# DECIPHERING BACTERIAL CATABOLIC GENES FOR PAH-DEGRADATION

**A thesis submitted in partial fulfilment of the requirements for the award of degree of**

**Doctor of Philosophy**
in
**Environmental Engineering**

**By**

**SAKSHI**

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**DEPARTMENT OF ENVIRONMENTAL ENGINEERING**
**DELHI TECHNOLOGICAL UNIVERSITY**
**DELHI-110 042, INDIA**

**July, 2021**

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**(Regn. No. 2K16/PhD/EN/02)**

Under the supervision of

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**July, 2021**

## DECLARATION

I hereby declare that the research work presented in this thesis entitled "Deciphering Bacterial Catabolic Genes for PAH-Degradation" is original and carried out by me under the supervision of Dr. A.K. Haritash, Associate Professor, Department of Environmental Engineering, Delhi Technological University, Delhi, and under the co-supervision of Prof. S. K. Singh, Professor and Head, Department of Environmental Engineering, Delhi Technological University, Delhi, and being submitted for the award of Ph.D. degree to Delhi Technological University, Delhi, India. The contents of this thesis have not been submitted either in part or whole to any other University or institute for the award of any degree or diploma.

Sakshi

Sakshi

Date: 20th July, 2021

Place: Delhi

दिल्ली प्रौद्योगिकी विश्वविद्यालय
**DELHI TECHNOLOGICAL UNIVERSITY**
*(Formerly Delhi College of Engineering)*

Date: 20th July, 2021

## CERTIFICATE

This is to certify that the Ph.D. thesis entitled "Deciphering Bacterial Catabolic Genes for PAH-Degradation" being submitted by Ms. Sakshi for the award of the degree of Doctor of Philosophy in Environmental Engineering, Delhi Technological University, Delhi, India, is a bonafide record of original research work carried out by her under our guidance and supervision. The results embodied in this thesis have not been submitted to any other university or institution for the award of any degree or diploma.

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Sakshi

Sakshi

# ABSTRACT

Polyaromatic hydrocarbons (PAHs) are considered as hazardous organic priority pollutants. PAHs are immense public concern and critical environmental challenge around the globe due to their toxic, carcinogenic, and mutagenic properties, and their ubiquitous distribution, and persistence in the environment. The knowledge about the harmful effects of PAHs on the ecosystem along with human health has resulted in the interest of researchers on the degradation of these compounds. Whereas physico-chemical treatment of PAHs is cost and energy-prohibitive, bioremediation *i.e.* degradation of PAHs using microbes is becoming an efficient and sustainable approach. A broad range of microbes including bacteria, fungi, and algae has been found to have the capability to transform/degrade PAHs under both aerobic and anaerobic conditions. Microbial genetic makeup containing genes encoding catabolic enzymes is responsible for the PAH-degradation mechanism. The degradation capacity of microbes may be induced by exposing them to higher PAH concentration, resulting in genetic adaptation or changes responsible for high efficiency towards removal/degradation. In order to devise efficient bioremediation strategies for PAH-degradation, the identification and study of the metabolic potential of microbial species are essential. The goal of this study was to isolate PAH-degrading bacterial strains from petroleum-contaminated soil that can utilize PAHs (three-ring (phenanthrene, anthracene, and fluorene) and four-ring (pyrene)) as their sole carbon source. PAH-utilizing bacterial strains were isolated from petroleum-contaminated soil from the siding area, Bijwasan supply location of Bharat Petroleum Corporation Limited in Delhi, India. Seven bacterial strains with different morphology were isolated and acclimatized under a mixture of four PAH compounds in a concentration range of 50mg/L to 10,000mg/L. Two Gram-positive bacterial strains (DTU-1Y and DTU-7P) were found to be resistant to high PAH (10,000mg/L) exposure. The 16S rRNA gene sequencing of these two strains identified DTU-1Y as *Kocuria flava* and DTU-7P as *Rhodococcus pyridinivorans.* Both the isolated strains belonged to the actinobacteria phylum, a group of gram-positive bacteria. Most of the species belonged to the actinobacteria phylum representing a role in the degradation of xenobiotic compounds such as polychlorinated biphenyls, pesticides, dioxins, PAHs, etc. The strain *K. flava* DTU-1Y could degrade 53-63% of 10 mg/l of phenanthrene, anthracene, fluorene, and pyrene in 15 days of incubation period. whereas *R. pyridinivorans* had degradation efficiency in the range of 56-66%. The consortium of both strains exhibited almost similar degradation efficiency as

observed individually indicating that there was no inhibitory or synergistic effect of these strains over each other. Catechol 2,3-dioxygenase (C23O), dehydrogenase, and peroxidase enzyme activities during PAH-degradation coincided with degradation of PAHs, highlighting the role of these enzymes in catabolizing PAHs. This is the first investigation confirming the participation of C23O, dehydrogenase, and peroxidases enzyme of these two strains in PAH-degradation. The bacterial strains reported in this study were also examined for catabolic gene expression during PAH-degradation and the evolutionary relationship of isolated bacterial strains with known PAH-degrading bacterial strains having PAH-catabolic genes/enzymes involved in PAH-bioremediation was also confirmed.

The degradation efficiency of both strains (monoculture and consortium) was more than 53% for three-ring and four-ring PAHs (10 mg/L) when used individual PAH compound or in a mixture of PAHs. The degradation efficiency of isolated bacterial strains for a PAH compound was not affected by the presence of other PAH compounds. It was observed that the degradation efficiency for individual PHE, ANT, and PYR of the selected bacterial strains *i.e. K. flava* DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium was almost similar to the degradation efficiency for respective PAHs in the mixture of the PAHs (PHE, ANT, FLU and PYR). In the case of FLU, degradation by *K. flava* DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium was found slightly lower in the mixture of PAHs (PHE, ANT, FLU, and PYR) as compared to degradation of FLU individually. Both the isolated strains having the ability to degrade three-ring and four-ring PAHs showed C23O, peroxidase, and dehydrogenase activity during PAH-degradation when present as a single PAH compound or a mixture of PAHs. The C23O enzyme may be responsible for the initiation of hydrolysis through meta-cleavage of benzene ring; whereas dehydrogenases and peroxidases might catalyze further oxidation of PAHs into simpler non-toxic forms. The presence of C23O, which catalyzes PAH-degradation *via* meta-cleavage, indicated C23O involvement in the initial ring cleavage of pyrene and based on enzyme activity a probable degradation pathway of pyrene was suggested. The catabolic gene expression for naphthalene dioxygenase (*NAH*) and catechol 2,3-dioxygenase (*C23O*) was confirmed in isolated strains in the present study and significant catabolic gene expression during degradation of PAHs concluded that *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P are efficient PAH-degraders and can be used for the development of an efficient bioremediation method for cleaning of PAH-contaminated environmental matrix.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| 1,3-DMNAP | 1,3-Dimethylnaphthalene |
| 16S rRNA | 16S Ribosomal Ribonucleic Acid |
| 1D-PAGE | One Dimensional Polyacrylamide Gel Electrophoresis |
| 1-MNAP | 1-Methylnaphthalene |
| 1-MPHE | 1-Methylphenanthrene |
| 2DE | Two-dimensional Electrophoresis |
| 2-MNAP | 2-Methylnaphthalene |
| 3,6-DMPHE | 3,6-Dimethylphenanthrene |
| *A.thaliana* | *Arabidopsis thaliana* |
| ACEN | Acenaphthene |
| ACEY | Acenaphthylene |
| ANT | Anthracene |
| AOPs | Advanced Oxidation Processes |
| ATF | Aviation Turbine Fuel |
| ATSDR | Agency for Toxic Substances and Disease Registry |
| B(a)A | Benzo(a)anthracene |
| B(a)F | Benzo(a)fluoranthene |
| B(a)P | Benzo(a)pyrene |
| B(a)P-E | Benzo(a)Pyrene Equivalents |
| B(b)C | Benzo(b)chrysene |
| B(b)F | Benzo(b)fluoranthene |
| B(e)P | Benzo(e)pyrene |
| B(ghi)P | Benzo(g,h,i)perylene |
| B(j)F | Benzo(j)fluoranthene |
| B(k)F | Benzo(k)flouranthene |
| B. Pt. | Boiling Point |
| BCD | β-cyclodextrin |
| BLAST | Basic Local Alignment Search Tool |
| BPCL | Bharat Petroleum Corporation Limited |
| C12O | Catechol 1,2-Dioxygenase |
| C23O | Catechol 2,3-Dioxygenase |

| | |
|---|---|
| CAS | Chemical Abstracts Service |
| CHRY | Chrysene |
| COR | Coronene |
| CSIR-IMTECH | Council of Scientific and Industrial Research-Institute of Microbial Technology |
| $C_T$ | Cycle Threshold |
| CYC | Cyclopenta(c,d)pyrene |
| D(ac)P | Dibenzo(ah)pyrene |
| D(ah)A | Dibenz(ah)anthracene |
| D(ah)P | Dibenzo(ah)pyrene |
| DCM | Dichloromethane |
| DMBA | 7,12-di-methylbenz(a)anthracene |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic acid |
| FA | Fulvic Acid |
| FLA | Fluoranthene |
| FLU | Fluorene |
| FO | Furnace Oil |
| GEMs | Genetically Engineered Microorganisms |
| HA | Humic Acid |
| HMW | High Molecular Weight |
| HPCD | Hydroxypropyl- β –cyclodextrin |
| HPLC | High Pressure Liquid Chromatography |
| HSD | High Speed Diesel |
| IARC | International Agency for Research on Cancer |
| IPCS | International Programme on Chemical Safety |
| IPYR | Indeno(1,2,3-cd)pyrene |
| *K. flava* | *Kocuria flava* |
| $K_{ow}$ | Octanol/Water Partition Coefficients |
| LB | Luria-Bertani |
| LC-MS/MS | Liquid Chromatography Tandem Mass Spectrometry |
| LDO | Light Diesel Oil |
| LMW | Low Molecular Weight |
| LnP | Lignin Peroxidase |

| | |
|---|---|
| M. Pt. | Melting Point |
| MCAGCC | Marine Corps Air Ground Combat Center |
| MCD | Methyl- β –cyclodextrin |
| MEGA | Molecular Evolutionary Genetics Analysis |
| MG | Microbial Growth |
| MNAP | Methylnaphthalene |
| MnP | Manganese Peroxidase |
| MOPS | 3-(N-morpholino) Propane Sulfonic Acid |
| MS | Motor Spirit |
| MSM | Mineral Salt Medium |
| MTO | Mineral Turpentine Oil |
| MUSCLE | Multiple Sequence Comparison by Log- Expectation |
| NAH | Naphthalene Dioxygenase |
| NAP | Naphthalene |
| NCBI | National Center for Biotechnology Information |
| NCP | National contingency plan |
| NIOSH | National Institute for Occupational Safety and Health |
| NK | Natural Killer |
| NM | Not Mentioned |
| NM | Not Mentioned |
| NMAM | NIOSH Manual of Analytical Methods |
| NSO | Nitrogen, Sulphur, and Oxygen Compounds |
| NTC | No Template Control |
| OSE | Oil Spill Eater |
| PAHs | Polycyclic Aromatic Hydrocarbons |
| PCBs | Polychlorinated Biphenyls |
| PCR | Polymerase Chain Reaction |
| PERY | Perylene |
| PHE | Phenanthrene |
| PMS | Peroxymonosulfate |
| POP | Persistent Organic Pollutants |
| PYR | Pyrene |
| *R. pyridinivorans* | *Rhodococcus pyridinivorans* |
| RCF | Relative Centrifugal Force |

| | |
|---|---|
| RHD | Ring Hydroxylating Dioxygenase |
| RT-qPCR | Reverse Transcription Polymerase Chain Reaction |
| SD | Standard Deviation |
| SKO | Superior Kerosene Oil |
| TCA | Tricarboxylic Acid |
| TERI | Tata Energy Research Institute |
| TF | Triphenyl Formazan |
| TTC | Triphenyl Tetra-zolium Chloride |
| USEPA | United States Environmental Protection Agency |
| UV | Ultraviolet |
| V. P. | Vapor Pressure |
| WGPAH | Working Group on Polycyclic Aromatic Hydrocarbons |
| YE | Yeast Extract |

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# CHAPTER 1
# INTRODUCTION

With increasing awareness about the adverse effects of hazardous chemicals on human health and the environment, the remediation of such chemicals has received more attention globally. Environmental pollution caused by the dumping of solid waste, untreated industrial effluents, persistent organic pollutants (POPs) like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and pesticides, is a critical problem. These compounds, directly or indirectly released into the environment, are found to be very toxic and lead to contamination of water, soil, and air. Soils contaminated with organic pollutants and inorganic contaminants have high potential health risks because such contaminants can enter the food chain (Xiaojun *et al.,* 2007). Studies have established that toxicity increases with an increase in the complexity of the chemical structure of the contaminant (Patnaik, 1999). Moreover, since complex/branched structure results in chemical stability, such pollutants are stable, resistant to biodegradation, and as a result, concentrate in the environment which leads to accumulation of toxicity. Soil contaminated with hazardous petroleum hydrocarbons can adversely affect human health in several ways. Therefore, it is high time to adopt effective measures to reclaim contaminated sites and control other sites from being getting contaminated. If no efforts are made to clean the contaminated sites, then the harmful effects of such sites will persist in the environment, and add up to the Brownfield list.

The PAHs are found to have critical effects on all forms of life, so it is important to overcome the hazards associated with these compounds. There are several physical (solvent extraction, air sparging, thermal desorption, microwave heating, vitrification, electrokinetic treatment, etc.); chemical (oxidation using Fenton's reagent, ozone, photocatalytic degradation); and biological (microbial, landfarming, composting, phytoremediation, etc.) methods being used to remediate the contaminated sites. The selection of treatment method is subject to the type of contaminant, soil type, and interferences present. Although physical and chemical methods are effective and efficient, most of them are energy, cost, or chemical-intensive. Moreover, these methods are responsible for the generation of secondary pollutants. Keeping in view, the limitations associated with physicochemical treatment, the biological methods are gaining popularity owing to their eco-friendly nature and bioconversion of toxic pollutants into harmless/nontoxic chemical species (Sakshi *et al.,* 2019).

### 1.1 Polycyclic aromatic hydrocarbons: Physical and chemical properties

Environmental pollution is the alteration in the state of the environment that has adverse effects on human health and other living organisms, and that affects the quality of life. With increasing awareness of the adverse effects of hazardous chemicals on human health and the environment, the remediation of such chemicals has received more attention of researchers. PAHs are hazardous ubiquitous organic pollutants and are widely distributed in the environment; 16 PAHs among them are categorized as priority environmental pollutants and some of them are categorized as probable human carcinogens by the United States Environmental Protection Agency (USEPA) due to their toxic, carcinogenic, and mutagenic nature. PAH compounds are composed of fused benzene rings (simple to complex structural configuration) in linear, angular, or cluster arrangements. These pollutants have varying physico-chemical, and toxicological characteristics according to their molecular weight. PAHs may be classified based on the number of benzene rings. Low molecular weight (LMW) PAHs consist of two or three benzene rings such as naphthalene, phenanthrene, and anthracene; and high molecular weight (HMW) PAHs consist of more than three benzene rings such as pyrene, benzo(a)pyrene (B(a)P), chrysene, etc. PAHs have high melting and boiling points, low vapor pressure, and very low aqueous solubility. Vapor pressure and aqueous solubility of PAHs tend to decrease with increasing molecular weight. HMW-PAHs with lower vapor pressure will tend to be associated with the particulate phase, while LMW-PAHs with higher vapor pressures will be associated with the vapor phase (Clar, 1964; Patnaik, 1999) in the atmosphere. PAHs are detrimental persistent organic pollutants, while HMW-PAHs are even more harmful to the environment and human health. Increasing stability and hydrophobicity with an increase in molecular weight of HMW-PAH are the two primary factors that contribute to their persistence in the environment.

### 1.2 Sources, occurrence and fate of PAHs

Generally, PAHs are produced from anthropogenic as well as natural sources. Forest fires, volcanic eruptions, exudates from trees (pine needles), and oil seeps are some natural sources; and burning of fossil fuel, coal tar, crude oil or petroleum spill *i.e.* oil spillage and leakage, high-temperature industrial processes, municipal solid waste incineration, vehicular emissions and petroleum refinery effluent, smoke from wood-burning stoves, creosote waste materials and manufactured gas plant (coal gasification), etc. are some anthropogenic sources (Haritash and Kaushik, 2009; Abdel-shafy and Mansour, 2016; Sharma and Jain, 2019). The natural and anthropogenic sources of PAHs are associated with pyrogenic, petrogenic, and

biogenic activities in the environment. Pyrogenic PAHs are formed during the combustion of petroleum and pyrolysis (organic substances are exposed to high temperatures ranging from 350°C to more than 1200°C under a limited supply of oxygen). The pyrolytic conversion of coal into coke and/or coal tar; and the catalyst-induced thermal cracking of plastic or secondary products of petroleum refinery into the mixture of simpler hydrocarbons has been a common practice. Some unintentional pyrogenic processes occur during incomplete combustion of motor fuel in cars and trucks, and incomplete combustion of wood during forest fire. Petrogenic PAHs are released into the environmental matrix during crude oil extraction, its transportation, storage, and disposal, and the use of crude oil and petroleum products in other associated activities. Apart from anthropogenic activities, some PAHs are produced and released from natural sources. Such PAHs are synthesized by certain pine and coniferous trees, and some termites (Stogiannidis and Laane, 2015; Hussain *et al.,* 2018).

The organic pollutants do not degrade easily and their toxicity and persistence increase with the increase in molecular weight. The persistence of PAHs in the environment depends on various aspects such as physico-chemical properties of PAHs, environmental matrix, including the concentration, and bioavailability. PAHs are reported in all constituents of the environment *i.e.* air, water, soil, and vegetation thereby getting an entry into the food chain. The emissions from volcanoes, forest fires, residential wood burning, and exhaust from vehicles are responsible for the release of PAHs into the atmosphere. Vehicular emission is the dominant source of PAHs in the air. Since PAHs have lower vapor pressure, therefore, they can readily condense on suspended particulate matter and travel to remote places with the wind. PAHs associated with respirable dust have a significant adverse effect on the population resulting in respiratory disorders (Haritash and Kaushik, 2011; Kaushik *et al.,* 2012). Most PAHs do not dissolve easily in water (low aqueous solubility), they stick to solid particles and settle down, and subsequently, soil acts as the ultimate depository of PAHs. Leakage of underground storage tanks and pipelines, spill at petroleum wells and seepage are some sources of soil and groundwater contamination. The soil-bound PAHs may also be washed into water surface bodies through surface run-off and industrial discharge. The environmental fate of PAHs is determined by the presence of processes like adsorption, leaching, microbial degradation, photo-dissociation, volatilization, and chemical breakdown (Wild and Jones, 1995).

### 1.3 Bioremediation: Role of microbes in PAH degradation

Removal of PAHs from polluted sites is essential and the application of microorganisms to degrade PAHs (bioremediation) has become a popular method over the period. Bioremediation is considered an eco-friendly and sustainable remediation technique and has recently gained considerable interest all around the globe (Reddy and Adams, 2015). It is the transformation of the hazardous compounds to less hazardous/non-hazardous forms with less input of chemicals and energy, and microbes are agents for catabolic activity during microbial remediation. It involves the breakdown of organic compounds into inorganic minerals, $H_2O$, $CO_2$ (aerobic), or $CH_4$ (anaerobic). The biodegradation of a pollutant and its rate depends on the environmental conditions (pH, temperature, and nutrients), number and type of the microorganisms (biomass concentration and enzyme activities), and nature of chemical compound (density, solubility, structure of the contaminant) being degraded. Various microorganisms (algae, bacteria, and fungi) have been studied for their ability to degrade PAHs. The microorganisms produce a number of enzymes to detoxify and mineralize PAHs eventually leading to the degradation. The microorganisms isolated from contaminated sites show more degradation ability as they have got adapted to the polluted environment and can survive in the presence of the pollutant. A very challenging task in bioremediation processes is the confirmation of the first step, *i.e.*, identification of microorganism to be used for degradation and determination of the end products. A number of bacterial species have been investigated for proficient PAH-biodegradation, and it essentially depends on the catalytic action of enzymes produced by them. Mono-oxygenases, dioxygenases (catechol 1,2-dioxygenase), dehydrogenases, peroxidases, and lipases are the dominant enzymes responsible for the degradation of PAHs (Cerniglia, 1992; Lyu *et al.,* 2014; Pandey *et al.,* 2012).

### 1.4 Research gap

Most of the studies on microbial degradation of PAHs report the degradation efficiency under controlled laboratory conditions. Moreover, similar species of microorganisms may respond differently towards the degradation of PAHs depending on the exposure to the contaminant, period for adaptation, and above all expression of genes responsible for PAH-degradation. Therefore, it is important to screen the strains and understand the genetic/molecular level changes for the successful implementation of bioremediation. Most of the studies investigated the degradation efficiency of isolated microorganisms but enhancing the efficiency through gradually increasing exposure and

adaptation are scanty. Since catabolic enzymes are involved in the PAH-degradation pathway, the genes encoding respective enzymes in different microbial species may be identified using molecular techniques to explore their potential toward PAH-removal under dynamic environmental conditions. Most of the studies investigated the degradation of PAHs but simultaneous analysis of enzyme activity during degradation is limited and the specific role of enzymes in establishing the mechanism of degradation is overlooked in many of the studies. In addition, the 16S ribosomal ribonucleic acid (16S rRNA) sequencing has turned out as a useful technique in determining phylogenetic or evolutionary relationship among PAH-degrading species because of their conservative nature (Wang *et al.,* 1995). Evolutionary relationships among bacterial species having the ability for PAH-degradation may also help to elucidate links between catabolic capabilities and understand how these bacterial species are evolved. Since a number of the earlier studies lack analysis of expression of PAH-catabolic genes (Hegazi *et al.,* 2007; Zeinali *et al.,* 2007; Farahat and El-Gendy, 2008; Zhang *et al.,* 2009), scaling up such studies under different environmental conditions could be difficult. In order to conclude the efficacy of bacterial strains, molecular or genetic level studies have become important. Most of the studies involving genetic investigation generally identify genes encoding the catabolic enzymes, but the expression of genes has not been reported in earlier literature.

### 1.5 Objectives of the present study

Considering the bioremediation as a sustainable remediation technique for PAH-degradation, and realizing the importance of genetic make-up/gene expression of bacterial species under contaminated/stress conditions, the present study is devised with the following objectives.

i. Isolation, characterization, and identification of PAH-degrading bacterial strains from a contaminated site.

ii. Determining the efficiency of isolated bacterial strains to degrade different PAHs.

iii. Isolation and sequencing of microbial 16S rRNA and screening of enzymes involved in PAHs-degradation.

iv. Molecular analysis for detection of catabolic genes, and to study the expression of individual genes relating to degradation of PAHs.

### 1.6 Scope and relevance of the study

Studies carried out on the degradation of PAHs show variation in degradation efficiency (Badejo *et al.,* 2013) since these species are isolated from different environments and represent different genetic compositions. The molecular investigation and enzymatic assay can help in the identification of microorganisms having the capability of PAH-degradation. Since catabolic enzymes are involved in PAH-removal, so, enzymatic assay during degradation may provide a better understanding of the metabolic pathway and can help in the design of an efficient remediation technique for PAH-contaminated sites. The present study is aimed at the isolation and characterization of bacterial strains from aged petroleum-contaminated soil to assess their capability to degrade PAHs (phenanthrene, anthracene, fluorene, and pyrene). This study represents scientific relevance in terms of

i. Generation of the database of bacteria that can potentially treat hydrocarbon contaminated environment.
ii. Analysis of the evolutionary relationship among soil-PAH degrading bacterial strains isolated from petroleum-contaminated soil with other PAH-degrading bacterial strains based on 16S rRNA diversity.
iii. Inference of the catabolic enzymes *i.e.* catechol 2,3-dioxygenase, dehydrogenase, and peroxidase in biodegradation of PAHs
iv. Expression of catabolic genes (naphthalene dioxygenase (*NAH*), PAH-ring hydroxylating dioxygenase (*PAH-RHD*), and catechol 2,3-dioxygenase (*C23O*)) during PAH-degradation.

The screening of enzymes and identification of genetic make-up would provide a better understanding of the degradation mechanism and can help to design an efficient cleaning strategy. The analysis of enzyme activity may help identify the enzymes directly taking part in the transformation of PAHs. Other fast-growing microorganisms representing activity for similar enzymes may also be put through screening for their role in degradation. Sometimes it has been found that the genes associated with PAH removal could be present in the isolated bacteria but not expressed, so it is interesting to find the expression of the PAH catabolic gene. The results along with the analysis of the expression of the gene involved in PAH degradation will allow to improve the bacteria-assisted bioremediation of polluted sites.

# CHAPTER 2
# REVIEW OF LITERATURE

Organic pollutants associated with petroleum such as PAHs are considered hazardous pollutants due to their toxic, mutagenic, and carcinogenic effects. They have varying physico-chemical and toxicological characteristics according to their molecular weight. PAHs are highly stable ubiquitous organic compounds consisting of benzene rings. These organic compounds are hydrophobic and their hydrophobicity increases with an increase in molecular weight. High molecular weight PAHs containing more than three benzene rings are more hydrophobic with a higher octanol-water coefficient indicating their low water solubility (Table 2.1). These pollutants do not degrade easily and their toxicity and persistence increase with the increase in molecular weight. PAHs are produced from anthropogenic as well as natural activities (Table 2.2). PAHs are found in all constituents of the environment *i.e.* air, water, soil, and enter the food chain. Soil is the major sink for a broad range of harmful contaminants. Due to the hydrophobic nature with low water solubility, PAHs have the tendency to bind with organic matter present in soil/ sediments, hence they tend to stick to solid particles which ultimately settle down over soil. These organic pollutants are relatively stable contaminants showing recalcitrant nature in soils, and it is difficult to degrade them as compared to many other organic contaminants. PAHs are ubiquitously present around the globe and have contaminated the soil in different geographical regions of the world (Table 2.3). Considering the toxic properties and persistence of PAHs in soil, reclamation of PAH-contaminated soils becomes imperative (Haritash and Kaushik, 2009; Sakshi *et al.,* 2019). Therefore, reclamation/remediation of PAH-polluted soils is essential and the hazards related to PAHs can be solved/overcome using conventional methods for removal, alteration, or isolation of the pollutant. PAHs may undergo adsorption, photolysis, volatilization, chemical degradation, and microbial degradation (Wild and Jones, 1995). There are a number of physical (solvent extraction, air sparging, thermal desorption, microwave heating, vitrification, electrokinetic treatments); chemical (oxidation using Fenton's reagent, ozone, photocatalytic degradation); and biological (microbial, landfarming, composting, phytoremediation) methods being used to remediate the contaminated site (Fig. 2.1). The selection of treatment method is subject to the type of contaminant, soil type, interferences present, and risk analysis associated with the techniques. Although physical and chemical methods are effective and efficient, most of them are energy, cost, or chemical-intensive.

Moreover, these methods are responsible for the generation of secondary pollutants. Keeping in view, the limitations associated with physico-chemical treatment, the biological methods (bioremediation and phytoremediation) in which microorganisms or plants are used to remove/degrade PAHs are gaining popularity owing to their eco-friendly nature and bioconversion of toxic pollutants into harmless/nontoxic chemical species.

Bioremediation in which various microorganisms like bacteria, fungi, yeasts, and algae are used to degrade PAHs from contaminated sites has recently gained huge interest all around the world because it is an efficient, eco-friendly, cheap, and sustainable remediation technique (Reddy and Adams, 2015; Agrawal *et al.,* 2019; Dhanya and Kalia, 2020). Microorganisms have degradative potential by which contaminants are converted into less toxic compounds or water and carbon dioxide (Alexander, 1994; Kumar and Bharadvaja, 2019). It is a feasible remediation technique in which microorganisms adapt to the contaminated environment and efficiently transform the contaminants into harmless/ non-toxic byproducts. Microbial catabolic activity for PAH-degradation depends strongly on microbial adaptations, previous contaminant exposure, the bioavailability of contaminant, environmental conditions (pH, temperature, and nutrients), number and type of the microorganisms (biomass concentration and enzyme activities), nature or physicochemical properties (density, solubility, structure), and concentration of PAH being degraded (Singh and Ward, 2004). The degradation efficiency of microbes is the outcome of its genetic makeup responsible for the degradation and expression of the catabolic genes under the given set of conditions. The degradation capacity of microbes can be stimulated by exposing them to high PAH concentration which results in genetic adaptation for higher degradation efficiency. A number of bacterial species such as *Janibacter, Microbacterium, Gordonia, Rhodococcus, Pseudomonas,* and *Bacillus* (Zhang *et al.,* 2009; Lara-Severino *et al.,* 2016; Qi *et al.,* 2017; Subashchandrabose *et al.,* 2019a; Rabodonirina *et al.,* 2019) and white-rot fungi such as *Phanerochaete chrysosporium*, and *Pleurotus ostreatus* (Bishnoi *et al.,* 2007; Pozdnyakova *et al.,* 2018) have been investigated for proficient PAH-biodegradation. Although various studies have already been done to investigate microbial degradation efficiency but it is still important to understand microbial degradation pathways at the molecular level. Knowledge of metabolic and genetic mechanisms for microbial degradation of PAHs is essential to devise a proficient bioremediation process.

**Table 2.1 Physicochemical properties, carcinogenic classification, and half-life of major PAHs**

| S. No. | Name | Chemical formula | Chemical structure | CAS registry No. [1] | B. Pt. (◦C)[a] | M. Pt. (◦C)[a] | V. P. (Pa at 25 ◦C)[a] | Aq. Sol. (mg/l)[b] | Log $K_{ow}$ [c] | IARC group[a] | Half-life (days)[d] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | Naphthalene | $C_{10}H_8$ | | 91-20-3 | 218 | 80.2 | 11 | 31.7±0.26 | 3.35 | NE | 5.66 |
| 2. | Fluorene | $C_{13}H_{10}$ | | 86-73-7 | 295 | 116.7 | 0.08 | 1.98±0.04 | 4.18 | 3 | 15.14 |
| 3. | Anthracene | $C_{14}H_{10}$ | | 120-12-7 | 342 | 216.4 | $1\times10^{-3}$ | 0.073±0.005 | 4.54 | 3 | 123 |
| 4. | Phenanthrene | $C_{14}H_{10}$ | | 85-01-8 | 340 | 100.5 | $2\times10^{-2}$ | 1.29 ± 0.07 | 4.57 | 3 | 14.97 |
| 5. | Fluoranthene | $C_{16}H_{10}$ | | 206-44-0 | 375 | 108.8 | $1.2\times10^{-3}$ | 0.26±0.002 | 5.22 | 3 | 191.4 |
| 6. | Pyrene | $C_{16}H_{10}$ | | 129-00-0 | 150.4 | 393 | $6.0\times10^{-4}$ | 0.135±0.005 | 5.18 | 3 | 283.4 |
| 7. | Benzo[a]anthracene | $C_{18}H_{12}$ | | 56-55-3 | 438 | 158 | 0.548 | 0.014±0.0002 | 5.91 | 2A | 343.8 |
| 8. | Benzo[a]pyrene | $C_{20}H_{12}$ | | 50-32-8 | 495 | 179 | $4\times10^{-7}$ | 0.0038±0.00031 | 5.98 | 2A | 421.6 |
| 9. | Benzo[ghi]perylene | $C_{22}H_{12}$ | | 191-24-2 | 500 | 277 | $6\times10^{-8}$ | 0.00026±0.00001 | 7.10 | 3 | 517.1 |

Note: CAS- Chemical Abstracts Service, B.Pt.- Boiling point, M.Pt.- Melting point, V.P.- Vapor pressure, Aq. Sol.- Aqueous solution, Log $K_{ow}$ - Octanol/water partition coefficients, IARC- *International Agency for Research on Cancer*

a- WGPAH-2001

b- Mackay and Shiu, 1977

c- Michele *et al.,* 1985; Howard *et al.,* 2005

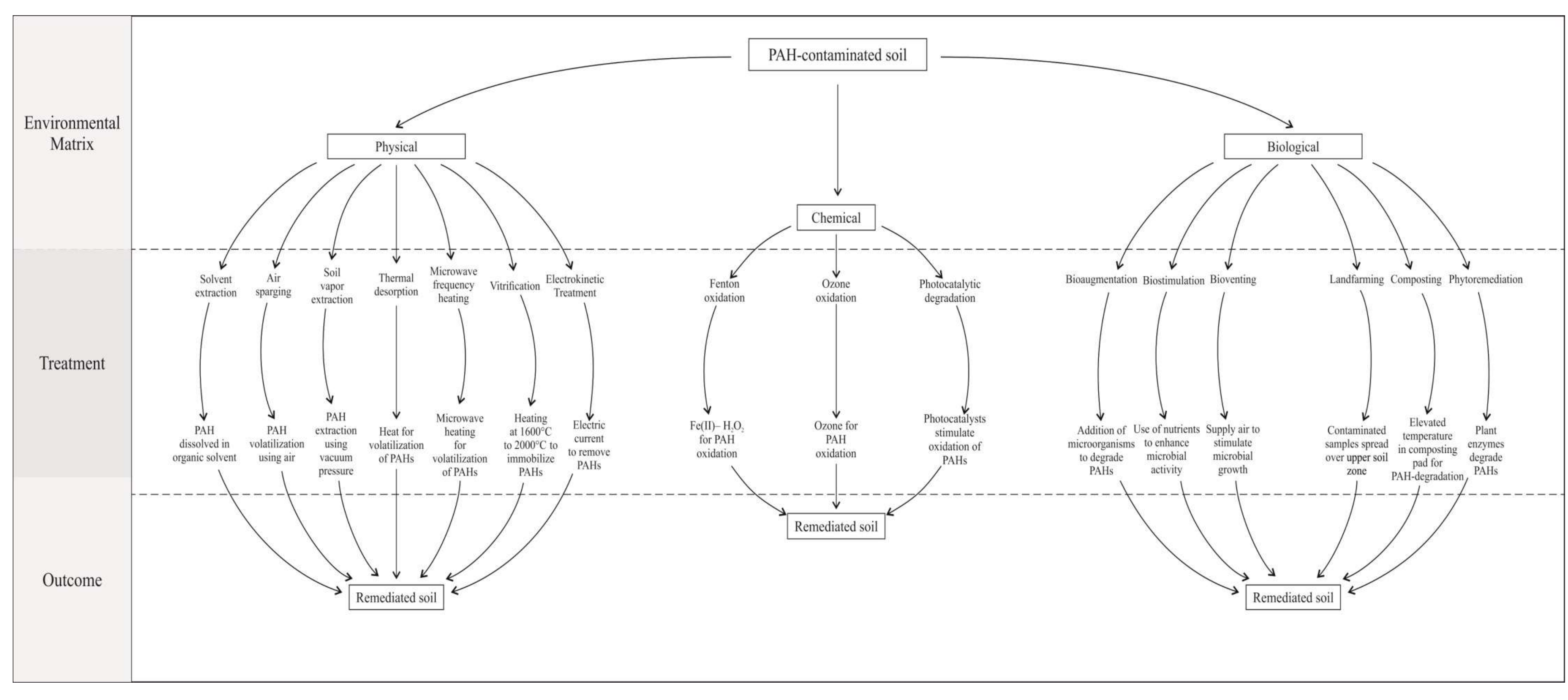



**Fig. 2.1 Physical, chemical, and biological methods for treatment of hydrocarbon-contaminated soil (Sakshi *et al.,* 2019)**

**Table 2.2 Sector-wise classification of sources of PAHs in contaminated soil**

| S. No. | Type of Sources | Activities | Process | Reference |
|---|---|---|---|---|
| 1. | Pyrogenic (associated with the combustion of wood, petroleum product, coal) | Industrial (Anthropogenic) activities | Wood burning | Wilson and Jones, 1993 |
| | | | Burning of tires | Downard *et al.,*, 2015 |
| | | | Burning of fossil fuel | McRae *et al.,*, 2000 |
| | | | Burning of tobacco | Haussmann, 2012 |
| | | | Burning of agricultural waste | Lai *et al.,* 2009 |
| | | | Combustion of oil, diesel, coal, and oil products | Kaushik *et al.,* 2012 |
| | | Natural activities | Volcanic eruption | Kozak *et al.,* 2017 |
| | | | Forest fire | Denis et al., 2012 |
| 2. | Petrogenic (associated with substances originate from crude oil/ petroleum) | Industrial (Anthropogenic) activities | Oil spill | Soriano *et al.,* 2006 |
| | | | Outlets from oil refinery | Pettersen *et al.,*1997 |
| | | | Petroleum handling facilities like kerosene tank, generating plant, petrol stations, mechanic workshops, leaking pipeline, and airport fuel dump | Nganje *et al.,* 2007 |
| | | | Petroleum oil industries and industrial activities<br>Creosote, asphalt production | Varjani *et al.,*2017 |
| | | | Used engine oil, jet fuel, kerosene | Abdel-Shafy and Mansour,2016<br>Kaushik and Haritash, 2006;<br>Kaushik *et al.,* 2012 |
| | | Natural activities | Manufactured gas plants | Abdel-Shafy and Mansour,2016 |
| | | | Oil seeps | Pampanin and Sydnes, 2013 |
| 3. | Biogenic | Natural activities | Surface waxes of leaves and insect cuticles | Oleszczuk and Baran, 2005 |
| | | | The wood of tropical forests | Krauss *et al.,* 2005 |
| | | | PAH synthesis in termite organisms | Krauss *et al.,* 2005 |
| | | | Pine needles | Ratola *et al.,* 2006 |

**Table 2.3 Polyaromatic hydrocarbons in soils and sediments of different geographical regions of the world**

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| **India** (ASIA) | Agra (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)F, CHRY, B(b)F, B(k)F, B(a)P, and B(ghi)P | 3190-28540 | NM; NM | Masih and Taneja, 2006 |
| | Delhi (Vehicular traffic soil) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 1062-9652 | NM; NM | Agarwal, 2009 |
| | Assam and Arunachal Pradesh (Soil from Indian Himalayan Region) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 15.3-4762 | NM; NM | Devi *et al.,* 2016 |
| **China** (ASIA) | Beijing (Urban soils, <10 m from roads) | NAP, ACEY, ACEN, FLU, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, IPYR, D(ah)A, and B(ghi)P, | 1470-6610 | NM; NM | Chu *et al.,* 2003 |
| | Macao (coastal sediments) | PHE, ANT, PYR, MPHE , FLA, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, B(e)P, IPYR, D(ah)A, and B(ghi)P, | 294 - 12 741 | NM; 0.42-1.7 | Mai *et al.,* 2003 |
| | Hong Kong (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 7.0-410 | 4.62-7.52; 0.532-2.92 | Zhang *et al.,* 2006 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| **China** (ASIA) | Hong Kong (Mangrove swamps sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 356-11,098 | NM; 1.23-7.50 | Tam *et al.*, 2001 |
| | North and tropical forests in South China (forest Soils) | ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, CHRY, B(a)A, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 24.81-4539 | NM; 2.007-52.375 | Syed *et al.*, 2017 |
| **Russia** (ASIA) | Moscow and the south Taiga near Moscow (Grassland and forest soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, B(e)P, PERY, D(ah)A, B(ghi)P and COR | 59-1350 | 3.8-5.9; 1-6.8 | Wilcke *et al.*, 2005 |
| **Thailand** (ASIA) | Bangkok (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, B(e)P, PERY, D(ah)A, B(ghi)P, and IPYR | 12-380 | 3.6-7.4; 1.04±0.69-2.03±1.94 | Wilcke *et al.*, 1999b |
| **South Korea** (ASIA) | Korean peninsula (Paddy soils and Upland soils) | NAP, ACEY, ACEN, FLU, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 23.3-2834 | NM; NM | Nam *et al.*, 2003 |
| **Korea** (ASIA) | Kyeonggi Bay (sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, PERY, D(ah)A, B(ghi)P, and IPYR | 9.1–1400 | NM; 0.08-1.2 | Kim *et al.*, 1999 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| **Taiwan** (ASIA) | Gao-ping River (sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 8 to 356 | NM; NM | Doong and Lin, 2003 |
| **Germany** (EUROPE) | Stephanskirchen (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, PERY, and B(e)P | 400-470,000 | 4.82-12.21; 1.2-44.5 | Wilcke *et al.,* 1997 |
| **Switzerland** (EUROPE) | Swiss soil monitoring network (NABO) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 50-619 | 3.9-7.2; 1.0-22.7 | Bucheli *et al.,* 2004 |
| Poland (EUROPE) | Upper Silesia (Agricultural soil) | FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 28-2447 | 3.8-6.3; 1.45-17.20 | Maliszewska-Kordybach, 1996 |
| Sweden (EUROPE) | Karlstad (gasworks plant soil), and Stockholm (wood tar production site ) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, and B(ghi)P | 270-2601000 | 4.7-8.63; 2.0±0.3-49.1-0.4 | Arp *et al.,* 2014 |
| **France** (EUROPE) | Coke oven plant and metallurgy site | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 78000-2651000 | 7.5-8.35; 7.7±2.1-33±0.6 | Arp *et al.,* 2014 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| Belgium (EUROPE) | Gasworks site | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 296880 | 7.57; 2.5 | Arp *et al.,* 2014 |
| Slovak Republic (EUROPE) | Ziar/Central Slovakia (Forest soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, B(e)P, PERY, D(ah)A, B(ghi)P, and IPYR | 40,000-200,000 | 3.85-4.30; 0.81-2.46 | Wilcke *et al.,* 1996 |
| Czech Republic (EUROPE) | Bohemian mountains (Forest soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, B(e)P, PERY, B(ghi)P, and IPYR | 2000-30,000 | NM; 4.3-46.5 | Wilcke and Zech, 1997 |
|  | Valasske Mezirici (agricultural and forest soil) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 860-10840 (agricultural) 7660-79390 (forest soil) | 4.62-7.12; 1.78-3.82 3.24-4.03; 3.45-19.20 | Plachá, *et al.,* 2009 |
| Spain (EUROPE) | Tarragona County, Catalonia (soil and vegetation samples) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, PERY, D(ah)A, B(ghi)P, and IPYR | 112-1002 | 7.08-8.22; 2.6-9.5 | Nadal *et al.,* 2004 |
| Italy (EUROPE) | Chioggia and Ancona (sediments of Adriatic Sea) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 24.1–501.1 | NM; NM | Magi *et al.,* 2002 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| Tunisia (AFRICA) | Sfax (Sfax–Kerkennah coastal zone sediments) | NAP, PHE, ANT, FLA, PYR, B(k)F, CHRY, B(a)P, PERY, B(a)A, and B(ghi)P | 110-10,720 | NM; NM | Zaghden *et al.,* 2007 |
| **South Africa** (AFRICA) | South Africa (Soils from industrial, residential, and agricultural areas) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 44-39,000 | NM; 0.0000001-0.00000049 | Nieuwoudt *et al.,* 2011 |
| **Nigeria** (AFRICA) | Okobo (Soil from the vicinity of a coal mine) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 0-10 | NM; 4-8 | Ugwu and Ukoha, 2016 |
| ***United States*** (NORTH AMERICA) | New Orleans, Louisiana (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(j)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 731-2927 | NM; NM | Mielke *et al.,* 2004 |
| **Canada** (NORTH AMERICA) | Kitimat fjord (sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 1000-10000000 | NM; 1.4-30 | Simpson *et al.,* 1996 |
| **Cuba** (NORTH AMERICA) | Havana (Soils of Mayabeque) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 20-106 | 5.3-8.2; 1.26-4.70 | Sosa *et al.,* 2017 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| **Mexico** (NORTH AMERICA) | San Nicolás (soils from brick manufacturing site) | NAP, ACEY, FLU, ANT, PHE, B(a)A, CHRY, FLA, PYR, B(b)F, B(k)F, B(a)P, and D(ah)A | 7-1384 | NM; NM | Barrán-Berdón *et al.,* 2012 |
| | Uberlândia (Urban soils) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(j)F, B(k)F, B(a)P,PERY, D(ah)A, B(ghi)P, and IPYR | 7-390 | 4.6-8.4; 0.37-2.34 | Wilcke *et al.,* 1999a |
| **Brazil** (SOUTH AMERICA) | Argentinean Pampas to the Patagonian Steppe (Uppermost topsoil and the first subsoil horizons) | NAP, 2-MNAP, 1-MNAP, 1,3-DMNAP,ACEY, ACEN, FLU, PHE,ANT, 1-MPHE, 3,6-DMPHE, FLA, PYR, B(a)A, CHRY, B(b)F, B(j)F, B(k)F, B(a)P, B(e)P, PERY, D(ah)A, B(ghi)P, COR, and IPYR | 2.4-38 | NM; NM | Wilcke *et al.,* 2014 |
| **Argentina** (SOUTH AMERICA) | South Central Chile (Laja River Basin soil) | NAP, FLU, PHE, ANT, FLA, PYR, B(a)A, and CHRY | 600-4243 | NM; NM | Barra *et al.,* 2005 |
| **Chile** (SOUTH AMERICA) | Sydney Harbour (Surface sediment) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, PERY, D(ah)A, B(ghi)P, and IPYR | 100-380,000 | NM; NM | McCready *et al.,* 2000 |
| **Australia** | Kogarah, Sydney (Surface natural soils of Urban area) (NS-surface natural soil, RDS-road deposited sediments and WS-water sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, PERY, D(ah)A, B(ghi)P, and IPYR | NS-400-7490: RDS-1650-4000; WS-490-5190 | NM; 1.8-3.8<br>NM; 0.8-13.0<br>NM; 0.3-3.0 | Nguyen *et al.,* 2014 |

| Country | Location | PAHs* | Total PAH range (ng $g^{-1}$) | Soil pH; TOC/OC (%) | References |
|---|---|---|---|---|---|
| **Australia** | McMurdo Station (Surface soil) | NAP, ACEY, ACEN, PHE, FLU, ANT, FLA, PYR, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(e)P, D(ah)P, D(ac)P, B(b)C, and COR | 1000,000-27000,000 | NM; NM | Mazzera *et al.,* 1999 |
| **Ross Island** (ANTARCTICA) | Scott Base, Marble Point, and the Wright Valley (surface and subsurface soil) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 41-8105 | NM; NM | Aislabie *et al.,* 1999 |
| **McMurdo Sound - Dry Valley Region** (ANTARCTICA) | James Ross Island (soil) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 34.9-171 | NM; 0.09-0.34 | Kla´nova *et al.,* 2008 |
| **Antarctic Peninsula** (ANTARCTICA) | James Ross Island (sediments) | NAP, ACEY, ACEN, FLU, PHE, ANT, FLA, PYR, B(a)A, CHRY, B(b)F, B(k)F, B(a)P, D(ah)A, B(ghi)P, and IPYR | 1.4-205 | NM; 0.04-1.71 | Kla´nova *et al.,* 2008 |

*NAP-Naphthalene, MNAP-Methylnaphthalene, 2-MNAP-2-methylnaphthalene, 1-MNAP-1-methylnaphthalene, 1,3-DMNAP-1,3-dimethylnaphthalene, ACEY-Acenaphthylene, ACEN-Acenaphthene, FLU-Fluorene, PHE-Phenanthrene, 1-MPHE-1-methylphenanthrene, 3,6-DMPHE-3,6-dimethylphenanthrene, ANT-Anthracene, FLA-Fluoranthene, B(j)F-benzo(j)fluoranthene, PYR-Pyrene, B(a)A-Benzo(a)anthracene, CHRY-Chrysene, B(b)C-Benzo(b)chrysene, B(a)F-Benzo(a)fluoranthene, B(b)F-Benzo(b)fluoranthene, B(k)F-Benzo(k)flouranthene, B(a)P-Benzo(a)pyrene, D(ah)A-Dibenz(ah)anthracene, D(ah)P-Dibenzo(ah)pyrene, D(ac)P-Dibenzo(ah)pyrene B(ghi)P-Benzo(g,h,i)perylene, IPYR-Indeno(1,2,3-cd)pyrene, B(e)P-Benzo(e)pyrene, PERY-Perylene, and COR-Coronene.
$ NM: Not Mentioned

A number of studies report the isolation of microbial species for PAH-degradation, and analysis of metabolic and genetic mechanisms for PAH-bioremediation and several catabolic genes encoding PAH-degrading enzymes (to detoxify and mineralize PAHs eventually leading to its degradation) have been identified. Various catabolic enzymes such as mono-oxygenases, dioxygenases such as catechol 1, 2-dioxygenase, and lipases are involved in the transformation/degradation of PAHs like phenanthrene, pyrene, anthracene, fluorene, and B(a)P (Lyu *et al.,* 2014; Pandey *et al.,* 2012). Various genes such as *nah*, *phn*, *nid,* etc. encoding catabolic enzymes are responsible for PAH-degradation and these genes were found upregulated after PAH exposure (Dore *et al.,* 2003, Darmawan *et al.,* 2015; Lyu *et al.,* 2014). The main aim of these studies is to understand the genetic mechanism for PAH-degradation which is facilitated by the catabolic genes code for specific enzymes involved in the PAH degradation pathway.

Presently, advanced genomics, proteomics, transcriptomics, and metabolomics are some high-throughput technologies that help researchers in better understanding the metabolic ability of microorganisms for biodegradation and how the genetic material of a microbe is responsible for degradation in different environmental conditions (Gupta *et al.,* 2015; Pérez-Llano *et al.,* 2018). Genetic investigation of microbial species responsible for PAH-degradation help in identifying the genes responsible for degradation and phylogenetic analysis helps in identifying the other species with similar genetic makeup. This cannot only help to identify potential microbial species but also arrive at the evolutionary relationship of different species possessing the PAH-catabolic genes. There are a number of species such as *Gordonia bronchialis*, *Gordonia namibiensis*, *Gordonia rubripertincta*, *Gordonia sputi*, *Williamsia muralis*, and *Corynebacterium efficiens* which were found to have catechol 1,2-dioxygenase gene (Shen *et al.,* 2009) and it has been studied that catechol 1,2-dioxygenase gene is involved in PAH-degradation (Zhou *et al.,* 2013a, b; Hesham *et al.,* 2014; Cao *et al.,* 2015). These species having catechol 1,2-dioxygenase gene involved PAH degradation were not studied for PAH-degradation but these species could have an evolutionary relationship with PAH-degrading bacterial species and may also have a role in PAH-degradation due to the presence of catechol 1,2-dioxygenase gene. Studies on the degradation of PAHs using the newly-identified species have a possibility of an enhanced level of degradation. The combination of already studied species with the new species can help understand the inhibition and co-metabolism existing among them. Since the genetic code regulates the production of catabolic enzymes, the combination of microbes may be based on enzymes being responsible for the catalysis of PAH-degradation to target reduce time and cost during

remediation of contaminated sites. Further, the genes coding specific cleaving enzymes may be introduced into the desired microorganism. Moreover, genetic investigation opens the possibility of the introduction of catabolic genes which may produce enzymes responsible either for ring-opening in the initial stage or mineralization in the later stage. The genetic investigation may, therefore, be a black box full of information relevant to the degradation of hydrocarbons. Therefore, understanding catabolic pathways, mechanisms, and responsible gene encoding catabolic enzymes is important for effective removal/degradation of PAHs using the bioremediation technique. Likewise, genetic information assists researchers to select appropriate genes to develop genetically engineered microorganisms (GEMs) having high PAH-degradation efficiency.

## 2.1 Ecotoxicity of PAHs

As PAHs are found in all environmental matrices, it has critical effects on all forms of life. PAH-toxicity is due to the formation of reactive metabolites. All PAHs are not found to have similar toxicity because of their different structure which affects PAH-metabolism. Another factor that determines PAH-toxicity is the amount or effective dose of PAHs that actually interact with the target site (ATSDR, 1995; 2009). A number of studies have been intended at assessing the risk of PAH-exposure on human health such as cancer (White *et al.,* 2016; Ugochukwu *et al.,* 2018), cardiovascular problems (Hu *et al.,* 2018), and oxidative stress (Lu *et al.,* 2016). Toxic response of PAHs (Fig. 2.2) can be considered at three levels *i.e.* response of organism (death, impaired growth, developmental abnormalities, and edema), mode of action (carcinogenicity, immunotoxicity, teratogenicity, and mutagenicity), and the mechanism of toxic action (specific biochemical event) (Meador *et al.,* 2008; Carls *et al.,* 2009; ATSDR, 2009). The accidental oil spills can even cause damage to the sea and shoreline organisms or marine ecosystem (Saul *et al.,* 2005). Fish are highly sensitive to the toxic effects of PAHs. Some observed effects include narcosis, mortality in all life stages, decrease in growth, cardiac dysfunction, a variety of deformities, and tumors of the skin and liver, cataracts, damage to immune systems, bioaccumulation, and trophic transfer (Logan, 2007). PAHs are toxic to bird embryos, young birds, and adult birds. For embryos, effects include death, developmental abnormalities, and a variety of cellular and biochemical responses. For adult and young birds, effects include reduced egg production, reduced growth, increased organ weights, and a variety of biochemical responses (Albers, 2006). PAH exposure in human beings can cause adverse health problems. short-term exposure of PAHs can cause eye and skin irritation, nausea, and vomiting, and long-term exposure of PAHs can

cause skin, lung, bladder, gastrointestinal, breast, colon, and prostate cancer, deoxyribonucleic acid (DNA), liver and kidney damage, and gene mutation causing cell-damaging (Kim *et al.,* 2013). PAHs have been extensively studied for their carcinogenic and mutagenic effects and many PAHs have also been found to show teratogenic as well as immunotoxic effects.

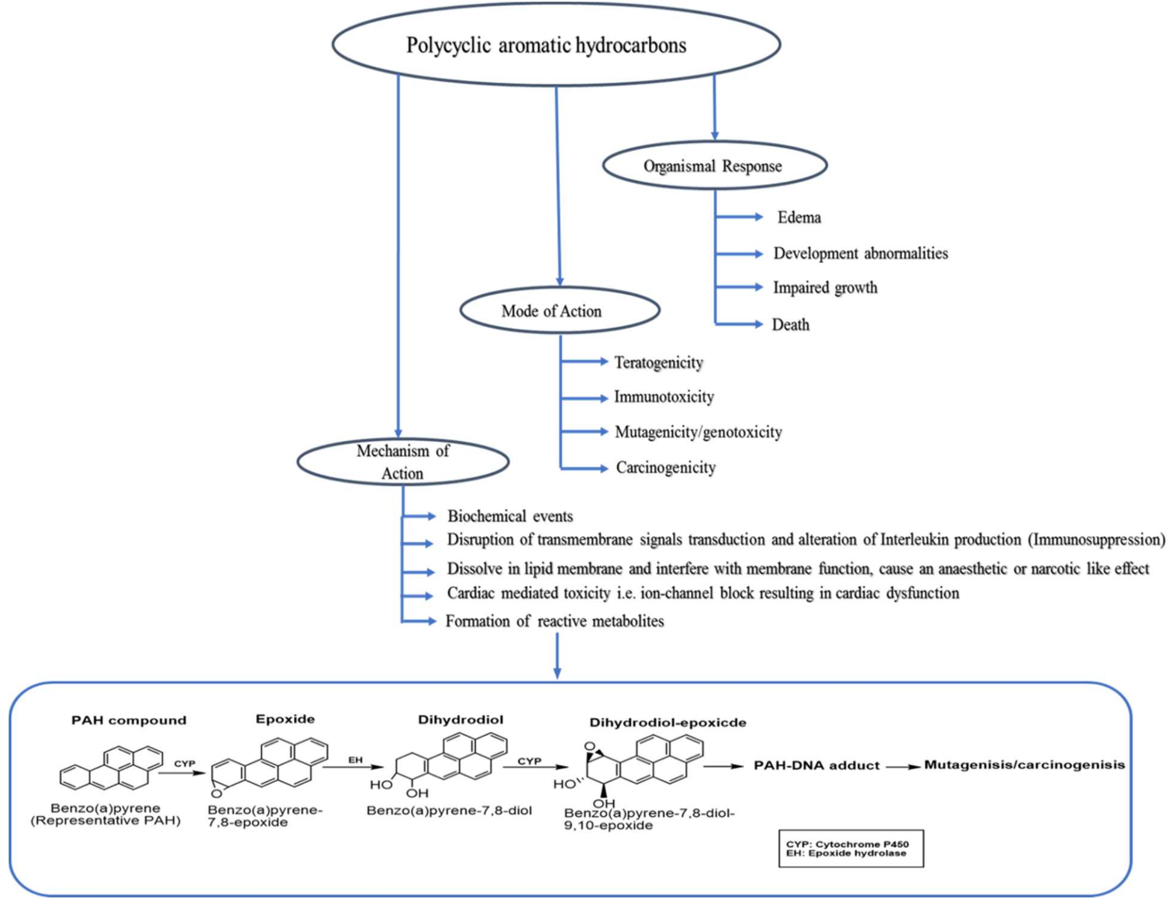



**Fig. 2.2 Toxic response of polycyclic aromatic hydrocarbons over organisms (Sakshi and Haritash, 2020)**

### 2.1.1 Teratogenicity

PAHs have been found to have embryotoxic or teratogenic effects and described in experimental animals exposed to different PAHs such as benzo(a)anthracene, B(a)P, and naphthalene (Wassenberg and Di-Giulio, 2004). B(a)P, a well-known carcinogen was found to have a toxic effect on developing chick embryos. Retarded growth *i.e.* lower embryonic weight, and decreased crown-rump length was observed when chick embryos were exposed with B(a)P at different development stages via the yolk sac route. Abnormalities such as

twisted legs with shortening of bones, abdominal edema, blisters, and short neck were observed in some chick embryo survivors after B(a)P exposure (Anwer and Mehrotra, 1988). It has been investigated that high-level ingestion of B(a)P in mice during pregnancy results in birth defects and reduced body weight in progeny (Kristensen *et al.,* 1995). PAH birth effects have been evaluated on African-American women residing in Washington Heights, Central Harlem, and the South Bronx, New York, and it was observed that transplacental exposure to PAH pollution during pregnancy results in adverse birth effects such as lower birth weight and smaller head circumference (Perera *et al.,* 2003).

### 2.1.2 Immunotoxicity

The immune system is the common target for PAH-toxic effects and due to this reason, PAH-immunogenicity/immunotoxicity has been widely examined in different studies using animal models. The effects of PAHs on the immune system of animals have been reviewed significantly (White Jr, 1986). B(a)P and the prototype methylated PAH *i.e.* 7,12-di-methylbenz(a)anthracene (DMBA) are the most extensively studied PAHs and it was reviewed that B(a)P exposure could suppress the humoral immune response and DMBA exposure could suppress the cell-mediated immune response, natural killer (NK) cell activity and humoral immune response. Immunotoxicity of PAHs on human beings has been evaluated and it has been examined that human T cells are highly sensitive to suppression of mitogen effects of PAHs. B(a)P and DMBA are found highly immunotoxic in the human system and showed suppression of T lymphocyte mitogenesis (Davila *et al.,* 1996). It has been evaluated that PAHs are involved in the immune-suppression mechanism in rodents (IPCS, 2010).

### 2.1.3 Mutagenicity/ Genotoxicity

PAHs have been investigated for their mutagenic or genotoxic effects. Genotoxicity has been found to play a crucial role in the carcinogenicity process. Most of the parent PAHs have not been found genotoxic but their intermediate metabolized diol-epoxides introduce genotoxic damage as they react with DNA and form adducts (PAH-DNA adduct) leading to base pair substitution and mutation. The DNA destruction caused by the reactive PAH-metabolites involves DNA covalent binding results in the formation of stable or depurinating adducts and the oxidative damage of DNA (Denissenko, 1996; Xue and Warshawsky, 2005; Gamboa *et al.,* 2008). Adduct formation may have an effect on DNA repair mechanisms and cause carcinogenesis due to mutation in DNA. It was examined that B(a)P diol epoxide bind

with DNA and causes cancer (Denissenko, 1996). Molecular examinations have shown that PAH-DNA adducts in DNA obstruct polymerase replication activity and contribute to enhancing DNA damage by lowering repair activity (Hsu *et al.,* 2005).

### 2.1.4 Carcinogenicity

All PAHs are not found to have similar carcinogenicity, and some parent PAHs are weak carcinogens as compared to their metabolites (ATSDR, 2009). The International Agency for Research on Cancer (IARC) has summarized PAHs in carcinogenic group (WGPAH, 2001). Although PAHs have been found to have toxic effects on living organisms, but there is a major concern about their reactive metabolites such as epoxides and dihydrodiols as they have the tendency to bind with cellular proteins and DNA. This results in biochemical disruption and cell damage which leads to mutation, developmental deformities, and cancer (Armstrong *et al.,* 2004). Therefore, PAH-induced carcinogenesis may result when metabolites bind with DNA and form PAH-DNA adduct at a site critical for cell differentiation or growth regulation (ATSDR, 2009). It has been investigated that B(a)P diol epoxide adduct formation occurs at several guanine positions of the p53 tumor suppressor gene which are the same positions where cancer mutations occur during lung cancer (Denissenko, 1996). Occupational studies revealed that workers having long-term exposure to sites containing mixed PAHs have shown an increased risk of skin, lung, gastrointestinal, and bladder cancer (Bach *et al.,* 2003; Armstrong *et al.,* 2004). During lab-scale investigations it has been found that long-period exposure of animals with PAHs has resulted in the development of lung cancer from PAH-inhalation, stomach cancer from PAHs consumption through food, and skin cancer through skin contact with PAHs (USEPA, 2008). Seven PAH compounds *i.e.* benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(k) fluoranthene, chrysene, dibenz(ah)anthracene, and indeno(1,2,3-cd)pyrene have been classified as probable human carcinogens (USEPA, 2008).

## 2.2 Properties of contaminated soils

Soil is the major sink for a broad range of harmful contaminants and these contaminants may affect the physical, chemical, and biological properties of soil. Crude oil is the major source of PAHs which may easily diffuse and get absorbed inside soil particles. Soil is extremely sensitive to different contaminants and its behavior changes under different environmental conditions. Soil contamination may affect geotechnical properties as well as the biological properties of soil.

### 2.2.1 Physico-chemical properties

Properties of soil are affected by different activities occurring on land and different types of contaminants, crude oil accidental spillage, and leakage, etc. Longer contamination may affect geotechnical properties such as Atterberg limits, permeability, hydraulic conductivity, strength parameters, consolidation, compaction and shear strength Effects on properties of soil may be due to partial or full replacement of the soil pore liquid with PAH, and chocking of soil pores with contaminant due to which soil aeration and water infiltration could reduce.

The degree of effects of PAH is regulated by certain important parameters of soil *viz.* grain size of soil particles, organic carbon associated, and to some extent pH of the soil. A number of studies have confirmed that binding of PAHs takes place preferentially on finer grain size *i.e.* silt and clay (Magi *et al.,* 2002). The clay particles have more surface area and as a result, more bonding sites resulting in adsorption of PAHs firmly on the finer fraction of soil. Fine particles also result in less porosity and hence lesser movement of adsorbed contaminants over the period of time which results in persistent toxicity and long-term effects. Some of the studies have also confirmed that HMW-PAHs bind firmly to the soil compared to LMW-PAHs (García-Alonso *et al.,* 2008). Since the inter-particle spaces in fine soil are less, the porosity-mediated effects are minimum resulting in the non-transfer of HMW-PAHs from one location to another due to their hydrophobic nature. Some of the studies have also reported that within-the-particle movement of PAHs from the surface to the core also takes place (Bogan and Trbovic, 2003). The intra-particle movement is ascribed to be the function of organic carbon associated with the soil particles. The two types of domains which interact with contaminant are the surface layer of humic acid and fulvic acid (HA-FA), and a core formed of a hard layer of humin. The HA-FA layer is porous, flexible, and lipophilic, and the humin layer is rigid, hard, and glassy (Xing and Pignatello, 1997). The humin-bound PAHs are firmly bonded to the soil which has been subjected to diagenetic alteration (derived from weathering of sedimentary rocks) (Lueking *et al.,* 2000). On the other hand, soils that have not been subjected to diagenetic alterations have organic matter with soft carbon fraction (HA-FA). Therefore, the effect, as well as treatment strategy of contaminated soil, significantly depends upon the grain size, its piedogenetic profile, and the type/fraction of organic carbon associated with the soil. The pH of soil also, to some extent, regulates the degree of contamination. The HA-FA-rich acidic soils bind preferably with PAHs resulting in higher and persistent contamination whereas high/basic pH results in frequent dissociation of soil particles which does not favor the binding of PAHs (Saba *et al.,*

2010). Thus, organic carbon-rich fine-grained acidic soil is more prone to PAH contamination and its effect; and is difficult to remediate.

### 2.2.2 Biological Properties

The biological activities *i.e.* microbial biomass and enzymatic activities of soil are highly sensitive to environmental pressure/parameters (Labud *et al.,* 2007). Since microbes have the ability to degrade hazardous contaminants, they are involved in remediation and soil self-purification processes. PAHs are toxic even to microbes and contaminated soil has lower self-purification capacity due to a decrease in the microbial activity of soil (Hreniuc *et al.,* 2015). Various experiments have been done under controlled environments to investigate the adverse effect of petroleum hydrocarbon (PAHs) contamination on soil microorganisms and metabolic activities. Microbial activity may be inhibited due to the presence of a high concentration of organic contaminants. Soil contamination with crude oil may develop anaerobic conditions in soil by blocking soil pores with consequent effects on microbial communities of soil (Sutton *et al.,* 2013). By long term contamination of soil by petroleum hydrocarbon and at high concentration of total petroleum hydrocarbons there is a decline in microbial biomass and soil enzyme activity owing to toxicity induced by high molecular weight hydrocarbons *i.e.* PAHs (soil enzymes - dehydrogenase and urease), whereas at low petroleum hydrocarbon concentration there is no effect on microbial biomass and enzymes (Verrhiest *et al.,* 2002; Lipińska *et al.,* 2014). Soil microbial diversity may be affected by PAHs contamination as PAHs may have toxic effects towards microorganisms present in the soil. In an investigation, it was observed that PAH contamination has a significant effect on soil bacterial community structure (Khomarbaghi *et al.,* 2019). In certain cases, PAH contamination may lead to complete loss of specific microbial species and if any particular species from contaminated soil have been damaged then certain essential soil functions may be lost (Muckian *et al.,* 2009). A study was carried out to investigate the effect of pyrene on the bacterial richness and microbial diversity in soil. It has been reported that after pyrene contamination microorganism population belonging to phyla *Chlorflexi, Alphaproteobacteria, Actinobacteria*, *Deltaproteobacteria,* and *Crenarchaeota* were extensively reduced (Ren *et al.,* 2015). The effect of three PAH mixture (phenanthrene, fluoranthene, and benzo(k)fluoranthene ) on the indigenous microbial species of natural fresh water sediment was investigated. It was reported that due to a heavy dose of PAHs microbial activity was adversely affected. At low PAH concentration *i.e.* 30mg PAH/kg no negative effect on microbes was observed but at 300mg PAH/kg harmful effects of PAHs *i.e.*

reduction in bacterial density and partial inhibition of the enzyme activity was observed as compared to the control 0mg PAH/kg (Verrhiest *et al.,* 2002). It has been studied that there is a significant effect of oil contamination on the biological activity of the soil, a strong decline in dehydrogenase and urease enzyme activity was observed in chainsaw oil (containing a complex mixture of PAHs, highly toxic.) contaminated soil. It was also found that there is a major influence of chainsaw oil on earthworm biomass and density (Klamerus-Iwan *et al.,* 2015).

## 2.3 Remediation of PAH-contaminated soils

Today, remediation of polyaromatic hydrocarbon (diesel/crude oil) contaminated soils is a global concern due to adverse risk to public health. Reclamation/remediation of PAH-polluted soils is essential and it can be done by different methods which involve removal/isolation or alteration of the contaminant. Various physical, chemical, thermal, and biological remediation techniques (ex-situ and in-situ) have been developed for soil reclamation. In situ remediation process takes place at the contaminated site, whereas ex-situ remediation is an alternative to in-situ remediation where contaminants are treated off-site. There are some advantages of in-situ remediation. It has lower cost, lower risk factor, limited involvement of human, and environmental surroundings can help in remediation process to transform the contaminants. For ex-situ remediation, contaminated soil is excavated (dig and haul) from the site and transfer to another location for treatment so it requires mechanical as well as civil work. It is costly, time-consuming, more human involvement, direct exposure to the contaminant, and due to digging it can harm underground utilities. There are some regulatory constraints also for ex-situ remediation. Some of these remediation techniques are solvent extraction, ultraviolet (UV) oxidation, photochemical or photocatalytic degradation, bioremediation, and phytoremediation. The selection of suitable remediation technique for contaminated soils depends on several factors such as type of contaminant, future use of contaminated soil, type and properties of soil, budget, etc.

### 2.3.1 Physical treatment

#### 2.3.1.1 Solvent Extraction/ Soil Washing

PAHs have a high tendency to get absorb on organic matter present in soil due to their hydrophobic nature. Solvent extraction/ soil washing is a separation or cleanup technique that is used to separate compounds based on their solubility. This technique can be used for

PAH removal from contaminated soils. Different organic solvents (individual solvent or mixture of solvents), cyclodextrins (β-cyclodextrin (BCD), hydroxypropyl- β -cyclodextrin (HPCD) and methyl- β -cyclodextrin (MCD)), and vegetable oils can be used for extraction of PAHs from soils. The extraction of PAHs from soils is a two-step process. The first step is desorption of compound from soil and the second step involves leaching (elution of compound into extraction fluid) of the desorbed compound. The efficiency of solvent extraction is influenced by the nature of the solvent used in the extraction process and the ratio of the mass of contaminated soil to the volume of solvent (Silva *et al.,* 2005; Viglianti *et al.,* 2006). PAHs from extremely contaminated soils obtained from the manufactured gas site can be extracted effectively using a solvent mixture containing ethanol or 2-propanol with 1-pentanol and water. The solvent mixture 1-pentanol (5%), water (10%), and ethanol (85%) is highly efficient and more effective than a single solvent in the removal of extractable PAH (19 PAH having two to six aromatic rings e.g. Naphthalene, 2-Methyl naphthalene, Acenaphthene, Fluorene, Phenanthrene, Fluoranthene, Pyrene, Benzo(e)pyrene, Benzo(a)pyrene, Benzo(ghi)perylene, etc.) from the soil. The extraction efficiency of this mixture is up to 95% when a 4ml solvent mixture is used for 1g soil for one hour in three crosscurrent wash (extraction) stages (Khodadoust *et al.,* 2000). The organic solvent mixture of Cyclohexane and ethanol (3:1) is suitable for fluoranthene extraction from contaminated soil. Its extraction efficiency is approximately 93% (Rababah and Matsuzawa, 2002a). Similarly, it has been documented that a solvent mixture of ethyl acetate (50%), acetone (40%), and water (10%) is suitable for the extraction of hydrocarbons from soil (Silva *et al.,* 2005). A major concern in this technique is the toxic nature of solvents and the liquid phase *i.e.* the solvent containing desorbed PAHs. Therefore, the selection of solvents is crucial and solvent containing desorbed PAHs exposed to other treatment for complete degradation of extracted PAHs.

#### 2.3.1.2 Organic and Mineral Soil Amendments

The organic and mineral soil amendment is an important process for the reclamation of contaminated soils. These amendments in the contaminated soils help in attaining high remediation efficiency as many biological, chemical, and physical processes start after these amendments. Compost, manures, organic by-products, etc. are organic amendments and foundry sand, gypsum, coal combustion products, volcanic ashes, etc. are mineral amendments that are found to be useful for pollutant degradation (Fernández-Luqueño *et al.,* 2017). In an investigation activated carbon and olive mill waste compost were used as

amendments for PAH contaminated soil and enhanced degradation of total PAHs was observed (García-Delgado *et al.,* 2019). Similarly, it was studied that sand can be used as an efficient amendment as sand amendment increases both oxygen and proton passage which increases soil porosity, reduce Ohmic resistance, and increase charge output. The sand amendment was found to be an effective method to accelerate the degradation of PAHs by bio-electrochemical treatment (Li *et al.,* 2015). In order to allow soil amendments effectively improve remediation of contaminated soils, it is essential to find different amendments which can improve soil porosity, increase microbial activity, and increase pollutant mineralization in different soils while retaining soil functions.

### 2.3.2 Thermal Treatment

Thermal treatment for soil remediation uses heat to destroy contaminants. Organic chemicals such as PAHs can be destroyed or volatilize by heat, these contaminants changes into gases which results in increased mobility, and these gases can be collected in wells for ex-situ treatment. Different techniques have been used under thermal remediation such as thermal desorption, microwave frequency heating, and vitrification.

#### 2.3.2.1 Thermal Desorption

Thermal desorption is a process in which heat is applied to increase the vapor pressure of organic contaminants results in the volatilization of contaminants and release of them from contaminated sites such as soil (Rushton *et al.,* 2007). In this process, volatilized contaminants are carried away or sweep by gas for secondary treatment or removal. High PAHs removal efficiencies could be obtained using thermal desorption. Soil contaminated with different PAHs from a manufacturing gas plant treated by thermal desorption process at laboratory scale. After thermal treatment at maximum temperatures above 450°C, the concentration of different PAHs is reduced to below 0.05mg/kg dry weight. For dibenzo(a,h)anthracene efficiency is around 87% at temperature 250°C, and for fluoranthene and pyrene efficiency is nearly 100% with the temperature above 350°C (Renoldi *et al.,* 2003). The subsurface soil of a wood treatment plant contaminated with benzo(a)Pyrene Equivalents (B(a)P-E) was treated by thermal desorption at field scale. Approximately 12,385 $m^3$ (16,200 cubic yards) of predominantly silty soil containing 30.6 mg/Kg B(a)P-E was treated to a maximum depth of 32 m. After 130 days of post-treatment the remaining B(a)P-E concentration was 0.059 mg/Kg, which is equivalent to 99.8% PAH removal. Therefore, thermal desorption is highly efficient at the field scale for PAH-remediation (Baker *et al.,*

2007). Temperature increase from 100 °C to 250 °C, results in an increase in removal efficiency of diesel from 47% to 100% from sand (Falciglia *et al.,* 2011). Similar laboratory studies also showed that the efficiency of diesel removal from the soil is increased during thermal desorption and removal efficiency depends on soil composition, temperature, temperature time, and concentration of contaminants (Piña *et al.,* 2002; Tatàno *et al.,* 2013).

#### 2.3.2.2 Microwave Frequency Heating

Microwave frequency heating is an effective thermal remediation technique in which microwave energy is converted into thermal energy to eliminate contaminants through volatilization via heating (Rushton *et al.,* 2007). This technique has been effectively proposed for soil remediation (Falciglia *et al.,* 2013). Around 99% diesel oil could be removed within 10 min from contaminated soil using microwave-induced thermal treatment in a modified domestic microwave oven with power 800W and frequency 2.45GHz (Li *et al.,* 2008). A domestic microwave oven with a power 700 W and frequency of 2.45 GHz was used to treat heavy oil (diesel fuels and marine fuels) contaminated soil, and it was found that up to 92.5% diesel and 89.5% marine fuel removed from soil in 20–150s (Chang *et al.,* 2011). 75.6–98.4% petroleum removal was achieved within 3.5h at field-scale remediation of petroleum hydrocarbon contaminated soil using an on-site microwave heating system using the antenna of 4m, with power 2 kW and frequency of 2450 MHz (Chien, 2012). On the other hand, using a modified domestic microwave with a power 1000W and frequency of 2450 MHz, maximum of 95% diesel removed from soil in 5–60 min (Falciglia *et al.,* 2013). Other similar studies also investigated the efficiency of this technique for oil removal from soil. Using this technique up to 100% PAHs could be removed in 60min from soil artificially contaminated with different PAHs (FLU, PHE, ANT, PYR, B(a)A, etc.) when microwave frequency 2.45GHz applied at 1000W for the remediation process. Total removal was achieved for biphenyl and fluorene whereas up to 90% removal was achieved for phenanthrene and anthracene and 50-80% removal was achieved for other PAHs (Falciglia *et al.,* 2017).

#### 2.3.2.3 Vitrification

Vitrification is a thermal technique that can be used for in-situ soil remediation. This technique uses very high temperatures *i.e.* 1600°C to 2000 °C to melt and immobilize contaminants in soil. Heat is delivered to the soil through electric current via molybdenum electrodes. In this process, high temperatures melt the contaminants as well as the soil. After

the melting process electrodes are turned off, allowing fusion of contaminants with soil, and then after cooling both are converted into a glass-like solid (Shearer *et al.,* 1991). Vitrification product *i.e.* glass-like solid is a chemically stable, leach resistant, and crystalline material. Vitrification can be used to treat various organic contaminants such as petroleum products (Hinchee and Smith, 1992). Fly ash is a major source of PAHs, after the vitrification process in a coke bed furnace different PAHs (NAP, ACEY, ACEN, FLA, PYR, CYC, B(a)A, CHYR, B(b)F, B(k)F, B(e)P, B(a)P, PERY, IPYR, B(b)C, B(ghi)P, and COR) present in fly ash significantly dropped from 69.6μg to 3.46ng, which results in high efficiency of PAH depletion. The lowest percent removal of individual PAH is higher than 99.9%. Therefore, PAHs in fly ash are fully destroyed in the vitrification process due to very high temperatures (Kuo *et al.,* 2003).

The thermal methods as mentioned above *i.e.* thermal desorption, microwave frequency heating, and vitrification are essentially taking place under anaerobic conditions since oxygen in the subsurface is absent. The added advantage of thermal processes is the absence of the formation of secondary toxic pollutants that are formed sometimes during PAH-oxidation. Some toxic oxidized products such as epoxides and dihydrodiols are found to be formed and have higher toxicity than the parent PAHs (Cerniglia and Sutherland, 2010)

### 2.3.3 Electrokinetic Treatment

Electrokinetic technique is an in-situ remediation technique where direct electric current is used to remove organic and other contaminants (inorganic and heavy metal) from the soil (Huang *et al.,* 2012; Karaca *et al.,* 2016). During this method, low voltage direct current electric potential is applied through electrodes (anode and cathode). Low voltage electric current causes mobilization of contaminants and their transportation toward electrodes placed inside contaminated soil matrix. Contaminants collected on these electrodes are pumped out for further treatment. Different transport mechanisms are induced by electric current such as Electromigration, electro-osmosis, electrophoresis, and diffusion (Acar *et al.,* 1995). It has been found that using upward electrokinetic remediation process 67% of phenanthrene could be removed after 6 days from contaminated soil (Wang *et al.,* 2007). Electrokinetic treatment combined with different surfactants and complexing agents has been used to increase the desorption and solubility of contaminants. It is observed that 70% phenanthrene was removed after 30 days from kaolin clay using electrokinetic treatment combined with1% Tween 80 and 0.1 M ethylenediaminetetraacetic acid (EDTA) (Alcántara *et al.,* 2012).

### 2.3.4 Chemical treatment

Remediation of PAH contaminated soil by chemical treatment includes methodologies that chemically degrade or transform the contaminants. The chemical treatment process to degrade/transform contaminants involves oxidation using chemical oxidants such as hydrogen peroxide and ozone.

#### 2.3.4.1 Chemical Oxidation

Chemical oxidation treatment involves redox (oxidation/ reduction) reactions that involve electron transfer from one chemical to another chemical. This treatment converts hazardous contaminants into less toxic or nonhazardous compounds. In recent days, advanced oxidation processes (AOPs) involving different oxidants have been used for the treatment of PAHs contaminated soils. Different types of oxidants such as hydrogen peroxide, persulfate, ozone, Fenton's reagent, persulfate, peroxymonosulfate, and potassium permanganate have been used for the remediation of oil-contaminated soil via chemical oxidation reactions (Goi *et al.,* 2006; Do *et al.,* 2009; Do *et al.,* 2010; Yen *et al.,* 2011). Chemical oxidation reactions have been used for the treatment of oil or PAHs contaminated soils, different oxidants can be added to soil to oxidize contaminants (Tsai and Kao, 2009; Rivas, 2006).

***Fenton's reagent***

Fenton's reagent ($Fe(II)–H_2O_2$) for Chemical oxidation remediation uses hydrogen peroxide as the oxidant in the presence of ferrous sulfate to generate free radicals *i.e.* hydroxyl radicals (OH•).

$$Fe(II) + H_2O_2 \rightarrow Fe(III)OH^{2+} + HO\bullet$$

Hydroxyl radicals are powerful oxidants (Fenton, 1894). The use of Fenton's reagent for the chemical oxidation process has been found effective for the remediation of soil contaminated by polycyclic aromatic hydrocarbons (PAHs). Fenton oxidation treatment efficiency on PAH contaminated soils has been found in different laboratory-scale experiments. Soil samples contaminated with fluoranthene, benzo(b)fluoranthene, and benzo(a)pyrene were treated using the Fenton oxidation process. The soil samples were mixed with water to form suspension (2g soil sample in 10ml water) in which 0.01M Fe(II) was added with successive addition of $H_2O_2$. The suspension was then magnetically stirred for a 24 hr process to allow Fenton oxidation at room temperature with no pH adjustment. After 24 hr oxidation process, high efficiency for PAH removal was observed, with removal of 85.7% fluoranthene, 87.4% benzo(b)fluoranthene, and 88.6% benzo(a)pyrene (Flotron *et al.,* 2005). About 24 different

PAHs (2-6 ring) in different nine contaminated soil samples were effectively degraded using Fenton's reagent. PAH degradation efficiency was 40-86% (20g soil in 10ml water) in presence of 30% $H_2O_2$ and 4mM Fe(II) at 70°C and 3 pH (Jonsson *et al.,* 2006). So, the Fenton oxidation process could be used as an efficient remediation technique for PAH-contaminated sites.

***Ozone***

Ozone is one of the stronger oxidizing agent for chemical oxidation technique, which can be used for remediation of PAH-contaminated soil. During ozonation, the ozone molecule may directly attack double bonds and can form reactive hydroxyl radicals. Different intermediates such as quinone and biphenyl-type products are formed in the ring cleavage radical oxidation process (Yao *et al.,* 1998). In situ ozone treatment for soils can be done by injecting gaseous ozone which is the most advanced method and by injecting aqueous ozone because gaseous ozone is more effective than aqueous ozone. It was found that 20% of PAHs remain in the soil after gaseous ozone treatment and 40% of PAHs remain in the soil after aqueous ozone treatment (Masten and Davies, 1997). This is due to easy and more diffusivity of gaseous ozone, resulting in more contact between contaminants and ozone (oxidising agent). In an investigation, it has been shown that ozone could be used to remove phenanthrene from phenanthrene-spiked farm soils. At least 50% of phenanthrene level reduction in air-dried soils was achieved when soil samples were exposed to ozone at 20 ppm for 6 hr (O'Mahony *et al.,* 2006). From PAH contaminated soil 95% of phenanthrene removal was achieved with ozonation for 2.3hr at an ozone flux of 250 mg $hr^{-1}$, 91% of pyrene, and 50% of chrysene were removed using ozone flux of 600 mg $hr^{-1}$ for 4hr (Masten and Davies, 1997). Other similar studies also find the efficiency of ozone around 90-95% in the removal of total petroleum hydrocarbons or diesel fuel from sand or unsaturated soils (Shin *et al.,* 2005; Yu *et al.,* 2007)

***Other oxidants***

Several studies have investigated that other alternative oxidants like Persulfate/Fe (II), Peroxymonosulfate (PMS), Persulfate, $H_2O_2$, and permanganate can also be used for chemical oxidation treatment for diesel and fuel oil (Do *et al.,* 2009; Do *et al.,* 2010; Yen *et al.,* 2011). The feasibility of $KMnO_4$ (potassium permanganate) as an oxidant for removal of PAH from contaminated soil has been investigated and it has been found that there is

significant reduction in PAHs (benzo(a)pyrene-72.1%, pyrene-64.2%, phenanthrene-56.2%, and anthracene53.8%) present in soil (Brown *et al.,* 2003).

### 2.3.4.2 Photocatalytic degradation

Photocatalytic degradation is a process in which photocatalysts are used to stimulate oxidizing reactions *i.e.* photoreaction. This process is used for the treatment of petroleum contaminated soils to destroy organic contaminants in presence of the light radiation. To enhance the degradation rate of fluoranthene, a photocatalytic solar reactor was developed. During the photocatalysis process, both $TiO_2$ and $H_2O_2$ were used for fluoranthene degradation. Fluoranthene degradation efficiency was found 99% in presence of both $TiO_2$ and $H_2O_2$. However, lower fluoranthene degradation (83%) was observed when only $TiO_2$ was present as a catalyst (Rababah and Matsuzawa, 2002b). The photocatalytic degradation of phenanthrene, pyrene, and benzo(a)pyrene on soil surfaces using titanium dioxide $TiO_2$ in presence of UV light was investigated in a photodegradation chamber at 30°C. Photocatalyst $TiO_2$ (0.5%) accelerated the photodegradation process of phenanthrene, pyrene, and benzo(a)pyrene significantly as compared to degradation in absence of $TiO_2$ catalyst, their half-lives being reduced from 533.15 to 130.77 hr, 630.09 to 192.53 hr and 363.22 to 103.26 hr, respectively. There is a synergistic effect of UV light and $TiO_2$ catalyst for PAHs degradation in soil (Zhang *et al.,* 2008). Phenanthrene degradation on soil surface using photocatalysis under UV-irradiation was investigated where nanometer anatase $TiO_2$ was used as photocatalyst. Soil samples spiked with phenanthrene, loaded with $TiO_2$ (0 wt.%, 1 wt.%, 2 wt.%, 3 wt.%, and 4 wt.%) exposed to UV light for 25h. It has been observed that $TiO_2$ significantly increases the degradation rate of phenanthrene with half-life reduced from 45.90 to 31.36 hr for 0 wt% and 4 wt% respectively (Gu *et al.,* 2012). So, $TiO_2$ is an efficient photocatalyst that has been used for the oxidation of PAHs through photocatalytic degradation for the treatment of oil-polluted soils.

### 2.3.5 Bioremediation: Microbial degradation of PAHs

At Present, different physical and chemical treatment processes have been used for remediation of PAH/petroleum polluted sites as remediation of these sites has become a critical challenge. These methods are expensive, complex, and form secondary contaminants. So, bioremediation is an emerging, cost-efficient, and environment-friendly sustainable technique for the reclamation of PAH-contaminated sites. A diverse group of naturally occurring aerobic and anaerobic microorganisms including bacteria (gram-positive and gram-

negative), fungi, and algae have been found to have the ability to utilize PAHs as energy source. Efficient PAH-biodegradation could be achieved when microbial species are present in favorable environmental conditions *w.r.t.* temperature, pH, nutrients, and metabolites (Boopathy, 2000). Naturally occuring microbial species having different PAH-degradation efficiencies have been investigated in several studies (Table 2.4).

The studies have revealed that the most prominently studied microorganisms for PAH-degradation are bacteria followed by fungi and algae. The removal/degradation rates of bacteria are placed above other microorganisms since these represent easy adaptability through molecular level changes leading to the production of catalytic enzymes suitable for the respective substrate. The smaller size, a larger number of cells increases the active surface area for efficient biodegradation of PAHs, relatively larger cell size of fungi and algae along with their susceptibility to environmental conditions like availability of nutrients, sunlight, moisture, etc. may restrict its efficacy towards potential PAH degradation. Since bacterial cells have an edge over other microbes for PAH degradation most of the commercially available products/technologies rely upon bacteria. Different experimental conditions and the microbial strains result in varying degradation efficiency for each microbe. It is noticeable that studies on biotransformation of PAHs have identified various microbial species having the capability to degrade LMW PAHs such as naphthalene, phenanthrene, and anthracene.

Moreover, microbial degradation has turned out to be a promising approach to remediate PAH-contaminated sites under aerobic as well as anaerobic conditions. Aerobic PAH-catabolism and its degradation pathways have been given more attention. Aerobic biotransformation pathways for PAH-degradation have been briefly discussed in different studies (Cerniglia, 1992, 1993; Seo *et al.,* 2009). It has been proposed that aerobic degradation requires molecular oxygen to start an enzymatic attack over PAH molecules. Usually, hydroxylation of aromatic ring is the first step in the aerobic metabolism of PAHs. Incorporation of molecular oxygen into the aromatic ring via action of dioxygenases/monoxygenases results in aromatic-ring oxidation and formation of dihydroxy aromatic intermediates. These intermediates get rearomatized by dehydrogenases. Intradiol or extradiol dioxygenases use these dihydroxy aromatic intermediates as substrate which results in *ortho* (intradiol dioxygenases) or *meta* (extradiol dioxygenases) ring cleavage between two hydroxyl groups using oxygen leading to formation of intermediates such as catechols. These intermediates of ring cleavage are then exposed to successive central pathways resulting in formation of intermediates of Kreb's cycle (Cerniglia, 1992, 1993; Samanta *et al.,* 2002; Johnsen *et al.,* 2005; Cao *et al.,* 2009).

**Table 2.4 PAH-degradation efficiency of different microorganisms**

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| Bacteria | *Mycobacterium sp.* PYR-1 | Oil-contaminated soil | Aerobic; 22-26; 14 | FLA (20) | 46 | Šepič *et al.,*1998 |
| | *Pasteurella sp*. IFA | Oil-contaminated soil | Aerobic; 22-26; 14 | FLA (20) | 24 | Šepič *et al.,*1998 |
| | *Neptunomonas naphthovorans* NAG-2N-126 | Creosote-contaminated Sediment | Aerobic; 20; 7 | NAP (5)<br>PHE (1) | 100<br>100 | Hedlund *et al.,*1999 |
| | *P.* aeruginosa 312A<br>*P.* aeruginosa 332C<br>*P.citronellolis*222A | Petrochemical sludge landfarming site | Aerobic; 38; 48 | ANT (250) | 71<br>24.4<br>51 | Jacques *et al.,*2005 |
| | *Pseudomonas putida* IR1 | Hydrocarbon-contaminated soil | Aerobic; 30; 7 | NAP (200)<br>PHE (200)<br>PYR (200) | 72 ± 2<br>60 ± 4<br>69 ± 3 | Kumar *et al.,*2006 |
| | *Sphingomonas sp*. GY2B | PAH- contaminated soil | Aerobic; 30; 2 | PHE (100) | 99.8 | Tao *et al.,*2007 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Bacteria** | *Pseudomonas citronellolis* 222A | Landfarm used for petrochemical effluent Treatment | Aerobic; 30; 48 | ANT (250) | 72 | Santos *et al.,*2008 |
| | *Janibacter anophelis* JY11 | Oil-contaminated soil | Aerobic; 30; 5 | PHE (500)<br>ANT (500)<br>PYR (500) | 98.5<br>82.1<br>97.7 | Zhang *et al.,*2009 |
| | *Gordonia sp*. strain BS29 | Diesel-contaminated soil | Aerobic; 25; 76 | PHE ( 183)<br>ANT ( 312)<br>PYR (178) | 96.1<br>39.4<br>97.8 | Franzetti *et al.,*2009 |
| | *Leclercia adecarboxylata* PS4040 | Oily sludge-contaminated soil | Aerobic; 30; 20 | PYR (200) | 61.5 | Sarma *et al.,*2009 |
| | *Pseudomonas stutzeri* ZP2 | Soil from oil refinery field | Aerobic; 37; 6 | PHE ( 250) | 96 | Zhao *et al.,*2009 |
| | *Bacillus subtilis* BMT4i | PAH-contaminated soil | Aerobic; 37; 28 | B(a)P (50) | 84.66 | Lily *et al.,*2009 |
| | Sulfate-reducing bacteria (SRB) | Anaerobic swine wastewater sludge | Anaerobic; 30; 21 | FLU (5)<br>PHE (5) | 88<br>65 | Tsai *et al.,*2009 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Bacteria** | *Streptomyces Sp.* | Bitumen-contaminated soil | Aerobic; 30 ± 1; 15 | FLU (100)<br>PHE (100)<br>ANT (100)<br>PYR (100) | 92<br>80<br>60.2<br>28 | Chaudhary *et al.*, 2011 |
| | *Sphingomonas koreensis* ASU-06 | Oil-contaminated soil | Aerobic; 30; 15 | NAP (100)<br>PHE (100)<br>ANT (100)<br>PYR (100) | 100<br>99<br>98<br>92.7 | Hesham *et al.*,2014 |
| | *Staphylococcus nepalensis* | Diesel-contaminated soil | Aerobic; 30; 5 | PYR (50) | 93.25 | Valsala *et al.*,2014 |
| | *Mesoflavibacter zeaxanthinifaciens* | Tokyo bay area | Aerobic; 25; 42 | B(a)P (100) | 86 | Okai *et al.*,2015 |
| | *Micrococcus luteus* | Petroleum-contaminated soil | Aerobic; 30; 15 | NAP (1)<br>PHE (1)<br>FLA (1)<br>PYR (1) | 68.7<br>62.9<br>61.4<br>61.3 | Haritash and Kaushik, 2016 |
| | *Kocuria rosea* | Petroleum-contaminated soil | Aerobic; 30; 15 | NAP (1)<br>PHE (1)<br>FLA (1)<br>PYR (1) | 59.8<br>54.6<br>53.8<br>53.3 | Haritash and Kaushik, 2016 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| Bacteria | *Stenotrophomonas sp.* | Plants growing at PAH-contaminated sites | Aerobic; 30;7 | NAP (100)<br>FLU (100)<br>PHE (100)<br>PYR (100)<br>B(a)P (10) | 98.0<br>83.1<br>87.8<br>14.4<br>1.6 | Zhu *et al.,*2016 |
| | *Pseudomonas sp.* | Plants growing at PAH-contaminated sites | Aerobic; 30; 7 | NAP (100)<br>FLU (100)<br>PHE (100)<br>PYR (100) | 95.3<br>87.9<br>90.4<br>6.9 | Zhu *et al.,*2016 |
| | *Raoultella ornithinolytica* | Diep and Plankenburg rivers | Aerobic; 37; 14 | ACEN (50)<br>FLU (50) | 98.6<br>99.9 | Alegbeleye *et al.,*2017 |
| | *Serratia marcescens* | Diep and Plankenburg rivers | Aerobic; 37;14 | ACEN (50)<br>FLU (50) | 95.7<br>97.90 | Alegbeleye *et al.,*2017 |
| | *Bacillus megaterium* | Diep and Plankenburg rivers | Aerobic; 30; 14 | ACEN (50)<br>FLU (50) | 90.20<br>98.40 | Alegbeleye *et al.,*2017 |
| | *Aeromonas hydrophila* | Diep and Plankenburg rivers | Aerobic; 35; 14 | ACEN (50)<br>FLU (50) | 99.9<br>99.5 | Alegbeleye *et al.,*2017 |
| | *Cellulosimicrobium cellulans* CWS2 | PAH-contaminated soil | Anaerobic; 30; 13 | B(a)P (10) | 78.8 | Qin *et al.,*2018 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Bacteria** | *Achromobacter xylosoxidans PY4* | Arabian Gulf shoreline | Aerobic; 37; 25 | PYR (100) | 80 | Nzila *et al.,* 2018 |
| | *Pseudomonas stutzeri* | Oil spill sites | Aerobic; 30±2; 72 | FLU (500)<br>PHE (500) | 64.97<br>86.32 | Rabodonirina *et al.,* 2019 |
| | *Bacillus simplex* | Oil spill sites | Aerobic; 30±2; 72 | FLU (500)<br>PHE (500) | 86.89<br>95.13 | Rabodonirina *et al.,* 2019 |
| | *Bacillus pumilus* | Oil spill sites | Aerobic; 30±2; 72 | FLU (500)<br>PHE (500) | 76.03<br>87.98 | Rabodonirina *et al.,* 2019 |
| **Fungal** | *Crinipellis campanella*<br>*Crinipellis perniciosa*<br>*Crinipellis stipitaria*<br>*Marasmiellus ramealis*<br>*Marasmius rotula* | Obtained from different Departments | Aerobic; 24; 14 | PYR (20) | 39<br>95<br>94<br>76.5<br>95 | Lange *et al.,*1996 |
| | *Penicillium sp.* 06 | Petrochemical-contaminated soil | Aerobic; 30; 28 | PHE(30)<br>ACEN (30)<br>FLU (30)<br>FLA (30) | 89<br>75<br>75<br>75 | Zheng and Obbard, 2003 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Fungi** | *Fusarium sp.* | Crude oil contaminated soil | Aerobic; 25; 15 | PHE (100)<br>PHE (200)<br>PYR (100)<br>PYR (200) | 83.7<br>70<br>74.6<br>32.1 | Li *et al.,*2005 |
| | *Fusarium sp.* E033 | Leaves of *Pterocarpus macrocarpus* | Aerobic; 32; 30 | B(a)P (100) | 70 | Chulalaksananukul *et al.,*2006 |
| | *Phanerochaete chrysosporium* | Petroleum contaminated soil | Aerobic; 30; 42 | PHE (5)<br>ANT (5)<br>PYR (5)<br>ACEN (5)<br>FLA (5) | 98.96<br>92.6<br>92.2<br>83.8<br>79.8 | Bishnoi *et al.,*2007 |
| | *Aspergillus fumigatus* | Contaminated soils near a gas station | Aerobic; 30; 5 | ANT (10) | 60 | Ye *et al.,*2011 |
| | *Merulius tremellosus* KUC9161 | Small pieces of mushrooms or wood | Aerobic; 27; 15 | PYR (50) | 83.6 | Lee *et al.,*2013 |
| | *Pleurotus eryngii* F032 | Decayed woods | Aerobic; 25; 23 | FLU (10)<br>FLU (20)<br>FLU (30) | 100<br>86.5<br>65.9 | Hadibarata *et al.,*2014 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Fungi** | *Peniophora incarnata* KUC8836 | Korea University Culture collection | Aerobic; 27; 14 | PHE (44.83±2.84)<br>FLA (27.63±1.56)<br>PYR (20.71±1.14) | 86.5<br>77.4<br>82.6 | Lee *et al.,*2015 |
| | *Trichoderma sp.* | Soils at the side of a freeway, gas station, and a lake in Japan | Aerobic; 28; 7 | PYR (10)<br>ANT (10)<br>FLA (10) | 37.4<br>36.4<br>14.3 | Mineki *et al.,*2015 |
| | *Fusarium sp.* | Soils at the side of a freeway, gas station, and a lake in Japan | Aerobic; 28; 7 | PYR (10)<br>ANT (10)<br>FLA (10) | 18.2<br>15.3<br>12.8 | Mineki *et al.,*2015 |
| | *Mycoaciella bispora* KUC8201 | Korea University Culture collection | Aerobic; 27; 14 | ANT (12.3± 0.86) | 61.8 | Lee *et al.,*2015 |
| | *Scopulariopsis brevicaulis* PZ-4 | PAH-contaminated soil | Aerobic; 28; 30 | PHE (100)<br>FLA (100)<br>PYR (100)<br>B(a)A (20) | 60<br>62<br>64<br>82 | Mao and Guan, 2016 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| **Fungi** | Aspergillus *ficuum* | Petroleum-contaminated soil | Aerobic; 30; 15 | NAP (1)<br>PHE (1)<br>FLA (1)<br>PYR (1) | 62.9<br>49.7<br>61.6<br>54.6 | Haritash and Kaushik, 2016 |
| | *Aspergillus fumigatus* | Petroleum-contaminated soil | Aerobic; 30; 15 | NAP (1)<br>PHE (1)<br>FLA (1)<br>PYR (1) | 73.7<br>61.9<br>60.7<br>59.6 | Haritash and Kaushik, 2016 |
| | *Penicillium* sp. CHY-2, | Antarctic soil | Aerobic; 20; 28 | NAP (100)<br>ACEN(100)<br>B(a)P (100)<br>NAP (500)<br>B(a)P (500) | 15<br>10<br>2<br>7<br>1 | Govarthanan *et al.,*2017 |
| | *Coriolopsis byrsina APC5* | Decayed wood surface | Aerobic; 27; 18 | PYR (20) | 96.1 | Agrawal and Shahi, 2017 |
| | *Cladosporium sp.* CBMAI 1237 | Marine sponges | Aerobic; 32; 21 | ANT (50)<br>FLU (50)<br>PHE (50)<br>FLA (50)<br>PYR (50) | 71<br>70<br>47<br>52<br>62 | Birolli *et al.,* 2017 |

| Microbial species | | Source | Condition; Temp (°C); Period (Days) | PAH Compound ($C_i$ - ppm) | Degradation Efficiency (%) | Reference |
|---|---|---|---|---|---|---|
| Algae | *Scenedesmus obliquus* ES-55 | Oil- contaminated soil | Aerobic; 20; 59 | PHE (14) | 42 | Safonova *et al.,*2005 |
| | *Selenastrum capricornutum* | Carolina Biological Supply Company (USA) | Aerobic; 22 ±2; 4 | PHE (1)<br>FLA (0.25)<br>PYR (0.1) | 94<br>100<br>100 | Chan *et al.,*2006 |
| | *Cyclotella caspia* | Tidal water (Futian Mangrove Forest) | Aerobic; 20; 6 | FLA (0.1)<br>FLA (0.2)<br>FLA (0.3) | 86.74<br>85.89<br>83.81 | Liu *et al.,*2006 |
| | *Skeletonema costatum* | Jiulong River | Aerobic; 20 ±1; 7 | PHE (0.95) | 16 | Hong *et al.,*2008 |
| | *Chlorella kessleri* | Botanic institute of Slovak Academy of Science, Bratislava. | Aerobic; *22±3;* 6 | B(a)A (0.1) | 29 | Takáčová *et al.,*2014 |
| | *Selenastrum capricornutum* | Carolina Biological Supply Company, USA | Aerobic; 22 ±2; 4 | B(ghi)P (0.1)<br>B(b)F (0.1)<br>B(k)F (0.1)<br>B(a)A (0.1)<br>B(a)P (0.1) | 13.3<br>74.1<br>55.1<br>79.4<br>64.1 | Luo *et al.,*2014 |

$C_i$ – Initial PAH-Concentration, NAP- Naphthalene, FLU- Fluorene, ANT- Anthracene, ACEN- Acenaphthene, PHE- Phenanthrene, FLA- Fluoranthene, PYR- Pyrene, B(a)A- Benzo[a]anthracene, B(b)F- benzo[b]fluoranthene, B(k)F- benzo[k]fluoranthene, B(a)P- Benzo[a]pyrene, CHRY- chrysene, B(ghi)P-benzo[g,h,i]perylene

There are several studies on PAH-catabolism, but little information is documented about anaerobic degradation pathways. Anaerobic PAH-biodegradation in different environmental matrices such as anaerobic sediments and water-logged soils where molecular oxygen is not present, requires alternate terminal electron acceptors like nitrate ($NO^{3-}$), sulfate ($SO_4^{2-}$), manganese (IV), $CO_2$, and Ferric iron ($Fe^{3+}$), etc. (Meckenstock *et al.,* 2004). It has been explained in a number of studies that anaerobic microbial species could grow on naphthalene and use it as an energy source under nitrate-reducing conditions (Rockne *et al.,* 2000). The pathways for degradation of low molecular weight PAHs like naphthalene and phenanthrene have been studied in detail (Zhang *et al.,* 2006). Some of the investigations have suggested that anaerobic PAH-catabolism is not limited to 2-ring PAHs, and PAHs having more than 2-rings can also be degraded anaerobically. In a study, up to 96% mineralization of phenanthrene after 20 days incubation was observed in a fluidized bed reactor with denitrifying enrichment culture (Rockne and Strand, 2001). Anaerobic degradation of fluorene, phenanthrene, and pyrene in soil by a mixed bacterial consortium has also been observed under denitrifying conditions (Ambrosoli *et al.,* 2005). Significant removal (78.8%) of B(a)P was observed by *Cellulosimicrobium cellulans* CWS2 under nitrate-reducing conditions (Qin *et al.,* 2018). Recently, metabolic pathways for anaerobic biodegradation of fluorene and phenanthrene have been proposed (Tsai *et al.,* 2009).

HMW PAHs having four or more benzene rings fused are found recalcitrant to microbial degradation for both aerobic and anaerobic pathways as chemical stability and persistence increase with the increase in molecular weight, even though some microbial species are found to have the capability of transformation of these compounds as well but at a relatively slow rate. Till now very few investigations have been done to study biotransformation of HMW-PAH. There is a significant concern for B(a)P degradation as it is a HMW-PAH having five benzene rings and it is highly carcinogenic, toxic, and teratogenic (Collins *et al.,* 1991). There are different aerobic and anaerobic transformation pathways proposed for the degradation of B(a)P by bacteria, fungi, and algae (Fig. 2.3). Limited information about metabolic pathways of B(a)P transformation by microbes is available and a limited number of B(a)P metabolites formed during B(a)P transformation have been characterized. cis-4,5-B(a)P-dihydrodiol, 4,5-chrysene-dicarboxylic acid, cis-4-(8-hydroxypyren-7-y1)-2-oxobut-3-enoic acid, 7,8-dihydroxy-pyrene-7-carboxylic acid, 6-phenoxy B(a)P radical, B(a)P 3,6-quinone, diethyl phthalate, and 2-acetyl-3-methoxy-benzoic acid are some B(a)P metabolites formed during degradation process. It has been studied that some of the B(a)P metabolites have carcinogenic properties as B(a)P can oxidize to epoxides

and dihydrodiols by monooxygenase in fungal metabolism. These two metabolites formed during fungal metabolism of B(a)P have a strong affinity for binding with DNA and are carcinogenic. So, due to toxic effects, the characterization of B(a)P metabolites is critical during its transformation (Sutherland, 1992). Microbial degradation of PAHs depends on microbial adaptation *i.e.* gradual development of PAH-degradation ability in microbes upon long-term exposure to PAHs. Still, complete metabolic pathways and genetic regulation of PAH-biodegradation are not known and it is to be ensured that no toxic metabolites are produced by the microbial species involved in bioremediation. In order to understand the biochemical transformation of PAHs, it is important to study the molecular level details of genetic make-up and the enzymes being regulated by the genetic material of the microbes involved.

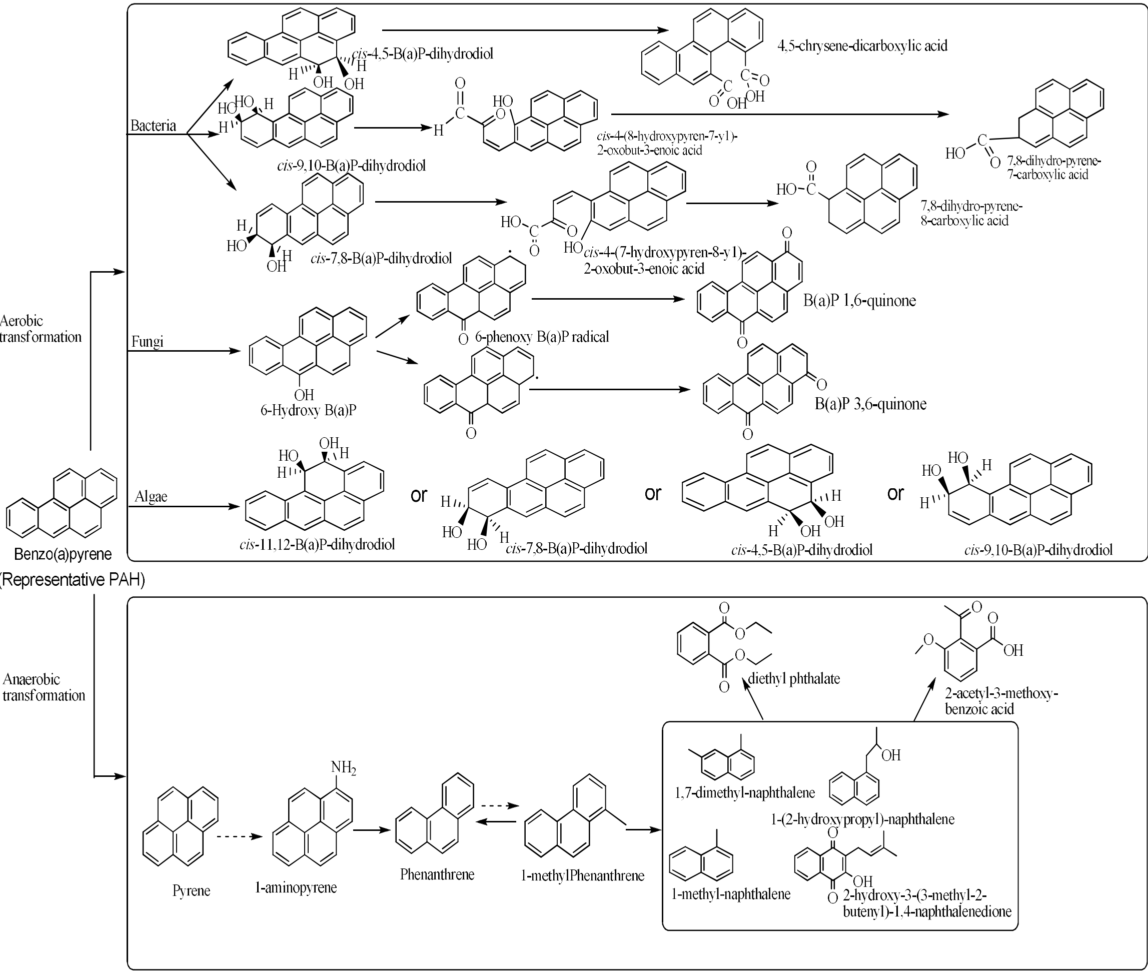



**Fig. 2.3 Aerobic and anaerobic pathways for microbial transformation of Benzo(a)pyrene (Sakshi and Haritash, 2020)**

### 2.4 Catabolic enzymes participating in PAH-degradation

Enzyme-mediated bioremediation of pollutants is an emerging technology, offering a more effective approach than other chemical treatments for PAH-bioremediation. In this process, microbial enzymes are used to transform toxic PAHs into less toxic metabolites. Once the catabolic genes are expressed in presence of PAHs and translated into catabolic enzymes, it results in the metabolism of PAHs. Pollutant-biodegradation involves successive steps using diverse enzymes (Abbasian *et al.,* 2015) and the enzyme systems involved in PAH-metabolism are different oxidases. Various catabolic enzymes such as oxygenases (mono-oxygenases and di-oxygenases), dehydrogenases, and fungal lignolytic enzymes such as LnP (lignin peroxidase), MnP (manganese peroxidase) and laccase are involved in PAH-transformation into $CO_2$ and $H_2O$ (Lyu *et al.,* 2014; Pandey *et al.,* 2012; Agrawal and Shahi, 2017; Al-Hawash *et al.,* 2018). The fungal lignolytic enzymes are involved in oxidation, and catalyze radical formation leading to bond- destabilization in hydrocarbons, eventually resulting in degradation (Ball and Jackson, 1995; Hofrichhter *et al.,* 1999). Dioxygenases generally consist of different subunits such as reductase, ferredoxin, and terminal oxygenase (Mallick *et al.,* 2011). Laccases and cytochrome P450 monooxygenases are oxido-reductases that have been isolated from various microbial species and extensively investigated for PAH-degradation in the last few decades (as some bacterial species can degrade PAHs using cytochrome P450-mediated pathway) (Hammel *et al.,* 1986; Moody *et al.,* 2004). The role of lignolytic enzymes such as laccases and peroxidases of wood-inhabiting white-rot fungus *Pleurotus ostreatus* and soil-inhabiting fungus *Agaricus bisporus* was determined for degradation of phenanthrene and anthracene. It was observed that lignolytic enzymes were responsible for the formation of quinone metabolites during the degradation process (Pozdnyakova *et al.,* 2018). As discussed earlier, RHDs (ring-hydroxylating dioxygenases) catalyzes the initial and critical PAH-oxidation step generating dihydrodiol products. Number of RHDs such as phthalate dioxygenase (Batie *et al.,* 1987), naphthalene dioxygenases (Parales *et al.,* 2000; Jouanneau *et al.,* 2006), Catechol dioxygenase (Hesham *et al.,* 2014), and biphenyl dioxygenase (Furusawa *et al.,* 2004) have been significantly characterized and identified.

The enzymatic catalytic action on PAHs is very effective compared to chemical catalytic action due to mild reaction conditions, more stereospecificity, and a high reaction rate (Venkata *et al.,* 2002). The knowledge of genes encoding catabolic enzymes should benefit researchers to enhance PAH-degradation performance by microbes. The catabolic enzymes could be used for genetic screening to investigate microorganisms with PAH-

degradation potential. Numbers of research studies have been done to investigate the role of the PAH-catabolic enzymes (Saito *et al.,* 1999, 2000; Mishra and Singh, 2014; Cao *et al.,* 2015). Gene cluster, *phd*EFABGHCD, encoding phenanthrene catabolic enzymes such as phenanthrene dioxygenase in *Nocardioides sp*. strain KP7 was investigated. The plasmid carrying gene phdABCD was introduced in *Escherichia coli* and the transformants carrying phdABCD genes for phenanthrene dioxygenase were proficient in phenanthrene oxidation (Saito *et al.,* 1999). Genetic analysis of *phd* genes from *Nocardioides sp*. strain KP7 reveals genetic organization, an unusual type of ferredoxin component, and phylogenetically diverged positions of the *phd* genes. The analysis suggested a novel class of aromatic ring-hydroxylating dioxygenases *i.e.* phenanthrene dioxygenase present in *Nocardioides sp*. strain KP7 involved in phenanthrene degradation (Saito *et al.,* 2000). Complete genome analysis of *C. indicus* $P73^T$ reveals many genes encoding various catabolic enzymes involved in PAH-metabolism. The genes encode ring hydroxylating dioxygenases, ring cleaving dioxygenases, and other enzymes such as ferredoxin reductase, ferredoxin, hydroxylase, decarboxylase, cytochrome P450, monooxygenase, dehydrogenase, hydratase, thiolase, and racemase involved in fluoranthene degradation. The enzymes coded by catabolic genes may also increase PAH-degradation ability of *C. indicus* $P73^T$ (Cao *et al.,* 2015). Differential expression of catabolic enzymes was also investigated to study its role in PAH-degradation. The differentiated ability of *Pseudomonas aeruginosa* PSA5 and *Rhodococcus sp.* NJ2 to degrade B(a)P by catabolic enzymes was analyzed. Salicylate hydroxylase, 2-carboxybenzaldehyde dehydrogenase, catechol 1,2-dioxygenase and catechol 2,3- dioxygenase were observed to be involved in B(a)P degradation, and found differentially expressed in both microbial species (Mishra and Singh, 2014). Many catabolic enzymes for PAH-degradation have been identified and isolated from a diverse group of microbial species and various novel degradation pathways have been revealed based on initial ring oxidation and ring cleavage products. High oxidative potential and expression of these enzymes may enhance the PAH-degradation ability of microbial species. However, a number of microbial species present in the environmental matrix can efficiently degrade PAHs at laboratory scale under optimum conditions, but most of these microbial species exhibit less efficiency under field conditions. So, microbial capabilities for PAH-degradation could be improved with bio-molecular engineering by altering genetic makeup and creating genetically engineered microorganisms.

### 2.5 Genes responsible for PAH-degradation

PAHs are a diverse group of pollutants that are widely dispersed in the environment and biodegradation of these organic compounds is stimulated by specific catabolic genes (genes involve in PAH-degradation). Even though various investigations for microbial degradation of pollutants have largely improved the understanding of the bioremediation process, the advanced genomics, proteomics, transcriptomics, and metabolomics studies allow researchers to analyze microbial metabolic ability and genetic insight for pollutant degradation. Genetic evaluation has revealed that microbial genes encoding catabolic enzymes are responsible for PAH-degradation (Laurie and Lloyd-Jones, 1999b). Proteomics and transcriptomics are efficient techniques to identify proteins and transcriptome, and their functions in PAH-degradation whereas, metabolomics is an approach used to identify and study metabolites produced during PAH-degradation. A number of investigations have been carried out to reveal the genetic mechanism for PAH-biodegradation and several PAH-catabolic genes for degradation pathways have been categorized which are confined as gene clusters (Kiyohara *et al.,* 1994; Laurie and Lloyd-Jones, 1999b; Singleton *et al.,* 2009). The aerobic, as well as anaerobic degradation of PAHs, has been evaluated in various studies and some catabolic genes have also been identified (Simon *et al.,* 1993; Khan *et al.,* 2001; Hesham *et al.,* 2014). However, relatively less information is available about degradation pathways, catabolic genes, or enzymes for PAH-degradation by anaerobic microbes. The complete study of the genetic makeup of microbes involved in PAH-degradation using high-throughput sequencing enables the understanding of PAH-degradation mechanisms. The complete genome study improves understanding of genetic basis or genes involved in PAH-degradation, secondary metabolite production, and other significant physiological functions. The 16S rRNA sequencing, polymerase chain reaction (PCR) or PCR amplification, DNA hybridization, gel electrophoresis and mass spectrometry for protein analysis, and metabolomic profiling are some of the techniques which have been used during PAH-biodegradation study for the identification of different microbes, their catabolic genes/ enzymes or metabolites produced during biodegradation. These techniques are becoming gradually helpful to identify and quantify metabolic abilities at the genetic level within a microbial species. The 16S rRNA sequencing help in the identification of microbial species isolated from different PAH-contaminated sites, whereas polymerase chain reaction (PCR) and DNA hybridization have been used for the detection of genes in different microbes involved in PAH-degradation (Hamann *et al.,* 1999). The genes may be located on either plasmids or genomic chromosomes (Menn *et al.,* 1993; Pinyakong *et al.,* 2003). However,

several catabolic genes engaged in PAH-degradation are discovered on plasmids (Johnsen *et al.,* 2005).

Diverse clusters of highly conserved PAH-catabolic genes were discovered in many research studies and various PAH-catabolic genes such as *nah, pah, nid, dox, phn, phd, nag, fln* etc. were reported (Table 2.5). Catabolic genes such as *nar, phd, nid* and *pdo* are common in the Gram-positive bacterial species *i.e. Rhodococcus*, *Mycobacterium,* and *Nocardioides* having the capability of PAH degradation, and *nah, nag, ndo, pah* and *phn* are common catabolic genes in the Gram-negative bacterial species *i.e. Pseudomonas, Ralstonia, Burkholderia*, *Sphingomonas,* and *Polaromonas* having PAH-degradation ability.

The *nahAc* gene is vastly conserved in numerous Gram-negative bacterial species, and it is due to environmental horizontal gene transfer which occurred among diverse microbial species (Stuart-Keil *et al.,* 1998). The aerobic microbial degradation of PAHs involves initial dioxygenation by dioxygenase enzyme such as Ring-hydroxylating dioxygenase (RHD) which yield cis-dihydrodiol products subsequently (Cerniglia, 1992; Peng *et al.,* 2008). A gene cluster encoding RHD related to PAH-degradation in the genome of *Rhodococcus sp.* P14 was identified and an increased expression level of this gene was observed when *Rhodococcus sp.* P14 was cultured with anthracene, pyrene, phenanthrene, or benz[a]-anthracene as a single carbon source for growth (Peng *et al.,* 2018). Various factors like gene structure, enzymes, substrates, and metabolites affect catabolic gene expression (Mishra *et al.,* 2001). Knowledge of PAH-catabolic genes in various microbial species may provide valuable information regarding the evolution of diverse degradation pathways and structure-function relationships of catabolic enzymes. There is a direct correlation between microbial degradation rate and catabolic gene copy number. Highly dynamic variations in *nahAc* gene copy numbers were found during naphthalene biodegradation in PAH-contaminated soils. Before naphthalene exposure, *nahAc* gene copy numbers were lower than the detection limits, but after 6 days of exposure with naphthalene gene copy number amplified over to 107 gene copies (Park and Crowley, 2006). Most catabolic genes necessary for complete PAH degradation may be common for different aromatic hydrocarbons and these genes code for enzymes involved in biodegradation.

The catabolic genes may be used to develop primer set for detection of unculturable microbial species involved in PAH-degradation. PCR amplification of genes with specific catabolic gene primers has been applied to identify and study the aromatic-dioxygenase genes in PAH-degrading microbial species (Laurie and Lloyd-Jones, 2000; C´ebron *et al.,* 2008). It has been observed that PCR amplification with primers which are designed from catabolic

gene *nahAc* in *Pseudomonas putida* PaW736 (NCIB 9816) can be used to detect naphthalene-degrading *Pseudomonas* species as well as other bacterial species like *Mycobacterium*, *Gordona*, *Sphingomonas, Rhodococcus,* and *Xanthomonas* capable to degrade HMW PAH such as fluoranthene and pyrene (Hamann *et al.,* 1999).

**Table 2.5 Clusters of highly conserved PAH-catabolic genes**

| PAH-catabolic gene | Microbial strain | Target PAH | References |
|---|---|---|---|
| ***Nah*** | *Pseudomonas putida* strains G7 | Naphthalene | Simon *et al.,*1993 |
| | *Sphingomonas yanoikuyae* B1 | Naphthalene, phenanthrene | Zylstra and Kim, 1997 |
| | *Lolium multiflorum* L | Pyrene | Khan *et al.,*2009 |
| | *Pseudomonas putida* KT2440 | Naphthalene | Fernández *et al.,*2012 |
| | *Sphingomonas koreensis* Strain ASU-06 | Naphthalene,phenanthrene, anthracene, pyrene | Hesham *et al.,*2014 |
| ***pah*** | *Pseudomonas putida* OUS82 | Naphthalene,phenanthrene | Takizawa *et al.,*1994; Kiyohara *et al.,*1994 |
| | *Pseudomonas aeruginosa* PaK1 | Naphthalene | Takizawa *et al.,*1999 |
| ***dox*** | *Pseudomonas* strain C18 | Naphthalene | Denome *et al.,*1993 |
| | *Arabidopsis thaliana* AT5G05600 | Phenanthrene | Hernández-Vega *et al.,*2017 |
| ***Phn*** | *Burkholderia RP007* | Naphthalene,Phenanthrene | Laurie and Lloyd-Jones, 1999a,b |
| | *Acidovorax* strain NA3 | Phenanthrene, naphthalene, chrysene,benz[a]anthracene, benzo [a]pyrene, | Singleton *et al.,* 2009 |
| | *Burkholderia sp.* Ch1-1 | Phenanthrene | Hickey *et al.,*2012 |

| PAH-catabolic gene | Microbial strain | Target PAH | References |
|---|---|---|---|
| ***Phn*** | *Delftia acidovorans* Cs1-4 | Phenanthrene | Shetty *et al.,*2015 |
| ***phd*** | *Nocardioides sp*. KP7 | Phenanthrene | Saito *et al.,*1999, 2000 |
| | *Nocardioides sp*. KP7 | Phenanthrene, 1-methoxynaphthalene | Chun *et al.,*2001 |
| | *Mycobacterium vanbaalenii* PYR-1 | Pyrene | Kim *et al.,*2007 |
| ***nag*** | *Ralstoniasp.* strain U2 | Naphthalene | Fuenmayor *et al.,*1998; Zhou *et al.,*2001 |
| | *Polaromonas naphthalenivorans* CJ2 | Naphthalene | Jeon *et al.,*2006 |
| | *Herbaspirillumsp*. strain RV1423 | Naphthalene | Jauregui *et al.,*2014 |
| ***nid*** | *Mycobacterium* sp. Strain PYR-1 | Anthracene,fluoranthene, pyrene,phenanthrene, and benzo[*a*]pyrene | Khan *et al.,*2001 |
| | *Mycobacterium vanbaalenii* PYR-1 | Naphthalene, Phenanthrene, Anthracene,fluoranthene, pyrene, Benz[a]anthracene | Kim *et al.,*2006 |
| | *Mycobacterium sp*. strain CH-2 | Phenanthrene, pyrene, and fluoranthene | Churchill *et al.,*2008 |
| ***nar*** | *Rhodococcus sp*. NCIMB12038 | Naphthalene | Larkin *et al.,*1999 |
| | *Rhodococcus* sp. NCIMB12038 | Naphthalene | Kulakov *et al.,*2000 |
| ***pdo*** | *Mycobacterium sp.* strain 6PY1 | Pyrene | Krivobok *et al.,*2003 |
| | *Mycobacterium sp*. strain CH-2 | Phenanthrene, pyrene, and fluoranthene | Churchill *et al.,*2008 |
| | *Lolium multiflorum* L | Pyrene | Khan *et al.,*2009 |

| PAH-catabolic gene | Microbial strain | Target PAH | References |
|---|---|---|---|
| ***C12O*** | *Pseudomonas sp*. CZ2 | Naphthalene | Zhou *et al.,*2013 |
| | *Sphingomonas koreensis* Strain ASU-06 | Naphthalene,phenanthrene, anthracene, pyrene | Hesham *et al.,*2014 |
| | *Celeribacter indicus* P73T | Fluoranthene | Cao *et al.,*2015 |
| | *Pseudomonas* aeruginosa, *Pseudomonas sp., Ralstonia sp.* | Phenanthrene, pyrene, fluoranthene | Sangkharak *et al.,* 2020 |
| ***C23O*** | *P. putida* OUS82, *P. putida* NCIB 9816, *P. stutzeri* AN11, *Sphingomonas yanoikuyae* DSM 6900, *P. puorescens*, *Sphingomonas sp*., *P. merzdocina* | Naphthalene and phenanthrene | Meyer *et al.,*1999 |
| | *Pseudomonas sp*. Strain ND6 | Naphthalene | Jiang *et al.,*2004 |
| | *Pseudomonas sp*. CZ2 and CZ5 | Naphthalene | Zhou *et al.,*2013 |
| | *Sphingomonas koreensis* Strain ASU-06 | Naphthalene,phenanthrene, anthracene, pyrene | Hesham *et al.,* 2014 |
| | *Pseudomonas* aeruginosa, *Pseudomonas sp., Ralstonia sp.* | Phenanthrene, pyrene, fluoranthene | Sangkharak *et al.,* 2020 |
| ***fld*** | *Sphingomonas sp.* LB126 | Fluorene | Wattiau *et al.,*2001 |
| ***fln*** | *Sphingomonas sp*. Strain LB126 | Fluorene | Schuler *et al.,*2008 |

Primers for PAH-dioxygenases such as *nah, phn,* and *pdo* have been used to examine microbial degradation of diffused street dust PAHs. It was observed that *pdo* gene was dominant among three catabolic genes in soil microcosms enriched in street dust PAHs (Johnsen *et al.,* 2006). So, PCR amplification with specific primers of the catabolic genes can

be used for determining the existence of these genes in the environment, and advanced genetic studies may be useful in the investigation of many other catabolic genes involved in PAH-degradation. Few investigations of PAH-catabolism by *Mycobacterium spp*. for proteomic studies have been done, and multiple dioxygenases and related enzymes were observed (Kim *et al.,* 2004, 2005; Lee *et al.,* 2007). Fluoranthene catabolism by *Mycobacterium* sp. JS14 was investigated and increased expression of fluoranthene catabolism-related proteins was observed in one dimensional polyacrylamide gel electrophoresis (1D-PAGE) and nano liquid chromatography tandem mass spectrometry (LC-MS/MS) (Lee *et al.,* 2007). PAH-induced proteins such as catalase-peroxidase, putative monooxygenase, dioxygenase small subunit, the small subunit of naphthalene-inducible dioxygenase, and aldehyde dehydrogenase were found using two-dimensional electrophoresis (2DE) and mass spectrometry analysis in *Mycobacterium vanbaalenii* PYR-1 cultivated in presence of different PAHs (pyrene, pyrene-4,5-quinolinephenanthrene, anthracene, and fluoranthene) (Kim *et al.,* 2004). Comparative metabolomic profile of *Sinorhizobium* sp. C4 during degradation of phenanthrene was investigated. Significant metabolome differences of *Sinorhizobium* sp. C4 were detected among the *Sinorhizobium* sp. C4 cultures with phenanthrene and natural carbon sources. This analysis signifies metabolic adaptation of C4 strain in response to phenanthrene as a substrate (Keum *et al.,* 2008).

The degradation of PAHs by microorganisms is facilitated by the enzymes produced by the microbes. The catabolic genes responsible for PAH-degradation code for specific enzymes which subsequently take part in the biochemical transformation of PAHs. Although genome, transcriptome, and proteome analysis are progressing rapidly in environmental microbiology and can give significant information, the study of metabolites produced during pollutant degradation is also important as a large number of metabolites are produced and interact with each other during pollutant biodegradation. So, metabolomics is a powerful tool in the elucidation of the biodegradation/biotransformation mechanism of organic pollutants in an environmental matrix. The degradation mechanism can easily be predicted based on the catalytic enzymes involved in the biotransformation of PAHs. Therefore, the study of microbial enzymes involved in PAH degradation is essential to understand the chemical nature and toxicity of the intermediate PAH compounds formed during microbial transformation.

## 2.6 Genetically engineered microorganisms (GEMs) for PAH-degradation

Nowadays, bioremediation for PAH-degradation has become an important area of study but sometimes it is very slow due to various biotic and abiotic factors. Microbial species show optimum ability for PAH-degradation in laboratory experiments, but exhibit less proficiency at field studies. Even though microbial species have the ability to use PAHs as their carbon source but enhancement of their catabolic efficiency for complete PAH-degradation is essential. It is feasible that enzymatic activities of these microbial species could be enhanced to increase PAH-biodegradation capability by modifying their genetic makeup with the aid of genetic engineering. So, the use of genetically engineered or modified microorganisms (Fig. 2.4) various types of pollutants such as PAHs can be potentially degraded.

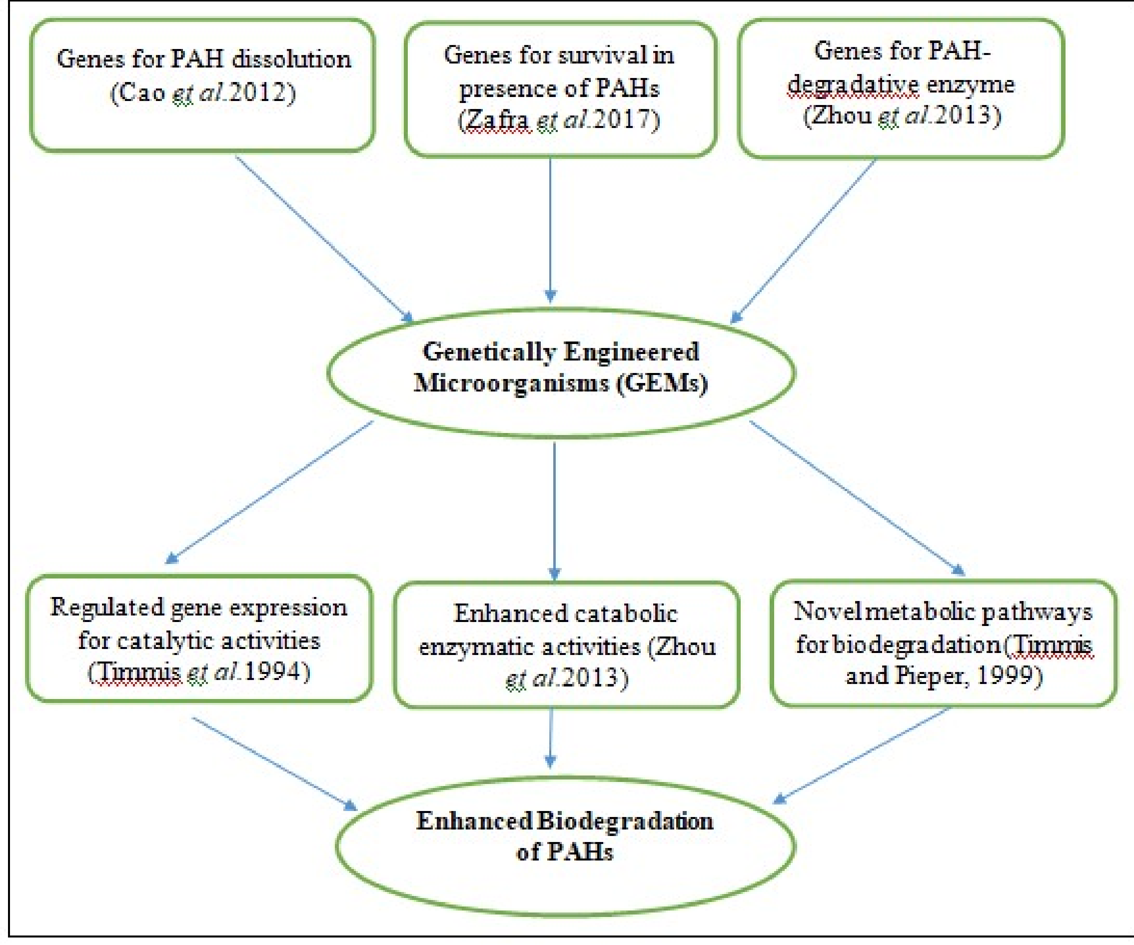



**Fig. 2.4 The systems approach for enhanced biodegradation of PAHs by genetic modification (Sakshi and Haritash, 2020)**

Genetically engineered microorganisms (GEMs) having novel metabolic activities have been extensively studied for PAH-degradation. GEMs are produced using genetic engineering by modification or manipulation of microorganism's genetic properties (Sayler and Ripp, 2000; Filonov *et al.,* 2005). DNA recombinant technology, gene duplication, genetic transposition, etc. are various genetic engineering techniques which have been developed for the design of new microorganism for removal of environmental contaminants. These techniques involve creating regulated gene expression for high catalytic activities under environmental stress (Timmis *et al.,* 1994). Sometimes indigenous microbial species are not able to tolerate contaminated environmental conditions. The degradation efficiency of such microorganisms might be less in a contaminated natural ecosystem as compared to laboratory-scale experiments. GEMs might be used as an alternative to degrade PAHs where indigenous microbial species do not show PAH-degradation ability.

Bioremediation of PAHs using genetically engineered microorganisms has entered a new era of research. Various investigations have shown that modified microorganisms are highly potential agents for PAH-degradation. Genetically engineered multi-plasmid microbial strains can degrade a number of aliphatic, aromatic, and polyaromatic hydrocarbons. Plasmid-specified degradation pathways can be stimulated in presence of an appropriate inducer and hydrocarbons are oxidized by this multi-plasmid microbial strain simultaneously. This multi-plasmid microorganism was found to grow more rapidly on crude oil than microorganism having one plasmid (Friello *et al.,* 1976). With the pBAD Thio Fusion system, the genes coding for dioxygenase from *Mycobacterium* sp. PYR-1 were cloned, sub-cloned, and over-expressed in *E. coli* bacterial species which are accountable for HMW PAH-degradation (Khan *et al.,* 2001). *Pseudomonas fluorescens* HK44 a recombinant strain that was genetically modified and constructed with naphthalene-catabolic plasmid pUTK21 also comprises transposon-based bioluminescence producing *lux* gene which is fused within naphthalene catabolic gene promoter. In presence of naphthalene, the recombinant strain has increased naphthalene catabolic gene expression and naphthalene biodegradation (King *et al.,* 1990; Sayler *et al.,* 1999; Ripp *et al.,* 2000). This recombinant strain *P. fluorescens* HK44 with naphthalene degrading capability was the first GEM which was used for field application in the USA (Sayler and Ripp, 2000). *Pseudomonas putida* strain KT2442 has been modified with plasmid pNF142::TnMod-OTc. Genetically modified *Pseudomonas putida* KT2442 (pNF142: TnMod-OTc) is found to be stable and has higher specific growth on naphthalene as compared to the natural host of pNF142 (Filonov *et al.,* 2005). A number of studies showed that the bioremediation activity of *Pseudomonas sp.* can be increased by

genetic engineering using different molecular techniques (Zhao *et al.,* 2011). Pollutant dissolution could also be enhanced using GEMs. The rhlABRI cassette with rhamnolipid-producing genes from *Pseudomonas aeruginosa* strain BSFD5 was cloned in a random transposon vector and transformed into *Pseudomonas putida* KT2440 chromosome, producing a GEM termed *Pseudomonas putida* KT2440-rhlABRI. This genetically engineered microorganism could firmly express rhlABRI cassette and yield rhamnolipid. It was observed that *Pseudomonas putida* KT2440-rhlABRI could enhance pyrene dissolution in natural soil and stimulates its degradation by native microbial species. Thus, *Pseudomonas putida* KT2440-rhlABRI has potential for increasing remediation of PAH-contaminated soils (Cao *et al.,* 2012).

Enhanced catabolic enzymatic activities in GEMs were observed for efficient PAH-degradation. The GEM constructed from *Pseudomonas sp*. CGMCC2953 which was isolated from oil-contaminated soil showed an enhanced ability for phenanthrene degradation. This constructed GEM contains C23O (catechol 2,3-dioxygenase) gene responsible for PAH degradation. The biodegradative C23O gene was effectively cloned into plasmid pK4 developed from plasmid pRK415. The recombinant microorganism, *Pseudomonas sp.* CGMCC2953-pK was found very efficient for phenanthrene degradation as C23O is the key enzyme for phenanthrene degradation. Therefore, increasing the activity of key enzymes like C23O is a proficient approach for PAH (phenanthrene) removal (Zhou *et al.,* 2013). The genetically engineered dioxygenase-producing bacterium *Pseudomonas putida* was developed using the catechol 2,3-dioxygenase (C23O) encoded gene (*nahH*). This gene was cloned into plasmid pUC18 for creating recombinant vector pUC18-nahH which was successfully transmitted into *Pseudomonas putida.* Significant enhancement in biodegradation of PAHs (phenanthrene and pyrene) by genetically engineered *Pseudomonas putida* was observed when compared with biodegradation by wild type (Mardani *et al.,* 2017). Similarly, a high level of tolerance and high efficiency of LMW and HMW PAHs degradation was observed by a PAH-degrading microbial consortium constructed with two genetically engineered fungal strains of *Aspergillus niger*. These GEMs express LiP (ligning peroxidase, LiP+5 strain) and MnP (manganese peroxidase, MnP+7 strain) genes from fungal strain *Phanerochaete chrysosporium*. These GEM strains exhibited prolonged survival in PAH-polluted soil and enhanced HMW-PAHs degradation (Zafra *et al.,* 2017). Though, different studies have shown that genetic engineering can be effective for pollutant-biodegradation and provides a significant opportunity for using native capabilities of microorganisms for construction of GEMs which are well adapted to polluted environment

and more efficient for PAH-degradation. But, unfortunately, these GEMs are applicable primarily in favorable laboratory environments and future prospective for GEMs in PAH-bioremediation at field scale is restricted by various challenges like gaining approval for the release of GEMs into the contaminated environment, its fate, monitoring, control, and risk assessment for ecosystem including human health. Therefore, genetic modification of the right microbial species with fast growth and effective biodegradation abilities without any risk to the environment is the vital step for attaining complete and safe removal of pollutants. So, further research for bioremediation using different microorganisms as well as GEMs is needed for improved reclamation of contaminated environment.

### 2.7 Genetic adaptation for PAH-biodegradation

Microbial degradation of PAHs has been substantially investigated and a number of microbial species have been found to have the proficiency to metabolize PAHs. Microbial degradation rate varies from one contaminated site to another contaminated site depending on environmental conditions, the structure of the contaminant, nutrients, and type of microorganism. The degradation rate of PAHs is distinct for individual microbial species and depends on PAH exposure under which microbial species have been adapted to the contaminant. Adaptation is the phenomenon that leads to increase in PAH-degradation efficiency after high dose PAH pre-exposure (Spain *et al.,* 1980). The microbial species which are isolated from contaminated sites are adapted to the polluted environment and can survive in presence of PAHs. Microbial adaptation depends on PAH concentration and the period of exposure. It occurs as an outcome of enhancement in the hydrocarbon-oxidizing capability of microorganisms which allows improved capacity for PAH-degradation. The adaptation potential depends on the interaction of microorganisms with soil and the bioavailability of contaminants (Kastner *et al.,* 1998).

Microbial adaptation to biodegradation of contaminants plays important role in the removal of environmental contaminants at an accelerated rate and in maintaining active microbial populations in the polluted environment. Specific enzyme induction/depression, new metabolic capabilities due to genetic changes, and selective enrichment of microbial species capable to transform the compounds are the mechanisms for adaptations of which enable microorganisms to grow in the presence of PAHs (Spain *et al.,* 1980; Spain and Veld, 1983; Leahy *et al.,* 1990). The main genetic mechanism for microbial adaptation is the amplification of genes responsible for the metabolism of contaminants by selective enrichment and insertion of gene copies in microorganisms *i.e.* gene transfer (Barkay and

Pritchard, 1988). In various investigations, microbial adaptation during PAH-biodegradation has been widely studied, and it affects PAH-biodegradation efficiency by improving the rate of PAH transformation (Walker and Colwell, 1975; Walker *et al.,* 1976; Sherrill and Sayler, 1980; Sayler *et al.,* 1983). Natural exposure of soil/sediment microbes to the high concentration of PAHs over a period of time may also result in the natural adaptation of exposed microbes for improved degradation efficiencies towards PAHs. Biodegradation of mixed hydrocarbon substrate with oil-contaminated sediment inoculum was found considerably higher as compared to biodegradation with non- oil-contaminated sediment inoculum (Walker and Colwell, 1975). A study (Walker *et al.,* 1976) reported that microbial species from the oil-contaminated Chesapeake Bay sediments provided relatively higher degradation of South Louisiana crude oil (saturated and aromatic hydrocarbons) than oil-free/unpolluted environment. PAH biodegradation is directly related to the historical background of environmental pollution of sampling sites. Phenanthrene biodegradation by indigenous microbial species was found to be higher in samples of polluted reservoirs receiving industrial waste as well as domestic waste from a nearby watershed and urban area when compared with reservoir moderately pristine and not receiving wastes from sources of PAHs (Sherrill and Sayler, 1980). The mineralization rate of PAHs by sediment microbial communities was also found significantly elevated after exposure of sediments to synthetic oil (Sayler *et al.,* 1983). During degradation of phenanthrene by *Sinorhizobium sp*. C4 significant metabolome modifications such as changes in the tricarboxylic acid (TCA) cycle, pyruvate metabolism, cofactor biosynthesis, fatty acid compositions, and polyhydroxyalkanoate biosynthesis of strain *Sinorhizobium sp*. C4 were observed when compared with metabolic responses of *Sinorhizobium sp.* C4 cultures with natural carbon source. These metabolomics modifications indicate microbial metabolic adaptation when phenanthrene is used as a substrate (Keum *et al.,* 2008). Whole-genome sequencing of *Pseudomonas aeruginosa* N002 isolated from crude oil contaminated soil of Geleky, Assam, India was done by whole-genome shotgun sequencing and the genome analysis of N002 strain revealed genetic adaptation of this bacterial strain for degradation of crude oil (Das *et al.,* 2015). Similarly, wild bacterial strains isolated from rhizospheric soil of *Morus alba* were adapted under different physico-chemical conditions such as UV exposure, varying temperature, pH, and pyrene concentration (10ppm to 1000ppm) in growth media. These bacterial strains were found to have increased growth with increased pyrene concentration at temperature of 35°C and pH 7 (Bibi *et al.,* 2017). These investigations suggest that high level PAH-exposure to microorganisms may change genetic makeup resulting in the adaptability of

microbial species by amplification of catabolic genes involved in the metabolism of PAHs and indicate that the improved efficiency for PAH-degradation is due to amplified gene expression from similar microorganisms. Therefore, the genetic change or adaptability may be responsible for the high PAH-biodegradation rate.

**2.8 Biostimulation: Regulating the contaminated environment**

As discussed, pollution due to PAHs is a global problem and a number of microbial species have been investigated for efficient PAH-degradation (Bayoumi, 2009; Mart´ınkov´a *et al.,* 2009). On-site bioremediation can be more efficient with appropriate conditions for microbial growth such as the proper supply of nutrients, surfactants, water, and oxygen. This process of supply of nutrients (nitrogen, phosphorous, and carbon, organic biostimulants), surfactants, water, and oxygen (electron acceptor) to stimulate microbial activity for contaminant degradation is known as biostimulation. It is one of the most significant approaches for the enhancement of bioremediation efficiency at PAH- contaminated sites (Pelaez *et al.,* 2013). The naturally occurring microbial species have been effectively used at the contaminated field and the effects of nutrient addition to stimulate bioremediation at the field scale have been extensively studied. Bioremediation using fertilizers at T/V Exxon Valdez oil spill in Prince William Sound, Alaska was investigated and higher phenanthrene mineralization was observed on remediated plots than on untreated plots (Lindstrom, 1991). Indigenous microbial populations of PAH-contaminated soil were used for ex-situ bioremediation (land-treatment process) of soil excavated from a creosoting plant comprising PAHs. Reduction from 290 mg/kg to ‹ 200 mg/kg of total 16 USEPA priority PAHs was observed. A high degradation rate was found for LMW PAHs (97% degradation of 2- ring PAHs, and 82% degradation of 3-ring PAHs) as compared to HMW PAHs (45%, 51%, and 35% degradation of 3, 4, and 6-ring PAH respectively) (Guerin, 1999). In another study, bioremediation of oil-contaminated Kuwaiti soil containing PAHs such as acenaphthene, fluoranthene, and pyrene was done using soil microcosms with $NH_4NO_3$ and $Na_2HPO_4$ supply as microbial degradation is facilitated by the addition of nutrients. Up to 30% PAHs removal (acenaphthene (20%), fluoranthene (30%), and pyrene (0%)) was observed after 90 days (Horinouchi *et al.,* 2000). Apart from PAHs, total petroleum hydrocarbons can also be treated by stimulation of the contaminated environment. Two plots A and B at the Indian refinery were chosen for in-situ bioremediation of petroleum hydrocarbon contaminated sites where the hydrocarbon-degrading native indigenous bacterial population in soil was extremely small. An indigenously chosen bacterial consortium containing five bacterial isolates and

nutrients was used to stimulate in-situ bioremediation. After one year of bioremediation, 92% and 89.7% of total petroleum were removed from plots A and B, respectively. At plot A the aromatic fraction of total petroleum hydrocarbons was reduced by 91.9% in 1 year and 94.8% aromatic fraction of total petroleum hydrocarbons was reduced at plot B in 345 days (Mishra *et al.,* 2001). Nutrients in the form of fertilizers may also found to improve bioremediation efficiency. A number of studies have been done to evaluate the effect of fertilizers on bioremediation. The bioremediation efficiency of natural attenuation and biostimulation at chronically diesel-oil contaminated soil with fertilizers (NPK) and without fertilizers in an Alpine Glacier skiing area has been studied (Margesin and Schinner, 2001). After 780 days, hydrocarbon contamination was decreased by (50 ± 4) % and (70 ± 2) % in contaminated unfertilized soil and soil with fertilizers, respectively. So, the presence of fertilizers (nutrients) enhances the biodegradation ability of microbes at contaminated sites. High biodegradation efficiency (62%) for crude oil contaminated soil was observed under the influence of biostimulation *i.e.* fertilized soil with nitrogen, phosphorus and potassium for 150 days as compared to unfertilized soil (47%) (Chaineau *et al.,* 2005). The bioremediation process combined with the addition of nutrients and surfactants, water irrigation, and proper air supply is highly efficient for PAHs removal at contaminated sites. On-site bioremediation by autochthonous bacterial species such as *Bacillus* and *Pseudomonas* with the addition of nutrients, surfactants, watering, and aeration was designed for 900 $m^3$ of PAH-contaminated soil to reduce PAH contamination. A significant PAH reduction *i.e.* 71.66% was observed in 48 days, and at the end of the treatment 94.4% total PAHs were degraded (after 161 days) when the treatment process was combined with nutrients and a commercially available surfactant (Pelaez *et al.,* 2013). In addition, there may be various factors related to biostimulation such as type and concentration of PAH compound, other nutrients present at the contaminated site, structure of the soil, environmental conditions such as moisture, temperature, organic matter, microflora, etc. which may affect bioremediation efficiency. Further research for biostimulation may be carried out to optimize conditions for severely PAH-contaminated sites.

The success rate of on-site bioremediation of severely PAH-contaminated sites is a challenge because microbial species are not able to grow at contaminated sites after inoculation. Even though PAH-bioremediation at field scale is a slow process and would take time to achieve the cleanup standards, however, this technique is still a preferred sustainable alternative for remediation of PAH-contaminated sites because it is cost-efficient and environment friendly. So, these microbial species having the capability of PAH-degradation

could be used for the formation of commercial products for reclamation of petroleum contaminated sites.

### 2.9 Commercial products for PAH or petroleum-bioremediation

Recently various research laboratories and companies are involved in the development of commercial products which could be used for bioremediation of PAH or petroleum contaminated sites. These products are developed based on previous findings of PAH-bioremediation techniques outlined and discussed in previous sections. Different commercial products such as nutrient additives have been developed for the cleanup of oil-contaminated sites. Inipol EAP22, Oil Spill Eater II (OSE II), BIOREN 1, and BIOREN 2 are some commercial bioremediation nutrient additives, listed on the National contingency plan (NCP) Product schedule (Jain and Bajpai, 2012). These products are nutrient/enzyme additives and consist of nutrients like nitrogen, phosphorus, readily available carbon, and vitamins for naturally occurring microbial species (Ladousse and Tramier, 1991; Zwick *et al.,* 1997; Le Floch *et al.,* 1997, 1999). Inipol EAP22 which is a nutrient additive was used in the cleanup of Exxon Veldez spill (Pritchard *et al.,* 1992) as it was the only commercially available bioremediation agent. Iniprol EAP22 was also used as a biodegradation process accelerator to study the degradation of hydrocarbon residuals from a highly aged hydrocarbon mixture deriving from an accidental spill in an oil refinery (Bergueiro-López *et al.,* 1996). To study nutrient products *i.e.* BIOREN 1 and BIOREN 2 activity, shoreline field experiments were done (Le Floch *et al.,* 1999) to clean oil-contaminated sediments, enhanced oil degradation was found initially during the first five weeks due to the presence of BIOREN 1 which contains a surfactant, and in presence of BIOREN 2, no effective oil degradation was found as it contains no surfactants. Oil Spill Eater II is a nutrient/enzyme additive, its efficacy for enhancing hydrocarbon biodegradation in a fuel contaminated zone was evaluated (Zwick *et al.,* 1997) in a field study which was carried out at a bioventing site in a Marine Corps Air Ground Combat Center (MCAGCC) in California. It was observed that Oil Spill Eater II might have enhanced microbial activities in the deeper soils at the contaminated site.

Microorganism-based products for bioremediation have also been developed. RemActiv Australian-made product of Ziltekis a liquid additive comprising selected microbial species (bacteria and fungi) and a specifically prepared nutrient mix, which is designed to enhance bioremediation of petroleum hydrocarbons in soil. BET BIOPETRO powder manufactured by BioEnviro Tech, Tomball, Texas, is another microbial product that contains bacterial product granules for bioremediation of heavy contamination by crude

hydrocarbon containing alkanes and aromatic contaminants in soil and water environments. Tata Energy Research Institute (TERI), New Delhi has developed another oil-degrading bacterial system *i.e.* TERI'S Oilzapper for bioremediation of oil-contaminated sites (Mishra *et al.,* 1999). The bacterial system of Oilzapper was developed by combining five different bacterial strains having the ability to degrade aliphatic hydrocarbons, aromatic hydrocarbons, asphaltene, and NSO (nitrogen, sulphur, and oxygen compounds) fractions of oil. Oilzapper is carrier-based bacterial species that was used over a field at the Mathura oil refinery and promising results were attained. Oilivorous-S is another carrier-based bacterial species consortium developed by TERI for oil degradation. It is more effective at oil-contaminated sites with high sulfur content. There are various agencies as clients of Oilzapper and Oilivorous-S such as Indian Oil Corporation Ltd, Bharat Petroleum Corporation Ltd, Hindustan Petroleum Corporation Ltd, Oil and Natural Gas Corporation Ltd, Oil India Ltd, Indian Petrochemicals Corporation Ltd, Reliance Industries Ltd, Abu Dhabi National Oil Company, Abu Dhabi, Kuwait Oil Company, Kuwait, etc. (Randhawa and Kaushal, 2014). Bacterial species present in these products feed on hydrocarbons present in the oil, and the harmful hydrocarbon waste produced from oil refineries, which are transformed/mineralized and transform into non-toxic products such as $CO_2$ and $H_2O$.

# CHAPTER 3
# MATERIALS AND METHODS

Contamination of the environment due to PAHs has become a serious threat; removal or degradation of PAHs is not easy and the presence of PAHs in the environmental matrix is a major problem and will continue to be an environmental hazard unless proper treatment method is not developed and used. Therefore, the need to clean up these pollutants (PAHs) from the environment is an issue of global concern and the development of effective methods for the removal of hydrocarbons is a crucial task. At present different physical and chemical methods are being used for PAH removal which are costly, therefore biological treatment using naturally occurring microorganisms can be used for PAH removal from different oil or PAH contaminated sites. This study deals with the use of microorganisms isolated from long-term petroleum-contaminated soil to degrade different PAHs (phenanthrene, anthracene, fluorene, and pyrene) along with the analysis of enzymatic assay and the expression of the gene involved in PAH degradation.

## 3.1 Materials

### 3.1.1 Chemicals and Reagents

Two-ring PAH *i.e.* Naphthalene (NAP), three-ring PAHs *i.e.* phenanthrene (PHE), anthracene (ANT), fluorene (FLU), and four-ring PAH *i.e.* pyrene (PYR) (all with ≥98% purity), and HPLC grade acetonitrile used as mobile phase for high pressure liquid chromatography (HPLC) were procured from Sigma-Aldrich, India and SRL chemicals, India**.** All the chemicals used to prepare Luria-Bertani (LB) medium (NaCl, yeast extract, tryptone) mineral salt medium (MSM) ($KNO_3$, $KH_2PO_4$, $K_2HPO_4.3H_2O$, $(NH_4)_2SO_4$, NaCl, $MgSO_4.7H_2O$, $CuSO_4.7H_2O$, $FeSO_4.7\ H_2O$) and agar (used for the preparation of solid medium) were of analytical grade and purchased from SRL chemicals, India and CDH, India. The n-Hexane, dichloromethane (DCM), silica (100-200 mesh size), sodium sulphate used for PAH extraction, and acetone used for the preparation of PAH-stock solutions (NAP, PHE, ANT, FLU, PYR, and mixed PAHs) were purchased from Thermo Fisher Scientific, India and RANKEM, Avantor Performance Materials, India. Triphenyl tetra-zolium chloride (TTC), triphenyl formazan (TF) dye (to establish a standard curve for absorbance ($OD_{484}$) vs. TF concentration), and glucose used to measure the dehydrogenase activity were procured from CDH, India, Alfa Aesar, India, and Sigma-Aldrich, India. Methanol used to extract the

TF from bacterial cells to study dehydrogenase activity was purchased from SRL chemicals, India.

Petroleum contaminated soil sample (Fig. 3.1) was collected aseptically from 5cm to 10cm below the soil surface of siding area (rail track from where petroleum is unloaded), Bijwasan supply location- Bharat Petroleum Corporation Limited (BPCL) in Delhi, India, and placed inside the sterilized storage box. The collected soil sample was transported to the laboratory for future investigation, stored at 4°C until use.

**Fig. 3.1 Petroleum contaminated soil sample collected from siding area**

The stock solutions (5000 mg/L) of different PAHs (NAP, PHE, ANT, FLU, and PYR) were prepared in acetone; 125mg of each PAH was dissolved in 25ml acetone in a volumetric flask. A stock solution of mixed PAHs (10,000 mg/L) was prepared by dissolving 50mg of each PAH (NAP, PHE, ANT, FLU, and PYR) in 25ml acetone in a volumetric flask. These stock solutions were capped tightly and stored under the dark until use.

A TTC-glucose reagent was prepared by dissolving 1 g glucose and 2 g TTC in 100 mL distilled water and stored at 4°C in the dark until use.

The stock solution of TF of 0.2µmol/mL was prepared by dissolving 0.3g of TF in 500ml methanol to establish a standard curve of TF.

The potassium phosphate buffer (0.1M) of pH 7.0 was prepared by dissolving 9.3 g $K_2HPO_4$ and 6.3 g $KH_2PO_4$ in 1L distilled water and the potassium phosphate buffer (0.1M) of pH 7.5 was prepared by dissolving 12.8 g $K_2HPO_4$ and 3.6 g $KH_2PO_4$ in 1L distilled water. Tris buffer (1M, pH 7.2) was prepared by dissolving 121.14 g of Tris base in 1L distilled water.

### 3.1.2 Preparation of growth medium

Luria-Bertani (LB) medium and mineral salt medium (MSM) were used during the present study. pH value (7.0-7.2) of the media was adjusted with 1N NaOH and deionized water was used to prepare media. LB medium and MSM used during the study were sterilized before use by autoclaving at 121ºC temperature and 15 psi pressure for 20 min. Agar was added before autoclaving to prepare solid medium plates.

**Luria-Bertani (LB) medium**

This medium consists of the following ingredients (g/L):

| | |
|---|---|
| NaCl | 10.0g |
| Yeast extract | 5.0g |
| Tryptone | 10.0g |

**Mineral salt medium (MSM)**

This medium consists of the following ingredients (g/L):

| | |
|---|---|
| $KNO_3$ | 3.0g |
| $KH_2PO_4$ | 0.5g |
| $K_2HPO_4.3H_2O$ | 0.5g |
| $(NH_4)_2SO_4$ | 2.0g |
| NaCl | 1.0g |
| $MgSO_4.7H_2O$ | 0.008g |
| $CuSO_4.7H_2O$ | 0.002g |
| $FeSO_4.7\ H_2O$ | 0.002g |

The agar (2%) was added in a 1.0 L medium for preparing solid LB and MSM plates.

### 3.2 Methodology

#### 3.2.1 Isolation of bacterial strains

Bacterial strains used in this study were isolated from petroleum-contaminated soil. The enrichment and isolation of PAH-degrading microbes from petroleum-contaminated soil were done using LB medium by traditional methods based on serial dilution (Cappuccino and Sherman, 2013). Soil suspension (1:10 w/v) prepared in Erlenmeyer's flask by dissolving soil in normal saline (0.9% NaCl) was kept in a shaking incubator at 30°C for 48 hours. The supernatant of soil suspension was collected and serially diluted to $10^{-4}$ order, which was used (1ml) for inoculation on LB agar medium, incubated at 30°C for 3 days for bacterial growth. The bacterial strains with the best growth, based on different colony morphology were repeatedly streaked over new LB agar plates at regular intervals of 7 days; incubated at 30°C every time in order to obtain pure cultures.

#### 3.2.2 Adaptation and screening of PAH degrading bacterial strains

Sensitivity levels of different isolated strains to mixed PAHs were determined using the spray plate technique on LB plates to screen PAH-degrading bacterial strains (Kiyohara *et al.,* 1982). The obtained pure bacterial strains were exposed to a dose of 50 mg/L of mixed PAHs by inoculating them on different LB agar plates which were uniformly sprayed with PAHs solution (50 mg/L) containing NAP, PHE, ANT, FLU, and PYR. The solvent *i.e.* acetone was allowed to evaporate leaving a fine coating of mixed PAHs on LB agar surface, later inoculated with obtained pure bacterial stains using an inoculating loop and incubated at 30°C for 7 days with close observation each day. Bacterial strains which survived on LB plates sprayed with mixed PAHs (50 mg/L) were selected and exposed to higher doses of mixed PAHs by sub-culturing them with steadily decreasing the concentration of yeast extract (0.5% to nil), and progressively increasing concentration of mixed PAHs from 50 mg/L to 10,000 mg/L (NAP, PHE, ANT, FLU, and PYR each with equal concentration). The pure cultures were initially subjected to a dose of 50 mg/L with 0.5% yeast extract for 7 days; 200 mg/L with 0.25% yeast extract for the next seven days; similarly with 500 mg/L with 0.25% yeast extract, 1000 mg/L with 0.25% yeast extract, 1000 mg/L without yeast extract for seven days each time. Colonies obtained at this stage were further exposed to higher PAH concentrations *i.e.* 1500 mg/L, 2000 mg/L, 3000 mg/L, 4000 mg/L, 5000 mg/L, 7000 mg/L, and 10000 mg/L without yeast extract for seven days of incubation each time. In such a way, isolated bacterial strains were exposed to high doses of PAHs under static incubation conditions at 30°C, suitably acclimatized and well adapted for PAH-degradation as these

bacterial strains used PAHs as sole carbon and energy source when no yeast extract was present in the medium. Finally, the screened potent bacterial strains *i.e.* DTU-1Y and DTU-7P having the ability to grow on a medium containing mixed PAHs (10,000 mg/L) were grown on MSM to confirm the assimilation of PAHs as the sole carbon and energy source, and selected for further study. The bacterial isolates were streaked on LB agar slants and stored at 4°C for preservation. Regular sub-culturing was done after every 20-25 days.

### 3.2.3 Characterization of isolated bacterial stains

**Morphology of bacterial colonies:** The selected potent bacterial strains were identified by color, morphology, and Gram staining.

**Color and morphology of bacterial stains**: Shape, size, color, the elevation of the bacterial colony, and their appearance were observed on LB agar medium plates after 48hr incubation period.

**Gram staining**: A smear of isolated bacterial cultures from LB agar media after 48 hr incubation period was made separately on clean glass slides with the help of a sterile loop. The smear on glass slides was air-dried and heat-fixed. Primary stain *i.e.* crystal violet was applied to heat-fixed smear of bacterial isolates then Gram's iodine (trapping agent) was added followed by the addition of alcohol for rapid decolorization. After the decolorization step counterstaining was done with safranin. The gram-stained cells were observed under the optical microscope.

**Biochemical characterization and molecular identification:** Biochemical characterization to study metabolic capabilities and molecular identification of isolated bacterial strains by 16S rRNA gene sequencing of isolates were done at Council of Scientific and Industrial Research-Institute of Microbial Technology (CSIR-IMTECH), Chandigarh, India. Nucleotide BLAST (Basic Local Alignment Search Tool) (Altschul *et al.,* 1990) search analysis was done to identify the strains. The 16s rRNA gene sequences of the isolated bacterial strains obtained in this study were deposited at National Center for Biotechnology Information (NCBI).

### 3.2.4 Inoculum preparation to study PAH-degradation

The inoculum was prepared using a pure culture of each selected bacterial isolate, which had shown viable growth at a high dose (10,000 mg/L) of mixed PAHs when no yeast extract was present in the growth medium. The selected bacterial strains considered as potent isolates were grown in 50 ml MSM broth to attain the exponential growth phase at 30°C.

When cell density of the suspension was found to be OD = 0.6 at 600 nm, 1% (v/v) of MSM broth containing bacterial strain was used as inoculum for degradation of PAHs.

### 3.2.5 Microbial degradation assay

Assessment of PHE, ANT, FLU, and PYR degradation was carried individually, using a single compound and as a mixture of all four PAHs in MSM broth. The experiments were performed in the laboratory ensuring sterile and controlled conditions during 15 days of the incubation period. Each degradation study was carried out using sterilized 250ml Erlenmeyer's flasks containing 100 ml MSM broth supplemented with PHE, ANT, FLU, and PYR (dissolved in acetone) to attain a final concentration of 10 mg/L for each PAH compound, and a mixture of all four PAHs (2.5 mg/L of each PAH) separately with respect to PAH-degradation as reported in earlier studies (Hedlund *et al.,* 1999; Ye *et al.,* 2011; Mineki *et al.,* 2015; Qin *et al.,* 2018; Subashchandrabose *et al.,* 2019a and 2019b). As, generally PAHs are present in soil/sediments with very low concentration (< 10mg/L) (Magi *et al.,* 2002; Wilcke *et al.,* 2005; Devi *et al.,* 2016; Sosa *et al.,* 2017), the PAH-degradation studies were performed at PAH concentration of 10mg/L which is considered as a high dose for contamination in any environmental component *i.e.* soil, water, and sediments. The MSM broth were inoculated aseptically with 1% (v/v) of bacterial monocultures of DTU-1Y and DTU-7P (OD = 0.6 at 600 nm) individually, as well as the combination of both strains (1:1 (v/v) each having $OD_{600}$ of 0.6). Abiotic controls *i.e.* blank were prepared in the same way with no bacterial inoculums. All flasks containing monoculture, consortium, and control were incubated at a temperature of 30°C and shaking at 120 rpm under dark conditions for 15 days (Fig. 3.2 ) in an orbital shaking incubator as reported in earlier studies (Rabodonirina *et al.,* 2019). All the experiments were performed in triplicates for the biodegradation of PAHs. A volume of 5ml was extracted from each experiment at regular time intervals of 0, 3, 6, 9, 12, and 15 days to analyze the residual PAH concentration in the medium using high-performance liquid chromatography (HPLC) (Bishnoi *et al.,* 2007, Haritash and Kaushik, 2016).

The percentage of PAHs degradation was calculated using the following relation (1)

$$\text{Percentage degradation} = \frac{(C_i - C_f)_s - (C_i - C_f)_b}{(C_i)_s} \times 100 \quad (1)$$

Where $C_i$ is the initial concentration and $C_f$ is the final concentration of PAH compound in the sample (s) and blank (b), on a particular day.

**Fig. 3.2 Erlenmeyer's flasks containing MSM broth supplemented with PAHs, inoculated aseptically with bacterial monocultures and combination of isolated strains, and abiotic controls**

Biomass growth of each strain in monoculture and in the consortium was monitored during degradation study at regular intervals by measuring the increase of absorbance at $OD_{600}$ over a spectrophotometer (Labtronics, model LT-290) as spectrophotometric (turbidimetric) analysis is the standard method (Cappuccino and Sherman, 2013) to examine microbial growth in liquid medium.

### 3.2.6 HPLC for analysis of residual PAHs

A volume of 5ml of sample from each flask was taken for analysis over HPLC (Shimadzu, LC-20AD) equipped with degassing unit (DGU-20$A_{3R}$), binary pump, Ultraviolet-visible (UV/VIS) detector (SPD-20A), autosampler (SIL-20$A_{HT}$), communications bus module (CBM-20A), analytical column (Inertsil® ODS-3V, 5µm 4.6×250mm), column oven (CTO-10$AS_{VP}$) and a data station. Standard chromatograms of PHE, ANT, FLU, and PYR were prepared and used to calculate residual PAH concentration during degradation. The stock solutions of each PAH were diluted to attain the concentration of 2, 4, 6, 8 and 10mg/L, and used to prepare the standard curve (Annexure I). Residual PAHs were extracted with dichloromethane (DCM) in three cycles (5, 3, 2ml), by vigorous shaking for 5 minutes in each cycle, using a separating funnel to ensure complete PAH-

extraction, and the organic phase extracts were pooled. The extracts (10ml organic phase) were dried using the column prepared by preheated (120 -$140^0$C, 3-4hr) anhydrous silica and sodium sulphate (80:20) to remove water content/aqueous phase. The extracts were reduced in volume and concentrated by placing them under dark conditions for evaporating DCM. Finally, extracted residual PAHs were dissolved in 1ml acetonitrile and quantified by HPLC (Fig. 3.3). A gradient mixture of acetonitrile and water (80:20) was used as a mobile phase at a flow rate of 1ml/min in binary mode with 10µL sample injection volume, and UV/VIS-Detector wavelength was set at 254 nm for chromatographic analysis of PAHs as reported in the standard method given by National Institute for Occupational Safety and Health (NIOSH, Manual of Analytical Methods (NMAM), Fourth Edition, Polynuclear Aromatic Hydrocarbons by HPLC: METHOD 5506, Issue 3, dated 15 January 1998, Annexure II).

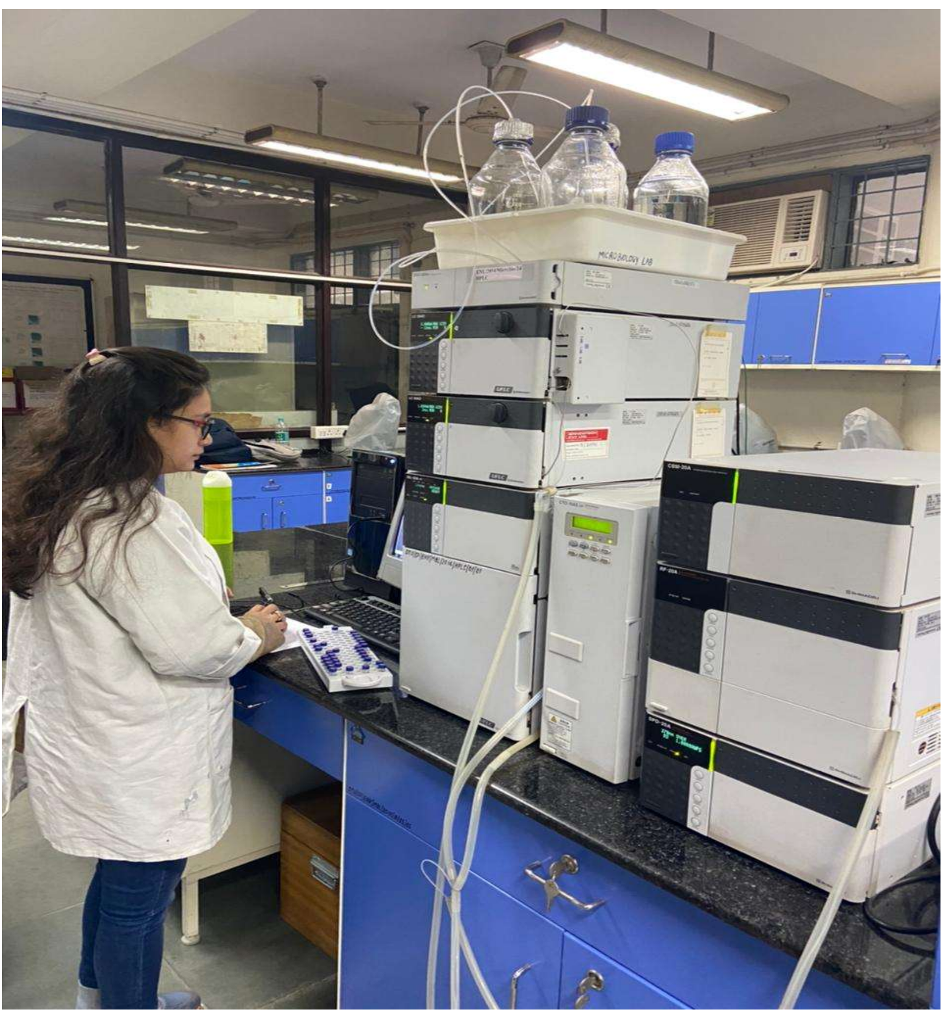



**Fig. 3.3 HPLC (Shimadzu, LC-20AD) system used for the analysis of residual PAHs**

### 3.2.7 Phylogenetic analysis

Preliminary identification of the other bacterial species having similarity with isolated bacterial strains was done using nucleotide BLAST of 16S rRNA gene sequence of isolated strain against 16S ribosomal RNA sequences database on the National Centre for Biotechnology Information (NCBI) (https://blast.ncbi.nlm.nih.gov). The identified bacterial species from nucleotide BLAST having similarity (> 90%) with isolated bacterial strains were searched for the presence of catabolic genes or catabolic enzymes with their ability for PAH-degradation in the existing literature. For phylogenetic tree construction, 16S rRNA gene sequences of bacterial species having similarity with isolated strains and having the ability to degrade PAHs (based on earlier reports) were selected and retrieved from NCBI in FASTA format. MEGA X (Molecular Evolutionary Genetics Analysis) was used for phylogenetic tree analysis (Hall, 2013). Multiple sequence alignment of these retrieved sequences with the sequence of isolated strain was done using Multiple Sequence Comparison by Log- Expectation (MUSCLE) in MEGA X (Kumar *et al.,* 2018) and used to construct a phylogenetic tree by neighbor-joining analysis (Saitou and Nei, 1987) using a maximum composite likelihood model and 1000-bootstrap replications.

### 3.2.8 Enzyme Extraction and assay

The catabolic enzymatic activities during PAH-degradation were monitored using a double beam UV–vis spectrophotometer (Lab India make UV 3092 model) (Fig. 3.4).

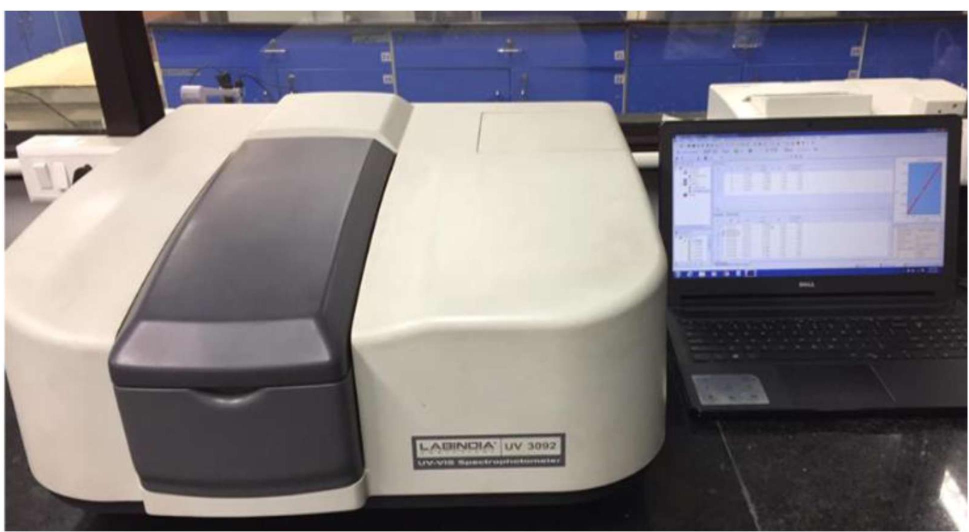



**Fig. 3.4 Double beam UV-Vis Spectrophotometer (Lab India make UV 3092 model) used for determining the catabolic enzymatic activities**

For extraction of crude enzymes after every three-day intervals to study catabolic enzymes *i.e.* dioxygenase (catechol 1,2-dioxygenase (C12O), catechol 2,3-dioxygenase (C23O)), dehydrogenase, and peroxidase activity, 10ml of bacterial cell suspension from each flask were subjected to cell disruption using sonication (Labman scientific instruments LMUC –9, with frequency 40 kHz) maintaining the temperature of 10°C for 10 min (short impulses of 30-40s with pauses 10-20s to maintain low temperature). Sonicated samples were centrifuged at 12000 RCF (Relative centrifugal force) at 4°C for 15 minutes, the supernatant of each sample carrying enzymes was stored at 4°C and used for further enzyme activity assay while cell debris remained as sediments were removed (Singh *et al.,* 2013; Cao *et al.,* 2015).

### 3.2.8.1 Catechol 1,2-Dioxygenase and Catechol 2,3-dioxygenase assay

Catechol 1,2-Dioxygenase (C12O) and catechol 2,3-dioxygenase (C23O) activities were assayed using catechol as a substrate, by recording an increase in absorbance at 260nm and 375nm respectively. The assay mixture for enzyme activity contained 2.8 ml of 0.1M potassium phosphate buffer of pH 7.5, 0.1 ml of 0.01M catechol, and 0.1 ml crude enzyme in a quartz cuvette with 1cm light path. The increase in absorbance at 260nm due to the formation of cis-cis muconic acid ($\varepsilon_M = 1.6\times10^4$ mol$^{-1}$lcm$^{-1}$); and at 375nm due to formation of 2-hydroxymuconic semialdehyde ($\varepsilon_M = 4.4\times10^4$ mol$^{-1}$lcm$^{-1}$) by enzymatic reaction were monitored (Nozaki, 1970; Nakazawa and Nakazawa, 1970; Fernandez–Lafuente *et al.,* 2000). The least count of the increase in absorbance measurement was 0.001, and one unit of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

### 3.2.8.2 Peroxidase assay

The peroxidase activity was monitored in a 3mL reaction mixture containing crude enzyme, 0.1M potassium phosphate buffer of pH 7.0, 5 mM guaiacol, and 0.6mM hydrogen peroxide as the substrates. The increase in absorbance at 470 nm due to the formation of tetraguaiacol ($2.66 \times 10^4$ mol$^{-1}$lcm$^{-1}$) due to enzymatic reaction was monitored (Whitaker, 1972). The least count of the increase in absorbance measurement was 0.001, and one unit of activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per minute.

#### 3.2.8.3 Dehydrogenase assay

The dehydrogenase activity was examined using 2,3,5-triphenyl tetrazoilum chloride (TTC, as the artificial electron acceptor), which was reduced to red-colored triphenyl formazan (TF).

**Standard Curve of Triphenyl Formazan (TF)**

A standard curve was developed to determine the concentration of TF (µmol/mL) corresponding to an absorbance measured at 484 nm to study dehydrogenase enzyme activity. A stock solution of 0.2µmol/mL was prepared by dissolving 0.03g TF in 500 mL methanol. The TF stock solution was diluted with methanol to produce TF concentrations ranging from 0.0008 µmol/mL to 0.1µmol/mL. The absorbance of each solution was measured with a spectrophotometer (Lab India make UV 3092 model) at 484 nm. The absorbance readings ($OD_{484}$) were plotted against the TF concentration (µmol/mL) as shown in Figure (3.5) the following linear best-fit Equation ($R^2 = 1$) was obtained:

Equation: Ab = K1*(conc.) + K0

K0 = 0.00868

K1 = 20.6903

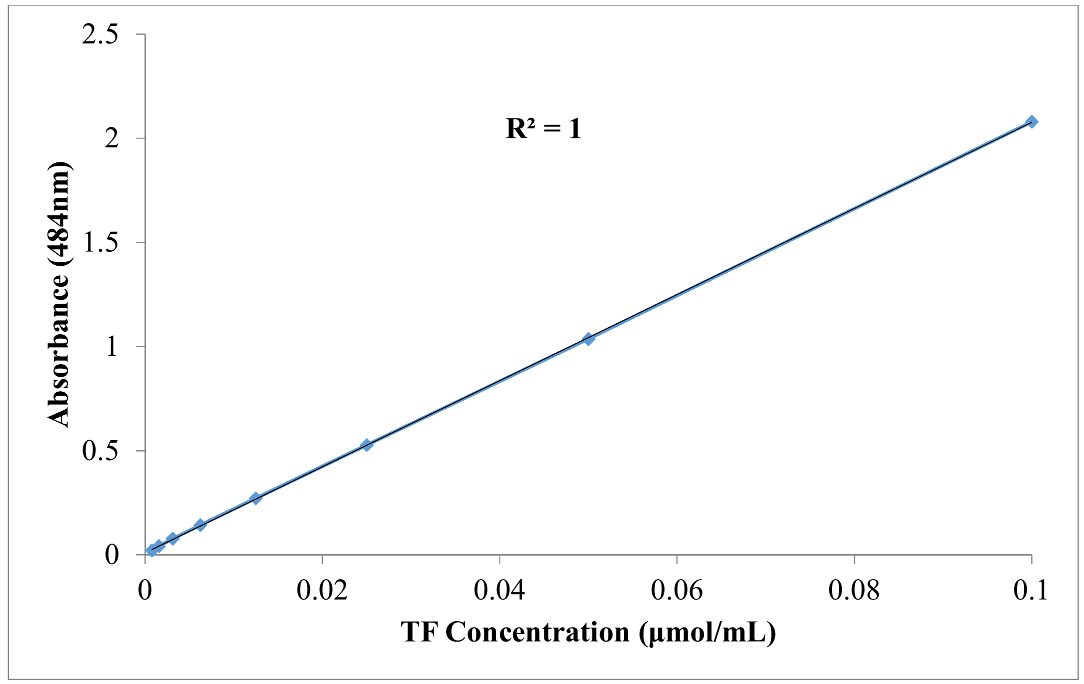



**Fig. 3.5 The standard curve of triphenyl formazan**

For dehydrogenase enzyme assay, 1mL of bacterial cell suspension from each flask was transferred into test tubes containing tris buffer (2.5ml) and 1ml TTC-glucose solution, after every three-day interval. The tubes were swirled to mix the contents and incubated at 30 °C for 1hr in a temperature-controlled oven (Yamato, DKM400). After 1hr incubation, the reaction product (TF) was extracted from bacterial cells with methanol. The product of enzyme activity (TF) was centrifuged at 10,000 rpm at 4 °C for 10 min, the supernatant from each tube was discarded and the cell pellets were dissolved in 2.5ml methanol to aid TF extraction from bacterial cells. Samples were centrifuged again and the supernatant was decanted in another tube. Two more extractions were done with methanol to attain complete TF extraction. TF concentration was measured using TF standard curve at $OD_{484}$ using UV-Vis spectrophotometer (Lab India make UV 3092 model) (Ghaly and Mahmoud, 2006; Burdock *et al.,* 2011). One unit of enzyme activity was defined as the amount of 1 µmol product formed in 1 minute.

### 3.2.9 Kinetics of biodegradation

Biodegradation kinetics of PHE, ANT, FLU, and PYR by the isolated strains was evaluated using first-order kinetics presented in formula (Equation 2) (Mohd-Kamil *et al.,* 2013; Kachieng'a and Momba, 2017).

$$Ct = C_0 e^{-Kt} \quad (2)$$

Where $C_0$ is the initial concentration of PAHs in control, Ct is the PAHs concentration in the medium at time t, K is the first-order rate constants of the degradation process and t is time (days) (Zahed *et al.,* 2011; Agarry *et al.,* 2013).

A linear relation of ln[PAHs] against t was used to determine the value of k from its slope (Equation. 3)

$$\ln[PAH] = -kt + \ln[PAHo] \quad (3)$$

The half-life, t/2 (the time required for a concentration of any substrate to reduce to one-half of its initial concentration) was calculated using formula (Equation 4)

$$t1/2 = \ln(2)/k \quad (4)$$

### 3.2.10 Expression of catabolic genes

The isolated bacterial strains having the capability for PAH-degradation were studied for catabolic gene expression involved in the degradation process. The genes such as catechol 2,3 dioxygenase (*C23O*), PAH-ring hydroxylating dioxygenase (*PAH-RHD*), and naphthalene dioxygenase (*NAH*) involved in the degradation process were studied. For gene expression study during mixed PAH-degradation 25ml of bacterial cell suspension from each flask were taken aseptically on 15$^{th}$ day of the incubation period. The collected bacterial cell suspension was submitted to Superworth Biodiscoveries, Delhi, India, for gene expression analysis by quantitative reverse transcription polymerase chain reaction (RT-qPCR). For catabolic gene expression analysis by RT-qPCR, gene-specific primers giving an amplicon of 150-300 bp and anneals at 60°C temperature (Table 3.1) were designed using IDT (Integrated DNA Technologies) real-time assay design (https://www.idtdna.com/scitools/Applications/RealTimePCR/).

**Table 3.1 The primers used for RT-qPCR analysis in the present study**

| S. No. | Name | Sequence | Length | Start | End | Amplicon Length |
|---|---|---|---|---|---|---|
| 1 | 16SrRNA Fwd | AATTCCTGGTGTAGCGGTG | 19 | 568 | 586 | 245 |
| 2 | 16S rRNA Rev | CCTTTGAGTTTTAGCCTTGCG | 21 | 812 | 792 | |
| 3 | *NAH* Fwd | ATCGGCAACTTCCTGATGG | 19 | 406 | 424 | 158 |
| 4 | *NAH* Rev | TTGTAGCTCTCGTCCTTGAAC | 21 | 563 | 543 | |
| 5 | *C23O* Fwd | CACATCCACATCAAGGTCTCC | 21 | 706 | 726 | 171 |
| 6 | *C23O* Rev | GTAGTCGTACTGAACCTCGTTG | 22 | 876 | 855 | |
| 7 | *PAH-RHDα* Fwd | CGGCGCCGACAAYTTYGTNGG | 21 | 641 | 661 | 292 |
| 8 | *PAH-RHDα* Rev | GGGGAACACGGTGCCRTGDATRAA | 24 | 933 | 909 | |

* Fwd- Forward, Rev- Reverse

#### 3.2.10.1 RNA isolation

Total RNA from isolated bacterial strains was isolated using the Trizol method of RNA isolation (Rio *et al.,* 2010) followed by DNase treatment to remove contaminations of DNA molecules. The collected bacterial samples were centrifuged at 12000 RCF at 4°C for 15 minutes, the supernatant was discarded and the bacterial culture pellet was dissolved in liquid nitrogen. Bacterial pellets were lysed by adding 1 mL of TRIzol per 2ml of bacterial culture. The lysed cells were resuspended in TRIzol by pipetting several times, or by vortexing. The samples were kept for 5 min at room temperature and 0.2 mL of chloroform was added per 1.0 mL of TRIzol. The samples were mixed vigorously for 20-30 seconds. The mixture was allowed to stand for 2-3 min at room temperature. Chloroform addition promotes phase separation. The mixture was centrifuged at 10,000 rpm in a centrifuge (SPINWIN™ MC-03) for 10 min at room temperature. Two phases i.e. an upper clear one containing RNA and a lower red one that contains protein were visible. Most of the DNA molecules were residing at the interface. The upper clear phase was transferred to a fresh tube, without disturbing the interface, and; 0.5 mL of isopropanol was added per 1 ml of the clear phase and mixed vigorously by rapid shaking. All the tubes were kept at rest for 10 min and the isopropanol was allowed to precipitate the total RNA. The precipitated RNA was collected by centrifugation at 12000 rpm speed in a microcentrifuge (SPINWIN™ MC-03) for 10 min at 4°C. The supernatant was carefully decanted. Any remaining liquid was removed with a pulled Pasteur pipette (Abdos, P31209) and the purified RNA was stored at -20°C. The quality of purified RNA samples was analyzed on 1.2 % denaturing agarose gel run in MOPS (3-(N-morpholino) propane sulfonic acid) buffer and quantified using NanoDrop 8000 Spectrophotometer (Thermo Scientific).

#### 3.2.10.2 Analysis of gene expression

The 500 ng of total RNA was used in the cDNA preparation reaction using the iScript kit (BioRad) following the manufacturer's protocol. Total cDNA was diluted up to10 ng/µl, and a total of 50 ng was used in a 20 µl reaction mixture using Power SYBR® Green PCR Master Mix (Life Technologies), and the reaction was performed on StepOnePlus™ Real-time PCR system (Thermo Fisher Scientific, Applied Biosystems™). The total 20 µl reaction mixture for RT-qPCR contained 10 µl 2X Sybr green buffer, 5 µl cDNA diluted to 10 ng/µl), 1µl primer of 10 µM forward, 1µl primer of 10 µM reverse, and 3µl nuclease-free water. For each reaction, three technical replicates were set in a 96 well format. Simultaneously, a no template control (NTC) for each set of primer was kept to put a check for contaminants or

non-specific amplicons or primer dimmers, if any. The thermal cycling program was set as 10 min at 95 °C (enzyme activation), 10 sec at 95 °C (cyclic denaturation), and 30 sec at 60 °C (annealing/extension) for 40-45 cycles, which includes data acquisition. A dissociation curve (Melting Curve) analysis was performed from 55°C-95°C in increments of 0.1 °C, each lasting for 5 sec, to ensure the presence of a specific product. The cycle threshold ($C_{T)}$ (Livak and Schmittgen 2001) values for each gene were recorded to examine gene expression. The RNA concentration in different samples was normalized using housekeeping gene 16S rRNA transcript abundance for both microbial samples.

All the experiments for the present study were performed in triplicates at the laboratory scale. The calculations during the present study for determining percent degradation, enzyme activity, the mean, standard deviation, and rate kinetics were performed on Microsoft Excel software. The standard curves were also drawn using Microsoft Excel software.

# CHAPTER 4
# RESULTS AND DISCUSSION

Based on the experiments performed for the isolation of microbes, two strains of bacteria were used for degradation of selected PAHs. The isolated strains could effectively degrade PAHs individually as well as in consortium. The catabolic enzyme activity and gene expression towards PAH-degradation was also reported during the experiments.

## 4.1 Isolation and identification of PAH-degrading microorganisms

The PAH-degrading bacterial strains were isolated from petroleum-contaminated soil collected from the siding area, Bijwasan supply location- BPCL, Delhi, by enrichment culture technique. The collected petroleum-contaminated soil was continually polluted with various petroleum products like motor spirit (MS), high speed diesel (HSD), aviation turbine fuel (ATF), light diesel oil (LDO), furnace oil (FO), mineral turpentine oil (MTO), and superior kerosene oil (SKO) since 2004. Different bacterial colonies were seen on LB agar plates inoculated with petroleum-contaminated soil and these colonies were first purified by repeated streaks on LB agar. Total seven different bacterial strains *i.e.* DTU-1Y, DTU-2Or, DTU-3W, DTU-4P, DTU-5Wr, DTU-6Tl, and DTU-7P having varied colony shapes and color were isolated from the soil. These seven bacterial strains were exposed to increasing concentrations of mixed PAHs (50-10,000 mg/L containing NAP, PHE, ANT, FLU, and PYR) gradually on LB plates by spray plate method to assay their growth (Table 4.1). The results showed that the growth of some of these isolated bacterial strains was inhibited or slow in presence of PAHs. Among these strains, DTU-2Or, DTU-3W, DTU-4P, and DTU-6Tl were found to have moderate growth on LB plates when only mixed PAHs up to 4000 mg/L concentration were present as the sole carbon source. Two potent Gram-positive strains DTU-1Y (forming yellow, aerobic, smooth, non-motile, coccoid wet colonies on LB-agar) and DTU-7P (forming antique pink, aerobic, smooth, non-motile, coccoid wet colonies on LB-agar) were able to grow on LB plate without yeast extract and with high PAHs concentration (10,000 mg/L) indicating that the strains were able to degrade PAHs as the sole carbon and energy source, and were selected for further study. The isolated bacterial strains were also tested for growth on minimal salt medium (MSM) with PAHs (10.0 mg/L) as the sole carbon source, and these strains showed similar growth as observed on LB medium without yeast extract (Table 4.1). This confirmed that the strains were able to use PAH

compounds as source of carbon. These two strains were able to display good growth in presence of a mixture of PAHs as well.

**Table 4.1 Utilization of mixed PAHs* as a carbon source for growth by isolated bacterial stains**

| Strains / *PAHs Conc. (mg/l) | Medium | DTU-1Y | DTU-2Or | DTU-3W | DTU-4P | DTU-5Wr | DTU-6Tl | DTU-7P |
|---|---|---|---|---|---|---|---|---|
| 50 | LB (0.5% YE) | ++++ | ++++ | ++++ | ++++ | - | ++++ | ++++ |
| 200 | LB (0.25% YE) | ++++ | ++++ | ++++ | ++++ | - | ++++ | ++++ |
| 500 | LB (0.25% YE) | ++++ | ++ | ++ | ++ | - | ++ | ++++ |
| 1000 | LB (0.25%YE) | ++++ | ++ | ++ | ++ | - | ++ | ++++ |
| 1000 | LB (W/o YE) | ++++ | ++ | ++ | ++ | - | ++ | ++++ |
| 1500 | LB (W/o YE) | ++++ | ++ | ++ | ++ | - | ++ | ++++ |
| 2000 | LB (W/o YE) | ++++ | ++ | + | ++ | - | ++ | ++++ |
| 3000 | LB (W/o YE) | ++++ | ++ | + | ++ | - | + | ++++ |
| 4000 | LB (W/o YE) | ++++ | ++ | - | + | - | - | ++++ |
| 5000 | LB (W/o YE) | ++++ | + | - | + | - | - | ++++ |
| 7000 | LB (W/o YE) | ++++ | - | - | - | - | - | ++++ |
| 10,000 | LB (W/o YE) | ++++ | - | - | - | - | - | ++++ |
| 10 | MSM+ 25%LB (W/o YE) | ++++ | - | - | - | - | - | ++++ |
| 10 | MSM+ 10%LB (W/o YE) | ++++ | - | - | - | - | - | ++++ |
| 10 | MSM+ 5%LB (W/o YE) | ++++ | - | - | - | - | - | ++++ |
| 10 | MSM (W/o LB) | ++++ | - | - | - | - | - | ++++ |

*: Mixture of Naphthalene, phenanthrene, anthracene, fluorene and pyrene; LB- Luria Bertani; YE- yeast extract; MSM- Minimal salt medium; W/o-without; (++++): Good growth; (++): moderate growth; (+): less growth; (-): no growth.

### 4.1.1 Biochemical characterization of isolated bacterial strains

Biochemical characteristics of isolated strains DTU-1Y and DTU-7P to study their metabolic capabilities were done at CSIR-IMTECH. Both isolated strains were negative for oxidase, gelatinase, Methyl red reaction, Voges–Proskauer tests. In biochemical tests, both the strains were positive for nitrate reduction, urease, amylase, catalase, Tween 80, and D-ribose tests (Table 4.2). The biochemical tests signify that the isolated strains have the ability to hydrolyse urea and starch, ferment carbohydrates as carbon source and reduce nitrate to nitrite or nitrogen gas. The strains do not have cytochrome c-oxidase and the ability of the oxidation of glucose.

**Table 4.2 Biochemical characterization of isolated bacterial strain**

| Characteristic | DTU-1Y | DTU-7P |
|---|---|---|
| Nitrate reduction | + | + |
| Urease | + | + |
| Oxidase | -- | -- |
| Phosphatase | + | -- |
| β-Galactosidase | + | -- |
| Amylase | + | + |
| Catalase | + | + |
| Methyl red reaction | -- | -- |
| Voges–Proskauer | -- | -- |
| Lactose fermentation | + | -- |
| Arabinose utilization | + | -- |
| Gelatinase | -- | -- |
| Maltose | + | -- |
| Tween 80 | + | + |
| D-ribose | + | + |
| D-xylose | + | -- |
| D-Sorbitol | + | + |
| Salicin | -- | + |
| D-Fructose | + | + |

### 4.1.2 Molecular identification of isolated bacterial strains

The 16S rRNA gene sequencing for molecular identification of isolated bacterial strains was done using the Sanger sequencing method and based on the whole 16S rRNA sequence (Table 4.3) analysis of pure bacterial strains by nucleotide BLAST search by CSIR-IMTECH. It was found that DTU-1Y strain was having similarity with *Kocuria sp.* and identified as *Kocuria flava* with 99.58% similarity, and DTU-7P strain was having similarity with *Rhodococcus sp.* and identified as *Rhodococcus pyridinivorans* with 98.58% similarity (Table 4.4). The position of identified bacterial strains in phylogeny was confirmed by constructing a phylogenetic tree for both strains using MEGA X (Molecular Evolutionary Genetics Analysis) (Kumar *et al.,* 2018) by the neighbor-joining algorithm (Saitou and Nei, 1987) using a maximum composite likelihood method and 1000-bootstrap replications. The phylogenetic tree indicated that DTU-1Y strain and *Kocuria flava* shared one cluster, and DTU-7P and *Rhodococcus pyridinivorans* shared one cluster (Fig. 4.1). The 16s rRNA gene sequences of the strains obtained in this study were deposited to NCBI (National Center for Biotechnology Information), *Kocuria flava* DTU-1Y with sequence accession number: MN841976, and *Rhodococcus pyridinivorans* DTU-7P with sequence accession number: MN841977. Similar observations about the screening, selection, and molecular identification of PAH-degrading microorganisms have been reported in other studies for isolation of *Sphingomonas koreensis* and *Achromobacter denitrificans* (Hesham *et al.,* 2014; Mawad *et al.,* 2016). *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P exhibited growth on LB agar plates without yeast extract when provided with 10,000 mg/L mixed PAHs containing NAP, PHE, ANT, FLU, and PYR as the carbon source for their growth. These isolated species from genus *Kocuria* and *Rhodococcus* have been reported for degradation of aromatic pollutants (Ahmed *et al.,* 2010; Kumari *et al.,* 2012; Kundu *et al.,* 2013). It has been reported that *Kocuria flava* CMG2028 capable of growing on PAHs and was found to have PAH-degradation ability when grown on naphthalene as the sole carbon source, and 53% naphthalene degradation was observed in 10 days when 500 mg/mL naphthalene was provided (Ahmed *et al.,* 2010). *Rhodococcus pyridinivorans* has not been studied for PAH degradation yet but it has been reported that this species has the ability to transform or degrade toxic compounds such as total petroleum hydrocarbons and nitrotoluene (Kumari *et al.,* 2012; Kundu *et al.,* 2013). *Rhodococcus pyridinivorans* has been found to have catabolic enzymes which are responsible for PAH degradation such as catechol 1,2 dioxygenase and catechol 2,3 dioxygenase (Kundu *et al.,* 2013). The results from this study

also confirm that these two isolated strains are capable of growing on the high concentration of PAHs and assimilate the compounds as the sole carbon source.

**Table 4.3 Detailed 16S rRNA gene sequence data for DTU-1Y and DTU-7P**

| S.No. | Bacterial strain | 16S rRNA gene sequence |
|---|---|---|
| 1. | DTU-1Y | > DTU-1Y<br>TCGAACGCTGAAGCTCCAGCTTGCTGGGGTGGATGAGTGGCGAACGGGTGAGTA<br>ATACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAA<br>TACTGGATACGACTCCTCACGCATGGTGGGGTGTGGAAAGGGTTTTACTGGTTTT<br>GGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGA<br>CGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACGGGACTGAGACACGGC<br>CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT<br>GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA<br>GCAGGAAGAAGCCACAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTAC<br>GTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGG<br>CGTAAAGAGCTCGTAGGCGGTTTTCGCGTCTGCTGTGAAAGCCCGGGGCTCAAC<br>CCCGGGTCTGCAGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAA<br>TTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACGATGGCGAAGG<br>CAGGTCTCTGGGCTGTTACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAG<br>GATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGGA<br>CATTCCACTTCTCCGCGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTAC<br>GGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGA<br>GCATGCGGATTAATTCGATGCAACGCGAGAACCTTACCAAGGCTTGACATCCAC<br>CGGACCGCACTGGAGACAGTGCTTCCCTTCGGGGCTGGTGGACAGGTGGTGCAT<br>GGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAAGAGCGCAA<br>CCCTCGTTCTATGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGCCGG<br>GGTCAACTCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCT<br>TGGGCTTCACGATGCTACAATGGCCGGTACAAAGGGTTGCGATACTGTGAGGTG<br>GAGCTAATCCCAAAAAGCCGGTCTCAGTTCGGATTGAGGTCTGCAACTCGACCT<br>CATGAAGTCGGAGTCGCTAGTAATCGCAGACAGCAACGCTGCGGTGAATACGTT<br>CCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTTGGTAACACCCGAAG<br>CCGGTGGCCTAACCCCTTGTGGGAGGGAGCCGTCGAAGGTGGGACCGGCATTGG<br>GACTAAGTCGTAAC |
| 2. | DTU-7P | > DTU-7P<br>GCAGTCGAACGATGAAGCCCAGCTTGCTGGGTGGATTAGTGGCGAACGGGTGAG<br>TAACACGTGGGTGATCTGCCCTGCACTCTGGGATAAGCCTGGGAAACTGGGTCT<br>AATACCGGATATGACCTCGGATGCATGTCCTGGGGTGGAAAGTTTTTCGGTGCA<br>GGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGA<br>CGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGACTGAGACACGGC<br>CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCT<br>GATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA<br>CCCAGACGAAGCGCAAGTGACGGTAGTGGGAGAAGAAGCACCGGCCAACTACG<br>TGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGC<br>GTAAAGAGCTCGTAGGCGGTTTGTGCGTCGTCTGTGAAATCCCGCAGCTCAACT<br>GCGGGCTTGCAGGCGATACGGGCAGACTCGAGTACTGCAGGGGAGACTGGAATT<br>CCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGTGGCGAAGGC<br>GGGTCTCTGGGCAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAG<br>GATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTT<br>CCTTCCACGGATCCGTGCCGTAGCCAACGCATTAAGCGCCCCGCCTGGGGAGTA<br>CGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG<br>AGCATGTGGATTAATTCGATGCAACGCGAAAACCTTACCTGGGTTTGACATGTA<br>CCGGACGACTGCAGAGATGTGGTTTCCCTTGTGGCCGGTAGACAGGTGGTGCAT<br>GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGACGCAA<br>CCCTTGTCCTGTGTTGCCAGCACGTGATGGTGGGGACTCGCAGGAGACTGCCGG<br>GGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCC<br>AGGGGCTTCACACAGCTACAATGGTCGGTACAGAGGGGCCTGCGATACCGGTGG<br>AGGGTGGAGAGCGGAAATCCCCTTAAAAGCCGGGTCTTCAATTTCGGATATCGG<br>GGG |

**Table 4.4 Nucleotide BLAST search of isolated strains DTU-1Y and DTU-7P with the nearest type strains**

| S. No. | Bacterial strain | Name | Strain | Pairwise Similarity (%) |
|---|---|---|---|---|
| **1.** | **DTU-1Y** | *Kocuria flava* | HO-9041 | 99.58 |
| | | *Kocuria oceani* | FXJ8.095 | 98.52 |
| | | *Kocuria turfanensis* | HO-9042 | 98.30 |
| | | *Kocuria sediminis* | FCS-11 | 98.19 |
| | | *Kocuria salina* | Hv14b | 98.14 |
| | | *Kocuria polaris* | CMS 76or | 97.95 |
| | | *Kocuria rosea* | DSM 20447 | 97.88 |
| | | *Kocuria dechangensis* | NEAU-ST5-33 | 97.66 |
| | | *Kocuria aegyptia* | YIM 70003 | 97.46 |
| | | *Kocuria atrinae* | P30 | 97.38 |
| **2.** | **DTU-7P** | *Rhodococcus pyridinivorans* | DSM 44555 | 98.94 |
| | | *Rhodococcus biphenylivorans* | TG9 | 98.54 |
| | | *Rhodococcus gordoniae* | DSM 44689 | 98.21 |
| | | *Rhodococcus lactis* | DW151B | 98.05 |
| | | *Rhodococcus rhodochrous* | NBRC 16069 | 97.65 |
| | | *Rhodococcus artemisiae* | YIM 65754 | 97.08 |
| | | *Rhodococcus electrodiphilus* | - | 96.49 |
| | | *Rhodococcus ruber* | DSM 43338 | 96.35 |
| | | *Rhodococcus coprophilus* | NBRC 100603 | 96.35 |
| | | *Rhodococcus phenolicus* | DSM 44812 | 96.02 |

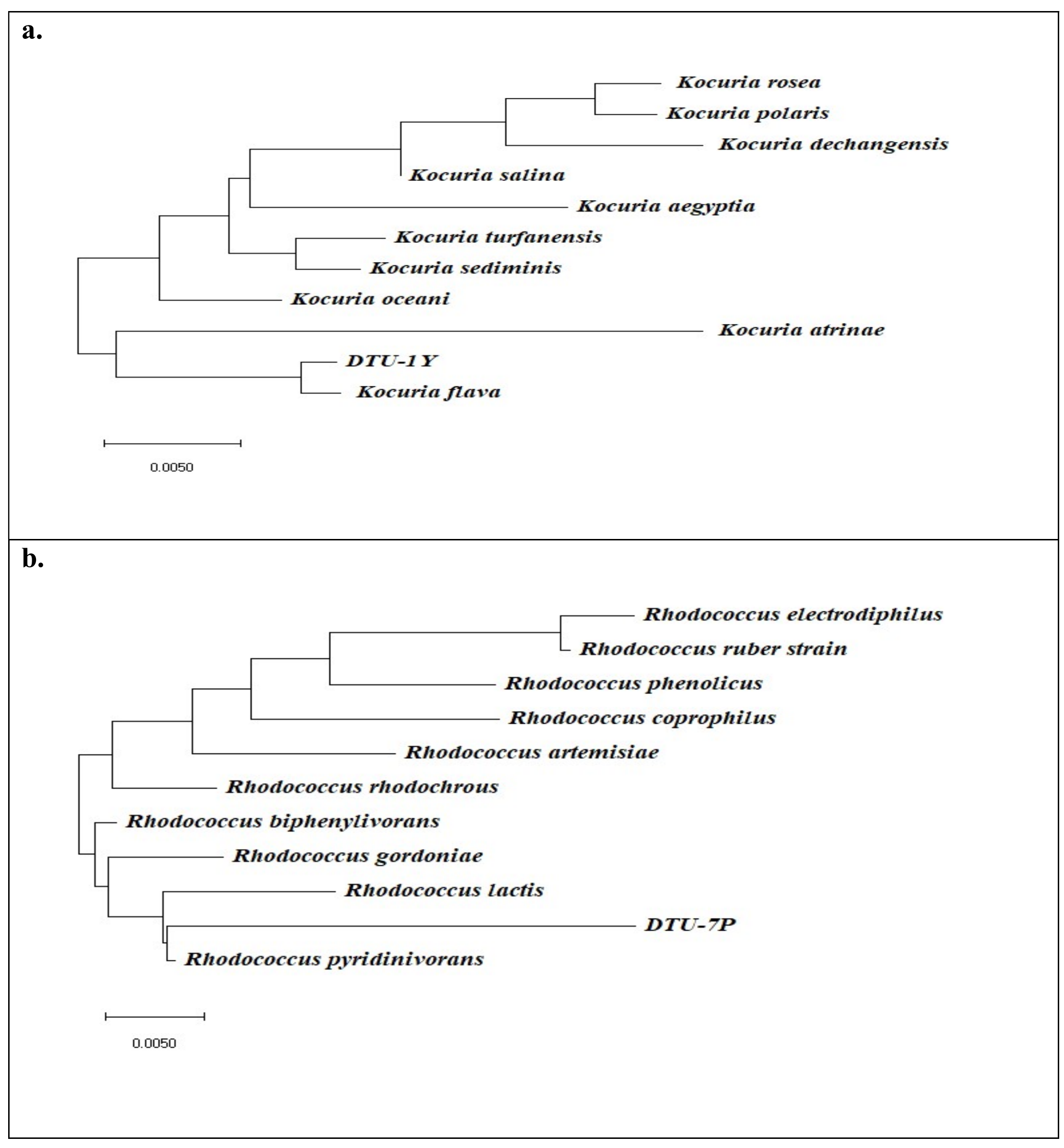



**Fig. 4.1 Phylogenetic analysis of 16S rRNA gene sequence of isolates DTU-1Y (a) and DTU-7P (b) with most closely related species in MEGA X by Neighbor-Joining method with 1000 replicates (bootstrap test). The scale bar corresponds to approximately 0.0050 changes per nucleotide position.**

## 4.2 Biodegradation of PAHs

Various bacterial strains have been found to have the ability to utilize more than one type of PAH for their growth. A number of studies have been carried out to investigate the degradation of PAHs by microbial species and a few genera of bacterial species having the ability to utilize different PAHs as carbon-and energy source have been isolated and studied

(Lara-Severino *et al.,* 2016; Haritash and Kaushik, 2016; Mawad *et al.,* 2016). Two bacterial strains *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P were selected based on their growth at high concentration of mixed PAHs. The degradation efficiency of isolates, as monocultures and in a consortium, for three-ring PAHs (PHE, ANT, and FLU) and four-ring PAH (PYR) was examined individually as well as in a mixture of PAHs at an initial PAH concentration of 10 mg/L. Biodegradation experiments monitored at 0, 3, 6, 9, 12, and 15 days of incubation in an incubator shaker at a temperature of 30°C under the dark conditions with constant shaking at 120 rpm demonstrated that the two bacterial isolates were able to degrade three-ring PAHs ranging from 55%-66% and four-ring PAH ranging from 53%-56% after 15 days of incubation at 30°C.

### 4.2.1 Biodegradation of three-ring PAHs (phenanthrene, anthracene, and fluorene)

The degradation efficiency of the two isolates *i.e. K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P as monocultures and in a consortium for three different PAHs i.e. PHE, ANT, and FLU was examined. Biodegradation experiments demonstrated that the two bacterial isolates (*Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P) were able to efficiently degrade three-ring PAHs with efficiency ranging from 55%-66% after 15 days of incubation at 30°C. As far as available data, it is the first study where *R. pyridinivorans* DTU-7P is reported to use PHE, ANT, and FLU as carbon and energy source and *K. flava* DTU-1Y to have the ability to use ANT and FLU. Both strains exhibited potential in utilizing all selected PAHs as carbon source as indicated by their growth on the substrate; illustrated in Table 4.5; and Fig. 4.2, 4.3, and 4.4. Degradation of PHE, ANT, and FLU by monocultures and consortium was recorded from the 3$^{rd}$ day, and more than 40% degradation was achieved within first nine days of incubation. It was observed that strain *K. flava*-DTU-1Y degraded 55.13%, 59.01%, 63.46%; strain *R. pyridinivorans* DTU-7P degraded 62.03%, 64.99% 66.79%; and consortium of both strains degraded 61.32%, 64.72%, 66.64% of 10 mg/L of PHE, ANT, and FLU, respectively, in liquid MSM cultures within 15 days. There was no significant difference in degradation efficiencies of *R. pyridinivorans* DTU-7P and consortium indicating that there is no synergistic or inhibitive effect of both isolated strains over each other. Monitoring of bacterial growth curve during PAH degradation has notable significance in understanding biodegradation capabilities of the bacterial strain. The biomass of both bacterial strains gradually increased during the 3$^{rd}$ to 6$^{th}$ day of incubation and after that maximum biomass accumulation was achieved. Relatively more growth of both strains was observed on ANT and FLU compared to PHE. The

maximum degradation rate was observed in the logarithmic growth phase of bacterial isolates. The degradation rate for PHE was found to be slightly low for both the bacterial strains as monocultures and as consortium. It was observed that the degradation efficiency of monoculture *R. pyridinivorans* DTU-7P is more as compared to monoculture *K. flava* DTU-1Y and consortium for all the PAHs (Fig. 4.2, 4.3, and 4.4), however insignificant difference was observed in the degradation efficiencies of *R. pyridinivorans* DTU-7P and consortium. The strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium had maximum degradation efficiency for FLU (63.46%, 66.79%, and 66.64% respectively) as compared to the other two PAHs. Minimal degradation was observed for PHE (55.13%) by *K. flava.* Comparing the degradation among isolated strains, minimum degradation for PHE was observed for both the strains and consortium. The minimum degradation for PHE might be due to its stable chemical structure because biodegradability primarily depends on the complexity of chemical structures as well (Bamforth and Singleton, 2005). The benzene rings in ANT are fused linearly having four resonance structures, whereas, in PHE, the rings are fused at an angle having five resonance structures. The extra resonance in PHE makes it more stable than ANT (Poater *et al.,* 2018). The degradation profile of PAHs based on initial and final concentration determined over HPLC confirmed that complete degradation of PAHs has been observed, and no formation of secondary metabolites was noted (Fig. 4.5).

Upon comparison with other studies, bacterial species belonging to the genus *Rhodococcus* were found to have catabolic activity for PAHs (Song *et al.,* 2011; Goswami *et al.,* 2017), *R. pyridinivorans* has not been investigated for PAH degradation. *Rhodococcus sp*. P14, isolated from crude oil-contaminated sediment was reported to degrade 43% PHE in 30 days when supplemented at a concentration of 50 mg/L (Song *et al.,* 2011); and *R. opacus* degraded ANT within 7 days (Goswami *et al.,* 2017). In the present study, *R. pyridinivorans* DTU-7P strain has the potential in utilizing three-ring PAHs as the sole source of carbon and energy with 62.02%, 64.99%, and 66.79% degradation efficiency for PHE, ANT, and FLU respectively, after 15 days of incubation. *K. flava* CMG2028 has been reported to grow in presence of PHE (Ahmed *et al.,* 2010) but it has not been reported for three-ring PAH degradation till date. In other studies, *K. rosea* has been reported to degrade 54.6% PHE (1 mg/L) in 15 days (Haritash and Kaushik, 2016), and 46-54% of ANTH (0.27 mg/L) within 2 days (Lara-Severino *et al.,* 2016). The strains used in the present study represent slightly higher degradation efficiency compared to similar strains reported in the previous studies.

**Table 4.5 Degradation efficiency (%) and growth of biomass during biodegradation of PHE, ANT, and FLU with *K. flava* DTU-1Y. *R. pyridinivorans* DTU-7P, and consortium (± represents the standard deviation of replicates)**

| Microbial sp. | Time (Day) | Phenanthrene | | Anthracene | | Fluorene | |
|---|---|---|---|---|---|---|---|
| | | Degradation (%) ± SD | Microbial Growth ($OD_{600}$) | Degradation (%) ± SD | Microbial Growth ($OD_{600}$) | Degradation (%) ± SD | Microbial Growth ($OD_{600}$) |
| ***K. flava*** **DTU-1Y** | 0 | 00.00±0.0 | 0.03±0.01 | 00.00±0.0 | 0.09±0.04 | 00.00±0.0 | 0.04±0.02 |
| | 3 | 15.13±4.0 | 0.44±0.20 | 25.89±3.5 | 0.68±0.3 | 16.54±2.9 | 0.68±0.31 |
| | 6 | 21.90±2.2 | 0.57±0.25 | 37.72±3.6 | 0.83±0.37 | 43.27±2.6 | 0.74±0.33 |
| | 9 | 42.96±2.8 | 0.49±0.22 | 49.42±4.3 | 0.81±0.36 | 52.81±2.7 | 0.72±0.33 |
| | 12 | 53.93±2.6 | 0.47±0.21 | 52.99±3.3 | 0.8±0.36 | 59.95±2.4 | 0.68±0.3 |
| | 15 | 55.13±3.4 | 0.46±0.21 | 59.01±2.9 | 0.77±0.34 | 63.46±2.9 | 0.66±0.3 |
| ***R. pyridinivorans*** **DTY-7P** | 0 | 00.00±0.0 | 0.03±0.01 | 00.00±0.0 | 0.09±0.04 | 00.00±0.0 | 0.04±0.02 |
| | 3 | 14.04±3.2 | 0.42±0.19 | 26.13±4.4 | 0.69±0.31 | 27.10±3.7 | 0.53±0.24 |
| | 6 | 28.13±3.6 | 0.54±0.24 | 38.79±2.4 | 0.75±0.34 | 46.84±2.2 | 0.62±0.28 |
| | 9 | 44.58±3.8 | 0.52±0.23 | 48.77±2.3 | 0.72±0.33 | 52.73±4.4 | 0.58±0.26 |
| | 12 | 61.14±2.9 | 0.49±0.22 | 52.84±2.3 | 0.72±0.35 | 62.96±1.9 | 0.53±0.24 |
| | 15 | 62.03±2.6 | 0.46±0.21 | 64.99±2.6 | 0.72±0.38 | 66.79±3.6 | 0.51±0.23 |
| ***K. flava*** **DTU-1Y +** ***R. pyridinivorans*** **DTY-7P** | 0 | 00.00±0.0 | 0.03±0.02 | 00.00±0.0 | 0.07±0.03 | 00.00±0.0 | 0.05±0.02 |
| | 3 | 20.38±3.3 | 0.61±0.27 | 32.41±4.7 | 0.67±0.3 | 25.81±2.7 | 0.61±0.27 |
| | 6 | 36.19±3.8 | 0.73±0.33 | 42.68±2.1 | 0.78±0.35 | 44.18±1.6 | 0.72±0.32 |
| | 9 | 43.79±3.3 | 0.72±0.32 | 47.29±4.2 | 0.76±0.34 | 50.11±2.8 | 0.7±0.31 |
| | 12 | 56.77±4.3 | 0.71±0.32 | 52.19±4.6 | 0.76±0.34 | 59.99±1.1 | 0.66±0.3 |
| | 15 | 61.32±1.5 | 0.68±0.3 | 64.72±3.9 | 0.75±0.33 | 66.64±3.4 | 0.64±0.29 |

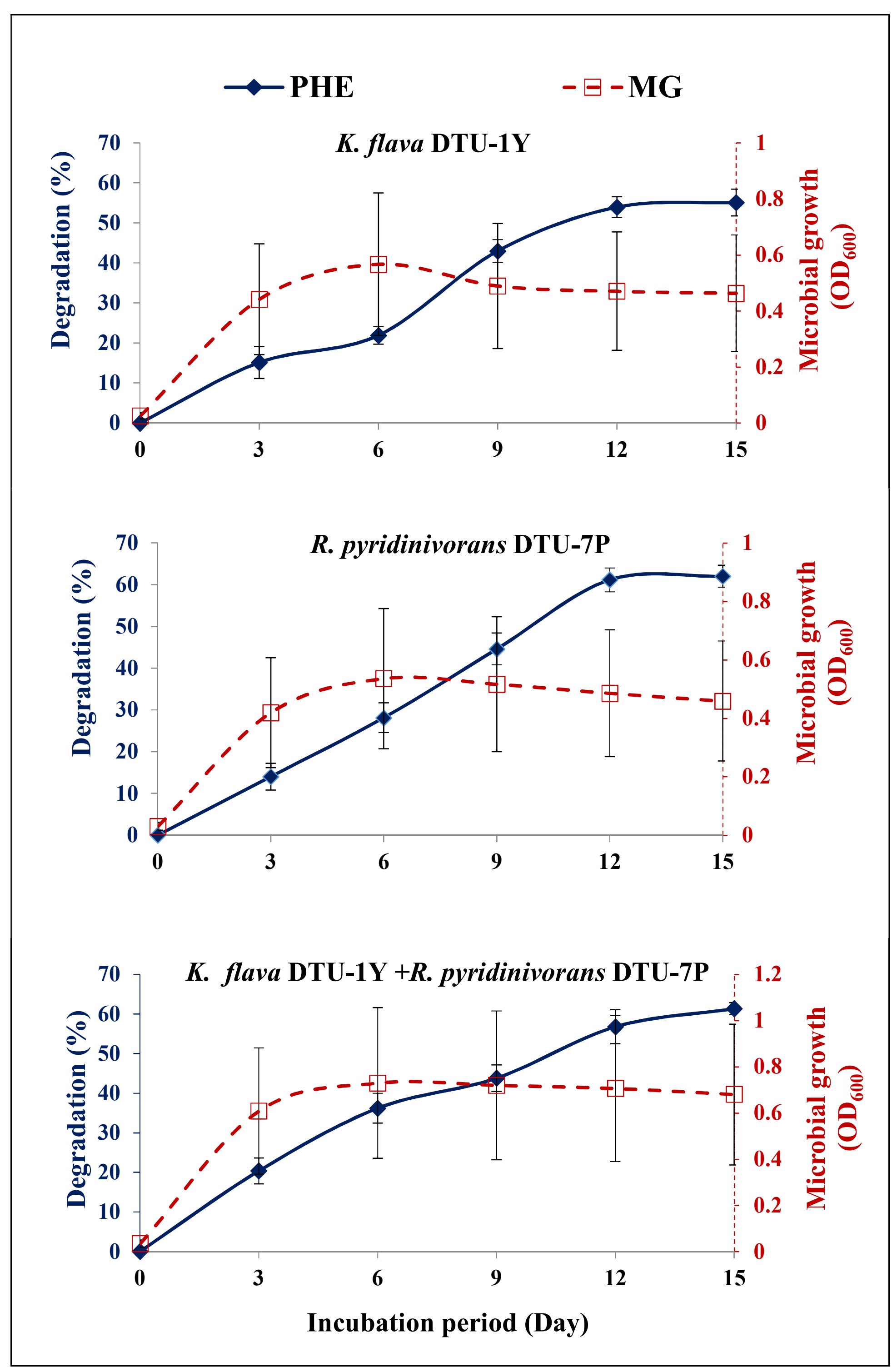



**Fig. 4.2 Dynamics of microbial growth (MG) during biodegradation of phenanthrene (PHE) by bacterial strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P and consortium in 15 days. (Solid symbol represents degradation; hollow symbol is for bacterial growth ($OD_{600nm}$) and error bars represents the standard deviation of replicates)**

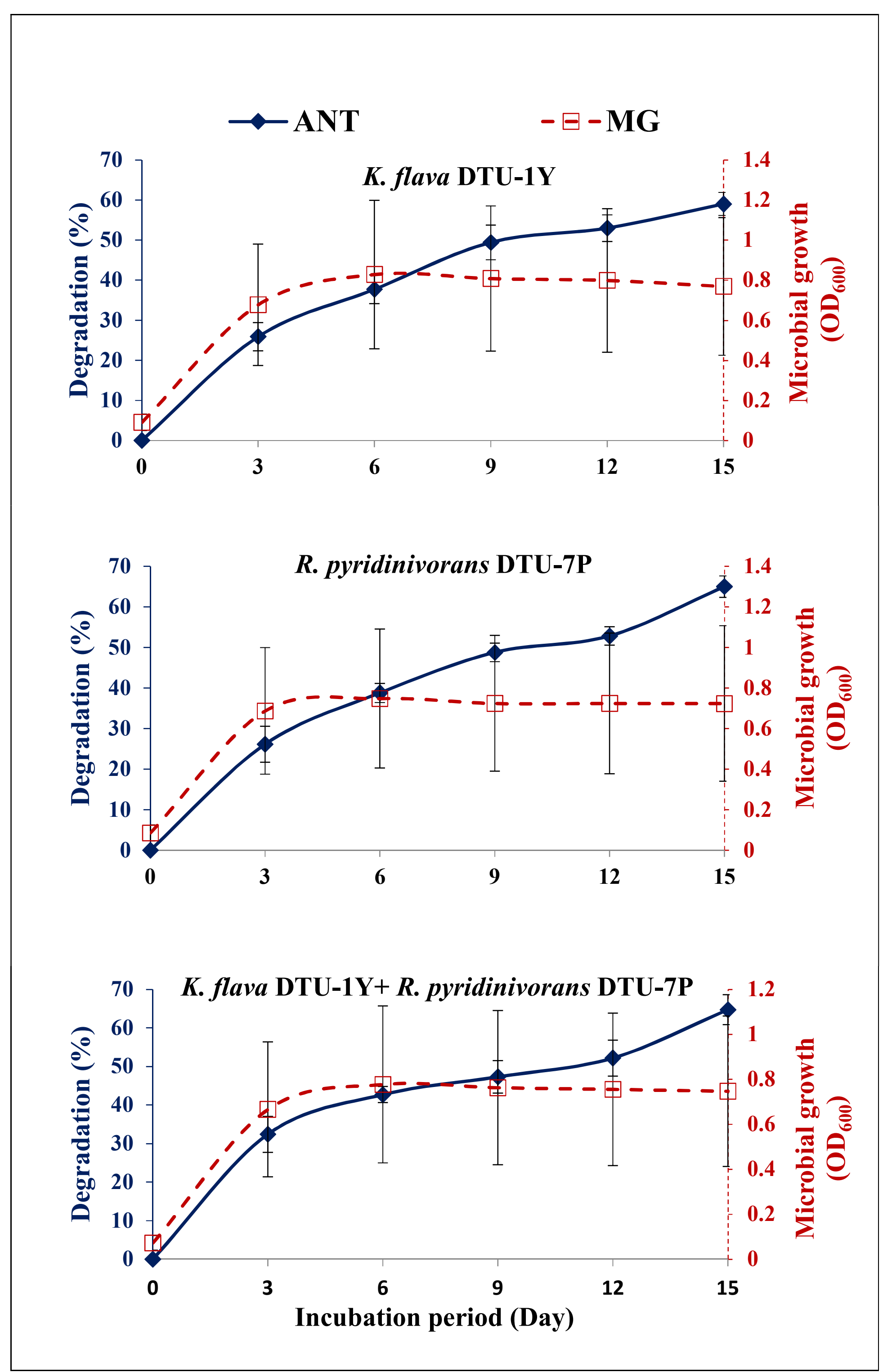



**Fig. 4.3 Dynamics of microbial growth (MG) during biodegradation of anthracene (ANT) by bacterial strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P and consortium in 15 days. (Solid symbol represents degradation; hollow symbol is for bacterial growth ($OD_{600nm}$) and error bars represents the standard deviation of replicates)**

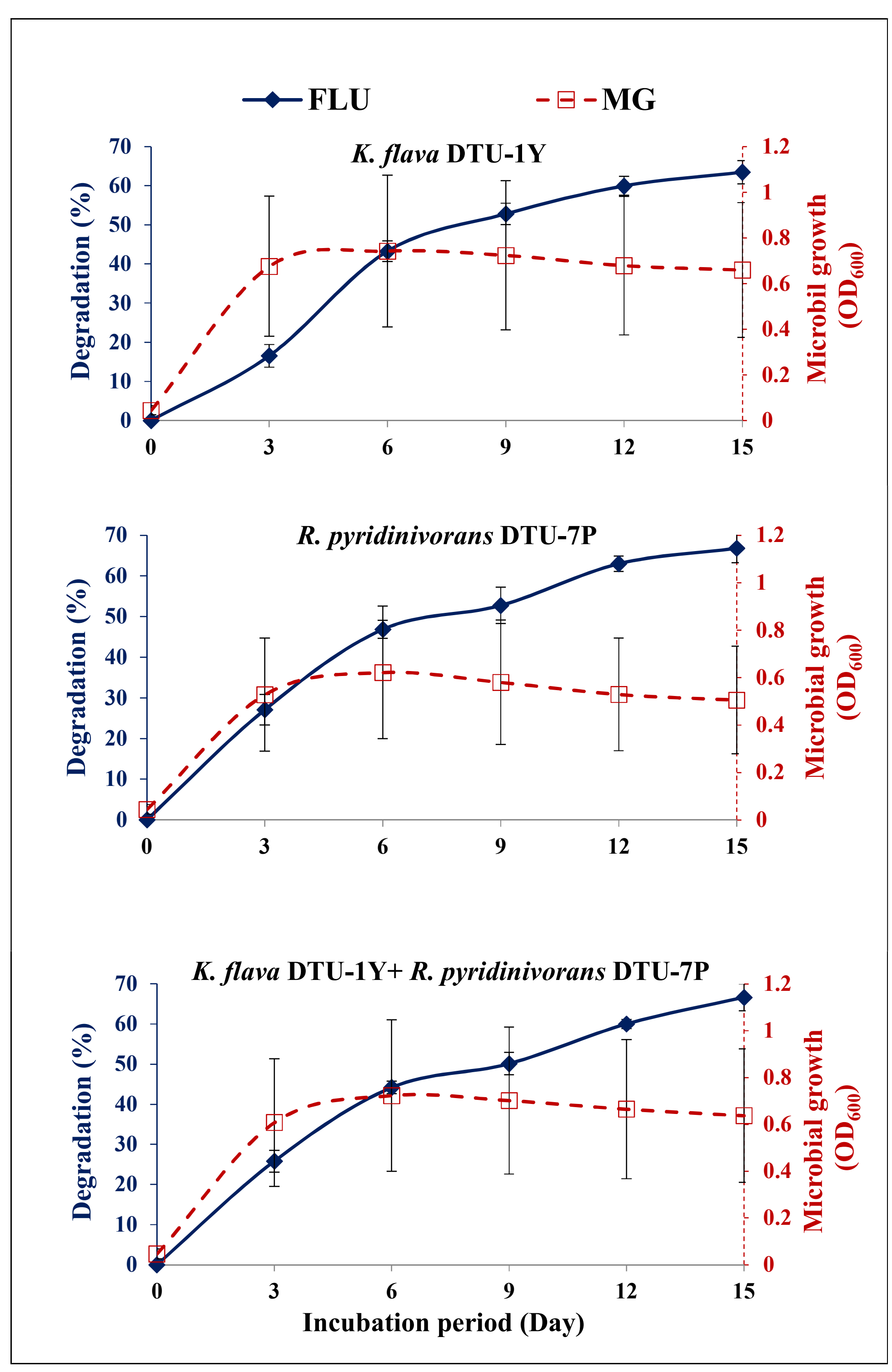



**Fig. 4.4 Dynamics of microbial growth (MG) during biodegradation of fluorene (FLU) by bacterial strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P and consortium in 15 days. (Solid symbol represents degradation; hollow symbol is for bacterial growth ($OD_{600nm}$) and error bars represents the standard deviation of replicates)**

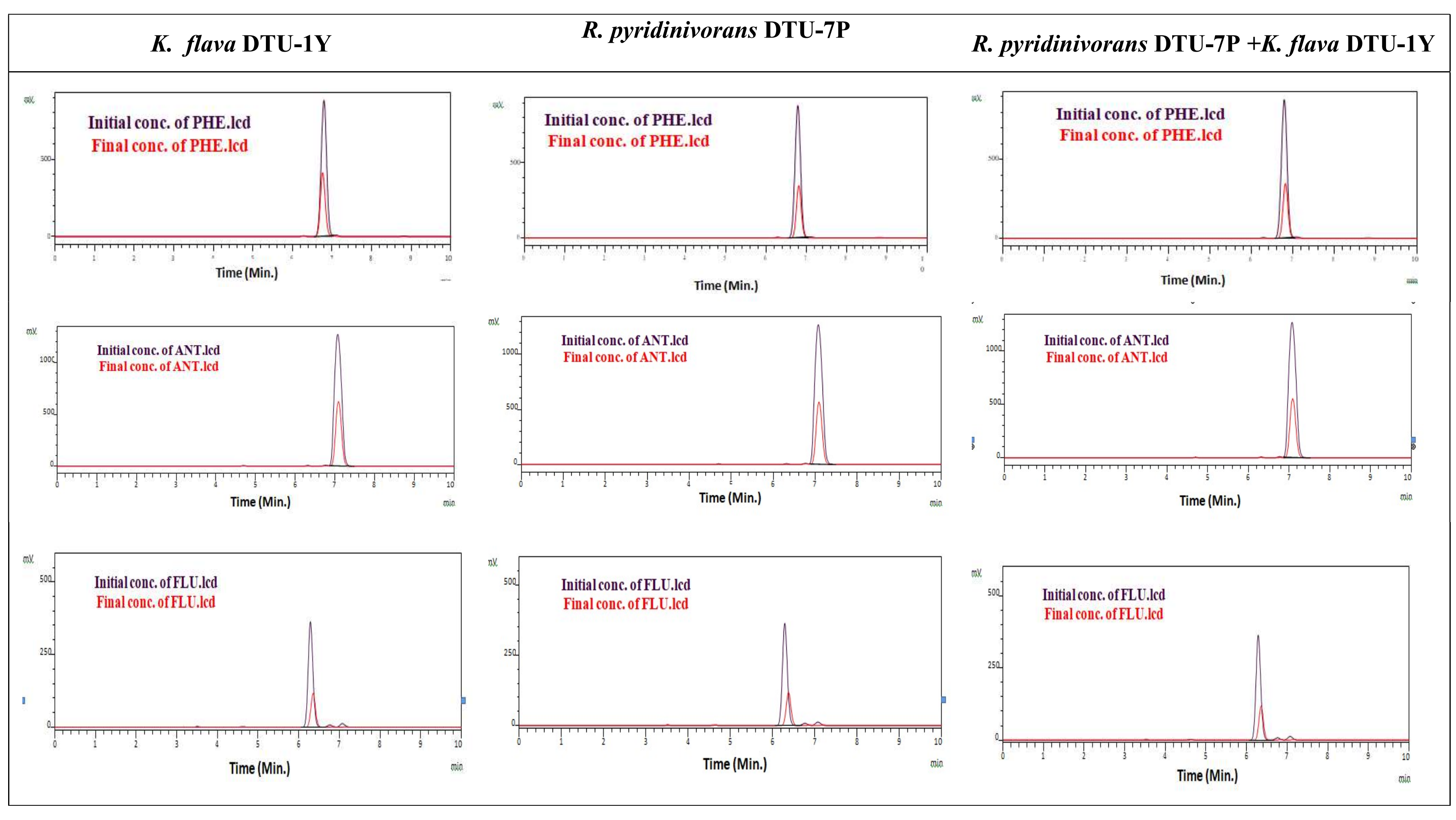



**Fig. 4.5 Initial and final HPLC chromatograms of PHE, ANT, and FLU during microbial degradation**

### 4.2.2 Biodegradation of four-ring PAH (pyrene)

The degradation of PYR was evident with both the isolates. Experiments revealed that the two isolates have the ability to efficiently degrade PYR ranging from 53%-56% after an incubation period of 15 days. It is the first investigation where the two isolates *K. flava* and *R. pyridinivorans* are reported to use PYR; a HMW-PAH as carbon and energy source for their growth. Percent degradation of PYR was examined during microbial growth of pure cultures in MSM containing 10 mg/L PYR. Both isolated strains revealed potential in utilizing PYR as carbon source as signified by their growth on PYR as substrate in MSM (Fig. 4.6). Degradation of PYR by monocultures and consortium of both isolates was recorded at a 3-day interval, and significant degradation was attained during the first six days of incubation. It was noticed that the degradation efficiency of monoculture *K. flava* for PYR is slightly less as compared to monoculture *R. pyridinivorans*, and consortium. Within 15 days of incubation, *K. flava*, *R. pyridinivorans,* and consortium degraded 53.79%, 56.22%, and 56.45% of 10 mg/L of PYR, respectively, in liquid MSM (Table 4.6). Pyrene degradation by the consortium revealed that there is no synergistic or inhibitive effect of the strains over each other. The possible reason for almost similar maximal degradation could be inter-specific competition for the substrate (PYR) in case of degradation by the consortium. Although the competition may be treated as mutually inhibitive effect, but the outcome in terms of PYR degradation remained unaffected, thereby classifying the interaction in consortium as neutral. Assessment of bacterial growth during PAH-degradation has notable significance to understand the biodegradation abilities of bacterial strains. As a part of this study, the microbial growth of each isolated strain was monitored during PYR-degradation. The biomass of both the isolates gradually increased during the first six days of incubation and after that the growth stabilized gradually. The maximum rate of PYR-degradation was monitored during the logarithmic growth phase of isolates, and more than 25% degradation was achieved within first 6 days. Analysis of initial and final concentration of treated media over HPLC confirmed significant pyrene degradation within 15 days (Fig. 4.7).

Whereas a number of studies have been done for PYR degradation using various bacterial species (Mawad *et al.,* 2016; Subashchandrabose *et al.,* 2019a), but degradation by *K. flava* and *R. pyridinivorans* have not been investigated. However, other bacterial species belonging to the genus *Kocuria* and *Rhodococcus* have been studied for pyrene degradation. *K. rosea* isolated from oil-contaminated soil has been reported to degrade 53.3% pyrene (1 mg/L) in 15 days (Haritash and Kaushik, 2016), and *Rhodococcus wratislaviensis* has been reported to degrade 40% pyrene (10.11 mg/L) in 7 days (Subashchandrabose *et al.,* 2019a).

An enteric bacterium *Leclercia adecarboxylata* PS4040, isolated from oily sludge-contaminated soil sample was also reported for 61.5% degradation of pyrene (300 mg/L) within 20 days when it was used as a sole carbon and energy source (Sarma *et al.,* 2009). *Mycobacterium* strains (NJS-1 and NJS-P) isolated from PAH-contaminated farmland soil were reported to degrade PYR (48.55 ± 5.76% to 54.14 ± 7.43%) within 2 months of incubation period (Zeng *et al.,* 2010). These studies signify the role of native bacterial species isolated from PAH-contaminated sites, in PYR-degradation. Although the PYR-degradation ability of *K. flava* and *R. pyridinivorans* examined in the present study is found to be similar to other strains reported in the literature when studied under controlled laboratory conditions, but the degradation may reduce when these microbes are applied under open field conditions indicating that the bacterial species and environmental conditions can affect the degradation significantly.

**Table 4.6 Degradation efficiency (%) and growth of biomass during biodegradation of PYR with *K. flava* DTU-1Y. *R. pyridinivorans* DTU-7P, and consortium (± represents the standard deviation of replicates)**

| Microbial sp. | Incubation time (Day) | Degradation (%) ± SD | Microbial Growth ($OD_{600}$) |
|---|---|---|---|
| ***K. flava* DTU-1Y** | **0** | 00.00±0.0 | 0.19±0.09 |
| | **3** | 17.80±1.8 | 0.70±0.32 |
| | **6** | 25.98±3.0 | 0.87±0.39 |
| | **9** | 35.70±2.9 | 0.86±0.38 |
| | **12** | 47.37±3.0 | 0.84±0.38 |
| | **15** | 53.76±3.4 | 0.83±0.37 |
| ***R. pyridinivorans* DTY-7P** | **0** | 00.00±0.0 | 0.20±0.09 |
| | **3** | 19.77±3.9 | 0.64±0.29 |
| | **6** | 29.75±1.8 | 0.72±0.32 |
| | **9** | 39.93±2.8 | 0.69±0.31 |
| | **12** | 44.92±2.2 | 0.66±0.30 |
| | **15** | 56.22±4.9 | 0.65±0.29 |
| ***K. flava* DTU-1Y + *R. pyridinivorans* DTY-7P** | **0** | 00.00±0.0 | 0.21±0.09 |
| | **3** | 20.06±1.6 | 0.88±0.39 |
| | **6** | 25.06±3.6 | 0.94±0.42 |
| | **9** | 44.22±2.1 | 0.90±0.40 |
| | **12** | 49.44±1.6 | 0.87±0.39 |
| | **15** | 56.45±1.3 | 0.86±0.39 |

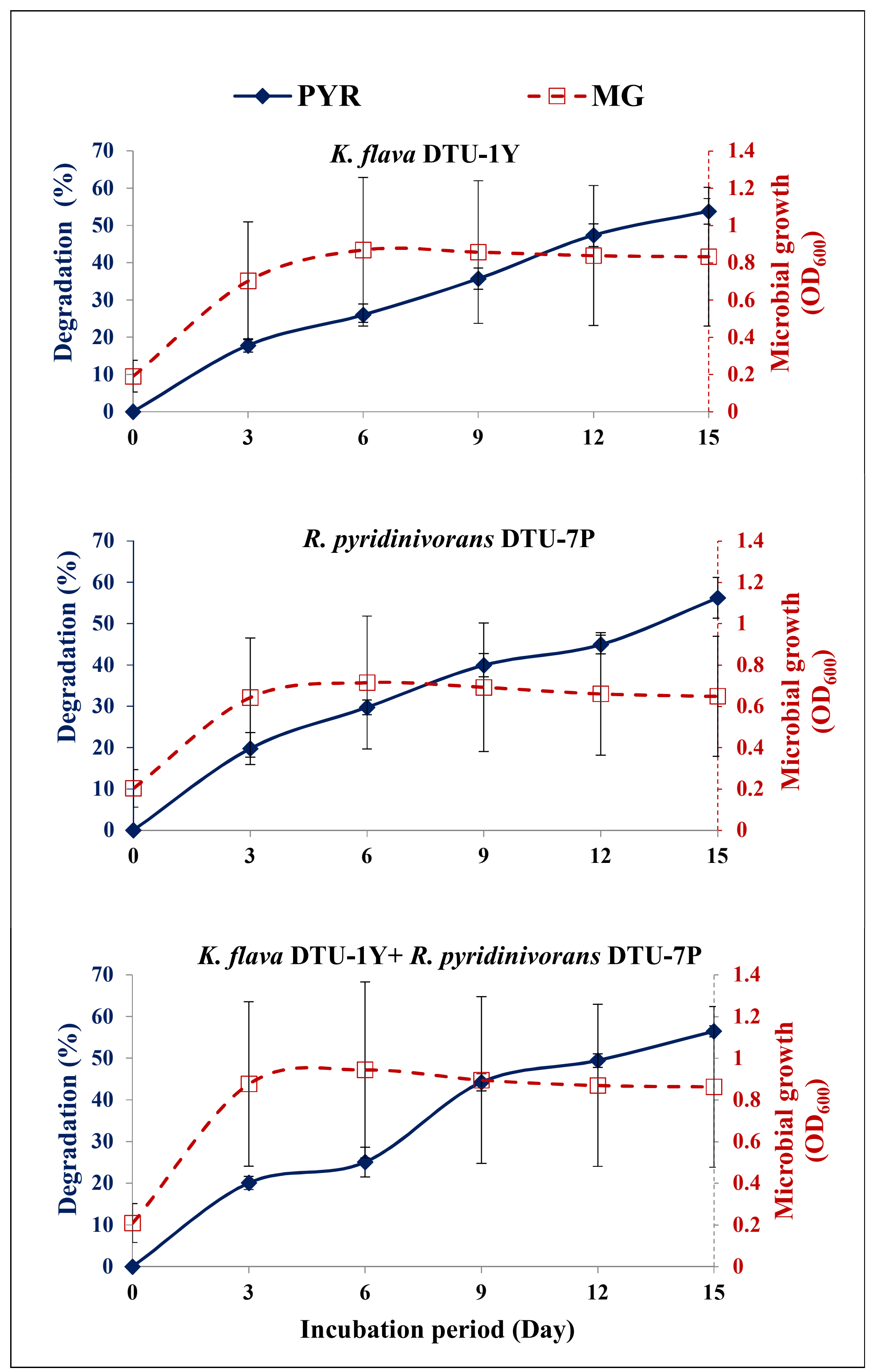



**Fig. 4.6 Dynamics of microbial growth (MG) during biodegradation of pyrene (PYR) by bacterial strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P and consortium in 15 days. (Solid symbol represents degradation; hollow symbol is for bacterial growth ($OD_{600nm}$) and error bars represents the standard deviation of replicates)**

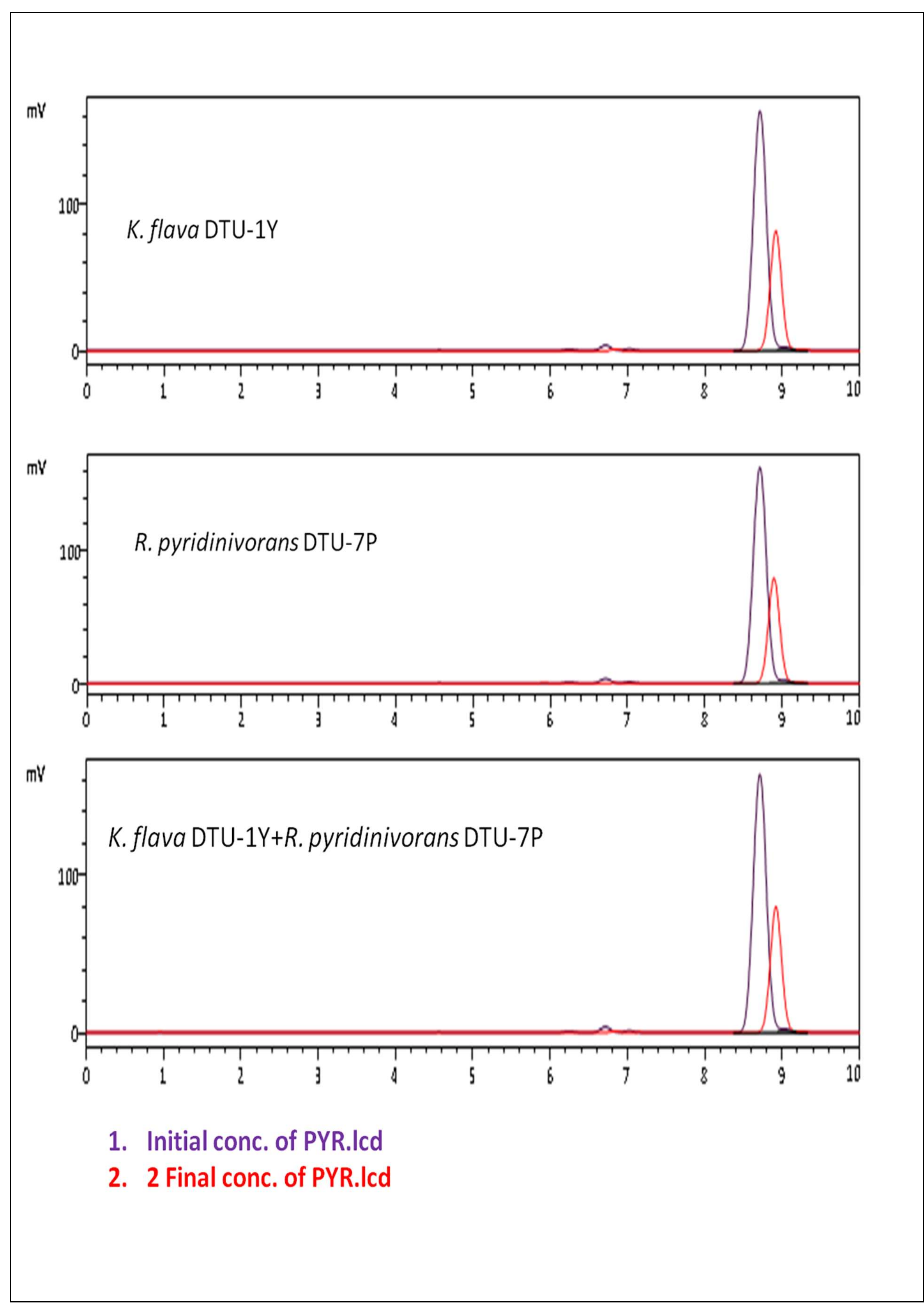



**Fig. 4.7 Initial and final HPLC chromatograms of PYR during microbial degradation**

### 4.2.3 Degradation of mixed PAHs

The degradation of mixed PAHs was evident with bacterial strains *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P in the present investigation. Degradation experiments show that these bacterial strains have the ability to efficiently degrade a mixture of PAHs containing low molecular weight PAHs *i.e.* PHE, ANT, FLU as well as high molecular weight PAHs *i.e.* PYR ranging from 56%-59% after 15 days of incubation period at 30°C. As far as available data, this is the first report to examine the capability of *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P to use PAH-mixture as carbon and energy source for their growth. Degradation of mixed PAHs by bacterial strains was examined during microbial growth in MSM containing 10 mg/L mixed PAHs. Both the strains were found to have great potential in utilizing mixed PAHs as carbon source as indicated by their growth on a mixture of PAHs as substrate in MSM (Fig. 4.8). Degradation of mixed PAHs by monocultures and consortium of both isolates was significant during the first nine days of incubation. It was investigated that the degradation efficiency of monoculture *K. flava* DTU-1Y is less as compared to monoculture *R. pyridinivorans*-DTU-7P and consortium. After 15 days of incubation *K. flava*-DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium degraded 55.6%, 59.5%, and 59.1% of 10 mg/L of mixed PAHs, respectively, in liquid MSM (Table 4.7). The strain *R. pyridinivorans*-DTU-7P had maximum degradation efficiency for mixed PAHs, however, there is no significant difference in the degradation efficiencies of *R. pyridinivorans*-DTU-7P and consortium. Bacterial growth assessment during degradation has a significant role to understand the degradation abilities of bacterial strains. The biomass of both the bacterial strains increased steadily during the first $6^{th}$ to $9^{th}$ day of incubation. Maximum growth of biomass of *K. flava*-DTU-1Y and *R. pyridinivorans*-DTU-7P was observed during $6^{th}$ -$9^{th}$ day. The degradation of mixed PAHs follows steady increase during the incubation period of 15 days. Stabilization of microbial biomass/growth from $12^{th}$ day onward resulted in gradual stabilization of degradation as well. This may be attributed to limited/scarce availability of growth nutrients (by the $15^{th}$ day) in the MSM used for degradation studies. HPLC analysis of initial and final concentration confirmed significant degradation of mixed PAHs within 15 days (Fig. 4.9). The graphs clearly show continuous considerable degradation. The maximum rate of degradation was achieved during the logarithmic growth phase of isolates, and more than 43% degradation was achieved within 9 days.

The range of degradation efficiency of three-ring PAHs (ANT, PHE, and FLU) and four-ring PAH (PYR) was 53%-61% during 15 days of the incubation period. The strain *K.*

*flava* DTU-1Y degraded 54.11%, 60.75%, 53.26% of 2.5 mg/L of PHE, ANT, and FLU, respectively, *R. pyridinivorans*-DTU-7P degraded 59.97%, 61.13%, 61.21% of 2.5 mg/L of PHE, ANT, and FLU, respectively whereas, consortium of both strains degraded 59.84%, 60.80%, 60.34% of 2.5 mg/L of PHE, ANT, and FLU, respectively. The *K. flava*-DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium degraded 53.17%, 55.87%, 55.70% of 2.5 mg $L^{-1}$ of PYR, respectively (Table 4.7). The consortium of both strains exhibited similar degradation efficiency with *R. pyridinivorans*-DTU-7P and slightly higher degradation efficiency as compared to *K. flava* DTU-1Y indicating that there was no inhibitory or synergistic effect of these strains over each other.

Both the isolated strains were found to have the ability to utilize PHE, ANT, FLU, and PYR as the sole source of carbon and energy when present as a single PAH compound or as a mixture of PAHs. The degradation efficiency of both strains (monoculture and consortium) was more than 53% for three-ring and four-ring PAHs (10 mg/L) when used individual PAH compound or in a mixture of PAHs. The degradation efficiency of 55.13%, 59.01%, and 63.46% for PHE, ANT, and FLU (10 mg/L) respectively, was observed with *K. flava* DTU-1Y, whereas, *R. pyridinivorans*-DTU-7P could degrade 62.03%, 64.99%, and 66.79% of PHE, ANT, and FLU (10 mg/L). The 61.32%, 64.72%, and 66.64% degradation of 10 mg/L of PHE, ANT, and FLU was observed with consortium of both strains within 15 days of incubation. The *K. flava*-DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium degraded 53.79%, 56.22%, and 56.45% of 10 mg $L^{-1}$ of PYR, respectively. On the other hand, in a mixture of PAHs, *K. flava*-DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium degraded 55.6%, 59.5%, and 59.1% of 10 mg/L of mixed PAHs, respectively, in liquid MSM within 15 days of incubation (Fig. 4.10). In the present study, it was observed that the degradation efficiency of isolated bacterial strains for a PAH compound was not affected by the presence of other PAH compounds. It was observed that the degradation efficiency of the selected bacterial strains *i.e. K. flava* DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium for PHE (54.11%, 59.97%, and 59.84, respectively), ANT (60.75%, 61.13%, and 60.80%, respectively ), and PYR (53.17%, 55.87%, and 55.70%, respectively) in the mixture of the PAHs (PHE, ANT, FLU and PYR) was found almost similar to the degradation efficiency of *K. flava* DTU-1Y and *R. pyridinivorans*-DTU-7P and consortium for respective PAHs *i.e.* for PHE (55.13%, 62.03%, and 61.32%, respectively), ANT (59.10%, 64.99%, and 64.72%, respectively), and PYR (53.17%, 55.87%, and 55.70%, respectively) when present as a single compound within 15 days of the incubation period (Table 4.7 and Fig. 4.10).

**Table 4.7 Degradation efficiency (%) and growth of biomass during biodegradation of mixed PAHs (PHE, ANT, FLU and PYR) with *K. flava* DTU-1Y. *R. pyridinivorans* DTU-7P, and consortium (± represents the standard deviation of replicates)**

| Microbial sp. | Incubation time (Day) | Microbial Growth ($OD_{600}$) | Mixed PAHs (PHE, ANT, FLU and PYR; 10mg/L) | Phenanthrene in a mixture of PAHs (2.5mg/L) | Anthracene in a mixture of PAHs (2.5mg/L) | Fluorene in a mixture of PAHs (2.5mg/L) | Pyrene in a mixture of PAHs (2.5mg/L) |
|---|---|---|---|---|---|---|---|
| | | | Degradation (%) ± SD | Degradation (%) ± SD | Degradation (%) ± SD | Degradation (%) ± SD | Degradation (%) ± SD |
| ***K. flava* DTU-1Y** | **0** | 0.04±0.01 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 |
| | **3** | 0.40±0.06 | 22.70±2.6 | 22.77±4.1 | 32.18±5.3 | 13.41±3.6 | 18.49±4.6 |
| | **6** | 0.51±0.05 | 31.60±1.8 | 32.80±2.2 | 41.97±2.1 | 18.92±1.4 | 27.59±4.2 |
| | **9** | 0.48±0.06 | 43.50±1.9 | 40.31±4.3 | 53.88±2.2 | 36.86±1.9 | 39.99±3.0 |
| | **12** | 0.43±0.03 | 51.92±3.1 | 49.91±3.4 | 59.63±3.9 | 45.57±0.5 | 49.96±4.1 |
| | **15** | 0.42±0.03 | 55.64±2.6 | 54.11±2.8 | 60.75±4.2 | 53.26±3.6 | 53.17±4.1 |
| ***R. pyridinivoran*s DTY-7P** | **0** | 0.04±0.01 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 |
| | **3** | 0.40±0.07 | 30.70±3.0 | 30.09±7.1 | 30.18±3.7 | 33.94±4.5 | 29.96±1.4 |
| | **6** | 0.47±0.08 | 41.07±3.9 | 43.90±4.0 | 41.00±4.0 | 38.91±4.1 | 39.35±3.8 |
| | **9** | 0.50±0.10 | 50.84±2.2 | 50.84±1.7 | 52.46±3.2 | 50.84±3.0 | 48.87±2.2 |
| | **12** | 0.49±0.11 | 56.83±3.1 | 57.00±2.6 | 59.13±3.8 | 57.20±3.7 | 53.55±4.5 |
| | **15** | 0.46±0.08 | 59.49±2.2 | 59.97±2.1 | 61.13±4.4 | 61.21±4.2 | 55.87±5.4 |
| ***K. flava* DTU-1Y + *R. pyridinivoran*s DTY-7P** | **0** | 0.03±0.01 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 | 00.00±0.0 |
| | **3** | 0.43±0.02 | 28.64±0.9 | 25.83±2.3 | 27.94±2.7 | 33.63±3.5 | 29.51±1.3 |
| | **6** | 0.47±0.01 | 39.29±1.0 | 42.79±2.8 | 38.32±1.9 | 36.57±2.1 | 38.16±3.1 |
| | **9** | 0.60±0.02 | 47.90±0.8 | 47.83±1.9 | 48.27±2.9 | 48.54±3.6 | 47.14±2.3 |
| | **12** | 0.59±0.02 | 54.00±1.1 | 53.31±1.3 | 55.81±3.0 | 54.18±2.7 | 52.59±2.7 |
| | **15** | 0.51±0.02 | 59.14±1.5 | 59.84±1.1 | 60.80±3.6 | 60.34±2.8 | 55.70±1.5 |

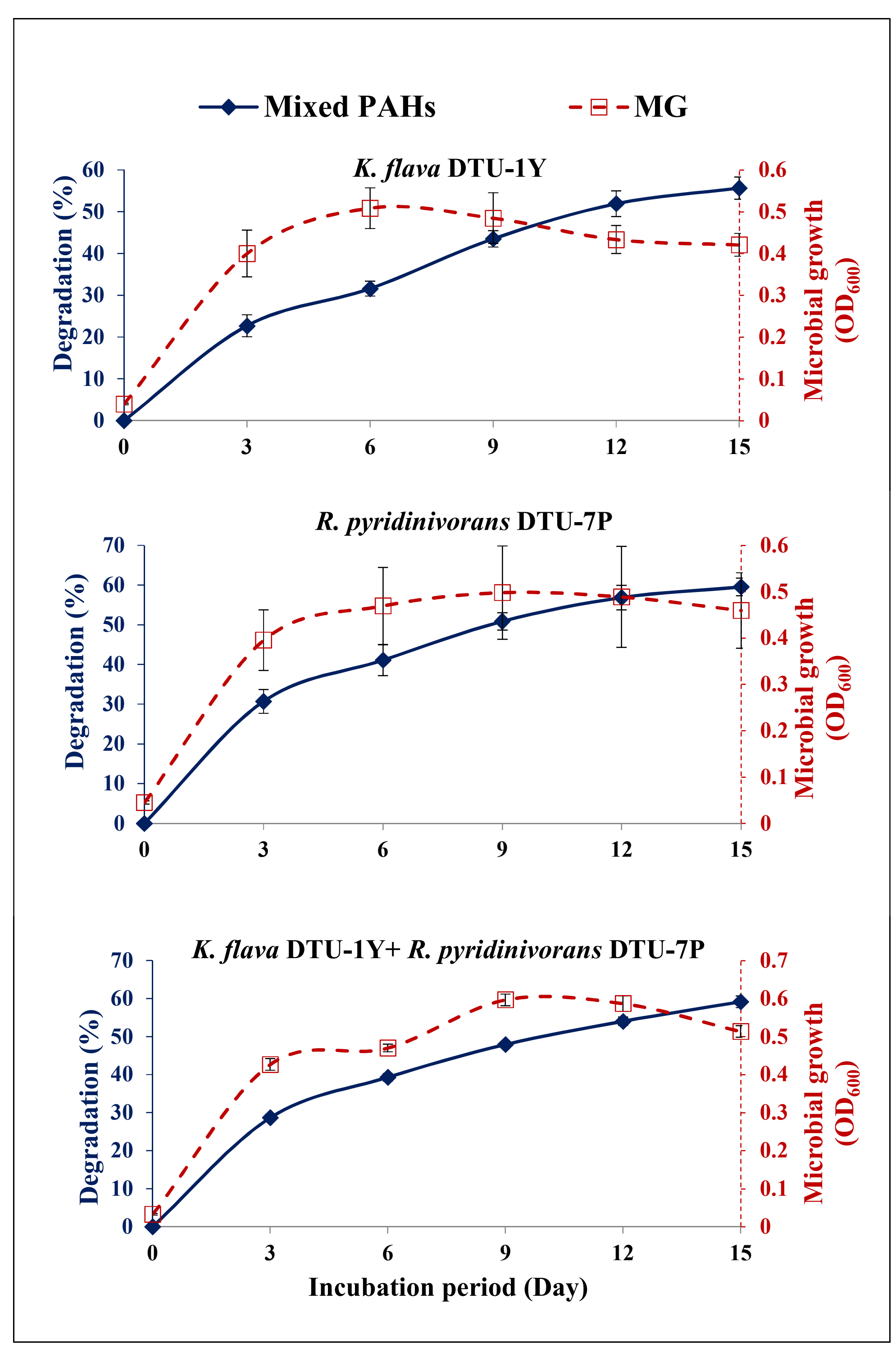



**Fig. 4.8 Dynamics of microbial growth (MG) during biodegradation of mixed PAHs (PHE, ANT, FLU and PYR) by bacterial strain *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P and consortium in 15 days. (Solid symbol represents degradation; hollow symbol is for bacterial growth ($OD_{600nm}$) and error bars represents the standard deviation of replicates)**

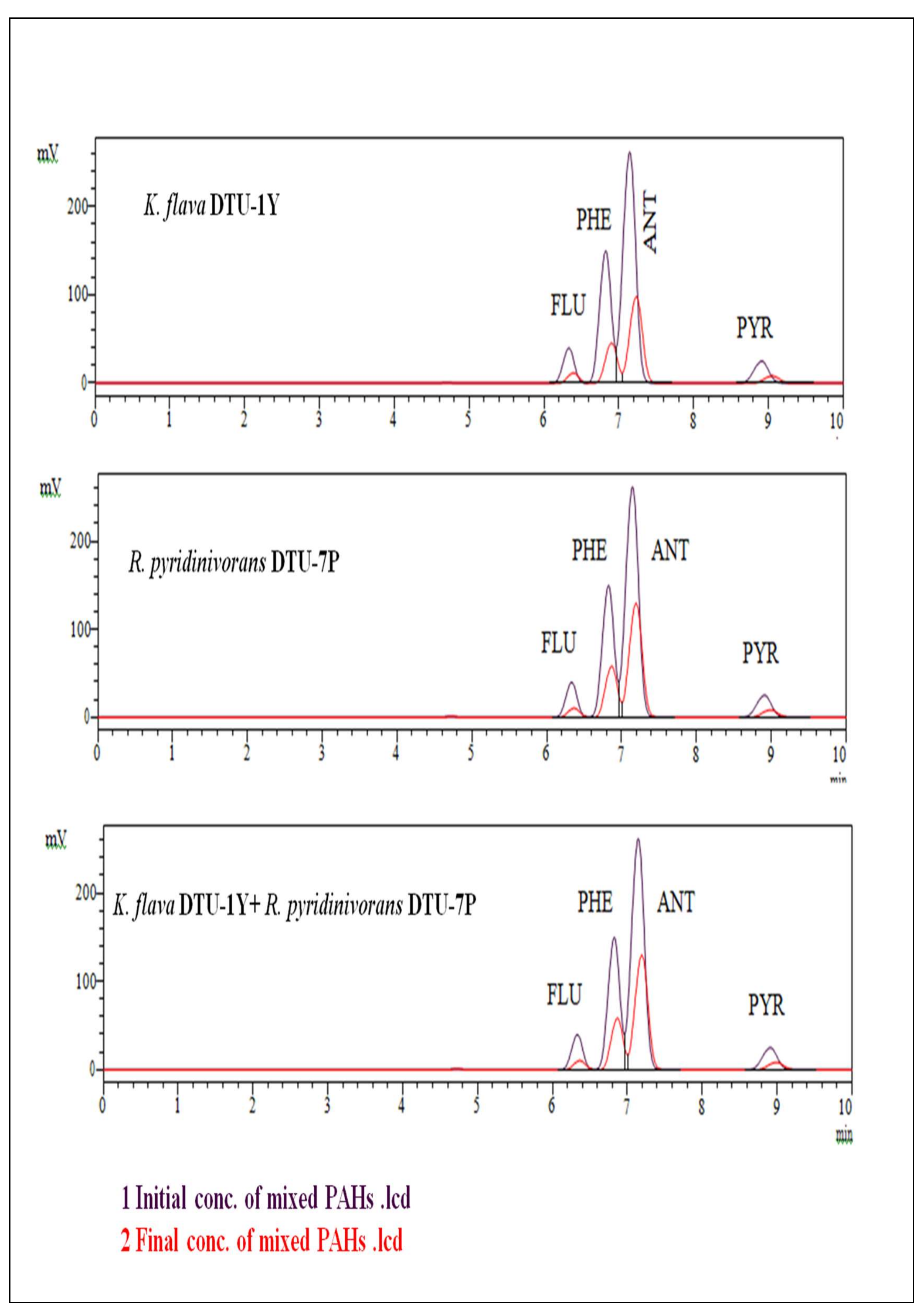



**Fig. 4.9 Initial and final HPLC chromatograms of mixed PAH-biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

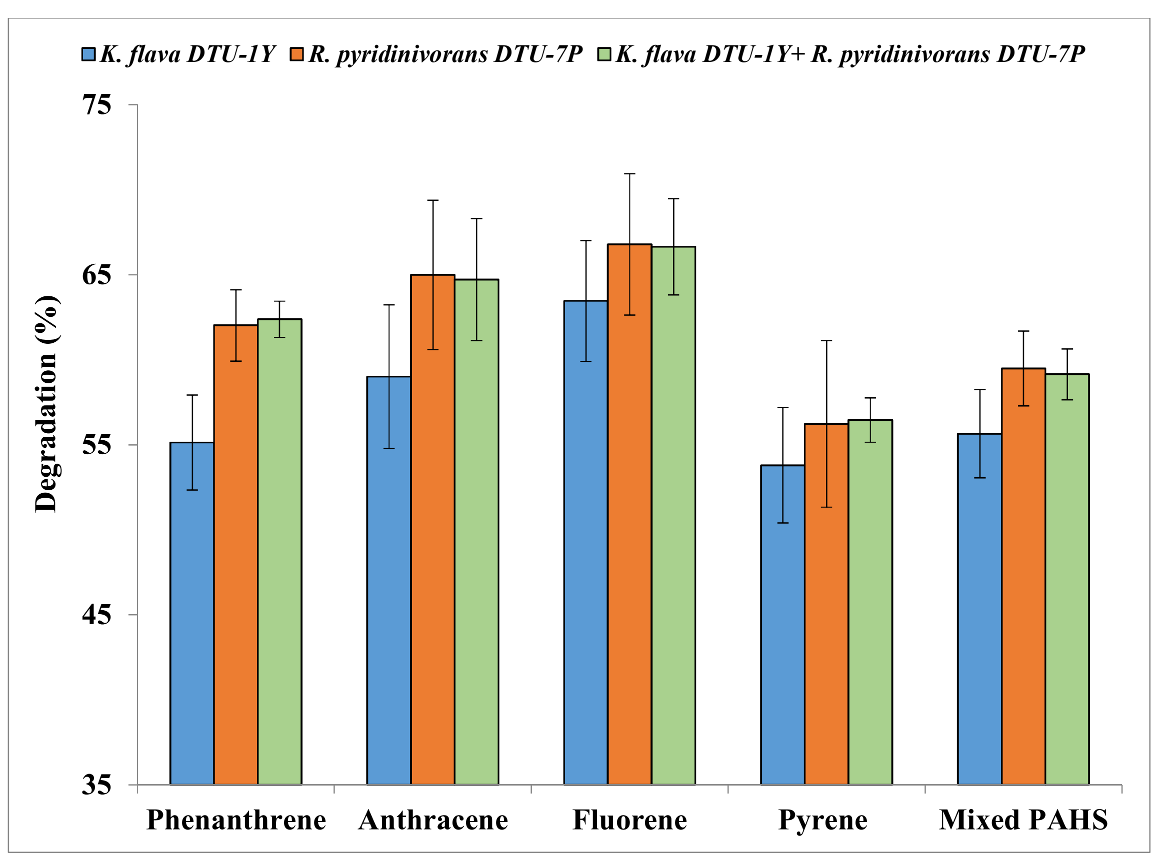



**Fig. 4.10 Comparison of Degradation efficiency of *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P for PHE, ANT, FLU, and PYR as individual PAH compound and as a mixture of PAHs at initial PAH concentration of 10 mg/L**

In case of FLU, degradation by *K. flava* DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium (53.26%, 61.21%, and 60.34%, respectively) was found slightly lower in the mixture of PAHs (PHE, ANT, FLU, and PYR) as compared to degradation of FLU individually, using *K. flava* DTU-1Y, *R. pyridinivorans*-DTU-7P, and consortium (63.46%, 66.79%, and 66.64%, respectively) (Table 4.7 and Fig.4.11). Slightly lower FLU-degradation may be due to competitive inhibition (Herwijnen et al., 2013). This may be due to the preferred utilization of PHE, ANT, and PYR as against FLU as carbon source owing to their structural simplicity. Generally, the innermost 6-C (benzene) ring can easily react with available oxygen in the presence of oxidases resulting in the formation of quinone, while the steric congestion at 5-C ring in FLU results in relatively lesser reactivity with oxygen molecule resulting in slightly lower microbial assimilation of FLU compared to PHE, ANT, and PYR.

The present study confirms the ability of isolated bacterial strains to utilise PAHs simultaneously when present as an individual compound as well as in a mixture of PAHs. In this study, the interaction between two isolated strains may be positive as there is no inhibitory effect observed on degradation efficiency when used in a consortium for degradation of individual PAH as well as mixture of PAH.

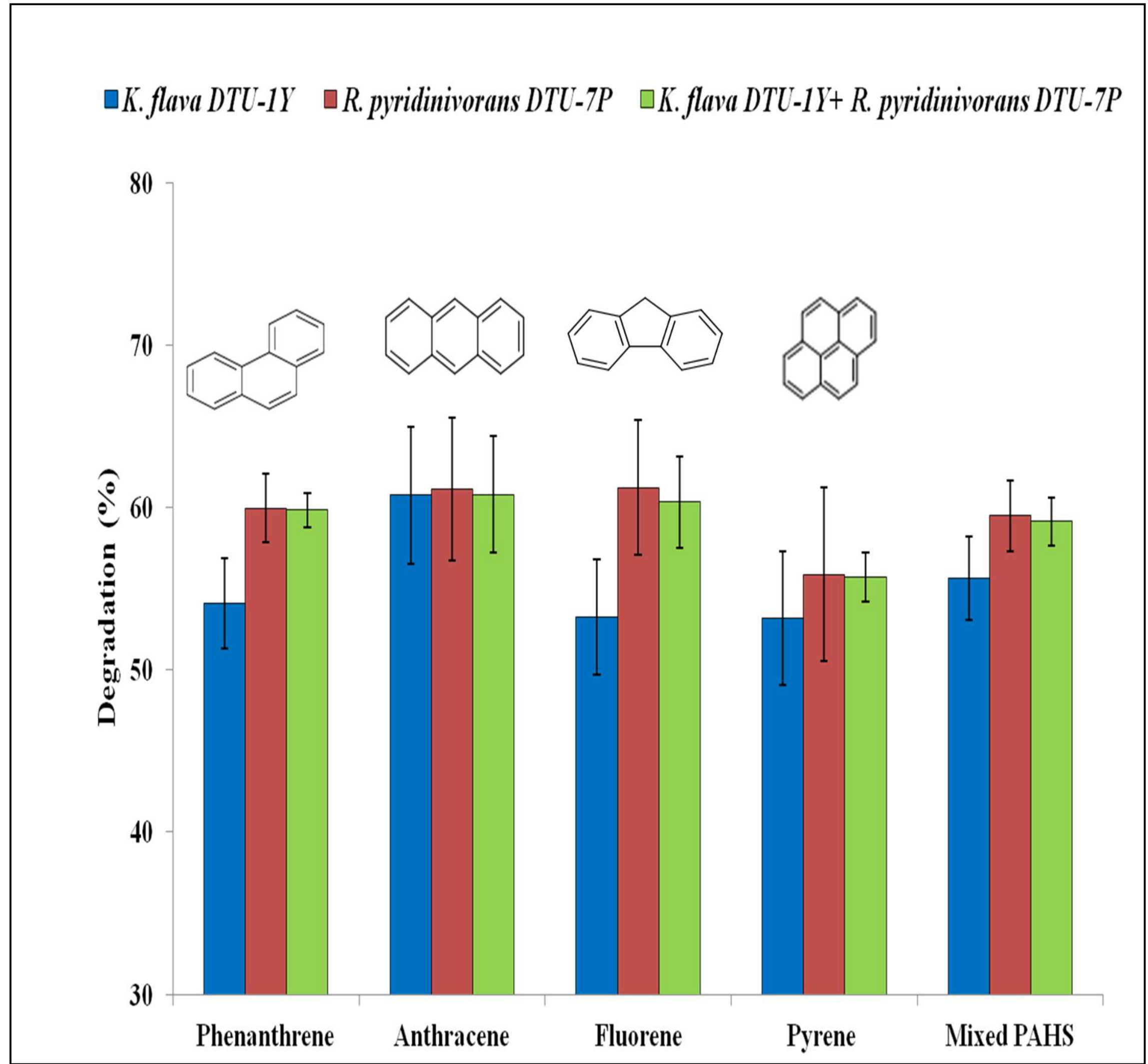



**Fig. 4.11 Degradation of phenanthrene, anthracene, fluorene, and pyrene individually (when present in a mixture of PAHs) and mixed PAHs by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium after 15 days of incubation period at initial concentration of 10 mg/L**

### 4.3 Evolutionary relationship of isolated strains with other bacterial species

The isolated bacterial strains *i.e. Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P were used for similarity search with other PAH-degrading bacterial strains having catabolic genes or catabolic enzymes involved in the PAH-degradation pathway. About 1000 highly similar sequences of different bacteria for each isolated strain were found separately using nucleotide BLAST search against 16S ribosomal RNA sequences database. A closest phylogenetic relative of these 1000 bacterial species similar to isolated strains *i.e. Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P respectively, were searched for their ability for PAH-degradation and presence of PAH-catabolic genes/enzymes from previous studies. About 18 different bacterial species among 1000 species similar to *Kocuria flava* DTU-1Y were found to have the ability for PAH-degradation (Table 4.8). Some of these species were investigated for the presence of catabolic genes/enzymes such as dehydrogenase, monooxygenase, catechol dioxygenase, and aromatic-ring-hydroxylating dioxygenase in different studies (Kallimanis *et al.,* 2009; Sowada *et al.,* 2014; Jin *et al.,* 2017; Salam *et al.,* 2017). Phylogenetic tree for analysis of the evolutionary relationship of *Kocuria flava* DTU-1Y with these identified 18 different bacterial species was constructed (Fig. 4.12). *Kocuria flava* DTU-1Y was found to be closest with *Kocuria rosea* with 0.02591 evolutionary divergence as compared to other species; and distantly related with *Rhodococcus rhodochrous* with 0.09406 evolutionary divergence. For *Rhodococcus pyridinivorans* DTU-7P, 16 different bacterial species were found to have the ability for PAH-degradation (Table 4.9). Some of these species were investigated for the presence of catabolic genes/enzymes such as catechol dioxygense, *nah*, and monooxygenase in different studies (Uz *et al.,* 2000; Kurniati *et al.,* 2016; Subashchandrabose *et al.,* 2019a). Phylogenetic tree for analysis of evolutionary relationship of *Rhodococcus pyridinivorans* DTU-7P with these identified 16 different bacterial species was constructed (Fig. 4.13). *Rhodococcus pyridinivorans* DTU-7P strain was found to be closest with *Rhodococcus rhodochrous* with 0.02333 evolutionary divergence, and distantly related with *Kocuria flava* with 0.11234 evolutionary divergence. The evolutionary distances for estimating divergence during phylogenetic tree construction were computed using the Maximum Composite Likelihood method (Tamura *et al.,* 2004) and are in the units of the number of base substitutions per site. All the 18 and 16 different bacterial strains which are closest phylogenetic relatives of *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P, respectively, belonged to the actinobacteria phylum. The bacterial species belonging to actinobacteria phylum are

widely distributed in aquatic as well as terrestrial ecosystems. Actinobacteria are a group of gram positive bacteria present in a high number in soils metabolising complex organic matter. In environment the actinobacteria can be used for bioremediation of contaminated sites as they have the potential to remove organic and inorganic pollutants (Alvarez *et al.,* 2017). The group of bacteria belonging to actinobacteria is predominant in soils contaminated with PAHs. A high number of catabolic genes are present in this group of bacteria responsible for PAH-degradation resulting in the predominance of actinobacteria in historically PAH-contaminated sites (Kim *et al.,* 2008; Kweon *et al.,* 2010).

This is the first report to study evolutionary relationship of isolated bacterial strains from petroleum contaminated sites, with other known PAH-degrading bacteria having catabolic genes/enzymes capable of metabolising PAHs. The isolated strains were found to have an evolutionary relationship with bacterial species having PAH-catabolic genes/enzymes indicated that these strains may also have the catabolic gene/enzymes and can play an important role in bioremediation of PAH-contaminated sites. Since these strains were isolated from a 20-year-old petroleum-contaminated site which is still in use and is fairly contaminated, the continuous exposure and survival of these strains is a testimony to adaptation and PAH-metabolism under stressed environmental conditions. The natural screening is expected to have isolated the bacterial species which can withstand high PAH exposure and assimilate them simultaneously. Secondly, the isolated strains, when exposed to a very high concentration of mixed PAHs, could survive without a source of carbon (in MSM) under laboratory conditions which highlights their resilience to a PAH-contaminated environment. The phylogenetic analysis, too, identified the strains as having closest proximity to *K. flava* and *R. pyridinivorans* which have been reported as PAH-degrading strains in earlier studies (Lara-Severino et al., 2016**;** Li *et al.,* 2016; Qin *et al.,* 2018). Besides, 34 phylogenetically similar species have been reported for PAH-degradation under different laboratory studies. The 16S rRNA gene sequences might be helpful in identifying the evolutionary similar microbes possessing the property of PAH degradation and the presence of PAH catabolic genes/enzymes in phylogenetically similar species further strengthens the scientific belief that the isolated species may also possess PAH-degrading genes.

**Table. 4.8 Nucleotide BLAST search showing homology of *K. flava* DTU-1Y with other bacterial species having PAH degradation ability**

| S.No | Bacterial Strain | Phylum | Accession Number | Pairwise NCBI seq. similarity (% homology) | Enzymes/Genes | PAH compound (Ci- mg/l) | Degradation efficiency (%; days) | Reference |
|---|---|---|---|---|---|---|---|---|
| 1. | *Kocuria rosea* | Actinobacteria | NR_044871.1 | 96.99 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.,* 2012 |
| | | | | | NM | Anthracene (0.27) | 46–54; 2 | Lara-Severino *et al.,* 2016 |
| | | | | | NM | Naphthalene (1)<br>Phenanthrene (1)<br>Fluoranthene (1)<br>Pyrene (1) | 60;15<br>55;15<br>54;15<br>53;15 | Haritash and Kaushik, 2016 |
| 2. | *Pseudarthrobacter phenanthrenivorans (Arthrobacter phenanthrenivorans)* | Actinobacteria | NR_074770.2 | 94.97 | 1-hydroxy-2-naphthoate dioxygenase; 2-carboxybenzaldehyde dehydrogenase | Phenanthrene (NM) | NM; NM | Kallimanis at al., 2009 |
| 3. | *Micrococcus luteus* | Actinobacteria | NR_075062.2 | 94.06 | Monooxygenase gene | Benzo[a]pyrene (NM) | NM; NM | Sowada at al., 2014 |
| | | | | | NM | Naphthalene (1)<br>Phenanthrene (1)<br>Fluoranthene (1)<br>Pyrene (1) | 69;15<br>63;15<br>61;15<br>61;15 | Haritash and Kaushik, 2016 |
| 4. | *Janibacter anophelis* | Actinobacteria | NR_043218.1 | 92.67 | NM | Phenanthrene (500)<br>Anthracene (500)<br>Pyrene (500) | 99; 5<br>82; 5<br>98; 5 | Zhang at al., 2009 |
| 5. | *Cellulosimicrobium cellulans* | Actinobacteria | NR_119095.1 | 92.17 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.,* 2012 |
| | | | | | NM | Benzo[*a*]pyrene (10) | 79; 13 | Qin *et al.,* 2018 |
| 6. | *Brevibacterium casei* | Actinobacteria | NR_119071.1 | 90.63 | NM | Benzo[b]fluoranthene (NM)<br>Indeno[1,2,3cd]pyrene (NM)<br>Benzo[a]anthracene (NM) | 100; 30<br>100; 30<br>98;60 | Farahat and El-Gendy, 2008 |
| 7. | *Cellulomonas hominis* | Actinobacteria | NR_029288.1 | 92.59 | NM | Naphthalene (NM) | NM; NM | Benedek *et al.,* 2013 |
| | | | | | NM | Phenanthrene (500) | 97; 7 | Hegazi *et al.,* 2007 |

| S.No | Bacterial Strain | Phylum | Accession Number | Pairwise NCBI seq. similarity (% homology) | Enzymes/Genes | PAH compound (Ci- mg/l) | Degradation efficiency (%; days) | Reference |
|---|---|---|---|---|---|---|---|---|
| 8. | *Cellulomonas humilata* | Actinobacteria | NR_026226.1 | 91.63 | NM | Naphthalene (NM) | NM; NM | Benedek *et al.,* 2013 |
| 9. | *Microbacterium arabinogalactanolyticum* | Actinobacteria | NR_044932.1 | 91.68 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.*, 2012 |
| | | | | | NM | Anthracene (10) | 69; 5 | Ntougias *et al.,* 2015 |
| 10. | *Microbacterium testaceum* | Actinobacteria | NR_026163.1 | 91.60 | NM | Anthracene (0.27) | 46-54; 2 | Lara-Severino *et al.*, 2016 |
| 11. | *Microbacterium esteraromaticum* | Actinobacteria | NR_026468.1 | 91.54 | Catechol dioxygenase | Pyrene (50) | 89; 21 | Salam *et al.,* 2017 |
| 12. | *Microbacterium paraoxydans* | Actinobacteria | NR_025548.1 | 91.13 | Aromatic-ring-hydroxylating dioxygenase | Fluoranthene (100) | 92; 25 | Jin *et al.,* 2017 |
| 13. | *Microbacterium oleivorans* | Actinobacteria | NR_042262.1 | 91.33 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.*, 2012 |
| 14. | *Plantibacter flavus* | Actinobacteria | NR_025462.1 | 91.33 | NM | Naphthalene (NM) | 16-39; NM | Lumactud *et al.,* 2016 |
| 15. | *Herbiconiux ginsengi* | Actinobacteria | NR_043879.1 | 91.43 | NM | Benzo[*a*]pyrene (45) | 60-70; 42 | Okai *et al.,* 2016 |
| 16. | *Microbacterium imperiale* | Actinobacteria | NR_026161.1 | 91.12 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.*, 2012 |
| 17. | *Rhodococcus rhodochrous* | Actinobacteria | NR_037023.1 | 91.04 | C23O gene/ catechol2,3-dioxygenase | Naphthalene (5) | 50; 5 | Meyer *et al.,* 1999 |
| 18. | *Microbacterium hydrocarbonoxydans* | Actinobacteria | NR_042263.1 | 91.06 | NM | Phenanthrene (10)<br>Naphthalene (10) | NM; NM<br>NM; NM | Al-Awadhi *et al.*, 2012 |
| | | | | | Catechol 1,2 dioxygenase (C12O) and catechol 2,3dioxygenase (C23O) | Naphthalene (NM) | 87; 13 | Muthukamalam *et al.,* 2017 |

NM: not mentioned; seq.: sequence

**Table 4.9 Nucleotide BLAST search showing homology of *R. pyridinivorans* DTU-7P with other bacterial species having PAH degradation ability**

| S.No | Bacterial Strain | Phylum | Accession Number | Pairwise NCBI seq. similarity (% homology) | Enzymes/Genes | PAH compound (Ci- mg/l) | Degradation efficiency (%; days) | Reference |
|---|---|---|---|---|---|---|---|---|
| 1. | *Rhodococcus rhodochrous* | Actinobacteria | NR_037023.1 | 96.85 | C23O gene/ catechol2,3-dioxygenase | Naphthalene (5) | 50; 5 | Meyer *et al.,* 1999 |
| 2. | *Rhodococcus ruber* | Actinobacteria | NR_026185.1 | 95.32 | Protocatechuate 3,4-dioxygenase | Naphthalene (500) | 100; 6 | Li *et al.,* 2016 |
| 3. | *Rhodococcus aetherivorans* | Actinobacteria | NR_118619.1 | 94.82 | ethB (cytochrome P450) | Naphthalene (8.3) | 6; 56 | Auffret *et al.*, 2009 |
| 4. | *Rhodococcus rhodnii* | Actinobacteria | NR_118610.1 | 94.70 | NM | Phenanthrene (50)<br>Anthracene (50) | NM; NM<br>NM; NM | Leneva *et al.*, 2009 |
| 5. | *Rhodococcus wratislaviensis* | Actinobacteria | NR_118605.1 | 93.70 | NM | Naphthalene (8.3) | 100; 56 | Auffret *et al.*, 2009 |
| | | | | | 2,3-dihydroxybiphenyl 1,2-dioxygenase; 4-nitrophenol 2-monooxygenase; 4-hydroxybenzoate 3-monooxygenase; extradiol dioxygenase; naphthalene dioxygenase | Phenanthrene (49.9)<br>Pyrene (10.11)<br>Benzo[a]pyrene (10) | 100; 7<br>40; 7<br>28; 7 | Subashchandrabose *et al.,* 2019a |
| 6. | *Rhodococcus soli* | Actinobacteria | NR_134799.2 | 93.62 | Aromatic dioxygenase | Phenanthrene (10)<br>Pyrene (10) | 90; 7<br>90;7 | Lee *et al.,* 2018 |
| 7. | *Rhodococcus opacus* | Actinobacteria | NR_026186.1 | 93.37 | Catechol 1,2-dioxygenase | Naphthalene (2000) | NM; NM | Uz *et al.,* 2000 |
| | | | | | NM | Anthracene (50-500) | 68%-81%; 7 | Goswami *et al.*, 2017 |
| 8. | *Nocardia otitidiscaviarum* | Actinobacteria | NR_117344.1 | 92.12 | NM | Pyrene (500)<br>Phenanthrene (500)<br>Anthracene (500) | 40; 4<br>55; 4<br>10; 4 | Zeinali *et al.,* 2007 |
| 9. | *Rhodococcus baikonurensis* | Actinobacteria | NR_024784.1 | 93.20 | NM | Fluoranthene (NM) | NM; NM | Li *et al.,* 2013 |
| 10. | *Gordonia polyisoprenivorans* | Actinobacteria | NR_117829.1 | 92.19 | Catechol 1,2-dioxygenase; catechol 2,3-dioxygenase | Anthracene (250) | NM; NM | Silva *et al.,* 2013 |

| S.No | Bacterial Strain | Phylum | Accession Number | Pairwise NCBI seq. similarity (% homology) | Enzymes/Genes | PAH compound (Ci- mg/l) | Degradation efficiency (%; days) | Reference |
|---|---|---|---|---|---|---|---|---|
| **11.** | *Gordonia paraffinivorans* | Actinobacteria | NR_028832.1 | 91.79 | NM | Naphthalene (500)<br>Phenanthrene (500)<br>Anthracene (500)<br>Pyrene (500) | 90; 7<br>67; 7<br>42; 7<br>35; 7 | Qi *et al.,* 2013 |
| **12.** | *Gordonia cholesterolivorans* | Actinobacteria | NR_044445.1 | 91.47 | *nah*Ac | Pyrene (50) | 96; 7 | Kurniati *et al.*, 2016 |
| **13.** | *Corynebacterium urealyticum* | Actinobacteria | NR_074344.1 | 90.57 | NM | Phenanthrene (350) | 82; 20 | Mohd-Kamil *et al.,* 2013 |
| **14.** | *Corynebacterium variabile* | Actinobacteria | NR_025314.1 | 92.28 | *Nah* | Total aromatic hydrocarbons (NA) | 19; 12 | Gurav *et al.,* 2017 |
| **15.** | *Saccharothrix xinjiangensis* | Actinobacteria | NR_029060.1 | 91.17 | NM | Pyrene (NA) | NM; NM | Hu *et al.,* 2004 |
| **16.** | *Kocuria flava* | Actinobacteria | NR_044308.1 | 89.49 | NM | Naphthalene (500) | 53; 10 | Ahmed *et al.*, 2004 |

NM: Not mentioned; seq.: sequence

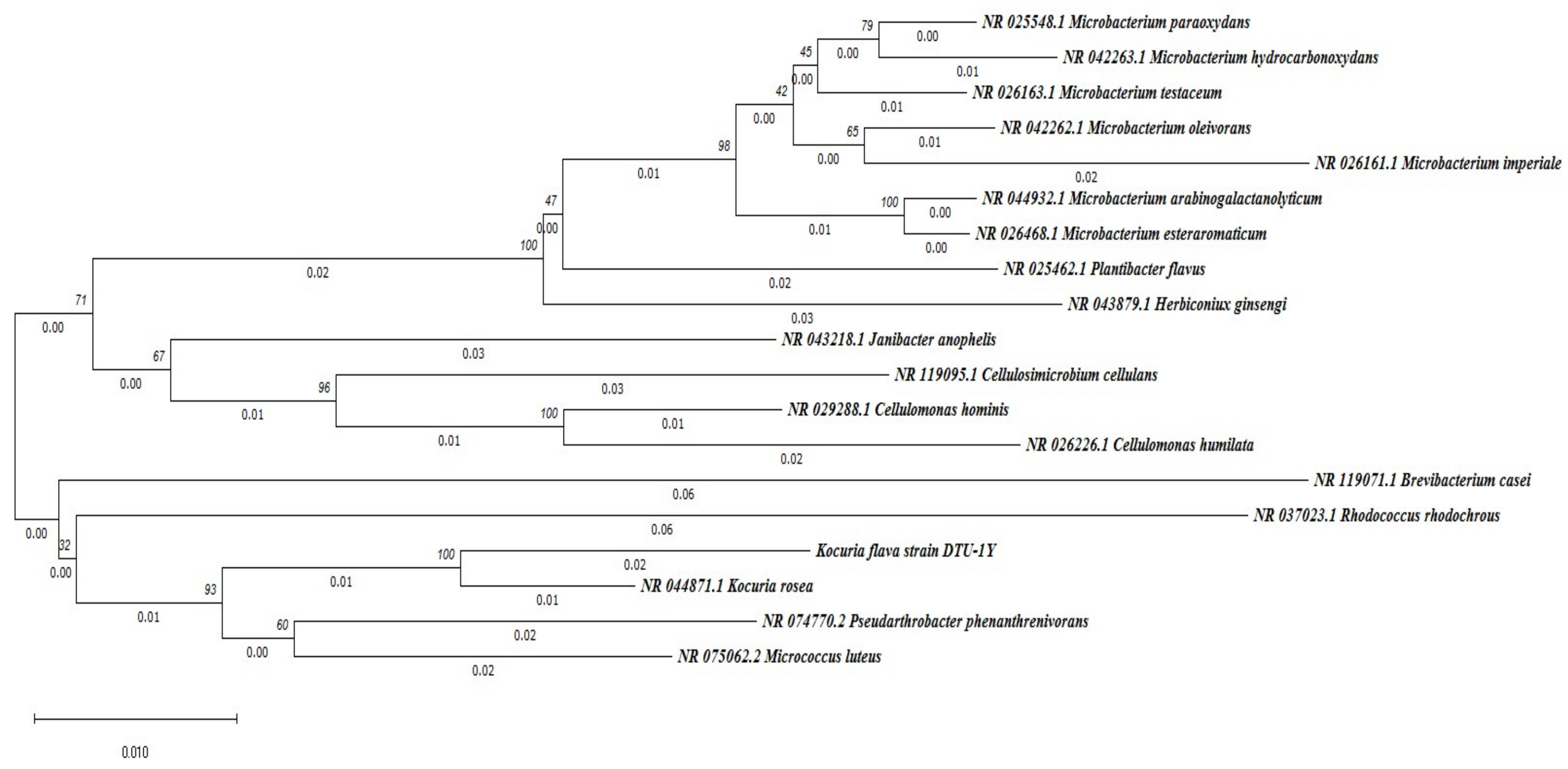



**Fig. 4.12 Phylogenetic tree created by neighbour-joining method based on comparison of 16S rRNA sequences of *Kocuria flava* DTU-1Y and some of its similar bacterial species having ability for PAH-degradation. The tree was generated with 1000 replicates and the number at the nodes represent values for bootstrap probabilities. The scale bar corresponds to approximately 0.010 change per nucleotide position**

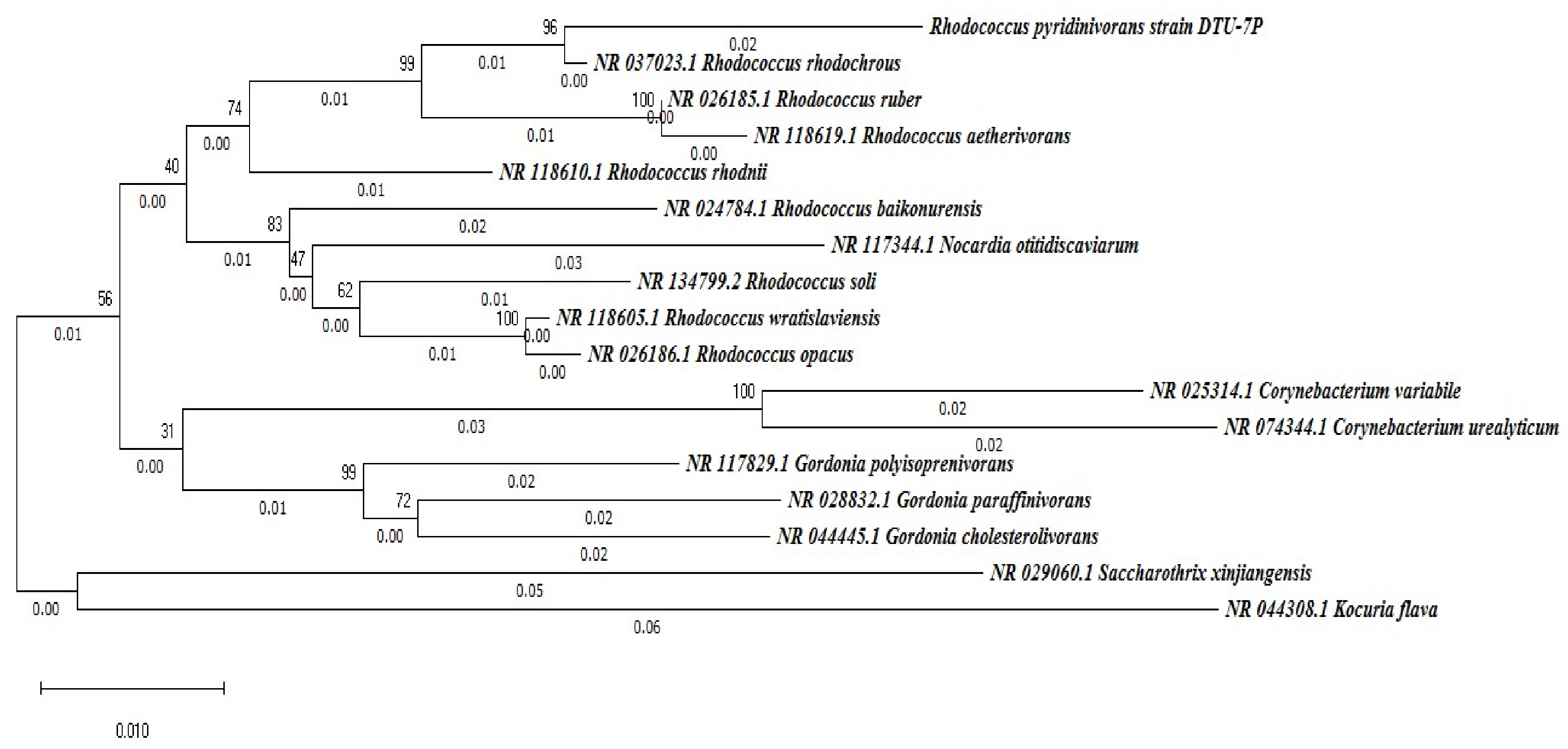



**Fig. 4.13 Phylogenetic tree created by neighbour-joining method based on comparison of 16S rRNA sequences of *Kocuria flava* DTU-1Y and some of its similar bacterial species having ability for PAH-degradation. The tree was generated with 1000 replicates and the number at the nodes represent values for bootstrap probabilities. The scale bar corresponds to approximately 0.010 change per nucleotide position**

### 4.4 Catabolic enzyme activity during PAH-degradation

In the present study, the activities of catabolic enzymes such as C12O, C23O, dehydrogenase, and peroxidase were investigated at 0, 3, 6, 9, 12, and 15 day when samples were analyzed for PAH degradation to examine the role of catabolic enzymes during three-ring and four-ring PAH-degradation. None of the isolates was found to exhibit C12O activity during degradation in the present study. The extent of C23O and peroxidase activity was observed to steadily increase during the initial phase of PAH-degradation. The activity of peroxidase was observed to be higher than the activity of C23O and dehydrogenase enzymes.

The levels of C23O, dehydrogenase, and peroxidase activity during degradation of three-ring PAHs (PHE, ANT, and FLU) were observed to increase during the initial phase of degradation and slightly decrease at the $12^{th}$ and $15^{th}$ day of incubation (Fig. 4.14, 4.15, and 4.16). The C23O activity for *K. flava* was maximum during the $6^{th}$, $9^{th}$, and $12^{th}$ days of culturing for PHE ($5.7\times10^{-4}$ µmoles/ml/min), ANT ($5.7\times10^{-4}$ µmoles/ml/min), and FLU ($2.6\times10^{-4}$ µmoles/ml/min), respectively, whereas for *R. pyridinivorans* C23O showed its highest levels during $6^{th}$-$9^{th}$, $12^{th}$ and $6^{th}$ days of culturing for PHE ($3.8\times10^{-4}$ µmoles/ml/min), ANT ($4.0\times10^{-4}$ µmoles/ml/min) and FLU ($3.4\times10^{-4}$ µmoles/ml/min), respectively. Peroxidase activity for *K. flava* as well as for *R. pyridinivorans* was maximum during the $9^{th}$ day of incubation for all three PAHs. In the case of the consortium the enzyme activity was maximum at $9^{th}$ day for PHE ($5.5\times10^{-4}$ µmoles/ml/min for C23O, $17.4\times10^{-4}$ µmoles/ml/min for peroxidase), $12^{th}$ and $6^{th}$ day for ANT ($2.1\times10^{-4}$ µmoles/ml/min for C23O, $32.89\times10^{-4}$ µmoles/ml/min for peroxidase); as well as for FLU ($2.5\times10^{-4}$ µmoles/ml/min for C23O, $14.0\times10^{-4}$ µmoles/ml/min for peroxidase), respectively. Dehydrogenase activity for both the strains was maximum during the $6^{th}$ and $9^{th}$ day of incubation for all three PAHs and almost similar dehydrogenase activity was observed during the initial phase of degradation for all three PAHs. It was observed that dehydrogenase activity decreased during the $12^{th}$ and $15^{th}$ days of incubation (Table 4.10, 4.11, and 4.12). The C23O and peroxidase activity was observed to steadily increase during the initial phase of PYR-degradation, and it started stabilizing/decreasing by the $12^{th}$ day of incubation (Fig. 4.17). The C23O activity for *K. flava* as well as for *R. pyridinivorans* was maximum during the 12th day (3.41 $\times10^{-4}$ µmoles/ml/min). Peroxidase activity for *K. flava* and *R. pyridinivorans* was maximum during the $9^{th}$ day (9.40 $\times10^{-4}$ µmoles/ml/min and 16.92 $\times10^{-4}$ µmoles/ml/min, respectively). However, the peroxidase activity was maximum on the $9^{th}$ day (19.74 $\times10^{-4}$ µmoles/ml/min) for the consortium. It was observed that during the $12^{th}$ and $15^{th}$ days of incubation dehydrogenase activity decreased (Table 4.13).

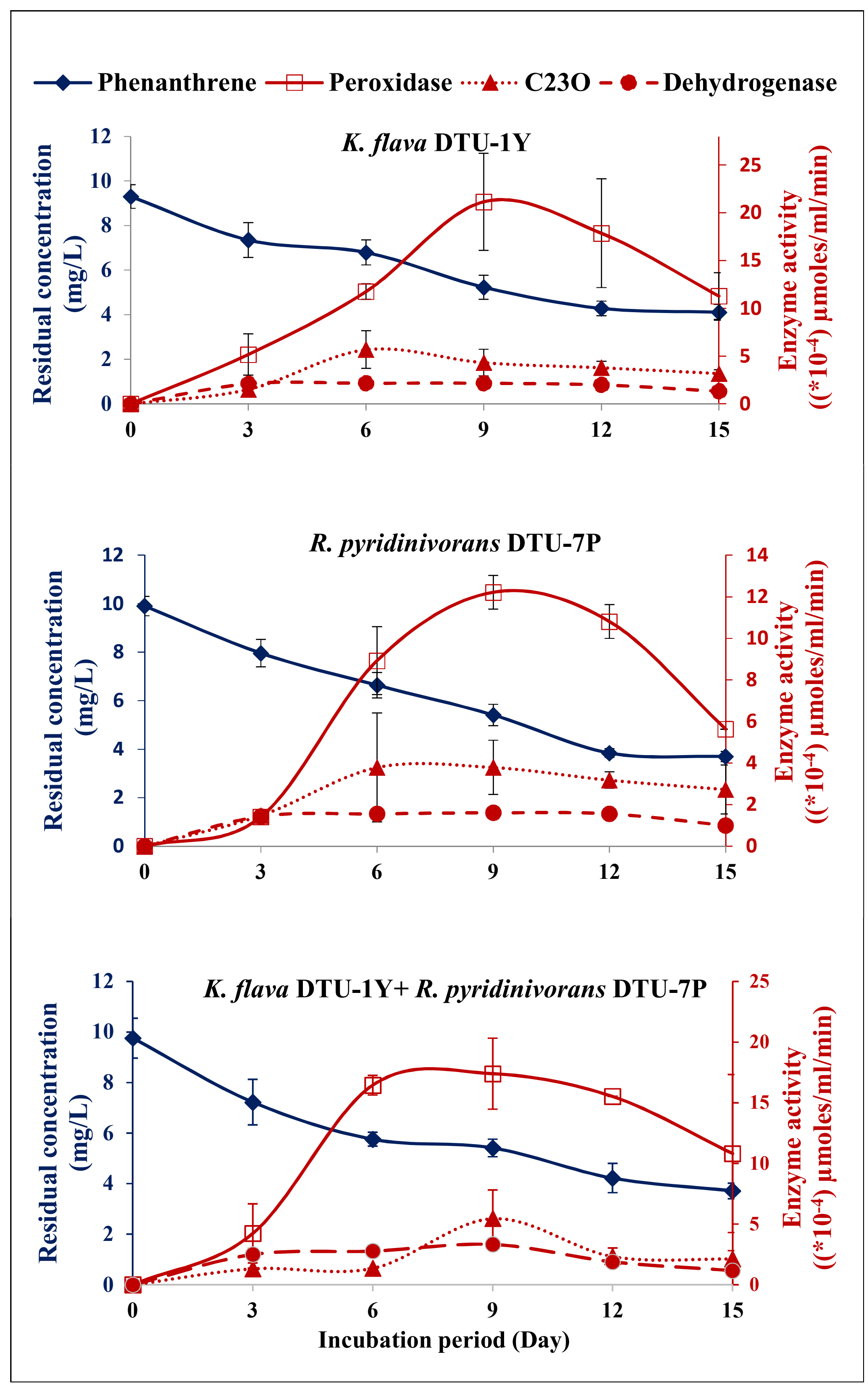



**Fig. 4.14 Catabolic enzyme activities ((*$10^{-4}$) µmoles/ml/min) with average of residual phenanthrene (mg/L) during biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

**Table. 4.10 Catabolic enzyme activity ((*$10^{-4}$) µmoles/ml/min) during biodegradation of phenanthrene (PHE) by isolated bacterial strains**

| Microbial sp. | Time (Days) | Phenanthrene | | | |
|---|---|---|---|---|---|
| | | Average Residual Conc. (mg/L; Replicates=4) | Average Enzyme Activity ((*$10^{-4}$) µmoles/ml/min; Replicates=3) | | |
| | | | C23O | Peroxidase | Dehydrogenase |
| *K. flava* DTU-1Y | 0 | 9.30±0.5 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.35±0.8 | 1.52±0.47 | 5.17±2.15 | 2.11±0.10 |
| | 6 | 6.79±0.6 | 5.68±1.97 | 11.75±0.81 | 2.17±0.17 |
| | 9 | 5.23±0.5 | 4.32±1.38 | 21.15±5.08 | 2.17±0.17 |
| | 12 | 4.28±0.3 | 3.79±0.66 | 17.86±5.70 | 2.00±0.17 |
| | 15 | 4.11±0.4 | 3.18±0.39 | 11.28±2.44 | 1.33±0.17 |
| *R. pyridinivorans* DTU-7P | 0 | 9.90±0.4 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.96±0.6 | 1.48±0.20 | 1.41±0.00 | 1.44±0.10 |
| | 6 | 6.64±0.5 | 3.79±2.62 | 8.93±1.63 | 1.56±0.10 |
| | 9 | 5.41±0.4 | 3.79±1.31 | 12.22±0.81 | 1.61±0.10 |
| | 12 | 3.84±0.2 | 3.18±0.39 | 10.81±0.81 | 1.56±0.10 |
| | 15 | 3.70±0.2 | 2.73±1.18 | 5.64±0.00 | 1.00±0.17 |
| *Consortium (R. pyridinivorans* DTU-7P+ *K. flava*DTU-1Y*)* | 0 | 9.75±0.8 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.22±0.9 | 1.32±0.07 | 4.23±2.44 | 2.50±0.17 |
| | 6 | 5.75±0.3 | 1.36±0.00 | 16.45±0.81 | 2.78±0.10 |
| | 9 | 5.41±0.3 | 5.45±2.36 | 17.39±2.93 | 3.33±0.17 |
| | 12 | 4.22±0.6 | 2.32±0.70 | 15.51±0.00 | 1.89±0.10 |
| | 15 | 3.71±0.3 | 2.12±0.69 | 10.81±6.51 | 1.17±0.17 |

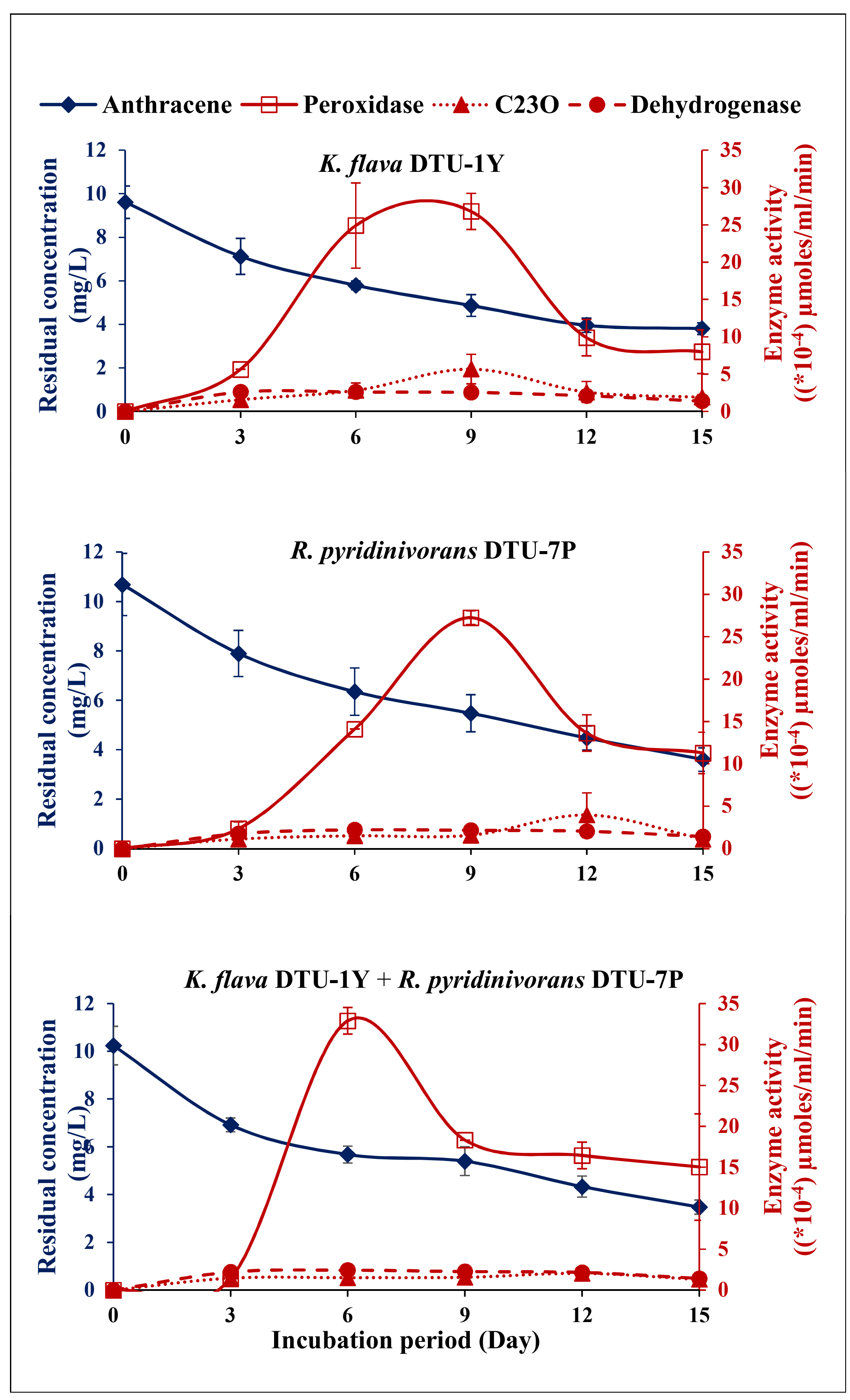



**Fig. 4.15 Catabolic enzyme activities (($*10^{-4}$) µmoles/ml/min) with average of residual anthracene (mg/L) during biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

**Table. 4.11 Catabolic enzyme activity ((*$10^{-4}$) µmoles/ml/min) during biodegradation of anthracene (ANT) by isolated bacterial strains**

| Microbial sp. | Time (Days) | Anthracene | | | |
|---|---|---|---|---|---|
| | | Average Residual Conc. (mg/L; Replicates=4) | Average Enzyme Activity ((*$10^{-4}$) µmoles/ml/min; Replicates=3) | | |
| | | | C23O | Peroxidase | Dehydrogenase |
| *K. flava* DTU-1Y | 0 | 9.61±0.7 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.12±0.8 | 1.59±0.20 | 5.64±0.00 | 2.61±0.25 |
| | 6 | 5.79±0.2 | 2.84±0.98 | 24.91±5.7 | 2.61±0.25 |
| | 9 | 4.86±0.5 | 5.68±1.97 | 26.79±2.44 | 2.56±0.10 |
| | 12 | 3.96±0.3 | 2.60±1.41 | 9.87±2.44 | 2.11±0.10 |
| | 15 | 3.80±0.3 | 1.91±0.38 | 7.99±2.93 | 1.39±0.10 |
| *R. pyridinivorans* DTU-7P | 0 | 10.69±1.3 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.90±0.9 | 1.14±0.39 | 2.35±0.81 | 1.78±0.25 |
| | 6 | 6.35±1.0 | 1.52±0.33 | 14.10±0.00 | 2.22±0.10 |
| | 9 | 5.48±0.7 | 1.59±0.20 | 27.26±0.81 | 2.17±0.17 |
| | 12 | 4.48±0.5 | 3.98±2.60 | 13.63±2.15 | 2.06±0.10 |
| | 15 | 3.61±0.5 | 1.14±0.23 | 11.28±2.44 | 1.44±0.10 |
| *Consortium* (*R. pyridinivorans* DTU-7P+ *K. flava*DTU-1Y) | 0 | 10.24±0.8 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 6.92±0.3 | 1.48±0.20 | 1.41±0.00 | 2.22±0.10 |
| | 6 | 5.68±0.4 | 1.53±0.41 | 32.89±1.63 | 2.44±0.10 |
| | 9 | 5.40±0.6 | 1.58±0.11 | 18.33±0.00 | 2.28±0.10 |
| | 12 | 4.33±0.4 | 2.06±0.19 | 16.45±1.63 | 2.17±0.17 |
| | 15 | 3.48±0.3 | 1.32±0.07 | 15.04±6.51 | 1.44±0.10 |

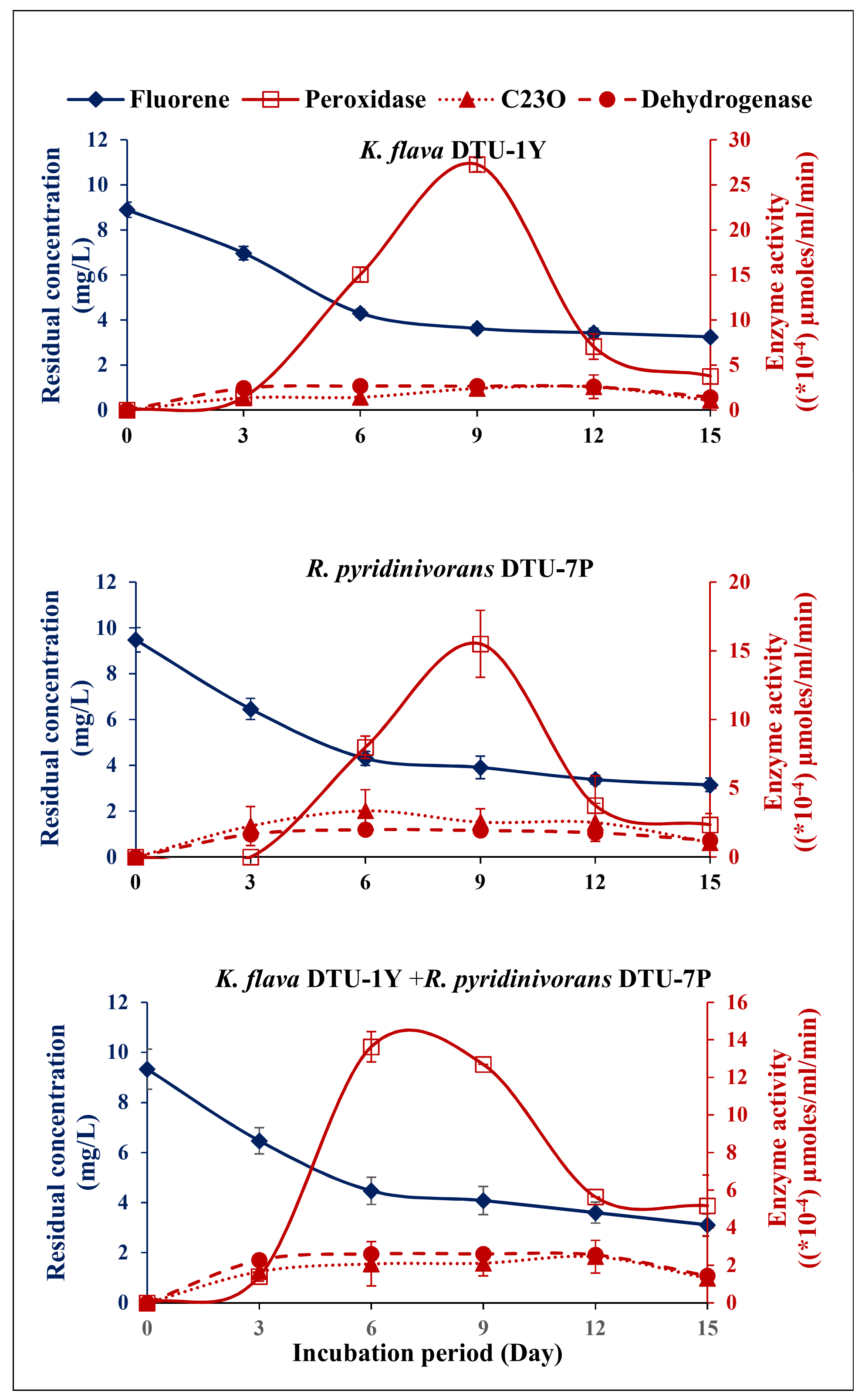



**Fig. 4.16 Catabolic enzyme activities ((*10$^{-4}$) µmoles/ml/min) with average residual fluorene (mg/L) during biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

**Table. 4.12 Catabolic enzyme activity ((*$10^{-4}$) µmoles/ml/min) during biodegradation of fluorene (FLU) by isolated bacterial strains**

| Microbial sp. | Time (Days) | Fluorene | | | |
|---|---|---|---|---|---|
| | | Average Residual Conc. (mg/L; Replicates=4) | Average Enzyme Activity ((*$10^{-4}$) µmoles/ml/min; Replicates=3) | | |
| | | | C23O | Peroxidase | Dehydrogenase |
| *K. flava* DTU-1Y | 0 | 8.89±0.3 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 6.97±0.3 | 1.36±0.59 | 1.41±0.00 | 2.44±0.10 |
| | 6 | 4.31±0.2 | 1.44±0.67 | 15.04±0.81 | 2.67±0.17 |
| | 9 | 3.62±0.2 | 2.42±0.26 | 27.26±0.81 | 2.67±0.17 |
| | 12 | 3.43±0.2 | 2.58±1.31 | 7.05±1.41 | 2.61±0.25 |
| | 15 | 3.25±0.2 | 1.09±0.31 | 3.76±0.81 | 1.44±0.10 |
| *R. pyridinivorans* DTU-7P | 0 | 9.48±0.5 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 6.46±0.5 | 2.27±1.42 | 0.00±0.00 | 1.67±0.17 |
| | 6 | 4.31±0.3 | 3.37±1.53 | 7.99±0.81 | 2.00±0.17 |
| | 9 | 3.91±0.5 | 2.55±0.96 | 15.51±2.44 | 1.94±0.10 |
| | 12 | 3.38±0.2 | 2.52±1.38 | 3.76±2.15 | 1.78±0.10 |
| | 15 | 3.15±0.3 | 1.06±0.30 | 2.35±0.81 | 1.22±0.10 |
| *Consortium* (*R. pyridinivorans* DTU-7P+ *K. flava*DTU-1Y) | 0 | 9.33±0.8 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 6.47±0.5 | 1.67±0.52 | 1.41±0.00 | 2.28±0.10 |
| | 6 | 4.47±0.5 | 2.08±1.18 | 13.63±0.81 | 2.61±0.10 |
| | 9 | 4.08±0.6 | 2.12±0.69 | 12.69±0.00 | 2.61±0.10 |
| | 12 | 3.60±0.4 | 2.46±0.87 | 5.64±0.00 | 2.56±0.10 |
| | 15 | 3.11±0.4 | 1.31±0.24 | 5.17±1.63 | 1.44±0.10 |

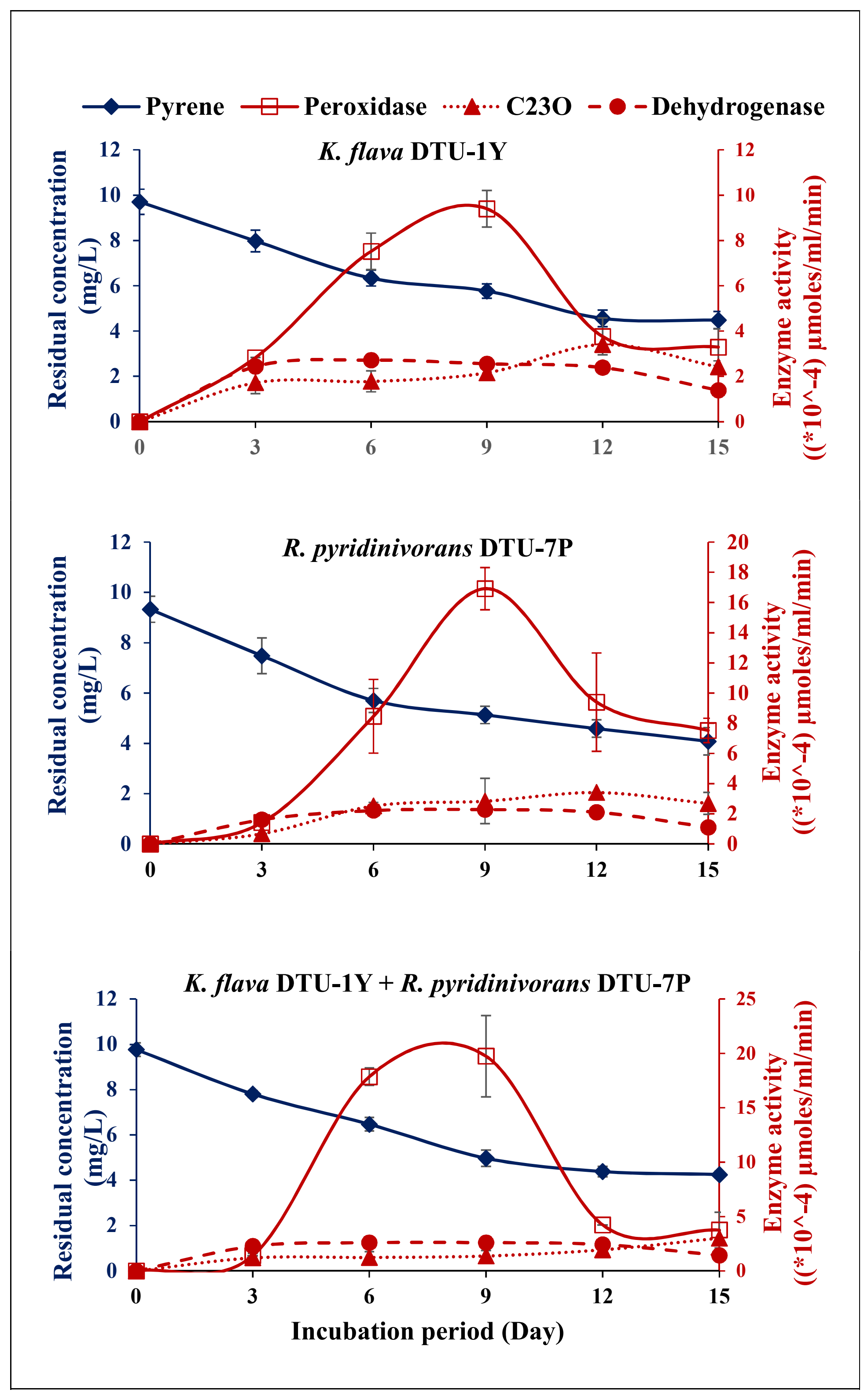



**Fig. 4.17 Catabolic enzyme activities ((*$10^{-4}$) µmoles/ml/min) with average concentration residual pyrene (mg/L) during biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

**Table. 4.13 Catabolic enzyme activity ((*$10^{-4}$) µmoles/ml/min) during biodegradation of pyrene (PYR) by isolated bacterial strains**

| Microbial sp. | Time (Days) | Pyrene | | | |
|---|---|---|---|---|---|
| | | Average Residual Conc. (mg/L; Replicates=4) | Average Enzyme Activity ((*$10^{-4}$) µmoles/ml/min; Replicates=3) | | |
| | | | C23O | Peroxidase | Dehydrogenase |
| *K. flava* DTU-1Y | 0 | 9.71±0.6 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.98±0.5 | 1.72±0.49 | 2.82±0.00 | 2.44±0.19 |
| | 6 | 6.34±0.4 | 1.78±0.46 | 7.52±0.81 | 2.72±0.10 |
| | 9 | 5.77±0.3 | 2.16±0.19 | 9.40±0.81 | 2.56±0.10 |
| | 12 | 4.56±0.4 | 3.41±0.00 | 3.76±0.81 | 2.39±0.10 |
| | 15 | 4.49±0.4 | 2.42±0.26 | 3.29±0.81 | 1.39±0.10 |
| *R. pyridinivorans* DTU-7P | 0 | 9.33±0.5 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.48±0.7 | 0.68±0.00 | 1.41±0.00 | 1.61±0.10 |
| | 6 | 5.71±0.5 | 2.52±0.23 | 8.46±2.44 | 2.22±0.10 |
| | 9 | 5.13±0.3 | 2.84±1.50 | 16.92±1.41 | 2.28±0.10 |
| | 12 | 4.59±0.3 | 3.41±0.00 | 9.40±3.26 | 2.11±0.10 |
| | 15 | 4.08±0.5 | 2.69±0.73 | 7.52±0.81 | 1.11±0.10 |
| *Consortium (R. pyridinivorans* DTU-7P+ *K. flava*DTU-1Y*)* | 0 | 9.77±0.3 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | 3 | 7.81±0.1 | 1.21±0.40 | 1.41±0.00 | 2.28±0.10 |
| | 6 | 6.47±0.3 | 1.25±0.52 | 17.86±0.81 | 2.61±0.19 |
| | 9 | 4.97±0.4 | 1.38±0.50 | 19.74±3.73 | 2.61±0.19 |
| | 12 | 4.39±0.2 | 1.95±0.00 | 4.23±0.00 | 2.44±0.10 |
| | 15 | 4.25±0.2 | 3.03±0.66 | 3.76±1.63 | 1.44±0.10 |

The range of C23O, dehydrogenase, and peroxidase activity during degradation of mixed PAH was noted to progressively increase during the initial phase of 0-9 days, and decrease to some extent at the $12^{th}$ and $15^{th}$ day of incubation (Fig. 4.18). Maximum activity for C23O, dehydrogenase, and peroxidase was observed during the $9^{th}$ day of incubation for *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P. For *K. flava* DTU-1Y maximum catabolic enzyme activity of C23O, dehydrogenase, and peroxidase was $10.8\times10^{-4}$ µmoles/ml/min, $8.93\times10^{-4}$ µmoles/ml/min, and $2.22\times10^{-4}$ µmoles/ml/min respectively, for *R. pyridinivorans* DTU-7P maximum C23O, dehydrogenase, and peroxidase activity was $11.93\times10^{-4}$ µmoles/ml/min, $12.69\times10^{-4}$ µmoles/ml/min, and $1.94\times10^{-4}$ µmoles/ml/min, respectively. While for consortium, C23O, dehydrogenase, and peroxidase activity were maximum on the $9^{th}$ day ($10.8\times10^{-4}$ µmoles/ml/min, $10.34\times10^{-4}$ µmoles/ml/min, and $1.94\times10^{-4}$ µmoles/ml/min, respectively). The activity of peroxidase was observed to be higher than the activity of C23O and dehydrogenase enzymes for both bacterial strains (Table 4.14).

Catabolic enzymes such as dioxygenase and peroxidase have been found to have an important role in the oxidation of PAHs (Mester and Tien, 2000; Pinyakong *et al.,* 2000). Dehydrogenases are known to be associated with the transformation of PAHs to dihydrodiol intermediates (van der Waarde *et al.,* 1995; Margesin *et al.* 2000; Sakshi and Haritash, 2020) which have relatively less chemical stability and are prone to further oxidation. Based on the activity of enzymes, C23O may be responsible for the initiation of hydrolysis through meta-cleavage of benzene ring; whereas dehydrogenases and peroxidases might catalyze further oxidation of PAHs into simpler non-toxic forms. The dehydrogenase enzyme has been reported for its role in the transformation of PAHs to dihydrodiols which are relatively less stable than PAHs (van der Waarde *et al.,* 1995). The chemically reactive PAH-dihydrodiols are easily oxidised by peroxidases into secondary metabolites broken down in Kreb's cycle (Cerniglia, 1993). The observations of this study show that C23O and peroxidase enzymes in both isolates have the opportunity to use these isolates in biodegradation. The notable activity of dehydrogenases in the present study is an indicator of the possible formation of diols making the ring susceptible to opening by the subsequent activity of dioxygenases and peroxidases. There is a significant role of ring-cleaving dioxygenase in the biodegradation of aromatic compounds by aromatic ring oxidation *via* $O_2$ incorporation into the aromatic nucleus (Goyal and Zylstra, 1996). In this study, C23O enzyme activity was observed indicating meta-cleavage by C23O enzyme during PAH-degradation by both isolates (Meyer *et al.,* 1999). There is much information available on C23O enzyme activities during utilization of naphthalene (Shamsuzzaman, 1974a,b; Kiyohara and Nagao, 1978), and for

peroxidase activity during PAH degradation mainly fungal species have been studied (Collins and Dobson, 1996; Pozdnyakova, 2012; Kadri *et al.,* 2017), while little information is available for C23O and peroxidase activities during three-ring PAH degradation. C23O and peroxidase, both, are known to be involved in PAH-oxidation. Peroxidase activity has not been studied for bacterial species during PAH-degradation. To our knowledge, this is the first report to determine the dynamic patterns of the catabolic enzymes (C23O, dehydrogenase, and peroxidase), simultaneously, from two different bacterial strains during PAH-degradation.

Previously, Meyer *et al.*, (1999) investigated bacterial strains *Pseudomonas* and *Sphingomonas* for the presence of C23O, which catalyze key steps of phenanthrene degradation *via* meta-cleavage. In this study also, C23O activity was found during the degradation of each PAH which points towards the involvement of C23O in the initial degradation process. In contrast, PAH oxidation by peroxidase enzyme has been examined widely for various fungal species, which were found to be involved in the initial steps of degradation. Studies by Pozdnyakova, (2012) and Kadri *et al.,* (2017) have extensively reviewed fungal enzyme (peroxidase) involved during PAH degradation. Collins and Dobson, (1996) have also studied the oxidation of fluorene and phenanthrene by Manganese peroxidase activity in *Trametes (coriolus) versicolor* cultures. Similarly, Acevedo *et al.,* (2010) has investigated peroxidase activity for degradation of PHE, ANT, and FLU. Different bacterial strains were evaluated for degradation of pyrene mediated by the C23O enzyme in earlier literature. Bacterial species of *Pseudomonas* YL8*, Ochrobactrum intermedium*, and *Rhodococcus pyridinvorans* (Singh *et al.,* 2013); *Achromobacter denitrificans* (Mawad *et al.,* 2016)*, Pseudomonas aeruginosa* N6P6, and *Pseudomonas pseudoalcaligenes* NP103 (Mangwani *et al.,* 2017) have been evaluated for the presence of C23O, which catalyzes PYR-degradation *via* meta-cleavage. In this study also, C23O activity was observed during PYR-degradation which may indicate C23O involvement in the initial ring cleavage of PYR (Fig. 4.19). The findings of this study signify that *K. flava* and *R. pyridinivorans* have the ability to degrade PYR, and have a possibility for use in degradation of HWM-PAH. On the basis of results obtained, it is proposed that the PYR-degradation mechanism in the isolated strains could be mediated by C23O (dioxygenase enzyme), which may probably involve in the initial degradation of HWM-PAHs, and subsequently, dehydrogenase and peroxidase enzyme may play a role to continue the degradation of intermediates of HWM-PAHs.

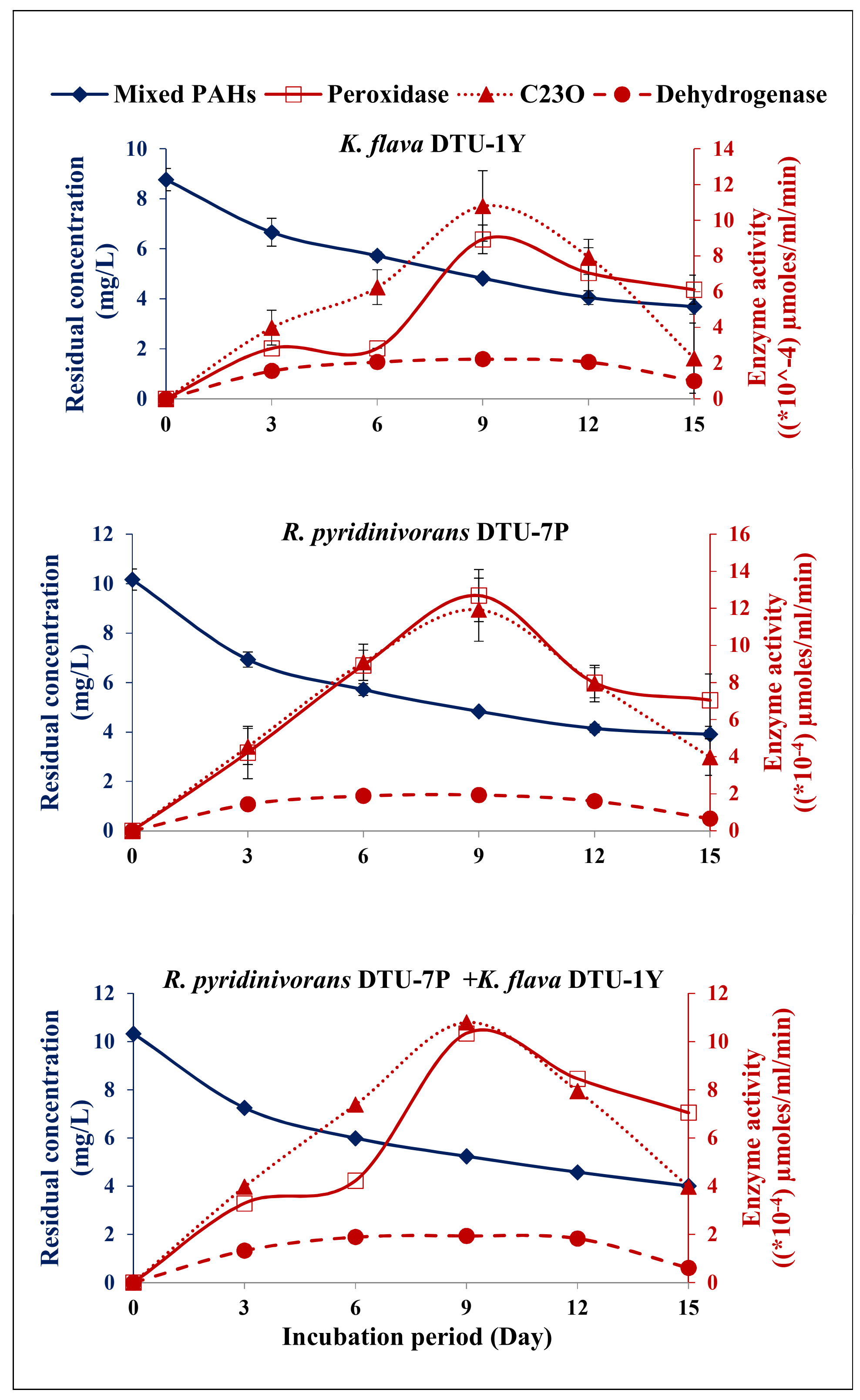



**Fig. 4.18 Catabolic enzyme activities ((*$10^{-4}$) µmoles/ml/min) with average residual mixed PAHs (phenanthrene, anthracene, fluorene, and pyrene) during biodegradation by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium**

**Table. 4.14 Catabolic enzyme activity ((*$10^{-4}$) µmoles/ml/min) during biodegradation of mixed PAHs by isolated bacterial strains**

| Microbial sp. | Time (Days) | Mixed PAHs (phenanthrene, anthracene, fluorene and pyrene) | | | |
|---|---|---|---|---|---|
| | | Average Residual Conc. (mg/L; Replicates=4) | Average Enzyme Activity ((*$10^{-4}$) µmoles/ml/min; replicates=3) | | |
| | | | C23O | Peroxidase | Dehydrogenase |
| ***K. flava*** **DTU-1Y** | **0** | 8.77±0.4 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | **3** | 6.66±0.6 | 3.98±0.98 | 2.82±0.00 | 1.56±0.10 |
| | **6** | 5.73±0.1 | 6.25±0.98 | 2.82±0.00 | 2.06±0.25 |
| | **9** | 4.81±0.1 | 10.80±1.97 | 8.93±0.81 | 2.22±0.10 |
| | **12** | 4.05±0.3 | 7.95±0.98 | 7.05±1.41 | 2.06±0.10 |
| | **15** | 3.68±0.3 | 2.27±1.97 | 6.11±0.81 | 1.00±0.17 |
| ***R. pyridinivorans*** **DTU-7P** | **0** | 10.17±0.4 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | **3** | 6.93±0.3 | 4.55±0.98 | 4.23±1.41 | 1.44±0.10 |
| | **6** | 5.72±0.2 | 9.09±0.98 | 8.93±0.81 | 1.89±0.10 |
| | **9** | 4.83±0.1 | 11.93±1.70 | 12.69±1.41 | 1.94±0.10 |
| | **12** | 4.15±0.2 | 7.95±0.98 | 7.99±0.81 | 1.61±0.10 |
| | **15** | 3.91±0.2 | 3.98±0.98 | 7.05±1.41 | 0.67±0.17 |
| ***Consortium*** ***(R. pyridinivorans*** **DTU-7P+** ***K. flava*****DTU-1Y*****)*** | **0** | 10.33±0.3 | 0.00±0.00 | 0.00±0.00 | 0.00±0.00 |
| | **3** | 7.25±0.1 | 3.98±0.98 | 3.29±0.81 | 1.33±0.17 |
| | **6** | 5.99±0.2 | 7.39±0.98 | 4.23±2.44 | 1.89±0.19 |
| | **9** | 5.24±0.1 | 10.80±1.97 | 10.34±2.15 | 1.94±0.19 |
| | **12** | 4.58±0.2 | 7.95±0.98 | 8.46±1.41 | 1.83±0.44 |
| | **15** | 4.01±0.1 | 3.98±0.98 | 7.05±1.41 | 0.61±0.25 |

Fundamentally, the biodegradation of PAHs depends on the catabolic action of microbial enzymes. Various catabolic enzymes *viz.* mono-oxygenases, dioxygenases, dehydrogenase, peroxidases, and lipases have been reported responsible for PAH-degradation (Cerniglia 1992; Lyu *et al.,* 2014; Pandey *et al.,* 2012). In this study, the ability of two isolated bacterial strains to degrade LMW-PAHs as well as HMW-PAHs was confirmed by the presence of catabolic enzyme activity during the PAH-degradation study at regular intervals of 3 days within 15 days of incubation period. Both the isolated strains having the ability to degrade three-ring and four-ring PAHs exhibited C23O, peroxidase, and dehydrogenase activity during PAH-degradation when present as a single PAH compound or a mixture of PAHs. The two strains *K. flava*-DTU-1Y and *R. pyridinivorans*-DTU-7P attributed to produce and express catabolic enzymes for PAH-degradation and there is no inhibitive effects of enzymes produced by the two strains when used in a consortium. The results of this study demonstrated that using the isolated strains *K. flava*-DTU-1Y and *R. pyridinivorans*-DTU-7P individually as well as a consortium of the strains, is a potential approach for efficient degradation of LMW-PAHs as well as HMW-PAHs when present alone or as a mixture at contaminated sites.

**Fig. 4.19 Probable pathway for degradation of pyrene by *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P**

### 4.5 Kinetics of microbial degradation of PAHs

The kinetic study is a significant factor playing a part to understand the biological degradation process for the development of proficient remediation methods for PAH-polluted sites. Information on PAH-bioremediation kinetics is very imperative, as it finds out the remaining concentration of PAH at any given time and estimates the expected time required for complete remediation. During the biodegradation kinetics study, the plots of the natural logarithm of PAH concentration versus incubation period were linear. The plots between *ln* [PAHs] versus incubation period were found to fit the Monod first-order kinetic equation *i.e.* $ln\ C = -kt + A$, where C is the PAH concentration, 't' represents incubation time, 'k' is the first-order rate constant and 'A' is a constant. In this study, the plots of the natural logarithm of PAH concentration against incubation period for degradation in liquid MSM with *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium represented first-order kinetics.

The plots of the natural logarithm of PHE concentration against incubation period (Fig. 4.20) were linear with $R^2$ values of 0.97, 0.98, and 0.97, respectively, for *K. flava* DTU-1Y*, R. pyridinivorans* DTU-7P, and consortium The rate constant k calculated during the PHE-degradation study was 0.0568 $day^{-1}$, 0.0697 $day^{-1}$, and 0.062 $day^{-1}$ respectively, and half-life was 12.2, 9.9, and 10.9 days respectively (Table 4.15). The plots of the natural logarithm of ANT concentration versus incubation period (Fig. 4.21) were linear with $R^2$ values of 0.96, 0.99, and 0.96, respectively, for *K. flava* DTU-1Y*, R. pyridinivorans* DTU-7P, and consortium The rate constant k calculated during the ANT-degradation study was 0.0626 $day^{-1}$, 0.0694 $day^{-1}$, and 0.0653 $day^{-1}$ respectively, and half-life was 11.1, 9.9, and 10.6 days respectively. Similarly, the plots of the natural logarithm of FLU concentration against incubation period (Fig. 4.22) were linear with $R^2$ values of 0.88, 0.90, and 0.92, respectively, for *K. flava* DTU-1Y*, R. pyridinivorans* DTU-7P, and consortium The rate constant k calculated during the FLU-degradation study was 0.0698 $day^{-1}$, 0.0719 $day^{-1}$, and 0.0699 $day^{-1}$ respectively, and half-life was 9.9, 9.6, and 9.9 days respectively. The plots of the natural logarithm of PYR concentration against incubation period (Fig. 4.23) were linear with $R^2$ values of 0.96 for *K. flava* DTU-1Y*, R. pyridinivorans* DTU-7P, and consortium The rate constant k calculated during the PYR-degradation study was 0.0537 $day^{-1}$, 0.0543 $day^{-1}$, and 0.0586 $day^{-1}$ respectively, and half-life was 12.9, 12.8, and 11.8 days respectively.

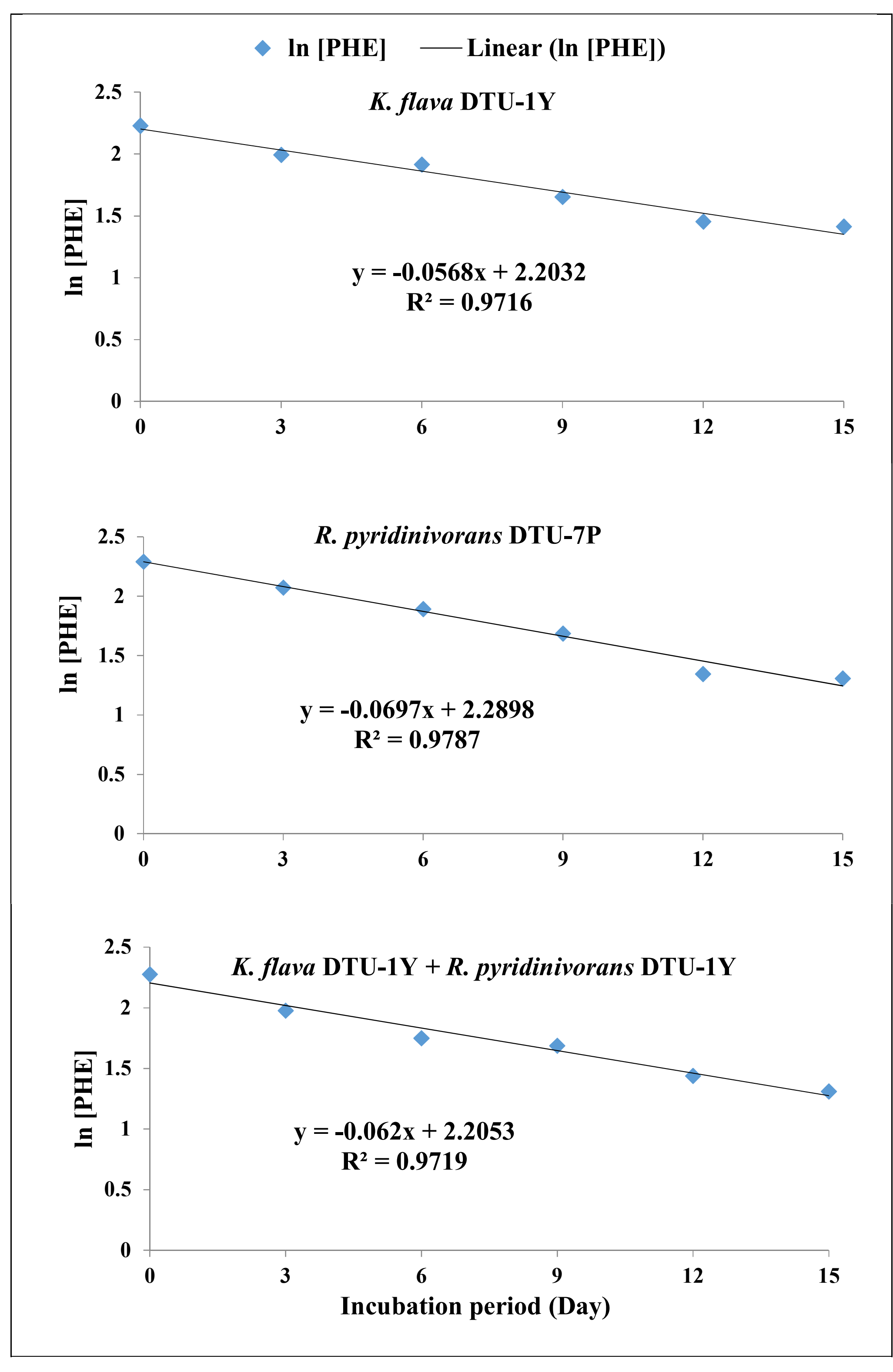



**Fig. 4.20 Plot of the natural logarithm of phenanthrene concentration versus incubation period for degradation of anthracene in liquid MSM with *K. flava*. *R. pyridinivorans*, and consortium in 15 days**

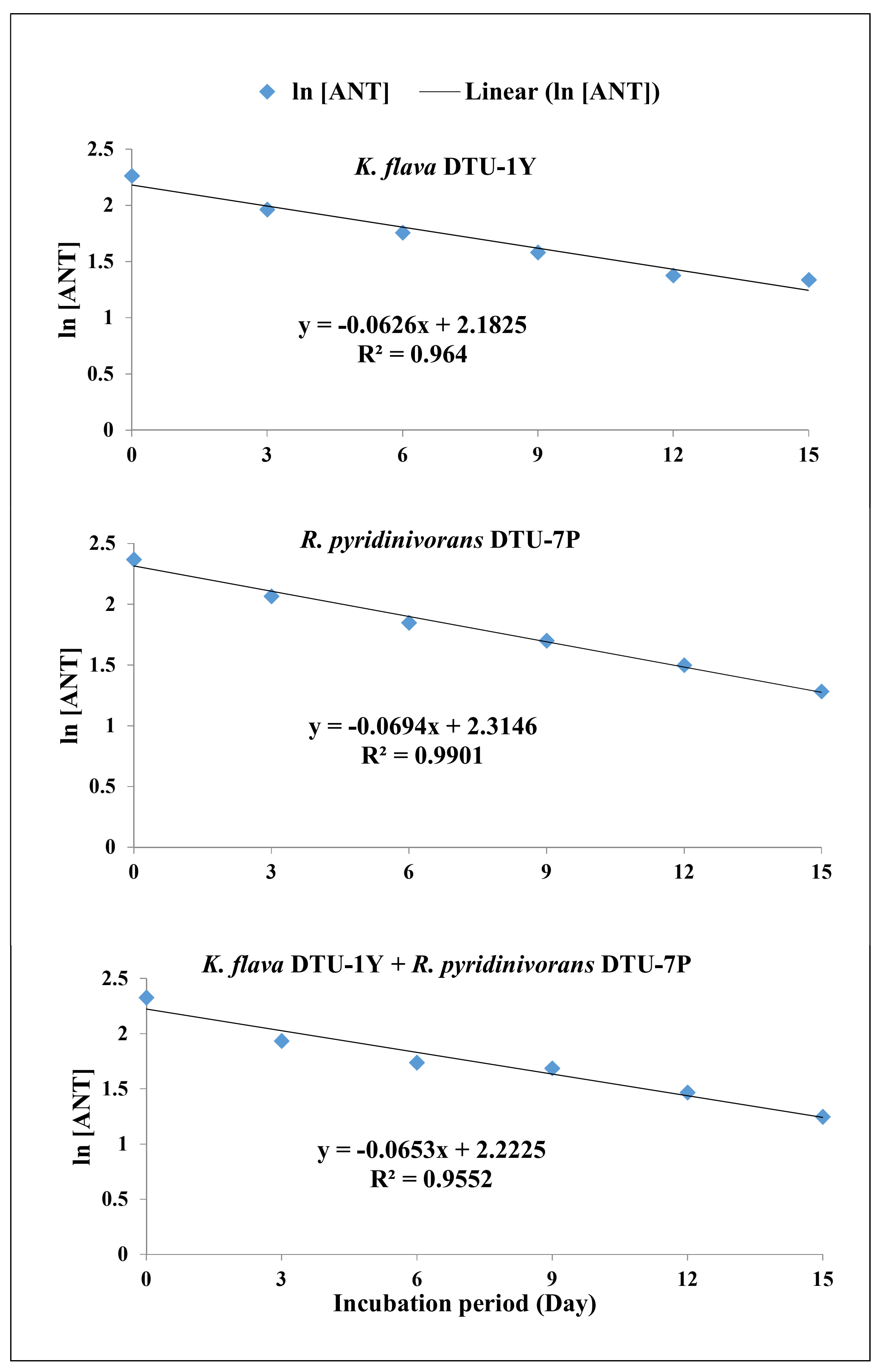



**Fig. 4.21 Plot of the natural logarithm of anthracene concentration versus incubation period for degradation of anthracene in liquid MSM with *K. flava*. *R. pyridinivorans*, and consortium in 15 days**

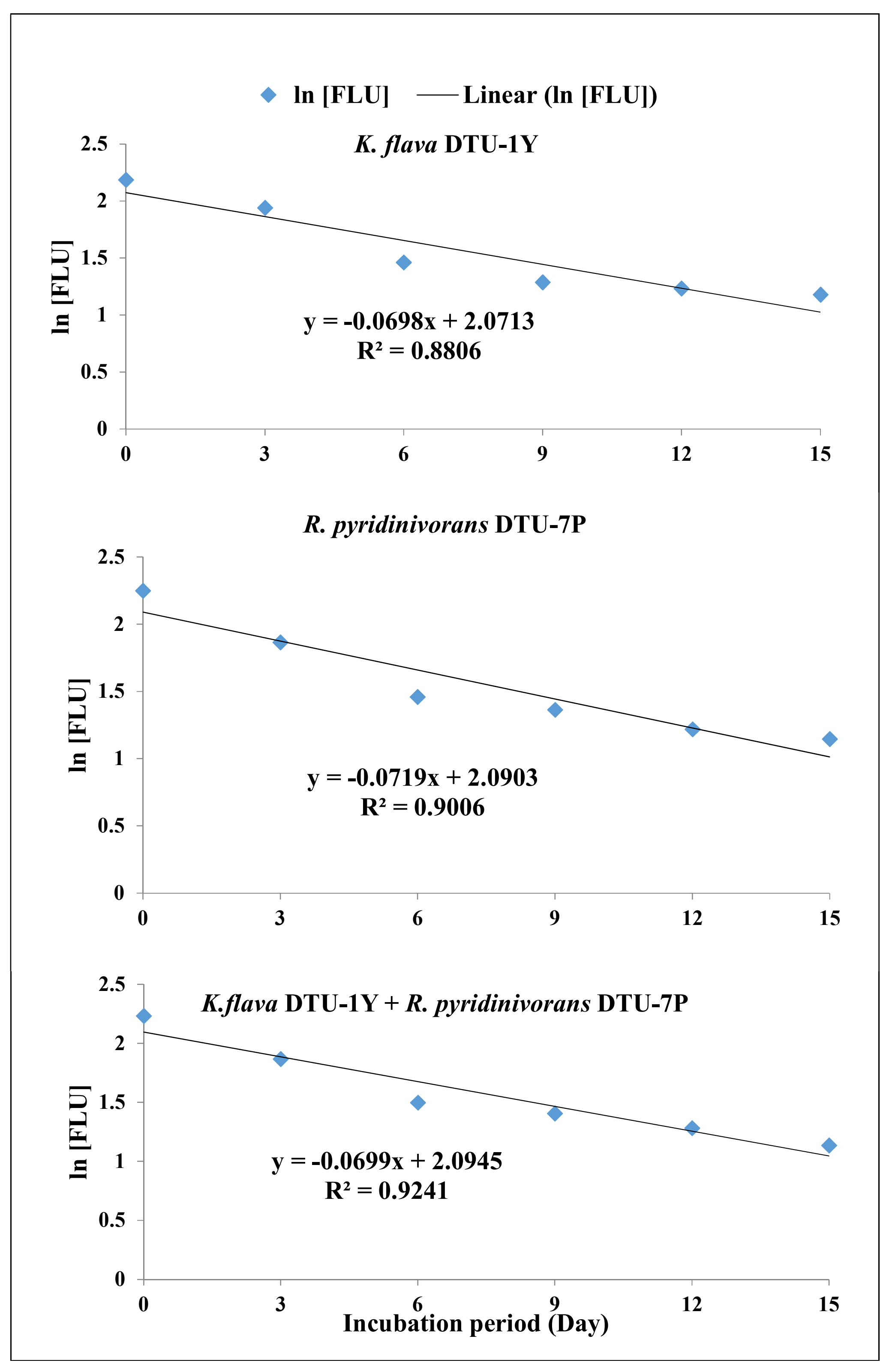



**Fig. 4.22 Plot of the natural logarithm of fluorene concentration versus incubation period for degradation of fluorene in liquid MSM with *K. flava*. *R. pyridinivorans*, and consortium in 15 days**

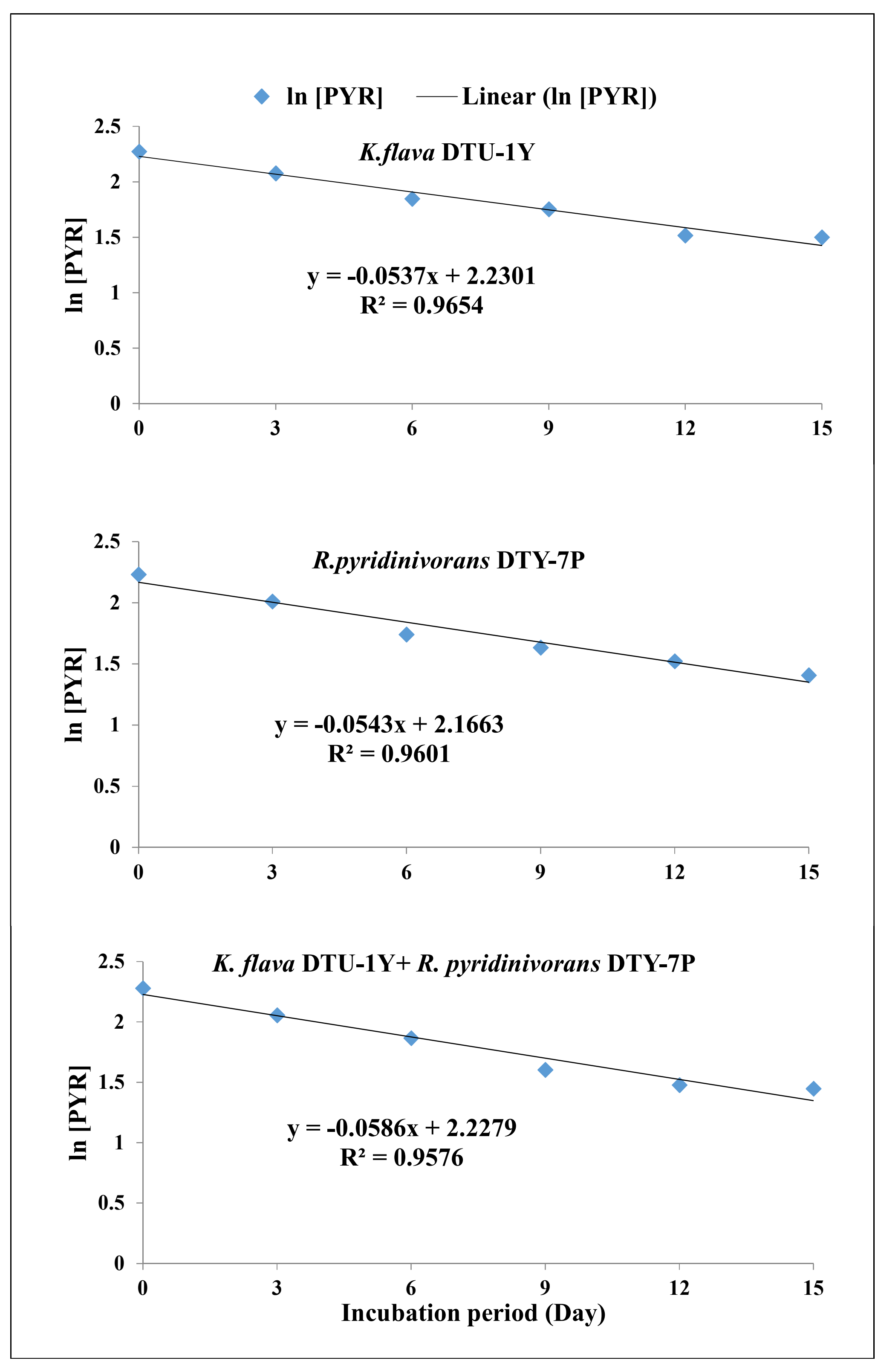



**Fig. 4.23 Plot of the natural logarithm of pyrene concentration versus incubation period for degradation of pyrene in liquid MSM with *K. flava*. *R. pyridinivorans*, and consortium in 15 days**

The plots of the natural logarithm of mixed PAH concentration versus incubation period (Fig. 4.24) were linear with $R^2$ values of 0.98, 0.93, and 0.95, respectively for *K. flava* DTU-1Y*, R. pyridinivorans* DTU-7P, and consortium The rate constant k calculated during mixed PAH-degradation study was 0.0572 $day^{-1}$, 0.0618 $day^{-1}$, and 0.0595 $day^{-1}$ respectively, and half-life were 12.1, 11.2, and 11.6 days respectively (Table 4.15). These linear plots signify first-order reaction kinetics similar to a study by Dutta *et al.,* (2017) where first-order reaction kinetics is studied during degradation of mixed PAHs. The half-life of similar periods (days) has been reported in other studies as well (Ping *et al.,* 2017; Aldaby and Mawad, 2019) indicating that the microbes used in the present study have also got significant potential towards PAH-degradation. Several studies have reported results similar to this study stating that first-order reaction kinetics is observed during degradation of a single substrate (Atuanya and Chakrabarti, 2004; Mohd-Kamil *et al.,* 2013; Kachieng'a and Momba, 2017). Based on the chemical kinetics of PAH-degradation, consortium represented slightly higher efficiency compared to *K. flava* and *pyridinivorans*. The difference may be attributed to the slightly higher growth rate/cell biomass of consortium although the enzyme activity was almost similar to that of other species.

**Table. 4.15 First-order kinetic function, results of degradation constant (K), and half-life (t/2) during biodegradation of PAHs by *K. flava* DTU-1Y, *R. pyridinivorans* DTU-7P, and consortium in 15 days**

| S. No. | PAH | Microbial sp. | First-order kinetic function | $R^2$ | K (day−1) | t/2 (days) |
|---|---|---|---|---|---|---|
| 1. | PHE | ***K. flava*** | y = -0.0568x + 2.2032 | 0.9716 | 0.0568 | 12.2 |
| | | ***R. pyridinivorans*** | y = -0.0697x + 2.2898 | 0.9787 | 0.0697 | 9.9 |
| | | **Consortium** | y = -0.0620x + 2.2053 | 0.9719 | 0.0620 | 10.9 |
| 2. | ANT | ***K. flava*** | y = -0.0626x + 2.1825 | 0.9640 | 0.0626 | 11.1 |
| | | ***R. pyridinivorans*** | y = -0.0694x + 2.3146 | 0.9901 | 0.0694 | 9.9 |
| | | **Consortium** | y = -0.0653x + 2.2225 | 0.9552 | 0.0651 | 10.6 |
| 3. | FLU | ***K. flava*** | y = -0.0698x + 2.0713 | 0.8806 | 0.0698 | 9.9 |
| | | ***R. pyridinivorans*** | y = -0.0719x + 2.0903 | 0.9006 | 0.0719 | 9.6 |
| | | **Consortium** | y = -0.0699x + 2.0945 | 0.9241 | 0.0699 | 9.9 |
| 4. | PYR | ***K. flava*** | y = -0.0537x + 2.2301 | 0.9654 | 0.0537 | 12.9 |
| | | ***R. pyridinivorans*** | y = -0.0543x + 2.1663 | 0.9601 | 0.0543 | 12.8 |
| | | **Consortium** | y = -0.0586x + 2.2279 | 0.9576 | 0.0586 | 11.8 |
| 5. | Mixed PAHs | ***K. flava*** | y = -0.0572x + 2.1102 | 0.9813 | 0.0572 | 12.1 |
| | | ***R. pyridinivorans*** | y = -0.0618x + 2.1898 | 0.9357 | 0.0618 | 11.2 |
| | | **Consortium** | y = -0.0595x + 2.2248 | 0.9544 | 0.0595 | 11.6 |

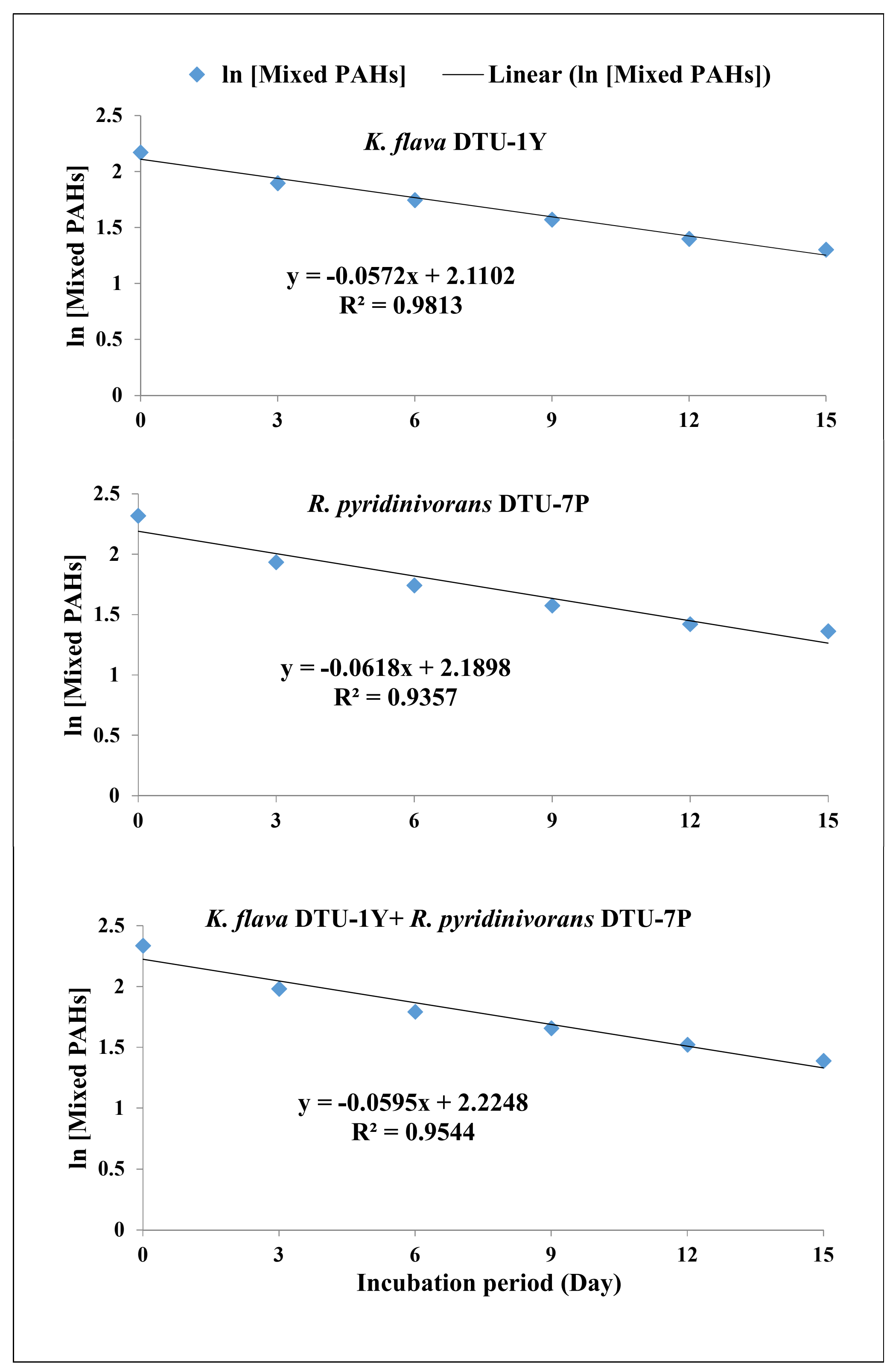


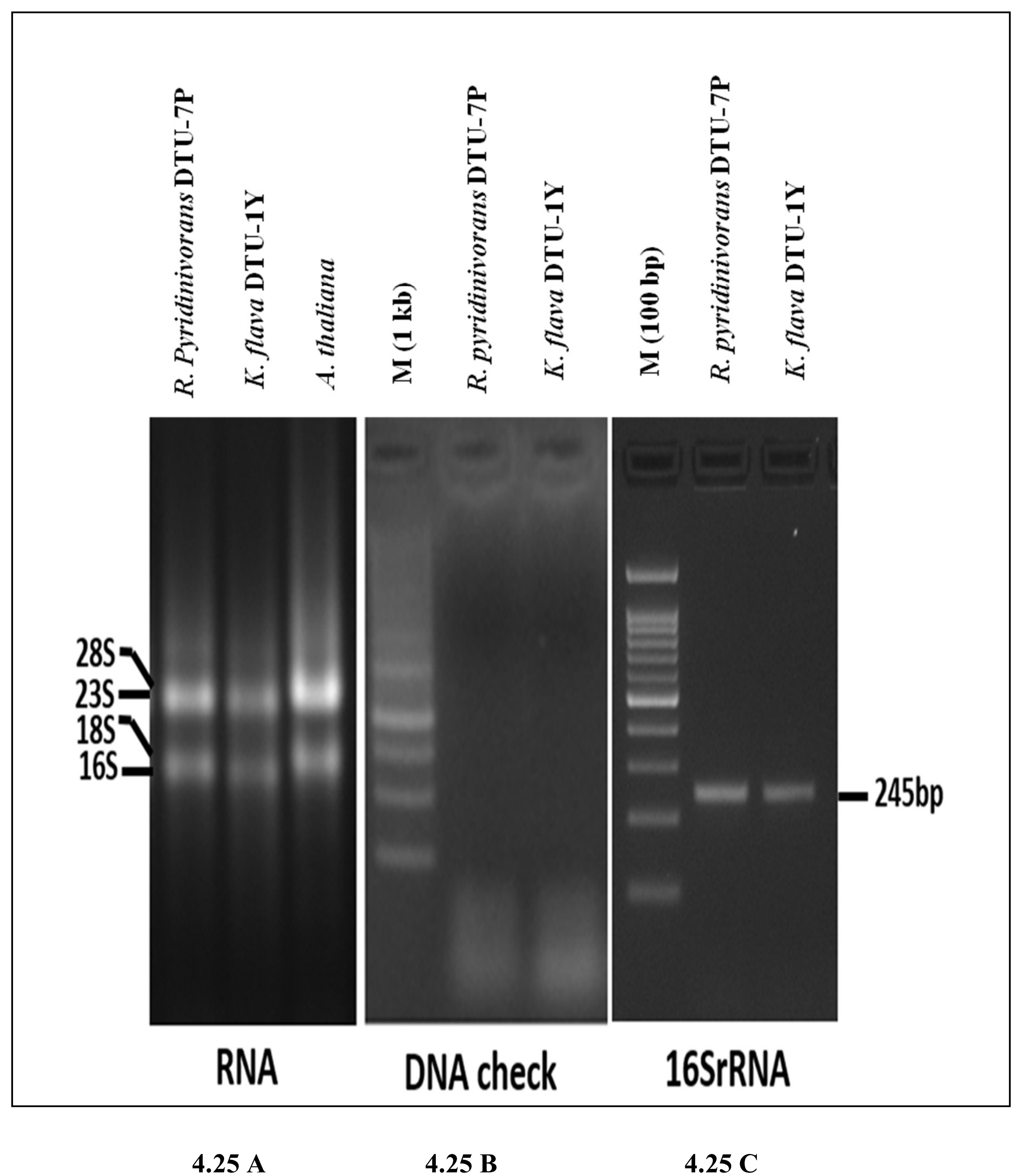

**Fig. 4.24 Plot of the natural logarithm of mixed PAHs concentration vs. incubation period for degradation of mixed PAHs in liquid MSM with *K. flava*. *R. pyridinivorans*, and consortium in 15 days**

### 4.6 Catabolic gene expression during PAH-degradation

In the present study, the catabolic genes *i.e. NAH* (naphthalene dioxygenase), *C23O* (Catechol 2,3-dioxygenase) and *PAH-RHD* (PAH-ring hydroxylating dioxygenase) along with the 16S rRNA gene as a housekeeping gene were analyzed to investigate the expression level of the selected genes in two selected bacterial strains during PAH-degradation. The isolated RNA from both bacterial strains was examined over 1.2% MOPS (3-(N-morpholino) propane sulfonic acid) containing denaturing Agarose gel. Two distinct bands, 23S, and 16S rRNA molecules were in each of the RNA samples of both strains which ensure the good quality of RNA for cDNA synthesis (Fig. 4.25 A). As the isolated RNA had been given treatment with DNase to remove the residual DNA, the presence of DNA contamination was checked using 16S rRNA gene-specific primers and it was observed that there is no amplicon in the samples (Fig. 4.25 B) indicating DNA free preparation of RNA samples. The RNA samples from each strain were converted to cDNA, followed by analysis for the presence of the 16S rRNA transcript. In both samples, a specific transcript of 245 bp size (Fig. 4.25 C) was observed indicating that cDNA could be used for expression analysis of the selected genes. The RT-qPCR assay was performed using three technical replicates of each sample for which cDNA synthesis was performed separately and independently. The amplification plot and melting curve plots were examined for catabolic genes (*C23O*, *PAH-RHD,* and *NAH*) (Fig. 4.26). This is the first study that shows significant gene expression of the catabolic genes during mixed PAH-degradation by selected bacterial strains. It was observed that among the three genes, two *i.e. C23O* and *NAH* were yielding a specific and single melting curve (Fig. 4.26 A, 4.26 C), whereas, *PAH-RHD* yield multiple peaks (Fig. 4.26 B). *PAH-RHD* gene in both *R. pyridinovorans* and *K. flava* did not exhibit any gene expression (their melting curves exhibited a mixture of peaks). The *C23O* and *nah* gene expression exhibited in *R. pyridinovorans* and *K. flava* strains during degradation of PAHs.

The RT-qPCR technique was used for catabolic gene expression analysis during mixed PAH-degradation in this investigation. Similarly, RT-qPCR has been used in various previous studies on degradation of aromatic pollutants to examine the catabolic gene expression, which is induced in the presence of substrates. The qRT-PCR technique was used to study the expression of aromatic ring-cleaving dioxygenase genes in *Mycobacterium gilvum* PYR-GCK strain during pyrene degradation (Badejo *et al.,* 2013), catabolic gene expression in four different strains of *Mycobacterium sp.* during phenanthrene and pyrene degradations as single and mixed-PAH was studied (Hennessee and Li, 2016). An increased expression level of a catabolic gene related to PAH-degradation was examined in

*Rhodococcus* sp. P14 when cultured with anthracene, pyrene, phenanthrene, or benz[a]-anthracene as sole carbon source for growth (Peng *et al.,* 2018).




**4.25 A** **4.25 B** **4.25 C**

**Fig. 4.25 Agarose gels showing quality of RNA isolated (4.24 A); Check for DNA contamination in RNA preparation (4.24 B) and a control RT-PCR for 16S rRNA showing amplification of specific size amplicons (4.24 C)**

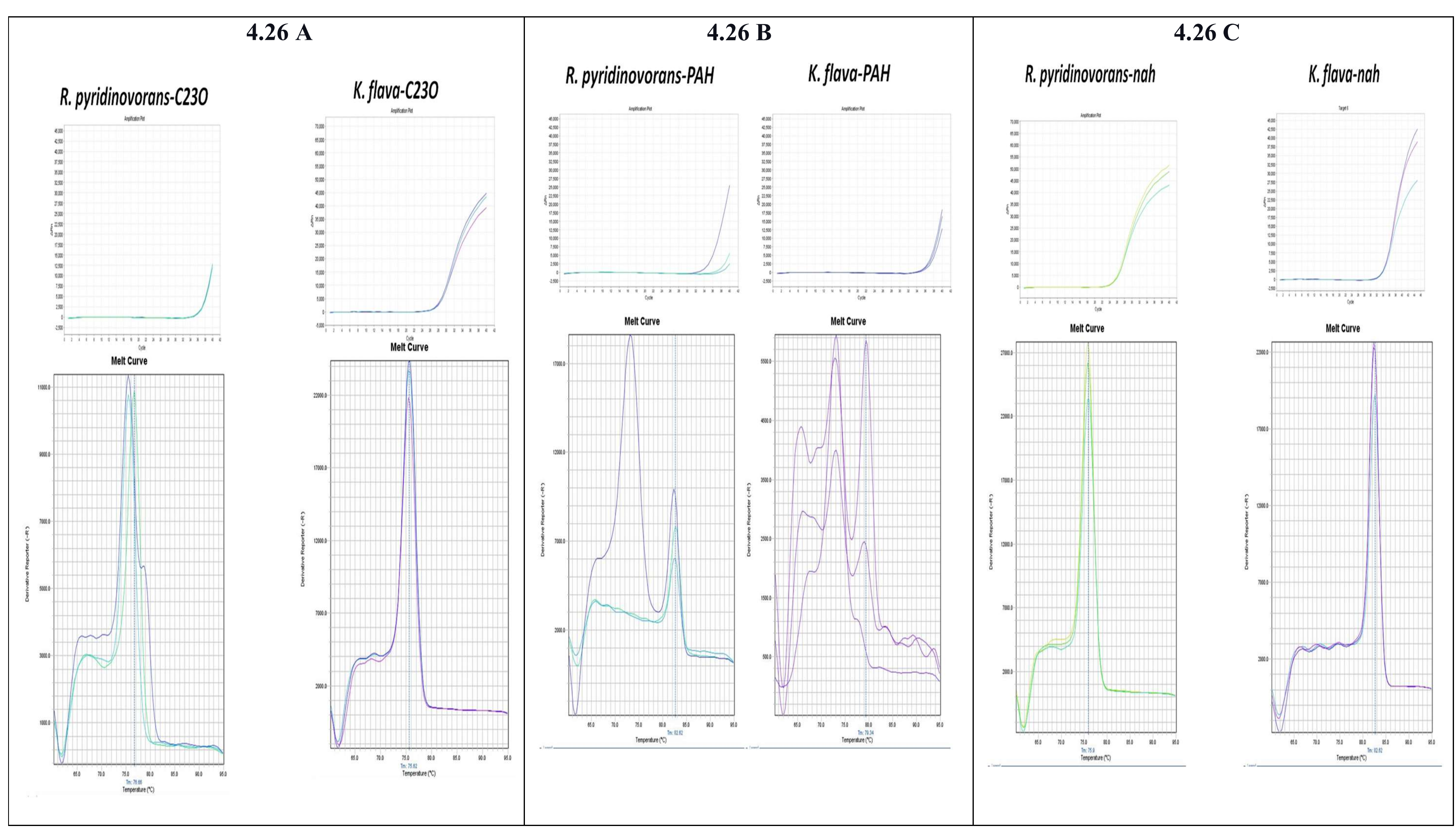



**Fig. 4.26 Melting curves and amplification plots showing the specific and non-specific amplifications of *C23O* (4.26 A), *PAH-RHD* (4.26 B) and *NAH* (4.26 C) in *R. pyridinivorans* DTU-7P and *K. flava* DTU-1Y samples**

**Table. 4.16 $C_T$ values of the genes analyzed along with standard deviation**

| Gene Name | ***R. pyridinivorans* DTU-7P** | **SD** | ***K. flava* DTU-1Y** | **SD** |
|---|---|---|---|---|
| ***16S rRNA*** | 24.71855 | 0.203929 | 24.60683 | 0.310787 |
| ***C23O*** | 34.77323 | 0.176670 | 28.64123 | 0.120169 |
| ***NAH*** | 25.05444 | 0.394907 | 32.85457 | 0.274250 |

The $C_T$ (cycle threshold) values (Table 4.16) of the catabolic genes were also analyzed in *R. pyridinovorans* DTU-7P and *K. flava* DTU-1Y. In case of the housekeeping gene, *i.e.* 16SrRNA, both of the strains exhibited similar level of expression which is relevant for the housekeeping gene (internal control) to normalize the catabolic gene expression. After calculating the log (base 2) expression values using $2^{-ddCT}$ method (Livak and Schmittgen 2001), it was observed that the *C23O* gene showed a 6.02 log fold higher expression in *K. flava* DTU-1Y in comparison to *R. pyridinovorans* DTU-7P, and *NAH* gene exhibited a 7.9 log fold higher expression in *R. pyridinovorans* DTU-7P in comparison to *K. flava* DTU-1Y. The study concluded that the *C23O* gene expressed higher activity in *K. flava* DTU-1Y*,* and *NAH* gene expressed higher activity in *R. pyridinivorans* DTU-7P. The outcomes of gene expression analysis indicated that the presence and expression of catabolic genes for PAH-degradation in isolated bacterial strains may be suitable for the bioremediation process, and can make opportunities available for degradation/removal of PAHs from contaminated environment.

# CHAPTER 5
# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

Based on the results obtained for degradation of PAHs, enzyme activity and expression of catabolic genes, following conclusions are drawn from the present study:

I. The growth of *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P at a very high concentration of PAHs (10,000 mg/L) indicated that both the isolated strains are resistant/resilient to the PAH-contaminated environment. Such microorganisms growing at contaminated sites have adapted for PAH-degradation over the period of exposure to a high dose of PAHs. The continuous exposure and survival of the strains *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P have developed the capabilities to degrade PAHs upon gradually increasing exposure to PAHs since these strains were isolated from a 20-year-old petroleum-contaminated site which is still in use and is fairly contaminated. It is the testimony to adaptation and PAH-metabolism under stressed environmental conditions. The natural screening is expected to select the bacterial species which can withstand high PAH exposure and assimilate them simultaneously and can be a potential tool for remediation of oil/PAH-contaminated sites.

II. The two bacterial isolates *i.e. Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P, and consortium of the isolates can efficiently degrade three-ring PAHs (phenanthrene, anthracene, and fluorene) as well as four-ring PAHs (pyrene) when present as a single PAH compound or as a mixture of PAHs. The degradation efficiency of isolated bacterial strains for a PAH compound is not affected by the presence of other PAH compounds. The degradation efficiency of the selected bacterial strains for PAHs in the mixture is almost similar to the degradation efficiency for PAHs when present as a single compound confirming the ability of isolated bacterial strains to utilize PAHs when present as an individual compound as well as in a mixture of PAHs.

III. PAH-degradation by the consortium of *Kocuria flava* DTU-1Y and *Rhodococcus pyridinivorans* DTU-7P revealed that there is no synergistic or inhibitive effect of the strains over each other. The possible reason for almost similar maximal degradation could be inter-specific competition for the substrate (PAHs) in case of degradation by consortium. Although the competition may be treated as the mutually inhibitive effect, but the outcome in terms of PAH-degradation remained unaffected, thereby classifying the interaction in consortium as neutral. The isolated strains could be used for bioremediation of PAHs contaminated sites without any inhibitory/synergistic effects as the interaction between two isolated strains may be positive as there is no inhibitory effect observed on degradation efficiency when used in a consortium for degradation of individual PAH as well as a mixture of PAHs.

IV. Phylogenetic analysis for evolutionary relationship revealed that the isolated strains are related to different known PAH-degrading bacterial strains which have catabolic genes/ enzymes involved in the degradation pathway. The evolutionary relationship of isolated bacterial strains with other known PAH-degrading bacteria having catabolic genes/enzymes capable of metabolizing PAHs indicated that these strains may also possess the catabolic gene/enzymes responsible for PAH-degradation and can play an important role in the bioremediation of PAH-contaminated sites.

V. The exposure to a high concentration of PAHs under laboratory conditions can further help express the catabolic enzymes responsible for PAH-degradation making both the isolated strains potential candidates for remediation of hydrocarbon-contaminated sites. Catabolic enzyme activity of dioxygenase (C23O), dehydrogenase, and peroxidase enzymes were found to play a crucial role in the microbial transformation of three-ring and four-ring PAHs through meta-cleavage of benzene ring by C23O and subsequent transformation/degradation dehydrogenase and peroxidase enzyme produced by both the isolated bacteria.

VI. The degradation of PAHs follows first-order rate kinetics during enzyme-catalyzed PAH-removal, it is fairly expected that hydrocarbon contaminated environment can initiate/enhance enzyme activity in these microbes resulting in efficient removal of PAHs.

VII. For successful biodegradation of PAHs, profound knowledge of various aspects of the degradation mechanism is required at the molecular level that allows the use of these contaminants as carbon source by microbial species. The genetic studies help in the elucidation of degradation pathways which involve catabolic genes encoding catabolic enzymes responsible for PAH-degradation.

VIII. Gene expression may be induced by gradually increasing exposure to contaminants as the main genetic mechanism for microbial adaptation is the amplification of genes responsible for the metabolism of contaminants. Significant catabolic gene expression for naphthalene dioxygenase (*NAH*) and catechol 2,3-dioxygenase (*C23O*) during degradation of PAHs concluded that *K. flava* DTU-1Y and *R. pyridinivorans* DTU-7P are efficient PAH-degraders and can be used for the development of an efficient bioremediation method for cleaning of PAH-contaminated environmental matrix.

## 5.2 Recommendations

Based on the observations of the present study, the following recommendations are made:

I. The bacterial strains *Kocuria flava* and *Rhodococcus pyridinivorans* can be used for the degradation of PAHs and may be a suitable candidate for remediation of PAH-contaminated sites. The consortium of isolated bacterial strains can also be used without any possibility of inhibition although the difference in degradation efficiency is marginally low.

II. Further studies on the degradation of PAHs by microbial species should target contaminated sites for isolation of microbes since natural adaptation over a long period of time, has already taken place and gene expression in such microbes is also expected.

III. Taking into consideration that PAHs are found as a mixture in the environment, the bacterial strains *Kocuria flava* and *Rhodococcus pyridinivorans* can be used for the degradation of other high molecular weight PAHs with 5-6 benzene rings as the isolates possess catabolic enzymes and genes responsible for PAH-degradation.

IV. The genetic studies for bioremediation of PAHs are limited to low molecular weight PAHs (two to three rings) and investigations on high molecular weight PAHs (four or more rings) are still limited. The genetic analysis seems to be required on high-molecular-weight PAH-degradation.

V. Further research may be done to find new catabolic genes, enzymes, and metabolic pathways. The catabolic genes, so studied, may be identified in other species (online database) and the degradation capability of other microbes may be studied but after adaptation.

VI. Using the genetic information suitable PAH-catabolic genes can be used to create genetic-engineered microorganisms (GEMs) having desirable properties for PAH-biodegradation.

**5.3 Scope for future work**

The contamination of the environment with PAHs is inescapable in some sectors such as the petroleum industry. So, the selection of microbial species for bioremediation of PAH-contaminated sites for field-scale application is very important for PAH removal. For designing an efficient bioremediation technique the exact mechanism of degradation of PAHs may be undertaken considering the roles of enzymes individually and in combination. Further studies on the expression of fungal and algal genes involved in the degradation of PAHs may be undertaken. Microbial degradation of PAHs under field conditions may be undertaken to identify the inhibitors. Studies on other remediation methods coupled with microbial degradation may be undertaken to enhance the rate of PAH-degradation. Suitable PAH-catabolic genes may be used to create genetic-engineered microorganisms (GEMs) having desirable properties for biodegradation. However, future prospective for GEMs in PAH-bioremediation at field scale is restricted by various challenges like gaining approval for the release of GEMs into the contaminated environment, its fate, monitoring, control, and risk assessment for the ecosystem. Future investigation is needed to find the conditions and factors that may enhance bioremediation efficiency at the field scale with the aid of molecular techniques.

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# Annexure I

**Standard curve of PAHs**

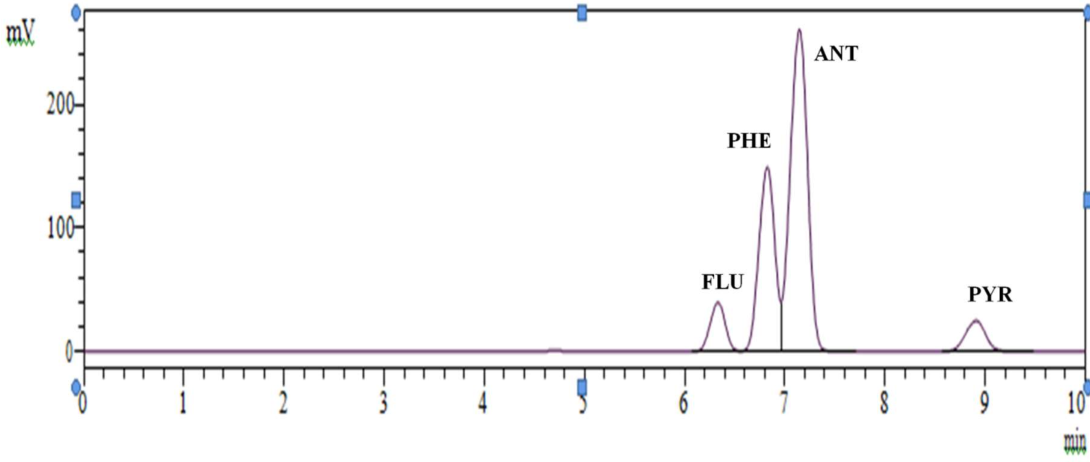
The Standard curve for PHE, ANT, FLU, and PYR using HPLC were prepared. The stock solutions of each PAH were diluted to attain the concentrations of 2, 4, 6, 8 and 10 mg/l and were used to prepare standard chromatograms using:

Mobile phase: 80:20 (Acetonitrile: Water)

Wavelength: 254nm

Flow rate: 1ml/min

Sample injection volume: 10µL



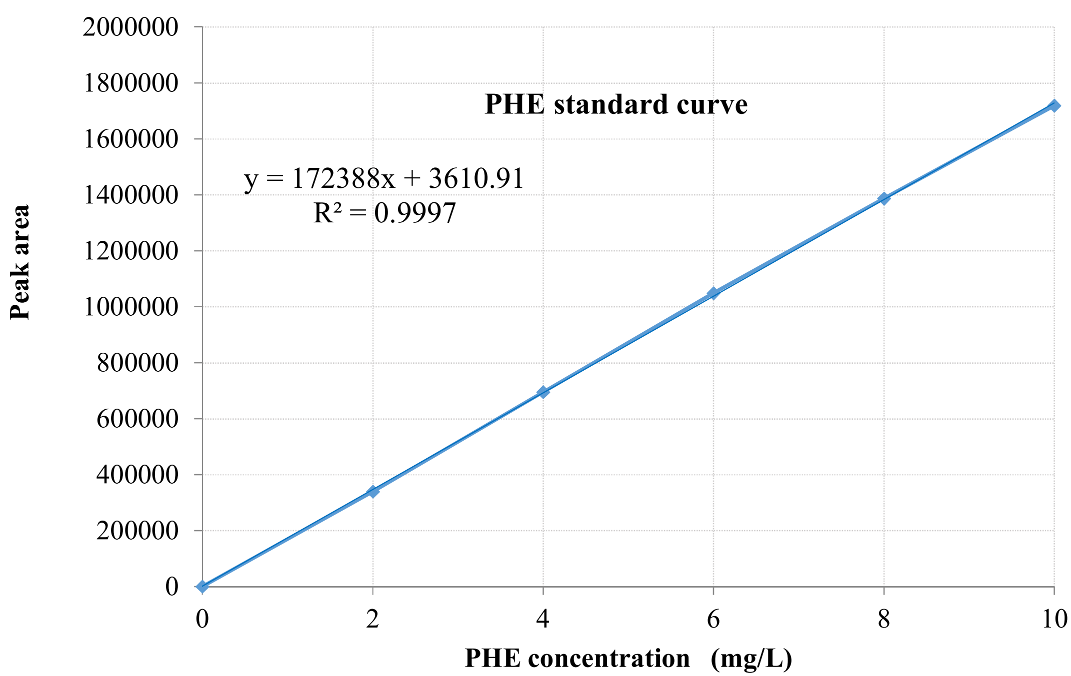

**Chromatogram for retention time of PAHs (ANT, PHE, FLU, and PYR) (Mobile phase: 80:20 (Acetonitrile: Water), Wavelength: 254nm, Flow rate: 1ml/min, Sample injection volume: 10µL)**

- Retention time of FLU: 6.3 min
- Retention time of PHE: 6.7 min
- Retention time of ANT: 7.1 min
- Retention time of PYR: 8.9 min

**Standard curve of PHE**

**Retention time of PHE: 6.7 min**

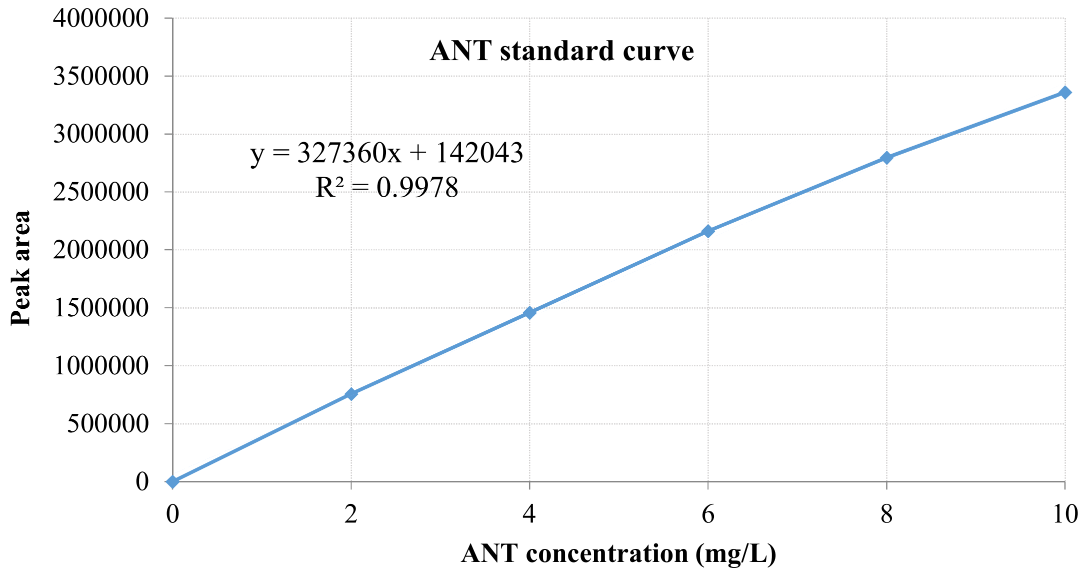
| PHE Concentration (mg/L) | Peak Area |
|---|---|
| 0 | 0 |
| 2 | 340321 |
| 4 | 695564 |
| 6 | 1048538 |
| 8 | 1386586 |
| 10 | 1718693 |



**Standard curve of ANT**

**Retention time of ANT: 7.1 min**

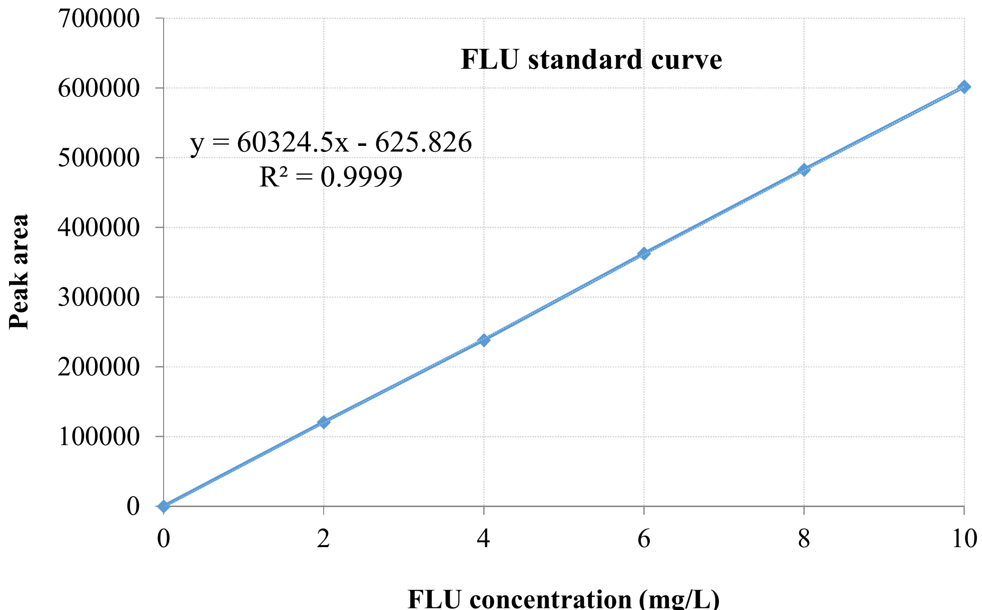
| ANT Concentration (mg/L) | Peak Area |
|---|---|
| 0 | 0 |
| 2 | 755790 |
| 4 | 1456882 |
| 6 | 2162774 |
| 8 | 2795487 |
| 10 | 3360091 |



**Standard curve of FLU**

**Retention time of FLU: 6.3 min**

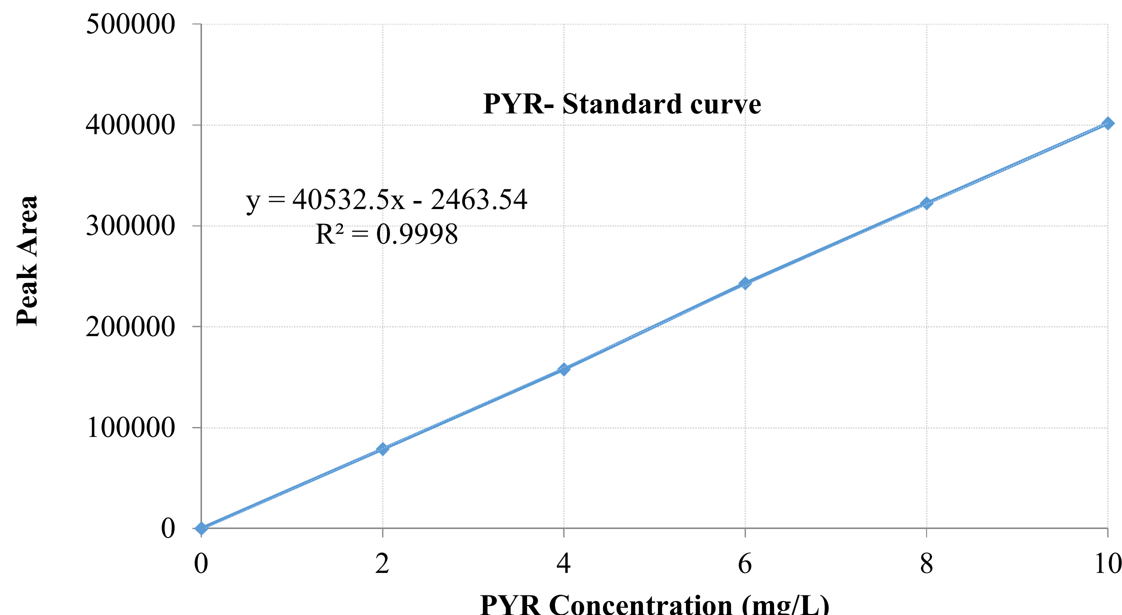
| FLU Concentration (mg/L) | Peak Area |
|---|---|
| 0 | 0 |
| 2 | 120796 |
| 4 | 238487 |
| 6 | 362666 |
| 8 | 482743 |
| 10 | 601913 |



**Standard curve of PYR**

**Retention time of PYR: 8.9 min**

| PYR Concentration (mg/L) | Peak Area |
|---|---|
| 0 | 0 |
| 2 | 78765 |
| 4 | 157687 |
| 6 | 243049 |
| 8 | 322447 |
| 10 | 401711 |



## POLYNUCLEAR AROMATIC HYDROCARBONS by HPLC 5506

Formulae: Table 1 MW: Table 1 CAS: Table 2 RTECS: Table 2

| METHOD: 5506, Issue 3 | EVALUATION: PARTIAL | Issue 1: 15 May 1985<br>Issue 3: 15 January 1998 |
|---|---|---|
| OSHA: Table 3<br>NIOSH: Table 3<br>ACGIH: Table 3 | PROPERTIES: Table 1 | |

**Compounds**

| acenaphthene | benzo[ghi]perylene | fluorene |
|---|---|---|
| acenaphthylene | benzo[a]pyrene | indeno[1,2,3-cd]pyrene |
| anthracene | benzo[e]pyrene | naphthalene |
| benz[a]anthracene | chrysene | phenanthrene |
| benzo[b]fluoranthene | dibenz[a,h]anthracene | pyrene |
| benzo[k]fluoranthene | fluoranthene | |

**NAMES & SYNONYMS:** Polycyclic aromatic hydrocarbons, PAHs; also see Table 2.

| SAMPLING | | MEASUREMENT | |
|---|---|---|---|
| SAMPLER: | FILTER + SORBENT TUBE (37-mm, 2-µm, PTFE + washed XAD-2, 100 mg/50 mg) | TECHNIQUE: | HPLC, FLUORESCENCE/UV DETECTION |
| FLOW RATE: | 2 L/min | ANALYTE: | compounds listed above |
| VOL-MIN: | 200 L | EXTRACTION: | 5 mL acetonitrile; ultrasonic bath, 30 to 60 minutes |
| -MAX: | 1000 L | INJECTION VOLUME: | 10 to 50 µL |
| SHIPMENT: | transfer filters to culture tubes; wrap sorbent and culture tubes in Al foil; ship @ 0 °C | MOBILE PHASE: | acetonitrile/water gradient @ ambient temperature, 1 mL/min |
| SAMPLE STABILITY: | unknown; protect from heat and UV light | COLUMN: | 250 x 4.6-mm, reversed-phase, 5-µm $C_{18}$ |
| FIELD BLANKS: | 3 to 10 field blanks per set | DETECTOR: | UV @ 254 nm; fluorescence @ 340 nm (excitation), 425 nm (emission) |
| MEDIA BLANKS: | 6 to 10 media blanks per set | CALIBRATION: | standards in acetonitrile |
| **ACCURACY** | | RANGE: | see EVALUATION OF METHOD |
| RANGE STUDIED: | not determined | ESTIMATED LOD: | see EVALUATION OF METHOD |
| BIAS: | not determined | PRECISION ($\bar{S}_r$): | see EVALUATION OF METHOD |
| OVERALL PRECISION ($\hat{S}_{rT}$): | not determined | | |
| ACCURACY: | not determined | | |

**APPLICABILITY:** This method is applicable to samples that can be extracted with acetonitrile. This method is not applicable to samples that require a different extraction solvent or contain large amounts of highly adsorptive particulate matter, e.g., fly ash or diesel soot; also, this method is not applicable to asphalt fume samples.

**INTERFERENCES:** Any compound that elutes at the same HPLC retention time may interfere. Heat, ozone, $NO_2$, or UV light may cause sample degradation.

**OTHER METHODS:** This revises P&CAM 206 and 251 [1]. Method 5515 uses the same sampling technique, with gas chromatographic measurement [2]. Method 5800 uses the same sampling technique, and a flow-injection method to determine total polycyclic aromatic compounds at two different sets of fluorescent wavelengths [3].

**REAGENTS:**

1. Water, distilled, deionized, degassed.
2. Acetonitrile, HPLC grade, degassed.
3. PAH test mixture,* a liquid standard containing the PAHs except benzo[e]pyrene (EPA 610 Polynuclear Aromatic Hydrocarbons,Supelco, Cat. No. 4-8743; or equivalent).
4. Benzo[e]pyrene,* solid (Supelco, Cat. No. 44-2475; or equivalent).

* See SPECIAL PRECAUTIONS

**EQUIPMENT:**

1. Sampler:
   a. Filter. 37-mm, 2-µm pore size, PTFE membrane filter laminated to PTFE, (Zefluor, Pall Gelman Sciences, Cat. No. P5PJ037; SKC Inc., Cat. No. 225-17-07; or equivalent filter), cellulose spacer ring, 37-mm OD, 32-mm ID, (SKC Inc., Cat. No. 225-23; or equivalent) in a 37-mm cassette filter holder.
      NOTE: If sampling is to be done in bright sunlight, use opaque or foil-wrapped cassettes to prevent sample degradation.
   b. Sorbent tube, washed XAD-2 resin (front = 100 mg; back = 50 mg) (ORBO 43, Supelco, Cat. No. 2-0258; or equivalent), connected to filter with minimum length of PVC tubing. Plastic caps are required after sampling.
      NOTE: If pressure drop is excessive or pump fails, use a larger diameter sorbent tube with XAD-2 resin (ORBO 42 Large, Supelco, Cat. No. 2-0264U; or equivalent).
2. Personal sampling pump capable of operating for 8 h at 2 L/min, with flexible connecting tubing.
3. Aluminum foil.
4. Refrigerant, bagged.
5. Culture tubes, PTFE-lined screw cap, 13-mm x 100-mm.
6. Forceps.
7. Syringe filters, 0.45-µm, 25-mm, PTFE (Acrodisc-CR, Pall Gelman Sciences, Cat. No. 4219; or equivalent).
8. Pipet, 5-mL.
9. Syringe or micropipets, 1- to 100-µL.
10. Ultrasonic bath.
11. HPLC, with gradient capability, fluorescence (excitation @ 340 nm, emission @ 425 nm) and UV (254 nm) detectors in series, electronic integrator, and a 250 x 4.6-mm $C_{18}$ column (Vydac 201TP, The Separations Group, Hesperia, CA, Cat. No. 201TP54; or equivalent).
12. Volumetric flasks, 10- and 100-mL.
13. Recommendation: lighting in laboratory should be incandescent or UV-shielded fluorescent.

**SPECIAL PRECAUTIONS:** Treat all polynuclear aromatic hydrocarbons as carcinogens. Samples and unused standards are considered toxic waste. Dispose of in an appropriate manner. Counter tops and equipment should be checked regularly with a "black light" for fluorescence as an indicator of contamination by PAHs.

**SAMPLING:**

1. Calibrate each personal sampling pump with a representative sampler in line.
2. Take personal samples at 2 L/min for a total sample size of 200 to 1000 L.
3. Immediately after sampling, transfer the filter carefully with forceps to a culture tube. Hold filter at edge to avoid disturbing the collected sample. Cap the tube and wrap in aluminum foil.
   NOTE: This step is necessary to avoid loss of analytes by sublimation.
4. Cap the sorbent tube and wrap in aluminum foil.
5. Ship to laboratory in insulated container with bagged refrigerant.

**SAMPLE PREPARATION:**

NOTE: UV light may degrade PAHs; therefore, recommend using yellow, UV-absorbing shields for fluorescent lights or use incandescent lighting.

6. Refrigerate samples upon receipt at laboratory.
7. Extract PAH from filters.
   a. Add 5.0 mL of acetonitrile to each culture tube containing a filter. Similarly, add 5.0 mL of acetonitrile to each culture tube containing the media and reagent blanks. Cap the tubes.
   b. Place capped tubes in an ultrasonic bath for 30 to 60 min.
8. Desorb PAH from sorbent.
   a. Score each sorbent tube with a file in front of the front (larger) sorbent section. Break tube at score line.
   b. Transfer front glass wool plug and front sorbent section to a culture tube. Transfer back sorbent section, and the middle glass wool plug to a second culture tube.
   c. Add 5.0 mL acetonitrile to each culture tube. Cap the tubes.
   d. Place capped tubes in an ultrasonic bath for 30 to 60 min.
9. Filter all sample extracts through an 0.45-µm syringe filter.

**CALIBRATION AND QUALITY CONTROL:**

10. Calibrate daily with at least six working standards.
    NOTE: If a benzo[e]pyrene standard is needed, weigh desired amount and add to a known volume of the PAH test mixture.
    a. Dilute aliquots of the PAH test mixture (containing benzo[e]pyrene if needed) with acetonitrile in 10-mL volumetric flasks. The concentration range should cover most of the PAH concentrations in the samples.
    b. During analysis, intersperse working standards with samples and blanks.
    c. Prepare calibration graphs (peak area vs. µg of each PAH per sample).
11. Recovery and desorption efficiency.
    a. Determine recovery (R) from filters and desorption efficiency (DE) from sorbent tubes at least once for each lot of filters and sorbent tubes used in the range of interest.
       (1) Filters. Using a microliter syringe or a micropipette, spike four filters at each of five concentration levels with a mixture of the analytes. Allow the filters to dry in the dark overnight. Analyze the filters (steps 7, 9, and 13 through 15). Prepare graphs of R vs. amounts found.
       (2) Sorbent tubes. Transfer an unused front sorbent section to a culture tube. Prepare a total of 24 culture tubes in order to measure DE at five concentration levels plus blank in quadruplicate. Using a microliter syringe or micropipette, add calibration stock solution directly to sorbent. Cap culture tubes and allow to stand overnight. Desorb and analyze (steps 8, 9, and 13 through 15). Prepare graphs of DE vs. amounts found.
    b. Check R and DE at two levels for each sample set, in duplicate. Repeat determination of R or DE graphs if checks do not agree to within ±5% of R or DE graph.
12. Analyze at least three field blanks for each sample medium.

**MEASUREMENT:**

13. Set HPLC according to manufacturer's instructions, conditions on page 5506-1 and steps 14 and 15.
14. Inject sample aliquot (10 to 50 µL). Start mobile phase gradient:
    a. Linear gradient from 60% acetonitrile/40% deionized water to 100% acetonitrile at 1 mL/min over 20 min.
    b. Hold at 100% acetonitrile for 20 min.
    c. Linear gradient to initial condition, 5 min.
15. Measure peak areas for each analyte using the appropriate detector as specified in Table 1.

    NOTE 1: The order of elution for the PAHs appears in Table 4.

    NOTE 2: If peak area is above the calibration range, dilute with acetonitrile, reanalyze, and apply dilution factor in calculations.

    NOTE 3: If sample has many interferences, additional sample cleanup may be necessary.

**CALCULATIONS:**

16. Read the mass, µg (corrected for R or DE) of each analyte found on the filter (W) and front sorbent ($W_f$) and back sorbent ($W_b$) sections, and on the average media blank filter (B) and front sorbent ($B_f$) and back sorbent ($B_b$) sections from the calibration graphs.
17. Calculate concentration, C (mg/m³), as the sum of the particulate concentration and the vapor concentration in the actual air volume sampled, V (L).

$$C = \frac{(W + W_f + W_b - B - B_f - B_b)}{V}, \ mg/m^3$$

NOTE 1: µg/mL = mg/m³

NOTE 2: $W_f$ and $W_b$ include analyte originally collected on the filter as particulate, then volatilized during sampling. This can be a significant fraction for many PAHs (e.g., anthracene, fluoranthene, fluorene, naphthalene, phenanthrene).

**EVALUATION OF METHOD:**

The UV detector is used to analyze for some PAHs (see Table 1), and the remaining PAHs are analyzed by a fluorescent detector, which gave better sensitivity for some PAHs. The ranges of the limit of detection (LOD) and the limit of quantitation (LOQ) values for the 17 PAHs are reported in Table 4 [4]. The LOD and LOQ values varied because of differences in the detectors used and the concentrations of the standards. Therefore, it is important that the LOD and LOQ values be determined for each set of samples. The LOQs are the lower end of the analytical ranges. The upper end of the analytical ranges were not determined.

This method was evaluated by means of a user check [5]. An independent laboratory prepared spiked filters and sorbent tubes for a recovery and desorption efficiency study (see Table 4). For the filters, except naphthalene, the recovery results were greater than or equal to 75%. Since naphthalene is fairly volatile under ambient conditions, this may account for the poor recovery results. For the sorbent tubes, only four of the 17 analytes had desorption efficiencies that were greater than or equal to 75%. During the user check, the sorbent tubes were extracted by adding 5 mL acetonitrile and were allowed to stand for 30 minutes with occasionalswirling. In more recent quality control experiments, the desorption efficiencies were often better for some analytes (see Table 4) [4]. These results were achieved using an ultrasonic bath for 30 to 60 minutes. The results indicated the importance of preparing media spikes for recovery and desorption efficiency studies for each set of samples; moreover, the results reenforce this need when using new lots of media.

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**METHOD REVISED BY:**

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**TABLE 1. FORMULAS AND PHYSICAL PROPERTIES.**

| COMPOUND (by M.W.) | FORMULA | WEIGHT | DETECTOR | MELTING POINT (°C) | BOILING POINT (°C) | REFERENCE |
|---|---|---|---|---|---|---|
| 1. NAPHTHALENE | $C_{10}H_8$ | 128.17 | UV | 80.2 | 218 | [8] |
| 2. ACENAPHTHYLENE | $C_{12}H_8$ | 152.20 | UV | 92.5 | 280 | [8] |
| 3. ACENAPHTHENE | $C_{12}H_{10}$ | 154.21 | UV | 93.4 | 279 | [8] |
| 4. FLUORENE | $C_{13}H_{10}$ | 166.22 | UV | 115 | 295 | [8] |
| 5. ANTHRACENE | $C_{14}H_{10}$ | 178.23 | UV | 215 | 340 | [8] |
| 6. PHENANTHRENE | $C_{14}H_{10}$ | 178.23 | UV | 99.2 | 340 | [8 |
| 7. FLUORANTHENE | $C_{16}H_{10}$ | 202.26 | FL | 108 | 384 | [8] |
| 8. PYRENE | $C_{16}H_{10}$ | 202.26 | FL | 151 | 404 | [8] |
| 9. BENZ[a]ANTHRACENE | $C_{18}H_{12}$ | 228.29 | FL | 167 | 435 | [7] |
| 10. CHRYSENE | $C_{18}H_{12}$ | 228.29 | UV | 258 | 448 | [8] |
| 11. BENZO[b]FLUORANTHENE | $C_{20}H_{12}$ | 252.32 | FL | 168 | – | [7] |
| 12. BENZO[k]FLUORANTHENE | $C_{20}H_{12}$ | 252.32 | FL | 217 | 480 | [8] |
| 13. BENZO[a]PYRENE | $C_{20}H_{12}$ | 252.32 | FL | 177 | 495 | [8, 8] |
| 14. BENZO[e]PYRENE | $C_{20}H_{12}$ | 252.32 | FL | 178 | 311 | [8] |
| 15. BENZO[ghi]PERYLENE | $C_{22}H_{12}$ | 276.34 | FL | 278 | – | [7] |
| 16. INDENO[1,2,3-cd]PYRENE | $C_{22}H_{12}$ | 276.34 | FL | 164 | – | [7] |
| 17. DIBENZ[a,h]ANTHRACENE | $C_{22}H_{14}$ | 278.35 | FL | 270 | 524 | [7, 8] |

**TABLE 2. SYNONYMS, CAS AND RTECS NUMBERS.**

| COMPOUND (alphabetically) | SYNONYMS, CAS and RTECS Numbers* |
|---|---|
| 1. ACENAPHTHENE | CAS # 83-32-9; RTECS # AB1000000 |
| 2. ACENAPHTHYLENE | acenaphthalene; CAS # 208-96-8; RTECS # AB1254000 |
| 3. ANTHRACENE | CAS # 120-12-7; RTECS # CA9350000 |
| 4. BENZ[a]ANTHRACENE | 1,2-benzanthracene; benzo[b]phenanthrene; 2,3-benzophenanthrene; tetraphene; CAS # 56-55-3; RTECS # CV9275000 |
| 5. BENZO[b]FLUORANTHENE | 3,4-benzofluoranthene; 2,3-benzofluoranthene; benz[e]acephenanthrylene; B(b)F; CAS # 205-99-2; RTECS # CU1400000 |
| 6. BENZO[k]FLUORANTHENE | 11,12-benzofluoranthene; CAS # 207-08-9; RTECS # DF6350000 |
| 7. BENZO[ghi]PERYLENE | 1,12-benzoperylene; CAS # 191-24-2; RTECS # DI6200500 |
| 8. BENZO[a]PYRENE | 3,4-benzopyrene; 6,7-benzopyrene; B(a)P; BP; CAS # 50-32-8; RTECS # DJ3675000 |
| 9. BENZO[e]PYRENE | 1,2-benzopyrene; 4,5-benzopyrene; B(e)P; CAS # 192-97-2; RTECS # DJ4200000 |
| 10. CHRYSENE | 1,2-benzophenanthrene; benzo[a]phenanthrene; CAS # 218-01-9; RTECS # GC0700000 |
| 11. DIBENZ[a,h]ANTHRACENE | 1,2,5,6-dibenzanthracene; CAS # 53-70-3; RTECS # HN2625000 |
| 12. FLUORANTHENE | benzo[jk]fluorene; CAS # 206-44-0; RTECS # LL4025000 |
| 13. FLUORENE | CAS # 86-73-7; RTECS # LL5670000 |
| 14. INDENO[1,2,3-cd]PYRENE | 2,3-phenylenepyrene; CAS # 193-39-5; RTECS # NK9300000 |
| 15. NAPHTHALENE | naphthene; CAS # 91-20-3; RTECS # QJ0525000 |
| 16. PHENANTHRENE | CAS # 85-01-8; RTECS # SF7175000 |
| 17. PYRENE | benzo[def]phenanthrene; CAS # 129-00-0; RTECS # UR2450000 |

* Data from [6, 8, and 9].

**TABLE 3. EXPOSURE LIMITS*.**

| COMPOUND | OSHA† | NIOSH‡ | ACGIH§ |
|---|---|---|---|
| 1. ANTHRACENE | 0.2 mg/m³ | – | |
| 2. BENZ[a]ANTHRACENE | – | – | suspect human carcinogen |
| 3. BENZO[b]FLUORANTHENE | – | – | suspect human carcinogen |
| 4. BENZO[a]PYRENE | 0.2 mg/m³ | – | suspect human carcinogen |
| 5. CHRYSENE | 0.2 mg/m³ | potential occupational carcinogen‡ | animal carcinogen |
| 6. NAPHTHALENE | 10 ppm; STEL 15 ppm | 10 ppm; STEL 15 ppm | 10 ppm; STEL 15 ppm |
| 7. PHENANTHRENE | 0.2 mg/m³ | – | – |
| 8. PYRENE | 0.2 mg/m³ | – | – |

* This table only includes the compounds with established exposure limit values.
† Information from [10].
‡ Information from [11].
§ Information from [12].

TABLE 4. LOD AND LOQ VALUES, AND RECOVERY DATA.

| COMPOUND (by elution order) | Range of values† | | Recoveries (%)† | |
|---|---|---|---|---|
| | LOD (µg per sample) | LOQ (µg per sample) | Filters | Sorbent tubes |
| 1. NAPHTHALENE | 0.20 - 0.80 | 0.39 - 2.6 | 49.6 | 68.5 |
| 2. ACENAPHTHYLENE | 0.090 - 2.0 | 0.28 - 6.6 | 98.2 | 98.2 |
| 3. ACENAPHTHENE | 0.20 - 5.0 | 0.58 - 16. | -- | -- |
| 4. FLUORENE | 0.030 - 0.30 | 0.099 - 0.28 | 95.0 | 95.0 |
| 5. PHENANTHRENE | 0.0070 - 0.060 | 0.023 - 0.19 | 99.0, 90.4* | 84.0, 92.5*, 82.6* |
| 6. ANTHRACENE | 0.0010 - 0.090 | 0.023 - 0.30 | 81.8, 94.4* | 72.8, 96.2*, 72.9* |
| 7. FLUORANTHENE | 0.0020 - 0.090 | 0.0066 - 0.30 | 94.9, 90.4* | 73.0, 93.5*, 81.7* |
| 8. PYRENE | 0.0010 - 0.30 | 0.0036 - 0.99 | 94.4, 76.1* | 84.9, 77.0*, 75.9* |
| 9. BENZ[a]ANTHRACENE | 0.0010 - 0.090 | 0.0042 - 0.30 | 86.6, 92.7* | 82.4, 95.0*, 72.3* |
| 10. CHRYSENE | 0.0070 - 0.20 | 0.023 - 0.37 | 94.6, 89.9* | 82.7, 89.6*, 74.0* |
| 11. BENZO[e]PYRENE | 0.0060 - 0.80 | 0.020 - 2.6 | 110 | 48.3 |
| 12. BENZO[b]FLUORANTHENE | 0.0030 - 0.20 | 0.011 - 0.66 | 94.8 | 84.2 |
| 13. BENZO[k]FLUORANTHENE | 0.0020 - 0.040 | 0.0054 - 0.13 | 103 | 53.2 |
| 14. BENZO[a]PYRENE | 0.0020 - 0.10 | 0.0051 - 0.33 | 101, 88.1* | 50.4, 91.6*, 68.4* |
| 15. DIBENZ[a,h]ANTHRACENE | 0.0040 - 0.60 | 0.014 - 2.0 | 76.5 | 61.0 |
| 16. BENZO[ghi]PERYLENE | 0.0030 - 0.50 | 0.011 - 1.7 | 76.5 | 61.0 |
| 17. INDENO[1,2,3-cd]PYRENE | 0.0090 - 0.20 | 0.027 - 0.66 | 91.6 | 36.5 |

* Data from [4].
† Data from [5].

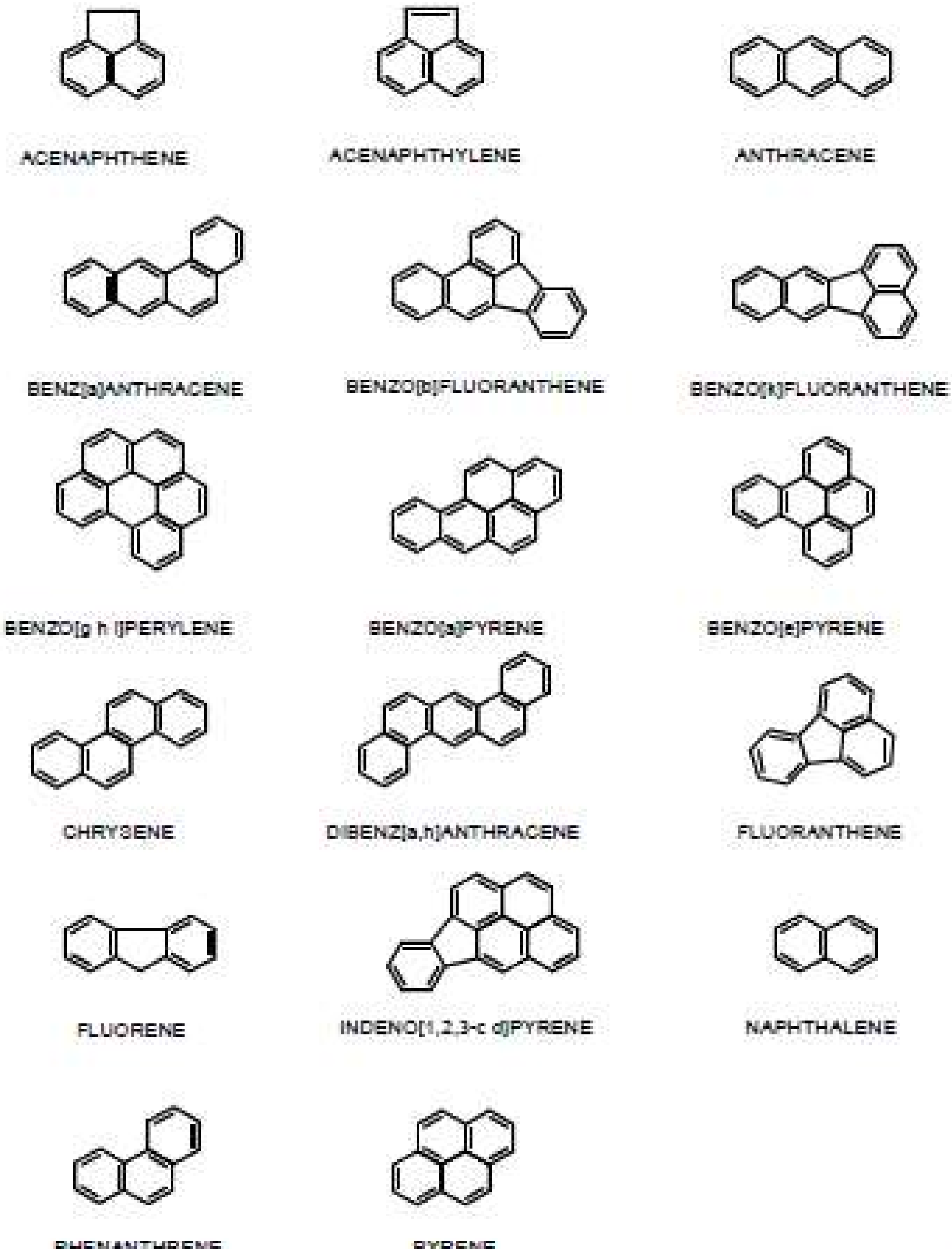


Figure 1. Structure of the PAHs.

**Annexure III**

# LIST OF PUBLICATIONS

1. Sakshi, Singh, S.K., Haritash, A.K. (2018). Environmental Biotechnology for Control of Environmental Pollution. *Int J Adv Res* 6(11), 816-819. DOI:10.21474/IJAR01/8064.

2. Sakshi, Singh, S.K., Haritash, & A.K. (2019). Polycyclic aromatic hydrocarbons: soil pollution and remediation. *Int J Environ Sci Technol*. https ://doi.org/10.1007/s1376 2-019-02414 -3.

3. Sakshi, & Haritash, A.K. (2020). A comprehensive review of metabolic and genomic aspects of PAH- degradation. *Arch Microbiol*. https://doi.org/10.1007/s00203-020-01929-5.

4. Sakshi, Singh, S.K., & Haritash, A.K. (2020). Evolutionary Relationship of Polycyclic Aromatic Hydrocarbons Degrading Bacteria with Strains Isolated from Petroleum Contaminated Soil Based on 16S rRNA Diversity. *Polycycl Aromat Compd*, DOI: 10.1080/10406638.2020.1825003.

5. Sakshi, Singh SK, Haritash AK (2021). Catabolic enzyme activities during biodegradation of three ring PAHs by novel DTU-1Y and DTU-7P strains isolated from petroleum contaminated soil. *Arch Microbiol*. https://doi.org/10.1007/s00203-021-02297-4.

6. Sakshi, Singh, S.K., Haritash, A.K. (2021). Catabolic enzyme activity and kinetics of pyrene degradation by novel bacterial strains isolated from contaminated soil. *Environ Technol Innov*. https://doi.org/10.1016/j.eti.2021.101744.

**Annexure IV**

# CURRICULUM VITAE

**Name:** Sakshi
**Date of Birth:** 14th December 1990

## ACADEMIC QUALIFICATIONS

| Year | Degree/ Certificate | Institute/ Board | CGPA / % |
|---|---|---|---|
| 2013-2015 | M. Tech, Bioinformatics | Delhi Technological University (Formerly DCE) | 8.46/10 |
| 2012 | B. Tech, Biotechnology | National Institute of Technology (NIT), Jalandhar | 7.84/10 |
| 2008 | Twelfth Grade | C.B.S.E. | 83.25% |
| 2006 | Tenth Grade | C.B.S.E. | 92.4% |

**Presentations made in International conferences**

- Sakshi, Singh SK, Haritash AK (2019) Effect of petroleum hydrocarbons on Soil properties and its biotransformation. Sustainable Technologies for Environmental Management (STEM-2019), DTU, 25th -26th March, 2019
- Sakshi, Singh SK, Haritash AK (2018) Polyaromatic hydrocarbons: soil pollution and bioremediation. Go Green summit at Manila, Philippines, 23rd -24th March, 2018
- Sakshi, Singh SK, Haritash AK (2017) Environmental Biotechnology for Control of Environmental Pollution. International conference on Emerging Areas of Environmental Science and Engineering (EAESE-2017), GJU Hisar, 16th -18th February.

**Presentations made in National conferences**

- Sakshi, Singh SK, Haritash AK (2018) Microbial degradation of polythene: opportunities & challenges. National conference on Beating the Plastic Hazard: Challenges & Strategies, 4th June 2018.

## TRAINING UNDERTAKEN

- 6 week Industrial training in IGIB (Institute of Genomics and Integrative Biology, CSIR),Delhi on Project Title-"Expression and purification of fungal recombinant allergen",
  Guide: Dr. Naveen Arora, Scientist E II May-July,2011
- Industrial training in Northern Railway Hospital, New Delhi, Work: Immunology, Microbiology, Biochemistry
  Guide; Dr.Meenakshi Agarwal May-July,2010

## PROJECT

- 6 month project in IGIB (Institute of Genomics and Integrative Biology, CSIR),Delhi on Project Title-"Comparative genomic analysis and functional annotation of different strains of *Trichophyton rubrum*".

  Guide: Dr. Bhupesh Taneja, Senior Scientist Jan-June,2015
- M.Tech Project- "Analysis of Expression level of NKG2D in different cancers and study comparative binding of NKG2D receptor with different NKG2D ligands".

  Guide: Dr.Asmita Das Jan 2014-Dec 2014
- B.Tech Project- "Extraction and characterization of potent microbe from soil sample that can degrade pesticide Chlorpyrifos".

  Guide: Dr. Shailu Dalal July 2011- May 2012

## ACHIEVEMENTS

- Awarded DTU Fellowship for Ph.D. in 2016.
- Qualified GATE (Biotechnology) in 2016.
- Qualified GATE (Biotechnology) in 2013, Awarded MHRD Fellowship.