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Biodegradation of Methyl *tert*-butyl Ether by Microbial Consortium*

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Abstract: In this study, the feasibility of applying the microbial consortium was assessed for natural biodegradation evaluation by isolated microorganisms of activated sludge, wastewater and sediment in the aqueous solutions. The consortia were isolated from a variety of sources, generally from petroleum, chemical and urban wastewater treatment plants. The capacity of the consortium to degrade and grow on Methyl *tert*-butyl Ether (MTBE) as a sole carbon and energy source was investigated. Enrichment was conducted in batch reactor, fitted with a screw cap and rubber septum. MTBE concentration was measured in headspace by gas chromatography. Degradation was determined by MTBE removal. The aerobic microbial consortium able to MTBE removal was adapted in laboratory for 4 months. The consortium was capable of degrading concentration as great as 1000 mg L^{-1} MTBE.

Key words: MTBE, biodegradation, microorganisms, activated sludges, consortium

Introduction

Organic chemical mixtures are present in wastewaters from industrial and municipal sources as well as in contaminated groundwater. Common examples of chemical mixtures that often become pollutants include gasoline and other petroleum fuels, pesticides and wood-treating substances (Singleton, 1994). The bioremediation of these substances has become an alternative to the traditional physical and chemical methods that can be costly and produce hazardous products (Pruden *et al*., 2001). Combining methanol and isobutylene makes Methyl *tert*-butyl Ether (MTBE). The MTBE is very water soluble with a low sorption partition coefficient and thus is highly mobile in both groundwater and surface water. The pollutant is also moderately volatile, which can lead to redistribution and further contamination of the zone, surface soils and sediments. The fuel additive MTBE has become a widespread environmental contaminant within the past 30 years (Burbano *et al*., 2002). MTBE was the second most common volatile organic compound detected in wells monitored in urban areas nationwide between 1985 and 1995 (Bradley *et al*., 2001). MTBE is considered a potential human carcinogen (Cirvello *et al*., 1995). Although it is know to be less toxic than many other gasoline constituents, concerns have been raised about the potential for acute effects from inhalation and the long-term effects from drinking water contamination. MTBE has been shown to biodegrade under various conditions including aerobic, anaerobic and cometabolic conditions, however it is not well understood under which geochemical conditions degradation occurs (Hanson *et al*., 1999; Kazumi *et al*., 1997; Squillace *et al*., 1996). Several researchers described successful mineralization of MTBE in laboratory-scale research. Researchers are currently investigating the potential for cometabolism of MTBE at the field-scale (Garnier *et al*., 1999).

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Microorganisms were isolated from a variety of sources, generally from petroleum or chemical plant wastewater bioreactors. Microbial cell yields tend to be lower on MTBE than those observed for aromatic hydrocarbons (0.1-0.2 g cells g^{-1} MTBE). Biodegradation rates tend to be slower than those observed for the aromatic hydrocarbons (Reardon *et al*., 2000). Aerobic biodegradation of MTBE is demonstrable. Additional research is necessary to clarify the microorganisms involved in the process, factors that impact cell yield and biodegradation rates and the degradation pathway (Solano-Serena *et al*., 2000). Information on the pathway of anaerobic MTBE has not yet been investigated. Investigation of the anaerobic biodegradation of MTBE is still in the early stages and more research is necessary to fully understand this process.

Materials and Methods

Sampling

Sediment, sludge and wastewater were obtained from Tehran and Isfahan refineries contaminated aquifer. The sediments were generally sandy in nature with some clay aggregates and smelled of hydrocarbons. Other samples were obtained from a wastewater treatment plant located in Tehran, Shosh wastewater treatment plant. Lastly, a sample was collected from the garden of Tarbiat Modarres University. Sediment incubations were prepared by adding 50 g of sediment and 75 mL of water in sterile 160 mL serum bottles as previously described (Landmeyer *et al*., 2001). All chemicals were of the highest purity available (Sigma).

Culture Condition

The microorganisms used in this study were enriched from activated sludge, sediments and wastewater. Initially, 10 mL of the samples were diluted to 100 mL with mineral salts medium that had been sterilized (autoclaved at 121°C for 15 min) and spiked with different concentrations of MTBE. The mineral medium consisting of the following components (g L^{-1}): $MgSO_4 \cdot 7H_2O$, 0.25; KNO_3, 0.5; $CaCl_2 \cdot 2H_2O$, 0.009; KH_2PO_4, 0.5; K_2HPO_4, 0.5; NaCl, 1.0 and 1.0 mL L^{-1} of trace elements solution was periodically refreshed. The trace elements solution contained (g L^{-1}): $FeCl_2 \cdot 4H_2O$, 1.5; $CuCl_2 \cdot 2H_2O$, 0.015; $NiCl_2 \cdot 6H_2O$, 0.025; $MnCl_2 \cdot 4H_2O$, 0.1; $CoCl_2 \cdot 6H_2O$, 0.12; $ZnCl_2$, 0.07; $NaMoO_4 \cdot 2H_2O$, 0 .025; H_3BO_3, 0.06; EDTA $\cdot 4H_2O$, 5.2; the final pH was 4.2 (Hristova *et al*., 2003). The pH was adjusted with NaOH to 7.0 and checked with litmus paper or pH meter. The culture was shaken continuously at 150 rpm in an incubator at 20°C under aerobic conditions. MTBE were added every 3 days during a 3 week enrichment period. A subculture of the enrichment was used to measure the bacterial concentration and the absorbance once a day for seven successive days for the growth curve. The bacterial concentration (count) was determined by the pour-plate technique. The culture was maintained continuously and subsequently served as a source of inoculum for samples. A spectrophotometer (Phillips) was used to measure the absorbance for 1 mL bacterial suspensions. The bacteria count after a serial dilution was measured by the pour-plate method in a nutrient agar plate incubated at 25°C. By multiplying the bacteria count by the inverse of the diluted fraction, the bacterial concentration (i.e., bacteria count per milliliter) was obtained. Finally, growth curves were obtained based on bacteria count per milliliter and absorbance with relation to time.

Analytical Techniques

MTBE concentrations and TBA (*tert* butyl alcohol) were determined using a Philips PU-4410 gas chromatograph equipped with a flame ionization detector as possible compounds from MTBE

biodegradation in the headspace of the vials. The compounds were separated on a %10 SE30 packed column (1.5 m, 4 m ID). Column temperature was adjusted isothermally at 50°C, injector at 180°C and detector at 200°C. Nitrogen gas (30 mL min^{-1}) was used as the carrier gas. Fluid samples (4 mL from each well) were stored in serum bottles with Teflon-lined caps and were analyzed for baseline values (i.e., MTBE, DO, pH and temperature). Static headspace analysis is based on the partitioning of analyses from an aqueous or solid sample to air in a closed system (headspace vial). This method is suitable for compounds that show sufficiently high air-water partitioning (quantified by the Henry's Law constant). Hundred microliter of headspace was injected and analyzed into the chromatograph using a wetted 500 µL gas-tight syringe. The trap inside the concentrator was packed with an appropriate adsorbent and maintained at ambient temperature. The liquid sample was swept with purge gas to release analyses from the sample and deposit them on the concentrator trap. The concentrated analyses on the trap were rapidly heated and the heated trap was then back flushed with gas to deliver a tight band of organic analyses to the GC for analysis for concentrations of MTBE. A similar serum bottle, which was contained 1% of NaCN as a microbial respiration inhibitor was used as a control medium, to monitor any MTBE loss from volatilization and diffusion from septum.

Effect of Humic Substances and Yeast Extract

Humic and fulvic acids were extracted from a well humified organic soil and purified following a standard procedure of the Canadian Society of Soil Sciences (Carter, 1993). The extracted humic substances and yeast extract were test for enhancing of MTBE removal.

Results and Discussion

In an attempt to reduce the atmospheric environmental consequences of fuel burning in internal combustion engines, oxygenate chemicals are being introduced into gasoline. Oxygenates are organic compounds that are designed to increases the oxygen content of gasoline. One of the most commonly used oxygenates is MTBE. It is by far the most popular ether oxygenate due to its low cost, ease of production, transfer and blending characteristics. The atmosphere is not the only area where additives and combusted fuels are released. Since MTBE is soluble in water, it is likely to reach groundwater and surface waters. If the use of MTBE reduces carbon monoxide emissions but causes human health problems or ecotoxicity from ground and surface water contamination, alternative oxygenates may need to be investigated for use. Recent literature provided evidence that while single species cultures have some problems in biodegrade MTBE, multiple species growing in a consortium would degrade MTBE to produce CO_2 (Francois *et al*., 2002). MTBE and TBA degradation has been reported in the presence of all environmentally relevant terminal electron acceptors, however, except for ozic conditions, results are controversial in literature or very limited studies have been carried out so far (Bruce *et al*., 2002). Under methanogenic conditions, TBA degradation has not been observed and it is currently widely accepted that TBA is an accumulating dead-end product of MTBE degradation under such conditions (Finneran and Lovley, 2001). Present results indicate that MTBE may slowly be metabolized to CO_2 aerobically in the environment, but when conditions preclude then its metabolites from exerting toxicity to the population of microorganisms capable of degrading it. The rate and extent of degradation of organic components in gasoline will influence the type of clean-up efforts which will be appropriate when it is spilled. MTBE does effect the microbial degradation of hexadecane, but only when present in relatively high

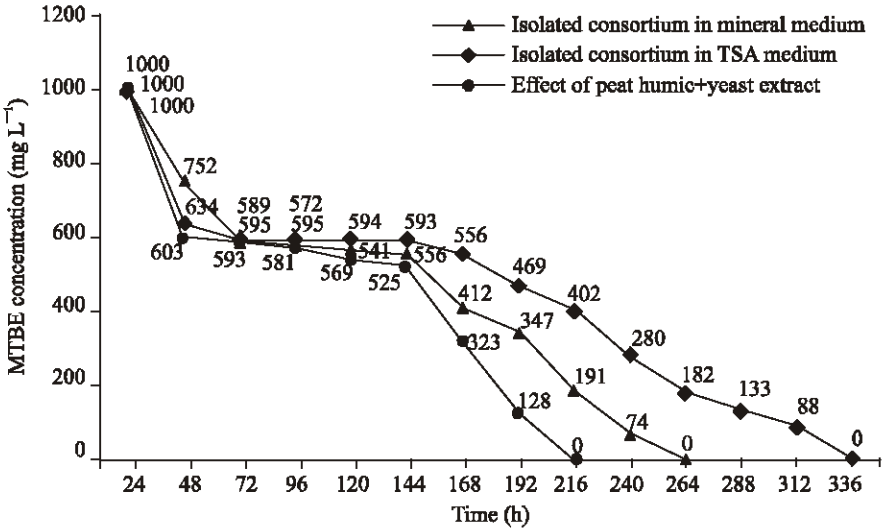

Fig. 1: The results of MTBE-degrading consortium and the effect of yeast extract and peat humic on MTBE biodegradation.

concentrations. We found that a low biomass carbon-limited consortium utilized oxygen when small amounts of MTBE were added as the sole carbon source. This provides indirect evidence that MTBE is capable of being metabolized by microbial populations since oxygen is the terminal electron acceptor in aerobic metabolism (Kane *et al*., 2001). The combined results from the enrichment culture and mineralization experiments indicate that while aerobic metabolism of MTBE may occur to some extent by consortia, it occurs at a very slow rate. MTBE or its metabolites appear to inhibit metabolism of MTBE at concentrations exceeding 1000 ppm when no other carbon sources are present. Thus it is unlikely that MTBE can serve as a sole carbon source for microorganisms. The MTBE-degrading consortium was obtained after 4 months enrichment. Present results showed that MTBE had a weak inhibitory effect on the biodegradation of MTBE at a concentration of 1000 mg L^{-1}. However, the mechanism of inhibition is not clear. In summary, when MTBE was added to a carbon-limited enrichment consortium, oxygen levels in the culture vessel decreased, providing indirect evidence of MTBE metabolism (Steffan *et al*., 1997; Deeb *et al*., 2000). Bacterial population incubated in MTBE at 1000 mg L^{-1} were found to biodegrade 99% of the MTBE present within 240 h. Simultaneous production of TBA, the primary metabolite of MTBE, was also observed. Mass balances of MTBE and TBA showed that TBA accumulated at a rate slower than that at which MTBE was being biodegraded. This observation suggested that a portion of the TBA being produced was simultaneously being degraded along with MTBE. MTBE at 1000 mg L^{-1} was completely degraded within 250 h. It has been suggested that the same enzyme degrades TBA and MTBE. Consequently, the effect of TBA on the MTBE biodegradation rate is of interest. In the presence of equal molar concentrations of TBA and MTBE, the rate of MTBE biodegradation by bacterial population was 80% of that of the control. The inhibitory effect of TBA increased with increasing TBA concentrations. The MTBE degradation rate of cultures incubated with a high concentration of TBA was 15% of that of the control. The selected microbial consortium can also degrade completely TBA as sole source of carbon and energy, without lag period during initial batch cultures. Additional research

is needed to optimize the growth conditions of these organisms so as to obtain the best MTBE remediation rates (Steffan *et al*., 1997). In addition, bioreactors using selected microbial consortium to treat MTBE-contaminated water must be designed so as to minimize competitive inhibition effects among MTBE, TBA and butane. The activated sludge sample were able to degrade 100% of 1000 mg L^{-1} of MTBE within 250 h. After the substrate was exhausted, the adaptation phase was extended by periodic additions of substrate over a 2-month period. 100 mg L^{-1} of yeast extract and 200 mg of Peat humic support growth of microbial consortium by itself, it clearly had a stimulatory effect on MTBE consumption. A similar effect of 100 mg L^{-1} of yeast extract and 200 mg of Peat humic was observed and had a stimulatory effect on MTBE consumption. The results of MTBE-degrading consortium and the effect of yeast extract and Peat humic on MTBE biodegradation are shown in Fig. 1. In cultures able to degrade MTBE, the greatest amounts of MTBE removal were seen to occur in the first few days of the biodegradation assays. No biomass aggregates were observed during all the batch cultures, but the attached biomass was observed (the concentration of the initial attached biomass was about 0.11 g L^{-1} of dry weight). Although MTBE-degrading bacteria have been isolated, there are still unanswered questions about which specific members of the microbial community are capable of degrading MTBE, the enzymatic pathways and metabolic pathways involved and most fundamentally, the differences between degradation enzymes that explains the restrictions in substrate utilization in bacteria. In addition, because it is well known that both single species and consortia living in hydrocarbon contaminated sites will easily degrade hexadecane to CO_2. We found that a low biomass carbon-limited consortium utilized oxygen when small amounts of MTBE were added as the sole carbon source. This provides indirect evidence that MTBE is capable of being metabolized by microbial populations since oxygen is the terminal electron acceptor in aerobic metabolism (Salanitro *et al*., 1994). That microorganisms would follow this conversion pathway is not unreasonable and such a pathway may not be detected Suflita and Mormile found that in anaerobic degradation, oxygenates containing a *terti*ary or quaternary carbon atom proved more recalcitrant than unbranched or moderately branched analogs but would degrade eventually (Hristova *et al*., 2001). Other studies have shown the MTBE is completely recalcitrant to degradation. Our results indicate that MTBE may slowly be metabolized to CO_2 aerobically in the environment, but only when conditions preclude it or its metabolites from exerting toxicity to the population of microorganisms capable of degrading it. The rate and extent of degradation of organic components in gasoline will influence the type of clean-up efforts which will be appropriate when it is spilled. MTBE does effect the microbial degradation of hexadecane, but only when present in relatively high concentrations. In summary, when MTBE was added to a carbon-limited enrichment consortium, oxygen levels in the culture vessel decreased, providing indirect evidence of MTBE metabolism.

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