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Isolation and characterization of hydrocarbon degrading bacterial isolate from oil contaminated sites

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Abstract

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic environmental pollutants that have accumulated in the environment due to a variety of anthropogenic activities. We isolated 14 different bacteria from various petroleum contaminates sites, using enrichment technique, and studied for utilization of various hydrocarbons. The bacteria were capable to grow on various hydrocarbon like diesel, petrol, lubricating oil, toluene, naphthalene and kerosene. The tests were conducted to detect the biodegradation of diesel, by a monooxygenase biodegradation pathway, and it was observed that diesel was degraded within 12 hours. Gram negative bacterial isolate RP12 was found to be the best isolate growing on majority of hydrocarbons and degrading diesel.

Keywords: Polycyclic aromatic hydrocarbons; biodegradation; diesel degradation; monooxygenase biodegradation pathway

1. Introduction

Petroleum contamination resulting from leaking above ground and underground storage tanks, spillage during transport of petroleum products, abandoned manufactured gas sites and various industrial processes is hazardous to soil and water ecosystems, and is expensive to remediate. There is an increased interest in promoting environmental methods in the process of cleaning oil-polluted sites. These methods are less expensive and do not introduce additional chemicals to the environment. Compared to physiochemical

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methods, bioremediation offers a very feasible alternative for the decontamination of oil spills. This technique is considered an effective technology for treatment of oil pollution [1]. Bioremediation is an effective, economical and environmentally friendly treatment method in which microbes are used to degrade hydrocarbons. Factors influencing the rate of microbial growth include soil moisture, temperature, population diversity, pH, oxygen supply and nutrient levels. Concentrations of carbon, nitrogen and phosphorus are a major factor in effective remediation of hydrocarbons in soils. 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 [2]. The microorganisms can be obtained originally by enrichment culture procedures, where maximum specific growth rate or maximum final cell concentration can be used as the selection criterion. Petroleum hydrocarbons can be degraded by microorganisms such as bacteria, fungi, yeast and microalgae [3, 4]. However, bacteria play the central role in hydrocarbon degradation. The present work was aimed at determining the hydrocarbon degrading microorganisms present in the oil contaminated sites from the Gujarat, India and to study the growth responses of the bacterial isolates to different hydrocarbons along with their characteristics.

2. Experimental procedures

Oil contaminated soil samples were collected from the oil production sites of Essar Oil LTD., Vadinar-Jamnagar, and from Oil and Natural Gas Corporation (ONGC) Point, Andada- Ankleshwar (Gujarat- India). Samples were collected in sterile zip-lock polythene bags and transferred to laboratory for further study and stored at 4^oC. one gram soil samples from each source was suspended and vortexed with 10 ml of sterile normal saline. The suspension was allowed to settle down and 2.5 ml of supernatant was used as inoculum, in 50 ml of Luria Bertani broth containing 1% hydrocarbons (diesel, petrol, kerosene, lubricating oil, napthalene and toluene) added seperatly in each flask. The flasks were incubated for 48 hours at 37^oC on a rotary shaker at 100 rpm. After this step 50 ml broth was centrifuged at 5000 rpm for 10 min. The cell pellets were obtained. The cell pellets were washed with 0.1M phosphate buffer solution (pH-6.8) twice. After that the cell pellet was suspended in Bushnell Hass (BH) medium. Pure hydrocarbon degrading strains were isolated on petroleum agar plates. 0.1 ml of above suspended culture was spread on petroleum agar plates. In the preparation of petroleum agar plates 0.1ml of sterile hydrocarbon solution were evenly spreaded, so that a film of hydrocarbon got absorb over the entire surface of agar medium in the petriplates and then inoculum was spread with spreader on the medium (each plate contained one of the hydrocarbon; i.e. diesel, petrol, kerosene, lubricating oil, napthalene and toluene). The plates were incubated at 37^oC for one week in an incubator. Pure and representative colonies were transferred to nutrient agar plates as well as to nutrient agar slants for preservation. The isolated strains were preserved in 25% v/v glycerol solution at -20°C. For day to day experimentation strains were maintained on nutrient agar slants at 4^oC in refrigerator and sub-cultured at an interval of 30 days.

Growth on diesel was determined by the 'hole-plate diffusion method' on Bushnell Hass (BH) agar medium [5]. Colonies of the different bacterial isolates were transferred into 50 ml BH Medium [6], supplemented with 0.05% (v/v) Hydrocarbon solutions, filter sterilized through 0.45μ membranes and incubated at 37°C in a water bath shaker at 150 rpm for 30 days. Bacterial growth was determined at intervals by measuring the optical density (OD_{540}). The test of was also conducted to detect the biodegradation of diesel, by a monooxygenase biodegradation pathway [7]. The test was performed at 28ºC in a glass test tubes containing: 20μl of 0.05mol/l 2,6-dichlorophenolindophenol (DCPIP) (HiMedia, India); 30μl of 0.05mol/l 5-methyl-phenazinium methylsulphate (5-MPMS) (HiMedia, India); 25μl of 0.1% (v/v) diesel; 5μl of 0.15M NAD solution (HiMedia, India) and 25μl of pre-washed bacterial cells. Change in color was visually

compared with four controls: no diesel (substrate); no NAD$^+$; no cells and heat-killed cells. The reaction was monitored up to 12 hours with intermittent observation after 1, 2, 6 and 12 hours.

3. Results and Discussion

Hydrocarbon degrading bacteria were enriched and isolated from the oil contaminated soil from different areas on the nutrient agar (NA) medium as well as on the petroleum agar medium. Fourteen colonies showing different morphological characteristics on the NA plates were selected for further characterization. Results of grams staining showed that majority of the hydrocarbon degrading bacteria in this study (RP1, RP2, RP4, RP12, DE5, DE7ii, DE8ii, OW13 and OW14) belonged to Gram negative coccobacilli strains, followed by Gram positive cocci and only RP2 was Gram positive bacilli.

Hole-plate diffusion assay using diesel as a carbon source showed bacterial growth around the holes containing the diesel. Distilled water was used as control. Out of 14 cultures 9 had shown the growth around the holes, which were RP1, RP2, RP4, RP12, DE5, DE7ii, DE8ii, OW13 and OW14. Growth of hydrocarbon degrading bacteria using different hydrocarbons as carbon source (Agar Ditch Method) was analyzed. The bacterial growth was observed at perpendicular of the ditches containing different hydrocarbons as shown in Figure 1.
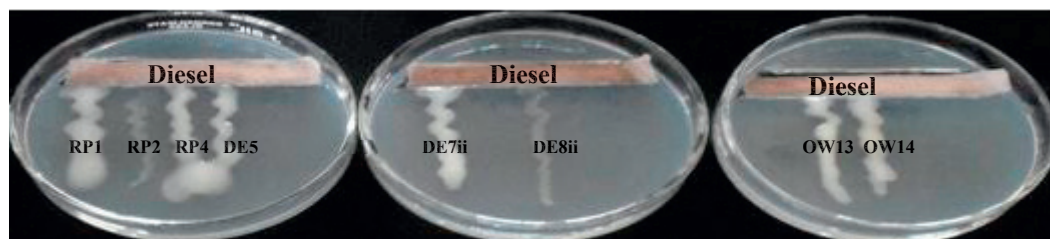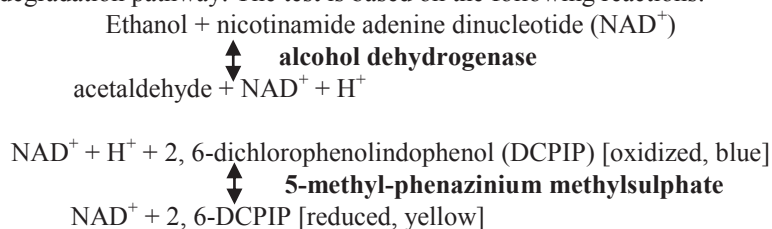

Fig. 1. Growth response of bacterial isolate in presence of different hydrocarbons by the Agar Ditch method.

As shown in figure 2(A-D), a significant difference was observed in growth of bacterial strains isolated from different ecological sources. It was observed that the isolates RP12, RP2, DE8ii, OW13 and OW14 were found to be more potent hydrocarbon degrading strains. Other isolates showed the degradation but in less extent than above mentioned isolates. All the isolates showed different length of lag phase during growth on different hydrocarbons. Better growth was observed when diesel or kerosene was used as a carbon source, as compared to toluene or lubricating oil, and very little growth was observed in presence of petrol or naphthalene. Among these potent isolates, RP12 showed the shortest lag phase. The potent hydrocarbon degrading isolates (based on hole-plate diffusion method) were selected for the diesel degradation assay, by a monooxygenase biodegradation pathway. The test is based on the following reactions:

Ethanol + nicotinamide adenine dinucleotide (NAD$^+$)

$\updownarrow$ **alcohol dehydrogenase**

acetaldehyde + NAD$^+$ + H$^+$

NAD$^+$ + H$^+$ + 2, 6-dichlorophenolindophenol (DCPIP) [oxidized, blue]

$\updownarrow$ **5-methyl-phenazinium methylsulphate**
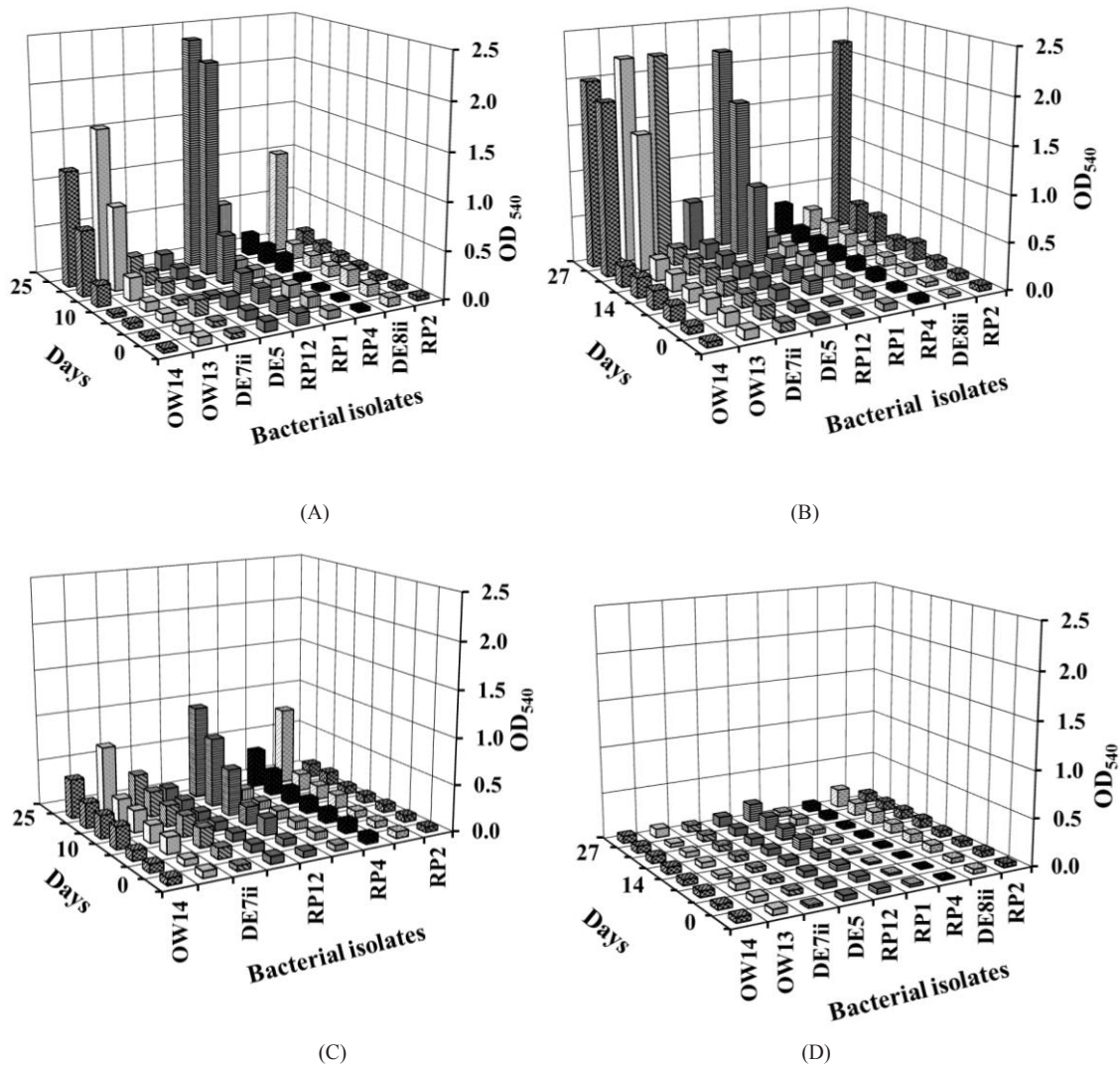
NAD$^+$ + 2, 6-DCPIP [reduced, yellow]

Fig. 2. Growth characteristics of different isolates on hydrocarbons: Diesel (A), Kerosene (B), Lubricating oil (C) and Toluene (D).

During incubation time of 1, 2, 6 and 12 hours, the color change was observed from blue to other colors. Except the isolate RP4, all other isolates i.e. RP2, RP12, DE8ii, OW13 and OW14 gave the yellow color from blue, indicating positive test for diesel degradation. Growth of bacteria on diesel was intended for the induction of mixed function oxygenases and other catabolic enzymes essential for biodegradation. The most potent hydrocarbon degrading isolates were tested and from them RP2, RP12, DE8ii, OW13 and OW14 were found able to degrade diesel as indicated by a color change from blue to yellow. Running the test without NAD+ and heating the cells for 10 min at 90 °C gave a negative color reaction which indicates the enzymatic nature of the reaction, and support the main idea that diesel can be degraded biologically and the test adopted in this study can be applied for screening organisms for their ability to degrade hydrocarbons [5]. Overall, Isolate RP12, a Gram negative coccobacilli, was observed to be quite potent to utilize different hydrocarbons and degrade them. Further identification of bacterium RP12 and gas chromatographic studies related to

degradation of hydrocarbons are needed.

Acknowledgements

References

[1] Malatova K. Isolation and characterization of hydrocarbon degrading bacteria from environmental habitats in western New York state, Master's Thesis in Chemistry, 2005; Department of Chemistry, Rochester Institute of Technology, Rochester, NY.

[2] Lyle G,. Whyte L, Charles WG. Biodegradation of Petroleum Hydrocarbons by Psychrotrophic Pseudomonas Strains Possessing Both Alkane (alk) and Naphthalene (nah) Catabolic Pathways, *Appl Environ Microbiol* 1997;**63**: 3719–23.

[3] Riser-Roberts E. Bioremediation of Petroleum Contaminated Sites. Boca Raton (FL): *CRC Press Inc*; 1992.

[4] Bundy JG, Paton GI, Campbell CD. Combined microbial community level and single species biosensor responses to monitor recovery of oil polluted soil. *Soil Biol Biochem* 2004;**36**:1149-59.

[5] Saadoun I. Isolation and characterization of bacteria from crude petroleum oil contaminated soil and their potential to degrade diesel fuel. *J Basic Microbiol* 2002; **42**:420–8.

[6] Leadbetter ER, Foster JW. Studies of some methane utilizing bacteria. *Arch Microbiol* 1958;**30**:91–118.

[7] Jacobs CJ, Prior BA, Dekock MJ. A rapid screening method to detect ethanol production by microorganisms. *J Microbiol Methods* 1983;**1**:339–42.