BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS USING INDIGENOUS SOIL MICROORGANISMS

By

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DEDICATION

To my beloved parents whose faith in my abilities and tireless endeavors
has been a steady source of inspiration for me in all walks of life

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LIST OF ABBREVIATIONS

Abbreviation	Stands for
PAH	Polycyclic aromatic Hydrocarbons
GC	Gas Chromatography
pH	Power of hydrogen
°C	Degrees Centigrade
ml	Millilitre
g/L	Gram per litre
mg/L	Milligram per litre
ng/L	Nanogram per litre
lb/cm²	Pound per centimeter square
rpm	Rotations per minute
hrs	Hours
H_2SO_4	Sulfuric Acid
FID	Flame Ionization Detector
kPa	kilopascal
cm/sec	centimeter per second
mL/min	Millilitre per minute
µl	Microlitre
MR	Methyl red test
PCP	Polychlorinated biphenyl
ppb	Parts per billion
ppt	Parts per trillion

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ABSTRACT

In Pakistan detoxification of polycyclic aromatic hydrocarbons (PAHs) contaminated sites require development of degradation system. Present research work is a laboratory scale study conducted to investigate the degradation of two polycyclic aromatic hydrocarbons i.e. anthracene and phenanthrene, which present a high pollution potential and health risk and have been used as a model for degradation studies on PAHs because of their relative toxicity.

This study aimed to evaluate anthracene and phenanthrene degradation potential by *pseudomonas* sp.The organism was able to grow on media containing anthracene and phenanthrene. It was found that the biomass of the organisms decreased with the increase of PAH concentration in the cultures. *Psudomonas* bacteria showed more growth on anthracene as compared to phenanthrene, which also indicates that anthracene is less toxic to bacterial culture than phenanthrene.

Analysis of degradation by *Pseudomonas* sp estimated by Gas chromatography revealed that with increase in concentration the degradation rate decreased. 350 mg/L of anthracene showed 57.02 % degradation, while 250 mg/L showed 82.344 %, for phenanthrene 250 mg/L showed 53.106 % degradation, while 100 mg/L showed 58.363 %. anthracene showed more degradation than phenanthrene. The maximum degradation was observed in first 3 to 4 days, which reduced with the passage of time due to lowered bacterial count.

Chapter 1

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic xenobiotic fused-ring aromaticcompounds consisting of hydrocarbon molecules of two or more fused benzene and/or pentacyclic rings in linear, angular or cluster formation (Cerniglia, 1992). They constitute a group of priority environmental pollutants, which are ubiquitous contaminants in soils and sediments and are of environmental concern because of their toxic, mutagenic and/or carcinogenic effects (Mallick *et al*., 2007). PAHs are formed, in large part, from the incomplete combustion of fossil fuels and are present in petroleum products and wood preservatives such as creosote (Maliszewska-Kordybach, 1999), there is also the accidental spilling of processed hydrocarbons and oils, coal liquefaction and gasification, or organic oil seepage and surface run-offs from forest/brush fires and natural geologic processes (Freeman and Cattell, 1990).

PAH contaminated sites are mostly found in or near cities, thus representing a considerable public health hazard. PAH's also possess physical properties, such as low aqueous solubility and high solid water distribution ratios, which stand against their ready microbial utilization and promote their accumulation in the solid phases of the terrestrial environment (Johnsen *et al.,* 2004). The aqueous solubility of PAH decreases approximately one order of magnitude for each additional ring. Thus the low aqueous solubility limits their bioavailability and thus the efficiency of a bioremediation process. Since PAHs exhibit toxic, mutagenic and carcinogenic properties, there is serious concern about their environmental presence, especially their potential for bioaccumulation in many food chains (Fujikawa *et al.,* 1993).

Exposures to PAHs have been linked to skin, lung, liver, intestine, and pancreas cancers (Doyle *et al.,* 2008). Although PAHs may fade away from the environment through adsorption, chemical degradation, photolysis, and volatilization, microbial degradation is the major process to remove PAH contamination in the environment (Yuan *et al.,* 2002).

Improvement in quality of life requires efforts to combat such PAH wastes by identifying cost effective and nature friendly remediation techniques. Development in the past few decades have made microbial system and bioremediation programs the method of choice over traditional methods such as land filling, incineration, excavation, etc. for the treatment of contaminated soils and water (Labana*et al.,* 2005). Biodegradation of organic matter and toxic chemicals in soil and water is most efficient, cost effective and nature friendly approach for removal of these compounds (Furukawa *et al.,* 1998). The basis of the process is to enhance the rate of natural microbial degradation of contaminants by providing the existing microorganisms with specific characteristics, which allows them to degrade the desired contaminant at a quick rate.

Ideally, bioremediation results in complete mineralization of contaminants into water and carbon dioxide without the buildup of intermediates (Frazar, 2000). Biodegradation has been successfully employed for the bioremediation of many pollutants such as pentachlorophenol, diesel oil, pesticides, herbicides and polycyclic aromatic hydrocarbons provided the environmental conditions are optimized (Mathava and Ligy, 2006).

Among different polycyclic aromatic hydrocarbons (PAHs) being released into the environment, phenanthrene and anthracene are important in terms of damage they are causing. Uptil now no significant research has been carried out in the country

regarding biodegradation. Therefore this study is being conducted to investigate biodegradation of polycyclic aromatic hydrocarbons i.e. phenanthrene and anthracene in laboratory conditions using indigenous soil culture and the influence of factors such as phenanthrene and anthracene concentration, temperature on breakdown and mineralization of polycyclic aromatic hydrocarbons.

Objectives of the Study

Bioremediation is defined as the process where organic wastes are biologically degraded under controlled conditions to a harmless state, or to levels below concentration limits established by regulatory authorities (Muller *et al.,* 1997). In this process, microorganisms, fungi, green plants or their enzymes use their natural biological activity to attack pollutants and to return the natural environment altered by contaminants to its original condition.

The study is specifically designed to evaluate the degradation potential of microorganisms for various concentrations of phenanthrene and anthracene in nutrient broth. Phenanthrene and anthracene concentration and temperature are optimized for achieving maximum bacterial growth and subsequent degradation during the process.

The specific objectives of the present study were:

1. Isolation, identification, and chemical characterization of indigenous soil microorganism having polycyclic aromatic hydrocarbon degradation potential, using the enrichment technique.
2. Biodegradation of phenanthrene and anthracene (PAH’s) using indigenous soil microorganisms by using Orbital Shaker technique.

3. Quantitative analysis using gas chromatography to correlate the reduction in phenanthrene and anthracene (PAH's) with the corresponding growth of microbes in order to determine the rate and extent of biodegradation.

Overall this research work is an initiative in developing remediation methodologies for cleaning up of contaminated sites thus improving the overall quality of life and environment.

Chapter 2

REVIEW OF LITERATURE

A current environmental concern is the contamination of soil with PAH's due to pesticide discharges, fossil fuel or other organic material combustion, the accidental spilling of processed hydrocarbons and oils, coal liquefaction and gasification, organic oil seepage and surface run-offs from forest/brush fires and other sources.

PAHs are relatively neutral and stable molecules, including one or more benzene rings, substituted or not in a linear or angular layout. PAHs have low solubility (Moore and Ramamoorthy, 1984) and low volatilities, except small components like naphthalene. The physical and chemical properties of selected PAHs are given in Table 2.1.

Name of Compound	Phenanthrene	Anthracene
Molecular Weight (g/mole)	178.2	178.2
Molecular Formula	$C_{14}H_{10}$	$C_{14}H_{10}$
Melting Point (°C)	100	280
Boiling Point (°C)	338	340
[a] Carcinogenic Potential	(-)	(-)
Aqueous Solubility µg/L at 25°C	73	1290
Physical Appearance	Brown Crystals	White Flakes

[a]National Academy of Science (1972) (-); indicates Non Carcinogenic.

Table 2.1. Physical and Chemical Properties of Selected PAHs

PAHs show long half life in geological media. A few PAHs are used to make pesticides, plastic and dyes. Others are contained in asphalt used in road construction. PAHs are found in the environment in the air, water and soil. There are more than 100 different PAH compounds that cause health effects.

Growing awareness about the quality of life demands that the improvement in the quality of environment by establishing cost effective nature friendly remediation technologies for treating PAH contaminated sites. Among the different methods being used nowadays, bioremediation has gained popularity due to its nature friendly approach. It has become a technology of the modernized word. Keeping in view the prevailing situation of toxic waste in the country, this research is a preliminary step for establishing biodegradation system at laboratory scale for handling PAHs waste.

2.1 PAHs Contamination

Since PAHs exhibit toxic, mutagenic and carcinogenic properties, there is serious concern about their environmental presence, especially their potential for bioaccumulation in many food chains (Fujikawa*et al.,* 1993). The US Environmental Protection Agency has identified 16 PAH compounds as priority pollutants whose levels in industrial effluents require monitoring (Heitkamp and Cerniglia, 1988). Possible fates for PAHs released into the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation and adsorption on soil particles. The principle processes for their successful removal are currently believed to be microbial transformation and degradation (Gibson *et al.,* 1975).

There has been increasing interest in the bioremediation of terrestrial and aquatic PAH-contaminated environments. Interest in the biodegradation mechanisms and environmental fate of the pollutants is prompted by their ubiquitous distribution and

their potential deleterious effects on human health (Kastner*et al.,* 1997).The concentration of polycyclic aromatic hydrocarbons in the environment varies widely, depending on the level of industrial development, proximity of the contaminated sites to the production source and the mode of PAH transport. Reported soil and sediment PAH concentrations range from 1 μg/kg to over 300 g/kg (Kanalay*et al.,* 2000).

Since PAHs are genotoxic and carcinogenic, they represent considerable environmental concerns (Phalhmannand Pelkonen, 1987). Their mutagenicity varies with the number of aromatic rings. PAHs released into the environment could be removed by many processes, including volatilization, photo-oxidation,chemical oxidation, bioaccumulation, and adsorption on soil particles. However, the principal process for successful removal and elimination of PAHs from the environment is microbial transformation and degradation (Wilson and Jones, 1993). In PAH contaminated soil, microorganisms capable of utilizing and degrading hydrocarbons would be present and could be employed for PAH elimination (Catallo and Portier, 1992). Potential biodegradation strains isolated from hydrocarbon-contaminated environments have been found as active as or even higher than those originating from non contaminated soil, since certain bacteria could acclimatize and adapt to the contaminated environments (Chaineau*etal.,* 1999). Several different bacterial genera, including species of *Pseudomonas*, *Alcalgenes*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas* (Han *et al.,* 2003) and *Cycloclasticus* (Dyksterhouse *et al.,* 1995) arecapable of degrading PAHs. Numerous fungal species havealso been found to degrade PAHs of both low and highmolecular weight (Mueller *et al.,* 1997). For bacterial isolates, the majorityhave enriched their ability to grow on low molecular PAHs(2- or 3-ring PAHs). Nevertheless, some studies have shown that bacteria such as *Mycobacterium*, *Rhodococcus*, *Alcaligenes*, *Pseudomonas*, and *Sphingomonas*are

able to grow on the four-ring PAHs (Dagher*et al.,* 1996). Moreover, numerous otherlower molecular weight PAHs facilitated to degrade highermolecular weight PAHs, when lower and higher PAHswere co-metabolized (Mueller *et al.,* 1997).

The interaction betweendifferent microorganisms under mixed-culture conditionssuch as co-metabolism or antagonism might also be important,and biodegradation of toxic organic compounds such asPAHs by mixed culture could be different from that of asingle culture (Kobayashi and Ritmann, 1982).

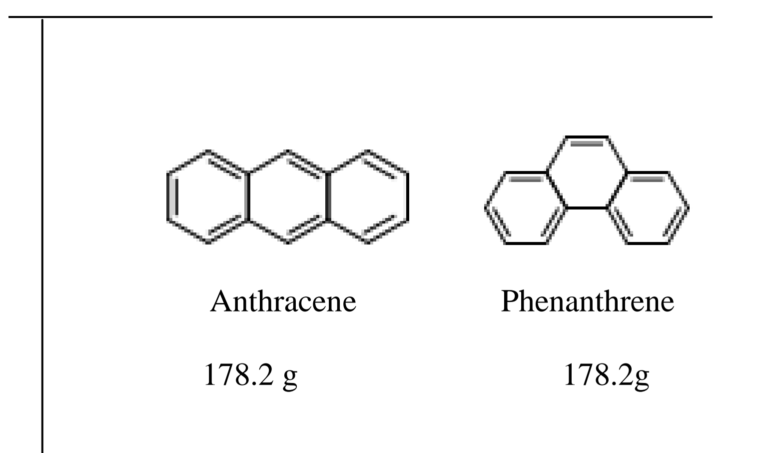

Figure 2.1 Molecular Structure and Molecular Mass of Anthracene and Phenanthrene

2.2 Sources of PAHs in the Environment

PAHs are listed as priority pollutants, being found in air, water and soil. They are formed naturally during the incomplete combustion of organic matter or by many anthropogenic activities, such as the petrochemical industry and oil refining. Sources of PAHs can be broadly classified as pyrogenic (combustion origin) or petrogenic(petroleum origin). The presence of high molecular PAHs indicates likely pyrogenic like benzo(α)pyrene, benzo(*k*)fluoranthene. While the presence of low

molecular PAHs indicates likely petrogenic like naphthalene, anthracene and phenanthrene.

When PAHs are discharged into the atmosphere they will partition between particulate and gaseous phases. These PAHs contributing to wet and dry decomposition are a function of their vapor exchange across air water interface. Wet and dry deposition has been reported as major transport processes for atmospheric PAHs to the aquatic environment (Terzi and Samara, 2005).

Seasonal changes may also have an effect on deposition rates of PAHs. Seasonal differences in environmental conditions such as rain characteristics, temperature, wind speed and possible changes and source area contributions are likely to affect the deposition characteristics of PAHs in any area.

PAHs in ground water can occur in both particulate and soluble forms, although studies have identified particulate form as being the most predominant (Pitt *et al.,* 1999). Residues of PAHs in ground water are usually due to leaching of the top soil. Lateral movements of water from industries or urbanized cities is a major source of high PAH concentration in ground water. Masih *et al.* (2008) observed from their study in Agra, India that the total concentration of PAH in ground water range from 13.2 to 64.3 ng/L.

Although PAHs may fade away from the environment through adsorption, chemical degradation, photolysis, and volatilization, microbial degradation is the major process to remove PAH contamination in the environment (Yuan *et al.,* 2002).

2.3 Health Effect of PAHs

PAH exposure occurs by inhalation, ingestion and dermal contact and PAHs are highly lipid-soluble and quickly absorbed by the gastro-intestinal tract of mammals. PAH metabolism in the human bodyproduces epoxide compounds with mutagenic andcarcinogenic properties, cases of lung, intestine, liver,pancreas and skin cancer being reported (Samanta *et al.,* 2002).

Health effect from chronic or long term exposure to PAH may include cataracts, kidney and liver damage and Jaundice. Naphthalene a specific PAH can cause breakdown of red blood cells if inhaled or ingested in large amounts.

Animals exposed to PAHs over long periods in laboratory studies have developed lung cancer from inhalation, stomach cancer from ingesting PAHs in food and in skin contact. Long term studies of workers exposed to mixtures of PAHs and other workplace chemicals have shown an increase rate of skin, lung, gastrointestinal and bladder cancer. The studies have also reported asthma like symptoms, lung function abnormalities, chronic bronchitis and decreased immune functions. (Saba and Hashmi, 2010).

2.4 Biodegradation Techniques

Biological treatments are cheaper than alternatives such as incineration, storage, or soil washing (Cookson, 1995). Bioremediation is a technique that enhances the natural rate of biodegradation of pollutants through reactions carried out by selected microorganisms (Providenti *et al.,* 1993).

Considerable attention has been focused on the potential use of microorganisms to remediate soils contaminated with persistent organic pollutants (Bogardt *et al.,* 1992). Since PAHs are hydrophobic compounds with low solubility in water they have a tendency to bind with organic matter or soil, limiting their availability to

microorganisms. Despite these properties many bacterial strains have been isolated for their ability to transform, degrade and utilize PAHs as a source of carbon and energy (Lee *et al.,* 2001).

The contamination of the environment with hydrocarbons provides serious problems for many countries. Scientists have conducted research on cost-effective clean-up techniques with minimal long-term damage to the environment. Biodegradation of hydrocarbon-contaminated soils, which exploits the ability of microorganisms to degrade and/or detoxify organic contamination, has been established as an efficient, economic, versatile and environmentally sound treatment. The extent of hydrocarbon biodegradation in contaminated soils is critically dependent upon three factors: a) the creation of optimal environmental conditions to stimulate biodegradative activity, b) the predominant petroleum hydrocarbon types in the contaminated matrix and c) the bioavailability of the contaminants to microorganisms. Additionally, the degradation is also affected by the molecular composition of the hydrocarbons (Marques-Rocha *et al*., 2001). Mehrasbi*et al*. (2003) investigated biodegradation of petroleum hydrocarbons. In the laboratory experiments, during 5 months, the activities of petroleum hydrocarbon-degrading microorganisms and dehydrogenase activity of soil was determined. Gas chromatographic analysis showed the biological decontaminations for gas oil, kerosene and synthetic mixture (gas oil, kerosene and furnace oil) was 60 %, 36 % and 55 %, respectively.

Bacteria are the class of microorganisms actively involved in the degradation of organic pollutants from contaminated sites. A number of bacterial species are known to degrade PAHs. Most of them, representing biodegradation efficiency, are isolated from contaminated soil or sediments. Long-term petrochemical waste discharge harbours bacteria capable of degrading PAH to a considerable extent.

2.5 Polycyclic aromatic hydrocarbons degradation

By far the most important route for the environmental degradation of phenanthrene and anthracene is microbial degradation. This is because a variety of microorganisms are capable of degrading the compounds in different habitats and circumstances.

2.5.1 Anthracene Degradation

Anthracene is one among more than 100 polycyclic aromatic hydrocarbons (PAHs) that are chosen for the study of PAH degradation because of its relative toxicity. These chemical compounds show a skeleton of carbon and hydrogen atoms arranged in two or more aromatic rings of low solubility that makes them resistant to nucleophilic attack Johnsen*et al.* (2004) aimed to evaluate anthracene degradation by *Pseudomonas sp*. by isolating it from a 14-year-old petrochemical sludge land farming site. Three isolates were selected from twenty six by the best growth in anthracene and two of them were identified by 16S rRNA gene sequencing as *Ps. aeruginosa* and *Ps. citronellolis*. They showed better growth at pH 7.0 and 30 °C in medium containing up to 2 g/Lanthracene. They were also able to grow in medium containing phenanthrene, pyrene, gasoline and diesel oil. Analysis of anthracene degradation estimated by gas chromatography showed that *Ps. aeruginosa* isolate 312A had the highest rate ofdegradation (up to 3.90 mg/L per day), degrading 71% of the anthracene added to the medium (250 mg/L) after 48 days. *Ps. citronellolis*222A showed an intermediate level of degradation (51%), but *Ps. aeruginosa* 332C degraded only 24.4%. Isolate 312A was also responsible for the highest phenanthrene and pyrene degradation after 48 days.

Figure 2.2 shows the generalized biodegradation pathway of anthracene as proposed by many studies (Ouyang, 2011).

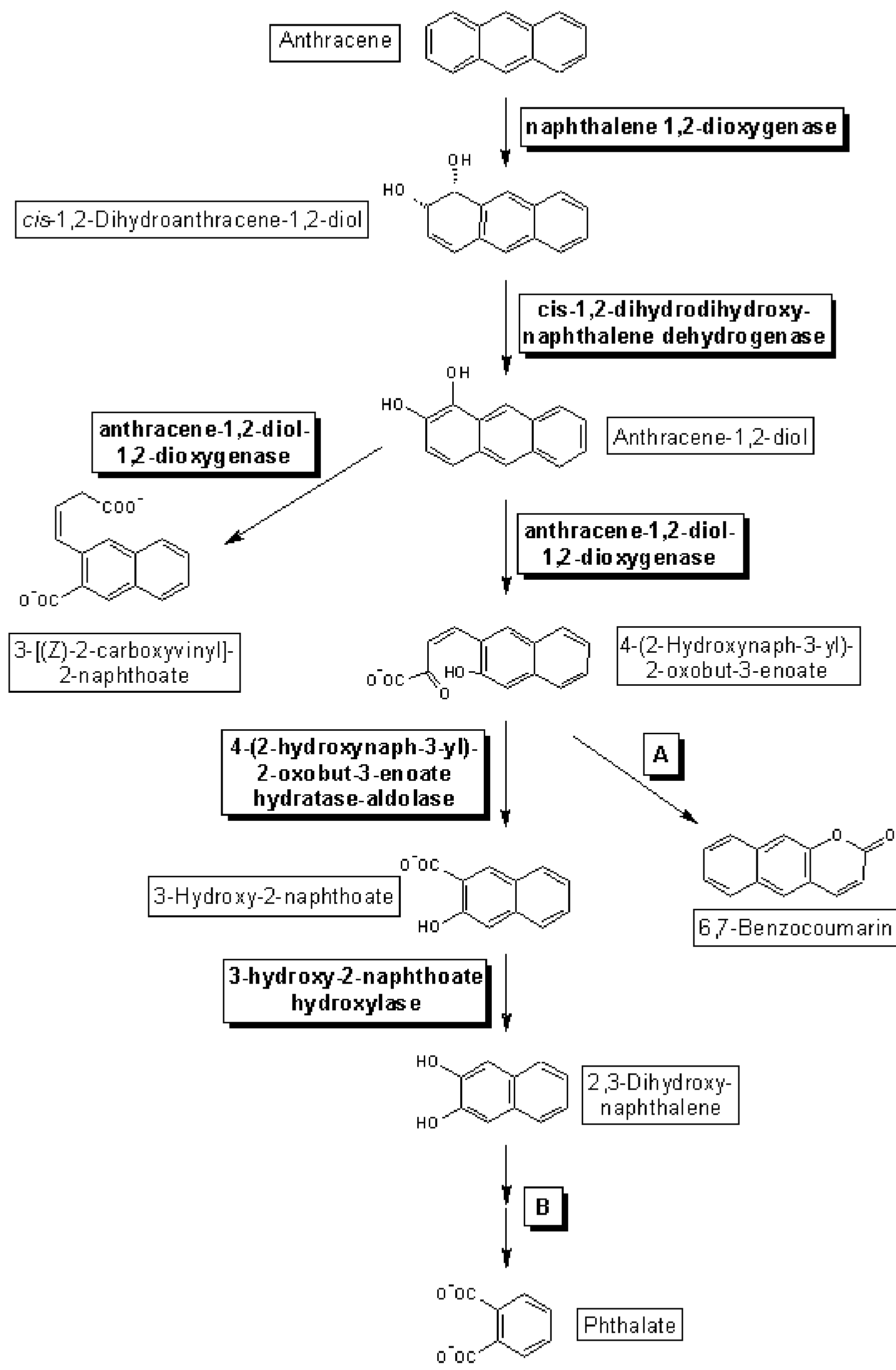

Figure 2.2 Biodegradation pathway of Anthracene

2.5.2 Phenanthrene Degradation

Kim *et al.* (2005) investigated the Degradation of phenanthrene by bacterial strains isolated from soil in oil refinery fields in Korea. The degradation of phenanthrene, a model PAH compound, by microorganisms isolated from oil-contaminated soil in oil refinery vicinity sites, was examined. The optimal conditions for phenanthrene biodegradation were determined to be 30°C and pH 7.0. A significantly positive relationship was observed between the microbial growth and the rate of phenanthrene degradation.

Janbandhu and Fulekar in 2010 isolated and characterize high-efficiency PAH-degrading microbial consortium from 3 decade old petrochemical refinery field located in Nagpur, Maharashtra with history of PAH disposal. Based on biochemical tests and 16S rDNA gene sequence analysis the consortium was identified as *Sphingo bacterium sp., bacillus cereus* and a novel bacterium *achromobacter insolitus MHF* ENV IV with effective phenanthrene degrading ability. The biodegradation data of phenanthrene indicates about 100%, 56.9% and 25.8% degradation at the concentration of 100 mg/L, 250 mg/L and 500 mg/l respectively within 14 days.

Ping Zhao *et al.* 2008 identified a bacterial strain *Pseudomonas stutzeri* ZP2 w with phenanthrene degrading ability. It is the first time that *P. stutzeri*is reported to process the capability for phenanthrene degradation. The strain was isolated from soil samples contaminated with polycyclic aromatic hydrocarbon (PAH) containing waste from an oil refinery field in Shanghai, China. Strain *P. stutzeri*. ZP2 can utilize naphthalene, phenanthrene and tween 80 as its sole carbon source and can degrade phenanthrene very fast, 6 days for 96% phenanthrene at 250 ppm concentration. The optimal growth conditions of strain ZP2 was determined to be at pH 8.0, 37 °C, respectively. The

results also indicate that strain ZP2 can remove more than 90% of phenanthrene at any concentrations ranged from 250 to 1000 ppm in 6 days.

Figure 2.3 shows the generalized biodegradation pathway of phenanthrene as proposed by many studies (Ouyang, 2011).

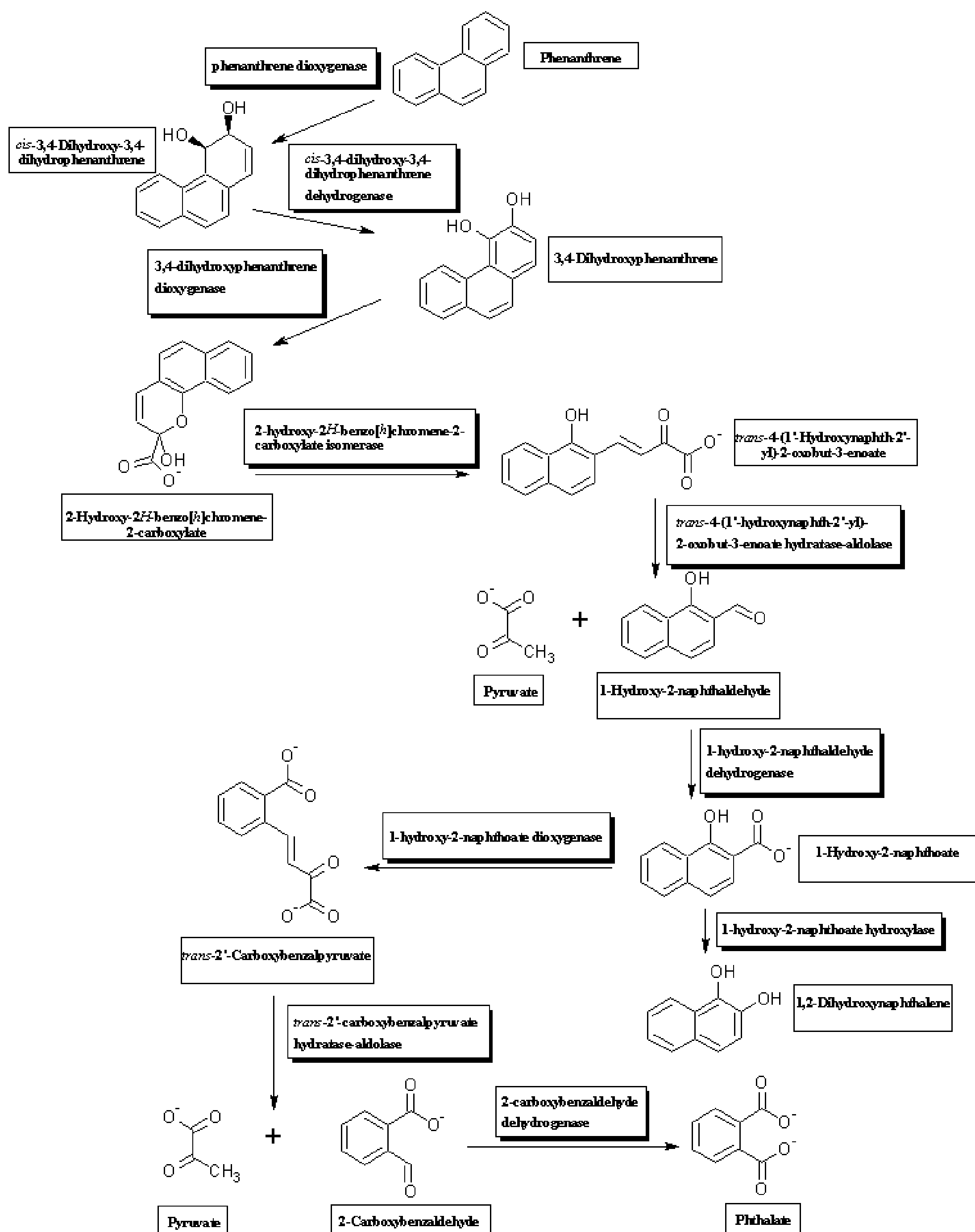

Figure 2.3 Biodegradation pathway of Phenanthrene

From the literature review it is evident that PAHs are potential contaminants and area of growing concern in anthropogenic activities. The aim of this research work was to develop a biodegradation system by isolating and identifying PAH degrading indigenous soil microorganisms. Further to analyze degradation potential of microorganisms at significantly higher concentrations of PAHs. The lab scale study will be beneficial for establishment of optimal environmental conditions for bioremediation system in the field.

Chapter 3

MATERIALS AND METHODS

Present study was carried out in the Environmental Microbiology and Biotechnology Research laboratory, Institute of Environmental Sciences and Engineering at National University of Sciences and Technology.

Biodegradation studies of anthracene and phenanthrene were conducted by using indigenous soil bacterial culture in orbital shaker. The increase in growth of microorganisms determined the utilization potential of polycyclic aromatic hydrocarbons by microorganisms and resulted in decrease of organic matter. The quantitative analysis using gas chromatography was carried out to correlate the reduction in polycyclic aromatic hydrocarbons with the corresponding growth of microbes in order to determine the rate and extent of biodegradation.

3.1 Chemicals and Standards

The commercial grade polycyclic aromatic hydrocarbons used in this study were purchased/obtained from Merck. The reliability of the experiment was maintained by using commercial sample as they represent the actual situation to which the microorganism is likely to be exposed in the environment. The standard analytes of anthracene and phenanthrene were purchased from Chiron chemicals, Australia.

3.2 Concentration of PAHs

For growth studies different concentrations of anthracene and phenanthrene were used in nutrient broth to determine the utilization and biodegradation of bacteria. Concentration of anthracene 250 mg/l and 350 mg/l and phenanthrene 100 mg/l and 250 mg/l in nutrient broth were used during the research work.

3.3 Preparation of Culture Media

3.3.1 Washing and sterilization of glassware

For experiments, all glassware was dipped in solution of chromic acid for 24hrs, then washed thoroughly with tap water and then finally rinsed with distilled water. For sterilization they were then wrapped in aluminum foil and autoclaved at 121°C and 15 lb/cm² pressure for 15 minutes. The sterilized glassware was then dried in oven at 171°C for two hours before use (Prescott *et al.,* 2003).

All glassware for gas chromatographic analysis was prepared prior to sampling. Preparation of glassware included washing with phosphate free detergent, then washing with chromic acid and then rinsing with distilled water followed by n-hexane.

3.3.2 Preparation of Medium

Nutrient agar and Nutrient Broth (Merck KGA) were prepared according to the manufacturer's instructions. It was used for the isolation and growth studies of culture. Nutrient broth medium after preparation was dispensed in 500 ml flask for growth studies. Nutrient agar medium 400 ml was prepared in 500 ml flask, for slants and media plates. These were then autoclaved at 121°C and 15 lb/cm² pressure for 15 minutes. Nutrient agar plates and slants were prepared in aseptic conditions of laminar flow hood. Sterility of media was checked for 24 hours by incubating them at 35 °C and was used for growth studies of microorganisms. Slants were used for inoculums preparation, used in degradation studies (Kao *et al.,* 2005).

3.4 Isolation of anthracene and phenanthrene degrading bacteria

3.4.1 Bacterial Culture

Pseudomonas specie was used for the degradation studies. The bacterial culture used in this study is designated as IES-*Ps*-1 was available at the Institute of Environmental Sciences and Engineering (IESE), through earlier work of Hashmi (2001) and Ishaq (2009). However for present research work pure culture of *Pseudomonas* was also isolated.

3.4.2. Isolation of Bacteria

The *Pseudomonas* specie capable of degrading anthracene and phenanthrene was isolated using soil enrichment technique. Wet unsieved soil was collected from polycyclic aromatic hydrocarbon contaminated soil from agricultural land near Attock oil refinery, Morgah Rawalpindi.

The sample was sieved and then diluted to make 1/10 dilution. Dilution was shaken well then 100 µl of sample was poured in nutrient agar plates and spread with a sterilized spreader. The plates were placed in incubator at 35°C for 24 to 48 hours. Number of colonies appeared after the incubation.

3.4.3. Purification and Enrichment of Bacterial Culture

The isolated bacteria were purified by repeated streaking on nutrient agar plates. Colonies appeared after incubation at 37°C after 24 hours. These colonies were again sub cultured on nutrient agar plates for further purification for screening.

Figure 3.1 illustrates the isolation and identification of PAH degrading bacterial culture

Soil (10gms) + 250 ml autoclaved distilled water + Anthracene and Phenanthrene (1.5mg/l)

↓ Incubated at 37°C in Orbital shaker for 14 days

Sub cultured from flask onto nutrient agar

↓

Observation of growth on nutrient agar

↓

Culture identification

↙ ↘

Microscopy

↓

Gram's staining

Biochemical tests

↓

Catalase test

Citrate utilization

Methyl red test

Indole test

Figure 3.1 Schematics of isolation and identification of PAH degrading bacterial culture

3.5. Identification and Characterization of *Pseudomonas*

Identification of and characterization of *pseudomonas* was done up to genus level using Gram's Staining and biochemical differentiation tests as described by Collin and Lyne (1985).

3.5.1. Gram staining

Gram staining is a method of differentiating bacterial species into two large groups; gram positive and gram negative based on the physical and chemical properties of their cell walls. Gram's staining was done by preparing a smear of bacterial isolates by first putting a drop of distilled water on the slide. Small amount of inoculum was then spread with the help of inoculating loop, then it was air dried and after that it was heat fixed by passing through the flame two or three times, then smear was flooded with crystal violet for 1-2 minutes. After that it was washed with distilled water, smear was then flooded with iodine for 1-2 minutes and washed with water. After iodine treatment ethanol (70%) was added in the smear for 3-5 minutes. At last smear was stained with safranine for 30-45 seconds and finally smear was washed with distilled water and air dried. Drops of oil immersion was put on the slide and covered with cover slip. It was then observed under microscope

3.5. 2. Methyl red test

This test was performed to check the ability of an organism to produce and maintain stable acid products from glucose fermentation. A positive test shows pink to red color.

The medium for this test was prepared by dissolving all the required nutrients in distilled water pH was set at 6.9 after that media was poured into two sets of test tubes. These test tubes were then covered with cotton plugs and then they were autoclaved at 121 ^{0}C and 151b/cm^{2} pressure for 15 minutes. The medium was allowed

to cool, and then the bacterial strains were inoculated. One set of inoculated test tubes was placed in incubator at 37 ^{0}C for 24 hours. At last results were observed after adding 50 µl of methyl red in each test tube and change in color was recorded.

3.5.3. Citrate Utilization Test

Simmons' Citrate Agar is a defined, selective medium that tests for an organism's ability to use citrate as a sole carbon source and ammonium ions as the sole nitrogen source. The medium was prepared by dissolving all the required nutrients in distilled water. pH was set at 6.9 and it was autoclaved at 121 ^{0}C and 15 lb/cm^2 pressure for 15 minutes. It was then poured in sterilized petri plates for solidification. The medium was allowed to cool, and then the bacterial cultures were streaked on it. One plate was set as control. These were then placed in incubator at 37 ^{0}C for 24-48 hours. Growth as well as change in color of medium was observed.

3.5.4. Catalase test

Catalase test is used to detect the presence of enzyme catalase. Catalase is the enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen. The catalase test was performed by taking cultures of bacteria on a glass slide. A drop of 3% hydrogen peroxide was dropped on it. Bubbling indicates the presence of catalase enzyme.

3.6 Biodegradation Studies

3.6.1 Preparation of Inoculum for Biodegradation Study

The inocula for the experiment of anthracene and phenanthrene degradation study were prepared by growing acclimatized culture on nutrient agar slants, incubated for 24 hours at 37°C. The culture slants were washed with 10ml of phosphate buffer having pH 7 and used as inoculums as degradation experiment. (Qiu *et al.,* 2007)

3.6.2 Acclimatization of *Pseudomonas* in Nutrient Broth using lower concentrations of anthracene and phenanthrene

Early experiments were conducted with different concentrations (100, 250 and 400 mg/L) of anthracene and phenanthrene to study the growth pattern of *pseudomonas* in relation to anthracene and phenanthrene in nutrient broth. All experiments were carried out in triplicate.

3.6.3 Anthracene and Phenanthrene Degradation in Orbital Shaker

The degradation potential of *pseudomonas* for anthracene and phenanthrene was performed using the orbital shaker. In 500 ml conical flasks, 400 ml of nutrient broth with two concentrations of anthracene (250 mg/L and 350 mg/L) and phenanthrene (100 mg/L and 250 mg/L) dissolved in 10 ml of dimethylsulfoxide was inoculated with 10 ml of inoculum on orbital shaker at temperature 37° C and 120 rpm (Janbandhu and Fulekar, 2011).

Orbital Shaker	Settings
Speed	120 rpm
Total Volumetric Load	400 ml
Temperature	37° C
Retention Time	14 days.
Sample Collection	24 hrs.
Size of Inoculum	15 ml ($3x10^8$ Mc Farlands turbidometric index)

Table 3.1: Technical Data for Orbital Shaker

3.7 Sampling Procedure

After every 24 hours, 15 ml sample was extracted from the experimental flasks and filtered under sterilized conditions of laminar flow hood. 2 to 3 drops of diluted H_2SO_4 were added to stop any further degradation process. Samples were then refrigerated.

3.8 Bacterial Growth Studies

To study the bacterial growth different dilutions were made of inocula up to 10^{-6} and plating was done on nutrient agar. These plates were then placed in incubator at 37^0C for 24 hours. After incubation the viable colonies were counted using the method described by Collins and Lynes (1985).

3.9 Gas Chromatographic Analysis

Gas chromatographic analysis was performed using a Shimadzu 2010 series gas chromatograph coupled with FID detector. The column used was fused to silica capillary column whose length is 30 m, inner diameter of 0.32 mm, thickness is 0.5 µm. Figure shows the overall schematic representation of GC working with FID. In injector split injection mode was used and the split ratio was 50. Carrier gas was helium. The pressure of the gas was 46.9 kPa and total flow was 54 mL/min. Out of this total flow the column flow was 1 ml/min. Linear velocity for the gas flow was 23.8 cm/sec. makeup flow of nitrogen gas was 30 mL/min. Figure 3.2 represents diagram of GC working on FID detector.

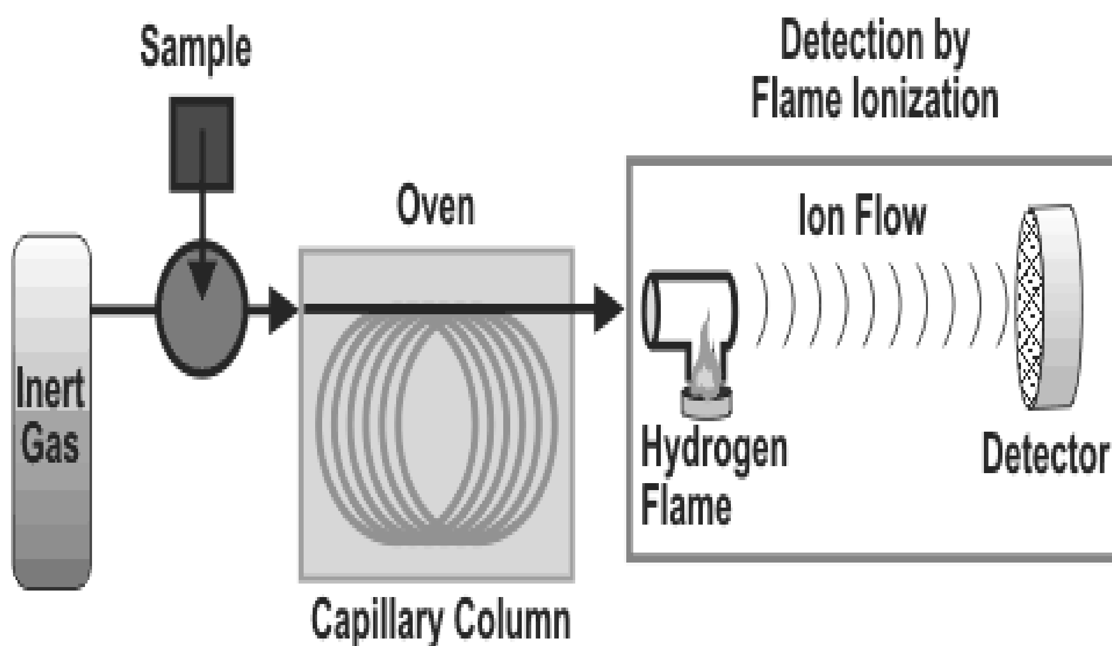

Figure 3.2 Gas Chromatography working on FID

3.10 Standard Dilutions Preparation

Anthracene and phenanthrene dilutions were prepared by dissolving 100 µl of standard analyte in 1 ml of GC grade n-hexane. Liquid injections of 1 µl were given to instrument (GC 2010) to check the signal and retention time of both analytes.

3.11 GC Programming

Shimatzu GC 2010 was used for the analysis. FID detector was used. The GC separation column used was a fused silica capillary column. The column has internal diameter of 0.32mm and a length of 30 meters. The internal lining of siloxanes is 1µL.

Before running actual samples, the instrument was optimized by altering various conditions. These included the injector temperature, column oven temperature, detector temperature, gas flow rates, split ratio etc. Preliminary studies include response check of GC to concentrations of analytes. 1µL stock solution of each standard stock solution was injected into the GC injection port with a glass syringe. To achieve maximum separation and resolution of individual peaks, GC conditions that are injector temperature, column oven temperature, detector temperature, gas flow rates, split ratio were altered. The temperature program were developed and evaluated for signal resolution and noise level. Table 3.2 shows gas chromatographic conditions.

Parameters	Conditions
Carrier gas	Helium
Makeup Gas	Nitrogen
Carrier Gas Flow	1ml/min
Oven Temperature	280°C
Initial Temperature	200°C
Final Temperature	280°C
Gradient of temperature rise	10°C/min
Temperature of Injector	250°C
Temperature of Detector	280 °C
Dimensions of Column	
Length	30m
Inner Diameter	0.32mm
Thickness	0.5 µm
Filling Material	5% diphenyl, 95% Dimethyl polysiloxane

Table 3.2: Gas Chromatographic Conditions

3.12 Liquid-Liquid Extraction

Liquid-liquid extraction, also known as solvent extraction and partitioning, is a method to separate compounds based on their relative in immiseable liquids, usually water and an organic solvent. Liquid-liquid extraction is an inexpensive technique performed using separatory funnel. It is the extraction of a substance from one liquid phase into another liquid phase. Figure shows liquid-liquid extraction phenomenon.

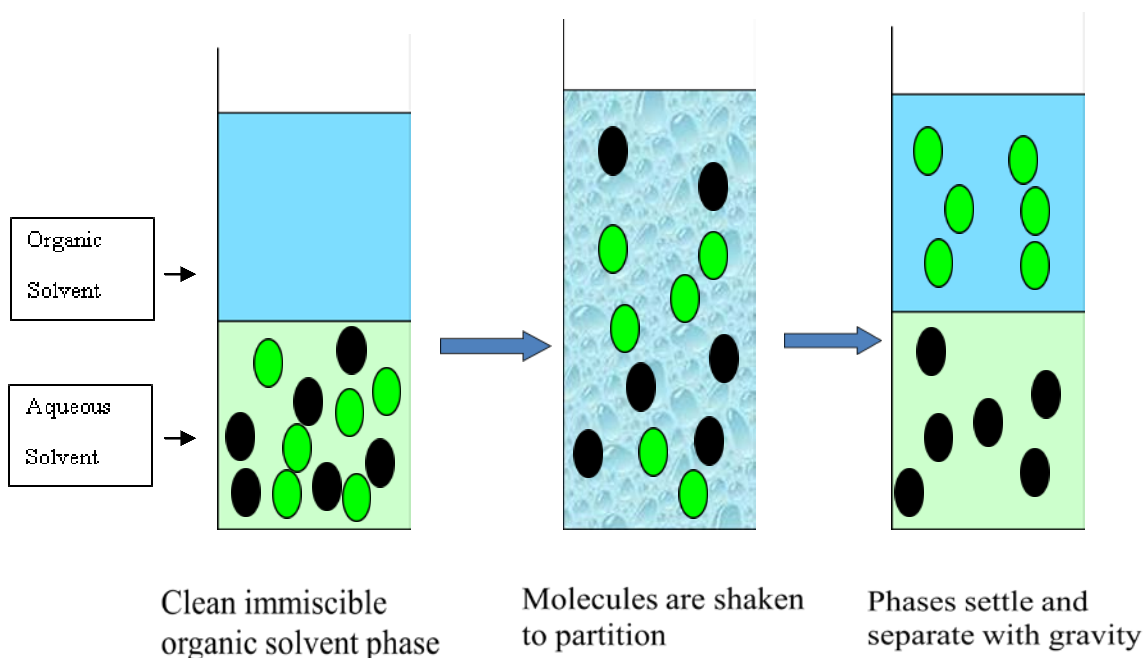

Figure 3.3: Liquid-liquid extraction phenomenon

The extraction procedure was carried out by liquid-liquid extraction method (US EPA, 1994). The apparatus for this method consisted of a 50 ml volume separating funnel mounted on a retort stand. The separatory funnel was thoroughly washed with distilled water and chromic acid and dried in a muffle furnace at high temperature. Prior to use separatory funnel is rinsed with n-hexane and then allowed to completely dry.

15 ml of nutrient broth to be extracted was transferred to the separatory funnel and 15 ml of n-hexane was added to it. These two were shaken vigorously for 5 minutes and allowed to separate and settle. After 10 minutes the aqueous layer was removed and the process repeated with the organic layer. The organic solvent was then passed through hydrophilic syringe filter cartridges so if any water remained behind it could also be removed.

The samples collected after liquid-liquid extractions were kept in glass vials. They were stored at 4°C in sample storage refrigerator. The samples were mostly analyzed few hours after extraction.

3.13 Sample Analysis

Firstly the syringe was immersed in the glass vials and sample was extracted. 1 µl of the extract was injected into the GC for analysis. The chromatogram obtained showed the peaks of anthracene and penanthrene.

Single point external standard method was used for quantification of analytes. This method makes use of the response factor that was calculated for zero hour sample analyte of known concentration, and then concentration of other samples was calculated according to it.

Chapter 4

Results and Discussion

Work on biodegradation studies were conducted to study the removal of anthracene and phenanthrene, in nutrient broth medium by *Pseudomonas* culture and to determine the influence of Polycyclic aromatic hydrocarbon concentration on bacterial growth and subsequent removal of organic matter. The extent of biodegradation of the Polyaromatic Hydrocarbons was conducted using gas chromatography.

4.1 Isolation of Anthracene and Phenanthrene Degrading Bacteria

Enrichment technique was used for the isolation of anthracene and phenanthrene degrading bacteria from soil under sterilized conditions. Bacterial colonies were observed on nutrient agar, microscopic observations revealed these colonies as Gram negative.

4.2 Identification and Chemical Characterization of Anthracene and Phenanthrene Degrading Bacteria

In the present study the soil isolates were identified up to genus level. No attempts were made for speciation. Gram staining technique was carried out to study the morphological characteristics and MR, Citrase and Catalase tests were performed for chemical identification. On the basis of morphological, cultural and biochemical characterization, the strain was identified as a member of genus *Pseudomonas* according to the "Bergeys Manual of Determinative Bacteriology" (1994) as shown in Annexure 1. The characteristics of *Pseudomonas* are listed in table 4.1.

This work supports the findings reported by other researchers, that *Pseudomonas* are Gram negative , rod shaped, highly oxidative metabolically versatile and have been

reported to degrade toxic organic chemicals, aromatic hydrocarbons, oil and petroleum products and pesticides (Ramanthan and Lalithakumari, 1999 ; Anjali Janbandhu and Fulekar, 2011).

Tests	*Pseudomonas*
Shape	Thin short Rods
Gram Stain	Gram Negative
Growth on Nutrient Agar	Colonies are round, Smooth & convex
Motility	Motile
Nitrate Reduction	Positive
Indole Production	Negative
Oxidase and Catalase	Positive
MR reaction	Negative
Citrate Utilization	Positive

Table 4.1 Characteristics of *Pseudomonas*

4.3 Bacterial Acclimatization to Anthracene and Phenanthrene

The isolated organism was designated as *Pseudomonas*, and its ability to grow on anthracene and phenanthrene was tested. Initial experiments were carried out to study the adaptation and acclimatization of bacterial culture in anthracene and phenanthrene. Different concentrations of anthracene and phenanthrene (100, 250, and 400 mg/L) and control was used to study the growth of inoculated culture for 3 days. The *Pseudomonas* species showed positive growth in presence of polycyclic aromtic hydrocarbons.

There have been many reports of PAH's degradation by different bacteria species including *Pseudomonas species*. (Johri *et al., 1*999). He-Ping Zhao *et al.* (2008)

identified a bacterial strain *Pseudomonas stutzeri* with phenanthrene degrading ability. The *Pseudomonas stutzeri* strain can utilize phenanthrene as its sole carbon source and can degrade it very fast, 6 days for 96% phenanthrene at 250ppm concentration. Johnsen *et al.* (2005) aimed to evaluate anthracene and phenanthrene degradation by *Pseudomonas* sp. The bacterial isolate was able to grow in medium containing Phenanthrene, and Anthracene. Oluwafemi *et al.,* (2009) reported that *P. aeruginosa* exhibited fair degradative ability on Anthracene.

It has been reported that several different bacterial genera, including species of *Pseudomonas* are capable of degrading polycyclic aromatic hydrocarbons. The majority of bacterial isolates including *Pseudomonas* have ability to grow on low molecular PAHs (2- or 3-ring) and are also able to grow on the four-ring PAH (Cerniglia, 1992 ; Mueller *et al.,* 1997).

From the biodegradation aspect in natural environment, microorganisms that have degradation capability and also show chemotaxis towards a compound would be more efficient for bioremediation than non-chemotactic microorganisms (Samanta *et al.,* 2002). There have been several reports in the literature regarding chemotaxis of *P. aeruginosa* and *P. putida*, (Adler, 1975 ; Harwood, *et al.,* 1984)

Based on the result of present studies and those of other researchers, it is concluded that *Pseudomonas* is able to grow in the presence of anthracene and phenanthrene.

4.4 Growth Studies using orbital shaker

4.4.1 Effect of different concentrations of Anthracene and Phenanthrene

In the present study bacterial growth for two different concentrations of anthracene and phenanthrene in nutrient broth was observed. For growth studies standard plate count method as described by Collins and Lynes (1985) was used. Figure 4.7 and 4.8

shows the growth of *Pseudomonas* with different concentrations of anthracene and phenanthrene

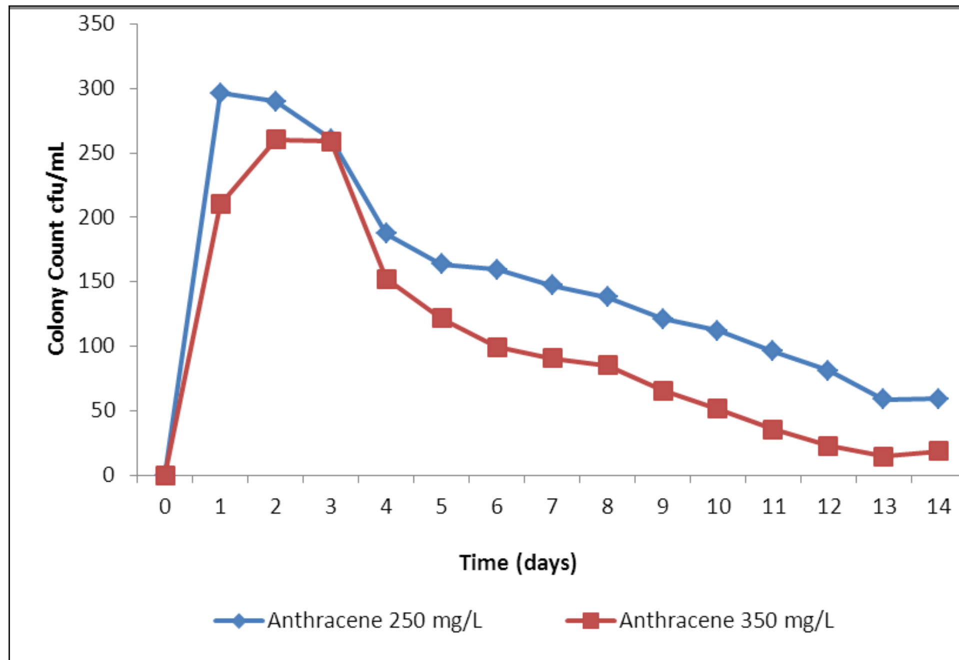

Figure 4.7 Growth of *Pseudomonas* with different concentrations of Anthracene

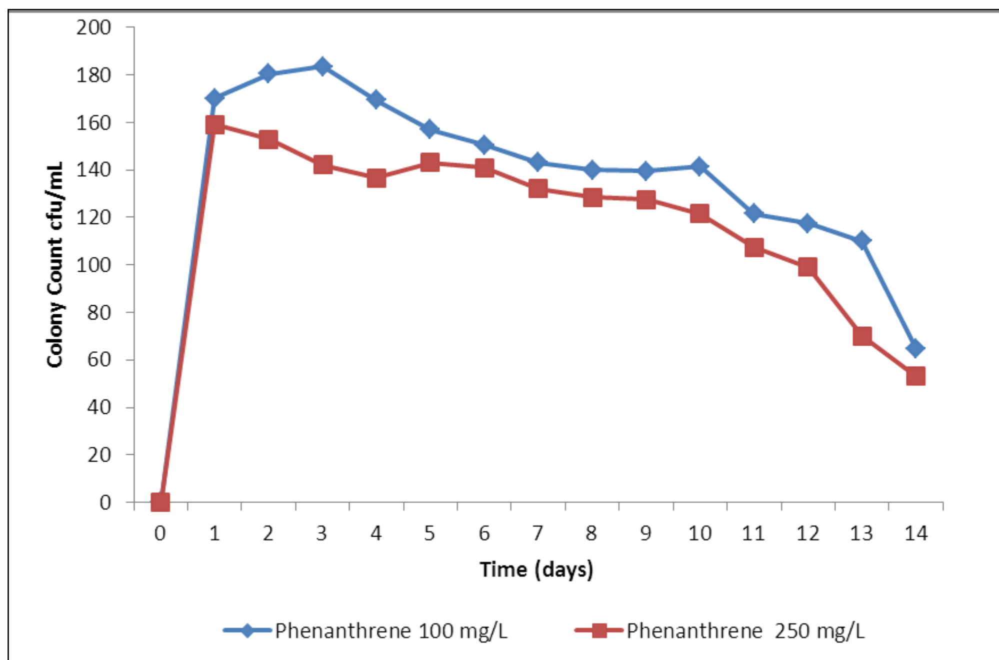

Figure 4.8 Growth of *Pseudomonas* with different concentrations of Phenanthrene

Reports by previous works make it clear that Polycyclic aromatic Hydrocarbons exhibit high toxicity to growth of microorganisms. The toxicity of higher concentration limits the microbial growth and subsequent degradation by microbes (Labana *et al.,* 2005). Jalani and Khan (2006), while studying the effect of cypermethrin concentration on growth of *Pseudomonas* species, reported that pesticide degradation up to 51 % is possible if the initial concentration is 80mg/L and with increase in concentration to 125 mg/L only 22 % of degradation was observed. Yuan *et al*. (2000, 2001) reported that biodegradation rate decreased at higher phenanthrene concentrations due to increased toxicity of phenanthrene metabolites. Yuan *et al.* (2000) also reported that biodegradation of polycyclic aromatic hydrocarbons, by mixed bacterial culture, and found slow degradation rate at higher concentration rate due to increase level of toxicity.

Also Johnsen *et al*. (2004) reported that Microbial degradation of PAHs and other hydrophobic substrates is believed to be limited by the amounts dissolved in the water phase. Kao *et al.* (2005) investigated the effects of different pentachloro phenols (PCP) concentration on biodegradation by *Pseudomonas mendocina*. The experiments were undertaken using six different concentrations i.e. 20, 40, 80, 100, 150 and 320 mg/L. The result revealed that at lower concentrations, microorganisms had shorter lag phase of 6 days and approximately 99% of PCP removal was possible. At concentration of 150 mg/L the microorganisms required more time for acclimatization and complete removal of PCP that was 12 days where at 320 mg/L researchers observed that there was no removal even after 20 days of incubation. It was concluded that the growth of the bacteria was inhibited by the toxic effect of the higher PCP concentration.

These studies make it clear that microorganisms show tolerance against a certain concentration of polycyclic aromatic hydrocarbons and beyond that limit polycyclic aromatic hydrocarbons exhibit toxicity towards microbial growth. In short, Figure 4.1 shows the growth pattern of Pseudomonas with different concentrations of anthracene and phenanthrene in incubation times of 14 days. Among the different two concentrations of anthracene (250 mg/L and 350 mg/L), the lower concentration showed higher bacterial growth. Also in phenanthrene (100 mg/L and 250 mg/L) the lower concentration showed more bacterial growth. It was also concluded that *Psudomonas* bacteria showed more growth on anthracene as compared to phenanthrene, which also indicates that anthracene is less toxic to bacterial cultures than phenanthrene.

4.5 Sample Collection

15ml of samples were collected after every 24 hours from orbital shaker in degradation studies. 2 to 3 drops of diluted sulphuric acid was added to stop further degradation and samples were stored and refrigerated at 4°C.

4.6 Gas Chromatographic Analysis

Standard Analytes were purchased from Chiron i.e. Anthracene and Phenanthrene. Stock solutions for the standard analytes were prepared by dissolving in GC grade n-hexane. The stock solutions were run on gas chromatograph to detect their signal and retention time. Signals were observed by injecting one µL of stock solution into the injection port of gas chromatograph.

Temperature of injector, column and detector were also adjusted according to the signals. A temperature ramp for the column was designated to get the well resolved and separate peaks for the individual analytes. The temperature of injector was adjusted at 250°C according to the nature of the test analyte and its boiling point. The

test analytes are all volatile compounds so the temperature was adjusted a little high to their boiling points. Detector temperature was 280°C. The reason for the high temperature was to make the detector (FID) more sensitive for the analytes to be studied. If the analyte is sensitive more it will detect the analyte in ppb or even ppt concentration. (Saba and Hashmi, 2010).

The initial temperature of column oven was 200°C with a hold time of 1 minute. Then a temperature ramp of 10 ° C rise per minute was given to the column. Final temperature of the column was 250 ° C with 2 min hold time. The hold time is given to flush out the remaining traces so that the column may become ready for the next injection. This practice was used to get clear and get reproducible result.

Helium gas was used as carrier gas for the analysis. It flowed through the column throughout the analysis and the flow rate was 1mL/min. Nitrogen gas was used only at the time of sample analysis.

Stock solutions of both analytes were injected one by one. Single point calibration curve was used. Table 4.2 shows retention time of stock solution of standard analytes prepared.

Analyte	Retention Time (min)
Anthracene	7.1
Phenanthrene	6.9

Table 4.2 Retention time of stock solution of standard analytes

Table 4.2 represents chromatogram representing the GC analysis of anthracene and phenanthrene. The retention time of anthracene is 7.1 minutes and phenanthrene is 6.9 minutes. GC grade n-hexane was used as a solvent for all the solutions due to several reasons. Firstly all solvents used in this study were non polar in nature and n-hexane is also non polar. Therefore the test analytes were easily dissolved in n-hexane. Boiling point of n-hexane 69.9°C and its retention time is 1.4 minutes, which is lowest among both the analytes studied therefore it elutes earlier.

4.7 Reproducibility Graphs

Reproducibility of analytes was calculated. Each compound was injected four to five times. Average retention time was calculated.

4.8 Sample Analysis

Sample analysis was done using gas chromatography. Mehrasbi *et al.* (2003) reported biodegradation of petroleum hydrocarbons in 3 media, differing in the kind of petroleum fractions. In the laboratory experiments, during 5 months, the activities of petroleum hydrocarbon-degrading microorganisms and dehydrogenase activity of soil was determined. Gas chromatographic analysis showed the biological decontaminations for gas oil, kerosene and synthetic mixture were degraded up to 60 %, 36 % and 55 %, respectively.

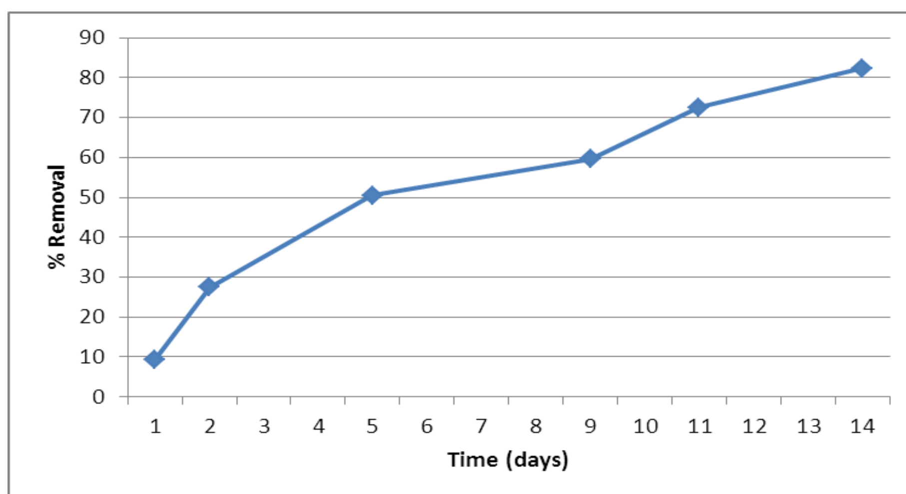

Figure 4.9 Percentage degradation of Anthracene at 250mg/L

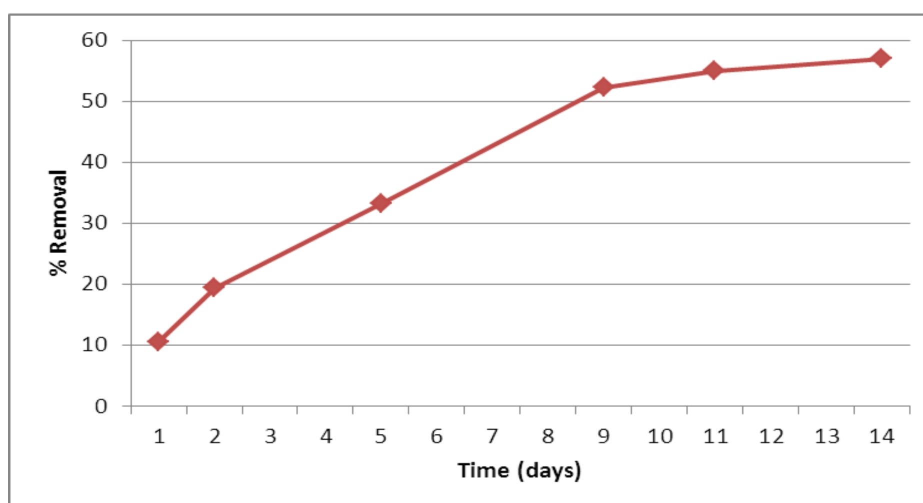

Figure 4.10 Biodegradation of Anthracene at 350mg/L

Figure 4.13 shows the biodegradation of 350 mg/L of anthracene and Figure 4.14 shows 250mg/L of anthracene in 14 days. The 350 mg/L of anthracene showed 57.02% degradation, while 250 mg/L showed 82.3 %. Higher concentration of anthracene showed less degradation as compared to lower concentrations.

Jacques *et al.,* (2005) investigated the analysis of anthracene degradation estimated by gas chromatography showed that *Ps. aeruginosa* isolate showed highest rate of degradation upto 3.90 mg/L per day, degrading up to 71% of the anthracene after 48 days.

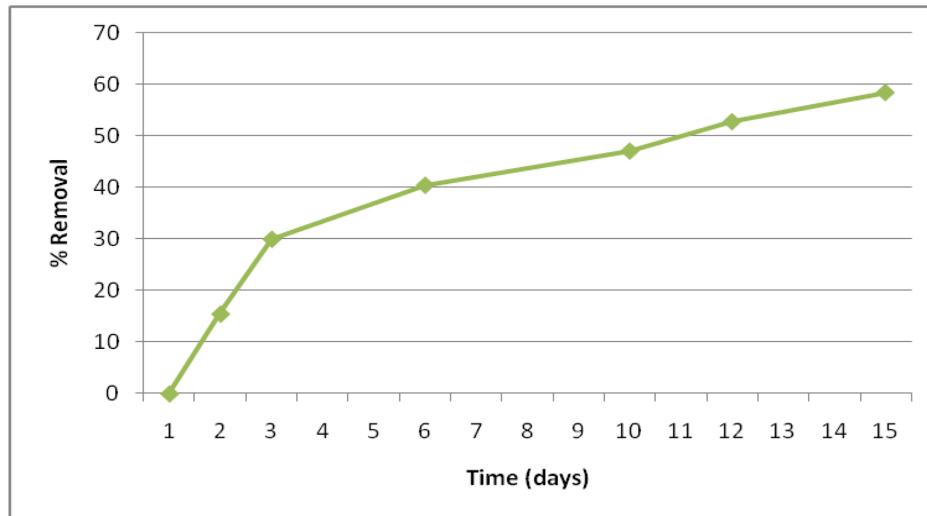

Figure 4.11 Biodegradation of Phenanthrene at 100mg/L

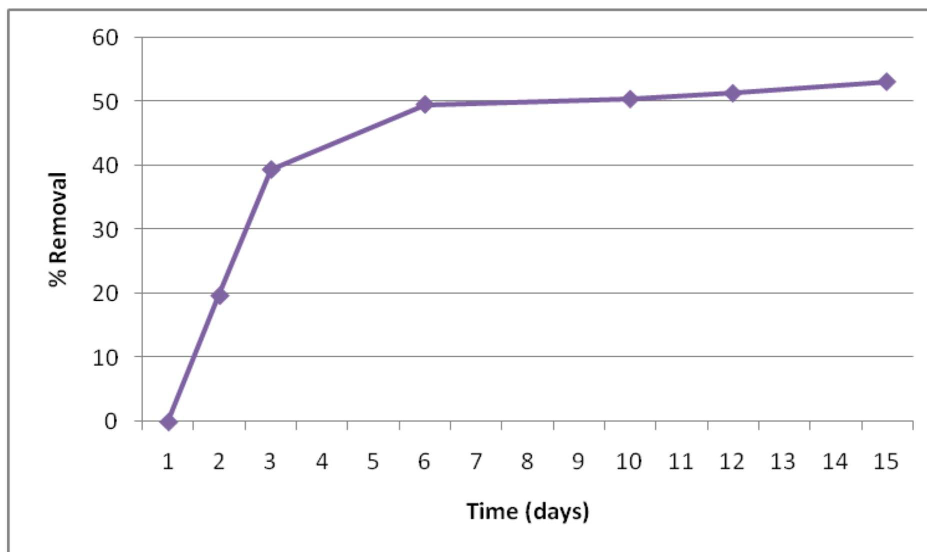

Figure 4.12 Biodegradation of Phenanthrene at 250mg/L

Figure 4.15 shows the biodegradation of 250 mg/L of phenanthrene and Figure 4.16 shows 100mg/L of phenanthrene in 14 days. The 250 mg/L of phenanthrene showed 53.1 % degradation, while 100 mg/L showed 58.3 %. phenanthrene as compared to anthracene showed less degradation.

The degradation curve of anthracene and phenanthrene with relation to the different concentrations is listed in Annexure 2.

Jeong-Dong *et al.,* 2004 reported that high concentration of phenanthrene inhibited the growth of microbial strains and biodegradation of phenanthrene. Janbandhu *et al.* 2011 reported that the biodegradation data of phenanthrene indicates about 100 %, 56.9 % and 25.8 % degradation at the concentration of 100 mg/L, 250 mg/L and 500 mg/L respectively within 14 days.

In short it could be reported that anthracene showed more degradation than phenanthrene. The lower concentrations of both anthracene and phenanthrene degraded more, whereas high concentrations showed slower degradation process. Also that maximum degradation was in the first 3 to 4 days. With increase in time the degradation slowed because the bacterial count also got lower.

Chapter 5

CONCLUSIONS

Polycyclic aromatic hydrocarbons (PAHs) represent a large group of organic pollutants, which have contaminated the environment through improper disposal of materials such as creosote, coal tar and hydrocarbon fuels. Currently bioremediation is recognized as an environmentally friendly technique for detoxification of polycyclic aromatic hydrocarbon contaminated sites and has become an important topic for research.

The overall goal of this work was to study biodegradation of polycyclic aromatic hydrocarbons (PAHs) using phenanthrene and anthracene as model PAHs.

The outcomes of this study are discussed below:

1. Pure culture isolated from soil by enrichment technique was identified as *Pseudomonas sp*.

2. *Pseudomonas sp* showed more growth on anthracene as compared to phenanthrene, which also indicated that anthracene is less toxic to bacterial cultures than phenanthrene.

3. The investigation of polycyclic aromatic hydrocarbon degradation revealed that with increase in concentration the degradation rate decreases.

4. The 350 mg/L of anthracene showed 57.02% degradation, while 250 mg/L showed 82.34 %, while 250 mg/L of phenanthrene showed 53.10 % degradation, while 100 mg/L showed 58.36 %.

5.

Chapter 6

SUGGESTIONS FOR FUTURE WORK

1. Degradation by *Pseudomonas* culture at higher concentration by optimizing process parameters is a subject for further research.

2. Studies can be conducted on the interaction between different species, mixture of polycyclic aromatic hydrocarbons and effect of different environmental factors on biodegradation.

CHAPTER 7

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Annexure 1

ISOLATION, MORPHOLOGICAL AND CHEMICAL CHARACTERIZATION OF *PSEUDOMONAS* BACTERIA

Figure 4.1. Isolation of PAH degrading bacterial strain

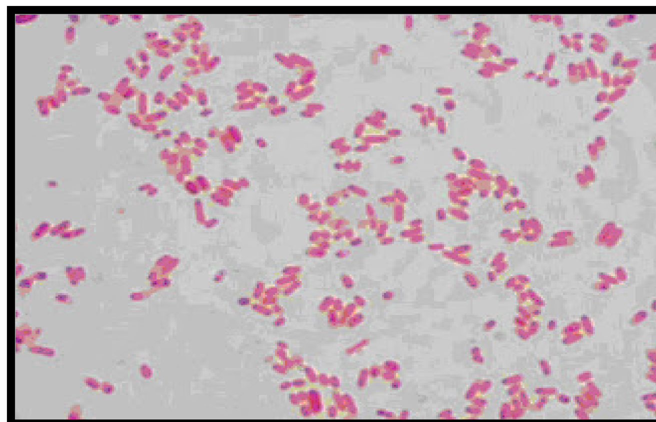

Figure 4.2. PAH degrading bacteria culture, stained with Gram's Method

Figure 4.3. Indole test for bacterial culture

Figure 4.4. Catalase test for bacterial culture

Figure 4.5. M.R test for bacterial culture

Figure 4.6. Citrate test for bacterial culture

Annexure 2

DEGRADATION CURVE OF ANTHRACENE AND PHENANTHRENE

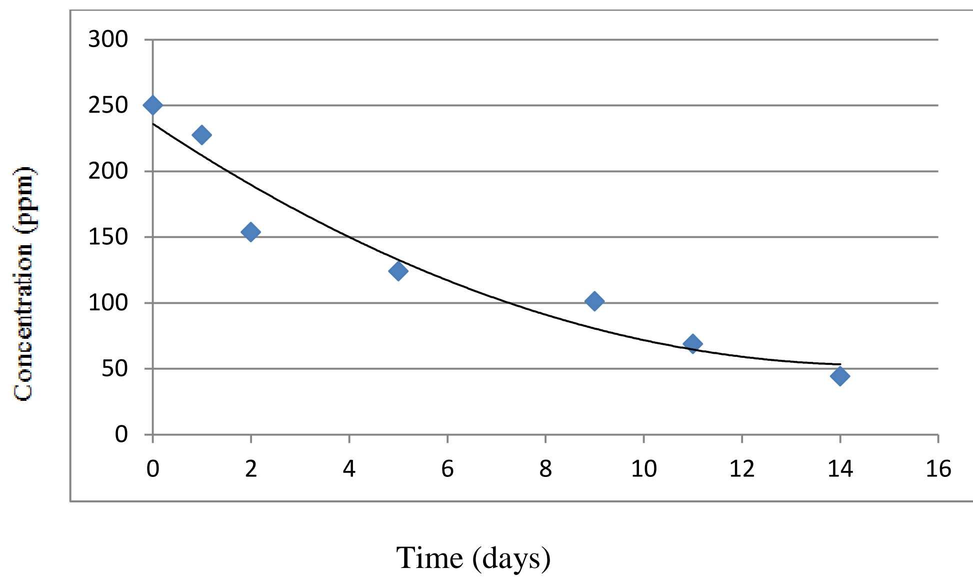

Figure 4.13Biodegradation of Anthracene at 250mg/L

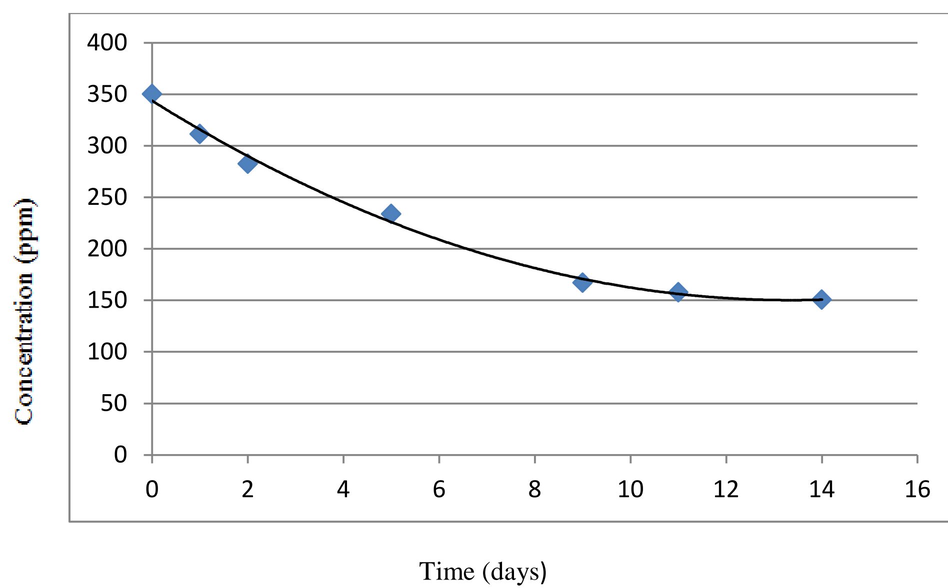

Figure 4.14 Biodegradation of Anthracene at 350mg/L

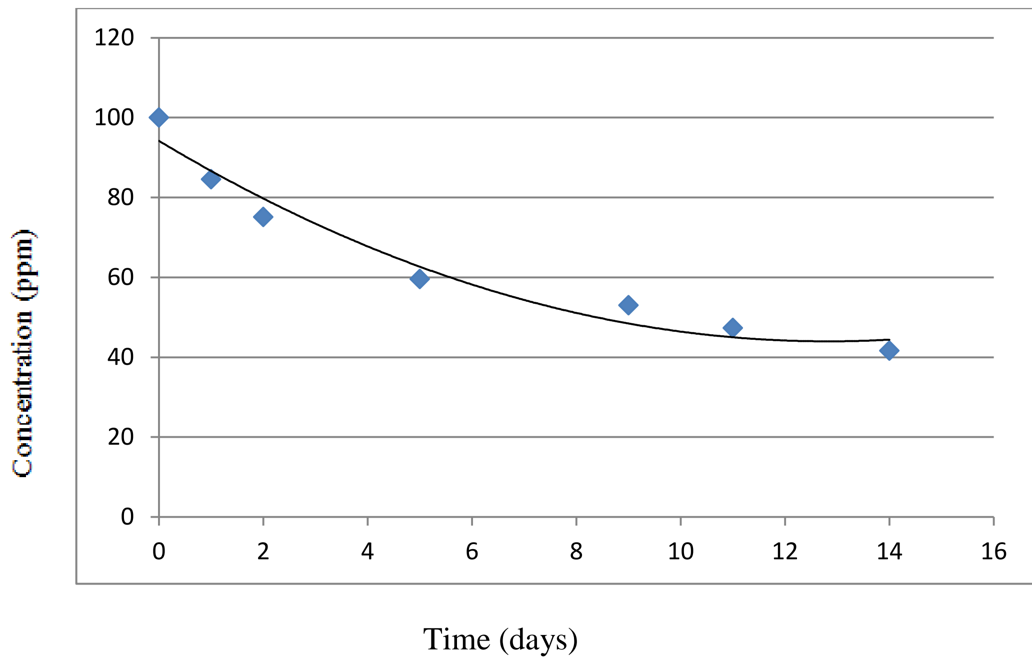

Figure 4.15 Biodegradation of Phenanthrene at 100mg/L

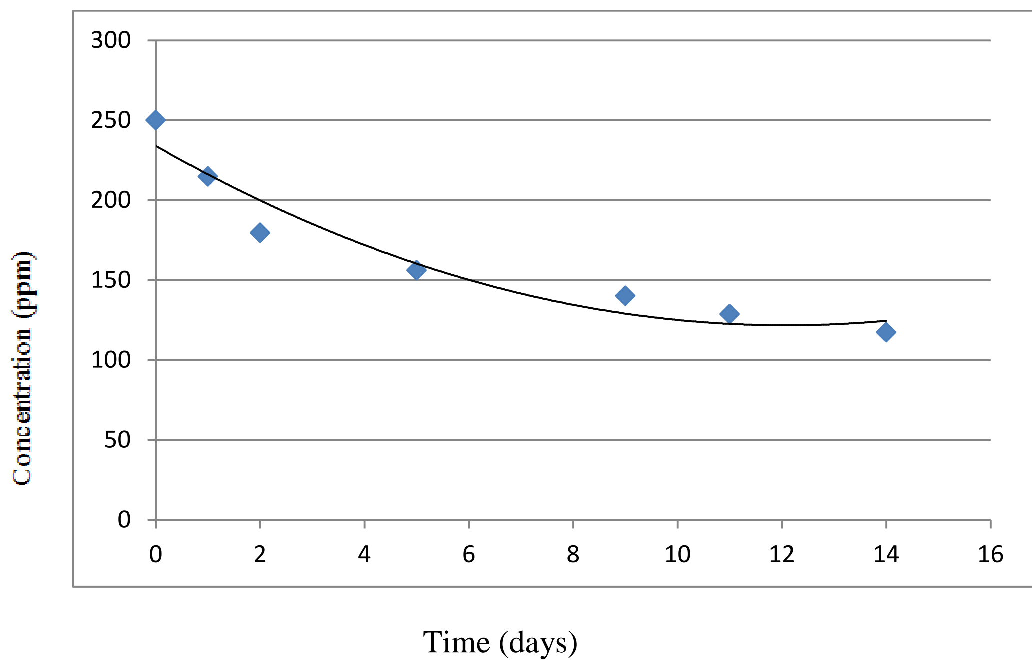

Figure 4.16 Biodegradation of Phenanthrene at 250mg/L