ORIGINAL ARTICLE

Application of terpene-induced cell for enhancing biodegradation of TCE contaminated soil

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Abstract

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Trichloroethylene (TCE), a chlorinated solvent, is a major water pollutant originating from spillage and inappropriate disposal of dry cleaning agents, degreasing solvents, and paint strippers. Due to its wide-spread contamination and potential health threat, remediation technology to clean-up TCE is necessary. Aerobic biodegradation of TCE is reported to occur via cometabolism, by which TCE degrading bacteria utilize other compounds such as toluene, phenol, and methane as growth substrate and enzyme inducer. Although toluene is reported to be the most effective inducer, it is regulated as a hazardous material and should not be applied to the environment. The objectives of this study were to identify an alternative enzyme inducer as well as to apply the induced bacteria for degradation of TCE in contaminated soil. We investigated the effect of terpenes, the main components in volatile essential oils of plants, on induction of TCE degradation in *Rhodococcus gordoniae* P3, a local Gram (+) bacterium. Selected terpenes including cumene, limonene, carvone and pinene at various concentrations were used in the study. Results from liquid culture showed that 25 mg l^{-1} cumene-induced *R. gordoniae* P3 cells resulted in 75% degradation of 10 ppm TCE within 24 hrs. Soil microcosms were later employed to investigate the ability of cumene to enhance TCE biodegradation in the environment.

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There were two bioremediation treatments studied, including bioaugmentation, the inoculation of cumene-induced *R. gordoniae* P3, and biostimulation, the addition of cumene to induce soil indigenous microorganisms to degrade TCE. Bioaugmentation and biostimulation were shown to accelerate TCE reduction significantly more than control treatment at the beginning of study. The results suggest that cumene-induced *R. gordoniae* P3 and cumene can achieve rapid TCE biodegradation.

Key words : TCE, terpene, biodegradation, bioremediation, cometabolism

บทคัดย่อ

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การประยุกต์ใช้เซลล์ที่ชักนำด้วยสารเทอร์ปีนเพื่อส่งเสริมกระบวนการย่อยสลายทางชีวภาพของทีซีอีที่ปนเปื้อนในดิน

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ไตรคลอโรเอธิลีน (ทีซีอี) เป็นตัวทำละลายที่มีคลอรีนเป็นองค์ประกอบ และเป็นมลพิษทางน้ำที่สำคัญ โดยมีที่มาจากการหกหรือการกำจัดสารจำพวกน้ำยาซักแห้ง สารทำความสะอาดไขมัน และน้ำยาขัดสีที่ไม่เหมาะสม เนื่องจากทีซีอีมีการปนเปื้อนเป็นบริเวณกว้างและอาจจะก่ออันตรายต่อสุขภาพ จึงจำเป็นต้องมีเทคโนโลยีบำบัดเพื่อใช้ทำความสะอาดสารนี้ การย่อยสลายทางชีวภาพของทีซีอีในสภาวะที่มีอากาศนั้น มีการรายงานว่าเกิดขึ้นโดยกระบวนการโคเมตาบอลิสม โดยแบคทีเรียที่ย่อยสลายทีซีอีจะใช้สารโทลูอีน ฟีนอล และมีเธน เป็นสารตั้งต้นสำหรับการเจริญเติบโตและการชักนำเอนไซม์ แม้ว่าโทลูอีนจะเป็นสารชักนำที่มีประสิทธิภาพดีที่สุด แต่มันไม่ควรนำไปใช้ในสิ่งแวดล้อมเนื่องจากถูกจัดว่าเป็นสารอันตราย จุดมุ่งหมายของการทดลองนี้คือ การหาสารชักนำเอนไซม์ตัวอื่น และการใช้แบคทีเรียที่ถูกชักนำมาย่อยสลายทีซีอีในดินที่ปนเปื้อน โดยเราได้สำรวจผลของสารเทอร์ปีนซึ่งเป็นส่วนประกอบหลักในน้ำมันหอมระเหยของพืช ต่อการชักนำกระบวนการย่อยสลายทีซีอีโดยแบคทีเรีย *Rhodococcus gordoniae* P3 ซึ่งเป็นแบคทีเรียแกรมบวกที่พบในท้องถิ่น สารเทอร์ปีนที่นำมาทดสอบคือ คิวมีน ไลโมนีน คาร์โวน และไพนีน ที่ความเข้มข้นต่าง ๆ ผลการทดลองจากอาหารเลี้ยงเชื้อเหลวพบว่า คิวมีนที่ความเข้มข้น 25 มก/ลิตร สามารถชักนำเซลล์ของ *R. gordoniae* P3 ให้ย่อยสลาย 75 % ของทีซีอีความเข้มข้น 10 ส่วนในล้านส่วน ในเวลา 24 ชั่วโมง หลังจากนั้นได้ทำการศึกษาโดยใช้ชุดทดลองดินขนาดเล็ก เพื่อตรวจสอบความสามารถของคิวมีนในการส่งเสริมการย่อยสลายทางชีวภาพของทีซีอีในสิ่งแวดล้อม ทั้งนี้มีวิธีบำบัดทางชีวภาพ 2 วิธี คือ วิธี Bioaugmentation โดยการเติม *R. gordoniae* P3 ที่ชักนำด้วยคิวมีน และวิธี Biostimulation โดยการเติมคิวมีนเพื่อชักนำจุลินทรีย์ที่มีอยู่ในดินให้ย่อยสลายทีซีอี การบำบัดด้วยวิธี Bioaugmentation และวิธี Biostimulation ช่วยเร่งให้ปริมาณทีซีอีลดลงเร็วกว่าชุดควบคุมอย่างมีนัยสำคัญในช่วงแรกของการทดลอง จึงสรุปได้ว่า *R. gordoniae* P3 ที่ชักนำด้วยคิวมีน และคิวมีน ช่วยให้การย่อยสลายทางชีวภาพของทีซีอีเกิดขึ้นอย่างรวดเร็ว

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TCE, a chlorinated solvent, has been widely used as an ingredient in industrial cleaning solutions and as a degreasing agent. Due to its recalcitrance and chemical properties, widespread contamination of terrestrial subsurface environmental media has occurred. Since TCE is a known animal and suspected human carcinogen (Infante and Tsongas, 1987), considerable effort has been

focused on methods to remediate this contamination. Bioremediation is one of the most promising technologies for cleaning up soil and groundwater contamination because of its low cost and its potential for the destruction of the pollutant.

Aerobic biodegradation of TCE occurs through a process termed cometabolism, meaning that the bacteria degrade TCE fortuitously by a non-specific enzyme. In order to produce the required enzyme, a substrate must be provided with an inducer, for example methane and aromatic compounds such as toluene and phenol. Accordingly, bioremediation trials have been carried out by injection of methane (Semprini, 1997), toluene (Fries *et al.*, 1997), and phenol (Hopkins *et al.*, 1993) to stimulate indigenous bacteria at the TCE contaminated sites.

Of all the inducers, TCE degradation by toluene- and phenol-induced cells is the most effective and has been extensively studied (Fries *et al.*, 1997). However, toluene may not be suitable for inducing TCE degradation in the environment because it is a classified hazardous substance that should not be used as a soil or water amendment. On the other hand, phenol addition may be questioned since chlorination of groundwater containing phenol produces chlorinated phenols that cause taste and odor problems (Hopkins *et al.*, 1993). To prevent this problem, alternative compounds that are non-toxic, environmentally friendly and cheap are required for TCE cometabolism.

Terpenes are the main components in volatile essential oils of plants, for example limonene from lemon oil, carvone from peppermint oil, and pinene from pine oil. Plant terpenes have structures that are analogous to many commercially produced chemicals and have been reported to stimulate microbial degradation of xenobiotic compounds such as polychlorinated biphenyls (PCB), toluene and phenol (Crowley *et al.*, 2001, Singer *et al.*, 2003). To date, the application of plant derived compounds for TCE degradation is limited and the only plant terpene that has been studied is cumene (isopropylbenzene) (Dabrock *et al.*, 1992, Pflugmacher *et al.*, 1996). The reports showed that *Pseudomonas* sp. JR1 and *Rhodococcus erythropolis* BD2, were able to oxidize TCE after induction by cumene. However, the utilization of plant terpenes for degradation of TCE in contaminated soil has never been studied, and therefore prevents the development of TCE bioremediation program.

The objectives of this study were to investigate the ability of plant terpene as an alternative inducer for TCE biodegradation and to apply terpene-induced cell for TCE bioremediation. The efficiency of four plant terpenes including cumene, limonene, carvone and pinene (Figure 1) on induction of the TCE degradative pathway in *Rhodococcus gordoniae* P3, a local Gram (+) bacterium is reported. We discovered that 25 mg l^{-1} cumene was suitable for inducing TCE degradation in bacterial liquid cultures. The study then examined the effectiveness of TCE bioremediation strategies using cumene-induced *R. gordoniae* P3 cells (bioaugmentation treatment) and cumene solution (biostimulation treatment) added to TCE amended soil microcosm.

Materials and Methods

Chemicals

TCE (99.5% purity) and four types of purified terpene solution (99.0% carvone, 96.0% limonene, 97.0% pinene, 99.0% cumene) were obtained commercially from Merck, USA. TCE solutions were prepared by dissolving aliquots of TCE in N,N-dimethylformamide (Merck, USA) to obtain the desired concentration. Hexane (Fisher Scientific, Inc.) and triton x-100 (Fluka Chemical Industrial) were used for extraction of TCE from liquid cultures and soil samples. All solvents were reagent grade.

Microorganism and culture maintenance

This study used *R. gordoniae* P3, a gram (+) bacterium isolated from petroleum-contaminated soil collected from Bangkok area (Luepromchai, 2004). The bacterium generally utilizes toluene

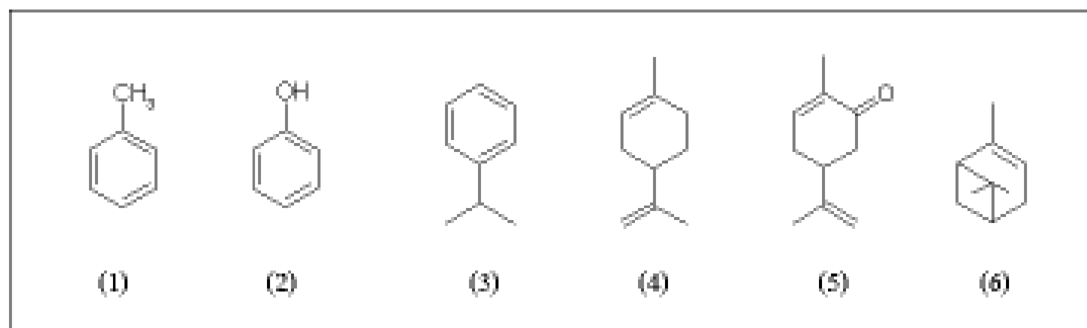

Figure 1. Structures of xenobiotic compounds commonly used as substrate and inducer for supporting TCE degradation; (1) Toluene; (2) Phenol, and structures of terpenes used as inducer in this study; (3) Cumene; (4) Limonene; (5) Carvone; and (6) Pinene.

as the sole carbon source thus it was cultured on mineral salts (MS) agar and incubated in toluene-equilibrated glass box. Approximately every two to three weeks, the culture was transferred to a new medium. Mineral salts (MS) medium was used to supply essential nutrients for the bacteria and prepared according to Focht (1994).

Terpene-induced cell preparation

Terpene-induced *R. gordoniae* P3 cells for TCE degradation assay were prepared as follows. Liquid culture bacteria were grown in a 250-ml Erlenmeyer flask containing 100 ml mineral salts (MS) medium, by which toluene (substrate) was supplied by hanging an Eppendorf tube containing 200 µl toluene above the medium. The culture was incubated on an orbital shaker at 200 rpm, room temperature for 24 hrs. Then, the bacteria were induced by aliquoting the culture to a second 250-ml Erlenmeyer flask (the optical density (OD) at 600 nm should be about 0.02-0.04 at the beginning) that contained 100 ml glucose-MS medium (4 g l^{-1}). Stock solution of terpene (limonene, carvone, pinene and cumene prepared in N,N-dimethylformamide) was added directly to the flask to give a final concentration of 5, 10, 25, and 50 mg l^{-1}; and the culture incubated again for 18 hrs. Induced-bacteria cells were harvested using refrigerated centrifuge at 4,800 rpm for 10 minutes. The supernatant was discarded, and the bacterial cells were washed by resuspending them in 10 ml fresh MS medium. One ml of each sample was removed, diluted with 9 ml of mineral salts (MS) medium, mixed thoroughly, and the optical density adjusted to 1.0 at 600 nm (approximately 10^8 CFU ml^{-1}).

TCE biodegradation in liquid culture

TCE biodegradation assay was carried out in liquid bacterial culture by a method adapted from Nelson *et al.* (1987) and Luu *et al.* (1995). Bacterial cells were induced with terpenes for 18 hrs as described above, then 5 ml of the induced cell suspensions (OD_{600} = 1.0) were transferred to 50-ml serum bottle sealed with Teflon-lined rubber septum and aluminum crimp cap. Oxygen was provided only at the beginning of study to minimize the loss of TCE by volatilization. TCE stock solutions were prepared in N,N-dimethylformamide, and aliquots were added with gas-tight syringe to give a final TCE concentration of 10 ppm. The bottles were shaken at 200 rpm, room temperature for 24 hrs. The experiment was done in triplicate. TCE degradation was monitored by measuring TCE concentrations in the aqueous phase of the test bottles by hexane extraction and gas chromatography as described below. The 100% baseline used to determine the

extent of TCE degradation was determined from the average of the triplicate, first-hour samples.

TCE bioremediation in soil microcosm

Soil sample for bioremediation study was collected from an uncontaminated region (agricultural soil) located in Nontaburi province. The soil was pulverized by passage through a 2-mm sieve. Prior to the treatment, the soil was analyzed and found to be free of TCE. Selected soil properties were determined by the System Development of Soil and Water Analysis Subgroup, Agricultural Chemistry Research Group, Department of Agriculture. The properties were summarized as follows: soil texture (%); sand: silt: clay = 8.6: 31.4: 60.0, pH = 4.13, organic carbon (%) = 1.46, organic matter (%) = 2.52, soil moisture content (%) = 3.3, nitrogen (%) = 0.126, phosphorus (ppm) = 142, and maximum water holding capacity (%) = 43.25.

The bioremediation experiments consisted of bioaugmentation, biostimulation, and control (no treatment). Soil microcosms consisted of 5 g non-sterilized soil in 50-ml serum bottles. TCE was spiked into the bottles using gas-tight syringe to give a final concentration of 100 ppm. In bioaugmentation treatments, *R. gordoniae* P3 were grown on glucose-MS medium which contained 25 mg l^{-1} cumene for 24 hrs (see terpene-induced cell preparation) before adding to the soil microcosms to give a final concentration of 10^8 CFU g^{-1} soil. In the biostimulation treatments, only cumene solution was added directly to soil microcosm. Soil moisture content of each microcosm was adjusted by addition of cell-containing medium or cell-free medium. Excess O_2 was provided at the beginning of study, and then the microcosm was sealed with Teflon-lined rubber septum. During incubation at room temperature and dark condition, the samples were taken by sacrificing certain microcosms at 0, 4, 7, 10, and 14 days.

TCE extraction and analysis

TCE extraction and analysis procedure was modified from Leahy *et al.* (1996). For liquid cultures, 2 ml aliquots of liquid culture were transferred into a new 8-ml vial and then 2 ml of hexane and 400 µl of 10% Triton x-100 were added, mixed and shaken at 200 rpm for 2 hrs. For soil sample, 10 ml hexane and 3 ml 15% Triton x-100 solution were added directly to the sacrificed soil microcosm (in 50-ml sealed serum bottles) with gas-tight syringe and shaken for 2 hrs at 200 rpm. After shaking, the vials were frozen at -4 °C to solidify the lower aqueous layer, and then the solvent fraction was transferred with a Pasteur pipette to an 8 ml vial where 2-3 g of anhydrous sodium sulfate was added to dewater the sample. TCE dissolved in solvent fraction was transferred to gas chromatography (GC) auto sampler vials for analysis using gas chromatography (GC). The extraction efficiency during the experiment in liquid cultures and soil microcosm were 90 ± 10%. Recovery rates were consistent for all samples.

GC analysis was performed with a Hewlett-Packard 6890 equipped with an electron capture detector (GC-ECD) and a HP-5 (5% Phenyl Methyl Siloxane) fused-silica capillary column (30 m × 0.32 mm ID; thickness, 0.25µm). The following operating conditions were used: injector temperature 250 °C, detector temperature 250 °C, initial column temperature 80 °C then, programmed at 80 °C to 100 °C at a rate of 25 °C min^{-1} (4 min), and 100 °C to 150 °C at a rate of 40 °C min^{-1} (2 min), and 150 °C to 300 °C at a rate of 90 °C min^{-1}(2 min). A post column temperature of 80 °C was held for 2 min. The carrier gas was helium with gas flow of 20 ml min^{-1}, and a 40:1 injector split ratio. The make-up gas was N_2 at 70 ml min^{-1}.

Quantifying of toluene degraders in soil

Toluene-utilizing bacteria were used to represent the bacteria that are capable of TCE degradation. The number of toluene utilizing bacteria in treated soil was determined by spread plate technique. The procedure consisted of diluting 1 g soil sample with a series of sterile MS medium and spread on MS agar. Triplicate of each dilution were done. Spread plates were in-

cubated at room temperature in a glass box supplied with toluene as the sole carbon and energy source for 1 wk. Then, bacteria colonies were counted and the results were averaged.

Results

Biodegradation of TCE in liquid culture by terpene-induced *R. gordoniae* P3

Four plant terpenes, namely carvone, cumene, limonene, and pinene were screened for their ability to induce TCE degradative pathway in *R. gordoniae* P3 compared to control treatments. These control treatments consisted of heat-killed cells and non-induced cells (glucose-grown cells). Decreasing of TCE in heat-killed cells treatment indicated the effect of non-enzymatic processes i.e. TCE adsorption on bacterial cells and volatilization, whereas non-induced cells treatment represented the possibility of TCE degradation by constitutive enzymes (non-induced enzymes). In this study, percent TCE degradation was calculated from the difference between the amount of TCE in first-hour sample (100% baseline) and the remaining TCE after 24-hr incubation.

TCE degradation by terpene-induced cells ranged from 53-76%, whereas heat-killed cells and non-induced cells led to 42% and 58% decrease of TCE, respectively (Table 1). Cumene at 10-50 mg l^{-1}, limonene at all concentrations, carvone at 5-25 mg l^{-1}, and pinene at 5-10 mg l^{-1} had no harmful effect on *R. gordoniae* P3 cells because the induced cell treatments degraded TCE sig-

Table 1. Percent TCE degradation in liquid culture containing *Rhodococcus gordoniae* P3 cells with various inducers after 24-hr incubation*.

Treatments	Amount of Inducers (mg l^{-1})	% TCE degradation
Heat-killed cells	0	42^{a}
Non-induced cells	0	58^{A}
Toluene-induced cells	50	74^{bA}
Cumene-induced cells	5	60^{aA}
	10	72^{bA}
	25	76^{bB}
	50	68^{bA}
Limonene-induced cells	5	67^{bA}
	10	61^{bA}
	25	66^{bA}
	50	61^{bA}
Carvone-induced cells	5	72^{bA}
	10	65^{bA}
	25	64^{bA}
	50	59^{aA}
Pinene-induced cells	5	62^{bA}
	10	63^{bA}
	25	53^{aA}
	50	60^{aA}

*** Comparisons between treatment and heat-killed cells are significantly different (LSD, P <0.05) if marked with different small letters. Comparisons between treatment and non-induced cells are significantly different (LSD, P <0.05) if marked with different capital letters.**

nificantly more than heat-killed cells. However, high amount of terpene may be toxic to bacterial cells as shown by the significantly decrease of percent TCE degradation by *R. gordoniae* P3 cells induced with carvone at 50 mg l^{-1} or pinene at 25 and 50 mg l^{-1}. The results also showed that considerable amount of TCE (42%) was lost through abiotic process.

When comparing the extent of TCE degradation between induced cells and non-induced cells, it was found that only *R. gordoniae* P3 induced with 25 mg l^{-1} cumene could degrade TCE significantly more than the non-induced cells (Table 1). Percent TCE degradation by *R. gordoniae* P3 induced with 25 mg l^{-1} cumene and non-induced cells were 76% and 58%, respectively. The results suggested that *R. gordoniae* P3 was probably using non-specific enzymes to degrade TCE; however the bacteria produced specific TCE degrading enzyme when 25 mg l^{-1} cumene was presented in the culture.

Toluene was reported to be the most effective inducer. However, our results showed that percent TCE degradation of toluene-induced cells was comparable to 25 mg l^{-1} cumene-induced cells, which was 74% and 76%, respectively. In addition, toluene-induced cells degraded TCE significantly more than heat-killed cells, but it was not effective when compared to non-induced cells.

Bioremediation of TCE contaminated soil using cumene-induced *R. gordoniae* P3 and cumene solutions.

Biodegradation of 100 ppm TCE contaminated soil was studied in microcosm containing 5 g spiked soil with 30% moisture content. The percent moisture content was selected based on a preliminary study that showed moisture content at 10, 30, and 45% had no significant effects on the extent of TCE degradation in soil microcosms (Figure 2). Meanwhile, the amount of moisture affected the mixing of soil with added bacteria as well as the percent TCE recovery. We found that moderate mixing and a higher amount of TCE degradation was achieved from 30% moisture content (data not shown). Percent TCE remaining in soil microcosm were used to determine the amount of TCE losses due to biodegradation and any other loss mechanisms that may be associated

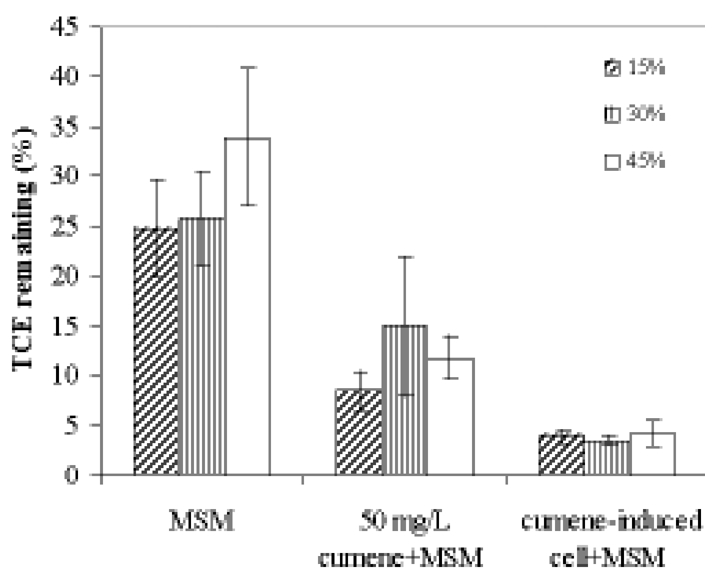

Figure 2. Percent TCE remaining in soil microcosms with 15, 30, and 45% moisture contents after 14-day incubation.

with the treatment. The value was determined from the difference between initial TCE (100%) concentration and the amount of TCE recovered at designated time.

Bioaugmentation treatment was done by adding *R. gordoniae* P3 induced with 25 mg l^{-1} cumene to TCE contaminated soil microcosms. At the beginning of the study, TCE decreased rapidly in the bioaugmented soil, and only 30% of TCE was remained after 4-day incubation (Figure 3). The amount of TCE decreased gradually later on and became stable at 5% after 10 days. At day 4 and 7, percent TCE remaining in bioaugmentation treatments (30% and 15%) were significantly lower than control treatments, which ranged from 75-85% after 4-day and 35-55% after 7-day incubation.

Biostimulation treatments were conducted by addition of cumene at 25, 50, and 100 ppm to TCE contaminated soil microcosms. Cumene as an alternative TCE enzyme inducer was expected to stimulate indigenous soil microorganisms to degrade TCE. Amount of TCE reduction between the treatments with various cumene concentrations was not significantly different at any time point (Figure 4). During the first 4 days, TCE rapidly decreased to approximately 40% of its initial concentration in all biostimulation treatments. The amount of TCE in biostimulation treatments at this stage was significantly lower than in control treatments. Later, the amount of TCE gradually decreased until less than 5% remained in the soil after 10 days.

Control treatments consisted of microcosms with dry soil (non-amended soil microcosm) and MSM (microcosms with MS medium to maintain 30% moisture content). Reduction of TCE in dry soil microcosms indicated the effect of abiotic process, whereas MSM microcosms represented the effects of intrinsic bioremediation that might have occurred. Amount of TCE remaining in control dry soil was significantly higher than in bioaugmentation and biostimulation treatments at all time points, even though TCE gradually decreased to 40% at day 14 (Figures 3 and 4). On the other hand, percent TCE remaining in control MSM microcosms was not significantly different from biostimulation soil after 7 days and from

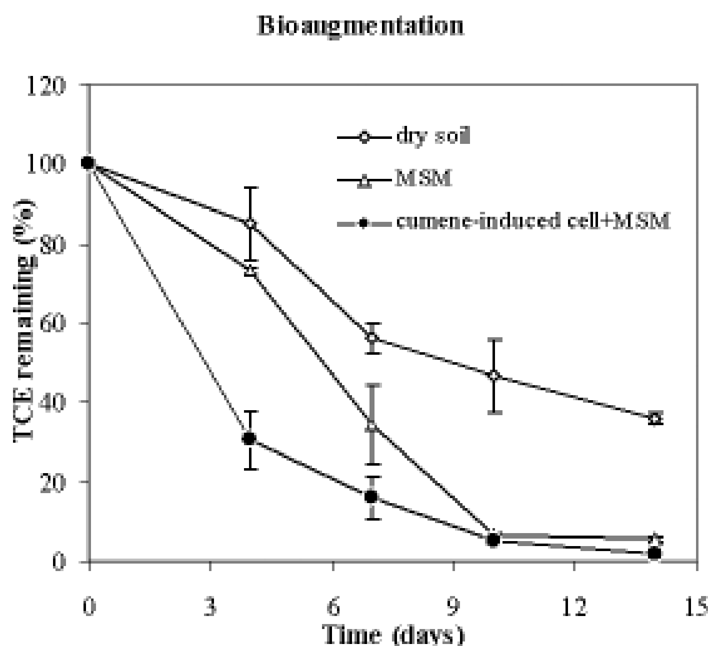

Figure 3. Percent TCE remaining in the bioaugmented soil microcosms.

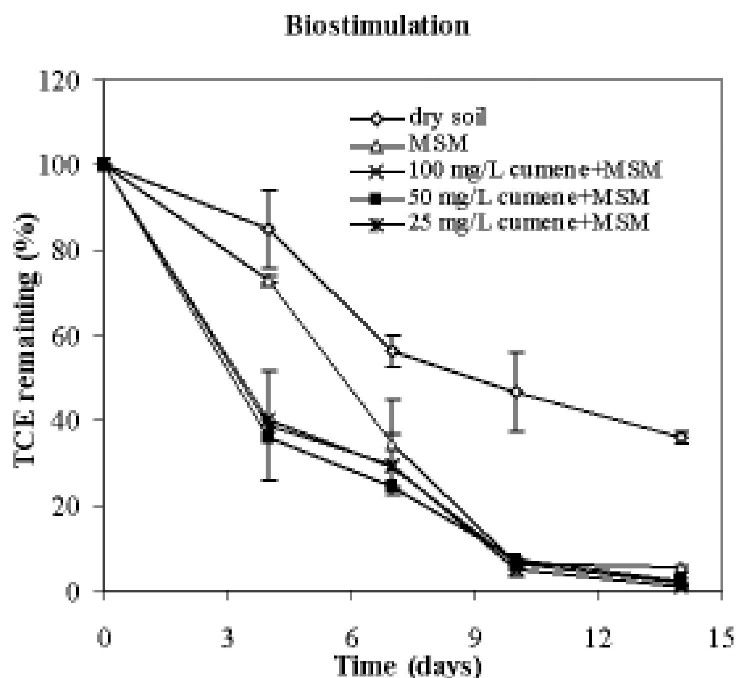

Figure 4. Percent TCE remaining in the biostimulated soil microcosms.

bioaugmentation soil after 10 days. Percent TCE remaining in these microcosms was 35% at day 7 and less than 10% at day 10.

Densities of toluene degraders in the bioremediated soil

Population densities of toluene degraders were used to indicate the survival of added bacteria in bioaugmentation treatment as well as represent the increasing number of TCE degrading bacteria after biostimulation. The results showed that toluene degrader populations in every treatment increased from approximately 10^6 CFU g^{-1} soil at the beginning of study to 10^{14} CFU g^{-1} soil at day 14 (Table 2). The extent of bacterial proliferation was affected by each treatment. Toluene degrader populations were found in bioaugmentation treatment (6×10^6 CFU g^{-1} soil) to be greater than in any treatments at the beginning of the study, whereas more bacteria were found in biostimulation treatments (6.2-6.9 $\times 10^{14}$ CFU g^{-1} soil) after 14 day incubation. When compared between treatments, the densities of bacteria in control and bioaugmentation treatments were significantly different only at day 0, but became comparable at approximately 5×10^{14} CFU g^{-1} soil after incubation. The amount of toluene degraders in MSM control and bioaugmentation treatment was significantly different from that in all biostimulation treatments (cumene at 25, 50, and 100 mg l^{-1}) at day 0, 4, and 14.

Discussion

The result showed that cumene was able to induce TCE co-metabolic pathway of *R. gordoniae* P3; in particular 25 mg l^{-1} cumene could induce *R. gordoniae* P3 to degrade 75% of 10 ppm TCE within 24 hrs. Cumene is a terpene that is found in variety of essential oils from plants as well as foodstuffs such as cumin seed and curry. The compound is also a constituent of crude oil and finished fuels. From Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/index.html),

Table 2. Number of toluene-degrading bacteria found in the treated soil microcosms*.

Time (Days)	Treatment ($\times 10^8$ CFU g^{-1})				
	Control[1]	Bioaugmentation[2]	Biostimulation		
			25 ppm Cumene	50 ppm Cumene	100 ppm Cumene
0	0.01^{A}	0.06^{B}	0.02^{C}	0.02^{C}	0.02^{C}
4	9.77^{A}	14.50^{B}	3.60^{C}	8.90^{A}	7.00^{AC}
10	$82{,}700^{A}$	$102{,}000^{A}$	$93{,}000^{A}$	$92{,}300^{A}$	$93{,}000^{A}$
14	$5{,}400{,}000^{AB}$	$5{,}100{,}000^{B}$	$6{,}930{,}000^{C}$	$6{,}430{,}000^{C}$	$6{,}200{,}000^{AC}$

*** Comparisons between treatments within each time period are significantly different (LSD, P <0.05) if marked with different capital letters.**
[1] Control treatment contained MS medium only.
[2] Bioaugmentation treatment was done by adding *R. gordoniae* P3 induced with 25 mg l^{-1} cumene.

cumene is not classified as human carcinogen. Once exposed, it is metabolized primarily to the secondary alcohol and readily excreted out of the body. If released to soil, cumene is expected to volatilize and undergo considerable biodegradation in soil environments. Due to its benign and non-persistent nature, the compound would be a potential alternative to toluene for biodegradation and bioremediation of TCE.

The ability of cumene on TCE induction was similar to previous reports (Dabrock *et al.*, 1992; 1994; Pflugmacher *et al.*, 1996). They found that cumene inducing *R. erythropolis* BD2 cells degraded 71% of 50 µM TCE after 20-hr incubation. Cumene is able to induce TCE degradation probably because the bulky isopropyl residue adjacent to the double bond on the benzene ring may mimic the two chlorines in the TCE molecule (Dabrock *et al.*, 1992). Although other terpenes could not enhance TCE biodegradation significantly, this may be because the concentrations of certain terpene used in the study were not appropriate. Optimization of terpene concentrations and screening of more terpene varieties are suggested for future study.

The ability of *R. gordoniae* P3 on TCE degradation after induction by both cumene and toluene suggested a greater similarity of their structures than between any other terpenes. The results also showed that non-induced *R. gordoniae* P3 was able to degrade TCE but to lower extent than with cumene-induced cells. Thus suggests that the bacteria may use non-specific enzymes to degrade TCE. TCE mineralization or the conversion of TCE to carbon dioxide and chloride ions by cumene-induced *R. gordoniae* P3 was studied by monitoring the increase of chloride ion concentration in liquid culture (Suttinun, 2004). Consequently, *R. gordoniae* P3 would be a prospective candidate for the bioremediation of soil contaminated with TCE.

Biodegradation of TCE contaminated soil occurred considerably in the beginning of this study, in which 30% and 40% of TCE remained in bioaugmented soil and biostimulated soil after 4-day incubation, respectively. On the other hand, 75-85% of TCE remained in control treatments (dry soil and MSM amended soil) during the same period. The results suggest that the addition of cumene-induced *R. gordoniae* P3 and cumene solutions resulted in rapid TCE biodegradation. However, the degradation rate of TCE decreased gradually and the percent TCE remaining in both bioaugmentation and biostimulation treatments was not significantly different from the control MSM amended soil after 10 days. This suggests

that moist soil has intrinsic ability to biodegrade TCE but it requires a longer incubation period. The results were similar to Borch *et al.* (2003), who showed that TCE, PCE, and tetrachloromethane can be biodegraded in water unsaturated topsoil.

Population densities of toluene degraders in the bioaugmentation treatment were significantly different from the control MSM soil only at the beginning of the study. The result suggested that cumene-induced *R. gordoniae* P3 could not grow well in the soil microcosms. However, the reduction of TCE in bioaugmentation treatment implied that these bacteria were still active after addition into the soil. Highest numbers of TCE degrading bacteria were found in biostimulation treatments at the end of the study and expected to be a result of cumene and MSM addition. Increased number of indigenous soil bacteria enhanced TCE degradation in the soil microcosms. The effect of terpene on enhanced bacterial growth was similar to previous studies. Hernandez *et al.* (1997) demonstrated that soil enriched with plant residues rich in terpene, i.e. orange peel, ivy leaves, pine needles or eucalyptus leaves, resulted in 10^5 times more biphenyl utilizers (10^8 CFU g^{-1}) than unamended soil (10^3 CFU g^{-1}) and simultaneously induced polychlorinated biphenyls (PCB) degradation. This suggests that one of the benefits of using plant terpene to stimulate TCE biodegradation is to increase the population densities of TCE degrading bacteria in soil.

At the end of the study period, there was no significant difference in TCE reduction between bioaugmentation and biostimulation treatments. For bioaugmentation, this is probably due to nutrient limitation, suppression by predators and parasites, poor transport of bacteria through soil and low substrate concentration, which contribute to poor survival and activity of inoculum. For biostimulation, the high volatilization and relatively low solubility of cumene made it difficult to maintain the enzyme responsible for TCE degradation. In addition, aging effect and adsorption of TCE may occur in the treated soil. The limitation of biodegradation in soil phase caused by the limited rate of TCE desorption from soil into the aqueous phase (aging effect) led to lower availability for uptake by microorganisms (Fan and Scow, 1993).

From this study, the improvements of its efficiency may require; (1) a second addition of terpene (inducer) during incubation period to maintain the enzyme responsible for initiating TCE biodegradation process, (2) the use of a variety of bacteria consortium to improve their survival and abilities to break down TCE and other contaminants, and (3) the repeated application of the degrading bacteria. Bacteria reapplication was reported to increase the transportation of bacteria through soil (Gilbert and Crowley, 1998). In addition, it will be important to investigate physical and chemical interactions between the volatile organic compounds and the soil matrix with respect to their impact on biodegradation.

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