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Isolation and characterization of alkane degrading bacteria from petroleum reservoir waste water in Iran (Kerman and Tehran provenances)

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ABSTRACT

Petroleum products spill and leakage have become two major environmental challenges in Iran. Sampling was performed in the petroleum reservoir waste water of Tehran and Kerman Provinces of Iran. Alkane degrading bacteria were isolated by enrichment in a Bushnel–Hass medium, with hexadecane as sole source of carbon and energy. The isolated strains were identified by amplification of 16S rDNA gene and sequencing. Specific primers were used for identification of alkane hydroxylase gene. Fifteen alkane degrading bacteria were isolated and 8 strains were selected as powerful degradative bacteria. These 8 strains relate to *Rhodococcus jostii*, *Stenotrophomonas maltophilia*, *Achromobacter piechaudii*, *Tsukamurella tyrosinosolvens*, *Pseudomonas fluorescens*, *Rhodococcus erythropolis*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* genera. The optimum concentration of hexadecane that allowed high growth was 2.5%. Gas chromatography results show that all strains can degrade approximately half of hexadecane in one week of incubation. All of the strains have alkane hydroxylase gene which are important for biodegradation. As a result, this study indicates that there is a high diversity of degradative bacteria in petroleum reservoir waste water in Iran.

1. Introduction

Petroleum hydrocarbons are the most common environmental pollutants and oil spills pose a great hazard to terrestrial and marine ecosystem. Oil pollution may arise either accidentally or operationally whenever oil is produced, transported, stored, processed or used at sea or on land. Oil spills are a major menace to the environment as they severely damage the surrounding ecosystem (Head et al., 2006).

The traditional methods to cope with oil spills are confined to physical containment. Biological methods can have an edge over the physicochemical treatment in removing spills as they offer a biodegradation of oil fraction by microorganisms (Chaillan et al., 2004; Hanson et al., 1997).

Microbiological decontamination of oil derivatives in polluted environments is claimed to represent an efficient, economic and versatile alternative to physicochemical treatment (Emtiazi et al., 2009). The rate of biodegradation depends on oil concentration, alkanes length, biosurfactant and type of microorganisms (Cappello et al., 2012a). It has been observed that the saturated components of crude oil (alkanes) are particularly the alkanes of intermediate length (C_{10}–C_{20}) which are biodegraded more readily (Subarna et al., 2002). The rate of uptake and mineralization of many organic compounds depend on the concentration of the compound, high concentration of hydrocarbon which causes inhibition of biodegradation. It is done by nutrient or oxygen limitation or through toxic effects exerted by volatile hydrocarbons (Luis et al., 2000; Chaneau et al., 2005; Emtiazi et al., 2005).

The growth of microorganisms on hydrocarbons is often accompanied by the emulsification of the insoluble carbon source in the culture medium. In most cases, this has been due to the production of extra cellular emulsifying agents, during the breakdown of hydrocarbons. These processes aid microorganisms to grow on and metabolize crude oil (Cappello et al., 2012b).

Alkane hydroxylase is a key enzyme in alkane degradation. This enzyme which introduces oxygen atom is derived from molecular oxygen which is in the alkane substrate and plays an important role in crude oil bioremediation. Alkane hydroxylase genes are

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classified into three groups through phylogenetic analysis. The alkane hydroxylase group (I) is encoded as *alk-B* gene which catalyzes medium chain length (C_6–C_{12}) *n*-alkanes. The gene classified group (II) is encoded *as alk*-M which catalyzes long chain alkanes >C_{12} and are possessed by *Acinetobacter*. The gene classified group (III) is encoded as *alk-B* which is unknown for substrate specificity, alkane oxidation pathway and oxidation system (Kohno et al., 2002; Heissblanquet et al., 2005).

Oil reservoirs have a complex mixture of microbial community which is related to type of hydrocarbons. Waste water produced from these oil reservoirs makes many environmental problems. The elimination of pollutant from this waste water is important for quality control of the oil reservoirs (Ghanavati et al., 2008).

The aims of this study were to isolate and characterize hexadecane degrading bacteria from petroleum reservoirs waste water in two different regions of Iran. In this study, we report some bacterial strains capable of efficiently growing on hexadecane.

2. Materials and methods

2.1. Sampling

To isolate of alkane hydrocarbon degrading bacteria from contaminated soils and petroleum reservoirs waste water, samples were collected from 16 different sites in Tehran and Kerman petroleum reservoirs regions (36°15, N; 44°15, E). Sixteen samples had been collected into sampling, 5 samples have collected from Kerman Petroleum reservoirs waste water, the other 5 samples from Tehran petroleum reservoirs and the remaining 6 samples had been collected from soil, contaminated to hydrocarbons. The soil samples were collected from 1 to 12 cm below the surface of soil using a sterile knife. Waste water samples were collected from a depth of 3 cm, in sterile 100 ml bottles and had been transported on ice to the laboratory for isolation (Hassanshahian et al., 2012b).

2.2. Isolation and selection of alkane degrading bacteria

A synthetic Bushnell Haas Mineral Salts medium (BHMS) was used for the isolation of alkane degrading bacteria (Kohno et al., 2002). BHMS medium contain ($g\,l^{-1}$) KH_2PO_4, 1; K_2HPO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.02; NH_4NO_3, 1; and 2 droplet of $FeCl_3$ 60%. The pH adjusted to 7 (Cappello et al., 2012b). BHMS medium was supplemented with 1% (v/v) hexadecane as sole carbon source and energy. Portion of soil (1 g) or waste water (1 ml) samples were added to 250 ml Erlenmeyer flasks containing 100 ml BHMS medium and the flask incubated for 10 days at 30 °C on rotary shaker (INFORS AG, Switzerland) operation at 180 rpm. Then 5 ml aliquots were removed to a fresh BHMS medium. After a series of four further subcultures, inoculums from the flask were streaked out and phenotypically different colonies were purified on BHMS agar medium. Phenotypically different colonies obtained from the plates were transferred to a fresh BHMS medium with and without hexadecane to eliminate autotrophs and agar-utilizing bacteria. The procedure was repeated and isolated, only exhibiting pronounced growth on hexadecane were stored for further characterization (Chaillan et al., 2004; Hassanshahian et al., 2012a).

2.3. Identification of isolates

2.3.1. Biochemical characterization

To identify and characterize the isolated bacteria, biochemical tests such as gram staining, oxidation/fermentation, production of acid from carbohydrates, hydrolysis of gelatin and citrate were carried out according to the Bergey's manual of Systematic Bacteriology (Holt et al., 1998).

2.3.2. Molecular identification of bacteria and detection of alkane hydroxylase gene (alk-B) in the isolated bacteria

Analysis of 16S rDNA was performed to the taxonomic characterization of isolated strains. Also, the purified DNA extracts were subsequently screened by PCR to detect catabolic *alkB* gene that encodes enzymes involved in alkane degradation pathways. Total DNA extraction of bacterial strains was performed with the CTAB method (Winnepenninckx et al., 1993). The bacterial 16S rDNA loci was amplified using the forward domain specific bacteria primer, Bac27_F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer Uni_1492R (5′-TACGYTACCTTGTTACGACTT-3′). Alkane hydroxylase gene was detected by *alk*-3F (5′-TCGAGCA-CATCCGCGGCCACCA-3′) and *alk*-3R (5′-CCG TAG TGCTCGACG-TAGTT-3′) primers (Kohno et al., 2002; Hassanshahian et al., 2010). The amplification reaction was performed in a total volume of 50 μl consisting, 1*X* solution Q (Qiagen, Hilden, Germany), 1*X* Qiagen reaction buffer, 1 μM of each forward and reverse primer, 10 μM dNTPs (Gobco, Invitrogen Co., Carlsbad, CA), and 2 U of Qiagen Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a thermacycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept, 95 °C for 5 min (1 cycle); 94 °C for 1 min and 72 °C for 2 min (35 cycles), followed by 72 °C for 10 min at the end of final cycle (Troussellier et al., 2005). PCR products were visualized by gel electrophoresis using a horizontal 2% agarose gel (Sigma, St. Louis, MO) with 1*X* TBE buffer. Gels were stained in a solution of ethidium bromide and visualized with a UVP UV transilluminator (UVP Inc., San Gabriel, CA) (Kohno et al., 2002; Sei et al., 2003).

The 16S rDNA amplified was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Analysis and phylogenetic affiliates of sequences was performed as previously described (Yakimov et al., 2006; Maidak et al., 1997).

2.4. Growth and hexadecane removal assay

Growth curves of bacteria in the study were routinely assessed indirectly by turbidity measurement ($O.D_{600}$ nm) in a UV–visible spectrophotometer (Shimadzu UV-160, Japan). The hexadecane removal assay was carried out by Gas Chromatography Flame Ionization Detector (GC-FID) analysis (Hassanshahian et al., 2012a).

2.5. Extraction of hexadecane and gas chromatography analysis

Residual hexadecane in liquid was extracted using a liquid–liquid extraction technique with acetone/hexane (1:1) and was analyzed by GC-FID for residual hexadecane (Chaneau et al., 2005). First, liquid was placed in the refrigerator until the hexadecane becomes a solid. After 4 h, 5 ml of hexane and 5 ml of acetone was added and was stirred vigorously for 2 min. The liquid obtained was centrifuged for 10 min, 5000 rpm. The upper liquid was then passed through a PFTE filter (0.22 μm). Then, hexadecane concentrations were determined by GC-FID. In order to avoid losses of hexadecane during extraction, the whole sample was extracted directly in the experimental flasks. Then, hexadecane concentrations were determined by GC-FID. 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 injected volume was 2 μl and dodecane ($C_{12}H_{26}$) was used as internal standard (Chaneau et al., 2005).