

The effect of soil pH on degradation of polycyclic aromatic hydrocarbons (PAHs)

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Abstract

The environmental fate of polycyclic aromatic hydrocarbons (PAH) is a significant issue, raising interest in bioremediation. However, the physio-chemical characteristics of PAHs and the physical, chemical, and biological properties of soils can drastically influence in the degradation. Moreover, PAHs are toxic and carcinogenic for humans and their rapid degradation is of great importance. The process of degradation of pollutants can be enhanced by manipulating abiotic factors.

The effect of soil pH on degradation of PAHs with a view to manipulating soil pH to enhance the bioremediation of PAH's was studied. The degradation rate of key model PAHs (Phenanthrene, Anthracene, Fluoranthene, and Pyrene) was monitored in J Arthur Brower's topsoil modified to a range of pH between pH 4.0 and pH 9.0 at half pH intervals. Photo-catalytic oxidation of PAHs in the presence of a catalyst (TiO₂) under UV light at two different wavelengths was studied. The degradation of PAHs during photo-catalytic oxidation was carried out at varying soil pH, whilst the degradation rate of each individual PAH was monitored using HPLC. It was observed that pH 6.5 was most suitable for the photo-degradation of all the PAHs, whilst in general acidic soil had greater photo-degradation rates than alkaline soil pH. Photo-degradation of PAHs at 375 nm exhibited greater degradation rates compared to 254 nm. Phenanthrene at both the wavelengths had greater degradation rate and pyrene has lower degradation rate of the four PAHs.

Pure microbial cultures were isolated from road-side soil by shaken enrichment culture and characterized for their ability to grow on PAHs. Bacterial PAH degraders, isolated via enrichment were identified biochemically and by molecular techniques using PCR amplification and sequencing of 16S rDNA. Sequences were analyzed using BLAST (NCBI) and their percentage identity to known bacterial rDNA sequences in the GeneBank database (NCBI) was compared. The 6 bacterial strains were identified as *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp. Similarly, fungal PAH degraders were identified microscopically and with molecular techniques using PCR amplification and sequencing of 18S rDNA and identified as *Aspergillus niger* and *Penicillium freii*.

Biodegradation of four PAHs with two and four aromatic rings were studied in soil with inoculation of the six identified bacteria and two identified fungi over a range of pH. It was observed that pH 7.5 was most suitable for the degradation of all the PAHs maintained in the dark. A degradation of 50% was observed in soil pH 7.5 within first three days which was a seventh of the time taken at pH 5.0 and pH 6.5 (21 days). Greater fungal populations were found at acidic soil pH and alkaline soil pH, in comparison with neutral pH 7.0. *Penicillium* sp. was found to be more prevalent at acidic pH whilst *Aspergillus* sp. was found to be more prevalent at pH 7.5-8.0. Bacterial populations were greater at pH 7.5 which was highly correlated with soil ATP levels. It was therefore evident that the greatest rates of degradation were associated with the greatest bacterial population. Soil enzyme activities in general were also greatest at pH 7.5.

The converse effect of pH was found with fastest rate of photo-catalytic degradation at the optimal conditions were observed at acidic condition in soil pH 6.5 whilst, the results obtained during biodegradation at the optimal conditions exhibits fastest rate of degradation at alkaline conditions particularly at pH 7.5. Thus, manipulation of soil pH to 7.5 has significant potential to dramatically increase the degradation rate of PAHs.

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Declaration

This dissertation describes research conducted in School of Life Sciences, and School of Pharmacy, University of Hertfordshire between January 2009 and January 2012 under supervision of Dr. David Naseby and Dr. Avic Hall. I certify that the research described is original and that any parts of the work that have been conducted by collaboration are clearly indicated. I also certify that I have written all the text herein and have clearly indicated by suitable citation any part of this dissertation that has already appeared in publication.

Signature:

Date:

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Abbreviations

ABTS: 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
ATP: Adenosine 5'triphosphate
ANT: Anthracene
bp: basepair
CFU: Colony Forming Unit
CHR: Chrysene
COD: Chemical oxidation process
DMAB: 3-Dimethylaminobenzoic acid
DNA: deoxyribonucleic acid
EB: elution buffer
Fig: figure
FLT: Fluoranthene
HMW: High molecular weight PAH
HPLC: High performance liquid chromatography
HSP: high scoring pair
kb: kilobase(s)
K_m: Michaelis constant
LAC: laccase oxidation
LiP: lignin peroxidase
LMW PAH: Low molecular weight PAH
LSD: least significant data difference
MEA: Malt Extract Agar
MBTH: 3-methyl-2-benzothiazolionone hydrazone hydrochloride
MnP: Mn-dependent peroxidase
mRNA: messenger RNA
min: minutes
NOM: Natural Organic matter
OP: Oxidation process
OD: optical density
O-F: oxidation- fermentation test
PAH: Polycyclic aromatic hydrocarbons
PHE: Phenanthrene
PCR: polymerase chain reaction
PYR: Pyrene
RNA: ribonucleic acid
SOM: Soil Organic matter
SDS: sodium dodecyl sulphate
SDW: sterile distilled water
TiO₂: Titanium oxide
V_{max}: Enzyme maximum rate
WRF: white rot fungi
WHC: water holding capacity

Chapter: 1.0

General Introduction

1.0 Introduction

1.1 General Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a large and diverse group of organic compounds. They are stable aromatic hydrocarbon molecules with two or more fused benzene and/or pentacyclic rings in linear, angular or cluster formation (Muckian *et al.*, 2007). PAHs are formed due to anthropogenic pyrolytic processes and also due to natural events such as volcanic eruptions and bush fires. Moreover, they are major constituents of crude oil thus, PAHs are distributed ubiquitously in the environment (LaFlamme & Hites, 1978). PAHs produced in the environment are transported over long distances in air before they settle in the atmosphere, on soil surfaces, vegetation, sea and inland waters (Van Jaarsveld *et al.*, 1997).

In recent years, various industrial and/or agricultural processes have been releasing pollutant compounds into the environment (Gianfreda *et al.*, 2004). These pollutants have negative effects on environmental quality and human health. However, their carving on environment is due to the rapid industrial enterprise of agribusiness, expanded chemical industry and the need to generate cheap forms of energy (Borazjani *et al.*, 1997, Borazjani *et al.*, 2000 & Vidali, 2001). The removal of polycyclic aromatic hydrocarbons (PAHs) from the environment has attracted considerable attention owing to their widespread environmental distribution, toxicity, and carcinogenicity (Kou *et al.*, 2009). Microbial degradation is used as a primary method for the elimination of PAHs from the environment. Although many microorganisms are able to utilize these organic compounds as a substrate and energy source for their growth, these compounds, especially high molecular weight PAHs, are considered as

recalcitrant molecules due to their low solubility in water. Moreover, they are suspected to be carcinogenic and therefore their rapid remediation is required (Urgun-Demirtas *et al.*, 2006).

In recent years, photo-catalysis is one of the most attractive methods for PAHs degradation with respect to solar energy utilization (Kou *et al.*, 2009). Due to the utilization of clean solar energy and no use of any additional chemicals, heterogeneous photo-catalytic processes have gained much interest. To date, most of the studies are dominated by Titanium oxide (TiO₂) as a catalyst because of its stability and relatively high activity.

Most of the evident environmental factors, which vary from site to site (such as bioavailability of the contaminants, soil pH and nutrient availability), can influence the process of bioremediation by inhibiting growth and activity of the pollutant-degrading microorganisms (Bamforth & Singleton, 2005a). Multidisciplinary use of soil, water and sediments leads towards cost effective and efficient remediation techniques. Bioremediation is considered to be one of the important tools for the treatment of contaminants from the environment (Kuiper *et al.*, 2004). Furthermore, degradation of PAHs in contaminated soils are highly influenced by various environmental factors such as pH, water availability, oxygen level, salinity, temperature, PAH bioavailability, nutrient requirements of microbes and adaptation of the microorganism's population (Balba *et al.*, 1991).

Among all the environmental abiotic factors, studies have rarely focused on soil pH and degradation. Soil pH, an abiotic factor, plays an important role in degradation of PAHs. Most PAH-contaminated sites are not at the optimal pH for bioremediation. As pollutants are directly linked to the pH of contaminated sites, the transformation of PAHs under acidic or basic conditions cannot be carried out by indigenous

microorganisms. The *in-situ* microorganisms at a contaminated site may be not only tolerant of the site conditions, but may also have the potential to metabolise PAHs in sub-optimal conditions. These facts constitute the necessity for studying the soil pH as important factor (Leahy & Colwell, 1990). The efficiency of bioremediation process depends on the environmental conditions and is generally low (Yan *et al.*, 1998). Considering these conditions in this study, effect of soil pH as an abiotic factor was investigated to monitor the environmental conditions suitable for the promotion of bioremediation. Also, compared to other methods of degradation, soil pH adjustment is an easy process by liming and is a cost effective approach.

Aims and Objectives

Aims and objectives

Aim: The effect of soil pH on degradation of polycyclic aromatic hydrocarbons (PAHs)

Photo-catalytic oxidation

Aim: To study the effect of soil pH on photo-catalytic degradation of polycyclic aromatic hydrocarbons (PAHs).

Objectives:

- To monitor PAH photo-catalytic degradation rate in experimental soil at varying pH.
- To determine usefulness of heterogeneous photo-catalytic process at two wavelengths using TiO₂ for the degradation of PAHs present in soil.

Enrichment isolation and characterization

Aim: Isolation, characterization of PAH degrading bacteria and fungi- and identification by PCR amplification by 16S rDNA for bacteria and 18S rDNA for fungi

Objectives:

- Isolation of PAH degrading organisms via shaken enrichment culture
- Identification of isolated bacterial strains degrading PAHs – using PCR amplification and sequencing of 16S rDNA
- Identification of isolated fungal strains degrading PAHs – using PCR amplification and sequencing of 18S rDNA

- **Biodegradation**

Aim: To study the effect of soil pH on biodegradation of polycyclic aromatic hydrocarbons (PAHs).

Objectives:

- To monitor PAH biodegradation rate using HPLC analysis in experimental soil at varying pH.
- Quantify the bacterial and fungal populations and identification of predominant isolates from varying soil pH.
- To study microbial activity by soil ATP measurement at different pHs in the presence of PAHs.
- To determine soil enzyme activity using buffer pH (5.5, 7 and 8.5) for β -glucosidase, L-arginine ammonification, acid/alkaline phosphatase (C: N: P) source, manganese dependent peroxidase (MnP), lignin peroxidase (LiP), laccase activity at varying soil pH

Intra/extra cellular enzyme studies

Aim: To study the ligninolytic enzymes from fungi and dioxygenase enzyme from bacteria involved in PAH degradation and their kinetics studies

Objectives:

- Purification and characterisation of ligninolytic and dioxygenase intra/extra cellular enzymes involved in PAH degradation
- Optimization of cultural conditions with respect to pH and temperature for ligninolytic and dioxygenase enzyme production involved in PAH degradation
- Biochemical characterization of ligninolytic and dioxygenase enzyme

- To investigate relative activity of the enzymes at varying pH and temperature
- To study enzyme kinetics using Michaelis–Menten kinetics equation and calculate V_{\max} and K_m by adding a reciprocal Lineweaver-Burk plot.

Chapter: 2.0

Literature Review

2.0 Literature review

Removal of pollutants from the environment can be carried out through remediation processes using one or more of the following: microbial degradation, photo catalytic oxidation, chemical oxidation, volatilization and sedimentation (Gao *et al.*, 1998). It has been observed that industrial and agricultural processes release various polluting compounds in the environment (Gainfreda *et al.*, 2005). These polluting compounds are responsible for negative effects on environmental quality and human health. It is therefore important to control the release of pollutants and understand their fate and effects once they enter the soil. Biodegradation is an inexpensive and an effective approach to degrade and remove pollutants from contaminated soils. In biodegradation, organisms are used to break down and thereby detoxify dangerous chemicals in the environment. As the microbial community structure has been suggested to be important in the decomposition of pollutants (Beulke *et al.*, 2005), there are different methodologies to degrade these pollutants from the environment.

The chemical pollutants can be classified into inorganic and organic pollutants as described below:

2.1 Inorganic pollutants

Inorganic chemical pollutants in the environment are found naturally. However, due to human activities they may be more concentrated and released into environment. The inorganic pollutants of primary concern are heavy metals such as cadmium, copper, mercury, lead, zinc etc and nutrient pollutants such as nitrogen, phosphates, sulphates (Sandrin & Maier, 2003).

2.2 Organic pollutants

In recent years, many organic compounds are used in day to day life. These organic compounds are produced for different uses such as pesticides, plasticizers, lubricants, refrigerant, fuels, solvents and preservatives (Liu, 2010). Some of these organic compounds are biologically harmful even in very small concentrations but some are relatively inert and harmless. Some of these pollutants that enter into soil may inhibit or kill soil organisms, thereby perturbing the balance of the soil community. However, some may also be transported from the soil to air, water or vegetation where they may come into physical contact, could be inhaled or ingested by number of organisms (Beulke *et al.*, 2005).

The key types of organic pollutants are:

Aliphatic hydrocarbons

Alicyclic hydrocarbons

Aromatic hydrocarbons

2.2.1 Aliphatic hydrocarbons

The Aliphatic hydrocarbon group consists of alkanes, alkenes, and alkynes. The alkanes saturated hydrocarbons (i.e., methane) are fairly inert and generally inactive in atmospheric photochemical reactions (Leahy & Colwell, 1990).

2.2.1a Saturated hydrocarbons: If all the hydrogen atoms attached to carbon bonds are together in a chain, molecule is said to be saturated. For example: $\text{H}_3\text{C}-\text{CH}_3$ (ethane) (Krafft & Crooks, 1988).

2.2.1b Unsaturated hydrocarbons: If two adjacent carbon atoms each lose a hydrogen atom, a double bond forms between them. Such a molecule is said to be unsaturated (Krafft & Crooks, 1988). For example: $\text{H}_2\text{C}=\text{CH}_2$ (ethylene, ethene).

Aliphatic and aromatic hydrocarbons are differentiated as they provide a useful method for categorizing these compounds. For example, although anaerobic biodegradation of aromatic hydrocarbons has been reported, it is uncommon and relatively slow compared to aerobic biodegradation (Leahy & Colwell, 1990). Andrews & Novak (2001) studied the pH effect and the ferrous ion effect on carbon tetrachloride degradation by *Methanosarcina thermophila*. Carbon tetrachloride is considered to be simple unsaturated aliphatic hydrocarbon. Their studies suggest that unsaturated aliphatic hydrocarbons are degraded easier and faster as the pH increases. However, saturated aliphatic hydrocarbons degrade slowly with increase in pH (Weathers *et al.*, 1997).

2.2.2 Alicyclic hydrocarbons

Alicyclic hydrocarbons are made up as cyclic saturated carbon chains. Most common alicyclic hydrocarbons occur naturally. For example, alicyclic hydrocarbons are a major component of crude oil, comprising 20 to 67% by volume. Examples of complex, naturally occurring alicyclic hydrocarbons include camphor, which is plant oil; cyclohexyl fatty acids, which are components of microbial lipids. Rios-Hernandez *et al.*, (2003) studied “Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer.” Ethylcyclopentane (ECP) which is an alicyclic hydrocarbon was used in the research conducted to study its metabolism by sulfate-reducing bacterial enrichment. Moreover, the research suggests that (ECP)-alicyclic hydrocarbons are anaerobically activated by addition of fumarate. Alkylsuccinate derivatives are obtained as by-products under sulfate-reducing conditions.

2.2.3 Aromatic hydrocarbons

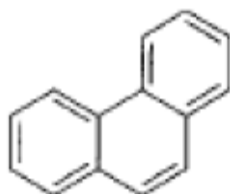
Aromatic hydrocarbons are found in petroleum components and its refined products. Naturally occurring aromatic hydrocarbons consists of benzene and substituted derivatives of benzene. Benzene is an aromatic compound exhibiting similar chemical behaviour and is one of the simplest forms of petrochemicals (Krafft & Crooks, 1988). Naphthalene is considered as one of the simplest representative of polycyclic aromatic hydrocarbons and benzene, toluene and ethyl benzene are among the other important aromatic petroleum hydrocarbons (Wrenn *et al.*, 1998).

Benzene exhibits important properties and is a naturally occurring of aromatic hydrocarbons. The elemental composition of benzene is organic compound with molecular composition of C_6H_6 with six-member ring and with three carbon-carbon double bonds. Also, due to delocalised nature of bonding, benzene is represented with ring inside hexagonal arrangements of carbon atoms (Wilson & Jones, 1993). It is structurally similar to cyclic alkenes and is cyclic in nature. It is colourless and highly flammable. Benzene is considered as an aromatic hydrocarbon and is naturally occurring constituent of crude oil. It is, however, unusually stable and does not readily participate in reactions that are characteristic of alkenes (Wrenn *et al.*, 1998). Many chemical compounds are originated from benzene by substituting one or more of its hydrogen atoms with some other functional group. The resulting effect on the reactivity of these molecules that distinguishes aromatic hydrocarbons from unsaturated aliphatic hydrocarbons depends on the relative stability of benzene and its derivatives (Rios-Hernandez *et al.*, 2003).

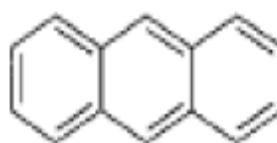
2.3 PAHs

Polycyclic aromatic hydrocarbons (PAHs) are a class of stable organic molecules which consist of hydrogen and carbon molecules. As PAHs are commonly found widespread contaminants, these are of environmental concern (Uyttebroek *et al.*, 2007). The structure of PAHs compounds appears flat and consists of carbon and hydrogen atoms (Cutright, 2006). However, other atoms like sulphur, nitrogen and oxygen get readily substituted in the benzene ring and get converted to heterocyclic aromatic compounds. These heterocyclic aromatic compounds are commonly grouped together with PAHs (Wilson & Jones, 1993). Also, unsubstituted PAHs are non-polar, neutral and hydrophobic compounds that are randomly scattered during energy conversion and industries dealing with petroleum (Juhasz *et al.*, 2000).

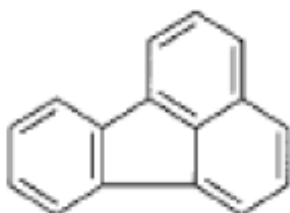
PAHs possess different chemical and physical properties due to their chemical structure. The examples of PAHs structures are shown in figure 1 (Johnsen *et al.*, 2005).



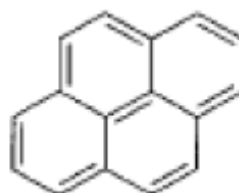
Phenanthrene



Anthracene



Fluoranthene



Pyrene

Fig. 1: Structure of representative PAHs

The PAH molecules exhibit biochemical persistence due to the dense cloud of π electrons on both sides of ring structures. Hence, PAHs compounds are more resistant to nucleophilic attack (Haritash & Kaushik, 2009).

PAHs compounds exhibit various noxious and hazardous properties. Thus, PAH compounds are toxic, potential mutagens and carcinogens that result in the fate of PAHs in the environment contributing towards environmental concerns (Martens, 1995).

PAHs in the environment are found commonly through two sources.

Natural sources

Artificial (anthropogenic) sources

Natural sources include forest and rangeland fires, oil seeps, and volcanic eruptions. Anthropogenic sources include exudates from coal tar, wood, garbage, refuse, and used lubricating oil and oil filters discharge. Soil, air and water act as the ultimate depository of PAHs. PAHs are found to be major pollutants of air and soil (Haritash & Kaushik, 2009).

PAHs are formed due to thermal decomposition of various organic molecules in the environment. PAHs are produced as a result of incomplete combustion at high temperature (500-800⁰C) or when subjected to low temperature (100⁰C- 300⁰C) for long durations. PAHs appear to be colourless in nature with white/pale yellow solids. They have low solubility in water with low vapour pressure and with high melting and boiling points (Johnsen *et al.*, 2005).

2.4 Formation of PAHs

PAHs are formed due to incomplete combustion of organic compounds (Keith & Telliard, 1979). Different types of combustion, such as domestic, industrial and

agricultural, contribute to their emissions. Waste combustion is (due to the heterogeneous mix) a potential source for different types of PAHs. The chemical and physical formation of PAHs is very complex and many decades of scientific study have been dedicated to this. The different compounds involved during formation of PAHs include:

- Unburned fuel and lubricating oil
- Fuel that has undergone pyrolysis but has not passed through a flame zone
- PAHs produced by combustion in flame (Longwell, 2007).

Hydrophobicity and stability of PAHs results in recalcitrance of high molecular weight (HMW) PAHs in the environment (Kanaly & Harayama, 2000). Thus, presence of dense clouds of π electrons on each side of aromatic rings restricts the PAHs to limited nucleophilic attack leading to biochemical stability (Johnsen *et al.*, 2005). Solubility in water or polar solvents is prevented by the hydrophobic nature of PAHs and results in less bioavailability (Boocham *et al.*, 2000). However, to enhance the biodegradation process it is necessary to increase the bioavailability of PAHs and therefore, solubility in water and polar solvents have to be increased (Field *et al.*, 1995).

2.5 Sources of PAHs

Industrial and daily human activities such as coal processing, wood, crude oil and natural gas combustion for heating, vehicles, cooking and smoking, or even natural processes such as carbonization are responsible for the incomplete combustion of organic materials. This incomplete combustion of organic material further augments or produces PAHs through pyrolytic processes (Hati *et al.*, 2009). Among the several PAHs; benzoic[a]pyrene (Bap), anthracene, fluoranthene, phenanthrene and

naphthalene are the main PAHs that are produced in the environment. Apart from these, some other PAHs such as heterocyclic aromatic compounds (e.g. carbazole and acridine), and nitro-PAHs (for example: pyrene) are also generated through incomplete combustion (Neff, 1985).

2.6 Toxicity of PAHs

PAHs exert deleterious effects on human health. According to US Environmental Protection Agency among all known PAHs Benzo[a]pyrene is recognised as the most dangerous pollutant due to its extreme carcinogenic potential. It is also a major component of smoke released from the cigarettes.

PAHs have very high lipid solubility and hence are quickly absorbed into the gastrointestinal tract of humans (Gibson & Subramanian, 1984). Moreover, PAHs have been investigated to be carcinogenic, mutagenic and teratogenic along with potential endocrine- disrupting properties. The mutagenic effects of PAHs are responsible for tumour initiation (Lee & Hosomi, 2001). Foods may contain PAHs in varying quantities especially when some food is exposed to high temperatures. Studies have suggested that PAHs are found in oil, fats and cereals. Furthermore, they are also obtained from cooked meat and vegetables (Eriksson *et al.*, 2003). PAHs distributed in soil harm human health as they may pass into the food chain, therefore, PAHs contaminated soil are important source and of great concern with respect to human health (Tao *et al.*, 2006).

Skin is another major route of absorption of PAHs which accounts for 75% of the total PAHs (specifically pyrene) absorbed. Absorption rates of PAHs are fast due to high potential for biomagnifications in the food chain. In general, Cerniglia, (1992) states, “the greater the number of benzene rings, the greater the toxicity of the PAH.”

The relative acute toxicity of PAHs can be measured using LD₅₀ values (the lethal dose in 50% of the population tested). PAHs are also suspected carcinogens however, they are not considered to be genotoxic until mammalian enzymes are ‘activated’ from reactive epoxide and quinones. The Cytochrome P450 monooxygenase enzyme responsible for degradation is mostly found in fungi which oxidises the aromatic ring to form epoxide and diol-epoxide reactive intermediates. Due to the oxidation by cytochrome P450 enzymes the genotoxic effect comes into consideration and the aromatic ring forms epoxide and diol-epoxide reactive intermediates (Harvey, 1996). During oxidation processes these intermediates combine with DNA, or attack DNA and undergo oxidation or hydrolysis. Further intermediates combine and attack DNA and form covalent adducts with DNA causing mutation which may lead to tumour formation (Bamforth & Singleton, 2005b).

2.7 Chemical and physical properties of PAHs

PAHs are a group of compounds containing carbon and hydrogen. Most PAHs consist of a “bay-region”, a “K-region” and an “L-region” (figure 2).

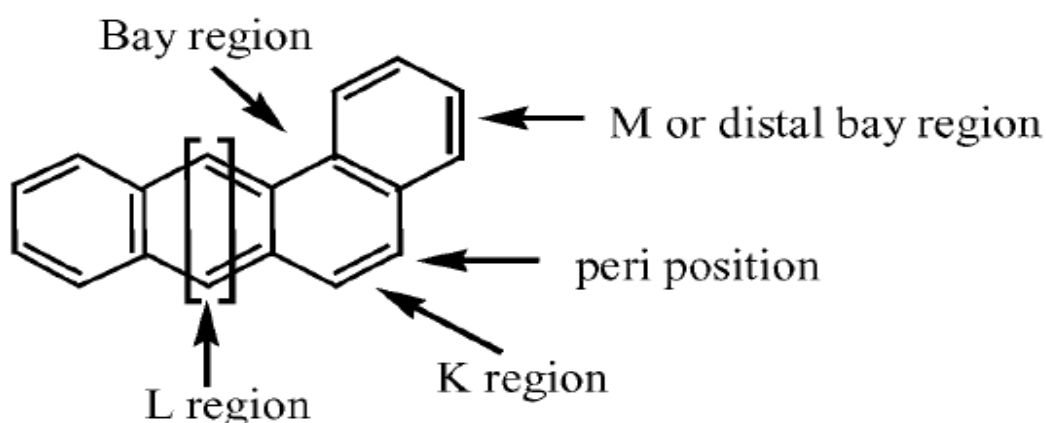


Fig. 2: Different region of biological activity in PAHs

The bay- region is an internal open inner corner for an example, open inner corner of phenanthrene structure. The K-region is an external closed corner and L-region represents the pair of opposed anthracenic point atoms (Aramandla *et al.*, 2004). These bay- and K-region epoxides are chemically reactive. Thus, they are developed metabolically and biologically. Phenanthrene is the simplest aromatic hydrocarbon composed of these regions. The bay-region of phenanthrene is a sterically hindered area between carbon atoms 4 and 5 and the K-region is the 9, 10 double bond, which is the most olefinic aromatic double bond with high electron density (figure 2) (Sikkema *et al.*, 1995).

Low-molecular weight (LMW) PAHs is relatively volatile, soluble and degrades more quickly. Whereas, high molecular weight (HMW) PAHs are more resistant to microbial degradation due to their strong anchoring to soils. Due to the solid state of PAHs compounds, high molecular weight PAHs compounds are hydrophobic and these PAHs are very toxic to whole cells (Cerniglia, 1992). The chemical properties, of a PAH molecule depends on the molecular size including the number of aromatic rings, and molecule topology or the pattern of ring linkage. Ring linkage patterns in PAHs may occur in a way such that the tertiary carbon atoms are centers of two or three interlinked rings for example the case of linear kata-annelated PAH anthracene or the peri-condensed PAH pyrene (Kanaly & Harayama, 2000). Some properties of selected PAHs are presented in Table 1.

Table 1: Chemical and physical properties of selected PAHs

Compound	C atoms	MW	MP* (°C)	BP* (°C)	SW* (mg/l)	CAS	AS* (mg/l)
Naphthalene	10	128	80.2	218	30	91-20-3	30
Phenanthrene	14	178	100	339	1.2	85-01-8	1-2
Anthracene	14	178	217	340	0.7	120-12-7	0.015
Pyrene	16	202	150	393	0.1	129-0-0	0.12-0.18
Fluoranthene	16	202	108	383	0.2	206-44-0	0.25

MW*-Molecular weight SW*-Solubility in water (Haritash & Kaushik, 2009).
MP*-Melting point BP*-Boiling point
AS*-Aqueous solubility in water

2.8 Persistence of PAHs in the environment

The persistence of PAHs in the environment is determined by various factors. These factors include chemical structure, the concentration, and dispersion of PAH, as well as the bioavailability of the co-existing contaminants. Apart from these factors environmental factors such as soil type and texture, pH and temperature are other important factors that control the persistence of PAHs in the environment (Boyle *et al.*, 1998). Thus, the long environmental persistence of higher molecular weight PAH

molecules are due to higher hydrophobicity and toxicity. The persistence of PAHs is also influenced with the ‘age’ of the co-existing contaminants in the soil matrix. If the age of coexisting contaminants is higher the persistence of PAHs is longer. However, PAHs in the environment can be removed through a natural techniques using microbial degradation or using physical or chemical processes as shown in figure 3 (Hatzinger *et al.*, 1995).

Phenanthrene has shown reduced biodegradability due to depletion of oxygen and increase of anaerobicity in the environment. However, work has shown that biodegradation of PAHs can occur even in anaerobic conditions in the absence of molecular oxygen (Rockne & Strand, 1998) but the PAH degradation under anaerobic conditions has limited efficiency (Bamforth & Singleton, 2005a).

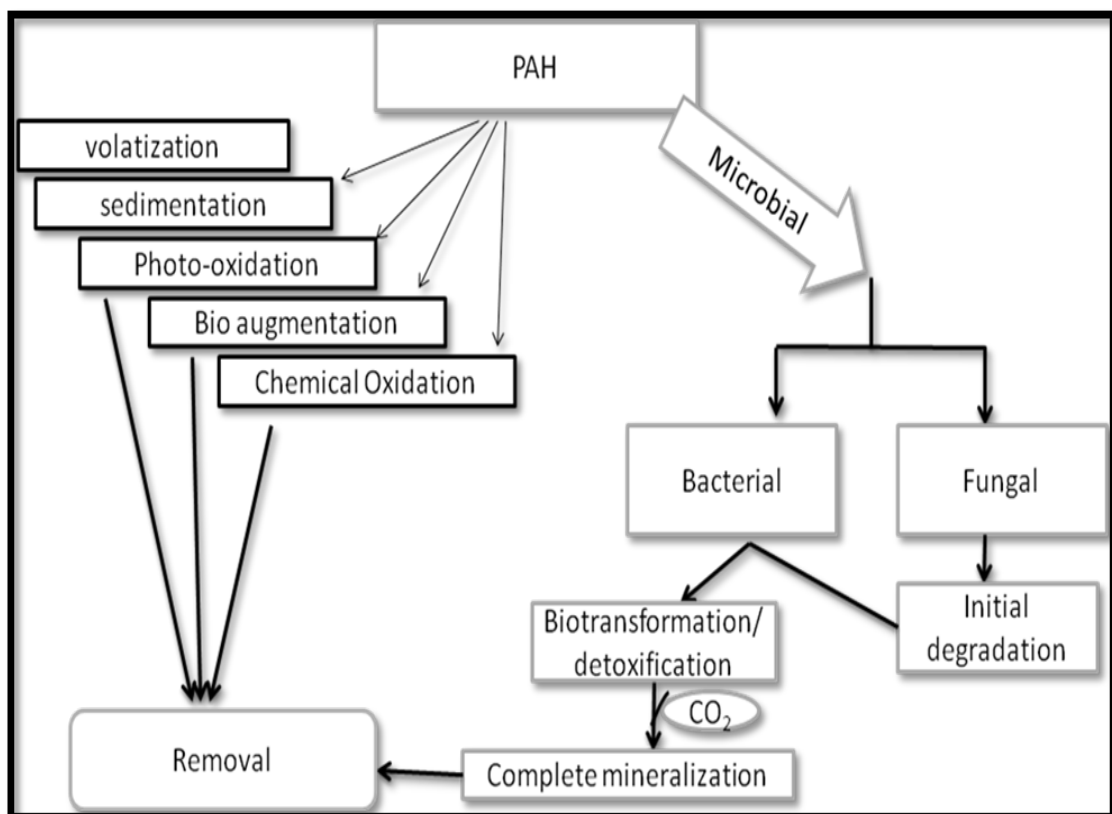


Fig. 3: Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons.

In the environment the fate of PAHs is dependent upon the extent of loss of contaminants due to mechanisms of biodegradation (Rockne *et al.*, 2000). PAH biodegradation processes can be considered as a removal of anthropogenic pollutants as well as a normal process of carbon cycles from the environment. The fate of organic contaminants in the environment is associated with both abiotic and biotic factors including chemical oxidation, volatilization, and photo-oxidation (Pathak *et al.*, 2009).

2.8.1 Chemical oxidation

Chemical oxidation is an evolving technology that involves the introduction of chemical oxidants into subsurface soil and groundwater to destroy organic contaminants (ESTCP, 1999). It is always difficult to remediate organic compounds such as solvents and polycyclic aromatic hydrocarbons present in contaminated soils and groundwater. Chemical oxidation can be carried out with different oxidants such as gaseous ozone, Fenton's reagent, potassium permanganate (KMnO₄) and persulfate.

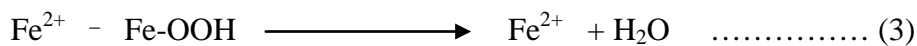
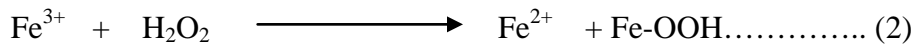
Gaseous ozone process is an effective agent and it is used for the treatment of soils and sediments contaminated by PAHs (Haapea & Tuhkanen, 2006; Masten & Davies, 1997; Rivas, 2006 & U.S. EPA, 1998). Fenton's reagent and potassium permanganate are liquid oxidants, used for degradation of PAHs and other pollutants from the environment (Rivas, 2006; Watts *et al.*, 2002).

Fenton's reaction is carried out in presence of hydrogen peroxide so as to enhance radical formation. Hydrogen peroxide acts as a catalyst sequentially increasing the peroxide oxidative strength (Rivas, 2006). Fenton's reaction includes hydrogen

peroxide's decomposition into hydroxyl radicals along with ferrous iron enhancing reaction.

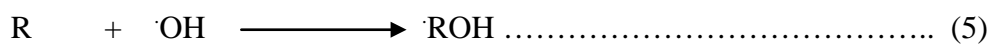
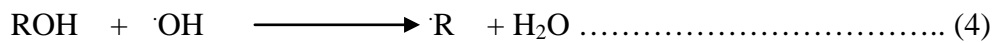


The catalytic reaction is in presence of Fe (III) reduction, which leads to Fe (II) regeneration:



Iron is found as Fe (III) in different environmental conditions, therefore, acidic pH or different chelating agents (such as citric acid, ethylene diamine, tetra-acetic acid (EDTA), and catechol) are used to increase the availability of Fe (II). The increase in availability of iron enhances Fenton's reactions (Sun & Yan, 2007).

Hydroxyl radicals play important role in degradation which can be due to its ability to either abstract hydrogen (reaction 4) or to add hydroxyl groups (reaction 5).



(Ferrarese *et al.*, 2007)

Potassium permanganate (KMnO₄) is one of the strongest oxidizing agents having an oxidation potential of 1.7eV (Ferrares *et al.*, 2008). It has been investigated, that most of the petroleum hydrocarbons remediation is effective with help of (KMnO₄) and widely applied for *in situ* and *ex situ* remediation (Sun & Yan, 2007).

Simonnot *et al*, (2000) studied oxidation processes with Fenton's reagent vs. potassium permanganate (KMnO₄). The research suggested that Fenton's reagent in a chemical degradation is one of the upgraded oxidation processes and KMnO₄ is one of

the strong oxidizing agents. The experimental conditions, proved to be efficient in remediation of phenanthrene and pyrene in PAHs contaminated soils.

2.8.2 Volatilization

The process of conversion of a chemical substance from a liquid or solid state to a gaseous or vapour state by the application of heat, by reducing pressure, or by a combination of both is called volatilization. However most of the compounds are degraded slowly and thus tend to accumulate in the environment (Urgun-Demirtas *et al.*, 2006).

Organic contaminants are present in soil and their transportation to air is one of the important processes in volatilization. Therefore, it affects and controls the transport and fate of organic pollutants present in soil (Liu *et al.*, 2010). Liu *et al.*, (2011) investigated that volatilization of PAHs can be inhibited with respect to solubilisation of PAHs by micelles. However, sorbed surfactant formation drastically inhibited solid-vapour volatilization of PAHs.

2.8.3 Photo-oxidation

Photo-oxidation is potentially one of the important and prevalent ways for PAH modification along with bioremediation in the environment. Photo-catalysis is a process that uses a catalyst, for example titanium oxide (TiO₂) which facilitates a photoreaction to degrade the toxic compounds. TiO₂ induced-photo catalyzed degradation of a variety of all organic substrates is gaining attention due to its potential to degrade PAHs from the environment (Wen *et al.*, 2002). Apart from microbial degradation of PAHs, photo-catalysis is another efficient process, and can be used for the elimination of PAHs. Upon absorbing sunlight, a PAH can be rapidly transformed

to a variety of compounds, most of which are oxidation products (Mallakin *et al.*, 2000). The principle of photo-catalytic oxidation is shown in figure 4.

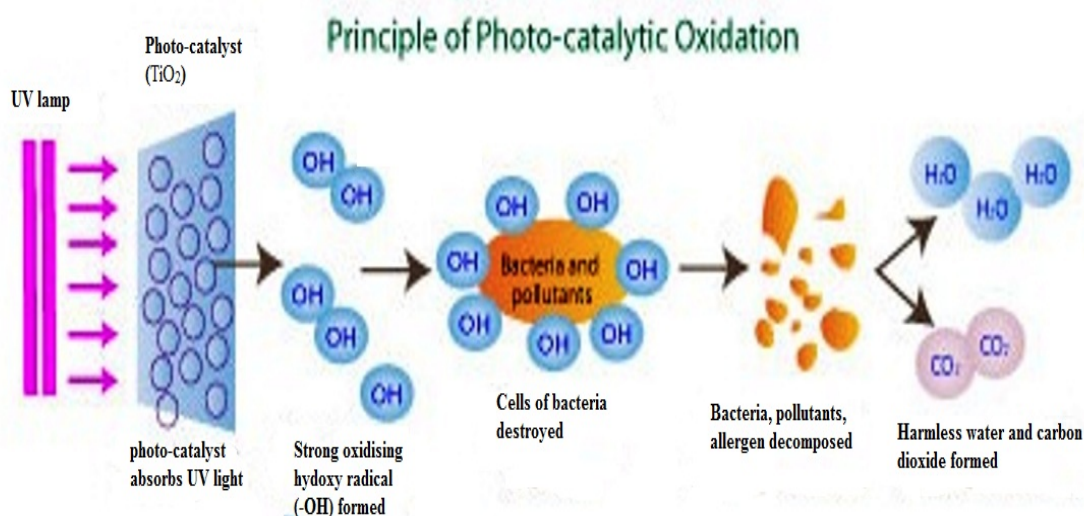


Fig. 4: Principle of photo-catalytic oxidation (www.peakpureair.com)

It has been found that when aromatic compounds are exposed to UV light, partially oxidised intermediates of aromatic compounds are produced which are more susceptible to degradation than their parent ones. Because of this property of aromatic compounds, photo-degradation has been recommended as an early stage strategy for biodegradation (Mueller *et al.*, 1997). Photo-degradation of PAHs in the presence of catalytic solution is considered as an oxidative process which is further augmented in the presence of photo-inducers. The polarity of the solvent is directly proportional to the rate of the degradation process. Thus, higher the polarity of the solvent faster is the degradation process. It has been suggested that reactive oxygen species and hydroxyl radicals do not play an important role in PAH photolysis. PAHs photo-decomposition initiated by photo-ionization results in the production of PAH radical cations and hydrated electron which further destroys PAH in the presence of water (Zeep & Schlotzhauer, 1979; Zeep, 1982).

However, photo-catalytic oxidation, volatilization and chemical oxidation are primary degradation processes which can be applied in the degradation of PAHs of *in situ* contaminated soil (Zepp, 1982).

2.9 Bioremediation

The main purpose of bioremediation is to de-toxify the toxic compounds by microbial degradation in the environment. The degradation particularly includes organic and can be defined as the use of microorganisms to remove environmental pollutants of soils, waters, and sediments (Gogoi *et al.*, 2003). The bioremediation processes may be enhanced/facilitated by various applications to remove pollutants, to treat polluted environments is one of the most efficient practices toward a healthier environment (Gogoi *et al.*, 2003; VanGestel *et al.*, 2003). In the bioremediation process, microorganisms are used to degrade the toxic compounds. However, biodegradation rates of hydrocarbons in soil can be defined by many factors, for example: microorganism type, nutrients, pH, temperature, moisture, oxygen, soil properties, and contaminant concentration (Semple *et al.*, 2001; Ghazali *et al.*, 2004).

2.10 Types of bioremediation

2.10.1 *In-situ* bioremediation

In-situ bioremediation technologies are used to enhance the mechanisms that degrade PAH as well as various eminent polluting compounds in contaminated soil and groundwater. Generally, *in-situ* bioremediation technologies employ engineered systems to enhance the effects of naturally occurring degradation mechanisms (Fiedler, 2000). *In-situ* bioremediation includes:

2.10.1a Biostimulation provides nutrients and suitable physiological conditions for the growth of the indigenous microbial populations. Thus, during the degradation of contaminants metabolic activities are increased.

2.10.1b Bioaugmentation means introduction of specific blends of laboratory-cultivated microorganisms into a contaminated environment or into a bioreactor to initiate the bioremediation process (Fahnestock *et al.*, 1998). Degradation carried out by microbial populations use carbon, nitrogen and phosphorous as sole source of nutrient. However, phosphate and nitrogen are two of the nutrients in the soil with limiting concentration (USEPA, 1994).

2.10.2 Types of *In-situ* Bioremediation

2.10.3 Intrinsic Bioremediation

In order to degrade contaminants from the environment, the bioremediation process depends on different intrinsic factors. Intrinsic bioremediation is defined as the degradation of contaminants without alteration or amendment, to achieve *in-situ* bioremediation. In monitored natural attenuation (MNA) sites, intrinsic bioremediation may play a role that helps broaden the term defined by the US National research Council (NRC) and EPA as “biodegradation, dispersion, chemical or biological stabilization, transformation or destruction of contaminant” (Sturman *et al.*, 1995).

2.10.4 Engineered *in-situ* Bioremediation

In-situ bioremediation techniques include many selection procedures at contaminated sites. During remediation of a contaminated site the identification of degradation mechanisms provides enhancement of technologies that are more beneficial for use at the site. The steps in selection and implementation of *in-situ* bioremediation are:

- Evaluation of site characteristics
- Identification of site conditions and engineering solutions
- Identification of primary reactants
- Perform test based on treatability (bench-scale)
- Perform design system, field test, and implementation (Fiedler, 2000).

2.11 Ex-situ Bioremediation

The main metabolic activities and genetic features of microbial degradation need to be evaluated for a successful bioremediation approach. The microbial degradation at contaminated site can be evaluated considering the design and implementation suitable for microbial detoxifying strategies. It thus helps in monitoring the effectiveness of the bioremediation and understanding the metabolic and genetic features (Andreoni & Gianfreda, 2007). There are some aspects that help for easier *ex-situ* bioremediation and the most concerning aspects are:

- (a) Pollution of the environment by two classes of aromatic pollutants namely, polycyclic aromatic hydrocarbons (PAHs) and volatile aromatics are collectively indicated as BTEX (benzene, toluene, ethylbenzene, and xylene)
- (b) The main metabolic pathways and the genetic basis of their microbial degradation
- (c) The biological strategies to reduce or to eliminate their contamination, and
- (d) More advanced monitoring techniques to evaluate the effectiveness of a bioremediation process (Andreoni & Gianfreda, 2007).

2.12 The contaminated soil matrix

Air, water and soil are major natural resources. Soil is a complex mixture of air (25%), water (25%), minerals that come from rocks below or nearby surfaces (45%),

and organic matter (5%) (figure 5). The organic matter present in soil is due to remains of plants and animals that use the soil and living organism that reside in soil (Bollag & Liu, 1990). The type of soil depends on the clay, sand and silt present in it. However, other factors like climate, vegetation, time, the surrounding terrain, and even human activities (e.g. farming, grazing, gardening, etc.), are also important in influencing the soil that is formed and the types of soil that occur in a particular landscape (Pritchard & Bourquin, 1985).

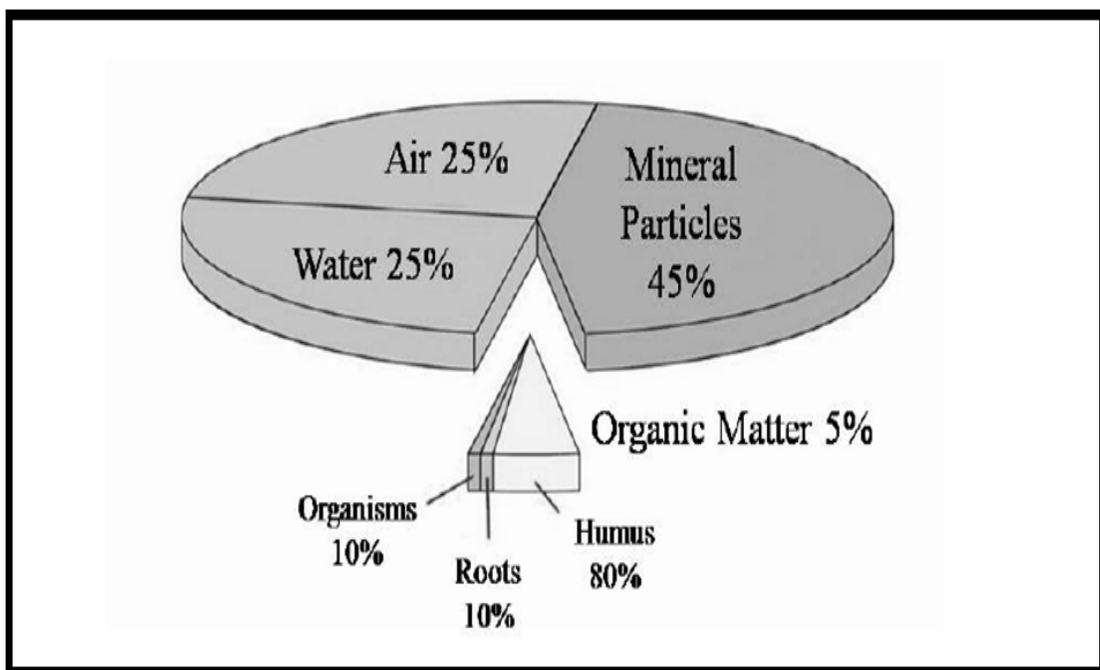


Fig. 5: Soil contains four basic components: mineral particles, water, air, and organic matter. Organic matter can be further sub-divided into humus, roots and living organisms (<http://www.physicalgeography.net>).

Thus, soil quality depends in part on its natural composition, and also on the changes caused by human use and management (Larson & Pierce, 1991).

2.13 Fate of organic contaminants in the soil environment

An organic contaminant (OC) in the soil environment may be lost by both biological and physical-chemical pathways in soil. Living organisms carry out biological degradation. The abiotic processes include leaching or volatilization,

accumulation within the soil biota or sequestration within the soil mineral and organic matter fractions as shown in figure 6 (Lifongo & Nfon, 2009; Bollag & Liu, 1990).

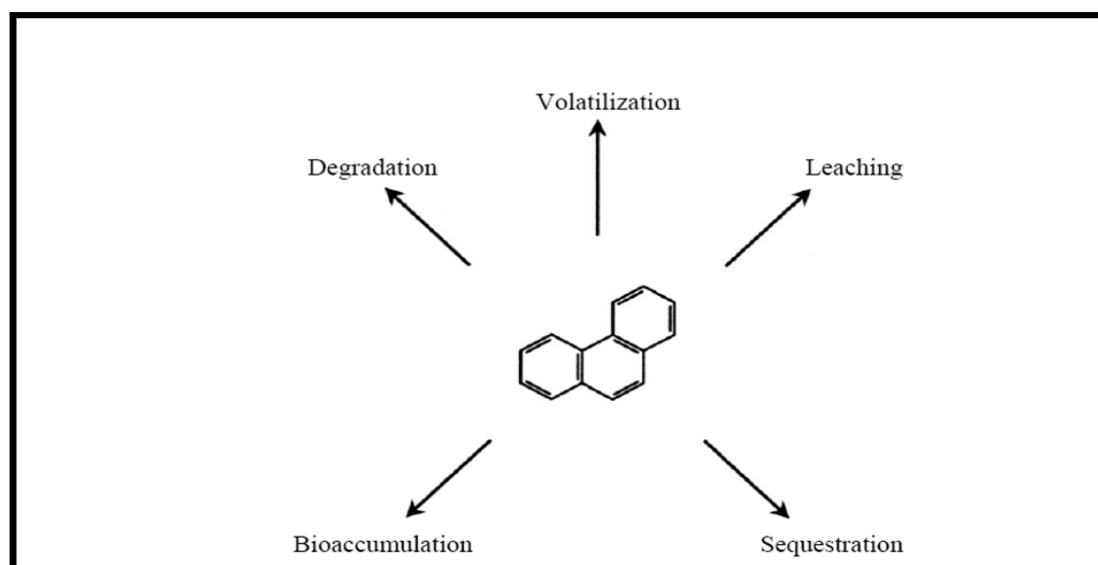


Fig. 6: Assumed fate of behaviour of a model organic contaminant (phenanthrene) in soil (Semple *et al.*, 2003).

2.14 Remediation of PAHs in soil systems

Biodegradation is one of the most common mechanisms harnessed in order to treat organic contaminants. Soil bioremediation is dependent upon desorption from the solid to the liquid phase of contaminant. Limited oxygen or nutrients, lack of bioavailability however, limit the bioremediation process. Under denitrifying conditions, anaerobic degradation of some PAHs occurs in the presence of excess nitrate (Mihelcic & Luthy, 1988). The hydrophobicity of these compounds constitutes the main factor that determines their persistence in the environment; in particular they tend to be strongly absorbed by soil particles with low bioavailability and possibly accumulate in the food chain as well (Mollea *et al.*, 2005).

In the soil matrix, as the contaminants are released, they bind to the surface and become sequestered into the soil matrix. Due to sequestering of contaminants,

sorption strength increases over time. Further, sorption strength reduces the susceptibility of the contaminants to remediation. Soil organic matter (SOM) is not homogenous; it consists of varying proportions of combustible residues, non-aqueous phase liquids, and natural organic matter (NOM), all of which vary in their affinity for contaminants (Jonsson *et al.*, 2007). Mass transfer rates of the PAH molecule is an independent factor. Degradation rates often depend on the mass transfer rates of the PAH from the solid phase to the water phase. Enhanced mass transfer can be achieved by the consumption of minor substrate molecules, thereby altering the surface area and resulting in a higher dissolution rate (Tiehm, 1994).

2.15 Microbial metabolism of PAHs

Prokaryotes are considered as major decomposers of organic compounds in an ecosystem (Campbell & Reece, 2005). Enzymes present in bacteria, digest these organic compounds and make them available for absorption. Higher molecular weight PAHs are often oxidized in variety of organic compounds by white rot fungi providing more advantages compared to bacteria (Pointing, 2001).

PAH degradation is carried out by white rot fungi under aerobic conditions, however, in bacteria it occurs under aerobic and anaerobic conditions. Bacteria and white rot fungi have different biochemistry for the catabolism of organo-pollutants (Pointing, 2001). Organo-pollutants are degraded by bacteria as their cells use a source of carbon and nitrogen as nutritional benefits, while PAHs and toxic pollutants are co-metabolically oxidised/ transformed with no energy supply to their cells. As a result white rot fungi require additional nutrients to manage their cellular activities (Pointing, 2001; Boochan *et al.*, 2000).

Microorganisms that degrade PAHs are universally distributed in the natural environment, such as in soils (bacteria and non-ligninolytic fungi) and woody materials (ligninolytic fungi). A comprehensive listing of microorganism genera capable of PAH degradation is tabulated in table 2.

Table 2: Bacterial and fungal genera that contain PAHs- degrading species

Bacterial genera that contain PAHs-degrading species			
<i>Achromobacter</i>	<i>Azotobacter</i>	<i>Escherichia</i>	<i>Phorm</i>
<i>Acidovorax</i>	<i>Bacillus</i>	<i>Flavobacterium</i>	<i>Proteus</i>
<i>Acinetobacter</i>	<i>Beijerinckia</i>	<i>Gordonia</i>	<i>Pseudomonas</i>
<i>Actinomyces</i>	<i>Beneckea</i>	<i>Klebsiella</i>	<i>Rhodococcus</i>
<i>Aerobacter</i>	<i>Brevibacterium</i>	<i>Lactobacillus</i>	<i>Sarcina</i>
<i>Aeromonas</i>	<i>Clavibacter</i>	<i>Leucothrix</i>	<i>Serratia</i>
<i>Agmenellum</i>	<i>Clostridium</i>	<i>Marinobacter</i>	<i>Sphaerotilus</i>
<i>Agrobacterium</i>	<i>Coccochloris</i>	<i>Micrococcus</i>	<i>Sphingomonas</i>
<i>Alcaligenes</i>	<i>Comamonas</i>	<i>Microcoleus</i>	<i>Spirillum</i>
<i>Alteromonas</i>	<i>Corynebacterium</i>	<i>Moraxella</i>	<i>Streptomyces</i>
<i>Anabaena</i>	<i>Curtobacterium</i>	<i>Mycobacterium</i>	<i>Thermoleophilum</i>
<i>Aphanocapsa</i>	<i>Cycloclasticus</i>	<i>Nocardia</i>	<i>Vibrio</i>
<i>Art hroba cter</i>	<i>Cytophaga</i>	<i>Nostoc</i>	<i>Xanthomonas</i>
<i>Aureobacterium</i>	<i>Enterobacter</i>	<i>Oscillatoria</i>	
<i>Azospirillum</i>	<i>Erwinia</i>	<i>Peptococcus</i>	

Fungal genera that contain PAHs-degrading species			
<i>Absidia</i>	<i>Cunninghamella</i>	<i>Mucor</i>	<i>Saccharomyces</i>
<i>Acremonium</i>	<i>Debaromyces</i>	<i>Neurospora</i>	<i>Saccharomycopsis</i>
<i>Allischeria</i>	<i>Dendryphiella</i>	<i>Oidiodendrum</i>	<i>Saprolegnia</i>
<i>Aspergillus</i>	<i>Emericellopsis</i>	<i>Paecilomyces</i>	<i>Scedosporium</i>
<i>Aureobasidium</i>	<i>Epicoccum</i>	<i>Panaeolus</i>	<i>Scopulariopsis</i>
<i>Basidiobolus</i>	<i>Eupenicillium</i>	<i>Penicillium</i>	<i>Smitium</i>
<i>Bjerkandera</i>	<i>Fusarium</i>	<i>Peronospora</i>	<i>Sordaria</i>
<i>Botrytis</i>	<i>Gilbertella</i>	<i>Pestalotia</i>	<i>Sporobolomyces</i>
<i>Candida</i>	<i>Gliocladium</i>	<i>Phanerochaete</i>	<i>Syncephalastrum</i>
<i>Cephalosporium</i>	<i>Gonytrichum</i>	<i>Phlyctochytrium</i>	<i>Talaromyces</i>
<i>Choanephora</i>	<i>Graphium</i>	<i>Phycomyces</i>	<i>Tetracoccosporium</i>
<i>Circinella</i>	<i>Hansenula</i>	<i>Phytophthora</i>	<i>Thamnidium</i>
<i>Cladosporium</i>	<i>Helicostylum</i>	<i>Pichia</i>	<i>Torulopsis</i>
<i>Claviceps</i>	<i>Helminthosporium</i>	<i>Pleurotus</i>	<i>Trametes</i>
<i>Cokeromyces</i>	<i>Humicola</i>	<i>Psilocybe</i>	<i>Trichoderma</i>
<i>Conidiobolus</i>	<i>Hypochytrium</i>	<i>Ramaria</i>	<i>Trichosporon</i>
<i>Corioloopsis</i>	<i>Lep hot richus</i>	<i>Rhizophlyctis</i>	<i>Varicospora</i>
<i>Coriolospora</i>	<i>Linderina</i>	<i>Rhizopus</i>	<i>Verticillium</i>
<i>Crinipellis</i>	<i>Luiworthia</i>	<i>Rhodospordium</i>	<i>Zoophthora</i>
<i>Cryptococcus</i>	<i>Mortierella</i>	<i>Rhodotorula</i>	<i>Zygorhynchus</i>

(Al-Turki, 2009).

The large molecular weight (LMW) PAHs in contaminated soil can be effectively treated by bioremediation. In the bioremediation processes, the native microbes are of a great interest as these organisms may be expected to be adapted to the soil environment. These microorganisms when used in particular soil environments and with optimized abiotic conditions are more likely to out-compete the introduced microorganisms (Silva *et al.*, 2009). Microbial degradation of PAH by aerobic mechanisms is expressed by three fundamental mechanisms. The specific details of

degradation metabolism by bacterial and fungal are shown in figure 7.

Several environmental factors influence the rate of PAH degradation. Low oxygen concentrations limit PAH degradation that requires oxygenase activity. Generally, PAH degradation is limited by low water solubility, bioavailability and soil pH. Some PAH metabolites, particularly epoxide, dihydrodiols and quinones, affect the survival and viability of microorganisms, since they are cytotoxic and genotoxic. It has been found that the pH of culture media affects the levels of the two constitutive PAH o-quinone reductases particularly a pyrene-degrading enzymes (Kim *et al.*, 2005).

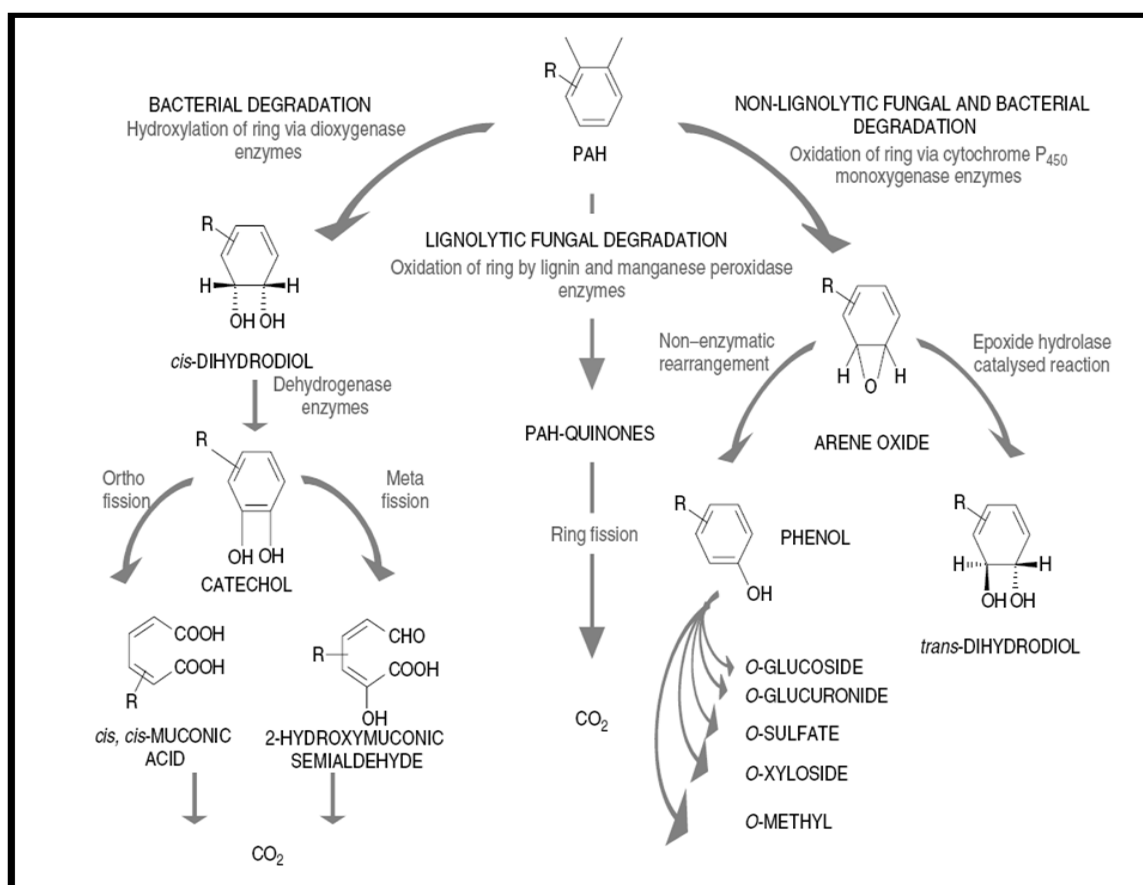


Fig. 7: Three main pathways for polycyclic aromatic hydrocarbon degradation by fungi and bacteria (Bamforth and Singleton, 2005b).

2.16 Bacterial metabolism of PAHs

Active degradation of organic pollutants from the contaminated site in the environment is performed by bacteria. Natural processes of degradation via bacteria can however be carried out more rapidly by altering abiotic factors at these contaminated site (Haritash & Kaushik, 2009).

The principal mechanism for the aerobic bacterial metabolism of PAHs is illustrated in figure 7. The initial step involves oxidation of the benzene ring by the action of dioxygenase enzymes to form cis-dihydrodiols (Parales *et al.*, 1998; Kanaly & Harayama, 2000). Further, re-aromatization occurs and these cis-dihydrodiols are dehydrogenated to form dihydroxylated intermediates. These dihydroxylated intermediates are exposed to cleavage by dioxygenase via ortho or meta cleavage resulting in production of carboxylic acid cycle (Parales *et al.*, 1998) which can then be further metabolised via catechols to carbon dioxide and water (figure 7). There is a large diversity of bacteria that are able to oxidise PAH using dioxygenase enzymes, including organisms from the genera *Pseudomonas* and *Rhodococcus*. A few bacteria such as *Mycobacterium* species are also capable of oxidising PAHs by the action of the cytochrome P450 monooxygenase enzyme to form trans-dihydrodiols (Kelley *et al.*, 1990). Naphthalene, the simplest form of PAHs is the model PAH studied mostly (Parales *et al.*, 1998). It is the most soluble PAH (Annweiler *et al.*, 2000). Davies & Evans (1964) were first to report the biochemical sequence and enzyme kinetics responsible for naphthalene degradation. The common naphthalene degradation pathway is shown in figure 8. Naphthalene is converted as a metabolite to cis-1, 2-dihydroxy-1, 2-dihydro-naphthalene. Further, oxidation of naphthalene helps in conversion of cis-1, 2-dihydroxy-1, 2-dihydro-naphthalene to 1, 2-dihydroxy-naphthalene (Cerniglia, 1984; Mrozik *et al.*, 2003). Naphthalene-cis-dihydrodiol

dehydrogenase is catalyzed and requires NAD^+ as an electron acceptor. The next step leads to conversion of naphthalene-cis-dihydrodiol dehydro-genase to cis-2-hydroxybenzalpyruvate. Further, a series of dioxygenases convert cis-2-hydroxybenzalpyruvate to salicylate and pyruvate. Oxidation of salicylate is carried out by salicylate hydroxylase to catechol leading to ortho or meta fission. This ortho or meta fission depends on bacterial metabolism (Dagley & Gibson., 1965). The naphthalene degradation pathway is commonly found in prokaryotic cells and was described first in bacteria (Mrozik *et al.*, 2003).

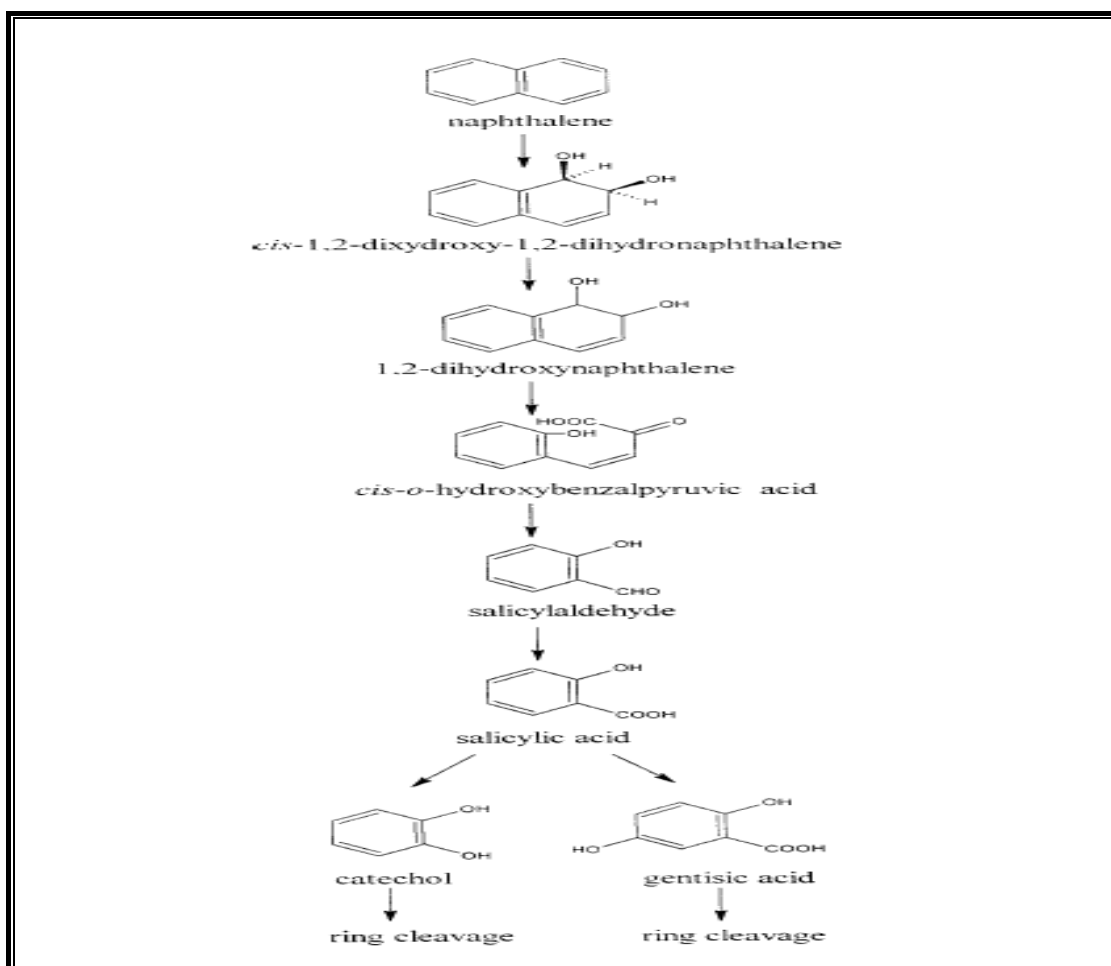


Fig. 8: Naphthalene degradation pathway (Bamforth and Singleton, 2005b).

2.18 Degradation of anthracene

Anthracene is a tricyclic aromatic hydrocarbon, found widely in the environment. It has been a model substrate for various studies on degradation of PAHs (Moody *et al.*, 2001).

Anthracene is oxidised in the positions 1, 2 and gets converted to cis-1, 2-dihydroxy-1, 2-dihydroanthracene. It is further converted to 1, 2-dihydroxyanthracene which uses NAD^+ dependent dihydrodiol dehydrogenase. Moreover, oxidation of 1, 2-dihydroxyanthracene undergoes ring fission to form a cis-4-(2-dihydroxynaphth-3-yl)-2-oxobut-enoic acid as a product. This product gets converted to 2-hydroxynaphthoic acid. Further, the fission helps the metabolic product to form salicylate and catechol through 2, 3-dihydroxynaphthalene (Cerniglia, 1984; Evans *et al.*, 1965). The schematic proposed pathway for anthracene degradation is exhibited in figure 10.

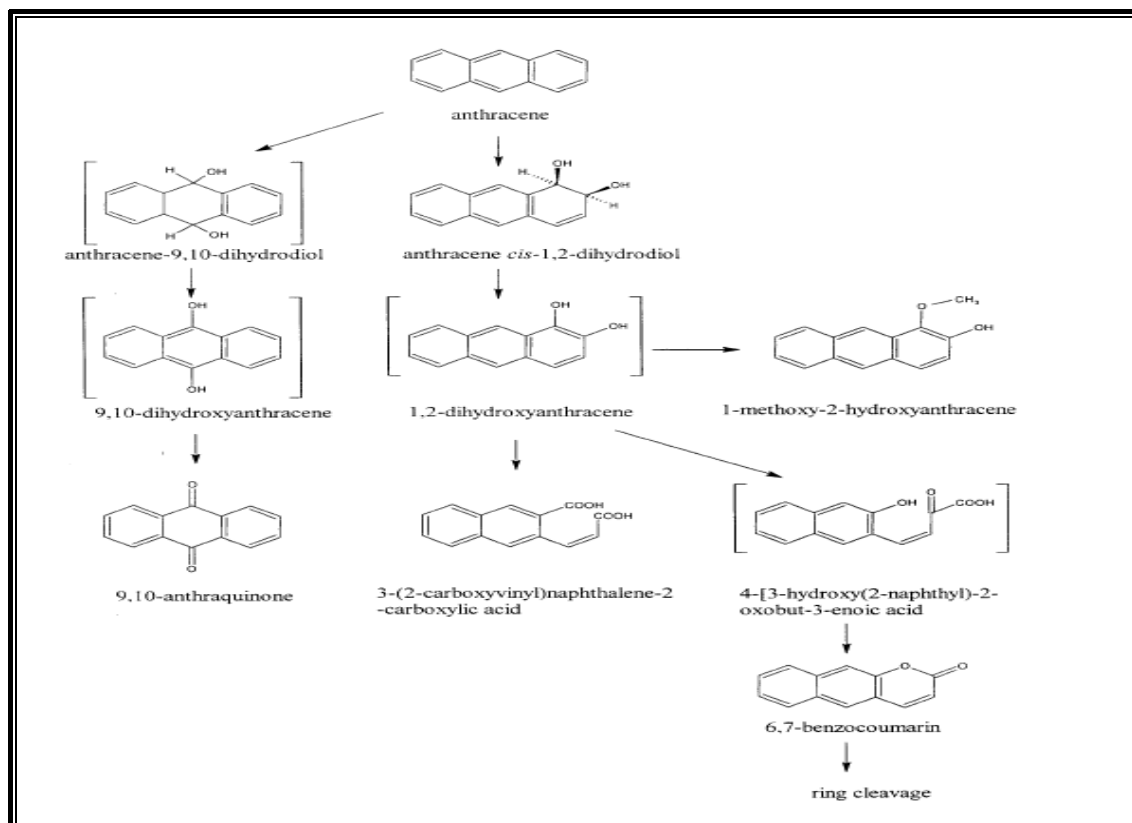
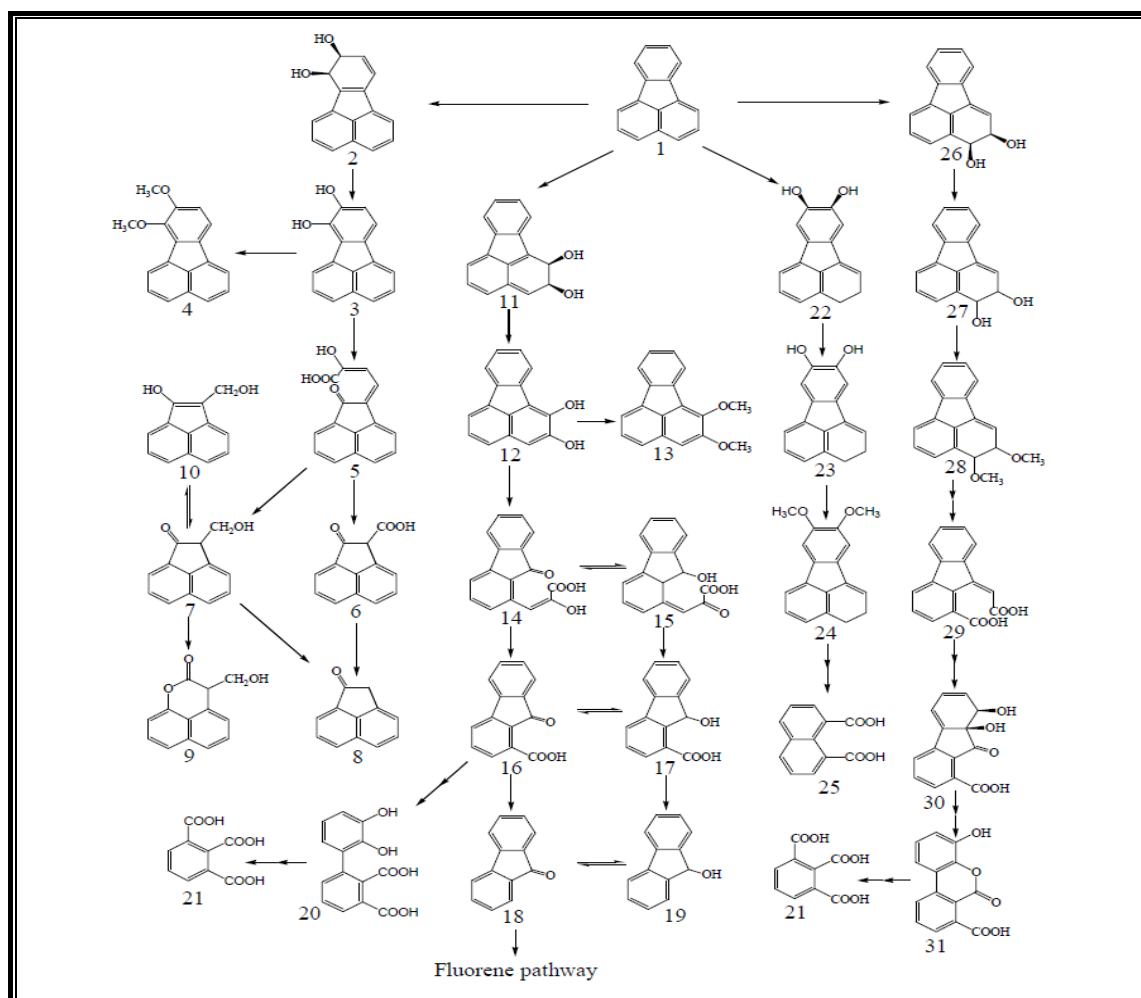


Fig. 10: Anthracene degradation pathway (Bamforth and Singleton, 2005b).

2.19 Degradation of fluoranthene



Compound designations: 1, fluoranthene; 2, *cis*-7,8-fluoranthene dihydrodiol; 3, 7,8-dihydroxy-fluoranthene; 4, 7,8-dimethoxyfluoranthene; 5, 2-hydroxy-4-(2-oxo-2*H*-acenaphthylen-1-ylidene)-but-2-enoic acid; 6, 2-oxo-acenaphthene-1-carboxylic acid; 7, 2-hydroxymethyl-2*H*-acenaphthylen-1-one; 8, 1-acenaphthenone; 9, 3-hydroxymethyl-3*H*-benzo[*de*] chromen-2-one; 10, 2-hydroxymethyl-acenaphthylen-1-ol; 11, *cis*-1,2-fluoranthene dihydrodiol; 12, 1,2-dihydroxy-fluoranthene; 13, 1,2-dimethoxyfluoranthene; 14, 9-fluorenone-1-(carboxy-2-hydroxy-1-propenol); 15, 9-fluorenone-1-carboxy-3-propenyl-2-one; 16, 9-fluorenone-1-carboxylic acid; 17, 9-fluorenone-1-carboxylic acid; 18, 9-fluorenone; 19, 9-fluorenone; 20, 2',3'-dihydroxybiphenyl-2,3-dicarboxylic acid; 21, benzene-1,2,3-tricarboxylic acid; 22, *cis*-8,9-fluoranthene dihydrodiol; 23, 8,9-dihydroxy-fluoranthene; 24, 8,9-dimethoxyfluoranthene; 25, naphthalene-1,8-dicarboxylic acid; 26, *cis*-2,3-fluoranthene dihydrodiol; 27, 2,3-dihydroxy-fluoranthene; 28, 2,3-dimethoxyfluoranthene; 29, 9-carboxymethylene-9*H*-fluorene-1-carboxylic acid; 30, *cis*-1,9a-dihydroxy-1-hydrofluorene-9-one-8-carboxylic acid; 31, 4-hydroxybenzochromene-6-one-7-carboxylic acid.

Fig. 11: Fluoranthene degradation pathway (Seo *et al.*, 2009).

2.20 Degradation of pyrene

Pyrene- four ring structures are a pericondensed PAH. Pyrene degradation and pathways for degradation along with their metabolites has been reported by many investigations.

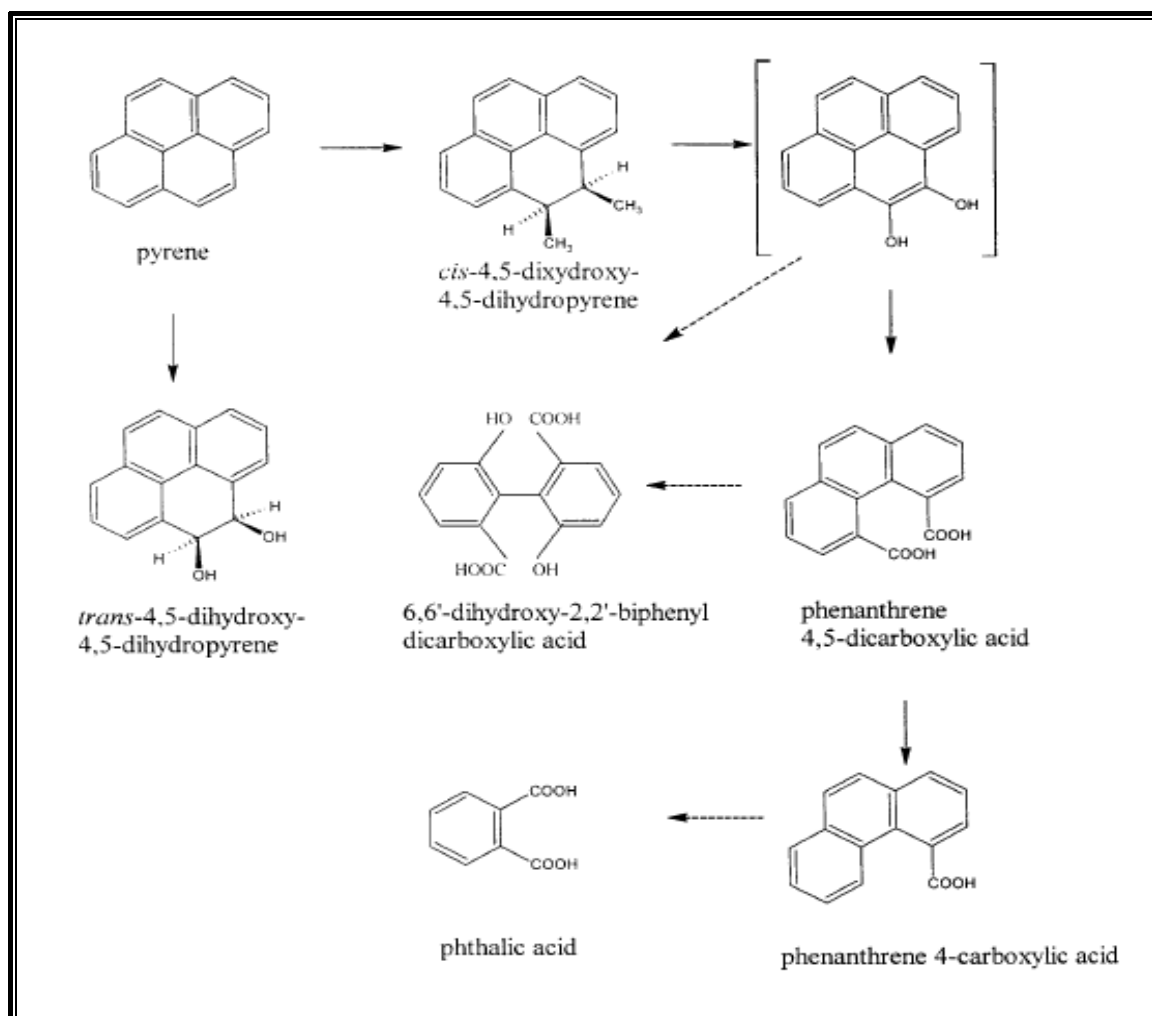


Fig. 12: Pyrene degradation pathway (Bamforth and Singleton, 2005b).

2.21 Mycoremediation

Mycoremediation involves fungi that degrade toxic compounds from the environment. Most mycoremediation studies have been carried out and investigated on artificially contaminated soils spiked with organic pollutants. Due to this, it is very important to research the use of fungal remediation under non-sterile and sterile

conditions and with soils samples from contaminated sites (D'Annibale *et al.*, 2006). Bio augmentation is the process in which the indigenous bacteria and fungi as well as alternative bio stimulation can favour contaminant degradation. It deals with specified historically and/or heavily contaminated sites (Bastiaens *et al.*, 2000).

2.22 Fungal metabolism of PAHs and fungal enzymes involved in PAH degradation

Filamentous fungi play an important role in degradation and detoxification of polycyclic hydrocarbons, including condensed aromatic ring systems as well as other xenobiotic compounds, present in polluted environments. Some of these compounds are very harmful and carcinogenic.

2.23 Lignin degradation (Dissimilation of aromatic polymers)

Lignin is a branched polymer with a complex three-dimension structure formed by the oxidative polymerization of substituted p-hydroxycinnamyl alcohols which form the basic phenyl-propane units. Lignin which is one of the important components that provides mechanical support to plants (Crawford & Crawford, 1976). Combination of p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol together contribute to lignin formation. It is an organic polymer due to cellulose in woody plants that accounts nearly about 50% of the carbon content of plants (Dix & Webster, 1995).

2.24 White rot fungi

White rot fungi are found mainly on the wood of living trees, decaying wood and causing damage to unprotected wood in buildings (Dix & Webster, 1995). Lignin oxidation in white rot fungi causes the wood to take on the diagnostic white or

bleached appearance. Furthermore, lignin and carbohydrates in wood are metabolized by white rot fungi (Kirk & Farrell, 1987). White rot fungi belong to basidiomycota that use wood as a nutrient source and degrade lignin component of wood with the help of ligninolytic enzymes. It is not a primary metabolism, as oxidation of lignin yields no energy supply to their cells (Pointing, 2001). White rot fungi degrade some anthropogenic recalcitrant organic pollutants. They are capable of degrading various pollutants like synthetic dyes, synthetic polymers, pesticides, chlorophenols, polychlorinated biphenyls, and wood preservatives due to the non specificity of their ligninolytic enzymes (Urairuj *et al.*, 2003; Pointing, 2001; Novotny *et al.*, 2004).

The fungal ligninolytic system consists of three major enzymes: lignin peroxidase LiP (EC 1.11.1.14), manganese peroxidase MnP (EC 1.11.1.13) and laccase activity LAC (EC 1.10.3.2) (Cajthaml *et al.*, 2008).

Fungi can metabolise PAH compounds using Cytochrome P450 monooxygenase enzyme. Cytochrome P450 monooxygenase enzyme-mediated reaction oxidises the aromatic ring to form epoxide and diol-epoxide reactive intermediates. Thus, fission occurs in diol- epoxide releasing further CO₂ in the environment (Bamforth & Singleton, 2005b).

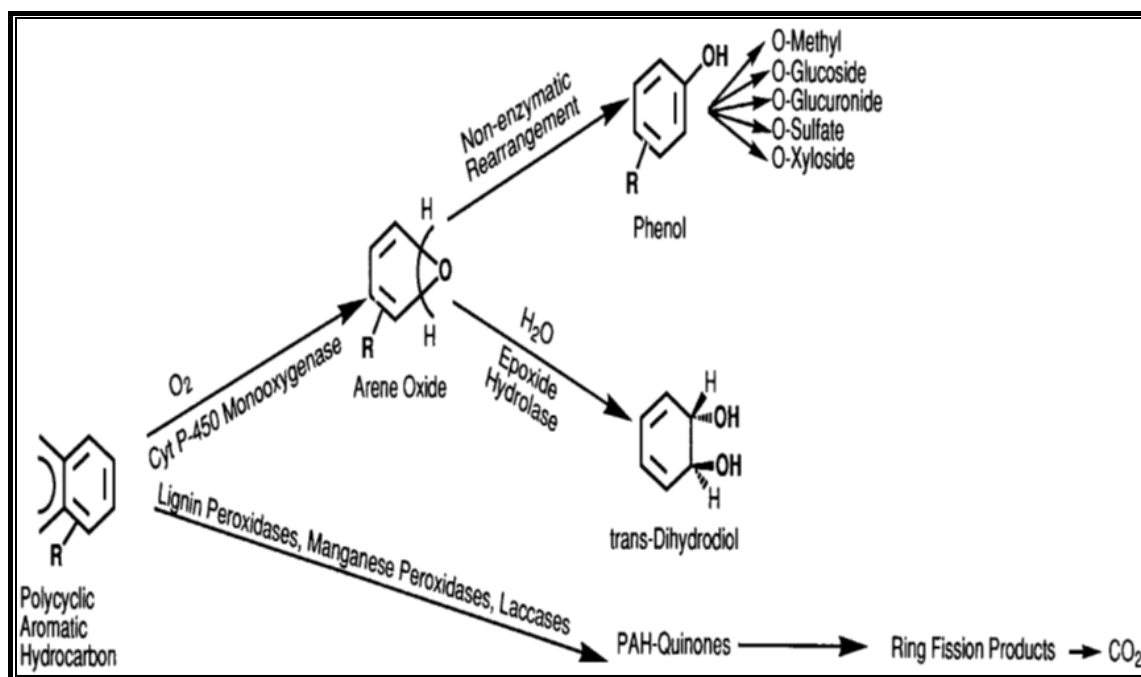


Fig. 13: Pathways for the fungal metabolism of polycyclic aromatic hydrocarbons (Cerniglia, 1992).

2.25 Microbial biomass and activity

Microbial metabolic activity in soil can be evaluated using CO_2 evolution as an indicator of microbial respiration. Moreover, the relation between respiration and biomass (qCO_2) is a parameter considered to be a good indicator of the amount of pollution and the efficiency of bioremediation in contaminated soils (Silva *et al.*, 2009), further leading to lower biomass yield found in most of contaminated soils (Sirguy *et al.*, 2008). Arginine ammonification is one of the methods to measure the microbial activity which serves as a convenient test for microbial activity in soil samples. Although a wide range of methods are available to determine the microbial activity, soil ATP measurements, arginine ammonification may be used as a convenient, inexpensive and relatively fast method for routine estimation of microbial activity potentials (Alef & Kleiner, 1987).

2.26 Factors affecting the bioremediation of PAHs

Stacking and composting of soil from contaminated site is one of the classic examples for successful remediation. The efficiency of bioremediation on a bench scale and under ideal laboratory conditions have been investigated in many studies, often at specified neutral pH and mesophilic temperatures. However, the environmental factors vary from site to site (such as bioavailability of contaminant, nutrient availability, temperature and soil pH) and regulate the process of bioremediations (Bamforth & Singleton, 2005b).

2.26.1 Temperature

The effect of temperature is considered as one of the factors influences during degradation of PAHs. As temperature increases, the solubility of PAH molecule increases. During different seasons of the year, temperature at most contaminated sites is not helpful for bioremediation (Margesin & Schinner, 2001). However, Erickson *et al.*, (2003) suggests that oxygen solubility decreases with increase in temperature. This results in decreased metabolic activity of aerobic microorganisms. Different populations of hydrocarbon-utilizing microorganisms which are adapted to ambient temperatures are selected and expected in different climates and seasons. In some Arctic and temperate regions, soil temperature is below 10°C all year-round, and availability of oxygen in wet conditions is limited. The, cost of increasing the temperature may be prohibitive, so low temperatures need to be optimized (Eriksson *et al.*, 2003).

2.26.2 Oxygen

Organic contaminants can be metabolized to achieve remediation both under aerobic and anaerobic conditions. Most work has concentrated upon the dynamics of aerobic metabolism of PAHs (Gibson *et al.*, 1968). Oxygen is necessary for the action of microbial mono- and dioxygenase enzymes in the initial process as carried out during aerobic PAH metabolism.

There is still a debate and issues have been raised as to whether the benefits of anaerobic bioremediation are outweighed by the negatives. This has been carried out using hydrogen peroxide, sodium nitrate and perchlorate (Coates *et al.*, 1999). However, it has also been reported that rates of anaerobic PAH degradation under denitrifying conditions were comparable to those under aerobic conditions (McNally *et al.*, 1998). This suggests that the dominant *in situ* microbial community consist of PAH-degrading microorganisms and that bioremediation was not limited by low numbers of PAH-degrading microorganisms rather than adverse environmental conditions. Oxidation of the substrate by oxygenase is involved due to catabolism of alicyclic and aromatic hydrocarbons by bacteria and fungi. Therefore, the route of microbial oxidation of hydrocarbons in the environment makes it necessary for aerobic conditions. The rates of microbial oxygen consumption and, type of soil, are fairly dependent on the availability of oxygen. The oxygen availability when soil is waterlogged is low and the high availability of utilizable substrates can also lead to oxygen depletion (Genthner *et al.*, 19997).

2.26.3 Nutrient availability

For cellular metabolism during degradation of PAHs microorganisms require mineral nutrients for successful growth. Different mineral nutrients such as nitrogen,

phosphate and potassium (N, P and K) are required for growth and so help to readily degrade carbon source. Therefore, contaminated land may need to be supplemented with various nutrients, generally nitrogen and phosphates to stimulate *in situ* microbial activity and further enhance bioremediation strategies (Brandli *et al.*, 2008).

The ratio of C: N: P in microbial biomass has been estimated for optimal growth and hence for the bioremediation process (Bamforth & Singleton, 2005a). However, a recent study due to nature of the pollutants in contaminated sites, where organic carbon levels are often high, available nutrients can rapidly deplete during microbial metabolism. The failure in the bioremediation of high molecular weight PAHs has been reported by Bamforth & Singleton, (2005a) due to the temporary increased long duration inhibition of functionally important organisms (Bamforth & Singleton, 2005a).

2.26.4 Bioavailability

PAH compounds have low bioavailability because they are hydrophobic (Genthner *et al.*, 1997). Bioavailability depends on the rate and extent of biodegradation and is the most important factor in bioremediation (Muller *et al.*, 1997). In addition, in the presence of different soil matrices rapid sorption to mineral surfaces such as clays and organic matter such as humic and fulvic acids are carried out by PAHs. As long as PAH compounds are in direct contact with soil, sorption becomes stronger and chemical and biological extractability of contaminants is lowered. This phenomenon is called ‘ageing’ of the contaminant. Therefore the bioavailability of a pollutant is linked to its persistence in an environment (Vandyke *et al.*, 1991).

PAHs are chemicals with low water solubility that are resistant to biological, chemical and photolytic breakdown. The higher the molecular weight of the PAH, the

lower its solubility, and due to lower solubility, the accessibility of PAHs compounds during the metabolism by the microbial biota is lower (Fewson, 1988). Various surface-active agents for example, surfactants or detergents, help in release of PAHs from the surface of minerals and organic matter. These can be monitored as microbial respiration rates (mineralisation) of ¹⁴C-labelled contaminants, the bioluminescence of microorganisms such as lux microorganisms and/or lux-tagged pollutants. The surface active compounds contain both a hydrophobic and hydrophilic moiety. A 'bridge' between the hydrophobic PAH molecule and the hydrophilic microbial cell is formed due to these hydrophobic and hydrophilic moieties and this maintains the bioavailability (Bamforth & Singleton, 2005a).

2.26.5 Salinity

The mineralization of phenanthrene and naphthalene in fine soil sediments is positively correlated with salinity and the rate of mineralization. It has been reported in general that correlation decreases with the increase in salinity. In a study of soil containing salt in high quantities, it has been shown that rates of hydrocarbon metabolism decreased with increase in salinity in the range of 3.3 to 28.4% and the results are attributed to a general reduction in microbial metabolic rates (Leahy & Colwell, 1990). Thus, there are few published studies which deal with effects of salinity on the microbial degradation of hydrocarbons.

2.26.6 Water activity

The water activity or water potential of soils can range from 30% to 45% water saturation. The hydrocarbon biodegradation in an acidic soil may therefore be limited by the available water for microbial growth and metabolism.

2.26.7 pH

For bioremediation, most of PAHs contaminated sites are not at the optimal pH. For example, treated workplaces sites frequently hold substantial amount of destruction waste such as concrete and brick. Leaching processes will however, increase the pH of the soil and result in limited favourable conditions for microbial metabolism. On the other hand the oxidation and leaching of coal will create an acidic environment by the release and oxidation of sulphides (Kastner *et al.*, 1998). As the pollutants are frequently linked with the pH of contaminated sites, the indigenous microorganisms may not be able to transform PAHs under the prevalent acidic or alkaline conditions. Therefore, it is common practice to adjust the pH at these sites, for example by the addition of lime (Alexander, 1995). Many pollutants determine the fate of soil pH, However soil acidity is also affected by microbial activity, where fungi are found to be more active in acidic pH while bacteria tend to be active in neutral/alkaline pH (Brady & Weil, 2002). Few investigations show phenanthrene degradation, although bacterial and fungal growth is significant, since phenanthrene removal was only 40% at pH 5.5 after 16 days, whereas at neutral pH values, phenanthrene removal was 80%. If an acid pH is considered as an important abiotic factor that requires lime, suggests that consortium of both fungi and bacteria are necessary to accomplish removal (Kastner *et al.*, 1998). These findings show that future research would benefit from the isolation and characterisation of PAH degrading microorganisms from both acidic and alkaline environments.

The degradation of PAHs can be enhanced and carried out faster by optimising the pH conditions. The various findings related to pH of soil at various contaminated sites indicate that *in situ* microorganisms are tolerant of the site conditions. These may also have the potential to metabolise PAHs in sub-optimal conditions (Bamforth &

Singleton, 2005a). Most heterotrophic bacteria and fungi favour a pH near neutrality, with fungi being more tolerant of acidic conditions. Extremes in pH, as can be observed in some soils, would therefore be expected to have a negative influence on the ability of microbial populations to degrade hydrocarbons (Leahy & Colwell, 1990). Low water solubility and dissolution rates of hydrophobic substrates are always limiting factors during the process of biodegradation. The rate of mass transfer can be enhanced in order to increase the degradation rate. However, the mass transfer can be achieved by the use of small substrate particles, thereby increasing the surface area and resulting in a higher dissolution rates (Tiehm, 1994).

2.26.8 Soil pH determination

Soil pH is determined by electrometric and colorimetric methods. These methods are found to be rapid and accurate.

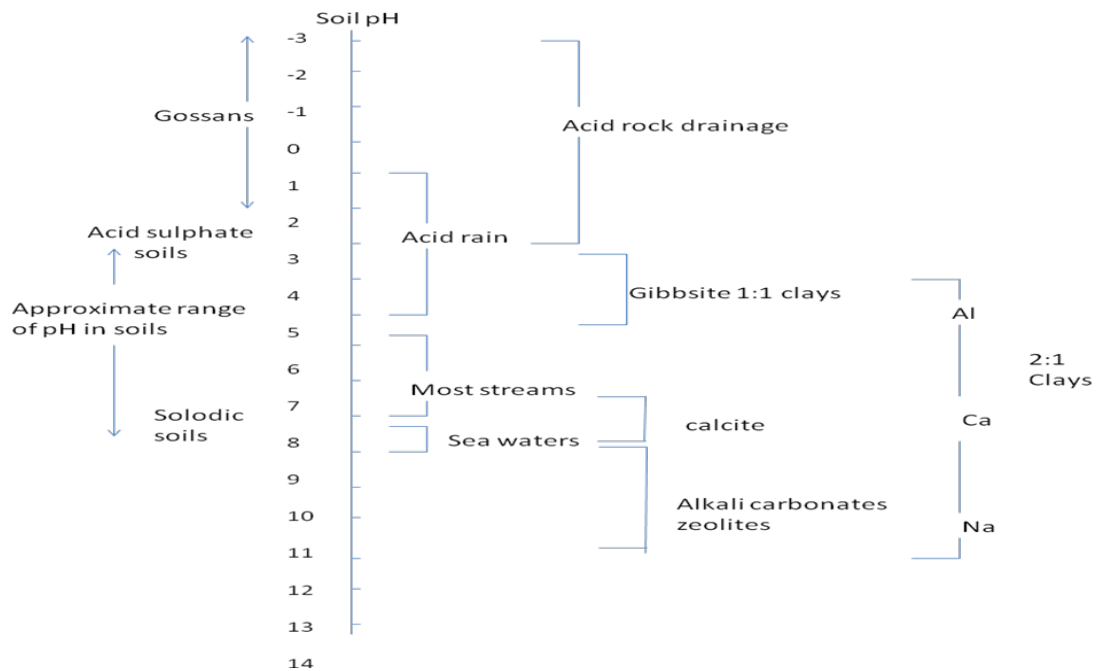


Fig. 14: Soil and other materials approximate pH ranges (Chesworth, 2008).

Table 3: Interpretation of field pH

Field pH rating	Soil pH	Field interpretation
Extremely acid	4.0	Free acid present, oxidation of iron sulphides (FeS ₂) to sulphuric acid (H ₂ SO ₄).
Strongly acid	5.0 5.5	Soluble aluminium (Al ³⁺) present
Moderately acid	6.0	Common range of soil pH for crop production on non-calcareous soils.
Slightly acid	6.5	Exchangeable cations dominated by Ca ²⁺ , Mg ²⁺ and K ⁺
Neutral soil pH	7.0	
Slightly alkaline	7.5	
Moderately alkaline	8.0	Free CaCO ₃ can be present
Strongly alkaline	8.5	Sodic soils with potential for excessive Na ⁺

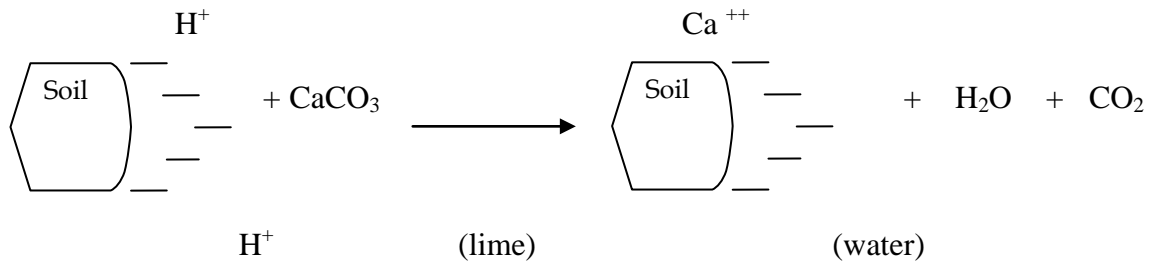
(Chesworth, 2008).

2.27 Liming process in soil

The liming process is the use of calcium and magnesium to enrich soil by the addition of different substances such as limestone, hydrated lime, and chalk. These substances help neutralise the soil acidity and increase the soil activity suitable for microorganisms (Chesworth, 2008).

Soil pH is affected by several factors such as decomposition of organic matter, precipitation, native vegetation, nitrogen fertilization and flooding. Lime reduces soil acidity with respect to Ca⁺ ion and lime replaces two H⁺ ions on the cations exchange

complex. The H^+ ions combine with OH^- (hydroxyl) ions to form water. The pH increases because the acidity source (H^+) has been reduced;



The more mixing of lime to the soil, the more efficient is the neutralization of soil acidity (Chesworth, 2008). Various sites are contaminated by different pollutants like pentachlorophenol, diesel oil, herbicides, poly aromatic hydrocarbons (Kastner *et al.*, 1998) petroleum products (Balba *et al.*, 1991) and munitions compounds (Funk *et al.*, 1997). These contaminated sites are mainly cleaned by endemic or added microbial cells by degradation of these dangerous chemicals. Further techniques like photocatalytic oxidation are carried out to enhance the degradation process or as an initial step towards degradation. Besides, the addition of microorganisms in soil might exhibit low degradative activity due to high concentrations of polluting chemicals (Awasthi *et al.*, 2000).

Chapter: 3.0

General Materials and Methods

3.0 Generic materials and methods

3.1 Experimental soil

Commercially available J. Arthur Bower's topsoil (J. Arthur Bower's Ltd.) was used throughout the experiments. The reason for using J. Arthur Bower's topsoil was to provide a constant soil of neutral pH for all experimental studies.

Table 3.1: Characteristic of experimental soil

Characters	Content
Soil Name	J. Arthur Bower's topsoil
pH	7
Water holding capacity (WHC)	41%
Sand content	10%
Silt content	75%
Clay content	15%

3.2 pH adjustment of soil

The pH of J. Arthur Bowers topsoil is 7.0. This study required soil pH of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.5, 8.0 & 8.5 respectively. For pH adjustment 1 M HCl (Sigma) and Na₂CO₃ (0.01 M) (sigma) were prepared, mixed with 10 ml of distilled water and 5 g of soil in centrifuge tube, was vortexed for 2 min, left at room temperature for 30 min and added to the soil to make J. Arthur Bower's topsoil more acidic and basic (Kastner *et al.* 1998).

Similarly, the pH adjustment was confirmed with 1000 g of soil and the amount of 1 M HCl (Sigma) and 0.01 M Na₂CO₃ (Sigma) required to adjust the pH was monitored.

3.3 PAHs standard solution preparation

In this study phenanthrene, anthracene, fluoranthene and pyrene purchased from

Sigma-Aldrich Germany, were used throughout experimental work as model PAHs. A standard curve was plotted using the following concentrations 50, 40, 30, 20, 10 ppm and 1ppm respectively. Each of these individual PAHs 100 mg was dissolved in 1000ml of acetonitrile to make 100 ppm of standard stock solution which was further diluted to 50, 40, 30, 20, 10 ppm and 1 ppm concentrations to produce a standard curve for HPLC analysis. Carbazole (Sigma) was used as an internal standard.

3.4 Preparation of PAHs solution

The PAH compounds used in the experiments exist as crystalline solids at room temperature. 100 mg of each PAH was added to 1000 ml of n-hexane and used to contaminate J. Arthur Bowers topsoil giving a final concentration of 100 ppm.

3.5 Contamination of soil with PAHs solution

The weight of the pots containing J. Arthur Bower's topsoil before adding the n-hexane along with PAHs was measured. J. Arthur Bower's topsoil containers contaminated with these four PAHs were kept overnight in a fumehood in order to evaporate the n-hexane. The weight of the soil was measured that until dry to ensure n-hexane was completely evaporated.

3.6 HPLC analysis

An HPLC pump Dionex P680 (Dionex) was connected with UV detector (UVD 170 U) (Dionex) set at 252 nm, also the pump and detector was connected to a computer equipped with Chromeleon chromatography management software version 6.6. The Chromeleon chromatography software was used for quantification of PAHs throughout the experiment. A 4 µm particle size silicon column (C16 and C18) of

35cm length was used with a mobile phase of 90% acetonitrile and 10% of HPLC grade water (de-ionized water) with the flow rate set isocratically at 0.8 ml/min. PAH concentrations in samples were calculated against standard curves and the percentage of PAH remaining in the soil samples were calculated using the internal standard as a correction factor. The mean values were calculated for replicates and standard deviations were quantified. Remaining PAH percentages were calculated by dividing the PAH residue concentration by the PAH original concentration.

3.6.1 Mobile phase preparation for HPLC

Acetonitrile: de-ionised water (milli-Q water) was used in a ratio of 90:10 and degassed under vacuum. Fresh mobile phase was prepared daily to run the HPLC samples.

3.6.2 Preparation of extraction solvent

Extraction solvent was prepared using Carbazole (200 mg) dissolved in 1 litre of acetonitrile. 1.5 ml of extraction solvent was added to 0.5 g of soil sample prior to extraction.

3.6.3 Extraction method

Extraction was carried out using bench top rotor (Sigma Aldrich) for 15 mins. The samples were centrifuged for universal rpm and supernatant was transferred to fresh 2 ml Eppendorf tube for HPLC analysis.

3.7 Statistical analysis

The data obtained from experiments were used for statistical analysis. The final

graphed values are represented as mean (Standard deviation) SD. Statistical analysis was carried out performing calculations, analyzing and visualizing data in SPSS Statistic version 16.0. And all the graphs were plotted in Microsoft office Excel 2007 and graph pad prism.

Data analyses were carried out using SPSS Statistic version 16.0. Least significant difference (LSD) and Tukey's HSD between two different wavelengths was calculated using variance post hoc test in SPSS analysis. Post hoc test including LSD (least Significant difference) and Tukey's HSD at the different time points and each individual pH was performed using SPSS Statistics version 16.0.

Chapter: 4.0

Photo-catalytic oxidation of PAHS

Aim: To study the effect of soil pH on photo-catalytic degradation of polycyclic aromatic hydrocarbons (PAHs).

4.1 Introduction

In the natural environment, PAHs undergo an important reaction called photolysis (Sigman & Zingg, 1994; Sigman *et al.*, 1998). Photocatalysis (also called photolysis) is a process which uses catalysts such as Titanium dioxide (TiO_2) which facilitates photoreaction in order to degrade the toxic compound. TiO_2 a photo-catalyst is a chemical compound that, in presence of various wavelengths of UV light becomes highly reactive. TiO_2 induced photo-catalytic degradation of a variety of organic substrates is gaining attention due to its potential to degrade PAHs, specifically the PAHs in the environment (Wen *et al.*, 2002).

Photo-catalytic oxidation (PCO) of PAHs occurs either in solution or in solid phase and also when catalyst is adsorbed onto solid substances. However, recently it has been investigated that photo-catalytic degradation of PAHs may occur in aqueous TiO_2 suspensions (Wen *et al.*, 2002). It has been found that when aromatic compounds are exposed to UV light, partially oxidised intermediates of the aromatic compounds are produced which are more susceptible to degradation than their parent compounds. Because of this property of aromatic compounds, photo-degradation has been recommended as an early stage strategy for biodegradation (Mueller *et al.*, 1997). Photo-degradation of PAHs in the presence of a catalytic solution is considered as an oxidative process which has been further augmented in the presence of photo-inducers. The polarity of the solvent is directly proportional to the rate of the degradation process hence, the higher the polarity of the solvent, the faster the degradation process. Thus, PAH photo-decomposition initiated by photo-ionization results in the production of PAH radical cations and hydrated electrons which further destroy PAH in the

presence of water (Zeep & Schlotzhauer, 1979; Zepp, 1982). PCO, one of the many advanced oxidation processes, relies on the generation of $\bullet\text{OH}$ by photo-catalysts (e.g. titanium dioxide semiconductor, TiO_2) to trigger oxidative degradation (Zhang *et al.*, 2008). TiO_2 , a semiconductor can be used in photo-catalysis when exposed to ultraviolet (UV) light irradiation, due to its ability to transfer electrons and promote oxidation or reduction which plays a vital role in photo-catalysis (Zhang *et al.*, 2008).

A number of studies on the adsorption of PAHs on silica, alumina and other surfaces have been reported. However, the present work focuses on the possible advantages of various photo-catalytic processes using TiO_2 for the degradation of PAHs present in soil.

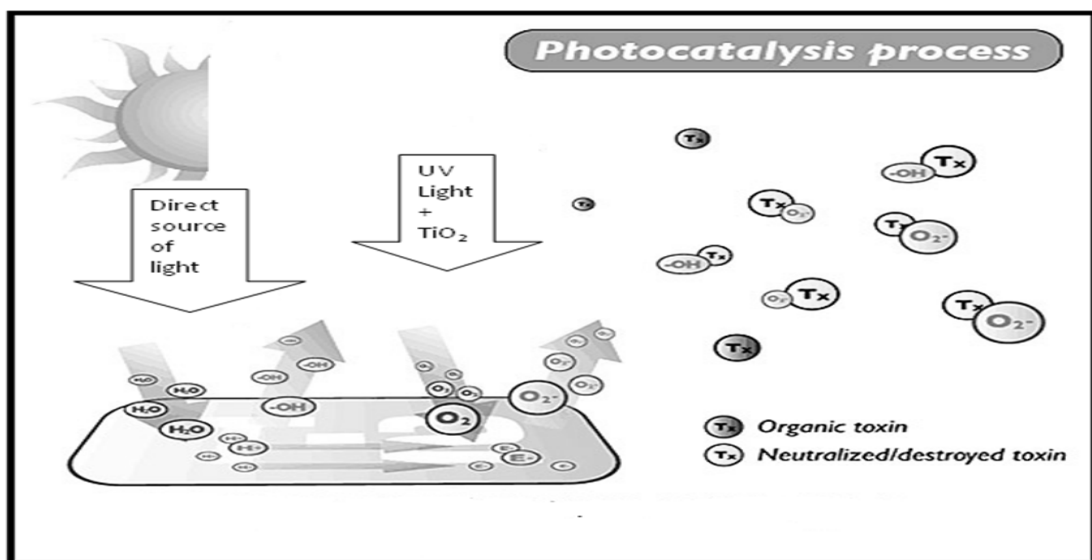


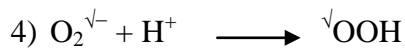
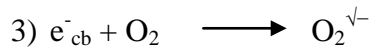
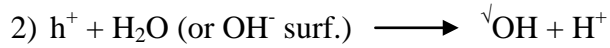
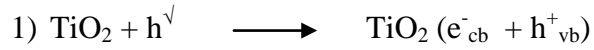
Fig. 4.1: Photo-catalysis in presence of catalyst:

The energy from light/UV light in presence of catalyst gets charged and de-toxicify with oxidation reactants: hydroxyl radicals and superoxide anions. This reactance decomposes toxic organic substances through oxidation (eco-cleanse.net).

Under UV irradiation (figure 4.1), TiO_2 particles absorb UV light whose energy is equal or greater than 3.2 eV of the band gap energy and generate electron/hole pairs (Eq. (1). Holes (h^+) in the valence band are subsequently trapped by OH^- ions or H_2O to yield OH^\bullet radicals (Eq. (2), while the electrons (e) in the conduction band are

trapped by pre-adsorbed O₂ molecules to yield superoxide radicals (O₂^{•-} species) which can interact with protons to generate OOH radicals (Eqs. (3) (Zhang, 2006).

Equations:



Strong oxidation activity is represented by the electron-hole, OH[•], and OOH[•]. Almost all of the organic compounds can be completely mineralized due to such a strong oxidation activity. One of the most preferred or important research process, photo-catalytic oxidation, depends on generation of OH[•] by a photo catalyst in the presence of catalyst (e.g. titanium dioxide, TiO₂) in order to perform oxidative degradation under UV irradiation. Thus, in a photo-catalytic process, OH[•] and/or OOH[•] radicals play a vital role and thereby reaction occurs directly between the electron/hole pair and organic substrate on the surface (Woo *et al.*, 2009).

However, very few studies have investigated the photo-catalytic degradation of PAHs on soil surfaces using TiO₂ as the catalyst under UV irradiation. Investigating photo-catalytic degradation using a catalyst under varying abiotic conditions particularly soil pH, to enhance the degradation process is one of the objectives of this particular study.

4.2 Materials and methods

4.2.1 Chemicals

The test PAHs, namely phenanthrene (PHE), anthracene (ANT), fluoranthene (FLU) (Sigma) and pyrene (PYR) (Fluka) were used throughout the experiment. Acetonitrile (HPLC grade), n-hexane was the solvent used (Fisher Scientific, UK). Particles of TiO₂ were purchased from Sigma Aldrich, UK.

Table 4.1: pH adjustment of Arthur Bower's topsoil

Soil pH	Acidic pH Amount of 1M HCL	Basic pH Amount of 0.1M Na₂CO₃
pH 5	260 µl + 10ml de-ionized water + 5 g of soil	-
pH 5.5	190 µl + 10ml de-ionized water + 5 g of soil	-
pH 6	132 µl + 10 ml de-ionized water + 5 g of soil	-
pH 6.5	90 µl + 10 ml de-ionized water + 5 g of soil	-
pH 7	-original pH	-original pH
pH 7.5	-	40 µl + 10 ml de-ionized water + 5 g of soil
pH 8	-	54 µl + 10 ml de-ionized water + 5 g of soil
pH 8.5	-	80 µl + 10 ml de-ionized water + 5 g of soil
pH 9	-	98 µl + 10 ml de-ionized water + 5 g of soil

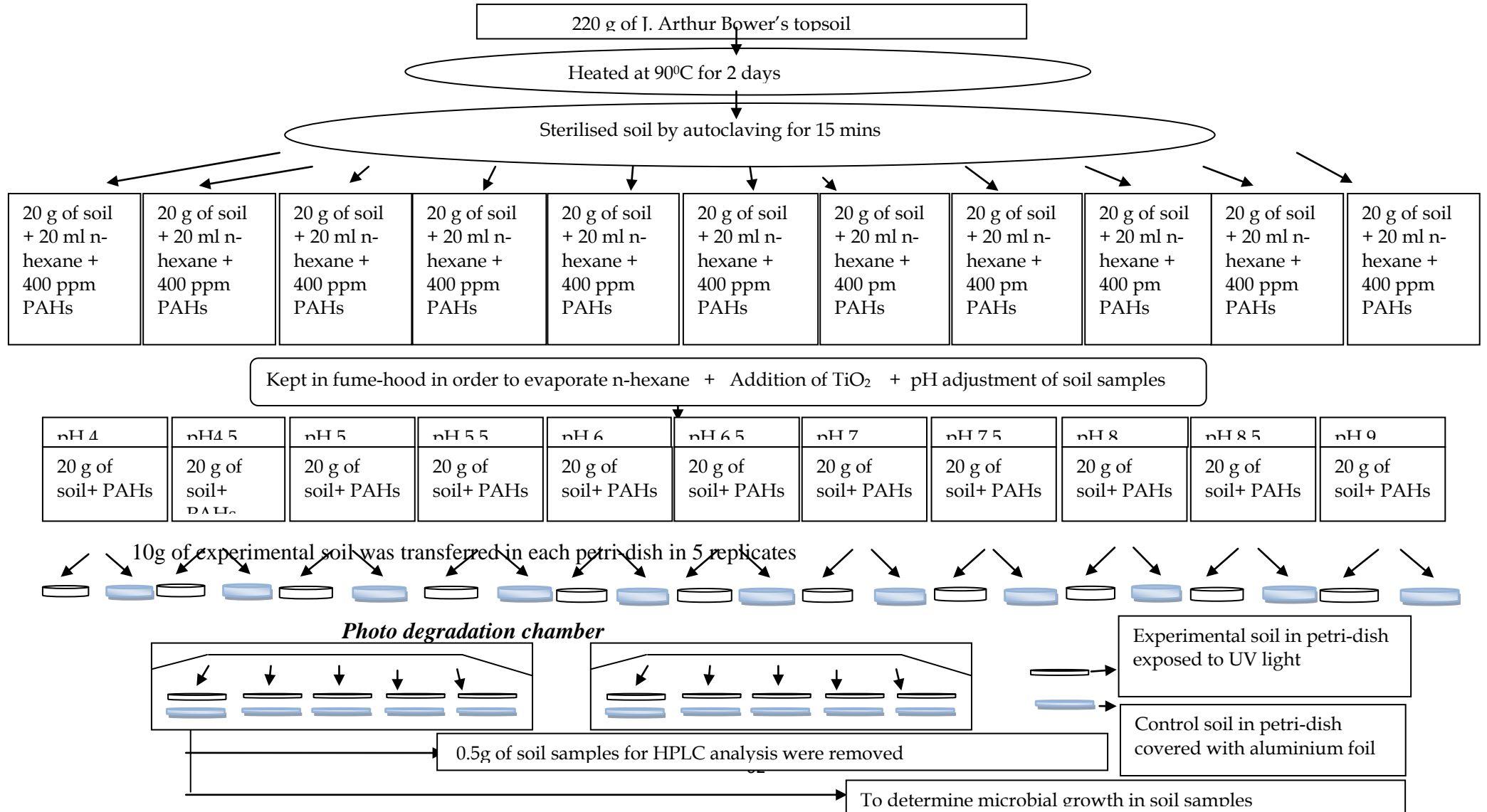
Based on table 4.1, calculations were performed for larger volume (120 g) of experimental soil samples.

4.2.2 Standard curve for photo-catalytic oxidation

a) PAHs standard solution:

In this experiment phenanthrene (Sigma Aldrich), anthracene (Sigma Aldrich), fluoranthene (Sigma Aldrich), pyrene (Fluka) were used as model PAHs. A standard curve was made using the following concentrations 50, 40, 30, 20, 10 ppm and 1 ppm of each PAH. 100 mg of each of these individual PAHs were dissolved in 1000 ml of acetonitrile to make 100 ppm of standard stock solutions which were further diluted to produce a standard curve for HPLC analysis. Carbazole (Sigma Aldrich) was used as the internal standard.

Fig. 4.2 Schematic representation of experimental design



4.2.3 Photo-catalytic degradation of PAHs

See figure 4.2 for a schematic representation of the experimental design.

4.2.4 Photo degradation chamber

Photo degradation studies were performed in a chamber as shown in figure 4.2. The UV irradiation intensity was 1041 Wcm^{-2} . The UV lamps (Phillips ATLD 20W, Model UVA, UVB and UVC) were set at wavelengths of 254, and 375 nm. 90 mm plastic petri dishes containing the experimental soil samples were placed under the UV light for photo irradiation. The distance between the lamps and soil samples was 120 mm. Temperature within the chamber was maintained at 20°C throughout all the experiments.

4.2.5 Dissolution of PAH compounds and TiO_2 as catalyst in J. Arthur Bower's topsoil

The PAH compounds used in these experiments exist as crystalline solids in a glass bottle at room temperature. 20 mg of each PAH was added to 20 ml of n-hexane in 500 ml a sterile conical flask and used to contaminate 20 g of J. Arthur Bower's topsoil giving a final concentration of 100 ppm.

To study the influence of soil pH, the pH of PAH contaminated soil sample was adjusted to range from pH 4.0 to pH 9.0 at half pH intervals (as described in section 4.2.1) and 2% aqueous TiO_2 (Sigma Aldrich) was added. The weight of the pots containing J. Arthur Bower's topsoil before adding the n-hexane along with PAHs was measured. J. Arthur Bower's topsoil contaminated with PAHs were kept overnight in fumehood to evaporate the n-hexane. The weight of the soil was measured again to make sure n-hexane had completely evaporated until the contaminated soil was dried. All the processes were performed in a fumehood which was switched on.

4.2.6 Monitoring pH and moisture content of the experimental soil

140 g of soil was transferred to seven different pots in order to monitor the pH of the soil along with PAH. Furthermore, deionised water was added to maintain a 30% moisture content of the soil and the pH was adjusted as described in section 4.2.1.

4.2.7 Sampling analysis and sampling point

From the eleven different pots containing soil of each pH treatment, 20 g of soil was transferred into five petri-dishes resulting in 5 replicates. All replicates were maintained at 20⁰C in UV light chamber throughout the experiment. Treated samples from the Petri-dish were removed at 0, 24, 48, 72, 96, 120 hours respectively.

4.2.8 Samples for HPLC analysis and PAH extraction

0.5 g of treated sample, from the 5 replicates of petri-dishes was transferred into 1.5 ml Eppendorf tubes. PAHs were extracted from the soil by adding 1.5 ml of acetonitrile to the eppendorf containing 200 ppm carbazole as an internal standard to 0.5 g of soil before analysis by HPLC. Samples were mixed well using round vortex mixer fitted with multi sample holder which holds a total of 12 samples (Sigma Aldrich) for 5 minutes prior to HPLC analysis.

4.2.9 HPLC analysis

As described in section 3.6 (General materials n methods).

4.2.10 Mobile phase preparation for HPLC

As described in section 3.6.1 (General materials n methods).

4.3 Results

Photo-catalytic degradation of PAHs

Objective: To monitor PAH photo-catalytic degradation rate in experimental soil at varying pH.

4.3.1 HPLC analysis of PAH

To study the effect of abiotic factors particularly soil pH on the rate of photo-degradation, HPLC analysis was employed.

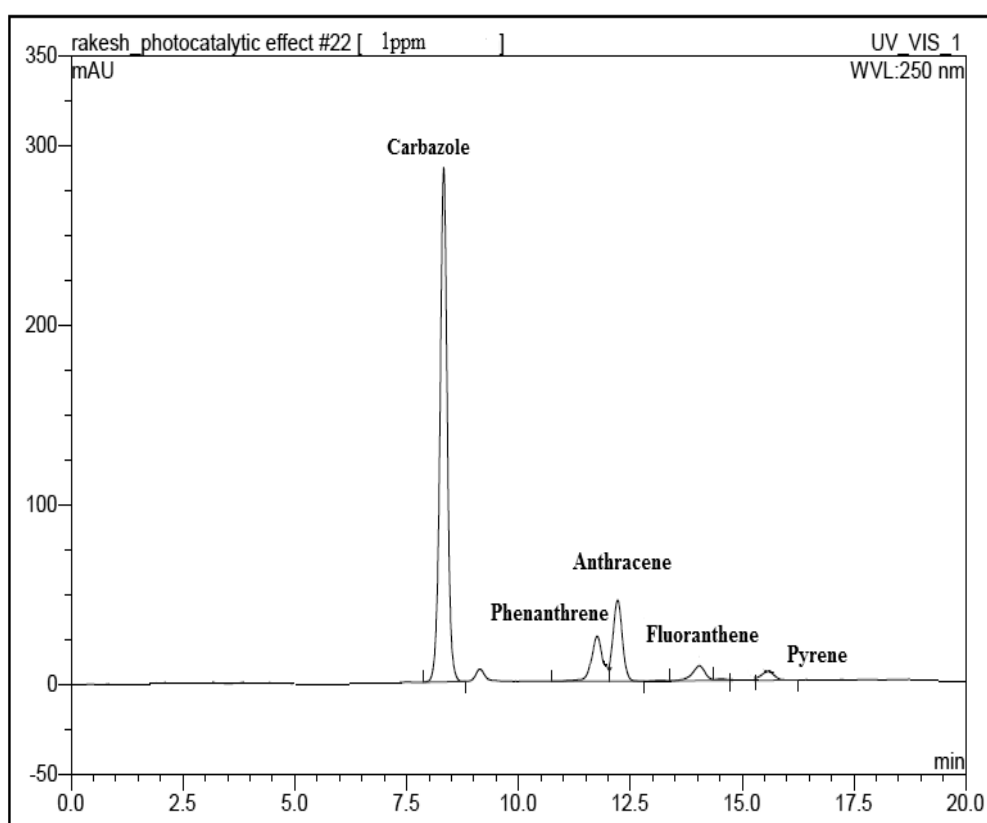


Fig. 4.3: Standard Chromatogram for HPLC analysis of four PAHs (concentration 1 ppm) and carbazole (20 ppm) with peak height against time.

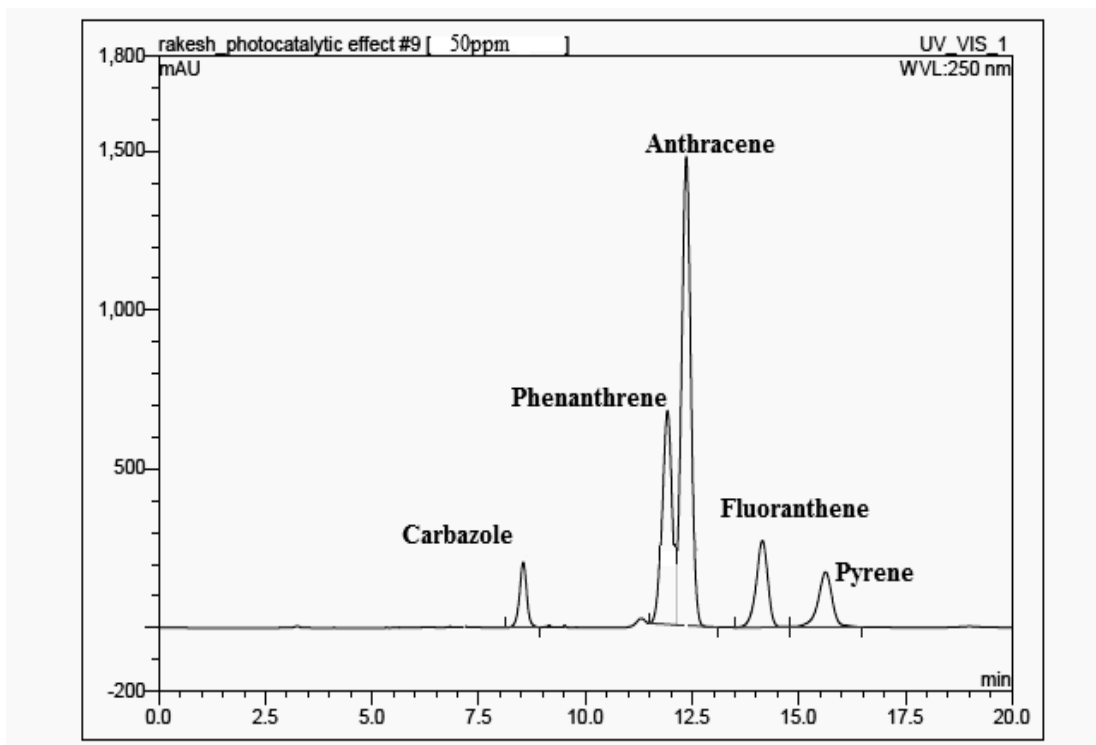


Fig. 4.4: Standard chromatogram for HPLC analysis of four PAHs (concentration 50 ppm) and carbazole (20 ppm) with peak height against time.

Figures 4.3 and 4.4 show HPLC standards for 1 ppm and 50 ppm concentration of the four different PAHs dissolved in acetonitrile. The four PAHs present in the contaminated soil were also extracted using acetonitrile. Carbazole was the internal standard representing the first peak in both chromatograms at 20 ppm with a retention time of 8min. In figure 4.3 carbazole peak area was 300 mAU/min, similarly the peak area in figure 4.4 was 300 mAU/min with no difference in retention time. Therefore, constant results were found for carbazole. Phenanthrene follows carbazole with a retention time of 11 min and peak area of 30 mAU/min for 1 ppm and 700 mAU/min for 50 ppm. Anthracene is the third peak and second PAH to elute with a retention time of 12.5 mins. Anthracene, and phenanthrene peaks merge in both chromatograms before reaching the x axis. The split peak facility of the chromeleon software was implemented to statistically attribute peak area to these two PAHs. Anthracene at 1

ppm resulted in a peak area of 50 mAU/min and 1500 mAU/min for 50 ppm. The fourth peak and third PAH to elute was fluoranthene at 13 mins with a peak area of 20mAU/min for 1ppm and peak area of 300 mAU/min for 50ppm with no differences seen in retention time. The final PAH to elute was pyrene with a peak area of 10 mAU/min for the 1ppm concentration whereas the peak area for pyrene in 50 ppm concentration was 200 mAU/min.

4.3.2 Standard graph for polycyclic aromatic hydrocarbons

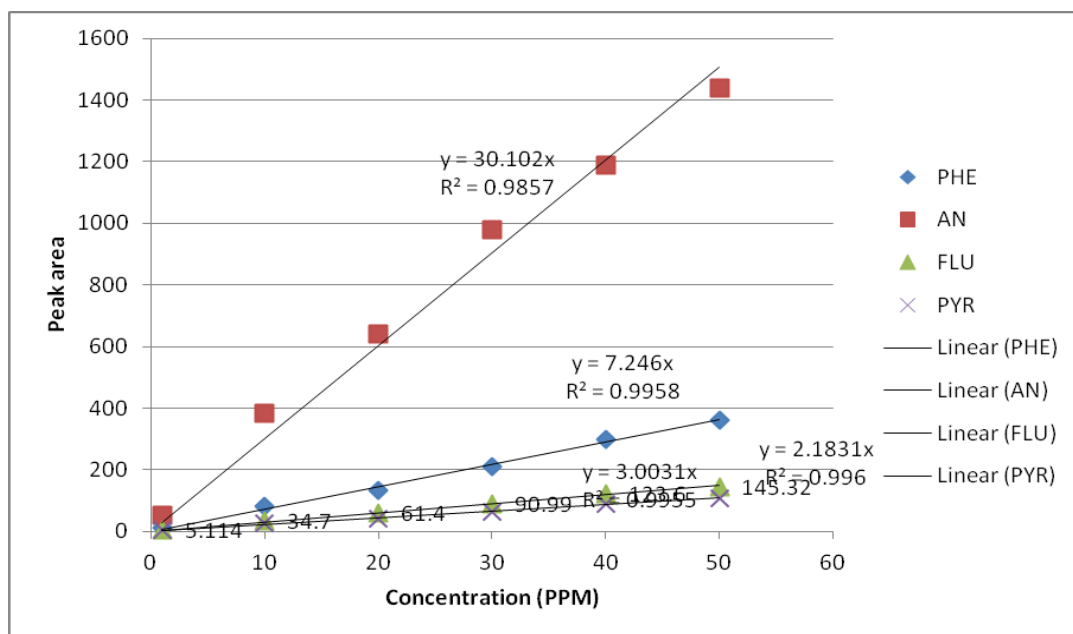


Fig. 4.5. HPLC standard curve of four PAH showing peak area against concentration. PAH used and their symbol abbreviations are (PHE) Phenanthrene; (AN) Anthracene; (FLU) Fluoranthene; (PYR) Pyrene

The peak areas obtained from running standards of the four PAHs at 1 ppm, 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm were used to plot standard curves of peak area against the PAH concentration (figure 4.5). The chromeleon software was used to calculate a linear regression for each PAH (figure 4.5). All PAH have r^2 (regression coefficient) values above 0.97 whilst the slope was estimated and displayed as Y values. The r^2 value was 0.987 for phenanthrene, 0.978 for fluoranthene, 0.979 for

pyrene and r^2 values for anthracene was 0.983 respectively. The Y value was around 35x for anthracene, 14x for phenanthrene, whilst fluoranthene and pyrene were much lower at 6.42x and 4.08x respectively.

In order to have a full evaluation of the extraction efficiency of the four PAHs, 100 ppm of each individual PAH was added to J. Arthur Bower's topsoil and extracted with acetonitrile. The re-extraction efficiency of the four PAHs obtained from these samples ranged from 52.81 to 74.69 % (table 4.2).

Table 4.2: Extraction efficiency of four PAHs from J. Arthur Bowers topsoil

PAH used	Amount of PAH added (ppm)	% efficiency for experimental values.
Phenanthrene	100	74.69
Anthracene	100	68.42
Fluoranthene	100	64.98
Pyrene	100	52.81

4.3.3 Degradation of polycyclic aromatic hydrocarbons over time

Figures 4.6 to 4.13 exhibit the degradation curves obtained for the four different PAH in treated soil samples at varying pH under UV irradiation at 254 nm and 375 nm. PAH remaining is displayed as a percentage of the HPLC quantification results obtained after re-extraction at time 0. The control at both wavelengths exhibited little degradation figure (4.6 to 4.13 A) in contrast to the samples exposed to UV light figure (4.6 to 4.13 B). Greater degradation was more evident at 375 nm (figure 4.6 to 4.9) than 254 nm (figure 4.10 to 4.13) for all PAHs with a significance value of $P < 0.05$ obtained (Post hoc test including LSD and Tukey's test). Phenanthrene showed the highest degradation followed by anthracene, pyrene and fluoranthene. At 375 nm phenanthrene was 80-85% degraded and around 60- 65% degraded at 254 nm. The photo-catalytic degradation rate of anthracene was slower than phenanthrene whilst its degradation rate generally increased at acidic pH with most rapid rate evident at pH 6.5. At 375 nm anthracene was 75-80% degraded and at wavelength 254 nm degradation was 65-70%. Fluoranthene followed after anthracene was 70% degraded at 375 nm and 65% at 254 nm. Around 65% degradation was observed for pyrene at 375 nm and 60% degradation observed at 254 nm.

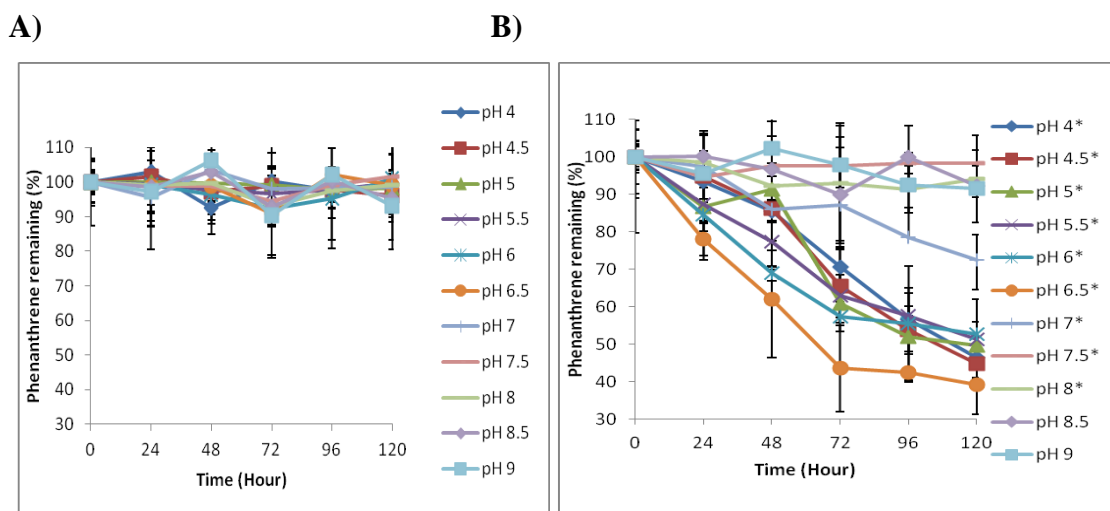


Fig. 4.6: Percentage phenanthrene remaining in J. Arthur Bower's topsoil at different pH over time during exposure to UV light at 375 nm in the presence of TiO₂.

A): Percentage phenanthrene remaining in control samples not exposed to UV light.
 B): Percentage phenanthrene remaining in experimental sample exposed to UV light at 375 nm. *P<0.05 indicates significant difference between control and experimental sample.

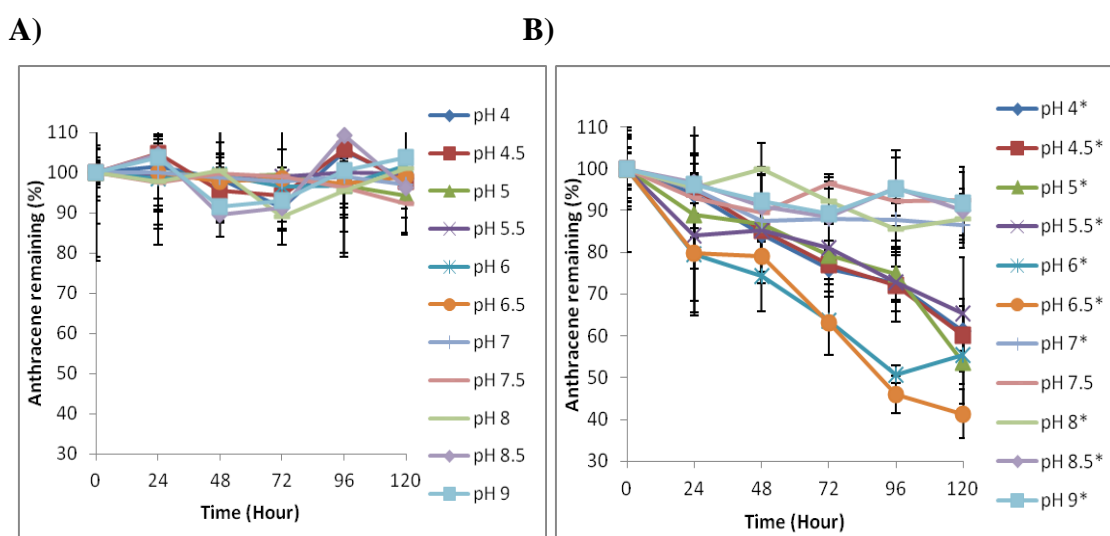


Fig. 4.7: Percentage anthracene remaining in J. Arthur Bower's topsoil at different pH over time during exposure to UV light at 375 nm in the presence of TiO₂.

A): Percentage anthracene remaining in control samples not exposed to UV light.
 B): Percentage anthracene remaining in experimental sample exposed to UV light at 375 nm. *P<0.05 indicates significant difference between control and experimental sample.

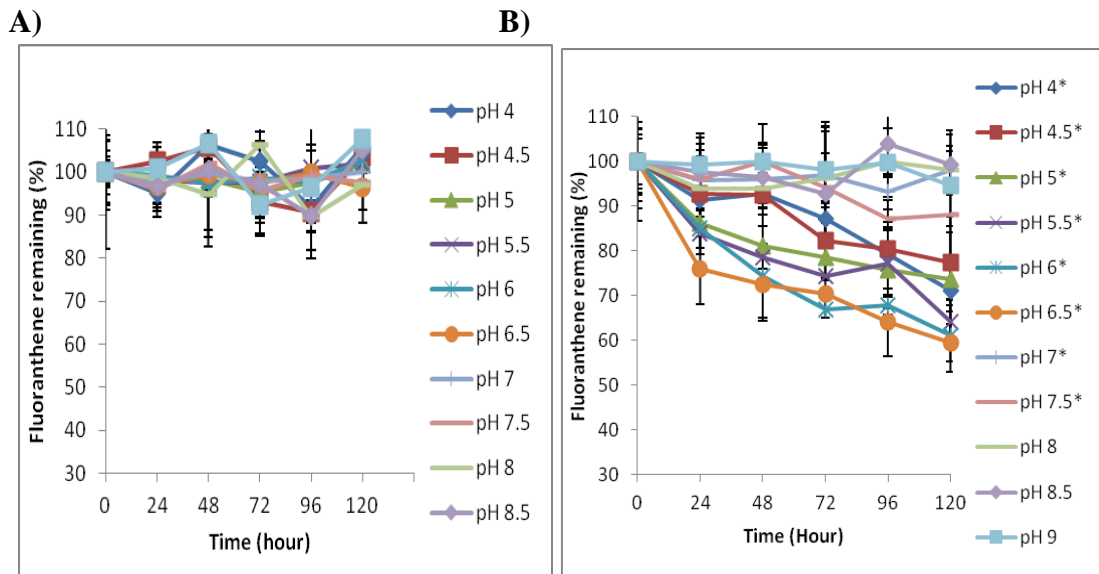


Fig. 4.8: Percentage fluoranthene remaining in J. Arthur Bower's topsoil at different pH over time exposure to UV light at 375 nm in the presence of TiO_2 .

A): Percentage fluoranthene remaining in control samples not exposed to UV light. B): Percentage fluoroanthene remaining in experimental sample exposed to UV light at 375 nm. * $P < 0.05$ indicates significant difference between control and experimental sample.

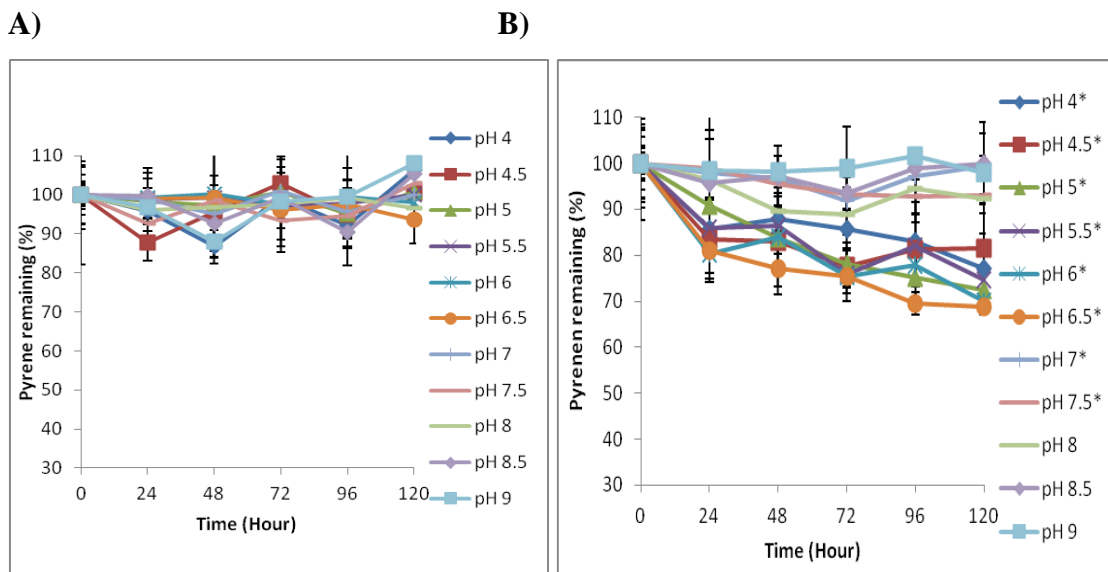


Fig. 4.9: Percentage pyrene remaining in J. Arthur Bower's topsoil at different pH over time during exposure to UV light at 375 nm in the presence of TiO_2 .

A): Percentage pyrene remaining in control samples not exposed to UV light. B): Percentage pyrene remaining in experimental sample exposed to UV light at 375 nm. * $P < 0.05$ indicates significant difference between control and experimental sample.

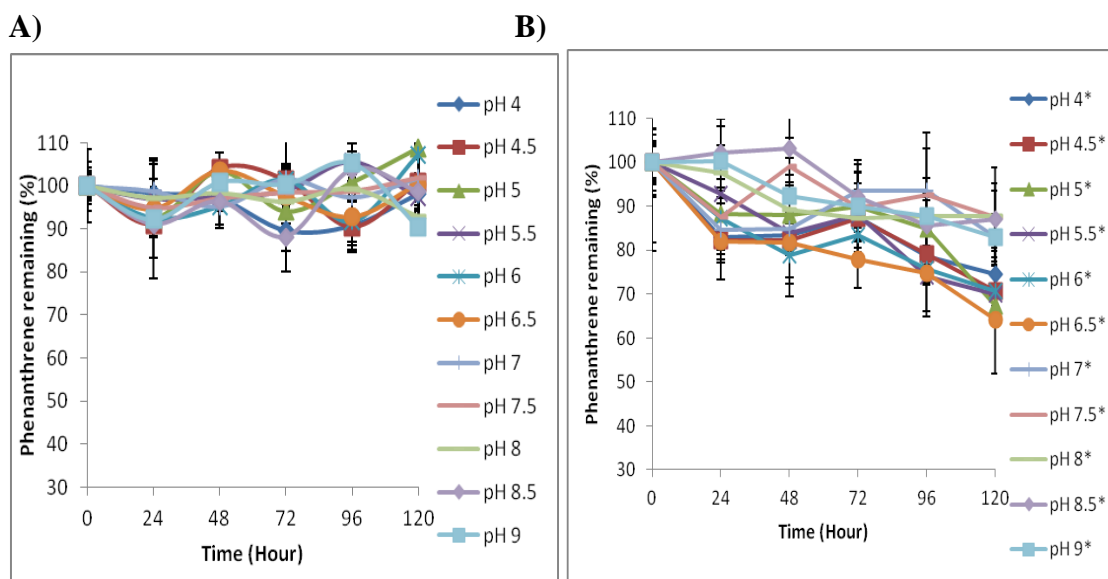


Fig. 4.10: Percentage phenanthrene remaining in J. Arthur Bower's topsoil at different pH over time during exposure to UV light at 254 nm in the presence of TiO₂.

A): Percentage phenanthrene remaining in control samples not exposed to UV light.
 B): Percentage phenanthrene remaining in experimental sample exposed to UV light at 254 nm. *P<0.05 indicates significant difference between control and experimental sample.

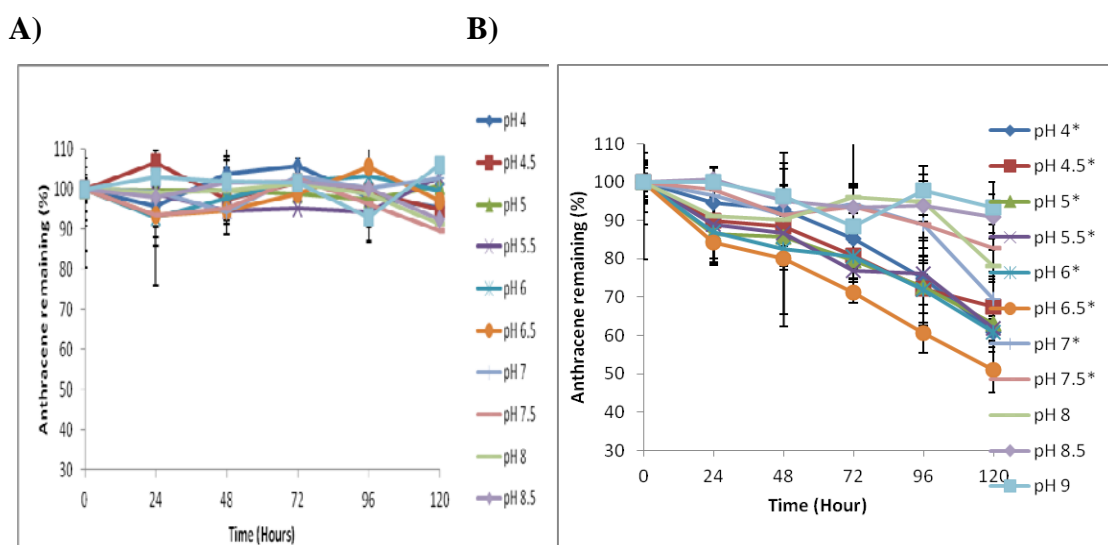


Fig. 4.11: Percentage anthracene remaining in J. Arthur Bower's topsoil at different pH over time during exposure to UV light 254 nm in the presence of TiO₂.

A): Percentage anthracene remaining in 254nm control samples not exposed to UV light.
 B): Percentage anthracene remaining in experimental sample exposed to UV light at 254 nm. *P<0.05 indicates significant difference between control and experimental sample.

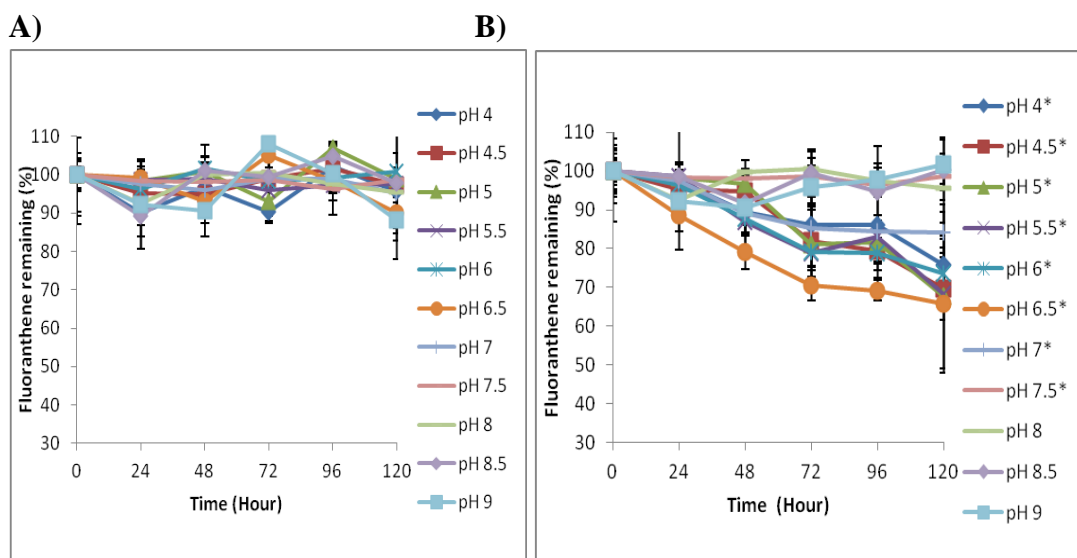


Fig. 4.12: Percentage fluoranthene remaining in *J. Arthur Bower's* topsoil at different pH over time during exposure to UV light 254 nm in the presence of TiO_2 .

A): Percentage fluoranthene remaining in control samples not exposed to UV light. B): Percentage fluoranthene remaining in experimental sample exposed to UV light at 254 nm. * $P < 0.05$ indicates significant difference between control and experimental sample.

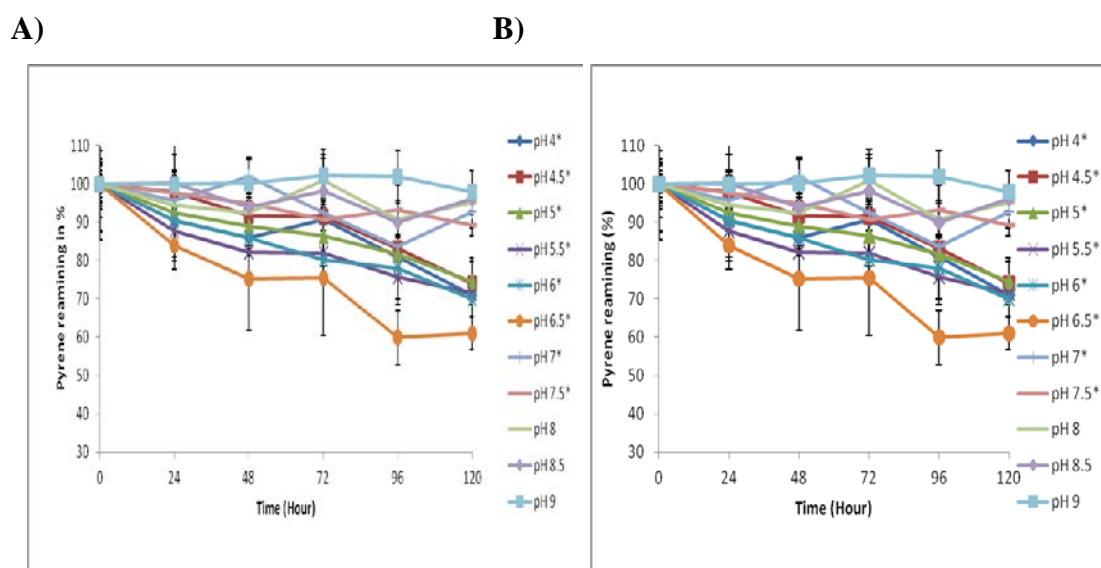


Fig. 4.13: Percentage pyrene remaining in *J. Arthur Bower's* topsoil at different pH over time during exposure to UV light at 254 nm in the presence of TiO_2 .

A): Percentage pyrene remaining in control samples not exposed to UV light. B): Percentage pyrene remaining in experimental sample exposed to UV light at 254 nm. * $P < 0.05$ indicates significant difference between control and experimental sample.

Degradation rate was studied using HPLC for each PAH by calculating the remaining percentage of individual PAH. Phenanthrene, anthracene, fluoranthene and

pyrene percentage were constant in all controls with very low degradation rates observed not exposed to UV irradiation. Greater degradation was observed at 375 nm compared to 254 nm with significance $P < 0.05$. Time 0, was plotted at 100% in order to show the remaining percentage of individual PAHs in soil pH.

Table 4.3: Degradation of polycyclic aromatic hydrocarbons effect of soil pH over time.

	Highest degradation rate		Lowest degradation rate	
	375 nm	254 nm	375 nm	254 nm
Wavelength	375 nm	254 nm	375 nm	254 nm
Phenanthrene	pH 6.5	pH 6.5	pH 8	pH 8.5
Anthracene	pH 6.5	pH 6.5	pH 9	pH 8
Fluoranthene	pH 6.5	pH 6.5	pH8	pH 9
Pyrene	pH 6.5	pH 6.5	pH 9	pH 9

It was demonstrated in this study that photo catalytic degradation rates were greater in acidic soil pH (pH 5.0, 5.5, 6.0, 6.5) when compared to alkaline soil pH (7.5, 8.0, 8.5, and 9.0) and neutral soil pH (7.0) for each of individual phenanthrene, anthracene, flouranthene and pyrene. In figure 4.6-4.13, pH 6.5 exhibits greater degradation rate followed by pH 5.5, pH 6.0, pH 5.0, pH 4.5 and pH 4. However, at alkaline soil pH lower degradation rates were evident. Among alkaline soil pH greater degradation was measured in pH 7.5 followed by pH 8.5, pH 8.0 and pH 9.0.

Thus, UV irradiation by two different wavelengths in experimental soil in the presence of TiO_2 resulted in greater degradation at soil $pH < 6.5$, whereas lower degradation resulted under alkaline conditions $pH > 6.5$.

In general, results obtained during photo-catalytic degradation exhibited high influence on soil pH with highest rate of degradation obtained for low-molecular weight (LMW) PAH (phenanthrene and anthracene) when compared to high molecular weight PAH (fluoranthene and pyrene).

4.4 Discussion

Objective: To monitor PAH photo-catalytic degradation rate in experimental soil at varying pH.

Soil pH is one of the most important factors that influences the degradation processes in soils (Ferrarese *et al.*, 2008).

4.4.1 Standard chromatograms for HPLC

Standard curves were prepared using the HPLC analysis. Carbazole was used at the same concentration (20 ppm) in all experiments as an internal standard to monitor reproducibility of results. Concentrations of PAHs used for standard curves were 1ppm, 10 ppm, 20 ppm, 30 ppm, 40 ppm, 50 ppm respectively; as the concentration in experimental samples would not reach levels above 50 ppm and the lower limit of detection is in the region of 1 ppm.

The peaks appearing on the chromatogram were observed based on the number of rings and molecular weights of the PAHs. The order of peaks was phenanthrene followed by anthracene, fluoranthene and at last pyrene. The peak of phenanthrene and anthracene appear to be merged in the chromatogram (section 4.3.1 in figure 4.3 & 4.4). The split peak facility of the chromeleon software was implemented to separate the peaks of the two PAHs (phenanthrene and anthracene) and was reproducible with standard PAH solutions. Anthracene resulted strong signals and appeared with larger peaks due to the linearity of the molecule as UV detection is greater for linear molecules (Irwin *et al.*, 1997).

Phenanthrene and anthracene are three ring compounds with molecular weight of 178.23 and appear before fluoranthene and pyrene (molecular weight 202.26) (Haritash & Kaushik, 2009). Although, anthracene and phenanthrene are stereoisomers,

anthracene is a linear molecule while phenanthrene is non-linear, resulting in an anthracene being more hydrophobic and thus eluting from the column a little slower in the 90:10 acetonitrile: deionised water mobile phase. Peak area of anthracene at a particular retention time gives better symmetry to the molecule which consequently leads to less solubility in extraction solution. Therefore, in general anthracene is expected to degrade slower than phenanthrene followed by fluoranthene and then by pyrene based on their solubility/ hydrophobicity and molecule size (Song *et al.*, 2002). In addition to this, phenanthrene has low molecular weight and is a three ring compound with high solvent solubility (Irwin *et al.*, 1997). Whereas, fluoranthene and pyrene have lower solvent solubility (Haritash & Kaushik, 2009). Pyrene is a fused four ring compound with very low extractability. The low solvent extractability of pyrene might be due to its high hydrophobicity (sorption partition coefficient $\log K_{oc}$: 4.88; water solubility= 0.13 mg l⁻¹) (Irwin *et al.*, 1997).

4.4.2 Standard curve

With respect to chromatogram obtained in the results displayed in figure 19, a standard curve was constructed to study the PAHs peak area in the standard solution based on their retention time. A linear standard curve (figure 4.5) was produced for each PAH with the regression coefficient of 0.97 for phenanthrene, 0.97 for fluoranthene, 0.97 for pyrene and 0.98 for anthracene. The value of the regression coefficient obtained for each calibration curve shows that the correlation between relative peak area and concentration was linear and reproducible within selected concentration range. The Y value representing linear regression equation for phenanthrene was 14.32x and for anthracene was 35.12x, whilst for fluoranthene and

pyrene the values were much lower at 6.42x and 4.08x respectively. Thus, the data obtained from standard chromatogram were reliable and accurate (figure 4.5).

4.4.3 Extraction efficiency

Contaminated J. Arthur Bower topsoil was used to examine the extraction efficiency (table 4.2) of the PAHs using HPLC analysis. All four PAHs were extracted with the greatest extraction rate found for phenanthrene (table 4.2). The total PAH recovered was phenanthrene 74.69%, anthracene 68.42%, fluoranthene 64.98%, and pyrene 52.81%. The extraction efficiency of phenanthrene was highest and of pyrene was the lowest. Alef & Kleiner, (1987) has suggested different extraction efficiency might be due to the poor contact of solvent and soil. PAHs with high-molecular weight may have stronger adsorption and formation of non-extractable residues especially within a complex substrate such as soil (Song *et al.*, 2002). Recovery obtained for phenanthrene and pyrene was consequentially different. In general, relative recovery rates obtained for each PAH were as expected as the molecular weights of phenanthrene and anthracene are the same and for fluoranthene and pyrene are the same. However water solubility and molecular structures are different with greater linearity for anthracene and pyrene resulting in reduced solubility compared to phenanthrene and fluoranthene respectively (Song *et al.*, 2002). Phenanthrene and anthracene are three ring compounds with molecular weight of 178.23 and fluoranthene and pyrene are four ring structures (molecular weight 202.26) (Haritash & Kaushik, 2009). Recovery rates obtained for each individual PAH correlates with the number of aromatic rings and molecular weight of the PAHs.

4.4.4 Photo-catalytic degradation

Soil pH is considered as an important parameter due to amphoteric nature of most semiconductor oxides. The surface-charged particles present in the soil in presence of catalyst are influenced by the photo-semiconductor particles (Kot-Wasik *et al.*, 2004). Therefore, the effect of pH on rate of photo catalytic degradation needs to be considered.

UV irradiation accelerated the photo degradation of phenanthrene, anthracene, fluoranthene and pyrene in this study. Some studies suggest that naphthalene, acenaphthene, anthracene, fluoranthene all undergo efficient photo-catalytic degradation by TiO₂ (Das *et al.*, 1994, Wen *et al.*, 2005). Studies reported by Hoffman *et al.*, (1995) indicated that when TiO₂ is irradiated with light energy greater than its band gap energy (3.2eV), induction (b) and electron (e⁻) and valence band holes (h⁺) are generated. Thus, organic compounds reduces or react with electron acceptors such as O₂, reducing it to superoxide radical anion O₂^{•-} with the help of the photo-generated electrons. The H₂O molecules which are photo-generated holes are adsorbed to OH⁻ radicals at the surface of TiO₂ (Hoffman *et al.*, 1995). On the basis of adsorption of H₂O molecules photo-catalytic processes using TiO₂ could be an effective photo catalytic detoxification method for PAH contaminated soil.

This study demonstrated that photo-catalytic degradation rates were higher in acidic soil and lower in alkaline soil than in neutral soil for phenanthrene, anthracene, fluoranthene and pyrene. This is supported by the work of Zhang *et al.*, (2008) who suggested that H⁺ was favourable for high molecular weight PAH degradation using TiO₂ under UV light, however the same study also suggested that OH⁻ made low molecular weight PAHs (example: phenanthrene) become more degradable. Similar results for PAH photo-catalytic degradation was found by Funk *et al.*, (1997).

Moreover, the lack of degradation at high pH is supported by the work of Zhang *et al.*, (2008) who reported that in pesticide contaminated soil, “raising soil pH by adding $\text{Ca}(\text{OH})_2$ did not significantly alter the photo-catalytic degradation of Diuron when compared to the soil that received no lime.”

In this study, higher degradation rates were obtained of phenanthrene and lower degradation rates of pyrene. Similar results were indicated exhibiting high molar absorptivity and disappearance quantum yield for phenanthrene and pyrene as suggested by Hoffman *et al.*, (1995). The most efficient degradation of PAHs in various contaminated sites is recorded with UV irradiation in presence of the catalyst, TiO_2 (Wilson & Jones, 1993). In these studies, photo-catalytic oxidation degradation was carried out at varying soil pH at 375 nm and 254 nm respectively. The control (soil samples with TiO_2 not exposed to UV light) at both wavelengths exhibited little degradation (figure 4.6 A to 4.13 A) in comparison to the samples exposed to UV light (figure 4.6 B to 4.13 B). During photo-catalytic degradation, 375 nm resulted in greater degradation of each individual PAH compared to 254 nm. Phenanthrene had the highest degradation followed by anthracene, pyrene and last fluoranthene at 375 nm. Phenanthrene exhibited 65% of degradation after five days and 60- 65% was degraded at 254 nm.

The photo catalytic degradation rate of anthracene was slower than phenanthrene whilst its degradation rate generally increased with acidic pH with most rapid rate evident at pH 6.5. At 375 nm anthracene exhibited 55-60% degradation and at 254 nm degradation was 55-60%. Degradation of fluoranthene followed after anthracene exhibiting 60% degradation at 375 nm and 45% at 254 nm. Around 45-50% degradation rate was observed for pyrene at 375 nm and 45% degradation observed at 254 nm.

In all figures (section 4.3.4) for photo-catalytic oxidation, it was observed that soil pH 6.5 gave the fastest rate of photo catalytic degradation in comparison to all other pH. The second greatest rate of degradation was found at pH 6.0. Acid pH resulted in higher degradation rates compared to alkaline pH of soil. Comparatively acidic pH from 4 to pH 6.5 exhibited greater degradation as OH^- and OOH^- radicals which plays important role are highly generated, whilst little degradation was evident at neutral and alkaline pH 7.0 to pH 9.0 where, OH^- and OOH^- radicals might be less. Similarly, Han *et al.*, (2005) reported consistently greater degradation of phenol in acidic soil pH during investigating photo-catalytic oxidation. However, the current investigation suggests soil pH is an important parameter that needs to be monitored in order to control the degradation as high pH led to low photo-catalytic degradation rates.

Chapter: 5.0

Biodegradation of PAHs

Aim: To study the effect of soil pH on biodegradation of polycyclic aromatic hydrocarbons (PAHs).

5.1 Introduction

Biodegradation is the chemical dissolving of organic and non-organic pollutants by use of micro-organisms or other biological agents (Das *et al.*, 1994). In recent years, biodegradation of pollutants by microbes has received significant interest as mankind attempts to reduce contamination and construct a pollution free environment. Various pollutants are transformed by microbial populations through enzymatic or metabolic processes during biodegradation (Leahy & Colwell, 1990). In a biodegradation process the final end product obtained is either carbon dioxide or methane. In the environment, natural populations of bacteria and fungi carry out biodegradation of hydrocarbons to produce these end products. It is one of the primary mechanisms which lead to the elimination of hydrocarbon pollutants. However, different environmental parameters restrict the degradation process (Leahy & Colwell, 1990).

Biodegradation leading to bioremediation and biotransformation techniques have been shown to transform a large range of compounds including hydrocarbons, pharmaceuticals substances, polychlorinated biphenyls (PCBs), poly-aromatic hydrocarbons (PAHs) (Das *et al.*, 1994). Degradation of hydrocarbons may be carried out aerobically and anaerobically (AL-Turki, 2007). Anaerobic degradation of hydrocarbons are reported to be scarce compared to aerobic degradation and most of the reports focus on small aromatic molecules (for example, benzene, toluene and xylene) (Smith, 1990). PAHs are important pollutants in the environment, (for example: phenanthrene is considered as human skin photo-sensitizer and mild allergen) and require rapid remediation. The interest in understanding PAHs toxicity is due to the microbial population degradation reactions, physiochemical processes and fate of

these compounds in groundwater and soil sediments systems (Mrozik *et al.*, 2003). Various micro-organisms carry out biodegradation of PAHs. To date 60 genera of bacteria and 80 genera of fungi have been shown to carry out degradation of hydrocarbons. Some of these species degrade aliphatic hydrocarbons, others degrade aromatic hydrocarbons and some are capable of degrading both aliphatic and aromatic hydrocarbon molecules (Whyte *et al.*, 1997; AL- Turki, 2007). However, Boonchan *et al.*, (2000) indicate various bacteria and fungi do not use PAHs as energy and carbon sources but co-metabolically transform these compounds to detoxified metabolites. In their studies it was suggested that fungi detoxify PAHs polluted soils and sediments due to extracellular lignin-degrading enzyme production and thus, are capable of degrading some five-benzene-ring PAHs (Boonchan *et al.*, 2000). The first milestone in the biodegradation of high molecular weight (HMW) PAHs was reached in late 1980s. Heitkamp & Cerniglia, (1988) were first to publish work on extensive degradation of PAHs with four aromatic rings from their studies on isolation of bacteria from the environment. In their studies, isolation of gram positive rod shaped bacteria from sediments collected from oil fields degraded HMW PAHs co-metabolically. The PAHs included were fluoranthene, pyrene, 3-methylcholanthrene, 6-nitrochrysene, and Benzo(a)Pyrene. Therefore, bioremediation may be a relatively easy and cost effective method to decontaminate polluted sites. Degradation carried out using microbial populations enhance biodegradation rate considerably (Eriksson *et al.*, 2003; Lal & Khanna, 1996). It has been suggested that optimal soil conditions and their maintenance regulates the survival of micro-organisms providing higher degradation rates (Hadibarata & Tachibana, 2009). The types of organisms involved in degradation demonstrate enormous adaptability and diversity by using different organic molecules as a sole carbon source and however, they have different abilities

depending on biomass and source of energy in order to carry out the degradation of specific hydrocarbons (Davies & Weestlake, 1979).

5.1.1 Microbial biomass and soil ATP determination

In order to investigate the effect of various environmental soil microbiological and biochemical processes, many researchers have used soil ATP (adenosine 5'-triphosphate) and ATP related estimates (Naseby & Lynch, 1997; Wen *et al.*, 2005). ATP content not only helps in measuring the biomass, but also can be used as an index of microbial activity in soil (Wen *et al.*, 2005).

Adenosine 5'triphosphate is the most labile constituent of cells that consists of a 6-aminopurine base attached to β -D-ribofuranose due to a glycosidic linkage. Three phosphate groups are esterized by higher anhydride bonds to 5'-carbon of the ribose sugar. Also, due to the O-P-O-P-O-P backbone of triphosphate, it is positively charged and becomes unstable and a negative oxygen cloud around it repels itself. In general, the pyrophosphate bond carries the energy (Wen *et al.*, 2005). Similarly, chemical reaction of extracellular ATP in soil is upgraded by organic complexes, inorganic compounds and enzymes. Thus, release from dead cells results in rapid hydrolysis of ATP (Webster *et al.*, 1985).

The amount of ATP is found to be different in all the research reported, which may be due to the different soil types used by different authors. Jenkinson & Oades (1979) reported values from 0.64 to 7.00 $\mu\text{g ATP g}^{-1}$ soil, Nielsen & Eiland, (1980) 0.08-0.40 $\mu\text{g ATP g}^{-1}$ soil, 0.002-0.028 $\mu\text{g ATP g}^{-1}$ soil and 0.11 $\mu\text{g ATP g}^{-1}$ wet soil respectively. An easy, fast and reproducible technique without using any toxic reagents was suggested by Eiland, (1983) to determine the total amount of ATP in soil.

Various factors such as compound properties, soil characteristics and environmental factors and abiotic factors are highly responsible (Jones *et al.*, 1989). Toxic pollutants (PAHs) are often localised in soil, however toxic pollutants released in to the atmosphere may become attached to molecules in air and settle in soil. Bioavailability of pollutants results in eco-toxicity and the degradation of hydrophobic organic pollutants is a major issue in soil (Hatzinger *et al.*, 1995). Bio-degradation processes can be enhanced by considering the enzymes present in soil and their active role a varying pH values. Understanding of a range of enzyme activities in the ecosystem under different soil management practices may lead to an enhanced degradation process (Dick, 1994; Dick, 1992). Several soil metabolic processes have been determined with respect to enzymes present in soil. Varying enzyme levels have been found due to soil type with different organic contents, composition, organisms present and intensity of the biological activities (Makoi & Ndakidemi, 2008).

5.1.2 Enzymes produced by soil microorganisms

Soil enzymes are proteins which catalyze different chemical reactions by increasing the rate of reaction. In a soil enzyme reaction, molecules at an initial level considered as substrates, are converted molecules are termed as products as shown in figure 5.1. Different types of chemical reactions in the biological systems of cell require enzymes to gain considerable rate.

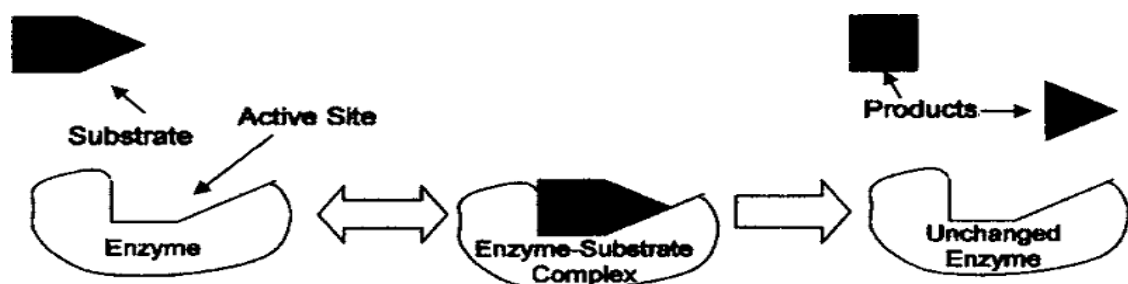


Figure 5.1: *Enzymatic reaction process* (Ruggaber & Talley, 2006)

Various soil enzymes originate from different plants and organisms that grow in soil and a major origin of soil enzymes is generally from microbial populations present in soil (Baldrian *et al.*, 2000). Enzymes in soil show variation or alteration due to various biotic and abiotic factors in environment. These factors include microbial communities, temperature, pH, nutrient availability and chemicals already present in soil (Chun, 20011; Kourtev *et al.*, 2002). Enzymes at different range of temperatures may not respond with required high efficiency.

White rot fungi secrete different types of enzymes. Secretions from white rot fungi may contain lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase respectively. Each of these enzyme classes show different oxidative activities and are implicated as pollutant degraders (Bogan & Lamar, 1996; Baldrain *et al.*, 2000). LiP enzymes are seen to be less effective during PAHs degradation, as in the absence of LiP different genera of white rot fungi, other than *Phanerochaete* have found to produce MnP and laccase activity. Similarly *Ceriporiopsis subvermispora*, *Phlebia brevispora*, *Stereum hirsutum*, *Panus tigrinus*, *Rigidoporus lignosus* and *Ganoderma valesiacum* are reported to follow the same pattern (Bogan & Lamar, 1996). *Trametes versicolor* producing laccase enzyme has been reported in effective degradation of anthracene and Benzo[α]pyrene (Collins *et al.*, 1996).

Soil pH is important for enzyme activity. Bonomo *et al.*, (2001) reported enzyme activities that are effected due to soil pH. The electrostatic properties for enzyme surface reaction regardless of binding and catalysis, changes due to sensitivity of amino acid functional groups (Bonomo *et al.*, 2001). For example crude laccase from *Bacillus subtilis* in dye decolourisation has optimum pH of 6.8 and temperature of 60⁰C. In contrast to laccase activity in fungi, activity of *Bacillus subtilis* WD₂₃ also has high activity at alkaline pH 9.0 (Chun *et al.*, 2011). This suggests that soil pH and

temperature are of importance in degradation. Most research carried out on fungal laccase activity indicated that optimal soil pH varies from 3 to 7 which depend on different fungal species (Sulistyaningdyah *et al.*, 2004). The effect of soil pH on degradation of PAHs has been poorly documented and none of these studies have focused on the effect of soil pH on biodegradation of PAHs along with the enzyme activities and microbial biomass measurements by soil ATP content. A number of studies have been focused on degradation of PAHs with little work on limited soil pHs and no one covering a wide range of soil pH. Therefore, considering all the factor studies was conducted on soil pH effect.

5.2 Materials and methods

5.2.1 Soil

Commercially available J. Arthur Bower's topsoil was used throughout the experiment. The reason for using J. Arthur Bowers topsoil was to provide a constant soil of neutral pH for all experimental studies.

Table 5.1: Soil texture

Soil Name	pH	Water holding capacity (WHC)
J. Arthur Bower's topsoil	7	41%

5.2.2 Moisture content of the soil

Water-holding capacity is dependent on the organic matter in the soil and soil particle size. Normal field soil water-holding capacity is 60-80% of its total capacity; that is, 60-80% of the water filled pore spaces are filled. This corresponds to the optimal biological activity for water-holding capacity. When the water-holding capacity falls below 24-18%, organisms could suffer from dryness; and when the capacity is over 80%, they begin to suffer from a depletion of soil oxygen (Zhang *et al.*, 2006). With respect to studies carried out, the J. Arthur Bowers topsoil was dried in an oven maintained at 90°C for two days filled in two steel trays covered with aluminium foil. The moisture holding capacity of the J. Arthur Bowers topsoil was determined using the calculation described in appendix III section III.1.

5.2.3 pH adjustment of soil

The natural pH of J. Arthur Bowers topsoil is 7.0. This study required soil pH of 5.0, 5.5, 6.0, 6.5, 7.5 & 8 respectively. For pH adjustment 1 M HCl and Na₂CO₃ (0.01 M) (table 5.2) were added in 5 g of topsoil along with de-ionized water used to

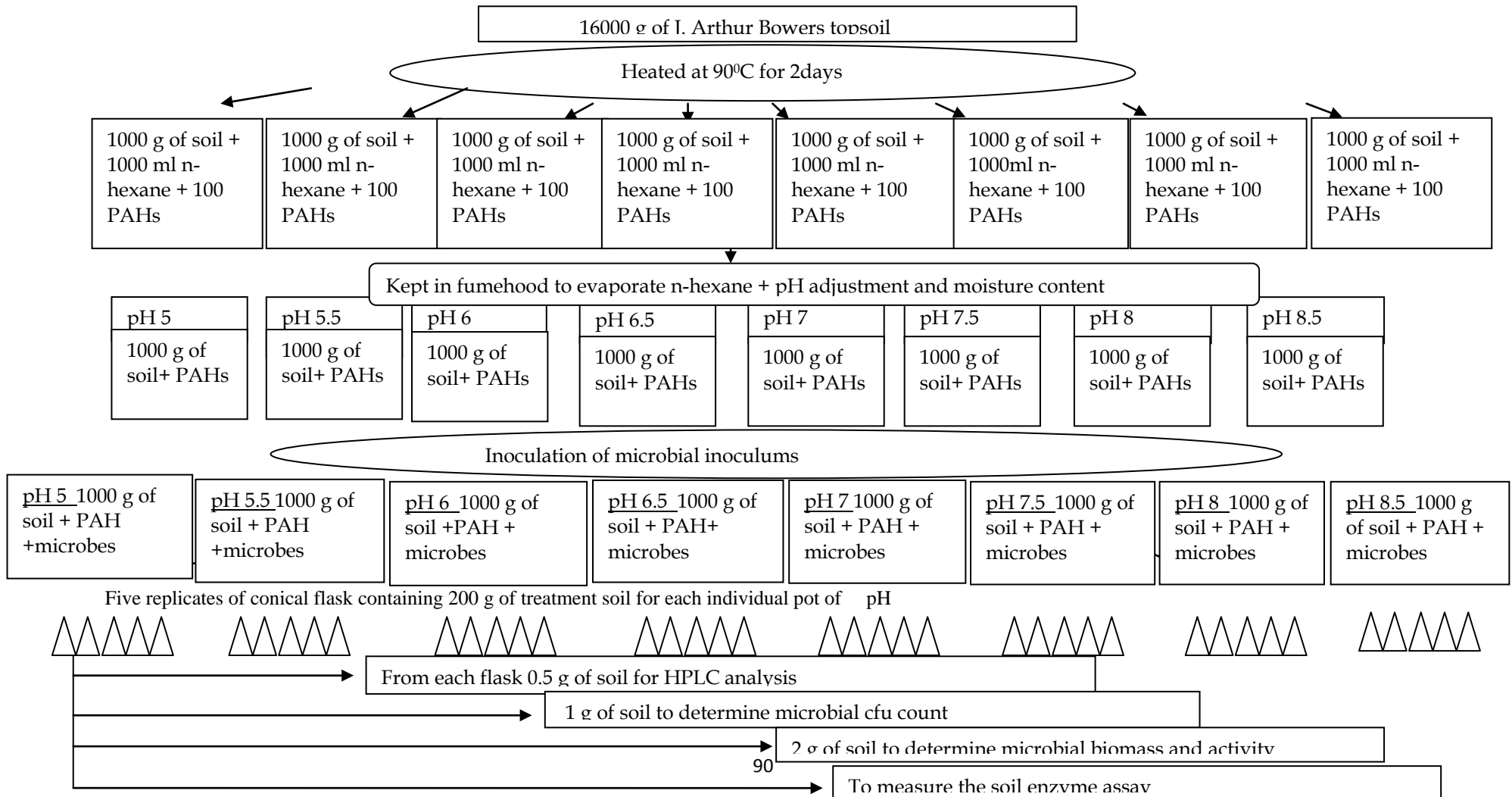
maintain 30% of moisture content of the soil as mentioned in section 5.2.2 (Kastner, 1998). Based on table 5.2, calculations were performed for larger volumes of experimental soil samples.

Table 5.2: pH adjustment of J. Arthur Bower's topsoil

Soil pH	Acidic pH Amount of 1M HCL	Basic pH Amount of 0.1M Na₂CO₃
pH 5	260 µl + 10 ml de-ionized water + 5 g of soil	-
pH 5.5	190 µl + 10 ml de-ionized water + 5 g of soil	-
pH 6	132 µl + 10 ml de-ionized water + 5 g of soil	-
pH 6.5	90 µl + 10 ml de-ionized water + 5 g of soil	-
pH 7	-original pH	-original pH
pH 7.5	-	40 µl + 10 ml de-ionized water + 5 g of soil
pH 8	-	54 µl + 10 ml de-ionized water + 5 g of soil
pH 8.5	-	80 µl + 10 ml de-ionized water + 5 g of soil

5.2.4 PAH degradation experiment

Fig. 5.2 Schematic representation of Experimental Design



5.2.5 Biodegradation monitoring HPLC

Figure 5.2 is a schematic representation experimental design for the test samples (with microbial inocula). Similar, experimental design was performed for control samples (without microbial inocula).

5.2.6 Dissolution of PAH compounds in J Arthur Bower's topsoil

As described in chapter 3, section 3.5 .

5.2.7 Monitoring pH and moisture content of the experimental soil and addition of bacterial and fungal inocula

1.6 kg of soil each was transferred in two different sterile trays and from each tray containing 1.6 kg of soil was further separated into 0.2 kg and transferred to seven different pots (as a control-without microbial inocula) and similarly 0.2 kg of sterile soil was transferred into seven different pots (as a test samples- with microbial inocula) to monitor the pH of the soil along with bacterial and fungal inocula. Deionised water was added to maintain 30% moisture content of the soil, pH adjusted as described in section 5.2.3.

5.2.8 Inoculation of microbial inocula into experimental samples

The initial population of known count of bacteria and spores for fungi (obtained from isolates described in appendix I: sections I.3.6) was determined by spread plate technique to prepare inoculum. 1.1 kg of the contaminated soil was adjusted to pH 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5 each and adjusted to 30% water holding capacity (WHC) using sterile distilled water.

5.2.9 Sample analysis and sampling point

From the seven different pots containing soil of each pH treatment, 200 g of soil were further transferred into 500 ml conical flasks covered with sponge bungs and replicated 5 times. All replicates were maintained at 20⁰C in a dark incubator throughout the experiment. Samples from the conical flask were removed after 0, 1, 2, 3, 4, 5, 6, 7 and 8 weeks.

5.2.10 Samples for HPLC analysis

0.5 g of treated sample, from each of the 5 replicates were placed into 1.5 ml Eppendorf tubes. PAHs were extracted from soil by adding 1.5 ml of acetonitrile containing 20 ppm carbazole as an internal standard. Samples were mixed vigorously using a round vortex mixer fitted with multi sample holder which holds a total of 12 samples (Sigma Aldrich) for 5 minutes prior to HPLC analysis.

5.2.11 Samples for bacterial and fungal cfu count

1 g of treated soil from each of the 5 replicates in conical flasks was added to the universal bottles containing 9 ml of ringer's solution to perform serial dilution to quantify bacterial and fungal cfu per gram of soil.

5.2.12 Samples for microbial activity

2 g of treated soil from the 5 replicates of conical flasks were transferred in pairs (one marked as control) to 15 ml centrifuge tubes for each individual replicate (figure 5.2). Moisture content was monitored every three days and water loss was compensated for by the addition of de-ionized water throughout the experiment (Kastner *et al.*, 1998).

5.2.13 Samples for soil enzyme activity assay

Soil samples from each replicate of each treatment were added to 15 ml centrifuge tubes in pairs (one marked as control- without microbial inocula and one as test samples- with microbial inocula) for various assays as described. 1 g of soil samples were measured for phosphatase extraction, 3 g of soil samples were measured for laccase extraction, 3 g of soil samples were measured each in centrifuge tubes for manganese peroxidase and lignin peroxidase enzyme extraction. 2 g of soil samples for L-arginine ammonification enzymatic extraction. Each of these samples was measured on a weekly basis starting from week 0 to the termination of the experiment.

5.2.14 HPLC analysis

As described in chapter 3, section 3.6.

5.2.15 Preparation of standard samples for HPLC analysis

As described in chapter 3, section 3.3.

5.2.16 Isolation of bacterial and fungal populations from experimental soil

Objective: Quantify the bacterial and fungal c.f.u and identification of predominant isolates from varying soil pH.

5.2.16a Bacterial strains and treatment:

The bacterial strains used were isolated from PAH contaminated soils as mentioned in (appendix I: sections I.3.6). The six identified bacterial strains used as inocula were *Pseudomonas putida* strain, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp.. The bacteria were introduced to a final concentration of 2×10^6 cells/g of soil. Constant numbers of bacteria populations were evaluated at 10^6 dilutions. These bacterial isolates were grown in nutrient broth in conical flask for 48 hours on a shaker (120 rpm). The bacterial cells were collected by centrifugation (5000 rpm for 10 mins). Bacterial cells were washed twice with sterile water to remove excess nutrients and further used as inocula into the sterile soils for experimental samples.

The bacteria were grown on nutrient agar (Sigma Aldrich) for 2 days at 28°C and each plate subsequently was harvested in 10 ml of sterile distilled water using sterile glass spreader.

5.2.16b Fungal culture inoculations into soil samples

Two fungal identified strains *Aspergillus niger* and *Penicillium freii* were used as fungal inocula (appendix I: sections I.3.6). Fungal inocula were prepared by making suspension of spores from the old culture (grown on MEA in petridishes [2×10^4]) by washing it into 4 ml of sterile distilled water (SDW). The fungal mycelia were

removed by filtration from the spore suspension using sterile glass wool. Also, the concentration of the spores per volume of the suspension was estimated using haemocytometer (Fisher Scientific).

5.2.17 Media and culture conditions

Media and culture conditions used are described in appendix II section II.1

5.2.18 Isolation method

1 g of soil from the treated samples were taken with a spatula and added to 9 ml of sterile ringer's solution. Dilution series of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6} were made up by adding 1 ml of 10^{-n} dilution and add to 9 ml of autoclaved ringers solution to make up $10^{-(n+1)}$ dilution. Each dilution (100 μ l) was spread on bacterial and fungal plates in five replicates for each dilution series and incubated at desired temperatures. Bacterial plates were incubated at 28⁰C for 48 hrs and fungal plates at 22⁰C for 4 days (Loeffler, 2000). The known numbers of colonies obtained for a particular dilution were counted and the population per gram of soil was calculated.

5.2.19 Determination of Microbial Activity: (ATP concentration measurements)

Objective: To study microbial activity in experimental soil over 70 day's duration in the presence of PAHs.

5.2.20 Soil ATP extraction

7 ml of extraction buffer per gram of soil was transferred in a polyethylene centrifuge tube and was mixed well. All the samples were sonicated for 2×30 sec on a sonicator (Heat systems, Ultrasonic processor, Model, XL2015) set at level 5. The samples were on ice during and after the sonication procedure. After the sonication step all the samples were kept on rotator shaker (15000 rpm) (Sigma) for 30 min at 4⁰C following by centrifugation was carried out for 20 mins at 15000 rpm at 4⁰C.

Table 5.3: Extraction buffer:

Reagents	Constituents per ml
Phosphoric acid	0.066 ml
Urea	120 mg
DMSO	0.2 ml
Adenosine	1.8 mg
EDTA	7.84 mg
Polyethylene 10 Lauryl ether	7.5 µl
Antifoam	0.0075 ml

(Webster *et al.*, 1985)

5.2.21 Sample treatment and measurement

0.2 ml of sample supernatant was added to the centrifuge tube along with 1.8 ml of tricine buffer (pH 10). Titration to pH 7.6 to pH 7.8 with 5 M ethanol amine, using phenol red as an indicator was carried out. The amount of ethanolamine required was recorded and titration for fresh samples without addition of phenol red was performed.

5.2.22 Soil enzyme assay

5.2.23 Manganese peroxidase enzyme extraction at three pH

3 g samples were taken from each flask and added to 15 ml centrifuge tubes. 8ml of 50 mM phosphate buffer adjusted to pH 5.5, 7 or 8.5 was added to the soil samples labeled with pH 5.5, pH 7 and pH 8.5 and placed on a rotary shaker (100 rpm maintained 25⁰C for an hour). A centrifugation step was performed at 6000 g for 15 min and supernatant was collected in fresh centrifuge tubes. A similar process was followed for all the control samples in 5 replicates without microbial inocula in experimental soil samples.

5.2.24 LiP enzyme extraction at three pH

8ml of 50 mM sodium acetate buffer (along with 1% polyvinylpolpyrrolidone) with different volumes of 1 M NaOH for the alkaline pH and 0.5 M, 1 M HCl for the acidic pH) was added to each experimental soil sample and kept on a rotary shaker (100 rpm maintained 25⁰C for an hour). Further, centrifugation was performed at 6000 g for 15 min and the supernatant was collected in fresh tubes.

5.2.25 Laccase enzyme extraction

Buffer A containing (0.1 M sodium acetate, 0.005 M CaCl₂, 0.05% tween 80 and 1% polyvinylpolpyrrolidone with different volume of 1 M NaOH for the alkaline pH and 0.5 M, 1 M HCl for the acidic pH) was added to each sample and kept on a rotary shaker (100 rpm maintained 25⁰C for an hour). Further, centrifugation step was performed at 6000 g for 15 min and supernatant was collected in fresh tubes.

5.2.26 Alkaline phosphatase enzyme extraction

The method for alkaline phosphatase enzyme extraction was followed by modification of enzyme assay at three pH in the method of Naseby & Lynch, (1997). 25 mM p- nitrophenyl phosphate oxidation was used as a substrate for acid/alkaline phosphatase enzyme assay. 1 g of experimental soil samples and controls were treated similarly with sodium orthophosphate buffer containing NaN_3 (sodium azide) at different pH (5.5, 7 and 8.5) of buffer. All the samples were centrifuged (6000 rpm for 15 minute).

5.2.27 L-arginine ammonification extraction at three pH

2 g samples of treated soil from each of the 5 replicates of individual conical flask containing contaminated soil of pH 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5 were transferred to 15ml sterile centrifuge tubes. 0.5 ml of an arginine solution (0.2%) was added drop wise. The soil samples were further extracted using three buffers adjusted to pH 5.5, 7 and 8.5 respectively. Two sets of samples were taken; one set was incubated at 4°C and second was incubated at 30°C for two hours.

5.2.28 Manganese Peroxidase (MnP) enzymes assay

MnP activity was measured in PAHs contaminated soil with few modifications in the procedure by Vyas *et al.*, (1994). 3 g of experimental soil was collected in centrifuge tubes from each flask in 5 replicate and enzyme extraction was performed at three pH's as described in section 5.2.23.

Reaction mixture in a final volume of 2ml which contained supernatant along with 100 μM succinate lactate buffer (adjusted at pH 5.5, 7 and 8.5) containing 0.1 μM of 3-methyl-2-benzthiazolinone hydrazone hydrochloride (MBTH), 5 μl of 3-

dimethylaminobenzoic acid (DMAB), 0.2 μM of MnSO_4 and 0.1 μM H_2O_2 were used. The enzyme activity was terminated during the assay by using cold 0.4 M NaHCO_3 . Similar procedures were followed for all the replicates and all the samples for buffer pH 7 and 8.5 respectively. All the assayed samples were loaded onto 96 well plates (Fisher Scientific) and also absorbance at 590 nm was monitored with the UV spectrophotometer.

MnP activity was measured as amount of enzyme capable of oxidizing one μmol of ABTS min^{-1} . One unit of enzyme activity is defined as the activity that produces 1 μM of the product per min under assay conditions.

5.2.29 Lignin peroxidase (LiP) enzyme assay

LiP activity was measured in PAHs contaminated soil followed by extraction process and activity procedure followed by few modifications by D' Annibale *et al.*, (2006). The extraction process was carried out as described in section 5.2.24.

Reaction mixture in a final volume of 2 ml for LiP enzyme assay was followed similar to MnP activity mentioned D' Annibale *et al.*, (2006). LiP assay was performed with similar procedure for MnP assay except that H_2O_2 was omitted. LiP activity was terminated during the assay by using cold 0.4 M NaHCO_3 . All the assayed samples were loaded onto 96 well plates (Fisher Scientific) and absorbance at 401 nm was monitored with the UV spectrophotometer.

LiP activity was measured as amount of enzyme capable of oxidizing one μmol of ABTS min^{-1} . One unit of enzyme activity is defined as the activity that produces 1 μmol of the product per min under assay conditions.

5.2.30 Laccase enzyme assay

Laccase activity was measured in PAHs contaminated soil followed by extraction with a modification of the method of D' Annibale *et al.*, (2006). Enzyme extraction process was performed as described in section 5.2.25

1 mM of ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) oxidation was monitored as a substrate to measure laccase activity at 25⁰C. 50 µl of ABTS substrate was added to each of the 5 replicates of individual soil pH sample extract and were assayed with respective Britton- Robinson buffer (0.1 M boric acid, 0.1 M phosphoric acid, 0.1 M acetic acid and pH adjusted to 5.5, 7 and 8.5 with HCl and NaOH) buffer adjusted at pH 5.5, 7 and 8.5 respectively. Laccase activity was terminated during the assay by using cold 0.4 M NaHCO₃. All the assayed samples were loaded onto 96 well plates (Fisher Scientific) and absorbance at 401 nm was monitored with the UV spectrophotometer

Laccase activity was measured as the amount of enzyme capable of oxidizing one µmol of ABTS min⁻¹. One unit of enzyme activity is defined as the activity that produces 1 µmol of the product per min under assay conditions.

5.2.31 Acid and alkaline phosphatase assay

Phosphatase activity was measured for Acid and Alkaline phosphatase. Enzyme extraction was carried out as described in section 5.2.26

The supernatant was collected in fresh 15 ml centrifuge tube with addition of substrate (25 mM p-nitrophenyl phosphate). During reaction the samples were kept in shaking water bath, set at 200 strokes/min for 1 hour maintained at 37⁰C. Termination of enzyme activity was carried out by addition of 1 ml of cold 0.4 M NaHCO₃ followed by centrifugation (4000 rpm for 15 min). Each sample (100µl) was loaded onto 96 well

plate and colour change and also, absorbance at was measured using UV spectrophotometer.

The enzyme activity is expressed as the amount of enzyme capable of oxidizing 1 μ mol p- nitrophenyl phosphate.

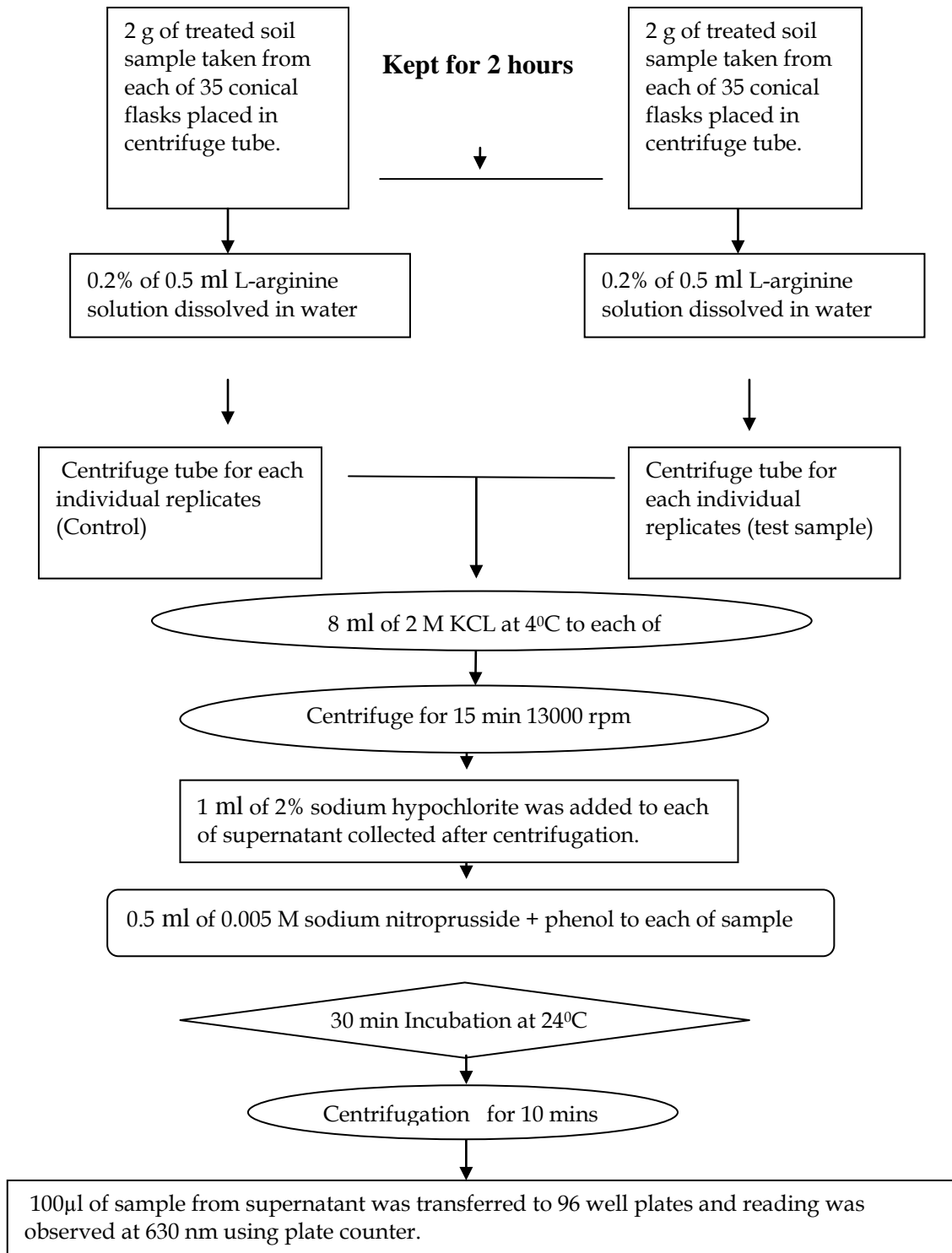
5.2.32 β – Glucosidase enzyme assay

β – Glucosidase activity was measured in PAHs contaminated soil followed by improved extraction process and activity procedure followed from D' Annibale *et al.*, (2006). Enzyme extraction process was performed as described in section 5.2.26.

1 mM of o-nitrophenyl- β -D-glucopiranoside (ONPG) oxidation was monitored as a substrate to measure β -Glucosidase activity at 25⁰C. 50 μ l of p-nitrophenyl- β -D-glucopiranoside substrate was added to each 5 replicate of individual soil pH sample extract and were assayed with respective 50 mM phosphate buffer. β – Glucosidase was terminated during the assay by using cold 0.4 M NaHCO₃. All the assayed samples were loaded onto 96 well plates (Fisher Scientific) and also absorbance at 401 nm was monitored with the UV spectrophotometer.

5.2.33 L-arginine ammonification

Fig. 5.3 Schematic representation of L-arginine ammonification



5.2.34 Methodology for microbial activity in presence of PAHs in soil at different pH

Microbial activity using L-arginine ammonification is a simple, fast and inexpensive technique to determine the microbial activities of the microbial population in soil, based on ammonia liberation by addition of arginine was carried out (Alef, 1987). As represented in figure 48 five replicates after extraction process were incubated and immediately mixed with 8ml of 2 M KCl at 4⁰C and centrifuged for 15 min at 13000 rpm. This treatment efficiently removed most of the ammonia adsorbed to soil particles. After centrifugation the 5 ml supernatant was mixed with 0.5 ml of 0.005% phenol nitroprusside and 0.5 ml sodium hypochlorite. After incubation of samples at room temperature for 30 min, the extinction coefficient was measured at 630 nm. The arginine ammonification rate was measured in micrograms of NH₄⁺ liberated per gram soil per hour (Alef & Kleiner, 1987). The arginine ammonification rate was calculated as described in appendix III section III.3 and it is expressed as μgNH₃/g soil d.w/h.

5.3 Results

Objective: To monitor PAH biodegradation in experimental soil at varying pH.

5.3.1 HPLC analysis of PAH

To study the effect of abiotic factors particularly soil pH on the rate of biodegradation, HPLC analysis was employed.

5.3.2 Standard graph for polycyclic aromatic hydrocarbons

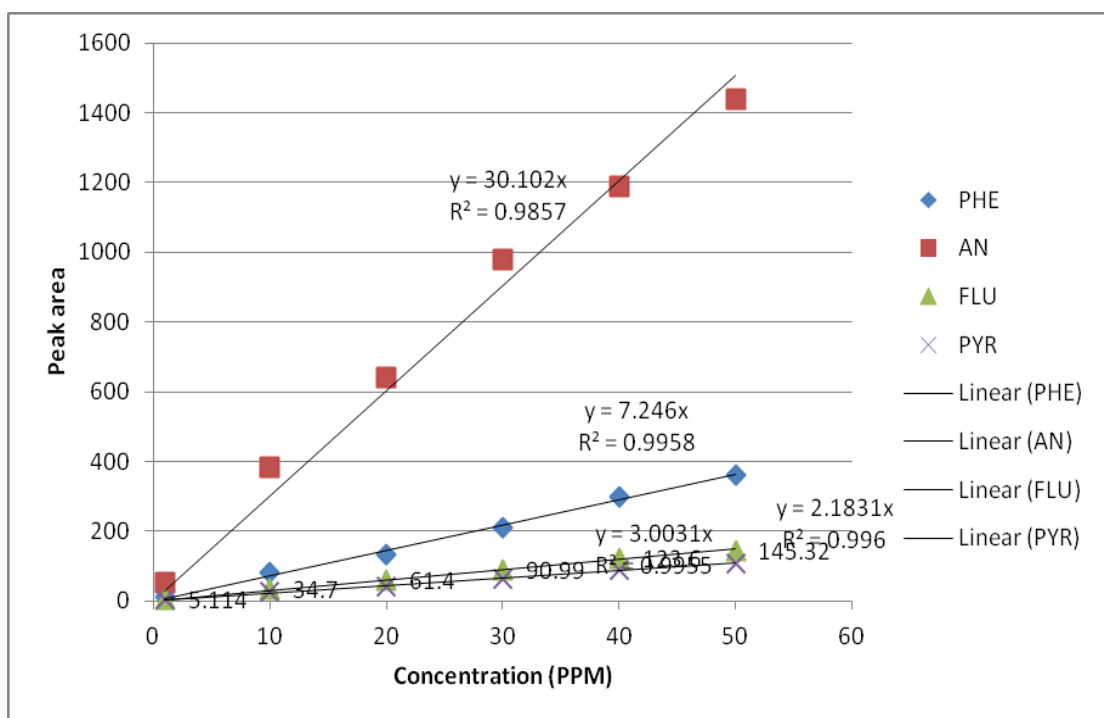


Fig. 5.4: HPLC standard curve of four PAH showing peak area against concentration. PAH used and their symbol abbreviations are (PHE) Pheanthrene; (AN) Anthracene; (FLU) Flouranthene; (PYR) Pyrene

Table 5.4: Extraction efficiency of four PAHs from J. Arthur Bowers topsoil

PAH used	Amount of PAH added (ppm)	% efficiency
Phenanthrene	100	62.08
Anthracene	100	53.63
Fluoranthene	100	51.19
Pyrene	100	50.68

In order to have a full evaluation of the extraction efficiency of the four different PAH, 90 ppm of each individual PAH was added to J. Arthur Bowers topsoil and extracted with acetonitrile. The re-extraction efficiency of the four PAHs obtained from these samples ranged from 50.68 to 62.08%. The highest value was obtained for phenanthrene (62.08), and lowest percentage obtained was (50.68) for pyrene (table 5.4).

5.3.3 Degradation of polycyclic aromatic hydrocarbons

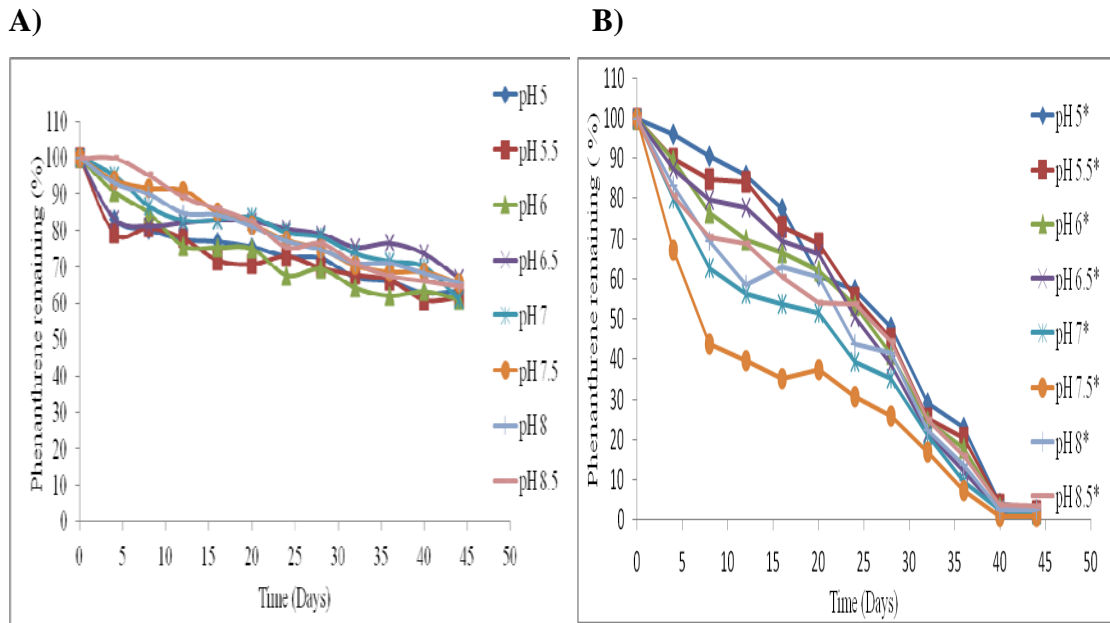


Fig. 5.5: Percentage phenanthrene remaining in *J. Arthur Bower's* topsoil at varying pH over time.

A): without microbial inocula B): with microbial inocula. *P<0.05 indicates significant difference between control and experimental sample.

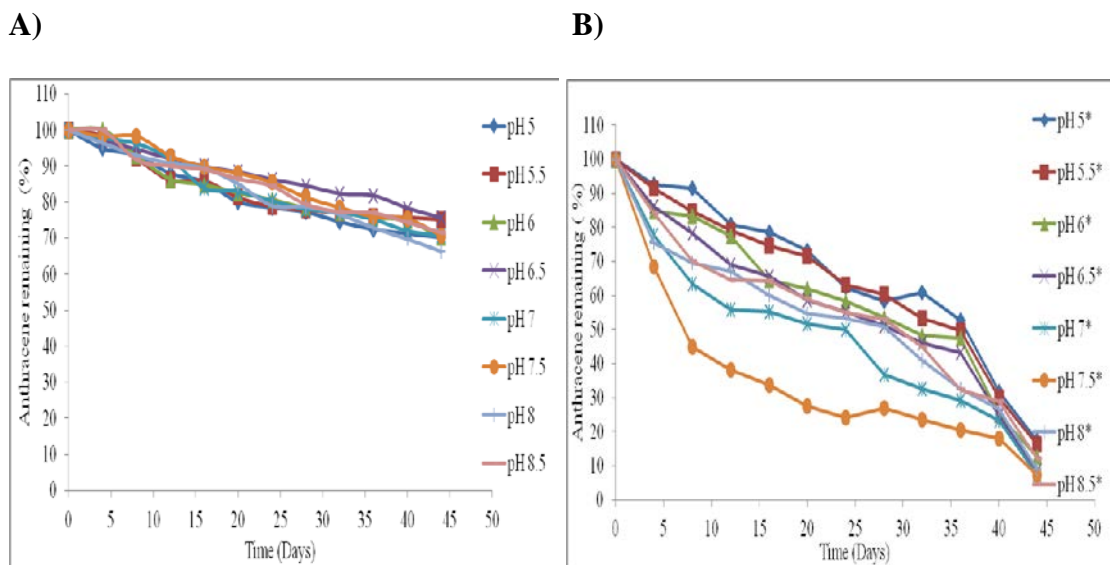


Fig. 5.6: Percentage anthracene remaining in *J. Arthur Bower's* topsoil at varying pH over time.

A): without microbial inocula B): with microbial inocula. *P<0.05 indicates significant difference between control and experimental sample.

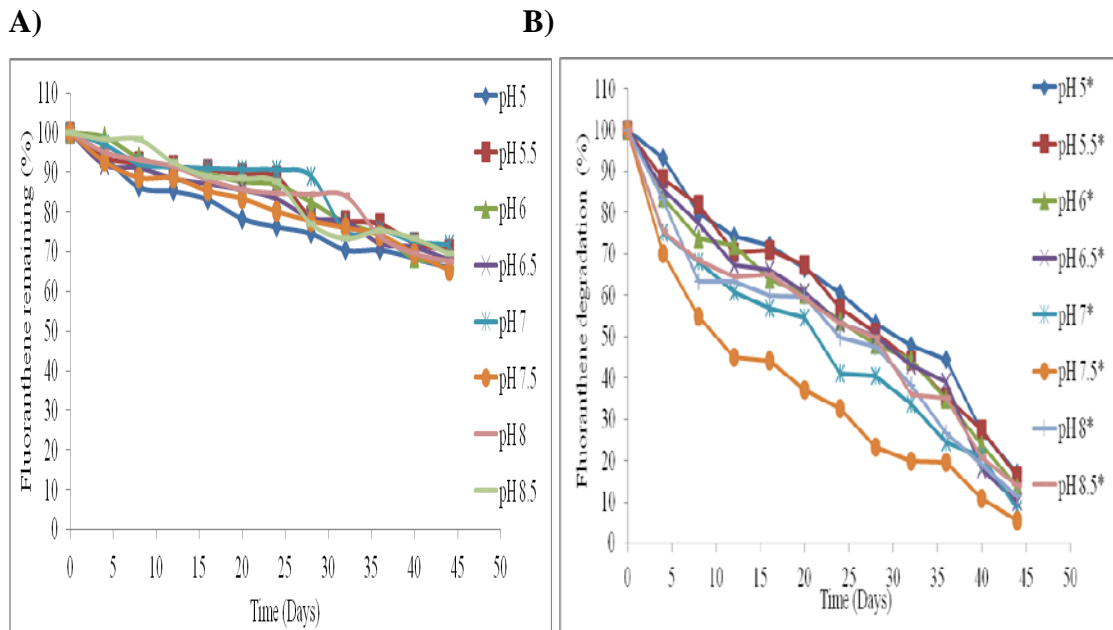


Fig. 5.7: Percentage fluoranthene remaining in J. Arthur Bower's topsoil at varying pH over time.

A): without microbial inocula B): with microbial inocula. *P<0.05 indicates significant difference between control and experimental sample.

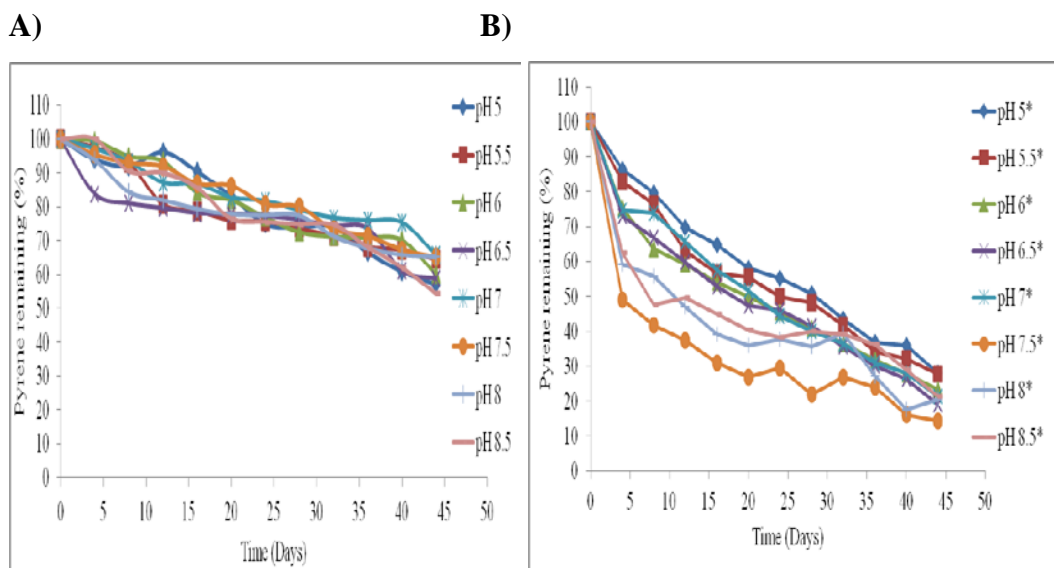


Fig. 5.8: Percentage pyrene remaining in J. Arthur Bower's topsoil at varying pH over time.

A): without microbial inocula B): with microbial inocula. *P<0.05 indicates significant difference between control and experimental sample.

Figure 5.5, 5.6, 5.7 and 5.8 exhibits degradation of phenanthrene, anthracene, fluoranthene and pyrene used as model PAHs in the soil with and without microbial inocula. All the control soil samples were without microbial inocula; whilst experimental samples were inoculated with a consortium of microbes (six bacterial strains used as inocula were *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp. and 2 fungal strains *Aspergillus* sp. and *Penicillium* sp.). PAH remaining is displayed as a percentage of the HPLC quantification results obtained after re-extraction on day 0. Degradation of each PAH was observed for 45 days, alkaline soil pH resulted in higher degradation rates compared to acidic soil pH, as soil pH increases, and rate of biodegradation also increases. Interestingly, soil pH 7.5 exhibited the highest degradation rate for all four PAHs and soil pH 5 exhibited lowest rate of degradation for all four PAHs.

Table 5.5: Degradation rates of four PAHs (values expressed in percentage)

	Time point	pH5	pH5.5	pH6	pH6.5	pH7	pH7.5	pH8	pH8.5
Phenanthrene	0	100	100	100	100	100	100	100	100
	5	96.1	90.0	89.5	87.4	79.9	67.2	83.0	80.6
	10	90.5	84.9	76.5	79.7	62.6	43.7	69.3	70.4
	15	85.7	84.2	69.7	77.8	56.1	39.5	58.6	68.7
	20	77.3	73.0	66.4	69.3	53.7	35.1	63.0	60.4
	25	60.7	68.8	62.1	66.3	51.3	37.4	60.6	54.0
	30	57.2	55.4	53.3	50.5	39.3	30.5	43.8	53.8
	35	48.1	45.1	41.2	38.8	35.2	25.8	41.4	44.3
	40	29.1	25.34	24.5	21.8	21.1	16.9	22.4	24.7
	45	23.1	20.6	18.0	12.1	9.7	7.2	13.6	15.9
	50	4.11	3.7	3.1	2.2	2.5	1.0	2.6	3.9
Anthracene	0	100	100	100	100	100	100	100	100
	5	92.5	91.5	84.7	86.0	77.7	68.5	75.3	84.1
	10	91.5	84.8	83.2	78.37	63.4	44.8	69.5	70.0
	15	80.7	79.1	77.5	69.0	55.8	38.1	67.1	64.6
	20	78.4	74.7	64.5	65.5	55.1	33.1	60.1	64.3
	25	73.1	71.5	61.9	58.5	51.6	27.5	54.7	59.0
	30	62.3	63.1	58.5	55.3	49.8	24.0	53.3	54.9
	35	58.2	60.2	53.4	51.1	36.8	26.9	50.7	53.1
	40	60.7	53.4	48.2	46.2	32.4	23.4	40.8	44.8
	45	52.8	49.5	47.4	43.0	29.2	20.4	32.6	32.2
	50	31.5	29.9	25.1	24.5	23.3	17.9	26.6	28.8
Fluoranthene	0	100	100	100	100	100	100	100	100
	5	93.3	88.1	83.6	85.7	75.0	70.0	83.0	75.5
	10	79.9	82.0	73.8	77.5	68.1	54.7	63.3	68.5
	15	74.4	70.4	72.0	72.0	67.3	60.7	45.0	63.4
	20	72.1	71.0	64.0	66.2	56.7	44.2	59.7	65.0
	25	66.4	67.2	60.2	60.8	54.5	37.0	59.7	59.2
	30	60.5	56.8	53.8	53.6	41.1	32.6	49.8	53.3
	35	53.2	51.0	48.2	49.9	40.4	23.2	47.6	49.9
	40	47.9	44.0	44.4	43.0	33.6	19.9	38.1	35.8
	45	44.5	35.4	34.9	39.0	24.5	19.5	26.7	35.0
	50	26.8	27.8	23.7	17.8	20.9	10.9	18.9	20.8
Pyrene	0	100	100	100	100	100	100	100	100
	5	86.4	82.8	75.3	72.9	74.6	49.1	59.2	62.5
	10	79.6	76.4	63.5	66.7	73.7	41.7	55.7	47.7
	15	69.7	63.0	59.2	59.8	65.5	37.3	47.1	49.6
	20	64.8	56.5	53.9	52.8	57.6	30.8	39.2	44.9
	25	58.0	55.3	49.8	47.2	51.7	27.0	35.9	40.4
	30	55.8	50.0	45.6	46.1	44.3	29.4	37.7	38.3
	35	50.8	48.1	40.8	41.5	40.0	21.8	35.6	40.0
	40	43.5	41.7	36.5	35.4	36.6	26.7	39.4	39.0
	45	36.8	34.2	31.7	29.9	31.0	23.9	27.2	36.3
	50	35.8	32.1	27.6	26.3	28.1	16.0	17.7	28.8

The greatest rate of biodegradation was observed for phenanthrene and lowest biodegradation rate for pyrene (table 5.5). Phenanthrene degradation rate was followed by anthracene, fluoranthene and pyrene respectively in line with the molecular weight and number of rings in the structure. Little phenanthrene degradation was evident in soil samples without microbial inocula (figure 5.5), whilst 100% degradation of phenanthrene was evident over 40 days with the microbial inocula. Degradation was most rapid at pH 7.5 followed by pH 7, pH 8, pH 6.5, pH 8, pH 6, pH 8.5, pH 5.5 and pH 5 respectively.

Anthracene degradation was observed over 45 days, exhibiting lowest degradation evident in soil samples without microbial inocula when compared to soil samples with microbial consortia. Higher biodegradation rate for anthracene was observed in soil pH 7.5 followed by soil pH 7, 8, 8.5, 6, 6.5, 5.5, 5. Anthracene was degraded earlier than fluoranthene and pyrene with 95% biodegradation.

Approximately 90% of fluoranthene was degraded by the soil with microbial inocula over 45 days with highest degradation rate at soil pH 7.5 and lower degradation for soil pH 5. Similarly 80% pyrene biodegradation was observed over 45 days with faster degradation at soil pH 7.5 and slower degradation observed at soil pH 5. DT_{50} (half life) the point of 50% degradation was tabulated for each individual PAH (table 5.6).

Table 5.6: DT_{50} of PAH remaining at different soil pH (days)

Soil pH	Phenanthrene (days)	Anthracene (days)	Fluoranthene (days)	Pyrene (days)
5	28	35	39	37
5.5	28	32	34	33
6	26	30	34	32
6.5	24	26	29	28
7	10	10	18	21
7.5	5	5	5	5
8	15	25	28	27
8.5	25	31	36	34

** DT_{50} – Time taken for 50% of the PAH to be degraded (Half life)*

Table 5.6 exhibit soil pH 7.5 resulted in 50% of biodegradation for each PAH in the first 5 days. Soil pH 7 resulted in 50% of phenanthrene and anthracene biodegradation in 10 days and 18 and 21 days for fluoranthene and pyrene respectively. A 50% of biodegradation at soil pH 6.5 was observed over 24 days for phenanthrene, 26 days for anthracene, and 29 days for fluoranthene and 28 days for pyrene respectively. Soil pH 6 resulted in 50% of phenanthrene biodegradation in 26 days followed by anthracene, fluoranthene and pyrene degradation in 30, 32 and 34 days respectively. Soil pH 6 and soil pH 5.5, resulted in 50% of biodegradation for phenanthrene in 28 days, anthracene after 32 days, and pyrene in 33 days and last fluoranthene in 34 days. Soil pH 5 resulted in 50% of biodegradation over 28 days for phenanthrene and 35, 39 and 37 days for anthracene, fluoranthene and pyrene respectively. At soil pH 8, 50% biodegradation occurred in 15 days for phenanthrene, 25 days for anthracene, and 28 days for fluoranthene and 27 days for pyrene. Soil pH 8.5 resulted in 50% of phenanthrene biodegraded over 25 days, anthracene over 31 days, and fluoranthene over 36 days and pyrene over 34 days respectively. However pyrene was observed to

degrade faster than fluoranthene whilst its degradation rate generally increased with increasing pH with the most rapid rate evident at pH 7.5.

In general, soil pH 7.5 exhibited greatest and fastest rates of biodegradation for all the four PAH's over 45 days. Phenanthrene was degraded fastest followed by anthracene, fluoranthene and pyrene respectively.

Objective: Quantify the bacterial and fungal populations and identification of predominant isolates from varying soil pH.

5.3.4 Total bacterial and fungal populations over 70 days in PAH contaminated soil at varying pH.

5.3.4a Bacterial populations

The six identified bacterial strains (*Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp.) and 2 fungal strains (*Aspergillus niger* and *Penicillium freii*) isolated by enrichment culture (appendix I: sections I.3.6) were used as a inoculums for the biodegradation experiment. The studies reported by Lal & Khanna (1996); Bharathi & Vasudevan (2001); and Rahman *et al.*, (2002) have identified most of the genera as hydrocarbon-degrading micro-organisms.

The total bacterial and fungal populations were evaluated during the degradation experiment. Figure 5.9 exhibits the bacterial populations over 70 days incubation time at varying soil pH.

A)

Log C.F.U/g of soil

B)

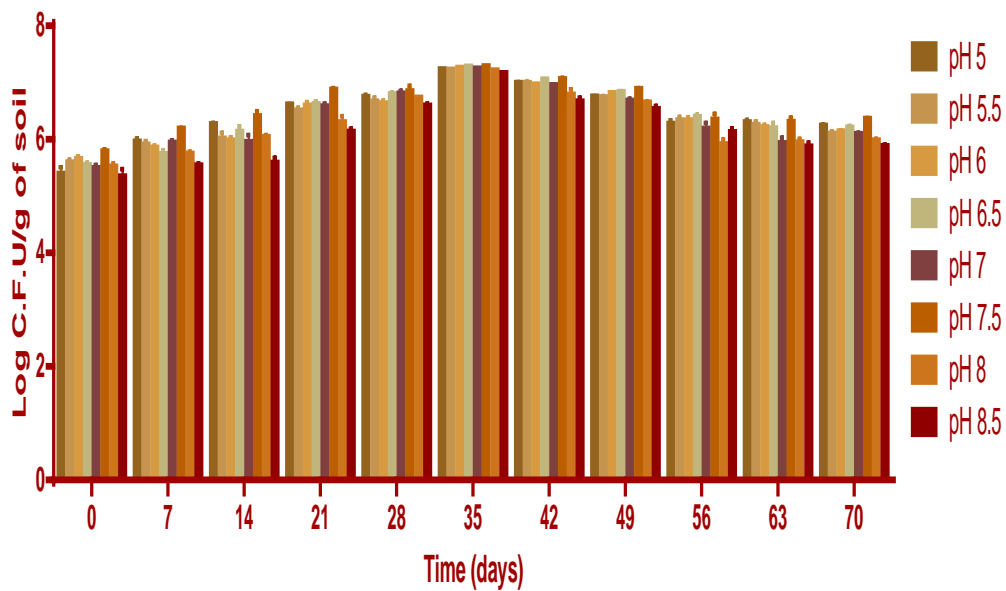


Fig. 5.9: Bacterial populations in J. Arthur Bower's topsoil inoculated with a microbial consortium at varying pH over time.

A) Soil inoculated without microbial consortium **B)** Soil with microbial consortium.

In figure 5.9 B), the lowest bacterial populations were obtained at the start of experiment. Bacterial populations at day 7, 14, 21, increased whilst the highest population was evident at day 35. All the time points (from 0 to 70 days) had the greatest bacterial population exhibited at soil pH 7.5 (figure 5.9 B). The bacterial

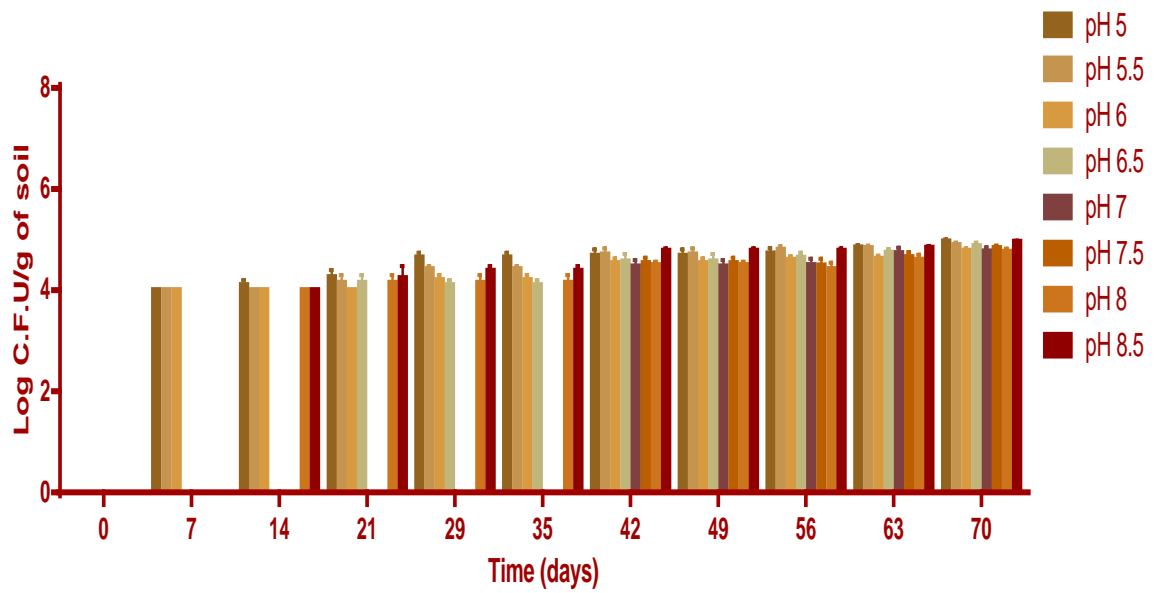
population dropped from its peak at day 35, dropping each week until the end of the experiment at day 70. The bacterial populations measured in soil samples without microbial inocula were ($\log 2 \times 10^3$) below the detection limits at day 0 and after 7 days (figure 5.9A). Bacteria were evident in soil without microbial inocula figure 54 (A), indicating recolonisation of the soil from 14 to 70 day of incubation.

Bacterial culture before adding as inocula were grown in nutrient broth (pH 7.2) suggests that they were more favourable to grow at soil pH 7.5 and bacterial population had a pH stress across all other pH range except for soil pH 7.5 when added as inocula. In general, bacterial population in soil samples with microbial inocula increased by at least 2 log units (from 10^4 to 10^6 cells per gram of soil).

5.3.4b Fungal populations

During biodegradation of PAH at varying soil pH, the total fungal population was evaluated. The fungal c.f.u counts per gram of soil exhibited higher difference in soil samples with and without microbial inocula. *Aspergillus* and *Penicillium* strains exhibited increased population count by at-least one log (from 10^4 to 10^5 cells per ml) in soil sample with microbial inocula. A total number of fungal c.f.u count measured per gram of experimental J. Arthur Bower's topsoil have mentioned in figure 5.10.

A)



B)

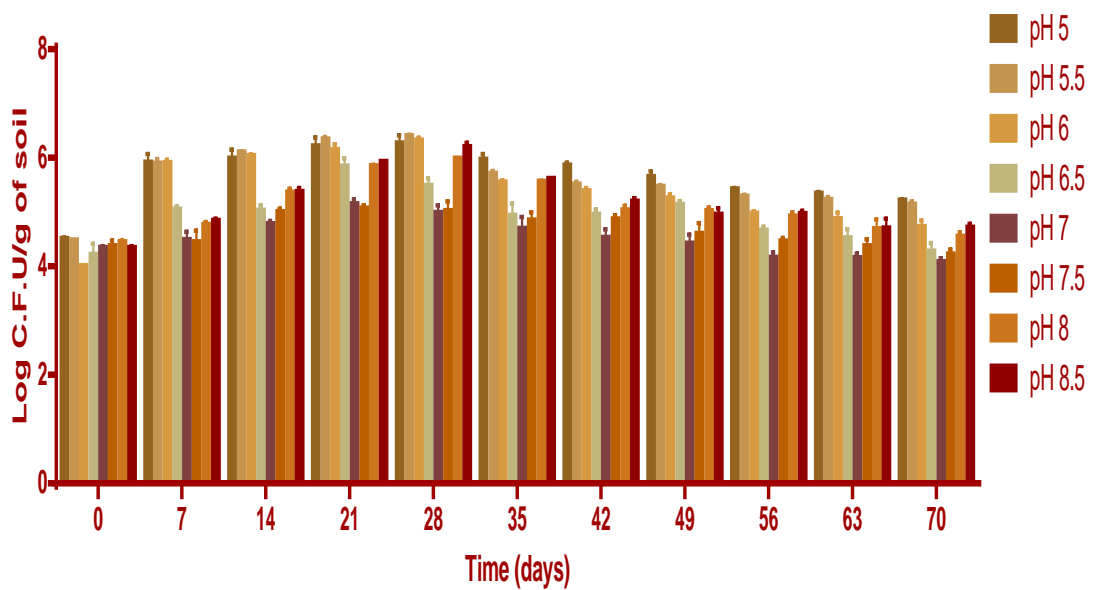


Fig. 5.10: Fungal population (CFU/g of soil) in PAH contaminated soil at varying soil pH.

A) Experimental soil inoculated without microbial consortium **B)** Control soil samples with microbial consortium

Figure 5.10(A) and 5.10 (B) exhibit fungal populations in each individual week and

for each soil pH respectively. The greatest fungal populations were found in day 29 followed by time point of days 21, 14 and 42. The highest population was found in soil pH 5 after 21 and at 29 day of incubation.

In figure 5.10 (B) the greater fungal populations were found at low soil pH acidic conditions. However, alkaline soil pH 8 and 8.5 had higher populations compared to neutral soil pH. Soil pH 7.0 and 7.5 had the lowest fungal populations. Table 5.6 exhibits the *Penicillium* and *Aspergillus* populations studied during biodegradation at 14 days.

Interestingly, *Penicillium* species predominated at acidic soil pH and with lower *Aspergillus* populations whereas at alkaline conditions of (pH 8 and 8.5) *Aspergillus* were predominant and *Penicillium* was not detected (table 5.7).

Table 5.7: *Penicillium* and *Aspergillus* populations after 14 days

pH of soil	<i>Penicillium</i> colonies*	<i>Aspergillus</i> colonies*
5	3.27	2.85
5.5	4.38	3.2
6	4.05	3.65
6.5	3.76	2.47
7	2.08	Not detected
7.5	Not detected	3.81
8	Not detected	3.96

* Values expressed as log c.f.u/g soil.

Objective: To study microbial biomass by soil ATP measurement at different pHs in the presence of PAHs.

5.3.5 ATP soil concentrations over 70 days in PAH contaminated soil at varying pH

ATP measurement was performed to investigate the microbial activity during biodegradation over the 70 days incubation time. The measurement of soil ATP was conducted using the methods of Naseby *et al.*, (1997).

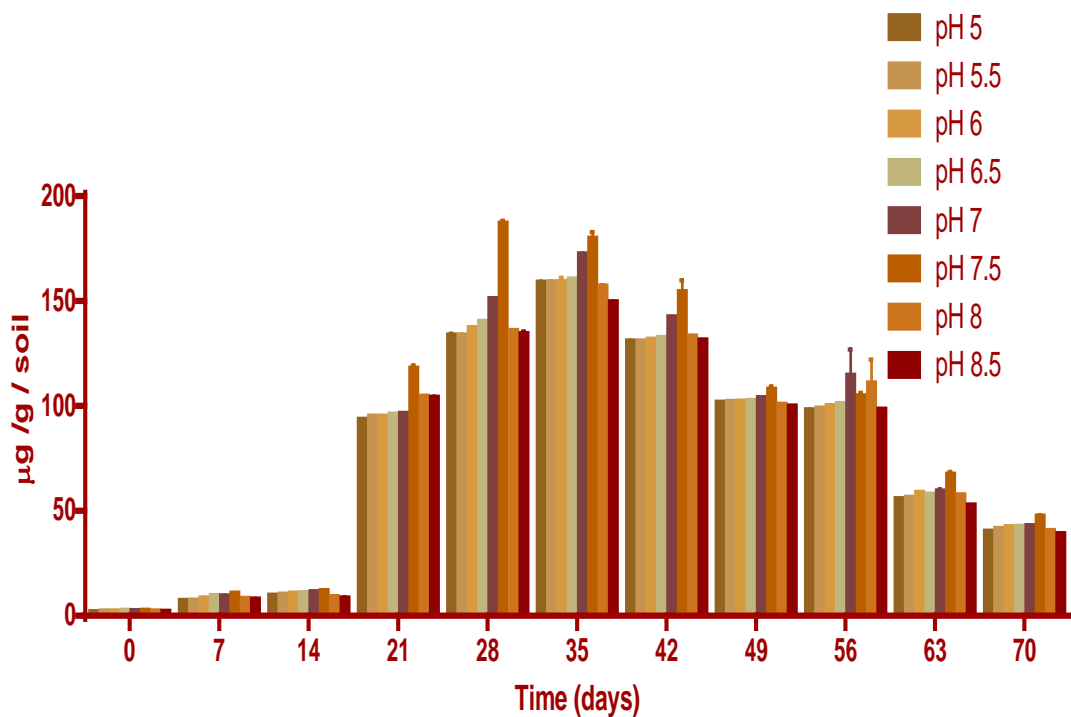


Fig. 5.11: Total microbial ATP measurements in J. Arthur Bower's topsoil at varying soil pH over time.

Figure 5.11 represents the soil ATP content during the degradation of polycyclic aromatic hydrocarbons from day 0. Over day 0, 7 and 14 a small increase in ATP from 0.7 µg/g/soil to 39 µg/g/soil was observed followed by a dramatic increase to around 80 µg/g/soil. Across all the time points the highest ATP concentrations were observed at soil pH 7.5 which peaked 180 µg/g/soil at day 29 and 176 µg/g/soil at day 35 whereas, the lowest microbial activity was observed at soil pH 5 (0.12 µg/g/soil) at the

start. The ATP concentration conversion to biomass was followed according to Naseby & Lynch, (1997) and calculations for ATP activity are shown in appendix III section III.6.

Soil concentrations in (figure 5.11) were highly correlated (correlation coefficient 0.9) to bacterial populations (figure 5.9 B) over 70 days of incubation period. ATP content continued to increase to a peak of around 150 $\mu\text{g/g/soil}$ at day 35; it then decreased gradually from day 42 till day 70 where the ATP concentration was around 50 $\mu\text{g/g/soil}$.

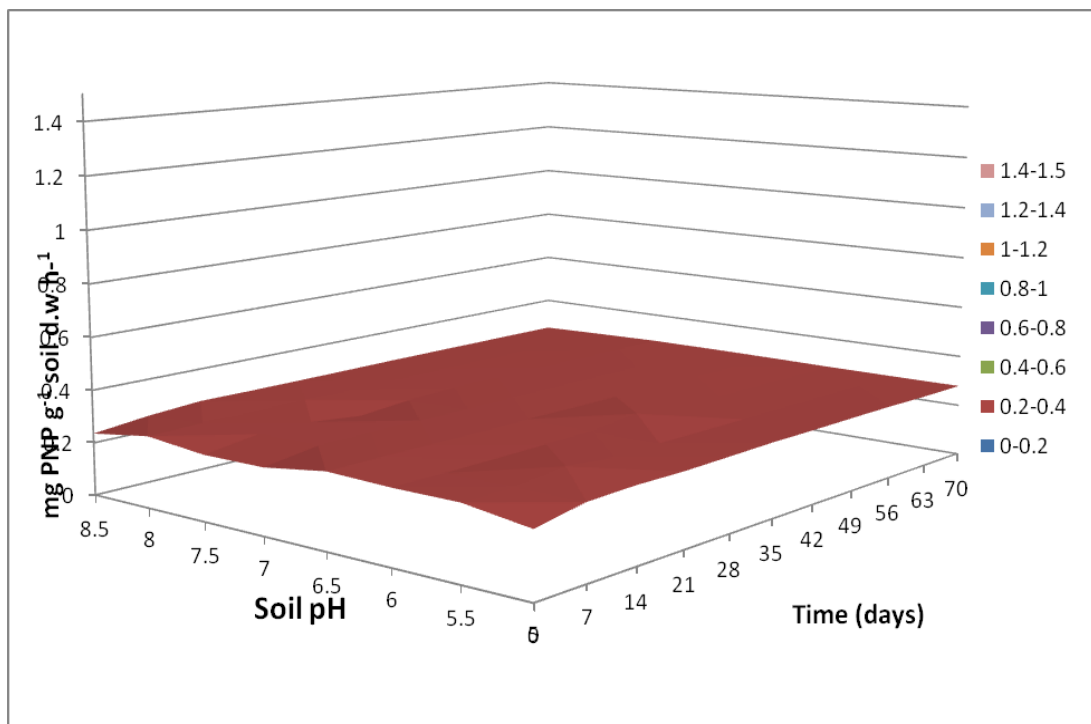
5.3.6 Soil enzyme activity

Objective: To determine soil enzyme activity using buffer pH (5.5, 7 and 8.5) for β -glucosidase, L-arginine ammonification, acid/alkaline phosphatase (C: N: P) cycles, manganese peroxidase (MnP), Lignin peroxidase (LiP), Laccase activity at varying soil pH.

5.3.6a Phosphatase soil activity

Soil enzymes are originated mainly through micro-biota present in the soil. In this experiment soil enzyme activities were studied at varying soil pH as enzymes mostly vary with environmental factors and co-existing chemicals. Studying the enzymes at varying soil pH and assay pH will aid understanding of the effect of soil pH on enzyme activities and the production of enzymes with different pH optima.

A)



B)

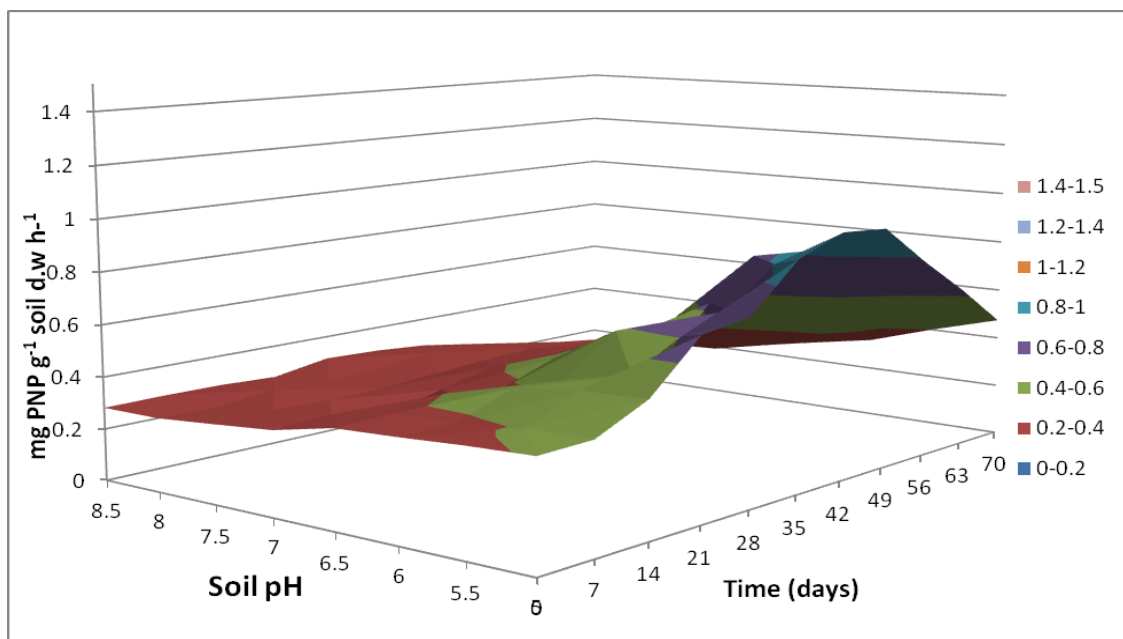
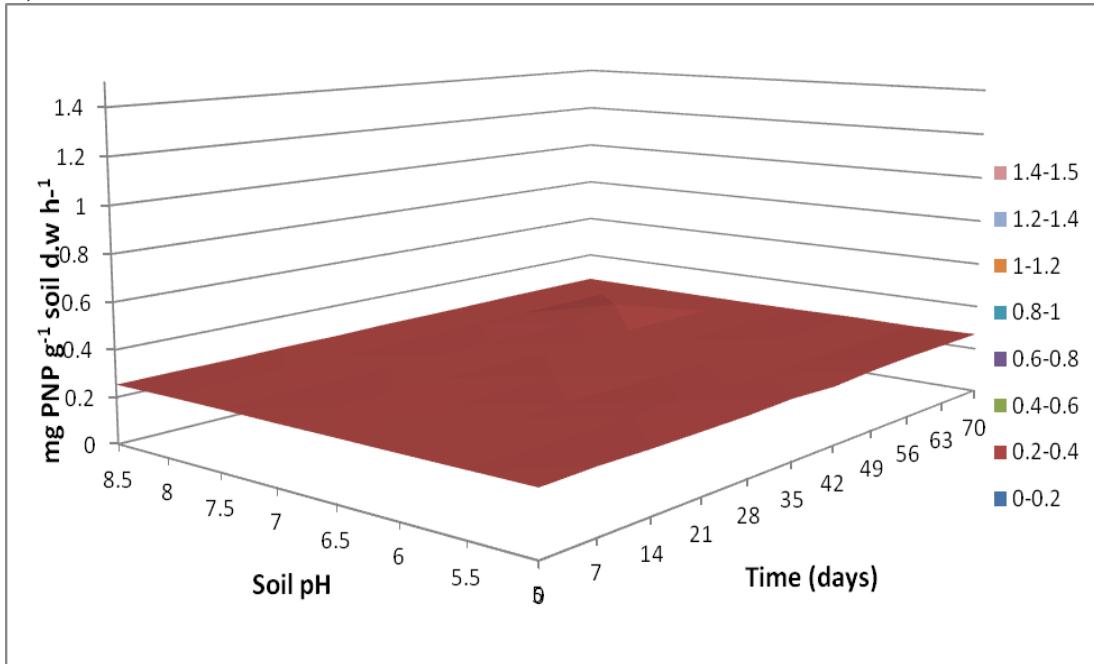


Fig. 5.12: Phosphatase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

A): Phosphatase activity in control samples without microbial inocula.

B): Phosphatase activity in experimental sample inoculated with microbial strains.

A)



B)

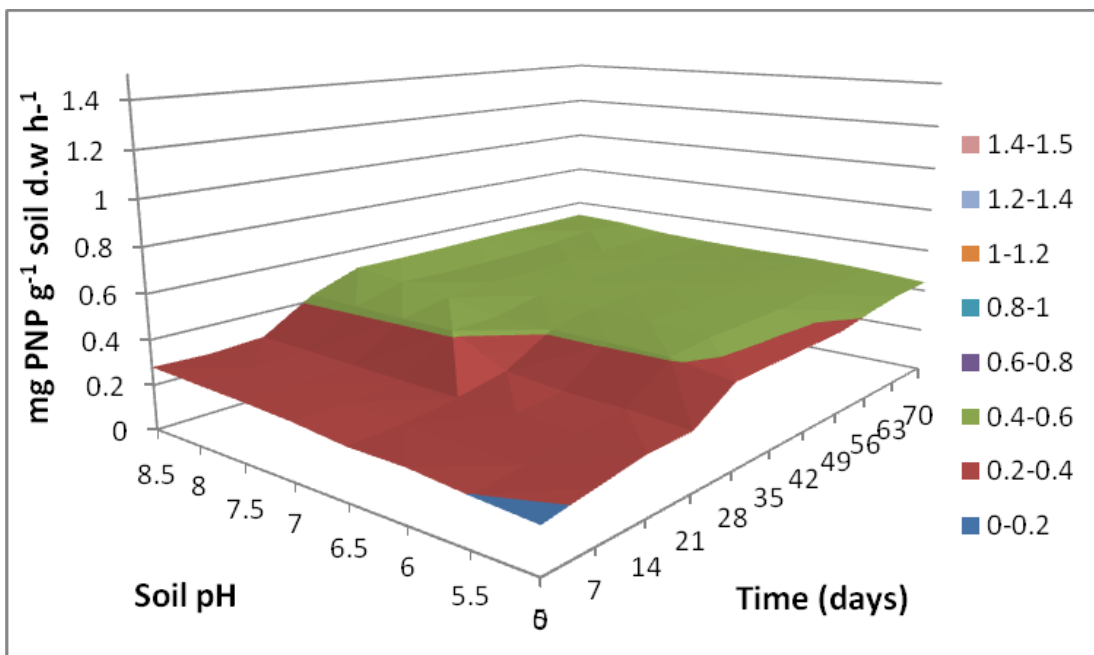


Fig. 5.13: Phosphatase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 7

A): Phosphatase activity in control samples without microbial inocula.

B): Phosphatase activity in experimental sample inoculated with microbial strains.

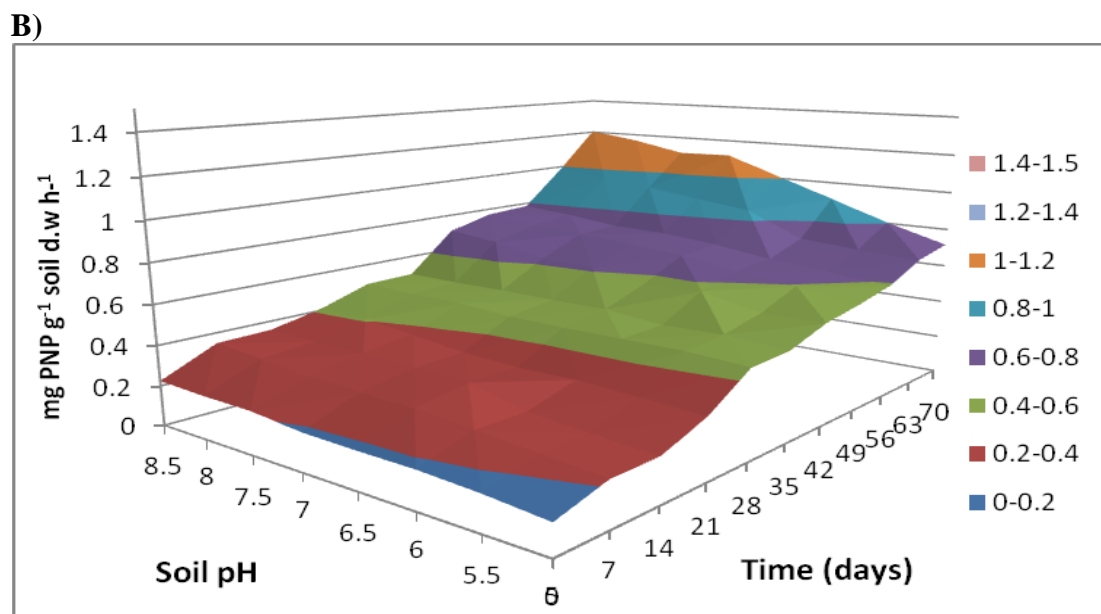
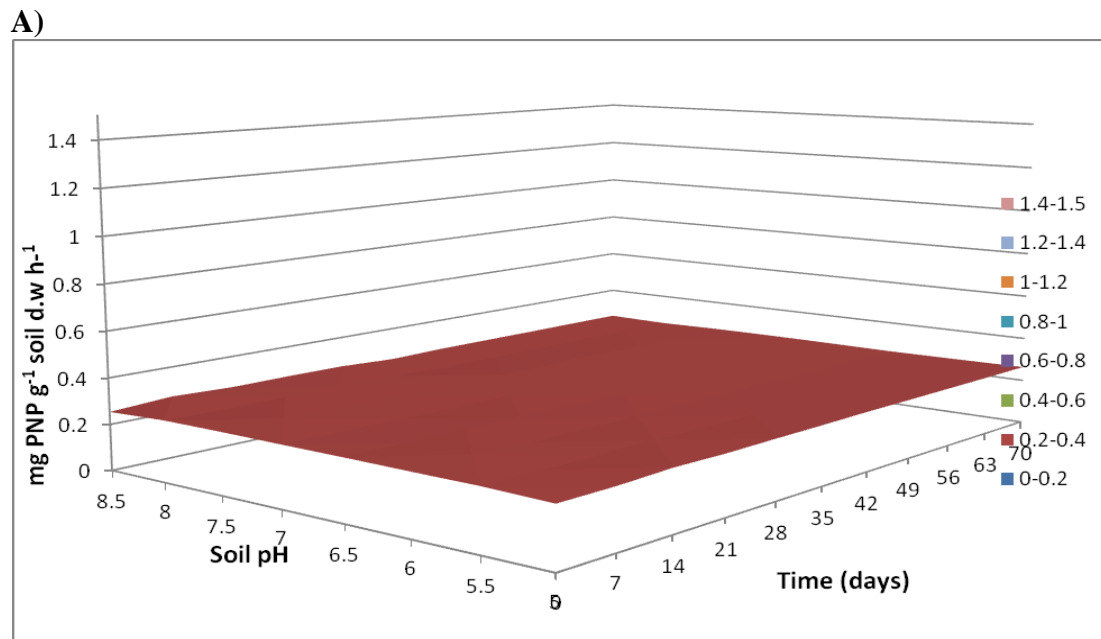


Fig. 5.14: Phosphatase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH adjusted to 8.5.

A): Phosphatase activity in control samples without microbial inocula.

B): Phosphatase activity in experimental sample inoculated with microbial strains.

Phosphatase activities were determined with buffer pH 5.5, 7 and 8.5 respectively. Activities were measured at varying soil pH over the 70 days incubation. Soil samples without microbial inoculation resulted in similar low phosphatase activities (0.2 mg pNP g⁻¹ soil d.w h⁻¹) across the three buffer pH varying buffer pH 5, 7 and 8.5 resulted in same activity levels (figure 5.12 A, 5.13 A, 5.14 A). The phosphatase activities in soil samples with microbial inocula resulted in higher levels of activities over varying soil pH.

Soil inoculated with microorganisms resulted in differences in phosphatase activities over time and pH at acidic soil pH when compared to neutral and alkaline soil pH. Furthermore at acidic pH 5.5 activities increased over the time from day 0 a peak of 0.8 mg pNP g⁻¹ soil d.w h⁻¹ at day 42 respectively. However, soil pH 8.5 and 8 exhibited low acid phosphatase activity of around 0.2-0.4 mg pNP g⁻¹ soil d.w h⁻¹ from day 0 – day 42 and fell to 0.2 mg pNP g⁻¹ soil d.w h⁻¹ by day 70. In general, as the soil pH increased the acid phosphatase activity decreased (figure 5.12 B).

Phosphatase activity measured at buffer pH 7 exhibited low levels of activity (approximately 0.2 mg pNP g⁻¹ soil d.w h⁻¹ in all the soil pH from 0 to 14 days across all soil pH (figure 37 B). However, higher levels of activity (approximately 0.4-0.6 mg pNP g⁻¹ soil d.w h⁻¹) were observed from day 21 at neutral and alkaline soil pH and from day 28 for pH 7, 6.5, 6, 5.5 and pH 5 which reached 0.2- 0.4 mg pNP g⁻¹ soil d.w h⁻¹ by day 56 (figure 5.13 B).

Phosphatase activity measured at buffer pH 8.5 (figure 5.14 B) exhibited initially low activity levels (around 0.2 mg pNP g⁻¹ soil d.w h⁻¹) for all soil pH. However, activity levels gradually increased to around 0.4-0.6 mg pNP g⁻¹ soil d.w h⁻¹ at day 28 with slight lower activity obtained in acidic soil pH 5, 5.5 and 6 (figure 5.14 B). Higher

activity was observed in alkaline soil pH 6.5, 7, 7.5, 8 and 8.5. Phosphatase activity measured at pH 8.5 continued to increase over time especially at alkaline and neutral pH reaching a peak of 1-1.2 mg pNP g⁻¹ soil d.w. h⁻¹ at pH 8.5 at 70 days whilst at the same time point at soil pH of 5.5 the activity was between 0.6-0.8 mg pNP g⁻¹ soil d.w h⁻¹ (figure 5.14 B).

5.3.6b β -glucosidase soil activity

β -glucosidase activity was performed using three buffer at varying pH 5.5, 7 and 8.5 respectively. All the soil samples contaminated with PAH for biodegradation by mixed microbial cultures exhibited β -glucosidase activity in soil. The highest activities were measured around 0.04-0.06 mg pNP g⁻¹ soil d.w h⁻¹ at varying soil pH over the 70 days incubation.

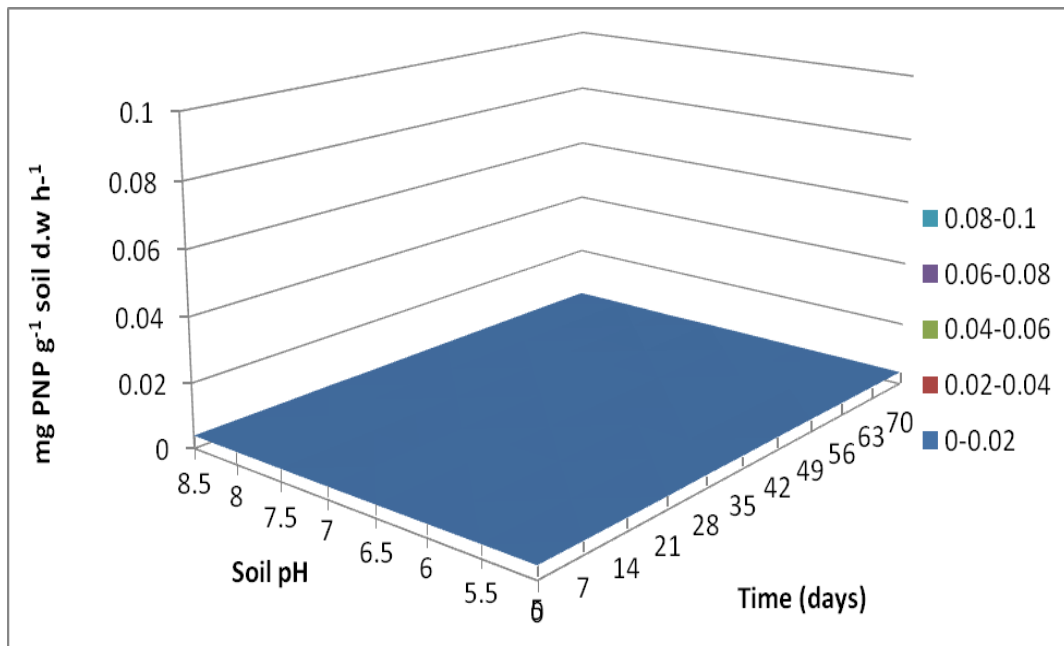
A difference in β -glucosidase was obtained in soil samples without microbial inocula and samples with microbial inocula. Soil samples without microbial inocula had lower β -glucosidase activity of 0.02 mg pNP g⁻¹ soil d.w h⁻¹. Soil inoculated with microorganisms resulted in slight differences in β -glucosidase activities over time at acidic soil pH when compared to neutral and alkaline soil pH. Furthermore at acidic pH 5.5 activities increased over the time from day 0 to a peak of 0.04 mg pNP g⁻¹ soil d.w h⁻¹ at day 7 (figure 5.15 B). Whilst, all soil pH is resulted in constant activities of around 0.04-0.06 mg pNP g⁻¹ soil d.w h⁻¹ from day 7 to a day 70.

β -glucosidase activity measured at buffer pH 7 exhibited similar levels of activity (approximately 0.04 mg pNPg⁻¹ soil d.w h⁻¹) at soil pH 6, 6.5, 7and 7.5 from 0 to 70 days. However, soil pH 5 and 5.5 exhibited lower activity levels around 0.02-0.04 mg pNP g⁻¹ soil d.w h⁻¹ from 0 to 35 days which increased over the time from day 35 to a

peak of 0.04- 0.06 mg pNP g⁻¹ soil d.w h⁻¹ (figure 5.16 B). Soil pH 8 and 8.5 exhibited β -glucosidase activity with a peak around 0.004- 0.006 mg pNP g⁻¹ soil d.w h⁻¹ over time 0 to 14 days however, activity low levels around 0.002-0.006 mg pNP g⁻¹ soil d.w h⁻¹ were obtained from 14 to 28 days.

β -glucosidase activity measured at buffer pH 8.5 (figure 5.17 B) exhibited (approximately 0.004-0.005 mg pNP g⁻¹ soil d.w h⁻¹ in soil samples with microbial inocula across all the soil pH over time 0 to 56 days. The activity levels decreased across all the soil pH around 0.04 – 0.02 mg pNP g⁻¹ soil d.w h⁻¹ from 56 to 70 days.

A)



B)

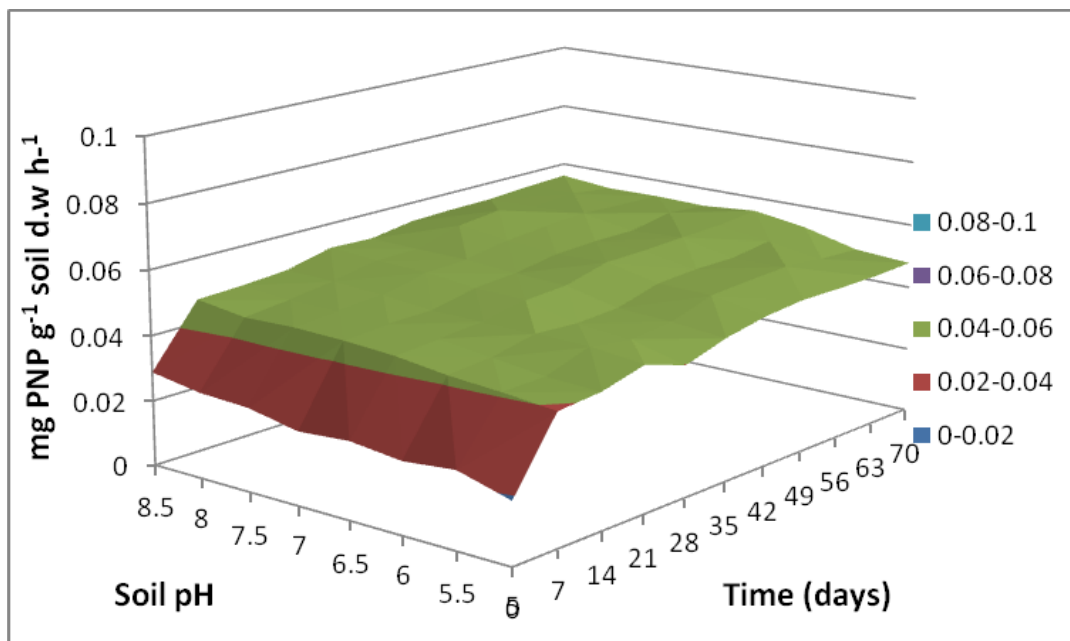
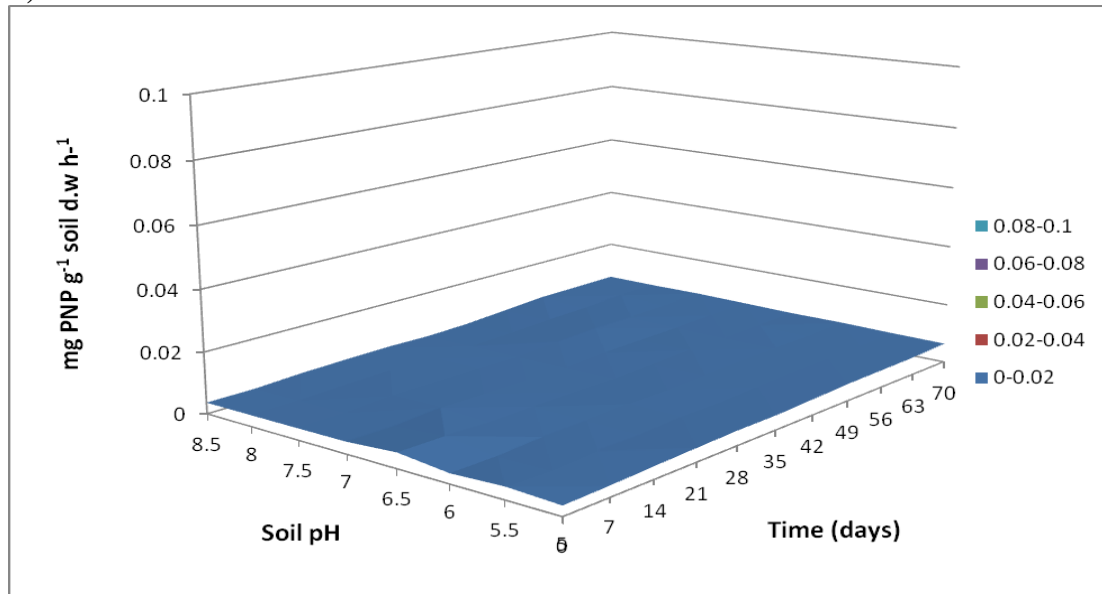


Fig. 5.15: β -glucosidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

A): β -glucosidase activity in control samples without microbial inocula.

B): β -glucosidase activity in experimental sample inoculated with microbial strains.

A)



B)

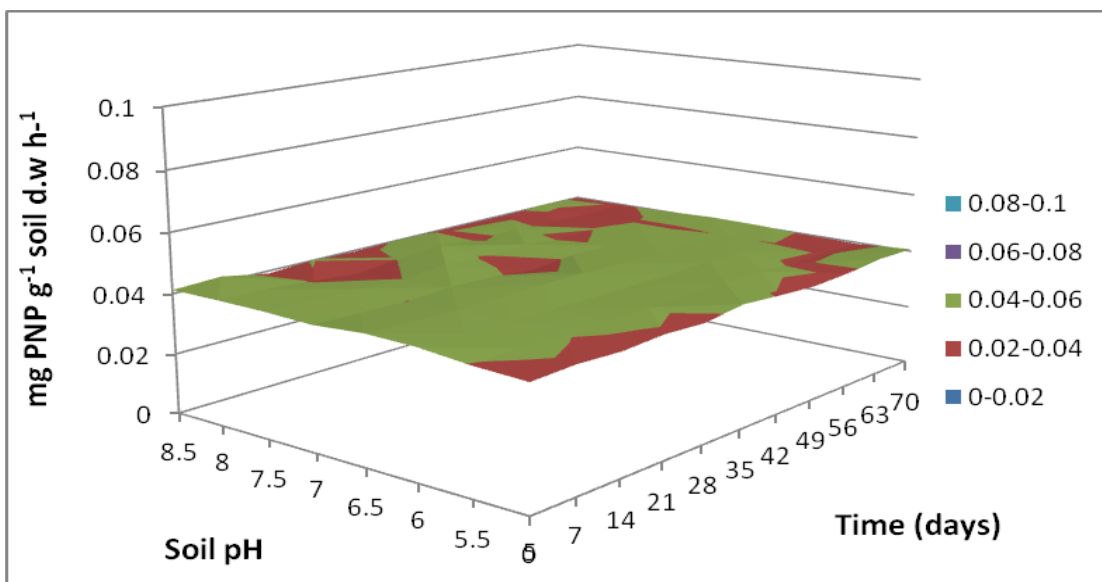
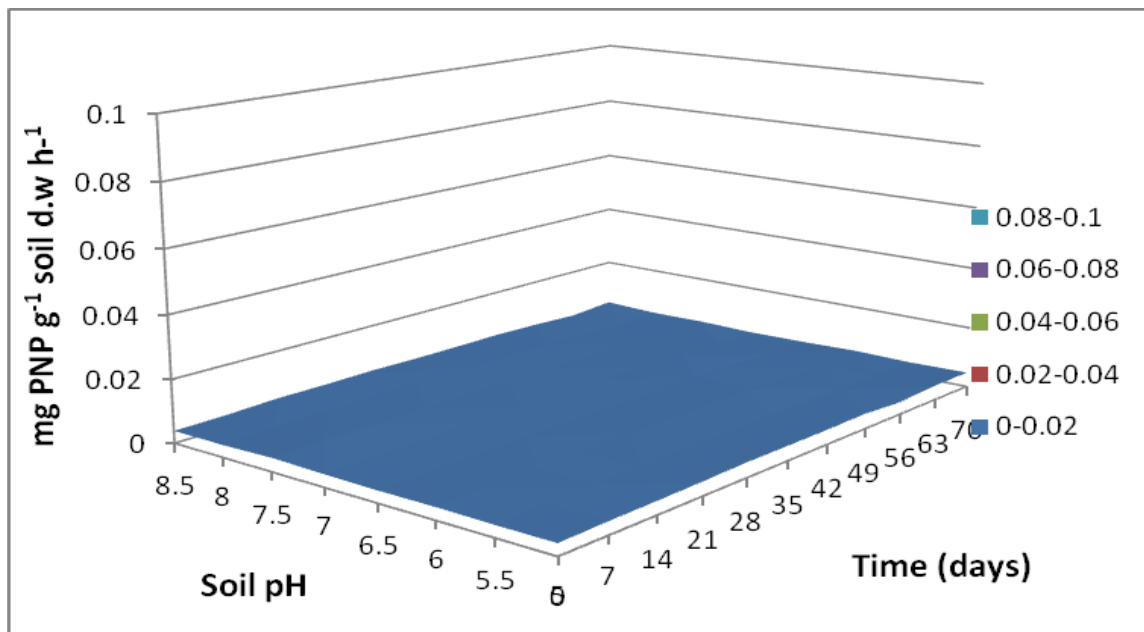


Fig. 5.16: *β -glucosidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 7.*

A): β -glucosidase activity in control samples without microbial inocula.

B): β -glucosidase activity in experimental sample inoculated with microbial strains.

A)



B)

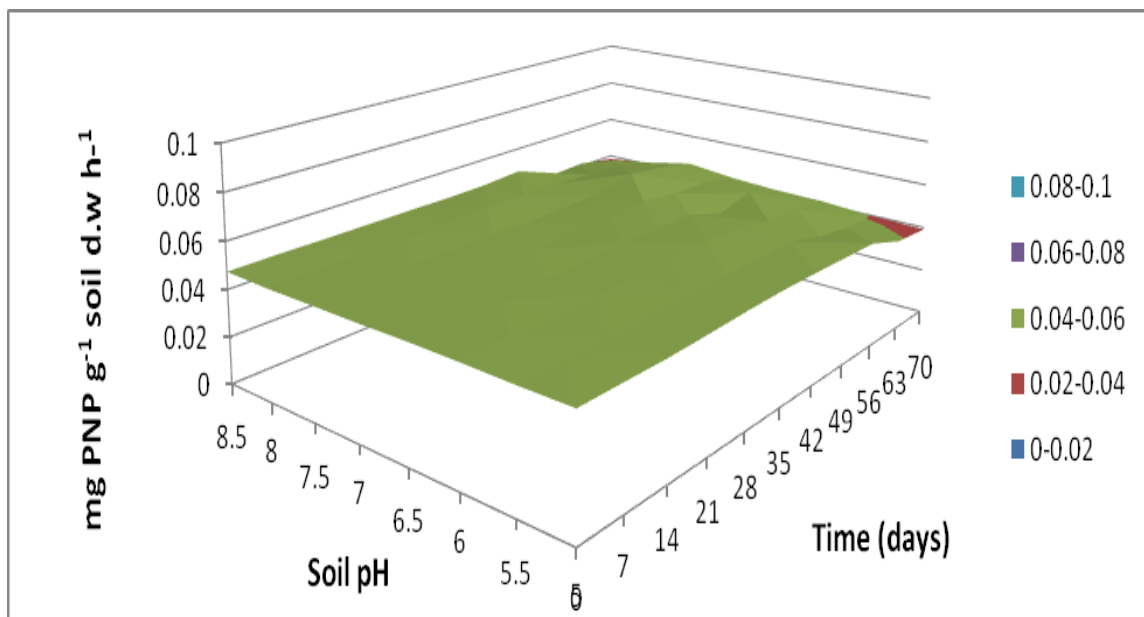


Fig. 5.17: β -glucosidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 8.5.

A): β -glucosidase activity in control samples without microbial inocula.

B): β -glucosidase activity in experimental sample inoculated with microbial strains.

5.3.6c L-arginine ammonification soil activity

L-arginine ammonification activity was measured in PAH contaminated J. Arthur bower's topsoil at varying soil pH.

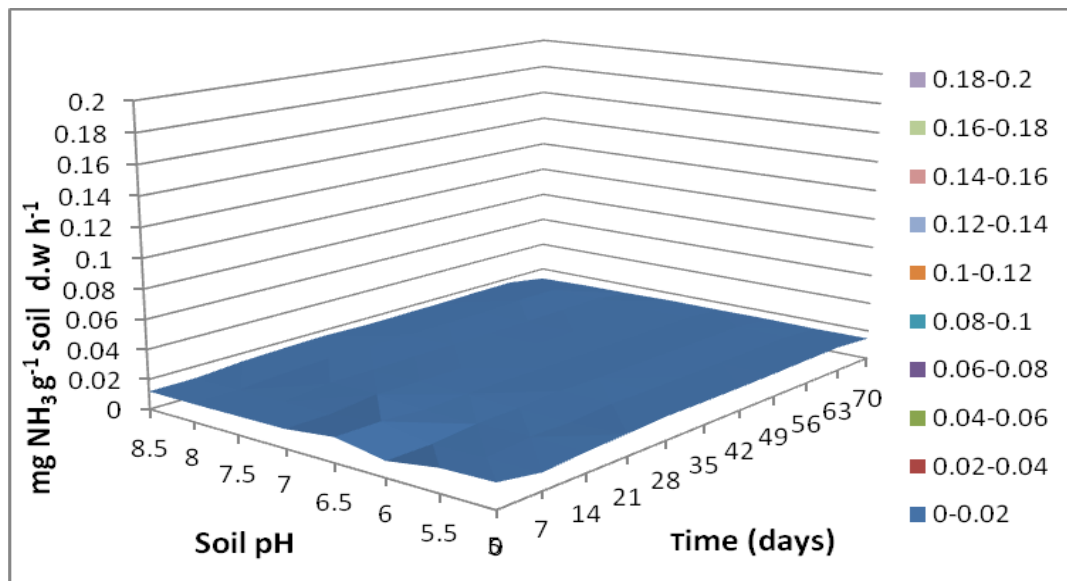
L-arginine ammonification activities exhibited higher activities (approximately 0.18-0.2 mg NH₃ g⁻¹ soil d.w h⁻¹) in samples with microbial inocula over 70 days of incubation (figure 63B). Soil samples with microbial inocula were evident with least lower activities of around 0.12- 0.14 mg NH₃ g⁻¹ soil d.w h⁻¹ across acidic soil pH whereas high activities were exhibited by alkaline soil pH (around from 0.14- 0.16 mg NH₃ g⁻¹ soil d.w h⁻¹ from 0 to 21 days. Soil pH 5, 5.5, 6, and 6.5 activities increased to 0.14- 0.16 mg NH₃ g⁻¹ soil d.w h⁻¹ from 35 to 56 days with further increase of 0.18- 0.2 mg NH₃ g⁻¹ soil d.w h⁻¹ from 56 to 70 days respectively.

L-arginine ammonification activity measured at buffer pH 7 exhibited high activities in soil samples with microbial inocula compared to samples without microbial inocula. Initially activity levels were increased from day 7, 14 and 21 exhibiting 0.12-0.14 mg NH₃ g⁻¹ soil d.w h⁻¹ at soil pH 5, 5.5 and 6 whereas, activities at alkaline pH exhibited 0.14-0.16 mg NH₃ g⁻¹ soil d.w h⁻¹ from 0 to 28 days (figure 5.19 B). Soil pH 5, 5.5, 6 and 6.5 exhibited 0.16 mg NH₃ g⁻¹ soil d.w h⁻¹ between 28 to 56 days whereas, activity levels at soil pH 7, 7.5, 8 and 8.5 were 0.18 mg NH₃ g⁻¹ soil d.w h⁻¹ from 28 to 56 days. A decrease in activity to 0.14 mg NH₃ g⁻¹ soil d.w h⁻¹ resulted across all the soil pH after 63 day.

L-arginine activity measured at buffer pH 8.5 exhibited low levels of activities (approximately 0.16 mg NH₃ g⁻¹ soil d.w h⁻¹) across all the soil pH except for pH 8.5 which exhibited slightly different activity of 0.18 mg NH₃ g⁻¹ soil d.w h⁻¹ respectively over the incubation of 0 to 28 days (figure 5.20 B). Soil pH 5 and 5.5 exhibited similar

activity measurements up to 49 days, whereas activities across all the soil pH increased to $0.18 \text{ mg NH}_3 \text{ g}^{-1} \text{ soil d.w h}^{-1}$. Activities measured across soil pH 5, 5.5, 6 and 6.5 increased to $0.18 \text{ mg NH}_3 \text{ g}^{-1} \text{ soil d.w h}^{-1}$ from day 49 to day 70 however for soil pH 7, 7.5, 8 and 8.5 activity levels increased to $0.2 \text{ mg NH}_3 \text{ g}^{-1} \text{ soil d.w h}^{-1}$ from 49 to 70 days of incubation.

A)



B)

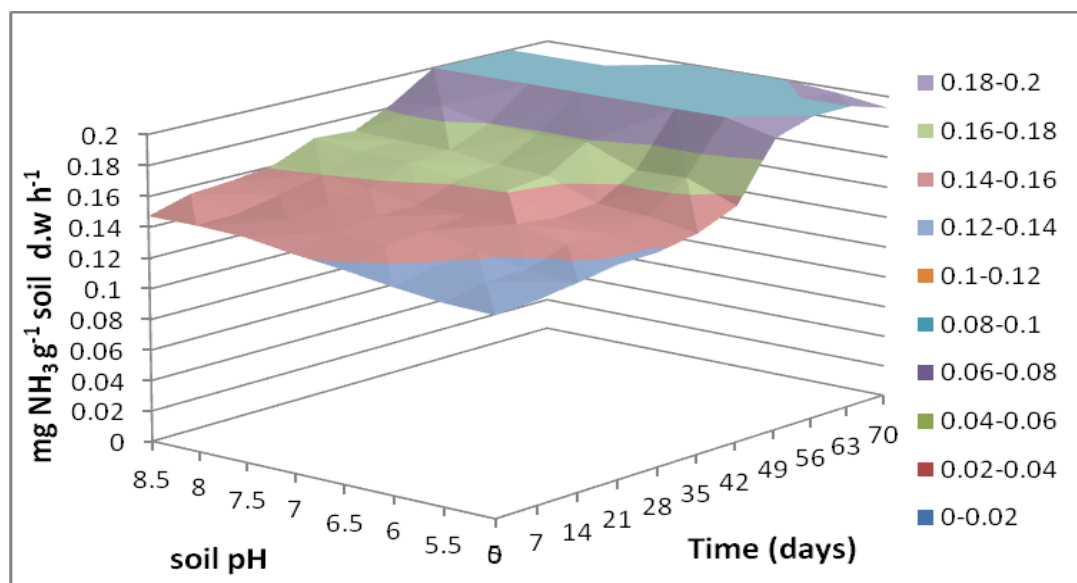
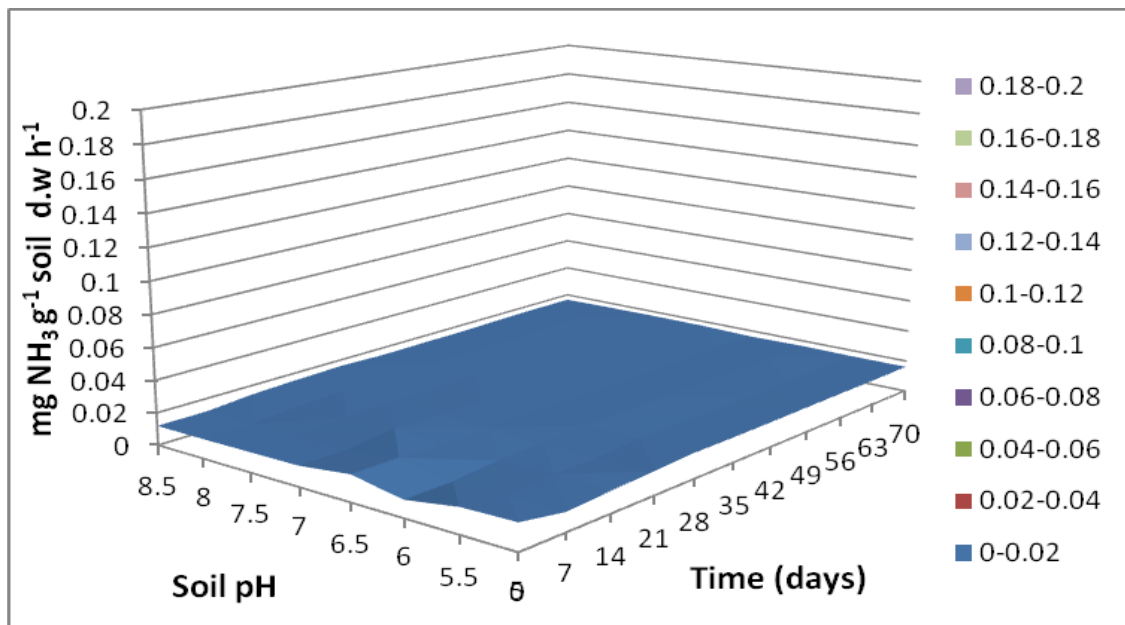


Fig. 5.18: L-arginine ammonification activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

A): L-arginine ammonification activity in control samples without microbial inocula.

B): L-arginine ammonification activity in experimental sample inoculated with microbial strains.

A)



B)

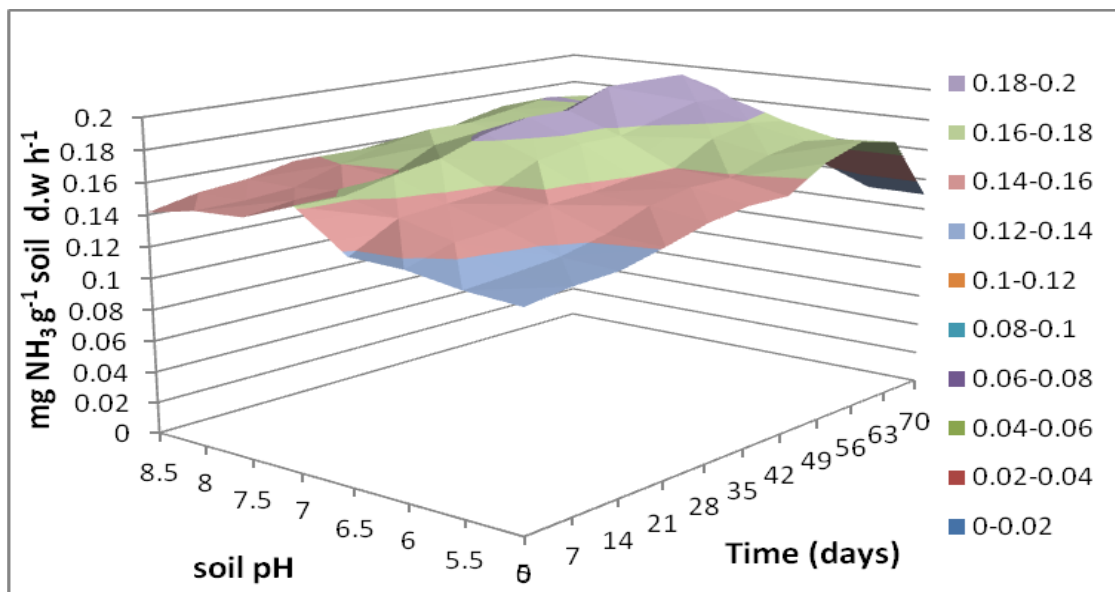


Fig. 5.19: L-arginine ammonification activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 7.

- A): L-arginine ammonification activity in control samples without microbial inocula.
- B): L-arginine ammonification activity in experimental sample inoculated with microbial strains.

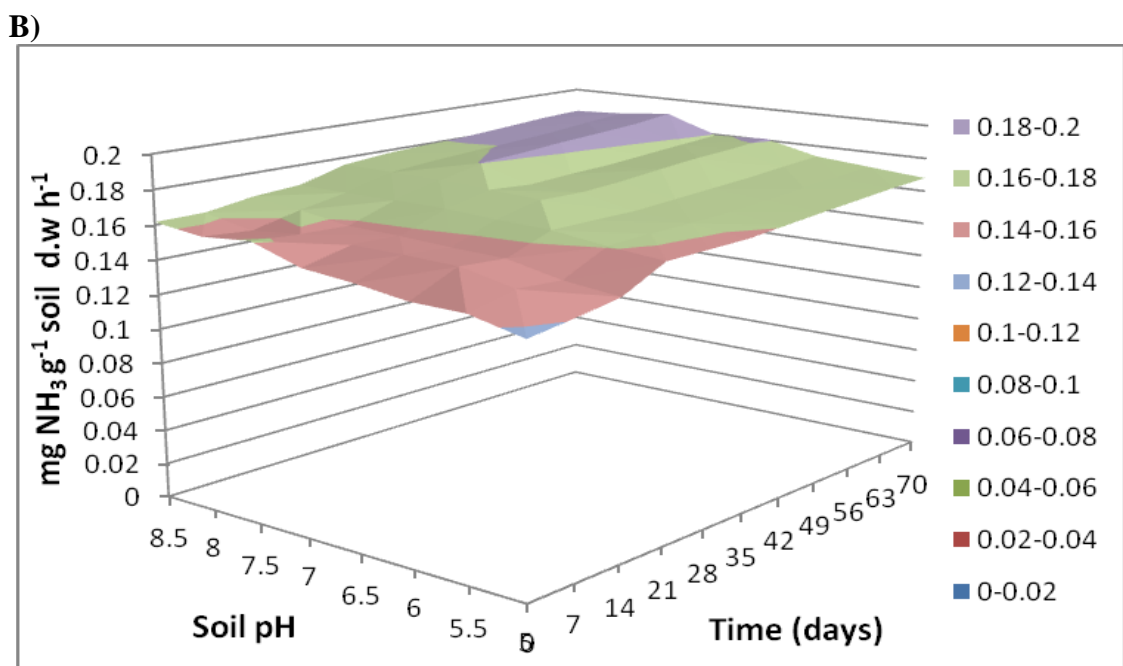
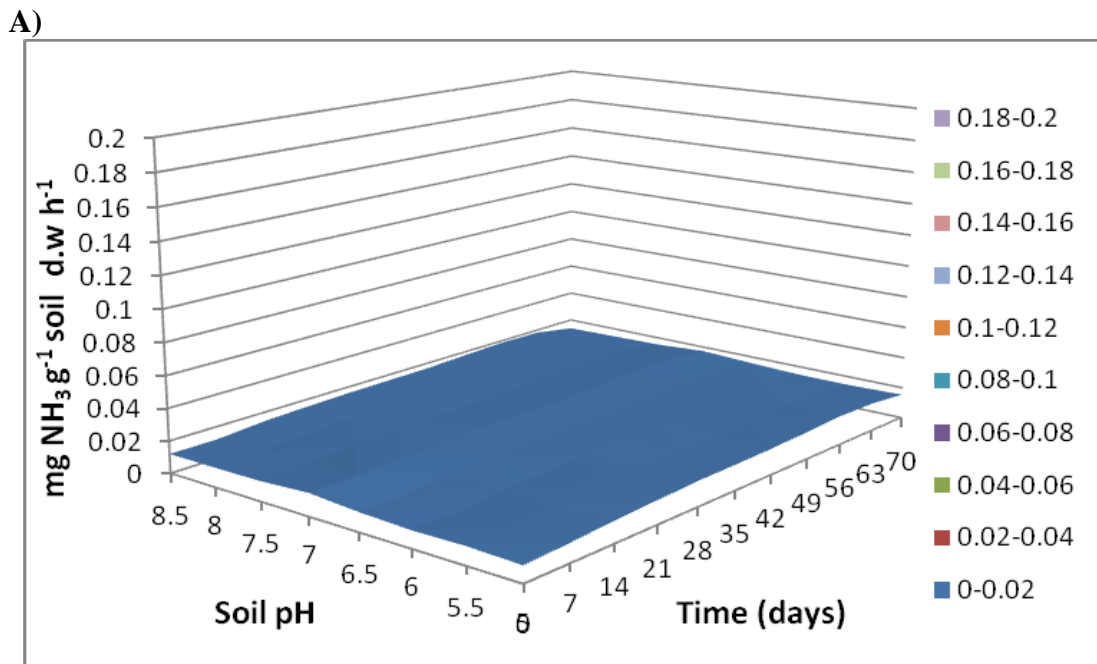


Fig. 5.20: L-arginine ammonification activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 8.5.

- A): L-arginine ammonification activity in control samples without microbial inocula.
 B): L-arginine ammonification activity in experimental sample inoculated with microbial strains.

5.3.6d Laccase soil activity

Laccase activity was measured in PAH contaminated J. Arthur Bower's topsoil at varying soil pH. Activity was measured at three buffer pH 5.5, 7 and 8.5 respectively. Laccase activities measured in soil samples without microbial inocula exhibited lower levels of activity compared to soil with microbial inocula. Activities measured at three buffer pH in soil without microbial inocula exhibited between 0.02-0.04 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} over 70 days of incubation (figure 5.21 A).

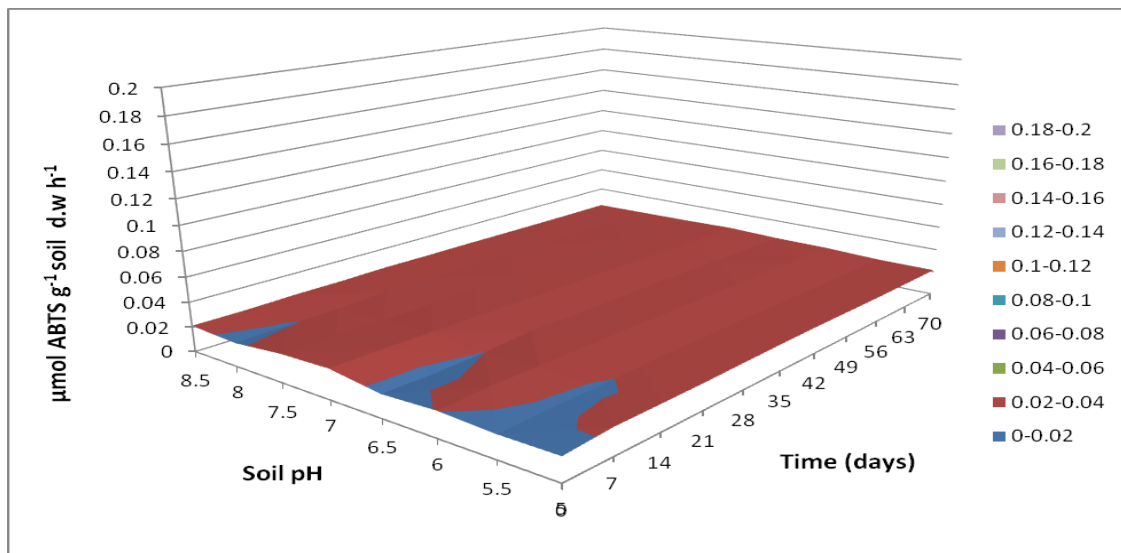
Laccase activities measured in soil samples with microbial inocula had 0.08 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from 0 to 49 days at acidic soil pH whereas, 0.08 $\mu\text{mol g}^{-1}\text{h}^{-1}$ activity was measured in alkaline soil pH from 0 to 28 days of incubation. Acidic soil pH exhibited 0.06 $\mu\text{mol g}^{-1}\text{h}^{-1}$ of activity from 49 to day 70 whereas; in alkaline condition activity was 0.04 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from 35 to 70 day (figure 5.21 B).

Laccase activity measured at buffer pH 7 was 0.02 to 0.04 $\mu\text{mol g}^{-1}$ d.w h^{-1} across all the soil pH in soil samples without microbial inocula. The activity levels in soil with microbial consortia were low in acidic and alkaline (approximately 0.1 to 0.12 $\mu\text{mol g}^{-1}$ soil d.w h^{-1}) whereas neutral soil pH exhibited 0.14 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from 0 to 49 days (figure 5.22 B). From 49 to 70 days of incubation laccase activities increased across all the soil pH to 0.14 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} . The activity at neutral soil pH was increased to 0.2 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from 49 to 70 day respectively.

Laccase activities measured at buffer pH 8.5 were low compared to activities measured at other two buffer pH. The highest activity in soil inoculated with microbial consortium was 0.1 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} activities at neutral soil pH from day 49 to 70. Soil pH 5, 5.5, 6 and 6.5 exhibited 0.04-0.06 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from days 7 to 70 days, whereas alkaline soil pH 8 and 8.5 exhibited 0.06 $\mu\text{mol g}^{-1}$ soil d.w h^{-1} from day

0 to 14 which were further increase to $0.08 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ from 14 to day 70 respectively. Activity in soil pH 7 and 7.5 was $0.08 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ from day 0 to 49 with a slight increase to $0.1 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ from day 49 to 70 (figure 5.23 B).

A)



B)

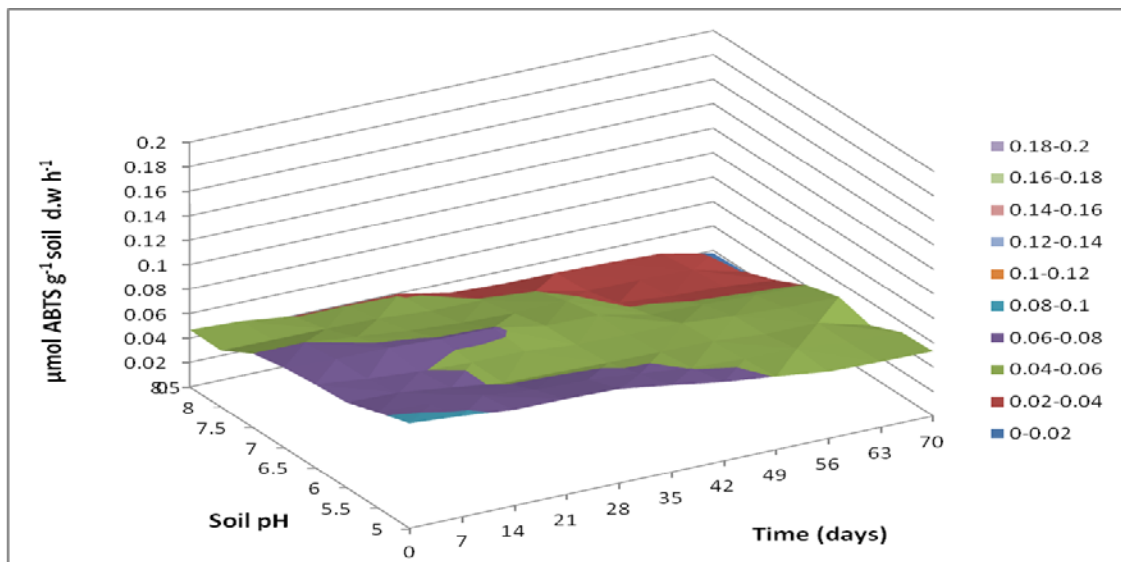
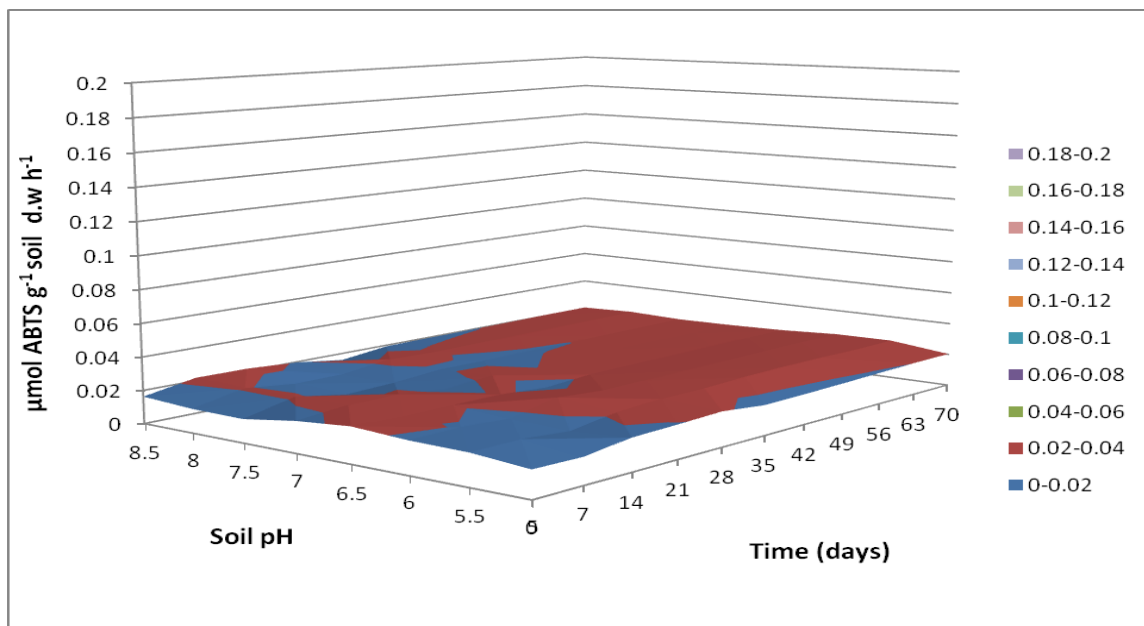


Fig. 5.21: Laccase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

A): Laccase activity in control samples without microbial inocula.

B): Laccase activity in experimental sample inoculated with microbial strains.

A)



B)

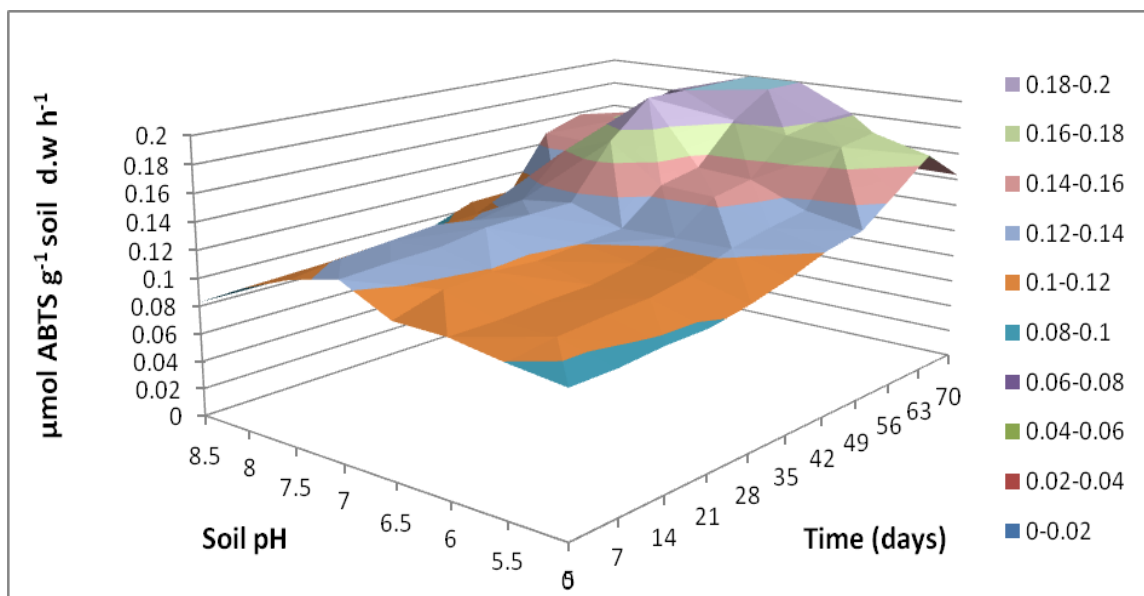
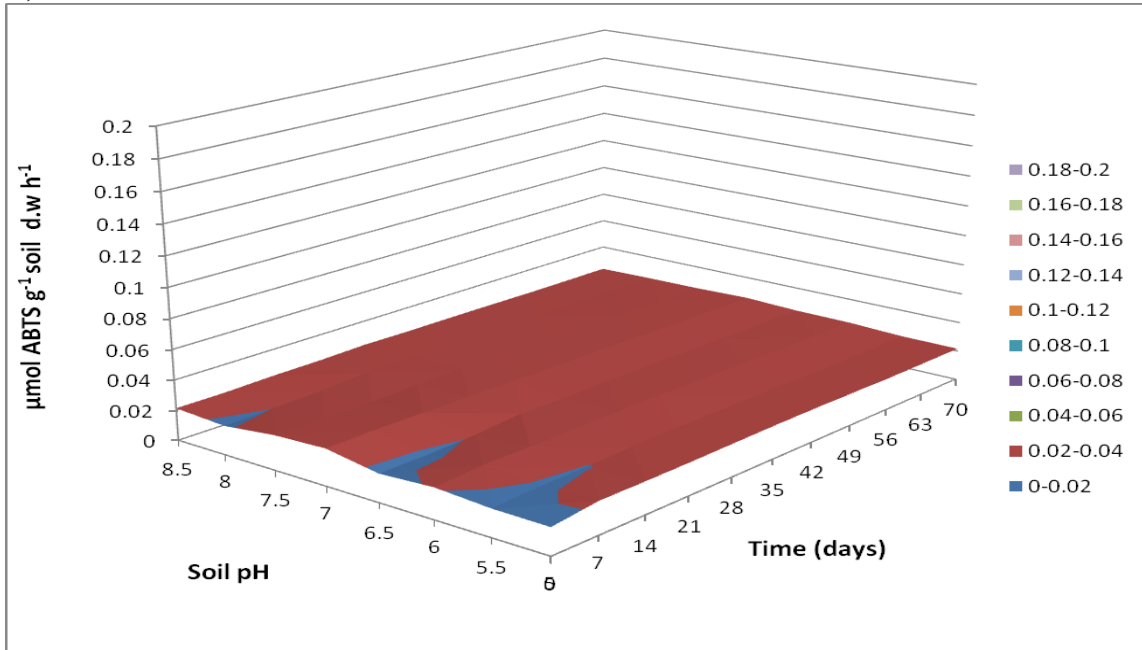


Fig. 5.22: Laccase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 7.

A): Laccase activity in control samples without microbial inocula.

B): Laccase activity in experimental sample inoculated with microbial strains.

A)



B)

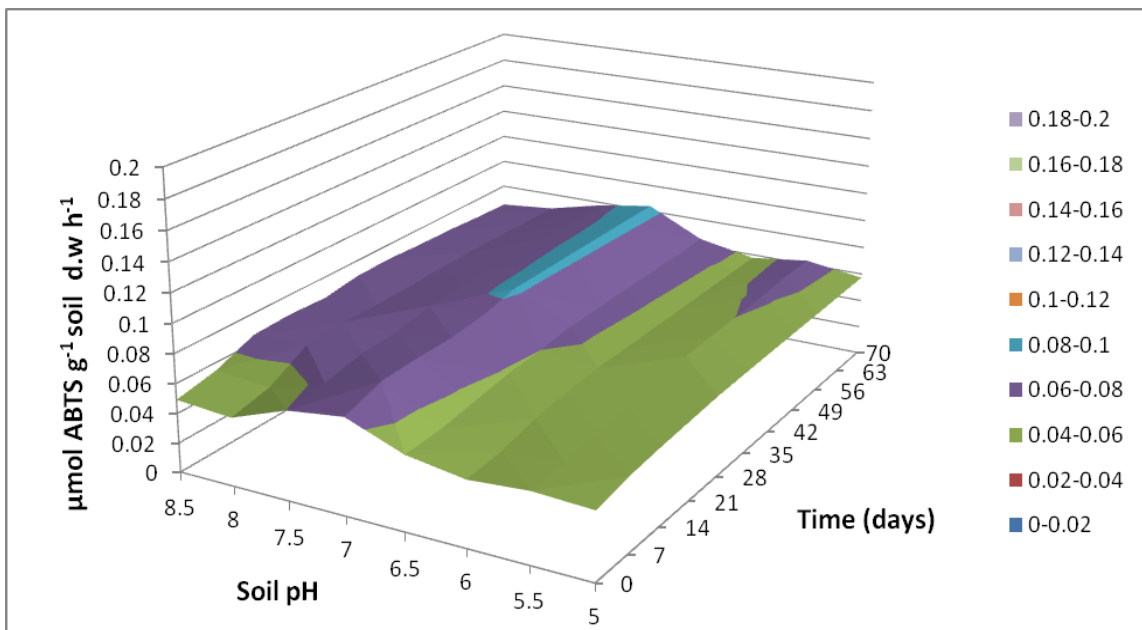


Fig. 5.23: Laccase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 8.5.

A): Laccase activity in control samples without microbial inocula.

B): Laccase activity in experimental sample inoculated with microbial strains.

5.3.6e Lignin peroxidase soil activity

Lignin peroxidase activities were measured in PAH contaminated soil samples at buffer pH 5.5, 7 and 8.5 respectively. Activities measured using buffer pH 5.5 in soil without microbial inocula was $0.4 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ from 0 to 35 day which further increased to $0.6 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ from 35 to 70 day (figure 5.24 A). Activities measured using buffer pH 7 (figure 5.25 A) and 8.5 (figure 5.26 A) without microbial inocula were $0.4 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ across all soil pH's.

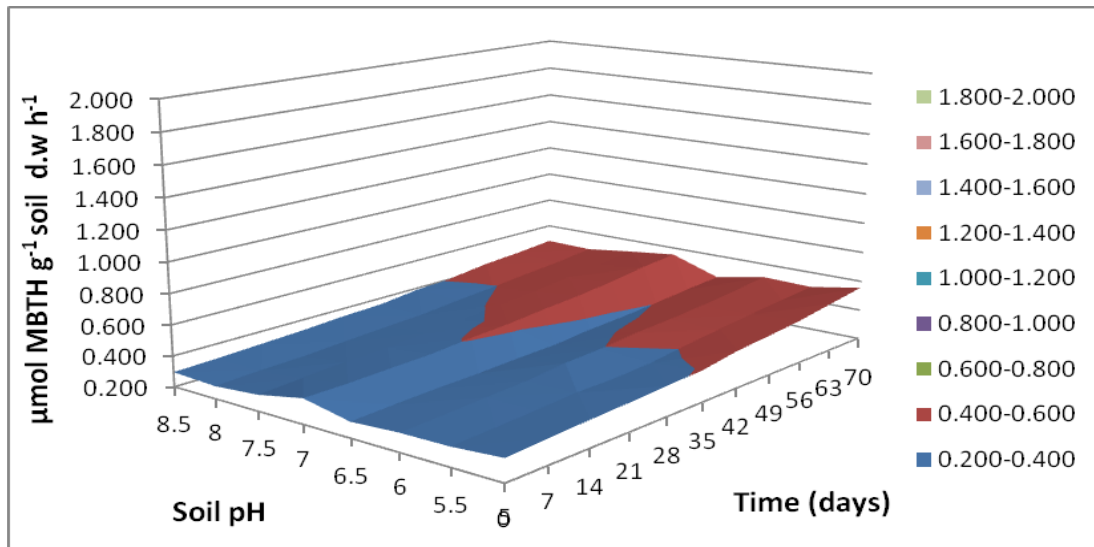
LiP activity measured at buffer pH 5.5, (figure 5.24 B) exhibited greater activity levels of $1.8 \mu\text{mol g}^{-1}\text{h}^{-1}$ at acidic soil pH of 5, 5.5, 6 and 6.5 from 0 day to 70 days of incubation in soil with microbial inocula. The activity measured across neutral soil pH 7 and 7.5 was between $1.2\text{-}1.4 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ with a slight decrease in activity levels from day 0 to 49 day. Soil pH 8 and 8.5 exhibited lower activity levels of between 0.6 and $1.0 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$.

LiP activity measured using buffer pH 7 exhibited activities of between $0.8\text{-}1.0 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ across all the soil pH from day 0 to 21 days. However, the activity level increased from day 21 to 70 days further to at least $1.2 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$ across all the soil pH (figure 5.25 B). After day 63 a slight increase in activity was observed in acidic soil pH 5 and 5.5 to $1.4 \mu\text{mol g}^{-1} \text{soil h}^{-1}$ of LiP release.

LiP activity measured using buffer pH 8.5 exhibited low levels of LiP peroxidase released in soil samples without microbial inocula (approximately $0.4 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$) compared to soil samples with microbial inocula with highest peak of activity (approximately $1.0 \mu\text{mol g}^{-1}\text{h}^{-1}$) (figure 5.26 B). LiP activity was similar in acidic and alkaline conditions (approximately $0.8 \mu\text{mol g}^{-1} \text{soil d.w h}^{-1}$) and comparably lowers

than at neutral soil pH (approximately $1.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$) from day 0 to 63 day. Lower activities were evident from day 63 to 70 day across all the soil pH of $0.6 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ at acidic soil pH 5, 5.5 and 6 whereas the activity measured at neutral and acidic soil pH was $0.8 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$.

A)



B)

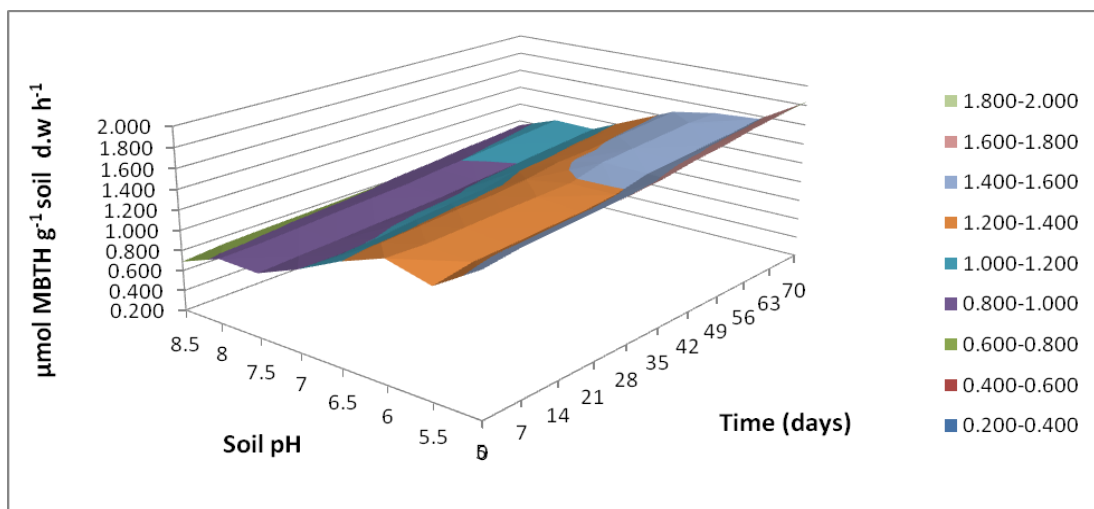
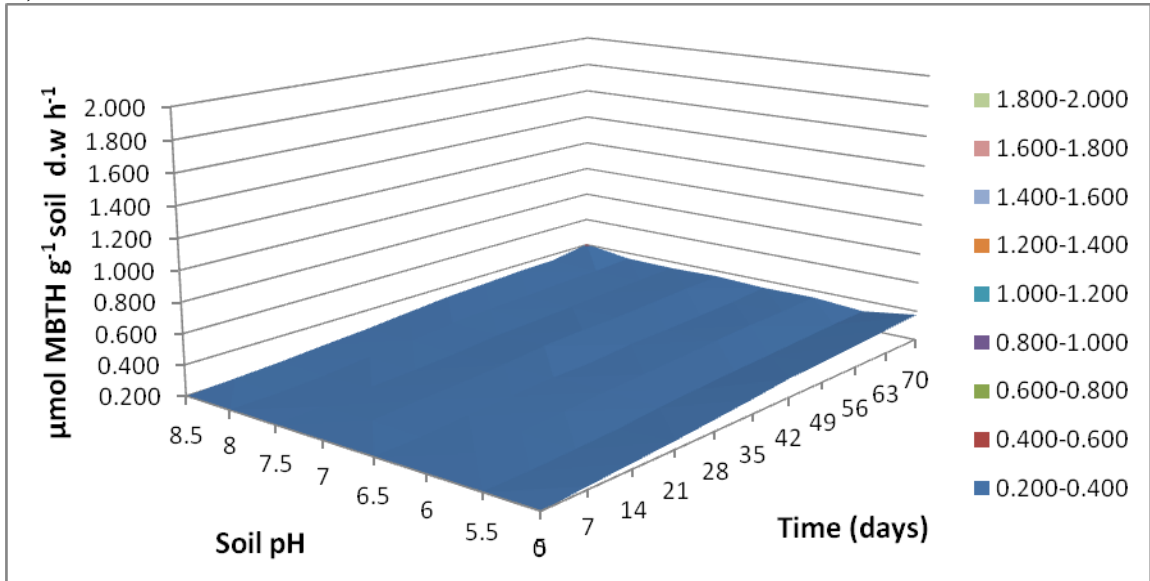


Fig. 5.24: Lignin peroxidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

- A): Lignin peroxidase activity in control samples without microbial inocula.
- B): Lignin peroxidase activity in experimental sample inoculated with microbial strains.

A)



B)

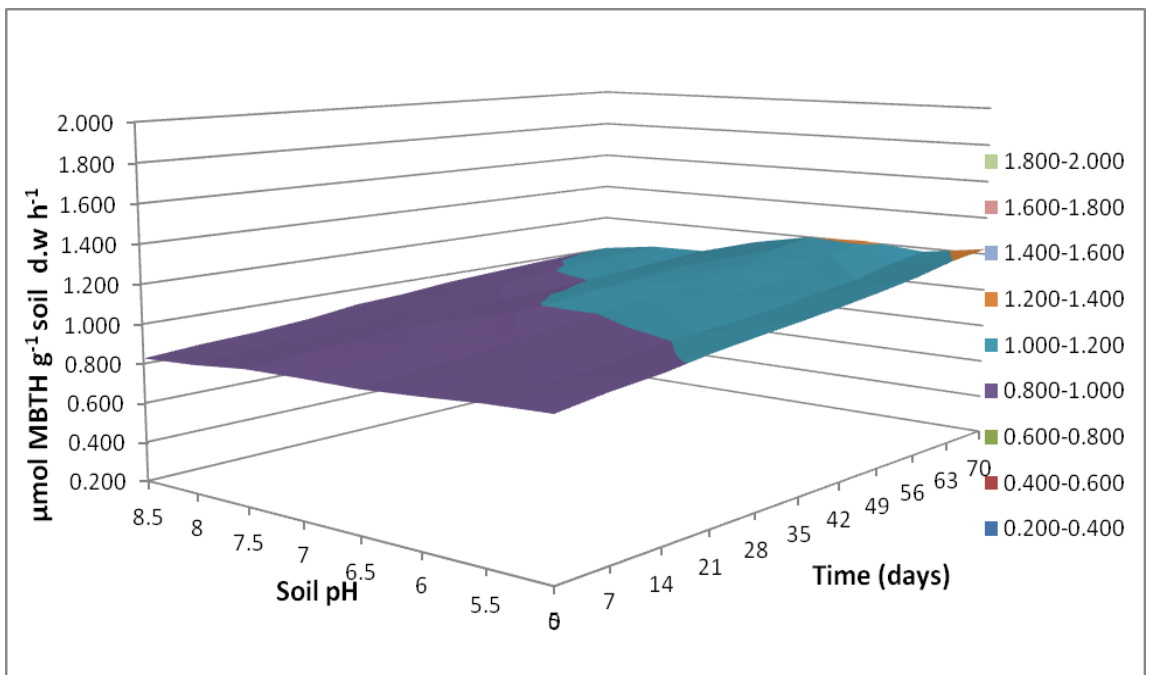
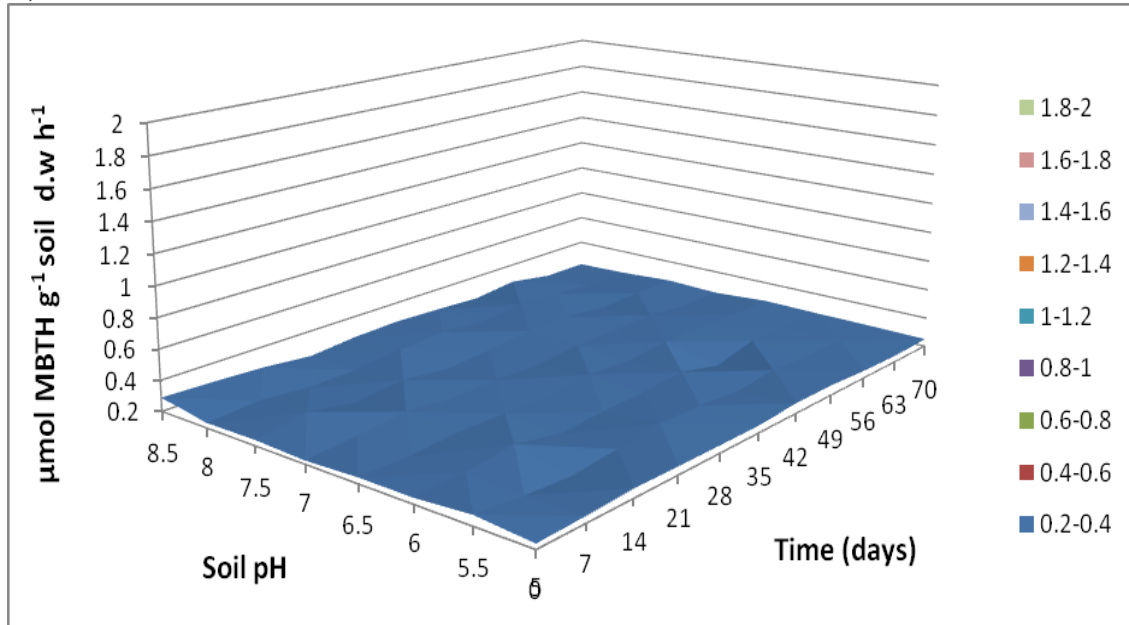


Fig. 5.25: Lignin peroxidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 7.

- A): Lignin peroxidase activity in control samples without microbial inocula.
- B): Lignin peroxidase activity in experimental sample inoculated with microbial strains.

A)



B)

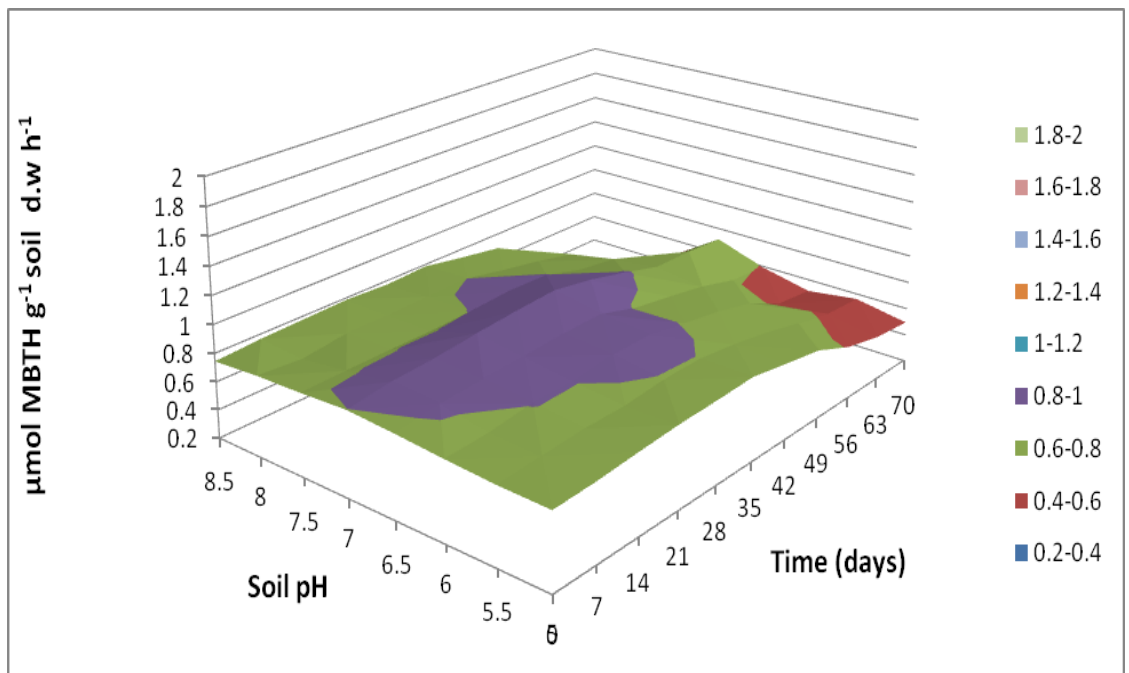


Fig. 5.26: Lignin peroxidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 8.5.

A): Lignin peroxidase activity in control samples without microbial inocula.

B): Lignin peroxidase activity in experimental sample inoculated with microbial strains.

5.3.6f Manganese peroxidase soil activity

Manganese peroxidase activity was measured in PAH contaminated J. Arthur Bower's topsoil with and without microbial inocula. Manganese peroxidase activity in soil with inoculated microbial consortium measured using buffer pH 5.5 initially exhibited $1.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ across all the soil pH from day 0 to 7. Soil pH 5, 5.5, 6 and 6.5 exhibited $2.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ whereas similar activity levels were measured at alkaline soil pH of 8 and 8.5 from day 7 to 49 respectively. The activity levels were high at soil pH 7 and 7.5 ($2.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$) from 7 to 49 days of incubation. Initial manganese peroxidase activity was higher in week 0 followed by day 7. From day 49 to 63 day of incubation a decrease was observed across all the soil pH to $2.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$. Subsequently activity at neutral soil pH (7 and 7.5) was similar to previous days of incubation (approximately $2.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$) whereas the decrease in activity levels were observed at acidic and alkaline soil pH exhibiting $1.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ from 63 to 70 days (figure 5.27 B).

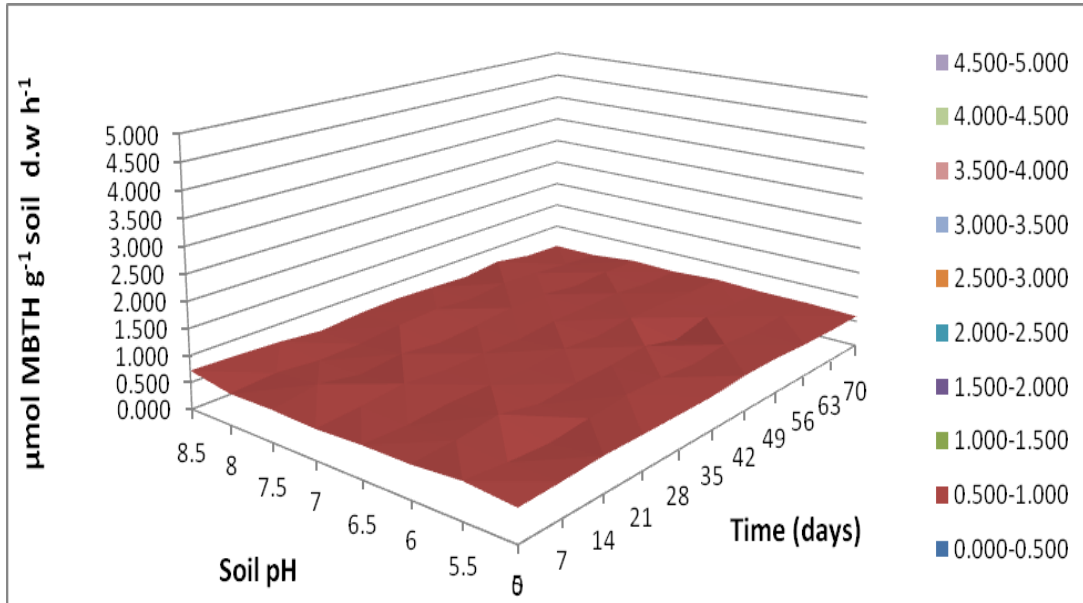
Manganese peroxidase activities measured using buffer pH 7 exhibited a high peak of $3 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ across all the soil pH from day 0 to 14 days. A decrease across acidic and alkaline soil pH was observed to $2 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ from 14 to 49 days whereas at neutral soil pH (7 and 7.5) activity was $2.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ from 14 to 49 days. Activity of $2 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ was found across all the soil pH from 49 to 63 days. Manganese peroxidase activity decreased at acidic soil pH $1.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ from days 63 to 70 (figure 5.28 B).

MnP activity measured at buffer pH 8.5 was of $2.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ across all the soil pH except for alkaline soil pH 8 and 8.5 were $1.5 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ from day 0 to 63 days. Activity from day 63 to 70 slightly increased in acidic (pH 5, .5.5, 6 and

6.5) as well as neutral (pH 7 and 7.5) exhibiting $3.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ (figure 5.29

B).

A)



B)

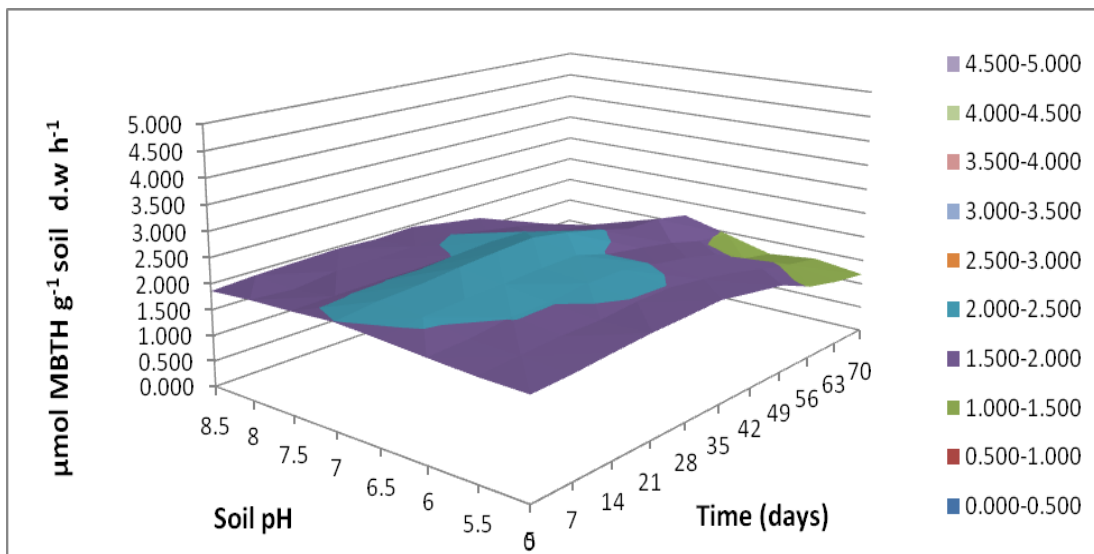


Fig. 5.27: Manganese peroxidase activity of J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 5.5.

A): Manganese peroxidase activity in control samples without microbial inocula.

B): Manganese peroxidase activity in experimental sample inoculated with microbial strains.

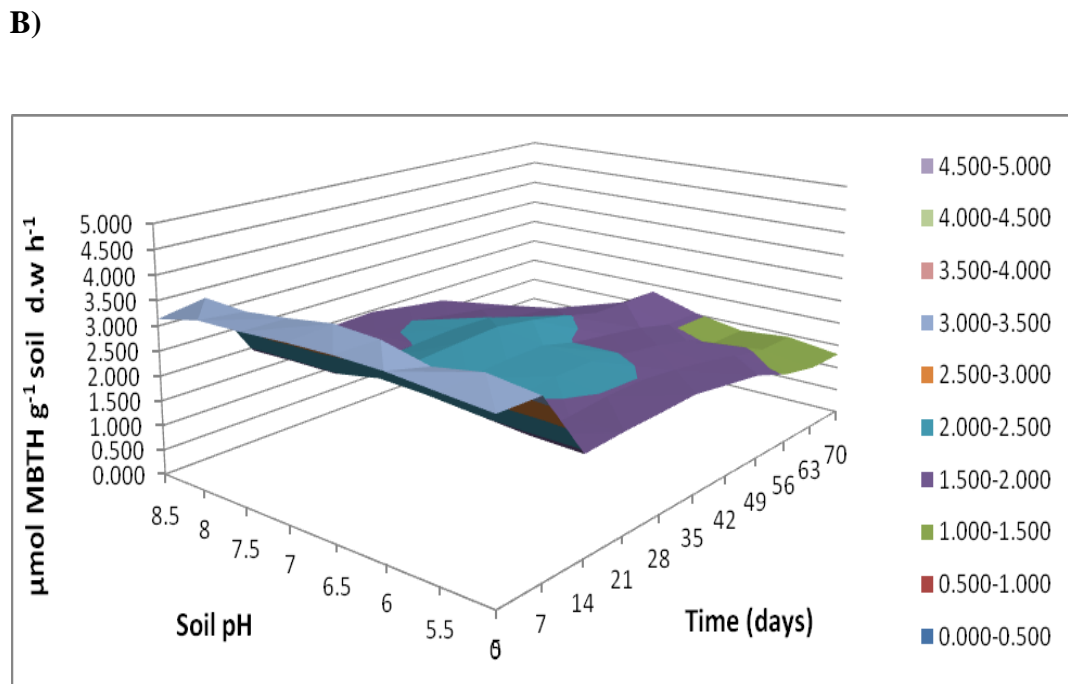
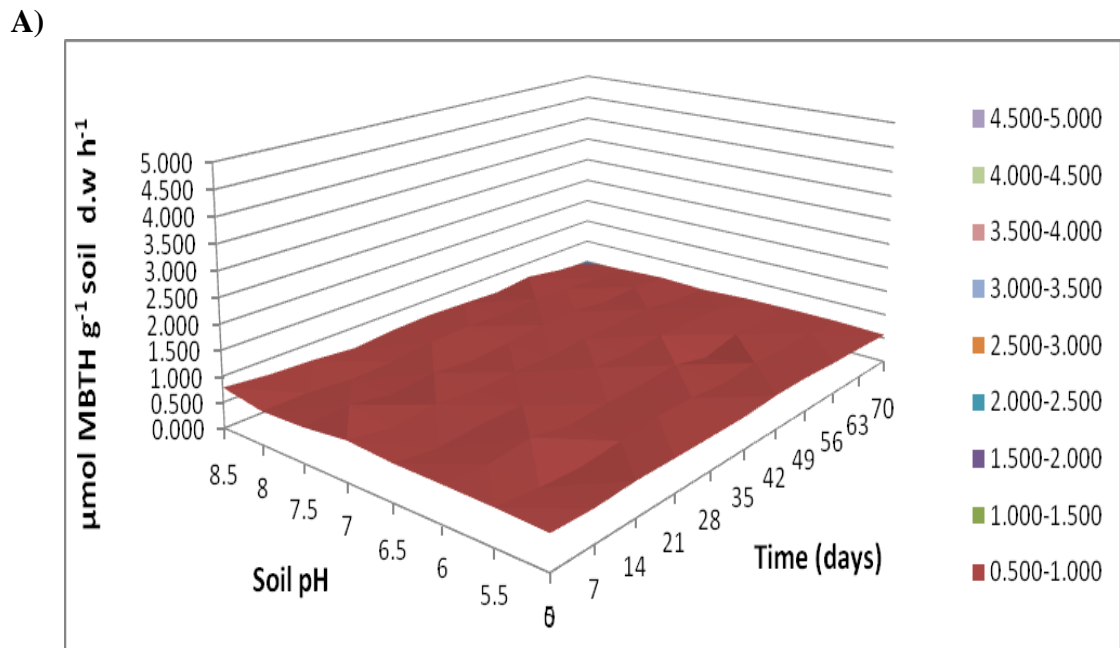
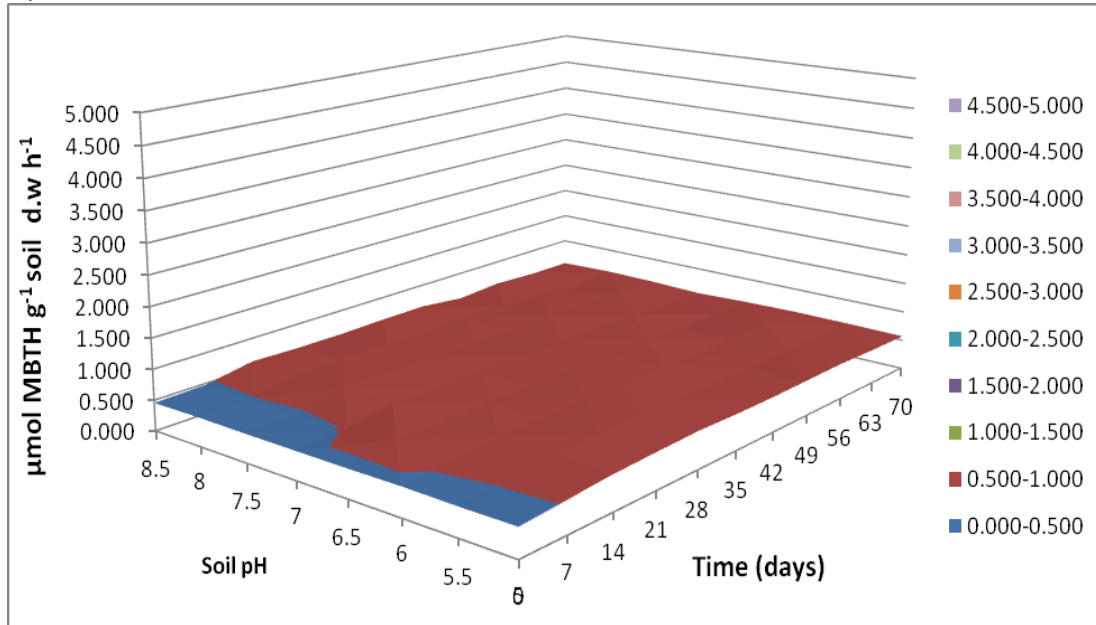


Fig. 5.28: Manganese peroxidase activity in *J. Arthur Bower's* topsoil at varying soil pH over time measured at buffer pH7.

A): Manganese peroxidase activity in control samples without microbial inocula.

B): Manganese peroxidase activity in experimental sample inoculated with microbial strains.

A)



B)

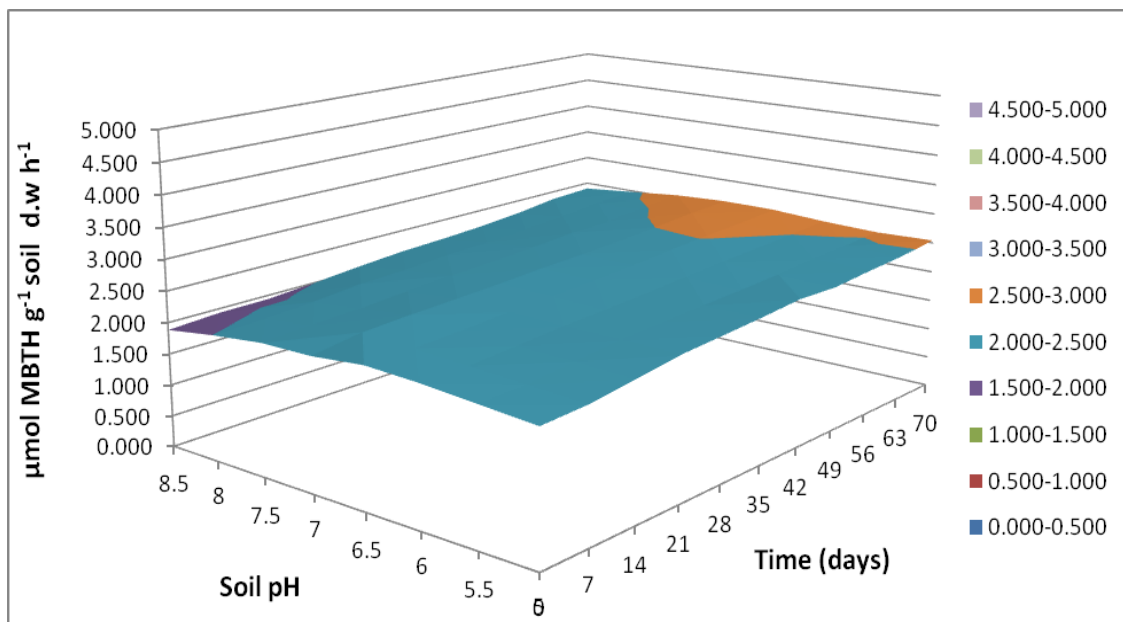


Fig. 5.29: Manganese peroxidase activity in J. Arthur Bower's topsoil at varying soil pH over time measured at buffer pH 8.5.

A): Manganese peroxidase activity in control samples without microbial inocula.

B): Manganese peroxidase activity in experimental sample inoculated with microbial strains.

5.4 Discussion

Soil pH has been suggested to be one of the most important factors that influences degradation processes in soils (Margesin & Schinner, 2001; Funk *et al.*, 1997). The measurement of the effect of soil pH on biodegradation of PAH was achieved using Dionex HPLC fitted with UV detector (UVD 170 U) set at 252 nm, connected to a PC equipped with Chromeleon chromatography software version 6.6. Studies were carried out using four PAHs.

5.4.1 PAH analysis

A standard curve of peak area against PAH concentrations was constructed. A linear standard curve (figure 5.4) was produced for each PAH with an r value (regression coefficient) for phenanthrene of 0.97, fluoranthene 0.97, pyrene 0.97 and for anthracene 0.98. The r values obtained for each calibration curve exhibits that the correlation between relative peak area and concentration was linear and reproducible within the concentration range selected. The Y value representing linear regression equation for phenanthrene was $14.32x$ and for anthracene was $35.12x$, whilst for fluoranthene and pyrene values were much lower at $6.42x$ and $4.08x$ respectively. Similar results were found repeatedly for all the PAH standards. Thus, the data obtained from standard chromatogram were reliable, accurate and reproducible (figure 5.4).

5.4.2 PAH degradation and microbial populations over 70 days in PAH contaminated soil

This study concentrated on monitoring the effect of soil pH on the efficacy of biodegradation of hydrocarbons. The reason for using J. Arthur Bowers topsoil was to provide a constant soil for all experimental studies.

Figure 5.5 exhibits the degradation rate of phenanthrene at varying soil pHs. The greatest rate of PAH's degradation was at pH 7.5, whilst the slowest was at pH 5, 5.5 and pH 8.5. The greatest rate of PAH degradation were obtained for phenanthrene, whilst slowest were obtained for pyrene. Similarly, anthracene, fluoranthene and pyrene exhibited greatest rate of PAH's degradation at soil pH 7.5 (figure 5.6 B, 5.7 B and 5.8 B).

Degradation of PAHs *in situ* is often slow, and research over the last two decades has demonstrated that these compounds very often are persistent (Eriksson *et al.*, 2003). The persistence may be due to several factors such as nutrients, bioavailability of PAHs (sorption to particles), temperature, oxygen, pH and presence of PAH-degrading micro-organisms. The greater degradation rates at soil pH of 7.5 represents the optimal pH for degradation for all the four PAHs studied.

Table 5.5 exhibited the degradation time for 50% of each PAH (DT₅₀) which gives an overview of the conditions most conducive to degrade and the relative recalcitrance of each PAH. It was observed that pH 7.5 was most suitable for the degradation of all PAHs as 50% degradation was observed for all in soil pH 7.5 within the first seven days which is at least a seventh of the time taken at pH5, pH 5.5 and 8.5 (36 days). Furthermore, soil pH 8, exhibited 50% degradation within 15 days for phenanthrene, 25 days for anthracene, and 28 days for fluoranthene and 27 days for pyrene. Soil pH

5.5 exhibited rate of phenanthrene degradation within 28 days, 32, 34 and 33 days for anthracene, fluoranthene and pyrene respectively. Whilst this study indicates that another key factor is adjustment of pH to optima for higher stimulation of four PAH as a carbon source used as growth factor by microbes. A simple explanation is that at neutral condition nutrients with H^+ and OH^- ions are equal whereas, nutrients with cationic and anionic charges at acidic and alkaline conditions are not equal. Nutrient availability at soil pH 7.5 is greater since cations and anions are balanced in soil, whereas nutrient availability at acidic soil pH is less due to greater number of anions (negative charge) that have ionic interaction with H^+ ions. However, under alkaline conditions more of ionic interactions with OH^- are present in soil, which makes alkaline conditions unstable (Chesworth, 2008). Thus, lower degradation rates were observed at acidic and alkaline conditions compared to neutral soil pH. As literature suggests greater population size or biomass arising can be attributed to the additional carbon source present in that particular pH (Kastner, 1998). Also, microbial degradation of PAHs and other hydrophobic substrates is believed to be limited by the amounts available in the aqueous phase (Ogram *et al.*, 1985; Bosma *et al.*, 1997). Therefore, the most soluble PAH should be degraded quicker than others as exhibited by phenanthrene in this study observed at all pH's other than the most rapid at pH 7.5. The effect of pH has been rarely studied for most organic pollutants of soil. However, Chang *et al.*, (2002) confirmed degradation of PAHs with optimal incubation conditions maintained at alkaline soil pH, i.e. pH 8 and at $30^{\circ}C$ along with addition of acetate, lactate, or pyruvate under anaerobic conditions. Kastner *et al.*, (1998) suggested a shift in pH from 5.2 to 7.0 increased anthracene and pyrene degradation by *Sphingomonas paucimobilis* BA 2 strain. Thus in this study, microbes isolated at three adjusted pH exhibited metabolism necessary for higher degradation rates at soil pH

7.5. However, a pH of 5 did not lead to total inhibition of activity whilst a shift from soil pH 7 to pH 7.5 resulted in increased pyrene and anthracene degradation. Alva & Peyton, (2003) also indicated that biodegradation of catechol is greater in alkaline soil pH than in acidic pH. This is particularly important as catechol is a diol, which is obtained as the product of the first step of PAH degradation by mono or dioxygenase enzymes present in fungi and bacteria. Yuan *et al.*, (2001) also suggested biodegradation of phenanthrene is greater under aerobic conditions that occur with greater degradation efficiency for biodegradation maintained at 30⁰C.

In general in this study soil pH 7.5 exhibited greater degradation over 70 days with a lower half life (50% of PAH degradation tabulated in table 5.5) degradation.

Biodegradation is an inexpensive and easy process to detoxify toxic contaminants from the environment (Hadibarata & Tachibana, 2009). It has been observed that various algae, fungi, cyanobacteria and heterotrophic bacteria, play an important role in PAH degradation (Kim *et al.*, 2005). Soil conditions influence the survival of microbes and their active participation in degradation process (Hadibarata & Tachibana, 2009).

In this study, bacterial populations at varying pH over a 70 days period during the degradation of soil PAHs were studied (figure 5.9 B). Initially the bacterial population on 0 day was low, with slight increase in bacterial populations at 7 and 14 days. However, the number of bacteria on day 21, 28 and 35 exhibited increased followed by a decrease on 42, 49, 56, 63 and 70 respectively. The highest bacterial count was obtained on day 35 and lowest bacterial count on 0 day and 70 day. Soil pH 7.5 exhibited high bacterial populations followed by soil pH 7.0 and soil pH 6.5 (figure 5.9 B). Lower bacterial populations were obtained in soil pH 5, 5.5 and soil pH 8.5 respectively. At soil pH 7.5 the nutrient availability is greater, due to equal number of H⁺ and OH⁻ ions whilst, the anionic due to OH⁻ is in greater number and H⁺ ions are in

greater number. However, more of ionic interaction with OH^- present at alkaline soil pH make alkaline conditions unstable (Chesworth, 2008). Thus, greater population size or biomass arising is because of the additional carbon source present in that particular pH (pH 7.5) (Kastner, 1998), if the resulting organisms can act readily on both substrates using the suitable monitored abiotic condition. Bacterial populations were evident in soil without microbial inocula figure 5.9 (A) however lower than inoculated soil, indicating recolonisation of the soil from 14 day to 70 day of incubation from low levels of surviving bacteria or due to the non-sterile conditions or apparatus used during sampling points or addition of sterile water. The bacterial populations in microbial inoculated soil were greatest at soil pH 7.5 which also resulted in the greatest degradation rates suggesting that bacteria were involved in the biodegradation of polycyclic aromatic hydrocarbons. Therefore, it may be that microbial community particularly bacteria was more prevalent and active in degradation at pH 7.5. Conversely, fungal populations were greatest at acidic soil pH and with some evident at alkaline soil suggests degradation at lower pH might be initiated by fungal populations.

Figure 5.10 (A) and (B), represents the fungal populations over 70 days are greatest at acidic soil pH and with some evident at alkaline soil pH but were not detected at neutral soil pH. Fungi in general grow actively at acidic pH 5 and 5.5 (Cajthaml, 2008). However, the degradation was fastest at pH 7.5 – (figure 5.10) bacterial populations were greatest at pH 7.5 (appendix III section 5.5 for the table of bacterial c.f.u from soil with microbial inocula). Thus, the research carried out indicates that fungi are more tolerant to acidic soil pH and bacteria being more tolerant to neutral soil pH. However this study also found an increase in the fungal population at basic pH in comparison with neutral pH with different predominant fungi found in comparison to acidic pH. *Penicillium* species predominated at acidic soil pH and with lower

Aspergillus populations whereas at alkaline conditions of (pH 8.0 and 8.5) *Aspergillus* were predominant and *Penicillium* was not detected (table 5.6). It is in very rare conditions that fungal growth is measured at alkaline conditions. Also, Novotny *et al.*, (1999) have reported growth of *Pleurotus ostreatus* in alkaline conditions, suggesting oxidative enzyme production and PAH removal in soil by mycelium of white rots fungi.

Moreover, tremendous diversity in fungal population has been studied by Hadibarata & Tachibana, (2009) exhibiting its adaptability for using different carbon sources; but fungal ability to degrade PAHs as carbon source may be different. Similar fungal species were used in work reported by Orth, (1991) who screened and identified *Aspergillus niger* and *Aspergillus versicolor* that performed rapid onset and highest extent (98%) of PAH biodegradation.

Research in the biodegradation mechanisms and environmental fate of polycyclic aromatic hydrocarbons (PAHs) has been prompted by the ubiquitous distribution of the organisms and their potentially deleterious effects on human health (Silva *et al.*, 2009). Since the 1970s, research on the biological degradation of PAHs demonstrated that bacteria, fungi and algae possess catabolic abilities that are being utilised for the remediation of PAH-contaminated soil and water (Juhasz & Naidu, 2000). Hydrocarbon degradation by microbial communities depends on the composition of the community and its adaptive response to the presence of hydrocarbons. Bacteria and fungi are the key agents of degradation, with bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments (Leahy & Colwell, 1990). The extent to which bacteria, yeasts, and fungi participate in the biodegradation of hydrocarbons has been the subject of only limited study, but appears to be a function of the ecosystem and local environmental

conditions. The bio-degradation of polycyclic aromatic hydrocarbons in the environment is a complex process, which engages quantitative and qualitative aspects depending on the nature and amount of the hydrocarbons present, the ambient and seasonal environmental conditions, and the composition of the autochthonous microbial community (Leahy & Colwell, 1990).

In general, according to the review of literature with respect to other studies indicated fungi plays active role in the degradation at acidic pH however, bacteria play role in degradation at neutral soil pH (Kastner *et al.*, 1998). This study indicates that soil pH 7.5 is favourable for mixed bacterial populations which resulted in greater biodegradation whilst also soil pH 5.0, 5.5 and pH 8.5 are less favourable with lower c.f.u counts and lower biodegradation rates.

5.4.3 Microbial activity (ATP concentrations)

Objectives: To study microbial activity by soil ATP measurement at different pHs in the presence of PAHs.

The liberation of adenosine diphosphate (ADP) and phosphate ion due to decomposition of adenosine triphosphate (ATP) is due to the activity of microbial cells, catalyzed by ATPases enzymes (Weibull *et al.*, 1962). ATPase enzymes are microbial integral proteins that correlate well with the widely used measurement of water content present in the soil showing its potential as indicator for microbial activity (McDonnell & Russell, 1999). ATP allows an autonomous measurement of quantity/amount of microbial biomass in soil regardless of biomass activity. However, early time point soil sample incubation results in ATP-to-biomass C ratios (Denome *et al.*, 1993). Likewise, ATP measurement by improved procedures Naseby *et al.*, (1997) & Webster *et al.*, (1984) proved to be a simple, easy and reproducible method without use of non-toxic reagents thus, resulted in measuring total amount of ATP (figure 5.11) and microbial biomass (appendix III section III.6) in experimental soil.

Amendment of soils with carbon sources usually results in elevated microbial metabolic activities. In order to understand the correlation of ATP as an indicator of microbial activity, this method was compared with the c.f.u of bacterial and fungal samples. The correlation value between bacterial c.f.u and soil ATP concentrations was high ($r= 0.9$) whilst correlation to fungal c.f.u was low ($r = 0.2$) across the degradation time curve indicating that soil microbial activity levels are closely linked to bacterial populations whereas, fungi initiated degradation at acidic pH representing as an important contribution to microbial activity. Moreover, (figure 5.11) day 0 exhibits lower ATP levels followed by day 7 and 14. ATP concentrations were converted to

biomass using biomass conversion factor from Naseby *et al.*, (1997) $C = 171 \times$ soil ATP content (conversion data tabulated in appendix III section III.6 & III.7). L-arginine process was helpful in measurement of ammonia liberated in soil sample also with its potential as indicator for microbial activity (Alef, 1987). Interestingly, the correlation to ATP biomass and ammonification biomass was (0.8) demonstrated that ATP biomass was correlated and closely linked to bacterial population ($r = 0.9$) Thus, the correlation values obtained suggests ATP biomass is more competent indicating greater correlation values for measurements of microbial biomass. Mostly ATP concentrations reported in literature might be lower due to loss during hydrolysis (chemical or enzymatic) in the extraction process or by adsorption on soil colloids resulting in lowered measurement of ATP reaction was suggested by Denome *et al.*, (1993). The fate of PAHs is determined by the soil pH which is due to the impact of microbial activity and microbial communities at varying pH. The soil acidity is based on factors involved with active acidity, residual acidity and exchangeable acidity (Brady & Weil, 2002). Active acidity involves H^+ and Al^{+3} ions in the soil, residual acidity is neutralised by limestone or other alkaline materials and exchangeable acidity consists of aluminium and hydrogen cations that are easily replaced by other cations. Since many sites in the UK are of neutral soil pH or below it may be necessary to adjust the soil pH to improve the intrinsic degradation rates. Also, total bacterial populations are greater in alkaline soil pH, particularly in soil at pH 7.5 (Bastiaens *et al.*, 2000; Ho *et al.*, 2000; Johnsen *et al.*, 2005). Moreover, this study indicates higher fungal populations are also possible at basic pH than neutral and interestingly this population is made up of a different group of organisms from those at acidic pH. Tsai *et al.*, (1997) indicates microbial biomass, and ATP concentration by addition of glucose to soil that are found to obtain with substantial change in short time. It is also

notable that degradation rates were at their lowest at pH 6 and 6.5, where fungal populations and bacterial population at pH 6 and 6.5 were evident to be lower.

Soil pH 7.5 is highly suitable for the degradation of PAHs with greater microbial populations, and greater ATP concentrations as well as microbial biomass with an increasing degradation rate up to seven fold. These results indicate that soil pH is one of the most important abiotic factors limiting the degradation of PAHs.

5.4.4 Soil enzyme activity

The enzyme assays performed allowed study of a range of enzyme activities with small soil quantities over a range of different pH. The soil enzyme studies followed the methods of Naseby & Lynch, (1997) allowed seven different enzymes to be studied and made it possible to study all the activities in same amount of time required for less enzymes by other methods.

Assays for β -glucosidase, L-arginine ammonification, acid/alkaline phosphatase representing carbon: nitrogen: phosphorous cycle (C: N: P) activities and manganese peroxidase, lignin peroxidase and laccase involved in PAH degradation were performed. Each of these activities was measured at buffer pH 5.5, 7 and 8.5 regardless of soil pH during bio-degradation. Activities at acidic, neutral and alkaline buffer pH would reflect the effect of soil pH providing variation between enzyme activities at varying soil pH in order to enhance the bio-degradation rate in the environment. Enzyme activities affected by soil pH may be due to the sensitivity of amino acids, electrostatic properties and reaction centre (Bonomo *et al.*, 2001).

Acid/alkaline phosphatases are important in the P cycle (Phosphorous cycle) (Makoi & Ndakidemi, 2008). In natural soil the enzyme activities are impaired due to absorption and immobilization between soil particles and organic matter with respect to soil type (Naseby *et al.*, 1997).

For acid/alkaline phosphatase activity, acidic soils were found to be more predominant for acid phosphatase enzyme activity whereas alkaline and neutral soils were observed with greater activities for alkaline phosphatase. The soil pH effects varied with microbial inoculation and without microbial inoculation soil samples with the phosphatase activity significantly greater in soil with microbial inoculation. Greater phosphatase activities (approximately $1.2 \text{ mg pNP g}^{-1} \text{ soil d.w. h}^{-1}$) were exhibited

using buffer pH 8.5 (figure 5.12 B) compared to activity measured at acidic and neutral buffer pH. However, the majority of acid phosphatase activity and alkaline phosphatase activity is indicated. In general, acid phosphatase is predominantly associated with fungal origin whilst alkaline phosphatase is more likely due to bacterial origin (Naseby & Lynch, 1997). In this case, the acid and alkaline phosphatase, exhibits independent effects regardless of inocula. Naseby & Lynch, (1997) supported similar work, indicating rhizosphere acid phosphatase was not significant by difference to that of bacterial inoculation and substrate addition did not make a trend with soil depth for phosphatase activity. Thus, acid phosphatase activity is more closely related to nutrient availability at acidic soil pH, which exhibited lower activity and demonstrated loss in higher carbon amount, resulting in reduced phosphate extract. Soil phosphatase activity is decreased as the inorganic soluble phosphate increases (Tabatabai 1970; Tadano *et al.*, 1993). Therefore, the available phosphate in the soil with microbial inocula might be greater at alkaline soil pH due to greater bacterial population (greater bacterial population evident at soil pH 7.5) causing an overall increase in activity at alkaline soil pH. Moreover, the available phosphate increase might be due to an increase in available carbon at alkaline soil pH compared to acidic soil. In specific, as the ratio of carbon to phosphate available in soil is increased, the microbial phosphates demand for phosphorous also increases. It indicates degradation and enzyme activity co-relationship as greater bacterial populations were also evident at soil pH 7.5 along with greater phosphatase activity at pH 7.5. Similar research by Mobley *et al.*, (1984) exhibited co-relationship between the soil phosphatase activity and degradation of PAH in soil indicating ratio of carbon to phosphate available in soil samples. Effect of soil pH on enzyme activities was studied since 1990s to understand the phosphatase activity concept (Dick & Tabatabai, 1984; Dick &

Tabatabai, 1992; Naseby *et al.*, 1997). The acid and alkaline phosphatase activity by *Bordetella bronchispetica* was found at an optimum temperature of 37⁰C. Moreover, studies suggested acid phosphatase at optimum of pH 4.8 and alkaline phosphatase at pH 9 exhibited greater activity in clinical and non-clinical isolates of *Bordetella bronchispetica* (Mobley *et al.*, 1984).

The liberation of ammonia from arginine is due to the activity of microbial cells, and extracellular enzymes. Ammonia liberated from arginine correlates well with the widely used measurement of water content present in soil demonstrating its potential as indicator for microbial activity (Alef, 1987). Decomposition of arginine by organisms involves four pathways. These pathways include arginine deiminase, arginine transmidinase, and arginine decarboxylase and arginase-urea amidolyase. In all of these pathways, apart from arginine transmidinase, the end product obtained is ammonium (Abdelal, 1997). In this study it was observed that L-arginine ammonification activity measured at buffer pH 8.5 was higher than buffer pH 7 and 5.5. Also, at buffer pH 5.5, initially activity was low, but as the incubation time was increased, activity too increased (figure 5.18). Microbial population and activity depends on soil pH. Haemmerli *et al.*, (1986), suggests higher arginine ammonification production as buffer pH increases. However, arginine determinations are not recommended for low pH soil characteristics that cause high mobile aluminium content with negative effect on microbial activity (Mirjana & Licina, 2002). The amount of soil biomass has been shown to be proportional to arginine ammonification rates under specific conditions (Hund *et al.*, 1998). Microbial biomass was also measured using L-arginine ammonification process. Also, biomass measurement by ATP and L-arginine ammonification was compared. Thus, greater biomass for both the procedures was

exhibited similarly on day 35 and the lowest biomass was on day 0 and day 7 respectively.

β -glucosidase activities were measured at buffer pH 5.5, 7 and 8.5 and these showed significantly greater activity with microbial inocula (figure 5.15 B, 5.16 B, 5.17 B). Also, greater activity was measured in buffer pH 8.5 (approximately $0.06 \text{ mg pNP g}^{-1} \text{ soil d.w. h}^{-1}$) at alkaline soil pH and the lower activity rates obtained in buffer pH 7 (approximately $0.04 \text{ mg pNP g}^{-1} \text{ soil d.w. h}^{-1}$) is potentially associated with bacteria respectively. β -glucosidase plays a key role in soil catalysing the hydrolysis and biodegradation of β -glucosidase involved in plant debris decomposition in the ecosystem (Makoi & Ndakidemi, 2008)

Manganese peroxidase is an extracellular peroxidase and is a Mn-induced enzyme that oxidizes Mn^{2+} to Mn^{3+} respectively. Fungal manganese peroxidase also results in production of hydrogen peroxide by oxidation of substrate such as NAD(P)H, dithiothreitol and dihydroxymaleic acid (Castillo *et al.*, 1994). In this study, greater soil fungal manganese peroxidase activities were observed in buffer pH 5.5 followed by activity measured at buffer pH 7 and 8.5 respectively.

Fungal lignin peroxidase results in oxidation of lignin. After incubation of microbial inocula in soil, over 0 to 70 days under pH 5, 5.5, 6 and 6.5, greater activity levels of lignin peroxidase found were $1.8 \mu\text{mol g}^{-1} \text{ h}^{-1}$. Similarly lignin peroxidase activity found at neutral soil pH 7-7.5 was between 0.6 and $1.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$ and at alkaline soil pH 8 and 8.5 was between 0.6 and $1.0 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$. Thus, acidic soil pH exhibited greater fungal lignin peroxidase activity compared to neutral and acidic soil pH.

Fungal laccase activities measured at three buffer pH exhibited greater activity rates for buffer pH 5.5 (figure 5.21 B). Acidic soil pH 5.0, 5.5, 6.0, 6.5 exhibited greater

laccase activity ($0.06 \mu\text{mol g}^{-1}\text{h}^{-1}$) from 49 to day 70 whereas in alkaline soil pH 7.0, 7.5, 8.0 and 8.5 exhibited lower activity levels ($0.04 \mu\text{mol g}^{-1} \text{ soil d.w h}^{-1}$) over 35 to 70 days. Studies reported with laccase on Benzo[α]pyrene degradation indicated that the greatest activity was at pH 4.0, but decreases rapidly by either increasing or decreasing the pH of the moderated soil value from 3.0 to 7.0. Laccase activity was almost lost at pH 7.0 (Xuanzhen *et al.*, 2010).

Studies involving soil enzymes are biologically important for degradation of PAHs (Cerniglia, 1984). Three different functions in biological degradation of PAHs involves (i) Yield of carbon and energy by assimilative biodegradation for degrading organisms is carried out by mineralization of the compound. (ii) Intracellular detoxification of PAHs by making it water-soluble as a pre-requisite for elimination of the compounds. Broadly, the initial step includes developing ring fission and carbon assimilation in bacteria leads to intracellular oxidation and hydroxylation whereas in fungi, detoxification is an initial step (Cerniglia, 1984). (iii) Co-metabolism- representing degradation of PAHs by cell metabolism without generating energy and carbon. Thus, a non-specific enzymatic reaction, with a substrate competing with the structurally similar primary substrate for the enzyme's active site is termed as co-metabolism. Benzo(a)pyrene degradation by bacteria growing on pyrene represents an example of co-metabolism (Boonchan *et al.*, 2000). Also, Keck *et al.* (1989) noted that: "In the case of a pure culture, co-metabolism is a dead-end transformation without benefit to the organism. In a mixed culture or in the environment, however, such an initial co-metabolic transformation may pave the way for subsequent attack by another organism." In spite of considerable effort, only a very limited number of bacteria have been isolated that can grow in pure cultures on PAHs with five or more aromatic rings (high molecular weight (HMW) PAHs). A possible reason is the high retention of these

compounds by the solid soil phase, resulting in mass-transfer rates of HMW-PAHs to the bacterial cells being too low to match the cells' basic metabolic requirements. The low bioavailability of PAH may have prevented the evolution of suitable enzymatic pathways in soil bacteria. According to Perry, (1979) recalcitrant compounds generally do not serve as growth substrates for any single microbial organism, but are thought to be oxidized in a series of steps by consortia of microbes. Inoculation with selected microbes, exhibited significant difference in phosphatase, B-glucosidase, ammonification and oxidase activities compared to soil without microbial inoculation. This study suggests that altering pH may be most effective method to remediate soil contaminated with PAHs.

Interestingly, with respect to photocatalytic degradation experimental results (chapter 4), the converse effect of pH was found with fastest rate of biodegradation observed at acidic condition in soil pH 6.5 whilst, the results obtained in this report during biodegradation degradation exhibits fastest rate of degradation at alkaline conditions particularly at pH 7.5. However, biodegradation rates at optimum conditions were greater than photo-catalytic oxidation, for example only 65% of phenanthrene was degraded by photo-catalytic oxidation after 5 days, and at the same time point 90% of phenanthrene was biodegraded at pH 7.5 after 5 days.

The biodegradation experiments carried out and photo-catalytic oxidation (chapter 4) overall suggests,

- a) Optimal conditions for photo-catalytic degradation and biodegradation are different and show converse degradation effect of soil pH.
- b) Biodegradation carried out exhibits higher and rapid degradation in comparison to that of photo-catalytic degradation.

Chapter: 6.0

Isolation and purification of enzymes involved in PAH degradation

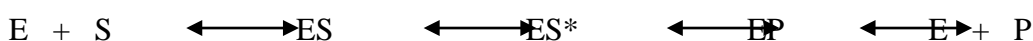
6.1 Introduction

Aim: To study the ligninolytic enzymes from fungi and dioxygenase enzyme from bacteria involved in PAH degradation and their kinetics studies

In the environment, degradation of PAHs by organisms depends on the complexity of the PAH's chemical structure and the enzymatic capacity of the indigenous microbial population including their response to chronic exposure to aromatic hydrocarbons (Heitkamp *et al.*, 1988). Heitkamp *et al.*, (1988) reported in their studies that degradation of PAHs with two or three aromatic rings is relatively easy as compared to PAHs with four or more aromatic rings. It has been observed that PAHs with four or more aromatic rings are often genotoxic and recalcitrant.

Identified mixed bacterial and fungal cultures degrade PAHs in the soil and release CO₂ and water without any excretion of intermediary substrates. During degradation, different reactions between PAHs and soil particles occur and they undergo co-metabolic side-reactions resulting in metabolites. These metabolites possess high resistance in comparison to parental compounds which makes it difficult to break down these metabolites and thus carbon constituents are released (Johnsen *et al.*, 2005). Soil organic compounds may be covalently associated with PAHs metabolites which may further modulate PAH to carbon mineralization (Richnow *et al.*, 1997).

Furthermore, Enzymes interact with specific substrate forming an enzyme-substrate complex which is extended into transition state [ES*] which further dissociates into product and free enzyme.



(Whiteley & Lee, 2006)

During PAH degradation, degradation process is initiated in bacteria by molecular oxygen with the help of atoms which are present in aromatic nucleus. This process is

further catalysed by NADH dependent dioxygenase enzyme (Iwabuchi & Harayama, 1997). The PAH degradation process by bacterial dioxygenase enzyme require NADH as a co-factor formed by cell-bounding process (Johnsen *et al.*, 2004). Dioxygenase is incorporates with two atoms of oxygen. Bacterial enzymes involved in cleavage of double bonds of aromatic compounds are characterized as non-haem iron dioxygenases (Wallis & Chapman, 1990). Non-haem iron dioxygenases include catechol dioxygenase which are divided on cleavage pattern. One catechol dioxygenases contain Fe (III) that cleaves catechol in an intradiol fashion whereas another catechol dioxygenase contains Fe (II) that cleaves in an extradiol fashion (Wallis & Chapman, 1990). Extradiol cleavage is performed by catechol 1, 2-dioxygenases of meta-substituted substrates (for example 3-methylcatechol or 3-methoxycatechol substrates) (Strachan *et al.*, 1998).

As mentioned above micro-organisms degrade PAH compounds aerobically by converting them into more reactive dihydroxylated intermediates. These intermediates such as catechol or protocatechuate are further subjected to intra or extra-diol ring cleavage which is carried out by molecular oxygen (Strachan *et al.*, 1998). Most bacteria degrade PAHs particularly phenanthrene through one of two routes through a common intermediate, 1-hydroxy-2-naphthoate (figure 6.1). In general, 1-hydroxy-2-naphthoate is oxidized to 1, 2-dihydroxynaphthalene which gets degraded via salicylate (Iwabuchi & Harayama, 1997). The other route of degradation is also via 1-hydroxy-2-naphthoate, which is cleaved and is further metabolized via o-phthalate (Evans, 1965; Iwabuchi & Harayama, 1997; Kiyohara, 1976). Different genes and enzymes involved in PAHs degradation use salicylate which is identified and characterized in many strains (Denome *et al.*, 1993; Kiyohara *et al.*, 1983).

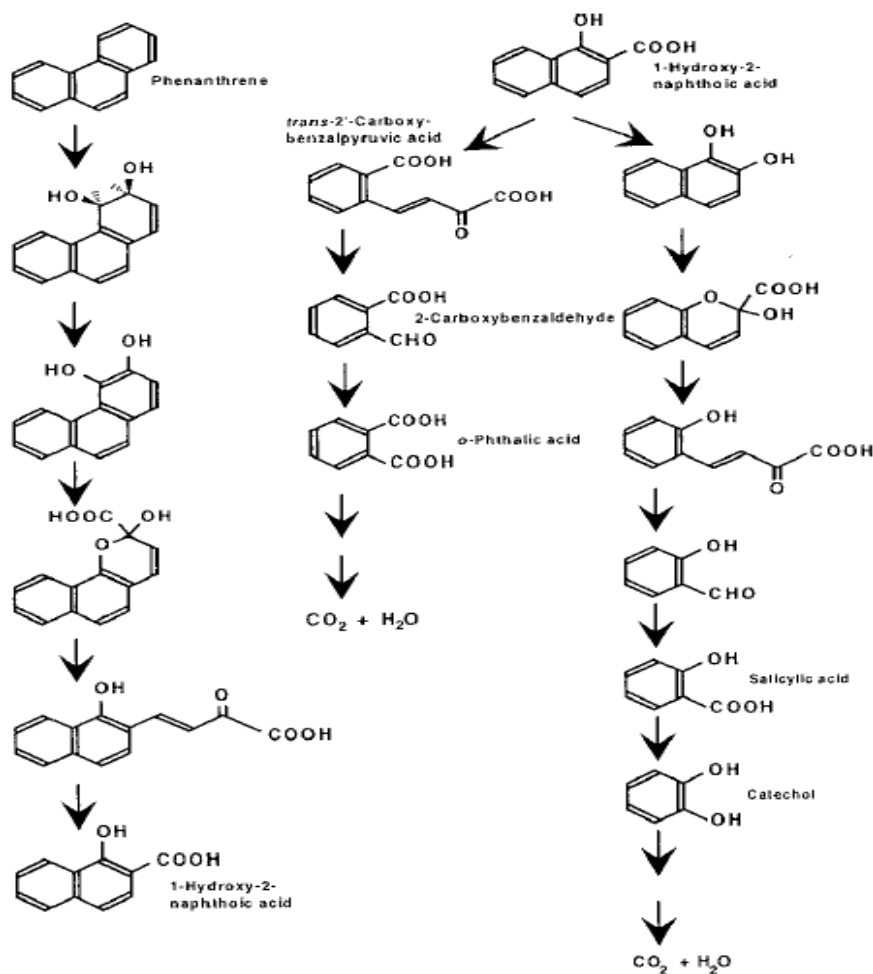


Fig. 6.1: Pathway representing degradation of phenanthrene (Iwabuchi & Harayama, 1997).

During degradation of PAH by fungal ligninolytic enzyme, HMW-PAHs in soil are more likely to be attacked by fungal exo-enzymes in comparison to the intracellular bacterial enzymes. The majority of immobile HMW- PAH are diffused with these fungal exo-enzymes. Most of the research has suggested fungi as important bio-remediating agents (Reddy, 1995; Cameron & Aust, 1999; Pointing, 2001). Studies have found *Penicillium* spp., and white rot fungi (*Phanerochaete chrysosporium*) to be more attractive organisms for bioremediation of polluted sites as these exhibit extracellular oxidative enzymatic systems (Gianfreda & Rao, 2004). Lignin is degraded by white rot fungi. White rot fungi posses high enzymatic activities as it

oxidizes lignin extra-cellularly and are considered as ligninolytic fungi. However, non-ligninolytic enzymes such as dehydrogenases, may also be involved in the transformation of polluting substances (Cameron & Aust, 1999).

Various peroxidases, lignin peroxidase (LiP), Mn-dependent peroxidase (MnP), and phloxidase and laccase (Lac), constitute an enzyme systems present in white-rot fungi (Gianfreda & Rao, 2004). H_2O_2 dependent LiP oxidizes various aromatic structures and produces radicals that form aryl cations. Also, LiP is considered to have high oxidation-reduction potential that oxidize xenobiotics (Gianfreda & Rao, 2004). Moreover, manganese peroxidases catalyze H_2O_2 dependent oxidation of various phenolic substrates and aromatic compounds (Wallis *et al.*, 1990). Also, laccase mediates oxidation of various phenolic substrates to phenoxy radicals using molecular oxygen (Gianfreda *et al.*, 2005). Various non phenolic substrates have also been oxidized by laccase with either with the help of 2, 2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) or with the help of different substrates mediatorsthat are present in the reaction mixture (Pointing, 2001). In all the developments and studies of PAH bioremediation, a key advance has been reported based on the ecologically distinct group of wood and litter degrading white rot fungi. *Phanerochaete chrysosporium*. One of the species included in white rot fungi, has gained early attention with reference to bioremediation with all these enzymes (Barr & Aust, 1994; Cavaleiri & Rogan, 1985; Paszcynski *et al.*, 1985). Biochemical data (Bogan & Lamar, 1995; Haemmerli *et al.*, 1986; Hammel *et al.*, 1992), liquid-culture studies (Bogan & Lamar, 1995; Bumpus, 1989; Hammel *et al.*, 1992) and bench scale solid-phase experiments (Orth *et al.*, 1991; Morgan *et al.*, 1993) have studied the importance of *P. chrysosporium* and its extracellular enzymes capable of PAH degradation. However

less attention has been focussed on the enzyme systems of *Penicillium* spp. and *Aspergillus* spp.

The aim of the work was to isolate, characterize and purify manganese peroxidase and laccase enzymes produced by fungi and dioxygenase, 1, 2- catechol- dioxygenase produced by bacteria isolated during shaken aqueous enrichment (appendix I: sections I.3.6). Enzyme kinetic studies were carried out using Michaelis –Menten equation.

6.2 Materials and methods

6.2.1 Bacterial and fungal strains

The six identified bacterial strains (*Pseudomonas putida* strain, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp.) and two identified fungal strains (*Aspergillus niger* and *Penicillium freii*) isolated by enrichment culture (appendix I: sections I.3.6) were used for further enzymatic studies.

6.2.2 Production/Growth and culture conditions -exposure of cultures to PAHs as the sole carbons source

6.2.2a Bacterial growth

All bacterial cultures were grown in 50 ml of nutrient broth (Sigma Aldrich) and minimal salt broth in 250 ml Erlenmeyer flasks in three replicates kept on a rotary shaker maintained at 28⁰C for 24-48 hrs at 150 rpm. The bacterial cultures were then harvested and re-suspended in 50 ml of fresh nutrient broth and minimal salt medium containing 50 mg of each of phenanthrene, anthracene, fluoranthene and pyrene. PAH crystals were dissolved in acetone before adding to flasks to ensure equal distribution. Each flask was left overnight in fume-hood capped with a sterile cotton wool bung to evaporate acetone. Cultures were inoculated using sterile wire loop in each flask maintaining sterile conditions. After inoculation with culture the flasks were incubated in a rotary shaker at 150 rpm for 4 days maintained at 28⁰C.

6.2.2b Fungal growth

Fungal strains were also grown in three replicates in 50 ml of ME broth (Sigma Aldrich) and minimal salt broth in 250 ml Erlenmeyer flask along with 50 mg of each

individual PAH. PAH crystals were dissolved in acetone before adding to flasks to ensure equal distribution. Each flask was left overnight in fume-hood capped with a sterile cotton wool bung to evaporate acetone. The fungal cultures inoculated cultures were kept on rotary shaker for 7 days at 150 rpm maintained at 22⁰C.

6.2.3 Isolation of PAH degrading bacterial enzymes

Cultures were harvested to prepare cell- free extracts for all the samples. Centrifugation (1200 rpm) was performed and 10ml of supernatant was preserved at 4⁰C on nutrients agar slopes, for extracellular enzyme assay. Cell-free extracts were washed with 0.1M NaCl. After washing, pellet was re-suspended in 50 mM Tris HCL buffer with pH 7.8, containing 10% (v/v) glycerol, 10% (v/v) ethanol and 0.5 mM dithiothreitol. The cell suspension was transferred to 15 cm centrifugation tube and cells were disrupted with an ultrasonic oscillator (Heat systems, Ultrasonic processor, Model, XL2015) set at level 5, 50W in an ice bath for 30seconds. Bacterial particulate matter was removed by centrifugation for 10 mins at 8000 rpm maintained at 4⁰C. The supernatant was filtered using Whatman filter paper (0.45 µm) samples were either stored at -20⁰C or used immediately. The Bradford assay was performed to measure the protein concentrations using bovine serum albumin as a standard (Bradford, 1976).

6.2.4 Isolation of PAH degrading fungal ligninolytic enzymes

Fungal ligninolytic cultures were transferred by centrifugation (8000 rpm) and cell-extracts were washed using 100 ml of sodium phosphate buffer (0.1 mol/L, pH 6.5) containing 0.1 mol/L NaCl, was added to each flask. The supernatant collected was used for extracellular enzyme assays. The fungal mycelium in 100 ml of sodium phosphate buffer was crushed gently using glass rod and agitated at a room

temperature for 45 min on rotator shaker at 120 rpm. The enzyme extract were filtered using sterile nylon cloth and centrifuged at 8000 rpm for 20 min at 24⁰C. All the filtrates were either stored at -20⁰C or used immediately for purification of enzymes. The Bradford assay was performed to measure the protein concentrations using bovine serum albumin as a standard (Bradford, 1976).

6.2.5 Enzyme extraction

Culture broths were harvested and filtered through Whatman filter paper (0.45 µm). The filtrates were then centrifuged at 8000 rpm for 15 min at 4 ± 8⁰C and immediately washed with 0.1 M NaCl. Cell suspensions in 0.1 M NaCl were re-suspended in 20 mM phosphate buffer (pH 7.3) containing 10% (v/v) glycerol, 10% (v/v) ethanol and 0.5 mM dithiothreitol. The pellet obtained was disrupted with an ultrasonic oscillator (Heat systems, Ultrasonic processor, Model, XL2015) set at level 5, 50 W in an ice bath for 30 seconds. The pellets were separated into centrifuge tubes for enzyme assay and for preserved at -20⁰C. Activities for fungal manganese peroxidase were measured before purification. One unit of specific activity is termed as the amount of enzyme that produces 1 nmol product/min/(mg protein). Also, in this enzyme assay protein concentration was based on total cellular protein that was estimated by Bradford method using bovine serum albumin as a standard.

6.2.6 Catechol 1, 2-dioxygenase enzyme assay

The catechol 1, 2-dioxygenase enzyme assay was performed from procedure by Nadaf & Ghosh, (2011) with few modifications. by containing 5 µL of catechol 1, 2 dioxygenase, 20 µL of 10 mM catechol (Sigma Aldrich) as a substrate and final volume adjusted to 1 mL with 50 mM sodium phosphate buffer (pH 7.0). The enzyme

activity was measured at 260 nm. Also, one international unit (IU) of the enzyme activity was defined as the amount of the enzyme required to catalyze the formation of 1 μ mol of product per min per mg protein at 25°C. The protein amount was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

6.2.7 Manganese peroxidase assay

For manganese peroxidase enzyme assay enzyme was followed from the procedure by Sadhasivam *et al.*, (2008). The enzyme substrate used was 3-methyl-2-benzothiazolionone hydrazone hydrochloride (MBTH) and 3-Dimethylaminobenzoic acid (DMAB) (Sigma Aldrich). The enzyme assay was performed with total volume of 2ml consisting of 160 μ l of 100 mM succinate-lactate buffer (pH 4.5), 300 μ l of 6.6 mM DMAB, 100 μ l of 1.4 mM MBTH, 30 μ l of 20 mM MnSO₄, and 100 μ l of supernatant. Further the enzyme substrate reaction was activated by addition of 10 μ l of 10 mM H₂O₂ and the deep purple compound colorization was measured at 590 nm. One unit is defined as the amount of enzyme needed to form 1 μ mol of product in 1 min per mg protein.

6.2.8 Laccase assay

For laccase enzyme assay, procedure by Verdin *et al.*, (2004) was followed. Laccase activity in the supernatant collected were measured by oxidation of 2; 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma Aldrich) Childs and Bardsley, 1975). The reaction mixture contained 5mM ABTS in 0.1M sodium acetate buffer (pH 5) and an appropriate enzyme. One international unit (IU) of the enzyme activity was defined as the amount of the enzyme required to catalyze the formation of

1 μmol of product per min per mg protein at 24°C . The protein amount was determined by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).

6.2.9 Purification for fungal ligninolytic enzyme

Purification of ligninolytic enzyme was followed from the procedure by Sadhasivam *et al.*, (2008). Two isolated fungal strains were grown under the optimized culture conditions in presence of PAHs which relatively helped to obtained purified manganese peroxidase using Sephadex G-25 column. 100 ml of sodium phosphate buffer (0.1mol/L, pH 6.5) containing 0.1 mol/L NaCl, were added to each flask containing fungal cultures. The fungal mycelium were crushed gently with a sterile glass rod and agitated on a rotary shaker 180rpm maintained at 24°C for 45 min. the enzyme extracts were filtered through a membrane filter Whatman paper ($0.45\ \mu\text{m}$) and used for purification process using fraction collector.

6.2.10 Purification by fraction collector/sephadex G-25

The enzyme purification was performed using column sephadex G-25 (Pharmacia), which had been equilibrated with 20 mM sodium phosphate buffer pH 5.0. Elution of the proteins was achieved with a flow rate of $1\ \text{ml}\ \text{min}^{-1}$ by a gradient with 0 – 2M NaCl. Elution of proteins in this column was equilibrated with the same buffer with 100 mM NaCl. Fractions were collected and specific activity was determined by the ratio of total activity/proteins content. Purification yield was measured by total activity for each step and the initial one. The purification fold was calculated by the ratio between specific activities at each purification step.

6.2.11 Extraction of catechol 1, 2-dioxygenase

Purification of catechol 1, 2-dioxygenase were followed from procedure by Nadaf & Ghosh, (2011) with few modifications. As conditions mention in section for harvesting cells, similar procedure was used. Further, the harvested cells were washed twice with 30 ml of 50 mM sodium phosphate buffer (pH = 7.0) and re-suspended in the same buffer (pH = 7.0) and then homogenized by sonication (Heat systems, Ultrasonic processor, Model, XL2015) set at level 5, 50 W in an ice bath for 30 seconds. Sonicated sample was centrifuged for 15 min at 13000 rpm at 4°C. After centrifugation, the supernatants were filtered using Whatman filter paper (0.45 µm) and samples were eluted using sephadex G-25 column (Pharmacia). The elution was carried out at a flow rate of 1ml min⁻¹. Each eluted fractions were analyzed at 280 nm for protein concentrations. The fractions having protein were tested for catechol 1, 2-dioxygenase activities at 260 nm. Purification yield was measured by total activity for each step and the initial one. The purification fold was calculated by the ratio between specific activities at each purification step.

6.2.12 Protein estimation: Bradford's assay

Protein estimation was performed for all the samples to determine the protein concentrations using Bradford's assay. Protein concentration in all the purification steps was estimated by Bradford method with bovine serum albumin as a standard (Bradford 1976). The detailed procedure is described in appendix IV section: IV.2

6.2.13 SDS-page analysis

Protein confirmation in purified enzymes was performed using SDS-PAGE, analysis according to the method of Laemmli using Bio-Rad Protien apparatus

(50X100X1.5mm), using 10% (w/v) polyacrylamide separating gels and 4% (w/v) polyacrylamide stacking gel. Detailed procedure for resolving gel and stacking gel preparation is described in appendix IV.

Table 6.1: Resolving gel (for 2 gels): 10%

Reagents	Volumes
Acrylamide stock 30%	3.33 ml
1.875M Tris/HCl pH 8.8	2.00 ml
Sterile distilled water	4.67 ml
SDS 10%	100 μ l
APS	50 μ l
TEMED	5 μ l

Before addition of TEMED to resolving gel reagents in the flask, were degassed

Table 6.2: Stacking gel (for 2 gels); 4%

Reagents	Volumes
Acrylamide stock 30%	1.33 ml
0.6M Tris/HCl pH 6.8	1.04 ml
Sterile distilled water	7.63 ml
SDS 10%	100 μ l
APS	50 μ l
TEMED	5 μ l

6.2.14 Coomassie Staining and destaining

The SDS page gels were stained with 0.1% coomassie brilliant blue R-250. Standard markers with broad range gel electrophoresis were used (Bio-Rad 161-0317) for the estimation of molecular size of purified protein bands.

6.2.15 Characterization of Enzyme

All the enzyme assays were performed with the enzyme eluted from sephadex column G-25 (Phamecia). Various temperature conditions at varying pH were performed.

6.2.15a Relative pH

The pH of the buffer was adjusted within a range of 5.0 to 9.5 to determine the relative pH activity for enzymes. These activities were performed by adjusting 0.1 M acetate buffer with a range of pH 5.0 to 5.5, phosphate buffer for pH and 6.0, 6.5, 7 and Glycine-NaOH buffer for pH 7.5, 8, 8.5, 9, 9.5. Each substrate for specific enzyme activity was dissolved in above mentioned buffers (20 mg/ml concentrations) according to pH and further standard enzyme assay were performed as described in section (6.2.7 & 6.2.8).

6.2.15b Relative temperature

Relative enzyme activity for temperature procedure by Jordaan and Leukes, (2003) was followed with a few modifications. Relative temperatures were regulated using spectrophotometer (UV 160 A) along with the water bath. The temperatures to perform assay were equilibrated 10 minutes prior to assay time points. For all the enzyme activity were measured by incubating substrate along with buffers at different

temperatures in the range of 10-70⁰C at pH 7.0 in 0.1 M phosphate buffer and enzyme assays were performed as described in section (6.2.7 & 6.2.8).

6.2.16 Enzyme kinetics

The enzyme kinetics studies were carried out with the procedure by Wallis & Chapman, (1990) with a few modifications. The partially purified enzymes were characterized kinetically. Kinetic studies were performed in 0.2 M phosphate buffer (pH 7) and at 25 ± 1⁰C apart from optimum pH experiment (which was performed in phosphate buffer at varying pH range from pH 5 to pH 9). Enzyme activities were measured following the formation of reaction products and also enzymes were incubated with various concentration of the substrate. V₀ (initial rate, μmol of product produced per min) against the substrate concentration graphs were plotted to calculate the V_{max} and K_m values. Further initial rates versus substrate concentrations graphs were plotted to confirm the Michaelis – Menten kinetics equation. Also, reciprocal plot of reciprocal reaction velocities versus reciprocal substrate concentrations represented as Lineweaver-Burk was plotted with same data to yield V_{max} and K_m values.

6.4 Results

6.3.1 Production for intracellular/extracellular enzymes

In this study, organisms involved in the degradation of PAHs were investigated for the enzymes they possess and the kinetics of these enzymes. Cell free extracts were purified for MnP and Laccase (in fungi) which are ligninolytic enzymes involved in PAH degradation. Catechol 1, 2-dioxygenase (in bacteria) was purified from cell extracts and kinetics along with optimal condition studied. The six identified bacterial strains (*Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp.) and two identified fungal strains (*Aspergillus niger* and *Penicillium freii*) were isolated as described in appendix I: sections I.3.6 and were compared for the production of manganese peroxidase, laccase and catechol 1, 2-dioxygenase enzyme in the presence of PAHs either intracellularly or extracellularly.

6.3.2 Production of fungal manganese peroxidase and laccase enzyme

Production of manganese peroxidase and laccase were studied by *Aspergillus niger* and *Penicillium freii* in the presence of PAH. In figure 6.2 & 6.3 it was observed that both the fungal strains exhibited greater extracellular production of manganese peroxidase (approximately 900 $\mu\text{mol ml}^{-1}\text{h}^{-1}$ for *Aspergillus niger* and 1300 $\mu\text{mol ml}^{-1}\text{h}^{-1}$ for *Penicillium freii*) compared to intracellular activity measured (approximately 300 $\mu\text{mol ml}^{-1}\text{h}^{-1}$ for *Aspergillus niger* and 380 $\mu\text{mol ml}^{-1}\text{h}^{-1}$ for *Penicillium freii*) as concentration per litre of original culture. Moreover, the fungal laccase activity for *Penicillium freii* exhibited a greater total extracellular activity (2000 $\mu\text{mol ml}^{-1}\text{h}^{-1}$) than that of *Aspergillus niger* (1300 $\mu\text{mol ml}^{-1}\text{h}^{-1}$). Thus, similar results exhibiting greater *Penicillium freii* extracellular activity were obtained for laccase enzyme production.

Thus, *Penicillium freii* was found to have greater manganese peroxidase and laccase activity compared to *Aspergillus niger* (figure 6.2 & 6.3).

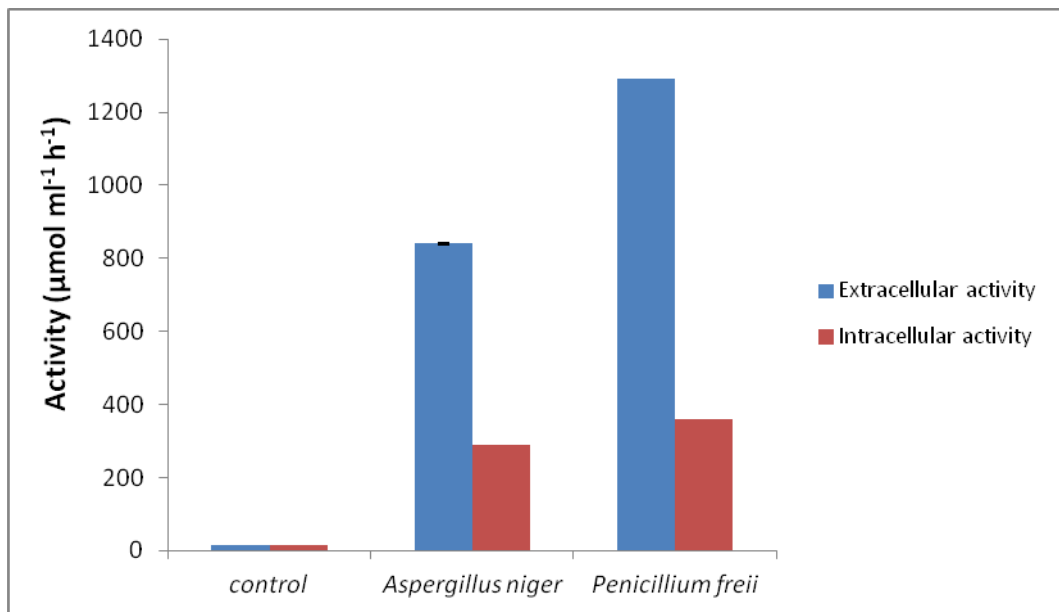


Fig. 6.2: Fungal manganese peroxidase activity

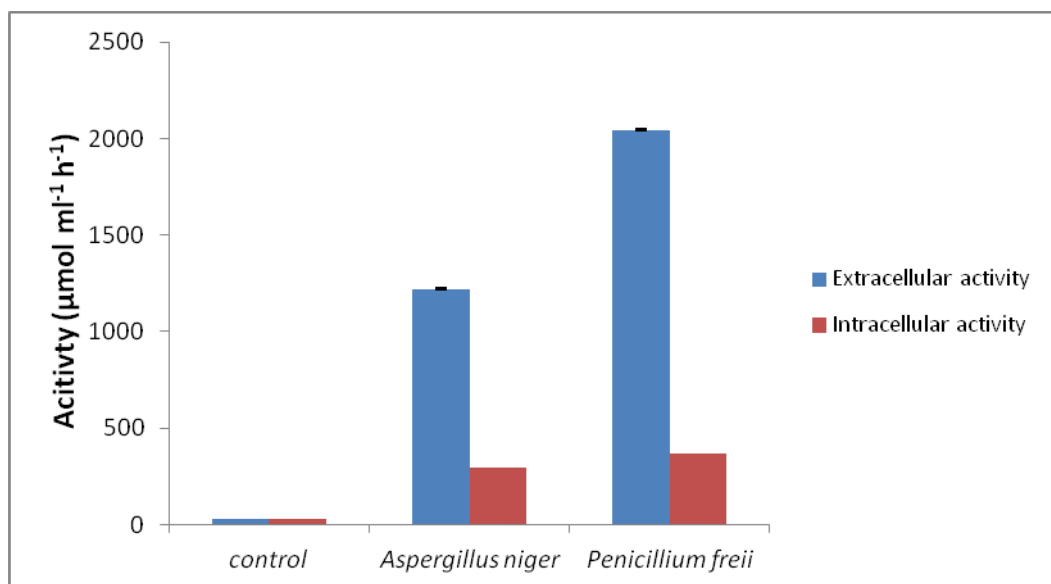


Fig. 6.3: Fungal laccase activity

6.3.3 Production of catechol 1, 2-dioxygenase

The six distinct identified bacterial cultures isolated during enrichment (appendix I: sections I.3.6) were used for production catechol 1, 2-dioxygenase enzyme. All the cultures were grown in the presence of four PAHs (phenanthrene, anthracene, fluoranthene and pyrene).

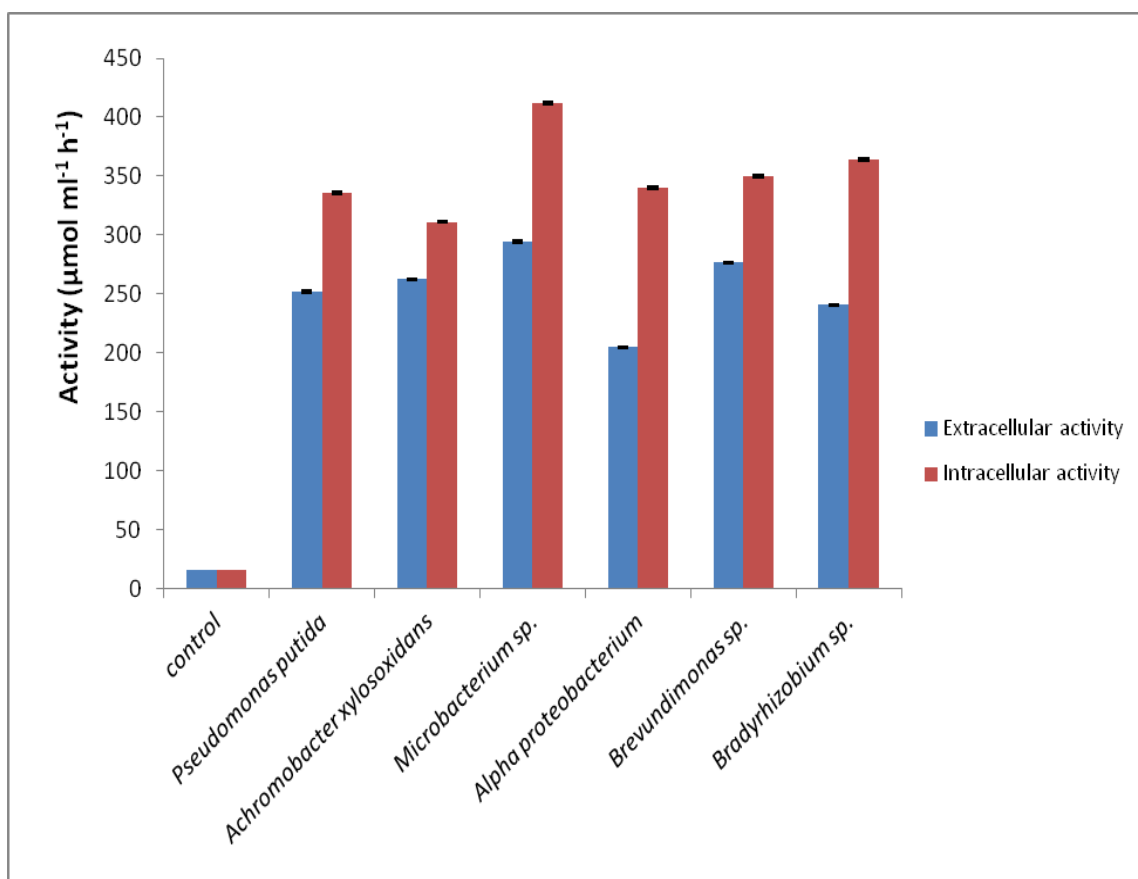


Fig. 6.4: Production of bacterial catechol 1, 2-dioxygenase activity by 6 different bacterial strains

Figure 6.4 exhibits the catechol 1, 2-dioxygenase activity of by six identified bacterial cultures in the presence of PAH which was determined extracellularly and intracellularly. It was observed that all the cultures exhibited greater intracellular activity compared to extracellular activity. *Pseudomonas putida* exhibited 250 µmolml⁻¹h⁻¹ extracellular and 340 µmolml⁻¹h⁻¹ of intracellular catechol 1, 2-dioxygenase activity

in presence of PAHs. *Achromobacter xylosoxidans* exhibited $250 \mu\text{molml}^{-1}\text{h}^{-1}$ of extracellular activity similar to that of *Pseudomonas putida*. However, intracellular activity exhibited by *Achromobacter xylosoxidans* was $310 \mu\text{molml}^{-1}\text{h}^{-1}$ compared to *Pseudomonas putida*. *Microbacterium* sp. exhibited highest level of activity (approximately $420 \mu\text{molml}^{-1}\text{h}^{-1}$ of intracellular activity and $300 \mu\text{mol ml}^{-1}\text{h}^{-1}$ of extracellular activity) followed by *Bradyrhizobium* sp. ($390 \mu\text{molml}^{-1}\text{h}^{-1}$ intracellular activity and $240 \mu\text{molml}^{-1}\text{h}^{-1}$ extracellular activity) which was isolated at alkaline conditions (appendix I: sections I.3.6). Lower levels of activity (approximately $320 \mu\text{mol ml}^{-1}\text{h}^{-1}$ of intracellular and $200 \mu\text{molml}^{-1}\text{h}^{-1}$ of extracellular activity) were exhibited by *Alpha proteobacterium* isolated at alkaline conditions and also (approximately $320 \mu\text{molml}^{-1}\text{h}^{-1}$ of intracellular activity and $260 \mu\text{molml}^{-1}\text{h}^{-1}$ of extracellular activity) exhibited by *Brevundimonas* sp. isolated at neutral pH conditions (appendix I: sections I.3.6).

6.3.4 Purification of ligninolytic enzymes

Purification of laccase and manganese peroxidase was performed for *Aspergillus niger* and *Penicillium freii* isolates described in appendix I: sections I.3.6 were used to study the ligninolytic enzyme activities (figure 6.5 & 6.6).

The manganese peroxidase and laccase activities were measured for the oxidations of MBTH and ABTS substrates respectively. However, the each of the substrate are either used for manganese peroxidase and laccase activities of both the enzymes and are expressed considering a change in OD values (optical density) per minute and were measured within the first minute of reaction initiation.

In figure 6.5, protein concentrations along with manganese peroxidase activity for *Aspergillus niger* and *Penicillium freii* were plotted against the fractionated samples collected at a flow rate of 1 ml min⁻¹. The protein concentration measured for each fractionated samples using sephadex G-25 column for *Aspergillus niger* exhibited two non-symmetrical peaks, the first of 1.8 mg/ml obtained peaking at 30th fractionated sample and a second 4.5 mg/ml peaking at the 64th fractionated sample. Similarly, the manganese peroxidase activity measured for each fractionated samples for *Aspergillus niger* exhibited two non-symmetrical peaks. Similarly first peak obtained at 30th fractionated sample was 3.8 IU/ml and second peak obtained at 64th fractionated sample was 6.9 IU/ml.

The protein concentration of each fractionated sample collected using sephadex G-25 for *Penicillium freii* exhibited similar symmetrical peaks compared to *Aspergillus niger*. First peak was at the 38th fractionated sample (2 mg/ml protein) with a manganese peroxidase activity of 4 IU/ml and the second peak was obtained for 70th fractionated sample (3 mg/ml protein) with manganese peroxidase activity of 6 IU/ml

(figure 6.5). Fractions with the highest activity were used for SDS/Page electrophoresis analysis to determine the molecular size of protein.

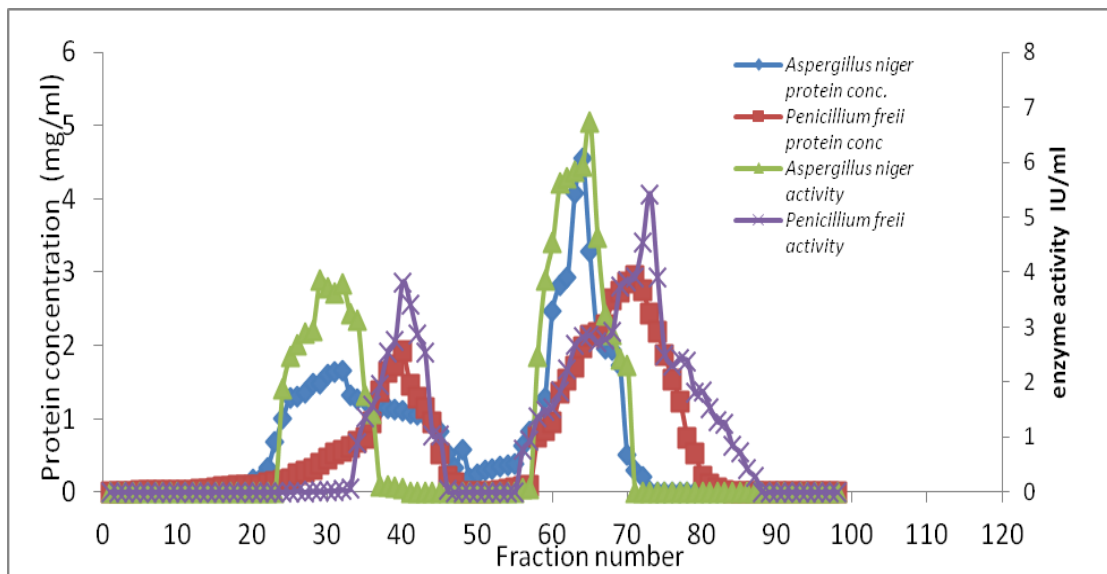


Fig. 6.5: Elution profile of fungal manganese peroxidase from sephadex G-25 column.

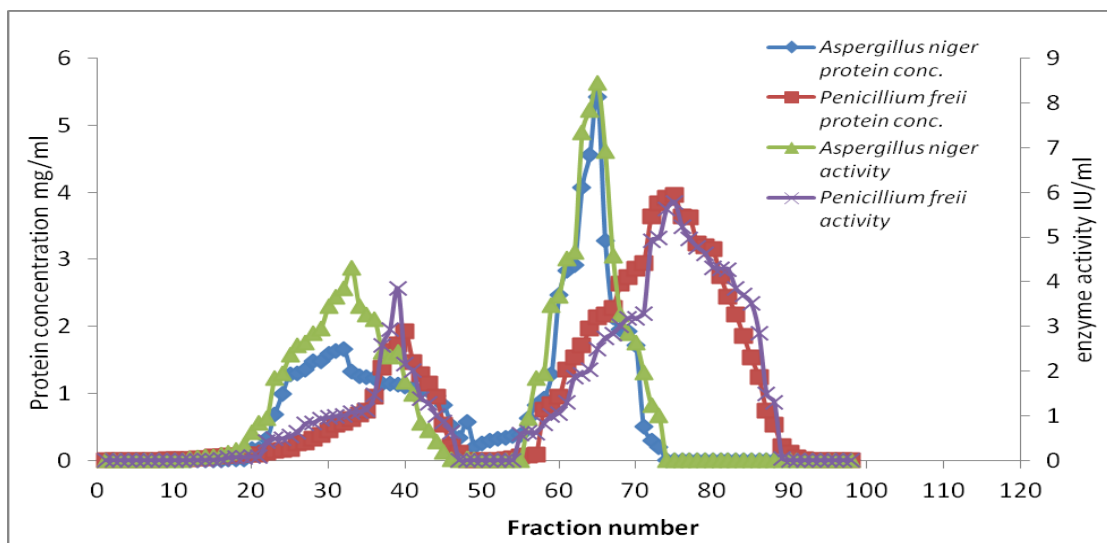


Fig. 6.6: Elution profile of fungal laccase from sephadex G-25 column.

The fungal laccase was purified using sephadex G-25 column for *Aspergillus niger* and *Penicillium freii* (figure 6.6). The protein concentration along with fungal laccase activity was plotted against fractionated samples collected at flow rate of 1 ml min⁻¹.

The protein concentration measured for *Aspergillus niger* exhibited two peaks

(approximately 1.8 mg/ml and 5.9 mg/ml) obtained for fractionated sample number 32 and 65 respectively. The laccase activity measured for *Aspergillus niger* 4 IU/ml and 8.2 IU/ml for fraction 32 and 65 respectively. Similarly, the protein concentration measured for *Penicillium freii* exhibited two peaks of 2 mg/ml and 4.3 mg/ml obtained at the 40 and 78 fractionated samples collected. The laccase activity obtained for *Penicillium freii* exhibited 3.8 IU/ml and 6 IU/ml respectively (figure 6.6).

A single-step procedure was followed to purify ligninolytic manganese peroxidase and laccase and catechol 1, 2-dioxygenase using sephadex G-25 column (Pharmecia). The activity of manganese peroxidase and laccase enzyme measured at different stages of purification is summarised in table 6.3 & 6.4:

Table 6.3: Purification of fungal manganese peroxidase

Organisms		Volume (ml)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
<i>Aspergillus niger</i>	Cell-free extract	100	239.57	288.63	100	1.00
	sephadex G-25	5	5.503	573.3	60.92	1.98
<i>Penicillium freii</i>	Cell-free extract	100	291	359.62	100	1.00
	sephadex G-25	5	12.10	23.2	65.91	1.73

Table 6.4: Purification of fungal laccase

Organisms		Volume (ml)	Protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
<i>Aspergillus niger</i>	Cell-free extract	100	219.6	296.87	100	1.00
	sephadex G-25	5	49.20	558.3	69.52	1.88
<i>Penicillium freii</i>	Cell-free extract	100	240.7	367.96	100	1.00
	sephadex G-25	5	64.49	589.7	71.4	1.602

The purification for fungal manganese peroxidase and laccase is tabulated in table 6.3 & 6.4. The specific activity of manganese peroxidase using *Aspergillus niger* was 296.87 IU/mg in the crude extract which increased to 573.3 IU/mg after purification using sephadex G-25 and for *Penicillium freii* the specific activity in crude extract obtained was 367.96 which increased to 623.2 IU/mg after purification. Thus, the fold purification for manganese peroxidase resulted in 1.98 for *Aspergillus niger* and 1.73 for *Penicillium freii* along with 60.92 and 65.91 % purification yield. The laccase enzyme resulted in a specific activity of 558.3U/mg of specific activity for *Aspergillus niger* and 589.7 IU/mg for *Penicillium freii* after purification using sephadex G-25. The percentage yield obtained for laccase was 69.52 & 71.4 along with fold purification of 1.88 for *Aspergillus niger* and 1.602 for *Pencillium freii* respectively. Manganese peroxidase and laccase purification was confirmed by loading the fractionated samples on SDS Page gel electrophoresis analysis.

6.3.5 Fungal manganese peroxidase and laccase SDS-page confirmation

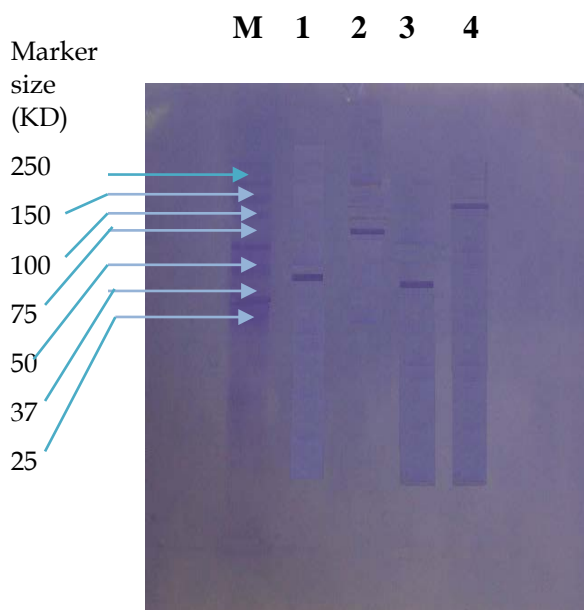


Fig. 6.7: SDS-Page analysis of purified fungal manganese peroxidase and laccase sample after elution from sephadex G-25 column

M represents Marker (250 kDa) (Bio-rad). Lane 1, 2, 3 and 4 represents protein fractionated samples collected using sephadex G-25 analysed on 10% resolving gel electrophoresis. Electrophoresis was performed for 40 minutes followed by Coomassie blue staining and destaining of gel for 30 minutes.

Figure 6.7 represents fungal manganese peroxidase and laccase confirmation for four different fractionated samples using sephadex G-25 by SDS-Page gel electrophoresis. Fractionated samples with higher absorbance readings were used for activity measurements and further SDS/Page electrophoresis at (150V) was performed to determine the molecular sizes of purified protein.

In figure 6.7, a protein band for manganese peroxidase was obtained at 48 kDa and 42 kDa, whereas for laccase activity the purified protein band was obtained at 64 kDa and 69 kDa respectively. Substrates MBTH and ABTS, either or both can be used to measure the manganese peroxidase or laccase activity.

6.3.6 Purification of bacterial catechol 1, 2 dioxygenase using fraction collector and sephadex G-25

Purification of bacterial catechol 1, 2-dioxygenase was performed for six bacterial isolates (*Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp.) isolates from enrichment culture (appendix I: sections I.3.6) (figures 6.8 & 6.9).

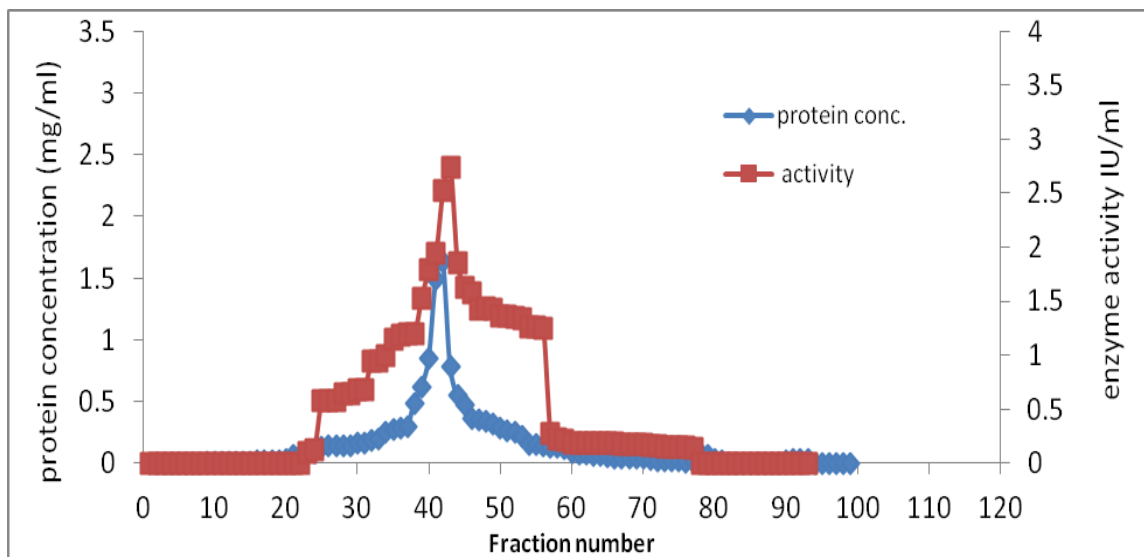


Fig. 6.8: Elution profile of *Pseudomonas putida* catechol 1, 2-dioxygenase from a sephadex G-25 column

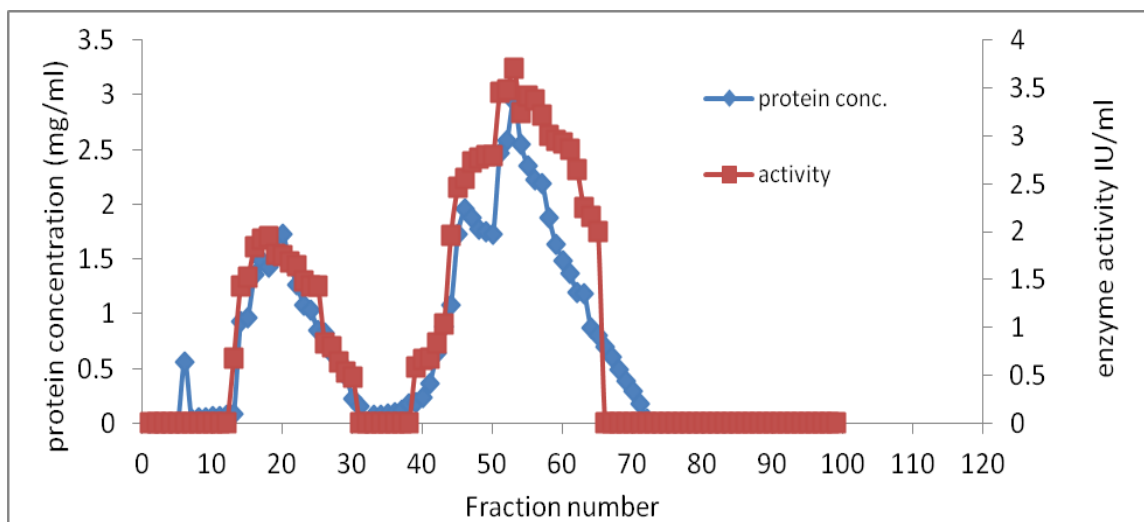


Fig. 6.9: Elution profile of *Achromobacter xylosoxidans* catechol 1, 2-dioxygenase from a sephadex G-25 column

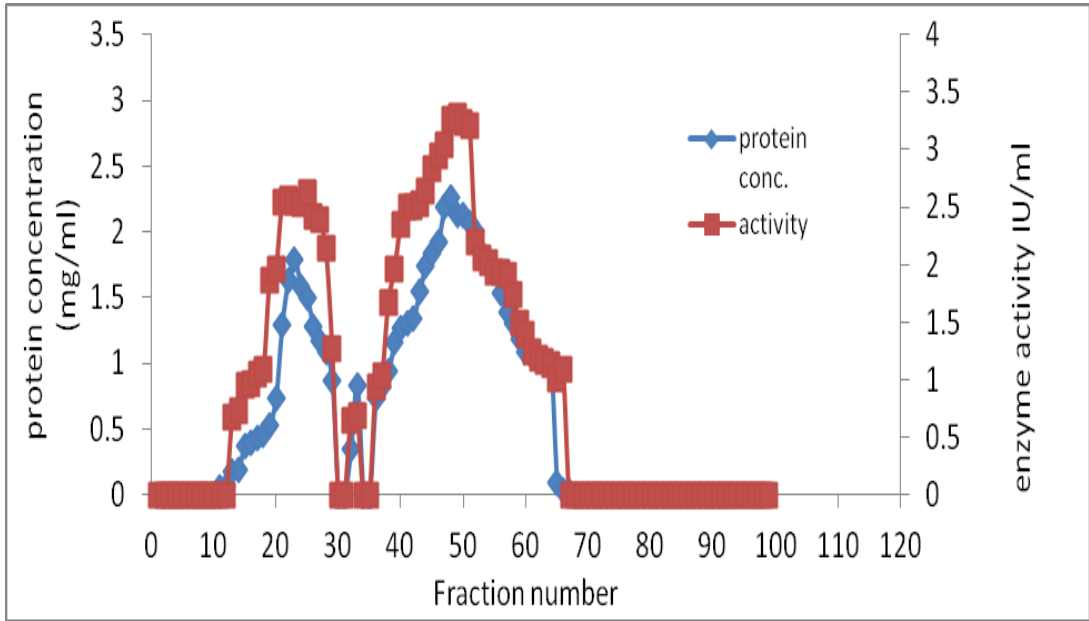


Fig. 6.10: Elution profile of *Microbacterium sp. catechol 1, 2-dioxygenase* from a sephadex G-25 column

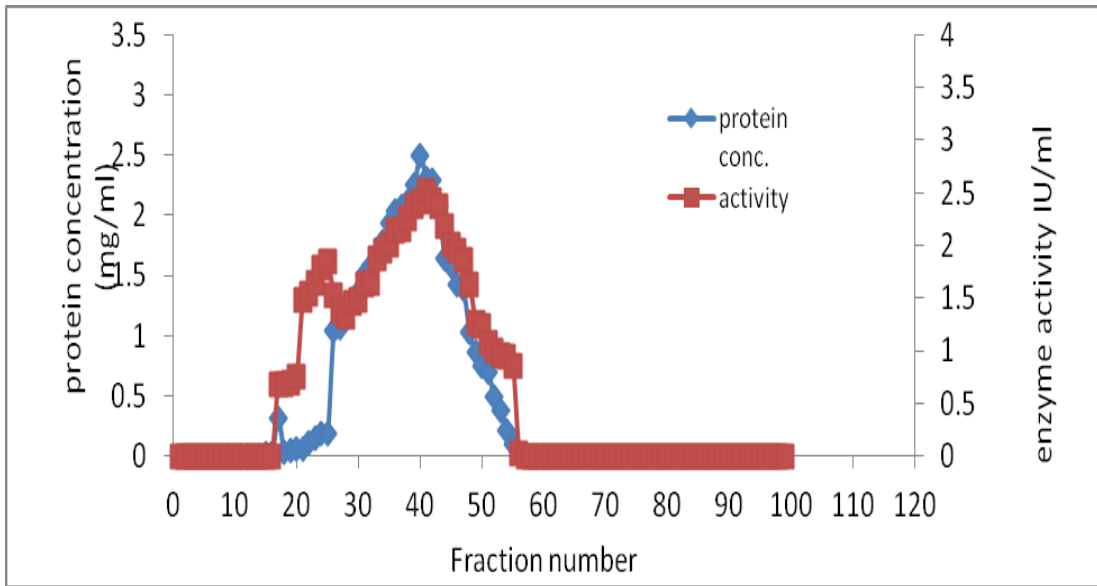


Fig. 6.11: Elution profile of *Alpha proteobacterium catechol 1, 2-dioxygenase* from a sephadex G-25 column

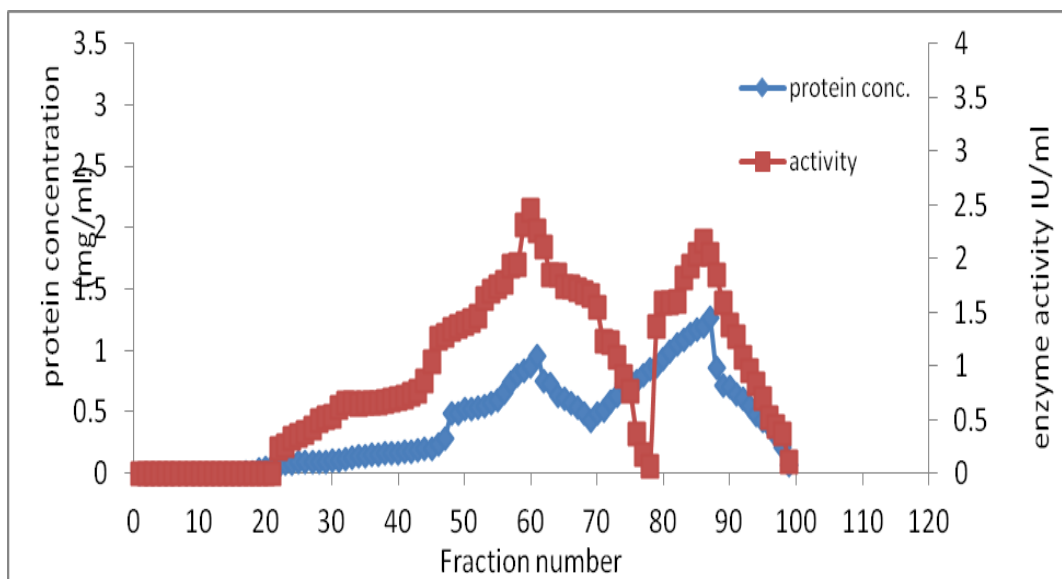


Fig. 6.12: Elution profile of *Brevundimonas sp. catechol 1, 2-dioxygenase* from a *sephadex G-25* column

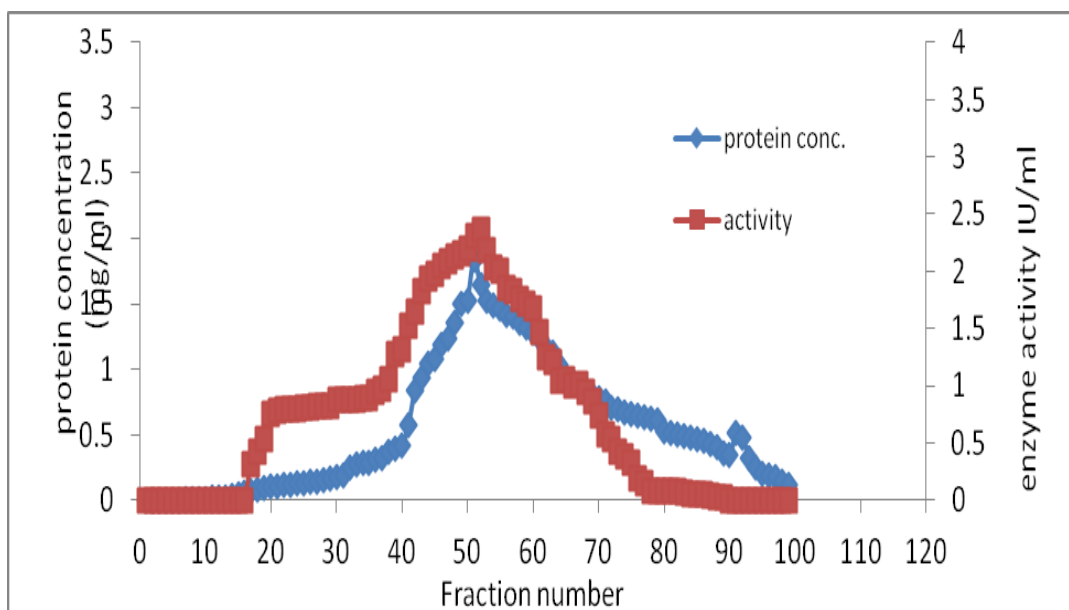


Fig. 6.13: Elution profile of *Bradyrhizobium sp. catechol 1, 2-dioxygenase* from a *sephadex G-25* column

Protein purification was performed for bacterial catechol 1, 2-dioxygenase using 6 distinct isolates grown in minimal salt medium supplemented with PAHs. Figures (6.8-6.13) depict purification of catechol 1, 2-dioxygenase by fractionation.

A symmetrical peak of bacterial catechol 1, 2-dioxygenase activity along with protein concentrations for each fractionated samples has been exhibited in figure 6.8, 6.10 and 6.12 respectively. Elution profile of *Pseudomonas putida* catechol 1, 2-dioxygenase exhibited in figure 6.8 represents the catechol 1, 2 dioxygenase activity along with protein concentrations measured against each fractionated sample collected using sephadec G-25 column. A single peak purification was formed for *Pseudomonas putida* with highest obtained at fraction 43 exhibiting catechol 1, 2-dioxygenase activity of 4IU/ml and protein concentration of 1.7 mg/ml. However, two peaks were formed of for *Achromobacter xylosoxidans* with protein concentrations of approximately 1.8 mg/ml peaking at fraction 20 and 3 mg/ml peaking at fraction 52 and catechol 1, 2-dioxygenase activity of 2.0 IU/ml for first peak and 3.6 IU/ml for the second peak (figure 6.9). Similarly two peaks were also obtained for *Microbacterium* sp. with greater protein concentrations, first peak was approximately 1.8 mg/ml peaking at fraction 24 and second peak of 2.4 mg/ml peaking at fraction 50. These peaks were collected using sephadex G-25 and the catechol 1, 2-dioxygenase activity obtained for these fractionated samples were 2.5 IU/ml and 3.5 IU/ml (figure 6.10). A single peak was evident for *Alpha proteobacterium* with protein concentration of 2.5 mg/ml peaking at fraction 40 and catechol 1, 2 dioxygenase activities of 2.5 IU/ml (figure 6.11). A two peaks protein concentration was formed for *Brevundimonas* sp. exhibiting 1 mg/ml for first peak and 1.5 mg/ml for second peak and catechol 1, 2-dioxygenase activity of 2.6 IU/ml and 2.2 IU/ml for 60th and 87th fractionated sample (figure 6.12). A single peak was exhibited using *Bradyrhizobium* sp. with a protein concentration of 2mg/ml and the catechol 1, 2 dioxygenase activity of 2.5 IU/ml peaking at fraction 53 respectively.

A single-step procedure was followed to purify catechol 1, 2-dioxygenase enzyme. The activity of catechol 1, 2-dioxygenase enzyme measured at different stages of purification is summarised in table 6.5:

Table 6.5: Purification of bacterial catechol 1, 2-dioxygenase enzymes

Organisms	Volume (ml)	Protein (mg)	Specific activity U/mg	Yield (%)	Purification fold	
<i>Pseudomonas Putida</i>	Crude extract	100	253.14	335.53	100	1.00
	sephadex G-25	5	14.60	482.6	12	1.43
<i>Achromobacter xylooxidans</i>	Crude extract	100	262.31	311.12	100	1.00
	sephadex G-25	5	20.64	408.7	22	1.31
<i>Microbacterium</i> sp.	Crude extract	100	240.61	412.14	100	1.00
	sephadex G-25	5	9.04	696.5	62	1.68
<i>Alpha proteobacterium</i>	Crude extract	100	294.17	340.27	100	1.00
	sephadex G-25	5	20.70	509.4	25	1.49
<i>Brevundimonas</i> sp.	Crude extract	100	276.54	349.76	100	1.00
	sephadex G-25	5	6.30	525.1	43	1.50
<i>Bradyrhizobium</i> sp.	Crude extract	100	04.68	364	100	1.00
	sephadex G-25	5	18.004	582.8	15	1.60

Catechol 1, 2-dioxygenase activities were measured, in crude extract for each bacterial culture and after purification step, tabulated in table 6.5. For *Pseudomonas putida* catechol 1, 2-dioxygenase purification obtained was around 3IU/ml (figure 6.8) with 12% purification yield and 1.43 purification fold, whilst the specific activity was

335.53 U/mg in crude extract which increased to 482.6 U/mg after purification using sephadex G-25 (table 6.5). Figure 6.9 exhibits two peaks of activity for *Achromobacter xylosoxidans* with a purification yield of 22.58% and purification fold of 1.31, whilst the specific activity was 311.12 U/mg in a crude extract which increased to 408.7 U/mg after purification using sephadex column G-25. Similarly (figure 6.10) exhibits two peaks of purified catechol 1, 2 dioxygenase for *Microbacterium* sp. with specific activities of 412.14 U/mg in crude extract which increase to 696.5 U/mg after purification using sephadex G-25. Also, purification fold measured was 1.68 with highest purification yield of 62% compared to other bacterial strains. A single symmetrical peak was formed for the *Alpha proteobacterium* strain with the specific activity of 340.27 U/mg in crude extract which increased upto 509.4 U/mg after purification using sephadex G-25. Similarly, two peaks formed for *Brevundimonas* sp. resulted in a specific activity of 349.76U/mg which increased to 525.1 U/mg after purification using sephadex G-25 and a purification yield of 43% and purification fold of 1.50. A single peak formed for *Bradyrhizobium* sp. (figure 6.13) resulted with increased specific activity of 582.8 U/mg (table 6.5) after purification using sephadex column G-25 and purification yield obtained was 15% with 1.60 fold purification. After the purification of catechol 1, 2-dioxygenase, *Microbacterium* sp. produced highest specific activity followed by *Bradyrhizobium* sp., *Brevundimonas* sp. and *Alpha proteobacterium* and least specific activity was obtained from *Pseudomonas putida* exhibiting 4.82 U/mg (table 6.5). The fractions with the greatest activity were used for analysis by SDS/Page electrophoresis.

6.3.7 Bacterial catechol 1, 2-dioxygenase SDS-page confirmation

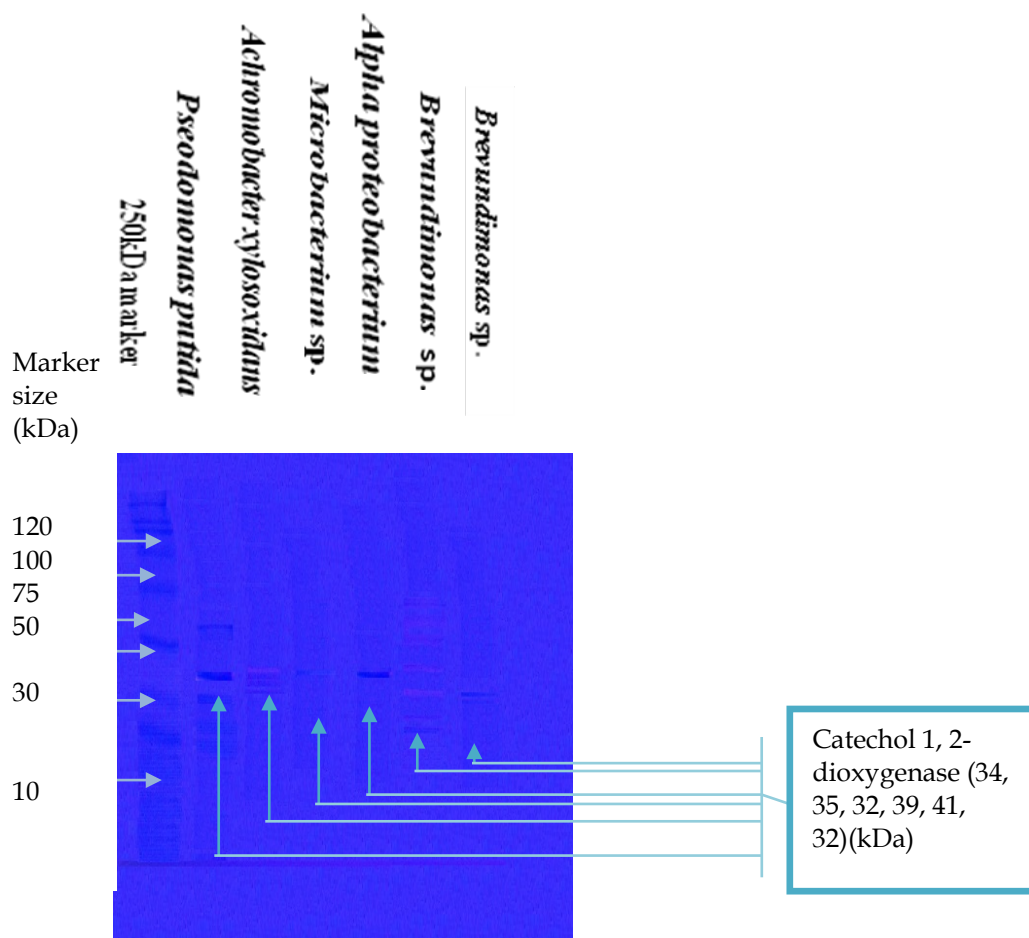


Fig. 6.14: SDS-Page analysis of purified bacterial catechol 1, 2-dioxygenase sample after elution from sephadex G-25column

Electrophoresis was performed for 40 mins followed by Coomassie blue staining and destaining of gel for 30 mins.

Figure 6.14 represents bacterial catechol 1, 2-dioxygenase analysis by SDS-Page gel electrophoresis. Fractionated samples with higher absorbance readings were used for activity measurement for SDS/Page electrophoresis to estimate molecular sizes of purified proteins.

In figure 6.14, bands with molecular size of 34, 35, 32, 39, 41 and 32 kDa were obtained for *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium sp.*, *Alpha proteobacterium*, *Brevundimonas sp.* and *Bradyrhizobium sp.* respectively.

6.3.8 Characterization of enzymes

The enzyme activities for the bacterial and fungal cultures with respect to effect of pH and temperature were analysed. A pH range of 5 to 9.5 and temperature range of 20⁰C to 70⁰C were analysed.

6.3.8a Effect of pH

The relative pH for MnP, laccase and catechol 1, 2-dioxygenase enzymes were estimated by measuring the activities of these enzymes at 40⁰C in pH ranging from 5 to 9 with half pH intervals using 0.1M of acetate buffer for pH 5 and 5.5, phosphate buffer for pH 6, 6.5, 7 and 7.5 and Glycine-NaOH buffer for pH 8, 8.5, 9 and 9.5 respectively. The results are displayed as a percentage of the activity of the pH which gave the highest activity.

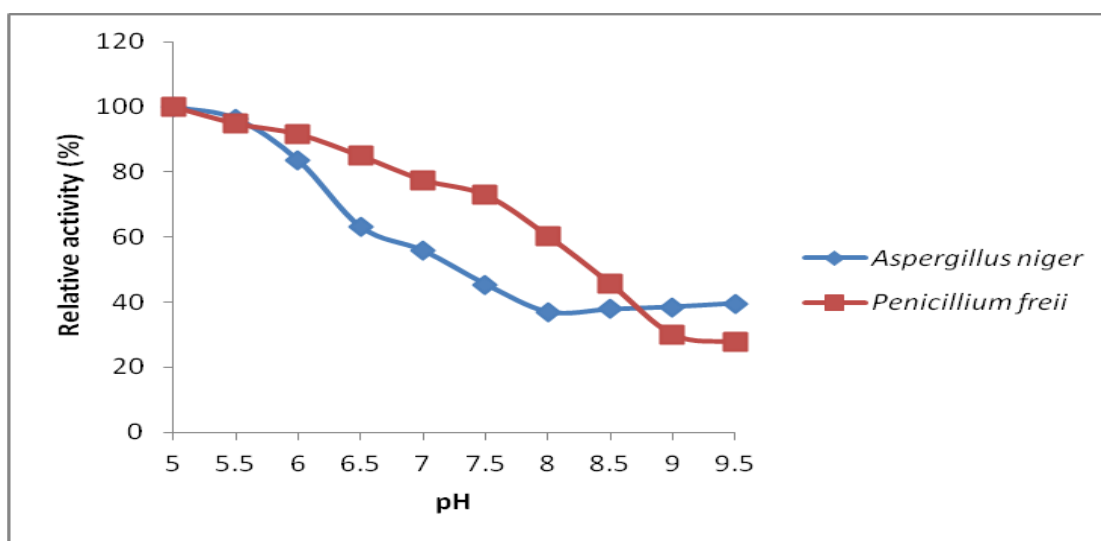


Fig. 6.15: Effect of pH on fungal manganese peroxidase (MnP) activity

The pH with the greatest MnP activity was at pH 5 for both strains (figure 6.15). Manganese peroxidase activity for both *Aspergillus niger* and *Penicillium freii* was gradually decreased with increase in pH however, relative activity of *Aspergillus niger* was decreased more rapidly between pH 5.5 and 8. Between pH 9 and 9.5 *Aspergillus niger* relative activity was greater than *Penicillium freii*.

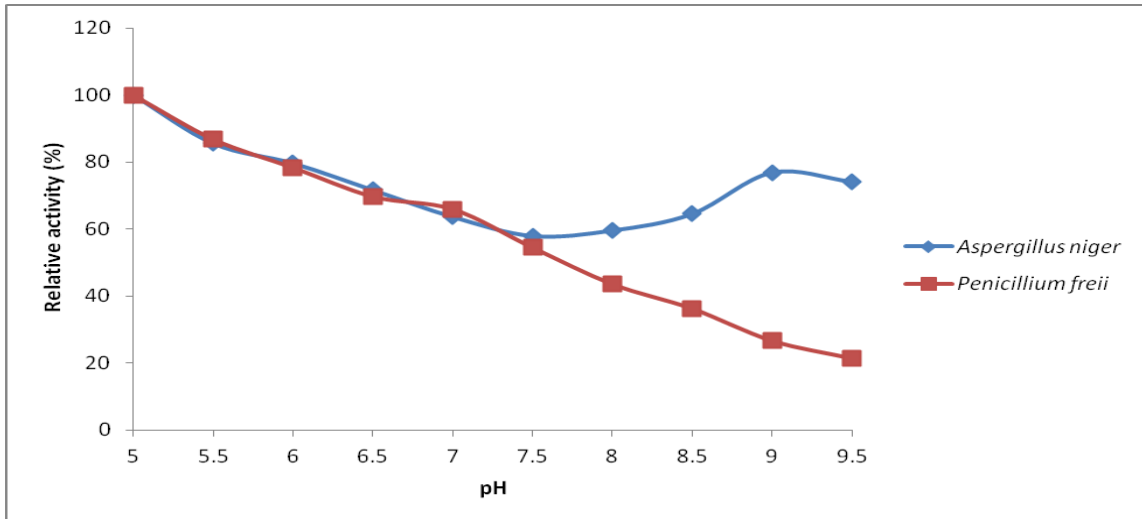


Fig. 6.16: Effect of pH on fungal laccase activity

The fungal laccase activity for both *Aspergillus niger* and *Penicillium freii* was greatest at pH 5 and gradually decreased for both strains to pH 7.5 where relative activity was around 60% of the activity at pH 5. However, laccase activity measured for *Aspergillus* strain was found to increase from pH 7.5 to pH 9, whilst relative activity of laccase for *Penicillium freii* continued to decrease to around 20% at pH 9.5 (figure 6.16). Relative laccase activity for *Aspergillus niger* increased to a peak of around 80% at pH 9.0.

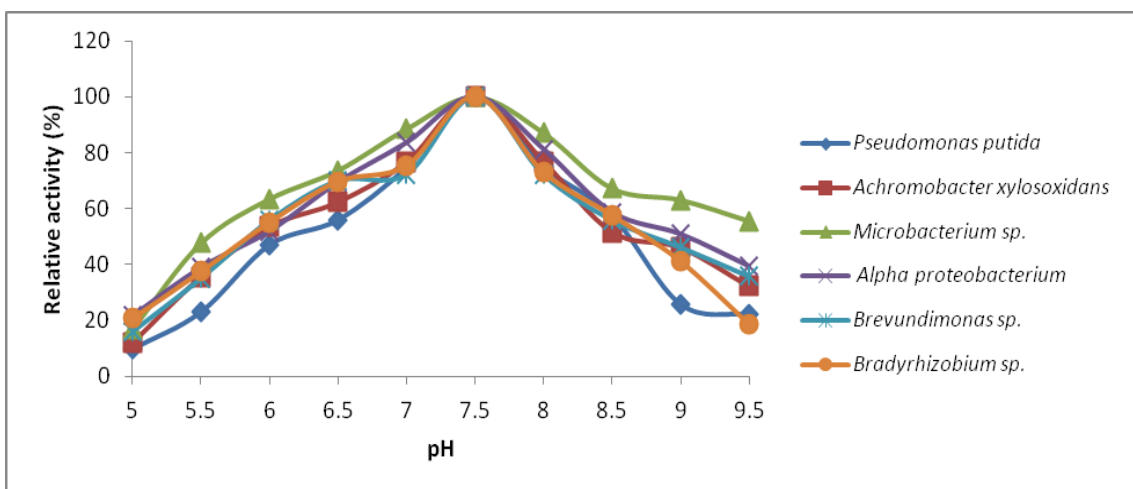


Fig. 6.17: Effect of pH on bacterial catechol 1, 2-dioxygenase activity

The effect of pH on catechol 1, 2-dioxygenase activity was measured using catechol as a substrate. The relative pH obtained for catechol 1, 2-dioxygenase activity was pH 7.5 for all the bacterial cultures. *Microbacterium* sp. exhibited higher relative activity compared to all other cultures across the pH range. Initially the relative activities obtained were lowest at pH 5 and as the pH increased the catechol 1, 2-dioxygenase activity also increased. Beyond the peak at pH 7.5 the relative activity decreased to between 25% and 55% at pH 9.5. The lowest activities for all the cultures were obtained at pH 5 and pH 9.5 respectively. Moreover, acidic pH and alkaline pH resulted in lower catechol 1, 2-dioxygenase activity.

6.3.8b Effect of temperature

Temperature activity for fungal manganese peroxidase, laccase and bacterial catechol 1, 2-dioxygenase was carried out by incubating the substrate at a temperature range of 20°C to 70°C.

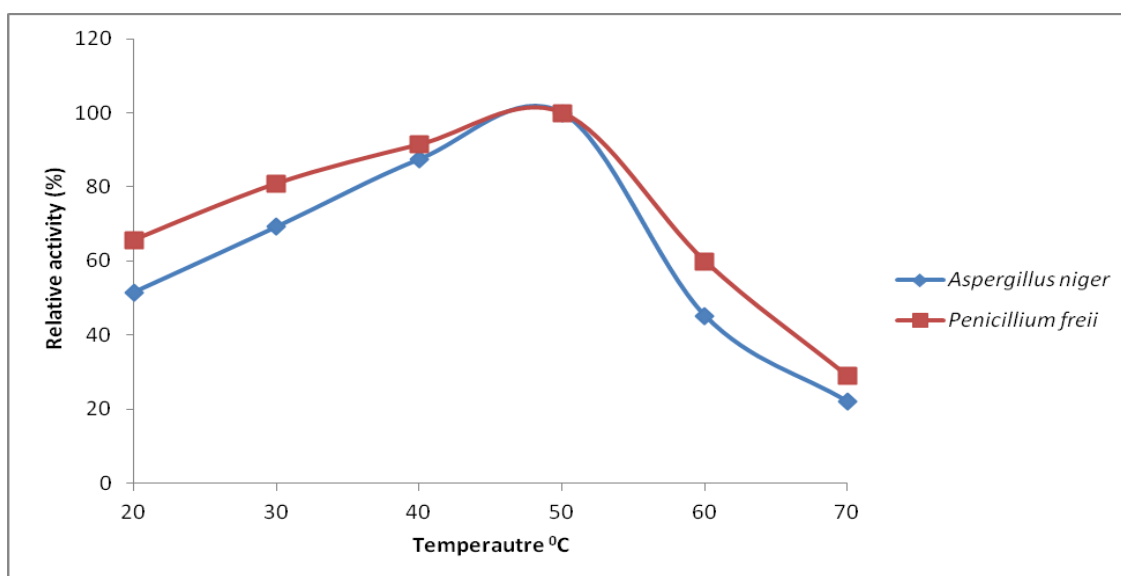


Fig. 6.18: Effect of temperature on fungal manganese peroxidase (MnP) activity

The effect of temperature on manganese peroxidase relative activity was studied. The maximum relative activity was obtained at 50°C (figure 6.18). Manganese peroxidase relative activity increased at the temperature increased from 20°C upto 50°C dropped at 60°C and 70°C. Greater relative activity of manganese peroxidase was obtained for *Penicillium freii* compared to *Aspergillus niger* (figure 6.18) at temperatures higher and lower than 50°C.

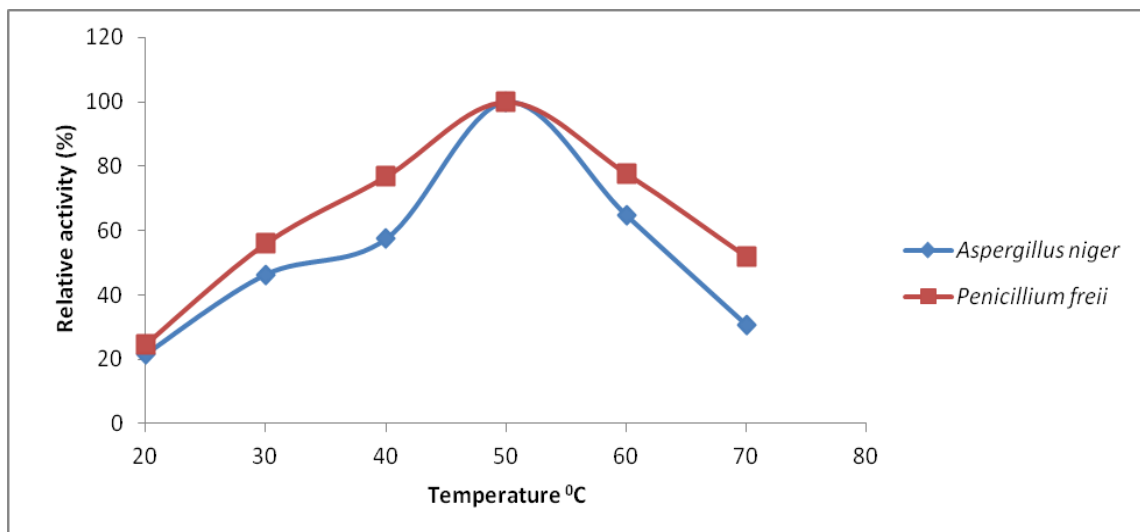


Fig. 6.19: Effect of temperature on laccase (Lac) activity

The laccase activity measured with respect to temperature is exhibited in figure 6.19. Laccase activity was found to be highest at 50°C. *Penicillium freii* has greater relative activity when compared to *Aspergillus niger* at temperatures above and below 50°C. The relative activity of both fungal strains dropped to approximately 20% of the peak at 20°C and 70°C.

6.3.8c For catechol 1, 2-dioxygenase

The effect of temperature on catechol 1, 2-dioxygenase activity was measured using catechol as a substrate for the enzyme assay. The activities were measured using six identified cultures at a temperature range of 20-70°C. The maximum activities for all

cultures were obtained at 30⁰C. However, as the temperature increased beyond 30⁰C a gradual drop was recorded. *Microbacterium* sp. exhibited highest relative enzyme activity compared to other cultures beyond 30⁰C whilst, *Bradyrhizobium* sp. had the lowest relative activity.

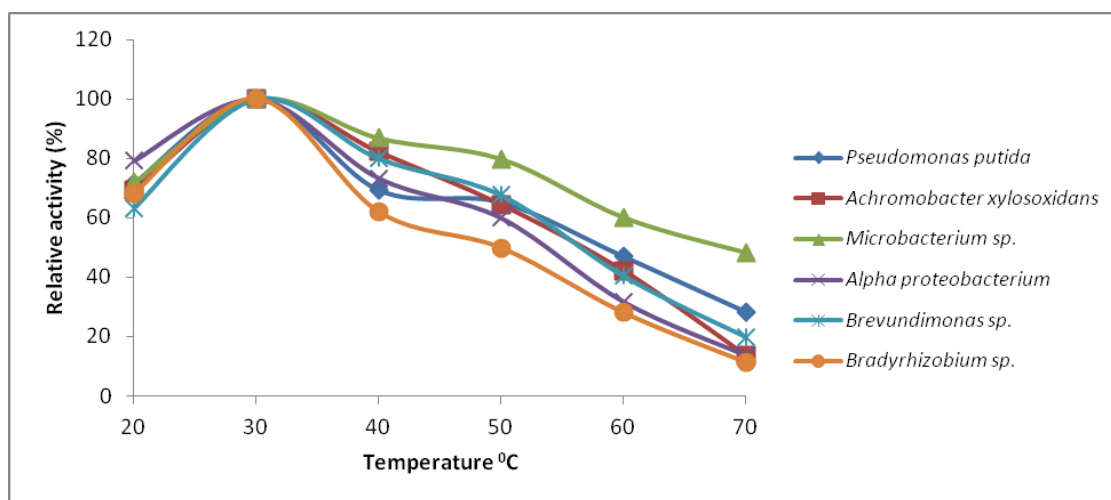


Fig. 6.20: Effect of temperature on catechol 1, 2-dioxygenase activity

In general, relative pH and temperature for fungal manganese activity was pH 5 and 50⁰C respectively. Similarly, the relative pH and temperature for fungal laccase activity was pH 5 and 50⁰C, whilst the relative pH and temperature for bacterial catechol 1, 2-dioxygenase was pH 7.5 and 30⁰C.

6.3.9 Enzyme kinetics

Kinetic studies were performed for purified fungal manganese peroxidase, laccase and bacterial catechol 1, 2-dioxygenase. Various concentrations of substrate (1-50mM) were used to incubate the enzymes to calculate the V_{max} and K_m values by plotting graph of V_0 (initial rate, mole of ABTS, MBTH and catechol produced per min) against the substrate concentration. Graphs were plotted to confirm whether enzymes follow the Michaelis- Menten kinetics by considering initial rates versus substrate

concentrations. Reciprocal graphs were plotted to determine Lineweaver –Burk plot (figure 6.21 and 6.22).

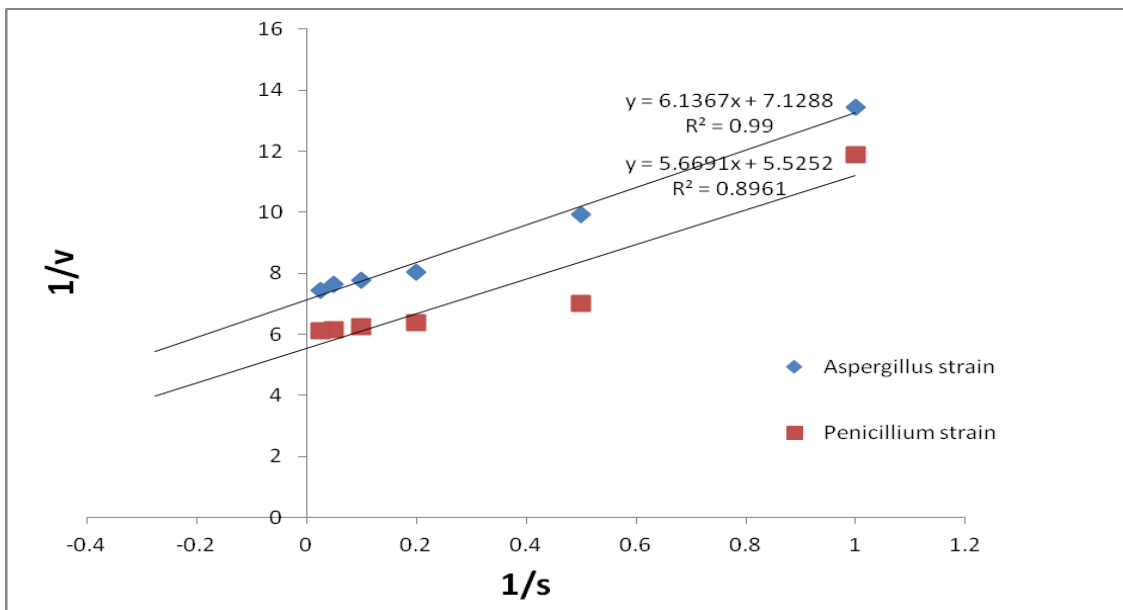


Fig. 6.21: Lineweaver –Burk plot of fungal manganese peroxidase (MnP)

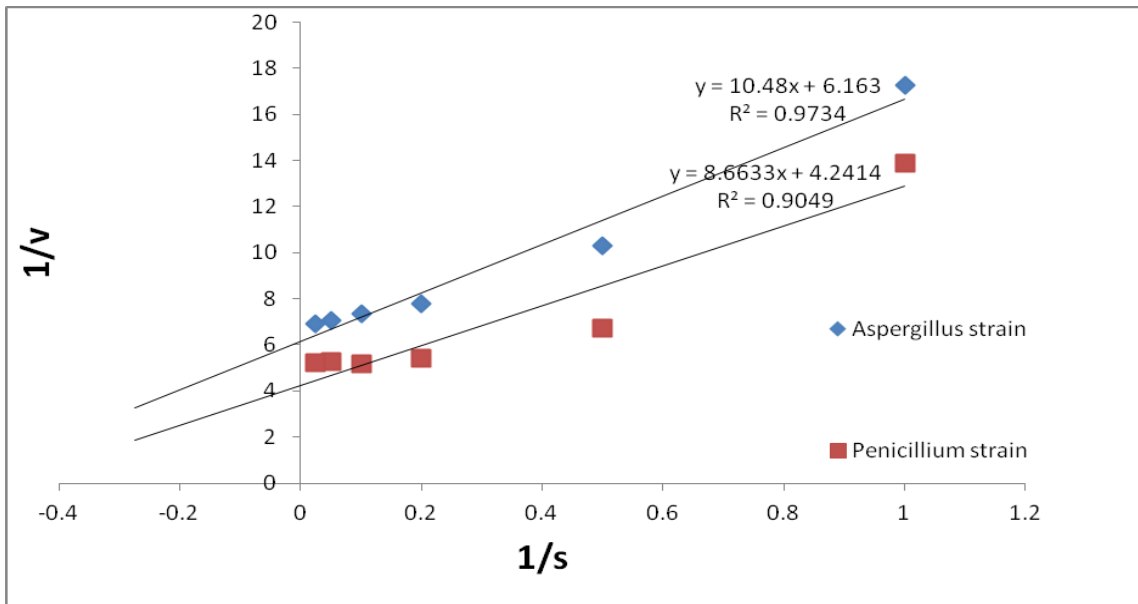


Fig. 6.22: Lineweaver –Burk plot of fungal laccase using fungal strains

Figure 6.21 & 6.22 exhibits a set of double reciprocal plots (Lineweaver- Burk plot) one calculated for *Aspergillus niger* and one for *Penicillium frei* at different

concentration of ABTS and MBTH as a substrate ($1/s$ represents reciprocal substrate concentrations). The series of concentrations of substrate (1-50 mM) incubated was used to calculate the V_{\max} and K_m values by plotting graph of V_0 (initial rate, mole of ABTS, MBTH and catechol produced per min) against the substrate concentration. The V_{\max} and K_m values obtained for *Aspergillus niger* and *Penicillium freii* are described in table 6.6 and for bacterial cultures are described in table 6.7:

Table 6.6: Enzyme kinetics for fungal strains

Enzyme	Organisms	V_{\max} (mM/min)	K_m (mM)
MnP	<i>Aspergillus niger</i>	0.1402	0.8575
	<i>Penicillium freii</i>	0.181	1.0261
Lacasse	<i>Aspergillus niger</i>	0.162	1.700
	<i>Penicillium freii</i>	0.2358	2.0425

The Lineweaver Burk plot exhibits linear lines with separate intercept on the $1/V_0$ axis however with different slopes (figure 6.22 & 6.23). The intercept of $1/V_0$ axis equals $1/V_{\max}$ and it is known that when V_{\max} is lower, K_m is greater for different fungal organism which is said to be competitive. The V_{\max} and K_m values were calculated for manganese peroxidase and laccase activity from Lineweaver Burk plot figure 6.22 & 6.23. For manganese peroxidase, V_{\max} 0.1402 mM/min and K_m 0.8575 mM were calculated for *Aspergillus niger*. Similarly, 0.181mM/min V_{\max} and 1.0261 mM K_m were calculated for *Penicillium freii* from figure 6.22 & 6.23. V_{\max} and K_m calculated for laccase activity exhibited 0.162 mM/min and 1.7mM for *Aspergillus niger* and 0.2358 mM/min and 2.04 mM for *Penicillium freii*. From L-B plot it is clear the two fungal strains are competitive exhibiting manganese peroxidase and laccase activity for different substrate concentrations.

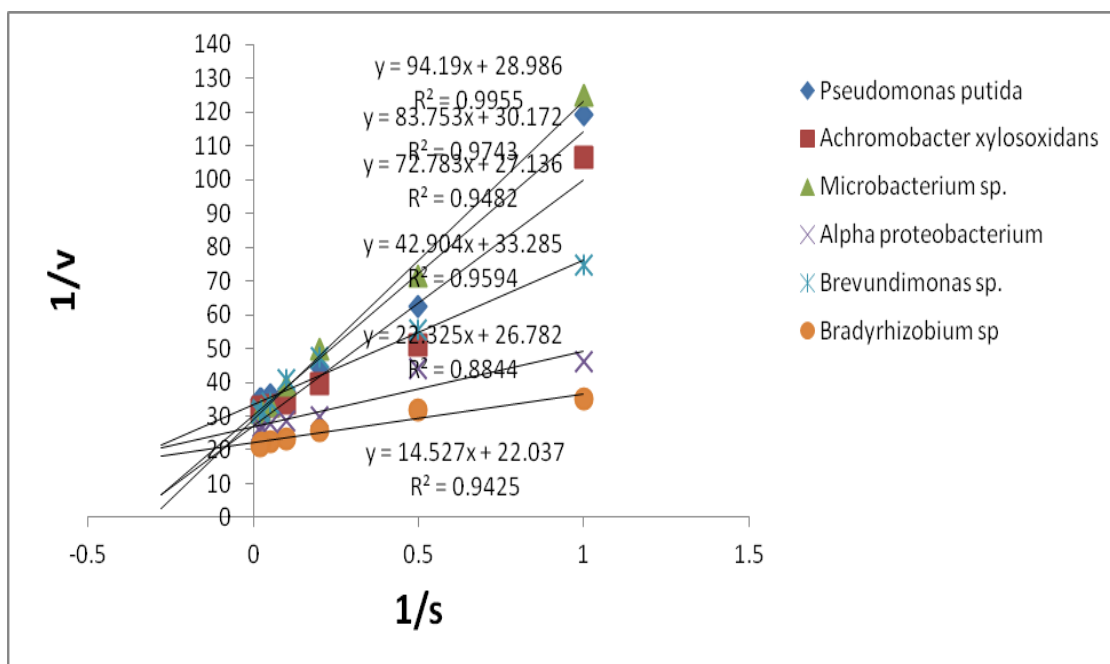


Fig. 6.23: Lineweaver –Burk plot of bacterial catechol 1, 2-dioxygenase

Table 6.7: Enzyme kinetics by bacterial strains

Organisms	V_{max} (mM/min)	K_m (mM)
<i>Pseudomonas putida</i>	0.0033	2.7
<i>Achromobacter xylosoxidans</i>	0.0036	2.6824
<i>Microbacterium sp.</i>	0.0045	0.6592
<i>Alpha proteobacterium</i>	0.0037	0.8336
<i>Brevundimonas sp.</i>	0.003	1.289
<i>Bradyrhizobium sp.</i>	0.0034	3.2499

Figure 6.23 exhibits a set of reciprocal plots (Lineweaver- Burk plot) one obtained for six bacterial isolates calculated at different catechol concentration as a substrate ($1/s$ represents reciprocal substrate concentrations). The series of concentrations of substrate (1-50 mM) incubated was used to calculate V_{max} and K_m values by plotting graph of V_0 (initial rate, mole catechol produced per min) against the substrate concentration. A set of linear lines were obtained with separate intercept on the $1/V_0$

axis however with different slopes (figure 6.23). Intercept of $1/V_0$ axis equals $1/V_{max}$ and it is known that when V_{max} is lower, K_m is greater for different bacterial organism which is said to be competitive. V_{max} and K_m values for *Pseudomonas putida* were 0.0331 mM/min and 2.7 mM (table 6.7) calculated from figure 6.23. V_{max} exhibited by bacterial isolates *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp. calculated were 0.0369 mM/min, 0.045 mM/min, 0.0373 mM/min, 0.03 mM/min and 0.034 mM/min and K_m calculated were 2.7 mM, 2.68 mM, 0.659 mM, 0.833 mM, 1.28 mM and 3.24 mM respectively. Lower K_m value was obtained for *Microbacterium* sp. and higher K_m value was obtained for *Bradyrhizobium* sp. however, higher V_{max} was obtained for *Microbacterium* sp. and lower V_{max} was obtained for *pseudomonas putida* and *Bradyrhizobium* sp. The Lineweaver-Burk plot represents the six identified bacterial isolates are competitive exhibiting catechol 1, 2 dioxygenase activities for different substrate concentrations.

6.4 Discussion

Most studies of PAH degradation focus on two main groups of organisms: soil bacteria and ligninolytic fungi that carry out oxidation and mineralization of PAH compounds (Baldrian *et al.*, 2000).

6.4.1 Production of fungal and bacterial intracellular/extracellular enzymes

Enzyme studies in this thesis were conducted in presence of PAHs added to shaken cultures of *Penicillium freii* and *Aspergillus niger* and resulted in increased manganese peroxidase and laccase ligninolytic enzyme activities (figure 6.2 & 6.3). Manganese peroxidase and laccase ligninolytic enzymes of fungi play an important role in PAH degradation which were studied by extracting purified cell free enzymes intracellularly and extracellularly from crude extracts. All the fungal ligninolytic enzymes are rarely reported with *Aspergillus* spp. and *Penicillium* spp. The white-rot fungi have been reported as principal organisms that produce several extracellular enzymes which include laccase, manganese peroxidase and lignin peroxidase. Besides white-rot fungi, other organisms are also involved in degradation and de-colorization of different recalcitrant organic compounds (Kalmis *et al.*, 2008; Kirk & Farrell, 1987). In this study, both fungal strains (*Aspergillus niger* and *Penicillium freii*) were found to produce manganese peroxidase and laccase activities in liquid culture medium. Optimum enzyme extraction after performing enzyme assay were determined greater in extracellular fungal crude extract whereas; in intracellular activities it was lower (figure 74 & 75). Also, Baldrian *et al.*, (2000) has reported that, initial reaction of PAH degradation is mostly initiated by extracellular ligninolytic enzymes which include laccase, lignin peroxidase and MnP (Mn-dependent peroxidase enzymes). Thus,

extracellular fungal extract results in PAH degradation, for example laccase reached the greater enzyme activity of $2000 \mu\text{mol ml}^{-1}\text{h}^{-1}$ after 8 days of growth which is highest than that of manganese peroxidase activity of $1200 \mu\text{mol ml}^{-1}\text{h}^{-1}$ intracellularly using *Penicillium freii* and *Aspergillus niger* (figure 6.2 & 6.3). Thus, extracellular fungal activities were greater compared to intracellular enzyme activities. Also, an activity measurement contributes to initiation of PAH degradation reaction at initial levels by extracellular ligninolytic fungal enzymes. Lower intracellular manganese peroxidase activity was observed due to formation of mycelial pellets and O_2 limitation that is responsible for mycelia and denaturation of enzymes caused by high agitation speeds of 100 to 200 rpm, required by fungi for constant consumption of carbon source (Venkatadri and Irvine, 1990). *Penicillium freii* was more compatible with higher activity compared to *Aspergillus niger*. Also, work carried out in this report is comparable to the research reported by Giarfreda *et al.*, (2005) reporting 8 times higher enzyme activity in popular sawdust extracellularly than that of fir sawdust intracellularly by *Pleurotus ostreatus* cultures. However, studies reported by Bogan & Lammar, (1996) indicates *Phanerochaete laevis* HHB-1625 grown in presence of PAH in nitrogen limited liquid medium has complements of extracellular ligninolytic enzymes. Manganese peroxidase activity was found to be strongly regulated whereas low levels of laccase activity were detected and no LiP was found in the culture medium.

As catechol 1, 2-dioxygenase is the aromatic ring-cleavage enzyme of catechol dioxygenase present in bacteria and is mostly characterized based on biochemical and structural properties. Also, among various aromatic compounds catechol 1, 2 dioxygenases play a vital role in the degradation pathway and are found to be ubiquitous in bacterial cultures (Brodrick *et al.*, 1991; Latus *et al.*, 1995; Sauret-Ignazi

et al., 1996). Thus, studies related to isolation, production and purification of dioxygenases enzyme indicate that they help in complete removal of aromatic compounds from industrial and domestic environment suggesting conservation of biodiversity (Nadaf & Gosh, 2011).

In this research reported, catechol 1, 2-dioxygenase enzyme activities in presence of PAH were studied intracellularly as well as extracellularly in a crude extract using 6 different distinct bacterial cultures in minimal medium. All the bacterial strains exhibited catechol 1, 2-dioxygenase activity. Moreover, in these studies catechol 1, 2-dioxygenase production (figure 6.4) exhibited greater intracellular activity for all the bacterial strains compared to extracellular enzyme activities. Among all the bacterial strains greater intracellular activity of $450 \mu\text{mol ml}^{-1}\text{h}^{-1}$ was obtained for *Microbacterium* sp. and extracellular activity of $300 \mu\text{mol ml}^{-1}\text{h}^{-1}$ was obtained for *Microbacterium* sp.. Similar results were reported by Lee *et al.*, (2003) indicating catechol 1, 2-dioxygenase activity intracellularly and extracellularly during fermentation condition by two strains of *Pseudomonas* species. Also, *Ralstonia* sp. Ba-0323 strain producing 1.6mg/ml of catechol from sodium benzoate in a 20 hours growing culture was studied for extracellular and intracellular production of dioxygenase indicating greater bacterial intracellular activities (Wang *et al.*, 2001).

Studies involving fungal manganese peroxidase and laccase activities in this thesis exhibit greater extracellular activities than that of intracellular activities. Also, Cajthaml *et al.*, (2007) has reported ligninolytic enzymes of *Irpex lacteus* indicate greater extracellular ligninolytic activity in microsomal fraction of biomass grown in complex and N-limited medium. However, bacterial catechol 1, 2-dioxygenase extracted intracellularly and extracellularly exhibited greater activities for intracellular enzymes compared to extracellularly. Difference between intracellular and

extracellular activities in these studies exhibits substantial difference for bacterial and fungal strains. This substantial difference may be due to Na⁺/K⁺ ATPase in majority of organism which sustains high potassium level and low sodium levels which accounts for low chemical volatility within cells (Masten & Davies, 1997). After the production of intra/extracellular enzymes for fungal manganese peroxidase, laccase and bacterial catechol 1, 2-dioxygenase activities, single step purification was studied.

6.4.2 Purification of ligninolytic enzymes

Fungal manganese peroxidase and laccase catalyses MBTH and ABTS that was purified by a single step purification using a mixture of crude ligninases (laccase and manganese peroxidase) obtained from 8 day old incubated cultures of *Penicillium freii* and *Aspergillus niger*, grown in presence of PAHs. Crude enzyme solutions after determining enzyme activity, loaded onto a sephadex G-25 column imparted single step enzyme purification. Protein fractions eluted at a flow rate of 0.5ml/min were expressed with two peaks, exhibiting enzyme activity in IU/ml and protein concentration in mg/ml for each fractionated sample (figure 6.5 and 6.6). Subsequently, the peak point with highest protein concentration for fractionated sample was assayed for laccase and manganese peroxidase activities. The manganese peroxidase and laccase activities along with their effects were mostly evaluated, as these enzymes play important role in degradation of recalcitrant compounds (Steffen *et al.*, 2003). The purification to homogeneity of manganese peroxidase and laccase, using a single step purification procedure is tabulated in table 6.3 & 6.4. Purification fold for manganese peroxidase activity measured for *Aspergillus niger* exhibited was 1.98 and for *Penicillium freii* was 1.74fold. In sephadex G-25 column chromatography, specific activity was increased to 573.3U/mg for *Aspergillus niger* and for *Penicillium*

freii specific activity measured was increased to 623.2 U/mg. However, laccase exhibited greater purification fold of 1.88 for *Aspergillus niger* and 1.60 for *Penicillium freii* and specific activities 558.3 U/mg for *Aspergillus niger* and 589.7 U/mg for *Penicillium freii* when compared to manganese peroxidase enzyme. This suggests that manganese peroxidase purification from both fungi have different values in both enzymes i.e. *Aspergillus niger* was less stable compared to *Penicillium freii* which was evident from the purification yields. Similar results were indicated by Steffen *et al.*, (2003) indicating comparison between *Agapanthus praecox* and *S. coronilla* suggesting *A. praecox* has lower purification yields than that of *S. coronilla*. In 1883, laccases were first described from Japanese lacquer tree *Rhus vernicifera* (More *et al.*, 2011). Moreover, laccase activity in *T. versicolor* 951022 has been reported with much higher specific activity of 91,443 IU/mg using ABTS as a substrate (Han *et al.*, 2005). The specific activity reported in this thesis has slightly lower values compared to literatures. However, specific activities were found to be different depending on the substrate and conditions of assay. Also, the unit for activity measurement is defined differently by other research groups (Han *et al.*, 2005; Eggert *et al.*, 1996; Yaver *et al.*, 1996; Xiao *et al.*, 2003).

6.4.3 Purification of catechol 1, 2-dioxygenase

The purification and characterization of catechol 1, 2-dioxygenase from *Rhodococcus* sp. NCIM 2891 was first reportedly performed with single step purification process by Nadaf and Ghosh (2011). The catechol 1, 2-dioxygenase enzyme purification to homogeneity was performed using single step purification process by sephadex G-25 column. Elution of protein fractionations along with catechol 1, 2-dioxygenase activity plotted versus fraction numbers for each bacterial

culture strain using sephadex G-25, were purified (figure 6.8, 6.9, 6.10, 6.11, 6.12 and 6.13). The protein fractions were eluted at a flow rate of 0.5 ml/min. Subsequently, peak points with highest protein concentration for the fractionated sample along with highest catechol 1, 2-dioxygenase activity were observed and molecular sizes were confirmed by SDS-Page analysis. The purity to homogeneity obtained by single step purification using sephadex G-25 for all cultures, exhibited increased specific activity of about 482.6, 408.7, 696.5, 509.4, 525.1, 582.8 U/mg whereas, purification fold values obtained for 6 cultures were 1.43, 1.31, 1.69, 1.49, 1.50 and 1.60 respectively. Similarly, Briganti *et al.*, (1997) has summarized the enzyme purification with more similar values to those obtained in this thesis.

6.4.4 SDS-page confirmation

The manganese peroxidase activity from both fungi after purification step, appeared as a purified protein bands on SDS-page with a molecular weight between 41 to 48kDa, as a common molecular size reported in literatures (Steffen *et al.*, 2003). Researchers found similar results where *A. biporus* strain producing manganese peroxidase was found with molecular weight around 40kDa which were grown on solid compost (Latus *et al.*, 1995). Similarly, with white-rot fungi particularly *Phanerochaete chrysosporium* producing manganese peroxidase were evident with molecular weight around 45kDa (Glenn *et al.*, 1983) and *Bjerkandera* sp. BOS55 (Mester & Field, 1998) were reported with molecular weight around 46 kDa and (44-45 kDa). In addition to this, molecular weight of laccase for both fungi was appeared as a single band on SDS-page with molecular weight of 64 kDa (figure 6.7). Laccase isolated from Basidiomycota that include *Trametes* spp. has been generally represented as monomeric protein with 50 and 80 kDa molecular mass (Levin & Forchiassin,

2003). To confirm, the protein band as a laccase, molecular size was analysed in figure 85 outcome in the range represented as monomeric protein for *Trametes* spp. Moreover, molecular sizes of proteins evident for fungal enzymes were purified and activity measured using ABTS and MBTH as a substrates. Both of these substrates are suggested in literature reviews and could express their enzymatic function with either manganese peroxidase or laccase activities which makes it difficult to confirm the particular band for specific activity of protein analysed on SDS-page gel electrophoresis (figure 6.7) (Jordaan and Leukes, 2003).

In this research work, SDS-page analysis for catechol 1, 2-dioxygenase enzyme from six identified bacterial cultures were analysed and molecular weight around 34, 35, 32, 39, 48, 32 kDa has been obtained. Similar results have been reported by researchers where they found molecular weight of catechol 1, 2-dioxygenase around 30 kDa for *Rhodococcus* sp. NCIM 2891 and *Ralstonia* sp. Ba-0323 (Nadaf & Ghosh, 2011; Wang *et al.*, 2001). Thus, results obtained in this study are comparable with the results of other researchers.

6.4.5 Characterization of purified enzymes

6.4.5a Effect of pH

The effect of pH was studied for purified enzymes for manganese peroxidase, laccase and catechol 1, 2-dioxygenase at a range of 5.0 to 9.0 with half intervals. Optimum pH for greater relative activity of manganese peroxidase was observed at pH 5 using ABTS as a substrate. At pH greater than 5.0, decreased manganese peroxidase relative activity for both *Aspergillus niger* and *Penicillium freii* were observed (figure 6.15).

Interestingly, *Aspergillus niger* exhibited slight increase in relative activity at alkaline pH of 7.5, 8, 8.5 and 9, whilst, relative activity for *Penicillium freii* continued to decrease to around 20% at pH 9.5 and relative activity of laccase for *Aspergillus niger* increased to a peak of around 80% at pH 9.

Interestingly, in chapter 5 (Biodegradation of PAHs) increase in the fungal population of one of predominant fungi (*Aspergillus niger*) was found at alkaline and acidic pH in comparison to neutral pH.

Moreover, the relative activity of laccase measured at different pH 5 was found greater as compared to relative activity of laccase at pH 9.5. Also, as the pH was increased the relative activity of laccase was gradually decreased (figure 6.16). Also, gradual decrease in laccase was found with increased pH, (figure 6.16) and this gradual decrease with increased may be due to the difference in redox potential between the reducing substrate and active site of the enzymes (Sadhasivam *et al.*, 2008) present in *Aspergillus niger* and *Penicillium freii*. Moreover, research conducted on *Chalara paradoxa* CH32, *Cerena unicolor*, *Trichoderma atroviride* and *Coriolus hirsutus* suggested that the optimal pH range for fungal laccase was from 4.0 to 6.0 (Robles *et al.*, 1999). All the ligninolytic enzymes including manganese peroxidase has been

reported with optimum pH in acidic range. However, industry activities particularly leading to pulping and bleaching mainly exhibit alkaline conditions and thus, the waste generated through these sources is alkaline (O'Mahony *et al.*, 2006)

The specific relative pH of bacterial catechol 1, 2-dioxygenase was obtained at pH 7.5 (figure 6.17). Nadaf & Ghosh, (2011) has also reported similar results where they found relative pH 7.5, for bacterial catechol 1, 2-dioxygenase. However, their studies are focused on phenol degradation whereas, in these studies catechol 1, 2-dioxygenase was studied in PAH degrading organisms.

6.4.5b Effect of temperature

The temperature effect was conducted to characterize the fungal manganese peroxidase, fungal laccase and bacterial catechol 1, 2-dioxygenase by incubating their relative substrates within a temperature range of 20-70⁰C. The relative temperature obtained for all the enzymes corresponded well to the values available in literature. The specific relative activity for manganese peroxidase was obtained at 50⁰C (figure 6.18) and the specific relative activity for laccase was also obtained at 50⁰C (figure 6.19). Both strains exhibited greater specific relative temperature for laccase and manganese peroxidase activity at 50⁰C. A research by Baborova *et al.*, (2006) suggests that, the specific relative temperature and pH of manganese peroxidase activity was slightly greater for *L. lacteus* although, stability at elevated temperature was low. Also, the laccase specific temperature values obtained for *Penicillium freii* and *Aspergillus niger* corresponds well with the temperature reported for *Botrytis cinerea* (Zouari *et al.*, 1987). Rogalski, (1991) has reported the relative temperature for laccase from *Fomes fomentarius* is 52⁰C while laccase from *Chaetomium thermophile* is reported with specific relative temperature between 50 and 60⁰C (Chefetz *et al.*, 1998).

The relative temperature obtained for bacterial catechol 1, 2-dioxygenase using all 6 distinct bacterial cultures was 30⁰C (figure 6.20). The thermal effect of catechol 1, 2-dioxygenase reported by Nadaf & Ghosh (2011) suggests that enzyme effect is greater when exposed to temperature lower than 40⁰C. Similarly, specific relative temperature of 30⁰C was obtained for *Pseudomonas aeruginosa* in studies related to aromatic degradation (Chung *et al.*, 2003).

6.4.6 Enzyme kinetics

Enzyme kinetics studies were conducted to determine the specific formation of metabolites by six distinct bacterial and two distinct fungal cultures. The main kinetic parameters V_{max} (maximum enzyme velocity) and K_m (affinity constant) were determined for manganese peroxidase, laccase (ligninolytic enzymes) and for bacterial catechol 1, 2-dioxygease enzyme by plotting lineweaver Burk plot.

Kinetic studies for fungal manganese peroxidase and laccase were studied using MBTH and ABTS as a substrate with different concentrations (1 mM to 50 mM) (table 6.3 & 6.4). The Lineweaver Burk plot exhibits linear lines with separate intercept on the $1/V_0$ axis however with different slopes (figure 6.21 & 6.22). The intercept of $1/V_0$ axis equals $1/V_m$ however, when V_{max} was lower, K_m was greater for different fungal organism which is said to be competitive. The kinetic studies for fungal manganese peroxidase, exhibited V_{max} 0.1402 mM/min and K_m 0.8575 mM for *Aspergillus niger* and 0.181mM/min V_{max} and 1.0261 mM K_m for *Pencillium freii* from figure 6.21 & 6.22. Fungal laccase activity exhibited 0.162 mM/min V_{max} and 1.7 mM K_m for *Aspergillus niger* and 0.2358 mM/min V_{max} and 2.04 mM K_m for *Pencillium freii*. The lower K_m value represents condition where enzyme saturated with substrate does not convert much of substrate to product per unit of time. Also, larger the K_m lower is the

affinity of substrate towards enzyme. Thus, lower the V_{\max} more enzyme converts substrate to product per unit of time. In this study, higher K_m values were evident for laccase activity compared to manganese peroxidase activity.

In general, laccases exhibit non-specificity to their substrates and are able to oxidize a wide range of aromatic compounds from contaminated soil and water (Sadhasivam *et al.*, 2008). Thus, V_{\max} and K_m at different concentrations suggest a different behavior of enzyme for their substrates. The V_{\max} value obtained for *Chaetomium thermophilum* is 2.6 $\mu\text{mol}/\text{min}/\text{mg}$ for the ABTS as a substrate (Chefetz *et al.*, 1998). The V_{\max} and K_m parameters for purified laccase from *Pleurotus* sp. using ABTS indicated by More *et al.*, (2011) were 0.25 mM and 0.33 $\mu\text{mol}/\text{min}$ respectively. Similar values were obtained for laccase from *Melanocarpus albomyces* with ABTS concentration of 0.28 mM.

The K_m value obtained for laccase from *Pleurotus sajor-caju* was 0.092mM using ABTS as a substrate (Lo and Buswell, 2001). However, the K_m values representing catalytic constants obtained for *Aspergillus niger* are lower whereas for *Penicillium freii* are either greater or equivalent to the reported studies in literatures. This indicates lower substrate specificity for *Aspergillus niger* and greater substrate specificity for *Penicillium freii* for fungal laccase.

Bacterial catechol 1, 2-dioxygenases kinetic values obtained for six different bacterial strains exhibit linear lines with separate intercept on the $1/V_0$ axis however with different slopes (figure 6.23). The greater V_{\max} and lower K_m values were obtained for *Microbacterium* sp. (V_{\max} : 0.04538 and K_m : 0.6892) and the minimum V_{\max} and greater K_m values were obtained for *Bradyrhizobium* sp. (V_{\max} : 0.0345 and K_m : 3.2499) (figure 6.23). The V_{\max} and K_m values obtained in this study exhibit slightly lower values when compared to literature. The K_m value obtained by Nadaf and Ghosh, (2011)

were 5 $\mu\text{mol/ml}$ with V_{max} 2.5 U/mg of protein. The lower values may be due to lower affinity for substrate (catechol) which is formed during assimilation of culture broth in presence of PAHs (Nadaf & Ghosh, 2011). Also, the K_m and V_{max} values for hydroxyquinol 1, 2-dioxygenase obtained were 7 μM and V_{max} was 5.25 U/mg using hydroxyquinol as a substrate from *Escherichia coli* (Daubaras *et al.*, 1996). The Michaelis constant (K_m) of an enzyme is a measure of the affinity of the enzyme for its substrate. The value of K_m for a particular enzyme is defined as the substrate concentration at which half of the enzyme molecules are complexed with substrate. Under these conditions, at any instant, half of the total enzyme molecules are capable of catalysis. The substrate concentration required to drive half of the enzyme molecules into an enzyme substrate (ES) complex that depends on the ability of the enzyme to bind its substrate. Thus, an enzyme with a high affinity for its substrate will have a low K_m i. e, 50% of the enzyme molecules will have bound substrate at a relatively low concentration of substrate. In contrast, an enzyme with a low affinity for its substrate has a high K_m value because a relatively high concentration of substrate is required to drive 50% of the enzyme molecules into complexes with substrate. Since the Michaelis-Menten constant measures the affinity between enzyme and substrate, the values suggested that fungal laccase had a greater affinity for *Penicillium freii* and bacterial catechol 1, 2-dioxygenase had lower affinity for five bacterial strains apart from *Microbacterium* sp. (Table 6.7).

Thus, the K_m and V_{max} values calculated by adding a reciprocal plot (Lineweaver-Burk plot) confirmed that data obtained follows Michaelis- Menten kinetics.

Chapter: 7.0

General Discussion

Conclusion

Future Prospects

References

Appendix

7.1 General Discussion

In recent years, developments using organisms to decontaminate polluted soil have been employed within remediation strategies. The effect of soil pH has not been investigated, therefore this thesis focused upon evaluating degradation of PAHs using photo-catalytic degradation and biodegradation processes at varying pH.

The effect of soil pH (pH range 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) on photo-catalytic degradation was studied and rate of degradation at two different wavelengths were monitored using HPLC analysis (chapter 5). Soil pH 6.5 was most suitable for the photo-degradation of PAHs, whilst in general acidic soil had greater photo-degradation rates than alkaline soil pH. Photo-catalytic oxidation, depends on generation of OH^\cdot by a photo catalyst in the presence of catalyst (e.g. titanium dioxide, TiO_2) in order to perform oxidative degradation under UV irradiation (Zhang *et al.*, 2008). Thus, Woo *et al.*, (2009) suggest in a photo-catalytic process, OH^\cdot and/or OOH^\cdot radicals play a vital role and these radicals are highly generated in presence of charged catalyst, thereby reaction occurs directly between the electron/hole pair and organic substrate on the surface. Therefore, at soil pH 6.5, TiO_2 might be charged generating high number of OH^\cdot and/or OOH^\cdot radicals. PAH degradation was greater for UV irradiation at 375 nm compared to 254 nm. The greater degradation rates at 375 nm may be due to characteristic of a catalyst which has a better spectral match between its emission spectrum and absorption spectrum of the light source (Zhang *et al.*, 2008). A good spectral match between them always results in fast and greater photo-catalytic degradation (Zhang *et al.*, 2008). Phenanthrene at both the wavelengths had greater photo-catalytic degradation rate representing good spectral match and pyrene has lower photo-catalytic degradation rate of the four PAHs.

Six distinct bacterial cultures and two distinct fungal cultures were isolated by shaken

enrichment using road side soil with PAHs as the sole carbon source. This procedure was performed with media at varying pH conditions to isolate organisms at acidic, neutral and alkaline conditions. Bacterial PAH degraders, isolated via enrichment were identified biochemically and by molecular techniques using PCR amplification and sequencing of 16S rDNA. Sequences were analyzed using BLAST (NCBI) and their percentage identity to known bacterial rDNA sequences in the GeneBank database (NCBI) was compared. The six bacterial strains were identified as *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp. Similarly, fungal PAH degraders were identified microscopically and with molecular techniques using PCR amplification and sequencing of 18S rDNA and identified as *Aspergillus niger* and *Penicillium freii*. The studies reported by Lal & Khanna (1996); Bharathi & Vasudevan (2001); and Rahman *et al.*, (2002) have identified above mentioned genera as hydrocarbon-degrading micro-organisms. Biodegradation of four PAH was studied at varying soil pH, and the results generated suggest that soil pH plays important role in degradation process (chapter 6). Greater rate of degradation were observed at soil pH 7.5 for all of the four PAHs. HPLC analysis in this work showed that phenanthrene biodegradation was 100% in 45 days and that biodegradation was faster than the other pHs studied.

Bacterial populations were greater at pH 7.5 which was highly correlated (correlation value = 0.97) with soil ATP levels. It was therefore evident that the greatest rates of degradation were associated with the highest bacterial population. Soil enzyme activities in general were also greatest at soil pH 7.5. However, greater fungal populations were found at acidic soil pH and alkaline soil pH, in comparison with neutral pH 7.0. The fungal populations when correlated with soil ATP levels showed

lower correlation (0.23) suggesting bacterial populations were more actively involved in PAHs degradation at pH 7.5 compared to fungal populations. Also, fungal populations suggest they might be active in PAH degradation at acidic and slightly at alkaline soil pH as fungal soil enzyme activities were found greater at acidic and slightly at alkaline soil pH. *Pencillium freii* was found to be more prevalent at acidic pH whilst *Aspergillus niger* was found to be more prevalent at pH 7.5-8.0.

Soil enzyme activity measurements at three different pH conditions were studied for oxidase, and the carbon: nitrogen: phosphorous cycles in soil samples with microbial inocula as test samples and soil samples without microbial inocula used as control. Soil bacterial enzymes such as phosphatase, β -glucosidase and L-arginine ammonification exhibited greater soil activities at buffer pH 7.5 indicating greater overall microbial activity and involvement of bacterial populations at soil pH 7.5, Whereas, fungal manganese peroxidase, laccase and lignin peroxidase exhibited greater soil activities at acidic buffer pH among varying soil pH.

In general, the overall results obtained during the biodegradation experiment indicates that soil pH 7.5 results in higher degradation rates which correlates with higher bacterial populations, higher soil ATP levels and greater soil enzyme activities measured at varying soil pH. Therefore, soil pH is an important parameter that could be manipulated for successful enhancement of biodegradation process.

In this thesis, comparison between biodegradation and photo-catalytic degradation with respect to soil pH over the time suggests biodegradation rates are greater than photo-catalytic degradation at all soil pHs. For example, at soil pH 7.5 (greatest biodegradation rates; chapter 5) exhibits 50% biodegradation in seven days. Whilst, in photo-catalytic degradation at soil pH 6.5 (optimum for photo-catalytic oxidation; chapter 4) 40% photo-catalytic degradation rate were obtained in 20 days. In general,

as a new finding comparison between biodegradation and photo-catalytic degradation process has been reported in this thesis. Thus, biodegradation experiments suggest greater degradation rates which could be cost effective and easier to perform in compared to photo-catalytic degradation. As optimal conditions for photo-catalytic degradation and biodegradation are different, combining their effects may prove difficult.

Oxidase and dioxygenase enzyme studies were purified and characterised (chapter 6). Results were obtained for ligninolytic enzyme such as MnP and laccase in fungi and dioxygenase enzyme such as catechol 1, 2-dioxygenase enzyme in bacteria. Single step purification from crude extract was carried out using sephadex G-25 column. Protein concentration was determined by Bradford assay and size confirmation of purified enzymes was obtained by SDS-page gel electrophoresis.

The single step purification using sephadex-G25 by fractionation for manganese peroxidase resulted in fold purification of 1.98 for *Aspergillus niger* and 1.73 for *Penicillium freii* along with 60.92 and 65.91 % purification yield. The percentage yield obtained for laccase was 69.52 & 71.4 along with fold purification of 1.88 for *Aspergillus niger* and 1.602 for *Pencillium freii* respectively. Manganese peroxidase and laccase purification was confirmed by loading the fractionated samples on SDS Page gel electrophoresis analysis. A protein band obtained at 48 kDa and 42 kDa confirms for manganese peroxidase when compared to molecular sizes suggested in literature, whereas the purified protein band obtained at 64 kDa and 69 kDa indicated for laccase activity. Substrates, MBTH and ABTS either or both can be used to measure the manganese peroxidase or laccase activity. After the purification of catechol 1, 2-dioxygenase, *Microbacterium* sp. produced the highest specific activity followed by *Bradyrhizobium* sp., *Brevundimonas* sp. and *Alpha proteobacterium* and

least specific activity was obtained from *Pseudomonas putida*. Protein bands with molecular size of 34, 35, 32, 39, 41 and 32 kDa were obtained for *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp. and *Bradyrhizobium* sp. respectively.

Kinetic studies were performed for purified fungal manganese peroxidase, laccase and bacterial catechol 1, 2-dioxygenase. Various concentrations of substrate (1-50mM) were used to incubate the enzymes to calculate the V_{\max} and K_m values by plotting graph of V_0 (initial rate, mole of ABTS, MBTH and catechol produced per min) against the substrate concentration. Thus, the K_m and V_{\max} values were calculated by adding a reciprocal plot Lineweaver- Burk plot (chapter 8). The lower K_m value represents condition where enzyme saturated with substrate does not convert much of substrate to product per unit of time (Sadhasivam *et al.*, 2007). Also, larger the K_m the lower is the affinity of enzyme to substrate. Thus, larger the V_{\max} the more the enzyme converts substrate to product per unit of time. In this study, higher K_m values were evident for fungal laccase activity compared to fungal manganese peroxidase activity. Also, the greater V_{\max} and lower K_m values were obtained for *Microbacterium* sp. (V_{\max} : 0.04538 and K_m : 0.6892) and the minimum V_{\max} and greater K_m values were obtained for *Pseudomonas putida* sp. (V_{\max} : 0.0345 and K_m : 3.2499).

Relative enzyme activity for pH and temperature effect along with enzyme kinetics studies for different substrate concentrations was studied. The fungal laccase and manganese peroxidase relative activities for both *Aspergillus niger* and *Penicillium freii* were greatest at pH 5 and gradually decreased for both strains to pH 7.5 where relative activity was around 60% of the activity at pH 5. However, laccase activity measured for *Aspergillus* strain was found to increase from pH 7.5 to pH 9, whilst relative activity of laccase for *Penicillium freii* continued to decrease to around 20% at

pH 9.5. Relative laccase activity for *Aspergillus niger* increased to a peak of around 80% at pH 9.0. Moreover, the relative pH activity obtained for catechol 1, 2-dioxygenase activity was pH 7.5 for all the bacterial isolates which correlates with the optimal pH for biodegradation of PAHs in soil (chapter 6).

In general, the six distinct bacteria and two distinct fungi isolated via shaken enrichment culture were confirmed as PAH degraders by performing biodegradation. Also, these organisms were reported by Lal & Khanna (1996); Bharathi & Vasudevan (2001); and Rahman *et al.*, (2002) as hydrocarbon-degrading micro-organisms. Studying enzyme kinetics in this thesis for isolated organisms has revealed the catalytic mechanism which suggests *Microbacterium* sp. (V_{max} : 0.04538 and K_m : 0.6892) with active sites of PAH-degrading enzymes and its metabolism that inhibit the enzyme.

This thesis shows that soil pH is an important factor in degradation as shown by significant difference in degradation rates. Interesting results were obtained as soil pH 7.5 results in greater degradation of PAHs. It is also shown that bacterial populations were greater at pH 7.5 and greater soil ATP levels were also measured at soil pH 7.5. Therefore bacterial population was associated with the greatest rates of degradation at soil pH 7.5. Soil enzyme activities in general were also greatest at soil pH 7.5. Thus, results obtained in this thesis have met all objectives.

As most of the soil in the UK and Europe are acidic (Adamson *et al.*, 1996), adjusting soil pH to 7.5 by liming for example, may enhance biodegradation both *in-situ* and *ex-situ*.

7.2 Conclusion

Soil pH has significant impact on photo-catalytic degradation and biodegradation of PAHs monitored in J Arthur Bower's topsoil.

The effect of soil pH on photo-catalytic degradation of key model PAHs (phenanthrene, fluoranthene, anthracene and pyrene) were monitored over a pH range pH 4.0 and pH 9.0 with half pH interval. A wavelength of 375 nm resulted in a greater degradation rate compared to 254 nm in presence of TiO₂ used as a catalyst. Greater degradation rates were obtained at acidic soil pH and lower degradation rates were obtained at alkaline soil pH. Soil pH 6.5 was found to be optimum pH for photo degradation whilst pH 8.5 and pH 9.0 resulted in the lowest photo-catalytic degradation of PAHs. Of the PAH studied phenanthrene exhibited greatest degradation rates and lower degradation rates were obtained for pyrene. Thus, photo-catalytic degradation is found not only depend on soil pH but on chemical nature of PAH, and wavelengths of UV light.

Shaken enrichment culture allowed isolation of pure microbial cultures from road-side soil. Each of these microbial cultures was characterized for their ability to use PAHs as sole carbons source and were confirmed to grow on PAHs. Biochemical and molecular techniques like PCR amplification and sequencing of 16S rDNA were performed to identify the PAH degrading bacteria. Six distinct bacterial strains were identified using BLAST analysis by comparing the sequences and their percentage identity to known bacterial rDNA sequences in the GeneBank database (NCBI). The six distinct bacteria were identified as *Pseudomonas putida*, *Achromobacter xylosoxidans*, *Microbacterium* sp., *Alpha proteobacterium*, *Brevundimonas* sp., *Bradyrhizobium* sp. Similarly fungal PAH degraders were identified microscopically and with molecular techniques using

PCR amplification and sequencing of 18S rDNA and were identified as *Aspergillus niger* and *Penicillium freii*.

Biodegradation of PAHs were studied with isolates from above. These isolates were inoculated into J Arthur Bower's topsoil and rate of PAH degradation was monitored. Over the range of soil pH 5.0 to pH 8.0 with half pH intervals, soil pH 7.5 exhibited greatest rate of biodegradation. Lower rates of degradation were obtained in soil pH 5.0 and 6.5 respectively. The half life of PAHs owing biodegradation was strong at soil pH 7.5 (3 days) whilst soil pH 5 and 6.5 had a half life of 21 days which is seven times greater than soil pH 7.5. During biodegradation greater bacterial populations were evident at alkaline conditions (soil pH 7.5) where as greater fungal populations were evident at acidic conditions (pH 5.0 and 5.5). Interestingly, the populations of fungi were greater at acidic soil pH but the *Aspergillus* was also isolated at alkaline soil pH. After microscopic identification *Pencillium* sp. was found to be predominant at acidic soil pH and *Aspergillus* sp. was predominant at alkaline soil pH.

Correlation between bacterial population and soil ATP levels were found higher ($r = 0.9$) when compared to fungal populations and soil ATP levels ($r = 0.2$). Thus, greater degradation rates obtained at soil pH 7.5 also had greater bacterial populations indicating bacteria played major role in degradation of PAHs where as greater fungal populations at acidic soil pH indicated that fungal populations were more active at acidic soil pH in degradation of PAHs. Moreover, soil enzyme activity significantly correlated with greater activity levels at soil pH 7.5 for L-arginine ammonification, alkaline phosphatase and β -glucosidase. Whereas, manganese peroxidase, lignin peroxidase and laccase soil enzyme exhibited greater activities at acidic soil pH. As oxidase and laccase soil enzyme activities were significantly correlated to PAH

degradation rates confirming fungi actively participated in degradation of PAH at acidic soil pH whereas bacterial population played major role at soil pH 7.5.

PAH degrading fungal (manganese peroxidase and laccase) and bacterial (catechol 1, 2-dioxygenase) enzyme were isolated and characterized to study the enzyme kinetics.

In this study, both the fungal strains (*Aspergillus niger* and *Penicillium freii*) were found to produce manganese peroxidase and laccase activities in liquid culture medium. Activities were greater in extracellular fungal crude extract than in intracellular activities. The purified fungal manganese peroxidase, fungal laccase and bacterial catechol 1, 2-dioxygenase was obtained by single step purification using sephadex G-25 column. The size of the purified fungal and bacterial enzymes was confirmed by SDS page.

The fungal ligninolytic and bacterial enzyme activities were characterized with respect to pH and temperature. The greatest activity relative temperature obtained for bacterial catechol 1, 2-dioxygenase for all six distinct identified bacterial cultures was 30⁰C and pH obtained was 7.5 (figure 6.19). Thus, PAH degrading bacterial populations (chapter 5) were greater at soil pH 7.5 during biodegradation resulting in greater degradation rates at pH 7.5 and bacterial enzyme activities also exhibited greater relative activity at pH 7.5

Both the fungal strains exhibited greater relative activity for laccase and manganese peroxidase activity at 50⁰C. The greatest activity of manganese peroxidase and fungal laccase relative activity was observed at pH 5.0. However, laccase activity measured for *Aspergillus niger* was found to increase from pH 7.5 to pH 9.0, whilst laccase relative activity for *Penicillium freii* continued to decrease to around 20% at pH 9.5. Interestingly, greater fungal populations measured during biodegradation (chapter 5) were found at acidic soil pH 5.0 and 5.5. *Aspergillus niger* resulted in increased fungal

ligninolytic activity at pH 7.5 to pH 9.0 and greater populations *Aspergillus* sp. populations were obtained at alkaline soil pH during biodegradation than that of neutral pH.

Enzyme kinetics were studied using various concentrations of substrate (1-50mM) to calculate the V_{max} and K_m values by plotting a graph of V_0 (initial rate, mole of ABTS, MBTH and catechol produced per min) against the substrate concentration. Lower K_m value was obtained for *Microbacterium* sp. and higher K_m value was obtained for *Bradyrhizobium* sp. however, the higher V_{max} was obtained for *Microbacterium* sp. and lowest V_{max} was obtained for *Pseudomonas putida* and *Bradyrhizobium* sp.

In general, with respect to biodegradation and photo-catalytic degradation the converse effect of pH was found at the optimal conditions. Biodegradation rate was greatest at alkaline conditions particularly at pH 7.5 whilst, Photocatalytic degradation exhibited greatest degradation rate at acidic conditions particularly at pH 6.5. Biodegradation rates at pH 7.5 were highest than photo-catalytic degradation. 65% of phenanthrene was degraded by photo-catalytic oxidation after 5 days, and at the same time point 90% of phenanthrene was biodegraded at pH 7.5 after 5 days.

Thus, manipulation of soil to pH 7.5 suggests significant potential for the remediation of PAHs essential enhancement for successful biodegradation process.

7.3 Future Prospects

Biodegradation of PAHs (chapter 6) was studied using J. Arthurs Bowers topsoil, so as to maintain the constant reproducibility through the experiments. However, biodegradation experiment in future required to study on different types of soil, so as to confirm the impact of variation in soil pH on biodegradation process.

Identified mixed microbial populations were inoculated to study the rate of degradation of four PAHs, however, in future individual microbial inocula can help to study the degradation rates carried out by individual microbes.

Studies involving thermalstabilization of PAH degrading enzymes with respect to effect of salts, effect of pH, deactivation rate constant can be investigated.

References

7.4 References

- Abdel, A. T. (1997). Arginine catabolism by microorganism. *Annual Review of Microbiology* **33**: 139-168.
- Abd-Elsalam, H. E. and Hafez, E. E. (2009). Isolation and Identification of Three-Rings Polyaromatic Hydrocarbons (Anthracene and Phenanthrene) Degrading Bacteria. *American-Eurasian Journal of Agriculture & Environmental Science* **5**(1): 31-38.
- Abliz, P., Fukushima, K., Takizawa, K. and Nishimura, K. (2004). Identification of pathogenic dematiaceous fungi and related taxa based on large subunit ribosomal DNA D1/D2 domain sequence analysis. *FEMS Immunology Medical Microbiology* **40**(1): 41-49.
- Acevedo, F., Pizzul, L. Castillo, M. D., Gonzalez, M. E., Cea, M., Gianfreda, L. and Diez, M. C. (2009). Degradation of polycyclic aromatic hydrocarbons by free and nanoclay-immobilized manganese peroxidase from *Anthracophyllum discolor*. *Chemosphere* **80**(3): 271-278.
- Adamson J. K., Rowland A. P. and Scott W. A. (1996). Changes in soil acidity and related variables over 25 years in the north Pennine Uplands. UK. *Soil Use Management* **12**(2):55-61
- Al-Turki, A. I. (2009). Microbial polycyclic aromatic hydrocarbons degradation in soil. *Research Journal of Environmental Toxicology* **3**: 1-8.
- Alef, K. and Kleiner D. (1987). Applicability of Arginine Ammonification as Indicator of Microbial Activity in Different Soils. *Biology and Fertility of Soils* **5**(2): 148-151.
- Alexander, M. (1995). How toxic are toxic chemicals in soil? *Environmental Science Technology* **29**(11): 2713-2717.
- Alva, V. A. and Peyton, B. M. (2003). Phenol and catechol biodegradation by the haloalkaliphile *Halomonas campisalis*: influence of pH and salinity. *Environmental Science Technology* **37**(19): 4397-4402.

- Andreoni, V. and Gianfreda, L. (2007). Bioremediation and monitoring of aromatic-polluted habitats. *Applied Microbiology Biotechnology* **76**(2): 287-308.
- Andrews, E. J. and Novak, P. J. (2001). Influence of ferrous iron and pH on carbon tetrachloride degradation by *Methanosarcina thermophila*. *Water Resources* **35**(9): 2307-2313.
- Annweiler, E., Richnow, H. H., Antranikian, G., Hebenbrock, S., Garms, C., Franke, S., Francke, W. and Michaelis, W. (2000). Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophile *Bacillus thermoleovorans*. *Applied Environmental Microbiology* **66**(2): 518-523.
- Anzai, Y., Kim, H., Park, J. Y., Wakabayashi, H. and Oyaizu, H. (2000). Phylogenetic affiliation of the pseudomonads based on 16S rRNA sequence. *International Journal Systematic Evolutionary Microbiology* **50** (4): 1563-1589.
- Aramandla, R., Walker, S. A., Hood, D. B., Guillen, M. D., Schneider, K. and Weyand, E. H. (2004). Bioavailability and risk assessment of orally digested polycyclic aromatic hydrocarbons. *International Journal Toxicology* **23**(5): 301-333.
- Atagana, H. I. (2009). Biodegradation of PAHs by fungi in contaminated-soil containing cadmium and nickel ions. *African Journal of Biotechnology* **8**(21): 5780-5789.
- Atlas, R. M. (1991). Bioremediation of fossil fuel contaminated soils. *In situ Bioreclamation*: 14-32.
- Awasthi, N., Ahuja, R. and Kumar, A. (2000). Factors influencing the degradation of soil-applied endosulfan isomers. *Soil Biology & Biochemistry* **32**(11-12): 1697-1705.
- Baborova, P., Moder, M., Baldrian, P., Cajthamlova, K. and Cajthaml, T. (2006). Purification of a new manganese peroxidase of the white-rot fungus *Irpex lacteus*, and degradation of polycyclic aromatic hydrocarbons by the enzyme. *Research Microbiology* **157**(3): 248-253.
- Balba, A. M., Elshibiny, G. and Elkhatib, E. S. (1991). Effect of Lead Increments on the Yield and Lead Content of Tomato Plants. *Water Air and Soil Pollution* **57-8**: 93-99.
- Baldrian, P., Der Wiesche, C., Gabriel, J, Nerud, F. and Zadrazil, F. (2000). Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic

aromatic hydrocarbons by *Pleurotus ostreatus* in soil. *Applied Environmental Microbiology* **66**(6): 2471-2478.

Bamforth, S. M. and Singleton, I. (2005a). Naphthalene transformation by the *Pseudomonas* at an elevated pH. *Journal of Chemical Technology & Biotechnology* **80**(7).

Bamforth, S. M. and Singleton, I. (2005b). Bioremediation of polycyclic aromatic hydrocarbons: current knowledge and future directions. *Journal of Chemical Technology and Biotechnology* **80**(7): 723-736.

Barathi, S. and Vasudevan, N. (2001). Utilization of petroleum hydrocarbons by *Pseudomonas fluorescens* isolated from a petroleum-contaminated soil. *Environmental International* **26**(5-6): 413-416.

Barr, D. P. and Aust, S. D. (1994). Mechanisms white rot fungi use to degrade pollutants. *Environmental Science Technology* (28): 78A-87A.

Bastiaens, L., Springael, D., Wattiau, P., Harms, H., DeWachter, R., Verachtert, H. and Diels, L. (2000). Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. *Applied Environmental Microbiology* **66**(5): 1834-1843.

Bauchop, T. and Elsdon, S. R. (1960). The growth of micro-organisms in relation to their energy supply. *Journal General Microbiology* **23**: 457-469.

Beller, H. R. and Spormann, A. M. (1997). Anaerobic activation of toluene and o-xylene by addition to fumarate in denitrifying strain T. *Journal Bacteriology* **179**(3): 670-676.

Beulke, S., Van Beinum, W., Brown, C. D., Mitchell, M. and Walker, A. (2005). Evaluation of simplifying assumptions on pesticide degradation in soil. *Journal of Environmental Quality* **34**(6): 1933-1943.

Bishnoi, K., Kumar, R. and Bishnoi, N. R. (2008). Biodegradation of polycyclic aromatic hydrocarbons by white rot fungi *Phanerochaete chrysosporium* in sterile and unsterile soil. *Journal of Scientific & Industrial Research* **67**(7): 538-542.

- Bogan, B. W. and Lamar, R. T. (1996). Polycyclic aromatic hydrocarbon-degrading capabilities of *Phanerochaete laevis* HHB-1625 and its extracellular ligninolytic enzymes. *Applied Environmental Microbiology* **62**(5): 1597-1603.
- Bogan, B., Schoenike, B., Lamar, R. T. and Cullen, D. (1996). Expression of lip genes during growth in soil and oxidation of anthracene by *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **62**(10): 3697-3703.
- Bollag J. M. and Liu S.Y. (1990). Biological transformation processes of pesticides. In H.H. Cheng (ed.) Pesticides in the environment: process, impact and modelling. *Soil Science Society of America*, Madison, Wisconsin, 169-185.
- Bonde, T. A., Nielsen, T. H., Miller, M. and Sorensen, J. (2001). Arginine ammonification assay as a rapid index of gross N mineralization in agricultural soils. *Biology and Fertility of Soils* **34**(3): 179-184.
- Bonomo, R. P., Cennamo, G., Purrello, R., Santoro, A. M. and Zappala, R. (2001). Comparison of three fungal laccases from *Rigidoporus lignosus* and *Pleurotus ostreatus*: correlation between conformation changes and catalytic activity. *Journal Inorganic Biochemistry* **83**(1): 67-75.
- Boonchan, S., Britz, M. L. and Stanley, G. A. (2000). Degradation and mineralization of high-molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. *Applied and Environmental Microbiology* **66**(3): 1007-1019.
- Borazjani, H., Diehl, S. V. and Stewart, H. A. (1997). Production of compost from furniture manufacturing woodwastes. *Forest Products Journal* **47**(2): 47-48.
- Borazjani, R. N., May, L. L., Noble, J. A., Avery, S. V. and Ahearn, D. G. (2000). Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against *Acanthamoeba* spp. *Applied and Environmental Microbiology* **66**(3): 1057-1061.
- Borneman, J. and Hartin, R. J. (2000). PCR primers that amplify fungal rRNA genes from environmental samples. *Applied and Environmental Microbiology* **66**(10): 4356-4360.
- Bosma, T., Middeldrop, P. M., Schraa, G. and Zender, A. B. (1997). Mass transfer limitation of biotransformation: quantifying bioavailability. *Environmental Science & Technology* (31): 248-252.

- Bossert, I., Kachel, W. M. and Bartha, R. (1984). Fate of hydrocarbons during oily sludge disposal in soil. *Applied Environmental Microbiology* **47**(4): 763-767.
- Boyle, D., Wiesner, C. and Richardson, A. (1998). Factors affecting the degradation of polyaromatic hydrocarbons in soil by white-rot fungi. *Soil Biology & Biochemistry* **30**(7): 873-882.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**: 248-254.
- Brady, N. C. and Weil R. R. (2002). The nature and properties of soils. *Upper Saddle River, N.J.*, Prentice Hall.
- Brandli, R. C., Hartnik, T., Henriksen, T. and Cornelissen, G. (2008). Sorption of native polyaromatic hydrocarbons (PAH) to black carbon and amended activated carbon in soil. *Chemosphere* **73**(11): 1805-1810.
- Broderick, J. B. and O'Halloran T. V. (1991). Overproduction, purification, and characterization of chlorocatechol dioxygenase, a non-heme iron dioxygenase with broad substrate tolerance. *Biochemistry* **30**(29): 7349-7358.
- Brookes, P. C., Tate, K. R. and Jenkinson, D. S. (1983). The Adenylate Energy-Charge of the Soil Microbial Biomass. *Soil Biology & Biochemistry* **15**(1): 9-16.
- Bumpus, J. A., Tien, M., Wright, D. and Aust, S. D. (1985). Oxidation of persistent environmental pollutants by a white rot fungus. *Science* **228**(4706): 1434-1436.
- Cajthaml, T., (2008). Degradation of PAHs by ligninolytic enzymes of *Irpex lacteus*. *Folia Microbiologica* **53**(4): 289-294.
- Cajthaml, T., Moder, M., Kacer, P., Sasek, V. and Popp, P. (2002). Study of fungal degradation products of polycyclic aromatic hydrocarbons using gas chromatography with ion trap mass spectrometry detection. *Journal Chromatography A* **974**(1-2): 213-222.

Cameron, M. D. and Aust S. D. (1999). Degradation of chemicals by reactive radicals produced by cellobiose dehydrogenase from *Phanerochaete chrysosporium*. *Archives Biochemistry Biophysics* **367**(1): 115-121.

Campbell, N. and Reece, J. (2005). *Biology* 7th edition, AP. San Francisco, CA: Pearson, Education Inc.

Cassago, A., Panepucci, R., Baiao, A. and Henrique-Silva, F. (2002). Cellophane based mini-prep method for DNA extraction from the filamentous fungus *Trichoderma reesei*. *BMC Microbiology* **2**: 14.

Castillo, M. P., Stenstrom, J. and Ander, P. (1994). Determination of manganese peroxidase activity with 3-methyl-2-benzothiazolinone hydrazone and 3-(dimethylamino)benzoic acid. *Analytical Biochemistry* **218**(2): 399-404.

Cavaliere, E. and Rogan E. (1985). Role of radical cations in aromatic hydrocarbon carcinogenesis. *Environmental Health Perspectives* **64**: 69-84.

Cerniglia, C. E. (1984). Microbial metabolism of polycyclic aromatic hydrocarbons. *Advance Applied Microbiology* **30**: 31-71.

Cerniglia, C. E. (1990). Biodegradation of Organic Contaminants - Overview and Examples with Polycyclic Aromatic-Hydrocarbons. *Abstracts of Papers of the American Chemical Society* **199**: 136-ENVR.

Cerniglia, C. E. (1992). Biodegradation of polycyclic aromatic hydrocarbons. *Springer. Advance Applied Microbiology* **3**: 351-368.

Cerniglia, C. E. and Crow S. A. (1981). Metabolism of aromatic hydrocarbons by yeasts. *Archives Microbiology* (129): 9-13.

Chang, B. V., Shiung, L. C. and Yuan, S. Y. (2002). Anaerobic biodegradation of polycyclic aromatic hydrocarbon in soil. *Chemosphere* **48**(7): 717-724.

Chefetz, B., Chen, Y. and Hadar, Y. (1998). Purification and characterization of laccase from *Chaetomium thermophilum* and its role in humification. *Applied Environmental Microbiology* **64**(9): 3175-3179.

Cheng, H. R. and Jiang N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letter* **28**(1): 55-59.

Chesworth, W. (Eds.), (2008). Encyclopedia of soil science. *Springer* The Netherlands.

Chun, L. W., Zhao, M., Lei, L., Xing, D. W. and Tailun L. (2011). Characterization of spore lacase from *Bacillus subtilis* WD₂₃ and its use in dye decolorisation. *African Journal of biotechnolgy* **10**(11): 2186-2192.

Chung, T. P., Tseng, H. Y. and Jung, R. S. (2003). Mass transfer effect and intermediate detection for phenol degradation in immobilized *Psuedomonas putida* systems. *Process Biochemistry* (38): 1497-1507.

Church, T. W. (1993). Cleaning up the mess: Implementation strategies in superfund. *Brookings Institution Press*.

Coates, J. D., Michaelidou, U., Bruce, R. A., O'Connor, S. M., Crespi, J. N. and Chenbach, L. A. (1999). Ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria. *Applied Environmental Microbiology* **65**(12): 5234-5241.

Collins, P. J., Kotterman, M., Field, J. A. and Dobson, A. (1996). Oxidation of Anthracene and Benzo[a]pyrene by Laccases from *Trametes versicolor*. *Applied Environmental Microbiology* **62**(12): 4563-4567.

Conant, R. T., Paustian, K. and Elliott, E. T. (2001). Grassland management and conversion into grassland: Effects on soil carbon. *Ecological Applications* **11**(2): 343-355.

Contin, M., Corcimar, S., De Nobili, M. and Brookes, P. C. (2000). Temperature changes and the ATP concentration of the soil microbial biomass. *Soil Biology & Biochemistry* **32**(8-9): 1219-1225.

Crawford, D. L. and Crawford, R. L. (1976). Microbial degradation of lignocellulose: the lignin component. *Applied Environmental Microbiology* **31**(5): 714-717.

Cutright, T. J. and Hwang, S. (2006). Polycyclic Aromatic Hydrocarbons (PAHs). *Encyclopedia of Chemical Processing*: 2291-2299.

D'Annibale, A., Rosetto, F., Leonardi, V., Federici, F. and Petruccioli, M. (2006). Role of autochthonous filamentous fungi in bioremediation of a soil historically contaminated with aromatic hydrocarbons. *Applied and Environmental Microbiology* **72**(1): 28-36.

Daane, L. L., Harjono, I., Zylstra, G. J. and Haggblom, M. M. (2001). Isolation and characterization of polycyclic aromatic hydrocarbon-degrading bacteria associated with the rhizosphere of salt marsh plants. *Applied Environmental Microbiology* **67**(6): 2683-2691.

Dagley, S. and Gibson, D. T. (1965). The Bacterial Degradation of Catechol. *Biochemistry Journal* **95**: 466-474.

Das, S., Muneer, M. and Gopidas, K.R. (1994). Photocatalytic degradation of wastewater pollutants. Titanium-dioxidemediated oxidation of polynuclear aromatic hydrocarbons. *Journal Photochemistry Photobiology A: Chemistry*: 77-83.

Daubaras, D. L., Saido, K. and Chakrabarty, A. M. (1996). Purification of hydroxyquinol 1,2-dioxygenase and maleylacetate reductase: the lower pathway of 2,4,5-trichlorophenoxyacetic acid metabolism by *Burkholderia cepacia* AC1100. *Applied Environmental Microbiology* **62**(11): 4276-4279.

Davies, J. I. and Evans, W. C. (1964). Oxidative metabolism of naphthalene by soil pseudomonads. The ring-fission mechanism. *Biochemistry Journal* **91**(2): 251-261.

Davis, J. S. and Weestlake, D. S. (1979). Crude oil utilization by fungi. *Canadian Journal of Microbiology* (25): 146-156.

Dellaporta, S., Wood, J. and Hicks, J. B. (1983). A plant DNA mini-preparation: version II. *Plant Molecular Biology* (1): 19-21.

Denome, S. A., Stanley, D. C., Olson, E. S. and Young, K. D. (1993). Metabolism of dibenzothiophene and naphthalene in *Pseudomonas* strains: complete DNA sequence of an upper naphthalene catabolic pathway. *Journal Bacteriology* **175**(21): 6890-6901.

Dick, W. A. and Tabatabai, M. A. (1984). Kinetic parameters of phosphatase in soils and organic waste materials. *Soil Science* (137): 7-15.

Dick, W. A. and Tabatabai, M. A. (1992). Significance and potential uses of soil enzymes. *Soil microbiology ecology*: 95-127.

Dix, N. J. & Webster, J. (1995). Fungal Ecology. *Chapman Hall*: 179-183.

Do, N. and Adams, R. P. (1991). A simple technique for removing plant polysaccharide contaminants from DNA. *Biotechniques* **10**(2): 162, 164, 166.

Eggert, C., Temp, U. and Eriksson, K. E. (1996). The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: purification and characterization of the laccase. *Applied Environmental Microbiology* **62**(4): 1151-1158.

Eiland, F. (1983). A simple method for quantitative determination of ATP in soil. *Soil Biology & Biochemistry* **15**: 665-670.

Eriksson, M., E., Sodersten, E., Yu, Z., Dalhammar, G. and Mohn, W. W. (2003). Degradation of polycyclic aromatic hydrocarbons at low temperature under aerobic and nitrate-reducing conditions in enrichment cultures from northern soils. *Applied Environmental Microbiology* **69**(1): 275-284.

ESTCP (1999). Technology Status Review: In situ Oxidation. *ESTCP*: 1-42.

Evans, W. C., Fernley, H. N. and Griffiths, E. (1965). Oxidative Metabolism of Phenanthrene and Anthracene by Soil Pseudomonads. the Ring-Fission Mechanism. *Biochemistry Journal* **95**: 819-831.

Fahnestock, F. M., G. B. Wickramanavake, et al. (1998). Biopile Design, Operation, and Maintenance Handbook for Treating Hydrocarbon Contaminated Soil. *Battelle Press*.

Faust and Rosmarie, A. (1993). Oak Ridge National Laboratory, Chemical Hazard Evaluation Group. *Toxicity Summary for Phenanthrene*.

Ferrarese, E., Andreottola, G., and Oprea, I. A. (2008). Remediation of PAH-contaminated sediments by chemical oxidation. *Journal Hazard Materials* **152**(1): 128-139.

Fewson, C. A. (1988). Biodegradation of Xenobiotic and Other Persistent Compounds - the Causes of Recalcitrance. *Trends in Biotechnology* **6**(7): 148-153.

Fiedler, L. (2000). Engineered Approaches to In Situ Bioremediation of Chlorinated Solvents: Fundamentals and Field Applications. *Stroming media: report*.

Field, J. A., Boelsma, F., Baten, H. and Rulkens, W. H. (1995). Oxidation of anthracene in water/solvent mixtures by the white-rot fungus, *Bjerkandera* sp. strain BOS55. *Applied Microbiology Biotechnology* **44**: 234-240.

Funk, G. D., Johnson, S. M., Smith, J. C., Dong, X. W., Lai, J. and Feldman, J. L. (1997). Functional respiratory rhythm generating networks in neonatal mice lacking NMDAR1 gene. *Journal of Neurophysiology* **78**(3): 1414-1420.

Gao, J. P., Maguhn, J., Spitzauer, P. and Kettrup, A. (1998). Sorption of pesticides in the sediment of the Teufelsweiher Pond (Southern Germany). I: Equilibrium assessments, effect of organic carbon content and pH. *Water Research* **32**(5): 1662-1672.

Genthner, B. R. S., Townsend, G. T., Lantz, S. E. and Mueller, J. G. (1997). Persistence of polycyclic aromatic hydrocarbon components of creosote under anaerobic enrichment conditions. *Archives of Environmental Contamination and Toxicology* **32**(1): 99-105.

George-Okafor, U., Tasié, F. and Muotoe-Okafor, F. (2009). Hydrocarbon Degradation Potentials of Indigenous Fungal Isolates from Petroleum Contaminated Soils. *Journal of Physical and Natural Sciences* **3**(1).

Ghazali, F. M., Rahman, R., Salleh, A. and Basri, M. (2004). Biodegradation of hydrocarbons in soil by microbial consortium. *International Biodeterioration & Biodegradation* **54**(1): 61-67.

Gianfreda, L., Luz Mora, M. and Cristina, M. (2005). Restoration of polluted soils by means of microbial and enzymatic processes. *Review article*: 20-40.

Gianfreda, L. and Rao, M. A. (2004). Potential of extra cellular enzymes in remediation of polluted soils: a review. *Enzyme and Microbial Technology* **35**(4): 339-354.

Gibson, D. T., Koch, J. R. and Kallio, R. E. (1968). Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. *Biochemistry* **7**(7): 2653-2662.

Gibson, D. T. and Subramanian V. (1984). Microbial degradation of aromatic hydrocarbons. *Molecular degradation of organic compounds*: 181-252.

Glenn, J. K., Morgan, M. A., Mayfield, M. B., Kuwahara, M. and Gold, M. H. (1983). An extracellular H₂O₂-requiring enzyme preparation involved in lignin biodegradation by the white rot basidiomycete *Phanerochaete chrysosporium*. *Biochemistry Biophysics Research Communication* **114**(3): 1077-1083.

Gogoi, B. K., Dutta, N. N., Goswami, P. and Mohan, T. K. (2003). A case study of bioremediation of petroleum-hydrocarbon contaminated soil at a crude oil spill site. *Advances in Environmental Research* **7**(4): 767-782.

Guerin, W. F. and Jones, G. E. (1989). Estuarine ecology of phenanthrene- degrading bacteria. *Estuarine Coastal Shelf Science* **29**: 115-130.

Haapea, P. and Tuhkanen, T. (2006). Integrated treatment of PAH contaminated soil by soil washing, ozonation and biological treatment. *Journal of Hazardous Materials* **136**(2): 244-250.

Hadibarata, T. and Tachibana, S. (2009). Microbial Degradation of Crude Oil by Fungi Pre-Grown on Wood Meal. *Interdisciplinary Studies on Environmental Chemistry* **2**: 317-322.

Haemmerli, S. D., Leisola, M. and Fiechter, A. (1986). Polymerization of lignins by ligninases from *Phanerochaete chrysosporium*. *FEMS Microbiology Letter* (35): 33-36.

Hammel, K. E., Gai, W. Z., Green, B. and Moen, M. A. (1992). Oxidative degradation of phenanthrene by the ligninolytic fungus *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* **58**(6): 1832-1838.

Han, M. J., Choi, H. T. and Song, H. G. (2005). Purification and characterization of laccase from the white rot fungus *Trametes versicolor*. *Journal of Microbiology* **43**(6): 555-560.

Haritash, A. K. and Kaushik, C. P. (2009). Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials* **169**(1-3): 1-15.

Harvey, R. G. (1996). Mechanisms of carcinogenesis of polycyclic aromatic hydrocarbons. *Polycyclic Aromatic Compounds* **9**(1-4): 1-23.

Hati, S. S., Dimari, G. A., Egwu, G.O. and Ogugbuaja, V. O. (2009). Polycyclic aromatic hydrocarbons (PAHs) contamination of synthetic industrial essential oils utilized in Northern Nigeria. *African Journal of Pure and Applied Chemistry* **3**(5): 086-091.

Hatzinger, P. B. and Alexander, M. (1995). Effect of Aging of Chemicals in Soil on Their Biodegradability and Extractability. *Environmental Science & Technology* **29**(2): 537-545.

Heitkamp, M. A. and Cerniglia, C. E. (1988). Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. *Applied Environmental Microbiology* **54**(6): 1612-1614.

Hesham, A. L., Alamri, S. A., Khan, S., Mahmoud, M. E. and Mahmoud, H. M. (2009). Isolation and molecular genetic characterization of a yeast strain able to degrade petroleum polycyclic aromatic hydrocarbons. *African Journal of Biotechnology* **8**(10): 2218-2223.

Hilyard, E. J., Jones-Meehan, J. M., Spargo, B. J. and Hill, R. T. (2008). Enrichment, isolation, and phylogenetic identification of polycyclic aromatic hydrocarbon-degrading bacteria from Elizabeth River sediments. *Applied Environmental Microbiology* **74**(4): 1176-1182.

Ho, Y., Jackson, M. M., Yang, Y., Mueller J. G. and Pritchard, P. H. (2000). Characterization of fluoranthene- and pyrene-degrading bacteria isolated from PAH-contaminated soils and sediments and comparison of several *Sphingomonas* spp. *Journal of Industrial Microbiology and Biotechnology* **24**: 100-112.

Hoffman, M. R., Martin, S. T., Choi, W. and Bahnemann D. W. (1995). Environmental application of semiconductor photocatalysis. *Chemical Reviews* **95**: 69-96.

Hwang, S. and Cutright, T. J. (2004). Evidence of underestimation in PAH sorption/desorption due to system nonequilibrium and interaction with soil constituents. *Journal of Environmental Science Health And Toxic Hazard Substance Environmental Engineering* **39**(5): 1147-1162.

Ilori, M. O. and Amund, D. I. (2000). Degradation of anthracene by bacteria isolated from oil polluted tropical soils. *Journal of Biosciences* **55**(11-12): 890-897.

Irwin, R. J., Vanmouwerik, M., Stevens, M., Seese, L. and Basham, N. D. (1997). Environmental contaminants encyclopedia. National Park Service, *Water Resources Division*, Fort Collins, Colorado.

Iwabuchi, T. and Harayama S. (1997). Biochemical and genetic characterization of 2-carboxybenzaldehyde dehydrogenase, an enzyme involved in phenanthrene degradation by *Nocardioides* sp. strain KP7. *Journal of Bacteriology* **179**(20): 6488-6494.

Jackson, H. L. and Mckckusick B. C. (1955). Organic syntheses. *John Wiley and Sons* **56**: 62.

Jacques, R. J., Okeke, B. C., Bento, F. M., Peralba, M. C. and Camargo, F. A. (2009). Improved enrichment and isolation of polycyclic aromatic hydrocarbons (PAH)-degrading microorganisms in soil using anthracene as a model PAH. *Current Microbiology* **58**(6): 628-634.

Jain, M. A., Tiwari, V., Guleria, R.S. and Verma, R.K. (2002). Qualitative Evaluation of Mycobacterial DNA extraction protocols for polymerase chain reaction. *Molecular biology Today* (3): 43-50.

Jenkinson, D. S. and Oades, J. M. (1979). A method for measuring adenosine triphosphate in soil. *Soil Biology & Biochemistry* **11**: 193-199.

Jerina, D. M., Selander, H., Yagi, H., Wells, M. C., Davey, J. F., Mahadevan, V. and Gibson, D. T. (1976). Dihydrodiols from Anthracene and Phenanthrene. *Journal of the American Chemical Society* **98**(19): 5988-5996.

Johnsen, A. R., Wick, L. Y. and Harms, H. (2005). Principles of microbial PAH-degradation in soil. *Environmental Pollution* **133**(1): 71-84.

Jones, K. C., Stratford, J. A., Tidridge, P., Waterhouse, K. S. and Johnston, A. E. (1989). Polynuclear aromatic hydrocarbons in an agricultural soil: long-term changes in profile distribution. *Environmental Pollution* **56**(4): 337-351.

Jonsson, S., Persson, Y., Frankki, S., van Bavel, B., Lundstedt, S., Haglund, P. and Tysklind, M. (2007). Degradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soils by Fenton's reagent: A multivariate evaluation of the importance of soil characteristics and PAH properties. *Journal of Hazardous Materials* **149**(1): 86-96.

Jordaan, J. and Leukes, W. D. (2003). Isolation of a thermostable laccase with DMAB and MBTH oxidative coupling activity from a mesophilic white rot fungus. *Enzyme and Microbial Technology* **33**(2-3): 212-219.

Juhasz, A. L. and Naidu, R. (2000). Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *International Biodeterioration & Biodegradation* **45**(1-2): 57-88.

Kalmis, E., I. Yasa, et al. (2008). Ligninolytic enzyme activities in mycelium of some wild and commercial mushrooms. *African Journal of Biotechnology* **7**(23): 4314-4320.

Kamal, S., K. M. Ajay, et al. (2008). A simple and efficient method for extraction of genomic DNA from tropical tuber crop. *African Journal of biotechnology* **8**: 1018-1022.

Kanally, R. A. and Harayama, S. (2000). Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *Journal of Bacteriology* **182**(8): 2059-2067.

Kastner, M., Breuer-Jammali, M. and Mahro, B. (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied and Environmental Microbiology* **64**(1): 359-362.

Kastner, M., Breuer-Jammali, M. and Mahro, B. (1998). Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Applied Environmental Microbiology* **64**(1): 359-362.

Keck, J., Sims, R. C. and Coover, M. (1989). Evidence for cooxidation of polynuclear aromatic hydrocarbons in soil. *Water Resources* (23): 1467-1477.

Keith, L. H. and Telliard, W. A. (1979). Priority Pollutants I-a Perspective View. *Environmental Science & Technology* **13**(4): 416-423.

Kelley, I., Freeman, J. P. and Cerniglia, C. E. (1990). Identification of metabolites from degradation of naphthalene by a *Mycobacterium* sp. *Biodegradation* **1**(4): 283-290.

Khan, F. I., Husain, T. and Hejazi, R. (2004). An overview and analysis of site remediation technologies. *Journal of Environmental Management* **71**(2): 95-122.

Kim, Y. H., Freeman, J. P., Moody, J. D., Engesser, K. H. and Cerniglia, C. E. (2005). Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbaalenii* PYR-1. *Applied Microbiology and Biotechnology* **67**(2): 275-285.

Kirk, T. K. and Farrell, R. L. (1987). Enzymatic "combustion": the microbial degradation of lignin. *Annual Reviews Microbiology* **41**: 465-505.

Kiyohara, H., Sugiyama, M., Mondello, F. J., Gibson, D. T. and Yano, K. (1983). Plasmid involvement in the degradation of polycyclic aromatic hydrocarbons by a *Beijerinckia* species. *Biochemistry Biophysics Resources Communication* **111**(3): 939-945.

Kluck, C. and G. Achari (2004). Chemical oxidation techniques for *in situ* remediation of hydrocarbon impacted soils. *Environmental Engineering Case study*.

Kot-Wasik D., Dabrowska D. and Namiesnik J. (2004). Photodegradation and biodegradation study of benzo[a]pyrene in different liquid media. *Journal of Photochemistry Photobiology A: Chemistry* **168**: 109-115.

Kou, J., Li, Z., Yuan, Y., Zhang, H., Wang, Y. and Zou, Z. (2009). Visible-light-Induced photocatalytic oxidation of polycyclic aromatic hydrocarbons over tantalum oxynitride photocatalysts. *Environmental Science Technology* **43**(8): 2919-2924.

Kourtev, P., Enrenfield K. J. and Huang W. (2002). Enzyme activities during litter decomposition of two exotic and two native plant species in hard wood plants of new jersey. *Soil Biology and Biochemistry* (34): 1207-1218.

Krafft, M. E. and Crooks. III W. J. (1988). Saturated and unsaturated hydrocarbons; Review article. *Journal of Organic Chemistry* **53**: 3158-3163.

Kuiper, I., Lagendijk, E. L., Bloemberg, G. V. and Lugtenberg, B. J. (2004). Rhizoremediation: a beneficial plant-microbe interaction. *Molecular Plant Microbe Interaction* **17**(1): 6-15.

Kumar, V. and Kothiyal, N. C. (2011). Distribution behavior and carcinogenic level of some polycyclic aromatic hydrocarbons in roadside soil at major traffic intercepts within a developing city of India. *Environmental Monitoring Assessment* **29**(6): 104-106.

Kurata, S., Kanagawa, T., Magariyama, Y., Takatsu, K., Yamada, K., Yokomaku, T. and Kamagata, Y. (2004). Reevaluation and reduction of a PCR bias caused by reannealing of templates. *Applied Environmental Microbiology* **70**(12): 7545-7549.

LaFlamme, R. E. and Hites, R. A. (1978). The global distribution of polycyclic aromatic hydrocarbons in recent sediments. *Geochimica et Cosmochimica Acta* **42**: 289-303.

Lal, B. and Khanna, S. (1996). Degradation of crude oil by *Acinetobacter calcoaceticus* and *Alcaligenes odorans*. *Journal of Applied Bacteriology* **81**(4): 355-362.

Larson, W. E. and Pierce, F. J. (1991). Conservation and enhancement of soil quality. In: Evaluation for Sustainable Land Management in the Developing World. *IBSRAM Proceedings* **12**(2): 175-203.

Latus, M., Seitz, H., Eberspacher, J. and Lingens, F. (1995). Purification and Characterization of Hydroxyquinol 1,2-Dioxygenase from *Azotobacter* sp. Strain GP1. *Applied Environmental Microbiology* **61**(7): 2453-2460.

Leahy, J. G. and Colwell, R. R. (1990). Microbial-Degradation of Hydrocarbons in the Environment. *Microbiological Reviews* **54**(3): 305-315.

Lease, C., Bentham, R., Gaskin, S. and Juhasz, A. (2011). Isolation and Identification of PyreneMineralizing *Mycobacterium* spp. from Contaminated and Uncontaminated Sources. *Applied and Environmental Soil Science* **2011**: 11.

Lee, B. D. and Hosomi, M. (2001). Fenton oxidation of ethanol-washed distillation-concentrated benzo(a)pyrene: reaction product identification and biodegradability. *Water Resources* **35**(9): 2314-2319.

Lee, K., Park, J. W. and Ahn, I. S. (2003). Effect of additional carbon source on naphthalene biodegradation by *Pseudomonas putida* G7. *Journal of Hazardous Materials* **105**(1-3): 157-167.

Levin, L. A. and Forchiassin A. (2003). Degradation of organic pollutants by the white rot basidiomycete *Trametes trogii*. *International Biodeterioration Biodegradation* **52**: 1-5.

Lifongo, L. and Nfon, E. (2009). Evaluating the fate of organic compounds in the Cameroon environment using a level 111 multimedia fugacity model. *African Journal of Environmental Science & Technology* **3**(11): 376-386.

Liu, W. W., Yin, R., Lin, X. G., Zhang, J., Chen, X. M., Li, X. Z. and Yang, T. (2010). Interaction of biosurfactant-microorganism to enhance phytoremediation of aged polycyclic aromatic hydrocarbons (PAHS) contaminated soils with alfalfa (*Medicago sativa* L.). *Huan Jing Ke Xue* **31**(4): 1079-1084.

Loeffler, J., Henke, N., Hebart, H., Schmidt, D., Hagemeyer, L., Schumacher, U. and Einsele, H. (2000). Quantification of fungal DNA by using fluorescence resonance energy transfer and the Light Cycler system. *Journal of Clinical Microbiology* **38**(2): 586-590.

Longwell, J. (2007). The Formation of polycyclic aromatic hydrocarbons by combustion. *International Journal of toxicology* **19**(1): 1339-1350.

MacGillivray, A. R. and Shiaris, M. P. (1993). Biotransformation of polycyclic aromatic hydrocarbons by yeasts isolated from coastal sediments. *Applied Environmental Microbiology* **59**(5): 1613-1618.

Mahfeey, W. R., Gibson, D. T. and Cerniglia, C. E. (1988). Bacterial oxidation of chemical carcinogens: formation of polycyclic aromatic acids from benz[a]anthracene. *Applied and Environmental Microbiology* **54**: 2415-2423.

Makkar, R. S. and Rockne, K. J. (2003). Comparison of synthetic surfactants and biosurfactants in enhancing biodegradation of polycyclic aromatic hydrocarbons. *Environmental Toxicology and Chemistry* **22**(10): 2280-2292.

Makoi, J. H. and Ndakidemi, P. A. (2008). Selected soil enzymes: Examples of their potential roles in the ecosystem. *African Journal of Biotechnology* **7**(3): 181-191.

Mallakin, A., Dixon, D. G. and Greenberg, B. M. (2000). Pathway of anthracene modification under simulated solar radiation. *Chemosphere* **40**(12): 1435-1441.

Margesin, R. and Schinner, F. (2001). Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology* **56**(5-6): 650-663.

- Martens, D. A. (1995). Enhanced degradation of polycyclic aromatic hydrocarbons in soil treated with an advanced oxidative process--Fenton's reagent. *Journal of Soil Contamination* **4**(2): 175-190.
- Masten, S. J. and Davies, S. R. (1997). Efficacy of in-situ ozonation for the remediation of PAH contaminated soils. *Journal of Contaminant Hydrology* **28**(4): 327-335.
- Mayer, A. M. and Staples, R. C. (2002). Laccase: new functions for an old enzyme. *Phytochemistry* **60**(6): 551-565.
- McDonnell, G. and Russell, A. D. (1999). Antiseptics and disinfectants: activity, action, and resistance. *Clinical Microbiology Reviews* **12**(1): 147-179.
- McNally, D. L., Mihelcic, J. R. and Lueking, D. R. (1998). Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions. *Environmental Science & Technology* **32**(17): 2633-2639.
- Mester, T. and Field, J. A. (1998). Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by *Bjerkandera* species strain BOS55 in the absence of manganese. *Journal Biology Chemistry* **273**(25): 15412-15417.
- Mihelcic, J. R. and Luthy, R. G. (1988). Degradation of Polycyclic Aromatic Hydrocarbon Compounds under Various Redox Conditions in Soil-Water Systems. *Applied and Environmental Microbiology* **54**(5): 1182-1187.
- Mirjana, K. and Licina, V. (2002). The determination of potential ammonification in soil by arginine method. *Journal of Agricultural Sciences* **47**(2): 129-135.
- Mobley, D. M., Chengappa, M. M., Kadel, W. L. and Stuart, J. G. (1984). Effect of pH, Temperature and Media on Acid and Alkaline-Phosphatase Activity in Clinical and Nonclinical Isolates of *Bordetella-Bronchiseptica*. *Canadian Journal of Comparative Medicine-Revue Canadienne De Medecine Comparee* **48**(2): 175-178.
- Moenne-Loccoz, Y., Tichy, H., O'Donnell, A., Simon, R. and O'Gara, F. (2001). Impact of 2,4-diacetylphloroglucinol-producing biocontrol strain *Pseudomonas fluorescens* F113 on intraspecific diversity of resident culturable fluorescent pseudomonads associated with the roots of field-grown sugar beet seedlings. *Applied Environmental Microbiology* **67**(8): 3418-3425.

Mollea, C., Bosco, F. and Ruggeri, B. (2005). Fungal biodegradation of naphthalene: microcosms studies. *Chemosphere* **60**(5): 636-643.

Moody, J. D., Freeman, J. P., Doerge, D. R. and Cerniglia, C. E. (2001). Degradation of phenanthrene and anthracene by cell suspensions of *Mycobacterium* sp. strain PYR-1. *Applied Environmental Microbiology* **67**(4): 1476-1483.

More, S. S. and Malini, S. (2011). Isolation, Purification, and Characterization of Fungal Laccase from *Pleurotus* sp. *Enzyme Resources*: 248735.

Morgan, P., Lewis, S. T. and Watkinson R. J. (1991). Comparison of abilities of white-rot fungus to mineralise selective xenobiotic compounds. *Applied Microbiology and Biotechnology* (34): 693-696.

Morrison, R. T., Boyd, R. N. and Boyd, R. K. (1973). Organic Chemistry. *Organic Chemistry 6th Edition*: 196-204.

Mrozik, A., Piotrowska-Seget, Z. and Labuzek, S. (2003). Bacterial degradation and bioremediation of polycyclic aromatic hydrocarbons. *Polish Journal of Environmental Studies* **12**(1): 15-25.

Muckian, L., Grant, R., Doyle, E. and Clipson, N. (2007). Bacterial community structure in soils contaminated by polycyclic aromatic hydrocarbons. *Chemosphere* **68**(8): 1535-1541.

Mueller, J. G., Devereux, R., Santavy, D. L., Lantz, S. E., Willis, S. G. and Pritchard, P. H. (1997). Phylogenetic and physiological comparisons of PAH-degrading bacteria from geographically diverse soils. *Antonie Van Leeuwenhoek* **71**(4): 329-343.

Mueller, J. G., Lantz, S. E., Blattman, B. O. and Chapman, P. J. (1991). Bench-scale evaluation of alternative biological treatment processes for the remediation of pentachlorophenol and creosote-contaminated material: slurry phase bioremediation. *Environmental Science Technology* (25): 1055-1061.

Murray, M. G. and Thompson, W. F. (1980). Rapid Isolation of High Molecular-Weight Plant DNA. *Nucleic Acids Research* **8**(19): 4321-4325.

- Nadaf, N. H. and Ghosh, J. S. (2011). Purification and characterization of catechol 1, 2-dioxygenase from *Rhodococcus* sp. NCIM 2891. *Research Journal Environmental Earth Science* **3**: 608-613
- Naseby, D. C. and Lynch, J. M. (1997). Rhizosphere soil enzymes as indicators of perturbations caused by enzyme substrate addition and inoculation of a genetically modified strain of *Pseudomonas fluorescens* on wheat seed. *Soil Biology & Biochemistry* **29**(9-10): 1353-1362.
- Naseby, D. C. and Lynch, J. M. (1998). Establishment and impact of *Pseudomonas fluorescens* genetically modified for lactose utilization and kanamycin resistance in the rhizosphere of pea. *Journal of Applied Microbiology* **84**(2): 169-175.
- Neff, J. M. (1985). Polycyclic aromatic hydrocarbons. *Fundamentals of aquatic toxicology*, Taylor and Francis: 416-425.
- Neilson, R., Robinson, D., Marriott, C. A., Scrimgeour, C. M., Hamilton, D., Wishart, J., Boag, B. and Handley, L. L. (2002). Above-ground grazing affects floristic composition and modifies soil trophic interactions. *Soil Biology & Biochemistry* **34**(10): 1507-1512.
- Neumann, B., Pospiech, A. and Schairer, H. U. (1992). Rapid isolation of genomic DNA from gram-negative bacteria. *Trends Genet* **8**(10): 332-333.
- Nielsen, J. D. and Eiland, F. (1980). Investigations on the relationship between P-fertility, phosphatase activity and ATP content in soil. *Plant and Soil* (57): 95-103.
- Novotny, C., Erbanova, P., Sasek, V., Kubatova, A., Cajthaml, T., Lang, E., Krahl, J. and Zadrazil, F. (1999). Extracellular oxidative enzyme production and PAH removal in soil by exploratory mycelium of white rot fungi. *Biodegradation* **10**(3): 159-168.
- Novotny, C., Svobodova, K., Erbanova, P., Cajthaml, T., Kasinath, A., Lang, E. and Sasek, V. (2004). Ligninolytic fungi in bioremediation: extracellular enzyme production and degradation rate. *Soil Biology & Biochemistry* **36**(10): 1545-1551.
- Ogram, A. V., Jessup, R. E., Ou, L. T. and Rao, P. S. (1985). Effects of sorption on biological degradation rates of (2,4-dichlorophenoxy) acetic acid in soils. *Applied Environmental Microbiology* **49**(3): 582-587.

- O'Mahony, M. M., Dobson, A. D., Barnes, J. D. and Singleton, I. (2006). The use of ozone in the remediation of polycyclic aromatic hydrocarbon contaminated soil. *Chemosphere* **63**(2): 307-314.
- Orth, A. B., Denny, M. and Tien, M. (1991). Overproduction of lignin-degrading enzymes by an isolate of *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* **57**(9): 2591-2596.
- Pan, T. T., Feng, Z. N., Lee, S. W., Moore, P. K. and Bian, J. S. (2006). Endogenous hydrogen sulfide contributes to the cardioprotection by metabolic inhibition preconditioning in the rat ventricular myocytes. *Journal of Molecular Cell Cardiology* **40**(1): 119-130.
- Pandey, R. N., Adams, R. P. and Flournoy, L. E. (1996). Inhibitions of random amplified polymorphic DNAs (RAPDs) by plant polysaccharides. *Plant Molecular Biology Reporter* (14): 17-22.
- Parales, R. E., Emig, M. D., Lynch, N. A. and Gibson, D. T. (1998). "Substrate specificities of hybrid naphthalene and 2,4-dinitrotoluene dioxygenase enzyme systems." *Journal of Bacteriology* **180**(9): 2337-2344.
- Paszczyn', A. S., Huynh, V. B. and Crawford, R. (1985). Enzymatic activities of an extracellular, manganese-dependent peroxidase from *Phanerochaete chrysosporium*. *FEMS Microbiology Letters* (29): 37-41.
- Pathak, H., Kantharia, D., Malpani, A. and Madamwar, D. (2009). Naphthalene degradation by *Pseudomonas* sp HOB1: In vitro studies and assessment of naphthalene degradation efficiency in simulated microcosms. *Journal of Hazardous Materials* **166**(2-3): 1466-1473.
- Perry, J. J. (1979). Microbial cooxidations involving hydrocarbons. *Microbiology Revolution* **43**(1): 59-72.
- Peterson, R. R. M. and Bridge, P. D. (1994). Introduction. In: *Biochemical Techniques for Filamentous Fungi*. CAB International (Wallingford, Oxon): 1-4.
- Pointing, S. B. (2001). Feasibility of bioremediation by white-rot fungi. *Applied Microbiology Biotechnology* **57**(1-2): 20-33.

Pothuluri, J. V. and Cerniglia, C. E. (1994). Microbial metabolism of polycyclic aromatic hydrocarbons. *Biological degradation and bioremediation of toxic chemicals*, London: Chapman and Hall: 92-124.

Potin, O., Veignie, E. and Rafin, C. (2004). Biodegradation of polycyclic aromatic hydrocarbons (PAHs) by *Cladosporium sphaerospermum* isolated from an aged PAH contaminated soil. *FEMS Microbiol Ecology* **51**(1): 71-78.

Pritchard, P. H. and Bourquin, A. W. (1985). Microbial toxicity studies In: *Fundamentals of Aquatic Toxicology*, G. M. Rand and S. R. Petrocelli. *Hemisphere Press, N. Y.*: 177-217.

Rahman, K. S., Thahira-Rahman, J., Lakshmanaperumalsamy, P. and Banat, I. (2002). Towards efficient crude oil degradation by a mixed bacterial consortium. *Bioresource Technology* **85**(3): 257-261.

Reddy, C. A. (1995). The potential for white-rot fungi in the treatment of pollutants. *Current Opinion in Biotechnology* **6**(3): 320-328.

Rehmann, L., Prpich, G. P., and Daugulis, A. J. (2008). Remediation of PAH contaminated soils: Application of a solid-liquid two-phase partitioning bioreactor. *Chemosphere* **73**(5): 798-804.

Reid, B. J., Jones, K. C. and Semple, K. T. (2000). Bioavailability of persistent organic pollutants in soils and sediments--a perspective on mechanisms, consequences and assessment. *Environmental Pollution* **108**(1): 103-112.

Richnow, H. H., Seifert, R., Hefter, J., Link, M., Francke, W., Schaefer, G. and Michaelis, W. (1997). Organic pollutants associated with macromolecular soil organic matter: Mode of binding. *Organic Geochemistry* **26**(11-12): 745-758.

Rios-Hernandez, L. A., Gieg, L. M. and Sufflita, J. M. (2003). Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. *Applied Environmental Microbiology* **69**(1): 434-443.

Rivas, F. J. (2006). Polycyclic aromatic hydrocarbons sorbed on soils: A short review of chemical oxidation based treatments. *Journal of Hazardous Materials* **138**(2): 234-251.

- Robles-Martinez, F. and Gourdon, R. (1999). Effect of baling on the behaviour of domestic wastes: laboratory study on the role of pH in biodegradation. *Bioresource Technology* **69**(1): 15-22.
- Rockne, K. J., Chee-Sanford, J. C., Sanford, R. A., Hedlund, B. P., Staley, J. T. and Strand, S. E. (2000). Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. *Applied and Environmental Microbiology* **66**(4): 1595-1601.
- Rockne, K. J. and Strand, S. E. (1998). Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environmental Science & Technology* **32**(24): 3962-3967.
- Rogalski, J., Wojtas-Wasilewska, M., Apalovic, R., Leonowicz, A. (1991). Affinity chromatography as a rapid and convenient method for purification of fungal laccases. *Biotechnology Bioengineering* **37**(8): 770-777.
- Ruggaber, T. P. and Talley, J. W. (2006). Enhancing bioremediation with enzymatic processes: A Review. *Practice Periodical of Hazardous, Toxic, and Radioactive Waste Management* **10**(2): 73-85.
- Sadhasivam, S., Savitha, S., Swaminathan, K. and Lin, F. H. (2008). Production, purification and characterization of mid-redox potential laccase from a newly isolated *Trichoderma harzianum* WL1. *Process Biochemistry* **43**(7): 736-742.
- Sandrin, T. R. and Maier, R. M. (2003). Impact of metals on the biodegradation of organic pollutants. *Environmental Health Perspectives* **111**(8): 1093-1101.
- Sardans, J. and Penuelas, J. (2005). Drought decreases soil enzyme activity in a Mediterranean *Quercus ilex* L. forest. *Soil Biology & Biochemistry* **37**(3): 455-461.
- Sarma, P. M., Bhattacharya, D., Krishnan, S. and Lal, B. (2004). Degradation of polycyclic aromatic hydrocarbons by a newly discovered enteric bacterium, *Leclercia adecarboxylata*. *Applied Environmental Microbiology* **70**(5): 3163-3166.
- Sauret-Ignazi, Gagnon, J., Beguin, C., Barrelle, M., Markowicz, Y., Pelmont, J. and Toussaint, A. (1996). Characterisation of a chromosomally encoded catechol 1,2-dioxygenase (E.C. 1.13.11.1) from *Alcaligenes eutrophus* CH34. *Archives Microbiology* **166**(1): 42-50.

Schlotzhauer, R. Z. (1979). Photoreactivity of selected aromatic hydrocarbons in water. *Ann Arbor Science Publisher*: 141-157.

Semple, K. T., Morriss, A. J. and Paton, G. I. (2003). Bioavailability of hydrophobic organic contaminants in soils: fundamental concepts and techniques for analysis. *European Journal of Soil Science* **54**(4): 809-818.

Semple, K. T., Reid, B. J. and Fermor, T. R. (2001). Impact of composting strategies on the treatment of soils contaminated with organic pollutants. *Environmental Pollution* **112**(2): 269-283.

Seo, J. S., Keum, Y. S. and Li, Q. X. (2009). Bacterial Degradation of Aromatic Compounds. *International Journal of Environmental Research and Public Health* **6**(1): 278-309.

Shen, G., Lu, Y., Zhou, Q. and Hong, J. (2005). Interaction of polycyclic aromatic hydrocarbons and heavy metals on soil enzyme. *Chemosphere* **61**(8): 1175-1182.

Siddiqi, M. A., Ye, D. Y., Elmarakby, S. A., Kumar, S. and Sikka, H. C. (1994). Microbial-Metabolism of Polycyclic Aromatic-Hydrocarbons (Pah) and Aza-Pah. *Polycyclic Aromatic Compounds* **7**(1-3): 115-122.

Sigman, M. E., Schuler, P. F., Ghosh, M. M. and Dabestani, R. T. (1998). Mechanism of pyrene photochemical oxidation in aqueous and surfactant solutions. *Environmental Science & Technology* **32**(24): 3980-3985.

Sigman, M. E. and Zingg, S. P. (1994). Aquatic and Surface Photochemistry. *CRC Press*: 197-206.

Sikkema, J., Debont, J. M. and Poolman, B. (1995). Mechanisms of Membrane Toxicity of Hydrocarbons. *Microbiological Reviews* **59**(2): 201-222.

Silva, I. S., Dos Santos, E. D., de Menezes, C. R., de Faria, A. F., Franciscon, E., Grossman, M. and Durrant, L. R. (2009). Bioremediation of a polyaromatic hydrocarbon contaminated soil by native soil microbiota and bioaugmentation with isolated microbial consortia. *Bioresource Technology* **100**(20): 4669-4675.

- Silva, I. S., Grossman, M. and Durranta, L. R. (2009). Degradation of polycyclic aromatic hydrocarbons (2-7 rings) under microaerobic and very-low-oxygen conditions by soil fungi. *International Biodeterioration & Biodegradation* **63**(2): 224-229.
- Simonnot, M. O., Castel, C., Nikolai, M., Rosin, C., Sardin, M. and Jaufferet, H. (2000). Boron removal from drinking water with a boron selective resin: is the treatment really selective? *Water Resources* **34**: 109-116.
- Sirguey, C., Silva, P. E., Schwartz, C. and Simonnot, M. O. (2008). Impact of chemical oxidation on soil quality. *Chemosphere* **72**(2): 282-289.
- Smith, M. R. (1990). The biodegradation of aromatic hydrocarbons by bacteria. *Biodegradation* **1**(2-3): 191-206.
- Somerville, C. C., Knight, I. T., Straube, W. L. and Colwell, R. R. (1989). Simple, rapid method for direct isolation of nucleic acids from aquatic environments. *Applied Environmental Microbiology* **55**(3): 548-554.
- Song, Y. F., Jing, X., Fleischmann, S. and Wilke, B. M. (2002). Comparative study of extraction methods for the determination of PAHs from contaminated soils and sediments. *Chemosphere* **48**(9): 993-1001.
- Starmer, W. T., Phaff, H. J., Ganter, P. F. and Lachance, M. A. (2001). *Candida orba* sp. nov., a new cactus-specific yeast species from Queensland, Australia. *International Journal Systematic Evolutionary Microbiology* **51**(Pt 2): 699-705.
- Steffen, K. T., Hatakka, A. and Hofrichter, M. (2003). Degradation of benzo[a]pyrene by the litter-decomposing basidiomycete *Stropharia coronilla*: role of manganese peroxidase. *Applied Environmental Microbiology* **69**(7): 3957-3964.
- Strachan, P. D., Freer, A. A. and Fewson, C. A. (1998). Purification and characterization of catechol 1,2-dioxygenase from *Rhodococcus rhodochrous* NCIMB 13259 and cloning and sequencing of its catA gene. *Biochemistry Journal* **333** (Pt 3): 741-747.
- Sturman, P. J., Stewart, P. S., Cunningham, A. B., Bouwer, E. J. and Wolfram, J. H. (1995). Engineering Scale-up of in-Situ Bioremediation Processes - a Review. *Journal of Contaminant Hydrology* **19**(3): 171-203.

Stylidi, M., Kondarides, D. I. and Verykios, X. E. (2003). Pathways of solar light-induced photocatalytic degradation of azo dyes in aqueous TiO₂ suspensions. *Applied Catalysis B-Environmental* **40**(4): 271-286.

Sulistyaningdyah, Ogawa, J., Tanaka, H., Maeda, C. and Shimizu, S. (2004). Characterization of alkaliphilic laccase activity in the culture supernatant of *Myrothecium verrucaria* 24G-4 in comparison with bilirubin oxidase. *FEMS Microbiology Letters* **230**(2): 209-214.

Sun, H. W. and Q. S. Yan (2007). Influence of Fenton oxidation on soil organic matter and its sorption and desorption of pyrene. *Journal of Hazardous Materials* **144**(1-2): 164-170.

Tabatabai, M. A. and Bremner, J. M. (1970). Factors affecting soil aryl sulphatase activity. *Science Society of America Proceedings* **34**(4): 427-429.

Tadano, T., Ozawa, K., Satai, M., Osak, M. and Matsui, H. (1993). Secretion of acid phosphatase by the roots of crop plants under phosphorus deficient conditions and some properties of the enzyme secreted by lupine roots. *Plant Soil* **156**: 95-98.

Tang, Y. B., Yang, X., Chen, F. Y., Jiang, R. L. and Wang, X. G. (2011). Screening, identification and degrading gene assignment of a chrysene-degrading strain. *African Journal of Biotechnology* **10**(34): 6549-6557.

Tao, S., Jiao, X. C., Chen, S. H., Liu, W. X., Coveney, R. M., Zhu, L. Z., Luo, Y. M. (2006). Accumulation and distribution of polycyclic aromatic hydrocarbons in rice (*Oryza sativa*). *Environmental Pollution* **140**(3): 406-415.

Tian, L., Ma, P. and Zhong, J. J. (2002). Kinetics and key enzyme activities of phenanthrene degradation by *Pseudomonas mendocina*. *Process Biochemistry* **37**(12): 1431-1437.

Tiehm, A. (1994). Degradation of Polycyclic Aromatic-Hydrocarbons in the Presence of Synthetic Surfactants. *Applied and Environmental Microbiology* **60**(1): 258-263.

Torres, E., Bustos-Jaimes, I. and Le Borgne, S. (2003) Potential use of oxidative enzymes for the detoxification of organic pollutants'. *Applied Catalysis: Environmental* **46**: 1-15.

Tsai, C. S., Killham, K. and Cresser, M. S. (1997). Dynamic response of microbial biomass, respiration rate and ATP to glucose additions. *Soil Biology & Biochemistry* **29**(8): 1249-1256.

Tu, C., Teng, Y., Luo, Y. M., Sun, X. H., Deng, S. P., Li, Z. G., Liu, W. X. and Xu, Z. H. (2011). PCB removal, soil enzyme activities, and microbial community structures during the phytoremediation by alfalfa in field soils. *Journal of Soils and Sediments* **11**(4): 649-656.

Tumaikina Iu, A., Turkovskaia, O. V. and Ignatov, V. V. (2008). Degradation of hydrocarbons and their derivatives by a microbial association based on Canadian pondweed. *Prikl Biokhim Mikrobiology* **44**(4): 422-429.

U.S., E. (1998). Field applications of in situ remediation technologies: chemical oxidation. Office of solid waste and emergency response. *Technology innovation office US*, Report EPA 542-R-98-008.

Urairuj, C., Khanongnuch, C. and Lumvong, S. (2003). Ligninolytic enzymes from tropical endophytic Xylariaceae. *Fungal Diversity* **13**: 209-219.

Urgun-Demirtas, M., Stark, B. and Pagilla, K. (2006). Use of genetically engineered microorganisms (GEMs) for the bioremediation of contaminants. *Critical Reviews in Biotechnology* **26**(3): 145-164.

USEPA. (1994). In-Situ Biodegradation Treatment. *Engineering Bulletin USEPA/540/S-94/502*

Uyttbroek, M., Vermeir, S., Wattiau, P., Ryngaert, A. and Springael, D. (2007). Characterization of cultures enriched from acidic polycyclic aromatic hydrocarbon-contaminated soil for growth on pyrene at low pH. *Applied and Environmental Microbiology* **73**(10): 3159-3164.

Van Gestel, K., Mergaert, J., Swings, J., Coosemans, J. and Ryckeboer, J. (2003). Bioremediation of diesel oil-contaminated soil by composting with biowaste. *Environmental Pollution* **125**(3): 361-368.

Vandyke, M. I., Lee, H. and Trevors, J. T. (1991). Applications of Microbial Surfactants. *Biotechnology Advances* **9**(2): 241-252.

- VanJaarsveld, A. J., Vanpul, W. J. and De Leeuw, F. A. (1997). Modelling transport and deposition of persistent organic pollutants in the European region. *Atmospheric Environment* **31**: 1011-1997.
- Venkatadri, R. and Irvine, R. L. (1990). Effect of Agitation on Ligninase Activity and Ligninase Production by *Phanerochaete chrysosporium*. *Applied Environmental Microbiology* **56**(9): 2684-2691.
- Verdin, A., Sahraoui, A. H. and Durand, R. (2004). Degradation of benzo[a]pyrene by mitosporic fungi and extracellular oxidative enzymes. *International Biodeterioration & Biodegradation* **53**(2): 65-70.
- Vidali, N. (2001). Bioremediation. A review. *Pure Applied Chemistry* **73**: 1163-1172.
- Vinas, M., Sabate, J., Espuny, M. J. and Solanas, A. M. (2005). Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Applied and Environmental Microbiology* **71**(11): 7008-7018.
- Wallis, M. G. and Chapman, S. K. (1990). Isolation and partial characterization of an extradiol non-haem iron dioxygenase which preferentially cleaves 3-methylcatechol. *Biochemical Journal* **266**(2): 605-609.
- Walter, M., Boyd-Wilson, K. H., McNaughton, D. and Northcott, G. (2005). Laboratory trials on the bioremediation of aged pentachlorophenol residues. *International Biodeterioration & Biodegradation* **55**(2): 121-130.
- Wang, C. L., Takenaka, S., Murakami, S. and Aoki, K. (2001). Production of catechol from benzoate by the wild strain *Ralstonia* species Ba-0323 and characterization of its catechol 1,2-dioxygenase. *Bioscience Biotechnology Biochemistry* **65**(9): 1957-1964.
- Watts, R. J., Stanton, P. C., Howsawkung, J. and Teel, A. L. (2002). Mineralization of a sorbed polycyclic aromatic hydrocarbon in two soils using catalyzed hydrogen peroxide. *Water Resources* **36**(17): 4283-4292.
- Weathers, L. J., Parkin, G. F. and Alvarez, P. J. (1997). Utilization of cathodic hydrogen as electron donor for chloroform cometabolism by a mixed, methanogenic culture. *Environmental Science & Technology* **31**(3): 880-885.

- Webster, J. J., Hampton, G. J., Wilson, J. T., Ghiorse, W. C. and Leach, F. R. (1985). Determination of Microbial Cell Numbers in Subsurface Samples. *Ground Water* **23**(1): 17-25.
- Webster, J. and Dix, J. E. (1994). Fungal Ecology. *Chapman & Hall*. 179-184.
- Weibull, C., Greenwalt, J. W. and Low, H. (1962). The hydrolysis of adenosine triphosphate by cell fractions of *Bacillus megaterium*. I. Localization and general characteristics of the enzymic activities. *Journal of Biology and Chemistry* **237**: 847-852.
- Weissenfels, W. D., Beyer, M., Klein, J. and Rehm, H. J. (1991). Microbial-Metabolism of Fluoranthene - Isolation and Identification of Ring Fission-Products. *Applied Microbiology and Biotechnology* **34**(4): 528-535.
- Wen, G., Voroney, R. P., Curtin, D., Schoenau, J. J., Qian, P. Y. and Inanaga, S. (2005). Modification and application of a soil ATP determination method. *Soil Biology & Biochemistry* **37**(11): 1999-2006.
- Wen, S., Zhao, J., Sheng, G., Fu, J., Peng, P. (2002). Photocatalytic reactions of phenanthrene at TiO₂/water interfaces. *Chemosphere* **46**(6): 871-877.
- Wenger, A. F. (1992). Transcultural nursing and health care issues in urban and rural contexts. *Journal of Transcultural Nursing* **3**(2): 4-10.
- West, P. A., Okpokwasili, G. C., Brayton, P. R., Grimes, D. J. and Colwell, R. R. (1984). Numerical taxonomy of phenanthrene-degrading bacteria isolated from the Chesapeake Bay. *Applied Environmental Microbiology* **48**(5): 988-993.
- Whiteley, C. G. and Lee, D. J. (2006). Enzyme technology and biological remediation. *Enzyme and Microbial Technology* **38**(3-4): 291-316.
- Whyte, L. G., Bourbonniere, L. and Greer, C. W. (1997). Biodegradation of petroleum hydrocarbons by psychrotrophic *Pseudomonas* strains possessing both alkane (alk) and naphthalene (nah) catabolic pathways. *Applied Environmental Microbiology* **63**(9): 3719-3723.
- Wilson, S. C. and Jones, K. C. (1993). Bioremediation of soil contaminated with polynuclear aromatic hydrocarbons (PAHs): a review. *Environmental Pollution* **81**(3): 229-249.

Woo, O. T., Chung, W. K., Wong, K. H., Chow, A. T. and Wong, P. K. (2009). Photocatalytic oxidation of polycyclic aromatic hydrocarbons: Intermediates identification and toxicity testing. *Journal of Hazardous Materials* **168**(2-3): 1192-1199.

Wrenn, B. A., Suidan, M. T., Strohneier, Eberhart, L. and G. J. Wilson. (1998). Influence of tide and waves on washout of dissolved nutrients from the bioremediation zone of a coarse-sand beach: Application in oil-spill bioremediation. *Spill Science & Technology* (4): 99-106.

Wu, L., Ma, L. Q. and Martinez, G. A. (2000). Comparison of methods for evaluating stability and maturity of biosolids compost. *Journal of Environmental Quality* **29**: 424-429.

Xiao, X., Zhang, J., Zhang, Wang, Q., Tan, Y., Guo, Z., Yang, R., Qiu, J. and Zhou, D. (2011). Two methods for extraction of high-purity genomic DNA from mucoid Gram-negative bacteria. *African Journal of Microbiology Research* **5**(23): 4013-4018.

Xiao, Y. Z., Tu, X. M., Wang, J., Zhang, M., Cheng, Q., Zeng, W. Y., Shi, Y. Y. (2003). Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. *Applied Microbiology Biotechnology* **60**(6): 700-707.

Xuanzhen, L., Xiangui, L., Rui, Y., Yucheng, W., Hajyan, C. and Jun, Z. (2010). Optimization of laccase-mediated benzo[a] pyrene oxidation and the bioremediation application in aged polycyclic aromatic hydrocarbons in contaminated soil. *Journal of Health Science* **56**(5): 534-540.

Yan, S. C., Ouyang, S. X., Gao, J., Yang, M., Feng, J. Y., Fan, X. X., Wan, L. J., Li, Z., S., Ye, J. H., Zhou, Y. and Zou, Z. G. (1998). A room-temperature reactive-template route to mesoporous ZnGa₂O₄ with improved photocatalytic activity in reduction of CO₂. *Angewandte Chemie International Edition* **49**(36): 6400-6404.

Yaver, D. S., Xu, F., Golightly, E. J., Brown, K. M. Brown, S. H., Rey, M. W., Schneider, P., Halkier, T., Mondorf, K., Dalboge, H. (1996). Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa*. *Applied Environmental Microbiology* **62**(3): 834-841.

Yuan, S. Y., Chang, J. S., Yen, J. H. and Chang, B. V. (2001). Biodegradation of phenanthrene in river sediment. *Chemosphere* **43**(3): 273-278.

Zander, M. (1983). Physical and chemical properties of polycyclic aromatic hydrocarbons. A. Biorseth (ed.), *Handbook of polycyclic aromatic hydrocarbons*: 1-26.

Zang, S., Li, P., Li, W., Zhang, D. and Hamilton, A. (2007). Degradation mechanisms of benzo[a]pyrene and its accumulated metabolites by biodegradation combined with chemical oxidation. *Chemosphere* **67**(7): 1368-1374.

Zepp, R. G. (1982). Experimental approaches to environmental photo-chemistry (Ed.), *Hand Book of Environmental chemistry*. Springer Verlag **2**(B): 19-41.

Zepp, R. G. and Schlotzhauer, P. F. (1979). Photoreactivity of selected aromatic hydrocarbons in water. Polycyclic aromatic hydrocarbons. *Ann Arbor Science Publisher*: 141-158.

Zhang, L. H., Li, P. J., Gong, Z. Q. and Li, X. M. (2008). Photocatalytic degradation of polycyclic aromatic hydrocarbons on soil surfaces using TiO₂ under UV light. *Journal of Hazardous Materials* **158**(2-3): 478-484.

Zhang, Y., Liu, S., Ma, J. (2006). Water-holding capacity of ground covers and soils in alpine and sub-alpine shrubs in western Sichuan. *China: Elsevier* **26**(9): 2775-2781.

Zouari, N., Romette, J. L. and Thomas, D. (1987). Purification and properties of two laccase isoenzymes produced by *Botrytis cinerea*. *Applied Biochemistry Biotechnology* **15**: 213-225.

Appendix

Appendix I
Screening and Characterization of
PAH degrading organisms

Aim: Isolation, Identification and Characterization of PAH degraders—using PCR amplification and sequencing of 16S rDNA for bacterial strains and 18S rDNA for fungal strains

I.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment as a result of various natural and anthropogenic activities (Guerin & Jones, 1988). PAHs presence in the environment is a major toxicological concern for living organisms. As a result, practical remediation technologies that are inexpensive need to be developed to eliminate the PAHs in contaminated soils (Lease *et al.*, 2011).

In recent years, possible economic strategies for remediation of toxic inorganic compounds and PAH-contaminated soil have been encouraged by the success of bioremediation techniques (Lease *et al.*, 2011). A number of studies on PAH degradation have been reported by various researchers indicating the catabolic diversity of bacterial, fungal and algal PAH degradation by shaken enrichment cultures (Cerniglia, 1992; Wilson and Jones, 1992; Lease *et al.*, 2011). Also, these studies suggest that a major role in the degradation of PAHs is carried out by bacteria and fungi as a natural process at contaminated sites. Natural processes to degrade toxic compounds via organisms can however be carried out more rapidly by altering abiotic factors at the contaminated site (Haritash and Kaushik, 2009). A lists of micro-organisms capable of PAH degradation is provided in (section 3.25; Table 2).

An enrichment culture is defined as a specific medium for the growth of particular micro-organism. It is mostly suitable for environmental cultures that support growth of organisms by inhibiting growth of other organisms (Bass-Becking, 1934). Thus, enrichment culture is considered as efficient processes to isolate pure hydrocarbon

degrading micro-organisms. Also, microbes capable of PAH degradation by shaken enrichment technique was reported by Elsalam *et al* (2009). In the research reported by Elsalam *et al* (2009), PAH degradation and the genes involved in PAH degradation by *Pseudomonas*, *Sphingomonas* and *Nocardioides* species, were studied. These studies used shaken enrichment culture and reported increased degradation rate for phenanthrene and anthracene by *Escherichia coli* (EF105548), Soil bacterium strain (EF105549), *Alcaligenes* sp. (EF105546) relative degradation efficiency obtained in 17hrs was 42.45, 48.44 and 40.45% respectively (Elsalam *et al.*, 2009). Sarma *et al.*, (2004) reported an enteric bacterial strain, PS4040 that degrades high molecular weight (HMW), four-benzene-ring PAH using pyrene as sole carbon source in shaken enrichment culture. Moreover, *Mycobacterium* species were isolated by Lease *et al* (2011) via shaken enrichment culture to study the degradation of phenanthrene and pyrene.

Previous studies have suggested that fungal mediated degradation of PAHs by shaken enrichment is more rapid compared to bacterial populations (Bishnoi *et al.*, 2007). Various white rot fungi (WRF) like *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* are highly efficient in PAH degradation by producing extracellular enzymes. These WRF produce lignin peroxidase and manganese peroxidase which are actively involved in PAH degradation (Bishnoi *et al.*, 2007). Also, Bishnoi *et al.*, (2007) have reported degradation of phenanthrene, anthracene, acenaphthene, fluoranthene and pyrene in sterilized and unsterilized soil through enrichment with *P. chrysosporium*.

Studies have been reported shaken enrichment culture using yeasts responsible for PAH degradation. However, a number of studies have reported that many yeasts such as *Saccharomyces cerevisiae*, *Debaromyces hansenii*, *Candida lipolytica*, *C.*

guilliermondii, *C. maltosa* and *Pichia anomala* cannot utilize HMW PAHs as their sole carbon sources (Cerniglia & Crow, 1981; McGillivray & Sharis, 1993; Zinjarde & Pant, 2002 & Pan *et al.*, 2004). However, Hesham *et al.*, (2009) reported that a yeast strain AH70 isolated from petroleum contaminated soil by enrichment in mineral salts medium were capable of degrading low molecular weight (LMW) and HMW PAHs with an efficiency of 89.67% for naphthalene, 60.77% for pyrene, 77.21% for phenanthrene and 55.53% for benzo (a) pyrene over 10 days.

In this study, PAH enrichment cultures were prepared to isolate PAHs degrading micro-organisms from contaminated sources. The major purpose of enrichment culture is that it provides a relatively easy way to isolate pure cultures (particular PAHs degraders in this study) among mixed culture in the environments (O'Leary, 1989). Isolated organisms were identified morphologically and by sequencing 16S rRNA for bacterial culture and 18S rDNA for fungal cultures. The sequences were subjected to Blast in order to compare the sequences from the genebank database for particular species identification. This extensive available database makes the task of species identification much easier (Kurtzman, 2001; Starmer *et al.*, 2001; Wesselink *et al.*, 2002) and serves as a reliable and practical criteria for identification (Abliz *et al.*, 2004).

I.2 Materials and methods

Objectives: Isolation of PAH degrading organisms via shaken aqueous enrichment culture.

I.2.1 Sample collection for enrichment culture

Road side soil was collected from the entrance gate of University of Hertfordshire, college Lane, AL10 9AB by the traffic lights. These were obtained using a 22-cm hand-dug soil auger and put in labeled polyethylene bags and transported to the laboratory. The pH of the soil was 7.2, with a 38% water holding capacity.

The presence of PAH contamination in the soil samples was confirmed by solvent extraction (chapter 3: section 3.4) using round vortex mixer fitted with multi sample holder which holds a total of 12 samples (Sigma Aldrich) for 5 minutes prior to HPLC analysis.

I.2.2 Media

The minimal medium contained salts and trace element solution. The composition of mineral medium was adapted from Bastiaens *et al.* (2000) described in appendix II (section II.1) with pH adjustment as tabulated in table 3.1:

Table I.1: pH adjustment of minimal medium

Minimal salt medium pH adjustment	1M HCl (µl)	0.01M Na ₂ CO ₃ (µl)
5.5	200	-
7.0	Original pH	Original pH
7.5	-	1000

I.2.3 Addition of PAHs solvent into minimal salt medium

99 ml of pH adjusted minimal media (minimal salt medium composition (appendix II section II.1) was added to Erlenmeyer flasks in 5 replicates each containing 1 ml of

trace element solution. 100 mg of each individual PAH: phenanthrene, anthracene, fluoranthene and pyrene respectively were added to (1000 ml) of acetone and 10 ml of acetone was added to the flasks containing 100 ml of broth. Each of these flasks covered with cotton plug bung were kept in fume-hood in order to evaporate the acetone. Evaporation of acetone was confirmed by measuring the weights of flasks.

I.2.4 1st Shaken enrichment

5 g of the road side soil samples (described in appendix I.2.1) were inoculated into each flask in 5 replicates. The PAHs in each flask acted as a sole carbon and energy source for the growth of the organisms. The flasks were incubated aerobically at 28⁰C for 14 days with shaking at 175 rpm.

I.2.5 2nd Shaken enrichment

Second enrichment was followed from the flasks incubated for 1st enrichment. 5 ml of broth from the 1st enrichment were transferred into the appropriate fresh minimal salt medium as described in appendix I.2.2. Similar incubation conditions were followed as described in appendix I.2.4.

I.2.6 3rd Shaken enrichment

3rd enrichment was performed into the appropriate fresh minimal salt medium followed as described in appendix I.2.4 and I.2.5.

PAH-degrading bacterial isolates, 100 µl was plated from second and third shaken enrichments were diluted and plated on nutrient agar (Sigma Aldrich) plates for bacterial isolates and fungal isolates on malt extract agar (MEA) plates containing 50 mg rose bengal.

Also, after pure culture isolation, growth of isolated pure bacterial colonies was confirmed by spraying PAH solution on minimal salt agar plate while pure isolated fungal colonies isolated on MEA were confirmed for growth in presence of PAHs by spraying PAH dissolved solution in acetone plates along with plates without PAH (control).

I.2.7 Biochemical test

Biochemical tests were performed on PAH-degrading bacterial isolates obtained described in section (5.2.6). The procedure was adapted with modification from Cowan & Steel, (1996) for gram staining reactions, catalase and oxidase test and oxidation or fermentation (O-F) test as described in appendix II section II.2

Objective: Identification of bacterial strains degrading PAHs – using PCR amplification and sequencing of 16S rDNA

I.2.8 DNA extraction from isolated bacteria

The bacterial cultures isolated from shaken enrichment technique, described in appendix I.2.4 were preserved on nutrient agar slopes maintained at 4⁰C. Bacterial isolates from nutrient agar slopes were grown overnight in 100 ml of nutrient broth (Sigma Aldrich) and further used for DNA extraction.

I.2.9 DNA extraction (Phenol: Chloroform method)

The improved phenol: chloroform method was used for genomic DNA (gDNA) extraction according to the method of Cheng & Jiang (2006) and Neumann *et al.*, (1992) with the elimination of lysis step (SDS/lysozyme or proteinase K steps). The bacterial DNA was isolated from Gram-negative and Gram positive bacteria grown in 100 ml of nutrient broth. The (100 ml) cell suspension was centrifuged in sterile bottles for 10 mins at 8000 g. The supernatant was removed, and the 1.5 ml of bacterial cells was transferred to sterile 2 ml eppendorf tubes. These bacterial cells were washed twice with 400 µl of STE buffer followed by micro-centrifugation for 1 min at 6000 rpm. The composition of STE buffer is described in table I.2:

Table I.2: STE buffer preparations

Reagents	Concentration
NaCl	100 mM
Tris/HCL	10 mM
EDTA (pH 8.0)	1 mM
SDW (sterile distilled water)	-

After the washing step the bacterial cells were re-suspended in 200 µl TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0). Further, 100 µl Tris containing phenol with (pH

8.0) was added to eppendorf tubes containing TE buffer along with bacterial cells. All the samples were vortex mixed for about 60 s followed by centrifugation at 13000 g for 5 mins at 4⁰C to separate aqueous phase from the organic phase. From all the samples 160 µl of aqueous phase was transferred to a clean 1.5 ml eppendorf tubes. 40 µl of TE buffer was added to make 200 µl and mixed with 100 µl chloroform and centrifuged for 5 min at 13000 g at 4⁰C. The chloroform extraction step was performed to purify the lysate the step was repeated 3 times until a white interface completely disappeared from the samples. 160 µl of the aqueous layer from at the top of the samples were transferred to clean 1.5 ml eppendorf tubes. 40 µl of TE buffer along with 5 µl of RNase (Sigma Aldrich) (at 10 mg/ml) was added and incubated at 37⁰C for 10 mins so as to ensure no RNA was present in the samples. After incubation, 100 µl of chloroform was added to the tubes and mixed well followed by centrifugation at 13000 g for 5 mins at 4⁰C. The purified DNA present in aqueous phase was transferred to the fresh 1.5 ml eppendorf tube. Cold absolute alcohol (375 µL) was added to the mixture, mixed gently and left overnight to precipitate the DNA. The mixture was then centrifuged for 2 minutes. The supernatant was discarded and the pellet was washed with 1 mL of 70% ethanol before centrifuging for 2 minutes. The pellet was dried for 5 minutes using speed vacuum to remove any residual ethanol. The resultant DNA pellet was subsequently re-suspended in TE buffer pH 8 and stored at -20⁰C.

I.2.10 Concentration and purity of DNA

The purified DNA was quantified by using the Biophotometer plus (EppendorfTM). Eppendorf UVette^R specially designed cuvettes were used. 5 µl of isolated DNA was dissolved in 95 µl of sterile distilled water with distilled water used as a blank. For absorbance of double stranded DNA the wavelength used was 260 nm. The

concentration and purity of DNA was determined by measuring the absorbance at 230, 260, 280 and 320 nm on the eppendorf spectrometer.

I.2.11 Agarose gel electrophoresis

DNA isolated from all the bacterial samples was visualised by electrophoresis based on molecular weight of DNA samples which was compared with DNA marker.

Materials required

Agarose: Gel running buffer 1X (10.9 g/l Tris base; 0.93 g/l disodium-EDTA (Sigma); 5.6 g/l boric acid; pH 8.2)

Samples: Minigel stop mix/Loading dye (1X TBE, 20% w/v sucrose; 10% Ficoll; 10 mM EDTA; 0.25% w/v bromophenol blue)

Trackit 100 bp and 1 Kb ladder from Invitrogen™ Life technologies.

Methods

The 0.8% Agarose gel concentration was prepared by mixing 0.25 g of Agarose with 30 ml of 1X gel running buffer. This mixture was heated in the microwave for 1 min and swirled to mix the agarose well and was allowed to cool before pouring into the gel former with a comb placed in the gel former and allowed to set. After the gel had set the comb was taken out of the gel former and the tapes on the side of the former were taken off. The gel tank was filled with the gel running buffer. The sample was mixed with the loading dye (10:3) and was loaded in the wells and then the samples were allowed to run at 80V.

I.2.12 Staining and visualization of gel

The gel was then stained in freshly prepared 0.5 µg/ml ethidium bromide solutions for 10 – 15 minutes and thereafter destained in running buffer alone to wash the excess

ethidium bromide for 15 – 20 minutes. It was visualised under UV light using UVP gel documentation imaging and GENE Scan system.

I.2.13 Polymerase chain reaction

All DNA samples stored at -20⁰C were used for further PCR amplification. PCR amplification was carried out using universal primers that amplify 16S rDNA. The PCR reactions and universal primers were followed by modification of the method of Hoefel *et al.*, (2005). The bacterial 16S rDNA were amplified using universal primers (specifically for environmental isolates) 27F, 5'-AGAGTTTGATYMTGGCTCAG-3' and 1492R, 5'- TACGGYTACCTTGTTACGACT-3'. PCR reactions were performed on a TGradient thermo cycler machine. The reagents for PCR reaction from Sigma Aldrich and Invitrogen are tabulated in table I.3.

Table I.3: Components of PCR reaction

Reagent concentration	Concentrations of reagents used	Final volume in µl for 6 samples
SDW		232.8
PCR buffer (Sigma Aldrich) [®]	10X	30
MgCl ₂ (Sigma Aldrich) [®]	50 mM	18
dNTP (Sigma Aldrich) [®]	10 mM	6
Primer 1 (Sigma Aldrich) [®]	25 µM	6
Primer 2 (Sigma Aldrich) [®]	25 µM	6
Taq pol (Invitrogen)	5 U/µl	1.2
DNA template		1

Genomic DNA from bacterial samples was amplified using Thermal cycler/PCR machine (Eppendorf). The parameters for PCR reaction are:

Initial Denaturation cycle: 96⁰C for 10 min

The main cycle couple of 34 extensions cycles as below:

Main cycle: Denaturation: 94⁰C for 30 sec.

Annealing: 48⁰C for 1 min

Extension: 70⁰C for 2 min.

Final extension cycle: 72⁰C for 10 min.

PCR amplification products were first analyzed by electrophoresis in 1% (wt/v) agarose gels with ethidium bromide staining (Borneman & Hartin, 2000).

I.2.14 PCR purification method

The DNA fragments were then purified using either the QIAquick[®] PCR purification kit (Qiagen Ltd) or the Sigma Aldrich[®] GenElute PCR clean up kit (Sigma Aldrich) as per the manufacturer's instructions. Five volumes of buffer PB were added to one volume of PCR reaction mixture. The mixture was vortex-mixed thoroughly, then placed in a spin column with 2ml collection tube and centrifuged at 13000 rpm for 1 minute. The filtrate was discarded and the spin column was washed by adding 0.75 ml of buffer PE and centrifuged for 1 minute at 13000 rpm. The filtrate was discarded and the spin column was centrifuged once again at 13000 rpm for 1 minute to remove residual wash buffer. The collection tube was then replaced by a 1.5 ml eppendorf tube and 50µl of Elution buffer (EB) was added to the centre of the spin column. The sample was incubated at room temperature for 1 minute and the purified DNA fragment was eluted by centrifugation at 13000 rpm for 1 minute.

I.2.15 Sequencing and analysis

PCR product after amplification was sent for sequencing. The sequences were analysed using BLAST (NCBI), and the percentages homology to bacterial rDNA sequences in the GenBank database (NCBI) were compared.

Objective: Identification of fungal strains degrading PAHs – using PCR amplification and sequencing of 18S rDNA

I.2.16 Fungal microscopic identification

The fungal isolates were grown on MEA media containing 50 mg rose bengal added to 400 ml of MEA and samples of fungi were viewed at X100 magnification (Nikon, model YS100).

I.2.17 Fungal gDNA isolation

Fungal cultures (isolated from enrichment culture technique) preserved on slopes (4⁰) in cold room were inoculated into MEA broth. After inoculation the flask was incubated at 22⁰C with shaking for 5 days and fresh mycelium was used for DNA isolation.

I.2.18 Fungal gDNA extraction

The basic method of DNA isolation from fungus was followed using a Kit (Qiagen, DNeasy Plant or fungi mini, 69104-1KT, UK) as per the manufacturer protocol. Two different DNA isolation protocols were followed to extract the DNA from isolated fungus using phenol: chloroform method and using kit (Qiagen, DNeasy Plant or fungi mini) their results were compared. DNA isolation of the two different methods the (Qiagen, DNeasy Plant or fungi mini) kit provided the purest DNA samples and was used for further analysis.

I.2.18a Fungal gDNA extraction (Phenol: Chloroform method)

The DNA extraction procedure was adopted with some modification from the methods of Plaza, and Upchurch *et al.*, (2004).

Materials: liquid nitrogen, Micro centrifuge, eppendorf tubes, chloroform: isoamyl alcohol (24:1), RNAase (Sigma), β -mercaptoethanol (Sigma), TE buffer, 75% ethanol, isopropanol, small mortar and pestle.

Table I.4: CTAB extraction buffer

Tris HCL (1 M), pH 8.0	10 ml	0.1M
NaCl (5 M)	28 ml	1.4M
CTAB	2 g	2.0%
(0.5 M) EDTA, pH 8.0	4 ml	0.2M
Water	40.3 ml	

The samples containing the eppendorf tubes were kept in liquid nitrogen for 5 minutes and then kept in room temperature for 2-3 min. This step was repeated 3 to 4 times and then the material was ground with the help of small pestle mortar. In all the eppendorf tubes containing sample 400 μ l of CTAB extraction buffer and 4 μ l of β -mercaptoethanol were added. Then the tubes were kept in a water bath at 65⁰C for 20min. Then to each tube 400 μ l of chloroform: isoamyl alcohol (Sigma) was added and mixed on an orbital shaker for 15 min. All the tubes were then centrifuged at 13,000 rpm for 5 min. After centrifugation the top aqueous layer was transferred to a new eppendorf tube containing (400 μ l) of cold isopropanol. The tubes were kept at room temperature for 5min and then centrifuged at 13,000 rpm for 5 min. The supernatant was carefully discarded and the pellet was allowed to dry. The pellet was resuspended in (100 μ l) of TE buffer, and 4 μ l of RNAase was added and the tubes were kept at room temperature for 15 min. Then 500 μ l of 100% cold ethanol was added to each tube, inverted gently and kept at room temperature for 5 to 10 min. The tubes were centrifuged at 13000 rpm for 5 min. The supernatant was discarded and the pellet was allowed to dry. The pellet was again re-suspended in 100 μ l of TE buffer.

The UV spectrophotometric readings were taken for the samples as described in appendix I.2.10. Samples were stored at -20⁰C.

I.2.18b Fungal gDNA extraction (Qiagen DNeasy isolation Kit)

DNA was extracted from selected isolates, based on their observation under microscope (100X). Only four isolates were selected among the ones which had the same morphology and were identical under microscopic observation. The extraction was performed using fungal genomic DNA kit (Qiagen, DNeasy Plant or fungi mini, 69104-1KT, UK) according to the manufacturer's instructions.

Table I.5: Qiagen DNeasy KIT components

DNeasy fungi Kit Catalog no.	Mini (50) 69104	Mini (250) 69106	T^a Storage
DNeasy Mini spin columns	50	250	4 ⁰ C
QIAshredder mini spin columns	50	250	4 ⁰ C
Buffer AP1	40 ml	200 ml	Room temp (15- 25 ⁰ C)
Buffer AP2	18 ml	90 ml	Room temp (15- 25 ⁰ C)
Buffer AP3/E (concentrate)	30 ml	125 ml	Room temp (15- 25 ⁰ C)
Buffer AW (concentrate)	17 ml	81 ml	Room temp (15- 25 ⁰ C)
Buffer AE	2 × 12 ml	2×60 ml	Room temp (15- 25 ⁰ C)
RNase A (100mg/ml)	220 µl	5×220 µl	4 ⁰ C

I.2.19 Concentration and purity of fungal gDNA

The purified DNA was quantified and analysed as described in section 5.2.10 by using Biophotometer plus (EppendorfTM) instrument.

I.2.20 Agarose gel electrophoresis

DNA isolated from fungal samples was visualised by electrophoresis based on molecular weight of DNA samples which was compared with DNA marker as described in section 3.2.11.

I.2.21 Staining and visualization of gel

The gel was then stained in freshly prepared 0.5 µg/ml ethidium bromide solution as described in section I.2.12.

I.2.22 Polymerase chain reaction for fungal gDNA

All DNA preparations were amplified using ITS1 and ITS4 universal primers described by White *et al.*, (1990). A modification of the method of White *et al.*, (2005) using universal primers to amplify 18S rDNA was used to identify isolates. The first round of PCR reactions were performed using the 18S ribosomal DNA universal fungi primer set ITS1-F 5'-CTTGGTCATTTAGAGGAAGTAA-3' and ITS4, 5'-TCCTCCGCTTATTGATATGC-3'. PCR was performed on a TGradient thermo cycler machine.

Table I.6: PCR reaction components

Reagent concentration	Concentration of reagents	Final volume for in µl
SDW	-	151.2
PCR buffer	10X	20
MgCl ₂	50 mM	12
dNTP	10 mM	4
Primer 1	25 mM	4
Primer 2	25 mM	4
Taq pol	5 U/ µl	0.8
DNA template	-	1

Fungal genomic DNA was amplified using Thermal cycler/PCR machine (table 3.6)

from (Eppendorf). The parameters for PCR reaction are:

Initial Denaturation cycle: 96⁰C for 2 min

Following 34 extension cycle as mentioned bellowed.

Main cycle: Denaturation: 95⁰C for 30 sec

Annealing: 65⁰C for 30 sec

Extension: 70⁰C for 30 sec

Final extension cycle: 70⁰C for 7 min.

PCR amplification products were first analyzed by electrophoresis in 1% (wt/v) agarose gels and ethidium bromide staining (Borneman & Hartin, 2000).

I.2.23 PCR purification method

The DNA fragment was then purified using either the QIAquick[®] PCR purification kit (Qiagen Ltd) or the SIGMA ALDRICH[®] GenElute PCR clean up kit (Sigma Aldrich) as per the manufacturer's instructions. Five volumes of buffer PB were added to one volume of PCR reaction. The mixture was vortex-mixed thoroughly, then placed in a spin column with 2 ml collection tube and centrifuged at 13000 rpm for 1 minute. The filtrate was discarded and the spin column was washed by adding 0.75 ml of buffer PE and centrifuged for 1 minute at 13000 rpm. The filtrate was discarded and the spin column was centrifuged once again at 13000 rpm for 1 minute to remove residual wash buffer. The collection tube was then replaced by a 1.5 ml eppendorf tube and 50 µl of Elution buffer (EB) was added to the centre of the spin column. The sample was incubated at room temperature for 1 minute and the purified DNA fragment was eluted by centrifuging at 13000 rpm for 1 minute.

I.2.24 Sequencing and analysis

PCR product after amplification was sent for sequencing (GATC-Boxle[™]). The sequences were analysed using BLAST (NCBI) and the percentages homology to fungal rDNA sequences in the GenBank database (NCBI) were compared.

I.3 Results

Objective: Isolation of PAHs degrading micro-organisms by shaken enrichment technique

I.3.1 Enrichment culture technique

Isolation of PAH-degrading organisms was carried out via shaken aqueous enrichments followed by isolation onto plates containing PAHs resulting in pure cultures that were able to utilize one or more of phenanthrene, anthracene, fluoranthene and pyrene as the sole carbon sources. The total population in the enrichment cultures was enumerated on nutrient agar plate (table I.7) for bacteria and MEA for fungi at three pH's (table I.8).

Table I.7: Bacterial populations (c.f.u/ml) in shaken aqueous enrichment cultures

Broth pH	1 st Enrichment	2 nd Enrichment	3 rd Enrichment
5.5	6.949	6.755	5.602
7	7.397	6.832	6.00
8.5	6.838	6.491	5.778

Table I.8: Fungal populations (c.f.u/ml) in shaken aqueous enrichment cultures

Broth pH	1 st Enrichment	2 nd Enrichment	3 rd Enrichment
5.5	5.86	5.41	4.48
7	5.68	5.15	0
8.5	5.32	4.85	4.30

A total of six distinct bacterial and two fungal isolates from PAHs contaminated soil were isolated, after the third enrichment. Each of these isolates was characterised by various biochemical bacteriological and growth tests. The isolates were characterised by primary identification performing Gram staining, catalase, oxidase and oxidation and fermentation (O-F). Results recorded as described in table I.9.

Table I.9: Bacteriological characterisation performing biochemical tests

Isolate designation	pH isolated from	Gram reaction	Shape	Catalase tests	Oxidase test	O-F test	Preliminary identification
Culture 1	5.5	-	rod-shaped	+	+	Aerobic	<i>Pseudomonas</i> <i>Alcaligenes</i> <i>Burkholderia</i> <i>Flavobacterium</i> <i>Capnocytophaga</i> <i>Acidovorax</i>
Culture 2	7	-	rod-shaped	+	+	Aerobic	<i>Pseudomonas</i> <i>Alcaligenes</i> <i>Burkholderia</i> <i>Flavobacterium</i> <i>Capnocytophaga</i> <i>Acidovorax</i>
Culture 3	7	+	Irregular short rod-shaped	+	-	Anaerobic facultative	<i>Staphylococcus</i> <i>Micrococcus</i> <i>Rothia</i> ***
Culture 4	7	-	rod-shaped	+		Aerobic	<i>Pseudomonas</i> <i>Alcaligenes</i> <i>Burkholderia</i> <i>Flavobacterium</i> <i>Proteobacterium</i> <i>Capnocytophaga</i> <i>Acidovorax</i>
Culture 5	8.5	-	rod-shaped	+	+	Aerobic	<i>Pseudomonas</i> <i>Alcaligenes</i> <i>Burkholderia</i> <i>Flavobacterium</i> <i>Proteobacterium</i> <i>Capnocytophaga</i>

							<i>Acidovorax</i>
Culture 6	8.5	-	rod- shape d	+	+	Aer obic	<i>Pseudomonas</i> <i>Alcaligenes</i> <i>Burkholderia</i> <i>Flavobacteriu</i> <i>m</i> <i>Proteobactriu</i> <i>m</i> <i>Capnocytopha</i> <i>ga</i> <i>Acidovorax</i>

(+) represents positive test (-) represents negative test. ***represents organism is pleomorphic catalase variable, catalas test may not be helpful for differentiation

The bacteriological characterisation performing biochemical tests for isolates obtained via shaken enrichment cultures was further followed by 16S ribosomal DNA sequencing for bacteria. These isolates were used as inocula for PAH biodegradation experiment.

Objective: Identification of bacterial PAH degrading strains– using PCR amplification and sequencing of 16S rDNA

I.3.2 Bacterial DNA extraction (Phenol: Chloroform genomic DNA extraction)

Genomic DNA extraction was carried out by a modification of the method of Cheng and Jiang (2005). Extraction was performed by phenol chloroform method using only phenol without detergents (example: SDS, Triton X-100). Phenol is a strong oxidizing reagent and thus, is used directly to disrupt the cell wall in order to release the gDNA from the bacterial cells. The purification of gDNA carried out by chloroform to isolate the gDNA from the RNA and different proteins. Further precipitation of gDNA step using isopropanol gave reproducible yields of higher quality gDNA which was used for PCR analysis. A small aliquot of (8 μ l) was analysed by agarose gel electrophoresis (figure I.1). A sharp band of high molecular weight indicated the presence of genomic DNA.

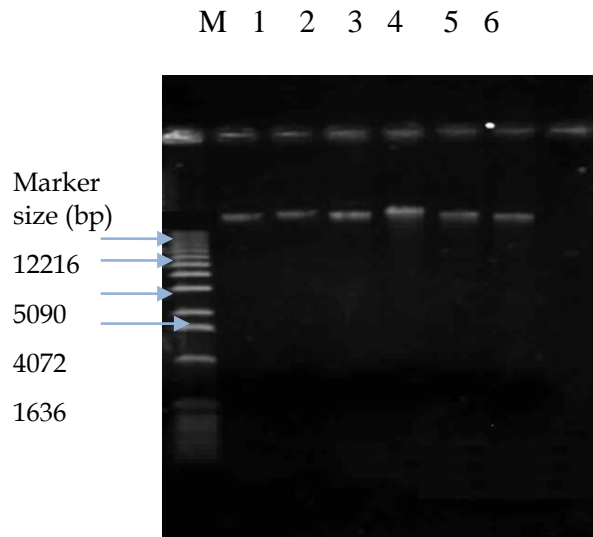


Fig. I.1: Agarose gel electrophoretic analysis of genomic DNA extracted from bacterial isolates.

M represents standard DNA markers (1 Kb) ladder. Lanes 1, 2, 3, 4, 5 and 6 represents the genomic DNA samples extracted from bacterial isolates.

I.3.3 Purity and concentration for bacterial DNA isolation

The absorbance at various wavelengths of 230, 260, 280 and 320nm (table I.10) was measured by estimating the purity and concentration of genomic DNA. A lower ratio at $A_{(260/230)}$ nm denotes contamination of DNA by polysaccharides and lower ratio at $A_{(260/280)}$ nm denotes contamination of gDNA by proteins (Kamal *et al.*, 2008; Xiao *et al.*, (2011)

I.3.4 Purity and concentration for Phenol: chloroform method

The values of purity and concentration of genomic DNA for each sample isolated using phenol: chloroform (table I.10) for $A_{(260/280)}$ and $A_{(260/230)}$ were more than 1.8. The specified absorbance ratios for the gDNA extracted from all isolates were recorded to be more than 1.6 and 1.8. Culture 1 was found to be 2.05 for $A_{(260/280)}$ and 1.96 for $A_{(260/230)}$ (table 14). Similarly, absorbance ratios for culture 2, 3, 4, 5 and 6 were 1.97, 1.87, 2.74, 2.07 and 2.11 for $A_{(260/280)}$ and 1.84, 1.62, 2.39, 1.98, 2.06 for $A_{(260/230)}$ indicating that the purity of all samples was high using phenol: chloroform method. Analysing these ratios further, it was observed that none of the samples were contaminated with proteins or phenol as the absorbance at 280 nm and 260 nm was high.

Table I.10: Purity and concentration for fungal DNA isolation (phenol: Chloroform method)

Sample number	A_(²⁶⁰/₂₈₀)	A_(²⁶⁰/₂₃₀)	A₂₃₀	A₂₆₀	A₂₈₀	A₃₂₀	Concentration (µg/mL)
Culture 1	2.0	1.9	0.3	0.03	0.27	0.08	995.2
Culture 2	1.9	1.8	0.0	1.05	1.00	0.04	701.3
Culture 3	1.8	1.6	1.0	0.72	1.21	0.00	1231.8
Culture 4	2.7	2.3	0.2	1.65	0.00	0.02	1430
Culture 5	2.0	1.9	1.0	0.72	1.21	0.00	1318.6
Culture 6	2.1	2.0	1.0	0.82	1.04	0.05	1625.6

Absorbance was measured at 230 nm, 260 nm, 280 nm and 320 nm. The absorbance ratios A_(²⁶⁰/₂₈₀) and A_(²⁶⁰/₂₃₀) and the concentrations were calculated by the spectrophotometer.

I.3.5 Optimized PCR reaction conditions

PCR reactions were carried out for isolated gDNA at varied annealing temperatures (42 to 55⁰C) and same quantity of 25 mM MgCl₂ table (I.11). A 1% agarose gel was prepared and the gel was run at 100V for 60 minutes for the analysis of PCR amplicons (figure I.2). The PCR conditions for the amplification are summarised in table I.11.

Table I.11: PCR reaction temperature optimization conditions

Reagent	Reagent volume (μL)					
	10	10	10	10	10	10
10x PCR reaction buffer	10	10	10	10	10	10
25mM MgCl_2	3	3	3	3	3	3
25mM Forward primer	1	1	1	1	1	1
25mM Reverse primer	1	1	1	1	1	1
10mM dNTP's	2	2	2	2	2	2
gDNA template	2	2	2	2	2	2
5U/ μL Taq polymerase	0.4	0.4	0.4	0.4	0.4	0.4
Sterile distilled water	40.6	40.6	40.6	40.6	40.6	40.6
Total	50	50	50	50	50	50
Annealing temperature	42°C	45°C	48°C	50°C	52°C	55°C

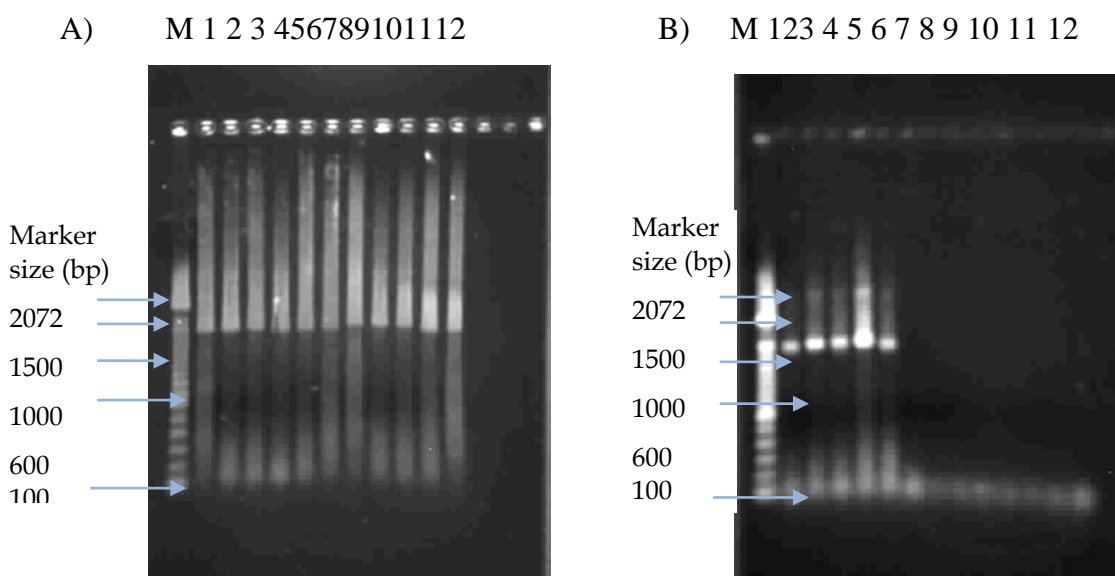


Fig. I.2: Agarose gel electrophoretic analysis of PCR optimization reactions:

A) Lanes 1, 2, 3, 4, 5 and 6 were samples containing 3.5 μL of 25 mM MgCl_2 at 42°C and, Lanes 7, 8, 9, 10, 11 and 12 at 45°C, B) Lanes 1, 2, 3, 4, 5 and 6 with 3 μL of 25 mM MgCl_2 at 48°C. Lane 7, 8, 9, 10, 11 and 12 at 50°C respectively. M represents standard DNA markers (1 kb). A 1% agarose gel was prepared and run at 100V for 60 minutes.

The PCR conditions for all the gDNA samples were optimized as amplification was confirmed by analysing samples on a standardized 1% agarose gel electrophoresis at 100volts for about 60 mins. Performing the PCR reactions at an annealing temperature of 42 to 48°C produced amplicons of roughly 1421 bp, 1476 bp, 1543 bp, 1472 bp, 1385 bp

and 1389 bp respectively when compared to marker (figure 3.2). Annealing temperatures of 50°C and above resulted in the production of amplicons (data not shown).

I.3.6 PCR product

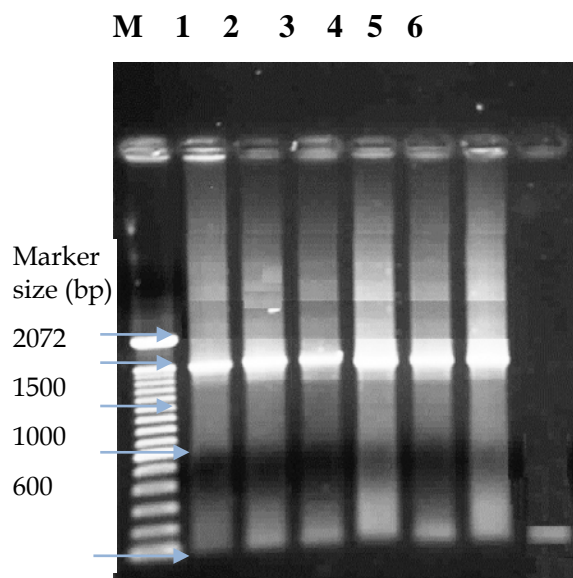


Fig. I.3: Agarose gel electrophoretic analysis of PCR product after the amplification of bacterial gDNA.

M represents standard DNA ladder (100 bp). Lane 1, 2, 3, 4, 5 and 6 represent PCR products of gDNA template and lane 7 represents the negative control containing master mix (table 5) without template DNA. A 0.8% agarose gel was prepared and run at 100V for 60 minutes.

The PCR product obtained after amplification were analysed on standardized 0.8% agarose gel electrophoresis. The product obtained when compared to marker (invitrogen) was around expected sizes of about 1421 bp, 1476 bp, 1543 bp, 1472 bp, 1385 bp and 1389 bp respectively. However, a smear showing primer dimer along with presence of nucleic acids contaminants or shearing of gDNA was present in all samples (figure I.3). Thus, further purification was necessary.

I.3.7 Purification of PCR product

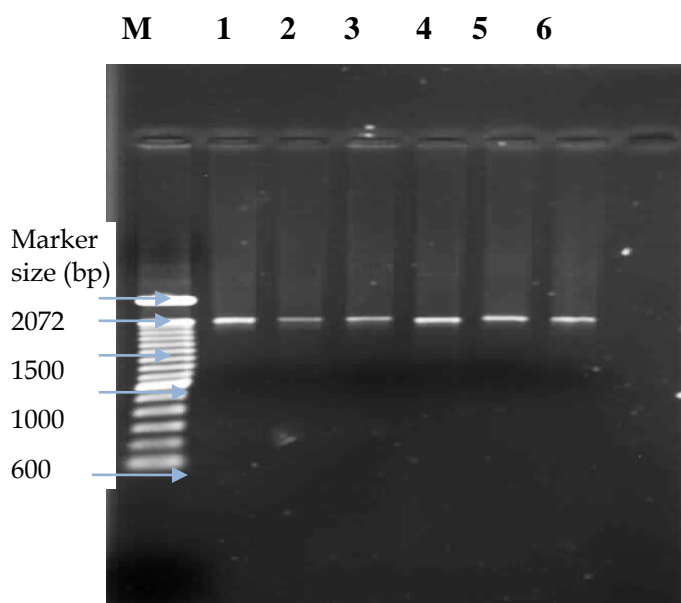


Fig. I.4: Agarose gel electrophoretic analysis of bacterial samples after the purification of PCR product.

M represents standard DNA ladder (100 bp). Lane 1, 2, 3, 4, 5 and 6 represents PCR product of gDNA template after purification procedure. A 0.8% agarose gel was prepared and run at 100V for 60 minutes for the analysis of PCR samples.

The PCR products obtained after amplification were subjected to purification in an attempt to purify PCR product from shearing of gDNA, primer dimer and nucleic acid contaminants. Purified samples were analysed on standardized 0.8% agarose gel electrophoresis at 100V for 60 minutes (figure I.4). The expected product sizes when compared to marker were 1421 bp, 1476 bp, 1543 bp, 1472 bp, 1385 bp and 1389 bp respectively. However no primer dimer as well as shearing gDNA was visible on agarose gel after purification analysis (figure I.4).

I.3.8 Identification and sequencing of 16S rDNA bacteria

The PCR products obtained after purification were sent for sequencing (sequencing data see appendix.II.1) (GATC-Boxle™). The sequences were analysed using BLAST (NCBI) and the percentages homology to bacterial rDNA sequences in the GenBank

database (NCBI) were compared using BLAST analysis and the accession numbers with detailed description of the most homogenous sequences are described in table I.12:

Table I.12: Identification of bacterial 16S rDNA sequences for *Apergillus* species using BLAST analysis against GeneBank database

Cultures	Accession number	Description	Max. Score*	Query coverage^a	E Value^b	Max. identity^c
Culture 1	<u>JQ247014.1</u>	<i>Pseudomonas</i> sp. SRI 360 16S ribosomal RNA gene, partial sequence.	520	100%	3e-144	98%
	<u>JQ012750.1</u>	<i>Pseudomonas putida</i> strain GMC1 16S ribosomal RNA gene, partial sequence.	520	100%	3e-144	98%
Culture 2	<u>AB680418.1</u>	<i>Achromobacter xylooxidans</i> gene for 16S rRNA, partial sequence, strain: NBRC 13495.	1170	98%	0.0	99%
	<u>HQ670710.1</u>	<i>Alcaligenes</i> sp.MG07 16S ribosomal RNA gene, partial sequence.	1170	98%	0.0	99%
Culture 3	<u>JN990375.1</u>	<i>Microbacterium foliorum</i> strain SR11_1385 16S ribosomal RNA gene, partial sequence.	545	100%	4e-153	96%
	<u>GU549407.1</u>	<i>Microbacterium</i> sp. D3(2010) 16S ribosomal RNA gene, partial sequence.	549	100%	4e-153	96%
Culture 4	<u>HM352335.1</u>	<i>Alphaproteobacterium</i>	1140	99%	0.0	98%

		CmLB11 16S ribosomal RNA gene, partial sequence.				
	<u>HM134009.1</u>	<i>Brevundimonas diminuta</i> strain IPSr105 16S ribosomal RNA gene, partial sequence.	167	100%	3e-39	98%
Culture 5	<u>JQ014523.1</u>	<i>Brevundimonas</i> sp. LC348 16S ribosomal RNA gene, partial sequence.	1423	98%	0.0	99%
	<u>GQ246696.1</u>	<i>Brevundimonas</i> sp. M2T2B5 16S ribosomal RNA gene, partial sequence.	52.0	100%	2e-04	91%
Culture 6	<u>JN578809.1</u>	<i>Bradyrhizobium</i> sp. DOA2 16S ribosomal RNA gene, partial sequence.	444	100%	1e-121	100%
	FN600560.2	<i>Bradyrhizobium</i> sp. GSM-467 partial 16S rRNA gene, isolate GSM-467.	444	100%	1e-121	100%

***Max. Score:** score of high scoring pairs, (HSPs), (Score of longest matching sequence)

^a**Query coverage:** percent of length coverage for the query

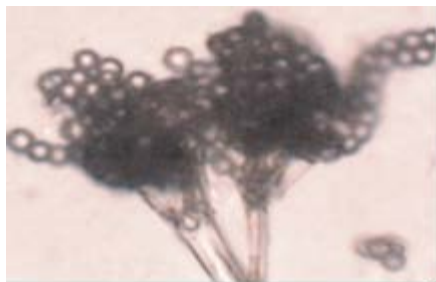
^b**E. Value:** number of hits one can "expect" to see by chance when searching a database of a particular size.

^c**Max. identity:** maximal percent Identity of the HSP

Objective: Identification of fungal strains degrading PAHs – using PCR amplification and sequencing of 18S rDNA

I.3.9 Fungal Microscopic Images

a)



Penicillium species

b)



Aspergillus species

Fig. I.5: Images of two fungi isolated from PAH enrichment culture

The fungal isolates were grown on MEA media and fresh grown specimens of fungi were viewed at X100 magnification (Nikon, model YS100). Image of *Penicillium* species shown in figure I.5a, was isolated from soil pH 5.5 four weeks after contaminating the soil. Similarly, the *Aspergillus* species image shown in figure I.5b was isolated from soil at pH 8.5 during week 4.

The morphological fungal microscopic identification for isolates obtained via shaken enrichment cultures was further followed by 18S ribosomal DNA sequencing for fungi.

I.3.10 Fungal gDNA extraction

Fungal cultures isolated during enrichment culture were preserved on malt extract agar and grown in malt extract broth for genomic DNA extraction. Two different DNA

isolation protocols were followed to extract the DNA from isolated fungi. The first method involved grinding fungal hyphae in liquid nitrogen, followed by phenol:chloroform extraction. The second method was performed using Qiagen, DNeasy Plant/ fungi mini kit.

I.3.11 Phenol: Chloroform genomic DNA extraction

Genomic DNA was isolated from fungal hyphae by breaking the cell wall in liquid nitrogen. A smear of fungal DNA was observed (figure I.6 A) on an agarose gel indicating the presence of genomic DNA. In the gel image of (figure I.6 A) lanes 1 & 2 are loaded with *Penicillium* gDNA extract and lanes 3 & 4 are loaded with *Aspergillus* gDNA. In lane 2 & 3 the presence of genomic DNA was indicated from above 12216 bp to 414 bp when compared with marker (Lane1). Similarly, the presence of gDNA in lanes 4 was evident from 12216bp to 414 bp. However, a smear of low molecular weight DNA indicated the presence of nucleic acid contaminants or shearing of gDNA in the samples. The gDNA samples were then subjected to purification in an attempt to purify the gDNA from the contaminating nucleic acid, and re-analyzed by agarose gel electrophoresis (figure 3.6 B). *Penicillium* and *Aspergillus* sp. samples did not contain genomic DNA after the purification step as shown on the agarose gel (figure I.6 B).

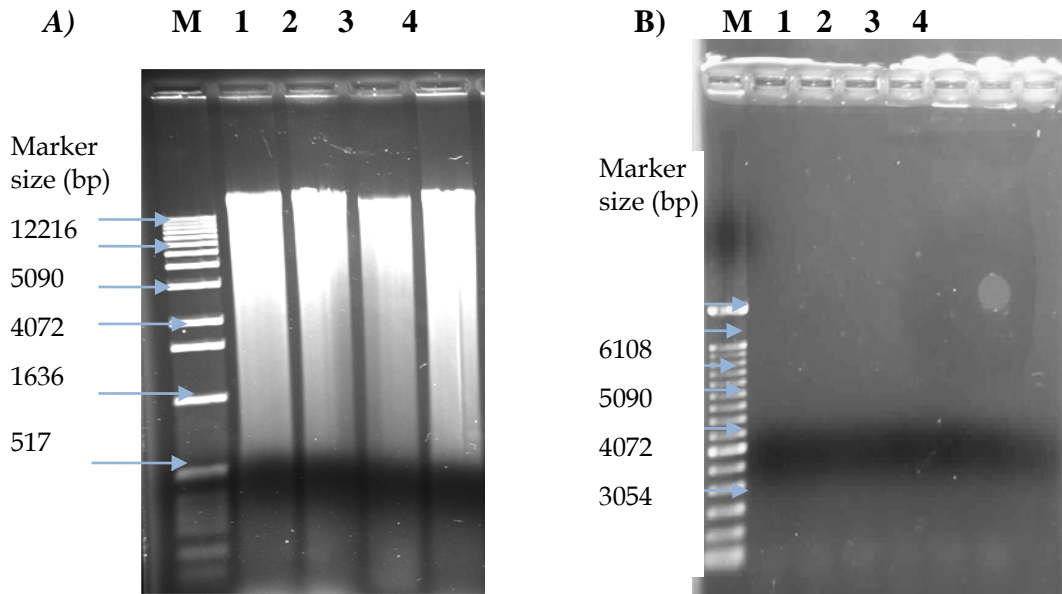


Fig. I.6: Agarose gel electrophoretic analysis of genomic DNA extracted from fungal isolates.

A) Lanes 1 & 2 represents the genomic DNA samples extracted from *Penicillium* species and 3, 4 and 5 represent the genomic DNA samples extracted from *Aspergillus* species. B) *Penicillium* and *Aspergillus* species gDNA sample after using purification cleanup kit. M represents standard DNA markers (100 bp) ladder.

I.3.12 Qiagen, DNeasy Plant or fungi mini kit genomic DNA extraction

Genomic DNA was isolated from fresh fungal mycelium grown in malt extract broth. A sharp band of high molecular weight indicated the presence of genomic DNA (figure I.7 A). However, a smear of DNA of low molecular weight indicated the presence of contaminants or sheared gDNA in the samples. *Penicillium* gDNA extract in lanes 1, 2, 3, & 4 produced sharp bands of gDNA at around 12216bp along with a smear to 512 bp when compared to marker (figure I.7 A). Similarly, *Aspergillus* gDNA extract in lanes 6, 7, 8, 9 &10 (figure I.7 A) had a sharp gDNA band along with a smear from 12216 bp to 512 bp. The gDNA samples were then subjected to a purification step in an attempt to purify the gDNA from the contaminating nucleic acid, and re-analyzed by agarose gel electrophoresis (figure I.7 B). A sharp DNA band of high molecular

weight was observed after the purification process as represented figure I.7b; whilst much of the smear of contaminating nucleic acid was lost indicating that the contaminants were precipitated out. The amount added in each well was (8 μ l) of original gDNA sample.

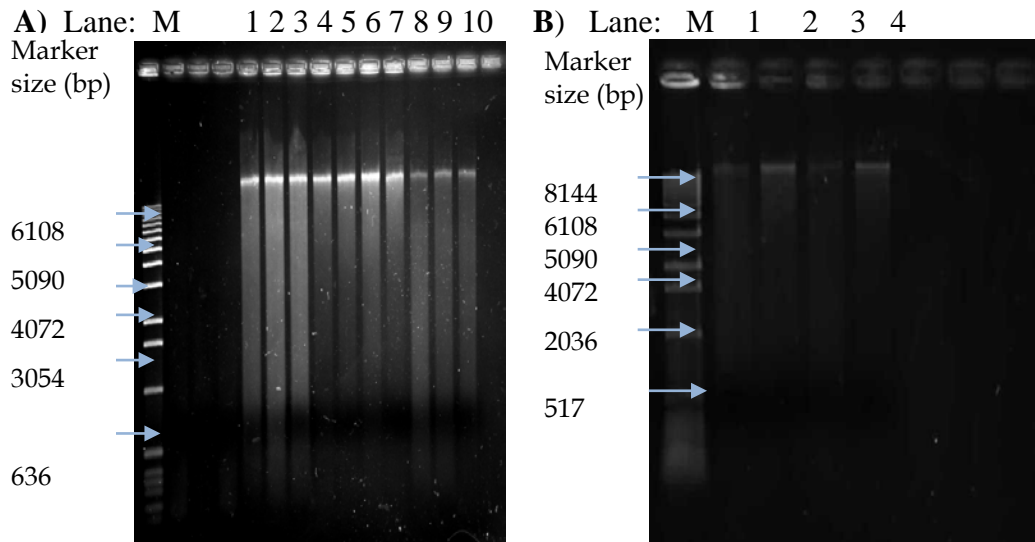


Fig. I.7: Agarose gel electrophoretic analysis of genomic DNA extracted from fungal isolates.

A) Lanes 1, 2, 3, 4 & 5 represent genomic DNA for *Penicillium* species sample and 6, 7, 8, 9 and 10 represent the genomic DNA for *Aspergillus* species extracted in triplets. B) Lanes 1 & 2 represents the genomic DNA sample after using purification cleanup kit for *Penicillium* species sample and Lane 3 & 4 represents genomic DNA after using purification cleanup kit for *Aspergillus* species samples. M represents standard DNA markers (1 kb) ladder.

I.3.13 Purity and concentration for fungal DNA isolation

The absorbance at various wavelengths of 230, 260, 280 and 320 nm (table I.13 & I.14) was measured to estimate the purity and concentration of genomic DNA.

Purity and concentration for Phenol: chloroform method

The values of purity and concentration of genomic DNA for each sample isolated using phenol: chloroform (table I.13) was too low for further PCR amplification. The specified absorbance ratios for sample *Penicillium* and *Aspergillus* were found to be less than 1.8 (table I.13) indicating that the purity of all samples was low using the phenol: chloroform method. Analysing these ratios further, it was observed that the contaminating factor were either proteins or phenol as the absorbance at 280 nm was high.

Purity and concentration for Qiagen, DNeasy method

The values for each sample isolated using Qiagen *DNeasy* kit (table I.14) were at least 1.8 for $A^{(260/280)}$ and $A^{(260/230)}$. The gDNA samples produced using Qiagen, *DNeasy* kit, absorbance ratios of *Penicillium* and *Aspergillus* as per (table I.14) for $A^{(260/280)}$ and $A^{(260/230)}$ respectively were achieved; indicating that the DNA was pure. However low purity and concentration was measured for DNA isolated through phenol: chloroform compared to the Qiagen *DNeasy* Plant or fungi mini kit method. A lot of DNA was lost during the precipitation process as evidenced by the drastic reduction in band intensity and concentration prior to purification step. This was due to the dilution of gDNA sample from (50 μ l) carrying out 1:10 dilution with (30 μ l) as the final volume during purification process. Therefore, the Qiagen *DNeasy* kit was used to isolate gDNA was used for all the subsequent work.

Table I.13: Purity and concentration for fungal DNA isolation (phenol: Chloroform method)

Sample number	A ^(260/280)	A ^(260/30)	A ₂₃₀	A ₂₆₀	A ₂₈₀	A ₃₂₀	Concentration (µg/mL)
<i>Pencillium</i> strain 1	1.2	2.1	0.31	0.03	0.27	0.08	42
	1.4	2.3	0.04	1.05	1.00	0.04	41
<i>Aspergillus</i> strain 1	1.3	1.6	1.00	0.72	1.21	0.00	74
	1.2	2.5	0.21	1.65	0.00	0.02	132

Absorbance was measured at 230 nm, 260 nm, 280 nm and 320 nm. The absorbance ratios A^(260/280) and A^(260/230) and the concentrations were calculated by the spectrophotometer.

Table I.14: Purity and concentration for fungal DNA isolation (using Qiagen, DNeasy Plant or fungi mini kit method)

Sample number	A ^(260/280)	A ^(260/230)	A ₂₃₀	A ₂₆₀	A ₂₈₀	A ₃₂₀	Concentration (µg/mL)
<i>Pencillium</i> strain 1	1.9	2.6	0.50	1.08	0.60	0.085	54
	1.8	2.7	1.06	2.41	1.37	0.043	121
<i>Aspergillus</i> strain 1	1.6	2.2	1.07	2.48	1.46	0.040	124
	1.7	2.3	0.72	1.84	1.03	0.033	92

Absorbance was measured at 230 nm, 260 nm, 280 nm and 320 nm. The absorbance ratios A^(260/280) and A^(260/230) and the concentrations were calculated by the spectrophotometer.

I.3.14 Optimized PCR reaction conditions

The PCR conditions for the amplification of one sample are summarised below in table I.15.

Table I.15: PCR reaction temperature optimization conditions for fungal samples

Reagent	Volume of reagent (μL)								
10x PCR reaction buffer	10	10	10	10	10	10	10	10	10
25mM MgCl₂	4	4	4	5	5	5	6	6	6
25mM Forward primer	1	1	1	1	1	1	1	1	1
25mM Reverse primer	1	1	1	1	1	1	1	1	1
10mM dNTP's	2	2	2	2	2	2	2	2	2
gDNA template	2	2	2	2	2	2	2	2	2
5U/μL Taq polymerase	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4
Sterile distilled water	39.6	39.6	39.6	38.6	38.6	38.6	37.6	37.6	37.6
Total	50	50	50	50	50	50	50	50	50
Annealing temperature	64° C	65 °C	66⁰ C	64 °C	65° C	66 °C	64 °C	65 °C	66° C

PCR reactions were carried out for isolated gDNA at varied annealing temperatures (64, 65 and 66⁰C) and varying quantities of 25 mM MgCl₂, (4 to 6 μl) table (I.15). A 1% agarose gel was prepared and the gel was run at 100V for 60 minutes for the analysis of PCR amplicons (figure I.8).

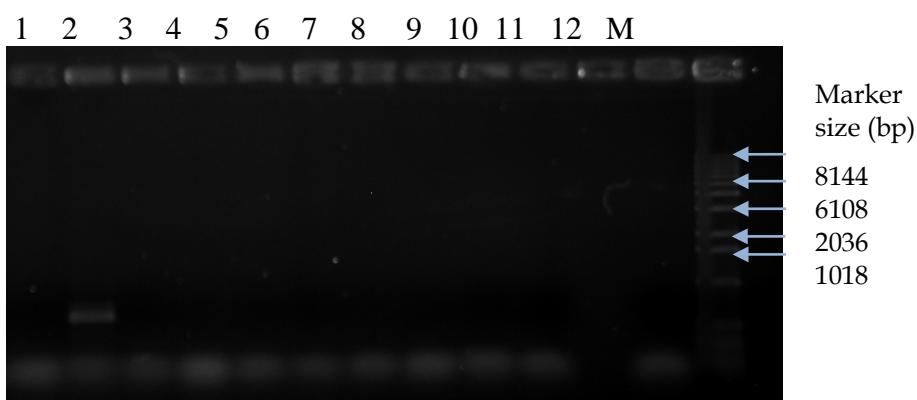


Fig. I.8: Agarose gel electrophoretic analysis of PCR reactions:

Lanes 1, 2 and 3 had samples containing 4μl, 5 μl and 6 μl of 25mM MgCl₂ at 65⁰C, Lanes 4, 5 and 6 with 4 μl, 5 μl and 6 μl of 25 mM MgCl₂ at 66⁰C, Lanes 7, 8 and 9 with 4 μl, 5 μl and 6 μl of 25 mM MgCl₂ at 64⁰C. Lane 10, 11, 12 were samples containing 6 μl of 25 mM MgCl₂ at 65⁰C, 65⁰C and 64⁰C respectively. M represents standard DNA markers (1 kb). A 1% agarose gel was prepared and run at 100V for 60 minutes.

Figure I.8 depicts a bright and clear single band of 746 bp at an annealing temperature of 65°C with 5 µl of 25 mM MgCl₂ in lane 2 (optimisation conditions: table I.15). No band was evident for all other samples (represented in lanes 1, 3-12).

I.3.15 PCR product

The purified genomic DNA was used as a template in the PCR reactions in order to amplify the gDNA template using specific forward and reverse primers. The expected sequences for each amplicon are described in appendix II section II.5

Several PCR reactions were performed in an attempt to amplify the gDNA template using the primers ITS1 and ITS4. The reactions were carried out at various annealing temperatures and also using gDNA templates prior to purification and after purification. Bands corresponding to the expected sizes (642 bp & 646 bp) of amplicon were observed in the reaction products (figure I.9); whilst primer dimers of less than 300 bp were observed, therefore a further purification step was conducted.

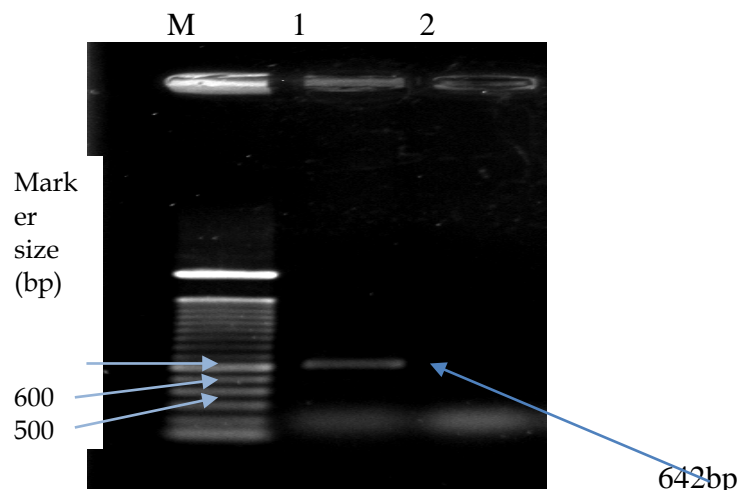


Fig. I.9: Agarose gel electrophoretic analysis of PCR product after the amplification of 18S rDNA from *Aspergillus* species.

M represents standard DNA ladder (100 bp). Lane 1 represents PCR product of *Aspergillus* gDNA template and lane 2 represent the negative control containing master mix (table 5) without template DNA. A 0.8% agarose gel was prepared and run at 100V for 60 minutes for the analysis of gDNA samples.

I.3.16 Purification of PCR product

PCR reaction product of all four samples after amplification were then purified (figure I.9) using the Qiagen PCR purification kit. A band of approximately 646 bp for *Pencillium* PCR product and 642 bp for *Aspergillus* PCR product were observed in lanes 1, 2, 3 and 4. Lane 5 contains negative control containing master mix without DNA template (figure I.10).

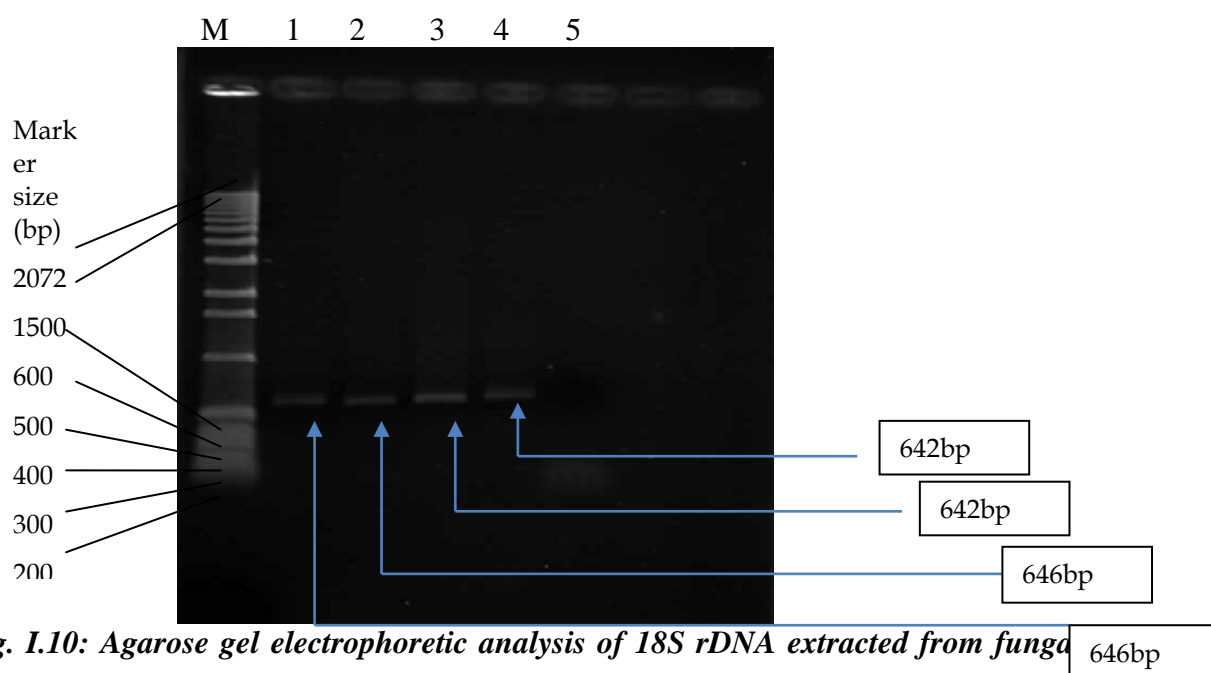


Fig. I.10: Agarose gel electrophoretic analysis of 18S rDNA extracted from fungal isolates.

M represents standard DNA ladder (100 bp). The PCR product in lane 1(646 bp), 2(646 bp), 3(642 bp) and 4(642 bp) represent the PCR product after purification of fungal samples. Lane 5 represents the negative control.

I.3.17 Identification and sequencing of 18S rDNA fungi

The PCR product obtained after amplification was 646 bp for *Pencillium* and 642 bp for *Aspergillus*. Samples were sent for sequencing (sequencing data see appendix. II section 1).

The DNA sequences determined in this study were compared to those in the GeneBank database using BLAST analysis and the accession numbers with detailed description of the most homogenous sequences are described in table 3.16 to 3.19:

Table 1.16: Identification of fungal 18S rDNA sequences for *Aspergillus* species using BLAST analysis against the GenBank database

Accession number	Description	Max. Score *	Query coverage^a	E Value^b	Max. identity^c
HQ014697.1	<i>Aspergillus niger</i> strain WM10.75 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	959	100%	0.0	99%
HQ014696.1	<i>Aspergillus niger</i> strain WM10.74 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	959	100%	0.0	99%
HQ315	<i>Aspergillus</i>	959	100%	0.0	99%

841.1	<i>tubingensis</i> internal transcribed spacer 1, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene , partial sequence				
HM801 881.1	<i>Aspergillus</i> sp. 06 SMR-2010 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	959	100%	0.0	99%

***Max. Score:** score of high scoring pairs, (HSPs), (Score of longest matching sequence)

^a**Query coverage:** percent of length coverage for the query

^b**E. Value:** number of hits one can "expect" to see by chance when searching a database of a particular size.

^c**Max. identity:** maximal percent Identity of the HSP

*Table I.17: Identification of fungal 18S rDNA sequences for *Aspergillus* species using Blast analysis against the GenBank database*

Accession number	Description	Max. Score*	Query coverage^a	E Value^b	Max. identity^c
JF411067.1	<i>Aspergillus tubingensis</i> strain CNU081066 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal	898	99%	0.0	96%

	RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence				
HQ89166 6.1	<i>Aspergillus niger</i> strain N internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	898	99%	0.0	96%
HQ72825 5.1	<i>Aspergillus tubingensis</i> isolate JH01 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	898	99%	0.0	96%
HQ01469 9.1	<i>Aspergillus niger</i> strain WM10.78 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	898	99%	0.0	96%

***Max. Score:** score of high scoring pairs, (HSPs), (Score of longest matching sequence)

^aQuery coverage: percent of length coverage for the query

^bE. Value: number of hits one can "expect" to see by chance when searching a database of a particular size.

^cMax. identity: maximal percent Identity of the HSP

Using BLAST (NCBI), the fungal rDNA sequences were compared to sequences in the GenBank database (NCBI) and the species were identified with 99% homology with a number of *Aspergillus niger* isolates and two *Aspergillus tubingensis* isolates with query coverage of 100%. The second *Aspergillus* isolate again showed homology with isolates of *Aspergillus tubingensis* (ITSII) and *Aspergillus niger*, but with a lower identity of 96% and a query coverage of 99% represented in table I.16 and I.17. However, the multiple sequence alignment including both *Aspergillus* isolates obtained using ClusterW (section I.3.18), shows that they have the same sequence with 100% homology and hence, the two *Aspergillus* species are the same strains over their overlapping query area.

1.3.18 CLUSTALW Multiple Sequence Alignments for *Aspergillus* spp.

```
gi|319429243|gb|HQ014697.1| -----AATATACAAGGTTTCCGTAGGTGAACCTGCGG
gi|329132758|gb|JF411067.1| TTTAGAGGAAGTAAAAGTCGTA-ACAAGGTTTCCGTAGGTGAACCTGCGG
** *****

gi|319429243|gb|HQ014697.1|  AAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGT
gi|329132758|gb|JF411067.1|  AAGGATCATTACCGAGTGCGGGTCCTTTGGGCCCAACCTCCCATCCGTGT
*****

gi|319429243|gb|HQ014697.1|  CTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTTCGGCCGCGGGG
gi|329132758|gb|JF411067.1|  CTATTATACCCTGTTGCTTCGGCGGGCCCGCCGCTTGTTCGGCCGCGGGG
*****

gi|319429243|gb|HQ014697.1|
GGGCGCCTTTGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAAC
gi|329132758|gb|JF411067.1|  GGGCGCCTTTGCCCCCGGGCCCGTGCCCGCCGGAGACCCCAACACGAAC
*****

gi|319429243|gb|HQ014697.1|  ACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAAC
gi|329132758|gb|JF411067.1|  ACTGTCTGAAAGCGTGCAGTCTGAGTTGATTGAATGCAATCAGTTAAAAC
*****

gi|319429243|gb|HQ014697.1|
TTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
gi|329132758|gb|JF411067.1|  TTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAA
*****

gi|319429243|gb|HQ014697.1|  TGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
gi|329132758|gb|JF411067.1|  TGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGA
*****

gi|319429243|gb|HQ014697.1|  ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
gi|329132758|gb|JF411067.1|  ACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTC
*****

gi|319429243|gb|HQ014697.1|  ATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCTCCCTCTCC
gi|329132758|gb|JF411067.1|  ATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCTCCCTCTCC
*****

gi|319429243|gb|HQ014697.1|
GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGC
gi|329132758|gb|JF411067.1|
GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGC
*****

gi|319429243|gb|HQ014697.1|  GTATGGGGCTTTGTACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACG
gi|329132758|gb|JF411067.1|  GTATGGGGCTTTGTACATGCTCTGTAGGATTGGCCGGCGCCTGCCGACG
*****

gi|319429243|gb|HQ014697.1|  TTTTCCAACCATTTTTTCCAGGTTGACCTCGGATCAGGTAGGGATACCCG
gi|329132758|gb|JF411067.1|  TTTTCCAACCATTTTTTCCAGGT-GACCTCGGATCAGGTAG-----
*****

gi|319429243|gb|HQ014697.1|  CTGAACTTAAGCATATCAATAAGCGGAGGT
gi|329132758|gb|JF411067.1|  -----
```

Table I.18: Identification of fungal 18S rDNA sequences for *Penicillium* species using BLAST analysis against the GenBank database

Accession number	Description	Max. Score*	Query coverage^a	E Value^b	Max. identity^c
AJ005479.1	<i>Penicillium freii</i> (IBT 3464) ribosomal internal transcribed spacers and the 5.8S ribosomal RNA gene (ITS1-5.8S-ITS2)	950	100%	0.0	97%
GU566214.1	<i>Penicillium</i> sp. BR 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	944	100%	0.0	97%
FJ389744.1	<i>Penicillium</i> sp. G4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	944	100%	0.0	97%
GU566234.1	<i>Penicillium aurantiogreiserum</i> strain D8 18S ribosomal RNA gene partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal	939	100%	0.0	97%

	transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene partial sequence				
--	--------------------------------------------------------------------------------------	--	--	--	--

***Max. Score:** score of high scoring pairs, (HSPs), (Score of longest matching sequence)

^a**Query coverage:** percent of length coverage for the query

^b**E. Value:** number of hits one can "expect" to see by chance when searching a database of a particular size.

^c**Max. Identity:** maximal percent Identity of the HSP

Table 1.19: Identification of fungal 18S rDNA sequences for Penicillium species using BLAST analysis against the GenBank database

Accession number	Description	Max. Score*	Query coverage ^a	E Value ^b	Max. identity ^c
GU5662 34.1	<i>Penicillium aurantiogriseum</i> strain D8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	922	100%	0.0	95%
GU5662 14.1	<i>Penicillium sp.</i> BR 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	911	100%	0.0	94%
AJ00547 9.1	<i>Penicillium freii</i> (IBT 3464) ribosomal internal	896	97%	0.0	95%

	transcribed spacers and the 5.8S ribosomal RNA gene (ITS1-5.8S-ITS2)				
GU5662 21.1	<i>Penicillium polonium</i> strain C6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	911	100%	0.0	94%

***Max. Score:** score of high scoring pairs, (HSPs), (Score of longest matching sequence) ^a**Query coverage:** percent of length coverage for the query

^b**E. Value:** number of hits one can "expect" to see by chance when searching a database of a particular size.

^c**Max. Identity:** maximal percent Identity of the HSP

Using BLAST (NCBI), the fungal rDNA sequences were compared to sequences in the GenBank database (NCBI) for *Penicillium* species and the species were identified with 97% homology with a number of *Penicillium freii* isolates and two *Penicillium aurantiogriseum* isolates with a query coverage of 100%. The second *Penicillium* isolate again showed homology with the internal transcribed spacer (ITS1) isolates *Penicillium Ferii* (ITSIII) and *Penicillium aurantiogriseum*, but with a lower identity of 95% and query coverage of 100% as shown in table I.18 and I.19. However, the multiple sequence alignment including both *Penicillium* isolates obtained for *Penicillium* sp. using Clusterw (section I.3.19), shows that they have the same sequence with 99% homology and hence, the two *Penicillium* species are the same strains over their overlapping query area.

I.3.19 CLUSTALW Multiple Sequence Alignments for *Pencillium* spp.

```
gi|3925736|emb|AJ005479.1| -----GGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAA
gi|291498422|gb|GU566234.1|  CTTGGTCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAA
*****
gi|3925736|emb|AJ005479.1|  CCTGCGGAAGGATCATTACCGAGTGAGGGCCCTTTGGGTCCAACCTCCCA
gi|291498422|gb|GU566234.1|  CCTGCGGAAGGATCATTACCGAGTGAGGGCCCTTTGGGTCCAACCTCCCA
*****
gi|3925736|emb|AJ005479.1|  CCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTTACTGGCCG
gi|291498422|gb|GU566234.1|  CCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCTTTACTGGCCG
*****
gi|3925736|emb|AJ005479.1|  CCGGGGGGCT-CACGCCCCCGGGTCCGCGCCCCGCCGAAGACACCCTCGAA
gi|291498422|gb|GU566234.1|  CCGGGGGGCTTACGCCCCCGGGCCCGCGCCCCGCCGAAGACACCCTCGAA
*****
gi|3925736|emb|AJ005479.1|  CTCTGTCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAACCTT
gi|291498422|gb|GU566234.1|  CTCTGTCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAACCTT
*****
gi|3925736|emb|AJ005479.1|  TCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG
gi|291498422|gb|GU566234.1|  TCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG
*****
gi|3925736|emb|AJ005479.1|  CGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACG
gi|291498422|gb|GU566234.1|  CGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACG
*****
gi|3925736|emb|AJ005479.1|  CACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATT
gi|291498422|gb|GU566234.1|  CACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATT
*****
gi|3925736|emb|AJ005479.1|  GCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCTCCGATTCCGGG
gi|291498422|gb|GU566234.1|  GCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCTCCGATTCCGGG
*****
gi|3925736|emb|AJ005479.1|  GGACGGGCCC GAAAGGCAGCGGCGGCACCGCGTCCGGTCCCTCGAGCGTAT
gi|291498422|gb|GU566234.1|  GGACGGGCCC GAAAGGCAGCGGCGGCACCGCGTCCGGTCCCTCGAGCGTAT
*****
gi|3925736|emb|AJ005479.1|  GGGGCTTTGTACCCGCTCCGTAGGCCCGGCCGGCGCTTGCCGATCAACC
gi|291498422|gb|GU566234.1|  GGGGCTTTGTACCCGCTCCGTAGGCCCGGCCGGCGCTTGCCGATCAACC
*****
gi|3925736|emb|AJ005479.1|  CAAATTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC
gi|291498422|gb|GU566234.1|  CAAATTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAAC
*****
gi|3925736|emb|AJ005479.1|  TTAAGCATATCAATAAGCGGAGGA
gi|291498422|gb|GU566234.1|  TTAAGCATATCAATAAGCGGAGGA
*****
```

I.4 Discussion

Objective: Isolation of PAHs degrading micro-organisms by shaken enrichment technique

I.4.1 Enrichment culture technique

An aqueous shaken enrichment method was used to isolate PAH-degrading bacteria and fungi from PAHs contaminated road-side soil. PAHs are one of the toxic contaminants found in road-side soil and dust particles. Road-side soil is considered as highly contaminated with atmospheric PAHs due to higher percentage of automobile emissions. Also, higher PAHs concentration in soil is reported suggesting traffic density, traffic behavior, road condition, meteorological condition, particulate matter present in environment and their deposition rates (Kumar & Kothiyal, 2011). In this method, PAHs were the sole carbon and energy sources used for the microbial growth.

The total of six bacterial strains and two fungal strains were isolated. Moreover, liquid enrichment culture also led to isolation of bacterial and fungal strains at varying pH specifically (acidic, neutral and alkaline). These isolates were confirmed as PAH-degrading organisms by growing them on PAH sprayed minimal medium agar plates (for bacteria) and malt extract agar along with rose bengal (for fungi) plates. The aqueous shaken enrichment method was performed for 6 weeks with each lasting 14 days (section I.2.6). The studies carried out by Jacques & Okeke, (2009) indicated the enrichment technique by reducing incubation time to 3 weeks compared to 6-12 weeks incubation does not limit the number of strains isolated as reported in other studies. Thus, less than 6 week of incubation time would affect the shaken enrichment technique to obtain the pure cultures of PAH-degraders (Jacques & Okeke, 2009).

Isolation of bacterial and fungal population confirmed by c.f.u counts and performing spread plate allowed pure cultures of PAH-degraders to be obtained. Higher bacterial population was observed in first enrichment when compared to second and third enrichment. During shaken enrichment cultures, bacteria grow slowly by adapting to crystalline PAH added as the sole carbon source resulting into growth curve characteristics (Johnsen *et al.*, 2005). Higher number of bacteria were found in neutral pH (pH 7), whereas lower populations were observed at acidic pH of basal salt medium (pH 5.5). However, acidic pH 5.5 resulted in high growth of *Penicillium* species and *Aspergillus* species also at alkaline pH (pH 8.5). Similar work was indicated by Bastiaens *et al.*, (2000) however, their studies reported isolation of PAHs degraders mostly bacteria via enrichment on the sorbing carrier and shaken liquid enrichment method. Thus, by performing two different enrichment methods they were able to isolate *Pseudomonas species*, *Sphingomonas species*, and *Mycobacterium* group and proved that both enrichment culture methods are useful to select and isolate new hydrocarbon degrading bacteria and fungi (Bastiaens *et al.* 2000).

Most of the studies are reported on temperature effect. Similarly, PAHs degradation under aerobic conditions and nitrate reducing conditions at low temperature in enrichment cultures were reported by Eriksson *et al.*, (2003). In their studies, shaken enrichment culture was performed at different low temperatures and research indicated that after 90 days 52-88% of PAHs were removed at 20⁰C. Also, 53% of PAHs removal was obtained at 7⁰C. However less research has been carried out with respect to pH effect and its importance.

Isolates obtained via shaken enrichment culture at acidic, neutral and alkaline conditions were identified using biochemical and molecular analysis. Also, these isolates were used for further experiments to investigate PAH degradation.

Objective: Identification of bacterial strains degrading PAHs – using PCR amplification and sequencing of 16S rDNA

I.4.2 Characterization of Bacterial cells and genomic DNA extraction

(Phenol: Chloroform genomic DNA extraction)

The enriched pure cultures were subjected to biochemical tests followed by genomic DNA isolation. These characterizations were carried out to identify the bacteria and fungi and to study whether representative strains isolated from the enrichments were distinct organisms that could be used for further experimental work. The bacterial strains were categorised into Gram positive and Gram negative. Culture 1, 2, 4, 5 and 6 groups were gram-negative bacteria and culture 3 was gram-positive strains. The cultures were examined for catalase and oxidase reaction, in which all the culture were catalase positive and culture 3, 5 & 6 were oxidase negative whilst culture 1 & 2 were oxidase positive. Based on biochemical tests performed, each of these bacterial cultures was identified in accordance to NHS (BSOP ID 1i1.4) issued by standards unit, evaluation and standards laboratory, centre for infection.

gDNA extraction was performed on 6 bacterial isolates using a method described by Cheng and Jiang (2006) followed by ethanol precipitation. The saturated phenol used during extraction, consisted of 72% phenol and 28% water, as phenol is weak acid, the solution was equilibrated with buffer to bring the pH to a particular target (pH 7.2), which is alkaline for DNA purification. Chloroform (isoamyl alcohol) was used to reduce the interphase- white fuzzy border formed during two phases partially help to denature the proteins. Low molecular weight DNA and RNA contaminates were removed by performing ethanol precipitation step and further confirmation of contaminants was observed by their respective absorbance readings in table I.9. DNA of high molecular weight recovered from environmental samples, was usually

concentrated by ethanol precipitation and requires no further purification step (Somerville *et al.*, 1998).

Phenol chloroform extraction provided high quality gDNA with no contamination of polysaccharides and phenolic compounds. Sharp single bands were observed in all the samples with high molecular weight and respective absorbance reading (table I.9).

The purified gDNA was further used as a template for PCR amplification to obtain sequences for identification of the particular organisms.

I.4.3 Purity and concentration for bacterial DNA isolation

Contamination with carbohydrates, phenolate ions, thiocyanates, and other organic compounds caused in DNA sample is determined by absorption at 230 nm (Xiao *et al.*, 2011).

PCR amplification needs sufficient purity of gDNA measured at A260 nm/ A230 nm (Jain *et al.*, 2002). The purity and concentration obtained for all the isolates were high as as tabulated in table I.9. A lower ratio at A260 nm/ 230 nm denotes contamination of DNA by polysaccharides and lower ratio at A260/280 nm denotes contamination of gDNA by proteins (Kamal *et al.*, 2008). Xiao *et al.*, (2011) in their studies used mucoid *Vibrio parahaemolyticus* and *Klebsiella pneumonia* as model organisms and showed the effective comparison of two DNA extraction methods. In their studies they used methoxy-ethanol in one method and ethyl ether in second method for removal of polysaccharides.

In this study, the phenol: Chloroform method for gDNA extraction showed little contamination with polysaccharides and phenolic compounds and other proteins as the ratios shown by the spectrophotometer readings obtained at A260 nm / A230 nm were 1.96, 1.84, 1.62, 2.39, 1.98, 2.06 respectively and at A260 / 280 nm were 2.05, 1.97,

1.87, 2.74, 2.07, 2.11 respectively resulting in gDNA was pure without significant contamination.

I.4.4 Bacterial PCR product

Initial attempts were performed to amplify gDNA using PCR (Thermal cycler/PCR machine from Eppendorf) resulting into no amplicons for PCR reactions performed. However, PCR reaction optimization was carried out using varied annealing temperatures with a constant 3 μ l of 25 mM magnesium chloride concentration. After optimization conditions amplicons of approximately the expected molecular size were obtained. The results obtained after PCR amplification confirmed the high purity and concentration of DNA template. Moreover the amplicons proved to be of working quality for further analysis. It also confirmed that the dNTP mixture, magnesium chloride, Taq polymerase and reaction buffer were working. Hence, the universal primers 27F, 5'-AGAGTTTGATYMTGGCTCAG-3' and 1492R, 5'-TACGGYTACCTTGTTACGACT-3' were proved to amplify the template DNA.

Bej *et al.*, (1991) reported denaturation temperature and re-annealing temperature are most crucial step for PCR amplification. Further, in the studies conducted with respect to PCR optimization the denaturation temperature was set at 94⁰C as a standard denaturation temperature. However, at very high temperatures DNA is highly prone to damage during the denaturation step. At high temperatures, cytosine is converted to uracil which is an analogue of thymine (Eckert & Kunel, 1991). Hence, such a base substitution can lead to errors in the amplified sequences. Thus, re-annealing temperature plays an important role in PCR amplification. Kurata *et al.*, (2004) reported the importance of re-annealing temperature resulting in 1:1 mixtures of genes in final PCR products. The specificity of PCR reaction is completely based on re-

annealing temperature. The re-annealing temperature is partially dictated by the primer pairs and therefore needs to be optimized (Kurata *et al.*, 2004). The re-annealing temperature was further optimized extensively for each of the samples. However, it was found that primers bind to non-target DNA producing multiple amplification products due to low annealing temperatures. If the temperature is too high the primers cannot anneal to the target template and therefore no amplification would be observed. Similar work was reported by Wu *et al.*, (2005) suggesting single- stranded mutagenic DNA synthesis at high annealing temperature and low annealing temperatures. Further, after optimization of PCR amplicons were observed at 48⁰C with 3 µl of 25mM MgCl₂ concentration (table I.12). However, larger volume of PCR amplified sample were loaded in each well as observed on agar gel electrophoresis (figure I.3). Amplification of four genomic DNA samples after purification resulted in amplicons that were approximately of the expected molecular sizes obtained by running the reaction sample on agarose gel electrophoresis was found with 1421 bp, 1476 bp, 1543 bp, 1472 bp, 1385 bp and 1389 bp sizes when compared to 100 bp marker (figure I.4).

I.4.5 Identification and sequencing of 16S rDNA

PCR reactions for all four samples were then purified using PCR purification kits. During PCR purification of product the primer dimer present in the amplified samples was lost. In this study the DNA sequences determined by GATAC (biolabs ltd., UK) using BLAST (NCBI) analysis, the rDNA sequences were compared to sequences in the GenBank database (NCBI) and bacterial PAH degraders were identified. Heitkamp and Cerniglia, (1988) were among first to document their studies on PAH degrading bacterium isolated from contaminated sites. Their studies involve degradation of

HMW PAHs and found 0.5 mg/litre degradation of fluoranthene, pyrene, 3-methylcholanthrene, 6-nitochrysene respectively.

In this study, a total of 6 bacterial cultures and 2 fungal cultures utilising phenanthrene, anthracene, fluoranthene and pyrene as a sole carbon source were isolated (section I.3.6). All cultures were isolated from same soil samples (described in section I.3.6) with enrichment set at a three pH's.

The culture 1 isolated at pH 5.5, after genomic DNA extraction and PCR reactions, the PCR product obtained after the purification of amplified region and sequencing of 1318 bp product was conducted and BLAST analysis undertaken. A match with 100% query coverage and 98% maximum identity was found with *Uncultured bacterium* isolate with 16S ribosomal RNA gene, partial sequence. The reverse sequence obtained showed size of 1562 bp and was found to match *Pseudomonas putida* strain 16S rRNA gene with 100% query coverage and 98% maximum identity. *Pseudomonas putida* is a gram negative soil bacterium which has been used in bioremediation of PAHs (Anzai *et al.*, 2000).

Culture 2 isolated at pH 7.0 found with sequencing of 1456bp product when blast analysis was undertaken. The sequence found to match *Achromobacter xylosoxidans* gene strain NBRC 13495, with 98% query coverage and 99% maximum identity. The reverse sequence (1440 bp) found to match as *Alcaligenes* sp. when compared to Genbank database with 98% query coverage and 99% maximum identity. Weissenfels *et al.*, (1990) reported *Alcaligenes denitrificans* strain, that biodegraded fluoranthene at a rate of 0.3 mg/ml per day.

Culture 3 isolated at pH 7.0 after sequencing (1385 bp) was identified as *Microbacterium foliorum* strain with 100% query coverage and 96% maximum identity when compared to Genbank database. The reverse sequence (1414 bp) also

was identified as *Microbacterium* sp. with 100% query coverage and 96% maximum identity. Similarly, Bastiaens *et al.*, (2000) studied two different enrichment methods for isolation of bacteria from PAH contaminated soil and sludge sample. In their studies one method helped in selection of *Sphingomonas* spp. whereas, the second lead to selection of *Micobacterium* spp. Also, shaken enrich and membranes containing sorbed PAH, both enrichment techniques proved as useful method to isolate PAH degraders (Bastiaens *et al.*, 2000). *Microbacterium* cultures simulating pyrene mineralization over 60% radio-labelled pyrene in 96 hours was reported by Kanaly & Harayama, (2000). Jackson *et al.*, (2000) suggest, *Sphingomonas* species are commonly found and is capable of degrading HMW PAHs in equal amount when compared to *Mycobacterium* species. However it has also been reported to oxidize fluoranthene through fluorenone indicating dual attack on fluoranthene molecules.

Culture 4 isolated at pH 7 was identified as an *Alpha proteobacterium* sp. over a 1451bp product with 99% query coverage and 98% maximum identity and the reverse sequence was identified as *Brevundimonas diminuta* strain with product size of 1295bp and 100% query coverage and 98% identity. *Brevundimonas diminuta* El-3.1 and *Pseudomonas fluorescens* El-2.1 has been reported by Tumaikina, (2006) as a crude oil degrading organisms isolated from Canadian pondweed. It was indicated that these organisms had a wide range and high degree of degradation activities with each of these organisms.

Culture 5 isolated at pH 8.5 was identified as a *Brevundimonas* sp. (1223 bp) product with query coverage 98% and 99% maximum identity. The reverse sequence (865 bp) also was identified as *Brevundimonas* sp. with 100% query coverage and 91% identity. *Brevundinonas* sp. is a Subclass of the *Proteobacteria* that consists of rRNA group

species which were transposed from *Proteobacteria* to genus *Brevundimonas* (Anzai *et al.*, 2000).

Culture 6 isolated at pH 8.5 was identified as *Bradyrhizobium* sp. over 1451 bp product with a 100% query coverage and 100% identity. The reverse sequence similarly was identified as *Bradyrhizobium* sp. over 783 bp sequenced product with 100% query coverage and 100% identity. Similarly, Tang, (2011) has reported *Bradyrhizobium* sp. as a predominant chrysene-degrading strain. Their studies indicated that *Bradyrhizobium* sp. isolated from activated sludge resulted about 85.2% of chrysene degradation within 8 days maintained at 35⁰C at pH 7.0.

Among above mentioned genera or groups of bacteria has been previously reported as PAH degraders, hydrocarbon and petroleum degraders (Mueller *et al.*, 1997; Kastner *et al.*, 1994; West *et al.*, 1984; Dyksterhouse *et al.*, 1995). Identification of the above described isolated and their capabilities of degradation were further demonstrated by biodegradation experiment. Moreover, studies reported by Lal and Khanna (1996); Bharathi and Vasudevan (2001); and Rahman *et al.*, (2002) have also identified most of the genera including *Pseudomonas putida*, *Brevundimonas*, *Proteobacterium* as hydrocarbon-degrading micro-organisms.

Characterization of fungi isolated and genomic DNA extraction

Objective: Identification of fungal strains degrading PAHs – using PCR amplification and sequencing of 18S rDNA

I.4.6. Genomic DNA isolation (Phenol: chloroform method)

The genomic DNA was extracted from the four fungal isolates by modification from the methods of Plaza, & Upchurch *et al.* (2004). The quality of the DNA obtained by phenol: chloroform procedure was not suitable for PCR amplification as demonstrated in figure I.12. A large smear of fungal gDNA was observed which includes low molecular weight fractions. This indicates DNA was sheared, resulting in a smear of DNA from low to high molecular weight. Therefore, it was necessary to eliminate the contaminants as an important step in the separation of a single band gDNA (Kamal *et al.*, 2008).

Qiagen, DNeasy Kit method

The DNA isolation from fungus was repeated using a Qiagen, DNeasy Plant or fungi mini kit according to the manufacturer's protocol. Sharp bands of high molecular weight genomic DNA were observed in lane 1 to 7 respectively (figure I.13). Yield and quality of extracted genomic DNA depends entirely on successful detection of DNA bands (Jain *et al.*, 2002). However, a smear of DNA of low molecular weight due to the presence of nucleic acid contaminants were observed in the samples in lane 8, 9, and 10 as demonstrated in figure 42A. As reported by various authors prior to precipitation of contaminants from gDNA samples due to polysaccharides, the phenolic compounds and other secondary metabolite adversely affect the purity and suitability of isolated DNA during ethanol precipitation for future

molecular use (Dellaporta *et al.*, 1983; Do & Adams, 1991; Pandey *et al.*, 1996; Weishing *et al.*, 1995). Most enzyme activity such as polymerases, ligases and endonucleases are inhibited by polysaccharides and hence are found to be more problematic (Do and Adams., 1991; Fang *et al.*, 1992; Scott & Playford, 1996). The gDNA samples were then purified using purification kit in an attempt to purify the gDNA from the contaminating proteins (figure I.13 B). A sharp DNA band of high molecular weight was observed after the purification process as represented in figure 42B whilst the low molecular weight DNA smear of contaminating proteins was lost indicating that the contaminants were precipitated out. The genomic DNA was highly pure depending on the purification step and removal of endogenous nucleases or other proteins.

The integrity of DNA isolated by Qiagen DNeasy kit was greater than the phenol: chloroform method as indicated by a clear single band without a smear.

3.4.1 Purity and concentration for fungal gDNA isolated

The absorbance at wavelengths of 230, 260, 280 and 320 nm (table I.5 & I.6) was estimated for the purity and concentration of genomic DNA.

Purity and concentration for Phenol: chloroform method

The ratio measured at A260nm/ A230nm needs to be sufficient to proceed with PCR amplification (Jain *et al.*, 2002). A lower ratio at A260nm/ 230nm denotes contamination of DNA by polysaccharides and lower ratio at A260/280nm denotes contamination of gDNA by proteins (Kamal *et al.*, 2008). The phenol: Chloroform method was contaminated with polysaccharides as shown by the spectrophotometer readings obtained at A260 nm / A230 nm of 1.2, 1.4, 1.34, 1.27 and A260 nm / A280 nm of 2.1, 2.31, 1.6, and 2.5 respectively (table I.13). Low purity and concentration

was measured for DNA isolated through phenol: chloroform method. The purity and concentration for genomic DNA should be greater than that of 1.8 µg/ml (Kamal *et al.*, 2008). Thus, values for each sample isolated using phenol: chloroform method was too low for further PCR amplification.

Purity and concentration for Qiagen, DNeasy method

PCR amplification needs sufficient ratio of gDNA measured at A_{260nm}/ A_{230nm} (Jain *et al.*, 2002). The values for each sample isolated using Qiagen *DNeasy* kit (table I.14) were at least 1.8 µg/ml for A (²⁶⁰/₂₈₀) and A (²⁶⁰/₂₃₀). The gDNA samples produced using Qiagen, *DNeasy* kit, absorbance ratios of *Penicillium* strain 1 & 2 and *Aspergillus* strain 1 & 2 as per (table 21) for A (²⁶⁰/₂₃₀) nm were 2.61, 2.74, 2.21, 2.38 nm respectively; and at A (²⁶⁰/₂₈₀) nm were 1.9, 1.87, 1.69 and 1.71 respectively, indicating that the DNA was pure without any contamination of protein and polysaccharides. Qiagen, *DNeasy* clean up kit was used to treat impurities and RNA present in gDNA samples in order to purify and get more efficient gDNA samples.

However low purity and concentration was measured for DNA isolated through phenol: chloroform compared to Qiagen, *DNeasy* Plant or fungi mini kit method. The values for ratio of A (260/230 nm) for each sample isolated using Qiagen kit was at or greater than 1.8 µg/ml compared to the phenol: chloroform method. Therefore, the yield and quality of the gDNA obtained using Qiagen *DNeasy* isolation kit procedure were suitable and used for all the subsequent work.

3.4.2 Polymerase Chain Reaction (PCR)

Two universal PCR primers were used to amplify the DNA isolates ITS1 and ITS4 respectively. Both primer pairs exhibited strong specificity for fungal rDNA sequencing. Results of DNA electrophoresis in 1% agarose gel are demonstrated

(figure I.10). Genomic DNA isolation in previous research has carried out based on gradient optimization for strong and reliable amplification product (Murray 1980). PCR products before the purification step are presented (figure I.11).

The PCR conditions for the amplification of one sample were optimized as tabulated in Table I.15 in order to carry out PCR amplification of isolated gDNA samples. According to Mullis, (1997) specific gDNA amplification is possible with the optimized conditions. As the conditions can be optimized by using varied annealing temperature and/or varied MgCl₂ concentration with different volumes (Murry, 1980). Thus, for specific amplification of isolated gDNA MgCl₂ concentrations and annealing temperatures (table 22) were optimised. Specifically, the amplicon was obtained at an annealing temperature of 65°C in lane 2 (figure I.12) with 5 µl of 25 mM MgCl₂ (table 22) with no amplicon obtained in other lanes loaded with other PCR samples at varied temperatures. Amplicons of approximate molecular sizes (642 bp) were obtained in lanes 1 (figure 44). However, primer dimer was present below 100 bp (figure I.13). Amplification of four genomic DNA samples further resulted in amplicons that were approximately of the expected molecular sizes ranging between 642 bp to 646 bp obtained in lane 1, 2, 3 & 4 (figure I.15). Amplified gDNA with similar range of molecular size was observed by White *et al.*, (1990) to identify California populations with an ITS-2 fragment size of about 658 bp and the Texas populations of ascomycetes with a size of about 1099 bp.

3.4.3 PCR Identification

PCR reactions for all four samples were then purified using PCR purification kits. During PCR purification of product the primer dimer present in the amplified samples was lost.

The PCR product obtained after the purification of amplified region was of 646 bp for *Pencillium* species and 642 bp for *Aspergillus* species. Samples were sequenced (sequencing data see appendix II: section II.6). Using BLAST (NCBI), the fungal rDNA sequences were compared to sequences in the GenBank database (NCBI) and the species were identified with 99% homology with a number of *Aspergillus niger* isolates and two *Aspergillus tubingenisis* isolates with query coverage of 100%. The second *Aspergillus* isolate again showed homology with isolates of *Aspergillus tubingenisis* (ITSII) and *Aspergillus niger*, but with a lower identity of 96% and query coverage of 99% represented in table I.17 and I.18. However, the multiple sequence alignment including the both *Apergillus* isolates obtained using ClusterW analysis (section I.3.18), show that they have same the sequence with 100% homology over their overlapping query area and hence, the two *Apergillus* isolates are the same strain. Similarly, using BLAST (NCBI), the fungal gDNA sequences were compared to sequences in the Genebank database (NCBI) for *Pencillium* species and the species were identified with 97% homology with a number of *Penicillium frei* isolates and two *Penicillium aurantiogriseum* isolates with query coverage of 100%. The second *Pencillium* isolate again showed homology with the internal transcribed spacer (ITSI) of isolates *Penicillium Ferii* (ITSIII) and *Penicillium aurantiogriseum*, but with a lower identity of 95% and query coverage of 100% as shown in tables I.19 and I.20. However, the multiple sequence alignment including the both *Pencillium* isolates obtained for *Pencillium* sp. using ClusterW analysis (section I.3.19), show that they have the same sequence with 99% homology over their overlapping query area (section I.1.9) and hence, the two *Pencillium* isolates are the same strain.

The fungal strains identified in this report provide evidence that universal PCR primers isolates ITS1 and ITS4 are very useful for identifying fungi from

environmental samples, such as soil and plant tissues. Thus, these primers are likely to be useful for construction of fungal rDNA libraries from the environmental samples, for denaturation gradient gel electrophoresis analysis or can be useful as fungus-specific hybridization probes (Borneman and Hartin, 2000). Biodegradation of hydrocarbons can be executed by various microorganisms. *Aspergillus* sp. and *Penicillium* sp. along with other species have been isolated previously as degraders of PAHs and degraders of other pollutants. They are common soil organisms. Interestingly, a study was conducted for biodegradation of PAHs between the complexity and recalcitrance of PAH compounds using number of *Aspergillus* spp. (Bastiaen *et al.*, 2000). Similarly, George-Okafor identified *Aspergillus niger* that provides better hydrocarbon degradation of PAHs with efficiency of (>98%) (George-Okafor, 2009). A similar study by Okaro, (2008) indicated that *Aspergillus fumigatus* is more efficient than *Rhizopus* spp. in the degradation of aromatics. Niu *et al.*, (1993) studied removal of toxic waste necessary for bioremediation process by using *Penicillium chrysogenum*. Similarly, *P. italicum* and *P. aurantiogriseum* was reported for fluoranthene degradation in presence of different cyclodextrins (Garon *et al.*, 2002). Hence, the species identified in this report may be important organisms in the bioremediation processes.

Appendix II

II.1 Media composition for shaken enrichment cultures

The composition of mineral medium was adapted from Bastiaens *et al.*, (2000) containing (per litre) 8.8 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 3.0 g of KH_2PO_4 , 1.0 g of NH_4Cl , 0.5 g of NaCl , 1.0 ml of 1 M MgSO_4 , and 1ml of a trace element solution ([per litre] 23 mg of $\text{MnCl}_2 \cdot 2\text{H}_2\text{O}$, 30 mg of $\text{MnCl}_4 \cdot \text{H}_2\text{O}$, 31 mg of H_3BO_3 , 36 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mg of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mg of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 30 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 50 mg ZnCl_2).

II.2 Biochemical test for bacterial isolates:

II.2.1 Gram staining

Isolates were grown for 24- 48 hrs in nutrient broth at 30°C . A bacterial smear was prepared by applying a drop of sterile water and inoculating the culture on glass slide and heat fixing it. Crystal violet stain was applied for 30seconds followed by wash step with running tap-water. The slide was flooded with Lugol's iodine for 30 seconds and immediately washed up with 95% alcohol. 95% alcohol was added until the slide appeared to be colourless. Further the slides were washed with tap-water followed by carbon fuchsin addition for 10 seconds. The final step was carried out by washing the slide with running tap-water.

The bacterial were observed at X1000 on microscope (Meiji EMT model number: 18089). There reaction to gram stain were observed

II.2.3 Catalase

Catalase test was performed using hydrogen peroxide (H_2O_2) reagent. The isolates were added to the reagent to confirm for the presence of catalase enzyme. The

presence of gas bubbles represents the presence of catalase enzyme resulting in oxygen release.

II.2.4 Oxidase

Oxidase test was performed using 1% of tetramethyl p-phenylenediamine aqueous solution. 10cm diameter Whatman filter paper was used in Petri dish and the drops of reagent was added to filter paper. The isolated culture was added to the filter paper using a wire loop. The appearance of dark purple colour within few seconds was considered as the indication of presence of cytochrome C enzyme resulting into position reaction test.

II.2.5 Oxidation or Fermentation (O-F) test

Oxidation or fermentation test was performed by preparation of O-F medium for testing the isolated cultures. The medium contained 5g of NaCl, 0.3g of K_2HPO_4 , 2g of peptone, 3g of agarose and 0.2% of bromothymole blue. The aqueous solution was made up to 100ml in distilled water. The medium prepared was heated to $95^{\circ}C$ and 1% of glucose was added to the medium. The medium was dispensed into universal bottles. The universal bottles were sterilized by autoclaving. After autoclaving (sterilization), the culture was stabbed into the universal bottle using a straight nichrome wire. Mineral oil (paraffin oil) was added to a duplicate universal bottle to create anaerobic conditions. The universal bottles were incubated for 14 days at $30^{\circ}C$. After incubation the tubes were observed for yellow colour indicating glucose oxidation. If both duplicates (with and without mineral oil) turned yellow the result was recorded as fermentation whereas, if the bottle did not turn yellow colour and bottle with mineral oil turned yellow the result was recorded as oxidation. Universal bottles with culture without mineral oil and one topped with mineral oil both turned

yellow representing fermentation whereas, bottle without mineral oil showing no yellow colour and bottle with mineral oil showing yellow colour indicated oxidation in the bottle. Thus, no colour change in either bottle showed that the bacteria do not metabolise glucose in either conditions.

II.3 Bacterial DNA isolation and PCR amplification

PRIMER DETAILS	PRIMER 1 (27F)	PRIMER 2 (1492R)
Sequence (5' to 3')	AGAGTTTGATYMTG GCTCAG	TACGGYTACCTTGT TACGACT
Complementary sequence		
Tm: Desalt adjusted	66	68
M.W	6168.5µg/mole	6380.7 µg/mole
GC content	45%	45%
Length	20	21

II.4 Bacterial sequencing results

Using BLAST (NCBI), the fungal rDNA sequences in the GenBank database (NCBI) were compared as followed:

II.4.1 *Pseudomonas* sp. 16S ribosomal RNA gene, partial sequence

GenBank: JQ247014.1

LOCUS JQ247014 1467 bp DNA linear BCT 07-FEB-2012

DEFINITION *Pseudomonas* sp. SRI 360 16S ribosomal RNA gene, partial sequence.

ACCESSION JQ247014

VERSION JQ247014.1 GI:374430961

SOURCE *Pseudomonas* sp. SRI 360

ORGANISM *Pseudomonas* sp. SRI 360

Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;

Pseudomonadaceae; *Pseudomonas*.

ORIGIN

1 gctcagattg aacgctggcg gcaggcctaa cacatgcaag tcgagcggat gacgggagct
61 tgctccttga ttcagcggcg gacgggtgag taatgcctag gaatctgcct ggtagtgggg
121 gacaacgttt cgaaaggaac gctaataccg catacgtcct acgggagaaa gtgggggatc
181 ttcggacctc acgctatcag atgagcctag gtcggattag ctagtggtg aggtaatggc
241 tcaccaaggc gacgatccgt aactggctg agaggatgat cagtcacact ggaactgaga
301 cacggtccag actcctacgg gaggcagcag tggggaatat tggacaatgg gcgaaagcct
361 gatccagcca tgccgcgtgt gtgaagaagg tcttcggatt gtaaagcact ttaagttggg
421 aggaagggca gtaagttaat accttgctgt tttgacgta ccgacagaat aagcaccggc
481 taactctgtg ccagcagccg cggtaataca gagggtgcaa gcgttaatcg gaactactgg
541 gcgtaaagcg cgcgtaggtg gttcgttaag ttggatgtga aagccccggg ctcaacctgg
601 gaactgcatc caaaactggc gagctagagt acggtagagg gtggtggaat ttctgtgta
661 gcggtgaaat gcgtagatat aggaaggaac accagtggcg aaggcgacca cctggactga
721 tactgacact gaggtgcgaa agcgtgggga gcaaacagga ttagatacc tggtagcca
781 cgccgtaaac gatgtcaact agccgttga atccttgaga ttttagtggc gcagctaacg
841 cattaagttg accgcctggg gactacggcc gcaaggttaa aactcaatg aattgacggg
901 ggccccaca agcgggtggag catgtggttt aattcgaagc aacgcgaaga acctaccg
961 gccttgacat gcagagaact tccagagat ggattggtgc ctccgggaac tetgacacag
1021 gtgctgcatg gctgtcgtca gctcgtgctg tgagatgtg ggtaagtcc cgtaacgagc
1081 gcaaccttg tcttagtta ccagcacgtt atggtgggca ctctaaggag actgccggtg
1141 acaaaccgga ggaaggtggg gatgacgtca agtcatcatg gcccttacgg cctgggctac
1201 acacgtgcta caatggtcgg tacagagggt tgccaagccg cgaggtggag ctaatctcac
1261 aaaaccgatc gtagtccgga tcgcagtctg caactcgact gcgtgaagtc ggaatcgcta
1321 gtaatcgca atcagaatgt cgcggtgaat acgttcccgg gccttgata caccgccctg
1381 cacaccatgg gactgggtg caccagagta gctagtctaa ccttcgggag gacggttacc
1441 acggtgtgat catgactggg tgagtcg

II.4.2 *Pseudomonas putida* strain YNA121 16S ribosomal RNA gene, partial sequence

GenBank: JN899561.1

LOCUS JN899561 897 bp DNA linear BCT 29-JAN-2012

DEFINITION *Pseudomonas putida* strain YNA121 16S ribosomal RNA gene, partial sequence.

ACCESSION JN899561

VERSION JN899561.1 GI:373880214

KEYWORDS .

SOURCE *Pseudomonas putida*

ORGANISM *Pseudomonas putida*

Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; *Pseudomonas*.

ORIGIN

1 acctcgcgta ggtggtttgt taagttggat gtgaaagccc ggggctcaac tgggaactgc
61 atccaaaact ggcaagctag agtacagtag aggggtggtgg aatttcatgt gtagcgggta
121 aatgcgtaga tataggaagg aacaccagtg gcgaaggcga ccacctggac tgatactgac
181 actgaggtgc gaaagcgtgg ggagcaaca ggattagata cctggtagt ccacgccgta
241 aacgatgtca actagccgtt ggaatccttg agattttagt ggcgcagcta acgcattaag
301 ttgaccgcct ggggagtacg gccgcaaggt taaaactcaa atgaattgac gggggcccgc
361 acaagcggtg gagcatgtgg ttaattcga agcaacgcga agaaccttac caggccttga
421 catgcagaga acttccaga gatggattgg tgccttcggg aactctgaca caggtgctgc
481 atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgtaacg agcgcaccc
541 ttgtccttag ttaccagcac gttatggtgg gcaacttaag gagactgccg gtgacaaaacc
601 ggaggaaggt ggggatgacg tcaagtcac atggccctta cggcctgggc tacacacgtg
661 ctacaatggt cggtagacag ggttgccaag ccgcgaggtg gagctaactt cacaaaaccg
721 atcgtagtcc ggatcgcagt ctgcaactcg actgcgtgaa gtcggaatcg ctagtaatcg
781 cgaatcagaa tgtcgcggtg aatacgttcc cgggccttgt acacaccgcc cgtcacacca
841 tgggagtggg ttgcaccaga agtagctagt ctaaccttcg ggaggacggt gacttag

**II.4.3 *Achromobacter xylooxidans* gene for 16S rRNA, partial sequence, strain:
NBRC 13495**

GenBank: AB680418.1

LOCUS AB680418 1456 bp DNA linear BCT 28-JAN-2012

DEFINITION *Achromobacter xylooxidans* gene for 16S rRNA, partial sequence,
strain: NBRC 13495.

ACCESSION AB680418

VERSION AB680418.1 GI:359803291

KEYWORDS .

SOURCE *Achromobacter xylosoxidans*

ORGANISM *Achromobacter xylosoxidans*

Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Alcaligenaceae; *Achromobacter*.

ORIGIN

1 attgaacgct agcgggatgc cttacacatg caagtcgaac ggcagcacgg acttcggtct
61 ggtggcgagt ggcgaacggg tgagtaatgt atcggaacgt gcctagtagc gggggataac
121 tacgcgaaag cgtagctaata accgcatacg ccctacgggg gaaagcaggg gatcgcgaaga
181 ccttgcaacta ttagagcggc cgatatcgga ttagctagtt ggtggggtaa cggctacca
241 aggcgacgat ccgtagctgg tttgagagga cgaccagcca cactgggact gagacacggc
301 ccagactcct acgggaggca gcagtgggga attttgaca atgggggaaa ccctgatcca
361 gccatcccgc gtgtgcgatg aaggccttcg ggttgtaaag cacttttggc aggaaagaaa
421 cgtcatgggc taataccccg tgaactgac ggtacctgca gaataagcac cggctaacta
481 cgtgccagca gccgcggtaa tacgtagggt gcaagcgtta atcggaatta ctggcgtaa
541 agcgtgcgca ggcgggtcgg aaagaaagat gtgaaatccc agagctaac tttggaactg
601 catttttaac taccgagcta gagtgtgca gagggagggt gaattccgcg ttagcagtg
661 aatgcgtag atatgcggag gaacaccgat ggccaaggca gcctcctggg ataactga
721 cgctcatgca cgaaagcgtg gggagcaaac aggattagat accctgtag tccacgcct
781 aaacgatgac aactagctgt tggggccttc gggccttggg agcgcagcta acgcgtgaag
841 ttgaccgct ggggagtacg gtcgcaagat taaaactcaa aggaattgac ggggaccgca
901 acaagcgggt gatgatgtgg attaattcga tgcaacgca aaaaccttac ctaccctga
961 catgtctgga attctgaaga gattcggaag tgctcgcaag agaaccggaa cacaggtgct
1021 gcatggtctg cgtcagctcg tgctgtgaga tgtggggtta agtcccgca cgagcgcaac
1081 ccttgctcatt agttgctacg aaagggcact ctaatgagac tgccgggtgac aaaccggagg
1141 aaggtgggga tgacgtcaag tcctcatggc ccttatgggt agggcttcac acgtcataca
1201 atggtcggga cagagggtcg ccaaccegcg agggggagcc aatcccagaa acccgatcgt
1261 agtccggatc gcagtctgca actcgactgc gtgaagtcgg aatcgctagt aatcgcgat
1321 cagcatgctc cggatgaatac gttcccgggt cttgtacaca ccgcccgtca caccatggga
1381 gtgggtttta ccagaagtag ttagcctaac cgnaaggggg gcgattacca cggtaggatt
1441 catgactggg gtgaag

II.4.4 *Alcaligenes* sp. MG07 16S ribosomal RNA gene, partial sequence

GenBank: HQ670710.1

LOCUS HQ670710 1440 bp DNA linear BCT 15-SEP-2011
DEFINITION *Alcaligenes* sp. MG07 16S ribosomal RNA gene, partial sequence.
ACCESSION HQ670710
VERSION HQ670710.1 GI:317513856
KEYWORDS .
SOURCE *Alcaligenes* sp. MG07

ORGANISM *Alcaligenes* sp. MG07

Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales;
Alcaligenaceae; *Alcaligenes*.

ORIGIN

1 catgcaagtc gaacggcagc acggacttcg gtctggtggc gaggggcga cgggtgagta
61 atgtatcgga acgtgcctag tagcggggga taactacgcg aaagcgtagc taataccgca
121 tacgccctac gggggaaagc aggggatcgc aagaccttcg actattagag cggccgatat
181 cggattagct agttggtggg gtaacggctc accaaggcga cgatccgtag ctggtttgag
241 aggacgacca gccacactgg gactgagaca cggeccagac tctacggga ggcagcagtg
301 gggaattttg gacaatgggg gaaaccctga tccagccatc ccgcgtgtgc gatgaaggcc
361 ttcgggttgt aaagcacttt tggcaggaaa gaaacgtcat gggctaatac cccgtgaaac
421 tgacggtacc tgagaataa gcaccggcta actacgtgcc agcagccgag gtaatacgtg
481 ggggtgcaagc gtaaatcgga attactgggc gtaaagcgtg cgcaggcggg tcggaagaa
541 agatgtgaaa tcccagagct taactttgga actgcatttt taactaccga gctagagtgt
601 gtcagaggga ggtggaattc cgcgtgtagc agtgaaatgc gtagatatgc ggaggaacac
661 cgatggcga cgcagcctcc tgggataaca ctgacgetca tgcacgaaag cgtggggagc
721 aaacaggatt agataccctg gtagtccacg ccctaaacga tgtcaactag ctgtggggc
781 ctccggcct tagtagcga gctaacgcgt gaagttgacc gcctggggag tacggtcgca
841 agattaaaac taaaggaat tgacggggac ccgcacaagc ggtggatgat gtggattaat
901 tcgatgcaac gcgaaaaacc ttacctacc ttgacatgc tggattccg aagagatttg
961 gaagtgtcgc caagagaacc ggaacacagg tgctgcatgg ctgtcgtcag ctcgtgtcgt
1021 gagatgttgg gtaagtccc gcaacgagcg caacccttgt cattagttgc tacgaaaggg
1081 cactetaatg agactgccgg tgacaaaccg gaggaagggt gggatgacgt caagtcctca
1141 tggcccttat gggtagggct tcacacgtca tacaatggtc gggacagagg gtcgccaacc

1201 cgcgaggggg agccaatccc agaaaccga tcgtagtccg gatcgcagtc tgcaactcga
1261 ctgcgtgaag tcggaatcgc tagtaatcgc ggcagcagcat gtcgcgggtga atacgttccc
1321 gggctttgta cacaccgcc gtcacacat gggagtgggt ttaccagaa gtagttagcc
1381 taaccgcaag gggggcgcatt accacggtag gattcatgac tggggtgaag tcgtaacaag

II.4.6 *Microbacterium foliorum* strain SR11_1385 16S ribosomal RNA gene, partial sequence

GenBank: JN990375.1

LOCUS JN990375 1385 bp DNA linear BCT 27-DEC-2011

DEFINITION *Microbacterium foliorum* strain SR11_1385 16S ribosomal RNA gene, partial sequence.

ACCESSION JN990375

VERSION JN990375.1 GI:365193008

SOURCE *Microbacterium foliorum*

ORGANISM *Microbacterium foliorum*

Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;

Micrococcineae; Microbacteriaceae; *Microbacterium*.

REFERENCE 1 (bases 1 to 1385)

ORIGIN

1 ctgcagtcga acggtgaaca cggagcttgc tctgtgggat cagtggcgaa cgggtgagta
61 acacgtgagc aacctgcccc tgactctggg ataagcgtg gaaacggcgt ctaactactg
121 atacgagtag cgaccgcatg gtcagttact ggaaagattt attggttggg gatgggctcg
181 cggcctatca gcttgttggg gaggtaatgg ctaccaagg cgtcgacggg tagccggcct
241 gagagggtga cgggccacac tgggactgag acacggccca gactcctacg ggaggcagca
301 gtggggaata ttgcacaatg ggcgcaagcc tgatgcagca acgccgcgtg agggatgacg
361 gccttcgggt tgtaaacctc ttttagcagg gaagaagcga aagtgacggg acctgcagaa
421 aaagcggcgg ctaactacgt gccagcagcc gcgtaatac gtagggcgca agcgttatcc
481 ggaattattg ggcgtaaaga gctcgtaggc ggtttgtcgc gtctgctgtg aaatccggag
541 gctcaacctc cggcctgcag tgggtacggg cagactagag tgcggtaggg gagattggaa
601 ttctggtgt agcggtgga tgcgcagata tcaggaggaa caccgatggc gaaggcagat
661 ctctgggccc taactgacgc tgaggagcga aagggtgggg agcaaacagg cttagatacc
721 ctggtagtcc accccgtaaa cgttgggaac tagttgtggg gtccattcca cggattccgt

781 gacgcagcta acgcattaag ttccccgct ggggagtagc gccgcaaggc taaaactcaa
841 aggaattgac ggggacccgc acaagcggcg gagcatcgcg attaattcga tgcaacgcga
901 agaaccttac caaggcttga catatacgag aacgggccag aaatggtaa ctctttggac
961 actcgtaaac aggtggtgca tggttgtcgt cagctcgtgt cgtgagatgt tgggttaagt
1021 cccgcaacga gcgcaaccct cgttctatgt tgccagcacg taatggtggg aactcatggg
1081 atactgccgg ggtcaactcg gaggaagggt gggatgacgt caaatcatca tgccccttat
1141 gtcttgggct tcacgcatgc tacaatggcc ggtacaaagg gctgcaatac cgcgaggtgg
1201 agcgaatccc aaaaagccgg tcccagttcg gattgaggtc tgcaactcga cctcatgaag
1261 teggagtgc tagtaatgc agatcagcaa cgctgcggtg aatacgttcc cgggtcttgt
1321 acacaccgcc cgtaagtca tgaaagtcgg taacacctga agccggtggc ctaacccttg
1381 tggag

II.4.7 *Microbacterium* sp. D3(2010) 16S ribosomal RNA gene, partial sequence

GenBank: GU549407.1

LOCUS GU549407 641 bp DNA linear BCT 14-MAR-2010

DEFINITION *Microbacterium* sp. D3(2010) 16S ribosomal RNA gene, partial
sequence.

ACCESSION GU549407

VERSION GU549407.1 GI:290782614

KEYWORDS .

SOURCE *Microbacterium* sp. D3(2010)

ORGANISM *Microbacterium* sp. D3(2010)

Bacteria; Actinobacteria; Actinobacteridae; Actinomycetales;

Micrococcineae; Microbacteriaceae; *Microbacterium*.

REFERENCE 1 (bases 1 to 641)

ORIGIN

1 actagtgtg gggccattc cacggattc cgtgacgcag ctaacgcatt aagtccccg
61 cctggggagt acgcccgcaa ggctaaaact caaaggaatt gacggggacc cgcacaagcg
121 gcgagcatg cggattaatt cgatgcaacg cgaagaacct taccaaggct tgacatatac
181 gagaacgggc cagaaatggt caactctttg gacactcgt aacaggtggt gcatggttgt
241 cgtcagctcg tctcgtgaga tgttgggtta agtcccgcaa cgagcgaac cctcgttcta
301 tgttccagc acgtaatggt gggaactcat gggatactgc cgggggtcaac tgggaggaag
361 gtggggatga cgtcaaatca tcatcccct tatgtcttgg gcttcacgca tgctacaatg

421 gccggtacaa agggctgcaa taccgcgagg tggagcgaat cccaaaaagc cgggccag
481 tcggattgag gtctgcaact cgacctcatg aagtcggagt cgctagtaat cgcagatcag
541 caacgctgcg gtgaatacgt tcccgggtct tgtacacacc gcccgcaag tcatgaaagt
601 cgtaacacc tgaagccggg gcctaaccct tgggagggag c

II.4.8 *Alpha proteobacterium* CmLB11 16S ribosomal RNA gene, partial sequence

GenBank: HM352335.1

LOCUS HM352335 1369 bp DNA linear BCT 31-MAY-2011

DEFINITION *Alpha proteobacterium* CmLB11 16S ribosomal RNA gene, partial
sequence.

ACCESSION HM352335

VERSION HM352335.1 GI:312839661

KEYWORDS .

SOURCE *alpha proteobacterium* CmLB11

ORGANISM *alpha proteobacterium* CmLB11

Bacteria; Proteobacteria; Alphaproteobacteria.

ORIGIN

1 tggcggcagg cctaacacat gcaagtcgaa cggacccttc ggggtagtg gcgacgggt
61 gagtaacacg tgggaacgtg ccttaggtt cggaatagct cctggaaacg ggtggtaatg
121 ccgaatgtgc ccttcggggg aaagattat gcctttaga gcggcccgcg tctgattagc
181 tagttggtga ggtaatggct caccaaggcg acgatcagta gctggtctga gaggatgacc
241 agccacattg ggactgagac acggcccaaa ctctacggg aggcagcagt ggggaatctt
301 gcgcaatggg cgaaagcctg acgcagccat gccgcgtgaa tgatgaaggt ctaggattg
361 taaaattctt tcaccgggga cgataatgac ggtaccgga gaagaagccc cggtaactt
421 cgtgccagca gccgcggtaa tacgaagggg gctagcgttg ctcggaatta ctgggcgtaa
481 agggcgcgta ggcgacatt taagtcaggg gtgaaatccc agagctcaac tctggaactg
541 cctttgatac tgggtgtctt gactgtgaga gaggtatgtg gaactccgag ttagaggtg
601 aaattcgtag atattcggaa gaacaccagt ggcgaaggcg acatactggc tcattactga
661 cgctgaggcg cgaaagcgtg gggagcaaac aggattagat accctggtag tccacgccgt
721 aaacgatgat tgctagtgtt cgggctgcat gcagttcggg gacgcagcta acgcattaag
781 caatccgcct ggggagtacg gtcgcaagat taaaactcaa aggaattgac gggggcccgc
841 acaagcgggt gagcatgtgg ttaattcga agcaacgcgc agaacttac cacctttga

901 catgcctgga ccgccagaga gatctggcct tctcttcgga gactaggaca caggtgctgc
961 atggctgtcg tcagctcgtg tcgtgagatg ttgggtaag tcccgcaacg agcgcaacc
1021 tcgccattag ttgccatcat ttagttggga actctaattg gactgccggt gctaagccgg
1081 aggaaggtgg ggatgacgtc aagtcctcat ggccettaca gggtaggta cacacgtgct
1141 acaatggcga ctacagaggg ttaatcctta aaagtcgtct cagttcggat tgcctctgc
1201 aactcgaggg catgaagttg gaatcgtag taatcgcgga tcagcatgcc gcggtgaata
1261 cgtcccggg cttgtacac accgcccgtc acaccatggg agttggttct acccgaagge
1321 gatgcgctaa ccagcaatgg aggcagtcga ccacggtagg gtcagcgac

II.4.9 *Brevundimonas* sp. LC348 16S ribosomal RNA gene, partial sequence

GenBank: JQ014523.1

LOCUS JQ014523 1223 bp DNA linear BCT 25-DEC-2011

DEFINITION *Brevundimonas* sp. LC348 16S ribosomal RNA gene, partial sequence.

ACCESSION JQ014523

VERSION JQ014523.1 GI:363992081

KEYWORDS .

SOURCE *Brevundimonas* sp. LC348

ORGANISM *Brevundimonas* sp. LC348

Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales;
Caulobacteraceae; *Brevundimonas*.

ORIGIN

1 tgcagtcgac ggacccttcg gggtagtgg cggacgggtg agtaacacgt gggaacgtgc
61 ctttaggttc ggaatagctc ctggaaacgg gtgtaatgc cgaatgtgcc ctccggggga
121 aagatttacc gccttagag cggcccgcgt ctgattagct agttggtgag gtaatggctc
181 accaaggcga cgatcagtag ctggtctgag aggatgacca gccacattgg gactgagaca
241 cggcccaaac tctacggga ggcagcagtg gggaaacttg cgcaatgggc gaaagcctga
301 cgcagccatg ccgcgtgaat gatgaagtc ttaggattgt aaaattctt caccggggac
361 gataatgacg gtaccggag aagaagcccc ggctaacttc gtgccagcag ccgcgtaat
421 acgaaggggg ctagcgttgc tcggaattac tggcgtaaa gggcgcgtag gcggacattt
481 aagtcagggg tgaatccca gagctcaact ctggaactgc ctttgatact ggggtctttg
541 agtgtgagag aggtatgtg aactccgagt gtagaggtga aattcgtaga tattcggag
601 aacaccagtg gcgaaggcga catactggct cactactgac gctgagggcg gaaagcgtgg
661 ggagcaaaca ggattagata ccttgtagt ccacgccgta aacgatgatt gctagttgct

721 gggctgcatg cagttcggg acgcagctaa cgcattaagc aatccgcctg gggagtacgg
781 tgcgaagatt aaaactcaaa ggaattgacg ggggcccgca caagcgggtg agcatgtggt
841 ttaatcgaa gcaacgcgca gaacctacc acctttgac atgcctggac cgccagagag
901 atctggcttt ctcttcggag actaggacac aggtgctgca tggctgtcgt cagctcgtg
961 cgtgagatgt tgggtaagt cccgcaacga gcgcaaccct cgccattagt tgccatcatt
1021 tagttgggaa ctctaatggg actgccggg ctaagccgga ggaaggtggg gatgacgtca
1081 agtcctcatg gcccttacg ggtgggctac acacgtgcta caatggcgac tacagagggt
1141 taatcctaa aagtcgtctc agttcggatt gtcctctgca actcgagggc atgaagttgg
1201 aatcgctagt aatcgcgatc agc

II.4.10 *Brevundimonas* sp. M2T2B5 16S ribosomal RNA gene, partial sequence

GenBank: GQ246696.1

LOCUS GQ246696 1376 bp DNA linear BCT 31-MAY-2010

DEFINITION *Brevundimonas* sp. M2T2B5 16S ribosomal RNA gene, partial sequence.

ACCESSION GQ246696

VERSION GQ246696.1 GI:261291226

KEYWORDS .

SOURCE *Brevundimonas* sp. M2T2B5

ORGANISM *Brevundimonas* sp. M2T2B5

Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales;
Caulobacteraceae; *Brevundimonas*.

ORIGIN

1 ggctcagagc gaacgctggc ggcaggccta acacatgcaa gtcgaacgga cccttcgggg
61 ttagtggcgg acgggtgagt aacacgtggg aacgtgcctt taggttcgga atagctcctg
121 gaaacgggtg gtaatccga atgtgccctt cgggggaaag atttatcgcc ttagagcgg
181 cccgcgtctg attagctagt tggtaggta atggctcacc aaggcgacga tcagtagctg
241 gtctgagagg atgaccagcc aactgggac tgagacacgg ccagactcc tacgggaggg
301 agcagtgggg aatcttgcgc aatgggcgaa agcctgacgc agccatgccg cgtgaatgat
361 gaaggtetta ggattgtaaa attctttcac cggggacgat aatgacggta cccggagaag
421 aagccccggc taacttcgtg ccagcagccg cggtataacg aaggggggcta gcgttgctcg

481 gaattactgg gcgtaaaggg cgcgtaggcg gatcgtaag tcagaggtga aatcccaggg
541 ctcaacctg gaactgcctt tgatactggc gatcttgagt atgagagagg tatgtggaac
601 tccgagtga gaggtgaaat tcgtagatat tcggaagaac accagtggcg aaggcgacat
661 actggetcat tactgacgct gaggcgcgaa agcgtgggga gcaaacagga ttagataccc
721 tggtagtcca cgccgtaaac gatgattgct agttgtcggg ctgcatgcag ttcggtgacg
781 cagctaacgc attaagcaat ccgcctgggg agtacggteg caagattaaa actcaaagga
841 attgacgggg gcccgcacaa gcggtggagc atgtggtta atcgaagca acgcgcagaa
901 ccttaccacc tttgacatg cctggaccgc cacggagacg tggctttctc ttcggagact
961 aggacacagg tgctgcatgg ctgtcgtcag ctctgtctgt gagatgttg gtaagtccc
1021 gcaacgagcg caaccctcgc cattagttgc catcatttag ttgggaactc taatgggact
1081 gccggtgcta agccggagga aggtggggat gacgtcaagt cctcatggcc cttacagggt
1141 gggctacaca cgtgctacaa tggcaactac agagggttaa tcctaaaag ttgtctcagt
1201 tcggattgct ctctgcaact cgagggatg aagtggaaat cgctagtaat cgggatcag
1261 catgcccgcg tgaatacgtt cccgggcctt gtacacaccg cccgtcacac catgggagtt
1321 ggttctacc gaaggcgatg cgtaaccgt aaggaggcag tcgaccacgt agtcag

II.4.11 *Bradyrhizobium* sp. DOA2 16S ribosomal RNA gene, partial sequence.

LOCUS JN578809 1361 bp DNA linear BCT 20-DEC-2011

DEFINITION *Bradyrhizobium* sp. DOA2 16S ribosomal RNA gene, partial sequence.

ACCESSION JN578809

VERSION JN578809.1 GI:363499004

SOURCE *Bradyrhizobium* sp. DOA2

ORGANISM [Bradyrhizobium sp. DOA2](#)

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;

Bradyrhizobiaceae; *Bradyrhizobium*.

ORIGIN

1 cagtcgagcg ggcgtagcaa tacgtcagcg gcagacgggt gagtaacgcg tgggaacgta
61 cttttggtt cggaacaaca cagggaaact tgtgctaata ccggataagc cttacgggg
121 aaagattat cgccgaaaga tcggcccgcg tctgattagc tagttggtga gtaaacggt
181 caccaaggcg acgatcagta gctggtctga gaggatgac agccacattg ggactgagac
241 acggcccaaa ctctacggg aggcagcagt ggggaatatt ggacaatggg ggcaaccctg
301 atccagccat gccgcgtgag tgatgaaggc ctaggggtg taaagctctt ttgtcggga
361 agataatgac ggtaccgcaa gaataagccc cggctaactt cgtgccagca gccgcggtaa
421 tacgaagggg gctagcgtt ctcggaatca ctgggcgtaa aggggtcgta ggccgggtctt
481 taagtcaggg gtgaaatcct ggagctcaac tccagaactg ctttgatac tgaagatctt
541 gagttcggga gaggtgagt gaactcgcgag ttagaggtg aaatttcgta gatattcgca
601 agaacaccag tggcgaaggc ggctcactgg cccgatactg acgctgagcg acgaaagcgt

661 ggggagcaaa caggattaga taccctggta gtccacgccg taaacgatga atgccagccg
 721 ttagtgggtt tactactag tggcgcagct aacgcttaa gcattccgcc tggggagtac
 781 ggtcgcaaga ttaaaactca aaggaattga cgggggcccc cacaagcggg ggagcatgtg
 841 gtttaattcg acgcaacgcg cagaacctta ccagcccttg acatgtccag gaccggtcgc
 901 agagatgtga ccctctcttc ggagcctgga acacaggtgc tgeatggctg tcgtcagctc
 961 gtgtcgtgag atgttgggtt aagcccgca acgagcgcaa cccccgtcct tagttgctac
 1021 catttagttg agcactctaa ggagactgcc ggtgataagc cgcgaggaag gtgggggatga
 1081 cgtcaagtcc tcatggcctt tacgggctgg gctacacacg tgctacaatg gcggtgacaa
 1141 tgggatgcta aggggcgacc cttcgcaaat ctcaaaaagc cgtctcagtt cggattgggc
 1201 tctgcaactc gagcccatga agttggaatc gctagtaatc gtggatcagc acgccacggt
 1261 gaatacgttc cggggccttg tacacaccgc ccgtcacacc atgggagttg gctttacctg
 1321 aagacggtgc gctaaccagc aatggaggca gccggccacg g

II.4.12 *Bradyrhizobium* sp. GSM-467 partial 16S rRNA gene, isolate GSM-467.

LOCUS FN600560 1300 bp DNA linear BCT 27-SEP-2010

DEFINITION *Bradyrhizobium* sp. GSM-467 partial 16S rRNA gene, isolate GSM-467.

ACCESSION FN600560

VERSION FN600560.2 GI:298285549

SOURCE *Bradyrhizobium* sp. GSM-467

ORGANISM [Bradyrhizobium sp. GSM-467](#)

Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales;

Bradyrhizobiaceae; *Bradyrhizobium*.

ORIGIN

1 agagttgat catggctcag agcgaacgct ggcggcaggc ttacacatg cagtcgagcg
 61 ggcgtagcaa tacgtcagcg gcagacgggt gagtaacgcg tgggaacgta ccttttgggt
 121 cggaaaca cagggaaact tgtgctaata ccggataagc cttacggggg aaagatttat
 181 cgccgaaaga tcggcccgcg tctgattagc tagttggtga ggtaacggct caccaaggcg
 241 acgatcagta gctggtctga gaggatgac agccacattg ggactgagac acggcccaaa
 301 ctctacggg aggcagcagt ggggaatatt ggacaatggg cgcaagcctg atccagccat
 361 gccgcgtgag tgatgaaggc ctaggggttg taaagctctt ttgtgcggga agataatgac
 421 ggtaccgcaa gaataagccc cggctaactt cgtgccagca gccgcggtaa tacgaagggg
 481 gctagcgttg ctcggaatca ctgggcgtaa aggggtgcgta ggcgggtctt taagtcaggg
 541 gtgaaatcct ggagctcaac tccagaactg cctttgatac tgaagatctt gagttcggga
 601 gaggtgagtg gaactgcgag ttagaggtg aaattcgtag atattcгаа gaacaccagt
 661 ggcgaggcg gctcactggc ccgatactga cgctgaggca cgaaagcgtg gggagcaaac
 721 aggattagat accctggtag tccacgccgt aaacgatгаа tgccagccgt tagtgggttt
 781 actcactagt ggcgcagcta acgcttaagc atccgcctgg ggagtacggg cgcaagatta
 841 aaactcaaag gaattgacgg gggcccgcac aagcgggtgga gcatgtggtt taattgacg
 901 caacgcgcag aaccttaca gcccttgaca tcccggctgc ggactccaga gatggagttc

961 ttcagttcgg ctggaccgga gacaggtgct gcatggctgt cgtcagctcg tgctgtgaga
 1021 tgttgggtta agtcccgcaa cgagcgcaac ccccgctctt agttgctacc attagttga
 1081 gcactctaag gagactgccg gtgataagcc gcgaggaagg tggggatgac gtcaagtctt
 1141 catggccctt acgggctggg ctacacacgt gctacaatgg cggtgacaat gggacgctaa
 1201 ggggcaacce ttcgcaaatc tcaaaaagcc gtctcagttc ggattgggct ctgcaactcg
 1261 agcccatgaa gtggaatcgc tagtaatcgt gtcagcacga

II.5 Fungal DNA isolation and PCR amplification

PRIMER DETAILS	PRIMER 1 (ITS1)	PRIMER 2 (ITS4)
Sequence (5' to 3')	CTTGGTCATTTAG AGGAAGTAA	TCCTCCGCTTAT TGTATGC
Complementary sequence	GAACCAGTAAAT CTCCTTCATT	AGGAGGCGAAT AACTATACG
Tm: salt adjusted	66°c	66°c
Tm: nearest neighbour	44°c	45°c
M.W	6814.4	6035.0
GC content	36%	45%
Length	22	20

II.6 Fungal sequencing results

Using BLAST (NCBI), the fungal rDNA sequences in the GenBank database (NCBI) were compared as followed:

II.6.1 *Aspergillus Niger*

Sequence obtained

```
acCCTGTTGCTTCGGCGGGCCCCGCCGCTTGTTCGGCCGCCGGGGGGGCGCCTTTGCCCCC
CGGGCCCCGTGCCCGCCGGaGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTG
AGTTGATTGAATGCAATCAGTTAAAACITTTCAACAATGGATCTCTTGGTTCCGGCATC
GATGAAGAACGCAGCGAAATGCGATAACTAATGTGAATTGCagaATTCAGTGAATCAT
cGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGC
```



```
GTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGG
ACGGGCCCCGAAAGGCAGCGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTG
TCACATGCTCTGTAGGATTGGCCGGCGCTGCCGacgTTTTCCAACCATTTTTTCCagGTT
GACCTCGGATcangtaGggaTACCCGCTGaacttaagCATAcaATAAGCGGAg
```

> [gb|HQ014697.1](#) Aspergillus niger strain WM10.75 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence
Length=612

Score = 959 bits (519), Expect = 0.0
Identities = 520/521 (99%), Gaps = 0/521 (0%)
Strand=Plus/Plus

Query 1
ACCCTGTTGCTTCGGCGGGCCCCGCCGCTTGTTCGGCCGCCggggggggCGCCTTTGCCCCCC 60

|||||
Sbjct 90
ACCCTGTTGCTTCGGCGGGCCCCGCCGCTTGTTCGGCCGCCGGGGGGGGCGCCTTTGCCCCCC 149

Query 61
GGGCCCCTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTT 120

|||||
Sbjct 150
GGGCCCCTGCCCGCCGGAGACCCCAACACGAACACTGTCTGAAAGCGTGCAGTCTGAGTT 209

Query 121
GATTGAATGCAATCAGTTAAAACCTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA 180

|||||
Sbjct 210
GATTGAATGCAATCAGTTAAAACCTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAA 269

Query 181
GAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTT 240

|||||
Sbjct 270
GAACGCAGCGAAATGCGATAACTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTT 329

Query 241
TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTG 300

|||||
Sbjct 330
TGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTG 389

Query 301
CCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCCGAA 360

|||||
Sbjct 390
CCCTCAAGCCCGGCTTGTGTGTTGGGTCGCCGTCCCCCTCTCCGGGGGGACGGGCCCCGAA 449

```

Query 361
AGGCAGCGGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTACATGCTCTGTA 420

|||||
Sbjct 450
AGGCAGCGGGCGGCACCGCGTCCGATCCTCGAGCGTATGGGGCTTTGTACATGCTCTGTA 509

Query 421
GGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTTTTTCCAGGTTGACCTCGGATCAN 480

|||||
Sbjct 510
GGATTGGCCGGCGCCTGCCGACGTTTTCCAACCATTTTTTCCAGGTTGACCTCGGATCAG 569

Query 481 GTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAG 521
          |||||
Sbjct 570 GTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAG 610

```

II.6.2 *Aspergillus tubingensis*

```

GannatgGTIGGAAAACGTCGGCAGGCGCCGgnntnnnnnnnnnnnnnaCAAAGCCCCATA
CGCTCGAggATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAGAGG
GGGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAG
GCATGCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGaTGATTCACT
GAATTCTGCAATTCACATTAGTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAAC
CAAGAGATCCATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACG
CTTTCAGACAGTGTTCGTGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCAAAGG
CGCCCCCGGCGGCCGACAAGCGGCGGGCCCGCggaagCAACAGGGTATAATAGACA
CGGATGGGAGGTTGGGCCAAAGGACCCGCACTCGGTAATGATCCTTCCgcaggttCACC
ta

```

```

> gb|HQ728255.1 Aspergillus tubingensis isolate JH01 18S
ribosomal RNA gene,
partial sequence; internal transcribed spacer 1, 5.8S ribosomal
RNA gene, and internal transcribed spacer 2, complete sequence;
and 28S ribosomal RNA gene, partial sequence
Length=631

```

```

Score = 898 bits (486), Expect = 0.0
Identities = 508/527 (96%), Gaps = 2/527 (0%)
Strand=Plus/Minus

```

```

Query 5 ATGGTTGGAAAACGTCGGCAGGCGCCGGNNN-Tnnnnnnnnnnnnnnn-
aCAAAGCCCCAT 62
          |||||
Sbjct 566
ATGGTTGGAAAACGTCGGCAGGCGCCGGCCAATCCTACAGAGCATGTGACAAAGCCCCAT 507

Query 63
ACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAGAGGG 122

```

Sbjct 506
ACGCTCGAGGATCGGACGCGGTGCCGCCGCTGCCTTTTCGGGCCCGTCCCCCGGAGAGGG 447

Query 123
GGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATG 182

|||||
Sbjct 446
GGACGGCGACCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATG 387

Query 183
CCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTTACTGAATTCT 242

|||||
Sbjct 386
CCCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTTACTGAATTCT 327

Query 243
GCAATTCACATTAGTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGAT 302

|||||
Sbjct 326
GCAATTCACATTAGTTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGAT 267

Query 303
CCATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACA 362

|||||
Sbjct 266
CCATTGTTGAAAGTTTTAACTGATTGCATTCAATCAACTCAGACTGCACGCTTTCAGACA 207

Query 363
GTGTTTCGTGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCAAAGGCGccccccccGGCG 422

|||||
Sbjct 206
GTGTTTCGTGTTGGGGTCTCCGGCGGGCACGGGCCCGGGGGCAAAGGCGCCCCCCCCGGCG 147

Query 423
GCCGACAAGCGGGCGGGCCCGCCGAAGCAACAGGGTATAATAGACACGGATGGGAGGTTGG 482

|||||
Sbjct 146
GCCGACAAGCGGGCGGGCCCGCCGAAGCAACAGGGTATAATAGACACGGATGGGAGGTTGG 87

Query 483 GCCCAAAGGACCCGCACTCGGTAATGATCCTTCCGCAGGTTACCTA 529


Sbjct 86 GCCCAAAGGACCCGCACTCGGTAATGATCCTTCCGCAGGTTACCTA 40

[clustalw.aln](#)

4.6.3 *Penicillium freii* (IBT 3464)

tgGGTCCAACCTCCCAccnnnnnttanntaCCTTGTGCTTCGGCGGGCCCGCCTTTACTGGC CGCCGGGGGGCTCACGCCCCGGGTCCGCGCCCGCCGAAGACACCCTCGAACTCTGT CTGAAGATIGAAGTCTGAGTGAAAATATAAATTATTTAAAACITTCACAACGGATCT CTTGGTTCGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCA

```
AATTCAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGG
CATGCCTGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCC
TCCGATTCCGGGGGACgGGCCCCGAAAGGCAGCGGGCGGCACCGCGTCCGGTCCTcgaGC
GTATGGGGCTTTGTACCCCGCTCcgtaggCCCGgccGgCGCTTGCCGATCAACCCAAATTTT
TATCcAGGTTGACCTcggatcnntaggGaTACCCCGCTGAACTTAagcATATCAATAagcGga
```

```
>  emb|AJ005479.1| Penicillium freii (IBT 3464) ribosomal internal
transcribed spacers
and the 5.8S ribosomal RNA gene (ITS1-5.8S-ITS2)
Length=609
```

```
Score = 950 bits (514), Expect = 0.0
Identities = 525/536 (98%), Gaps = 0/536 (0%)
Strand=Plus/Plus
```

```
Query 1
TGGGTCCAACCTCCCACCNNNNNTTANNNTACCTTGTTGCTTCGGCGGGCCCCGCCTTTAC 60
```

```
|||||
|||||
```

```
Sbjct 71
TGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCCGCCTTTAC 130
```

```
Query 61
TGGCCGCCGGGGGGCTCACGCCCCGGGTCCGCGCCCGCCGAAGACACCCTCGAACTCTG 120
```

```
|||||
```

```
Sbjct 131
TGGCCGCCGGGGGGCTCACGCCCCGGGTCCGCGCCCGCCGAAGACACCCTCGAACTCTG 190
```

```
Query 121
TCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAACCTTCAACAACGGATCTC 180
```

```
|||||
```

```
Sbjct 191
TCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAACCTTCAACAACGGATCTC 250
```

```
Query 181
TTGGTTCGGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAATT 240
```

```
|||||
```

```
Sbjct 251
TTGGTTCGGGCATCGATGAAGAACGCAGCGAAATGCGATACGTAATGTGAATTGCAAATT 310
```

```
Query 241
CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 300
```

```
|||||
```

```
Sbjct 311
CAGTGAATCATCGAGTCTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCC 370
```

```
Query 301
TGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCTCCGATT 360
```

```
|||||
```

```
Sbjct 371
TGTCCGAGCGTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCTCCGATT 430
```

```
Query 361
CCGGGGGACGGGCCCCGAAAGGCAGCGGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGC 420
```

```

|||||
Sbjct 431
CCGGGGGACGGGCCCGAAAGGCAGCGCGGCACCGCGTCCTCGAGCGTATGGGGC 490

```

```

Query 421
TTTGTACCCGCTCCGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTATCCAG 480

```

```

|||||
Sbjct 491
TTTGTACCCGCTCCGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTATCCAG 550

```

```

Query 481 GTTGACCTCGGATCNNNTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA
536

```

```

Sbjct 551 GTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGA
606

```

II.6.4 *Penicillium aurantiogriseum*

```

tgGGTTGATCGGCAAGCGCCGGCCGGGcnnnnnnnnnnnnngACAAAGCCCCATACGCTCG
AGGACCGGACGCGGTGCCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAatcGGAGGACG
GGGCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCC
CCCCGGAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTG
CAATTCACATTACGTATCGCATTTCGCTGCGTTCATCGATGCCGGAACCAagagaTC
CGTTGTTGAAAGTTTTAAATAATTTATATTTTCACTCnnnctTCAATCTTCagncnGagtTCGa
GGGTGTCTTcggCGGGCGCGGACCCGGGGGCGTGAGCCCCCGGCGGCCAGTAAAGGC
GGGCCCGCCgannnAACAAGGTAATAAACACGGGTGGGaggTTGGACCCaannnGCC
TACTcggTAATGATCCTTCCGcaggTTCACCTACGGAAACctgtnacnctTTTACTTCCTCT
AAATGAccAag

```

```

> gb|GU566234.1 Penicillium aurantiogriseum strain D8 18S
ribosomal RNA gene,
partial sequence; internal transcribed spacer 1, 5.8S ribosomal
RNA gene, and internal transcribed spacer 2, complete sequence;
and 28S ribosomal RNA gene, partial sequence
Length=624

```

```

Score = 911 bits (493), Expect = 0.0
Identities = 524/552 (95%), Gaps = 1/552 (0%)
Strand=Plus/Minus

```

```

Query 1
TGGTTGATCGGCAAGCGCCGGCCGGGcnnnnnnnnnnnnngACAAAGCCCCATACGCTC 60

```

```

|||||
Sbjct 552
TGGTTGATCGGCAAGCGCCGGCCGGGcctacggagcgggtgacaaagccccatagctc 493

```

```

Query 61
GAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAATCGGAGGACGG 120

```

```

|||||
Sbjct 492
GAGGACCGGACGCGGTGCCGCCGCTGCCTTTCGGGCCCGTCCCCCGGAATCGGAGGACGG 433

```

```

Query 121
GGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCG 180
|||||
Sbjct 432
GGCCCAACACACAAGCCGGGCTTGAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCG 373

Query 181
GAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCA 240
|||||
Sbjct 372
GAATACCAGGGGGCGCAATGTGCGTTCAAAGACTCGATGATTCACTGAATTTGCAATTCA 313

Query 241
CATTACGTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTT 300
|||||
Sbjct 312
CATTACGTATCGCATTTTCGCTGCGTTCTTCATCGATGCCGGAACCAAGAGATCCGTTGTT 253

Query 301
GAAAGTTTTAAATAATTTATATTTTCACTCENNCTTCAATCTTCAGNCNGAGTTCGAGGG 360
|||||
Sbjct 252
GAAAGTTTTAAATAATTTATATTTTCACTCAGACTTCAATCTTCAGACAGAGTTCGAGGG 193

Query 361 TGTCTTCGGCGGGCGCGGACCCGGGGCGTGA-
GCCCGCGGCGCCAGTAAAGGCGGGC 419
|||||
Sbjct 192
TGTCTTCGGCGGGCGCGGGCCCGGGGGCGTGAAGCCCCCGGCGCCAGTAAAGGCGGGC 133

Query 420
CCGCCGANNAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCCAANNNGCCCTCAC 479
|||||
Sbjct 132
CCGCCGAAGCAACAAGGTAAAATAAACACGGGTGGGAGGTTGGACCCAAGGGCCCTCAC 73

Query 480
TCGGTAATGATCCTTCCGCAGGTTACCTACGGAAACCTTGTNACGNCTTTTACTTCCTC 539
|||||
Sbjct 72
TCGGTAATGATCCTTCCGCAGGTTACCTACGGAAACCTTGTTACGACTTTTACTTCCTC 13

Query 540 TAAATGACCAAG 551
|||||
Sbjct 12 TAAATGACCAAG 1

```

Appendix III

Biodegradation

III.1 Calculations for Moisture content of soil:

1] To calculate the mass of dry Soil:

=mass of hillgard soil cup, filter paper, and soil - mass of cup and filter paper

=75.01 - 65.93

i.e. Mass of dry Soil is 9.0g

2] To calculate mass of saturated soil by following:

=mass of cup, filter paper, and saturated soil - mass of cup and filter paper

=81.52 - 65.93

i.e. Mass of saturated soil is 15.59g

3] To calculate mass of water contained in saturated Soil:

= mass of saturated soil - mass of dry soil

= 81.52 - 75.01

i.e. mass of water contained in saturated soil is 6.5g

4] To Calculate the Percentage of Water Holding Capacity

i.e.
$$\frac{\text{Mass of water contained in saturated Soil}}{\text{Mass of saturated Soil}} \times 100$$

=
$$\frac{6.51}{15.59} \times 100$$

= 41.75 %

III.2 HPLC-Analytical parameters for determination of rate of degradation of polycyclic aromatic hydrocarbons

The HPLC analytical parameters used were:

Detector: UV detector (UVD 170 U)

Column: C16 and C18

Flow rate: 0.8cm³/min

Mobile phase (solvent): A. Acetonitrile

B. De-ionized water (Milli-Q water)

Composition of mobile phase: A =70%, B= 30%

Temperature: Room temperature

Elution: Isocratic

Run time: 20 minutes

Data analysis: Chromeleon 32® Chromatography Manager 3.2.

III.3 Media and culture conditions

1) Bacterial culture- Nutrient agar (NA) media was used for isolation of bacterial population. 11.20g of nutrient agar (Sigma Aldrich) was weighed and added to 500cm³ of glass bottle. The 400cm³ sterile distilled water was added to the bottle containing nutrient agar powder and mixed well. The bottle containing media was kept for Autoclaving at 121⁰C for 15mins.

2) Fungal Culture- Malt extract agar (MEA):

For isolation of heterotrophic microorganisms in the environmental samples, MALT extract agar (MEA) (Sigma Aldrich) along with 50mg rose bengal (Sigma Aldrich) to prevent bacterial growth, was used for the isolation of fungi from the treated samples.

III.4 Soil enzyme activity

III.4.1 MnP enzyme activity rate is calculated as shown below and expressed as

$\mu\text{mol/g/min}$. $\mu\text{mol/g/min} = \frac{\text{O D value} \times \text{Total reaction volume}}$

$\text{E} \times \text{gram of soil} \times \text{incubation time}$

Where OD value = Optical density of ABTS oxidized by colour change

Total reaction volume = amount of sample analyzed (1ml)

E = extinction coefficient of p- ABTS

III.4.2 LiP enzyme activity rate is calculated as shown below and expressed as

$$\mu\text{M/g/min.} \quad \mu\text{mol/g/min} = \frac{\text{O D value X Total reaction volume}}$$

$$E \text{ X gram of soil X incubation time}$$

Where OD value = Optical density of ABTS oxidized by colour change

Total reaction volume = amount of sample analyzed (1ml)

E = extinction coefficient of p- ABTS (0.0865)

III.4.3 Laccase enzyme activity rate is calculated as shown below and expressed as

$$\mu\text{mol/g/min.} \quad \mu\text{mol/g/min} = \frac{\text{O D value X Total reaction volume}}$$

$$E \text{ X gram of soil X incubation time}$$

Where OD value = Optical density of ABTS oxidized by colour change

Total reaction volume = amount of sample analyzed (1ml)

E = extinction coefficient of p- ABTS (0.0865)

III.4.4 Acid/Alkaline phosphatase enzyme activity rate is calculated as shown below (see equation 2) and it is expressed as $\mu\text{mol/g/min}$.

$$\mu\text{mol/g/min} = \frac{\text{O D value X Total reaction volume}}{\dots\dots\dots} \quad (2)$$

$$E \text{ X gram of soil X incubation time}$$

Where OD value = Optical density of p- nitrophenol produced

Total reaction volume = amount of sample analyzed (1ml)

E = extinction coefficient of p- nitrophenol phosphate (0.0684)

III.4.5 L-arginine ammonification:

$$\mu\text{gNH}_3/\text{g soil/wk} = \frac{(\text{O.D Test} - \text{O.D Control}) \times (\text{ml KCl} + \text{ml Arginine})}{\dots\dots\dots} \quad (3)$$

$$(\text{grams of soil} \times \text{Time} \times e)$$

Where O.D Test = Optical density of Test sample (ammonia produced) at 24⁰c

O.D Control = Optical density of Control sample (ammonia produced) at 4⁰c

ml KCl = Volume of 2M KCl used (8ml)

ml Arginine = Volume of 0.2% Arginine (0.5 ml)

gram of soil = mass of soil used (2g)

Time = Incubation time (30mins)

e = Extinction coefficient of ammonium chloride (0.1018)

III.5 Log bacterial cfu/ g of soil

Day/ pH	5	5.5	6	6.5	7	7.5	8	8.5
0	5.45	5.60	5.66	5.56	5.79	5.51	5.53	5.30
7	5.97	5.92	5.86	5.76	6.19	5.94	5.75	5.45
14	6.26	6.06	5.99	6.18	6.44	6.01	6.04	5.62
21	6.61	6.52	6.61	6.63	6.87	6.59	6.35	6.15
28	6.75	6.69	6.64	6.80	6.88	6.81	6.73	6.60
35	7.23	7.23	7.26	7.28	7.23	7.24	7.21	7.16
42	6.99	6.99	6.96	7.06	7.06	6.95	6.82	6.69
49	6.75	6.74	6.81	6.83	6.88	6.63	6.65	5.54

III.6 Log fungal cfu/ g of soil

Day/pH	5	5.5	6	6.5	7	7.5	8	8.5
0	5.82	5.46	5.03	4.48	4.06	4.30	4.41	4.45
7	6.08	5.55	5.47	4.83	4.34	4.45	4.79	4.83
14	6.15	5.58	5.40	5.25	4.56	4.83	5.00	5.09
21	6.38	6.29	5.81	5.70	4.82	4.94	5.17	5.25
28	6.43	6.35	6.07	5.27	4.89	4.79	5.33	5.37
35	5.65	5.43	5.16	5.13	4.51	4.72	4.82	5.00
42	5.64	5.26	5.22	4.92	4.41	4.66	4.72	4.95
49	5.55	5.13	5.13	4.76	4.15	4.58	4.75	4.78
56	5.42	5.06	4.86	4.38	4.15	4.20	4.62	4.76
63	5.29	4.91	4.72	4.34	3.60	4.15	4.62	4.66
70	5.13	4.66	4.48	4.26	3.60	3.30	4.53	4.53

III.7 ATP measurements / g of soil

Day/pH	5	5.5	6	6.5	7	7.5	8	8.5
0	1.72	2.07	2.094	2.36	2.34	2.16	2.03	1.93
7	7.02	7.25	8.264	9.38	10.45	9.30	8.01	7.8
14	9.63	10.12	10.58	10.72	11.55	11.26	8.86	8.18
21	93.42	94.85	94.93	95.88	94.31	96.25	104.34	103.87
28	133.58	133.6	137.12	140.07	177.19	150.90	135.81	134.24
35	158.72	158.94	159.15	160.29	177.90	172.21	156.96	149.4
42	130.78	130.72	131.51	132.42	152.20	142.18	133.02	131.30
49	101.57	101.96	102.09	102.46	105.50	103.76	100.50	99.77
56	97.97	98.76	99.89	100.91	102.01	114.40	110.80	98.24
63	95.57	96.25	96.6	97.70	99.483	103.27	103.29	96.54
70	9.13	90.54	91.6	92.82	94.08	89.61	89.61	88.83

III.8 ATP biomass-

ATP biomass ($\mu\text{g biomass C g}^{-1}$ soil d.w) = 171 X C

Day/pH	5	5.5	6	6.5	7	7.5	8	8.5
0	294	354	358	403	400	370	347	330
7	1201	124	141	160	178	159	137	133
14	1648	173	1810	183	1975	1926	1515	1399
21	1597	162	1623	1639	1612	1645	1784	1776
28	2284	2285	2344	2395	3030	2580	2322	2295
35	2714	2717	2721	2741	3042	2944	2684	2554
42	2236	2235	2248	2264	2602	2431	2274	2245
49	1736	1743	1745	1752	1804	1774	1718	1706
56	1675	1688	1708	1725	1744	1956	1894	1679
63	1634	1645	1651	1670	1701	1766	1766	1650
70	1541	1548	1557	1580	1608	1587	1532	1519

Appendix IV

IV.1 Bradford assay procedure

Procedure

100 µl of each standard solution was pipette into a series of clean, dry test tubes. While, 100µl of water was pipette into a separate test tube as control blank. Then 5ml of the Bradford reagent was added to each test tube. The solutions were inverted gently and left to incubate for between 2 and 60 minutes. The spectrophotometer wavelength was set to 595nm and the control blank (with zero protein concentration) was used to zero the spectrophotometer. The other standard samples' Optical density (OD) was measured at 595nm and the samples with the lowest protein concentration recorded. Standard curve was plotted.

100µl of samples was pipette into a series of clean, dry test tubes. 100µl of sodium phosphate buffer (0.1mol/L, pH 6.5) containing 0.1mol/L NaCl was pipette into a separate test tube as control blank. And 5ml of the Bradford reagent was added to each test tube. The samples' OD was measured at 595nm. The standard graph was used to calculate the original concentration of protein in the fungal samples.

IV.2 Calculation of Protein concentration

The protein concentration of the bacterial and fungal samples was calculated from the equation of the standard curve of Bovine serum albumin standard solutions versus absorbance.

IV.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

SDS PAGE of purified samples after using Sphepadex G-25 for bacterial and fungal samples was carried out to determine the molecular weight. The molecular weight of purified enzymes were carried out using Bovine serum albumin

Solutions used

Tris/HCL (1.85M, pH 8.8):

About 70 ml 1.85M Tris was prepared and pH adjusted using HCl and made up to 100ml. Then it was kept at 4⁰C.

Tris/HCl (0.6M, pH 6.8)

About 70 ml 0.6M Tris was prepared; pH adjusted using HCL and made up to 100ml. kept at 4⁰C.

Acrylamide stock (30%)

Kept at 4⁰C.

TEMED (N,N,N,N-tetramethylenediamine)

Ammonium persulphate (APS) 10%

1 ml was prepared fresh.

SDS (10%)

10 ml was prepared and kept at room temperature.

Electrode buffer:

6g of Tris and 28.8g Glycine was dissolved in ~800ml water, then 2g of SDS was added and transferred to a litre plastic bottle and made up to volume and was kept at 4⁰C.

Sample buffer

The x1 sample buffer is used for a small amount of liquid or solid and x2 for when diluting a solution 2-fold.

Samples for SDS gels

SDS Gel Mixture: the following quantities were added in the Small Buchner flask to prepare the SDS gels;

IV.4 Calculation for table performed:

Protein concentration in sample =

$$\frac{\text{Abs (sample)} - \text{Abs (blank)}}{\text{Abs (control)} - \text{Abs (blank)}} \times \text{concentration of control}$$

$$\text{Fold purification} = \frac{\text{Specific activity}}{\text{Initial specific activity}}$$

$$\% \text{ yield} = \frac{\text{Total enzyme activity}}{\text{Initial total enzyme activity}} \times 100$$

Appendix – V Statistics

V.10.1 SPSS statistical analysis for the comparisson between-subject factors of two wavelengths among controls and experimental values:

Within-Subjects Factors

Measure: MEASURE_1

Phenanthrene	Dependent Variable
1	Time0
2	Time24
3	Time48
4	Time72
5	Time96
6	Time120

Between-Subjects Factors

	Value Label	N
1	control 375nm	5
2	experimental values 375nm	5
3	control 254nm	5
4	experimental value254nm	5

V.10.2 Post Hoc Tests

Table V.2 : Post Hoc Tests for Multiple Comparisons between-subject factors of two wavelengths

Measure:MEASURE_1					
(I)	(J)	Mean Difference (I-J)	Std. Error	95% Confidence Interval	
				Lower Bound	Upper Bound
control 375nm	experimental values 375nm	94.7571*	25.35043	26.8073	162.7069
	control 254nm	38.7339	25.35043	-29.2159	106.6837
	experimental value 254nm	105.5694*	25.35043	37.6196	173.5192
experimental values 375nm	control 375nm	94.7571*	25.35043	162.7069	-26.8073
	control 254nm	56.0232	25.35043	123.9730	11.9266
	experimental value 254nm	10.8123	25.35043	-57.1375	78.7621
control 254nm	control 375nm	38.7339	25.35043	106.6837	29.2159

	experimental values 375nm	56.0232	25.35043	-11.9266	123.9730
	experimental value254nm	66.8355	25.35043	-1.1143	134.7853
experimental value254nm	control 375nm	-10	25.35043	-	-37.6196
		5.5694 ⁺		173.5192	
	experimental values 375nm	10.8123	25.35043	-78.7621	57.1375
	control 254nm	-66.8355	25.35043	-	1.1143
control 375nm	experimental values 375nm	94.7571 ⁺	25.35043	43.5220	145.9922
	control 254nm	38.7339	25.35043	-12.5012	89.9690
	experimental value254nm	105.5694 ⁺	25.35043	54.3343	156.8045
experimental values 375nm	control 375nm	-94.7571 ⁺	25.35043	-	-43.5220
				145.9922	

	control 254nm	- 56. 02 32*	25.3 5043	- 107.2584	-4.7881
	experime ntal value254 nm	10. 81 23	25.3 5043	-40.4229	62.0474
control 254nm	control 375nm	- 38.7339	25.35043	-89.9690	12.5012
	experime ntal values 375nm	56.0232 *	25.35043	4.7881	107.2584
	experime ntal value254 nm	66.8355 *	25.35043	15.6004	118.0706
experimental value254nm	control 375nm	105.569 4*	25.35043	- 156.8045	-54.3343
	experime ntal values 375nm	- 10.8123	25.35043	-62.0474	40.4229
	control 254nm	66.8355 *	25.35043	118.0706	-15.6004
control 375nm	experime ntal values 375nm	94.7571 *	25.35043	24.3877	165.1265
	control 254nm	38.7339	25.35043	-31.6355	109.1033

	experimental value254nm	105.5694*	25.35043	35.2000	175.9388
experimental values 375nm	control 375nm	94.7571	25.35043	- 165.1265	-24.3877
	control 254nm	- 56.0232	25.35043	- 126.3926	14.3462
	experimental value254nm	10.8123	25.35043	-59.5571	81.1817
control 254nm	control 375nm	- 38.7339	25.35043	- 109.1033	31.6355
	experimental values 375nm	56.0232	25.35043	-14.3462	126.3926
	experimental value254nm	66.8355	25.35043	-3.5339	137.2049
experimental value254nm	control 375nm	- 105.5694*	25.35043	- 175.9388	-35.2000
	experimental values 375nm	- 10.8123	25.35043	-81.1817	59.5571

control 254nm	66.8355	25.35043	137.2049	3.5339
------------------	---------	----------	----------	--------

Based on observed means.

The error term is Mean Square(Error)
=3534.544.

*. The mean difference is significant at the .05 level.

V.11 Difference between each pH vs time points

One way between subjects ANOVA was conducted to compare the photo-catalytic effect and the difference between varying pH vs. Different time points is measured.

Table V .3: Difference between each pH vs. Time points

		MEASURE_1		
		N	Subset	
			1	2
Tukey HSD ^a	experimental value254nm	11	170.62 04	
	experimental values 375nm	11	181.43 27	
	control 254nm	11	237.45 59	237. 4559
	control 375nm	11		276. 1898
	Sig.		.055	.431

Means for groups in homogeneous subsets are displayed.

Based on observed means.

The error term is Mean Square(Error) = 3534.544.

a. Uses Harmonic Mean Sample Size = 11.000.

Between-Subjects Factors

	Value Label	N
Trea	1 Phenanthrene	6

tmen	2	Anthracene	6
t	3	Fluoranthene	6
	4	Pyrene	6

Tests of Between-Subjects Effects

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	pH4	558751.013 ^a	3	186250.338	1269.629	.000
	pH4.5	519805.238 ^b	3	173268.413	833.070	.000
	pH5	498034.030 ^c	3	166011.343	3893.007	.000
	pH5.5	558441.289 ^d	3	186147.096	776.376	.000
	pH6	1.258E6	3	419258.022	992.720	.000
	pH6.5	617487.865 ^f	3	205829.288	1201.793	.000
	pH7	525844.293 ^g	3	175281.431	4337.41	.000
	pH7.5	795463.583 ^h	3	265154.528	649.045	.000
	pH8	525696.931 ⁱ	3	175232.310	1553.770	.000
	pH8.5	581088.467 ^j	3	193696.156	2980.418	.000
	pH9	582843.928 ^k	3	194281.309	2979.267	.000
Intercept	pH4	916730.864	1	916730.864	6249.158	.000
	pH4.5	908509.594	1	908509.594	4368.088	.000
	pH5	854406.156	1	854406.156	20036.033	.000

	pH5.5	913896.250	1	913896.250	3811.646	.000
	pH6	1875468.802	1	1875468.802	4440.739	.000
	pH6.5	1067208.261	1	1067208.261	6231.199	.000
	pH7	872137.919	1	872137.919	21581.362	.000
	pH7.5	1155675.323	1	1155675.323	2828.860	.000
	pH8	864736.628	1	864736.628	7667.547	.000
	pH8.5	745223.808	1	745223.808	11466.816	.000
	pH9	744835.133	1	744835.133	11421.905	.000
Treatment	pH4	558751.013	3	186250.338	1269.629	.000
	pH4.5	519805.238	3	173268.413	833.070	.000
	pH5	498034.030	3	166011.343	3893.007	.000
	pH5.5	558441.289	3	186147.096	776.376	.000
	pH6	1257774.065	3	419258.022	992.720	.000
	pH6.5	617487.865	3	205829.288	1201.793	.000
	pH7	525844.293	3	175281.431	4337.401	.000
	pH7.5	795463.583	3	265154.528	649.045	.000
	pH8	525696.931	3	175232.310	1553.770	.000
	pH8.5	581088.467	3	193696.156	2980.418	.000
	pH9	582843.928	3	194281.309	2979.267	.000

;variance	pH4	2933.934	20	146.697		
	pH4.5	4159.759	20	207.988		
	pH5	852.870	20	42.643		
	pH5.5	4795.284	20	239.764		
	pH6	8446.651	20	422.333		
	pH6.5	3425.371	20	171.269		
	pH7	808.233	20	40.412		
	pH7.5	8170.608	20	408.530		
	pH8	2255.576	20	112.779		
	pH8.5	1299.792	20	64.990		
	pH9	1304.222	20	65.211		
Total	pH4	1478415.811	24			
	pH4.5	1432474.591	24			
	pH5	1353293.055	24			
	pH5.5	1477132.823	24			
	pH6	3141689.518	24			
	pH6.5	1688121.496	24			
	pH7	1398790.444	24			
	pH7.5	1959309.514	24			
	pH8	1392689.135	24			
	pH8.5	1327612.067	24			
	pH9	1328983.283	24			
Corrected Total	pH4	561684.947	23			
	pH4.5	523964.997	23			
	pH5	498886.900	23			
	pH5.5	563236.573	23			
	pH6	1266220.716	23			
	pH6.5	620913.235	23			
	pH7	526652.525	23			
	pH7.5	803634.190	23			
	pH8	527952.507	23			
	pH8.5	582388.259	23			
	pH9	584148.150	23			

V.12 Regression analysis

Variables Entered/Removed^b

Model	Variables Entered	Variables Removed	Method
1	Time120, Time0, Time72, Time48, Time96, Time24 ^a		Enter

a. All requested variables entered.

b. Dependent Variable: Treatment

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.645 a	.416	.321	2.63632

a. Predictors: (Constant), Time120, Time0, Time72, Time48, Time96, Time24

ANOVA^b

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	182.843	6	30.474	4.385	.002 ^a
	Residual	257.157	37	6.950		
	Total	440.000	43			

a. Predictors: (Constant), Time120, Time0, Time72, Time48, Time96, Time24

b. Dependent Variable: Treatment

Variables Entered/Removed^b

Model	Variables Entered	Variables Removed	Method
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1	pH9, pH6.5, pH5.5, pH7.5, pH7, pH8, pH8.5, pH4, pH5, pH6, pH4.5 ^a		Enter
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a. All requested variables entered.

b. Dependent Variable: Treatment

Model Summary

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.945 ^a	.894	.797	.78666

a. Predictors: (Constant), pH9, pH6.5, pH5.5, pH7.5, pH7, pH8, pH8.5, pH4, pH5, pH6, pH4.5

ANOVA^b

Model		Sum of Squares	df	Mean Square	F	Sig.
1	Regression	62.574	11	5.689	9.192	.000 ^a
	Residual	7.426	12	.619		
	Total	70.000	23			

a. Predictors: (Constant), pH9, pH6.5, pH5.5, pH7.5, pH7, pH8, pH8.5, pH4, pH5, pH6, pH4.5

b. Dependent Variable: Treatment

Coefficients^a

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.
	B	Std. Error	Beta		

1	(Constant)	5.509	1.194		4.614	.001
	pH4	-.005	.039	-.392	-.117	.909
	pH4.5	-.026	.093	-2.127	-.277	.786
	pH5	.159	.052	13.173	3.051	.010
	pH5.5	-.073	.035	-5.326	-2.071	.061
	pH6	.036	.065	2.725	.552	.591
	pH6.5	-.092	.031	-6.770	-2.990	.011
	pH7	-.003	.022	-.306	-.160	.876
	pH7.5	.005	.035	.366	.155	.879
	pH8	-.037	.043	-3.200	-.870	.401
	pH8.5	-.010	.022	-.989	-.473	.645
	pH9	.023	.031	2.275	.761	.461

a. Dependent Variable: Treatment