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Anaerobic benzene biodegradation by a pure bacterial culture of *Bacillus cereus* under nitrate reducing conditions

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Abstract

A pure culture using benzene as sole carbon and energy sources was isolated by screening procedure from gasoline contaminated soil. The analysis of the 16S rDNA gene sequence, morphological and physiological characteristics showed that the isolated strain was a member of genus *Bacillus cereus*. The biodegradation performance of benzene by *B. cereus* was evaluated, and the results showed that benzene could be efficiently biodegraded when the initial benzene concentration was below 150 mg/L. The metabolites of anaerobic nitrate-dependent benzene oxidation by strain *B. cereus* were identified as phenol and benzoate. The results of substrate interaction between binary combinations for benzene, phenol and benzoate showed that the simultaneous presence of benzene stimulated the degradation of benzoate, whereas the addition of benzene inhibited the degradation of phenol. Benzene degradation by *B. cereus* was enhanced by the addition of phenol and benzoate, the enhanced effects were more pronounced at higher concentration. To our knowledge, this is the first report that the isolated bacterial culture of *B. cereus* can efficiently degraded benzene under nitrate reducing conditions.

Key words: *Bacillus cereus*; nitrate reduction; anaerobic biodegradation; substrate interaction; phenol; benzoate
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Introduction

Benzene is among the most prevalent organic contaminants in groundwater and has been found in at least 816 of the 1428 National Priorities List (NPL) sites (Coates et al., 2002). Benzene can additionally cause hematological effects which may ultimately lead to aplastic anemia and development of acute myelogenous leukemia (ATSDR, 2004). Due to its toxicity and relatively high solubility, benzene represents a significant human health threat and is currently recognized as one of the most important contaminants in the United States. The USEPA has set a maximum permissible level of benzene in drinking water at 5 μg/L and set a goal of 0 μg/L for benzene in drinking and surface water. Consequently, benzene is often a single compound that drives the need for corrective action at sites contaminated with petroleum product releases (Da Silva and Alvarez, 2007).

Remediation of the benzene contaminated sites is desirable to avoid public health hazards. Bioremediation, expected to be an economical, energy efficient and environmentally sound approach to other remediation processes such as chemical or physical ones, has been developed as a useful clean-up technique. Aerobic bioremediation of benzene generally exhibits faster degradation rate than anaerobic systems (Corseuil et al., 1998). However, when soil and groundwater are contaminated with benzene, extensive anaerobic zones are frequently developed (Lovley, 1997). As a result, anaerobic bioremediation might be more appropriate to clean up some benzene contaminated sites.

In the last decade, studies have demonstrated that the degradative capacity of anaerobes is far greater than previously assumed and many hydrocarbon contaminants previously considered recalcitrant are now known to be biodegradable in the absence of oxygen (Burland and Edwards, 1999; Coates et al., 2001). Significant advances have been made towards understanding the bacterial characteristic and biochemical bases of anaerobic benzene degradation under nitrate reducing (Coates et al., 2001; Ulrich et al., 2005; Dou et al., 2008a, 2008b), Fe^{3+} reducing (Kunapuli et al., 2008; Anderson et al., 1998), sulfate reducing (Caldwell and Suflita, 2000; Kazumi et al., 1997; Lovley et al., 1995) and methanogenesis conditions (Ulrich et al., 2005; Ulrich and Edwards, 2003).

Up to now, only few pure cultures were reported to utilize benzene anaerobically. Coates et al. (2001) have successfully isolated the strains of RCB and JJ that degrade benzene coupled with chlorate, perchlorate, nitrate or oxygen reduction. Both of the isolates are members of

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the newly described *Dechloromonas* genus in the beta-subclass of the *Proteobacteria* (Chakraborty and Coates, 2005). Rooney-Varga et al. (1999) observed that *Geobacter* spp. could oxidize benzene under iron reducing conditions. Ulrich and Edwards (2003) found that *Desulfobacterium* sp. could degrade benzene under methanogenic conditions. Kasai et al. (2006) isolated two denitrifying *Azoarcus* strains (DN11 and AN9) with complex nutrients and oxygen or nitrate. Weelink et al. (2007) observed that the bacterium related to *Alicycliphilus denitrificans* K601 was able to degrade benzene coupled to chlorate reduction. On the basis of detected metabolites, the mechanism of benzene activation under anaerobic conditions has been suggested to occur via hydroxylation, carboxylation or methylation (Coates et al., 2001; Ulrich et al., 2005; Caldwell and Suflita, 2000; Chakraborty and Coates, 2005).

Metabolites that will occur during the degradation of benzene, therefore, the information about substrate interactions between the metabolites and benzene is essential to improve anaerobic bioremediation of benzene contaminated sites in the future. However, little attention has been given to this substrate interaction by the isolated pure culture. Furthermore, to help develop anaerobic remediation technologies, information regarding the degradation rates and characteristic for remediation of benzene contaminated sites is still required. Therefore, the detailed research is conducted to study benzene metabolites and the substrate interactions in the degradation of benzene by the isolated pure bacterium.

1 Materials and methods

1.1 Bacteria growth medium

Mineral salts medium (MSM) of the following composition NH_4Cl (1.0 g/L), KH_2PO_4 (1.0 g/L), $MgCl_2$ (0.1 g/L), $CaCl_2{\cdot}2H_2O$ (0.05 mg/L), $NaNO_3$ (1.5 g/L) was used in this study. The medium was supplemented with 0.1% of $Na_2S{\cdot}9H_2O$, vitamin solution (1%, *V/V*) and trace elements solution (1%, *V/V*). Trace elements solution contained in mg/L: 30 $CoCl_2{\cdot}6H_2O$, 0.15 $CuCl_2$, 5.7 H_3BO_3, 20 $MnCl_2{\cdot}4H_2O$, 2.5 $Na_2MoO_4{\cdot}2H_2O$, 1.5 $NiCl_2{\cdot}2H_2O$, and 2.1 $ZnCl_2$ (Hu et al., 2007). The vitamin solution contained in one litter of 20 mg biotin, 20 mg folic acid, 50 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg pantothenic acid, 1 mg cyanocobalamin, 50 mg *p*-aminobenzoic acid, and 50 mg thiotic acid (Zhang and Young, 1997). The final pH of the medium was adjusted between 6.8 and 7.2.

1.2 Isolation and identification of the benzene degrading microorganism

The isolation of benzene degrading strain was done with the mixed bacteria enriched from the gasoline contaminated soil. The screening procedure of the mixed bacteria was conducted as described in our previous work (Dou et al., 2008a). The pure culture was isolated by serial dilution using mineral salt medium mixed with molten agar and benzene. Each of the colonies produced was removed with an inoculating loop scraped over the agar surface and transferred to liquid medium to check for the growth ability at benzene initial concentration of about 150 mg/L. Controls consisted of uninoculated medium. The plates were inoculated and incubated at 20°C in an anaerobic glove box with a headspace consisting of pure nitrogen gas. The isolate was characterized and identified by standard morphological, physiological and biochemical plate and tube tests using the criteria and procedures described in Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984). Total DNA of the isolated strain was extracted using the DP302 DNA extraction kit (Tiangen Biotech, Beijing, China), then was purified with DNA purification kit (Dingguo Co. Ltd., Beijing, China). PCR amplification was performed using the universal primers 8F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACCTTGTTACGACTT-3′) in a 50-μL system, consisting of 25 μL PCR Master Mix, 2 μL 8F, 2 μL 1492R, 2 μL lysate and 19 μL ddH_2O. Amplifications were carried out for 28 cycles (94°C for 30 sec, 55°C for 40 sec, and 72°C for 90 sec) in a 1000-series thermal cycler (Bio-Rad, Hercules, USA) with an initial denaturation at 94°C for 6 min. The PCR products were sequenced using ABI3730 genetic analyzers (Sunbiotech Co. Ltd., Beijing, China). The sequences were analyzed by comparing with the standard of National Centre for Biotechnology Information (NCBI) for the identification of the bacteria.

1.3 Anaerobic biodegradation experiments

Five different initial benzene concentrations of approximately 10, 25, 50, 100 and 150 mg/L were used. In order to account for abiotic benzene degradation, control experiments containing no electron acceptor, no microorganisms were run in parallel. In addition, microcosms with electron acceptor and microorganisms but without benzene were also prepared. At the same time, a series of experiments with the binary combinations between benzene, phenol and benzoate with different initial concentrations of approximately 10, 30 and 50 mg/L were investigated. The sterile samples were established by autoclaving at 121°C for 3 hr before amendment of microorganisms and benzene.

Anaerobic biodegradation experiments were performed using 50-mL glass serum bottles, and the bottles were sealed with Teflon coated rubber stoppers (Alltech Associates, Inc., USA) and aluminum caps. The isolated culture was grown anaerobically to late exponential growth phase in 1.5 L of mineral medium containing benzene and nitrate. The culture was harvested anaerobically by centrifugation, washed, and resuspended in mineral salts medium.

Prior to inoculating the isolate, the flask with the isolate in it was homogenized so that the cell was distributed evenly throughout the liquor, and this procedure ensured that samples taken from the active flask were representative of the total flask contents. Different benzene concentrations were added to each mineral medium. The experiments were started by adding 2 mL of washed isolate to the glass serum bottles containing 40 mL mineral salt medium and benzene. All the experiments were conducted in triplicates in an anaerobic glove box filled with pure

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nitrogen gas. The maintenance of anaerobic conditions was examined by a preliminary experiment. The microcosms were continuously shaken on a rotary shaker at 20°C in darkness. Samples were periodically collected to measure the concentrations of benzene, nitrate and nitrite.

1.4 Chemical analysis

Benzene was analyzed by a model GC-14B gas chromatograph equipped with a capillary column (ULBON HR-1, 0.25 mm × 30 m, Shimadzu Corp., Japan), with a flame ionization detector (FID) (Shimadzu Corp., Japan). Injector, detector and column temperature was hold at 150, 150 and 100°C, respectively. Nitrogen gas served as carrier gas, and oxygen and hydrogen served as fuel gas for the FID.

Nitrate and nitrite were analyzed by ion chromatography (Dionex DX100, Sunnyvale, USA), using an Iopac ASI4 (4 mm × 250 mm) analytical column, the eluent was Na_2CO_3-$NaHCO_3$ (3.5 mmol/L, 1.0 mmol/L), and the flow rate was 1.2 mL/min.

Degradation intermediates were detected using TRACE GC 2000/TRACE MS (Thermo Finnigan, USA) equipped with a DB-5 fused silica capillary column (30 m × 0.25 mm, 0.25 μm) after extraction. The organic solvent of *n*-hexane was used to extract metabolites from the culture supernatant. After centrifugation of cultures for 10 min at 3000 r/min, the supernatant was collected, acidified with 6 mol/L HCl and extracted two times with *n*-hexane, which was then reduced to 0.5 mL under a stream of nitrogen gas. The sodium sulfate 1.5 g was added to the 0.5 mL solvent to reduce water content. 1.0 μL of the sample was analyzed by GC/MS. The initial column temperature of 40°C for 3 min, 15°C/min to 90°C, 5°C/min to 140°C, 25°C/min to 250°C for 10 min. Temperature of injector was 250°C. The identities of phenol and benzoate were confirmed by comparing the GC retention time and the mass spectra acquired from samples against spectra acquired from authentic standards (Sigma Aldrich, USA).

2 Results and discussion

2.1 Microbiological characteristics and identifications of the isolated pure culture

The benzene biodegradative bacterial strain was isolated after single colony purification from the gasoline contaminated sites. The cell of the strain was anaerobic, motile, gram positive, rod shaped, forming endospores and varying in size ((0.9–1.2) μm × (1.5–3.5) μm). Growth of the strain was observed at temperatures ranging from 10 to 40°C (lower temperatures were not tested), and the optimum temperature was 25 to 35°C. The biochemical and physiological characteristics and identifications of the strain are listed in Table 1. Comparison of the above microbiological properties with the relevant criteria and descriptions in Bergy's Manual of Systematic Bacteriology (Krieg and Holt, 1984), the strain was identified as a bacterium belonging to the genus *Bacillus* and was named *B. cereus*. The 16S rDNA (1543 bp) sequence of the isolate obtained in this work was aligned with the 16S rDNA sequence closely related to *B. cereus* retrieved from the NCBI nucleotide sequence databases (99.3% similarity).

Table 1 Biochemical and physiological characteristic of the strain

Tested item	Result	Tested item	Result
Oxidase	Negative	Denitrification	Positive
Catalase	Positive	Reduction of nitrate	Positive
Aminopeptidase	Positive	Citric acid utilization	Positive
Indole production	Negative	D-Glucose utilization	Positive
Phenylalanine deaminase	Negative	L-Arabinose utilization	Negative
Voges-Proskauer test	Positive	D-Xylose utilization	Negative
Gelatin hydrolysis	Positive	D-Mannitol utilization	Negative
Starch hydrolysis	Negative	Malonic acid utilization	Negative

2.2 Biodegradation of benzene and reduction of nitrate by pure bacteria

The isolated pure bacterium of *B. cereus* was examined for benzene degradation and nitrate transformation in liquid cultures under denitrifying conditions. The results are shown in Figs. 1 and 2.

As shown in Fig. 1, approximately 150 mg/L benzene could be completely degraded by *B. cereus* within 25 days and without a lag period, however, the loss of benzene in uninoculated control was insignificant (< 8%) during the whole incubation time (data not shown), which indicated that the possible abiotic effect on benzene removal was negligible. According to the data in Fig. 1, the biodegradation rates at each initial concentration could be calculated, and the results showed that the degradation rates increased with increasing the initial concentration within the range used in this study. Toxic effects were not observed on the isolate even if the concentration of benzene was up to 150 mg/L. However, in the previous research we found that benzene degradation rates by the mixed bacteria increased with increasing the substrate concentration and reached maximum values at the substrate concentration of 50 mg/L, and then decreased with the increase of substrate concentration, indicating substrate inhibition (Dou et al., 2008b). Furthermore, the maximum degradation rate by the purified isolate was (5.16 ± 0.87) mg/(L·day), compared to 1.46 mg/(L·day) by the mixed bacteria (Dou et al., 2008b).

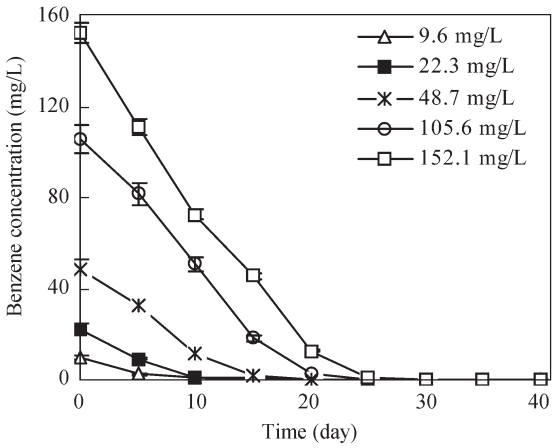

Fig. 1 Anaerobic biodegradation curves of benzene under different initial concentrations.

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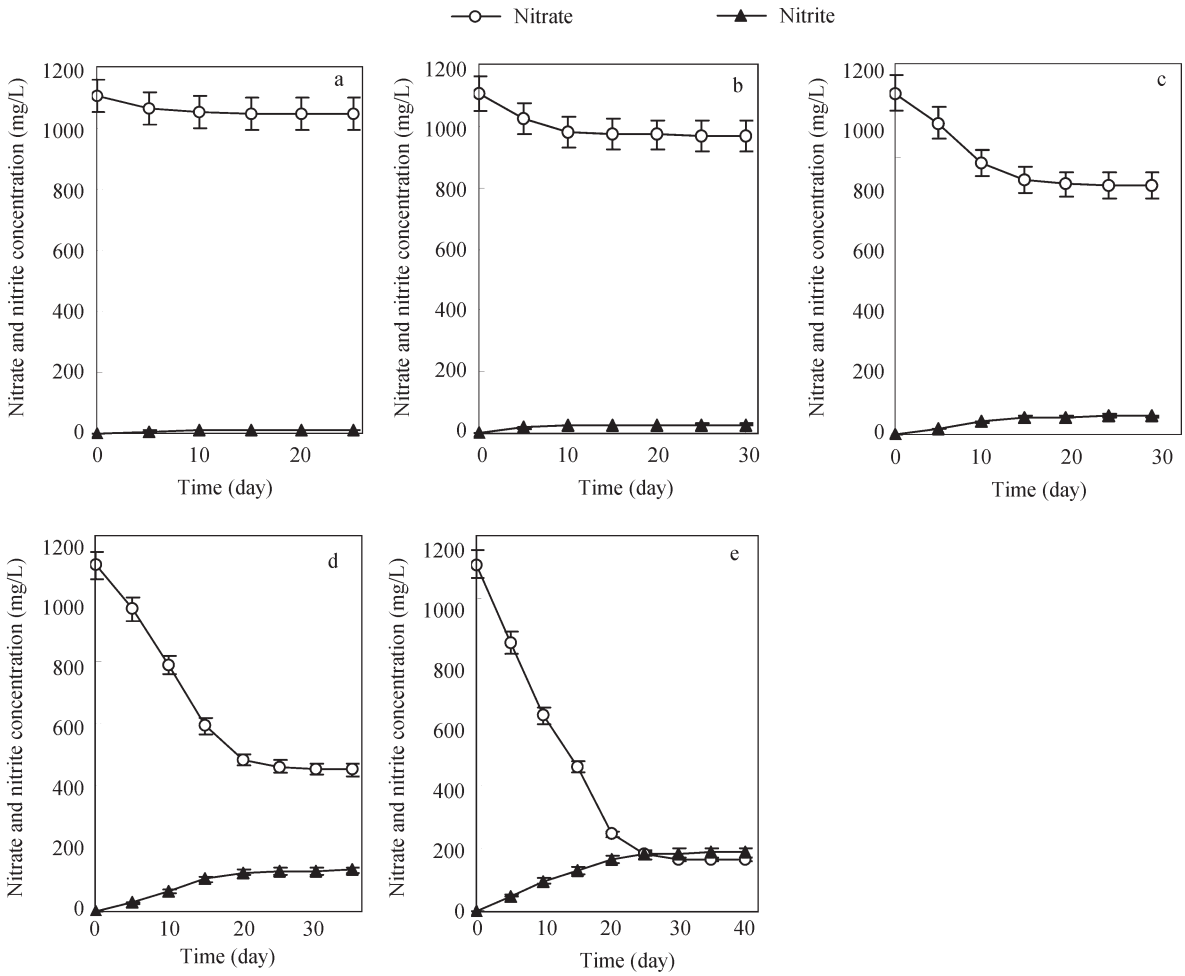

Fig. 2 Variation curve of nitrate and nitrite during anaerobic biodegradation of benzene. Initial benzene concentration: (a) 9.6 mg/L; (b) 22.3 mg/L; (c) 48.7 mg/L; (d) 105.6 mg/L; (e) 152.1 mg/L.

The isolated *Dechloromonas* strain RCB could degrade benzene with chlorate, nitrate and perchlorate reduction at a rate of around 2.50 mg/(L·day) (Coates et al., 2002). Other anaerobic enrichment cultures of *Desulfobacterium* sp. that degrade benzene with nitrate or sulfate reduction showed the degradation rates ranging from 0.08 to 5.85 mg/(L·day) (Ulrich and Edwards, 2003). Thus, it could be considered that the purified isolate obtained in this study was more efficient to degrade benzene under nitrate reducing conditions.

An interesting result is that benzene did not have toxic effect on the pure bacterium at the concentrations studied. This result was unexpected in light of previous research on BTEX degradation by the mixed cultures. In the previous study, an initial concentration of 80 mg/L of benzene showed an inhibition effect until the benzene was degraded to approximately 35 mg/L (Dou et al., 2008b). The reason for this phenomenon was that the accumulation of benzene in the membrane of the mixed cultures would inhibit bacterial growth, which led to the toxic effect at initial benzene concentration of 80 mg/L. However, the strain of *B. cereus* was isolated from the mixed cultures, and then the strain of *B. cereus* was adapted to high concentrations of benzene during the isolating procedure with the initial benzene concentration of about 150 mg/L.

Figures 1 and 2 show that the isolated culture exhibited nitrate removal along with a loss in benzene concentration. At the same time, the phenomena of nitrite accumulation were observed, whereas, no significant losses of nitrate or benzene were observed in the uninoculated benzene-containing control after 40 days of incubation. In the presence of nitrate, the isolate degraded 96% of benzene relative to the nitrate-free controls. These results clearly indicated that overall removal of benzene is attributable to biological mechanisms.

The half reaction for complete anaerobic oxidation of benzene to carbon dioxide with nitrate as the electron acceptors could be stated as follows:

$$C_6H_6 + 12H_2O \longrightarrow 6CO_2 + 30H^+ + 30e^- \quad (1)$$

$$NO_3^- + 6H^+ + 5e^- \longrightarrow 0.5N_2 + 6H_2O \quad (2)$$

$$NO_3^- + 2H^+ + 2e^- \longrightarrow NO_2^- + H_2O \quad (3)$$

Based on Reactions (1) and (2), the theoretical molar ratio of nitrate consumption to benzene degradation was 6. According to Reactions (1) and (3), the theoretical molar ratio of nitrate consumption to benzene degradation was 15. According to the experimental data illustrated in Figs. 1 and 2, the molar ratio of nitrate consumption to benzene degradation could be calculated, that is: nitrate of (9.88 ± 0.75) moles were consumed when 1 mole of benzene

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was degraded. The measured values were higher than the theoretical value of 6, that was expected assuming the complete reduction of nitrate to nitrogen gas with complete benzene mineralization, but were lower than the theoretical value of 15 that was calculated according to the assumption that nitrate was only reduced to nitrite. The reason for this phenomenon was that nitrate was reduced to nitrite, but only part of the nitrite was further reduced to nitrogen gas.

2.3 Identification of metabolites

GC-MS analysis of *n*-hexane extracts revealed that two metabolites were detected from samples within 5 and 10 days of inoculation, respectively. However, these metabolites were not detectable in *n*-hexane extracts from the supernatants of benzene-grown cultures within 25 days of incubation when benzene was completely degraded. Meantime, these metabolites were not observed in similar medium incubated without nitrate or the active isolated bacterium.

The mass spectra of these two metabolites are shown in Fig. 3. The metabolites identification was performed by comparison of GC retention time with authentic standards, and was confirmed by comparison of the mass spectra data from the MAINLIB library. One of the metabolites, which had a GC retention time of 7.67 min, and the characteristic fragment ions was at *m/z* 94, 66 and 40, the mass spectrum was identical to that of phenol. The other metabolite, which had a GC retention time of 13.22 min, yielded a different fragmentation pattern at *m/z* 122, 105, 77 and 51, the fragment ions were in agreement with the structure of benzoate. These metabolites were confirmed by using authentic standards of these two compounds that yielded the same fragmentary patterns. These results indicated that phenol and benzoate were intermediates of anaerobic nitrate-dependent benzene oxidation by strain *B. cereus*. There are several potential pathways for anaerobic metabolism of benzene, including hydroxylation to produce phenol, carboxylation to produce benzoate, and initial reduction of the ring to form cyclohexane (Grbic-Galic, 1990). Similarly, Caldwell and Suflita (2000) concluded that the formation of benzoate was involved in anaerobic benzene mineralization under iron-reducing and sulfate-reducing conditions, and they also demonstrated that the benzene was probably metabolized by an initial hydroxylation to form phenol, which was then converted to benzoate. The redox potential for benzene dehydrogenation to produce phenol ($C_6H_6 + H_2O \longrightarrow C_6H_5OH + 2H^+ + 2e^-$; $E^\circ = -0.09$ V) was not much high for the nitrate-reducing isolate, benzene was thus easily transformed to phenol. Because carboxylation of phenol was a suitable catabolic reaction with its relatively low energy gain, the phenol formed from benzene could be further carboxylated and dehydroxylated to form benzoate (Chakraborty and Coates, 2005).

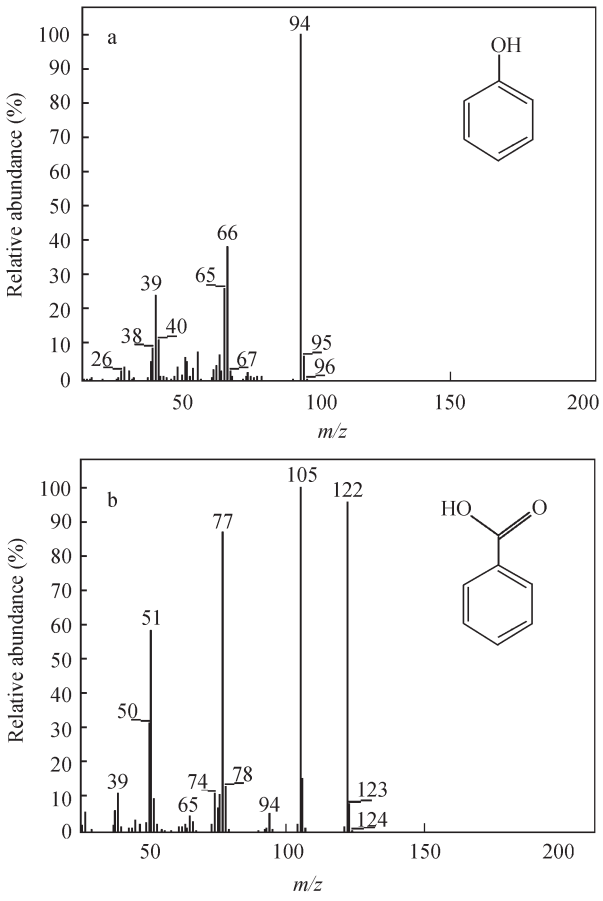

Fig. 3 Mass spectra of the metabolites. (a) metabolite identified as phenol; (b) metabolite identified as benzoate.

2.4 Test of substrate interactions in binary combinations between benzene, phenol and benzoate

The substrate interactions between the metabolites and benzene, and the effect of phenol and benzoate on benzene degradation were studied, and the results are shown in Figs. 4 and 5.

From Fig. 4, it could be observed that the biodegradation rates of phenol were slightly lower in the presence of benzene in comparison with phenol alone. On the contrary to phenol, the rates of benzoate degradation were higher in cell suspensions supplied with a mixture of benzene and benzoate than only with benzoate, indicating that benzene at the added concentration had a stimulated effect on the degradation of benzoate. The data in Fig. 4 demonstrated that both phenol and benzoate were degraded without a lag phase whether each of them was added alone or accompany with benzene. The observed immediate consumption of phenol and benzoate may be due to an adaptation of the isolated benzene-degrading culture. Phenol has hydrophobic properties and is easy to partition in lipid membranes, therefore, it is likely to enter the cell via diffusion (Sikkema et al., 1995). At the same time, there may exist enzyme system for the metabolism of free phenol in the benzene degrading bacterium. Therefore, phenol is likely to be a free intermediate and is biodegraded by the isolated benzene degrading bacterium. At the same time, nitrate was reduced without a lag phase upon the addition of these two intermediates alone or together with benzene (data not shown). The observed nitrate reduction and nitrite production starting immediately upon phenol or benzoate addition was another fact to confirm that phenol

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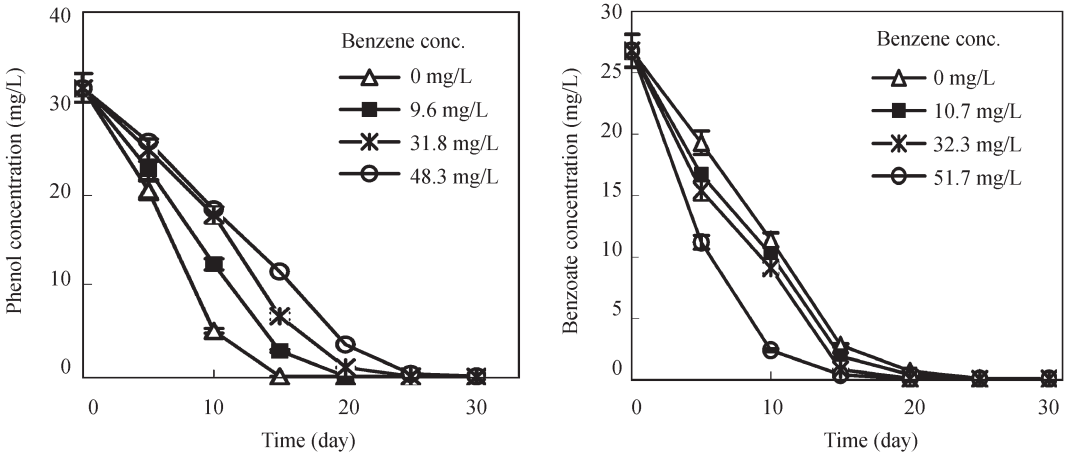

Fig. 4 Biodegradation of phenol and benzoate with the amendment of different concentrations of benzene. (a) phenol; (b) benzoate.

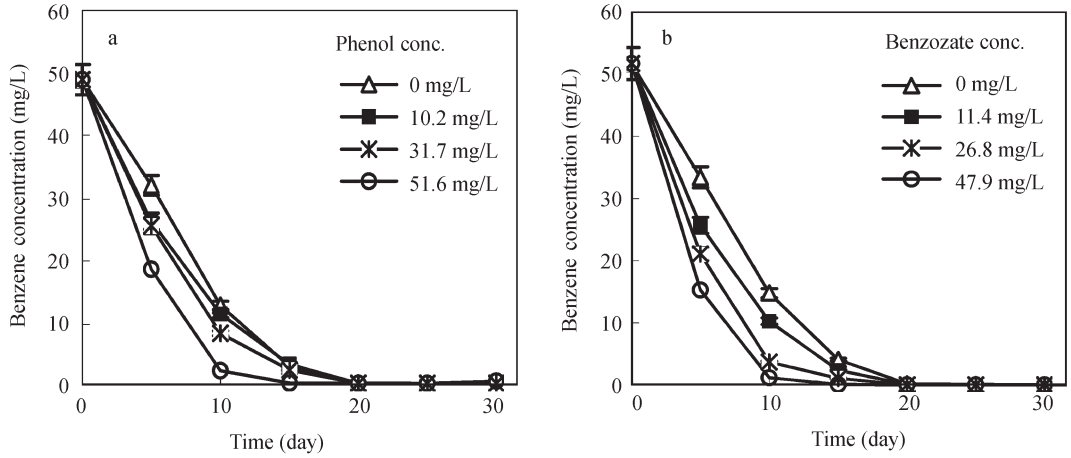

Fig. 5 Biodegradation of benzene with the amendment of different concentrations of phenol or benzoate.

and benzoate could be degraded by the bacterium. Thus, it could be concluded that in addition to being able to grow anaerobically with benzene as the sole electron and carbon source, the isolated strain *B. cereus* could also utilize phenol and benzoate for growth.

As shown in Fig. 5, the rate of benzene degradation in the presence of phenol was slightly higher than in assays with benzene alone, indicating that phenol at the added concentration of below 50 mg/L had stimulated effect on benzene degradation. As also can be seen from Fig. 5, benzene was degraded at a faster rate when benzoate was present simultaneously. Furthermore, the enhanced effect of the benzoate was more pronounced at higher concentration. One possibility might be that the presence of benzoate or phenol could stimulate the growth of the bacteria, and thus enhance the degradation rates of benzene. The reason for fortuitous growth may be that both benzoate and phenol were easily acceptable carbon sources for the isolated bacteria compared to benzene. Another possible explanation for the stimulated effect is through the induction of degradation enzymes by the presence of benzoate or phenol, and the enzyme systems could be involved in the reaction of anaerobic benzene degradation. Examining the intrinsic mechanism of benzoate-enhanced benzene degradation would be an attractive topic of further research and could reveal further points of enhancing benzene degradation rates.

3 Conclusions

The pure benzene degradation culture was isolated and identified as *B. cereus*. The experimental results showed that *B. cereus* could degrade 150 mg/L of benzene completely within 25 days. The isolated pure bacterium could transform benzene to phenol and benzoate, and then used phenol and benzoate as carbon and energy source. The simultaneous presence of benzene had a stimulated effect on the degradation of benzoate, whereas the addition of benzene had an inhibited effect on phenol degradation. The amendment of phenol or benzoate could stimulate the degradation of benzene. The results of the experiments showed that the isolated bacterium of *B. cereus* could potentially remediate a site contaminated with benzene using nitrate as terminal electron acceptors, highlighting its potential applicability to bioremediative technologies. Further study on the feasibility of the isolated pure bacterium under field conditions is required to promote the remediation of benzene contaminated sites under nitrate reducing conditions.

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