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Characterization and Evaluation of Polycyclic Aromatic Hydrocarbon (PAH) Degrading Bacteria Isolated from Oil Contaminated Soil

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants and biodegradation using microorganisms is the preferred and major route of PAH removal from contaminated environments. This study investigated the bacterial degradation of petrol and diesel in liquid media that were isolated from oil contaminated soils by enrichment technique. The isolates could use petrol and diesel as their sole carbon and energy source in Bushnell Hass Mineral Salts (BHMS) medium at 2% (v/v) concentration. A total of eight isolates were selected and characterized by using a variety of phenotypic and morphologic properties. Two isolates each showed highest growth in petrol and diesel containing media during screening were selected and characterized using 16S RNA sequencing. Molecular identification of the isolates assigned them to *Achromobacter* sp. and *Pseudomonas aeruginosa*. The selected isolates degraded petrol and diesel up to 31.9% and 34.4% respectively. This study indicates that the contaminated soil samples contain a diverse population of PAH-degrading bacteria and the use of *Achromobacter* sp. and *Pseduomonas aeruginosa* has the potential for bioremediation of PAH contaminated sites.

Keywords: Biodegradation; Polycyclic aromatic hydrocarbons; *Achromobacter*; *Pseudomonas*; Petrol, Diesel degradation

Introduction

Hydrocarbons play a special role amongst the contaminants polluting the environment due to their wide-scale distribution and hazardous physicochemical and biological properties. Hydrocarbons enter into the environment through waste disposal, accidental spills, losses during transport and storage. Polycyclic aromatic hydrocarbon (PAHs) compounds are among the most toxic components to plants and animals [1,2]. Petroleum hydrocarbons pose as a globally environmental pollutant [3] because of their hydrophobic nature and low volatility [4]. Petroleum constituents such as diesel oil are carcinogenic, mutagenic and potent immunotoxicants [5,6] Despite volatilization, leaching, chemical and photo oxidation are often effective in reducing the environmental level of PAHs [7], bioremediation using biological processes to ameliorate hydrocarbons from environment involves no secondary contamination and offers an effective technology for the treatment of oil pollution [8]. Besides, physicochemical treatments to the remediation of hydrocarbons are expensive and laborious [9].

Bioremediation of soil contaminated with petroleum oil has been commonly described globally [10-13] and is usually the preferred and major route of PAH removal from contaminated environments. Hydrocarbon degradative process is widely distributed among numerous genera or taxa [14] and biodegradation of hydrocarbons using microorganisms has been established as an efficient, economic and versatile approach [15-19]. Microorganisms degrade hydrocarbon contaminants and utilize the resulting compounds as nutrients and energy sources for growth and reproduction [20] and are depending on the chemical nature of the compounds and on environmental determinants [21]. The low aqueous solubility of PAHs makes them poorly available for microbial utilization [22] and identification of novel bacteria for the biodegradation of PAH in oil contaminated area is the need of the hour. The main objective of the present study was to characterize PAH degrading bacteria inhabiting the oil contaminated sites. From the samples, bacterial strains able to degrade petrol and diesel were isolated and characterized. Among the isolates, two efficient strains were selected and their degradation capacities of PAH compounds were evaluated.

Materials and Methods

Study cites

Samples of soil for this study were collected from two cites; oil station in Bangalore (13.04°N, 77.64°E) and Mumbai (19.39°N, 72.84°E).

Sample collection

Subsurface soils contaminated with petrol and diesel oil were picked from study sites in pre-sterilized sample bottles, labelled and transported. The samples were stored at -20°C in the Biotechnology laboratory at the Indian Academy Degree College, Bangalore.

Isolation and enrichment of PAH degrading bacteria

For culture enrichment, 1 g soil was inoculated into 100 ml of Bushnell Hass Mineral Salts (BHMS) medium ($MgSO_4$.$7H_2O$ - 0.2 g, $CaCl_2$- 0.02 g, KH_2PO_4- 1.0 g, K_2HPO_4 - 1.0 g, NH_4NO_3 - 1.0 g, 2 drops of 60% $FeCl_3$, pH - 7.0) supplemented with 0.5% petrol and diesel (v/v) as the sole carbon source. The mixture was incubated at 37°C at 130 rpm for 5 days. Bacteria from this culture were enumerated by total plate count method using serial dilution technique on BHMS agar medium supplemented with petrol and diesel. The

plates were incubated aerobically at 37°C for 48 h. The criterion for selection of petrol and diesel degrading strains was enhanced growth on plates with added test compound compared to the control plates without added substrate.

Phenotypic identification of the bacterial isolates

The bacterial colonies that grew on BHMS agar plates were sub-cultured on nutrient agar for pure culture preparation and identification. Eight bacterial isolates were identified on the basis of their colonial and cellular morphology; and biochemical characteristics. The colony colour, margin, form and elevation of the isolates were noted. Gram staining and biochemical tests including; indole, methyl red, Voges-Proskauer, catalase, oxidase, urease test, glucose fermentation and growth on King's media were also performed to identify the pure colonies. The pure colonies were preserved in glycerol broth (25% v/v) and for the day to day experiments, the bacteria were maintained on nutrient agar plates at 4°C in a refrigerator and sub-cultured at an interval of two weeks.

Screening of efficient PAH degrading bacteria

The efficacy of polycyclic aromatic hydrocarbon degradation was studied by gravimetric assay [23,24]. The bacterial isolates were inoculated into 100 ml of BHMS broth was added with 2% of the respective carbon sources (petrol and diesel) and the flasks were incubated for 5 days at 37°C at 125 rpm. After appropriate incubation, bacterial activities were stopped by adding 1N HCl. For extraction of oil, 50 ml of culture broth was mixed with 50 ml of petroleum ether:acetone (1:1) in a separating funnel and was shaken vigorously to get a single emulsified layer. Acetone was then added to it and shaken gently to break the emulsification, which resulted in three layers. The top layer containing petroleum ether mixed with oil and acetone was passed through anhydrous sodium sulphate followed by evaporation on a water bath. The gravimetric estimation of residual oil left after biodegradation was made by weighing the quantity of oil in a tarred beaker and the percentage of petrol and diesel oil degraded was determined as per the standard method [25]. All these screening experiments have done in triplicate. The average value of triplicate and standard error was calculated by Microsoft XL 2007. The isolates which showed better PAH degradation during the incubation were selected for a detailed analysis.

Genomic amplification of 16S rRNA

Genomic DNA was extracted from overnight grown bacterial cells using InstaGeneTM Matrix Genomic DNA isolation kit. The 16S ribosomal (rRNA) gene from the genomic DNA was amplified by PCR using the following primers; 5'- AGAGTTTGATCMTGGCTCAG-3' and 5'- TACGGYTACCTTGTTACGACTT-3' corresponding to the forward and reverse primers respectively. The amplification was done by initial denaturation of 94°C for 2 min followed by 35 amplification cycles of 94°C for 45 s, 55°C for 60 s; 72°C for 60 s and final extension at 72°C for 10 min in MJ Research Peltier Thermal Cycler. The purified PCR product was purified using Montage PCR Clean up kit (Millipore) and the product was sequenced using the 518F/800R primers.

DNA sequencing

Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). Single-pass sequencing was performed on each template using 5'-GGATTAGATACCCTGGTA-3' and 5'-CCGTCAATTCMTTTRAGTTT-3' primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Analysis of sequence data

The 16S rRNA gene sequence was compared with those from Genbank using n-BLAST program. A phylogenetic tree was constructed by the neighbour-joining method using MUSCLE 3.7 and PhyML 3.0 aLRT program [26]. For tree rendering, Tree Dyn 198.3 was used [27].

Results

Isolation of PAH degrading bacteria

Enrichment cultures initiated in BHMS medium containing 1% (v/v) petrol and diesel crude oil as the carbon and energy became turbid and the oil layer became clear indicating the degradation of the compounds. Colonies that have shown enhanced growth on BHMS agar plates added with petrol and diesel compared to control plates were selected. Five colonies from petrol degradation (P1, P2, P3, P4, P5) and three colonies from diesel degradation (D1, D2, D3) were picked and used for further characterization.

Phenotypic identification of PAH degrading bacterial isolates

The colony characteristics of the isolates are summarized in Table 1. The colonies were circular, smooth, elevated and 0.4-1.0 mm in diameter. All the strains were able to grow at 37°C and no pigmentation was observed. The cell morphology and biochemical characteristics are represented in Table 2.

Isolate	Size	Shape	Colour	Margin	Elevation	Pigmentation	Opacity
P1	0.5 mm	Circular	Green	Undulate	Convex	Negative	Translucent
P2	0.8 mm	Circular	Green	Undulate	Convex	Negative	Translucent
P3	0.7 mm	Circular	White	Lobate	Convex	Negative	Translucent
P4	0.4 mm	Circular	Green	Lobate	Convex	Negative	Translucent
P5	0.6 mm	Circular	White	Lobate	Convex	Negative	Translucent

D3	1.0 mm	Circular	White	Undulate	Convex	Negative	Translucent
D4	0.8 mm	Circular	White	Undulate	Convex	Negative	Translucent
D5	0.9 mm	Circular	White	Undulate	Convex	Negative	Translucent

Table 1: Phenotypic characters of the bacterial isolates.

Isolate	Gram reaction and cell shape	Catalse test	Oxidase test	Indole test	MR test	VP test	Glucose fermentation	Urease test	Growth on	
									King's B	King's A
P1	- rods	+	+	-	-	-	-	-	+	+
P2	- rods	+	+	-	-	-	-	-	+	+
P3	- coccobacilli	+	+	-	-	-	-	-	-	+
P4	- rods	+	+	-	-	-	-	-	+	+
P5	- coccobacilli	+	+	-	-	-	-	-	-	+
D3	- coccobacilli	+	+	-	-	-	-	-	+	+
D4	- coccobacilli	+	+	-	-	-	-	-	+	+
D5	- rods	+	+	-	-	-	-	-	-	+

Table 2: Gram's staining and biochemical parameters of the bacterial isolates.

PAH degrading ability of the isolates

Preliminary biodegradation assay was carried out to determine the PAH degradation capabilities of the indigenous bacterial isolates from oil contaminated environments. Petrol and diesel were added separately at 2% (v/v) to BHMS medium and the incubation was carried out for a period of 5 days for PAH degradation (Figure 1 and 2).

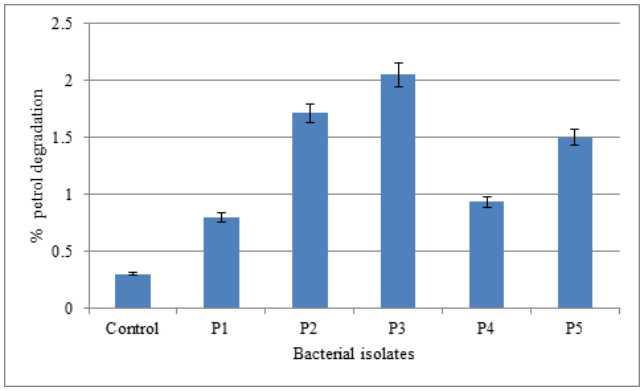

Figure 1: PAH degradation by the bacterial isolates during the five days of incubation in BHMS supplemented with petrol (2% v/v) at 37°C on a rotary shaker (125 rpm).

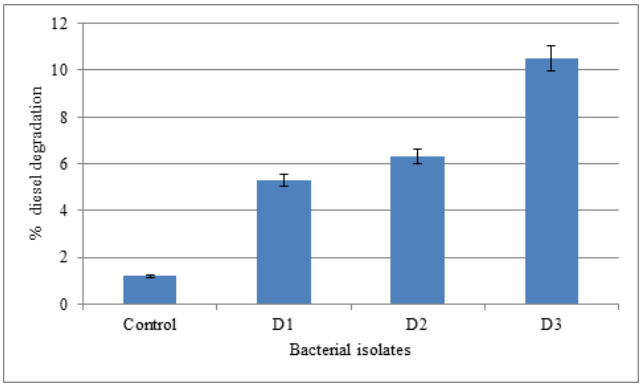

Figure 2: PAH degradation by the bacterial isolates during the five days of incubation in BHMS supplemented with diesel (2% v/v) at 37°C on a rotary shaker (125 rpm).

Isolates that exhibited highest degradation of PAH and were selected as efficient strains (P3 and D3) and the degrading ability was analyzed gravimetrically at 5, 10, 15 days of incubation period. The strain P3 was able to degrade petrol with increasing incubation period by recording maximum degradation (31.97%) at the end of 15 days incubation (Figure 3). The degradation rate was minimal till 10 days growth and the degradation rate was 88% increased at the end of incubation period. On the other hand, D3 strain exhibited higher degradation rate at 10th day growth period (34.4%). However, the degradation was significantly reduced with 8.16% degradation at the end of 15 days incubation which was lesser than 10 days degradation period (Figure 4).

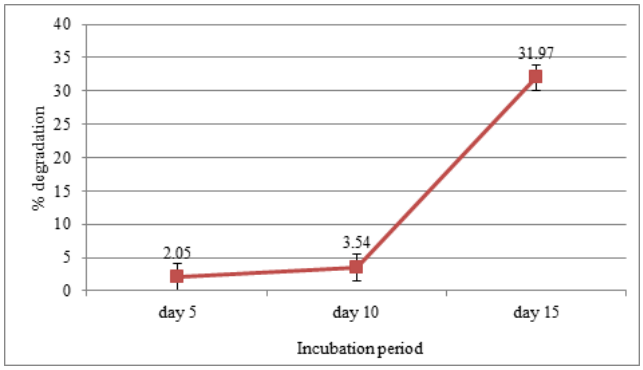

Figure 3: Petrol biodegradation percentage by the bacterial isolate (P3) during 15 days of incubation at 37°C on a rotary shaker (125 rpm).

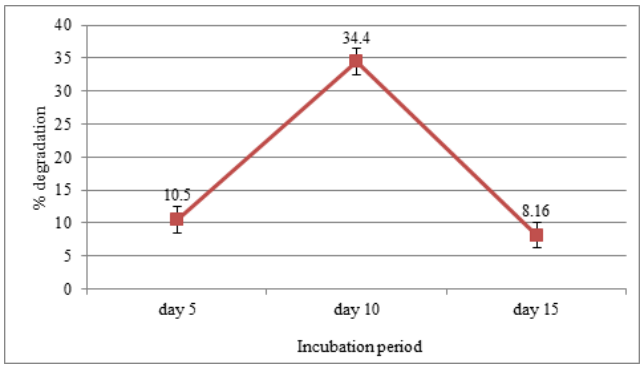

Figure 4: Diesel biodegradation percentage by the bacterial isolate (D3) during 15 days of incubation at 37°C on a rotary shaker (125 rpm).

Genotypic identification

The 16S rRNA gene sequences of P3 and D3 were accessed from the public GenBank data bases using the n-BLAST program. P3 which was Gram negative cocco bacilli was 94% identical to *Achromobacter*, suggesting that P3 was a strain of this species (Fig-5). D3 was Gram negative coccobacilli and 100% identical to *Pseudomonas aeruginosa* (Figure 6).

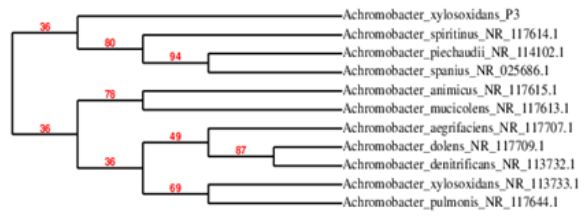

Figure 5: Phylogenetic tree constructed using neighbour joining method showing isolate P3 and its related *Achromobacter* sp.

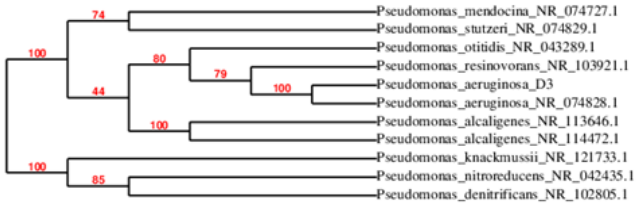

Figure 6: Phylogenetic tree constructed using neighbour joining method showing isolate D3 and its related *Pseudomonas aeruginosa*

Discussion

Polycyclic aromatic hydrocarbons (PAHs) contain two or more fused aromatic rings in linear, angular, or cluster arrangements [28,29] and are widespread organopollutants present in crude oil and fossil-fuel combustion products. Various new regulations have been introduced to control the environmental risks caused by petroleum products and research on remediation of contaminated soils is being increased [30]. The use of microorganisms to eliminate hydrocarbons from contaminated sites has a great potential [31-33]. Certain microorganisms use oil pollutants and microbial degradation of PAH has become the main mechanism for hydrocarbon derived pollution in the environment. Petroleum hydrocarbons are degraded not only by bacteria but by fungi, yeast and microalgae as well [34]. However, bacteria play an essential role in hydrocarbon degradation. In the present work, PAH degrading bacteria were isolated from soil in two different sites using enrichment culture procedure. A total of eight bacteria were isolated and preliminary screened for PAH degradation on BHMS media with 2% hydrocarbon as the sole carbon source individually (petrol and diesel). PAH degraders should use PAHs as sole carbon and energy source for use in bioremediation. This is important for minimizing the production of toxic, water-soluble degradation by-products and reducing the risk of isolates failing to survive at contaminated sites due to the lack of suitable growth substrates. Both the isolates from this research were able to grow well in the presence of petrol and diesel and exhibited significant degradation.

Novel molecular techniques have been extremely valuable in exploring the diversity of microbiota despite culture-based microbiological methods have provided important information about the microbial diversity [35]. The isolates were phenotypically identified and bacterial strains which exhibited relatively higher degradation were selected as most active strains. The tentative taxa and phylogenetic affiliation of the 16S rRNA of purified bacterial isolates were amplified by PCR and the bacterial 16S rRNA sequences were aligned with Blast search of NCBI databases. Partial 16S rRNA gene sequencing and database homology search for the isolates revealed their tentative close relationship to members of *Achromobacter* sp. and *Pseudomonas aeruginosa*.

Petroleum hydrocarbons can be degraded by microorganisms aerobically [36-38] and are readily isolated from oil contaminated sites [39]. Using natural populations of microorganisms for the removal of petroleum and other hydrocarbon pollutants from the environment is cheaper than other remediation technologies [40]. A wide range of hydrocarbon concentrations (0.5 - 6%) were used in biodegradation studies and varying growth was obtained [41-45]. In this study 2%

(v/v) was used as carbon and energy source for the degradation and based on preliminary screening of biodegradation, two native bacterial species namely *Achromobacter* and *Pseudomonas aeruginosa* were isolated. The experiments were carried out for a period of 15 days and the degrading ability of the isolates were gravimetrically determined at every 5 days interval. In general, the degradation rate was increased with incubation time and the results demonstrated that *Achromobacter* sp. have the greatest ability to degrade petrol while *Pseudomonas aeruginosa* demonstrated the greatest degrading ability on diesel. The rate of petrol degradation was maximum (31.9%) at the end of 15 days interval period by *Achromobacter*. Recently, *Achromobacter* sp has been reported to utilize polycyclic aromatic hydrocarbons [46-50]. PAH degradation by *Pseudomonas* strains is well characterized [51]. *P. aeruginosa* is a good candidate for bioaugmentation of petroleum contaminated soils [52] and its degradation of n-alkanes in diesel oil is reported [53]. Diesel oil is a complex mixture of alkanes and aromatic compounds that frequently are reported as soil contaminants [54]. Diesel degrading strain of *Pseudomonas aeruginosa* capable of growing in the presence of other hydrocarbons is reported earlier [55]. In this research, *Pseudomonas aeruginosa* was identified as potential diesel degrader with maximum degradation of 34.4% at the end of 10 days incubation period. However, with increasing incubation time, the rate of degradation was reduced which could be possible due to the release of by products would that inhibit the normal bacterial growth and dramatically reduce the hydrocarbon degradation rate.

Conclusion

The PAH degrading bacteria showed diverse capacities to degrade petrol and diesel and this study is an important step towards the development of bioremediation strategies for cleaning of sites contaminated with polycyclic aromatic hydrocarbons. Further studies are needed to define their bioremediation potential and elucidate the metabolic pathways involved in Petrol and diesel degradation to establish the safety of the by-products from PAH metabolism.

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