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Full Length Research Paper

Screening and characterization of petroleum-degrading bacterium

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Petroleum-degrading bacterium JY6 was isolated from petroleum-contaminated soils in DaQing oil field. It was identified as *Bacillus cereus* based on its morphological, physiological and biochemical characteristics, and analysis of its 16SrRNA gene. Biodegradation function of petroleum and oil degradation rates were studied, including different pH value, culture temperature, oxygen etc. Results show the optimum pH value and inoculation was 7.0 and 0.1% (v/v), respectively. 40 ml of liquid medium in 250 ml flask, shaking at 150 r/min and 32°C, petroleum-degrading rate was 75 to 77%. The petroleum-degrading bacterium might be a useful resource for bioremediation of oil-contaminated soils and biotreatment of oil wastewater.

Key words: Petroleum-degrading bacterium, screening, *Bacillus cereus*, bioremediation.

INTRODUCTION

With the development of petroleum exploitation and industry, oil pollution has become increasingly devastating. Although oil had brought huge economic efficiency, it has also brought series of environmental pollution. To eliminate the oil pollution and find out safe, economical and reliable treatment methods, domestic and foreign scientists have conducted a lot of theoretical and experimental research. There were many methods based on oil pollution treatment, some of which should be paid attention to, for example: physical technology, chemical technology, biological treatment, etc. Physical and chemical methods had the shortcomings of negative treatment, expensive and heavy secondary pollution (Wang, 1998). On the other hand, biological treatment is considered one of the most effective methods. It is highly efficient, economical, no second pollution, and has wide range of application (Ward and Van, 2003).

In recent years, scholars from various countries have studied the microbial degradation of oil pollution, mainly in the fields of the strain isolating (Wang et al., 2009; Li and Zhang, 2010; Grishchenkov, 2000), bacteria building (Guo and Qi, 2010; Han, 2010), the degradation characteristics of petroleum degrading strains (Zhang and Yang, 2010; Okpokwasili and Amanchukwu, 1988), immobilization of oil-degrading bacteria (Liu and Zhang, 2011; Liu et al., 2009) and so on. However, micro- organisms isolated so far by all researchers had poor adaptability on the environment, low performance of degradation and difficult to be applied to the bio- remediation of environments. Therefore, screening and separation of oil-degrading bacteria efficiently and studying its degradation characteristics were more significant.

In this study, we reported the screening and efficient separation of oil-degrading bacteria from Daqing oil field, and then studied the characteristics of JY6 and petroleum-degrading conditions. The results obtained herein might provide enough theoretical basis on oil pollution bioremediation.

MATERIALS AND METHODS

Culture conditions

The soil used in this study was from the Daqing oil field, machinery repair shop of Jilin Agricultural University. The oil-degrading medium consisted of (g/L): $NaNO_3$, 0.5; NaH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; $(NH_4)_2SO_4$, 0.5; $CaCl_2$, 0.02; KH_2PO_4, 1.0; petroleum, 0.9. The initial pH was adjusted to 7.0 using 0.1 M NaOH. Seed medium

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consisted of (g/L): Peptone, 10; yeast powder, 5; fructose, 10; K_2HPO_4, 2; and $FeSO_4 \cdot 7H_2O$, 0.3. The cells were cultivated at 150 rpm, 32°C for 22 h.

Bacterial isolation and medium

Bacterial strains were isolated from oil-contaminated soil samples from an oil field in Daqing, China. Then 5.0 g of oil contaminated soil samples were inoculated into 100 ml oil-degrading medium containing 2% (v/v) diesel oil as the sole source of carbon and energy, and incubated at 32°C, 150 rpm for three days. The cultures were enriched by five cycles of enrichment. Subsequently, 1 ml of the culture broth was diluted serially and inoculated on 2% agar selective medium, and 200 µL were sprayed on the surface of each plate. Each colony with a different morphology was characterized for petroleum hydrocarbon degradation.

Characterization of the bacteria

The 16S rRNA gene sequence of each strain was amplified using Taq DNA polymerase under standard reaction conditions with the primers 27F. The PCR products were sequenced with the primer 27F at Shanghai Sangon Biological Engineering Technology and Services Co. Ltd. The sequences were analyzed using the BLASTn (NCBI) and aligned with ClustalX software. Phylogenetic trees were constructed based on partial 16S rRNA gene sequences using neighbor-joining method.

Measure of oil degradation rate

The oil degradation rate was measured by UV spectrophotometry.

Effect of pH and temperature

The pH optimum for oil degradation rate was determined using the following methods: 5.0 ml of seed was inoculated into 95 ml oil-degrading medium, and pH was adjusted to 5, 6, 7, 8, 9 and 10, respectively with NaOH. This was cultivated at 150 rpm for two days and centrifuged at 2000 rpm. The supernatant was measured at OD600, with pH 7 as the control. Furthermore, the thermal optima for oil degradation rate was measured as follows: 5.0 ml seeds was inoculated into 95 ml oil-degrading medium in 250 ml Erlenmeyer flasks with temperatures adjusted to 25, 28, 31, 34, 37, and 40°C, respectively. This was cultivated at 150 rpm, for two days, and centrifuged at 2000 rpm. The supernatant was measured at OD600 using 34°C as the control.

Effect of dissolved oxygen

Five milliliters of seeds was inoculated into 95 ml oil-degrading medium and the rotation speed was adjusted to 50, 100, 150, 200, 250 and 300 rpm at pH 7.5 at 32°C for two days and centrifuged at 10000 rpm for 10 min. Finally, the supernatant was measured at OD_{600} using150 rpm as the control.

Effect of different additives

In this experiment, 5.0 ml of seed broth was inoculated into 90 ml oil-degrading medium, added with 1% k_2HPO_4, 1% $MgSO_4$, 0.25% NaCl, 0.5% NaCl and 1% Tween 60, respectively. The pH was 7.5 at 34°C for two days, centrifuged at 10000 rpm for 10 min and the supernatant was measured at OD_{600} with blank (inoculated seed broth with nothing added) as control.

RESULTS

Selection of high efficient oil degrading strains

Isolation of oil-degrading bacterium effectively was the foundation of studying the oil degrading mechanism and the bioremediation of soil. We collected 30 oil contaminated soil samples from Daqing Oil field and machinery repair shop of Jilin Agricultural University, and then got 20 strains by enrichment and purification for three times, and obtained six strains by oil degrading ability test.

Six bacteria grew well in the oil medium (Table 1). These could emulsify crude oil effectively, and after seven days culture, the bacterial density was up to 10^7 to 10^9 cpm/ml. These bacteria could use oil as the sole carbon source. The biological degreasing rate was 43.8 to 52.9% after seven days culture. The enzyme activity of the six bacteria was 0.133 to 0.184U. With the increase of enzyme activity, oil degradation ability will be increased, but the low lipase activity and high oil degradation rate indicated that lipase play a role, although not the main role on the oil degradation process. This may be related with the complex composition of oil. The degradation system therefore requires further investigation.

Characterization and identification

On the screening medium plate, strain JY6 formed a circular, smooth, transparent, pearly and white colony with approximately 1 mm in diameter after one day incubation at 36°C. More also, it was Gram-positive, rod-shaped and motility was well observed. Oxidase, contact enzyme, lecithinase, lipase, amylase and citrate salt test were positive. Methyl red (MR) and Voges-Proskauer (VP) tests were positive. Furthermore, acid but not gas was produced from glucose, xylose, arabinose, and mannitol. A 16S rRNA fragment of strain JY6 with 1450 bp in length was deposited. Multiple alignments revealed that its 16S rDNA sequence was closely related to that of *Bacillus cereus* (*B. cereus* strain Aj080319,TA2, G9842, Q0232, Q34, etc, with 99% of similarity). To identify the phylogeny of strain JY6, strains from different genera were chosen to construct the phylogenetic tree based on 16S rDNA sequences. Figure 1 indicates that the strain JY6 clustered closely with *B. cereus*. Hence, by examining morphological, biochemical characteristics and comparing its 16S rRNA sequence, strain JY6 was assigned to *B. cereus* and named *B. cereus* JY6.

Effect of pH

Effect of initial pH on degradation rate of crude oil was investigated. Figure 2 indicates that the initial pH value

Table 1. The physiological character and oil-biodegradation ratio of six strains.

Strain name	Collection location	The growth of bacteria and oil emulsion	Bacterium density after 7 days culture (cpm/ml)	Oil-degrading (%)	Enzyme activity (U/ml)	Strain characteristic
JY1	Machinery repair shop of Jilin Agricultural University	Completely emulsified and thick	5.3×10^7	43.8	0.156	White, dry surface, opaque, circular processes, uneven edges
JY2	Machinery repair shop of Jilin Agricultural University	Few of films, mostly floc	2.4×10^7	46.7	0.133	White, wet smooth, translucent, round, neat edge
JY3	Daqing oil field	Completely emulsified, thick and lots of foams	9.2×10^7	45.8%	0.176	White and transparent, wet smooth, round, neat edge
JY4	Daqing oil field	Completely emulsified, floc	1.2×10^7	45.0	0.164	White and transparent, wet smooth, round, neat edge
JY5	Daqing oil field	Few of films, floc	3.2×10^8	47.6	0.163	White and transparent, wet smooth, round, neat edge
JY6	Daqing oil field	Fully decentralized, floc, thick	3.6×10^9	52.9	0.184	White and transparent, wet smooth, round, uneven edges

significantly influenced the degradation rate of crude oil of JY6; the optimum pH value for growth and degradation rate of crude oil was 7.0.

Effect of temperature

Effect of temperature on the degradation rate of crude oil of JY6 was also investigated. Figure 3 indicates that temperature significantly influenced the growth and degradation rate of crude oil of JY6. An optimization test indicated that the optimal temperature was 32°C.

Effect of inoculation concentration

It can be seen from Figure 4 that the inoculum amount also affects the degradation rate of crude oil. As the concentration increases, the degradation rate was increased at first and then decreased. It had the maximum degradation rate of oil when the inoculation amount was 0.1% (v/v). This was probably because the oxygen and nutrients condition was not enough to sustain cell growth and metabolism when the inoculation amount was higher 0.1%.

Effect of different additives

Different additives of medium had a great impact on the degradation rate of crude oil. Potassium, magnesium and sodium are activators or cofactors of various enzymes. Figure 5 indicates that low concentrations of sodium ions promote the ability of oil degradation, while the high concentration of sodium ions inhibited the oil degradation. As a surfactant, Tween plays a significant role on oil degradation.

Verification experiment

Base on the single-factor experiment, we selected pH of 7.0, 32°C, inoculum amounts 0.1% (v/v), and added Tween 60 1% (v/v) for three times. The results showed that the degradation rate of crude oil was 77.2, 75.3 and 76.7%, respectively.

DISCUSSION

Petroleum is one of the most important sources of

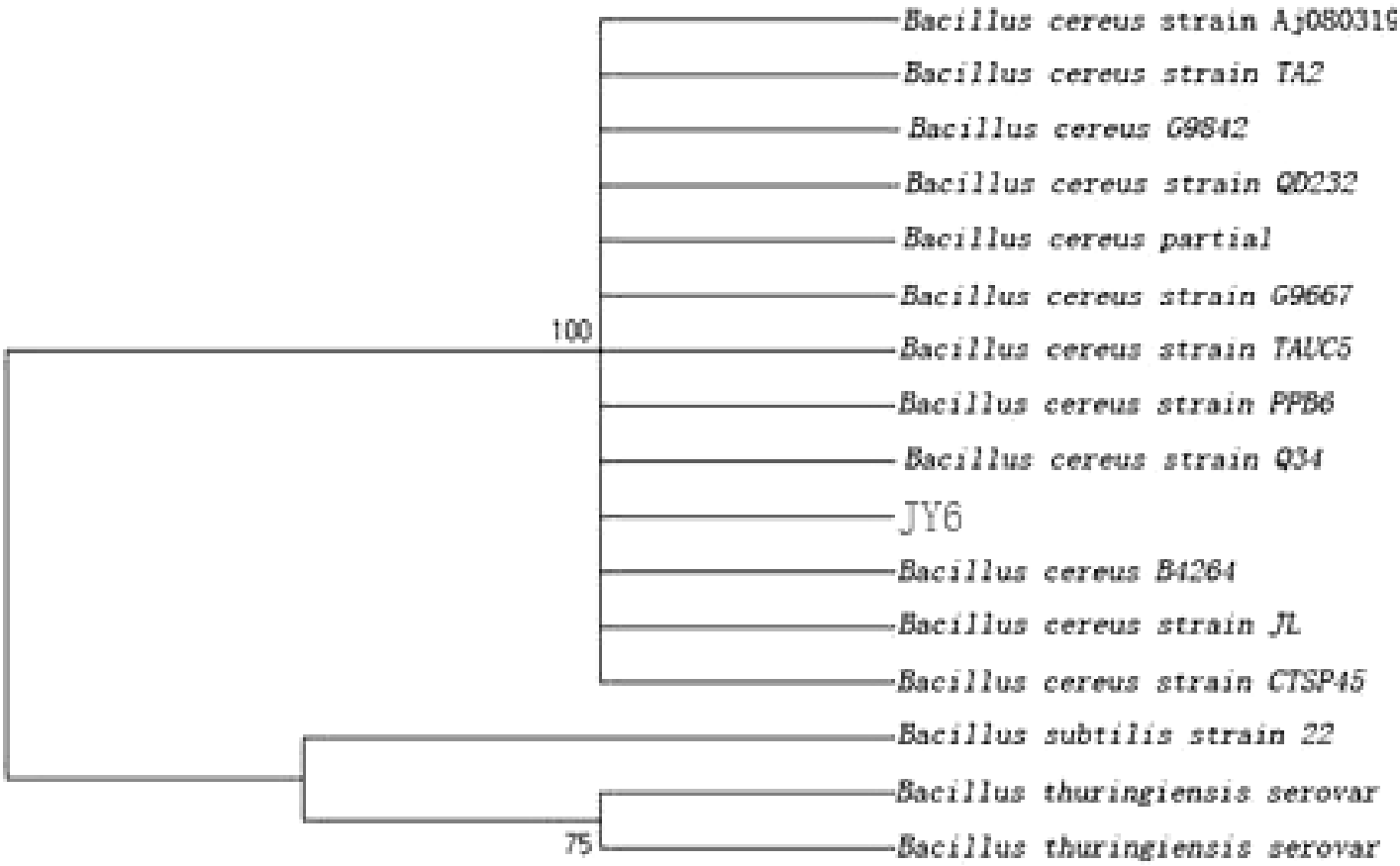

Figure 1. Phylogenetic tree for isolate JY6 and related strains based on the 16S rRNA sequence. The tree was constructed by the neighbor-joining approach.

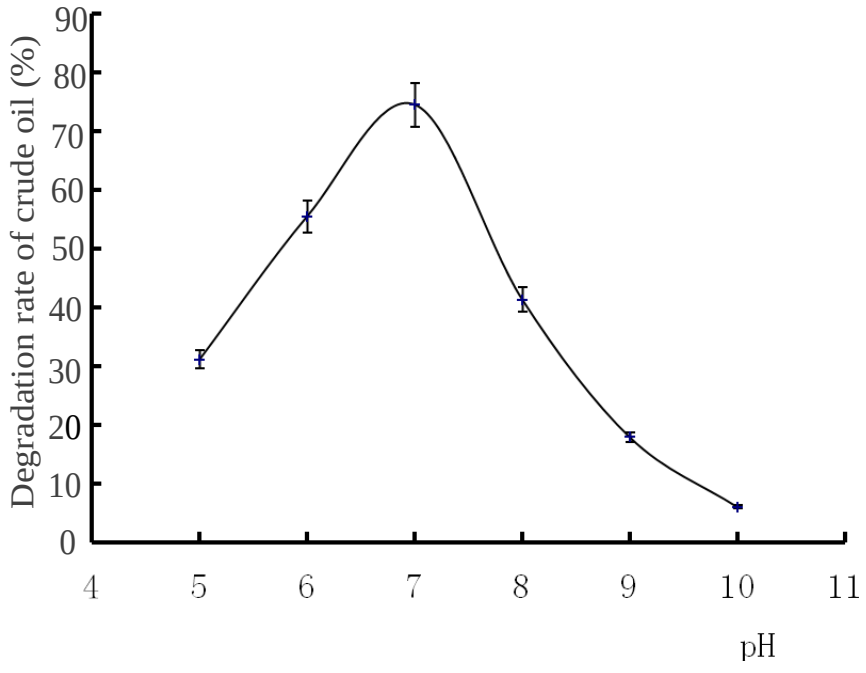

Figure 2. Effect of pH on the degradation rate of crude oil of JY6.

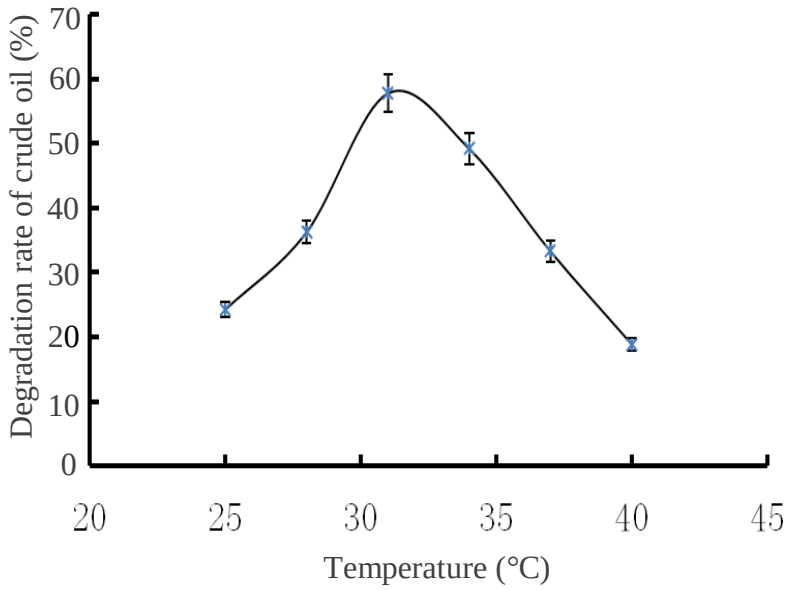

Figure 3. Effect of temperature on the degradation rate of crude oil of JY6.

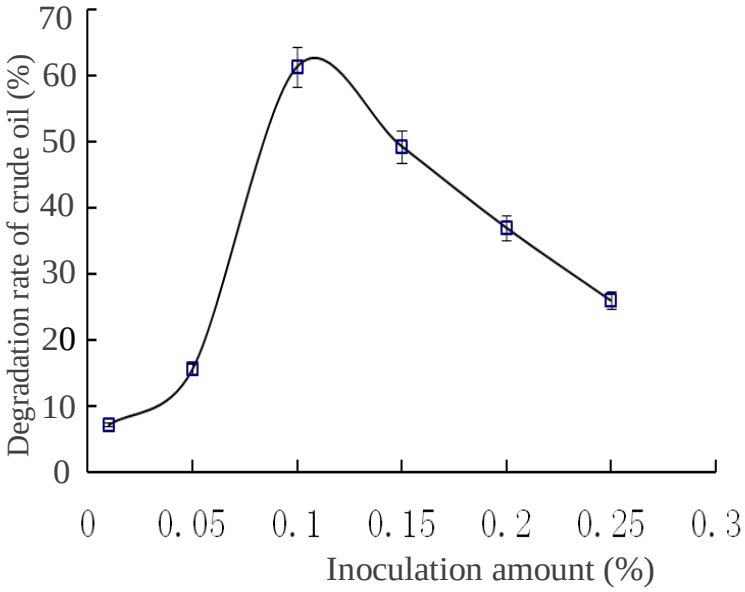

Figure 4. Effect of inoculation on the degradation rate of crude oil.

energy and also a backbone of the national economy. With the constant increase of domestic petroleum consumption, corresponding oil pollutant is produced heavily. The biological renovation has become an important method for treating the petroleum pollution.

Screening and identification

Microorganisms able to degrade oil were found widely in the nature (Zhang, 2011; Atlas and Atlas, 1991), including the *Rhodococcus rhodochrous* (Van Hamme and Ward, 2001), Nocard's bacillus (Hamamura and Arp, 2000), pseudomonad (van Beilen et al., 1994), etc. These microorganisms were obtained from lake (Duckworth, 1998), oil field (Borzenkov, 2006), reed rhizome rot (Borzenkov, 2006; Nazina, 2002), deep-sea sediments (Colquhoun, 1998), skin and gut of marine fish (Yumoto ., 2002), etc. The 16S rRNA, a kind of very conservative sequence of prokaryotic, play an important role in the modern microbial taxonomy (Rota, 2003; Ksiazek, 2003). In this study, based on the bacterial universal primers, we

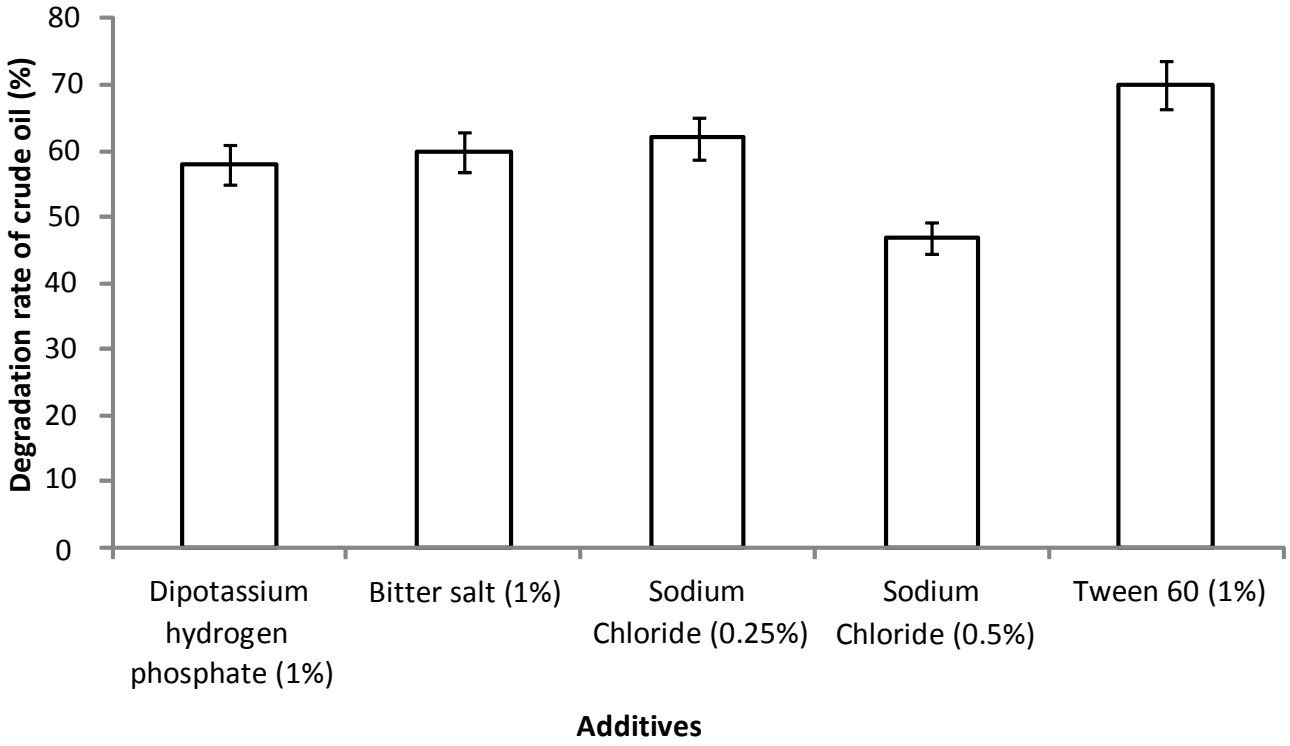

Figure 5. Effect of different additives on the degradation rate of crude oil.

amplified the 16SrRNA sequences of wild bacteria JY6 by polymerase chain reaction (PCR) method and constructed the phylogenetic tree. Results show that the wild bacteria JY6 attributed to *B. cereus*.

In recent years, many scholars had studied oil degradation bacterium but not *B. cereus*. Hao et al. (2002) isolated *Bacillus subtilis* SP4 from an oil reservoir, which could have transformed crude oil different aromatic non-hydrocarbon and asphaltene fractions etc., and also improve the physical and chemical properties of crude oil. In addition, Ma et al. (2006) isolated HBS-4 which could produce biosurfactants that degrades crude oil. After 12 h working, the crude oil asphaltene and aromatic components were converted and degraded and it was observed that the relative content of crude oil decreased by 2.89 and 17.39%. Furthermore, Song et al. (2004) isolated *Bacillus* sp. from oilfield wastewater which has the ability to degrade crude oil and utilize organic acids and biological surface. Strains Isolated from the Daqing oil field had good emulsifying and foaming properties, and can be applied into oil degradation (Wang et al., 2008).

Degrading characteristics of JY6

Degradation ability of *B. cereus* JY6 was lower than the study of Zhou et al. *(*2011), close to the level of that observed by Xu et al. (2010), and better than those reported by Lin., (1997) and Bao et al. (2010).

Conclusion

In this study, we isolated a new petroleum-degrading bacterium JY6 and studied the its characteristics. To date, no report is available on the isolation and production of petroleum-degrading by *B. cereus.* However, we have successfully isolated a new strain of *B. cereus* JY6 that is very useful for bioremediation on petroleum contaminated soil.

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