Evaluating MTBE Biodegradation with Hydrocarbon Metabolizing Cultures

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Abstract Methyl tert-butyl ether (MTBE) is added to reformulated gasoline to meet the 1990 Clean Air Act directives. Widespread use of MTBE in gasoline has resulted in groundwater contamination throughout the United States, increasingly impacting public drinking water supplies. Biodegradation of MTBE can offer an efficient and low-cost method of treating MTBE contaminated groundwater. It has been proposed that alkane-degrading bacterial cultures can co-metabolize MTBE. To evaluate the hypothesis that all alkane-degrading bacteria can co-metabolize MTBE, mixed cultures were enriched on iso-pentane, octane, decane, dodecane, tetradecane, pentadecane, and hexadecane alkanes as the sole carbon sources and then tested for their ability to co-metabolize MTBE. A toluene enrichment culture was also screened to see if aromatic-degrading cultures could co-metabolically degrade MTBE. Vials were sealed using Mininert caps and MTBE concentrations monitored by GC/FID headspace analysis. Microcosms were supplemented with enrichment substrate on day seven to test MTBE degradation stimulation. Degradation of MTBE to below detection limits of 0.1 mg/L in less than three days was observed by the iso-pentane enrichment culture. Partial but incomplete co-metabolism was observed in octane and decane cultures. Dodecane, tetradecane, pentadecane, and hexadecane cultures did not degrade MTBE. The toluene enrichment degraded MTBE to near below detection limits during the duration of the assay. Positive stimulation of MTBE degradation only occurred with the toluene culture, resulting in removal of MTBE below detection limits. The toluene enrichment was found capable of metabolizing iso-pentane. These experiments showed that of the alkanes tested, only bacteria able to grow on iso-pentane were efficient at MTBE co-metabolism. This suggests that MTBE degradation is limited to a specific subgroup of alkane degrading bacteria and possibly to bacteria able to degrade branched aromatic hydrocarbons. Continued studies with pure and mixed cultures will be performed to clarify interactions between alkane and aromatic metabolism, and MTBE biodegradation.

Introduction

Methyl *tert*-butyl ether (MTBE) is a synthetic additive originally introduced to replace lead as an octane-enhancer and anti-knocking agent in gasoline (API 1998). MTBE is now used as an oxygenate, to reduce automobile carbon monoxide emissions in fall and winter months, under programs mandated by the 1990 Clean Air Act Amendments. It is blended into gasoline at levels of 15% by volume to meet the federal standards of 2.0% oxygen by weight for reformulated gasoline. While alternative additives such as ethanol are available for increasing fuel oxygenation, they are not economically favorable to due to higher costs of synthesizing the alcohol. In addition, costs of transporting fuel additives are low for MTBE, which is produced at fuel refineries, while ethanol needs to be imported over greater distances (US EPA 1998).

Increased use of MTBE in reformulated gasoline has resulted in groundwater contamination throughout the United States, mainly from leaking underground storage tanks. High MTBE groundwater contamination has led to closures of drinking water well fields (Hitzig 1998). Even the very low concentrations of 35 μg/L of MTBE in water easily detectable and renders it unfit for human use (US EPA 1998). MTBE persists in groundwater due to the reduced exposure to air for volatilization and resistance to biodegradation (Yeh 1991).

Bacterial degradation of MTBE could provide an attractive solution to groundwater contamination. Such treatment can be performed *in-situ* at the site of contamination or *ex-situ* with biological water treatment reactors (Salanitro 2000, Stringfellow 2000). With *in-situ* bioremediation bacterial cultures naturally existing are metabolically stimulated with substrate addition to degrade the contaminant to non-toxic compounds. Alternatively selected or engineered bacterial cultures with the desired degradation ability are injected into the contaminated site and provided with the required metabolic stimulus to degrade the recalcitrant contaminants. In e*x-situ* bioremediation, the contaminated water is extracted from the ground and treated by selected bacteria. The effluent exiting the bioreactor is usually contaminant free, and is cycled can be pumped into the water table or treated further with downstream systems.

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. Understanding might suggest ways for stimulating faster growth of MTBE-degrading organisms

for either *in-situ* or *ex-situ* treatment systems (Prince 2000). Increased understanding of degradation processes can also potentially lead to engineered substrate specificity for bioremediation of other recalcitrant compounds and improved stability and efficiency of such processes, as well as other microbial biotechnology processes.

Cultures able to metabolize MTBE have been found in activated sludge, air and ground water treatment systems, soils, sediments, and even Gingko fruit (Solano-Serena 2000, Stringfellow 2000, Bradley 1999, Garnier 1999, Hanson 1999, Mo 1997, Steffan 1997, Salanitro 1994). Several pure strains of bacteria able to degrade MTBE have been described (Solano-Serena 2000, Garnier 1999, Hanson 1999, Steffan 1997, Mo 1997, Salinitro 1994). In such strains as PM1 that are able to utilize MTBE as the sole carbon source, studies show that the bacteria demonstrated a very slow growth rate of only 19% of ^{14}C-MTBE at 20 μg/ml to ^{14}C-labeled cells in 120 hours. Only 0.18 milligrams of cells were yielded per milligram of MTBE removed. Toxic activity of MTBE and high energetic requirements in breaking down MTBE has been to explain its low occurrence within the microbial community and the slow rates of metabolism (Prince 2000).

Many studies have demonstrated that alkane-degrading cultures can co-metabolize MTBE (Stringfellow 2000, Solano-Serena 2000, Bradley 1999, Garnier, 1999, Hyman 1999, Hyman 1998, Steffan 1997). With co-metabolism, the bacterial cultures degrade MTBE but receive little to no metabolic energy for cell growth. Studies in the molecular genetics of bacteria have shown high degrees in homology between the hydroxylase enzymes of alkane-degrading bacteria to AlkB, a key enzyme implicated MTBE degradation (Smits 1999). This homology suggests that alkane-degrading bacteria may be able to degrade MTBE. To evaluate the hypothesis that all alkane-degrading bacteria can co-metabolize MTBE, bacterial cultures were enriched on *iso*-pentane, octane, decane, dodecane, tetradecane, pentadecane, and hexadecane as sole carbon sources and then tested for their ability to co-metabolize MTBE.

Methods

Chemicals and Reagents All chemicals and reagents were of laboratory research grade. Minimal salts media was prepared by adding 1.0 g potassium phosphate monobasic (EM Science, 99% pure), 0.86 g sodium phosphate dibasic (JT Baker, 99%), 1.0 g ammonium

chloride (Sigma, > 98%), 0.12 g magnesium sulfate (Fischer, 99%), 0.06 g calcium chloride (Fischer, 99%), 2 ml trace metals solution, in 1 liter de-ionized water, with a final pH of 6.8.

The trace metals solution was made by combining 3.3 mg manganese sulfate (Mallinckrodt, 99% pure), 6.2 mg copper sulfate (EM Science, 99%), 7.6 mg zinc sulfate (EM Science, 99%), 11.7 mg sodium molybdate (Sigma, 99%) in one liter of 0.1 N HCl (Fischer, 99%).

MTBE stock solution was made by serially diluting research grade MTBE from EM Science (98% pure) to a final concentration of 76.4 mg/L in de-ionized water.

Enrichment substrates included *iso*-pentane from Aldrich (> 99.5% pure), octane, decane, dodecane, and hexadecane from Mallinckrodt (99%), tetradecane (99%) and toluene (99.5%) from EM Science, and pentadecane from Arcos Organics (99%).

Enrichment of Cultures Mixed bacterial cultures were enriched from oil and gas contaminated soils with *iso*-pentane, octane, decane, dodecane, tetradecane, pentadecane, hexadecane and toluene as sole carbon source. Soils were filtered with 100 mL of de-ionized water. Ten milliliters of soil filtrate was added with the minimal salts solution to a final volume of 250 mL in an Erlenmeyer flask with 0.5 mL of enrichment substrate. Bacterial cultures were maintained as draw-fill enrichments and provided with sufficient hydrocarbon to maintain active growth.

Analysis of MTBE MTBE concentrations in the headspace of the vials were determined using a gas chromatograph equipped with a flame ionization detector. MTBE was analyzed on a DB-WAX column (30 meter, 0.25 μm ID, J & W Scientific) isothermally at 40° C (injector at 150° C, detector at 225° C) by injecting 100 μl of microcosm headspace using a wetted 500 μl gas-tight syringe.

Assay for Co-Metabolic Degradation Co-metabolic biodegradation was measured using a high density, metabolically active culture in a microcosm batch assay. Fifty mL of log-phase culture was washed three times with minimal salts media. Optical density of the cultures was standardized to approximately 1.0 AU at 590 nm (20 mm pathlength) after washing.

Co-metabolic biodegradation assays were prepared in triplicate 20 x 120 mm culture tubes sealed with Mininert caps. Controls contained 9 ml of de-ionized water and 1 ml MTBE stock solution. Prior experience with dozens of assays had shown that MTBE biosorption was negligible and biomass controls are unnecessary. Treatment vials contained 4 ml DI water, 5 ml of washed culture, and 1 ml MTBE stock solution. MTBE degradation by the *iso*-pentane

enrichment was tested twice. Initial concentration of MTBE in all microcosms was approximately 7.6 mg/L. Initial cell concentration was approximately 125 mg/L. Microcosms were incubated on an orbital shaker at 25° C. After allowing one day for equilibration, MTBE concentrations were monitored by using headspace analysis by gas chromatography (see above). On day seven of the assay, the microcosm containing the octane, decane, dodecane, tetradecane, pentadecane, hexadecane and toluene enrichments were supplemented with 2 µl of their corresponding hydrocarbon to see if addition energy source would promote degradation activity. Optical density readings were used to monitor changes in culture density over the term of the assay.

Results

The cultures grown on dodecane, tetradecane, pentadecane, and hexadecane did not co-metabolize a significant amount of MTBE (Table 1). Octane and decane enrichments degraded some but not all of the MTBE added. Microcosms containing the octane-degrading bacteria removed approximately 15% of MTBE compared to the controls. Decane-degrading bacteria demonstrated a drop in MTBE concentration to roughly 75% of controls.

Table 1: Bacterial enrichment substrates and the final MTBE concentrations for each corresponding biodegradation assay.

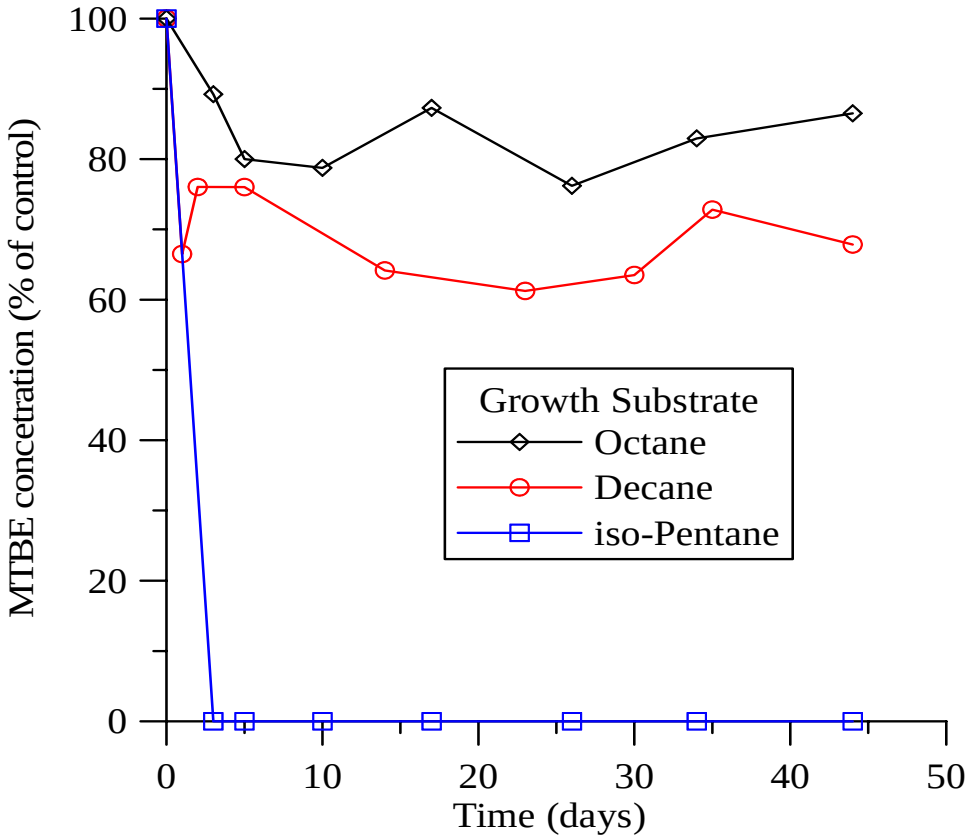
Growth Substrate	Chemical Formula	Percent MTBE Remaining
Iso-Pentane	C_5H_{12}	0.0
Toluene	$C_6H_5CH_3$	7.1
Toluene + co-substrate	$C_6H_5CH_3$	0.0
Octane	C_8H_{18}	86.5
Decane	$C_{10}H_{22}$	67.8
Dodecane	$C_{12}H_{26}$	98.2
Tetradecane	$C_{14}H_{30}$	96.7
Pentadecane	$C_{15}H_{32}$	91.4
Hexadecane	$C_{16}H_{34}$	100.0

In order to determine if the systems were energy limited, decane and octane was added on day seven to their respective vials. The additional co-substrate did not stimulate further MTBE degradation in octane and decane treatments. Additional co-substrate did not stimulate further MTBE degradation in dodecane, tetradecane, pentadecane, and hexadecane treatments either.

Rapid and complete MTBE degradation occurred with the *iso*-pentane enrichment culture (Figure 1). This assay was conducted twice in an attempt to measure kinetics. In the first *iso*-pentane degradation assay, MTBE was completely depleted to less than the detection limit of 0.1

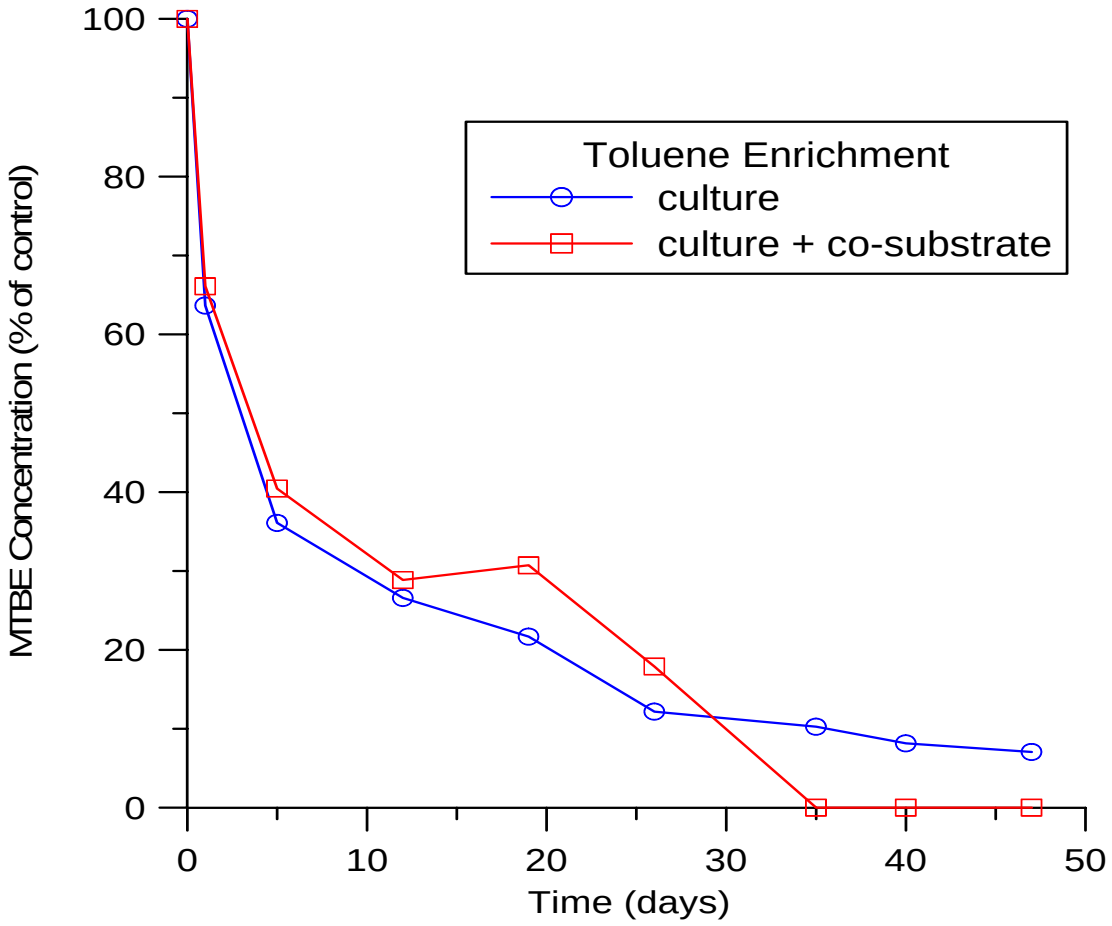
mg/L in less than three days (Figure 1). In a second assay, conducted to get a better measurement of the MTBE degradation kinetics by the *iso*-pentane enrichment, MTBE was degraded from 7.6 to 0.6 mg/L within twelve hours. On day two, the MTBE concentration was below the detection limit. In both experiments, the biomass in the microcosm did not show a significant change with the degradation of MTBE.

Figure 1: Degradation of MTBE by iso-pentane, octane, and decane enrichment cultures (n=3)



The culture enriched on toluene removed roughly 60% of available MTBE within the first five days (Figure 2). In vials injected with additional toluene on day seven, MTBE concentrations leveled off at approximately 2.3 mg/L for thirteen days. MTBE was then steadily depleted to below detection limits on day 35 of the assay. Vials of toluene culture without co-substrate addition only degraded MTBE to concentrations roughly 10% of controls.

Figure 2: Co-metabolic degradation of MTBE by a toluene enrichment culture. Co-substrate addition stimulated complete degradation of MTBE in treatment vials (n=3).



Discussion

Of the alkanes, only bacteria enriched on iso-pentane catalyzed complete degradation of MTBE. This suggests that MTBE degradation is limited to a specific class of alkane degrading bacteria. Many factors may contribute to the observed differences in MTBE degradation activity. The hydrocarbon structure and length originally metabolized by the enrichment culture may be a factor in its ability to degrade MTBE. Cultures growing on C_8 or greater linear alkanes removed far less MTBE than those grown on a C_5 branched alkane. MTBE co-metabolism decreased significantly as the alkane chain-lengths progressively increased (Table 1). The similarity between MTBE and *iso*-pentane structure may also account for the rapid degradation by the *iso*-pentane enrichment. Both *iso*-pentane and MTBE are smaller branched compounds, while the other alkane are more lengthy and strictly linear.

Bacteria enriched on toluene were able to completely degrade MTBE after provided with co-substrate. During the period that MTBE concentrations remained constant after co-substrate addition, the bacteria degraded the supplemented toluene and then the ether. Treatment vials not provided with additional toluene were not able to completely degrade the MTBE (Figure 2). Removal of MTBE only after toluene addition suggests a co-metabolic mechanism responsible for the degradation.

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. Cultures capable of MTBE biodegradation will show their greatest activity within the first few days after innoculation into the assay microcosms. Long-term monitoring shows that eventually degradation activity plateaus and that MTBE concentration reaches a fairly constant value.

The greater activity observed in the *iso*-pentane microcosms further suggests that the alkane hydroxylase of bacteria able to grow on branched hydrocarbons is more effective at co-metabolizing MTBE than the equivalent enzymes of bacteria that grow on linear alkanes. It should be noted however, that the *iso*-pentane degrading bacteria could grow on octane and still degrade MTBE rapidly (data not shown). The toluene enrichment was also found capable of degrading *iso*-pentane. Degradation of MTBE by toluene and *iso*-pentane cultures did not result in cell growth, as optical densities taken of these cultures remained fairly constant throughout the duration of the assay, suggesting the organism gains little or no metabolic energy from the co-metabolic reaction.

The results of this study support the hypothesis that MTBE degradation is a unique characteristic of a specific subgroup of alkane-degrading bacteria. Cultures grown on longer chained alkanes were less active towards MTBE, while cultures grown on shorter, branched alkanes were able to break down the ether. The ability to degrade *iso*-pentane was observed in all enrichments that were able to degrade MTBE and may possibly be a key characteristic in identifying organisms able to breakdown the ether. Further studies with pure and mixed alkane-degrading bacteria cultures will be conducted to elucidate the relationship between alkane degradation and MTBE co-metabolism.

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