2012 International Conference on Biological and Life Sciences
IPCBEE vol.40 (2012) © (2012) IACSIT Press, Singapore

Isolation and Characterization of Bacteria Degrading n-Hexadecane from Soil

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Abstract. Three n-hexadecane (HXD) degrading bacteria were isolated from petroleum and diesel oil-contaminated soils using enrichments process on HXD. The phylogenetic diversity of the isolates was evaluated by 16S rDNA gene sequence analysis which identified the isolates as *Pseudomonas aeruginosa* and *Acinetobacter radioresistens*. HXD degradation was evaluated by isolates. After 31 days, n- hexadecane concentration showed 55.27 to 85.67% decrease with *isolates* and the consortium. Results showed that mixed cultures are more efficient in biodegradation than the pure cultures. Our findings showed that the bacteria with different n-Hexadecane uptake mode also have different response to salinity. All of these bacteria were halotolerant. These finding suggests the selective pressure of natural ecosystems on evolution of more tolerant and more potentiated organisms in biodegradation.

Keywords: Isolation, Hexadecane, PCR, 16S rDNA.

1. Introduction

Petroleum hydrocarbons are the most frequent environmental pollutants. About 60 % of the marine-transported oil in the world is produced in Persian Gulf (Hasanshahian et al. 2012). Then oil pollution is an acute problem in this area. Contamination of soil and groundwater by diesel released from underground storage tanks is an important and extensive environmental problem in Iran. Hexadecane (HXD), $C_{16}H_{34}$, has been used as a model contaminant of diesel oil by many researchers (Bouchez-Naitali et al. 1999; Noordman et al. 2002). Biodegradation is a major mechanism that removes pollutants from the environment (Pepi et al. 2005). Many bacterial strains capable of degrading petroleum hydrocarbons have been isolated (Lu et al. 2006). Hasanshahian et al. isolated 25 petroleum degrading bacteria from petroleum contaminated sites in the Persian Gulf and the Caspian Sea. Most reports are enforced that n-Alkane -degrading bacteria isolated from oil-contaminated sites are mesophilic strains belonging to different bacterial species such as Acinetobacter (Daisuke et al. 2001), Nocardia sp. CF8, Planococcus alkanoclasticus (Engelhardt et al. 2001) and Ochrobactrum (Yuan et al. 2005).Wang et al. isolated a novel thermophilic Bacillus strain degrading long-chain n-alkanes (Wang et al. 2006). The aim of this study was to isolate and characterize HXD-degrading bacteria capable of tolerating high salinity to be used in bioremediation process. In this study, we report isolates capable of efficiently growing on HXD and growth in extreme environments.

2. Materials and Methods

2.1. Sampling Site and Isolation Conditions

The soil selected for this study was a chronically contaminated soil from 3 locations from Iran. Soils were sampled from 0–20 cm depth. Mineral salts medium (MSM) was prepared from minimal medium

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containing following minerals (in g l^{-1}): NH_4Cl, 4.0; KH_2PO_4, 2.5; NaCl, 0.5; $MgSO_4$, 0.3; $FeCl_3.6H_2O$, 0.03; $CaCl_2$, 0.01; and $MnCl_2.4H_2O$, 0.01 (Partovinia et al. 2010). The pH of the medium was adjusted to 7 (± 0.4). All chemicals used in this study were of analytical grade. To achieve n-hexadecane-degrading bacteria from contaminated soil, aliquots of soil (1 g) were added to 9 ml MSM medium supplemented with 1 ml of HXD as sole carbon and energy source. Cultures were vigorously shacked for 2 min. For pure culture preparation, subculturing was done on the 20 ml MSM agar plates and incubated for 7 days at 32 °C. Single colonies with different color and\or shape were selected and subcultured on the fresh MSM agar plates.

2.2. Genetic Identification

Genetic identification of isolated bacteria was performed according to nucleotide sequence of 16S ribosomal DNA (rDNA). Bacterial 16S rDNA was amplified with universal previously described primers 16F27 (5′-CCAGAGTTTGATCMTGGCTCAG-3′) and 16R1488 (5'-AGAGTTTGATCMTGGCTCAG-3') (Molina et al. 2009). PCR was performed in a reaction mixture with total volume of 25 µl, containing 20 µl sterile water, 2.5 µl 10x Taq polymerase buffer, 0.3 µl dNTPs (10 mmol l^{-1}), 1 U Taq DNA polymerase and 25 pmol l^{-1} from each primers. The PCR program consisted of initial denaturation (96 °C, 4 min), denaturation (94°C, 30 s), annealing (58 °C, 20 s), polymerization (72 °C, 50 s) and the final extension (72 °C, 8 min) with 30 cycles was carried using an Eppendorf personal thermal cycler. The PCR product was purified and bidirectional sequencing of fragments amplified by the PCR was performed using ABI 3730X capillary sequencer (Genfanavaran; Macrogen, Seoul, Korea). BLAST software was used to find nearly identical sequences for the 16S rDNA sequences determined. Nucleotide sequences of 16S rDNA determined for Acinetobacter radioresistens, Pseudomonas aeruginosa, Pseudomonas aeruginosa in this study, were deposited in GenBank database under the accession numbers JQ397280, JQ397281 and JQ397282, respectively.

2.3. Biodegradation and Salinity Tolerance Tests

In order to test the ability of isolated strains and the consortium to degrade HXD, 1 ml of overnight NB (Nutrient Broth) culture of strains (OD_{600}=1) were inoculated into 30-ml of the MSM supplemented with HXD (10000 mg l^{-1}) in 50-ml Erlenmeyer flasks. The flasks were sealed with rubber stoppers and incubated at 34°C, 120 rpm. Residual HXD was extracted using a liquid–liquid extraction technique with acetone/hexane (1:1) analyzed by GC-FID after 12 and 31 days. AHP-5MS (Agilent, USA) column (5% phenyl 95% methylpolysiloxane; 30 m length × 0.025 mm id × 0.25 µm film thickness) was used at a temperature program of 120°C for 1 min, increased to 180°C at 20°C /min, and held at 180°C for 5 min. Nitrogen was used as a carrier gas at a constant flow of 1.5 ml/min. Injector and detector temperatures were 250 and 270°C, respectively.

The effect of different salinity concentrations on bacterial population growth was determined by incubation of bacterial cells in 10 ml NB medium supplemented with 0, 2, 4, 6 and 8 (w/v) NaCl at 34°C, 120 rpm. The growth was monitored by determinations of OD600 after 3, 6, 9, 12 and 24 h.

3. Results

3.1. Isolation and Identification of Bacteria

Three isolates (*ASIa*, *ASIDa* and *ASIIDa*) were obtained which grew on MSM plates with HXD. On MSM plate, *ASIDa* and *ASIIDa* produced colorless, non-spreading, smooth, irregular, wet and transparent colonies, while *ASIa* colonies were cream, circular, non-spreading, wet and convex. Gram staining of isolates showed their gram negative characteristics. Sequence analysis based on 16S rDNA fragment and phylogenetic investigation using BLAST software identified *ASIa* as Acinetobacter radioresistens, while *ASIDa* and *ASIIDa* identified as Pseudomonas aeruginosa. The identity of isolated was confirmed by conventional bacteriological tests.

3.2. HXD Degradation and Salinity Tolerance Test

Fig. 1 shows the removal of 300 mg n-Hexadecane by isolated strains and consortium after 12 and 31 days incubation in MSM medium at 34°C, 120 rpm. It was observed that, in batch cultures, all the strains were able to grow using HXD (Fig. 1). After 31 days, n- Hexadecane concentration decreased to 134.18 mg

(55.27%) with *ASIa*, 52 mg (82.67%) with *ASIDa*, 79.47 mg (73.51%) with *ASIIDa* and 43 mg (85.67%) with the consortium.

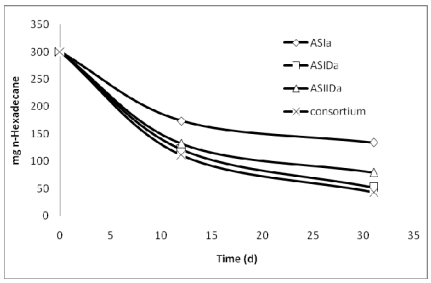

Fig. 1: The removal of HXD by strains and consortium in MSM supplemented with 300 mg of HXD.

The growth of bacterial strains under different salinity concentrations was evaluated. Fig. 2 shows average growth of strains (0, 2 and 4 % NaCl) at 0-24 h incubation. The growth of bacterial strains was measured at absorbance 600 nm.

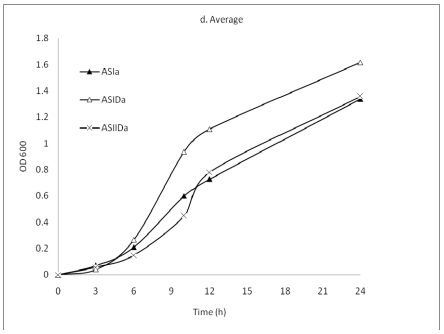

Fig. 2: Effect of salinity changes on the growth of strains.

4. Discussion

Three indigenous bacterial strains capable of HXD degradation were isolated and identified by 16S rDNA sequence analysis from polluted soils. The strains *ASIDa*, *ASIIDa* were identified as *Pseudomonas aeruginosa* and *ASIa* identified as *Acinetobacter radioresistens*.

After 31 days, HXD concentration (10g/l) showed 55.27%, 82.67%, 73.51% and 85.67% decrease with *ASIa*, *ASIDa*, *ASIIDa* and the consortium, respectively. Other investigators have shown 37% and 86.4% HXD biodegradation after 31 days of culture, by *Aspergillus niger* (Setti et al. 1993) and *Pseudomonas spp.* (Volke-Sepulveda et al. 2003) at an initial concentrations of 80 g/l and 12 g/l, respectively. This finding together with our recent data indicates that the mixed cultures were more effective in degrading HXD than the pure cultures. It was revealed that presence of each bacterium in culture medium had a cumulative enhancing effect on HXD degradation. It was also reported by other investigators that degradation of hydrocarbons by consortium is more effective than individual bacteria (Bharagava et al. 2009; Lu et al. 2006).

Salt is a common co-contaminant that can adversely affect the biodegradation of pollutants by microorganisms (Ulrich et al. 2009). In this study, the effect of salinity was investigated and it was revealed that bacteria show advanced growth in salinity ranging from 0% to 2%. Our findings indicate that *ASIa* and *ASIDa* were halotolerant but *ASIIDa* may be halophil.

5. Acknowledgements

The authors gratefully acknowledge the financial and technical support provided by the Tarbiat Modares University, Tehran, Iran.

6. References

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