Chemosphere 87 (2012) 1105–1110

Contents lists available at SciVerse ScienceDirect

Chemosphere

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

Isolation, identification and characterization of *Bacillus amyloliquefaciens* BZ-6, a bacterial isolate for enhancing oil recovery from oily sludge

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ARTICLE INFO

Article history:
Received 19 October 2011
Received in revised form 30 January 2012
Accepted 30 January 2012
Available online 24 February 2012

Keywords:
Oily sludge
Biosurfactants
Oil recovery
Bacillus amyloliquefaciens

ABSTRACT

Over 100 biosurfactant-producing microorganisms were isolated from oily sludge and petroleum-contaminated soil from Shengli oil field in north China. Sixteen of the bacterial isolates produced biosurfactants and reduced the surface tension of the growth medium from 71 to <30 mN m^{-1} after 72 h of growth. These bacteria were used to treat oily sludge and the recovery efficiencies of oil from oily sludge were determined. The oil recovery efficiencies of different isolates ranged from 39% to 88%. Bacterial isolate BZ-6 was found to be the most efficient strain and the three phases (oil, water and sediment) were separated automatically after the sludge was treated with the culture medium of BZ-6. Based on morphological, physiological characteristics and molecular identification, isolate BZ-6 was identified as *Bacillus amyloliquefaciens*. The biosurfactant produced by isolate BZ-6 was purified and analyzed by high performance liquid chromatography–electrospray ionization tandem mass spectrometry. There were four ion peaks representing four different fengycin A homologues.

1. Introduction

Oil refineries and petrochemical industries generate large amounts of solid waste. Of special concern is the oily sludge that accumulates at the bottom of crude oil storage tanks or is generated in water–oil separation systems (Mait et al., 2008). Oily sludge contains large amounts of benzene, phenol and polycyclic aromatic hydrocarbons which have highly toxic, mutagenic and carcinogenic effects on humans and pollute the environment. They are therefore classified as priority environmental pollutants by the US Environmental Protection Agency (USEPA, 1986) and are also on the List of Dangerous Solid Wastes in China. Improper disposal of oily sludge may contaminate soils and pose a serious threat to groundwater.

Landfill, coking treatment, solvent extraction and incineration are currently the main methods for disposal of oily sludge. However these methods are considered expensive or inadequate to meet current and future regulations (Conaway, 1999). The amount of oily sludge produced in Chinese refineries and oil fields has been estimated to be 450 kt yr^{-1} (Deng et al., 2007). Oily sludge contains various fractions of petroleum hydrocarbons (typically 10–50 wt%), solids (6–10 wt%) and water and is of widely varying composition. Recycling is the most desirable environmental option for handling oily sludge as the supply of oil declines and concern about environmental pollution increases. Separation and reclamation of oil from the sludge can minimize the disposal of pollutants and minimize environmental pollution.

Biosurfactants are complex biopolymers produced by microorganisms and they have desirable characteristics such as biodegradability, low toxicity, ecological acceptability and the capacity to be produced from renewable and cheaper substrates than many synthetic surfactants for oil industry applications (Banat et al., 2000). There has been a growing interest in biosurfactant applications in environmental remediation (Banat, 1995; Barathi and Vasudevan, 2001; Urum and Pekdemir, 2004; Bordoloi and Konwar, 2009; Joseph and Joseph, 2009). The potential application of biosurfactants in stimulating indigenous microorganisms for enhanced bioremediation of diesel-contaminated soil has been confirmed (Bordoloi and Konwar, 2009). Banat et al. (1991) described the application of microbial biosurfactants for the clean-up of oil storage tanks and the successful removal of oil from the bottom of the tanks.

Although a number of studies have been carried out on soil bioremediation and tank cleaning using biosurfactants, few assessments of their performance in recovering oil from oily sludge have been reported. The purpose of the present study was to isolate

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0045-6535/$ - see front matter © 2012 Published by Elsevier Ltd.
doi:10.1016/j.chemosphere.2012.01.059

biosurfactant-producing bacteria and evaluate their oil recovery efficiency from sludge. Furthermore, surface-active substances produced by the bacterial isolate with the highest oil recovery capacity were purified and identified.

2. Materials and methods

2.1. Media and oily sludge

Minimal medium (MM) used throughout the study contained (in g L^{-1}) $(NH_4)_2SO_4$ 5, glucose 2, KCl 1.1, NaCl 1.1, $FeSO_4$ 0.028, KH_2PO_4 1.5, K_2HPO_4 1.5, $MgSO_4$ 0.5 and 5 mL of trace element solution (TES). TES contained (in g L^{-1}) $ZnSO_4$ 0.2, $CaCl_2$ 0.24, $CuSO_4$ 0.25, and $MgSO_4$ 0.17. Solid MM plates were prepared by adding 20 g agar to 1000 mL MM. Blood agar was prepared with sheep's blood (5%) and LB medium. LB medium contained (in g L^{-1}) tryptone 10, yeast extract 5, and NaCl 10 and was adjusted pH to 7. Blood was added prior to pouring and the plates were allowed to solidify. Oily sludge used in this study was collected from Bingyi Collection Facility at Shengli oil field, Shandong province, China. The oil concentration of the sludge was 31% by weight.

2.2. Isolation of biosurfactant-producing bacteria

Biosurfactant-producing microorganisms were isolated from oily sludge and petroleum-contaminated soil from Shengli oil field using blood agar lysis and surface tension measurements (McInerney et al., 1990; Youssef et al., 2004). Briefly, an aliquot of 5 g of soil (sludge) sample was inoculated into 50 mL of MM medium containing 0.5 g crude oil and incubated aerobically at 25 °C on a reciprocal shaker at 150 rpm for 72 h. After incubation the medium was serially diluted with sterile water and plated on blood agar. The plates were incubated at 37 °C and observed after 48 h. Colonies producing biosurfactant (hemolytic zone around the colonies) were picked up. Isolation and purification procedures were carried out on LB plates by conventional spread plate techniques. The isolated strains were inoculated into 50 mL of LB medium and incubated at 25 °C with shaking at 180 rpm for 24 h. Then the surface tension of the culture medium of each strain was measured by the ring method at 25 °C using a Model ZL-2 digital tensiometer (Boshan Tongye Analytical Instrument, Shandong, China).

2.3. Oily sludge treatment experiment

Each isolated strain was inoculated into a 250 mL flask with 100 ml LB medium at 25 °C and incubated aerobically on a reciprocal shaker at 150 rpm for 48 h. The LB broth was then used in the following procedure. An aliquot of 10 g oily sludge sample was added to each LB broth and shaken at 25 °C and 180 rpm for 24 h. Unamended LB medium was also used as a control. The broth was allowed to settle for several hours and oil separation was observed visually. Oil was extracted and measured gravimetrically following the procedure recommended by USEPA test method 418 (USEPA, 1986). The oil recovery efficiency was estimated as follows:

$$\text{Oil recovery efficiency} = \frac{\text{Oil from liquid extraction}}{\text{Oil from liquid extraction} + \text{Oil from sludge}}$$

Fig. 1. The three phases separate of sludge after treated by the culture medium of BZ-6.

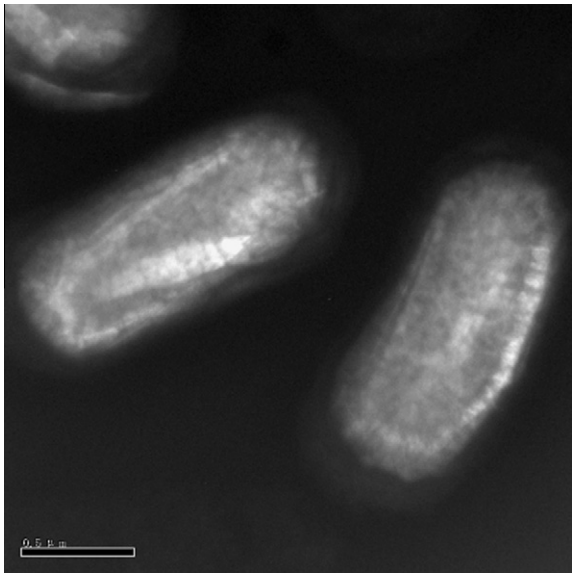


Fig. 2. Electron micrograph (TEM) of BZ-6.

Table 1
Surface tension and oil recovery efficiency of the 16 bacterial isolates.

Isolate	Surface tension (mN m^{-1})	Recovery efficiencies (%)	Isolate	Surface tension (mN m^{-1})	Recovery efficiencies (%)
SB-5	27.3	70	SO-4	29.6	50
SB-11	28.3	55	SO-9	29.3	48
SB-13	27.8	69	SO-12	28.2	56
SB-18	28.2	51	SO-18	29.4	42
SB-21	28.0	75	BZ-3	29.2	51
SS-8	29.7	39	BZ-6	28.5	88
SS-12	28.7	56	BZ-12	29.0	48
SS-15	27.4	78	BZ-14	30.0	42

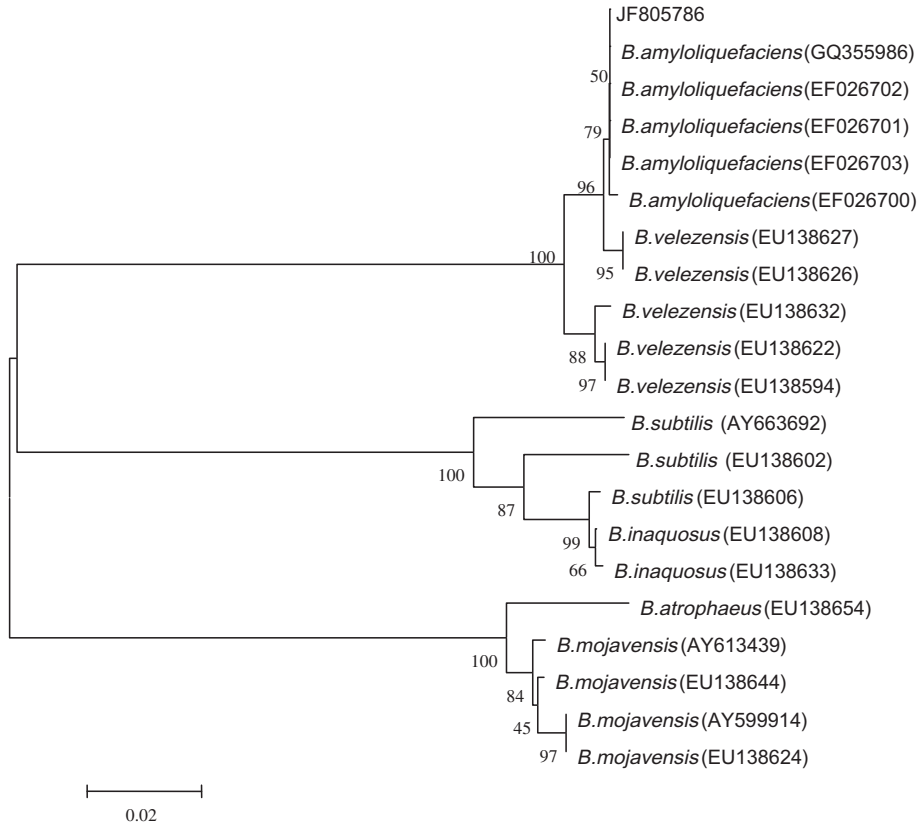


Fig. 3. Phylogenetic tree based on gyrA nucleotide sequences (690 bp) sequences. The scale bar corresponds to 0.02-estimated nucleotide substitution per sequence position. Bootstrap values from 1000 replicates.

2.4. Identification of strain BZ-6

The morphological properties of BZ-6 were examined by light microscopy and TEM. Biochemical and physiological properties of the isolate were analyzed using routine methods (Shen et al., 1999). 16S rDNA and gyrA nucleotide sequences were amplified from chromosomal DNA by PCR using universal oligonucleotide primers and sequenced as described by Lee et al. (2010). The sequences were then compared to the 16S rDNA and gyrA nucleotide sequences in the GenBank database by BLASTN. Multiple sequence alignment was done using CLUSTAL X software and a phylogenetic tree was constructed by the neighbor-joining method using MEGA (Version 4.1) software. The confidence level of each branch (1000 repeats) was tested by bootstrap analysis. The 16S rDNA and gyrA sequences of isolate BZ-6 were deposited in the GenBank database with accession numbers JF693628 and JF805786, respectively.

2.5. Purification of biosurfactant

The biosurfactant was extracted from the culture medium after removal of the bacteria by centrifugation at 10,000 rpm for 10 min at 4 °C. The supernatant pH was adjusted to 2 with 6 M HCl and flocculating sediment was observed. After settlement overnight at 4 °C, the liquid was centrifuged to collect the sediment at 10,000 rpm. The sediment was washed with acidified water at pH 2 and neutralized by dissolving in NaOH solution. The sediments were freeze-dried to form light brown loose crude biosurfactants. The crude biosurfactants were extracted in CH_2Cl_2 and desiccated through decreasing pressure. Then the dry biosurfactants were dissolved in dilute NaOH solution to form a foamy liquid. After filtration through Whatman No. 4 microspore membranes (15–20 μm pore diameter), the liquids were acidified to pH 2 with HCl to form a precipitate. Sediments were centrifuged and desiccated to obtain purified biosurfactant (Qin et al., 2005). The purified biosurfactants were further analyzed to determine their composition by electrospray ionization/collision-induced dissociation (ESI/CID) mass.

2.6. Biosurfactant HPLC/ESI/CID analysis

The purified biosurfactant was further separated by HPLC using a revered phase C18 analytical column (ODS-4.6 mm × 250 mm, Agilent, Santa Clara, CA) with mobile phases of 0.05% trifluoroacetic acid in acetonitrile (solvent A) and 0.05% trifluoroacetic in milliQ water (solvent B) at a flow rate of 200 μL min^{-1} using gradient elution with UV detection at 210 nm. The elution conditions were: 50–100% (A), 0–50 min, 50–0% (B), 0–50 min; 100% (A), 50–70 min, 0% (B) 50–70 min. ESI/CID mass spectrometry analysis was performed with a Surveyor-LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA). The electrospray source was operated at a capillary voltage of 15 V, a spray voltage of 5 kV and a capillary temperature of 275 °C. Helium was used as the collision gas for the CID experiment and the collision energy was set at 35% (Sun et al., 2006).

3. Results and discussion

3.1. Isolation of a high efficiency biosurfactant-producing strain

Surface tension measurement is the most direct approach for screening biosurfactant-producing bacteria. However, this method is time-consuming and is therefore inconvenient to use for screening large numbers of isolates (Youssef et al., 2004). In contrast, blood agar lysis has been recommended as a simple and easy

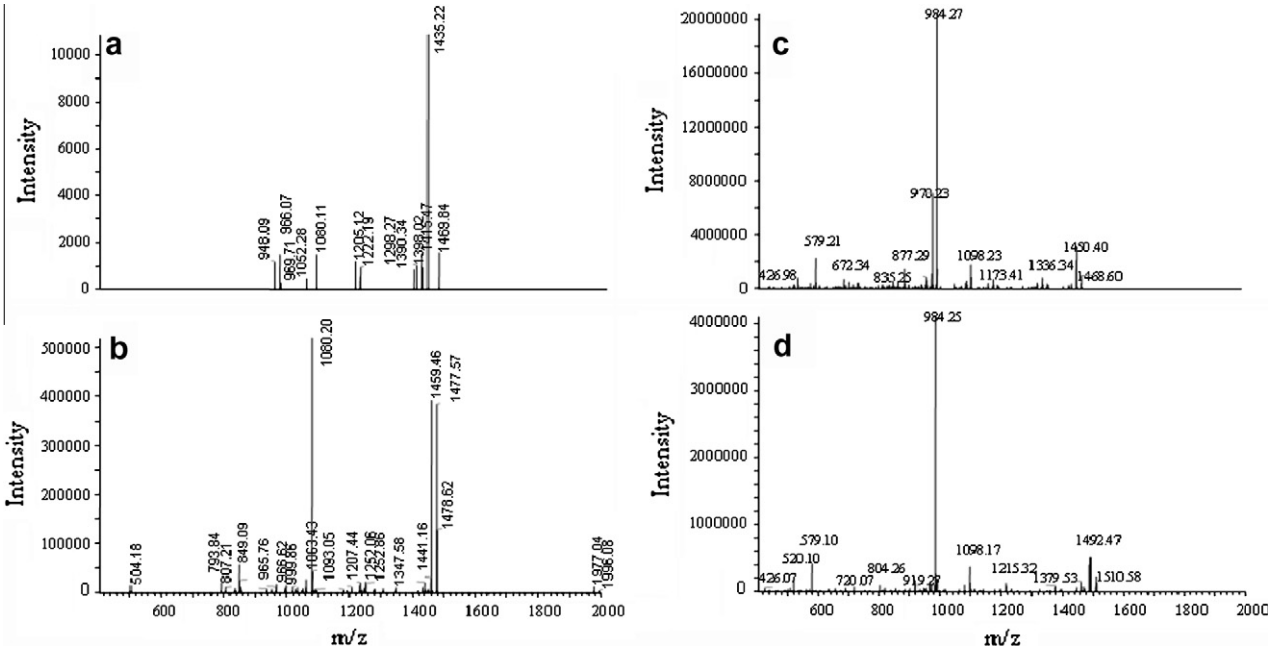
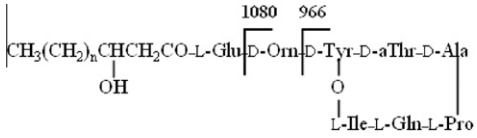
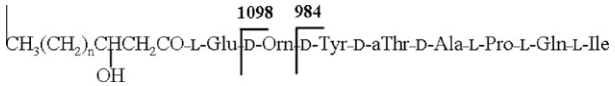


Fig. 4. CID spectra of the purified biosurfactant of *Bacillus amyloliquefaciens* BZ-6, (a–d) are the CID spectra of precursor ions of *m*/*z* 1435.2, 1477.5, 1468.6, and 1510.6, respectively.

Table 2
Summary of identified fengycin A homologues.

Mass peak (*m*/*z*)	Fingerprint product ions	Fengycin homologues	Structure
1435.2	1080, 966	Circular fengycin A (C14)	
1477.5	1080, 966	Circular fengycin A (C17)	
1468.6	1098, 984	Linear fengycin A (C15)	
1510.6	1098, 984	Linear fengycin A (C18)	

method to test for biosurfactant activity (Banat, 1993; Yonebayashi et al., 2000). Studies have also shown that a number of false positives (16%) when using blood agar lysis and the blood agar lysis diameter was not correlated with the surface tension ($r = -0.15$) (Youssef et al., 2004).

Blood agar lysis was used in the present study as a preliminary screening method and surface tension measurements were also carried out to confirm the results. More than 100 bacteria with hemolytic activity on blood agar plates were isolated from the petroleum contaminated soil and oily sludge. These isolates were further tested qualitatively for biosurfactant production by surface tension measurements. Of these, 16 strains reduced the surface tension of the growth medium from 71 to <30 mN m^{-1} after 48 h of growth in LB medium. Isolate SB-5 produced the lowest surface tension of 27.3 m Nm^{-1} (Table 1). These 16 isolates were selected for further study.

3.2. Oil recovery efficiency of different isolates

The fermentation broths of the 16 bacterial isolates were examined for their efficiencies of oil recovery from oily sludge. The oil recovery efficiency was only 12% in the control (LB medium only). The recovery efficiencies in the fermentation broths of the 16 isolates ranged from 39 to 88%, with isolate BZ-6 showing the highest efficiency (Table 1). BZ-6 was therefore considered to be the most effective isolate and was selected for further study.

There have been numerous studies on the washing of crude oil contaminated soil with surfactants but their purpose was only to clean the soil and not to recover the oil (Urum et al., 2006; Franzetti et al., 2009). After these treatments, further treatment is generally needed for oil-containing wastewater. In the present study the three phases oil, water and sediment were separated after the treatment. The sediments, without hydrocarbons, precipitate to the bottom, the oil extracted from the sludges rises to the surface and the water remains in the middle (Fig. 1) so that the oil can be recycled.

It is interesting to note that isolate SB-5, which produced the lowest surface tension (27.3 mN m^{-1}) did not have the highest oil recover efficiency as shown in Table 1. However, isolate BZ-6 showed the highest recover efficiency (88%) despite its surface tension of 28.5 mN m^{-1}. Similar observations were also described by Willumsen and Karlson (1997) who found an absence of correlation between the reduction in surface tension and emulsion formation. The explanation may be that surface tension is mostly dependent on the structure and concentration of biosurfactant. However, surface tension will not continue to decrease when the concentration of biosurfactant exceeds the critical micelle concen-

tration (Youssef et al., 2004). The broth of some isolates with high concentration of biosurfactant may display high oil removal efficiency even though they produce a relatively low surface tension.

3.3. Morphological and biochemical characteristics of BZ-6

The colonies of strain BZ-6 are cream in color, opaque and convex with a wrinkled surface and entire margins on solid LB plates. Cells are Gram-positive and rod-shaped (0.7–0.9 μm thick, 1.8–3.0 μm long) bearing a central/subterminal ellipsoidal spore and an electron micrograph (TEM) of BZ-6 is shown in Fig. 2. Physiological and biochemical tests indicated BZ-6 to be aerobic, catalase-positive, nitrate reduction-positive, indole-positive, capable of starch and gelatin hydrolysis, methyl red-negative and Voges–Proskauer-negative (Table 1).

3.4. Phylogenetic position of isolate BZ-6

Physiological and biochemical characters and 16S rRNA gene sequences (GenBank Accession No. JF693628) of BZ-6 were firstly used to identify the strain. It was found that BZ-6 has similar phenotypes and biochemical characters to *Bacillus*. The 16S rDNA sequence of strain BZ-6 also showed high similarities (>99%) (data not shown) to *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus vallismortis*, *Bacillus velezensis*, *Bacillus mojavensis*, *Bacillus amyloliquefaciens* and *Bacillus atrophaeus* in the similarity search. These species are regarded as members of the *B. subtilis* group and their discrimination on the basis of 16S rDNA sequence analysis has been questioned (Chun and Bae, 2000). The 16S rRNA gene sequences showed limited variation in the closely related species of *B. subtilis* group (e.g. *B. subtilis* and *B. amyloliquefaciens* showed more than 99% similarities) and prevents the resolution of strain and species relationship (Hutsebaut et al., 2006).

Chun and Bae (2000) demonstrated that the use of the gyrA sequence could accurately classify *B. subtilis* and related taxa, including *B. licheniformis*, *B. mojavensis*, *B. amyloliquefaciens*, and *B. atrophaeus*. Therefore the gyrA sequence and phylogenetic analysis were used to further identify the isolated bacteria. The gyrA sequence (Accession No. JF805786) of BZ-6 showed 100% homology with *B. amyloliquefaciens* and was most closely associated with *B. amyloliquefaciens* in the phylogenetic tree (Fig. 3). On the basis of morphological, physiological, biochemical characteristics, phylogenetic position and genes sequences of gyrA and 16S rDNA, the isolated strain BZ-6 was identified as *B. amyloliquefaciens*.

3.5. Structural characterization of the biosurfactant

The molecular mass of the purified compounds was measured using ESI-MS spectrometry, giving main peaks at *m*/*z* 1435.2, 1477.5, 1468.6, and 1510.6. Each of these ions was further selected as the precursor ion for further CID analysis. The results show that product ions of *m*/*z* 966 and 1080 were found in the CID spectra of precursor ions of *m*/*z* 1435.2 and 1477.5 (Fig. 4a and b). Product ions of *m*/*z* 984 and 1098 were found in the CID spectra of precursor ions of *m*/*z* 1468.6 and 1477.6 (Fig. 4c and d).

The ions of *m*/*z* 966 and 1080 can be considered as 'fingerprints' of circular fengycin A by comparing their mass data with those obtained in previous studies (Nishikiori et al., 1986; Bie et al., 2009). The ions of *m*/*z* 966 and 1098 are 'fingerprints' of linear fengycin A. They can be explained as neutral losses of (fatty acid–Glu) and (fatty acid–Glu–Orn), respectively, from the N-terminus segment of fengycin A (Table 2). Two peaks, at *m*/*z* 1435.2 and 1477.5, reveal differences of 3×14 Da, suggesting that the purified compound had a different carbon chain length (three $-CH_2-$ groups). Therefore, peaks at *m*/*z* 1435.2 and 1477.5 were protonated molecular ion peaks $[M+H]^+$ of circular fengycin A homologues and peaks at m/z 1468.6 and 1510.6 were those of linear fengycin A homologues with different carbon chain length. The assignment and structure of the fengycin A homologues are summarized in Table 2.

4. Conclusions

Over 100 bacterial strains with hemolytic activity on blood agar plates were isolated from the petroleum contaminated soil and oily sludge. Sixteen of these isolates were able to reduce the surface tension of the growth medium from 71 to <30 m Nm^{-1}. BZ-6 was the most efficient of the isolates in separating oil from the oily sludge, with a separation efficiency of 88% after 24 h treatment with the fermentation broth of the isolate. Isolate BZ-6 was selected for further study and was identified as *B. amyloliquefaciens*. The biosurfactant produced by BZ-6 was also analyzed and the results show that it includes four fengycin A homologues. This bacterial isolate may therefore have potential for the practical removal of oil from oily wastes that contaminate large oil fields and this merits further research.

Acknowledgements

We thank the Program of Innovative Engineering of the Chinese Academy of Sciences (KSCX2-YW-G-053 and KZCX2-YW-Q02-02), the National Natural Science Foundation of China (41001182) and the Environmental Protection Public Welfare Special Fund for Scientific Research (201009015) for generous financial support.

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