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Isolation and Initial Characterization of A Pure Cultures Capable to Degradation Methyl *tert*- Butyl Ether (MTBE)

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Abstract

Methyl *tert*-butyl ether (MTBE), a gasoline octane enhancer, was introduced as a substitute for lead tetraethyl over 30 years ago. Widespread use of MTBE in gasoline, has introduced MTBE into the environment compartments, mostly into the under ground and surface water and water as a second most frequently detected contaminant. In this study, we have isolated pure cultures from bacterial consortium capable to use MTBE as a sole carbon and energy source. MTBE biodegradation rate was measured in headspace by gas chromatography. Initial liner rates of biodegradation by Pinpoint and white strains were found 2.9 mg and 3 mg MTBE h^{-1} g^{-1} wet biomass, respectively. The results of 16S rDNA PCR disclosed similarities in the banding patterns between the cultures, and the known degrading strain PM1. The results of this study suggest promising perspectives for engineering the in situ bioremediation of MTBE.

Keywords: *Methyl tert-butyl ether (MTBE), Biodegradation, Pure strain, 16S rDNA, PCR*

Introduction

Methyl *tert*-butyl ether (MTBE), a gasoline octane enhancer was introduced in late 1970s as a substitute for lead tetraethyl. Owing to its low production cost and excellent blending characteristics, its production has rise dramatically, reaching to a value of over 33 million tons per year at present time (1, 2).

Widespread use of MTBE in gasoline, leaks of fuel from underground and surface tanks, spill or accidental runoff from petroleum facilities (including refineries, terminals, the pipelines and service stations)have introduced MTBE into the environment compartments, mostly into the under ground and surface water and water as a second most frequently detected contaminant (3, 4).

While MTBE is thought to be less harmful than other gasoline constituents such as benzene, there is still relatively incomplete knowledge on its health effects. Currently, the U.S. Environmental Protection Agency recommends 20-40 ppb as the indicative Health Advisory level for drinking water (1, 4).

The persistence of MTBE in the environment can be ascribed, on the one hand, to its physicochemical properties (i.e., its low adsorption on organic matter and its high solubility in water) and, on the other hand, to its molecular structure (it has both an ether bond and high steric hindrance, which makes it recalcitrant to microbial degradation) (5-7).

Unlike other gasoline components including BTEX, there are a few reports of microorganism in either pure or mixed culture capabale of biodegradation MTBE (4, 6, 8).

Salanitro et al. (9), were the first to report the bacterial consortium that were able to degrade 120 mg MTBE L^{-1} at rate of 34 mg g of cells^{-1} h^{-1}.

Scow et al. (10) isolated a pure bacterial strain, PM1, from a mixed microbial consortium, derived from a compost biofilter, that began re-

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moving MTBE after a 1 yr acclimation period (5). MTBE biodegradation rate by 2×10^6 cell ml^{-1} were 3.56 μg ml^{-1} h^{-1} for concentration 500 μg MTBE ml^{-1}.

In a previuos study (11), we isolated a mixed bacterial culture from activated sludge, which was capable of degrading the MTBE as a sole carbon and energy source. This consortium was developed after 4-month enrichment. Degradation rate was nearly 1.478 mg MTBE $h^{-1}g^{-1}$ wet biomass and did not change with the MTBE concentration (up to 500 mg L^{-1}). This consortium was used as a microbial source for pure culture isolation in this study.

Our objectives in this study were isolation and initial charactrization of the bacterial cultures capabale of using MTBE as a sole carbon and energy source.

Materials and Methods

Chemicals and Reagents Diluting a GC grade MTBE MERCK (99.9% pure) at the required concentration in aerated sterile Mineral Medium (MM) made the MTBE stock solution. All chemicals and reagents were analytical grade. The MM consisted of the following components (in g L^{-1}): $MgSO_4.7H_2O$, 0.25; KNO_3, 0.5; $CaCl_2.2H_2O$, 0.009; KH2PO_4, 0.5; K_2HPO_4, 0.5; NaCl, 1.0; and 1.0 ml/l of trace elements solution. The trace elements solution contained (in 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 (12, 13).

Isolation of pure culture A mixed bacterial culture capable of degrading MTBE was obtained from activated sludge (11). The consortium was plated on MM solidified with agarose (8 g L^{-1}). Plates were placed in the closed plastic box, incubated at the room temperature. MTBE was provided in gas phase in an initial concentration of 1 g L^{-1} (in H2O) and replenished every day to serve as sole carbon and energy source. Appearing colonies after one month were picked up and transferred to the general enriching medium. Isolated colonies were tested for their ability to grow in MM with 100 mg of MTBE L^{-1}. Cultures were incubated in 115 ml bottles sealed with the Teflon lined silicon septum (La-Pha-Pack) at 25 °C in the dark and shook continually on an orbital shaker (rotation of speed 130 rpm).

In order to establish cell line purity, cultures were maintained in MM with sufficient MTBE to maintain active growth. After MTBE consumption, culture was aerated, and then MTBE was added to the bottles. Every 10 days, half part of MM was filtered through 0.22 μm-pore-size Cellulose Acetate filters (Sartorius GmbH-Germany) and a new MM was added to the bottles. After one month, colonies were harvested by centrifugation at 4000 rpm for 15 min. The biomass was resuspended to obtain an initial concentration of 5 g wet biomass in L of MM, which contained about 150 mg MTBE L^{-1}. In each sterile bottle, 20 ml of above-mentioned mixture was added. A 95 ml of headspace volume was guaranteed sufficient air for aerobic degradation.

MTBE Removal Experiments The ability of any isolated colony, to degrade MTBE was evaluated by gas chromatograph (GC) at different times. MTBE and TBA (tert butyl alcohol) concentrations 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 meter, .4mm ID). Column temperature was adjusted isothermally at 50° C; injector at 180 °C and detector at 200 °C. Nitrogen gas (30 ml/ min) were used as the carrier gas. Samples (100 μl) were injected into the chromatograph, without further treatment. A similar flask, which was contained 1% of NaCN as a microbial res-piration inhibitor was used as a control medium, to monitor any MTBE loss from volatilization and diffusion from septum.

DNA extraction For DNA extraction, isolated colonies capable to degraded MTBE, were grown in LB Agar plates, each colony was sus-

pended in 100 µl of distilled water, an aliquot of 80 µl of NaOH (0.05 M) was added to 20 µl of bacterial cells suspension and the mixture was incubated at 60 °C for 45 min, followed by the addition of 6 µl of Tris/HCl (pH 7.0), achieving a final pH of 8.0. The resultant mixture was diluted 100 times and 5 µl of the diluted extract was used for PCR. The quality of extraction was checked by loading 5 µl of mixture on, 1% agarose gel.

PCR, gel electrophoresis, and 16S ribosomal RNA gene sequencing PCR amplification of the 16S rRNA gene was modified from a published protocol (14), DNase I treated distilled water and PCR master mix (which contains deoxynucleoside triphosphates (dNTPs), PCR buffer , and Taq polymerase) were used in all PCR reactions by adding 1 U of DNase I (Pharmacia, Uppsala, Sweden) to 40 µl of distilled water or PCR master mix, incubating the mixture at 25 °C for 15 min, and subsequently at 95 °C for 10 min to inactivate the DNase I. The bacterial DNA extract and control were amplified with 0.5 µM primers) LPW57, 5'-AGTTT GATCCTGGCTCAG-3'; and LPW58 -5', AGGCCCGGGAACGTATTCAC-3') (Gibco BRL, Rockville, Maryland, USA). The PCR mixture (50 µl) contained bacterial DNA, PCR buffer (10 mM Tris/HCl (pH 8.3, 50 mM KCl, 3 mM MgCl2, and 0.01% gelatin), 200 µM of each dNTP, and 1.0 U Taq polymerase (Boehringer Mannheim, Mannheim, Germany). The mixtures were amplified for 40 cycles at 94 °C for one min, 55 °C for one min, and 72 °C for two min, with a final extension at 72 °C for 10 min in an automated thermal cycler (Perkin-Elmer Cetus, Gouda, The Netherlands). DNase I treated distilled water was used as the negative control. An aliquot of 10 µl of each amplified product was electrophoresed in (1.0% w/v) agarose gel, with 1kb size marker (Boehringer Mannheim) in parallel. Electrophoresis in Tris-borate-EDTA buffer was performed at 100 V for 1.5 hours. The gel was stained with ethidium bromide (0.5 µg ml^{-1}) for 15 min, rinsed, and photographed under ultraviolet light illumination.

The rest of PCR product was gel purified using the QIA quick PCR purification kit (Qiagen, Hilden, Germany). Both strands of the PCR product were sequenced twice with an ABI 310 automated sequencer according to manufacturer's instructions (Perkin-Elmer, Foster City, California, USA), using the same set of primers (LPW57 and LPW58). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the GenBank database using the BLAST (Basic Local Alignment Search Tool) searching tool (15).

Results

MTBE biodegradation rate by a number of subcultures isolated from the mixed consortium on agarose, are presented in Fig. 1.

Most of the isolated cultures could not degrade MTBE. Only, Pinpoint and white cultures, after a long lag phase, degraded MTBE, and then were subjected for further analyses. A substantial decrease (10%) of MTBE concentration in the control sample may because of MTBE diffusion through the septum.

After streaking two colonies onto 0.1× TSA, colonies were transferred to MM with MTBE as a sole carbon and energy source. A very long lag phase was observed before the initiation of MTBE degradation by colonies (see Fig. 2). After the acclimatation phase, two colonies were found capable to use MTBE without any lag phase.

MTBE degradation rates by 5 g wet pure culture and consortium biomass in liter after enrichment in MM and presence of MTBE are presented in Fig. 3. Two cultures were capable to degrade MTBE concentrations up to 500 mg L^{-1} without any remarkable lag period. Initial liner rates of biodegradation by pinpoint and white strains were measured to be 2.9 mg and 3 mg MTBE h^{-1} g^{-1} wet biomass, respectively.

Unlike, the slow mineralization rate of MTBE by consortium (1.478mg MTBE h^{-1} g^{-1} wet biomass), two strains were found capable to biodegrade MTBE rapidly. We streaked strain onto

0.1×TSA plates. A number of colonies were isolated from two cultures.

The PCR results of the eubacterial 16S rRNA gene on DNA samples extracted from the colonies showed bands around 1.4 kb as it is expected. The results of sequence analysis on the amplified samples using BLAST in the Gene Bank Database, suggested isolated colonies as follows:

1- Pin point colony was *Stenotrophomonas* sp. JRL-2

2- Small transparent colony was uncultured *gamma proteobacterium* or *Acinetobacter* sp.'anoxic'

3- White colony was *Bacillus cereus* strain G3317

4- Large opaque colony was *Pseudomonas monteilii* or *Pseudomonas putida* strain ATCC 17390.

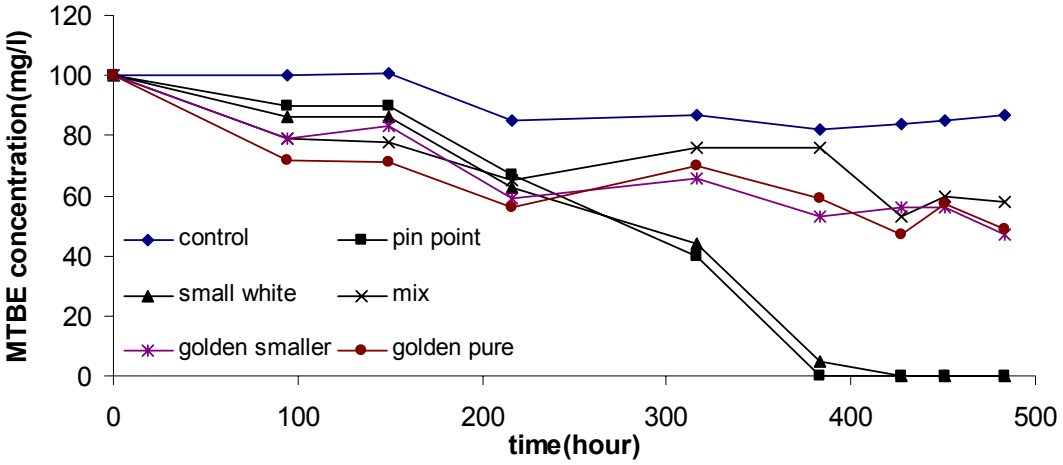

Fig. 1: MTBE biodegradation by some isolated cultures from the consortium

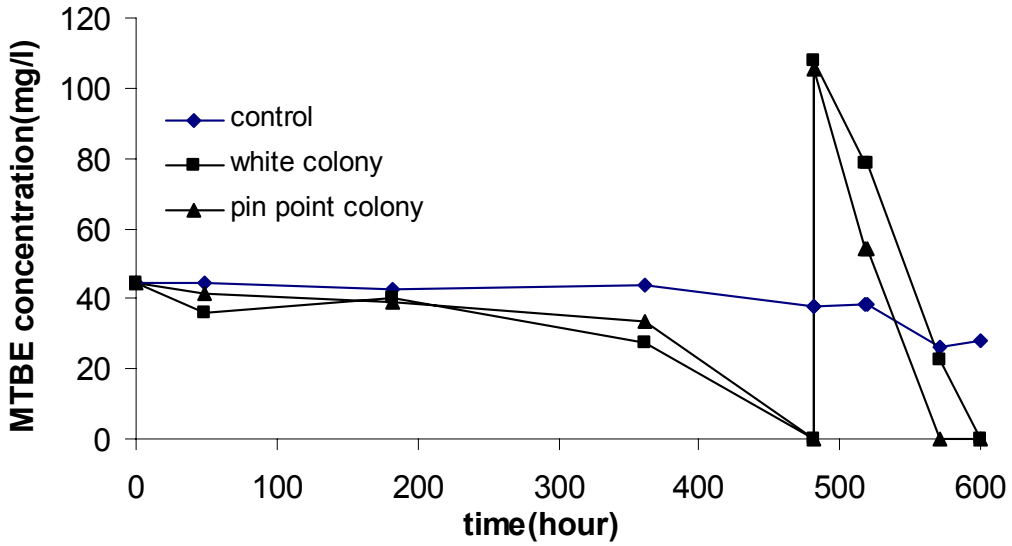

Fig. 2: MTBE biodegradation by pure cultures, after plating onto 0.1× TSA

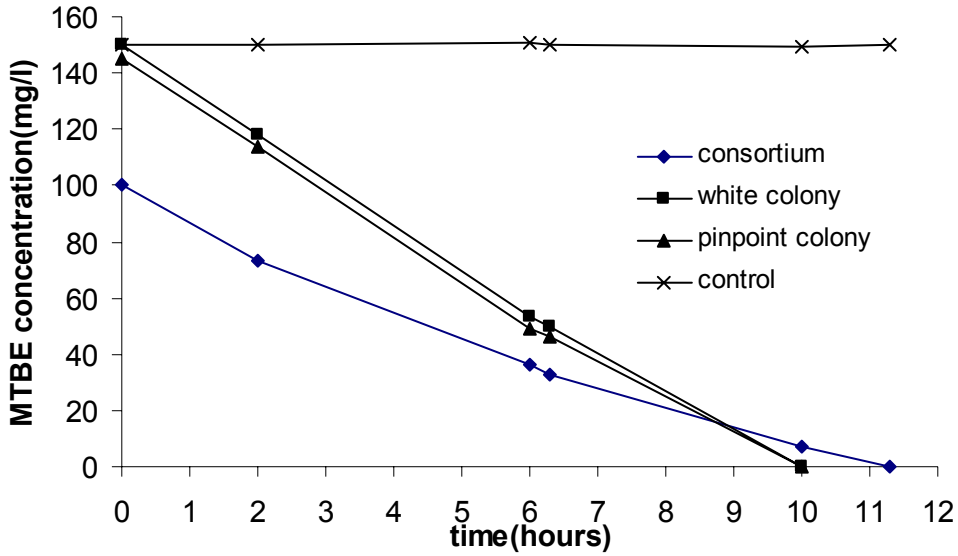

Fig. 3: Comparison of aerobic biodegradation of MTBE in pure cultures and consortium

Discussion

It has been a strenuous task to separate pure strain from the MTBE degrading consortium, by the usual separation procedure. First, because plating on general enriching medium suppresses degradation even by strains with demonstrated activity. This phenomenon could be explained by the fact that bacteria prefer to use easy carbon sources, and after growth on enriched medium, some MTBE biodegradation ca pability were lost, and long period of time required for degradation enzyme system to be developed.

Secondly, because most bacteria prefer to utilize easy carbon sources rather than recalcitrant MTBE, as a result, non-MTBE-degrading bacteria dominated and virtually inhibited the growth of MTBE-degrading bacteria that had a slow growth rate when MTBE was provided as sole carbon and energy source. According to previous studies, the specific growth rate of the bacterial consortium was about 0.0312-0.0375 day-1, or a doubling time 25-30 d (11).

As discussed earlier, while most isolated cultures from the consortium were unable to degrade MTBE, The pin point and white cultures were found to degrade MTBE more rapidly than a consortium.

These results indicated that two strains were the predominant population in microbial consortium and might play the dominant roles.

Despite of cultures enrichment in MM, in order to establishing cell line purity, result disclosed that there were more than two predominant members isolated from initial cultures. As mentioned, some strains were incapable of degrading MTBE alone, but they coexist with MTBE-degrading members for a long period, even after successive purification steps. It is still ambiguous what roles they play in the MTBE biodegradation or the general functioning of the consortium. Microscopic analysis of the isolated MTBE degrading colonies showed that, colonies were gram-negative strap array bacillus.

The results of 16S rRNA sequence analysis revealed similarities between the studied colonies and previously reported bacteria degrading MTBE, small transparent colony and the known degrading strain PM1 are in the β1 subgroup of Proteobacteria, meanwhile, akin to PM1, this strain when is grown in the liquid media produces flocs that make troubles in cell count.

We also checked the family of these bacteria and it seemed that they were from the families with degradation capability. However, further analyses are required to identify them exactly. MTBE biodegradation studies by these strains are currently investigated.

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