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Effect of different enrichment strategies on microbial community structure in petroleum-contaminated marine sediment in Dalian, China

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ABSTRACT

An oil spill occurred at Xingang Port, Dalian, China in 2010. Four years after this spill, oil contamination was still detected in samples collected nearby. In this study, the strains that evolved in the sediment were screened by high-throughput sequencing technology. Most of these strains were genera reported to have functions associated with crude oil biodegradation. The diversities and numbers of microbes were monitored through enrichment culturing; the dominant strains propagated at first, but the enrichment could not be continued, which indicated that the prolonged culture was not effective in the enrichment of the micro-consortium. Oxygen was also observed to affect the propagation of the dominant microbes. The results showed the role of culture strategies and oxygen in the enrichment of the petroleum-degrading microbes. Therefore, dominant strains could be screened by optimizing both the enrichment time and oxygen concentration used for culturing to facilitate oil biodegradation in the marine ecosystem.

1. Introduction

Petroleum and petroleum-derived products are important in industry and daily life, and a large amount of crude oil is extracted and transported around the world every year (Anisuddin et al., 2005). Spills and seepage during transport through pipelines, tankers and offshore oil production are the main causes for marine environment contamination by crude oil (Kvenvolden and Cooper, 2003), and they are deleterious to marine biodiversity (King et al., 2015).

One of the largest oil spills occurred in the Gulf of Mexico in 2010 (Valentine et al., 2010), and research considering people who were exposed to the crude oil due to their work or life environment showed that high concentrations of hydrocarbons were detected in their blood (Sammarco et al., 2015), which indicated that marine contaminants from crude oil not only destroyed the nature ecosystem but also poisoned human beings, which presents a challenge to addressing this problem.

Compared with physical and chemical treatments, bioremediation uses microbial communities to degrade contaminants without geographical limitation or secondary contamination to the environment (Biswas et al., 2015; Riser-Roberts, 1998). When pollution has occurred in the deep sea, the indigenous oil-degrading bacteria were found to be enriched by crude oil (Hazen et al., 2010). Previous studies showed that marine cyanobacteria have the ability to degrade crude oil (Raghukumar et al., 2001). Whereas some bacteria prefer to degrade alkanes (Rojo, 2009; Wentzel et al., 2007), others can use long-chain alkanes as a sole carbon source (Throne-Holst et al., 2007).

Developing an optimal method for the isolation and identification of the oil-degrading microbial consortium is a prerequisite for cleaning up the oil-contaminated marine environment by biodegradation; additionally, understanding the behaviors of the oil-degrading microorganisms is important for researchers to devise strategies for such a purpose. After the petroleum contamination in the Gulf of Mexico, the behavior of microorganisms was studied (Kostka et al., 2011). Compared with the control, the contaminated sediment was enriched for oil-degrading bacteria, which were primarily Gammaproteobacteria, including the genera of *Alcanivorax*, *Marinobacter*, *Pseudomonas* and *Acinetobacter* (Kostka et al., 2011).

In July 2010, an explosion occurred in the pipelines transporting crude oil from a tanker to the oil storage depot at Xingang Port, Dalian, China, and more than 1500 t of crude oil was poured into the sea, creating a slick of 180 km^2 in the Yellow Sea that spread quickly to an area of 430 km^2 within a week. Previous studies mostly focused on the diversity of microbes in different oil-contaminated samples and their oil-degrading efficiency (Wang et al., 2014; King et al., 2015; Macaulay, 2015; Rodriguez-R et al., 2015; Sun et al., 2015) and on isolating strains with biodegradation functions (Liu et al., 2016). However, little is known about the effect of the enriched culture and oxygen concentration on the diversities and propagation of the microbes, which is very

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Fig. 1. Sampling locations in the Dalian Xingang area [sample 3 (38°59′17.7″N, 121°55′4.2″E), sample 5 (38°54′46.4″N, 121°55′26.1″E), sample 16 (38°53′0.9″N, 121°47′44.3″E), sample 18 (38°50′38.21″N, 121°45′7.61″E)].

crucial for strain isolation and developing strategies for petroleum biodegradation. Thus, we attempted to fill this gap.

2. Materials and methods

2.1. Sampling sites and procedures

The samples, collected in October 2014 at the Xingang Port of Dalian, China, from marine sediment deposited at a depth of 10–40 m below sea level four years after the oil pipeline explosion accident that occurred on July 16, 2010, were stored in sterile valve bags and immediately transported to the lab for preservation at 4 °C. The temperature of the sampling sites was recorded at the same time, which was 15–18 °C. All samples were labeled by collection numbers, and the content of crude oil in the different samples was analyzed. Four samples labeled with 3, 5, 16 and 18, with different contents of crude oil, were selected for study [sample 3 (38°59′17.7″N, 121°55′4.2″E), sample 5 (38°54′46.4″N, 121°55′26.1″E), sample 16 (38°53′0.9″N, 121°47′44.3″E), sample 18 (38°50′38.21″N, 121°45′7.61″E)].

2.2. Enrichment of oil-degrading bacteria

Enrichment of oil-degrading bacteria was performed in an inorganic salt medium composed of (g): $MgSO_4 \cdot 7H_2O$ 0.7, NH_4NO_3 1.0, KCl 0.7, KH_2PO_4 2.0, Na_2HPO_4 3.0, and 10 mL trace element solution. These components were dissolved into sea water for a total volume of 1000 mL, and the pH of the medium was adjusted to pH 7.5. Meanwhile, 0.5% crude oil was supplemented as the sole carbon source. The trace element solution was composed of (mg/L) $CaCl_2$ 2.0, $FeCl_3 \cdot 6H_2O$ 50.0, $CuSO_4$ 0.5, $MnCl_2 \cdot 4H_2O$ 0.5, and $ZnSO_4 \cdot 7H_2O$ 10.0.

Each 250 mL Erlenmeyer flask was filled with 50 mL medium, which was autoclaved at 121 °C for 20 min. A sample of 10 g was inoculated into the sterilized medium to start the culture, which was maintained at 15 °C for 7 d, either statically or with shaking at 150 rpm. For the enrichment culture, 500 μL from the previous culture was transferred into 50 mL fresh medium, which was then cultured for 7 d under the same conditions, and 5 successive transfers were performed from the enrichment culture. The first, third, fourth and fifth cultures were selected for the analysis of microbial diversities and were designated groups A, C, D, and E. Each experiment was performed in duplicate, and the samples were mixed for the genome DNA extraction.

Table 1
Detailed information of four samples.

Sample no.	Concentration of crude oil (μg/mL)	Transfer time[a]				Inoculation condition	
		1	3	4	5	Static	Shaking
3	1109.85	A	C	D	E	1	29
5	903.00	A	C	D	E	2	30
16	2601.21	A	C	D	E	3	31
18	1235.94	A	C	D	E	4	32

[a] Note: Each enrichment culture was incubated for one week and then transferred into fresh medium and successively transferred five times. A. First enrichment culture; C. Third enrichment culture; D. Fourth enrichment culture; E. Fifth enrichment culture.

2.3. Genome DNA extraction, PCR amplification and sequencing

Total genome DNA was extracted with the FAST DNA® Spin Kit for soil (MP Biomedicals, LLC, Solon, OH, USA) following the manufacturer's protocol. The concentration and qualification of the total DNA were assessed by a NanoDrop ND 2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA), which was diluted to 1 ng/μL as a template for further PCR procedures. A pair of primers, 515F/806R, with a barcode for amplifying the V4 region of 16S rDNA was used for the PCR amplification (Bergmann et al., 2011). The PCR procedure was carried out as described previously (Caporaso et al., 2011). PCR products were mixed and loaded onto a 2% agarose gel to extract the fragments of 400–450 bp, which were then purified by GeneJET Gel Extraction Kit (Thermo Scientific).

The library was constructed with the NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations and index codes. The quality of the library was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. The library was sequenced on the Illumina MiSeq sequencing platform at Novogene (Beijing, China), and 250/300 bp paired-end reads were generated.

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