ORIGINAL ARTICLE

Comparison of an ability to degrade MTBE between mixed culture and monoculture isolated from gasoline contaminated soil

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Abstract

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Methyl tertiary butyl ether (MTBE) is an oxygenated compound used to enhance the octane index of gasoline and replace lead in gasoline. MTBE can reduce air pollution but causes water pollution due to its high water solubility and low sorption to soil and thus can easily contaminate the environment. Biodegradation is one of the promising techniques to reduce MTBE contaminated in the environment and MTBE degrader was proposed as an efficient method used to degrade MTBE. In this study, MTBE degraders were isolated from gasoline contaminated soil and then were evaluated with the hypothesis that MTBE degraders could improve biodegradation of MTBE in soil and mixed culture could degrade MTBE more rapidly than monoculture. Gasoline contaminated soil samples were taken from retail gas stations and a motorcycle repair shop in Khon Kaen University. Isolation of MTBE degrader was conducted by using Basal Salt Medium (BSM) containing 200 mg/L of MTBE as a carbon source. Mixed culture of MTBE degrader was

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successfully isolated under aerobic condition. Morphology study was conducted by streaking isolated mixed culture in solid medium, agar slant and identifying the cells shape under a microscope. It was found that this mixed culture was a gram negative bacteria with 7 different isolates. A comparison of the ability to degrade MTBE between mixed culture and monoculture was investigated in BSM containing 100 mg/L of MTBE. The results indicated that a mixed culture degraded MTBE more rapidly than monoculture i.e. 20% within 14 days. Monoculture, J4 and J7, were the most rapid MTBE degraders among the other monocultures in which they degraded 14% of MTBE in 14 days while monoculture J15 could degrade only 1% of MTBE. This preliminary result suggests that mixed cultures degrade MTBE more efficiently than monoculture.

Key words : Methyl tertiary butyl ether (MTBE), biodegradation, mixed culture, monoculture

บทคัดย่อ

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การเปรียบเทียบความสามารถในการย่อยสลาย MTBE ของกลุ่มจุลินทรีย์และจุลินทรีย์สายพันธุ์เดี่ยวที่แยกได้จากดินที่มีการปนเปื้อนแก๊สโซลีน

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Methyl Tertiary Butyl Ether (MTBE) เป็นสารประกอบประเภทอีเธอร์ที่นำมาเติมในน้ำมันเบนซินเพื่อเพิ่มค่าออกเทนแทนการใช้สารตะกั่ว ซึ่งสามารถช่วยลดมลพิษทางอากาศ แต่ทว่ากลับสร้างมลพิษทางน้ำให้เพิ่มมากขึ้น เนื่องจาก MTBE สามารถละลายน้ำได้ในปริมาณสูงจึงทำให้ MTBE ปนเปื้อนในสิ่งแวดล้อมได้ง่าย การกำจัด MTBE สามารถทำได้หลายวิธี ซึ่งวิธีทางชีววิทยาจัดเป็นทางเลือกหนึ่งที่ใช้ในการย่อยสลาย MTBE และการใช้จุลินทรีย์ที่มีความสามารถในการย่อยสลาย MTBE จัดเป็นวิธีหนึ่งที่คาดว่าสามารถเพิ่มประสิทธิภาพในการย่อยสลาย MTBE ได้ จึงทำให้งานวิจัยนี้สนใจที่ทำการคัดเลือกจุลินทรีย์ที่มีความสามารถย่อยสลาย MTBE จากดินบริเวณที่มีการปนเปื้อนแก๊สโซลีนและเปรียบเทียบประสิทธิภาพความสามารถในการย่อยสลาย MTBE ระหว่างกลุ่มจุลินทรีย์และจุลินทรีย์สายพันธุ์เดี่ยว ซึ่งในการทดลองนี้ได้เก็บตัวอย่างดินที่มีการปนเปื้อนแก๊สโซลีนจากบริเวณปั๊มน้ำมันย่อยและร้านซ่อมรถจักรยานยนต์ภายในมหาวิทยาลัยขอนแก่นจำนวน 3 แห่ง จากนั้นทำการคัดเลือกจุลินทรีย์ที่สามารถย่อยสลาย MTBE ได้ในอาหาร Basal salt medium (BSM) ที่มี MTBE ความเข้มข้น 200 มก./ลิตรเป็นแหล่งคาร์บอนให้แก่จุลินทรีย์ ผลการทดลองเบื้องต้นสามารถคัดเลือกกลุ่มจุลินทรีย์ที่มีความสามารถในการย่อยสลาย MTBE และเมื่อทำการศึกษาลักษณะทางสัณฐานวิทยาของกลุ่มจุลินทรีย์ในอาหารแข็ง อาหารวุ้นเอียง และลักษณะของเซลล์ภายใต้กล้องจุลทรรศน์ พบว่า กลุ่มจุลินทรีย์นี้ประกอบด้วยแบคทีเรียแกรมลบ ที่แตกต่างกันจำนวน 7 ชนิด โดยพบว่ากลุ่มจุลินทรีย์สามารถย่อยสลาย MTBE ที่มีความเข้มข้นเริ่มต้นเท่ากับ 100 มก./ลิตร ในอาหาร BSM ได้เร็วที่สุดเมื่อเปรียบเทียบกับจุลินทรีย์สายพันธุ์เดี่ยว คือประมาณ 20% ภายในเวลา 14 วัน ส่วนจุลินทรีย์สายพันธุ์เดี่ยวที่ย่อยสลาย MTBE ได้เร็วที่สุดคือ J4 และ J7 ซึ่งสามารถย่อยสลาย MTBE ได้ประมาณ 14% ภายในเวลา 14 วัน และสายพันธุ์ J15 ย่อยสลาย MTBE ได้ช้าที่สุดคือประมาณ 1% ภายในเวลา 14 วัน ผลการทดลองเบื้องต้นนี้ ชี้ให้เห็นว่าการทำงานร่วมกันของกลุ่มจุลินทรีย์ (mixed culture) ในการย่อยสลาย MTBE จะมีประสิทธิภาพดีกว่าการใช้จุลินทรีย์สายพันธุ์เดี่ยว (monoculture)

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Methyl *tert*-butyl ether (MTBE) or 2-methoxy-2-methyl-propane is a synthetic additive used to replace lead in gasoline as an octane booster (Okeke and Frankenberger, 2003). MTBE enters the environment via accidental release of gasoline (Mo *et al*., 1997). Due to its high water solubility (4.3×10^7 µg/L) and low sorption to soil, MTBE can contaminate groundwater near gas stations, gas storage tanks and filling terminals in the USA (Okeke and Frankenberger, 2003).

MTBE is stable in the environment and hazardous to human and animals. Mehlman (1995, 1998) reported adverse health effects of MTBE such as neurological disorders, respiratory problems and allergic diseases. The U.S. Environmental Protection Agency has listed MTBE as a possible human carcinogen (Cirvello *et al*., 1995) and established a drinking water advisory of 20-40 µg/L (Bradley *et al*., 1999).

Biodegradation is one of the promising techniques to reduce MTBE contaminated in the environment and MTBE degraders were reported as an efficient method used to degrade MTBE. MTBE degraders, monoculture, have been reported to be isolated from different sources. Mo *et al*. (1997) isolated three bacterial strains i.e. *Arthrobacter, Rhodococcus* and *Methylobacterium* from activated sludge and fruit of the Gingko tree. These cultures degraded up to 29% of an initial concentration of 200 mg/L of MTBE in 2 weeks and completely degraded MTBE, evolved as $^{14}CO_2$, about 8% within 7 days. Hanson *et al*. (1999) isolated a bacterial strain, PM1, from compost biofilter of Los Angles County Joint Water Pollution Control Plant. PM1 completely degraded 46% of MTBE, 20 mg/L, within 120 hrs. *Hydrogenophaga flava* ENV735 was isolated from soil or sludge of wastewater treatment plant in Hamilton, New Jersey, USA (Hatzinger *et al*., 2001). This isolate grew slowly on MTBE or *tert*-butyl alcohol as carbon and energy source, but growth on these substrates was greatly enhanced by the addition of a small amount of yeast extract. Degradation of MTBE by mixed culture were also reported by a number of workers (Salanitro *et al*., 1994; Cowan and Park, 1996; Borden *et al*., 1997; Landmeyer *et al*., 1998; Eweis *et al*., 1998; Bradley *et al*., 1999 and Kane *et al*., 2001).

The biological degradation of MTBE under aerobic conditions has been conducted by several workers. However, there is limited information on comparison of an ability to degrade MTBE between mixed culture and monoculture. In this study, we report a preliminary result of morphology of MTBE degraders, both mixed culture and its monoculture, isolated from gasoline contaminated soil. A comparison of the ability to degrade MTBE by mixed culture and its monoculture is also reported.

Materials and Methods

Chemicals

MTBE (99% purity) was purchased from Fluka Aldrich Co., Inc., France. Diethyl ether (DEE) (HPLC grade) was purchased from MERCK, Germany. Other chemicals were purchased from BDH chemical, England.

Microorganism media

Basal salt medium (BSM) (Mo *et al*., 1997) (in g/L) contained: 5.57 of Na_2HPO_4, 2.44 of KH_2PO_4, 2.00 of NH_4Cl, 0.20 of $MgCl_2.6H_2O$, 0.0004 of $MnCl_2.4H_2O$, 0.001 of $FeCl_3.6H_2O$ and 0.001 of $CaCl_2$. Basal salt agar was added with 15 g/L of agar. pH was adjusted to 7.0 and then autoclaved at 121 °C for 15 minutes. MTBE was added to BSM medium at the concentration of 200 mg/L for screening and 100 mg/L for degradation study.

Soil samples

Soil samples, 0-15 cm depth, were collected using soil probe with a diameter of 2.96 cm from three gasoline contaminated sites: 1) a motorcycle repair shop next to Dormitory Number 22, Khon-Kaen University; 2) a retail gasoline station across from Dormitory number 3, Khon Kaen University; and 3) a retail gasoline station next to Food Court, Khon Kaen University. The composite sample of

three cores was collected from each site and kept in plastic bag at 4 °C until ready to be used in the experiment.

Isolation of MTBE degrader

Enrichment technique was used to isolate MTBE degrader. Ten grams of each soil sample was dissolved in 100 ml of BSM containing 200 mg/L of MTBE as a C source. Flasks were incubated at 30 °C and shaken at 150 rpm for 10 days before transferred to a fresh medium containing 200 mg/L of MTBE. This was done 4 times or until media showed no sign of soil particles. The inoculum size used was 10%. After the last transfer, one mL of cell suspension was spread on BSM agar coated with 200 mg/L of MTBE and incubated at 30 °C for 24-48 hrs. Colonies grown on BSM agar were considered to be microorganisms capable of degrading MTBE or so-called MTBE degrader. The colonies then were 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.

Degradation of MTBE by isolated mixed culture and its monoculture

Isolated mixed culture and its monoculture, 10^6 cells/mL, were each inoculated into 50 mL of BSM containing 100 mg/L of MTBE (adapted from Mo *et al*., 1997) in serum bottles capped with butyl rubber and aluminum cap to examine the capability of degrading MTBE. The bottles were incubated at 30 °C and shaken at 200 rpm (adapted from Mo *et al*., 1997). Samples were taken at day 0, 3, 5, 7 and 14 to check for MTBE concentration using Gas Chromatography-head space technique. The end point of sampling date at Day 14 was selected because of the limited experimental time.

Analysis of MTBE using Gas Chromatography-head space technique

MTBE concentration in the sample was analyzed by adding 25 g of NaCl (Achten and Puttmann, 2000) into 100 ml of BSM medium containing MTBE. Then the sample was heated in a water bath at 80 °C for 40 min. Twenty-five μL of the head space sample was taken by using gas tight syringe and then analyzed for MTBE concentration using GC-17A Shimadzu Gas Chromatography-Flame Ionization Detector (GC-FID). The capillary column used was 30-m Rtx-VGC with an inner diameter of 0.45-mm (Restek Inc., USA). Helium was the carrier gas with a flow rate of 1.8 mL/min. Split mode with a ratio 1:2 was used. The GC column temperature was first held at 35 °C for 13 min, then increased to 180 °C at a rate of 30 °C/min and held for 10 min. Diethyl ether (DEE) was used as an internal standard. A standard curve was plotted between ratios of area of response of MTBE/DEE (y-axis) and relative known concentrations of MTBE in mg/l (x-axis). The coefficient of determination (r^2) of the standard curve used throughout the analysis was 0.99.

Results

Morphology of isolated mixed culture

Seven isolates were obtained after a mixed culture was streaked on BSM coated with 200 mg/L. The results indicated that all isolated monocultures were gram negative. Figure 1 shows the morphology of each monoculture under the microscope (magnification of ×1000) and Table 1 shows the details of morphology of monocultures under the microscope.

Degradation of MTBE by mixed culture and its monoculture

MTBE was degraded by mixed culture more rapidly than by monoculture. Twenty percent of MTBE was degraded in 14 days by mixed culture while the highest percentage of MTBE degraded by monocultures i.e., J4 and J7, was only 14. Table 2 reveals the percentage of MTBE degraded by mixed culture and its monoculture. Degradation of MTBE by mixed culture and its monoculture were fitted to a modified first-order kinetic model (Figure 2); $C = C_0 e^{-kt} + Ya$, where C is the mean

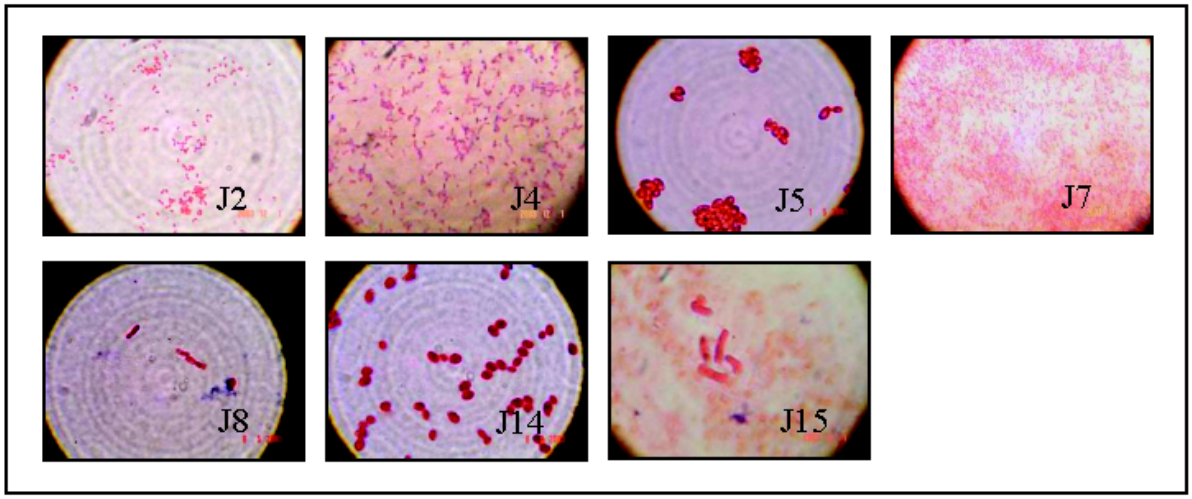

Figure 1. Morphology of isolated monocultures under microscope (magnification of ×1000).

Table 1. Morphology of monoculture under the microscope

Isolate Names	Colonies forming	Growth in agar slant	Morphology under microscope
J2	Orange, circular, spindle, erose, smooth, translucent, diameter of 1 mm	Filiform	Gram negative, cocci
J4	White, circular, covex, entire, smooth, translucent, diameter of 2-3 mm	Filiform	Gram negative, short rod
J5	White, circular, flat, entire, smooth, opaque, diameter of 1 mm	Beaded	Gram negative, oval
J7	Yellow, circular, covex, entire, smooth, translucent, diameter of 1-2 mm	Beaded	Gram negative, short rod
J8	Light brown with a dark brown spot in the middle, circular, covex, undulate, smooth, opalescent, diameter of 2-3 mm	Echinulate	Gram negative, rod
J14	Pink, circular, pulvinates, covex, entire, smooth, opaque, diameter of 1 mm	Filiform	Gram negative, oval
J15	Cream, circular, covex, undulate, radiately ridge, opaque, diameter of 3-4 mm	Beaded	Gram negative, rod

concentration of MTBE as a function of time in days (mg/L), Co is the initial MTBE concentration (mg/L), k was the rate constant (/day, t was time (days), and Ya was an asymptotic estimate of the concentration of MTBE that degrades very slowly over time (residual MTBE) (mg/L). Average initial MTBE concentration (at Day 0) was 104.15 mg/L. The mean concentrations used in the regression were weighted with inverse of the variance squared, S^{-2}. This method compensated for the non-constant variance and helped to improve estimation of parameters. The coefficients

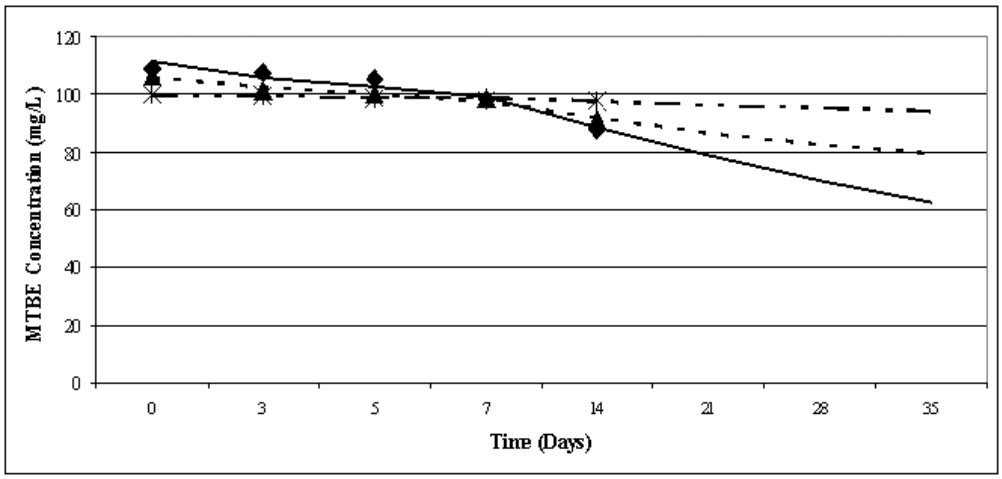

Figure 2. Dissipation of MTBE by mixed culture (♦) and its monoculture J7 (▲) and J14 (✳). Lines indicated MTBE concentrations fitted to the first order kinetic model.

Table 2. Percentage of MTBE degraded by mixed culture and its monoculture at Day 14

Isolate Names	MTBE concentration (mg/L)		Biodegradation (%)[a)]
	Day 0	Day 14	
J2	105.42	95.00	10
J4	103.05	88.02	14
J5	106.52	92.3	13
J7	106.49	91.44	14
J8	106.45	93.37	12
J14	100.11	97.76	2
J15	101.25	99.63	1
Mixed culture	109.13	87.63	20
Control (no inoculum)	97.98	97.43	0.5

[a)] **Biodegradation (%) was calculated from concentration of MTBE at day 14** $= \frac{\text{conc. at day 14 - conc. at day 0}}{\text{conc. at day 14}} \times 100$

of determination, r^2, ranged between 0.88-0.99 and indicated good fit of the data to the first-order kinetic model (data not shown).

Conclusions and Discussions

The results indicated that mixed culture degraded MTBE more efficiently than monoculture. Mo *et al*. (1997) and Okeke and Frankenberger (2003) reported similar trends. Mo *et al*. (1997) explained that this may be due to the fact that in mixed culture one strain may be able to degrade MTBE to a certain product and then another strain may take over and no toxicity or inhibition results. Okeke and Frankenberger (2003) stated that monoculture had advantage over mixed culture in

that monoculture isolates can be easily grown to high biomass density on high yield substrate better than mixed culture. In contrast, mixed culture must be grown on the main target compound to be maintained (Okeke and Frankenberge., 2003); and some target compounds such as MTBE produced low cell yields (Steffan *et al*., 2000).

In conclusion, according to our data, mixed culture was more attractive to be used to degrade MTBE residue in the environment than monoculture. Further studies will include the bioaugmentation of MTBE by isolated MTBE degraders (mixed culture and its isolate). The ability of these isolated microorganisms to degrade Ethyl Tertiary Butyl Ether will also be determined.

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