Isolation of a Bacterial Culture That Degrades Methyl t-Butyl Ether

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We have isolated a mixed bacterial culture (BC-1) which is capable of degrading the gasoline oxygenate methyl t-butyl ether (MTBE). BC-1 was developed from seed microorganisms present in a chemical plant biotreater sludge. This enrichment culture has been maintained in continuous culture treating high concentrations of MTBE (120 to 200 mg/liter) as the sole carbon source in a simple feed containing NH₄⁺, PO₄³⁻, Mg²⁺, and Ca²⁺ nutrients. The unit had a stable MTBE removal rate when maintained with a long cell retention time (ca. 80 to 90 days); however, when operated at a ≤50-day cell waste rate, loss of MTBE-degrading activity was observed. The following three noteworthy experimental data show that MTBE is biodegraded extensively by BC-1: (i) the continuous (oxygen-sparged) culture was able to sustain a population of autotrophic ammonia-oxidizing bacteria which could nitrify influent NH₄⁺ concentrations at high rates and obtain CO₂ (sole carbon source for growth) from the metabolism of the alkyl ether, (ii) BC-1 metabolized radiolabeled either (¹⁴CH₂O-MTBE) to ¹⁴CO₂ (40%) and ¹⁴C-labeled cells (40%), and (iii) cell suspensions of the culture were capable of degrading (substrate depletion experiments) MTBE to t-butyl alcohol, a primary metabolite of MTBE. BC-1 is a mixed culture containing several bacterial species and is the first culture of its kind which can completely degrade an alkyl ether.

Alkyl ethers such as methyl t-butyl ether (MTBE) are being used as octane enhancers in the reformulation of low-volatility unleaded gasoline blends and for reducing emissions of volatile organic compounds from engines (6, 11, 19). In general, alkyl ethers are a chemically stable class of compounds and there is little information on their biodegradability in soil, groundwater, and activated-sludge environments. MTBE can persist in groundwater from accidental spills of unleaded gasoline from underground storage tanks. However, no known naturally occurring microbial cultures exist to biotreat groundwater, wastewater, tank bottom wastes, or soils containing this ether. There are some data in the literature on the metabolism of diphenyl (3, 5) and cyclic (2) ethers (e.g., dioxane and furan) as sole carbon sources used for growth by soil bacterial (Erwinia, Rhodococcus, and Pseudomonas) species. Methoxybenzoate has been shown to be demethylated or demethoxylated under anaerobic conditions by an Acetobacterium organism (13). Alkyl ethers such as symmetric diethyl ether have been shown by Modrzkowski and Finnerty (7) to be only partially oxidized by an Acinetobacter strain in which the linkage is not cleaved and only the terminal carbons are utilized for growth. Definitive evidence for the growth on or oxidation of dimethyl ether by cells or cell extracts containing methane monoxygenases from methyloptrophs is lacking. Dimethyl ether does not support the growth of Methylococcus capsulatus (Texas) and was marginally oxidized in cell suspensions of the methyloptrophs (9). There are conflicting reports that methane monooxygenases of methyloptrophs can oxidize dimethyl ether (14, 17). Dimethyl ether has also been shown to inhibit methane oxidation in methyloptrophic cultures (9) and in soils (10). Recently, van den Wijngaard et al. (18) showed that microbes with dehalogenase activity isolated from saltwater sediments and activated sludge degraded (aerobically) bis(2-chloroethyl) ether and 2-chloroethyl vinyl ether. 2-Chloroethyl vinyl ether was dehalogenated to a hydroxy derivative, and then the ether bond was chemically cleaved to products which were used for growth. Studies on the biodegradability of MTBE in sludges and soils by Fujinara et al. (4) showed that 100 mg of MTBE or disopropyl ether per liter was not degraded in activated sludge (300 mg of cell solids per liter) in an oxygen uptake assay; also, MTBE did not significantly affect the sludge respiration rate of other gasoline hydrocarbons when blended (12%, wt/vol) with the fuel. Work by Moller and Arvin (8) confirmed that MTBE (10 mg/liter) or t-amy methyl ether (3 mg/liter) was not degraded in 60 days by microbes in an aquifer soil, topsoil, or activated sludges. In these experiments, MTBE at 200 mg/liter showed a weak inhibitory effect on the biodegradation of aromatic hydrocarbons (3.5 mg of benzene, toluene, ethylbenzene, and xylenes per liter). Recent studies by Sulfa and Mormile (16) on the anaerobic decomposition of gasoline oxygenates in landfill material showed that of several alkyl ethers tested (MTBE, t-amy methyl ether, ethyl t-butyl ether, disopropyl ether, ethyl ether, and propyl ether), only n-butyl methyl ether was metabolized under methanogenic conditions. The lack of alkyl ether degradation by indigenous microbes in soils and biosludges may be attributed to the very stable and chemically unreactive ether linkage, the inability of these compounds to be transported into cells, and/or the lack of inducible or existing enzyme activities (e.g., oxygenases and hydroxylases) which can attack the ether bond.

This report describes the isolation of an enrichment culture (mixed bacterial population) growing on MTBE as the sole carbon and energy source and its ability to metabolize ¹⁴CH₃O-MTBE to ¹⁴C-labeled cells and ¹⁴CO₂.

MATERIALS AND METHODS

Derivation of enrichment culture BC-1. Activated sludges from municipal, refinery, and chemical plant biotreaters were also used as microbial sources for isolating MTBE-degrading enrichments. Biosludge solids (100 to 200 ml) were added to 1
TABLE 1. Nitrification and biomass yields in BC-1 continuous culture degrading MTBE

<table>
<thead>
<tr>
<th>Nitrifying condition</th>
<th>Influent NH₄⁺ (mg/liter)</th>
<th>Effluent NO₃⁻ (mg/liter)</th>
<th>Cell dry wt (mg/liter)</th>
<th>Cell retention time (days)</th>
<th>Avg % MTBE removed</th>
<th>Cell yield (g of cells/g of MTBE utilized)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High NH₄⁺</td>
<td>120-125</td>
<td>390-450</td>
<td>2,500-2,580</td>
<td>80-90</td>
<td>80-90</td>
<td>0.21-0.24</td>
</tr>
<tr>
<td>Low NH₄⁺</td>
<td>10-20</td>
<td>50-70</td>
<td>2,020-2,340</td>
<td>80-85</td>
<td></td>
<td>0.23-0.28</td>
</tr>
</tbody>
</table>

* The results shown are averages of 4 weeks of data for each condition.

* Cell waste rates were 1.1 to 1.3 liters/week. Effluent cell concentrations under both conditions varied from 2 to 12 mg/liter and contributed 25 to 30% of the biomass loss from the unit.

* Influent and effluent MTBE concentrations varied from 160 to 210 and 3 to 40 mg/liter, respectively.

Influent and effluent MTBE concentrations varied from 120 to 175 and 50 to 60 mg/liter, respectively.

Batch substrate removal experiments. Utilization of MTBE and t-butyl alcohol (TBA), a possible major metabolite of MTBE, was assessed in batch removal assays with BC-1. Individual compounds were added (120 to 130 mg/liter) to 1 liter of BC-1 culture in a 1.5-liter vessel. The culture was flushed with sterile 100% O₂ for 2 to 5 min to achieve a dissolved-oxygen level of 20 mg/liter. The reaction vessel was stirred continuously at 22 to 25°C, and depletion of substrates was monitored by sampling the culture (2 to 3 ml) over a 24-h period. MTBE and TBA were analyzed by methods described below.

Radiolabeled-MTBE experiments. 14CH₃O-MTBE was custom synthesized by Amersham Corp. (Arlington Heights, Ill.), had a specific activity of 1.19 μCi/μmol, and was 99.3% pure by radiochromatography. Cultures were centrifuged, washed and resuspended in the same volume of sterile phosphate-buffered saline (PBS, 0.85% NaCl, 0.03 M Na₂HPO₄, 0.05 M KH₂PO₄, pH 7.2), and placed in 125-ml serum bottles sealed with Teflon-lined septa. 14CH₃O-MTBE was added to an initial concentration of 0.08 μCi/ml with MTBE at 2 ppm. Cultures were incubated at 30°C on a rotary shaker (150 to 200 rpm) for 7 days. The amount of 14CO₂ formed was determined by placing a 10-ml aliquot of the culture in a similar serum bottle, adjusting the pH to ≤2 with 6 N HCl, and then flushing the bottle for 1 h with a steady stream of N₂ into three gas washing bottles containing 100 ml of 0.1 M Ba(OH)₂. The Ba14CO precipitate (formed after coprecipitation with 0.1 M Na₂CO₃) was collected on 0.45-μm-pore-size Millipore filters, washed with PBS and dried, and the radioactivity was counted. After removal of 14CO₂, the culture was filtered with a 0.22-μm-pore-size Millipore filter, washed with PBS, and dried, and the 14C activity incorporated into the biomass (cells) was counted. The remaining radioactivity in the filtrate represented degraded 14CH₃O-MTBE and/or 14C-labeled metabolites. The efficiency of 14CO₂ trapping by the method described was confirmed in separate experiments in which NaH14CO₃ was added (0.06 μCi with CO₂ at 70 ppm) to PBS or azide-inhibited cultures, acidified (pH ≤2), and flushed into Ba(OH)₂ traps. Recovery of H14CO⁻ as Ba14CO₃ was 95 to 100% of the applied radioactivity. The 14C radioactivity was determined by placing 1-ml amounts of culture fluid (total initial 14C) and filtrates or filters containing Ba14CO₃ precipitates into glass scintillation vials containing 15 ml of Aquasol-2 Universal 2SC Cocktail (NEN Dupont Research Products, Boston, Mass.). Vials were counted in a Tri-Carb 2500 TR liquid scintillation analyzer (Packard Instrument Co., Meriden, Conn.).

Analysis of MTBE and TBA. Culture samples were analyzed for MTBE and TBA with a Hewlett-Packard 280 gas chromatography-flame ionization detection system. Compounds were separated on a Quadrex methyl silicone (1-μm-thick film) column at 150°C, with MTBE eluted as a single peak at 30 min. Detection times were 4 min for MTBE and 6 min for TBA. All results are expressed as percentages of the peak area.
capillary column 25 m long with a 0.025-mm inside diameter
(Alttech/Applied Science Labs, State College, Pa.). The
column temperature was initially 30°C for 3 min and was
programmed to rise to 70°C at 20°C/min. The carrier gas consisted
of helium (30 ml/min) and an N2 makeup gas. One-microliter
split samples were analyzed. The retention times of TBA and
MTBE were 3 and 3.8 min, respectively.

Chemicals. Common laboratory chemicals, e.g., salts, bases,
acids, and alcohols, used were purchased from Mallinckrodt or
Sigma Chemical Co. MTBE and TBA was obtained as ≥98%
pure material from Chem Service Inc., West Chester, Pa.

RESULTS AND DISCUSSION

Growth characteristics of BC-1 in a continuous cell recycle
unit. Data on the growth and metabolism of the BC-1 culture
in a solids recycle culture are given in Table 1. During the first
7 weeks, the culture oxidized most of the influent NH4+ (120
mg/liter) at high rates (ca. 400 mg/liter of effluent NO3-). This
initial observation suggested that the mixed culture in BC-1
utilizing pure O2 degraded MTBE at least partly to CO2 to
sustain autotrophic ammonia-oxidizing organisms.

When influent NH4+ and PO4-3 levels were reduced to
about 20 mg/liter (low-NH4+ condition), nitrification contin-
ued and NO3- concentrations stabilized at 65 to 70 mg/liter.
MTBE removal rates during and after this step change in
nutrient addition decreased from 80 to 90% to 60 to 65%,
suggesting that nitrifier populations may directly or indirectly
stabilize MTBE degradation in BC-1. The cell retention time
during the first 15 weeks was maintained at 80 to 90 days.
When the cell retention time was decreased to <50 days,
MTBE removal declined significantly to only 30% of the
influent concentration. This loss in MTBE removal indicates
that the growth of the MTBE-oxidizing organism(s) is very
slow (<0.01/day).

Biomass yields based on cell accumulation for the BC-1
culture calculated during phases of high and low nitrification
were 0.21 to 0.28 g of dry weight of cells per g of MTBE
utilized (Table 1). These low cell yields appear to be compara-
tible to those of anaerobic fermenting bacteria and autotrophs
(0.03 to 0.20 g/g) and are somewhat less than those of
aerobic-oxidizing cultures (0.3 to 0.6 g/g) growing on sugars,
alcohols, and fatty acids (1a). These results suggest that the low
growth rates and cell yields in BC-1 may be due to the slow
metabolism of a rate-limiting intermediate(s) and/or MTBE
may also be a metabolic or electron transport inhibitor or
uncoupler of ATP formation. Additional studies are required
to confirm these potential inhibitory effects on growth and
metabolism.

Metabolism of 14CH3O-MTBE. Results of the biodegrada-
tion of radiolabeled ether (2 mg/liter) by BC-1 are given in
Table 2. Less than 1 and 5% of the applied isotope was
recovered as 14CO2 and 14C-labeled cells, respectively, in the

abiotic (no-culture) control and cultures containing the respi-
ration inhibitor sodium azide (2%). About 80% of the
14CH3O-MTBE was incorporated into CO2 and cells, and the
remainder (ca. 15%) was undegraded ether and/or 14C-labeled
metabolites. Addition of 100 mg of NH4+ per liter to metab-
olizing cultures had no effect on stimulating or inhibiting
MTBE biotransformation (data not shown). These results
indicate that although nitrifiers are present in BC-1, the
MTBE-oxidizing system is apparently different from the NH3-
oxidase enzymes of these autotrophs. Little or no isolate
was metabolized to CO2 or cellular material (similar to the
azide-inhibited control) when BC-1 cultures were incubated
with 20 mg of 14CH3O-MTBE per liter (data not shown),
suggesting that at certain concentrations, MTBE may be more
an inhibitor than a substrate.

Substrate removal experiments. Results of batch substrate
depletion assays with BC-1 in the presence of MTBE and TBA
are shown in Fig. 1. MTBE (120 mg/liter) was rapidly de-
graded, within 4 h at a rate of 34 mg/g of cells per h. TBA was
formed as a transient metabolic product of MTBE breakdown.
The highest levels of TBA were reached after MTBE was
completely utilized. TBA alone and TBA formed from MTBE
decayed at a slower rate (14 mg/g of cells per h) than did
MTBE. These results provide additional evidence that BC-1
degrades MTBE to TBA as a primary intermediate. Detailed
mechanistic studies are required to determine the metabolic
pathway in BC-1 beyond TBA, as well as the nature of the
initial oxidative attack on MTBE.

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TABLE 2. Metabolism of 14CH3O-MTBE by BC-1

<table>
<thead>
<tr>
<th>Condition</th>
<th>% of applied 14CH3O-MTBE* in:</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CO2</td>
<td>Cells</td>
</tr>
<tr>
<td>Control (no cells)</td>
<td>0.2</td>
<td>4.1</td>
</tr>
<tr>
<td>BC-1 + 2% azide</td>
<td>0.9</td>
<td>5.1</td>
</tr>
<tr>
<td>BC-1</td>
<td>39.0</td>
<td>42.1</td>
</tr>
</tbody>
</table>

* Means of duplicate cultures did not differ by more than 10%.

* Cell dry weight was 2,440 mg/liter.