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Evaluation of heavy petroleum degradation using bacterial-fungal mixed cultures

Hassan Ghorbannezhad[a], Hamid Moghimi[a,*], Seyed Mohammad Mehdi Dastgheib[b]

[a] Department of Microbial Biotechnology, School of Biology, College of Science, University of Tehran, Tehran, Iran
[b] Microbiology and Biotechnology Research Group, Research Institute of Petroleum Industry, Tehran, Iran

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ABSTRACT

The use of potent microbial mixed cultures is a promising method for the bioremediation of recalcitrant compounds. In this study, eight molds, three yeasts, and four bacterial isolates were screened from an aged oil-polluted area. An oil degradation assay with various combinations including Bacterial Mixed Culture (BMC), Fungal Mixed Culture (FMC), Fungal-Bacterial Mixed Culture (TMC), and Sequential Fungal-Bacterial Mixed Culture (SMC) was investigated. The results indicated that the SMC culture had the highest yield of degradation (65.96%) in comparison with the degradation yields of TMC, FMC and BMC, which were 59.04%, 56.64%, and 47.56%, respectively. The degradation of saturates, aromatics, resins, and asphaltenes in the crude oil found using the Iatroscan system were, as follows: 64.21%, and 67.63% for aromatics, 72.90%, and 73.59% for saturates, and 53.88% and 58.25% for resins with respect to the TMC and SMC cultures as the superior mixed cultures. The growth rates of yeasts, molds, and bacteria in the TMC and SMC cultures were compared for further evaluation of the role of each microorganism in the degradation. Our findings support the use of mixed cultures in the bioremediation of recalcitrant petroleum pollution.

1. Introduction

Crude oil, a dark fossil fuel with a viscous liquid form, is a mixture of various simple and complex hydrocarbons with varying molecular weights, which is used for the production of different petrochemical products and fuels (Varjani, 2017). Based on their solubility in organic solvents, petroleum hydrocarbons are divided into four types, i.e., aliphatics, aromatics, resins, and asphaltenes (Chandra et al., 2013; Varjani, 2017). Aliphatic compounds have lesser toxicity than Polycyclic Aromatic Hydrocarbons (PAHs). The toxicity of resins and asphaltenes is not well known; however, due to their tolerance to degradation, they can disrupt the balance of an ecosystem (Moghimi et al., 2017). In an aged petroleum-contaminated soil, most of the recalcitrant compounds that have high molecular weights (HMW) are PAHs, such as pyrene, chrysene, and benzo-pyrene, or long-chain aliphatics, such as tetracosane, isoprenoids like pristine, and phytane (Varjani, 2017). Low bioavailability, hard degradability, and high toxicity of these compounds, particularly the HMW PAHs, can cause irreparable damages (Ortega-González et al., 2015; Varjani, 2017), such as the contamination of groundwater, changes in the ecosystem, disturbances in the metabolic reactions, hormone imbalance, and disruption of soil microbial communities (Nouri et al., 2017a; Souza et al., 2014; Varjani, 2017). Therefore, for the preservation of all living creatures and vital resources, quick and proper remediation of oil-contaminated areas is highly important.

A number of physicochemical methods have been devised with the aim of petroleum remediation; however, bioremediation methods are usually preferred because of their cost-effectiveness, and comparative environmental-friendliness (Fuentes et al., 2014). Although most of the previous studies have focused on the capability of bacteria in the degradation of petroleum, fungi have a great potential in this field as well (Adetutu et al., 2012; Moghimi et al., 2017). There are more than 60 genera of aerobic bacteria (such as *Bacillus*, *Geobacillus*, *Burkholderia*, *Mycobacterium*, and *Ralstonia*), some family of anaerobic bacteria (such as *Desulfobulbaceae*, *Desulfobacteraceae*, *Desulfarculaceae*, *Anaerolineaceae*, *Deferribacteres*, *Rhodospirillaceae*, and *Magnetovibrio*), and a large number of ligninolytic and non-ligninolytic fungi (such as *Bjerkandera*, *Irpex*, *Lentinus*, *Polyporus*, *Penicillium*, *Aspergillus* and *Candida*) that have the capability to degrade petroleum (Hadibarata et al., 2009; Matturro et al., 2017; Varjani, 2017). The ability of fungi to secrete enzymes, especially those involved in the decomposition of lignin has a great effect on the degradation of petroleum hydrocarbons, especially on the PAHs (Acevedo et al., 2011). Compared to fungal non-specific enzymes (such as laccase, manganese independent peroxidase, manganese

* Corresponding author.
E-mail address: hmoghimi@ut.ac.ir (H. Moghimi).

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peroxidase and lignin peroxidase), bacterial enzymes (such as AlkB related alkane hydroxylases, methane monooxygenases, bacterial P450 oxygenase system, and dioxygenases) are less effective in dealing with recalcitrant hydrophobic compounds (Acevedo et al., 2011; Varjani, 2017). In addition, the hyphal structure of fungi gives them a remarkable ability to penetrate the soil matrix, and degrade the pollutants (Acevedo et al., 2011; Masoudi et al., 2017). However, the use of fungi due to their slow growth and high oxygen requirement poses certain limitations (Ortega-González et al., 2015).

Owing to the diversity in petroleum hydrocarbons, a single microorganism cannot degrade all the petroleum compounds. The utilization of a mixed culture increases the variety of degradable substrates and create a system of commensalism and co-metabolism (Gojgic-Cvijovic et al., 2012); for example, the fungal extracellular enzymes can degrade less soluble compounds, and turn them into more soluble and less toxic materials, which can be consumed by the bacteria (Hadibarata et al., 2009). Moreover, a large number of fungi have a better growth rate and performance in the presence of a secondary carbon source. The simultaneous use of bacteria and fungi enables the consumption of carbon-based metabolites of bacteria, as a secondary carbon source, by the fungi (Chandra et al., 2013). The advantages of mixed cultures in the degradation of different pollutants including petroleum hydrocarbons have been proved by many researchers (Tyagi et al., 2011). However, it is still a debate as to which among the bacterial, fungal or fungal-bacterial mixed culture is the most superior. For instance, Wang et al. (2012), in their study, indicated that a mixed culture of fungi and bacteria has a better efficiency in the degradation of HMW PAHs, whereas Li et al. (2008) stated that a fungal mixed culture has a better performance in the degradation of HMW PAHs than the other mixed cultures.

This study was designed to evaluate the diversity of fungal and bacterial strains, which were isolated from an aged contaminated soil, and survey the capability of the microbial mixed culture in the degradation of heavy crude oil. In addition, the growth kinetics, and yields of degradation in fungal, bacterial, and fungal-bacterial mixed cultures were studied. Hence, this paper can help in gaining more precise insight into the degradation process of the fungal-bacterial mixed culture.

2. Materials and methods

2.1. Microbial isolation and culture conditions

The long-term presence of petroleum hydrocarbons in an area enables the microbial population to adapt, and enrich the number of degrading species. Therefore, the chance to isolate the potent strains in the degradation of petroleum hydrocarbons increases (Saadoun, 2002). Thus, in order to isolate the effective strains, soil samples were collected from an aged petroleum contaminated area located at 30°19′12″ N, 51°24′36″ E in Pazanan in the Kohgiluyeh and Boyer-Ahmad Province of Iran. The soils in this area got contaminated around the Pazanan oil field after its discovery in 1935, and the subsequent exploitation in 1963, which occurred due to pipeline leakages, and improper discharge of industrial oil wastes. The soil samples were obtained from a depth of 15–20 cm, poured into sterile containers, and transported to the laboratory within 48 h. After air drying in the dark, the soil samples were passed through a 2 mm sieve, and stored at 4 °C (Li et al., 2008). Before the isolation process, samples were incubated in 28 °C for 48 h in order to microbial acclimatization to the experimental conditions (Wang et al., 2012). One gram of the soil sample was added to 10 ml of the Ringer's saline solution, and shaken for 2 h at 120 rpm. Then, for enriching the potent microorganisms in the petroleum hydrocarbon degradation, 1 ml of the resulting mixture was added to 100 ml of the Bushnell Haas medium (BH medium) containing 0.2 g/l $MgSO_4$, 0.02 g/l $CaCl_2$, 1 g/l KH_2PO_4, 1 g/l K_2HPO_4, 1 g/l NH_4NO_3, and 0.05 g/l $FeCl_2$ supplemented by 1 g of heavy crude oil (as the only carbon source), and incubated for about 72 h (at 120 rpm, and 28 °C). Each medium (100 µl) was transferred to different plates, incubated at 28 °C for 7 days, and monitored every day (Ortega-González et al., 2015). The mold, yeast, and bacterial strains were isolated on PDA plates (200 g/l infusion form of Potato, 20 g/l Dextrose and 15 g/l Agar), containing 100 mg/l tetracycline, GYP plates (20 g/l Glucose, 5 g/l Yeast extract, 10 g/l Peptone, ang 15 g/l Agar) containing 50 mg/l rose bengal and 100 mg/l tetracycline (Nouri et al., 2017a), and R2A plates (0.250 g/l casein ezymatic hydrolate, 0.250 g/l peptone, 0.500 g/l casein acid hydrolate, 0.500 g/l yeast extract. 0.500 g/l glucose, 0.500 g/l starch soluble, 0.030 g/l K_2HPO_4, 0.500 g/l $MgSO_4{\cdot}5H_2O$, 0.030 g/l $C_3H_3NaO_3$, and 15 g/l agar) containing 50 mg/l cycloheximide, respectively. The isolates were divided based on their colonization and microscopic morphologies.

2.2. Crude oil degradation by the isolated strains

All the isolated fungal and bacterial strains were examined for evaluating their crude oil degradation activity. Accordingly, the bacterial and yeast pre-cultures were prepared by adding a full loop of pure cultures of each strain into 50 ml flasks containing 10 ml of the Bushnell Haas (BH) medium with 1% glucose (as the carbon source), and 0.1% crude oil (as the inducer) (Rahman et al., 2002); the contents were incubated at 28 °C and 120 rpm. When the absorbance of the pre-cultures reached 0.6 at 600 nm, 1.0 ml of each culture was centrifuged at 4000 rpm for 10 min, washed twice with sterilized Ringer's saline solution, and then re-suspended in 1 ml of sterile Ringer's saline solution. The preparation of the molds inoculum was carried out in the following order: at first, the spores were prepared on a PDA medium, and then a suspension of the spores with a concentration of 10^4 ml^{-1} was prepared, which acted as the inoculum (Ortega-González et al., 2015). 1 ml inoculum of each strain was added to the 50 ml flasks containing 9 ml Bushnell Haas medium with 1% crude oil, which acted as the sole carbon source (Mukred et al., 2008). Afterward, all the cultures were incubated for 21 days on a rotary shaker under dark conditions (120 rpm at 28 °C). After 21 days, the samples were collected, and the remaining oil was extracted by adding 10 ml toluene, and measured by the gravimetric analysis method (Speight and Arjoon, 2012). Three replications were conducted for all the experiments. In addition, containing the BH medium with no inoculation were used as an abiotic control for calculating the crude oil loss due to photolysis, adsorption, or evaporation. At the end of the experiment, the strains that could degrade more than 30% of the crude oil were selected for the preparation of the three types of mixed cultures.

2.3. Molecular identification of the isolates

The molecular identification of the isolates was carried out by inoculating the fungal (mold and yeast strains) and bacterial strains in 100 ml flasks containing 20 ml Potato Dextrose Broth (PDB), and Luria Bertani Broth (LB Broth), respectively, and incubated for 48 h at 28 °C and 120 rpm. Subsequently, the bacterial cells and fungal mycelia were separated from the flasks by centrifugation at 4000 rpm for 10 min; they were washed twice using a sterilized Ringer's saline solution. The genomic DNA was extracted using liquid nitrogen, CTAB buffer (only for mold strains), and phenol–chloroform methods (Nouri et al., 2017b). Regarding the bacterial strains, the 16S rRNA gene was amplified with universal primers 9F (5′-AAGAGTTTGATCATGGCT CAG-3′), and 1541R (5′-AGGAGGTGATCCAACCGCA-3′) (Dastgheib et al., 2011); regarding the fungal strains, the region of the Internal Transcribed Spacers (ITS) of the ribosomal DNA (rDNA) was amplified with universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′), and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Kamyabi et al., 2017). Finally, the PCR products were sequenced by the Bioneer Corporation (South Korea). The similarities in the sequences of the 16S rDNA and ITS fragments were identified by running a BLAST search in the