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Isolation and Molecular Identification of Hydrocarbon Degrading Bacteria from Oil Contaminated Soils from Tamilnadu

KEYWORDS: Biodegradation, Polycyclic aromatic Hydrocarbons, Achromobacter xylosoxidans, aliphatic hydrocarbons, Pseudomonas medocina.

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ABSTRACT *Extensive hydrocarbons activities often result in the pollution of the environment, which could lead to disastrous consequences for the biotic and abiotic components in the ecosystem if not restored. Remediation of hydrocarbon- contaminated system could be achieved by either physiochemical or biological methods. Polycyclic aromatic hydrocarbons have different natural and anthropogenic sources and are widespread in the environment. They have deleterious effect on us. Because of structural complexity and hydrophobicity most of their members are chemically inert, so they are difficult to transform by any chemical reaction or to remove from the environment either by natural process of volatilization, photo oxidation or any other method. As a result they remain suspended in the environment and cause pollution. The present work was undertaken to assess, isolate and identify the hydrocarbon degrading bacteria associated with oil contaminated environmental soil. The samples were collected from soil near petrol, diesel pumps from different regions in Tamil Nadu.The samples were analyzed rmicrobiologically using standard microbiological techniques. These organisms were further characterized to determine their biodegrading ability on hydrocarbons as the sole source of carbon using culture dependent approach, the microbial growth were determined using UV Spectrophotometer at 600 nm. We have identified the following isolates using culture independent approach such as Achromobacter denitrificans, Bacillus flexus, Achromobacter xylosoxidans, Bacillus cereus, Pseudomonas medocina, Pseudomonas putida, Bacillus badius, Lysinibacillus xylanilyticus, and Exiguobacterium homiense. This results demonstrated that bacteria having the ability to degrade the PAHwith PAH degrading strains by an unknown mechanism. Therefore, understanding mechanism and interaction between these microbes may offer the opportunity to increase the degradation ability of soil-bound organic and inorganic condaminents.*

Introduction

Crude oil is composed of complex mixture of Alicyclic, Aliphatic and Aromatic hydrocarbons (Atlas et al., 1946). Hydrocarbons are organic compounds their structures consist of hydrogen and carbon. Hydrocarbons can be seen as linear linked, branched or cyclic molecules. They are observed as aromatic or aliphatic hydrocarbons. The first one has benzene (C6 H 16) in its structure, while the aliphatic one is seen in three forms: Alkanes, Alkenes and Alkynes (McMurry 2000). So far 175 kinds of hydrocarbon identified in raw oil that half of them are hydrocarbons with low boiling point whereas the remaining compound with high boiling point (Khosravi 2007) . In day today life the hydrocarbons are used in different forms such as gases, liquids, solids, waxes with low melting or polymer. Aromatic hydrocarbons are insoluble in water and are easily soluble in non-polar organic solvents (Survery et al., 2004). Polycyclic aromatic hydrocarbons (PAHs) are dangerous pollutants in the environment. They are very poisonous and cause many delitorius effect on human being.

Annually, around 6 million tons of crude oil from oily compounds enter the environment in worldwide which not only cause human health, but also damage our universe (Maola et al., 2002). Although some physical processes such as volatilization, leaching, chemical and photo oxidation are often effective in reducing the environmental level of PAHs (Heitmap et al., 1988). Besides, the physical processes are often limited to aquatic environments, whereas the miccrobial degradation can cleanup all type of environment. Microbial degradation is usually preferred and major route of PAH removal from contaminated area because of its cost effectiveness and inclusive cleanup (Pothuluri et al., 1994). Scientists are looking for particular ways to remove these pollutants from aquatic and soil environment. Nowadays, the use of microorganism and their products for the remedy of these pollutants has been taken into consideration. Many degradation study has been done using a series of these enzymes, and this process is called biodegradation. Bioremediation processs rely on the ability of microorganisms present naturally which are highly efficient due to their simplicity and cost effectiveness when compared to other technologies. It is established that selection of Hydrocarbon degrading microorganisms as with other chemicals occurs as a result of their previous exposure to this substances in the environment (Lewis et al., 1984) . However, these adaptations occur slowly and usually depend on the recalcitrance or biodegradability of the particular substance (Spain et al., 1980) . The PAH usually have low aqueous solubility and thus are poorly available for microbial utilization (Jonsen et al.,2005). Thus, the identification of novel bacteria using molecular finger printing techniques for the biodegradation of PAH in oil contaminated area is the need of the hour in the filed of microbial ecology. Particularly, the analysis of microbial communities that take part in in-situ hydrocarbon biodegradation activities has been a challenege to microbiologist. Several isolated microorganisms have been successfully utilized in major hazardous waste clean-up processes as for example, in industrial process streams and effulents (Levinson et al., 1994).Unfortunately, most of these studies were carried out in western countries and to a limited extend in India. In Tamilnadu, there is limited information on microbial degradation of polycyclic aromatic hydrocarbons. In this work, we report the isolation and molecular identification of polycylic aromatic hydrocarbon degrading bacteria from oil contaminated soils in Tamil Nadu.

Materials and Methodology

Sample Collection and Processing of Samples

Oil contaminated soil samples (includes petrol and diesel) were collected from different areas of Tamil Nadu viz Chennai Petroleum Corporation Limited situated in Chennai, Tamil Nadu, Oil spilled soil collection Tank Vilangudi, Madurai Tamil Nadu, Diesel filling station Kallakurichi, Villupuram, Tamil Nadu, Bus Depo (Disel), Trichy district,Tamil Nadu, Mechanical shed (Crude Oil) , Ariyalur District, Tamilnadu with a depth of 2-3 inches from the ground level using clean spatula. The soil was transferred into a sterile polythene bags and stored at 4^0C until using.

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Isolation of Hydrocarbon Degrading Bacteria
The bacteria were isolated by inoculating the soil on enrichment medium that contains the autoclaved mineral salt medium (MSM) supplemented with single hydrocarbon compound as sole carbon source (1% liquid petrol and oil). The medium contains K2HPO4 (1.8g/L); NH4CL (4g/L); MgSO4 .7H20 (0.2g/L); NaCl (0.1g/L); Na2S04.7H20 (0.01g/L); agar (20g/L); carbon source (1% petrol, oil); and distilled water (1L) with pH7.2. The medium was supplemented with 1% filter sterilized hydrocarbons (Petrol, oil) to serve as the only source of carbon and energy (Boboye et al., 2010). The medium was incubated at 37˚C for 5-10 days. After the incubation period the bacterial colonies were subcultured characterized acoording to Bergy's manual and 16s r DNA Sequencing. Pure cultures obtained by this procedure were stored in slants (enrichment medium with 15.0g/l pure agar).

Screening for Hydrocarbon degradative activity
Turbidometry analaysis was carried out to determine the bacterial growth by utilizing the hydrocabons (1% petrol and oil given as carbon source in MSM broth. This shows whether the bacterium possess the degrading activity of hydrocarbons like phenol, petrol, oil and diesel. The degrading activities of each isolates were obtained by using Mineral salt broth (MSB) in which 1% of each hydrocarbon (oil) was added and incubated at room temperature for 15 days. The growth of the bacterium was measured by taking the O.D at 600nm using a UV spectrophotometer (Systronics) from 0hrs-15days at regular intervals of 2 days against mineral salt medium as blank.

Biochemical characterization
Gram stain, Indole production, Methyl red, Citrate utilization test, Voges –Proskauer test, catalase, oxidase, urease test, starch Hydrolysis test, Nitrate reductase test, caesin Hydrolysis test, Gelatin liquefaction test, coagulase etc were performed with chosen isolate (Benson, 1990)

DNA extraction from pure cultures and PCR amplification
Genomic DNA was extracted from 1ml of bacterial culture, the culture was pelleted by centrifuging at 12,000rpm for 2 min. The pellet was treated with lysis solution and proteinase k and incubated at 60˚C for 30min. Nucleic acids were precipitated with isopropanol and followed by the ethanol treastment at 10,000 rpm for 10 mins. The pellet was washed with 1 ml of 70% (v/v) ethanol solution and dissolved in 0.1 ml of a TE buffer. The purity and quantity of DNA was examined by UV absorption spectrum and agarose gel electrophoresis. 16SrRNA PCR Amplification was performed with the help of the following primer sets: 16s- 8F (5' –GAGAGTTTGATCCTGGCTCAG-3') and 16s-1495R (5'- CGGCTACCTTGTTACTTC-3'). The following PCR conditions were followed (35 cycles of 3min at 94^{0}C, 1min at 50^{0}C, 2 min at 72^{0}C and 2min at 72^{0}C) and performed in a thermal cycler (Gradient Mastercycler, Eppenorff, USA). The amplified products were subsequently subjected to gel electrophoresis (Banglore Genei, India), stained with ethidium bromide and documented by gel documentation system.

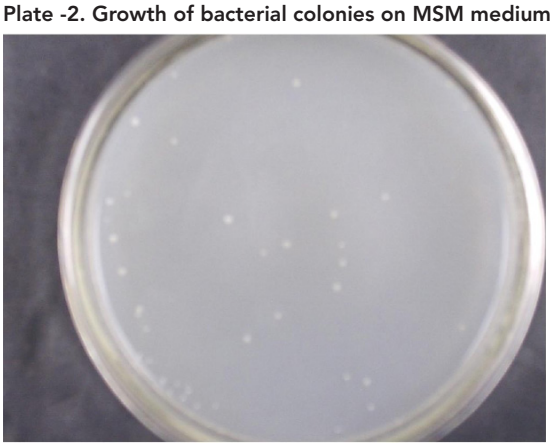
Sequencing of 16S rDNA and phylogenetic analysis:
The amplified 16S rDNA was subjected to agar gel electrophoresis and purified by Qiaquick gel extraction kit (Qiagen, USA). The purified PCR product was sequenced by dideoxy chain termination method using ABI Prism BigDye Terminator Cycle Sequencing Ready reaction kit as directed in the manufacturer protocol. Sequence reactions were electrophoresed and analysed by ABI Prism 3100 genetic analyser (Applied Biosystems, USA). The sequences were analysed using the CHECK CHIMERA and the SIMILARITY RANK programs of the Ribosomal Database project (Altrschul et al., 1990). The BLAST analysis was carried out (National Centre for Biotechnology information) to determine the closest bacterial sequences, the closest bacterial sequences were aligned using the Clustal W program (Shingler et al., 1996). Phylogenetic tree was constructed using Clustal W by distance matrix analysis and the neighbor –joining method (Saitou et al., 1987).The confirmed sequences were deposited in Genebank for public access. (accession numbers KJ452459, KJ452460, KJ452461, KJ452462, KJ452463, KJ452464, KJ452465, KJ452466, and KJ452467)

DGGE
Denatured gradient gel electrophoresis was carried out with D code system (Bio-Rad) according to the protocol outlined in the Bio-Rad manual. PCR products (25 µl of amplified products from soil DNA) were separated by DGGE using the linear gradient of urea and formamide (30–60% (v/v)) in an 8% acrylamide gel at 100 V and 60°C for 7 h. After separation the gels were stained with ethidium bromide and documented using the Gel Doc system. Gel portion containing the DNA band of interest was excised and recovered by conventional extraction method. The concentration of the DNA was determined using spectrophotometer. Depending on the concentration, this Purified DNA was used directly for sequencing reactions.

Results and Discussion:
Isolation and identification of hydrocarbon Degrading bacteria from oil contaminated soil:
The bacteria were isolated from five different types of contaminated soil samples on MSM medium. Further the samples were screened for the presence of hydrocarbon degrading bacteria on mineral salt medium with 1% of the hydrocarbons as the sole carbon source namely petrol and oil individually. Hydrocarbons are needed as a carbon source but it can be toxic to microorganisms due to the solvent effects of diesel and petrol that could destroy bacterial cell membrane. Many biodegradation studies were reported on diesel are carried out using lesser diesel concentration ranging from 0.5 to 1.5% . Number of colonies on mineral salt medium is lower when compared to the mother plate without hydrocarbons. This result showed that the bacteria grown on enriched medium were able to degrade the hydrocarbon source.

Plate:1.Isolation of PAH biodegrading bacteria by enrichment technique

Plate -2. Growth of bacterial colonies on MSM medium



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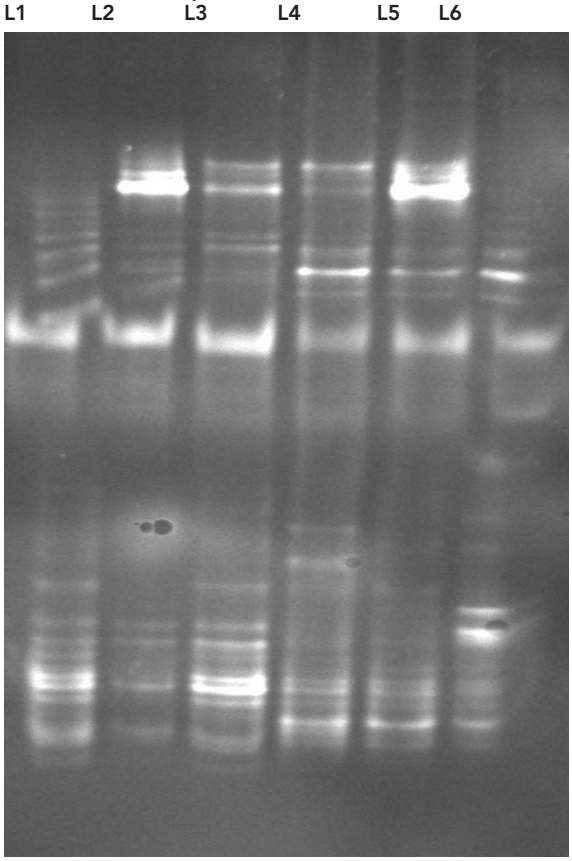
Biochemical analysis of the isolates :
As shown in Table 2 , Gram staining revelaed that all the isolates were belongs to Gram –negative except organism-2 are Gram positive. Catalase and nitrate reductase showed positive results with ten isolates, only organism-3 and 4 are positive results in starch hydrolysis.

16s rDNA gene sequence:
The isolate were further confirmed by 16s RDNA sequencing . Based on DNA extracts of isolate, (Fig-1), 16S rDNA which amplified by PCR using 35 cycles and primers 16sF and 16sR was got sequence result and listed in Table-2. The bacterial 16s rDNA sequences were aligned with Blast search of NCBI databases. The sequence aligned have 99% similarity with *Achromobacter denitrificans, Bacillus flexus, Achromobacter xylosoxidans, Bacillus cereus, Pseudomonas medocina, Pseudomonas putida, Bacillus badius, Lysinibacillus xylanilyticus, Exiguobacterium homiense,* respectively. These results highlight the different group of bacterial genera involved in hydrocarbon degradation . Many scientists studied the petroleum degradation by various *Pseudomonas* species and by *Bacillus species* (Adriano *et al.,* 2007) . It is evident from the study that when environment was contaminated with petroleum and oil components the proportion of hydrocarbon degrading microorganisms increases rapidly. High numbers of certain hydrocarbon degrading microorganisms from an environment implies that those organisms are the active degraders of that environment. The presence of oil degrading organisms in the polluted soil is clear indication that the indigenous microbes were carrying out their metabolic activity. The activities of these microorganisms could be responsible for the bioremedia tion of the environment.

Fig. 1 Showing the DGGE profiles of 16S rDNA oil contaminated soil samples.
L1 L2 L3 L4 L5 L6



L1- Madurai, L2-Chennai, L3-Kallakuruchi, L4-Thuraiyur, L5-Puthanampatti and L6-Ariyalur

PCR –DGGE analysis were performed to explore the bacterial diversity in all the oil contaminated soil samples. Bacterial 16S genes were amplified from DNA samples using conventional PCR. The same 16s samples was used for bacterial diversity analysis through PCR-DGGE method. DGGE results clearly stated the presence of distinguishable bands in resolving pattern (Fig. 1). In this present study, several soil DNA samples were isolated from different area. Contamination free DNA samples were subjected to bacterial specific 16S rDNA-targeted PCR-DGGE by v3 region specific primer with GC-clamp. The major DNA bands were purified, re-amplified and sequenced to identify its identity. We have identified the following bacteria *Bacillus, Psudomonas, Acromobacter* etc Fig. 1, revealed the overall microbial diversity from oil contaminated soil samples.

Hydrocarbons by Turbidometry:
Table-3 shows the O.D reading of biodegrading activity of each isolates on hydrocarbons. The O.D readings based on the turbidity of MSM broth at regular intervals of 2 days give the degradative activity on hydrocarbons by bacteria. The results demonstrated that Pseudomonas putida and Achromobacter xylooxidans have the greatest ability to degrade petrol contaminated soils while *Bacillus cereus* demonstrated the greatest ability to degrade oil. Our results showed that all the organisms maximally utilized all the hydrocarbon substrates (oil) when supplied as the sole source of carbon. and energy although, the level of utilization differs from one microbe to another (due to differences in their growth) and from one hydrocarbon substrate to the other, due to the obvious differences in their molecular sizes. The bacterium with the highest degrading activities on oil was *Pseudomonas putida* and *Bacillus badius* respectively. These degrading capabilities on different hydrocarbons revealed that the microorganisms isolated from the soil. The cells were able to multiply within the days of study, indicating that they were able to degrade and utilize the oil for their growth and development, hence the concomitant increase in the concentration of the broth. This gradual increase in the concentration of broth indicates bacterial growth hence degradation of hydrocarbons, mostly between days 2 and 3 and gradual decline in the concentration of the broth suggests decrease in the bacterial population and that the hydrocarbon has been degraded, mostly between days 13 and 15. The oil degrading microorganisms isolated from the contaminated soil were *Achromobacter denitrificans, Bacillus flexus, Achromobacter xylosoxidans, Bacillus cereus, Pseudomonas medocina, Pseudomonas putida, Bacillus badius, Lysinibacillus xylanilyticus,* and *Exiguobacterium homiense.*

Phylogenenetic study:
For the 16s rRNA gene sequence, ten highly homologous sequences were identified by blastin results and were downloaded and phylogenetic tree was constructed .(Figure-1). The nucleotide sequences of these ten bacterial strains were submitted to the Genebank with accession numbers KJ452459, KJ452460, KJ452461, KJ452462, KJ452463, KJ452464, KJ452465, KJ452466,and KJ452467.

Conclusion:
Bioremediation is one of the most rapidly growing areas of environmental Biotechnology, which has been used for the cleaning up of pollutants. This is because of its low costs and its public acceptability. Degradation of hydrocarbons through soil microorganism involves microorganisms which contain the specialized metabolic capacities. In contaminated soil there are many specialized microorganisms because of the adaptation of the microflorae to pollutant. It is evident from this study that, hydrocarbon degrading organisms are ubiquitous in environment and they can be isolated from hydrocarbon polluted sites. It has also been shown that ten bacterial strains from contaminated soil can be good petrol and oil degraders. This study can focus on more cost effective applications of native bacterial strains for petrol and oil degradation at large scale in industries, where it pose an alarming problem due to its detrimental health effets on

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different organisms and human beings.In conclusion of the present investigation demonstrates the feasibility of adopting sustainable and ecofriendly approach to minimize the hydrocarbon pollutants.

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Table: 1. Morphological and biochemical characteristics of bacteria isolated from oil polluted soil

Feature	Org1	Org2	Org3	Org4	Org 5	Org 6	Org7	Org8	Org9
Gram stain	-	+	-	-	-	-	-	-	-
Shape	cocci	cocci	rod	rod	rod	cocci	rod	cocci	rod
Indole	-	-	-	-	-	-	-	-	-
MR	-	-	-	-	-	-	-	-	-
VP	-	-	+	-	-	-	-	+	-
Citrate	-	-	+	+	-	-	+	+	+
Catalase	-	-	-	-	-	-	-	-	-
Oxidase	-	+	-	-	+	+	-	-	-
Urease	+	+	-	-	+	+	-	+	-
Starch Hydrolysis	-	-	+	+	-	-	-	-	-
NR	+	+	+	+	+	+	+	+	+
Caesin Hydrolysis	-	+	-	-	-	+	-	-	+
Coagulase	+	-	+	+	-	-	+	-	+
Gelatinase	+	+	+	-	+	+	-	+	-

Org-organism; +ve- positive; -ve – negative

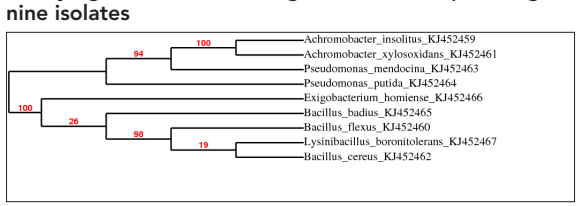
Table 2: Identification of bacteria by 16s r DNA sequencing

Organism	Sample Source	Identified by 16s sequencing
1	Soil	*Achromobacter denitrificans*
2	Soil	*Bacillus flexus*
3	Soil	*Achromobacter xylooxidans*
4	Soil	*Bacillus cereus*
5	Soil	*Pseudomonas medocina*
6	Soil	*Pseudomonas putida*
7	Soil	*Bacillus badius*
8	Soil	*Lysinibacillus xylanilyticus*
9	Soil	*Exiguobacterium homiense*

Table 3. OD at 600 nm of isolated bacterial samples

Name of organisms with inoculated in 10 ml MSM broth with 1% used engine oil	OD at 600nm (Zero hours incubation at 36^{0}c)	OD at 600nm (48hrs incubation at 36^{0}C)	OD at 600 nm (72hours incubation at 36^{0}C)
Achromobacter denitrificans	0.00	0. 300	0. 390
Bacillus flexus	0.00	0. 366	0.520
Achromobacter xylooxidans	0.00	0. 290	0.350
Bacillus cereus	0.00	0.360	0. 500
Pseudomonas medocina	0.00	0.411	0. 700
Pseudomonas putida	0.00	0.481	0.793
Bacillus badius	0.00	0. 410	0. 579
Lysinibacillus xylanilyticus	0.00	0.200	0. 258
Exiguobacterium homiense	0.00	0. 290	0. 340

1. Phylogenetic tree showing the relationship among the nine isolates



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