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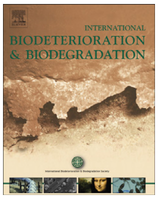

Isolation and characterization of crude-oil-degrading bacteria from oil-water mixture in Dagang oilfield, China

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ABSTRACT

Isolating novel crude-oil-degrading bacteria from oil-water mixture of oil production well and evaluating their degradation capacities are vitally important in the remediation of oil-polluted environments and crude oil exploitation. According to the molecular screening with degenerate primers of alkane hydroxylase gene (*alk B*), a strain *Acinetobacter* sp. LS-1 with *alk B* gene was isolated. This strain exhibited a 99.9% similarity with genus *Acinetobacter*. This *alk B* gene which is one of the key enzymes of metabolic process was identified. This gene sequence showed 98% similarity of its nucleotide and related amino acids to the genus *Marinobacter* but exhibited below 70% similarity to the genus *Acinetobacter*. This phylogenetic analysis indicated that *alk B* may have been transferred horizontally between bacteria. The isolated strain could utilize crude oil as the sole carbon, achieving a high degradation (70.3%) in 7 days. Microcalorimetric analysis of the metabolic processes for hexadecane degradation also demonstrated the ability of this strain to utilize hydrocarbons. Thus, this strain enables to degrade hydrocarbons as the sole carbon source from the gene level, combined with material and energy metabolism. These findings will benefit this strain in the remediation of oil-polluted environments and oil exploitation.

1. Introduction

Crude oil as a complex mixture contains a large number of distinctively different chemicals and is composed of four main fractions: saturated hydrocarbons, aromatic hydrocarbons, resins and asphaltenes (Hasanuzzaman et al., 2007). Hydrocarbon are relatively unreactive due to lack of functional groups and low water solubility (Hassanshahian et al., 2012). Accidental spillage such as pipeline rupture and tank leakage but sometimes also common oil production lead to serious oil pollution (Al-Saleh et al., 2009). Further deposition of partially combusted oil particles may be deposited upon exploitation, transportation and refining via oil born transport. Crude oil covered major world's energy requirements as well as demands for raw materials of the chemical industry, thus playing an important role in the fast development of the modern economy. On the other hand, crude oil is hazardous and could cause severe and long lasting damage to the environment and public health (Tang et al., 2010). As such, the remediation of crude oil contamination is a challenge for researchers in the field of applied and fundamental science. The stimulation of biological activities for a fast clearance of polluted environments may be an environmental friendly strategy for bioremediation of crude oil contaminated environments.

Plant (Yousaf et al., 2010), earthworm (Natal-da-Luz et al., 2012) and microbes were applied for the bioremediation of terrestrial or aquatic ecosystems polluted by crude oil. Microorganisms were given more attention in bioremediation of polluted environments as well as the oil exploitation considering their adaptation to the extreme environment (Whyte et al., 2002b), capabilities to produce biosurfactant (Zheng et al., 2012) and metabolic potential to

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degrade the hydrocarbons (Wang et al., 2011). Microorganisms are able to utilize hydrocarbons as the sole carbon and energy sources (Quek et al., 2006; Hassanshahian et al., 2012). It has been reported that some microbial species, for instance, *Pseudomonas spec.* (Grant et al., 2011), *Rhodococcus spec* (Song et al., 2011), *Mycobacterium spec.* (Nicolau et al., 2009), *and Alcanivorax spec.* (Liu et al., 2010) could degrade hydrocarbons. Since hydrocarbons are very hydrophobic compounds, hydrocarbon degrading microbes produce surfactants as a strategy to enhance the bioavailability of the hydrocarbon. So far, a large variety of microbes such as *Nocardia*, *Rhodococcus*, *Bacillus*, and *Pseudomonas* have already been identified as biosurfactants producers (Menezes Bento et al., 2005). Although a diverse range of hydrocarbon degrading microorganisms have already been isolated from oil polluted environments and crude oil, the mechanisms of microorganism-mediated degradation for hydrocarbons is considered to be complex. A large number of different factors could affect the utilization of hydrocarbons of which enzyme systems of aerobic hydrocarbons oxidation have been intensively studied. Enzymes systems for aerobic oxidation of hydrocarbons include *alk B*-type alkane hydroxylase, methane monooxygenase, dioxygenase and bacterial P450 oxygenase (Throne-Holst et al., 2007; Brash, 2009; Rojo, 2009). The most studied alkane degrading pathway is present in *Pseudomonas putida* Gpo1 organizing the *alk* gene into the octane utilizing plasmid (Baptist et al., 1963; Grant et al., 2011). This pathway is consist of three main subunits of alkane hydroxylase (Alk B), rubredoxin reductase (Alk T) and rubredoxin (Alk G) (Rojo, 2009; Liu et al., 2010). The first enzymatic step of this pathway involves an alkane hydroxylase which is an integral-membrane non-haem diiron monooxygenase hydroxylating alkanes at the terminal position (Smits et al., 1999; Rojo, 2009). Electrons from NADH are then carried by rubredoxin reductase via rubredoxin to Alk B (van Beilen and Funhoff, 2007). Alkane hydroxylases were found in both Gram-negative and Gram-positive microbes with high diversity (Shanklin et al., 1994). There are other different degradation pathways for alkane hydroxylase genes located in the bacterial chromosome (Tani et al., 2001; Mara et al., 2012). Analysis of *alk*⁻ mutants revealed that degrading genes were in the two different plasmids pAV1 and pAV2 (Mengoni et al., 2007).

The goal of this research is to isolate microorganisms from oil-water mixture and to characterize their metabolic capabilities. These include enrichment and isolation of bacteria, 16S rRNA gene sequence analysis, cloning of *alk B* gene, and studying the thermal effect of metabolic reactions during hexadecane degradation. The isolation of hydrocarbon-degrading microbes and investigation of functional genes and metabolic capability could be useful to understand their metabolic mechanisms for future biotechnological applications.

2. Materials and methods

2.1. Sampling

For isolating and screening crude oil-degrading bacteria, the sample of oil-water mixture was collected from the oil production well (NO.Xi-51-5-1: 38°41′, N; 117°22′, E) in Dagang oilfield, near Bohai Sea, southeast of Tianjin in the northeast China.

2.2. Isolation and selection of crude-oil degrading bacteria

1.5% unvarnished oil-water mixture as the inoculum was inoculated and enriched in two 100-ml Erlenmeyer flasks containing 30 ml mineral salts medium (MSM). One liter MSM medium contains 5.0 g of NaCl, 5.0 g of KH_2PO_4, 1.0 g of $(NH_4)_2SO_4$, 1.0 g of $K_2HPO_4 \cdot 3H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 2.0 g of $NaNO_3$, 0.020 g of $FeCl_2 \cdot 4H_2O$, and 0.020 g $CaCl_2$, with the pH adjusted to 7.2, which is sterilized under 121 °C for 30 min (Liu et al., 2010). The Erlenmeyer flasks were incubated for 7 days at 30 °C and 180 rpm on the rotary shaker. The culture was enriched by four consecutive inoculations of 1.5-ml inoculum to 100 ml Erlenmeyer flasks with 30 ml fresh MSM medium. Following enrichment, parts of the medium were plated onto MSM medium containing 2.0% of agarose, as well as 1.5% of crude oil, and incubated at 30 °C for 3–7 days (Wang et al., 2011). Finally, different pure colonies obtained from the plates were stored in LB medium (contained 10 g of NaCl, 10 g of tryptone and 5.0 g of yeast extract LB at pH 7.0) with 15% of glycerol at −80 °C for later molecular screening and characterization.

2.3. Identification

2.3.1. Classical physiochemical identification

Crude-oil-degrading bacteria was identified and characterized by following the standard protocols in Bergey's Manual of Systematic Bacteriology (Bergey, 1934) and the Identification Manual of Systematic Bacteriology published in China (Dong and Cai, 2001). Morphological character, growth character (pH and temperature), and enzyme activity (oxidase and catalase) were performed to identify this bacteria.

2.3.2. 16S rRNA gene sequence analysis

Total DNA of crude-oil-degrading bacteria was extracted using the SDS method for 16S rRNA gene sequence analysis (Lee et al., 2003). The 16S rRNA region was amplified by PCR using the universal forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ and the reverse primer 5′-TACGGCTACCTTGTTACGACTT-3′. The amplification reaction was performed in a total volume of 25 μl containing 15.5 μl sterile deionized water, 2.0 μl Mg^{2+} (25 mM), 2.0 μl dNTP (2.5 mM), 1.0 μl of each forward and reverse primer (25 μM), 0.5 μl DNA template, 0.5 μl *Taq*DNA polymerase (5 u μl^{-1}), and 2.5 μl 10 × buffer solution (20 mM). Amplification for 35 cycles was performed in GeneAmp® PCR System 9700, while the temperature profile for PCR was 95 °C for 5 min predenaturation (1 cycle), 94 °C for 30 s denaturation, 53 °C for 1 min annealing and 72 °C for 2 min extension (35 cycles), 72 °C for 10 min extension after the final cycle. PCR amplicons were checked by running 1.0% agarose gel electrophoresis, purified with Tiangel Midi Purification Kit (Sangon Biotech (Shanghai) Co., Ltd., China), and then cloned into pMD18T-vector for sequencing by Sangon (Sangon Biotech (Beijing) Co., Ltd., China).

2.4. Cloning of the alkane hydroxylase gene

Different single colonies growing on the solid MSM medium were removed with a sterile inoculating loop and re-suspended 100 μl of sterile deionized water in micro-centrifuge tubes, then 100 μl of chloroform/isoamyl alcohol (24:1) was added to the suspensions, and centrifuged at 16,000 g for 5 min at 4 °C after vortexing 5 s in the end. The upper aqueous phase was used as DNA template for PCR to screen for the alkane hydroxylase gene from different single colonies. The forward primer 5′-AAYACNGCNCAYGARCTNGGNCAYAA-3′ and the reverse primer 5′-GCRTGRTGRTCNGARTGNCGYTG-3′ were used to amplify *alk B* (Wang et al., 2011). The PCR was carried with a total volume of 25 μl containing 15.5 μl sterile deionized water, 0.5 μl DNA template, 2.0 μl Mg^{2+} (25 mM), 2.0 μl dNTP, 1.0 μl of each forward and reverse primer (25 μM), 0.5 μl *Taq*DNA polymerase (5 u μl^{-1}), 2.5 μl 10 × buffer solution (20 mM). The amplified condition was 94 °C for 5 min, 30 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 1.0 min) and a final extension at 72 °C for 10 min. After the procedure, the total product was run in 1.0% agarose gel, stained with the nucleotide dye of GoldView (BLKW Co., Ltd., China), and visualized under UV light to