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ORIGINAL RESEARCH

Identification and isolation of hydrocarbon degrading bacteria from the soils contaminated with industrial effluents

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ABSTRACT

Biodegradation of petrol by microorganisms isolated from the soils of Industrial area was studied. In order to isolate hydrocarbon utilizing organisms, enrichment medium supplemented with 1% petrol was inoculated with the above soil samples. The isolated organisms were identified by morphological characters, cultural studies and biochemical properties. The identified organisms were further studied to determine the biodegrading activities on hydrocarbons using petrol as the sole source of carbon. The growth of isolates was determined using calorimeter at 595 nm. The bacteria were identified at genus level as *Bacillus* sp. by 16S rRNA sequencing and showed that they were able to grow on petrol.

KEY WORDS: petrol, degrading, bacteria, effluents

Introduction

Hydrocarbons are considered as the world's most widely used primary energy and fuel resources. Many microorganisms have the ability to utilize hydrocarbons as sole sources of carbon as energy for metabolic activities and these microorganisms are omnipresent and widely distributed in nature. The microbial utilization of hydrocarbons depends on the chemical nature of the compounds within the petroleum mixture and on environmental determinants (Adeline *et al.*, 2009). Hydrocarbons enter into the environment through waste disposal, accidental spills, as pesticides and via losses during transport, storage, and use (Atlas, 1981). Diesel engine oil, which is one of the major products of crude oil, constitutes a major source of pollution in our environment. With the combined dependence on diesel engine oil by some vehicles and generators, greater quantities are being transported over long distances. Therefore diesel engine oil

can enter into the environment through wrecks of oil tankers carrying diesel oil, cleaning of diesel tanks by merchants, war ships carrying diesel oil and motor mechanics (Hill and Moxey, 1980).

Diesel oil spills on agricultural land generally reduce plant growth. Suggested reasons for the reduced plant growth in diesel oil contaminated soils range from direct toxic effect on plants (Baker, 1982) and reduced germination to unsatisfactory soil condition due to insufficient aeration of the soil because of the displacement of air from the space between the soil particles by diesel engine oil (Zahir *et al.*, 2001).

Bioremediation processes rely on the ability of microorganisms present naturally which are highly efficient due to their simplicity and cost-effectiveness when compared to other technologies. Bioremediation is a mineralization of organic chemicals, leading ultimately to the formation of CO₂, H₂O and biomass (Dua *et al.*, 2002). But the rate of

degradation of hydrocarbons in nature is limited due to their hydrophobic property which leads to their limited solubility in ground water and tendency to partition to the soil matrix, Bioremediation is an effective economical and environmentally friendly treatment method in which microbes are used to degrade hydrocarbons. Microorganisms which biodegrade the various components of petroleum hydrocarbons such as polynuclear aromatic hydrocarbons (PAHs), naphthalene, monoaromatic hydrocarbons such as toluene, or aliphatic hydrocarbons such as the *n*-alkanes, can be readily isolated from the environment, particularly from petroleum-contaminated sites (Lyle *et al.*, 1997; Ronald, 1981).

One of the most important characteristics of hydrocarbon degrading bacteria is the ability of emulsifying hydrocarbons in solution by producing surface active agents such as biosurfactants (Hommel, 1990; Desai and Banat, 1997). Biodegradation is carried out largely by diverse bacterial populations mostly by *Pseudomonas* species (Dubey, 2009). The purpose of this research was to identify the feasibility of bacteria growing in soils of industrial area to degrade hydrocarbons like petrol and diesel and to detect the presence of catechol 2,3 dioxygenase enzyme in the identified bacteria.

Materials and Methods

Sample collection

The study includes three types of samples to isolate the hydrocarbon degrading bacteria. Soil sample extending from the ground surface to a depth of 10–20 cm were collected in sterilized plastic containers from the Industrial areas of GVK, Biological E limited (effluent after treatment) and near parker in Patancheru. Patancheru was one of the largest industrial zones which is located at 17.53°N 78.27°E in Telangana, on the Hyderabad-Sholapur highway and around 18 km from HITEC City. The soil samples were duly labeled and transported to laboratory aseptically and stored at 4°C for further analysis.

Isolation of bacterial cultures

One gram soil sample from each source was suspended and vortexed with 10ml sterile distilled water. The suspension was allowed to settle down and 5 ml of supernatant was

used as inoculum in 100ml Mineral salts broth containing 1% Petrol. The flasks were incubated for 48 hrs at 37 °C on a rotary shaker at 100rpm. The broth was centrifuged at 5,000 rpm for 15 min and the cell pellets were obtained. The cell pellets were washed with 0.1 M phosphate buffer solution (pH 6.8) (Geetha *et al.*, 2013). The cell pellets were used to inoculate Bushnell Hass (BH) medium supplemented with 1% petrol as a single carbon source. Composition of BH medium is MgSO₄·7H₂O -0.2 g; K₂HPO₄ -1g; KH₂PO₄ -1g; FeCl₃ -0.05 g; NH₄NO₃ -1g; CaCl₂ -0.02g, pH -7.2; Agar -20g; Distilled water -1000ml.

The medium without hydrocarbon was sterilized by autoclaving at 121°C for 15 min. The medium was supplemented with 1% filter sterilized hydrocarbon of petrol to serve as the only source of carbon and energy (Olukunle and Adetuyi, 2010). The medium was incubated at 37°C for 10–15 days. After the incubation the bacterial colonies that were grown on the medium were identified by staining, cultural and biochemical characterization. The pure and representative colonies were subcultured onto nutrient agar slants and preserved at 4 °C in refrigerator.

Morphological characterization of isolates

Isolates were examined after growth on Nutrient medium for Grams reaction and cell morphology. Some biochemical characteristics of the cultures as growth on carbon sources, starch hydrolysis, gelatin liquefaction and IMViC tests were studied according to Bergey's Manual of Determinative Microbiology.

Determination of bacterial biodegradative activity

Turbidometry was used to determine bacterial growth by utilizing the hydrocarbons of 1% petrol given as carbon source in Mineral salts broth. The degrading activities of each isolate were obtained by using MS broth adding 1% of hydrocarbon of petrol and kept for incubation for about 25 days at 37 °C. The growth of bacterium was measured by taking O.D readings at 595 nm from 0 hrs -25 days at regular intervals of 5 days against mineral salt broth as blank (Nikhil *et al.*, 2013).

Isolation of Genomic DNA for identification of the isolate

DNA was extracted from 0.5ml bacterial cultures and was pelleted by centrifuging at 12,000 rpm for 30sec suspending

it in phosphate buffer solution. The pellet was treated with 30µl of DBM to clear the lysate and mixed by inverting the tube 3-4 times and kept for incubation at room temperature for 2-3 min followed by centrifugation at maximum speed for 30sec. The supernatant was discarded and the pellet was washed with 400µl of 70% ethanol. Centrifugation was done at maximum speed for 60 sec to remove the traces of 70% ethanol completely. 15µl of Elution Buffer was added to the pellet, mixed and incubated at room temperature for 2-3 min. After Centrifugation at Maximum speed for 60 sec at room temperature the elute was stored at -20° C. The purity of DNA was examined by running on 1% agarose gel electrophoresis.

Amplification of 16S region of bacterial genomic DNA

The DNA isolated was amplified using 16s rDNA universal primers -Bac8F & 1492 R and sequenced for the identification of bacterial strain at molecular level. Upon obtaining amplicons of 1.5kb, the products were desalted (purified), and were sequenced. The 16S r-RNA gene sequence of each sample was aligned with the database (NCBI) using an alignment tool- BLAST (Basic Local Alignment Search Tool).

Results and Discussion

The hydrocarbon degrading bacteria were isolated from soils contaminated with industrial effluents from different areas on the Bushnell Hass (BH) agar medium and nutrient agar (NA) medium. 10 colonies showing different morphological characteristics on BH medium were selected for further characterization. Out of 10 cultures 5 had shown the growth on mineral salt medium with 1% of hydrocarbons as sole carbon source namely petrol. The most potent bacterial petrol degraders were identified by observing morphological characters and biochemical tests. Different types of biochemical tests were done such as Gram's staining, Indole test, Methyl red test, VP test, Citrate utilization test, Urease test, Nitrate reduction test, Gelatinase test, starch hydrolysis test, Catalase test and H₂S production test etc. Hydrocarbons can be toxic to microorganisms due to the solvent effects of diesel and petrol as they destroy bacterial cell membrane. The studies reported on diesel using the concentrations 0.5 to 1.5 %. The studies showed that Pseudomonas and Bacillus degrade alkanes, n – paraffins

and aromatics. (Mittal and Singh, 2009).

Morphological, cultural and biochemical characteristics of isolates illustrated in **Table 1** as follows (*A-Acid producer, G –gas producer, +ve – positive, -ve –negative.*).

Feature	S1	S2	S3	S4	S7
Gram Stain	+ve	+ve	+ve	+ve	+ve
Shape	ROD	ROD	ROD	ROD	ROD
Spore	+ve	+ve	+ve	+ve	+ve
Catalase	+ve	+ve	+ve	+ve	+ve
Starch Hydrolysis	+ve	+ve	-ve	-ve	-ve
Indole	-ve	-ve	-ve	-ve	-ve
MR	-ve	-ve	-ve	-ve	+ve
VP	-ve	+ve	-ve	-ve	-ve
Glucose	A	NA	A	AG	AG
Lactose	NA	NA	NA	A	G
Citrate	+ve	+ve	-ve	+ve	+ve
Nitrate reduction test	+ve	-ve	-ve	+ve	+ve
Gelatinase	+ve	+ve	-ve	_ve	+ve
H₂S gas production	-ve	-ve	-ve	-ve	-ve
Urease	-ve	-ve	-ve	-ve	+ve
Growth on MacConkey Agar	No growth	No growth	No growth	No growth	No growth

16s rDNA sequence

The genomic DNA from each sample was amplified for 16S r-RNA gene using universal primers- Bac8F and 1492R. Upon obtaining amplicons of 1.5kb, the products were desalted (purified), and were sequenced using Sanger's method. The bacterial 16S r-RNA gene sequences of each sample was aligned with the database (NCBI) using an alignment tool- BLAST (Basic Local Alignment Search Tool).

The genome was scanned for the presence of Catechol 2,3 dioxygenase coding gene, in a Genes and Genomes database- KEGG (Kyoto Encyclopedia for Genes and Genomes)

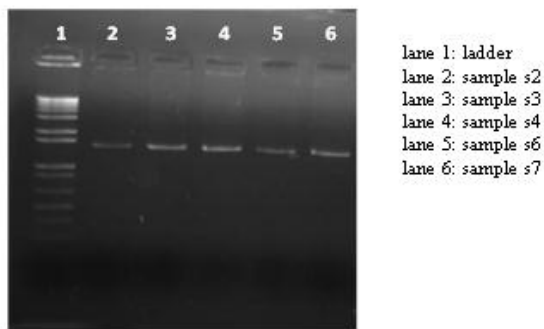


Figure 1: Agarose gel showing 16S amplicons

The sequence alignment gave 98% similarity with *Bacillus subtilis*, *B.pumilis*, *B.altitudinis*, *B.licheniformis* and *B.safensis* respectively. These results highlight the different species of *Bacillus* are able to degrade hydrocarbons. Boboye *et al* (2010) reported the degradation of hydrocarbons by *Bacillus* species and other group of bacteria. Likewise Ojo (2006) reported hydrocarbon degradation by *B.megaterium*, *B.brevis* and *B.pumilis*.

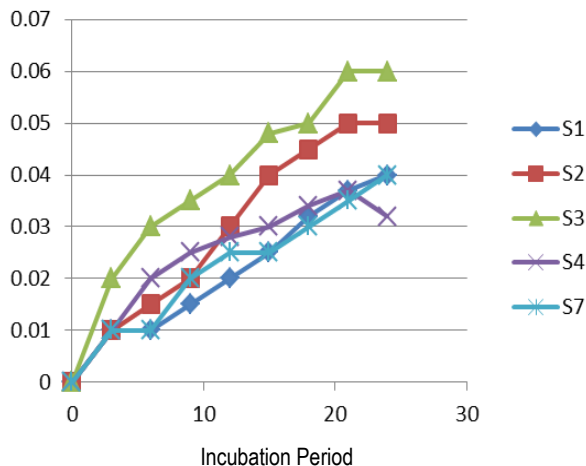


Figure 2 : Graph showing the growth curve (O.D) values of bacteria on petrol degrading broth for a period of 25 days of incubation (S1-*Bacillus subtilis*,S2- *Bacillus pumilis* ,S3- *Bacillus altitudinis*,S4- *Bacillus licheniformis*,S7-*Bacillus safensis*)

The ability of members of genus *Bacillus* of *B.megaterium* and *B.cereus* to degrade hydrocarbons of petrol and diesel has been reported by Jyothi *et al* (2012) and *B.pumilis* degrade diesel by Mandri & Lin (2007) and Singh *et al* (2008), pyrene by Khanna *et al* (2011), pyrene & phenanthrene by Yaliani *et al* (2012) and poly aromatic

hydrocarbons (Patowary *et al.*, 2015).The species of *B. fusiformis* also degrade petroleum hydrocarbons provided ions and optimum conditions for their growth.(Zhao *et al.*, 2011).

The below analyses show that all the given isolates Possess the gene coding for Catechol 2,3 dioxygenase. The identifiers or entry numbers can be used to retrieve the sequences for the gene in the respective organism.

Name of the isolate	Organism (PCR and BLAST)	Presence of Catechol 2,3 dioxygenase	KEGG identifier
S1	<i>Bacillus subtilis</i>	Yes	K07104
S2	<i>Bacillus pumilus</i>	Yes	K07104
S3	<i>Bacillus altitudinis</i>	Yes	K07104 QR42_04240
S4	<i>Bacillus licheniformis</i>	Yes	K07104
S7	<i>Bacillus safensis</i>	Yes	QR42_04240 K07104

Table 2: Identification of bacteria by 16S rDNA sequencing

Conclusion

The study produced promising results were in the 5 isolates of genus *Bacillus* were characterized. These isolates have been identified and characterized based on the cultural, staining, biochemical and genetic i.e. 16s RNA analysis as *Bacillus subtilis*, *B.pumilis*, *B.altitudinis*, *B.licheniformis* and *B.safensis* respectively. These have been shown as of special relevance as petrol degrading microbes. Some of the strains of genus *Bacillus* were identified in this study through qualitative analysis as promising strains for petrol degradation. The degrading ability demonstrated by these organisms is a clear indication that they possess a gene of Catechol 2, 3 dioxygenase for degradation of hydrocarbons.

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