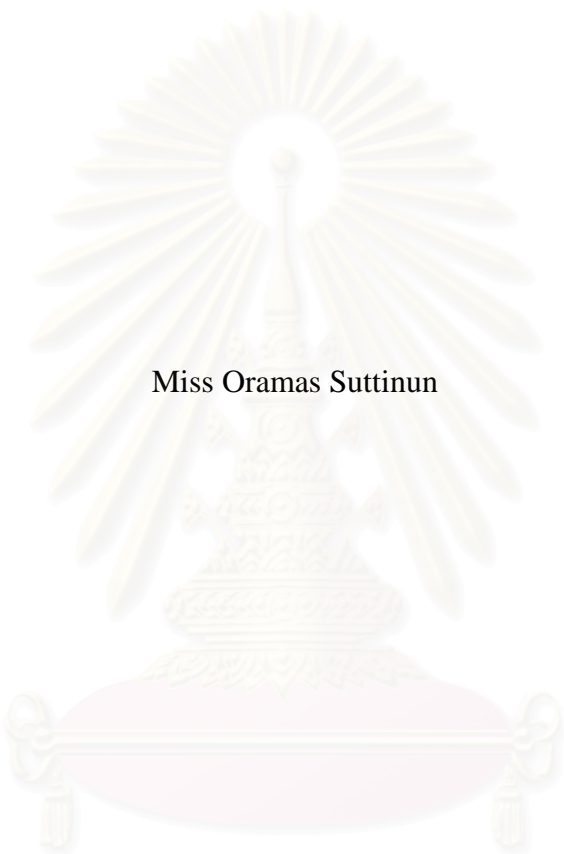# BIODEGRADATION OF TRICHLOROETHYLENE
USING PLANT TERPENES AS INDUCER

Miss Oramas Suttinun

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Environmental Management (Inter-Department)
Graduate School
Chulalongkorn University
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การย่อยสลายทางชีวภาพของสารไตรคลอโรเอธิลีน
โดยใช้สารเทอร์ปีนเป็นตัวชักนำ

นางสาวอรมาศ สุทธินุ่น

Thesis Title	Biodegradation of trichloroethylene using plant terpenes as inducer
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อรมาศ สุทธินุ่น : การย่อยสลายทางชีวภาพของสารไตรคลอโรเอธิลีนโดยใช้สารเทอร์ปีนเป็นตัวชัก นำ (BIODEGRADATION OF TRICHLOROETHYLENE USING PLANT TERPENES AS INDUCER) อ. ที่ปรึกษา : ดร.เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาร่วม : ศ.ดร.ปีเตอร์ บี เลเดอร์แมน, 96 หน้า. ISBN 974-17-4357-2

สารไตรคลอโรเอธิลีน (ทีซีอี) ถูกนำมาใช้อย่างกว้างขวางในอุตสาหกรรมเพื่อเป็นตัวทำละลายและ สารชะล้างไขมัน เนื่องจากการกำจัดที่ไม่เหมาะสม ส่งผลให้ทีซีอีเกิดการปนเปื้อนเป็นบริเวณกว้างและอาจจะ ก่ออันตรายต่อสุขภาพ จึงจำเป็นต้องมีเทคโนโลยีเพื่อบำบัดสารนี้ การย่อยสลายทางชีวภาพของทีซีอี ในสภาวะที่มีอากาศนั้น มีรายงานว่าเกิดขึ้นโดยขบวนการโคเมตาบอลิสม โดยแบคทีเรียที่ย่อยสลายทีซีอี จะใช้สารฟีนอล โทลูอีน และมีเธน เป็นสารตั้งต้นสำหรับการเจริญเติบโตและการชักนำเอนไซม์ แต่เนื่องจาก สารดังกล่าวจัดเป็นสารอันตรายจึงไม่เหมาะสมในการนำไปใช้ในพื้นที่ปนเปื้อน จุดมุ่งหมายของการศึกษานี้ คือการหาสารชักนำเอนไซม์ตัวอื่น และการใช้แบคทีเรียที่ถูกชักนำมาย่อยสลายทีซีอีในดินที่ปนเปื้อน การทดลองนี้ได้นำสารเทอร์ปีนซึ่งเป็นส่วนประกอบหลักในน้ำมันหอมระเหยของพืชมาใช้ชักนำขบวนการ ย่อยสลายทีซีอีในแบคทีเรีย *Rhodococcus gordoniae* P3 และแบคทีเรีย *Pseudomonas* sp.T1 ซึ่งเป็น แบคทีเรียแกรมบวกและแกรมลบ ที่คัดแยกจากดินในท้องถิ่น ตามลำดับ สารเทอร์ปีนที่นำมาทดสอบ คือ คิว มีน ไลโมนีน คาร์โวน และไพนีน ที่ความเข้มข้นต่างๆ ผลการทดลองพบว่าเทอร์ปีนที่มีประสิทธิภาพใน การชักนำขบวนการย่อยสลายทีซีอีได้ดีที่สุดคือ คิวมีน โดยเซลล์ *R. gordoniae* P3 ที่ชักนำด้วยคิวมีน ความเข้มข้น 25 มิลลิกรัมต่อลิตร และ *Pseudomonas* sp.T1ที่ชักนำด้วยคิวมีนความเข้มข้น 10 มิลลิกรัมต่อ ลิตร สามารถย่อยสลายทีซีอีความเข้มข้น 10 ส่วนในล้านส่วน ได้ 76% และ 61% ภายในเวลา 24 ชั่วโมง ตามลำดับ นอกจากนี้พบว่าแบคทีเรียสามารถย่อยสลายทีซีอีอย่างสมบูรณ์ (mineralization) โดยอัตราการ ลดลงของทีซีอีมีความสัมพันธ์กับความเข้มข้นของคลอไรด์อิออนที่เกิดขึ้นในอาหารเหลว เนื่องจาก *R. gordoniae* P3 สามารถย่อยสลายทีซีอีได้ดีกว่า จึงนำไปใช้ในการย่อยสลายทีซีอีในดินที่ปนเปื้อน ซึ่ง ทำการศึกษาโดยใช้ชุดทดลองดินขนาดเล็ก เพื่อตรวจสอบความสามารถของคิวมีนและแบคทีเรียที่ชักนำด้วย คิวมีนในการส่งเสริมการย่อยสลายทางชีวภาพของทีซีอีในสิ่งแวดล้อม ทั้งนี้มีวิธีบำบัดทางชีวภาพ 2 วิธี คือ วิธี Bioaugmentation โดยการเติมเซลล์ *R. gordoniae* P3 ที่ชักนำด้วยคิวมีนความเข้มข้น 25 มิลลิกรัมต่อ ลิตร และวิธี Biostimulation โดยการเติมคิวมีนที่ความเข้มข้น 25 50 และ 100 ส่วนในล้านส่วน เพื่อชักนำจุลินท รีย์ที่มีอยู่ในดินให้ย่อยสลายทีซีอี การบำบัดดินที่ปนเปื้อนทีซีอีด้วยวิธีการดังกล่าวช่วยเร่งให้ปริมาณทีซีอีลด ลงเร็วกว่าชุดควบคุมอย่างมีนัยสำคัญ โดยเฉพาะอย่างยิ่งในช่วงแรกของการทดลอง ในระยะ 4 วันแรกของ การทดลองพบว่าทีซีอีความเข้มข้น 100 ส่วนในล้านส่วน ลดลง 60% และ70% หลังจากบำบัดด้วยวิธี Biostimulation และ Bioaugmentation ตามลำดับ

สหสาขาวิชา การจัดการสิ่งแวดล้อม   ลายมือชื่อนิสิต...

สาขาวิชา การจัดการสิ่งแวดล้อม   ลายมือชื่ออาจารย์ที่ปรึกษา.......................................

ปีการศึกษา 2546   ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...................................

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KEY WORD: TRICHLOROETHYLENE / TERPENE /BIODEGRADATION / BIOREMEDIATION / CO-METABOLISM

ORAMAS SUTTINUN: BIODEGRADATION OF TRICHLOROETHYLENE USING PLANT TERPENES AS INDUCER. THESIS ADVISOR: EKAWAN LUEPROMCHAI, Ph.D., THESIS CO-ADVISOR: PROF. PETER B. LEDERMAN, Ph.D., 96 pp. ISBN 974-17-4357-2

Trichloroethylene (TCE) has been used extensively as a solvent and degreasing agent. Due to its widespread contamination and potential health threat, remediation technology to clean-up TCE is necessary. TCE co-metabolism by aerobic bacteria is well established using phenol, toluene or methane as growth substrate and enzyme inducer. However, these compounds should not be applied to a TCE contaminated site because they are regulated as hazardous materials. 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. Terpenes, the main components in volatile essential oils of plant associated with plant characteristic fragrances were investigated for their ability to induce TCE degradation in *Rhodococcus gordoniae* P3 (Gram positive) and *Pseudomonas* sp. T1 (Gram negative), the isolated bacteria found in Bangkok soil. Selected terpenes, including cumene, limonene, carvone and pinene at various concentrations, were used in this study. The most effective terpene for enzyme induction was cumene, in which *Rhodococcus gordoniae* P3 induced with 25 mg l^{-1} cumene and *Pseudomonas* sp.T1 induced with 10 mg l^{-1} cumene degraded 76% and 61% of 10 ppm TCE in 24 hours, respectively. Moreover, the bacteria were able to completely degrade TCE (mineralization), which was indicated by a correlation between TCE reduction and chloride ion accumulation in the bacterial culture. Since, *Rhodococcus gordoniae* P3 degraded TCE more efficiently, it was selected for of the biodegradation of TCE contaminated soil. Soil microcosms were later conducted to investigate the ability of cumene and cumene induced cells for TCE bioremediation. There were two bioremediation treatments; bioaugmentation, the inoculation of 25 mg l^{-1} cumene-induced *Rhodococcus gordoniae* P3, and biostimulation, the addition of 25, 50, and 100 ppm cumene to induce the indigenous soil microorganisms to degrade TCE. Bioaugmentation and biostimulation were shown to significantly accelerate TCE reduction when compared to control treatment, especially at the beginning of the test period. Approximately 60% and 70% of 100 ppm TCE was reduced during the first 4 days of incubation in biostimulation and bioaugmentation treatment, respectively.

Inter-department Environmental Management   Student's signature

Field of study Environmental Management   Advisor signature............................

Academic year 2003   Co-advisor's signature……………..

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

1,2-DCA	=	1,2-dichloroethane
ATSDR	=	Agency for Toxic Substances and Disease Registry
CAHs	=	chlorinated aliphatic hydrocarbons
CB	=	chlorobenzene
CEPA	=	Canadian Environmental Act
CFC	=	chlorofluorocarbon
CM	=	chloromethane
DCE	=	dichloroethene
DCM	=	dichloromethane
DNAPL	=	dense non-aqueous phase liquid
ERTC	=	Environmental Research and Training Center
MSM	=	mineral salts medium
NADH	=	nicotinamide adenine dinucleotide
O.D.	=	optical density
PAHs	=	polycyclic aromatic hydrocarbon
PCBs	=	polychlorinated biphynyl
PCE	=	tetrachloroethylene
ppb	=	part per billion
ppm	=	part per million
TCE	=	trichloroethylene
TEI	=	Thai Environment Institute
TOD	=	toluene dioxygenase

LIST OF ABBREVIATIONS (Cont.)

TOM   =   toluene ortho-monooxygenase

TRI   =   Toxic Chemical Release Inventory

VC   =   vinyl chloride

CHAPTER 1

INTRODUCTION

1.1 Statement of Problem

The widespread use of industrial chemicals has often caused contamination of large volumes soil and groundwater as a result of improper manufacture and disposal as well as accidental leakage and spills. Contamination of subsurface environments with chlorinated aliphatic hydrocarbons (CAHs), especially trichloroethylene (TCE) has become an important environmental problem in many countries. TCE poses a significant risk to humans when it enters drinking water supplies, as it is a known animal and suspected human carcinogen (Infante *et al*., 1987). In addition, TCE is a dense non-aqueous phase liquid (DNAPL). It does not move with the groundwater flow but instead moves downward by gravitational force through an aquifer until reaching an impermeable confining layer. Therefore, it is difficult to predict its migration through the subsurface and to apply remediation strategies (Russell *et al*., 1992). TCE was first synthesized during the preparation of tetrachloroethane in 1864. The new volatile compound was subsequently used as a degreaser for metal parts. It is also used as a solvent or raw material to make other chemicals. TCE can be found in some household products, including typewriter correction fluid, paint removers, adhesives, and spot removers (ATSDR, 1997).

In Thailand, a large number of factories were built in the last decade. These factories often used TCE in cleaning and degreasing processes. TCE is also used as a substitute for chlorofluorocarbon (CFC), a banned chemical related to greenhouse effect (TEI, 1998). Therefore, there is high potential of TCE to be released into the environment. TCE was found in water and soil samples taken from selected factories that utilized it in their processes (ERTC, 2001). With the evidence of long-range transport, deposit, and persistence of this chemical, it is expected that TCE would be a major environment problem in the future. Recently, Thai government has issued groundwater quality standard and soil quality standard draft for the treatment of TCE from industrial sector. Therefore, appropriate clean-up technologies are necessary.

Various methods have been used to clean up soil and groundwater pollution, such as air stripping, carbon adsorption, soil venting, surface bioreactors, and vacuum extraction. However, these processes do not eliminate the contamination problem; TCE is only transferred from one medium to another.

Bioremediation is one of the most promising technologies to clean up soil and groundwater contamination because of its low cost and its potential for complete degradation of the pollutants (Arp *et al*., 2001). This method is considered as a clean technology because it uses microbes to convert hazardous chemicals to environmentally benign products such as water, carbon dioxide, biomass, and salts. However, the success of bioremediation is site specific and the development of technology requires information on TCE biodegradation process, especially under local

condition. The study of TCE is limited in Thailand. This prompts the need to establish fundamental knowledge of TCE biodegradation as well as to develop bioremediation approaches to clean up contaminated sites.

Anaerobic bacteria can reductively dechlorinate TCE (Vogel and McCarty, 1985), but this process is often slow and incomplete (Kleopfer *et al*., 1985; Parsons and Lage, 1985), potentially leading to a buildup of more toxic substances such as vinyl chloride, which requires further degradation. Aerobic biodegradation of TCE, however, is quicker and leads to the production of harmless by-products, making it an attractive choice for bioremediation.

Biodegradation of trichloroethylene (TCE) under aerobic condition has been studied using co-metabolism with aliphatic and aromatic hydrocarbon-degrading bacteria such as methane oxidizing bacteria (Little *et al*., 1988), ammonia oxidizing bacteria (Hyman *et al*, 1995), phenol oxidizing bacteria (Hopkins *et al*., 1993), and toluene oxidizing bacteria (Fries *et al.,* 1997). Various compounds have been reported as growth substrates and inducers for TCE cometabolism, for example methane, toluene, phenol, ethylene, isopropylbenzene, and ammonia (Arp *et al*., 2001). Among these chemicals, toluene and phenol are known as the most effective substrates for induction of TCE degradation. However, the amendments of these compounds to TCE contaminated site is prevented by their toxicity, thus a non-toxic alternative compound is required.

Plant terpenes, the main components in volatile essential oils of plants, 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). Examples of plant terpenes are limonene from lemon oil, carvone from peppermint oil, cumene from cumin seed and pinene from pine oil. The compounds are considered environmentally friendly and cheap from their natural origin. To date, the utilization of plant terpenes for TCE degradation is limited and the only plant terpenes that has been studied in bacterial liquid cultures is cumene (isopropylbenzene) (Dabrock *et al*. 1992, 1994; Pflugmacher *et al*., 1996). Nevertheless, the utilization of plant terpenes for degradation of TCE in contaminated soil has never been studied, and therefore prevented the development of TCE bioremediation program. The results of this study would open the prospect of applying plant terpenes and native bacteria for TCE bioremediation as well as for other chlorinated pollutants.

1.2 Objectives

The main objectives of this study were to investigate the ability of plant terpenes as an alternative inducer for biodegradation of TCE and to apply a selected terpene for bioremediation of TCE contaminated soil under aerobic condition. The specific objectives were:

1.2.1 To screen for a plant terpene and optimize its concentration that provides the highest ability/rate in induction of TCE co-metabolic pathway in bacterial liquid cultures.

1.2.2 To determine the TCE biodegradation rate of a selected bacterial liquid cultures using a selected plant terpene with its optimum concentration and to confirm complete mineralization of TCE by measuring chloride ion formation.

1.2.3 To develop bioremediation treatments i.e. biostimulation and bioaugmentation techniques to clean up TCE-contaminated soil using a selected plant terpene and bacterial culture.

1.3 Hypotheses

A particular plant terpene can induce TCE co-metabolic pathway in certain bacteria and it can be applied for bioremediation of TCE contaminated soil.

1.4 Scopes of study

1.4.1 Screening of plant terpenes and optimizing their concentrations

Four types of purified terpene solutions (limonene, carvone, pinene and cumene), obtained commercially were screened for the ability to induce TCE co-metabolism with two separate toluene-degrading bacteria, *Rhodococcus gordoniae* P3

and *Pseudomonas* sp. T1. The concentrations of each terpene were 5, 10, 25, and 50 mg l^{-1}. A terpene and its concentration that showed highest induction ability/rate was selected for further study.

1.4.2 Study of biodegradation rate of selected bacterial cultures

TCE biodegradation rate of a selected bacterial strain was studied using a selected terpene with its optimum concentration to induce TCE co-metabolic pathway. Chloride ions formation was monitored in order to confirm the mineralization of TCE.

1.4.3 Bioremediation treatments in soil microcosm

Bioremediation of TCE contaminated soil was conducted in microcosms contained 5 g TCE spiked soil. The treatments were consisted of:

1.4.3.1 Bioaugmentation: Inoculation of bacteria induced with the selected terpene. .

1.4.3.2 Biostimulation: Addition of the selected terpene at different concentrations to stimulate indigenous soil bacteria.

1.4.3.3 Control treatment: A series of control microcosms were consisted of sterilized soil, dry non-sterilized soil,and non-sterilized soil with 30% moisture content (amended with MS medium).

1.5 Methodology

        1.5.1 Microorganisms: Two bacterial types, *Rhodococcus gordoniae* P3 (Gram positive) and *Pseudomonas* sp. T1 (Gram negative) were isolated recently from petroleum contaminated soil collected from Bangkok area. These bacteria generally used toluene as a sole carbon source.

        1.5.2 Terpenes: Four types of purified terpene solutions, cumene, limonene, pinene and carvone obtained commercially were used to screen for the ability to induce TCE co-metabolism.

        1.5.3 Substrates and media: Glucose-mineral salts medium (glucose-MS medium) was used for growing bacterial cells as well as providing carbon and energy source for the induced bacteria.

        1.5.4 Soil: TCE-uncontaminated soil was collected from agricultural area in Nontaburi, Thailand.

        1.5.5 Incubation conditions: TCE degradation experiment was carried out in 50-ml serum bottle sealed with Teflon-lined rubber septum and aluminum crimp caps. During incubation, liquid culture samples were shaken at 200 rpm, room temperature, whareas soil samples were kept in dark at room temperature.

CHAPTER 2

THEORETICAL BACKGROUND AND LITERATURE REVIEWS

2.1 Trichloroethylene (TCE)

2.1.1 Use
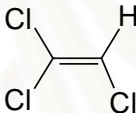
TCE, a chlorinated aliphatic hydrocarbon (CAH) was first synthesized during the preparation of tetrachloroethane in 1864. Due to its unique properties and solvent effects, it has been widely used as an ingredient in industrial cleaning solutions and as a "universal" degreasing agent. TCE can also be found in some household products, including typewriter correction fluid, paint removers, adhesives, and spot removers. Other than its main use as a solvent to remove grease from metal parts, it is also used as raw material to make other chemicals (ATSDR, 1997) .

Usage patterns of TCE in Thailand have increased in the last two decades resulting from a rapid development of industrial sector. The amount of TCE imported to the country has been increased annually from 3522.6 tons in 2001 to 7363.6 tons in 2002 (Thai Customs Department). TCE was mainly imported for cleaning and degreasing operations and for substitution of chlorofluorocarbon (CFC). In 1998, Thailand Environment Institute (TEI) surveyed 476 factories and found that 16% of the factories used TCE in their degreasing process, while only 1% of those factories used PCE for the same purpose (TEI, 1998).

2.1.2 Properties

TCE is also known as trichloroethene, ethylenetrichloride and by other trade names in industry (ATSDR, 1997). The summary of its relevant physico-chemical properties is presented in Table 2.1 below.

Table 2.1 Physical and chemical properties of TCE (Adapted from http://www.atsdr.cdc.gov/toxprofiles/tp19-c3.pdf)

Property	Characteristic
Structure	
Formula	C_2HCl_3
Molecular weight	131.40
Color	Clear, colourless
Physical state	Liquid (at room temperature)
Melting point	-87.1^oC
Boiling point	$86.7\ ^oC$
Density at 20^oC	1.465 g/mL
Odor	Ethereal; chloroform- like; sweet
Odour threshold: Air	100 ppm
Solubility: Water at 20^oC 25^oC Organic solvents	1.070 g/L 1.366 g/L Miscible with many common organic solvents (such as hexane, ether, and chloroform)
Partition coefficients: Log Kow Log Koc	2.42 2.03-2.66
Vapour pressure at 25oC	74 mm Hg
Henry's law constants: at 20^oC at 25^oC	0.020 atm-m^3/mol 0.011 atm-m^3/mol

2.1.3 Sources and Environmental Fate

According to the U.S. Environmental Protection Agency's Toxic Chemical Release Inventory, the biggest source of TCE in the environment is evaporation from factories that use it to remove grease from metals. TCE can also enter the air and water when it is disposed of at chemical waste sites. TCE can be released into the soil through industrial discharges and landfill leachate (TRI, 1997). Volatilization to the atmosphere is the primary means of TCE elimination from the soil. The process is relatively rapid, but more slowly than from surface waters. TCE is readily mobile in the soil. The mobility is primarily affected by the organic carbon content, which affects sorption to the soil (CEPA, 1993). Most TCE deposited in surface waters or on soil surfaces volatilizes into the atmosphere, however TCE has high mobility in soil and this may result in substantial percolation to subsurface regions before volatilization occurred (CEPA, 1993).

TCE is referred to as dense non-aqueous phase liquids (DNAPL). Because its density is higher than water, it sinks into the soil subsurface by displacing water from soil pores and eventually sinking through the groundwater while leaving behind residual pockets that can contribute to long term contamination (Anderson and Andersen, 1996). In these subsurface environments, TCE is only slowly degraded and is relatively persistent. Under anaerobic conditions, TCE may be biotransformed into dichloroethylene and ultimately to a more potent carcinogen such as vinyl chloride (Parsons *et al*., 1984).

2.1.4 Potential Health Effects

People who breathe moderate levels of trichloroethylene may have headaches or dizziness. It is possible that some people who breathe high levels of trichloroethylene may develop damage to some of the face nerves. Some studies with mice and rats have suggested that high levels of TCE may cause liver or lung cancer. People exposed to high levels of TCE in drinking water or in workplace air over long periods have showed evidence of increased cancer incidents. However, these results are inconclusive because cancer could have been caused by other chemicals as well (ATSDR, 1997).

2.1.5 Regulations

In the United States, Environmental Protection Agency (EPA) has set a drinking water standard for TCE to 5 ppb. TCE levels in the workplace are regulated by the Occupational Safety and Health Administration (OSHA). The occupational exposure limit for an 8 hour workday, 40 hour workweek, is an average concentration of 100 ppm TCE in air. The 15 minute average exposure in air that should not be exceeded at any time during a workday is 300 ppm. The OSHA standards are based on preventing central nervous system effects after TCE exposure (ATSDR, 1997).

In Thailand, according to the notification of the National Environmental Board No. 20, B.E. 2543 (2000), issued under the Enhancement and Conservation of National Environment Quality Act B.E. 2535 (1992), published in the

Royal Government Gazette, Vol. 117 Special part 95 D, dated September 15, B.E.
2543 (2000), TCE level in groundwater should not exceed 5 ppb. In addition, soil
quality standard draft has been established and no more than 28 ppm and 61 ppm TCE
should be found in residential soil and industrial soil, respectively (PCD, 2000).

2.2 Fundamental of Chlorinated Hydrocarbon Biodegradation

Microbial organisms use a wide variety of metabolic processes to
generate energy and maintain cellular growth. These processes involve the transfer of
electrons from an electron donor (food source) to an electron acceptor. Chlorinated
compounds are generally degraded by several processes, including:

(1) Direct oxidation (electron donor reactions)

The reaction typically takes place under aerobic conditions, where the
microorganisms utilize less chlorinated compounds as a primary (growth) substrate.
Only few chlorinated solvents can be used as growth substrates for example; vinyl
chloride (VC); dichloroethene (DCE); dichloromethane (DCM); chloromethane
(CM); 1,2-dichloroethane (1,2-DCA); and chloroethane (Bradley *et al*., 1997).

(2) Reductive dechlorination (electron acceptor reactions)

The reaction typically takes place under anaerobic conditions, where the
chlorinated solvent acts as an electron acceptor for the bacteria, and one chlorine atom
is replaced with a hydrogen atom as the result of reaction. Reductive dechlorination is
the most important reaction for highly chlorinated compounds such as

tetrachloroethylene (PCE). The chemical reactions are less favorable as the molecule loses chlorine atoms (Alexander, 1994).

(3) <u>Co-metabolism</u>

It is a fortuitous modification of one molecule (co-metabolized substrate or co-substrate) by an enzyme which routinely acts on another (primary substrate) molecule. The primary substrate supports growth of the microorganism, while the co-metabolized substrate is usually altered only slightly and does not enter catabolic and anabolic pathways of the microbial cells. Therefore, the responsible organism does not benefit from co-metabolic reactions and microbial growth does not occur from the reaction. Co-metabolism of chlorinated solvents presents the cells with a new set of compounds (product). Some of these compounds are toxic to cells, others are stable products that are expelled from the cell, and in a few case the cells utilize the products (Alvarez-Cohen and McCarty, 1991). In addition, products from co-metabolism process may be mineralized by orther organisms (Madsen, 1991). Co-metabolism has been reported to take place with trichloroethylene (TCE), dichloroethylene (DCE) and vinyl chloride (VC), in which the less chlorinated compounds (e.g. VC) would react faster than the higher chlorinated compounds.

Monooxygenases or dioxygenaes are enzymes responsible to the initiation of aerobic co-metabolism of chlorinated compounds (Figure 2.1). These oxidative enzymes generally catalyze a reaction that incorporates O_2 into the target substrate such as CH_4, NH_3, or toluene. This oxidation reaction requires an energy source such as nicotinamide adenine dinucleotide (NADH) to incorporate the O_2. It

was suggested that these enzymes lack the ability to efficiently distinguish their original substrate from certain chlorinated solvents. The lack of substrate specificity results in a chemical reaction in which oxygen is incorporated into the solvent molecule forming and unstable molecule such as TCE epoxide (Alvarez-Cohen and McCarty, 1991). The unstable molecule will spontaneously degrade to one of several chloroacetic acids, such as dichloroacetic acid. These chloroacetic acids are soluble in water and will slowly degrade to CO_2, chloride, and water (Figure 2.1).

Many reasons have been developed to explain co-metabolism, that is, why a chlorinated compound does not support growth but is converted to products that accumulate. There are three theoretical reasons suggested by Alexander (1994); (1) the initial enzyme or enzymes convert the substrate to an organic product that is not further transformed by other enzymes in the microorganism to yield the metabolic intermediates that ultimately are used for biosynthesis and energy production, (2) the initial substrate is transformed to products that inhibit the activity of late enzymes in mineralization or that suppress growth of the organisms, (3) the organism needs a second substrate to bring about some particular reactions.

Co-metabolism tends to be an unsustainable process under stagnant conditions because of substrate competition and enzyme inhibition and inactivation. Competition occurs between substrates, such as CH_4, NH_3, or toluene, and chlorinated solvents for binding on the active site of the nonspecific oxygenase enzyme (Semprini et al.,1990). The TCE oxidation byproducts such as TCE epoxide may result in the inactivation of the oxygenase activity caused by damage to the enzymes. However,

inhibition and inactivation may be overcome by addition of substrates (Alvarez-Cohen and McCarty, 1991).
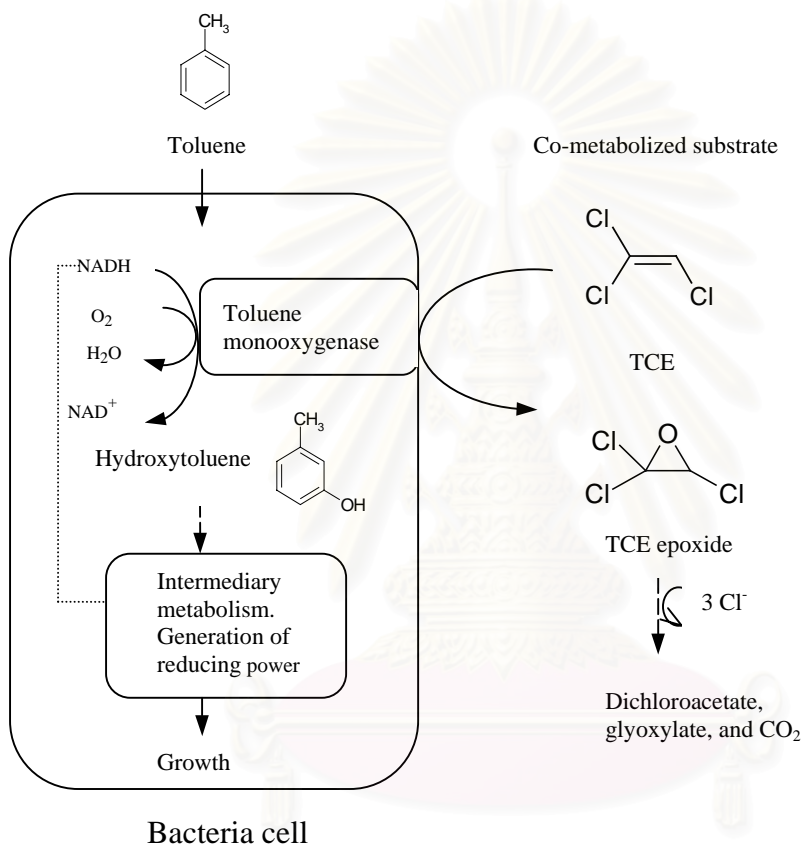
Figure 2.1 Example of TCE co-metabolic pathway by toluene monooxygenase enzyme. The microorganism utilizes toluene as primary substrate while oxidizes TCE to an epoxide and later to CO_2. (modified from http://www.wiley-vch.de/books/biotech/pdf/v11b_aero.pdf)

2.3 Co-metabolism of TCE

The contamination of TCE is frequently detected in the environment. It suggests that environmental conditions are probably not conducive to TCE degradation. Since Wilson and Wilson (1985) firstly investigated biotransformation of TCE in soil by adding a mixture of natural gas (0.6%, v/v methane) to an unsaturated soil column (containing indigenous microbes), methanotrophic populations were increased and TCE was degraded extensively to carbon dioxide. Without exposure to methane, there was no statistically significant degradation of TCE. Therefore, it can be suggested that TCE does not serve as a primary (growth) substrate for microorganisms and is biodegraded under aerobic conditions only through the process known as co-metabolism or co-oxidation (Little *et al*. 1988).

An impressive body of work on TCE biodegradation has been carried out. A number of studies demonstrated that TCE are transformed co-metabolically by a group of aliphatic and aromatic hydrocarbon-degrading bacteria. Generally, co-metabolism of TCE requires chemical compounds such as benzene, toluene, ethylbenzene, xylene, phenols or methane that the microorganisms can utilize to produce energy as well as to induce for TCE degrading enzymes. In aerobic condition, these miocroorganisms were reported to completely mineralize TCE to CO_2 and H_2O (Ensley, 1991; Semprini, 1997). Example of TCE concentrations used for the biodegradation study in liquid cultures were 0.4 mg l^{-1} (Little *et al*., 1988), 3 - 24 mg l^{-1} (Hyman *et al.,* 1991; 1995), 1 mg l^{-1} (Fries *et al*., 1997), and 1 mg l^{-1} (Vannelli *et al.,* 1990), while the amounts of TCE in soil studies were 0.1-10 mg of TCE l^{-1} of soil

solution (Fan and Scow, 1993), 50 to 60 mg of TCE l^{-1} of soil solution (Mu and Scow, 1994).

Little *et al*. (1988) first observed TCE biodegradation by pure cultures of methane oxidizing bacteria strains 46-1 that isolated from groundwater samples taken from monitoring wells. When incubated in pure culture, strain 46-1 converted up to 40% of added TCE to biodegradation products in methane-limited batch culture incubated for 20 days, while sterile and active cultures of non-TCE-degrading bacteria as control culture showed less than 2% TCE loss. The maximum rate of TCE degradation occurred during the active phase of cell growth (days 2 to 6) and dropped to zero after methane was depleted. They also reported that the final byproducts consisted of 3.4 to 4.0% cell bound materials and 40.1 to 42.7 % of TCE was completely converted to CO_2. Most of the converted TCE appeared as water-soluble breakdown products. The water-soluble byproducts are further degraded by heterotrophic microorganisms to carbon dioxide. The enzyme system responsible for both methane and TCE oxidation has been identified as methane monooxygenase (MMO).

Vannelli *et al*. (1990) studied the aerobic transformation of several halogenated aliphatic compounds including TCE using cell suspension of *Nitrosomonas europaea,* ammonia-oxidizing bacterium. They found that most or the entire test compound had disappeared within 24 hours. The reaction is suggested to be at least dependent on or probably catalyzed by the ammonia oxygenase. Furthermore, the rate of nitrite production from ammonia was decreased when TCE was degraded.

Hopkins *et al*. (1993) investigated the ability of aerobic groundwater microorganisms, especially phenol oxidizing bacteria to co-metabolically degrade TCE. They found that microcosms amended with phenol were effective in removing 60-70% TCE. Fries *et al.* (1997) conducted the experiment by using phenol and toluene oxidizing bacteria from the Moffett Field aquifer that grew in response to toluene, phenol and TCE additions. The results showed that TCE was metabolized at a high initial rate and then the degradation was decreased over time for a number of strains. More than 50% of gram negative bacteria grown on toluene showed less than 50% cometabolism capacity when toluene was the primary carbon source, whereas more than 50% gram negative bacteria co-metabolized TCE when grown on phenol and the toluene oxygenase enzymes acted to induce this degradation process.

Luu *et al*.(1995) monitored TCE mineralization by resting cell suspensions of *Pseudomonas cepacia* G4 PR1 and phenol induction. The new chloride-free medium was developed to reduce the concentration of chloride ions to the extent that the chloride ions generated from TCE mineralization may be detected with chloride ion electrode. They found that TCE degradation was similar in both glucose minimal medium (M9/glucose medium) and chloride-free minimal medium (MCl medium) in which 89 % of 10 mg of TCE l^{-1} degraded in 6 hours. In addition, the extent of complete TCE mineralization was determined by monitoring the increase in chloride ion concentration, 77 % and 59 % mineralization presented in MCl medium and M9 medium, respectively.

Hinchee *et al.* (1994) found that toxicity of the transformation product affects the methanotropic transformation of CAHs. The activity of resting cells has been found to decrease by transformation product toxicity in proportion to the amount of contaminant transformed. They also concluded that hydrogeological, hydrogeochemical and microbial variations could potentially support variations in degradation of chlorinated compounds in the environment due to variations in transport and availability of bacteria, nutrients, and electron acceptors.

Roberts et al. (1989) found that competition between the growth substrate and the non-growth substrate for the active site of the relevant enzyme was very likely to occur and could considerably influence the transformation of the non-growth compound. Alvarez-Cohen and McCarty (1991) reported that methane monooxygenase (MMO) was responsible for both methane oxidation and TCE epoxidation. Methane and TCE were considered to be competitive substrate such that in the presence of both compounds TCE transformation rates were reduced.

Futamata *et al.* (2001) compared the whole-cell kinetics between phenol and TCE-degrading activities, indicating that phenol was a much preferred substrate for the degradative enzymes, rather than TCE. This suggested that TCE is not efficiently degraded in the presence of phenol and they also reported that the phenol concentration should be carefully determined in phenol amended biostimulation.

Fries *et al.* (1997) compared the effects of phenol and toluene on microbial populations in a TCE-contaminated aquifer and they suggested that phenol

may be a better co-substrate than toluene, due to its lower toxicity. However, 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 compound that is non-toxic, environmentally friendly, and cheap is required.

2.4 TCE Bioremediation

Nowadays, remediation of TCE contaminated sites relies primarily on physical and chemical processes (e.g. air stripping and carbon adsorption) of contaminated groundwater and soil. Unfortunately, the technologies are typically expensive and do not always attain the desired treatment goals. The most promising technology for clean up soil and groundwater contamination is bioremediation because of its low cost and its potential for complete degradation of the pollutants rather than transferring them from one part of the environment to another (Wilson and Wilson., 1985, Arp *et al*., 2001).

The objective of bioremediation is to exploit biological processes to clean up contaminated site. During bioremediation, xenobiotic compound is broken down by microorganism into less complex compounds, and ultimately to water, and either carbon dioxide or methane. This process can occur under both anaerobic and aerobic conditions but most of organic pollutants degrade at a faster rate under aerobic conditions. To increase biodegradation rate, bioremediation can be performed as follows:

(1) *Biostimulation* is to supply essential nutrients or inducer to stimulate indigenous microorganisms in soil.

(2) *Bioaugmentation* is to inoculate microorganisms capable of degrading target pollutants to facilitate biodegradation, either with or without nutrients, into the contaminated environment. The inoculant may be wild-type or genetically engineered microorganisms and may be applied either as a single microbial species or a consortium of several species.

Actual application of TCE bioremediation is still limited in practice but is rapidly gaining popularity. Different methods of application have been studied in both field and laboratory scale, for example growing indigenous microbes in situ, injecting bacteria grown in reactor into the soil subsurface, and adding soluble substrate (i.e. phenol and toluene) into contaminated aquifer. In some cases, direct addition of gaseous substrate (i.e. propane and methane) was used for TCE treatments. Ex situ bioremediation may be implemented to treat soil and groundwater as well.

The biostimulation trials included the injection of methane (Semprini *et al.*, 1990), toluene (Hopkins and McCarty, 1995) and phenol (Hopkins *et al.*, 1993; Hopkins and McCarty, 1995) into aquifers to stimulate indigenous bacteria. Example of bioaugmentation trial was studied by Duba *et al.* (1996). They used methanotrophic bacteria suspended in groundwater and injected into an aquifer. After withdrawal of the contaminated groundwater, 98% TCE decreased during the first 50 hours incubation.

For contaminated soil, Fan and Scow (1993) reported that toluene supported the degradation of 1 mg of TCE per l of soil solution in silt loam soils and was not degraded in the absence of toluene. Usually 60 to 75% of the initial 1mg of TCE per l of soil solution was degraded, whereas 100% of the toluene disappeared. The addition of phenol also stimulated the degradation of TCE, while glucose did not induce the oxygenases responsible for TCE degradation, so did not support TCE degradation. Temperature and soil moisture also strongly influenced the degradation of TCE in soil. The initial rates of TCE degradation at 32, 25, 18 ^{0}C were similar, at 10 ^{0}C the rate of TCE degradation were lower than other temperatures. There was virtually no degradation of TCE or toluene at soil moisture levels of 2.5-5%, whereas there was little difference in the rate and extent of toluene or TCE degradation at levels of 16, 25, and 30%.

Lee $et.\ al.$ (2002) studied the effect of sorption and desorption resistance on aerobic TCE biodegradation in aqueous and soil-slurry phase. In aqueous phase, the TCE degradation rates were clearly increased in microcosms when toluene was added multiple times and related with toluene dioxygenase (TOD) enzyme activity over time, indicating that TCE co-metabolism occurred by the microbial population. The TCE biodegradation rate was approximately 0.31 mg of TCE l^{-1} h^{-1} for the first 2 h with 27mg l^{-1} toluene addition. After toluene was depleted from the system, the TCE degradation rate slowed considerably to 0.035 mg of TCE l^{-1} h^{-1}. In soil-slurry experiments, freshly contaminated soil, TCE degradation rate of approximately 0.15 mg TCE kg^{-1} h^{-1} was observed during 39 hours of incubation and then TCE degradation rate decreased considerably to 0.00059 mg TCE kg^{-1} h^{-1}. The TCE degradation rates in soil-

slurry microcosms containing the desorption-resistant fraction of TCE-contaminated soil were approximately 0.00032 mg TCE kg^{-1} h^{-1}. It is clear from these results that mass transfer into the aqueous phase limited bioavailability of TCE in the contaminated soil.

The application of bioremediation depends on type of pollutants or pollutant mixtures present and the type of microorganism present. Brigmon *et al.* (1998) constructed the microcosm to assess the extent of attenuation possible via biodegradation and sorption in rhizosphere soils at the Savannah River Site (USA). Sorption was an important removal mechanism during the first week of incubation, resulting in as much as 90% of the TCE removal from the aqueous phase. Diffusional losses appeared to be a dominant removal mechanism during the remainder of the experiment. The potential to enhance TCE removal by stimulating methanotrophic activity (through methane and oxygen addition) and stimulating anaerobic activity by addition of readily degradable electron donor (methanol) were not successful. The sorption results indicated that natural attenuation may represent a viable remediation option for the TCE plumes as it passes through the rhizosphere.

Another bioremediation approach is intrinsic bioremediation, which is a passive remedial approach that depends upon natural processes to degrade and dissipate contaminants in soil and groundwater. Natural attenuation processes include physical, chemical, and biological transformation e.g. aerobic/anaerobic biodegradation, cometabolism, dispersion, volatilization, oxidation, reduction, and adsorption (Kho and Prosser, 1999). Recently, intrinsic bioremediation of TCE

contaminated aquifer has been studied more extensively. Kho and Prosser (1999) evaluated a contaminated site at Robins Air Force Base in Georgia, USA, which contains a mixture of trichloroethylene TCE and chlorobenzene (CB). Results from the field investigation suggest that an intrinsic bioremediation process is occurring, which caused the decrease in TCE and CB concentrations, and increase in TCE degradation byproducts e.g., dichloroethylene isomers (DCEs), and vinyl chloride (VC) concentrations. Microcosm results suggest that CB can serve as the primary substrate electron donor, and enhance TCE biodegradation to less-chlorinated compounds under both aerobic cometabolism and reductive dechlorination conditions.

2.5 Plant Terpenes

Terpenes are the largest class of natural products. They function in plant as membrane sterols, carotenoids, pigments, biocides, insect attractants, growth hormones, and signal compounds. Terpenes are based on polymerization of 5-carbon isoprene units. This 5 carbon unit is polymerized into compounds with 10, 15, 20 or more carbons, that undergo modifications to yield the final compound. Monoterpenes consist of 2 isoprene units, while diterpenes and triterpenes contain 4, 6 isoprene units, respectively (Crowley *et al*., 2001). To date, several thousand of these compounds have been identified, of which more than one hundred terpenes are known. These compounds, the main components in volatile essential oils of plants, are widely distributed throughout vegetation types (Amaral *et al.*, 1997). For example limonene from orange peel and carvone from mint leaves are important aroma compounds for use

in foods and beverages. All of the terpenes synthesized by plants are broken down through natural processes.

Chemical characteristics of terpenes (Langenheim, 1994).

(1) Terpenes are largest group of plant chemicals. They have a common biosynthetic origin.

(2) Terpenes pathway perhaps best suited to generate great structural diversity and complexity of compounds, thus generating enormous potential for mediating ecological interactions.

(3) Terpenes are broadly lipophilic, but considerable water solubility demonstrated for some oxygenated terpenes.

(4) Some compounds are volatile, in varying degrees, and others are nonvolatile.

Several terpenes have structures that are analogous to many aromatic chemicals (Figure 2.2). They were reported to stimulate microbial degradation of xenobiotic compounds such as polychlorinated biphenyls (PCB), toluene, and phenol (Crowley *et al*., 2001, Singer *et al*., 2003). Crowley *et al*. (2001) proposed that they beneficially affect xenobiotic degradation by at least three mechanisms, including the selective enrichment of degrader organisms, enhancement of growth-linked metabolism, and the induction of co-metabolism in certain microorganisms that carry degradative genes and plasmids. Since plant terpenes are often effective at very low concentrations and are 'environmentally friendly', there would be fewer concerns about

introducing them into subsurface soil or aquifers. The use of these compounds to remediate the environment is therefore a promising technology (Singer *et al.,* 2003).
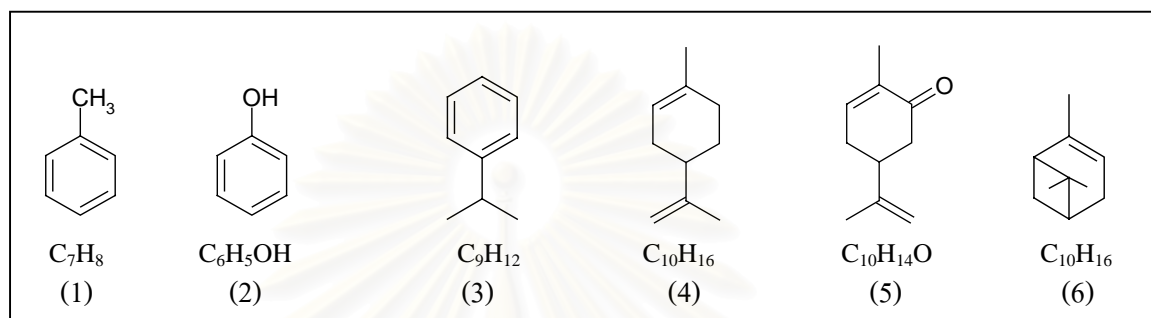
Figure 2.2 Structures and formulas of xenobiotics commonly used as substrate and inducer for supporting TCE degradation; (1) Toluene; (2) Phenol, and structures of terpenes used as inducer in this study; (3) Cumene; (4) Limonene; (5) Carvone; and (6) Pinene.

The induction of co-metabolism in certain microorganisms by terpenes probably because terpenes have structures that are analogous to many substrates commonly used as sole carbon source and/or enzyme inducer. For example, Gilbert and Crowley (1997) screened several terpene compounds, including carvone, cumene, carvacrol, thymol, limonene, cymene and cinnamic acid for their ability to induce polychlorinated biphenyls (PCB) biodegradation in *Arthrobacter* sp. Carvone, the principal component prepared from spearmint extract, was able to induce the biotransformation of 62% of Aroclor 1242 at concentration as low as 50 mg l^{-1}. Carvone had higher solubility among other substrates, thus increasing its bioavailability to utilize by microorganisms. The authors also suggested that no

growth was detected in *Arthrobacter* sp. when carvone was provided as the sole carbon source at 100, 200, 300, 450, 500 mg l^{-1} or greater, suggesting that the induced metabolism of carvone by *Arthrobacter* sp. B1B was principally a detoxification mechanism. A subsequent study by the same researchers (1998) investigated the efficacy of repeated applications of carvone-induced *Arthrobacter* sp. strain B1B onto Aroclor-1242-contaminated soil, resulting in the removal of 27 ± 6 % of PCB after 17 applications (9 weeks). Later, Singer *et al.* (2000) developed an effective treatment for decontamination of PCB-contaminated soils using two carvone-induced bacteria species, *Arthrobacter* sp. strain B1B and *Rasstonia eutruphus* H850 and sorbitan trioleate, a non-toxic surfactant added to improve bioavailability of PCB. The results showed over 60% removal of PCBs from the contaminated soil.

Koh *et a*l. (2000) showed that a monoterpene, carvone, could induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Alcaligenes eutrophus*.

Tandlish *et al*. (2001) evaluated the effect of two terpenes, carvone and limonene, as potential inducers of the PCB degradative pathway in *Pseudomonas stutzeri* in the presence of glucose, biphenyl, glycerol and xylose as a sole carbon and energy sources. Interesting results were obtained for PCB biodegradation when xylose was used as the sole carbon source and carvone as a possible inducer. In this case, 7-37% of the individual PCB congeners were degraded from the system without carvone addition, while 30-70% of congeners were removed after supplementing with 10 mg l^{-1} and 20 mg l^{-1} carvone, independent of the added concentration.

Plant terpenes can be applied directly using plant materials such as mint leaves for carvone and orange peel for limonene. Hernandez *et al.* (1997) demonstrated that soil enriched with either orange peel, ivy leaves, pine needles or eucalyptus leaves resulted in 10^5 times more biphenyl utilizers (10^8 g^{-1}) than non-amended soil (10^3 g^{-1}) and simultaneously inducing Aroclor 1242 degradation. Five distinctly different isolates obtained from these soils were further studied with respect to growth on pure terpenes and metabolism of PCBs. The most effective strains were *Cellulomonas* sp. T109 and R. *rhodochrous* T100, which metabolized 83% and 80% of Aroclor 1242, respectively, after growth on cymene and limonene, respectively. Dzantor and Woolston (2001) investigated the effect of pine needles and orange peel-amended soil combined with the planting of various plant species on the degradation of Aroclor 1248 (PCB). PCB losses were observed in orange peel (54-59% loss) and pine needles (44-55% loss) amend soil treatments, including all planted and unplanted treatments.

To date, the utilization 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, 1994; Pflugmacher *et al*., 1996). The reports showed that *Pseudomonas* sp. JR1 and *Rhodococcus erythropolis* BD2, isolated from enrichments with cumene as sole carbon and energy source, were able to oxidize TCE after induction by cumene. They also suggested that increasing the initial TCE concentration resulted in increasing initial rates of TCE degradation. Initial TCE oxidation rate increased proportional to concentration of substrate from 3 to 24 mg l^{-1}. The two bacteria exhibited pH-optimum ranging from 5.5-8.0. The effect of

temperature was varied between 10-40 $^{\circ}$C and found that at 20-30 $^{\circ}$C was the optimum temperature.

However, the utilization of plant terpenes for degradation of TCE in contaminated soil has never been studied, and therefore prevented its development for TCE bioremediation. This study will investigate the ability of various plant terpenes on induction of TCE co-metabolism, and then examine the effectiveness of bioremediation treatments that utilize a selected terpene and local bacterial isolates. The results would open the prospect of applying plant derived compounds and native bacteria for TCE bioremediation as well as for other chlorinated pollutants.

CHAPTER 3

METHODOLOGY

3.1 Methodology Outline

3.1.1 Liquid cultures study

(1) First part

- Screened plant terpenes and optimizing their concentrations for induction of TCE co-metabolism in *Rhodococcus gordoniae* P3 and *Pseudomonas* sp.T1.

(2) Second part

- Studied biodegradation rate of a selected bacterial culture using the selected terpene with its optimum concentration.

- Measured chloride ion formation during TCE degradation.

3.1.2 Soil microcosm study

- Applied the selected terpene and terpene induced bacteria to TCE-contaminated soil microcosms and studied TCE biodegradation.

3.2 Materials and Methods

3.2.1. Chemicals

99.5% trichloroethylene (TCE), four types of purified terpene solutions (99.0% carvone, 96.0% limonene, 97.0% pinene, 99.0% cumene), N,N-dimethylformamide were obtained commercially from Fluka Chemical Industrial. TCE and terpene solutions were prepared by dissolving aliquots of TCE and terpenes in N, N-dimethylformamide to obtain the desired contaminant concentration. 99.9% Hexane (from Fisher Scientific, Inc.) and triton x-100 (from Fluka Chemical Industrial) were used for extraction of TCE from bacterial cultures and soil microcosm. 99.5% toluene purchased from Merck was supplied as carbon and energy source for culture maintenance. The mineral salts (MS) medium was used for isolation, TCE degradation experiments and determination of living toluene-degrading bacteria by means of dilution plate count. MS medium was prepared according to Focht (1994) with details in Appendix A. All chemicals were analytical reagent grade and obtained from Merck.

3.2.2. Microorganisms

Two types of bacteria were isolated from petroleum contaminated soil collected from Bangkok area using toluene enrichment cultures technique. *Rhodococcus gordoniae* P3 (Gram (+)) was isolated earlier by Dr.Ekawan Luepromchai, while *Pseudomonas* sp.T1, (Gram (-)) was isolated in this study.

(1) Enrichment culture technique and the isolation of pure cultures

An Enrichment culture technique was used to isolate bacteria from soil. These bacteria were capable of utilizing toluene as sole carbon sources and energy. Enrichment cultures were prepared by using 1 g petroleum contaminated soil in a 250-ml Erlenmeyer flask that contained 100 ml of mineral salts medium, added the substrate (150-200 µl toluene) in the Eppendorf tube suspended at the top of the flask (Figure 3.1), and placed on the orbital shaker at 200 rpm, room temperature, for 24 hours. Then a sample was removed with an inoculating loop. The suspension was streaked according to standard microbiological procedures onto sterile mineral salts agar plates. After streaking these plates, Petri plates are inverted and placed on the equilibrated glass box with toluene supplied in a small beaker as sole carbon source. The plates were incubated at room temperature and then transferred to new agar plates until the presence of single isolated colonies were noted.

Figure 3.1 Erlenmeyer flask with the Eppendorf tube suspended at the top of the flask used for enrichment cultures.

(2) Screening and identification of enrichment culture isolates

Enrichment culture isolates were screened for the ability to grow on the enrichment hydrocarbon (toluene) as a sole source of carbon and energy. This was performed as follows. Strains were grown on MS agar and removed a loopful of culture on plate to a 250-ml Erlenmeyer flasks containing 100 ml mineral salts (MS) medium, added the substrate (150-200 µl toluene) to the Eppendorf tube suspended at the top of flask, incubated on orbital shaker at 200 rpm and room temperature. After 24 hours incubation, pure culture isolates were characterized and identified to genus and species using molecular biology method (Luepromchai and Suttinun, 2003). Indole agar indicator plates can be used to roughly distinguish between TCE-degrading bacteria and other bacteria by giving different color formation on the agar plates. 5 mg indole crystals (from Fluka Chemical Industrial) were added to the lid of the Petri dish after 12-18 hours of bacteria growth, and compared the test results to published color formation on the agar plates.

(3) Culture Maintenance

It was necessary to maintain the ability of enzyme production by bacteria throughout the course of study. All bacteria were maintained on mineral salts (MS) agar and incubated in toluene equilibrated glass box or stock cultures were either best maintained by removing a loopful of culture from an isolated colony on a plate and mixing it in 0.5 ml of sterile MS medium or adding 0.5 ml liquid cultures,

which was then added to Eppendorf tube containing 0.5 ml 25% glycerol in water, and stored at -81 ^{0}C.

(4) Substrate utilization

Substrate utilization of *Rhodococcus gordoniae* P3 and *Pseudomonas* sp.T1 were tested in liquid media separately supplied with cumene, limonene, pinene, carvone, toluene, or glucose as a sole carbon source and shaken at 200 rpm for 24 hours. Then, growth of the bacteria was monitored from changes in turbidity.

(5) Growth conditions and inoculum preparation

The liquid cultures were grown in a 250-ml Erlenmeyer flasks containing 100 ml mineral salts (MS) medium, added the substrate (toluene) to the Eppendorf tube suspended at the top of flask (Figure3.1). The flasks should be inoculated with an amount sufficient to give slight turbidity, incubated on orbital shaker at 200 rpm and room temperature for 24 hours. Then the bacteria were induced by aliquoting to a second 250-ml Erlenmeyer flasks (the absorbance or optical density (OD) should be about 0.02-0.04 at 600 nm at the beginning) containing 4 g l^{-1} glucose-mineral salts (MS) medium, in which stock solution of terpenes (limonene, carvone, pinene and cumene mixed in N,N-dimethylformamide) was added separately to a final concentration of 5, 10, 25, or 50 mg l^{-1} and incubated overnight at room temperature using an orbital shaker at 200 rpm. Bacteria cells were grown until the mid-to-late-log phase (Appendix A), harvested by using a refrigerated centrifuge at

4800 rpm for 10 minutes. The supernatant was discarded. The bacteria cells were washed by resuspending them in about 10 ml of mineral salts (MS) medium. A ml each sample was removed, diluted with 9 ml of mineral salts (MS) medium, mixed thoroughly, and adjusted the absorbance to 1.0 at 600 nm (approximately 10^8 CFU ml^{-1} based on Focht (1994)).

3.3 TCE Biodegradation Experiments

3.3.1 Bacterial liquid cultures study

3.3.1.1 Screening for a terpene type with optimum concentration in each bacterial strain

(1) Test-bottles

50 ml serum bottles (Figure 3.2) were used as test bottles for experiment. They were sealed with Teflon-lined rubber septum (20 mm) and aluminum crimp caps.
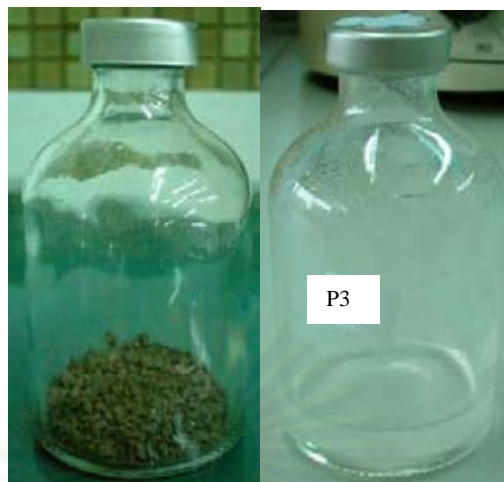
Figure 3.2 50-ml serum bottles .containing 5 ml liquid media and 5 g soil samples capped with Teflon-lined rubber septum and aluminum crimp caps.

(2) Bacterial liquid cultures incubations and conditions

TCE biodegradation experiment was carried out in liquid bacterial culture by a method adapted from Nelson *et al.* (1987) and Luu *et al*. (1995). The study was performed on each *Rhodococcus gordoniae* P3, gram positive (+) and *Pseudomonas* sp. T1, gram negative (-) by resting cell assay. After induction with each four terpenes at different concentration for 24 hours as described above, 5 ml of induced cell suspensions were transferred to 50-ml serum bottles sealed with Teflon-lined rubber septum. O_2 were 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 a gas-tight syringe to give the desired final TCE concentration of 10 ppm. The bottles were shaken 24 hours at room temperature and 200 rpm. The experiment was done in triplicate. After incubation, the

samples were analyzed for the remaining TCE. Control samples were bottles containing heat-killed cells autoclaved at 121 ^{o}C for 15 min and non-induced cells (cells grown on glucose only).

(3) Experimental design

The experimental design is outlined in Figure3.4. For each terpene tested, four sets of triplicate experiments (carvone, cumene, limonene or pinene) were prepared and 2 sets of control treatments (heat-killed cell and non-induced cell) were performed on each *Rhodococcus gordoniae* P3 and *Pseudomonas* sp.T1. This examined the ability of four plant terpenes at different concentration (5, 10, 25, or 50 mg l^{-1}) to induce TCE degradation. After all sets of terpene-induced cells were analyzed for TCE remaining, the average rate of TCE degradation was compared between these sixteen sets and control treatments on each bacterial strain. Combinations of terpene type with optimum concentration and the bacterial strain which provide highest TCE degradation rate that resulted in the lowest remaining TCE were selected for further study.
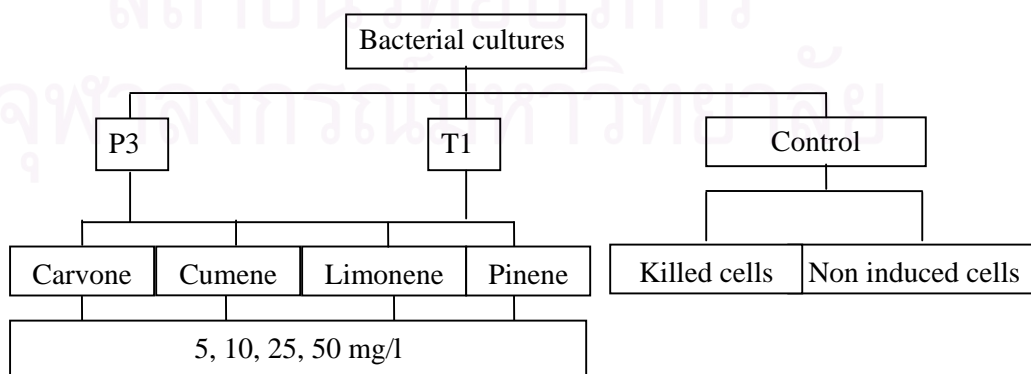
Figure 3.3 Diagram of bacterial liquid cultures experimental design

3.3.1.2 Determination of biodegradation rate and chloride formation in bacterial liquid cultures

(1) Incubations and conditions

TCE degradation rates were monitored by resting cell assay. The experiment consisted of liquid cultures of *Rhodococcus gordoniae* P3 grown in 250-ml Erlenmeyer flasks containing 4 g l^{-1} glucose-MS medium (adjusted to O.D.= 1.0 at 600 nm), induced with 25 mg l^{-1} cumene obtained from the methodology 3.3.1.1. Then, cumene-induced cells were prepared as inoculum preparations mentioned previously. 5 ml of cumene-induced cell suspensions were added to 50-ml serum bottles sealed with Teflon-lined rubber septum. TCE solutions were added with a gas-tight syringe to give the desired final TCE concentration of 10 ppm. The bottles were shaken at 200 rpm and room temperature.

(2) Sampling time

During 24 hours incubation, triplicates of each samples were sacrificed by opening the seal at 0, 2, 4, 6, 9, and 24 hours. The samples were analyzed for the amount of TCE remaining and the data was plotted against sampling time. The chloride ion generated was measured with an ion-sensitive chloride combination electrode.

3.3.2 Soil microcosm study

(1) Soil

Soil was collected from an uncontaminated area (agricultural soil) located in Nontaburi province, Thailand. All debris was removed and the soil sample was then air dried. After that the dried soil sample was pulverized by passage through U.S. standard sieve No.10 (2.0 mm.). Sieved soil was hexane extracted, and the extract was analyzed by GC-ECD, and found to be free of TCE. Selected soil properties (soil texture, %organic carbon, %organic matter, total nitrogen (N), available phosphorus (P), and maximum water holding capacity) other than pH and soil moisture content were determined by the System Development of Soil and Water Analysis Subgroup, Agricultural Chemistry Research Group, Department of Agriculture before conducting the experiment. Properties of the soil are shown in Appendix B.

(2) Soil microcosm preparation and application of amendments to soil microcosms

Bioremediation treatment was conducted using soil microcosm (a laboratory vessel set up to resemble as closely as possible the conditions of a natural environment). Five grams dry weight of uncontaminated, non-sterile soil were added to 50-ml serum bottles capped with Teflon-lined rubber septum, and then spiked TCE with a gas-tight syringe to a give final concentration of 100 ppm. In bioaugmentation

treatments, *Rhodococcus gordoniae* P3 cultures were grown on 4 g l^{-1} glucose-MS medium which also contained the optimum concentration of cumene at 25 mg l^{-1} (See 3.3.1.1) for 24 hours before adding to the soil microcosms at O.D = 1.0 (approximately of 10^8 CFU g soil^{-1}) (See growth condition and inoculum preparation). In the biostimulation treatments, only cumene was added directly to soil microcosm. Control treatment consisted of sterilized soil by autoclaving 121 ^{o}C, 15 min, three times for three consecutive days, non-sterilized soil (no moisture addition) and non-sterilized soil added with MS medium. O_2 was provided only at the beginning of study then the microcosm was sealed with Teflon-lined rubber septum and aluminum crimp caps. Teflon tape and parafilm was placed over punctured septum to minimize possible losses of TCE through the puncture. All samples were incubated at room temperature and dark condition.

(3) Sampling time

During 2 weeks incubation, triplicate samples of each treated soil treatment and control treatment were taken by sacrificing test bottles at day 0, 4, 7, 10, and 14. TCE was extracted from the soil and analyzed by gas chromatography.

(4) Experimental design

The treatments consisted of one set of bioaugmentation (addition of 25 mg l^{-1}cumene-induced *Rhodococcus gordoniae* P3 to soil microcosm), three sets of biostimulation (addition of cumene solution at final concentration of 25, 50, and 100

ppm to soil microcosm), and three sets of control (sterilized soil, non-sterilized soil with no moisture addition and non-sterilized soil with MS medium addition to maintain 30% moisture) (Figure3.4). After incubation at given time, all sets of bioremediation treatment were analyzed for TCE remaining. The average TCE removal was compared between treated soil treatments and control treatments.
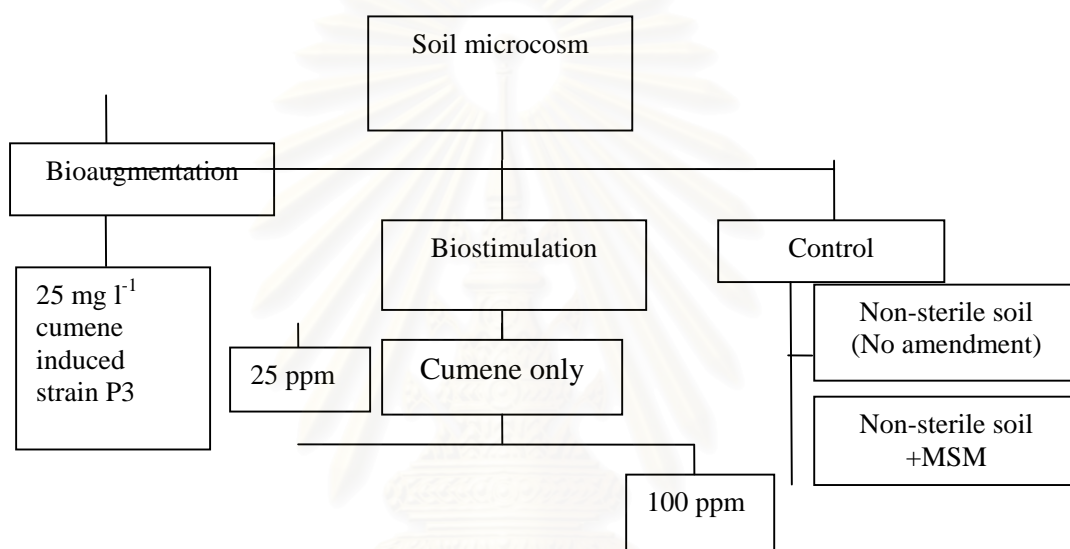
Figure 3.4 Diagram for soil microcosm experimental design.

3.4 Analytical Methods

3.4.1 Chemical analysis

Degradation of TCE was determined from the final TCE concentration as well as the production of chloride ions. TCE extraction and analysis procedure was modified from Leahy et al. (1996).

3.4.1.1 TCE extraction

For TCE extraction in bacterial liquid cultures, two-milliliter aliquots of TCE cell-suspension sample were transferred into sealed vials. Two ml of hexane, 400 µl of 10% Triton x-100 solution (surfactant) were added directly to each of the vials, mixed and shaken at 200 rpm for 2 hours. For soil samples, 10 ml hexane and 3 ml 15% Triton x-100 were added directly to the sacrificed soil microcosm (in 50-ml sealed serum bottles) with gas-tight syringe and shaken for 2 hours on an orbital shaker. The vials were then frozen at -4 ^{0}C to solidify the lower aqueous layer. Then the solvent fraction was transferred with Pasteur pipette to second vial where 2-3 g of anhydrous sodium sulfate was added to dewater the sample. TCE dissolved in the solvent fraction were transferred to gas chromatography (GC) auto sampler vials for analysis using gas chromatography (GC). The extractions efficiencies from liquid cultures and soil microcosm were approximately 90 ± 10 %.

3.4.1.2 GC analysis

TCE quantification was performed with external standard. GC analyses were 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 x 0.32 mm ID; thickness, 0.25µm). The following operating conditions were used: Injector temperature 250 ^{o}C, detector temperature 250 ^{o}C, initial column temperature 80°C then, programmed at 80 ^{o}C to 100 ^{o}C at a rate of 25 ^{o}C/min (4 min), and 100 ^{o}C to 150 ^{o}C at a rate of 40 ^{o}C/min (2 min), and 150 ^{o}C to

$300\,^{o}C$ at a rate of $90\,^{o}C$/min (2 min). Post column temperature $80\,^{o}C$ and hold for 2 min. The carrier gas was helium with gas flow of 20 ml/min, and a 40:1 injector split ratio. The make up gas was N_2 at 70 ml/min. TCE had a retention time of 3.775 min under these conditions. Each treatment was analyzed for concentration of TCE by comparing TCE recovered from cultures and soil to a standard curve of TCE.

3.4.1.3 Analysis of generated chloride ions

The suspension of selected resting cells and incubation conditions were prepared as the TCE biodegradation rate experiments, at 0, 2, 4, 6, 9, and 24 hours, triplicates of each sample were sacrificed by opening the seal. Most of the chlorine atoms in TCE eventually accumulated in the medium as Cl^- ions (Luu *et. al*., 1995). The concentration of chloride ions generated from TCE mineralization was analyzed by an ion-sensitive chloride combination electrode (model 94-17B, Thermo Electron Formerly Orion Research, Inc., USA). Chloride ion standard (1000 ppm sodium chloride) was used for calibration from concentration of 0.5 to 20 ppm chloride. 2% (v/v) ionic strength adjustor ($NaNO_3$) was added before measuring the 10 ml calibration standards and samples in a well-stirred 25-ml beaker.

3.4.2 Microbiological analysis

3.4.2.1 Viable plate count

TCE biodegradation process was studied from the relationship between decreasing of TCE and the increasing of TCE-degrader in soil microcosm. So, quantifying bacteria by dilution plate counts were performed. A number of toluene-degrading bacteria in treated soil were determined using MS medium agar plate. The procedure consists of diluting the bacteria with a series of sterile MS medium. The spread plate technique was used to prepare dilution plates. Generally, 0.1 ml of the diluted sample is pipetted onto the surface of a solidified agar medium in a Petri dish. The liquid is spread over the medium with a sterilized, bent, glass rod. Triplicates of each dilution are prepared. Spread plates were incubated at room temperature in glass box supplied with toluene as sole carbon source and energy. Then colonies were counted, results were averaged. A plate which had between 30 and 300 colonies was selected for counting. The number of bacteria per ml of culture was calculated by;

$$\text{Bacteria per ml of original solution (CFU/ml)} = \frac{\text{No. of colonies counted } \times \text{ dilution factor}}{\text{Vol. of a suspension added to a plate}}$$

3.4.2.2 Indole indicator test

Indole test was used to confirm the expression of its TCE-oxidizing enzyme (Luu *et al.*, 1995). In the test, 1 mM indole crystals were added directly to 250-Erlenmeyer flask to overnight bacteria cultures. The different color formed in media can be used to roughly distinguish the type of enzymes for example; a dark green-black for toluene dioxygenase, a light blue for toluene 4-monooxygenase, and a vivid red-orange for toluene 2-monooxygenase.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Characteristic of the TCE-Degrading Bacteria

4.1.1 Terpene utilization

Two types of TCE degrading bacteria were used in the study, namely *Rhodococcus gordoniae* P3 and *Pseudomonas* sp. T1. Both bacteria were isolated from petroleum contaminated soil collected from Bangkok area and generally using toluene as sole carbon source. The bacteria were tested for the ability to utilize four terpenes (cumene, limonene, carvone and pinene) as sole carbon and energy source. The level of growth was observed from an increased turbidity of cell culture after 24 hr incubation (Table 4.1).

The ability of *Rhodococcus gordoniae* P3 to utilize cumene, limonene, glucose, and toluene were similar, which indicated by equal level of growth (+++). Level of growth on cumene, limonene, glucose, and toluene as growth/primary substrate were higher than pinene (+), however the bacteria could not grow on carvone (-). The results, summaried in Table 4.1 suggested that cumene and limonene did not inhibit the growth of bacteria when supplied as vapor in flasks containing liquid medium. Since, no growth was observed from cells grown on carvone, it suggested that carvone may not be suitable as the sole carbon source for the bacteria.

This was also reported by Gilbert and Crowley (1997), who found that providing spearmint oil (containing carvone as principal component) as sole carbon source for growth of *Arthrobacter* sp. B1B resulted in cell lysis. Furthermore, carvone at high concentrations (100, 200, 300, 450, 500 mg of carvone l^{-1} or greater) were toxic to bacteria cells.

Table 4.1 Growth of *Rhodococcus gordoniae* P3 and *Pseudomonas sp.*T1 in various substrates after 24 hr incubation.

Substrate	Growth[1]	
	Rhodococcus gordoniae P3	*Pseudomonas sp.*T1
Cumene	+++	+++
Carvone	-	-
Pinene	+	-
Limonene	+++	-
Toluene	+++	+++
Glucose	+++	+++

[1] - , no growth; + to +++, growth, with the turbidity in cell culture increasing from + to +++.

These results also indicated that cumene and limonene were the best terpene to serve as sole carbon and energy sources for supporting growth of *Rhodococcus gordoniae* P3. From the results obtained on *Pseudomonas* sp. T1, cumene was the only one terpene to be utilized as sole carbon source (Table 4.1). The

ability of cumene to support *Pseudomonas* sp. T1 growth was similar to toluene and glucose.

Therefore, cumene was the only growth substrate suitable for both *Rhodococcus gordoniae* P3 and *Pseudomonas* sp. T1. This was probably because the structure of cumene was similar to toluene more than other terpenes (Figure 2.2). Cumene contain isopropyl residue adjacent to benzene ring, whereas toluene has methane residue at the same position. Furthermore, cumene has high solubility among terpenes used in this study, making it more available for uptake by bacteria than other terpenes. The types of bacteria may also influence substrate utilization of microorganisms as *Rhodococcus gordoniae* P3 could utilize limonene as well. The difference of cells structure between gram positive and gram negative may affect their efficiency in substrate utilization.

4.1.2 TCE degrading enzyme

Types of TCE-degrading enzymes in *Rhodococcus gordoniae* P3 and *Pseudomonas* sp. T1 were verified roughly by indole assay. The test was based on the transformation of indole (white color) to indigo (blue color) and other products by different TCE-degrading enzymes (Luu *et al*., 1995). Transformation of indole in the medium containing bacteria cells grown on various substrates were determined from color development after 24 hours incubation (Table 4.2). In the study, 1 mM indole was added to overnight culture of *Rhodococcus gordoniae* P3 and *Pseudomonas sp.* T1.

Table 4.2 Transformation of indole in the media containing bacteria cells grown on various substrates.

Type of Bacteria	Color of culture media				
	Cumene	Cumene + Glucose	Toluene	Toluene + Glucose	Glucose
Rhodococcus gordoniae P3	White	Light red/ Orange	Light red/ orange	Light red/ Orange	White
*Pseudomonas sp.*T1	White	Light blue/ Yellow	Light red/ orange	Deep red/orange	White

Indole was not transformed by *Rhodococcus gordoniae* P3 grown on the media containing cumene or glucose only, as the culture remained white. On the other hand, cells grown on a medium contained both cumene and glucose transformed indole to light red/orange product. This color also found in toluene grown cultures. The vivid red-orange was corresponded to toluene 2-monooxygenase (Luu *et al*., 1995). This enzyme was different from *Rhodococcus erythropolis* BD2, which suggested to be toluene dioxygenase (Dabrock *et. al*.,1992)

From these results, it was suggested that enzyme produced by *Rhodococcus gordoniae* P3 after cumene and toluene induction may be the same, referring to the development of similar colors. However, the induction ability of cumene on *Rhodococcus gordoniae* P3 was probably less than toluene, since the transformation of indole was distinct only when glucose was present in the medium.

Glucose was probably required to enhance the ability of cumene to induce enzyme production. Toluene grown cells provided similar result regardless of glucose. This was probably because the bacteria could utilize toluene effectively as both sole carbon source and inducer for TCE degrading enzyme production.

Similar to *Rhodococcus gordoniae* P3, cumene and toluene induced *Pseudomonas* sp.T1 transformed indole only when glucose was presented. Meanwhile, the color from cumene cells was different from toluene cells, which were light blue/yellow and deep red/orange, respectively. Based on their different color formation, cumene and toluene might induce for different enzymes in *Pseudomonas* sp.T1. Possible enzymes were toluene *para*-monooxygenase and toluene *ortho*-monooxygenase from the induction of cumene and toluene grown cells, respectively (Luu *et al*., 1995). No color was observed in non-induced cells (glucose grown cells).

4.2 Screening of Plant Terpenes as Inducer for the TCE Co-Metabolic Pathway

Four plant terpenes, including carvone, cumene, limonene, and pinene were screened for their ability to induce TCE degradative pathway in *Rhodococcus gordoniae* P3 and *Pseudomonas* sp. T1. To each bacterial type, different terpene solutions were added to produce final concentrations of 5, 10, 25 and 50 mg l^{-1}, respectively. The percentage of TCE degradation was calculated from the difference between the amount of TCE in first-hour sample (100% baseline) and the remaining of TCE after 24 hour incubation. Heat-killed cells and non-induced cells (glucose grown cells) were considered as control treatments. 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). TCE degradation by toluene-induced cells was also provided for comparison of terpene-induced cells.

TCE degradation by terpene-induced *Pseudomonas* sp.T1 ranged from 40.0-61.0%, whereas heated-killed cells and non-induced cells resulted in 41.7% and 49.0% decreasing of TCE, respectively (Table 4.3). Cumene at 10 and 50 mg l^{-1}, limonene at 25 mg l^{-1}, carvone and pinene at 50 mg l^{-1} had no harmful effect on *Pseudomonas* sp.T1 cells because the induced cell treatment showed percent TCE degradation significantly more than heat-killed cell treatment. The results also showed that considerable amount of TCE (41.7%) was lost through abiotic process.

Table 4.3 Averaged percentage TCE degradation after 24 h incubation in liquid cultures containing *Pseudomonas* sp. T1 cells with various inducers.

Treatments	Amount of inducers (mg l^{-1})	% TCE degradation*
Heat-killed cells	0	41.70^a
Non-induced cells	0	49.00^A
Toluene-induced cells	50	64.70^{bB}
Cumene-induced cells	5	48.50^{aA}
	10	61.00^{bB}
	25	49.80^{aA}
	50	55.35^{bA}
Limonene-induced cells	5	44.73^{aA}
	10	48.50^{aA}
	25	52.00^{bA}
	50	47.50^{aA}
Carvone-induced cells	5	48.00^{aA}
	10	40.00^{aB}
	25	49.99^{aA}
	50	51.04^{bA}
Pinene-induced cells	5	45.01^{aA}
	10	47.70^{aA}
	25	44.42^{aA}
	50	51.40^{bA}

* 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.

When compared the extent of TCE degradation between induced cells and non-induced cells, it was found that only *Pseudomonas* sp.T1 induced with 10 mg cumene l^{-1} could degrade TCE significantly more than the non-induced cells (Table 4.3). Percent TCE degradation by *Pseudomonas* sp.T1 induced with 10 mg cumene l^{-1} and non-induced cells were 61.0% and 49.0%, respectively. The results suggested that *Pseudomonas* sp.T1 may produce TCE degrading enzyme constitutively, however the amount of enzyme was higher when 10 mg cumene l^{-1} presented in the culture. *Pseudomonas* sp.T1 induced with 10 mg carvone l^{-1} showed 40.0% TCE degradation which was significantly lower than non-induced cell. This indicated that very low concentration of carvone may not be sufficient for enzyme induction.

Toluene-induced cells significantly degraded TCE more than heat-killed cells and non-induced cells. Toluene was reported to be the most effective inducer by previous studies. However, our result showed that percent TCE degradation of toluene induced cells was close to 10 mg l^{-1} cumene-induced cells, which were 64.70% and 61.0%, respectively.

Percent TCE degradation of terpene-induced *Rhodococcus gordoniae* P3 were summarized in Table 4.4 showed that cells induced with 25 mg l^{-1} cumene was the most effective treatment as shown by 76.01% TCE degradation which significantly different from percent TCE degradation in heat-killed cells and non-induced cells. Percent TCE degradations were 42.10% and 57.64% by heat-killed cell and non-induced cell, respectively. Cumene at 10 and 50 mg l^{-1}, limonene at all concentrations, carvone at 5-25 mg l^{-1} and pinene at 5-10 mg l^{-1} presented TCE

degradation significantly more than heat-killed cells. On the other hand, at 50 mg l^{-1} carvone and 25-50 mg l^{-1} pinene had no significant difference of TCE degradation from heat-killed cells. This can be concluded that at high concentrations of pinene and carvone were relatively toxic to bacteria cells. However, percent TCE degradation of terpene-induced *Rhodococcus gordoniae* P3 other than cells induced with 25 mg cumene l^{-1} were not significantly slower than non-induced cells.

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Toluene-induced cells presented a significant effect on TCE degradation compared to heat-killed cell and gave 74.43% of TCE degradation but had no significant difference from non-induced cell.

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 *Rhodococcus erythropolis* BD1 cells degraded 71.0% of 50 µM TCE (6.57 mg l^{-1}) after 20 hours incubation. Cumene is able to induce TCE degradation probably because the bulky isopropyl residue adjacent to the double bound on benzene ring (Figure 2.2) may mimic the two chlorines in the TCE molecule (Dabrock *et al.*, 1992). Although, other terpenes could not enhance TCE biodegradation significantly, it may be because the concentrations of certain terpene used in the study were not appropriate. Optimization of terpene concentrations as well as screening of more terpenes varieties would be suggested for future study.

Table 4.4 Averaged percentage TCE degradation after 24 h incubation in liquid cultures containing *Rhodococcus gordoniae* P3 cells with various inducers.

Treatments	Amount of inducers (mg l^{-1})	% TCE degradation *
Heat-killed cells	0	42.10^a
Non-induced cells	0	57.64^A
Toluene-induced cells	50	74.43^{bA}
Cumene-induced cells	5	60.33^{aA}
	10	72.06^{bA}
	25	76.01^{bB}
	50	67.69^{bA}
Limonene-induced cells	5	67.39^{bA}
	10	61.38^{bA}
	25	65.86^{bA}
	50	61.36^{bA}
Carvone-induced cells	5	72.13^{bA}
	10	65.41^{bA}
	25	64.28^{bA}
	50	59.22^{aA}
Pinene-induced cells	5	62.32^{bA}
	10	63.06^{bA}
	25	53.42^{aA}
	50	59.52^{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.

The ability of *Pseudomonas* sp.T1 and *Rhodococcus gordoniae* P3 to degrade TCE after induction by both cumene and toluene suggested that the structure of cumene was similar to toluene more than other terpenes. Otherwise, the bacteria may contain enzymes with broad substrate specificity. This study also showed that non-induced cell was able to degrade TCE but to lower extent than with cumene-induced cells. It suggests that the bacteria may produce certain TCE degrading enzymes constitutively.

Enzyme inhibition such as competitive inhibition which often existed between the growth-supporting substrate/inducer and TCE for oxidation by non-specific enzyme also must be considered. There was evidence of competitive inhibition in the response of low concentrations of TCE (approximately 40 μg l^{-1}) and phenol provided as substrate at concentration of 12.4 mg l^{-1} (Hopkins *et al.*, 1993).

However, it cannot be concluded from this work how terpenes affect TCE degradation. Terpenes affected bacteria in co-metabolism process by their interactions with microbial membranes because of their hydrophobicity (Amaral *et al.*, 1998). Besides, the effect of terpene on TCE biodegradation was probably selective at specific dosages on certain microorganisms. The same author also suggested that high concentrations of terpenes can disrupt electron transport and uncouple oxidative phosphorylation in bacteria. For example pinene at 270 mg l^{-1} disrupted the cytoplasmic membranes of the gram positive organism *Bacillus thuringiensis*, but the gram negative bacteria were more resistant to terpenes.

Results from this study were clear that 25 mg l^{-1} cumene induced *Rhodococcus gordoniae* P3 presented highest ability on induction TCE co-metabolic pathway (Table 4.3) and it was also best serve as growth substrate among terpenes used in this study (Table 4.1). 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. Based on data in the Hazardous Substances Data Bank (http://toxnet.nlm.nih.gov/index.html), cumene is not classified as human carcinogen. Once exposed, it 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. Consequently, cumene induced *Rhodococcus gordoniae* P3 would be a prospective candidate for further bioaugmentation of soil contaminated with TCE.

4.3 Rate of TCE Biodegradation Using the Selected Plant Terpene

Figure 4.1 showed the results of the TCE biodegradation study using *Rhodococcus gordoniae* P3 induced with 25 mg l^{-1} cumene and non-induced cells (glucose grown cells). During degradation, TCE concentration was decreased while the amount of chloride ion was increased from TCE mineralization. Biodegradation rate of TCE by cumene-induced *Rhodococcus gordoniae* P3 showed that TCE was decreased from approximately 3.53 mg l^{-1} of initial TCE concentration at time zero to approximately 1.96 mg l^{-1} after 9 hour incubation, while glucose grown cells degraded

TCE to approximately 3.1 mg l^{-1} at the same time period. The results were corresponding to 45 % and 12.14 % TCE reductions by cumene and glucose grown cells, respectively. From 9 to 24 hours, TCE decreasing by cumene-induced cells was relatively constant but at a lower rate than the first 9 hours. Approximately 1.94 mg l^{-1} TCE remained at the end of the study. Glucose-grown cells continued to show linear TCE reduction rate at the same period and approximately 2.41 mg l^{-1} TCE was remained in the culture at time 24 hours. The result from heat-killed cell treatment showed some TCE losses caused by abiotic process. However, TCE losses in this treatment were significantly less than glucose grown cells treatment (data not shown). TCE biodegradation rates obtained from figure 4.1 were given by

$$\Delta y/\Delta x_{cumene} = 0.0236x - 0.2813 \text{ (between 0-9 hours)}$$

$$\Delta y/\Delta x_{glucose} = -0.0386$$

Where, $\Delta y/\Delta x_{cumene}$ = TCE biodegradation rate of cumene-induced cell treatment between 0-9 hours; $\Delta y/\Delta x_{glucose}$ = TCE biodegradation rate of glucose grown cell treatment at designated time; x = Time

The TCE biodegradation rates were decrease with time from approximately 0.26 mg TCE l^{-1}h^{-1} to a concentration of near 0.07 mg TCE l^{-1}h^{-1} during the first 9 hours of cumeme-induced cells treatment (Figure 4.1), then a rate was close to zero at the end of incubation. On the other hand, a rate was relatively constant approximately of 0.04 mg TCE l^{-1}h^{-1} decreased during the experiment of glucose grown cell treatment.

The system probably needed a constant amount of enzyme inducer to maintain the production of enzyme to carry out TCE biodegradation activity. Cumene property with high volatilization makes it hard to maintain in the cultures for long periods (Gilbert and Crowley., 1997). Subsequently, TCE biodegradation process occurred intermittently during incubation period.
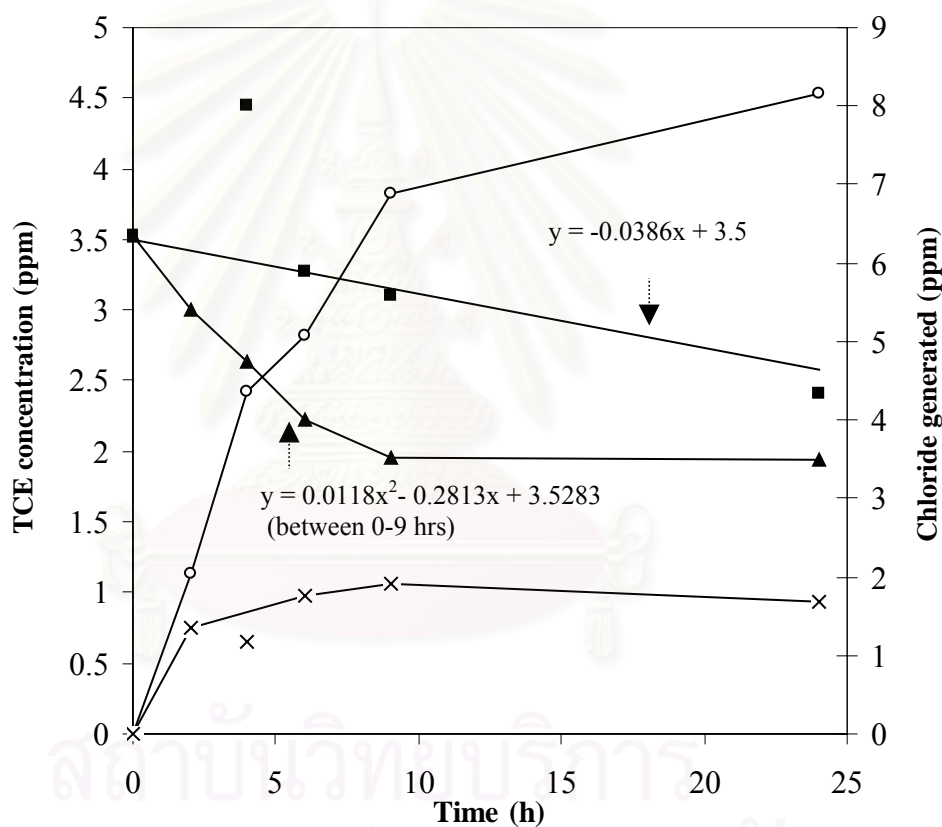

Figure 4.1 Degradation of TCE by *Rhodococcus gordoniae* P3 cells suspensions incubated with TCE for 24 hours, cumene- induced cells (▲) and glucose grown cells (■). Chloride formation of cumene-induced cells (o) and glucose grown cells (×)

In order to confirm the extent of complete TCE mineralization, chloride generated during TCE aerobic biodegradation process was determined. The result from Figure 4.1 indicated that during 9 hours incubation, cumene-induced cells readily degraded TCE, and simultaneously released high amount of chloride ion to media. Glucose grown cells presented a slight TCE decreasing, consequently a smaller amount of chloride was generated (Figure 4.1). It was possible that TCE degradation was carried out by constitutive enzymes or broad substrate enzymes.

There was evidence of positive correlation between TCE removal and chloride ion generation observed in this study. Accordingly, it can be shown that TCE losses relatively take place through mineralization process by conversion to carbon dioxide and chloride ions (Luu *et al.*, 1995). Dabrock *et al.* (1992) also reported that *Pseudomonas* sp. JR1 and *Rhodococcus erythropolis* BD2 degraded TCE with the presence of 3 mol of chloride per mol of TCE. Nevertheless, it cannot be exactly said that 3 mol of chloride created per mol of TCE in our study, because TCE losses may result from other processes such as volatilization, interaction between TCE and substrate, TCE adsorption to cells bacteria and etc.

4.4 Bioremediation of TCE in Soil Microcosm

4.4.1 Effect of soil moisture on the disappearance of TCE

The effect of soil moisture on TCE degradation was studied in treated microcosms with 15, 30, and 45% mixture soil moisture. The moisture content was adjusted by addition of cells containing medium or cell-free medium to the microcosm at the beginning of study. The effect of soil moisture content on the extent of TCE degradation was not significantly different when compared between treatments after 14 days incubation (Figure 4.2). This result was similar to a previous study (Fan and Scow, 1993). Meanwhile, the amount of moisture effected the mixing of soil with added bacteria as well as the percent TCE recovery. It was found that moderate mixing and a higher amount of TCE recovery was achieved from soil with 30% moisture content.
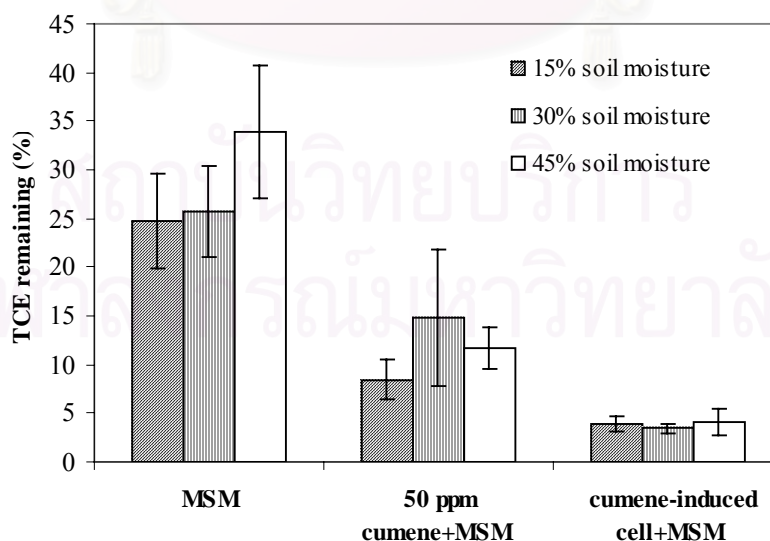

Figure 4.2 Percent TCE remaining in soil with various moisture contents after 14 days incubation.

4.4.2 Biodegradation of TCE contaminated soil using cumene

induced *Rhodococcus. gordoniae* **P3 and cumene solutions**

Biodegradation of 100 ppm TCE contaminated soil was studied in microcosm contained 5 g spiked soil with 30% moisture content. Approximately 45 ml of headspace was left in the bottle containing soil sample. All treatments were performed in the same manner, and then compared to control treatments. 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 with the treatment. The value was determined from the difference between initial TCE (100%) concentration and the amount of TCE recovered at designated time.

In order to investigate the effect of any amendments on TCE degradation, two bioremediation strategies, bioaugmentation and biostimulation, were performed.

Bioaugmentation treatment was done by adding *Rhodococcus gordoniae* P3 induced with 25 mg cumene l^{-1} to TCE contaminated soil microcosms. At the beginning of study, TCE decreased rapidly in the bioaugmented soil, and only 30% of TCE remained after 4 days incubation (Figure 4.3). 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%, respectively) were significantly lower than control treatments, which were ranged from 75-85% after 4 days and 35-55% after 7 days incubation. There was approximately 5% of

initial TCE concentration remained in the system after 10 days of incubation, showing

no significant different of TCE reduction between bioaugmentation treatment and

non-sterilized soil added with MS medium control treatment.
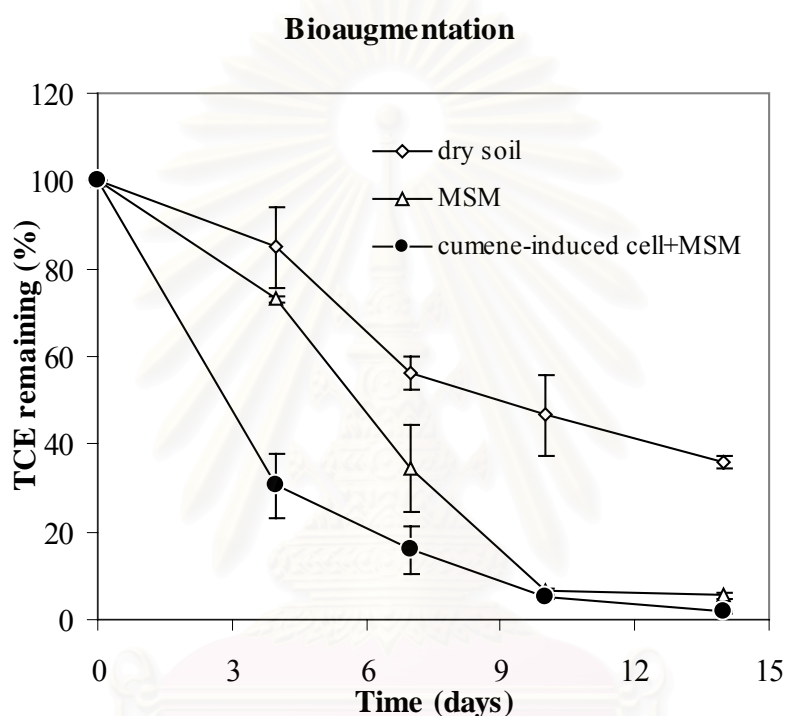
Figure 4.3 Percent TCE remaining in bioaugmented soil microcosms.

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 differences at all time points (Figure 4.4). During

the first 4 days, TCE decreased rapidly to approximately 40% of its initial

concentration remained in all biostimulation treatments. The amount of TCE in biostimulation treatments at this stage was significantly lower than control treatments. Later, the amount of TCE decreased gradually and less than 5% was remained in the soil after 10 days.
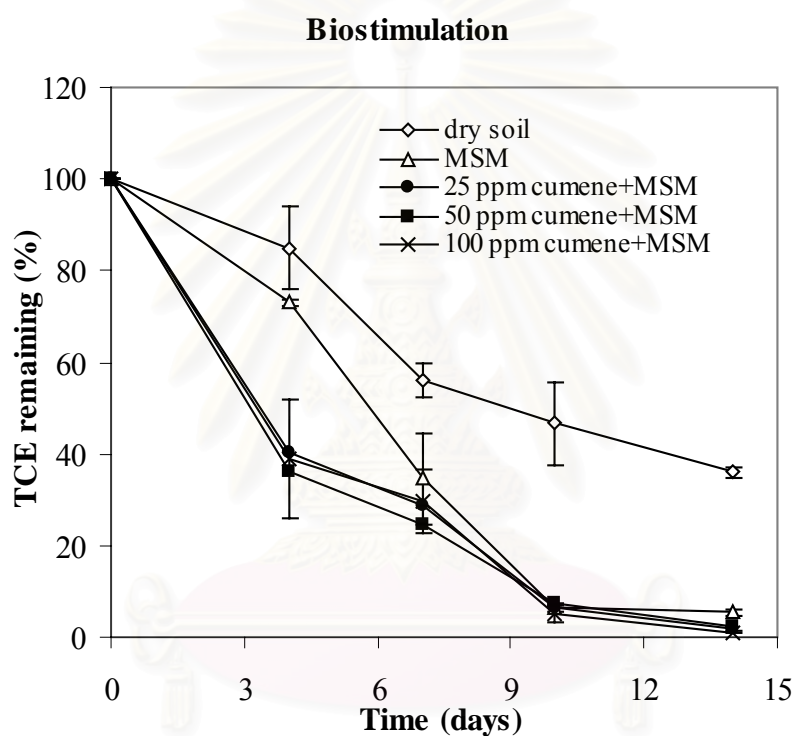
Biostimulation

Figure 4.4 Percent TCE remaining in biostimulated soil microcosms.

Control treatments consisted of microcosm with sterilized soil, non-sterilized soil amended with MS medium to maintain 30% moisture content and non-sterilized soil with no moisture adjustment (non-amended soil = dry soil). Reduction of TCE in sterilized soil and dry soil microcosms indicated the effect of abiotic process, whereas MS medium microcosms represented the effects of intrinsic

bioremediation that might have occurred. It was found that the sterilized soil treatment autoclaved 3 times showed highest the percentage of TCE decreasing among three control treatments (data not shown). Though, none of toluene degrading bacteria or any other soil bacterial were observed in autoclaved soil. This may be a consequence of changes in sorption properties of the soils caused by the autoclaving (Brigmon *et al.*, 1998). Additionally, abiotic process may also associate TCE losses in this treatment.

Amount of TCE remained in control dry soil was significantly higher than bioaugmentation and biostimulation treatments at any time points, even though TCE was gradually decreased to 40% at day 14 (Figure 4.3 and 4.4). On the other hand, percent TCE remaining in control MS medium microcosms was not significantly different from biostimulation soil after 7 days and from bioaugmentation soil after 10 days. TCE in this microcosm was remained at 35% at day 7 and less than 10% and day 10. TCE degradation of non-amended soil (dry soil) was significantly slower than all soil-slurry microcosms suggested that soil moisture strongly influenced TCE biodegradation. This may be because added moisture could facilitate TCE desorption from soil, resulting in more TCE soluble form. TCE in the liquid phase was considered to be best fortuitously transformed by microorganisms.

It can be concluded that biodegradation of TCE contaminated soil occurred considerably in the beginning of 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 treatment (dry

soil and MS medium amended soil) during the same period. The results suggested that cumene induced *Rhodococcus gordoniae* P3 and purified 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 MS medium amended soil after 10 days. This can be suggested that that moist soil has intrinsic ability to biodegrade TCE but it requires a longer incubation period. Organic matter in soil seemed to be available for microorganism activity and enzyme associated TCE degradation probably was inducible. The results were similar to Borch *et al*. (2003), who showed that TCE, PCE, and tetrachloromethane can be biodegraded in water unsaturated topsoil.

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, there was evident that the rate and extent of TCE degradation in soil microcosms was much lower than in liquid culture on the order of days. 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). Furthermore, soil properties may also affect TCE biodegradation process since they change the distribution of TCE among soil particles and modify its sorption, volatilization, and solubilization properties. This discussion was in agreement with Fan and Scow (1993) who suggested the amount of organic matter in soil correlates with its adsorptive capacity for organic chemicals.

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 for increasing 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.

4.5 Densities of Toluene-Degrading Bacteria in Treated Soil

Population densities of toluene-degrading bacteria were used to indicate the survival of added bacteria in bioaugmentation treatment as well as represent the increasing number of toluene-degrading bacteria after biostimulation. The results showed that toluene populations in every treatment were 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 4.5). The extent of bacteria proliferation was affected by treatments. Toluene populations were found in bioaugmentation treatment (6×10^6 CFU g^{-1} soil) more than any treatments at the beginning of study, whereas more bacteria were found in biostimulation treatments (6.2- 6.9×10^{14} CFU g^{-1} soil) after 14 day incubation. When compared between treatments, the densities of bacteria in control and bioaugmentation treatments were significantly differences at day 0, 4 but became comparable at approximately 5×10^{14} CFU g^{-1} soil after incubation. The number of toluene degraders in MS medium control and bioaugmentation treatment were significantly difference from all biostimulation treatments (cumene at 25, 50, and 100 ppm) only at day 0.

Population densities of toluene degraders in the bioaugmentation treatment were significantly different from the control MS medium soil at the beginning of study (day 0, 4). The result suggested that cumene-induced *Rhodococcus gordoniae* P3 could not grow well in the soil microcosms. However, the reduction of TCE in bioaugmentation treatment implied that the bacteria were still active after addition into the soil. At day 14, highest number of toluene-degrading bacteria were found in biostimulation treatments and expected to be a result of cumene and MSM addition. 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}soil) than unamended soil (10^3CFU g^{-1} soil) and simultaneously inducing polychlorinated biphenyls (PCB) degradation. This suggested that one of the benefits of using plant terpene to stimulate TCE

biodegradation is to increase the population densities of toluene degrading bacteria in soil. Corresponding to Crowley *et al.* (2001) who suggested that cumene may be one of the natural substrate for toluene-degrading bacteria.

Table 4.5 Number of toluene-degrading bacteria in the treated soil microcosms*.

Time	Treatment (x 10^8 CFU g^{-1})				
	Control[1]	Bioaugmentation[2]	Biostimulation		
(Days)			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 cumene induced *Rhodococcus gordoniae* P3.

Besides, a number of toluene-degrading bacteria were determined whether tested TCE concentration toxic to bacteria cells. Before exposure to TCE and cumene with no moisture adjustment, the initial population density of bacteria able to degrade toluene was 3.67×10^8 CFU g^{-1} soil and reached 4.67×10^{11} CFU g^{-1} soil at 18 days of incubation, while soil exposed to TCE was 8.77×10^{11} CFU g^{-1} of soil at the same period. This indicated that TCE concentration used for this study had no effect on the number of toluene-degrading bacteria. This can be discussed that bacteria may derive a small amount of energy caused by TCE biotransformation for supporting their growth and cell multiplication. The other possibility was that TCE may act as inducer for its own degradation. However, longer incubation (25 days) no toluene-degrading bacteria were detected in soil exposed to TCE. In contrast the number of toluene-degrading bacteria relatively increased in soil without TCE.

CHAPTER 5

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

5.1 Conclusions

The results from this study showed that cumene, a major terpene in cumin seed, significantly induced TCE degradation in *Pseudomonas* sp. T1 and *Rhodococcus gordoniae* P3 when compared with non-induced cells (glucose grown cells). These bacteria generally degrade TCE when growing on toluene. The ability of cumene on enzyme induction was suggested to be the analogous structure of cumene and toluene. Cumene concentration also affected biodegradation of TCE, 10 mg l^{-1} and 25 mg l^{-1} cumene showed highest ability on induction of TCE co-metabolism in *Pseudomonas* sp.T1, and *Rhodococcus gordoniae* P3, respectively. Moreover, the result also suggested that *Rhodococcus gordoniae* P3 showed higher ability on TCE degradation than *Pseudomonas* sp. T1.

Further evidence showed that complete mineralization of TCE to CO_2, water and chloride was taken place. The ability of *Rhodococcus gordoniae* P3 to mineralize TCE after induction by cumene was monitored by chloride ion formation. During degradation, TCE concentration was decreased while the amount of chloride ion was increased from the mineralization process.

Bioremediation techniques i.e. bioaugmentation and biostimulation were considered as potential means for clean-up TCE contaminated soil. The result showed that bioaugmentation and biostimulation significantly accelerated TCE reduction when compared to control treatment, especially at the beginning of study. Approximately 60% and 70% of 100 ppm TCE was reduced during the first 4 days of incubation in biostimulation and bioaugmentation treatment, respectively. Longer incubation resulted in no significant difference of TCE reduction between treated soil and control soil amended with MS medium.

5.2 Suggestions for Future Work

There are many factors affecting the amount of TCE removal, as indicated from this study. The percentage of actual TCE reduction may be overestimated because not only microbial activity caused TCE degradation, but abiotic process such as volatilization could also affected its diminishing. In addition, the sorption of TCE to soil and bacteria cells may influence the amount of TCE removal.

The effect of terpene on TCE in liquid and solid phase should be studied. For example, the distribution of organic compound between liquid and gas phases, its sorption to the soil, interaction between substrate and TCE, and etc. It may be possible to apply other forms of terpene such as plant extracted, plant residues or use of plants that produce terpenes (or other aromatic compounds) through their rhizosphere for supporting TCE or other xenobitic compounds degradation.

Based on the results of this study, further study is needed to extend the basic knowledge on TCE biodegradation and the mechanism of terpene as TCE inducer. This may be achieved by characterization of bacterial genes involved in the degradation of TCE. With the genes in hand, application of genetic engineering is become feasible, for example, to design gene probes for monitoring the added bacteria and other TCE degrading populations during bioremediation, to develop a TCE sensing bacteria for detection of its contamination, and to improve degrading activity and survival rate of TCE degrading bacteria.

Finally, larger scale bioremediation system should be conducted to optimize any conditions that would provide highest ability to degrade TCE before use in the real contaminated site. Future observation of systems with continuous feeding of effective terpenes, and systems using resting cells for their transformation ability, will help determine conditions where these different systems are best employed. Bioaugmentation of effective strains, both engineered and naturally occurring, may hold potential for enhancing both in situ and ex situ treatment. Additionally, use of carrier material may increase the potential for successful bioaugmentation by enhancing the survival of bacteria that have been inoculated into the soil because of their favorable characteristics for field application (Swadley, 2001).

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REFERENCES

Alexander, M.(1994). Biodegradation and bioremediation. Academic Press, San

    Diego, p 212.

Alvarez-Cohen, L., and McCarty, P.L. (1991). A co-metabolic biotransformation

    model for halogenated compounds exhibiting product toxicity. Environ Sci

    Technol 25:1381-1387.

Amaral, J.A., Ekins, A., Richards, S.R., and Knowles R. (1998). Effect of selected

    monoterpenes on methane oxidation, denitrification, and aerobic metabolism

    by bacteria in pure culture. Appl Environ Microbiol 64: 520-525.

Anderson, E.E., and Andersen, G.R. (1996). Ground water pollution primer: soil and

    groundwater pollution [online]. Available from

    http://cesunl.ce.vt.edu/enviro2/gwprimer.old/tce/tce.html [2003, March 25]

Arp, D.J., Yeager, C.M., and Hyman, M.R. (2001). Molecular and cellular

    fundamentals of aerobic co-metabolism of trichloroethylene. Biodegradation

    12: 81-103.

ATSDR (Agency for Toxic Substances and Disease Registry).(1997).Toxicological

    Profiles for trichloroethylene [online], Available from

    http://www.atsdr.cdc.gov/toxprofiles/tp19-c3.pdf .[2003, May 15].

Bradley, P.M. and Chapelle, F.H. (1997). Kinetics of dichloroethene (DCE) and vinyl

    chloride (VC) mineralization under methanogenic and Fe(III)-reducing

    conditions. Environ Sci Technol 31:2692-2696.

Brigmon, R.L., Bell, N.C., Freedman, D.L., and Berry C.J. (1998). Natural attenuation of trichloroethylene in rhizosphere soils at the Savannah River site. Journal of Soil Contamination 7:433-453.

CEPA (Canadian Environmental Act).(1993). Priority Substances List Assessment Report: Trichloroethylene [online], Available from http://www.hc-sc.gc.ca/hecs-sesc/exsd/pdf/trichloroethylene.pdf. [2003, January 15]

Crowley, D. E., E, Luepromchai, and Singer, A.C. (2001). Metabolism of xenobiotics in the rhizosphere, p. 333-352. *In* J. C. Hall, R. E. Hoagland, and R. B. Zablotowicz (ed.), Pesticide biotransformation in plants and microorganisms: similarities and divergences. American Chemical Society, Washington, DC.

Dabrock, B., Kebeler, M., Averhoff, B., and Gottschalk, G. (1994). Identification and characterization of a transmissible linear plasmid from *Rhodococcus erythropolis* BD2 that encodes isopropylbenzene and trichloroethylene catabolism. Appl Environ Microbiol 60:853-860.

Dabrock, B., Riedel, J., Bertram, J., Gottschalk, G. (1992). Isopropylbenzene (cumene) an new substrate for the isolation of trichloroethene-degrading bacteria. Arch Microbiol 158:9-13.

Duba, A.G., Jackson, K.J., Jovanovich, M.C., Knapp, R.B., and Taylor R.T. (1996). TCE remediation using in situ, resting-state bioaugmentation. Environ Sci Technol 30:1982-1989.

Dzantor, E.K., and Woolston, J.E. (2001). Enhancing dissipation of Aroclor 1248 (PCB) using substrate amendment in rhizosphere soil. J Environ Sci Health 36, 1861-1871.

Ensley, B.D. (1991). Biochemical diversity of trichloroethylene metabolism. <u>Annu Rev Microbiol</u> 45:283-299.

ERTC (Environmental Research and Training Centre). (2001). Study on contamination of chlorinated ethane in soil and groundwater in Thailand.

Fan, S., and Scow, K.M. (1993). Biodegradation of trichloroethylene and toluene by indigenous microbial populations in soil. <u>Appl Microbiol Biotechnol</u> 59:1911-1918.

Focht, D. D. (1994). Microbiological procedures for biodegradation research. <u>Methods of soil analysis, part 2. Microbiological and biochemical properties.</u> R. W. Weaver, J. S. Angle and P. S. Bottomley. Madison, WI, Soil Science Society of America.

Fries, M.R., Forney, L.J., and Tiedje, J.M. (1997). Phenol and toluene degrading microbial populations from an aquifer in which successful trichloroethylene cometabolism occurred. <u>Appl Environ Microbiol</u> 63:1523-1530.

Fries, M.R., Hopkins, G.D., McCarty, P.L., Forney, L.J., and Tiedje, J.M. (1997). Microbial succession during a field evaluation of phenol and toluene as the primary substrates for trichloroethlene cometabolism. <u>Appl Environ Microbiol</u> 63:1515-1522.

Fritsche, W. and Hofrichter, M. (2003). Aerobic degradation by microorganisms [online], Available from http://<u>www.wiley-vch.de/books/biotech/pdf/v11b_aero.pdf. 2003</u> . [2003, February 20]

Futamata, H., Harayama, S., Watanabe, K. (2001).Diversity in kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. <u>Appl Microbiol Biotechnol</u> 55:248-253.

Gilbert, E.S., and Crowley, D.E. (1997). Plant compounds that induce polychlorinated biphenyl biodegradation by *Arthrobacter* sp. Strain BIB. Appl Environ Microbiol 63:1933-1938.

Gilbert, E.S., and Crowley, D.E. (1998). Repeated application of carvone-induced bacteria to enhance biodegradation of polychlorinated biphenyls in soil. Appl Microbiol Biotechnol 50:489-494.

Hernandez, B.S., Koh, S.C., Chial, M., and Focht, D.D. (1997). Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphynyls in soil. Biodegradation 8:153-158.

Hinchee, R.E., Leeson, A., Semprini, L., and Ong, S.K. (1994.) Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds. Lewis Publishers, pp 1-500 cited from Curt Peterson (1999) aerobic degradation of TCE [online], Available from http://class.et.byu.edu/ce540/termpaper/1999-w/petersonc.pdf. [2003, May 20]

Hopkins, G.D., and McCarty, P.L. (1995). Field evaluation of in situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as primary substrates. Environ Sci Technol 29:1628-1637.

Hopkins, G.D., Semprini, L., McCarty, P.L. (1993). Microcosm and in situ field studies enhanced biotransformation of trichloroethylene by phenol-utilizing microorganisms. Appl Environ Microbiol 59:2277-2285.

Hyman, M.R. and Arp, D.J.(1991). Factors limiting aliphatic chlorocarbon degradation by *Nitrosomonas europaea*: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl Environ Microbiol 57:2986-2994.

Hyman, M.R., Russell, S.A., Ely, R.L., Williamson, K.J., Arp, D.J. (1995). Inhibition, Inactivation and recovery of ammonia-oxidizing activity in co-metabolism of trichloroethylene by *Nitrosomonas europaea*. Appl Environ Microbiol 61:1480-1487.

Infante, P.F., and Tsongas, T.A. (1987). Mutagenic and oncogenic effects of chloromethanes, chloroethanes and halogenated analogs of vinyl chloride. Environ Sci Res 25:301-327.

Kho, M.C.and Prosser, J. (1999). Intrinsic bioremediation of trichloroethylene and chlorobenzene: field and laboratory studies. Journal of hazardous materials 69:67-79.

Kleopfer, R.D., Easley, D.M., Hass, B.B., Delhl, T.G., Jackson, D.E., Wurrey. C.J. (1985). Anaerobic degradation of trichloroethylene in soil. Environmental science and technology 19:277-279.

Koh, C.S., Park, I.Y., Koo, M.Y., and So, S.J. (2000). Plant terpenes and lignin as natural cosubstrate in biodegradation of polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). Biotechnol Bioprocess Eng 5:164-168.

Langenheim, H.J. (1994). Higher plant terpenoids: A phytocentric overview of their ecological roles. J of Chem Ecol 20:1223-1280.

Leahy, J. G., Byrne, A. M. and Olsen, R. H.(1996). Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl Environ Microbiol 62:825-833.

Lee, S., Moe, W.M., Valsaraj, K. T., and Pardue, J. H. (2002). Effect of sorption and desorption resistance on aerobic trichloroethylene biodegradation in soils. Environmental Toxicology and Chemistry 21:1609-1617.

Little, C.D., Palumbo, A.V., Herbes, S.E., Lindstrom, M.E., Tyndall, R.L. and Gilmer, P.J. (1988). Trichloroethylene biodegradation by methane oxidizing bacterium. Appl Environ Microbiol 54:951-956.

Luepromchai, E. and O. Suttinun (2003). Substrate diversity of a new *Rhodococcus pyridinovorans* and its application for TCE biodegradation. SETAC Asia/Pacific | ASE Annual Meeting, Christchurch, New Zealand.

Luu, P.P., Yung, C.W., Sun, A.K., and Wood, T.K.(1995). Monitoring trichloroethylene mineralization by *Pseudomonas cepacia* G4 PR1. Appl Microbiol Biotechnol 44:259-264.

Madsen, E.L. (1991). Determining in situ biodegradation. Environ Sci Tech 25:1663-1673.

Mu, D.Y., and Scow, K.M. (1994). Effect of trichloroethylene (TCE) and toluene concentrations on TCE and toluene biodegradation and the population density of TCE and toluene degraders in soil. Appl Environ Microbiol 60:2661-2665.

Nelson, M. J. K., S. O. Montgomery, W. R. Mahaffey, and P. H. Pritchard (1987). Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl Environ Microbiol 53:949-954.

Parsons, F., and Lage, G.B. (1985). Chlorinated organics in simulated groundwater environments. Journal of American Water Works Association 77:52-59.

Parsons, F., Wood, R.P., and DeMarco, J.(1984). Transformation of tetrachloroethylene and trichloroethylene in microcosms and groundwater. J Am Water Works Assoc 76:56-59.

PCD (Pollution Control Department). (2000). Data contained in Pollution Control Department [online], Available from http://www.pcd.go.th/ [2003, December 5]

Pflugmacher, U., Averhoff, B., and Gottschalk, G. (1996). Cloning, sequencing, and expression of isopropylbenzene degradation genes from *Pseudomonas* sp. Strain JR1: identification of isopropylbenzene dioxygenase that mediates trichloroethene oxidation. Appl Environ Microbiol 62:3967-3977

Robert, P.V., L. Semprini, G.D. Hopkins, D. Grbic-Galic, P.L. McCarty, and Martin R. (1989). In Situ Aquifer Restoration of chlorinated Aliphatics by Methanotrophic Bacteria., USEPA, #CR-812220, pp.1-214 cited from Curt Peterson (1999) aerobic degradation of TCE [online], Available from http://class.et.byu.edu/ce540/termpaper/1999-w/petersonc.pdf. [2003, May 20]

Russell, H.H., Matthews, E.J., and Sewell, W.G. (1992). EPA Ground water issue: TCE removal from contaminated soil and ground water [online], Available from http://www.epa.gov/ada/download/issue/tce.pdf. [2003,September 2]

Semprini, L. (1997). Strategies for the aerobic co-metabolism of chlorinated solvents. Curr Opin Biotechnol 8:296-308.

Semprini, L., Roberts, P.V., Hopkins, G.D., and McCarty, P.L. (1990). A field evaluation of in-situ biodegradation of chlorinated ethenes. Ground Water 29:239-250.

Singer, A.C., Crowley, D,E., and Thomson, L.P. (2003). Secondary plant metabolites in phytoremediation and biotransformation. TRENDS in Biotechnology 21:123-130.

Singer, A.C., Gilbert, E.S., and Luepromchai, E. (2000). Bioremediation of polychlorinated biphenyl-contaminated soil using carvone and surfactant-grown bacteria. Appl Microbiol Biotechnol 54:838-843.

Swadley, A.L.(2001). Bioaugmented porous ceramics for degradation of trichloroethylene in groundwater: characterization and upscaling. NSF End-of-the-Year Report, Peliminary Rsults and Panning AY 2000-2001[online], Available from.http://www.mines.edu/fs_home/bmoska/schol/prog/Reports Sp_01/Swadley.pdf [2003, January 5]

Tandlich, R., Brezna, B., and Dercova, K. (2001). The effect of terpenes on the biodegradation of polychlorinated biphynyls by *Psudomonas stutzeri*. Chemosphere 44: 1547-1555.

TEI (Thailand Environment Institute). (1998). Study on utilization and management of volatile organic compounds (VOCs) in Thailand.

TRI. (Toxic Chemical Release Inventory). (1995). Data contained in theToxic Chemical Release Inventory (TRI) [online]. Available from http://toxnet.nlm.nih.gov/cgi-bin/sis/html/gen?TRI & search 79-01-6 [2003, January 13].

Vannelli, T., Logan, M., Arciero, M.D., Hooper, B.A.(1990). Degradation of halogenated aliphatic compounds by the ammonia-oxidizing bacterium *Nitrosomonas europaea.* Appl Environ Microbiol 56:1169-1171.
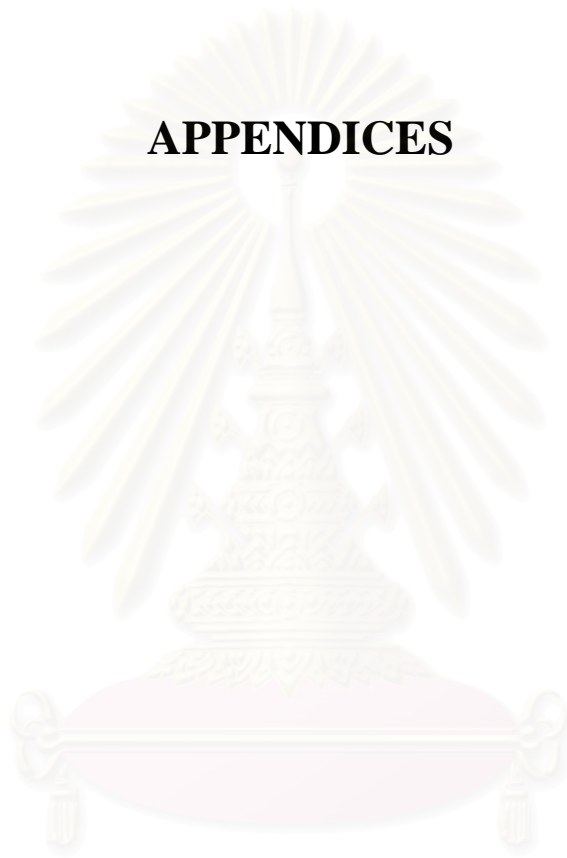
Vogel, T.M., and McCarty, P.L.(1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride and carbon dioxide under methanogenic conditions. <u>Appl Environ Microbiol</u> 49:1080-1083.

Wilson, J.T., And Wilson, B.H. (1985). Biotransformation of trichloroethylene in soil. <u>Appl Environ Microbiol</u> 29:242-243.

APPENDICES

APPENDIX A

The mineral salts medium (MSM)

MSM used for isolation, degradation experiments and quantifying bacteria was consisted of the following components per liter.

**Table A-1** Composition of MS medium used for this study

Stock solution	Additions, mL	Final concentration, mM
K_2HPO_4, 1M	10	10
NaH_2PO_4, 1M	3	3
$(NH_4)_2SO_4$, 1M	10	10
$MgSO_4$, 1M	1	1
$Ca(NO_3)_2$, 1M	0.1	0.1
$Fe(NO_3)_3$, 1M	0.01	0.01
Trace minerals	1	
$MnSO_4$	1 mM	0.001
$ZnSO_4$	1 mM	0.001
$CuSO_4$	1 mM	0.001
$NiSO_4$	0.1 mM	0.0001
$CoSO_4$	0.1 mM	0.0001
Na_2MoO_4	0.1 mM	0.0001

The second column represented the amount of each stock solution to be added to a final volume of 1 L, and the third column represented the final concentration. Add about 0.9 L of distilled water before adding any of the solutions above, or precipitates will form, and then fill to volume. The trace mineral solution was made up with all chemical listed. The final pH of medium = 7.25, 0.4% glucose (from Merck) was used as food supplement for bacterial growth. For solid media, 1.5 % agar (from HiMedia laboratories Limited, Mumbai, India) was added. The MS medium was autoclaved at 121 $^{\circ}$C for 15 min before use.

Counting viable toluene-degrading bacteria

The spread-plate technique was employed for dilution plate counts. The procedure consisted of diluting the bacteria with a series of sterile MS medium as illustrated in figure A-1.Dilutions were generally prepared in 9 ml MS medium, 1 g soil sample was added to create a 1:10 dilution. The further dilutions were needed by adding 0.1 ml from 1:10 dilution in an Efpendorf tube containing total of 0.9 ml MS medium to proceed 1:100 dilution of the original bacteria, more dilutions could be used if necessary by the same procedures indicated here (see figure A-1). Generally 0.1 ml of the diluted sample was pipetted onto the surface of a solidified agar medium in a Petri dish. The liquid was spread over the medium with a sterilized, bent, glass rod. To improve the accuracy of the methods, triplicate plates of each dilution were prepared. The spread plates were incubated librated glass box supplied with toluene as carbon and energy source. Then colonies were counted after 7 days incubation, results were averaged and estimated the number of viable bacteria per milliliter of the

original solution. Sometimes bacteria may grow from several bacteria clustered together. For this reason, the colony forming units were used for counting.
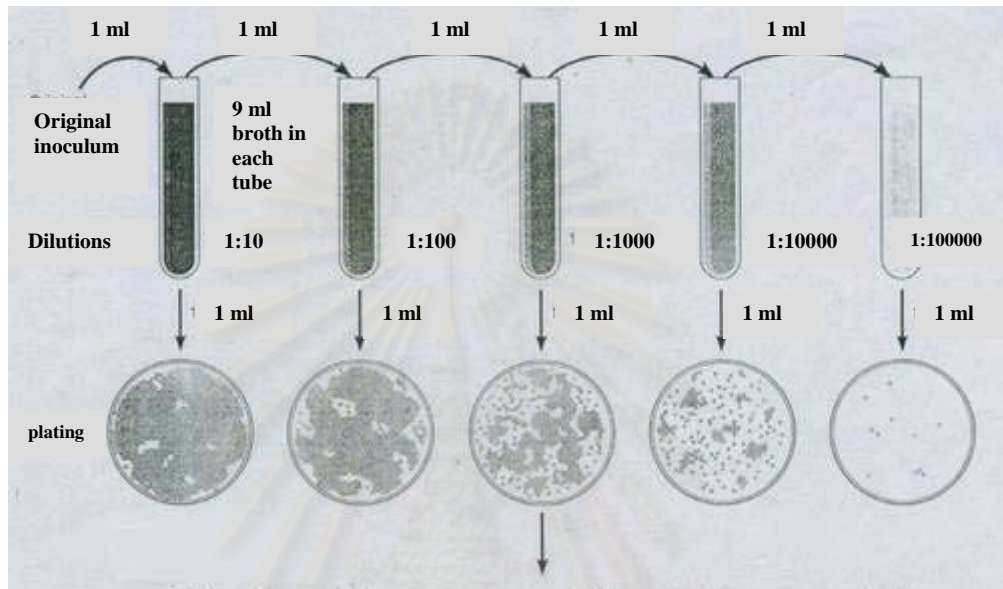
Calculation number of colonies on plate x reciprocal of dilution of sample = number of bacteria/ml

Figure A-1 Dilution plate counts procedures

Growth curve determination

Bacteria growth was monitored spectrophotometrically (A_{600}) as function of time.
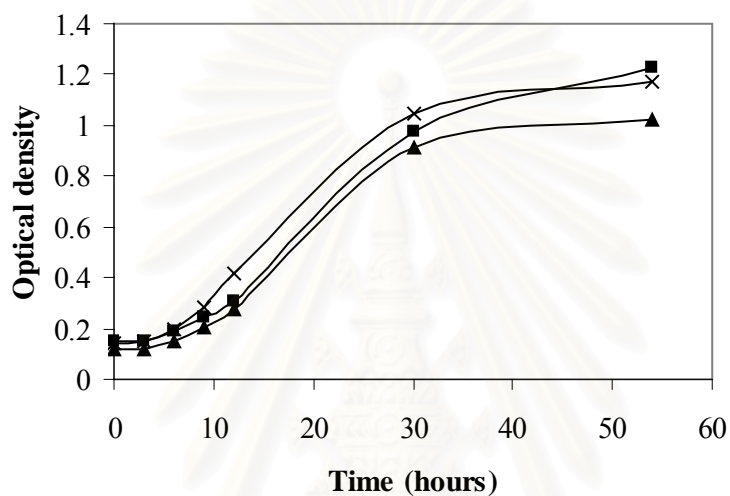

Figure A-2 Growth curve determination for *R. gordoniae* P3 grown on glucose (■), *R. gordoniae* P3 grown on toluene (▲) and *Pseudomonas* sp.T1grown on glucose (×)

APPENDIX B

Determination of soil characteristics

(1) pH

5 g of soil was weighed in a beaker and 5 ml of distilled water was added to give a ratio of 1:1 (soil: water). Then the soil slurry was stirred for about an hour. Then the pH was measured using pH meter.

(2) Moisture content

5 g of soil was weighed in dried crucible (A). Then the crucible and its content were dried at 103-110 ^{0}C for 24 hours in an oven. At the end of 24 hours allowed it to cool down in desiccator and weighed the crucible and its content again (B). The moisture content was calculated based on the equation below.

$$\% \text{ moisture} = \frac{(A-B)}{A} \times 100$$

The methods of determination of soil characteristics other than pH and moisture content were analyzed by System Development of Soil and Water Analysis Subgroup, Agricultural Chemistry Research Group, Department of Agriculture. The results are presented below.

Table B-1 Summary of soil properties

| Soil properties	Results
1. Soil texture (%)	
(1.1) Sand	8.6
(1.2) Silt	31.4
(1.3) Clay	60.0
2. pH	4.13
3. Moisture content (%)	
(3.1) At the beginning of study	3.3
(3.2) Mixture moisture content	30
4. Organic matter (%)	2.52
5. Organic carbon (%)	1.46
6. Nitrogen (%)	0.126
7. Phosphorus (ppm)	142
8. Maximum water holding capacity (%)	43.25

APPENDIX C

Calibration curve of TCE and chloride ions

    A calibration curve was developed for both liquid cultures and soil by dissolving TCE in hexane, then the stock standard were diluted to obtain the desired concentration (triplicate per each). The calibration standards were analyzed similar to sample procedures. Calibration curve was shown in FigureC-1-3.

The equation shown on the graph:

$$y = 1207.5x + 1.2449$$
$$R^2 = 0.9966$$
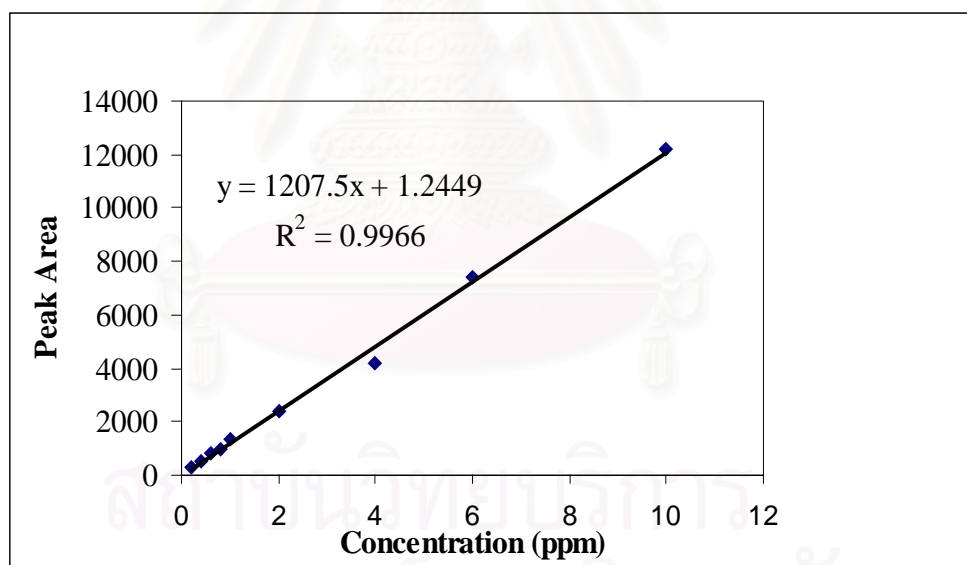
FigureC-1 Calibration curve of TCE for liquid cultures study
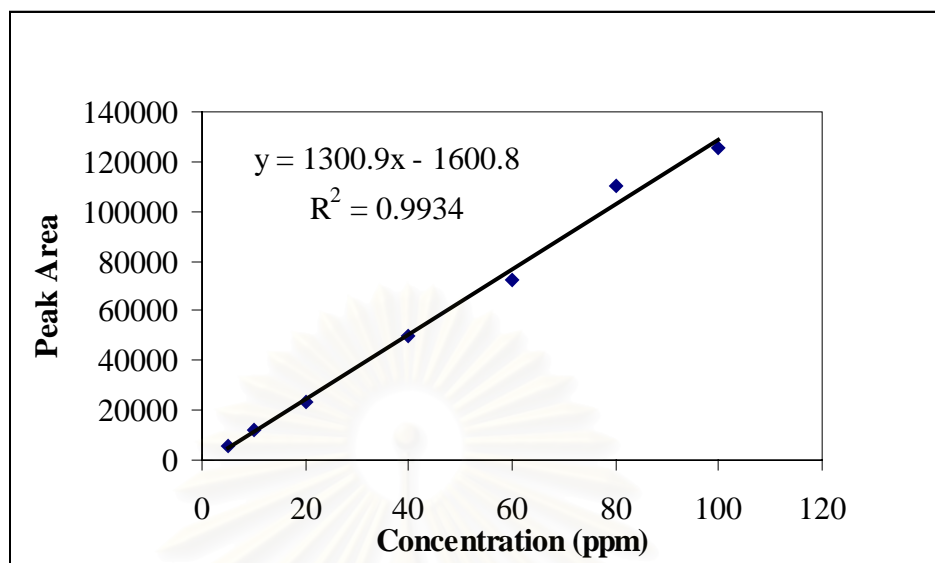
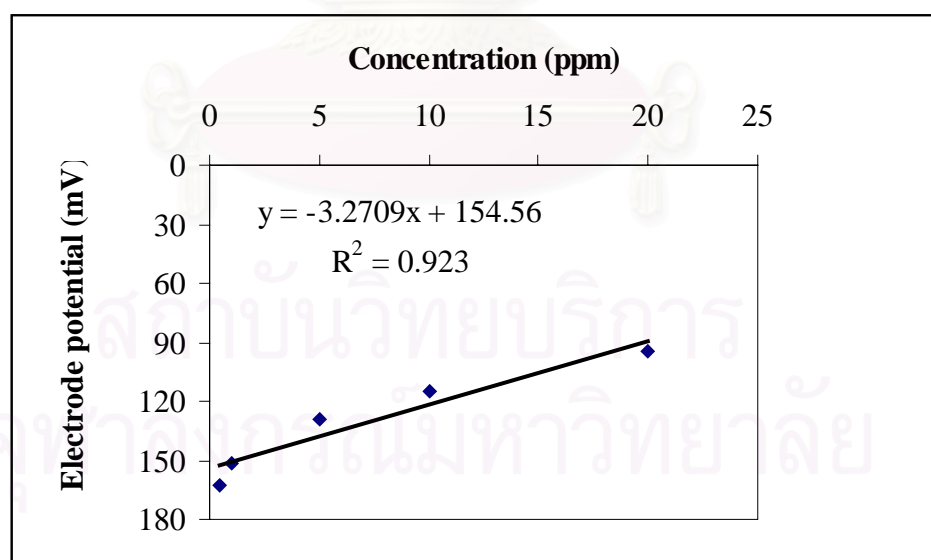**FigureC-2** Calibration curve of TCE for soil microcosm study

Figure C-3 Chloride electrode calibration curve

Extraction efficiency

TCE recoveries were determined for both liquid cultures and soil phase in triplicate samples experiment to determine the extraction procedures efficiencies by the following procedures. In liquid cultures, 2 ml hexane and 2 ml cell-free MS medium were added to extraction vials. Samples were spiked with TCE to give final concentration of 1, 5 and 10 ppm, then test vials were hexane extracted and injected to GC-ECD. For solid phase, 5 g non-sterilized, dry soil was added to 50 ml serum bottle with nothing addition and spiked with TCE to give a final concentration of 25, 50 and 100 ppm then TCE recoveries were analyzed the same procedures liquid cultures. The detail of TCE recovery efficiencies were presented in Table C-1-6.

Table C-1 % Recovery of TCE in liquid cultures spiked with 1 ppm TCE by using hexane extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	0.8566
2	0.8472
3	0.8489
Average	0.8509
% Recovery	85.09

Table C-2 % Recovery of TCE in liquid cultures spiked with 5 ppm TCE by using

hexane extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	3.9277
2	3.9783
3	4.5415
Average	4.1492
% Recovery	82.98

Table C-3 % Recovery of TCE in liquid cultures spiked with 10 ppm TCE by using

hexane extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	8.5325
2	9.0830
3	10.0357
Average	9.2170
% Recovery	92.17

Table C-4 % Recovery of TCE in soil spiked with 25 ppm TCE by using hexane

extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	22.1494
2	22.1380
3	21.9994
Average	22.0956
% Recovery	88.38

Table C-5 % Recovery of TCE in soil spiked with 50 ppm TCE by using hexane

extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	45.3578
2	46.8402
3	46.3600
Average	46.1860
% Recovery	92.38

Table C-6 % Recovery of TCE in soil spiked with 100 ppm TCE by using hexane

extraction and GC-ECD analysis

Sample	Concentration (ppm)
1	92.1138
2	82.2306
3	87.1700
Average	87.1714
% Recovery	87.18

Effect of concentration of surfactant on TCE extraction

    The effect of concentration and amount of chemical used on TCE extraction in solid phase were also determined by varying triton x-100 concentration (%, v/v): amount of triton x-100 (ml): amount of hexane (ml) as follows 15: 3: 10, 15: 3: 15, 10: 3: 10. All samples were analyzed for TCE remaining by GC-ECD as describe in the methodology. The result indicated that at ratio 15: 3: 10 provided highest TCE recovery, thus it was chosen for extraction in soil samples contaminated with TCE.

BIOGRAPHY

Miss Oramas Suttinun was born on April 15, 1978 in Suratthani, Thailand. She attended Suratthani School in Suratthani and graduated in 1996. She received her Bachelor's Degree from Thammasat University in the faculty of science and technology in March 2001. Her major study was Agricultural Technology and her minor one was Biotechnology. She pursued her Master Degree study in the International Postgraduate Programs in Environmental Management, Inter-Department of Environmental Management, Chulalongkorn University, Bangkok, Thailand in May 2002. She finished her Master Degree of Science in Environmental Management in May 2004.