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Petroleum Hydrocarbon Degradation Potential of *Ochrobactrum lupini* Isolated from BTEX Enrichment Soil

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ABSTRACT

Bioremediation is a process in which microorganisms metabolize contaminants either through oxidative or reductive processes. A total of thirty four bacterial cultures were isolated from hydrocarbon contaminated soil by enrichment media amended with 50 mg/L of benzene, toluene, ethylbenzene, *o-*, *m-* and *p*-xylene (BTEX), individually. The total cultivable bacterial isolates were screened for their growth on different concentrations of BTEX (50-250 mg/L). The number of BTEX utilizing bacteria decreased with the increase of BTEX concentration. The highest BTEX tolerant bacterial isolate (MS30) was chosen based on its higher colony size on solid basal salt medium amended with high BTEX concentration (250 mg/L). This bacterial culture had 16S rRNA gene sequence that was most similar to *Ochrobactrum lupini* with 97% similarity. Under laboratory conditions *O. lupini* was used for crude oil degradation in aqueous media and soil. The results show that *O. lupini* degraded 94.6% and 43.6% of total petroleum hydrocarbons (TPHs) in aqueous media and soil, respectively, in addition to a wide range of aliphatic and aromatic hydrocarbons. The knowledge of the potential of *O. lupini* to degrade hydrocarbons will increase the possibilities of developing models and strategies for removing hydrocarbon pollutants from the environment.

Key words: Soil bacteria, Hydrocarbons, BTEX, bioremediation, 16SrRNA

Introduction

Soil and groundwater contamination by petroleum hydrocarbons present has become a focus of great concern in all countries, due to its broad environmental distribution, which can reach soil, groundwater and air as a result of accidental oil spills, leaking underground storage tanks, oil extraction, and processing operations, producing a significant environmental burden (Lebrero *et al*., 2012). Petroleum hydrocarbons which constitute crude oil are categorized into four fractions: aliphatics, aromatics, resins and asphaltenes (Singh, 2006). The gasoline consists of relatively volatile hydrophobic hydrocarbons such as alkanes, cycloalkanes, Benzene, toluene, ethyl benzene and xylenes (BTEX), phenol and polycyclic aromatic hydrocarbons (PAH). These components are carcinogenic, mutagenic and toxic for human (Janbandhu and Fulekar, 2011). The high motility of these compounds causes a slow soil absorption and, consequently, a preferential water transport, thereby favoring the contamination of water reservoirs once they migrate fast in such medium (Nakhla, 2003).
Several conventional methods like land filling, incineration, air spurging, etc. have been used since early times for remediation of oily waste (Mandal *et. al*., 2007; Vidali, 2011), none of these methods is environment friendly solution (Sood and Baneari, 2009). Biodegradation by microorganisms is the primary mechanism in elimination of hydrocarbons and xenobiotic substances (Atlas, 1981). Bioremediation is non-disruptive, cost-effective and highly efficient method to remove petroleum components, including BTEX compounds (Atlas and Hazen, 2011). Several bacterial species, such as *Bacillus napthovorans*, *Halomonas eurihalina*, *Sphingomonas sp*., *Cycloclasticus sp.* and *Pseudoalteromonas sp.* (Zhuang *et al*., 2002; Martinez-Checa *et al*., 2002; Ye *et a*l., 1996; Geiselbrecht *et al*., 1998; Hedlund and Staley, 2006), were used for petroleum hydrocarbons degredation. Sohn *et al.* (2004) isolated polyaromatic hydrocarbon (PAH) degrading bacterium (*Novosphingobium pentaromativorans sp*. nov.) from estuarine sediment. The purpose of this study was 1) to isolate and identify the BTEX utilizing bacteria as a sole carbon source, 2) determine the efficiency of BTEX tolerant bacteria for petroleum hydrocarbon degradation in aqueous media and soil.

Materials and Methods

Bacterial isolation and enrichment culture

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The bacterial strains used in this study were isolated from contaminated soils collected from sites near gas stations. Soil samples were stored in closed containers at 4°C prior to use. Five grams of soil samples was added to 100 ml MBSM containing 1g of NH_4NO_3, 0.5g KH_2PO_4, 5.24g of $K_2HPO_4.3H_2O$, 0.2g of $MgSO_4.7H_2O$, 2 ml of 1% $CaCl_2$, 200 µl of 1% $FeCl_3$, 200 µl of 5%NaCl, 5 ml of 1% yeast extract, and 5 ml of trace elements solution contained in mg/L: 30 $CoCl_2 \cdot 6H_2O$, 0.15 $CuCl_2$, 5.7 H_3BO_3, 20 $MnCl_2 \cdot 4H_2O$, 2.5 $Na_2MoO_4 \cdot 2H_2O$, 1.5 $NiCl_2 \cdot 2H_2O$ and 2.1 $ZnCl_2$ (Hu *et al*., 2007; Li *et al*., 2010). In addition 50 mg/L of benzene, toluene, ethylbenzene, p-xylene, o-xylene, m-xylene, or BTEX mixture (1.1.1.1.1.1) was added to soil as a sole source of carbon. Soil incubated with shaking at 150 rpm for 7 days at 28°C. After five cycles of enrichment, 1 ml of the culture was serially diluted and 100 µl was spread on 1.5% agar MBSM plates amended with 50 mg/L BTEX, and incubated for 7 days. The selected bacterial isolates based on their colony morphology and size were cultivated overnight in LB broth.

Selection of BTEX degrading bacteria

Bacteria cultures grown in LB media were harvested and washed twice with 0.85% NaCl solution to remove the remaining carbon source. The washed bacterial cells were cultivated on agar MBSM plates amended with (50, 100, 150, 200, and 250 mg/L) of Benzene, Toluene, Ethyl benzene, p-xylene, o-xylene, m-xylene and/or BTEX mixture, and incubated at 30˚C. The highest tolerant bacterial isolate (250 mg/L) was selected for further investigations.

Bacterial identification

Genomic DNA of selected bacterial isolates were extracted according to Ausubel *et al.* (1987). Oligonucleotide primers were used to amplify the 16S rRNA gene fragments (Abou-Shanab *et al*., 2010). A Perkin-Elmer 377 DAN sequencer in combination with Dye Deoxy Terminator Cycle Sequencing Kit was used for sequencing the amplified PCR products. A BLAST search of GenBank database was used to identify named bacterial species.

Growth on crude oil

The selected isolate was grown on MBSM containing 2% crude oil. The growth curve of the selected isolate was determined by counting the colonies forming unit (CFU) per ml at Zero time and after 2, 3, 6, 7, and 10 days incubation. One ml of the culture was diluted to 10^{-6} and 100µl was streaked on LB agar plates and incubated for 24hr at 30˚C. The percentage of crude oil degradation was determined gravimetrically (Sakalle and Rajkumar, 2009).

Gas chromatographic analysis of residual crude oil

The ability of selected strain to aerobically degrade aliphatic and aromatic components of crude oil was determined in 100 ml flasks containing 25ml of MBSM and in Falcon tube (50 ml) contains 20g soil, both MBSM and soil amended with 4% crude oil and inoculated with 2ml of bacterial suspension with optical density 0.9 at 600nm. Un-inoculated flask and tube were used as control and experiment was incubated at 30°C, 150 rpm/min for 30 day. The residual crude oil was extracted by solvent mixture (acetone/methylene chloride) (1: 1, vol/vol). The organic layer was taken out and evaporated at room temperature. The extracted residue was re-suspended in 1 ml n-hexane from which 1 µl was injected in a GC for analysis (Ho-Sang and Oh-Seung, 2000).

Results and Discussion

Bacterial isolation and enrichment culture

The biodegradation of organic compounds becomes more efficient when the microorganisms are pre-selected and more adapted to target pollutants. The enrichment step was important for biodegradation success because selected microorganisms adapted to BTEX (Kataoka, 2001). Total culturable BTEX utilizing bacteria as a colony forming unit/ g soil was shown in Table 1. The highest number was obtained using O-Xylene (24 x 10^6) and benzene 23 x 10^6) as a sole source of carbon. Out of these cultures thirty four bacterial isolates were selected based on colony morphology and size for further investigations.

Selection and identification of BTEX utilizing bacteria

The bacterial isolates were screened to grow on different concentrations of BTEX compounds (from 50 to 250 mg/L). It is particularly important to study substrate interaction at different concentration since substrate toxicity is experienced by the cells, especially at high concentration. Substrate inhibition due to critical concentration is also said to be cell strain dependent. The inhibitory effect of the organic compounds increase with the increase of their concentration (Figure 1). The o-xylene was found to be markedly recalcitrant and persistent compared to other BTEX compounds (Littlejohns and Daugulis, 2008).

Table 1: The total bacterial count (CFU/g soil) on MBSM agar plate amended with 50 mg/l of individual benzene, toluene, xylene, ethyl-benzene and BTEX mixture

Carbon source	Bacterial Count (CFU/g soil) x 10^6
Control	0.5
Benzene	23
Toluene	7
Ethylbenzene	7
O-Xylene	24
P-Xylene	9
m-Xylene	4
BTEX-mixture	9

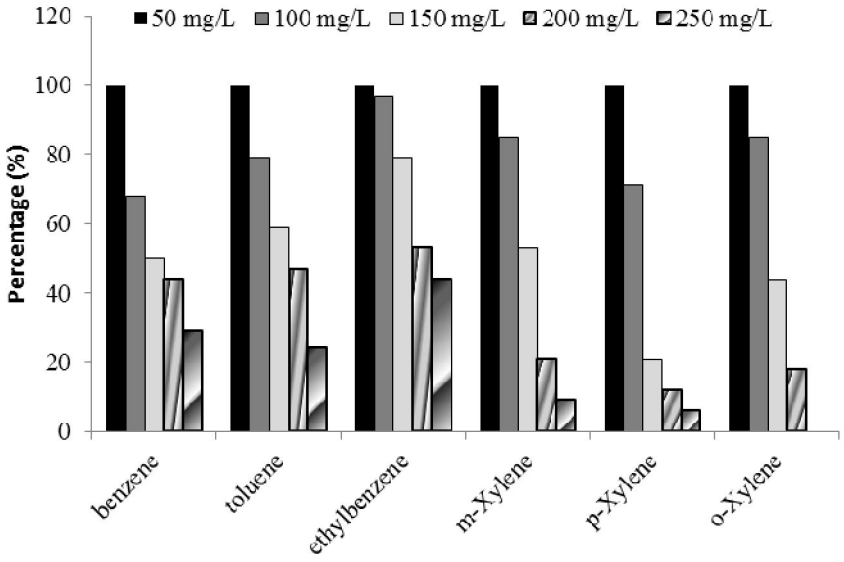

Fig. 1: Susceptibility of thirty four bacterial isolates to the different concentrations of organic compounds.

The isolate MS30 was identified as *Ochrobactrum lupini* based on 16SrRNA. The identification of this isolate was confirmed by the phylogenetic tree (Figure 2). This isolate was able to grow on 250 mg/L and selected for further investigations. EL-Sayed *et al*., 2003 isolated a bacterial strain AS1 belonging to the genus *Ochrobactrum*, from an enriched phenol-activated sludge in Egypt. The ability of the bacterial cell to degrade a wide variety of aromatic compounds makes it a good candidate for use in biodegradation of hazardous wastes (Zhang *et al*., 2013).

Fig. 2: A neighbor-joining tree showing the phylogenetic relationships of strain MS30 to the closely related strains.

Growth rate of O. lupini on crude oil

The growth rate of *O. lupini* on 2% crude oil as a sole source of carbon was illustrated in Figure 3. The log 10 cfu/ml was 10 after 3 day incubation at 30^{o}C. In addition, to evaluate the degradation potential of this

strain, crude oil degradation in culture media was determined gravimetrically after 13 day of incubation. *O. lupini* degraded more than 89% of crude oil (4%). This might account for the ability of this strain to survive in a given concentration of crude oil higher than the crude oil concentration was mentioned by Minoui, *et al*., 2008.

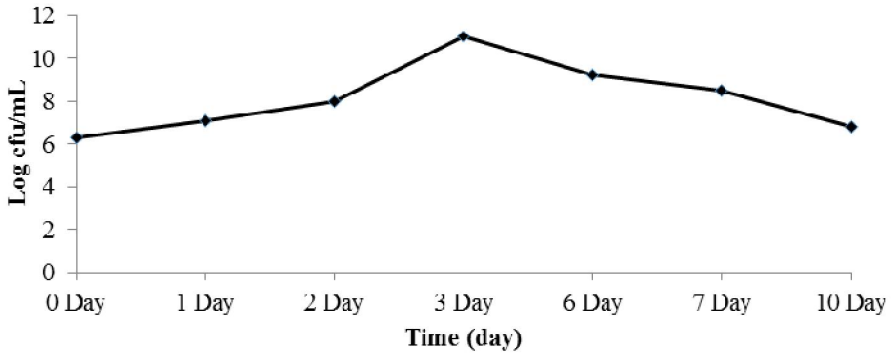

Fig. 3: The growth rate of *O. lupini* on liquid MBSM amended with 2% crude oil.

The degradation of some petroleum hydrocarbons

The results of petroleum hydrocarbons degradation in soil and aqueous media by *O. lupini* were shown in Figure 4. *O. lupini* degraded 94.6 and 43.6 % of TPHs in aqueous media and soil, respectively. Also it was capable of degrading wide a range of aliphatic hydrocarbons (C14-C30) in aqueous media and soil in addition of some aromatic hydrocarbons.

The successful use of microbial inocula in soils requires that the microorganisms contact the contaminant. Physical adsorption to soil particles may limit the transport of organisms and relatively low the crude oil degradation could be explained by possible water and nutrient limitations due to scare watering and no nutrient addition (Kuyukina and Ivshina, 2010; Mariano *et al*., 2007). Yirui *et al*., 2009 reported that *Ochrobactrum sp*. BAP5, degrading a wide range of PAHs, belongs to another versatile PAH-degrading bacterial strain with a potential ability for the bioremediation of PAHs in PAHs-contaminated soils and sediments.

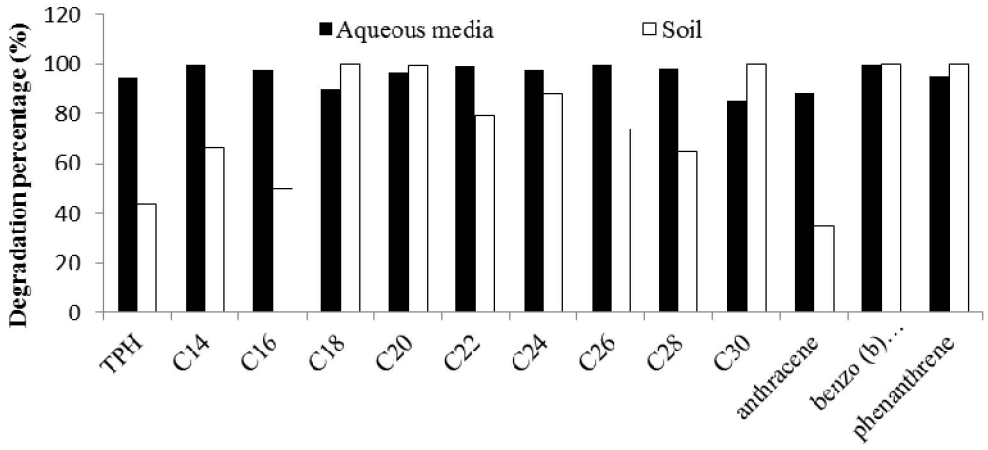

Fig. 4: Degradation percentage of total petroleum hydrocarbons (TPHs), some aliphatic and aromatic hydrocarbons by O. lupini in aqueous media and soil supplemented with 4 % crude oil after 30 day incubation.

Conclusion

A bacterial strain, *O. lupini* , capable of utilizing BTEX as sole carbon source and degrading a wide range of aliphatic and aromatic hydrocarbons. *O. lupini* was able to grow on MBSM plates amended with 250 mg/L benzene, toluene, ethylbenzene, *o-*, *m-* and *p*-xylene individually. The log number of bacterial cultures

was decrease with the increase of hydrocarbon concentrations. The GC analysis referred that *O. lupini* was a promising strain for decontaminate the environment from petroleum hydrocarbon contamination.

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