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Environmental impact statement

In this paper, the predominant bacterium *Acinetobacter sp.* D3-2 was isolated from oil-contaminated soil in Dongying, China. Morphological, biochemical and physiological characteristics of the strain were characterized. Environmental factors that could influence the regular growth of *Acinetobacter sp.* D3-2 were optimized. We then investigated the hydrocarbon-utilizing capability and also characterized the simultaneously produced biosurfactant. Furthermore, this study provides available capacity in crude oil degradation experiment. The results in shake flask trials showed that the strain could degrade *n*-alkane ranging from C9 to C38. The strain posed enormous potential in the process of oil pollution bioremediation. So, the predominant bacteria *Acinebacter sp.* D3-2 might be feasible for practical application in the future.

**Lipopeptide Biosurfactant Production bacteria *Acinetobacter sp.* D3-2 and its
Biodegradation of Crude Oil**

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Abstract: In this work, a hydrocarbon-degrading bacterium D3-2 isolated from petroleum contaminated soil samples was investigated on its potential effect of biodegradation of crude oil. The strain was identified as *Acinetobacter sp.* D3-2 based on morphological, biochemical and phylogenetic analysis. The optimum environmental conditions for the bacterium growth were determined to be pH 8.0, 3.0% (w/v) of NaCl concentration at 30°C. *Acinetobacter sp.* D3-2 could utilize various hydrocarbon substrates as sole carbon and energy source. From this study, we also found the strain had an ability to produce biosurfactant with the production of 0.52 g•L⁻¹. Surface tension of the culture broth was decreased from 48.02 to 26.30 mN•m⁻¹. The biosurfactant was determined to contain lipopeptide compounds based on the laboratory analyses. By carrying out crude oil degradation assay in Erlenmeyer flask experiment and analyzing hydrocarbon removal rate using gas chromatography, we found that *Acinetobacter sp.* D3-2 could grow at 30°C in 3% NaCl solution with a preferable ability

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to degrade 82% hydrocarbons, pointing out the fact that bioremediation did exist and play a profound role during the oil reparation process.

Key words: biodegradation, biosurfactant, *Acinetobacter sp.*, hydrocarbon degradation

1. Introduction

With the increasing demand for petroleum and associated products, hydrocarbon contamination in marine environment has been regarded as a critical international issue ^[18]. According to statistics, total petroleum released into the sea from all sources is approximately 1.3 Mt/year ^[15]. The huge amount of spilled oil can cause extensive ecological damage to marine and coastal environmental and have great impact on the local economy such as the explosion of the Deepwater Horizon drilling platform on April 20th, 2010. Crude oil is a complex mixture including aliphatics, aromatics and asphalt. Highly toxic petroleum compounds such as polycyclic aromatic hydrocarbons (PAHs) attracted wild attention regarding environmental and health concerns ^[27]. Technique of microbial remediation which is regarded as an economic and versatile alternative to physicochemical treatment is an efficient way to solve this problem ^[1, 10, 20, 23, 26, 31, 32]. Most of hydrocarbon-degrading microorganisms in common use were screened from nature. Oil contaminated site could enrich this kind of bacteria which were able to utilize different type of hydrocarbons as sole carbon and energy source. However, the interaction between petroleum contaminants and microorganisms is a complex process ^[8]. Environmental factors such as oil physical state, salinity, pH, DO, temperature and competitive with other microbial communities will also limit the performance of bioremediation agents in work.

Therefore, many experts are committed to make full use of hydrocarbon degrader with high efficiency in hydrocarbon biodegradation process. Guo et al. [7] isolated 21 strains from five different genera, which could achieve different degrading effect under different co-culture conditions. Bernabeu et al. [4] analyzed the evolution of oil from tar balls to coatings and found that water condition and salinity influenced oil degradation process on beaches. Liu et al. [11] found that alkane removal rate was corresponding to the concentration of NaCl in the medium and nutrient broth addition could enhance the biodegradation effect. Biosurfactant could increase the contact area between strains and crude oil in the medium [5, 16]. It had great potential to decontaminate polluted water and soils in bioremediation process [14, 22]. Some hydrocarbon-degrading bacteria could produce biosurfactant simultaneously during metabolizing hydrocarbons [24].

In this paper, the predominant bacterium *Acinetobacter sp.* D3-2 was isolated from oil-contaminated soil in Dongying, China. We then investigated the hydrocarbon-utilizing capability and also characterized the simultaneously produced biosurfactant. The aim of this study was to evaluate the practical application feasibility of *Acinetobacter sp.* D3-2 in the future.

2. Material and Methods

2.1 Culture Media and Microorganism

Enrichment medium used in this study contained 3 g beef extract, 10 g peptone, 5 g NaCl per liter. Mineral salt medium (MSM) [3] contained 3 g NaCl, 3 g Na₂HPO₄, 2 g KH₂PO₄, 5 g (NH₄)₂SO₄, 0.7 g MgSO₄·7H₂O, and 1mL trace salt solution per liter. The trace salt solution

included 2 mg CaCl_2 , 30 mg FeCl_3 , 0.5 mg CuSO_4 , 0.5 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 10 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per liter. The pH was adjusted to 7.0-7.2 with 1.0 M NaOH and 1.0 M HCl.

Hydrocarbon degrading bacteria were isolated from crude oil contaminated soil in Dongying (37.955°N, 118.247°E), China. We threw 10 g soil sample into 100 mL MSM in a 250mL Erlenmeyer flask added with 0.5% (w/v) crude oil as the sole source of carbon and then incubated the flask at 25°C with shaking at 130 rpm for 3 days (d), at last transferred 2 mL of culture solution into 100 mL fresh medium. After several recultivations, hydrocarbon degraders were enriched. The strains were separated by MSM flat agar covered with a thin layer of sterilized crude oil. Plates were cultured at 36°C for 5 d.

Pure colonies were picked up and transferred individually into 100 mL MSM with 0.5% (w/v) crude oil as sole carbon. After 7 d culture at 25°C with shaking at 130 rpm, residual crude oil was extracted by hexane ^[28] and determined by UV analysis. Bacteria with strong ability of degrading crude oil were selected and stored as the final isolates at -80 °C in glycerol.

The crude oil which was purchased from reservoir of Shengli oilfield had a viscosity 22.2 mPa•s (50°C, 50 rpm), freezing point 23.0°C, density 0.8552 g•cm³, and no wax. Referring to previous research reported by Wang et al. ^[30], for the purpose of reducing volatile hydrocarbon losses during the experiments, crude oil was then put into fume hood for 24 h to reduce volatile hydrocarbon losses during our experiments.

2.2 Physiology and Biochemistry Characterization

D3-2 was selected as candidate in following bioremediation experiments based on the efficiency of crude oil utilization. The strain was cultivated in 100 mL enrichment medium for 24 hours (h) at first. All flasks were incubated at 25 °C with shaking at 130 rpm for 3-5 d. Then the bacterium was enriched and ready to use. It was identified by means of physiology and biochemistry characteristics such as cell morphology, colony morphology and substrate utilizing ability according to Bergey's manual ^[6].

2.3 16S rRNA Gene Sequencing and Phylogenetic Analysis

RNA was extracted using the TIANamp Bacteria RNA Extraction Kit (Tiangen Biotech Co., Ltd., Beijing, China). To amplify the 16S rRNA gene, a polymerase chain reaction (PCR) was performed using a pair of primers: the forward primer 27F (5' -AGAGTTTGATCMTGGCTCAG-3') and the reverse primer 1492R (5' -GGCTACCTTGTTACGACTT-3').

In 25 uL PCR reaction system, there were 1 uL RNA template, 0.5 uL 27F, 0.5 uL 1492R (final concentration 0.4 $\mu\text{M}\cdot\text{L}^{-1}$), 2 uL dNTP (final concentration of each dNTP 0.2 $\text{mM}\cdot\text{L}^{-1}$), 2 uL MgCl_2 (final concentration 2.0 $\text{mM}\cdot\text{L}^{-1}$), 2.5 uL of 10×PCR buffer and 0.2 uL Taq polymerase. The PCR mixtures were preheated at 94 °C for 5min prior to running the following cycles: 94 °C, 30s; 55 °C, 1min; 72 °C, 1min. A PCR was run for 30 cycles in a RNA thermal cycle. At the end of the final cycle, a chain-elongation step at 72 °C for 10 min was programmed.

The sequencing was completed by Genscript Biotech Co., Ltd., Nanjing, China. The 16S rRNA sequences of strain D3-2 was subjected to a similarity search BLAST on the NCBI

website (<http://www.ncbi.nlm.nih.gov>) and deposited into GenBank with an accession number of HQ731046. Phylogenetic tree was constructed using the software MEGA 4.0.

2.4 Optimization of Growth Conditions

In order to find out the suitable growth conditions for microorganism, optimum temperature, pH and salinity were determined in the way of shaking flask experiments. 100 mL MSM was added into 250 mL Erlenmeyer flask along with 0.35% (w/v) crude oil as sole source of carbon. First, experiments were carried out at various temperatures ranging from 15 to 40°C, pH=8 and NaCl 20 g•L⁻¹. Then the experiments were conducted at various pH ranging from 5.0 to 10.0, with NaCl 20 g•L⁻¹ at optimum temperature. Finally, salt tolerance of strain D3-2 was tested in MSM under optimum temperature and pH, containing 5 to 50 g•L⁻¹ of NaCl. All of the flasks were sterilized at 120 °C for 30 min before inoculation. Incubation was carried out for 7 d. Cell density used to evaluate optimum growth conditions was determined using ultraviolet and visible spectrophotometer at wavelength of 600 nm.

2.5 Detection and Identification of Biosurfactant

In order to investigate biosurfactant - producing ability of the microorganism, strain D3-2 was fermented in MSM with vegetable oil as the sole source of carbon. The fermentation broth was filtered and centrifuged at 9000 rpm for 20 min. After the pH was adjusted below 2.0 by adding concentrated hydrochloric acid ^[21], 50 mL of the solution was taken from the supernatant and stored at -4 °C in refrigerator for 24 h. Surface tension of fermentation broth was analyzed by Automatic Surface Tensiometer (BZY-2, Hengping

instrument and meter company, Shanghai).

The other cell-free supernatant was used to extract biosurfactant produced by strain D3-2. Equal volume of ethyl acetate was added to supernatant and the organic phase was collected after 2 h of sufficient extraction. Most of ethyl acetate solvent was removed from the extract by a vacuum distillation apparatus in rotary evaporation. The remaining about 2 mL of ethyl acetate solution was placed in fume hood for about 3 d. Biosurfactant formed naturally when the ethyl acetate was evaporated gradually. Certain KBr (chromatographic grade) mixed with biosurfactant product was compressed to form an almost transparent disc. The functional group of biosurfactant was detected by AVATER 360 FT-IR spectrophotometer (Nicolet). And the spectra were obtained from 500 to 4000 wave numbers (cm^{-1}).

2.6 Hydrocarbon Degradability Test of the Strain

Hydrocarbon-utilization ability of strain D3-2 was examined on varieties of hydrocarbon substrates in flask-scale. The trials were carried out in 100 mL MSM inoculated with 2% (v/v) microorganism after the medium sterilized at 120 °C for 30 min. The amount of respective sole carbon source using in this study was 0.005% (w/v) of solid hydrocarbons or 0.1% (v/v) of liquid substrates ^[17]. Hydrocarbon substrates included toluene, xylene, naphthalene, paraffin oil, *n*-hexadecane, *n*-tetradecane, cyclohexane, diesel oil, hexane and crude oil. All of the Erlenmeyer flasks were incubated at optimum temperature for 14 d. Cell density was used to monitor the utilization of each hydrocarbon and it was tested by ultraviolet and visible spectrophotometer in the wavelength of 600 nm. It was used to reflect

the microbial growth condition and monitor the utilization of each hydrocarbon substrate.

2.7 Biodegradation of Crude Oil

The crude oil degradation test of strain D3-2 was conducted in MSM supplemented with crude oil as the sole source of carbon under the optimum condition with $30 \text{ g} \cdot \text{L}^{-1}$ NaCl. 250 mL Erlenmeyer flasks were filled with 100 mL MSM under the principle of equal with 0.5% (w/v) crude oil. After sterilization at 120°C for 30 min, microorganism culture solution (2%, v/v) was added into flask. Cells were incubated at optimum temperature with shaking at 130 rpm for 28 d. The residual crude oil ^[28] remained in the culture medium was measured by gas chromatography analyses.

The *n*-alkane distributions were analyzed by a Shimadzu (Kyoto, Japan) GC-2010 with the FID detector. DB-5 capillary chromatographic column (30 m×0.32 mm I.D.) and DB-5 MS capillary column (30 m×0.25 mm I.D.) were used for GC-FID and GC-MS respectively. The carrier gas was nitrogen. The injector temperature was 290°C and detector temperatures were maintained at 300°C . Details of experiment condition and quality control could refer to our previous papers reported by Sun et al ^[25].

3. Results

3.1 Isolation and Identification of Strain D3-2

The strain D3-2 was isolated from soil sample polluted by crude oil. Morphological, biochemical and physiological characteristics of the bacteria was then investigated. The strain was rod-shape, Gram-negative and failed to ferment sugar, which also had milky-white

colony with wet surface and round smooth edges on the agar. Flagella and the production of spore were not observed. Physiological experiments confirmed that D3-2 was oxidase negative, catalase negative and did not liquefy gelatin, while was methyl red test positive, V-P test positive and could hydrolyze starch and cellulose.

Strain D3-2 was identified by the 16S rRNA gene sequence with a 1404 - bp fragment which was aligned with sequences from the GenBank database and checked manually. It was 99.9% similar to the 16S rRNA gene of genus *Acinetobacter* sp. (No.GU566321). A phylogenetic tree was constructed with software MEGA using NJ method (Fig. 1). Based on the above characteristics, strain D3-2 was identified as a strain of *Acinetobacter* sp..

3.2 Investigation of Environmental Factors

3.2.1. Effect of Temperature

In the continuous experiment, growth conditions of *Acinetobacter* sp. D3-2 were optimized. Biomass concentration was evaluated by determining the absorption in the wavelength of 600 nm. Biomass of *Acinetobacter* sp. D3-2 increased as the temperature progressed. The maximum absorption occurred at 30 °C, bacterial growth decreased when the temperature was higher than 30 °C (Fig. 2). It could be inferred that activities of the enzymatic was reduced at high temperature. As a result, the optimum temperature for the growth of *Acinetobacter* sp. D3-2 was 30 °C.

3.2.2. Effect of Initial pH

The pH was also an important environmental factor, which influenced the stability and solubility of enzymatic compounds ^[4]. The amount of bacteria increased significantly along

with the increase of pH till about 8.0. When the pH became higher than 8.0, biomass concentration of *Acinetobacter sp.* D3-2 began to decline (Fig.3). Most of the microorganisms could maintain their high bioactivity in neutral environment, but the optimum pH suitable for *Acinetobacter sp.* D3-2 was neutral to alkali around 8.0. It was primarily due to the original soil sample obtained from oil-polluted site. The neutral-alkali environment in Dongying had contributed to the enrichment of hydrocarbon-degrading bacteria with better alkali resistance.

3.2.3. Effect of Initial Salinity

Salinity was essential for balance of osmotic pressure between inside and outside of cell wall. *Acinetobacter sp.* D3-2 was tested under different concentrations of NaCl. Biomass concentration increased gradually with the amount of NaCl used. The peak value was observed at the NaCl concentration of $30 \text{ g} \cdot \text{L}^{-1}$ (Fig. 4). As the NaCl concentration continued to increase, biomass concentration showed a decrease process obviously. As a result, the optimum initial concentration of NaCl was determined to be $30 \text{ g} \cdot \text{L}^{-1}$.

3.3 Characterization of Biosurfactant Produced by *Acinetobacter sp.* D3-2

Surface tension of culture broth was tested both in sterile control and the inoculated flask in order to detect the possibility of biosurfactant production. After 7 d culture, surface tension in the control flask was determined to be $48.02 \text{ mN} \cdot \text{m}^{-1}$. In the inoculated flask, surface tension decreased dramatically to $26.30 \text{ mN} \cdot \text{m}^{-1}$. The result suggested that biological emulsion was produced in the metabolism process of *Acinetobacter sp.* D3-2.

The crude biosurfactant molecules produced by this strain were obtained primarily by the

chemical isolation method ^[21]. After the fermentation, broth was filtered and centrifuged; the supernatant with pH of 2.0 by adding concentrated hydrochloric acid ^[21] was put into refrigerator for one day. When the supernatant was taken out, white precipitation was appeared in the solution. It could be primarily illustrated that lipopeptide compounds were included in the biosurfactant which was produced by *Acinetobacter sp.* D3-2 ^[12].

After the evaporation of ethyl acetate, coarse product of biosurfactant was obtained with a 0.52 g•L⁻¹ value. IR spectrogram was presented in Fig. 5. The strong characteristic absorption peak in 3432cm⁻¹ indicated the group of –O-H. The absorptions at 2927cm⁻¹ and 2854cm⁻¹ were due to group of –C-H in alkane. It illustrated that aliphatic chain existed in the biosurfactant compound. Ester was included in the structure because of the absorption at 1717 cm⁻¹ which presented for the –C=O in group of –COOR. The absorption at 1654 cm⁻¹ was the representative peak of –C=O in –CONH. It showed that peptide unit was contained in the structure of biosurfactant compound ^[5, 12]. In summary, biosurfactant compound was produced simultaneously in the metabolic process of *Acinetobacter sp.* D3-2. Based on the above analysis, the biosurfactant product extracted from fermentation broth included lipopeptide compounds. Although the biosurfactant had a low yield, this biological behaviour could strengthen the biodegradation effect to some extent.

3.4 Utilization of Hydrocarbon Substrates

Substrate-utilizing ability of *Acinetobacter sp.* D3-2 was examined under various kinds of hydrocarbons. The strain could grow very well in the presence of diesel oil, crude oil, and vegetable oil (Fig. 6). All of the three kinds of organic matters were complex mixtures

containing different substrates. It confirmed that *Acinetobacter sp.* D3-2 could utilize wide range of substrates for its growth. The strain could degrade hexadecane, tetradecane, cyclohexane and paraffin oil well but grew fairly in naphthalene. Aromatic compounds were difficult to be degraded in oil polluted marine environment. *Acinetobacter sp.* D3-2 grew slightly in phenol but could utilize a certain degree of toluene and xylene as energy source. It reflected the potential of *Acinetobacter sp.* D3-2 in bioremediation of petroleum contaminants. In summary, *Acinetobacter sp.* D3-2 could grow better in various hydrocarbon substrates even in some aromatics. Crude oil was a complex mixture containing *n*-alkane, branch alkane, aromatics, cycloalkanes, isoprenoids, asphaltene and so on. Wide range of hydrocarbon substrates utilization provided certain feasibility to *Acinetobacter sp.* D3-2 in future practical application.

3.5 Degradation of Crude Oil

The components changes of the crude oil were detected by gas chromatography (Fig. 7) . In the sterile control flask, most components of crude oil decreased slightly and a few of lighter ones disappeared after 28 d culture. The abundance of each *n*-alkane in inoculated flask was remarkably lower than the case in the control experiment. *Acinetobacter sp.* D3-2 could degrade *n*-alkane ranging from C9 to C38. GC fingerprints reflected that most *n*-alkane and major important aromatics were removed from the medium. Biodegradation degree of *Acinetobacter sp.* D3-2 was estimated using *n*-C17 and *n*-C18 ^[30]. Pristane and phytane were not susceptible to biodegradation effect. Ratio of pristane/phytane was steady either in the control or in the inoculated experiment. The ratios of *n*-C17/pristane and *n*-C18/phytane in

the inoculated flask decreased obviously, the losses were only 11.7% and 13.0% after 28 d culture, respectively. The consequences showed that over 82% alkane hydrocarbons were degraded by *Acinetobacter sp.* D3-2. Aromatic compounds were also susceptible to biodegradation. Incomplete oxidation of the aromatic under the effect of co-metabolism had played an important role in aromatic utilization. However, the degradation rate decreased along with the complexity of the molecular instruction increased. The results were in accordance with the pure hydrocarbon degrading test in section 3.4. It demonstrated that *Acinetobacter sp.* D3-2 could hold available capacity in crude oil degradation experiment. The strain posed enormous potential in the process of oil pollution bioremediation.

4. Discussion

Various microorganisms have the potential to use different hydrocarbons as their energy source. Indeed, more than 200 bacterial, algal, and fungal genera, encompassing over 500 species, have been recognized as capable of hydrocarbon degradation ^[9]. The oil degradation capacity of the microorganisms was affected by various factors, such as the kinds of microorganism, the composition of crude oil and the environmental factors, including pH, temperature, salinity, nutrients and so on. Previously native bacteria or exogenous microorganisms were usually applied during the bioremediation process in petroleum contaminated environment ^[29]. Although the native bacteria were adapted to the complex field conditions, the lack of the required enzymatic activity sometimes limit the oil degradation rates. Based on these characteristics, exogenous microorganisms were more adapted in the field for bioremediation process, especially the marine biosurfactant producers.

They can degrade the petroleum hydrocarbon compounds, and produce the biosurfactant which can increase the emulsification of crude oil, thus more conducive to the degradation of crude oil.

In the current study, we examined the potential of *Acinetobacter sp.* D3-2 isolated from oil-contaminated soil in Dongying, China. Morphological, biochemical and physiological characteristics of the strain were characterized. Based on the results of phylogenetic analysis, strain D3-2 was identified as *Acinetobacter sp.*. Environmental factors that could influence the regular growth of *Acinetobacter sp.* D3-2 were optimized. Biosurfactant was found to be produced simultaneously. The surface tension of the inoculated flask was found to be the lowest ($26.30 \text{ mN} \cdot \text{m}^{-1}$) indicating its powerful surface tension-reducing property. The biosurfactants produced by the strains of *Acinetobacter sp.* are a mixture of different lipopeptide species or isoforms^[13], and the concentration was $0.52 \text{ g} \cdot \text{L}^{-1}$. The lipopeptide of the biosurfactant was further confirmed by the IR spectra, which was consistent with the reference^[5,12].

The degradation of crude oil of *Acinetobacter sp.* D3-2 was performed in shake flask trials and the results showed that could degrade *n*-alkane ranging from C9 to C38. It had great performance on the petroleum hydrocarbons, after 28 d biological treatment, ratios of *n*-C17/pristane and *n*-C18/phytane in the inoculated flask decreased obviously. The consequences showed that over 82% alkane hydrocarbons were degraded by *Acinetobacter sp.* D3-2. However, ratio of pristane/phytane was steady either in the control or in the inoculated experiment and the degradation rate decreased along with the complexity of the

molecular instruction increased. Aromatic compounds were also susceptible to biodegradation, which the incomplete oxidation of the aromatic under the effect of co-metabolism had played an important role in aromatic utilization. These results were consistent with previous findings^[29] and confirmed that the predominant bacteria *Acinebacter* sp. D3-2 might be feasible for practical application in the future.

Furthermore, this study provides available capacity in crude oil degradation experiment. The strain posed enormous potential in the process of oil pollution bioremediation. But the emulsifying properties of the lipopeptide was not studied in the paper, and further studies of biological activity of lipopeptides are needed to determine their applicability as antibacterial, antifungal, antiviral or anticancer agents.

5. Acknowledgements

This study was supported by "The National Natural Science Foundation of China"(41376084); "Program for New Century Excellent Talents in University"(NCET-11-0464); "Program for Innovative Research Team in University (IRT1289) "; "The development program of science and technology in Shandong province"(2011GHY11522); "The Open Foundation of Key Laboratory of Marine Spill Oil Identification and Damage Assessment Technology of SOA"(201104). This is MCTL Contribution No. 33.

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Fig. 1 Phylogenetic tree of *Acinetobacter sp.* D3-2

Fig. 2 Cell growth of *Acinetobacter sp.* D3-2 under different temperatures. All data were carried out in

triplicates. Error bars were mean of SD.

Fig.3 Cell growth of *Acinetobacter sp.* D3-2 under different pH. All data were carried out in triplicates.

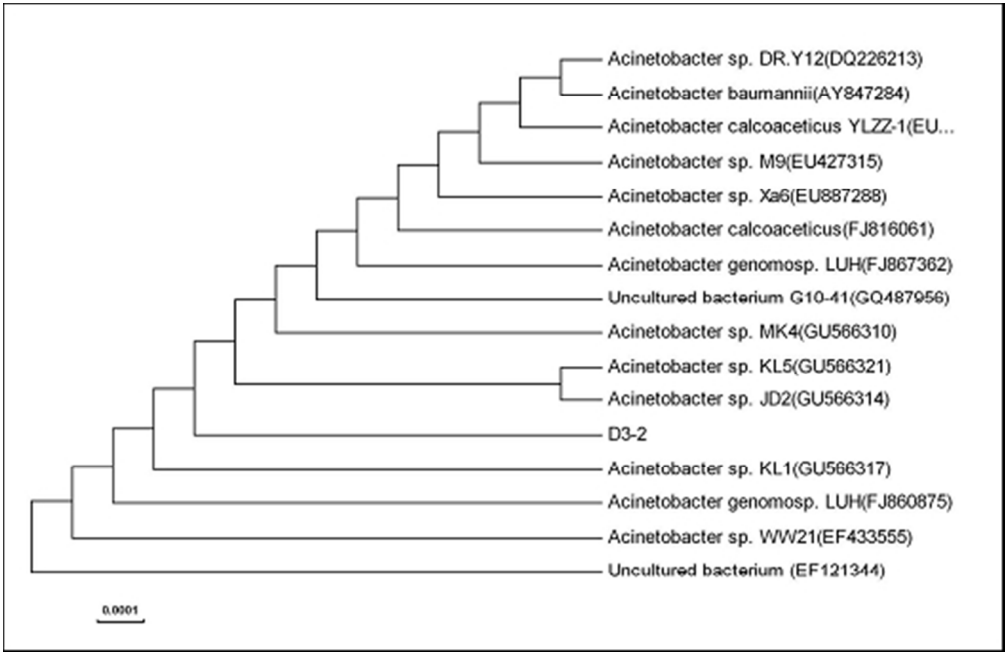
Error bars were mean of SD.

Fig. 4 Cell growth of *Acinetobacter sp.* D3-2 under different saline conditions. All data were carried out in triplicates. Error bars were mean of SD.

Fig. 5 FT-IR spectra of biosurfactant extracted from fermentation broth of *Acinetobacter sp.* D3-2.

Fig. 6 Cell growth of *Acinetobacter sp.* D3-2 with different hydrocarbon substrates.

Fig.7 Gas chromatograph of crude oil in degradation assay. a) WC, weathered source oil; b) SC, the sterile control at 28 d; c) PC, the positive control inoculated with *Acinetobacter sp.* D3-2 at 28 d. Pr and Ph represent pristine and phytane respectively.



139x90mm (96 x 96 DPI)

