Studies on Biodegradation of High Molecular Weight Polycyclic Aromatic Hydrocarbons by Bacteria and Characterization of Metabolites and Genes

Thesis
Submitted to
Academy of Scientific and Innovative Research
For the Award of the Degree of

Doctor of Philosophy
In
Science

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2019

Preface

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Pollutants released into the environment through both natural and anthropogenic activities cause threat to human health and environment. Toxic chemicals enter in the environment and food chain through oil refineries activities, industrial effluents, sewage wastes, agricultural wastes and automobiles that cause health hazards. Different physical and chemical methods such as oxidation, photochemical reaction, incineration and mechanical methods have been used for remediation of toxic compounds but these are not cost-effective, not eco-friendly, need more energy and even produce more toxic compounds. Degradation of such toxic compounds has become a challenge to remove these from the environment with suitable techniques. The use of microorganisms is a good approach for removal of recalcitrant compounds in an ecofriendly manner. Aerobic and anaerobic bacteria, fungi and microalgae have unique properties to utilize polycyclic aromatic hydrocarbons (PAHs) as a sole source of carbon and energy. Bacteria available in the natural environment need to be stimulated for enhancing their degradation ability.

Chapter 1 reviews the literature on PAHs and other pollutants which exist in the environment, their structure, source and distribution. Anaerobic, aerobic bacteria and their genes involved in PAHs degradation are reported.

Chapter 2 describes the isolation and identification of bacteria responsible for degradation of polycyclic aromatic hydrocarbons from Mathura oil contaminated soil of Mathura oil which contains various toxic hydrophobic compounds such as PAHs and Lucknow tyre dump site soil. Bacteria for PAHs degradation were identified and characterized on the basis of morphological and biochemical characteristics. A total of five PAHs degrading bacteria were isolated by spray plate clearance assay method in

presence of pyrene and phenanthrene. Bacteria were identified on the basis of 16S rRNA phylogeny, namely, *Ochrobactrum anthropi*, *Pseudomonas mendocina*, *Microbacterium esteraromaticum*, *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. Growth of bacteria was optimized in the pH range 4.0 to 7.5 in presence of phenanthrene as the sole carbon source for further studies.

Chapter 3 describes the degradation of HMW-PAHs (phenanthrene, pyrene and benzo(a)pyrene) degradation by *Stenotrophomonas maltophilia* IITR87 and *Ochrobactrum anthropi* IITR07. Degradation was enhanced upto 25 to 60% in presence of chemical surfactant Triton X-100 as compared to biosurfactant rhamnolipid. Four metabolites each of pyrene and phenanthrene by *S. maltophilia* IITR87 and *O. anthropi* IITR07 respectively was detected by GC-MS/MS on the basis of their retention time and mass spectrum.

Chapter 4 describes the presence of PAH degrading genes in *S. maltophilia* IITR87 and *O. anthropi* IITR07. Occurrence of multi-component naphthalene dioxygenase (*nid*) genes, phenanthrene degrading (*phd*) genes, and phthalate degrading (*pht*) genes involved in complete degradation of HMW-PAHs such as phenanthrene and pyrene was amplified. The gene sequences were submitted to NCBI GenBank with accession numbers KM379097 (*nid*A), KM379098 (*nid*B), KM387408 (*nid*D), MH355577 (*pht*Aa) and MH355578 (*pht*B). Localization of *nid*A gene was also confirmed by Southern hybridization.

Chapter 5 describes the application and development of bacterial consortium using five different species of bacteria for bioremediation of PAHs from oily sludge / crude oil contaminated soil. Acute toxicity of crude oil at different concentrations (0.25, 0.50, 1.0 1.5 and 2.0%). was evaluated in *Eisenia fetida* for 14 days. Hundred percent mortality

was observed at 1.5 and 2.0% concentrations of crude oil. Based on the results of acute toxicity an experiment was designed for determining long term exposure for ecotoxicity study. Soil microcosm study using consortium of bacteria amended with earthworms exposed to 1% crude oil in six different groups was performed for 45 days.

Summary

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The present study focussed on isolation and identification of bacteria involved in degradation of high molecular weight (3-5 rings) polycyclic aromatic hydrocarbons. Soil samples collected from two different locations, namely, Mathura refinery and a tyre waste dump site, were used for screening bacteria involved in degradation. Based on a functional, substrate clearance methods on minimal medium agar using the target substrate, five different bacteria were isolated. These degradative bacteria were identified on the basis of 16S rRNA gene sequence analysis and identified as *Ochrobactrum anthropi* IITR07, *Pseudomonas mendocina* IITR46, *Microbacterium esteraromaticum* IITR47, *Pseudomonas aeruginosa* IITR48 and *Stenotrophomonas maltophilia* IITR87. On the basis of morphological and biochemical characteristics, the gram negative strains identified were IITR07, IITR46, IITR48, and IITR87, while strain IITR47 was gram positive. The 16S rRNA gene sequences of bacteria were submitted in NCBI where showed 99% similarity to those available in Genbank and the culture was deposited in Microbial Type Culture Collection (MTCC) and GenBank, Institute of Microbial Technology, Chandigarh. These five PAHs degrading bacteria were tested in the pH range 4.0-7.5 to optimize and find out the ability of the bacteria to biodegrade under diverse conditions.. All the isolates showed better growth at pH 7.5 except IITR47 which showed optimum growth at pH 6.5. These five isolates were used to develop a consortium of bacteria and used for further studies as described in the thesis.

An attempt has been made in this study to characterize metabolites and elucidate degradative pathway of PAHs and bacteria involved in degradation of PAHs. In particular, the metabolites were identified for the degradation of phenanthrene and

pyrene by the using *Ochrobactrum anthropi* IITR07 and *Stenotrophomonas maltophilia* IITR87 respectively.

Stenotrophomonas maltophilia IITR87 was found to grow and degrade using pyrene as a sole source of carbon and energy. Strain IITR87 used 0.38 mM of pyrene in 25 days and attained OD_{600} 0.35 in presence of 0.49 mM pyrene. Metabolites obtained from degradation of phenanthrene and pyrene was identified using gas chromatography and mass spectrometry. Four metabolites each were identified on degradation of phenanthrene by *O. anthropi* IITR07 and pyrene degradation by *S. maltophilia* IITR87. Partial phenanthrene and pyrene pathways were elucidated which suggest that various enzymes present in these bacteria help in biodegradation. Aqueous solubility of PAHs was found to be negligible because of the hydrophobic property of the compounds, and their solubility tends to decrease on increase in molecular weight. The solubility of phenanthrene, pyrene and benzo(a)pyrene increased as the concentration of chemical surfactant, triton X-100 and biosurfactant, rhamnolipid increased. On this basis 1232 µM and 928 µM (CMC) concentrations of both the surfactants were taken for further degradation studies. Degradation rate of phenanthrene was enhanced (there was an increase of 25 to 60%) in presence of triton X-100 than in the presence of rhamnolipid. The results indicate that these surfactants may be useful in enhancing bioavailablity of these compounds to degradative bacteria involved in bioremediation of PAHs from contaminated sites.

Genes present in bacteria for PAH, particularly for phenanthrene and pyrene degradation was investigated. Genetic studies revealed that naphthalene dioxygenase large subunit (*nid*A), dioxygenase small subunit (*nid*B) and dehydrogenase (*nid*D) enzymes were involved in initially activating pyrene and phenanthrene to form

dihydrodiol metabolites. Further, dihydrodiol compounds was found to undergo conversion into cis 4,5- dihydroxypyrene. *phd*F and *phd*I gene coding for extradiol dioxygenase and 1-hydroxy-2-naphthoate dioxygenase respectively was PCR-amplified resulting in the product size of 891 bp and 1086 bp in *Stenotrophomonas maltophilia* IITR87 and *Ochrobactrum anthropi* IITR07 respectively. Amplification of phthalate dehydrogenase (*pht*Aa) and phthalate dihydrodiol dehydrogenase (*pht*B) genes was also seen in *Stenotrophomonas maltophilia* IITR87 of expected product size 1209 bp and 440 bp indicating their activities in lower metabolic pathway. The *pht*Aa and *pht*B gene sequences showed 99% similarity with the genes present in *M. vanbaalenii* PYR-1 for pyrene degradation. Amplification of expected product sizes of extradiol dioxygenase (*phd*F) and 1-hydroxy-2-naphthoate dioxygenase (*phd*I) genes in *S. maltophilia* and *O. anthropi* confirmed the presence of phenanthrene degrading genes, leading to the formation of lower pathway metabolites of phenanthrene biodegradation.

Developing and optimizing a bacterial consortium which is able to biodegrade PAHs in liquid medium and in soil microcosm was studied in this thesis. As a prerequisite for field remediation process, ecotoxicity assessment using *Eisenia fetida* was evaluated. In microcosm, bacterial consortium formed contained all the five bacteria isolated and identified namely *Ochrobactrum anthropi*, *Pseudomonas mendocina, Microbacterium esteraromaticum*, *Pseudomonas aeruginosa,* and *Stenotrophomonas maltophilia*. In the microcosm study, the bacterial consortium augmented with rhamnolipid might prove to be significant in facilitating the large-scale remediation of oil sludge contaminated sites, as compared to treatment with individual bacterium. Here, 97% degradation of naphthalene was achieved by the consortium of bacteria in 45 days, while 80% degradation was obtained by *Microbacterium esteraromaticum* in 45 days.

The soil microcosm studies were performed in six different groups (G1-G6) with bacterial consortium treated with 1% crude oil amended with earthworm, *Eisenia fetida* for 45 days. Seven selected PAHs, namely, naphthalene, acenaphthylene, phenanthrene, fluoranthene, pyrene, chrysene and benzo(a)pyrene were degraded by bacterial consortium. On day 15, application of earthworms along with microbial consortia spiked crude oil (G3) showed an increased degradation pattern (10-64%) of selected PAHs. On day 30, 20-89% PAHs degradation was observed in crude oil spiked soil amended with earthworms and bacterial consortium (G3). Similar degradation pattern was observed in application of earthworms in crude oil spiked group (G4) on day 15 and 30. 35-99% degradation of PAHs was observed in crude oil spiked soil amended with earthworms and bacterial consortium (G3) on day 45. Biosurfactant added crude oil with microbial consortia (G5) resulted in 31-97% degradation of selected PAHs. It was evident from the soil microcosm study that the percent degradation of selected PAHs was directly proportional to the duration of exposure. Minimum 45 days incubation is recommended to degrade the PAHs under laboratory conditions.

The aim of the present study was to develop a bacterial consortium which could remediate the oily sludge from the contaminated sites. The role of metabolites and genes involved in biodegradation has to be taken holistically, before undertaking large scale biodegradation studies. Thus, the overall results suggest that microbial degradation is a tool to develop an efficient, cheap, and eco-friendly method for safeguarding environment by restoration of contaminated, hazardous sites.