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Cometabolic biodegradation of methyl *tert*-butyl ether by a soil consortium: Effect of components present in gasoline

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A soil consortium was tested for its ability to degrade reformulated gasoline, containing methyl *tert*-butyl ether (MTBE). Reformulated gasoline was rapidly degraded to completion. However, MTBE tested alone was not degraded. A screening was carried out to identify compounds in gasoline that participate in cometabolism with MTBE. Aromatic compounds (benzene, toluene, xylenes) and compounds structurally similar to MTBE (*tert*-butanol, 2,2-dimethylbutane, 2,2,4-trimethylpentane) were unable to cometabolize MTBE. Cyclohexane was resistant to degradation. However, all *n*-alkanes tested for cometabolic activity (pentane, hexane, heptane) did enable the biodegradation of MTBE. Among the alkanes tested, pentane was the most efficient (200 μg/day). Upon the depletion of pentane, the consortium stopped degrading MTBE. When the consortium was spiked with pentane, MTBE degradation continued. When the ratio of MTBE to pentane was increased, the amount of MTBE degraded by the consortium was higher. Finally, diethylether was tested for cometabolic degradation with MTBE. Both compounds were degraded, but the process differed from that observed with pentane.

Key Words——biodegradation; cometabolism; gasoline; methyl *tert*-butyl ether

Introduction

Fuel oxygenates are organic additives designed to increase the oxygen and octane contents of gasoline. The most common are methyl *tert*-butyl ether (MTBE) and ethanol. Others include ethyl *tert*-butyl ether (ETBE), *tert*-amyl methyl ether (TAME), isopropyl ether, and *tert*-butanol (TBA). The massive production of MTBE, 17 billion pounds were generated in the United States in 1995 (Anon, 1996), combined with its mobility, persistence, and toxicity, makes it the second most common groundwater pollutant in the U.S. (Squillace et al., 1996). MTBE has also attracted attention because of its presence in urban air (Grosjean et al., 1997) coming mainly from gas stations. Furthermore, MTBE has been reported to be a carcinogen in animals (Belpogi, 1995).

Much research has been undertaken on the biodegradability of MTBE and other fuel oxygenates under aerobic (Cowan and Park, 1996; Fayolle et al., 1998; Mo et al., 1996; Salanitro et al., 1994) and anaerobic (Mormile et al., 1994; Suflita and Mormile, 1993) conditions. MTBE is chemically stable, and its tertiary carbon structure and ether linkage are two characteristics that theoretically hinders biological attack. It has been reported (Fortin and Deshusses, 1999; Salanitro et al., 1994) that a mixed bacterial cul-

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ture is able to mineralize MTBE. The degradation of MTBE was shown to proceed through the formation of TBA, which was then also degraded by the culture. Compounds similar in structure to MTBE and present as additives in gasoline have also been studied. Mo et al. (1996) reported the biodegradation of MTBE, ETBE, and TAME by mixed cultures and by pure isolates. Studies on the enrichment of mixed cultures degrading MTBE, ETBE, TAME, TBA, and *tert*-amyl alcohol (TAA) as the sole carbon and energy sources have also been reported (Cowan and Park, 1996). Kinetic parameters and stoichiometric characteristics of the degradative ability were measured by Cowan and Park (1996). The authors also reported the effects of oxygen and temperature on the biodegradation of MTBE (Park and Cowan, 1997). Cometabolic biodegradability of MTBE has recently been demonstrated. Pure microorganisms are able to degrade MTBE in the presence of propane (Steffan et al., 1997), butane and ether (Hardison et al., 1997), and pentane (Garnier et al., 1999).

About 70% of the compounds present in reformulated gasoline are aromatic (such as benzene, toluene, and xylenes), alkanes (such as pentane, hexane, and heptane), and MTBE. Among the former compounds are those with a tertiary carbon structure (such as MTBE; 2,2,4-trimethyl pentane (224 TMP); 2,2-dimethyl butane (22 DMB); etc. ...). They represent a large fraction (20 to 40%, [v/v]) of those found in reformulated gasoline. MTBE may maintain 30% (v/v) of the compounds present in gasoline (Stelljes, 1997). It is of great interest to determine how MTBE would be degraded in the presence of a mixture of compounds such as those present in gasoline because the latter often represents a leading source of environmental contamination.

In this study, an evaluation was made of the ability of a soil consortium to achieve a complete degradation of gasoline. The degradation of MTBE was observed only by cometabolism. Therefore the identification of some of the compounds present in gasoline that participate in the cometabolic biodegradation of MTBE was investigated. The cometabolic activity of MTBE with pentane, the most efficient compound tested, was further characterized.

Materials and Methods

Microcosm construction. Different soil samples were obtained and mixed from different contaminated gasoline service stations in Mexico and further enriched in microcosms. The setup was adapted from Mu and Scow (1994). Microcosms were constructed by using 125-ml serum bottles that contained gasoline-contaminated soil (2 g), 20 ml of a mineral salt solution (Whittenbury et al., 1970) with 1 g/L KNO_3 as nitrogen source, and 5 µl gasoline as a sole source of carbon and energy. The bottles were stoppered with Teflon Mininert valves. Microcosms were then incubated at 30°C on a rotatory shaker at 250 rpm. Air and carbon sources were renewed when the CO_2 concentration was more than 3%. The culture in the microcosm was enriched by 10 successive dilutions (1 ml of solution from the consortium able to degrade reformulated gasoline was added into a new bottle). This enriched consortium was used in all further studies.

Microcosm preparation for cometabolism studies. A mineral salt solution (20 ml) was added to each serum bottle with Teflon-lined rubber septa and autoclaved. A 2-ml inoculum (0.25 mg of dry biomass per bottle) was then added. Bottles were stoppered and each growth substrate to be tested was added (5 µl) along with MTBE (2 µl) with a gas-tight syringe and incubated as above. The degradation of substrates, MTBE, O_2, and the production of CO_2 were monitored daily by headspace gas chromatography. Total MTBE concentration in the bottle was obtained from the headspace and the partition coefficient.

Chemicals. The following compounds were from Aldrich Chemical Co. (Milwaukee, WI, USA): benzene (99%, d=0.874); cyclohexane (99.5%, d=0.893); diethyl ether (DEE) (99.8%, d=0.709); 22 DMB (99%, d=0.649); heptane (99%, d=0.684); hexane (95%, d=0.659); isopropanol (99.5%, d=0.78); MTBE (98%, d=0.740); pentane (99%, d=0.626); TBA (99%, d=0.77); toluene (99.8%, d=0.865); 224 TMP (99.8%, d=0.626); xylenes (98.5%, d=0.953). The lead-free reformulated gasoline (Magna Sin) was from PEMEX, Mexico.

Analyses. Gasoline concentration (from the headspace of each microcosm) was measured by taking a 200-µl gas sample and injecting it in a Gas Chromatograph (Hewlett-Packard (HP), model 5890 series II) connected to a flame ionization detector (FID). An HPI column (methyl silicon gum) 5 m×0.53 mm was used. The GC was operated as follows: For analysis of compounds present in total gasoline, injector temperature was set at 210°C, the detector at 210°C, and the col-

umn at 180°C. The retention time was from 1.5 to 5 min; For analysis of MTBE and other compounds listed above, the injector temperature was set at 150°C, the detector at 210°C, and the column at 60 or 180°C. Nitrogen was used as a carrier gas with a flow rate of 1.7 ml/min.

Oxygen and CO_2 concentrations were measured by using a gas chromatograph GOW MAC (series 550 thermal conductivity detector with a concentric column CTR-1, Alltech, USA) with helium as carrier gas at a flow rate of 65 ml/min. The operating temperatures were injector, 30°C; detector, 70°C; and column, 30°C.

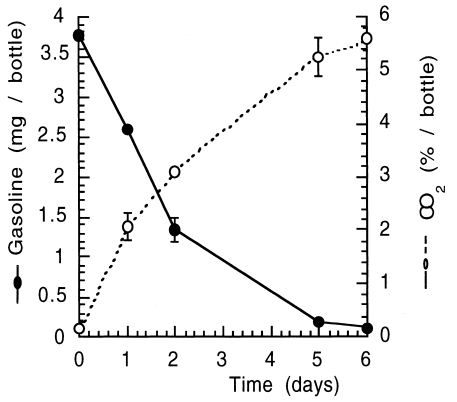

Fig. 1. Degradation of reformulated gasoline by the consortium.

Results

A consortium enriched from contaminated soil was able to mineralize reformulated gasoline, which included MTBE, when used as sole source of carbon and energy, producing CO_2 (Fig. 1). After 6 days of incubation, 97% of total reformulated gasoline was degraded. When MTBE was added alone to this consortium, no significant degradation occurred within one week.

The compound(s) responsible for this observed cometabolic activity was (were) unknown. To identify some of the potential compounds present in gasoline that participate in the cometabolism with MTBE, a screening was carried out as shown in Fig. 2. Complete substrate utilization was observed for all aromatic substrates tested such as benzene, toluene, and xylenes. These compounds are in reformulated gasoline at concentrations (v/v) of 0.8, 5.2, and 4.5%, respectively. Cyclohexane was, however, resistant to degradation. Xylenes were removed very rapidly (about 80% in 24 h), followed by toluene (100% in 2 days) and benzene (100% in 3 days) (Fig. 2). In all four cases, MTBE remained constant after 5 days of incubation.

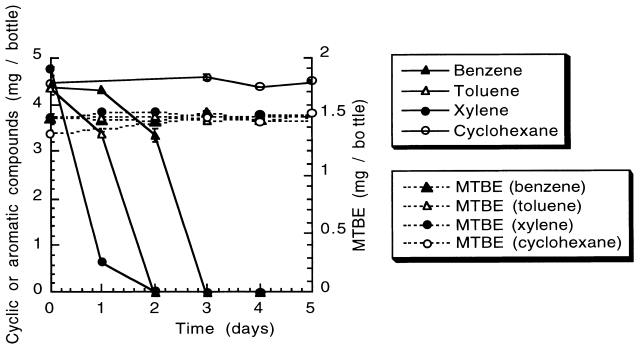

Fig. 2. Cometabolic degradation of MTBE with specific cyclic and aromatic compounds present in gasoline.

For the three *n*-alkane substrates tested (initial concentration of 5 µl per bottle), pentane, hexane, and heptane, complete substrate utilization was observed (Table 1). These compounds are in gasoline at concentrations (v/v) of 4.7, 2.7, and 1.1%, respectively. The compound enabling the most efficient degradation of MTBE through cometabolic activity was pentane (Table 1). In the presence of hexane, the cometabolic degradation of MTBE was much lower than with pentane, as shown in Table 1. The degradation rate was 42 µg/day, about 5 times less than with pentane. With heptane, the degradation rate was 87 µg/day, 2 to 3 times less than with pentane, as shown in Table 1. A more in-depth study was conducted to test the cometabolic relationship between pentane and MTBE, as shown in Fig. 3. A reduction in the concentration of MTBE (initial concentration of 1.48 mg per bottle) was observed along with the degradation of pentane during the first 4 days of incubation (Fig. 3), in bottles 1 and 2. As soon as all the pentane was consumed, MTBE degradation was halted and the concentration remained constant thereafter. Bottle 1 was respiked with pentane demonstrating cometabolic degradation of MTBE in this microcosm. Once again, a reduction of MTBE was observed as pentane in bottle 1 was respiked (Fig. 3). The MTBE degradation rate was 200 µg/day, and the cometabolism rate (MTBE consumed/pentane consumed) was 10% (Table 1). It was also observed that when pentane was respiked (Fig. 3), MTBE concentration increased slightly in the gas phase, which was probably due to variations of the MTBE solubility in water with pentane. The effect on

Table 1. Microcosm screening studies in presence of MTBE (2 µl): Effect of different cometabolic substrates (5 µl).

Growth substrates	Initial concentration and percentage of degradation after 4 days	Degradation of MTBE (mg/bottle)	Final percentage of CO_2 (%/bottle)	ΔMTBE/Δt (µg/day)	ΔMTBE/Δsubstrate (w/w)
Aromatic and cyclic substrates					
Benzene	4.37 mg (100%)	0	6.55±0.44	0	
Toluene	4.32 mg (100%)	0	5.91±0.13	0	
Xylenes	4.76 mg (100%)	0	5.76±0.02	0	
Cyclohexane	4.46 mg (0%)	0	0.90±0.1	0	
Alkane substrates					
Pentane	3.13 mg (100%)	0.225±0.005	3.75±0.05	200	0.10
Hexane	3.30 mg (100%)	0.054±0.003	3.44±0.09	42	0.01
Heptane	3.42 mg (100%)	0.104±0.019	3.35±0.35	87	0.02
Structurally similar substrates					
TBA	ND	0	0.40±0.02	0	
22 DMB	3.24 mg (0%)	0	0.92±0.01	0	
224 TMP	3.13 mg (0%)	0	0.33±0.02	0	
Other					
Isopropanol	3.9 mg (100%)	0	3.54±0.06	0	
DEE	3.0 mg (85%)[a]	1.230±0.23	5.26±0.16	83	0.45

[a] Degradation after 17 days.

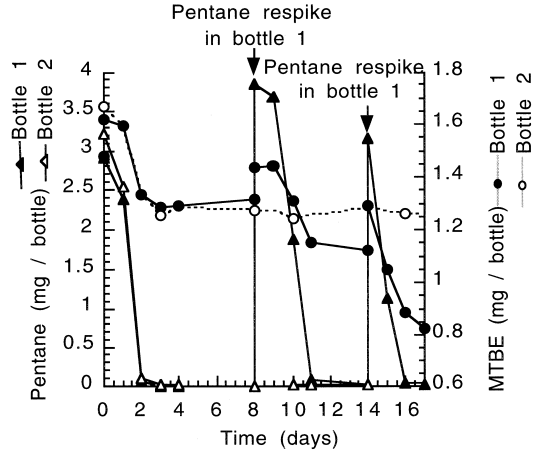

Fig. 3. Cometabolic degradation of MTBE in presence of pentane.

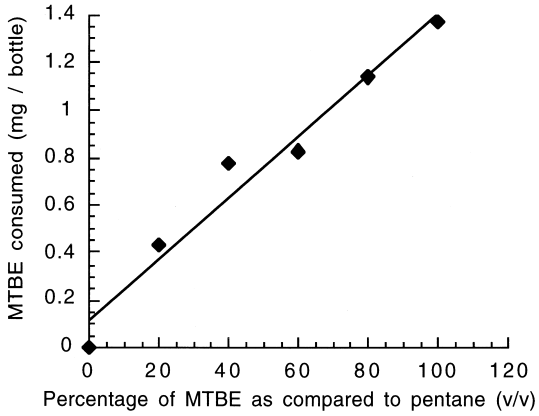

Fig. 4. Effect of varying ratios of MTBE to pentane on cometabolism.

cometabolism of varying proportions of MTBE to pentane was studied. In doing so, we added a fixed amount of pentane (10 µl) to each bottle (0.25 mg of initial biomass), and the amount of MTBE was varied from 2 to 10 µl, representing 20 to 100% of MTBE in relation to pentane (v/v). The results are shown in Fig. 4. When the ratio of MTBE to pentane was increased, the amount of MTBE degraded was higher. Each data point is from a 3-day culture; the degradation of pentane was complete at that point.

When substrates structurally similar to MTBE were tested for cometabolic activity with MTBE, no utilization was observed for 224 TMP, 22 DMB, and TBA, as shown in Table 1. MTBE was not degraded, indicating the absence of cometabolism with the tested compounds. The production of CO_2 was lowest when compared with the other compounds tested (Table 1). It is to be noted that CO_2 levels were high only when substrates were utilized (Table 1).

Compounds with a tertiary carbon structure were screened for cometabolism in the presence of pen-

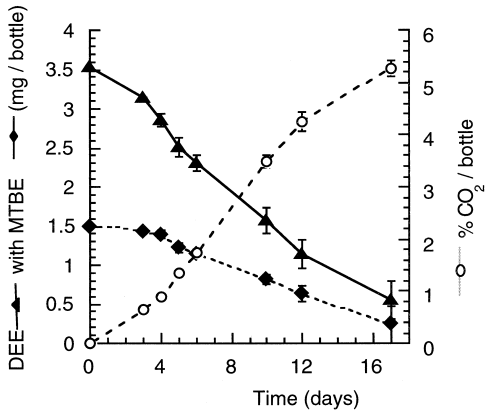

Fig. 5. Cometabolic degradation of MTBE with DEE as substrate.

tane. Both 22 DMB and 224 TMP were cometabolized by the consortium in the presence of pentane with rates of 0.112±0.027 mg 22 DMB/bottle and 0.050± 0.013 mg 224 TMP/bottle, respectively. These values were lower than that found for MTBE (Table 1).

Finally, DEE was tested for cometabolism with MTBE. DEE is an industrial solvent not present in gasoline. Over 17 days, 85% of the DEE was degraded (around 175 μg/day), which was much slower than the *n*-alkanes that were fully degraded in 3 days. MTBE was cometabolized (Fig. 5) with an average degradation rate of 83 μg/day (Table 1).

Discussion

MTBE alone was not degraded when used as a sole source of carbon and energy. However, the enriched soil consortium used in our studies was able to degrade gasoline to completion (Fig. 1). MTBE was therefore degraded through a cometabolic process. The soil consortium was able, in the presence of MTBE, to grow on aromatic compounds such as benzene, toluene, and xylenes as the sole carbon and energy sources. However, the consortium was unable to degrade MTBE in the presence of any of these tested compounds (Fig. 2). A cyclic compound (cyclohexane) was also tested. The consortium was unable to grow on this compound alone, and no cometabolic activity with MTBE was observed (Fig. 2). Further studies with this consortium (Garnier et al., 1999) showed that the cometabolic activity could be attributed to a *Pseudomonas aeruginosa* strain.

The results of the present study demonstrate that cometabolism does, however, exist between MTBE and *n*-alkanes, the best being with pentane (Table 1). Cometabolic studies were undertaken by Steffan et al. (1997), who also observed that the degradation of MTBE was achieved in the presence of propane. Hardison and co-workers (1997), on the other hand, demonstrated cometabolic activity with MTBE with a different alkane, namely butane.

The cometabolic interaction between pentane and MTBE was further analyzed (Fig. 3). MTBE was degraded as long as pentane (Fig. 3) was present and consumed (rapid degradation rate of 200 μg/day and a cometabolism rate of 0.1; Table 1). When pentane was completely consumed, MTBE degradation stopped (Fig. 3). When the proportion of MTBE to pentane was increased, a stronger biodegradation rate (Fig. 4) was obtained. These experiments suggest that MTBE was degraded by an alkane-induced enzyme probably produced in the early steps of the pentane degradation pathway. MTBE alone was degraded only when the consortium was spiked with pentane. Hardison et al. (1997) reported a fungal cometabolism between MTBE and butane and suggested the existence of one or several very similar enzymes that are responsible for MTBE degradation in cometabolism.

All the compounds tested that have a carbon with three methyl group structures such as 22 DMB, 224 TMP, TBA, and MTBE were unable to be used by the consortium as sole carbon and energy sources. It is to be noted that in the presence of isopropanol (a two-methyl group structure) the consortium was able to grow (Table 1). Both TBA and isopropanol have a hydroxy group in common. Therefore the structure with a carbon with three methyl groups seemed to be limiting biodegradation.

In the presence of pentane, MTBE, 22 DMB, and 224 TMP could be degraded by cometabolism with varying rates. Previous studies showed that the first attack of MTBE resulted in the accumulation of TBA (Salanitro et al., 1994) indicating that the trimethyl group was not attacked (TBA was not detected in the headspace analysis, probably because of its low concentration and high solubility). It was observed that as the molecular weight increased (MTBE≧22 DMB≧224 TMP), the rate of cometabolic degradation decreased significantly.

The consortium was able to degrade DEE (an ether not present in gasoline) but very slowly, requiring 17 days (Fig. 5). When cometabolic activities MTBE/DEE and MTBE/pentane were compared, a better degrada-

tion rate (ΔMTBE/Δt) with pentane (Table 1) was observed. Nevertheless, the cometabolic rate (ΔMTBE/Δsubstrate) is better with DEE (Table 1). This could be due to differences in cometabolic affinities in the two systems. MTBE and DEE both have an ether bond in common. Cometabolic activity could be due to an induction of an alternative enzymatic system, as observed with the slower uptake rates than pentane. The importance of this ether linkage in the cometabolic process has not been developed and could be an area of further research.

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