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Degradation of MTBE and TBA by a new isolate from MTBE-contaminated soil

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Abstract

Methyl *tert*-butyl ether (MTBE), a gasoline additive, possesses serious problems to the environmental health. In the present study, a bacterial culture named A-3 which could effectively degrade MTBE was isolated from the MTBE contaminated soil. The isolate was identified as *Chryseobacterium* sp., a new species capable of degrading MTBE. In order to enhance its degradation ability, selected environment factors were investigated. The results showed that the optimal temperature was in the range of 25–30°C, the pH was 7.0, the inoculum size was 2×10^8 CFU/ml and the optimal concentration of MTBE was from 50 to 100 mg/L. The maximum MTBE utilization rate (v_{max}) was 102 nmol MTBE/(mg cell protein·h). Furthermore, it was found that the isolate could also degrade *tert*-butyl alcohol (TBA). The degradation rates of TBA were much faster than those of MTBE. The additional TBA would lead to the decrease of the initial MTBE degradation rate and the inhibitory effect of TBA increased with the increase of TBA concentration. Similar protein profiles at least seven peptides were demonstrated after SDS-PAGE analysis of crude extracts obtained from the cells growing in MTBE and TBA culture.

Key words: methyl *tert*-butyl ether (MTBE); biodegradation; *tert*-butyl alcohol (TBA)

Introduction

Since 1970's, methyl *tert*-butyl ether (MTBE) has been added to gasoline in response to octane ratings demand and the phase-out of lead. Unfortunately, MTBE is odorous and also dissoluble in water. Besides these, its low biodegradability with more and more leakage of groundwater storage tanks and spills happening leads it to refusal of groundwater source. It has been reported that MTBE exposure would result in risks of kidney and liver tumors in rats, but there have been no human studies on determination of long-term effect of MTBE exposure (Belpoggi *et al*., 1995). In 1997, Environmental Protection Agency of the United States proposed an advisory safety level of 20–40 μg/L MTBE in groundwater, and the compound was classified as a potential carcinogen (USEPA, 1997).

The widespread use of MTBE has already caused a large number of contaminations in groundwater and drinking water sources in USA and Europe (Johnson *et al*., 2000). On this situation, some states had already banned the application of MTBE. Whereas, the demand for MTBE keeps growing and there is no evidence to slow down in the coming decades in China. The high production cost of ethanol gasoline and the fact that it cannot be transported by pipeline conveniently and must be carried in trucks or rail cars will limit the growth of ethanol blended gasoline in the coming decades. So, MTBE will still be a reality in China for a long time, which will result in serious environmental problems. However, few studies have reported the situation of MTBE pollution and the relevant remediation technologies (Chen, 2004).

Conventionally, several non-biological MTBE-treatment technologies have been developed in the west countries. Air stripping and active carbon adsorption have been proposed for the actions of groundwater and drinking water cleanup (Sutherland *et al*., 2004). However, they are expensive and ineffective because of the low affinity of MTBE to the sorbents. Biological treatment of soil and groundwater is expected to be the cost-effective technique. The first reported culture capable of degrading MTBE was the mixed culture BC-1 described by Salanitro *et al*. (1994). Since then, a number of mixed cultures (Kane *et al*., 2001) and pure strains capable of using MTBE as sole carbon and energy source (Mo *et al*., 1997; Hatzinger *et al*., 2001) or degrading it through co-metabolic process (Hardison *et al*., 1997; Steffan *et al*., 1997) have been obtained and identified. *tert*-Butyl alxohol (TBA) is the key intermediate product of MTBE degradation. In addition, in contrast to MTBE, TBA is almost exclusively used as a

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fuel oxygenate.

In China, the studies on MTBE and TBA removal by bioremediation techniques are still at their infancy for both fundamental and on-site application. In this study, a pure culture, the *Chryseobacterium* sp. A-3, capable of degrading MTBE and TBA isolated from the MTBE contaminated soil was characterized. This work would provide a new insight into the process of MTBE biodegradation.

1 Materials and methods

1.1 Soils samples

Soil samples taken at 5–20 cm depth from ground surface, were collected from a MTBE plant in Liaoning, China. Samples were then stored at 4°C and analyzed soon in a week.

1.2 Chemicals

MTBE (99.5%) and TBA (99%) were purchased from Dima Technology Inc. All other chemicals were analytical grade and obtained commercially.

1.3 Acclimation of microorganism for degradation MTBE

Ten grams of soil sample were dissolved in 100 ml sterilized water, and shaken at 150 r/min for 3 h, and then left standing for 30 min. The supernatant 10 ml was then incubated into BSM (base synthetic medium) containing MTBE as the sole carbon source to enrich cultures. BSM (Mo *et al*., 1997) medium was composed of (g/L): 5.57 of Na_2HPO_4, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2{\cdot}6H_2O$, 0.0004 of $MnCl_2{\cdot}4H_2O$, 0.001 of $FeCl_3{\cdot}6H_2O$ and 0.001 of $CaCl_2$. BSM agar was added with 15–20 g/L of agar. The pH of the medium was adjusted to 7.0 and then the medium was autoclaved at 121°C for 15 min. Flasks were incubated at 25°C and shaken at 150 r/min for 10 d. Thereafter, 10% of the enriched culture was transferred to a fresh medium. The adaptation phase was extended by successive additions of MTBE (the content of MTBE increased from 50 mg/L to 200 mg/L in the medium) over a 2-month period. After that, 0.1 ml of the cell suspension was spread on BSM agar coated with 100 mg/L of MTBE and incubated at 30°C. Colonies grown on BSM agar were isolated several times to ensure that the single colony is pure. Morphology of each isolate was studied using a microscope.

1.4 Identification of MTBE degrading monoculture by 16S rDNA analysis

Bacteria strain was grown aerobically in Luria-Bertani medium. Total genomic DNA was extracted as described by Ausubel *et al*. (1995). 16S rDNA amplification was performed by PCR using the forward-primer 799f: 5′-TAGATACCCTGGTAGTCC-3′ and the reverse-primer 1492r: 5′-GGTTACCTTGTTACGACTT-3′. PCR reactions were carried out in a thermal robocycler with 1 cycle of 3 min at 95°C; 30 cycles of 45 s at 95°C; 45 s at 53°C, 2 min at 72°C and 10 min at 72°C (Marcia *et al*., 2004). The percentage of identity was determined using the EMBL/GenBank database with the Blast alignment tool.

1.5 Degradation assays of MTBE and TBA

Cells were grown in shake flasks containing Luria Bertani (LB) rich medium with MTBE or TBA. LB medium was composed of (g/L): 10 of Trypton, 5 of yeast extraction, 10 of NaCl. The pH of the medium was adjusted to 7.0 and then the medium was autoclaved at 121°C for 15 min. The bacteria were collected by centrifuging at 4000×*g* for 15 min and washed twice with phosphate buffer (20 mmol/L, pH 7.0) and suspended in BSM to an optical density of 0.1 at 600 nm wave length. MTBE or TBA was then added to the culture as aqueous solution depending on the desired finial concentration by using plastic syringes filtered with steriled membranes (0.22 μm). The degradation experiments were performed in 250-ml serum vials sealed with Teflon-faced silicon septa and aluminum crimps at ambient temperature (25–28°C) shaking (150 r/min) for 14 d. The volume of the vials headspace was sufficient to prevent any limitation to O_2. Killed control samples were prepared by adding 10 mmol/L $HgCl_2$ to replicate vials.

Degradation was determined by direct-liquid-injection gas chromatographic with flame ionization detection (PerkinElmer Inc.). Assay samples 1 μl were filtered through a 0.22-μm membrane filter to remove the cells. Operational parameters for the GC were injector temperature 200°C, detector temperature 300°C, oven temperature 80°C. Nitrogen was used as carrier gas at a flow rate of 1 ml/min, and H_2 and air flow rate were controlled at 45 and 460 ml/min, respectively. The area under the curves was quantified by comparing with the standards from dilutions of pure MTBE or TBA. All the experiments were conducted in triplicate.

1.6 Cell extraction and gel electrophoresis

Strain A-3 was incubated in BSM for one week on an orbital shaker (150 r/min) at room temperature 20°C with 100 mg/L MTBE or TBA added after pre-grown in LB medium. Total cellular proteins were analyzed using dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis on polyacrylamide gel (Stacking gel, 5%; Running gel, 12%) (Ausubel *et al*., 1995). Low protein molecular weight markers were used to determine the size of peptides in the gel.

2 Results and discussion

2.1 Isolation and identification of microorganism for degradation MTBE

The microbial consortium obtained from the soil of MTBE plant was performed by periodically adding MTBE over a 2-month period. After several enrichment steps in BSM liquid medium containing MTBE as the sole carbon, a yellow-pigmented strain A-3, which showed a faster rate of MTBE removal, was isolated. Microscopic analysis of the strain A-3 showed that this microorganism

is a gram-negative, nonsporulated and capsulated rod. 16S rDNA sequence comparison with sequences in the GenBank database showed a 99% nucleotide sequence similarity with *Chryseobacterium* sp. PSR10 (accession number DQ118018.1) (Chaturvedi and Goel, 2005).

2.2 Effect of temperature and pH on MTBE degradation

Figure 1a illustrates the influence of temperature on MTBE degradation at an initial MTBE concentration of 50 mg/L. MTBE biodegradation rates increased with the increase of temperature. The MTBE degradation ratio were 43.5%, 48.9%, 52.3%, 52.6% and 42.1% at the temperature 15, 20, 25, 30 and 35°C, respectively. The optimal temperature was between 25 and 30°C. The strain studied in the present study was able to degrade MTBE at a low temperature, which was important for the groundwater bioremediation.

The influence of the pH of the reaction solution on the MTBE degradation was also investigated at an initial MTBE concentration of 50 mg/L and the temperature was at 30°C (Fig.1b). The degradation was investigated with different pH from 5.0 to 8.0. The result showed that the pH values were either higher than 7.5 or lower than 6.5, the rate of degradation was low, for the enzyme involved the degradation may lower activity in acid or condition. Then the optimal pH was 7.0.

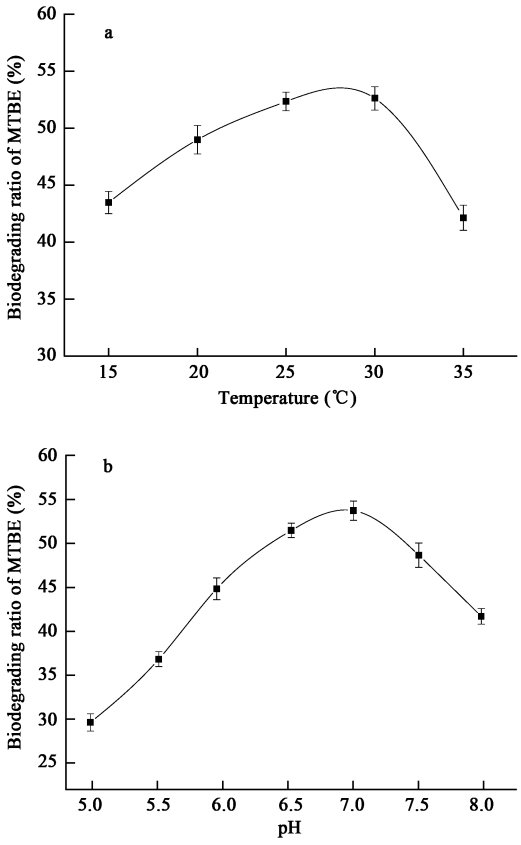

Fig. 1 Biodegradation of MTBE by strain A-3 as functions of temperature (a) and pH (b) at an initial MTBE concentration of 50 mg/L.

2.3 Effect of inoculum size on MTBE degradation

The effect of inoculum size on MTBE degradation was tested. Cells were grown as shake cultures at 30°C in LB medium were supplemented with MTBE at pH 7.0 in 250 ml flasks at 150 r/min. The bacteria were collected by centrifuging at 4000×g for 15 min and washed twice with phosphate buffer (20 mmol/L, pH 7.0). The different inoculum sizes were transferred into BSM medium with 50 mg/L MTBE. The initial absorbance at 600 nm wave length were 0.033, 0.073, 0.105 and 0.163, corresponding the cell density were 9×10^7, 1.4×10^8, 2×10^8 and 4.5×10^8 CFU/ml, respectively. These inoculum sizes were confirmed at the beginning of the experiment by plate count. As shown in Fig.2, MTBE degradation by *Chryseobacterium* sp. A-3 increased with the increase of inoculum size. When optical density at 600 nm was higher than 0.1, the increase of MTBE degradation ratio was not obvious. Besides, the higher innoculum size would consume the more oxygen, so the optimum optical density at 600 nm choose 0.1, and the corresponding innoculum size was 2×10^8 CFU/ml.

2.4 Effect of MTBE initial concentration on the degradation using *Chryseobacterium* sp. A-3

The degradation of MTBE by strain A-3 was studied at five initial concentration levels of MTBE (Fig.3). An initial inoculum of 2×10^8 CFU/ml of strain A-3 was added. The results showed that the degradation rate of MTBE was 6.28, 19.6, 43.1, 90.4, and 64.3 nmol MTBE/(mg cell protein·h) for the initial MTBE concentrations of 10.1, 25.3, 50.1, 101.6 and 202.4 mg/L, respectively. There was insignificant MTBE disappearance in the controls. Results demonstrated the rate of degradation increased with increasing initial MTBE concentration when it was below 100 mg/L. According to the degrading kinetics analysis, it could be fit to the model of Hakdane. The parameters were obtained by experiments, the maximum substrate utilization rate was 102 nmol MTBE/(mg cell protein·h), the half-saturation concentration was 171.3 mg/L and the inhibitory constant was 64.4 mg/L. The experimental results showed that the optimal concentration of MTBE in the present study was from 50 mg/L to 100 mg/L.

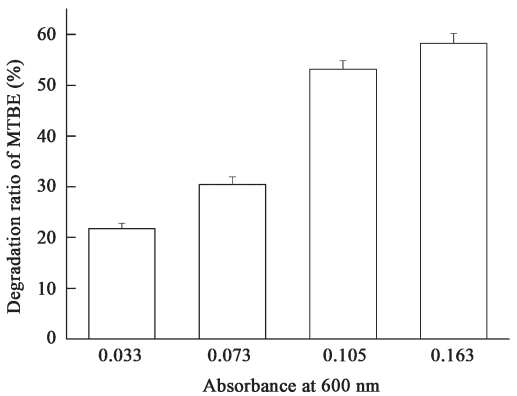

Fig. 2 Biodegradation of MTBE by strain A-3 as a function of inoculum size.

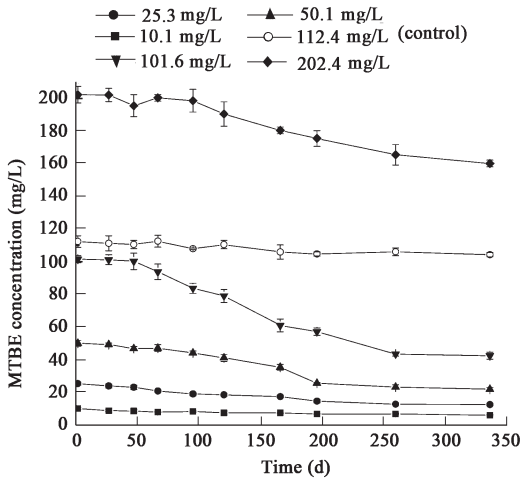

Fig. 3 The effect of initial concentration of MTBE on the degradation of MTBE using *Chryseobacterium* sp. A-3. Strain A-3 was incubated with MTBE at different initial concentrations. Values were obtained from means of three samples. Error bars represent standard deviation of the mean.

2.5 TBA degradation by *Chryseobacterium* sp. A-3

TBA was the intermediate product of MTBE degradation in most of MTBE-degrading cultures. The degradation of TBA by *Chryseobacterium* sp. A-3 was studied at the TBA initial concentrations of 10, 25, 50 and 100 mg/L, respectively. In contrast to the MTBE degradation, the TBA degradation ability by strain A-3 was obviously higher than that of MTBE (Table 1). This behavior of strain A-3 was similar to *Mycobacterium austroaficanum* IFP 2012 and UC1 (François *et al*., 2002; Amy and Makram, 2004).

2.6 Effect of TBA addition on the biodegradation of MTBE by *Chryseobacterium* sp. A-3

The effect of TBA on biodegradation MTBE was investigated for *Chryseobacterium* sp. A-3. Cells were cultivated in medium containing 50 mg/L of MTBE and 5, 25, 50, 100 mg/L of TBA, respectively. The results of MTBE and TBA degradation are shown in Table 2. Samples containing only MTBE or TBA were taken as controls. The result showed that the additional TBA would lead to a decrease in the initial MTBE degradation rate and the inhibitory effect of TBA increased with the increase of TBA concentrations. In the presence of equal concentrations of TBA and MTBE, the rate of MTBE biodegradation by *Chryseobacterium* sp. A-3 was 56% of that of the control. These findings suggested that a competition between the two compounds occurred if MTBE and TBA co-existed. From the results, it can be assumed that the same enzyme possibly responsible for MTBE and TBA metabolism and they may be competing for the same active site. The presence of TBA had a negative impact on the MTBE degradation rate, the similar result was also reported about butane-oxidizing *Arthrobacter* (ATCC 27778) bacteria (Liu *et al*., 2001). In the case of *M. austroafricanum* IFP 2012, the addition of TBA did not have any significant effect on MTBE degradation rate, while, the presence of MTBE clearly limited TBA degradation (François *et al*., 2002).

Table 1 Ability of *Chryseobacterium* sp. A-3 to degrade MTBE and TBA

Initial concentration (mg/L)	MTBE degradation rate (d^{-1})	TBA degradation rate (d^{-1})
10	0.0187	0.0381
25	0.0435	0.0703
50	0.0752	0.0916
100	0.0968	0.1137

Table 2 Effect of TBA addition on biodegradation of MTBE by *Chryseobacterium* sp. A-3

Series number	Concentration of MTBE (mg/L)	Concentration of TBA (mg/L)	MTBE degradation rate (%)	TBA degradation rate (%)
1	50	–	56.4	–
2	50	5	45.2	42.4
3	50	25	39.9	51.2
4	50	50	31.5	54.0
5	50	100	27.3	57.1
6	–	50	–	73.6

2.7 Induction of MTBE and TBA degradation

To further evaluate the induction of MTBE and TBA degradation, and to investigate enzymes involved in MTBE or TBA metabolism, strain A-3 was grown on LB and was then incubated into BSM for one week with 100 mg/L MTBE or TBA. Cells were then harvested, and total cell proteins were separated by SDS-PAGE. The protein patterns of cellular extracts after growth on MTBE and on TBA were quite similar (Fig.4). Both of them produced at least seven polypeptides (approximately 85, 55, 40, 27, 24, 17, 11 kDa), which were not produced or expressed at much higher levels than that in LB-grown cells. Hatzinger *et al*. (2001) found that at least two polypeptides (approximately 60 kDa and 40 kDa) induced by TBA in the *Hydrogenophaga flava* ENV735, and Ferreira *et al*. (2006) reported that there were ten molecular masses of 67, 64, 63, 55, 50, 27, 24, 17, 14 and 11 kDa were induced in the protein profiles of crude extracts after growing in MTBE

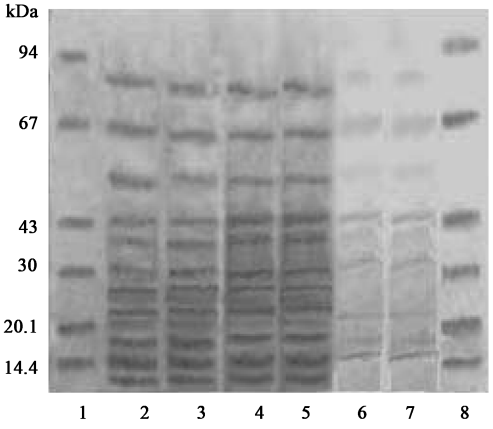

Fig. 4 SDS-PAGE gel of strain A-3 proteins after grown on different substrates. Proteins of crude extracts of cells grown on MTBE (lanes 2 and 3), TBA (lanes 4 and 5), LB (lanes 6 and 7) were analyzed. Lanes 1 and 8 were protein markers. Protein size in kilodaltons (kDa) is shown in the left of the figure.

in the strain of *Mycobacterium austroafricanum* IFP 2012. Thus, the protein involved in MTBE and TBA metabolism by *Chryseobacterium* sp. A-3 may be different from that in *Hydrogenophaga flava* ENV735 and *Mycobacterium austroafricanum* IFP 2012.

3 Conclusions

Chryseobacterium sp. A-3 could degrade both MTBE and TBA. A temperature range of 25–30°C, reach pH 7.0 and an inoculum size of 2×10^8 CFU/ml were the optimal conditions to reach the maximal degradation of MTBE in this batch experimental system. TBA was more easily degraded than MTBE by this new strain A-3. The additional TBA would lead to decrease in the initial MTBE degradation rate. On the basis of these results, it can be assumed that the same enzyme was possibly responsible for MTBE and TBA metabolism and it might be competing for the same active site. Furthermore, similar protein profiles at least seven peptides were demonstrated after SDS-PAGE analysis of crude extracts obtained from the cells growing in MTBE and TBA culture.

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