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Bioremediation treatment of MTBE and ETBE in contaminated soils

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Abstract

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Three Methyl Tertiary Butyl Ether (MTBE) degradative consortia were isolated from gasoline-contaminated soil namely: mKMS, mKGS1 and mKGS2. These consortia were tested for the ability to degrade Ethyl Tertiary Butyl Ether (ETBE) at the concentration of 100 mg/L and to degrade a mixture of MTBE and ETBE in the Nutrient Broth (NB) media at the concentration of 50 mg/L each. The results showed that mKGS1 was the best degraders in which 74% of MTBE, 25% of ETBE and 16% of MTBE and 23% of ETBE in the mixture were degraded, within 30 days. mKGS1 was then further used in the bioaugmentation and biostimulation experiments. Degradation of MTBE increased from 34% to 61% after 70 days when mKGS1 was amended in soil mixed with the combination of MTBE and ETBE (at 50 mg/L each). However, mKGS1 did not significantly help the ETBE degradation when it was amended in soil (biostimulation technique). One percent glucose significantly stimulated the degradation of MTBE by the indigenous microorganisms. The presence of mKGS1 and an addition of 1% glucose as extra carbon source improved the degradation of MTBE, from 42 to 51%, suggesting mKGS1 played an important role in the degradation of MTBE.

Key words : MTBE, ETBE, mixed culture, biodegradation, bioaugmentation, biostimulation, bioremediation

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การฟื้นฟูดินที่มีการปนเปื้อน MTBE และ ETBE โดยวิธีทางชีวภาพ

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กลุ่มจุลินทรีย์ที่มีความสามารถในการย่อยสลาย Methyl Tertiary Buthyl Ether (MTBE) ได้ถูกคัดแยกจากดินที่มีการปนเปื้อนด้วยน้ำมันเบนซิน (Gasoline) โดยแยกได้ 3 กลุ่มคือ mKMS mKGS1 และ mKGS2 เมื่อทำการทดสอบความสามารถของจุลินทรีย์ทั้ง 3 กลุ่มในการย่อยสลาย MTBE และ Ethyl Tertiary Buthyl Ether (ETBE) ที่ความเข้มข้น 100 มก./ลิตร และสารผสมระหว่าง MTBE กับ ETBE ที่ความเข้มข้นละ 50 มก./ลิตร ในอาหาร Nutrient broth (NB) ผลการทดลองพบว่า กลุ่มจุลินทรีย์ mKGS1 มีความสามารถในการย่อยสลายสูงสุด โดยสามารถย่อยสลาย MTBE ได้ 74% ETBE 25% และสารผสมระหว่าง MTBE กับ ETBE ได้ 16% และ 23% ภายในเวลา 30 วันตามลำดับ ดังนั้น mKGS1 จึงถูกนำมาใช้เติมลงไป (bioaugmentation) ในดินที่มีการปนเปื้อนด้วยสารที่มีส่วนผสมของ MTBE, ETBE หรือ MTBE กับ ETBE เมื่อผ่านไป 70 วันพบว่าการเติม mKGS1 สามารถช่วยเพิ่มการย่อยสลาย MTBE ในดินที่มีการปนเปื้อนด้วย MTBE และ ETBE ที่ความเข้มข้นอย่างละ 50 มก./ลิตร ได้ คือจาก 34% เป็น 61% แต่ไม่เพิ่มการย่อยสลายของ ETBE เมื่อใช้เทคนิคการกระตุ้น (biostimulation) โดยการเติมกลูโคส (1%) เพื่อเป็นแหล่งคาร์บอนเสริม พบว่า mKGS1 สามารถเร่งการย่อยสลาย MTBE โดยจุลินทรีย์ที่อยู่ในดินได้ และเมื่อเติม mKGS1 และเติมกลูโคส (1%) ลงในดินพบว่าการย่อยสลาย MTBE มีค่าเพิ่มขึ้นจาก 42% เป็น 51% ชี้ให้เห็นว่ากลุ่มจุลินทรีย์ mKGS1 มีบทบาทสำคัญในการย่อยสลาย MTBE

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Methyl Tertiary Butyl Ether (MTBE) is a common octane-enhancing lead replacement in gasoline to provide a cleaner burning fuel with reduced vehicle exhaust emission (U.S. EPA, 2001). The other oxygenated substance used is Ethyl Tertiary Butyl Ether (ETBE). The route of MTBE to enter the environmental is the accidental release of gasoline (Mo *et al*., 1997). MTBE and ETBE are very low absorbed in soil but very soluble in water. MTBE was found in groundwater with the concentration up to 5,000 mg/L (McCauluou and Slater, 2002). The Iowa state survey reported 635 soil samples were contaminated with MTBE at the average contamination level of 122.5 mg/kg (Iowa Department of Natural Resource, 2000).

As MTBE and ETBE can replace lead in gasoline and are biodegraded but they may pose significant problems for soil, groundwater and human health risk as a possible carcinogen, the effective remediation technique to clean up MTBE and ETBE in the environment deserved attention. One of bioremediation treatments for cleaning up soil, groundwater and waste contaminated with hazardous waste and pollutants is bioaugmentation. Salanitro *et al*. (2000) reported that MTBE was rapidly degraded to the concentration of less than 10 μg/L at Port Hueneme site, at the soil depth of 25-30 ft, by the use of MTBE mixed culture (MC-100) and pure culture (SC-100). The other bioremediation treatment is biostimulation. This technique is conducted by stimulating indigenous microorganism with carbon source to accelerate biodegradation rate. Hatzinger *et al*. (2001) found that pure culture ENV735 was able to mineralize MTBE when 0.1% sucrose was used as an extra carbon source. In contrast, a study by François *et al*. (2002) found that an addition of yeast extract and glucose did not assist growth and degradation of MTBE by *Mycobacterium austroafricanum* IFP 2012. Okeke and Frankenberger (2003) reported that when ethanol was used as an additional carbon source, MTBE removal rate by Iso2A was

increased.

Biodegradation of MTBE (Salanitro *et al*., 1994; Mo *et al*., 1997; Hanson *et al*., 1999; Fortin *et al*., 2001; Hatzinger *et al*., 2001; François *et al*., 2002, Okeke and Frankenberger, 2003; Charathirakup *et al*., 2004), ETBE (Khroune *et al*., 2001a) and combination of MTBE and ETBE (Steffan *et al*., 1997; Khroune *et al*., 2001a; Khroune *et al*., 2001b) by pure and mixed cultures have been reported. Not only the MTBE could be degraded by MTBE degraders but also the other oxygenated compounds (Table 1). Kharoune *et al*. (2001a) reported that ETBE degrading bacteria (E_1 and E_2) could also degrade MTBE and at the faster rate of 19.7 (E_1) and 14.0 mg/L.day (E_2) for ETBE and 1.8 (E_1) and 1.4 mg/L.day (E_2) for MTBE. Metabolites of MTBE and ETBE resulted from degradation by propane-oxidizing bacteria was Tertiary Buthyl Ether (TBA) (Steffan *et al*., 1997).

Previous works have reported the biodegradation of MTBE by MTBE degraders using mixed cultures and pure culture but only a few have reported on MTBE degraders that can also degrade ETBE. In addition, there is little information on bioaugmentation and biostimulation of MTBE degraders capable of degrading ETBE. Therefore, this research examined the ETBE degradation ability of MTBE degraders isolated from gasoline contaminated soil and its use in bioaugmentation. The biostimulation of indigenous microorganism using 1% glucose with and without the presence of MTBE degrader, KGS1, was also conducted.

Materials and Methods

Chemicals

MTBE (99% purity) was purchased from Fluka Aldrich Co., Inc., France. ETBE (98% purity) was purchased from Sigma Chemical Co., USA. Other chemicals were purchased from BDH chemical, England.

Media composition

Basal Salt Media (BSM) contains (in g/L): Na_2HPO_4, 5.57; KH_2PO_4, 2.44; NH_4Cl, 2.00; $MgCl_2.6H_2O$, 0.20; of $MnCl_2.4H_2O$, 0.0004; $FeCl_3.6H_2O$, 0.01; and $CaCl_2$, 0.001 (modified from Mo *et al*., 1997). Nutrient broth (NB) contains (in g/L) beef extract, 3; and peptone, 5.

Isolation of MTBE degradative consortia

Gasoline-contaminated soils were collected from 3 sites; 1) a motorcycle repair shop next to Dormitory Number 22, Khon Kaen University (KMS); 2) a retail gasoline station opposite

Table 1. Oxygenated compounds degraded by microorganisms

Oxygenated compounds	References
ETBE	Steffan *et al*. (1997), Khroune *et al*. (2001a), Khroune *et al*. (2001b) and François *et al*. (2002)
Tertiary Amyl Methyl Ether (TAME)	Steffan *et al*. (1997), Khroune *et al*. (2001a), Khroune *et al*. (2001b) and François *et al*. (2002)
Tertiary Butyl Alcohol (TBA)	Salanitro *et al*. (1994), Steffan *et al*. (1997), Hatzinger *et al*. (2001), Khroune *et al*. (2001a), Khroune *et al*. (2001b) and François *et al*. (2002)
Tertiary Amyl Alcohol (TAA)	Khroune *et al*. (2001b) and François *et al*. (2002)
Tertiary Butyl Formate (TBF)	Khroune *et al*. (2001a) and Khroune *et al*. (2001b)
tetrahydrofuran (THF)	Fortin *et al*. (2001)
Diehtyl Ether (DEE)	Fortin *et al*. (2001)
methanol	François *et al*. (2002)
formate	François *et al*. (2002)
BTEX (benzene, toluene, ehtylbenzene and xylene	Fortin *et al*. (2001)

Dormitory number 3 Khon Kaen University (KGS1); and 3) a retail gasoline station next to Food Court, Khon Kaen University (KGS2). The samples were taken from soil at 0-15 cm depth using soil probe with a diameter of 2.96 cm. The composite sample of three cores was collected from each site and kept in plastic bag at 4ºC until ready to be used in the experiment.

Enrichment technique was used to isolate MTBE degradative consortia. Ten grams of each soil samples (KMS, KGS1 and KGS2) were dissolved in 100 ml of BSM (Mo *et al*., 1997) containing 200 mg/L of MTBE as a sole carbon source. The soil suspensions were incubated at 30ºC and shaken at 150 rpm for 10 days before transferred to a fresh medium containing 200 mg/L of MTBE. The procedure was repeated 4 times or until media showed no sign of soil particles. After the last transfer, one milliliter of soil-suspension medium was plated on BSM agar coated with 200 mg/L of MTBE and incubated at 30ºC for 24-48 h. Colonies grown on BSM agar was considered to be microorganisms capable of degrading MTBE or MTBE degradative consortia. The colonies were then streaked on BSM agar coated with 200 mg/L MTBE to isolate the single colony. Morphology of each isolate was studied under a microscope and checked for Gram staining. Only Gram negative rod isolates were then identified using API 20NE system (bioMerieux sa, France) due to a limitation of the system that is able to identify only Gram negative bacteria.

Degradation of MTBE, ETBE and combination of MTBE and ETBE by isolated degradative consortia

The ability of three isolated degradative consortia to degrade MTBE and ETBE were examined in Nutrient Broth (NB) containing 100 mg/L of MTBE or 100 mg/L of ETBE or combination of 50 mg/L MTBE and 50 mg/L ETBE. The inoculum (10%) had an initial OD_{550} of 1.0. The 25 mL serum bottles were capped with Silicone/Teflon and aluminum cap to prevent MTBE from vaporizing. The bottles were incubated at 30ºC and were shaken at 200 rpm. Samples were taken at day 0, 1, 2, 4, 8, 12, 18 and 30, and kept at -20ºC prior to the extraction. Extraction of MTBE and ETBE in media and the analysis of MTBE concentration followed the method previously described by Charathirakup *et al*. (2004).

Bioaugmentaion and Biostimulation techniques

Bioaugmentation was conducted by adding the mKGS1 (5% inoculum) at an initial OD_{550} of 0.3 into 10 g soil mixed with MTBE or ETBE or combination of MTBE and ETBE to achieve a final concentration of 50 mg/kg soil in 25 mL serum bottles capped with Silicone/Teflon and aluminum cap. The samples were sacrificed at day 0, 14, 28, 42, 56 and 70. Residuals of MTBE and ETBE in soil were extracted by heating the vial containing soil samples in a heat block at 80ºC for 40 min. Fifty µL of the head space was taken by using gas tight syringe and then analyzed for MTBE and ETBE concentrations using GC-17A Shimadzu Gas Chromatography-Flame Ionization Detector (GC-FID) (Charathirakup *et al*., 2004). Percent recovery of MTBE and ETBE by this extraction technique was 102.65% and 101.2%, respectively, and then their concentrations were analysed using Gas Chromatography-head space technique (Charathirakup *et al*., 2004).

Biostimulation was examined by adding 1% glucose into 10 g soil sample as extra carbon source. MTBE or ETBE or combination of MTBE and ETBE was then mixed with soil sample to achieve a final concentration of 50 mg/kg soil in all treatments. Controls were autoclaved soil added with 1% glucose. In addition, the combined treatment of bioaugmentation and biostimulation using 1% glucose and the KGS1 on the degradation of MTBE, ETBE and combination of MTBE and ETBE was also examined.

Results and Discussions

Degradation of MTBE, ETBE and combination of MTBE and ETBE by consortia

Three MTBE degradative consortia (mKMS, mKGS1 and mKGS2) were isolated from gasoline-contaminated soil by enrichment technique using

MTBE as a sole carbon source. In 30 days, 100 mg/L of MTBE was degraded 79% 74% and 48% by mKMS, mKGS1 and mKGS2, respectively (Table 2). These MTBE degradative consortia were then checked for their ability to degrade ETBE in media. It was found that these MTBE degradative consortia could also degrade ETBE in which 18% 25% and 23% of 100 mg/L of ETBE was degraded by mKMS, mKGS1 and mKGS2, respectively (Table 2). This may due to the fact that ETBE and MTBE has a similar structure. The only difference was the alkyl side chain resulted in the ability of MTBE degradative consortia to capable in degrading ETBE. Apparently, MTBE was degraded by these MTBE degradative consortia much more than ETBE (Table 2). We speculated that it was due to fact that mixed culture was enriched from media containing MTBE as a sole carbon source for 6 weeks prior to being used in MTBE or ETBE degradation, thus these MTBE degradative consortia could adapt themselves to degrade MTBE better than ETBE.

The ability of MTBE degradative consortia to degrade a combination of MTBE and ETBE in media was also examined. The results indicated that all three MTBE degradative consortia could degrade ETBE better than MTBE (Table 2) when MTBE and ETBE were together in the mixture. This may due to the fact that ETBE has -ethyl group that is easier to be degraded than -methyl group in MTBE structure (Khroune *et al*., 2001b) so MTBE degradative consortia was able to degrade ETBE more than MTBE. These researchers also reported that when the mixture of MTBE and ETBE were presented in media, ETBE was simpler to use than MTBE. This was because of the accessibility of the thoxy carbon of CH_3CH_2O-ETBE was easier than the methoxy carbon of CH_3O-MTBE, due to the proximity of the ether bond. Growth of mKGS1 (Figure 1) suggested that MTBE, ETBE, MTBE and ETBE in the mixture were used as a sole carbon source for mKGS1.

Since the mKGS1 showed the highest overall reduction of MTBE, ETBE and the combination of MTBE and ETBE (Table 2), the mKGS1 was used to study in the bioaugmentation and biostimulation experiments. In addition, mKGS1 was streaked on the BSM agar coated with 200 mg/L of MTBE to separate each single colony. The single colonies obtained were Gram stained. Only Gram-negative rod strains were picked out and then identified using API 20 NE system due to a limitation of the system that is able to identify only Gram negative bacteria.

According to the manufacturer of API 20NE system (bioMerieux sa, France), a percentage of identification by API 20NE system greater than 80% was acceptable. The range of percentages for the strains identified in this study ranged from 88.1 to 99.9%. The results idicated that the consortia mKGS1 comprised 6 single colonies, namely *Ochrobacterium anthropi* (IsoJ1), *Agrobacterium radiobacter* (IsoJ2 and IsoJ3), *Pseudomonas putida* (IsoJ5), *Sphingomonas paucimobilis* (Iso

Table 2. Biodegradation of MTBE, ETBE combination of MTBE and ETBE by three isolates mixed culture

Mixed Culture	Biodegradation of MTBE and ETBE (%) in 30 days			
	Initial concentration = 100 mg/L		Initial concentration = 50 mg/L	
			Combination of MTBE and ETBE	
	MTBE	ETBE	MTBE	ETBE
mKMS	79	18	10	25
mKGS1	74	25	16	23
mKGS2	48	23	3	19
Control (no inoculum)	2	1	2	2

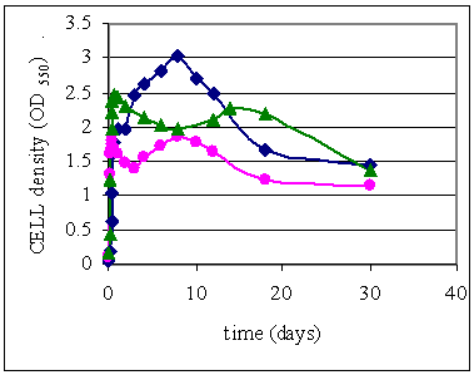

Figure 1. Growth of mKGS1 in NB containing 100 mg/L of MTBE (◆), 100 mg/L of ETBE (●) and combination of 50 mg/L of MTBE and 50 mg/L of ETBE (▲)

J8) and *Pseudomonas aeruginosa* (IsoJ9). Some of these single colonies had been reported as MTBE degraders i.e. *Sphingomonas* sp. (Okeke and Frankenberger, 2003), *Pseudomonas putida* (Steffan *et al*., 1997) and co-metabolize MTBE i.e., *Pseudomonas aeruginosa* (Garnier *et al*., 1999).

Bioaugmentation

In this study soil mixed with MTBE, ETBE or the combination of MTBE and ETBE was amended with the consortia mKGS1 to investigate its ability to accelerate the rate of biodegradation of MTBE or ETBE. A very low percentage of degradation of MTBE (5%), ETBE (10%) in autoclaved soil compared to high degradation of MTBE, ETBE and combination of MTBE and ETBE in autoclaved soil non-amended and amended with mKGS1 suggested biological degradation of MTBE and ETBE in soil was the important process (Table 3). The results indicated that the addition of mKGS1 into soil increased MTBE degradation from 16% to 63% but did not increase ETBE degradation (Table 3), from 74% to 75%, suggesting bioaugmentation was applicable technique to remediate soil contaminated with MTBE. MTBE degradation in the media containing combination of MTBE and ETBE showed a similar trend (Table 3) in the increase of MTBE degradation in the mixture (from 34% to 61%) but there was a slight increased in degradation of ETBE in the mixture (from 52% to 70%). Degradation of ETBE in soil was higher than MTBE both with and without mKGS1 (Table 3), that may due to an ionic strength in ETBE being lower than in MTBE thus ETBE was more bioavailable to the indigenous microorganism in soil and the mKGS1 than MTBE. These results were consistant with the results from the study on a degradation of MTBE, ETBE and combination of MTBE and ETBE by consortia in which when MTBE and ETBE were combined in the solution, mKGS1 could degrade ETBE better than MTBE (Table 2).

Biostimulation

This experiment examined the effect of 1% glucose as an extra carbon source to stimulate the indigenous microorganism in soil microcosm and to stimulate the isolate mKGS1 in order to accelerate the degradation of MTBE and ETBE. The results indicated that 1% glucose significantly stimulated only the degradation of MTBE, from 16% to 42% (Table 4). A positive effect of nutrients was reported by Moreels *et al*. (2004), in which the nutrients and 8 mg/L of dissolved oxygen increased the rate constant (k) of P450 and P600 microcosm in the degradation of MTBE in soil from 0.050 day^{-1} and 0.0074 day^{-1} to 0.097 day^{-1} and 0.043 day^{-1}, respectively. They explained that the extra carbon source added might have led to a larger number of MTBE degraders in these microcosm resulted in a higher MTBE degradation rates in P450 and P600. The presence of mKGS1

Table 3. Bioaugmentation of mKGS1 on degradation of MTBE and ETBE in contaminated soils

	Degradation of MTBE and ETBE (%)			
			Combination of MTBE and ETBE	
	MTBE	ETBE	MTBE	ETBE
Autoclaved soil	5	10	3	7
without isolate mKGS1	16	74	34	52
(non-autoclaved soil)				
with isolate mKGS1	63	75	61	70
(non-autoclaved soil)				

Table 4. Effect of 1% glucose on biodegradation of MTBE, ETBE and combination of MTBE and ETBE in soil (biostimulation)

	Degradation of MTBE and ETBE (%)			
			Combination of MTBE and ETBE	
	MTBE	ETBE	MTBE	ETBE
1% glucose/Autoclaved soil	11	9	6	7
no glucose without isolate mKGS1	16	74	34	52
1% glucose without isolate mKGS1	42	73	46	67
1% glucose with isolate mKGS1	51	71	69	82

with an addition of 1% glucose improved the degradation of MTBE, from 42 to 51%, suggesting mKGS1 played an important role in the degradation of MTBE (Table 4). Enhancement of MTBE degradation in the mixture of MTBE and ETBE was evident when 1% glucose and mKGS1 were added (Table 4) suggesting a positive effect of the combined treatment of bioaugmentation and biostimulation. However, it is worth noting that the percentage of degradation of MTBE (63%) in bioaugmentation study, no 1% of glucose added, (Table 3) was slightly higher than in the biostimulation study (51%), 1% glucose added (Table 4). Hatzinger *et al*. (2001) found that pure culture ENV735 was able to mineralize MTBE when 0.1% sucrose was used as an extra carbon source. Okeke and Frankenberger (2003) reported that when ethanol was used as an additional carbon source, MTBE removal rate by Iso2A was increased. However, François *et al*. (2002) reported that an addition of yeast extract and glucose did not assist growth and degradation of MTBE by *Mycobacterium austroafricanum* IFP 2012.

Conclusion

Three groups of mixed culture i.e. mKMS, mKGS1 and mKGS2 were isolated from three gasoline-contaminated soils. These isolates were capable of degrading both MTBE and ETBE. Due to the highest overall reduction of MTBE and ETBE by mKGS1, it was selected to augmend the soil mixed with MTBE, ETBE and a combination of MTBE and ETBE. Results showed that the degradation of MTBE and ETBE in soil was higher in soil amended with the mKGS1 than without the mKGS1 indicating a successful bioaugmentation. An addition of 1% glucose stimulated the indigenous microorganism to degrade MTBE. The combined treatment of biostimulation and bioaugmentation showed superior results of MTBE degradation.

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