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Bioremediation of marine oil pollution by *Brevundimonas diminuta*: effect of salinity and nutrients

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ABSTRACT

Brevundimonas diminuta was isolated from the oil-contaminated seawater in Dalian, China. The effects of salinity and nutrients (nitrogen and external carbon) on diesel oil biodegradation were investigated. This strain could utilize diesel oil as the sole source of carbon and energy and gave a biodegradation rate of 45% over 6 d under the salinity of 3.38% (w/w). Under the saline condition, nutrient addition has been proved to be an effective strategy to enhance oil biodegradation in marine environment. The addition of soluble starch and methanol exhibited a significant stimulative effect on oil biodegradation. The optimum external carbon source was soluble starch. Based on this, the optimum carbon source to external carbon source (C/EC) ratio in the test range was 2:1. Under the optimum conditions, diesel oil biodegradation rate increased from 44 to 65%. The optimum nitrogen source and C/N ratio were found to be $NaNO_3$ and 20:1, respectively. Under the optimum condition, the diesel oil biodegradation rate increased to 88%. The polymeric compounds formed in the culture after nitrogen addition comprise a number of amphoteric functional groups. The results suggest that *B. diminuta* has considerable ability for bioremediation process of oil in marine environment.

Keywords: Brevundimonas diminuta; Bioremediation; Oil pollution; Salinity; Nutrient

1. Introduction

Marine pollution has become a global concern in recent years. Large amounts of petroleum products are released into marine environment from natural sources, production, transport, and conflicts [1,2]. It is estimated that between 1.7 and 8.8 million metric tons of oil are released into the world's water every year [3]. The traditional available treatment processes used to decontaminate the polluted areas have been limited in their application. Physical collection methods such as booms, skimmers, and adsorbents are generally the first priority of responders but they typically recover not more than 10–15% of the spilled oil, while chemical methods such as the use of surfactants or dispersants usually have toxic effects on the existing biota in the polluted area [4]. The use of micro-organisms to decompose petroleum pollutants has been recognized as a promising method since its successful application after the 1989 *Exxon Valdez* oil spill [5–7].

As for the bioremediation of oil in seawater, the environmental factors such as nutrients and salinity

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could have large impacts on cell growth and biodegradation rate [8]. Nutrient limitation is generally correlated to the low background levels of nitrogen and phosphorus and imbalances in the amount of carbon, nitrogen, and phosphorous caused by spilled oil. Adding nutrients at a certain concentration is a commonly used strategy in the bioremediation of oil-contaminated seawater, and it was showed that the supply of carbon sources enhanced bacteria's respiration as compared to those supplied with petroleum hydrocarbons and minerals only [9]. As the composition of petroleum is so complicated, the optimal nutrient types and concentrations vary widely based on the oil properties and the environmental conditions [10]. At the same time, the salinity in sea also requires that strains used for bioremediation of oil in seawater should be salt tolerance. Many isolated strains have been reported to be capable of biodegrading petroleum hydrocarbons such as *Bacillus subtilis* and *Pseudomonas aeruginosa*, but the rate of petroleum degradation is low under high-salinity condition in marine environment [11–13]. Komarova et al. [14] isolated *R. erythropolis* strains from deep sea and coastal sediment which could overcome osmotic shock by increasing the synthesis of free amino acid. Another *R. erythropolis* INMI 100 was noted for its utilization of turbine oil and a mixture of paraffins (C14-C18) at 0.5, 2, and 5% NaCl concentrations [15].

On the other hand, since petroleum is a complex mixture of many hydrophobic compounds such as alkanes, aromatics, resins, and asphaltenes, the bioavailability of the hydrophobic hydrocarbons to micro-organisms is poor. Therefore, enhancement of the bioavailability of petroleum hydrocarbons is crucial to their efficient and rapid degradation [16]. In recent years, many reports show microbial surfactants obtained from a wide range of oil-degrading micro-organisms are promising agents for the bioremediation of oil contaminants, as they could emulsify various hydrocarbons, enhance the solubility of hydrocarbons, and also shield bacterial cells from direct exposure to toxic substances [17]. In this sense, it has been reported that biosurfactants and other natural emulsifying agents are important tools for the biotreatment of hydrocarbon-polluted environments [18].

The aim of this work was to explore the effects of environmental factors such as salinity and nutrients on biodegradation of diesel oil in seawater by a newly isolated marine bacterial, namely *Brevundimonas diminuta* in shake flask and microcosm experiments. The additional goal of this work was to obtain information whether there is a relation between cell hydrophobicity and the degree of hydrocarbon biodegradation.

2. Materials and methods

2.1. Chemicals

$0^{\#}$ diesel oil purchased from Shanghai Dongpu Petrochemical Co., Ltd (China). Sea salt was purchased from Shanghai Heqing Chemical Industry Co., Ltd (China). All other reagents were of analytical grade and were obtained from Sigma-Aldrich (USA).

2.2. Bacterial strain and culture

B. diminuta was isolated from the oil-contaminated seawater in Dalian, China and was identified by sequencing of 500-bp length 16S rDNA. The bacterial strain was grown in LB medium at 25℃ for 3 d. The cells were harvested by centrifugation at 8,000×*g* for 5 min and washed three times with sterilized phosphate buffer (0.05 M). The cell concentration was 0.0090 g dry weight/mL. Artificial seawater medium was used for the growth and diesel oil biodegradation of the isolate. AS medium contained 1.75 g of sea salt and 50 mL of distilled water. The pH was adjusted to 7 before sterilization.

2.3. Experimental design

For the biodegradation under saline conditions, cells were cultivated in 150-mL Erlenmeyer flasks containing 1.25, 1.5, 1.75, 2.00, and 2.25 g of sea salt of 50 mL of distilled water, respectively. 1 mL of inocula and 100 μL of diesel oil were added in the medium (1,600 mg/L diesel oil). The flasks were agitated at 25℃ and 150 rpm on an orbital shaker. Samples were taken to measure cell density and concentration of residual oil after 6 d of incubation.

In order to evaluate the effects of external carbon sources (glucose, soluble starch, and methanol) on biodegradation of diesel oil, 800 mg C/L of each of the carbon sources was aseptically added to AS medium and the cultivation conditions were the same as above.

In order to evaluate the effects of nitrogen sources (NH_4Cl, NH_4NO_3, $NaNO_3$) on biodegradation of diesel oil, 80 mg N/L of each of the nitrogen sources was aseptically added to AS medium and the cultivation conditions were the same as above. Based on the result of optimal nitrogen source, different carbon to nitrogen ratios (2, 8, 14, 20) were investigated the effect on biodegradation efficiency. The cultivation conditions were the same as the experiments conducted above.

At the end of the degradation test, all of the aqueous samples were collected and extracted with carbon

tetrachloride to measure residual concentrations of diesel oil by ET1200 Oil Analyzer of infrared spectrometer (Shanghai, China). The biodegradation was calculated as $(C_0 - C)/C_0 \times 100\%$ (%), where C_0 is the initial concentration of diesel oil, C is the concentration of diesel oil after biodegradation.

The alkane fraction was analyzed by GC/MS (Agilent5975C), which was equipped with DB-5MS column (30 m × 0.25 mm × 0.25 μm). Helium gas was used as carrier and set at a constant flow rate of 0.8 mL/min. The column temperature was programmed to 100℃ at 15℃/min and increase from 100 to 170℃ at 10℃/min then increase from 170 to 300℃ within 3 min. The injector temperature was 250℃ and the transfer line temperature was 280℃. Individual components present in the alkane fraction were determined by matching the retention time with authentic standards.

2.4. Microbiological analyses

The growth of the micro-organism was characterized by the optical density (OD) of the solution at 600 nm, measured on a Unico UV-4802 spectrophotometer (China). Cell surface hydrophobicity (CSH) was assessed by microbial adherence to the hydrocarbon method (MATH). The cells were washed twice and suspended in a buffer solution (16.9 g/L K_2HPO_4, 7.3 g/L KH_2PO_4) to give an OD at 600 nm. The cell suspension (4 mL) with 1.5 mL of xylene was vortex-shaken for 60 s in 10-mL screw capped test tube. After shaking, organic phase and aqueous phase were allowed to separate for 15 min [4]. The OD of the aqueous phase was then measured at 600 nm in a spectrophotometer. The CSH value was calculated as follows: $CSH = [(A_0 - A_1)/A_0] \times 100\%$, where A_0 and A_1 represent the initial and the final optical densities at 600 nm of the aqueous phase, respectively. The procedure was represented three times.

The metabolite produced by *B. diminuta* was determined by the HPLC method. The thallus was separated by filtration and the filtrate was centrifuged at 5,000*g* for 10 min to show a visible emulsion layer. Both the thallus and 1 mL of emulsified liquid were lyophilized to get the dry production for FT-IR analysis (Nicolet 5700, USA).

3. Results and discussions

3.1. Effect of salinity on biodegradation

For practical application of bioremediation in contaminated seawater, micro-organisms have to overcome the salinity, osmotic pressure of seawater and maintain their biodegradability. The growth of *B. diminuta* under varying salinity conditions was shown in Figs. 1 and 2. The acclimatized cultures could tolerate salinity up to 3.38% sea salt. In general, it was noted that the turbidity (Fig. 1) and absorbance at 600 nm (Fig. 2) of the medium decreased with the salinity above 3.38%. The growth profile showed good correlation with the extent of oil degradation determined after 6 d (Fig. 3). Significant degradation of diesel oil (45%) was observed up to a salt concentration of 3.38% where the cell density was high, whereas at the higher salt concentrations of 3.85 and 4.31% where the cell density was low, the loss of diesel oil decreased to 39 and 32%, respectively. Since the typical salinity in marine environments is 3–3.5%, *B. diminuta* can be used for in suit oil bioremediation in marine environments.

3.2. Effect of external carbon source on biodegradation

The culture of *B. diminuta* using diesel oil as carbon source was investigated with or without nitrogen source ($NaNO_3$, 80 mg N/mL). From Fig. 4, we can see petroleum hydrocarbons can be a source of energy and nutrients for some microbial groups, but they may not be easily utilized by the oil degraders resulting in a long lag phase when there is no other nutrient. Biodegradation of refractory organic compounds can be positively or natively affected by the external addition of organic carbon sources and is dependent on types of added carbon sources [19]. Xie et al. [20] reported that the glucose supplementation can be an effective way to enhance atrazine biodegradation. Although the effects of exogenous carbon sources on

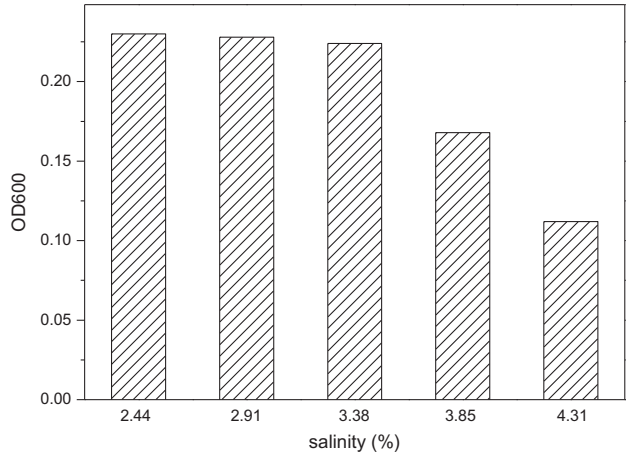

Fig. 1. OD of the medium with different saline conditions after 6-d incubation.

Fig. 2. Visual changes of the medium after 6-d incubation under different saline conditions from left to right: 2.44, 2.91, 3.38, 3.85, and 4.31%.

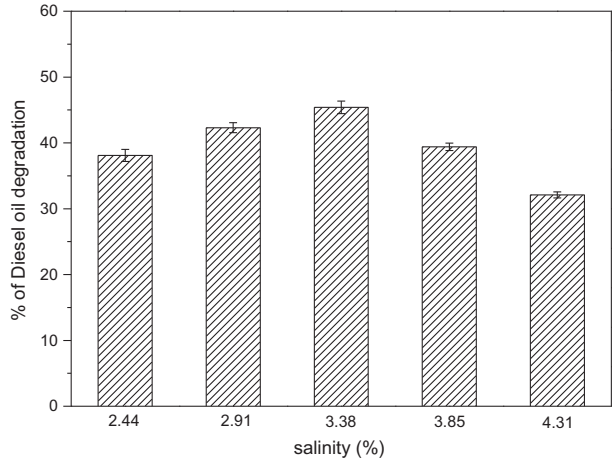

Fig. 3. The six-day biodegradation rates of diesel oil under different saline conditions.

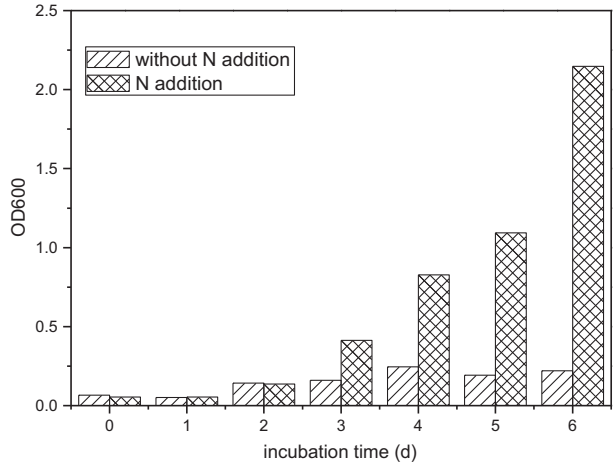

Fig. 4. Effect of nitrogen source on cell growth of *B. diminuta*.

some refractory organic compounds have been studied, the information on carbon impact on oil biodegradation is still lacking. In this study, three kinds of external carbon source were used to evaluate their effect on diesel oil biodegradation (Fig. 5).

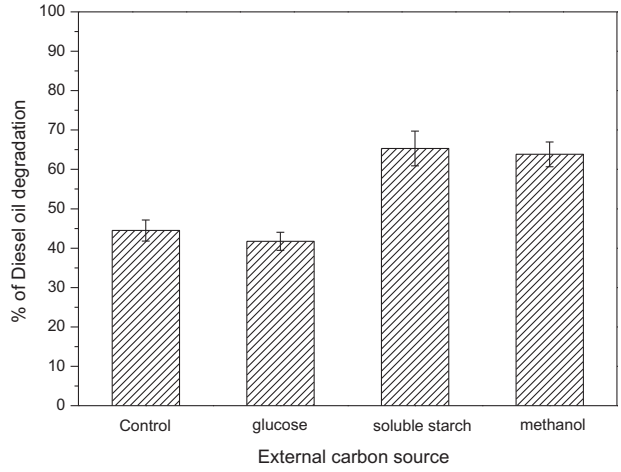

Fig. 5. Effect of external carbon source on diesel oil biodegradation.

Results showed that soluble starch gave the highest biodegradation rate of 65%. The addition of glucose gave a biodegradation of 42%, which is lower than the control group (without external carbon source addition). For the bacteria using oil as carbon source, the addition of a simple carbon source can promote their growth and thus enhance the oil biodegradation. At the same time, the external carbon source may inhibit their utilization of oil due to a higher bioavailability than oil. It may also partly explain the negative effect of carbon supplementation on biodegradation by the isolates. Both soluble starch and methanol can be the effective external carbon source to enhance diesel oil biodegradation. Using the best external source (soluble starch), the carbon to external carbon ratios (C/EC) ranging from 2 to 20 were studied by keeping a constant diesel oil concentration of 1,600 mg/L (Fig. 6). In the studied range, the highest biodegradation rate (about 65%) was observed when the C/EC ratio was 2. With the increasing of C/EC ratio, the biodegradation ratio maintained at around 50%. Concluded from above, choosing the right external carbon source and the appropriate C/EC ratio is an effective way to enhance oil biodegradation.

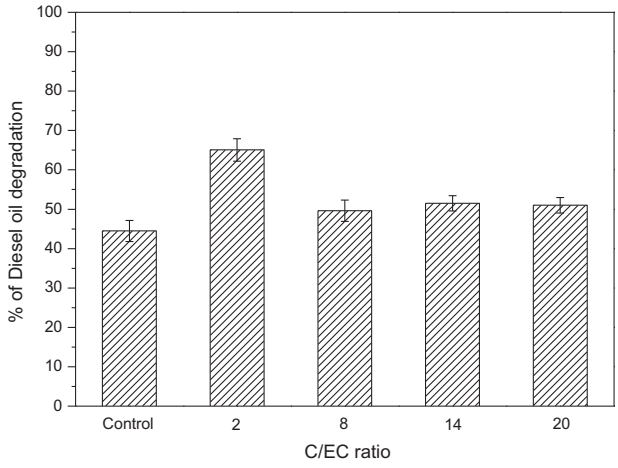

Fig. 6. Effect of carbon to external carbon ratio on diesel oil biodegradation.

3.3. Effect of nitrogen source on biodegradation

Inorganic nitrogen source can be easily utilized for most micro-organisms in nutrients limited environment [21]. The choice of nitrogen source has been reported to affect the enhanced effectiveness of bioremediation. Ramstad and Sveum [22] compared the effect of different forms of nitrogen on biodegradation of crude oil and found that nitrate had the most pronounced effect in stimulating oil degradation. However, Jackson and Pardue [23] found that addition of ammonia appeared to be more effective than nitrate in stimulating degradation of crude oil. In this study, as shown in Fig. 7, the best nitrogen source for diesel oil biodegradation was $NaNO_3$ giving a biodegradation rate of 87.8% which was significantly improved compared with the control group. The trend is as follows: $NaNO_3 > NH_4NO_3 > NH_4Cl$. It is probably because acid production associated with ammonia metabolism may inhibit oil biodegradation. Using the best nitrogen source ($NaNO_3$), the effect of carbon to nitrogen ratios was studied (Fig. 8). In theory, approximately 150 mg of nitrogen is utilized in the conversion of 1 g of hydrocarbon to cell materials [24]. In this study, the best C/N ratio was 20 which gave a diesel oil biodegradation rate of 88%. Kanaly et al. [25] and Vyas and Dave [26] reported that the addition of excess nutrients beyond certain limit in bioremediation would have no impact on cell growth and biodegradation process and excess nutrient content can be toxic to cell growth. Thus, the biodegradation rate was not increased even if the concentration of nitrogen was increased to the C/N ratio of 5. The effectiveness of using the best nitrogen source and C/N ratio in stimulating biodegradation in the tests is illustrated in gas chromatographs traces (Fig. 9). The samples clearly suggest that the bacterial strain used in this study was able to break the compounds present in diesel oil and biodegradation in the flasks with bacteria and nitrogen was faster and more extensive than in the untreated control or with only bacteria.

Comparison of biodegradation of diesel oil and microbial growth suggested that the biodegradation of diesel oil was in related with an increase in the population of oil degrader. In addition, when comparing the treatments between nitrogen addition and the control group, one noticed the significant stimulation of the microbial growth that was achieved by the application of nitrogen in the treatments (Fig. 4). After a

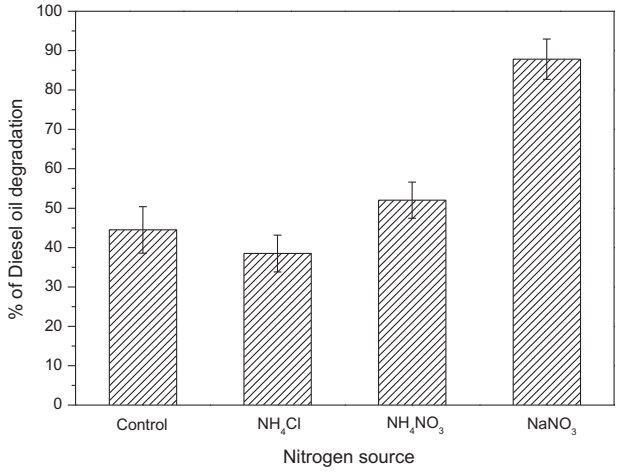

Fig. 7. Effect of nitrogen source on diesel oil biodegradation.

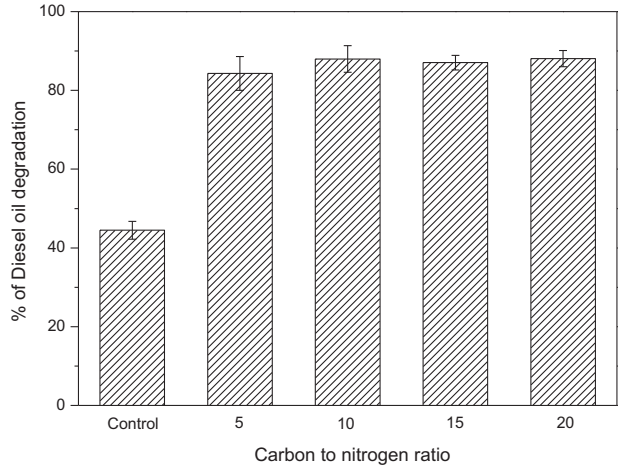

Fig. 8. Effect of carbon to nitrogen ratio on diesel oil biodegradation.

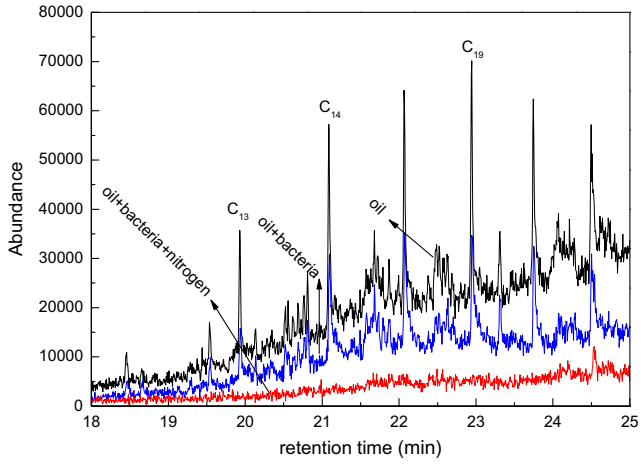

Fig. 9. GC–MS analysis of diesel oil biodegradation at day 6.

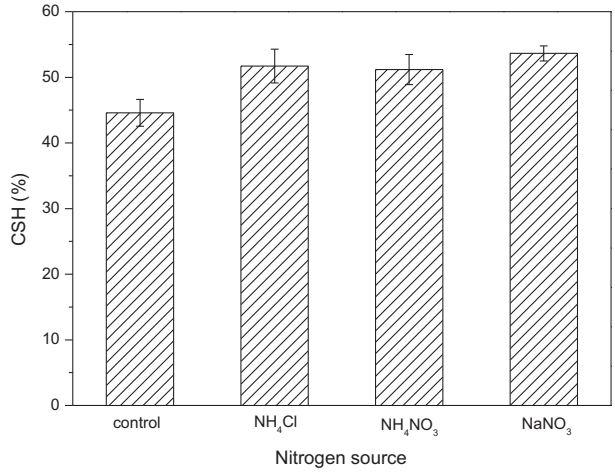

Fig. 10. Effect of nitrogen source on CSH.

lag phase of 2 d, a rapid cell growth was observed in the flask with nitrogen addition. However, the microbial growth in the control solution was slowly increasing with the OD value not more than 0.25. The above results confirm the fact that in natural seawater we can have significant microbial growth only if the essential nutrients are present.

In the previous studies, effect of nitrogen source on enhancement of oil biodegradation mostly focused on its improvement to microbial growth [4,9,27,28]. However, the changes of metabolism caused by nitrogen source, which were little mentioned in earlier studies, were also an important aspect to enhance biodegradation [8]. Cell surface properties are important factors that determine the rate of degradation of hydrophobic substrates. It has been reported that micro-organisms with higher cell-surface hydrophobicity (CSH) have potential superiorities in petroleum bioremediation [29–31]. In this study, different kinds of nitrogen source (NH_4Cl, NH_4NO_3, $NaNO_3$) were added at the concentration of 80 mg/L to observe its effect on CSH (Fig. 10). Results showed that the addition of nitrogen can improve CSH of *B. diminuta*. The best nitrogen source for CSH was $NaNO_3$ which equally possessed the highest biodegradation rate of diesel oil. The type of nitrogen source had little effect on the CSH. After four days' incubation, a kind of flavescent polymer was formed and floating on the surface of culture medium, which cannot be observed when there is no nutrient added in the solution (Fig. 11). The appearance of polymer may be associated with cell density during the culture. The FT-IR spectroscopy was used to elucidate the molecular composition of the polymer (Fig. 12). It can be clearly observed characteristic absorbance bands of peptides at 3,298 cm^{-1} (the NH-stretching mode); at 1,656 cm^{-1} resulting from the stretching mode of the CO–N bond; and at 1,545 cm^{-1} from the deformation mode of the N–H bond combined with C–N stretching mode. In

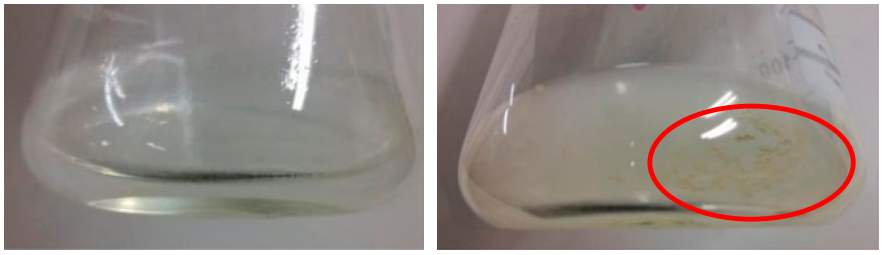

Fig. 11. Appearance of flavescent polymer in AS medium (A) without N addition and (B) N addition.

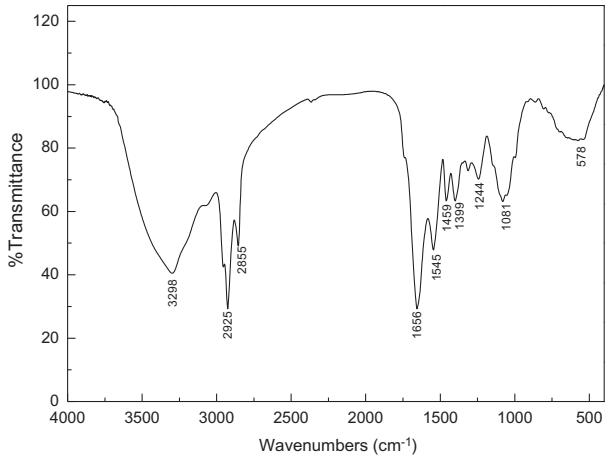

Fig. 12. FT-IR spectrum of the flavescent polymer presented in AS medium.

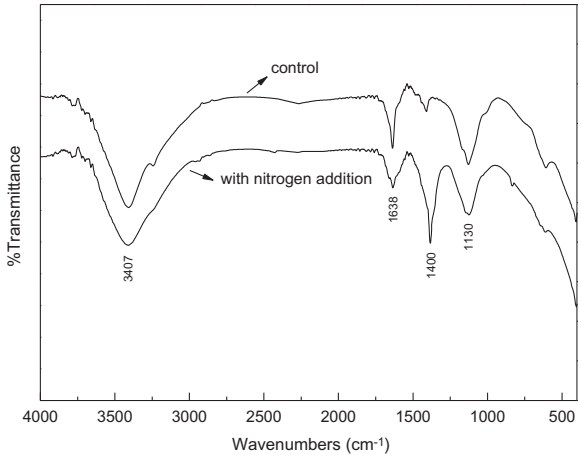

Fig. 13. FT-IR spectrum of compounds in emulsion layer of culture medium.

addition, it is also clear that the presence of aliphatic chains (–CH_3; –CH_2–) is represented by the bands between 2,850–2,930 cm^{-1} and 1,465–1,368 cm^{-1}. The presence of ester carbonyl bond is detected in the range at 1,300–1,000 cm^{-1}. The absorption peak at 578 cm^{-1} indicates the presence of C–O–S group [32]. The above information from the respective wave numbers confirmed the glycolipid nature of the polymer. Based on the above results, the polymer was tentatively identified as a glycolipid. The emulsion layer was observed when the culture medium was filtrated and centrifuged. The FT-IR analysis of the emulsion layer revealed the changes caused by nitrogen addition (Fig. 13). The most important bands were located at 3,407 cm^{-1} (the NH-stretching mode), 1,638 cm^{-1} (the stretching mode of the CO–N bond), 1,400 cm^{-1} (the carbonyl group in –COOH), and 1,130 cm^{-1} (the characteristic for C–O–C bond) [33,34]. The absorption peak at 1,400 cm^{-1} was increased and stronger than the peak at 1,638 and 1,130 cm^{-1} with the addition of nitrogen. It indicated that the quantity of hydrophobic group such as –COOH was increased, which meant the uptake ability of hydrophobic substrate like diesel oil was improved.

4. Conclusions

In this work, we have demonstrated the diesel oil degrading potential of *B. diminuta* isolated from the oil-contaminated seawater in Dalian. Biodegradation rates under different salinities showed *B. diminuta* had a good toleration of the salinity in seawater and can be applied to in situ bioremediation of marine oil spill. Addition of nitrogen increased the strain's density and positively affected its growth. Moreover, nitrogen supplementation could alter the characteristics of the process of metabolism to make the strain more easily assimilate petroleum hydrocarbon. The optimal nutrient condition to enhance diesel oil biodegradation was the supplementation of $NaNO_3$ at a C/N ratio of 20. Addition of external carbon source was also an effective way to enhance biodegradation. The optimal nutrient condition was the supplementation of soluble starch at a C/EC ratio of 2.

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