Enhanced cometabolic degradation of methyl tert-butyl ether by a Pseudomonas sp. strain grown on n-pentane

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Abstract. When methyl tert-butyl ether (MTBE) is added as oxygenates it increases the octane number and decreases the release of nitric oxide from the incomplete combustion of reformulated gasoline. The extensive use of MTBE allowed it to be detectable as a pollutant in both ground-level and underground water worldwide. The present study focuses on the isolation and characterization of MTB-degrading microorganisms by cometabolism based on the results of growth on different carbon sources. It also focuses on the kinetic analysis and the continuous degradation of MTBE. A bacterial strain WL1 that can grow on both n-alkanes (C5-C8) and aromatics was isolated and named *Pseudomonas sp*. WL1 according to the 16S rDNA sequencing analysis. Strain WL1 could cometabolically degrade MTBE in the presence of n-alkanes with a desirable degradation rate. Diverse n-alkanes with different lengths of carbon chains showed significant influence on the degradation rate of MTBE and accumulation of tert-butyl alcohol (TBA). When strain WL1 cometabolically degraded MTBE in the presence of n-pentane, higher MTBE-degrading rate and lower TBA-accumulation were observed (Vmax = 38.1 nmol/min/mgprotei, Ks = 6.8 mmol/L). In the continuous degrading experiment, the removal efficiency of MTBE by *Pseudomonas sp*. WL1 did not show any obvious decrease after five subsequent additions.

1. Introduction

Cometabolism is defined as the capability of microorganisms to transform the non-growth substrate in the presence of a growth substrate or another biodegradable substrate [1]. It helps amplify the range of biodegrading substrates for microorganisms. Until now, our knowledge about the complete mechanism of cometabolism has been limited. Some studies suggest that microorganisms utilize the primary substrate (growth-supporting substrate) for cell growth, and that they also induce enzymes for the transformation of the growth substrate. In turn, this can also catalyze the conversion of the non-growth substrate [2]. Cometabolism has been confirmed in the elimination of alkane, aromatics, and chlorinated compounds in nature [3, 4], including MTBE.

MTBE is the most frequently used gasoline additive because of its favorable characteristics, such as low production cost, a high octane rating, and the ability to mix with other gas components at any ratio. With the widespread increase in reports of MTBE contamination in underground water, great concern has been raised about its elimination [5]. Due to the presence of a stable ether bond and sterically hindered tertiary carbon in its molecular structure, MTBE is relatively recalcitrant to microbial degradation [6]. It has been reported that both direct-metabolism and cometabolism could potentially degrade MTBE. Only a few microorganisms can grow with MTBE as the sole carbon source, including *Methylibium petrol EIP hilum* PM1 [7], *Aquincola tertiaricarbonis* L108 [8], and

Hydrogenophaga flava ENV735 [9]. Most MTBE-degrading microorganisms transform MTBE via cometabolism. Various compounds present in the gasoline are able to use the cometabolic growth substrates for MTBE degradation (some of these are normal and branched alkanes [6, 10, 11], and aromatics [12]). Smith et al. reported that *Pseudomonas mendocina* KR-1 cometabolically degraded MTBE with an average degradation rate of 61.1 nmol/min/$mg_{protein}$, when grown on *n*-alkanes (C_5-C_8). Although MTBE was consumed, stoichiometric accumulation of *tert*-butyl alcohol (TBA) was observed [13]. Two MTBE-degrading strains, *Pseudomonas aeruginosa* BM-B-450 and *Pseudomonas citronellolis* BM-B-447, were isolated from *n*-pentane-adapted consortium with different kinetic parameters for MTBE, which suggested the cooperative degradation in the consortium [6].

One accepted hypothesis of metabolic pathway is that MTBE integrates with one oxygen atom with the help of monooxygenase and produces tert-butyl formate (TBF), which is subsequently hydrolyzed to TBA. Non-specific monooxygenase enzymes are responsible for the initial oxidation of MTBE, including cytochrome P450 monooxygenases [14, 15] and alkane hydroxylase [16, 17]. Furthermore, alkane hydroxylases and their close homologs appear to be widely distributed, especially among gram-negative organisms that utilize n-alkanes as growth substrates [18]. This may help interpret why alkanes are the preferred cometabolic substrates for the degradation of MTBE. Smith et al. found that an alkane hydroxylase expressed by n-octane grown P. putida GPo1 was responsible for MTBE oxidation [16].

The growing reports of the worldwide contamination of aquifer by MTBE have raised concerns regarding the development of effective elimination techniques of MTBE. Among these, biological treatments are recognized as cost-effective and environmentally friendly options. Therefore, the present study's objective was to isolate the single strain that could degrade MTBE cometabolically from the consortium grown on n-octane. From there, we studied the cometabolic characterizations of this strain, including the range of the cometabolic substrate, the kinetic characterization, and the continuous degradation of MTBE. Though there were a few reports on the cometabolism of MTBE with single Pseudomonas strains grown on either n-alkanes or aromatics, the single strain isolated in this study could grow on both n-alkanes (C5-C8) and aromatics (toluene and xylene). The amplified range of growth substrates of the isolated strain will undoubtedly increase biomass production and improve its adoption in a further application. These results provide a basis for further efforts to eliminate MTBE from natural environments.

2. Materials and methods

2.1. Strains and media

Microbial consortium RS was cultivated in salty mineral medium (MSM) with n-octane and MTBE [19]. The MSM [20] for MTBE degradation was designed as follows (g/L): KH_2PO_4 0.9, $Na_2HPO_4{\cdot}12H_2O$ 6.5, $(NH4)2SO_4$ 0.4, $MgSO_4{\cdot}7H_2O$ 0.2, $CaCl_2{\cdot}2H_2O$ 0.01, $FeSO_4{\cdot}7H_2O$ 0.001, and 1mL original stock solution containing the following trace elements in 1 L of deionized water: 0.1 g of H_3BO_3, 0.4 g of $CoCl{\cdot}6H_2O$, 0.25 g of $ZnCl_2$, 1 g of $MnSO_4{\cdot}H_2O$, 0.25 g of $Na_2MoO_4{\cdot}2H_2O$, 0.1 g of $NiSO_4{\cdot}6H_2O$, and 0.25 g of $CuCl_2{\cdot}2H_2O$. The pH of the medium was adjusted to 7.0 and the medium was then autoclaved at 115 °C for 15 min. The single strain used in this study was isolated from mixed culture RS [19]. The whole-grown mixed RS culture was diluted and smeared on MSM (n-octane + MTBE) agar plates for screening single colonies. Various individual colonies were first cultivated in LB broth overnight and then cultivated for a second analysis. Cell growth was measured by monitoring the optical density of the culture at 600 nm (OD600) in a Model 680 Microplate Reader (BioRad, CA, USA).

2.2. Chemicals

Xi'an Standard Gas Station supplied all gaseous alkanes (methane, ethane, propane, and n-butane). N-pentane (99% purity), n-hexane (99% purity), n-heptane (99% purity), n-octane (99% purity), MTBE

(99% purity) and TBA (99% purity) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.3. Identification of the isolated strain

Genomic DNA of a single, isolated strain was extracted with the Bacterial Genomic DNA Extraction Kit (Generay Biotech, Shanghai, China) per the manufacturer's instructions. Fragments of 16S rDNA were amplified from the DNA extraction with universal primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The amplification reaction mixtures (50 μL) contained 1μL of template DNA, 1.5 U of Takara TaqTM polymerase (Takara Biotech, Dalian, China), 5 μL of 10× PCR buffer, 2μL each of 10 μM forward and reverse primer, and 5μL of 2.5mM stock dNTP mixture. Amplifications were carried out on Long Gene® Thermal Cycler A300 (Long Gene, Hangzhou, China) under the following conditions: 94 ^{o}C for 5 min, then 40 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min. The PCR product after purification was cloned onto a TA cloning vector pMD-18T (Takara Biotech, Dalian, China), and sent to Sangon (Sangon Biotech, Shanghai, China) for Sanger sequencing.

Phylogenetic analysis of the 16S rDNA sequence was performed on MEGA 5.1 software [21]. Alignments with the Gen Bank database were generated based on the Clustal method [22]. A phylogenetic tree was constructed via a neighbor-joining method.

2.4. The cometabolic culture conditions of the microorganisms

All the cometabolic MTBE-degrading experiments were conducted in triplicate in 125 mL serum bottles sealed with Teflon Mininert valves with 20 mL of MSM. The carbon source was added to the bottles with syringes at a concentration of 50 mg/L in the presence or absence of MTBE (10 mg/L). The bottles were incubated at 30°C with shaking at 150 rpm. Cell growth was measured by monitoring the optical density of the culture at 600 nm (OD600).

The continuous degrading experiments were performed as described above. Strain WL1 was cultivated in MSM with n-pentane (50 mg/L) and MTBE (10 mg/L) as the substrates. The n-pentane and MTBE were spiked at the time their concentrations were below 5% of initial levels. This addition was conducted four subsequent times.

2.5. Kinetic determination

For the kinetic experiments of the isolated strain, new cells were harvested from the culture with various initial concentrations of MTBE. Cell samples were heated to 65 °C in 3 M sodium hydroxide for 30 minutes. Then, the insoluble material was centrifuged in a Thermo Scientific Sorvall ST 40 Centrifuge before determining its protein content by Folin phenol method. Bovine serum albumin (BSA) was used as a standard. The rate of MTBE oxidation was derived from the final TBA concentration, assuming that the rate of oxidation of MTBE remained constant for the first 30 minutes and that no further oxidation of TBA occurred during the reaction period. The kinetic constants were derived via computer-fitting of the data by nonlinear regression to a single substrate-binding model $y = Vmax \cdot [x/(Ks + x)]$ with Origin version 6.1.

2.6. Analytical method

MTBE and TBA concentrations were analyzed by a headspace solid-phase dynamic extraction-gas chromatography-mass spectrometry (HS-SPDE-GC/MS) method. Liquid culture was collected immediately for HS-SPDE analysis on a CTC CombiPAL-xt auto sampler (Chromtech, Idstein, Germany) equipped with a PDMS/AC syringe (90% polydimethylsiloxane and 10% activated carbon coated) as the aforementioned method [19]. The sample absorbed on the syringe was automatically injected into a Trace GC ULTRA (Thermo Finnigan, Milano, Italy) gas chromatograph fitted with an HP-5MS capillary column (30 m, 0.32 mm ID, 1 μm film) and a TraceISQ (Thermo Finnigan, TX, USA) mass spectrometric detector. The injection was pulsed split less for 1 minute. The oven

temperature was maintained at 40°C for 3 minutes and ramped to 120°C at 10°C/min. The flow rate of carrier gas (Helium 5.0) was 1.0 mL/min. The interface and ion source temperatures remained at 280 °C and 230 °C, respectively. The mass spectrometry was operated in electron impact mode at 70 eV in selected ion monitoring (SIM) mode at m/z 73 and 53 for MTBE and TBA, respectively. The MTBE and TBA were quantified using standards of known concentrations [19].

3. Results and discussion

3.1. The isolation and identification of MTBE-cometabolic-degrading strain

One of the key factors that constrain the development of biodegradation of MTBE is the extremely low biomass of microorganisms grown on MTBE. The potential alternative is cometabolism, confirmed during the process of MTBE elimination. Cometabolism describes the transformation of a non-growth substrate when the microbes are cultivated with another compound as a growth substrate. Four single colonies were isolated from a mixed culture with n-octane as the sole carbon source. To determine the single strain with higher biomass production on optimum growth substrate, n-alkanes, and aromatics were tested for their capability to support the growth of the isolated strains. As shown in Table 1, all four strains could grow on the aromatic compounds tested. However, all the isolated strains were unable to use any gaseous n-alkanes (C1 to C4) but grew well on the liquid n-alkanes tested (C5 to C8) with a much higher biomass. Among the four single strains, strain WL1 showed the maximum biomass concentration cultivated with n-alkanes. Furthermore, the final biomass production of WL1 cultivated with different n-alkanes decreased with the length of carbon chain of n-alkanes. MTBE or TBA could not be utilized as the growth substrate of *Pseudomonas sp*. WL1.

Table 1. The cell growth of the four different single strains on potential growth substrates.

Potential growth subatrates[a]	OD_{600} for 5 days cultivation[b]			
	WL1	WL3	WL4	WL6
n-Alkanes				
Methane	<0.01	<0.01	<0.01	<0.01
Ethane	<0.01	<0.01	<0.01	<0.01
Propane	<0.01	<0.01	<0.01	<0.01
n-Butane	<0.01	<0.01	<0.01	<0.01
n-Pentane	0.285 ± 0.013	0.132 ± 0.019	0.192 ± 0.018	0.086 ± 0.003
n-Hexane	0.238 ± 0.010	0.196 ± 0.033	0.227 ± 0.024	0.124 ± 0.007
n-Heptane	0.211 ± 0.013	0.123 ± 0.006	0.175 ± 0.016	0.136 ± 0.018
n-Octane	0.175 ± 0.026	0.145 ± 0.013	0.184 ± 0.026	0.179 ± 0.021
Aromatics				
Benzene	0.022 ± 0.008	0.045 ± 0.004	0.062 ± 0.006	0.086 ± 0.011
Toluene	0.074 ± 0.013	0.031 ± 0.008	0.095 ± 0.004	0.075 ± 0.017
o-Xylene	0.094 ± 0.006	0.051 ± 0.006	0.033 ± 0.007	0.096 ± 0.006
p-Xylene	0.088 ± 0.018	0.062 ± 0.016	0.038 ± 0.009	0.114 ± 0.021
m-Xylene	0.082 ± 0.003	0.057 ± 0.012	0.045 ± 0.003	0.089 ± 0.014
MTBE and its metabolites				
MTBE	<0.01	0.012 ± 0.005	<0.01	<0.01
TBA	<0.01	<0.01	<0.01	<0.01

[a] Triplicate cultures of mixed ERS were grown for 5 days in the presence of each substrate at an initial substrate concentration of 0.02% vol/vol (the initial value of OD_{600} was 0.01).
[b] All optical densities reported are the means ± SD.

Finally, a pure strain *Pseudomonas sp*. WL1, characterized as being gram-positive, short, and rod-shaped, was selected for further study. The 16S rDNA analysis revealed that strain WL1 belongs to the Pseudomonas genus. *Pseudomonas plecoglossicida* NBRC 103162 was found to be phylogenetically closest to the isolated strain WL1, showing 99% similarity between the nucleotides of the 16S rDNA

genes (figure 1). The 16S rDNA sequence of the isolated WL1 strain was stored in NCBI GenBank under the accession number of KC217655.

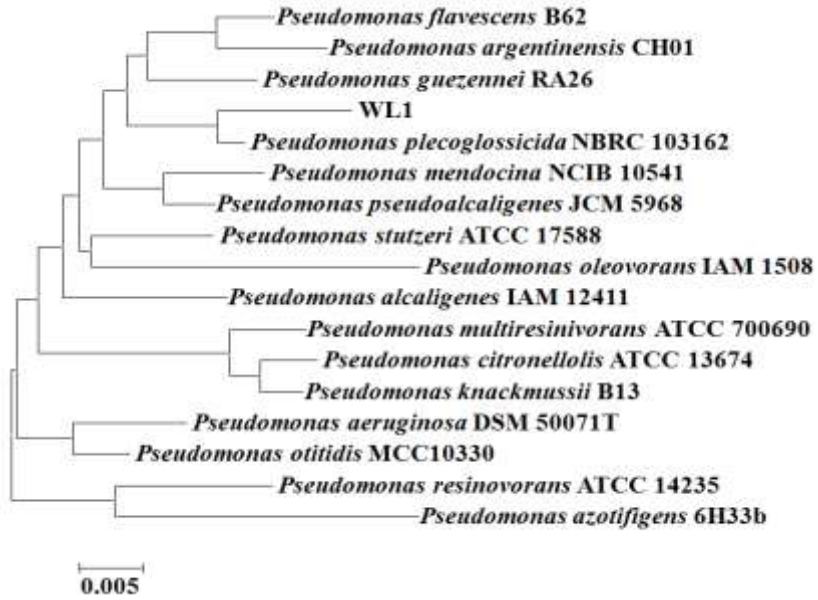

Figure 1. Phylogenetic tree of *Pseudomonas* sp.
WL1 based on 16S rDNA sequences.

The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. All positions containing gaps and/or missing data were eliminated from the dataset (complete deletion option).

Based on the reports of previous studies, Pseudomonas genus was found in numerous environmental niches and characterized as capable of degrading various pollutants, such as polycyclic aromatic hydrocarbon (PAH) [23, 24], benzene [25], and alcohols [26, 27]. Additionally, the higher the biomass production, the lower the nutritional demand of Pseudomonas genus strains to make them meet the requirements of industrial biotechnology [28]. Furthermore, lots of typical strains from the Pseudomonas genus have been sequenced for whole-genome, including P. aeruginosin PAO1 [29], P. putida KT2440 [30] and P. mendocina NBRC 14162 [31]. The thick substrates scope and easy accessibility of genetic bioinformations make Pseudomonas genus strains ideal for further use in biotechnology and bioremediation.

3.2. Cometabolic degradation of MTBE with different n-alkanes as growth substrate

Cometabolism is defined as a microorganism's capability to transform any kind of non-growth substrate in the obligatory presence of a growth substrate [2]. Cometabolism has been confirmed in the degrading processes of many pollutants, such as chlorinated compounds [32, 33], aromatic hydrocarbons [34, 35], and gasoline additives [36], including MTBE [10, 14].

Various studies have identified that microorganisms can cometabolically degrade MTBE efficiently when cultured with a variety of growth substrates, such as alkanes, aromatics, and alicyclics [10, 37]. Though strain WL1 was isolated from a mixed culture inoculated with n-octane as the sole carbon source, other n-alkanes and aromatics were tested for their capability to support the cometabolical degradation of MTBE (table 2). For the aromatic compounds tested, *Pseudomonas sp*. WL1 was able to grow on toluene or xylene (low biomass yield), accompanied by the slow degradation of MTBE. All the n-alkanes tested in this study (C5-C8) could support the growth of *Pseudomonas sp*. WL1 with a much higher degradation rate than that of aromatic compounds. Further, as the carbon number of the n-alkanes increased, both the degradation rate of n-alkanes and MTBE (from 178.4

mgMTBE/gprotein/h to 59.3 mgMTBE/gprotein/h) decreased. After two days' cultivation, low levels of TBA accumulation (less than 7%) were observed for all the n-alkanes tested. The simplest interpretation of this phenomenon is that strain WL1 could synthesize the enzyme that can catalyze the oxidation of TBA. This result is similar to the result reported by Morales et al. [6]. Pseudomonas aeruginosin showed no TBA accumulation when the initial ratio of MTBE and n-pentane was below 0.7, indicating complete MTBE mineralization. In the reported MTBE-degrading-microorganisms by cometabolism, the oxidation of MTBE and TBA could be catalyzed by the same monooxygenase [38] or different ones [16]. These results were similar to the reports for MTBE cometabolism by Garnier et al. [39]. All n-alkanes (n-pentane, n-hexane, and n-heptane) tested for cometabolic activity did support the degradation of MTBE. Among them, n-pentane was the most efficient with a deterioration rate of (200 μg/d). In another study, the cometabolic degradation rate of MTBE increased with the carbon number of the n-alkanes used as the growth substrate by P. mendocina KR-1 [13]. Although MTBE was transformed at a high cometabolic degrading rate by P. mendocina KR-1 (61.1 nmol/min/mgprotein), the corresponding accumulation of TBA was observed for all n-alkanes tested (C5 to C8). This indicated the absence of the enzyme catalyzed in the transformation of TBA in strain KR-1.

A cometabolic coefficient (CC) was used to represent cometabolic efficiency. The CC value is defined as the ratio of degraded MTBE to the consumed cometabolic substrate [6]. The lowest and highest CC values of 0.03 and 0.72 for *Pseudomonas sp*. WL1 were obtained from the growth substrate of toluene and n-pentane, respectively (table 2). A lower CC value for aromatic substrates was compatible with a lower degradation rate of MTBE with different alkanes. When cultivated with n-alkanes, the CC value by *Pseudomonas sp.* WL1 decreased in correlation to an increase in the growth substrate's carbon number. The shorter-chain alkane (more soluble than the longer one) could be transferred to the microorganisms more quickly; this may be the potential reason why the CC value obtained from shorter-chain alkane was higher than that from longer-chain alkane.

Table 2. Specific degradation rates and products by *Pseudomonas* sp. WL1 on diverse substrates.

Carbon source[a]	Biomass ($mg_{protein}$ /d)	Degradation rate of substrates[b] ($mg_{substrate}/g_{protein}/h$)	Degradation rate of MTBE[b] ($mg_{MTBE}/g_{protein}/h$)	CC ($mg_{MTBE}/mg_{substrate}$)	Residual TBA[c] (mg/L)
Aromatic substrate					
Benzene	0.3 ± 0.1	3.6 ± 0.2	0	-	-
Toluene	8.3 ± 0.7	247.3 ± 7.9	4.6 ± 0.1	0.03	0.12
Xylene	9.5 ± 0.5	435.8 ± 10.5	12.3 ± 0.9	0.11	1.34
n-Alkanes					
n-Pentane	15.2 ± 1.1	1393.2 ± 78.1	178.4 ± 8.4	0.72 ± 0.2	0.14 ± 0.1
n-Hexane	14.6 ± 1.4	1096.7 ± 56.4	130.2 ± 9.2	0.56 ± 0.1	0.56 ± 0.1
n-Heptane	12.2 ± 0.4	841.7 ± 53.9	72.3 ± 7.1	0.44 ± 0.1	0.67 ± 0.1
n-Octane	10.9 ± 0.8	681.6 ± 45.8	59.3 ± 6.9	0.26 ± 0.1	0.26 ± 0.1
Others					
MTBE	-	-	-	-	9.5 ± 0.2
TBA	-	-	-	-	9.8 ± 0.3

[a] Microbial consortium ERS were cultivated in glass serum vials (125 mL) contained mineral salt medium (20 mL) on diverse carbon source (0.02%, w/v%) in the presence (20 μmol) of MTBE. After 2 days' growth, the concentrations of biomass were examined. All the cultures on diverse carbon sources were performed in triplicate. All the values were the means of triplicate ± SD.

[b] After 2 days cultivation, the amounts of residual carbon source and MTBE in each culture were determined by SPDE-GC-MS. The degradation rates of growth substrate and MTBE were calculated, respectively.

[c] After 2 days cultivation, the amount of TBA produced in each culture was determined by SPDE-GC-MS.

3.3. The kinetic characteristics of cometabolism of MTBE by Pseudomonas sp. WL1 on n-alkanes

The oxidation of TBA, the first stable metabolite detected in MTBE degradation, is the limited step of the mineralization of MTBE. The apparent TBA accumulation during the first period of MTBE oxidation by n-alkane-grown cells indicated that the kinetics of MTBE oxidation could be detected directly by determining the rates of accumulation of TBA. Values of Ks and Vmax for MTBE oxidation were determined for pure culture WL1 grown on n-pentane and n-octane, respectively (figure 2). When cultivated with the equivalent concentration of MTBE and n-alkane with different carbon numbers (C5 and C8) under the same conditions, the pure culture of *Pseudomonas sp*. WL1 showed different kinetic parameters. For example, n-pentane-oxidizing WL1 cells exhibited a Vmax value of 38.1 nmol/min/mgprotein and Ks value of 6.8 mmol/L for MTBE, whereas n-octane-grown WL1 cells had a Vmax value of 19.6 nmol/min/mgprotein and a Ks value of 17.2 mmol/L. The potential interpretation for the variable kinetic parameters of WL1 culture for MTBE on different n-alkanes would be the water solubility of each substrate (C5>C8). The Henry's Law constant of n-pentane and n-octane was 8.1×10-4 M/atm and 3.14×10-4 M/atm (at 25 °C), respectively [40]. When incubated with the same initial concentration of n-pentane or n-octane, the higher dissolved quantity of n-pentane facilitated its capture by the WL1 cells and then induced the transcription of alkane monooxygenase, improving the degradation of n-pentane and MTBE.

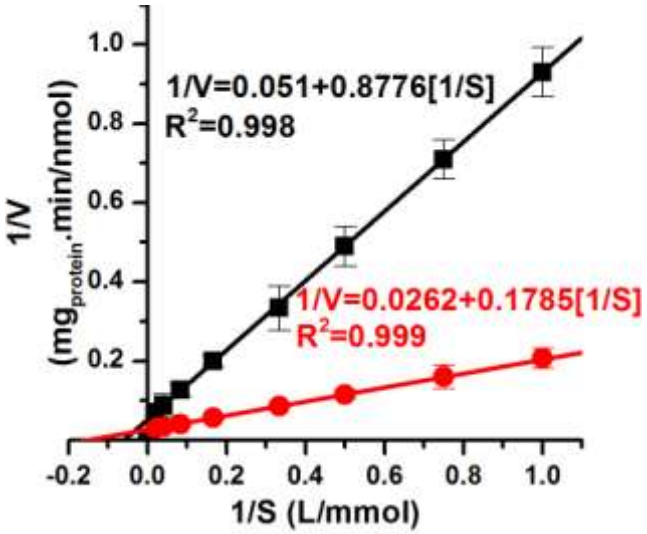

Figure 2. Kinetics of MTBE oxidation by pure strain.
WL1 with *n*-pentane and *n*-octane as a substrate cometabolic.

Values represent means of triplicate samples, and error bars represent the standard deviation of the average. The figure shows the average rate of MTBE oxidation by strain WL1 on n-pentane (filled circles) or n-octane (filled squares).

Another of the study's key findings was that the Ks value for MTBE of strain WL1 was much higher than the values of cometabolically MTBE-degrading microorganisms on the growth substrate of n-alkanes. For instance, the Ks value for MTBE obtained from Arthrobacter sp. ATCC27778 [41] on butane was 24 μmol/L, and another n-alkanes-oxidizing P. mendocina KR-1 had a Ks value of 12.95 μmol/L for MTBE [13]. The clearest interpretation of this result is that variable alkane monooxygenases responsible for the transformation of MTBE are involved in these strains. Particular affinity (aºMTBE = Vmax/Ks) is generally used as an index reflecting substrate specificity [6]. The value for Pseudomonas sp. WL1 (aºMTBE = 0.34 L/gprotein/h) on n-pentane was almost four times higher than the value on n-octane (aºMTBE = 0.07 L/gprotein/h), mainly due to the significant difference in Ks values. Similar aºMTBE values were obtained from P. mendocina KR-1 (0.28 L/gprotein/h), which also has higher Ks values [13]. Though higher aºMTBE values were calculated

for Xanthobacter sp. (1.27 L/gprotein/h) [42] and M. vaccae JOB5 (1.08 L/gprotein/h) [18], TBA accumulation was observed as occurring hand in hand with the MTBE degradation process.

3.4. Degradation of MTBE by Pseudomonas sp. WL1 grown on n-pentane in continuous culture

Further research on the cometabolic degradation of MTBE with *n*-pentane as the growth substrate was conducted to determine the continuous degrading ability of strain WL1. MTBE (10 mg/L) and *n*-pentane (50 mg/L) were added when the residual concentrations of both MTBE and *n*-pentane in the cultures fell below 5% of their initial concentrations. Figure 3 below shows the degradation curves of MTBE and *n*-pentane after five subsequent feeding and degrading cycles. Pure strain WL1 can cometabolically degrade MTBE (10 mg/L) at a rate of 45.3-52.5 $mg_{MTBE}/g_{protein}/h$ with *n*-pentane (50 mg/L) as the growth substrate. A distinct lag phase was observed in the MTBE-degradation curve (in comparison with the *n*-pentane-degradation curve), where *n*-pentane transformed itself immediately once it was added. The first degradation cycle of MTBE and *n*-pentane was completed in two days. After additional, *n*-pentane degraded continuously, the removal of MTBE followed. We observed that the degradation rate of MTBE did not decrease after the fifth (and final) addition.

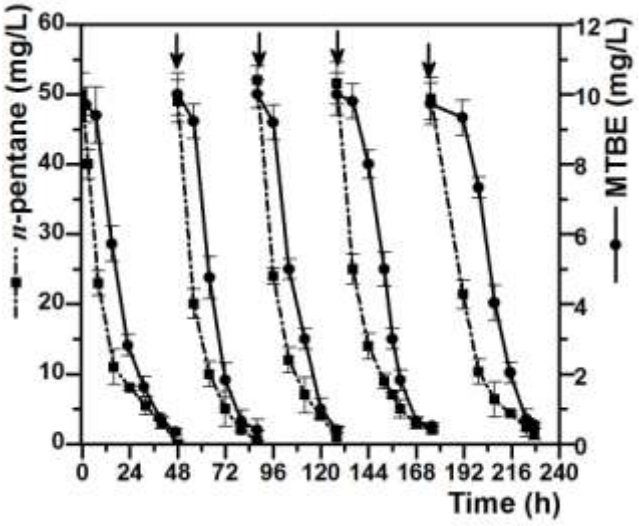

Figure 3. Cometabolic degradation of MTBE with n-pentane as growth substrate. Vertical arrows indicate the additions of MTBE and n-pentane. Values represent means of triplicate samples, and error bars represent standard deviation of the mean.

These results demonstrate that *Pseudomonas sp*. WL1 had the cometabolic capability for the continuous degradation of MTBE when cultivated with n-pentane as the growth substrate. As reported by Fortin et al., a mixed culture F-consortium can degrade MTBE in the presence or after incubation of diethyl ether (DiEE) after two respikes. MTBE would only be transformed after the complete degradation of the cometabolic carbon source, indicating that MTBE biodegradation undergoes some inhibition [43]. Volpe *et al*. inoculated a microbial consortium in a batch reactor wby repeatedly feeding it with MTBE and oxygen. After several instances of feeding and depletion, a significant decrease of the timeframe of complete degradation of MTBE was observed [37].

MTBE has most often been detected in fields of gasoline-contaminated soil and/or in groundwater. This detection is often accompanied with the detection of other gasoline components, such as alkanes. This confirms the growth substrates of MTBE-degrading microorganisms. Various studies have been conducted to solve the problem of MTBE elimination. Both n-alkanes (C5-C8) and aromatics (toluene and xylene) could be used as the growth substrates for MTBE degradation by the *Pseudomonas sp*. WL1 that was isolated in this study. This finding broadens the range of cometabolites for MTBE in a single strain. No visible TBA accumulation was observed during the process of MTBE degradation in the presence of n-pentane by WL1, suggesting complete MTBE mineralization. In the continuous degrading experiment, the removal efficiency of MTBE by *Pseudomonas sp*. WL1 did not show a noticeable decrease after five respikes. The present work seeks to provide a microbial source and

theoretical groundwork for further bioremediation of MTBE-contaminated aquifers, a topic of real environmental significance.

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References

[1] Tran N H, Urase T, Ngo H H, Hu J Y and Ong S L 2013 Insight into metabolic and cometabolic activities of autotrophic and heterotrophic microorganisms in the biodegradation of emerging trace organic contaminants *Bioresource Technol* **146** 721-31
[2] Arp D J, Yeager C M and Hyman M R 2001 Molecular and cellular fundamentals of aerobic cometabolism of trichloroethylene *Biodegradation* **12** 81-103
[3] Criddle C S 1993 The kinetics of cometabolism *Biotechnology and bioengineering* **41** 1048-56
[4] Horvath R S 1972 Microbial co-metabolism and the degradation of organic compounds in nature *Bacteriological Reviews* **36** 146-55
[5] Johnson R, Pankow J, Bender D, Price C and Zogorski J 2000 Peer reviewed: MTBE-To what extent will past releases contaminate community water supply wells? *Environmental Science & Technology* **34** 210A-7A
[6] Morales M, Nava V, Velasquez E, Razo-Flores E and Revah S 2009 Mineralization of methyl tert-butyl ether and other gasoline oxygenates by Pseudomonads using short n-alkanes as growth source *Biodegradation* **20** 271-80
[7] Kane S R, Chakicherla A Y, Chain P S, Schmidt R, Shin M W, Legler T C, Scow K M, Larimer F W, Lucas S M, Richardson P M and Hristova K R 2007 Whole-genome analysis of the methyl tert-butyl ether-degrading beta-proteobacterium Methylibium petroleiphilum PM1 *J. Bacteriol* **189** 1931-45
[8] Rosell M, Barcelo D, Rohwerder T, Breuer U, Gehre M and Richnow H H 2007 Variations in 13C/12C and D/H enrichment factors of aerobic bacterial fuel oxygenate degradation *Environmental Science & Technology* **41** 2036-43
[9] Streger S H, Vainberg S, Dong H and Hatzinger P B 2002 Enhancing transport of hydrogenophaga flava ENV735 for bioaugmentation of aquifers contaminated with methyl tert-butyl ether *Appl. Environ. Microbiol* **68** 5571-9
[10] Salazar M, Morales M and Revah S 2012 Biodegradation of methyl tert-butyl ether by cometabolism with hexane in biofilters inoculated with Pseudomonas aeruginosa *Journal of Environmental Science and Health. Part A, Toxic/hazardous Substances & Environmental Engineering* **47** 1017-26
[11] Steffan R J V S, Condee C W, McClay K and Hatzinger P B 2000 *Biotreatment of MTBE with a new bacterial isolate*
[12] Steffan R J, McClay K, Vainberg S, Condee C W and Zhang D L 1997 Biodegradation of the gasoline oxygenates methyl tert-butyl ether, ethyl tert-butyl ether, and tert-amyl methyl ether by propane-oxidizing bacteria *Appl. Environ. Microb.* **63** 4216-22
[13] Smith C A, O'Reilly K T and Hyman M R 2003 Cometabolism of methyl tertiary butyl ether and gaseous n-alkanes by Pseudomonas mendocina KR-1 grown on C5 to C8 n-alkanes *Appl. Environ. Microbiol.* **69** 7385-94
[14] Malandain C, Fayolle-Guichard F and Vogel T M 2010 Cytochromes P450-mediated degradation of fuel oxygenates by environmental isolates *FEMS Microbiology Ecology* **72** 289-96
[15] Chauvaux S, Chevalier F, Le Dantec C, Fayolle F, Miras I, Kunst F and Beguin P 2001 Cloning of a genetically unstable cytochrome P-450 gene cluster involved in degradation of the

pollutant ethyl tert-butyl ether by Rhodococcus ruber *J. Bacteriol* **183** 6551-57
[16] Smith C A and Hyman M R 2004 Oxidation of methyl tert-butyl ether by alkane hydroxylase in dicyclopropylketone-induced and n-octane-grown Pseudomonas putida GPo1 *Appl. Environ. Microbiol* **70** 4544-50
[17] Johnson E L and Hyman M R 2006 Propane and n-butane oxidation by Pseudomonas putida GPo1 *Appl. Environ. Microb.* **72** 950-52
[18] Nava V, Morales M and Revah S 2007 Cometabolism of methyl tert-butyl ether (MTBE) with alkanes *Reviews in Environmental Science and Biotechnology* **6** 339-52
[19] Li S, Li D and Yan W 2015 Cometabolism of methyl tert-butyl ether by a new microbial consortium ERS *Environmental Science and Pollution Research International* **22** 10196-205
[20] Li S, Zhao H, Li Y, Niu S and Cai B 2013 Complete nucleotide sequence of plasmid pND6-2 from Pseudomonas putida ND6 and characterization of conjugative genes *Gene* **512** 148-56
[21] Kumar S, Nei M, Dudley J and Tamura K 2008 MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences *Briefings in Bioinformatics* **9** 299-306
[22] Larkin M A, Blackshields G, Brown N P, Chenna R, McGettigan P A, McWilliam H, Valentin F, Wallace I M, Wilm A, Lopez R, Thompson J D, Gibson T J and Higgins D G 2007 Clustal W and Clustal X version 2.0 *Bioinformatics* **23** 2947-48
[23] Li S, Zhao H, Li Y, Niu S and Cai B 2012 Complete genome sequence of the naphthalene-degrading Pseudomonas putida strain ND6 *J. Bacteriol* **194** 5154-55
[24] Ferreira L, Rosales E, Sanroman M A and Pazos M 2015 Preliminary testing and design of permeable bioreactive barrier for phenanthrene degradation by Pseudomonas stutzeri CECT 930 immobilized in hydrogel matrices *J. Chem. Technol. Biot.* **90** 500-06
[25] Hasan S A and Jabeen S 2015 Degradation kinetics and pathway of phenol by Pseudomonas and Bacillus species *Biotechnol Biotec. Eq.* **29** 45-53
[26] Vallon T, Simon O, Rendgen-Heugle B, Frana S, Muckschel B, Broicher A, Siemann-Herzberg M, Pfannenstiel J, Hauer B, Huber A, Breuer M and Takors R 2015 Applying systems biology tools to study n-butanol degradation in Pseudomonas putida KT2440 *Eng. Life Sci.* **15** 760-71
[27] Wang X Q, Wu C, Liu N, Li S J, Li W, Chen J M and Chen D Z 2015 Degradation of ethyl mercaptan and its major intermediate diethyl disulfide by Pseudomonas sp strain WL2 *Appl. Microbiol Biot.* **99** 3211-20
[28] Poblete-Castro I, Becker J, Dohnt K, dos Santos V M and Wittmann C 2012 Industrial biotechnology of Pseudomonas putida and related species *Applied microbiology and biotechnology* **93** 2279-90
[29] Winsor G L, Van Rossum T, Lo R, Khaira B, Whiteside M D, Hancock R E and Brinkman F S 2009 Pseudomonas Genome Database: facilitating user-friendly, comprehensive comparisons of microbial genomes *Nucleic Acids Research* **37** D483-8
[30] Nelson K E, *et al.* 2002 Complete genome sequence and comparative analysis of the metabolically versatile Pseudomonas putida KT2440 *Environmental Microbiology* **4** 799-808
[31] Roach D J, Burton J N, Lee C, Stackhouse B, Butler-Wu S M, Cookson B T, Shendure J and Salipante S J 2015 A Year of Infection in the Intensive Care Unit: Prospective whole genome sequencing of bacterial clinical isolates reveals cryptic transmissions and novel microbiota *PLoS Genetics* **11** e1005413
[32] Broholm K, Ludvigsen L, Jensen T F and Ostergaard H 2005 Aerobic biodegradation of vinyl chloride and cis-1,2-dichloroethylene in aquifer sediments *Chemosphere* **60** 1555-64
[33] Elango V, Kurtz H D and Freedman D L 2011 Aerobic cometabolism of trichloroethene and cis-dichloroethene with benzene and chlorinated benzenes as growth substrates *Chemosphere* **84** 247-53
[34] Jones M D, Rodgers-Vieira E A, Hu J and Aitken M D 2014 Association of Growth Substrates and Bacterial Genera with Benzo[a]pyrene Mineralization in Contaminated Soil *Environ Eng.*

Sci. **31** 689-97
[35] Hadibarata T, Kristanti R A, Fulazzaky M A and Nugroho A E 2012 Characterization of pyrene biodegradation by white-rot fungus Polyporus sp S133 *Biotechnol Appl. Bioc.* **59** 465-70
[36] Fayolle F, Vandecasteele J P and Monot F 2001 Microbial degradation and fate in the environment of methyl tert-butyl ether and related fuel oxygenates *Appl. Microbiol Biot.* **56** 339-49
[37] Volpe A, Del Moro G, Rossetti S, Tandoi V and Lopez A 2009 Enhanced bioremediation of methyl tert-butyl ether (MTBE) by microbial consortia obtained from contaminated aquifer material *Chemosphere* **75** 149-55
[38] Smith C A, O'Reilly K T and Hyman M R 2003 Characterization of the initial reactions during the cometabolic oxidation of methyl tert-butyl ether by propane-grown Mycobacterium vaccae JOB5 *Appl. Environ. Microbiol* **69** 796-804
[39] Garnier P M and Auria R 2000 Cometabolic biodegradation of methyl tert-butyl ether by a soil consortium: Effect of components present in gasoline *The Journal of general and applied microbiology* **46** 79-84
[40] Sander R 2015 Compilation of Henry's law constants (version 4.0) for water as solvent *Atmos. Chem. Phys.* **15** 4399-981
[41] Liu C Y, Speitel G E and Georgiou G 2001 Kinetics of methyl t-butyl ether cometabolism at low concentrations by pure cultures of butane-degrading bacteria *Appl. Environ. Microb.* **67** 2197-201
[42] Hyman M, Kwon P, Williamson K and O'Reilly K 1998 Cometabolism of MTBE by alkane-utilizing microorganisms *First International Conference on Remediation of Chlorinated and Recalcitrant Compounds* **3** 321-6
[43] Fortin N Y, Morales M, Nakagawa Y, Focht D D and Deshusses M A 2001 Methyl tert-butyl ether (MTBE) degradation by a microbial consortium *Environ Microbiol* **3** 407-16