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Aerobic degradation of methyl *tert*-butyl ether by a *Proteobacteria* strain in a closed culture system

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Abstract

The contamination of methyl *tert*-butyl ether (MTBE) in underground waters has become a widely concerned problem all over the world. In this study, a novel closed culture system with oxygen supplied by H_2O_2 was introduced for MTBE aerobic biodegradation. After 7 d, almost all MTBE was degraded by a pure culture, a member of β-*Proteobacteria* named as PM1, in a closed system with oxygen supply, while only 40% MTBE was degraded in one without oxygen supply. Dissolved oxygen (DO) levels of the broth in closed systems respectively with and without H_2O_2 were about 5–6 and 4 mg/L. Higher DO may improve the activity of monooxygemase, which is the key enzyme of metabolic pathway from MTBE to *tert*-butyl alcohol and finally to CO_2, and may result in the increase of the degrading activity of PM1 cell. The purge and trap GC-MS result of the broth in closed systems showed that *tert*-butyl alcohol, isopronol and acetone were the main intermediate products.

Key words: methyl *tert*-butyl ether; biodegradation; β-*Proteobacteria*; intermediate products

Introduction

The fuel oxygenate methyl *tert*-butyl ether (MTBE) is one of the organic chemicals with the highest production volume worldwide. In 1999, about 21×10^6 t were produced globally (Krayer von Krauss and Harremoes, 2001). The occurrence and fate of MTBE in the environment continues to be a major scientific and political issue. Over only a few years of extensive use, MTBE has become one of the most frequently detected underground water pollutants in USA (Baehr *et al.*, 1999), and similar findings are emerging in Europe (Klinger *et al.*, 2002). The poor state of fuel storage system led to more than 400000 leaking underground storage tank sites in USA identified by USEPA since 1988 (Small *et al.*, 2002). In Europe, although in some countries stricter storage facility regulations were implemented as early as in the 1970s, there are still a growing number of reports about point-source releases of gasoline containing MTBE (Hansen *et al.*, 2002). Similar situations are emerging in China. MTBE was detected in more and more samples of underground water because of the rapid increase of petrol usage. For example, about 18 μg/L of MTBE was detected in well water samples near highway gasoline stations in East China, Zhejiang Province.

With the current practice of amending gasoline with up to 15% by volume MTBE, the contamination of groundwater by MTBE has become widespread. As a result, the bioremediation of MTBE-impacted aquifers has become an active area of research. As reviewed by Deed *et al.* (2000), a number of cultures isolated from diverse environments can either partially degrade or completely mineralize MTBE. MTBE is either utilized as sole carbon and energy source or degraded co-metabolically by cultures grown on alkanes. In a review by Schmidt *et al.* (2004), which was about the degradation pathway of MTBE and *tert*-butyl alcohol (TBA), monooxygenase was considered as the first enzyme, by which MTBE degradation was started and the ether bond was cleaved. TBA and formaldehyde was regarded as the dominant detectable intermediates. TBA could be further degraded to the following chemicals: 2-methyl-2-hydroxy-1-propanol, 2-hydroxyisobutyric acid, 2-propanol, acetone, or hydroxyacetone and eventually, to carbon dioxide.

Although some research found that MTBE biodegradation could occur in anaerobic conditions, in which the terminal electron acceptors could be SO_4, Fe(III), Mn(IV), and NO_3, respectively, the mineralization efficiency of MTBE to CO_2 in surface water-sediment microcosm was lower than that of aerobic biodegradation using oxygen as terminal electron acceptor (Bradley *et al.*, 2001; Finneran and Lveley, 2001). As for *in situ* MTBE biodegradation, native aerobic MTBE-degrading microorganisms can be stimulated and promoted by dissolved oxygen (DO), which was released into the anaerobic MTBE plume by diffusion through the walls of oxygen-pressurized polymeric tubing placed in contact with the flowing groundwater (Wilson *et al.*, 2002). Although microbial communities indigenous to groundwater can degrade MTBE under aerobic and

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anaerobic conditions, oxygen addition could improve the MTBE degradation activity and the growth of MTBE-degrading bacteria (Hristova *et al.*, 2003). Therefore, oxygen is necessary for MTBE degradation by bacteria, especially in closed system or subsurface.

Several bioreactors with good oxygen supply were introduced into the methodology study of MTBE removal from MTBE-contaminated influent under aerobic conditions, which resulted in significant removal efficiency (Fiorenza and Rifai, 2003). So far, more than six kinds of bioreactor have been evaluated for MTBE degradation, e.g. biotrickling filters (Fortin and Deshusses, 1999), biomass concentrator reactor (Zein *et al.*, 2004), laboratory-scale biofilter made of a natural fiber (kenaf) mat (Hu *et al.*, 2004), aerobic fluidized bed reactor (Pruden *et al.*, 2003), batch and continuous upflow fixed-biofilm reactors (Acuna-Askar *et al.*, 2000), continuously stirred tank reactor (Wilson *et al.*, 2001), upflow fixed-bed reactor (Kharoune *et al.*, 2001). In the above studies, the inoculum was mix cultures with oxygen supplied by air, in which led to high cell density in the bioreactors. However, few literature have been found discussing the effect of oxygen supply on MTBE degradation by pure bacteria in closed systems. In this study, the effect of oxygen supply by H_2O_2 on MTBE degradation by PM1, and intermediate metabolites produced in "cannula", a novel simple closed system that is to ensure both the oxygen supply and the avoidance of MTBE volatilization, were discussed.

1 Materials and methods

1.1 Materials

MTBE was HPLC grade product from Merck Schuchardt OHG (Hohenbrrunn, Germany). Hexane, cyclohexane, TBA, isopropanol and acetone were from local chemicals company. H_2O_2 (30% purity) was from Yuanda Peroxide Ltd. (Shanghai, China). Glucose, peptone, yeast extract, and agar were from Jonya Marine Biological Engineering Co. Ltd. (Shanghai, China).

1.2 Microorganism and incubation conditions

PM1 is a member of β-*Proteobacteria*, originally isolated from a biofilter in southern California, USA (Hanson *et al.*, 1999). The strain was cultivated on tryptic soy agar (TSA) slant for 48 h at 28°C to activate the cells. The composition of TSA is as follows (g/L): tryptone 17, yeast extract 6, sodium chloride 5, dipotassium hydrogen phosphate 2.5, glucose 2.5, algar 20, with the pH of 7.2 after sterilization at 121°C. One loop of slant was inoculated into 30 ml of TSA liquid medium without agar in 250 ml flask. The culture was incubated at 30°C overnight on a rotatory shaker at 160 r/min. PM1 cells were harvested from the broth after centrifugation at 4000 r/min for 20 min. Then the cells were washed for 3 times with sterile saline to prepare cell suspension. The absorption at 600 nm (A_{600}) measured by spectrophotometer was employed to detect the cell density in suspension according to the relationship between A_{600} and the cell density measured by TSA plate numbering. Cell density of the broth could also be detected by similar methods.

The basic salty medium (BSM) for MTBE degradation by PM1 was designed as follows (g/L): Na_2HPO_4 5.57, KH_2PO_4 2.44, NH_4Cl 2.0, $MgCl_2 \cdot 6H_2O$ 0.2, $MnCl_2 \cdot 4H_2O$ 0.0004, $FeCl_3 \cdot 6H_2O$ 0.001, $CaCl_2$ 0.001, and with the pH of 7.0.

1.3 Closed system deigned for MTBE degradation by PM1

The "cannula", i.e., the closed system (Fig.1), was composed of one small test tube (13 mm×150 mm) with 5 ml BSM containing MTBE and one big test tube (18 mm×200 mm) closed by rubber plug. In between the two test tubes was 30% H_2O_2 solution.

1.4 MTBE degradation by PM1 in closed system

One milliliter of PM1 cell suspension was inoculated into 5 ml BSM medium containing MTBE in the smaller tube of the closed system. The culture was then incubated at 30°C on a rotatory shaker at 160 r/min. To avoid the effect of possible MTBE leakage after sampling, three "cannula" were designed for each sampling point, and each "cannula" was removed from the incubator to stop incubation after sampling.

1.5 Assay of MTBE and DO

The change of MTBE concentration in BSM was detected by DAI-GC/FID (GC-14B, Shimadazu, Japan). The conditions of GC were as follows: pure nitrogen as carry gas with the flow rate of 1.0 ml/min, FID detector, PEG-20M capillary column (30 m×0.32 mm×0.33 μm), polyehtylene as immobilized phase, column temperature 55°C, FID detector temperature 150°C and gasifying chamber temperature 140°C.

DO in the closed system was measured by DO analysis apparatus with polarographic membrane dissolved oxygen electrode (JPB-607, Shanghai Fine Instruments Ltd., China) at the culture time of 0 h, 48 h, 120 h, and 168 h, respectively. The electrode was immediately inserted into the inner tube of "cannula" after the rubber plug was taken off so as to avoid DO change in the broth caused by the entrance of oxygen. Every sample was prepared in duplicate for each sampling time point. So eight "cannula" were designed under each condition. After sampling, each "cannula" would not be put back for culture.

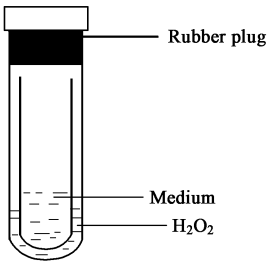

Fig. 1 Diagram of closed system for MTBE degradation by PM1.

1.6 Analysis of intermediate products

The analysis of intermediate products was carried out by the method described by Schmidt (2003) utilizing purge and trap (P&T) GC-MS (HP GC6890/MS 5973, HP-INNOWax column, USA). Instrumental conditions were as follows: the injector temperature was 200°C with splitless injection mode; pure helium as carry gas with flow rate of 1.0 ml/min, oven temperature increased from 45°C to 90°C at the rate of 5°C/min and kept at 90°C for 3 min.

2 Results and discussion

2.1 DO analysis in closed system

DO in the closed system for MTBE degradation was measured to identify the effect of H_2O_2 on oxygen supply. As shown in Table 1, DO in the broth respectively with PM1 and without H_2O_2 in between decreased gradually from 7.8 to 3.7 after 168 h; while DO in the broth with PM1 and H_2O_2 in between kept high levels (4.9, 5.9 and 5.4 respectively at 48 h, 120 h and 168 h). So H_2O_2 showed favorable effect on oxygen supply in the closed system.

2.2 MTBE degradation by PM1 in closed system

The effect of oxygen supply on MTBE degradation by PM1 in closed system is shown in Fig.2. It has been found that MTBE degradation was more rapid and complete when oxygen was continuously supplied throught H_2O_2 in the closed system, and after 168 h, no MTBE could be detected. Meanwhile, only 40% MTBE was degraded after 168 h in the closed system without oxygen supply. These results coincide with the DO situation in Table 1, it meant that H_2O_2 in between could keep DO level in the medium without PM1 as high as that of pure water, and could also improve DO level of the broth with PM1, increasing from 3.7–4.2 mg/L to 4.9–5.9 mg/L. It suggests that the DO level of 3.7–4.2 mg/L might be the critical level for the basic metabolism of cells, and that once DO is above the critical level, the aerobic MTBE degradation might be improved significantly. As pointed out by previous researchers (Fiorenza and Rifai, 2003; Smith and O'Reilly, 2003; Johnson *et al.*, 2004), MTBE degradation was firstly catalyzed by monooxygenase, which was one of the key enzymes in the process of MTBE degradation. So oxygen supply and high DO level play an important role in MTBE degradation. The oxygen supply also could accelerate the cell growth in the closed system. After 120 h, it was detected that the cell density increased about five times in the closed system with continuous oxygen supply by H_2O_2, while no significant change of cell density was detected without continuous oxygen supply. However, MTBE could be degraded by PM1 with very low rate after 120 h without H_2O_2, as shown in Fig.2. It suggested that low DO (but equal to or above the critical level) might result in only the low activity of MTBE monooxygenase, and MTBE degradation took place slowly. Actually, complete degradation also could be achieved after 10–15 d.

PM1 cell concentration of the inoculum also had effect on MTBE degradation. As shown in Fig.3, high cell concentration resulted in high degradation efficiency. The higher cell concentration is, the smaller time required for completely degrading MTBE. MTBE degradation usually results from the induction and improvement of inoculum cell (i.e., resting cells) activity by MTBE. In addition, the acceleration of degradation might result from the gradual increase of cell density because of adequate metabolite and energy that were supplied by MTBE degradation. Therefore, high inoculum cell density could result in higher

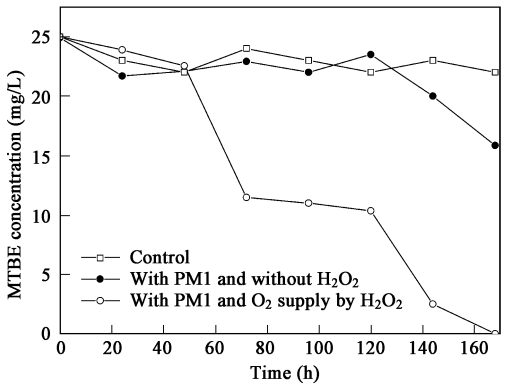

Fig. 2 Methyl *tert*-butyl ether (MTBE) degradation by PM1 in the closed system seeded with 1 ml of inoculum (cell concentration: 10^7 cfu/ml) into 5 ml medium.

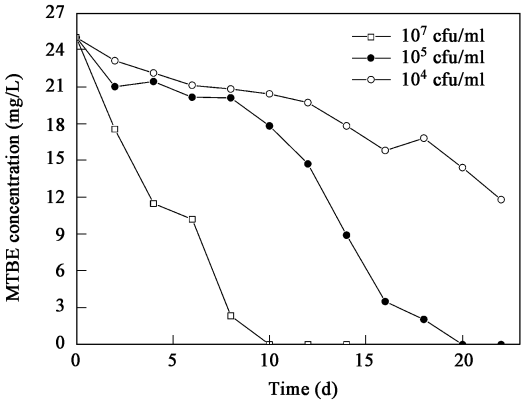

Fig. 3 Effect of cell concentration of inoculum on MTBE degradation by PM1 in closed system with 1 ml of inoculum added into 5 ml medium.

Table 1 Comparison of DO change in closed system with/without PM1 and oxygen supply

Samples/conditions	DO change (mg/L)			
	0 h (25°C)	48 h (25°C)	120 h (27.7°C)	168 h (27.7°C)
Broth with PM1 and H_2O_2 in between	7.8	4.9	5.9	5.4
Broth with PM1 and without H_2O_2 in between	7.8	4.2	3.8	3.7
Medium without PM1 and with H_2O_2 in between	7.8	7.6	7.9	7.8
Pure water at the same temperature	7.85	7.85	7.9	7.9

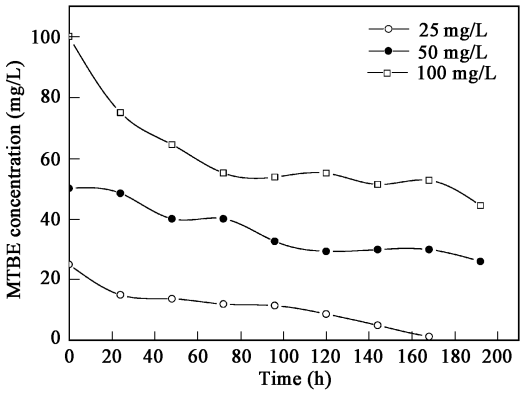

Fig. 4 Effect of initial concentration on MTBE degradation in closed system with 1 ml of inoculum (1.5×10^7 cfu/ml) added into 5 ml medium.

MTBE degradation rate than low inoculum cell density.

The initial MTBE concentration also showed significant effect on MTBE degradation as shown in Fig.4. Low initial concentration resulted in high degradation rate, i.e., all MTBE could be completely degraded after 192 h with initial MTBE concentration of 25 mg/L. Meanwhile, only 48% and 56% MTBE was degraded after 192 h with initial MTBE concentrations of 50 and 100 mg/L, respectively. MTBE is not good carbon source and might be harmful to cell with high concentration. So the activity of MTBE degradation by PM1 might be partly inhibited, and MTBE degradation was not complete at high concentrations in the same reaction time, even when high DO was kept by H_2O_2 in the closed system.

2.3 Effect of co-metabolic substances on MTBE degradation by PM1 in closed system

As reported by Pruden *et al.* (2001) and Deeb *et al.* (2001), the degradation effects of MTBE under various substrate conditions were different. The addition of diethyl ether, diisopropyl ether, ethanol and BTEX was found to have no effect on MTBE biodegradation in well-mixed reactors containing polyethylene porous pot for biomass retention. The presence of ethylbenzene or xylenes in the mixtures with MTBE completely inhibited MTBE degradation. However, Hyman *et al.* (2000) and Liu *et al.* (2001) reported that the presence of alkanes or isoalkanes in polluted aquifers could improve MTBE degradation. In this study, considering that the boiling points of methane, ethane and pentane are so low that these substances easily evaporate and that the experiment results will be hard to analyze, only hexane and cyclohexane were selected as co-metabolic substances to evaluate the effect of co-metabolism on MTBE degradation.

Hexane and cyclohexane were added into BSM to reach the final concentration of 20 mg/L, while that of MTBE was 15 mg/L. The incubation conditions were similar to those of 3.3 in closed system. Fig.5 shows that the addition of hexane or cyclohexane inhibited MTBE degradation by PM1 in the closed system. It suggested that hexane and cyclohexane could be more easily utilized by PM1 than MTBE, and that no MTBE degradation was required for the metabolism and growth of PM1.

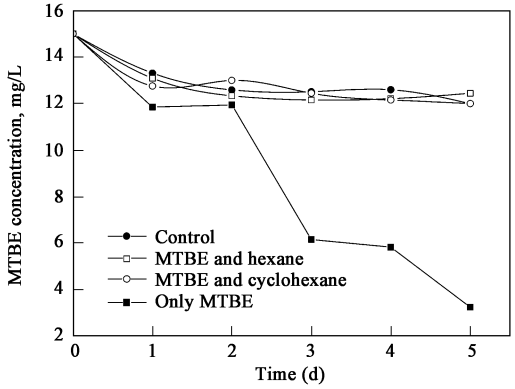

Fig. 5 Effect of co-metabolic substances on MTBE degradation by PM1 in closed system.

2.4 Analysis of intermediate products

The P&T GC/MS retention time of MTBE and its possible metabolites such as TBA, acetone and isopropanol were detected before the broth analysis by GC/MS. By comparing the retention time, possible metabolites in the broth of MTBE degradation could be revealed. As shown in Fig.6, P&T GC-MS results of the broth in closed system suggested that TBA, isopropanol and acetone were the key intermediate products. According to their molecular structure, the possible relation of these metabolites could be speculated as follows: MTBE was degraded directly to TBA, and then TBA was degraded to isopropanol through some unknown or undetectable intermediate products. Isopropanol was further transferred to acetone directly or through other unknown or undetectable intermediate products. Finally, acetone could be transferred to pyruvate, which would be easy to enter TCA cycle to supply required substances and energy for cell growth and basic metabolism. This possible metabolic pathway was similar to what of Steffan and Meclay (1997) speculated.

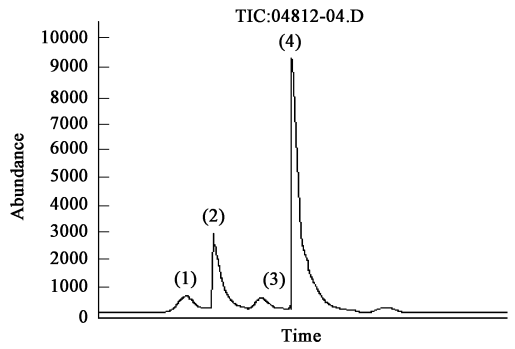

Fig. 6 P&T GC/MS spectra of the broth of MTBE degradation by PM1 in closed system. (1) isopropanol; (2) acetone; (3) TBA; (4) MTBE.

3 Conclusions

The closed system with oxygen supplied by H_2O_2 was favorable for MTBE degradation in aqueous phase. Higher DO could be achieved during the whole degradation period than that achieved in the closed system without oxygen

supplied by H_2O_2. After 168 h, almost all MTBE with original concentration of 25 mg/L could be degraded by *Proteobacteria* sp. PM1 in the closed system with oxygen supply, while only 40% MTBE was degraded in the one without oxygen supply. In the closed system with oxygen supplied by H_2O_2, the main intermediate metabolites of MTBE degradation by PM1 were TBA, isopropanol and acetone.

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