

## Biodegradation of Ethyl *tert*-Butyl Ether (ETBE) by *Rhodococcus erythropolis* ET10

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We isolated an ethyl *tert*-butyl ether (ETBE)-degrading soil bacterium, designated strain ET10, which was identified as *Rhodococcus erythropolis* based on morphological, physiological, and 16S rRNA gene analyses. *Rhodococcus erythropolis* ET10 was able to utilize ETBE as a sole source of growth energy and carbon. Twenty micromoles of ETBE in a 60-mL bottle containing 10 mL growth medium was completely degraded by strain ET10 in 14 days. Approximately 65% of ETBE was degraded after 19 days in cultures containing 50  $\mu\text{mol}$  of ETBE bottle<sup>-1</sup>. We determined that *tert*-butyl alcohol (TBA) was the main metabolite in these cultures. The maximum degradation rate ( $V_{\text{max}}$ ) and half-saturation constant ( $K_m$ ) of ETBE were 7.80  $\mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$  and 2.5 mM, respectively. Strain ET10 was also able to degrade ETBE in four types of soil. Our results suggest that strain ET10 may be useful for the bioremediation of ETBE-contaminated soil.

**Key words:** ETBE, TBA, *Rhodococcus erythropolis*,  $V_{\text{max}}$ ,  $K_m$

### 1. Introduction

Ethyl *tert*-butyl ether (ETBE) is an oxygenate additive that increases the octane rating of gasoline and has been used as a substitute for methyl *tert*-butyl ether (MTBE) in France, Spain, and Germany. ETBE is manufactured from isobutylene and ethanol, which is often derived from plants, including corn and sugar cane<sup>9,10</sup>. The use of ETBE derived from bioethanol contributes to reducing the levels of carbon dioxide in the exhaust of gasoline vehicles and is expected to help limit global warming. However, ETBE has toxic effects on the livers of male and female mice<sup>16</sup>, although the data of acute toxicity and carcinogenicity are not enough in the present state of affair<sup>15</sup>, and ETBE contamination has been detected in the drinking water of several areas in the Netherlands<sup>21</sup> and in groundwater in Düsseldorf, Germany<sup>19</sup>. As ETBE has poor biodegradation characteristics, it takes long time to clean up the ETBE-contaminated soil and underground water. There are several reports on the ETBE assimilating-bacteria belonging to *Gordonia terrae*<sup>4,8</sup>, *Comamonas testosteroni*<sup>12</sup>, *Rhodococcus equi*<sup>4</sup>, *Rhodococcus aetherivorans*<sup>1</sup>, and *Aquicola tertiarycarbonis*<sup>18</sup>. Therefore, the mineralization of ETBE by bacteria represents an effective method for the elimination of ETBE<sup>1</sup>. However, there is little report on the application of microorganisms to clean up the ETBE contaminated environment.

In the present study, we isolated and characterized an

ETBE-degrading bacterium from soil. Following the identification of this strain, its ETBE degradation products and activities were evaluated. Moreover, ETBE degradation and *tert*-butyl alcohol (TBA) production were investigated in four types of soil amended with this strain.

### 2. Materials and methods

#### 2.1. Media

MM medium was used for culturing bacteria present in the soil samples and contained the following components (in mg L<sup>-1</sup>): NH<sub>4</sub>Cl, 2,140; K<sub>2</sub>HPO<sub>4</sub>, 1,170; KH<sub>2</sub>PO<sub>4</sub>, 450; MgSO<sub>4</sub>·7H<sub>2</sub>O, 120; FeSO<sub>4</sub>·7H<sub>2</sub>O, 28; Ca (NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 4.8; CaCO<sub>3</sub>, 100; MnSO<sub>4</sub>·4-6H<sub>2</sub>O, 0.6; H<sub>3</sub>BO<sub>3</sub>, 0.05; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 0.1; (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>·4H<sub>2</sub>O, 0.01; Co(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, 0.6; NiSO<sub>4</sub>·7H<sub>2</sub>O, 0.06; and CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.06; in Milli-Q water. Prepared MM medium was sterilized by autoclaving at 121°C for 20 min.

#### 2.2. Chemicals

ETBE was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TBA and lactose were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Reagent-grade agar, lactose, starch, sodium acetate trihydrate, sodium succinate, glycerol, sucrose, and glucose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). TaKaRa Ex Taq<sup>®</sup> HS was purchased from (TaKaRa Bio

Inc., Siga, Japan). Purification of PCR products was used by ExoSAP-IT (GE Healthcare Tokyo, Japan). BigDye® Terminators v1.1 Cycle Sequencing Kit was used as sequencing reagent (Applied Biosystems, Tokyo Japan). Purification of the sequencing reaction was carried out by illustra™ AutoSeq G-50 Dye Terminator Removal Kit (GE Healthcare, Tokyo Japan).

### 2.3. Isolation and identification of ETBE-degrading bacteria

Ten soil samples were collected from various locations in Ibaraki Prefecture, Japan. For each sample, 0.1 g of soil was placed into a 60-mL vial containing 10 mL MM medium supplemented with 5 µmol ETBE and 5 mg glucose. The vials were sealed with butyl rubber caps and crimped with aluminum rings, and then incubated at 30°C with shaking at 120 rpm for approximately 10 to 40 days. ETBE degradation was evaluated using the headspace gas method and a gas chromatography-mass spectrometry (GC-MS) system (GC-2010/GCMS-QP2010; Shimadzu Co., Kyoto, Japan) equipped with a Rtx®-5MS capillary column (0.25 mm i.d. × 30 m; Restek Co., PA, USA). For measurement of ETBE or TBA concentration, 50 µL of headspace gas was injected into GC-MS in all experiments. ETBE or TBA-concentration was calculated by use of calibration curve as a concentration per bottle. ETBE concentration in water was calculated by using Henry's constant of 1.62 L atm mol<sup>-1</sup> (at 30°C).

Following the long-term incubation period, 0.1 mL of the growth cultures was streaked onto MM agar plates (2% agar) by use of a spreader. Ten microliters of ETBE (undiluted solution) was added to the surface of the agar plate. The plates were then sealed with Parafilm M® film (Structure Probe, Inc. and SPI Supplies, PA, USA) and incubated statically for 3 days at 30°C. Growing individual colonies were selected and inoculated into 60-mL vials containing 10 mL MM medium supplemented with 5 µmol ETBE (5 µmol ETBE bottle<sup>-1</sup>). The vials were incubated at 30°C with shaking at 120 rpm for 7 days. ETBE degradation was evaluated using the GC-MS headspace gas method, as described above. To isolate individual strains, one µL of ETBE-degrading culture was streaked onto an MM agar plate to which 10 µL ETBE was added. The purified colonies were then maintained on agar slant cultures. From this process, one strain was purified and was named as ET10. Identification of the microorganism was performed by testing for oxidation/fermentation (OF) of glucose, oxidase, catalase, motility, and spore formation, and Gram staining, colony morphology, and 16S rRNA gene analyses.

Genomic DNA was extracted by the method of Molecular Cloning of Sambrook *et al.* For the sequence analysis, the 16S rRNA gene was amplified by PCR using 27FP (GAGTTTGATCMTGGCTCAG), 518RFP (CCAGCAGC CGCGTAAT) and 1392RP (ACGGGCGGTGTGTRC) as the primers. The PCR mixture (50 µL total volume) consisted of 200 µM of each primer, 200 µM of deoxynucleotide phosphate, TaKaRa Ex Taq® HS DNA polymerase 0.25 µL,

the DNA sample and PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>). The DNA amplification was conducted by use of thermal cycler TaKaRa MP3000 (TaKaRa Bio Inc., Siga, Japan), at one cycle 94°C 3 min., 30 cycle 94°C 30 sec. 55°C 1 min. 72°C 1 min. and last one cycle 72°C 7 min. DNA sequence was measured by ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Tokyo Japan). Phylogenetic tree was plotted by a NCBI BLAST search.

### 2.4. Determination of ETBE degradation products

To investigate the ETBE degradation products produced by strain ET10, approximately 0.1 mg of strain ET10 was inoculated into a 60-mL vial containing 10 mL MM medium supplemented with 5 µmol ETBE. The culture was incubated at 30°C with shaking at 120 rpm for 6 days, and ETBE and its degradation products were determined by the headspace GC-MS method.

### 2.5. Effect of ETBE concentration on its degradation

Approximately 0.1 mg of strain ET10 was inoculated into a 60-mL vial containing 10 mL MM medium supplemented with 2.5 to 50 µmol ETBE. After sealing the vials with butyl rubber caps and crimping with aluminum rings, the cultures were incubated at 30°C with shaking at 120 rpm for 19 days. ETBE degradation and TBA production were evaluated by the headspace GC-MS method. Bacterial growth was measured turbidimetrically at 660 nm (OD<sub>660</sub>) using a spectrophotometer (UV Mini-1240; Shimadzu Co., Kyoto, Japan) and was converted to dry weight (mg L<sup>-1</sup>).

### 2.6. Effect of carbon substrate on specific growth rate and maximum yield

Approximately 0.3 mg of strain ET10 was inoculated into a 60-mL vial containing 10 mL MM medium supplemented with 5 µmol ETBE and 500 mg L<sup>-1</sup> of one of the following test organic substrates: glucose, lactose, sucrose, starch, acetic acid, succinic acid, or glycerol. The culture was incubated at 30°C with shaking at 120 rpm for 9 days. Bacterial growth was measured turbidimetrically at 660 nm (OD<sub>660</sub>) using a spectrophotometer.

Conversion efficiency (C) from substrate concentration (S) to maximum cell yield (Y) was calculated as follows.  
 $C (\%) = Y (\text{mg l}^{-1}) / S (\text{mg l}^{-1}) \times 100$

### 2.7. Kinetics of ETBE degradation

Strain ET10 was pre-cultured in a 60-mL vial containing 10 mL MM medium supplemented with 50 µmol ETBE at 30°C for 7 days. The culture was centrifuged at 1,400 × g for 30 min and then washed five times with phosphate buffer (pH 7.2). Kinetic experiments were performed using resting cells. Five milligrams of washed strain ET10 cells was inoculated in 10 mL phosphate buffer containing 0.5 to 100 µmol ETBE (0.5 to 100 µmol bottle<sup>-1</sup>), and the resulting culture was incubated at 30°C for 7 h with shaking at 120 rpm. To stop the ETBE degradation reaction, 200 µL of 1,000 mg L<sup>-1</sup> hydrazine was added to the culture, and ETBE level was then

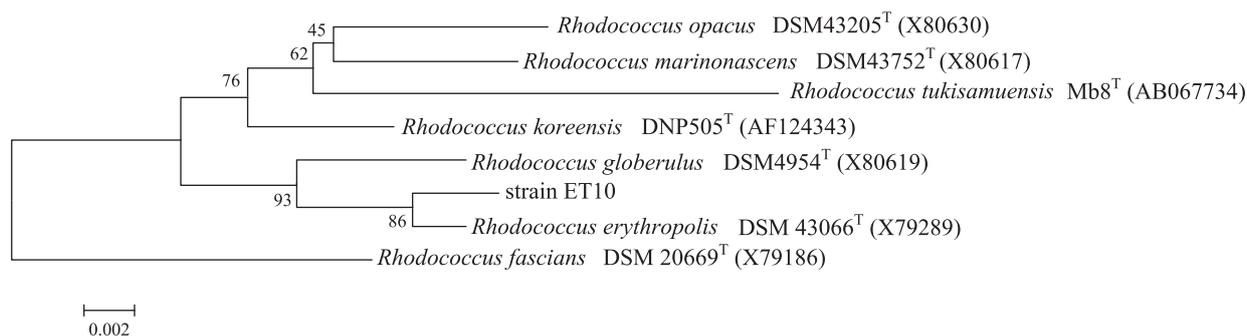


Fig. 1. 16S rRNA dendrogram showing the phylogenetic position of the ETBE-degrading soil isolate, strain ET10. The numbers above the branching points represent the bootstrap values (expressed as percentages). The bar equals 0.002 inferred sequence divergence.

measured by GC-MS. The maximum ETBE degradation rate ( $V_{\max}$ ;  $\mu\text{mol mg dry cells}^{-1} \text{min}^{-1}$ ) and half-saturation constant of ETBE ( $K_m$ ; mM) were determined from Lineweaver-Burk plots.

## 2.8. ETBE degradation and TBA production in four soil types

ETBE degradation and TBA production by strain ET10 was evaluated using four types of soil: fine sand (Hokota City, Ibaraki Prefecture, Japan), clay (Mashiko Town, Tochigi Prefecture, Japan), Andosol (Kanuma City, Tochigi Prefecture, Japan) and Kanuma soil (Kanuma City, Tochigi Prefecture, Japan). For each sample, 11.6 g of soil, 5  $\mu\text{mol}$  ETBE, and approximately 2.8 mg of strain ET10 were added to each 60-mL vial. The soil microcosms were incubated at 30°C with shaking at 120 rpm for 21 days. ETBE degradation and TBA production were determined by the headspace GC-MS method.

## 2.9. Effect of organic substances on ETBE degradation and TBA production in soil

The effects of organic substances on ETBE degradation and TBA production by strain ET10 were investigated using Andosol soil collected in Kanuma City, Tochigi Prefecture, Japan. For the analysis, 11.6 g of soil sample, 5  $\mu\text{mol}$  ETBE, 10 mL of a 500 mg  $\text{L}^{-1}$  solution of either glucose, starch, glycerol, or lactose, and 0.7 mg of strain ET10 were added to 60-mL vials. These soil microcosms were incubated at 30°C with shaking at 120 rpm for 19 days, and ETBE degradation and TBA production were then determined by the headspace GC-MS method.

## 3. Results

### 3.1. Isolation and identification of an ETBE-degrading bacterium

An ETBE-degrading bacterium was isolated from soil and designated strain ET10. The isolated strain was Gram positive, non-motile and non-spore forming, and appeared coccoid to rod-shaped. Formed colonies were smooth and pale white in color. Strain ET10 was negative for oxidation and fermentation in the OF test. Tests for oxidase and cata-

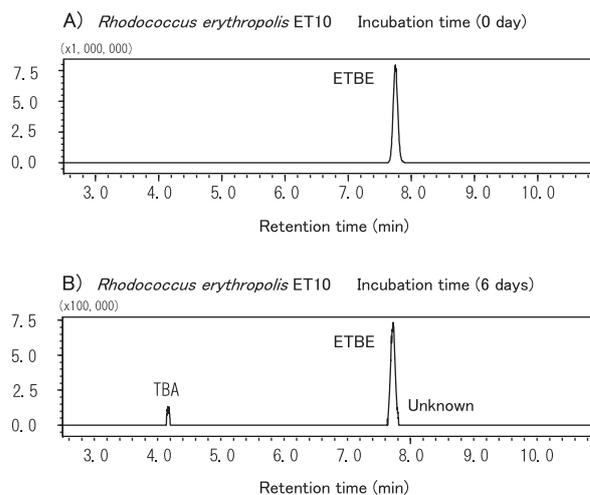


Fig. 2. ETBE and its degradation products produced by strain ET10.

lase were negative and positive, respectively.

A phylogenetic tree was constructed based on the 16S rRNA sequences of strain ET10 and the most closely related bacterial species (Fig. 1). Strain ET10 displayed the highest homology to *Rhodococcus erythropolis* DSM 43066<sup>T</sup> (X79289) (99.9%) (Fig. 1). From these characteristics, it was determined that strain ET10 was a *R. erythropolis*.

### 3.2. ETBE degradation products

Fig. 2 shows the gas chromatogram of headspace gas after culturing strain ET10 for 21 days in medium supplemented with ETBE. The retention time of TBA standard was similar to that of the main ETBE degradation product (4.2 min). Moreover, typical MS peaks at 29, 31, 41, 43, and 59  $m/z$  for TBA standard were observed for the TBA metabolite produced by strain ET10 (data not shown). Therefore, it was confirmed that the main ETBE degradation product was TBA. In addition to the main ETBE degradation product, a peak that eluted at a retention time of approximately 8.4 min was observed (Fig. 2); however, this substance was not identified.

### 3.3. Effect of ETBE concentration on ETBE degradation

The effects of ETBE concentration on its degradation, TBA production, and cell yield were next examined (Fig. 3). ETBE at 2.5, 5, 10, and 20  $\mu\text{mol bottle}^{-1}$  was completely degraded by strain ET10 in 6, 14, 19, and 14 days, respectively. At 50  $\mu\text{mol ETBE bottle}^{-1}$ , approximately 65% of ETBE was degraded in 19 days. The degradation rate decreased with increasing ETBE concentration.

TBA production increased with the increase of incubation time. Maximum TBA production by strain ET10 was

observed after 6 days at low ETBE concentrations (2.5, 5, and 10  $\mu\text{mol ETBE bottle}^{-1}$ ) and after 14 and 19 days at the highest examined ETBE concentrations (20 and 50  $\mu\text{mol ETBE bottle}^{-1}$ ) (Fig. 3). The levels of TBA increased with increasing ETBE concentration. However, TBA was not degraded by strain ET10. At 2.5, 5, 10, 20, and 50  $\mu\text{mol ETBE bottle}^{-1}$ , maximum cell yields of 53, 56, 69, 88, and 104  $\text{mg L}^{-1}$ , respectively, were observed. Bacterial cell yield increased with increasing concentration of ETBE added to the cultures (Fig. 3).

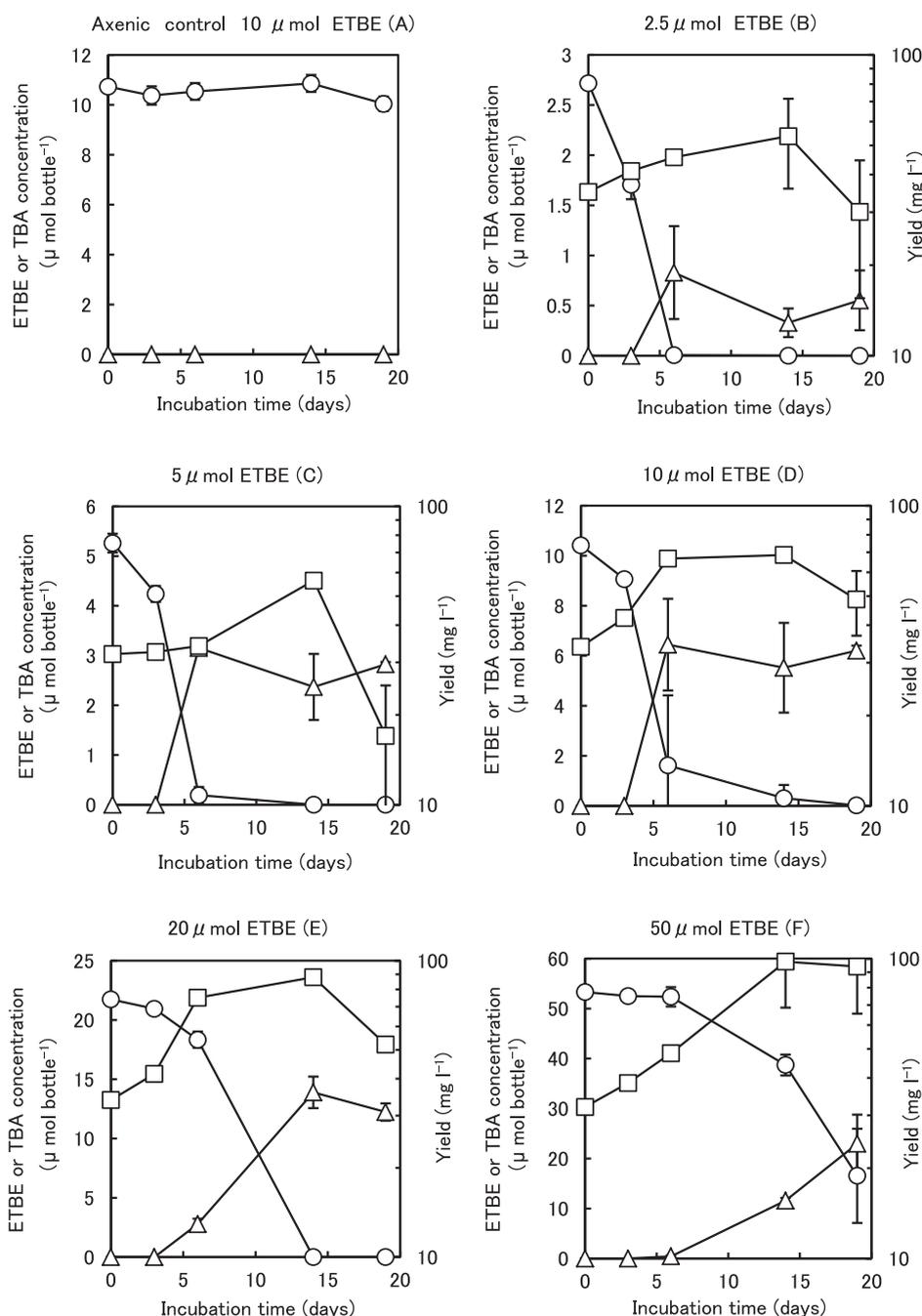


Fig. 3. Effect of ETBE concentration on the degradation of ETBE, production of TBA, and growth of strain ET10.  $\circ$ , ETBE;  $\triangle$ , TBA; and  $\square$ , Yield. Values are presented as means ( $n=3$ ), and error bars represent standard deviation.

Table 1. Effect of carbon substrate on the specific growth rate and maximum yield of *Rhodococcus erythropolis* ET10.

Substrates	Specific growth rate (day <sup>-1</sup> )	Maximum cell yield (mg L <sup>-1</sup> )	Conversion efficiency (%)
ETBE (50 μmol 10 mL <sup>-1</sup> = ca. 510 mg L <sup>-1</sup> )	0.10	104	20.4
Lactose (500 mg L <sup>-1</sup> )	0.08	9	1.8
Succinic acid (500 mg L <sup>-1</sup> )	0.13	94	18.8
Starch (500 mg L <sup>-1</sup> )	0.14	53	10.6
Glucose (500 mg L <sup>-1</sup> )	0.15	223	44.6
Glycerol (500 mg L <sup>-1</sup> )	0.15	274	54.8
Sucrose (500 mg L <sup>-1</sup> )	0.18	236	47.2
Acetic acid (500 mg L <sup>-1</sup> )	0.25	117	23.4

### 3.4. Specific growth rate and yield

Table 1 shows the effects of organic substances on the specific growth rate (day<sup>-1</sup>) and maximum cell yield (mg L<sup>-1</sup>) of strain ET10. The highest specific growth rate was 0.25 day<sup>-1</sup> in cultures supplemented with acetic acid, while the highest cell yield of 274 mg L<sup>-1</sup> was observed with glycerol. Cultures of strain ET10 supplemented with glycerol also displayed the highest conversion efficiency of cell yield (54.8%). The conversion efficiency of ETBE (20.8%) was similar in the presence of acetic acid (23.4%) and succinic acid (18.8%).

### 3.5. $V_{max}$ and $K_m$ of strain ET10

Fig. 4 shows a Lineweaver-Burk plot determined by the ETBE degradation rate (μmol mg dry cells<sup>-1</sup> min<sup>-1</sup>)<sup>-1</sup> of strain ET10 at various ETBE concentrations (mM)<sup>-1</sup>. The maximum degradation rate ( $V_{max}$ ) and half-saturation constant ( $K_m$ ) of ETBE were 0.13 μmol mg dry cells<sup>-1</sup> min<sup>-1</sup> (7.80 μmol mg dry cells<sup>-1</sup> h<sup>-1</sup>) and 2.5 mM, respectively.

### 3.6. ETBE degradation and TBA production in soil

ETBE degradation and TBA production by strain ET10 were examined in four types of soil (Fig. 5). Strain ET10 completely degraded the 5 μmol ETBE added to the fine sand, clay, Andosol, and Kanuma soils after 4 days. TBA production increased with ETBE degradation and reached a maximum at 7 days for four soil types. TBA was completely degraded in the fine sand, clay, and Kanuma soils by 21 days. In the absence of strain ET10, a slight reduction of ETBE was observed in the four soil types; however, TBA production was not detected. The reduction of ETBE was likely caused by its absorption to soil particles.

Fig. 6 shows the effect of organic substances on ETBE degradation by strain ET10 in Andosol. In the absence of organic substances, ETBE was degraded within 13 days. ETBE was degraded in 2, 4, 9, and 9 days in Andosol supplemented with glycerol, glucose, lactose, and starch, respectively (Fig. 6A). The ETBE degradation rate by strain ET10 clearly increased following the addition of organic substances. TBA generated as a metabolite was also degraded (Fig. 6B).

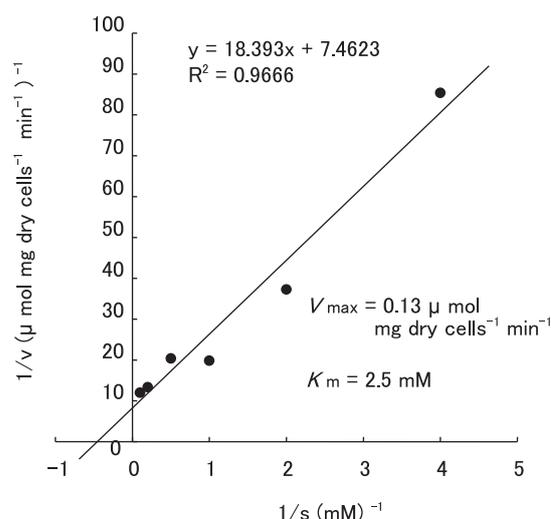


Fig. 4. Determination of  $V_{max}$  and  $K_m$  for ETBE degradation by strain ET10.

## 4. Discussion

Numerous microorganisms, including *Gordonia terrae* IFP 2001<sup>4,8)</sup>, *Comamonas testosteroni* E1<sup>12)</sup>, Centre for Disease Control group A-5 E2<sup>12)</sup>, a microbial consortium<sup>13)</sup>, *Rhodococcus equi* IFP 2002<sup>4)</sup>, *Rhodococcus aetherivorans* IFP 2017<sup>1)</sup>, *Aquicola tertiarycarbonis* L108<sup>18)</sup>, and a mixture of filamentous microorganisms<sup>11)</sup>, are able to assimilate ETBE as an energy and carbon source (Table 2). Here, we isolated an ETBE-assimilating bacterium, strain ET10, which was identified as *R. erythropolis*. Among strains of *R. erythropolis* identified to date, ET10 is the first that is capable of assimilating ETBE. It is well known that members of the genus *Rhodococcus* have high metabolic versatility and catabolize a wide range of compounds. Thus, *Rhodococcus* spp., such as strain ET10, may be useful for the bioremediation of ETBE and other harmful chemicals in contaminated environments.

The degradation rates of ETBE by *G. terrae* IFP 2001 and *R. equi* IFP 2002<sup>4)</sup> are 3.72 and 1.37 μmol mg protein<sup>-1</sup> h<sup>-1</sup>, respectively. We determined that the degradation rate of ETBE by strain ET10 is 7.80 μmol mg dry cells<sup>-1</sup> h<sup>-1</sup> (corresponding to 15.6 μmol mg protein<sup>-1</sup> h<sup>-1</sup>), a value that represents the highest ETBE degradation rate among pure

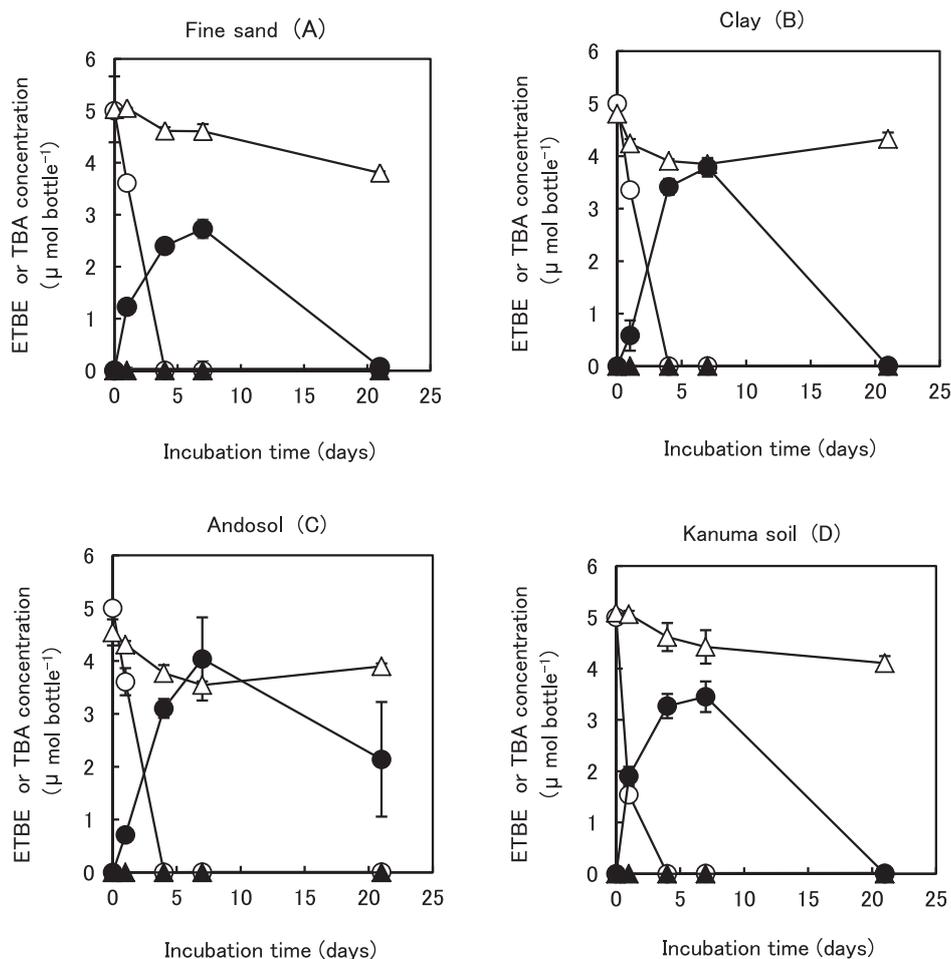


Fig. 5. ETBE degradation and TBA production by strain ET10 in four types of soil.

○, ETBE concentration with strain ET10; ●, TBA concentration with strain ET10; △, ETBE concentration without strain ET10; ▲, TBA concentration without strain ET10. Values are presented as means ( $n=3$ ), and error bars represent standard deviation.

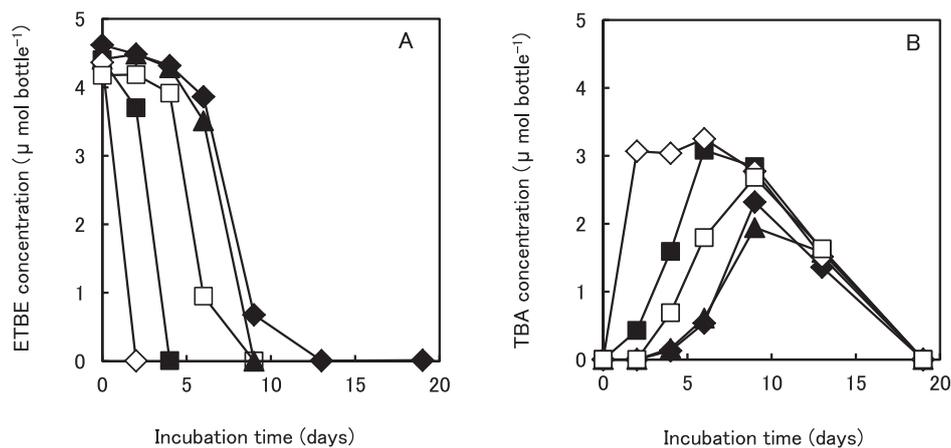


Fig. 6. Effect of organic substances on the degradation of ETBE (A) and production of TBA (B) by strain ET10 in Andosol.

◆, Without organic substances; ■, glucose; ▲, starch; ◇, glycerol; □, lactose

cultures of ETBE-assimilating bacteria reported to date (Table 2).

It has been demonstrated that *Mycobacterium vaccae* JOB5 co-metabolically degrades ETBE with propane or 2-propanol at rates of 1,250 and 1,000  $\mu\text{g L}^{-1} \text{h}^{-1}$ , respec-

tively<sup>20</sup>. Moreover, propane-oxidizing strain ENV425 co-metabolically degrades ETBE with propane, 2-propanol, ethanol or acetone at rates of 324, 174, 174, and 50  $\mu\text{g L}^{-1} \text{h}^{-1}$ , respectively<sup>20</sup>. Another propane-oxidizing strain, ENV420, is capable of co-metabolically degrading ETBE in the presence

Table 2. Summary of ETBE degradation rates of various bacteria.

Microorganism	Type of metabolism	Metabolic or cometabolic carbon sources	ETBE degradation rate	Reference
<i>Gordonia terrae</i> IFP 2001	Assimilation	ETBE	$V_{\max} = 3.72 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	4)
<i>Gordonia terrae</i> IFP 2001	Assimilation	ETBE	$3.54 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	8)
<i>Comamonas testosteroni</i> E1	Assimilation	ETBE	$0.03 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	12)
Centre for disease control group A-5 E2	Assimilation	ETBE	$0.02 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	12)
Microbial consortium	Assimilation	ETBE	$0.93 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	13)
<i>Rhodococcus equi</i> IFP 2002	Assimilation	ETBE	$V_{\max} = 1.37 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	4)
<i>Rhodococcus aetherivorans</i> IFP 2017	Assimilation	ETBE	$0.12 \mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$	1)
<i>Aquicola tertiarycarbonis</i> L108	Assimilation	ETBE	$V_{\max} = 1.11 \mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$	18)
Mixture of filamentous microorganisms (batch tests)	Assimilation	ETBE	$V_{\max} = 0.21 \mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$	11)
Mixture of filamentous microorganisms (upflow fixed-bed reactor)	Assimilation	ETBE	$V_{\max} = 0.06 \mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$	11)
<i>Rhodococcus erythropolis</i> ET 10	Assimilation	ETBE	$V_{\max} = 7.80 \mu\text{mol mg dry cells}^{-1} \text{ h}^{-1}$ 100% of $167 \text{ mg L}^{-1}$ for 14 days ( $497 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	This study
<i>Mycobacterium vaccae</i> JOB5	Cometabolism	Propane	100% of $30 \text{ mg L}^{-1}$ for 24 h ( $1,250 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
	Cometabolism	2-Propanol	80% of $30 \text{ mg L}^{-1}$ for 24 h ( $1,000 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
Propane oxidizing bacterial strain ENV425	Cometabolism	Propane	26% of $30 \text{ mg L}^{-1}$ for 24 h ( $324 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
	Cometabolism	2-Propanol	14% of $30 \text{ mg L}^{-1}$ for 24 h ( $174 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
	Cometabolism	Ethanol	14% of $30 \text{ mg L}^{-1}$ for 24 h ( $174 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
	Cometabolism	Acetone	4% of $30 \text{ mg L}^{-1}$ for 24 h ( $50 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
Propane oxidizing bacterial strain ENV420	Cometabolism	Ethanol	31% of $30 \text{ mg L}^{-1}$ for 24 h ( $390 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	20)
MTBE degradable consortia mKMS	Cometabolism	Nutrient broth	18% of $100 \text{ mg L}^{-1}$ for 30 days ( $25 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	2)
MTBE degradable consortia mKGS1	Cometabolism	Nutrient broth	25% of $100 \text{ mg L}^{-1}$ for 30 days ( $35 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	2)
MTBE degradable consortia mKGS2	Cometabolism	Nutrient broth	23% of $100 \text{ mg L}^{-1}$ for 30 days ( $32 \mu\text{g L}^{-1} \text{ h}^{-1}$ )	2)
<i>Pseudomonads</i> containing consortium	Cometabolism	n-pentane	$V_{\max} = 0.78 \mu\text{mol mg protein}^{-1} \text{ h}^{-1}$	17)

$V_{\max}$ : Maximum degradation rate

of ethanol at a rate of  $390 \mu\text{g L}^{-1} \text{ h}^{-1}$ . The ETBE-degrading consortia mKMS, mKGS1, and mKGS2 co-metabolically degrade ETBE with nutrient broth at rates of 25, 35, and  $32 \mu\text{g L}^{-1} \text{ h}^{-1}$ , respectively<sup>2)</sup>. As the degradation rate of ETBE by strain ET10 was found to be  $497 \mu\text{g L}^{-1} \text{ h}^{-1}$ , it is clear that strain ET10 has a relatively high ETBE degradation capacity.

Several studies have investigated ETBE degradation enzymes. Notably, cytochrome P-450 has been demonstrated to function as a catalyst for monooxygenase in *G. terrae* IFP 2001 after induction with ETBE<sup>3,8)</sup>. It is reported that *R. ruber* IFP 2001, *R. zopfii* IFP2005, and *Gordonia* sp. IFP 2009 are able to degrade ETBE with cytochrome P-450 and CYP249<sup>14)</sup>. Although cytochrome P-450 appears to play an important role in ETBE degradation, we could not detect cytochrome P-450 in strain ET10. Therefore, further investigation of the mechanisms of ETBE oxidation, including the role of cytochrome P-450, are needed.

In MM medium, strain ET10 was able to degrade ETBE but not to degrade TBA that was derived from ETBE (Fig. 3). However, in the case of the four examined soil microcosms, ETBE and its main metabolite TBA were degraded with strain ET10 (Fig. 5).

*R. aetherivorans* IFP 2017, *G. terrae* IFP 2001, and *R. equi* IFP 2002 also transform ETBE to TBA, whereas TBA is not utilized by these bacteria<sup>1)</sup>, as the tertiary alcohol

structure of TBA is resistant to degradation<sup>4,5)</sup>. In contrast, *Mycobacterium austroafricanum* IFP 2015<sup>6)</sup> and 2012<sup>7)</sup>, and *Aquicola tertiarycarbonis* L108 are able to grow on TBA<sup>18)</sup>. As we observed TBA degradation in soil, it is possible that indigenous TBA-degrading bacteria are present in the four soil types examined here. Notably, glycerol, glucose, lactose, and starch promoted ETBE degradation by strain ET10 (Fig. 6). Taken together, our results suggest that strain ET10 may be useful for the clean-up of ETBE-contaminated environments, particularly soil, where organic substances are limited.

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