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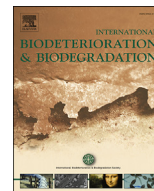

Degradation of asphaltenes by two *Pseudomonas aeruginosa* strains and their effects on physicochemical properties of crude oil

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ABSTRACT

Microbes can enhance oil recovery through the degradation of some heavy fractions of crude oil. Two *Pseudomonas aeruginosa* strains (Gx and Fx) using crude oil as the carbon source were isolated from oil-contaminated soils. The potential of Gx and Fx for oil displacement was assessed by testing their ability to degrade pure asphalt and crude oil asphaltenes. Approximately ~10% of pure asphalt and 59–72% of crude oil asphaltenes were degraded using cell-containing fermentation broths. The content of lighter fractions (saturates and aromatics, maximum 11%) increased and the content of heavier fractions (resins and others, maximum 75%) decreased in the degraded oil compared with the controls. The relative quantity of vaporizable fractions (230 °C) increased after treatment, 10% by Gx and 19% by Fx. The oil viscosity (35 °C) was reduced by nearly half from 76.5 m Pa•s, and ~90% of oil adsorbed on filter paper was removed after treatment. Gx and Fx produced surfactants with crude oil as the sole carbon source, and the oil-spreading diameter ranged from 15 to 17 cm. In conclusion, biosurfactant-producing *P. aeruginosa* strains Gx and Fx could efficiently degrade recalcitrant asphaltenes in crude oil and are therefore candidate strains for microbial enhanced oil recovery.

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1. Introduction

Microbial enhanced oil recovery (MEOR) is a promising method of oil recovery that has high efficiency, low cost, no environmental pollution, and easy operation (Song et al., 2015). The mechanisms of MEOR mainly include the degradation of high-molecular-weight (HMW) hydrocarbons and the production of biosurfactants (Barker et al., 2003). Asphaltenes are the heaviest and most polar fraction of crude oil. High levels of asphaltenes are present in heavy oils, resulting in a high viscosity of crude oil (Batzle et al., 2006; Guiliano et al., 2000). Viscosity reduction by asphaltene reduction is one of the approaches to enhance heavy oil recovery (Lavania et al., 2012).

Asphaltenes have complex structures constituted by interacting systems of polyaromatic sheets bearing alkyl side-chains. Earlier studies showed that it is extremely difficult to degrade crude oil asphaltenes biologically. For example, Chaineau et al. (1995) reported that saturates and aromatics were almost completely degraded, while asphaltenes were totally retained in an oil-containing soil seven months after activation of indigenous microorganisms. Uraizee et al. (1997) believed that high levels of asphaltenes can prevent the transport of biodegradable fractions in oil droplets toward the oil–bacteria interface and thus reduce the biodegradation efficiency of oil fractions.

Recently, asphaltenes have proven to be biodegradable (Uribe-Alvarez et al., 2011; Lavania et al., 2012; Wang and Li, 2011; Asadollahi et al., 2016). A microbial consortium (*Corynebacterium* sp., *Bacillus* sp., *Revibacillus* sp., and *Staphylococcus* sp.) using asphaltenes as the sole carbon and energy source was found in the Maya crude oil (Pineda-Flores et al., 2004). A fungal strain (*Neosartorya fischeri*) and a bacterial strain (*Garciaella petrolearia*) were shown to grow with asphaltenes as the sole carbon source and they preferentially degraded asphaltenes and aromatics in crude oil (Uraizee et al., 1997; Uribe-Alvarez et al., 2011). However, the efficiency of biodegradation of asphaltenes is relatively low compared with those of saturates and aromatics, two light fractions of crude oil (Das and Mukherjee, 2007; Potter and Duval, 2001; Rojas-Avelizapa et al., 2002). Therefore, screening of bacteria using asphaltenes as the carbon source and evaluation of their ability to

Abbreviations: MEOR, Microbial enhanced oil recovery; HMW, High-molecular-weight; ADE, Asphalt degradation efficiency; VRR, Viscosity reduction rate; ORE, Oil removal efficiency; LMW, Low-molecular-weight.

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degrade crude oil are important work for selecting microorganisms with oil displacement activity. The ability of microorganisms in asphaltene degradation is particularly important for MEOR in high-asphaltene heavy oil reservoirs.

Microorganisms with oil displacement activity mainly act at the oil–water interface. However, the hydrophobicity of crude oil prevents oil dispersion; this directly reduces the surface area of microbial cells in contact with oil droplets and thereby attenuates the effects of microbial activity on oil properties (Tian et al., 2000). Emulsification of oil can greatly improve oil dispersion and thus increase the chance of contact between microbial cells and oil beads, promoting the adsorption and degradation of crude oil hydrocarbons by microorganisms (Tian et al., 2000). The use of co-solvents or surfactants can facilitate emulsification, but may also lead to environmental pollution (Ricomartínez et al., 2013). Thus, screening of biosurfactant-producing microbes that can degrade crude oil hydrocarbons has been a hot topic for research on MEOR.

Pseudomonas aeruginosa is a hydrocarbon-degrading bacterium commonly found in oil reservoirs and it can synthesize surfactant (rhamnolipid) with crude oil as the sole carbon source. Several studies reported that *P. aeruginosa* produced rhamnolipid to reduce the surface tension of crude oil and maintain a high emulsifying capacity in fermentation broth (Pornsunthorntawee et al., 2008; Dubey et al., 2012). The production of rhamnolipid reached 55.1 g L^{-1} when *P. aeruginosa* was growing with sunflower oil as the carbon source (Xia et al., 2007). Additionally, *P. aeruginosa* was found to degrade crude oil and the efficiency of degradation of crude oil reached 64.5% after 40 days of incubation (Zhao and Ma, 2007). The surfactants produced by *P. aeruginosa* facilitated the degradation of crude oil (Thavasi et al., 2011; Zhang et al., 2012a).

Existing studies on *P. aeruginosa* have mainly focused on a metabolic product, rhamnolipid. Less work investigated this bacterium in degrading the resins and asphaltenes of crude oil. For example, Mohammad et al. (2007) found that *P. aeruginosa* was unable to utilize long-chain hydrocarbons alone; nevertheless, long-chain hydrocarbons became degradable after being dissolved in crude oil. Das and Mukherjee (2007) showed that *P. aeruginosa* could degrade only a small amount of heavy fractions such as asphaltenes in crude oil. Xu et al. (2015) found that *P. aeruginosa* could efficiently decompose medium-chain and long-chain *n*-alkanes (C10–C26) of the crude oil as its sole carbon source. Ramadass et al. (2016) found that *P. aeruginosa* was capable of degrading diesel oil, *n*-alkanes or hexadecane. So far, there have been no reports on the ability of *P. aeruginosa* to degrade pure asphalt or its compositional changes during bacterial degradation.

In the present study, oil-utilizing aerobic bacterial strains were isolated from crude oil and oil-contaminated soil samples. Two strains were found to efficiently degrade the heavy fractions of crude oil and produce surfactants with crude oil as the sole carbon source. The potential of these two strains for oil displacement was assessed by testing their ability to degrade pure asphalt and crude oil asphaltenes. This study provides candidate strains for potential use in MEOR.

2. Materials and methods

2.1. Crude oil, soil and asphalt

For bacterial isolation, four crude oil samples were collected from well Chang 6 in Yanchang Oilfield in Northern Shaanxi, China. Two oil-contaminated soil samples were obtained near the well-head. Details on the source of oil and soil samples are available in our recent publication (Zhang et al., 2016a).

For pure asphalt degradation test, heavy traffic asphalt was purchased from BAOLIRUS International Investment Co., Ltd. (Yangling, Shaanxi, China). The asphalt penetration index was 91 (0.1 mm); the softening point was 46 °C; and, the ductility was 45 cm at 10 °C and 102 cm at 15 °C.

For oil degradation test, a crude oil sample was collected from well Chang 6 in Yanchang Oilfield. The sample contained: saturates, 612.6 mg g^{-1}; aromatics, 83.6 mg g^{-1}; resins, 51.6 mg g^{-1}; asphaltenes, 54.3 mg g^{-1}; and others, 69.1 mg g^{-1}. The oil viscosity was 76.5 mPa s at 35 °C and a shear rate of 2028 s^{-1}. The API (American Petroleum Institute) of the oil was 23.9.

2.2. Bacterial isolation, screening and identification

2.2.1. Bacterial isolation

A 10.0 g portion of each oil-contaminated soil sample was weighed into 250 mL flasks, each containing 90 mL of sterile water and serially diluted to 10^{-3}. The 10^{-1} to 10^{-3} dilutions of soil suspensions (0.1 mL each) were spread on plates containing the isolation medium (agar, 10 g; NaCl, 5.0 g; water, 1000 mL; autoclaved at 121 °C for 30 min before use; a sterilized filter paper covered with crude oil was tightly attached onto the surface of the solidified medium). The inoculated plates were incubated at 37 °C under aerobic conditions for 7 days. Bacterial colonies with rapid growth and large diameter were picked and purified. Meanwhile, the crude oil sample was thoroughly agitated and a 0.1 mL aliquot was spread on plates containing the isolation medium. Bacterial strains were isolated and purified as described above. All strains were preserved on slants of beef extract–peptone medium (Cheng and Xue, 2012).

2.2.2. Bacterial screening

For primary screening, the purified strains were inoculated into plates containing the primary screening medium (agar, 10.0 g; crude oil, 20.0 g; KNO_3, 5.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 1.0 g; water, 1000 mL, with pH adjusted to 7.0; autoclaved at 121 °C for 30 min before use). The inoculated plates were incubated at 37 °C under aerobic conditions for 7 days. Bacterial growth was observed and recorded during 3–7 days of incubation.

For secondary screening, strains with rapid growth on the primary screening medium were selected and transferred into 250 mL narrow-mouth glass bottles containing 100 mL of basal mineral medium ($NaNO_3$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KH_2PO_4, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; water, 1000 mL, with pH adjusted to 7.0) supplemented with 2.0 g of crude oil (autoclaved at 121 °C for 30 min). The inoculated bottles were incubated at 37 °C with oscillation at 120 rpm for 10 days. Crude oil adhesion to the glass wall was observed during incubation. Additionally, the emulsification time of crude oil, the diameter of oil spreading, and the pH change of aqueous phase were measured.

2.2.3. Bacterial identification

Colony morphology was observed by naked eye and cell morphology was examined by scanning electron microscopy (Gudiña et al., 2012). 16S rDNA sequence analysis was conducted as previously described (Li et al., 2007). The retrieved sequences were subjected to Blast searches in the NCBI database (http://www.ncbi.nlm.nih.gov/) and deposited in the Genbank under accession numbers KT189160 and KT189159. Phylogenetic trees were constructed by the neighbor-joining method using MEGA 4.0 (Tamura et al., 2007).

2.3. Pure asphalt degradation test

A 3 g portion of pure asphalt was accurately weighed and dissolved in 30 mL of carbon tetrachloride to prepare the asphalt solution. Five drops of asphalt solution were added onto each pre-

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