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Full Length Research Paper

Isolation and characterization of a biosurfactant-producing bacterium from Daqing oil-contaminated sites

Tong Liu[1#], Jumei Hou[1#], Yuhu Zuo[1]*, Sining Bi[1] and Jing Jing[2]

[1]Institute of Plant Pathology and Applied Microbiology, Heilongjiang Bayi Agricultural University, Daqing, Heilongjiang, 163319, P. R. China.
[2]School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai, 200240, P. R. China.

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Biosurfactants are biodegradable, non-toxic and ecofriendly compounds released by microorganisms. Most of the microorganisms were isolated from contaminated sites by petroleum hydrocarbon and industrial wastes. In this study, a biosurfactant-producing strain was isolated from Daqing oil-contaminated sites in China by enrichment culture, hemolytic activity assay, hydrolyzing oil activity assay, improved degreasing effect assay, and emulsification activity measurement. The strain was identified as *Pseudomonas* sp. BS1 based on its physiological characteristics and analysis of its 16S rRNA gene sequence. The identified isolate is capable of producing glycolipids or other anionic surfactants as determined in our analysis of a phenotypic assay using CTAB. The biosurfactant was isolated from the culture supernatant and identified as dirhamnolipids (Rha-Rha-C_{10}-C_{10}) by high-performance liquid chromatography–mass spectrometry. The culture filtrate and cell-free supernatant produced by this isolate were found to be highly effective in oil displacement from oil sand, suggesting that the isolate has potential use in enhanced oil recovery.

Key words: Biosurfactant, *Pseudomonas* sp., dirhamnolipids, microbial enhanced oil recovery.

INTRODUCTION

A surfactant is an important chemical raw material that can be derived from monosodium glutamate, which has a special and important function in the oil and food industries, as well as in environmental engineering. However, almost all surfactants are chemically derived from petroleum and are highly toxic and non-degradable, thereby causing serious environmental pollution problems (Franzetti et al., 2006). Biosurfactants are a group of surface-active molecules with hydrophilic and hydrophobic moieties. As alternative surfactants, biosurfactants have outstanding advantages, such as high biodegradability, low toxicity, environmental compatibility, high selectivity, and specific activity at extreme temperatures, pH, and salinity, among others. Biosurfactants have been shown to have a variety of applications, including enhancing crude oil recovery from oil reservoirs, mobilizing heavy crude oil transport in pipelines, and cleaning oil sludge from oil storage facilities. They are also used in soil/sand bioremediation, remediation of organics and metals, and as emulsifiers in agriculture and medicine in biological control (Desai and Banat, 1997; Lang and Wullbrandt, 1999).

Numerous microorganisms such as yeast, bacteria, and filamentous fungi have been widely used in the production of biosurfactants (Mata-Sandoval et al., 2000). These microorganisms have been found in contaminated sites containing petroleum hydrocarbon byproducts and industrial waste. For example, *Cupriavidus* sp. BSNC28C isolated from hydrocarbon-contaminated environments can reduce surface tension in the culture medium by up to 37.1 mNm^{-1} (Ruggeri et al., 2009). *Pseudomonas aeruginosa* B189 isolated from a milk factory exhibited higher surfactant activities than the artificial surfactants

*Corresponding author. E-mail: zuoyhu@163.com. Tel: 86-0459-8997858. Fax: 86-0459-8997856.

[#]These authors contributed equally to this work.

such as SDS and Tween 80 (Thanomsub et al., 2007). Thus, the isolation of bacteria-producing biosurfactants from the environment can provide excellent materials and resources.

The main objectives of this study are to (1) isolate a strain capable of producing biosurfactants from Daqing petroleum-contaminated sites; (2) identify the isolate by analyzing the 16S rDNA sequence, as well as the biochemical and physiological characteristics of the isolate; (3) identify the biosurfactant produced by the isolate by high-performance liquid chromatography–mass spectrometry (HPLC-MS); (4) determine the effectiveness of oil displacement from oil sand by assay; and (5) investigate potential applications in the oil industry.

MATERIALS AND METHODS

Collection of soil samples and enrichment culture

Petroleum-contaminated soil samples were collected from 90 different points at the Daqing oil field. Three soil samples were separated from each site. Each sample was loaded into sterile 250 ml Erlenmeyer flasks. The mouth of the flasks was covered immediately with sterile cotton wool. The samples were then transported to the laboratory in an ice bucket for isolation of micro-organisms that produce biosurfactants.

About 1 g of soil sample was inoculated into 50 ml minimal salt medium (MSM) in a 250 ml Erlenmeyer flask. The medium contained 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.01 g $FeSO_4$, 1.5 g $NaNO_3$, and 0.002 g $CaCl_2$ per liter. $(NH_4)_2SO_4$ (1.5 g) was supplemented with 5% (v/v) crude oil as the sole carbon source. Inoculation was performed with shaking (180 rpm) at 37°C for 7 days. Every 5 days, 2.5 ml of enrichment culture was transferred into fresh MSM.

Screening for biosurfactant producers

The isolated strain was screened by hemolytic activity and hydrolyzing oil activity assays. Hemolytic activity assay on blood agar plates (peptone: 10 g/L; yeast extract: 3 g/L; NaCl: 5 g/L; sheep blood: 100 ml/L) is a primary method for screening microorganisms capable of producing biosurfactants, according to Youssef et al. (2004). The isolates present in the clearing zone were re-screened by hydrolyzing oil activity assay on oil agar plates, following the method of Morikawa et al. (1993). Potential biosurfactant-producing stains were inoculated into the MSM, and the cultured filter was further analyzed by improved degreasing effect assay and emulsification activity measurement. Improved degreasing effect assay was carried out following Bi et al. (2009). The emulsification activity was determined by adding 5 ml of kerosene and an equal volume of cell-free supernatant to a 20 ml tube. The sample was homogenized in a vortex at high speed for 2 min and allowed to settle for 24 h. The emulsification index was then calculated by dividing the measured height of the emulsion layer by the total height of the liquid layer, and then multiplying the obtained value by 100.

Identification of biosurfactant-producing bacterium

Morphological, biochemical, and physiological characteristics were analyzed to identify the isolate, according to the method proposed by Buehanan and Gibbons (1974). To identify the isolate more accurately, we analyzed partial 16S rRNA gene nucleotide sequences corresponding to positions 27-1492 of the *Escherichia coli* 16S rRNA gene. Genomic DNA was obtained following the method of Ferrara et al. (2006). The universal oligonucleotide primers 27F and 1492R were used to amplify the 16S rRNA gene. Polymerase chain reaction (PCR) amplifications were performed with 0.5 µl Extensor Hi-Fidelity PCR Enzyme Mix (5 U/L), 5 µl 10× buffer, 4 µl $MgCl_2$ (25 mM), 1 µl dNTP (10 mM), 0.5 µl primer 27F (50 µM), 0.5 µl of the primer 1492R, 1 µl of template DNA (50 to 100 ng), and 37.5 µl ddH_2O. The PCR reaction conditions are as follows: 1 cycle at 94°C for 5 min; 30 cycles at 92°C for 1 min, 50°C for 1 min, and 72°C for 2 min; and a final cycle at 72°C for 5 min. The amplification products obtained after gel electrophoresis were purified using a QIAamp Mini Kit. The purified product was ligated to pMD19-T vector, transformed into *E. coli* DH5, and sequenced. The 16S rRNA gene nucleotide sequences obtained were compared with known sequences in GenBank using the BLAST program. Sequence alignment was carried out using ClustX (version 2.0), and the phylogenetic trees were drawn using the software MEGA version 4 by the neighbor joining method.

Production, isolation and identification of rhamnolipids

The identified strain was initially maintained on *Pseudomonas* isolation agar at 30°C for 24 h. Random single colonies were transferred into the MSM with the addition of 200 g/ml cetyltrimethylammonium bromide (CTAB, Sigma), 5 g/ml methylene blue, and 1.5% (w/v) agar, as described by Siegmund and Wagner et al. (1991). A colony showing a dark blue halo was selected and grown in Kay's minimal medium at 30°C for 5 days with shaking at 250 rpm.

The culture filtrate was centrifuged at 8,000 × g for 10 min at 4°C to remove the cells and the debris. The pH of the supernatant obtained was adjusted to 2.0 using 12 M hydrochloric acid, and then stored overnight at 4°C. The precipitates were collected by centrifugation at 8,000 × g for 20 min, and then extracted three times with a chloroform–methanol (2:1, v/v) mixture. The mixture was evaporated, leaving behind an oil-like appearance as the crude biosurfactant. The crude biosurfactants were dissolved in menthol and filtered by silica gel (50 Å) column chromatography with the elution of menthol. The elution fractions were collected and assayed with CTAB plates. The active fractions were analyzed by HPLC-MS (ZQ4000/2695, America Waters Company). Chromatographic separation was performed with a Waters C18 column (250 × 4.6 mm, 5 µm) with the elution of acetonitrile: water (90:10) at a rate of 1 ml/min. The injected volume was 10 µl. The separation was monitored at 280 nm. The molecular weight scan was 200 to 1500 with 50 V of CID.

Microbial enhanced oil recovery (MEOR)

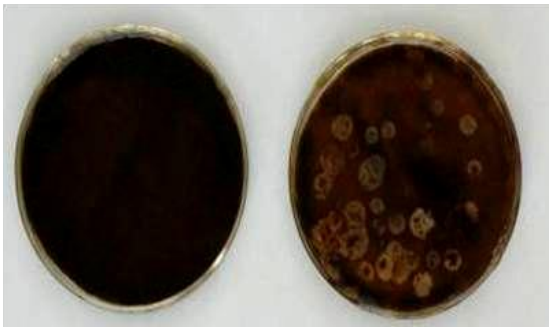
The MEOR process was carried out by the sand pack method described by Abu-Ruwaida et al. (1991). Hydrocarbon saturated sand pack column was treated with the culture filtrate and cell-free supernatant at 30°C. Distilled water served as the control. The oil displacement rate (%) = (M2/M1) × 100 (M_1: oil content in the sand (g); M_2: wash out oil content (g)). The assay was repeated three times with three replications for each treatment.

RESULTS

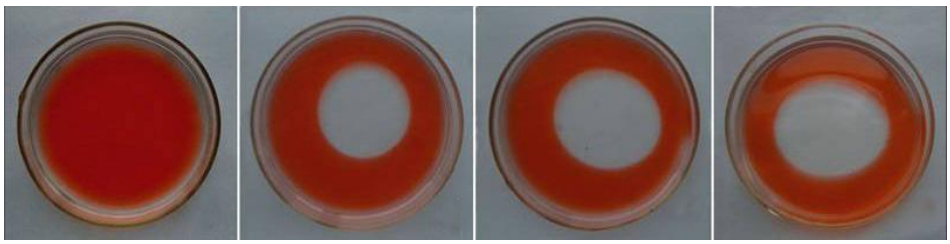
Isolation and identification of biosurfactants-producing bacterium

Twenty – three isolates from 270 samples of oil-

A



B



C

Figure 1. (A) Hemolytic zone of biosurfactant from isolates on blood agar plate; (B) Hydrolyzing oil spot of biosurfactant from isolates on oil ager plate; (C) Degreasing effect of fermentation broth on Sudan red plate containing liquid paraffin. CK: 100 µl water on plates containing liquid paraffin with Sudan red as control; 1, 2, 3: 100 µl cultured filter of different isolate on plates containing liquid paraffin with Sudan red

contaminated soil collected from the Daqing oil field in 90 different areas showed an obvious hemolytic zone on the blood agar plate (Figure 1A). Among the isolates, 16 formed a hydrolyzing oil spot on the oil agar plate (Figure 1B), and were considered potential biosurfactant-producing isolates. Moreover, as determined by improved degreasing effect assay (Figure 1C), three isolates among the potential biosurfactant-producing isolates exhibited an obvious oil displacement zone (>5.0 cm). One strain was determined as the best potential biosurfactant producer by emulsification activity measurement (43%). This isolate was characterized as Gram negative having a slender rod with rounded ends. The morphology of the colonies, as well as the physiological and biochemical characteristics of the strain, is shown in Table 1.

Because of intrinsic limitations, the biochemical and physiological features enabled only preliminary identification (Huang et al., 2010). The final identification was confirmed by combining similar results from 16S rDNA sequencing and analysis of biochemical and physiological characteristics. The 16S rDNA sequence was deposited in NCBI with the accession number GQ 281048 and retrieved from the classifier program Ribosomal Database Project II. Phylogenetic analysis indicated that the isolate was closer to *Pseudomonas* sp.

Table 1. Physiological and biochemical characteristics of the isolate.

Physiological and biochemical indexes	Reaction
Methyl red	+
V.P text	-
Activity of H_2O_2	+
Sugar fermentation tests	+
Sucrose	+↑
Glucose	+
Starch hydrolysis test	+
Indole test	-
H_2S test	+
Nitrate reduction test	+
Gelatin hydrolysis test	+

Notes: Physiological and biochemical responses of isolate: "+" positive, "–" negative, "↑"gas.

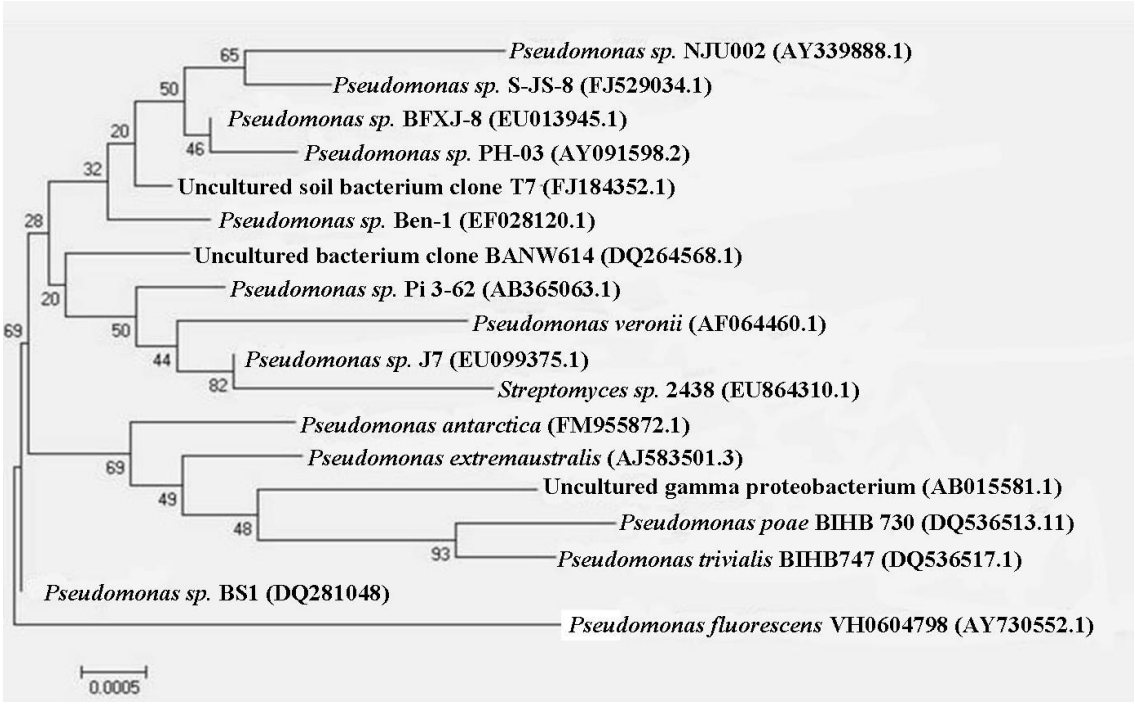


Figure 2. NJ bootstrapping phylogenetic tree of isolate and their closest NCBI (BLASTn) strains based on the 16S rRNA gene sequences.

(Figure 2) and is probably a species belonging to this genus. It was therefore named *Pseudomonas* sp. BS1.

Rhamnolipid assay, isolation and identification of rhamnolipids

A wide range of different *Pseudomonas* species produce various types of biosurfactant–rhamnolipids. Bacterium can be screened for rhamnolipid production using CTAB–methylene blue indicator plates. The results of the current work showed a dark blue halo after 48 h of incubation at 30°C, whereas the other strains did not show a positive reaction, demonstrating that the isolate can produce biosurfactant–rhamnolipids. The crude rhamnolipids were isolated by a combination of acid deposition, organic solvent extraction, and column chromatography. The active fractions were identified by HLPC-MS. The results showed that active fractions exhibited a peak at 4.79 min. A molecular ion 649.5 and special ion peak 479.1, 339.2

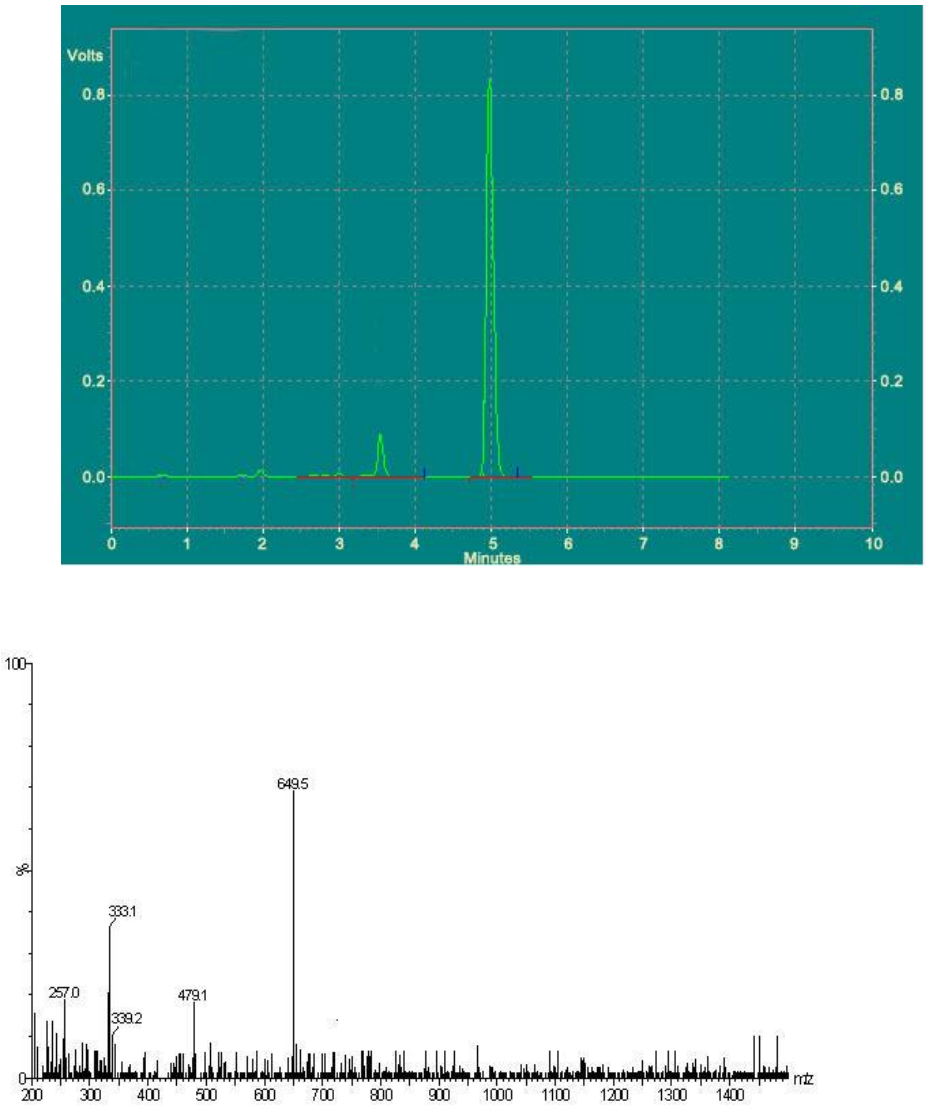


Figure 3. HPLC-MS figure of dirhamnolipids(Rha-Rha-C_{10}-C_{10}) produced by *Pseudomonas* sp. BS1.

and 333.1 were observed (Figure 3). The results clearly indicate that the strain can produce dirhamnolipids (Rha-Rha -C_{10}-C_{10}).

MEOR

The oil displacement rates caused by the fermentation broth and culture filtrate (except for the cell) at 30°C were 83.5 and 83.3%, respectively, whereas those caused by bacterial cell suspensions and distilled water were 16.8 and 16.6%, respectively. The results showed that both the fermentation broth and culture filtrate (except for the cell) produced by *Pseudomonas* sp. BS1 was highly effective in recovering crude oil from the sand pack column.

DISCUSSION

Kiran et al. (2010) suggested that the single screening method is unsuitable for identifying all types of biosurfactants, and recommended that more than one screening method should be included during primary screening to identify potential biosurfactant producers. In the present study, therefore, hemolytic activity assay, hydrolyzing oil activity assay, improved degreasing effect assay, and emulsification activity measurement were used to screen the biosurfactant producer. Strain *Pseudomonas* sp. Showed positive results in all of the

four screening methods used. Thus, we confirm that this bacterium can produce biosurfactants with positive responses. *Pseudomonas* sp. are known to produce different types of rhamnolipids. For example, *P. aeruginosa* produces a mixture of homologues including RL1, RL2, RL3, and RL4 (Syldatk and Wager et al., 1987). *Pseudomonas chlororaphis* produces mono-rhamnolipid-type Rha-C_{10}-C_{12}, Rha-C_{12}-C_{12}, and Rha-C_{10}-C_{10} (Gunther et al., 2005). Thanomsub found that *P. aeruginosa* B189 produces Rha-Rha-C_{10}-C_{10} and Rha-C_{10}-C_{12}. *Pseudomonas fluorescens* NCIMB 11712 was also found to produce a methylpentose monosaccharide (Caldini et al., 1995). A simple method using CTAB-methylene blue indicator plates can be used to screen rhamnolipids produced by a wide range of *Pseudomonas* species, as well as other types of bacteria. We conjecture that *Pseudomonas* sp. BS1 may produce rhamnolipids, and first assayed the strain by this method. The results demonstrated that the strain showed positive reaction on the indicator plates, thereby providing a direction for further identification of rhamnolipids.

Rhamnolipids are primary representatives of biosurfactants and generally include one to two molecules of rhamnose and one to two molecules with different carbon chain lengths of saturated or unsaturated fatty acids. Many similar homologues were generated through linking between the various chemical structures in the biological synthesis process (Lang and Wullbrandt et al., 1999). Therefore, it is difficult to completely separate the individual ingredients of the rhamnolipids using the microorganisms from culture filtration by traditional separation methods, such as column chromatography and thin layer chromatography. These methods may also result in the loss of some components during the complicated operation process. Such techniques pose difficulties in generating comprehensive information on chemical composition. In recent years, a developmental HPLC-MS technique that features effective separation and identification of glycolipid produced by *P. aeruginosa* (Li et al., 2011) has been reported. Therefore, we identified rhamnolipids produced by *Pseudomonas* sp. BS1 using the HLPC-MS technique. Our research showed that *Pseudomonas* sp. BS1 mainly produced dihamnolipids (Rha-Rha-C_{10}-C_{10}).

In the current work, the highest level of rhamnolipid production by *Pseudomonas* sp. BS1 was 0.9 g/L (data not shown). The optimization of some factors, such as carbon substrate, temperature, pH, pressure, and salinity, affected rhamnolipid production; this influence makes *Pseudomonas* sp. BS1 a more attractive candidate for commercial rhamnolipid production.

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