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≪ Research Paper ≫

Evaluation of Intrinsic Bioremediation of Methyl Tert-butyl Ether (MTBE) Contaminated Groundwater

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ABSTRACT

This paper reported the use of real-time polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and the culture-based method in the intrinsic bioremediation study at a petroleum contaminated site. The study showed that phenol hydroxylase gene was detected in groundwater contaminated with benzene, toluene, ethylbenzene, xylene isomers (BTEX) and methyl tert-butyl ether (MTBE). This indicated that intrinsic bioremediation occurred at the site. DGGE analyses revealed that the petroleum-hydrocarbon plume caused the variation in microbial communities. MTBE degraders including *Pseudomonas sp.* NKNU01, *Bacillus sp.* NKNU01, *Klebsiella sp.* NKNU01, *Enterobacter sp.* NKNU01, and *Enterobacter sp.* NKNU02 were isolated from the contaminated groundwater using the cultured-based method. Among these five strains, *Enterobacter sp.* NKNU02 is the most effective stain at degrading MTBE without the addition of pentane. The MTBE biodegradation experiment indicated that the isolated bacteria were affected by propane. Biodegradation of MTBE was decreased but not totally inhibited in the mixtures of BTEX. *Enterobacter sp.* NKNU02 degraded about 60% of MTBE in the bioreactor study. Tert-butyl alcohol (TBA), acetic acid, 2-propanol, and propenoic acid were detected using gas chromatography/mass spectrometry during MTBE degraded by the rest cells of *Enterobacter sp.* NKNU02. The effectiveness of bioremediation of MTBE was assessed for potential field-scale application.

Keywords : Real-time polymerase chain reaction (PCR), Denaturing gradient gel electrophoresis (DGGE), Methyl tert-butyl ether (MTBE) degradation, *Enterobacter sp.* NKNU02

1. Introduction

Gasoline constituents such as benzene, toluene, ethylbenzene, and xylene isomers (BTEX) are regulated by many nations. Gasoline contains approximately 10-20% of BTEX. The residual amount of BTEX persists in a pure liquid phase [commonly referred as non-aqueous-phase liquids (NAPLs)] within pore spaces or fractures at many gasoline spill sites. The slow dissolution of residual BTEX results in a contaminated plume of groundwater. In addition to BTEX, methyl tert-butyl ether (MTBE), naphthalene, 1,3,5-trimethylbenzene (1,3,5-TMB), and 1,2,4-trimethylbenzene (1,2,4-TMB) are also toxic to humans (Deeb et al., 2003; Chen et al., 2008). Among those gasoline constituents, MTBE is the most commonly used oxygenate due to its low cost, convenience of transfer, and ease of blending and production. MTBE has been the most commonly used high octane additive to gasoline since 1990. Currently, MTBE has become a prevalent groundwater contaminant with its wide usage.

MTBE is a highly water soluble compound and its biodegradation rate is low in many cases. Consequently, a MTBE plume typically results in longer remediation periods (Fischer et al., 2004). Because MTBE is a possible human carcinogen, U.S. Environmental Protection Agency (USEPA) has set its advisory level for drinking water at 20-40 μg/L (USEPA, 1997). MTBE was classified as the Class IV toxic chemical substances by Taiwan Environmental Protection Administration (TEPA) (TEPA, 1999). The Class IV toxic chemical substances are defined as those chemical substances that may pollute the environment or endanger human health. Thus, MTBE was regulated under the

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amendment of "Soil and Groundwater Pollution Remediation Act" in 2012 by TEPA.

The unique characteristics of MTBE make its behavior in the subsurface different from BTEX. The biodegradability of MTBE is low due to the stable ether linkage and tert-butyl structure. Thus, it usually migrates a longer distance than BTEX and results in more difficult for remediation at gasoline-contaminated sites. Given that it is often not possible to locate and remove the residual BTEX or MTBE, remediation must focus on preventing further migration of the dissolved contamination. This plume control must be maintained for a long period of time. Therefore, some more economic approaches are desirable for groundwater remediation to provide for long-term control of contaminated groundwater (Deeb et al., 2003; Okeke and Frankenberger, 2003).

Many physical and chemical remediation technologies such as air sparging, chemical oxidation or pump and treat have been used to treat MTBE-contaminated groundwater. However, bioremediation is a more attractive option because of its economic benefit. Therefore, biological treatment systems, including intrinsic and enhanced approaches, offer the possibility of a cost-effective destruction technology for groundwater remediation. MTBE is biodegradable under both aerobic and anaerobic conditions. Based on the above description, in situ aerobic bioremediation is a feasible technology to clean up MTBE-contaminated sites if MTBE degrader exists at the site.

The purpose of this paper is to provide a review of the past work performed in the petroleum contaminated site (Kao et al., 2010; Chen et al., 2011). A series of study include the following tasks: (1) assessment of the potential of intrinsic bioremediation at a petroleum contaminated aquifer by quantitative real-time polymerase chain reaction (PCR), (2) determination of the dominant native microorganisms at different locations of the contaminated aquifer through microbial identification via denaturing gradient gel electrophoresis (DGGE) and culture-based methods, (3) isolation of the potential MTBE-degrading bacteria from the petroleum contaminated site, and (4) to analyze the mechanisms of MTBE utilization by the potential MTBE-degrading bacteria.

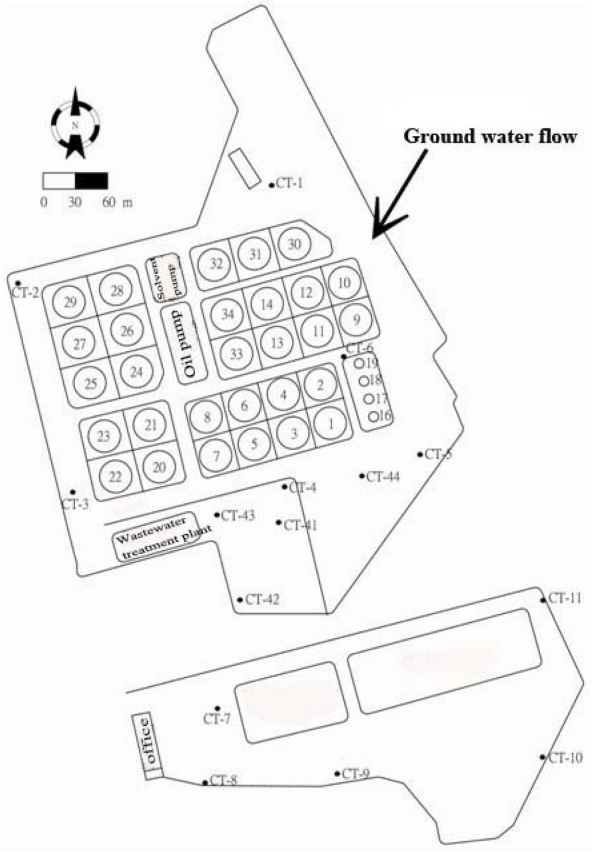


Fig. 1. The contaminant source area, groundwater flow direction, and the sampling locations in the petroleum contaminated site.

2. Materials and methods

2.1. Gasoline contaminated site

A petroleum company owned storage tank facility located in southern Taiwan was selected for this study. Leakage of gasoline from a pipeline resulted in the groundwater contamination. On-site borings encountered up to 25 m of mostly brownish to graying, fine to medium sand to silty sand. The average groundwater elevation within the shallow aquifer is approximately 3 to 4 m below land surface. Groundwater in the unconfined aquifer flows to the southwest. The measured effective porosity is 0.3. The average hydraulic conductivity for the surficial, unconfined aquifer is 2.9×10^{-5} m/sec. The groundwater flow velocity is 3.7×10^{-7} m/sec. The measured groundwater temperature in the surficial aquifer varies from 17 to 29ºC. Fig. 1 illustrates the locations of representative monitor wells, and groundwater flow direction in the early stage of the studies (Kao et al., 2010).

Table 1. Groundwater monitoring for four monitor wells in the gasoline contaminated site

Monitoring well	Cl^- (mg/L)	NO_2^- (mg/L)	NO_3^- (mg/L)	PO_4^{3-} (mg/L)	SO_4^{2-} (mg/L)	S^{2-} (μg/L)	CH_4 (mg/L)	Alkalinity (mg/L as $CaCO_3$)	COD[a] (mg/L)	Total Fe (mg/L)	Fe^{2+} (mg/L)	NH_3-N (mg/L)
CT-2	6.91 ± 1.2	N.D.[b]	7.7 ± 2.2	N.D.[b]	9.6 ± 3.2	5.0 ± 1.3	0.02 ± 0.01	156 ± 13	10.4 ± 4.4	1.5 ± 0.4	N.D.[b]	0.31 ± 0.07
CT-4	0.93 ± 0.72	N.D.[b]	0.1 ± 0.03	N.D.[b]	3.7 ± 2.3	17.0 ± 5.7	14.2 ± 1.5	298 ± 32	14.9 ± 6.3	19.0 ± 3.6	12 ± 4.6	0.10 ± 0.03
CT-41	N.D.[b]	N.D.[b]	0.3 ± 0.15	N.D.[b]	2.8 ± 1.1	34.0 ± 4.6	6.2 ± 4.1	247 ± 27	23.8 ± 5.7	26.5 ± 6.4	6 ± 1.3	0.3 ± 0.15
CT-7	0.77 ± 0.14	N.D.[b]	5.6 ± 1.6	N.D.[b]	7.5 ± 3.5	6.0 ± 1.8	0.7 ± 0.2	187 ± 19	17.3 ± 6.5	2.0 ± 0.3	0.2 ± 0.1	5.6 ± 1.6

a Chemical Oxygen Demand.
b Not detected.

Monitoring well	MTBE (μg/L)	Benzene (μg/L)	Toluene (μg/L)	Ethylbenzene (μg/L)	m,p-Xylene (μg/L)	o-Xylene (μg/L)	1,3,5-Trimethyl benzene (μg/L)	1,2,4-Trimethyl benzene (μg/L)	TOC[a] (mg/L)
CT-2	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	8.8
CT-4	255 ± 49	399 ± 41	22 ± 13	60 ± 24	24 ± 8	5.1 ± 1.3	32 ± 4.3	104 ± 9.2	18.9 ± 3.4
CT-41	25 ± 17	35 ± 12	N.D.[b]	3.0 ± 1.4	N.D.[b]	N.D.[b]	3 ± 0.7	N.D.[b]	19.1 ± 6.4
CT-7	7 ± 2.5	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	N.D.[b]	17.7 ± 2.9

a Total organic carbon.
b Not detected.

Groundwater samples were collected from four representative monitor wells CT-2, CT-4, CT-7, and CT-41, which were located at the background area, source area, mid-plume area, and down gradient area, respectively. All selected monitoring wells were sampled quarterly during the investigation period from 2007 to 2009. Table 1 shows the results of groundwater analyses in four representative wells.

2.2. DNA extraction and conventional PCR

DNA extractions were performed with 0.5 g of the groundwater solids using Power Soil® extraction kit (MoBiol, USA). The PCR primers for aromatic oxygenase genes and multiplex PCR protocols were adopted from the previously published paper (Baldwin et al., 2003). The individual primer sets were allowed to amplify of fragments of naphthalene dioxygenase (NAH), toluene dioxygenase (TOD), toluene monooxyhenase (TOL), ring-hydroxylating toluene monooxygenase (RMO), phenol hydroxylase (PHE), and biphenyl dioxygenase (BPH4). All PCR experiments included negative controls containing no temple and reactions with DNA extracts from appropriate positive control strains was described previously (Baldwin et al., 2008).

2.3. Real-time PCR with SYBR green I

Real time PCR was performed on a LightCycler® 1.5 Instrument (Roche, USA). The LightCycler® FastStart DNA Master SYBR Green I kit (Roche, Germany) was used for the real time PCR reaction. All of the procedures including annealing and polymerization temperatures, primers concentrations, and $MgCl_2$ concentration for qPCR were done following the procedures of the manufactures.

2.4. Denaturing gradient gel electrophoresis (DGGE)

Total bacterial DNA from 1 g of collected groundwater samples were extracted with a Power Soil® extraction kit (MoBiol, USA). Denaturing gradient gel electrophoresis was performed with D-Code® universal mutation detection system (BioRad Lab., USA). PCR product were loaded on to 8% (w/v) polyacrylamide gels with a denaturing gradient of 40% to 60% (100% denaturant according to 7 M urea plus 40% formamide in 1 × TAE buffer) and were run for 7 h at 150 V and at a constant temperature in 1 × TAE buffer (pH 7.4). After electrophoresis, the gels were stained with SYBE Safe® (Invitrogen, UK), and then were put in a shaker at 150 rpm for 10 min, followed by excision bands from the gel with a pipette tip under UV illumination. The excised bands were directly placed in 1.5 mL micro-centrifugation tubes containing 50 μL ultrapure DNase/RNase–free sterile water and incubated at 4°C for overnight to elute DNA. Ten microliters of the supernatants was used as template for the re-amplification (as described above). All sequences were compared to those in the GeneBank data-

base and Ribosomal database (Kao et al., 2010).

2.5. Enrichment of MTBE-degrading cultures

Groundwater in well CT-4 was used to enrich MTBE-degrading microorganisms in sterile FTW minimal salts to which 100 mg/L of MTBE (Sigma, USA) was added. The pH of the medium was adjusted to 7.4. The aerobic culture was incubated at ambient temperature (25ºC) with orbital shaking (150 rpm) for 4 weeks. Thereafter, 1 mL of the culture was transferred onto fresh sterile FTW enrichment medium including 100 mg/L MTBE, and further incubated for 2 weeks. Subsequently, this enrichment culture was used for isolation of single strains.

2.6. MTBE degrading monocultures

To isolate single strains from the enrichment culture, 1 mL aliquots of the enrichment culture were centrifuged (10,000 rpm, 10 min) using an micro-centrifugation tube. The supernatant was removed and the residue was re-suspended in 50 μL of sterile FTW mineral salts medium by votex mixing. The resulting suspension were plating on agar plates which were made from the mixtures of FTW medium, 1.5% agar and sterile MTBE (100 mg/L). Agar plates were incubated under aerobic conditions at 25ºC and colonies appearing within 3-4 days were isolated. The isolates were then pre-grown in FTW mineral salts media to which yeast extract (300 mg/L), ethanol (300 mg/L) and MTBE (100 mg/L) were added. Cells were harvested by centrifugation (5,000 rpm, 15 min) and washed in 40 mL FTW mineral salts solution. Subsequently, cells were re-suspended in the same medium to an in initial OD of approximately (2.0). This cell suspension (1 mL) was inoculated into 59 mL FTW mineral salts media in a 250 mL Erlenmeyer flask sealed with the rubber septa. Cultures were incubated (25ºC, 150 rpm) for the indicated days.

2.7. Banding analysis and phylogenic analysis

DGGE banding patterns were analyzed using Quantity One software (BioRad, USA). For cluster analysis, PCR-DGGE of one primer set was performed in duplicate. Calculation of the pair-wise similarities was based on the Dice correlation coefficient. Dendrograms were created using the algorithim of unweighted pair-group method using arithmetic averages (UPGMA) (Labbe et al., 2007). Database searches of these16S rDNA gene sequence determined were conducted by a BLAST program using the GenBank database. The profiled alignment technique of Clustal W was used to align the sequences. Phylogenetic trees were constructed by Neighbor-Joining method using MEGA 4 software (Saitou and Nei, 1987).

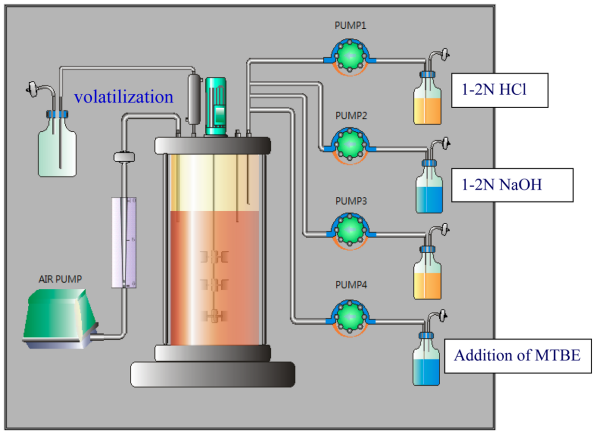


Fig. 2. The configuration of the bioreactor.

2.8. Statistic methods

Paired *t*-test was used for the evaluation of differences between the amounts of residual MTBE after biodegradation with or without pentane addition and their corresponding control.

2.9. Bacterial strain and growth condition

Enterobacter sp. NKNU02 was aerobically grown in a 5-L stirred tank bioreactor at the working volume of 4 L in FTW medium (g/L) (Fig. 2). The addition of 100 mg/L of MTBE into FTW medium was used as the sole carbon source. The aerobic culture in the bioreactor was incubated at 25ºC and pH 7.2. In the bioreactor, the pH was regulated by adding 1 N NaOH or 1 N HCl and the oxygen consumption was measured with an oxygen probe. Bacterial growth was measured by monitoring the optical density (OD) at 600 nm by the photospectrometer (Chen et al., 2011).

2.10. Analysis of MTBE and metabolites

Water samples were collected in 40 mL volatile organic analyte (VOA) vials with Teflon® lined septa. The VOA analyses were performed within two days (48 hours) of

sample collection. MTBE, tert-butyl alcohol (TBA), and tert-butyl formate (TBF) were analyzed by purge and trap coupling with gas chromatography/mass spectrometry. Five milliliters of water samples were concentrated by the purge and trap technique using a Tekmar Liquid Sample Concentrator 3100/Automatic Laboratory Sampler system. The purge-and-trap of aqueous samples was performed at ambient temperature. Nitrogen flow rate of 40 mL/min was adopted and purging time was 11 min followed by 4 min desorb time. The samples were analyzed using an Agilent 6890 gas chromatograph coupled with a 5973 N mass selective detector (MSD) and ChemStation for control and data acquisition. Analyte separation was achieved with a 0.32 mm i.d., 30 m, fused silica capillary column (5% diphenyl-95% dimethyl polysiloxane) with a 0.25 μm film thickness (HP-5MS, Agilent, USA). The temperature program included a 2 min hold time at 35°C, and temperature ramping at 8°C/min to 50°C, followed by a ramp to 70°C at 16°C/min, a ramp to 100°C at 22°C/min, and a final ramp to 220°C at 28°C/min. Helium was used as carrier gas at a flow rate of approximately 1.10 mL/min. The electron energy was set at 70 eV. The detector scanned from 45 to 260 amu. The electron multiplier voltage was 1235 eV.

3. Results and Discussion

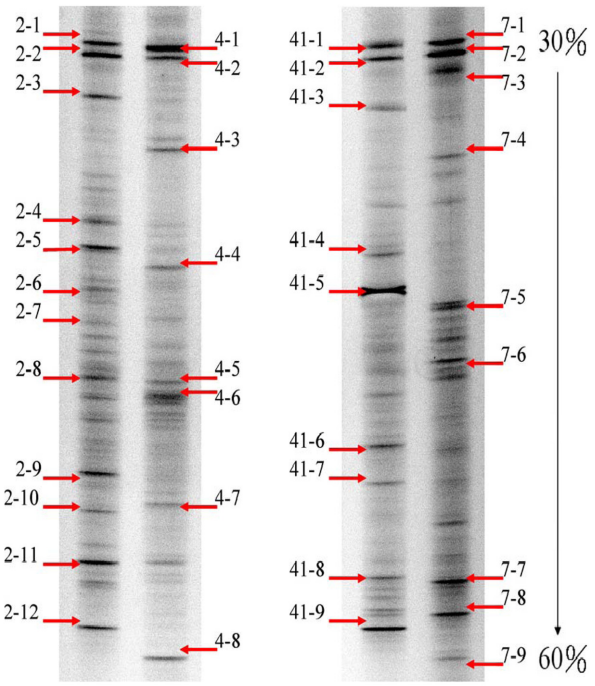
3.1. Evaluation of intrinsic remediation by Real-Time PCR

Field results indicated that the detected redox potential and dissolved oxygen near the source area were low, indicating that the reduced conditions in the most contaminated zone (Table 1). A higher concentration of methane was also detected in the area with a higher concentration of CO_2, reflecting that mixed anaerobic biodegradation process occurred in this area (Kao et al., 2010). Moreover, high CO_2 concentrations were observed in the plume, which suggests that significant microbial activity and natural bioremediation occurred in this area. The enumeration of aromatic oxygenase genes by real-time PCR was used for monitoring national attenuation at gasoline-contaminated sites. Aromatic oxygenase genes were detected in both currently impacted monitoring wells (CT-4 and CT-41), indicating that biodegradation could be a component of natural attenuation at both wells, although only the low copy numbers of PHE were detected. PHE, which catalyzes the further oxidation of hydroxylated intermediates of monoaromatic hydrocarbon catabolism, was also detected during the treatment of pulsed multi-phase extraction at a gasoline-contaminated sits (Baldwin et al., 2009). The detected genotypes of these aromatic oxygenase genes at different petroleum-contaminated sites could be correlated with the extent of gasoline contamination, which warrants further investigation (Baldwin et al., 2008). On the other hand, PHE was also detected in monitoring well CT-7 containing undetectable BTEX compounds. As in the similar study reported by Baldwin et al. (2008), the detection of aromatic oxygenase genes in historically impacted with the current BTEX concentrations below detection limits would be due to continual aromatic hydrocarbons flux and active biodegradation enabling low or no BTEX levels to be detected. In addition, no oxygenase genes were detected in the monitoring well CT-2 without BTEX contamination. The detection of these oxygenase genes may be the most beneficial for assessing BTEX biodegradation at petroleum-impacted sites (Baldwin et al., 2008).

Overall, quantification of aromatic oxygenase genes under natural attenuation demonstrated that aerobic BTEX-utilizing bacteria were present, suggesting that low BTEX degradation rates may have resulted from low oxygen availability (Nebr et al., 2009). It also provided a baseline to evaluate the impact of some strategies regarding enhanced bioremediation including the injection of oxygen-releasing materials (ORMs), multi-phase extraction, or in situ biosparging (Kao et al., 2010).

3.2. The bacterial community at the contaminated site

To determine if bacterial community patterns in environment were changed due to the petroleum contamination, the PCR-DGGE was performed to investigate these patterns on four different sampling wells (CT-2, CT-7, CT-41 and CT-4) (Fig. 3). Results indicated that these bacterial communities could be grouped into three major phylogenetic clusters. It was found CT-2 and CT-7, which were located at source and down-gradient areas, revealed similarly bacterial communities, whereas CT-41 and CT-4 displayed the divergent



Adopted from Kao et al., 2010.

Fig. 3. DGGE profiles of the PCR-amplified for V3 region of 16S rDNA in these four sampling wells.

communities from CT-2 and CT-7. Moreover, CT-41 and CT-4 were further grouped into two clusters, indicating that different levels of petroleum pollution caused the variations in bacterial communities.

Although a high number of bands appeared when performing PCR-DGGE analysis (Fig. 3), it was only possible to identify few bands by excising them from the DGGE. Weak and close bands were difficult to cut off the gel and could not be re-amplified or only produced multiple DNA sequences. Thus, only the prominent bands in four sampling wells were excised for DNA sequencing in this study. The V3 regions of 16S rDNA in these sequences were compared to the database of GenBank. An analyzed sequence whose similarity was less than 95% as compare to that in the known microorganisms could suggest a potentially novel genera (Labbe et al., 2007). The sequences of these novel bacteria were related to bacterium WCHB1-69 in CT-2 and CT-4, and *Novosphingobium capsulatum* strain 213 in CT-7.

A phylogenetic tree of these sequences was constructed in order to describe the relationships between all the sequences (Kao et al., 2010). Results revealed that there were two major separate phylogentic clusters. One cluster was classified as *Bacteroketes* containing bacterium WCHB1-69, and as *Proterobacteria* containing *Gallionella* sp, *Proteobacterium* EV221H2111601SAH33, *Geobacteraceae* bacterium clone RIFLESED22_110, bacterium LaC15L122, *Burkholderia vietnamiensis* strain WPB, *Aquaspirillum* sp. TG27, *Methylocaldum* sp. 5FB, *Novosphingobium capsulatum* strain 213, bacterium clone Chlplus_CL-120529_OTU-35, bacterium clone Neu2P1-42, *Geobacteraceae* bacterium clone RIFLESED22_110. In the other cluster, *Bacillus megaterium* strain DS8 belonging to *Firmicutes*, and *Chlorobi* bacterium 7025P4B42, *Spirochaetes* bacterium isolate DGGE gel band LPB108, bacterium clone GW12, *Spirochaetales* bacterium clone D15_39 belonging to *Spirochaetales* were grouped. Among them, *Geobacteraceae* have been reported to able to biodegrade petroleum with reduction of Fe (III) (Rooney-Varga et al., 1999; Li t al., 2002). Thus, the dominant microorganisms involving petroleum degradation could be exploited and isolated for their application on the bioremediation of gasoline-contaminated sites.

3.3. Isolation of MTBE-degrading bacteria from the gasoline contaminated site

As described above, MTBE-degrading bacteria was isolated from the gasoline-contaminated site by the culture-based method and identified them by 16S rDNA gene sequencing (Table 2). The sequences of the isolated bacteria have been submitted to GeneBank and assigned accession numbers for *Pseudomonas* sp. NKNU01(FJ158035), *Bacillus* sp. NKNU01 (FJ158036), *Klebsiella* sp. NKNU01 (FJ158037), *Enterobacter* sp. NKNU01 (FJ158038), *Enterobacter* sp. NKNU02 (FJ158039). These bacteria can grow on MTBE as the sole carbon under aerobic conditions, indicating the potential of bacteria existing in petroleum-contaminated sites to degrade MTBE. These bacteria were not detected in the dominant bands of DGGE, which could be related to the recalcitrance of MTBE to both aerobic and anaerobic biodegradation owing to the stability of the ether bond and the hindrance of the branched *tert*-butyl group (Sulfita and Mormile, 1993; Salanitro et al., 1994; Volpe et al., 2009).

From previous studies, it was found pure culture showed less activity for MTBE degradation. A mixed culture may be more suitable for treatment options than pure culture (Pruden et al., 2001). It could be the existence of many rea-

Table 2. Identification of MTBE-degrading bacteria grown in the defined medium (without carbon source) by 16S rRNA gene fragments via BLAST N research in GeneBank database

Species	Phylogenetic group	Related organism (accession no.)	Identity (%)
Pseudomonas sp. NKNU01	*γ-proteobacteria*	*Pseudomonas* sp. AHL 2 (AY379974)	100
Bacillus sp. NKNU01	*Firmicutes*	*Bacillus megaterium* (DQ789400)	99
Klebsiella sp. NKNU01	*γ-proteobacteria*	*Klebsiella pneumoniae* subsp. *pneumoniae* MGH 78578 (CP000647)	99
Enterobacter sp. NKNU01	*γ-proteobacteria*	*Enterobacter* sp. VET-7 (EU781735)	99
Enterobacter sp. NKNU02	*γ-proteobacteria*	*Enterobacter* sp. Px6-4 (EF175731)	99

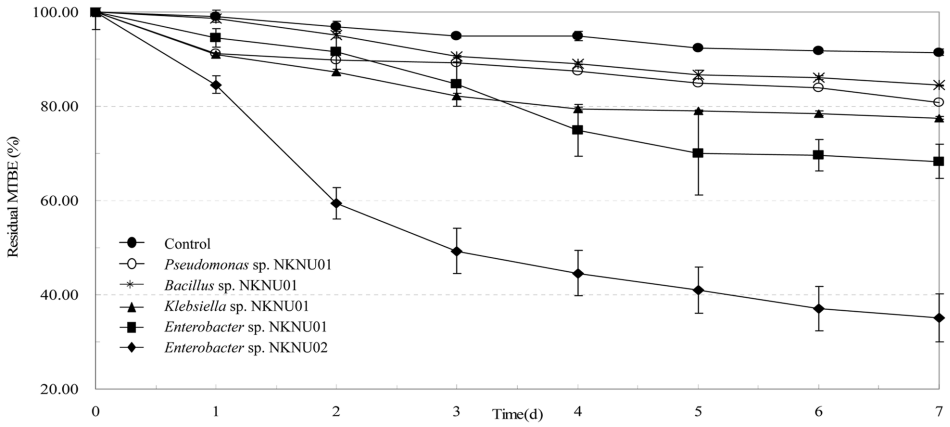


Fig. 4. MTBE degradation by different degraders.

sons. One possible scenario is that one strain may be able to degrade the compound to a certain product at which point another strain may take over and no toxicity or inhibition results.

3.4. The MTBE biodegradation capacity of *Enterobacter sp.* NKNU02

Although the MTBE-degrading ability of other four bacterial strains can be increased by the addition of pentane, *Enterobacter sp.* NKNU02 is the most effective strain at biodegrading MTBE without the addition of pentane among these five strains isolated from the contaminated site. The pentane was used as the cometabolic chemical to enhance MTBE degradation. The pure culture (*Enterobacter sp.* NKNU02) shown the best degradation potential about 29% of MTBE without adding pentane. *Enterobacter sp.* NKNU02 could degrade about 56% of MTBE without adding pentane in the batch experiment. *Bacillus sp*. NKNU01 and *Klebsiella sp.* NKNU01 could degrade about 22% of MTBE with adding pentane. Comparing with the batch experiments, bioreactor could enhance MTBE degradation significantly.

In the bioreactor experiment, *Enterobacter sp.* NKNU02 (at the resting cell state) can degrade up to 60% of the MTBE that is present in 7 days (Fig. 4). The percentage (%) was calculated from the ratio (the amounts of residual MTBE with bacterial inoculum divided by the initial MTBE) multiplied by 100 in Fig. 4. The initial MTBE concentration in each experiment was 100 mg/L. Error bars indicate the standard deviation of triplicate samples with the values of variation coefficient less than 10%. Similar degradation results were found, with 22-37% of MTBE being biodegraded after 5 day incubation with *Rhodococcus*, *Bacillus* and *Aureobacterium* (Zhang et al., 2009). The degradation of MTBE can be increased to 51% if *Pseudomonas putida* is pre-incubated with dicyclopropylketone (Smith and Hyman, 2004). Higher degradation of MTBE (78%) was found by *Achromobacter xylosoxidans* MCM1/1 in five days (Eixarch and Constani, 2010). The incubation temperature (30ºC) and seeded bacteria amounts (OD600 = 0.3) used in Eixarch and Constani's work (2010) are higher than the setting of Chen and coworker's experiments (25ºC and OD600 ≤ 0.1) (2011). The MTBE biodegradation capacity of *Enterobacter sp.* NKNU02 would at least be comparable to that of Achromobacter xylosoxidans MCM1/1 if the

incubation temperature and bacterial amounts were similar. The optimal temperature and bacterial amounts may increase the effective use of MTBE. As in the case of *Enterobacter sp.* NKNU02 for MTBE biodegradation in batch culture, the efficiency of MTBE removal with the amounts of seeded bacteria (OD = 0.2) is 1.66-fold higher than that with a lower starting amount of the same bacteria (OD = 0.1).

TBA is often found as an accumulating intermediate or dead-end product in lab studies using microcosms or isolated cell suspension (Schmidt et al., 2004). As expected, TBA was increasingly detected during the process of MTBE biodegradation by *Enterobacter sp.* NKNU02. Tert-butyl formate (TBF) can be produced from the hydrolysis of TBA by an esterase which was reported in Mycobacterium austroafricanum IFP 2012 and Mycobacterium vaccae JOB5 (Francois et al., 2003; Smith et al., 2003; Ferreira et al., 2006). However, TBF was not detected in our study. It may be rapidly degraded by an efficient esterase, or tert-butoxy methanol, the first intermediate in MTBE oxidation, or may be transformed into TBA via dismutation rather than dehydrogenation, as described by Ferreira et al (2006). Only one metabolic pathway has been described in some MTBE-degrading bacteria (Eixarch and Constani, 2010). Although the intermediates of MTBE degradation in *Enterobacter sp.* NKNU02 was determined in Chen et al.(2011), the details of this MTBE metabolic pathway need to be further elucidated.

3.5. The impact of BTEX on MTBE biodegradation

When mixtures of BTEX and MTBE were degraded together in our previous work, the degradation percentage of the individual compounds decreased in the order of toluene > benzene > MTBE > o-xylene = m/p-xylene (Chen et al., 2011). Also, in the presence of BTEX, the degradation capacity for MTBE was decreased from 60% in MTBE alone to 15% in presence of this mixture. The degradation of MTBE by *Rhodococcus sp.* EH831 was completely inhibited by the coexistence of BTEX (Lee and Cho, 2009). On the other hand, BTEX did not affect MTBE degradation by *Pseudomonas sp.* PM1 (Pruden and Suidan, 2004). The inhibitory effect of BTEX on the degradation of MTBE seems to greatly depend on the microorganisms, the concentration of the compounds, and the experimental system (e.g., batch or continuous) (Lee and Cho, 2009). Thus, it was found MTBE and BTEX (benzene, toluene, ethylbenzene, and xylenes) can coexist in gasoline-contaminated groundwater. MTBE-degradability of *Enterobacter sp.* NKNU02 could reduce about 16% of MTBE but inhibited by BTEX. However, it could also degrade BTEX including 36% of toluene and 32% of benzene.

4. Conclusion

MTBE can be used by bacteria either as a primary source of carbon in direct metabolism conditions, or co-metabolically when bacterial growth requires other substrates, usually an alkane. Also, it was reported that MTBE in organically poor soil can be inhibited by the addition of easily degradable organic compounds. In these studies described, DNA was extracted from petroleum-contaminated groundwater so bacterial communities were analyzed in these contaminated sites. This provides us insight into the evaluation for the possibility of the natural attenuation and enhanced bioremediation in petroleum-contaminated sites.

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