Role of microorganisms against hydrocarbon contamination; Bioremediation

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Introduction

The development of human civilization has changed its path since the industrial revolution. Since then began the use of hydrocarbon sources as the primary energy source of the world. The use of oil as fuel has led to intensive economic development worldwide. Even though these compounds contribute to the global economy on massive scale they in turn have perilous effects on the biotic and abiotic components of the ecosystem. In the stages of oil refinement, transportation, storage and on daily activities, unavoidable oil spills take place in small amounts. However, the accidental large oil spills draw the attention of the public to find remediation solutions. The methods of remediation can be physical, chemical or biological or may be a combination of two or more of these techniques. Hydrocarbon utilizing bacteria, fungi and cyanobacteria have been found in soil, marine and fresh water ecosystems (Okoh, 2002). Although several countries have already used methods including microorganisms for bioremediation of petroleum spills, it has not been previously used in Sri Lanka. Therefore, the objective was to isolate indigenous bacterial strains from hydrocarbons contaminated soils to assess their potential for bioremediation and to develop a bio-product for bioremediation.

Methodology

Three sites with soil contaminated by different petroleum hydrocarbons were identified in Ceylon Petroleum Corporation, Sapugaskanda, Kelaniya, Sri Lanka. A total of 18 soil samples (6 from each site) were collected randomly by simple soil sampling method (American Society for Testing and Materials, 1998). A weight of 10 g of soil was diluted in 90 ml of 0.1% sterile Sodium pyrophosphate solution containing 30 g of sterile glass beads. After shaking the mixture for 1 hour at 175 rpm, the soil extracts were serially diluted from 10^{0} to 10^{-8} and were vortexed for 1 minute. A volume of 120 µl of each dilution was spread on Luria Broth (LB) agar medium and was incubated at 28 °C for 7 days. The colonies appeared were inoculated on a Bushnell Haas (BH) liquid and solid mediums supplemented with 50 µl of hydrocarbons followed by an incubation at 28 °C for 7 days. The identified colonies were subjected to genomic DNA extraction using the Phenol-Chloroform method. The extracted genomic DNA samples were sent over to Macrogen, Korea for 16S rRNA sequencing.

The overnight grown bacterial cultures were centrifuged at 16000 g for 3 minutes at 4ºC. The pellet was resuspended in 200 µl of TE buffer and was vortexed and centrifuged at 16000 g for 1 minute at 4ºC and a volume of 1.5 µl of Protinase K was added and mixed. To this 20 µl (1/10) of 10% SDS was added, mixed well and incubated for 1 hour at 37 ºC. After the incubation, equal volume of Phenol: Chloroform (1:1) was added and centrifuged at 16000 g for 2 minutes at 4ºC. The aqueous layer was taken out without disturbing the protein layer and transferred into a fresh tube. A volume of 2V of 100% ice cold Ethanol and 0.1V Sodium acetate were added, mixed well and were incubated at 0 ºC for 1 hour. The solution mixture was centrifuged at 16000 g for 5 minutes at 4ºC. The supernatant was discarded and the pellet was dried and dissolved in 40 µl of nuclease-free water by tapping. For the selection of immobilizing agent, 10 g of autoclaved saw dust and rice husk each were

mixed with 7.5 ml of Yeast Extract Glucose (YEG) broth separately and was autoclaved. Then the washed, pure bacterial cells were inoculated on to autoclaved rice husk and saw dust at room temperature and were incubated at 30 °C at 150 rpm for 5-6 days in a shaking incubator. The immobilized samples were washed with sterile saline water for 3 times and were inoculated on BH agar plates with diesel.

Pure cultures of selected bacterial strains were inoculated with LB agar and were incubated over-night. A single colony of each bacterial strain was inoculated on 5 ml of LB broth. The cultured cells were centrifuged at 2000 g at 4°C for 10 minutes and the pellet was dissolved in 5 mL of phosphate buffer and re-centrifuged under the same conditions. Then the pellet was re-suspended in 5 ml of phosphate buffer. A mass of 14 g of autoclaved rice husk were mixed with 21 ml of YEG broth and was autoclaved. Then 2 ml of washed Bacterial cultures were inoculated on 2 g of autoclaved rice husk at room temperature separately and were incubated at 30 °C at 150 rpm for 5-6 days in a shaking incubator until a heavy culture develops. A volume of 20 ml of water was contaminated with 2 ml of diesel and 0.2 g of immobilized rice husk was added on top of the oil layers under sterile conditions. Turbidity and the time taken for the disruption of oil layer in the water were compared with a control.

Results and Discussion

A total of 10 bacterial strains were isolated from 18 samples of soil originating from three different places contaminated with petroleum hydrocarbons (Table 01).

Table 01: List of identified bacterial species and their risk assessment. The efficiency of the hydrocarbon degradation by the *A. oleivorans* and *P. odorifer* was evaluated individually.

Name of the Bacterial strain	Pathogenisity (Disease Caused)
Microbacterium oleivorans	Bacteremia
Ochrobactrum intermedium	Bacteremia
Pseudomonas aeruginosa	Opportunistic
Agrobacterium tumefaciens	Opportunistic
Achromobacter xylosoxidans	Opportunistic
Enterobacter aerogenes	Opportunistic
Raoultella ornithinolytica	Fish Pathogen
Acinetobacter calcoaceticus	Opportunistic
Paenibacillus odorifer	Non-Pathogenic
Acinetobacter oleivorans	Non-Pathogenic

Isolation was carried out using the traditional microbiological technique with petri dishes containing selective agars with hydrocarbons (petroleum, diesel and heavy fraction of refined petroleum) as the sole source of carbon. According to the experiment the rice husk was identified as the suitable immobilizing agent with compared to saw dust due to its floating ability, high binding affinity and high content of Silicon dioxide which delays biodegradation. The bacterial growth of immobilized saw dust on BH (Figure 1) medium was low compared to that of rice husk (Figure 2). A total of five bacterial strains (*M. oleivorans, P. aeruginosa* , *A. oleivorans* , *P. odorifer, R. ornithinolytica*) out of ten sequenced bacterial samples were identified as suitable candidates for the current research study due to their own specific properties of emulsifying and bioremediation capacities. Further, *A. oleivorans* and *P. odorifer* bacterial species were selected as the ideal candidates to introduce to the hydrocarbon contaminated sites in the natural environment for the bioremediation based on non pathogenic ability (Table 1

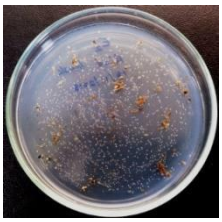

Figure 01: Immobilized saw dust on BH medium

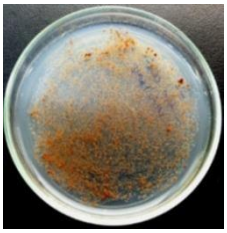

Figure 02: Immobilized rice husk on BH medium

The reduction in the hydrocarbon layer in the each tube was measured after 7 days of incubation period. The reduced height and volume of the hydrocarbon layer in tubes containing each bacterial strains and the mixture are given in table 2. It was observed that when introduce bacterial strains individually, showed similar efficiencies. However, there is a reduction in the efficiency of hydrocarbon degradation in cocktail consisting two bacterial strains. This may be due to the competition for the other requirements in the system. But, this may not be a critical limitation in the natural environment.

Table 02: Reduced height and volume if the hydrocarbon layer by each bacterial species

Bacterial species	Reduced height (mm)	Reduced Volume (mm^3) (Surface area 615 mm^2)
Paenibacillus odorifer	1.5	920 mm^3
Acinetobacter oleivorans	1.5	920 mm^3
Mixture of *Paenibacillus odorifer* and *Acinetobacter oleivorans* (Cocktail)	1	600 mm^3

The time period which was taken to carry out preliminary experiments was limited to four weeks and the results were assuring enough to prove that bioremediation of hydrocarbon. Although bioremediation is considered the ultimate resolution for remediation, it also holds some negative arguments. This method requires expert knowledge and labor-intensive field trials. Also there are certain regulatory boundaries to overcome before the application.

Conclusions

A. oleivorans and *P. odorifer* bacterial species showed significant potential in removing/detoxifying hydrocarbon contaminations in soil/water.

Acknowledgement

Financial assistance from ITI TG No. 11/5

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