DIVERSITY OF PYRENE DEGRADING MICROBES AND MOLECULAR ANALYSIS OF PYRENE DEGRADING PATHWAY

A Thesis

submitted in fulfillment of the requirements

for the award of the degree of

DOCTOR OF PHILOSOPHY

IN

BIOTECHNOLOGY

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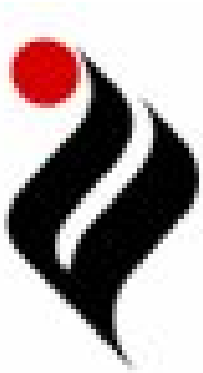

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CERTIFICATE

Certified that the thesis "**Diversity of Pyrene Degrading Microbes and Molecular Analysis of Pyrene Degrading Pathway**" which is submitted by **Ms. Purnima Khanna**, in fulfillment of the requirement for the award of the degree of Doctor of Philosophy in the Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is a record of the candidate's own independent and original research work carried out by her under our supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree.

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I hereby declare that the work which is being presented in this thesis "**Diversity of Pyrene Degrading Microbes and Molecular Analysis of Pyrene Degrading Pathway**" submitted by the undersigned for the award of the degree of Doctor of Philosophy in Department of Biotechnology and Environmental Sciences (DBTES), Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the supervision of **Dr. Dinesh Goyal**, Professor, Biotechnology & Environmental Sciences (DBTES), Thapar University, Patiala, Punjab, India and **Dr. Sunil Khanna**, Professor, Biotechnology & Bioinformatics, NIIT University, Neemrana, Rajasthan, India. The matter embodied in this thesis has not been submitted in part or full to any other university or institute for the award of any degree in India or Abroad.

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Place: Patiala

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Dedicated to my parents...

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

%	-	Percent
°C	-	Degree centigrade
µg	-	Microgram
µl	-	Microliter
BLAST	-	Basic Local Alignment Search Tool
bp	-	Base pair
cfu	-	Colony forming units
cm	-	Centimeter
DNA	-	Deoxyribonucleic acid
EC	-	Electrical Conductivity
EDTA	-	Ethylene diaminetetraacetic acid
g	-	Gram
GC	-	gas chromatography
GC-MS	-	gas chromatography equipped with mass spectrometry
HPLC	-	high performance liquid chromatography
hr	-	Hour
Kb	-	Kilo base
K_{ow}	-	Octanol:water partitioning coefficient
L	-	Litre
M	-	Molar
mg	-	Milligram
min	-	Minute
ml	-	Milliliter
mV	-	milli volts
NCBI	-	National Centre for Biotechnology Information
ND	-	not determined
nm	-	Nanometer
NM	-	No Match
OD	-	Optical density
PAH	-	Polycyclic aromatic hydrocarbon
PCR	-	Polymerase chain reaction
pH	-	Hydrogen ion concentration (negative log of)
rpm	-	revolutions per minute
RT	-	retention time
Tris	-	Tris-(hydroxymethyl)-aminomethane
TE	-	Tris EDTA
UV	-	Ultraviolet
v/v	-	Volume per volume
VIS	-	Visible
w/v	-	weight per volume

SYNOPSIS

INTRODUCTON

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitously distributed, diverse class of organic compounds with two or more fused aromatic rings in various structural configurations. As major components of fossil-fuels they enter the environment in many ways such as incomplete combustion and accidental spilling of hydrocarbons and oils, fallout from urban pollution, petroleum refining and coal liquefaction, and gasification processes (Vila and Grifoll, 2009). Due to their potentially deleterious effects on human health the US Environmental Protection Agency has listed 16 PAHs as priority pollutants to be monitored in industrial effluents (Yan *et al.,* 2004). Of the processes whereby these contaminants are removed from the environment, microbial degradation plays a major role in the decontamination of sediment and surface soils.

A variety of microorganisms such as *Acinetobacter*, *Bacillus* (Das and Mukherjee, 2007), *Mycobacterium*, (Kim *et al.,* 2007; Vila and Grifoll, 2009), *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Cycloclasticus*, *Pseudomonas*, *Ralstonia*, *Nocardia*, *Rhodococcus*, *Sphingomonas* and *Terrabacter*, (Zhou *et al.,* 2006) show characteristic metabolic removal of these xenobiotics. The two and three benzene ringed hydrocarbons are completely biodegraded in a variety of environments. Abundant genetic analysis and data on the biochemical mechanisms for naphthalene, phenanthrene and anthracene degradation have been reported in the past two decades (Zhou *et al.,* 2006; Heitkamp and Cerniglia 1989). In contrast, biodegradation of four and five ringed high-molecular-weight (HMW) PAHs (such as pyrene, Benz[*a*]pyrene), regarded as thermodynamically stable, recalcitrant and often genotoxic, requires more extensive study (Juhasz and Naidu, 2000).

PYRENE – The pericondensed PAH

Pyrene has been used as a model compound to study biodegradation of high-molecular-weight PAHs since it is structurally similar to several carcinogenic PAHs (Kim *et al.,* 2007). It is commonly found in the aromatic fraction of coal tar at concentrations ranging from 3.5 to 19,000 ppm (Irwin *et al.,* 1997). It is a toxic pollutant designated pursuant to section 307(a) (1) of the Clean Water Act and is subject to effluent limitations. Although it is not genotoxic, it acts as a pre-carcinogen i.e., it is transformed by cellular processes into a carcinogen (Juhasz and Naidu, 2000).

Human exposure to pyrene occurs primarily through smoking of tobacco, inhalation of air polluted by coal fires and by ingestion of food and water contaminated by combustion effluents (Yan *et al*., 2004). Due to high molecular weight (202.6 g mol^{-1}) and low water solubility (0.14 µg L^{-1} at 25ºC) it persists in soil for long periods (Kanaly and Harayama, 2000). Extensive bioremediation efforts have shown that bioremediation of coal-tar contaminated sites can lead to the degradation of HMW polycyclic aromatic constituents including pyrene (Rogers *et al.,* 2007).

Effective utilization of the capacity of diverse groups of soil bacteria to degrade or eliminate this carcinogenic pollutant from the environment was the rationale of my research work.

In the present study autochthonous, culturable, aerobic bacteria from two different PAH-contaminated sites were identified and characterized with respect to pyrene degrading capacities. Pyrene utilization pattern, concomitant activities during growth and its genetic basis in selected isolates was determined. One isolate was finally selected for pyrene metabolite identification and pathway prediction. The objectives of present study were as follows:

Objectives

- Biodiversity of pyrene degrading bacteria
- Elucidate the metabolic pathway of pyrene degradation in a selected isolate
- Genetic regulation of pyrene degradation in the selected isolate
- Removal of pyrene by selected bacterial isolate from soil contaminated with pyrene

Objective 1

Biodiversity of pyrene degrading bacteria

With an aim to investigate the role of bacteria in pyrene – a high-molecular-weight PAH – degradation the present study was conducted to isolate and characterize a diversity of aerobic soil bacteria, capable of growth on or biodegradation of pyrene. Bacteria were isolated from two PAH-contaminated soil samples, namely crude and diesel oil contaminated soil and coal-tar contaminated soil. 16S rDNA - RFLP analysis provided a picture of the genetic diversity in two microbial communities. The phylogenetic affiliations of bacterial isolates were determined using BLASTN and MEGA4 bioinformatics tools.

1. Development (selective enrichment) of bacterial consortia

Bacterial consortia CON-3 and THA-2 were isolated from crude oil and diesel oil contaminated soil samples, respectively, selectively enriched on crude oil obtained from Bombay High and screened for pyrene uptake capability. The consortia was grown in 100 ml Bushnell-Haas broth (BHB) basal medium (Toledo *et al.,* 2006) supplemented with crude oil (0.5 – 1 %; v/v) and pyrene (10 µg ml^{-1}) at 30 ^{o}C for 25 - 30 days initially. Gradual enrichment of pyrene in steps of 10 µg ml^{-1} up to 50 µg ml^{-1} and reduction of crude oil (upto 0.2 %; v/v) was done in the growth medium to selectively enrich the pyrene utilizing bacteria in the consortia. Glucose (0.1 – 0.5 %) was also added to increase the biomass. The rate of pyrene utilization was studied in batch cultures, as function of time, by spectrophotometric and high - performance liquid chromatographic (HPLC) analysis. It was observed that both consortia could biodegrade pyrene only in the presence of glucose (0.1 - 0.5 % w/v). Consortia CON-3 and THA-2 utilized 49 % and 30 % of 50 µg ml^{-1} pyrene, respectively over a 30 day incubation.

Bacterial diversity: Aliquots of each consortia, developed in BHB medium with crude oil (0.2 %; v/v), glucose (0.25 %; w/v) and pyrene (25 µg ml^{-1}) were plated on Luria-Bertani agar (LA) plates in triplicate and incubated at 30 ^{o}C for 48 - 72 hours. Pure cultures of twenty bacterial colonies, designated as PK-11 to PK-30, were replica plated on pyrene coated Bushnell-Haas agar containing 0.25 % (w/v) glucose (BHA+G0.25+P) plates and repeatedly transferred to BHB medium containing pyrene (25 µg ml^{-1}) and glucose (0.25 %; w/v) for 30 days. The bacterial isolates were found to utilize 25 µg ml^{-1} pyrene in the range 0 - 98 % in 30 days of growth. The difference

between total initial pyrene added and final residual pyrene was expressed as pyrene uptake. Isolates PK-23, PK-24 and PK-27 (belonging to THA-2 consortium) and isolates PK-16, PK-12, PK-13, PK-14 and PK-15 (belonging to CON-3 consortium) showing an uptake of 45 %, 39 %, 53 %, 51 %, 98 %, 61 %, 55 % and 54 % pyrene, respectively, over a 30 day incubation time were selected for exposure to increased amounts of pyrene such as 50 µg ml^{-1} and 75 µg ml^{-1} to check for maximum pyrene utilization limit. The high concentration of 75 µg ml^{-1} pyrene was found to inhibit the growth of all eight isolates even in the presence of 1.0 % (w/v) glucose, while 50 µg ml^{-1} pyrene did not inhibit growth of the isolates. Three bacterial isolates (PK-12, PK-13 and PK-14) found to utilize more than 50 % of 50 µg ml^{-1} pyrene and one non-pyrene utilizing isolate (PK-11) were selected for 16S rDNA analysis and phylogenetic identification.

2. Soil collection and characterization

Owing to the slow rate of pyrene uptake by consortium isolates, another source was selected to screen for pyrene degrading isolates. Soil contaminated with coal-tar was collected from a 15 year old hot pre-mix coal tar industrial plant site (Rakhra village, Patiala city, Punjab, India) in the month of August with a maximum air temperature of 40 – 45 ^{o}C. The top soil was collected from site (i) 1 - 2 m away and (ii) 3 - 4 m away from the coal-tar heating pre-mix plant. Two sub soil samples were collected from 0 - 25 cm soil depth from the same sites as above. Physical characterization of coal-tar contaminated soil (Zhou *et al.* 1996; Rayment and Higginson, 1992) showed that the top soil was hot (approx. 40 ^{o}C), loose, grey in color, dry to touch, having 1.2 - 2.9 % moisture content, 9.0 – 11.2 pH and 959 -1424 µS cm^{-1} electrical conductivity while the sub soil collected from 0 - 25 cm depth was very hot (approx. 45 ^{o}C), jet black, tightly packed with coal-tar and had 7.2 - 10.8 % moisture content, 9.5 – 9.9 pH and 431 – 1112 µS cm^{-1} electrical conductivity. Gas chromatography (GC-FID) analysis of acetone extracts of sub soil samples confirmed the presence of nine PAHs, in the decreasing order of Benzo[*g,h,i*]perylene, Dibenzo[*a,h*]anthracene, Indeno[*1,2,3-c,d*]pyrene, Pyrene, Acenaphthylene, Fluorene, Phenanthrene, Benzo[*k*]fluoranthene, Benzo[*b*]fluoranthene which belong to the class of "16 Priority Pollutants" as per the findings of Yan *et al*. (2004). Enumeration of bacteria (cfu gm^{-1} soil) at 37 °C and 45 °C by serial-dilution of 1 gm soil (top soil and sub soil) collected from site (i) 1 - 2 m away and (ii) 3 – 4 m away from the plant and standard agar plating (Cappuccino and Sherman, 1987) indicated that the sub-soil collected from site 1 - 2 m away had highest population of aerobic, mesophilic bacteria with thermotolerant properties ($\geq 3 \times 10^{8}$ cfu gm^{-1} soil). In contrast the top soil collected from same site had the lowest count of

bacteria (1 - 2 x 10^5 cfu gm^{-1} soil) amongst the four different soil samples collected. Based on the methods used in the present study hot, dry and loose top soils, having very low moisture content and high pH, do not seem to favor microbial growth and survival.

Bacterial diversity: The culturable diversity of bacteria present in coal-tar contaminated soil was evaluated by serial-dilution of 1 gm soil and agar plating on LA plates. A total of 229 autochthonous, culturable, aerobic bacterial strains were obtained, 91 colonies at 30°C and 138 colonies at 45°C. Of the total, pure cultures of only 27 bacterial isolates were able to colonize on pyrene coated BHA + 0.25 % (w/v) glucose (BHA+G0.25+P) plates during replica plating. For screening of pyrene utilizing isolates, the colonies were individually transferred in 5 ml BHB medium containing pyrene (25 µg ml^{-1}) with or without glucose (0.25 %; w/v) in test tubes and allowed to grow in shaking conditions (60 rpm). After 2 weeks of incubation, the turbid cultures were subcultured in flasks containing 50 ml BHB medium with 50 µg ml^{-1} pyrene with or without 0.5 % (w/v) glucose for 30 days of incubation. The bacterial strains grew on pyrene only in presence of glucose. Growth of isolates on 50 µg ml^{-1} pyrene in presence of 0.5 % (w/v) glucose was monitored to select 10 best growing isolates with maximum pyrene (50 µg ml^{-1}) utilization ability at 30 ^oC and 45 ^oC incubation temperatures for 16S rDNA analysis and phylogenetic identification.

3. Characterization of bacterial isolates

3.1 Morphological characterization by gram staining

Pyrene utilizing bacterial isolates PK1 – PK14 were analyzed microscopically by gram staining technique (Gram, 1884). Thirteen bacterial isolates showed gram positive character, only isolate PK-3 was observed to stain gram negative.

3.2 Molecular characterization by 16S rDNA analysis

16S rDNA gene sequencing is a rapid, accurate method of phylogenetic analysis as the gene is highly conserved ribosomal gene between different species of bacterial and archaebacteria. It is also the slowest evolving gene (Mignard and Flandrois, 2008). In addition to highly conserved primer binding sites, 16S rDNA gene sequences contain hypervariable regions that can provide species-specific signature sequences, useful for bacterial identification.

Genomic DNA of bacterial isolates (PK-1 to PK-10, PK-11 to PK-30) was extracted by ROSE (Rapid One Step Extraction) method (Steiner *et al.*, 1995) or by boiling lysis method (Krivobok *et al.*, 2003). Extracted DNA was used as a template to amplify 16S rDNA by PCR using universal bacterial primers 8/27-F and 1492-R (Baker *et al.* 2003). Nucleotide sequence of the primer 8/27-F is 5'-AGA GTT TGA TCC TGG CTC AG-3' and 1492-R is 5'-GGT TAC CTT GTT ACG ACT T-3'. PCR amplification resulted in sequences of about 1500 bp. PCR products of fourteen bacterial isolates (PK-1 to PK-14) showing diverse patterns with tetracutter *Alu* I, *Hin* 61, *Mbo* I and *Rsa* I restriction enzymes were chosen for cloning into pGEM-T Easy vector. The ligated products were transformed into *E. coli* DH5α cells by heat shock method (Cohen *et al*., 1972). The positive (recombinant) clones were screened by α-complementation and the plasmid DNA of the recombinant cells were isolated using the alkali lysis method (Birnboim and Doly, 1979). Plasmids containing insert were confirmed by *Eco* R1 restriction digestion and PCR with T7 (nucleotide sequence: 5'-ATT ATG CTG AGT GAT ATC CCG CT-3') and SP6 (nucleotide sequence: 5'-CAT AAG ATA TCA CAG TGG ATT TA-3') primers. The confirmed clones were sequenced by chain termination method (Sanger *et al*., 1977) using an Applied Biosystems automatic sequencer (Courtesy: Bangalore Genei Pvt Ltd, Bangalore, India). Sequence analysis was performed with BLAST program (Altschul *et al.,* 1997) using the nucleic acid sequences deposited in multiple data bases. The closest match to eleven isolates was *Bacillus* sp. (PK-1, PK-2, PK-4, PK-5, PK-6, PK-7, PK-8, PK-9, PK-12, PK-13, and PK-14) having nucleotide identities between 95 and 99 %, one isolate was *Pseudomonas* sp. (PK-3), one isolate was *Kocuria* sp. (PK-11) both having nucleotide identities 97 % and one isolate was *Rhodococcus* sp. (PK-10) having nucleotide identity above 99 %. Phylogenetic affiliations of the isolates were determined using CLUSTALW (Thompson *et al*. 1994) and MEGA4 (Tamura *et al*. 2007) software using neighbor-joining method (Saitou and Nei 1987). 16S rDNA characterization results showed that members of Firmicutes, Actinobacteria and Proteobacteria taxonomic clades were present in coal-tar contaminated soil while members of Firmicutes and Actinobacteria taxonomic clades were present in crude/diesel oil contaminated soil. The 16S rDNA gene sequences of fourteen bacterial isolates have been deposited with the NCBI GenBank database (accession numbers EU685813 - EU685826). Bacterial isolates *Bacillus licheniformis* (PK-6), *Bacillus pumilus* (PK-12) and *Bacillus firmus* (PK-14), showing maximum pyrene removal from growth medium have been deposited as MTCC 1005, 1002 and 1003, respectively, at Microbial Type Culture Collection library at IMTECH, Chandigarh (India).

Objective 2

Elucidate the metabolic pathway of pyrene degradation in a selected isolate

The objective was to gather information on bacterial catabolism of pyrene and elucidate the metabolic pathway in a single isolate. First the rate at which pyrene uptake and utilization occurs in eight pyrene utilizing isolates was studied by pulse chase experiments, time course experiments and media optimization studies. The effect of pyrene on bacterial growth was also determined. The results proved helpful in studying biochemistry of pyrene metabolism in the most efficient pyrene utilizing isolate *Bacillus* sp. (PK-6) MTCC 1005. The intermediate metabolites arising out of the degradation of pyrene were characterized by Gas Chromatograph coupled with Mass Spectrograph (GC-MS).

1. Pyrene Uptake Studies

1.1 Pulse Chase experiment

Log phase bacterial cells of eight pyrene utilizing isolates were washed twice and resuspended in 10 mM potassium phosphate buffer (pH 7.0). Bacterial cells equivalent to 2 mg protein (Itzhaki and Gil, 1964) were exposed to 100 µg pyrene in one set of flasks and incubated in phosphate buffer for 12 hours at 30 ^{o}C prior to pyrene addition in second set of flasks. At hourly intervals, over a time period of 7 hrs, percentage of pyrene uptake was measured (in triplicates) by solvent extraction of the withdrawn samples followed by spectrophotometric and HPLC analysis. Bacterial cells incubated for 12 hours in phosphate buffer prior to pyrene addition showed negligible pyrene uptake as compared to the log phase bacterial cells immediately exposed to pyrene. Log phase cells of isolates PK-12, PK-13 and PK-14 showed uniform and maximum pyrene uptake among the 8 isolates. However the percent utilization was very low 11 %, 13 % and 8 %, respectively, in 7 hours of incubation. Pyrene utilization appeared to be a slow mechanism and was subsequently studied over longer intervals of time.

1.2 Time Course experiment

Pyrene uptake pattern was studied in a time course experiment of 35 days. Bacterial isolates PK-12, PK-13, PK-14, PK-15, PK-16, PK-23, PK-24 and PK-27 showing > 35 % pyrene (25 µg ml^{-1}) uptake were grown in BHB medium containing 50 µg ml^{-1} pyrene and 0.5 % (w/v) glucose. At 7 day interval batch culture samples (in triplicate)

were withdrawn and residual pyrene determined. Pyrene uptake was found to be highest between 14 and 28 days and then stabilized till 35 days. *Bacillus pumilus* (PK-12) showed the highest pyrene uptake of 64 % from BHB medium containing glucose (0.5 %; w/v) after 35 days, closely followed by *Bacillus flexus* (PK-13) (55 %) and *Bacillus firmus* (PK-14) (53 %). These three isolates capable of utilizing more than 50 % pyrene (50 μg ml^{-1}), were selected for further studies.

1.3 Effect of pyrene on growth of* Bacillus sp. *(PK-12)

The effect of pyrene addition on growth of one pyrene utilizing isolate *Bacillus pumilus* (PK-12) was studied. Pyrene (50 μg ml^{-1}) was added to the growing culture in mid-log phase (~ 3 hrs in LB medium, ~ 20 - 24 hrs in BHB medium containing glucose) and growth was measured by optical density at 600 nm. The study indicated that pyrene addition in mid-log phase did not have any deleterious effect on the growth of the cells. In fact pyrene addition in BHB medium containing 0.5 % (w/v) glucose was favourable for the isolate growth.

2. Effect of media supplementation for enhanced pyrene utilization by bacterial consortia

Minimal BHB medium containing 50 μg ml^{-1} pyrene was supplemented either with glucose (0.5 %; w/v) or trace elements (Nitrilotriacetic acid, $MgSO_4$, $FeSO_4.7H_2O$, $CoCl_2$, $CaCl_2.2H_2O$, $ZnSO_4$, $CuSO_4.5H_2O$, $AlK(SO_4)$, H_3BO_3 and Na_2MoO_4) and trace vitamins (Pyridoxine HCl, Thiamine HCl, Riboflavin, Nicotinic acid, Calcium pentothenate, DL-α-Lipoic acid, Biotin and Folic acid) or with both glucose (0.5 %; w/v) and trace elements and vitamins or with glucose (1.0 %; w/v) alone. Growth cultures of CON-3 and THA-2 consortia were withdrawn in triplicates at 10 day intervals to study change in pyrene utilization rate. In the screening stage it had been observed that the consortia could grow on pyrene only in the presence of glucose. Addition of trace elements and vitamins enhanced the pyrene uptake by consortia CON-3 and THA-2 to 58 % and 49 %, respectively, both in the absence or presence of glucose, in 30 days. BHB medium with double glucose concentration of 1.0 % (w/v) stimulated the pyrene uptake to 63 % by consortium CON 3 and 56 % by consortium THA-2.

3. Effect of glucose on pyrene utilization by bacteria

Growth experiments with pyrene utilizing isolates PK1 to PK-5, PK-12 to PK-14 (growing at 30 °C) and PK-6 to PK-10 (growing at 45 °C) were undertaken in BHB

medium containing 50 µg ml^{-1} pyrene and 0.5 - 1.0 % (w/v) glucose. The enhanced growth of isolates was believed to accelerate the time course of pyrene uptake and metabolism by bacterial isolates. Therefore bacterial isolates were grown in BHB medium containing 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose and samples flasks were withdrawn after every 24 hrs interval of incubation time. Growth (OD 600 nm), pyrene **uptake** (spectrophotometric and HPLC analysis), biosurfactant activity (Barkay *et al.,* 1999; Jacques *et al*., 2007), increase in cell protein (Itzhaki and Gill, 1964) and percent glucose utilization (Plummer, 1988) were determined. Of all the isolates tested, *Bacillus* sp. (PK-6) removed the maximum (56 %) and *Bacillus* sp. (PK-9) the minimum amount (10 %) of pyrene while *Pseudomonas* sp. (PK-3) and *Rhodococcus* sp. (PK-10) removed 25 and 46 %, respectively, of pyrene in 4 days. It was observed that percent pyrene uptake, biosurfactant activity (OD 550 nm), increase in cell protein (mg ml^{-1}) and percent glucose utilized values were found to decrease for the three isolates, namely *Bacillus* sp. PK-6 (56.4 %, 1.96, 1.96 mg ml^{-1}, 0.60 %) > *Bacillus* sp. PK-12 (45.6 %, 1.11, 1.39 mg ml^{-1}, 0.351 %) > *Pseudomonas* sp. PK-3 (25.2 %, 0.43, 0.97 mg ml^{-1}, 0.18 %).

The kinetics of pyrene utilization by *Bacillus* sp. (PK-6) was studied at 24 hour intervals for 4 days in BHB medium containing 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 45 ^{o}C. It showed a start in pyrene uptake after one day of incubation. An uptake of 39 % pyrene (20 µg ml^{-1}) was observed from the spent culture after 2 days. Fifty percent of the pyrene was utilized by the third day, thereafter on the fourth day 6.6 % more pyrene disappeared from the growth medium which corresponded to uptake of 56 % (28 µg ml^{-1}) pyrene. The culture also showed maximum growth and biosurfactant activity among all bacterial isolates. It grew exponentially till 3 days and showed biosurfactant activity maxima after 4 days of growth. Therefore it may be suggested that the more opportunistic *Bacillus* sp. (PK-6) under optimal conditions is preferentially more capable of degrading pyrene.

4. Biosurfactant activity

Biosurfactant activity during pyrene utilization phase of growth was assessed for the selected pyrene utilizing isolates and one non-utilizing bacterial isolate using the standard emulsification assay (index) reported by Barkay *et al.* (1999) and Jacques *et al*. (2007). A 5 ml aliquot of the culture supernatant was taken in a test tube and vortexed with 2 % (v/v) Mobil oil (Racer 2T, HP Corporation Ltd., Mumbai) vigorously for 1 min. After 10 min the degree of dispersion of Mobil oil was measured

spectrophotometrically at 550 nm against a blank of uninoculated medium with 2 % (v/v) Mobil oil.

Biosurfactant activity was found to be concomitant with growth in all isolates but in varied expression levels. *Bacillus* sp. (PK-6) showed highest biosurfactant activity (OD 550 nm = 2) which correlated well with the highest > 50 % pyrene uptake. Second highest pyrene degrader *Bacillus* sp. (PK-7) showed moderate biosurfactant activity (OD = 1.6). *Bacillus* sp. (PK-1, PK-5, PK-12 and PK-14) and *Rhodococcus* sp. (PK-10) showed moderate 35 - 50 % pyrene uptake and medium biosurfactant activity (1 > OD > 2). *Bacillus* sp. (PK-2, PK-4, PK-9 and PK-13) having < 35 % pyrene uptake potential showed low biosurfactant activity (0.5 > OD > 1). *Pseudomonas* sp. (PK-3) showed minimum biosurfactant activity (OD = 0.43) in growth medium. The production of biosurfactants may be considered as part of the metabolism of indigenous bacteria in contaminated sites so as to create a **favorable local environment** for enhanced solubilization of pyrene, resulting in higher uptake and utilization of pyrene by bacteria, however it does not assure biodegradation (Das and Mukherjee 2007). Our results are in agreement with these observations.

5. Pyrene degradation

The intermediate metabolites arising out of the uptake and assimilation of pyrene by *Bacillus licheniformis* (PK-6) MTCC 1005 were analyzed by Gas Chromatograph coupled with Mass Spectrograph (GC-MS). The peaks obtained by GC-MS analysis of culture extracts pertained to catabolic products of pyrene. These were identified by probabilistic search (PBM) by comparing the fragmentation pattern and their abundance with the standard mass spectra of known compounds in NIST database library stored in an MS Chemstation library (Varian). *Bacillus* sp. (PK-6) culture on the second day of growth in BHB medium containing 50 µg ml^{-1} pyrene ($C_{16}H_{10}$; mol. wt. 202) and 1.0 % (w/v) glucose at 45 ^{o}C showed the production of Phenanthrene ($C_{14}H_{10}$; mol. wt.178), 9–Methoxyphenanthrene ($C_{15}H_{12}O$; mol. wt. 208) and Diisooctylphthalate ($C_{24}H_{38}O_4$; mol. wt. 390). Compounds 5, 6, 7, 8–Tetrahydro–1–naphthoic acid ($C_{11}H_{12}O_2$; mol. wt.176) and 1, 6, 7–Trimethyl–naphthalene ($C_{13}H_{14}$; mol. wt. 170) were detected on the third day and fourth day in the supernatant, respectively, while the concentration of 9-methoxyphenanthrene and phthalate declined. In addition 9, 10–Diphenylphenanthrene ($C_{26}H_{18}$; mol. wt. 330) was formed on day 2 and showed an increase on the 3rd day. However, no further change in its concentration was detected till the end of the experiment.

6. Pyrene analysis

i. ***Spectrophotometric analysis***: The metabolic activity in periodically withdrawn culture flasks was stopped by acidification to pH 2 using 6 N HCl (Vila *et al.*, 2001; Jacques *et al*., 2007) followed by hexane solvent extraction of non-utilized pyrene from spent growth medium (Khan *et al.,* 2001) at room temperature. Extraction efficiency was found to be in the range of 87 % $\pm$ 3 %. The solvent extract was dried in fume-hood chamber and redissolved in acetonitrile / cyclohexane. The absorbance of acetonitrile solvent extracts obtained from triplicate cultures was measured at 254 nm for pyrene (Bugg *et al.* 2000). Solvent extract from uninoculated medium was used as blank. Difference in amount of pyrene extracted from control (uninoculated) flask(s) and inoculated flask(s), withdrawn from incubation at different time intervals during experiment(s), denoted **uptake** of pyrene by the inoculated bacterial strain(s).

ii. ***High-Performance Liquid Chromatographic (HPLC) analysis:*** Spectrophotometric results were confirmed by quantifying the amount of pyrene by high-performance liquid chromatography. The residual pyrene in acetonitrile solvent extracts was quantified by reversed-phase HPLC using C18 column (dimensions: 33 x 4.6 mm). A linear gradient of 50 - 95 % methanol in MQ-water water was developed over 20 min at a flow rate of 1 ml min^{-1} (Heitkamp *et al*. 1988b; Stingley et al. 2004a; Kim *et al.* 2004c, 2006). Pyrene was identified by comparing characteristic absorption spectra (at 254 nm) and retention times to authentic pyrene (Wang et al. 2000), using a Perkin Elmer diode array detector with data display, and by analysis using Total Chrome Ver 6.0 software. Residual pyrene was quantified with the help of reduction in peak areas compared to uninoculated control or by multiplying its peak area (obtained by HPLC) with a regression factor, derived from the standard curve of pyrene.

iii. ***Gas chromatographic - Flame ionization detection (GC-FID) analysis:*** The total aromatic fractions present in coal-tar contaminated soil sample, obtained from 0 - 25 cm depth, was qualitatively estimated by capillary GC - FID. Five grams of sub soil was extracted thrice with 100 ml of hexane, air dried, resuspended in acetone and analyzed by Zebron ZB-5 capillary column

[dimensions: 30 m (l) x 0.25 mm (id) x 0.25 µm (ft)], in an oven programmed to initially hold at 50 °C for 2 min, then rise to 275 °C @ 4 °C min^{-1}, again rise to 320 °C @ 10 °C min^{-1} and finally held at 320 °C for 20 min (Juhasz, 1998). The gas flow rates were 40 ml min^{-1} for carrier gas nitrogen, 300 ml min^{-1} for air and 40 ml min^{-1} for hydrogen. Injection port and detector (FID) were maintained at 275 °C and 320 °C, respectively. Sample was split in 1:5 ratio.

iv. ***Gas chromatography-Mass spectrometry (GC-MS) analysis:*** Cyclohexane solvent extracts of the periodically withdrawn culture samples were 0.2 µm filtered and used for catabolic intermediate metabolites and product determination. Compounds were separated using a J&W DB-5ms capillary column [dimensions: 30 m (l) x 0.25 mm (id); film thickness: 0.25 µm] and helium as the carrier gas at 1 ml min^{-1} flow rate. The GC oven temperature was initially held at 70 °C for 1.5 min, programmed first to 200 °C @ 10 °C min^{-1}, then to 280 °C @ 5 °C min^{-1} and finally held at 280 °C for 5 min (Martin and Mohn, 1999). The mass spectrometer was operated at 70 eV of electron ionization energy. Mass spectra was analysed from 5^{th} min to 41^{st} min in 60 - 450 mass range. The ion trap temperature was 200 °C, transfer line temperature was 280 °C and emission current was 12 µamps. Injector and analyzer temperatures were set at 250 °C and 285 °C, respectively. MS scan was referred to NIST database library for identification of the peaks in chromatograph.

Objective 3

Genetic regulation of pyrene degradation in the selected isolate

To get a comprehensive picture of pyrene degradation, molecular level studies were conducted. The seat of pyrene metabolism and related genes in the bacterial cell of selected pyrene degrading isolates was established. A ring hydroxylating dioxygenase gene is reported to be involved in the first step of pyrene catabolism in *Mycobacterium* sp. (Brezna *et al.*, 2003). Present research goal was to detect Rieske centers, the conserved [Fe2-S2] cluster binding region of terminal dioxygenases within the genera *Bacillus*, *Pseudomonas* and *Rhodococcus*. With an understanding of the pathway reported for pyrene degradation in *Mycobacterium* sp. (Kim *et al.*, 2007) and biochemical data obtained above, a pathway for pyrene degradation in *Bacillus licheniformis* (MTCC 1005) has been proposed.

1. Plasmid profiling of pyrene utilizing isolates

To study the plasmid profile of pyrene utilizing isolates, plasmid detection was followed by Kado & Liu's (1981) method for high-molecular-weight plasmids, using transformed *E. coli* DH5α strain as a control. As well, Birnboim and Dolly's (1979) alkaline lysis method for low-molecular-weight plasmids was used. No high-molecular-weight plasmid or low-molecular-weight plasmid could be isolated from the pyrene utilizing bacterial strains. Therefore genes regulating pyrene metabolism are presumed to be chromosomally borne.

2. Polymerase chain reaction (PCR) amplification of Rieske centre(s)

PCR experiments using degenerate primers (mixture of similar primers having different bases at the variable positions) specific for Rieske gene (Brezna *et al.,* 2003) were conducted to amplify pyrene dioxygenase gene from pyrene utilizing bacterial isolates. Attempts were made to standardize the PCR protocol at different annealing temperatures, however positive results (amplicon bands corresponding to Rieske gene) could not be obtained in these experiments.

3. Biochemical pathway of pyrene degradation

This is the first report of pyrene metabolism by ***Bacillus* sp. (PK-6) MTCC No. 1005.** GC-MS profiling of growth extracts of *Bacillus* sp. (PK-6) identified Phenanthrene, substituted- phenanthrenes and naphthalenes, phthalate as possible pyrene metabolites. A gradual decrease in the pyrene concentration in growth medium along with formation of six compounds suggests that pyrene is degraded by *Bacillus* sp. (PK-6). The knowledge of bioinformatics was used to determine the chemical rules of biotransformations (like the University of Minnesota Biocatalysis/Biodegradation Database (UM-BBD; 2003 website http://umbbd.ahc.umn.edu/) along with the possible reaction mechanisms. With the help of these rules, the classification of pyrene degradation products into primary, secondary metabolites or final products could be verified. It is proposed that pyrene gets converted to phenanthrene which is successively transformed to 9–methoxyphenanthrene and 9, 10–diphenylphenanthrene. The intermediates of pyrene to phenanthrene conversion like pyrene *cis*-4, 5-dihydrodiol and 4, 5-dihydroxypyrene, as reported by Kim *et al.,* (2007) in *Mycobacterium vanbaalenii* PYR-1 were not detected in our study. The decreasing concentration (corresponding to peak area value) of 9-methoxyphenanthrene compound during incubation suggests that it is further metabolized to 5, 6, 7, 8–tetrahydro–1–naphthoic acid, leading to the formation of 1, 6, 7–trimethyl–

naphthalene. This compound, by a series of unknown steps, gets converted to phthalate which is believed to be further transformed via the β-ketoadipate pathway to tricarboxylic acid (TCA) cycle intermediates (Kim *et al.,* 2007, 2008). It is quite possible that 9, 10-Diphenylphenanthrene is a dead end metabolite. This is the first report of formation of five new intermediates during pyrene metabolism by *Bacillus* sp. (PK6). Based on these results a tentative pathway for pyrene degradation in *Bacillus* sp. MTCC 1005 is proposed.

Objective 4

Removal of pyrene by selected bacterial isolate from soil contaminated with pyrene

This study investigated the removal of pyrene in soil by bacteria *Bacillus* sp. (PK-6) MTCC 1005 isolated from coal-tar contaminated soil. Coal-tar contaminated soil supplemented with glucose (0.5 mg g^{-1} soil) under non-sterile conditions was inoculated with *Bacillus* sp. (PK-6), whereas in a separate experiment autoclaved garden soil was spiked with pyrene @ 0.1 mg g^{-1} and glucose @ 0.5 mg g^{-1} and inoculated with *Bacillus* sp. (PK-6) and incubated and checked for removal of pyrene. *Bacillus* sp. in association with the indigenous bacterial community in coal-tar contaminated soil removed same amount of pyrene (58 %) as the indigenous bacterial community alone (57 %) with no significant change in soil physico-chemical properties (pH, EC and organic carbon) over a time period of 4 weeks. However, *Bacillus* sp. (PK-6) could significantly remove pyrene (66 - 71 % of added pyrene) from autoclaved, pyrene-spiked garden soil as compared to uninoculated, autoclaved **control** with slight variation in soil physico-chemical properties. Glucose supplementation led to increase in the bacterial count along with high removal of pyrene.

1. Removal of pyrene from coal-tar contaminated soil

Coal-tar contaminated soil was not autoclaved so as to compare the pyrene removal capacity of coal-tar acclimatized, indigenous bacterial strains over inoculated *Bacillus* sp. (PK-6) over a time period of 28 days at 37 ^{o}C. It may be mentioned that coal-tar contaminated soil is the source for *Bacillus* sp. (PK-6) bacteria. The soil characteristics, namely pH, EC, moisture content, organic carbon, pyrene content and bacterial count per gram of soil, were determined at zero time of incubation and after 28 days of incubation. A moisture content of 35 $\pm$ 2 % was maintained in soil throughout the experiment. After 28 days, the pH remained constant in both soil

treatments, irrespective of uninoculation or inoculation with *Bacillus* sp. (PK-6) culture. The EC of *Bacillus* sp. (PK-6) inoculated soil treatment increased 1.6 times (from 600.57 $\pm$ 12.87 µS cm^{-1} to 989.67 $\pm$ 35.18 µS cm^{-1}) and of uninoculated soil in control treatment became double in 28 days (from 560.67 $\pm$ 4.91 µS cm^{-1} to 1112.33 $\pm$ 55.54 µS cm^{-1}). The organic carbon at zero time of incubation was 3.78 $\pm$ 0.04 % in control soil (CT) and 3.95 $\pm$ 0.00 % in CT-PK6 soil. After 28 days of incubation the organic carbon slightly increased to 3.82 $\pm$ 0.09 % in control treatment and to 3.98 $\pm$ 0.01 % in *Bacillus* sp. (PK-6) inoculated soil. A similar increase of two order magnitude in bacterial counts from 0.02 x 10^6 to 2.15 x 10^6 cfu g^{-1} and from 0.02 x 10^6 to 2.22 x 10^6 cfu g^{-1} were observed, respectively, in non-sterile coal-tar contaminated soil and *Bacillus* sp. (PK-6) inoculated coal-tar contaminated soil over a time period of 28 days. All values reported above are mean $\pm$ standard error of 3 replicates.

No lag phase in pyrene removal by bacteria was observed in uninoculated (control) soil treatment and *Bacillus* sp. (PK-6) inoculated soil treatment. Probably the non-sterile soil of both incubations had high count of well acclimatized, indigenous, pyrene utilizing microflora irrespective of reintroduction of *Bacillus* sp. (PK-6) in coal-tar contaminated soil. After 28 days of incubation uninoculated coal-tar contaminated soil showed **57 %** pyrene removal and with inoculation of *Bacillus* sp. (PK-6) **58 %** pyrene removal was observed.

In the present study, we attempted to investigate natural attenuation and the effect of reinoculation (bioaugmentation) on pyrene removal in non-sterile PAH-contaminated soil collected from a coal-tar contaminated site. The results showed that in 28 day incubation time marginally different counts of bacteria were recovered from the non-sterile and non-sterile plus *Bacillus* sp. (PK-6) inoculated soil treatments along with marginally different percentage of pyrene removal, suggesting that the extent of removal over this period was influenced by presence of indigenous pyrene acclimatized and pyrene metabolizing gene pool (natural attenuation) rather than introduced *Bacillus* sp. (PK-6) bacterial biomass (bioaugmentation).

2. Removal of pyrene from pyrene spiked garden soil

Garden soil was autoclaved three times at 121 ^oC for 1 hr so as to kill all active forms of microflora in it, spiked with pyrene @ 0.1 mg g^{-1} soil and glucose @ 0.5 mg g^{-1} soil followed by inoculation with *Bacillus* sp. (PK-6) to test its pyrene removal capacity in soil over a time period of 28 days at 37 ^oC. The soil characteristics, namely pH, EC, moisture content, organic carbon, pyrene content and bacterial count per gram of soil,

were determined at zero time of incubation and at the end of 28 days incubation. A moisture content of 35 + 2 % was maintained in soil throughout the experiment. After 28 days, the pH dropped from 8.2 to 6.8 in pyrene-spiked soil and 6.1 in soil amended with glucose and inoculated with *Bacillus* sp. (PK-6). A unit decrease in pH to 7.4 was observed in the autoclaved control soil after 28 days of incubation. The EC of control soil increased 1.4 times (from 151.43 $\pm$ 0.39 μS cm^{-1} to 205.33 $\pm$ 0.88 μS cm^{-1}) while maximum increase of 3.4 times the EC at zero time of incubation, i.e. from 166.00 $\pm$ 2.31 to 557.50 $\pm$ 1.50 μS cm^{-1} after 28 days was observed in soil amended with glucose and inoculated with *Bacillus* sp. (PK-6). The organic carbon at zero time of incubation was 0.08 $\pm$ 0.02 % in control (uninoculated) soil and 0.23 % in remaining soil treatments. After 28 days of incubation the organic carbon in uninoculated soil treatments remained the same while it increased in *Bacillus* sp. (PK-6) inoculated soil treatments (0.35 – 0.42 $\pm$ 0.00 %). Enumeration of bacteria from *Bacillus* sp. (PK-6) inoculated soil amended with 0.5 mg g^{-1} glucose (with and without pyrene) yielded more bacterial count per gram of soil than soil without glucose amendment in the course of 28 days of incubation. The bacterial counts in glucose amended and *Bacillus* sp. (PK-6) inoculated soils increased from 0.37 x 10^7 cfu g^{-1} to 9.00 x 10^7 cfu g^{-1} in 21 days of incubation (with pyrene amendment) and to 7.02 x 10^7 cfu g^{-1} in 14 days of incubation (without pyrene amendment) and thereafter declined to 2.20 x 10^7 cfu g^{-1} and 2.25 x 10^7 cfu g^{-1}, respectively, till 28 days of incubation. In contrast, the bacterial counts in *Bacillus* sp. (PK-6) inoculated soils (without glucose amendment) declined during the 28 day incubation period, from 0.37 x 10^7 cfu g^{-1} to 0.99 x 10^6 cfu g^{-1} (with pyrene amendment) and to 0.20 x 10^6 cfu g^{-1} (without pyrene amendment). No bacteria were detected in control soil, pyrene-spiked soil and glucose amended soil. Surprisingly a bacterial count of 0.07 x 10^6 cfu g^{-1} was detected in uninoculated soil spiked with pyrene and amended with glucose after 28 days of incubation. This could be probably due to some bacteria that survived the soil autoclaving process.

Pyrene removal in uninoculated pyrene-spiked soil was **13 %,** in pyrene-spiked and glucose amended soil was **34 %**, in pyrene-spiked and *Bacillus* sp. (PK-6) inoculated soil was **66 %** and in pyrene-spiked and glucose amended and *Bacillus* sp. (PK-6) inoculated soil was **71 %**. Glucose amendment in the noninoculated soil treatment probably stimulated some dormant forms of microbial community (which survived autoclaving process) resulting in 34 % pyrene removal. Overall soil treatment with pyrene degrading *Bacillus* sp. (PK-6) and soil amendment with glucose, both factors were found to favour maximum pyrene removal (71 %) over autoclaved pyrene-spiked garden soil.

In this study, the presence of easily metabolizable carbon source glucose significantly promoted the removal of pyrene from the soil due to increased number of pyrene degrading bacteria per gram of soil. Also the results have clearly shown that pyrene remaining in *Bacillus* sp. (PK-6) inoculated soils only accounted for about one-third of the total initial pyrene addition, suggesting that the extent of uptake over this period was influenced by bioaugmentation (introducing pyrene-degrading bacterial culture) and biostimulation (adding glucose).

Salient findings

1. Soil from coal-tar pre-mix industrial plant site showed PAH contamination of Acenaphthylene, Pyrene, Fluorene, Phenanthrene, Benzo[*b*]fluoranthene, Benzo[*k*]fluoranthene, Indeno[1,2,3-*c,d*]pyrene, Dibenzo[*a,h*]anthracene and Benzo[*g,h,i*]perylene which are listed by US Environmental Protection Agency as Priority Pollutants.

2. A culturable biodiversity of nearly 250 bacterial strains isolated from crude, diesel oil and coal-tar contaminated soil were screened for 50 μg ml^{-1} pyrene utilization abilities in basal medium containing 0.5 % (w/v) glucose. Bacterial consortia CON-3 and THA-2 were developed from crude, diesel oil contaminated soil by selective enrichment of pyrene for cometabolizing 50 μg ml^{-1} pyrene in the presence of 0.5 % (w/v) glucose. Thirteen pyrene utilizing bacterial isolates belonged to *Bacillus*, *Pseudomonas* and *Rhodococcus* genera and one pyrene non-utilizing isolate belonged to *Kocuria* genera. Members of Firmicutes (80 %, 75 %), Actinobacteria (10 %, 25 %) and Proteobacteria (10 %, nil) taxonomic clades were present in coal-tar contaminated soil and crude and diesel oil contaminated soil, respectively. Gram-positive bacteria may play more important roles than gram-negative isolates in the degradation of four-benzene ring compound pyrene.

3. Growth studies with bacterial isolates from crude oil contaminated soil show
   (a) Pure and enhanced pyrene utilization upon optimization of physiological conditions (media supplementation).
   (b) Pyrene concentrations of 25 and 50 μg ml^{-1} do not have deleterious effect on growth of log phase culture *Bacillus pumilus* (PK-12).

(c) Pyrene (50 μg ml^{-1}) uptake and degradation was a slow process, occurring between 14 and 28 days, stable thereafter.

4. *Bacillus licheniformis* (PK-6), isolated from coal-tar contaminated soil, showed maximum pyrene uptake (56.4 %) with maximum biosurfactant activity (OD_{550nm} = 1.96) in Bushnell-Haas broth medium in 4 days of incubation. *Bacillus pumilus* (PK-12), isolated from crude oil contaminated soil, showed moderate pyrene uptake (45.6 %) and biosurfactant activity (OD_{550nm} = 1.11). Isolates from coal-tar contaminated soil, *Rhodococcus rhodochrous* (PK-10) showed moderate pyrene uptake (46.3 %) but high biosurfactant activity (OD_{550nm} = 1.45) while *Pseudomonas oleovorans* (PK-3) showed low pyrene uptake (25.2 %) and biosurfactant activity (OD_{550nm} = 0.43). Pyrene was essentially cometabolized along with glucose utilization in all bacterial strains.

5. Six possible pyrene [$C_{16}H_{10}$] metabolites were identified from *Bacillus licheniformis* (PK-6) growth extracts - Phenanthrene [$C_{14}H_{10}$], 9,10-Diphenylphenanthrene [$C_{26}H_{18}$], 9-Methoxyphenanthrene [$C_{15}H_{12}O$], 5,6,7,8-Tetrahydro-1-naphthoic acid [$C_{11}H_{12}O_2$], 1,6,7-Trimethyl-naphthalene [$C_{13}H_{14}$] and Diisooctylphthalate [$C_{24}H_{38}O_4$].

6. Pyrene metabolism property of bacterial isolates is presumed to be chromosomally borne as no plasmids could be detected in bacterial cells.

7. Neither Rieske centers (gene) nor ring-hydroxylated products of pyrene catabolism could be detected by gene-specific PCR or GCMS analysis.

8. Pyrene degradation pathway is proposed in *Bacillus licheniformis* (PK-6) MTCC 1005 based on metabolites identified in this study.

9. Pyrene removal of 58 % was observed in non-sterile, *Bacillus* sp. (PK-6) inoculated coal-tar contaminated soil amended with 0.5 mg g^{-1} glucose in a time period of 28 days at 37 ^{o}C. In sterile, *Bacillus* sp. (PK-6) inoculated garden soil spiked with pyrene @ 0.1 mg g^{-1} and glucose @ 0.5 mg g^{-1} 71 % pyrene removal was observed. Glucose supplementation led to increase in the bacterial count along with high removal of pyrene.

10. Experimental studies in sterile garden soil and non-sterile coal-tar contaminated soil helped to demonstrate the pyrene removal potential of *Bacillus licheniformis* (PK-6) MTCC 1005, suggesting its possible use and advantage in bioremediation of sites contaminated with crude oil, coal-tar and a mixture of PAHs.

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CHAPTER 1

INTRODUCTON

Interest in the biodegradation mechanisms and environmental fate of polycyclic aromatic hydrocarbons (PAHs) is prompted by their ubiquitous distribution and their potentially deleterious effects on human health (Miller and Miller, 1981; Samanta *et al.,* 2002). PAHs are a large and diverse class of organic compounds with two or more fused aromatic rings in various structural configurations. As major components of coal tar and crude oil (Heitkamp *et al*., 1988a), these compounds enter the environment in many ways such as combustion and accidental spilling of hydrocarbons and oils, heat and power generation, refuse burning, fallout from urban pollution, petroleum refining (Zhang *et al*., 2004) and coal liquefaction and gasification processes (Cerniglia, 1984; Atlas and Cerniglia, 1995; Johnsen *et al*., 2005). Consequently, environmental levels of PAHs in industrialized countries across the globe have risen during the last century (Johnson and Larsen, 1985). According to the US Environmental Protection Agency (USEPA) (Irwin *et al.,* 1997) and State and Central Pollution Control Boards (Kumar *et al*., 2010) some of the PAHs are a threat to public health. Mammalian liver enzymes (cytochromes P450 monooxygenase and epoxide hydrolase) oxidize certain PAHs to fjord- and bay- region diolepoxides (Amin *et al*., 1995) which form covalent adducts with DNA (Samanta *et al.,* 2002). Because of this genotoxicity, the USEPA has listed 16 PAHs as priority pollutants to be monitored in industrial effluents (Keith and Telliard, 1979; Yan *et al.,* 2004). This interest has led to the development of technologies to contain and detoxify PAH-contaminated sites. Physico-chemical methods of removal of toxic chemicals from the environment are associated with grand expenditure (Bossert and Bartha, 1984). Their physico-chemical properties such as low water solubility, high adsorption coefficient, and high stability of the complex aromatic ring structure limit the application of conventional remediation techniques (Mohamed *et al*., 2006; Jacques *et al*., 2007). *In situ* bioremediation is an attractive process due to its cost effectiveness, versatility and the benefit of pollutant mineralization to carbon dioxide and water (Habe and Omori, 2003). So bio(remediation)-technologies are being developed to clean up PAH-contaminated environments.

As of today, microbial degradation is the major force affecting the persistence of chemicals in the natural ecosystems. In microbial cells, the PAHs seem to follow one of the two routes of degradation leading either to detoxification or deriving energy to run cellular processes (Kim *et al*., 2007). The degradation of petroleum and xenobiotic aromatic hydrocarbon contaminants may result from catabolism by individual strains of bacteria such as *Mycobacterium* (Heitkamp *et al*., 1988a; Churchill *et al*., 1999) *Bacillus*, *Pseudomonas* (Das and Mukherjee, 2007), *Cycloclasticus* (Geiselbrecht *et al*., 1998; Wang *et al.,* 2008), *Aeromonas*, *Beijerinckia*, *Flavobacterium*, *Nocardia*, *Corynebacterium*, *Burkholderia* (Cerniglia, 1984), *Paracoccus* (Zhang *et al*., 2004), *Stenotrophomonas* (Boonchan *et al*., 2000), *Sphingomonas* (Pinyakong *et al*., 2003; Leys *et al*., 2004; Tao *et al*., 2007) and fungi such as *Cunninghamella* (Pothuluri *et al*., 1990), *Phaenerochaete* (Hammel *et al*., 1986; Bumpus, 1989), *Aspergillus* (Sack *et al*., 1997a; Zang *et al*., 2007) or from combined metabolism by mixed bacterial communities called 'consortia' (Boonchan *et al*., 2000; Jacques *et al*., 2007; Lin and Cai, 2008).

As more studies are carried out, it is becoming increasingly evident that a vast array of microbial species (bacteria, fungi, algae, and cyanobacteria) have a diversity of tools to use PAHs either as sole carbon and energy sources or by cometabolic transformations (for reviews, Cerniglia, 1992, 1997; Kanaly and Harayama, 2000; Juhasz and Naidu, 2000). The metabolic pathways, enzymatic reactions, and genetic control of the catabolism of lower-molecular-weight PAHs (such as naphthalene, anthracene, and phenanthrene) have been well documented (Gibson and Subramanian, 1984; Heitkamp *et al.,* 1988b; Cerniglia and Heitkamp, 1989; Churchill *et al.,* 1999; Habe and Omori 2003). In comparison, little is known about the ability of pure microbial cultures to completely mineralize PAHs containing more than three fused benzene rings (such as pyrene, chrysene, benzo[*a*]pyrene). For the last two decades, research pertaining specifically to the microbial degradation of these PAHs has been advancing. Remediation studies show a low rate of microbial degradation of higher-molecular-weight PAHs which is attributed to their inability to degrade chemicals insoluble in water. The aqueous solubility of PAHs decreases almost logarithmically with increasing molecular mass, therefore high-molecular weight PAHs ranging in size from four to seven rings are of special environmental concern (Johnsen *et al.,* 2005). Some hydrocarbon-degrading microbes respond to these non soluble carbon sources by producing surface-active compounds, as

well as by changing cell surface properties such as cell surface hydrophobicity (Das and Mukherjee, 2007). Production of biosurfactant by cells will help to pseudo-solubilize hydrocarbons (Rosenberg and Ron, 1999), promote their bioavailability in the environment (Cameotra and Bollag, 2003) and ultimately facilitate its uptake by the micro-organism(s).

PYRENE – The pericondensed PAH (Kanaly and Harayama, 2000)

Pyrene, a model compound of high molecular weight PAH degradation, is found in sites contaminated with crude oil, coal tar and other complex mixtures of PAHs (Vila and Grifoll, 2009; Mohamed *et al.,* 2006; Kazunga and Aitken, 2000; Cerniglia, 1992). It consists of four condensed aromatic rings, is a toxic and persistent pollutant, and is released into the environment because of partial combustion of the fossil fuel (Heitkamp *et al*., 1988a) and other natural products such as tobacco (Bhide *et al*., 1984). Although it is not genotoxic, it acts as a pre-carcinogen i.e., it is transformed by cellular processes into a carcinogen (Penning *et al.,* 1999; Kim *et al.,* 2007 and references therein). It has a chemical structure found in several carcinogenic PAHs such as benzo[a]pyrene, indeno-(1,2,3-c,d)-pyrene, and 1-nitropyrene (Kanaly and Harayama, 2000); therefore has been used as an indicator for monitoring PAH-contaminated wastes, for potent carcinogens (Cerniglia *et al*., 1986). Pyrene is characterized by a low water solubility of 0.14 μg L^{-1} at 25 ºC (Cerniglia, 1993), an ionization potential (IP) of 7.50 eV and is a known substrate of lignin peroxidase enzyme (Hammel *et al*., 1986). A meticulous reading of literature has shown that bioremediation of crude oil (Boonchan *et al*., 2000; Calvo *et al*., 2002; Iwabuchi *et al*., 2002; Cubitto *et al*., 2004; Toledo *et al*., 2006) and coal tar (Erickson *et al*., 1993; Luthy *et al*., 1994; Bogan *et al*., 1999; Rogers *et al*., 2007) contaminated sites led to the degradation of pyrene together with other higher-molecular-weight polycyclic aromatic compounds. Although a number of bacterial isolates have been reported to grow on or mineralize pyrene, the majority of these isolates are nocardioform actinomycetes, such as members of the genera *Mycobacterium* (Heitkamp *et al*., 1988b; Grosser *et al*., 1991; Boldrin *et al.,* 1993; Kelley and Cerniglia, 1995; Schneider *et al.,* 1996; Vila *et al.,* 2001; Lopez *et al.,* 2005; Mutnuri *et al.,* 2005) and *Rhodococcus* (Walter *et al.,* 1991; Bouchez *et al.,* 1995).

In spite of the existing body of knowledge on pyrene metabolism, there are very few reports (Sarma *et al.,* 2004; Das and Mukherjee, 2007) of study of pyrene degradation in the Indian environment where rapid industrialization and unabated release by automobiles are loading the environment with cocktails of PAHs. Improving on the available bank of microbial resources (isolates) and information is crucial to the proper management of petroleum-polluted sites.

Therefore the rationale of my research work was to collect information about the prevalence of pyrene - catabolic bacterial genotypes in PAH - contaminated north Indian soils and effective utilization of the metabolic capacity of culturable soil bacteria to degrade or eliminate this carcinogenic pollutant from the environment, in pursuit of which, the complete and partial degradation of pyrene was investigated. The aim of the present study was to isolate aerobic, mesophilic bacterial consortia and monoculture bacterial isolates from crude oil and coal-tar contaminated soil with pyrene uptake capacities.

CHAPTER 2

REVIEW OF LITERATURE

Creosotes and the coal tars from which they are derived are typically comprised of a wide range of aromatic hydrocarbons, aliphatics, heterocyclic (nitrogen, sulphur and oxygen containing) compounds, phenols and amines (Rhodes, 1951; Nestler, 1974; Nishioka *et al*., 1986; Mueller *et al*., 1989b). Crude and refined oils have a similar composition; for example, a typical fuel oil (# 2) consists of 45 % cycloalkanes, 30 % linear (straight chain and branched) aliphatics, and 25 % aromatics (Arvin *et al*., 1988). Through bioremediation, removal or detoxification of all these compounds is sought. Indigenous microbial (bacterial, fungal) populations have been used due to their ability to degrade / detoxify hazardous chemicals such as benzene, toluene, xylene, polychlorinated biphenyls, haloaromatic compounds, PAHs etc. (Montgomery *et al*., 1997). These hydrocarbon wastes are present in the air, soil, sediments, ground or surface water and in due course enter the food chain. Several factors contribute to the fate of these organic compounds in the environment. These include the chemical, physical and biological conditions of the environment such as temperature, water, light, oxygen, gene-pool etc. (Leahy and Colwell, 1990; Bossert and Compeau, 1995). For brevity, this review will be restricted to bacterial degradation of polycyclic aromatic hydrocarbon compounds especially pyrene.

2.1 Physical properties of PAHs and Pyrene

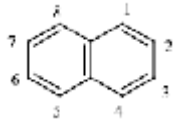
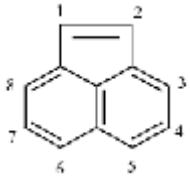
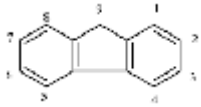
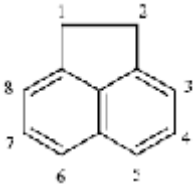
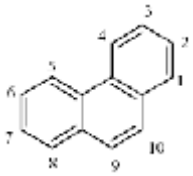
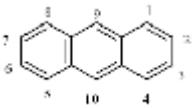
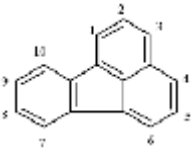
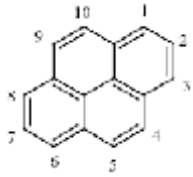
The term PAH generally refers to hydrocarbons containing two or more fused benzene rings in linear, angular or clustered arrangements (Sims and Overcash, 1983). PAHs are hydrophobic compounds and their persistence in the environment is chiefly due to their physicochemical properties such as water solubility, vapor pressure, Henry's law constant (Hs), octanol-water partition coefficient (K_{ow}), and organic carbon partition coefficient (K_{oc}). In general, PAHs have low water solubilities (Cerniglia, 1992). Generally, PAH solubility decreases and hydrophobicity increases with an increase in number of fused benzene rings (Juhasz and Naidu, 2000). The Henry's law constant is the partition coefficient that expresses the ratio of the chemical's concentrations in air and water at equilibrium and is used as an indicator of a chemical's potential to volatilize. In general,

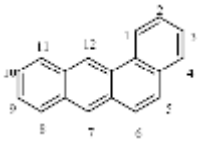
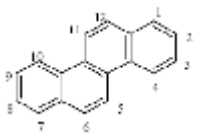
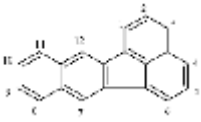
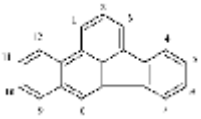
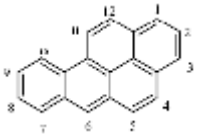
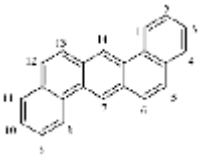
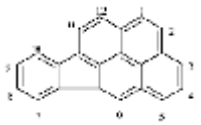
volatility decreases with an increasing number of fused rings (Wilson and Jones, 1993). The K_{ow} is used to estimate the potential for an organic chemical to move from water into lipid and has been correlated with bioconcentration in aquatic organisms (Lotufo, 1998). Some of the characteristics (e.g., Hs constant, and K_{ow} values) of the 16 PAHs are roughly correlated to their molecular weights. These properties are discussed by grouping these PAHs as follows:

- Low molecular weight compounds (128 - 178 g mol^{-1}) - acenaphthene, acenaphthylene, anthracene, fluorene, and phenanthrene;
- High molecular weight compounds (202 - 278 g mol^{-1}) – fluoranthene, pyrene, benz[*a*]anthracene, benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*g,h,i*]perylene, benzo[*a*]pyrene, benzo[*e*]pyrene, chrysene, dibenz[*a,h*]anthracene, and indeno[*1,2,3-c,d*]pyrene.

Pyrene is a four ring PAH where the fused rings are arranged in a symmetrical manner. It has a low aqueous solubility (0.077 - 0.14 mg l^{-1} at 25 ºC) and vapour pressure (3.33 x 10^{-4} Pa), and a high octanol:water partitioning coefficient (4.88 - 5.32), which suggests its preference for non-aqueous phases. **Table 1.1** outlines the physical-chemical properties of pyrene and some other PAHs.

Table 1.1 Structure, nomenclature and general physico-chemical properties of 16 polycyclic aromatic hydrocarbons (adapted from Juhasz and Naidu, 2000; Rogers *et al.*, 2002; Yan *et al.*, 2004; Singh *et al.*, 2007; http://en.wikipedia.org/wiki/Polycyclic_aromatic_hydrocarbon).

Polycyclic aromatic hydrocarbon (PAH)	Chemical Structure	Chem. Formula	Mol. wt.[a] (g mol^{-1})	Mp[b] (^oC)	Bp[c] (^oC)	Sol.[d] (mg l^{-1})	Log K_{ow}[e]	Vap. press. (Pa)
Naphthalene (NAPH)		$C_{10}H_8$	128.12	81.0	218	31.690	3.37	10.4
Acenaphthylene (ACEN)		$C_{12}H_8$	152.20	91.8	280	3.930	4.07	3.87
Fluorene (FLU)		$C_{13}H_{10}$	166.20	116-117	295	1.68 - 1.98	4.18	4.27×10^{-2}
Acenaphthene (ACE)		$C_{12}H_{10}$	154.21	93.4	279	1.930 - 3.420	3.98	5.96×10^{-1}
Phenanthrene (PHEN)		$C_{14}H_{10}$	178.20	101	340	1.00 - 1.29	4.45 -4.46	9.07×10^{-2}
Anthracene (ANTH)		$C_{14}H_{10}$	178.20	216	340	0.045 - 0.076	4.45	2.27×10^{-3}
Fluoranthene (FA)		$C_{16}H_{10}$	202.26	111	250	0.206 - 0.260	4.90 -5.33	6.67×10^{-4}
Pyrene (PYR)		$C_{16}H_{10}$	202.26	149	360	0.077 - 0.140	4.88 -5.32	3.33×10^{-4}

Benzo[*a*]anthracene (BaA)		$C_{18}H_{12}$	228.29	158	400	0.0057 - 0.0140	5.61	2.93×10^{-6}
Chrysene (CHRY)		$C_{18}H_{12}$	228.30	255	488	0.0018 - 0.0028	5.16 -5.61	8.40×10^{-5}
Benzo[*k*]fluoranthene (BkF)		$C_{20}H_{12}$	252.30	-	-	0.00076 – 0.00430	6.06	8.79×10^{-9}
Benzo[*b*]fluoranthene (BbF)		$C_{20}H_{12}$	252.30	-	-	0.0012 - 0.0140	6.04	6.67×10^{-5}
Benzo[*a*]pyrene (BaP)		$C_{20}H_{12}$	252.30	179	496	0.0023 – 0.0038	6.04 -6.06	7.47×10^{-7}
Dibenzo[*a,h*]anthracene (DBA)		$C_{22}H_{14}$	278.35	262	524	0.0005	5.97 -6.84	1.33×10^{-8}
Indeno[1,2,3-*cd*]pyrene (IndPYR)		$C_{22}H_{12}$	276.30	163	536	0.00053 – 0.06200	6.58 -7.66	1.33×10^{-9} –1.33×10^{-4}
Benzo[*ghi*]perylene (BghiP)		$C_{22}H_{12}$	276.34	222	-	0.00026 – 0.00030	6.50 -7.23	1.37×10^{-8}

[a] Mol. wt.: molecular weight; [b] Mp: melting point; [C] Bp: boiling point; [d] Sol.: Aqueous solubility; [e] Log K_{ow}: logarithm of the octanol:water partitioning coefficient; Vap. press.: vapour pressure.

2.2 Production of PAHs

The major source of PAHs is from the combustion of organic material (Cerniglia and Yang, 1984; Guerin and Jones, 1988a). PAHs are formed naturally during thermal geologic production and during burning of vegetation in forest and bush fires (Cerniglia, 1993; Wilson and Jones, 1993). Anthropogenic sources, particularly from fuel combustion, pyrolytic processes, spillage of petroleum products, waste incinerators and domestic heaters (Freeman and Cattell, 1990), are significant sources of PAHs in the environment. In industrial countries, anthropogenic combustion activities are a principal source of PAHs in soils where they arise from atmospheric deposition. This had lead to an increase in soil PAH concentration over the last 100 - 150 years (Jones *et al*., 1989a, b).

2.3 Occurrence of PAHs in the environment: Source and composition

The release of PAHs into the environment is wide-spread since these compounds are ubiquitous products of incomplete combustion. PAHs have been detected in a wide variety of environmental samples, including air (Freeman and Cattell, 1990), soil (Jones *et al*., 1989a, b, c; Wilson and Jones, 1993), sediments (Guo *et al*., 2005), water (Cerniglia and Heitkamp, 1989), oils, tars (Perwak *et al*., 1982; IARC 1985) and foodstuffs (Lijinsky, 1991). Industrial activities, such as processing, combustion and disposal of fossil fuels, are usually associated with the presence of PAHs at highly contaminated sites. PAH contamination on industrial sites is commonly associated with spills and leaks from storage tanks and with the conveyance, processing, use and disposal of these fuel/oil products (Wilson and Jones, 1993). PAHs are also a major constituent of creosote (approximately 85 % PAH by weight) and anthracene oil, which are commonly used pesticides for wood treatment (Bumpus, 1989; Walter *et al*., 1991). As such, PAH contamination is frequently associated with wood treatment activities (Mueller *et al*., 1991a, 1993; Vanneck *et al*., 1995; Sims and Overcash, 1983).

2.3.1 PAHs in air

PAHs are directly released to the atmosphere from both natural and anthropogenic sources. The primary natural sources of airborne PAHs are forest fires and volcanoes (Maliszewska-Kordybach, 1999; NRC, 1983). Residential burning of wood is the largest source of atmospheric PAHs (Ramdahl *et al*., 1982); releases are primarily the result of inefficient combustion and uncontrolled emissions (NRC, 1983; Samanta *et al*., 2002). Other important stationary anthropogenic sources include industrial power generation,

incineration (Shane *et al*., 1990; Wild and Jones, 1995); the production of coal tar, coke, and asphalt; and petroleum catalytic cracking (Guerin, 1978; Perwak *et al*., 1982; Maliszewska-Kordybach, 1999). Tobacco smoke, convective kerosene heaters, gas cooking and heating appliances may be important sources of PAHs in indoor air (NRC, 1986; Hoffmann and Hoffmann, 1997). PAH concentrations of 3000 μg m^{-3} have been measured in flue emissions from small residential stoves (Freeman and Cattell, 1990). Stationary sources account for about 80 % of total annual PAH emissions; the rest are from mobile sources. The most important mobile sources of PAHs are gasoline and diesel-powered engines (Johnson, 1988; Maliszewska-Kordybach, 1999). Motor vehicles like diesel automobiles, trucks and buses contribute to atmospheric PAH pollution through exhaust condensate and particulates, tire particles and lubricating oils and greases (Wakeham *et al*., 1980). During the combustion of fossil fuels, diesel powered vehicles are the major source of lighter PAHs to the atmosphere, whereas gasoline vehicles are the dominant source of higher molecular weight PAHs, including pyrene.

Results from ambient air monitoring programs have shown that PAH concentrations are usually of the order of a few nanograms per cubic metre of air (Ramadahl *et al*., 1982). Peters *et al*. (1981) estimated that a total of 11,031 metric tons of PAHs were released to the atmosphere in the United States on an annual basis, with 36 % of the total coming from residential heating, 6 % from industrial processes, 1 % from incineration, 36 % from open burning, 1 % from power generation, and 21 % from mobile sources. This estimate can be compared to that of Ramdahl *et al*. (1982), who reported that a total of 8,598 tons of PAHs were emitted to the atmosphere annually from the following sources: residential heating 16 %; industrial processes 41 %; incineration 1 %; open burning 13 %; power generation 5 % and mobile sources 25 %.

The composition of PAH emissions varies with the combustion source. For example, emissions from residential wood combustion contain more acenaphthylene than other PAHs (Perwak *et al*., 1982), whereas auto emissions contain more benzo[*g,h,i*]perylene and **pyrene** (Santodonato *et al*., 1981; Rogge *et al*., 1993a). PAHs in diesel exhaust particulates are dominated by three- and four-ring compounds, primarily fluoranthene, phenanthrene, and **pyrene** (Kelly *et al*., 1993; Rogge *et al*., 1993a; Westerholm and Li, 1994). Phenanthrene and benzo[*g,h,i*]perylene were the most frequently detected PAH in fly ash samples collected from municipal refuse and solid waste incinerators,

respectively (Shane *et al*., 1990; Wild and Jones, 1995). Fluoranthene, benzo[*a*]fluoranthene, benzo[*g,h,i*]perylene, indeno[*1,2,3-c,d*]pyrene, phenanthrene, and chrysene and benz[*a*]anthracene were predominant in emission samples collected from a municipal and medical / pathological waste incinerator while **pyrene**, fluoranthene, and phenanthrene were predominant in emission samples from a pilot scale rotary kiln incinerator charged with polyethylene (Williams, 1994). In coal tar pitch emissions, concentrations of phenanthrene and **pyrene** have been reported to be 20-80 times greater than the concentrations of benzo[*a*]pyrene and benzo[*g,h,i*]perylene (Sawicki, 1962). Chrysene, **pyrene**, and fluoranthene were dominant among the PAHs found in fine particle emissions from natural gas home appliances (Rogge *et al*., 1993b). Cigarette mainstream smoke contains a wide variety of PAHs with reported concentrations of benzo[*a*]pyrene ranging from approximately 5-80 ng per cigarette; side stream smoke concentrations are significantly higher with side stream / mainstream concentration ratios for benzo[*a*]pyrene ranging from 2.5 to 20 (Hoffmann and Hoffmann, 1997; IARC, 1983).

2.3.2 PAHs in water

The main sources of PAHs in water are deposition of atmospheric PAHs (Jensen, 1984), municipal waste water discharge (Barrick, 1982), urban storm water runoff (Pitt *et al*., 1995), runoff from coal storage areas (Iwamotu and Nasu, 2001), effluents from wood treatment industries (USDA, 1980; DeLeon *et al*., 1986), oil spills (Van Hamme *et al*., 2003), and petroleum processing (Guerin, 1978). The majority of the aromatic compounds in surface waters are believed to result from atmospheric deposition (Santodonato *et al*., 1981). Prahl *et al*. (1984) found that combustion derived PAHs adsorbed to suspended sediments in rivers accounted for the major portion of PAHs in coastal waters, and Kanaly and Harayama (2000) have identified industrial effluents, road runoff, and oil spills as the major contributors in water bodies. Morselli and Zappoli (1988) reported elevated PAH levels in refinery waste waters, with concentrations for most PAHs in the range of 400 ng l^{-1} (benzo[*b*]fluoranthene) to 16,000 ng l^{-1} (phenanthrene).

2.3.3 PAHs in sediments

As PAHs are characterized by low water solubilities and high octanol-water partitioning coefficients, their concentrations in water are extremely low. However, sediments may be

considered a good reservoir for PAHs. Sediment studies have revealed an increasing PAH burden since the late 1800s, with a peak in the 1950s (Hites *et al*., 1980) especially in the urban areas (Jones *et al*., 1989a, 1989b). A rapid increase in PAH concentration from 1900, peaking around the 1950s has been reported; however, a decrease in PAH concentration occurred after the 1950s. The rise and fall in sediment PAH concentrations was attributed to the increase in industrial activity at the turn of the century, followed by an increasing reliance on oil and gas fuels from the mid 1940s. It is generally believed that if substituted PAHs (such as alkyl derivatives) predominate in sediments then the source of PAHs is petroleum based; however, if unsubstituted PAHs predominate, then it is likely that the PAHs arise from combustion processes (Meador *et al*., 1995). Due to their hydrophobic nature, PAHs accumulate in fine grain sediments, partitioning to organic carbon-coated particles (Meador *et al*., 1995). PAH may accumulate in sediments due to atmospheric deposition, urban run-off, marine seeps of petroleum hydrocarbons, off-shore production or petroleum transportation, sewage disposal or boating (Juhasz and Naidu, 2000). The concentration of PAHs in sediments may range over several orders of magnitude (from a few mg kg^{-1} up to g kg^{-1}) depending on the proximity of the waterway to industrial activity, water currents and water usage.

2.3.4 PAHs in soils

Anthropogenic combustion of fossil fuels and atmospheric deposition of PAHs has contributed to the dispersal of PAHs throughout the environment (Greenberg *et al*., 1985; Thomas, 1986; Juhasz and Naidu, 2000). Other potential sources of PAHs in soil include sludge disposal from public sewage treatment plants, automobile exhaust, irrigation with coke oven effluent, leachate from bituminous coal storage sites, and use of soil compost and fertilizers (White and Lee, 1980; Santodonato *et al*., 1981; Perwak *et al*., 1982; Stahl *et al*., 1984). A study by Jones *et al*. (1989a) on PAH concentrations in soil, reported an increase in PAH abundance near urban centres. The principal sources of PAHs in soils along highways and roads are vehicular exhausts and emissions from wearing of tires and asphalt. PAHs may also be released to soils at landfill sites (Black *et al*., 1989), former gas works sites (Bewley *et al*., 1989; Tiehm *et al*., 1997; Bogan *et al*., 1999) and industrial sites, including creosote production (Ellis *et al*., 1991; Vinas *et al*., 2005), wood-preserving (Weissenfels *et al*., 1990; Mueller *et al*., 1991a), and coking plants (Yrjaniheikki *et al*., 1995; Preuss *et al*., 2003). These industrial activities heavily and increasingly burden the soil with PAHs and also influence the concentrations of

Table 1.2 Concentration of selected PAHs in contaminated soils and sediment associated with industrial activity (adapted from Juhasz and Naidu, 2000).

PAH	PAH concentration (mg kg^{-1} soil) in PAH-contaminated soils/sediments (Reference):									
	Creosote production site			Wood preserving site (Mueller *et al.*, 1991a)	Superfund site		Gas works (Bewley *et al.*, 1989)	Petrochemical works (Juhasz, 1998)	Manufacturing gas plant site	
	(Ellis *et al.*, 1991)	(Juhasz *et al.*, 2005)	(Vinas *et al.*, 2005)		Mississippi, USA (USEPA, 1995a)	Minnesota, USA (USEPA, 1995b)			(Erickson *et al.*, 1993)	(Bogan *et al.*, 1999)
Naphthalene	1131			3925	673	6494		186	97	
Acenaphthylene	33			49	79	3651			28	28
Acenaphthene		844	151	1368	705	21,319	2	43	49	2
Fluorene	650	472	182	1792	32	2497	225	87	14	4
Phenanthrene	1595	1305	496	4434	266	7902	379	156	26	51
Anthracene	334	158	114	3307	2	1440	156	53	11	58
Fluoranthene	682	2050	693	1629	419	10,053	2174	137	73	195
Pyrene	642	1659	387	1303		9481	491	99	47	173
Chrysene	614		144	481	305	2392	345		15	52
Benz[*a*]anthracene		301	108	171	496	1670	317	33	16	88
Benzo[*a*]pyrene		101	21	82	224	536	92	15	14	106
Benzo[*b*]fluoranthene / Benzo[*k*]fluoranthene		349	82	140	513	2271	498		21	99
Dibenz[*a,h*]anthracene		< 10			27	192	2451	12	33	
Benzo[*g,h,i*]perylene		34								
Indeno[*1,2,3-c,d*]pyrene		46		23	64	120	207		7	46
Total PAH	**5863**	**7767**	**2724**	**18,704**	**3815**	**70,633**	**7337**	**821**	**451**	**974**

individual PAHs associated with contaminated soils. For example, in soil from a creosote production site, Ellis *et al*., (1991) reported a total PAH concentration of 5863 mg kg^{-1} and pyrene was detected at 642 mg kg^{-1}. At a French manufacturing gas plant (MGP) site, pyrene concentration in soil was 173 mg kg^{-1} (Table 1.2).

2.3.5 PAHs in marine organisms

Uptake of PAHs by marine organisms is dependent on the bioavailability of the PAHs (i.e. partitioning of the compound between sediment, water and food), and physiology of the organism (Meador *et al*., 1995). Biological processes such as an organism's size, ingestion rate, growth rate, membrane permeability, ventilatory rate, gut residence time and osmoregulation influence PAH uptake (Baumard *et al*., 1999). Environmental factors, such as temperature, oxygen content, pH and salinity can also influence the uptake of PAHs by marine organisms due to their effect on the bioavailability of the compounds. In addition, changes in the organism's behavior, seasonal rhythms, nutritional quality and stress can also influence PAH uptake. Table 1.3 outlines the occurrence of PAHs in a number of marine organisms (fish, molluscs, crustaceans, sea sponges, algae and diatoms) from around the world. PAHs may also be eliminated from the organism by passive diffusion or by excretion of polar metabolites produced from the metabolism of the parent compound (Meador *et al*., 1995).

2.3.6 PAHs in plants

PAHs may also accumulate in vegetation that could indirectly cause human exposure through food consumption (Juhasz and Naidu, 2000). Airborne PAHs may deposit on plant parts and may get transformed to β-*O*-glucoside and β-*O*-glucuronide conjugates that can penetrate the cell (Nakajima *et al*., 1996); however, the fates of these transformation products and their parent compounds in leaves have not been fully elucidated. A number of factors like atmospheric PAH concentrations, temperature and wind conditions, the type of PAH and the plant species can influence the accumulation of PAHs in plants (Wagrowski and Hites, 1997). Wagrowski and Hites (1997) estimated that the PAH burden in rural vegetation was up to 10 times lower than in urban vegetation samples which correlated to the low atmospheric PAH concentration in rural areas as compared to urban areas. They also calculated that up to 160 tonnes of total PAHs per year flowed from the atmosphere to vegetation. Fluoranthene, pyrene, and chrysene were-

Table 1.3 Total PAH concentration detected in a number of marine organisms from around the world (adapted from Juhasz and Naidu, 2000)

Organism	Source	Total PAH ($ng\ g^{-1}$)	Reference
Fish[a] *Pseudopleuronectes americanus, Scophthalmus aquosus, Arophysate chuss*	NY Bight	14 - 536 [d]	Humason and Gadbois (1982)
Molluscs[b] *Mytilus edulis Mytilus edulis, Mytilidae, Littorina littorea, Patella vulgate, Cardium edule*	Scotland, Norway, Gulf of Naples (Italy)	54 – 2803 [d], 500 - 225,000 [e]	Bjorseth *et al*. (1979); Mackie *et al*. (1980); Knutzen and Sortland (1982); Cocchieri *et al*. (1990)
Crustacean [a] *Cancer irroratus*, *Homarus americanusa*	NY Bight, Nova Scotia	52 - 1600 [d]	Humason and Gadbois (1982)
Sea Sponge[b] *Halichondria panicea*	S. Norway	769 [e]	Knutzen and Sortland (1982)
Diatom [b] *Navicula phyllepta*	Netherlands	454 [e]	Stronkhorst *et al*. (1994)
Algae *Fucus vesiculosus, Fucus serratus*	S. Norway	284 - 4665 [e]	Knutzen and Sortland (1982)

[a] PAH concentrations were determined from edible tissue.
[b] Whole organisms minus the shell.
[d] PAH concentrations were determined per wet weight.
[e] PAH concentrations were determined per dry weight.

- found in concentrations of 1.2, 2.0, and 2.9 $\mu g\ g^{-1}$, respectively, in composite samples of green leaves from 62 plant species in the Los Angeles area; corresponding values for dried leaf samples were 0.47, 1.1, and 1.9 $\mu g\ g^{-1}$ (Rogge *et al*., 1993d).

2.3.7 PAHs in other environmental media

Significant amounts of PAHs are contained in crude oil and some of its derivatives (e.g. diesel oil, bitumen, lubricating oils) and in coal products (e.g. coal tar, creosote, anthracene oil) (Smith *et al*., 1999). PAHs are present at 1-2 weight percent in crude oils (Guerin, 1978). Actual PAH concentrations in crude oil depends on the geological source

of the oil (IARC, 1989). For example, the NRC (1985) has reported concentrations of seven individual carcinogenic PAHs ranging from 1.2 µg g^{-1} for banzo[*a*]pyrene to 23 µg g^{-1} for chrysene in a South Louisiana crude oil and from 0.5 µg g^{-1} for benzo[*a*]pyrene to 6.9 µg g^{-1} for chrysene in a Kuwait crude oil. PAHs are also found in refined petroleum products including gasoline, kerosene, diesel fuel, some heating oils, and motor oil (Guerin, 1978). A coal-tar sample has been found to contain approximately 0.007 mg kg^{-1} benz[*a*]anthracene, 3 mg kg^{-1} benzo[*b*]fluoranthene, 4 g kg^{-1} chrysene, and 30 mg kg^{-1} benzo[*a*]pyrene (Perwak *et al*., 1982). High temperature coal-tar contained 1,000 mg kg^{-1} dibenz[*a,h*]anthracene (IARC, 1985). Creosote has been reported to contain 21 % phenanthrene, 10 % fluorene, 10 % fluoranthene, 9 % acenaphthene, **8.5 % pyrene**, 3 % chrysene, 3 % naphthalene, and 2 % anthracene (Lorenz and Gjovik, 1972).

PAHs have been detected in many food products including vegetables, fruits and smoked or broiled meat and fish. The concentrations in uncooked foods largely depend on the source of the food. For example, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, and chrysene have been detected in vegetables grown near a heavily traveled road (Wang and Meresz, 1982). The method of cooking like the time of cooking, distance from the heat source, and the drainage of fat during cooking (e.g., cooking in a pan versus on a grill) can also influence the PAH content of food. For example, charcoal broiling increases the amounts of PAHs in meat. Smoked meat products processed with natural wood smoke have higher total PAH and total carcinogenic PAH concentrations than those processed with liquid smoke flavorings.

PAHs have also been reported to occur in chewing tobacco, snuff, and in tobacco smoke. Smoking one pack of cigarettes a day has been estimated to result in exposure to carcinogenic PAHs of up to 5 µg per day (Menzie *et al*., 1992). Reported concentrations of **pyrene** and some PAHs in various types of tobacco smoke are shown in Table 1.4. The data show concentrations of pyrene in cigarette mainstream smoke ranging between 50 and 270 ng per cigarette (IARC, 1983). Concentrations of PAHs in sidestream smoke are significantly higher than in mainstream smoke.

Table 1.4 Concentrations of some PAHs in Tobacco Smoke (Adapted from a tabulation of data from several studies in IARC (1983), Guerin *et al*. (1992) and http://www.atsdr.cdc.gov/toxprofiles/tp69-c5.pdf).

Compound	Cigarette main stream smoke (µg/100 cigarettes)	Cigarette side stream smoke (µg/100 cigarettes)	Cigarette smoke-polluted environments (µg/m^3)	Cigar smoke (µg/100 g)	Pipe smoke (µg/100 g)
Anthracene	2.3–23.5			11.9	110.0
Benz(*a*)anthracene	0.4–7.6	4–20	0.1–100	2.5–3.9	
Benzo(*b*)fluoranthene	0.4–2.2		0.1–35[b]		
Benzo(*j*)fluoranthene	0.6–2.1				
Benzo(*k*)fluoranthene	0.6–1.2				
Benzo(*g,h,i*)fluoranthene	0.1–0.4				
Benzo(*g,h,i*)perylene	0.3–3.9	9.8	0.4–17		
Benzo(*a*)pyrene	0.5–7.8	2.5–19.9	0.4–760	1.8–5.1	8.5
Benzo(*e*)pyrene	0.2–2.5	13.5	0.4–18		
Chrysene	0.6–9.6		2.6–16		
Dibenz(*a,h*)anthracene	0.4		<0.1–13		
Fluoranthene	1–27.2	126	0.2–99	20.1	
Fluorene	present				
Indeno(1,2,3-*c,d*)pyrene	0.4–2.0		0.6–1		
Phenanthrene	8.5–62.4		4–87	115	
Pyrene	5–27	39–101	0.8–66	17.6	75.5

sidestream smoke = smoke that comes from the end of a lighted cigarette, pipe, or cigar; *mainstream smoke* = smoke that is exhaled by a smoker.

PAH concentrations in fly ash and bottom ash samples from domestic municipal incinerators ranged from not detected to 7,400 μg kg^{-1}, with phenanthrene the most abundant and frequently detected compound (Shane *et al*., 1990). Machado *et al*. (1993) reported the total concentrations of 16 PAHs (all PAHs in Table 1.4 except benzo[*e*]pyrene) in asphalt and coal tar pitch to be 122 and 300 μg g^{-1}, respectively. The PAH content of asphalt and coal tar pitch fumes increased with increasing generation temperature.

Tire wear particles, brake lining particles, and paved road dust from a residential area had total PAH concentrations of 226.1, 16.2, and 58.7 μg g^{-1}, with maximum concentrations of individual PAHs of 54.1 μg g^{-1} pyrene, 2.6 μg g^{-1} benzo[*g,h,i*]perylene, and 9.4 μg g^{-1} pyrene, respectively (Rogge *et al*., 1993c). Combined particle- and vapor-phase emissions from scrap tire fires have been reported to contain average total PAH concentrations of 3.2 mg m^{-3} (Fullana *et al*., 2000). Tire pyrolysis oil, which may be used as a fuel, contains high levels of PAHs, with average total PAH concentrations ranging from 14,540 μg g^{-1} to over 100,000 μg g^{-1} (10 %) of oil (Williams and Taylor, 1993).

2.4 PAH toxicity

Combustion of fossil fuels is always incomplete and generates PAHs as a class of most spread mutagenic and carcinogenic particulate environmental pollutants (Yan *et al*., 2004). It has been found that low molecular weight (1 - 3 ringed) PAHs are acutely toxic (Sims and Overcash, 1983), while higher molecular weight PAHs are considered to be genotoxic (Lijinsky, 1991; Cerniglia, 1992; Juhasz and Naidu, 2000; Pagnout *et al*., 2006). PAHs have been shown to produce carcinogenic tumours in experimental animals when administered orally, by skin application, inhalation or food consumption, subcutaneous and/or intramuscular administration (Collins *et al*., 1991). Tests in cultured mammalian cells have shown that PAHs are active in assays for DNA binding, sister chromatid exchange, chromosomal aberrations, point mutations and transformations (Hollstein *et al*., 1979; de Serres and Ashby, 1981). Further it has been suggested that concomitant exposure to chemicals and light can cause toxic reactions, also termed as phototoxicity (Davies, 1980; Arfsten *et al*., 1996; Gocke, 2001; Baudouin *et al*., 2002). Because of the multiple aromatic ring systems in PAHs, these compounds can absorb light energy in the UV region (320 – 400 nm) and for some in the visible region (400 – 700 nm) (Dabestani and Ivanov, 1999), forming reactive species and causing damages to

human cellular components. Since PAHs are ubiquitous in the environment and concomitant exposure to PAHs and light by humans is inevitable, photomutagenicity of these compounds are of human health importance. Concomitant exposure to PAHs and light can cause DNA single strand cleavage, oxidation of DNA bases, and formation of DNA covalent adducts (Rogers *et al*., 2002).

Yan *et al*. (2004) studied 16 PAHs listed as USEPA priority toxic pollutants in Ames test using *S. typhimurium* TA102 concomitantly under 30 minutes of light (UV + visible) irradiation. As shown in Table 1.5, 11 of the 16 PAHs tested are photomutagenic, of which 6 PAHs (anthracene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*g,h,i*]perylene, indeno[1,2,3-*cd*]pyrene, and pyrene) are identified as strongly photomutagenic or caused bacterial death, 5 PAHs (acenaphthene, acenaphthylene, benzo[*k*]fluoranthene, chrysene, and fluorene) are weakly photomutagenic and remaining 5 PAHs are not photomutagenic. Interestingly, some of the mutagenic and / or carcinogenic PAHs (like benzo[*b*]fluoranthene, dibenz[*a,h*]anthracene, and fluoranthene) are not photomutagenic and some of the non-mutagenic and / or non-carcinogenic PAHs (acenaphthene, acenaphthylene, anthracene benzo[*g,h,i*]perylene, fluorene, and pyrene) are photomutagenic. Only benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*k*]fluoranthene, chrysene, and indeno[1,2,3-*c,d*]pyrene are both photomutagenic and mutagenic. This photomutagenicity of PAHs indicates that PAHs most abundant in the environment, especially acenaphthene, acenaphthylene, anthracene, benzo[*g,h,i*]perylene, fluorene, and pyrene, that are not mutagenic in cells through metabolic activation, can be activated by light irradiation leading to adverse human health effects, e.g., DNA damage or DNA covalent adducts formation. This result may affect the way PAH risk assessment is conducted.

Some recent studies on PAH degradation by bacteria also revealed formation of genotoxic intermediates with metabolic activation of PAHs. During PAH degradation by *Mycobacteria* quinones from nonenzymatic oxidation of dihydroxy-PAHs and *trans*-dihydrodiols, resulting from the epoxidation of PAHs by a P450- dependent monooxygenase, were detected in the culture media (Cerniglia and Heitkamp, 1990; Heitkamp *et al*., 1988b; Vila *et al*., 2001). Quinones and epoxide derivates are the biologically active forms of PAHs and are known for their genotoxic potentials on mammals (Dipple *et al*., 1999).

Table 1.5 Comparison of toxicity of the 16 PAHs on the list of US EPA 126 priority pollutants (adapted from Rogers *et al.*, 2002; Yan *et al.*, 2004; Singh *et al.*, 2007).

PAH [EPA #[A]] CAS #	No. of rings	Photomutagenicity (μg) [B]	Carcinogenicity [C]	Mutagenicity
Naphthalene [55] 91-20-3	2	- (864)	I [D]	-
Acenaphthylene [77] 208-96-8	3	+ (10)	I [D]	-
Fluorene [80] 86-73-7	3	+ (2.2)	I [D]	-
Acenaphthene [1] 83-32-9	3	+ (10)	0	-
Phenanthrene [81] 85-01-8	3	- (300)	I	-
Anthracene [78] 120-12-7	3	++ **(2.4)**	0	-
Fluoranthene [39] 206-44-0	4	- (2.7)	0	+
Pyrene [84] 129-00-0	4	++ **(2.7)**	0	-
Benzo[*a*]anthracene [72] 56-55-3	4	++ **(3.0)**	++	+
Chrysene [76] 218-01-9	4	+ (3)	+	+
Benzo[*k*]fluoranthene [75] 207-08-9	5	+ (3.4)	++	+
Benzo[*b*]fluoranthene [74] 205-99-2	5	- (3.4)	++	+
Benzo[*a*]pyrene [73] 50-32-8	5	++ **(3.4)**	++	+
Dibenzo[*a,h*]anthracene [82] 53-70-3	5	- (3.7)	++	+
Indeno[1,2,3-*cd*]pyrene [83] 193-39-5	6	++ **(0.07)**	++ [D]	+
Benzo[*ghi*]perylene [79] 101-24-2	6	++ **(3.7)**	I	-

[A] Numbers are from the EPA list of priority pollutants (Yan *et al.*, 2004).

[B] Photomutagenicity symbols are: (+) photomutagenic; (++) strongly photomutagenic. The doses given in brackets, underlined and boldfaced indicate bacterial death which means that more than 90% of the bacteria died based on viable bacterial counts.

[C] Carcinogenicity symbols are: (0) no evidence of carcinogenicity; (I) inadequate evidence for evaluation; (+) limited evidence of carcinogenicity; (++) sufficient evidence of carcinogenicity in experimental animals.

[D] From EPA web site for animal carcinogenicity study.

2.5 Fate of PAHs in the environment

The fate of PAHs and other organic contaminants in the environment is associated with both abiotic and biotic processes, including volatilization, photooxidation, chemical oxidation, bioaccumulation, and microbial transformation (**Figure 1.1**). Microbial activity has been deemed the most influential and significant cause of PAH removal (Cerniglia, 1993).

2.5.1 Abiotic factors

When aromatic hydrocarbons enter previously uncontaminated soil some physical changes take place such as evaporation of volatile hydrocarbons from mixtures, and seepage of heavier non-volatile fractions into the soil. The hydrocarbons in soil may be subjected to auto-, photo- or thermal oxidation. These partially oxidized hydrocarbons are available to biological systems (Bossert and Bartha, 1984). For instance Miller *et al.* (1988) demonstrated that photolysis of benzo[*a*]pyrene primes its degradation in activated sewage sludge.

Water immiscible hydrocarbons, which do not evaporate, and the oxidized intermediates of PAHs comparatively more soluble than their parent compounds, move down in the soil and eventually reach the water table. The movement of contaminant hydrocarbon depends on the porosity or texture of the soil (Bossert and Bartha, 1984).

2.5.2 Biological factors

In a heterogeneous environment like soil, microorganisms capable of hydrocarbon degradation determine their fate. Under ideal conditions, given that the necessary gene pool is available, hydrocarbons may be completely mineralized to CO_2 and water. However, such conditions may not always exist. Partial metabolism of hydrocarbons may lead to formation of harmless or less toxic or more toxic intermediates (McGill *et al.*, 1981).

2.5.2.1 Bacterial versus fungal metabolism of hydrocarbons

Although ubiquitous in ecosystems, the fraction of the total heterotrophic community represented by the hydrocarbon-utilizing bacteria and fungi is highly variable, with reported frequencies ranging from 6 % to 82 % for soil fungi, 0.13 % to 50 % for soil -

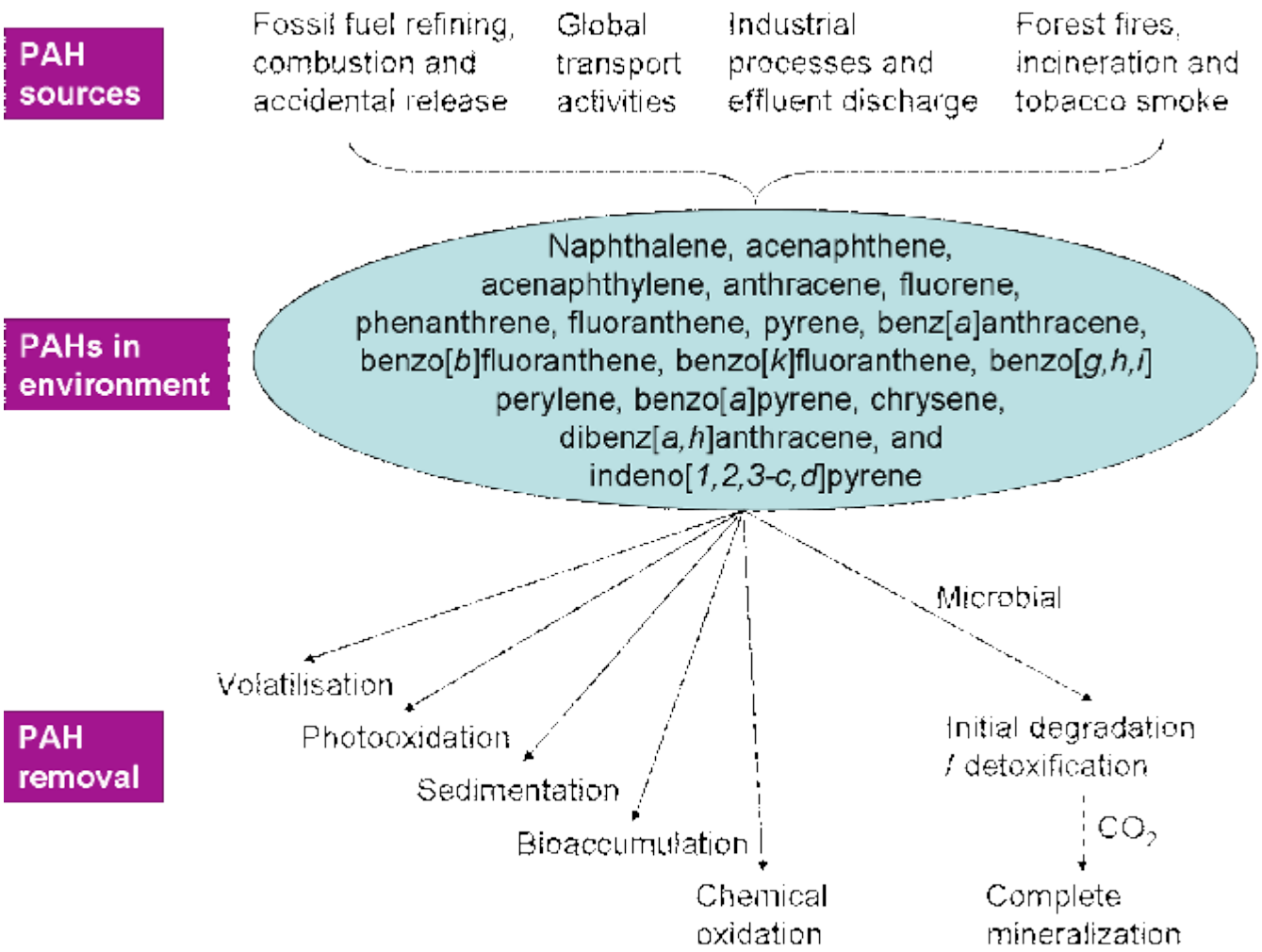


Figure 1.1 Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons (adapted from Cemiglia, 1993; Juhasz, 1998; Kanaly and Harayama, 2000).

- bacteria (Jones *et al*., 1970; Pinholt *et al*., 1979). While individual organisms can metabolize only a limited range of hydrocarbon substrates, assemblages of mixed populations with overall broad enzymatic capacity are required to degrade complex mixtures of hydrocarbons such as crude oil and completely bioremediate PAH-contaminated sites (Boonchan *et al*., 2000).

Fungi on one hand have hyphal structures that can penetrate into soil matrix and have greater access to more hydrocarbons than bacteria. Fungi also produce extracellular enzymes that can act on hydrocarbons in the soil itself. It is therefore considered that fungi may play an important role in degrading hydrocarbon contaminants during the initial stages of bioremediation. Bacteria, on the other hand, exhibit more versatile metabolism and can play a role during the later stage of bioremediation when the hydrocarbons are more heterogeneous in nature, i.e., when partially degraded PAHs of various ring sizes are present along with parent PAHs. Degradation by bacteria is often faster than that by fungi. In a comparative study of hydrocarbon degradation by bacteria and fungi in soil, Song *et al.* (1986) observed that 82 % of *n*-hexadecane mineralization in a sandy loam was attributed to bacteria and only 13 % was attributed to fungi.

2.5.2.2 Algal metabolism of hydrocarbons

The extent of algal and protozoan involvement in hydrocarbon biodegradation is largely unknown. Cerniglia *et al.* (1980a, b) observed that prokaryotic and eukaryotic photoautotrophic marine algae, nine cyanobacteria, five green algae, one red alga, one brown alga and two diatoms could metabolize naphthalene. Bacterial degradation of PAHs generally involves dioxygenases, but in green algae like *Oscillatoria* sp., *Agmenellum quadruplicatum* etc. PAH degradation involves cytochrome P450 and conversion of PAHs to arene oxides. However, a fresh water alga, *Selenastrum capricornutum*, when grown on benzo[*a*]pyrene, forms cis-dihydrodiols (Warshawsky *et al*., 1995). This suggests that in certain algal species dioxygenase enzymes play an important role in PAH degradation. While the oxidation of naphthalene by a number of algae and cyanobacteria has been reported, relatively few studies have demonstrated the degradation of high molecular weight compounds by these organisms.

Table 1.6 An exhaustive survey of common PAHs oxidised by different species of bacteria.

Naph: Naphthalene, Ace: Acenaphthene, Phen: Phenanthrene, Anth: Anthracene, FA: Fluoranthene, Pyr: Pyrene, Chry: Chrysene, BaA: Benz[*a*]anthracene, DBA: Dibenz[*a,h*]anthracene, BaP: Benzo[*a*]pyrene

	Bacteria	Naph	Ace	Phen	Anth	FA	Pyr	Chry	BaA	DBA	BaP	References
1.	*Achromobacter xylosoxidans*			+								Andreoni *et al*. (2004)
2.	*Acidovorax delafieldii*			+								Samanta *et al*. (1999)
3.	*Acidovorax sp.*	+		+				+	+		+	Singleton *et al*. (2009)
				+								Singleton *et al*. (2005)
							+					Singleton *et al*. (2006)
4.	*Acinetobacter calcoaceticus*	+										Ryu *et al*. (1989)
				+	+	+	+	+				Lal and Khanna (1996)
5.	*Acinetobacter lwoffii*			+								Sisto *et al*. (2008)
6.	*Aeromonas sp.*			+								Kiyohara *et al*. (1976)
7.	*Alcaligenes denitrificans*	+		+								Weissenfels *et al*. (1990)
		+		+		+	+		+			Weissenfels *et al*. (1991)
8.	*Alcaligenes eutrophus*		+									Selifonov *et al*. (1993)
9.	*Alcaligenes faecalis*	+				+						Toledo *et al*. (2006)
				+								Kiyohara *et al*. (1982, 1990)
10.	*Alcaligenes paradoxus*		+									Selifonov *et al*. (1993)
11.	*Alcaligenes sp.*			+								Andreoni *et al*. (2004)
12.	*Aquamicrobium defluvium*			+								Andreoni *et al*. (2004)
13.	*Arthrobacter*			+								Keuth and Rehm (1991)

	polychromogenes											
14.	*Arthrobacter sp.*			+	+							Savino and Lollini (1977)
15.	*Arthrobacter sulphurous*			+								Samanta *et al*. (1999)
16.	*Bacillus cereus*			+			+					Kazunga and Aitken (2000)
							+					Lin and Cai (2008)
				+	+		+					Jacques *et al*. (2007)
17.	*Bacillus megaterium*						+					Lin and Cai (2008)
18.	*Bacillus pumilus*	+		+			+					Toledo *et al*. (2006)
							+					Gauthier *et al*. (2003)
19.	*Bacillus subtilis*	+		+								Toledo *et al*. (2006)
							+					Das and Mukherjee (2007)
							+				+	Hunter *et al*. (2005)
									+			Mahaffey *et al*. (1988)
20.	*Brevibacterium sp.*			+								Samanta *et al*. (1999)
21.	*Burkholderia cepacia*					+	+		+	+		Juhasz *et al*. (1997)
22.	*Caulobacter sp.*						+					Jones *et al*. (2008)
23.	*Comamonas testosterone*	+		+	+							Goyal and Zylstra (1996)
24.	*Corynebacterium renale*	+										Dua and Meera (1981)
25.	*Cycloclasticus pugetii*			+	+							Dyksterhouse *et al*. (1995)
26.	*Cycloclasticus sp.*	+	+	+	+							Geiselbrecht *et al*. (1998)
27.	*Cycloclasticus spirillensus*						+					Wang *et al*. (2008)
28.	*Enterobacter sp.*	+										Toledo *et al*. (2006)
				+								Kastner *et al*. (1998)
29.	*Gordonia polyisoprenivorans*			+	+		+					Jacques *et al*. (2007)
30.	*Leclercia adecarboxylata*						+					Sarma *et al*. (2004)
31.	*Methylobacterium sp.*			+								Andreoni *et al*. (2004)

32.	*Microbacterium sp.*			+							Zhang *et al*. (2004)
				+	+		+				Jacques *et al*. (2007)
							+				Gauthier *et al*. (2003)
33.	*Micrococcus luteus*	+		+							Toledo *et al*. (2006)
34.	*Micrococcus sp.*			+							Ghosh and Mishra (1983)
35.	*Mycobacterium austroafricanum*			+		+	+			+	Bogan *et al*. (2003)
36.	*Mycobacterium flavescens*					+					Dean-Ross *et al*. (2002)
							+				Dean-Ross and Cerniglia (1996), Brezna *et al*. (2003)
37.	*Mycobacterium fortuitum*			+	+		+				Jacques *et al*. (2007)
38.	*Mycobacterium montefiorense*						+				Uytttebroek *et al*. (2007)
39.	*Mycobacterium sp.*	+	+	+		+	+				Vila *et al*. (2001)
		+									Kelly *et al*. (1991), Walter *et al*. (1991)
			+			+	+				Churchill *et al*. (1999)
			+	+	+	+	+				Karabika *et al*. (2008)
				+		+	+				Boldrin *et al*. (1993), Dandie *et al*. (2004), Pagnout *et al*. (2006)
				+	+	+	+		+		Vila and Grifoll (2009)
				+							Guerin and Jones (1988b)
					+						van Herweijnen *et al*. (2003)
						+					Heitkamp and Cerniglia (1988), Kelly and Cerniglia (1991),

											Rehmann *et al*. (2001), Lopez *et al*. (2006), Zhou *et al*. (2007)
					+	+					Lopez *et al*. (2005)
											Grosser *et al*. (1991), Rehmann *et al*. (1998), Habe *et al*. (2004b), Krivobok *et al*. (2003), Brezna *et al*. (2003), Liang *et al*. (2006), Sho *et al*. (2004), Kim *et al* (2003), Jimenez and Bartha (1996), Gauthier *et al*. (2003), Cheung and Kinkle (2005a, b), Cottin and Merlin (2007)
						+					Kim *et al*. (2006)
						+		+		+	Schneider *et al*. (1996)
										+	Warshawsky *et al*. (2007)
			+	+		+				+	Pizzul *et al*., (2007)
40.	*Mycobacterium vanbaalenii*	+	+			+					Heitkamp and Cerniglia (1988)
		+	+	+	+	+					Kim *et al*. (2006)
			+	+							Moody *et al*. (2001)
			+			+					Khan *et al*. (2001), Kim *et al*. (2004b, 2005)
			+	+	+	+					Kim *et al*. (2004a)
			+								Stingley *et al*. (2004a)
				+							Moody *et al*. (2001), Kim *et al*. (2004a, 2006)

						+						Kelly *et al*. (1993), Sepic *et al*. (1998), Kweon *et al*. (2007)
							+					Heitkamp *et al*. (1988a, 1988b), Brezna *et al*. (2003), Kim *et al* (2007)
											+	Moody *et al*. (2004)
41.	*Neptunomonas naphthovorans*	+	+									Hedlund *et al*. (1999)
42.	*Nocardia sp.*			+								Iwabuchi *et al*. (1998)
43.	*Nocardioides sp.*			+								Saito et. (2000)
44.	*Paenibacillus*						+					Daane *et al*. (2001)
45.	*Paracoccus sp.*			+	+	+	+	+				Zhang *et al*. (2004)
				+		+	+					Guo *et al*. (2005)
46.	*Pasteurella sp.*					+						Sepic *et al*. (1998)
47.	*Porphyrobacter sp.*						+					Gauthier *et al*. (2003)
48.	*Pseudomonas aeruginosa*	+										Eaton and Chapman (1992)
							+					Bordas *et al*. (2005), Das and Mukherjee (2007)
49.	*Pseudomonas alcaligenes*					+						Hickey *et al*. (2007)
50.	*Pseudomonas fluorescens*	+										Izmalkova *et al*. (2005)
		+		+	+							Foght and Westlake (1996)
51.	*Pseudomonas marginalis*	+			+			+				Burd and Ward (1996)
52.	*Pseudomonas mendocina*			+								Tian *et al*. (2003)
53.	*Pseudomonas paucimobilis*	+										Kuhm *et al*. (1991)
				+								Mueller *et al*. (1990b)
		+			+	+						Mueller *et al*. (1990a)

54.	*Pseudomonas putida*	+										Dunn and Gunsalus (1973), Jeffrey *et al.* (1975), Filonov *et al.* (1999), Basu *et al.*(2006)
		+	+									Kiyohara *et al.* (1994)
		+		+								Yang *et al.* (1994)
55.	*Pseudomonas saccharophila*	+		+								Stringfellow and Aitken (1995)
				+							+	Kazunga and Aitken (2000)
						+	+	+	+			Chen and Aitken (1999)
56.	*Pseudomonas sp.*	+										Davies and Evans (1964), Williams *et al.*, (1975), Ensley *et al.* (1982), Barnsley (1975); Barnsley (1983a), Garcia-Valdes *et al.* (1988), Zhang *et al.* (2004), Singleton *et al.* (2005)
		+		+								Ma *et al.* (2006)
			+									Chapman (1979)
				+								Samanta *et al.* (1999), Prabhu and Phale (2003), Zhang *et al.* (2004)
				+	+							Evans *et al.* (1965)
					+							Kumar *et al.* (2010)
		+		+	+	+						Foght and Westlake (1988)
							+					Obayori *et al.* (2008)
57.	*Pseudomonas stutzeri*	+		+								Stringfellow and Aitken

												(1995)
				+			+					Kazunga and Aitken (2000)
58.	*Ralstonia sp.*	+										Singleton *et al*. (2005)
59.	*Rhizobium galegae*			+								Andreoni *et al*. (2004)
60.	*Rhodococcus aetherovorans*			+								Andreoni *et al*. (2004)
61.	*Rhodococcus rhodochrous*			+								Iwabuchi *et al*. (2002)
62.	*Rhodococcus sp.*	+										Grund *et al*. (1992), Allen *et al*. (1997), Larkin *et al*. (1999)
				+		+	+					Guo *et al*. (2005)
					+							Tongpim and Pickard (1996), Dean-Ross *et al*. (2001)
					+	+	+	+				Walter *et al*. (1991)
63.	*Selenastrum capricornutum*										+	Warshawsky *et al*. (2007)
64.	*Sphingomonas paucimobilis*			+			+					Kastner *et al*. (1998)
						+	+	+	+	+		Ye *et al*. (1996)
65.	*Sphingomonas sp.*	+		+								Tao *et al*. (2007)
				+								Johnsen *et al*. (2002), Johnsen and Karlson (2004)
					+							Kastner *et al*. (1998)
				+		+	+					Guo *et al*. (2005)
						+						Zhou *et al*. (2007)
							+					Singleton *et al*. (2006)

							+				Demaneche *et al*. (2004)
66.	*Sphingomonas yanoikuyae*		+			+				+	Kazunga and Aitken (2000)
				+							Kim *et al*. (1997)
										+	Rentz *et al*. (2008), Warshawsky *et al*. (2007)
67.	*Stenotrophomonas acidaminiphila*		+								Andreoni *et al*. (2004)
68.	*Stenotrophomonas maltophilia*		+		+	+	+	+	+	+	Boonchan *et al*. (2000)
			+			+				+	Dandie *et al*. (2004)
					+	+		+	+	+	Juhasz *et al*. (2000)
69.	*Streptomyces flavovirens*		+								Sutherland *et al*. (1990)
70.	*Streptomyces griseus*		+								Trower *et al*. (1988)
71.	*Streptomyces sp.*	+									Trower *et al*. (1988)
72.	*Terrabacter*				+						Zhou *et al*. (2007)
73.	*Thermus sp.*					+					Feitkenhauer *et al*. (2003)
74.	*Vibrio sp.*		+								Kiyohara and Nagao (1978), West *et al*. (1984), Geiselbrecht *et al*. (1996), Zylstra *et al*. (1997)

Table 1.7 An exhaustive survey of common PAHs oxidised by different species of fungi.

Naph: Naphthalene, Ace: Acenaphthene, Phen: Phenanthrene, Anth: Anthracene, FA: Fluoranthene, Pyr: Pyrene, Chry: Chrysene, BaA: Benz[*a*]anthracene, DBA: Dibenz[*a,h*]anthracene, BaP: Benzo[*a*]pyrene

	Fungi	Naph	Ace	Phen	Anth	FA	Pyr	Chry	BaA	DBA	BaP	References
1.	*Absida glauca*	+										
2.	*Agrocybe aegerita*			+			+					Sack *et al*. (1997b)
3.	*Aspergillus niger*			+			+					Sack *et al*. (1997a)
											+	Zang *et al*. (2007)
4.	*Bjerkandera adjusta*			+	+	+	+					Schutzendubel *et al*. (1999)
						+	+	+				Valentin *et al*. (2007)
					+						+	Field *et al*. (1992)
5.	*Bjerkandera sp.*										+	Kotterman *et al*. (1998)
6.	*Crinipellis stipitaria*						+					Lambert *et al*. (1994), Lange *et al*. (1994)
7.	*Cunninghamella bainieri*	+										Ferris *et al*. (1973)
8.	*Cunninghamella elegans*		+									Pothuluri *et al*. (1992)
				+	+							Cerniglia and Yang (1984)
				+								Cerniglia *et al*. (1989)
					+							Cerniglia (1982)
						+						Pothuluri *et al*. (1990)
									+			Cerniglia *et al*. (1994)
											+	Cerniglia and Gibson (1979, 1980a, b)
		+										Cerniglia and Gibson (1977), Cerniglia *et al*. (1982a)
9.	*Cyclothyrium sp.*	+		+	+		+				+	da Silva *et al*. (2004)

10.	*Fusarium oxysporum*		+	+		+					Jacques *et al*. (2007)
11.	*Kuehneromyces mutabilis*		+			+					Sack *et al*. (1997b)
12.	*Laetiporus sulphureus*		+			+					Sack *et al*. (1997b)
13.	*Penicillium glabrum*					+					Wunder *et al*. (1997)
14.	*Penicillium janthinellum*					+	+	+	+	+	Boonchan *et al*. (2000)
15.	*Phanerochaete chrysosporium*					+					Hammel *et al*. (1986)
				+			+				Bogan *et al*. (1996)
				+						+	Field *et al*. (1992)
										+	Zheng and Obbard (2002)
			+								Sutherland *et al*. (1991), Bumpus (1989), Hammel *et al*. (1992), Brodkorb and Legge (1992)
16.	*Phanerochaete laevis*		+	+				+		+	Bogan and Lamar (1996)
17.	*Pleurotus ostreatus*						+	+	+	+	Baldrian *et al*. (2000)
			+	+		+				+	Bezalel *et al*. (1996a)
			+								Bezalel *et al*. (1996b)
				+		+					Bezalel *et al*. (1996c)
			+	+	+	+					Schutzendubel *et al*. (1999)
18.	*Pleurotus sp.*					+					Lang *et al*. (1996)
19.	*R. solani*			+							Sutherland *et al*. (1992)
20.	*Stropharia coronilla*									+	Steffen *et al*. (2003)
21.	*Trametes versicolor*		+								Collins and Dobson (1996)
		+									Johannes *et al*. (1998)
				+							Johannes *et al*. (1996)
				+						+	Field *et al*. (1992), Collins *et al*. (1996)
			+			+					Sack *et al*. (1997b)

Table 1.8 PAHs oxidised by different species of cyanobacteria and algae (adapted from Cerniglia, 1992; Juhasz and Naidu, 2000).

Naph: Naphthalene, Ace: Acenaphthene, Phen: Phenanthrene, Anth: Anthracene, FA: Fluoranthene, Pyr: Pyrene, Chry: Chrysene, BaA: Benz[*a*]anthracene, DBA: Dibenz[*a,h*]anthracene, BaP: Benzo[*a*]pyrene

	Algae	Naph	Ace	Phen	Anth	FA	Pyr	Chry	BaA	DBA	BaP	References
1.	*Agmenellum quadruplicatum*			+								Narro *et al*. (1992a)
		+										Cerniglia *et al*. (1979), Narro *et al*. (1992a)
2.	*Amphora sp.*	+										Cerniglia *et al*. (1980b, 1982b)
3.	*Anabaena sp.*	+										Cerniglia *et al*. (1980b, 1982b)
4.	*Aphanocapsa sp.*	+										Cerniglia *et al*. (1980b, 1982b)
5.	*Chlamydomonas angulosa*	+										Cerniglia *et al*. (1980b, 1982b)
6.	*Chlorella autotrophica*	+										Cerniglia *et al*. (1980b, 1982b)
7.	*Chlorella sorokiniana*	+										Cerniglia *et al*. (1980b, 1982b)
8.	*Coccochloris elabens*	+										Cerniglia *et al*. (1980b, 1982b)
9.	*Cylindrotheca sp.*	+										Cerniglia *et al*. (1980b, 1982b)
10.	*Dunaliella*	+										Cerniglia *et al*.

	tertiolecta		(1980b, 1982b)
11.	*Microcoleus chthonoplastes*	+	Cerniglia *et al*. (1980b, 1982b)
12.	*Navicula sp.*	+	Cerniglia *et al*. (1980b, 1982b)
13.	*Nitzschia sp.*	+	Cerniglia *et al*. (1980b, 1982b)
14.	*Nostoc sp.*	+	Cerniglia *et al*. (1980b, 1982b)
15.	*Oscillatoria sp.*	+	Cerniglia *et al*. (1980d), Narro *et al*. (1992b)
16.	*Porphyridium cruentum*	+	Cerniglia *et al*. (1980b, 1982b)
17.	*Ulva fasciata*	+	Cerniglia *et al*. (1980b, 1982b)

Protozoa by contrast, have not been shown to utilize hydrocarbons. Rogerson and Berger (1981) cultured freshwater and soil protozoa on petroleum-degrading yeasts and bacteria either in the presence or absence of partially degraded crude oil and found no effect of crude oil on the growth of protozoa.

2.6 Biodegradation of PAHs

The persistence of PAHs in the environment depends on their physical and chemical characteristics. PAHs are degraded by photo- and chemical- oxidation, but biological transformation is probably the prevailing route of PAH loss (Mueller *et al*., 1990b). The recalcitrance of PAHs to microbial degradation increases directly with the molecular weight and the octanol: water partition coefficient (log K_{ow}) (Cerniglia, 1992). The microbial metabolism of PAHs containing up to three rings, naphthalene (Wodzinski and Bertolini, 1972; Cox and Williams, 1980; Aranha and Brown, 1981; Heitkamp *et al*., 1987; Kelly *et al*., 1990; diGrazia *et al*., 1991; Narro *et al*.,1992b; Guerin and Boyd, 1992), phenanthrene (Evans *et al*., 1965; Barnsley, 1975, 1983a, 1983b; Kiyohara *et al*., 1976,1982, 1990; Kiyohara and Nagao, 1978; Ghosh and Mishra, 1983; Guerin and Jones, 1988a, 1988b; Sutherland *et al*., 1990; Weissenfels *et al*., 1990; Keuth and Rehm, 1991), anthracene (Jerina *et al*.,1976), fluorene (Mihelcic and Luthy, 1988; Grifoll *et al*., 1992, 1994, 1995; Monna *et al*.,1993; Trenz *et al*., 1994) has been studied extensively. Their degradation rates, metabolic pathways, enzymatic and genetic regulation have been well documented. Over the last 2 decades, biodegradation of the larger, more recalcitrant, PAHs containing four or more fused benzene rings has been intensively studied, however there is still a paucity of information on the biodegradation of high molecular weight PAHs. Among a variety of microorganisms studied, bacteria, especially mycobacteria, oxidize the greatest variety of PAHs, including naphthalene, acenaphthene, anthracene, fluorene, phenanthrene, **pyrene**, fluoranthene, benz[*a*]anthracene and benzo[*a*]pyrene (Heitkamp and Cerniglia, 1989; Grosser *et al*., 1991; Boldrin *et al*., 1993; Pothuluri and Cerniglia, 1994; Schneider *et al*., 1996; Churchill *et al*., 1999; Vila *et al*., 2001; Moody *et al*., 2001, 2003, 2004; Kweon *et al*., 2007).

Bacteria initially oxidise aromatic hydrocarbons to *cis*-dihydrodiols as shown in Figure 1.2 (Evans *et al*., 1965; Jerina *et al*., 1976; Cerniglia, 1984; Heitkamp *et al*., 1988b; Kelly *et al*., 1991). The oxidation of these compounds involves the dioxygenase enzyme mediated incorporation of two oxygen atoms into the aromatic nucleus of substrate

molecule. Characteristically, bacterial dioxygenases are multicomponent enzyme systems (Albaiges *et al*., 1983). The initial ring oxidation is usually the rate-limiting step in the biodegradation reaction of PAHs (Cerniglia, 1992). In the successive steps *cis*-dihydrodiols are re-aromatised through a dehydrogenase enzyme leading to the formation of catechols (Atlas, 1981; Gibson and Subramanian, 1984) which are substrates for other dioxygenases that bring about enzymatic cleavage of the aromatic ring. Catechol can be oxidised via *ortho* or *meta* pathways. The *ortho* pathway involves cleavage of the bond between carbon atoms of the two hydroxyl groups to yield *cis*, *cis*-muconic acid. On the other hand, the *meta* pathway involves cleavage of the bond between a carbon atom with a hydroxyl group and the adjacent carbon atom with a hydroxyl group to yield 2-hydroxymuconic semialdehyde (**Figure 1.2**). Ring cleavage results in the production of succinic, fumaric, pyruvic and acetic acids and aldehydes, all of which are utilized by the microorganism for the synthesis of cellular constituents and energy (Wilson and Jones, 1993). A by-product of these reactions is the production of carbon dioxide and water.

Once the initial hydroxylated aromatic ring of the PAH is degraded (to pyruvic acid and carbon dioxide), the second ring is then attacked in the same manner (Atlas and Bartha, 1981). However, many high molecular weight PAHs, such as **pyrene**, are only degraded with difficulty or not at all, due to their low water solubility, high resonance energy and toxicity (Cerniglia, 1992; Wilson and Jones, 1993). **Pyrene** may be degraded, however, via a co-oxidation or co-metabolism mechanism using less recalcitrant compounds as co-substrate (Beam and Perry, 1973).

2.6.1 Biochemical aspects of PAH degradation

2.6.1.1 PAH metabolism via cis-diols

In most bacteria and some algae, the main mechanisms of PAH degradation involve oxidation by dioxygenase to form *cis*-dihydrodiols. These are converted to diphenols, which are cleaved by other dioxygenases. Phenanthrene, acenaphthene, acenaphthylene, fluorene, anthracene, fluoranthene and **pyrene** are reported to be metabolized via *cis*-diols.

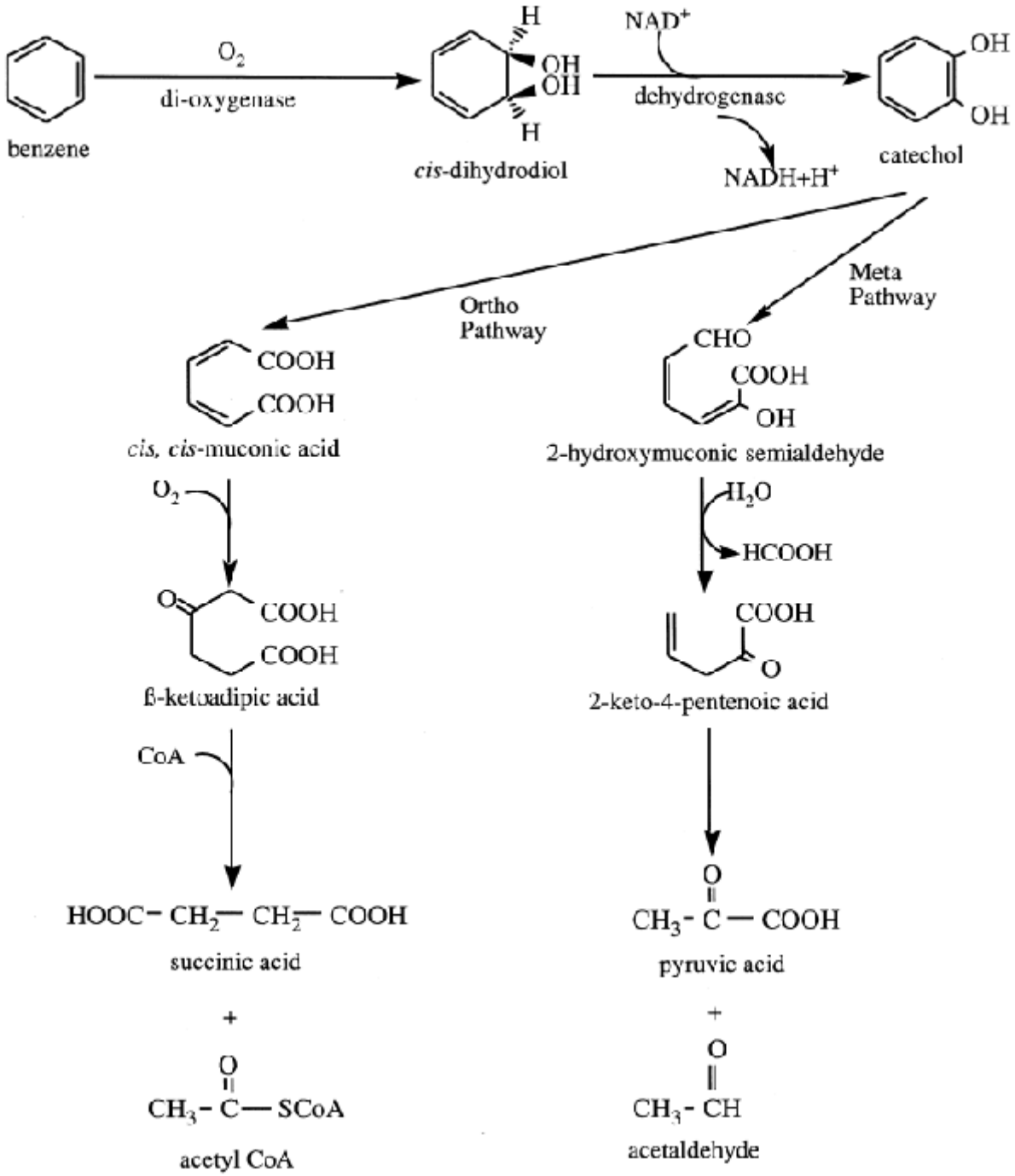


Figure 1.2 Microbial metabolism of the aromatic ring by ortho or meta cleavage (adapted from Cerniglia, 1984; Rochkind-Dubinsky *et al*., 1987; Juhasz and Naidu, 2000).

2.6.1.2 PAH metabolism via trans-diols

Degradation of PAHs via *trans*-dihydrodiols occurs mainly in mammalian systems, fungi, certain bacteria, cyanobacteria involving cytochrome P450 monooxygenases and epoxide hydrolase. The major product formed due to the action of monooxygenases is arene oxide. Invariably this route of metabolism helps microbe to detoxify the PAHs, whereas metabolism via *cis*-dihydrodiol leads to mineralization. The fungus *Cunninghamella elegans* metabolizes acenaphthene and anthracene to *trans*-diols (Cerniglia and Yang, 1984; Pothuluri *et al*., 1992).

2.6.1.3 Five- or more ringed HMW PAH degradation

Currently, there is only limited information regarding the biodegradation of PAHs with five or more rings in environment. Most studies have focused on the five-ring benzo[*a*]pyrene (B*a*P) molecule. Its concentrations are often highest in soils and sediments. Many studies have documented the environmental recalcitrance of B*a*P (Bossert and Bartha, 1986; Cerniglia, 1992; Juhasz and Naidu, 2000). Turnover times of greater than 3.3 years in oil contaminated sediments and greater than 60 years in uncontaminated sediments have been reported for B*a*P (Goodin and Webber, 1995). In soils that readily accommodated mineralization of three- and four-ring PAHs, Carmichael and Pfaender (1997b) showed that only 2 - 9 % of [^{14}C] B*a*P at 136 ng g^{-1} was mineralized in 8 weeks while in a soil from a previously contaminated coal gasification site, 25 % of [^{14}C] B*a*P at 84 ng g^{-1} was mineralized to $^{14}CO_2$ during a 225 day incubation including a 28 day lag period (Grosser *et al*., 1991). In another study, it was shown that after an incubation period of 100 days, approximately 40 % of [^{14}C] B*a*P at 67 to 80 µg g^{-1} was cometabolized to $^{14}CO_2$ when the indigenous soil microbiota was provided with a suitable cosubstrate (Kanaly *et al*., 1997; Kanaly and Bartha, 1999).

So far all reported B*a*P biotransformations by bacteria have occurred under cometabolic conditions. *Mycobacterium* sp. strain PYR-1 biotransformed 24.7 % of 0.5 ppm B*a*P to aqueous and organic-extractable metabolites when grown with peptone, yeast extract, and soluble starch but B*a*P mineralization was not detected (Heitkamp and Cerniglia, 1988; Kelley and Cerniglia, 1995). Schneider *et al*. (1996) published the only paper which described the identification of B*a*P ring fission products. The PAH-degrading versatility of *Sphingomonas paucimobilis* EPA505 was demonstrated by Ye *et al*. (1996). After 16 hrs of incubation with 10 ppm of a HMW PAH mixture, the resting cells of EPA505 mineralized B*a*P, benzo[*b*]fluoranthene, and dibenz[*a,h*]anthracene to 33.3, 12.5, and

7.8 % $^{14}CO_2$, respectively. No mineralization was detected for the six-ring PAH dibenz[*a,h*]pyrene.

2.6.2 Genetic aspects of PAH degradation

The bacterial degradation of two and three ringed PAHs (naphthalene, phenanthrene) has been studied and the biochemical pathways for their biodegradation along with their molecular basis have been thoroughly investigated. Literature pertaining to the degradation of LMW PAHs is reviewed here to have an in depth understanding of the molecular mechanisms involved in the degradation of PAHs. The forthcoming sections would focus on **pyrene** degradation.

Goyal and Zylstra (1996) cloned a gene cluster encoding phenanthrene dioxygenase and *cis*-phenanthrene dihydrodiol dehydrogenase from *Commamonas testosteroni* GZ39. Interestingly, the genes in this phenanthrene and naphthalene degrading strain showed no homology to the *nah* genes responsible of naphthalene degradation. Also, the cloned gene from GZ39 failed to hybridize with genes from *C. testosteroni* strain GZ42 isolated from the same environment indicating that there exists a great diversity of microorganisms performing similar functions. Similarly, Kim *et al.,* (1996) isolated genes involved in naphthalene degradation from the genomic DNA of *Sphingomonas yanaiknae* B1. This strain contained degradative genes on a megaplasmid (230 kb).

Several bacteria that oxidize aromatic hydrocarbon to *cis*-dihydrodiol also oxidize indole to indigo, because of the combined activities of tryptophanase and dioxygenase (Wackett and Gibson, 1983). Investigators screened recombinants expressing dioxygenases by the appearance of blue colonies on Luria-Bertani agar plates containing indole. Using this approach, Yang *et al.,* (1994) isolated genes involved in naphthalene, fluorene and phenanthrene degradation from a *Pseudomonas putida* strain and demonstrated that it is possible for a single gene cluster to degrade a variety of PAH compounds. Similarly Zylstra *et al.* (1994) found that the genes cloned for naphthalene degradation also metabolized fluorene, phenanthrene and pyrene. Contrary to this Kiyohara *et al.* (1994) demonstrated that *Pseudomonas putida* OUS82 used different metabolic pathways for naphthalene and phenanthrene metabolism.

2.7 Anaerobic degradation of aromatic hydrocarbons

Anaerobic metabolism is a vital process with respect to bioremediation of PAH contaminated sediments, water-logged soils and aquifers (Johnsen *et al*., 2005). With its

unique biochemistry now being uncovered, it is also vital with respect to biomimetic catalyst development (Van Hamme *et al*., 2003). Under anaerobic conditions, aromatic hydrocarbons are difficult to biodegrade because of the stability of the ring structures (Hu *et al*., 2007). However a diverse set of bacteria (no fungi have been studied to date), recently isolated for their ability to metabolize various aromatic hydrocarbons (Coates *et al*., 1997; Annweiler *et al*., 2002; Eriksson *et al*., 2003; Kodama and Watanabe, 2003), form an excellent framework from which to elucidate the underlying biochemical and molecular mechanisms driving anaerobic hydrocarbon metabolism. Work with microbial consortia in the field, in enrichment cultures, and in microcosms has illustrated that hydrocarbons such as toluene (Elshahed and McInerney, 2001), alkylbenzenes (Haner *et al*., 1997; Phelps and Young, 1999), benzene (Burland and Edwards, 1999; Hu *et al*., 2007), naphthalene and phenanthrene (Meckenstock *et al*., 2000; 2004; Rockne *et al*., 2000; Rogers *et al*., 2007), fluorene and fluoranthene (Coates *et al*., 1997), anthracene and **pyrene** (McNally *et al*., 1998), methylnaphthalene and tetralin (Annweiler *et al*., 2002), > C6 *n*-alkanes (Van Hamme *et al*., 2003 and references therein), branched alkanes (Bregnard *et al*., 1997), and hydrocarbon mixtures (Grishchenkov *et al*., 2000) can be metabolized under anaerobic conditions. These reactions may take place under Fe (III)-reducing, denitrifying, and sulfate-reducing conditions, by anoxygenic photosynthetic bacteria, or in syntrophic consortia of proton-reducing and methanogenic bacteria. Rockne *et al*. (2000) demonstrated anaerobic growth on naphthalene (as the sole source of carbon and energy) by pure cultures of *Pseudomonas* sp. and *Vibrio* sp. when coupled to dissimilative nitrate reduction. McNally *et al*. (1998) also isolated *Pseudomonas* sp. which showed phenanthrene, anthracene and **pyrene** degradation under denitrifying conditions. Coates *et al*. (1997) showed anaerobic oxidation of fluorene, phenanthrene and fluoranthene to carbon dioxide under sulfate-reducing conditions in ocean sediments. Other terminal electron acceptors shown to be used during anaerobic hydrocarbon metabolism include manganese oxides (Langenhoff *et al*., 1997), soil humic acids (Cervantes *et al*., 2001), and fumarate in a fermentative oxidation process (Meckenstock, 1999).

Toluene has been the most studied hydrocarbon with respect to enzymatic and genetic characterizations in the denitrifying bacteria *Azoarcus* sp. (T), *Thauera aromatica* (K172), and *Thauera* sp. (T1) (Biegert *et al*., 1996; Beller and Spormann, 1998; Leuthner *et al*., 1998; Heider *et al*., 1999; Achong *et al*., 2001). In the proposed pathway, fumarate

addition to toluene is mediated by benzylsuccinate synthase to form benzylsuccinate. Following this unusual addition reaction, a series of modified β-oxidation reactions are thought to convert benzylsuccinate to benzyl-CoA (Biegert *et al*., 1996; Beller and Spormann, 1998), which is a central intermediate in the anaerobic degradation of aromatic compounds (Harwood and Gibson, 1997). It is suggested that addition of CoA residue modifies the electron clouds of benzene which facilitate downstream degradation reactions (Villemur, 1995). Benzylsuccinate synthase has been purified from *Azoarcus* sp. (T) and *T. aromatica* (K172) and the genes encoding this enzyme have been cloned and sequenced in same bacterial species (Leuthner and Heider, 1998; Achong *et al*., 2001; Hermuth *et al*., 2002). Together, these studies suggest that the potential for PAH-degradation in anaerobic environments may be greater than previously recognized (Johnsen *et al*., 2005)**.**

2.8 Pyrene degradation

A great deal of work has been carried out in trying to rationalize the persistence of pyrene in the environment. As more studies are carried out, it is becoming increasingly evident that a large number of bacteria and fungi have the ability to use pyrene as sole carbon and energy source. For strains not able to utilize pyrene and other PAHs with more than four rings, such as benzo[*a*]pyrene as a sole carbon and energy source, cometabolic transformations have been characterized (Cerniglia, 1992, 1997; Kanaly and Harayama, 2000; Juhasz and Naidu, 2000).

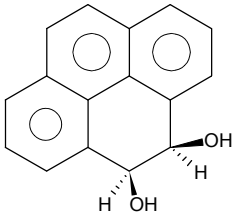
2.8.1 Bacterial degradation

It was not until the late 1980s that the first milestone in the biodegradation of pyrene was reached. In 1988, Heitkamp and Cerniglia published the first study on the isolation of a bacterial isolate, *Mycobacterium* sp. that could extensively degrade pyrene cometabolically. They described it as an aerobic Gram-positive rod from sediment below an oil field which could mineralize up to 63 % of the original amount of pyrene in two weeks in pure culture with organic nutrients. The bacterium could also mineralize a number of other PAHs besides pyrene, such as naphthalene, phenanthrene, fluoranthene, 1-nitropyrene, 3-methyl-cholanthrene and 6-nitrochrysene to carbon dioxide (Heitkamp *et al*., 1988a; Cerniglia and Heitkamp, 1990). Pyrene-induced *Mycobacterium* cultures mineralized over 60 % of radiolabeled pyrene in 96 h. Pyrene *cis*- and *trans*-4,5-dihydrodiols and pyrenol were identified as the initial ring-oxidation products, while 4-

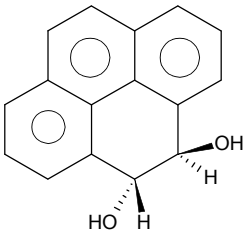
phenanthroic acid, 4-hydroxyperinaphthenone, cinnamic and phthalic acids were identified as ring fission products of pyrene. The various identified metabolites of pyrene degradation are shown in Figure 1.3. $^{18}O_2$ studies showed that the formation of *cis*- and *trans*-4,5-dihydrodiols were catalyzed by dioxygenase and monooxygenase enzymes, respectively (Heitkamp *et al*., 1988b). Chloramphenicol treatment demonstrated that the enzymes responsible for pyrene degradation were inducible at the transcription level. Sediment microcosms inoculated with the *Mycobacterium* showed enhanced mineralization of various PAHs, including pyrene and benzo[*a*]pyrene (Heitkamp and Cerniglia, 1989). Although the degradation of pyrene by this strain was cometabolic, increase in organic nutrients to the microcosm inhibited pyrene degradation. This most likely occurred due to the utilization of nutrients by rapidly growing indigenous -

Figure 1.3 Structures of identified pyrene metabolites produced by the *Mycobacterium* sp. (adapted from Heitkamp *et al*., 1988b)

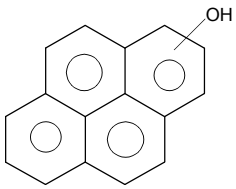
RING-OXIDATION PRODUCTS:



***cis*-4,5-Pyrenedihydrodiol**

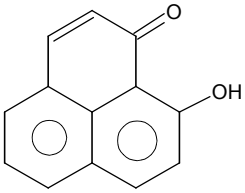


***trans*-4,5-Pyrenedihydrodiol**

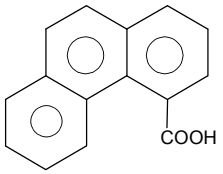


Pyrenol

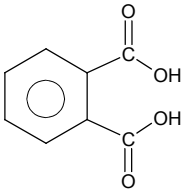
RING-FISSION PRODUCTS:



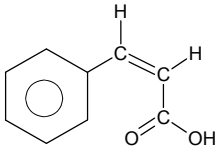
4-Hydroxyperinaphthenone



4-Phenanthroic acid



Phthalic acid



Cinnamic acid

microorganisms instead of the inoculant *Mycobacterium* species.

In hydrocarbon contaminated soils collected from abandoned coal gasification plants, Grosser *et al*. (1991) isolated *Mycobacterium* sp. strain RJGII-135, which could mineralize 10 to 48 % of pyrene in soil as sole carbon source in 60 days. Enhanced mineralization of pyrene to a level of 55 % within 2 days, compared with a level of 1 % for the indigenous population, was demonstrated by RJG11-135 after its reintroduction into soil following growth in pure culture on pyrene. The study also showed that mineralization of PAH was not dependent on high levels of microbial biomass and activity in the soils. The presence of a gene pool for degradation of recalcitrant compound(s) is of more significant importance than concentration of cells to be used for mineralization experiments. Herbes (1981) and Grosser *et al*., (1991) proposed that continuous but controlled discharge of PAHs in soil helps enrich the native PAH degrading population. Increased inputs of PAHs in sediments in the vicinity of a coal coking wastewater discharge increased the ability within the native microbial community to utilize certain PAHs (Herbes, 1981). The strain RJGII-135 was also capable of cometabolic benz[*a*]anthracene and benzo[*a*]pyrene metabolism (Schneider *et al*., 1996). Degradation studies with pyrene showed three metabolites formed by *Mycobacterium* sp. RJGII-135, including 4,5-phenanthrene-dicarboxylic acid, not previously isolated, 4-phenenthrene-carboxylic acid and 4,5-pyrene-dihydrodiol (Schneider *et al*.,1996).

In another study the indigenous *Mycobacterium* community structures in soil samples taken from heavily contaminated and less contaminated areas at four different sites were compared (Cheung and Kinkle, 2001). The TGGE profiles of 16S rRNA genes obtained for *Mycobacterium* phylotypes from heavily contaminated soils were less diverse than those from less contaminated soils. Results also suggested that the addition of degradative strains may stimulate the bioremediation of contaminated sites. Comparison of the levels of pyrene mineralization observed and TGGE profiles obtained from (i) non-sterilized, uninoculated (i) non-sterilized, inoculated and (i) sterilized, inoculated soils suggested that competition with indigenous microbiota, besides toxicity of PAHs might be a significant factors affecting the degradative activity of strain RJGII-135. It was observed that pyrene-amended soils, inoculated with strain RJGII-135 and uninoculated, experienced both increases and decreases in the population sizes of the inoculated strain

and indigenous *Mycobacterium* populations during incubation (Cheung and Kinkle, 2001).

Walter *et al*. (1991) isolated bacterium *Rhodococcus* sp. strain UW1 from contaminated soil which was capable of utilizing pyrene and chrysene as sole sources of carbon and energy. The strain UW1 had a growth rate of 0.023 h^{-1} on pyrene and mineralized 72 % of 500 µg ml^{-1} pyrene within 2 weeks when grown at pH 7.0 and 30 ^{o}C. It showed a maximum pyrene utilization rate of 0.08 mg ml^{-1} day^{-1}, while growing with a doubling time of 30 h. From the GC-MS analysis, they proposed re-cyclization of the initial meta-ring fission product of pyrene to form a water-soluble metabolite with molecular formula of $C_{16}H_{10}O_4$. However, they were unable to determine the position (i.e., 1,2- or 4,5-) of initial ring cleavage reactions on pyrene. Bouchez *et al*. (1995) used six bacterial strains, including two *Rhodococcus* spp. capable of growth on pyrene and fluoranthene, to investigate the degradation of PAHs in binary mixtures. All individual strains were capable of cometabolic degradation of PAHs, and inhibition and synergistic interactions were observed. Inhibition was most commonly observed when the added PAH was more water soluble than the PAH added originally. It was also observed that mineralization yields were higher and biomass yields were lower for HMW PAH-degrading bacteria than for LMW PAH-degrading bacteria (Bouchez *et al*., 1996).

Pyrene-utilizing *Mycobacterium gilvum* BB1 (DSM 9487) was isolated from another former coal gasification site by Boldrin *et al*. (1993). Exponential growth with solid pyrene and fluoranthene, as the sole sources of carbon and energy and at the rate of 0.056 and 0.040 h^{-1}, respectively, was obtained in fermentor cultures (Boldrin *et al*., 1993). *Mycobacterium* sp. BB1 was also used to examine the effects of various culture conditions on the biodegradation of PAHs, including the degradation of pyrene at low defined oxygen concentrations (Fritzsche, 1994), utilization of pyrene and fluoranthene in presence of non-ionic surfactants (Tiehm, 1994) and the utilization of PAHs in mixtures (Tiehm and Fritzsche, 1995). Protocols for enhancement of PAH degradative capacities in soil microcosm studies by the application of surfactants, so as to reduce the physicochemical barrier between the pollutant and the microorganisms, were followed by workers like Tiehm (1994), Thibault *et al*. (1996), Jimenez and Bartha (1996) and Volkering *et al*. (1998). Nonionic surfactants which were not utilized preferentially as growth substrates and which were not toxic to *Mycobacterium* sp. BB1 were found to

enhance the degradation of fluoranthene and pyrene (Tiehm, 1994). Thibault *et al*. (1996) isolated two pyrene-utilizing *Pseudomonas* strains B-24 and K-12 from contaminated soils and tested four surfactants for their effectiveness in desorbing pyrene and effect on degradation of pyrene in soil inoculated with pyrene degraders. It was observed that presence of surfactant increased mineralization of pyrene from 46 to 80 % under unsaturated conditions. However, soil slurries were most effective when pyrene degraders were inoculated without the surfactant, indicating that the surfactant had some biostatic properties inhibiting mineralization. Working with a *Mycobacterium* sp. soil isolate that utilized pyrene as a sole source of carbon and energy, Jimenez and Bartha (1996) demonstrated detergent- and solvent-augmented mineralization of pyrene. In untreated mineral salts solution, the isolate mineralized 50 % of a 250 µg ml^{-1} concentration of radiolabelled pyrene in 2-3 days. Detergent below the critical micelle concentration (CMC) increased the pyrene mineralization rate to 154 %, but above the CMC, the detergent severely inhibited pyrene mineralization. They also showed that microbial cells which physically adhered to hydrophobic solvent (paraffin oil, squalene, tridecylcyclohexane, and *cis*-9-tricosene at 0.8 %; v/v) droplets containing pyrene in mineral medium were capable of mineralizing pyrene 8.5 times faster than suspended cells in the aqueous phase.

In soil screenings for PAH-degrading bacteria, *Gordona* sp. strain BP9 and *Mycobacterium* sp. strain VF1 were isolated from soils contaminated with fuel or coal-tar oil and were found to be capable of growing on a solidified medium sprayed with pyrene as the sole carbon source (Kastner *et al*., 1994). Reintroduction of BP9 into soil, after growth on 200 µg ml^{-1} of pyrene in a pure culture, showed a sixfold increase in pyrene metabolism compared to native uninoculated soil (Kastner *et al*., 1998). Dean-Ross and Cerniglia (1996) isolated *Mycobacterium flavescens* PYR-GCK (ATCC 700033) from polluted sediments, which had the highest reported pyrene mineralization efficiency. It mineralized 63 % of 50 µg ml^{-1} pyrene as sole source of carbon and energy within 24 h at a utilization rate of 0.56 µg ml^{-1} day^{-1} with a doubling time of 9.6 h. The bacterium could also mineralize phenanthrene and fluoranthene, besides pyrene, to carbon dioxide (Dean-Ross and Cerniglia, 1996). Metabolites produced during growth on pyrene were identified as 4,5-dihydroxy-4,5-dihydropyrene, 4-phenanthroic acid, phthalic acid and 4,5-phenanthrenedioic acid.

A *Mycobacterium* sp., strain KR2 which was able to utilise pyrene as sole source of carbon and energy was isolated from a PAH contaminated soil originating from the area of a former gaswork plant (Rehmann *et al*., 1998). The isolate metabolised up to 60 % of the pyrene added (0.5 mg ml^{-1}) within 8 days at 20 °C. *Cis*-4,5-pyrene dihydrodiol, 4,5-phenanthrene dicarboxylic acid, 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde, phthalic acid, and protocatechuic acid (3,4-dihydroxybenzoic acid) were identified as degradation products. *Mycobacterium* sp. strain CH1 isolated from PAH-contaminated freshwater sediments could utilize pyrene as the sole carbon and energy source but with a lag phase of three days. The lag phase decreased to less than one day when it was grown in the presence of phenanthrene or fluoranthene (Churchill *et al.,* 1999). *Mycobacterium* sp. strain AP1 grew in mineral medium with pyrene as the sole source of carbon (Vila *et al*., 2001). After 6 days of growth, the bacterium decreased the amount of pyrene in the growth medium from 180 µg ml^{-1} to 50 µg ml^{-1} while producing, for the first time in literature, metabolite 6,6-dihydroxy-2,2-biphenyl dicarboxylic acid. Another isolate, *M. austroafricanum* strain, GTI-23, was found capable of utilizing a wide range of PAHs like phenanthrene, fluoranthene, pyrene, fluorene and benzo[*a*]pyrene (Bogan *et al*., 2003). It was isolated from manufactured gas plant site soil and showed extensive and rapid mineralization of 300 µg ml^{-1} pyrene as sole source of carbon and energy, in liquid cultures and pyrene-amended soil. Pizzul *et al*. (2007) reported isolation of an efficient PAH degrader from an agricultural soil, designated as *Mycobacterium* LP1. In liquid culture, it degraded phenanthrene (58 %), pyrene (24 %), anthracene (21 %) and benzo[*a*]pyrene (10 %) present in mixture (initial concentration 50 µg ml^{-1} each) and phenanthrene (92 %) and pyrene (94 %) as sole carbon sources after 14 days of incubation at 30 ^{o}C. In soil, *Mycobacterium* LP1 mineralised ^{14}C-phenanthrene (45 %) and ^{14}C-pyrene (65 %) after 10 days.

An alkaliphilic *Mycobacterium* sp. strain MHP-1 isolated from a soil sample, could utilize pyrene as sole carbon and energy source. At pH 9 it metabolized about 50 % of pyrene in 7 days. 4,5-phenanthrenedioic acid, 4-phenanthroic acid and phthalic acid were identified as metabolic intermediates (Habe *et al.,* 2004b). Pyrene-utilizing *Mycobacterium* sp. cultures growing at pH 2 to pH 3 have been enriched from extremely acidic environments using pyrene as sole carbon source (Uyttebroek *et al*., 2007). The bacterial community of the acidic soils was proved to be dominated by only one *Mycobacterium* species, which was highly related to *M. montefiorense* – related populations. Even enrichment at pH 7

from the acidic soils resulted in an *M. montefiorense* – related population (Uyttebroek *et al*., 2007). In contrast, the Mycobacterium community of the neutral soil was dominated by several *Mycobacterium* species (Leys *et al*., 2005).

Until the mid 90s it was suggested that nocardioform bacteria (e.g. *Rhodococcus*, *Nocardia*, *Mycobacteria* and *Gordona*) may play a crucial role in the degradation of high molecular weight PAHs like pyrene in soils. This contemplation (observation) was based on screening results by researchers for pyrene-degrading bacteria from soil samples (Heitkamp *et al*., 1988a; Grosser *et al*., 1991; Walter *et al*., 1991; Boldrin *et al*., 1993; Kastner *et al*., 1994; Dean-Ross and Cerniglia, 1996; Jimenez and Bartha, 1996) and on the observation that there were very few reports of bacteria capable of growing on the high molecular weight PAHs which are outside the nocardioform actinomycetes group (Thibault *et al*., 1996). Though the importance of nocardioform bacteria in the degradation of four-ringed PAHs (especially pyrene), with respect to understanding of the process of pyrene degradation, cannot be challenged to date, the works of Trzesicka-Mlynarz and Ward (1995); Juhasz *et al.* (1997); Geiselbrecht *et al*. (1998); Kazunga and Aitken (2000); Boonchan *et al*. (2000); Daane *et al*. (2001); Gauthier *et al*. (2003); Sarma *et al*. (2004); Das and Mukherjee, (2007); Obayori *et al*. (2008) [discussed below] indicate that the microbial degradation of high molecular weight PAHs is not restricted to the actinomycetes group.

Three *Burkholderia cepacia* strains isolated from PAH-contaminated soils by Juhasz *et al*. (1997) and reclassified as *Stenotrophomonas maltophilia* on the basis of 16S rRNA gene analysis (Juhasz, 1998) were capable of utilizing pyrene as a sole carbon and energy source. All three cultures showed growth and pyrene degradation at concentrations up to 1000 µg ml^{-1} demonstrating their high tolerance of pyrene compound; however the proportion of pyrene degraded declined with increasing pyrene concentrations (250, 500 and 1000 µg ml^{-1}). An initial concentration of 250 µg ml^{-1} of pyrene was degraded to undetectable levels within 7-10 days. Thereafter pyrene degradation ceased when ~400 µg ml^{-1} of the initial pyrene in all three cultures had been utilized, probably due to toxicity of the intermediates formed. The three pyrene-utilizing, Gram-negative *S. maltophila* isolates were also capable of slowly degrading fluoranthene, benz[*a*]anthracene and dibenz[*a*,*h*]anthracene, demonstrating their high tolerance of high molecular weight PAHs (Juhasz *et al*., 2000). It was observed that growth on these

compounds was not possible without prior exposure to pyrene, or some other easily metabolizable PAH. A similar behavior was observed in *Mycobacterium* sp. RJGII-135 by Grosser *et al*. (1991). In *Mycobacterium* sp. B1, the presence of pyrene is responsible for benzo[*a*]pyrene degradation (Gauthier *et al*., 2003). The results suggested that catabolic operons or enzymes involved in high molecular weight PAH degradation are induced only when a particular metabolite or substrate is present. It may be possible that pyrene, or metabolites produced during its degradation, induced the synthesis of enzymes necessary for the high molecular weight PAH catabolism. This argument agreed with earlier suggestions by Heitkamp and Cerniglia (1989) that the production of degradable PAH metabolites by a *Mycobacterium* sp. may be important for enhancing the degradation of PAHs in the environment. Later Juhasz (1998) reclassified the *Burkholderia cepacia* strain VUN 10,003 as *Stenotrophomonas maltophilia* on the basis of 16S rRNA gene analysis.

Pseudomonas putida, *P. aeruginosa* and *Flavobacterium* sp. were isolated from a soil-derived mixed culture which was capable of metabolizing pyrene and fluoranthene when supplemented with other forms of organic carbon (Trzesicka-Mlynarz and Ward, 1995). These strains, when recombined into a mixed culture, were found to degrade PAHs in a fashion similar to that of the original culture. Cometabolism of pyrene has been observed in *Pseudomonas saccharophila* (Chen and Aitken, 1999). *Pseudomonas aeruginosa* present indigenously in pyrene aged soils, produced rhamnolipids biosurfactants and showed substantial degradation of pyrene and phenanthrene (Hwang and Cutright, 2002; Bordas *et al*. 2005). Recently Obayori *et al*. (2008) have isolated pyrene degrading *Pseudomonas* sp. LP1, LP5 and LP6 strains from the oil-polluted sub-Saharan soils, capable of utilizing pyrene as sole source of carbon and energy. *Cycloclasticus* strains isolated from marine sediments were capable of partially degrading 1 µg ml^{-1} pyrene or fluoranthene cometabolically when provided with 10 µg ml^{-1} phenanthrene (Geiselbrecht *et al*., 1998). Wang *et al*. (2008) also isolated an efficient pyrene-degrading consortium from the Pacific Ocean sediment, in which Cycloclasticus bacterium played a key role. Bacterial strains *Rhodococcus opacus* (HCCS), *Paracoccus versutus* (SPNT), *Sphingomonas* sp. (MWFG) were isolated from the consortia enriched from sediments in seven mangrove swamps in Hong Kong SAR (Guo *et al*., 2005). The total concentrations of 16 PAHs in these sediments ranged from 169 to 1058 ng g^{-1}. The isolates were capable of using pyrene (and other four-ring PAHs) as the sole carbon and energy source. Gram-

negative *Paracoccus versutus* (SPNT) was the most effective isolate and able to degrade 10 µg ml^{-1} pyrene completely within 10 days incubation, though with a lag phase of around 2 days, while strains *Rhodococcus opacus* (HCCS) and *Sphingomonas* sp. (MWFG) showed only 20 % pyrene degradation. A second *Paracoccus* strain, designated as Ophe1, was isolated from Greek soils contaminated with PAH-containing waste from the oil refinery industry by Zhang *et al*. (2004). It exhibited a very broad substrate profile, being able to utilize anthracene, phenanthrene, fluorene, fluoranthene, chrysene, and pyrene, as well as cresol compounds and *n*-alkanes as sole carbon source.

Aitken *et al*. (1998) described the broad PAH substrate ranges of 11 bacteria isolated from PAH-contaminated soils by enrichment on phenanthrene as a sole carbon source. None of these organisms was capable of mineralizing pyrene, yet all could remove pyrene from solution. Out of these 11, 4 strains, namely *Pseudomonas stutzeri* strain P16, *Bacillus cereus* strain P21, *Sphingomonas yanoikuyae* strain R1 and *Pseudomonas saccharophila* strain P15 transformed pyrene to non-mineral products, *cis*-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione. It was suggested that the first intermediate, *cis*-4,5-dihydro-4,5-dihydroxypyrene, was oxidized to 4,5-dihydroxypyrene, which subsequently auto-oxidized to pyrene-4,5-dione. At their aqueous saturation concentrations, both *cis*-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione had the potential to accumulate in PAH-contaminated systems and severely inhibit phenanthrene and benzo[*a*]pyrene mineralization by strains P15 and R1 (Kazunga and Aitken, 2000). Therefore such products could significantly influence the removal of other PAHs in the soil system (Kazunga and Aitken, 2000).

Boonchan *et al*. (2000) attempted to degrade four and five-benzene ringed PAHs using a bacterial fungal co-culture of *Stenotrophomonas maltophilia* VUN 10,010 and *Penicillium janthinellum* VUO 10,201. The axenic bacterial culture could substantially grow on pyrene as sole carbon source; however growth on benzo[*a*]pyrene was observed only when pyrene (250 µg ml^{-1}) was added to the basal salt medium. When basal salt medium containing pyrene was co incubated with bacterial-fungal co-culture, a higher rate of pyrene mineralization and fungal biomass yield was achieved as compared to when *S. maltophilia* VUN 10,010 and *P. janthinellum* VUO 10,201 were individually inoculated. A mutual dependence under these conditions was suggested. Later Dandie *et al*. (2004) suggested that VUN 10,010 is a mixed culture containing at least two strains

(*S. maltophilia* and *Mycobacterium* sp. 1B) that are involved in PAH degradation. Daane *et al*. (2001) have determined pyrene degrading capacities in rhizosphere-associated phenanthrene-degrading *Paenibacillus* strain PR-P1 and *Arthrobacter* sp. strain PR-P3, isolated from salt marsh ecosystems. *Paenibacillus* sp. is an endospore forming bacteria, while *Arthrobacter* sp. was non-spore forming bacteria. Both were able to survive the adverse conditions at contaminated site. Seven novel isolates, namely *Pusillimonas* sp., *Alcaligenes* sp., *Mycobacterium* sp., *Pseudomonas* sp., *Parvibaculum* sp., *Stappia* sp., and *Microbacterium* sp. were identified as key groups of PAH-degrading strains present in contaminated aquatic sediments by enrichment techniques (Hilyard *et al*., 2008). In addition, PAH-degrading strains belonging to *Mycobacterium* and *Novosphingobium* groups were also represented from same source by molecular analysis. These isolates were capable of mineralizing phenanthrene and fluoranthene, besides pyrene.

Gauthier *et al*. (2003) characterized four high molecular weight (HMW) PAH-degrading bacteria, *Mycobacterium gilvum* B1, *Bacillus pumilus* B44, *Microbacterium esteraromaticum* B21, and *Porphyrobacter* B51, the latter two isolates were newly reported, from an enriched microbial consortium (Villemur *et al*., 2000) degrading HMW PAHs (pyrene, chrysene, benzo[a]pyrene and perylene) in soil slurry. Isolates B1 and B51 showed complete degradation of 113 µg ml^{-1} pyrene in 7–24 days while B21 degraded approximately 50 % of pyrene in 12 days. However, after 12 days the pyrene degradation rate slowed. Isolate B44 was the least effective with 10–20 % degradation of pyrene. In contrast, strains of *B. pumilus*, *B. subtilis*, *Micrococcus luteus*, *Alcaligenes faecalis* and *Enterobacter* sp., isolated from solid waste oil samples, were characterized (Toledo *et al*., 2006) with capacities to remove PAHs (naphthalene, phenanthrene, fluoranthene or pyrene) as sole carbon and energy source, and also to produce biosurfactants. In their experiments Toledo *et al*. (2006) showed that *Bacillus* strains grew better on PAHs compared with strains of *Micrococcus*, *Alcaligenes* and *Enterobacter*. Pyrene supported growth of five *B. pumilus* strains.

Bacillus subtilis, isolated from PAH contaminated soil and identified by fatty acid methyl ester (FAME) analysis, was adjudged as a potential degrader of pyrene and benzo[a]pyrene by Hunter *et al*. (2005). It was found that after a four-day incubation period at 30 ^{o}C in 20 µg ml^{-1} pyrene or benzo[*a*]pyrene, *B. subtilis* was able to transform

approximately 40 % and 50 % pyrene and benzo[*a*]pyrene, respectively. Another member of *Bacillus* genera, *B. cereus*, was isolated on anthracene from petrochemical sludge contaminated soil for its PAH (pyrene, anthracene and phenanthrene) degrading property and emulsification capacity (Jacques *et al*., 2007). The isolate could utilize pyrene in monoculture and also in mixed culture with *Mycobacterium fortuitum*, *Microbacterium* sp., *Gordonia polyisoprenivorans*, two *Microbacteriaceae* bacteria, and a fungus identified as *Fusarium oxysporum*, isolated from the same source. Lin and Cai, (2008) suggested that mangrove sediments worldwide are huge banks for PAH biodegraders. They isolated pyrene degrading *Bacillus cereus* and *Bacillus megaterium* strains from a PAH-degrading microbial consortium enriched from the sediment samples of Huian mangroves. Pyrene-degrading plasmids were extracted from the consortium and used to transform *Escherichia coli* DH5a cells. Within three weeks 85.7 % of the original pyrene concentration (50 mg l^{-1}) was degraded by cloned cells, while only 2.0 % degradation was observed by the normal *E. coli* cells.

A new enteric bacterium, identified as *Leclercia adecarboxylata* strain PS4040, was isolated from subsurface soils collected from an oily-sludge storage pit at the Digboi oil refinery**, in the northeastern region of India** by Sarma *et al*. (2004). The site has been exposed to petroleum hydrocarbons for more than 100 years. This rod-shaped, motile, Gram-negative bacterium can grow in minimal growth medium using pyrene as a sole source of carbon at 30 ^{o}C. The concentration of pyrene in the culture decreased from 200 to 77 µg ml^{-1}, indicating 62 % degradation of pyrene by strain PS4040, in 20 days. The strain PS4040 was also capable of degradation of catechol, naphthalene, fluorene, and fluoranthene (73, 53, 41, and 48 %, respectively) under similar growth conditions, except for anthracene which did not support growth. Das and Mukherjee (2007) isolated *Bacillus subtilis* DM-04 and *Pseudomonas aeruginosa* (M) and nonmucoid (NM) strains from a petroleum-contaminated soil sample of **north-east India**. Both strains were capable of secreting biosurfactant in the culture medium while growing on pyrene and their pyrene (2.5 mg ml^{-1}) utilizing efficiency was 48 % and 32 %, respectively, after 96 hrs of growth. Further, the biosurfactants produced by the bacteria under study were capable of enhancing the solubility of pyrene in aqueous media and can influence the cell surface hydrophobicity of the biosurfactant-producing strains that results in a higher uptake of pyrene.

In order to understand the processes contributing to pyrene (and other PAHs) degradation Stringfellow and Alvarez-Cohen (1999) investigated the relationship between biodegradation and biosorption of PAHs phenanthrene, pyrene and fluoranthene to activated sludge bacterial biomass. They found that PAH biosorption varied with bacterial genus and species. Bacteria with the highest sorption capacity (K_p) belong to the Nocardioforms, and PAH sequestration by high K_p, non-degraders had a significant impact on PAH biodegradation. It was suggested that although biosorption can decrease the rate of PAH biodegradation in the short term, it can also result in the removal of PAHs from the wastewater and PAH retention in the treatment system where it may be ultimately biodegradaed.

Low bioavailability of PAHs at ambient temperatures may be the reason for slow degradation. Extreme thermophilic pyrene degradation by mixed and pure cultures of aerobic, moderately thermophilic bacteria, *Bacillus* spp. and *Thermus* spp. at 65 ^{o}C was reported by Feitkenhauer *et al*. (2003). *Thermus brockii* was observed to metabolize up to 40 mg l^{-1} h^{-1} pyrene as the sole carbon and energy source in a 2-liter stirred bioreactor at 70 ^{o}C. High temperature hydrocarbon degradation is a very promising, inexpensive biodegradation technique for treating industrial effluents that are often discharged at 50-130 ^{o}C, with temperatures frequently exceeding 80 ^{o}C.

The pH of culture media and buffers may also influence the rate of pyrene degradation in bacteria (Kim *et al*., 2003, 2004b). pH affected the levels of the two constitutive PAH o-quinone reductases in pyrene-degrading *Mycobacterium* sp. strain PYR100 (Kim *et al*., 2003), and *M. vanbaalenii* PYR-1 also displayed similar enzyme activities (Kim *et al*., 2004b). In tryptic soy broth, *M. vanbaalenii* PYR-1 grew more rapidly at pH 7.5 ($\mu' = 0.058\ h^{-1}$) than at pH 6.5 ($\mu' = 0.028\ h^{-1}$). However, resting cells suspended in phosphate buffers with the same pH values displayed a shorter lag time for the degradation of pyrene (and phenanthrene) at pH 6.5 (6 h) than at pH 7.5 (48 h). The one-unit pH drop increased the degradation rates fourfold. An acidic condition (pH 6.5) seemed to render the mycobacterial cells more permeable to hydrophobic molecules of pyrene (and phenanthrene), resulting in higher substrate concentrations in the cytosol (Kim *et al*., 2005). Rapid mineralization of radiolabelled pyrene (60 % in 17 h) at pH 3 by *Mycobacterium* sp., enriched from soil at pH 7, was observed by Uyttebroek *et al*. (2007). Similarly, Grosser *et al*. (1991) observed that a pyrene-degrading *Mycobacterium* sp. in

acidic soil (pH 4.4) mineralized pyrene more rapidly without a significant lag time, compared with that in slightly alkaline soil (pH 7.8). Pyrene degradation in historically petroleum contaminated soil was limited by inorganic nutrients, as shown by Cheung and Kinkle (2005a). Soil amendments with nutrients (KNO_3 and KH_2PO_4) enhanced pyrene mineralization.

Mycobacteria are known to possess extremely lipophilic cell surfaces (Bogan *et al*., 2003), which may make them better suited to the direct uptake of highly hydrophobic hydrocarbons, including pyrene and other high-molecular weight PAHs (Kelley and Cerniglia, 1995; Schneider *et al*., 1996) and highly branched aliphatic hydrocarbons (Solano-Serena *et al*., 2000). Hydrophobicity of *Mycobacterium* sp. strains PYR-1, ATCC 33464, GTI-23 was considerably higher than various strains of PAH-degrading soil bacteria (*Acidovorax*, *Burkholderia*, *Pseudomonas* and *Sphingomonas*). *Mycobacterium* sp. strain PYR-1 had approximately the same hydrophobicity value as strain GTI-23 (52 Vs 48 %) isolated from manufactured gas plant site soil. The suitability of *M. vanbaalenii* PYR-1 and *M. austroafricanum* GTI-23 for growth on pyrene and its degradation was attributed to the property of high cell wall hydrophobicity of these bacteria (Bogan *et al*., 2003). Furthermore, the authors observed *M. austroafricanum* strain GTI-23 capable of utilizing a wide range of PAHs like phenanthrene, fluoranthene, pyrene, fluorene and benzo[*a*]pyrene, similar to *Mycobacterium* sp. PYR-1. Bastiaens *et al*. (2000) using hydrophobic membranes containing sorbed PAHs, have enriched and recovered PAH-degrading bacteria exclusively belonging to *Mycobacterium* spp. from PAH-contaminated soil and sludge samples while, in liquid enrichment cultures, mainly *Sphingomonas* sp. were isolated. The ability of members of this group to degrade PAH could be explained by the hydrophobicity of the cell surfaces, which allows the close contact with or adhesion to low water soluble or lipophilic molecules and which favours the uptake process (Bastiaens *et al*., 2000).

With an aim to better understand the microbial affinity for, ability to grow on, or to biotransform PAHs at the interface or in the aqueous phase in a two-liquid-phase (TLP) culture system (Deziel *et al*., 1999), Gauthier *et al*. (2003) grew three bacterial strains in TLP cultures using silicone oil as the organic phase. *Porphyrobacter* B51 isolate was found to grow strongly in the interfacial fraction in the presence of naphthalene vapours and phenanthrene compared with cultures without LMW PAHs. The growth of isolates

Mycobacterium gilvum B1 and *Microbacterium esteraromaticum* B21 was better in the aqueous phase than in the interfacial fraction for cultures with naphthalene vapours. The effect of naphthalene on the growth and pyrene degradation performance of isolate B21, in TLP cultures was also determined. B21 cultured in presence of naphthalene showed little degradation (10 - 15 %) in contrast to cultures without naphthalene which showed more than 80 % degradation. Presence of LMW PAH - naphthalene vapours had a stimulatory effect on isolate growth in TLP cultures but did not stimulate the HMW PAH degradation. This could be the result of inhibition of the induction of HMW PAH-degrading enzymes, competitive inhibition of the PAH-transport system or preferential degradation of the LMW PAH by the microorganisms.

2.8.1.1 Molecular characterization of pyrene degradation in bacteria:

Several different species of bacteria have the ability to degrade pyrene and high molecular weight PAHs (Kanaly and Harayama, 2000). However, Gram-positive bacteria play more important roles than Gram-negative isolates in environmental degradation of high molecular weight PAHs, especially in degradation of the four-ring compound pyrene (Heitkamp *et al*., 1988a, b; Walter *et al*., 1991; Grosser *et al*., 1991; Boldrin *et al*., 1993; Dean-Ross and Cerniglia, 1996; Schneider *et al*., 1996; Rehmann *et al*., 1998; Churchill *et al*., 1999; Vila *et al*., 2001; Kim *et al*., 2003; Bogan *et al*., 2003; Habe *et al*., 2004b; Liang *et al*., 2006; Uyttebroek *et al*., 2007; Das and Mukherjee, 2007). Most of our knowledge on the genetics of pyrene degradation comes from molecular studies in the past ten years on actinomycetes bacteria, *Mycobacterium* and *Rhodococcus* strains (Wang *et al*., 2000; Khan *et al*., 2001; Brezna *et al*., 2003; Krivobok *et al*., 2003; Sho *et al*., 2004; Stingley *et al*., 2004a, b; Kim *et al*., 2004a, b, 2007, 2008).

Mycobacterium sp. strain PYR-1 has been shown to mineralize anthracene, fluoranthene, **pyrene**, 1-nitropyrene, phenanthrene, and benzo[*a*]pyrene (Heitkamp *et al*., 1988a, b; Heitkamp and Cerniglia, 1989; Kelley and Cerniglia, 1995). It is known to have an inducible system for PAH degradation (Heitkamp *et al*., 1988a). Studies indicate that homology between mycobacterial genes for PAH-degrading enzymes and those known from other genera (e.g. the *nah* operon) is apparently very low (Churchill *et al*., 1999). Proteomics has been used to identify PAH-catabolic genes in strain PYR-1 (Wang *et al*., 2000; Khan *et al*., 2001; Kim *et al*., 2004a). At least six major proteins were found to be over expressed in *Mycobacterium* cells after phenanthrene and pyrene exposure (Wang *et*

al., 2000; Khan *et al*., 2001). The first pyrene-inducible gene, designated *katG*, was cloned and characterized from *Mycobacterium* sp. strain PYR-1 by Wang *et al*. (2000). The 81-kDa catalase-peroxidase (KatG) protein, recovered during 2 - 8 hrs of induction with pyrene, was sequenced using a gene-walking technique. The deduced *katG* sequence showed significant homology to *katGII* of *M. fortuitum* and clustered with catalase-peroxidase proteins from other *Mycobacterium* species in a phylogenetic tree. The *katG* gene was expressed in *Escherichia coli* to produce a protein with catalase-peroxidase activity. Since the induction of this catalase-peroxidase occurred in pyrene-induced cultures and the exposure of these cultures to hydrogen peroxide reduced pyrene metabolism, Wang *et al*. (2000) suggested that the catalase-peroxidase (KatG) enzyme played an important role in preserving optimal pyrene metabolism by strain PYR-1 and/or its cell viability.

The second major pyrene-induced protein isolated from *Mycobacterium* sp. PYR-1 culture had a molecular mass of 50 kDa and was identified by N-terminal sequencing as a dioxygenase (Khan *et al*., 2001). From the genomic library of strain PYR-1 three dioxygenase-positive clones, each containing the same 5,288 bp DNA insert with three genes of the dioxygenase system was obtained. Three open reading frames (ORFs), exhibiting homology to those encoding polypeptides from several multicomponent dioxygenases, were identified. Based on sequence homology, predicted polypeptides from the three ORFs were designated naphthalene-inducible dioxygenase (iron-sulfur protein, large [α]-subunit), aromatic dioxygenase (iron-sulfur protein, small [β]-subunit), and aldehyde dehydrogenase; corresponding gene designations were *nidA* (1,461 bp ORF), *nidB* (510 bp ORF), and *nidD* (1,368 bp ORF), respectively as shown in Figure 1.4. The N-terminal amino acid sequence of the 50 kDa protein (12 residues [TTETTGTADATD]) found at the start of the *nidA* gene product, indicated that the 50 kDa PAH-induced protein was the *nidA* (naphthalene-inducible dioxygenase large subunit) gene product. Translation of all three gene locations, in the genetic order *nidDBA*, and calculated molecular mass values of the predicted polypeptides are shown in Figure 1.4. Analysis of the translated ORF of the large subunit showed a Rieske center iron-sulfur binding site, having highly conserved consensus sequence $CXHRGX_8GNX_5CXYHG$. Four histidines and three tyrosines near the middle of the polypeptide may contribute to a potential iron-binding site. Amino acid sequence of substrate binding site was found to be divergent, thus explaining the wide substrate range of PAHs that are degraded by *Mycobacterium* sp.

PYR-1. The DNA sequence of the large [α]- and small [β]-subunits showed a high degree of homology with known PAH ring-hydroxylating dioxygenases genes, *NidA* (42 %) and *NidB* (43 to 46 %) from *Rhodococcus* sp. I24 (Treadway *et al*., 1999) and *PhDA* (50 %) and *PhDB* (56 %) from *Nocardioides* sp. KP7 (Saito *et al*., 2000). The *nidA* and *nidB* genes sequence was distantly related (less than 30 %) to classic bacterial ring-hydroxylating genes such as the *nahAc*, *ndoB* (Kurkela *et al*., 1988) and *phnAc* (Laurie and Lloyd-Jones, 1999) genes from *Pseudomonas* and *Burkholderia* species. The *nidA* and *nidB* dioxygenase genes, successfully cloned, subcloned and overexpressed in *E. coli* with the pBAD/Thio Fusion vector system, from *Mycobacterium* sp. PYR-1 were responsible for initiation of the pyrene degradation pathway that proceeds through pyrene 4,5-dihydrodiol (Khan *et al*., 2001).

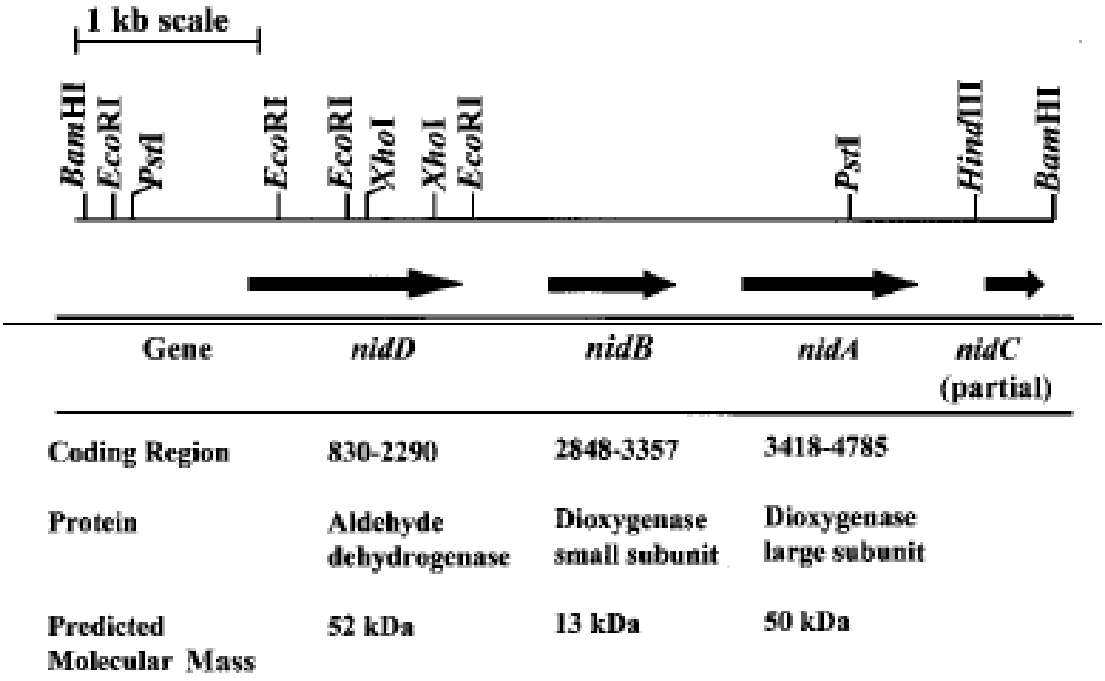


Figure 1.4 Restriction map, gene organization, and predicted *nidABD* gene products from the cloned 5,288 bp fragment from *Mycobacterium* sp. strain PYR-1 (Adapted from Khan *et al*., 2001).

Nine kb upstream of the *nidDBA* BamHI fragment, Stingley *et al*. (2004b) found a putative operon containing genes that encode enzymes involved in the degradation of phthalate. A putative regulatory protein gene (*phtR*), transcribed from the opposite strand,

is upstream of the putative operon. The operon consists of large (*phtAa*) and small (*phtAb*) dioxygenase subunits, an unknown ORF (*phtU*), a dihydrodiol dehydrogenase (*phtB*), a dioxygenase ferredoxin subunit (*phtAc*) and a dioxygenase ferredoxin reductase (*phtAd*). Phthalate has been isolated as an intermediate metabolite in the degradation of pyrene, phenanthrene and fluoranthene in *M. vanbaalenii* PYR-1 (Heitkamp *et al*., 1988b; Kelley *et al*., 1993; Moody *et al*., 2001, 2002, 2003, 2004). *Mycobacterium vanbaalenii* PYR-1 *pht* operon gene products share 53 - 78 % identity and 66 - 88 % similarity with their counterparts in *Terrabacter* sp. DBF63 (Habe *et al*., 2003) and *A. keyseri* 12B (Eaton, 2001).

It was found that *NidA* and *NidB* genes in *Mycobacterium* sp. PYR-1 have conserved homologues in a number of *Mycobacterium* species that degrade PAHs (Brezna *et al*., 2003; Krivobok *et al*., 2003; Sho *et al*., 2004; Habe *et al*., 2004a, b). Southern DNA-DNA hybridization using *nidA* and *nidB* probes from PYR-1 revealed that there was more than one copy of *nidA* and *nidB* genes in *Mycobacterium vanbaalenii* PYR-1 (Khan *et al*., 2001), *M. gilvum* BB1 (Boldrin *et al*., 1993), *M. flavescens* PYR-GCK (Dean-Ross and Cerniglia, 1996) and *M. frederiksbergense* FAn9T (Willumsen *et al*., 2001). However, only one copy of each gene was observed in *Mycobacterium* sp. PAH2.135 (RJGII-135). *M. vanbaalenii* PYR-1 had at least three copies of the terminal dioxygenase gene (Brezna *et al*., 2003, Stingley *et al*., 2004a). Among them, *nidAB* and *phtAaAb* were shown to transform the PAHs phenanthrene and pyrene into their respective cis-dihydrodiols (Khan *et al*., 2001, Stingley *et al*., 2004a) and phthalate into phthalate cis-3,4-dihydrodiol (Stingley *et al*., 2004b), respectively. The fourth copy of terminal dioxygenase gene was cloned with the aid of PCR and PYR-1 genome library screening by Kim *et al*. (2004a, 2006). The substrate specificities for four-ring HMW PAHs pyrene and fluoranthene and transformation rates of the enzyme expressed in *E. coli* was examined by Kim *et al*. (2006). The *pht* operon is also conserved within PAH degrading *Mycobacterium* sp. PAH2.135 (RJGII-135), *M. flavescens* PYR-GCK (ATCC 700033), *M. gilvum* BB1 (DSM 9487), *M. frederiksbergense* FAn9 (DSM 44346), *Rhodococcus* sp. (Dean-Ross *et al*., 2001) and *M. austroafricanum* GT1-23.

Pyrene-induced proteins in *Mycobacterium* sp. 6PY1 protein extracts were isolated and identified by Krivobok *et al*. (2003). The bacterium was grown on pyrene or alternate carbon sources (acetate, benzoate, and phenanthrene) and the respective soluble protein

extract profiles were analyzed by two-dimensional gel electrophoresis. A total of 16 distinct polypeptides appeared to be specifically synthesized in pyrene grown cells of strain 6PY1, two polypeptides were absent in phenanthrene-induced cells, five of sixteen polypeptides were missing in benzoate-grown cells while none of the above polypeptides were expressed in acetate grown cells. The three findings – first, most of the pyrene-specific polypeptides were also detected upon induction with phenanthrene; second, phenanthrene-grown cells found to be as active as pyrene-grown cells in mineralizing pyrene; and third, a majority of the pyrene-specific proteins found in strain 6PY1 were similar in sequence to enzymes responsible for phenanthrene degradation in *Nocardioides* sp. KP7 (see below) suggested that the same enzymes were involved in the catabolism of the two PAHs (Krivobok *et al*., 2003). Likewise, Molina *et al*. (1999) observed that when *Mycobacterium* strains were exposed to phenanthrene, de novo protein synthesis was not required for rapid mineralization of pyrene.

Pyrene-induced catabolic enzymes (designated P) were tentatively identified by peptide sequence analysis as hydratase-aldolase (P15), aldehyde dehydrogenase (P22), *trans*-2-carboxybenzalpyruvate hydratase-aldolase (P13), 1-hydroxy-2-naphthoate dioxygenase (P18), thiosulfate sulfur transferase (P12) and two distinct ring-hydroxylating dioxygenases (P6-P21, P8) as shown in **Table 1.9** (Krivobok *et al*., 2003). The first set of dioxygenase genes from 6PY1 were designated *pdoB1A1* or *pdo1* (**Figure 1.5**) and displayed high similarity to the *nidBA* genes from strain PYR-1 (Khan *et al*., 2001) as shown in **Table 1.9**. The second distinct dioxygenase gene system was designated *pdoA2B2* or *pdo2*; its component α, β-subunit showed 73 and 62 % similarity with the *phdA*, *phdB* genes of the phenanthrene dioxygenase from *Nocardioides* sp. KP7 (Saito *et al*., 2000). Upstream of *pdoA2*, a gene exhibiting 88% identity with an extradiol dioxygenase from *Nocardioides* sp. KP7 (*phdF* gene product) (Saito *et al*., 2000) was identified. The two dioxygenase gene systems from strain 6PY1 were cloned and overexpressed in *E. coli*. The terminal dioxygenase components from *Mycobacterium* sp. 6PY1 were found to be active in *E. coli* with the complementation of nonspecific ferredoxin and reductase components from the host. Enzyme Pdo1 was found to be overproduced in 6PY1 cells grown on benzoate, phenanthrene, or pyrene and absent in acetate-grown cells by immunoblot analysis of cell extracts. It could catalyze the dihydroxylation of both pyrene and phenanthrene. In contrast, Pdo2 could be detected

only in PAH-grown cells and preferentially oxidized phenanthrene. Therefore the two dioxygenases showed markedly different selectivities towards pyrene and phenanthrene.

TABLE 1.9 Sequence analyses of pyrene-induced polypeptides from *Mycobacterium* sp. 6PY1 and possible functions based on sequence similarities (Adapted from Krivobok *et al*., 2003).

Polypeptide (*Mr*)	Best database Match (Accession no.)	% Amino acid identity[B]	Possible function
P6 (19.0)	NidB (AF249302)	99	β Subunit of arene dioxygenase
P8 (21.0)	PhdB (AB031319)	62	β Subunit of arene dioxygenase
P12 (37.0)	ThtR (O05793)	89	Thiosulfate sulfur transferase
P13 (37.2)	PhdJ (D89988)	58	*trans*-2-Carboxylbenzalpyruvate hydratase-aldolase
P15 (39.0)	PhdG (AB031319)	72	Hydratase-aldolase
P18 (45.4)	PhdI (AB000735)	40	1-Hydroxy-2-naphthoate Dioxygenase
P21 (54.0)	NidA (AF249301)	98.5	α Subunit of arene dioxygenase
P22 (58.0)	PhdH (AB031319)	90	Aldehyde dehydrogenase

Under similar growth conditions, pyrene dioxygenase genes were also characterized from another member of the same genus *Mycobacterium* strain S65 (Sho *et al*., 2004). This pyrene (and phenanthrene, fluoranthene) degrading bacterium was isolated from jet-fuel contaminated airport soil. Growth on pyrene and phenanthrene induced two separate loci (Accession no. AF546905 and AF546904) encoding two large subunit ring-hydroxylating dioxygenases (designated *pdoA/X* and *nidA/X* gene products), two alcohol dehydrogenases (designated *pdoC/H* and *nidC/H* gene products), and one unknown orf (*orfP6* and *orfN4*). The *pdo* locus also included *pdoB*, which was predicted to function as a small subunit ring-hydroxylating dioxygenase. The large subunit of

both dioxygenases displayed 99 % and 89 % similarity to the *nidA* gene (Accession no. AF249301) from *Mycobacterium vanbaalenii* PYR-1 (Khan *et al*., 2001). The genetic

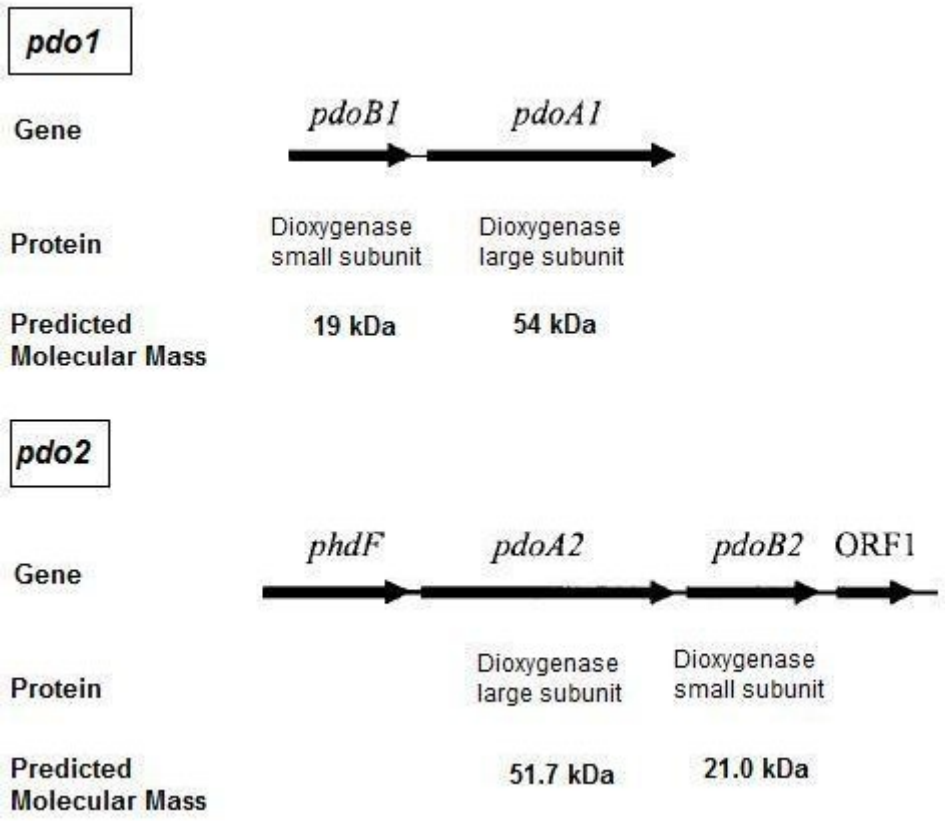


Figure 1.5 Map representing the two loci containing the dioxygenase encoding genes from *Mycobacterium* sp. 6PY1 (Adapted from Krivobok *et al*. 2003).

organizations of pyrene degradation genes in both loci and the functional description are shown in comparison to those in PYR-1 in figure 1.6. The possession of two distinct PAH-degrading gene clusters in strain 6PY1 (Krivobok *et al*., 2003) and S65 (Sho *et al*., 2004) serve to explain why mycobacteria enjoy advantage of more efficient pyrene degradation over other non-actinomycete bacteria and how mycobacteria develop the genetic abilities to degrade pyrene and other high-molecular-weight PAHs. *Mycobacterium* sp. MHP-1 was found to possess aromatic-ring dioxygenase genes, which are highly homologous to *nidAB* genes from pyrene-degrading neutrophilic *Mycobacterium* sp. PYR-1 (Habe *et al.,* 2004b).

Finally, an integrated omics approach using metabolomic, genomic, and whole-cell proteomic analyses was undertaken by Kim *et al*. (2007, 2008) to comprehensively

elucidate the pyrene metabolism in *M. vanbaalenii* PYR-1 and establish a basis for the understanding of dynamic aspects of cellular processes involved in PAH degradation. Many genome-predicted proteins were identified, and more detailed roles were suggested with respect to the degradation of pyrene. Based on genomic and proteomic data, 27 enzymes were unambiguously identified necessary for constructing a complete pathway for pyrene degradation; genes/ORFs were assigned to each enzymatic step required for the pathway as shown in figure 1.7 (colored arrows) and 1.8. The bacterium degrades pyrene to central intermediates through *o*-phthalate and the β-ketoadipate pathway. Fourteen proteins are responsible for the degradation of pyrene to phthalate, six proteins are responsible for the degradation of phthalate to protocatechuate, seven proteins are responsible for the lower pathway from protocatechuate to acetyl coenzyme A (acetyl-CoA) and succinyl-CoA, and three proteins are responsible for the transformation of pyrene to 1,2-dimethoxypyrene (Figure 1.7). Proteomic analysis also revealed that 18 enzymes in the pathway were upregulated more than twofold, when the organism was grown with pyrene; three copies of the terminal subunits of ring-hydroxylating oxygenase (NidAB2, MvanDraft_0817/0818, and PhtAaAb), dihydrodiol dehydrogenase (MvanDraft_0815), and ring cleavage dioxygenase (MvanDraft_3242) were detected only in pyrene-grown cells.

2.8.1.2 Chemical characterization pathway for pyrene

Bacterial degradation of PAHs under aerobic conditions begins with oxidation of the aromatic ring, catalyzed by dioxygenases (Mueller *et al*., 1996; Kim *et al*., 2004a). In this reaction, both atoms of molecular oxygen are incorporated into the PAH to form cis-dihydrodiol metabolites. Aromatic ring-hydroxylating dioxygenases are multicomponent enzyme systems consisting of an electron transport chain and a terminal dioxygenase (Mason and Cammack, 1992). The terminal dioxygenase is composed of large (α) and small (β) subunits (Mason and Cammack, 1992; Kauppi *et al*., 1998; Khan *et al*., 2001; Brezna *et al*., 2003). The α subunit is the catalytic component and contains two conserved regions: the [Fe_2-S_2] Rieske center and the mononuclear iron binding domain, which are involved in the consecutive electron transfer to the dioxygenase molecule (Parales *et al*., 1999; Khan *et al*., 2001). Both α and β subunits are necessary for function and in determining the substrate specificity of the dioxygenase (Hurtubise *et al*., 1998).

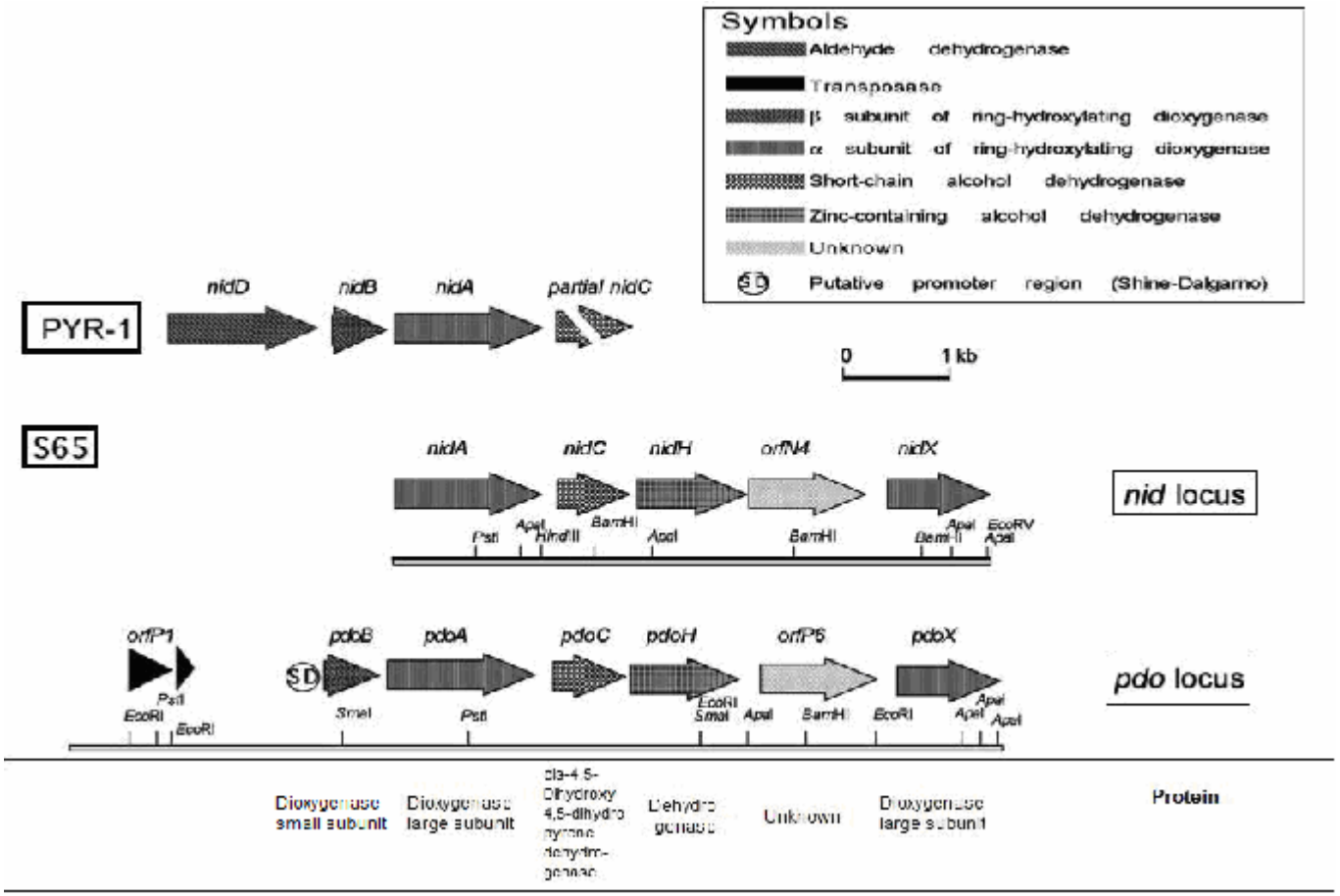
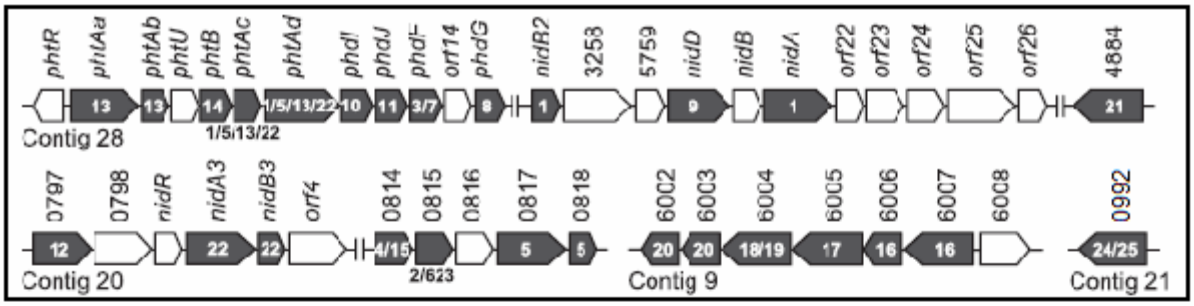


Figure 1.6 Genetic organization of the loci encompassing pyrene degradation genes identified in *Mycobacterium* sp. S65. The positions and orientations of the putative genes are shown by shaded arrows (Adapted from Sho *et al*., 2004).

Figure 1.7 Organization of the gene clusters involved in the catabolism of pyrene in *Mycobacterium vanbaalenii* PYR-1. Arrows represent genes/ORFs; shaded arrows represent gene products (proteins) involved in pyrene degradation; numbers within arrows correspond to enzymatic steps in pyrene degradation pathway (refer section 2.8.1.2. Figure 1.8); numbers above the arrows indicate the ORF locus tag numbers from the PYR-1 draft genome (http://img.jgi.doe.gov); vertical lines indicate that genes are not adjacent in the genome (Adapted from Kim *et al*., 2007).



ORF locus / Gene	Product	Reference
phtR	*phl* operon regulatory protein	Stingley et al. 2004b
3236/*phtAa*	Phthalate 3,4-dioxygenase, large [α] subunit	Stingley et al. 2004b, Kim et al. 2007
3237/*phtAb*	Phthalate 3,4-dioxygenase, small [β]subunit	Stingley et al. 2004b, Kim et al. 2007
3238/*phtB*	Phthalate 3,4-dihydrodiol dehydrogenase	Stingley et al. 2004b, Kim et al. 2007
phtAc	Phthalate dioxygenase, ferredoxin subunit	Stingley et al. 2004b, Kim et al. 2007
3239/*phtAd*	Phthalate dioxygenase, ferredoxin reductase	Stingley et al. 2004b, Kim et al. 2007
3240*phdI*	1-hydroxy-2-naphthoate dioxygenase	Kim et al. 2007
3241/*phdJ*	trans-2'-Carboxybenzalpyruvate hydratase-aldolase	Kim et al. 2007
3242/*phdF*	Ring cleavage dioxygenase	Kim et al. 2007
3244/*phdG*	Hydratase-aldolase	Kim et al. 2007
3257/*nidB2*	Pyrene/phenanthrene dioxygenase small [β] subunit	Khan et al., 2001, Kim et al. 2004a, Kim et al. 2007
3259/*nidD*	Aldehyde dehydrogenase	Khan et al., 2001, Kim et al. 2004a, Kim et al. 2007
3261/*nidA*	Pyrene/phenanthrene dioxygenase large [α] subunit	Khan et al., 2001, Kim et al. 2004a, Kim et al. 2007
4884	β-Ketoadipyl-CoA thiolase	Kim et al. 2007
0797	2-Carboxylbenzaldehyde dehydrogenase	Kim et al. 2007
nidR	Putative transcriptional regulator	Kim et al. 2006
0800/*nidA3*	Fluoranthene / pyrene dioxygenase, large subunit	Kweon et al. 2007/ Kim et al. 2006, Kim et al. 2007
0801/*nidB3*	Fluoranthene / pyrene dioxygenase, small subunit	Kweon et al. 2007/ Kim et al. 2006, Kim et al. 2007
orf4	alcohol dehydrogenase	Kim et al. 2006
0814	Decarboxylase	Kim et al. 2007
0815	Dihydrodiol dehydrogenase	Kim et al. 2007
0817/*pdoA2*	Phenanthrene ring-hydroxylating oxygenase, α subunit	Kim et al. 2007
0818/*pdoB2*	Phenanthrene ring-hydroxylating oxygenase, β subunit	Kim et al. 2007
6002	β-Ketoadipate succinyl-CoA transferase, β subunit	Kim et al. 2007
6003	β-Ketoadipate succinyl-CoA transferase, α subunit	Kim et al. 2007
6004	γ-Carboxymuconolactone decarboxylase/ β-Ketoadipate enol-lactone hydrolase	Kim et al. 2007
6005	β-Carboxy-cis,cis-muconate cycloisomerase	Kim et al. 2007
6006	Protocatechuate 3,4-dioxygenase, α subunit	Kim et al. 2007
6007	Protocatechuate 3,4-dioxygenase, β subunit	Kim et al. 2007
0992	Catechol *O*-methyltransferase	Kim et al. 2004c

Early studies on the catabolism of pyrene by a *Mycobacterium* sp. PYR-1 led to the identification of several ring oxidation products, including pyrene 4,5-dihydrodiol and 4-phenanthroic acid (Heitkamp *et al*., 1988b). Later studies conducted with other *Mycobacterium* strains identified pyrene-4,5-dione and phenanthrene 4,5-dicarboxylic acid as intermediate metabolites (Dean-Ross and Cerniglia, 1996; Schneider *et al*., 1996; Liang *et al*., 2006). Based on these findings, a pathway of pyrene degradation by *Mycobacterium* species was proposed which likely involves a dioxygenase for catalysis of the initial attack on the C4 and C5 positions, followed by dehydrogenation to produce 4,5-dihydroxy-pyrene (Figure 1.8). Dehydrogenation was followed either by *ortho* cleavage to give the first ring-cleaved product, phenanthrene 4,5-dicarboxylic acid (Heitkamp *et al*., 1988b; Dean-Ross and Cerniglia, 1996; Schneider *et al*., 1996; Vila *et al*., 2001) or pyrene-4,5-dione may be formed following the non-enzymatic autooxidation of 4,5-dihydroxy-pyrene (Kazunga and Aitken, 2000; Khan *et al*., 2001). Pyrene-4,5-dione may be reduced back to 4,5-dihydroxypyrene by quinone reductase (PQR) as reported for *Mycobacterium* sp. strain PYR100 and *M. vanbaalenii* PYR-1 (Kim *et al*., 2003, 2004b) or may be oxidized to phenanthrene-4,5-dicarboxylic acid by quinone oxidase as reported for *Mycobacterium* sp. KMS (Liang *et al*., 2006). Then 4,5-dicarboxyphenanthrene appeared to undergo further metabolism, either through a reductive decarboxylation reaction to produce 4-phenanthroic acid (Cerniglia, 1992), or through a substitution of the carboxyl group with hydroxide ion (Vila *et al*., 2001). The latter reaction could be followed by an oxidation reaction to produce 4-carboxy-5-hydroxyphenanthrene-9,10-dihydrodiol which could be further metabolized via 4-carboxyphenanthrene-5,9,10-triol and 2,6,6′-tricarboxy-2′-hydroxybiphenyl to 2,2′-dicarboxy-6,6′-dihydroxybiphenyl, as proposed by Vila *et al*. (2001). Alternatively dioxygenation reaction of 4-carboxyphenanthrene to form 4-carboxyphenanthrene-cis-3,4-dihydrodiol, which was preferably re-aromatized to eliminate the carboxyl group occupying a pseudoaxial position at the Bay-region (Rehmann *et al*., 1998) to give phenanthrene-cis-3,4-dihydrodiol. This intermediate was further transformed to 3,4-dihydroxyphenanthrene, which was subsequently acted upon by enzymes known to be involved in phenanthrene degradation (Cerniglia, 1992; Dean-Ross and Cerniglia, 1996; Rehmann *et al*., 1998; Krivobok *et al*., 2003). Cis-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid was formed through an extradiolic ring fission reaction of 3,4-dihydroxyphenanthrene resulting from

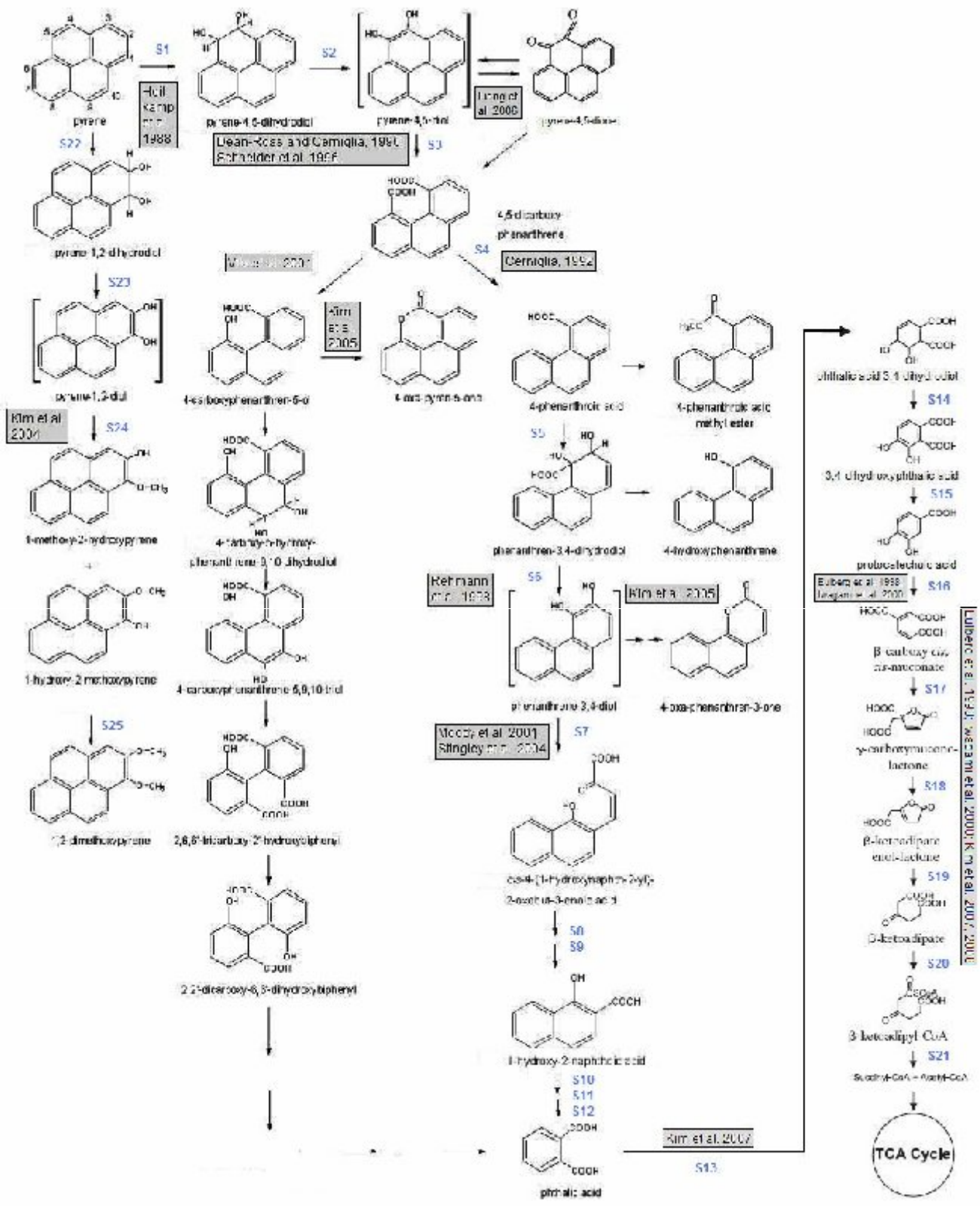


Figure 1.8 Proposed pathways for the degradation of pyrene by *M. vanbaalenii* PYR-1. The absolute configurations of pyrene-dihydrodiols are not included and the transient catechol intermediates are shown in brackets. S denotes step (Adapted and modified from Heitkamp *et al*., 1988b; Cerniglia, 1992; Dean-Ross and Cerniglia, 1996; Schneider *et al*., 1996; Rehmann *et al*., 1998; Vila *et al*., 2001; Moody *et al*., 2001; Krivobok *et al*., 2003; Stingley *et al*., 2004a, b; Kim *et al*., 2004b, 2005, 2007, 2008; Liang *et al*., 2006).

the Bay-region oxidation of phenanthrene (Moody *et al*., 2001; Stingley *et al*., 2004a). The flanking alkyl chain was further metabolized to produce 1-hydroxy-2-naphthoic acid (Kim *et al*., 2005) and successively phthalic acid. Phthalic acid was then degraded to protocatechuic acid (3,4-dihydroxybenzoic acid) (Stingley *et al*., 2004b; Liang *et al*., 2006; Kim *et al*., 2007, 2008).

Non-K-region PAH detoxification (i.e. catechol-O-methyltransferase (COMT)-mediated PAH catechol oxidation to produce monomethoxyhydroxy-PAHs and dimethoxy-PAHs) was reported in *Mycobacterium* sp. PYR-1 by Kim *et al*. (2004b). At pH 6.5, *M. vanbaalenii* PYR-1 produced higher levels of the *O*-methylated derivatives of non-K-region pyrene diols (hydroxymethoxypyrenes and 1,2-dimethoxypyrene) in the cytosol. Other non-K-region products, such as cis-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid, 1,2-dicarboxynaphthalene and two benzocoumarin-like compounds (4-oxa-pyren-5-one; Mw=220, 4-oxa-phenanthren-3-one; Mw=196), were also detected in the culture fluids (Kim *et al*., 2005). The non-K-region PAH oxidation at acidic pH might be a significant burden to the bacterial cell due to the accumulation of PAH substrates and their toxic metabolites in high concentrations in the cytosol.

Recently, Wang *et al*. (2008) have reported that the intermediates of pyrene degradation by *Cycloclasticus* sp. P1 are unusual in that they are different with those previously reported (Dean-Ross and Cerniglia, 1996; Kim *et al*., 2005). Based on the compounds identified by gas chromatography-mass spectrometry analysis and combined with the salicylate pathway, a novel catabolic pathway was proposed in nonactinomycetes *Cycloclasticus* sp. P1 bacterium (**Figure 1.9**). The first step probably starts from oxidation to form pyrene-4, 5-dihydrodiol, exclusively catalyzed by PAH dioxygenase enzyme system - *PhnA1A2A4A3* (Wang *et al*. 2008), then transformed to cyclopenta-(def)phenanthrenone (the main detected intermediate) via an unknown process, and then, transformed to a lactone which is inferred by the fluoranthene pathway (Lee *et al*., 2007). Subsequently, the lactone is transformed through some kind of ring cleavage to form 4-phenanthrenol (another detected intermediate), which enters the phenanthrene pathway via 3,4-dihydroxyphenanthrene; further degradation goes through salicylate to catechol. Phthalate, a metabolic intermediate of PAHs in previously described Mycobacterial studies, was not detected in the study of Wang *et al*. (2008). This fact was consistent with the observation that isolate P1 could not use phthalate for its growth.

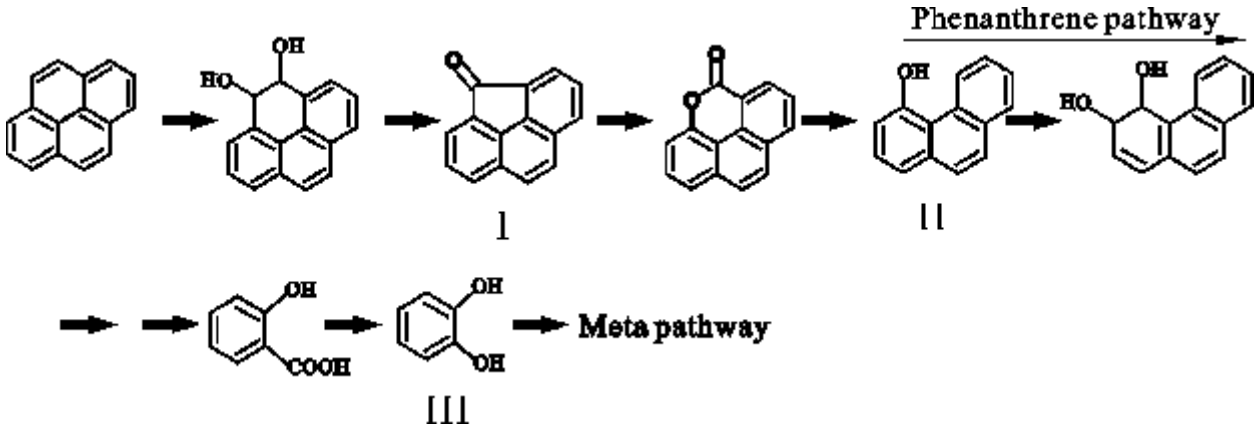


Figure 1.9 A proposed pathway of pyrene degradation by *Cycloclasticus* sp. P1. Intermediates: I, cyclopenta(def)phenanthrenone; II, 4-phenanthrenol; III, catechol (Adapted from Wang *et al*., 2008)

2.8.2 Fungal degradation

Fungal degradation of pyrene has also been investigated by some research workers. In der Wiesche *et al*. (1996) demonstrated degradation of pyrene when the white rot fungi *Dichomitus squalense* and *Pleurotus* sp. were grown in solid-state fermentations. The degradation was higher when experimental soil microorganisms were also added to this fermentation indicating synergistic degradation. The degradation by either individual fungi or soil microorganisms was found to be lower compared to that achieved by their mixture.

Lambert *et al*. (1994) isolated two basidiomycete, *Crinipillis stipitaria* strains JK364 and JK375, that cometabolized pyrene in the presence of yeast extract, malt extract and glucose. Under these conditions *C. stipitaria* JK375 could metabolize pyrene to 1-hydroxypyrene, 1,6-dihydroxy and 1,8-dihydroxy pyrene, whereas the second strain *C. stipitaria* JK364 could metabolize pyrene to 1-pyrenesulfate and 4,5-dihydro 4,5-dihydroxy pyrene. This study clearly demonstrated that the ability to degrade pyrene using a particular degradation pathway is strain specific. Lange *et al*. (1994) found that *Crinipellis* and some of the other related genera of basidiomycete fungi metabolize pyrene to yield different metabolic products.

Bezalel *et al*. (1996 a, b, c) demonstrated that *Pleurotus osteratus* could mineralize 0.4 % of radiolabeled pyrene cometabolically. In addition to pyrene, this fungus also mineralized 7 % of catechol, 3 % of phenanthrene, 0.19 % of benz[*a*]pyrene in 11 days. Although this fungal strain had lignolytic activity, there was no correlation between its activity and degradation of the PAHs.

Fungal co-metabolism of pyrene in the presence of 10 mg ml^{-1} of glucose by *Kuehneromyces mutabilis* was investigated by Sack *et al*. (1997a). In liquid culture *K. mutabilis* mineralized 1.4 % of pyrene, while another fungus, *Agrocybe aegerita*, could mineralize 3.3 % of pyrene. The intermediates purified from these fungi belonged to the K-region (4,5- carbon on pyrene) and the degradation products formed were strain specific. *Aspergillus niger* isolated from hydrocarbon-contaminated site was found to degrade pyrene to 1-pyrenol when grown in malt agar supplemented with pyrene. Non-basidiomycete fungi like *Penicillium* sp. also degrade pyrene cometabolically with 1 % dextrose to 1-pyrenol, 1,6- and 1,8-pyrenediols within 24 - 48 hrs (Sack *et al*., 1997b).

Cerniglia *et al*. (1992) studied the cometabolism of pyrene by the zygomycete fungus *Cunninghamella elegans* and identified 1,6- and 1,8-pyrene quinones; glucose conjugates of 1-pyrenol and 1,6- and 1,8-pyrenediols were detected as degradation intermediates. Degradation of PAHs in mammals is mediated by microsomal cytochrome P450 monooxygenase giving rise to *trans*-dihydrodiols. The detection of *trans*-dihydrodiols in several fungal species suggests that the degradation of PAHs in these fungi proceeds via cytochrome P450.

In certain other fungi like *Cunninghamella baineri*, extracellular lignin peroxidase is involved in pyrene degradation. Incorporation of ^{18}O into quinones from H_2O^{18} provides a clear proof (Cerniglia, 1992).

2.8.2.1 Metabolism of pyrene via trans-dihydrodiol

Cunninghamella elegans metabolizes pyrene to 1-hydroxypyrene, glucoside conjugates and 1,6- and 1,8-pyrenequinones. Some *Mycobacterium* spp. also produce *trans*-4,5-dihydrodiol and 1-pyrenol as degradation intermediates. 1-pyrenol is thought to be a precursor for the formation of methoxypyrene, nitropyrene and pyrene sulphates (Cerniglia *et al*., 1986).

2.8.2.2 Metabolism to quinones

Certain white rot fungi produce the lignolytic enzyme lignin peroxidase. These enzymes convert PAHs into quinones. For example the fungus *Phaenerochaete chrysosporium* degrades pyrene to 1,6-diquinones and 1,8- diquinones (Hammel *et al*., 1986).

2.9 Factors affecting pyrene degradation

Degradation of pyrene may be influenced by physical, chemical, biological or environmental factors. The lack of essential nutrients (such as nitrogen, phosphorus, potassium) or growth substrates, suboptimum temperatures, oxygen availability or pH may not allow pyrene degradation to occur. Pyrene may not be accessible due to its **low water solubility** or it may be present at a toxic concentration to bacteria, fungi or algae. In addition, pyrene may not be able to be transported into the cell, may not be a substrate for the available enzymes or may not be an inducer for the appropriate transport or degradative enzymes (Kanaly and Harayama, 2000; Juhasz and Naidu, 2000).

Boldrin *et al*. (1993) reported a **growth rate** of 0.056 h^{-1} for a *Mycobacterium* sp. grown on pyrene, and Walter *et al*. (1991) revealed that *Rhodococcus* sp. UW1 had a growth rate of 0.023 h^{-1}. Growth rates on pyrene measured for the pseudomonad isolates K-12 and B-24 were 0.014 and 0.013 h^{-1}, respectively (Thibault *et al*., 1996). Pyrene crystal size was shown to greatly influence pyrene degradation rates by the *Mycobacterium* sp. and *Pseudomonas* strains. The smaller the crystal size, the higher is the microbe growth rate. It has been suggested that crystal size can greatly influence the rate of dissolution from the solid phase into the aqueous phase (Walter *et al*., 1991; Thibault *et al*., 1996).

Bioavailability of pyrene could be another rate determining factor for its degradation. The covalent attachment of pyrene to clays and humic materials may affect the bioavailability, transport, biological activity and degradation of the compound in the soil (Gauthier *et al*., 2003). Adsorption of pyrene onto soil particles is due to its hydrophobicity and is tempered in part by the fraction of organic carbon present in the soil (Thibault *et al*., 1996). Weissenfels *et al*. (1992) and Erickson *et al*. (1993) observed a decrease in the mineralization of high molecular weight PAHs with increasing residence time of PAHs in soils. This decrease in PAH degradation was attributed to the association

of PAHs to soil organic matter. Such associations may result in a reduction in the rate and extent of PAH degradation due to the slowing of PAH desorption from soil organic matter into the soil aqueous phase (Grosser *et al*., 1991). Mechanisms like van derWaals forces, chemical binding, H-bonding, ion exchange, covalent bonding or ligand exchange (Khan and Ivarson, 1982; Koskinen and Harper, 1990) may be responsible for the sorptive process; however, these mechanisms are not fully understood. Besides, environmental factors such as pH, temperature, type and quality of clay minerals, amount of organic matter in soil and the type and concentration of solutes in the surrounding solution can also influence the sorption of pyrene. In addition, the extent of contaminant retention is also directly related to the octanol/water (K_{ow}) partitioning coefficient. As pyrene is characterized by a large log K_{ow} (= 5.18) its tendency to sorb onto the organic soil fraction and become unavailable is high.

Persistence of pyrene in the polluted environment may also be affected by production of toxic or dead-end **metabolites**, metabolite repression, and accumulation of by-products resulting in **cytotoxicity**, the presence of preferred substrates, and / or the lack of cometabolic or inducer substrates (Molina *et al*., 1999; Juhasz *et al*., 2002). Competition for enzymes involved in PAH oxidation or transport and blockage of enzyme induction may also inhibition PAH biodegradation (Bouchez *et al*., 1995; Stringfellow and Aitken, 1995; Shuttleworth and Cerniglia, 1996). Heitkamp and Cerniglia (1988) have reported that a significant amount of PAHs undergo biotransformation to more polar metabolites with unique chemical and toxicological characteristics differing from those of unmetabolized PAHs. In *Pseudomonas saccharophila* (P15) and *Sphingomonas yanoikuyae* (R1) the pyrene metabolite *cis*-4,5-dihydro-4,5-dihydroxypyrene inhibited phenathrene metabolism but had little effect on *Pseudomonas stutzeri* (P16) and *Bacillus cereus* (P21) (Kazunga and Aitken, 2000). In addition, the above metabolite and its oxidation product, pyrene-4,5-dione, inhibited benzo[*a*]pyrene mineralization in the sensitive strains. In a follow-up study, the strains were found to form the dead-end product fluoranthene-2,3-dione as a cometabolic product of flouranthene when grown on phenanthrene. Phenanthrene removal was inhibited by this metabolite in *Sphingomonas* sp. (R1) but not in the three other strains studied. Mineralization of benz[*a*]anthracene, benzo[*a*]pyrene, and chrysene was also inhibited in R1, while only benzo[*a*]pyrene metabolism in P15 was affected. Cytotoxicity was partly responsible for this observed inhibition (Kazunga *et al*., 2001). Thus, depending on the strains, transformation products

from one PAH may affect the removal of other PAHs (Chen and Aitken, 1999; Juhasz *et al*., 2002).

2.10 Techniques to improve pyrene degradation

A number of approaches may be applied to bioremediation of pyrene to overcome the abovementioned limiting factors and to improve microbial pyrene degradation in contaminated soils and sediments.

2.10.1 Enhancing the metabolic capabilities

The studies outlined in Table 1.6 and 1.7 illustrate that a number of bacteria and fungi have the potential to degrade pyrene, however these organisms may not always be present in soils where pyrene removal is required. The survival of bacteria in soils can be affected by competition with indigenous bacteria, as well as competition for nutrients (Grosser *et al*., 1991). In addition, the extent of pyrene degradation by these organisms may be limited to the production of polar metabolites which may pose a greater toxicological threat due to their increased toxicity, solubility and mobility (Heitkamp and Cerniglia, 1988; Kim *et al*., 2005).

2.10.1.1 Bioaugmentation

Bioaugmentation is a way to enhance the biodegradative capacities of contaminated sites by inoculation of bacteria with the desired catalytic capabilities (Iwamoto and Nasu, 2001). Mueller *et al*. (1989a, b) and Trzesicka-Mlynarz and Ward (1996) proposed that bioaugmentation is especially important for sites containing high PAH concentrations, sites which contain a significant proportion of high molecular weight PAHs and for recently polluted soils which do not have an adapted microbial population. In studies of mycobacterial-contaminant degradation described by Bogan *et al*. (2003) the results provided clear evidence of *Mycobacterium* sp. GTI-23's ability to survive and degrade PAH in a non-sterile soil system. Pyrene mineralization continued for several weeks in soils inoculated with GTI-23, while no mineralization occurred in its absence. Cheung and Kinkle (2001) have also reported survival of *Mycobacterium* sp. RJGII-135 in soil systems for more than 80 days.

2.10.1.2. Bacterial fungal co-cultures for cooperative catabolism of PAHs

A wide phylogenetic spectrum of microorganisms has been studied for their degradative abilities. Bacteria known to mineralize high molecular weight PAHs, like pyrene, fluoranthene and chrysene, as sole carbon and energy sources, are *Rhodococcus* sp. (Walter *et al*., 1991), *Burkholderia cepacia* (Juhasz *et al*., 1997), *Stenotrophomonas maltophilia* (Boonchan *et al*., 1998), *Mycobacterium* spp. (Heitkamp *et al*., 1988b; Kastner *et al*., 1994; Bouchez *et al*., 1995), *Alcaligenes denitrificans* (Weissenfels *et al*., 1990), and *Sphingomonas paucimobilis* (Mueller *et al*., 1990a, b; Ye *et al*., 1996). In contrast to bacteria, fungi generally do not utilize PAHs as their sole carbon and energy source but transform these compounds cometabolically to detoxified metabolites (Sutherland, 1992). Extensive studies have focused on white rot fungi such as *Phanerochaete chrysosporium* (Barclay *et al*., 1995) *Pleurotus ostreatus* (Bezalel *et al*., 1996a) and *Trametes versicolor* (Anderson and Henrysson, 1996; Collins *et al*., 1996) able to degrade PAHs and detoxify PAH-polluted soils and sediments due to the production of extracellular lignin-degrading enzymes. Non lignolytic fungi, such as *Cunninghamella elegans*, *Penicillium janthinellum*, and *Syncephalastrum* sp., can transform a variety of PAHs, including pyrene, chrysene, and benzo[*a*]pyrene, to polar metabolites (Pothuluri *et al*., 1994; Launen *et al*., 1995; Kiehlmann *et al*., 1996; Wunder *et al*., 1997).

Meulenberg *et al*. (1997), Sack *et al*. (1997b) and Kotterman *et al*. (1998) suggested that PAH degradation in nature is a consequence of sequential breakdown by fungi and bacteria, with fungi performing the initial oxidation step. The improved mineralization rate of a combined culture may be due to the greater bioavailability to the bacterial community of water-soluble compounds arising from fungal preoxidation of PAHs (Kotterman *et al*., 1998). Boonchan *et al*. (2000) observed that a bacterial fungal co-culture of *S. maltophilia* VUN 10,010 and *P. janthinellum* VUO 10,201 showed increased pyrene degradation rate, considerable microbial growth on five benzene-ring PAHs and benzo[*a*]pyrene mineralization when these compounds were provided as a sole carbon and energy source, while no significant microbial growth or benzo[*a*]pyrene mineralization was observed with axenic cultures. In fact *P. janthinellum* VUO 10,201 alone could not grow on pyrene as a sole carbon source. The increased PAH degradation and mineralization in the presence of the two microorganisms is proposed to be related to

the presence of metabolic complementarities among the coculture members leading to mutual dependence and benefit under these conditions (Boonchan *et al*., 2000).

Another hypothesis that explains the increase in PAH degradation in the presence of bacterial fungal coculture is the **increase in PAHs bioavailability** to the bacterial isolates due the fungal hyphae which can act as dispersion vectors of the bacteria in the soil (Jacques *et al*., 2007). Kohlmeier *et al*. (2005) and Wick *et al*. (2007) discussed that the movement of bacteria in soil is practically negligible due to dependence on water films, the tortuosity and small diameter of the soil pores. Concomitant to this, the distribution of the hydrophobic contaminants in the soil is heterogeneous, resulting in low bioavailability of PAHs. The presence of fungi in the soil allows the formation of a group of hyphae around of which is formed a film of water that allows the bacterial degraders to move to the contaminant. This phenomenon is magnified predominantly in the presence of fungi with hydrophilic hyphae and mobile bacteria. The presence of the fungi *Pythium ultimum* and of the *Pseudomonas putida* PpG7 in the soil resulted in bacterial displacements of 0.8 cm d^{-1}, which significantly increased the degradation of phenanthrene. In the absence of fungus, bacterial displacement was not observed and phenanthrene degradation was very less.

2.10.2. Enhancing bioavailability

A common approach to enhance bioavailability of PAHs to biodegrading microorganisms is to attempt increasing the apparent solubility of hydrophobic hydrocarbons by treatments such as **addition of synthetic surfactants** (Thibault *et al*., 1996; Tiehm *et al*., 1997; Volkering *et al*., 1995; Jimenez and Bartha, 1996) or **biosurfactants** (Rosenberg, 1986; Desai and Banat, 1997; Barkay *et al*., 1999; Jacques *et al*., 2007). The application of surfactants in soil systems can increase the rate of dissolution and desorption of pyrene by enhancing the rate of mass transfer from solid and sorbed phases. This has been observed for surfactants in excess of their critical micelle concentration (CMC) (Wilson and Jones, 1993). Further Tiehm (1994) reported that non-ionic surfactants with long ethoxylate chains are non-toxic to bacteria and not utilized preferentially as growth substrates; therefore enhance the degradation of fluoranthene and pyrene.

Rhamnolipids produced by *Pseudomonas aeruginosa* (Van Dyke *et al*., 1993), sophorose lipids by *Torulopsis bombicola* (Oberbremer *et al*., 1990), trehalose dimycolipids by

Rhodococcus erythropolis (Bruheim *et al*., 1997), and lichenysins by *Bacillus* sp. (Yakimov *et al*., 1995) are potent biosurfactants known to reduce surface tension (from 72 to $\leq$ 30 dynes cm^{-1}) and have low (µg ml^{-1}) critical micelle concentrations (CMC), which increase apparent solubilities of hydrophobic hydrocarbons by their solubilization into the hydrophobic core of micelles (Barkay *et al*., 1999). Alasan, a high-molecular-weight bioemulsifier produced by *Acinetobacter radioresistens* KA53 bacteria, enhanced the aqueous solubility and biodegradation rates of phenanthrene, fluoranthene and pyrene by 7, 16 and 20-fold, respectively (Barkay *et al*., 1999). Rhamnolipid biosurfactants from *Pseudomonas aeruginosa* 57SJ enhanced pyrene removal from an artificially contaminated soil (Bordas *et al*., 2005). This pyrene remobilization took place independently of the soil organic carbon solubilization.

Recently Pizzul *et al*. (2007) have also proposed the use of environmentally friendly **solvents** to increase solubility of PAHs. According to Pannu *et al.* (2004) the naturally occurring, non-toxic, cost-effective and biodegradable **vegetable oil**, peanut oil, may be used as a contaminated soil remediation amendment at concentrations of 2.5 – 20 % (v/v) and in the pH range of 6 to 7 to remove anthracene from garden soil with extraction efficiency greater than 90 %. Peanut oil amendment (0.1 % – 0.2 %; v/v) increased high molecular weight PAH biodegradation by 15 % – 80 % with a mixed bacterial culture and a pure culture of *Comamonas testosteroni* in aqueous media and in PAH-contaminated weathered soil slurry systems (Pannu *et al.*, 2003). The addition of **paraffin oil** to a slurry type bioreactor doubled the mineralization of pyrene by a *Mycobacterium* strain (Jimenez and Bartha, 1996). Due to the hydrophobic surface of their cell walls, the bacteria adhered to the emulsified solvent droplets, which also contained the dissolved pyrene, and in this way the uptake was enhanced.

2.10.3 Cometabolism

As mixtures of PAHs are often present in contaminated soils, cometabolic degradation might be of importance for bioremediation. The microbial degradation of pyrene in the presence of a more easily metabolized substrate has been observed by a number of investigators (Heitkamp and Cerniglia, 1988; Wang et al., 1990; Walter *et al*., 1991; Weissenfels *et al*., 1991; Geiselbrecht *et al*., 1998; Hwang and Cutright, 2002; Sho *et al*., 2004; Gennaro *et al*., 2008), but only a few reports describe the degradation of pyrene as

a sole carbon source (Grosser *et al*., 1991; Boldrin *et al*., 1993; Kastner *et al*., 1994; Dean-Ross and Cerniglia, 1996; Juhasz *et al*., 1997; Vila *et al*., 2001; Bogan *et al*., 2003; Krivobok *et al*., 2003; Sarma *et al*., 2004**).** According to Cerniglia, (1992) pyrene cannot be utilised as a sole carbon and energy source, so a growth substrate must be supplied to initiate growth of the microorganisms and to induce the production of catabolic enzymes. The type of growth substrate / inducer supplied can markedly influence the extent of pyrene degradation. Chen and Aitken (1999) have proposed that application of lower pathway PAH metabolites stimulates the induction of high molecular weight PAH-degrading capabilities in bacteria.

2.11 Rationale

Effective utilization of the capacity of diverse groups of soil bacteria to degrade or eliminate this carcinogenic pollutant from the environment was the rationale of my research work. In the present study autochthonous, culturable, aerobic bacterial diversity from two different PAH-contaminated sites were identified and characterized with respect to pyrene degrading capacities. Pyrene utilization pattern, concomitant activities during growth and its genetic basis in selected isolates was determined. One isolate was finally selected for pyrene metabolite identification and pathway prediction.

2.12 Objectives

- Biodiversity of pyrene degrading bacteria
- Elucidate the metabolic pathway of pyrene degradation in a selected isolate
- Genetic regulation of pyrene degradation in the selected isolate
- Removal of pyrene by selected bacterial isolate from soil contaminated with pyrene

CHAPTER 3

MATERIALS AND METHODS

3.1 Chemicals

3.1.1 General (analytical) chemicals

Boric acid, bovine serum albumin (Fraction V), bromophenol blue, calcium chloride, cupric sulphate $CuSO_4.5H_2O$, potassium acetate, potassium chloride, potassium hydroxide, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, safranin, sodium dodecyl sulphate, sodium hydroxide were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai (India). Ammonium molybdate $[(NH_4)_6Mo_7O_{24}.4H_2O]$, antimony potassium tartarate, ascorbic acid, bromocresol green, crystal violet, diphenylamine, ferrous ammonium sulphate $[Fe(NH_4)_2(SO_4).6H_2O]$, HgO, methyl red, potassium dichromate ($K_2Cr_2O_7$), (KH_2PO_4), potassium iodide, K_2SO_4, salicylic acid, sodium bicarbonate ($NaHCO_3$), sodium fluoride (NaF), sodium thiosulphate ($Na_2S_2O_3$), concentrated hydrochloric acid (HCl), concentrated sulphuric acid (H_2SO_4), orthophosphoric acid (H_3PO_4; 85 %), glacial acetic acid were obtained from E. Merck (India) Limited, Mumbai. Mobil oil (Racer 2T oil) manufactured by Hindustan Petroleum Corporation Ltd., Mumbai was obtained from Petrol Filling Station, Patiala. Solvents used for high-performance liquid chromatography analysis like acetone, acetonitrile, chloroform, cyclohexane, hexane (fraction from petroleum), methanol were of highest purity (> 99.8 %) and purchased from Merck Limited (India). Gas cylinders of nitrogen, air, hydrogen and helium were purchased from Lalit Gas Agency, New Delhi.

3.1.2 Bacteriological media and components

Luria-Bertani broth, Bushnell Haas broth, agar-agar growth media were obtained form Himedia Laboratories Pvt Ltd, Mumbai. Crude oil (C.O.) was procured from a petrochemical plant at Bombay High, India. Pyrene (Pyr) was purchased from Merck - Schuchardt, Germany. Glucose, glycerol, sodium chloride, trace elements (Nitrilotriacetic acid, $MgSO_4$, $FeSO_4.7H_2O$, $CoCl_2$, $CaCl_2.2H_2O$, $ZnSO_4$, $CuSO_4.5H_2O$, $AlK(SO_4)$, H_3BO_3, Na_2MoO_4), trace vitamins (Pyridoxine HCl, Thiamine HCl, Riboflavin, Nicotinic acid, Calcium pentothenate, DL-α-Lipoic acid, Biotin, Folic acid), were obtained from

E. Merck (India) Limited, Mumbai. Ampicillin was purchased from HiMedia Laboratories Pvt. Ltd., Mumbai (India). Milli-Q^{TM} (MQ) water (Millipore, Eschborn, Germany) was the universal solvent.

3.1.3 Molecular biology chemicals

Agarose, EDTA, tris (hydroxymethyl amino ethane) base, polyvinylpolypyrrolidone, phenol were obtained from Sigma Chemicals Co., St Louis, Missouri, U.S.A. All oligonucleotide primers for PCR reaction were manufactured from Qiagen Operon GmBH (Germany). Lysozyme, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal), isopropyl β-D-1-thiogalactopyranoside (IPTG), $MgCl_2$, deoxyribonucleotide triphosphates (dATP, dGTP, dTTP, dCTP), *taq* DNA polymerase, Type II restriction enzymes (*Alu* I, *Hin* 61, *Mbo* I, *Rsa* I, *Eco* RI), DNA molecular weight markers (1.0 kb DNA ladder, Lambda / Hind III DNA ladder, GeneRuler TM DNA Ladder Mix, GeneRuler 100 bp DNA ladder) were purchased from MBI Fermentas Life Sciences, USA. Ethidium bromide and N,N′-dimethyl-formamide were purchased from HiMedia Laboratories Pvt. Ltd., Mumbai (India). Ethyl alcohol, isoamyl alcohol and isopropyl alcohol were obtained from E. Merck (India) Limited, Mumbai.

3.1.4 Glassware and plasticware

Erlenmeyer flasks (500-ml, 250-ml, 100-ml, 50-ml capacity), glass-stoppered conical flasks (50-ml capacity), volumetric flasks (50-ml), HPLC solvent vacuum filtration assembly, Kjeldahl digestion flasks, spherical flat-bottomed distillation flasks, Beakers (250-ml, 100-ml), Burettes (50-ml), pipettes (10-ml), test tubes (150 mm × 18 mm), microscopic slides, microbiological spreaders, glass cuvettes, Quartz cuvettes, extraction crucibles (100-ml capacity), Reagent bottles (1000-ml), glass beads were purchased from Borosil Glass Works Ltd., India and Schott Duran®, Germany. HPLC and GC-MS injection syringes (50-µl and 10-µl, respectively) were obtained from Hamilton, USA.

Plastic-ware like eppendorf tubes (0.2, 0.5, 1.5, 2.0-ml) were purchased from Greiner Bio-One GmbH, Germany. Sterile, disposable petri dishes (60 and 90-mm diameter), polypropylene tubes (50-ml), Falcon tubes (50-ml), Filter funnels (diameter: 9 to 11-cm), float, storage vials (5-ml, 2-ml), Pasteur pipettes (3-ml) were purchased from Tarsons Products Pvt. Ltd. Kolkata.

Filtration units like syringe filters (Millex-GV, 0.22-µm) were purchased from Millipore, Whatman No. 42 filter paper (diameter: 125-mm) from Whatmann International Ltd Maidstone, England and disposable syringes (2-ml) from the local market.

3.1.5 Bacterial strains and plasmids

E. coli DH5α was used for 16S rRNA gene cloning studies.

The transforming plasmid was pGEM®-T Easy Vector, a high copy no *E. coli* plasmid, 3,018-bp in length, containing ampicillin resistance (Amp^r) gene as selection marker and procured from Promega Corporation, Madison, WI, USA at a concentration of 50 ng ml^{-1}.

3.2 COLLECTION OF PAH CONTAMINATED SOIL

3.2.1 Collection of crude and diesel oil contaminated soils

Crude oil and diesel oil contaminated soil samples were previously collected from a refinery and diesel oil depot, respectively, located in Patiala (Punjab), India (Kumar, 2007). Soil samples were brought to lab and used for microbiological studies.

3.2.2 Collection of coal-tar contaminated soil

1. Coal-tar contaminated soil samples were collected from a 15 year old hot pre-mix coal tar industrial plant site (Rakhra village, Patiala city, Punjab, India) in the month of August, 2006 with a maximum air temperature of 40 – 45 ^oC.
2. Two top soil samples, first 1-2 m away and the second 3-4 m away from the coal-tar plant, labeled NP and AP, respectively, were collected using sterile spatula in sterile sample bags.
3. Two sub soil samples (0-25 cm soil depth), first 1-2 m away and the second 3-4 m away from the coal-tar plant, labeled F and B, respectively, were collected using a soil augur.
4. All soil samples were brought to laboratory, and analyzed for physico–chemical characteristics such as temperature, color, moisture content, pH (Zhou *et al.,* 1996), electrical conductivity (Rayment and Higginson, 1992) and enumeration of bacterial count by serial-dilution of 1 gm soil and standard agar plating (Cappuccino and Sherman, 1987). The subsoil sample B was analyzed for presence of PAHs.

3.3 SOIL ANALYSIS

3.3.1 Physico - chemical analysis of coal-tar contaminated soil

3.3.1.1 pH

Soil pH was determined potentiometrically in a soil - water suspension of 1:5 ratio using an electronic pH meter as per the method given by Zhou *et al*. (1996).

Requirements

1. *Soil sample*
2. *Analytical weighing balance*
3. *pH meter (Thermo Orion Model 290)*
4. *Distilled water*
5. *Pipettes*
6. *Buffer solutions for calibrating the pH meter*

Procedure

1. The pH meter was calibrated over the range of pH 4.0, 7.0 and 9.2 using the manufacturer's instructions.
2. Ten gram (10 g) soil sample was weighed, transferred to a 100-ml capacity beaker and 50 ml distilled water was added to the sample.
3. The sample was stirred vigorously with glass rod for 5 minutes and kept undisturbed for 10 minutes followed by stirring again. All samples were analyzed in duplicate.
4. The samples were allowed to stand undisturbed for 30 minutes.
5. The pH of soil - water suspensions were read using pH meter, ensuring that the electrode tip remained in the suspension and did not touch the soil settled at the bottom of the beaker.

Precautions

1. While measuring pH of a soil /liquid slurry the electrode must be protected to prevent insertion to the very bottom of the slurry-containing vessel.
2. Abrasion of the sensing surfaces should not occur as it will decrease the life of the electrode and lead to inaccurate pH readings.
3. The pH meter must be calibrated routinely at three points with buffer solutions of pH 4.0, 7.0, 9.2 before measuring the pH of a soil sample.

3.3.1.2 Electrical conductivity

Electrical conductivity was measured in the laboratory as per the method given by Rayment and Higginson (1992) in a 1:5 soil-to-water suspension. The international unit of measurement for EC is seimen m^{-1} (S m^{-1}).

Requirements

1. *Soil sample*
2. *Analytical weighing balance*
3. *Conductivity meter (Thermo Orion Model 125 A^{+})*
4. *Pipettes*
5. *Distilled water*
6. *Calibration solution (0.01 M KCl solution):* Dissolved 0.7456 g KCl in 1 litre of water. This solution has a conductivity of 1413 µS cm^{-1} at 25 ^{o}C.

Procedure

1. Ten gram (10 g) soil sample was weighed, transferred to a 100-ml capacity beaker and 50 ml of distilled water was added.
2. The sample was stirred vigorously with glass rod for 5 minutes and kept undisturbed for 10 minutes followed by stirring again. All samples were analyzed in duplicate.
3. The samples were allowed to stand undisturbed for 30 minutes until the soil settled completely.
4. The conductivity meter calibrated with the 0.01 M KCl was inserted into the soil supernatant and the conductivity was read in µS cm^{-1}.

Precaution

1. The same soil suspension was used for pH measurement after (not before) taking the conductivity measurement.

3.3.1.3 Moisture content

Moisture in soil was determination in percentage water content as per the method of Black (1965).

Requirements

1. *Soil sample*
2. *Analytical weighing balance*
3. *Aluminium Keen boxes*
4. *Temperature controlled oven*
5. *$CaCl_2$ Dessicator*

Procedure

1. Duplicate soil samples (10 gm) were placed in pre-weighed aluminium keen boxes.
2. Samples were heated in an oven at 50 ^{o}C for 24 hrs and then cooled to room temperature in desiccators.
3. The weight change was noted, and moisture content calculated using the formula:

$$\text{Water content (\%)} = \frac{\text{Change in weight}}{\text{Initial weight (10 gm)}} \text{ X } 100$$

3.3.1.4 Determination of PAHs present in contaminated soil

The different PAHs present in coal-tar contaminated soil were detected by Gas chromatographic – Flame ionization detection (GC-FID) analysis as described in Materials and Methods section 3.3.1.4.

3.3.2 Microbiological analysis

3.3.2.1 Enumeration of bacteria

Bacterial enumeration was carried out by standard plate count method on Luria-Bertani agar (LA) (Cappuccino and Sherman, 1987).

Requirements

1. *Glass test tubes*
2. *Vortex mixer*
3. *Autopipettes and sterile microtips*
4. *Petri plates*
5. *Glass spreader*

6. *70 % alcohol*
7. *BOD Incubator*
8. *Luria-Bertani Agar* (Sambrook *et al.,* 1989)

Composition	**(g l^{-1})**
Peptone / Bactotryptone	10
Bactoyeast extract	5
Sodium chloride	5
Agar	15 - 20
pH	7.0 $\pm$ 0.2

9. *Saline solution (0.85 %; w/v):* Dissolved 0.85 g NaCl in 100 ml of distilled water.

Procedure

1. One gram of soil was added to 10 ml of sterile 0.85 % saline (NaCl) in a glass test tube and vortexed for 5 minutes. Tubes were allowed to stand undisturbed for 30 minutes.
2. One milliliter of this soil suspension was serially diluted five times using 9 ml of sterile 0.85 % saline. Similarly set up for all soil samples.
3. The soil suspension in tubes was allowed to stand for 15 minutes. Next, 0.1 ml of each dilution was aseptically spread onto three sets of LA plates using a sterilized spreader.
4. Replicate plates were incubated at 30 ^{o}C, 45 ^{o}C and 60 ^{o}C, and colonies were counted after 24 hrs and 48 hrs.

3.4 BIODIVERSITY OF PYRENE DEGRADING BACTERIA

3.4.1 Culture media preparation

All media were prepared in double distilled, deionized water. Basal media used in the study were liquid Bushnell Haas Broth (BHB) and solid Bushnell Haas Agar (BHA). The nutrient rich media used in the study were Luria-Bertani broth (LB) and Luria-Bertani Agar (LA). The medium was sterilized by autoclaving at 121 ^{o}C, 15 lbs, for 20 min. Wherever required, autoclaved crude oil (C.O.) and filter-sterilized glucose (G) were aseptically added to sterilized media prior to inoculation.

*3.4.1.1 Bushnell Haas Broth / Agar * (Toledo et al., 2006)*

Composition	**(g l^{-1})**
$MgSO_4.7H_2O$	0.2
$CaCl_2.2H_2O$	0.02
KH_2PO_4	1
K_2HPO_4	1
$NH_4NO_3.6H_2O$	1
$FeCl_3$	0.05
* Agar	15 - 20
pH	7.0 $\pm$ 0.2

Glucose (G) stock solution (20 %; w/v)

Composition	
Glucose	20 g
Milli Q Water	100 ml

Filter sterilized before use.

Pyrene (P) stock solution (5 %; w/v)

Composition	
Pyrene	50 mg
Acetone (HPLC grade)	1 ml

Filter-sterilized before use.

3.4.1.2 Bushnell Haas Broth + Glucose (BHB+G) medium

Filter-sterilized glucose stock solution (G) was aseptically added to sterile BHB medium prior to inoculation at working concentration of 0.25 % (G0.25), 0.5 % (G0.5), 0.75 % (G0.75) or 1.0 % (G1.0).

3.4.1.3 Bushnell Haas Broth + Pyrene (BHB+P) medium (Vila et al., 2001)

Pyrene stock solution was aseptically added to autoclaved BHB medium at working concentration of 25 µg ml^{-1} or 50 µg ml^{-1} or 75 µg ml^{-1}, followed by shaking on an orbital shaker (120 rpm) at 30 ^oC for 12 hrs before inoculation to allow for acetone evaporation.

3.4.1.4 Bushnell Haas Agar + Pyrene (BHA+P) medium (Kiyohara et al., 1982; Khan et al., 2009)

Pyrene coating of BHA media plates was done by uniformly spreading 0.1 ml of pyrene stock solution over the surface of the media plate. The acetone immediately vaporized at ambient temperature and a white, thin layer of pyrene remained on the entire surface.

3.4.1.5 Bushnell Haas Agar + Glucose (BHA+G0.25) medium

Filter-sterilized glucose stock solution (G) was aseptically added to autoclaved molten BHA medium at working concentration of 0.25 % (w/v). Poured the media aseptically in sterile petri plates and allowed to solidify.

3.4.1.6 Bushnell Haas Agar + Glucose + Pyrene (BHA+G0.25+P) medium

Bushnell Haas Agar + Glucose (BHA-G0.25) media plates were prepared and pyrene coating was done as described by Kiyohara *et al.* (1982).

3.4.2 Microbiological methods

3.4.2.1 Bacterial consortia from crude and diesel oil contaminated soil

Bacterial consortia were isolated from the crude oil and diesel oil contaminated soil samples (section 3.2.1), respectively and used for the study.

Requirements

1. *soil sample*
2. *70 % ethanol*
3. *Petri plates*
4. *Analytical weighing balance*
5. *Orbital shaker-cum-incubator*

Procedure

1. Ten gram soil samples were added to autoclaved 100 ml BHB + C.O. (1 %; v/v) medium in Erlenmeyer flasks (250-ml capacity) and incubated at 30 ^{o}C with

continuous aeration in a orbital shaker at 120 rpm (revolutions per min) in the dark. After 3 weeks of incubation the mixed culture of crude oil utilizing bacteria obtained from crude oil and diesel oil contaminated soil samples were named C and T, respectively.

2. Whole flask contents (100 ml consortium culture) were centrifuged at 10,000 rpm, for 5 minutes, and the supernatant discarded. The cell pellet was resuspended in 10 ml of sterile 50 % glycerol to make a homogenous cell suspension. This culture stock in glycerol, stored at –20 ^{o}C or –80 ^{o}C, was known as 'consortium mother culture'.
3. In routine experimental studies, for inoculum preparation, a loopful of the thawed mother culture, serially diluted upto 10^{-2} dilution, was streaked on LA plates, to get isolated colonies. An isolated colony from this culture plate was used to inoculate 5 ml sterile LB in test tubes. Each culture plate was used as inoculum only for a month and stored at 4 ^{o}C. Alternatively 5 µl of the thawed mother culture was used to inoculate LB (5 ml) in a test tube. After overnight incubation at 30 ^{o}C on a rotary shaker (60 rpm), 2 % (v/v) inoculum was used to inoculate LB or BHB +Pyrene media in shake flasks. After one month, started afresh with mother culture.

3.4.2.2 Selective enrichment of bacterial consortia

Development of bacterial consortia from crude oil (C) and diesel oil (T) contaminated soil was carried out by selective enrichment procedures using pyrene to selectively enrich the pyrene utilizing bacteria.

Requirements

1. *Consortium mother culture C and T*
2. *Autopipette and sterile microtips*
3. *Orbital shaker-cum-incubator*
4. *Autoclaved BHB medium in 250-ml Erlenmeyer flask*
5. *Autoclaved crude oil (C.O.)*
6. *Filter sterilized pyrene (P) stock solution (5 %; w/v)*

7. *Filter sterilized glucose (G) stock solution (20 %; w/v)*

Procedure

1. Ten milliliters of the consortium mother culture was inoculated in 100 ml of BHB + C.O. (0.5 %; w/v) + P (10 µg ml^{-1}) medium and grown with orbital shaking (120 rpm) at 30 ^{o}C.
2. After 4 weeks incubation, 10 ml of the saturated culture was again transferred into 100 ml BHB + C.O. (0.3 %; w/v) + G (0.2 %; w/v) +P (20 µg ml^{-1}) medium and incubated. The consortia were unable to grow in presence of pyrene alone, therefore glucose was provided as a co-substrate. Glucose also served to increase the biomass level (Tian *et al.*, 2003).
3. In successive three growth cycles of 30 days each, the growth medium BHB was enriched with pyrene concentration in steps of 25, 35 and 50 µg ml^{-1} and glucose concentration in steps of 0.25, 0.35 and 0.5 % (w/v), respectively, while C.O. concentration was brought down from 0.3 to 0.2 % (w/v) and maintained thereafter. After 5 months, the consortia were designated CON-3 and THA-2, respectively, and maintained in BHB + C. O (0.2 %; w/v) + G (0.5 %; w/v) + P (50 µg ml^{-1}) medium and preserved as 50 % glycerol stocks at -20 ^{o}C and -80 ^{o}C.
4. A 5 µl aliquot of thawed glycerol stock was used to inoculate LB (5 ml) in a test tube and incubated at 30 ^{o}C on a rotary shaker (60 rpm) for 24 hrs. Subcultured 2 % (v/v) inoculum in 100 ml BHB + G (0.5 %; w/v) + P (50 µg ml^{-1}) medium in 250-ml Erlenmeyer flasks with orbital shaking (120 rpm) at 30 ^{o}C for 4 weeks. The percentage of pyrene uptake by consortia CON-3 and THA-2 from growth medium was determined by spectrophotometric analysis (as described in section 3.5.4.2) and High-Performance Liquid Chromatographic (HPLC) analysis (as described in section 3.5.4.3).

3.4.2.3 Isolation and growth of pyrene utilizing bacterial isolates from consortia

Pyrene utilizing bacteria from consortia CON-3 and THA-2 were isolated by standard agar plating techniques (Cappuccino and Sherman, 1987).

Procedure

1. Luria-Bertani agar (LA), pyrene coated BHA (BHA+P) and pyrene coated BHA plus 0.25 % (w/v) glucose (BHA+G0.25+P) plates were prepared as described in section 3.4.1.
2. Aliquots (0.1 ml) of enriched CON-3 and THA-2 consortia, developed in BHB + C.O. (0.2%; v/v) + G (0.25 %; w/v) + P (25 µg ml^{-1}) for 30 days, were plated on LA plates (in triplicate) and incubated at 30 ^{o}C for 48 – 72 hrs.
3. Morphologically different, discrete bacterial colonies were picked and replica plated on LA, BHA+P and BHA+G0.25+P plates to get pure cultures of pyrene utilizing bacterial isolates.
4. Individual colonies were purified by repetitive streaking on the same medium (Jacques *et al*., 2007) and LA medium (Lin and Cai, 2008).
5. Discrete colonies from BHA+P and BHA+G0.25+P plates were transferred to 25 ml of BHB + G (0.25 %; w/v) +P (25 µg ml^{-1}) medium and incubated at 30 ^{o}C for 30 days. Growth was measured spectrophotometrically (as described in section 3.5.3.1) and pyrene uptake was determined by HPLC analysis (as described in section 3.5.4.3).
6. Isolates showing more than 50 % uptake of 25 µg ml^{-1} pyrene, were designated Pyr^{25} isolates, and subcultured at 30 ^{o}C first in 100 ml of BHB + G (0.5 %; w/v) +P (50 µg ml^{-1}) medium for 30 days and subsequently in 100 ml of BHB + G (0.75 %; w/v) +P (75 µg ml^{-1}) medium for 30 days. Pyrene uptake was determined after each growth cycle by HPLC analysis (as described in section 3.5.4.3). Isolates showing more than 50 % uptake of 50, 75 µg ml^{-1} pyrene were broadly designated Pyr^{50} and Pyr^{75} isolates, respectively.
7. Bacteria growing at 25, 50 and 75 µg ml^{-1} of pyrene were preserved in 50 % glycerol stocks at -20 ^{o}C and -80 ^{o}C.

3.4.2.4 Isolation of pyrene utilizing bacterial isolates from coal-tar contaminated soil

Bacteria present in coal-tar contaminated soil were isolated by serial-dilution and agar plating techniques (Cappuccino and Sherman, 1987).

Procedure

1. Luria-Bertani agar (LA), pyrene coated BHA (BHA+P) and pyrene coated BHA + 0.25 % (w/v) glucose (BHA+G0.25+P) plates were prepared as described in section 3.4.1.

2. One gram of soil sample was suspended by vortexing in 10 ml of sterile 0.8 % saline (NaCl solution). One milliliter (1 ml) of this suspension was serially diluted five times using 9 ml of sterile 0.8 % saline.
3. Aliquots (0.1 ml) of each resting soil suspension were plated onto two sets of LA plates. One set of plates was incubated at 30 ^{o}C and a second at 45 ^{o}C.
4. After 24 - 48 hrs, morphologically distinct, isolated bacterial colonies were picked and replica plated on LA, BHA+P and BHA+G0.25+P plates to get pure cultures of pyrene utilizing bacterial isolates.
5. Individual colonies were purified by repetitive streaking on the same medium (Jacques *et al*., 2007) and LA medium (Lin and Cai, 2008).
6. Discrete bacterial colonies visible on the BHA+P and BHA+G0.25+P plates at 30 ^{o}C, 45 ^{o}C were transferred to 5 ml of BHB +P (25 µg ml^{-1}) and BHB + G (0.25 %; w/v) +P (25 µg ml^{-1}) media in test tubes and incubated at respective incubation temperatures with continuous shaking at 60 rpm. Bacterial strains were unable to use pyrene alone as carbon and energy source for growth, so glucose was provided as co-substrate in growth medium. The culture tubes were checked at regular intervals for growth (turbidity) of the isolates.
7. Subcultured isolates showing growth into 50 ml BHB + G (0.5 %; w/v) +P (50 µg ml^{-1}) medium for 30 days and measured growth at regular intervals of time (as described in section 3.5.3.1). Isolates showing maximum growth with 50 µg ml^{-1} pyrene, designated Pyr^{+} isolates, were preserved in 50 % glycerol stocks at -20 ^{o}C and -80 ^{o}C and maintained on LA plates by routine patching / streaking techniques for further experimental work.

3.4.3 Morphological characterization of pyrene utilizing bacterial isolates

Pyrene utilizing bacterial isolates PK1 – PK10 and PK12 – PK14 were analyzed microscopically by gram staining technique (Gram, 1884).

Requirements

1. *Glass slide with cover slip*
2. *Inoculation needle*
3. *Laminar air-flow*
4. *Compound Microscope*
5. *Aqueous Crystal Violet (1%)*

Composition

Crystal violet	1 g
Distilled water	100 ml

6. *Gram's Iodine*

Composition

Potassium iodide	2 g
Distilled water	100 ml

7. *Decolorizer: acetone (50%) and alcohol (50%)*
8. *Aqueous safranin (2%)*

Composition

Safranin	2 g
Distilled water	100 ml

Procedure

1. A drop of bacterial culture was placed on a slide with the help of an inoculation needle and spread evenly in the center of the slide. The smear was dried and heat-fixed.
2. The slide was placed on a staining rack and flooded with crystal violet for about 1 minute.
3. The stain was washed gently with iodine solution and stained with fresh iodine solution for 1 minute followed by washing in tap water or by dipping in a beaker containing water.
4. A few drops of decolorizer were added and continued until color ceased to come out of the preparation. This took 5 seconds to 1 minute.
5. Slides were washed repeated gently with water as in step 4.
6. Cells were counter-stained with dilute aqueous safranin for 10 - 30 seconds.
7. Again the slide was washed with water and dried with absorbent paper and left to dry by evaporation.
8. The dry slide was examined under the microscope directly without a cover slip first under low power and then under higher magnification.

3.4.4 Molecular *characterization of pyrene utilizing bacterial isolates*

3.4.4.1 Isolation of genomic DNA

Genomic DNA was extracted from bacterial isolates (PK1 – 10, PK 11 – PK30) either by ROSE (Rapid One Step Extraction) method as described by Steiner *et al.* (1995) or by boiling lysis method of Krivobok *et al.* (2003).

DNA isolation by ROSE method (Steiner *et al.*, 1995)

Requirements

1. *Rose Solution:*

Composition	**(g L^{-1})**
EDTA	0.37
Tris-HCl	0.12
SDS	1.00
Polyvinylpolypyrrolidone	1.00

2. *TE buffer:*

Composition	
Tris HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Procedure

1. A bacterial colony was picked from a freshly grown plate and transferred into 20 ml LB in a 250-ml flask. The culture was incubated for 16 - 20 hrs at 30 or 45 ^{o}C in an orbital shaker (120 rpm).
2. Bacterial cells from 2.0 ml stationary phase culture were harvested in sterile eppendorf tubes at 8000 rpm for 10 minutes. The media from the cell pellets was decanted and the tubes were allowed to stand in an inverted position for 1 minute to allow the last traces of media to drain away. The cell pellet was washed using chilled TE buffer.
3. Bacterial cell pellets were resuspended in 0.2 ml Rose solution without frothing and incubated at 90 ^{o}C for 20 minutes with intermittent shaking.
4. The eppendorf tubes were kept in an ice-bath for 5 minutes, then 1 ml of chilled Sevag (chloroform: isoamyl alcohol 24:1) was added into the tubes. The tube

contents were mixed gently, kept at room temperature for 30 minutes and centrifuged at 8000 rpm for 10 minutes.

5. The upper aqueous phase was transferred to a sterile eppendorf tube. DNA was precipitated by adding 2.5 volumes of chilled ethanol and incubating at -20 °C for 30 -60 min.
6. The DNA fibers obtained by centrifuged were washed with 100 µl 70% (v/v) ethanol, air dried and redissolved in 50 µl TE (pH 8.0) or sterile Milli-Q water. DNA was stored at -20 ^{o}C for further use.

DNA isolation by boiling lysis method (Krivobok *et al.*, 2003)

Procedure

1. Bacterial colonies of pyrene-utilizing soil isolates from a freshly grown plate were transferred into 20 ml of LB broth in a 250-ml capacity flask. Cultures were incubated for 16 - 20 hrs at 30 or 45 ^{o}C in an orbital shaker (120 rpm).
2. Aliquots (1.5 - 2.0 ml) of saturated culture were poured into eppendorf tubes and centrifuged at 10,000 rpm for 15 minutes. The supernatants were discarded and tubes were allowed to stand in an inverted position for 1 minute to allow the last traces of media to drain away. The cell pellets were washed using chilled TE buffer (pH 8.0).
3. Bacterial cell pellets was resuspended by vigorous vortexing in 0.1 ml sterile nuclease free MQ water and incubated for 10 minutes in a water bath, previously set at 95 ^{o}C.
4. Contents in the tube were immediately chilled in an ice-bath for 5 minutes and then centrifuged at 10,000 rpm for 15 minutes to pellet down the cell debris.
5. The supernatant containing genomic DNA was transferred to a sterile eppendorf tube and stored at -20 ^{o}C.

3.4.4.2 Analysis and quantification of DNA

DNA was quantified by measuring optical density on a UV spectrophotometer (Sambrook *et al.,* 1989) and the DNA samples were analyzed by horizontal agarose gel electrophoresis using aid of DNA molecular wt. markers (1.0 kb DNA ladder, Lambda / Hind III DNA ladder).

A. Quantification of DNA

Requirements

1. *Quartz cuvettes*
2. *Milli-Q water*
3. *Autopipette and microtips*

Procedure

1. The concentration of DNA was measured spectrophotometrically by reading the optical density at 260 nm using UV-Vis spectrophotometer and quartz cuvettes. One optical density unit at 260 nm in 1 cm cuvette corresponds to approximately 50 µg ml^{-1} of double stranded DNA (Sambrook *et al.,* 1989).
2. The purity of the DNA sample from contaminating polysaccharides and proteins was evaluated by the ratio between A260 and A280 nm (A_{260}/A_{280}). Pure DNA samples were indicated by a value closer to or higher than 1.8.

B. Agarose gel electrophoresis of DNA

Requirements

1. 0.*5 X TBE buffer*

Composition	**(g L^{-1})**
Tris base	5.4 g
Boric acid	2.75 g
EDTA (0.5 M; pH 8.0)	2 ml

2. *6 X gel loading dye*

Composition	
EDTA (0.5 M; pH 8.0)	8 ml
Bromophenol blue	0.20 g
Sucrose	40 g or

Glycerol	30 g
Milli-Q water	100 ml

3. *Ethidium Bromide (EtBr) stock solution*

Composition

EtBr	5 mg
Milli-Q water	1 ml

Procedure

1. Agarose gel (0.7 %; w/v) was prepared in 0.5 X TBE buffer and submerged in the same buffer in a horizontal electrophoresis chamber.
2. Aliquots of DNA sample mixed with 6 X gel loading dye were loaded into the wells of the submerged agarose gel using a micropipette. DNA molecular weight markers like 1 kb DNA ladder and/or λ *Hind* III digest DNA ladder were also loaded.
3. Electrophoretic fractionation was carried out at a constant current of 120 mA, 60 - 70 V for 45 minutes – 1 hr.
4. The agarose gel was stained in EtBr solution (working concentration = 0.5 µg ml^{-1}) for 10 minutes, viewed under UV light on a UV transilluminator and photographed using Biorad Gel Documentation system.

3.4.4.3 Polymerase chain reaction (PCR) amplification of 16S ribosomal RNA gene

Genomic DNA extracted from bacterial isolates (as described in section 3.4.4.1) was used for the amplification reaction. Universal bacterial primers were adapted from Suzuki and Giovanni, (1996) and Baker *et al.* (2003) for the amplification of 16S rRNA gene.

Requirements

1. *Sterile eppendorf tubes (0.2-ml)*
2. *Autopipettes and sterile microtips*
3. *Spinwin (Tarsons)*
4. *GeneAmp® PCR system 9700 (Applied Biosystems)*
5. *Deoxyribonucleotides dATP, dGTP, dCTP, dTTP*
6. *Taq DNA Polymerase*
7. *Universal Bacterial primers:*

⇒ **E8/27-F** (*E. coli* nucleotide position 8 to 27): 5'-AGA GTT TGA TCC TGG CTC AG-3'.

⇒ **E1492-R** (*E. coli* nucleotide position 1492 to 1551): 5'-GGT TAC CTT GTT ACG ACT T-3'.

8. *Sterile Milli-Q water*

Procedure

A. Amplification

1. PCR reactions were carried out in a final volume of 50 µl containing 1x reaction buffer 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 10 ng of genomic DNA, and 1.25 units of *Taq* DNA polymerase in thin-walled 0.2-ml reaction tubes placed in a GeneAmp® PCR system 9700.
2. Positive (enzyme missing) and negative (template missing) controls were included.
3. The PCR program was carried out as follows: initial denaturation at 92 ^{o}C for 2 min 10 sec, followed by 36 cycles of denaturation at 92 ^{o}C for 1 min 10 sec, annealing at 48 ^{o}C for 30 sec, extension at 72 ^{o}C for 2 min 10 sec, followed by a final extension at 72 ^{o}C for 6 min 10 sec.

B. Analysis

4. PCR reaction product (amplicons) was analyzed by agarose (0.7 %; w/v) gel electrophoresis of a 5 µl aliquot from the total PCR reaction, followed by UV transillumination of the EtBr -stained gel as described in section 3.4.4.2.
5. The total concentration of PCR product was estimated by comparison to DNA mass standard 1 kb DNA ladder on agarose gel.
6. PCR reaction products were stored at -20 °C. Amplified 16S rDNA products were analyzed by RFLP, cloned and sequenced.

3.4.4.4 Restriction Fragment Length Polymorphism (RFLP) analysis of 16S rDNA

16S rDNA PCR products (1-2 µg DNA) were subjected to restriction digestion by Type II tetracutter enzymes *Alu* I, *Hin* 61, *Mbo* I, *Rsa* I followed by gel electrophoresis to separate fragments according to size. Based on the restriction fragment length polymorphism (RFLP) patterns in agarose gels, the bacterial isolates were categorized into 14 different phylogenetic groups, wherein members of a group had similar RFLP

pattern for one restriction enzyme but different pattern from members of another group. One member isolate from each phylogenetic group, showing pyrene utilization abilities was identified by 16S rDNA cloning and sequencing.

Requirements

1. *Autoclaved 0.5-ml eppendorf tubes*
2. *Autopipette and sterile microtips*
3. *Float*
4. *Spinwin (Tarsons)*
5. *Circulating water bath*
6. *6X gel loading dye*

Procedure

1. The restriction buffer and plasmid DNA sample tubes were briefly centrifuged to collect contents at the bottom of the tubes.
2. Restriction digest reactions were set up as described below in autoclaved 0.5-ml eppendorf tubes.

Table 2.1 Restriction digestion conditions

Components (stock concentration)	Standard reaction	Control
Enzyme assay buffer (10 X)	2 µl (1X)	2 µl (1X)
Restriction enzyme (10 units/µl) [A]	0.5 µl	-
PCR product [B]	X µl	X µl
Sterile MQ water to a final volume of	20 µl	20 µl

[A] One enzyme unit is defined as the amount of enzyme required to produce a complete digest of 1 µg of lambda DNA in a reaction volume of 50 µl in 60 minutes under optimal conditions of salt, pH and temperature.

[B] Concentration of DNA = 1 - 2 µg

3. The eppendorf tube contents were mixed by gentle tapping, spun down briefly and incubated in a circulating water bath set at 37 °C for 3 hrs.
4. The restriction digestion reaction was stopped by adding 0.5 M EDTA (pH 8.0) to a final conc. of 10 mM. Alternatively 4-5 µl 6X gel-loading dye was added and mixed by vortexing briefly. The digested DNA samples were analyzed on 1 % agarose gels.

3.4.4.5 Cloning of 16S rRNA gene

The PCR products, amplified from genomic DNA, were directly ligated into the pGEM® T Easy vector system and transformed into *E. coli* DH5α cells.

Ligation

Requirements

1. *Vector:* pGEM®-T Easy Vector (supplied at concentration of 50 ng ml^{-1}; Figure 2.1).
2. *Insert:* PCR product (75 ng)

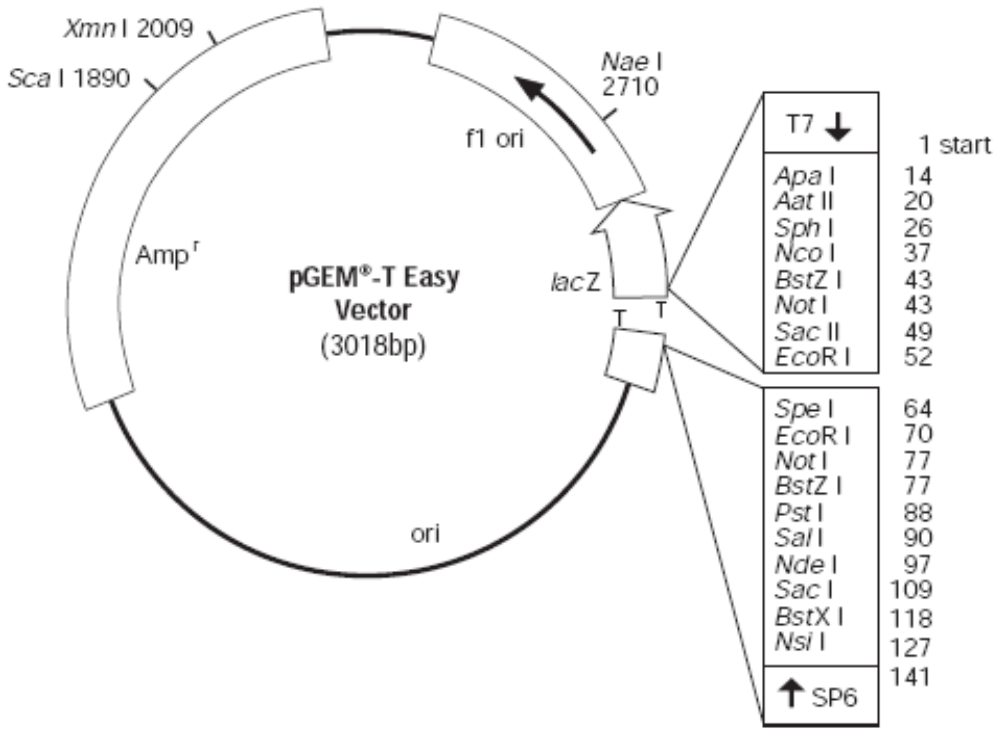


Figure 2.1 Diagrammatic representation of pGEM® T Easy vector system

Calculations

A 3:1 molar ratio of the PCR product (Insert) DNA to the Vector was optimized for ligation reaction by the vector manufacturer Promega Corporation, Madison, WI, USA. To calculate the appropriate amount of PCR product to include in the ligation reaction used the following equation:

$$\frac{\text{Nanogram of vector x size of insert (kb) x insert: vector molar ratio (3:1)}}{\text{Size of vector (kb)}} = \text{ng of insert}$$

$$\Rightarrow \frac{50 \text{ ng x } 1.5 \text{ kb}}{3.0 \text{ kb}} \text{ X } \frac{3}{1} = 75 \text{ ng insert}$$

$$\Rightarrow \quad 75 \text{ ng insert}$$

Procedure

1. The pGEM®-T Easy Vector, PCR product and Control Insert DNA tubes were briefly centrifuged to collect contents at the bottom of the tube.
2. Ligation reactions were set up as described in table below in autoclaved 0.5-ml eppendorf tubes.
3. Rapid Ligation Buffer (2X) was vortexed vigorously before each use.

Table 2.2 Ligation reaction conditions

Components (stock concentration)	Standard	Positive control
Rapid Ligation buffer (2X)	5 µl (1X)	5 µl (1X)
pGEM-T Easy vector (50 ng/µl)	1 µl	1 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl	1 µl
PCR product (75 ng) [A]	X µl	-
control insert DNA (4 ng/µl)	-	2 µl
Sterile MQ water to a final volume of	10 µl	10 µl

[A] Molar ratio of PCR product: vector optimized at 3:1

4. The reactions were mixed by pipetting and incubated overnight at 4°C (or for 1 hr at room temperature) for maximum number of transformants.

3.4.4.6 Transformation of E. coli DH5α by heat shock method

The protocol used for transformation involved **$CaCl_2$-induction method** described by Cohen *et al*. (1972). $CaCl_2$ treatment induces a transient state of "competence" in the recipient *E. coli* bacterial cells, during which they are able to take up various DNA molecules such as plasmid DNA, bacteriophage DNA, chromosomal DNA fragments etc. Competence usually arises at a specific stage of growth of a culture, typically late log phase and is highly dependent on the growth medium and the degree of aeration of culture. The transforming plasmid in this experiment is pGEM®-T Easy Vector (a high copy no *E. coli* plasmid, 3,018 bp in length, containing ampicillin resistance (Amp^r) gene as selection marker was procured from Promega Corporation, Madison, WI, USA).

Requirements

1. *Autoclaved, chilled 50-ml polypropylene tubes*
2. *Autopipettes and sterile microtips*
3. *Sterile eppendorf tubes (1.5-ml capacity)*
4. *Float*
5. *Glass spreader*
6. *Laminar air-flow*
7. *Glass cuvettes*
8. *UV-Vis spectrophotometer*
9. *Orbital shaker-cum-Incubator*
10. *Refrigerated centrifuge (Hitachi, Japan)*
11. *Circulating water bath*
12. *Ice bath*
13. *E.coli DH5α glycerol stock*
14. *Plasmid DNA sample*
15. *LB broth in 250-ml flasks*
16. *Luria-Bertani agar (LA) plates*
17. *Ampicillin stock solution*

    Composition

Ampicillin	50 mg
Milli-Q water	100 ml

    Filter-sterilized and stored at -20 ^{o}C.

18. *$CaCl_2$ (0.1 M) solution*

    Composition

$CaCl_2$	11.09 g
Milli Q water	100 ml

    Filter-sterilized and stored at -20 ^{o}C.

19. *IPTG stock solution (1 M)*

    Composition

Isopropyl β-D-1-thiogalactopyranoside (IPTG)	1.2 g
Milli-Q water	5 ml

    Filter-sterilized and stored at 4 °C.

20. *X-Gal (50 mg ml^{-1})*

Composition

5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal)	100 mg
N,N´-dimethyl-formamide	2 ml

Covered with aluminum foil and stored at –20 °C.

21. *LA-ampicillin plates* - Autoclaved LA medium and allowed to cool to 50 °C before adding ampicillin to a final concentration of 50 µg ml^{-1}. Plates were prepared with 30 - 35 ml of this medium and stored at 4 °C for up to one month.
22. *LA +ampicillin +IPTG +X-Gal plates* – Prepared the LA-ampicillin plates as above; then 10 µl of 1 M IPTG and 20 µl of 50 mg ml^{-1} X-Gal was spread over the surface of an LA-ampicillin plate and allowed to absorb for 30 minutes at 37 °C prior to use.

Procedure

1. *E.coli* DH5α cells were taken from glycerol stock and streaked on Luria-Bertani agar (LA) plates and incubated at 37 °C overnight.
2. A single *E. coli* DH5α colony was picked from a freshly grown plate and transferred into 20 ml LB broth in a 250-ml flask. Cultures were incubated for 16-20 hrs at 37 ^{o}C with vigorous shaking in an orbital shaker (120 rpm).
3. Aseptically 200 µl of the above-saturated culture was transferred into 20 ml fresh LB broth in a 250-ml flask and incubated with vigorous shaking (120 rpm) at 37 ^{o}C for 2-3 hrs. The growth of culture was determined by measuring the OD_{600} every one-hour.
4. The culture, with a final OD_{600} ~ 0.5, was transferred to sterile, disposable, ice-cold 50-ml polypropylene tubes and cooled to 4 ^{o}C by storing the tubes on ice for 10 minutes.
5. Bacterial cell pellet was obtained by centrifugation at 7,000 rpm for 15 minutes at 4 ^{o}C, media was decanted and the tubes allowed to stand in an inverted position for 1 min to allow the last traces of media to drain away. The cell pellet was resuspended in 10 ml filter-sterilized ice-cold 0.1 M $CaCl_2$, stored on ice for 15 min and again recovered by centrifugation. The fluid was decanted from the cell pellets and allowed the last traces of fluid to drain away.

6. The cell pellet was resuspended in 1 ml ice-cold 0.1 M $CaCl_2$ and incubated on ice for 2 - 3 hrs to induce competence in the *E. coli* cells.

7. Aliquots (100 µl) of competent cell suspension were transferred to sterile, chilled eppendorf tubes (1.5-ml capacity). The ligated plasmid DNA sample (~100 ng/5 µl or less; as described in section 3.4.4.5) was added to each tube. In control eppendorf tubes, the competent bacterial cells received no plasmid DNA at all. The eppendorf tube contents were mixed gently and stored on ice for 30 minutes for binding of the plasmid to the competent cells.

8. The eppendorf tubes were incubated in a circulating water bath, preheated to 42 ^{o}C, with the help of a float, for exactly 2 minutes without shaking. Then the tubes were immediately transferred into an ice bath and the cells were chilled for 2-3 minutes.

9. Sterile LB + Ampicillin (50 µg ml^{-1}) medium (1 ml) was added to each tube and incubated for 1.5 hrs at 37 °C with shaking (~150rpm) to allow the bacteria to recover from heat shock and to express the antibiotic resistance marker Amp^{r} encoded by the plasmid.

10. LA +ampicillin +IPTG +X-Gal media plates were prepared and incubated for 30 mins at 37 °C.

11. The eppendorf tubes containing transformed bacterial cells were centrifuged at 5,000 rpm for 10 minutes and the supernatant was drained off. The cell pellet was resuspended in 0.5 ml LB medium and aliquots (0.1 ml) of this transformed cell suspension were spread onto five LA +ampicillin +IPTG +X-Gal plates. Left the plates in laminar air-flow until the liquid was absorbed.

12. The plates were inverted and incubated at 37 ^{o}C for 16 - 24 hrs. Upon appearance of white (transformed cells) and blue (non-transformed cells) bacterial colonies, the plates were kept in refrigerator at 4 °C overnight so as to intensify the white and blue color of the colonies and facilitate differentiation between recombinants and non-recombinants.

3.4.4.7 Screening of transformants for inserts

Cloning of 1.5 kb 16S rDNA insert in the pGEM®-T Easy Vector interrupted the coding sequence of β-galactosidase and therefore recombinant clones were identified by colour

screening on LA +ampicillin +IPTG +X-Gal indicator plates as white colonies (α-complementation). Plasmid DNA was extracted from the recombinant cells using Alkaline Lysis method (Birnboim and Doly, 1979) and the presence of insert was confirmed by PCR reaction using T7 and SP6 primers and *Eco* R1 restriction digestion.

3.4.4.8 Isolation of plasmid DNA

The plasmid pGEM® -T Easy vector was isolated from transformed *E. coli* DH5α cells by the standard alkaline lysis method as described by Birnboim and Doly (1979).

Requirements

1. *Solution I*

Composition	
Glucose	50 mM
EDTA	10 mM
Tris-HCl (pH 8.0)	25 mM

2. *Solution II:* (freshly prepared)

Composition	
NaOH	0.2 M
SDS	1.0 % (w/v)

3. *Solution III*

Composition	
Potassium acetate (5.0 M)	60 ml
Glacial acetic acid (11.5 ml)	11.5 ml
MQ water	100 ml (final volume)

pH ~ 5.0 (3.0 M with respect to potassium and 5.0 M with respect to acetate.

4. *Tris saturated Phenol* - Phenol was saturated with 50 mM Tris buffer; pH 8.0
5. *TE buffer*

Composition	
Tris HCl (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM

Procedure

1. A single white bacterial colony of transformed *E. coli* DH5α was picked using sterile toothpick from LA + ampicillin (50 μg ml^{-1}) plate, transferred into 5 ml LB

+ ampicillin (50 μg ml^{-1}) medium in a cotton plugged test tube and incubated for 16 - 20 hrs at 37 ^{o}C in an orbital shaker (120 rpm).

2. The bacteria were subcultured on LA + Ampicillin plates with proper naming / numbering and incubated overnight at 37 °C.
3. A 2.0 ml aliquot of this saturated bacterial culture was taken in duplicate autoclaved eppendorf tubes, centrifuged at 4 ^{o}C, 8000 rpm for 5 minutes in a refrigerated centrifuge, and the supernatant discarded, leaving the bacterial pellets as dry as possible.
4. Bacterial pellets were resuspended in 200 μl ice-cold Solution I by vigorous vortexing to ensure that the bacterial pellets were thoroughly dispersed in this solution. An aliquot (50 μl) of 10 mg ml^{-1} freshly prepared lysozyme was added to the eppendorf tubes and kept at room temperature for 5 minutes.
5. Freshly prepared Solution II (200 μl) was added to the eppendorf tubes, capped tightly and the tube contents were mixed by gentle inversion of the tubes ten to twelve times rapidly. Vortexing was avoided here and the tubes were kept for 2 minutes at room temperature.
6. Ice-cold Solution III (300 μl) was added to the eppendorf tubes and the tubes were inverted slowly ten to twelve times to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 10-15 minutes and then centrifuged at 12,000 rpm for 10 minutes at 4 ^{o}C.
7. The supernatants were transferred carefully to sterile eppendorf tubes and added 400 μl of phenol: chloroform: isoamyl alcohol (24:25:1). The tube contents were mixed by inverting the tubes for 2 minutes and then centrifuged at 12,000 rpm for 10 minutes.
8. The upper aqueous layers were transferred to fresh eppendorf tubes and mixed well with equal volumes of isopropanol for precipitation of DNA. The mixture was allowed to stand at room temperature for 5-10 minutes and then centrifuged at 10,000 rpm for 10 minutes at 4 ^{o}C. The supernatants were discarded and the eppendorf tubes were allowed to stand in an inverted position on a paper towel for 1 minute to allow all of the supernatant to drain away. Any adhering drops of fluid on the walls of the tube were also removed.

9. The plasmid DNA pellets were washed with 100 µl of 70 % ethanol and centrifuged at 12,000 rpm for 5 minutes.
10. Plasmid DNA pellets were air-dried, dissolved in 50 µl of TE (pH 8.0) or sterile Milli-Q water and stored at –20 ^{o}C.

3.4.4.9 PCR amplification of cloned 16S rRNA gene using vector specific primers

Recombinant plasmid DNA extracted from transformed *E. coli* DH5α cells (as described in section 3.4.4.8) was used as template for the amplification reaction. Primers designed from T7 and SP6 polymerase promoter region of pGEM®-T Easy cloning vector system were used for the amplification and sequencing of cloned 16S rDNA.

Requirements

1. *Sterile eppendorf tubes (0.2-ml)*
2. *Autopipettes and sterile microtips*
3. *Spinwin (Tarsons)*
4. *GeneAmp® PCR system 9700 (Applied Biosystems)*
5. *Deoxyribonucleotides dATP, dGTP, dCTP, dTTP*
6. *Taq DNA Polymerase*
7. *Vector sequence specific primers:*
   - ⇒ **T7 -** forward primer (plasmid DNA binding site 3002-6)**:** 5'-ATT ATG CTG AGT GAT ATC CCG CT-3'.
   - ⇒ **SP6** - reverse primer (plasmid DNA binding site 136-158)**:** 5'-CAT AAG ATA TCA CAG TGG ATT TA-3'**.**
8. *Sterile Milli-Q water*

Procedure

A. Amplification

1. PCR reactions were carried out in a final volume of 25 µl containing 1X reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.5 µM of each primer, 1 ng of plasmid DNA, and 1.25 units of *Taq* DNA polymerase in thin-walled 0.2-ml reaction tubes placed in a GeneAmp® PCR system 9700.
2. Positive (enzyme missing) and negative (template missing) control PCR reactions were included.

3. The PCR program was carried out as follows: initial denaturation at 94 ^{o}C for 3 min, followed by 36 cycles of denaturation at 92 ^{o}C for 1min, annealing at 55 ^{o}C for 30 sec, extension at 72 ^{o}C for 1 min, followed by a final extension at 72 ^{o}C for 5 min.

B. Analysis

4. The amplification products were visualized by agarose (1.0 %; w/v) gel electrophoresis of a 5 µl aliquot from the total PCR reaction, followed by UV transillumination of the EtBr -stained gel as described in section 3.4.4.2.
5. The presence of fluorescent DNA band in the gel indicated presence of insert in the plasmid DNA sample.
6. The PCR reaction products were stored at -20 °C.

3.4.4.10 Restriction digestion analysis of cloned plasmid DNA

Recombinant plasmid DNA isolated from transformed *E. coli* DH5α cells (as described in section 3.4.4.8) was assayed for the presence of insert DNA by restriction digestion using a Type II hexacutter enzyme *Eco* RI.

Requirements

1. *Autoclaved 0.5-ml eppendorf tubes*
2. *Autopipette and sterile microtips*
3. *Float*
4. *Spinwin (Tarsons)*
5. *Circulating water bath*
6. *6X gel loading dye*

Procedure

1. The restriction buffer and plasmid DNA sample tubes were briefly centrifuged to collect contents at the bottom of the tubes.
2. Restriction digest reactions were set up as described below in autoclaved 0.5-ml eppendorf tubes.

Table 2.3 Restriction digestion conditions

Components	Standard reaction	Control

(stock concentration)		
Enzyme assay buffer (10 X)	2 µl (1X)	2 µl (1X)
Restriction enzyme (10 units μl^{-1}) [A]	0.5 µl	-
Plasmid DNA [B]	X µl	X µl
Sterile MQ water to a final volume of	20 µl	20 µl

[A] One enzyme unit is defined as the amount of enzyme required to produce a complete digest of 1 µg of lambda DNA in a reaction volume of 50 µl in 60 minutes under optimal conditions of salt, pH and temperature.

[B] Concentration of plasmid DNA = 1 - 2 µg

3. The eppendorf tube contents were mixed by gentle tapping, spun down briefly and incubated in a circulating water bath set at 37 °C for 3 hrs.
4. The restriction digestion reaction was stopped by adding 0.5 M EDTA (pH 8.0) to a final conc. of 10 mM. Alternatively 4-5 µl 6X gel-loading dye was added and mixed by vortexing briefly. The digested DNA samples were analyzed on 1 % agarose gels.

3.4.4.11 DNA Sequencing, analysis and submission

The insert DNA in plasmid was sequenced on both strands by using T7 and SP6 primers. The sequence was generated by chain termination method (Sanger *et al*., 1977) using an Applied Biosystems automatic sequencer (Courtesy: Bangalore Genei Pvt Ltd, Bangalore, India). Sequence analysis and homology search was performed with BLASTN program (Altschul *et al.,* 1990) using the nucleic acid sequences deposited in multiple databases like NCBI GenBank database and Ribosomal Database (RDB) II. Sequences obtained in the study were combined into a database along with the most similar 16S rDNA sequences retrieved from GenBank, and used to build a distance tree using the neighbor-joining algorithm (Saitou and Nei, 1987) and MEGA package version 4 (Tamura *et al*., 2007).

The 16S rRNA gene sequences of fourteen bacterial isolates have been submitted to the NCBI GenBank database under accession numbers EU685813 to EU685826. Bacterial isolates *Bacillus licheniformis* (PK-6), *Bacillus pumilus* (PK-12) and *Bacillus firmus* (PK-14), showing maximum pyrene removal from growth medium have been

deposited at Microbial Type Culture Collection library at IMTECH, Chandigarh (India) with culture collection number MTCC 1005, 1002 and 1003, respectively.

3.5 ELUCIDATE THE METABOLIC PATHWAY OF PYRENE DEGRADATION IN A SELECTED ISOLATE

3.5.1 Pyrene uptake studies

3.5.*1.1 Pulse chase experiment*

Pulse-chase experiments were undertaken with PK-12, PK-13, PK-14, PK-15, PK-16, PK-23, PK-24, PK-27 isolates, capable of > 35 % utilization of 25 µg ml^{-1} pyrene. Log phase bacterial cells defined in terms of their protein content were hourly monitored for capacity to uptake 100 µg pyrene.

Requirements

1. *Erlenmeyer flasks (1 L)*
2. *Glass-stoppered conical flasks (50-ml)*
3. *Autopipettes and sterile microtips*
4. *Orbital shaker-cum-incubator*
5. *Refrigerated centrifuge (Hitachi, Japan)*
6. *10 mM Potassium phosphate buffer*

Composition

K_2HPO_4 (0.1 M stock solution)	61.5 ml
KH_2PO_4 (0.1 M stock solution)	38.5 ml

The 0.1 M stock solutions were combined in above-mentioned volumes and the final volume was made upto 1 liter with distilled H_2O. The pH of buffer at 25 ^oC was 7.0 $\pm$ 0.2.

Procedure

1. Bacterial isolates were cultured in 10 ml BHB + G (0.5 %; w/v) medium from 100 µl glycerol stock at 30 ^oC in an orbital shaker (120 rpm) for 24 hrs. From the 24 hr cultures, subcultures were grown in 1 L Erlenmeyer flasks containing 500 ml LB medium till mid-log phase of growth (~ 12 - 14 hrs).

2. Bacterial cells were harvested (centrifugation at 10,000 rpm for 15 minutes), washed in 10 mM phosphate buffer (pH 7.0 $\pm$ 0.2) and resuspended in the same buffer.
3. The total protein content of cell suspension was estimated using biuret method of Itzhaki and Gil (1964) as described in following section 3.5.3.2. Bacterial cell suspension equivalent to 2 mg protein was pipetted in sterile 50-ml capacity glass-stoppered conical flasks and the final volume made up to 5 ml using 10 mM phosphate buffer.
4. In one set of flasks the pulse-chase reaction was immediately initiated by addition of 100 µg pyrene and incubation at 30 ^{o}C with orbital shaking (120 rpm).
5. The second set of flasks were first incubated for 12 hrs at 30 ^{o}C to starve the mid-log phase cells in phosphate buffer, then pyrene addition was done followed by incubation as above.
6. At hourly intervals, culture flasks (in triplicates) from both sets were withdrawn, metabolic activity was stopped by acidification (as described in section 3.5.4.1) and percentage of pyrene uptake measured in triplicates by spectrophotometric (as described in section 3.5.4.2) and HPLC analysis (as described in section 3.5.4.3).
7. The percentage of pyrene uptake by bacterial isolates, over a period of 7 hrs, was calculated as residual pyrene concentration in inoculated flasks relative to pyrene concentration in control flasks without inoculum (Sarma *et al*., 2004; Jacques *et al*., 2007).

3.5.*1.2 Time-course experiment*

Pyrene uptake pattern was studied in a time course experiment of 35 days. Bacterial cultures PK-12, PK-13, PK-14, PK-15, PK-16, PK-23, PK-24 and PK-27 were monitored in this experiment for pattern of 50 µg ml^{-1} pyrene uptake, from log to stationary growth phase.

Requirements

1. *Erlenmeyer flasks (250-ml)*
2. *Autopipettes and sterile microtips*
3. *Orbital shaker-cum-incubator*

Procedure

1. Inoculum preparation: Bacterial isolates were cultured in 100 ml BHB + G (0.5 %; w/v) medium from 200 µl glycerol stock at 30^{o}C in an orbital shaker (120 rpm) for 24 hrs.
2. After 24 hrs, for each bacterial isolate, 5 % inoculum was sub-cultured in a batch of sixteen Erlenmeyer flasks containing 100 ml BHB + G (0.5 %; w/v) + P (50 µg ml^{-1}) medium and incubated at 30 ^{o}C, 120 rpm.
3. One culture flask from each batch was withdrawn at zero time (designated as 'positive control') to determine initial pyrene concentration. In addition, a flask containing the same amount of pyrene but without any microbial inoculum was used as the 'negative control' to determine abiotic losses.
4. Culture flasks (in triplicate) from each batch were withdrawn every 7th day upto 35 days, solvent-extracted and pyrene uptake quantified by spectrophotometric and HPLC analysis (as described in sections 3.5.4.2 and 3.5.4.3., respectively). The utilization percentage of pyrene was calculated as the difference in residual pyrene concentrations between the uninoculated (control) and inoculated flasks.

3.5.*1.3 Effect of pyrene on growth of* Bacillus sp. *(PK-12)*

The effect of pyrene addition on growth of one pyrene utilizing isolate was studied.

Requirements

1. *Glass test tubes (150 mm × 18 mm)*
2. *Autopipettes and sterile microtips*
3. *Test tube rotary shaker-cum-incubator*
4. *Glass cuvettes*
5. *UV-Visible spectrophotometer*

Procedure

1. *Bacillus* sp. (PK-12) inoculum was developed in 10 ml BHB + G (0.5 %; w/v) medium from 100 µl glycerol stock at 30 ^{o}C in an orbital shaker (120 rpm) for 24 hrs. The inoculum was subcultured in test tubes containing 5 ml BHB + P (50 µg ml^{-1}), LB and BHB + G (0.5 %; w/v) media in triplicate and allowed to grow at 30 ^{o}C with shaking (60 rpm).

2. At mid-log phase of growth (~ 3 hrs in LB, ~ 20 - 24 hrs in BHB + G medium) pyrene (50 µg ml^{-1}) was added to half of the LB and BHB + G culture tubes. The other half culture tubes not receiving pyrene served as 'control'. All culture tubes were again incubated at 30 ^{o}C with shaking.
3. Growth was spectrophotometrically determined at 600 nm from the zero time of inoculation until measurements remained constant (i.e. 24 hrs in LB medium and 200 hrs in BHB medium).
4. The experiment was performed in triplicate and mean and standard error (SE) values were calculated using Microsoft Excel 2003.

3.5.*1.4 Effect of media supplementation for enhanced pyrene utilization by bacterial consortia*

The effect of media supplementation on rate of pyrene uptake and absolute / optional requirement of glucose for pyrene metabolism by consortia CON-3 and THA-2 was studied.

Requirements

1. *Erlenmeyer flasks (250-ml)*
2. *Trace element (TE) solution (50 X stock)* (Van Hamme et al., 2000).

Composition	**mg ml^{-1}**
Nitrilotriacetic acid *	15
$MgSO_4$,	5
$FeSO_4.7H_2O$	1
$CoCl_2$	1
$CaCl_2.2H_2O$	1
$ZnSO_4$	0.1
$CuSO_4.5H_2O$	0.1
$AlK(SO_4)$	0.1
H_3BO_3	0.1
Na_2MoO_4	0.1

* Dissolved in distilled water by adjusting the pH to 6.5 with 10 N KOH

Stock solution was sterilized by autoclaving at 121 ^{o}C for 15 min and stored at 4 ^{o}C.

3. *Trace vitamins (V) solution (1000 X stock)* (Pfennig, 1978)

Composition	mg ml^{-1}
Pyridoxine HCl	10
Thiamine HCl	5
Riboflavin	5
Nicotinic acid	5
Calcium pentothenate	5
DL-α-Lipoic acid	5
Biotin	2
Folic acid	1

Stock solution was filter-sterilized and stored at 4 ^{o}C.

Procedure

1. Bushnell Haas broth (100 ml) containing 50 µg ml^{-1} pyrene was prepared in four sets of Erlenmeyer flasks. To one set of flasks, labeled '0.5G', added glucose (0.5 %; w/v), to the second set, labeled 'TEV' flasks, added 2 ml of trace elements 50 X stock solution and 0.1 ml of trace vitamins 1000 X stock solution. In the third set of flasks, labeled '0.5G +TEV' added both glucose (0.5 %; w/v) and trace elements and vitamins solutions. In the fourth set of flasks, labeled '1.0G', added glucose (1.0 %; w/v).

2. Bacterial consortia CON-3 and THA-2 were cultured in 10 ml BHB + G (0.5 %; w/v) medium from 100 µl glycerol stock at 30 ^{o}C in an orbital shaker (120 rpm) for 24 hrs.

3. Consortia were subcultured @ 10 % (v/v) inoculum in each of the four different media flasks, namely 0.5G, TEV, 0.5G +TEV and 1.0G, and incubated in triplicate at 30 ^{o}C with continuous shaking on an orbital shaker (120 rpm). Uninoculated media flasks served as controls.

4. Growth cultures of CON-3 and THA-2 consortia and uninoculated media (control) flasks were withdrawn at 10 day interval for 30 consecutive days, solvent extracted and quantified by HPLC (as described in section 3.5.4.3.) for pyrene utilization.

5. The percentage of pyrene uptake by bacterial consortia was calculated as residual pyrene concentration in inoculated flasks relative to pyrene concentration in control flasks without inoculum (Sarma *et al*., 2004; Jacques *et al*., 2007). The experiment

was performed in triplicate and mean and standard error (SE) values were calculated using Microsoft Excel 2003.

3.5.*1.5 Effect of glucose on pyrene utilization by bacteria*

Bacterial isolates from crude, diesel oil (PK-11 to PK-14) and coal-tar (PK-1 to PK-10) contaminated soil, capable of maximum uptake and utilization of pyrene, were used for the study. The incubation temperature for PK-1 to PK-5 and PK-12 to PK-14 isolates was 30 ^{o}C and for PK-6 to PK-10 isolates was 45 ^{o}C.

Requirements

1. *Erlenmeyer flasks (250-ml)*
2. *Glucose stock solution (20 %; w/v)*
3. *Autopipettes and sterile microtips*
4. *Orbital shaker-cum-incubator*

Procedure

1. Bacterial isolates were cultured in 10 ml BHB + G (0.5 %; w/v) medium from 100 µl glycerol stock in an orbital shaker (120 rpm) for 24 hrs. Subcultures were grown in Erlenmeyer flasks containing 100 ml BHB + G (1.0 %; w/v) + P (50 µg ml^{-1}) medium using 7 % (v/v) inoculum for three successive cycles.
2. Glucose was added at a concentration of 1.0 % (w/v) for enhanced growth of the isolates and increased cell biomass so that the time course of pyrene uptake and metabolism by bacterial isolates could be accelerated.
3. Culture flasks were withdrawn after every 24 hrs interval of time and culture samples used to determine growth (as described in section 3.5.3.1), pyrene uptake by spectrophotometric (as described in section 3.5.4.2) and HPLC (as described in section 3.5.4.3) analysis, biosurfactant activity (as described in section 3.5.1.6), increase in cell protein by biuret method (as described in section 3.5.3.2) and percent glucose utilization by DNS method (as described in section 3.5.3.3).

3.5.*1.6 Biosurfactant activity*

Biosurfactant activity during pyrene utilization phase of growth was assessed for the bacteria isolated from crude, diesel oil (PK-11 to PK-14) and coal-tar (PK-1 to PK-10)

contaminated soil using the standard emulsification assay (index) reported by Barkay *et al.* (1999) and Jacques *et al*. (2007). The incubation temperature for PK-1 to PK-5 and PK-12 to PK-14 isolates was 30 ^{o}C and for PK-6 to PK-10 isolates was 45 ^{o}C.

Requirements

1. *Glass tube (150 mm × 18 mm)*
2. *Mobil oil (Racer 2T, Hindustan Petroleum Ltd, India)*
3. *Vortex mixer*
4. *Orbital shaker – cum – incubator*
5. *Centrifuge (Hitachi, Japan)*
6. *Autopipette, microtips*
7. *Stopwatch*
8. *Glass cuvettes*
9. *UV - Vis spectrophotometer (Hitachi model U-2900, Japan)*

Procedure

1. Bacterial isolates were inoculated in BHB + G (1.0 %; w/v) + P (50 µg ml^{-1}) medium in triplicate Erlenmeyer flasks (250-ml capacity) for 4 days with orbital shaking (120 rpm).
2. One culture flask was withdrawn every 24 hrs and culture was centrifuged at 10000 rpm, 4 ^{o}C for 30 min.
3. A 5 ml aliquot of the culture supernatant was mixed by vigorous vortexing with 100 µl Mobil oil (~ 2 %; v/v) in a glass tube for 1 min and kept undisturbed for 10 min.
4. The degree of dispersion of Mobil oil and stability of the emulsion was measured in triplicates spectrophotometrically at 550 nm against a blank of uninoculated medium with 2 % (v/v) Mobil oil.

3.5.2 Pyrene degradation

The mechanism of pyrene breakdown and assimilation as carbon source was evaluated in bacterial isolate *Bacillus licheniformis* (PK-6) MTCC 1005. GC-MS technique was used for intermediate metabolites and product determination (Stingley *et al*., 2004a; Liang *et al*., 2006; Karabika *et al.*, 2008).

Requirements

1. *Erlenmeyer flasks (250-ml)*
2. *Laminar air-flow*
3. *Orbital shaker – cum – incubator*
4. *Autopipette, microtips*

Procedure

1. Bacterial culture was developed in 10 ml BHB + G (0.5 %; w/v) medium from 100 µl glycerol stock in an orbital shaker (120 rpm) for 24 hrs.
2. BHB medium (100 ml) containing 50 µg ml^{-1} pyrene and 1 % (w/v) glucose was inoculated with the culture using 7 % (v/v) inoculum and incubated in triplicate at 45 °C for 4 days. Flasks containing uninoculated medium served as 'control'.
3. At every sampling point of 24 hrs the respective triplicate flasks containing growing culture samples were withdrawn, processed by liquid-liquid extraction (as described in 3.5.4.1) and subjected to GC-MS analysis (as described in section 3.5.4.5).
4. Amount of pyrene extracted from control flasks (without inoculum) before and after completion of experiment was used to determine extraction efficiency.
5. The peaks obtained by GC-MS analysis of culture extracts pertained to catabolic products of pyrene. These were identified by probabilistic search (PBM) by comparing the fragmentation pattern and their abundance with the standard mass spectra of known compounds in NIST database library stored in the Varian MS Chemstation library.

3.5.3 General techniques

3.5.3.1 Measurement of growth

Growth was measured spectrophotometrically.

Requirements

1. *Blank (uninoculated media)*
2. *Laminar air flow*

3. *Autopipette, microtips*
4. *Glass cuvettes*
5. *UV-Vis spectrophotometer (Hitachi model U-2900, Japan)*

Procedure

1. Culture samples (2 ml) were aseptically withdrawn from culture flask in to glass cuvettes.
2. Optical density was measured at 600 nm against a media blank using spectrophotometer.

3.5.3.2 Estimation of protein

Protein was determined by Biuret method of Itzhaki and Gill, (1964) using a standard solution of bovine serum albumin (Fraction V).

Requirements

1. *Test tubes (150 mm × 18 mm)*
2. *Autopipette, microtips*
3. *Vortex mixer*
4. *Stopwatch*
5. *Quartz cuvettes*
6. *UV-Vis spectrophotometer (Hitachi model U-2900, Japan)*
7. *Protein sample (bacterial culture sample)*
8. *Bovine serum albumin (Fraction V) solution*

Composition	
Bovine serum albumin (Fraction V)	1 mg
Distilled water	1 ml

9. *Biuret reagent*

Composition	**(g l^{-1})**
NaOH	300
$CuSO_4.5H_2O$	2.1

The components were dissolved in distilled water in separate beakers. First NaOH was dissolved in 500 ml distilled water in a beaker kept in ice-bath followed by addition of cupric sulphate solution to the beaker with continuous stirring. The

final volume was adjusted to 1 L with distilled water. Biuret reagent was discarded if black or reddish precipitates persisted.

Procedure

1. An aliquot of the bacterial culture / cell suspension was taken in a test tube (in triplicate) and volume made up to 2 ml using distilled water. This served as protein sample. Alternatively, for protein standard curve, aliquots of bovine serum albumin (Fraction V) solution were taken in test tubes and volume made up to 2 ml using distilled water.
2. The protein sample was mixed with 1 ml biuret reagent and vortexed vigorously. In case of 'reagent blank' the protein sample was replaced by 2 ml distilled water.
3. Precisely after 10 min the optical density was measured in quartz cuvettes at 310 nm against a reagent blank using UV-Visible spectrophotometer.
4. A standard graph of optical density against protein concentration was prepared.
5. Using the standard graph, protein content in protein sample was calculated.

3.5.3.3 Estimation of glucose

Glucose was determined by 3,5-Dinitrosalicylic acid (DNS) method (Plummer, 1988) using a standard solution of glucose.

Requirements

1. *Test tubes (150 mm × 18 mm)*
2. *Autopipette, microtips*
3. *Vortex mixer*
4. *Stopwatch*
5. *Glass cuvettes*
6. *UV-Vis spectrophotometer (Hitachi model U-2900, Japan)*
7. *Boiling water bath*
8. *Marbles*
9. *Sugar standard solution: Dissolved 1 g glucose in 1 L distilled water.*
10. *Sodium hydroxide (2 M) solution*
11. *Sodium potassium tartrate stock solution*

Composition

Sodium potassium tartrate	300 g

Distilled water	500 ml

12. 3,5-Dinitrosalicylic acid stock solution

Composition

3,5-Dinitrosalicylic acid	10 g
NaOH (2 M)	200 ml

Heated the mixture to dissolve.

13. *Dinitrosalicylic acid reagent:* Prepared fresh by mixing solutions (11) and (12) and made up the volume to 1 litre with distilled water.

Procedure

1. An aliquot of the bacterial culture supernatant was taken in a clean and dry test tube (in triplicate) and volume made up to 3 ml using distilled water. This served as sugar sample. Alternatively, for standard curve, aliquots of sugar standard solution were taken in test tubes and volume made up to 3 ml using distilled water.
2. The sugar sample was mixed with 1 ml dinitrosalicylic acid reagent and vortexed vigorously. In case of 'reagent blank' the sugar sample was replaced by 3 ml distilled water.
3. Each tube was covered with a marble and placed in a boiling water bath for 5 min and then cooled to room temperature.
4. The optical density was measured in glass cuvettes at 540 nm against a reagent blank using UV-Visible spectrophotometer.
5. A standard graph of optical density against sugar concentration was prepared.
6. Using the standard graph, the initial and residual glucose content in sugar samples was calculated.

Precaution

1. DNS reagent must be prepared just before use.
2. The test tubes must be cooled to room temperature properly before readings since the optical density in sensitive to temperature.

3.5.4 Bioanalytical techniques

Pyrene uptake was monitored by liquid-liquid extraction of residual pyrene from growth medium (inoculated or noninoculated) followed by spectrophotometric and chromatographic (HPLC, GC-MS) analysis.

3.5.4.1 Sample preparation by liquid - liquid extraction

Metabolic activity in periodically withdrawn culture flasks was stopped by acidification (Heitkamp *et al.*, 1988b; Vila *et al.*, 2001; Jacques *et al.*, 2007) followed by solvent extraction of non-utilized pyrene from spent growth medium (Cerniglia, 1992; Khan *et al.,* 2001; Kim *et al.,* 2005; Lopez *et al.*, 2006; Jacques *et al.,* 2007; Cottin and Merlin, 2007) at room temperature. The culture was neither heated (Liang *et al.*, 2006) nor sonicated to prevent any degradation of metabolites. Extraction efficiency was found to be in the range of 87 % $\pm$ 3 %.

Requirements

1. *HCl (6 N)*
2. *Acetone (HPLC grade)*
3. *Hexane (fraction from petroleum) (HPLC grade)*
4. *Acetonitrile (HPLC grade)*
5. *Cyclohexane (HPLC grade)*
6. *Measuring cylinder*
7. *Glass crucible*
8. *Pasteur pipette (3-ml)*
9. *Sample storage cryovials (1-ml, 2-ml, 5-ml)*
10. *0.2-µm syringe filters (Millipore)*
11. *Orbital shaker*
12. *Autopipette, microtips*

Procedure

1. The spent culture medium in periodically withdrawn flask(s) was acidified to pH 2 using 6 N HCl and mixed with hexane solvent, 10 ml (in pulse chase expt) or 50 ml (time course expt) at a time, by vigorous vortexing in an orbital shaker (150 rpm) for 15 – 20 minutes.
2. The liquid – solvent mixture in flask(s) was allowed to settle in -20 ^{o}C to facilitate layer separation. The upper solvent layer containing pyrene was carefully pipetted out

in a labeled glass crucible. This extraction procedure was repeated atleast three times to obtain high extraction efficiency.

3. The pooled hexane extract was completely evaporated to dryness in a fume hood chamber, redissolved in 3 ml acetone, collected in 5-ml capacity storage vial and stored at -20 ^{o}C for spectrophotometric and chromatographic (HPLC, GC-MS) analysis.

3.5.4.2 Spectrophotometric analysis

Pyrene uptake was monitored spectrophotometrically as per the method of Bugg *et al.* (2000).

Requirements

1. *Acetonitrile (HPLC grade)*
2. *Sample storage cryovials (5-ml)*
3. *Autopipette, microtips*
4. *Quartz cuvettes*
5. *UV-Vis spectrophotometer (Hitachi model U-2900, Japan)*

Procedure

1. Pyrene extracts in acetone solvent obtained from triplicate culture samples were air-dried and redissolved in 5 ml acetonitrile (ACN) solvent.
2. The optical density of acetonitrile extracts was measured in a quartz cuvette at 254 nm using UV-visible spectrophotometer.
3. Solvent extract from uninoculated medium was used as blank or control to determine extraction efficiency.
4. Difference in amount of pyrene extracted from control (uninoculated) flask(s) and inoculated flask(s), withdrawn from incubation at different time intervals during experiment(s), denoted uptake of pyrene by the inoculated bacterial strain(s).

3.5.4.3 High - Performance Liquid Chromatographic (HPLC) analysis

Spectrophotometric results were confirmed by quantifying the amount of pyrene by reverse-phase high-performance liquid chromatography. The protocol has been developed from Heitkamp *et al*. (1988b), Boldrin *et al*. (1993), Schneider *et al*. (1996), Boonchan *et al*. (2000), Wang *et al*. (2000), Stingley *et al*. (2004a) and Kim *et al.* (2004b, 2006).

Requirements

1. *Perkin-Elmer liquid chromatographic system (LC 200 Series) containing, a Perkin-Elmer binary solvent system pump, a Rheodyne manual injector and a Perkin-Elmer Diode array detector, all controlled by Perkin-Elmer TotalChrom ver 6.2.1 chromatographic software.*
2. *Analytical HPLC column: Perkin-Elmer RP-C18, dimensions = 33 [length] x 4.6 mm (inside diameter); 5-µm particles.*
3. *Guard column: Perkin-Elmer, dimensions = 25 by 4.6 mm (inside diameter); 7-µm particles.*
4. *Vacuum filtration assembly*
5. *Vacuum pump*
6. *Sonicator*
7. *Sample injection syringe (50-µl capacity) (Hamilton, USA)*
8. *Autopipette, microtips*
9. *Acetonitrile (HPLC grade)*
10. *Methanol (HPLC grade)*
11. *Milli-Q water*
12. *0.45-µm solvent filtration membrane (Millipore)*
13. *Measuring cylinder*

Procedure

1. Pyrene extracts in acetone solvent obtained from triplicate culture samples were air-dried and redissolved in 5 ml acetonitrile (ACN) solvent.
2. A 0.02 ml aliquot of ACN extract was injected onto guard column and analytical column.
3. A linear gradient of 50 - 95 % methanol in MQ-water was developed over 20 min at a flow rate of 1 ml min^{-1} (Heitkamp *et al*., 1988b; Stingley *et al*., 2004a; Kim *et al.,* 2004b, 2006)**.**
4. The UV A_{254} of the eluate was monitored for all injections (Boldrin *et al*., 1993; Schneider *et al*., 1996; Boonchan *et al*., 2000; Kim *et al*., 2004b).
5. Pyrene was identified by comparing characteristic absorption spectra (at 254 nm) and retention times to authentic pyrene (Wang *et al*., 2000) and quantified with the help of

reduction in peak areas compared to uninoculated control or by multiplying the peak area obtained with a regression factor, derived from the standard curve of pyrene.

6. Amount of pyrene extracted from control flask (without inoculum) after completion of experiment was considered as 100 %. The percentage of pyrene uptake by bacterial isolates was calculated relative to pyrene concentration in control flasks (Sarma *et al*., 2004; Jacques *et al*., 2007). Pyrene extraction efficiency was found to be 87 % $\pm$ 3 %. The minimum limit of detection by HPLC analysis was 0.1 µg ml^{-1} pyrene.

3.5.4.4 Gas chromatographic (GC) – Flame ionization detection (FID) analysis

The total aromatic fractions present in coal-tar contaminated soil sample, obtained from 0 - 25 cm depth, were analyzed by capillary GC-FID. The protocol has been adopted and modified from Mueller *et al.* (1989a), Boldrin *et al.* (1993), Willsch and Radke (1995), Juhasz (1998) and Mishra *et al.* (2001).

Requirements

1. *Gas chromatograph apparatus equipped with a Flame ionization detector (Agilent, Palo Alto, CA), all controlled by NuChrom software.*
2. *Analytical GC column: Zebron ZB-5 capillary column, dimensions = 30 m (length) x 0.25 mm (inside diameter) x 0.25 µm (coating film thickness)]*
3. *Gas cylinders of nitrogen, air and hydrogen*
4. *Sample injection syringe (10-µl capacity) (Hamilton, USA)*

Procedure

A. Sample preparation:

1. Five grams of sub soil was mixed with 100 ml of hexane (fraction from petroleum) solvent by vigorous vortexing in an orbital shaker (150 rpm) at room temperature for 30 minutes.
2. The soil-solvent mixture was allowed to settle to facilitate layer separation. The solvent extract was collected in a glass crucible. This extraction procedure was repeated atleast three times.
3. The pooled hexane extracts were completely evaporated to dryness in a fume hood, redissolved in 5 ml of acetone, transferred into 5-ml capacity storage vials and stored in -20 ^{o}C for GC analysis.

B. Analysis:

1. Acetone extract (10 µl) was analyzed by ZB-5 capillary column, in an oven programmed to initially hold at 50 °C for 2 min, then rise to 275 °C at a rate of 4 °C min $^{-1}$, again rise to 320 °C at a rate of 10 °C min $^{-1}$ and finally hold at 320 °C for 20 min (Juhasz, 1998).
2. Nitrogen was used as the carrier gas (40 ml min^{-1}), while air (300 ml min^{-1}) and hydrogen (40 ml min^{-1}) were supplied for the flame ionization detector.
3. The operating temperature of the flame ionization detector was 320 °C and that of the injector was 275 °C.
4. The injector was used in the split mode, with a split ratio of 1:5.
5. PAHs were determined by reference to pure standards (Sigma-Aldrich) dissolved in acetone (10 µg ml^{-1} each): naphthalene, acenaphthene, acenaphthylene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, indeno[*1,2,3-c,d*]pyrene, dibenz[*a,h*]anthracene and dibenz[*g,h,i*]perylene.

3.5.*4.5 Gas chromatography-Mass spectrometry (GC-MS) analysis*

GC-MS analysis was performed for catabolic intermediate metabolites and product determination (Juhasz, 1998; Martin and Mohn, 1999; Stingley *et al.*, 2004a; Karabika *et al.*, 2008).

Requirements

1. *Varian CP 3800 Gas chromatograph apparatus equipped with a Varian Saturn 2200 GC/MS/MS mass detector, 8400 series autosampler, all controlled by Varian Saturn GC/MS Workstation version 6.40 software.*
2. *Analytical GC column: DB-5ms capillary column (J&W Scientific, Folsom, California), dimensions = 30 m [length] x 0.25 mm [inside diameter]; 0.25 µm film thickness).*
3. *0.2-µm syringe filters (Millipore)*
4. *Helium gas cylinder*
5. *Sample injection syringe (10-µl capacity) (Hamilton, USA)*

Procedure

1. Acetone extracts were dried, redissolved in 1 ml of cyclohexane (CHX) solvent, filtered through 0.2-µm syringe filters and analyzed chromatographically by GC-MS (Stingley *et al.*, 2004a).
2. Compounds in CHX extracts were separated using a DB-5ms capillary column and helium as the carrier gas at 1 ml min^{-1} flow rate.
3. The GC oven temperature was initially kept at 70 °C for 1.5 min, programmed first to 200 °C at a rate of 10 °C min^{-1}, then to 280 °C at a rate of 5 °C min^{-1} and final hold at 280 °C for 5 min (Martin and Mohn, 1999; Karabika *et al*., 2008).
4. The mass spectrometer was operated at 70 eV of electron ionization energy. Mass spectrum was analysed from 5^{th} min to 41^{st} min in 60 - 450 mass range.
5. The ion trap temperature was 200 °C, transfer line temperature was 280 °C and emission current was 12 µamps.
6. The injector and analyzer temperatures were held at 250 and 285 °C, respectively for complete run.
7. Data was collected and integrated with a personal computer using the Varian Saturn GC/MS Workstation version 6.40.
8. MS scan was referred to NIST database library for identification of the peaks in chromatograph.

3.6 GENETIC REGULATION OF PYRENE DEGRADATION IN THE SELECTED ISOLATE

3.6.1 Plasmid profiling of pyrene utilizing isolates

To study the plasmid profile of pyrene utilizing isolates, followed Birnboim and Doly's (1979) alkaline lysis method for low-molecular-weight plasmids (as described in section 3.4.4.8), as well as Kado & Liu's (1981) method for high-molecular-weight plasmid detection, using transformed *E. coli* DH5α strain as control.

3.6.1.1 Isolation of plasmid DNA (Kado and Liu, 1981)

Requirements

1. *E buffer*

Composition

Tris acetate	50 mM
EDTA	2 mM

Buffer pH was adjusted to 7.9 using glacial acetic acid

2. *Lysis buffer*

Composition

SDS	3 %
Tris	50 mM

Buffer pH was adjusted to 12.5 using 2 N NaOH

Procedure

1. A single bacterial colony was picked from a freshly grown LA plate, transferred into 5 ml LB medium in a cotton plugged test tube and incubated for 16-20 hrs at 30 ^{o}C or 45 ^{o}C (respective incubation temperature) in an orbital shaker (120 rpm).
2. Harvested cells from 2.0 ml of the saturated culture by centrifugation at 10,000 rpm for 5 minutes. The supernatant was discarded, leaving the bacterial pellet as dry as possible.
3. The bacterial pellet was resuspended in 0.1 ml of E buffer by vigorous vortexing to ensure that the bacterial pellet is thoroughly dispersed in this solution.
4. Lysis buffer (0.2 ml) was added to the eppendorf tube and closed the tube tightly. The tube contents were mixed by gentle inversion of the tube ten to twelve times rapidly. Vortexing was avoided here and the tubes were incubated at 65 ^{o}C for 30 minutes.
5. The crude lysate was cooled at 4 ^{o}C and 0.3 ml of phenol: chloroform: isoamylalcohol (24:25:1) was added. The contents were mixed by inverting the tube for 2 minutes and then centrifuged at 10,000 rpm for 25 minutes at 4 ^{o}C.
6. The supernatant, containing plasmid DNA, was carefully transferred to a fresh sterile eppendorf tube and stored at –20 ^{o}C for further use.

3.6.1.2 Analysis of DNA

The DNA samples were analyzed by horizontal agarose gel electrophoresis (section 3.4.4.2) using aid of DNA molecular wt. markers (1.0 kb DNA ladder, Lambda / *Hind* III DNA ladder).

3.6.2 Polymerase chain reaction (PCR) amplification of Rieske centre(s)

Presence of Rieske center (the conserved [Fe_2-S_2] cluster binding region) of terminal PAH dioxygenases was determined in the pyrene-utilizing isolates belonging to *Bacillus*, *Pseudomonas* and *Rhodococcus* genera, isolated from PAH-contaminated soils. PCR experiments using degenerate primers specific for Rieske gene (Brezna *et al.,* 2003) were conducted to amplify the gene.

What are Degenerate primers?

The need to design degenerate primers arises when PCR primer sequences are deduced from amino acid sequences as the exact nucleotide sequence of their target is unknown. However, because of the degeneracy of the genetic code, the deduced sequences may vary at one or more positions. A common solution in such cases is to use a degenerate primer. A degenerate primer is defined as a mixture of similar primers that have different bases at the variable positions. A wide range of melting temperatures is typically encountered with these primers (Löffert *et al*., 2008). Since only a low proportion of the primer molecules in a degenerate primer pool match the target sequence, amplification efficiency may decrease. One possibility to enhance PCR efficiency is to increase primer concentration, although there will be a greater risk of mispriming and generation of nonspecific PCR products. It is advised to begin PCR with a primer concentration of 0.2 µM. In case of poor PCR efficiency, increase primer concentration in increments of 0.25 µM until satisfactory results are obtained (Löffert *et al*., 2008).

Requirements

1. *Sterile eppendorf tubes (0.2-ml)*
2. *Autopipettes and sterile microtips*
3. *Spinwin (Tarsons)*
4. *GeneAmp® PCR system 9700 (Applied Biosystems)*
5. *Autoclaved Milli-Q water*
6. *Deoxyribonucleotides dATP, dGTP, dCTP, dTTP*
7. *Taq DNA Polymerase*
8. *Genomic DNA:* extracted from pyrene-utilizing bacterial isolates (as described in section 3.4.4.1).

9. *A set of degenerate primers:* designed from the conserved [Fe_2-S_2] cluster binding region (Rieske center) of terminal PAH dioxygenase genes (adapted from Brezna *et al.*, 2003) for the amplification of Reiske centre.

   Degenerate primer designation and sequence:

   ⇒ **Rf1** (*Mycobacterium* sp. nucleotide position 289 - 302 *): 5'-TG**Y** **M**G**N** CA**Y** **M**G**N** GG -3'

   ⇒ **Rr1** (*Mycobacterium* sp. nucleotide position 360 - 348 *): 5'- CCA **N**CC **R**TG **R**TA **NSW** **R**CA-3'

   (Degenerate nucleotides: **N**=G, A, T, C; **S**=G, C; **W**= A, T; **M**=A, C; **Y**=C, T; **R**= A, G)

   * Position relative to *nidA* gene from *Mycobacterium vanbaalenii*, GenBank accession number AF249301).

Procedure

A. Amplification:

1. PCR reactions were carried out in a final volume of 20 µl containing 1 X reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 1.5 µM of each primer, 0.01 µg template DNA, and 1.5 units of *Taq* DNA polymerase in thin-walled 0.2-ml reaction tubes placed in a GeneAmp® PCR system 9700.
2. Positive (enzyme missing) and negative (template missing) controls were included.
3. PCR program was as follows: initial hold of 3 min at 95 ^oC was followed by 40 cycles of 1 min denaturation at 95 ^oC, 1 min annealing at 53 ^oC and 1 min extension at 72 ^oC, followed by a final hold at 72 ^oC for 7 min.

B. Analysis:

4. PCR reaction product (amplicon) was analyzed by agarose (2 %; w/v) gel electrophoresis of the total PCR reaction, followed by UV transillumination of the EtBr -stained gel as described in section 3.4.4.2.
5. The total concentration of PCR product was estimated by comparison to DNA mass standards GeneRuler TM DNA Ladder Mix and/or GeneRuler 100 bp DNA ladder on agarose gel.
6. PCR reaction products were stored at -20 °C for further use.

3.6.3 Biochemical pathway of pyrene degradation

3.6.3.1 Biochemistry

Growth experiments conducted in section 3.5.1.5 helped to determine the change in pyrene concentration in growth medium. GC-MS profiling of growth extracts of bacterial isolate *Bacillus* sp. (PK-6) MTCC 1005 as described in section 3.5.2 led to the identification of possible metabolites.

3.6.3.2 Bioinformatics

The knowledge of bioinformatics was used to determine the chemical rules of biotransformations from the University of Minnesota Biocatalysis / Biodegradation Database (UM-BBD, 2003; website http://umbbd.ahc.umn.edu/) along with the possible reaction mechanisms. With the help of these rules, the classification of pyrene degradation products into primary, secondary metabolites or final products could be verified. Based on the results obtained a tentative pathway for pyrene degradation in *Bacillus* sp. (PK-6) MTCC 1005 was proposed.

3.7 REMOVAL OF PYRENE BY SELECTED BACTERIAL ISOLATE FROM SOIL CONTAMINATED WITH PYRENE

Removal of pyrene from coal-tar contaminated soil (collected from industrial site) and garden soil spiked with pyrene by *Bacillus* sp. (PK-6) MTCC 1005 at room temperature conditions was studied.

3.7.1 Soil collection

3.7.1.1 Coal-tar contaminated composite soil

1. Coal-tar contaminated soil samples were collected from a Hot-mix coal-tar Industrial Plant site (Patiala, Punjab) from four different areas. Presence of pyrene in soil was confirmed by GC-FID analysis (section 3.3.1.4).
2. Completely dried soil samples were sieved through 0.5 mm mesh and thoroughly mixed in equal ratios.

3.7.1.2 Garden soil

1. Garden soil was collected from STEP, Thapar University campus, crushed and air-dried.
2. Completely dried soil was sieved through 0.5 mm mesh, autoclaved three times at 121 ^{o}C for 1 hr.

3.7.2 Characterization of soil

3.7.2.1 pH

Soil pH was determined potentiometrically in a soil - water suspension of 1:5 ratio using an electronic pH meter as per the method given by Zhou *et al*. (1996) as described in 'Materials and Methods' section 3.3.1.1.

3.7.2.2 Electrical conductivity

Electrical conductivity was measured in µS cm^{-1} as per the method given by Rayment and Higginson (1992) in a 1:5 soil-to-water suspension as described in 'Materials and Methods' section 3.3.1.2.

3.7.2.3 Moisture content

Moisture in soil was determination in percentage water content as per the method of Black (1965) as described in 'Materials and Methods' section 3.3.1.3.

3.7.2.4 Organic carbon

Organic carbon was estimated as per the chromic acid oxidation method given by Walkley and Black (1934). This method used a temperature of approximately 120 ^{o}C, obtained in the heat-of-dilution reaction of concentrated H_2SO_4 acid (Allison, 1965), to drive the oxidation of active forms of organic C in **test sample** to CO_2 but not the more inert forms. The unreduced $Cr_2O_7^{2-}$ was measured by titration.

Requirement

1. *Erlenmeyer flasks (500-ml)*
2. *Glass pipettes (10-ml)*
3. *Dispensers (10 and 20-ml)*
4. *Burette (50-ml)*

5. *Analytical balance*
6. *Orthophosphoric acid (H_3PO_4; 85 %)*
7. *Concentrated sulphuric acid (H_2SO_4; 96 %)*
8. *Sodium fluoride (NaF)*
9. *Standard 1 N potassium dichromate solution* – 49.04 g of dried (105 ^oC) $K_2Cr_2O_7$ was dissolved in water and diluted to 1 litre.
10. *0.5 N ferrous ammonium sulphate (Fe^{2+}) solution* – 196.1 g of $Fe(NH_4)_2(SO_4).6H_2O$ was dissolved in 800 ml of water containing 20 ml of concentrated H_2SO_4 and diluted to 1 litre. The Fe^{2+} in this solution oxidized slowly on exposure to air so it was standardized against the dichromate daily.
11. *Diphenylamine indicator* – 0.5 g of diphenylamine was dissolved in a mixture of 20 ml water and 100 ml concentrated sulphuric acid.

Procedure

1. One gram (1 g) of sample was weighed for light brown colored STEP garden soil and 0.5 g for dark grey coal-tar contaminated soil and transferred to a 500-ml Erlenmeyer flask. Ten milliliters (10 ml) of 1 N $K_2Cr_2O_7$ was added by means of a pipette and the flask was swirled for mixing the soil and reagent.
2. Concentrated H_2SO_4 (20 ml) was added by means of dispenser, swirled gently to mix and allowed to stand undisturbed for 30 minutes.
3. The suspension was diluted with 200 ml distilled water to provide a clearer suspension for viewing the endpoint.
4. To the mixture 10 ml of 85 % H_3PO_4 and 0.5 g of NaF was added. H_3PO_4 and NaF are added to complex Fe^{3+} which would interfere with the titration endpoint.
5. Diphenylamine indicator (1 ml) was added to the flask.
6. The flask contents were ultimately titrated with 0.5 N Fe^{2+} solution to a grassy-green endpoint. The colour of the solution at the beginning was dark blue, depending on the amount of unreacted $Cr_2O_7^{2-}$ remaining, which shifted to a clear blue-violet before the endpoint and then changed sharply to grassy-green at the end point.
7. A reagent blank was also run following the above procedure without soil. The reagent blank was used to standardize the Fe^{2+} solution.
8. The soil was tested in triplicate and mean and standard error (SE) values were calculated using Microsoft Excel 2003.

Precaution

1. The flask contents must be swirled gently to mix. Excessive swirling must be avoided as it would result in organic particles adhering to the sides of the flask out of the solution.
2. Diphenylamine indicator should be added just prior to titration to avoid deactivation by adsorption onto clay surfaces.
3. If less than 5 ml of FAS solution is required to back-titrate the excess $Cr_2O_7^{2-}$ there must be insufficient $Cr_2O_7^{2-}$ present, and the analysis should be repeated either by using a smaller sample size or doubling the amount of $K_2Cr_2O_7$ and H_2SO_4.

Calculation

Organic carbon in soil sample(s) was calculated using the formula:

$$\% C = \frac{(B - S) \times M \text{ of } Fe^{2+} \times 0.003 \times 100}{\text{grams of soil}}$$

B = volume (ml) of ferrous ammonium sulphate (Fe^{2+}) solution used to titrate blank

S = volume (ml) of ferrous ammonium sulphate (Fe^{2+}) solution used to titrate soil sample

M = Molarity

0.003 = $^{12}/_{4,000}$, the milliequivalent weight of C in grams

3.7.2.5 Available phosphorus

Available phosphorus in the soil samples was estimated by the sodium bicarbonate method given by Olsen *et al*. (1954). The detection limit of this method is approximately 2.0 mg kg^{-1} (air dried soil basis) and varies plus or minus 12 %.

Requirements

1. *Erlenmeyer extraction flasks (100-ml)*
2. *Filter funnels (9 to 11-cm)*

3. *Whatman No. 42 (or equivalent) filter paper (9 to 11-cm)*
4. *Volumetric flasks (50-ml)*
5. *Glass pipettes*
6. *Orbital shaker*
7. *UV-VIS spectrophotometer*
8. *Extracting Solution* (0.5 M $NaHCO_3$; pH 8.5)

   Composition

Sodium bicarbonate	42 g
Distilled water	1 L

   Dissolved sodium bicarbonate in distilled water and made up the volume to 1 litre. A magnetic stirrer was used to dissolve $NaHCO_3$. The pH was adjusted to 8.5 with I N NaOH.
9. *Acid Molybdate Stock Solution*

   Reagent A

   i) 12 g of ammonium molybdate, $(NH_4)_6Mo_7O_{24}.4H_2O$, was dissolved in 250 ml of distilled water
   ii) 0.291 g of antimony potassium tartarate was dissolved in 100 ml of distilled water
   iii) Both solutions were added to 1000 ml of 2.5 M H_2SO_4 (148 ml of concentrated H_2SO_4 per litre of water - ADDED ACID TO WATER).
   iv) Mixed thoroughly and diluted to 2000 ml with distilled water.
   v) The reagent was stored in a glass, pyrex bottle in a dark, cool place.

   Reagent B

   i) 1.058 g of ascorbic acid was dissolved in 200 ml of reagent A and mixed.

   NOTE: This reagent must be freshly prepared since it will not keep good for more than 24 hrs.

10. *Stock standard P solution*: 50 ppm

    i) 0.2197 g of dry potassium dihydrogen phosphate (KH_2PO_4) was dissolved in about 25 ml of distilled water.
    ii) The above solution was diluted to a final volume of 1000 ml with extracting solution and was stored in the dark under refrigeration.

    NOTE: Stock standard P solution was stable for 6 months.

11. *Working Standard P solution*: 1 ppm

    20 ml of (50 ppm P) solution was diluted to 1000 ml with extracting solution.

Procedure

1. A 2.5 g soil sample was weighed into a 100-ml Erlenmeyer flask followed by the addition of 50 ml extracting solution.
2. The solution was kept on a shaker at 200 rpm, at 24 to 27 ^{o}C, for 30 minutes and then filtered through Whatman No. 42 filter paper.
3. A 10 ml aliquot of the filtrate was transferred to a 100-ml beaker or Erlenmeyer flask followed by addition of 1 ml of 2.5 M H_2SO_4, 15.5 ml of distilled water, 8 ml of Reagent B and another 15.5 ml of distilled water. The flask was agitated for thorough mixing of contents.
4. The flask contents were then allowed 10 minutes for color development. Optical density was read on a spectrophotometer at 882 nm.
5. A blank was prepared same as above but without soil.
6. A standard curve was prepared by pipetting a 10 ml aliquot of each of the working P standards, developed color and read intensity in the same manner as with the soil extracts. In the standard graph, intensity was plotted against concentration of the working P standards. Available phosphorus concentration in soil extract was determined from intensity and standard graph.
7. For the standard curve: 0, 2, 5, 10, 15 and 20 ml of standard P solution was placed in 50-ml volumetric flasks separately. Ten milliliters of extracting solution, 1.0 ml of 2.5 M H_2SO_4, 8 ml Reagent B was added and the final volume was made upto 50 ml. The P concentrations of these solutions were 0, 2, 5, 10, 15 and 20 ppm, respectively (Table 2.4). After 10 minutes, the P concentration was read at 882 nm.
8. The soil was tested in triplicate and mean and standard error (SE) values were calculated using Microsoft Excel 2003.

Precaution

1. The blue color developed was stable for ~2 hrs.

Calculations

P concentration in filtrate (ppm) was converted to P concentration in soil using the formula:

P in soil (ppm) = P in extract (ppm) x 20 (the standard soil to solution ratio)

P in soil (lb $acre^{-1}$) = P in extract (ppm) x 40 (the standard soil to solution ratio).

Table 2.4 Working standard solution for the Olsen test (Frank *et al.,* 1998).

Volume of 50 ppm stock P solution (ml)	**Final volume** (ml)	**Concentration of working P standard** (ppm P)	**Equivalent P concentration in soil** (ppm P)
1	50	1.0	20.0
2	50	2.0	40.0
3	50	3.0	60.0
4	50	4.0	80.0
5	50	5.0	100.0
10	50	10.0	200.0
15	50	15.0	300.0
20	50	20.0	400.0

3.7.2.5 Total nitrogen

Total nitrogen was estimated as per the Kjeldahl method given by Piper, (1960).

Requirements

1. *Kjeldahl flask*
2. *Analytical balance*
3. *Digestion chamber*
4. *Glass beads*
5. *Round flat-bottomed distillation flasks*
6. *100-ml beaker*
7. *Glass burette*
8. *Glass pipette*
9. *Concentrated H_2SO_4.*
10. *0.02 N H_2SO_4*
11. *Sulphuric-Salicylic acid = 1 g salicylic acid mixed with 30 ml sulphuric acid*

12. *Sodium thiosulphate ($Na_2S_2O_3$)*
13. *4 % boric acid*
14. *Mixed indicator* - 0.066 g of methyl red and 0.099 g of bromocresol green dissolved in 100 ml of ethyl alcohol.
15. *50 % NaOH*
16. *Digestion mixture*

Composition	
HgO	10 g
$CuSO_4$	5 g
K_2SO_4	100 g

Procedure

1. Five gram soil sample was taken in a Kjeldahl flask and mixed thoroughly with sulphuric-salicylic acid followed by 5 g of sodium thiosulphate. The flask was heated for 5 minutes followed by cooling and addition of 10 g digestion mixture.
2. The flask contents were mixed well and kept in the digestion chamber at 100 °C for 2 - 4 hrs. During sample digestion, color change of flask contents was monitored from dark brown to greenish white.
3. After the contents were cooled 300 ml distilled water was added to the flask.
4. A 20 ml aliquot of the digested sample, 15 - 20 ml of 50 % NaOH and glass beads were added to a distillation flask through the open end of the condensor attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml of 4 % boric acid and 2 drops of mixed indicator till the end-point color changed from pink to green.
6. The distillate was titrated against 0.02 N H_2SO_4 till the endpoint (green to pink) was obtained.
7. The soil was tested in triplicate and mean and standard error (SE) values were calculated using Microsoft Excel 2003.

Calculation

$$\text{Total nitrogen (\%)} = \frac{(T - B) \times \text{Normality of } H_2SO_4 \times 1.4 \times 300}{\text{Weight of soil sample}}$$

where T is the titre value for sample and B is for blank.

3.7.3 Experimental set up

3.7.3.1 Coal-tar contaminated soil experiment

Requirements

1. *Composite soil sample*
2. *Analytical balance*
3. *BOD Incubator*
4. *250-ml glass beakers*
5. *Measuring scale*
6. *Spatula*
7. *70 % (v/v) alcohol*
8. *Sampling bags*
9. *Marker*
10. *Saline solution (0.85 %; w/v)* – dissolved 0.85 g of sodium chloride (NaCl) in 100 ml of distilled water and autoclaved at 121 ^{o}C for 15 min.
11. *Glucose stock solution (20 %; w/v)* – dissolved 20 g of glucose in 100 ml distilled water and filter-sterilized.

Procedure

1. Coal-tar contaminated soil (80 g) was weighed in 250-ml glass beaker. The height of soil column in each setup was 2 cm and diameter 6.5 cm.
2. The beakers were labeled (in triplicate) as CT and CT-PK6.
3. A 10 ml aliquot of sterile 0.85 % (w/v) saline was thoroughly mixed in soil using a spatula, previously sterilized with 70 % (v/v) ethanol.
4. The beaker was covered with transparent, punched polybag and the set up was kept at 37 ^{o}C.
5. An aliquot (0.2 ml) of 20 % glucose stock solution was added to all soil beakers and mixed thoroughly using a sterile spatula.
6. *Inoculum development*: *Bacillus* sp. (PK-6) MTCC 1005 culture was developed from 100 µl of glycerol stock in duplicate flasks containing 100 ml LB medium plus

pyrene (50 µg ml^{-1}) at room temperature (37 ^{o}C) in an orbital shaker (120 rpm). The culture growth (A_{600}) was determined after 18 - 20 hrs. Log phase culture with OD_{600} equal to ~1 was harvested by centrifugation at 8,000 rpm, 4 ^{o}C for 15 min. The cell pellet was resuspended in 50 ml of 0.85 % saline + glucose (0.2 %) solution. Bacterial count (cfu ml^{-1}) in cell suspension was determined by preparing serial dilutions of cell suspension in saline and spread plating 0.1 ml aliquot of each dilution on triplicate LA plates followed by incubation at 37 ^{o}C for 24 hrs.

7. Aliquots (3 ml) of bacterial cell suspension were added to the soil beakers labeled CT-PK6. *Bacillus* sp. (PK-6) inoculum was thoroughly mixed in soil using a sterile spatula.
8. Biotic soil control, labelled CT, contained PAHs and indigenous microflora but did not receive *Bacillus* sp. (PK-6) inoculum.
9. Soil samples (20 g) were withdrawn at zero time of incubation from each beaker to determine the physico-chemical parameters like pH, EC, moisture content, total organic carbon, available phosphorus, total nitrogen, initial pyrene concentration and cfu per gram of soil.
10. The beakers were covered and incubated at 37 ^{o}C. Light was restricted from the soil cultures.
11. Saline was periodically (every 1–2 days) added to all soil treatments to replace evaporative losses. A moisture content of 35 $\pm$ 2 % in soil was maintained throughout the experiment.
12. Soil samples (5 g) were withdrawn every 7 days, till 28 days to determine change in pyrene concentration and bacterial count (cfu ml^{-1}) of soil. After completion of experiment again noted pH, EC and total organic carbon.

3.7.3.2 Pyrene spiked garden soil experiment

Requirements

1. *Garden soil*
2. *Analytical balance*
3. *Incubator at 37 ^{o}C*
4. *Black polybags*
5. *Measuring scale*
6. *Spatula*

7. *70 % alcohol*
8. *Sampling bags*
9. *Marker*
10. *Saline solution (0.85 %; w/v)* – 0.85 g of sodium chloride (NaCl) was dissolved in 100 ml of distilled water and autoclaved at 121 ^{o}C for 15 min.
11. *Glucose stock solution (20 %; w/v)* – 20 g of glucose was dissolved in 100 ml distilled water and filter-sterilized.
12. *Pyrene stock solution (4 mg ml^{-1})* – 100 mg pyrene was dissolved in 25 ml acetone solvent (HPLC grade) and stored at -20 ^{o}C.

Procedure

1. Autoclaved garden soil (1000 g) was weighed in double-layered black polybags. The inner bag was punched at sides and bottom while the outer bag was not punched. The height of soil column in each setup was 7 cm, length was 13 cm and breadth was 7.5 cm.
2. The bags were labeled (in triplicate) as S, S-P, S-G, S-P-G, S-B, S-P-B, S-G-B and S-P-G-B.
3. Sterile saline (0.85 %; w/v) was thoroughly mixed in soil using a spatula, pre-sterilized with 70 % ethanol.
4. A 25 ml aliquot of 4 mg ml^{-1} pyrene stock solution was added to the soil in bags labeled S-P, S-P-G, S-P-B and S-P-G-B and thoroughly mixed with the help of sterile spatula. The soil bag was covered with inverted black polybag and the set up kept at 37 ^{o}C for 48 hrs to allow for acetone evaporation.
5. A 2.5 ml aliquot of 20 % glucose stock solution was added to the soil in bags labeled S-G, S-P-G, S-G-B and S-P-G-B and thoroughly mixed with sterile spatula.
6. *Inoculum development*: *Bacillus* sp. (PK-6) MTCC 1005 culture was developed from 100 µl of glycerol stock in duplicate flasks containing 100 ml LB medium plus pyrene (50 µg ml^{-1}) placed at room temperature (37 ^{o}C) in an orbital shaker (120 rpm). The culture growth (A_{600}) was determined after 18 - 20 hrs. Log phase culture with OD_{600} equal to ~1 was harvested by centrifugation at 8,000 rpm, 4 ^{o}C for 15 min. The bacterial cell pellet was resuspended in 50 ml of saline + glucose (@ 0.2 %; w/v) solution. Bacterial count (cfu ml^{-1}) in cell suspension was determined by preparing serial dilutions of cell suspension in saline and spread plating 0.1 ml aliquot of each dilution on triplicate LA plates followed by incubation at 37 ^{o}C for 24 hrs.

7. A 7 ml aliquot of bacterial cell suspension was added to the soil in bags labeled S-B, S-P-B, S-G-B and S-P-G-B and thoroughly mixed with the help of sterile spatula.
8. Abiotic soil controls contained pyrene and glucose but did not receive *Bacillus* sp. (PK-6) inoculum.
9. Soil samples (20 g) were withdrawn at zero time of incubation from each set up to determine the physico-chemical parameters like pH, EC, moisture content, total organic carbon, available phosphorus, total nitrogen, initial pyrene concentration and cfu per gram of soil.
10. The soil bags were covered and incubated at 37 ^{o}C. Light was restricted from the soil. Saline was periodically (every 1–2 days) added to soil to replace evaporative losses. A moisture content of 35 $\pm$ 2 % in soil was maintained throughout the experiment.
11. Soil samples (5 g) were withdrawn every 7 days, till 28 days to determine change in pyrene concentration and cfu per gram of soil. After completion of experiment again noted pH, EC and total organic carbon.

3.7.4 Pyrene analysis

The extent of pyrene removal from pyrene spiked garden soil and coal-tar contaminated soil was monitored by solid-liquid extraction of residual pyrene from periodically withdrawn soil samples (inoculated or uninoculated) followed by spectrophotometric analysis.

Requirements

1. *Erlenmeyer extraction flasks (50-ml)*
2. *Glass pipette (10-ml)*
3. *Orbital shaker*
4. *Glass crucibles*
5. *Sample storage cryovials (5-ml)*
6. *UV-VIS spectrophotometer*
7. *Quartz cuvettes*
8. *Hexane (fraction from petroleum)*
9. *Acetone*
10. *Acetonitrile*

Procedure

1. Pyrene contaminated soil sample (5 g garden soil, 0.5 g coal-tar contaminated soil) was weighed in a 50-ml Erlenmeyer flask and vigorously mixed with 10 ml hexane (fraction from petroleum) solvent in an orbital shaker (150 rpm) at room temperature for 30 minutes.
2. The soil - solvent mixture was allowed to settle to facilitate solid-liquid phase separation. The solvent extract was collected in a glass crucible and this extraction procedure was repeated atleast three times.
3. The pooled hexane extracts were completely evaporated to dryness in a fume hood, redissolved in 5 ml acetone, collected in storage vials and stored in -20 ^{o}C till further analysis.
4. Acetone extracts obtained from duplicate samples were dried and redissolved in acetonitrile (3 - 5 ml) for spectrophotometric analysis as per the method of Bugg *et al.* (2000) as described in section 3.5.4.2.
5. Amount of pyrene extracted from control soil sample (without inoculum) at zero time of incubation was considered as 100 percent. Amount of pyrene extracted from control soil sample after completion of experiment was considered as abiotic loss of pyrene and/or loss of pyrene due to indigenous soil microbial activity. The percentage of pyrene removal by inoculated bacterial culture *Bacillus* sp. (PK-6) was calculated relative to pyrene concentration in control soil (Sarma *et al*., 2004; Jacques *et al*., 2007).

3.7.5 Enumeration of bacteria

Bacterial enumeration was carried out according to the standard plate count method of Cappuccino and Sherman, (1987) on Luria-Bertani agar (LA) at 37 ^{o}C as described in "Materials and Methods" section 3.3.2.1.

CHAPTER 4

RESULTS

4.1 BIODIVERSITY OF PYRENE DEGRADING BACTERIA

With an aim to investigate the role of bacteria in pyrene – a high-molecular-weight PAH – degradation the present study was conducted to isolate and characterize a diversity of aerobic soil bacteria, capable of growth and/or utilization of pyrene. Bacterial isolation was done from two PAH-contaminated soil samples, namely crude and diesel oil contaminated soil and coal-tar contaminated soil. 16S rDNA - RFLP analysis provided a picture of the genetic diversity in two microbial communities. The phylogenetic affiliations of bacterial isolates were determined using BLASTN and MEGA4 bioinformatics tools.

4.1.1 Physico-chemical characterization

Two physical parameters, depth of soil and distance from contamination source, were considered while selecting sites for soil sample collection (Figure 3.1 A, B). Firstly, the coal-tar heating plant had sampling points in its front and back side. The top layer soil was observed to get contaminated with coal-tar due to spillage during sampling. Therefore two top soil samples (0 cm soil level), one 1 - 2 m away and the second 3 - 4 m away from the coal-tar plant, labeled NP and AP, respectively, were collected. Secondly the spillage sites were regularly overlaid with fresh non-contaminated soil. Therefore two sub soil samples (0 - 25 cm soil depth), first 1-2 m away and the second 3 - 4 m away from the coal-tar plant, labeled F and B, respectively, were collected (Table 3. 1).

Physical characterization of coal-tar contaminated soil showed that the top soil was hot (~ 40 ^{o}C), loose, grey in color, dry to touch, having 1.2 - 2.9 % moisture content, 9.01 - 11.24 pH and 959 - 1424 µS cm^{-1} electrical conductivity. The sub soil was hot (~ 45 ^{o}C), jet black in color, tightly packed with coal-tar and had 7.2 - 10.8 % moisture content, 9.48 - 9.88 pH and 431 - 1112 µS cm^{-1} electrical conductivity (Table 3.1).

(A)

(B)

Figure 3.1 Collection of coal-tar contaminated soil samples A) 1-2 m away and B) 3-4 m away from the coal-tar heating unit. The hole was dug upto 25 cm depth. (Courtesy: Coal-tar pre-mix plant, Rakhra village, District Patiala, Punjab).

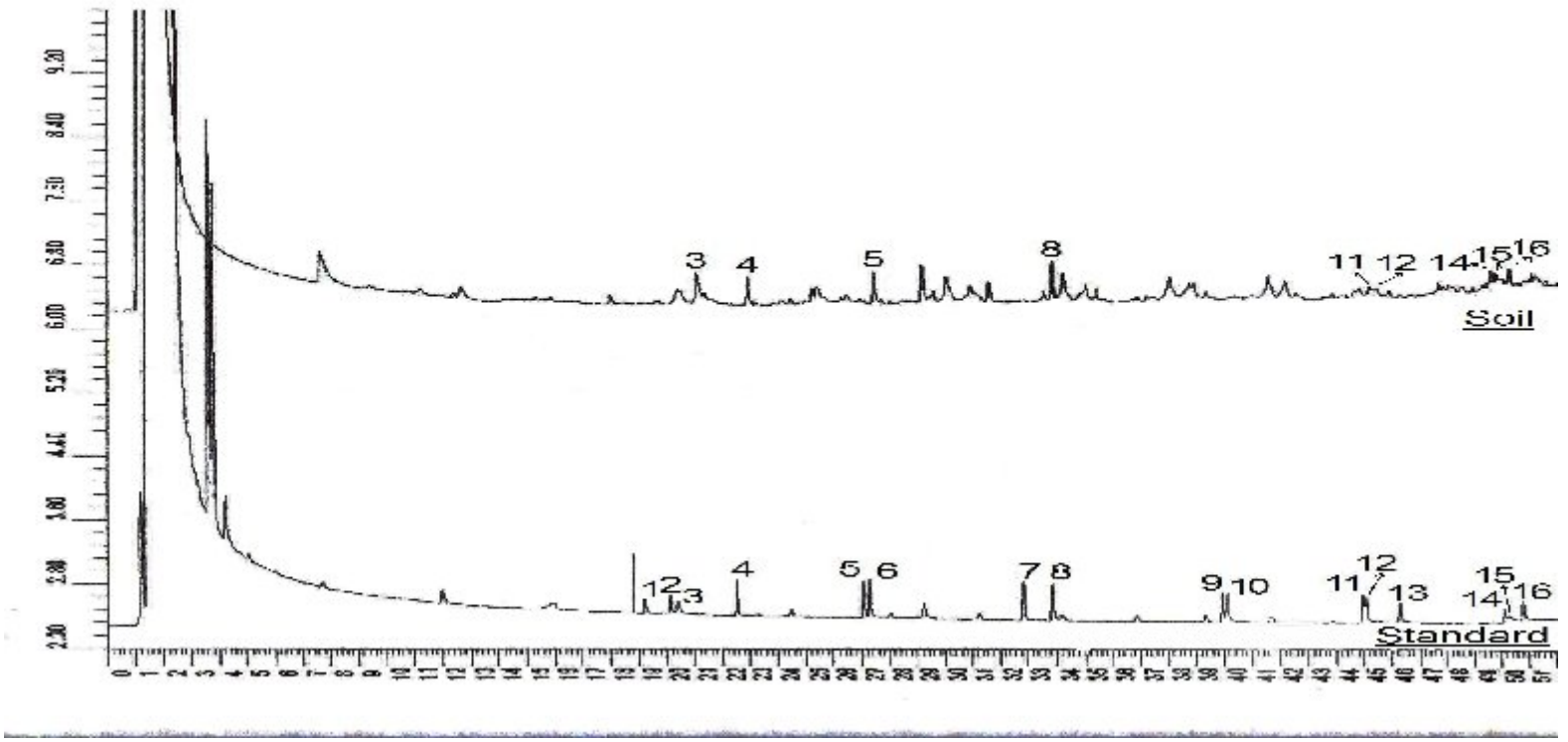
Table 3.1 Properties of coal-tar contaminated soil

Location	Soil depth	Physico-chemical properties *			Total bacterial count *		
					cfu g $^{-1}$ (30 ^{o}C)	cfu g $^{-1}$ (45 ^{o}C)	cfu g $^{-1}$ (60 ^{o}C)
		Moisture (%)	pH	EC (micro Siemens cm $^{-1}$)	48 hr (x 10^8)	48 hr (x 10^8)	48 hr (x 10^8)
1 – 2 m away from plant	Top soil (NP)	2.86 ± 0.02	11.24 ± 0.09	958.67 ± 8.44	0.002	0.001	0
	0 - 25 cm (F)	10.78 ± 0.04	9.88 ± 0.06	430.67 ± 6.72	3.960	3.800	0
3 – 4 m away from plant	Top soil (AP)	1.23 ± 0.08	9.01 ± 0.09	1423.67 ± 4.97	0.630	0.311	0
	0 – 25 cm (B)	7.15 ± 0.07	9.48 ± 0.01	1112.33 ± 6.10	0.418	0.199	0

* values are mean ± SE, no. of replicates, n = 3

Enumeration of bacteria at 37 °C, 45 and 60 °C indicated that the sub soil (F) had the highest culturable bacterial load (≥ 3 x 10^8 cfu gm^{-1} soil). Aerobic, mesophilic and culturable bacteria were abundant in the sub soil F (Table 3.1), an area with high moisture content and mildly alkaline pH. However the top soil (NP) collected from same area had the lowest count of bacteria (1 - 2 x 10^5 cfu gm^{-1} soil) probably due to high soil pH. The sub soil sample (B) and top soil sample (AP) collected 3-4 m away from heating plant, had lower bacterial counts 2 - 4 x 10^7 cfu gm^{-1} soil and 3 - 6 x 10^7 cfu gm^{-1} soil, respectively as compared to soil sample (F). Though top soil AP had the lowest record of moisture content among the four soil samples, its mildly alkaline pH helped to support bacterial counts similar to sub-soil B. Hot, dry and loose top soil NP also had low moisture content but its high pH did not favor bacterial growth.

Gas chromatographic (GC-FID) analysis of solvent extracts of sub soil samples showed the presence of nine PAHs: Benzo[*g,h,i*]perylene, Dibenzo[*a,h*]anthracene, Indeno[*1,2,3-c,d*]pyrene, Pyrene, Acenaphthylene, Fluorene, Phenanthrene, Benzo[*k*]fluoranthene and Benzo[*b*]fluoranthene (Figure 3.2).



16 PAHs Standard:	1= Naphthalene	2= Acenaphthene
3= Acenaphthylene	**4= Fluorene**	**5= Phenanthrene**
6= Anthracene	7= Fluoranthene	**8= Pyrene**
9= Benzo[*a*]anthracene	10= Chrysene	**11= Benzo[*b*]fluoranthene**
12= Benzo[*k*]fluoranthene	13= Benzo[*a*]pyrene	**14= Indeno[*1,2,3-c,d*]pyrene**
15= Dibenzo[*a,h*]anthracene	**16=Benzo[*g,h,i*]perylene**	

Figure 3.2 Gas chromatographic (GC) analysis of coal-tar contaminated soil. PAHs found in soil are represented in shaded boxes.

4.1.2 Isolation of bacteria from PAH contaminated soils

4.1.2.1 Crude and diesel oil contaminated soil

Bacterial consortium CON-3 was isolated from soil contaminated with crude oil from refinery wastes while consortium THA-2 was isolated from soil contaminated with diesel oil from a local oil depot. A total of five gradual enrichments of pyrene in steps of 10 µg ml^{-1} upto 50 µg ml^{-1}, over a time period of five months, were carried out to develop the HMW-aromatic hydrocarbon degradation phenotype and to selectively enrich the pyrene-utilizing bacterial isolates in consortia CON-3 and THA-2. The consortia capacity to uptake pyrene from growth medium was evaluated by spectrophotometry and HPLC after 30 days of incubation in BHB containing 50 µg ml^{-1} of pyrene (Figure 3.3).

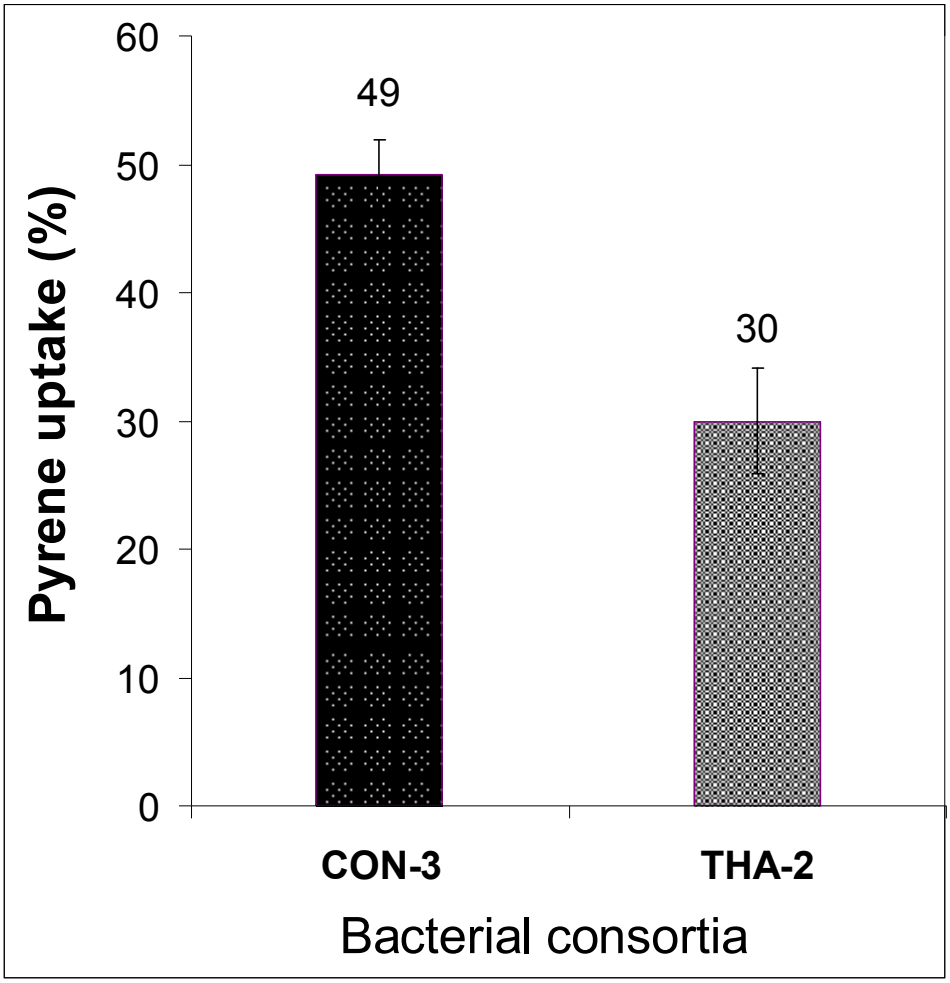


Figure 3.3 Pyrene uptake by bacterial consortia CON-3 and THA-2 in BHB medium containing 0.5 % (w/v) glucose and 50 µg ml^{-1} pyrene at 30 ^{o}C, 120 rpm in 30 day incubation time. (Values are mean + SE, no. of replicates n = 3)**.**

Consortia CON-3 and THA-2 were able to utilize 49 % and 30 % pyrene, respectively, in 30 days of growth. It was also observed that both consortia could grow on pyrene only in the presence of glucose. No growth and pyrene degradation was observed in the absence of glucose in BHB medium by both consortia.

4.1.2.2 Bacterial isolates from microbial consortia from fuel oil

After three enrichment transfers, ten bacteria were isolated from each developed bacterial consortium CON-3 and THA-2. Pure cultures of the twenty bacterial isolates, designated as PK-11 to PK-30, were obtained after four transfers. During initial screening in BHB + G (0.25 %; w/v) + P (25 µg ml^{-1}) medium the bacterial isolates from CON-3 consortium were found to utilize pyrene in the range 6 - 98 % at 30 °C in 30 day incubation time. Comparatively, bacterial isolates from THA-2 consortium were found to utilize pyrene in a lower range of 0 - 53 % in similar incubation conditions (Figure 3.4).

Eight isolates showing an utilization uptake of more than 35 % pyrene were designated Pyr^{25} isolates, namely PK-23 (45 %), PK-24 (39 %), PK-27 (53 %), PK-12 (98 %), PK-13 (61 %), PK-14 (55 %), PK-15 (54 %) and PK-16 (51 %), and were exposed to 50 µg ml^{-1} and 75 µg ml^{-1} pyrene to check for maximum pyrene utilization limit. Glucose concentration in growth medium was 0.5 % and 0.75 % (w/v), respectively. Pyrene concentration 75 µg ml^{-1} inhibited the growth of all isolates even in the presence of 1.0 % (w/v) glucose, while 50 µg ml^{-1} pyrene could support good growth of the isolates (Figure 3.5 A, B). Three bacterial isolates PK-12, PK-13 and PK-14 found to utilize more than 50 % of 50 µg ml^{-1} pyrene (designated Pyr^{50}) and one non-pyrene utilizing isolate PK-11 (designated Pyr^{00} isolate) were selected for molecular characterization studies.

4.1.2.2 Coal-tar contaminated soil

A total of 229 autochthonous, culturable, aerobic bacterial strains were isolated from coal-tar contaminated soil by means of standard serial-dilution technique, at two incubation temperatures 30 ^{o}C and 45 ^{o}C. Ninety one (91) colonies were obtained at 30 °C and one hundred and thirty eight (138) colonies were obtained at 45 °C on LA plates. Of the total,

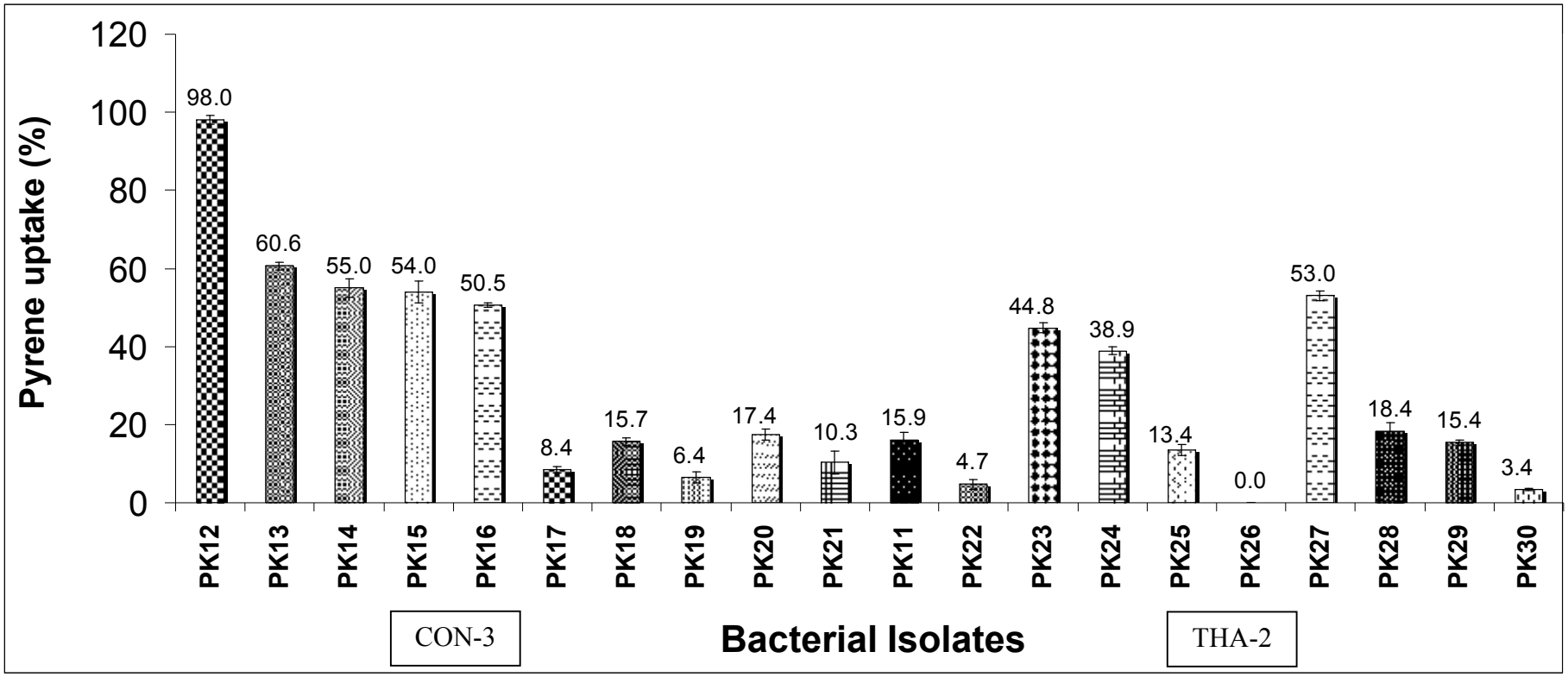


Figure 3.4 Pyrene uptake efficiency of bacterial isolates from fuel oil contaminated soil in BHB medium containing 0.25 % (w/v) glucose and 25 µg ml^{-1} pyrene at 30 ^{o}C with continuous aeration (120 rpm) in 30 day incubation time. Bacteria were isolated from crude oil utilizing consortia CON-3 and THA-2 (Values are mean + SE, no. of replicates, n = 3)**.**

pure cultures of only 27 bacterial isolates were able to grow on pyrene coated BHA (BHA+P) and pyrene coated BHA + 0.25 % (w/v) glucose (BHA+G0.25+P) plates. Each of the 27 colonies was transferred in 5 ml of BHB +P (25 µg ml^{-1}) and BHB + G (0.25 %; w/v) +P (25 µg ml^{-1}) media in test tubes and allowed to grow in shaking conditions (60 rpm). After 2 weeks of incubation, the turbid cultures were subcultured in flasks containing 50 ml of BHB +P (50 µg ml^{-1}) and BHB + G (0.5 %; w/v) +P (50 µg ml^{-1}) media for 30 day incubation time. The bacterial strains grew on pyrene only in presence of glucose. Growth of isolates on 50 µg ml^{-1} pyrene in presence of 0.5 % (w/v) glucose was monitored to select the 10 best growing isolates with maximum pyrene (50 µg ml^{-1}) utilization ability (designated Pyr^{50} isolates) at 30 °C and 45 °C incubation temperatures (Figure 3.6 A, B) for molecular characterization studies.

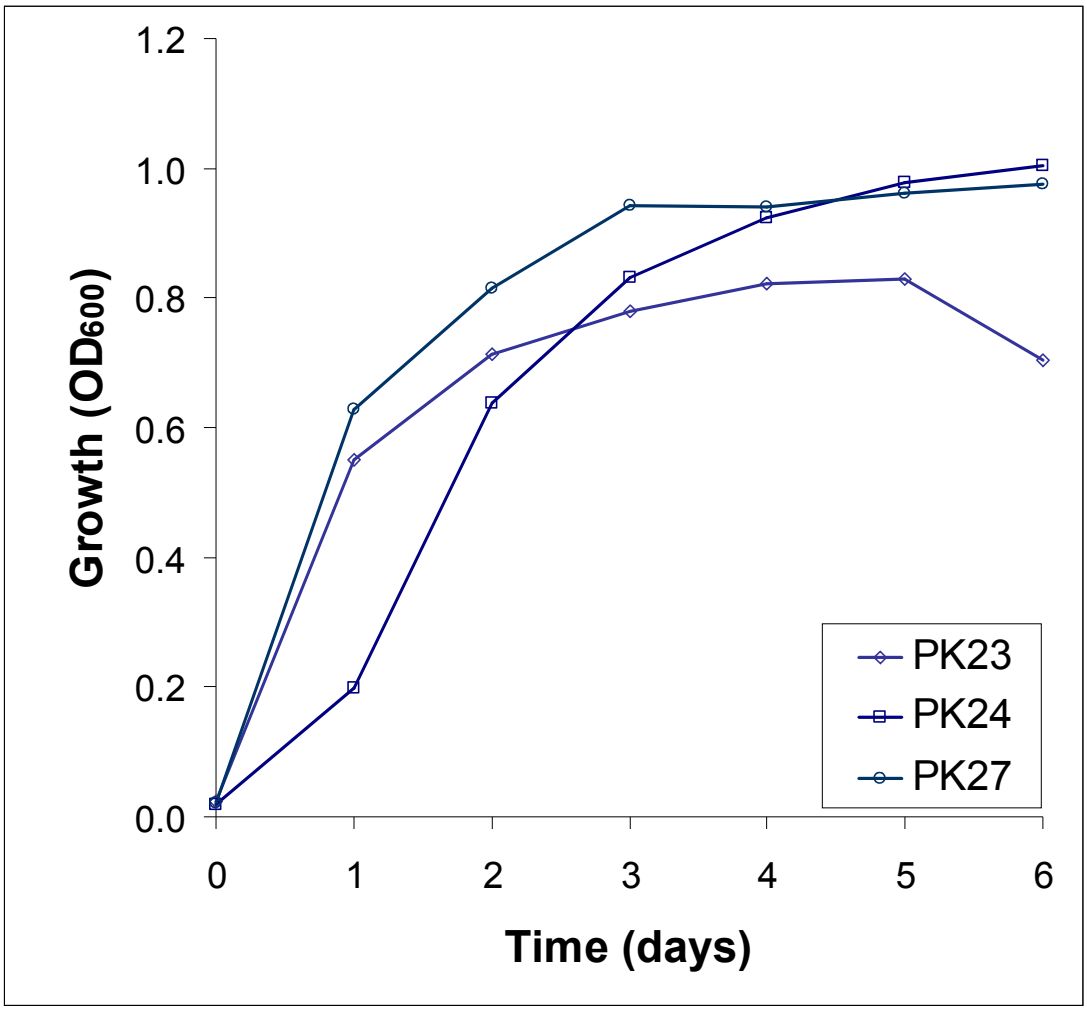


Figure 3.5 A Growth of bacterial isolates from THA-2 consortium exposed to 50 µg ml^{-1} pyrene in BHB + glucose (0.5 %; w/v) medium at 30 °C (no. of replicates, n = 1)**.**

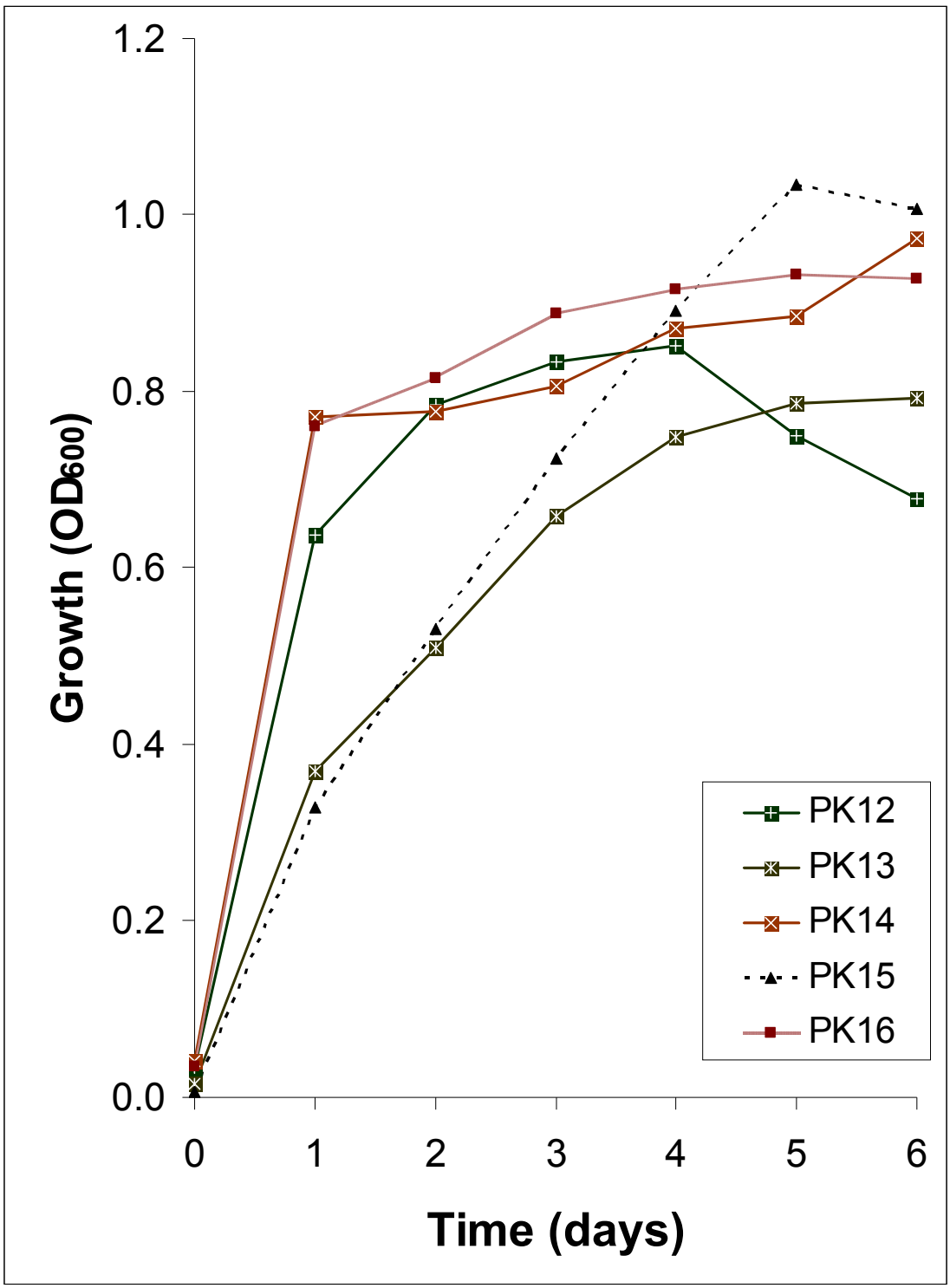


Figure 3.5 B Growth of bacterial isolates from CON-3 consortium exposed to 50 µg ml^{-1} pyrene in BHB + glucose (0.5 %; w/v) medium at 30 ^{o}C (no. of replicates, n = 1)**.**

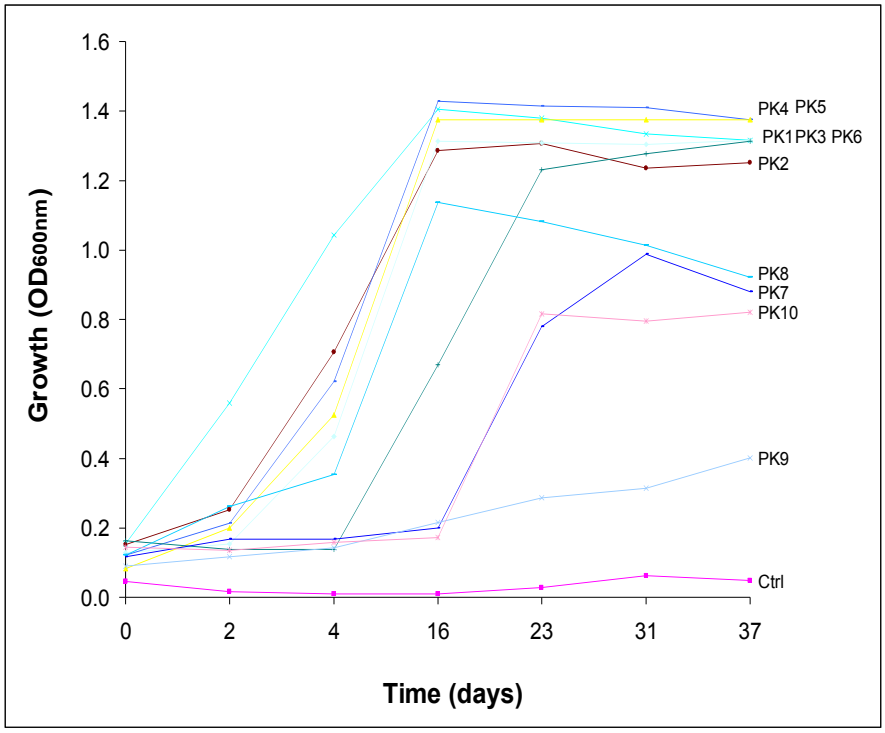


Figure 3.6 A Growth profile of bacteria from coal-tar contaminated soil exposed to 50 µg ml^{-1} pyrene in BHB at 30 ^{o}C and 45 ^{o}C (no. of replicates, n = 1)**.**

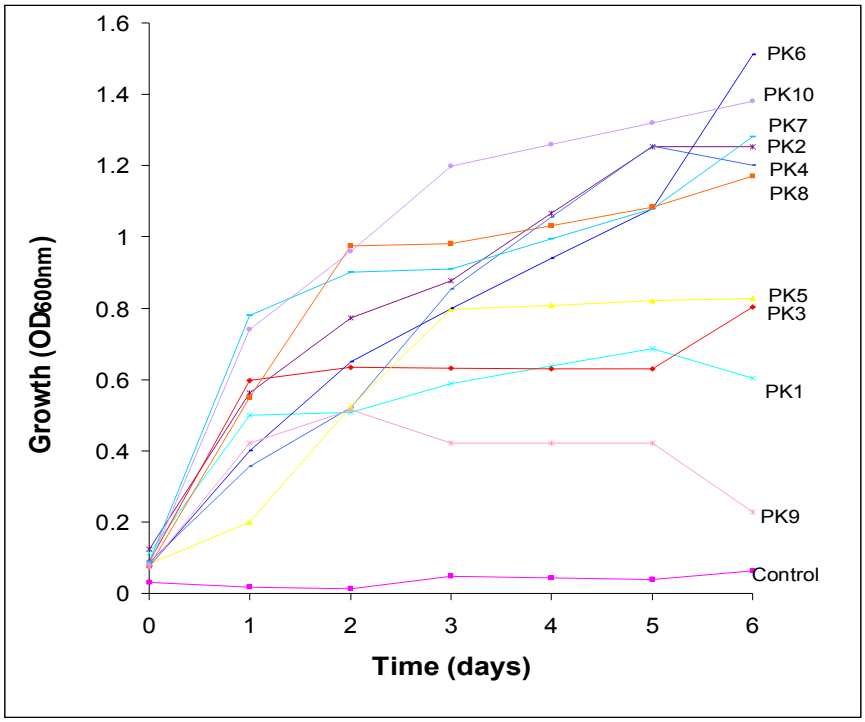


Figure 3.6 B Growth profile of bacteria from coal-tar contaminated soil exposed to 50 µg ml^{-1} pyrene in BHB + glucose (0.5 %; w/v) medium at 30 ^{o}C and 45 ^{o}C (no. of replicates, n = 1)**.**

4.1.3 Characterization of bacteria isolates

4.1.3.1 Morphological characterization by Gram staining

The Gram staining character of bacterial isolates capable of growth on pyrene (50 µg ml^{-1}) is described in Table 3.2. Thirteen bacterial isolates showed Gram positive character, only isolate PK-3 was observed to stain Gram negative.

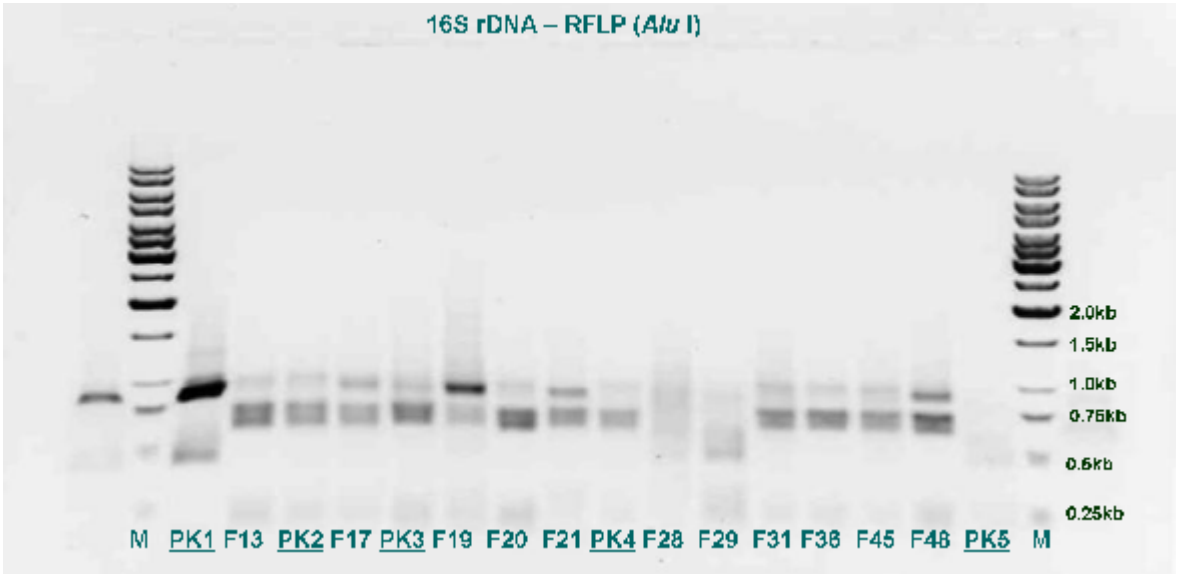
4.1.3.2 Molecular characterization by 16S rDNA analysis

A. 16S rDNA restriction enzyme digests:

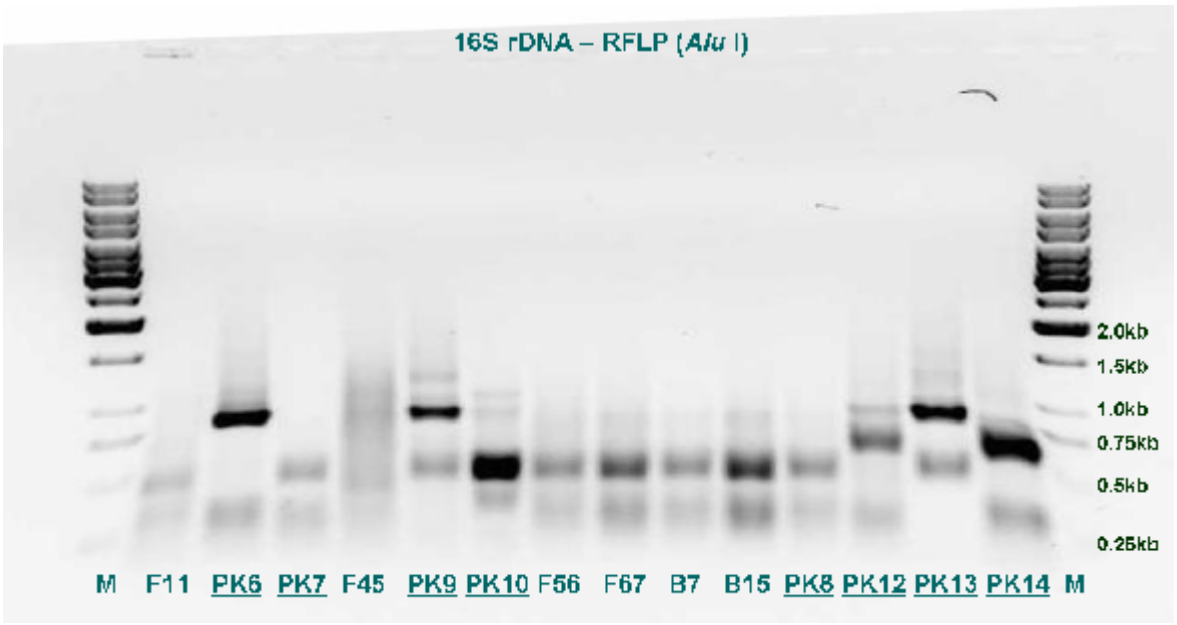
The phylogenetic affiliation of bacterial isolates capable of growth on pyrene (50 µg ml^{-1}) was determined by DNA sequence analysis of PCR amplified 16S ribosomal DNA. First RFLP analysis of 16S rDNA PCR product (~ 1500 bp) digested with *Alu* I, *Hin* 61, *Mbo* I and *Rsa* I enzymes was used as a screening tool to determine unique isolates. The digestion patterns for different isolates were compared visually in 1.0 % agarose gels. A large number of experiments were performed encompassing digestion patterns for all the cultured pyrene-utilizing (Pyr^{50}) isolates from crude oil and coal-tar contaminated soil (Figure 3.7 A, B, 4.8 A, B, 4.9 A, B and 4.10 A, B). These gels were examined visually, and an isolate was considered unique based on the presence of a unique banding pattern. Isolates which shared a banding pattern were grouped together, and then a single isolate from the group was chosen as a representative strain for sequencing analysis.

Amongst the first group of pyrene-utilizing isolates from crude oil and diesel oil contaminated soil four isolates, PK-11 to PK-14, were found to have different restriction enzyme banding patterns. Shared RFLP patterns (for *Hin* 61, *Mbo* I and *Rsa* I enzymes) were seen between the isolates PK-12, PK-28, PK-20, and PK-21. The second group of pyrene-utilizing isolates from coal-tar contaminated soil, containing 27 isolates, was found to have 10 isolates (PK-1 to PK-10) exhibiting unique RFLP patterns. Shared banding patterns were seen between the isolates F29 and PK-5; between F13, PK-2, F17, F20, PK-4, F31, F36, and F45; and between PK-7, F45, F56, F67, B7, B15, and PK-8.

Thus the isolates PK-11 to PK-14 (from crude / diesel oil contaminated soil) and PK-1 to PK-10 (from coal-tar contaminated soil) represented different phylotypes.

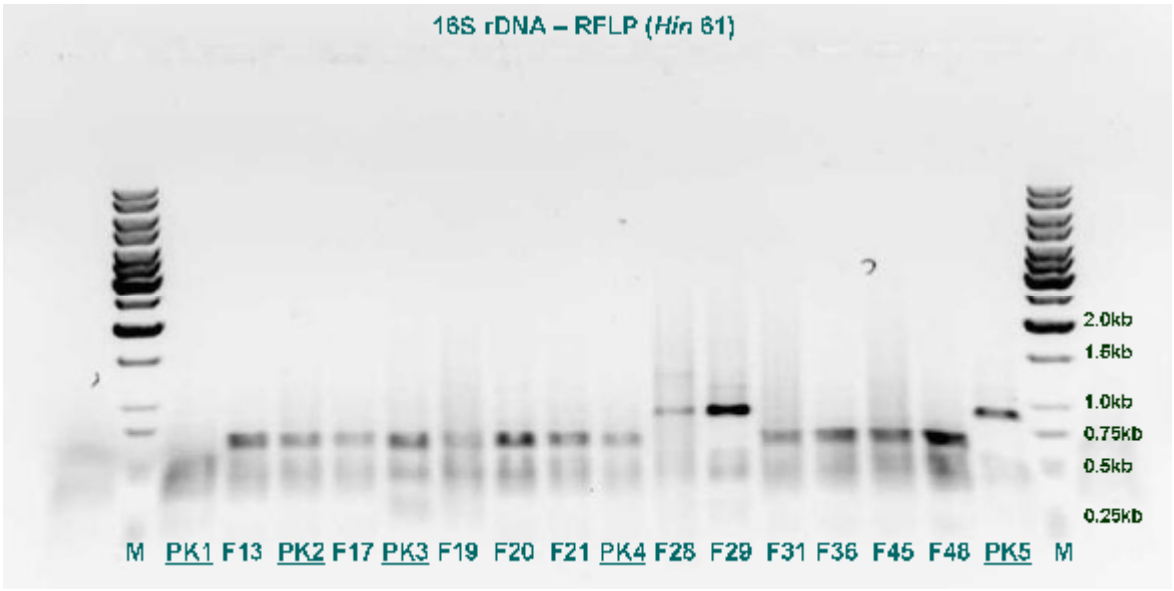


A

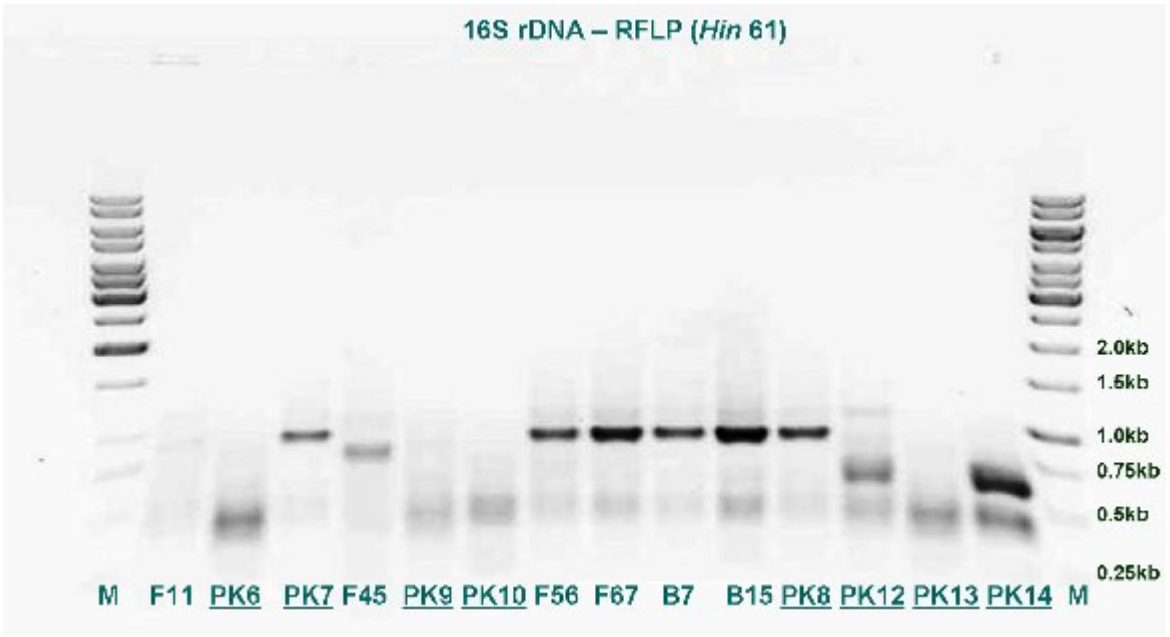


B

Figure 3.7 Restriction Fragment Length Polymorphism (RFLP) pattern with *Alu* I restriction enzyme of 16S rRNA gene amplified from genomic DNA of bacterial strains isolated from (A) coal-tar contamianted soil, (B) coal-tar contamianted soil (F11 to PK-8) and crude oil contaminated soil (PK-12 to PK-14).

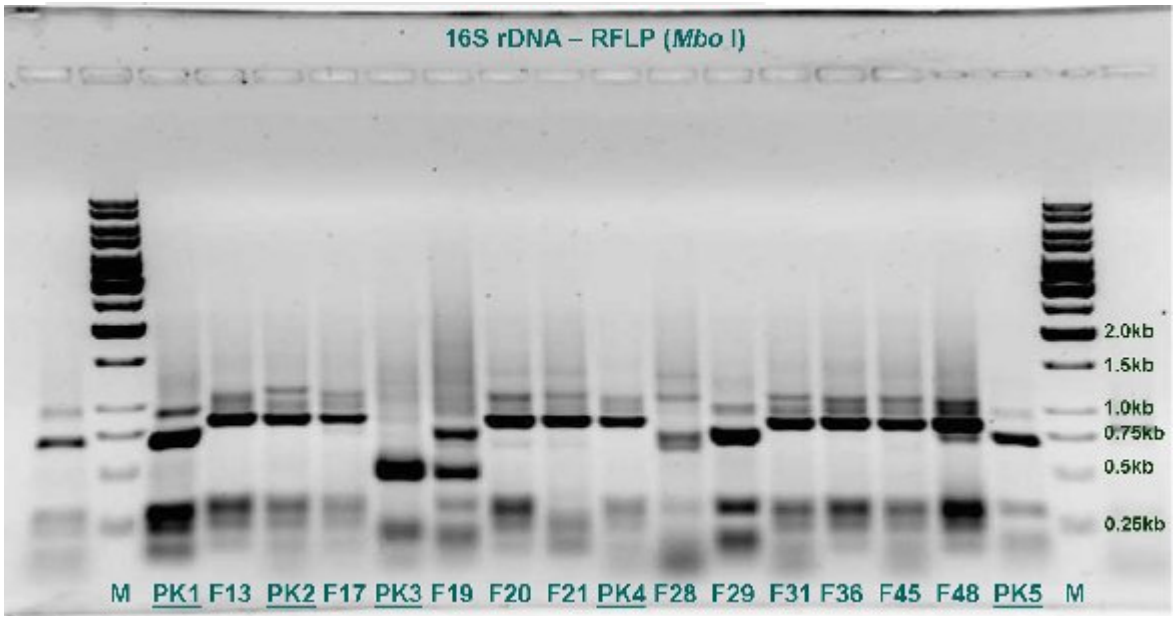


A

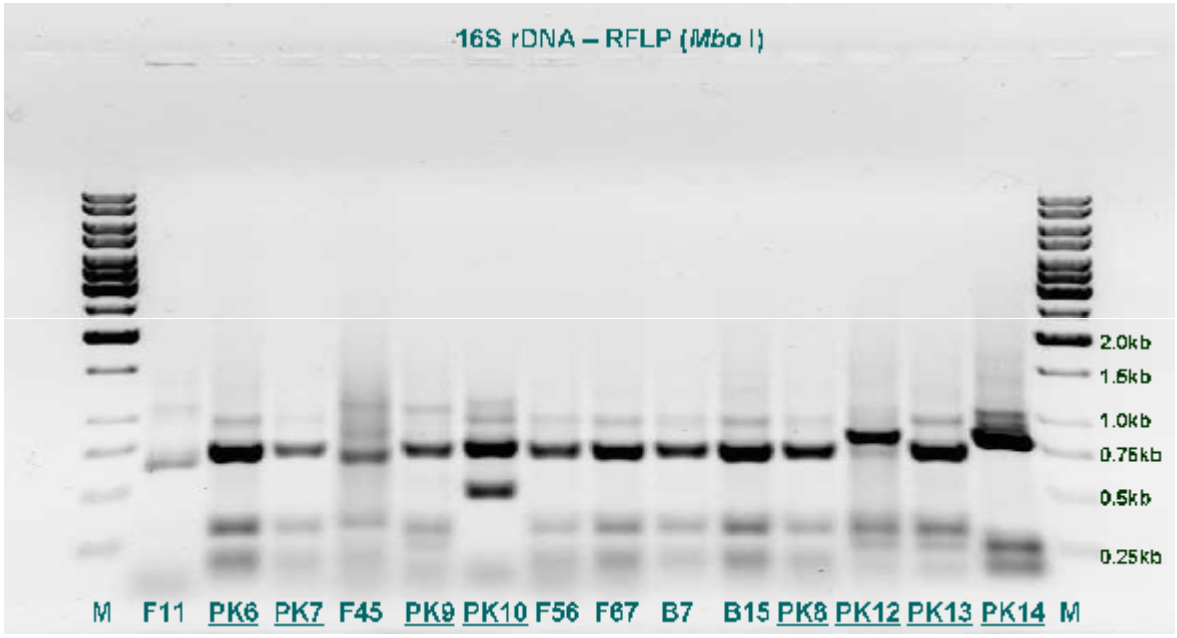


B

Figure 3.8 Restriction Fragment Length Polymorphism (RFLP) pattern with *Hin* 6I restriction enzyme of 16S rRNA gene amplified from genomic DNA of bacterial strains isolated from (A) coal-tar contamianted soil, (B) coal-tar contamianted soil (F11 to PK-8) and crude oil contaminated soil (PK-12 to PK-14).



A



B

Figure 3.9 Restriction Fragment Length Polymorphism (RFLP) pattern with *Mbo* I restriction enzyme of 16S rRNA gene amplified from genomic DNA of bacterial strains isolated from (A) coal-tar contamianted soil, (B) coal-tar contamianted soil (F11 to PK-8) and crude oil contaminated soil (PK-12 to PK-14).

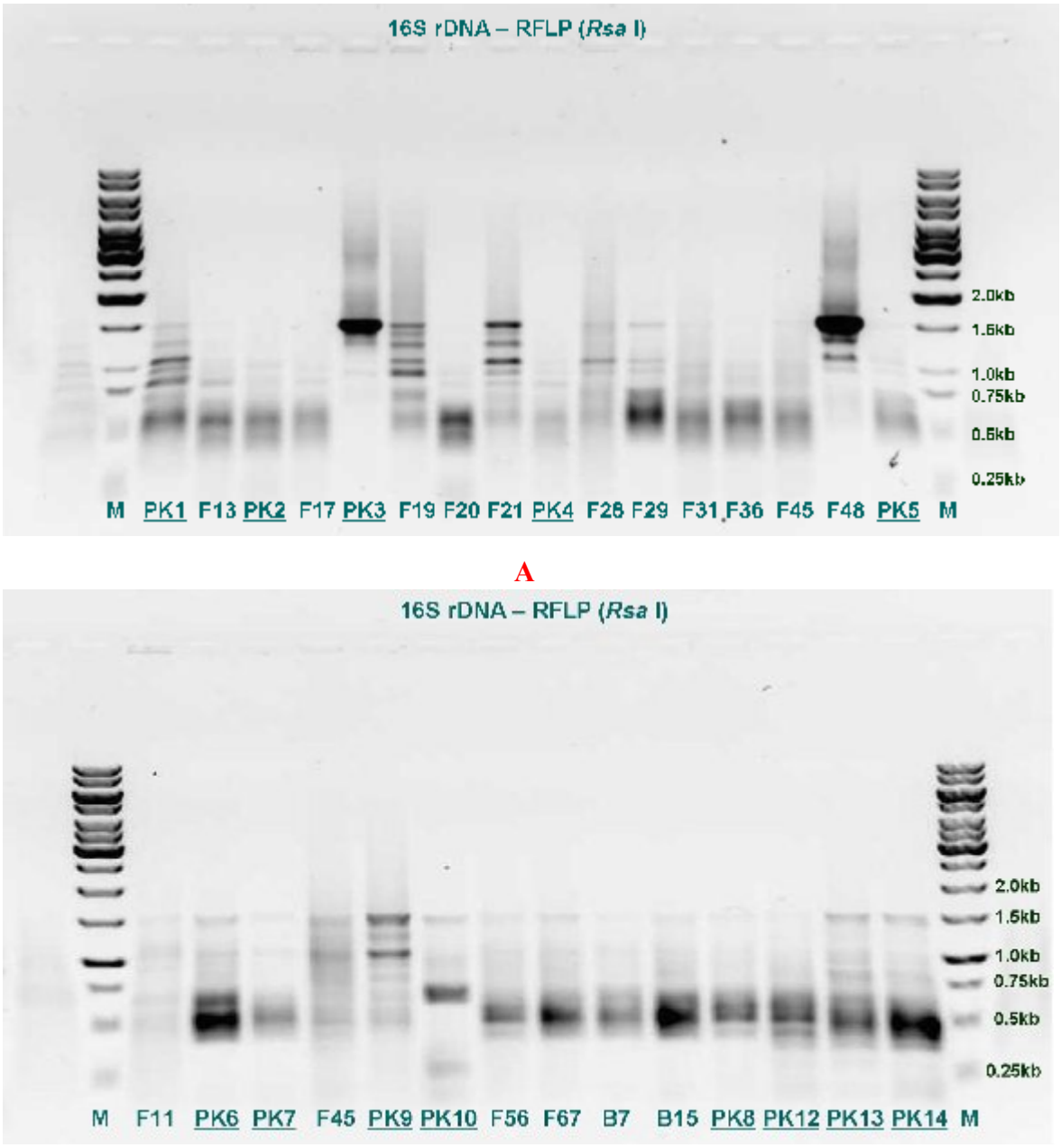


Figure 3.10. Restriction Fragment Length Polymorphism (RFLP) pattern with *Rsa* I restriction enzyme of 16S rRNA gene amplified from genomic DNA of bacterial strains isolated from (A) coal-tar contamianted soil, (B) coal-tar contamianted soil (F11 to PK-8) and crude oil contaminated soil (PK-12 to PK-14).

B. 16S rRNA gene analyses:

The unique isolates from each of the fourteen major groups were further identified by complete sequencing of their 16S rRNA gene. The 16S rDNA of unique soil isolates was ligated into pGEM-T Easy vector system (Figure 3.11) and transformed in *E. coli* DH5α bacterial cells. The white transformant colonies obtained on X-Gal +IPTG plates (Figure 3.12) were confirmed for the presence of plasmid with insert by standard plasmid isolation (Figure 3.13), *Eco* R1 restriction digestion (Figure 3.14 A) and PCR using vector specific T7 and SP6 primers (Figure 3.14 B).

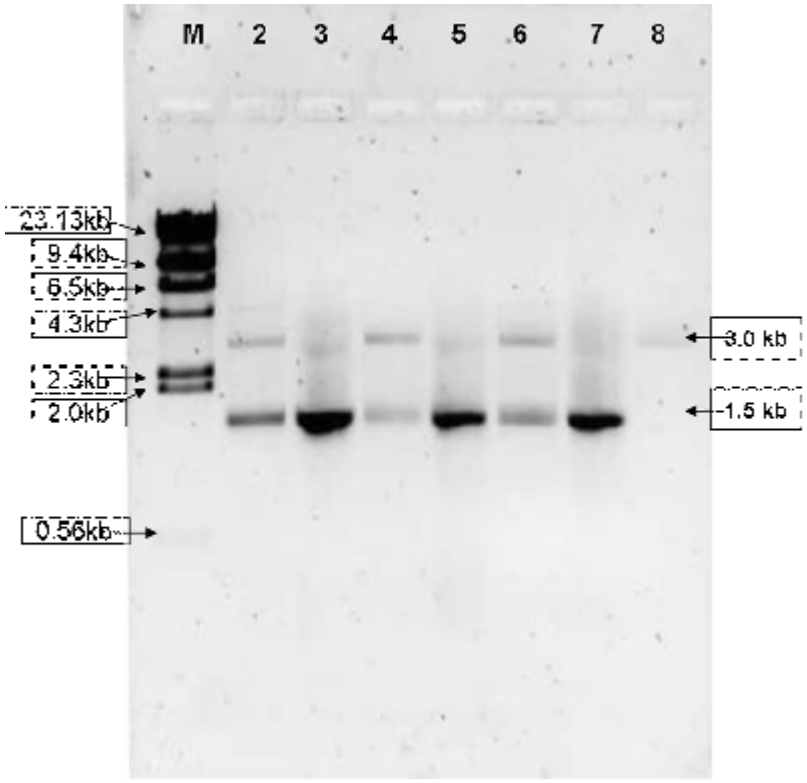


Figure 3.11 Agarose gel electrophoresis of 16S rRNA gene ligated in pGEM-T Easy vector system.

Lane M: λ Hind III DNA ladder; Lane 2, 4, 6: Ligation products for PK-1, PK-2 and PK-3 soil isolates, respectively; Lane 3, 5, 7: 16S rDNA amplicon for PK-1, PK-2 and PK-3 soil isolates, respectively; Lane 8: Positive control ligation reaction.

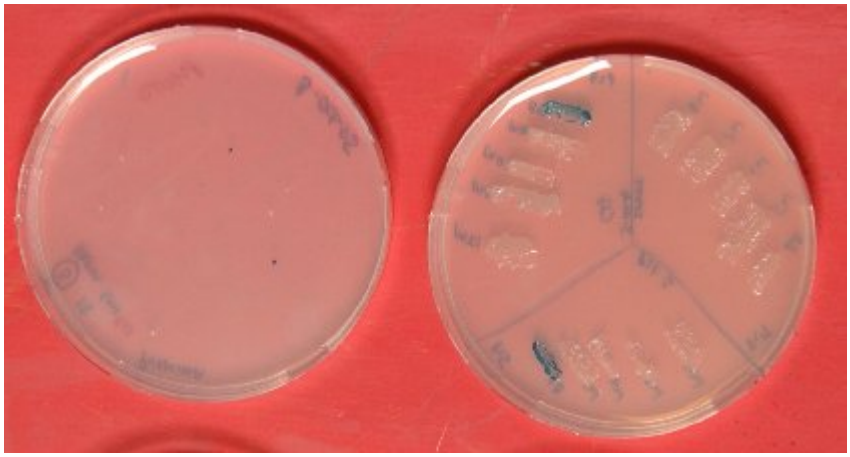


Figure 3.12 Transformed *E.coli* DH5α colonies on LA +Ampicillin +X-Gal +IPTG plates. The white colonies bear 16S rDNA (insert) ligated in pGEM-T plasmid (vector) while the blue colonies bear vector without insert.

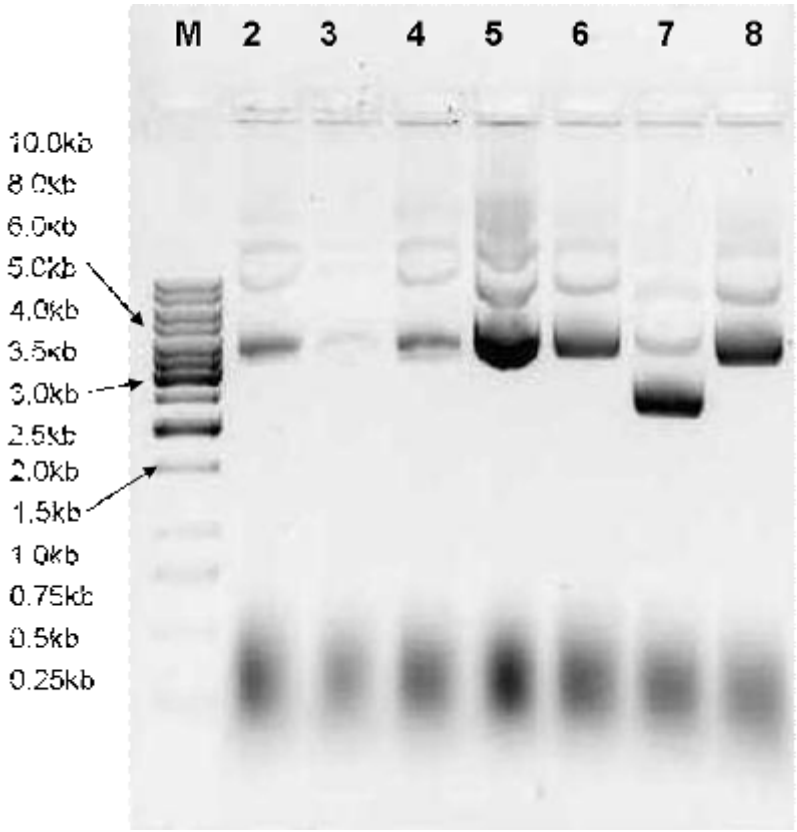
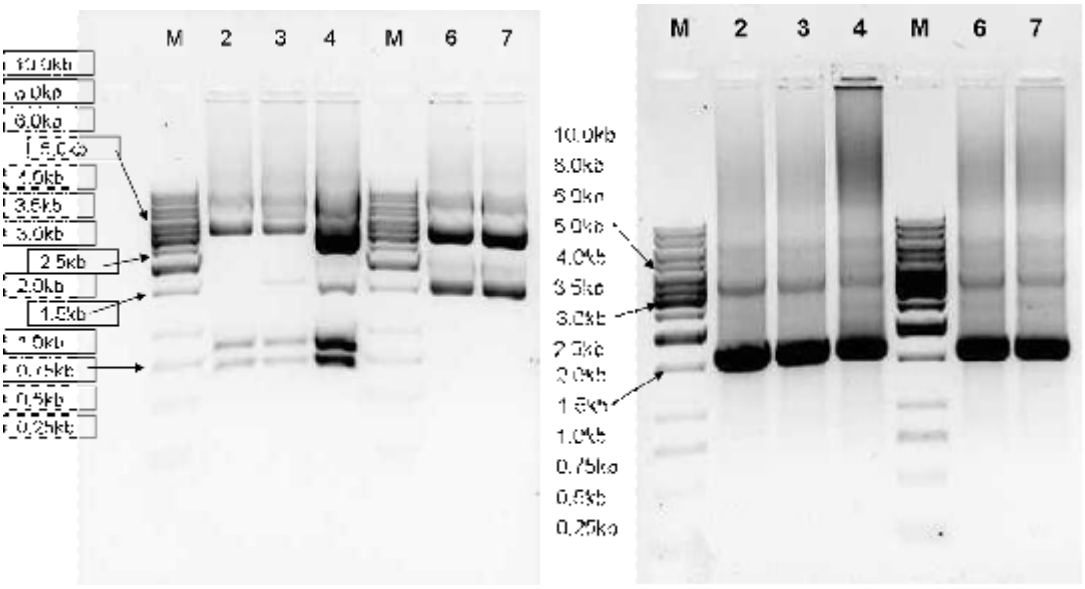


Figure 3.13 Agarose gel electrophoresis of recombinant pGEM-T plasmid DNA (containing 16S rDNA) isolated from *E.coli* DH5α clones corresponding to soil isolate PK-1 (Lane 2, 3, 4), PK-2 (Lane 5) and PK-3 (Lane 6, 7, 8). Lane M corresponds to 1 kb DNA ladder.



A B

Figure 3.14 Agarose gel electrophoresis of (A) *Eco* RI restriction digestion reaction and (B) PCR reaction using T7 and SP6 primers of recombinant pGEM-T plasmid DNA isolated from *E.coli* DH5α clones corresponding to soil isolate PK-1 (Lane 2, 3), PK-2 (Lane 4) and PK-3 (Lane 6, 7). Lane M corresponds to 1 kb DNA ladder.

4.1.3.3 16S rDNA sequence analysis and submission

Isolates PK-1 to PK-14 represented a diversity of aerobic, culturable, mesophilic, and pyrene utilizing bacteria from coal-tar, crude and diesel oil contaminated soils, except for the isolate PK-11 which showed poor pyrene utilization ability. The 16S rRNA gene sequences (~1500 bp) obtained for all 14 soil isolates were submitted to the NCBI GenBank database and provided accession number as follows:

Bacillus sp. PK-1 16S ribosomal RNA gene, partial sequence

GenBank: EU685820.1

FASTA Graphics

Go to:

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JUN-2008
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  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
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  JOURNAL   Unpublished
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  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
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1441 tggggtaacc ttttggagcc agccgcctaa ggtgggacag atgattgggg tgaagtcgta
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Bacillus sp. PK-2 16S ribosomal RNA gene, partial sequence

GenBank: EU685821.1

FASTA Graphics

Go to:

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JUN-2008
DEFINITION  Bacillus sp. PK-2 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU685821
VERSION     EU685821.1  GI:189913526
KEYWORDS    .
SOURCE      Bacillus sp. PK-2
  ORGANISM  Bacillus sp. PK-2
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1510)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1510)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Pseudomonas sp. PK-3 16S ribosomal RNA gene, partial sequence

GenBank: EU685822.1

FASTA Graphics

Go to:

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JUN-2008
DEFINITION  Pseudomonas sp. PK-3 16S ribosomal RNA gene, partial
sequence.
ACCESSION   EU685822
VERSION     EU685822.1  GI:189913527
KEYWORDS    .
SOURCE      Pseudomonas sp. PK-3
  ORGANISM  Pseudomonas sp. PK-3
            Bacteria; Proteobacteria; Gammaproteobacteria;
Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1  (bases 1 to 1499)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1499)
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  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-4 16S ribosomal RNA gene, partial sequence

GenBank: EU685826.1

FASTA Graphics

Go to:

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JUN-2008
DEFINITION  Bacillus sp. PK-4 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU685826
VERSION     EU685826.1  GI:189913531
KEYWORDS    .
SOURCE      Bacillus sp. PK-4
  ORGANISM  Bacillus sp. PK-4
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1515)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1515)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-5 16S ribosomal RNA gene, partial sequence

GenBank: EU685823.1

FASTA Graphics

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JUN-2008
DEFINITION  Bacillus sp. PK-5 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU685823
VERSION     EU685823.1  GI:189913528
KEYWORDS    .
SOURCE      Bacillus sp. PK-5
  ORGANISM  Bacillus sp. PK-5
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1516)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1516)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
FEATURES             Location/Qualifiers
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Bacillus sp. PK-6 16S ribosomal RNA gene, partial sequence

GenBank: EU685825.1

FASTA Graphics

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JUN-2008
DEFINITION  Bacillus sp. PK-6 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU685825
VERSION     EU685825.1  GI:189913530
KEYWORDS    .
SOURCE      Bacillus sp. PK-6
  ORGANISM  Bacillus sp. PK-6
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1511)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1511)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-7 16S ribosomal RNA gene, partial sequence

GenBank: EU685824.1

FASTA Graphics

Go to:

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JUN-2008
DEFINITION  Bacillus sp. PK-7 16S ribosomal RNA gene, partial sequence.
ACCESSION   EU685824
VERSION     EU685824.1  GI:189913529
KEYWORDS    .
SOURCE      Bacillus sp. PK-7
  ORGANISM  Bacillus sp. PK-7
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1516)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
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  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1516)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-8 16S ribosomal RNA gene, partial sequence

GenBank: EU685817.1

FASTA Graphics

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JUN-2008
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  ORGANISM  Bacillus sp. PK-8
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1558)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1558)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-9 16S ribosomal RNA gene, partial sequence

GenBank: EU685818.1

FASTA Graphics

Go to:

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JUN-2008
DEFINITION  Bacillus sp. PK-9 16S ribosomal RNA gene, partial sequence.
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VERSION     EU685818.1  GI:189913523
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  ORGANISM  Bacillus sp. PK-9
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1568)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1568)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Rhodococcus sp. PK-10 16S ribosomal RNA gene, partial sequence

GenBank: EU685819.1

FASTA Graphics

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JUN-2008
DEFINITION  Rhodococcus sp. PK-10 16S ribosomal RNA gene, partial
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ACCESSION   EU685819
VERSION     EU685819.1  GI:189913524
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SOURCE      Rhodococcus sp. PK-10
  ORGANISM  Rhodococcus sp. PK-10
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            Corynebacterineae; Nocardiaceae; Rhodococcus.
REFERENCE   1  (bases 1 to 1485)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from coal-
tar
            contaminated soil
  JOURNAL   Unpublished
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REFERENCE   2  (bases 1 to 1485)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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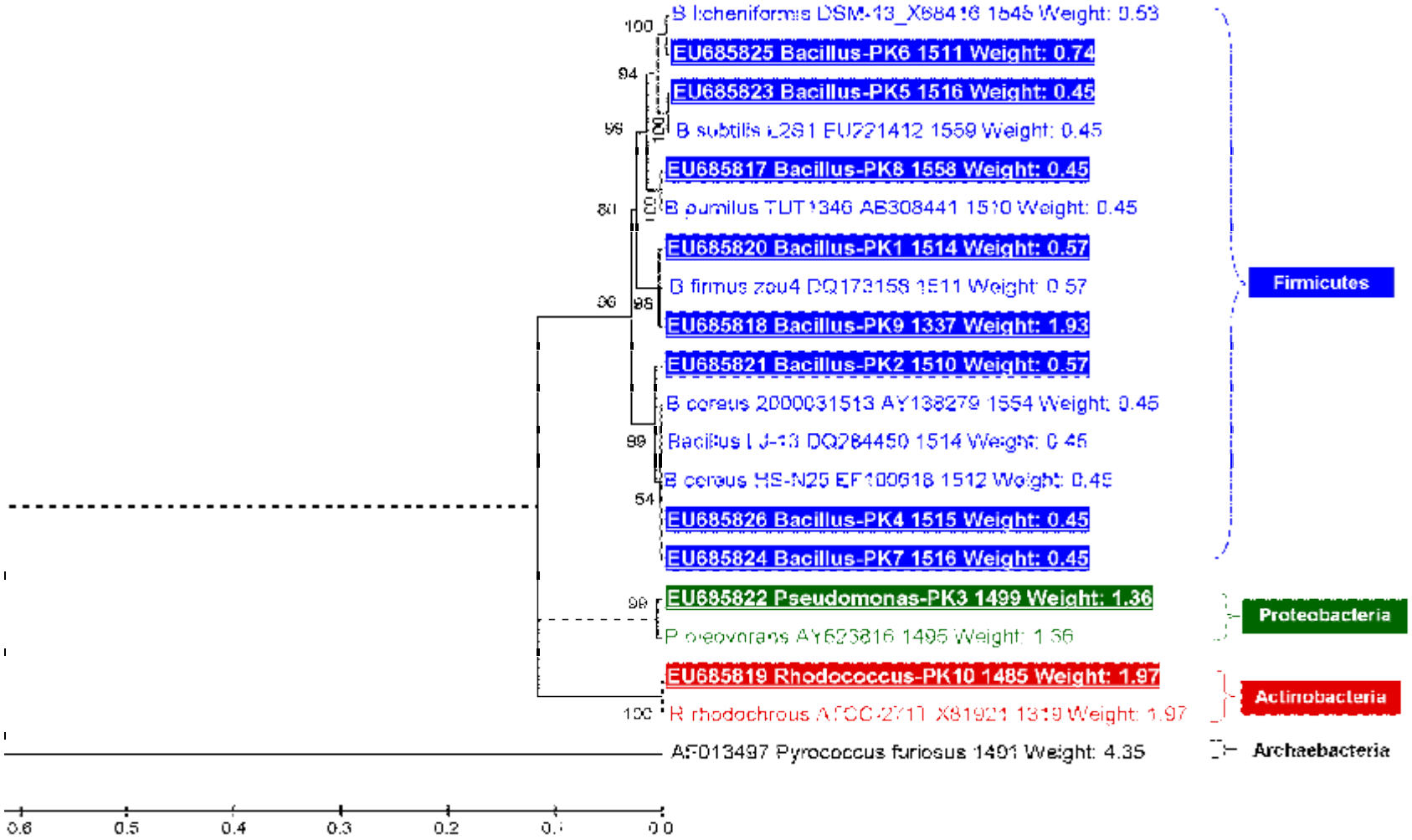

Figure 3.15 Phylogenetic dendrogram based on a comparison of the 16S ribosomal DNA sequences of pyrene utilizing bacteria isolated from coal-tar contaminated soil and some of their closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicates the percentages of bootstrap sampling derived from 1000 replications. Isolates characterized in the study are indicated in colored boxes. Bar, 10 inferred nucleotide substitutions per 1000 nucleotides.

Kocuria sp. PK-11 16S ribosomal RNA gene, partial sequence

GenBank: EU685813.1

FASTA Graphics

Go to:

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JUN-2008
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ACCESSION   EU685813
VERSION     EU685813.1  GI:189913518
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SOURCE      Kocuria sp. PK-11
  ORGANISM  Kocuria sp. PK-11
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REFERENCE   1  (bases 1 to 1525)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from crude
oil
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1525)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-12 16S ribosomal RNA gene, partial sequence

GenBank: EU685814.1

FASTA Graphics

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JUN-2008
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ACCESSION   EU685814
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SOURCE      Bacillus sp. PK-12
  ORGANISM  Bacillus sp. PK-12
            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1511)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from crude
oil
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1511)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
Box No.
            32, Patiala, Punjab 147004, India
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Bacillus sp. PK-13 16S ribosomal RNA gene, partial sequence

GenBank: EU685815.1

FASTA Graphics

Go to:

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JUN-2008
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            Bacteria; Firmicutes; Bacillales; Bacillaceae; Bacillus.
REFERENCE   1  (bases 1 to 1514)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Diversity of pyrene utilizing bacteria isolated from crude
oil
            contaminated soil
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1514)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
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  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
Environmental
            Sciences, Thapar University, Thapar Technology Campus, Post
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Bacillus sp. PK-14 16S ribosomal RNA gene, partial sequence

GenBank: EU685816.1

FASTA Graphics

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JUN-2008
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  TITLE     Diversity of pyrene utilizing bacteria isolated from crude
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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1512)
  AUTHORS   Khanna,P., Goyal,D. and Khanna,S.
  TITLE     Direct Submission
  JOURNAL   Submitted (30-APR-2008) Department of Biotechnology &
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Based on a BLASTN search of NCBI GenBank database, the closest match to eleven isolates was *Bacillus* sp. (PK-1, PK-2, PK-4, PK-5, PK-6, PK-7, PK-8, PK-9, PK-12, PK-13 and PK-14) having nucleotide identities between 95 and 99 %, one isolate was *Pseudomonas* sp. (PK-3), one isolate was *Kocuria* sp. (PK-11) both having nucleotide identity 97 % and one isolate was *Rhodococcus* sp. (PK-10) having nucleotide identity 99 % (Table 3.2). Phylogenetic affiliations of the isolates as shown in Figure 3.15, 3.16 were determined using CLUSTALW (Thompson *et al*., 1994) and MEGA4 (Tamura *et al*., 2007) software using neighbor-joining method (Saitou and Nei, 1987). The study showed that members of Firmicutes (*Bacillus* sp.), Actinobacteria (*Rhodococcus* sp.) and Proteobacteria (*Pseudomonas* sp.) taxonomic clades were present in coal-tar contaminated soil, while Firmicutes (*Bacillus* sp.) and Actinobacteria (*Kocuria* sp.) taxonomic clades were present in crude/diesel oil contaminated soil. Bacterial isolates (PK-6), (PK-12) and (PK-14) have been deposited at Microbial Type Culture Collection (MTCC) library at IMTECH, Chandigarh (India).

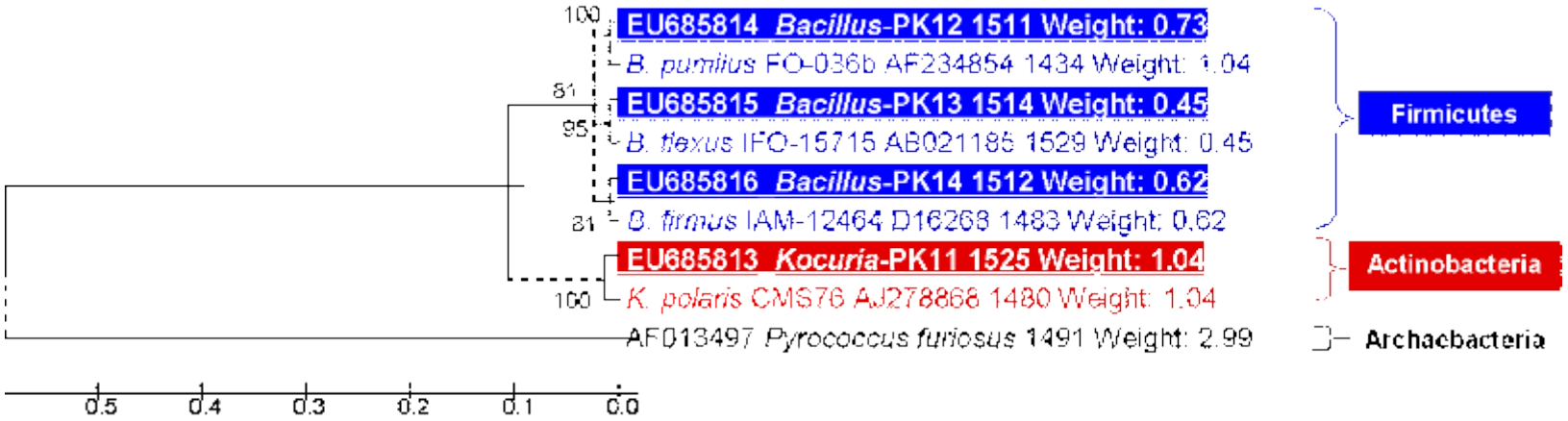

Figure 3.16 Phylogenetic dendrogram based on a comparison of the16S ribosomal DNA sequences of pyrene utilizing bacteria isolated from CON-3 and THA-2 consortia (from crude and diesel oil contaminated soil, respectively) and some of their closest phylogenetic relatives. The tree was created by the neighbor-joining method. The numbers on the tree indicate the percentages of bootstrap sampling derived from 1000 replications. Isolates characterized in the study are indicated in bold and underline font. Bar, 10 inferred nucleotide substitutions per 1000 nucleotides.

Table 3.2 Characterization of pyrene utilizing bacteria isolated from coal-tar contaminated soil (PK-1 - PK-10) and crude and diesel oil contaminated soil (PK-11 - PK-14).

Bacterial isolates	Gram Character	Molecular Characterization		
		NCBI match (Accession no) Max identity	GenBank Accession No.	MTCC No.
PK-1	+	*Bacillus firmus* (DQ173158) 99 %	**EU685820**	-
PK-2	+	*Bacillus cereus* (AY138279) 99 %	**EU685821**	-
PK-3	-	*Pseudomonas oleovorans* (AY623816) 97 %	**EU685822**	-
PK-4	+	*Bacillus cereus* (EF100618) 98 %	**EU685826**	-
PK-5	+	*Bacillus subtilis* (EU221412) 97 %	**EU685823**	-
PK-6	+	*Bacillus licheniformis* (T) DSM-13 (X68416) 95 %	**EU685825**	**1005**
PK-7	+	*Bacillus* sp. (DQ284450) 96 %	**EU685824**	-
PK-8	+	*Bacillus pumilus* (AB308441.1) 99 %	**EU685817**	-
PK-9	+	*Bacillus firmus* (DQ173158) 99 %	**EU685818**	-
PK-10	+	*Rhodococcus rhodochrous* (T) ATCC-271T (X81921) 99 %	**EU685819**	-
PK-11	+	*Kocuria polaris* (T) CMS76 (AJ278868) 97 %	**EU685813**	-
PK-12	+	*Bacillus pumilus* (T) FO-036b (AF234854) 99 %	**EU685814**	**1002**
PK-13	+	*Bacillus flexus* (T) IFO-15715 (AB021185) 98 %	**EU685815**	-
PK-14	+	*Bacillus firmus* (T) IAM-12464 (D16268) 98 %	**EU685816**	**1003**

4.2 ELUCIDATE THE METABOLIC PATHWAY OF PYRENE DEGRADATION IN A SELECTED ISOLATE

The objective was to gather information on bacterial catabolism of pyrene and elucidate the metabolic pathway. The rate at which its uptake and utilization occurs was studied by pulse chase experiments, time course experiments and media optimization studies. The effect of pyrene on bacterial growth was determined. The results proved helpful in studying the biochemistry of pyrene metabolism in the most efficient pyrene utilizing isolate. The intermediate metabolites arising out of the degradation of pyrene were characterized by Gas Chromatograph coupled with Mass Spectrograph (GC-MS).

4.2.1 Pyrene uptake by bacterial isolates from consortia

4.2.1.1 Pulse chase experiment

Pulse-chase experiments revealed that bacterial isolates (PK-23, PK-24, PK-27, PK-12, PK-13, PK-14, PK-15 and PK-16) had the ability to uptake pyrene from growth medium. Bacterial cells (2 mg protein) grown till mid-log phase in LB medium, resuspended and starved for 12 hrs in 10 mM phosphate buffer (pH 7.0 $\pm$0.2) and then exposed to 100 µg pyrene in phosphate buffer for 7 hrs showed negligible pyrene uptake since initial and final concentration of residual pyrene in culture flasks was same. In comparison, bacterial cells (2 mg protein) grown till mid-log phase in LB medium, washed in 10 mM phosphate buffer (pH 7.0 $\pm$0.2) and directly exposed to 100 µg pyrene in phosphate buffer showed 0 % (PK-14), 0.2 % (PK-27), 1.0 % (PK-12, PK-16), 2.3 % (PK-23, PK-24), 3.2 % (PK-15) and 5.5 % (PK-13) uptake in 1 hr with gradual increase up to 7.9 % (PK-14), 2 % (PK-27), 10.9 % (PK-12), 7.1 % (PK-16), 6.3 % (PK-23), 5.3 % (PK-24), 7.3 % (PK-15) and 13.4 % (PK-13), respectively, in 7 hrs (Table 3.3; Figure 3.17). The minimum limit of detection by HPLC analysis was 0.1 µg ml^{-1} pyrene. Though the uptake of pyrene as substrate increased in percentage uniformly, however the uptake of pyrene was slow and very little.

Table 3.3 Pyrene uptake by bacteria in 10 mM phosphate buffer containing 100 µg pyrene over a time period of 7 hrs (values are mean, number of replicates = 3)**.**

Time (hr)	Pyrene uptake (%)							
	PK-13	PK-12	PK-14	PK-15	PK-16	PK-23	PK-24	PK-27
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	**5.5**	1.0	0.0	3.2	1.0	2.3	2.4	0.2
2	6.4	5.4	0.0	3.7	5.6	4.3	2.6	0.3
3	7.1	7.4	0.0	5.4	5.7	6.4	2.6	0.5
4	8.7	8.5	0.0	5.9	5.7	7.7	3.0	1.1
5	10.3	9.3	0.7	6.2	5.7	7.0	4.6	1.8
6	13.3	10.6	4.6	6.7	6.4	6.9	5.1	1.9
7	**13.4**	11.0	7.9	7.3	7.1	6.3	5.3	2.0

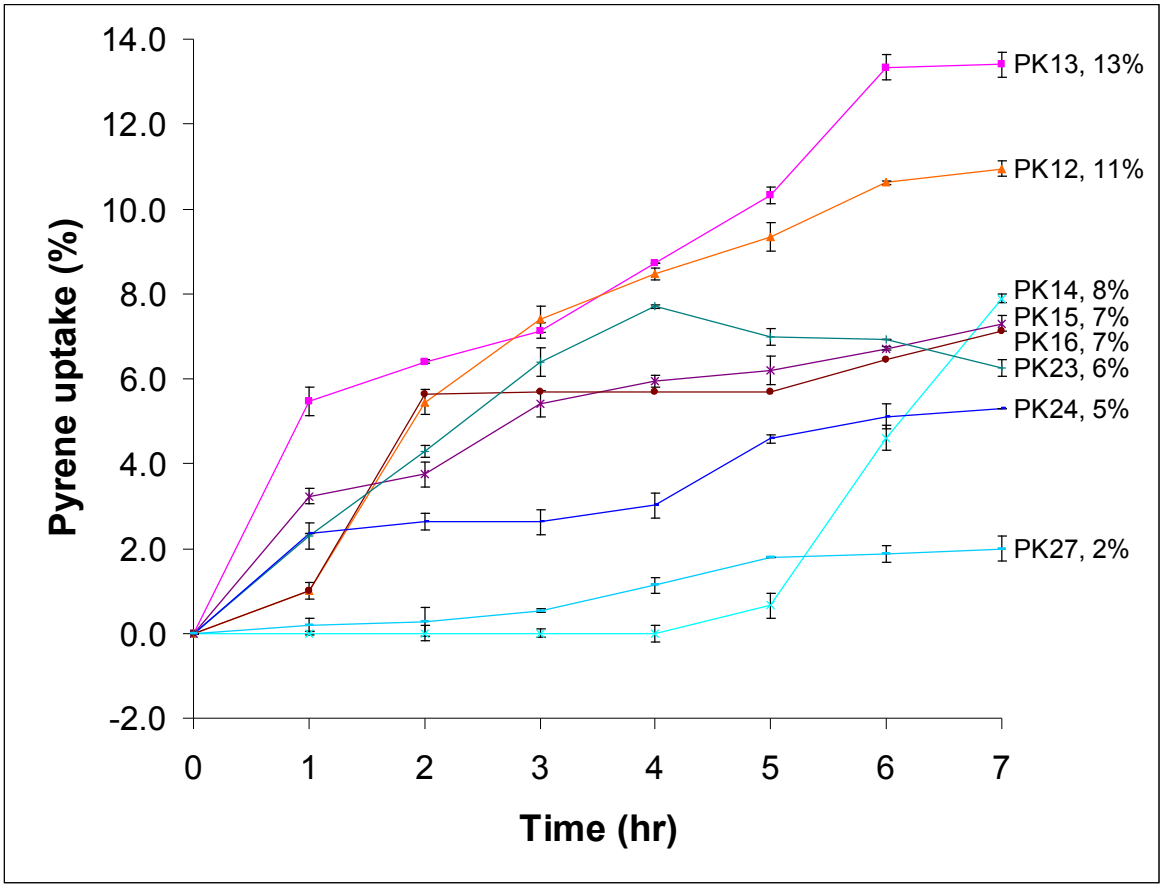

Figure 3.17 Pulse chase experiment: Pyrene uptake by washed bacterial cells in 10 mM phosphate buffer containing pyrene (100 µg) over a time period of 7 hrs (values are mean ± SE, number of replicates = 3).

4.2.1.2 Time-course experiment

In a 35 days time-course study it was observed that bacterial isolates PK-12, PK-13, PK-14, PK-15, PK-16, PK-23, PK-24 and PK-27 cometabolized 50 µg ml^{-1} pyrene (in the presence of 0.5 %; w/v glucose) by 18, 13, 18, 11, 17, 0, 10, 15 %, respectively, after 14 days of incubation (Table 3.4; Figure 3.18) which increased to 59, 53, 50, 50, 47, 17, 28 and 45 %, respectively, as compared to negligible change in abiotic control after 28 days. Thereafter pyrene uptake for three bacterial isolates PK-15, PK-16 and PK-23 remained constant till 35 days, while for five isolates PK-12, PK-13, PK-14, PK-24 and PK-27 it (uptake) increased upto 64 %, 55 %, 53 %, 30 and 49 %, respectively. Pyrene uptake was found to be highest between 14 and 28 days (Figure 3.19) and then stabilized till 35 days. With reference to growth curve of these bacterial isolates shown in Figure 3.5 A, B the time course experiment demonstrated that maximum extent of pyrene uptake corresponded to stationary phase of growth for all isolates.

Table 3.4 Pyrene uptake by bacteria isolated from crude oil contaminated soil, grown in BHB + glucose (0.5 %; w/v) + pyrene (50 µg ml^{-1}) medium at 30 °C over a time course of 35 days (values are mean, number of replicates = 3).

Time (days)	Pyrene uptake (%)								
	PK-12	**PK-13**	**PK-14**	**PK-15**	**PK-16**	**PK-23**	**PK-24**	**PK-27**	Abiotic control
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
7	13.5	7.0	18.1	6.8	12.8	0.0	5.5	14.3	0.0
14	17.7	13.1	18.2	10.7	16.6	0.0	10.1	14.9	0.0
21	30.0	17.4	22.2	12.7	25.5	1.5	11.0	14.9	0.0
28	59.2	52.5	50.4	50.0	46.9	16.9	27.9	44.8	0.0
35	**64.1**	**55.1**	**52.9**	**50.0**	**47.4**	**16.9**	**29.7**	**48.6**	**0.0**

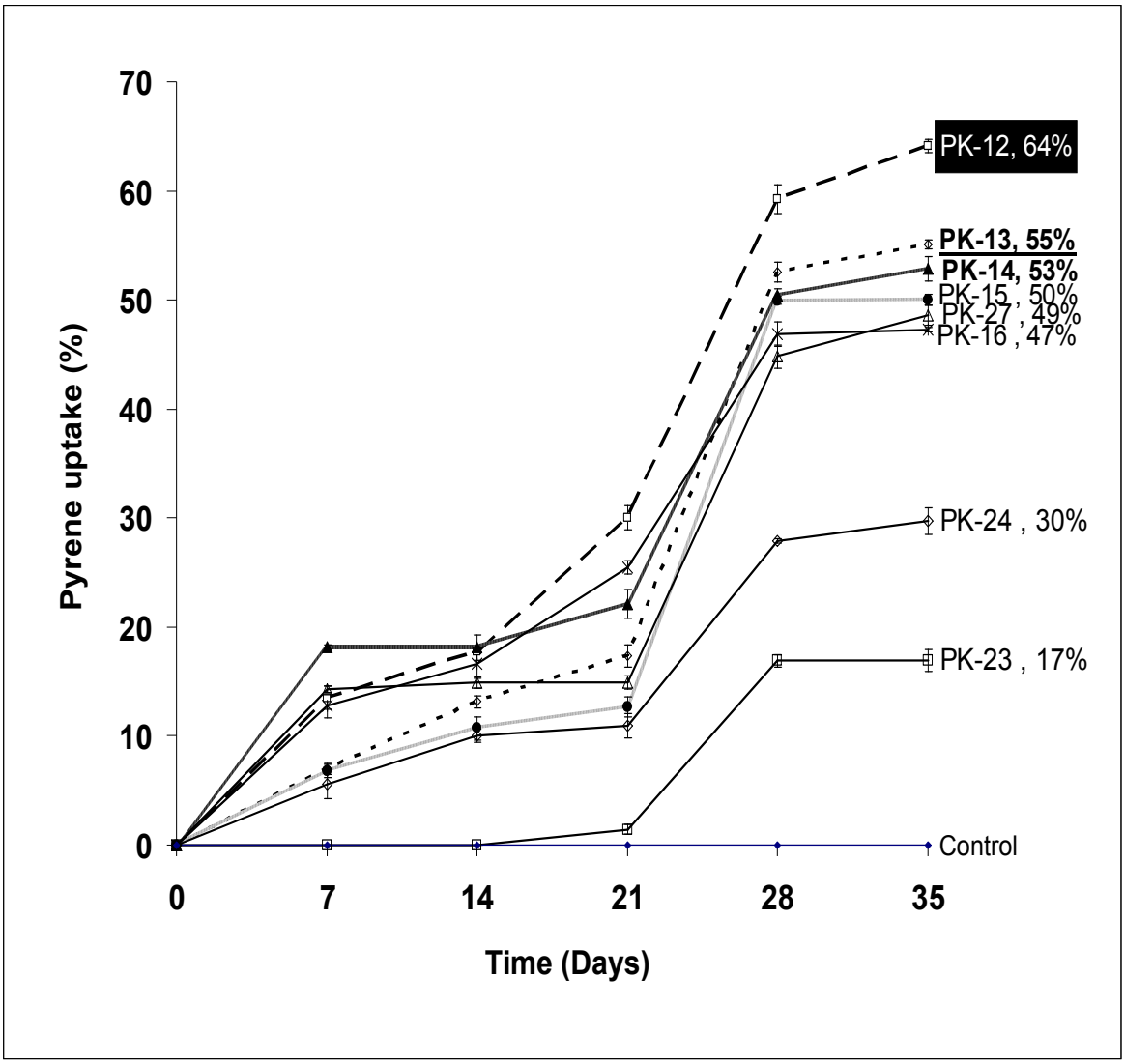

Figure 3.18 Uptake of pyrene by bacteria isolated from crude oil contaminated soil, grown in BHB + glucose (0.5 %; w/v) + pyrene (50 µg ml^{-1}) medium at 30 ^{o}C over a time -course of 35 days (values are mean ± SE, number of replicates = 3)**.**

Bacillus pumilus (PK-12) showed the highest pyrene uptake of 64 % from BHB medium containing glucose (0.5 %; w/v) after 35 days, closely followed by *Bacillus flexus* (PK-13) (55 %) and *Bacillus firmus* (PK-14) (53 %). These three isolates capable of utilizing more than 50 % pyrene (50 µg ml^{-1}), were selected for further studies.

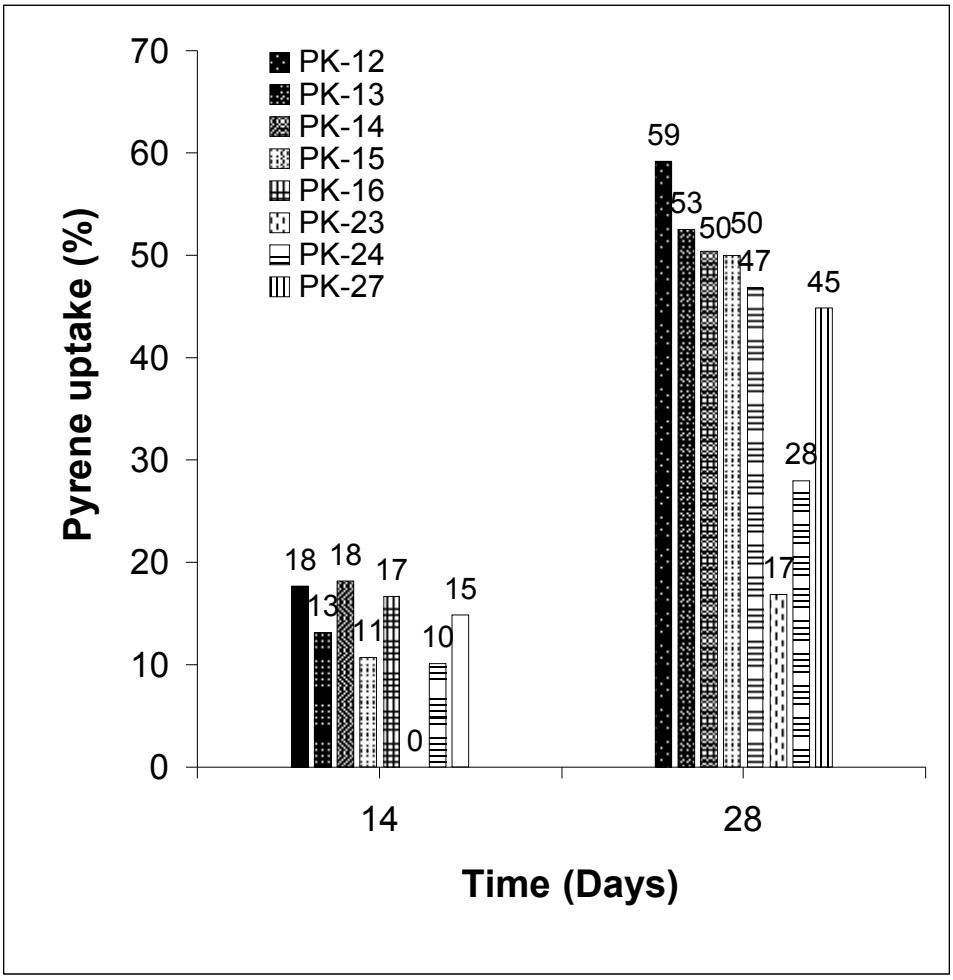

Figure 3.19 Pyrene uptake by bacteria isolated from crude oil contaminated soil, grown in BHB + glucose (0.5 %; w/v) + pyrene (50 µg ml^{-1}) medium at 30 ^{o}C between 14 and 28 day incubation period.

4.2.1.3 Effect of pyrene on growth of Bacillus *sp. (PK-12)*

The effect of pyrene addition on growth of *Bacillus pumilus* (PK-12) was studied. Pyrene (50 µg ml^{-1}) was added to the growing culture in mid-log phase and growth was measured by optical density at 600 nm. Growth measurements suggest that the growth of *B. pumilus* was not negatively affected by 50 µg ml^{-1} pyrene in BHB +Glucose (0.5 %) and LB medium. Optical density measurements taken after every hour of growth indicated that *B. pumilus* reached exponential phase within the first 6 hrs of growth in LB and within 60 hrs in BHB +Glucose (0.5 %; w/v) and became stationary thereafter in each growth medium.

Table 3.5 Effect of pyrene (50 µg ml^{-1}) addition on growth of *Bacillus pumilus* (PK-12) in different basal media over a time period of 200 hrs (values are mean of 3 replicates; Standard deviation was in range 0 - 6 %).

BHB +P: Bushnell Haas broth, containing pyrene, G: glucose (0.5 %; w/v), [A] : Pyrene added when culture entered log phase of growth

Time (hr)	Growth ($OD_{600\ nm}$)		
	BHB + P	BHB + G	BHB + G + P
0	0.117	0.118	0.118
1	0.117	0.119	0.119
2	0.126	0.124	0.124
3	0.132	0.117	0.117
4	0.147	0.136	0.136
5	0.136	0.113	0.113
6	0.118	0.115	0.115
12	0.127	0.130	0.130
27	0.389	0.524	0.524 [A]
35	0.370	0.702	0.702
50	0.277	0.759	0.759
57	0.333	0.806	0.877
79	0.306	0.783	0.937
106	0.297	0.825	0.932
122	0.353	0.853	0.953
148	0.305	0.821	1.064
176	0.305	0.892	1.066
200	0.305	0.766	1.016

Pyrene alone as carbon source did not support good growth (max OD_{600} = 0.38; Table 3.5; Figure 3.20). Pyrene addition in BHB +glucose (0.5 %; w/v) medium to mid-log phase culture increased maximum optical density at 600 nm from 0.89 to 1.06 (Table 3.5; Figure 3.20).

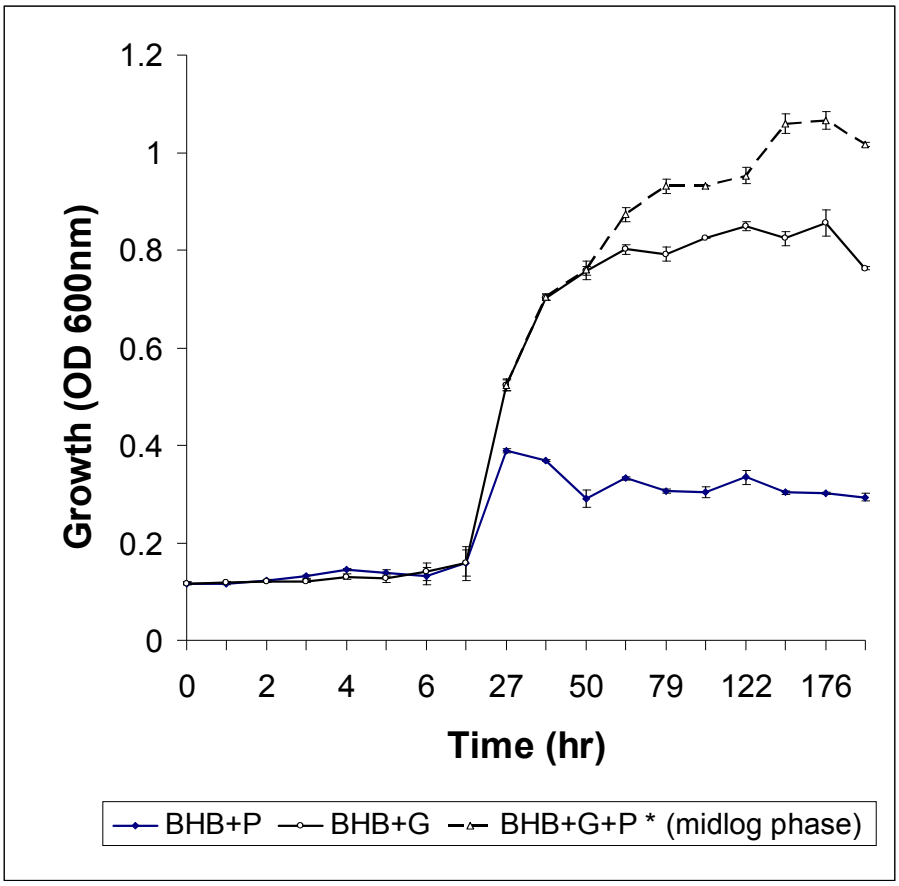

Figure 3.20 Growth of *Bacillus pumilus* (PK-12) in different basal media over a time period of 200 hrs (values are mean ± SE, number of replicates, n = 3)**.**

BHB: Bushnell Haas broth medium, P: pyrene (50 µg ml^{-1}), G: glucose (0.5 %; w/v), P *: Pyrene added at mid-log phase of growth.

Growth of bacterial cells in LB medium (max OD_{600} = 1.98) was not affected by pyrene addition in mid-log phase (max OD_{600} = 2.17; Table 3.6; Figure 3.21). The study indicated that pyrene addition in mid-log phase did not have any deleterious effect on the growth of the cells. In fact pyrene addition in BHB medium containing 0.5 % (w/v) glucose was favourable for the isolate growth.

Table 3.6 Effect of pyrene (50 µg ml^{-1}) addition on growth of *Bacillus pumilus* (PK-12) in different LB media over a time period of 24 hrs (values are mean of 3 replicates; Standard deviation was in range 0-7 %)**.**

[A] implies pyrene added when culture entered log phase of growth

Time (hr)	Growth ($OD_{600\ nm}$)	
	LB	**LB + P [A]**
0	0.197	0.197
1	0.307	0.307
2	0.502	0.502
3	1.109	1.109 [A]
4	1.573	1.609
5	1.769	1.849
6	1.929	2.012
7	1.969	2.014
8	1.991	2.101
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12	2.069	2.075
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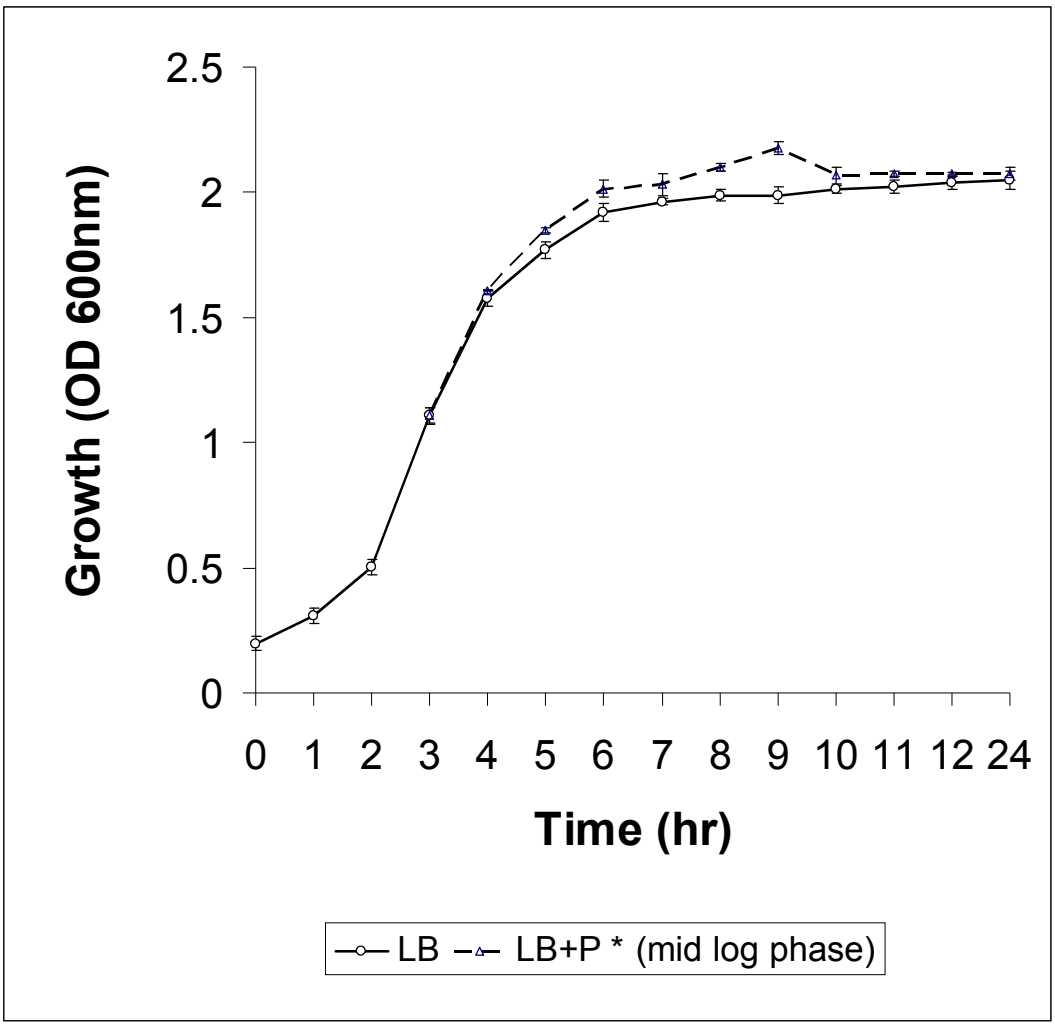


Figure 3.21 Growth of *Bacillus pumilus* (PK-12) in different LB media over a period of 24 hrs (values are mean ± SE, number of replicates, n = 3).

LB: Luria-Bertani broth, P *: Pyrene (50 µg ml^{-1}) added at mid-log phase of growth.

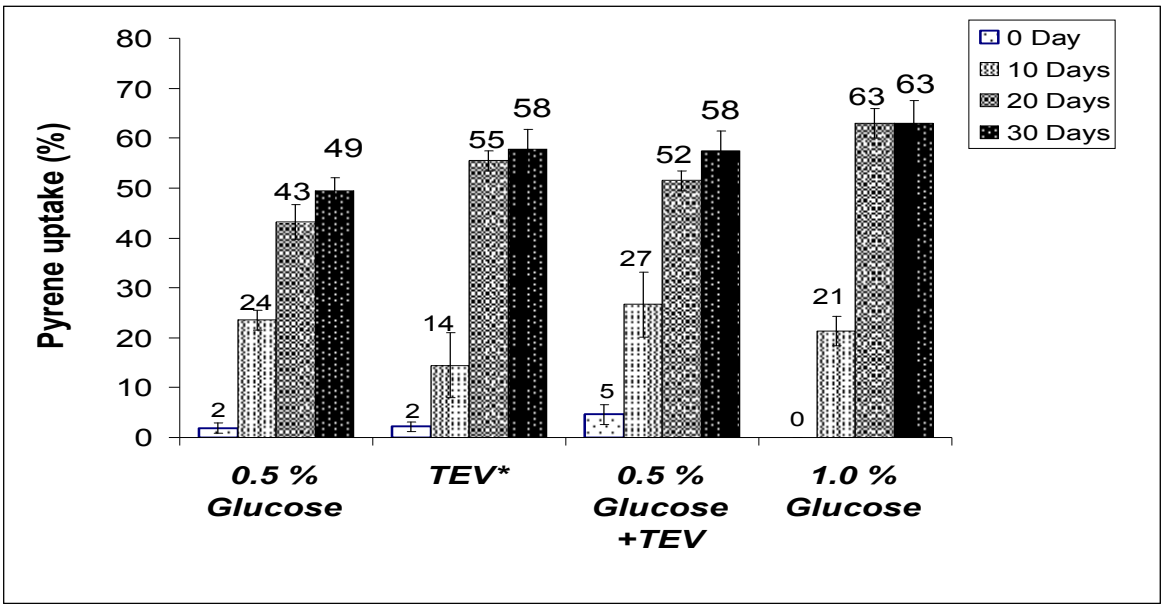
4.2.1.3 Effect of media supplementation for enhanced pyrene utilization by bacterial consortia

The effect of media supplementation on rate of pyrene uptake and absolute / optional requirement of glucose for pyrene metabolism by consortia CON-3 and THA-2 was studied.

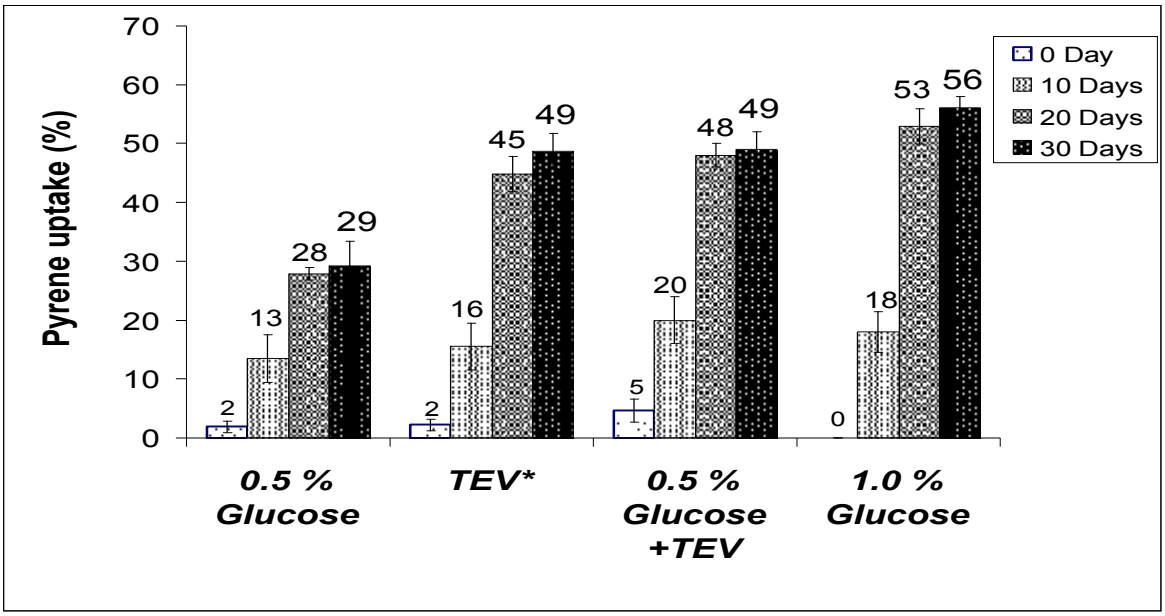
Four different media combinations were prepared for the study. To first set of Erlenmeyer flasks containing BHB media (100 ml) and pyrene (50 µg ml^{-1}) added glucose (0.5 %; w/v) and labeled '0.5G', to the second set, labeled 'TEV' flasks, added trace elements and vitamins, to the third set of flasks, labeled '0.5G +TEV', added both glucose (0.5 %; w/v) and trace elements and vitamins and to the fourth set of flasks, labeled '1.0G', added glucose (1.0 %; w/v). All four sets of media were inoculated with CON-3 and THA-2 consortia (in triplicates), and uninoculated media served as control. Media supplementation with trace elements and vitamins was found to have enhanced the pyrene uptake from 49 to 58 % by consortium CON-3 (Table 3.7; Figure 3.22 A) and from 29 to 49 % by consortium THA-2 (Table 3.7; Figure 3.22 B) in 30 days, both in the absence and presence of glucose. BHB medium with double glucose concentration of 1.0 % (w/v) stimulated the pyrene uptake to 63 % by consortium CON 3 and 56 % by consortium THA-2 in 30 days.

Table 3.7 Effect of media supplementation for enhanced pyrene utilization by bacterial consortia isolated from fuel oil contaminated soil at 30 °C (values are mean, no of replicates, n = 3)**.**

Time (days)	Pyrene Uptake (%)							
	CON-3				THA-2			
	0.5 % glucose	TEV	0.5 % glucose + TEV	1.0 % glucose	0.5 % glucose	TEV	0.5 % glucose + TEV	1.0 % glucose
0	2	2	5	0	2	2	5	0
10	24	14	27	21	13	16	20	18
20	43	55	52	63	28	45	48	53
30	49	58	58	63	29	49	49	56



(A)



(B)

Figure 3.22 Pyrene uptake by bacterial consortia (A) CON-3 and (B) THA-2 grown in BHB plus pyrene (50 µg ml^{-1}) medium supplemented with either 0.5 % (w/v) glucose or trace elements and trace vitamins (TEV) or 1.0 % (w/v) glucose at 30 ^{o}C (values are mean ± SE, number of replicates, n = 3).

4.2.1.4 Effect of glucose on pyrene utilization by bacteria

An increase in growth was observed when glucose concentration in BHB +pyrene (50 µg ml^{-1}) medium was increased from 0.5 % to 1.0 % (w/v) (Table 3.8; Figure 3.23).

Table 3.8 Effect of glucose (0.5 – 1.0 %) supplementation on growth of bacterial isolates from (A) crude oil contaminated soil (PK-12 to PK-14) and (B) coal-tar contaminated soil (PK-1 to PK-10). Symbol (-) implies not determined.

Time (days)	Growth ($OD_{600\ nm}$)					
	PK-12		PK-13		PK-14	
	0.5%	1%	0.5%	1%	0.5%	1%
0	0.033	0	0.016	0	0.041	0
1	0.638	1.006	0.370	0.081	0.771	0.803
2	0.786	1.037	0.510	0.526	0.777	1.587
3	0.833	1.166	0.659	0.559	0.807	1.880
4	0.852	0.982	0.748	0.559	0.871	1.833
5	0.751	-	0.787	-	0.885	-
6	0.679	-	0.793	-	0.974	-

Time (days)	Growth ($OD_{600\ nm}$)									
	PK-1		PK-2		PK-3		PK-4		PK-5	
	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%
0	0.113	0.113	0.123	0.123	0.088	0.088	0.086	0.086	0.082	0.082
1	0.500	1.096	0.563	0.772	0.598	0.762	0.355	0.367	0.199	0.579
2	0.508	1.549	0.772	1.375	0.634	1.141	0.522	0.909	0.524	0.717
3	0.589	1.563	0.877	1.596	0.632	1.496	0.854	1. 256	0.797	1.105
4	0.638	1.581	1.065	1.619	0.631	1.765	1.055	1.676	0.809	1.326
5	0.687	-	1.253	-	0.629	-	1.255	-	0.820	-
6	0.603	-	1.252	-	0.803	-	1.200	-	0.828	-

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Time (days)	Growth ($OD_{600\ nm}$)									
	PK-6		PK-7		PK-8		PK-9		PK-10	
	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%	0.5%	1%
0	0.076	0.076	0.088	0.088	0.076	0.076	0.073	0.073	0.080	0.080
1	0.399	0.500	0.780	0.783	0.550	1.061	0.421	0.471	0.740	0.758
2	0.650	1.061	0.900	1.352	0.974	1.377	0.514	1.127	0.960	1.172
3	0.800	1.541	0.910	1.455	0.981	1.355	0.421	1.120	1.198	1.344
4	0.940	1.686	0.995	1.465	1.032	1.363	0.421	1.114	1.259	1.474
5	1.080	-	1.080	-	1.083	-	0.421	-	1.320	-
6	1.510	-	1.280	-	1.170	-	0.228	-	1.380	-

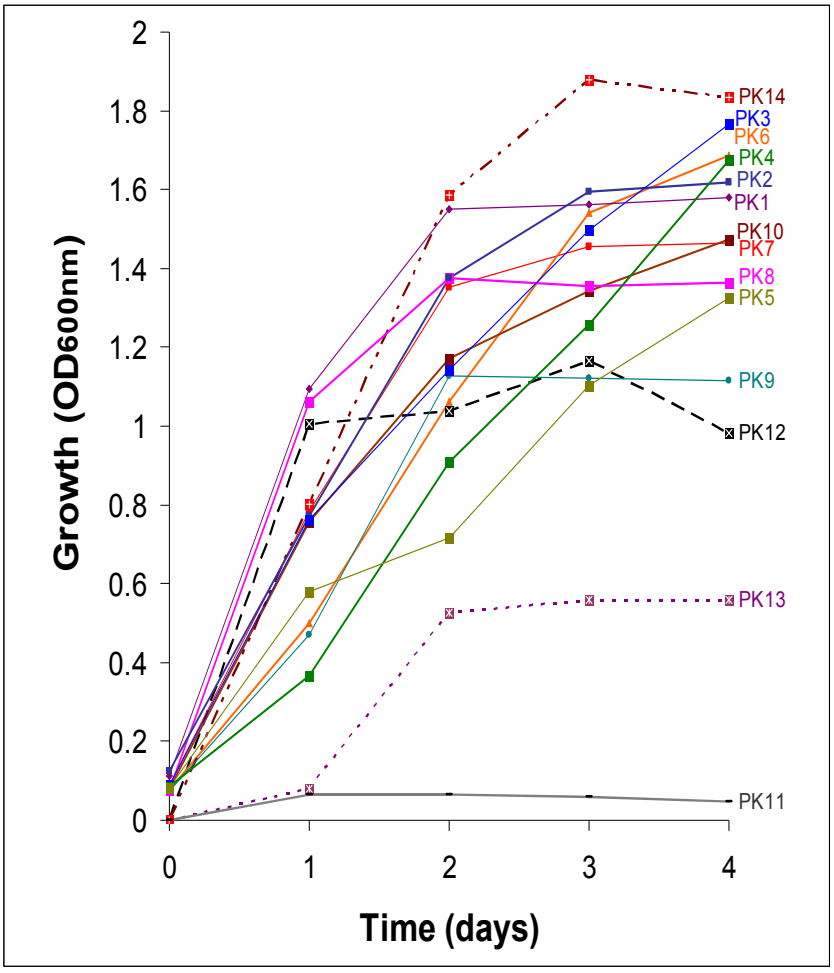

Figure 3.23 Growth of bacterial isolates from coal-tar and crude oil contaminated soil in BHB plus pyrene (50 µg ml^{-1}) medium supplemented with 1.0 % (w/v) glucose at 30 °C.

Pyrene uptake by the bacteria *Bacillus pumilus* (PK-12), *B. flexus* (PK-13) and *B. firmus* (PK-14) in the presence of glucose in basal growth medium was studied by spectrophotometric observation at 254 nm and HPLC analysis of residual pyrene in solvent extracts of spent medium. In a time course study, *Bacillus pumilus* (PK-12), *B. flexus* (PK-13) and *B. firmus* (PK-14) showed 13.5, 7.0 and 18.1 % uptake of pyrene, respectively from BHB media containing 50 µg ml^{-1} pyrene and 0.5 % (w/v) glucose in 7 days (Table 3.4). When the glucose concentration in BHB medium was doubled to 1.0 % (w/v) *Bacillus* sp. PK-12, PK-13 and PK-14 showed increased and rapid cometabolic uptake of pyrene which was 45.6, 18.5 and 36.5 %, respectively in 4 days (Table 3.9 A). A stimulatory effect on bacterial uptake of pyrene was observed with increase in the glucose concentration in basal medium.

Pyrene utilizing bacterial community in coal-tar and crude oil contaminated soils was represented by three major soil bacterial groups *Bacillus*, *Rhodococcus* and *Pseudomonas*. Of all the isolates tested, *Bacillus* sp. (PK-6) removed maximum (56.4 %) and *Bacillus* sp. (PK-9) minimum amount (9.8 %) of pyrene while *Pseudomonas* sp. (PK-3) and *Rhodococcus* sp. (PK-10) removed 25.2 and 46.3 %, respectively, of pyrene in four days (Fig 3). The isolates can be arranged in the decreasing order of 50 µg ml^{-1} pyrene uptake (expressed in percentage) as *Bacillus* sp. PK-6 (56.4 %) > *Bacillus* sp. PK-7 (53.4 %) > *Rhodococcus* sp. PK-10 (46.3 %) > *Bacillus* sp. PK-12 (45.6 %) > *Bacillus* sp. PK-5 (43.8 %) > *Bacillus* sp. PK-1 (40.6 %) > *Bacillus* sp. PK-14 (36.5 %) > *Bacillus* sp. PK-4 (33.0 %) > *Bacillus* sp. PK-8 (27.0 %) > *Pseudomonas* sp. PK-3 (25.2 %) > *Bacillus* sp. PK-2 (20.9 %) > *Bacillus* sp. PK-13 (18.5 %) > *Bacillus* sp. PK-9 (9.8 %) as shown in Table 3.9 A, F.

4.2.1.5 Biosurfactant activity

Standard emulsification assay (index) of Barkay *et al.* (1999) and Jacques *et al.* (2007) was followed to monitor biosurfactant activity by the above 14 pyrene utilizing bacteria, namely PK-1 to PK-14.

Table 3.9 A. **Effect of 1.0 % (w/v) glucose supplementation on pyrene (50 µg ml^{-1}) uptake by bacterial isolates (PK-1 to PK-10) isolated from coal-tar contaminated soil and (PK-12 to PK-14) from crude oil contaminated soil, grown in BHB medium.**

Time (days)	Pyrene uptake (%)													
	Bacillus sp. PK-1	*Bacillus* sp. PK-2	*Pseudomonas* sp. PK-3	*Bacillus* sp. PK-4	*Bacillus* sp. PK-5	*Bacillus* sp. PK-6	*Bacillus* sp. PK-7	*Bacillus* sp. PK-8	*Bacillus* sp. PK-9	*Rhodococcus* sp. PK-10	*Bacillus* sp. PK-12	*Bacillus* sp. PK-13	*Bacillus* sp. PK-14	*Kocuria* sp. PK-11
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	10.4	0.0	0.0	6.1	14.8	0.0	15.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
2	20.9	0.0	0.0	12.1	29.6	39.4	19.6	18.2	0.2	16.2	0.0	0.0	22.9	0.0
3	30.7	10.4	12.6	22.6	36.7	50.0	25.4	24.0	9.3	32.8	19.6	0.0	31.9	0.0
4	40.6	20.9	25.2	33.0	43.8	**56.4**	53.4	27.0	**9.8**	46.3	45.6	18.5	36.5	0.0

Table 3.9 B. **Biosurfactant activity produced by bacterial isolates (PK-1 to PK-10) isolated from coal-tar contaminated soil and (PK-12 to PK-14) from crude oil contaminated soil, grown in BHB medium.**

Time (days)	Biosurfactant activity ($OD_{550\ nm}$)													
	Bacillus sp. PK-1	*Bacillus* sp. PK-2	*Pseudomonas* sp. PK-3	*Bacillus* sp. PK-4	*Bacillus* sp. PK-5	*Bacillus* sp. PK-6	*Bacillus* sp. PK-7	*Bacillus* sp. PK-8	*Bacillus* sp. PK-9	*Rhodococcus* sp. PK-10	*Bacillus* sp. PK-12	*Bacillus* sp. PK-13	*Bacillus* sp. PK-14	*Kocuria* sp. PK-11
0	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.15	0.10	0.10
1	0.66	0.43	0.16	0.28	0.73	0.49	0.56	0.44	0.49	1.11	1.06	0.15	0.22	0.16
2	0.89	0.48	0.27	0.48	0.82	1.67	1.24	1.12	0.55	1.21	1.09	0.64	1.19	0.27
3	0.98	0.68	0.32	0.85	0.91	1.78	1.54	1.20	0.66	1.32	1.23	0.65	1.58	0.32
4	1.01	0.72	**0.43**	0.97	1.02	**1.96**	1.61	1.33	0.66	1.45	1.11	0.55	1.54	0.26

Of all the isolates tested, *Bacillus* sp. (PK-6) showed maximum and *Pseudomonas* sp. (PK-3) minimum biosurfactant activity in growth medium. *Rhodococcus* sp. (PK-10) also showed high biosurfactant activity (Figure 3.24 A). The isolates can be arranged in the decreasing order of biosurfactant activity produced (expressed in terms of OD_{550}) as *Bacillus* sp. PK-6 (1.96) > *Bacillus* sp. PK-7 (1.61) > *Bacillus* sp. PK-14 (1.54) > *Rhodococcus* sp. PK-10 (1.45) > *Bacillus* sp. PK-8 (1.33) > *Bacillus* sp. PK-12 (1.11) > *Bacillus* sp. PK-5 (1.02) > *Bacillus* sp. PK-1 (1.01) > *Bacillus* sp. PK-4 (0.97) > *Bacillus* sp. PK-2 (0.72) > *Bacillus* sp. PK-9 (0.66) > *Bacillus* sp. PK-13 (0.55) > *Pseudomonas* sp. PK-3 (0.43) as shown in Table 3.9 B.

It was observed that both pyrene uptake values and biosurfactant activity values are together found in decreasing order for eight isolates, namely *Bacillus* sp. PK-6 (56.4 %, 1.96) > *Bacillus* sp. PK-7 (53.4 %, 1.61) > *Rhodococcus* sp. PK-10 (46.3 %, 1.45) > *Bacillus* sp. PK-12 (45.6 %, 1.11) > *Bacillus* sp. PK-5 (43.8 %, 1.02) > *Bacillus* sp. PK-1 (40.6 %, 1.01) > *Bacillus* sp. PK-4 (33.0 %, 0.97) > *Pseudomonas* sp. PK-3 (25.2 %, 0.43) as shown in Table 3.9 F. Five isolates, *Bacillus* sp. PK-14 (36.5 %, 1.54), *Bacillus* sp. PK-8 (27.0 %, 1.33), *Bacillus* sp. PK-2 (20.9 %, 0.72), *Bacillus* sp. PK-13 (18.5 %, 0.55) and *Bacillus* sp. PK-9 (9.8 %, 0.66) fall out of this decreasing order of pyrene uptake and biosurfactant activity.

1.2.1.6 Total cell protein

Of all the isolates tested, *Bacillus* sp. (PK-7) showed maximum and *Pseudomonas* sp. (PK-3) showed minimum cell protein in growth medium. *Rhodococcus* sp. (PK-10) also showed high cell protein (Figure 3.24 B). The isolates can be arranged in the decreasing order of cell protein (expressed in mg ml^{-1}) as *Bacillus* sp. PK-7 (2.25 mg ml^{-1}) > *Rhodococcus* sp. PK-10 (2.20 mg ml^{-1}) > *Bacillus* sp. PK-6 (1.96 mg ml^{-1}) > *Bacillus* sp. PK-2 (1.64 mg ml^{-1}) > *Bacillus* sp. PK-4 (1.59 mg ml^{-1}) > *Bacillus* sp. PK-8 (1.44 mg ml^{-1}) > *Bacillus* sp. PK-1 (1.42 mg ml^{-1}) >*Bacillus* sp. PK-12 (1.39 mg ml^{-1}) > *Bacillus* sp. PK-5 (1.25 mg ml^{-1}) > *Bacillus* sp. PK-9 (1.18 mg ml^{-1}).> *Bacillus* sp. PK-14 (1.13 mg ml^{-1}) >*Bacillus* sp. PK-13 (1.03 mg ml^{-1}) >*Pseudomonas* sp. PK-3 (0.97 mg ml^{-1}) as shown in Table 3.9 C.

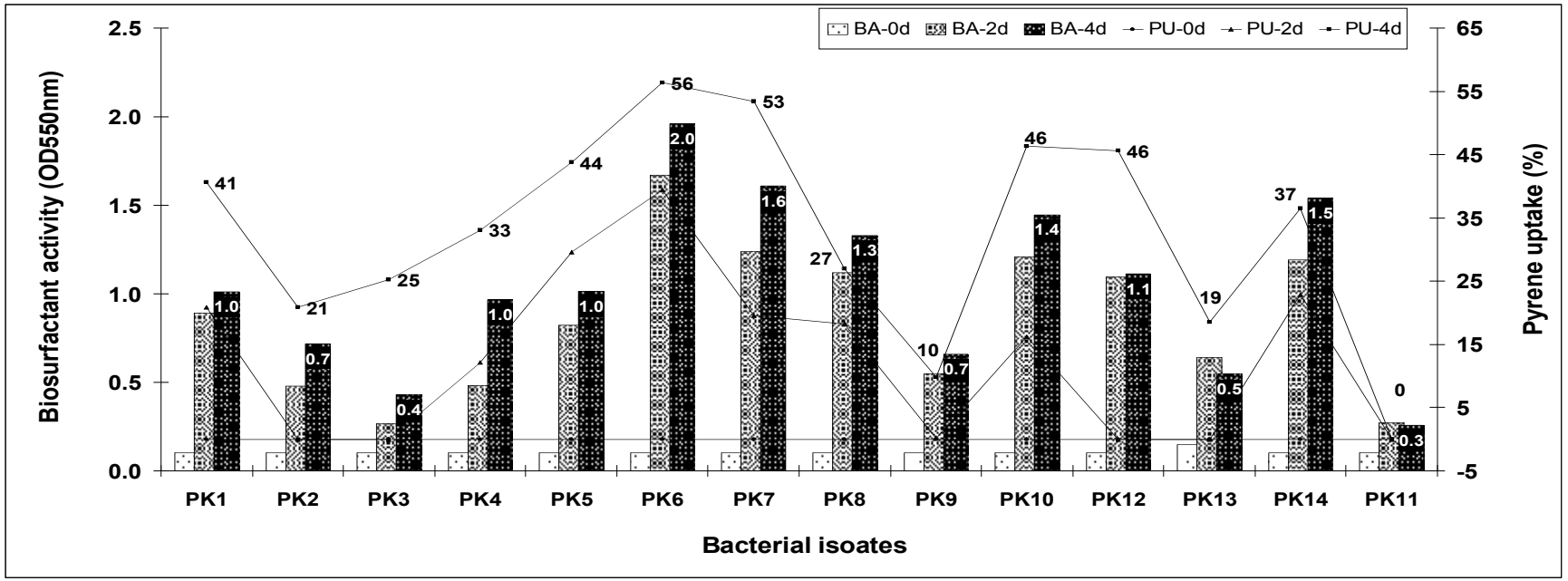

Figure 3.24 A. Pyrene uptake (PU) and biosurfactant activity (BA) profile of bacteria from coal-tar and crude oil contaminated soils in BHB medium supplemented with 1 % (w/v) glucose and 50 µg ml^{-1} pyrene.

Table 3.9 C. Cell protein produced in 4 day growth of bacterial isolates (PK-1 to PK-10) isolated from coal-tar contaminated soil and (PK-12 to PK-14) isolated from crude oil contaminated soil. The growth medium BHB contained pyrene (50 µg ml^{-1}) and glucose (1.0 %; w/v).

Time (days)	Protein (mg ml^{-1})														
	Ctrl	*Bacillus* sp. PK-1	*Bacillus* sp. PK-2	*Pseudo monas* sp. PK-3	*Bacillus* sp. PK-4	*Bacillus* sp. PK-5	*Bacillus* sp. PK-6	*Bacillus* sp. PK-7	*Bacillus* sp. PK-8	*Bacillus* sp. PK-9	*Rhodo coccus* sp. PK-10	*Bacillus* sp. PK-12	*Bacillus* sp. PK-13	*Bacillus* sp. PK-14	*Kocuria* sp. PK-11
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.0
1	0.00	1.07	0.78	0.66	0.75	0.85	1.51	1.74	0.97	0.97	1.40	1.25	0.78	0.74	0.62
2	0.00	1.19	0.96	0.68	1.01	1.08	1.66	1.93	1.23	0.97	1.70	1.29	0.83	0.99	0.68
3	0.00	1.26	1.23	0.90	1.08	1.14	1.73	2.13	1.43	1.12	2.10	1.35	0.88	1.06	0.80
4	0.00	1.42	1.64	**0.97**	1.59	1.25	1.96	**2.25**	1.44	1.18	2.20	1.39	1.03	1.13	0.82

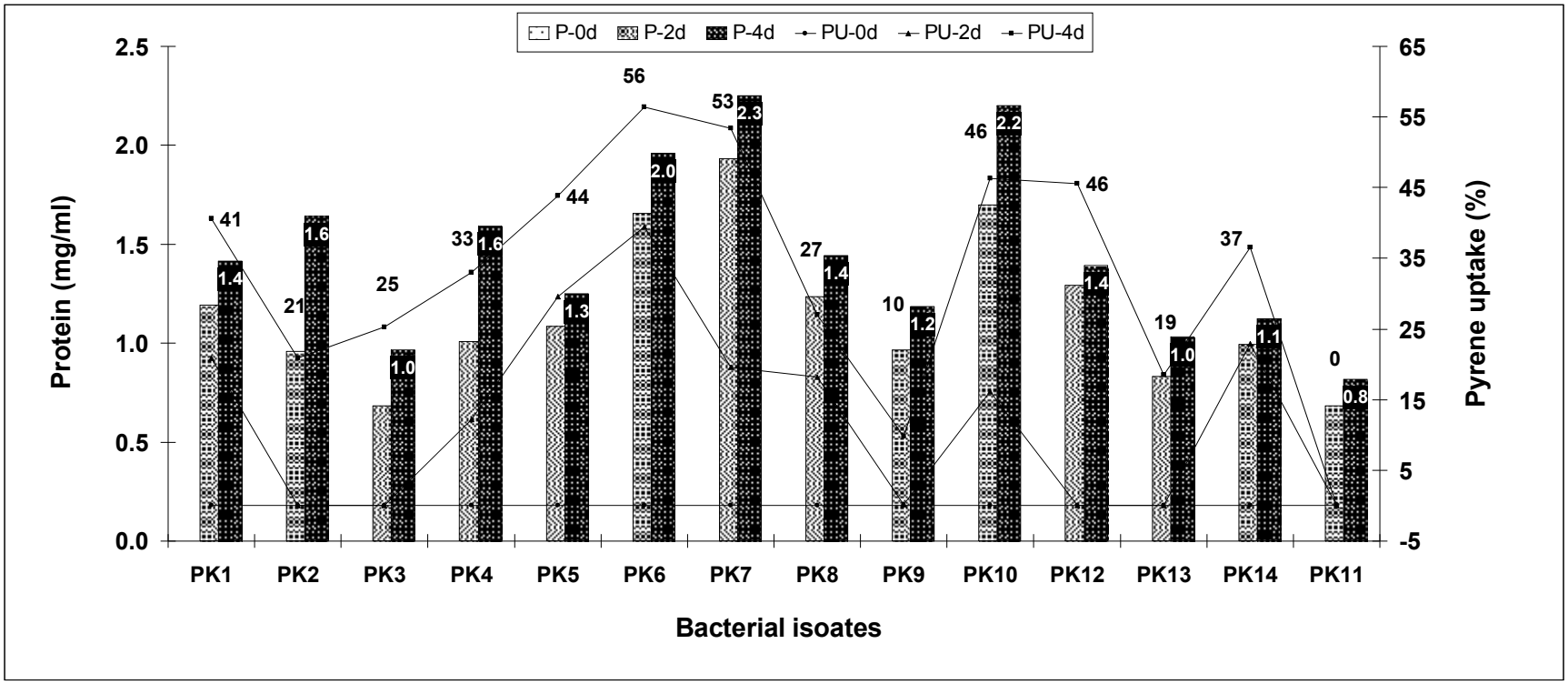

Figure 3.24 B. Pyrene uptake (PU) and Total cell protein (P) profile of bacteria isolated from coal-tar and crude oil contaminated soils in BHB medium supplemented with 1 % (w/v) glucose and 50 µg ml^{-1} pyrene.

Table 3.9 D. Residual glucose concentration after 4 days in growth medium cultured with bacterial isolates (PK-1 to PK-10) from coal-tar contaminated soil and (PK-12 to PK-14) from crude oil contaminated soil. The growth medium BHB contained pyrene (50 µg ml^{-1}) and glucose (1.0 %; w/v).

Time (days)	Residual glucose (%)														
	Ctrl	*Bacillus* sp. PK-1	*Bacillus* sp. PK-2	*Pseudomonas* sp. PK-3	*Bacillus* sp. PK-4	*Bacillus* sp. PK-5	*Bacillus* sp. PK-6	*Bacillus* sp. PK-7	*Bacillus* sp. PK-8	*Bacillus* sp. PK-9	*Rhodococcus* sp. PK-10	*Bacillus* sp. PK-12	*Bacillus* sp. PK-13	*Bacillus* sp. PK-14	*Kocuria* sp. PK-11
0	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
1	1.00	0.78	0.86	0.89	0.83	0.79	0.51	0.68	0.63	0.91	0.45	0.84	0.91	0.75	0.92
2	1.00	0.68	0.84	0.84	0.82	0.73	0.43	0.58	0.59	0.91	0.45	0.71	0.91	0.54	0.99
3	1.00	0.58	0.60	0.82	0.73	0.63	0.43	0.48	0.48	0.91	0.44	0.71	0.90	0.44	0.89
4	1.00	0.46	0.57	0.82	0.65	0.60	**0.40**	0.46	0.46	**0.87**	0.42	0.65	0.85	0.42	0.89

Table 3.9 E. Glucose utilized in 4 days of growth of bacterial isolates (PK-1 to PK-10) isolated from coal-tar contaminated soil and (PK-12 to PK-14) from crude oil contaminated soil. The growth medium BHB contained pyrene (50 µg ml^{-1}) and glucose (1.0 %; w/v).

Time (days)	Glucose utilized (%)														
	Ctrl	*Bacillus* sp. PK-1	*Bacillus* sp. PK-2	*Pseudomonas* sp. PK-3	*Bacillus* sp. PK-4	*Bacillus* sp. PK-5	*Bacillus* sp. PK-6	*Bacillus* sp. PK-7	*Bacillus* sp. PK-8	*Bacillus* sp. PK-9	*Rhodococcus* sp. PK-10	*Bacillus* sp. PK-12	*Bacillus* sp. PK-13	*Bacillus* sp. PK-14	*Kocuria* sp. PK-11
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1	0.00	0.22	0.14	0.11	0.17	0.21	0.49	0.32	0.37	0.09	0.55	0.16	0.09	0.25	0.08
2	0.00	0.32	0.16	0.16	0.18	0.27	0.57	0.42	0.41	0.09	0.55	0.29	0.09	0.46	0.09
3	0.00	0.42	0.40	0.18	0.27	0.37	0.57	0.52	0.52	0.09	0.56	0.29	0.10	0.56	0.11
4	0.00	0.54	0.43	0.18	0.35	0.40	**0.60**	0.54	0.54	**0.13**	0.58	0.35	0.15	0.58	0.11

It wais observed that both pyrene uptake values and cell protein are together found in decreasing order for five isolates, namely *Bacillus* sp. PK-6 (56.4 %, 1.96 mg ml^{-1}) > *Bacillus* sp. PK-12 (45.6 %, 1.39 mg ml^{-1}) > *Bacillus* sp. PK-5 (43.8 %, 1.25 mg ml^{-1}) > *Bacillus* sp. PK-14 (36.5 %, 1.13 mg ml^{-1}) > *Pseudomonas* sp. PK-3 (25.2 %, 0.97 mg ml^{-1}) as shown in Table 3.9 F. Eight isolates, *Bacillus* sp. PK-7 (53.4 %, 2.25 mg ml^{-1}), *Rhodococcus* sp. PK-10 (46.3 %, 2.20 mg ml^{-1}), *Bacillus* sp. PK-1 (40.6 %, 1.42 mg ml^{-1}), *Bacillus* sp. PK-4 (33.0 %, 1.59 mg ml^{-1}), *Bacillus* sp. PK-8 (27.0 %, 1.44 mg ml^{-1}), *Bacillus* sp. PK-2 (20.9 %, 1.64 mg ml^{-1}), *Bacillus* sp. PK-13 (18.5 %, 1.03 mg ml^{-1}) and *Bacillus* sp. PK-9 (9.8 %, 1.18 mg ml^{-1}) fall out of this decreasing order of pyrene uptake and cell protein.

4.2.1.7 Glucose utilization

Of all the isolates tested, *Bacillus* sp. (PK-6) showed minimum and *Bacillus* sp. (PK-9) showed maximum residual glucose concentration in growth medium after 4 days of incubation (Table 3.9 D). In other words, *Bacillus* sp. (PK-6) showed maximum and *Bacillus* sp. (PK-9) showed minimum glucose utilization in growth medium. *Pseudomonas* sp. (PK-3) showed high residual glucose i.e., low glucose utilization (Figure 3.24 C). The isolates can be arranged in the increasing order of residual glucose (decreasing order of glucose utilization) (expressed in percentage) as *Bacillus* sp. PK-6 (0.600 %) > *Rhodococcus* sp. PK-10 (0.580 %) > *Bacillus* sp. PK-14 (0.578 %) > *Bacillus* sp. PK-1 (0.544 %) > *Bacillus* sp. PK-7 (0.543 %) > *Bacillus* sp. PK-8 (0.537 %) > *Bacillus* sp. PK-2 (0.430 %) > *Bacillus* sp. PK-5 (0.400 %) > *Bacillus* sp. PK-12 (0.351 %) > *Bacillus* sp. PK-4 (0.347 %) > *Pseudomonas* sp. PK-3 (0.180 %) > *Bacillus* sp. PK-13 (0.150 %) > *Bacillus* sp. PK-9 (0.130 %) as shown in Table 3.9 E.

It is observed that both pyrene uptake values and glucose utilized values are together found in decreasing order for seven isolates, namely *Bacillus* sp. PK-6 (56.4 %, 0.60 %) > *Bacillus* sp. PK-7 (53.4 %, 0.54 %) > *Bacillus* sp. PK-12 (45.6 %, 0.35 %) > *Bacillus* sp. PK-4 (33.0 %, 0.35 %) > *Pseudomonas* sp. PK-3 (25.2 %, 0.18 %) > *Bacillus* sp. PK-13 (18.5 %, 0.15 %) > *Bacillus* sp. PK-9 (9.8 %, 0.13 %) as shown in Table 3.9 F. Six isolates, *Rhodococcus* sp. PK-10 (46.3 %, 0.58 %), *Bacillus* sp. PK-5 (43.8 %, 0.40 %), *Bacillus* sp. PK-1 (40.6 %, 0.54 %), *Bacillus* sp. PK-14 (36.5 %, 0.58 %), *Bacillus* sp. PK-8 (27.0 %, 0.54 %) and *Bacillus* sp. PK-2 (20.9 %, 0.43 %) fall out of this decreasing order of pyrene uptake and glucose utilized.

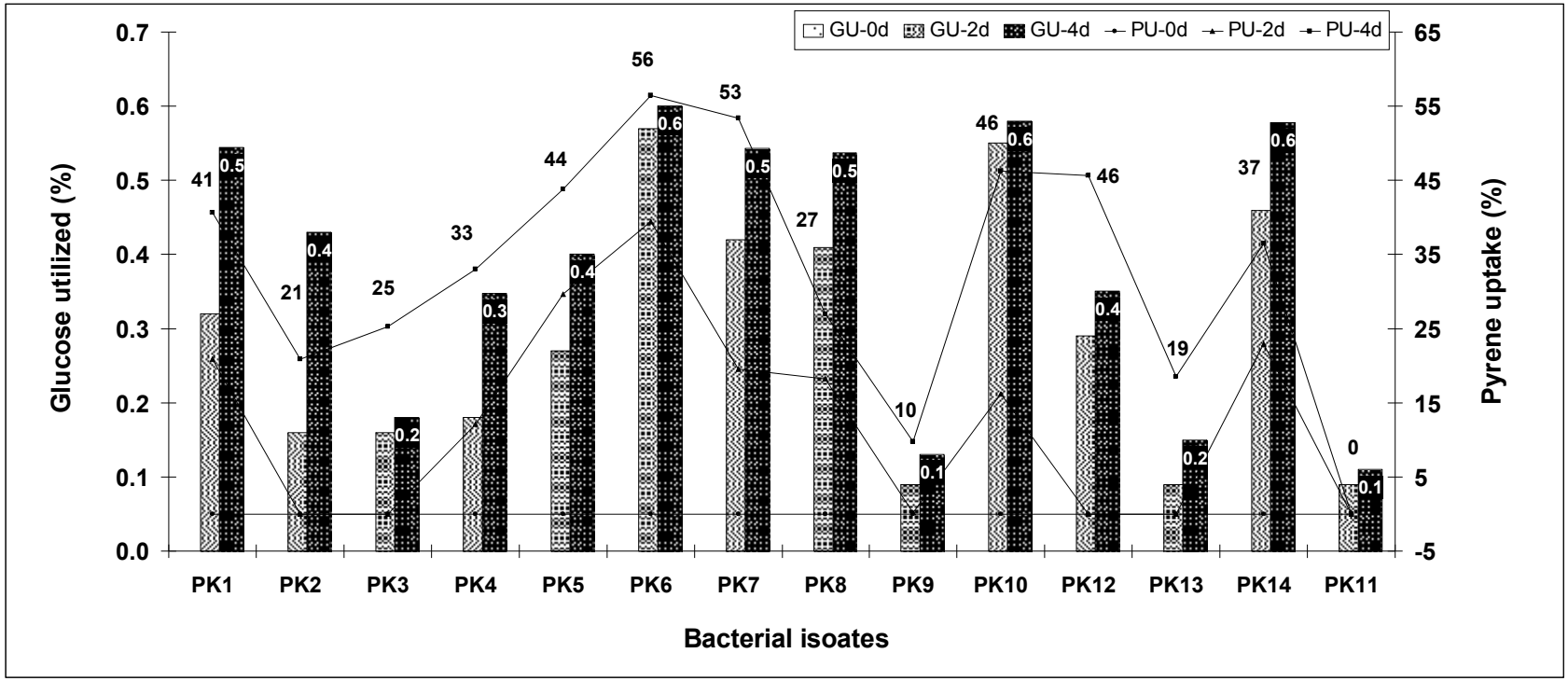

Figure 3.24 C. Pyrene uptake (PU) and Glucose utilization (GU) profile of bacterial isolates from coal-tar and crude oil contaminated soils in BHB medium supplemented with 1 % (w/v) glucose and 50 µg ml^{-1} pyrene.

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Table 3.9 F. Pyrene uptake, biosurfactant activity, total cell protein and glucose utilization profile of bacterial isolates (PK-1 to PK-14) isolated from coal-tar and crude oil contaminated soils when grown for 4 days in BHB medium containing pyrene (50 µg ml^{-1}) and glucose (1.0 %; w/v).

S. No. [A]	ISOLATE	Pyrene uptake (%)	Biosurfactant activity ($OD_{550\ nm}$)	Protein (mg ml^{-1})	Glucose utilized (%)
1	*Bacillus* sp. PK-6	56.4	**1.96**	**1.96**	**0.60**
2	*Bacillus* sp. PK-7	53.4	**1.61**	2.25	**0.54**
3	*Rhodococcus* sp. PK-10	46.3	**1.45**	2.20	0.58
4	*Bacillus* sp. PK-12	45.6	**1.11**	**1.39**	**0.35**
5	*Bacillus* sp. PK-5	43.8	**1.02**	**1.25**	0.40
6	*Bacillus* sp. PK-1	40.6	**1.01**	1.42	0.54
7	*Bacillus* sp. PK-14	36.5	1.54	**1.13**	0.58
8	*Bacillus* sp. PK-4	33.0	**0.97**	1.59	**0.35**
9	*Bacillus* sp. PK-8	27.0	1.33	1.44	0.54
10	*Pseudomonas* sp. PK-3	25.2	**0.43**	**0.97**	**0.18**
11	*Bacillus* sp. PK-2	20.9	0.72	1.64	0.43
12	*Bacillus* sp. PK-13	18.5	0.55	1.03	**0.15**
13	*Bacillus* sp. PK-9	9.8	0.66	1.18	**0.13**
	No of isolates correlating with pyrene uptake	-	**8**	**5**	**7**

[A] S. No. corresponds to decreasing order of pyrene uptake among bacterial isolates

4.2.1.8 Kinetics of pyrene utilization in bacteria

The kinetics of pyrene utilization by fourteen bacterial isolates was studied at 24 hr intervals for 4 days in BHB liquid medium containing 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose. In four days isolate *Bacillus* sp (PK-1) could uptake 41 % pyrene at 30 ^{o}C (Figure 3.25 A). The bacterium showed a linear pyrene uptake from first day of incubation. An uptake of 20.9 %

pyrene (10.4 µg ml^{-1}) was observed from the spent culture after 2 days. After third day of incubation the uptake of pyrene increased to 30.7 % (15.4 µg ml^{-1}). Thereafter on the fourth day residual pyrene in the medium was 29.7 µg ml^{-1} which corresponds to uptake of 40.6 % (20.3 µg ml^{-1}) pyrene as shown in Figure 3.25 A. The culture grew exponentially until the end of day 2 and showed BA maxima after 3 days of growth.

In four days isolate *Bacillus* sp (PK-2) could uptake 20.9 % pyrene (Figure 3.25 B). The bacterium showed a linear pyrene uptake from second day of incubation. An uptake of 10.4 % pyrene (5.2 µg ml^{-1}) was observed from the spent culture after 3 days. After the fourth day of incubation, residual pyrene in the medium was 39.6 µg ml^{-1} which corresponds to uptake of 20.9 % (10.4 µg ml^{-1}) pyrene as shown in Figure 3.25 B. Growth of isolate PK-2 was in log phase till 3rd day of incubation, thereafter the culture entered stationary phase (similar to PK-7). BA was observed to reach maximum after fourth day, though there was not much change after third day of incubation.

In four days isolate *Pseudomonas* sp. (PK-3) could uptake 25 % pyrene (Figure 3.25 C). The bacterium showed a linear pyrene uptake from second day of incubation (similar to PK-2). An uptake of 12.6 % pyrene (6.3 µg ml^{-1}) was observed from the spent culture after 3 days. After fourth day of incubation residual pyrene in the medium was 37.4 µg ml^{-1} which corresponds to uptake of 25.2 % (12.6 µg ml^{-1}) pyrene as shown in fig C. The isolate PK-3 reached growth maxima and BA maxima after 4 days (similar to PK-4, PK-6 and PK-10), was stationary thereafter. The biosurfactant activity maxima was noticeably very low.

The pattern of pyrene utilization by *Bacillus* sp (PK-4) was slow but similar to PK-1 i.e. a linear graph, showing 33 % uptake of pyrene in four days (Figure 3.25 D). The isolate PK-4 showed an uptake of 6.1 % pyrene (3.0 µg ml^{-1}) from the growth medium after 24 hrs and 12.1 % (6.1 µg ml^{-1}) pyrene uptake after 2 days of incubation. After third day of incubation the uptake of pyrene increased to 22.6 % (11.3 µg ml^{-1}). Thereafter on the fourth day residual pyrene in the medium was 33.5 µg ml^{-1} which corresponds to uptake of 33.0 % (16.5 µg ml^{-1}) pyrene as shown in Figure 3.25 D. The culture attained growth maxima and biosurfactant activity maxima in 4 days, unlike PK-1 which showed exponential growth during 2 days and maximum BA was attained in 3 days.

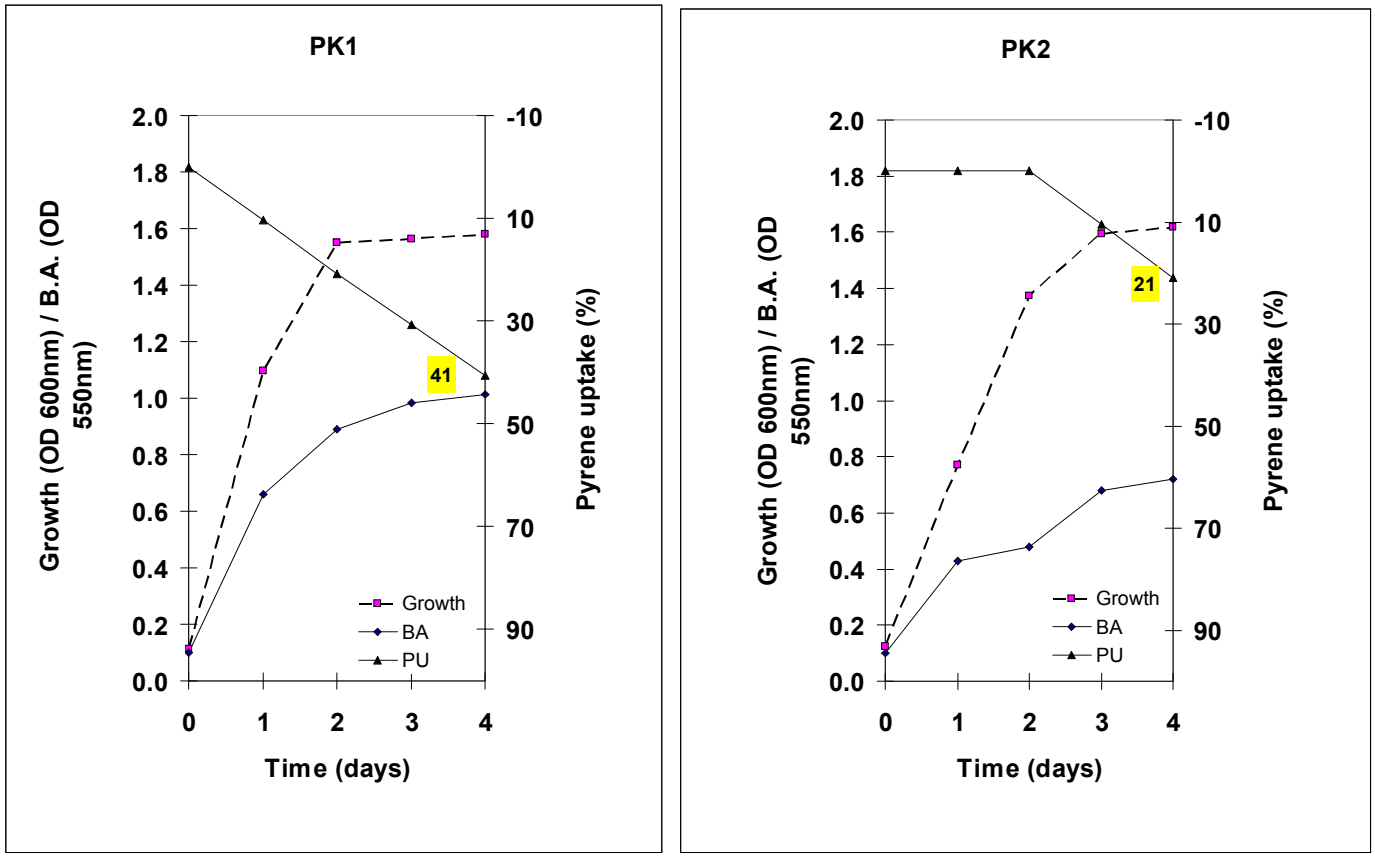


Figure 3.25 Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C of (A) *Bacillus firmus* (PK-1) and (B) *Bacillus cereus* (PK-2).

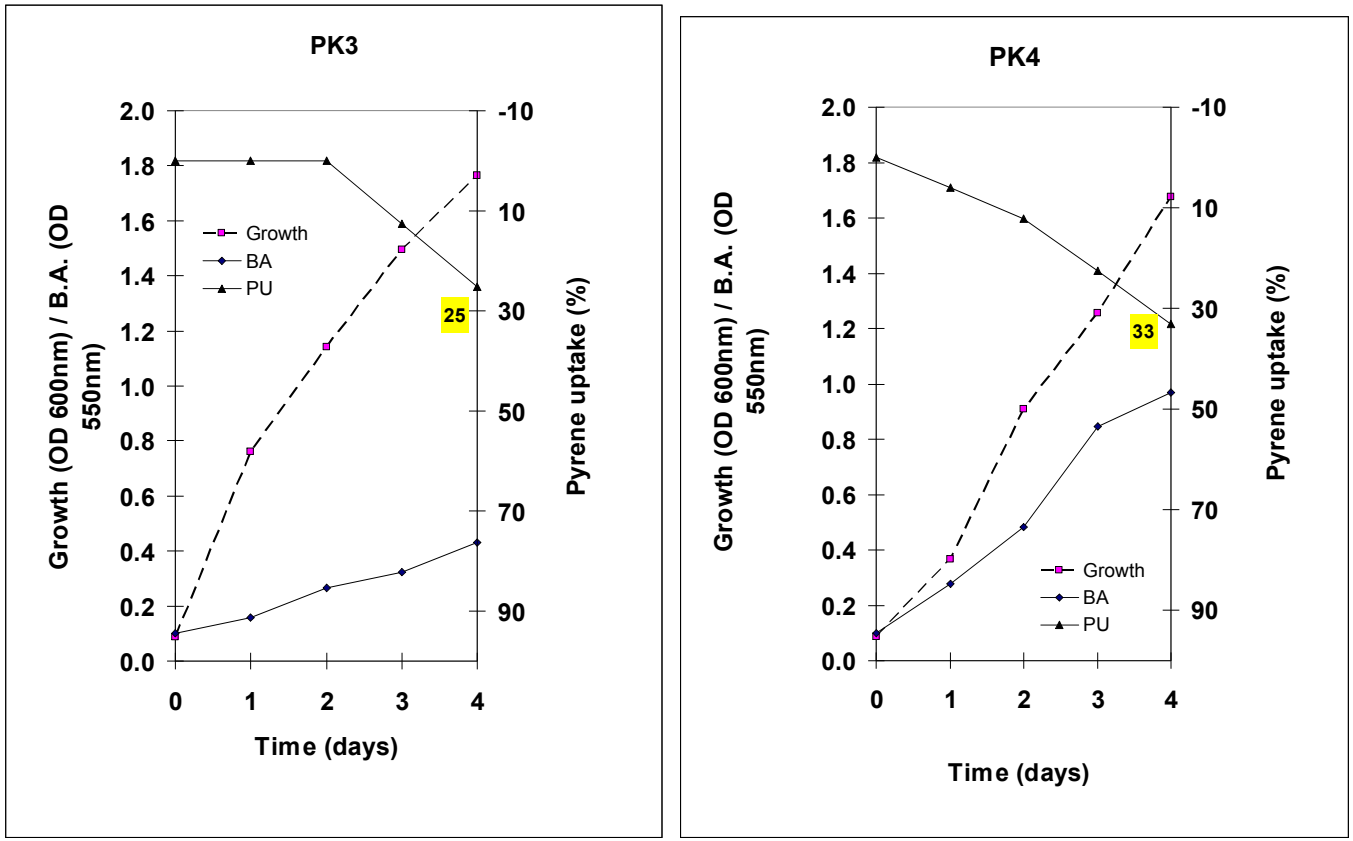


Figure 3.25 Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C of (C) *Pseudomonas oleovorans* (PK-3) and (D) *Bacillus cereus* (PK-4).

The pattern of pyrene utilization by *Bacillus* sp (PK-5) was linear (similar to PK-1, PK-4), showing 44 % uptake of pyrene in four days (Figure 3.25 E). The isolate PK-5 showed an uptake of 14.8 % pyrene (7.4 µg ml^{-1}) from the growth medium after 24 hrs, 29.6 % (14.8 µg ml^{-1}) after 2 days and 36.7 % (18.4 µg ml^{-1}) after 3 days of incubation. After four days the uptake of pyrene reached 43.8 % (21.9 µg ml^{-1}) corresponding to residual pyrene of 28.1 µg ml^{-1} in the growth medium as shown in Figure 3.25 E. The culture attained growth maxima and biosurfactant activity maxima in 4 days, unlike PK-1, similar to PK-4.

The maximum pyrene uptake and utilization was found in *Bacillus* sp. (PK-6), with uptake reaching 56 % after four days (Figure 3.25 F). Uptake began after one day of incubation in BHB medium containing 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 45 ^{o}C. After 2 days, 39.4 % pyrene (19.7 µg ml^{-1}) uptake was observed and, after three days the residual pyrene content decreased to 50.0 % (implying an uptake of 25 µg ml^{-1} pyrene). Thereafter on the fourth day residual pyrene in the medium was 21.8 µg ml^{-1} which corresponds to uptake of 56.4 % (28.2 µg ml^{-1}) pyrene as shown in Figure 3.25 F. The culture also showed the most growth and biosurfactant activity of all of the bacterial isolates. It grew exponentially till 3 days and showed BA maxima after 4 days of growth.

The pattern of pyrene utilization by *Bacillus* sp (PK-7) was linear (similar to PK-1, PK-4 and PK-5), showing 53 % uptake of pyrene in four days (Figure 3.25 G). The isolate PK-7 could utilize 15.2 % pyrene (7.6 µg ml^{-1}) in 24 hrs. After 2 days, 19.6 % pyrene (9.8 µg ml^{-1}) uptake and after 3 days, 25.4 % (12.7 µg ml^{-1}) pyrene uptake was observed from the culture medium. Thereafter on the fourth day the residual pyrene in the medium was 23.3 µg ml^{-1}, corresponding to an uptake of 53.4 % (26.7 µg ml^{-1}) pyrene as shown in Figure 3.25 G. The growth profile of PK-7 was similar to PK-1. The culture entered stationary phase after 2 days and nearly maximum BA was attained in 3 days.

Bacillus sp. (PK-8) showed slow cometabolic uptake of 27 % pyrene in 4 days (Figure 3.25 H). Pyrene uptake began after one day of incubation (like PK-6) and 18.3 % (9.2 µg ml^{-1}) of the pyrene had been utilized by the second day of growth.

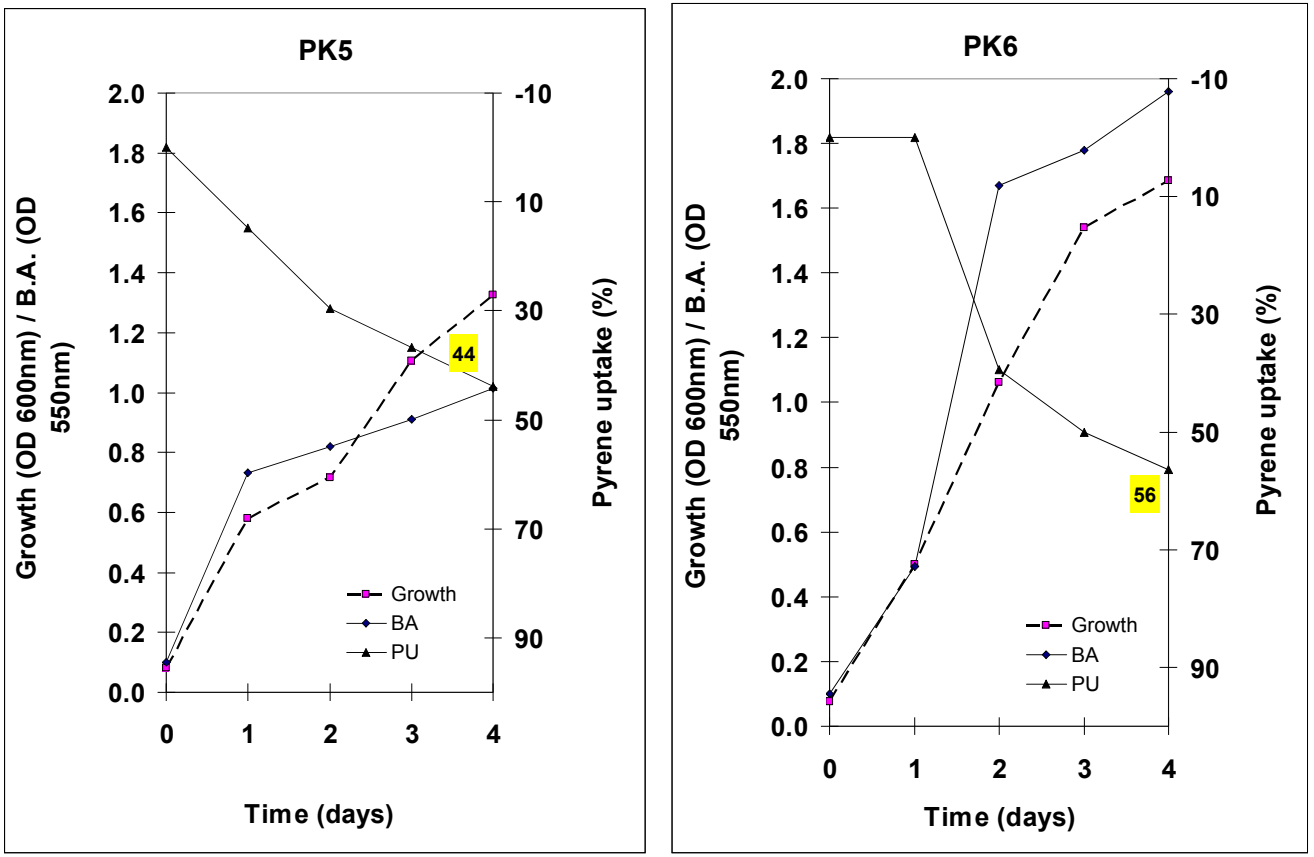


Figure 3.25 Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C of (E) *Bacillus subtilis* (PK-5) and (F) *Bacillus licheniformis* (PK-6).

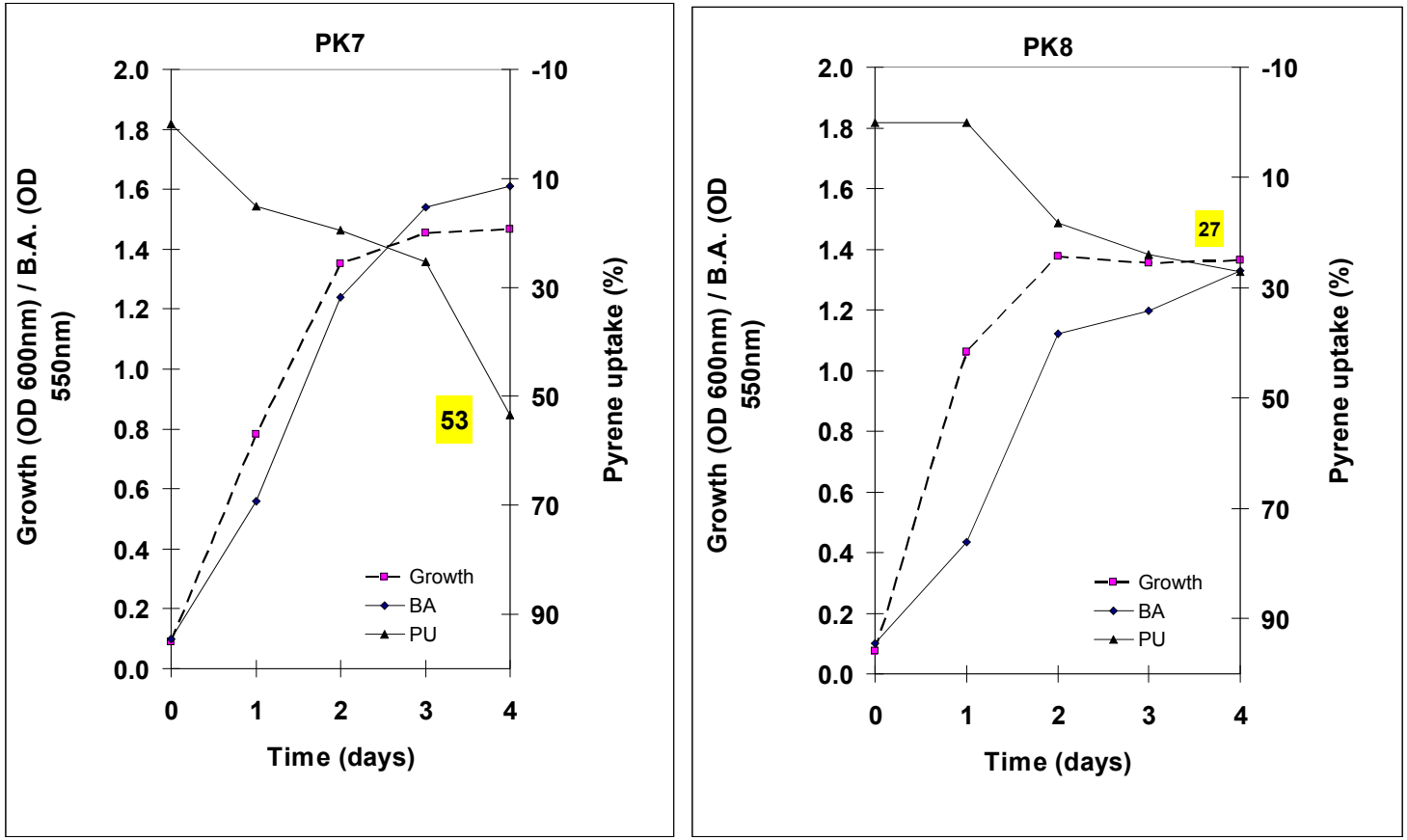


Figure 3.25 Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 45 ^{o}C of (G) *Bacillus* sp. (PK-7) and (H) *Bacillus pumilus* (PK-8).

After three days 24.0 % (12.0 µg ml^{-1}) pyrene uptake was observed. Finally on the fourth day the residual pyrene in the medium was 36.5 µg ml^{-1} which corresponded to uptake of 27.0 % (13.5 µg ml^{-1}) pyrene as shown in Figure 3.25 H. *Bacillus* sp. (PK-8) reached exponential growth maxima in 2 days and entered stationary phase thereafter (like PK-1). The culture showed BA maxima after 4 days of growth.

In four days isolate *Bacillus* sp (PK-9) could uptake minimum pyrene i.e. 10 % (Figure 3.25 I). The bacterium showed pyrene uptake after second day of incubation (similar to PK-2 and PK-3). After 3 days, 9.3 % pyrene (4.7 µg ml^{-1}) uptake was observed and, after 4 days the residual pyrene in the medium was 45.1 µg ml^{-1} which corresponded to 9.8 % (4.9 µg ml^{-1}) pyrene uptake as shown in Figure 3.25 I. The culture grew exponentially for 2 days and entered stationary phase thereafter (similar to PK-1, PK-8). The trend of biosurfactant activity was similar to that of PK-2 and BA maxima was attained after 3 days of growth.

Rhodococcus sp. (PK-10) showed moderate cometabolic uptake of 46 % pyrene in 4 days (Figure 3.25 J). A linear pattern of pyrene uptake began after one day of incubation (similar to PK-6 and PK-8). After 2 days, 16.2 % (8.1 µg ml^{-1}) pyrene uptake and, after 3 days 32.8 % (16.4 µg ml^{-1}) pyrene uptake was observed. After four days 26.9 µg ml^{-1} residual pyrene was detected in the spent medium which corresponded to uptake of 46.3 % (23.2 µg ml^{-1}) pyrene as shown in Figure 3.25 J. *Bacillus* sp. (PK-8) reached growth maxima and BA maxima after 4 days of growth (like PK-6).

Kocuria sp. (PK-11) was incapable of pyrene uptake from BHB medium containing 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C in 4 days (Figure 3.25 K).

Bacillus sp. (PK-12) showed cometabolic, linear uptake of 46 % of 50 µg ml^{-1} pyrene in 4 days (Figure 3.25 L). Pyrene uptake began after two days of incubation (similar to PK-2, PK-3 and PK-9). After 3 days, about 20 % (9.8 µg ml^{-1}) of the pyrene had been utilized and, after four days 45.6 % (22.8 µg ml^{-1}) pyrene uptake was observed, -

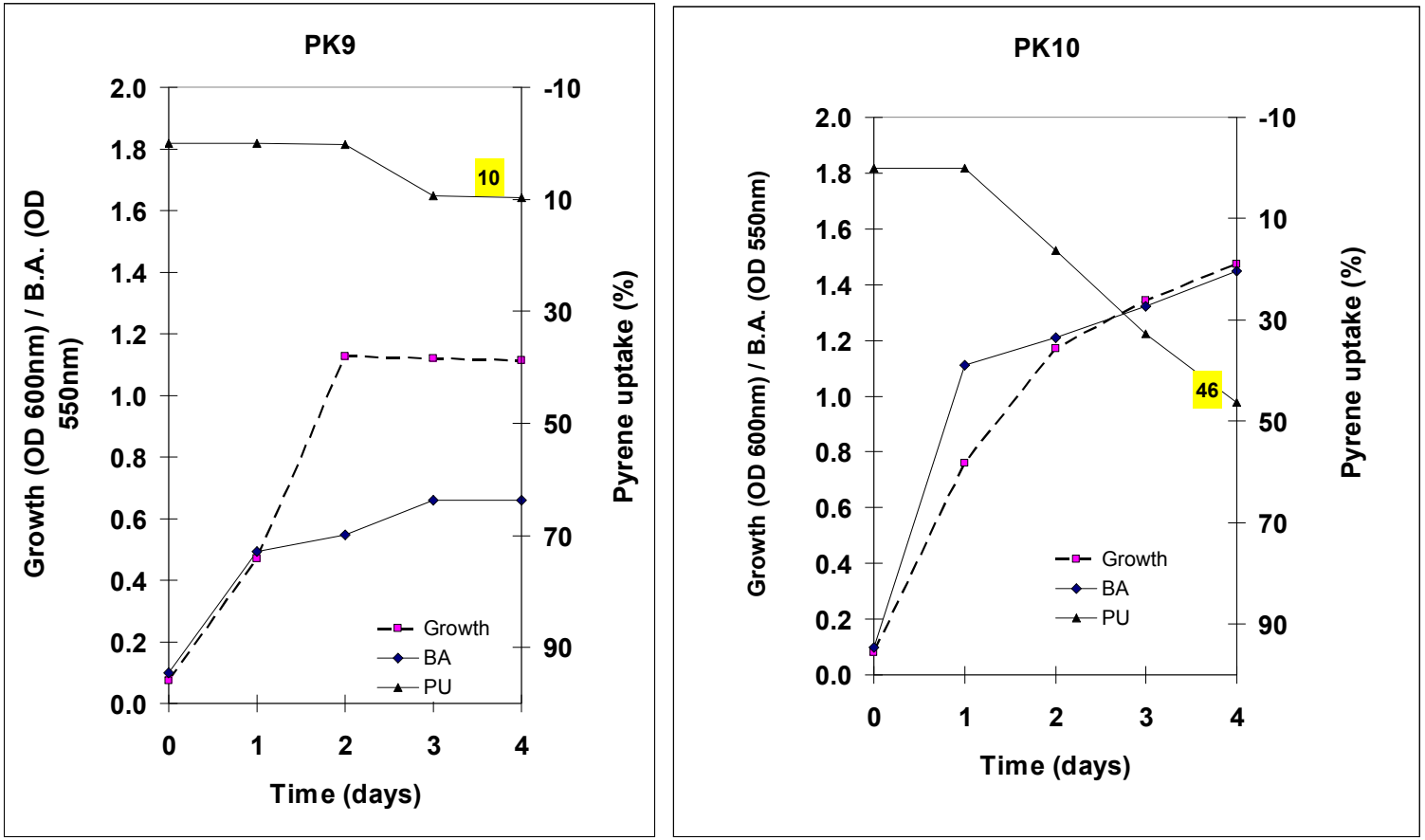


Figure 3.25 **Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 45 ^{o}C of (I) *Bacillus firmus* (PK-9) and (J) *Rhodococcus rhodochrous* (PK-10).**

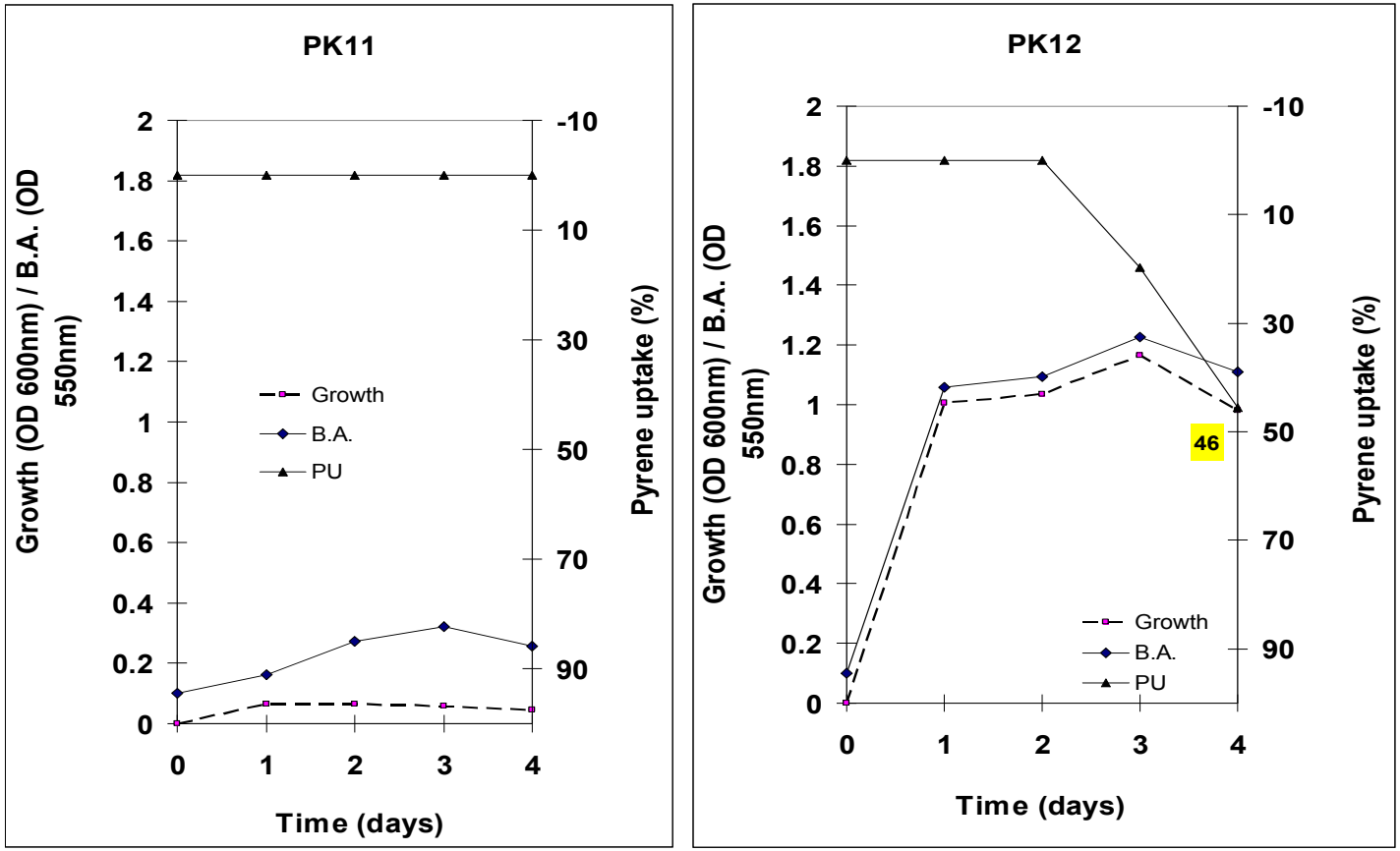


Figure 3.25 Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C of (K) *Kocuria polaris* (PK-11) and (L) *Bacillus pumilus* (PK-12).

- corresponding to 27.2 µg ml^{-1} residual pyrene present in the spent medium. It can be noted in Figure 3.25 L that the total pyrene uptake by *Bacillus* sp. (PK-12) occurred during stationary phase of growth and BA. Also the isolate showed parallel trend for growth and biosurfactant activity in medium, attaining maxima of both by the third day of incubation. Both growth and BA declined thereafter.

Bacillus sp. (PK-13) showed slow cometabolic uptake of 19 % pyrene in 4 days (Figure 3.25 M). Among all of the isolates, it showed longest lag period of 3 days for uptake of pyrene. After 4 days, uptake of 18.5 % (9.3 µg ml^{-1}) pyrene was observed in the growth medium, which corresponded to 40.7 µg ml^{-1} residual pyrene in culture medium. Pyrene uptake by isolate PK-13 started during stationary phase of growth and BA, just as in case of isolate PK-12. Both growth and biosurfactant activity maxima were attained on 2^{nd} day. BA appeared in growth medium of PK-13 only after one day of incubation (unlike all other isolates), increased till second day, was stable till third day and declined thereafter till the fourth day. Growth was logarithmic till second day and became stationary thereafter (similar to PK-9).

Bacillus sp. (PK-14) showed moderate cometabolic, almost linear uptake of pyrene, equal to 37 % of 50 µg ml^{-1} from growth medium in 4 days (Figure 3.25 N). Uptake of pyrene began after one day of incubation (similar to PK-6, PK-8 and PK-10) and after 2 days, 22.9 % (11.5 µg ml^{-1}) pyrene uptake was observed. After 3 days PU was 31.9 % (16.0 µg ml^{-1}) while after four days PU was 36.5 % (18.3 µg ml^{-1}) corresponding to residual pyrene 31.7 µg ml^{-1} present in the spent medium. It can be noted in Figure 3.25 N that a major percentage of the total pyrene uptake by *Bacillus* sp. (PK-14) occurred during exponential phase of growth and BA in the medium. Growth and BA maxima were reached after three days of incubation (similar to PK-7).

4.2.2 Pyrene degradation

The physiological mechanism of pyrene assimilation as co-carbon source has been evaluated in fourteen bacterial monocultures, in the above mentioned results.

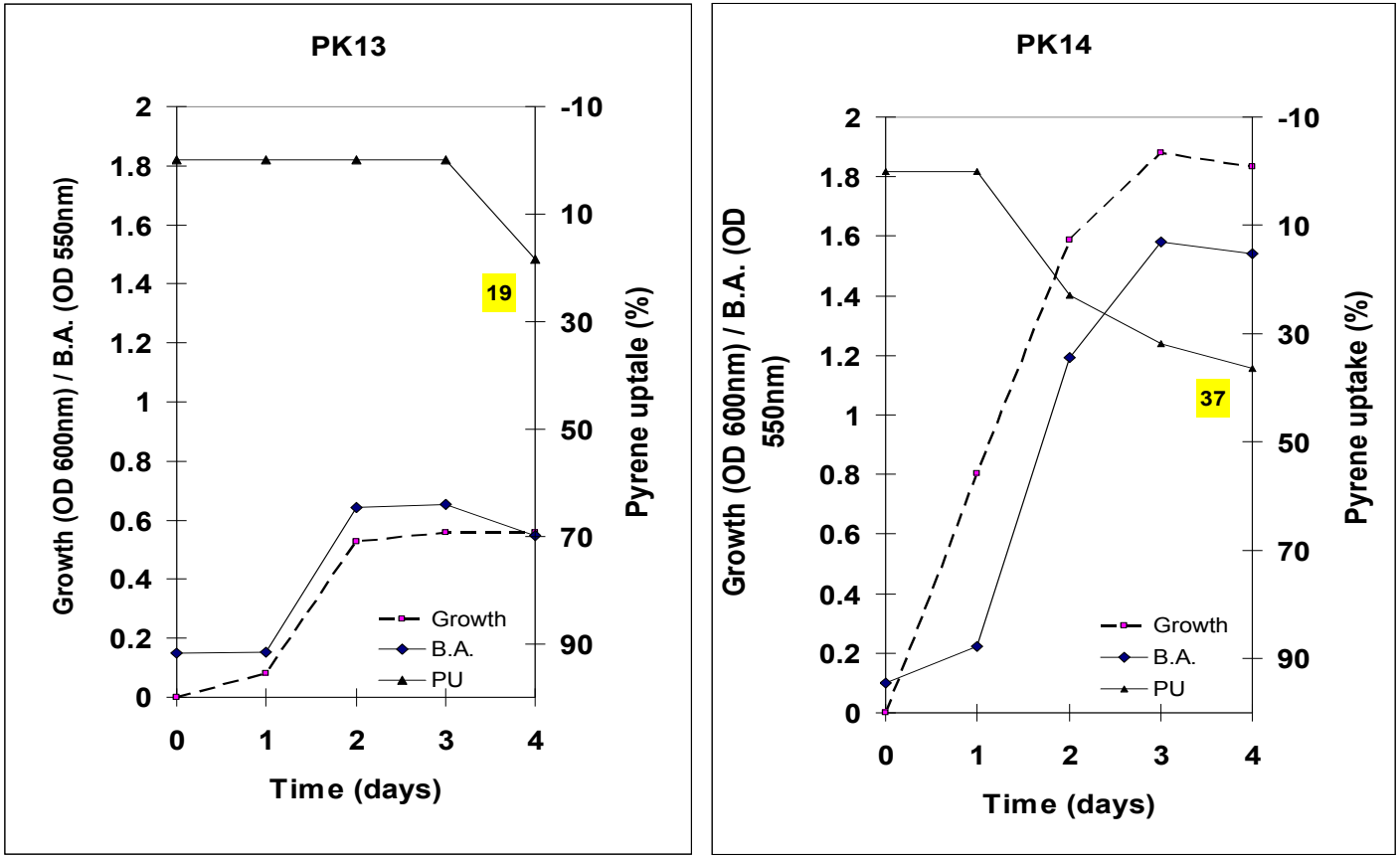


Figure 3.25 **Pyrene uptake (PU) pattern, biosurfactant activity (B.A.) and growth in BHB medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 30 ^{o}C of (M) *Bacillus flexus* (PK-13) and (N) *Bacillus firmus* (PK-14).**

Based on residual pyrene analysis in solvent extracts of spent medium, by spectrophotometric (OD_{254}) and HPLC analysis it was determined that *Bacillus* sp. (PK-6), under optimal conditions of 1.0 % (w/v) glucose supplementation in BHB medium and incubation at 45 ^{o}C, was the most capable pyrene-degrading isolate (Figure 3.24 A). Further, GC-MS was used for intermediate metabolites and product determination. The GC-MS chromatogram plots for day 2, 3 and 4 solvent extracts of spent culture media of *Bacillus* sp. (PK-6) were superimposed, as shown in Figure 3.26 and it confirmed the gradual decrease in pyrene signal (RT = 17.5 minutes). Therefore, it may be suggested that *Bacillus* sp. (PK-6) MTCC 1005 is preferentially more capable of pyrene uptake among all isolated bacterial monocultures from crude/diesel oil and coal-tar contaminated soils.

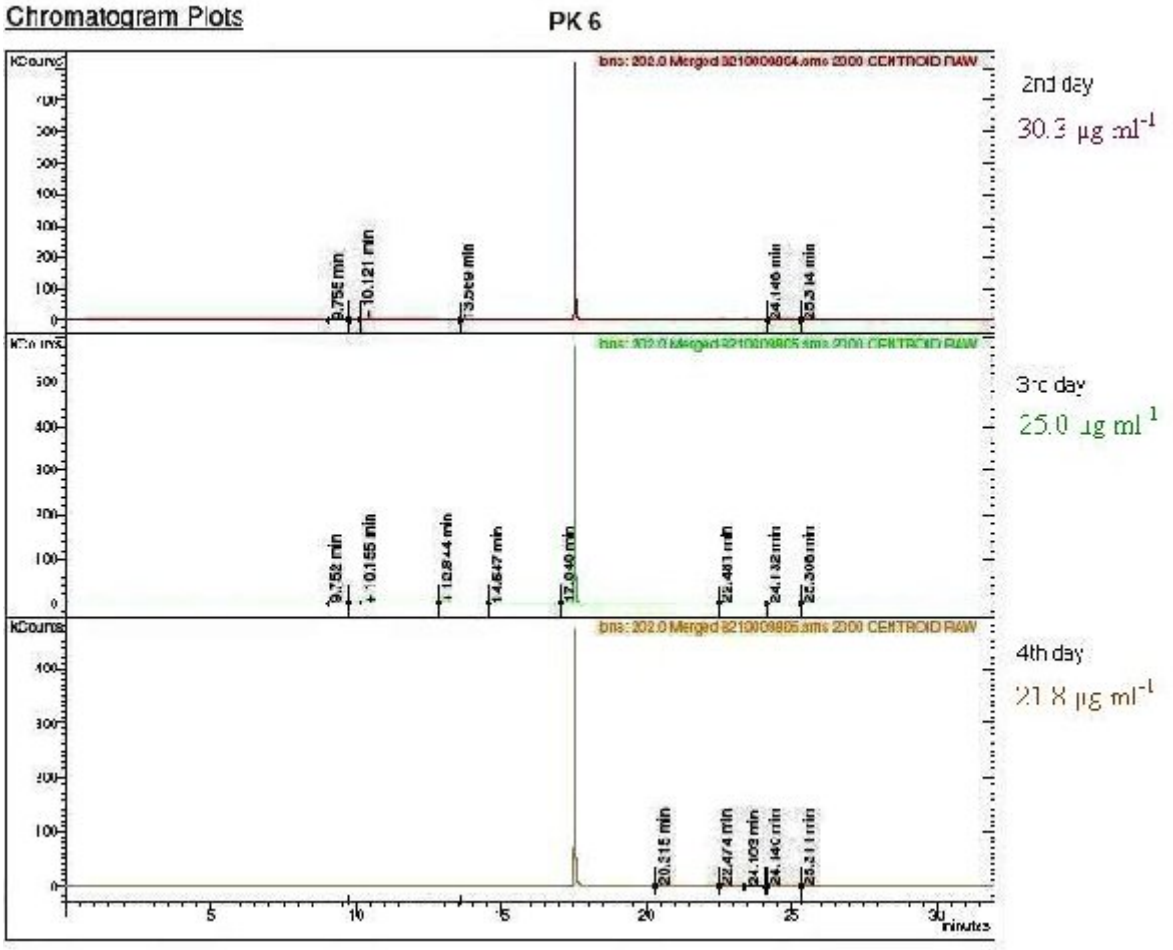


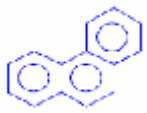
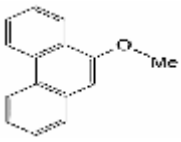
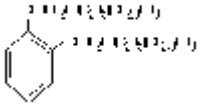
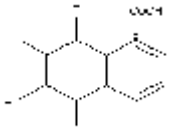
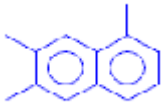
Figure 3.26 Gas chromatography – Mass spectrometry (GC-MS) profile of *Bacillus* sp. (PK-6) culture extract, showing the gradual decrease in pyrene signal (17.5 min) indicating pyrene uptake by the isolate from culture medium.

The peaks obtained by GC-MS analysis of culture extracts pertained to catabolic products of pyrene. These were identified by probabilistic search (PBM) by comparing the fragmentation pattern and their abundance with the standard mass spectra of known compounds in NIST database library stored in an MS Chemstation library (Varian). Besides pyrene ($C_{16}H_{10}$; mol. wt. 202; Figure 3.27 A), signals for 6 other compounds were obtained in the mass spectrogram (Table 3.10), namely Phenanthrene ($C_{14}H_{10}$; mol. wt.178; Figure 3.27 B), 9-Methoxyphenanthrene ($C_{15}H_{12}O$; mol. wt. 208; Figure 3.27 C), Diisooctylphthalate ($C_{24}H_{38}O_4$; mol. wt. 390; Figure 3.27 D), 5,6,7,8-Tetrahydro-1naphthoic acid ($C_{11}H_{12}O_2$; mol. wt.176; Figure 3.27 E), 1,6,7-Trimethyl-naphthalene ($C_{13}H_{14}$; mol. wt. 170; Figure 3.27 F) and 9,10-Diphenylphenanthrene ($C_{26}H_{18}$; mol. wt. 330; Figure 3.27 G).

GCMS analysis of *Bacillus* sp. (PK-6) culture supernatant on the second day (Figure 3.28 A) of growth in BHB +Glucose (1.0 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C showed the production of Phenanthrene, 9-Methoxyphenanthrene and Phthalate (Table 3.10). 5,6,7,8-Tetrahydro-1-naphthoic acid was detected in the third day solvent extract (Figure 3.28 B) and 1,6,7-Trimethylnaphthalene was detected in the fourth day supernatant extract (Figure 3.28 C), while the production of 9-methoxyphenanthrene and phthalate declined. In addition 9,10-Diphenylphenanthrene was formed on day 2 and showed an increase on the 3rd day. However, no further change in its concentration was detected till the end of the experiment. Phthalate was detected in the culture medium extracts of second day, thereafter its concentration declined in third and fourth day extracts. A gradual decrease in the residual pyrene concentration along with formation of these new six compounds suggests that pyrene is degraded by *Bacillus* sp. (PK-6). As the culture prior to extraction was neither heated nor sonicated therefore residual pyrene and its degradation products are extracellular.

Table 3.10 Gas chromatography – Mass spectrometry (GCMS) profile of *Bacillus* sp. (PK-6) isolated from coal-tar contaminated soil grown for 4 days in BHB +glucose (1 %; w/v) +pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

NM: not found in list of first 10 match, [A] Peak area could not be determined as the analyte's amount was too low

Compound Identified				Peak Area		
Mol. wt., Formula	**Structure**	**RT (min)**	**Name of Match**	**2 day**	**3 day**	**4 day**
202, $C_{16}H_{10}$		**17.5**	**Pyrene**	**18,70,000**	**11,31,100**	**9,35,000**
178, $C_{14}H_{10}$		**14.1**	**Phenanthrene**	**2,124**	**1,820**	**Nil**
330, $C_{26}H_{18}$		**25.3**	**9,10-Diphenylphenanthrene**	**29,032**	**63,113**	**63,494**
208, $C_{15}H_{12}O$		**17.3**	**9-Methoxyphenanthrene**	**17,051**	**11,434**	**10,894**
390, $C_{24}H_{38}O_4$		**21.8**	**Diisooctylphthalate**	**1,78,029**	**14,625**	**15,275**
176, $C_{11}H_{12}O_2$		**22.4**	**5,6,7,8-Tetrahydro-1-naphthoic acid**	Nil	**Yes** [A]	NM
170, $C_{13}H_{14}$		**11.3**	**1,6,7-Trimethyl-naphthalene**	Nil	Nil	**Yes** [A]

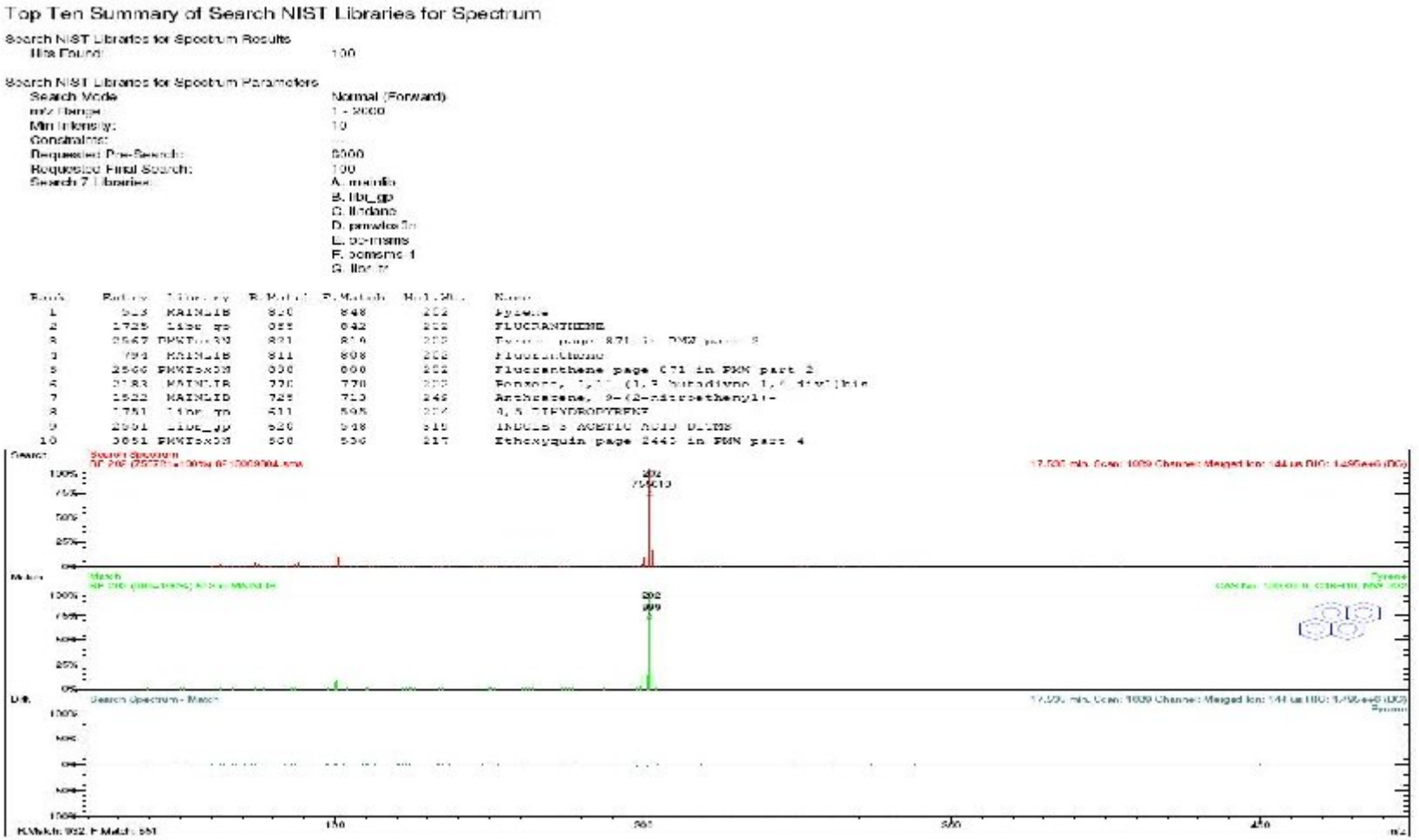


Figure 3.27 A Mass spectra of compound **Pyrene (RT** = 17.5 min).

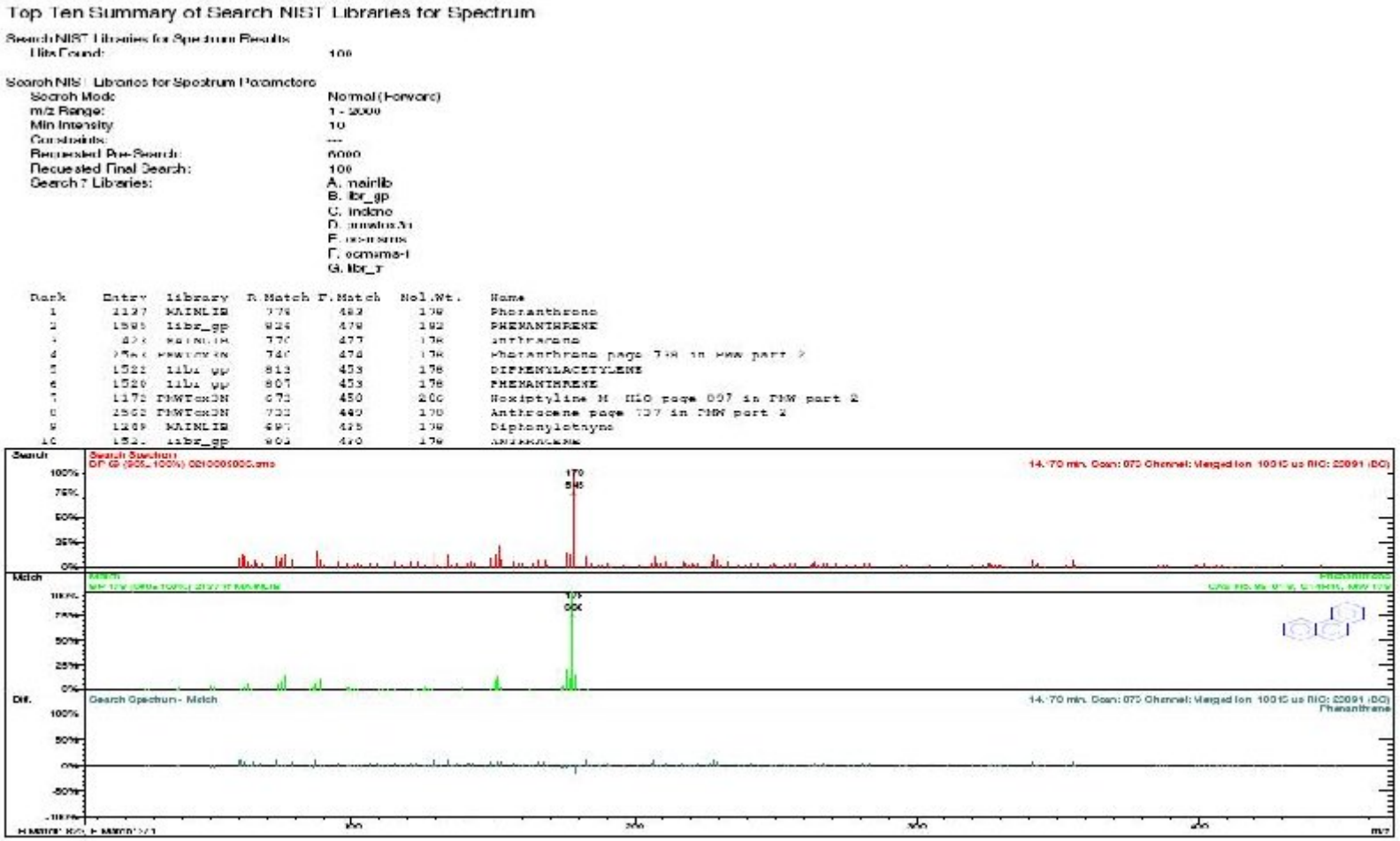


Figure 3.27 B Mass spectra of compound **Phenanthrene (RT = 14.1 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

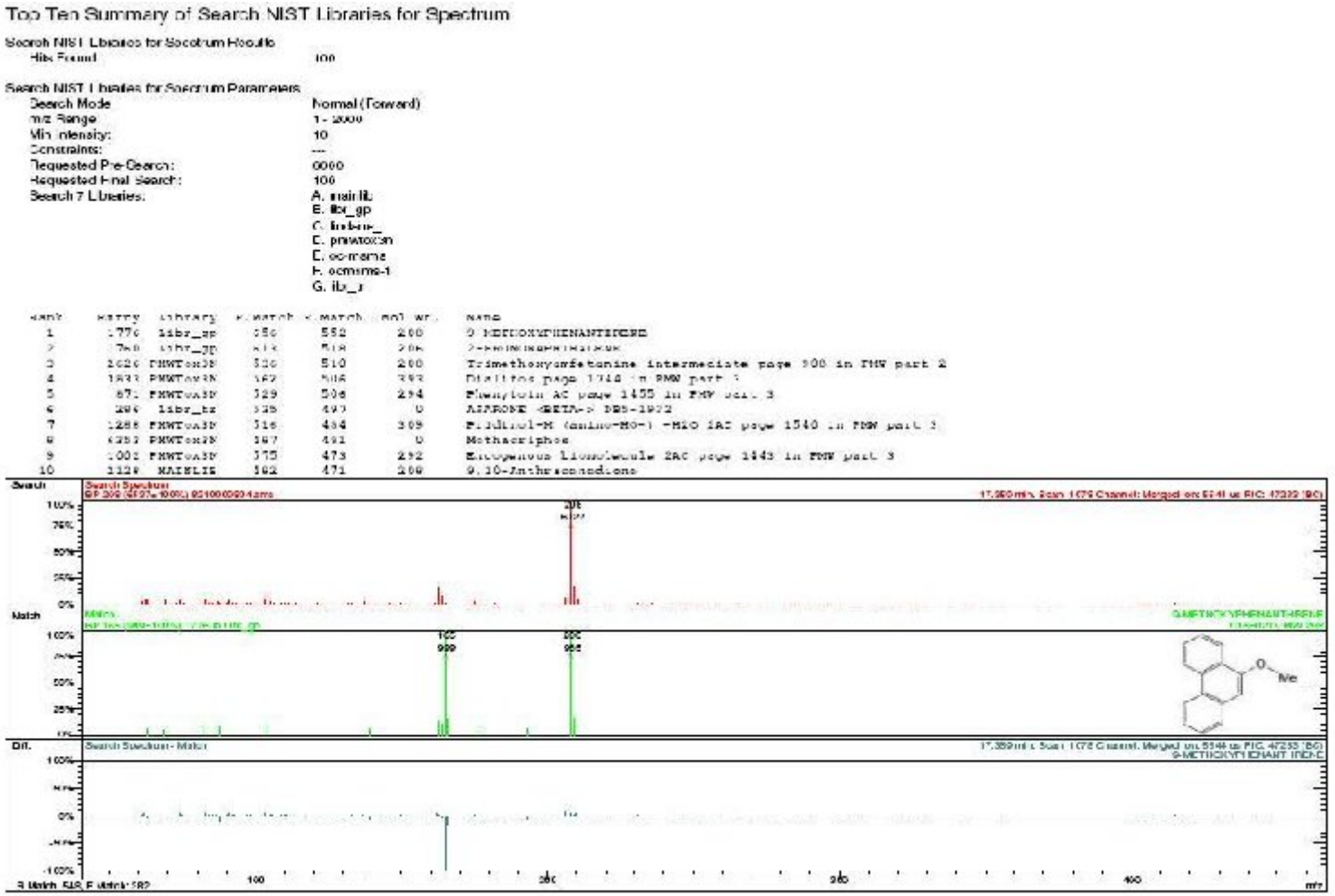


Figure 3.27 C Mass spectra of compound **9-Methoxyphenanthrene (RT = 17.3 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

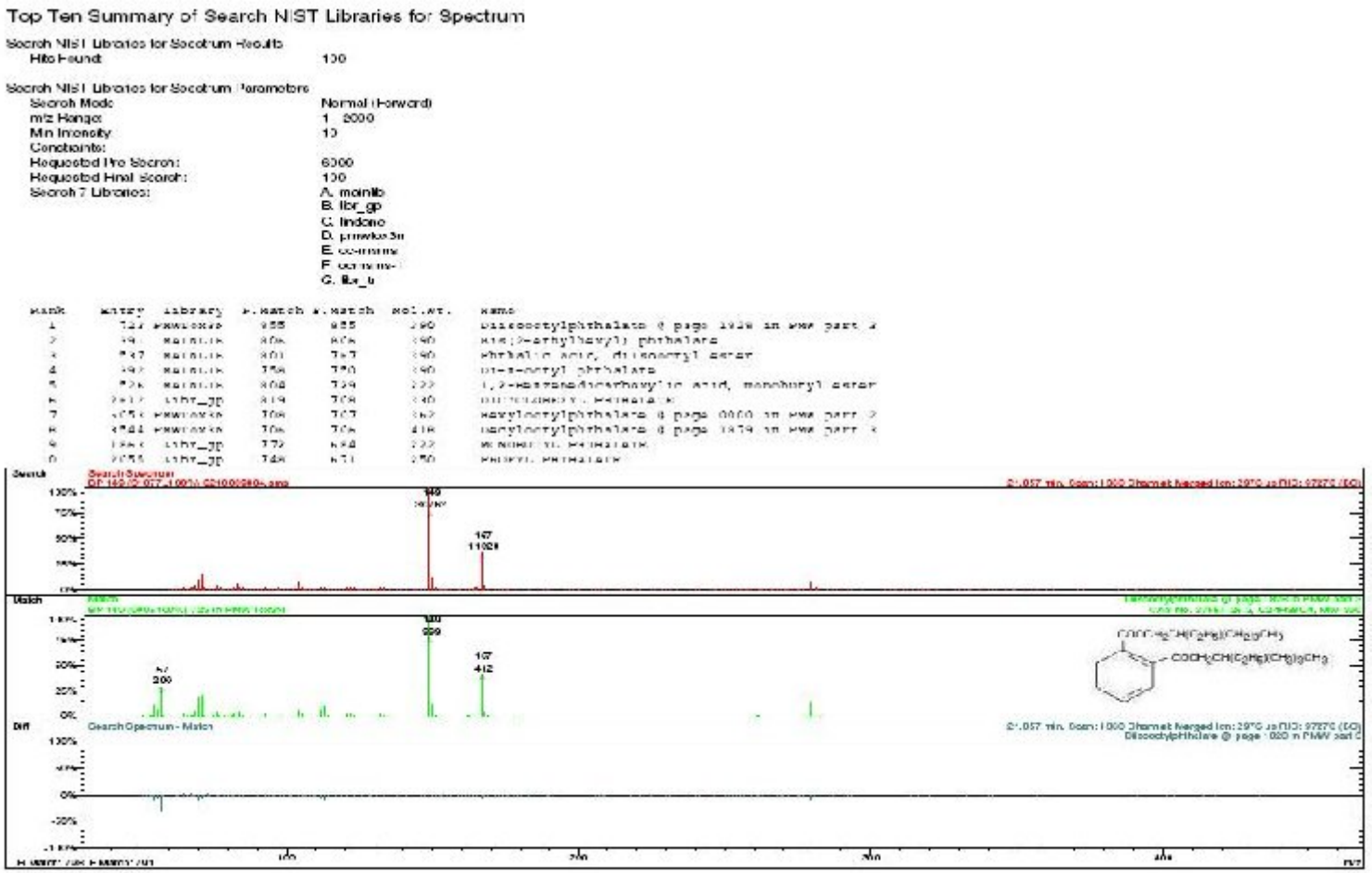


Figure 3.27 D Mass spectra of compound **Diisooctylphthalate (RT = 21.8 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

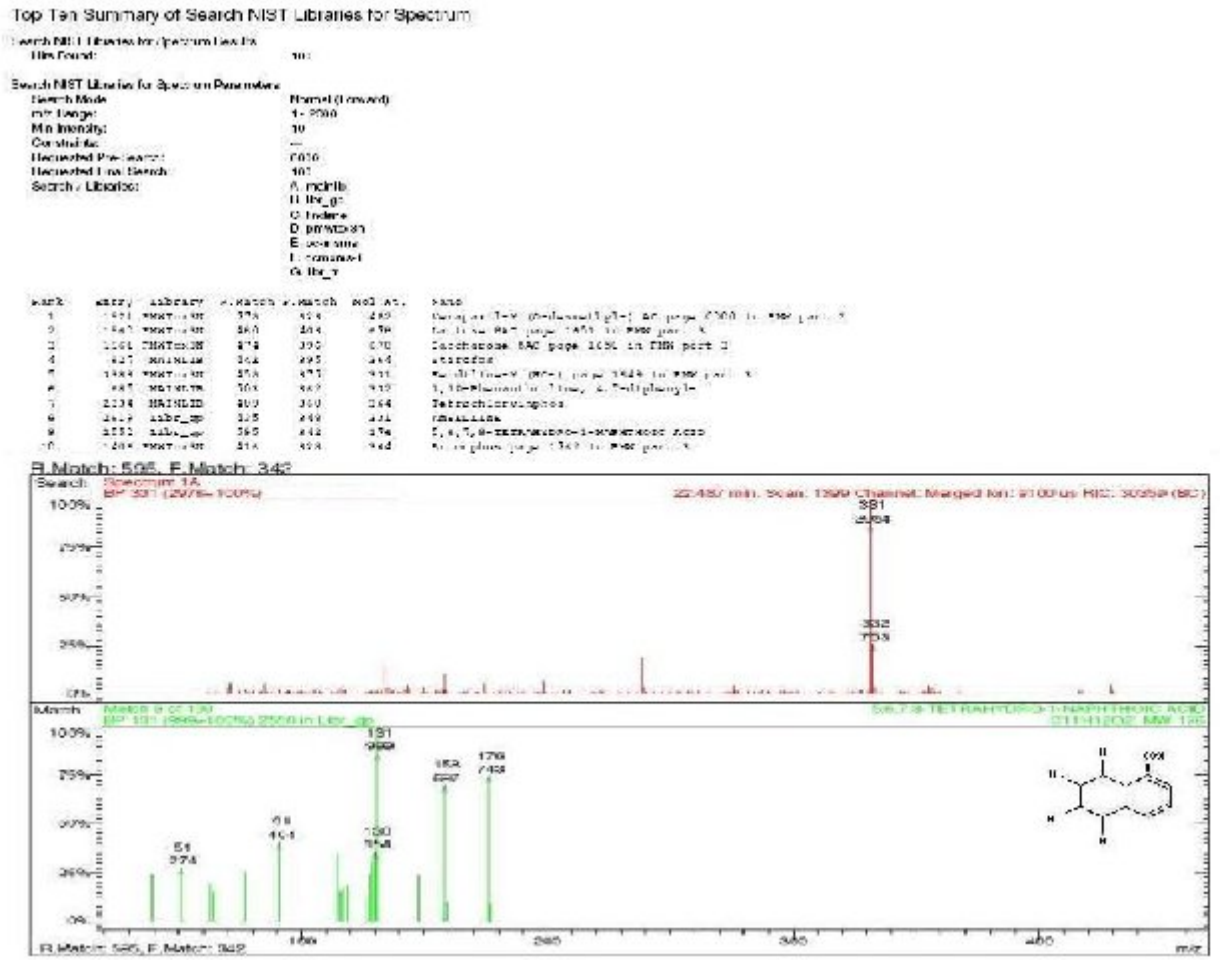


Figure 3.27 E Mass spectra of compound **5,6,7,8-Tetrahydro-1-naphthoic acid (RT = 22.4 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

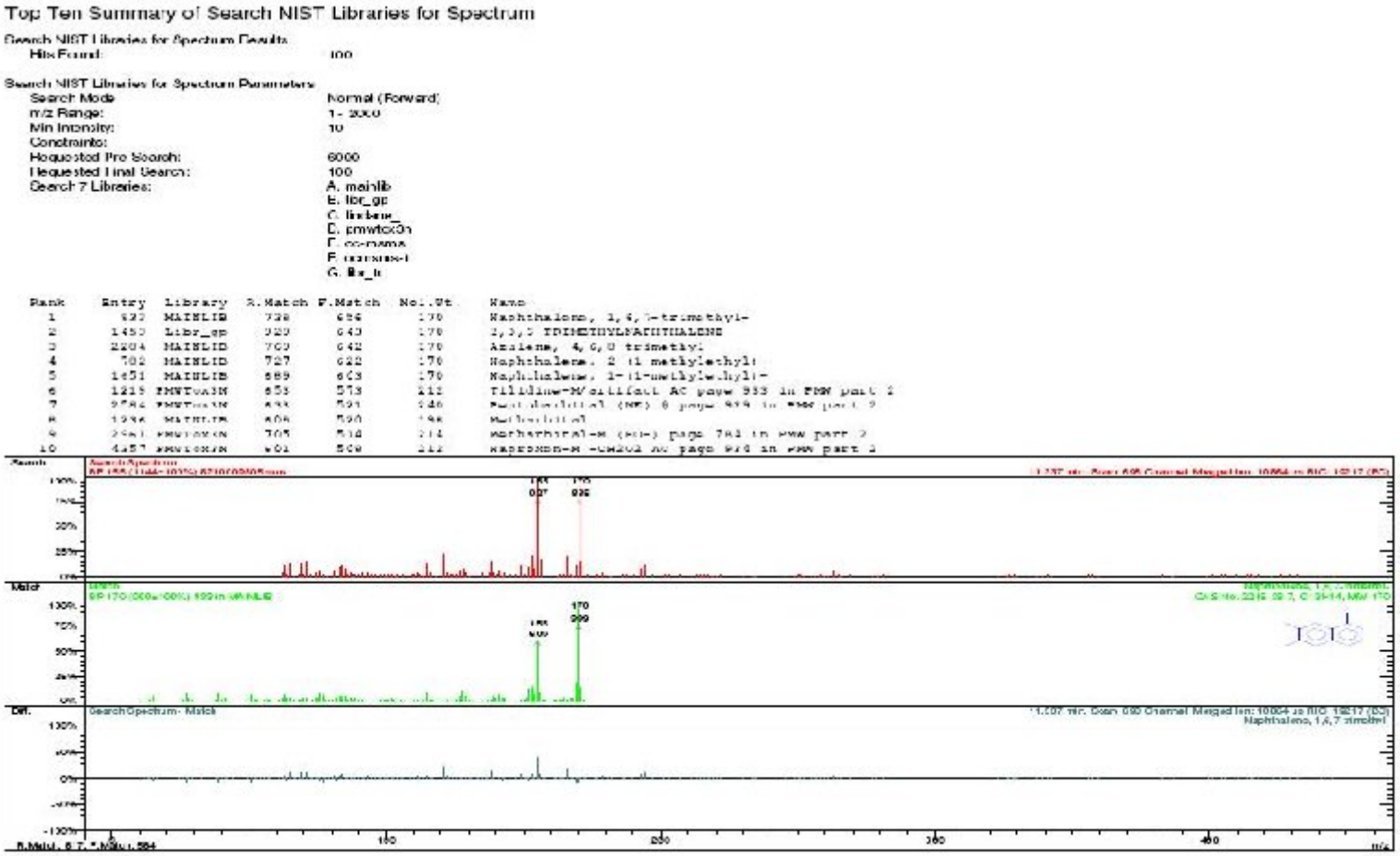


Figure 3.27 F Mass spectra of compound **1,6,7-trimethyl-naphthalene (RT = 11.3 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

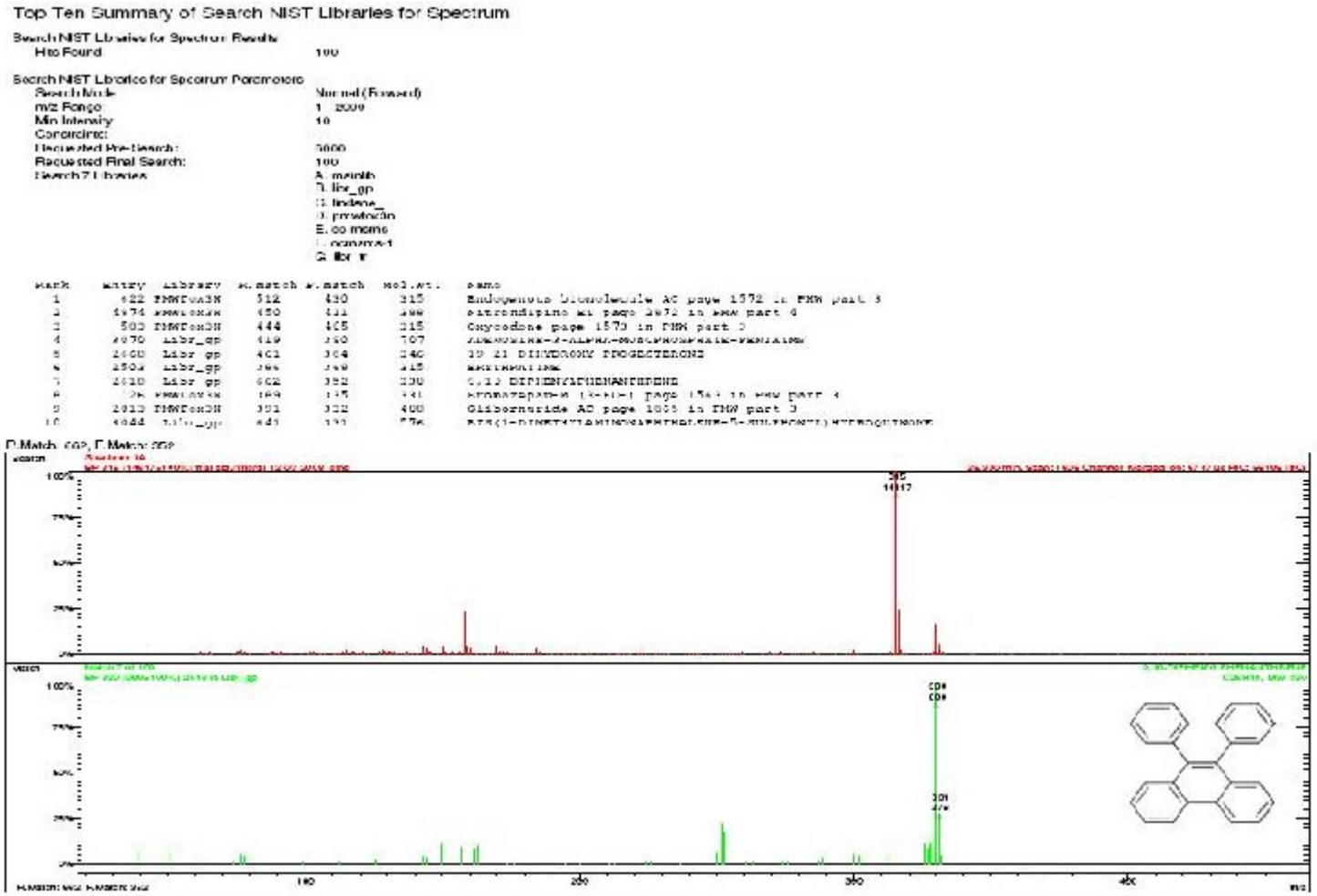


Figure 3.27 G Mass spectra of compound **9,10-Diphenylphenanthrene (RT = 25.3 min)** formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

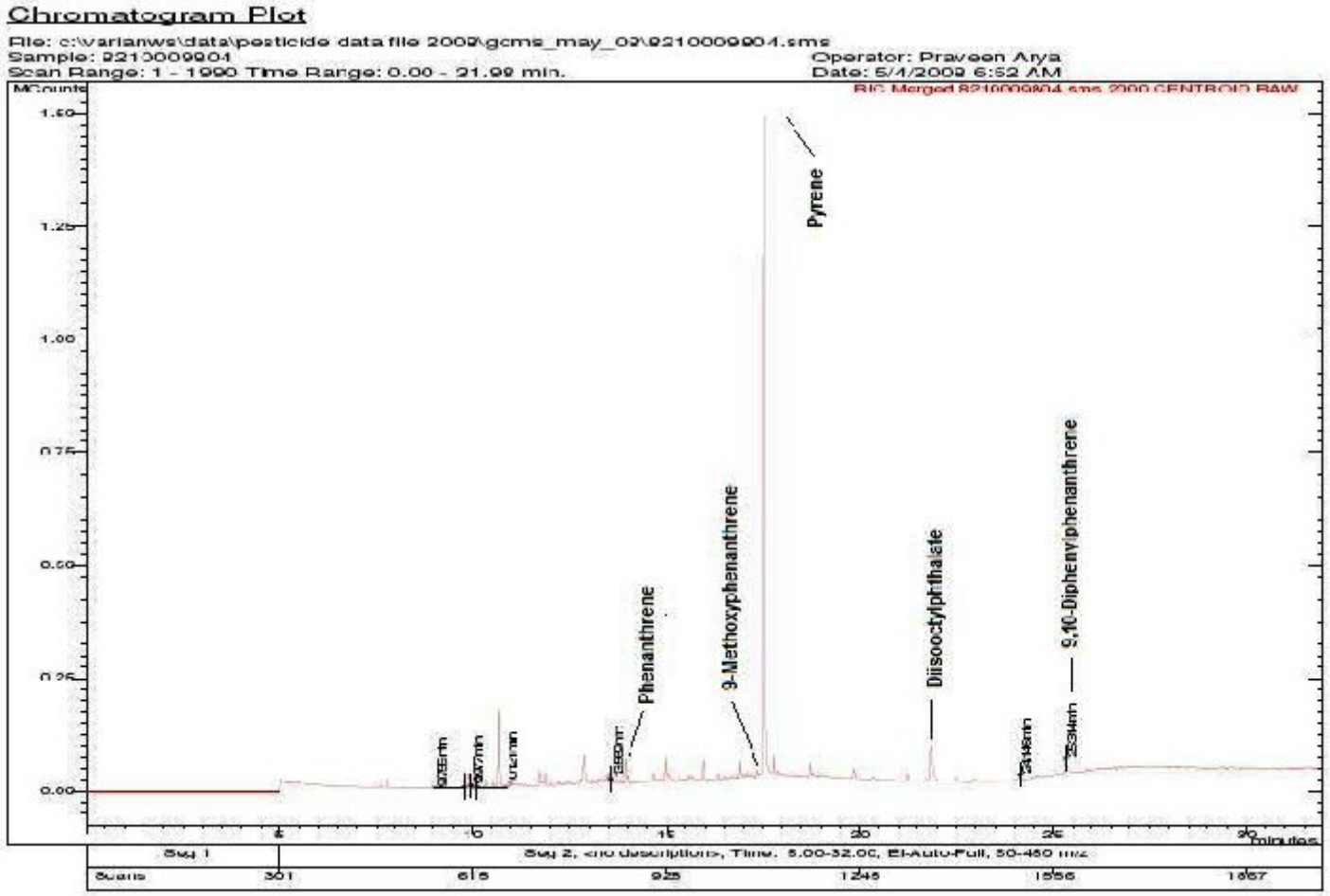


Figure 3.28 A Gas chromatography – Mass spectrometry (GCMS) separation of hexane-soluble metabolites formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown for 2 days in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

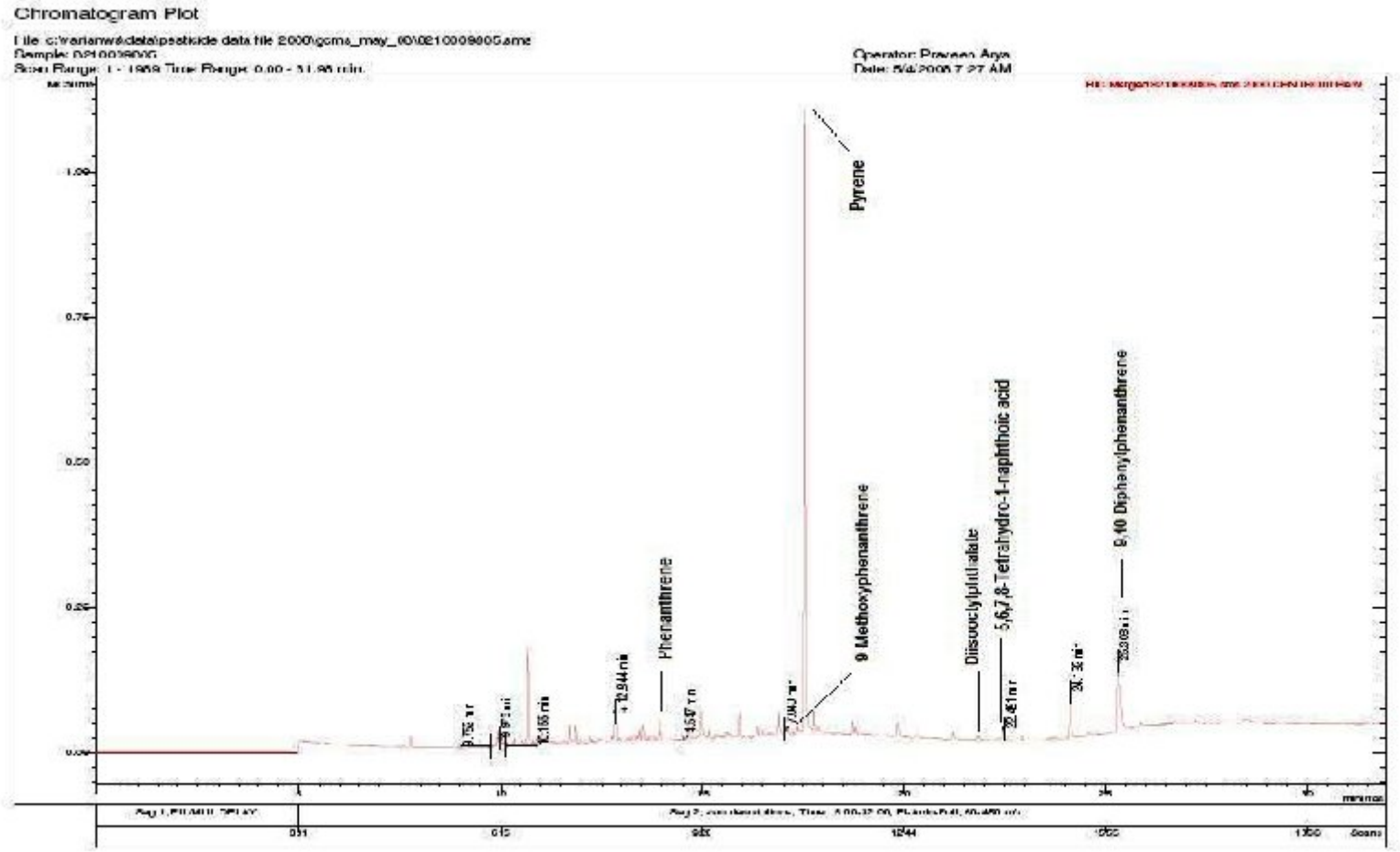


Figure 3.28 B Gas chromatography – Mass spectrometry (GCMS) separation of hexane-soluble metabolites formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown for 3 days in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

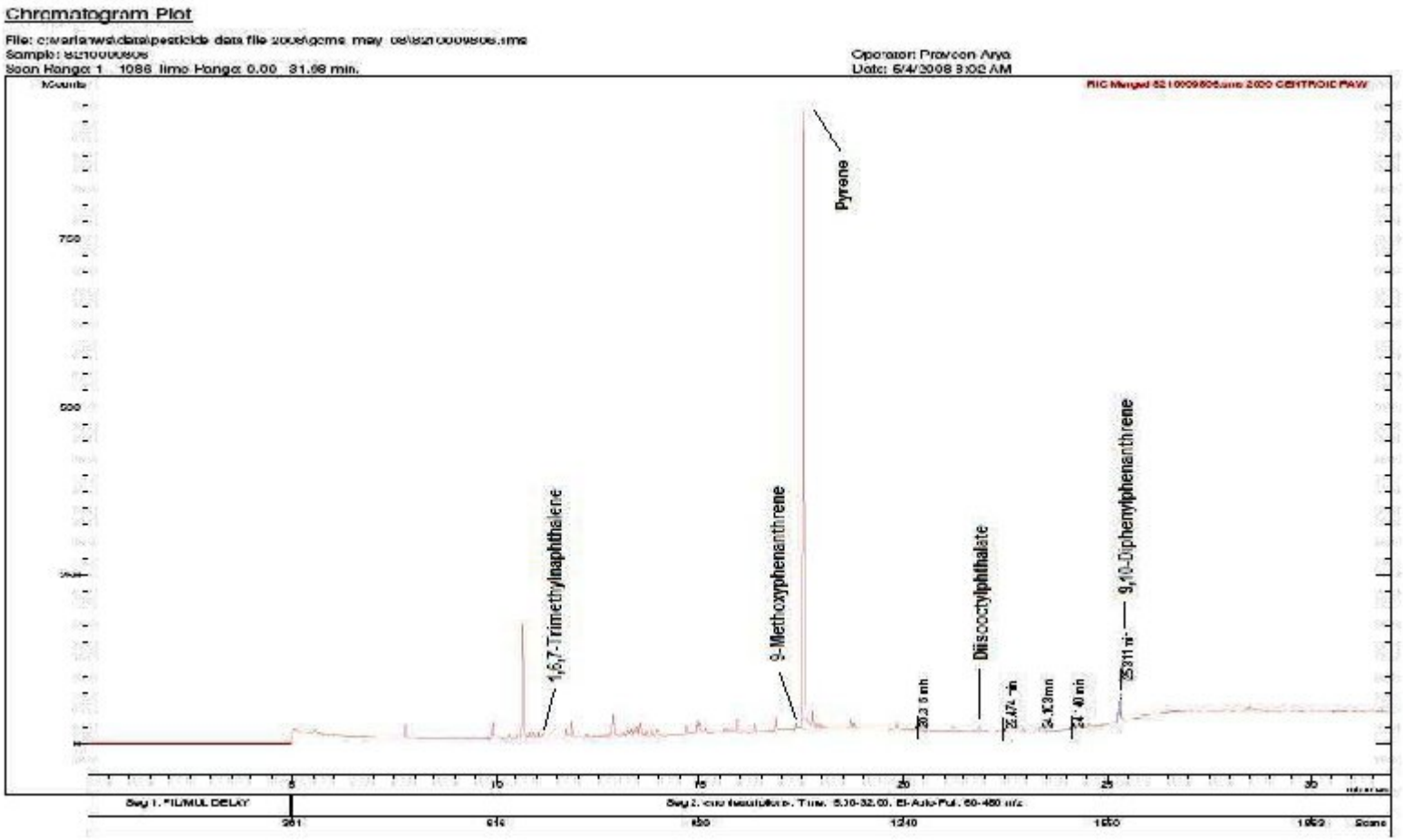


Figure 3.28 C Gas chromatography – Mass spectrometry (GCMS) separation of hexane-soluble metabolites formed from pyrene by *Bacillus* sp. (PK-6). The bacterium was grown for 4 days in BHB +Glucose (1 %; w/v) +Pyrene (50 µg ml^{-1}) medium at 45 ^{o}C.

4.3 GENETIC REGULATION OF PYRENE DEGRADATION IN THE SELECTED ISOLATE

To get a comprehensive picture of pyrene degradation molecular level studies were conducted. The seat of pyrene metabolism and related genes in the bacterial cell of selected pyrene degrading isolates was established. A ring hydroxylating dioxygenase gene is reported to be involved in the first step of pyrene catabolism in *Mycobacterium* sp. (Brezna *et al.,* 2003). Present research goal was to detect Rieske centers, the conserved [Fe2-S2] cluster binding region of terminal dioxygenases within the genera *Bacillus*, *Pseudomonas* and *Rhodococcus*. With an understanding of the pathway reported for pyrene degradation in *Mycobacterium* sp. (Kim *et al.,* 2007) and biochemical data obtained above, a pathway for pyrene degradation in *Bacillus licheniformis* (MTCC 1005) has been proposed.

4.3.1 Plasmid profiling of pyrene utilizing isolates

The genes encoding degradative enzyme for pyrene metabolism could be borne on the genome or on extra-chromosomal genetic element like plasmid. In order to study the plasmid profile of the pyrene- utilizing bacterial isolates, we followed the standard alkaline lysis method of Birnboim and Doly, (1979) for low-molecular-weight plasmids as well as the protocol of Kado and Liu (1981) for high-molecular-weight plasmid detection. No plasmid could be isolated from the pyrene utilizing bacterial strains as shown in Figure 3.29 A, B.

4.3.2 Polymerase chain reaction (PCR) amplification of Rieske centre(s)

All of the unique isolates, as determined by RFLP, were tested for the presence of Rieske center of terminal PAH dioxygenase genes encoding pyrene dioxygenase enzyme. A large number of polymerase chain reactions were performed, with efforts to optimize PCR conditions by varying the

a) $MgCl_2$ concentration in the range of 1.5 mM – 2.0 mM
b) primer concentration in the range of 0.6 µM – 2.0 µM
c) Annealing temperature in the range of 50 – 60 ^{o}C
d) Genomic DNA in the range of 10 – 50 ng.

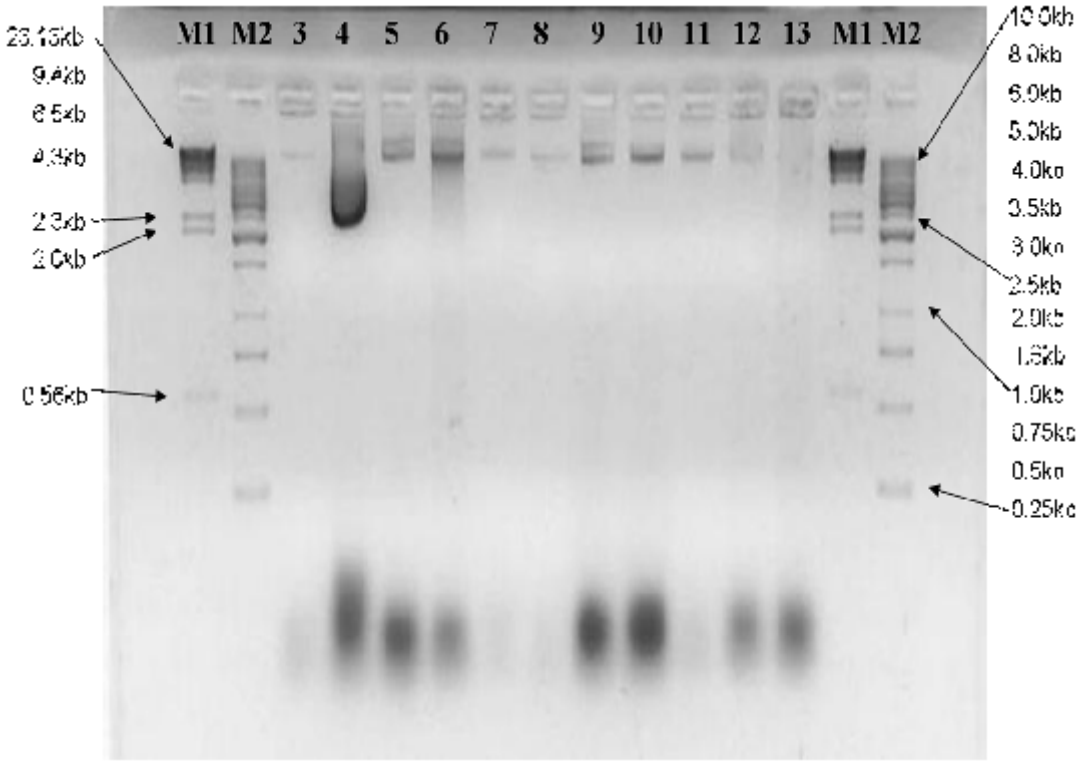


Figure 3.29 A Plasmid DNA isolation from pyrene utilizing bacterial isolates by the standard alkaline lysis method of Birnboim and Doly (1979).

Lane M1 = λ Hind III DNA Ladder, Lane M2 = 1 kb DNA Ladder, L 3 = *E. coli* DH5α, L 4 = Transformed *E. coli* DH5α, L 5 = *Bacillus* sp. (PK-6), L 6 = *Bacillus* sp. (PK-7), L 7 = *Rhodococcus* sp. (PK-10), L 8 = *Pseudomonas* sp. (PK-3), L 9 = *Bacillus* sp. (PK-12), L10 = *Bacillus* sp. (PK-13), L11 = *Bacillus* sp. (PK-14), L12 = *Bacillus* sp. (PK-8), L13 = *Bacillus* sp. (PK-9).

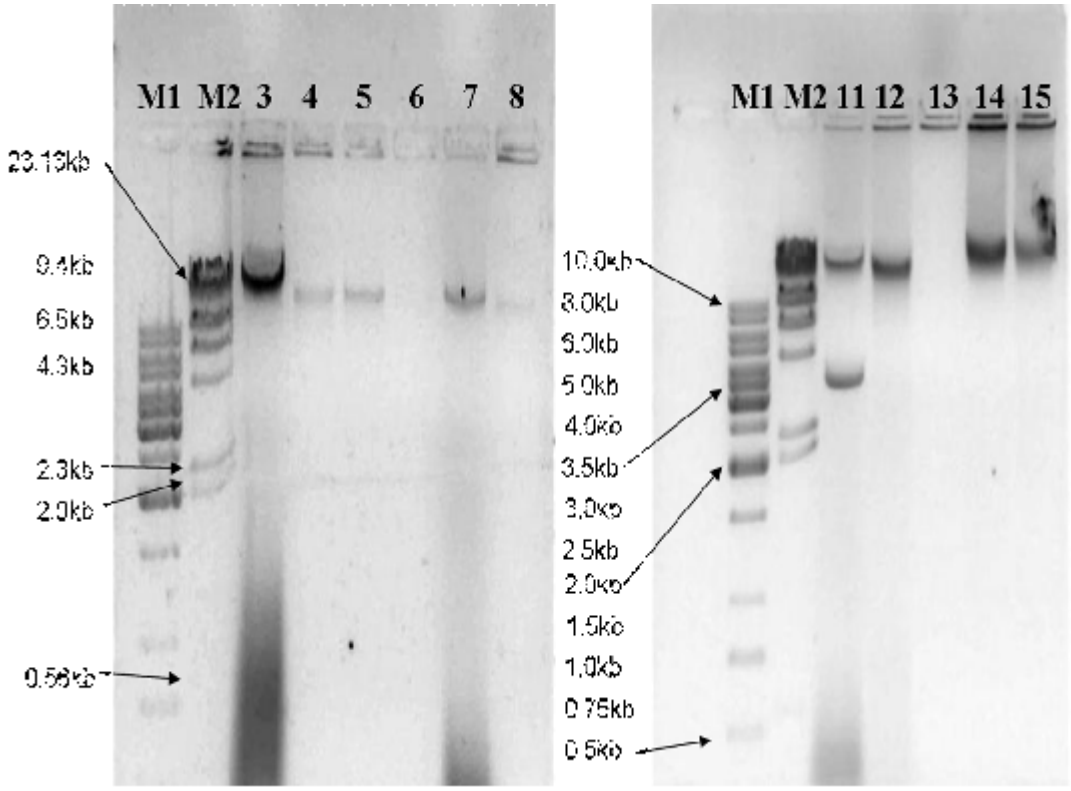


Figure 3.29 B Plasmid DNA isolation from pyrene utilizing bacterial isolates by the protocol of Kado and Liu (1981).

Lane M1 = 1 kb DNA Ladder, Lane M2 =λ Hind III DNA Ladder, L 3 = *E. coli* DH5α, L 4 = *Bacillus* sp. (PK-6), L 5 = *Bacillus* sp. (PK-7), L 6 = *Rhodococcus* sp. (PK-10) L 7 = *Pseudomonas* sp. (PK-3), L 8 = *Bacillus* sp. (PK-12), L 11 = Transformed *E. coli* DH5α, L12 = *Bacillus* sp. (PK-13), L13 = *Bacillus* sp. (PK-14), L14 = *Bacillus* sp. (PK-8), L15 = *Bacillus* sp. (PK-9).

The data, however, revealed no amplification results. None of the tested pyrene utilizing isolates, representing the three major bacterial groups (*Pseudomonad*, *Rhodococcus*, and *Bacillus*) showed presence of Rieske centres.

4.3.3 Biochemical pathway of pyrene degradation

This is the first report of pyrene metabolism by *Bacillus* sp. (PK-6) MTCC No. 1005. GC-MS profiling of growth extracts of bacterial isolate *Bacillus* sp. (PK-6) identified Phenanthrene, substituted- phenanthrenes and naphthalenes, phthalate as possible pyrene metabolites. A gradual decrease in the pyrene concentration in growth medium along with formation of six compounds suggests that pyrene is being degraded by *Bacillus* sp. (PK-6). This is the first report of formation of five new intermediates during pyrene metabolism by *Bacillus* sp. (PK-6). Based on these results a tentative pathway for pyrene degradation in *Bacillus* sp. MTCC 1005 is proposed.

It is proposed in *Bacillus* sp. (PK-6) that pyrene gets converted to phenanthrene which is successively transformed to 9–methoxyphenanthrene and 9, 10–diphenylphenanthrene (Figure 3.30). The decreasing concentration (corresponding to peak area value in Table 3.10) of 9-methoxyphenanthrene compound during incubation suggests that it is further metabolized to 5, 6, 7, 8–tetrahydro–1–naphthoic acid, leading to the formation of 1, 6, 7–trimethyl–naphthalene. This compound by a series of unknown steps gets converted to phthalate which is believed to be further transformed via the β-ketoadipate pathway to tricarboxylic acid (TCA) cycle intermediates (Kim *et al.,* 2007). It is quite possible that 9, 10-Diphenylphenanthrene formation is a dead end metabolite which is then not further utilized.

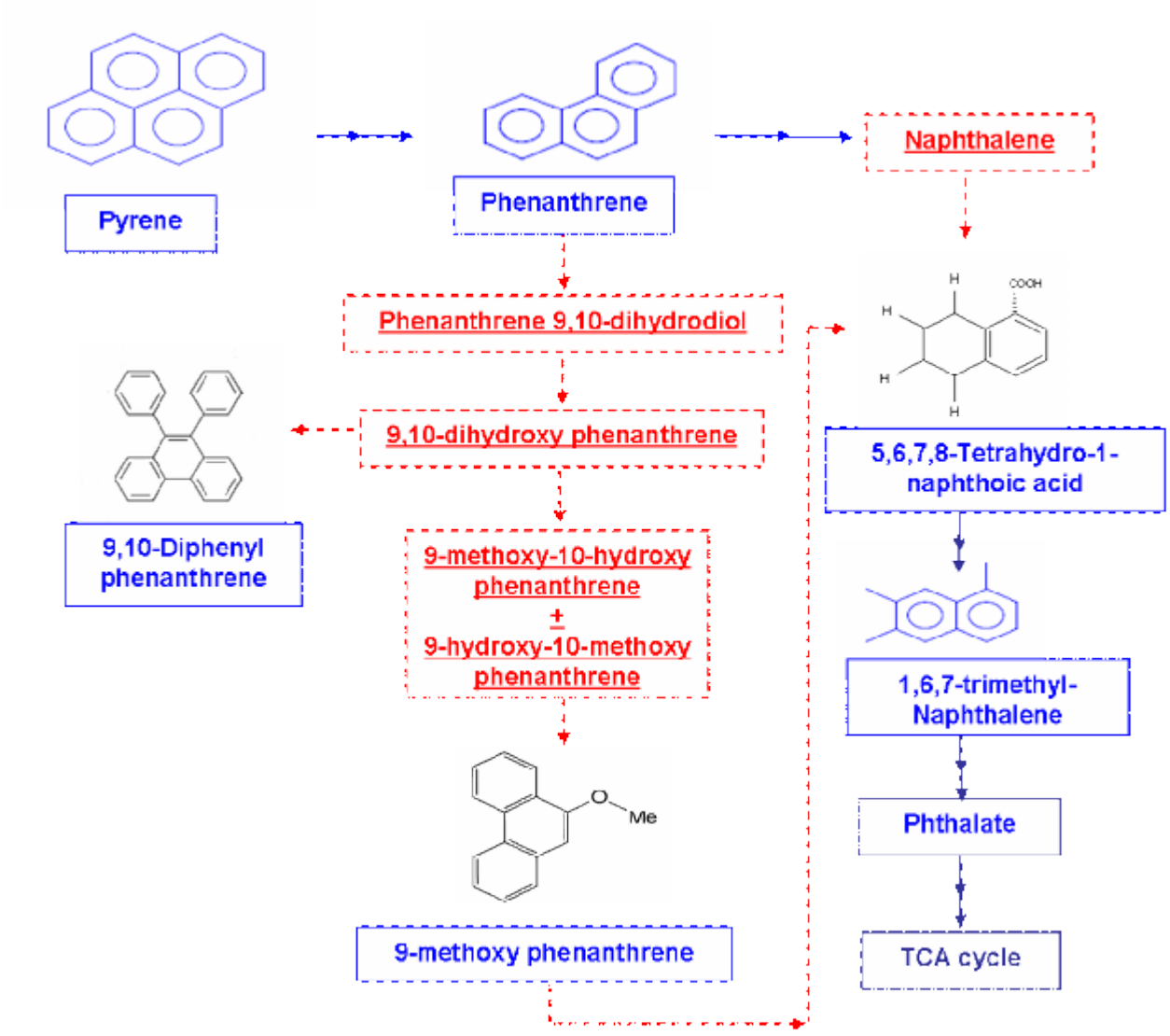


Figure 3.30 Proposed pathway for the degradation of pyrene by *Bacillus* sp. (PK-6) MTCC1005 based on isolated metabolites identified in the supernatant by GC-MS when *Bacillus* sp. was grown in BHB liquid medium supplemented with 50 µg ml^{-1} pyrene and 1.0 % (w/v) glucose at 45 ^{o}C (Underlined compounds are hypothetical intermediates. Arrows between metabolites indicate multiple steps, not single reactions.)

4.4 REMOVAL OF PYRENE BY SELECTED BACTERIAL ISOLATE FROM SOIL CONTAMINATED WITH PYRENE

This study investigated the removal of pyrene in soil by bacteria *Bacillus* sp. (PK-6) MTCC 1005 isolated from coal-tar contaminated soil. Coal-tar contaminated soil supplemented with glucose (0.5 mg g^{-1} soil) under non-sterile conditions was inoculated with *Bacillus* sp. (PK-6), whereas in a separate experiment autoclaved garden soil was spiked with pyrene @ 0.1 mg g^{-1} and glucose and inoculated with *Bacillus* sp. (PK-6) and incubated and checked for removal of pyrene. *Bacillus* sp. in association with the indigenous bacterial community in coal-tar contaminated soil removed same amount of pyrene (58 % $\pm$ 0.01; no. of replicates, n=3) as the indigenous bacterial community alone (57 % $\pm$ 0.01; no. of replicates, n=3) with no significant change in soil physico-chemical properties (pH, EC and organic carbon) over a time period of 4 weeks. However, *Bacillus* sp. (PK-6) could significantly remove pyrene (71 % of added pyrene) from pyrene-spiked garden soil as compared to control with slight variation in soil physico-chemical properties. Glucose supplementation led to increase in the bacterial count alongwith high removal of pyrene.

4.3.1 Soil characterization

A composite mixture of coal-tar contaminated soil (CT) collected from 4 different locations in a Hot-mix Coal-tar Industrial Plant (Patiala, Punjab) was used for this study. This soil was grayish-black in color due to the presence of coal-tar, sandy with fine texture. The pH of CT soil was alkaline, 8.0 $\pm$ 0.03, electrical conductivity was high 580.6 $\pm$ 19.95 µS cm^{-1}, organic carbon was in the range of 3.87 $\pm$ 0.08 %, available phosphorus 1.20 $\pm$ 0.05 ppm and total nitrogen of 0.02 % (Table 3.11).

Garden soil (S) collected from STEP, Thapar University campus, Patiala (Punjab, India) was autoclaved three times at 121 ^{o}C for 1 hr before setting up an experiment. The soil was brown colored, sandy loam soil with alkaline pH 8.4 $\pm$ 0.02, electrical conductivity of 164.3 $\pm$ 5.42 µS cm^{-1}, organic carbon in the range of 0.17 $\pm$ 0.06 %, available phosphorus 0.82 $\pm$ 0.07 ppm and total nitrogen of 0.01 % (Table 3.11).

Table 3.11 Physico-chemical characterization of soils

	Soil (Location)	Physico-chemical properties					
		Moisture (%)	pH	EC ($\mu S\ cm^{-1}$)	Organic Carbon (%)	Available Phosphorus (ppm)	Total Nitrogen (%)
1	S (STEP, Thapar University, Patiala)	36.5 ± 0.48	8.4 ± 0.02	164.3 ± 5.42	0.17 ± 0.06	0.82 ± 0.07	0.01 ± 0.00
2	CT (Coal-tar Hot-mix Industrial Plant, Patiala)	35.0 ± 0.58	8.0 ± 0.03	580.6 ± 19.95	3.87 ± 0.08	1.20 ± 0.05	0.02 ± 0.00

4.3.2 Soil studies

3.8.2.2 Coal-tar contaminated soil experiment

Non sterile compostite coal-tar contaminated soil supplemented with glucose @ 0.5 mg g^{-1} soil (CT) was inoculated with *Bacillus* sp. (PK-6) which has been isolated from the same soil and also the presence of pyrene was confirmed by gas chromatography (see section 4.1.1) (Figure 3.2). Inoculum of *Bacillus* sp. (PK-6) was developed in LB medium containing pyrene (50 µg ml^{-1}). After 18 hrs of growth, a unit OD at 600 nm corresponded to 0.58 x 10^6 cfu ml^{-1} in saline cell suspension of which 3 ml was inoculated in CT-PK6 treatment (in triplicates). The average bacterial count per gram of soil at zero time of incubation was 0.022 x 10^6 cfu g^{-1}. A moisture content of 35 $\pm$ 2 % was maintained throughout the experiment.

pH: The pH of control (CT) and *Bacillus* sp. (PK-6) inoculated soil (CT-PK6) was in alkaline range of pH 8.00 $\pm$ 0.02 to 7.94 $\pm$ 0.02 (no. of replicates, n=3) at zero time of incubation (Table 3.12). There was no change in soil pH after 28 days of incubation.

Electrical conductivity: The electrical conductivity of control (CT) and *Bacillus* sp. (PK-6) inoculated soil (CT-PK6) was 560.7 and 600.57 $\pm$ 12.87 µS cm^{-1}, respectively at zero time of incubation (Table 3.12). After 28 days of incubation the final EC of soil was observed to be 1112.33 $\pm$ 55.54 and 989.67 $\pm$ 35.18 µS cm^{-1}, respectively. The EC of *Bacillus* sp. (PK-6) inoculated soil increased 1.6 times (from 600.57 $\pm$ 12.87 µS cm^{-1} to 989.67 $\pm$ 35.18 µS cm^{-1}), while the EC of soil in control treatment became double in 28 days (from 560.67 $\pm$ 4.91 µS cm^{-1} to 1112.33 $\pm$ 55.54 µS cm^{-1}). All values reported are mean $\pm$ standard error of 3 replicates.

Organic carbon: The organic carbon at zero time of incubation was 3.78 $\pm$ 0.04 % in control soil (CT) and 3.95 $\pm$ 0.00 % in CT-PK6 soil (Table 3.12). After 28 days of incubation the organic carbon slightly increased to 3.82 $\pm$ 0.09 % in control treatment and to 3.98 $\pm$ 0.01 % in *Bacillus* sp. (PK-6) inoculated soil.

Table 3.12 Characterization of physico-chemical properties of non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) at 0 day and after 28 days of incubation at 37 ^{o}C (values are mean ± SE of 3 replicates)**.**

Coal-tar contaminated soil	pH		Electrical conductivity ($\mu S\ cm^{-1}$)		Organic carbon (%)	
	0 day	**28 day**	**0 day**	**28 day**	**0 day**	**28 day**
Control (CT)	8.00 ± 0.02	8.03 ± 0.01	560.67 ± 4.91	1112.33 ± 55.54	3.78 ± 0.04	3.82 ± 0.09
CT-*Bacillus* sp. (PK-6)	7.94 ± 0.02	7.94 ± 0.01	600.57 ± 12.87	989.67 ± 35.18	3.95 ± 0.00	3.98 ± 0.01

Bacterial enumeration: Viable cell count in non-sterile, glucose-amended coal-tar contaminated soil (CT) and non-sterile, glucose amended, *Bacillus* sp. (PK-6) inoculated soil (CT-PK6) was studied in a time-course of 28 days at 37 ^{o}C (Table 3.13; Figure 3.31). Serial dilutions of 1 gm soil from the soil treatments CT and CT-PK6 after weekly interval were plated on LA plates and photographed after 24 hrs (Table 3.14). After 7 days of incubation the control soil (CT) showed bacterial count of 0.95 x 10^{6} cfu g^{-1} , while in soil inoculated with *Bacillus* sp. PK6 (CT-PK6), an average bacterial count of 0.99 x 10^{6} cfu g^{-1} was obtained. After 14 days of incubation the bacterial count in CT treatment soil was 1.33 x 10^{6} cfu g^{-1} compared to count of 1.40 x 10^{6} cfu g^{-1} in CT-PK6 treatment soil. After 21 days of incubation the bacterial count in CT treatment soil increased to 1.73 x 10^{6} cfu g^{-1} and in CT-PK6 treatment soil increased to 1.95 x 10^{6} cfu g^{-1}. After 28 days of incubation it was observed that the bacterial count further increased in both soil treatments. Bacterial count in control coal-tar contaminated soil amended with glucose (CT) treatment was 2.15 x 10^{6} cfu g^{-1} and in similar soil inoculated with *Bacillus* sp. (PK-6) was 2.22 x 10^{6} cfu g^{-1}.

Table 3.13 Bacterial enumeration in non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) over a time period of 28 days (values are mean of 3 replicates)**.**

Time (days)	Bacterial count (x 10^6 cfu g^{-1})	
	Control (CT)	CT-*Bacillus* sp. (PK-6)
0	0.022	0.022
7	0.950	0.994
14	1.330	1.400
21	1.730	1.950
28	2.146	2.220

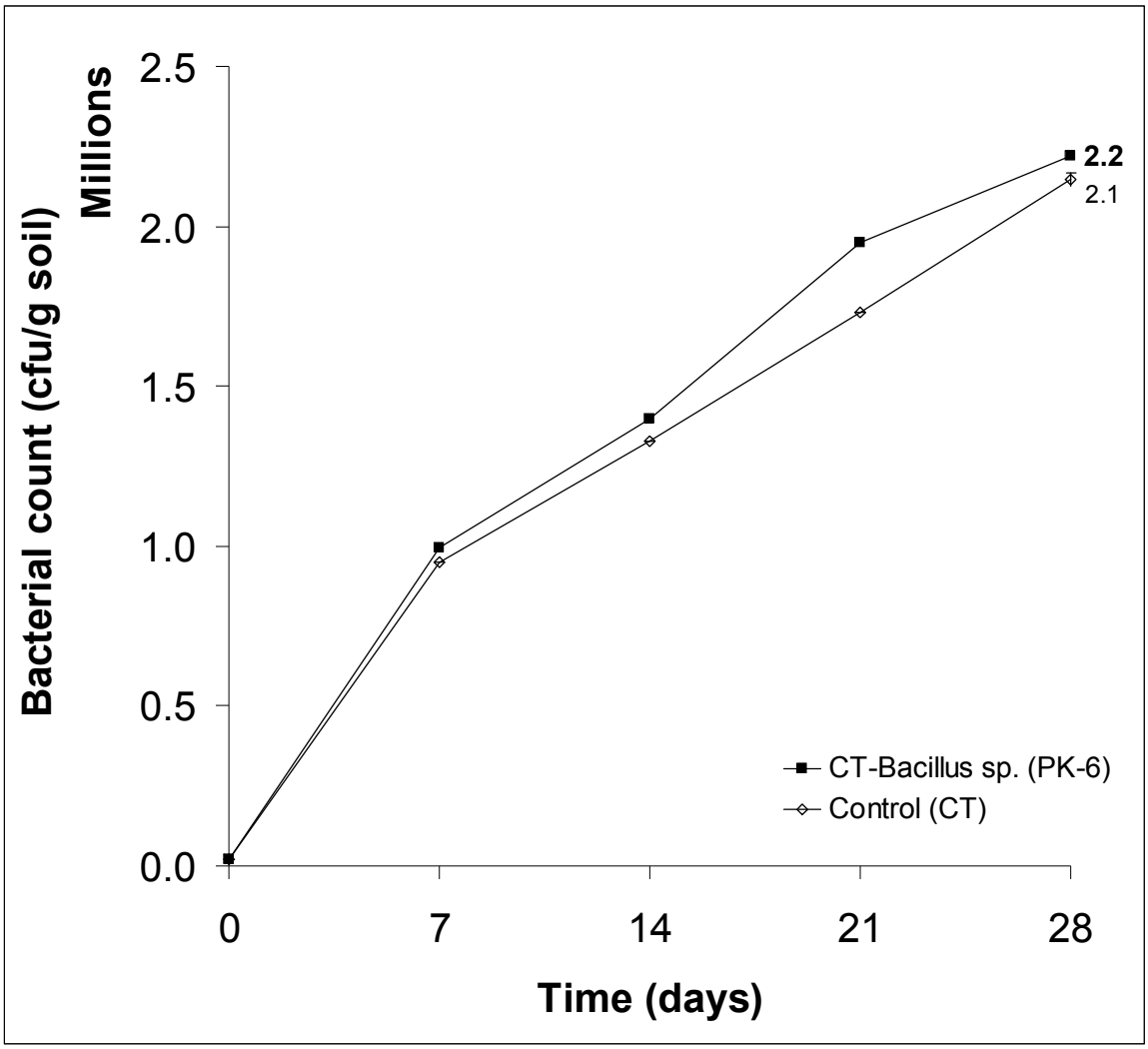
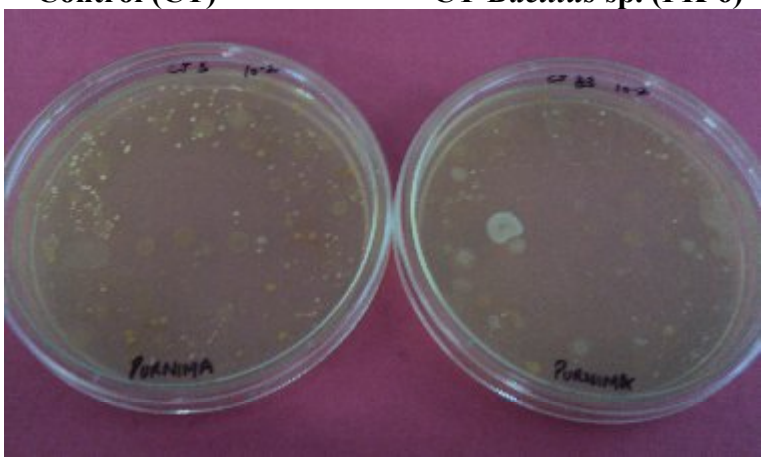


Figure 3.31 Enumeration of bacteria in non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) over a time period of 28 days (values are mean ± SE of 3 replicates)**.**

Table 3.14 Bacterial enumeration on LA medium from non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) at 0, 14 and 28 days of incubation at 37 ^{o}C.

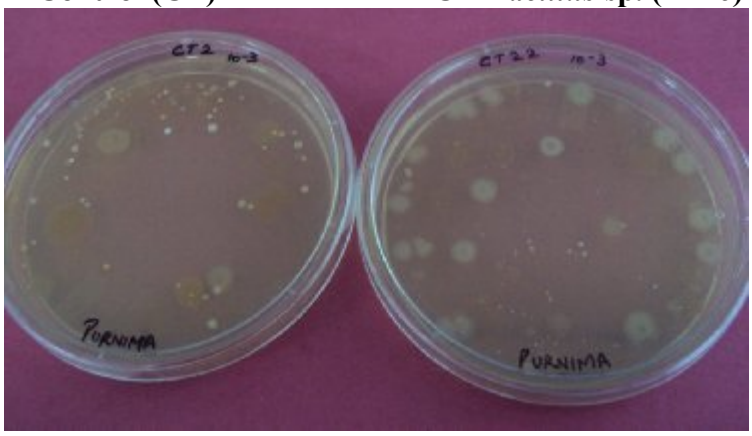
0 Day incubation

Control (CT) **CT-*Bacillus* sp. (PK-6)**



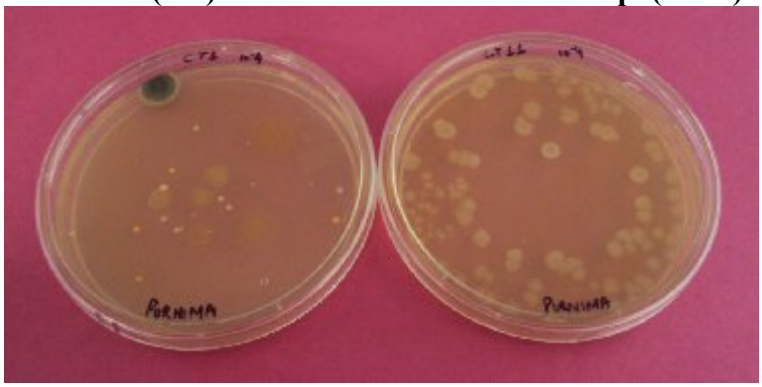
14 Day incubation

Control (CT) **CT-*Bacillus* sp. (PK-6)**



28 Day incubation

Control (CT) **CT-*Bacillus* sp. (PK-6)**



Thus after an incubation at 37 ^{o}C for 28 days, an increase of two order magnitude in bacterial counts from 0.02 x 10^6 to 2.15 x 10^6 cfu g^{-1} and from 0.02 x 10^6 to 2.22 x 10^6 cfu g^{-1} were observed, respectively, in non-sterile coal-tar contaminated soil (CT) and *Bacillus* sp. (PK-6) inoculated coal-tar contaminated soil (CT-PK6) over a time period of 28 days. Overall the bacterial count in CT-PK-6 soil amended with 0.5 mg g^{-1} glucose was similar to control (CT) with marginal increase (Table 3.13; Figure 3.31).

Pyrene removal: Pyrene removal capacity (Table 3.15) of the indigenous bacteria present in coal-tar contaminated soil (CT) versus the combination of indigenous bacteria and *Bacillus* sp. (PK-6) in CT-PK6 soil has been studied in this experiment. No lag phase in pyrene removal was observed in control (CT) and *Bacillus* sp. (PK-6) inoculated soil treatments (CT-PK6) as shown in Figure 3.32. After 14 days, the residual pyrene in non-sterile uninoculated control soil (CT) was 76 % (Table 3.15) indicating 24 % pyrene removal (Figure 3.32), while in bacillus sp. PK-6 inoculated soil (CT-PK-6) only 16 % pyrene had been removed (i.e. 84 % residual pyrene). Therafter, the rate of pyrene removal in inoculated soil (CT-PK6) showed increase and after 28 days of incubation CT soil showed 43 % and CT-PK6 soil showed 42 % residual pyrene. It implied 57 % $\pm$ 0.01 (no. of replicates, n=3) pyrene removal in coal-tar contaminated soil (CT) and marginally higher pyrene removal (58 % $\pm$ 0.01; no. of replicates, n=3) with *Bacillus* sp. (PK-6) inoculation (CT-PK6) in 28 days at 37 ^{o}C. The indigenous microflora -

Table 3.15 Pyrene analysis in non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) over a time period of 28 days (values are mean $\pm$ SE of 3 replicates)**.**

Time (days)	Residual pyrene (%) in soil			
	Control (CT)		CT-*Bacillus* sp. (PK-6)	
	$OD_{254\ nm}$	%	$OD_{254\ nm}$	%
0	0.57 $\pm$ 0.03	**100**	0.57 $\pm$ 0.01	**100**
7	0.54 $\pm$ 0.03	**94**	0.51 $\pm$ 0.02	**90**
14	0.43 $\pm$ 0.03	**76**	0.48 $\pm$ 0.01	**84**
21	0.40 $\pm$ 0.05	**71**	0.35 $\pm$ 0.04	**61**
28	0.25 $\pm$ 0.01	**43**	0.24 $\pm$ 0.01	**42**

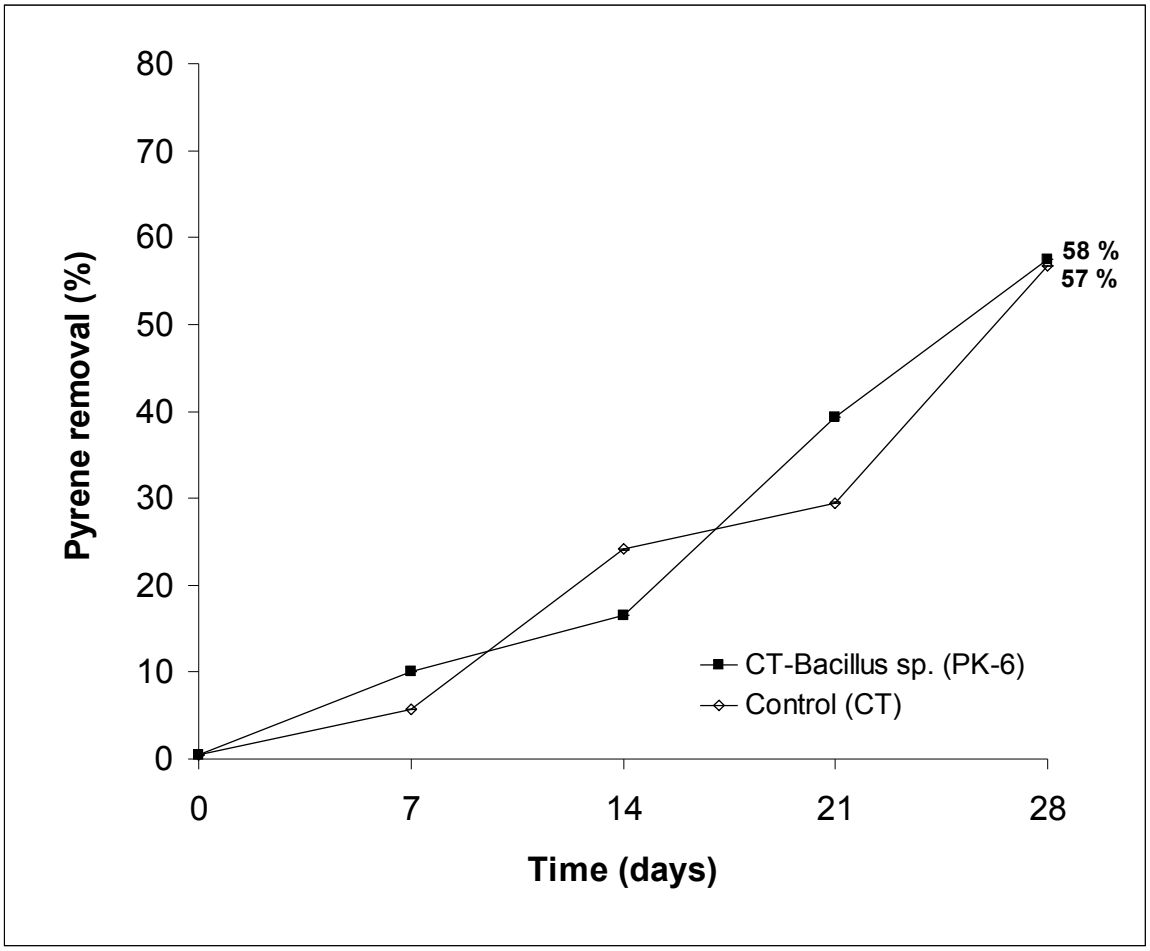


Figure 3.32 Pyrene analysis in non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) over a time period of 28 days. The mean of triplicate samples is plotted for each incubation condition as the percentage of pyrene removal.

- in coal-tar contaminated soils (CT and CT-PK6) showed high pyrene removal rates. Coal-tar contaminated soil augmentation with pyrene utilizing *Bacillus* sp. (PK-6) MTCC 1005 was found to marginally favour pyrene removal over the indigenous soil microbial community.

A correlation is sought in Figure 3.33 between enumeration of bacteria from soil treatments and percentage of pyrene removed by the respective bacterial species present therein. In the *Bacillus* sp. (PK-6) inoculated soil (CT-PK6), during the first 14 days of incubation, bacterial population increased from 0.02 x 10^6 cfu g^{-1} to 1.40 x 10^6 cfu g^{-1} soil and caused a 16 % reduction (spectrophotometric) in the pyrene content of soil. From this point to the end of incubation (28 days), the pyrene content decreased by 42 % more, while the bacterial population increased to 2.22 x 10^6 cfu g^{-1} soil. In the uninoculated soil (CT), during the first 14 days of incubation, indigenous bacterial count increased from 0.02 x 10^6 cfu g^{-1} to 1.33 x 10^6 cfu g^{-1} soil and caused a 24 % reduction in pyrene (spectrophotometric). From this point to the end of incubation (28 days), the pyrene content decreased by 33 % more, while the bacterial population increased to 2.15 x 10^6 cfu g^{-1} soil. The figure shows that in 28 days of incubation marginally higher counts of bacteria were recovered from soil incubation in CT-PK6, which removed slightly higher percentage of pyrene as compared to control CT.

4.3.2.1 Pyrene spiked garden soil experiment

To study the effect of inoculation of *Bacillus* sp (PK6) in soil on removal of pyrene, in an experiment autoclaved garden soil (S) was spiked with pyrene (P) @ 0.1 mg g^{-1} soil and supplemented with glucose (G) @ 0.5 mg g^{-1} soil and was inoculated with *Bacillus* sp. (PK-6) in various treatments over a period of 28 days (Table 3.16). Inoculum of *Bacillus* sp. (PK-6) was developed in LB medium containing pyrene (50 µg ml^{-1}) for 18 hrs, centrifuged to remove spent LB medium and bacterial cells (= 5.3 x 10^8 cfu ml^{-1}) were resuspended in 0.85 % (w/v) saline which was inoculated @ 7 ml per treatment. The bacterial count at zero time of incubation was 0.37 x 10^7 cfu g^{-1} soil. A moisture content of 35 $\pm$ 2 % was maintained throughout the experiment.

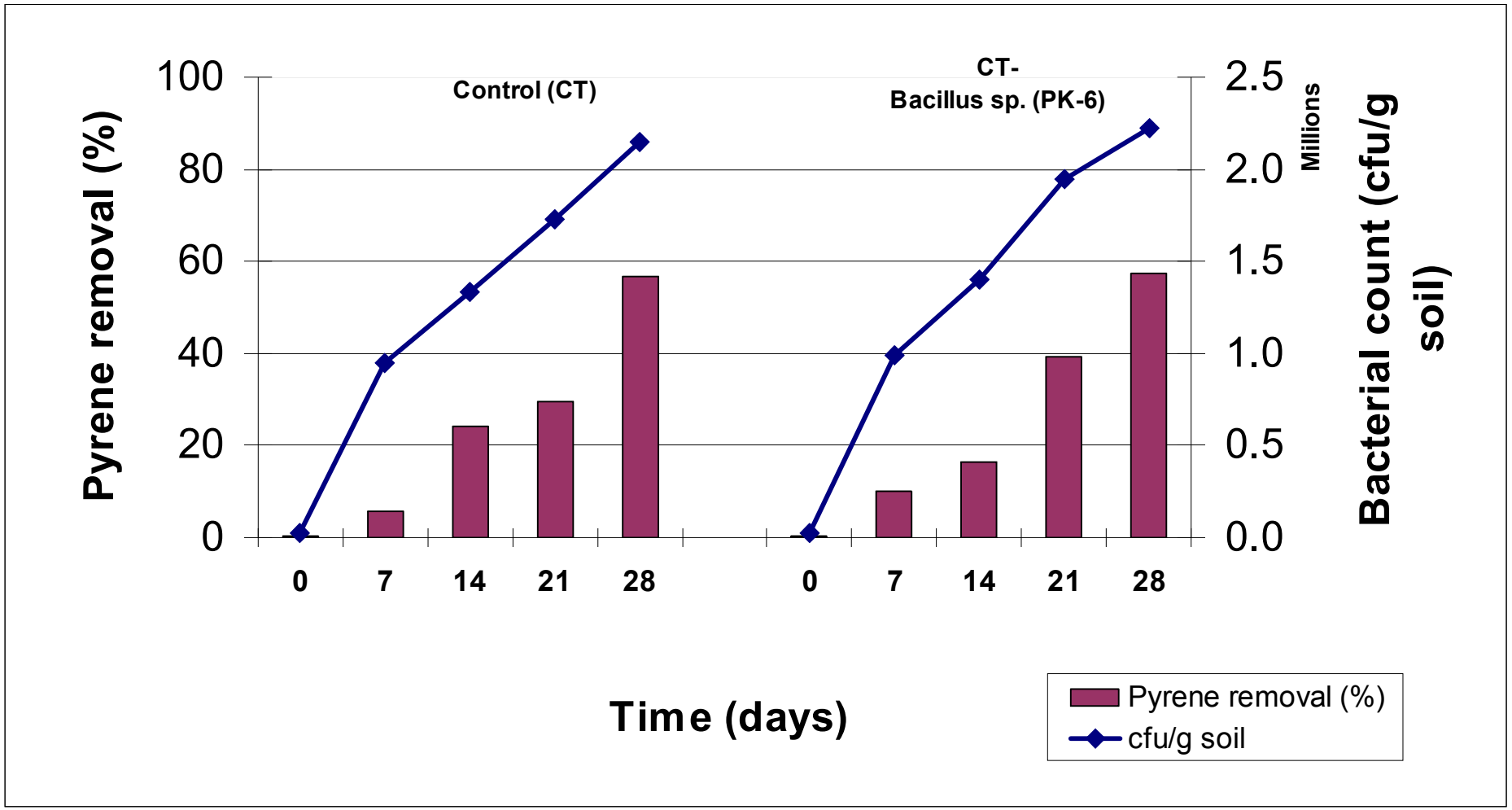


Figure 3.33 A comparative study of bacterial enumeration (represented by line curve) and pyrene analysis (represented by columns) in non-sterile coal-tar contaminated soil (CT) inoculated with *Bacillus* sp. (PK-6) over a time period of 28 days (values are mean of 3 replicates)**.**

pH: The pH of soil in all eight soil treatments was in alkaline range 8.08 ± 0.05 - 8.41 ± 0.07 at zero time (Table 3.16). After 28 days of incubation, the pH dropped to as low as 6.82 ± 0.06 in pyrene-spiked soil (S-P) and 6.07 ± 0.03 in soil amended with glucose and inoculated with *Bacillus* sp. PK-6 (S-G-B). A unit decrease in pH to 7.40 ± 0.02 was observed in the control soil (S) after 28 days of incubation.

Electrical conductivity: The electrical conductivity of soil in eight treatments was in the range of 151.43 ± 0.39 to 214.33 ± 1.20 µS cm^{-1} at zero time (Table 3.16). After 28 days of incubation the final EC of soil was observed to be in range of 205.33 ± 0.88 to 557.50 ± 1.50 µS cm^{-1}. The EC of control soil (S) increased 1.4 times (from 151.43 ± 0.39 µS cm^{-1} to 205.33 ± 0.88 µS cm^{-1}) and of pyrene-spiked soil (S-P) and glucose amended soil (S-G) became double in 28 days (from 156.03 ± 3.44 and 174.50 ± 3.93 µS cm^{-1} to 320.00 ± 1.53 and 339.33 ± 0.33 µS cm^{-1}, respectively). A 2.3 times increase in soil EC was reported in *Bacillus* sp. (PK-6) inoculated soil (S-B) and pyrene-spiked, PK-6 inoculated soil (S-P-B) treatment (from 214.33 ± 1.20 and 199.33 ± 4.70 µS cm^{-1} to 494.50 ± 0.50 and 460.00 ± 6.00 µS cm^{-1}, respectively). A 2.5 – 2.6 times increase in EC of soil was reported in pyrene-spiked, glucose amended, PK-6 inoculated soil (S-P-G-B) and pyrene-spiked, glucose amended non-inoculated soil (S-P-G) treatment (from 158.33 ± 1.20 to 397.50 ± 1.50 and from 162.33 ± 1.45 µS cm^{-1} to 416.00 ± 0.58 µS cm^{-1}, respectively). A maximum increase of 3.4 times the EC at zero time of incubation, i.e. from 166.00 ± 2.31 to 557.50 ± 1.50 µS cm^{-1} after 28 days was observed in soil amended with glucose and inoculated with *Bacillus* sp. PK-6 (S-G-B).

Organic carbon: The organic carbon at zero time of incubation was 0.08 ± 0.02 % in control (uninoculated) soil and 0.23 ± 0.00 % in remaining soil treatments (Table 3.16). After 28 days of incubation the organic carbon in S, S-P, S-G and S-P-G treatments remained same while it increased in *Bacillus* sp. (PK-6) inoculated soil treatments (0.35 – 0.42 ± 0.00 %).

Table 3.16 Characterization of physico-chemical properties of sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} **soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) at 0 day and after 28 days of incubation at 37 ^{o}C** (values are mean ± SE of 3 replicates)**.**

Garden soil	pH		Electrical conductivity (µS cm^{-1})		Organic carbon (%)	
	0 day	28 day	0 day	28 day	0 day	28 day
Soil control (S)	8.41 ± 0.07	7.40 ± 0.02	151.43 ± 0.39	205.33 ± 0.88	0.08 ± 0.02	0.10 ± 0.01
Soil-Pyrene (S-P)	8.15 ± 0.05	6.82 ± 0.06	156.03 ± 3.44	320.00 ± 1.53	0.23 ± 0.00	0.23 ± 0.00
Soil-Glucose (S-G)	8.35 ± 0.05	6.88 ± 0.04	174.50 ± 3.93	339.33 ± 0.33	0.20 ± 0.01	0.23 ± 0.02
Soil -Pyrene-Glucose (S-P-G)	8.33 ± 0.05	6.90 ± 0.03	162.33 ± 1.45	416.00 ± 0.58	0.23 ± 0.00	0.23 ± 0.00
Soil -Bacillus sp. PK-6 (S-B)	8.29 ± 0.07	7.37 ± 0.02	214.33 ± 1.20	494.50 ± 0.50	0.23 ± 0.00	0.38 ± 0.00
Soil –Pyrene-*Bacillus* sp. PK-6 (S-P-B)	8.15 ± 0.05	7.35 ± 0.03	199.33 ± 4.70	460.00 ± 6.00	0.23 ± 0.00	0.42 ± 0.00
Soil –Glucose-*Bacillus* sp. PK-6 (S-G-B)	8.19 ± 0.04	6.07 ± 0.03	166.00 ± 2.31	557.50 ± 1.50	0.23 ± 0.00	0.35 ± 0.00
Soil -Pyrene-Glucose-*Bacillus* sp. PK-6 (S-P-G-B)	8.08 ± 0.05	7.64 ± 0.03	158.33 ± 1.20	397.50 ± 1.50	0.23 ± 0.00	0.38 ± 0.00

Bacterial enumeration: Enumeration of bacteria from *Bacillus* sp. (PK-6) inoculated soil amended with 0.1 mg g^{-1} pyrene and 0.5 mg g^{-1} glucose (S-G-B, S-P-G-B) yielded more cfu of bacteria per gram of soil than soil without glucose amendment (S-B, S-P-B) as shown in Table 3.17 and Figure 3.34. Serial dilutions of 1 gm soil from the *Bacillus* sp. (PK-6) inoculated treatments S-B, S-P-B, S-G-B and S-P-G-B were plated on LA plates after 14 and 28 days of incubation and photographed after 24 hrs, as shown in Table 3.18. After 7 days of incubation it was observed that pyrene-spiked and glucose amended and *Bacillus* sp. (PK-6) inoculated soil (S-P-G-B) contained the highest number of organisms at 5.40 x 10^7 cfu g^{-1}, compared to 1.25 x 10^7 cfu g^{-1} for glucose amended and *Bacillus* sp. (PK-6) inoculated soil (S-G-B), 0.34 x 10^7 cfu g^{-1} for pyrene-spiked and *Bacillus* sp. (PK-6) inoculated soil (S-P-B) and 0.15 x 10^7 cfu g^{-1} for only *Bacillus* sp. (PK-6) inoculated soil (S-B). No culturable bacteria were detected in control (S) soil, pyrene-spiked soil (S-P), glucose amended soil (S-G) and pyrene-spiked and glucose amended soil (S-P-G). After 14 days of incubation it was observed that soil amended with pyrene and glucose and inoculated with *Bacillus* sp. PK-6 (S-P-G-B) contained 6.40 x 10^7 cfu g^{-1} compared to the higher count of 7.02 x 10^7 cfu g^{-1} in soil amended with glucose and inoculated with *Bacillus* sp. PK-6 (S-G-B) (Table 3.17; Figure 3.34). In contrast, bacterial counts in S-P-B soil decreased to 0.30 x 10^7 cfu g^{-1} and in S-B soil decreased to 0.11 x 10^7 cfu g^{-1}. A bacterial count of 0.01 x 10^6 cfu g^{-1} was detected in S-P-G soil. After 21 days of incubation it was observed that bacterial counts in soil amended with pyrene and glucose and inoculated with *Bacillus* sp. PK-6 (S-P-G-B) increased to 9.00 x 10^7 cfu g^{-1}, while it declined in soil amended with glucose and inoculated with *Bacillus* sp. PK-6 (S-G-B) to 5.58 x 10^7 cfu g^{-1}, in soil amended with pyrene and inoculated with *Bacillus* sp. PK-6 (S-P-B) to 0.22 x 10^7 cfu g^{-1}, in soil inoculated with *Bacillus* sp. PK-6 (S-B) soil to 0.53 x 10^6 cfu g^{-1}, in soil amended with pyrene and glucose (S-P-G) to 0.03 x 10^6 cfu g^{-1}. After 28 days of incubation it was observed that bacterial count declined in all soil treatments as compared to 21 day of incubation. Bacterial counts in *Bacillus* sp. (PK-6) inoculated soils amended with glucose (S-G-B) and pyrene (S-P-G-B) contained 2.25 x 10^7 and 2.20 x 10^7 cfu g^{-1}, respectively, while pyrene-spiked, *Bacillus* sp. PK-6 inoculated soil (S-P-B) showed 0.99 x 10^6 cfu g^{-1} and unamended soil inoculated with PK-6 (S-B) showed 0.20 x 10^6 cfu g^{-1} soil. In the uninoculated soil bacterial counts was 0.07 x 10^6 cfu g^{-1} in the pyrene-spiked, glucose amended soil (S-P-G).

Table 3.17 Bacterial enumeration in sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) over a time period of 28 days (values are mean of 3 replicates)**.**

Time (days)	Bacterial count (x 10^7 cfu g^{-1} soil)							
	Soil Control (S)	**Soil-Pyr (S-P)**	**Soil-Glu (S-G)**	**Soil-Pyr-Glu (S-P-G)**	**Soil-*Bacillus* sp. PK-6 (S-B)**	**Soil-Pyr-*Bacillus* sp. PK-6 (S-P-B)**	**Soil-Glu-*Bacillus* sp. PK-6 (S-G-B)**	**Soil-Pyr-Glu-*Bacillus* sp. PK-6 (S-P-G-B)**
0	-	-	-	-	**0.374**	**0.374**	0.374	0.374
7	-	-	-	-	0.149	0.340	1.252	5.400
14	-	-	-	0.001	0.111	0.300	**7.020**	6.400
21	-	-	-	0.003	0.053	0.220	5.580	**9.000**
28	-	-	-	0.007	0.020	0.099	2.246	2.200

Symbol (-) implies bacterial count was found to be nil.

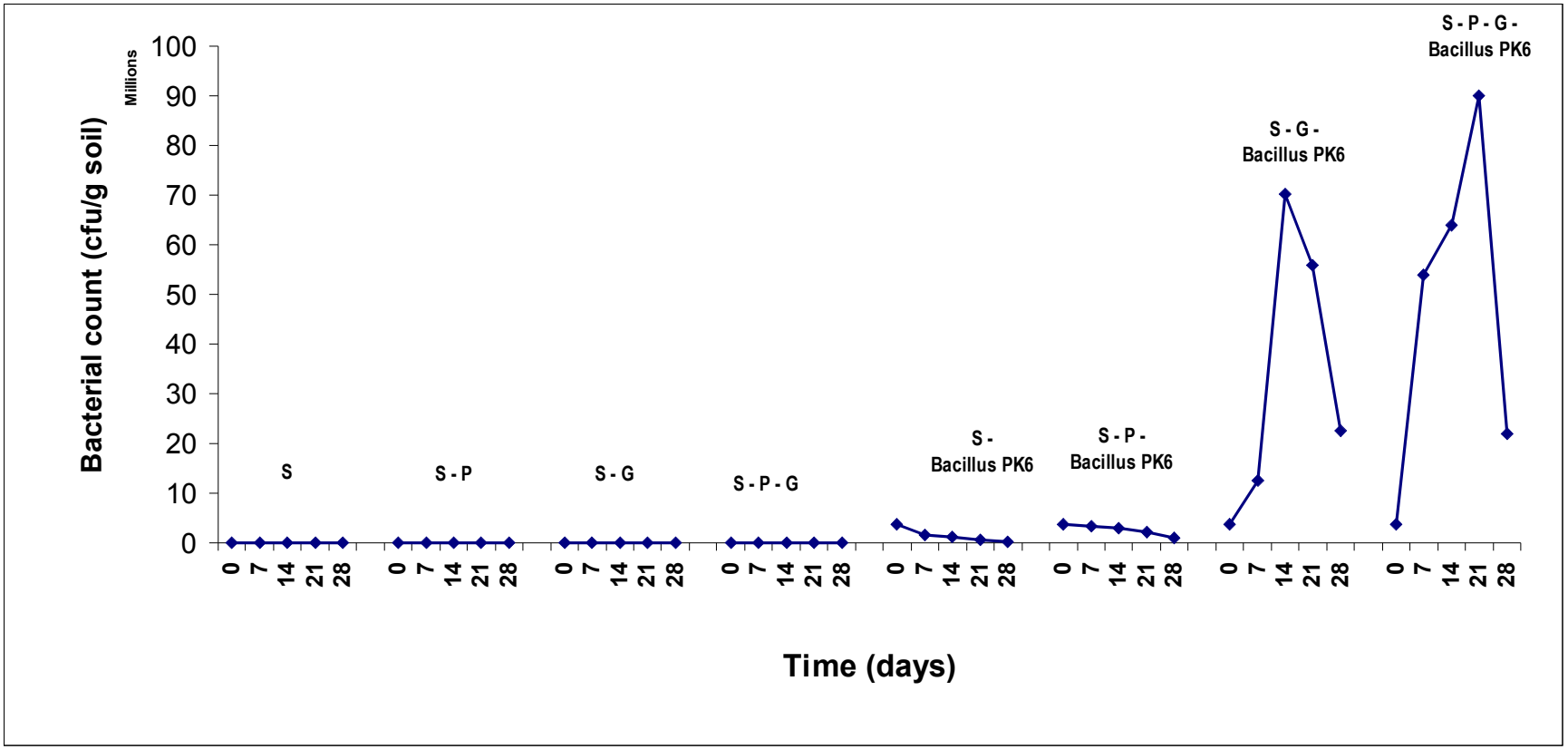
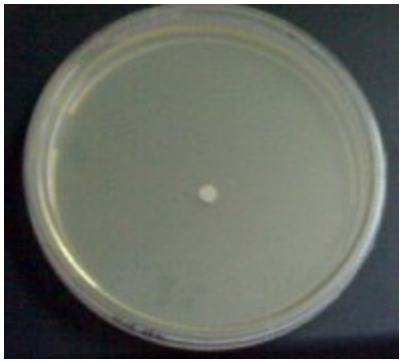
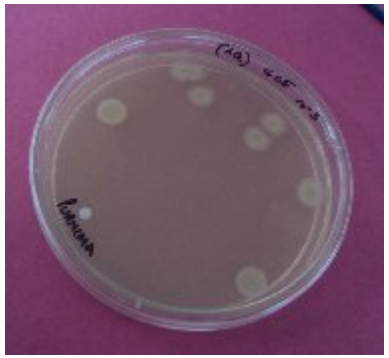
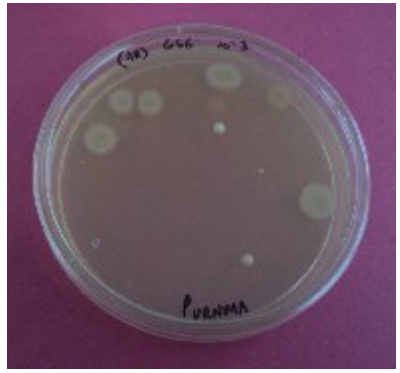
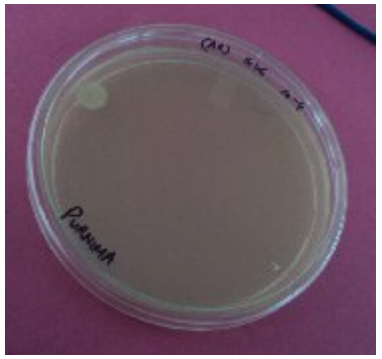
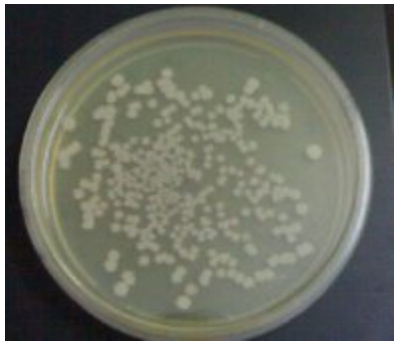
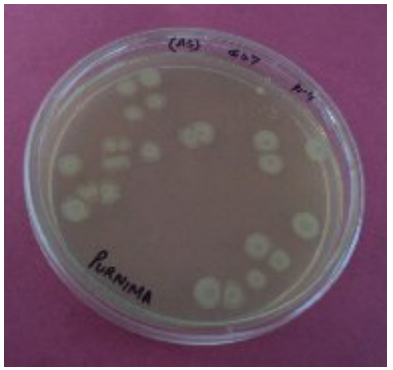
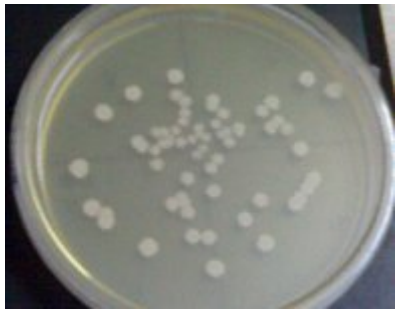
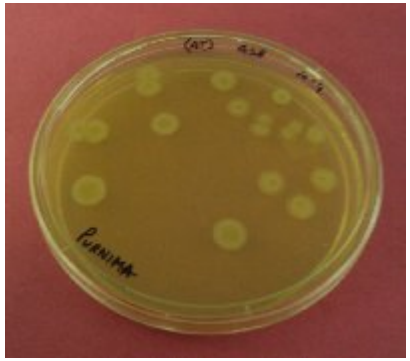
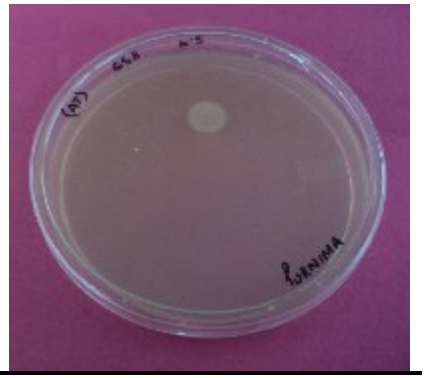


Figure 3.34 **Bacterial enumeration in sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1}, glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) over a time period of 28 days.**

Table 3.18 **Bacterial enumeration on LA medium from sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) after 14 and 28 days of incubation at 37 ^{o}C.**

Treatment	Dilution plated	14 day	Dilution plated	28 day
Soil-*Bacillus* sp. PK-6 (S-B)	10^{-4}		10^{-3}	
Soil-Pyrene-*Bacillus* sp. PK-6 (S-P-B)	10^{-4}		10^{-3}	

			10^{-4}	
Soil-Glucose-*Bacillus* sp. PK-6 (S-G-B)	10^{-4}		10^{-4}	
	10^{-5}		10^{-5}	

Soil-Pyrene-Glucose-*Bacillus* sp. PK-6 (S-P-G-B)	**10^{-4}**		**10^{-4}**	
	10^{-5}		**10^{-5}**	

Pyrene removal: Pyrene removal was monitored in pyrene (P) spiked sterile garden soil (S) and *Bacillus* sp. PK-6 (B) inoculated soil, with and without glucose (G) amendment, in four different treatments, over a 28 day incubation period at 37 ^{o}C. After 7 days the percent residual pyrene in *Bacillus* sp. (PK-6) inoculated soils amended with (S-P-G-B) and without glucose (S-P-B) were 67 $\pm$ 0.00 % and 85 $\pm$ 0.01 % (Table 3.19), impling 33 $\pm$ 0.00 % and 15 $\pm$ 0.01 % pyrene removal, respectively (Figure 3.35). A 15 $\pm$ 0.00 % and 1 $\pm$ 0.00 % pyrene removal was observed in uninoculated soil amended with and without glucose (S-P-G and S-P, respectively). After 14 days of incubation pyrene removal in S-P soil was 7 $\pm$ 0.00 %, in S-P-B soil was 23 $\pm$ 0.00 %, in S-P-G soil was 29 $\pm$ 0.01 % and in S-P-G-B soil was 47 $\pm$ 0.00 %. The two soil treatments with glucose showed higher pyrene removal than soils without glucose (S-P and S-P-B). However, after 21 days of incubation pyrene removal (45 $\pm$ 0.00 %) in S-P-B soil surpassed pyrene removal (32 $\pm$ 0.00 %) in S-P-G treatment. After 28 days the pyrene removal in pyrene-spiked soil (S-P) was 13 $\pm$ 0.00 %, in pyrene-spiked and glucose amended soil (S-P-G) was 34 $\pm$ 0.00 %, in pyrene-spiked and *Bacillus* sp. (PK-6) inoculated soil (S-P-B) was 66 $\pm$ 0.00 % and in pyrene-spiked and glucose amended and *Bacillus* sp. (PK-6) inoculated soil (S-P-G-B) was 71 $\pm$ 0.00 % (Figure 3.35). Therefore soil treatment with pyrene degrading *Bacillus* sp. (PK-6) and soil amendment with glucose, both factors were found to favour maximum pyrene removal over autoclaved pyrene-spiked garden soil.

A correlation is sought in Figure 3.36 between enumeration of bacteria and percentage of pyrene removed from soil by bacteria. The figure shows that the highest count of bacteria was recovered from the soil amended with glucose i.e. S-P-G-B treatment, which also displayed the highest pyrene removal.

Table 3.19 Pyrene analysis in sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) over a time period of 28 days (values are mean ± SE, n = 3)**.**

Time (days)	Residual pyrene (%) in soil							
	Soil-Pyrene (S-P)		**Soil-Pyrene-Glucose (S-P-G)**		**Soil-Pyrene-*Bacillus* sp. PK-6 (S-P-B)**		**Soil-Pyrene-Glucose-*Bacillus* sp. PK-6 (S-P-G-B)**	
	$OD_{254\ nm}$	**%**	$OD_{254\ nm}$	**%**	$OD_{254\ nm}$	**%**	$OD_{254\ nm}$	**%**
0	3.12 ± 0.00	**100**	3.12 ± 0.00	**100**	3.12 ± 0.01	**100**	3.12 ± 0.00	**100**
7	3.11 ± 0.00	**99**	2.67 ± 0.00	**85**	2.65 ± 0.01	**85**	2.10 ± 0.00	**67**
14	2.91 ± 0.00	**93**	2.23 ± 0.01	**71**	2.41 ± 0.00	**77**	1.64 ± 0.00	**53**
21	2.86 ± 0.02	**91**	2.14 ± 0.00	**68**	1.70 ± 0.00	**55**	1.02 ± 0.00	**33**
28	2.73 + 0.00	**87**	2.05 + 0.00	**66**	1.05 + 0.00	**34**	0.91 + 0.00	**29**

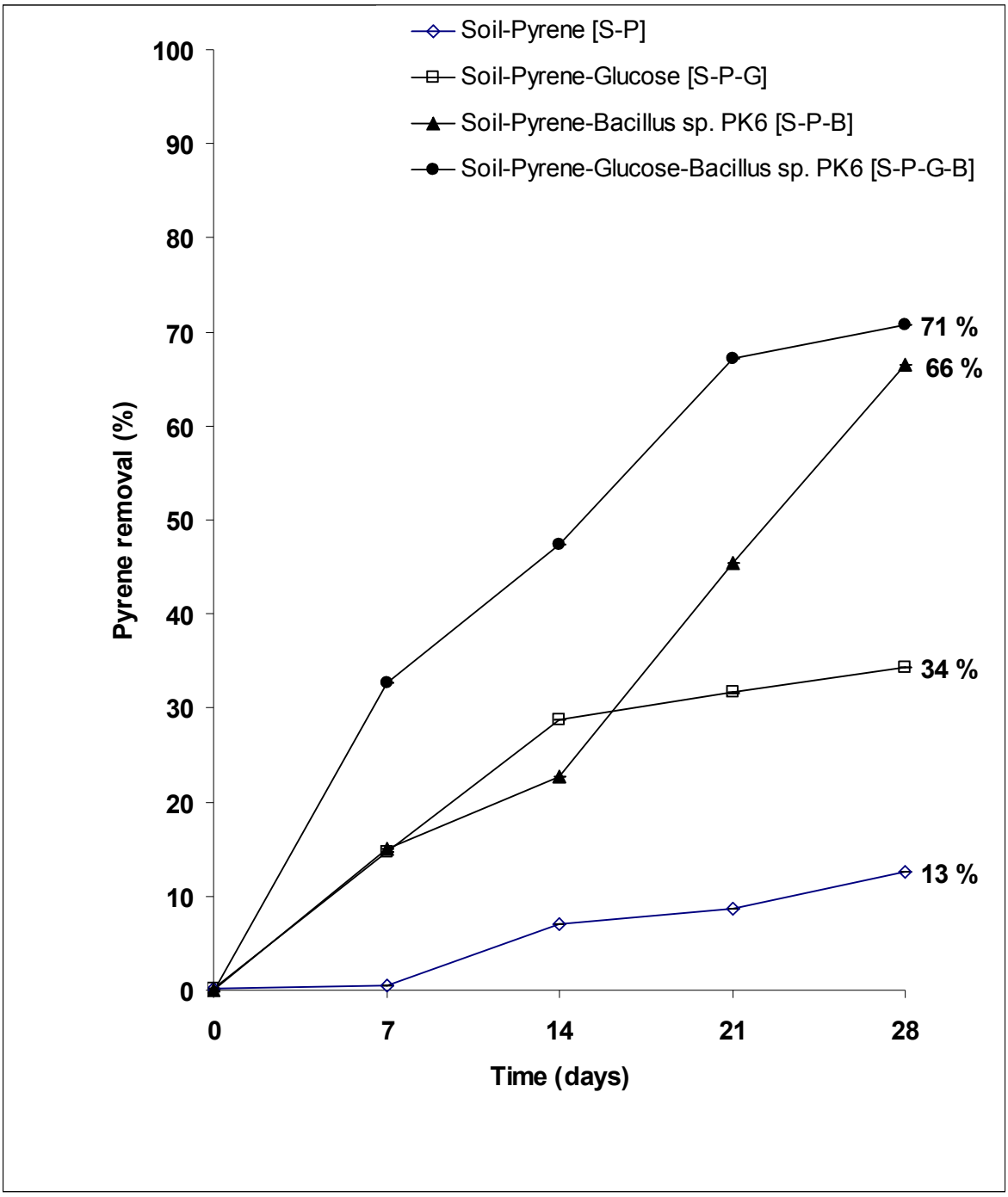


Figure 3.35 Pyrene analysis in sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) over a time period of 28 days. The mean of triplicate samples is plotted for each incubation condition as the percent of pyrene removed from soil.

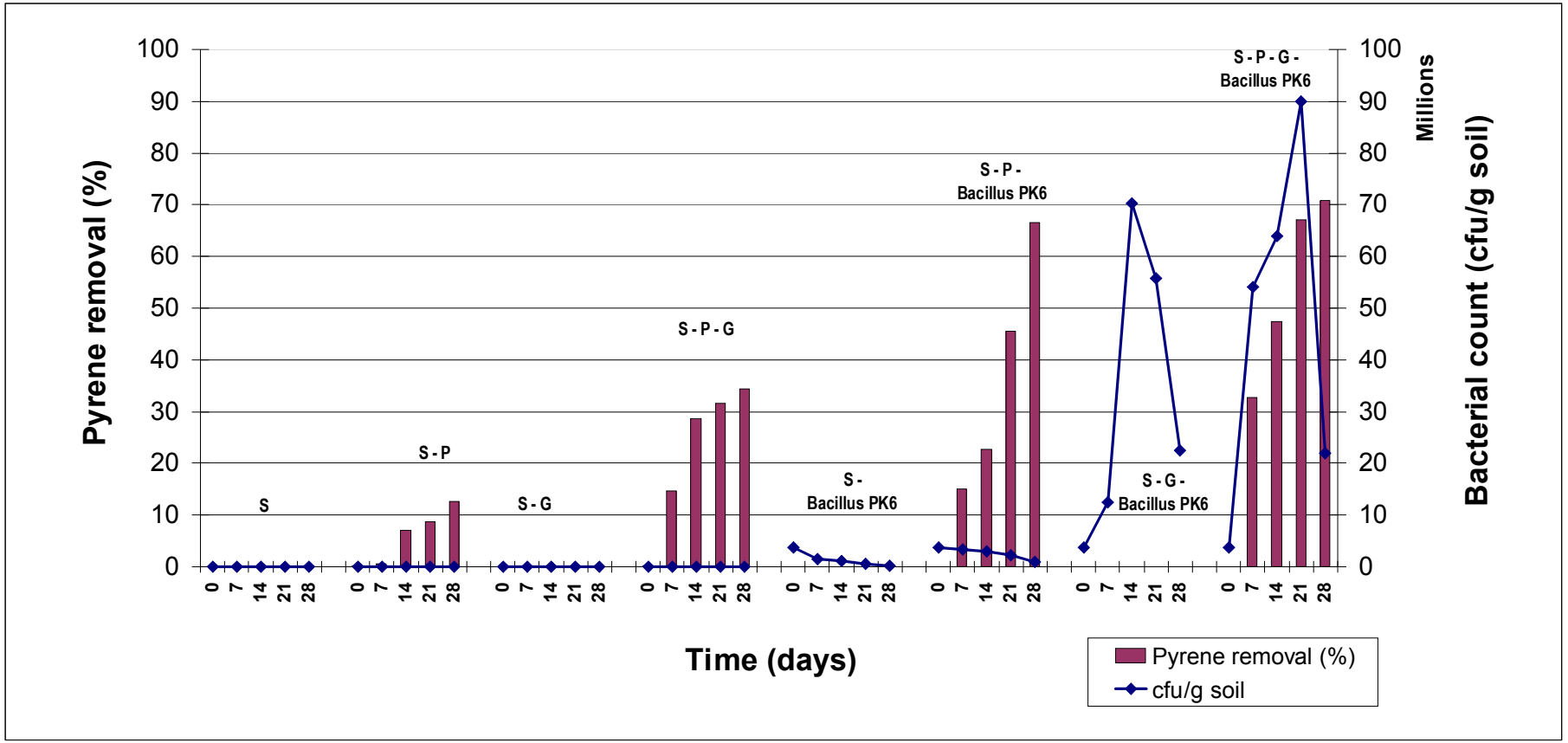


Figure 3.36 A comparative study of bacterial enumeration (represented by line curve) and pyrene analysis (represented by columns) in sterile garden soil (S) spiked with pyrene (P) @ 0.1 mg g^{-1} soil and glucose (G) @ 0.5 mg g^{-1} soil and inoculated with *Bacillus* sp. PK-6 (B) over a time period of 28 days (values are mean of 3 replicates)**.**

CHAPTER 5

DISCUSSION

With an aim to investigate the role of bacteria in pyrene – a high-molecular-weight PAH – degradation the present study was conducted to isolate, identify and characterize the autochthonous bacterial diversity from two different PAH-contaminated sites with respect to growth and pyrene utilization capacities. 16S rDNA - RFLP analysis provided a picture of the genetic diversity in two microbial communities. The phylogenetic affiliations of bacterial isolates were determined using bioinformatics tools. The second objective was to gather information on bacterial catabolism of pyrene and elucidate the metabolic pathway. The rate at which pyrene uptake occurs was studied by physiological optimization studies, time course experiments and pulse chase experiments. Effect of pyrene on bacterial growth was determined. The results proved helpful in studying the biochemistry of pyrene metabolism in the most efficient pyrene utilizing isolate. The intermediate metabolites arising out of the degradation of pyrene were characterized by Gas Chromatograph coupled with Mass Spectrograph (GC-MS). Molecular level studies were conducted to determine the seat of pyrene metabolism and related genes in the bacterial cell of selected pyrene degrading isolates. Ring hydroxylating dioxygenases are reported to be involved in the first step of pyrene catabolism in several actinomycetes bacterial strains (Brezna *et al*., 2003). Therefore the third goal was to detect Rieske centers, the conserved [Fe_2-S_2] cluster binding region of terminal dioxygenase genes within the pyrene-utilizing bacterial genera *Bacillus*, *Pseudomonas* and *Rhodococcus*. With an understanding of the pathway reported in literature for pyrene degradation in *Mycobacterium* sp. (Kim *et al*., 2007) and biochemical data obtained above, a pathway for pyrene degradation in *Bacillus* sp. (PK-6) MTCC 1005 has been proposed. Finally soil studies in sterile garden soil and non-sterile coal-tar contaminated soil were performed to demonstrate the pyrene removal potential of selected bacterial isolate, suggesting its possible use and advantage in bioremediation of sites contaminated with crude oil, coal-tar and a mixture of PAHs. The various techniques followed to meet these objectives are outlined in Figure 4.1.

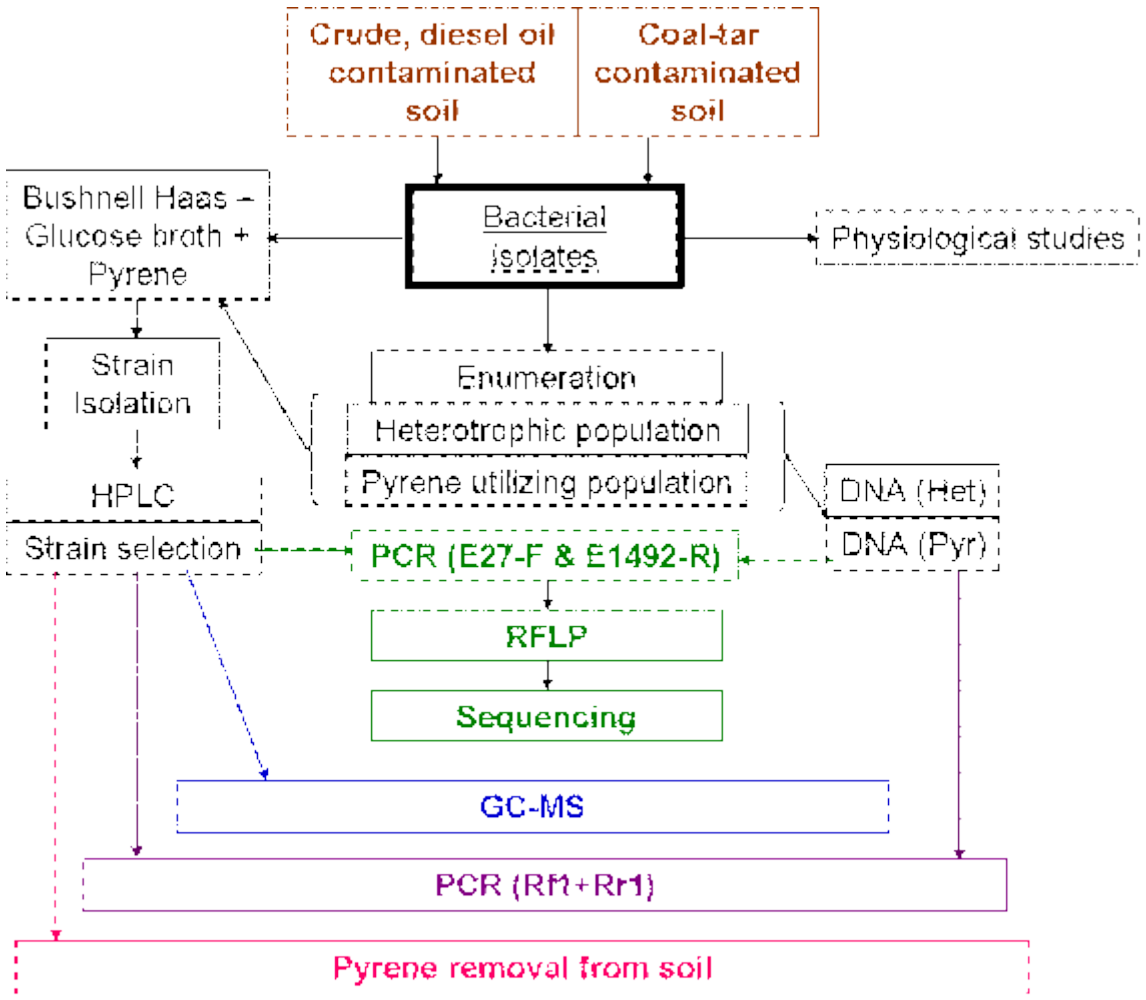


Figure 4.1 Flow chart diagram illustrating the protocols used in this study. For both samples types (bacterial consortia and soil isolates), biochemical, microbiological, and molecular analyses were done. DNA templates were extracted from strains cometabolizing pyrene. (a) Full-length 16S rDNA PCR fragments from the isolates were screened by restriction fragment length polymorphism (RFLP) analysis and different taxonomic units were sequenced. (b) Metabolic genes (plasmid / chromosome borne) were screened by rieske gene-specific PCR. (c) Pyrene utilization rates compared by HPLC and metabolic products identified by GC-MS. (d) Soil studies with *Bacillus* sp. (PK-6) MTCC 1005.

5.1 Biodiversity of pyrene degrading bacteria

Soil characterization

Coal-tar contaminated soil samples were collected from a 15 year old pre-mix industrial plant site, in addition to previously collected crude oil contaminated soil samples from refinery and diesel depot situated in Patiala, Punjab (India). Only coal-tar contaminated soil samples were physico-chemically characterized. It was observed that the soil samples collected from site 1-2 m away from coal-tar plant had more moisture content, higher pH and lower EC than soils collected from site 3-4 m away from the plant. Due to spillage of coal-tar during sampling in the proximity of the plant, lime was added regularly to soil therefore top soil (NP) showed strikingly high pH of 11.24. Also presence of high coal-tar impurity in soil near (1-2 m) plant served to increase the moisture content and decrease EC as compared to soil collected 3-4 m away from coal-tar plant. Aerobic, mesophilic and culturable bacteria were abundantly found in the sub soil samples, having high moisture content and mildly alkaline pH as compared to the top soils collected from the same area probably due to high pH of soil. These observations suggest that a correlation may exist between physico-chemical characteristics of PAH contaminated soil and its inherent bacterial activity (Trejo and Quintero, 2000). The alkaline pH of coal-tar contaminated soil samples can also be attributed to the land management practices, chemical composition, rates and time of application of coal-tar, lime addition and soil tillage, among others (Jacques *et al*., 2007). Nine PAHs, Benzo[*g,h,i*]perylene, Dibenzo[*a,h*]anthracene, Indeno[*1,2,3-c,d*]pyrene, Pyrene, Acenaphthylene, Fluorene, Phenanthrene, Benzo[*k*]fluoranthene and Benzo[*b*]fluoranthene were detected by gas chromatography (GC-FID) analysis in coal-tar contaminated sub soil samples which belonged to the class of "16 Priority Pollutants" (Yan *et al*., 2004).

Bacterial isolation from soil

Gradual enrichments of consortia CON-3 and THA-2 with increasing concentration of pyrene from 10 µg ml^{-1} upto 50 µg ml^{-1}, in steps of 10 µg ml^{-1}, and decreasing concentration of crude oil from 0.5 % upto 0.2 %, in steps of 0.1 %, in five months time period, were carried out to develop the HMW-aromatic hydrocarbon degradation phenotype and to selectively enrich the pyrene-utilizing bacterial isolates in. Enrichment methods used by Kanaly *et al*. (2002), Dagher *et al*. (1997), Juhasz *et al*. (1997) were compared for the isolation of pyrene-degrading bacteria from an industrially

contaminated soil by Gaskin and Bentham (2005). Enrichment with pyrene and the co-substrate diesel was the most successful method in terms of ability to select for diversity and abundance of culturable pyrene-degrading bacterial populations in liquid culture.

Twenty isolates were isolated from the two bacterial consortia CON-3 and THA-2, screened for growth in BHB medium containing three different concentrations (25, 50 and 75 μg ml^{-1}) of pyrene in 30 days at 30 °C. Pyrene concentration of 75 μg ml^{-1} was toxic to all these bacterial isolates and inhibited the growth even in the presence of 1.0 % (w/v) glucose. Pyrene concentration of 50 μg ml^{-1} in the BH medium supplemented with 0.5 % glucose was therefore used for molecular characterization studies. Bacterial isolates PK-12, PK-13 and PK-14 were identified by 16S rRNA gene analysis. Two hundred twenty-nine isolates from coal-tar contaminated soil were screened for their ability to grow in similar medium. Growth experiments demonstrated maximum growth and pyrene utilization by thirty isolates in BHB medium containing 25 and 50 μg ml^{-1} pyrene (in the presence of glucose). 16S rRNA gene digest patterns of thirty isolates were categorized into 13 unique groups, wherein members of a group had similar RFLP pattern for one restriction enzyme but different pattern from members of another group. Further one representative from each bacterial group, which showed maximum growth (OD 600 nm) in BHB liquid medium containing 50 μg ml^{-1} pyrene and 0.5 % (w/v) glucose, was chosen for further investigation. Thus, three bacterial isolates (members of CON-3 consortium) derived from crude oil contaminated soil, namely PK-12 to PK-14 (with optimum growth temperature 30 ^{o}C) and ten bacterial isolates from coal-tar contaminated soil, namely PK-1 to PK-5 (with optimum growth temperature 30 ^{o}C) and PK-6 to PK-10 (with optimum growth temperature 45 ^{o}C) capable of utilizing 50 μg ml^{-1} pyrene were identified by Gram staining and 16S rDNA cloning and sequencing To serve as control a non-pyrene degrader strain PK-11 (from THA-2 consortium) was also identified.

Bacterial characterization

Gram stain characterization showed one strain PK-3 to be Gram negative, while remaining isolates were Gram positive. This was confirmed by 16S rRNA gene cloning and sequence analysis results, whereby the Gram negative strain PK-3 belonged to *Pseudomonas* genera, one Gram positive strain PK-10 belonged to *Rhodococcus* genera and the remaining Gram positive pyrene-utilizing strains PK-1, PK2, PK-4 to PK-9 and

PK12 to PK14 belonged to *Bacillus* genera. The strain not able to utilize pyrene was identified as *Kocuria* sp. The study showed that members of Firmicutes (*Bacillus* sp.), Actinobacteria (*Rhodococcus* sp.) and Proteobacteria (*Pseudomonas* sp.) taxonomic clades were present in coal-tar contaminated soil, while Firmicutes (*Bacillus* sp.) and Actinobacteria (*Kocuria* sp.) taxonomic clades were present in crude oil contaminated soil. This observation is in agreement with reports of Calvo *et al*. (2002), Toledo *et al*. (2006), Zhou *et al.* (2006) and Rogers *et al.* (2007) which state that crude oil and coal-tar contaminated soils are huge reservoirs for PAH biodegrading bacteria belonging to Proteobacteria, Actinobacteria, Flavobacteria and Firmicutes.

Mostly the pyrene utilizing strains happen to be species of Gram-positive bacteria, *Mycobacterium* and *Rhodococcus* (Kanaly and Harayama, 2000). Heitkamp and Cerniglia (1988) reported the first *Mycobacterium* sp. PYR-1, from sediment near an oil field which could mineralize upto 63.0 % of the original amount of pyrene in two weeks in pure culture with organic nutrients. In soils found to mineralize pyrene, Grosser *et al*. (1991) isolated *Mycobacterium* sp. strain RJGII-135, which could mineralize 55 % pyrene within 2 days of reintroduction into soil following growth in pure culture on pyrene. Dean-Ross and Cerniglia (1996) isolated *Mycobacterium flavescens* that had the highest reported mineralization efficiency (62.6 %) of pyrene within 24 h. Walter *et al*. (1991) reported the first *Rhodococcus* sp. strain UW1 from contaminated soil at pH 7.0 and 30 ^{o}C. It mineralized 72 % of 500 µg ml^{-1} pyrene as sole source of carbon and energy within 2 weeks. *Bacillus* sp. is also reported to utilize pyrene (Kazunga and Aitken, 2000; Das and Mukherjee, 2007; Rogers *et al.,* 2007; Johnsen *et al.,* 2007; Lin and Cai, 2008) but the utilization potential and metabolic pathways have not been studied in depth. Kazunga and Aitken (2000) studied 4 strains, *Pseudomonas stutzeri* P16, *Bacillus cereus* P21, *Sphingomonas yanoikuyae* R1 and *Pseudomonas saccharophila* P15, isolated from PAH-contaminated soils by enrichment on phenanthrene as a sole carbon source. The strains could transform pyrene to non-mineral products. *Bacillus cereus* Py5 and *Bacillus megaterium* Py6, isolated from a PAH-degrading microbial consortium enriched from the sediment samples of Huian mangroves, China (Lin and Cai, 2008), were reported to degrade 65.8 % and 33.7 % of pyrene (50 µg ml^{-1}) within three weeks, respectively. *Bacillus subtilis* DM-04 has been reported by Das and Mukherjee (2007) to assimilate 48 % of 2500 µg ml^{-1} pyrene to cellular carbon post 4 days of growth at 55 ^{o}C incubation temperature.

All the low molecular weight (LMW) PAH dioxygenase genes were in Gram-negative bacteria, while the high molecular weight (HMW) PAH dioxygenase genes were in Gram-positive strains was suggested by Habe and Omori (2003). Our findings are in agreement as twelve pyrene utilizing isolates, PK-1 (*Bacillus firmus*), PK-2 (*B. cereus*), PK-4 (*B. cereus*), PK-5 (*B. subtilis*), PK-6 (*B. licheniformis*), PK-7 (*Bacillus* sp.), PK-8 (*B. pumilus*), PK-9 (*B. firmus*), PK-10 (*Rhodococcus rhodochrous*), PK-12 (*B. pumilus*), PK-13 (*B. flexus*) and PK-14 (*B. firmus*), isolated from crude oil and coal-tar contaminated soils, belong to Gram-positive category. Members of the genus *Bacillus* have been used in past studies for PAH biodegradation (Das and Mukherjee, 2007; Jacques *et al*., 2007; Lin and Cai, 2008; Toledo *et al*., 2006). Toledo *et al*. (2006) have attributed *Bacillus* strains with the property to colonize environments contaminated with PAHs. They isolated eight strains of *B. pumilus* from solid waste crude oil capable of growth in the presence of PAHs like naphthalene, phenanthrene or pyrene as sole carbon source. Another *B. pumilus* strain, isolated by enrichment from soil sample in Japan, has been described by Widada *et al*. (2002a), as being capable of utilizing PAHs. It could grow on naphthalene as a sole carbon source but not on phenanthrene. Calvo *et al*. (2002) isolated *B. pumilus* strains capable of growing in the presence of pyrene. Another *B. pumilus* strain has been isolated from waste electrical transformer oil by De Sisto *et al*. (2008). *B. flexus* is not reported to be involved in the degradation of any polycyclic aromatic hydrocarbons till date. However, polypropylene degradation in minimal medium with *B. flexus*, as part of mixed soil culture, for 12 months has been reported by Arkatkar *et al*. (2009). Mohamed *et al*. (2006) have isolated *B. firmus* as bacterial degraders of petroleum hydrocarbons from contaminated soils in Kuwait. This bacterium is also reported to reduce Cr (VI) from industrial effluents (Sau *et al*., 2008).

5.2 Elucidate the metabolic pathway of pyrene degradation in a selected isolate

Pyrene uptake

Bacterial consortium CON-3, isolated from soil contaminated with refinery waste crude oil, was able to utilize 19 % more pyrene as compared to consortium THA-2, isolated from soil contaminated with diesel from a depot in Patiala, Punjab (India) in 30 days of growth. Both consortia could grow on pyrene only in the presence of glucose.

Bioremediation techniques often include the addition of supplements to enhance biodegradation of the PAH by inoculated microorganisms (Carmichael and Pfaender, 1997b). Sometimes PAHs are degraded only in the presence of an additional co-substrate, sometimes another PAH, by cometabolism or by stimulation of biodegradation (Bouchez *et al*., 1999; Kanaly *et al*., 2000). Easily metabolizable, organic carbon sources (such as glucose) may play an important role in promoting the growth of a particular group of degraders (Wong *et al*., 2002; Lee *et al*., 2003). Minerals and vitamins are required for the growth and activity of bacteria. These essential nutrients might not be present at the contamination site, and a lack of any of them will inhibit the growth or reproduction of the microbes. They must be added to the site to assure the fastest, most efficient waste digestion (Mellor *et al*., 1996). Media enrichment with trace elements and vitamins stimulated pyrene uptake in 30 days to 58 % and 49 % by consortia CON 3 and THA-2, respectively, both in the absence or presence of glucose. BHB medium with double glucose concentration of 1.0 % (w/v) stimulated the pyrene uptake to 63 % and 56 % by consortia CON 3 and THA-2, respectively. This result favoured the absolute requirement of glucose for maximum utilization of pyrene. Therefore growth of both consortia on pyrene, in presence of glucose carbon source, is cometabolic in nature. In the recent past many reports on the use of PAHs as a growth substrate in the presence of glucose by degrading bacteria are available. De Sisto *et al*. (2008) used basal medium supplemented with 1 % yeast peptone glucose (YPG) and 1 % waste electrical transformer oil (ETO) to grow bacterial strains and check ability to use waste ETO as a sole carbon source. Tao *et al*. (2007) noted that addition of glucose at concentration 0.001 to 0.02 % in minimal medium, as second organic chemical, could enhance the cell growth of *Sphingomonas* sp. GY2B on 100 µg ml^{-1} phenanthrene. However glucose concentration in medium > 0.05 % inhibited the growth of strain GY2B. In contrast we have found that glucose concentration, as high as 1.0 % exerted a positive and stimulatory effect on pyrene utilization, by both consortia. Das and Mukherjee (2007) observed enhancement of pyrene utilization and growth of bacteria upon addition of 0.01 % glucose in the growth medium.

Time course and Pulse chase experiments

In accordance with observed pyrene uptake abilities of respective consortia, the bacterial isolates from CON-3 consortium had higher ability to uptake pyrene than member isolates of THA-2 consortium. PK-12 isolate of CON-3 consortium, identified as *Bacillus pumilus*

(PK-12) showed 98 % utilization of 25 µg ml^{-1} pyrene in BHB medium supplemented with 0.25 % glucose, while PK-27 (not identified) isolate of THA-2 consortium showed as high as 53 % utilization of pyrene in same medium. These results confirm the belief that soils contaminated with crude oils from refinery and diesel depot serve as abode for pyrene degrading bacteria (Matthew and Amund, 2000; Calvo *et al*., 2002; Toledo *et al*., 2006; Hamamura *et al*., 2006). However it may also be noted that as the source of contamination varies the bacterial diversity present in soil also varies. Communities of bacteria enriched from refinery waste contaminated soil are richer in bacteria capable of high / efficient pyrene uptake and utilization and therefore may be more adapted to HMW PAH degradation. A total of 8 bacterial isolates, five from the CON-3 consortium, namely PK-12 (98 %), PK-13 (61 %), PK-14 (55 %), PK-15 (54 %) and PK-16 (51 %), and three from the THA-2 consortium, namely PK-23 (45 %), PK-24 (39 %), PK-27 (53 %), showed more than 35 % uptake of 25 µg ml^{-1} pyrene. When these strains were exposed to a higher concentration of pyrene, i.e., 50 µg ml^{-1}, the percentage of pyrene utilization in 35 days decreased from 98 % to 64 % for isolate PK12, from 61 % to 55 % for isolate PK-13, from 55 % to 53 % for isolate PK-14, from 54 % to 50 % for isolate PK-15, from 51 % to 47 % for isolate PK-16, from 45 % to 17 % for isolate PK-23, from 39 % to 30 % for isolate PK-24 and from 53 % to 49 % for isolate PK-27. In addition to time course studies, attempts were made to study the hourly uptake pattern for pyrene by selected eight isolates in pulse chase experiments. It was observed that log phase bacterial cells grown in nutrient rich (LB) medium found pyrene molecule easy to uptake, while it is tough for minimal (BHB) medium grown cells and LB grown cells which have been starved overnight in 10 mM phosphate buffer (pH 7.0 + 0.2), to uptake pyrene as substrate. Bacterial cells of isolates PK-12, PK-13, PK-15, PK-24 and PK-27 showed uniform pyrene uptake among the 8 isolates. Isolates PK-12, PK-13 and PK-14 showed maximum uptake rates, 11 %, 13 % and 8 %, respectively. As these utilization rates were very low pyrene utilization appeared to be a slow mechanism and was subsequently studied in longer intervals of time. The isolates PK-12, PK-13 and PK-14 capable of more than 50 % pyrene uptake in 35 days, were selected for further studies.

Effect of pyrene on growth

In order to test the toxicity of pyrene to *Cycloclasticus* sp. bacterial cell growth, an overwhelming amount of pyrene had been added, upto 20 g l^{-1}. However, no growth inhibition was reported (Wang *et al*., 2008). In present study also the effect of pyrene

addition to growing culture of bacterial isolate PK-12, identified by 16S rDNA analysis as *Bacillus pumilus* (PK-12), was tested spectrophotometrically. Growth results obtained suggest that pyrene addition in mid-log phase to growing bacterial cells did not inhibit the cell growth. Though pyrene did not support growth as sole carbon and energy source, in glucose supplemented BHB medium pyrene addition led to enhanced growth as compared to its non-addition. *Bacillus subtilis*, isolated from PAH contaminated soil (Hunter *et al*., 2005) is reported to be capable of transforming approximately 40 % of 20 µg ml^{-1} pyrene in four-day incubation period at 30 ^{o}C. Growth physiology results suggested that the growth of *B. subtilis* was not affected by 20 µg ml^{-1} pyrene because its growth in presence of pyrene was almost identical to that in minimum medium alone.

Effect of glucose on pyrene utilization by bacteria

Media supplementation experiments with pyrene utilizing bacterial consortia CON-3, THA-2 and bacterial monocultures PK-1 to PK-14 showed increase in cell growth of isolates and total protein in the cultures. Glucose supplementation (@ 1.0 %; w/v) in BHB medium favoured growth and pyrene metabolic activity in all isolates capable of pyrene utilization. The presence of glucose, as a co-substrate, has been shown to enhance degradation of pyrene in liquid medium and soil slurry (Das and Mukherjee, 2007). Glucose is a versatile precursor for many biomolecules and acts as a growth inducer and co-source of carbon, energy and reducing power for microbes that leads to substantial increase in bacterial biomass with a corresponding increase in pyrene degradation (Das and Mukherjee, 2007). Addition of glucose resulted in considerably more growth (at least one order increase) of mesophiles (Mohammed *et al*., 2006). Glucose was found to increase the biomass level of enriched culture by Tian *et al.* (2003). Studies by Rentz *et al*. (2004) suggest that preferential utilization of a simple carbon source represses the degradation of complex compounds, leading to their accumulation in nature, thereby aggravating pollution. However Basu *et al*. (2006) reported cometabolic growth on aromatic compounds plus glucose in *Pseudomonas putida* CSV86, with utilization of aromatics in the first log phase and glucose in the second log phase. The present study results are in partial agreement with Basu *et al*. (2006) as the bacterial consortia cometabolize pyrene only in presence of glucose and glucose does not suppress pyrene utilization. The bacteria that have been reported to carry out cometabolic reaction include species of *Pseudomonas, Acinetobacter, Nocardia, Bacillus, Mycococcus, Methylosinus* and *Arthrobacter* (Churchill *et al*., 1999; Boonchan *et al*., 2000).

Bacillus spp. are capable of using numerous carbohydrates, but glucose is the most preferred carbon source (Stulke and Hillen, 2000) and often represses the expression and activity of catabolic systems that enable the utilization of secondary substrates. Our results however support that glucose supplementation does not suppress the activity of degrading enzymes, which are induced upon addition of aromatic compound (Basu *et al*., 2006). *Bacillus subtilis* DM-04 assimilated 48 % of 2500 µg ml^{-1} pyrene as sole carbon source post 4 days of growth at 55 ^{o}C (Das and Mukherjee, 2007). Pyrene utilization enhanced with addition of 100 mg l^{-1} glucose and the growth of bacteria in pyrene and glucose medium was significantly higher ($P < 0.01$) compared with growth on individual (pyrene or glucose) carbon source. *Mycobacterium* sp. B1, *Microbacterium* sp. B21 and *Porphyrobacter* B51 isolates degraded PAH more effectively in cultures when the aqueous phase contained Bushnell-Haas (BH) mineral salt medium and soil extract (Gauthier *et al*., 2003). Trypticase soy broth (TSB) was the richest and the least effective medium. Rich media probably inhibit PAH degradation at a genetic level (Guerin and Jones, 1988a, b) or because other easily degradable substrates are preferentially used by the microorganisms (Keuth and Rehm, 1991). However, *Bacillus pumilus* B44 was different and PAH degradation was more effective in the richest media, BH agar containing 0.05 % yeast extract (BHY) and TSB (Gauthier *et al*., 2003).

Of all the isolates tested, two *Bacillus* strains (PK-6 and PK-7) removed the greatest (56 % and 53 %, respectively) amount of pyrene, followed by *Rhodococcus* sp. (PK-10) and *Bacillus* sp. (PK-12) removing 46 % of pyrene while *Pseudomonas* sp. (PK-3) removed only 25 % of pyrene. *Bacillus* sp. (PK-9) removed the minimum amount (9.8 %) of pyrene. This diversity could be directly correlated to the contribution of these isolates to degradation of components of coal-tar and crude oil in soil. Dominance of Gram positive bacteria such as *Bacillus* and *Rhodococcus* rather than Gram negative *Pseudomonas* bacteria, which generally rapidly colonize in substrate rich conditions, is reflective of the unique ability of these strains in adaptation and utilization of a variety of PAHs under resource poor conditions. Such populations are generally more stable (Margesin *et al.,* 2007) and metabolize more varieties of PAH compounds than Gram-negative isolates, particularly HMW PAHs (Zhou *et al.,* 2006). The high abundance of *Bacillus* sp. among identified strains (Figure 3.15, 3.16) from coal-tar and crude oil contaminated soil and the greater tendency to remove pyrene from minimal growth medium (Table 3.9 F), among

all pyrene-utilizing isolates from coal-tar and crude oil contaminated soils, indicated that bacteria belonging to Firmicutes taxa could be important pyrene degraders *in situ*, in contrast to Actinobacteria and Proteobacteria. Das and Mukherjee (2007) have compared utilization potential of pyrene as the sole source of carbon by *Bacillus subtilis* DM-04 strain and *Pseudomonas aeruginosa* M and NM strains. *Bacillus subtilis* exhibited higher utilization and cellular assimilation of pyrene (48 %) compared with *P. aeruginosa* strains (32 %). It is interesting to observe that a similar utilization pattern was found among *Bacillus* sp. (PK-6, PK-7, PK-12, PK-5, PK-1, PK-14, PK-4 and PK-8 in decreasing order of pyrene utilization) with more pyrene uptake than *Pseudomonas* sp. (PK-3).

Biosurfactant activity

Biosurfactant activity was detected in 13 isolates concomitant with pyrene uptake (Figure 3.24 A). The Mobil oil emulsification index (A_{550}) for *Bacillus* sp. (PK6 and PK7), having first and second highest pyrene uptake capacities (56 % and 53 %), was 1.96 and 1.61, respectively. *Bacillus* sp. (PK8, PK4, PK14, PK1, PK5, PK12) and *Rhodococcus* sp (PK10) showed moderate 25-50 % (27, 33, 37, 41, 44, 46 and 46 %, respectively) pyrene uptake and medium biosurfactant activity ($1 > A_{550} > 1.5$; i.e. 1.33, 0.97, 1.54, 1.01, 1.02, 1.11 and 1.45, respectively). *Bacillus* sp. (PK9, PK13, PK-2) and *Pseudomonas* sp. (PK-3) having 10 – 25 % (10, 19, 21 and 25 %, respectively) pyrene uptake potential showed too low biosurfactant activity ($A_{550} < 1$; i.e. $A_{550} = 0.66$, 0.55, 0.72 and 0.43, respectively). The results suggest that a positive correlation exists between the production of biosurfactants and uptake of pyrene from the culture medium by bacterial isolates. In similar studies microbial growth on hydrocarbons has been found to be associated with the production of biosurfactants (Deziel *et al.,* 1996). They observed that in the presence of naphthalene, bacteria produced biosurfactants which promoted the solubility of naphthalene. Although Gutierrez-Rojas (2000) have stated that production of biosurfactants does not assure biodegradation, yet there are reports wherein biosurfactant production may be considered as part of the metabolism in indigenous bacteria in contaminated sites so as to create a favourable local environment. In one such study Das and Mukherjee (2007) have shown that biosurfactants secreted by *B. subtilis* DM-04, *P. aeruginosa* M and NM strains enhanced the apparent solubility of pyrene 5- to 7- fold, which resulted in its higher uptake and assimilation by the bacteria.

It is well known that microorganisms growing on hydrocarbons frequently produce biopolymers with emulsifying or surfactant activity (Toledo *et al*., 2006) so as to improve their ability to utilize these compounds (Ron and Rosenberg, 2002). In this study, emulsification assays were carried out to know the capacity of the genetically identified, pyrene utilizing bacteria to produce biosurfactant activity (BA). At the end of the assay, emulsification of Mobil oil resulted from the presence of biosurfactant activity in the culture medium. And this may be the reason that biosurfactant activity corresponded to the change in pyrene concentration in four days. The dynamics of biosurfactant activity in eight out of 13 pyrene metabolizing monocultures was consistent with that of pyrene concentration change, indicating that the pyrene uptake by the bacterial isolates *Bacillus* sp. PK-6, *Bacillus* sp. PK-7, *Rhodococcus* sp. PK-10, *Bacillus* sp. PK-12, *Bacillus* sp. PK-5, *Bacillus* sp. PK-1, *Bacillus* sp. PK-4 and *Pseudomonas* sp. PK-3 may be correlated or associated with the biosurfactant activity (Table 3.9 F). Further in these bacteria maximum PU is achieved after attaining BA maxima in the growth medium. Das and Mukherjee (2007) have also observed biosurfactant production in culture medium of *B. subtilis* DM-04 and two *P. aeruginosa* strains while growing on pyrene as sole carbon source. The biosurfactants secreted were believed to enhance the solubility of pyrene in aqueous media resulting in a higher uptake and utilization of pyrene (Das and Mukherjee, 2007). The increased PU rates and the emulsification capacities of the bacterial isolates *Bacillus pumilus* (PK-12) and *Bacillus firmus* (PK-14) indicate that they can be used for biotreatment and bioaugumentation of soils contaminated with PAHs.

Biosurfactants are compounds that affect interactions among pollutants, water and micro-organisms and may overcome the problem of limited bioavailability of hydrophobic PAHs (Barkay *et al.,* 1999), thereby sustaining bacterial growth (Das and Mukherjee, 2007). Bacteria, especially *Bacilli*, are known to have capabilities to produce bioactive compounds in growth medium which help to increase the dissolution rates of partially soluble or insoluble carbon sources (Das and Mukherjee, 2007). Jacques *et al*. (2007) have used an emulsification index for diesel oil to estimate surfactant activity produced by six bacterial isolates and one fungus isolate enriched from a petrochemical landfarm site and capable of utilizing a variety of PAHs like naphthalene, phenanthrene, anthracene and pyrene. Emulsion formation with or without the microbial cells, indicated the production and excretion of high-molecular-weight biosurfactants to the growth environment. In another report Barkey *et al*. (1999) routinely tested for emulsifying

activity of alasan preparations of *Acinetobacter radioresistens* KA53 in small volumes by measuring emulsion formation spectrophotometrically at 600 nm. In our study, biosurfactant activity was spectrophotometrically measured in terms of degree of emulsification of Mobil oil upon vigorous vortexing with the culture supernatant. The method used was found useful in predicting the preliminary mechanisms employed by bacteria, which helped to make PAHs bioavailable in aqueous medium. Our proposed assay for biosurfactant activity is quite practical and convenient to use, since it permits preliminary prediction of production of extracellular biopolymers with biosurfactant or bioemulsifier activities by bacteria on the basis of a simple and rapid test. The nature and role of these substances may be explored separately by established procedures, beyond the objectives of this study. This procedure could serve as a simple and rapid method for qualitative selection of bacterial isolates possessing ability to emulsify solid and water insoluble PAHs like pyrene.

Pyrene degradation

The strain best adapted to degrading pyrene, *Bacillus* sp (PK-6), was selected for identification of pyrene degradation products. The intermediate metabolites arising out of the uptake and assimilation of pyrene by the *Bacillus* sp. (PK6) were analyzed by Gas Chromatograph coupled with Mass Spectrograph (GC-MS). The fragmentation patterns obtained matched with standard mass spectra of compounds pyrene, Phenanthrene, 9-Methoxyphenanthrene, Diisooctylphthalate, 5,6,7,8-Tetrahydro-1naphthoic acid, 1,6,7-Trimethyl-naphthalene and 9,10-Diphenylphenanthrene. A gradual decrease in the residual pyrene concentration in growth medium along with formation of these six compounds suggests that pyrene is degraded by *Bacillus* sp. (PK-6). As the culture prior to extraction was neither heated nor sonicated therefore residual pyrene and its degradation products are extracellular. It has been suggested by Vila *et al.* (2001) that some "dicarboxylic acid compounds diffuse into the medium" and accumulate (Lopez *et al*., 2006). Cottin and Merlin (2007) showed "a transitory accumulation of three metabolites" in culture supernatants of *Mycobacterium* sp. 6PY1. In fact, the level of metabolites increased and gradually decreased thereafter. It was concluded that "the enzymes catalyzing PAHs have slow kinetics", which could be the justification for extracellular appearance of pyrene intermediates and products. In another study on the effect of pH on phenanthrene and pyrene degradation, Kim *et al*. (2005) have stated that "an acidic pH (of 6.5) seemed to render the mycobacterial (*M. vanbaalenii* PYR-1) cells

more permeable to hydrophobic (PAH) substrates", in comparison to pH 7.5. This principle of high cell permeability at acidic pH is the basis for liquid-liquid extraction procedure in our study.

Pyrene metabolism has been predominantly and extensively studied in *Mycobacterium* sp. and to lesser extents in *Rhodococcus* sp., *Pseudomonas* sp. and *Sphingomonas* sp. (Kanaly and Harayama, 2000). Bacterial pyrene metabolites identified to date by multiple analyses, including UV, infrared, mass, and NMR spectrometeries and gas chromatography are pyrene *cis*- and *trans*-4,5-dihydrodiol, 4-phenanthroic acid, pyrenol (1-hydroxypyrene), 4-hydroxyperinaphthenone, phthalic and cinnamic acids, 4,5-dihydroxypyrene, phthalate 3,4-dihydrodiol, 3,4-dihydroxyphthalate, β-ketoadipate in *Mycobacterium vanbaalenii* PYR-1 (Heitkamp *et al*., 1988b; Kim *et al*., 2007), protocatechuic acid in *Mycobacterium* sp. BB1 (Fritsche, 1994), 3,4-dihydroxyphenanthrene in *Pseudomonas* sp. XPW2 (Zylstra *et al*., 1994), 4,5-phenanthrene-dicarboxylic acid in *Mycobacterium* sp. RJGII-135 (Schneider *et al*., 1996) and *M. flavescens* (Dean-Ross and Cerniglia, 1996), *cis*-3,4-dihydroxyphenanthrene-4-carboxylate, 1-hydroxy-2-naphthoic acid, 2-carboxybenzaldehyde in *Mycobacterium* sp. KR2 (Rehmann *et al*., 1998), 6,6'-dihydroxy-2,2'-biphenyl dicarboxylic acid in *Mycobacterium* sp. AP1 (Vila *et al*., 2001), and pyrene-4,5-dione in *Mycobacterium* sp. KMS (Liang *et al*., 2006).

Reports on formation of metabolic intermediates of pyrene by *Bacillus* sp. are meagre. Kazunga and Aitken (2000) have shown *B. cereus* P21 to play an active role in pyrene metabolism when grown in minimum salts buffer. It transformed pyrene only into the first intermediate in aerobic degradation i.e., *cis*-4,5-Pyrene dihydrodiol. Further *Pseudomonas* sp. P15 and *Sphingomonas* sp. R1 metabolized *cis*-4,5-Pyrene dihydrodiol into pyrene-4,5-dione. Hunter *et al*. (2005) and Das and Mukherjee (2007) have isolated pyrene degrading *Bacillus subtilis* strains from PAH-contaminated soils but products of pyrene degradation by members of *Bacillus* genera have been reported by none. This is the first report of formation of five new intermediates during pyrene metabolism (degradation) by *Bacillus* sp. (PK-6) MTCC 1005.

Bacillus sp. can use pyrene, directly or cometabolically, therefore are considered as efficient PAH degraders (Hunter *et al.,* 2005) and important producers of biosurfactants,

which enhance biodegradation of aromatic compounds (Toledo *et al.,* 2006). Our results support considerable potential of *Bacillus* sp. in environmental and biotechnological applications of PAH biodegradation.

5.3 Genetic regulation of pyrene degradation in the selected isolate

Seat of pyrene metabolism in baceria

To get a comprehensive picture of pyrene degradation molecular level studies were conducted. The seat of pyrene metabolism and related genes in the bacterial cell of selected pyrene degrading isolates was established. No high-molecular-weight plasmid or low-molecular-weight plasmid could be isolated from the pyrene degrading strains. Therefore genes regulating pyrene metabolism are presumed to be chromosomally borne.

The PAH-degrading genes or gene clusters have been demonstrated to be located in the chromosome or plamids (Menn *et al*., 1993; Kiyohara *et al*., 1994; Cho and Kim, 2001; Zhang *et al*., 2003). Menn *et al*. (1993) reported that the naphthalene plasmid degradative enzyme system in *Pseudomonas fluorescens* strain 5R is involved in the degradation of anthracene and phenanthrene to naphthoic acids. Kiyohara *et al*. (1994) isolated and cloned a 25-kb DNA *Sal*I fragment from the chromosomal DNA of *Pseudomonas putida* OUS82, capable of utilizing phenanthrene and naphthalene. This fragment cloned in *E. coli* was found to harbour genes encoding enzymes that catalyze the upper pathway of naphthalene degradation. In a PAH degrading *Sphingomonas* sp. strain KS14 isolated from soil, Cho and Kim (2001) were able to detect a large degradative plasmid (>500 kb) responsible for the degradation of phenanthrene, naphthalene (as sole source of carbon and energy) and pyrene (cometabolically using phenanthrene as secondary growth substrate). A pyrene-degrading plasmid has been identified from a PAH-degrading bacterial strain ZL5, which was isolated from oil-contaminated soil of Liaohe Oil Field, China (Zhang *et al*., 2003). Pyrene-degrading plasmids were obtained from the PAH-degrading consortium YL by Lin and Cai (2008) but they were not able to report the same in member isolates *Bacillus cereus* and *Bacillus megaterium*. Therefore a solid foundation for the isolation of genes or gene clusters to uncover the metabolism pathways of pyrene degradation in bacterial systems other than mycobacteria is yet to be laid.

Pyrene metabolic gene

A ring hydroxylating dioxygenase gene is reported to be involved in the first step of pyrene catabolism in *Mycobacterium* sp. (Khan *et al*., 2001, Brezna *et al*., 2003, Krivobok *et al*., 2003, Sho *et al*., 2004; Kim *et al.,* 2006). The PAH dioxygenase genes reported till date include *nahAc* (Hedlund *et al.,* 1999; Wilson *et al.,* 1999; Marlowe *et al.,* 2002 ; Baldwin *et al.,* 2003), *phnAc* (Lloyd-Jones *et al*., 1999; Widada *et al*., 2002a, b), *pdoA2* (Krivobok *et al*., 2003), *pdoA/X* (Sho *et al*., 2004), *phtAa/phtA1* (Stingley *et al*., 2004b; Kim *et al*., 2007), *nidA* (Khan *et al*., 2001; Stach & Burns, 2002; Brezna *et al*., 2003; Kim *et al*., 2004a, 2007), *nidA3* (Kweon *et al*., 2007; Kim *et al*., 2006, 2007). Bacterial dioxygenases are enzymatic systems consisting of ferredoxin, ferredoxin reductase, and a terminal ring-hydroxylating dioxygenase composed of large [α] and small [β] subunits (Cerniglia, 1992). The [α] subunit of the terminal dioxygenase is more conservative than other components and is thought to be critical for substrate recognition (Kauppi *et al*., 1998). Evaluating the particular gene expressing this subunit could improve our understanding of the genetics of PAH-degrading bacteria and assist in monitoring active microbial communities during bioremediation (Habe & Omori, 2003; Dionisi *et al*., 2004). Keeping this point in mind, the present objective aimed at detecting Rieske centers, the conserved [Fe_2-S_2] cluster binding region of terminal dioxygenases within the genera *Bacillus*, *Pseudomonas* and *Rhodococcus*. Designed degenerate primers for Rieske centres in pyrene dioxygenase gene *nidA* from *Mycobacterium vanbaalenii*, GenBank accession number AF249301 (Brezna *et al*., 2003). Attempts to amplify Rieske gene from bacterial isolates were not successful. Further no pyrene metabolic genes have been reported in *Bacillus* sp. till now. More sophisticated studies may be pursued in this regard.

Biochemical pathway of pyrene degradation

From the various earlier mentioned reports on pyrene degradation, essentially in *Mycobacterium* species, it has been established that pyrene is initially hydroxylated on the C4 and C5 positions (K region) to give pyrene *cis*-4,5-dihydrodiol (Heitkamp *et al*., 1988b), followed by dehydrogenation and *ortho* cleavage to give phenanthrene 4,5-dicarboxylic acid (Dean-Ross and Cerniglia, 1996; Schneider *et al*., 1996; Vila *et al*., 2001). This intermediate is further decarboxylated to 4-phenanthroate (Heitkamp *et al*., 1988b; Krivobok *et al*., 2003). These intermediates of pyrene to phenanthrene conversion, as reported in *Mycobacterium* sp., were not detected in our study. The subsequent

intermediate in *Mycobacterium* sp., *cis*-3,4-dihydroxyphenanthrene-4-carboxylate, is produced by a second dioxygenation reaction (Rehmann *et al*., 1998). Rearomatization then formed 3,4-dihydroxyphenanthrene, which is subsequently decomposed either to cinnamic acid (Dean-Ross and Cerniglia, 1996) or to 1-hydroxy-2-naphthoate (Krivobok *et al*., 2003; Kim *et al*., 2007). A different naphthoate, namely 5,6,7,8-tetrahydro-1-naphthoic acid was identified in our study during incubation with *Bacillus* sp. (PK-6). In *Mycobacterium* sp. the next enzymatic reaction included intradiol ring cleavage dioxygenation resulting in the production of phthalic acid (Kim *et al*., 2007). It is at this important step when similarity can be seen in pyrene metabolite formation in *Mycobacterium* sp. and *Bacillus* sp. Both *Mycobacterium vanbaalenii* PYR-1 (Kim *et al*., 2007) and *Bacillus* sp. (PK-6) (our study) degraded pyrene to central intermediates through *o*-phthalate metabolite. Further biochemical mechanisms are little known in members of the genus *Mycobacterium*. But in other Gram-positive bacteria *Rhodococcus opacus* 1CP (Eulberg *et al*., 1998) and *Streptomyces* sp. 2065 (Iwagami *et al*., 2000) it is known that phthalate gets metabolized to protocatechuate, which is further transformed via the β-ketoadipate pathway (Ornston and Stanier, 1966) to tricarboxylic acid (TCA) cycle intermediates (Kim *et al*., 2008). Therefore, we assume that *Bacillus* sp. (PK-6) likely utilizes the same pathway for the utilization of phthalate intermediate.

With an understanding of the pathway reported for pyrene degradation in *Mycobacterium vanbaalenii* PYR-1 (Kim *et al*., 2007) and biochemical data obtained in current research work, a pathway for pyrene degradation in *Bacillus* sp. (PK-6) has been proposed (Figure 3.30). The breakdown starts with conversion of pyrene to phenanthrene which is successively transformed to 9-methoxyphenanthrene and 9,10-diphenylphenanthrene. The intermediates of pyrene to phenanthrene conversion like pyrene *cis*-4, 5-dihydrodiol and 4, 5-dihydroxypyrene, as reported by Kim *et al.* (2007) in *Mycobacterium vanbaalenii* PYR-1 were not detected in our study. The compound 9-methoxyphenanthrene is further metabolized to 5,6,7,8-tetrahydro-1-naphthoic acid, leading to the formation of 1,6,7-trimethyl-naphthalene. This compound by a series of unknown steps gets converted to phthalate which is believed to be further transformed to TCA cycle intermediates (Kim *et al.,* 2007). It is quite possible that 9,10-diphenylphenanthrene is a dead end metabolite which is then not further utilized. Dean-Ross and Cerniglia (1996) have also proposed that a dioxygenase was capable of hydroxylating pyrene at 1,2- position, which led to the formation of 4-hydroxyphenanthrenone and its further metabolism was terminated.

Huesemann (1997) suggested that incomplete biodegradation of non-ionic organic contaminants is caused by bioavailability limitations and also by the inherent resistance of certain compounds to break down in the micro-environment. The latter may have toxic effect on microbial populations, thereby leading to dead end pathways (Gutierrez-Rojas, 2000).

5.4 Removal of pyrene by selected bacterial isolate from soil contaminated with pyrene

The bacterial isolate *Bacillus* sp. (PK-6) adjudged in this study to be the most efficient pyrene utilizer and degrader among a community of 14 bacterial isolates from crude oil and coal-tar contaminated soils could be used for practical field application for effective *in situ* bioremediation of pyrene-contaminated sites. To validate this statement we undertook soil studies.

A variety of methods are currently employed for the remediation of PAH-contaminated soils. **Natural attenuation** is one such method, whereby naturally occurring physical, chemical, and biological processes are monitored but otherwise allowed to govern contaminant removal without human intervention (Jones *et al*., 2008). **Bioaugmentation** is the addition of microorganisms with specific degradative capacities for enhanced bioremediation as compared to natural attenuation (Juhasz, 1998). Bioaugmentation is especially important for sites containing high PAH concentrations, sites which contain a significant proportion of high molecular weight PAHs and for recently polluted soils which do not have an adapted microbial community (Juhasz, 1998). If the speed of decontamination is a prime factor, the addition of a microbial community with known degradative capabilities can be used to start the remediation process with little or no lag period (Forsyth *et al.,* 1995). **Biostimulation** encourages the growth of indigenous microorganisms acclimated for the removal of specific contaminants through the addition of nutrients or other amendments to increase rates of contaminant degradation (Carmichael and Pfaender, 1997b; Hwang and Cutright, 2002; Roling *et al*., 2002; Vinas *et al*., 2005). For example, *ex situ* biostimulation in slurry (S)-phase bioreactors has proven effective in PAH-contaminated soil remediation (Huesemann *et al*., 2004; MacLeod and Daugulis, 2003; Mueller *et al*., 1991b; Singleton *et al*., 2005).

Pyrene removal from coal-tar contaminated soil

In the coal-tar contaminated soil experiment we investigated natural attenuation and the effect of reinoculation (bioaugmentation) treatment methods on pyrene removal in composite coal-tar-contaminated soil from a coal-tar pre-mix plant over a time period of 4 weeks. Further the significance of *Bacillus* sp. (PK-6) MTCC 1005 reinoculation in the soil system from which it was isolated over indigenous microbial community was evaluated. The pH of soil after 28 days of incubation remained constant in both soil treatments, irrespective of uninoculation or inoculation with *Bacillus* sp. (PK-6) culture. Perhaps soil contaminated with coal-tar has buffering agents, which prevent any change in pH upon microbial activity in soil over 28 days of incubation. Uninoculated and inoculated coal-tar contaminated soils both showed loss of pyrene within 4 weeks of incubation. Pyrene removal of 58 % was observed in soil treatment inoculated with *Bacillus* sp. (PK-6). In addition, the indigenous bacteria present in the nonsterile soil were also responsible for the loss of 57 % pyrene seen in the uninoculated soil treatments. The *Bacillus* sp. (PK-6) inoculated soil treatments did not show extra loss of pyrene, indicating that reinoculation of strain PK-6 (**bioaugmentation)** into source coal-tar contaminated soil did not enhance pyrene transformation over the indigenous microflora. This result is in contrast to report of Kastner *et al*. (1998) in which bacterial degraders were added to soil contaminated with PAHs and a six fold increase in the degradation of pyrene was observed.

No lag phase in pyrene removal was observed in any of the two soil incubations probably because both incubations had high count of indigenous pyrene utilizing microflora with high pyrene removal rates irrespective of reintroduction of *Bacillus* sp. (PK-6) in soil. The disappearance of half of the total pyrene initially present from the *Bacillus* sp. (PK-6) inoculated soil treatments over a 28 day time period cannot be attributed to degradative abilities of *Bacillus* alone because the indigenous, coal-tar acclimatized microbial community also showed similar results. The failure of specifically inoculated pyrene-degrading *Bacillus* sp. (PK-6) to remove more pyrene from soil could be due to soil's chemical, physical and biological complexity which could have reduced the allochthonous introduced population by antagonistic relationship (biotic factors) like competition from the autochthonous populations, as well as by physiological stresses caused by abiotic factors such as pH, availability of water and air, temperature and in the

specific case of PAHs, the bioavailability of C and energy sources (Jacques *et al*., 2008). The coal-tar contaminated soil study shows that there is no overall advantage of cultivated bacterial species towards the removal of pyrene in the soil system from which it has been isolated.

Bacterial counts were similar in indigenous coal-tar contaminated soil and *Bacillus* sp. (PK-6) re-inoculated coal-tar contaminated soil; also the removal of pyrene after 28 days in both soil treatments was marginally different. However between 14 and 21 days of incubation, the pyrene removal rate in the *Bacillus* sp. (PK-6) inoculated soil condition exceeded that in control uninoculated treatment. At the same time bacterial count in inoculated soil treatment is also more. This observation may suggest some interesting positive correlation between bacterial counts in soil and corresponding pyrene removal rate. A number of factors like soil type, nutrient status, and PAH exposure history can affect microbial community dynamics or the rate or extent of substrate removal (Carmichael and Pfaender, 1997a; Hwang and Cutright, 2003; Vinas *et al*., 2005). Owing to the relatively long incubation time in the soil experiment (28 days) and the complex nature of coal-tar in terms of PAH content, we cannot be certain that the bacterial counts recovered from coal-tar contaminated soil in both the treatments resulted from growth on pyrene itself or whether growth might have occurred on one or more products of coal-tar metabolism. With increase in molecular weight, PAHs show increased hydrophobicity (Kanaly and Harayama, 2000) and decreased bioavailability due to their absorption into the organic matter, strong binding to soil particles and sequestration inside micropores (Hatzinger and Alexander, 1995).

Pyrene removal from pyrene-spiked garden soil

In the garden soil experiment the effects of bioaugmentation (introduction of pyrene-degrading bacterial species) remediation strategy and biostimulation (addition of organic nutrient glucose) treatment method on pyrene removal over a time period of 4 weeks was evaluated. *Bacillus* sp. (PK-6) MTCC 1005 was inoculated in soil for uptake and removal of pyrene. Pyrene was removed to a greater extent in *Bacillus* sp. (PK-6) inoculated soil, amended with and without glucose (71 % and 66 %, respectively) as compared to uninoculated soil, amended with and without glucose (34 % and 13 %, respectively), suggesting that the extent of uptake over a period of 28 days was influenced by **bioaugmentation** in soil. Pyrene removal efficiency for pyrene-spiked garden soil (71 %)

was higher than for coal tar contaminated soil (58 %). Pyrene removal, accounting to more than two-thirds of the total pyrene addition, in *Bacillus* inoculated (with or without glucose amendment) soil treatments might be attributable to the pyrene uptake and degradation capability of the introduced bacterium. Kastner and Mahro (1996) reported complete degradation of 100 mg kg^{-1} of pyrene within 50 days of incubation. Jacques *et al*. (2008) worked with 250 mg kg^{-1} pyrene concentration in soil and showed 92 % mineralization of pyrene. It is also noteworthy that in spite of decline in bacterial count from day 21, removal of pyrene substrate lasted the experimental period. According to Khan *et al*. (2009) the decline in bacterial numbers could be probably due to substrate limitations.

It has been proposed by Vinas *et al*. (2005) that moisture content and nutrient addition are important with regard to substrate removal. In this study the presence of glucose is believed to have significantly promoted the removal of pyrene from the soil due to enhanced bacterial community size, increased number of pyrene degraders and corresponding metabolic activity in soil. Corresponding increases in bacterial counts during the first 14 to 21 days of incubation was observed only in glucose amended and *Bacillus* sp. (PK-6) inoculated soils, while in *Bacillus* sp. (PK-6) inoculated soils without glucose amendment only decline in bacterial counts was observed during the 28 day incubation period. Further, glucose amendment in the noninoculated soil treatment probably stimulated the dormant members of the microbial community (which survived autoclaving process) for pyrene removal. Easily metabolizable carbon sources contribute to the degradation of PAHs by increasing the size of microbial population, promoting microbial activity, and modifying microbial community diversity in the soil (Das and Mukherjee, 2007). Although pyrene has a highly complex and symmetric aromatic ring structure, which is tough to degrade, the potential for soil-associated glucose to be used as primary carbon source increases the bacterial community's (inoculated and indigenous) ability to cometabolize the substrate. In addition, soil treatments amended with glucose displayed a greater extent of removal over 28 days than those without glucose amendment (71 % in *Bacillus* inoculated soil amended with pyrene and glucose versus 66 % in similar soil amended with only pyrene, and 34 % in uninoculated soil amended with pyrene and glucose versus 13 % in similar soil amended with only pyrene; Figure 3.35) is consistent with the effects of nutrient amendment reported in a previous study by Potter *et al.* (1999) on the treatment of soil from contaminated site in compost reactors. In this study, adding

glucose to the pyrene-spiked soil inoculated with *Bacillus* sp. (PK-6) increased the rate of pyrene removal, suggesting that the uptake of pyrene over this time-period was influenced by biostimulation in soil.

The highest count of bacteria per gram of soil was recovered from the garden soil treatment which was amended with glucose and pyrene and inoculated with *Bacillus* sp. (PK-6); the same treatment also displayed maximum pyrene removal. As per the previous findings the treatment method and/or nutrient status alone may be predictive of extent of substrate removal when bioavailability does not govern degradation kinetics (Carmichael and Pfaender, 1997a, b; Hwang and Cutright, 2002; Potter *et al.,* 1999); we have also found that a positive correlation exists between bacterial enumeration and extent of pyrene removal from soil.

As a first step towards bioremediation of PAH contaminated sites, the present study attempts to identify the most relevant pyrene degrading bacteria in two, fuel oil and coal-tar, contaminated systems. In the second step, physiological, biochemical, genetic and field determinants that define the capacity of bacterial system(s) to biologically mediate removal of pyrene were evaluated. The results support considerable potential of members of the *Bacillus* genera in pyrene mobilization and degradation. It may also be suggested that the bacteria used in this study could be found suitable for practical field applications for effective *in situ* bioremediation of pyrene-contaminated sites.

CHAPTER 6

SUMMARY

6.1 BIODIVERSITY OF PYRENE DEGRADING BACTERIA

In the present study, we isolated aerobic, mesophilic pyrene utilizing bacteria from crude, diesel oil and coal-tar contaminated soil. We were particularly interested in isolating novel pyrene degrading bacteria for two reasons. First, the ability of a bacterium to survive adverse conditions like long term exposure to priority pollutants in PAH contaminated soils would be an attractive feature for remediation of pyrene contaminated sites. Secondly, only a few studies have examined the degradation of pyrene by bacteria, and none, to our knowledge, involve north-Indian pyrene contaminated soils.

1. The coal-tar contaminated soil collected from coal-tar pre-mix industrial plant site contained nine polycyclic aromatic hydrocarbons (PAHs) - Acenaphthylene, Pyrene, Fluorene, Phenanthrene, Benzo[*b*]fluoranthene, Benzo[*k*]fluoranthene, Indeno[1,2,3-*c,d*]pyrene, Dibenzo[*a,h*]anthracene and Benzo[*g,h,i*]perylene which are listed by US Environmental Protection Agency as Priority Pollutants.
2. A biodiversity of nearly 250 bacterial strains isolated from crude, diesel oil and coal-tar contaminated soil were screened for 50 μg ml^{-1} pyrene utilization abilities in Bushnell-Haas broth (basal medium) containing 0.5 % (w/v) glucose. Bacterial consortia CON-3 and THA-2 were developed from crude, diesel oil contaminated soil by selective enrichment of pyrene for cometabolizing 50 μg ml^{-1} pyrene in the presence of 0.5 % (w/v) glucose.
3. 16S ribosomal DNA sequencing technique was used to identify the selected bacterial isolates. Thirteen pyrene utilizing bacterial isolates belonged to *Bacillus*, *Pseudomonas* and *Rhodococcus* genera and one pyrene non-utilizing isolate belonged to *Kocuria* genera. Members of Firmicutes (80 %, 75 %), Actinobacteria (10 %, 25 %) and Proteobacteria (10 %, nil) taxonomic clades were present in coal-tar contaminated soil and crude and diesel oil contaminated soil, respectively. Gram-positive bacteria may play more important roles than Gram-negative isolates in the degradation of four- benzene ring compound pyrene. Therefore this study specifically

addressed the issue of pyrene degradation not being restricted to the actinomycetes group of bacteria.

6.2 ELUCIDE THE METABOLIC PATHWAY OF PYRENE DEGRADATION IN A SELECTED ISOLATE

The goal of this research was to provide a more complete understanding of pyrene degradation by Gram positive, nonactinomycetes bacterial isolates enriched from crude oil and coal-tar contaminated sites. This study examined the processes contributing to the fate of pyrene in selected bacteria. Several conclusions can be drawn from this work:

1. *Bacillus pumilus* (PK-12), isolated from crude oil contaminated soil, was able to cometabolize maximum 64 % of 50 μg ml^{-1} pyrene in basal medium containing 0.5 % (w/v) glucose in 35 days while in pulse-chase studies, as assessed by HPLC analysis, log phase *B. pumilus* cells were able to uptake 11.0 % of 100 μg pyrene as pure substrate in 7 hrs at 30 ^{o}C.
2. Growth and pyrene utilization enhanced upon increased supply of glucose (0.5 to 1.0 %; w/v) in the growth medium. *Bacillus pumilus* (PK-12) showed 13.5 % pyrene uptake from basal medium containing 0.5 % (w/v) glucose in 7 days, while 45.6 % pyrene uptake was observed in 4 days in presence of 1.0 % (w/v) glucose.
3. Growth studies with bacterial isolates from crude oil contaminated soil show:
    a. Bacterial cells grown in nutrient rich medium showed better pyrene uptake than minimal medium grown cells.
    b. Pyrene (50 μg ml^{-1}) uptake and utilization was a slow process, occurred maximally between 14 and 28 days and was stable thereafter,
    c. A stimulatory effect on bacterial growth and uptake of pyrene with increase in the glucose concentration in basal medium,
    d. Pyrene concentrations of 25 and 50 μg ml^{-1} did not have any deleterious effect on the growth of *Bacillus pumilus* (PK-12). In fact the addition of pyrene during mid log phase in basal medium containing 0.5 % (w/v) glucose is favourable for the isolate growth.
4. *Bacillus licheniformis* (PK-6), isolated from coal-tar contaminated soil, showed maximum pyrene uptake (56.4 %) and biosurfactant activity (OD_{550nm} = 1.96) in Bushnell-Haas broth medium in 4 days of incubation. *Bacillus pumilus* (PK-12)

showed moderate pyrene uptake (45.6 %) and biosurfactant activity (OD_{550nm} = 1.11). Bacterial isolates *Rhodococcus rhodochrous* (PK-10) showed moderate pyrene uptake (46.3 %) but higher biosurfactant activity (OD_{550nm} = 1.45) while *Pseudomonas oleovorans* (PK-3) showed low pyrene uptake (25.2 %) and biosurfactant activity (OD_{550nm} = 0.43). Pyrene was essentially cometabolized along with glucose utilization in all bacterial strains.

5. Biosurfactant activities can be measured reproducibly; a positive correlation exists between the production of biosurfactant activity and uptake of pyrene from the culture medium by eight bacterial isolates (*B. licheniformis* PK-6 > *Bacillus* sp. PK-7 > *R. rhodochrous* PK-10 > *B. pumilus* PK-12 > *B. subtilis* PK-5 > *B. firmus* PK-1 > *B. cereus* PK-4 > *P. oleovorans* PK-3) and therefore can be incorporated into models that describe the fate of hydrophobic PAHs in aqueous systems.
6. Biosurfactant activity was examined by measuring the degree of emulsification of mobil oil in culture supernatant. Results suggest that biosurfactant activity by the tested bacteria involves an extracellular phenomenon for enhancing pyrene bioavailability and uptake into the bacterial cell.
7. Identified six possible pyrene [$C_{16}H_{10}$] metabolites from *Bacillus licheniformis* (PK-6) growth extracts - Phenanthrene [$C_{14}H_{10}$], 9,10-Diphenylphenanthrene [$C_{26}H_{18}$], 9-Methoxyphenanthrene [$C_{15}H_{12}O$], 5,6,7,8-Tetrahydro-1-naphthoic acid [$C_{11}H_{12}O_2$], 1,6,7-Trimethyl-naphthalene [$C_{13}H_{14}$] and Diisooctylphthalate [$C_{24}H_{38}O_4$].

These results help to comprehend the fundamental processes governing bacterial degradation of pyrene in aqueous media. Knowledge of the relative roles of glucose, biosurfactant activity in relation to biodegradation and the physiology of bacterial processes contribute to a better understanding of the fate of pyrene in efficient bacterial systems.

6.3 GENETIC REGULATION OF PYRENE DEGRADATION IN THE SELECTED ISOLATE

To get a comprehensive picture of pyrene degradation molecular level studies were conducted so as to isolate pyrene metabolic (ring hydroxylating dioxygenase) gene from the selected isolate.

1. The seat of pyrene metabolic genes in the selected pyrene degrading isolates is presumed to be chromosomally borne as no plasmid could be detected in bacterial cells.
2. Attempt was made to detect Rieske centers, the conserved [Fe_2-S_2] cluster binding region of terminal dioxygenases within the genera *Bacillus*, *Pseudomonas* and *Rhodococcus*; however neither Rieske centers (gene) nor ring-hydroxylated products of pyrene catabolism could be detected by gene-specific PCR or GCMS analysis, respectively.
3. Pyrene degradation pathway is proposed in *Bacillus licheniformis* (PK-6) MTCC 1005 based on metabolites identified in this study.

6.4 REMOVAL OF PYRENE BY SELECTED BACTERIAL ISOLATE FROM SOIL CONTAMINATED WITH PYRENE

In this study we evaluated the pyrene removal potential of *Bacillus licheniformis* (PK-6) MTCC 1005 in sterile and non-sterile, pyrene spiked and glucose amended soil systems and determined if biostimulation (with glucose) and bioaugmentation (with *Bacillus*) of the contaminated soil would enhance pyrene removal. Knowledge gained herein can be used to provide insight into the efficient bioremediation of pyrene contaminated soils in North India.

1. In the non-sterile composite coal-tar contaminated soil study, similar counts of bacteria per gram of soil were recovered from the uninoculated and *Bacillus* inoculated soil treatments along with marginally different percentage of pyrene removal (57 and 58 %, respectively) in 28 days of incubation. The results suggest that pyrene removal in non-sterile pyrene contaminated soil is influenced by presence of indigenous, pyrene acclimatized and pyrene metabolizing gene pool rather than the introduced *Bacillus* sp. (PK-6) bacterial biomass (bioaugmentation).
2. In the sterile pyrene-spiked (@ 0.1 mg g^{-1}) garden soil study, amendment of soil with glucose (@ 0.5 mg g^{-1}) significantly promoted the increase in count of pyrene degrading bacteria per gram of soil, thereby leading to high removal of pyrene (71 %) from soil in 28 days of incubation. Pyrene removal from pyrene spiked and glucose amended and *Bacillus* inoculated soil treatment, accounting to about two-third of the total pyrene addition, suggests that pyrene removal in sterile

pyrene contaminated soil is influenced by bioaugmentation (introducing pyrene-degrading bacterial culture) and biostimulation (adding glucose).

The pyrene removal potential of selected bacterial isolate *Bacillus licheniformis* (PK-6) MTCC 1005 in sterile, pyrene-spiked garden soil suggests its possible use and advantage in bioremediation of sites contaminated with crude oil, coal-tar and a mixture of PAHs.

CHAPTER 7

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LIST OF RESEARCH PUBLICATIONS AND CONFERENCE PRESENTATIONS

Refereed Publications:

1. **G. Purnima Khanna**, Dinesh Goyal and Sunil Khanna. (2011) **Pyrene Biodegradation by *Bacillus* sPp. Isolated from Coal tar-Contaminated Soil**. *Bioremediation J* (SCI index - ISSN No.: 1088-9868; Impact factor: not available). 15(1): 12 - 25.

2. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. (2011) **Pyrene Degradation by *Bacillus pumilus* Isolated from Crude oil Contaminated Soil.** *Polycyclic Aromatic Compounds* (SCI index - ISSN No.: 1040-6638; Impact factor: 0.741). 31(1): 1 - 15.

3. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. (2011) **Characterization of Pyrene Utilizing *Bacillus* sp. from Crude oil Contaminated Soil.** *Brazilian Journal of Microbiology* (SCI index - ISSN No.: 1517-8382; Impact factor: 0.548) (Under review)

4. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. (2011) **Pyrene Removal by *Bacillus* sp. from pyrene contaminated soils.** *Water, Air and Soil Pollution* (Manuscript In Preparation).

5 **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. (2011) **Bacterial degradation of polycyclic aromatic hydrocarbons.** *Bioresource Technology* (Manuscript In Preparation).

Conference Presentations:

National

1. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. **Characterization of coal-tar contaminated soil and screening for pyrene utilizing bacteria.** *Abstracts, 48th Annual Conference of Association of Microbiologists of India,*

IIT Chennai, India. December (2007) pp. 280.

2. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. **Pyrene utilization by *Bacillus pumilus***. *Abstracts, 48th Annual Conference of Association of Microbiologists of India,* IIT Chennai, India. December (2007) pp. 332.

International

3. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. **Pyrene Utilization Potential of Bacterial Diversity Characterized from Coal-tar Contaminated Indian Soil**. *Abstracts, 10th International In Situ and On-Site Bioremediation Symposium,* Baltimore, Maryland, USA. May (2009) pp. 90.

4. **Purnima Khanna**, Dinesh Goyal and Sunil Khanna. **Pyrene Degradation by *Bacillus licheniformis* MTCC 1005**. *Abstract, 5th European Bioremediation Conference,* Chania, Crete, Greece, July 4-7 (2011) (Communicated).

Bacteria 16S rDNA Sequences Deposited with NCBI GenBank Database:

1. **Purnima Khanna,** Dinesh Goyal and Sunil Khanna (2008) **Diversity of pyrene utilizing bacteria isolated from crude oil contaminated soil**: Four 16S ribosomal RNA gene partial sequences published in National Center for Biotechnology Information (NCBI) GenBank vide Accession no. **EU685813 upto EU685816** (http://www.ncbi.nlm.nih.gov)

2. **Purnima Khanna,** Dinesh Goyal and Sunil Khanna (2008) **Diversity of pyrene utilizing bacteria isolated from coal-tar contaminated soil**: Ten 16S ribosomal RNA gene partial sequences published in National Center for Biotechnology Information (NCBI) GenBank vide Accession no. **EU685817 upto EU685826** (http://www.ncbi.nlm.nih.gov)