Characterization of Benzo[a]pyrene and Pyrene Degrading Bacterial strains Isolated from Hydrocarbon contaminated Sites

SUMMARY OF THE THESIS

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SUMMARY

Polycyclic aromatic hydrocarbons (PAHs) are organic contaminants that play a major role in environmental pollution due to their large-scale distribution and inherent toxic physicochemical properties. The major hydrocarbons are persistent and cause a toxic effect on the biological system due to their covalent bond formulation with genetic material (DNA and RNA) and proteins. This results in nucleic acid structural mutations and also chromosomal damage. The specific properties of PAHs are low bioavailability, hydrophobicity, and thermodynamic stability; this feature inhibits the degradation efficiency of these pollutants. Besides, the few metabolic products of PAHs, such as epoxides, are more toxic than their parent compound, which can cause a lethal effect on a biological system. Therefore, several PAHs are reported to be mutagenic, carcinogenic, and genotoxic because of their above-mentioned characteristics. The carcinogenicity of PAHs increased with an increase in their molecular weight. Hence, various PAHs compounds, pyrene, benzopyrene, benzo anthracene, and dibenzo anthracene can show increasing toxic effects due to the increase of their molecular weight. Anthropogenic activities are major sources of release of PAH in the surrounding environment (air, water, and soil). They are generated due to their incomplete combustion of various fossil fuels. PAHs are used in the combustion of engines (diesel and petrol), power stations, and industrial, domestic, and gas manufacturing plants, which is the main source of PAH emissions. When hydrocarbons and their crude compounds come in contact with the surface and environment, these compounds evaporate, disperse, and adsorb into suspended particles and a few get photo-oxidized also. Therefore, PAHs pose a severe threat to the environmental and human health of their bioremediation and detoxification from the contaminated site.

Pyrene and benzo[a]pyrene is a group of chemical polycyclic aromatic hydrocarbons (PAHs) having 4 and 5 fused aromatic rings respectively, and are ubiquitous environmental pollutants of natural biogenesis and anthropogenic processes and they are highly persistent. Pyrene and benzo[a]pyrene are also characterized as the priority pollutants of 16 PAHs listed by USEPA in 1983. The contact with pyrene, benzo[a]pyrene causes lung disease, nephrotic syndrome, liver

damage, mutagenic, carcinogenic, and genotoxic. Therefore, it is very important to reduce the pyrene and benzo[a]pyrene and its relevant hydrocarbons from the environment. Proteobacteria and Actinobacteria are reported as the most abundant, potential, and diverse group of bacteria for pyrene degradation from the PAHs contaminated site. Among the proteobacteria, alpha-proteobacteria are more abundant than beta and gamma-proteobacteria. Certain bacterial species are also reported to convert toxic PAHs into non-toxic metabolites and minerals because they use these compounds as energy sources required for their multiplication and growth. Several microorganisms are resistant to hydrocarbon compounds due to their versatile nutritional properties. They have also reported bioaccumulation and biomagnifications as special features of biomagnifications.

But, the rapid global industrialization, oil spilling and petroleum refinery plants created adverse effects on the environment. This has caused an environmental imbalance and has been caused by climate change and global warming. The conventional methods of wastewater treatment and land do not meet the required standards of the environment for waste disposal. Therefore, researchers have attempted in the past few decades to develop novel technologies for the removal of hazardous and complex pollutants from the discharge of petroleum and hydrocarbon waste.

But, yet suitable technologies are still warranted. However, to adapt to the microbial population in the environment, some potential microbial species have been reported for bioremediation of the polluted environment for eco-restoration due to their specialized cellular structure and adaptation. Keeping in view these polluting characteristics, bioremediation has been reported as a green-polluting, feasible, and novel technology for the eco-restoration of hydrocarbon-polluted sites. Due to these properties, aromatic and metallic complex compounds of hydrocarbon waste persist for several years in the environment and cause toxicity to flora and fauna. Since the bioremediation of hydrocarbon waste is regulated by the specific bacterial community present in the hydrocarbon-contaminated soil and water areas. But, the detailed nature of bacterial communities growing around hydrocarbons and PAHs polluted sites is not much known so far.

Hence, the present study has focused on the role of pyrene and benzo[a] pyrene-degrading bacteria during bioremediation which were isolated from petrochemical plant effluent and hydrocarbon-contaminated soil. These properties of bacteria for specific aromatic hydrocarbons (pyrene and benzo[a]pyrene) degradation may be utilized to remove and detoxify waste hydrocarbons for eco-restoration of a polluted environment. The present thesis is divided into eleven chapters. The first chapter is an introduction to the topic. This chapter has imbibed the background information and rationale of the title of the thesis and this information has been described in detail in this chapter. In the second chapter, global and national states of the art of the problems have been reviewed to understand the problems with the development of new technologies. The chapter has also highlighted to understand the problem of pyrene and benzo[a]pyrne, hydrocarbon waste i.e. organic pollutants, heavy metals, and their environmental impact. Some bacterial species and consortiums have also been reported for their ability to degrade the various pollutants in hydrocarbon waste. The degradation abilities of various bacteria have been reported in this chapter. The roles of some bacteria for bioremediation potential in different conditions are also mentioned. Though various attempts have been reported for the degradation of hydrocarbon waste in laboratory stages by using different bacterial consortiums, all these studies have been reported at very low concentrations of pyrene and beno[a]pyrene. Therefore, the present study has focused on aerobic, anaerobic, and metabolic pathways of pyrene and benzo[a]pyrene, at high concentrations for feasibility of technology by bacteria.

Further, chapter three has focused on five objectives as per the topic of the thesis. Chapter fourth has focused on the analysis of the physicochemical properties of different collected hydrocarbon waste. The study focused on the organic pollutants by using GC-MS (gas chromatography-mass spectroscopy) techniques and other metals by using AAS methods, BOD(biological oxygen demand), COD (chemical oxygen demand), phosphate and all by using different techniques were identified which were disturbed according to CPCB(central pollution control board) limits. Different unknown recalcitrant complex by using FTIR analysis which, are a major source of environmental pollutants and health hazards functional group are identified and in this technique functional group accessed on 3500-700 cm -1 wavenumber and in GC-MS analysis [1] Benzopyrano[4,3-c] isoquinoline-5,12(11H)-dione, 2-

Benzylidene-3-oxo-4-(octylsulfanyl)-2,3-dihydrothiophene-1-dioxide, 1-Nitro-9-hydroxy-10-methyl-perhydro-naphthalene, Phthalic acid ,cyclohexyl pentyl ester, Hexamethyl-hexahydro-anthracene or phenanthrene, 1,2-bibenzoylbenzo[e]indolizine, and 3,7,13,17-tetraethyl-2,8,12,18-tetramethylporphyrin in soil sample and Silane ,(1-cyclohexene-1-yloxy)trimethyl-(CAS) , 1,2-benzodicarboxylic acid, Benzene 1,2,4-triol tri(trimethyl silyl) and Dihydro-1,2-hydroxy-4-propy 1,6,6,11-trimethyl-2H,6H,12H-benzo tripyran-2-one were found in petrochemical effluent plant; which were prominent organic compounds in the category of mutagenic and androgenic compounds characteristics, detected in both sample. In the soil sample, hydrocarbons contained were high as compared to the liquid sample in my research.

In addition, the presence of various activities in germinating seeds at different concentrations of both samples (in petrochemical effluent conc. are 10, 25, 50, 75,100 % and soil conc. are 50, 100%) indicated the contribution of toxic properties of hydrocarbons, as resulting in a phytotoxic effect on seed germination with mung. Mung seeds were not germinated due to the high concept of heavy metals and aromatic hydrocarbons present in samples.

The fifth chapter describes the isolation, screening, and characterization of most potential benzo[a]pyrene, pyrene degrading bacterial strains. The findings of the study revealed that pyrene and benzo[a]pyrene i.e. *Staphylococcus aureus (LOP-9)* and *Mycobacterium vanbaalenii* (GWP-2) for the degradation of pyrene, which were pyrene degraded upto 2000ppm whereas, *Stutzerimonas stutzeri* (LOBP19A) bacteria, which was benzo[a]pyrene degraded up to 1000ppm from two different collected sample sites. Its degrading metabolic products were identified by GCMS and degradation of pyrene and benzo[a]pyrene analyzed by HPLC, which is LOP-9 and GWP-2 bacteria have 99.3% and 97.9% respectively, LOBP-19A has 87.5% degrading abilities. Optimization (temperature, conc. of pyrene and benzo[a]pyrene, NaCl conc., pH, carbon and nitrogen sources) and screening of enzymes (MnP, Laccase, and Catechol dioxygenase) were also done. These bacteria showed the potential for pyrene and benzo[a]pyrene degradation which has supported the bacterial-assisted other heavy metal and various polycyclic aromatic hydrocarbons degradation. These bacteria may be used as biotechnological tools for eco-friendly and cost-effective polluted sites of aromatic waste as a green technology.

The sixth chapter showed the metagenomic analysis of the microbial profile of benzo[a]pryene, pyrene degrading bacterial strains, and molecular analysis of the isolated pyrene and benzo[a]pyrene degrading bacterial strains. The sequence analysis of the 16s rRNA V3–V4 hypervariable region with the Illumina MiSeq platform showed sample 1 liquid was 69299 and sample 2 solid was 38638 in number of OTUs sequences read derived from two different samples respectively. The major genus detected in sample 1 liquid sample was uncultured-*bacillus* (40%), *paracoccus*, and *pseudomonas* (14%). In the sample 2 solid were *prevotella* 24%, *bacillus* 15%, and *paracoccus* 14%. Our results suggested that pyrene and benzo[a]pyrene degrading bacterial communities associated with *Staphylococcus aureus* (LOP-9) and *Mycobacterium vanbaalenii* (GWP-2) and *Stutzerimonas stutzeri* (LOBP19A) were substantially different in richness, diversity and relative abundance of taxa compared to both samples. The metagenomic functional pathway that is the biosynthesis of amino acids, carbon metabolism, methane metabolism, arginine, and proline, TCA cycle were abundantly presented in both samples. The comparative study was also described between liquid and solid samples and found that various unique bacterial genera were presented in both samples which helped in the degradation of hydrocarbons and can help in designing appropriate biodegradation of aromatic compounds studies for eco-restoration of polluted sites.

The seventh chapter has focused on the characterization of benzo[a]pyrene and pyrene degrading enzymes and the analysis of metabolic products by HPLC and GC-MS. The enzymatic assays were performed for the degradation of benzo[a]pyrene and pyrene by using isolated bacterial strains and degrading enzymes present in the extracellular and intracellular crude extracts. These extracts, named EE and IE, respectively, were obtained from cultures of *Staphylococcus aureus* (LOP-9) and *Mycobacterium vanbaalenii* (GWP-2), and *Stutzerimonas stutzeri* (LOBP-19A). MnP (Magnese Peroxidase) assay (EC 1.11.1.13), Laccase assay (EC 1.10.3.2), and Catechol 2,3- dioxygenase assay (EC 1.13.11.2) are produced by these isolated bacteria and identified by using GC-MS analysis, the chemical name of enzymes described in GCMS NIST library which have given in GCMS table of metabolites of bacterial isolates. Isolated bacteria degrade pyrene and benzo[a]pyrene due to the secretion of these enzymes.

The eighth chapter has focused on the development of the bacterial consortia for the degradation of benzo[a]pyrene, pyrene from hydrocarbon waste. *Mycobacterium vaanbaalenii* GWP-2 (ON715011) *Staphylococcus aureus* LOP-9(ON715121) and *Stutzerimonas stutzeri* (LOBP-19A) OP389146 total three bacteria were selected for the development of two bacterial consortia with the suitable conc. of pyrene and benzo[a]pyrene has given as carbon sources of bacteria and then checked degradation rate of pyrene and benzo[a]pyrene after 30 and 50 days respectively by using HPLC and degraded metabolites were identified by using GC-MS techniques. The degradation rate was compared with control as a standard of benzo[a]pyrene(1000ppm) and pyrene(2000ppm). We found that benzo[a]pyrene was 99.62% and 93.8% pyrene degraded by bacterial consortia. In LOP-9 and GWP-2 consortia, Naphthalene, Cyclohexane 1,3,5-trimethyl-2-octadecyl, 1,4-benzodicarboxylic acid, Terephthalic acid, 9-octadecenoic acid, Bisphenol , 9,12-octadecadienoic acid, Silane diethyldecylcloxydodecyloxy and Diethyl (pentafluorobenzyloxy) tetradecycloxy and second LOBP-19A and LOP-9 degrading metabolites as 1,2,3,4-tetramethyl-5-(chloromethyl)benzene, 5,6,7,8-tetrahydro-8,8-dimethyl-2-indolizinecarboxylic acid methyl ester, Butanedioic acid (succinic acid), Benzo[a]pyrene-1,6-dione, Benzene,[3-chloro-2-propenyl)oxy]-(CAS), 3-acetoxy-3,7-dimethyloocta-1,6-diene, t-butyl 3-(3-methyl-1-butenoxy) propanoate, 2,5-bis(bromomethyl)-1,4-dihexylbenzene, 1-nitro-4-octanol, 3-(6,6-dimethyl-5-oxohept-2-enyl)-cycloheptanone, Propenoic acid, 1-heptacosanol, Hexadecane,2-methyl-(CAS), Heptadecanoic acid,dimethyl ester, 6,7-dimethoxy-3,4-dihydroisoquinoline-N-oxide, Tridecanoic acid, trimethyl ester, 1,2-dimethylpropyl trifluoroacetate, 1,2-dibenzoylbenzo[e]indolizine, Benzeneacetic acid, 2,3-bis [trimethylsilyl]oxy propyl stearate, Dithioerythritol, 3,5,7-tri(trimethylsiloxy)-2-[3,4-di(trimethylsiloxy)phenyl]-4H-1-benzopyran-4-one and 5[4-(acetylthio)butyl]-15butyl-10,20-diphenylporphyrin were identified by using GC-MS techniques. Functional groups of both consortia were also identified by FTIR. That is C-H Stretching (alkenes, alkanes), C-O carboxy esters, ethers, aromatics, alcohol ether, and C-O bond alkanes, a hydroxyl group(O-H). So this study may play an important role in other PAHs degradation and these bacterial consortia may be useful for pyrene and benzo[a]pyrene degradation. Thus, the present study gives a prominent idea about the promising PAHs degrading bacteria under environmental conditions are a very cost-effective and environmentally sustainable development.

Chapter Nine has summarized the whole thesis work and findings with research output systematically. Chapter ten has compiled all the cited references for the thesis which are relevant to the topic and they are cited in each chapter. Each chapter has cited recent references based on methodology and results. The available references are cited on biodegradation of pyrene and benzo[a]pyrene and other PAHs compounds and degrading enzymes and their degrading metabolites, impacting soil, water, and plant growth reported at polluted sites. The complete thesis has 316 cited references.

The last chapter has listed all the scientific output based on the thesis work. There are two original research papers published in a peer-reviewed journal of international repute. One review paper is also published based on the topic of the thesis and has been published in an international journal. A certificate of participation and oral presentation award has been provided at international conferences.