Marine Pollution Bulletin 106 (2016) 301–307

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Isolation, identification, and crude oil degradation characteristics of a high-temperature, hydrocarbon-degrading strain

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ARTICLE INFO

Article history:
Received 29 May 2015
Received in revised form 24 September 2015
Accepted 30 September 2015
Available online 16 March 2016

Keywords:
Bacillus licheniformis
Crude oil
Biodegradation
Emplastic
High temperature
Biosurfactant

ABSTRACT

In this work, a hydrocarbon-degrading bacterium Y-1 isolated from petroleum contaminated soil in the Dagang Oilfield was investigated for its potential effect in biodegradation of crude oil. According to the analysis of 16S rRNA sequences, strain Y-1 was identified as *Bacillus licheniformis*. The growth parameters such as pH, temperature, and salinity were optimised and 60.2% degradation of crude oil removal was observed in 5 days. The strain Y-1 showed strong tolerance to high salinity, alkalinity, and temperature. Emplastic produced by strain Y-1 at high temperatures could be applied as biosurfactant. Gas chromatography analysis demonstrated that the strain Y-1 efficiently degraded different alkanes from crude oil, and the emplastic produced by strain Y-1 promoted the degradation rates of long-chain alkanes when the temperature increased to 55 °C. Therefore, strain Y-1 would play an important role in the area of crude oil contaminant bioremediation even in some extreme conditions.

1. Introduction

With the increasing demand for oil, and oil products, in various fields, petroleum hydrocarbon pollution (PHP) is becoming more serious. Such pollution damages both marine, and terrestrial ecosystems, it has attracted much attention (Bao et al., 2012; Oren, 1990). PHP results from the exploitation and transport of oil, and the processing of oil products. In the exploitation and processing of oil, large volumes of waste water and residues containing oil are produced, and crude oil spillages happen frequently in the transporting of oil (Deng et al., 2014). Oil spills often result in both immediate and long-term environmental damage (Ricardo et al., 2013; Suneel et al., 1996). Statistics shows that about 1.3 million tonnes (Bao et al., 2012) of oil have been spilled in the oceans: PHP not only significantly damages the ecosystem in ocean waters and coastal soils, but also influences economic development and the lives of residents.

Microbial biodegradation technology has gained increasing acceptance in the remediation of oil pollution. Compared with traditional physico-chemical treatments, biodegradation is more environmentally friendly, cost-effective, and efficient (Das and Mukherjee, 2007; Zhang et al., 2012). Numerous studies have proved that microbial biodegradation technology serves potentially significant applications (Wentzel et al., 2007). In the remediation of xenobiotic pollutants (such as oil), natural microbial populations are considered the most basic and reliable bioremediation mechanism (Cappello et al., 2007). Many microorganisms can degrade short-chain petroleum hydrocarbons. However, microorganisms which can degrade long-chain petroleum hydrocarbons and complex polycyclic aromatic hydrocarbons (PAHs) are key to the remediation of oil pollution (Kenzo et al., 2008).

The effectiveness of microbial biodegradation is often limited by the low bioavailability of hydrocarbons to microorganisms (Nitschke and Pastore, 2006; Van et al., 2003). Biosurfactants are surface active molecules produced by certain strains, which either adhere to the cell surface or are excreted extracellularly in the growth culture medium (Suneel et al., 1996). During the culturing process, certain microorganisms secrete biosurfactants into the medium which can increase the surface area of hydrophobic substrates by reducing surface tension of the culture, and further increase their bioavailability (Chandankere et al., 2014; Ramos et al., 1991; Rufino et al., 2008). So attention, to date, is focused on hydrocarbon degrading microorganisms with biosurfactant-producing capabilities, and several species have been provided, such as *Bacillus*, *Alcaligenes*, *Pseudomonas*, and *Corynebacterium* to this end (Calvo et al., 2004; Rengathavasi et al., 2011; Toledo and Gonzalez, 2008; Zhang et al., 2010).

Dagang Oilfield in Tianjin, China is next to the Bohai Sea and the soil close to the mined oil fields suffers from heavy oil pollution and salinisation. Therefore, there are high requirements impinging upon the microbial consortium responsible for remediating oil pollution in

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http://dx.doi.org/10.1016/j.marpolbul.2015.09.053
0025-326X/

the area. Here, a *Bacillus licheniformis* Y-1 was isolated from a soil with heavy oil-contamination in the Dagang Oilfield. As the bacterial strain shows high degradation capacity for oil, salt, a high alkali bearing capacity, and high temperature resistance, it has research and application significance. The gene of the strain was identified, the growth and its oil degradation behaviour were optimised, and the degradation characteristics of the strain for petroleum hydrocarbons were analysed.

2. Materials and methods

2.1. Crude oil

The crude oil used was collected from the oil pipeline of Dagang Oilfield (latitude: 38° 49′ N; longitude: 117° 31′ E). After standing for 48 h, the oil was separated from water and the supernatant crude oil was collected and reserved. 0# diesel was obtained from the Sinopec gas station in Huayuan, Tianjin, China.

2.2. Media

T-medium was used as the nutrient medium. The original inorganic salt medium contained: 10 g of NaCl, 1 g of NH_4NO_3, 0.5 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 0.2 g of $CaCl_2$, 1 mL of trace element solution, and 1 L of distilled water. The pH of the medium was 7.5.

The trace element solution was composed of: 1 g of $CuSO_4$, 1 g of $MnSO_4$, 1 g of $FeSO_4 \cdot 7H_2O$, and 1 L of distilled water.

All the above culture media were sterilised at 121 °C for 20 min. The reagents used in the experiments were purchased from Tianjin Jiangtian Chemical Technology Co., Ltd, Tianjin, China.

Crude oil medium was prepared as follows: 5 g of crude oil sterilised by filtration through a membrane with an aperture size of 0.45 μm was added to 1 L of sterilised original inorganic salt medium. Afterwards, the medium was not sterilised further and the oil was applied as single carbon unit to the medium.

2.3. Isolation and screening of strains

2.3.1. Isolation of strains

The soil sample DG-2 was heavily polluted by the oil spillage, and collected from the area (latitude: 38° 49′ N; longitude: 117° 31′ E) near the pumping unit of the Dagang Oilfield in Tianjin, China. As the production platform was close to Bohai Sea, the soil samples contained high salt and alkali levels. Then, 10 g of soil sample DG-2 was soaked in 100 mL of bacteria-free water. Then it was stood, after being shocked sufficiently, and 10 mL of supernatant was collected and added to 200 mL of the original inorganic salt medium. Then, the medium was cultured at 30 °C on a thermostatically controlled shaking table and rotated at 120 rpm for 5 d. Owing to the soil being heavily polluted by the oil spill, the saturated solution contained certain petroleum hydrocarbons, there was therefore no need to add oil as a carbon source in the enrichment culture.

After being cultured for 1 day, the clear solution in the conical flask became muddy. When it was cultured for 2 days, white strips formed by the aggregation of mycelia were observed on the inner wall of the conical flask. On the fifth day, the culture solution became more turbid.

The solution, after being cultured for 5 days, was smeared on the plate with the T-medium, and cultured at 30 °C for 24 h. After bacterial colonies grew, streaking was conducted repeatedly to obtain pure strains. Afterwards, the pure stains were transferred to the medium in a test tube and preserved at 4 °C.

2.3.2. Screening of the strain

The hydrocarbon-degrading capacity of the purified strains was detected by comparing their emulsifying effectiveness, and by gravimetric method, to screen strains with high hydrocarbon-degrading capacity for further study.

The purified strains were inoculated in the liquid T medium to be cultured at 30 °C for 48 h. Afterwards, 10 mL of bacterial liquid was centrifuged at 3000 rpm for 15 min. After discarding the supernatant, the mycelia were washed using bacteria-free water and centrifuged. The sample was then washed and thrice centrifuged. Then the nutrients in the mycelia were removed and the mycelia were inoculated into a 200 mL conical flask filled with crude oil medium containing 1 g of crude oil, to be cultured at 30 °C for 5 days. When the culturing period had finished, 50 mL of n-hexane was applied to extract the oil in the conical flask repeatedly until all the oil had been recovered. The extraction liquid was dehydrated using anhydrous sodium sulphate (which had been calcined at 600 °C for 6 h and added to the n-hexane; then the container was sealed with liquid) and the mycelia in the extraction liquid were removed by filtration through a filter membrane with an aperture of 0.45 μm. Then, the treated extraction liquid was poured into a water bath cauldron to evaporate the n-hexane at 40 °C. After the extraction liquid was allowed to dry naturally under a fume hood for 8 h, the residue was weighed. The degradation rate for petroleum hydrocarbon was calculated using the following formula, where M_1 was the mass of the petroleum hydrocarbon in the experimental group with bacterium solution and cultured for 5 days and M_2 was the mass of petroleum hydrocarbon before degradation (Deng et al., 2014), and the degradation rate η was given by:

$$\eta = \frac{M_2 - M_1}{M_2} \times 100\%.$$

2.4. Identification of strain

2.4.1. Morphological and biochemical characteristics

The morphological properties of strain Y-1 was examined by optical microscopy。 The typical biochemical and physiological characteristics of strain Y-1, including Gram staining, spore formation, Catalase activity, Anaerobic growth, V-P test, Gelatin hydrolysis, Propionate utilisation, Acid from glucose, Acid from arabinose, Acid from xylose and Acid from mannitol were systematically analysed according to Bergey's Manual of Determinative for Bacteriology (Holt et al., 1998).

2.4.2. PCR amplification of 16S rDNA

PCR amplification was performed for the 16sRNA gene by adopting universal primers 27F and 1541R. The 100 μL of reaction system contained 0.8 μL of Taq (5 U/μL), 10 μL of 10 × PCR Buffer (Mg^{2+}Plus), 8 μL of dNTP Mixture (2.5 mmol/L/sample), 2.5 ng of template DNA, 2 μL of primer 1 (10 μmol/L), 2 μL of primer 2 (10 μmol/L), and the rest was ultrapure water. The PCR amplification was conducted at 95 °C for 5 min; 20 cycles at 95 °C for 1 min, 58 °C for 1 min, −0.5 °C/cycle, and 72 °C for 1 min; 72 °C for 5 min; 15 cycles at 94 °C for 1 min, 48 °C for 1 min, and 72 °C for 1 min; 72 °C for 5 min; the reserved at 4 °C. The product of PCR was sequenced by the Huada Gene Technology Company, China (Ma et al., 2007). The specific sequence obtained was submitted to GenBank, where the homology of the sequence was compared with known 16sRNA through BLAST. Analysis of the 16SrDNA gene sequence data was performed using the software package Clustal-1.81 and MEGA version 6.0. Clustal-1.81 was used for sequence alignment. The phylogenetic tree was inferred using neighbour-joining methods through MEGA version 6.0.

2.5. Growth curve of the degrading bacteria

The screened strains were inoculated into the conical flask filled with 200 mL of T medium. Then the flask was placed on the same shaking table and rotated at 120 rpm to culture the strains at 30 °C. As the inoculation started, the strains were sampled every 2 h to determine the mycelia concentration therein. In other words, a UV spectrophotometer was adopted to measure the value of OD_{600}.