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Enrichment and identification of naphthalene-degrading bacteria from the Persian Gulf

Mehdi Hassanshahian *, Negar Amini Boroujeni

Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, Iran

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ABSTRACT

Naphthalene is a ubiquitous pollutant of the marine environment, and naphthalene biodegradation has been receiving constant scientific consideration. For cleanup of aromatic contaminated sites, bioremediation methods are considered as economical and safe approaches for the marine environment. The aims of this research are isolation and characterization of naphthalene-degrading bacteria from some marine samples of the Persian Gulf. Fifty four naphthalene-degrading bacteria were isolated from marine samples (sediment and seawater) that are enriched in ONR7a medium with naphthalene as the only carbon source. Some screening tests such as growth at high concentration of naphthalene, bioemulsifier production and surface hydrophobicity were done to select the best and prevalent strains for naphthalene degradation. Determination of the nucleotide sequence of the gene encoding for 16S rRNA shows that these isolated strains belong to these genera: *Shewanella*, *Salegentibacter*, *Halomonas*, *Marinobacter*, *Oceanicola*, *Idiomarina* and *Thalassospira*. These strains can degrade half of the percentage of naphthalene in 10 days of incubation. This research is the first report on isolation of these genera from the Persian Gulf as naphthalene-degrader.

1. Introduction

Asphaltenes, heterocyclic, aliphatics and aromatics are four oil components which are regarded as main chemical pollutants. Among these four groups, cyclic aromatic compounds are regarded as stable compounds due to having benzene rings and since they afflict great harm to mammals, they are of great concern (Ben Said et al., 2008; Bayat et al., 2015). In fact they are regarded as one of the toxic and mutagen compounds which can cause cancer and can easily get solved and replaced with in fatty tissues. These compounds can be found abundantly in water and land ecosystems. They pollute the environment and as a result natural sources are wasted (Bayat et al., 2016; Hassanshahian et al., 2014b). The main factors involved in the entrance of these compounds to the environment include: industrial sewage waste water, home sewage, oil material extractions, and the factories involved in making medicine and drugs, paint, plastic and insecticides. Naphthalene is the first in cyclic aromatic compounds which is a common pollutant in water and its poisonous feature is proved since it can hinder mitochondrial respiration (Dabestani and Ivanov, 1999; Johnsen et al., 2005). One way to reduce pollutants, especially PAHs, is biologic approach and applying microorganisms which is known as bioremediation. Bacteria have different decomposing enzymes, a feature which makes them outstanding among all other microbes (Cappello et al., 2012; Khazi et al., 2010).

Biological methods have an edge over the physicochemical treatment regimes in removing spills since they offer in situ biodegradation of PAH compounds and oil fractions by the microorganisms (Ghanavati et al., 2008). Microbial degradation is the major route through which PAHs are removed from contaminated environments (Hassanshahian et al., 2012a). Bacterial biodegradation of lower molecular weight PAHs is well characterized, but less information is available on the degradation of higher molecular weight PAHs although they serve as growth substrates for a number of soil bacteria (Kom et al., 2012; Hassanshahian et al., 2012b).

Naphthalene, the simplest PAH, has long been used as a model compound in PAH bioremediation studies. Common naphthalene-degrading bacteria include *Pseudomonas* spp., *Vibrio* spp., *Mycobacterium* spp., *Marinobacter* spp., and *Sphingomonas* spp. Although many naphthalene degrading bacteria have been isolated, these bacteria may thrive in one environment but may not be able to compete with other microorganisms in another environment as environmental conditions will impose a selection pressure on specific types of bacteria (Nair et al., 2008; Hassanshahian et al., 2013).

The Persian Gulf is an important marine environment in south of Iran that supplies approximately 60% of the marine-transported oil in the world. This area was polluted with crude oil during the 1990 to 1991 Persian Gulf War. Since the ecological impacts of oil pollution are truly serious in these areas, bioremediation strategy seems necessary to efficiently eliminate environmental pollutants. PAH bioremediation is considered an effective and environmentally benign cleanup technology as it involves the partial or complete bioconversion of these

* Corresponding author.
E-mail addresses: mshahi@uk.ac.ir, hasanshahi@gmail.com (M. Hassanshahian).

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pollutants to microbial biomass, carbon dioxide and water (Hassanshahian et al., 2010; Radwan et al., 2005).

The main objective of the present study was to characterize some naphthalene degrading bacteria from different zones of the Persian Gulf that are chronically polluted with heavy crude oil. Also identification of these bacteria and biodegradation ability of these isolates are another aims of this research.

2. Material and methods

2.1. Seawater and sediment samples

For isolation of naphthalene-degrading bacteria, marine environmental samples were collected from various sites in the Persian Gulf. Three oil contaminated stations were selected for sampling. These three sampling sites are located at Khark island (39°15, N; 24°15, E), Bushehr shoreline (47°31, N; 39°16, E) and Aboumosa island (59°15, N; 14°15, E). The samples included contaminated seawater and marine sediment. Sediment samples (100 g) were taken from 1 to 12 cm below the surface using a sterile knife and seawater samples were collected from a depth of 15 cm in sterile 1000-ml bottles and transported on ice to the laboratory for isolation on the same day.

2.2. Isolation and selection of naphthalene-degrading bacteria

Naphthalene-degrading bacteria were isolated in ONR7a medium supplemented with 200 ppm of naphthalene as sole carbon source and energy. ONR7a contained (per liter of distilled water) 40 g of NaCl, 11.18 g of $MgCl_2 \cdot 6H_2O$, 3.98 g of Na_2SO_4, 1.46 g of $CaC1_2 \cdot 2H_2O$, 1.3 g of TAPS0 {3-[N tris(hydroxymethyl) methylamino]-2 hydroxypropanesulfonic acid}, 0.72 g of KCl, 0.27 g of NH_4Cl, 89 mg of $Na_2HP0_4 \cdot 7H_20$, 83 mg of NaBr, 31 mg of $NaHCO_3$, 27 mg of H_3BO_3, 24 mg of $SrCl_2 \cdot 6H_20$, 2.6 mg of NaF and 2 mg of $FeCl_2.4H_20$. For solid media, bacterial agar (15 g/l) was added to the solution (Hasanshahian and Emtiazi, 2008).

The seawater was filtered by sterile 0.2 μm paper filter and then the paper filter was added to the ONR7a medium with 5 ml of seawater. Also, portions of sediments (10 g) were added to Erlenmeyer flasks containing 100 ml of medium and the flasks were incubated for 7 days at 30 °C on a rotary shaker (180 rpm, INFORS AG). Then 5 ml was transported to fresh medium. After a series of four further subcultures, inoculums from the flask were streaked out, and phenotypically different colonies on ONR7a agar were purified. Phenotypically different colonies obtained from the plates were transferred to fresh medium with and without naphthalene to eliminate autotrophic and agar utilizing bacteria. The procedure was repeated, and only isolates exhibiting pronounced growth on naphthalene were stored in stock media with glycerol at −20 °C for further characterization (Hassanshahian et al., 2014a).

2.3. Growth rate and naphthalene removal assay by bacterial strains

Growth curves of the isolates were routinely assessed indirectly by turbidity measurement as (O.D. at 600 nm). To estimate the remaining naphthalene after time course standard graphs were prepared using 1 to 10 (ppm) of naphthalene. The naphthalene removal assay was carried out using calibration curve of naphthalene at 276 nm (Shamsuzzaman and Barnsley, 1974).

2.4. Identification of the isolates

2.4.1. Biochemical characterization

The following characteristics were determined according to the "Bergey's Manual of Determinative Bacteriology": the gram stain, motility, starch hydrolysis, indole, H_2S production, catalase and oxidase, oxidation/fermentation, reduction of nitrate, growth and acidification of carbohydrates tests were performed (Holt et al., 1998).

2.4.2. Molecular identification

Analysis of 16S rRNA was performed to taxonomically characterize the isolated strains. Total DNA of bacterial strains was extracted with the CTAB method. PCR amplification of 16S rRNA genes was performed using the general bacteria primer 27F (5-AGAGTTTGATCCTGGCTCAG-3) and universal reverse primer 1492R (5-TACGYTACCTTGTTACGACTT-3) (Cappello et al., 2012; Hassanshahian et al., 2014c). The amplification reaction was carried out in a total volume of 25 μl consisting, 2 mM $MgCl_2$ (1 μl), 10X PCR reaction buffer (200 mM Tris; 500 mM KCl) (2.5 μl), 2 mM each dNTP (2 μl), 0.15 mM each primer (1 μl), 1 U (0.5 μl) Taq DNA polymerase (Qiagen, Hilden, Germany) and 2 μl of template DNA (50 pmol). Total volume was brought up to 15 μl using sterile MilliQ water. PCR program consisted of 35 cycles was performed in a thermal cycler GeneAmp 5700 (PE Applied Biosystem, Foster City, CA, USA). The temperature profile for PCR was kept, 94 °C for 5 min, 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, 30 cycles; then 72 °C for 10 min and finally storage at 4 °C. The 16S rRNA amplified was sequenced with Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic Analyzer, Applied Biosystems). Similarity rank from the Ribosomal Database Project RDP) and FASTA Nucleotide Database Query was used to determine partial 16S rRNA sequences to estimate the degree of similarity to other 16S rRNA gene sequences. Analysis and phylogenetic affiliates of sequences were performed as following the protocol described by Yakimov et al. (2007). Phylogenetic tree was drawn by MEGA5 software with neighbor joining method.

2.5. Measure of emulsification activity (E_{24}) and bacterial adhesion to hydrocarbon (BATH)

The emulsification activity (E_{24}) was determined by the addition of hexadecane, to the same volume of cell free culture broth, mixed with a vortex for 2 min and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm). The bacterial adhesion to hydrocarbon (BATH) assay was used to recognize changes in cell surface hydrophobicity during growth on minimal salt medium with 200 ppm naphthalene (Rosenberg et al., 1980). Bacterial adhesion to hydrocarbon was carried out according to a procedure explain by Pruthi and Cameotra (1997).

2.6. Growth assessment of isolated bacteria in different concentrations of naphthalene

In order to determine the bacteria growth curve in different concentrations of naphthalene ONR7a medium was supplemented with various concentrations of naphthalene (200, 300, 400, 500 and 600 ppm). The flasks were incubated for 7 days at 30 ± 1 °C on a rotary shaker, operating at 180 rpm (Shaker INFORS AG, Switzerland). Growth was routinely assessed indirectly by turbidity measured as optical density (OD600 nm) in a UV–visible spectrophotometer (Shimadzu UV-160, Japan) (Nnamchi et al., 2006; Khazi et al., 2010).

2.7. Gas chromatography (GC) of residual naphthalene

Residual naphthalene in culture medium was calculated using gas chromatography (GC). After seven days of incubation, 5 ml of culture medium broth was used to assay the remaining naphthalene. Therefore, 2 ml of ethyl acetate/methanol (89:11) was added in the culture medium broth and the mixture was extracted twice by agitating in the vortex for 5 min. Supernatant was removed and put into vessel containing anhydrous sodium sulfate. Extracts were condensed by evaporation of the

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