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ORIGINAL PAPER

Characterization of a Biosurfactant-producing Strain *Rhodococcus* sp. HL-6

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Abstract
An effective biosurfactant-producer and hydrocarbon degrading bacterial strain, Rhodococcus sp. HL-6 was isolated from Xinjiang oil field using diesel oil as sole source of carbon. The produced biosurfactant (BS) had the ability to decrease the surface tension (ST) of distilled water from 72.5 to 30.7 mN/m, with the critical micelle concentration (CMC) of 40 mg/L. Subsequently, the BS characterization was made by thin-layer chromatography (TLC) and infrared spectra analysis. The stability was determined by EI_{24} value measurement over a certain pH (4-10), temperature (4-100 ºC) and salt concentration (0-5 % w/v) ranges. The glycolipid quality of the BS was further determined. To deduce the role of solid bioagent in diesel oil degradation, a comparison of solid bioagent and liquid bioagent was investigated. The study suggested application of the HL-6 BS as an appropriate candidate for bioremediation of crude oil contaminants. To our knowledge, this is the first report showing detailedly introduce the surface properties and biological activity of glycolipid biosurfactant producing strain (Rhodococcus sp.).

Keywords: *Rhodococcus sp., Biodegradation, Biosurfactant, Bioremediation*

1. Introduction

Biosurfactants are microbial-derived surface active molecules produced by a variety of microorganisms, which either adhere to cell surface or are excreted extracellular in the growth culture medium. Recently, biosurfactants have received much attention in numerous environmental and industrial applications. As a result, there is an intensive search for microbes which are capable of producing bios

urfactants for bioremediation and microbial enhanced oil recovery (MEOR) purposes (1, 2) Biosurfactants can enhance the degradation of hydrocarbons by two mechanisms, namely by increasing substrate bioavailability for the microorganisms and by interacting with the surface of the bacterial cell to increase the hydrophobicity of the surface, thereby allowing hydrophobic substrates to associate more easily with bacterial cells (3). By reducing surface and interfacial tensions, biosurfactants increase the surface areas of insoluble compounds, leading to an increased mobility and bioavailability of hydrocarbons. Taken together, biosurfactants ultimately enhance the biodegradation and removal of hydrocarbons. Therefore, the addition of biosurfactant - producing bacteria to a culture system can be expected to enhance hydrocarbon biodegradation by mobilization, solubilization or emulsification (4). In this study, a highly efficient indigenous diesel oil degrading strain was isolated from soil sampled in Xin Jiang oilfield, China and a *Rhodococcus* sp. strain HL-6 was identified based on its 16S rDNA. The physico-chemical properties of the BS and stability studies were also investigated. The study suggested application of the HL-6 BS as an appropriate candidate for bioremediation of crude oil contaminants. This is the first time to detailedly introduce the surface properties and biological activity of glycolipid biosurfactant producing strain (*Rhodococcus* sp.).

2. Materials and Methods

2.1. *Bacterial strains, culture media and chemicals*

Hydrocarbon-degrading strain HL-6 was isolated from the oil-contaminated soil of Xin Jiang oilfield by serial batch enrichments in mineral medium (MM) containing 3.48 g l^{-1} KH_2PO4, 1.5 g l^{-1} Na_2HPO_4, 4 g l^{-1} $(NH_4)_2SO_4$, 0.7 g l^{-1} $MgSO_4{\cdot}7H_2O$, 0.01 g l^{-1} yeast extract and 1% diesel oil as sole source of carbon. Diesel oil was purchased from Tianjin Science and Technology Co., Ltd. (China). Squalane were purchased from Sigma (USA) or Fluka (Switzerland). All other reagents were of analytical grade and were obtained from various commercial sources.

2.2. *Species identification*

Species identification was carried out using the Biolog identification system, the DNA sequence of a 1500-bp fragment of the 16S rRNA gene of strain HL-6, and by physiological and biochemical characterization. For sequencing, the 16S rRNA gene was amplified from genomic DNA of strain HL-6 using the following primer pair: 27f, 5′-AGA GTT TGA TCC TGG CTC AG-3′ and 1541r, 5′-AAG GAG GTG ATC CAG CC-3′.The sequences were subjected to a similarity search on the BLAST database, and deposited into the GenBank database under the accession No. JQ839141.

2.3. *Diesel oil degradation test*

Overnight cultures of HL-6 (LB, 25 ºC) were collected by centrifugation (12,000 ×g, 5 min) and washed twice with minimal medium. Culture suspension was adjusted at OD_{600} = 0.05 in 50 ml minimal medium. After being supplemented with 1% (v/v) diesel oil as the sole carbon source, the cells were incubated at 25ºC with shaking at 200 rpm. The remaining hydrocarbons were extracted with equal volume hexane at 12 h intervals. Each sample included 20 ppm (w/v) squalane as an internal standard. The amount of diesel oil was quantified by Gas Chromatography (GC) (5).

2.4. *Cell surface properties of Rhodococcus sp. HL-6*

Contact angle measurements

Hydrophobicity of *Rhodococcus* sp. cells was determined by water contact angle measurements on bacterial lawns deposited on membrane filters as described by (6).

Salt aggregation test (SAT)

Series of ammonium sulfate solutions of various molarities (0.02 to 4.0) were mixed with equal volumes of bacterial suspensions on a glass slide and observed for aggregation for 1 min at room temperature. The concentration of the lowest molarity in a mixture with bacterial cells which gives visual bacterial cell clumping is scored as a value for bacterial surface hydrophobicity (7).

Cell surface hydrophobicity determination

Cell surface hydrophobicity of *Rhodococcus* sp. HL-6 was accessed by the bacterial adhesion to hydrocarbons (BATH) assay (8).

2.5. *Characterization of BS producing by Rhodococcus sp. HL-6*

Determination of Surface Tension (ST)

The determination of ST was carried out in the cell-free broth obtained by centrifugation of the cultures at 9000×g for 20 min at room temperature, using a Sigma 700 digital surface tensiometer (KSV Instruments LTD, Finland) and working on the principle of the Du Nuoy ring method.

Measurement of emulsification activity

The emulsification activity was determined using a modification of the method described by (9). Briefly, 6 ml of culture filtrate was added to 4 ml of diesel oil in a screw-cap tube and this was vortexed at high speed for 1 min. The emulsion stability was determined after letting

the mixture stand for 24 h, and then the emulsification index (EI_{24}, %) was calculated by dividing the measured height of the emulsion layer by the height of the oil/hydrocarbon phase and multiplying by 100.

Stability of BS with different pH, temperature, salinity and hydrocarbons

To determine the effect of pH on BS stability, pH of the cell free broth was adjusted to different values ranged from (4 to 10) using 1 M HCl and 1 M NaOH. The thermal stability of the BS was determined by maintaining the supernatant at constant different range of temperature from 4 to 100 ˚C for 30 min and cooled at room temperature. The effect of addition of different concentration of NaCl on the activity of the BS was studied; the specific concentration of NaCl was (1 to 5% w/v). The effect of different hydrocarbons on BS stability was investigated by using hexane, tridecane, hexadecane, benzene, toluene, xylene and diesel oil (10).

2.6. *Purification and Biochemical Characterization of BS*

To obtain BS, the cell-free supernatant obtained was acidified by concentrated hydrochloric acid to pH 2. The solution was left overnight at 4°C. Extraction was done by a mixture of chloroform and methanol (3:1, v/v). The bottom organic layer was collected and the solvent was removed on a rotary evaporator. Purification of BS in the extract was done on a 20 cm×1 cm glass chromatographic column with silica gel (60-120). The samples were eluted first with chloroform to remove yellow pigment, then with a mixture of chloroform and methanol (5:2, v/v). Then the fraction eluted was pooled and the solvent was evaporated to dryness to remove the organic solvent. The isolated BS was analyzed by thin-layer chromatography as described by (11).

2.7. *Determination of critical micelle concentration (CMC)*

The BS produced after a growth of 72 h was serially diluted to obtain different concentration of BS in cell free broth. The critical micelle concentration (CMC) was determined by measuring the ST of the solutions at room temperature and plotted against BS concentration. These experiments were conducted in triplicates (12).

2.8. *Carbohydrate, protein and lipid estimation*

The carbohydrate content of the BS was determined by the phenol-sulfuric acid method (13) using D-glucose as a standard. The protein content was determined by the method (14) using bovine serum albumin as a standard. The lipid content was estimated by following the procedure (15).

2.9. *Proteinase K treatment*

The BS was treated with Proteinase K (10mg/ml) at 58 ºC for 30 min then inactivated Proteinase K at 75 ºC for 30 min. Afterwards, the EI_{24} was determined as described above.

2.10. *Fourier transform infrared spectroscopy (FTIR)*

FTIR is useful for identifying the types of chemical bonds (functional groups) that are present in an unknown mixture. Infrared absorption spectra were recorded on a Shimadzu FTIR system 8400 spectrometer in the 4000-400 cm^{-1} spectral region with spectral resolution.

2.11. *Biodegradation test with liquid and solid bioagent*

Immobilization of bacteria

The bacterial cells were collected by centrifugation at 12,000×g for 5min, and then washed with MM buffer twice. The suspended cells were added to the mixture which containing zeolites (the final bacteria1 concentration was 4×10^9 cells/g), and stirred to be uniform. The liquid reagent was lyophilized for the preparation of reagent in powder form, and store at -20°C.

SEM test of solid bioagents

The zeolites (before and after absorption) were observed by a Model S-2400 (Hitachi,

Japan) scanning electron microscope to verify the zeolite adsorption effect.

Biodegradation test with liquid and solid bioagent

The harvested cells of equal amount were transferred into the seed liquid or immobilized to zeolites, then inoculated to the flasks which containing MM media with diesel oil as sole carbon source for 7 days. The degradation rate of diesel oil was detected by GC analysis every day.

3. Results and Discussion

Isolation and identification of the hydrocarbon-degrading bacteria

Hydrocarbon-degrading strain was enriched and separated by culturing oil-contaminated soils sampled from Xinjiang oil field. The obtained isolate was found to be an aerobic, Gram-positive, non-mobile, irregular rod. Eventually, based on 16S rDNA sequence and phylogenetic analysis, this bacterium was identified as *Rhodococcus* sp. HL-6 (Fig.1), which showed 99.6% homology with *R. erythropolis* EPWF (GenBank accession no. AY822047).

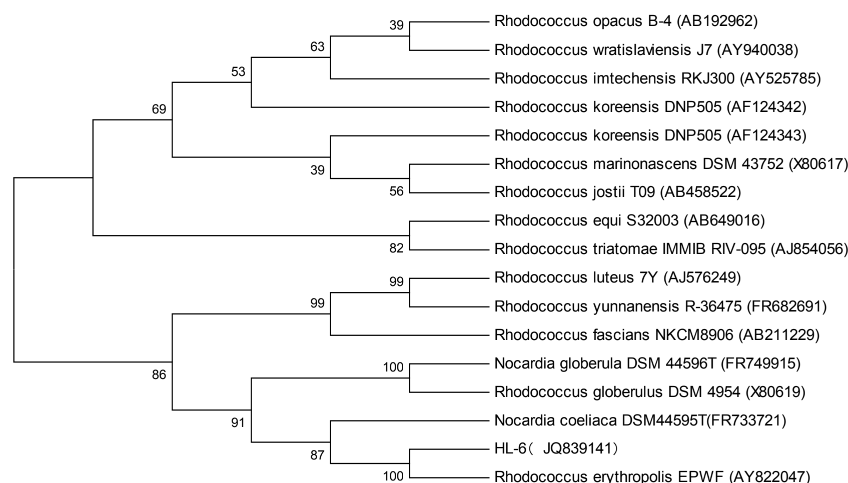


Figure 1. Phylogenetic tree based on the comparison of 16S rDNA sequence of other actinomycetes which have closed relationship of 16S rDNA.

Hydrocarbon degradation character of *Rhodococcus* sp. HL-6

All diesel oil components showed obvious reduction and the degradation rate was 78.5%. Diesel oil is mainly composed of saturated hydrocarbons with medium-length chains; therefore, strain HL-6 was considered to have good capacity to degrade saturated hydrocarbons with medium-length chains.

Cell surface properties

Hydrophobic interactions play a key role in the adhesion of microorganisms to surfaces. The hydrophobic nature of bacterial surfaces is a cell growth factor in hydrophobic substrates, such as hydrocarbons. Contact between the cell and hydrophobic compound is a necessary, as the first step in the degradation of hydrocarbon is the introduction of oxygen into molecules through cell-associated oxygenases (16). A number of methods to evaluate cell surface hydrophobicity have been developed; in this present study we examined three methods for evaluating *Rhodococcus* sp. HL-6 surface hydrophobicity including contact angle, salt aggregation and BATH. With the contact angle method, a hydrophilic surface is indicated by an angle of 23.9° of *E.coli*, and a hydrophobic surface is indicated by an angle of 116.2° of

Rhodococcus sp. HL-6 (Fig.2). With the SAT, the choice of growth substrate influenced the surface hydrophobicity. After growth in MM with 1% glucose, the SAT value was >4.0 mol/L; after growth in MM with 1% diesel oil, the SAT value was only 0.2 mol/L. This indicated that hydrocarbons such as hydrophobic matrix could induce the changes of bacteria cell surface hydrophobicity (Fig.3). In general if the BASH>70%, that showed high bacterial cell surface hydrophobicity; if BASH<30%, showed high bacterial cell surface hydrophilic. As shown in Table.1, cell surface hydrophobicity (CSH) increased from 24h and reached the maximum value 76.71% at 96h with diesel oil as the sole substrate. Then with the culture time prolonged, the cell surface hydrophobicity decreased rapidly. This trend was in consistent with the literature (17). Comparison of their values obtained with varied substrates is indicative of the lower cell surface hydrophobicity in growth on hydrophobic substrate than on hydrophilic substrate as shown in Table 2.

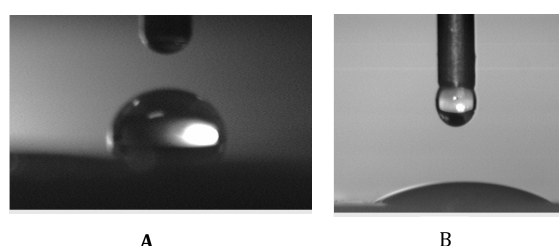


Figure 2. Result of the contact angle test (A) HL-6; (B) *E.coli*

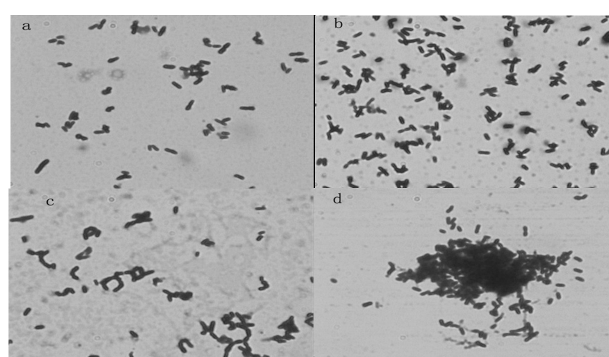


Figure 3. Photomicrographs of bacterial HL-6 in different carbon resource (1000×); a,b: Treated with $(NH_4)_2SO_4$ in Glucose; c,d: Treated with $(NH_4)_2SO_4$ in diesel oil

Table 1 Changes of bacterial cell surface hydrophobicity during the growth

Growth process	24h	48h	72h	96h	120h
CSH (%)	48.08	58.51	65.99	76.71	46.73

Table 2 Effect of carbon resource on bacterial cell surface hydrophobicity

Carbon resource	**glycerol**	**ethanol**	**liquid paraffin**	**diesel oil**	**glucose**	**xylose**	**galactose**	**fructose**	**sucrose**	**mannose**	**maltose**	**lactose**
CSH (%)	5.66	12.62	83.64	72.56	27.38	40.35	0	0	16.92	0	0	0

BS stability regarding surface tension (ST) and emulsification

An essential parameter characterizing the amount and efficiency of BS produced by microorganisms is the ST of culture broth (18). The ST values found in this work varied in the range of 30.7 mN/m to 57.9 mN/m with different substrates, which is approximately twice lower than the ST of the reference solution (Fig.4). That indicates a high surface activity of synthesized BS with liquid paraffin or diesel oil as substrate. In addition to surface activity, good emulsification property is critical for BS to be promising in different environmental and industrial applications. An emulsion is formed when one liquid phase is dispersed as microscopic droplets in another (continuous) liquid phase. Most microbial surfactants are substrate specific and solubilise or emulsify different hydrocarbons at different rates (19). The

inadequate emulsification of some hydrocarbons may be due to the inability of the BS to stabilise the microscopic droplets. Environmental factors, such as pH, salinity and temperature, also affect the activity and stability of BS and it is therefore important to study the influence of these variables when considering specific applications for such compounds (20). In the present study, the ability of BS obtained from *Rhodococcus* sp. HL-6 was investigated at different environmental conditions. The effects of pH, salinity and temperature on the emulsification activity of the cell-free supernatant against diesel oil were shown in Fig.5a, b and c respectively. Initially, when the pH was set to 4 and 6, the emulsification activity of cell-free supernatant was 0% and 72%. This suggested that the activity of HL-6 BS was limited to acidic pH. The optimum emulsification activity (EI_{24}=100%) was observed at pH 8 and 10. The pH increase has a positive effect on emulsion stability, since the more alkaline solution provides the better conditions for prolonged stability. The NaCl of BS-containing culture media in normal concentration was changed from 1% to 5%. When the NaCl concentration was lower than 4%, the emulsification activity of fermentation liquid kept 60% above. A maximum emulsification activity was showed at 2% NaCl concentration. There are some reports about disruptive effects of salts on oil-water emulsions and on emulsifying activity of surfactants (21). This provides a clue as to why BS emulsifying ability gradually declines when salt concentration in medium increases. This special ionic strength tolerance offers the BS more suitability for oil-related applications most of which are in moderately saline conditions such as marine environment. The temperature was one of the critical parameter that has been controlled in bioprocess. The stability of the BS from *Rhodococcus* sp. HL-6 was tested over a wide temperature range. The BS proved stable during 30min of incubation at temperatures of 4-55 °C, but only 35% at temperature of 100°C and this clearly indicated moderately thermostable in nature. The synthetic surfactants such as sodium dodecyl sulfate exhibited a significant loss of emulsification activity, beginning at 60 °C. Although our data did not show the expected thermostability for the BS, the EI remained in acceptable range from 4 to 75 °C, indicating its effectiveness at oil cleanup enhancement in all seasons and most climates. The BS from *Rhodococcus* sp. HL-6 showed above 80% emulsification activity with different hydrocarbons (Fig.6). Thus, our results demonstrate that this BS has good emulsifying effects for alkanes and aromatic hydrocarbons.

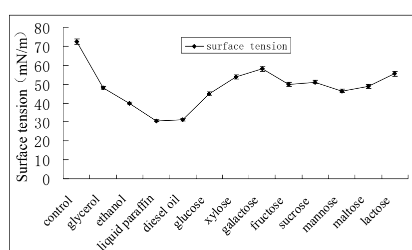
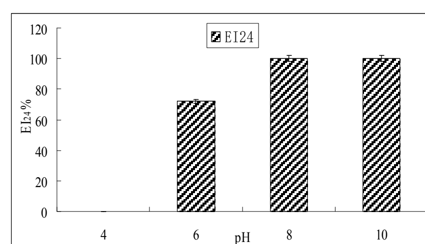
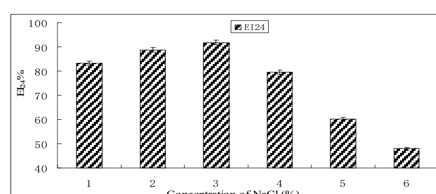


Figure 4. Effect of carbon resource on surface tension of the culture





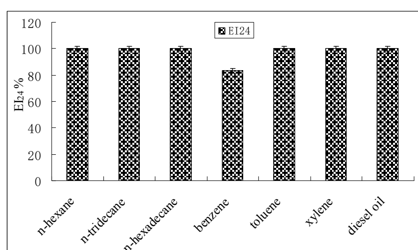


Figure 6. Effect of substance on emulsification activity

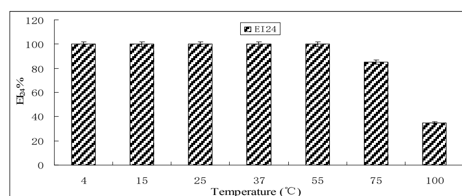


Figure 5. Effects of different environmental conditions on the emulsification activity (A) pH; (B) Salinity; (C) temperature

Characterization of BS

The biosurfactant purified from the cell-free broth was analyzed by thin-layer chromatography (Fig.7), the band stained with Molish reagent and Ammonium molybdate-perchloric acid showing positive reactions to sugars and lipid groups. Staining the TLC plate with ninhydrin, indicated no protein or amino acids moiety in HL-6 BS which is consistent with the resistance of its surface activity to the proteinase K treatment. The BS produced by *Rhodococcus* sp. HL-6 was a glycolipid comprising 17% (w/w) carbohydrate and 79% (w/w) lipid on a dry weight basis. Thus, the BS from *Rhodococcus* sp. HL-6 is glycolipid with less carbohydrate component. Biosynthesis of glycolipids, as surface active agents, has been reported for a variety of bacteria. Bryant has reported the isolation of glycolipid BS from *Rhodococcus* sp. strain H13A. The glycolipids were diglycosyl glycolipids and trehalose containing glycolipids (22). Kuyukina et al. also described the determination of polar lipids and mostly glycolipids as *Rhodococcus* BS using MTBE extraction (23). Moreover, Peng et al. reported that *Rhodococcus erythropolis* strain 3C-9 BS consisted of at least 12 free fatty acids of chain lengths from C9 to C22; and 2 kinds of glycolipids: a glucolipid and a trehalose lipid (24). Based on these findings we may assume that extracellular production of fatty acids and glycolipids and not glycoproteins as surface active agents, is a general characteristic of BS producing *rhodococci*.

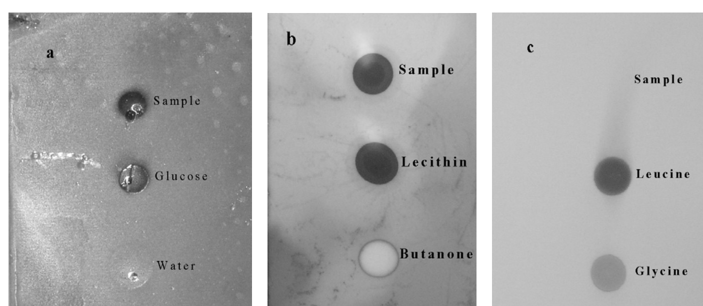


Figure 7. Qualitative analysis of biosurfactant by staining: a. Sulfuric acid-anthrone colouring; b. Ammonium molybdate-perchloric acid colouring; c. ninhydrin colouring

Critical micelle concentration determination

The CMC is an important characteristic of a BS and is defined as the BS concentration requisite to form micelle. Upon reaching the CMC, the ST remains relatively constant due to the interface saturation with the surfactant. Distilled water was found to have the ST of 72.5 mN/m and the addition of BS reduced its ST to 30.7 mN/m. Fig. 8 represents the measurement of ST as a function of BS concentration. At this point, CMC of the HL-6 BS was 40 mg/L and is in close agreement with the literature for BS from B. methylotrophicus (R. Chandankere & al. [25]). Furthermore, there was no significant change in the ST till the

end of the cultivation. This observation might be due to the attainment of CMC by the BS. Generally, the ability to reduce ST to below 35 mN/m is one of the criteria in selecting BS-producing microorganisms and our HL-6 fulfills this requirement.

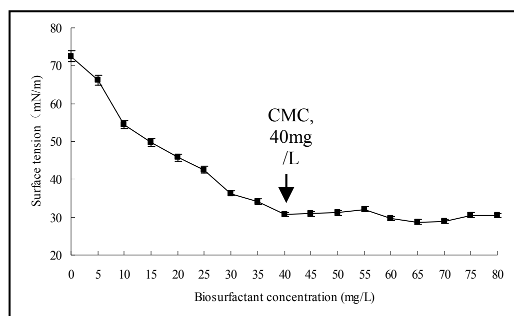


Figure 8. Effect of increasing BS concentrations on surface tension

Chemical analysis of BS

The BS was further presumed to be glycolipid in nature by FTIR analysis, as the characteristic absorption bands corresponding to typically functional groups of glycolipids could be observed for the BS (Fig.9). The FTIR spectrum in Fig.9 depicts -OH hydrogen bond stretching of 3386 cm^{-1}, the absorption band observed at 2,926 cm^{-1}confirmed the presence of C-H stretching vibrations of hydrocarbon chain of alkyl (CH_2-CH_3)groups, C=O stretching vibrations of carbonyl group at 1632 cm^{-1} and C-O-C ester stretching between 1,076 and 1,241 cm^{-1}. The absorption peak observed at 530cm^{-1} known to be characteristics of sugar derivatives. Overall, detection of carbohydrate and lipid compounds, the high repetition of O-H and C=O functional groups present in the FTIR spectrum and protein absence in the BS samples infer that the BS produced by strain HL-6 probably belongs to glycolipids category.

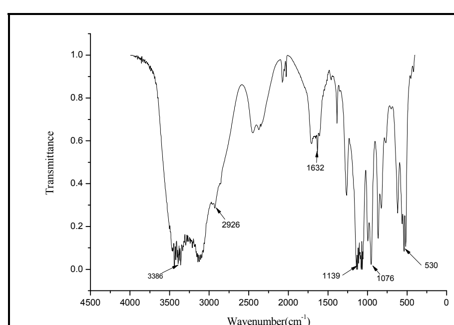


Figure 9. Fourier transform infrared spectroscopy spectra of the BS

Laboratory experiment on biodegradation of diesel oil with solid and liquid bioagent

As shown in Fig.10, zeolites with and without absorbing were detected by SEM. HL-6 was adsorbed on zeolites, illustrated the use of zeolite as the carrier of biosorption for preparing solid bioagent was feasible. In order to verify the degradation results, a verification test was performed using the mineral medium with diesel oil as the carbon source for 7 days at 25 ^{o}C. The results shown in Fig. 11 indicate that the degradation rate of diesel oil was 82.8% with liquid bioagent and 74.6% with solid bioagent. This is only about 11% greater than the degradation rate between liquid and solid bioagent. This also proved that bioremediation using solid bioagent for oil pollution had a good application potential.

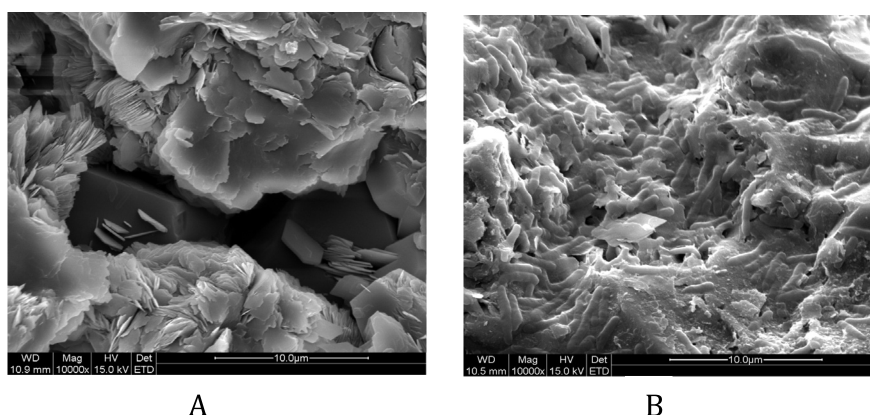


Figure 10. The SEM photo of solid bacterial reagent A: The blank of zeolite; B：The adsorption sample of zeolite

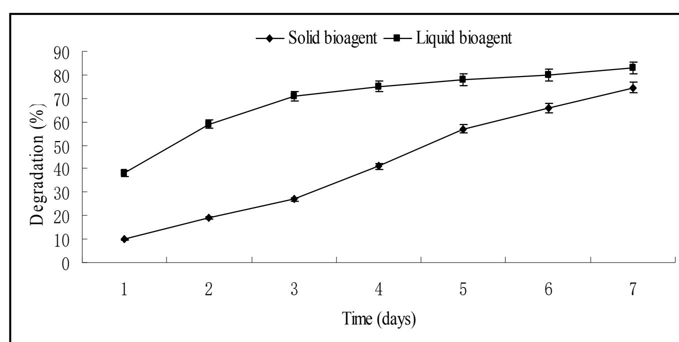


Figure 11. Contrast experiment of solid bioreagent and liquid bioreagent

From the results above it can be conclude that the biosurfactant produced by *Rhodococcus* sp. HL-6 was glycolipid in nature. This BS possesses high surface activity that could lower the surface tension of water to 30.7 mN/m at CMC of about 40 mg/L and exhibited excellent emulsification activities against different hydrocarbon substrates. The stability of the BS under varying pH, temperature and salinity is also another attractive characteristic for its applications in a diversity of environments. All these favorable properties facilitate the strain as an efficient tool in various biotechnological, industrial, and environmental applications, particularly in the remediation of crude oil contamination sites.

4. Acknowledgements

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