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Biodegradation of marine crude oil pollution using a salt-tolerant bacterial consortium isolated from Bohai Bay, China

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ABSTRACT

This study aims at constructing an efficient bacterial consortium to biodegrade crude oil spilled in China's Bohai Sea. In this study, TCOB-1 (*Ochrobactrum*), TCOB-2 (*Brevundimonas*), TCOB-3 (*Brevundimonas*), TCOB-4 (*Bacillus*) and TCOB-5 (*Castellaniella*) were isolated from Bohai Bay. Through the analysis of hydrocarbon biodegradation, TCOB-4 was found to biodegrade more middle-chain n-alkanes (from C17 to C23) and long-chain n-alkanes (C31–C36). TCOB-5 capable to degrade more n-alkanes including C24–C30 and aromatics. On the basis of complementary advantages, TCOB-4 and TCOB-5 were chosen to construct a consortium which was capable of degrading about 51.87% of crude oil (2% *w*/*v*) after 1 week of incubation in saline MSM (3% NaCl). It is more efficient compared with single strain. In order to biodegrade crude oil, the construction of bacterial consortia is essential and the principle of complementary advantages could reduce competition between microbes.

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1. Introduction

Crude oil is one of the most important compounds in both energy and chemical industry. Nevertheless, due to the natural seeps and anthropogenic activities, widespread releases of petroleum hydrocarbons have been the most common organic pollutant for the marine environment (Prince et al., 2013; Janbandhu and Fulekar, 2011; Sebiomo et al., 2010). In June 2011, a serious crude oil spill occurred in China's Bohai Penglai 19–3 oil field, with 763 barrels of crude oil flowing into the Bohai Sea and 2620 barrels of mineral oil-based mud stranding under the sea (Liu et al., 2015; Liu and Zhu, 2014). As a result, the Bohai Sea marine aquaculture is severely affected. Because of immediate and long-term environmental damage (Wiese et al., 2004; Hii et al., 2009), marine crude oil pollution has been regarded as an increasingly serious international concern.

Crude oil is a complex mixture of paraffinic, cycloparaffinic and aromatic hydrocarbons as well as nitrogen-, oxygen-, sulfur-containing compounds and traces of a variety of metal-containing organic and inorganic compounds (Haritash and Kaushik, 2009; Balba et al., 1998; Bachmann et al., 2014). It is constitutive of hydrocarbons of various molecular weights and other organic compounds. According to their polarizability and polarity, crude oil is always separated into four fractions (saturated, aromatic, resin and asphaltene (SARA)) (Wu et al., 2013). Many compounds exist in crude oil show large variations in physicochemical properties (Head et al., 2006; Kristensen et al., 2015).

Many methods including physical, chemical and thermal ones have been applied on marine oil pollution (Seddighi and Hejazi, 2015; Gao et al., 2014; Bacosa et al., 2015; Nwadiogbu et al., 2016). Microbial bioremediation attracts people's attention among the present disposal ways due to its economy, efficiency and no second contamination (Kuyukina et al., 2013; Roy et al., 2014; Ferradji et al., 2014; Chandankere et al., 2014). So throughout the last several decades, diverse crude oil degrading bacteria have been isolated from oil-polluted locations (Chandankere et al., 2014; Pasumarthi et al., 2013; El-Sheekh et al., 2013; Lin et al., 2014; Hassanshahian et al., 2014a). The general degradation order by microbes is recognized as n-alkanes > monoaromatic compounds (including benzene, toluene, ethylbenzene and xylenes known as BTEX) > branched and cyclic alkanes > polycyclic aromatic compounds (negative correlation between degradation and number of aromatic rings) (Wang et al., 1998), while microbial consortium capable of degrading small aromatics and PAHs faster than alkanes is also found (Bacosa et al., 2010).

Generally speaking, oil-degrading bacterial consortia have higher biodegradation efficiency than one kind of bacterium (Chen et al., 2014; Bao et al., 2012) because single microbe may not degrade a wide range of complex compounds of crude oil (Balba et al., 1998; Suja et al., 2014). To construct an efficient bacterial consortium to degrade complicated crude oil is essential for bioremediation especially bioaugmentation, while studies on the mixture of crude oil degrading bacteria were paid little attention. The methods used to construct oil-degrading bacterial consortium are not systematic or advisable.

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Malik and Ahmed performed biodegradation of crude oil (OGDCL, Pakistan) using the consortium of fifteen (15) indigenous bacterial isolates. The consortium comprised of *Bacillus* sp. (07), *Micrococcus* sp. (03), *Staphylococcus* sp. (01), *Pseudomonas* sp. (02), *Alcaligens* sp. (01) and *Psychrobacter* sp. (01) (Malik and Ahmed, 2012). Varjani et al. degrade about 28% crude oil (3% *v*/*v*) utilizing HUBC bacterial consortium consisting of *Ochrobactrum* sp. (01), *Pseudomonas aeruginosa* (03) and *Stenotrophomonas maltophilia* (02) in 12 days (Varjani et al., 2015). In Kumar's work, the 10 days of degradation rate of spent engine oil by the mixed bacterial consortium reached a maximum of 52% while pure cultures of *Ruegeria* sp., *Pseudoalteromonas* sp., *Acinetobacter* sp. and *Exiguobacterium* sp. were 41%, 40%, 28% and 24%, respectively (Ganesh Kumar et al., 2014). All of the bacterial consortia used in these works show excellent results. However the interaction between these bacteria is indistinct. In Mehdi and Giti's study, the result highlights the reduced biodegradation capability of the consortium, suggesting an unfavorable interaction between *Alcanivorax* and *Thalassolituus* genera (Hassanshahian et al., 2014b), there is a competitive relationship between crude oil degrading bacteria. Accordingly, more kinds of bacteria don't mean higher degradation efficiency; it has important theoretical and practical value to seek out appropriate way to construct efficient marine oil-degrading bacterial consortia.

There are three main parts in this study: (1) isolate and identify bacteria which can biodegrade crude oil from Bohai bay; (2) analyze the hydrocarbon biodegradation and associate the bacteria into consortia to bioremediate crude oil; and (3) assess the effectiveness of bacterial consortia.

2. Materials and methods

2.1. Chemicals

N-hexane, dichloromethane, MeOH and chemicals used in mineral salt are purchased from Tianjin Jiangtian Chemical Technology Co., Ltd. The silica gel (100–200 mesh) was purchased from Qingdao Dingkang Silica Gel Co., LTD. The crude oil used in this study was provided by China Offshore Environmental Service Ltd. The composition of crude oil was analyzed using methods described in Section 2.5. Gravimetric analysis showed the saturated fraction, aromatic fraction, resin fraction and asphaltene fraction in crude oil is 77.89%, 14.92%, 3.83%, and 3.36%, respectively.

2.2. Nutrient requirements

The LB medium contained 5.0 g of yeast extract, 10.0 g of peptone, and 10.0 g of NaCl per liter. For solid media, Agar (15–20 g/l) was added into the solution. The basic medium used for screening was mineral salt medium (MSM) with crude oil as the sole carbon source. The MSM contained 29.0 g of NaCl, 3.0 g of Na_2HPO_4, 1.0 g of KH_2PO_4, 1.0 g of NaH_2PO_4, 1.0 g of KNO_3, 1.0 g of NH_4Cl, 0.7 g of $MgSO_4 \cdot 7H_2O$, and 1.0 mL of trace salt solution per liter. The trace salt solution was defined as 20 mg of $CaCl_2$, 30 mg of $FeCl_3$, 0.5 mg of $CuSO_4$, 0.5 mg of $MnSO_4 \cdot H_2O$, and 10 mg of $ZnSO_4 \cdot 7H_2O$ per liter. The pH was adjusted to 7.0–7.2 with 1.0 M NaOH before sterilization in 121 °C for 20 min.

2.3. Isolation and identification of bacteria

Seawater sample ($T \approx 8$ °C, salinity = 2.9%, pH = 7.6, TN = 2.0 mg/L, TP = 0.11 mg/L, petroleum = 0.13 mg/L) was collected at Bohai Bay and enriched in mineral salt medium (MSM) supplemented with crude oil (10,000 ppm) as carbon source. The mixture was incubated at 30 °C on a constant temperature shaker (200 rpm) for 7 days then 15 mL inoculum was transferred to a fresh mineral salt medium and incubated for another cycle. The procedure was repeated three times. After consecutive transfers, hydrocarbon degraders were isolated by plating on LB solid plates. Phenotypically different colonies obtained from the plates were purified and stocked in slants.

Analysis of 16S rRNA gene sequences of isolated strains was performed. Total DNA extraction of bacterial strains was performed by Sangon Biotech (Shanghai) Co., Ltd. The bacterial 16S rRNA loci were amplified using the forward domain specific bacteria primer, Bac27_F (5′-AGAGTTTGATCCTGG CTCAG-3′) and reverse primer, 1492R (5′-GGTTACC TTGTTACGACTT-3′). The amplification reaction was performed in a total volume of 25 μL consisting template (1 μL), 10× buffer (with Mg^{2+}) (2.5 μL), 2.5 mM each dNTP (1 μL), 10 μM F (0.5 μL), 10 μM R (0.5 μL) and enzyme (0.2 μL). The double distilled water was added for remaining of reaction. The temperature profile for PCR was kept, 94 °C for 4 min, 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, 30 cycles, then 72 °C for 10 min and finally stored at 4 °C. BLAST software (http://www.ncbi.nlm.nih.gov) was used to find near identical sequences for the determined 16S rRNA sequences and the MEGA6 software was used to draw the phylogenetic tree.

2.4. Biodegradation of crude oil by pure strains

Biodegradation of crude oil with bacteria was conducted at a 100-mL scale for 7 days in the constant temperature shaker at parameters 200 rpm and 30 °C. Triplicates of bacteria samples and controls were established. 100-mL flasks containing 50 mL MSM were prepared and sterilized. Crude oil was dissolved in n-hexane firstly and added into each flask, and the final content of crude oil is 2% *w*/*v*. Then 2% *v*/*v* inoculum of bacteria (optical density 1.0 at OD600) was added into the flasks, except for three flasks that were kept as controls which added sterile water instead.

2.5. Oil extraction and analyses

After 7 days of incubation, the samples were kept frozen at −20 °C until analysis. The whole volume was extracted three times using 20 mL of n-hexane. Sample clean-up and fractionation were performed according to the previously described protocols (Gao et al., 2014; Grace Liu et al., 2011). The extract was estimated by weighing the dry residual after evaporation of the solvent.

The variation of the different constituent fractions of oil, i.e. saturates, aromatics, resins and asphaltenes was analyzed. The dry extract of total residual oil was suspended in 10 ml of n-hexane by sonication (5 min, 40 kHz). After filtration of the n-hexane solution on solvent-washed pre weighed PVDF transfer membrane filters, the insoluble fraction contained the asphaltenes of the oil were retained on the filters and then dried in a drying oven and weighed.

The soluble fraction was separated by a 15 cm length × 1 cm diameter solid ± liquid chromatography column which were filled with activated (8 h, 110 °C) silica-gel (100–200 mesh). There was successive elution of the saturates, aromatics and resins of the oil with 60 ml of n-hexane, 60 ml of a 60–40 *v*/*v* mixture of n-hexane/dichloromethane and 60 ml of MeOH, respectively. After evaporation of the solvents, each fraction was weighed and the degradation efficiency of the four constituent fractions (saturates, aromatics, resins and asphaltenes) was calculated following the equation.

Four Constituent Fractions degradation efficiency

$$= \left(1 - \frac{\overline{Q}}{\overline{Q0}}\right) \times 100\%$$

$\overline{Q}$ average value of quality in the samples,
$\overline{Q}_0$ average value of quality in the control.

Bacosa et al. found that biodegradation was the key process for n-alkanes, while it was photooxidation for PAHs (Bacosa et al., 2015). It makes sense to analyze the variation of the saturated hydrocarbons

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