high aqueous solubility with a relatively hydrophilic exterior and a relatively hydrophobic interior “hole”. Cuypers *et al.* (2002) showed that cyclodextrin macromolecule is able to form inclusion complexes with PAHs, thus subsequently enhancing their water solubility. Boving *et al.* (2000) from their study concluded that CD possesses solubilization power in between that of surfactants and miscible organic solvents, with additional advantages of being less toxic and high biodegradable.

Reid *et al.* (2004) demonstrated an excellent potential with use of hydroxypropyl-β-cyclodextrin (HPCD) in non-exhaustive extraction and determination of extractable fraction of hydrocarbon available in soil.

Ramsay *et al.* (2005) examined the effect of the CD, HPCD on phenanthrene mineralization of a Fe (III)-reducing consortium; in this study they found low concentrations of HPCD (0.05-0.5 g l^{-1}) enhanced phenanthrene mineralization compared to that of control (without HPCD). At a higher HPCD concentration (5.0 g l^{-1}), though the initial rate of mineralization was faster, it ceased within 25 d, and the extent of mineralization was even 17% lower than the control.

In another study, Wang *et al.* (2005) tested the impact of HPCD on the bioavailability and biodegradation of pyrene by *Burkholderia* CRE 7. The authors observed that biodegradation of pyrene by *Burkholderia* CRE 7 in medium containing HPCD (10 g l^{-1}) initiated after approximately 15 weeks and only 14% (w/w) of the pyrene was biodegraded by the end of the experiment.

Recently, Doick *et al.* (2006) estimated phenanthrene availability to bacteria over a wide concentration range (0-100 mg kg^{-1} of soil) and an extended period of ageing (123 d). The results demonstrated accuracy and reproducibility of the HPCD-extraction technique to predict phenanthrene bioavailability in soils with differing physico-chemical parameters or soil organic matter contents or the presence of a secondary PAHs contaminant.

2.3.2. Biosurfactants

Biosurfactants are surface active agents produced by yeast or bacteria from various substrates, including sugars, oils and aliphatic hydrocarbons. Biosurfactants are grouped as glycolipids, lipopeptides, phospholipids, fatty-acids, polymeric and particulate compounds. The hydrophobic part of the molecule is based on long-chain fatty acids, hydroxy fatty acids or α-alkyl-β-hydroxy fatty acids, and the hydrophilic portion can be a carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid or alcohol. Most extensively studied biosurfactants are (i) the rhamnolipids from *Pseudomonas aeruginosa,* (ii) sophorolipids produced by *Candida bombicola* and (iii) *Bacillus subtilis* produced lipopeptide called surfactin. They can be potentially as effective with some distinct advantages over the highly used synthetic surfactants, including high specificity, biodegradability and biocompatibility, and has been used in biodegradation of PAHs to enhance bioavailability (Mulligan, 2005).

Vipulanandan and Ren (2000) compared the solubilisation of naphthalene by a bacterial rhamnolipid against the synthetic surfactants SDS (anionic surfactant) and Triton X-100 (non-ionic surfactant). Although the biosurfactant at 10 g l^{-1} concentration increased the solubility of naphthalene by 30 times, biodegradation of naphthalene at 30 mg l^{-1} initial concentration took abnormally long time (40 d) compared to just 100 h for Triton X-100 (10 g l^{-1}). It appeared that the biosurfactant was used as a carbon source instead of the naphthalene, which was however not the case for Triton X-100. On the contrary, naphthalene in the presence of SDS was not at all degraded by the *Pseudomonas* sp.

Dean *et al.* (2001) investigated the bioavailability of phenanthrene in soils to two microbial strains known to biodegrade the PAH. Either rhamnolipid or a biosurfactant-producing strain of *P. aeruginosa* was added for the experiment, and results were mixed and difficult to interpret. While strain R in the experiments showed enhanced biodegradation when the surfactant was added, strain P5-2 did not show any effect. Addition of rhamnolipid enhanced release of phenanthrene, but did not necessarily enhance its biodegradation. Simultaneous inoculation with the biosurfactant producer did not affect mineralization by isolate P5-2, but significantly enhanced phenanthrene mineralization by the strain R.

Chang *et al.* (2004) studied effects of trehalose lipid biosurfactants produced by *Rhodococcus erythropolis* on the solubilization and biodegradation of phenanthrene using a known PHE degrader (isolate P52) in batch experiments. The addition of biosurfactants at 20-fold the CMC increased the apparent solubility of phenanthrene by more than 30 fold. Addition of biosurfactant increased either rate or both rate and extent of phenanthrene degradation in three different degradation systems.

Wong *et al.* (2004) tested bioavailability and biodegradation of PAHs in presence of two biosurfactants produced from *Pseudomonas aeruginosa* strain P-CG3 and *Pseudomonas aeruginosa* ATCC 9027. Addition of biosurfactant inhibited the biodegradation of phenanthrene in mineral salts medium by isolate *Bacillus* sp. B-UM. Degradation of phenanthrene diminished with increasing surfactant concentrations, and was completely inhibited for both the biosurfactants at concentrations greater than their respective CMCs.

Shin *et al.* (2005) investigated the effect of the rhamnolipid biosurfactant on phenanthrene biodegradation and cell growth of two phenanthrene degrading isolated strain. Without the biosurfactant, large amounts of phenanthrene were degraded proportional to cell growth by both the strains. Upon the addition of rhamnolipid (240 mg l^{-1}), both phenanthrene degradation and cell growth of both these strains were reduced. The study also indicated that combined inhibitory and toxicity mechanism was different in the two strains; while toxicity to the first strain was due to rhamnolipid itself, in the other strain, toxicity was due to solubilised phenanthrene or the increased toxicity of rhamnolipid in the presence of the solubilised phenanthrene.

Das and Mukherjee (2007) studied the role of biosurfactants in enhancing bioavailability of pyrene in its differential utilization as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains. The biosurfactant secreted by the respective bacterial strains enhanced the apparent solubility of pyrene by factors of 5-7 and influenced the bacterial cell surface hydrophobicity resulting in higher uptake and utilization of pyrene by bacteria.

2.4. Solvent Aided Biodegradation of PAHs

To enhance biodegradation of PAHs by increasing its bioavailability, various approaches have been studied and attempted; of which, use of solvent seems the most important and also recent. However, use of solvent has emerged in two different dimensions- either water miscible or immiscible solvent, which is targeted for two different purposes, i.e., water miscible solvents as solubilising agent for PAHs in aqueous

phase at concentrations more than their intrinsic aqueous solubility and water immiscible solvent as PAHs loading vehicle at very high concentration levels.

The use of water-miscible organic solvents for PAH biodegradation also brings along with it major limitation of being highly toxic to degrading microorganism, and therefore these studies are only limited in literature. However, large no of studies relating to degradation of PAHs by ligninolytic enzymes from white rot fungi in water-miscible organic solvents have been reported.

Field *et al.* (1995) studied the oxidation of anthracene in the presence of co-solvents by the white-rot fungus *Bjerkandera* sp. *strain BOS55* using acetone and ethanol. Acetone and ethanol at 5% were toxic to this fungus when added at the time of inoculation. However, 9-day-old cultures could tolerate solvents up to 20% (v/v), in which case anthracene oxidation was attributed to extracellular peroxidases. Solvent additions of 11%-21% (v/v) acetone or ethanol increased the rate of anthracene bioconversion to anthraquinone in liquid medium by a factor of 2-3 compared to fungal cultures receiving 1%-3% of the solvent.

Lee *et al.* (2001) studied the effect of ethanol and acetone on PAHs biodegradation from contaminated soil, and observed that benzo[a]pyrene degradation for acetone pretreated soils was twice that of non-pretreated soils. The degradation rates of chrysene were found to be two times faster for solvent pretreatment than without solvent treatment. But there were no statistical differences between acetone-pretreated soils and ethanol-pretreated soils using 95% confidence interval.

Eibes *et al.* (2005) studied addition of different water miscible organic solvents (acetone, methyl-ethyl-ketone, methanol and ethanol) to increase the bioavailability of

anthracene for its subsequent degradation by the ligninolytic enzyme manganese peroxidase (MnP). Acetone at 36% (v/v) was selected as the best co-solvent that caused maximum solubilisation of anthracene and minimum loss of MnP activity. A nearly complete degradation (~100%) of anthracene was obtained following 6 h of time under optimal conditions.

Eibes *et al.* (2006) evaluated feasibility of MnP crude preparation from *Bjerkandera* sp. BOS55 in acetone (36% v/v) for *in vitro* degradation of three PAHs: anthracene, dibenzothiophene and pyrene. These compounds were degraded to a large extent (52%, 95% and 61% respectively), within 7 h, 24 h and 24 h, respectively, under optimized MnP-oxidative conditions of malonic acid concentration and H_2O_2 loading rate.

Pozdnyakova *et al.* (2006) studied catalytic activity of the yellow laccase from *Pleurotus ostreatus* D1 (YLPO) towards a range of PAHs in NaK-phosphate buffer with 1% (v/v) acetonitrile. YLPO degraded all the PAHs containing three to five rings with efficiencies of 91% for anthracene, 40% for pyrene, 95% for fluorene, 47% for fluoranthene, 82% for phenanthrene and 100% for perylene.

Hernandez *et al.* (2008) studied biodegradation of anthracene and pyrene by horseradish peroxidase (HRP) in organic media containing acetone. In this study, anthracene showed higher oxidation levels of 79% with the addition of 30% acetone compared to a moderate oxidation level of 56% in aqueous medium. In the case of pyrene, only slight improvement (32% degradation) was observed for 30% acetone compared to that in water (~20% degradation).

2.5. Two Phase Systems for PAH Biodegradation

Biodegradation of PAHs comes with some intrinsic and contrasting limitations related to its bioavailability. Addition of this substrate at too high a concentration could inhibit or even kill the degrading organisms, whereas at too low concentrations may lead to cell starvation resulting in a sub-optimal performance. Hence, it becomes important to have fine control over precise and constant delivery of PAHs to the microorganism. Conventional use of surfactant or water miscible organic phase although solubilises the total PAHs load, but at the same time results in a high toxicity levels of the PAHs. This situation becomes even more complicated when the surfactant or the water miscible phase are themselves amenable for biodegradation and/or toxic to microorganisms.

To overcome the limitations of these two systems, use of an immiscible and biocompatible organic solvent to dissolve large quantities of PAHs in two-phase partitioning bioreactor (TPPB) is being recently employed, where solvent containing PAHs is loaded with microbial cell-containing aqueous phase, so that the PAHs would partition at sub-inhibitory concentrations to the cells, as determined by its partition coefficient. In this system, partitioning is based on equilibrium considerations and real-time demand of the organisms (Daugulis, 2001), so that the cells consume PAHs, disequilibrium is created, thus causing more PAHs to be partitioned into the aqueous phase to maintain thermodynamic equilibrium. The TPPB system is also self-regulated as the pollutant delivery to the aqueous phase is only directed by the partitioning ratio between the two phases and the culture consumption rate (Daugulis, 1997). Hydrophobic nature of PAHs makes them ideal candidates for degradation in TPPB systems since high concentration and large surface area could be achieved by dissolving PAHs in the

dispersed organic phase (Daugulis and Janikowski, 2002). Two-liquid-phase bioreactors have the potential to resolve both limitations of bioavailability and toxicity PAHs by the enhancement of the mass-transfer rate and by the controlled delivery of compounds (Deziel *et al.*, 1999). Biphasic reactors support attached microbial growth at the aqueous-organic interface, which could lead to a direct uptake of the contaminant from the organic phase (Ascón-Cabrera and Lebeault, 1995[a]) and ultimately favoring the selection of xenobiotic-degrading organisms (Ascón-Cabrera and Lebeault, 1995[b]).

Marcoux *et al.* (2000) studied biodegradation of HMW PAHs in different water-immiscible organic phase by a microbial consortium, where highest PAH-degrading activity was observed with silicone oil as the water-immiscible phase compared to heptamethylnonane, paraffin oil, hexadecane and corn oil. Addition of surfactant (Triton X 100, Brij 35, rhamnolipid) or potential catabolic inducers (salicylate, benzoate and catechol) either did not improve or inhibited PAHs degradation in this two phase system. Also, it was observed that microorganisms from the interface were slightly more effective in degrading PAHs than those from the aqueous phase.

Guieysse *et al.* (2001) compared efficiency of monophasic and biphasic systems in biodegradation of pyrene and antharacene by a mixed culture of *Pseudomonas* sp. and *Sphingomonas* sp. Pyrene and antharacene were completely degraded within 3 d in biphasic reactor containing silicone oil, where as in monophasic reactor, the removal was only 93% after 4 d. The pyrene concentration decreased to 7% and 24% in the monophasic reactor and in the biphasic reactor, respectively, even after 12 d of cultivation. Most of the removal observed in the biphasic reactor occurred during the first

2 days of incubation, and the authors pointed out that adaptation of microorganism is required for efficient performance in TPPBs.

Janikowski *et al.* (2002) studied biodegradation of four LMW PAHs by *Sphingomonas aromaticivorans* B0695 in TPPBs in batch and fed batch mode using dodecane as substrate delivery solvent. The TPPB achieved complete biodegradation of naphthalene, phenanthrene, acenaphthene and anthracene at a volumetric consumption rate of 90 mg l^{-1} h^{-1} in approximately 30 h.

Daugulis and Janikowski (2002) investigated scale up performance of a TPPB for degradation of PAHs by *Sphingomonas aromaticivorans* in dodecane as solvent operated at two scales of 5 L and 150 L. Complete degradation of 15 g and 300 g of naphthalene and phenanthrene respectively in mixtures was achieved in 21 h with a volumetric PAH degradation rate of 238 mg l^{-1} h^{-1}, which is based on aqueous phase volume in the reactor.

Daugulis and McCracken (2003) used dodecane as delivery solvent in TPPB for degrading six PAHs in mixture by two species of *Sphingomonas*, presented both individually and as a consortium. The authors observed that the LMW PAHs naphthalene, phenanthrene and fluoranthene were degraded by the individual strains, and the consortium degraded all the PAHs almost completely.

2.5.1. Choice of solvents in TPPB system

Solvent selection is critical to the design of any TPPB system, as identity and quantity of solvent used can largely impact PAH mass transfer and degradation rates in the system; also, the selected solvent must be not-toxic to the organism, should have suitable physical and chemical properties (i.e. be immiscible, non-volatile, etc.) and

should be inexpensive (Marcoux *et al.* 2000). Moreover, the solvent should be non-bioavailable, as it is believed that additional carbon sources may interfere with the degradation of PAHs.

Vrionis *et al.* (2002) demonstrated that genetically engineered microorganism can be used to eliminate otherwise solvent bioavailability in TPPB system. In this study, the authors showed that while mutated *Pseudomonas putida* strain could be used with medium chain length alcohol (decanol) as delivery solvent in TPPB system, the native strain (ATCC 11172) could not be used owing to its ability to utilize such solvents as substrates. These authors also observed that mutated strain can continue to degrade phenol at similar rate as wild type with improved specific substrate utilization rate.

MacLeod and Daugulis (2003) successfully used a bioavailable solvent bis-ethylhexyl sebacate (BES) for degradation of pyrene and phenanthrene by *Mycobacterium PYR-1* in TPPB system at rates as high as 138 mg l^{-1} d^{-1} and 168 mg l^{-1} d^{-1}, respectively.

Muñoz *et al.* (2003) studied biodegradation of phenanthrene by defined algal-bacterial consortia in TPPB system, using either silicone oil or tetradecane as solvent. They observed that phenanthrene was best removed only in this TPPB system, indicating the potential of TPPB for toxicity reduction and for better acceptability. Phenanthrene was also found efficiently degraded at its highest rates when silicone oil was used rather than tetradecane; this aspect was attributed to sequestration of phenanthrene in tetradecane, which reduced its mass transfer to the aqueous phase.

Rehmann and Daugulis (2006) successfully used bis(2-ethylhexyl)sebacate and octadecene as delivery solvents for biodegradation of biphenyl by *Burkholderia*

xenovorans LB400 in a TPPB system. Their study indicated a very high efficiency of both the solvents, as about 6 g l^{-1} biphenyl was degraded within 25 h.

Vandermeer and Daugulis (2007) compared efficiency of silicone oil and dodecane for degradation of HMW PAHs by a defined microbial consortium of *Sphingomonas aromaticivorans* B0695 and *Sphingomonas paucimobilis* EPA505 in a TPPB system. Dodecane was found to sequester the HMW PAHs due to the inherent high solubility of the hydrophobic compounds in this solvent, and to circumvent this limitation, initial PAH loading concentration was sufficiently increased. And, when silicone oil was used, bioavailability of the HMW PAHs and extents of biodegradation improved, but the rates of degradation were lower than that obtained in the TPPB employing dodecane. These authors also showed that solvents with a high affinity for the target substrate sometimes exhibited contradicting features; while it may be advantageous to allow high initial substrate loadings in the system, it also possesses disadvantages as the solvent may potentially sequester the substrate from the aqueous phase rendering it non-bioavailable.

Eibes *et al.* (2007) studied degradation of anthracene by the enzyme manganese peroxidase (MnP) from the fungus *Bjerkandera* sp. BOS55 in TPPBs using silicone oil. The TPPB yielded near complete oxidation of anthracene at a conversion rate of 1.8 mg l^{-1} h^{-1} within 56 h at optimized agitation and organic phase volume fraction.

2.5.2. Interfacial area in TPPB system

The principal components in TPPBs are the substrate dissolved in the organic phase, inorganic nutrients dissolved in the aqueous phase, oxygen and microbial cells.

Thus, at least four phases are involved in this process, and air-liquid and liquid-solid interfaces exist in this system. The interfacial area between the two liquid phases is the most important factor in the system, which not only permits substrate transport from organic phase to aqueous phase, but also the microbial activity in the interfacial area and in the aqueous phase. Therefore, any variation in the interfacial area affects both the activity of suspended microorganisms in the aqueous phase and the activity of adhering microorganisms at the liquid-liquid interface.

Ascon-Cabrera and Lebeault (1995[a]) investigated the effect of interfacial area of an aqueous-organic biphasic system on growth kinetics of xenobiotic-degrading microorganisms. Substrate concentration in the aqueous phase was independent of the size of interfacial area at different agitator speeds and it decreased as the proportion of the silicone oil phase increased. In this study, the interfacial area increased during the culture process, reached maximum at 24 h; 50% of the biomass was found attached to the liquid-liquid interface forming a biofilm.

MacLeod and Daugulis (2005) studied interfacial effects in a TPPB system for degradation of phenanthrene and pyrene by *Mycobacterium PYR-1*. They observed that cell growth and PAH degradation rates were dependent on agitation, but not with substrate concentration, thus indicating the presence of an interfacial uptake mechanism in the study. It was also observed that *Mycobacterium PYR-1* was associated exclusively with the aqueous-organic interface (specifically, organic side of the interface) due to its high hydrophobicity.

2.5.3. Non-conventional two phase system

Selection of solvent for use in TPPBs requires that it is non-biodegradable, yet biocompatible. And, when both these criteria are not met with a given solvent, the choice becomes limited. Therefore, in recent past, attempts have been made to replace the organic phase with solid polymer as delivery agent, making the system as solid-liquid two phase partitioning reactor system. Solid polymers having high affinity for the target compound and partitioning behavior are preferred over water immiscible solvents in such systems.

Daugulis *et al.* (2003) used two solid polymers poly (ethylene-co-vinyl acetate) (EVA) and poly (styrene co-butadiene) (SB) as delivery agents for benzene to the cells of *Alcaligenes xylosoxidans* in a bioreactor. These authors determined the capacities of EVA and SB for benzene to be 4.19 mg g^{-1} and 9.45 mg g^{-1}, respectively. In a similar study,

Amsden *et al.* (2003) used EVA beads to reduce the aqueous concentration of phenol in a bioreactor from toxic levels (~2,000 mg l^{-1}) to sub-inhibitory levels (~750 mg l^{-1}). However, phenol absorbing capacity of these beads (14 mg g^{-1}) was higher compared to the earlier study by Daugulis *et al.* (2003).

More recently, Rehmann and Daugulis (2007) used a thermoplastic polymer Hytrel™, as solid delivery medium in solid-liquid two phase reactor systems for biphenyl degradation by *Burkholderia xenovorans* LB400, and they demonstrated experimentally that growth of the bacterium was limited due to reduced available surface area of the polymer.

2.6. Statistical Design of Experiments

Experiments are always considered a major tool for researchers to practically validate their theoretical hypotheses about scientific knowhow of a process or phenomenon, where the observation is expected to be correlated with some known associated process variable(s). In order to identify important factors and their contribution towards the observed response, simple traditional approach of using "one-variable-at-a-time" requires a large number of experiments to be performed which often fail to explain any significant interaction among the process variables (Ryan *et al.*, 2007).

Statistical design of experiments could be useful to gain more information from less number of experimental data compared to the conventional approach, where logically selected subset of experiments are executed and meaningful unbiased results are obtained, which can detect significance effects and draw valid and meaningful conclusions.

Factorial design of experiments

Factorial designs are widely used to study the interactions effects of several factors on the final response in experiments, where the levels of independent variables are altered with each experimental runs (Box *et al.*, 2005). The most popular 2-level full factorial design includes all possible factor combinations at two levels - 'low' and 'high' and coded as '-1' and '+1' for each of the input factors, and is known to be powerful in describing factor interactions in multi factor systems to avoid misleading conclusions (Bruns *et al.*, 2006).

Though the full factorial design is highly efficient in determination of all significant main and interaction effects on any response; the resource requirement increases exponentially with number of factors in study. The solution to this problem is to use an adequately chosen fraction of the total runs from the full factorial design, keeping it both balanced (where all treatment combinations have the same number of observations) and orthogonal (effects of each factor and the interaction of the two factors can be estimated independently of each other) (Ryan *et al.*, 2007).

On the other hand Plackett-Burman designs are very efficient screening designs requiring minimal number of experimental runs, and are useful for detecting large main effects, assuming all interactions are negligible.

Factorial design of experiments has largely been employed in studies related to PAH remediation. Pino *et al.* (2001) used a full factorial design to optimize the process time and surfactant concentration in extraction of PAHs from marine sediments with a micellar medium. The results from their study in spiked soil containing 13 PAHs including pyrene indicated that the surfactant concentration and interaction between surfactant concentration and extraction time were the most significant variable affecting the extraction process.

Oliveira *et al.* (2005) adopted a 2^3 full factorial experimental design to estimate the effects of bioaugmentation (using mixed culture), addition of fertilizer or mineral media and initial pH of the soil on biodegradation of pyrene. Remarkable decrease in the concentration of residual PAHs of the soil were observed under optimized condition in test runs carried out in PVC reactors.

Simonnot *et al.* (2006) used 2^{3-1} fractional factorial design to examine the effects of hydrogen peroxide, iron catalyst and reaction time on the Fenton's degradation efficiency of phenanthrene and pyrene. The authors observed best degradation for phenanthrene and pyrene by Fenton's process as follows: 98.1% for phenanthrene and 95.6% for pyrene at 540 mM H_2O_2, 27 mM Fe^{2+} and treatment time of 72 h.

Silva *et al.* (2008) used factorial design of experiments to determine the most effective treatment conditions in photo-Fenton process for the degradation of 16 PAHs in two soil samples artificially contaminated with diesel oil. The authors observed that best degradation conditions resulted in an overall PAH concentration reduction of 94.6% without any external source of iron, low hydrogen peroxide concentration, short exposure time and without any soil pH adjustment.

CHAPTER 3

MATERIALS AND METHODS

3.1. Chemicals and Reagents

Analytical grade pyrene used in this research work was purchased from Sigma-Aldrich Chemicals, India. Other PAHs, *viz.* naphthalene and anthracene, were purchased from Merck, Germany. Bushnell Hass (BH) media, routinely used in the pyrene biodegradation experiments, Nutrient broth (NB) and Brain Heart infusion (BHI) broth were purchased from Himedia laboratories, India. Other chemicals and surfactants of reagent grade and solvents of spectroscopic grade were purchased from Merck, India. Silicone oil (dimethylpolysiloxane) and low viscosity alginate were purchased from Loba Chemie, India. Polyvinyl alcohol (PVA) was supplied by Sigma chemicals, India

3.2. Microorganisms and Culture Conditions

3.2.1. *Mycobacterium frederiksbergense*

M. frederiksbergense (NRRL B-24126) was obtained from Microbial Genomics and Bioprocessing Research unit, USDA, Peoria, Illinois. Nutrient broth having the composition (g l^{-1}) beef extract: 1.5, peptic digest of animal tissue: 5.0, NaCl: 5.0 and yeast extract: 1.5, was used for routine growth (at 28°C and 140 rpm shaking) and maintenance of *M. frederiksbergense*. BH media (pH 7.0) having the composition (g l^{-1}) $MgSO_4 \cdot 7H_2O$: 0.2, $CaCl_2$: 0.02, KH_2PO_4: 1.0, K_2HPO_4: 1.0, NH_4NO_3: 1.0, $FeCl_3$: 0.05

and supplemented with trace elemental solution (2 ml l^{-1} of media) containing (g l^{-1}) KI: 0.30, $SnCl_2 \cdot 2H_2O$: 0.43, LiCl: 0.20, $CuSO_4 \cdot 5H2O$: 0.80, $AlK(SO_4)_2 \cdot H_2O$: 2.10, $NiCl_2$: 0.55, $CoCl_2 \cdot 6H_2O$: 0.85, boric acid: 0.60, $MnSO_4 \cdot H_2O$: 0.37 and $FeSO_4 \cdot 7H_2O$: 0.30, was used in the pyrene and other PAH degradation experiments using *M. frederiksbergense*.

3.2.2. *Mycobacterium vanbaalenii*

M. Vanbaalenii (NRRL B-24157), which was used to compare the pyrene degradation efficiency with that of *M. frederiksbergense*, was also obtained from Microbial Genomics and Bioprocessing Research unit, USDA, Peoria, Illinois. BHI broth having the composition (g l^{-1}) calf brain infusion: 200.0, beef heart infusion: 250.0, protease peptone: 10.0, dextrose: 2.0, NaCl: 5.0, disodium phosphate: 2.5, pH: 7.4±0.2, was used for growth and maintenance of *M. vanbaalenii*. BH media supplemented with trace elemental solution (as described earlier), was used in pyrene biodegradation experiments using this *Mycobacterium*.

3.2.3. Indigenous biosurfactant producing soil microbial culture

Hydrocarbon contaminated soil, collected from a gasoline filling station located in Guwahati, India, was used to obtain a microbial isolate capable of producing biosurfactant. For isolation purpose, 20 g of the contaminated soil was shaken with 100 ml carbon free minimal medium (CFMM) having the composition (g l^{-1}) NH_4NO_3: 3.0, Na_2HPO_4: 2.2, KH_2PO_4: 0.8, $MgSO_4 \cdot 7H_2O$: 0.01, $FeCl_3 \cdot 6H_2O$: 0.005 and $CaCl_2 \cdot 2H_2O$: 0.005, supplemented with pyrene and glucose at concentrations of 50 mg l^{-1} and 10 mM respectively, and at 27^{o}C with agitation (120 rpm) in a rotating orbital shaker for 12 h.

About 5 ml of the culture supernatant was then mixed with 45 ml of fresh CFMM, supplemented with pyrene and glucose, and allowed to grow under same culture conditions. Culture enrichment was obtained by extensive sub-culturing with pyrene and simultaneously decreasing the amount of glucose. Purity of the culture was ensured by plating a single colony from its previous culture onto nutrient agar plate.

3.3. Biodegradation Studies in Slurry Phase System using *M. frederiksbergense*

3.3.1. Single substrate condition

Pyrene biodegradation experiments using *M. frederiksbergense* were first carried out in 250 ml Erlenmeyer flask by taking the required amount of pyrene stock solution (25 g l^{-1}in acetone) in empty flasks to give a final pyrene content of 50 mg l^{-1} based on the final aqueous phase volume. Following evaporation of acetone, 100 ml BH media supplemented with trace elemental solution was added and autoclaved. 5 ml of an overnight grown (~16 h) culture was aseptically added into the flask and incubated at 28°C in shaker incubator set at 180 rpm. One ml samples were collected every day for 8 d to measure the residual pyrene concentration.

Pyrene biodegradation by *M. frederiksbergense* in this slurry phase system was also compared by performing the experiment in a 3 L autoclavable glass fermenter (Applikon® with ADI 1010 BioController and ADI 1025 BioConsole) equipped with pH, temperature, dissolved oxygen sensors for monitoring and/or control. Calculated amount of pyrene stock solution (25 g l^{-1} in acetone) was added to the empty fermenter vessels so as to give a final pyrene concentration of 50 mg l^{-1}. Following evaporation of acetone,

1.5 L BH media supplemented with trace elemental solution was added to the vessel and autoclaved. 50 ml of overnight grown *M. frederiksbergense* culture was aseptically added into the bioreactor as inoculum. The fermenter was operated at 28°C, pH 7.0, aeration rate of 1.5 vvm and agitation rate, 600 rpm. Duplicate samples, of 1.5 ml each, were collected every day for 12 d to measure residual pyrene concentration.

3.3.2. Mixed substrate conditions

Naphthalene and anthracene were chosen for studying their influence on pyrene biodegradation using *M. frederiksbergense.* To evaluate the influence of anthracene, naphthalene and pyrene on each other degradation by the bacterium, a 2^3 full factorial design was employed. The two levels of the factors used in the study were 1 and 50 mg l^{-1} concentrations of each of the PAHs. Table 3.1 presents the factor combinations in the experimental runs performed in this study.

Table 3.1: Experimental design concentration combinations of the PAHs in the slurry phase system to study pyrene biodegradation in mixture.

Run No.	Naphthalene (mg l^{-1})	Anthracene (mg l^{-1})	Pyrene (mg l^{-1})
1	1	1	50
2	50	1	50
3	50	50	1
4	1	50	1
5	50	1	1
6	1	50	50
7	50	50	50
8	1	1	1

All the experimental runs in duplicate were carried out in 250 ml Erlenmeyer flasks. To each flask requisite amounts of pyrene and anthracene from respective stock solutions (5 g l^{-1} in acetone) were separately dispensed. Upon evaporation of acetone in a fume hood, 100 ml BH media was added to give a pyrene concentration/content of 1 or 50 mg l^{-1} based on the aqueous phase volume, and both the PAHs were finely dispersed into the media as suspension slurry. After autoclaving the media at 15 psi for 15 min and allowing to cool down to room temperature, naphthalene was added aseptically from its filter sterilized stock solution (5 g l^{-1} in acetone) in such amount to give desired concentration of the compound as per the experimental design (Table 3.1). Biodegradation experiments were performed under agitation condition (180 rpm) at 28°C, and during the experiments, samples (1 ml) were collected every day for 8 d to measure residual anthracene, naphthalene and pyrene concentrations. For estimation of the PAHs, aqueous samples were extracted with ethyl acetate and analysed by synchronous fluorescence spectroscopy.

Results of PAHs degradation rate and removal efficiencies were also analysed in the form of analysis of variance (ANOVA) and Student's t-test using the statistical software package Minitab 15, PA, USA.

3.4. Biodegradation Studies in Surfactant Aided System

3.4.1. Pyrene degradation using Triton X 100 as the surfactant

In this surfactant aided system, pyrene degradation efficiency of *M. frederiksbergense* was initially compared with another widely studied potential pyrene degrader *M. vanbaalenii* using a two level Plackett-Burman screening design. While

Table 3.2 shows the factors and their levels used in the experimental design, Table 3.3 represents the combinations of the levels of the factors in each of the runs.

Table 3.2: Range and levels of factors used in Plackett-Burman design for comparing the effectiveness of *M. frederiksbergense* with *M. vanbaalenii* in degrading pyrene.

Factors	Level	
	-1	+1
Glucose, gm l^{-1}	0	1
M. vanbaalenii, %v/v	0	5
M. frederiksbergense, %v/v	0	5
Triton X-100, %v/v	0	0.5

Table 3.3: Design matrix showing factor combinations used in the Plackett-Burman design for comparing pyrene biodegradation efficiency between *M. frederiksbergense* and *M. vanbaalenii*.

Factors	Run number											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Glucose	-1	-1	+1	-1	+1	+1	-1	+1	+1	-1	-1	+1
M. vanbaalenii	+1	-1	-1	+1	+1	-1	-1	-1	+1	-1	+1	+1
M. frederiksbergens	+1	-1	+1	-1	+1	-1	+1	+1	-1	-1	+1	-1
Triton X-100	-1	+1	-1	-1	-1	-1	+1	+1	+1	-1	+1	+1

50 µl of pyrene stock solution (20 g l^{-1}) in acetone were used in all the duplicate experimental runs carried out in 150 ml Erlenmeyer flasks. Upon evaporation of acetone in a fume hood, 20 ml BH media was added, so that the total pyrene (both suspended and dissolved) in each flask were 50 mg l^{-1} based on the media volume. Glucose and Triton X-100 were added at final concentrations of 1 g l^{-1} and 0.5% v/v, respectively, when necessary and as per the design. After autoclaving the media at 15 psi for 15 min and allowing to attain room temperature, 1 ml of overnight grown *Mycobacterium* culture

was added to the flasks. Biodegradation experiments were performed under agitation condition (180 rpm) at 28°C. 100 µl samples from each flask were taken at start and end of the experiment, i.e. 5th day, and the samples were analyzed for total pyrene concentration. Sampling and analysis of total pyrene in this study were highly reproducible with less than 5% variation. The duplicate average results of % pyrene degradation in the study were statistically analyzed in the form of ANOVA and significance test of factors using the statistical software package Minitab 15, PA, USA.

3.4.2. Evaluation of different chemical surfactants

3.4.2.1. Determination of molar solubilization ratio of pyrene in presence of surfactant

Based on the chemical nature, i.e., cationic, anionic or non-ionic, five different surfactants, namely Triton X 100, Tween 80, Tween 20, cetyltrimethyl ammonium bromide (CTAB), sodium n-dodecyl sulfate (SDS), were tested for enhancing the aqueous solubility of pyrene in this study. To determine the extent of pyrene aqueous solubility in presence of these surfactants, molar solubilization ratio (MSR) of pyrene was analyzed; and towards this, six different standards for each of the five surfactants were prepared from respective stock solutions, containing 60 times of critical micellar concentration (CMC) each of these surfactants. To each of the standards, 50 mg l^{-1} pyrene was added, properly mixed and the resulting mixture filtered using 0.45 µm syringe filter to separate any insoluble pyrene. Soluble pyrene in surfactant solutions was determined using an earlier prepared standard calibration curve of pyrene concentration vs. synchronous fluorescence intensity. MSR values of pyrene due to the different surfactants

were then obtained from the following equation, which represented the slope of solubilised pyrene vs. surfactant concentration curves:

$$MSR = \frac{(S - S_{CMC})}{(C - CMC)} \quad \textbf{(3.1)}$$

where 'S' is the amount of pyrene solubilised at a surfactant concentration of 'C' and 'S_{CMC}' is the amount solubilised at 'CMC' value of the surfactant.

3.4.2.2. Pyrene degradation experiments

Pyrene biodegradation experiments in the study were first carried out in 250 ml Erlenmeyer flasks by taking 100 ml BH media supplemented with trace elemental solution; calculated amount of pyrene stock solution (25 g l^{-1} in DMSO) was added to the flasks so as to give a final pyrene concentration of 50 mg l^{-1}. Based on the previous experiments to determine MSR of pyrene in presence of the five surfactants, i.e., Triton X 100, Tween 80, Tween 20, CTAB and SDS, 2.32, 2.34, 4.64, 5.88 and 16.62 g l^{-1} of the surfactants respectively were added to the respective flasks for this pyrene biodegradation study. For carrying out the experiments, 5 ml of overnight grown culture was aseptically added into each of the flasks as inoculum and incubated in shaker incubator maintained at 180 rpm and 28°C. Samples were collected at definite intervals of time to analyze residual pyrene and surfactant concentrations.

To compare the pyrene biodegradation by the *Mycobacterium* in presence of the different surfactants batch shake flasks, experiments were also carried out in a 3 L autoclavable glass fermenter operated at 28°C, aeration rate of 0.5 vvm and agitation 400 rpm. The fermenter contained 1 L BH media supplemented with trace elemental solution, and calculated amount of pyrene stock solution (25 g l^{-1}in DMSO) was added so as to

give a final pyrene concentration of 50 mg l^{-1}. Predetermined amount of any of the three nonionic surfactants i.e., Triton X 100, Tween 80 and Tween 20 were added to solubilise pyrene for studying its biodegradation by *M. frederiksbergense*. Before starting the experiment, 50 ml of overnight grown culture (as inoculum) was aseptically added into the fermenter and samples were collected at definite time intervals during the experiment to analyze residual pyrene concentrations.

3.4.3. Evaluation of a biosurfactant produced by indigenous soil microbial culture

Kinetics of biosurfactant production by an indigenous soil microbial culture, mentioned earlier, was studied in CFMM supplemented with glycerol for 5 d. Samples were collected at 12 h interval, centrifuged at 13000 rpm at 4°C for 10 min, and the obtained cell pellet was dried at 50°C till constant weight. Surfactant concentration in the culture broth was analysed indirectly by its emulsifying activity against xylene and again checked with the amount of ammonium sulphate precipitate produced from the culture supernatant.

3.4.3.1. Isolation and partial purification of the biosurfactant

Biosurfactant production by the soil microbial culture was initially observed by formation of a stable emulsion when supernatant of the 3 d old culture was mixed with any organic solvent. For isolation of the biosurfactant, bacterial cells were removed from the culture broth by centrifugation (13000 rpm, 4^{o}C for 15 min) and the cell free broth was again filtered using 0.45 µm Millipore® filter to remove any extraneous matter present in the culture supernatant. The filtrate was then saturated with 50 % (w/v)

ammonium sulphate; the turbid suspension, obtained after overnight incubation at 4°C, was centrifuged. The precipitate so obtained was dissolved in water, dialyzed against deionized water, and lyophilized, thus yielding the partially purified biosurfactant.

3.4.3.2. PAHs solubilisation assay

All PAHs solubilisation analysis in this study was performed in double distilled water. Stock solutions (10 g l^{-1}) of pyrene and anthracene prepared in acetone, were distributed into eppendorf tubes to yield 500 µg of the PAH per tube. The tubes were left open inside an operating chemical fume hood to remove the solvent, and 1 ml of the partially purified biosurfactant solution in water of known amount was added. All experiments were performed in duplicates. The tubes were then capped and incubated overnight at 27°C with shaking. For determining the amount of PAH solubilised in the tubes, samples were filtered through 0.45 µm filter to remove any insoluble particulate PAH, and were analyzed for pyrene and anthracene.

Further, to determine the kinetics of solubilisation, 500 µg of anthracene or pyrene was crystallised in the bottoms of 1-ml quartz cuvette. After placing the cuvette in the holder of a spectrophotometer (SPEKOL 1200, Analytikjena®), 1 ml of pre-filtered (0.45µm, Millipore®) assay solution containing desired concentrations of the biosurfactant was added. Samples withdrawn at regular interval of 5 min for 3 h during the experiments were analysed for anthracene and pyrene concentrations taking biosurfactant solution as a control. The kinetics software package provided by the manufacturer was used in this experiment.

3.4.3.3. Emulsification activity and emulsion stability of the biosurfactant

Besides testing the biosurfactant towards solubilisation of PAHs, ability to form emulsion with different organic solvents and their stability was also investigated. For performing the study, emulsification method of Cirigliano and Carman (1984, 1985) was followed, according to which diluted biosurfactant solution (1ml) was mixed with 0.5 ml of an organic solvent, namely xylene, toluene etc. and vortexed for 2 min and allowed to stand for further 2 min. Turbidity of the resulting emulsion was measured at 600 nm by photo-emission diode spectrometer (SPEKOL 1200, Analytikjena®). One unit of emulsification activity of the biosurfactant was defined as the amount that resulted in an emulsion with OD_{600} of 1.0.

Stability of the emulsion formed were also analysed by allowing the emulsions to stand for about 30 min at room temperature and measuring the absorbance at 600 nm every 5 or 10 min for about 1 h. Stability of the emulsion was thus expressed as the decay constant (K_d) in the following equation (Kim *et al.* 2000).

$$K_d = \frac{d(\log A)}{dt} \qquad \textbf{(3.2)}$$

where, 'A' is the absorbance (OD_{600}) of the emulsion at time't'.

3.4.3.4. Effects of environmental factors on emulsification activity and its stability by the biosurfactant

To investigate the influence of pH on emulsification activity and stability of the biosurfactant, aqueous solutions containing the biosurfactant were adjusted to various pH in the range of 2-12 and incubated for 1 h at 4°C. 0.5 ml of xylene was added to each of

them, vortexed for 2 min and the resulting mixtures were tested for emulsion activity and stability, as described earlier.

Similar experiments were carried out to observe the effect of temperature on emulsification activity and stability of the biosurfactant. The temperature range studied was 20 to 100^{o}C.

3.4.4. Pyrene biodegradation using Tween 80 as the surfactant

3.4.4.1. Single substrate condition

Based on the earlier experiments, Tween 80 was chosen as the most effective surfactant compared to other chemical surfactants tested. The flasks containing varying concentrations of Tween 80 solubilised pyrene in the range of 0.1 to 10 mg l^{-1} were inoculated with 5 ml of the *Mycobacterium* culture and incubated for 48 h. Samples were collected at 12 h interval and analyzed for residual pyrene concentration.

To validate the pyrene biodegradation by *M. frederiksbergense* with the aid of the surfactant Tween 80, experiments were carried out in a 3 L fermenter containing 1 L BH media. Concentrations of pyrene in the experiments were kept at 1, 5, 10, 25 and 50 mg l^{-1} based on total aqueous phase volume. Samples were collected at definite time interval for 120 h and analyzed for residual pyrene concentration.

3.4.4.2. Mixed substrate condition

Pyrene biodegradation by the *Mycobacterium* in Tween 80 aided system was also performed in presence of the other PAHs namely, anthracene and naphthalene. In this mixture study, where all the three PAHs, including pyrene, were presented as substrates,

statistically valid 2^3 full factorial design of experiments, as performed earlier for slurry phase biodegradation study, was employed with the PAHs concentrations varying between 1 and 50 mgl^{-1}. The concentrations combinations of the PAHs adopted in the experimental runs are presented in Table 3.4.

All the experiments were carried out in 250 ml Erlenmeyer flasks in which required amount of pyrene and anthracene from their respective acetone stock solutions (5 g l^{-1} each), were separately dispensed kept in fume hood to evaporate the solvent, and subsequently, 100 ml BH media was added to the individual flasks. Before autoclaving, Tween 80 was added at a concentration of 4 g l^{-1} to solubilise the added PAHs; and thereafter, at normal room temperature, naphthalene at desired concentration was added aseptically from its filtered stock solution (5 g l^{-1} in acetone). All experiments were performed under agitation condition (180 rpm) at 28°C, and during the experiments 1 ml samples were collected every day for 8 d to measure residual anthracene, naphthalene and pyrene concentrations. Samples were extracted twice with equal volumes of ethyl acetate by vortexing for 1 min and then centrifuging at 10000 *g* for 10 min to allow phase separation. Ethyl acetate extracts were then pooled and the total volume adjusted to 1 ml. PAHs concentrations in all the samples were quantified using synchronous fluorescence spectroscopy, and the results of % PAHs removal were calculated as per the following equation:

$$q = \left[(C_i - C_f) / C_i \right] \times 100 \qquad \textbf{(3.3)}$$

where, q is the PAH degradation percentage, C_i and C_f are the initial and final PAH concentrations respectively, in mixture (mg l^{-1}).

Analysis of variance (ANOVA) was applied to estimate the effects of the PAHs and their potential interaction effects on the individual PAHs removal. All statistical analysis of the results in the study was done using the software MINITAB (Version 15, PA, USA).

Table 3.4: Experimental design showing concentration combination of the PAHs in the Tween 80 aided system to study pyrene biodegradation in mixture.

Run No.	Naphthalene (mg l^{-1})	Anthracene (mg l^{-1})	Pyrene (mg l^{-1})
1	1	1	50
2	50	1	50
3	50	50	1
4	1	50	1
5	50	1	1
6	1	50	50
7	50	50	50
8	1	1	1

3.5. Biodegradation Study in Two Phase Partitioning Bioreactor Systems

3.5.1. Selection of solvents

For selecting a suitable solvent that is biocompatible and non-biodegradable in this biodegradation study employing TPPB system, isopropyl myristate, n-hexadecane and silicone oil were examined. Soyabean oil was chosen as a positive control in the study by obtaining the growth of *M. frederiksbergense* in presence of the three solvents.

Five 125 ml flasks were prepared, each containing 40 ml of BH medium (with trace metal solution), 5 ml of previously grown *M. frederiksbergense* as the inoculum, 5 ml of any of the solvents and 2 ml of soybean oil; an additional flask containing all but no solvent served as the positive control. Another flask, which served as the negative control, contained neither soybean oil nor any solvent. All these flasks were incubated at 28°C, and at the end of 3 d the biomass was collected, washed to remove residual solvent and then dried at 100°C to constant weight. The relative metabolic activity due to the solvents in each flask was calculated by dividing the biomass from each of the flasks by the biomass obtained in the positive control. Biocompatible solvents were then tested for their use as a carbon source by *M. frederiksbergense*. The procedure followed was the same as above, except that soybean oil was omitted from the flasks other than the positive control**.**

3.5.2. Determination of volumetric oxygen mass transfer coefficient

In the TPPB system containing silicone oil and aqueous phase, the effect of adding the solvent on oxygen mass transfer coefficient (k_La) in the system was evaluated. And, in this hydrodynamic study, all the experiments for calculating the enhancement in oxygen mass transfer in the two liquid phase system were performed in a 3 L fermenter (Applikon® , Netherlands) of height 25 cm, internal diameter 13 cm and fitted with all necessary accessories for providing agitation and aeration, measurement of online DO and temperature in the system. The organic phase silicone oil had density 930 g l^{-1} and viscosity 10 cps at 25°C. The total working volume in the fermenter was 1 L. The ranges of operating conditions adopted in the experiments were as follows: silicone oil volume

fraction (X_{org}), 0-30%; agitation, 400-800 rpm and aeration, 0.5-2.5 l min^{-1}. Table 3.5 presents the different operating conditions combination adopted in the hydrodynamic study along with the estimated system parameters. Oxygen mass transfer coefficients in the system with and without silicone oil denoted as $k_L a_{TP}$ and $k_L a_A$ respectively, were calculated by the dynamic gassing out technique (Shuler and Kargi, 2002), using nitrogen gas for initially setting the DO content to zero at the start of each experimental run.

Table 3.5: Estimated system parameters at different operating conditions for $k_L a$ determination in the TPPB system.

Aeration (l min^{-1})	Agitation (rpm)	P_g/V (W m^{-3})	V_s (m s^{-1}) ($\times 10^{-4}$)
0.5	400	176.076	6.275
	600	631.520	
	800	1562.948	
1.5	400	133.495	18.827
	600	478.798	
	800	1184.977	
2.5	400	117.370	31.378
	600	420.966	
	800	1041.847	

3.5.3. Pyrene biodegradation experiments with the TPPB system

3.5.3.1. Single substrate condition

All biodegradation experiments in this study containing only pyrene as the single substrate (pollutant) were carried out in the same fermenter that was previously employed to investigate the hydrodynamics, i.e. enhancement in oxygen mass transfer in the system. Figure 3.1 is a schematic of the TPPB system employed in the study.

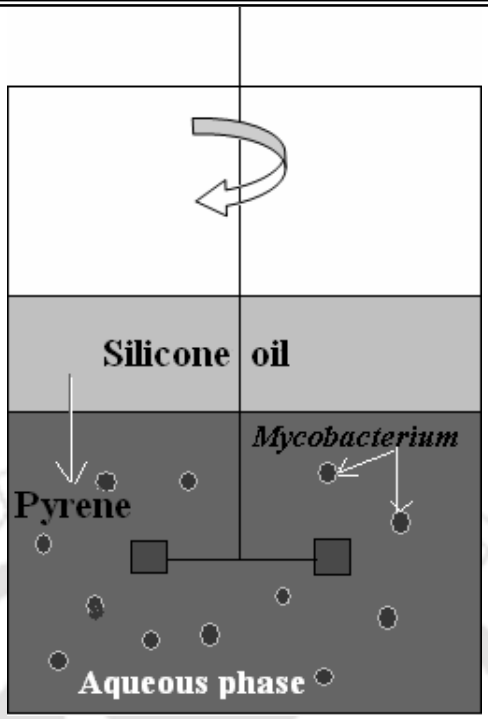


Figure 3.1: Schematic of the TPPB system employed in the pyrene biodegradation studies.

The operating conditions were kept optimum at 28°C, pH 7.0, aeration rate 1.5 vvm and agitation rate 600 rpm. While BH medium (1 L) supplemented with trace elemental solution constituted the aqueous phase of the system, organic phase was prepared by dissolving required amounts of pyrene in 250 ml of silicone oil and incubating the mixture in an ultrasonic water bath for 1 h, which gave different pyrene concentrations in the range of 200-1000 mg l^{-1} in silicone oil. Both the aqueous and organic phases were loaded together in the fermenter vessel and autoclaved. Fifty milliliters of overnight grown *M. frederiksbergense* culture was aseptically added into the bioreactor as inoculum. During its operation, duplicate samples of 1.5 ml each were withdrawn from the fermenter everyday for 15 d for measuring the residual pyrene concentration in the organic phase.

3.5.3.2. Mixed substrate condition

Pyrene biodegradation efficiency by the *Mycobacterium* in concomitant presence of other PAHs, naphthalene and anthracene, were also evaluated employing the developed TPPB system. The fermenter operating conditions were identical with those in the single substrate condition, as described earlier. However, organic phase was prepared by dissolving anthracene and pyrene each at previously found optimum concentration of 400 mg l^{-1} in silicone oil by the use of ultrasonic water bath for 1 h. Both aqueous and organic phases were loaded in the fermenter and autoclaved. Requisite amount of naphthalene was then added after dissolving in a small volume of pre-sterilized silicone oil. Fifty ml of overnight grown *M. frederiksbergense* culture was aseptically added into the bioreactor as inoculum. This mixed substrate experiment was run for 7 d, and during which duplicate samples of 1 ml each were withdrawn from the fermenter everyday for analyzing the residual PAHs concentrations in the organic phase.

3.6. Evaluation of a Non-Conventional TPPB System in Pyrene Biodegradation by *M. frederiksbergense*

In this study, a non-conventional TPPB system involving encapsulation of pyrene containing silicone oil in a suitable matrix was developed and evaluated of its performance in pyrene degradation by the *Mycobacterium*.

3.6.1. Standardization of pyrene encapsulation method

Five different approaches based on delivery vehicle of pyrene or encapsulating material were initially considered for preparation of suitable pyrene encapsulated beads.

Pyrene was delivered either in silicone oil or water miscible solvent or solubilised in surfactant solution. The encapsulating material used was either alginate or alginate-PVA mixture.

Table 3.6 shows the variations involved while preparing the five different pyrene encapsulated beads. The table shows that the variations were with respect to the solvent used to solubilise pyrene, encapsulating material, presence or absence of additives and composition of the gelling medium. Pyrene at 1 g l^{-1} was dissolved either in silicone oil (Type I, II, III and V) or DMSO (Type IV) as the organic solvent. The encapsulating material consisted of either alginate (2% w/v) (Type I and IV) or alginate-PVA mixture (2% w/v and 5% w/v respectively) (Type II, III and V), which was further mixed with pyrene containing organic solvent either by adding Triton X-100 for bead types I, II, III and IV or without adding any surfactant in case of the bead type V.

Table 3.6: Variations in the preparation of different pyrene encapsulated bead types in the non-conventional TPPB system.

	Bead Types				
	I	**II**	**III**	**IV**	**V**
Delivery phase	Silicone oil	Silicone oil	Silicone oil	DMSO	Silicone oil
Alginate (2% w/v)	+	+	+	+	+
PVA (5% w/v)	-	+	+	-	+
Surfactant	+	+	+	+	-
$CaCl_2$ (20% w/v)	+	+	+	+	+
Boric acid (Saturated)	-	-	+	-	+

The mixtures were then emulsified using a high-speed homogenizer (T25, IKA®, Germany) at 7,000 rpm for 10 min in an ice bath, and the resulting o/w emulsion was placed on an orbital shaker set at 150 rpm for 6 h to deaerate. Encapsulated beads were subsequently prepared by extruding the o/w emulsion in a drop wise manner into gently agitated 200 ml of chilled 20% w/v calcium chloride solution containing 0.2% Triton X-100 using a fine 21-gauge stainless steel needle from a distance of about 6 cm above the surface of the gelling solution. The gelling solution for bead types III and V contained saturated boric acid in addition to calcium chloride and Triton X-100. The prepared beads were allowed to harden for 12 h, washed with distilled water and air dried for 12 h at room temperature.

To estimate the pyrene encapsulation efficiency of the beads, the following relationship was used:

$$Encapsulation\ effeciency\ (\%) = \left[\left(M_i - M_d\right)/M_i\right] \times 100 \qquad \textbf{(3.4)}$$

where M_i is the initial mass of pyrene present in the hydrogel/mixture solution prior to the drop wise extrusion into gelling medium and M_d is the residual mass of pyrene measured in the gelling medium immediately after preparation of the pyrene-loaded beads.

Further, to select the best pyrene encapsulated bead among the five different bead types, pyrene release from the beads was carried out in 250 ml Erlenmeyer flasks each containing 100 ml of 10 CMC Triton X-100 solution. Accurately weighed dried beads known to contain 500 µg pyrene (based on mass balance) were added to the flasks, incubated at 28°C and shaken at 180 rpm in an orbital incubator shaker. Samples (1 ml) were taken from the flasks at regular intervals for 72 h; and for the every withdrawal of samples from the flasks, an equivalent volume (1 ml) of the fresh medium was added to

maintain a constant volume of the release medium in the flasks. Concentrations of pyrene in the samples were analyzed and the bead type yielding highest and sustained pyrene release was, therefore, chosen for further characterization study.

3.6.2. Characterization of the bead type V

3.6.2.1. Swelling behavior

Initially prepared beads of this type (V) were weighed (W_i) and placed in a beaker with a dissolution medium containing 10 CMC Triton X-100 solution and was agitated at 180 rpm on a shaker–incubator at 28°C. After every 10 min, beads were removed from the solution, blotted with Whatman® filter paper to remove excess water and re-weighed (W_f). The swollen beads were handled carefully in order to avoid breakage or erosion of the beads. Swelling - the percentage increase in weight of the beads due to the absorbed water was estimated using the following equation:

$$Swelling\ (\%) = \left[\left(W_f - W_i\right)/W_i\right] \times 100 \qquad \textbf{(3.5)}$$

3.6.2.2. Optical and scanning electron microscopy

To clearly distinguish silicone oil droplets in the encapsulated beads, cross-sections of the air dried beads was observed under stereo zoom microscope (Nikon, USA). Prior to this, the cross section of the beads was flooded with coomassie blue stain for 10 min, washed with distilled water to remove excess stain and air dried for 1 h. The morphology of the beads were also observed under a scanning electron microscope (Leo 1430 VP, UK), for which the beads were lyophilized for 12 h, dried under vacuum and

their cross-sections were mounted onto stubs using double-sided adhesive tape for vacuum coating with gold film using sputter coater (Edward, UK).

3.6.2.3. Pyrene release kinetics

To investigate the effect of surfactant concentrations in the release medium on pyrene release, calculated amount of the encapsulated beads containing 500 µg pyrene were taken in 250 ml Erlenmeyer flasks with different concentrations of Triton X-100 (2-10 CMC) in 100 ml release medium. Experiments were carried out in the same manner, as before for investigating the pyrene release profile from the beads. However, in this particular characterisation study, 0.5 ml silicone oil containing an equivalent amount of pyrene in 100 ml 10 CMC Triton X-100 solution taken in a 250 ml flask served as the control. Samples (1 ml) were collected from the sink medium for 24 h, and an equivalent volume of fresh medium was replaced for every sample withdrawal from the flask.

3.6.2.4. Reusability test of the bead type V

To check the feasibility of reusing the beads for reloading pyrene following its initial exhaustion, virgin silicone oil containing beads (without pyrene) and blank control beads (excluding both silicone oil and pyrene) were prepared as before and accurately weighed to 500 mg. The beads were then soaked in 100 ml unsaturated pyrene solution (50 mg l^{-1} in 10 CMC Triton X-100 solution) for 48 h in shaking incubator set at 28°C and 180 rpm. At the end of the incubation period, beads were washed with distilled water, vacuum dried and subsequently subjected to reveal its pyrene release behavior.

3.6.3. Pyrene biodegradation experiments using the bead type V

To demonstrate the utility of the developed non-conventional TPPB method for pyrene biodegradation applications, experiments were carried out using *M. frederiksbergense* with the pyrene encapsulated bead type V. Calculated amount of beads containing 100 µg pyrene were taken in 250 ml Erlenmeyer flasks with 100 ml BH media inoculated with 5% v/v overnight grown culture of *M. frederiksbergense*. An abiotic control flask containing an equivalent amount of beads with BH media was also included in the experiments. All the flasks were incubated in a shaker incubator set at 28°C and 180 rpm, and samples were collected at a regular interval for 5 d from the aqueous phase for measuring pyrene concentrations.

3.7. Analytical Techniques

3.7.1. Pyrene analysis

For estimating pyrene at concentrations less than 100 µg l^{-1}, stock solution (50 g l^{-1}) was prepared in dichloromethane (DCM) and synchronous fluorescence (SF) method was developed using a FluoroMax-3® (HORIBA Jobin Yvon, USA) spectrofluorimeter. Synthetic quartz cuvettes were of Hellma® type 104.045Q with a path length of 10 mm. For optimum selectivity and sensitivity of the SF analysis (Hua *et al.*, 2006, 2007) both the excitation and emission slits were set at 2 nm. All measurements were carried out at an integration time of 0.5 s and PMT voltage at 700 V.

Selection of SF parameters and calibration

Optimum wavelength offset and excitation wavelength in the range of 300-400 nm for pyrene in the SF method were found by changing the wavelength offset ($\Delta\lambda$) between 5 and 100 nm at initial intervals of 5 nm and subsequently at 1 nm interval. Scans with $\Delta\lambda$ less than 5 nm or more than 100 nm were, however, found not useful for determination of the PAH (Hua *et al.*, 2007).

Figure 3.2 shows the SF spectra of pyrene for a concentration of 50 µg l^{-1} in DCM, where maximum intensity of the pyrene emission peak was at $\Delta\lambda = 39$ nm. Any further increase or decrease in the value of $\Delta\lambda$ showed intensity less than the maximum, and, therefore, the SF peak selected for quantification of pyrene was obtained at an optimized excitation wavelength (λ_{opt}^{max}) = 335 nm and $\Delta\lambda_{opt}$ = 39 nm. All synchronous spectra were recorded in an excitation scale and the fluorescence intensity was expressed as either cps (counts per second).

The SF peak heights were used in the preparation of calibration curve with pyrene concentrations in the range 10-100 µg l^{-1}. Figure 3.3 is a calibration curve showing a perfect linear relationship (R^2 >0.99) between pyrene concentration and fluorescence intensity used for pyrene analysis in the study. These results also indicated that the maximum measurable pyrene concentration by the method could be 100 µg l^{-1}, which is much higher than the reported limit of 28.5 µg l^{-1} in minimal salt media (MSM) using the same technique (Zhang *et al.*, 2004).

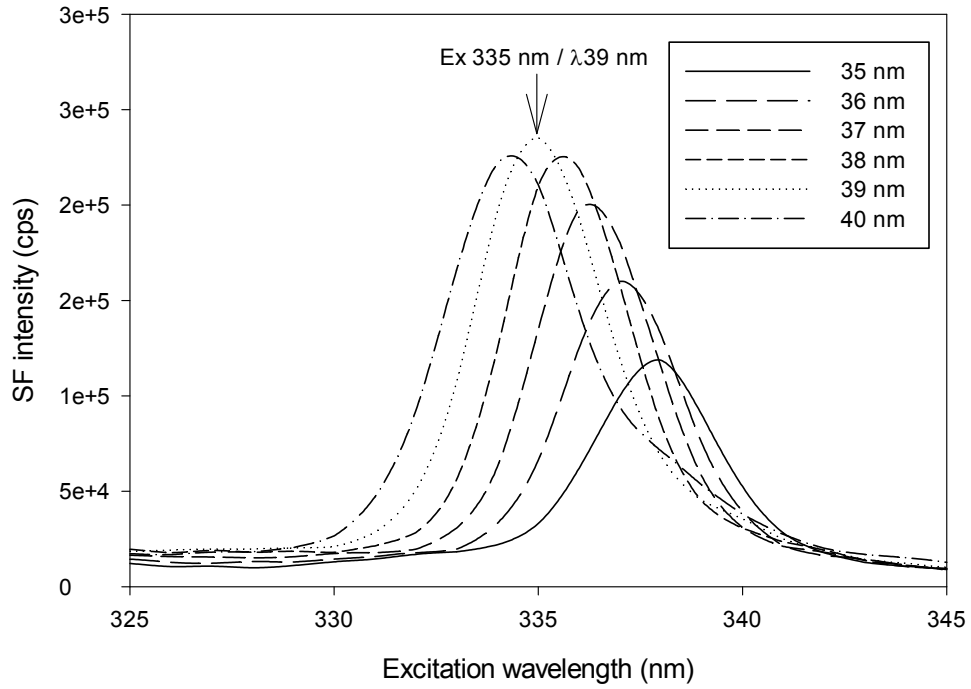


Figure 3.2: Synchronous-scan fluorescence spectra of pyrene (50 μg l^{-1} in DCM) at different wavelength offset (Δλ).

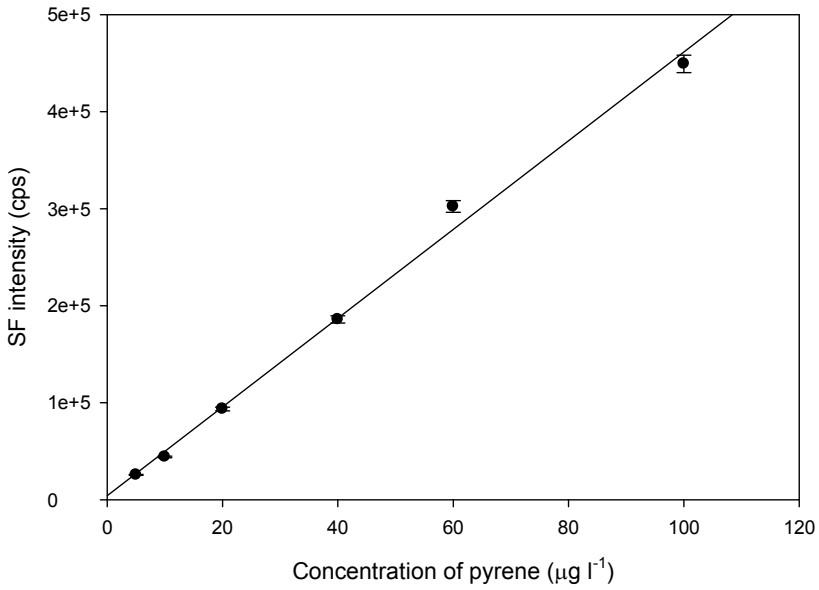


Figure 3.3: SF concentration calibration curve used in pyrene analysis at optimized synchronous parameters.

Specificity of pyrene estimation in a mixture of PAHs

The validity of the optimized $\Delta\lambda_{opt}$ and λ_{opt}^{max} in the developed SF method for pyrene analysis was further checked by estimating pyrene in presence of another PAH compound - naphthalene or anthracene (model 2 and 3 ring PAHs). Three test samples were prepared each containing 20 µg l^{-1} pyrene in DCM and two of them contained either naphthalene or anthracene (20 µg l^{-1}) in addition to pyrene. Synchronous spectra for all the samples (at $\Delta\lambda_{opt}$ and λ_{opt}^{max}) were recorded to validate the specificity of the method for analyzing pyrene in a mixture of PAHs. Figure 3.4 presents the SF profiles of pyrene in a mixture of PAHs, which shows that the presence of naphthalene or anthracene did not affect the pyrene SF signal position at its λ_{opt}^{max} 335 nm. However, the SF intensity of pyrene was slightly increased - by 4.7% with anthracene and 11% with naphthalene as compared to pyrene alone.

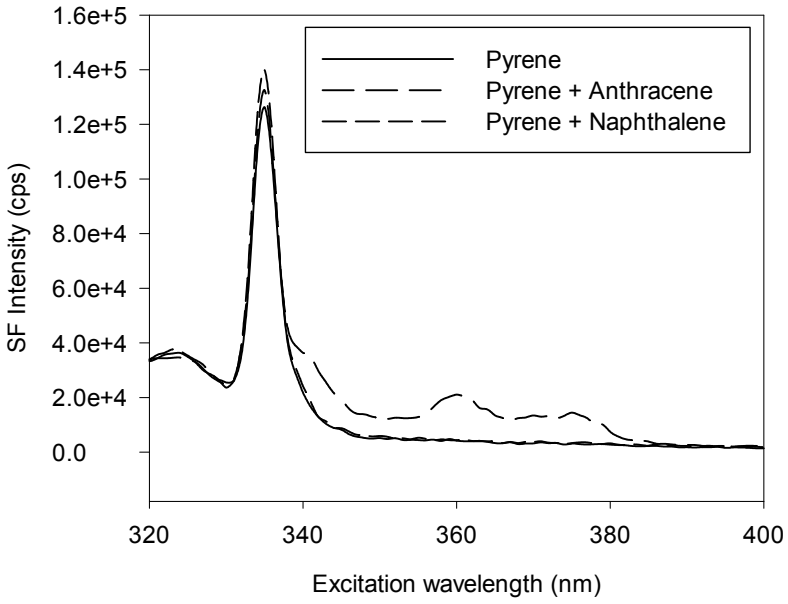


Figure 3.4: Interference to pyrene estimation by an equal concentration of anthracene or naphthalene.

Fixed wavelength fluorescence (FF) analyses were also performed to get a profile of pyrene along with the metabolites produced in biodegradation studies. For FF analysis of pyrene, excitation wavelength ($\lambda_{excitation}$) = 345 nm and emission wavelength ($\lambda_{emission}$) = 382 nm were chosen (Watson *et al.*, 2004).

3.7.2. Naphthalene and anthracene analysis in mixtures containing pyrene

Concentrations of other PAHs like naphthalene or anthracene were analysed using SF method and the detection conditions for these two PAHs were optimized by preparing individual standard solutions of naphthalene, anthracene and pyrene at equal concentrations in DCM. Synchronous scan was performed with these PAH containing standard solutions by changing the wavelength offset ($\Delta\lambda$) between 20 and 120 nm at intervals of 5 nm. Other fluorescence parameters were kept the same as those of the previously developed SF method for pyrene analysis. In the analysis of a PAH compound in ternary mixture, optimum wavelength offset ($\Delta\lambda$) and excitation wavelengths were assigned to the compound in such a way that the remaining two PAHs compounds interfere to the least extent. Thus, anthracene, naphthalene and pyrene were detected at $\Delta\lambda$ = 25, 35 and 35 nm and λ_{opt}^{max} = 377, 287 and 339 nm, respectively. Figure 3.5 illustrates the basis for which these settings of $\Delta\lambda$ and λ_{opt}^{max} were chosen for the analysis of these PAHs in mixture.

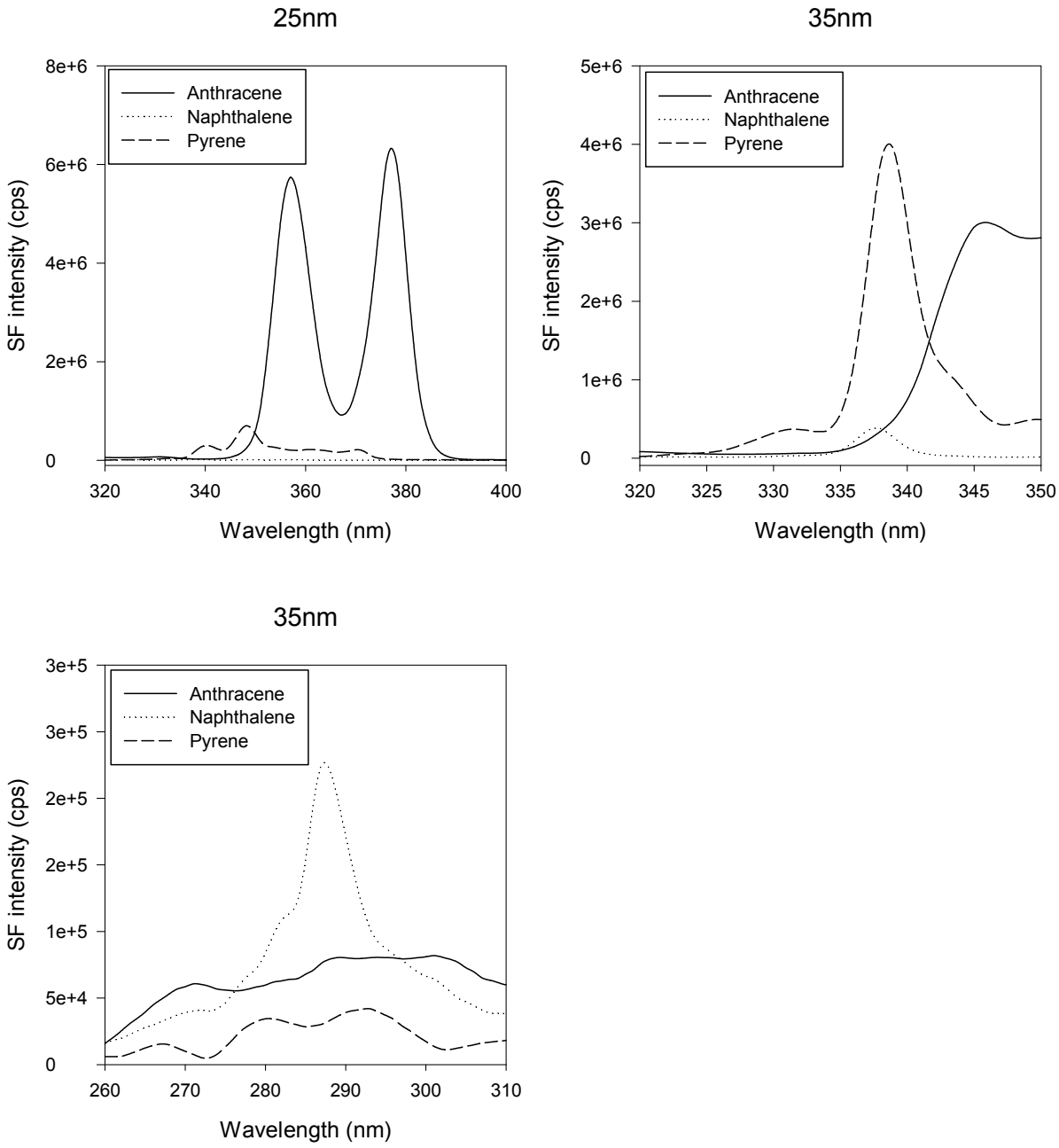


Figure 3.5: Selection of SF parameters for the identification of naphthalene, anthracene and pyrene in mixtures.

3.7.3. Analysis of surfactants

For analyzing the concentration of Tween 20, Tween 80, Triton X 100 and CTAB were determined with colorimetric method described by Hodgson *et al.* (2000) and

Woertz and Kinney (2004). In this method, 600 µl of the sample were mixed with 200 µl of reagent, which was constituted by mixing 30 g l^{-1} cobalt nitrate and 200 g l^{-1} ammonium thiocyanate, in 2 ml micro-centrifuge tube. The tubes were vortexed for 1 min and allowed to react at room temperature for 1 h. Samples were extracted with 1 ml DCM and the absorbance of DCM layer was determined at 650 nm. Concentrations of the surfactants were calculated on the basis of similarly prepared standard calibration curves between surfactant concentrations vs. OD_{650} (Figure 3.6), having R^2 of 0.9776, 0.9883, 0.9950 and 0.8187 for Tween 20, Tween 80, CTAB and Triton X 100, respectively.

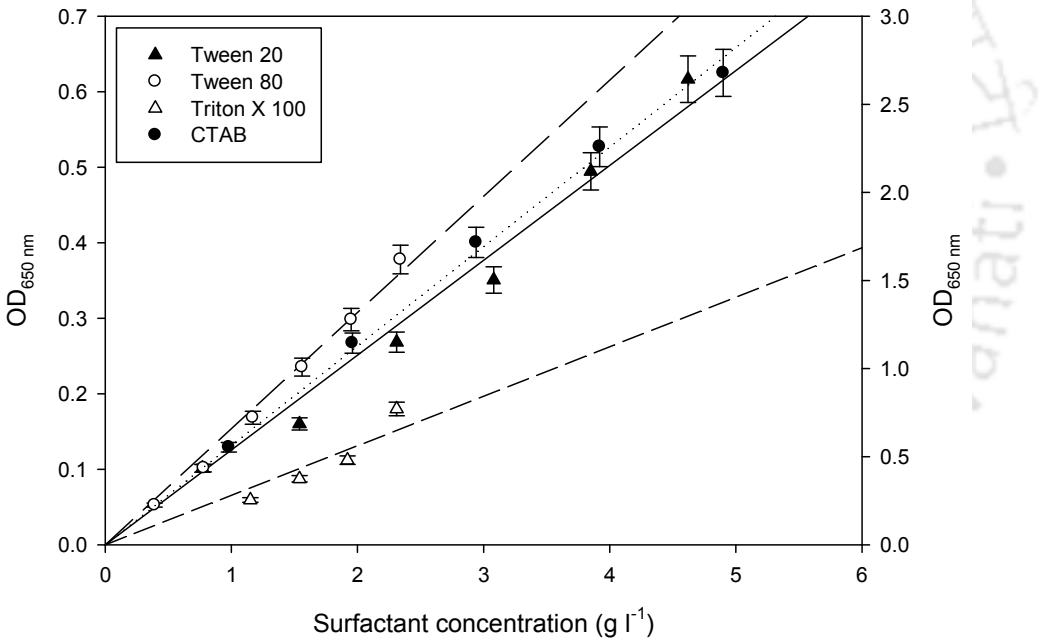


Figure 3.6: Calibration curves used in the analysis of synthetic surfactants by colorimetric method. (Secondary X- axis represents OD_{650} of CTAB).

Sodium n-dodecyl sulphate (SDS) concentration was measured by the colorimetric method for methylene blue active substances (Hayashi, 1975). Sample solution of volume 1 ml containing 0-10 mg l^{-1} of SDS was taken in 15 ml centrifuge tubes, and, when necessary, the samples were diluted. 0.5 ml methylene blue solutions

(0.5% stock in water diluted 100 fold with 0.7 mM phosphate buffer pH 7.2) were then added to each tube, mixed and 3 ml of chloroform was added followed by vigorous mixing. The two phases were separated by centrifugation at 10,000 rpm for 5 min at 4°C. Organic layer was carefully drawn out, and its absorbance at 655 nm was measured with Carry 100 Varian® spectrophotometer using proper blank. Concentrations of the surfactants were calculated on the basis of similarly prepared calibration curve (Figure 3.7).

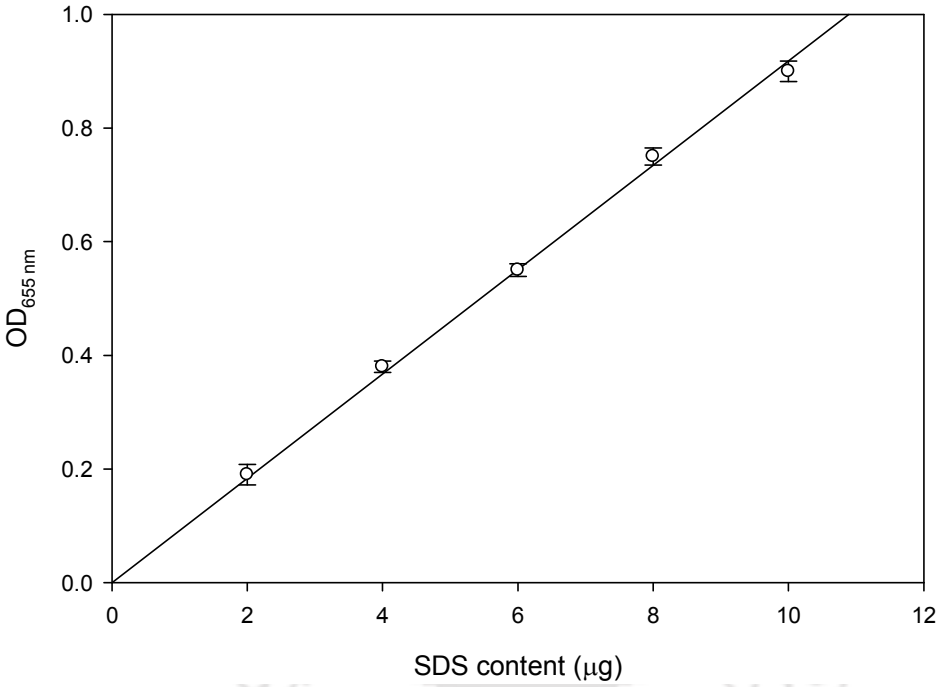


Figure 3.7: Calibration curve used in the colorimetric analysis of SDS.

3.7.4. Cell growth measurements

Quantification of the *Mycobacterium* biomass in term of its protein content was achieved based on a rapid and reliable protein extraction and estimation method (Makkar *et al.*, 1982; Meyers *et al.*, 1998). By this method, 1ml culture samples taken in

duplicates were centrifuged at 13,000 *g* for 15 min to produce compact cell pellets. Without re-suspending the cells, the pellets were washed with 1 ml of phosphate-buffered saline (PBS) of pH 7.0 and were centrifuged, as before. The pellets were then re-suspended in 0.1 ml of 1 M NaOH and the sealed tubes were placed in a boiling water bath for 10 min. The samples were neutralized by adding 0.1 ml of 1 M HCl, and the final volume adjusted to 1 ml with PBS (pH 7.0). Samples were centrifuged at 10,000 *g* for 30 min, and the supernatant was removed for measuring its absorbance (OD) at 230 and 260 nm. Protein concentrations in the samples were thus determined from the following equation (Makkar *et al.*, 1982)

$$\text{Protein concentration (mg l}^{-1}\text{)} = 183 \times OD_{230} - 75.8 \times OD_{260} \quad \textbf{(3.6)}$$

CHAPTER 4

RESULTS AND DISCUSSION

Among the various environmental pollutants, pyrene and other PAHs biodegradation has been found to be the most challenging owing to their low aqueous solubility and bioavailability. Very slow growth rate of microorganism capable of degrading pyrene further aggravate the situation. Hence, *Mycobacterium frederiksbergense*, which is considered as an efficient pyrene degrading organism, was employed to systematically evaluate the performance of different treatment systems in enhancing the efficiency and rate of pyrene degradation, in single and mixed substrate conditions.

An already known fact is that the *Mycobacterium* utilizes pyrene as the sole source of carbon and energy without any significant increase in its biomass amount (Johnsen *et al.*, 2002). In fact preliminary studies aiming at quantifying the growth of *M. frederiksbergense* in term of its protein content due to pyrene in the three systems evaluated in the work, revealed no appreciable increase in its growth. This observation is also supported in the literature, where *M. frederiksbergense* use HMW PAHs mainly for its cellular maintenance rather than its growth (Willumsen *et al.*, 2001). However in a highly enriched Trypticase soy broth agar in absence of pyrene or other PAHs, growth of this microorganism appears only after 5-7 days. Also, in the biodegradation of pyrene by *Mycobacterium*, there is no accumulation of any intermediate of the process (Giessing

and Johnsen, 2005). This aspect was also checked in one of our studies involving biodegradation of pyrene in surfactant aided system.

This chapter discusses the results of pyrene biodegradation in single and mixed substrate conditions employing three different systems. The chapter also compares the three systems with respect to their effectiveness in pyrene biodegradation and arrives at a cost benefit analysis of the three systems. Finally, based on the recommendation of cost benefit analysis, a non-conventional TPPB system was also developed with an aim to overcome certain disadvantages of conventional TPPB system.

4.1. Biodegradation of Pyrene in Slurry Phase System

Pyrene biodegradation experiments in slurry phase system were carried out in batch shake flask with a concentration of 50 mg l^{-1}, and the results were compared to that obtained by performing experiments in the fermenter. Of particular significance in this system is the low aqueous solubility of pyrene along with high dosing level (50 mg l^{-1}) in which pyrene is largely present as finely suspended solids. Biodegradation of pyrene in such slurry phase system essentially depends on the dissolution kinetics, which in turn is a function of operating conditions prevailing in the system. For complete degradation of added pyrene in such system, dissolution rate is desired be higher than the intrinsic biodegradation rate of soluble fraction of pyrene. The chosen pyrene concentration of 50 mg l^{-1} in the slurry system was also based on various literature reported data (Juhasz *et al.*, 2000).

4.1.1. Single substrate condition

Figure 4.1 illustrates the profile of pyrene degradation in the slurry phase system, which shows that in simple shake flask pyrene degradation had a lag phase of approximately 72 h and was not complete even at the end of 7 d. A net degradation rate of 19.86 mg l^{-1} d^{-1} was achieved during the active degradation period.

In the batch operated fermenter, pyrene was completely degraded within 200 h for the same initial concentration of 50 mg l^{-1}. Although, no initial lag phase in degradation was observed in the slurry phase fermenter, the overall degradation rate was only 6 mg l^{-1} d^{-1}.

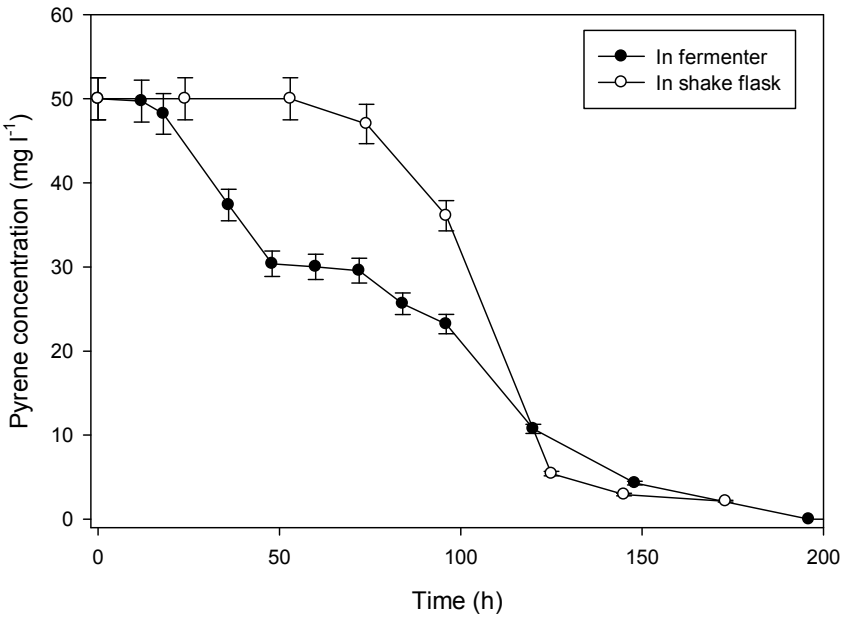


Figure 4.1: Pyrene degradation profiles (50 mg l^{-1}) in the slurry phase system.

The observed difference in pyrene biodegradation rate and pattern in shake flask and fermenter could well be attributed due to the difference in the operating conditions between the two. Higher agitation and aeration in the fermenter was sufficient to

overcome the rate limiting pyrene dissolution step, which was otherwise absent in the batch shake flask experiments. Prpich and Daugulis (2006) reported similar observation in biodegradation of chlorophenol using a microbial consortium, where degradation was found more rapid in a fully controlled fermenter than in shake flasks. The authors suggested that oxidation was a key component in such biodegradation, which is largely facilitated in a fermenter due to higher concentration of biomass and dissolved oxygen than those in shake flask

Pyrene degradation in slurry phase system has been widely studied. For instance, Habe *et al*. (2004) have reported 50% degradation of pyrene for an initial concentration of 1000 mg l^{-1} in shake flask within 7 d by an alkaliphilic *Mycobacterium* sp. Pagnout *et al.* (2006) in their study reported complete degradation of added pyrene (202 mg l^{-1}) within 4 d in shake flask using *Mycobacterium* sp. strain SNP11; however in the first 2 days, no appreciable degradation was observed. *Mycobacterium flavescens* is reported to degrade pyrene at a rate 0.56 mg l^{-1} d^{-1} when supplied in the form of slurry at 50 mg l^{-1} (Dean-Ross and Cerniglia, 1996). Compared to these reports, in the present study 50 mg l^{-1} pyrene was degraded in 8 d by *M. frederiksbergense* indicating better performance than *M. flavescens*. However, all the above literature data and the present study indicates certain general limitations of slurry phase pyrene biodegradation system in shake flask – prolonged lag period and/or incomplete degradation.

4.1.2. Mixed substrate condition

Factorial design of experiments has always been found useful to plan and execute investigation on the effect of one or more factors on such degradation studies. In this mixture study, the effect of other PAH compounds, naphthalene and anthracene, on

pyrene degradation was investigated employing 2^3 full factorial design of experiments. The two levels of the factors 1 and 50 mg l^{-1} represented slurry phase system containing the three PAHs in mixture.

Combined biodegradation profiles of pyrene along with naphthalene and anthracene in mixture in this system are presented in Figure 4.2. It is observed from the profiles that pyrene was more completely degraded (run no. 1, 2) when present at higher concentration (50 mg l^{-1}) along with a lag period of about 2 d. In contrast, experimental runs with low initial pyrene concentration (1 mg l^{-1}) displayed no lag period in its degradation; however, the degradation remained incomplete (run no. 5, 8). Interestingly, in experimental runs having anthracene at 50 mg l^{-1} concentration (run no. 3, 6 and 7), the pyrene removal efficiency was severely impaired. Results of PAHs degradation rate and efficiency during active degradation phase of all the three PAH compounds are summarized in Table 4.1. It is clear from the table that the extent of pyrene degradation varied from as low as 7% to as high as 95% in the different experimental runs, which depended on the concomitant presence of anthracene – lower level of anthracene favoring higher degradation efficiency of pyrene. However, the rate of pyrene degradation mostly depended on its initial concentration, and higher rates (0.64-0.88 mg l^{-1} h^{-1}) were observed only at its high initial concentration level (50 mg l^{-1}).

To validate the significance of the different PAHs and their interactions on their biodegradation, statistical analysis of the results in the form of analysis of variance (ANOVA) and Student's t-test was carried out for each PAH removal in the study. The ANOVA was used to investigate and model the relationship between PAHs biodegradation in mixture and their concentrations.

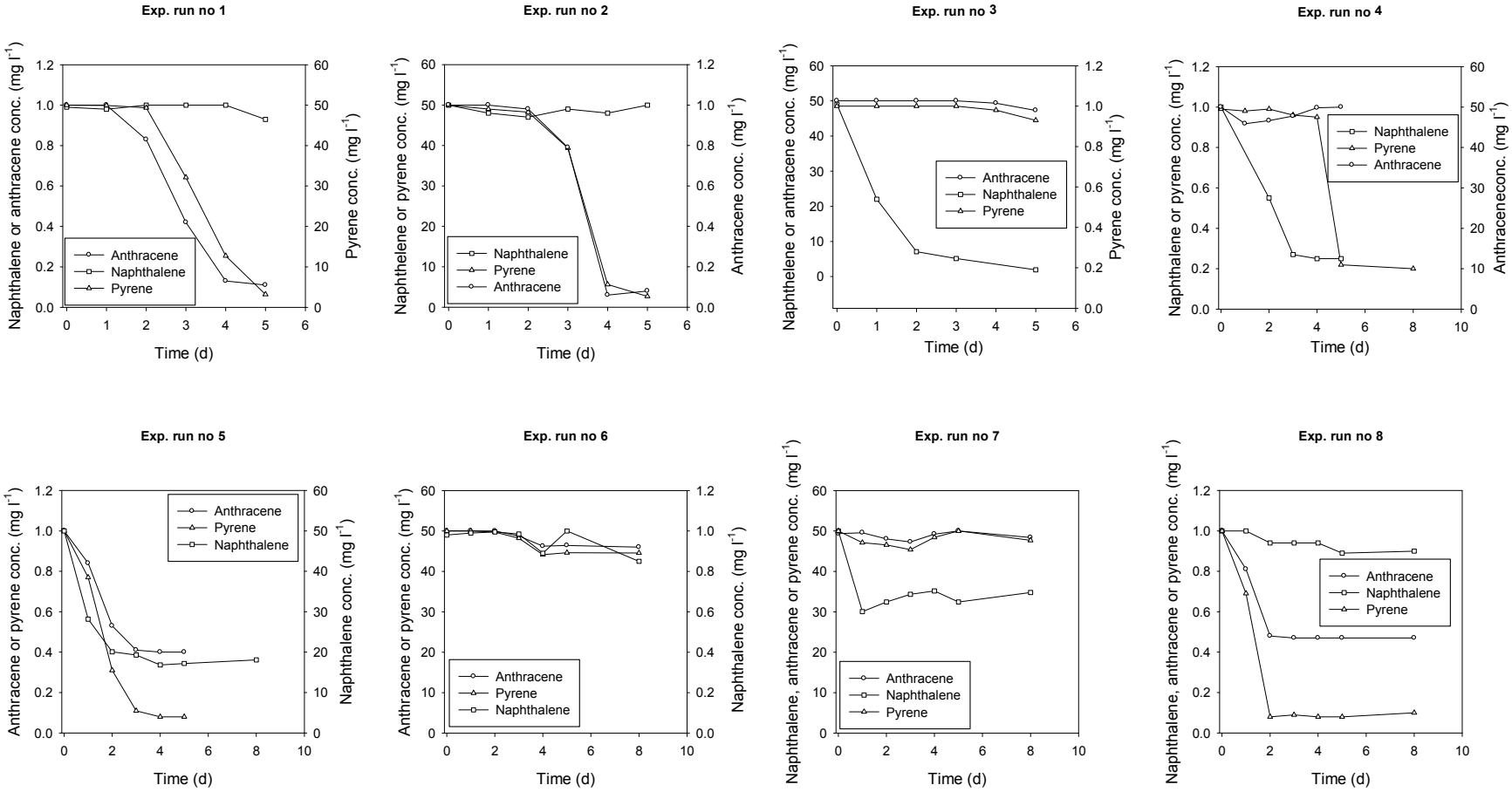


Figure 4.2: PAHs biodegradation profiles under mixed substrate conditions in the slurry phase system.

Table 4.1: PAHs degradation efficiency and rate obtained in the mixture study (slurry phase system).

Run No.	PAH concentration (mg l^{-1})			% Degradation						Degradation rate (mg l^{-1} h^{-1})		
	Naphthalene	Anthracene	Pyrene	Naphthalene		Anthracene		Pyrene		Naphthalene	Anthracene	Pyrene
				Exp.	Pred.	Exp.	Pred.	Exp.	Pred.			
1	1	1	50	7.00	-0.24	89.00	86.15	93.64	75.62	0.003	0.012	0.641
2	50	1	50	0.00	12.63	94.00	88.42	94.62	97.88	0.000	0.019	0.888
3	50	50	1	85.78	98.41	5.42	-0.15	7.00	10.26	0.893	0.084	0.001
4	1	50	1	73.00	65.75	0.00	-2.84	78.00	59.98	0.010	0.000	0.030
5	50	1	1	66.32	59.07	59.00	56.15	92.00	73.98	0.345	0.008	0.012
6	1	50	50	15.00	27.63	7.52	1.94	11.76	15.02	0.006	0.078	0.081
7	50	50	50	40.00	32.75	0.00	-2.84	26.88	8.86	0.833	0.000	0.28
8	1	1	1	6.00	18.63	52.00	46.42	92.00	95.26	0.002	0.010	0.019

Table 4.2 illustrates the results of ANOVA for PAHs removal in the mixture study, which reveals that the main effects for anthracene and naphthalene degradation were statistically significant with P values of 0.027, 0.082 respectively. However, 2- way interactions were not at all significant for the biodegradation of the three PAHs.

Table 4.2: ANOVA of (a) anthracene (b) naphthalene and (c) pyrene degradation in slurry phase system under mixed substrate condition.

(a)

Source	DF	SS	MS	F	P value
Main Effects	3	10572.9	3524.31	14.59	0.027
2-Way Interactions	3	663.5	221.17	0.92	0.528
Error	3	724.7	241.58		
Total	9	11961.2			

(R^2 = 93.94%, Adj. R^2 = 81.82%, PRESS = 7672)

(b)

Source	DF	SS	MS	F	P value
Main Effects	3	6871.69	2290.56	6.35	0.082
2-WayInteractions	3	594.58	198.19	0.55	0.683
Error	3	1082.95	360.98		
Total	9	8549.22			

(R^2 = 87.33%, Adj. R^2 = 62.00%, PRESS = 38022)

(c)

Source	DF	SS	MS	F	P value
Main Effects	3	8324.8	2774.93	2.70	0.218
2-Way Interactions	3	1672.7	557.58	0.54	0.686
Error	3	3080.9	1026.97		
Total	9	13078.4			

(R^2 = 76.44%, Adj. R^2 = 29.33%, PRESS = 61750)

To further understand which of the individual PAHs and their interactions played a crucial role on their biodegradations in mixture, Student's t-test was performed. The Student's t-test can be used as a tool to check the significance of the regression coefficient of the parameters. The estimated coefficients of individual and interaction terms are presented in Table 4.3 in which associated t and P values were used to indicate significance of these coefficient terms for removal of the PAHs from mixture. From the estimated regression coefficients, it can be elucidated that main effect of anthracene influenced its own removal in a significantly negative manner with a P value of 0.008. For naphthalene degradation, estimated effects of anthracene and pyrene were significant (with P values 0.088 and 0.051 respectively), but the effect of pyrene was negative. On the other hand, estimated effects of anthracene on pyrene degradation was significant (P = 0.071), but negative.

Table 4.3: Significance test of coefficients for individual PAHs removal from mixture in slurry phase system

	PAH removal								
	Naphthalene			**Anthracene**			**Pyrene**		
Term	*Coeff.*	*t*	P	*Coeff.*	*t*	P	*Coeff.*	*t*	P
Constant	39.33	6.55	0.007	34.16	6.95	0.006	54.61	5.39	0.013
Anthracene	16.81	2.50	**0.088**	-35.13	-6.39	**0.008**	-31.08	-2.74	**0.071**
Naphthalene	11.39	1.70	0.189	1.24	0.23	0.836	-6.86	-0.61	0.587
Pyrene	-21.14	-3.15	**0.051**	9.26	1.69	0.19	-5.26	-0.46	0.674
Naphthalene* Anthracene	-1.94	-0.29	0.791	-1.76	-0.32	0.769	-7.11	-0.63	0.575
Anthracene* Pyrene	-4.81	-0.72	0.526	-8.74	-1.59	0.210	-6.33	-0.56	0.615
Naphthalene* Pyrene	-6.89	-1.03	0.381	-1.87	-0.34	0.756	10.89	0.96	0.407

Based on the above results, the following regression equations for the PAHs removal from mixture were obtained:

$$\% \text{ naphthalene removal} = 17.21+0.84x_1+0.97x_2-0.36x_3-0.003x_1x_2-0.011x_1x_3-0.008x_2x_3 \quad \textbf{(4.1)}$$

$$\% \text{ anthracene removal} = 46.39+0.20x_1-0.98x_2+0.82x_3-0.003x_1x_2-0.003x_1x_3-0.014x_2x_3 \quad \textbf{(4.2)}$$

$$\% \text{ pyrene removal} = 96.82-0.44x_1-0.69x_2-0.41x_3-0.011x_1x_2+0.018x_1x_3-0.010x_2x_3 \quad \textbf{(4.3)}$$

where, x_1, x_2, x_3 are concentrations (in mg l^{-1}) of naphthalene, anthracene and pyrene, respectively. The predicted removals of the PAHs due to these regression model equations are also mentioned in Table 4.1, which suggests that both the experimental and model predicted removal values from each experimental run matched, though not closely, but reasonably well with each other.

Interaction of different PAHs such as naphthalene, anthracene, pyrene, fluorene, phenanthrene and fluoranthene on their biodegradation has been reported by Bouchez *et al.* (1995) in experiments involving the PAHs in pairs. Both, inhibition as well as synergism to a particular PAH removal in mixture was observed. In accordance to our results, these authors observed a good simultaneous utilization of both anthracene and pyrene from their binary mixtures. More or less extensive inhibition of degradation of a PAH was observed in the presence of a second one, specifically when the added PAH was more water-soluble. Likewise, a negative effect of naphthalene on anthracene biodegradation in the present study could also be attributed to the same phenomenon.

Thus, it is clear that although the *Mycobacterium* is quite capable of degrading pyrene, both in single and mixed substrate conditions, the organism could not efficiently degrade pyrene within a reasonable period of time, mainly owing to its low aqueous solubility in the slurry phase system. Hence, in order to improve the rate of pyrene and other PAHs degradation by the *Mycobacterium*, the next system evaluated was surfactant

aided system in which PAH solubility was enhanced several times. The following section discusses the results pertaining to this topic.

4.2. Pyrene Biodegradation in Surfactant Aided System

To overcome the solubility problem of pyrene and other PAHs in aqueous media for the degrading *M. frederiksbergense* to act upon, biodegradation study employing suitable surfactants were conducted.

4.2.1. Pyrene biodegradation with Triton X 100 as the surfactant

Triton X 100 has been the most widely used surfactant for PAH biodegradation (Kanaly and Harayama, 2000; Garon *et al.*, 2002; Makkar and Rockne, 2003). Hence, in the initial experiments with this surfactant aided pyrene biodegradation system, 5% Triton X 100 was used for solubilising pyrene at a concentration of 50 mg l^{-1}. Also, using this surfactant, the pyrene degradation efficiency by *M. frederiksbergense* was compared with *M. vanbaalenii*, which is reported to be an efficient degrader of pyrene in the literature. To carry out the experiments in this study, a statistically valid Pluckett-Burman design of experiments was applied. The factors chosen were the two *Mycobacterium* strains, Triton X 100 and additional carbon source glucose. The range and levels of the factors and the experimental combination were described in Chapter 3 (Table 3.2 and 3.3, respectively).

Interestingly, the levels of these factors in the designed experiment was chosen in such a way that each of the factor represented either presence or absence in the experimental flasks, so that significance of the individual as well as combined effects of

these factors on pyrene removal could be found out. Fig 4.3 is a bar chart showing % pyrene degradation efficiency obtained in the various experimental runs. From this figure, it is obvious that when both the strains were present, without any surfactant (run no 5), pyrene could be degraded completely. In the absence of all other factors, only *M. vanbaalenii* could degrade pyrene to the extent of 73% (run no 4); however, this efficiency was found to be still lower compared to that due to *M. frederiksbergense*, where 93% degradation efficiency could be achieved (run no 3). In absence of the two organisms, total pyrene concentration remained the same, thus showing that pyrene removal was due to biological degradation in the experiments. Despite the above observations, in the presence of Triton X 100, which was added to enhance the bioavailability of pyrene to the degrading microorganism(s), the % pyrene degradation fell drastically (run no. 8, 9 and 12).

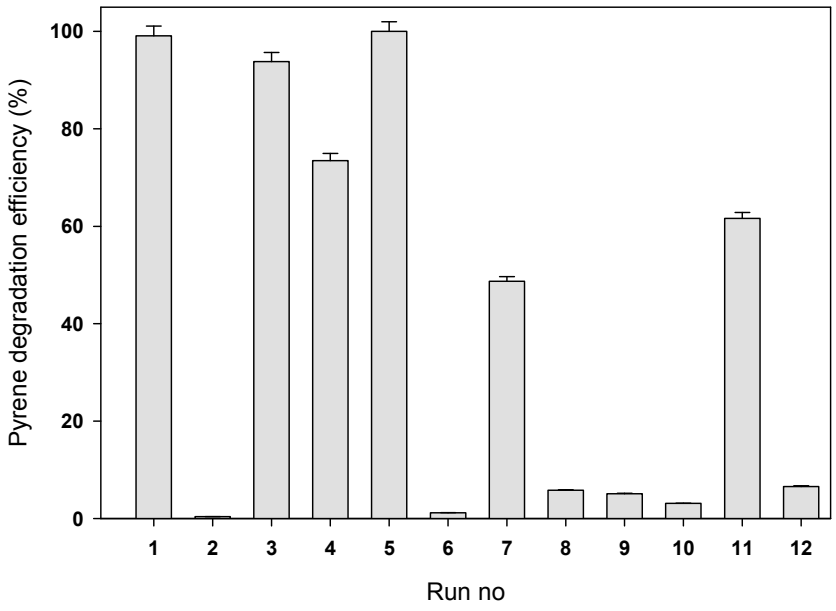


Figure 4.3: Pyrene degradation efficiency in the Triton X 100 aided system.

To illustrate the findings better, main effect plots of the factors - obtained by taking into consideration the change in response due to a change in level of a factor from low to high and by taking an average response for the other factors, were considered. Fig 4.4 shows main effect plot of the factors on pyrene biodegradation. As observed earlier and from the pyrene degradation efficiency values in Fig 4.3, *M. frederiksbergense* showed better efficiency than *M. vanbaalenii.* And quite contradictorily, both glucose and Triton X 100 exhibited significant negative effect on pyrene degradation.

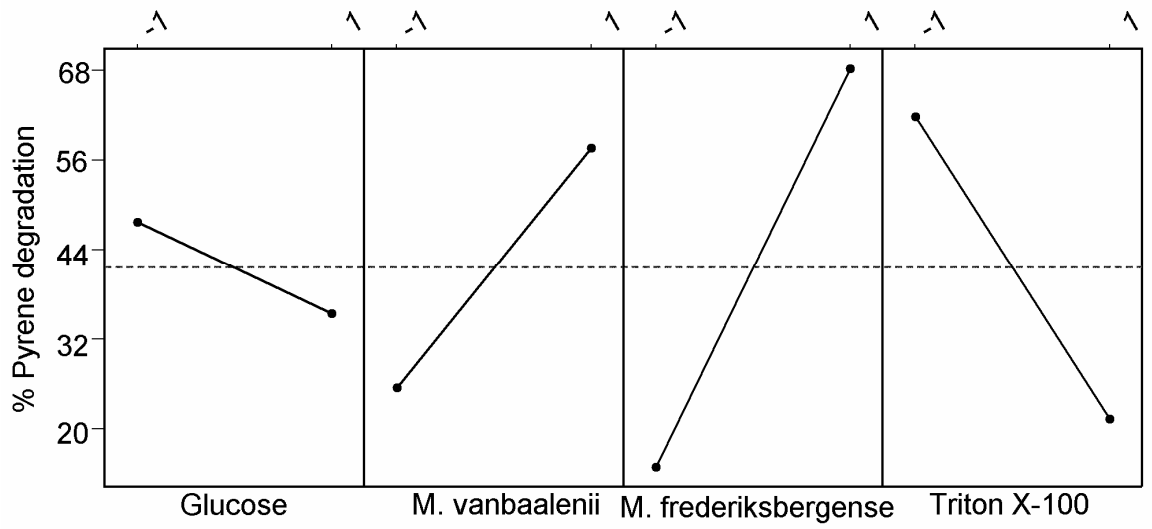


Figure 4.4: Main effect plots of the factors on pyrene degradation in the Triton X 100 aided system.

These findings on the effects of Triton X-100 and glucose on pyrene degradation are consistent with those of Allen *et al.* (1999) and Wong *et al.* (2002), respectively. Moreover, effect of Triton X 100 was found to be much greater than glucose. It should be mentioned here that in absence of Triton X 100 pyrene is soluble in the aqueous phase only to the extent of 0.01 mg l^{-1}, and its solubility could be enhanced several times at higher level of the surfactant (0.5% v/v). The larger negative effect of Triton X-100 on pyrene degradation could be hypothesized based on the enhancement of bioavailability

that in turn could have led to direct toxic effect on the microbial strain(s). To further confirm the observations on the effects of these factors on pyrene biodegradation, statistical analysis in the form of ANOVA and significance test of factors were performed.

Considerations from ANOVA

Table 4.4 shows ANOVA of percentage pyrene degradation obtained in this study in which main and interaction effect values and their significance levels are presented. The estimated F values were found to be higher than the corresponding critical F values for main and interaction effects ($F_{0.05}$ (4,1) = 224.583 and $F_{0.05}$ (6,1) = 233.986 respectively), indicating their significance on pyrene degradation. The ANOVA also shows that error term in the model is less (MS = 1.05), suggesting a good accuracy of the results obtained from the experiments.

Table 4.4: ANOVA of pyrene degradation in the Triton X 100 aided system.

Source	DF	SS	MS	F	P
Main Effects	4	11432.9	2858.2	3×10^3	0.014
2-Way Interactions	6	2604.2	2	411.83	0.038
Pure Error	1	1.1	1.05		
Total	11				

Considerations from the significance test of factors

Table 4.5 presents estimated linear and interaction effects, along with the corresponding P-values, on pyrene degradation in the study. The P values were used as a tool to check the significance of the coefficient terms of each factor and their interactions;

smaller the P value (<0.05) significance of the corresponding coefficient term in the model was considered high compared to others with a large P value. Associated P values of the coefficients of main effects of all the factors indicate a high significance in the model (> 95% confidence level). However, the coefficient terms of glucose and Triton X 100 showed large negative T-value, proving that these two factors were detrimental to pyrene degradation in the study. Apart from the coefficients of main effects of factors, the negative coefficient terms of interaction between glucose and Triton X-100 and that between the two microbial strains were also highly significant (P < 0.02) revealing repression in pyrene degradation due to combined effects of glucose and Triton X 100, and between the two *Mycobacterium* strains.

Table 4.5: Estimated effects and their coefficients on pyrene degradation efficiency in the Triton X 100 aided system.

Term	Effect	Coefficient	T	P
Constant		41.56	140.25	0.005
Glucose	-26.68	-13.34	-35.31	0.018
M. vanbaalenii	37.34	18.67	49.43	0.013
M. frederiksbergense	42.83	21.41	56.68	0.011
Triton X-100	-46.51	-23.25	-61.55	0.010
Glucose**M. vanbaalenii*	-4.24	-2.12	-5.62	0.112
Glucose* *M. frederiksbergense*	5.60	2.80	7.42	0.085
Glucose*Triton X-100	-26.16	-13.08	-34.62	0.018
*M. vanbaalenii***M. frederiksbergense*	-27.85	-13.93	-36.87	0.017
*M. vanbaalenii**Triton X-100	-0.92	-0.46	-1.22	0.438
*M. frederiksbergense**Triton X-100	-16.29	-8.15	-21.56	0.030

Overall, the results indicated the better performance of *M. frederiksbergense* over *M. vanbaalenii* in terms of pyrene degradation efficiency. And, presence of an alternative

easily available carbon source (e.g. glucose) negatively influenced the degradation efficiency. More importantly, with regard to the choice of the surfactant Triton X 100, although the pyrene aqueous solubility was enhanced several folds, the surfactant proved detrimental to pyrene degradation by *M. frederiksbergense*. Thus, it appeared that systematic investigation on the evaluation of different surfactants including Triton X 100 should be taken up before investigating further the pyrene degradation, in single and mixed substrate conditions, by the *Mycobacterium* in this surfactant aided systems.

4.2.2. Evaluation using different chemical surfactants

To determine the efficiency of different chemical surfactants on pyrene solubility in aqueous solutions, molar solubilization ratio (MSR) of pyrene due to the different surfactants were estimated in this study. MSR is generally referred to as the ratio of moles of a solute solubilised to the moles of surfactant present as micelles in a system, and is obtained from the slope of the solubility curve above its critical micelle concentration (Zhu and Feng, 2003). Figure 4.5 shows that the variation in pyrene intrinsic solubility was linear with the five surfactants, at concentrations above their CMC, with coefficient of determination (R^2) value above 0.90 in all the cases. Calculated MSR values of pyrene from the slopes of these linear plots obtained using the different surfactants indicated the following order of influence of the surfactants on pyrene solubility: Tween 80 > Tween 20 > Triton X 100 > CTAB > SDS. Similar efficiency scale has also been reported by Garon *et al.* (2002) for solubilization of fluorene and various PAHs compound using these surfactants.

The MSR values of pyrene obtained using the surfactants were also found close to the reported values in the literature (Mohamed and Mahfoodah, 2006; Prak and Pritchard, 2002). According to Tiehm (1994), nonionic surfactants of the alkylethoxylate type and the alkylphenolethoxylate type with an average ethoxylate chain length of 9 to 12 monomers, e.g. SDS, were toxic to PAH degrading *Mycobacterium sp.* and to several PAH degrading mixed cultures. Therefore, choosing the right surfactant based on MSR value seems to be important and also critical in surfactant-aided biodegradation of PAHs by microorganisms. In general, it is reported that surfactant having a high MSR value exhibits low toxicity on PAH degrading organism. In this study, Tween 80 was found to have higher MSR value compared to other surfactants, suggesting its better applicability over others.

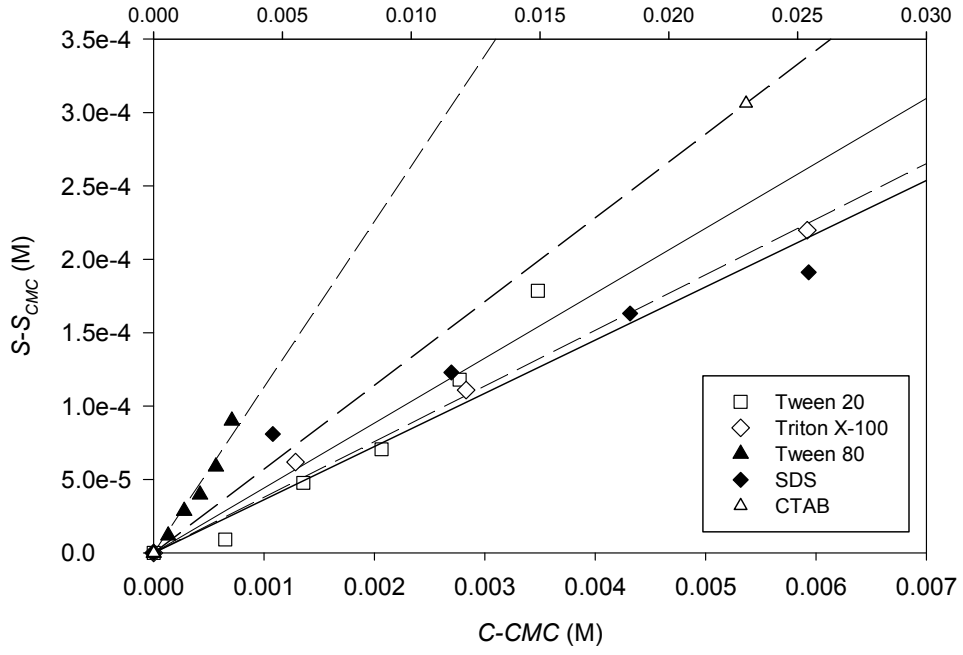


Figure 4.5: Molar solubilization profile of pyrene obtained using different surfactants. (Data for SDS and CTAB are presented in the secondary X-axis.)

As observed earlier in the previous experiments that Triton X 100 negatively influenced pyrene biodegradation by the *Mycobacterium*, further experiments were conducted to check the influence of all the chemical surfactants in this study on pyrene biodegradation.

Pyrene degradation profiles by *M. frederiksbergense* in presence of the different surfactants in shake flasks are shown in Figure 4.6. It is quite obvious from the figure that in presence of Tween 80, maximum pyrene degradation was achieved (about 90% degradation within 6 d) with more or less constant rate. When compared to slurry phase pyrene biodegradation study in shake flask, this pyrene biodegradation profile was same, but without lag phase. Although the results due to CTAB showed good degradation (about 60%) in the first two days, there was no further degradation; on the other hand, Triton X 100 showed only about 30% degradation in the first two days, confirming that it could not be an ideal surfactant for pyrene degradation using the *Mycobacterium*.

Surfactants, such as those used in our study, have been shown to be toxic to microorganisms (Bartha and Jimenez, 1996) or may change the physiological characteristics of degrading microbial population by altering the integrity of their cell membrane (Thibault *et al.*, 1996). Therefore, it is clear that the proper selection of surfactant is vital to any surfactant aided biodegradation systems. In this study, no degradation of the surfactants was observed, except for Tween 80 or Tween 20, where nearly 50% surfactant was utilized within 5 days with an initial lag period of 3 d (data not shown). Moreover, control experiments performed without the bacterium indicated that abiotic loss of pyrene or the surfactants were negligible.

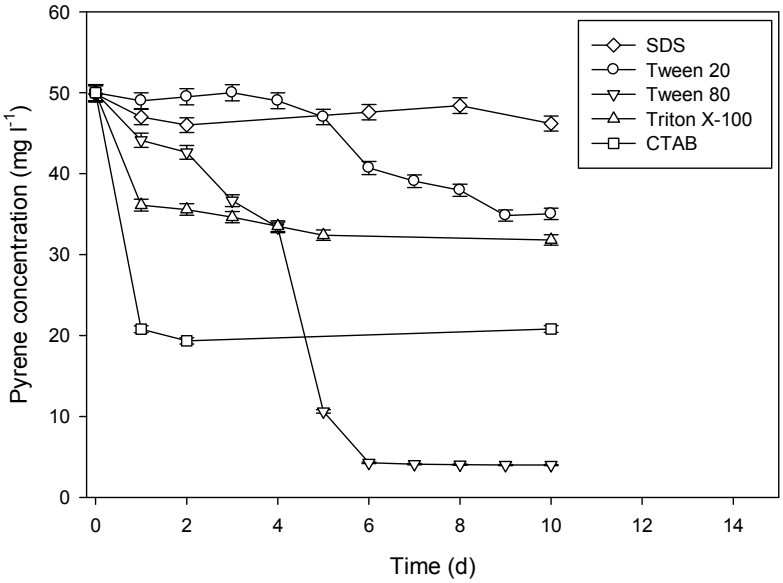


Figure 4.6: Pyrene degradation profiles obtained using different surfactants in shake flasks.

The results of pyrene degradation in presence of different non-ionic surfactants, *viz.* Triton X 100, Tween 80 and Tween 20, were also validated by conducting experimental trials in fermenter. Figure 4.7 illustrates the pyrene biodegradation profile obtained in these experiments. Although Triton X 100 favored only 30% pyrene degradation in batch shake flask, in these fermenter experiments the degradation efficiency was found to be higher (~80%). This difference in pyrene degradation with the surfactant could be due to better operating conditions, such as agitation and mixing, together with an increase in Triton X 100 micelle solubilised bioavailable pyrene concentration to the degrading mycobacterium in the fermenter than in batch shake flask. Also, because pyrene in the micellar phase is made available to the mycobacterium in the aqueous phase based on its equilibrium partitioning between the micellar and aqueous phases, which is a function of both real-time demand of the organism and operating

conditions in the system, toxicity effect of Triton X 100 (due to increased concentration of bioavailable pyrene) observed in an earlier experiment (section 4.2.1) with the *Mycobacterium*, was also overcome in the fermenter.

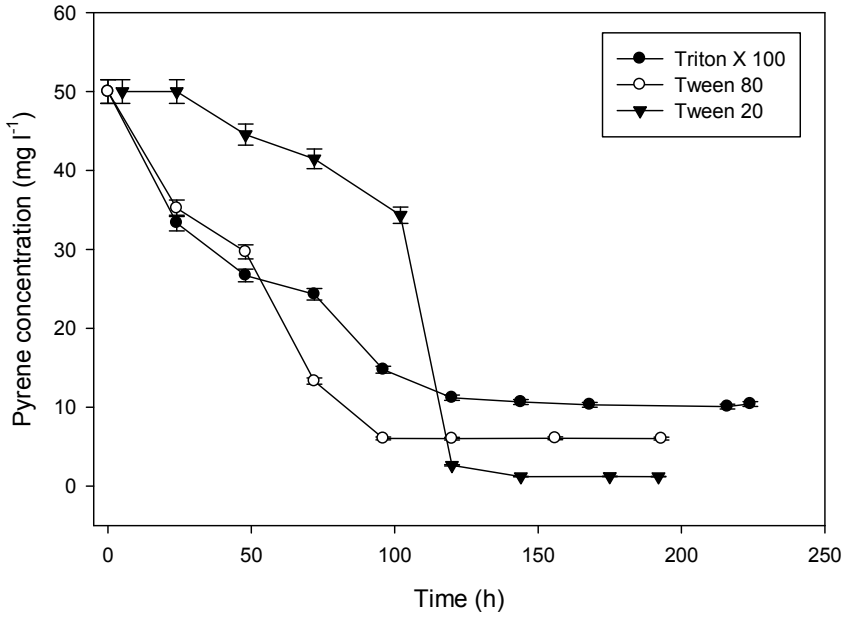


Figure 4.7: Pyrene degradation profile with the different non-ionic surfactants in the fermenter.

It is quite obvious from the Figure 4.7 that in presence of Tween 80 or Tween 20, about 88% and 96% degradations were achieved within 96 h and 120 h respectively. Between the two surfactants Tween 80 and Tween 20, pyrene degradation profile with the latter can be divided into two stages- first phase with a low degradation rate of about 0.15 mg l^{-1} h^{-1} (during initial ~100 h) and second phase with higher degradation rate of 1.77 mg l^{-1} h^{-1}.

Biodegradation of pyrene by *M. frederiksbergense* was also confirmed from the appearance of a new peak around 355 nm in synchronous fluorescence (SF) analysis, which was different from the pyrene specific peak at 335 nm, indicating the formation of

intermediates during the active pyrene degradation. Figure 4.8 shows normalized SF spectra of pyrene (with respect to 335 nm peak), which clearly shows an increase in intermediates to pyrene ratio (relative concentrations of intermediates to pyrene) over time. However, the un-normalized SF profile revealed that concentration of the intermediates decreased after reaching a peak at around 217 h.

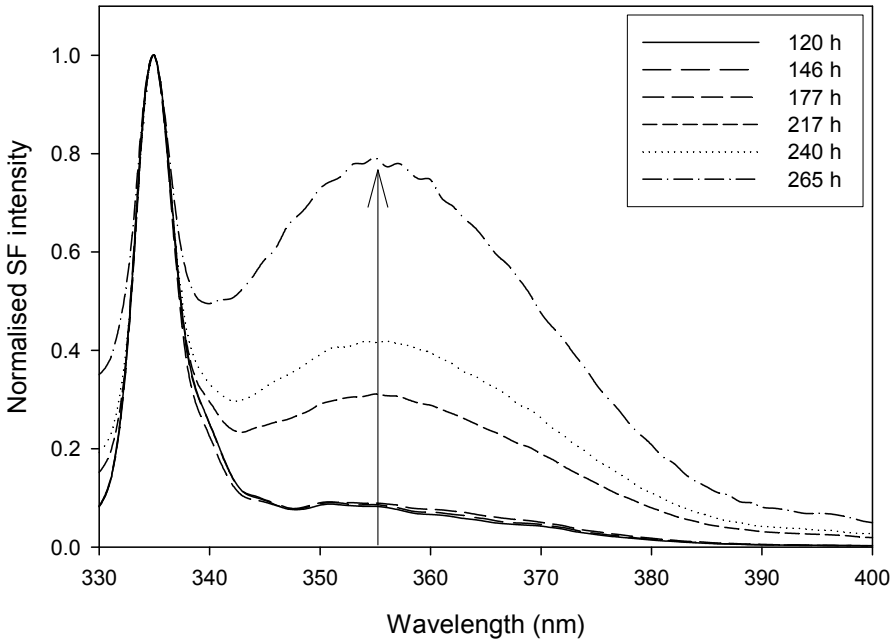


Figure 4.8: Appearance of pyrene metabolite peak (at 355 nm in normalized SF spectra) during its biodegradation in the Tween 20 aided system.

When fixed wavelength fluorescence analysis (FF) of the same samples was performed, it was found that up to a degradation time of 177 h the intensity remained nearly same, later increased to its maximum value at 217 h, and finally declined steadily (Figure 4.9). As, pyrene intermediates are known to have higher fluorescence intensity compared to the original pyrene on equal molar basis (Giessing *et al.*, 2003), this sudden increase in FF intensity further confirms the degradation of pyrene to result in the

formation of its intermediates in the early active degradation phase, which were subsequently mineralized towards the end leaving no trace of either pyrene or the intermediates. This is in agreement with the literature finding that pyrene can be completely mineralized without accumulation of its intermediates by *Mycobacterium frederiksbergense* (Giessing and Johnsen, 2005).

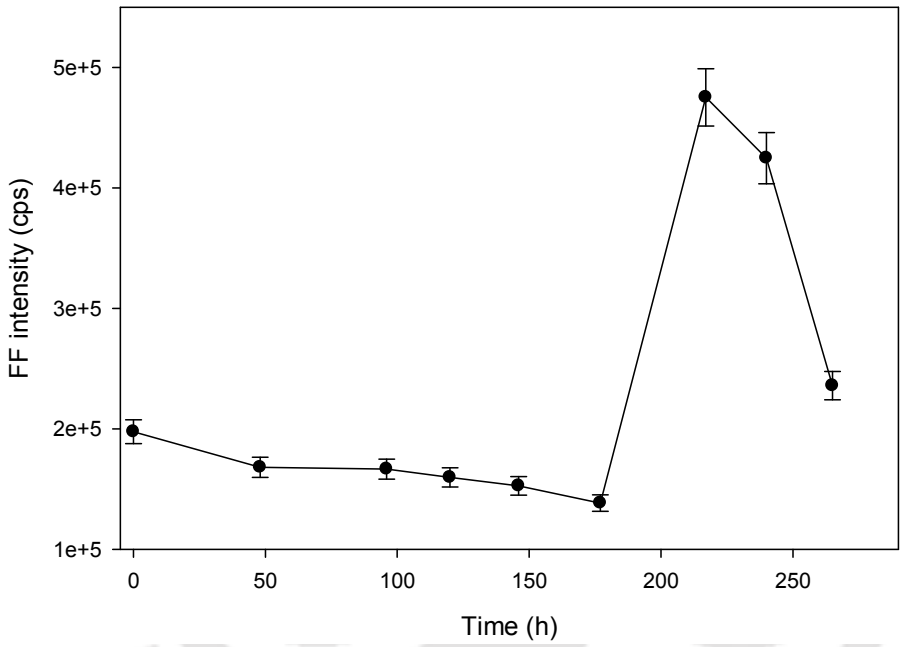


Figure 4.9: Combined FF intensity profile of pyrene along with its intermediates during biodegradation in the Tween 20 aided system.

Thus among all the surfactants evaluated for pyrene degradation by *M. Frederiksbergense*, both Tween 20 and Tween 80 showed equally good pyrene degradation efficiency within a reasonable duration of time. However due to absence of any lag phase in pyrene degradation using Tween 80, it was therefore chosen for further pyrene degradation in single and mixed substrate condition using the *Mycobacterium*.

4.2.3. Evaluation using a biosurfactant producing indigenous soil microbial culture

In contrast to using an additional chemical surfactant for the degradation of pyrene, it was envisaged that by using an indigenous culture capable of producing biosurfactant both simultaneous solubilization and biodegradation of pyrene could be achieved. With this aim, soil contaminated with petroleum hydrocarbon, collected from a nearby gasoline filling station in Guwahati, India, was used in isolation of the indigenous culture and evaluated of its potential to produce biosurfactant for solubilization and/or degradation of pyrene.

4.2.3.1. Culture growth and biosurfactant production

Following isolation of the indigenous soil culture by repeatedly growing in CFMM supplemented with pyrene and an alternate carbon source (glucose), the kinetics of its growth and biosurfactant production was investigated. It was observed that up to 12 h, there was no appreciable change in the emulsifying activity of the culture supernatant, but after this 12 h period and until 21st h, the activity increased exponentially followed by a slow decline (Figure 4.10). As the increase in the biosurfactant amount was synchronous with the growth of the soil culture, it is reasonable to say that the biosurfactant production was a growth associated one (Mulligan, 2005). Contrary to our results, the maximum biosurfactant production was achieved during stationary phase of growth of certain microbes (Sarubbo *et al.*, 2001; Patil and Chopade, 2001).

The effect of an alternate carbon source on the biosurfactant production by the culture was also carried out with respect to glucose and glycerol. The results showed that irrespective of whether pyrene was present or absent in the media, the culture showed

maximum biosurfactant production when grown on glycerol as the co-substrate (data not shown). This difference in emulsification activity of the supernatants obtained by growing the culture on the medium containing glucose and glycerol may be due to difference in the chemical composition of the two substrates, which also indicated that the soil culture is able to produce the biosurfactant without any inducer such as PAHs.

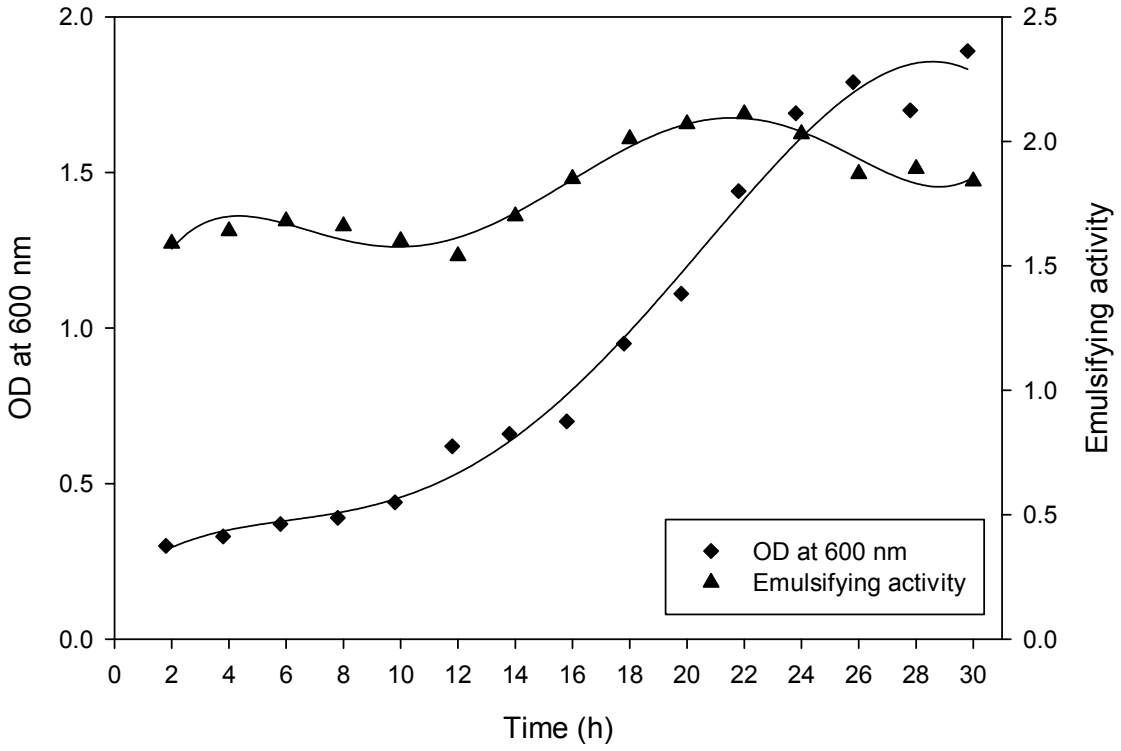


Figure 4.10: Biosurfactant production by the indigenous soil microbial culture and its growth.

However, the culture did not show any effect on pyrene degradation despite its ability to produce the biosurfactant. Nonetheless, to better understand the properties of the biosurfactant for possible PAH solubilization applications, further isolation, partial purification and characterizations of the product were carried out.

4.2.3.2. Isolation and partial purification of the biosurfactant

It is known that biosurfactant produced by microorganisms may be of various types based on their chemical composition (Mulligan, 2005), such as glycolipids, lipopeptides, phospholipids, fatty acids, neutral lipids, polymeric and particulate compounds. Preliminary investigations aimed at isolating the produced biosurfactant were based on chloroform-methanol (for lipid nature) and ammonium sulphate precipitation (for protein). It was found that a concentrated product was obtained only when the supernatant of culture grown on glycerol was subjected to ammonium sulphate precipitation. The precipitate so obtained was dialyzed overnight against distilled water to obtain the partially purified biosurfactant. The supernatant remaining after ammonium sulphate precipitation showed no emulsifying activity either with xylene or n-butanol indicating that the biosurfactant was completely extracted. It was found that the partially purified biosurfactant contained 10% protein (BSA equivalent) and 7% of carbohydrate content, as estimated with modified Lowry (Hartree, 1972) and phenol-sulphuric acid method (Herbert, 1971), respectively.

4.2.3.3. Properties of the partially purified biosurfactant

Kinetics of PAHs solubilization:

The low aqueous solubility of PAHs is one of the limiting factors affecting their bioavailability and subsequent mineralization. However, biosurfactants are known to increase solubility and consequently bioavailabity of such hydrophobic compounds for their biodegradation (Cameotra and Bollag, 2003). In an attempt to enhance the intrinsic

aqueous solubility of pyrene and anthracene, which are 0.175 mg l^{-1} and 0.08 mg l^{-1} respectively (Barkay *et al.*, 1999), aqueous solutions containing the biosurfactant at different concentrations were added with pyrene or anthracene. Figure 4.11 shows the apparent aqueous solubility of pyrene and anthracene in presence of the added biosurfactant.

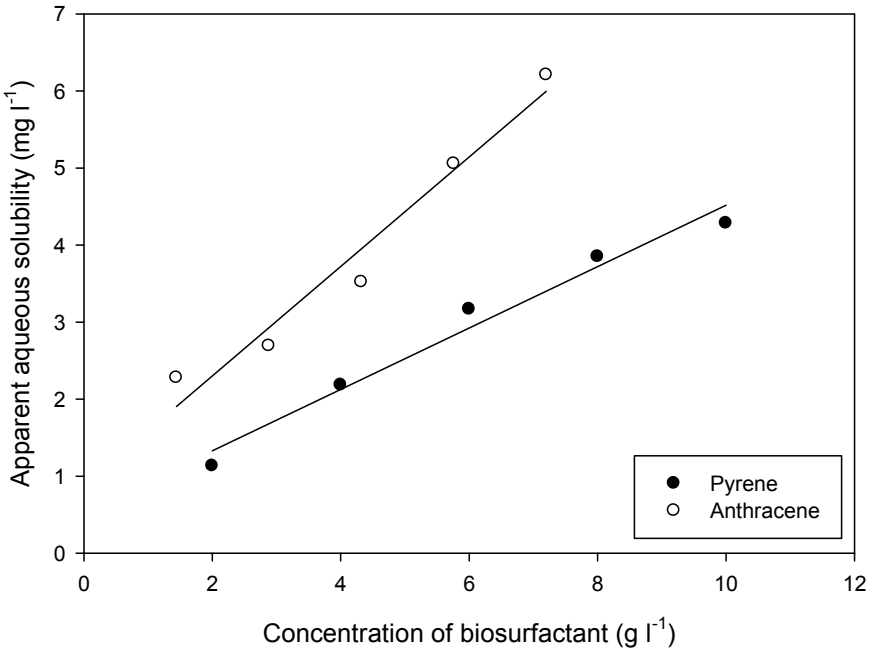


Figure 4.11: Apparent aqueous solubility of pyrene and anthracene due to the biosurfactant at different concentrations.

It is clear that the apparent solubility increased linearly with the biosurfactant concentration, and the correlation coefficient (R^2) was found to be 0.96 and 0.97 for the anthracene and pyrene solubility curves, respectively. While the aqueous solubility of anthracene improved by about 78 times in presence of 7.2 g l^{-1} of the biosurfactant, pyrene solubility enhanced 25 times in presence of 10 g l^{-1} of the product.

The increase in anthracene and pyrene concentration in their respective aqueous solutions was monitored to evaluate the effect of the biosurfactant on the solubilisation kinetic of these two PAHs. It was observed that the PAHs solubilisation was rapid, reaching half of its final magnitude within the first 120 min and 60 min for anthracene and pyrene respectively for a concentration of 7.2 g l^{-1} of the biosurfactant in each case (Figure 4.12).

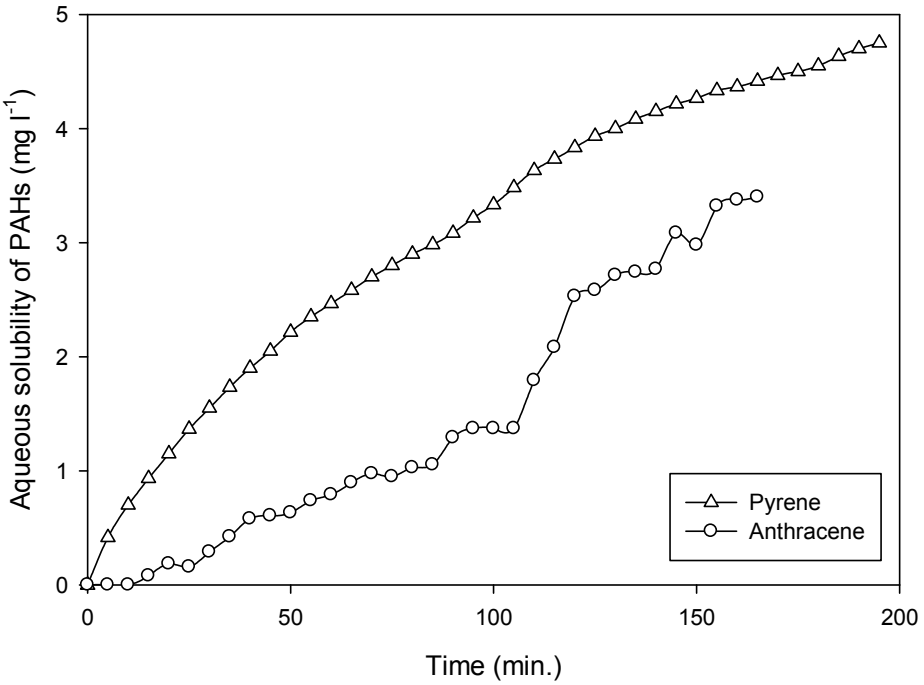


Figure 4.12: Solubilization kinetics of anthracene and pyrene with the biosurfactant.

From the slope of the initial anthracene and pyrene solubilisation kinetic curves, the observed rate of solubilisation of pyrene (0.0333 mg l^{-1} min^{-1}) was faster as compared to anthracene (0.0137 mg l^{-1} min^{-1}). And the final equilibrium aqueous concentrations of the PAHs obtained were 4.75 mg l^{-1} and 3.39 mg l^{-1} for pyrene and anthracene, respectively.

Emulsification activity and stability of the biosurfactant:

In general, emulsification property of a compound depends on the organic solvent used, e.g. aliphatic or aromatic. In this study specificity of emulsion formation was found to vary, depending on the carbon source used for growing the culture. The culture grown on medium containing glycerol as the sole carbon source showed emulsification activity against aliphatic hydrocarbons of n-butanol and n-hexane, whereas the activity was absent towards aromatic solvents such as benzene, toluene and xylene. However, when the culture was grown on carbon free mineral media (CFMM) supplemented with 50 mg l^{-1} pyrene along with 2% glycerol as the secondary carbon source, the biosurfactant showed emulsification activity against both aliphatic and aromatic hydrocarbons. Hence, the biosurfactant produced by using the medium containing CFMM supplemented with both pyrene and glycerol was used for further emulsification activity and stability studies.

Figure 4.13 shows the linear form of the emulsion stability profiles towards different organic solvents from which the decay constants (K_d) were calculated (Zhang and Miller, 1992), which are presented in Table 4.6. From the table, it could be seen that emulsification activity towards the different solvents followed the order: benzene > toluene > xylene > paraffin oil >kerosene > butane-1-ol > iso-amyl alcohol > petroleum ether > n-hexane > sunflower oil. Singh *et al.* (1990) reported a similar result, where biosurfactant in their study showed activity against various hydrocarbons, with the maximum towards aromatic compounds and the least toward normal paraffin. The stability of the emulsions formed with different organic solvent in the present study was observed to follow the order: benzene > xylene > kerosene > iso-amyl alcohol > sunflower oil > butane -1-ol > toluene > n-hexane > petroleum ether > paraffin oil**.**

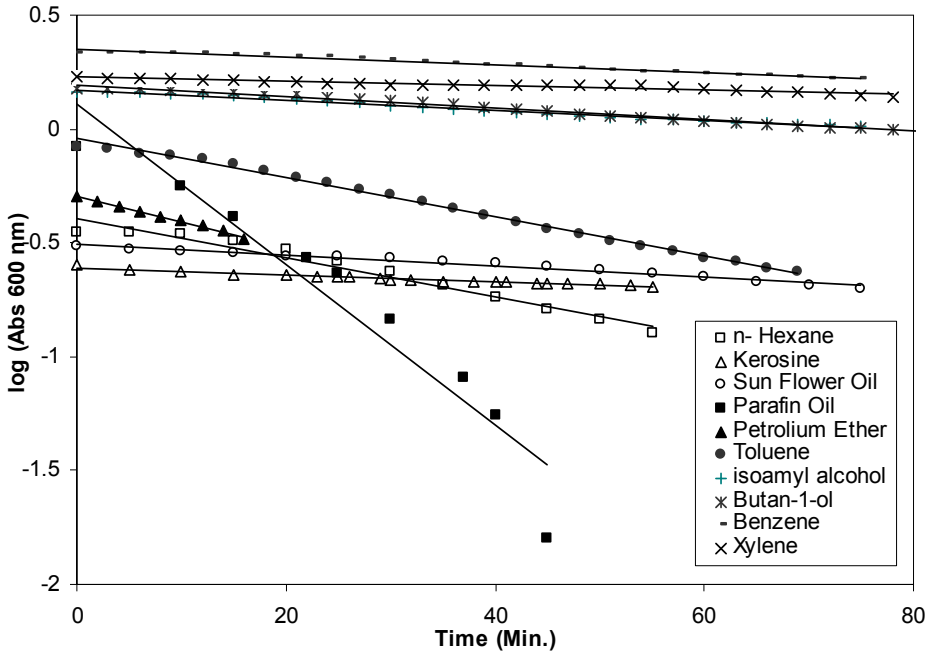


Figure 4.13: Emulsion stability profile towards the biosurfactant to different organic solvents.

Table 4.6: Emulsification activity of the biosurfactant towards the different organic solvents.

Organic solvent	Emulsification activity [a] ($A_{600\ nm}$)	Decay constant [b] (K_d)
n-Hexane	0.688	-12.52
Isoamyl alcohol	0.849	-3.312
Butane -1-ol	0.924	-3.60
Benzene	1.510	-1.008
Toluene	1.203	-12.38
Xylene	0.984	-1.296
Kerosene	0.949	-2.16
Sun-flower oil	0.445	-3.45
Paraffin oil light	0.980	-50.54
Petroleum Ether (30-40^{0}C)	0.843	-16.27

a-All absorbance values are average of three consecutive reading after allowing 10 min of standing time; **b-**For decay constant determination, after initial 1 h standing time, readings were taken at interval of 5 min and expressed in d^{-1}

Environmental factors affecting the emulsification activity of the biosurfactant:

The pH and temperature are known to be the important environmental factors that affect the performance of any biosurfactant. This particular aspect was validated for the biosurfactant in the present study by using xylene as the organic solvent which showed moderate emulsifying activity and best stability. Figure 4.14 shows emulsifying activity of the biosurfactant towards xylene at different pH, which reveals that the activity tends to be high at pH value above 6 with the highest activity at pH 12. However, the emulsions formed with xylene at lower pH were found to be more stable as compared to those formed at higher pH (Figure 4.15).

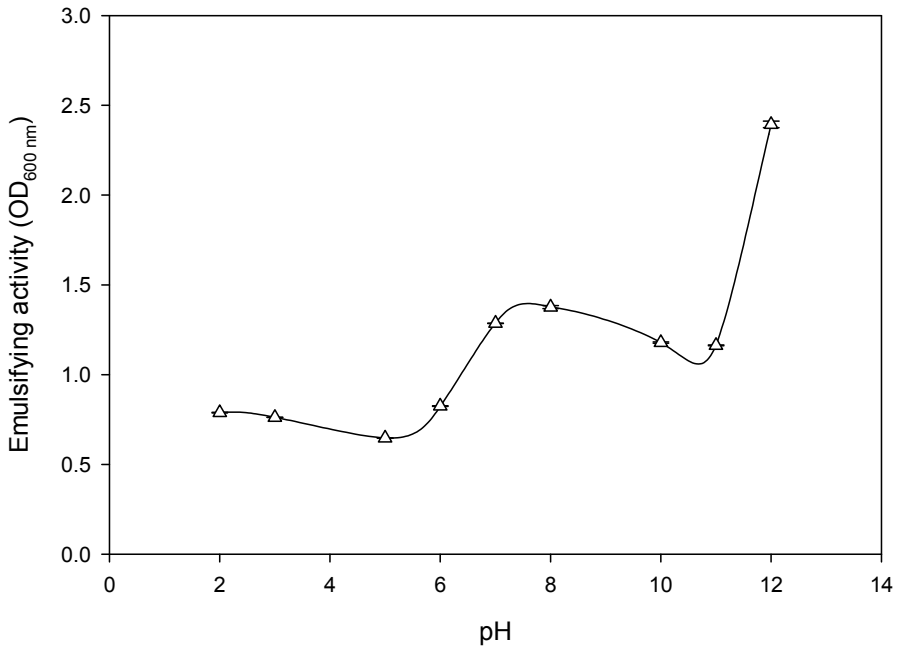


Figure 4.14: Emulsifying activity of the biosurfactant toward xylene at different pH.

In the literature, liposan has been shown to exhibit a high emulsifying activity in the pH range of 2 to 5 (Cirigliano and Carman, 1984), and in the case of a biosurfactant produced by *Bacillus stearothermophilus* VR-8, the activity was found high in the pH

range 2-8 (Gurjar *et al*., 1995). Another biosurfactant, Alasan, produced by *Acinetobacter radioresistens* is reported to show maximum activity in acidic range of pH 3.3 to 9.2 with the maximum activity at pH 5 (Navon-Venezia *et al*., 1995). Compared to the literature reported values of emulsifying activity and stability of biosurfactants, the present biosurfactant can be well said to possess good emulsification activity in a broad pH range of 6-12 with maximum activity in an alkaline scale of pH.

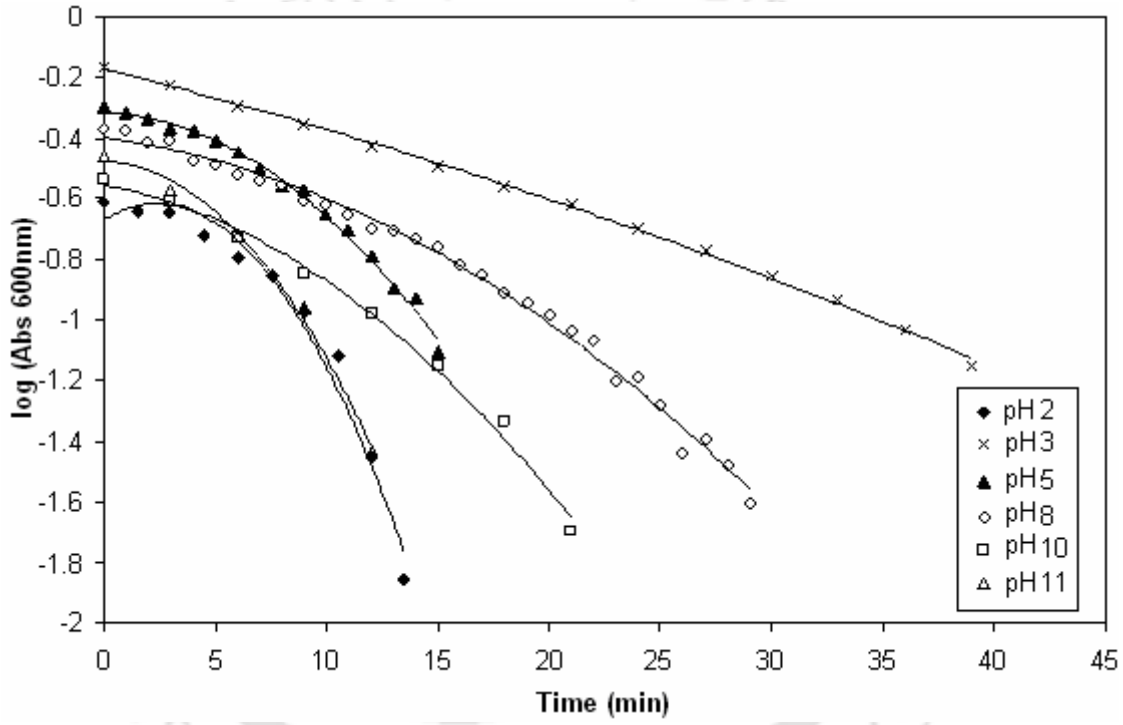


Figure 4.15: Stability profiles of the emulsion formed with xylene at different pH.

With respect to the emulsifying activity of the biosurfactant as a function of incubation temperature, it was inferred from Figure 4.16 that the emulsifying activity dipped at 50^{o}C from an initial value of 2.5 at 20^{o}C. As, the isolated biosurfactant has been shown to be composed of protein and carbohydrate moieties (section: 4.2.3.2.), conformational change of the protein moiety due to changes in temperature might have led to this observed decrease in emulsifying activity. And above 80^{o}C, the emulsifying

activity was appreciably high until the tested 100°C temperature. Similar increase in the emulsifying activity is also reported by Navon-Venezia *et al*. (1995) with the biosurfactant alasan.

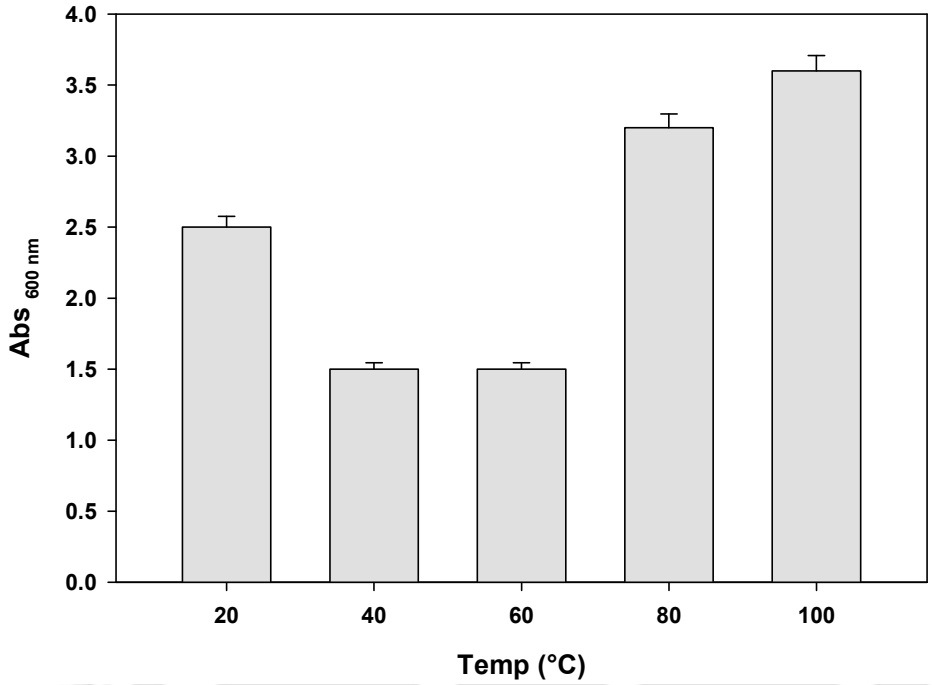


Figure 4.16: Effect of incubation temperature on the emulsifying activity of the biosurfactant towards xylene.

4.2.4. Pyrene biodegradation using Tween 80

From the previous results, it is conspicuous that although the biosurfactant produced by the indigenous soil culture was capable of solubilising PAH compounds with good emulsification activity and stability towards different organic solvents, the culture could not degrade pyrene even to a lesser extent. Therefore, further experiments in this surfactant aided system were conducted employing Tween 80 and with *M. frederiksbergense*, which far exceeded in their roles in pyrene degradation.

4.2.4.1. Single substrate condition

From the results of the calculated MSR values and pyrene degradation profile, Tween 80 showed good potential in the biodegradation of pyrene by *M. frederiksbergense* and therefore identified as the best among the five synthetic surfactants for further investigations. In this biodegradation study, flasks containing different concentrations of pyrene were solubilised in the aqueous media by adding suitable concentrations of Tween 80 as per the calculated MSR values. Figure 4.17 shows pyrene degradation profile by the *Mycobacterium* at different initial concentrations using Tween 80 as the surfactant.

It could be observed from the figure that at the indicated concentrations, near complete degradation was achieved within 30 h, but at 0.5 mg l^{-1} and 1.0 mg l^{-1} pyrene concentrations a lag period of 12 h at each of these concentrations was observed. The degradation rate during its active phase followed first order kinetics and was proportionate with the pyrene concentration in the media. During the active degradation phase, a maximum degradation rate of 1.34 mg l^{-1} d^{-1} was observed at 1 mg l^{-1} initial pyrene concentration. In our earlier study involving slurry phase system in shake flaks, the *Mycobacterium* showed a rate of 19.86 mg l^{-1} d^{-1} during active degradation phase for an initial total concentration of 50 mg l^{-1} with an initial lag period of 3 d. It can be noted here that, despite a large significant difference in initial pyrene concentration in slurry phase and the present surfactant aided systems, the observed degradation rates were not so. This very fact supports good improvement in pyrene degradation in surfactant aided system over the slurry phase system. Also, in comparison, *Mycobacterium flavescens* has

been reported to degrade pyrene at a rate of only 0.56 mg l^{-1} d^{-1} when supplied as a suspension at 50 mg l^{-1} (Dean-Ross and Cerniglia, 1996).

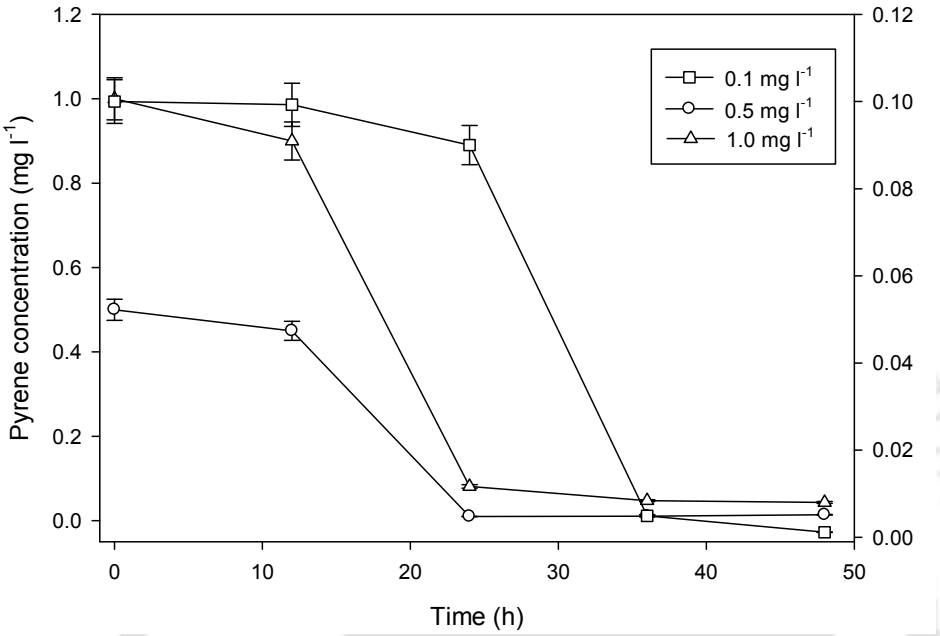


Figure 4.17: Pyrene degradation profile with different initial concentrations of Tween 80 in shake flask experiments. (Data for 0.1 mg l^{-1} initial pyrene concentration are shown in secondary Y-axis).

Similar to the previous slurry phase system containing pyrene as the single substrate, in the present surfactant aided system different concentrations of pyrene were solubilised using Tween 80 and its degradation by the *Mycobacterium* was investigated in a fermenter. Figure 4.18 shows pyrene degradation profile obtained in the fermenter experiments with different initial pyrene concentrations aided by the addition of Tween 80. It could be observed from the figure that near complete degradation was achieved within 50 h at all initial concentrations except at 50 mg l^{-1}, which took slightly longer time, but still lower in comparison to the previous slurry phase system. More interestingly, there was no lag period at any of the pyrene concentrations investigated.

The degradation rate during its active phase followed first order kinetics, and during this active phase, a maximum degradation rate of 17.7 mg l^{-1} d^{-1} was observed for 25 mg l^{-1} initial pyrene concentration. This observation also clearly supported that the pyrene degradation efficiency in this surfactant aided system is superior to that in the slurry phase system, where achieved degradation rate was only 6 mg l^{-1} d^{-1} with an initial pyrene concentration of 50 mg l^{-1}.

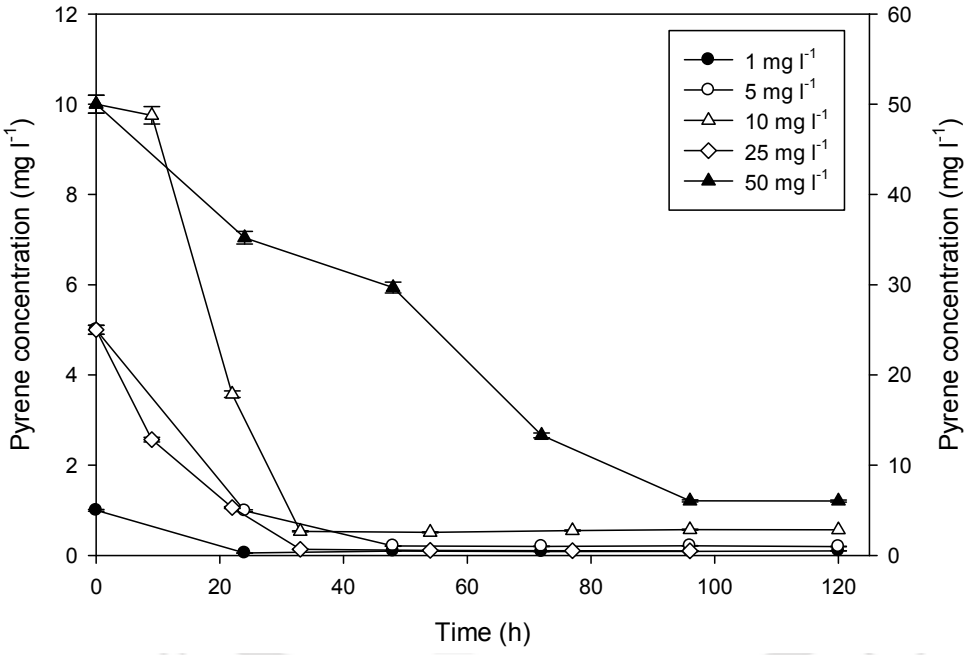


Figure 4.18: Pyrene degradation profiles obtained with for different initial concentrations of pyrene in the fermenter experiments (Tween 80 aided system). (Secondary Y-axis for 50 mg l^{-1} pyrene concentration data).

4.2.4.2. Mixed substrate condition

Bioremediation of sites contaminated with PAHs normally requires simultaneous metabolism of related compounds. PAHs do not display simple degradation patterns and rather exhibit substrate interactions among PAHs, which may be attributed to broad

specificity of enzymes involved in PAHs metabolism (Bouchez *et al.*, 1995). Hence, degradation rate or profile of single compound in laboratory cannot be simply extrapolated to simulate their degradation under actual field conditions due to presence of two or more PAHs in mixture.

To study the individual and interaction effects among the PAHs on their degradation in this Tween 80 aided system using *M. frederiksbergense,* experiments were carried out in shake flasks containing pyrene, naphthalene and anthracene at different levels as per the 2^3 full factorial design, for 8 d. Abiotic loss of the PAHs during autoclaving or during the experiment was negligible (<1%). Degradation pattern of these compounds in the different experimental runs are depicted in Figure 4.19, and the results of % PAH degradation efficiencies are summarized in Table 4.7.

From Table 4.7, it is seen that removals of anthracene, naphthalene and pyrene in the mixture varied in the range of 25.3-89%, 0-74.8% and 30-93.9%, respectively, and these removal values depended mainly on the low and high concentration levels of the PAHs. From these results, it is evident that the PAHs removal pattern was a function of the initial concentrations in the mixture. In Exp. run no. 8, where all the PAHs were present at their low level of concentrations (1 mg l^{-1}), anthracene and pyrene were removed better compared to naphthalene by the microbe: removal efficiencies of anthracene and pyrene being 89 and 81%, respectively, whereas naphthalene removal was low at 54%. However, when the concentrations of both anthracene and pyrene were increased to high level of 50 mg l^{-1} each, holding naphthalene at 1 mg l^{-1} (Exp. run no. 6), the removal of anthracene and pyrene changed only slightly to 86.4% and 87.6%, respectively, but naphthalene removal was totally inhibited (0%). This observation shows

that increasing the concentrations of anthracene and pyrene had antagonistic (inhibitory) effect on the removal of naphthalene in mixture in this surfactant aided system. When the concentration of naphthalene was increased from 1 to 50 mg l^{-1} keeping both anthracene and pyrene at 1 mg l^{-1} each, removal of both anthracene and pyrene decreased to 66 and 45%, respectively (Exp. run no. 5). Similarly, increase in anthracene concentration caused a decrease in removal efficiency of pyrene from 81 to 41% (Exp. run no. 4). Contrary to these findings, increase in pyrene concentrations resulted in higher anthracene degradation, particularly when anthracene was present at its higher level. Moreover, anthracene degradation in mixture depended on its relative concentration to pyrene – a lower ratio of anthracene to pyrene concentration favoring better anthracene degradation than vice-versa. At the highest concentration level (50 mg l^{-1}) of all the three PAHs in mixture (Exp. run no. 7), the removals of anthracene, naphthalene and pyrene were more or less equally good at 66.8, 88.4 and 88.5%, respectively. These degradation results indicated that an increase in the concentrations of any PAH from its low to high concentration level (1-50 mg l^{-1}) increased its own removals, but inhibited removal of other PAHs present in the mixture. However, exceptions were observed where increase in anthracene concentration inhibited its own removal and an increase in pyrene concentration stimulated anthracene removal.

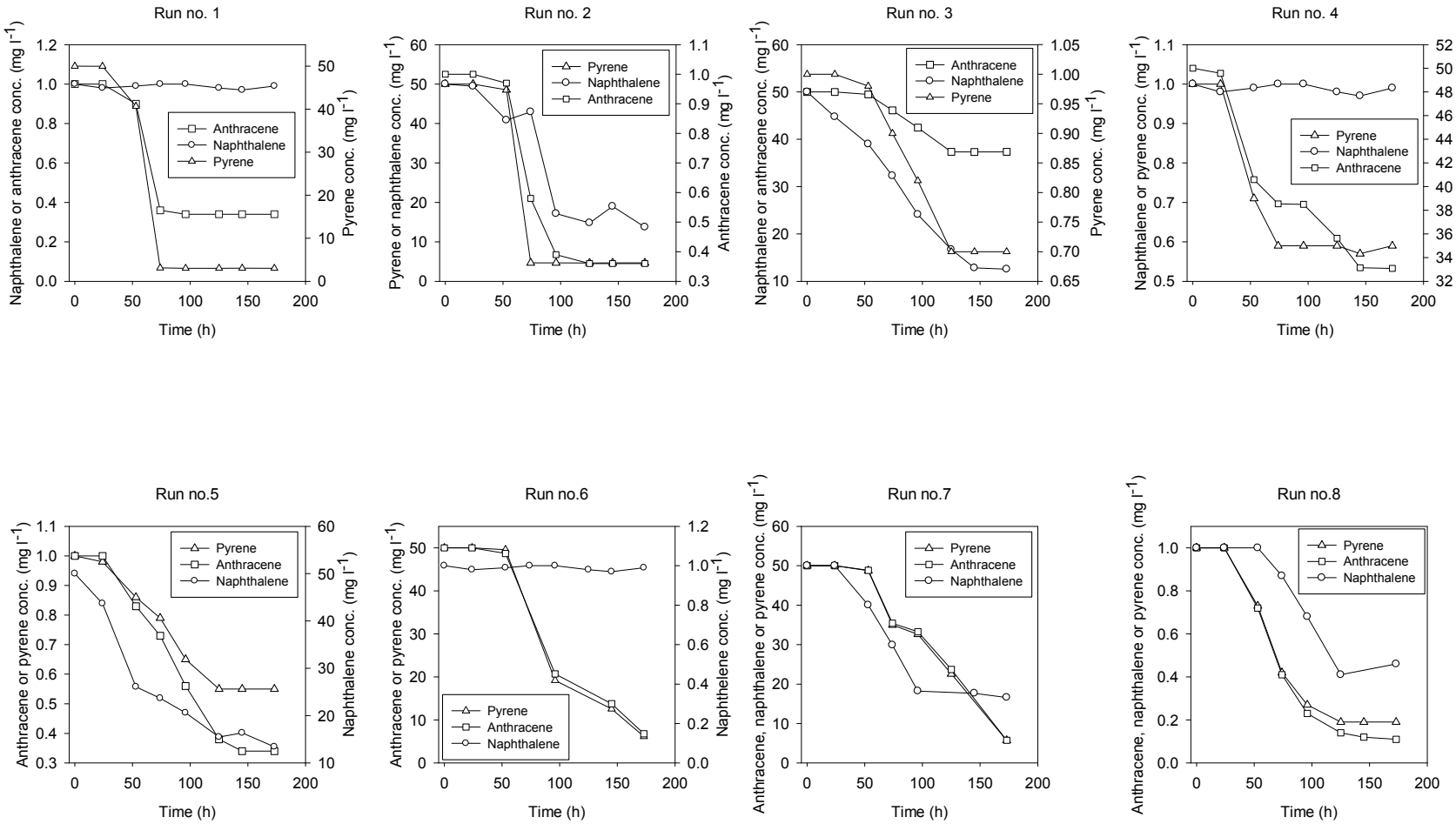


Figure 4.19 Degradation profiles of anthracene, naphthalene and pyrene in mixture using Tween 80 as the surfactant in the different experimental runs.

Table 4.7: Biodegradation efficiency of the PAHs in mixture in the Tween 80 aided system.

Run No.	PAH concentration (mg l^{-1})			% Degradation						Degradation rate (mg l^{-1} h^{-1})		
	Naphthalene	Anthracene	Pyrene	Naphthalene		Anthracene		Pyrene		Naphthalene	Anthracene	Pyrene
				Exp.	Pred.	Exp.	Pred.	Exp.	Pred.			
1	1	1	50	0.00	7.68	66.00	67.30	93.98	96.58	0.000	0.025	1.789
2	50	1	50	72.52	64.84	64.00	62.69	90.66	88.06	0.448	0.013	2.089
3	50	50	1	74.88	67.20	25.32	24.01	30.00	27.40	0.266	0.168	0.003
4	1	50	1	0.00	7.68	33.82	35.12	41.00	43.60	0.000	0.220	0.008
5	50	1	1	73.14	80.82	66.00	67.30	45.00	47.60	0.606	0.006	0.004
6	1	50	50	0.00	-7.68	86.40	85.09	87.68	85.08	0.000	0.349	0.362
7	50	50	50	66.82	74.50	88.48	89.78	88.56	91.16	0.441	0.638	0.358
8	1	1	1	54.00	46.32	89.00	87.69	81.00	78.40	0.008	0.008	0.010

The results from this surfactant aided biodegradation study also indicated that naphthalene does not significantly affect the degradation of pyrene or anthracene by the *Mycobacterium*. This result is, however, contradicting to the observation of McNally *et al.* (1999), who found naphthalene to stimulate pyrene degradation in mixture by a pure culture of *Pseudomonas putida* strain KBM-1 under aerobic conditions. In another study by Stringfellow and Aitken (1995), it was observed that degradation of phenanthrene was competitively inhibited by the presence of naphthalene by a *Pseudomonas species*. Because it is understood that different organisms take up different PAHs as their main substrates even when presented in a mixture, the non-interactive behavior of naphthalene with other PAHs in the present study could be reasoned due to the fact that naphthalene was not a preferred substrate for *M. frederiksbergense*.

Many studies have shown better ability of certain microbial consortia to simultaneously degrade both anthracene and pyrene than either of the two substrates (Bouchez *et al.*, 1995). On the other hand, other authors have also reported the inability of certain cultures to degrade these PAHs in mixture (Shuttleworth and Cerniglia, 1996). But, there exist only few reports in the literature where microorganisms have been shown to effectively degrade both anthracene and pyrene in individual as well as in mixture systems (Sartoros *et al.*, 2005). In general, co-metabolic degradation of HMW PAHs with LMW PAHs is found to result in lowering of utilization rate of the LMW PAHs compared to its individual degradation rate (Dean-Ross *et al.*, 2002).

M. frederiksbergense used in this biodegradation study is quite well studied to efficiently degrade pyrene or anthracene as the sole carbon source (Willumsen *et al.*, 2001). Hence, enhancement of anthracene degradation due to interaction with pyrene in

this study can neither be attributed to co-metabolism nor is in agreement with the general observation that LMW PAHs enhances removal of HMW PAHs. In view of this, it could be surmised that initial concentrations of the PAHs in mixture dictate to a large extent the degradation of these PAHs by *M. frederiksbergense*.

As before, to validate the roles played by the different PAHs and their interactions on their removals, statistical analysis of the results in the form of ANOVA and Student's t-test was carried out for each PAH removal case. The ANOVA was used to investigate and model the relationship between PAHs biodegradation efficiency in mixture and the individual PAHs and their two-way interactions. In general, the Fischer's 'F' value with a low probability 'P' value in the ANOVA table indicated high significance of the regression model (Montgomery, 2004). Table 4.8 illustrates the results of ANOVA of removal of the PAHs from mixture in this surfactant aided system.

The result from ANOVA suggest that only 2-way interaction effects for anthracene degradation were statistically significant ($F = 64.93$, $P = 0.091$). Accuracy and precision of the models, in the form of determination coefficient (R^2), adjusted R^2 and predicted residual error sum of squares (PRESS) shown in the ANOVA table, suggest that the models were highly efficient in predicting the experimental PAHs biodegradation data. However, among the three models, the model for naphthalene was found slightly less accurate than the models for anthracene and pyrene removal.

To further understand which of the individual PAHs and their interactions played a crucial role in their biodegradation in mixture, Student's t-test was performed, which is a tool to check the significance of the regression coefficients of the parameters. Table 4.9

presents the estimated coefficients of individual and interaction terms for the PAHs removal along with their associated t- and P- values.

Table 4.8: ANOVA of (a) anthracene (b) naphthalene and (c) pyrene biodegradation in mixture in the surfactant (Tween 80) aided system.

(a)

Source	DF	SS	MS	F	P
Main Effects	3	1477.49	492.50	36.29	0.121
2-way Interactions	3	2643.78	881.26	**64.93**	0.091
Residual Error	1	13.57	13.57		

(R^2 = 0.99, Adj. R^2 = 0.98, PRESS = 868)

(b)

Source	DF	SS	MS	F	P
Main Effects	3	7718.1	2572.7	5.45	0.303
2-way Interactions	3	840.7	280.2	0.59	0.715
Residual Error	1	471.9	471.9		

(R^2 = 0.95, Adj. R^2 = 0.63, PRESS = 30198)

(c)

Source	DF	SS	MS	F	P
Main Effects	3	4165.07	1388.36	25.67	0.144
2-way Interactions	3	626.22	208.74	3.86	0.354
Residual Error	1	54.08	54.08		

(R^2 = 0.98, Adj. R^2 = 0.92, PRESS = 3461)

Table 4.9: Statistical significance of the coefficients for naphthalene, anthracene and pyrene removal in mixture in the surfactant (Tween 80) aided biodegradation system.

	PAH removal								
	Naphthalene			Anthracene			Pyrene		
Term	**Coeff.**	**t**	**P**	**Coeff.**	**t**	**P**	**Coeff.**	**t**	**P**
Constant	42.67	5.56	0.113	64.88	49.81	0.013	69.74	26.82	0.024
Naphthalene	29.17	3.80	0.164	-3.93	-3.02	0.204	-6.18	-2.38	0.254
Anthracene	-7.25	-0.94	0.519	-6.37	-4.89	0.128	-7.93	-3.05	0.202
Pyrene	-7.84	-1.02	0.494	11.34	8.71	**0.073**	20.49	7.88	**0.080**
Naphthalene * Anthracene	6.26	0.81	0.565	2.32	1.78	0.325	3.65	1.40	0.394
Naphthalene * Pyrene	5.66	0.74	0.595	3.95	3.03	0.203	5.57	2.14	0.278
Anthracene * Pyrene	5.82	0.76	0.587	17.59	13.51	**0.047**	5.83	2.24	0.267

From the significance test of the regression coefficient values, it can be elucidated that the coefficients of main effect of pyrene (with t = 8.71, P = 0.073) and interaction between pyrene and anthracene (with t = 13.51, P = 0.047) influenced the removal of anthracene significantly in positive manner. In addition, the main effect of pyrene was also significant on its own removal, as revealed by its t and P values shown in the table (with t = 7.88, P = 0.080). The regression model equations relating the observed PAH removals with their initial concentrations are presented in the equations 4.4, 4.5, and 4.6 for naphthalene, anthracene and pyrene respectively.

$$\% \text{ naphthalene removal} = 47.22+0.68x_1-0.80x_2-0.80x_3+0.010x_1x_2+0.009x_1x_3+0.009x_2x_3 \quad (\mathbf{4.4})$$

$$\% \text{ anthracene removal} = 89.64-0.42x_1-1.10x_2-0.45x_3+0.003x_1x_2+0.006x_1x_3+0.029x_2x_3 \quad (\mathbf{4.5})$$

$$\% \text{ pyrene removal} = 79.39-0.64x_1-0.72x_2+0.35x_3+0.006x_1x_2+0.009x_1x_3+0.009x_2x_3 \quad (\mathbf{4.6})$$

where, x_1, x_2, x_3 are concentrations (in mg l^{-1}) of naphthalene, anthracene and pyrene, respectively. The predicted removals of the PAHs due to these regression model equations are also mentioned in Table 4.7, which suggests that both the experimental and model predicted removal values from each experimental run matched closely well with each other except for naphthalene removal.

Overall, in comparison to the previous slurry phase system, significant improvement in pyrene degradation rate and efficiency were observed in this surfactant aided system. However, it is fact that the current surfactant aided system also suffers from certain serious drawbacks. Firstly the load of pyrene to be biodegraded in such system is solely dictated by the surfactant under consideration that can limit its application for practical sized remediation problem. Secondly and more importantly, preferential uptake of surfactants over PAHs by the degrading microbes, even after careful selection cannot be simply ruled out. Hence, to overcome these limitations and to explore the full potential of the microorganism in pyrene biodegradation, a suitable two liquid phase partitioning bioreactor system was evaluated. The results of pyrene degradation in the TPPB system are discussed in the next section.

4.3. Pyrene Biodegradation in TPPB System

Substrate delivery in the biological treatment of PAHs is a crucial and the most significant challenge, where addition of the substrate at high concentration will inhibit or even kill the organisms, and substrate addition at too low concentration will cause the cells to starve resulting in a sub-optimal process performance or in an unavoidable extended lag phase. The situation becomes more complex for PAHs, as their aqueous

solubility are extremely low and their concentrations can not be precisely regulated with any conventional feedback control. In such scenario, sustained and controlled delivery of PAHs to the degrading microorganism is exceedingly important.

The most recent and promising technology for bioremediation of PAHs, including pyrene, seems to be use two-phase partitioning bioreactors system (TPPBs) characterized by an immiscible organic phase loaded with pyrene that partitions into the aqueous phase based on equilibrium considerations and real-time demand of the organisms (Daugulis, 2001), so that hydrophobic pyrene gets delivered in the aqueous phase at sub-inhibitory levels to the microorganisms. TPPB system is a two-step process that involves initial partitioning of pyrene from its highly soluble non-aqueous phase liquid (NAPL) to aqueous phase followed by subsequent microbial metabolism. Hydrophobic nature of pyrene and other PAHs makes them ideal candidates for degradation in TPPB systems, where high concentration and large surface areas can be achieved by dissolving in the dispersed organic phase (Daugulis and Janikowski, 2002; Marcoux *et al*., 2000).

4.3.1. Selection of solvents

The selection of organic solvent for TPPB system is crucial when dealing with a new contaminant and a new microbial strain; in addition to being non-volatile, inexpensive and readily available, the selected organic phase must be safe, stable, biocompatible and non bioavailable. The last two criteria are most crucial and must be accessed in context of the microorganism being employed in the TPPB system. The ability of the microorganism to grow and survive in an organic solvent is related with solvent polarity. Biocompatibility experiments with a range of solvents with different

polarity should therefore be conducted for the concerned microorganism. The selected solvent should have high solubility for the pyrene and at the same time mass partition coefficient should be as high as required for the aqueous phase concentration to remain below the inhibitory level.

The results in this solvent selection experiments are presented in Figure 4.20, which indicates that all solvents used in this study are biocompatible as relative metabolic activities (compared to the positive control containing only soybean oil) in presence of the solvents were greater than or nearly 1. In presence of silicone oil, relative metabolic activity was only slightly reduced compared to those of the other two solvents. Though isopropyl myristate was biocompatible, it was conceived that being bioavailable it might interfere with biodegradation of PAHs and therefore it was not selected for further investigations. Finally, based on economical consideration, silicone oil was considered as a better candidate than hexadecane.

In literature, silicone oil has preferentially been used in TPPB systems due to its hydrophobicity, chemical stability and resistance to hydrolytic and oxidative breakdown (Ascon-Cabrera and Lebeault, 1995[a,b]). Marcoux *et al.* (2000) reported superiority of silicone oil over 2,2,4,4,6,8,8-heptamethylnonane, paraffin oil, hexadecane and corn oil in PAH biodegradation by a microbial consortium. Moreover, silicone oil was found to be resistant to microbial attack and did not sequester PAHs. Recently, Vandermeer and Daugulis (2007) chosen silicone oil for improving the extent of degradation for the PAH mixture because of its superior PAH partitioning ability compared to dodecane.

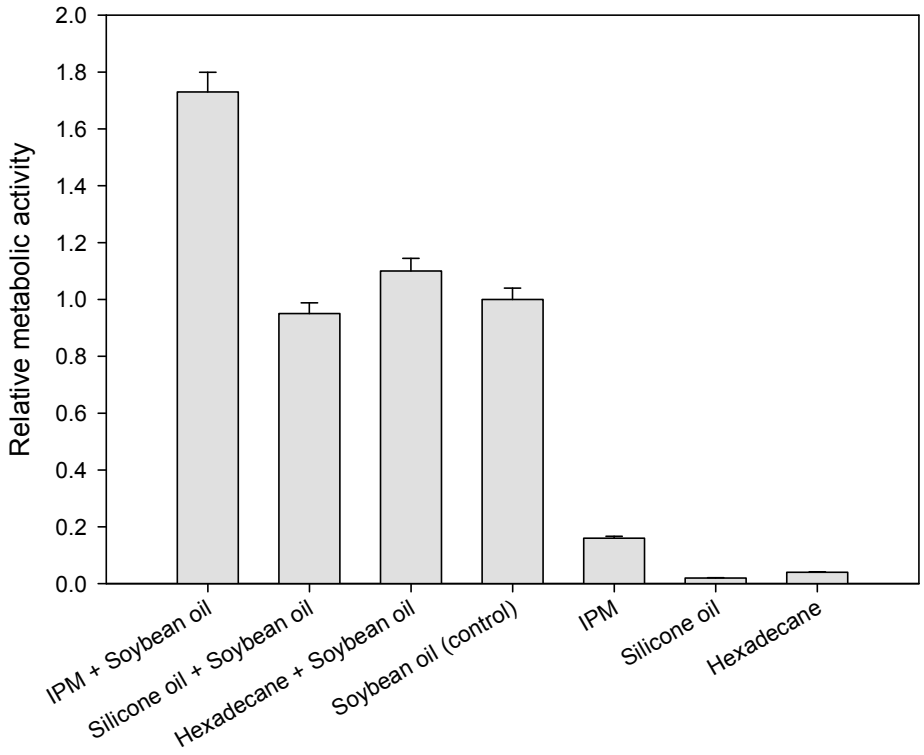


Figure 4.20: Relative metabolic activity of *M. frederiksbergense* in the presence of various solvents in the selection experiments.

The lower partitioning coefficient for silicone oil indicates that this solvent has a lower affinity for pyrene and therefore more likely to release pyrene into the aqueous phase based on real-time demand of the *Mycobacterium* (Villemur *et al.*, 2000).

Overall and based on the above findings, silicone oil was chosen as the organic phase for delivering pyrene in the TPPB system for its biodegradation by *M. frederiksbergense*. Prior to actual biodegradation experiments in this system, volumetric oxygen mass transfer coefficient in the two phase system was determined for optimizing the operating conditions, namely silicone oil fraction, agitation and aeration rate in the system.

4.3.2. Determination of volumetric oxygen mass transfer coefficient and enhancement factor in the two phase system containing silicone oil and water

For aerobic degradation of pyrene by the *Mycobacterium*, the mass-transfer rate of oxygen from the gas phase to NAPL or the aqueous phase is important, which is mainly governed by the size of the interfacial area. The interfacial area in turn is a function of surface averaged droplet diameter and dispersed-phase volume fraction (phase ratio). Optimal phase ratio which generates the highest interfacial area is influenced by the physical conformation of the bioreactor and the properties of the NAPL (density, viscosity, etc.). Another interlinked parameter known to influence performance of TPPB system is agitation rate and an increase in agitation intensity results in increase in the number of droplets. However, the distribution of drop sizes, as well as mean diameter of droplets are resultant of two simultaneous processes – break-up and coalescence of drops: high mixing rate not only produces smaller NAPL drops, but also multiply the probability of drop contact and coalescence. Further more, higher agitation may prevent contact of microorganisms with PAH-containing NAPL droplets or the shear stress could be detrimental to the microorganism. Therefore, choosing the best set of all these operating conditions has high significance on the success of a TPPB system.

In order to explain oxygen mass transfer process in two liquid phase systems, two types of models are employed: (i) homogeneous model and (ii) heterogeneous model. While a homogeneous model assumes a two phase system to be a single homogeneous liquid phase with weighted average uniform system properties, heterogeneous model considers mass transfer in both liquid phases separately. And, measurement of mass transfer rate in a two phase system with the homogeneous model requires estimation of

modified system average mass transfer coefficient and average driving force. Nielsen *et al.* (2003) proposed a correlation to predict overall volumetric oxygen transfer coefficient ($k_L a_{TP}$) for a two phase system as a function of operating conditions and organic-phase content as presented in Equation 4.7:

$$k_L a_{TP} = \delta . \left(\frac{P_g}{V} \right)^{\alpha} . (v_s)^{\beta} . (1 - X_{org})^{\gamma} \qquad \textbf{(4.7)}$$

where α, β, γ and δ are empirical constants P_g represents the power requirement of the aerated bioreactor, V represents the bioreactor working volume and v_s represents the superficial gas flow velocity through the reactor. This model uses weighing factor based on the volume-averaged oxygen saturation concentrations (to account for the dissolved oxygen solubility difference) to calculate mass transfer rate in the two phase dispersions.

An improved heterogeneous two phase mass transfer model was proposed by Nielsen *et al.* (2005), that describes the effective two liquid phase mass transfer coefficient ($k_L a_{TP}$) as a function of volumetric oxygen mass transfer coefficients involved in aqueous ($k_L a_A$) and organic phase ($k_L a_O$), respectively.

$$k_L a_{TP} = \frac{(k_L a_A \cdot V_A + k_L a_O \cdot D_{OA} \cdot V_O)}{(V_A + D_{OA} \cdot V_O)} \qquad \textbf{(4.8)}$$

The effective mass transfer coefficient ($k_L a_{TP}$) of the dispersed two-phase system was estimated by isolating $k_L a_A$ and $k_L a_O$ in single phase experiments. Though this effective mass transfer coefficient ($k_L a_{TP}$) is a function of X_{org} at any constant combination of agitation and aeration rates, it could be derived by the individual mass transfer process. The enhancement in oxygen mass transfer by the addition of an organic phase was estimated using the Equation 4.9.

$$\text{Enhancement factor } (E) = 1 + \left(\frac{k_L a_O . D_{OA}}{k_L a_A} - 1 \right) \cdot X_{org} \quad \textbf{(4.9)}$$

In the present study, the above heterogeneous oxygen mass transfer model (Equation 4.9) was adopted to evaluate the potential improvement in oxygen mass transfer in the two phase system consisting of silicone oil in water at various fractions in a fermenter operated under different sets of agitation and aeration speeds. The measured values of $k_L a_{TP}$ under different operating conditions of agitation and aeration in the reactor are displayed in Figure 4.21 using different organic phase fractions in the two phase system.

Between 0.5 and 2.5 l min^{-1} aeration rates, there was nearly a two fold increase in the observed mass transfer coefficient value. At low aeration rates, the effect of agitation rate was the same in both single and two liquid phase systems. However, at agitation rates of less than 400 rpm, large gas bubbles were observed in the reactor with two phase system and the two liquid phases were not well dispersed. Although liquid phase mixing is important in enhancing the oxygen mass transfer rate, shearing and dispersing of gas bubbles are also necessary for increasing gas–liquid mass transfer area and contact times, which relies on the agitation rate in the system. Addition of silicone oil, particularly at low X_{org} values, decreased the volumetric mass transfer coefficient of oxygen compared to single aqueous phase system. Similar results have been reported by Dumont *et al.* (2006), where the authors attributed hindrance to rate of oxygen mass transfer in two phases due to the organic phase silicone oil in the system. In a review work on gas absorption in oil-in-water systems, Dumont and Delmas (2003) reported that $k_L a_{TP}$ value in two phase systems can decrease, remain unaffected or increase depending upon the

nature of organic phase in the system. For instance, Hassan and Robinson (1977) found, for the same experimental conditions, that addition of hexadecane increased the $k_L a$ value, whereas the value was observed to decrease with the addition of dodecane in the two phase system. Not quite surprisingly, the authors could not explain these contrasting results in their study. Lekhal *et al.* (1997), in their study, performed gas-liquid mass transfer measurements by dynamic absorption in various single liquid phases and in liquid-liquid mixtures, and found that volumetric mass transfer coefficient $k_L a$ increased by adding a dispersed organic phase, up to a maximum of 3 to 4% of the system total volume.

Figure 4.21 also clearly indicates that the experimental value of $k_L a_{TP}$ depends on the organic fraction in the two phase system; hence, Equation 4.7 was also considered to predict the $k_L a_{TP}$ values at different operating conditions and organic fractions using nonlinear least-squares regression employing Matlab® curve fitting tools. The parameters δ, α, β, and γ were estimated to be 0.005122, 0.669, 0.443, and 4.504, respectively. Nielsen *et al*. (2002) in their work reported values of these empirical constants to be 650, 0.31, 0.70, and 1.70, respectively, using hexadecane as the second organic phase. Considering a different organic phase employed in the present study, such differences in the parameter estimates, particularly of γ, is not unlikely. Moo-Young and Blanch (1981) suggested typical values of α and β to be 0.7 and 0.3, respectively, for non-coalescing media for single-liquid phase system. The values of these two parameters indicate that the functional dependence of $k_L a_{TP}$ on operating conditions in two phase systems is only slightly different from that in single-phase system. Nevertheless, the model provided a good fit to the data, as evidenced from Figure 4.22, which shows predicted vs.

experimental values of $k_L a_{TP}$ with a line of slope equal to 0.95, with most of the predicted values corresponding well to the experimental data.

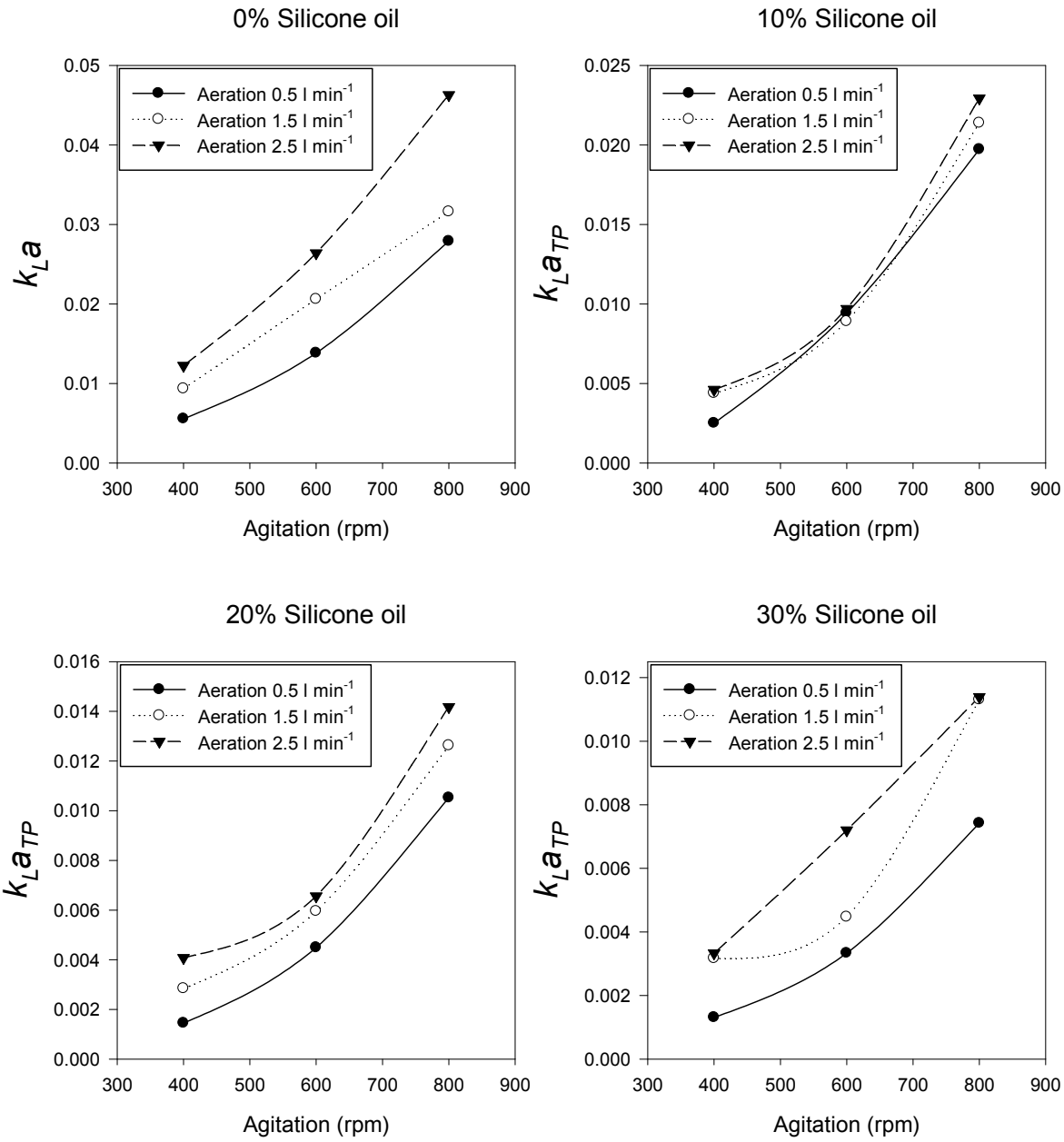


Figure 4.21: Observed volumetric oxygen mass transfer coefficient values in the two liquid phase system obtained at various sets operating conditions in the fermenter.

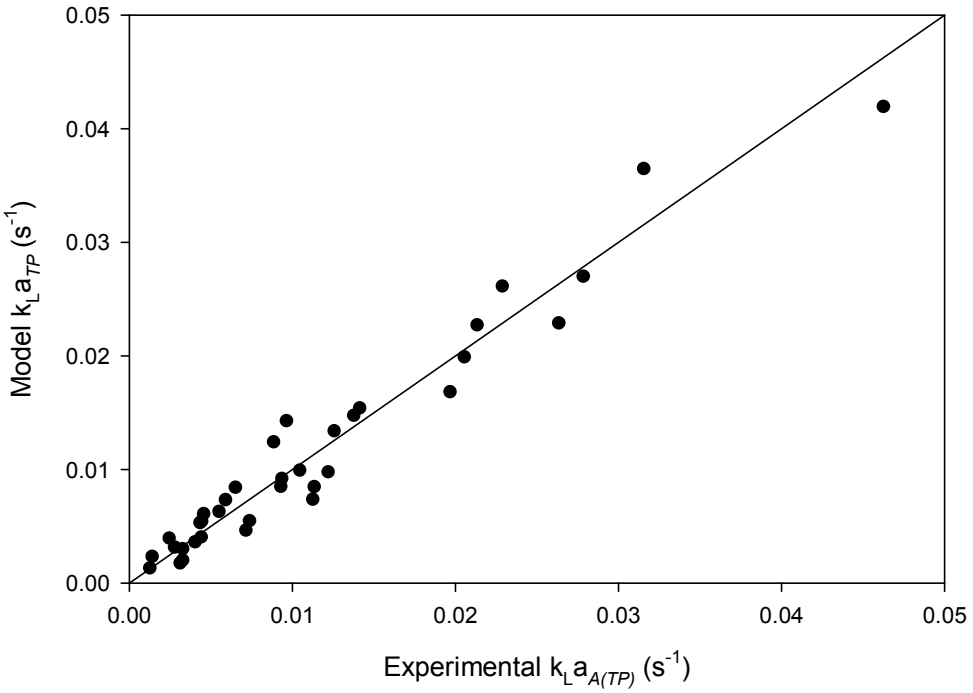


Figure 4.22: Experimental versus model predicted volumetric oxygen mass transfer coefficient in the two liquid phase system using Equation 4.7.

Enhancement factor

Values of $k_L a_A$ and $k_L a_O$ were used to estimate the enhancement factor using Equation 4.9, considering partition coefficient of oxygen between silicone oil and water (D_{OA}) value as 8.33 (Michael, 2005). Enhancement factor in the present work was calculated for each set of operating conditions i.e., silicone oil volume fraction, agitation and aeration rates. According to this model, the enhancement factor will always be greater than one, provided $k_L a_O . D_{OA} > k_L a_A$. Both, the above condition and the enhancement factors were observed to be satisfied at all the set of operating conditions adopted in this study, as also manifested in Figure 4.23.

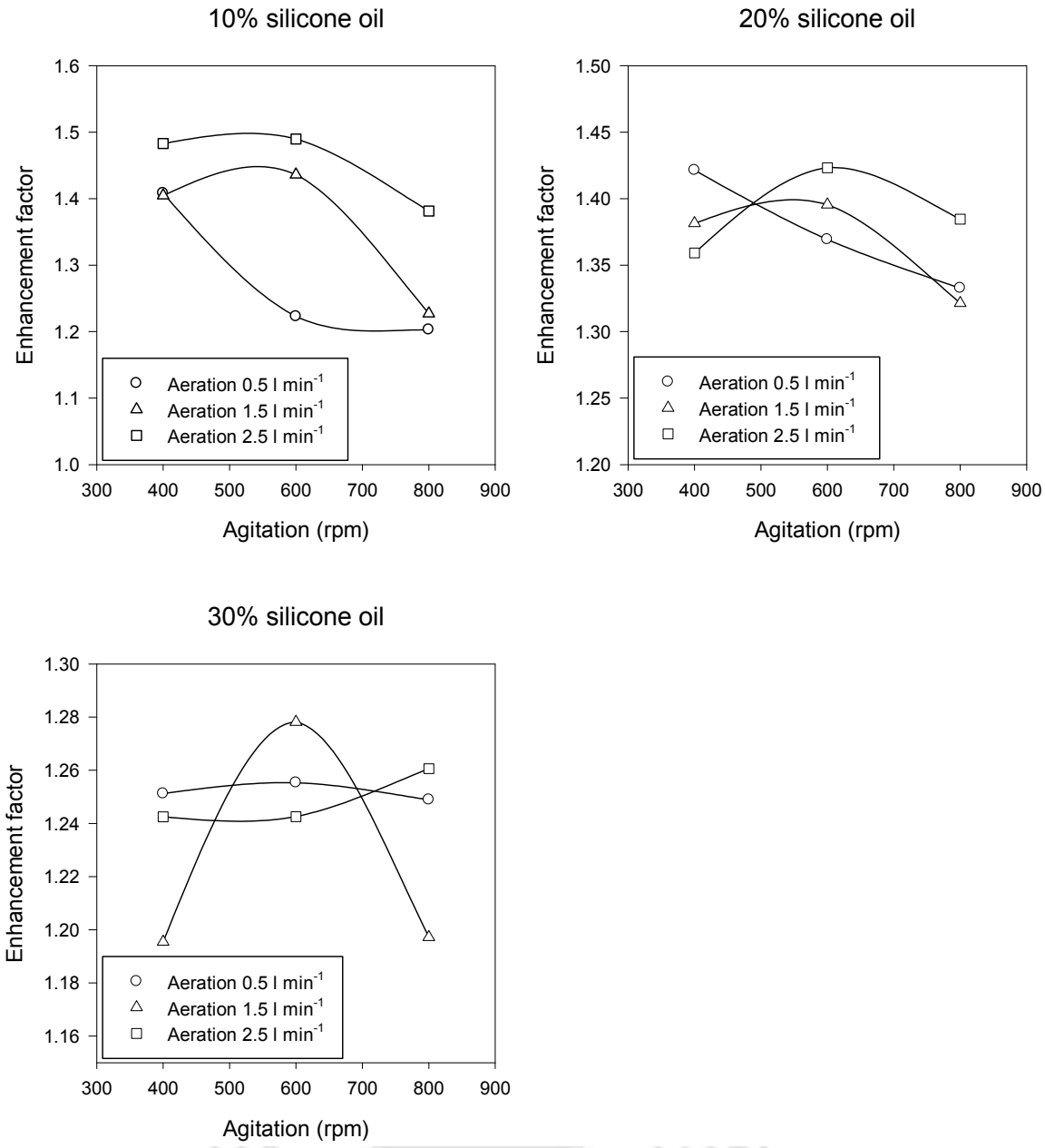


Figure 4.23: Calculated enhancement factor values of oxygen transfer in the two phase system at different operating conditions in the fermenter using the model of Nelson *et al.* (2005).

In general, the enhancement factor was found to decrease with higher silicone oil fraction in the two liquid phase system, particularly for an aeration rate of 2.5. However, variation in its values was found depending upon the combinations of aeration and agitation rates in the two liquid phase system: at 600 rpm, optimum value of

enhancement factor was observed at aeration rates of 1.5 and 2.5 l min^{-1}. These observations clearly indicate a high influence of operating conditions on the oxygen mass transfer in two liquid phase systems. Also, it could be said from these results, that in general, addition of any quantity of organic phase with a high affinity for dissolved oxygen will promote an enhanced total rate of oxygen transfer to the aqueous phases in a two liquid phase partitioning reactor system. It was thus concluded that for pyrene biodegradation experiments employing this system, the following set of conditions were applicable: silicone oil fraction: 20%, agitation rate: 600 rpm, aeration rate: 1.5 l min^{-1}

4.3.3. Pyrene biodegradation experiments in the TPPB system

4.3.3.1. Single substrate condition

Fig. 4.24 illustrates the pyrene degradation profile by *M. frederiksbergense* in the TPPB system, which clearly reveals that pyrene was completely degraded in 265, 240, 144 and 219 h for initial concentrations of 1000, 600, 400 and 200 mg l^{-1}, respectively. In all the cases, the entire degradation time could be divided into a variable duration lag phase and an active degradation phase. The initial lag phase was observed to vary from 2-7 d depending upon the pyrene concentration, particularly at concentrations above and below 400 mg l^{-1}. A probable reason for this lag phase might be that the *Mycobacterium* was not adapted to a biphasic environment as its maintenance was mainly performed in a mono-phasic system (Guieysse *et al.*, 2001).

Marcoux *et al.* (2000) have reported complete degradation of pyrene in 3-17 d in TPPB system using silicone oil as NAPL, with initial pyrene concentration of 80 mg l^{-1} in the organic phase. The authors did not, however, find any lag period in degradation

using the solvent; but when the solvent was changed the authors reported 3-d and 12-d of lag in pyrene degradation with heptamethylnonane and paraffin oil, respectively. During the active degradation phase in the present study, degradation rates were found to be 82, 139, 230 and 270 mg l^{-1} d^{-1} for the initial concentrations of 200, 400, 600 and 1000 mg l^{-1}, respectively. MacLeod and Daugulis (2003) in their study reported pyrene degradation rate of 138 mg l^{-1} d^{-1} with *Mycobacterium PYR-1*, which is so far the highest pyrene degradation rate reported till date. In the present study the degradation rates of pyrene at all initial concentrations (except at 200 mg l^{-1}) are much higher, suggesting superior performance of *M. frederiksbergense* in degrading pyrene employing the TPPB system.

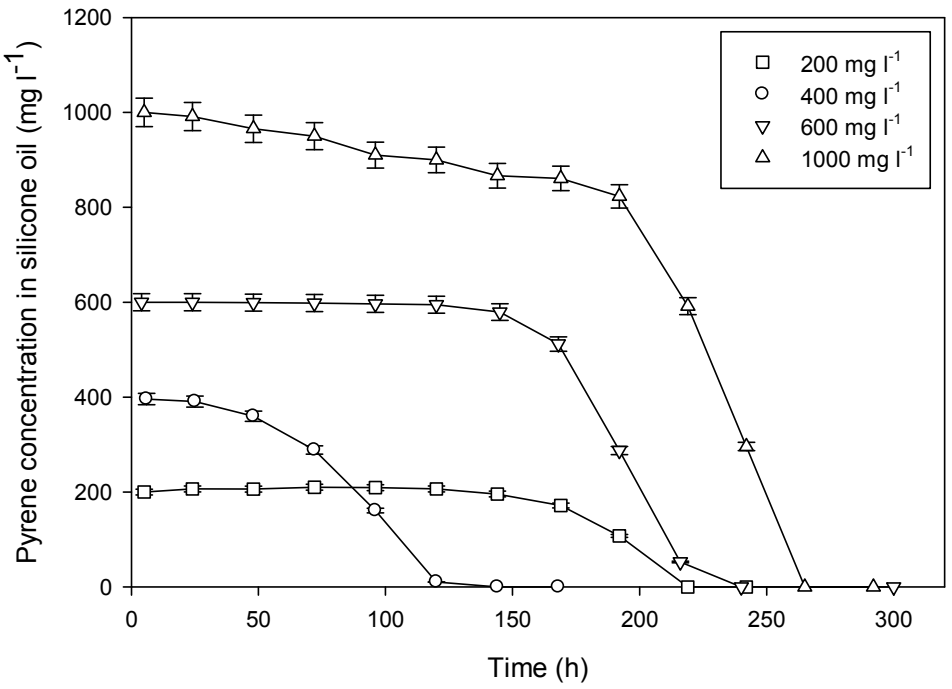


Figure 4.24: Pyrene degradation profiles obtained in the TPPB system.

It should be noted here that abiotic loss of pyrene in the current TPPB system was found negligible (data not shown), which is in accordance with the observations by other

authors regarding loss of pyrene due to evaporation (Lei *et al.*, 2007) or adsorption to reactor vessel surface (Knightes and Peters, 2003).

Microbial biomass concentrations in TPPB systems are measured, in general, by sampling the aqueous phase of TPPB, thereby ignoring the same in NAPL of such systems. Due to inhomogeneous nature of such TPPB systems, biomass estimation may lead to erroneous interpretation. Hence, in this study, models that consider substrate degradation profile as a function of time rather than biomass were used for modeling the pyrene degradation data.

To model the pyrene degradation profiles followed in this system, five different models belonging to two types, namely: Monod variants (I - simple Monod, II - logistic growth Monod, III - logarithmic growth Monod) and variants of mechanistic three-half-order models (IV - three-half order with linear growth and V - three-half-order with logarithmic growth) were chosen in this study. The mathematical forms of these models are presented in Table 4.10.

Non-linear least square analysis was performed using MATLAB® curve fitting tool to estimate the various kinetic parameters in these models by duly considering constraints for positive integer value or appropriate initial guess of the parameters. Table 4.11 presents the coefficient of determination (R^2) values obtained by fitting the various models considered in this study. The table clearly shows that the simple Monod model completely failed to fit the entire data. Though three-half order model (with linear growth) could fit the degradation profile of 400 mg l^{-1}, it however, failed to fit the other data. Monod variants with logarithmic growth could reasonably fit the kinetic data obtained for 1000 mg l^{-1} ($R^2 = 0.9152$) and 400 mg l^{-1} ($R^2 = 0.9231$) concentrations, but

not for other concentrations. And the exponential growth form of the three-half-order model provided the best fit for entire data in this study.

The kinetic parameters were thus estimated from this particular model and are given in Table 4.12. The value of k_1 (first-order proportionality constant) determined from the best fit three-half-order kinetic model are low if compared with those reported by Brunner and Focht (1984) and Scow *et al.* (1986) in their studies, but were close to the values reported by Metzger *et al.* (1999), where it was estimated to be 1.06×10^{-3} h^{-1}. Estimated k_1 in our study for an initial substrate concentration of 400 mg l^{-1} was maximum compared to those of other concentrations, and this value also seem to be consistent with the observation that the lag period in pyrene degradation is minimum at this concentration.

When similar degradation rates within a given concentration range are encountered in a TPPB system, the uptake mechanism could be said as an interfacial one (MacLeod and Daugulis, 2005). And, since in our study the degradation rates varied with initial concentration of pyrene in silicone oil phase, the interfacial based uptake mechanism was not found to be true. Due to very high partition coefficient of pyrene for silicone oil in TPPB system, no pyrene was found to precipitate during the experiments. Therefore, different aqueous phase pyrene concentration, depending on its concentration in silicone oil, could be attained for easy uptake by the microorganism growing in aqueous phase. Hence, it could be surmised that either “direct pollutant uptake from the organic phase” or “prior pollutant transfer to the aqueous phase before uptake” or a combination of these two could be a probable mechanism of pyrene uptake in this system.

Table 4.10: Kinetic models applied to fit the experimental data on pyrene degradation in the TPPB system.

Model		Mathematical form [a]	Necessary condition	Estimable parameters	Reference
I	Simple Monod	$t = \frac{Y_{x/s}(k_s + S)}{(\mu_{max} X_0 + \mu_{max} Y_{x/s} S_0)S - \mu_{max} Y_{x/s} S^2}$	Nil	k_s; $Y_{x/s}$; μ_{max}	Bandyopadhyay *et al.*, 1998
II	Logistic Monod	$S/S_0 = \frac{1 + (X_0/S_0)}{1 + (X_0/S_0)e^{[(\mu_{max}/k_s)(S_0 + X_0)t]}}$	$S_0 << K_s$	k_s; μ_{max}	Stephen and Martin, 1984
III	Logarithmic Monod	$S/S_0 = 1 + (X_0/S_0)(1 - e^{\mu_{max} t})$	$S_0 >> K_s$	μ_{max}	
IV	Three half order (linear growth)	$S/S_0 = e^{-k_1 t - ((k_2 t^2)/2)}$	Nil	k_1; k_2	Brunner and Focht, 1984
V	Three half order (exponential growth)	$S/S_0 = e^{-k_1 t - \frac{E_0}{\mu}(e^{-\mu t} - 1)}$	Nil	k_1; μ	

[a] E_0, Initial level of enzyme concentration (mg l^{-1}); k_1, First-order proportionality constant (h^{-1}); k_2, Second-order proportionality constant (h^{-2}); k_s, Half-saturation constant (mg l^{-1}); S, Substrate concentration at any time (mg l^{-1}); S_0, Initial Substrate concentration (mg l^{-1}); t, Time (h); X_0, Initial microbial biomass concentration (mg l^{-1}); $Y_{x/s}$, Yield coefficient (mg cell produced/mg of substrate degraded); μ, Specific growth rate (h^{-1}); μ_{max}, Maximum specific growth rate (h^{-1})

Table 4.11: Calculated coefficient of determination (R^2) values for the various models applied to the pyrene degradation data in the TPPB system.

Models	Initial pyrene concentration (mg l^{-1})			
	200	400	600	1000
I. Simple Monod	0.3475	0.4564	0.3322	0.4382
II. Logistic Monod	0.4593	0.9137	0.6701	0.7804
III. Logarithmic Monod	0.567	0.9231	0.8693	0.9152
IV. Three half order (linear growth)	0.5325	0.9333	0.6765	0.7254
V. Three half order (exponential growth)	0.9991	0.9929	0.9960	0.9971

Table 4.12: Estimated kinetic parameters from the best fit three-half-order model.

Estimated rate constant	Initial pyrene concentration (mg l^{-1})			
	200	400	600	1000
k_1 (h^{-1}) $\times 10^{-3}$	0.1219	3.258	0.134	0.874

4.3.3.2. *Mixed substrate condition*

In order to observe effect on the pyrene degradation by *M. frederiksbergense* in TPPB system due to presence of other PAHs – naphthalene and anthracene, experiments were performed using the TPPB system containing equal concentrations of the PAHs at 400 mg l^{-1}, a concentration which showed optimum degradation result for pyrene when presented individually in the system. Figure 4.25 illustrates the biodegradation profiles of the three PAHs in the mixture study. It was observed that the pyrene degradation in the mixture was associated with a lag period of about 88 h, which was, however, absent when presented as a single PAH substrate to the *Mycobacterium* employing this TPPB system, suggesting either competition among the PAHs for their biodegradation by the culture or

inhibition of the microorganism to degrade pyrene quickly due to an initial excess combined PAH load (concentration) in the system.

Similar appearance of initial lag period for pyrene degradation in five components PAHs mixture in TPPB system has been reported by Vandermeer and Daugulis (2007). Pyrene was degraded only to the extent of 64% at a rate of 43 mg l^{-1} d^{-1} in dodecane which increased to 84% with a rate of 35 mg l^{-1} d^{-1} when the solvent was replaced with silicone oil. In contrast to this finding, in the present study, pyrene was near completely degraded at the end of 150 h. In binary mixture containing naphthalene and anthracene in TPPB system, MacLeod and Daugulis (2003) observed slow and constant naphthalene degradation with a lag of 24 h and both anthracene and naphthalene leveled off at approximately 250 h to final degradation values of 95% and 75%, respectively.

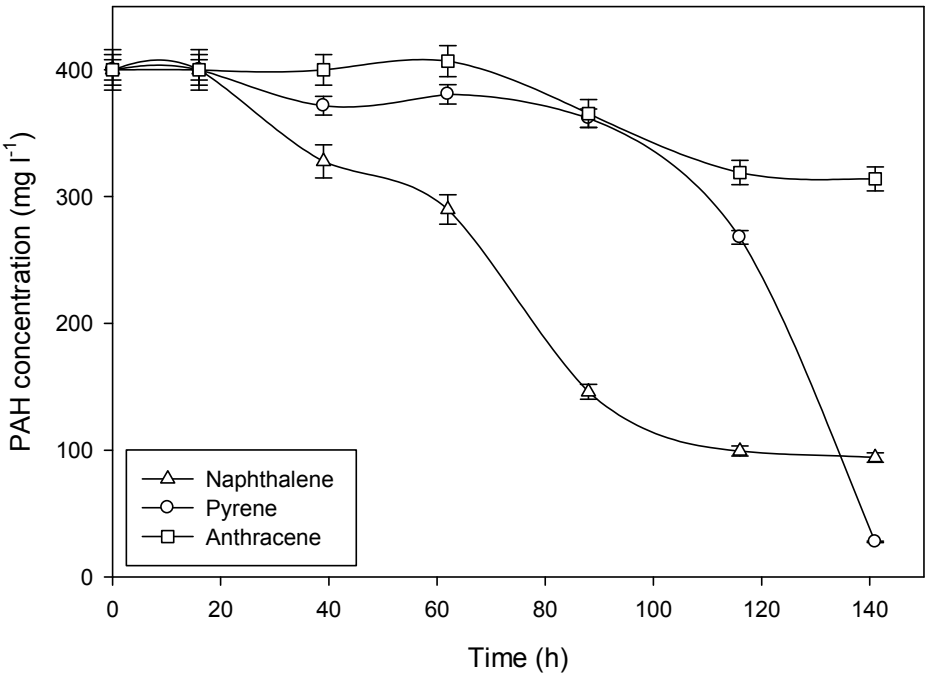


Figure 4.25: PAHs degradation in mixture in the TPPB system (initial concentrations of each PAH at 400 mg l^{-1}).

The mixture study clearly indicated that the utilization pattern of pyrene from PAHs mixture is not only a function of the relative concentrations of the individual PAHs in mixture, but also depends on the delivery system being employed. When the results are compared with slurry phase or surfactant aided system under mixed substrate conditions, significant improvement in pyrene degradation was observed. For slurry phase system containing naphthalene, anthracene and pyrene, each at 50 mg l^{-1}, no appreciable degradation of pyrene was noted even after 8 d, which was better in Tween 80 aided system; where about 88% pyrene was degraded in 7 d. In this present TPPB system containing PAHs in mixture, pyrene concentration, even at a very high value of 400 mg l^{-1}, was efficiently removed within relatively shorter time of 6 d.

4.4. Simple Cost Benefit Analysis of the Three Systems Evaluated in the Present Work

Cost effectiveness or cost-benefit analysis of any process involves, whether explicitly or implicitly, weighing the total expected costs in the process against the total expected benefits of one or more actions in order to choose the best or most desirable option. A hallmark of such analysis is that all benefits and all costs are expressed in money terms, and even adjusted for the time value of money. Cost benefit analysis has largely been used to select most viable options from multiple possible alternatives for achieving any particular goal. The approach is equally applicable while implementing any remediation project. Three treatment alternatives discussed in this thesis with respect to biodegradation of pyrene by *M. frederiksbergense* were subjected to a simple cost effectiveness analysis framework, so that the potential treatment methods could be

identified even from an economist point of view. Cost analysis of the three biodegradation systems investigated in this work was performed considering the cost of inoculum preparation, media ingredients and its preparation (if any), power requirements while running the batch experiments, either in shake-flask or in a fermenter. However, inputs like man power, initial setup, sample analysis etc were not taken into account to keep the analysis uncomplicated and simple for best comparison of the technologies investigated in this work.

Comparison among the three systems was also made from the standpoint towards the cost involved per unit amount of pyrene getting biologically degraded by *M. frederiksbergense* in single substrate condition. The cost and benefit analysis was thus completed in three stages: **(i)** determination of costs and benefits, **(ii)** measurement of costs and benefits and **(iii)** assessment of costs and benefits. In the cost analysis step, costs involved in operating the three processes were taken as the measurable direct cost and the amount of pyrene removed in each process as the measurable direct benefit. Time frame chosen for this analysis was limited to the batch pyrene biodegradation periods in the three processes. The power requirement in shaker or fermenter for inoculum preparation or batch biodegradation was estimated from detailed specifications of the equipments manufacturer; thus are presented in Table 4.13. Running cost for fermenter or shaker incubator was based on the LT commercial electricity tariff of Assam Electricity Regulatory Commission (AERC), India.

Table 4.14 details the preparation cost of inoculum for use in the slurry phase, surfactant aided or TPPB system. The cost for inoculum preparation included cost of nutrient broth media that was used for subculture and the power requirement for its

growth before using the freshly grown culture in biodegradation studies. It could be mentioned here that, the cost for power requirement was independent of the volume of inoculum to be prepared as it was always grown only in shaker incubator for 12 h.

Table 4.13: Power requirements for operating a fermenter or a shaker incubator.

Fermenter		**Shaker incubator**	
Unit/Components	**Power (kW)**	**Unit**	**Power (kW)**
Bioconsole	1.6	Complete system	3.45
Controller	0.07		
Agitator	0.04		
Chiller unit	0.9		
Air compressor	1.5		
Total power	**4.11**		**3.45**
Cost/h (in Rs.)*	**18.7**		**15.7**

* Electricity charges are based on LT-commercial tariff of AERC (Rs.4.55/kWh) (http://aerc.gov.in/15-CHAPTER-10-SCHEDULE%20OF%20TARIFF.pdf).

Table 4.14: Preparation cost of inoculums used in the three biodegradation systems.

Chemical / power Input	**For 50 ml**		**For 5 ml**	
	Amount	**Cost***	**Amount**	**Cost***
Nutrient broth	0.65 g	1.95	0.065 g	0.19
Power consumption in a shaker (12 h @ 3.45kW)	41.4 unit	188.37	41.4 unit	188.37
Total cost (in Rs.)		**190.32**		**188.56**

* All prices are inclusive of 12.5% VAT in Indian Rupees (1 USD = Rs.45), Electricity charges are based on LT-commercial tariff of AERC (Rs.4.55/kWh).

Table 4.15 details the preparation cost for the basic media used in the slurry phase, surfactant aided or TPPB system. The cost was highly variable depending on volume of the media irrespective of the type of system being used. In slurry phase

system, only BH media supplemented with trace metal solution accounted for the same. But, in surfactant aided system or in TPPB system, cost for surfactant and organic solvent respectively were additionally considered under the head pyrene delivery in Table 4.16.

Table 4.15: Cost involved in preparing the media for use in pyrene biodegradation employing the three different systems.

Chemical / power Input	**For 1500 ml**		**For 1000 ml**		**For 100 ml**	
	Amount	**Cost***	**Amount**	**Cost***	**Amount**	**Cost***
BH media	4.9 g	22.06	3.27 g	14.71	0.327 g	1.47
Trace element solution	3 ml	0.05	2 ml	0.03	0.2 ml	0.003
Total cost (in Rs.)		**22.11**		**14.74**		**1.473**

* All prices are in Indian Rupees inclusive of 12.5% VAT (1 USD = Rs. 45)

Table 4.16 comparatively illustrates the combined cost for performing the various steps in the three systems. A point worth noting here, this table compares the cost involved in each system with the best results only obtained from the various experimental runs, conducted either in shake flask or fermenter. In other words, experiments, in shake flask or fermenter, that did not yield desirable results, were not considered for this cost benefit analysis. These data clearly indicate that the TPPB system was the most economical among the three systems, involving minimal cost/unit of pyrene biodegraded, where only Rs. 30.00 was required for biodegradation of 1 mg of pyrene. However, the cost could be considerably lower, if we could have reused the solvents for TPPB systems. Though TPPB system seems much more economical when compared to other biodegradation system, it is evident from Table 4.16 that about 75% of the total cost is due the high power consumption in the system.

Table 4.16: Comparison of overall cost involved in degrading pyrene employing the different system using *M. frederiksbergense*.

System	Cost involved (in Rs.)					Pyrene removal (mg)	Cost/mg of pyrene removal (in Rs.)
	Inoculum preparation	Basic media preparation	Pyrene delivery	Power cost	Total cost		
Slurry phase system in shake flask	188.56	1.473	-	1884	2074	4.8 mg	**432**
Slurry phase system in fermenter	190.32	22.11	-	3740	3952	75 mg	**53**
Surfactant aided system in shake flask	188.56	1.473	0.375	2260	2450	4.5 mg	545
Surfactant aided system in fermenter	190.32	14.74	1.881	673.2	880	25 mg	35
TPPB system (in fermenter only)	190.32	22.11	550	2244	3006	100 mg	**30**

In view of the above, to achieve further cost reduction in the TPPB system, there is an urgent need to look for an alternative approach where power consumption cost could be considerably minimized. Therefore, it was envisaged to develop a non-conventional TPPB system, where solvent droplets could be properly stabilized or encapsulated inside a hydrophilic polymer, such that agitation power requirement for dispersion of the two phase could be kept minimal. Moreover, this approach would enable us to widen the choice of organic solvents for use in the encapsulated system, thereby overcoming solvent biocompatibility, bioavailability or toxicity problems.

The next section discusses the results obtained in such a non-conventional TPPB system that was developed for pyrene biodegradation by *M. frederiksbergense*.

4.5. Performance Evaluation of a Non-Conventional TPPB System

Previous results clearly manifested that the developed conventional TPPB system showed very high degradation rate in pyrene removal. The cost benefit analysis also revealed the potential of the TPPB system as the most cost efficient technique among the three systems studied. However, concerns on the feasibility of adopting a TPPB system lies in certain critical and crucial steps, such as solvent selection, recovery of solvent and its reuse for sustaining the process as a whole. In addition, requirements of constant high agitation rate along with aeration rate can be considered as some potential drawback of the system. In order to overcome these disadvantages with a conventional TPPB system, the development of certain non-conventional TPPB system may be essential. Hence, development of a pyrene-in-solvent encapsulated system for sustained controlled delivery

and biodegradation of pyrene by the *mycobacterium* growing in the aqueous phase was attempted.

4.5.1. Standardization of pyrene encapsulation method

Hydrogel polymers of alginate and PVA have been known to be used for encapsulation or entrapment of industrial enzymes, cells, drugs and even agrochemicals for control release delivery (Singh *et al.*, 2008), and are often reported to be more suitable for encapsulating hydrophobic compounds than hydrophilic compounds (Robinson and Lee, 1987; Ribeiroa *et al.*, 1999). Hence, alginate type beads were chosen for encapsulating pyrene to test their application in this study. Encapsulation efficiency of pyrene was found to be more than 99% with all the five different bead types. In addition, all the different bead types had a smooth surface without oil deposition upon drying at room temperature or lyophilization. These two basic properties suggest that the methods followed to prepare the different bead types are suitable for encapsulating hydrophobic pyrene in a hydrophilic alginate gel matrix. Similar successful entrapment of hydrophobic compounds in alginate micro spheres by emulsification internal-gelation technique has also been reported in the literature (Poncelet *et al.*, 1992).

Figure 4.26 shows the pyrene release profile observed with the different bead types prepared in the study. In all the bead types containing silicone oil as the delivery vehicle, the release was better compared to the bead type that used DMSO (type IV). This could be due to the fact that DMSO being more water soluble than silicone oil could have resulted in precipitation of pyrene inside the bead core thereby retarding its release into the medium. In case of the bead type II, where PVA was used along with alginate but not

treated with boric acid, a sudden burst release of pyrene was evident from its release profile (Figure 4.26), probably due to easy swelling of the beads owing to weak surface strength resulting from insufficient cross-linking. On the other hand, this behavior was not observed when boric acid was used to prepare the bead type III, which exhibited a better controlled release pattern than type IV. However, PVA-alginate bead (Type V) showed both sustained and highest release of pyrene into the medium containing 10 CMC Triton X-100 solution, which was only marginally lower than the theoretical maximum achievable pyrene concentration (~1.2 mg l^{-1}) calculated from the partition coefficient (~633) of pyrene between silicone oil and the surfactant solution. Hence, bead type V was chosen to be the most suitable among the five different bead types for further characterization and pyrene biodegradation experiments.

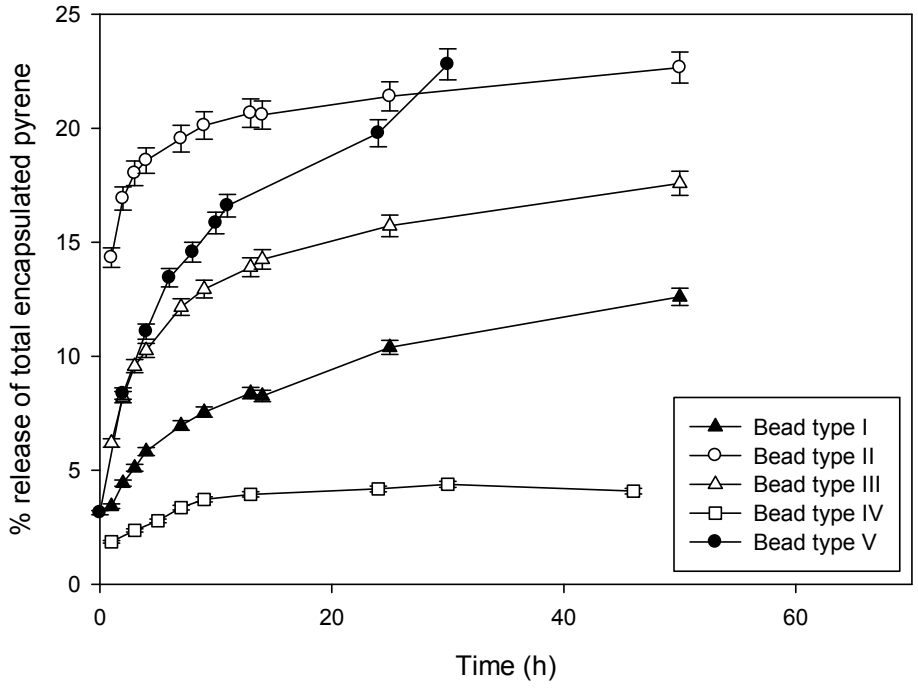


Figure 4.26: Cumulative release of pyrene as % of its total encapsulated amount from different bead types into 10 CMC Triton X 100 solutions.

4.5.2. Characterization of the bead type V

4.5.2.1. Swelling behavior

Swelling measurements were carried out to relate the observed phenomena of pyrene release with the extent of polymer hydration in the alginate-based bead type V. Figure 4.27 depicts the swelling behavior of the lyophilized beads, which indicates that the bead type V absorbed a maximum of about 85% w/w water in about 1 h. Tang *et al.* (2007) reported that oil encapsulation of hydrophobic additives (ibuprofen) inhibited equilibrium bead water uptake. Compared to the study by Tang *et al.*, equilibrium water uptake by the beads in our study was much lower owing to more hydrophobicity of silicone oil and pyrene used in the present study than sunflower oil and ibuprofen used by Tang *et al.*

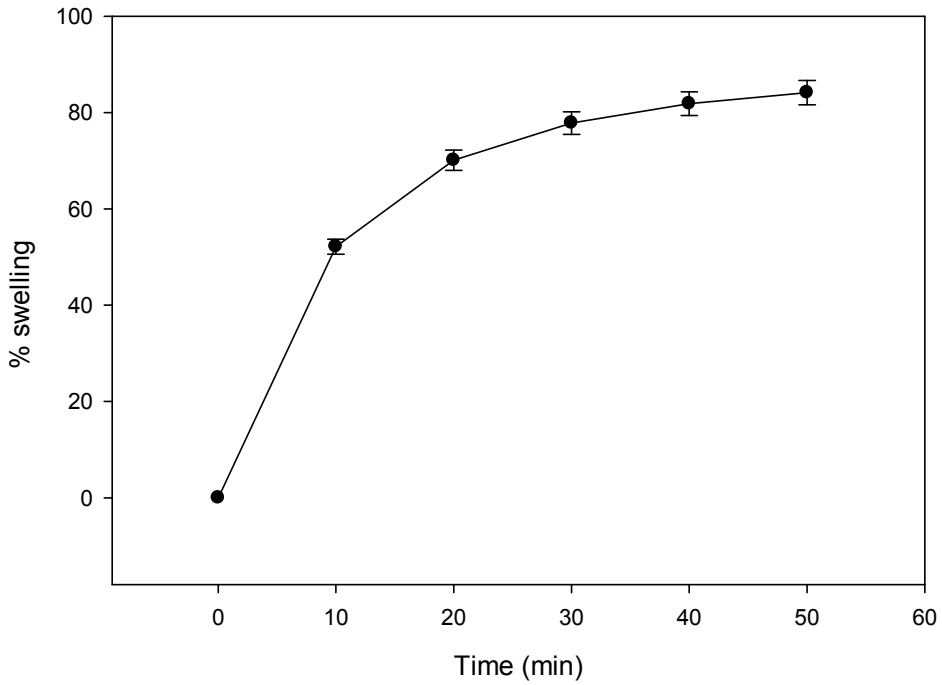


Figure 4.27: Swelling kinetics of the PVA-alginate based bead type V.

4.5.2.2. Optical and scanning electron microscopy

To reveal the internal details of the pyrene encapsulated beads, optical microscopy of the bead cross sections was performed. Figure 4.28 is an optical microscopy image of the bead cross sections revealing that emulsification of silicone oil in the PVA-alginate solution have resulted in a large number of tiny dispersed oil droplets within the bead matrices. The transparent oil droplets are also prominently observed against a blue background when treated with a colored dye solution.

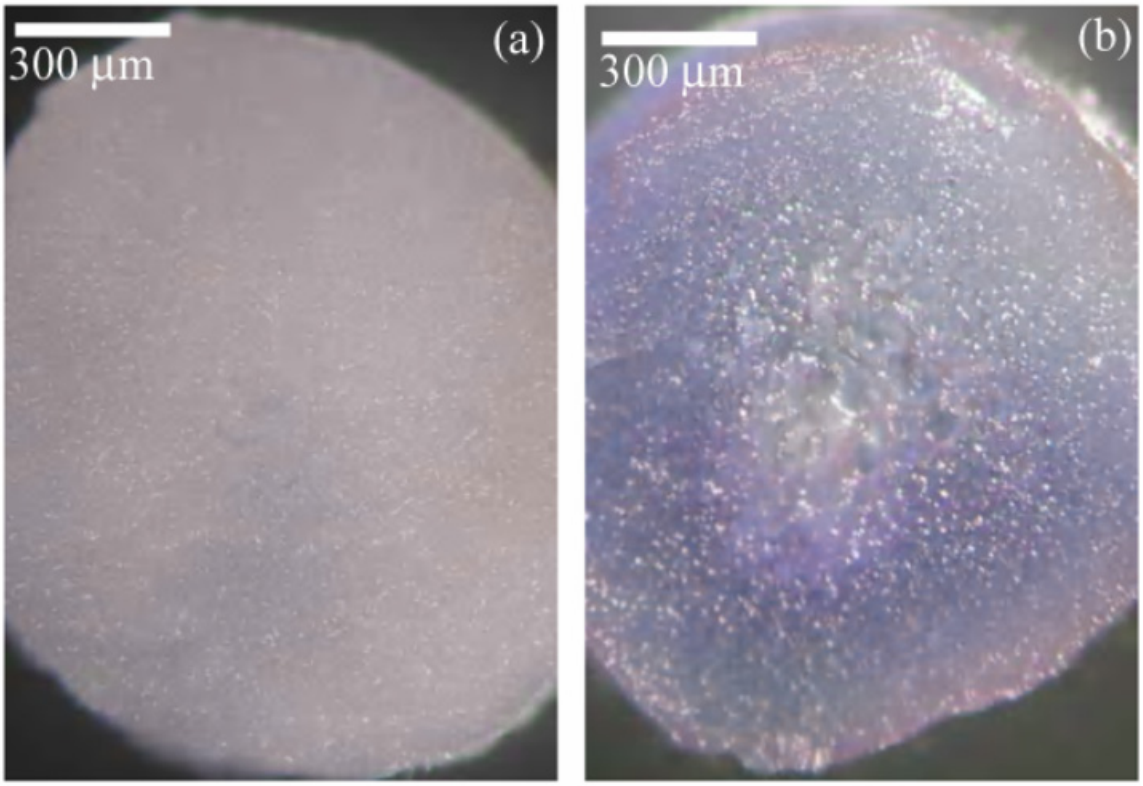


Figure 4.28: Cross sectional images of silicone oil encapsulated PVA-alginate bead: (a) untreated (b) coomassie blue treated.

SEM images of the spherical beads (~1.5 mm) shown in Figure 4.29 indicate porous internal hydrogel core and highly striated bead surfaces with an "orange peel" coat having thickness of about 100-150 µm. Similar observations have been reported by Tang *et al.* (2007) for beads prepared with alginate, sunflower oil and a drug of interest through emulsification/gelation process. Both these microscopy analyses confirm encapsulation of silicone oil containing pyrene inside the PVA-alginate bead and the nature of the beads.

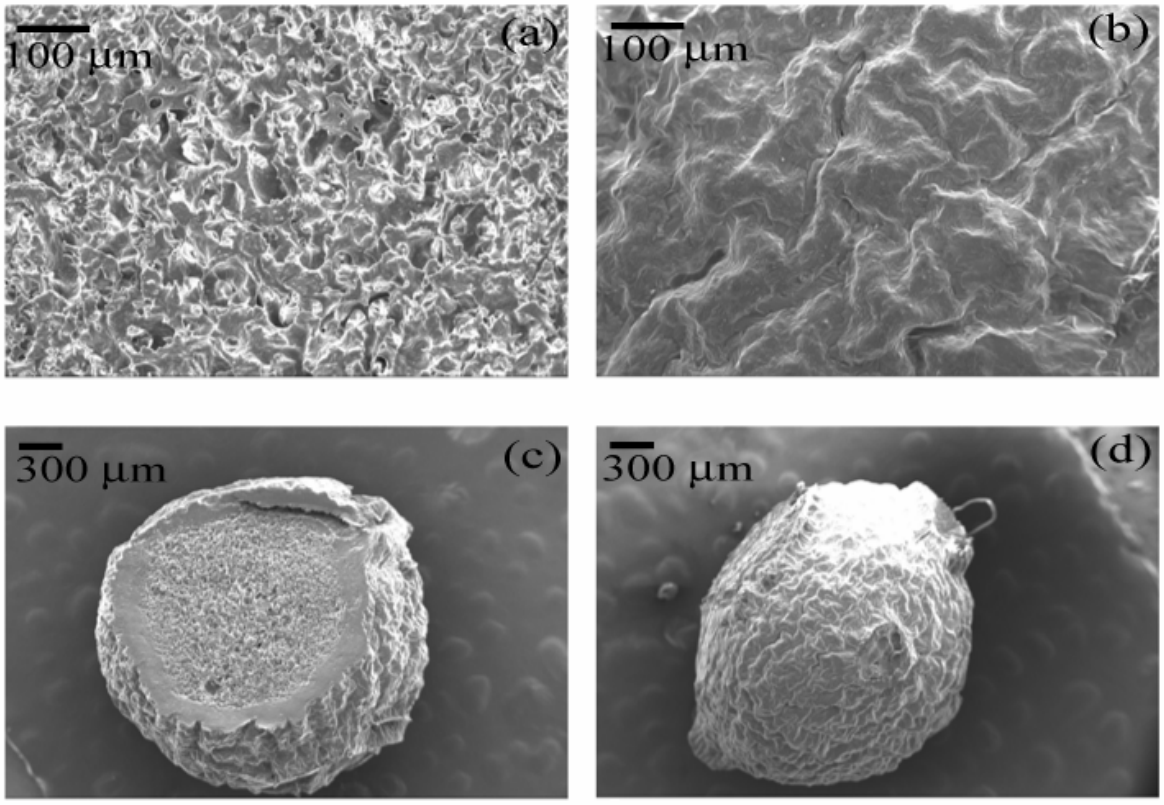


Figure 4.29: SEM images of the silicone oil encapsulated PVA-alginate bead (a) interior core (b) surface (c) cross section and (d) whole bead.

4.5.2.3. Pyrene release kinetics

To study the pyrene release kinetics, its release from encapsulated bead type V due to different surfactant concentration in the release medium was investigated. Figure

4.30 illustrates the pyrene release profile in presence of different surfactant concentrations, which clearly indicates that the rate and extent of pyrene release are dependent on surfactant concentration in the release medium. Higher surfactant concentration in the medium caused a higher extent of pyrene release, probably due to increased solubility and equilibrium saturation of pyrene. Moreover, extent of pyrene release from bead type V was comparable with the test system where free silicone oil containing equivalent amount of pyrene was simply mixed with surfactant solution. But in the latter case, the aqueous phase got immediately saturated unlike in the bead type V. This observation also demonstrates the fact that the encapsulation ensured more sustained and complete release of pyrene from the beads.

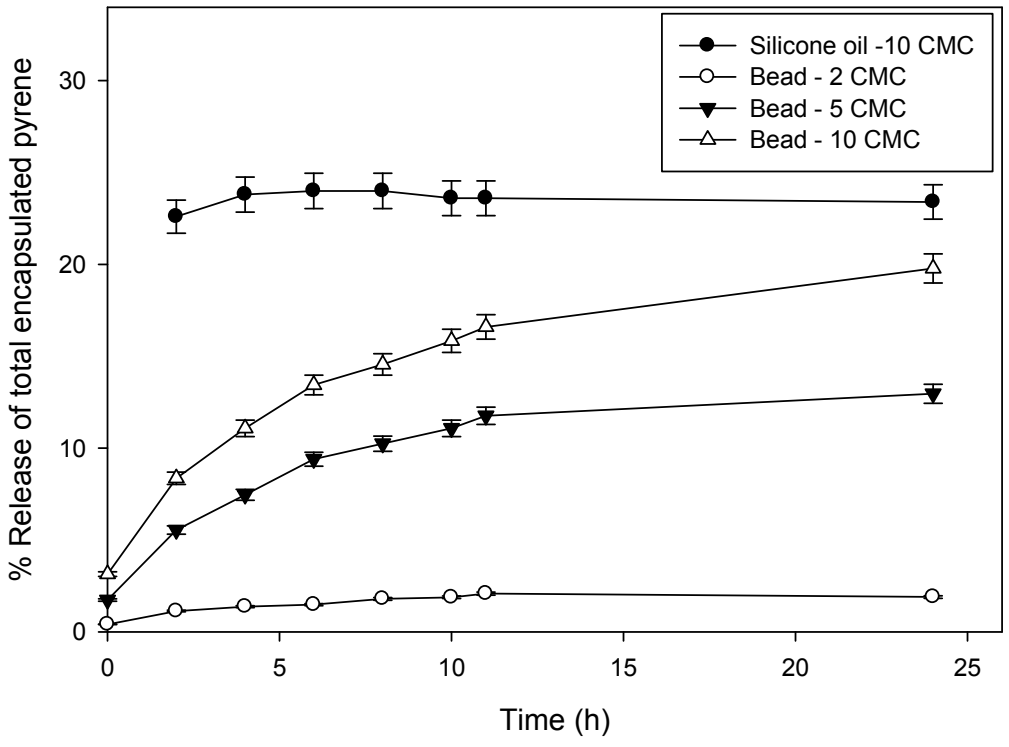


Figure 4.30: Pyrene release profile in different concentrations of Triton X 100 solution**.**

Modeling the pyrene release kinetics

In order to understand the pyrene release mechanism involved in the study and to estimate kinetic parameters affecting the pyrene release rate from the beads, pyrene release profiles (from bead type V) obtained using different concentrations of Triton X-100 were fitted into (i) a mechanistic (first order saturation kinetic) model and (ii) an empirical (Korsmeyer–Peppas,1983) exponential model. For fitting these two non-linear models to the experimental pyrene release data, Matlab® curve fitting tool was used during the model fitting exercise. A brief outline of these two models is provided below.

Considering pyrene movement from the hydrogel bead into the ambient release medium as a two step process involving (i) slow diffusion of pyrene from oil droplets into the swollen alginate matrix followed by (ii) faster diffusion into the release medium, the rate limiting step 1 can be modeled using first order saturation kinetics, described by the following equation:

$$M_t = M^* - \left[(M^* - M_0) e^{-k.t} \right] \quad \textbf{(4.10)}$$

where k is first order rate constant and M_0 and M^* are the respective initial and equilibrium saturation concentrations of pyrene (mg l^{-1}) in the surfactant containing aqueous phase; M_t is the amount of pyrene released at time t.

To understand whether the pyrene release behavior is diffusion controlled or erosion controlled; the empirical exponential model (Equation 4.11) due to Korsmeyer–Peppas (1983) can be used:

$$M_t / M_\infty = k' \cdot t^n \quad \textbf{(4.11)}$$

where k' is a constant incorporating the structural and geometric characteristics of the bead matrix, n is the empirical release exponent, indicative of the release mechanism whether it is diffusion controlled or not. M_t/M_∞ represent the fractional release of a solute normalized with respect to the equilibrium conditions.

Table 4.17 presents the results obtained by fitting these models to the experimental data, which reveals the estimated release rate constants and coefficient of determination (R^2) for the two models in predicting and explaining the observed pyrene release kinetics. It is observed from the table that the first order model was highly successful in predicting the pyrene release kinetics at all the surfactant concentrations with R^2 values greater than 0.96. Estimated equilibrium saturation concentrations of pyrene from this model (0.99, 6.52 and 9.89 mg l^{-1} at 2, 5 and 10 CMC of Triton X-100 in the release media, respectively) were proportional with the theoretical maximum pyrene solubility at these surfactant concentrations (1.41, 5.27 and 11.71 mg l^{-1} respectively).

Similar to the first order kinetics model, the empirical equation model was also highly accurate in predicting the pyrene release data with R^2 values greater than 0.94 at all surfactant concentrations in the release media. Values of the estimated release exponent 'n' (0.35±0.01) were nearly identical in all the cases strongly suggesting that the mechanism of pyrene release were consistent and independent of the surfactant concentrations in the release medium. Further, the values of the estimated release exponent, which were within 0.43, suggests that the pyrene release mechanism is diffusion controlled (Korsmeyer *et al.*, 1983).

Table 4.17: Release rate constants estimated by applying two kinetic models.

Kinetic Models	Estimated Parameter	Triton X-100 concentration in release media		
		2 CMC	5 CMC	10 CMC
$M_t / M_\infty = k' \cdot t^n$	k' (min^{-n})	0.10	0.08	0.08
	n	0.34	0.36	0.35
	R^2	**0.95**	**0.94**	**0.98**
$M_t = M^* - \left[(M^* - M_0)e^{-k.t}\right]$	k (min^{-1})	0.0042	0.0030	0.0026
	M^* (μg)	9.96	65.28	98.96
	R^2	**0.96**	**0.99**	**0.99**

4.5.3. Reusability test of bead type V

In order to study the feasibility of reusing the encapsulated beads, silicone oil containing PVA-alginate beads were soaked with pyrene saturated aqueous solution and pyrene release behaviour of such treated beads were studied after washing and drying. Pyrene release behavior of the treated beads, as shown in Figure 4.31, typically indicated an initial rapid release followed by a constant release rate that could be characterized into zero-order release kinetics. Similar biphasic release pattern from polymeric micro-particles has also been reported by Khang *et al.* (2001) and Hedberg *et al.* (2004). The extent of pyrene release from the control beads were much lower compared to the silicone oil containing beads, indicating negligible nonspecific adsorption of pyrene onto bead surface. This is also in accordance with the fact that control beads had no reservoir of silicone oil for trapping pyrene during the loading cycle.

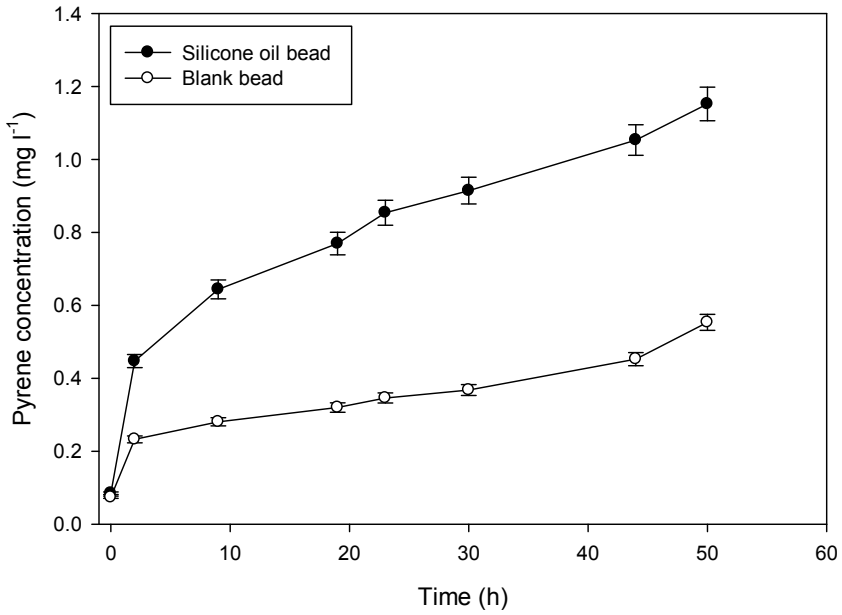


Figure 4.31: Pyrene release profile from silicon oil containing bead and control bead following initial loading of pyrene from aqueous solution.

4.5.4. Pyrene biodegradation experiments using the bead type V

The results of pyrene biodegradation using the encapsulated bead type V are presented in Figure 4.32. In case of the abiotic control, as expected, aqueous phase pyrene concentration increased initially and finally reached its saturation. However, in the flask containing *Mycobacterium*, pyrene concentration profile was different - an initial increase followed by a continuous fall. This result indicates that pyrene was degraded in the system immediately after getting released from the encapsulated beads. As pyrene concentration in the aqueous phase depends on rates at which pyrene is (i) released from the encapsulated beads and subsequently (ii) metabolized by the *Mycobacterium*, it is clear from these profiles that the biodegradation rate of pyrene was higher than its release rate from the encapsulated bead.

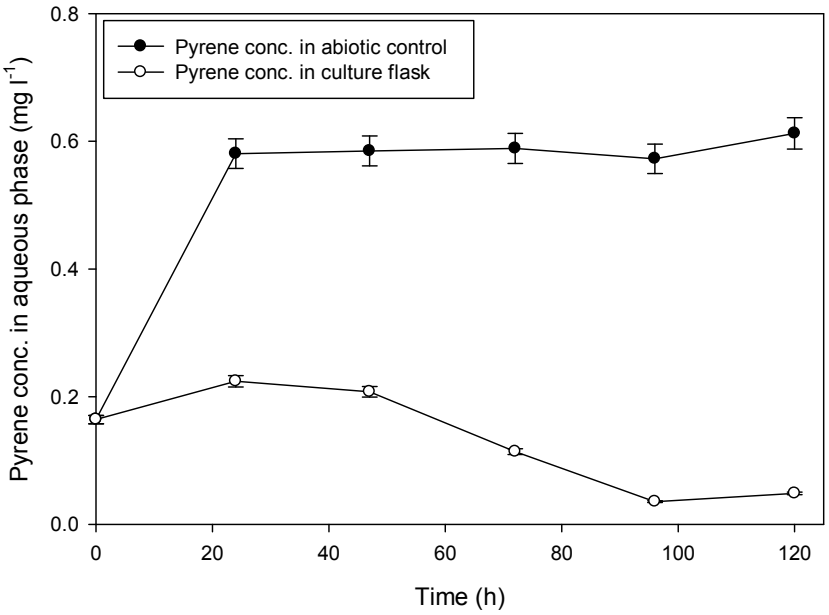


Figure 4.32: Aqueous phase pyrene concentration profiles in the biodegradation experiment using *M. frederiksbergense* with the bead type V.

Based on the aqueous phase volume and pyrene content of the beads in this present study, the initial pyrene concentration in the system could be assumed to be 1.0 mg l^{-1}, which was degraded within 4 d without any significant lag period with an estimated average rate of 0.25 mg l^{-1} d^{-1}. In our earlier surfactant (Tween 20) aided pyrene biodegradation study a degradation rate as high as of 10.22 mg l^{-1} d^{-1} was observed, but with a lag period of about of 5 d. However, the initial pyrene concentration in that study was 50 mg l^{-1}. In another study with an initial pyrene concentration of 1 mg l^{-1} with Tween 80, degradation rate of 1.3 mg l^{-1} d^{-1} was observed, but again with a lag of 12 h. Hence, it could be concluded that the present solvent encapsulated delivery system is more useful in terms of eliminating the lag period required to initiate pyrene biodegradation.

CHAPTER 5

SUMMARY AND CONCLUSIONS

- Pyrene was chosen as the model PAH compounds owing to its similar structure with most of the carcinogenic PAH compounds posing serious environmental and human health hazards.
- For degradation of pyrene, as single and mixed substrate conditions by *Mycobacterium frederiksbergense,* three different systems were evaluated.
- In the first slurry phase system, pyrene was shown successfully degraded by *M. frederiksbergense* in both shake flask and fermenter. In fermenter experiments, there was no lag in degradation; however, overall degradation rate was only 6 mg l^{-1} d^{-1}. A lag phase was prominent in shake flask and pyrene was incompletely degraded with a degradation rate of 19.86 mg l^{-1} d^{-1} during the active degradation period
- In presence of naphthalene or anthracene, slurry phase biodegradation in shake flasks showed that none of the main or interaction effects were important for pyrene degradation by the *Mycobacterium*. However, effects of anthracene or pyrene was significant (~90% confidence level) on naphthalene degradation. On the contrary, an increase in anthracene concentration was itself inhibitory toward its own degradation in the mixture.
- Five synthetic surfactants (1-cationic, 1-anionic and 3-nonionic) were evaluated of their possible use in surfactant aided system for biodegradation of pyrene. MSR values for pyrene estimated in the study were in the order Tween 80 > Tween 20 >

Triton X 100 > CTAB > SDS. For pyrene biodegradation employing this system, Tween 80 was found best when compared with the others, where maximum pyrene degradation was achieved (about 90% degradation within 6 d) with more or less constant rate. When the Tween 80 aided system was further evaluated at different pyrene concentrations (0.1-1 mg l^{-1}), a maximum degradation rate of 0.056 mg l^{-1} h^{-1} was observed at 1 mg l^{-1} initial concentration. In fermenter experiment involving Tween 80 and different concentrations of pyrene (1-50 mg l^{-1}), a maximum degradation rate of 0.737 mg l^{-1} h^{-1} was observed at 25 mg l^{-1} initial concentration. And about 88% pyrene was degraded within 96 h in these experiments

- . An indigenous microbial culture, isolated from a contaminated site, capable of producing a biosurfactant was evaluated of its potential to degrade pyrene. The biosurfactant showed good solubility enhancement of pyrene and anthracene, good emulsification activity and stability towards a range of organic solvent under different environmental conditions of pH and temperature. However, the culture failed to degrade pyrene even to the least extent.
- In presence of naphthalene or anthracene, Tween 80 aided pyrene biodegradation in shake flask showed that pyrene degradation efficiency varied between 30 and 94% in the experimental runs conducted as per the 2^3 full factorial design of experiment. Besides pyrene, anthracene was found to be degraded efficiently in this mixed substrate conditions.
- For developing a TPPB system for pyrene biodegradation by *M. frederiksbergense*, silicone oil was chosen as a suitable biocompatible solvent in solvent screening,

among three candidate solvents (IPM, silicone oil and hexadecane) by a screening procedure.

- In hydrodynamic study volumetric oxygen mass transfer coefficients in the two liquid phase system were determined at different silicone oil fraction, agitation and aeration rate in the fermenter. Based on enhancement in volumetric oxygen mass transfer coefficients due to added organic phase in the two phase system, the best sets of operating conditions viz. silicone oil fraction, agitation and aeration rates were determined for further pyrene biodegradation experiments in the two liquid phase system.
- Complete biodegradation of pyrene was achieved by *Mycobacterium frederiksbergense* in the developed TPPB system with utilization rates of 270, 230, 139, 82 mg l^{-1} d^{-1} for initial pyrene loading concentrations of 1000, 600, 400 and 200 mg l^{-1}, respectively. From the experimental findings, uptake of pyrene by the microorganism in TPPB was proposed to be a non-interfacial based mechanism.
- A simple cost-benefit analysis of the above three systems investigated in this work for pyrene biodegradation by *M. frederiksbergense* revealed the TPPB system to be the best, however, with some recommendation for further reduction in its cost by reducing power requirements for dispersing the two phase in the system.
- A non-conventional TPPB system based on pyrene-in-solvent encapsulated beads for sustained and controlled delivery for biodegradation of pyrene by the *Mycobacterium* was evaluated. In the study, standardization of bead type preparation based on pyrene release kinetics, characterisation of the suitable bead types and pyrene biodegradation experiments were successfully performed.

Scope for Future Work

1) Evaluation of TPPB system using *Mycobacterium frederiksbergense* for bioremediation of soil contaminated with mixture of PAHs including pyrene.
2) Development and evaluation of other non-conventional TPPB systems for pyrene biodegradation by *M. frederiksbergense*.
3) Genetic engineering of *M. frederiksbergense* to overcome solvent toxicity and biocompatibility problems for successful use in TPPB system.
4) Mechanistic modeling of pyrene biodegradation and mass transfer process involved in TPPB or modified TPPB systems.

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APPENDIX

Scientific Classification of *Mycobacterium frederiksbergense* (NRRL B-24126).

Kingdom	:	Bacteria
Phylum	:	*Actinobacteria*
Order	:	*Actinomycetales*
Suborder	:	*Corynebacterineae*
Family	:	*Mycobacteriaceae*
Genus	:	*Mycobacterium*
Species	:	*M. frederiksbergense*

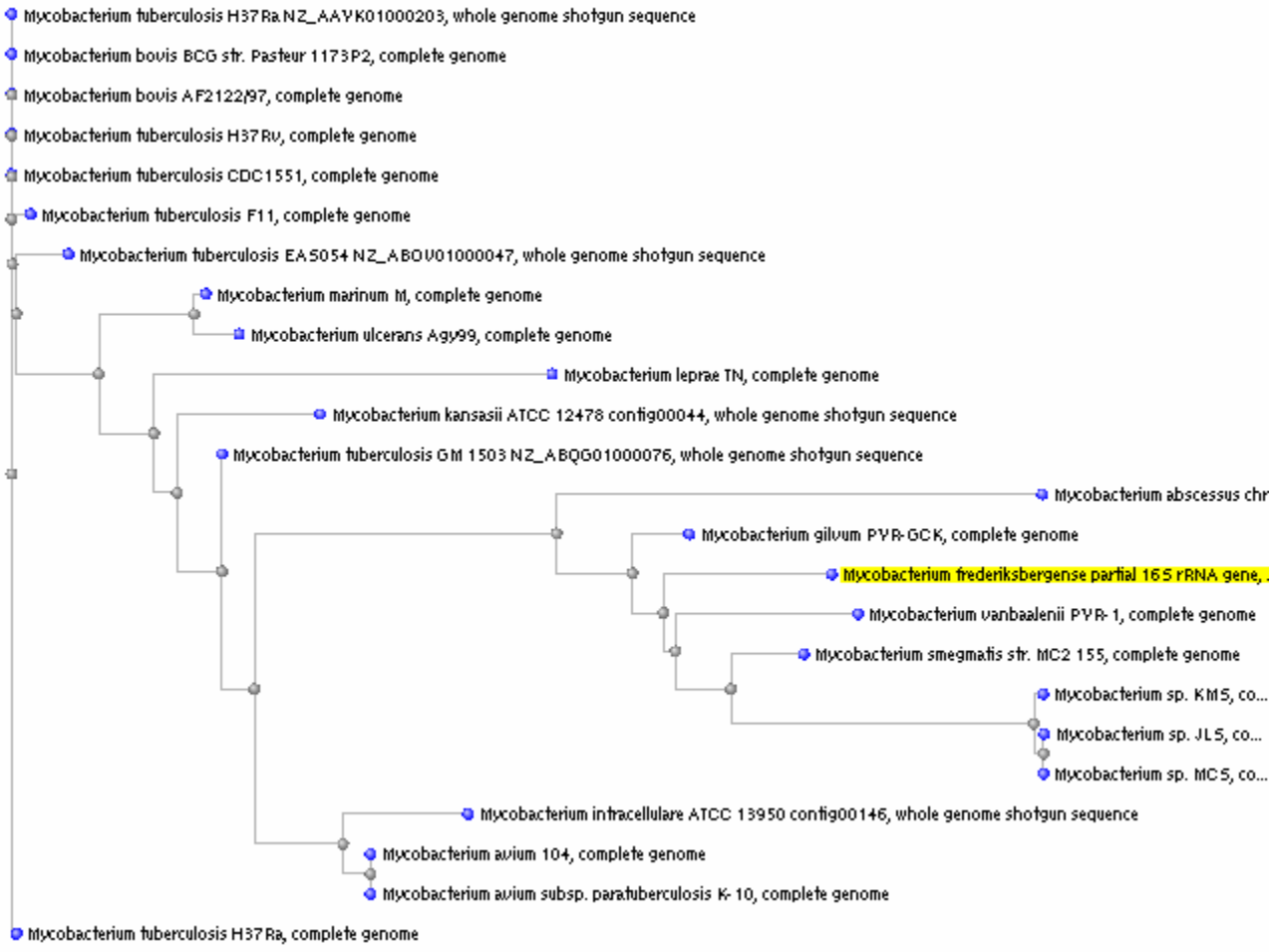
Physiological Description of *M. frederiksbergense*.

Gram stain	:	+
Acid-alcohol-fast	:	+
Motility	:	-
Spore formation	:	-
Shape	:	Rod
Colony morphology	:	Smooth
Growth Temperature	:	15-37°C
Cell surface hydrophobicity	:	+
Catalase	:	+
Nitrate reductase	:	+
Urease	:	-
Mineralization of PAHs as sole carbon	:	Phenanthrene, Fluoranthene, Pyrene
Unable to mineralize as sole carbon	:	Anthracene, Fluorene, Benzo[a]pyrene

Current sequence level information available in NCBI database.

Core subset of nucleotide sequence record	**:**	**19**
SmpB (smpB) gene	**:**	01
Elongation factor Tu (tuf) gene	**:**	01
tmRNA (ssrA) gene	**:**	01
65 kDa heat shock protein (hsp65) gene	**:**	01
65 kDa heat shock protein (hsp65) gene (strain OA128Y)	**:**	01
Superoxide dismutase (sod) gene	**:**	01
RNA polymerase (rpoB) gene	**:**	01
RNA polymerase (rpoB) gene (strain OA128Y)	**:**	01
Dioxygenase small beta subunit (nidB) gene	**:**	01
Dioxygenase large alpha subunit (nidA) gene	**:**	01
16S ribosomal RNA gene (isolate VM0458)	**:**	05
16S ribosomal RNA gene (clone K3840/4)	:	02
16S ribosomal RNA gene	**:**	02
Protein sequence data base entry	**:**	**09**
Dioxygenase small beta subunit	**:**	01
Dioxygenase large alpha subunit	**:**	01
SmpB	**:**	01
Elongation factor Tu	**:**	01
RNA polymerase	**:**	02
65 kDa heat shock protein	**:**	02
Superoxide dismutase	**:**	01

Distance tree view of *M. frederiksbergense*, 16S rRNA gene against 15 other *Mycobacterium* genome sequences available in NCBI (as on 6th January 2009).



LIST OF PUBLICATIONS

Published/Accepted in Referred International Journals

1. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2006) Production and properties of a biosurfactant applied to polycyclic aromatic hydrocarbon solubilization, *Applied Biochemistry and Biotechnology*, 134:129-141.

2. **Mahanty**, **B.,** Sarma, S.J. and Pakshirajan, K. (2007) Evaluation of different surfactants for use in pyrene biodegradation by *Mycobacterium frederiksbergense*, *International journal of Chemical Sciences*, 5:1505-1512.

3. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2008) Biodegradation of pyrene by *Mycobacterium frederiksbergense* in a two-phase partitioning bioreactor system, *Bioresource Technology*, 99:2694-2698.

4. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2008) Synchronous fluorescence as a selective method for monitoring pyrene in biodegradation studies, *Polycyclic Aromatic Compounds*, 28:213-227.

5. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2009) Pyrene encapsulated alginate bead type for sustained release in biodegradation: preparation and characteristics. *Polycyclic Aromatic Compounds*. 29:56-73.

6. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2009) Batch biodegradation of PAHs in mixture by *Mycobacterium frederiksbergense*: analysis of main and interaction effects, *Clean Technology and Environmental Policy*, (*Article in press*)

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1. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2009) Two liquid phase partitioning bioreactor system for biodegradation of pyrene: comparative evaluation and cost benefit analysis, *Journal of Chemical Technology & Biotechnology*, (*Under review*).

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1. **Mahanty**, **B.,** Dasu, V.V. and Pakshirajan, K. (2005) Production and properties of a biosurfactant from pyrene utilizing microbial culture, *46th Annual conference of 'Association of Microbiologist of India' December 8-10, 2005, Hyderabad, India, pp 21*.

2. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2006) Effect of glucose and triton X-100 on pyrene biodegradation by *Mycobacterium frederiksbergense* and *Mycobacterium vanbaalenii*: Plackett-Burman design, *59th Annual session of Chemical Engineering Congress 'CHEMCON 2006' December 27-30, 2006, Gujarat, India, pp 10.*

3. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2007) Effects of naphthalene, indole and biphenyl on pyrene degradation by *Mycobacterium vanbaalenii* and *Mycobacterium frederiksbergense*: A fractional factorial design approach, *International Conference on New Horizons in Biotechnology, November 26-29, 2007, Trivandrum, India.*

4. **Mahanty**, **B.,** Pakshirajan, K. and Dasu, V.V. (2008) Biodegradation of ternary PACs mixture by mycobacterium frederiksbergense, *ChEmference-2008, July 5-6, 2008, Kanpur, India.*