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Biodegradation of Methyl *tert*-Butyl Ether by *Mycobacterium* spp.

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Ethane-utilizing *Mycobacterium duvalii* TA5 and *Mycobacterium gilvum* TA27 and propane-utilizing *Mycobacterium chlorophenolicum* TCE 28 isolated from soils were able to degrade methyl *tert*-butyl ether (MTBE) cometabolically with various organic acids and carbohydrates as a carbon source. Among these strains, *M. duvalii* TA5 showed the highest MTBE degradation activity. *M. duvalii* TA5 degraded 45% of 22.7 mmol MTBE L^{-1} in 7 d cometabolically with 500 mg L^{-1} of glucose. The maximal MTBE degradation rate (*Vmax*) and the half-saturation constant (*K*m) of MTBE by resting cells of *M. duvalii* TA5 were 1,030 nmol min^{-1} mg dry $cells^{-1}$ and 6.8 mM, respectively. Among the various carbohydrates, glucose was the most efficient carbon source for MTBE degradation by *M. duvalii* TA5. *tert*-Butyl formate (TBF), *tert*-butyl alcohol (TBA), 2-hydroxy isobutyric acid (HIBA), 2-propanol, acetone, and hydroxyacetone (HA) were identified as metabolites of MTBE degradation by *M. duvalii* TA5. *M. duvalii* TA5 utilized both TBF and 2-propanol as sole energy and carbon sources. *M. duvalii* TA5 was able to degrade MTBE in the soil. The present study demonstrates that *M. duvalii* TA5 is effective in degrading MTBE and may be a useful tool for the bioremediation of MTBE-contaminated soil.

Key words: MTBE, TBA, *Mycobacterium*, *V*max, *K*m

1. Introduction

In the United States (U.S.), methyl *tert*-butyl ether (MTBE) has been used as a reformulated gasoline oxygenate additive, instead of lead, since the late 1970s. Because of its high usage, MTBE is found as a contaminant of soil and groundwater. The MTBE concentration of potable well water in several locations in the U.S. ranged from 20 to more than 200 mg L^{-1} [25]. In Southeastern Brazil the groundwater concentration of MTBE at a former industrial landfill site was 1.10 to 159 mg L^{-1} [17]. The minimal, average, and maximal groundwater concentrations of MTBE at a leaking underground storage tank site in Iowa exceeded 15 μg L^{-1}, 613 mg L^{-1}, and 99,400 mg L^{-1}, respectively[1]. MTBE was found at the relatively high concentration of 1.8 μg L^{-1} in human blood after heavy exposure to motor vehicle exhaust and gasoline fumes in Fairbanks, Alaska[18]. Within 10 h of exposure, approximately 0.9% of inhaled MTBE was excreted unchanged in human urine and 2.4% was excreted as urinary TBA[13]. MTBE may be carcinogenic[25], and MTBE contamination of drinking water in the U.S. has been found[26]. For this reason, the U.S. EPA established a drinking water advisory concentration of MTBE in the range of 20 to 40 μg L^{-1} [26]. MTBE has a high water solubility (51.3 g L^{-1} at 25°C), which complicates separating MTBE from water[25].

Bioremediation using MTBE-degrading bacteria has been proposed as a remedy for MTBE-contaminated environments. MTBE-degrading bacteria, which assimilate MTBE as the sole source of carbon and energy, include *Proteobacteria* sp. PM1[6], *Hydrogenophaga flava* ENV735[8], *Methylibium petroleiphilum* PM1[12], *Methylobacterium mesophilicum* Isolate 24[16], *Rhodococcus* sp. Isolate 33[16], *Arthrobacter ilicis* Isolate 41[16], and the mixed bacterial culture BC-1 (*Corynebacterium* sp., *Pseudomonas* sp., *Achromobacter* sp.)[20]. *Graphium* sp. ATCC 58400[7] and *Arthrobacter* sp. ATCC 27778[14] cometabolically degrade MTBE with n-butane, whereas *Mycobacterium vaccae*[10], *Xanthobacter* sp.[10], *Nocardia* sp. ENV421[24], *Nocardia* sp. ENV425 ATCC 55798[24] and *Mycobacterium vaccae* JOB5 ATCC 29678[24] cometabolically degrade MTBE with propane.

Possible pathways for MBTE degradation have been proposed by Hardison *et al.*[7] for the metabolism of MBTE to TBA and TBF by *Graphium* sp. ATCC 58400 and by Steffan *et al.*[24] for the metabolism of MTBE to pyruvic acid. However, few reports address the application of MTBE-degrading bacteria for the clean-up of contaminated environments. Whole-gene analysis of *Methylibium petroleiphilum* identified a megaplasmid carrying genes with an essential role in MBTE degradation[12]. An independent MTBE-regulating gene of degradation enzymes has also been identified[8]. A further investigation revealed the protein profile of *Mycobacterium*

austroafricanum IFP 2012 in the presence of MTBE and glucose by sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE)[15]. These genetic approaches to investigate the mechanisms underlying degradation of MBTE are continuing.

In this study, we investigated the characteristics of MTBE degradation by *Mycobacterium* spp. with the ability to assimilate ethane or propane. We also examined the pathways of MBTE degradation and the application of MBTE-degrading bacteria as a tool for the bioremediation of MTBE-contaminated soil.

2. Materials and Methods

2.1. Microorganisms and degradation experiments

For degradation experiments, we used the ethane-utilizing bacteria *Mycobacterium duvalii* TA5 and *Mycobacterium gilvum* TA27 isolated from tetrachloroethylene polluted soils. Both strains were able to utilize ethane, ethanol, and various other carbon compounds as their energy sources[27]. A propane-utilizing bacterium, *Mycobacterium chlorophenolicum* TCE 28, was isolated from field soil[11]. *M. duvalii* TA5, *M. gilvum* TA27, and *M. chlorophenolicum* TCE 28 are unable to utilize trichloroethylene (TCE) as the sole carbon source but can degrade TCE cometabolically with ethane.

The basal MM medium (in mg L^{-1}) was as follows: NH_4Cl, 2,140; K_2HPO_4, 1,170; KH_2PO_4, 450; $MgSO_4{\cdot}7H_2O$, 120; $FeSO_4{\cdot}7H_2O$, 28; $Ca(NO_3)_2{\cdot}4H_2O$, 4.8; $CaCO_3$, 100; $MnSO_4{\cdot}4\text{-}6H_2O$, 0.6; H_3BO_3, 0.05; $ZnSO_4{\cdot}7H_2O$, 0.1; $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.01; $Co(NO_3)_2{\cdot}6H_2O$, 0.6; $NiSO_4{\cdot}7H_2O$, 0.06; $CuSO_4{\cdot}5H_2O$, 0.06; and H_2SeO_4, 0.04 in water. The final pH of the medium was adjusted to 6.9.

MTBE degradation experiments were carried out as follows. *M. duvalii* TA5, *M. gilvum* TA27, or *M. chlorophenolicum* TCE 28 was inoculated into 60 mL serum bottles containing 12 mL MM medium with MTBE and various carbon sources. Propane, glucose, acetic acid, and succinic acid were used as carbon sources. Propane was injected into the headspace of the culture bottles to obtain a 10% (vol/vol) propane atmosphere. Glucose, acetic acid, and succinic acid were used at 500 mg L^{-1}.

In the experiment for the effect of glucose and MTBE concentration on MTBE degradation, glucose concentration from 100 to 2,000 mg L^{-1} and MTBE concentration from 15 to 300 μmol $bottle^{-1}$ were used. MTBE concentration was calculated by using Henry's law constant of 0.0225 (atm, 25°C)[25]. MTBE concentration of 100 mg L^{-1} is corresponding to 13.6 μmol 12 ml^{-1} or 15.0 μmol $bottle^{-1}$. The inoculums were precultured in MM medium with glucose at 500 mg L^{-1} and harvested by centrifugation. The isolated cells were washed three times with phosphate buffer (pH 7.0). The initial optical density (OD) at 660 nm of each strain was 0.02.

The MBTE-degrading ability of *M. duvalii* TA5 was assessed by using MTBE and the MTBE metabolites *tert*-butyl alcohol (TBA), *tert*-butyl formate (TBF), hydroxyacetone (HA), 2-propanol, and acetone. The culture bottles were sealed with butyl rubber caps and crimped by aluminum rings. The bacterial cultures were incubated at 30°C with shaking at 120 rpm. Bacterial growth was monitored by measuring the OD_{660} of the cultures.

2.2. Analysis of MTBE and MTBE metabolites

MTBE and the MTBE metabolites TBF, TBA, 2-propanol, and acetone were measured by means of a headspace gas chromatography-mass spectrometry (GC-MS) system (GC-17A/GCMS-QP5000, Shimadzu Co., Kyoto, Japan) equipped with a DB-1 capillary column (0.32 mm i.d.×30 m, J & W Scientific, California, USA) . MTBE concentration was caluculated by using Henry's law constant[25]. The degradation products generated by the metabolism of MTBE by *M. duvalii* TA5 were determined by the following procedure. *M. duvalii* TA5 was cultured in 12 mL MM medium containing 500 mg L^{-1} of glucose and 75 μmol MTBE in 60-mL serum bottles at 30°C, with shaking at 120 rpm, for 3 or 5 d. Culture broth was loaded and adsorbed onto a Waters Se-Pak Plus PS-2 cartridge column (Nihon Waters Corporation, Tokyo, Japan). The metabolites in the column were eluted with 10 mL 99.8% acetone and concentrated to 1 mL with a stream of nitrogen (N_2) gas. MTBE degradation and MBTE metabolites in the samples were analyzed by headspace GC-MS.

2.3. Kinetics of MTBE degradation

The kinetics of MTBE degradation were determined by using resting cells of *M. duvalii* TA5. Preparation of resting cells was as follows. Strain TA5 was cultured in basal MM medium containing 500 mg L^{-1} glucose at 30°C, with shaking at 120 rpm, for 5 d. The cells were harvested by centrifugation, washed with 0.1 M phosphate buffer (pH 7.0). MTBE degradation rates were determined as follows. MTBE and resting cells were added into 60 mL serum bottles containing 10 mL phosphate buffer (pH 7.0), and incubated at 30°C. Final resting cell concentration was 400 mg dry cells L^{-1}. MTBE was used at concentrations ranging from 0.1 mM to 30 mM. To determine the initial degradation rate, we measured the MTBE degradation amount at 0, 120, 300 and 450 minutes, periodically. MTBE degradation was stopped by the addition of 100 mg L^{-1} aminobenzotoriazole. The maximal rate of MTBE degradation (Vmax; nmol min^{-1} mg dry $cells^{-1}$) and the half-saturation constant for MTBE (Km; mM) were determined from a Lineweaver-Burk plot. The cell concentration of *M. duvalii* TA5 was converted into the dry weight equivalent by measuring the OD_{660}, where an OD value of 1 was equivalent to a dry weight of 580 mg dry cells L^{-1}.

2.4. MTBE degradation in soil

MTBE at 6 μmol and resting cells at 4.4 mg (9.8×10^9 cells) were added to a to 60-mL serum bottle with 13 g soil, with a water content of 38% and incubated at 30°C with shaking at 120 rpm for 20 d. Resting cells were

prepared by the same method as the kinetic experiment. The headspace gas was measured by GC-MS to analyze MTBE degradation and the production of TBA.

2.5. Chemicals

Acetone, glucose, acetic acid, succinic acid, and hydroxyacetone (HA) were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Pyruvic acid, 2-propanol, and 2-hydroxy isobutyric acid (HIBA) were purchased from Kanto Chemical Co. Inc. (Tokyo, Japan). MTBE and TBA were purchased from Merck (Darmstadt, Germany) and Junsei Chemical Co. Ltd. (Tokyo, Japan), respectively. TBF and 1-amonobenzotoriazol were purchased from Sigma-Aldrich (St. Louis, MO, USA), respectively. Propane (99.5%) was purchased from GL Sciences Inc. (Tokyo, Japan). All chemicals were purified reagent grade.

3. Results

3.1. Effects of carbon sources on MTBE degradation

The effects of different carbon sources on the ability of *M. duvalii* TA5, *M. gilvum* TA27, and *M. chlorophenolicum* TCE 28 to degrade MTBE are shown in Fig. 1. None of these three strains degraded MTBE in MM medium with 15.0 μmol MTBE bottle^{-1} (100 mg MTBE L^{-1}) nor could any utilize MTBE as the sole carbon and energy source (Fig. 1A). In the presence of 10% propane in the air phase, about 50%, 20%, and 20% of initial MTBE were degraded by, *M. chlorophenolicum* TCE 28, *M. duvalii* TA5, and *M. gilvum* TA27 in 6 d, respectively (Fig. 1 B). The highest level of MTBE degradation (80%) was observed by *M. duvalii* TA5 by using a mixture of glucose, acetic acid, and succinic acid, each at 500 mg L^{-1}, as the carbon and energy source. Under the same culture conditions, *ca*. 70% and 60% of MTBE were degraded by *M. gilvum* TA27 and *M. chlorophenolicum* TCE 28, respectively (Fig. 1C).

Fig. 2 shows the effect of 10% propane, acetic acid, succinic acid, and glucose as carbon sources on the degradation of MTBE by *M. duvalii* TA5. MTBE at 15.0 μmol bottle^{-1} was degraded by 20%, 30%, 30%, and 87% in 7 days with 10% propane, acetic acid, succinic acid, or glucose. This result demonstrates that glucose is the best carbon source for the degradation of MTBE by *M. duvalii* TA5.

3.2. Effect of glucose and MTBE concentration on MTBE degradation

Fig. 3 shows the effect of glucose (100–2,000 mg L^{-1}) and MTBE concentration (15, 75 and 300 μmol bottle^{-1}) on MTBE degradation, cell yield (OD_{660}) and pH by *M. duvalii* TA5 in 7 d incubation. The growth of *M. duvalii* TA5 increased with the increase of glucose concentration. The yield was about OD_{660} 1.4 at 2,000 mg L^{-1} of glucose in 7 d.

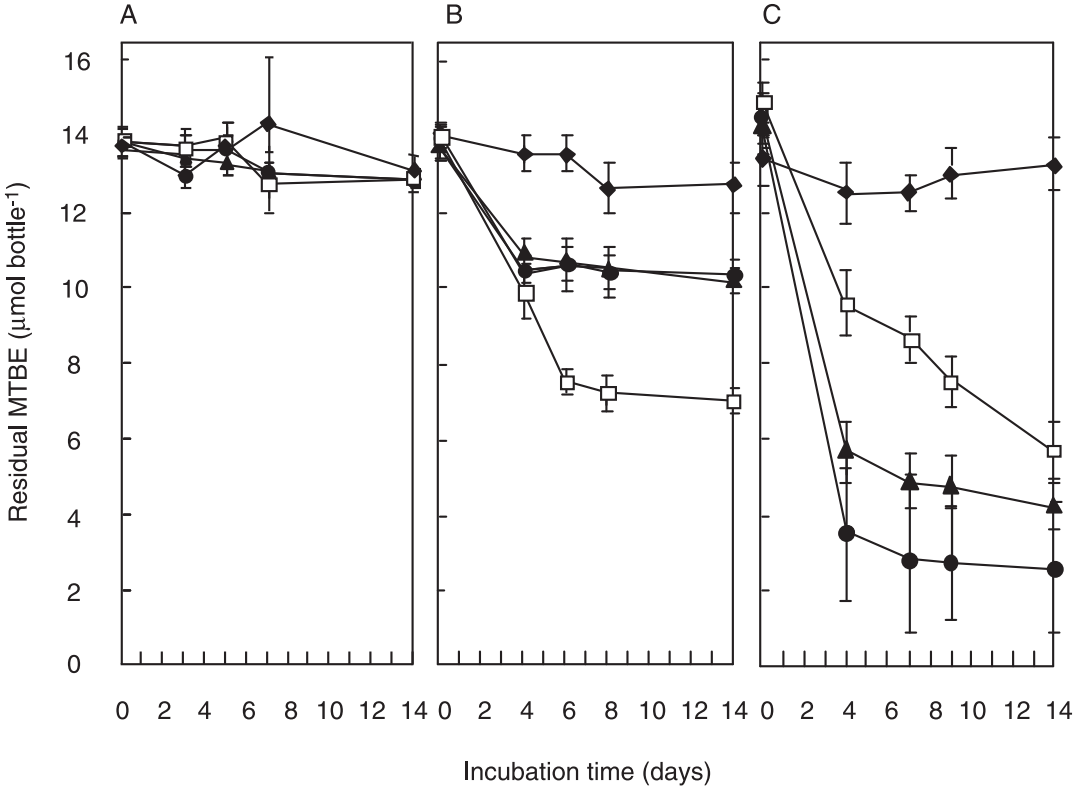

Fig. 1. Effect of carbon sources on MTBE degradation by *Mycobacterium duvalii* TA5, *Mycobacterium gilvum* TA27, and *Mycobacterium chlorophenolicum* TCE 28.
A, MM medium; B, MM medium with propane (10% in gas phase); C, MM medium with 500 mg L^{-1} each of glucose, acetic acid, and succinic acid. Symbols: ◆, without microorganism; ●, *M. duvalii* TA5; ▲, *M. gilvum* TA27; □, *M. chlorophenolicum* TCE 28
Values are means (n=3), and error bars represent standard deviation.

Good MTBE degradation was observed more than 1,000 mg L^{-1} of glucose. *M. duvalii* TA5 degraded 45% of 22.7 mmol MTBE L^{-1} (corresponding to 2,000 mg MTBE L^{-1} or 300 μmol $bottle^{-1}$) in 7 d with 500 mg L^{-1} of glucose, while MTBE was completely degraded at low concentration of 15 μmol $bottle^{-1}$ with 1,000 mg L^{-1} of glucose.

The reason was not clarified why MTBE was not completely degraded at the high concentration.

3.3. Analysis of MTBE and MTBE metabolites

The MTBE degradation products were separated by solid-phase extraction and analyzed by GC-MS. Fig. 4 shows the total ion chromatogram of MTBE and the MTBE metabolites resulting from MTBE degradation by *M. duvalii* TA5. Peaks A, B, and C appeared after 3 and 4 d of degradation, whereas peaks D and E occurred after 5 d. Peak B represents MTBE, and peak A was defined as TBA by the m/z of 39, 41, and 59 (data not shown). Peak C was determined to be TBF by the m/z of 41, 43, 56, 57, 59, and 87 (data not shown). Peak E was determined to be HIBA by the m/z of 40, 51, 69, 91, and 92 (data not shown); and peak D was identified as HA by the m/z of 43, 45, 58 and 74 (data not

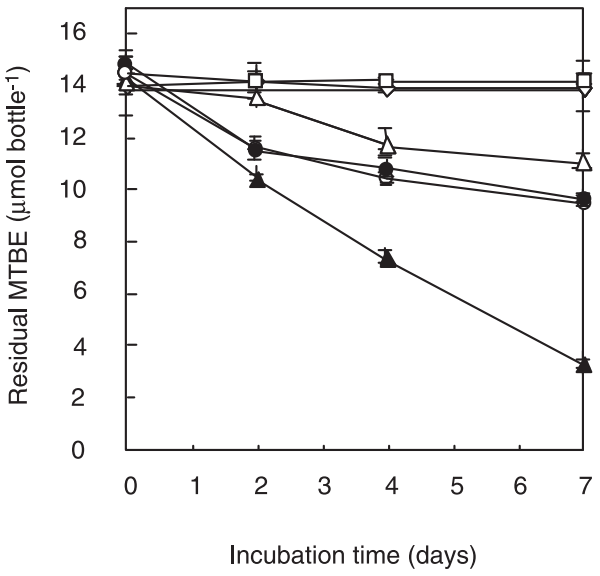

Fig. 2. Effect of carbon sources on MTBE degradation by *Mycobacterium duvalii* TA5. Symbols: □, without carbon source; △, 10% of propane in gas phase; ▲, 500 mg L^{-1} glucose; ○, 500 mg L^{-1} acetic acid; ●, 500 mg L^{-1} succinic acid.
Values are means (n=3), and error bars represent standard deviation.

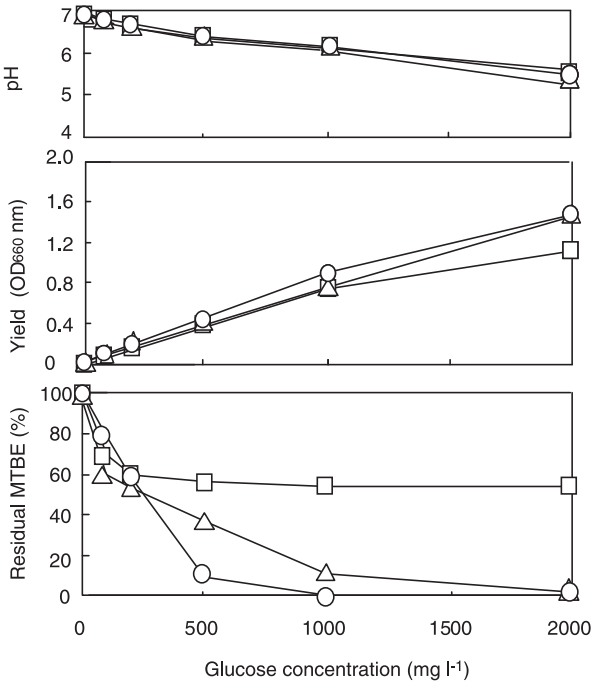

Fig. 3. Effect of glucose and MTBE concentration on MTBE degradation, yield and pH by *Mycobacterium duvalii* TA5 at 7 day incubation.
Symbols: MTBE (μmol $bottle^{-1}$) ○, 15; △, 75; □, 300

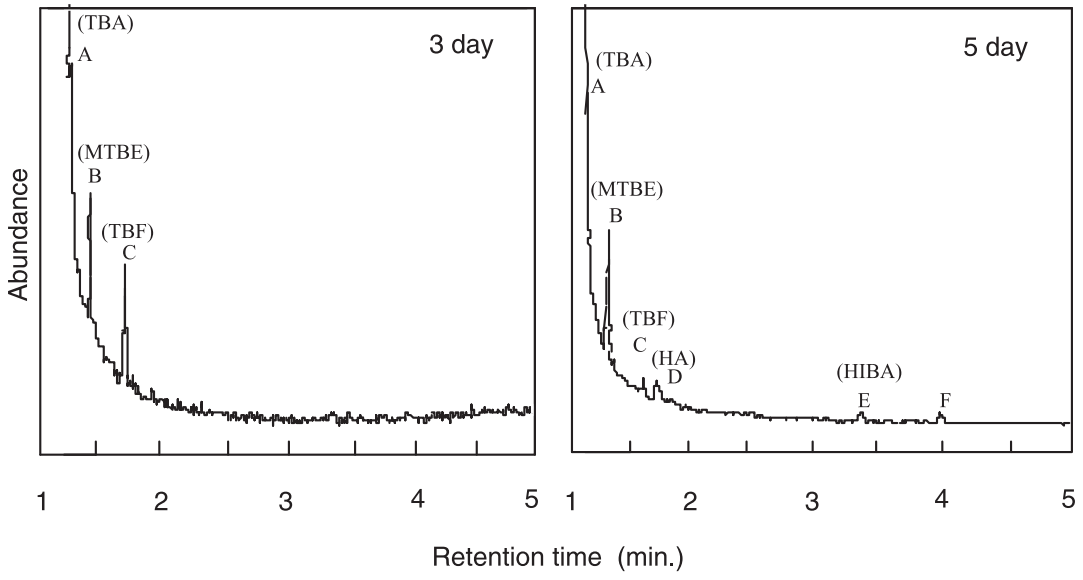

Fig. 4. Total ion chromatogram of MTBE degradation products produced by *Mycobacterium duvalii* TA5.

shown). Both TBF and TBA are products from the early stage of MTBE degradation, whereas HIBA and HA are products from the late stage of MTBE degradation.

3.4. Time course of MTBE degradation

Fig. 5 shows the time course of MTBE degradation and the formation of MTBE metabolites, including TBF, TBA, 2-propanol, and acetone, by *M. duvalii* TA5 with glucose. MTBE at 13.6 μmol bottle^{-1} was degraded by approximately 10% in 8 d. In this experiment, the MTBE degradation rate was low. It seemed that the activity of inoculated *M. duvalii* TA5 might be low. However, several metabolites were produced. After 2 d, 0.48 μmol bottle^{-1} TBF was produced and after 2 more days, the production of TBA increased and reached 1.56 μmol bottle^{-1} after 8 d. Small amounts of 2-propanol (0.24 μmol bottle^{-1}; m/z=45, 41, 27, and 59; data not shown) and acetone (0.10 μmol bottle^{-1} ; m/z=15, 39, 43, and 58; data not shown) were present after 2 d.

3.5. Degradation of MTBE metabolites

Fig. 6 shows the degradation of TBF, TBA, 2-propanol, and acetone (12 μmol bottle^{-1}, respectively) in MM medium with and without glucose by *M. duvalii* TA5. A decrease in the level of TBF of approximately 38% was observed without *M. duvalii* TA5 after 6 d (Fig. 6A). This finding is consistent with a report by Smith *et al.* (2003) that TBF is degraded by abiotic hydrolysis[22]. However, degradation of TBF by 75% and 99% was observed by *M. duvalii* TA5 alone and with glucose, respectively, after 6 d (Fig. 6A). Although TBA was not degraded by *M. duvalii* TA5 in the absence of glucose, about 50% of the TBA was degraded by *M. duvalii* TA5 in the presence of glucose after 4 d (Fig. 6B). *M. duvalii* TA5 degraded 2-propanol by *ca*. 82% in the presence of glucose but only by *ca*. 14% in the absence of glucose after 6 d (Fig. 6C). Also in the presence of glucose, *M. duvalii* TA5 degraded acetone by *ca*. 33% (Fig. 6D).

3.6. Degradation of MTBE metabolites and their degradation products

To determine degradation of MTBE metabolites and their degradation products, metabolite spike tests were performed by using TBF, TBA, 2-propanol, and acetone (12 μmol bottle^{-1}, respectively) in MM medium wituout glucose (Fig. 7). The production of TBA from TBF occurred, and the slight growth of *M. duvalii* TA5 ($DO_{660\ nm}$=0.030) suggested that *M. duvalii* TA5 assimilated TBF (Fig. 7A). In the case of TBA, a small amount of acetone was produced (Fig. 7B). In the case of 2-propanol, slight degradation of 2-propanol and acetone production accompanied slight growth of *M. duvalii* TA5 (DO_{660}=0.012, data not shown) (Fig. 7C). Acetone degradation was not clearely observed (Fig. 7D). From resuits of Fig. 6 and 7, it seems that *M. duvalii* TA5 is able to assimilate TBF, TBA, 2-propanol and acetone without glucose.

3.7. *V*max and *K*m values of MTBE degradation

*V*max and *K*m values of MTBE degradation for resting cells of *M. duvalii* TA5 are shown in Fig. 8. The *V*max and *K*m of MTBE degradation by *M. duvalii* TA5 were 1,030 nmol min^{-1} mg dry cells^{-1} and 6.8 mM, respectively.

3.8. MTBE degradation and TBA production in soil

Fig. 9 shows MTBE degradation and TBA production by *M. duvalii* TA5 in garden soil. The initial amount of MTBE was 5.6 μmol bottle^{-1}. MTBE was degraded by approximately 7% in the absence of *M. duvalii* TA5 after 10 d. However, in the presence of *M. duvalii* TA5 MTBE was degraded by approximately 60% after 10 d. Furthermore, TBA production reached the maximum level of 1.9 μmol bottle^{-1} in the presence of *M. duvalii* TA5 after 7 d and *ca.* 0.48 μmol bottle^{-1} in the absence of *M. duvalii* TA5 after 10 d.

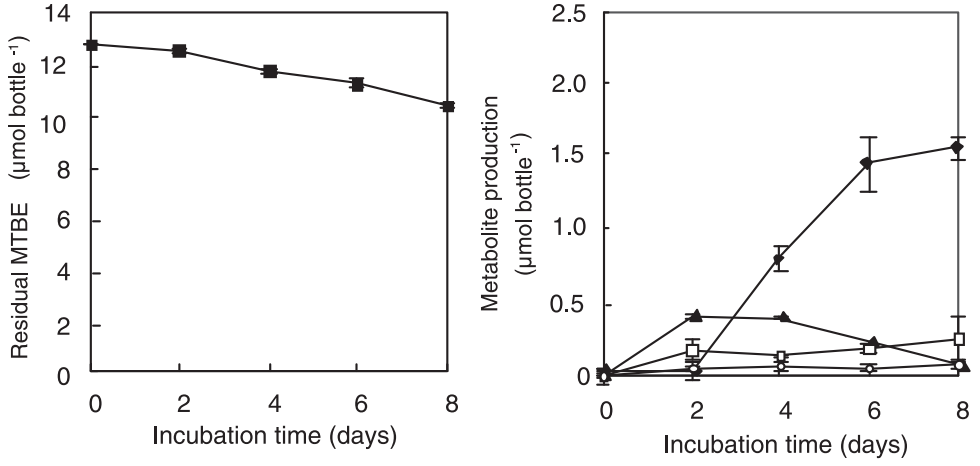

Fig. 5. MTBE degradation and metabolite produced by *Mycobacterium duvalii* TA5 in MM medium with 500 mg L^{-1} glucose and 12 μmol bottle^{-1} MTBE.
Symbols: ■ , MTBE; ▲ , TBF; ◆ , TBA; □ , 2-propanol; ○ , acetone.
Values are means (n=3), and error bars represent standard deviation.

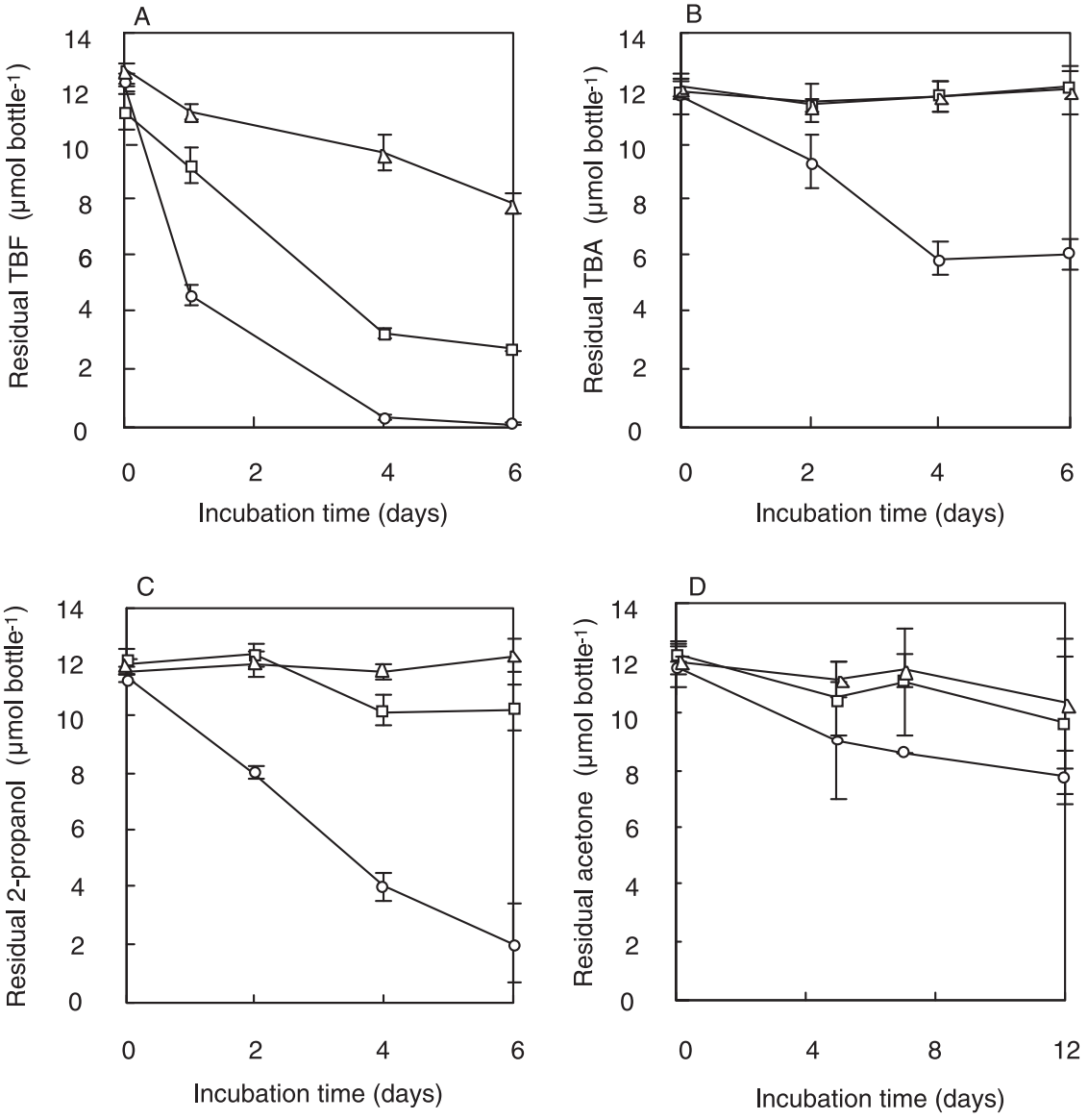

Fig. 6. Degradation of MTBE metabolites TBF, TBA, 2-propanol, and acetone by *Mycobacterium duvalii* TA5.
Symbols: ○, with glucose (500 mg L^{-1}); □, without glucose; △, without *M. duvalii* TA5 and glucose.
Values are means (n=3), and error bars represent standard deviation.

4. Discussion

Several strains of bacteria utilize MTBE as an energy and carbon source [6,8,16,20]. Some MTBE-degrading bacteria are unable to use MTBE as an energy and carbon source but can cometabolically degrade MTBE in the presence of pentane, *n*-alkane, or propane[5,7,24]. In the present study, *M. duvalii* TA5 cometabolically degraded MTBE in the presence of 10% propane in the headspace of the culture bottle. *M. duvalii* TA5 may utilize an alkane-degrading enzyme, such as propane monooxygenase, to degrade MTBE. Some *Mycobacterium* has cytochrome P-450[19]. *M. duvalii* TA5 is able to degrade MTBE with glucose. In this case, MTBE degaradation might be caused by the production of cytochrome P-450.

In the present study we determined the rates of MTBE degradation by various bacteria and calculated the *V*max and *K*m (Table 1). *Proteobacteria* sp. PM1 is reported to assimilate MTBE at a rate of 970 nmol d^{-1} mL^{-1} [6]. Three other bacterial strains, *Methylobacterium mesophilium* isolate 24, *Rhodococcus* sp. isolate 33, and *Artherobacter ilicis* isolate 41, also are capable of assimilating MTBE and were reported to degrade 200 mg L^{-1} MTBE by 29% (47.0 nmol d^{-1} mL^{-1}), 28% (45.4 nmol d^{-1} mL^{-1}), and 28% (45.4 nmol d^{-1} mL^{-1}), respectively, after 2 wk[16]. Here, *M. duvalii* TA5 cometabolocally degraded 45% of 2,000 mg MTBE L^{-1} (22.7 mmol MTBE L^{-1}) after 7 d (1,460 nmol day^{-1} mL^{-1}) in the presence of 500 mg L^{-1} glucose. Therefore, *M. duvalii* TA5 degraded MTBE at a rate 1.5 times higher than that of *Proteobacteria* sp. PM1 and about 30 times higher than those of *M. mesophilium* isolate 24, *Rhodococcus* sp. isolate 33, and *A. ilicis* isolate 41. MTBE

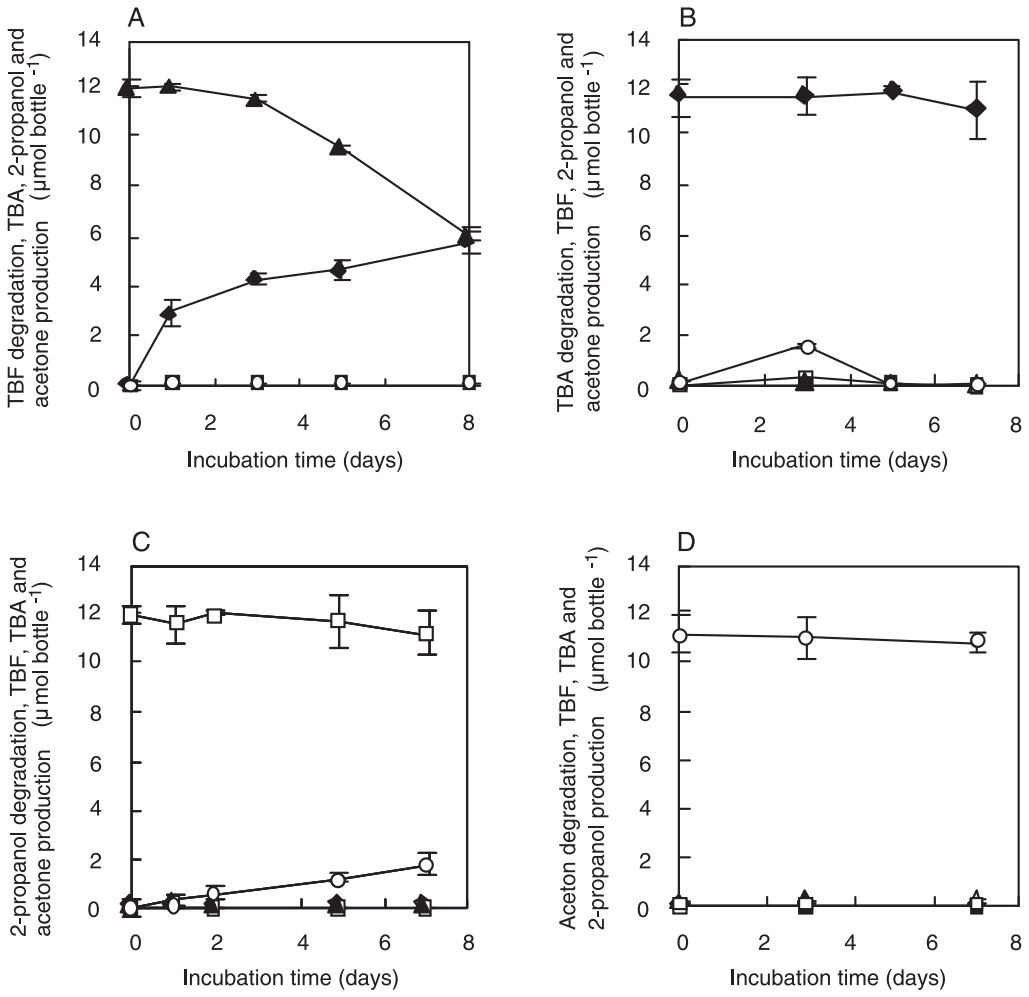

Fig. 7. Degradation of MTBE metabolites and their degradation products by *Mycobacterium duvalii* TA5.
M. duvalii TA5 was cultured in MM medium without glucose and with 12 μmol bottle^{-1} of metabolites. A, TBF; B, TBA; C, 2-propanol; D, acetone. Symbols: ▲, TBF; ◆, TBA; □, 2-propanol; ○, acetone.
Values are means (n=3), and error bars represent standard deviation.

was degraded cometabolically by *Nocardia* spp., *Mycobacterium vaccae* JOB5 ATCC 29678, *Xanthobacter* sp., *Graphium* sp. ATCC 58400, and *Arthrobacter* sp. ATCC 27778 at rates of 4.6, 12.2, 25.5, 0.18, and 3.39 nmol min^{-1} mg dry cell^{-1}, respectively. By comparison, the rate of MTBE degradation by *M. duvalii* TA5 was markedly higher, at 1,030 nmol min^{-1} mg dry cell^{-1}. The *V*max for MTBE degradation by *M. duvalii* TA5 was the highest among the bacterial strains assessed in the present study.

The rate of MTBE degradation by *M. duvalii* TA5 was low (3.2 nmol min^{-1} mg dry cell^{-1}) at a low concentration of MTBE (0.01 mM). *M. duvalii* TA5 does not have the ability to degrade MTBE at low concentrations (data not shown). The reason for this phenomenon is related to the degradation kinetics. The *K*m value of *M. duvalii* TA5 for MTBE is 6.8 mM, which is *ca.* 7, 5, and 3 times higher than the *K*m values of *Mycobacterium vaccae* (0.95 mM)[10], *Mycobacterium vaccae* JOB5 ATCC 2967 (1.4 mM)[21], and *Xanthobacter* sp. (2.4 mM)[10] for MTBE. The higher *K*m value of *M. duvalii* TA5 for MTBE compared with other bacterial strains indicates that *M. duvalii* TA5 has a lower

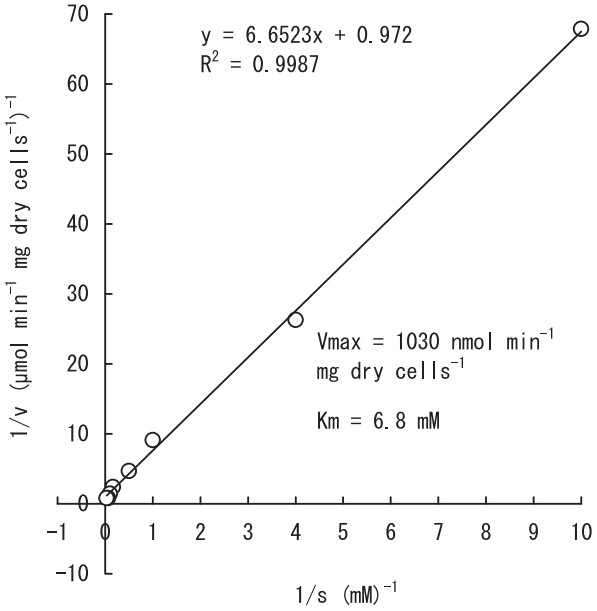

Fig. 8. Determination of *V*max and *K*m on MTBE degradation by *Mycobacterium duvalii* TA5.

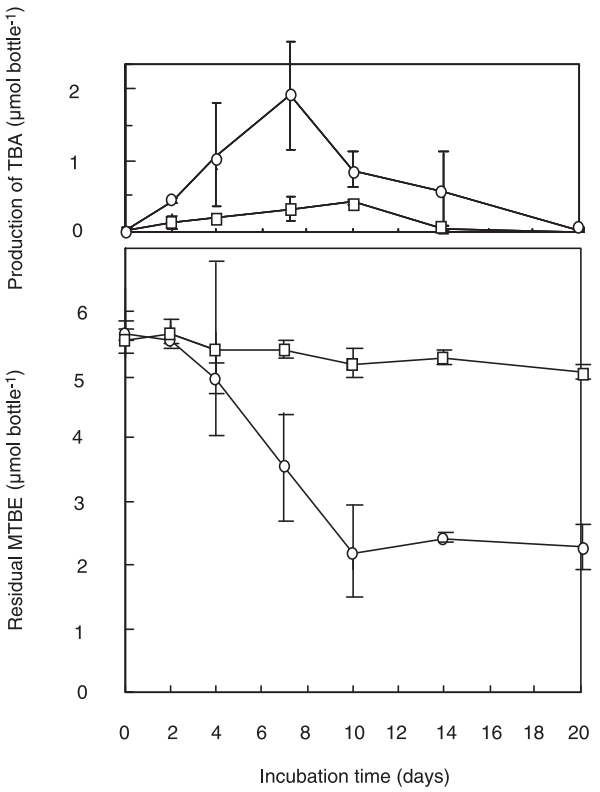

Fig. 9. MTBE degradation and TBA production by *Mycobacterium duvalii* TA5 in soil. Symbols: ○, with *M. duvalii* TA5; □, without *M. duvalii* TA5.
Values are means (n=3), and error bars represent standard deviation.

affinity for MTBE than do other bacteria.

We determined several MTBE metabolites. The MTBE degradation metabolites TBF, TBA, HIBA, and HA were analyzed by solid-phase extraction (Fig. 4). The production of TBF, TBA, 2-propanol, and acetone was quantified by headspace GC-MS analyses (Fig. 5). Our proposed pathway for the degradation of MTBE by *M. duvalii* TA5 is illustrated in Fig. 10. MTBE is degraded *via* the degradation intermediates TBF, TBA, HIBA, 2-propanol, acetone, and HA.

The following degradation pathways have been reported for various strains of bacteria. *Gordonia terrae* IFP 2007 is able to degrade MTBE to TBA[4]. *Graphium* sp.[10], *Graphium* sp. ATCC 58400[7], and *Pseudomonas mendocina* KR-1[22] have the ability to degrade MTBE via TBF to TBA. *Mycobacterium vaccae* JOB5 degrades MTBE to TBF, TBA, and 2-methyl-1,2-propanediol[21]. *Mycobacterium austroafricanum* IFP 2012 degrades MTBE via TBF and TBA to HIBA and CO_2[3]. Steffan *et al.* (1997) demonstrated that the native propane-oxygenating bacteria strain ENV425 (ATCC 55798) degraded MTBE via the intermediates TBF, TBA, 2-methyl-2-hydroxy-1-propanol, HIBA, 2-propanol, acetone, HA, and pyruvic acid[24]. Here, we show that *M. duvalii* TA5 degrades MTBE via TBF, TBA, HIBA, 2-propanol, acetone, and HA. This MTBE degradation pathway is similar to the degradation pathway reported for ENV425. Although we did not identify the degradation intermediates

Table 1. Summary of MTBE degradation rates of various bacteria

Organism	Culture condition	Type of metabolism	Metabolic or cometabolic carbon sources	MTBE degradation rate	Reference
Proteobacteria sp. PM1	Growing cell	Heterotrophic	MTBE	970 nmol d^{-1} mL^{-1}	6
Methylobacterium mesophilicum Isolate 24	Growing cell	Heterotrophic	MTBE	29% of 200 mg L^{-1} in 2 wk (47.0 nmol d^{-1} mL^{-1})	
Rhodococcus sp. Isolate 33	Growing cell	Heterotrophic	MTBE	28% of 200 mg L^{-1} in 2 wk (45.4 nmol d^{-1} mL^{-1})	16
Arthrobacter ilicis Isolate 41	Growing cell	Heterotrophic	MTBE	28% of 200 mg L^{-1} in 2 wk (45.4 nmol d^{-1} mL^{-1})	
Nocardia sp. ENV421	Resting cell	Cometabolic	Propane	9.2 nmol min^{-1} mg $protein^{-1}$	
Nocardia sp. ENV425 ATCC 55798	Resting cell	Cometabolic	Propane	4.6 nmol min^{-1} mg $protein^{-1}$	23
Mycobacterium vaccae JOB5 ATCC 29678	–	Cometabolic	Propane	Not reported	
Mycobacterium vaccae JOB5 ATCC 29678	Resting cell	Cometabolic	Propane	*V*max=24.4 nmol min^{-1} mg $protein^{-1}$, *K*m=1.4 mM	20
Mycobacterium vaccae	Resting cell	Cometabolic	Propane	*K*m=0.95 mM	10
Xanthobacter sp.	Resting cell	Cometabolic	Propane	*V*max=51 nmol min^{-1} mg $protein^{-1}$, *K*m=2.4 mM	
Mixed bacterial culture BC-1	Resting cell	Heterotrophic	MTBE	6.4 nmol min^{-1} mg dry $cells^{-1}$	19
Graphium sp. ATCC 58400	Resting cell	Cometabolic	n-Butane	10.5 nmol h^{-1} mg dry $weight^{-1}$ (0.18 nmol min^{-1} mg dry $cells^{-1}$)	7
Arthrobacter sp. ATCC 27778	Resting cell	Cometabolic	n-Butane	*V*max=0.43 mg^{-1} mg suspended $solids^{-1}$ d^{-1} (3.39 nmol min^{-1} mg dry $cells^{-1}$), *K*m=2.14 mg L^{-1} (0.02 mM)	14
Mycobacterium duvalii TA5	Growing cell	Cometabolic	Glucose or Propane	45% of 22.7 mmol L^{-1} in 7 d (1460 nmol d^{-1} mL^{-1})	This study
	Resting cell	Cometabolic	Glucose	*V*max=1030 nmol min^{-1} mg dry $cells^{-1}$, *K*m=6.8 mM	

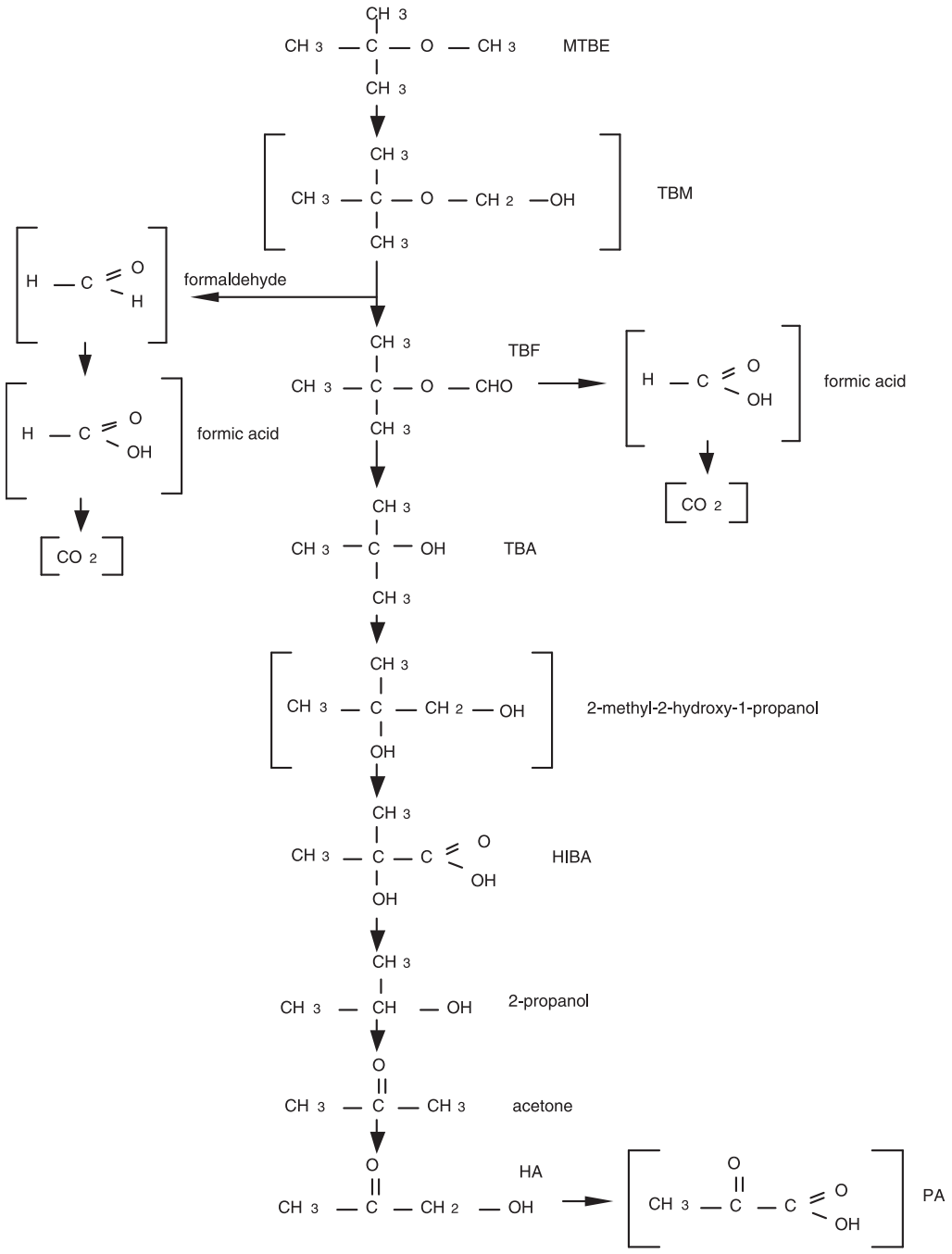

Fig. 10. Proposed degradation pathways of MTBE by *Mycobacterium duvalii* TA5.

2-methyl-2-hydroxy-1-propanol and pyruvic acid, we did confirm HA, 2-propanol, and acetone as MTBE degradation products.

Many studies highlight the potential of MTBE-degrading bacteria for the bioremediation of MTBE-contaminated soil and groundwater. Charathirakup *et al.* (2006) reported that 0.3 mg MTBE was degraded in a 10 g soil microcosm after 7 d incubation with the bacterial consortia mKGS1, consisting of 6 bacterial strains[2]. In an *in situ* bioaugmentation study, Hristova *et al.* (2003) reported that 0.11 mM (1.32 μmol 12 mL^{-1}) MTBE was completely degraded after 25 d by adding the MTBE-degrading bacteria PM1 with oxygen to MTBE-polluted groundwater at the Vandenberg Air Force Base (Lompoc, California)[9]. Spinnler *et al.* (2001) investigated the addition of a mixture of aerobic MTBE-degrading bacteria and oxygen for 6 month by means of a biobarrier system to the groundwater of a retail gasoline station polluted with MTBE in California[23].

In this experiment, we showed that *M. duvalii* TA5 degraded 53% of 5.6 μmol MTBE in bottle containing 13 g soil after 10 d. This MTBE degradation rate is almost same as the previously reported rates of MTBE-degrading bacteria. We demonstrated that *M. duvalii* TA5 is useful for the bioremediation of MTBE-contaminated soil and groundwater.

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