การย่อยสลายไตรคลอโรเอธิลีนแบบโคเมแทบอลิซึมโดย *Rhodococcus* sp. L4 ที่ถูกชักนำด้วยน้ำมันหอมระเหยจากพืชและสารองค์ประกอบของมัน

นางสาวอรมาศ สุทธินุ่น

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาการจัดการสิ่งแวดล้อม (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2551

TRICHLOROETHYLENE COMETABOLIC DEGRADATION BY *RHODOCOCCUS* SP. L4 INDUCED WITH PLANT ESSENTIAL OILS AND THEIR COMPONENTS

Miss Oramas Suttinun

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Environmental Management
(Interdisciplinary Program)
Graduate School
Chulalongkorn University
Academic Year 2008

Thesis Title	TRICHLOROETHYLENE COMETABOLIC DEGRADATION BY *RHODOCOCCUS* SP. L4 INDUCED WITH PLANT ESSENTIAL OILS AND THEIR COMPONENTS
By	Miss Oramas Suttinun
Field of Study	Environmental Management
Advisor	Assistant Professor Ekawan Luepromchai, Ph.D.
Co-Advisor	Professor Rudolf Müller, Ph.D.

Accepted by the Graduate School, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

..... Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Assistant Professor Manaskorn Rachakornkij, Ph.D.)

..... Advisor
(Assistant Professor Ekawan Luepromchai, Ph.D.)

..... Co-Advisor
(Professor Rudolf Müller, Ph.D.)

..... Examiner
(Associate Professor Jin Anotai, Ph.D.)

..... Examiner
(Tawan Limpiyakorn, Ph.D.)

..... External Examiner
(Assistant Professor Benjaphorn Prapagdee, Ph.D.)

อรมาศ สุทธินุ่น : การย่อยสลายไตรคลอโรเอธิลีนแบบโคเมแทบอลิซึมโดย *Rhodococcus* sp. L4 ที่ถูกชักนำด้วยน้ำมันหอมระเหยจากพืชและสารองค์ประกอบของมัน (TRICHLOROETHYLENE COMETABOLIC DEGRADATION BY *RHODOCOCCUS* SP. L4 INDUCED WITH PLANT ESSENTIAL OILS AND THEIR COMPONENTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. เอกวัล ลือพร้อมชัย, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ. ดร. รูดอร์ฟ มุลเลอร์,190 หน้า.

การปนเปื้อนของไตรคลอโรเอธิลีน (ทีซีอี) ในดินและน้ำใต้ดินได้กลายมาเป็นปัญหาสิ่งแวดล้อมสำคัญ เนื่องจากกความเป็นพิษและความคงทนของสารเคมีดังกล่าว การย่อยสลายทีซีอีแบบโคเมแทบอลิซึมโดยแบคทีเรียที่ย่อยสลายโทลูอีน ถือเป็นวิธีการบำบัดแบบชีวภาพที่มีประสิทธิภาพและประหยัด แต่เนื่องจากการใช้โทลูอีนอาจทำให้เกิดปัญหาสิ่งแวดล้อม การศึกษานี้จึงทดสอบน้ำมันหอมระเหยจากพืชและสารองค์ประกอบของมันหลายชนิด เพื่อใช้เป็นสารชักนำทางเลือกสำหรับการย่อยทีซีอีในแบคทีเรีย *Rhodococcus* sp. L4 ซึ่งเป็นแบคทีเรียที่สามารถย่อยสลายโทลูอีนได้ โดยใช้การทดลองแบบ resting cells assay พบว่าที่ความเข้มข้นเริ่มต้นของทีซีอี 14 ไมโครโมลาร์ แบคทีเรียที่ถูกเลี้ยงด้วยน้ำมันหอมระเหยจากมะนาว และตะไคร้สามารถย่อยสลายทีซีอีได้ 20 เปอร์เซ็นต์ และ 27 เปอร์เซ็นต์ ซึ่งต่ำกว่าเมื่อแบคทีเรียถูกเลี้ยงด้วยโทลูอีน (57 เปอร์เซ็นต์) ทั้งนี้ความสามารถในการย่อยทีซีอีเพิ่มขึ้นเป็น 36 เปอร์เซ็นต์ เมื่อแบคทีเรียถูกชักนำด้วยน้ำมันจากเมล็ดยี่หร่า การชักนำเอนไซม์ที่เกี่ยวข้องในการย่อยสลายทีซีอีเชื่อว่าเนื่องมาจากสารองค์ประกอบที่มีอยู่ในน้ำมันหอมระเหย ได้แก่ ซิตรอล คิวมินแอลดีไฮด์ คิวมีน และไลโมนีน โดยเฉพาะสารคิวมินแอลดีไฮด์และคิวมีน พบว่ามีประสิทธิภาพในการชักนำการย่อยสลายทีซีอีเทียบเท่ากับโทลูอีน ซึ่งค่าศักยภาพในการเปลี่ยนรูปทีซีอี (transformation capacity) ของแบคทีเรียที่ถูกชักนำด้วยสารเหล่านี้มีค่าระหว่าง 9.4 ถึง 15.1 ไมโครกรัมทีซีอีต่อมิลลิกรัมเซลล์ อย่างไรก็ตามเซลล์เหล่านี้สามารถย่อยทีซีอีได้เพียงระยะเวลาสั้น จึงได้ตรึง *Rhodococcus* sp. L4 ในวัสดุจากพืชที่มีน้ำมันหอมระเหยสูง ได้แก่ เมล็ดยี่หร่า เปลือกส้ม และใบตะไคร้ โดยเชื่อว่าจะช่วยผลิตและรักษาเอนไซม์ที่เกี่ยวข้องกับการย่อยสลายทีซีอี พบว่าเซลล์ที่ตรึงในเมล็ดยี่หร่ามีประสิทธิภาพในการยืดระยะการย่อยทีซีอีได้ดีกว่าวัสดุชนิดอื่น และสามารถทนต่อทีซีอีที่ความเข้มข้นสูง โดยมีค่าการเปลี่ยนรูปทีซีอีสูงสุดเท่ากับ 60 ไมโครกรัมทีซีอีต่อมิลลิกรัมเซลล์ นอกจากนั้นยังพบว่าเซลล์ที่ตรึงนี้สามารถนำกลับมาใช้ในการย่อยสลายทีซีอีได้ใหม่หลังจากนำไปเลี้ยงไว้ในอาหารเลี้ยงเชื้อ Mineral salts นาน 12 ชั่วโมง การศึกษาในระดับเอนไซม์พบว่าโทลูอีนไดออกซิจีเนส (ทีดีโอ) จาก *Rhodococcus* sp. L4 มีแอคทิวิตีลดลงในสภาวะที่มีทีซีอี อย่างไรก็ตามเอนไซม์สามารถกลับคืนสู่ภาวะปกติได้เมื่อนำทีซีอีออกจากระบบ และพบว่าการใส่คิวมีนลงไปสามารถป้องกันการเปลี่ยนสภาพของเอนไซม์ (enzyme inactivation) ได้ ผลการทดลองเหล่านี้สนับสนุนการศึกษาในระดับเซลล์และการตรึงเซลล์ที่ว่าการเติมสารองค์ประกอบน้ำมันหอมระเหยจำพวก คิวมีน คิวมินแอลดีไฮด์ ไลโมนีน หรือซิตรอล ซ้ำ ๆ จะช่วยพยุงรักษากิจกรรมการย่อยสลายทีซีอีในแบคทีเรีย *Rhodococcus* sp. L4

สาขาวิชาการจัดการสิ่งแวดล้อม ..... ลายมือชื่อนิสิต .....

ปีการศึกษา 2551 ..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

4789722020 : MAJOR ENVIRONMENTAL MANAGEMENT
KEYWORDS : TRICHLOROETHYLENE / COMETABOLIC DEGRADATION / BIODEGRADATION / *RHODOCOCCUS* / ESSENTIAL OILS / ESSENTIAL OIL COMPONENTS

ORAMAS SUTTINUN : TRICHLOROETHYLENE COMETABOLIC DEGRADATION BY *RHODOCOCCUS* SP. L4 INDUCED WITH PLANT ESSENTIAL OILS AND THEIR COMPONENTS. ADVISOR : ASSIST. PROF. EKAWAN LUEPROMCHAI, Ph.D., CO-ADVISOR : PROF. RUDOLF MÜLLER, Ph.D.,190 pp.

Contamination of soil and groundwater with trichloroethylene (TCE) has become an important problem because of its toxicity and persistence. Cometabolic degradation of TCE by toluene-degrading bacteria has the potential for being a cost-effective bioremediation technology. However, the application of toluene may pose environmental problems. In this study, several plant essential oils and their components were examined as alternative inducer for TCE cometabolic degradation in resting cells assay of a toluene-degrading bacterium, *Rhodococcus* sp. L4. Using the initial aqueous TCE concentration of 14 μM, lemon and lemongrass oil-grown cells were capable of 20% and 27 % TCE degradation, which were lower than that of toluene-grown cells (57 %). The ability of TCE degradation increased to 36% when the bacterium was induced with cumin oil. The induction of TCE-degrading enzymes was suggested to be due to the presence of citral, cumin aldehyde, cumene, and limonene in these essential oils. In particular, the efficiency of cumin aldehyde and cumene as inducers for TCE cometabolic degradation was similar to toluene. TCE transformation capacities (*Tc*) for these induced cells were between 9.4-15.1 μg of TCE mg cells^{-1}. However, these induced cells were able to effectively stimulate TCE degradation only for a short period. Immobilization of *Rhodococcus* sp. L4 on plant materials rich in essential oils i.e. cumin seeds, orange peels and lemon grass leaves was believed to help producing and maintaining TCE-degrading enzyme. Of all materials, cumin seeds-immobilized cells were more effective to sustain TCE degradation as well as to protect the bacteria from high TCE concentrations. A maximum *Tc* of 60 μg of TCE mg cells^{-1} was found from the cumin seeds-immobilized cells. Moreover, the immobilized cells could be reused for TCE biodegradation after a reactivation in mineral salts medium for 12 hrs. Enzymatic study found that the activity of toluene dioxygenase (TDO) enzyme from *Rhodococcus* sp. L4 was decreased in the presence of TCE. However, TDO activity could be recovered after removing TCE from the system. The addition of cumene to enzymatic reactions could protect TDO enzyme inactivation. These findings supported the whole-cells and immobilization study, in which the repeated addition of essential oil component i.e. cumene, cumin aldehyde, limonene and citral could maintain TCE-degrading activity of *Rhodococcus* sp. L4.

Field of study: Environmental Management Student's signature .....

Academic year : 2008 Advisor's signature .....

Co-advisor's signature .....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to two of the most contributors to my Ph.D. dissertation; firstly, I would like to express my profound gratitude, greatest appreciation to Assist. Prof. Dr. Ekawan Luepromchai for her precious time, kind supervision, enthusiastic support and encouragement throughout this research work. I also appreciated her for giving me an opportunity to work on this challenging research and to gain experience from research presentations at the international conferences. She is a source of inspiration for hard working. I would not be here today without her trust and support. I also would like to express my deep gratitude and respect to Prof. Dr. Rudolf Müller, my co-advisor, for providing me an opportunity to work in his laboratory at Institute of Technical Biocatalysis, Technical University Hamburg-Harburg, Germany. I am very thankful to him for his valuable suggestions and assistances.

I would like to express my sincere appreciation to Assist. Prof. Dr. Manaskorn Rachakornkij, Chairman of the dissertation committee, Assoc. Prof. Dr. Jin Anotai, Dr.Tawan Limpiyakorn, Assist. Prof. Dr. Benjaphorn Prapagdee, member of dissertation committee for valuable suggestions. Without the essential contributions of the chairman and the committee members, this dissertation would have never been completed.

I am very thankful to all laboratory staffs and officers of Institute of Technical Biocatalysis, Technical University Hamburg-Harburg, Environmental Research Institute of Chulalongkorn University (ERIC), National Center of Excellence for Environmental and Hazardous Waste Management (NCE-EHWM), especially Mr.Gerhard Schietke, Mr. Marco Berheide, Mrs. Areeya Rattanawanachai and Ms. Ramnaree Netwichean for their technical assistances.

Special and heartiest thanks to all my laboratory friends, Ms. Siraprapa Romyen, Mrs. Apaporn Siripornprasarn, Ms. Nutchanart Thongtamachart, Ms. Witchaya Kaewtip, Ms. Nichakorn Khondee, Mr.Katari Venkata Subbaraju and Mrs. Ana Gabriela Valladares for their guidances and untiring helps in all matters.

Finally, I wish to express my sincere gratitude to my family members, my parents and my brother, for their love and continued support at all times. This work would not have been possible without their moral supports.

CONTENTS

Pages

Pages

Pages

.

LIST OF TABLES

Table **Pages**

LIST OF FIGURES

Figure **Pages**

Figure **Pages**

Figure **Pages**

Figure **Pages**

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
ESO	=	Essential oil
ESOC	=	Essential oil component
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
TOM	=	toluene ortho-monooxygenase
TRI	=	Toxic Chemical Release Inventory
VC	=	vinyl chloride

CHAPTER I

INTRODUCTION

1.1 Statement of problem

Trichloroethylene (TCE), a chlorinated aliphatic hydrocarbon (CAH), is widely used as an ingredient in industrial cleaning solutions and as a universal degreasing agent in several manufactures such as electronic parts cleaning and in plastic processing. In Thailand, TCE is also used as a substitute for chlorofluorocarbon (CFC), a banned chemical related to greenhouse effect (TEI, 1998). Due to its unique properties and solvent effects, the uses of TCE are widespread. TCE was found in water and soil samples taken from selected factories that utilized it in their processes (Milintawisamai *et al*, 2001). In addition, TCE has often caused contaminations of large volumes of soil and groundwater as a result of improper handling, storage as well as from lack of legislation enforcement (TEI, 1998). With the evidence of long-range transport, deposit, and persistence of this chemical, TCE is classified as a major environmental problem. 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). Thai government has issued groundwater quality standard and soil quality standard for the treatment of TCE from residential, agricultural and industrial sectors in 2004. Therefore, appropriate clean-up technologies are necessary (PCD, 2004).

Various methods have been used to clean up soil and groundwater contaminated with TCE, such as air stripping, carbon adsorption, soil venting, UV/oxidation treatment process, 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 the TCE biodegradation process, especially under local conditions. 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 through cometabolic degradation/cometabolism, however, is quicker and leads to the production of harmless by-products, making it an attractive choice for bioremediation.

TCE cometabolic degradation occurred when nonspecific oxygenases (monooxygenases or dioxygenases) produced from oxidation of growth substrate such as isoprene, methane, ammonia, toluene, phenol, ethylene or isopropylbenzene catalyze the initial transformation of TCE, the non-growth substrate/co-substrate compound. Several aliphatic and aromatic hydrocarbon-degrading bacteria have been reported to degrade TCE while utilizing those growth substrates/inducers such as *Alcaligenes denitrificans* sp.

xylosoxidans JEv75, *Rhodococcus erythropolis* JE 77 (Ewers *et al*., 1990), *Methylosinus trichosporium* (Chu and Alvarez-Cohen, 1998), *Nitrosomonas europaea* (Hyman *et al*., 1995), *Comamonas testosterone* strain R5 (Futamata *et al*., 2001), *Pseudomonas* sp. JR1, *Rhodococcus erythropolis* BD1 (Dabrock *et al*., 1992) and *Pseudomonas putida*, *Burkholderia cepacia* G4 (Heald and Jenkins *et al*., 1994; Yeager *et al*., 2001). Among those substrates/inducers, toluene and phenol are known as the most effective compounds 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 essential oils contain several oil components that have structures 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 essential oils and their components are limonene from lemon oil, carvone from peppermint oil, citral from lemon grass oil, cumin aldehyde and cumene from cumin oil and pinene from pine oil. These compounds are considered environmentally friendly and cheap from their natural origin.

To date, the utilization of plant essential oils and their components for TCE degradation is limited and the only oil component that has been studied in bacterial liquid cultures is cumene (isopropylbenzene) (Dabrock *et al*., 1992; 1994; Pflugmacher *et al*., 1996). In this study, we therefore tested the ability of various plant essential oils and their components for induction of TCE biodegradation in *Rhodococcus* sp. L4, an isolate from

petroleum contaminated soil. Rhodococci are known to be potential candidates for bioremediation of contaminated sites (Bell *et al*., 1998).

TCE cometabolic degradation process is considered to be unsustainable. Several investigations with *Nitrosomonas europaea* (Hyman *et al*., 1995), *Burkholderia cepacia* G4 (Yeager *et al*., 2001), *Pseudomonas putida* F1 (Morono *et al*., 2004), and butane-oxidizing bacteria i.e. *Pseudomonas butanovora*, *Mycobacterium vaccae*, and *Nocardioides* sp. CF8 (Halsey *et al*., 2005) showed the inhibition and inactivation of TCE-degrading enzymes and the rapid disappearance of substrates (or inducers). Thus, TCE bioremediation is eventually limited. Another objective of this study was to protect and reactivate TCE-degrading enzymes in *Rhodococcus* sp. L4. Therefore, we immobilized *Rhodococcus* sp. L4 on plant materials rich in essential oils by attachment technique. These plant materials were expected to help producing and maintaining TCE-degrading enzyme activities as well as to sustain TCE cometabolic degradation. Use of plant materials for bacteria immobilization could be a new approach to develop the active immobilized cells for real environmental applications as well as for commercial purposes. Moreover, the study investigated the effect of TCE on activity and recovery of toluene dioxygenase from *Rhodococcus* sp. L4 at the enzyme level, which has never been studied before.

The results of this study were both basic and applicable information relevant to TCE cometabolic degradation. In-depth investigations of the potential of essential oil/its component for inducing, reactivating, and protecting TCE-degrading enzymes is useful to predict the activity of TCE-degrading bacteria in the environment. Furthermore, these basic data can be employed to improve and modify the biological processes for environmental application. The study of immobilization can increase bacterial survival and maintain TCE-

degrading activity during bioaugmentation which ensure a success TCE treatment technology.

1.2 Objectives

The main objectives of this study were to investigate the potential of plant essential oils/its components on inducing, reactivating, and protecting TCE-degrading enzymes and to develop a ready-to-use inoculum for TCE bioaugmentation. The specific objectives were:

1. To study the efficiency of various plant essential oils and their components on inducing *Rhodococcus* sp. L4 to cometabolize and dechlorinate TCE.

2. To develop an inoculum of *Rhodococcus* sp. L4 by immobilizing bacteria cells on plant materials rich in essential oils for TCE biodegradation.

3. To determine the effect of TCE on activity and recovery of the oxygenase from *Rhodococcus* sp. L4.

1.3 Hypotheses

Essential oils or their components can induce *Rhodococcus* sp. L4 for TCE cometabolic degradation but only for a short period. Consequently, the repeated addition of essential oil components is necessary for TCE degradation. The immobilization of *Rhodococcus* sp. L4 on plant materials rich in essential oils is a more convenient approach to sustain TCE cometabolic degrading activity because the continuously released essential oils could effectively induce, re-activate, and protect the target enzyme.

1.4 Scopes of study

1.4.1 Whole-cell (free cells) studies

(1) Four types of purified essential oil solutions (lemon, lemon grass, pine, and spearmint oil), a vapor phase of cumin oil from cumin seeds and four major components of lemon, lemon grass and cumin oil (limonene, citral, cumin aldehyde and cumene) obtained commercially were tested for the ability to degrade and dechlorinate TCE in *Rhodococcus* sp. L4. This bacterium originally utilized toluene as growth substrate and has been isolated in the Environmental Research Institute Laboratory by Assist. Prof. Dr. Ekawan Luepromchai. TCE transformation capacity (T_c) and rate (k_c) of each essential oil/its component-induced *Rhodococcus* sp. L4 were determined by resting cells assay. In addition, the chloride generation during TCE cometabolic degradation was determined to confirm TCE dechlorination. The TCE removal efficiency of these induced bacteria was compared with that when bacteria were grown on toluene as positive controls. Negative controls were sets of non-induced cells and killed cells, which represented TCE loss by abiotic processes.

(2) Non-induced resting cells exposed to chloramphenicol (a protein synthesis inhibitor) were incubated with toluene and TCE in order to prove the hypothesis that enzymes involved in TCE degradation are not constitutively produced. The test vials were compared to those without chloramphenicol exposure.

(3) Each essential oil component i.e. cumene, cumin aldehyde, citral or limonene was added repeatedly into the sample vials containing induced-*Rhodococcus* sp. L4 for protecting TCE-degrading enzymes and enhancing TCE degradation.

At the end of whole cell experiments, we selected a type of essential oil component i.e. cumene which provide the highest TCE degradation, as a carbon source of bacterial cultivation for further enzymatic studies.

1.4.2 Immobilization studies

(1) The screening of plant materials rich in essential oils for *Rhodococcus* sp. L4 immobilization and TCE degradation was performed. Three plant materials rich in essential oils (cumin seeds, orange peels, and lemon grass leaves) were investigated for their ability to sustain TCE cometabolic degradation. To ensure the benefit of essential oils, two immobilizing materials without essential oil (loofa sponge and activated carbon) were conducted.

(2) The optimization of cell immobilization was carried out in order to achieve a strong attachment between cells and materials.

(3) TCE transformation capacities by the immobilized cells on cumin seeds under various TCE concentrations were determined and compared to free cells in section 1.4.1 and other publications.

(4) The reusability of immobilized cells for TCE degradation was ultimately tested.

In this section, controls were sets of uninoculated materials and killed immobilized cells.

1.4.3 Enzymatic studies

A simple spectrophotometric assay based on the intermediate formation of a yellow dye (indoxyl) during the oxidation of indole to indigo (Jenkins and Dalton, 1985) was carried out to determine the activity of toluene dioxygenase (TDO) from

Rhodoccoccus sp. L4. The partially purified enzyme precipitated by ammonium sulfate was used throughout the study.

(1) Determining the effects of TCE on activity of TDO enzyme

(2) Determining the recovery of TDO activity after exposure to TCE

(3) Determining the ability of a selected essential oil component i.e. cumene to protect TCE-degrading enzymes during TCE degradation

In this section, controls consisted of samples without TCE or cumene.

CHAPTER II

THEORETICAL BACKGROUND AND LITERATURE REVIEW

2.1 General information of TCE

2.1.1 Use

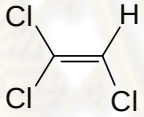
TCE (trichloroethylene) was first synthesized during the preparation of tetrachloroethane in 1864. Due to its unique properties and solvent effects, it has been widely used as dry cleaning agent and degreasing solvent in many countries e.g. the United States of America, Canada, France, Germany, Italy, Japan, Taiwan. TCE can also be found in some household products, including typewriter correction fluid, paint removers, adhesives, and spot removers. Moreover, TCE is also used as raw material to make other chemicals such as 1,1,1,2-tetrafluoroethane (ATSDR, 1997).

Usage patterns of TCE in Thailand have increased in the last two decades resulting from a rapid development of the industrial sector. The amount of TCE imported to the country has increased annually from 3522.6 tons in 2001 to 7363.6 tons in 2002 (Thai Customs Department) and its consumption increased continuously from about 5,797 – 7,841 tones per year during 2001 to 2005 (Sukhapan, 2007). TCE was mainly imported from Japan and EU countries for cleaning and degreasing operations (mostly in metal products and electronic parts cleaning) 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, ethylene trichloride and by other trade names in industry such as Trichloren, Chlorylen, Algylen, Trichloran, and Trielene (ATSDR, 1997). TCE belongs to a class of chlorinated aliphatic hydrocarbons (CAHs), which generally contain one or two carbon atoms and one to six chlorine atoms. 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^{o}C
Boiling point	86.7 ^{o}C
Density at 20^{o}C	1.465 g mL^{-1}
Odor	Ethereal; chloroform- like; sweet
Odor threshold: Air	100 ppm
Solubility: Water at 20^{o}C 25^{o}C Organic solvents	1.070 g L^{-1} 1.366 g L^{-1} Miscible with many common organic solvents (such as hexane, ether, and chloroform)
Partition coefficients: Log Kow Log Koc	2.42 2.03-2.66
Vapor pressure at 25^{o}C	74 mm Hg
Henry's law constants: at 20^{o}C at 25^{o}C	0.020 atm-m^3 mol^{-1} 0.011 atm-m^3 mol^{-1}

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 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 labeled 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 TCE in the environment

TCE has been found in at least 60 % of all hazardous waste sites identified as the National Priorities List (NPL) and are targeted for long term clean up (EPA, 1996). TCE is also one of the most frequently detected volatile organic chemicals (VOCs) in

groundwater in the United States (ATSDR, 1997). In Germany, trichloroethylene was detected in about 40% of the tested drinking-water samples with a concentration of more than 1 μg L^{-1} for 5.5% of the supplied residents (concentration range <0.001–21 μg L^{-1}) (WHO, 2000). In 1995, the Environment Agency of Japan reported that TCE and tetrachloroethylene (PCE) were major contaminants of 232 cases of soil and groundwater contamination, TCE concentration in groundwater from the contaminated site ranged from 5.3 to 6.5 mg L^{-1}, while the environmental quality standards for all kinds of soil in 1994 of TCE about 0.3 mg L^{-1}. (Environmental Agency of Japan, 1995).

A survey in Northern Industrial Estate, Lampoon province, Thailand found TCE contamination in soil and shallow groundwater at many locations where TCE was utilized in electronic appliance type production factories (Yaithavorn and Charnnarong, 1998). The observation of three selected factories that used TCE in their manufacturing processes found TCE contaminated in soil and groundwater from all factories at the concentration ranges from 0.03-970 mg L^{-1}. It was suggested that TCE may contaminate other factories in Thailand as well (Milintawisamai *et al.*, 2001). Recently, the illegal dumping of hazardous waste containing high concentrations of volatile organic compounds (VOCs) has been found in Pak-Chong, Nakhonratchasima in September, 2004. This site has been rented by General Environmental Conservation Public Company Limited (Genco), a waste management company, for collecting hazardous wastes from industrial sector before incineration. The groundwater collected from this site showed a high level of TCE, benzene, 1,1,2-trichloroethane. These air and groundwater pollutants may cause long term health effects to people living around the area (PCD, 2004). The environmental distribution of TCE was suggested from improper disposal or storage, poor

environmental awareness and lack of legislation enforcement. From these situations, TCE is expected to be a major environmental contaminant in the near future.

2.1.5 Potential health effects and regulations

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).

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 has been

established and no more than 28 ppm and 61 ppm TCE should be found in residential or agricultural soil and industrial soil, respectively (PCD, 2004).

2.2 Fundamental of chlorinated hydrocarbon biodegradation

Microorganisms 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) which forms sequential products of TCE, DCE, VC and ethane (Byl and Williams, 2000).

(3) Cometabolic degradation/co-metabolism

The term cometabolic degradation/co-metabolism was first studied in the 1950s and 1960s and focused on the microbial degradation of important industrial chemicals such as aromatics, pesticides and petroleum hydrocarbons (Dalton and Stirling, 1982; Arp *et al.* 2001). Cometabolic degradation/co-metabolism describes the ability of microorganisms to transform non-growth-supporting substrate (co-metabolized substrate or co-substrate), generally in the presence of a growth supporting substrate (primary substrate) (Arp *et al.*, 2001). 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 confronts 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 cells, 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 other 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.

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.

2.3 Enzymes involved in cometabolic degradation/co-metabolism of TCE

Several monooxygenases and dioxygenases have been reported to initiate TCE degradation, for example toluene 2-monooxygenase (T2MO), toluene 4-monooxygenase (T4MO), toluene-o-xylene monooxygenase (ToMO), toluene dioxygenase (TDO), soluble methane monooxygenase (MMO), and isopropylbenzene dioxygenase. Of all the enzymes, degradation of TCE by toluene oxygenases is the most effective and has been extensively studied. Leahy *et al*. (1996) reported that bacteria with different toluene oxygenase demonstrate different TCE degrading capabilities, from completely inactive strains to more active toluene monooxygenase-containing strains. In general, oxygenases are not constitutively produced in bacterial cells. Their production is induced by the presence of a particular growth or primary substrate such as isoprene, toluene, ammonia, propane, phenol, methane, or cumene (isopropylbenzene). The oxidative enzymes generally catalyze a reaction that incorporates O_2 into these compounds and the oxidation reaction requires an energy source such as nicotinamide adenine dinucleotide (NADH) to incorporate the O_2. The enzymes produced for the oxidation of these growth /primary substrates were suggested to 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 an unstable molecule such as TCE epoxide (Alvarez-Cohen and McCarty, 1991). The unstable molecule will be spontaneously decomposed to carbon monoxide, formic acid,

glyoxalic acid and chloroacetic acids e.g. dichloroacetic acid. These chloroacetic acids are soluble in water and will be slowly degraded to CO_2, chloride, and water (Figure 2.1).

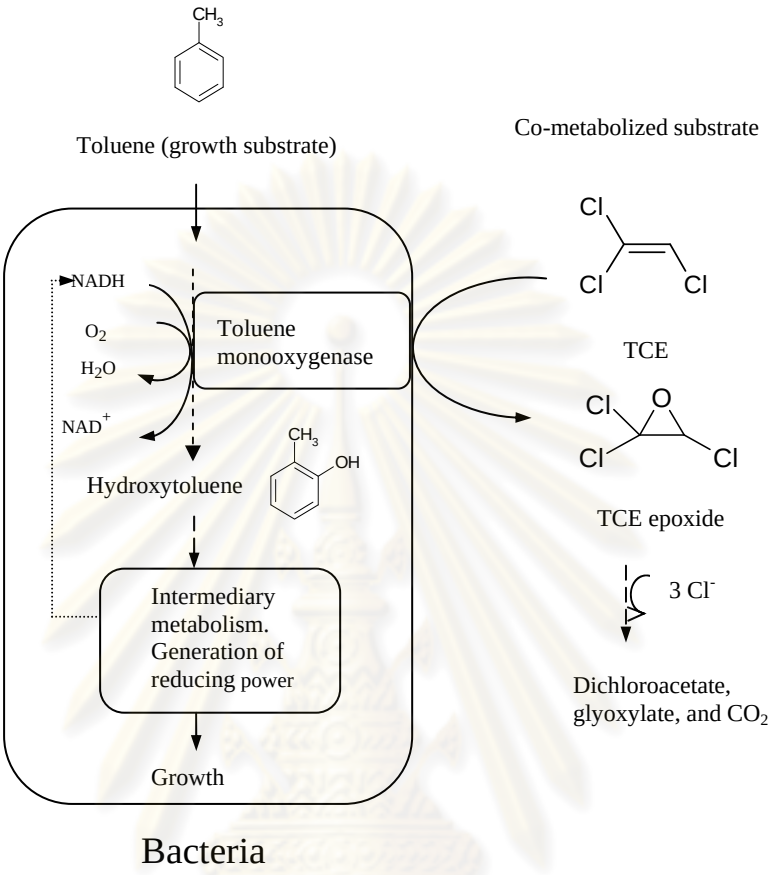


Figure 2.1 Example of TCE co-metabolic pathway by toluene monooxygenase enzyme. The microorganism utilizes toluene as primary substrate while oxidizes TCE to an epoxide and later to CO_2, dichloroacetate, glyoxylate (modified from http://www.wiley-vch.de/books/biotech/pdf/v11b_aero.pdf).

2.4 On the road to biodegradation and bioremediation of TCE

(1) Laboratory study

The contamination of TCE is frequently detected in the environment. It suggests that environmental conditions are probably not conducive to TCE degradation. Wilson

and Wilson (1985) firstly investigated the 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). They found that methanotrophic populations 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 cometabolic degradation/co-metabolism/co-oxidation (Little *et al.* 1988).

An impressive body of work on TCE biodegradation has been carried out. A number of laboratory studies demonstrated that TCE is transformed co-metabolically by a group of aliphatic and aromatic hydrocarbon-degrading bacteria such as methane oxidizing bacteria (Little *et al*., 1988; Alvarez-Cohen and McCarty, 1991), ammonia oxidizing bacteria (Hyman *et al*., 1995; Yang *et al*., 1999; Kocamemi and Cecen, 2007), phenol oxidizing bacteria (Hopkins *et al*., 1993; Futamata *et al*., 2001), butane oxidizing bacteria (Halsey *et al*., 2005) and toluene oxidizing bacteria (Wackett and Householder, 1989; Fries *et al.*, 1997; Morono *et al.*, 2004). These bacteria use ammonia, methane, propane, phenol, isoprene, isopropylbenzene, or toluene as primary substrate and/or inducer for TCE degrading enzymes. Most prevalent are Gram (-) bacteria such as *Pseudomonas*, *Burkholderia*, *Methylosinus*, *Alcaligenes*, and *Acinetobacter* (Arp *et al*., 2001; Milintawisamai *et al*., 2001). On the other hand, Gram (+) TCE degrading bacteria are rare. Only actinomycetes i.e. *Rhodococcus*, *Mycobacterium vaccae*, *Nocardioides* sp. CF8 and other filamentous bacteria are reported (Dabrock *et al*., 1994; Lee *et al*., 2000; Luepromchai, 2003; Halsey *et al*., 2005). In aerobic conditions, these microorganisms were reported to completely dechlorinate /mineralize TCE to CO_2 and H_2O.

Examples 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), and 1 mg L^{-1} (Fries *et al*., 1997; Vannelli *et al.*, 1990). The amounts of TCE in soil studies were 0.1-10 mg TCE soil solution L^{-1} (Fan and Scow, 1993) and 50 to 60 mg TCE soil solution L^{-1} (Mu and Scow, 1994). The concentrations of TCE in groundwater in the fields ranged from 0.05 to 5 mg L^{-1} (Hopkins and McCarty, (1995).

Little *et al*. (1988) first observed TCE biodegradation by pure cultures of methane oxidizing bacteria strains 46-1 that were 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 were 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 suspensions of *Nitrosomonas europaea*, *an* ammonia-oxidizing bacterium. They found that most or the entire test compound had disappeared within 24 hours. The reaction was suggested to be at least dependent on or

probably catalyzed by the ammonia oxygenase. Furthermore, the rate of nitrite production from ammonia 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 rates and then the degradation decreased over time for a number of strains. More than 50% of gram negative bacteria grown on toluene showed less than 50% cometabolic degradation 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 with 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 occured in MCl medium and M9 medium, respectively.

Lee *et. al*. (2002) studied the effect of sorption and desorption resistance on aerobic TCE biodegradation in microcosms aqueous phases. The TCE degradation rates were clearly increased in microcosms when toluene was added multiple times and correlated with toluene dioxygenase (TOD) enzyme activity over time, indicating that TCE co-metabolic degradation 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}.

(2) Field study

Nowadays, remediation of TCE contaminated sites relies primarily on physical and chemical processes such as air stripping, UV/oxidation treatment process, soil vapor extraction and carbon adsorption. 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 sites. During bioremediation, a 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 are degraded at a faster rate under aerobic conditions. To increase biodegradation rate, bioremediation can be performed by supplying the essential nutrients or inducers to stimulate indigenous microorganisms at the sites (biostimulation)

or by inoculating 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 (bioaugmentation).

The actual application of TCE bioremediation is still limited but is rapidly gaining popularity. Different methods of application have been studied. The in situ biostimulation trials included the injection of methane (Semprini *et al*., 1990), toluene (Hopkins and McCarty, 1995; McCarty *et al*., 1998) and phenol (Hopkins *et al*., 1993; Hopkins and McCarty, 1995) into aquifers to stimulate indigenous bacteria. Kuo *et al*. (2004) reported that injection of toluene vapor and air into TCE-contaminated aquifer enhanced indigenous bacteria and reduced potential clogging near the injection points due to excessive microbial growth. One example of bioaugmentation was studied by Duba *et al.*, 1996. They used methanotrophic bacteria (exogenous TCE-degrading bacteria) suspended in groundwater and injected into an aquifer. After withdrawal of the contaminated groundwater, 98% TCE disappeared during the first 50 hours.

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, an alternative compound that is non-toxic, environmentally friendly, and cheap is required.

In situ groundwater remediation may be achieved by using permeable reactive barriers (PRBs) in which reactive materials such as peat and vegetable oil are placed in the flow path of contaminated plumes (Kao *et al*., 2001; Hunter, 2005). The contaminant is removed by the natural movement of the plume through the PRB. Kao *et al*. (2001) reported that a barrier system containing oxygen-organic releasing material i.e. calcium peroxide and peat could remove up to 90% TCE from contaminated groundwater over a 4-month operating period. They suggested that the continuously released oxygen and organic substrates from oxygen-organic materials enhanced TCE degradation and biobarrier treatment had a potential for environmental application due to its low cost and because it is environmentally friendly. Ex situ bioremediation may be implemented to treat soil and groundwater as well. Shimomura *et al*. (1997) conducted a successful fluidized-bed bioreactor containing *Methylocystis* sp. M immobilized in calcium alginate gel beads for treatment of TCE.

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, co-metabolism, 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 cometabolic and reductive dechlorination conditions.

2.5 The limitations of TCE co-metabolic degradation

(1) Primary substrate (and/or inducer) utilization and disappearance

The rate of primary substrate utilization compared to the rate of TCE co-metabolism was studied. For the methane degrading bacteria, the rates of TCE

transformation are comparable to the rate of methane utilization while TCE transformation rates with phenol and toluene degrading bacteria are slower than that of toluene and phenol utilization. It was suggested that the use of toluene and phenol appeared to be less susceptible to TCE transformation toxicity than methane induction (Mars *et al*., 1996; Landa *et al*., 1994). It might be that the systems where primary substrate utilization is faster than TCE transformation, are able to tolerate TCE transformation product toxicity better. The faster rate of product formation might cause more destruction to the enzyme (Semprini *et al*., 1997).

Ely *et al*. (1995) observed the recovery of the ammonia-oxidizing bacteria, *Nirosomonas europae*, during TCE cometabolism in the presence of ammonia as a primary substrate. The results showed that the ability to recover depended on the amount of ammonia and TCE remaining. From their proposed enzyme based kinetic model for inactivation and recovery, they found that the systems would not recover above a certain ratio of TCE to ammonia, and below a certain ratio it would.

Our previous work revealed that TCE cometabolic degradation rate by cumene-induced cells was close to zero after 9 hours of incubation. The system probably needed a constant amount of enzyme inducer to maintain the production of enzyme to carry out TCE biodegradation activity (Suttinun *et al*., 2004). The high volatility of cumene makes it hard to maintain it in the system for long periods (Gilbert and Crowley, 1997). Subsequently, TCE biodegradation process occurred intermittently during incubation period.

(2) Inhibition, inactivation and cytotoxicity associated with TCE cometabolic degradation

Cometabolic degradation of TCE tends to be an unsustainable process under stagnant conditions because of substrate competition and enzyme inhibition and inactivation. The simultaneous presence of the two substrates for the same enzyme result in a competitive inhibition of the oxidation of the primary growth-supporting substrate such as methane, ammonia, phenol, or toluene, and TCE for binding on the active site of the nonspecific oxygenase (Semprini *et al*, 1990; Ensley, 1991).

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 substrates such that in the presence of both compounds TCE transformation rates were reduced.

There was competitive evidence of TCE degradation and phenol utilization in cultures of *Pseudomonas cepacia* G4 which indicated that the K_s values (represented affinity of enzyme for substrate) for both TCE and phenol are similar (5-10 µM). This competition results in an inhibition of the oxidation of the primary substrate as well as an inhibition the overall cometabolic process (Folsom *et al*., 1990).

Hyman *et al*. (1995) suggested in the presence of ammonia (NH_3) as primary substrate, TCE is a competitive inhibitor of ammonium oxidation by *Nitrosomonas europaea*. The K_i value (enzyme-inhibitor dissociation constant) for TCE (30 µM) is similar to the K_m for ammonia (40 µM). Kocamemi and Cecen (2007) examined the inhibitory effect of TCE on nitrification process with an enriched nitrifier culture. The results demonstrated that TCE inhibited ammonia oxidation and also influenced nitrification. The affinity of the TCE for the ammonia monooxygenase was found to be significantly higher than that of ammonia.

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 compared to 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.

Several laboratory works have been conducted to study the toxicity resulting from TCE cometabolic degradation observed as specific damage to the transforming enzyme itself or to general damage by influencing cellular respiration, viability, or the bacterial community (Halsey *et al*., 2005). 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; Morono *et al*. 2004).

Li and Wackett (1992) reported that for *Pseudomonas putida* F1, reaction products of TCE oxidation are diffusible and modify proteins and reduced nucleotides in solution. The major products of TCE oxidation by this bacteria were reported to be formate and glyoxylic acid, with formyl chloride and glyoxylyl chloride being proposed as initial oxidation products, respectively.

Hyman *et al*. (1995) examined the toxicity associated with TCE cometabolism. They found that *Nitrosomonas europaea* oxidized approximately 60 nmol of TCE per mg of protein before ammonia utilizing activity was completely inactivated by short-lived reactive intermediates generated during TCE oxidation.

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.

Additionally, some research groups reported that TCE degradation can result in injuries which remarkably affect basic cellular functions for example general respiratory activity and cell viability. Heald and Jenkins (1994) showed that TCE oxidation by *Pseudomonas putida* containing toluene dioxygenase resulted in a decrease in the growth rate of cultures and caused rapid cell death. They suggested that addition of dithiothreitol

(DTT) to assay mixtures increased the TCE removal capacity of cells by up to 67% but did not prevent TCE-mediated cell death.

Although, toluene oxidizing bacterium *Burkholderia cepacia* G4 was previously hypothesized to resist inactivation during TCE transformation, results of current studies showed that this bacterium is susceptible to cellular damage as a result of TCE oxidation. Newman and Wackett (1997) reported that TCE turnover by purified oxygenase from *Burkholderia cepacia G4* (toluene 2-monooxygenase) results in enzyme inactivation. It has been proposed that acyl chlorides created from hydrolysis or rearrangement of TCE epoxide cause damage by alkylating cellular constituents.

However, Yeager *et al*. (2001b) reported that there was a critical level of damage or a toxicity threshold that this organism can accumulate during TCE oxidation before cell culturability is affected significantly. It was suggested that some protective and/or DNA repair systems have mediated the ability of cells to survive following TCE degradation. Hyman *et al*. (1995) reported that TCE-inactivated cells did not recover as rapidly as light-inactivated cells. However, the recovery depended on the extent of inactivation of ammonia-oxidizing activity. The authors also suggested that such an inactivating effect on ammonia oxidizing activity could be recovered in a process requiring de novo protein synthesis.

Halsey *et al*. (2005) demonstrated that the primary toxic evidence from TCE oxidation by three butane oxidizers, *Pseudomonas butanovora*, *Mycobacterium vaccae*, and *Nocardioides* sp. CF8 carrying distinctly different butane monooxygenases (BMOs) appears to be loss of BMO activity. Both of *Nocardioides* sp.CF8 and *Pseudomonas*

butanovora sustained less than 5% of their initial BMO activity following exposure to 165 µM TCE. In contrast, *Mycobacterium vaccae* maintained 34% BMO activity even after longer TCE exposure. Culturability loss was observed only in *Pseudomonas butanovor.* It could be assumed that at higher initial concentration of TCE, this organism achieved the toxicity threshold proposed by Chu and Alvarez-Cohen (1999). The explanations for the other two bacteria which have no loss in viability is probably that the maximal rates of TCE degradation for both *Nocardioides* sp. CF8 and *M. vaccae* are lower than those of *P. butanovora* Therefore, first, it is possible that the repair of general cellular damage could overcome toxicity by a reactive intermediate such as TCE epoxide. Second, the BMOs of *M. vaccae* and *Nocardioides* sp. CF8 may catalyze the formation of different, non-toxic products during TCE transformation. Third, different ratios of the same products (including the toxic intermediates) may be formed, resulting in less accumulation of the more destructive intermediates.

Yang *et al*. (1999) studied the cometabolic efficiencies of TCE by ammonium monooxygenase under different environmental conditions and suggested that some amounts of NH_3 should be maintained in the system to keep the enzyme active to cometabolize TCE. Similarly, Morono *et al*. (2004) observed the restoration of nearly 100% of initial TCE degradation ability. Therefore, they proposed the loss of TCE transformation activity in a short period as pseudoinactivation. They also suggested that some of the TCE degradation products or TCE itself might remain in the active site of toluene dioxygenase (TDO) during TCE degradation and then inactivate the enzyme. Aromatic substrates such as toluene, benzene, and cumene can displace these inhibitors from the active site of TDO, thereby restoring enzyme activity. Moreover, a larger amount of TCE was degraded after cumene addition than after toluene or benzene addition.

Bacteria with different oxygenases showed different effects on the cells after transformation of TCE (Arp *et al*., 2001). In addition, different bacterial species with the same oxygenase respond to the TCE transformation process differently (Halsey *et al*., 2005).

From our previous work, terpenes were used as alternative enzyme inducers for biodegradation of TCE in both liquid and contaminated soil by *Rhodococcus* sp. P3. Nevertheless, the application of terpenes-induced *Rhodococcus* sp. P3 for cometabolic degradation of TCE has shown limited success, this is probably due to some limitations described above. The presence of terpenes and TCE simultaneously in the system might cause competition between two compounds. Subsequently, inhibition, inactivation and cytotoxicity resulting from TCE degradation occurred. Another explanation could be the disappearance of terpenes, since several terpenes are relatively low soluble in water and high volatilization makes it difficult to control and maintain them during TCE oxidation (Suttinun *et al*. 2004).

2.6 Plant essential oil and its component

(1) Introduction

Essential oils/volatile oils are aromatic oily liquids obtained from plant. They are distributed throughout vegetation types (Amaral *et al*., 1998) and widespread in nature. These oils are accumulated in all plant parts: flowers; leaves; roots; rhizome; wood; bark; fruit and seeds. Essential oils contain several components/compounds known as plant secondary metabolites i.e. compounds produced not necessary for cellular processes. These compounds function in plant as membrane sterols, carotenoids, pigments, biocides, insect

attractants, growth hormones, frost tolerance and signal compounds (Singer *et al*., 2003; Verpoorte and Memelink, 2002). The largest class of plant essential oil components are terpenes which 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 and 6 isoprene units, respectively (Crowley *et al*., 2001). All plant-derived compounds are broken down through natural processes. Most of them are broadly lipophilic, but considerable water solubility was demonstrated for some oxygenated compounds (Langenheim, 1994).

To date, several thousand of these compounds have been identified, of which more than one hundred essential oil components are known. Essential oil and its component hold potential interest applications particularly in food additives, flavor industries, fragrances, as well as in the chemical and pharmaceutical industries. Use of these compounds in environmental applications has received increasing attention due to good bioavailability, compatibility (environmentally friendly) in natural environments, their abundance in nature and economic advantage when compared to synthetic chemicals. Some essential oil components such as cumene have been used in metal cleaning applications to replace chlorinated solvents while minimizing regulated emissions to both air and water (OAIC, 1996).

(2) Motivation for utilization of essential oil in bioremediation

It has long been recognized that some essential oil and its component have antimicrobial properties (Burt, 2004). Several bacteria were capable of utilizing essential oil component as sole carbon source under aerobic condition. Duetz *et al*. (2001) reported that

Rhodococcus opacus PWD4 was able to transform limonene to trans-carveol and carvone and suggested this bacterium might contain toluene 2,3-dioxygenase. Cumene (isopropylbenzene/IPB) degradation in *Pseudomonas* sp. strain JR1 was initiated by a IPB 2,3-dioxygenase which oxidized the compound to 2,3-dihydro-2,3-dihydroxy-IPB and subsequently to 3-isopropylcatecol (3-IPC)(Pflugmacher *et al*., 1996).

Several essential oil components have structures that are analogous to many synthesized aromatic chemicals. 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). The induction of cometabolic degradation in certain microorganisms by plant essential oil and its components is probably because they have structures that are analogous to many substrates commonly used as sole carbon source and/or enzyme inducer. 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-derived compounds 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).

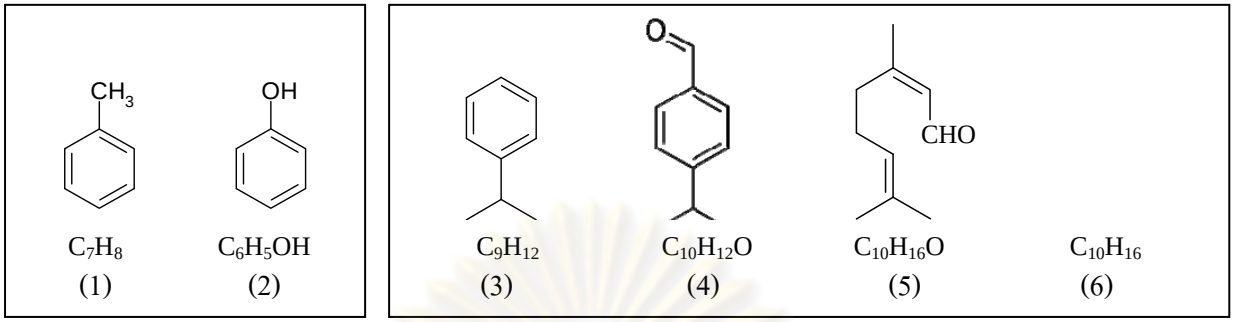


Figure 2.2 Structures and formulas of xenobiotic compounds commonly used as substrate and/or inducer for supporting TCE degradation; (1) Toluene; (2) Phenol, and structures of essential oil components used as inducer in this study and their plant sources; (3) Cumene ; (4) Cumin aldehyde; (5) Citral ; and (6) Limonene.

Gilbert and Crowley (1997) screened several essential oil components, including carvone, cumene, carvacrol, thymol, limonene, cymene and cinnamic acid for their ability to induce polychlorinated biphenyls (PCBs) biodegradation in *Arthrobacter* sp. Carvone, the principal component prepared from spearmint extract, was able to induce the biotransformation of 62% of Aroclor 1242 at concentrations as low as 50 mg L^{-1}. Carvone had higher solubility among other substrates, thus increasing its bioavailability to be utilized 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 *Ralstonia eutrophus* 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 essential oil component, carvone, could induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Alcaligenes eutrophus*.

Tandlish *et al*. (2001) evaluated the effect of two essential oil components, 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.

Essential oil and its component 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 Aroclor 1242 degradation was induced. Five distinctly different isolates obtained from these soils were further studied with respect to growth on

purified essential oil components and metabolism of PCBs. The most effective strains were *Cellulomonas* sp. T109 and *R. rhodochrous* T100, which metabolized 83% and 80% of Aroclor 1242 after growth on cumene 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) amended 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 essential oil component 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 ^{o}C and it was found that 20-30 ^{o}C was the optimum temperature. While Morono *et al*. (2006) demonstrated that cumene showed a negative correlation with TCE cometabolic degradation. Of all the toluene-degrading strains, the bacteria that can grow on cumene showed a comparably broad range of growth substrates but low ability to cometabolize TCE.

.

However, the utilization of plant essential oil and its component for TCE is still limited. In our previous study four essential oil components i.e. cumene, limonene,

carvone and pinene were found to induce TCE cometabolic degradation in resting cells suspension of *Rhodococcus* sp. P3. Among these compounds, cumene was the most effective inducer for TCE induction (Suttinun *et al*., 2004). Based on our findings, other essential oil components and their plant source materials are attractive for induction of TCE degradation in local bacterial isolate. The study would open the prospect of applying plant derived compounds and native bacteria for TCE bioremediation as well as for other chlorinated pollutants.

2.7 Cell immobilization

(1) Introduction

The original motivation of whole cell immobilization originates from research work applied to extracted enzymes. Due to several obvious benefits derived from cell immobilization, the techniques were rapidly extended to several purposes i.e. industrial applications (biosynthesis or bioconversions of variety of compounds in bioreactors), food and beverage processing applications (food quality analysis, brewing and winemaking), and environmental applications (treatment of wastewater, biosensors). Immobilization is a general term that describes many different forms of (1) the self-attachment of microorganisms to the material or (2) the artificial immobilization of microorganisms to the material (Cohen, 2001). Their details are as follows;

(1.1) Self-attachment immobilization

Microorganisms possess various structures used for attachment. These include fimbria (pili), capsules (glycocalyx), stalks, cell wall components and slimes. The principal structure in attachment is the glycocalyx which consists of extracellular

polysaccharides. Several mechanisms are involved in microbial attachment to a surface, e.g. electrostatic interactions, covalent bond formation, hydrophobic interactions. The extent of the attachment generally depends on environmental conditions, microbial species, material surface properties and fluid properties. The advantages of attachment systems are i.e. simplicity and cost effectiveness, low diffusion restriction, population changes within attached biofilms when waste sources changes with time

(1.2) Artificial immobilization

There are several methods for artificial immobilization of microorganisms to support materials as cited below

(1.2.1) Microencapsulation; this method consists of wrapping droplets containing microbes with a thin membrane. Materials used to construct microcapsules are for example, nylon and cellulose nitrate. The advantage of this technology is the low diffusion restriction of the thin membrane. However, the toxicity of the membranes may cause the loss of catalytic activity within the immobilized cells. Furthermore, cell growth and division or the gas produced may damage the encapsulation membrane.

(1.2.2) Membrane separation; basically, this technology aims to separate the microbes from the bulk fluid by the use of membrane sheets. The membrane will allow the substrates to penetrate to microbes, while preventing the microbes from fluid to be treated. This method usually has problems associated with membrane fouling and clogging. The chemicals used in conventional method of membrane cleaning may damage the microbial cells.

(1.2.3) Covalent bonding and covalent crosslinking; this know-how includes the creation of covalent bonds between reactive groups on outer surfaces of microbes and different ligands on materials. The coupling agents (e.g. glutaraldyde,

carbodiimine, isocyanate, and amino silane) are used to activate the ligands between those matrices. For covalent crosslinking technique, this involves the joining of microbes to form a large, three-dimensional complex structure which acts as material. The main problem of these techniques is the toxicity effect with microbes exposed to reactive groups. In addition, viable cells which are covalently bound will lead to cell leakage from the material.

(1.2.4) Entrapment within polymers; this technique consists of trapping microbes within a three-dimensional polymer matrix. The matrix pores will allow the penetration of substrates through the trapped microbes. This method is limited by higher diffusion restriction in some polymer materials. On the other hand, it preserves high viable biomass concentration, higher resistance to toxic compounds, greater plasmid stability.

Cassidy *et al*. (1996) reported that encapsulated cells have advantages over free cells, for instance, increased metabolic activity and metabolite production, protection from toxic substances and increased plasmid stability. In addition, another review paper addressed the advantages of various methods of cell immobilization in several aspects: a) higher reaction rates due to increased cell densities; b) possibilities for regenerating the biocatalytic activity; c) ability to conduct continuous operations at high dilution rate without washout; d) easier control of the fermentation process; e) long term stabilization of cell activity; f) reusability of the biocatalyst; and g) higher specific products yields (Junter and Jouenne, 2004).

(2) Support materials: selection criteria and their significance for cell immobilization

The limitation of immobilized cells for contaminants degradation not only depends on the associated bacteria but also on the lack of sufficient and effective surface area for suitable bacterial colonization. Albrechtsen *et al*. (1996) reported that biomass support materials should ideally meet a range of following criteria: 1) be standardized, and the product should not vary over time or between suppliers; 2) stimulate most of the physio-chemical properties of aquifer materials; 3) have reasonably large surface areas relative to their volume; 4) could be sterilized to avoid the activity of other bacteria; 5) should not sorb the contaminants; 6) have suitable hydraulic properties to avoid experimental clogging problems; 7) be easy to handle in the experiments. Leenen *et al*. (1996) investigated the characteristics of natural (alginate, carrageenan) and synthetic (polyvinyl alcohol, polyethylene glycol, polycarbamoyl sulphonate and polyacrylamide) support materials for application in domestic wastewater treatment systems. The solubility, biodegradability, stability, diffusivity and growth in the supports were investigated. They suggested that natural gels might be suitable material for some application since cells can grow in the support, the diffusion coefficients are high and the procedures are easy to scale-up. However, they are soluble, biodegradable and likely weak and therefore not suitable for treatment of wastewater. Synthetic supports, in contrast, do not dissolve, are non biodegradable and have good mechanical properties. Although the diffusivity is lower and immobilization procedures are more complicated, the synthetic gels are more promising materials for application in wastewater treatment.

(3) Potential of cell immobilization for environmental applications

There was a report which showed that viable *E. coli* cells entrapped in hydrogel micropatches photopolymerized within microfluidic systems can act as sensor for small molecules and as bioreactor for carrying out reactions (Heo *et al*., 2003). Furthermore, immobilized cells could degrade the contaminants at a higher initial concentration and for a longer period. These cells were also protected from harmful effects of toxic wastes. Immobilized cells could be stored for long periods without losing their degrading abilities (Quek *et al*., 2006).

Many different types of matrices have been applied to immobilizing cells for microbial protection and to improve the degradation of hazardous compounds. Immobilization by using biomass entrapment with various hydrogels is promising for the creation of active immobilized biocatalysts. Either natural biopolymers (polysaccharides such as alginate, carrageenan, agar etc. or proteins such as gelatin, collagen) or synthetic polymers (polyacrylates, polyurethanes, polyethers) can be used as the gel-forming agents. Among them, the cryogels of polyvinyl alcohol (PVA) have received increasing attention as carriers for cell immobilization mainly due to the very high operational stability of the gel basis (Lozinsky and Plieva, 1998).

Chen *et al*. (1998) investigated the operation features of municipal wastewater treatment processes using PVA-immobilized cell reactor. This system simultaneously performs the nitrification and denitrification. High removal efficiency, low excess sludge production, energy conservation, and small space were advantages for facilities. Cunningham *et al*. (2004) examined the potential of immobilized hydrocarbon-degrading microorganisms for bioremediation of diesel-contaminated soil. Polyvinyl alcohol (PVA)

was chosen due to its open sponge-like appearance, compared to other support materials their effective diffusivity is high. However, there remains a problem of the diffusivity of diesel because of its limited water solubility. In this study, therefore, the cells were pre-grown with aged diesel before immobilization. It was believed that this procedure would induce cells biosurfactant production that might enhance mass transfer. They found that after 32 days, the removal of diesel was the most successful, with greatest removal in a co-immobilization system containing PVA-entrapped microorganisms and a synthetic oil absorbent. Least success was obtained with a commercial liquid bioaugmentation agent containing surfactants and having a low pH, which also produced significant toxicity to cells. Moreover, the microbial number for the immobilized system were higher than that of control, suggesting that a degree of inoculation had occurred, or the bulking effect of the large pieces of PVA had improved the oxygen transfer of the soil. Chang *et al*. (2005) studied the application of PVA as alternative approach for wastewater treatment. The encapsulation of bacteria in these polymers has been reported providing high cell concentrations, thus presenting a high rate of nitrification. In addition, they investigated the effect of PVA characteristics on the solubility of the PVA gels because PVA is easily dissolved in water. As the saponification ratio (SR) and the molecular weight (MW) of PVA increased the solubility decreased. This results could be explained by inter-and intra-molecular hydrogen bonding between PVA molecules.

The encapsulation technique in different matrices has also been tested for immobilization of cells. Dias *et al*. (2000) studied acetonitrile degradation by free cells and cells immobilized in alginate, k-carrageenan and citric pectin. They found that alginate-immobilized cells degraded acetonitrile more efficiently than k-carrageenan or citric pectin-immobilized cells, a fact that can explain this is that the difference in porous

structures of the matrices probably permits a better growth of the cells in the alginate. Consequently, higher cell activity and performance to degrade this compound are achieved.

Recently, sol-gel technique has gradually gained attention for immobilization of many biomolecules including enzymes and whole cells. The process of this approach involves no high temperature and harsh chemical reaction and it has the ability to immobilize biomolecules without modifying their structures and functions. Chen *et al*. (2004) investigated the ability of *Methylomonas* sp. Strain GYJ3 immobilized by the sodium silicate silica-based sol-gel technique on propylene epoxidation. In a batch reaction system, they found that the immobilized cells can be used repeatedly for more than 25 times with no significant loss of the activity. Storing at 4^0C for 45 days maintained the activity of immobilized cells. It was proposed that this technique is very simple, efficient and cost-effective.

Pedrazzani *et al*. (2005) proposed a novel approach in which biomass was enclosed between polyester membranes coated by silica films for treatment of contaminated water. The results showed that no metabolic inhibition mechanisms of microorganism were observed and a sufficient degree of porosity allowed mass transfer through the membranes.

Although the practical application of the entrapped cells in poly gels has been widely studied, the limitation of gel stability and mass transfer has remained. Furthermore, immobilized cell preparation is relatively difficult and requires complex equipment which increases the cost of production. Attachment technique could be an

alternative way for application of cell immobilization. This can be obtained by spontaneous biomass adhesion onto porous support media such as polyurethane foam and other inorganic matrices. Quek *et al*. (2006) tried to identify hydrocarbon degraders that could be immobilized onto polyurethane foam (PUF) and maintain the ability to efficiently degrade alkanes in several petroleum products. Among four different microorganisms, *Rhodococcus* sp. F92 was efficiently immobilized onto PUF and the immobilized cells were able to degrade approximately 90% of the total n-alkanes in the petroleum products tested within 1 week at 30 ^{0}C. This organism also showed highest number of viable cells (10^{9} cells per ml PUF). They described that the efficient immobilization of F92 could also be due to the extracellular structures seen under SEM identified as exopolysaccharide fibers by Obuekwe and Al-Muttawa (2001). These fibers were responsible for forming a stable monolayer of cells and some cells were encapsulated in these fibers creating the attachment onto PUF surfaces. Rhodococci show low growth but great persistence in the environment and have been isolated from a wide variety of locations. With no catabolite repression, Rhodococci could degrade hydrocarbon even in the presence of more easily assimilable carbon sources thus, they are known to be versatile in their substrate specificity. Some of them are also indigenous in contaminated sites and are able to produce biosurfactant or emulsifiers with beneficial applications (Bell *et al*., 1998). A role of biosurfactant for mediating the removal of residual nonaqueous-phase liquids (NAPLs) may be to redistribute the entrapped contaminants following biodegradation in order to increase the bioavailability of the remaining contaminants (Herman *et al*. 1997). Owing to their ability mentioned above, Rhodocooci are increasingly holding attention in the field of bioremediation and biotechnology.

As the success emerged from this approach, various materials/matrices have been extensively studied to achieve a more feasible method for the real application. Natural materials could be one of attractive choices to avoid some of the limitations which occurred. Using natural materials as support carrier the ability of microorganisms to attach to surface, the use of irrelevant chemical substances in immobilization procedures has been minimized. Therefore, potentially low-technology and cheaper options would be attained. Obuekwe and Muttawa (2001) reported that two bacteria isolated from Kuwait oil lakes, *Arthrobacter* sp. and gram-negative bacillus immobilized with sawdust, styroform, or wheat bran exhibited retainable ability to degrade hydrocarbons after storage for 6 weeks at 45 ^{0}C. Viability was retained by immobilized *Arthrobacter* sp. and gram-negative bacteria at 45 ^{0}C for up to 6-12 months, respectively. Additionally, natural materials, loofa sponge, activated carbon and coconut fiber have been used as support material for cell immobilization. It was found that the microbial consortium immobilized on loofa sponge exhibited a high ability to simultaneously degrade and detoxify both carbendazim and 2,4-dichlorophenoxyacetic acid. Loofa sponge is renewable and biodegradable, the immobilization procedure is simple and it is affordable to use in developing countries (Pattanasupong *et al*., 2004). Regarding the advantages of loofa for immobilization, Ogbonna *et al*. (1994) also described that loofa is largely produced in most African and Asian countries, they are light, cylindrical in shape and made up of an interconnecting void within an open network of matrix support materials. With very high porosity, their potentiality as support material for cell immobilization is very high.

(4) Application of cell immobilization for TCE biodegradation and bioremediation

Uchiyama *et al*. (1994, 1995) entrapped the methanotrophs, *Methylocystis* sp., in agarose, alginate, k-carrageenan, polyurethane, photo-crosslinkable resin and polyelectrolyte complex for a bioreactor operation of TCE degradation, instead of using conventional method e.g. fixed-film or packed-bed reactor. They suggested that entrapped microbes would avoid the washed-out microbes from biofilms that might contaminate treated ground water. Cell immobilized in Ca-alginate, k-carrageenan, and agarose showed higher or almost the same degradation activity in comparison with free cells, while low activity was observed in the cells immobilized in photo-crosslinked resin, polyurethane, and polyelectrolyte complex. In repeated use, only the agarose-immobilized cells were not damaged and retained about 40% of the initial TCE degradation activity. Using agarose-immobilized cells, the maximum degradation rate (V_{max}) of TCE was 3.1 μg of TCE $mg^{-1}$of dry cells h^{-1}, while the optimum pH was 7.0 and the optimum temperature was 35 ^{0}C. Studies on the kinetics and the influence of cell density suggest that oxygen permeation was a rate-limiting step. Although a toxic effect caused by anaerobic degradation of TCE was observed, restoring of TCE degradation activity could be achieved by methane addition, suggesting that a two-step reactor system might be advantageous.

Shimomura *et al.* (1997) observed biodegradation of TCE by *Methylocystis* sp. strain M immobilized in gel beads in a fluidized-bed bioreactor which was supplied with a methane/air gas mixture. Approximately 80-90% of the influent TCE was degraded in the reactor. The degradation ability of the reactor was kept in a steady state for 10 days in this way, but the rate of recovery of TCE degradation activity gradually decreased. Within 24

h after supplying a methane/air mixture (2:8 v/v), the TCE degradability was restored. There was no corresponding decrease in methane consumption by the bacteria.

Radway *et al*. (1998) reported that foam embedded *Burkholderia cepacia* G4 removed up to 80% and 60% of 3mg L^{-1} TCE and 2 mg L^{-1} of benzene, respectively. Removal of TCE and benzene decreased more than 50% when easily metabolizable carbon sources were present, thus it appears that such easily utilizable carbon sources acted as competing substrate, effectively inhibiting TCE cometabolism as well as benzene utilization. In addition, TCE degradability was observed with G4 cells induced with phenol or benzene prior or after immobilization of cells. However, it was found that some of TCE and benzene were bound to the foam materials, and the question remained whether compounds absorbed in this manner are still available for bacterial degradation.

Chen *et al*. (2007) studied chitosan-bead immobilized *Pseudomonas putida* for TCE degradation in the presence of phenol as substrate. They found that the rate of TCE degradation in immobilization system was about 1/2 compared to that of free cells but the immobilized cells could tolerate higher TCE concentrations.

CHAPTER III

TRICHLOROETHYLENE COMETABOLIC DEGRADATION BY RHODOCOCCUS SP. L4 INDUCED WITH PLANT ESSENTIAL OILS

3.1 Introduction

Contamination of soil and groundwater with chlorinated solvents, especially trichloroethylene (TCE) has become an important problem because of its toxicity and persistence. Under aerobic conditions, TCE biodegradation mainly occurs through co metabolic degradation, in which TCE-degrading bacteria utilize methane, ammonia, propane, phenol, toluene or cumene as growth substrate (Alvarez-Cohen and Speitel, 2001). Consequently, bioremediation trials have been conducted by injecting methane (Moran and Hickey, 1997; Ohlen et al., 2005), phenol (Lee 2003; Chen et al., 2004), and toluene (Hopkins and McCarty, 1995; Tom Kuo et al., 2004) to promote the growth of TCE-degrading bacteria in contaminated soil and groundwater. Toluene-degrading bacteria are the largest group of aerobic TCE degraders and widely distributed in the environments (Morono et al., 2006). However, the application of toluene may pose potential regulatory problems, thus alternative compounds for the induction of toluene/TCE-degrading enzymes are required.

Singer et al. (2003) suggested that the introduction of plant secondary metabolites would be acceptable for enhancing bioremediation of subsurface soil and aquifers since these compounds are effective at low concentrations and are considered non-toxic. The most studied metabolites are components of plant essential oils, which have been reported to induce polychlorinated biphenyls (PCBs) degradation by several bacteria. For example,

carvone and limonene, the principal components of spearmint and lemon oil are able to induce PCB biotransformation in Arthrobacter sp. B1B, Alcaligenes eutrophus, and Pseudomonas stutzeri (Gilbert and Crowley, 1997; Koh et al., 2000; Tandlish et al., 2001). In addition, the application of plant materials rich in essential oil such as orange peels, ivy leaves, pine needles or eucalyptus leaves to contaminated soil was reported to stimulate the growth of biphenyl utilizers and simultaneously induce Aroclor 1242 and 1248 degradation (Hernandez et al., 1997; Dzantor and Woolston, 2001). The induction ability of these essential oil components is probably due to the similarity between their chemical structures and the pollutants (Crowley et al., 2001; Singer et al., 2003).

In this study, plant essential oils and their components were tested for the ability to induce TCE degradation in Rhodococcus sp. L4, a toluene-degrading bacterium isolated in Thailand. The utilization of plant essential oils as a substrate and/or inducer for TCE biodegradation has not been studied before. However, four essential oil components including cumene, limonene, carvone and pinene were previously tested for induction of TCE degradation in Rhodococcus gordoniae P3 (Suttinun et al., 2004). Cumene was more effective than other essential oil components. Similarly, Rhodococcus erythropolis BD2 utilized cumene as a substrate for TCE cometabolism (Dabrock et al., 1992; 1994). However, this compound can not be applied to the environment due to its potential toxicity (http://toxnet.nlm.nih.gov/).

To find alternative plant-derived inducers, we tested another strain of Rhodococcus. Bacteria of this genus are interesting since they can degrade a wide variety of recalcitrant compounds and have therefore gained considerable interest for use in bioremediation of contaminated sites (Larkin et al., 2005). The strain L4 was able to

cometabolize and dechlorinate TCE after growth on toluene. To replace toluene, Rhodococcus sp. L4 were grown in mineral media containing a selected plant essential oil with/without glucose before conducting the TCE degradation in a resting cells assay. The ability of plant essential oils to induce TCE degradation was further evaluated with their purified oil components. In addition, the repeated addition of oil components was performed for enhancing TCE degradation. The results confirmed that the components of plant essential oils were responsible for promoting TCE degradation. This study opens the prospect of applying plant essential oils, which are relatively safe and inexpensive, to stimulate TCE-degrading bacteria for TCE bioremediation.

3.2 Methodology overview

3.2.1 Inoculum preparation for TCE biodegradation by Rhodococcus sp. L4

(1) Carbon source: toluene or plant essential oil

(2) Inducer: plant essential oil or its component

3.2.2 Protein synthesis inhibition test

3.2.3 TCE biodegradation test

(1) Resting cells experiment by induced bacteria from 3.2.1

(2) Effect of TCE concentrations on its biodegradation

(3) Repeated addition of essential oil components for enhancing TCE degradation

3.3 Materials and methods

3.3.1 Chemicals

Plant essential oils including lemon oil, lemongrass oil, spearmint oil and pine oil were purchased from an aroma shop (Aromatherapy Hydration, Bangkok). Seeds of cumin (Cuminum cyminum L.) were obtained in one batch from its distributor (Nguan Soon, Bangkok). There was approximately 1.4-2.8% essential oil in cumin seeds (Beis et al., 2000; Jalali-Heravi et al., 2007). These essential oils contain several oil components that have previously been shown to be effective in stimulating xenobiotic degradation (Crowley et al., 2001). Toluene (99.5%) was purchased from Merck. TCE (99.5%) and eight essential oils components (cumene (99.0%), R-(+)-limonene (96.0%), cumin aldehyde (4-isopropyl benzaldehyde) (90.0%), (cis+trans) citral (95.0%), (+)-α-pinene (97.0%), (+)-carvone (99.0%), γ-terpinene (95.0%) and p-cymene (99.0%)) were obtained commercially from Fluka Chemical Industrial. TCE stock solutions were prepared by dissolving aliquots of TCE in N,N-dimethylformamide (Merck, USA) to obtain the desired concentration. All other chemicals were obtained from Fluka, Switzerland.

3.3.2 Bacterial strain, culture maintenance and growth conditions

Rhodococcus sp. L4 is deposited at the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR) and given the accession number TISTR 1542. The partial 16S rRNA gene sequence of Rhodococcus sp. L4 is available in GenBank under the accession number EF527237. The bacterium used in this study was maintained by culturing on mineral salts (MS) agar incubated in a glass box equilibrated with toluene (its enrichment substrate) vapor at room temperature.

Approximately every 10-14 days the culture was transferred to a new MS-agar for maintaining the ability of enzyme production by bacteria throughout the course of study.

MS medium is a chloride-free minimal medium prepared according to Focht (1994) with details in Appendix A. To allow the bacteria to acclimatize to the liquid media prior to use in the experiment, the pre-inoculum was prepared by culturing a loopful of Rhodococcus sp. L4 (on MS-agar) in a 250-mL Erlenmeyer flask containing 100 mL MS medium that was supplied with growth substrate in the vapor phase by adding 200 µL toluene (12.5 mmol L-1gas phase) in the Eppendorf tube fixed at the top of the flask (Fig. 3.1). The culture was incubated overnight at room temperature with shaking at 200 rpm.

3.3.3 Inoculum preparation for TCE biodegradation test when toluene or plant essential oil was used as sole carbon source for Rhodococcus sp. L4

Rhodococcus sp. L4 was cultured by transferring 10 mL late-log phase of pre-inoculum into a 250-mL Erlenmeyer flask containing 100 mL of MS medium. The optical density (OD) at 600 nm was about 0.06-0.08 at the beginning. The growth substrate was supplied as a vapour by adding 200 µL of toluene (12.5 mmol L-1 gas phase) or plant essential oil to a sterilized Eppendorf tube fixed at the top of the flask (Fig. 3.1). The vapor phase of cumin oil was achieved by placing 15 g of cumin seeds on a sterilized cheesecloth hung at the top of the flask (Fig 3.2). The cultures were incubated on an orbital shaker at 200 rpm, room temperature, for 24 h. Then, the cells were harvested by centrifugation at 2516 x g (7,500 rpm) for 10 min. Cells were washed twice and resuspended in MS medium to a OD600 of 2.0 before they were used as inoculum in the TCE biodegradation test as described below.

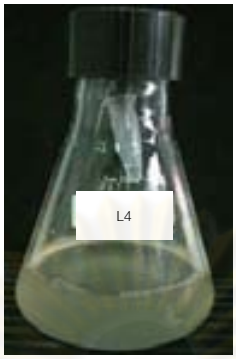


Figure 3.1 Erlenmeyer flask with toluene added into Eppendorf tube fixed at the top of the flask used for bacterial culture.

3.3.4 Inoculum preparation for TCE biodegradation test when plant essential oil or its component was used as inducer for Rhodococcus sp. L4

The tested compound was applied at a low concentration when used as inducer for Rhodococcus sp. L4. Ten mL pre-inoculum was transferred to 100 mL MS medium containing 20 mM glucose as carbon and energy sources. The optical density (OD) at 600 nm was about 0.06-0.08 at the beginning. Stock solutions of each essential oil component i.e. cumene, limonene, cumin aldehyde and citral prepared in N,N-dimethylformamide were added to the culture to give a final nominal concentration of 80-400 µM (the concentrations of plant essential oil components were calculated as if being completely dissolved in the aqueous phase). These concentrations showed no toxic effects on the bacteria and did not exceed their solubility. The optimum concentration of each essential oil component, 400 µM citral, 80 µM cumin aldehyde or 200 µM limonene/cumene was applied as inducer for Rhodococcus sp. L4 for TCE degradation and dechlorination assay. For cumin oil, 10 g of cumin seeds was put on sterilized cheesecloth and hanged at the top of the flask (Fig 3.2). The culture was incubated overnight and harvested by

centrifugation at 2516 x g (7,500 rpm) for 10 min. Cells were washed twice with MS medium and resuspended in 10 mL MS medium containing either essential oil components or cumin seeds as described above. The repeated addition of these compounds was used to ensure adequate enzyme induction. After 1 h, the induced-cells were harvested, washed, and resuspended in MS medium to a final OD600 of 2.0 before they were used as inoculum in the TCE biodegradation test as described below.

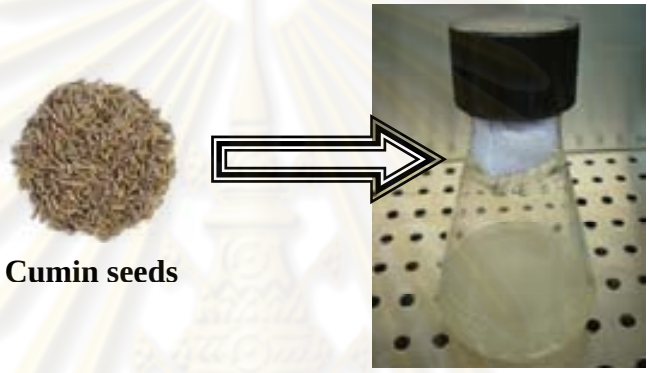


Figure 3.2 Erlenmeyer flask with 10 g of cumin seeds was put on sterilized cheesecloth and hung at the top of the flask. Essential oil of cumin seeds was supplied as carbon source and/or inducer for Rhodococcus sp. L4

3.3.5 Protein synthesis inhibition test

Chloramphenicol, a protein synthesis inhibitor was used to confirm that the enzymes involved in TCE biodegradation in Rhodococcus sp. L4 were not constitutively produced. At first, non-induced inoculum was prepared by growing cells on glucose-MS medium. The cells were harvested, washed, and resuspended in MS medium to a final density of 2.0 OD600. Then, chloramphenicol was added to the inoculum to a final concentration of 310 µM (100 µg mL-1). The suspension was shaken for 1 h before the

assay was performed. Another set of samples containing glucose-grown cells in the absence of chloramphenicol was used as control. Then, 1 mL of the inoculum was mixed with 1 mL MS media containing 0.4 µmol toluene and 0.16 µmol TCE. The initial aqueous concentrations of TCE and toluene were 14 and 45.5 µM, respectively. The aqueous concentrations of TCE and toluene in liquid-gas systems at 30 0C were calculated with a dimensionless Henry's constant of 0.49 (Gossett, 1987) and 0.34 (Yeager et al., 1999), respectively. During 25 h incubation, the amount of toluene and TCE was measured at intervals.

3.3.6 TCE biodegradation assay

(1) Resting cells experiment

TCE biodegradation experiments were carried out in liquid culture by a method adapted from Nelson et al. (1987) and Luu et al. (1995). Generally, the experiments contained resting cell suspensions and TCE in a 22-mL headspace vial sealed with a Teflon-lined silicone septum and aluminum crimp cap. A 10 µL TCE stock solution (0.16 µmol TCE) was introduced to the vials containing 1 mL MS medium before shaking at 200 rpm at room temperature for at least 2 h to achieve the equilibrium between gas and aqueous phase. Biodegradation experiment was initiated by adding 1 mL inoculums (prepared from 3.3.3 or 3.3.4) with a sterile microsyringe into the vials (final aqueous volume = 2 mL). The initial aqueous concentration of TCE was equal to 14 µM at the beginning of study. The aqueous concentrations of TCE in liquid-gas systems at 30 0C were calculated with a dimensionless Henry's constant of 0.49 (Gossett, 1987). There were re-equilibrations between gas and liquid phases during TCE degradation. The total TCE mass that remained in the system was calculated from the summation of TCE in

both aqueous and gaseous phases. The vials were incubated at 200 rpm at room temperature. Oxygen was provided by leaving 90% of air headspace in the tested vial only at the beginning of study to minimize the loss of TCE via volatilization. The amount of oxygen at the end of study detected by GC-TCD was approximately 14-29%, which implies sufficient oxygen was available during TCE biodegradation process in our study (Appendix B). Two sets of triplicate samples were made at each time point for measuring TCE and chloride ion concentrations separately. The reactions were stopped by adding one drop of 10 M H2SO4 to the vials before analysis. The control sets consisted of heat-killed cells and non-induced cells containing TCE, which represented abiotic loss and degradation by non-induced bacteria, respectively.

The final density of bacterial cells was 1.0 OD600 (0.4 mg dry cells mL-1). Cells dry weight was determined by measuring the mass difference between preweighed dry crucible with 10 mL of mineral salts (MS) medium added and those with 10 mL of bacterial culture added after both sets were incubated overnight at 103-1050C.

(2) Effect of TCE concentrations on its biodegradation

To observe the effect of increased TCE concentrations on its degradation, the resting cells assay described in (1) were tested with different concentrations of TCE i.e. 0.02, 0.08, 0.16, 0.3, 0.6 or 0.8 µmol TCE (2 to 68 µM initial aqueous concentration). TCE degradation was monitored at the beginning and at the end of incubation period.

(3) Repeated addition of essential oil components for enhancing TCE degradation

In resting cells experiment, the efficiency of TCE degradation was only around 35-55%, which was probably due to the loss of bacterial activity during TCE degradation. The repeated addition of each essential oil component might help the bacteria to produce

new enzymes or reactivate enzymes. Therefore, this experiment was performed according to (1) and each essential oil component was further added at 80-400 µM every hour.

3.3.7 Quantitative analysis of toluene, TCE, essential oil components and chloride ions

The amount of toluene and TCE was analyzed by the PerkinElmer TurboMatrix Automated Headspace Sampler with the Clarus 500 Gas Chromatography equipped with a flame ionization detector (Headspace GC-FID) and a HP-5 (5% Phenyl Methyl Siloxane) fused-silica capillary column (30 m x 0.32 mm ID; thickness, 0.25 µm). Sample vials were heated to 930C for 30 minutes for equilibration. The head-space pressure was 20 psi. The gas chromatography conditions were as follows: injector temperature 1500C, detector temperature 2500C, initial column temperature 400C (1.80 min) then, programmed at 400C to 550C at a rate of 450C min-1, and 550C to 1350C at a rate of 100C min-1. The carrier gas (nitrogen) pressure was 14 psi with a flow rate of 20 mL min-1. The retention time of TCE and toluene were 1.78 min and 2.05 min, respectively. Essential oil components i.e. cumene, limonene, cumin aldehyde and (cis+tran) citral showed retention time of 4.17, 5.69, 8.90 and 8.93+9.37 min, respectively. External standard quantitative calibrations were performed for the analysis of toluene, TCE, and essential oil components concentrations.

The concentrations of chloride ions generated from TCE dechlorination were monitored by an ion-sensitive chloride combination electrode (model 94-17B, Thermo Electron Formerly Orion Research, Inc., USA). A 1,000 ppm sodium chloride solution was used for calibration. Ionic strength adjustor, NaNO3 was added at 2% (v/v) before measuring the calibration standards and samples in a stirred beaker.

3.4 Results and discussion

3.4.1 TCE cometabolic degradation activity of toluene-grown Rhodococcus sp. L4

The resting cells of toluene-grown Rhodococcus sp. L4 were able to cometabolize TCE effectively. About 50 ± 5% of the initial TCE remained after 4-h incubation (Fig. 3.3a-b). The TCE was dechlorinated as seen from the gradual increase of chloride ions in this experiment. On the other hand, only 1-8% TCE was lost and nearly no chloride ions were generated by killed-cells and glucose-grown cells (Fig. 3.3a-b). Thus, TCE removal by abiotic processes and non-induced bacteria were considered to be insignificant in this study.

After 8-h incubation, about 0.09 µmol TCE was degraded by toluene-grown cells, while there were 0.24 µmol chloride ions produced. The molar ratio of TCE degraded to chloride ions was nearly 1:3, suggesting that the degraded TCE molecules were completely dechlorinated by toluene-grown Rhodococcus sp. L4.

The TCE transformation capacity (Tc) and the maximum specific rate (kc) of TCE cometabolic degradation (Alvarez-Cohen and McCarty, 1991) were 15.1 ± 1.04 µg TCE mg cells^{-1} and 0.14 ± 0.001 mg TCE mg cells^{-1} day^{-1} for toluene-grown Rhodococcus sp. L4 cells (Table 3.2). With these results our toluene-grown cells exhibit similar activities compared to other known TCE-degrading bacteria. There were a number of reports, which showed that the Tc and kc values by toluene-degrading bacteria were 5.2-8.5 µg TCE mg cells-1 and 0.17- 1.3 mg TCE mg cells-1 day-1, respectively (Heald and Jenkins,

1994; Landa et al., 1994; Chang and Alvarez-Cohen, 1996; Arcanlegi and Arvin, 1997; Kelly et al., 2000).

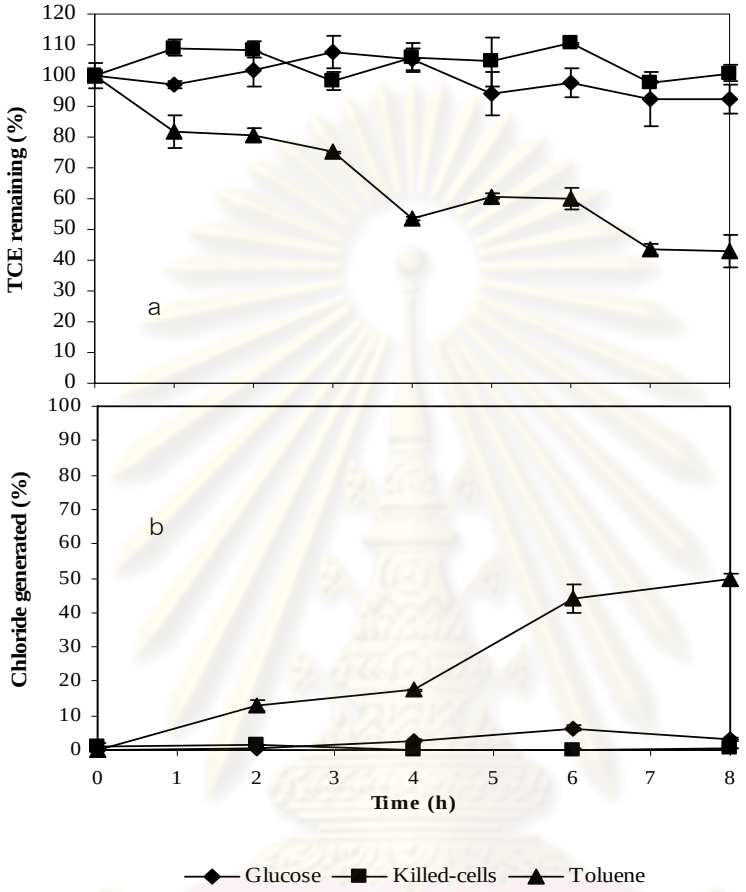


Figure 3.3 Time course of TCE degradation (a) and chloride formation (b) by killed-cells, glucose- and toluene-grown cells. All data are the average of triplicate samples with the standard deviations (error bars) given.

To confirm that Rhodococcus sp. L4 required enzyme induction before TCE degradation, chloramphenicol was added to the glucose-grown cells to inhibit protein synthesis. In the presence of chloramphenicol the cells could not degrade TCE or toluene (Fig. 3.4). In experiments without chloramphenicol the TCE decreased only after toluene was degraded. These results indicate that the enzymes involved in TCE biodegradation

were not constitutively produced. Moreover, the presence of toluene was necessary for the induction of enzymes involved in TCE degradation of Rhodococcus sp. L4.

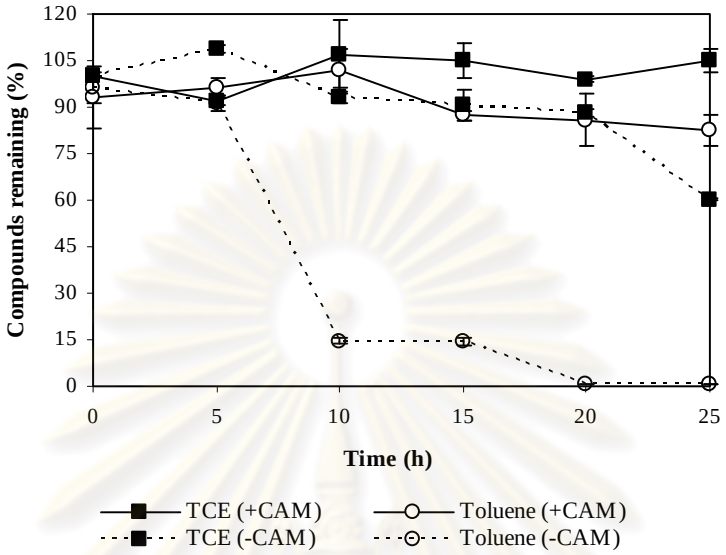


Figure 3.4 TCE and toluene degradation by glucose-grown cells with and without chloramphenicol (+/-CAM). All data are the average of triplicate samples with the standard deviations (error bars) given.

3.4.2 Effects of plant essential oils on growth of Rhodococcus sp. L4 and TCE cometabolic degradation

The toxicity of toluene prevents its use in contaminated sites. Therefore, we examined the efficiency of plant essential oils as its alternative. Five essential oils including lemon, lemon grass, cumin, pine, and spearmint oils were selected since they contain some oil components that have been reported as inducers for xenobiotic-degrading enzymes in bacteria (Table 3.1). Rhodococcus sp. L4 was able to utilize lemon oil as sole carbon source but with a slightly slower growth than with glucose or toluene (Table 3.1). The growth level of bacteria grown on lemongrass oil or cumin oil was about

60% and 20% compared to growth on glucose or toluene. No growth was detected when spearmint oil and pine oil were used as carbon source. The antibacterial activities of various plant essential oils have long been recognized in particular for the application as food preservatives (Burt, 2004). High concentrations of spearmint oil were toxic to Arthrobacter sp. B1B, a PCB-degrading bacterium and resulted in cell lysis (Gilbert and Crowley, 1997).

Lemon and lemongrass oil-grown cells were able to degrade 20 ± 6% and 27 ± 8% of 0.16 µmol TCE (14 µM initial aqueous concentration) after 8-h incubation, respectively (Table 3.1). The extent of TCE degradation was higher than with glucose-grown cells. The results suggested that some components in lemon and lemongrass oils induced TCE-degrading enzymes in Rhodococcus sp. L4. Nevertheless, toluene was the most effective substrate for TCE cometabolic degradation by this bacterium. This is probably due to the fact, that lemon and lemongrass oil contained many oil components and some of them might inhibit the production of TCE-degrading enzymes or might be toxic to the cells. To prevent the toxic effects of mixed oil components, four purified oil components, limonene, citral, cumin aldehyde, and cumene were selected for further study and their concentrations as inducer for Rhodococcus sp. L4 were optimized.

Table 3.1 Growth and TCE degradation of *Rhodococcus* sp. L4 after utilizing different compounds as sole carbon source

Substrate	Essential oil components (%)[a]	Growth[b]	% TCE degradation[c]
Plant essential oils			
Lemon oil	Limonene (31.67) and citral (2.35)	++++	20.4 ± 6.3
Lemongrass oil	Limonene (36.72) and citral (36.17)	+++	26.6 ± 7.8
Cumin oil	Terpinene (24.07), pinene (21.85), cymene (19.56), cumin aldehyde (16.10) and cumene (1.31)	+	ND
Pine oil	Pinene (64.11) and limonene (10.51)	-	ND
Spearmint oil	Carvone (55.10)	-	ND
Control			
Glucose	None	+++++	7.8 ± 4.7
Toluene	None	+++++	57.1 ± 5.2

[a] The concentrations of essential oil components were determined by GC analysis with the standard compounds as references. Only the major oil components or the one that has been reported as inducer for xenobiotic-degrading bacteria are shown.

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

[c] TCE degradation was determined after 8-h incubation by comparing with the initial TCE concentration. Values given are average of three samples ± standard deviation.

ND = Not determined; In these cases, there were not enough amount of bacteria to perform TCE biodegradation assay.

3.4.3 Efficiency of plant essential oil components as inducers for TCE cometabolic degradation

Limonene, citral, and cumin aldehyde were selected for further experiments because they are the major components of lemon, lemongrass, and cumin oils. In addition, our preliminary study showed that these oil components could induce TCE degradation in Rhodococcus sp. L4, while pinene did not have similar activity. Terpinene and cymene had much lower activity than that of cumin aldehyde. Although, cumene is a trace component in cumin oil, it was included here because several cumene enriched bacteria can degrade TCE (Dabrock et al., 1992; 1994; Pflugmacher et al., 1996; Morono et al., 2004; 2006). TCE degradation assay was carried out by using resting cells of Rhodococcus sp. L4, that had been grown in glucose-MS media containing the selected oil components. All of the induced cells were able to degrade TCE. Citral- limonene- and cumene-induced bacteria showed a similar trend in TCE degradation. There was a slight difference in TCE degradation when the oil concentrations were increased from 80 µM to 400 µM (Fig. 3.5a, b, d). Cumin aldehyde-induced cells showed lower TCE degradation when the oil concentrations were higher than 80 µM (Fig. 3.5c). The high concentration of cumin aldehyde was probably toxic to the cells. This may be the reason why Rhodococcus sp. L4 did not grow well on cumin oil (Table 3.1).

In the following experiment, either 400 µM citral, 80 µM cumin aldehyde or 200 µM limonene/cumene was applied as inducer for Rhodococcus sp. L4 before conducting TCE biodegradation assay. These concentrations were selected since they had the highest efficiency on inducing TCE cometabolic degradation (Fig. 3.5a-d). A significant reduction of TCE along with the production of chloride ion was observed after incubating the resting cells of induced Rhodococcus sp. L4 with TCE (Fig. 3.6a-b). TCE degradation

and dechlorination were different when the cells were induced with different oil components. Cumene-induced cells showed the highest TCE degradation. About 53 ± 3% of 0.16 µmol TCE (14 µM initial aqueous concentration) was degraded after 8-h incubation (Fig. 3.6a-b). The efficiency of cumene-induced cells was comparable to toluene-grown cells (Table 3.1). Limonene-, citral- and cumin aldehyde-induced cells degraded 36 ± 4%, 40 ± 9% and 49 ± 7% of TCE, respectively.

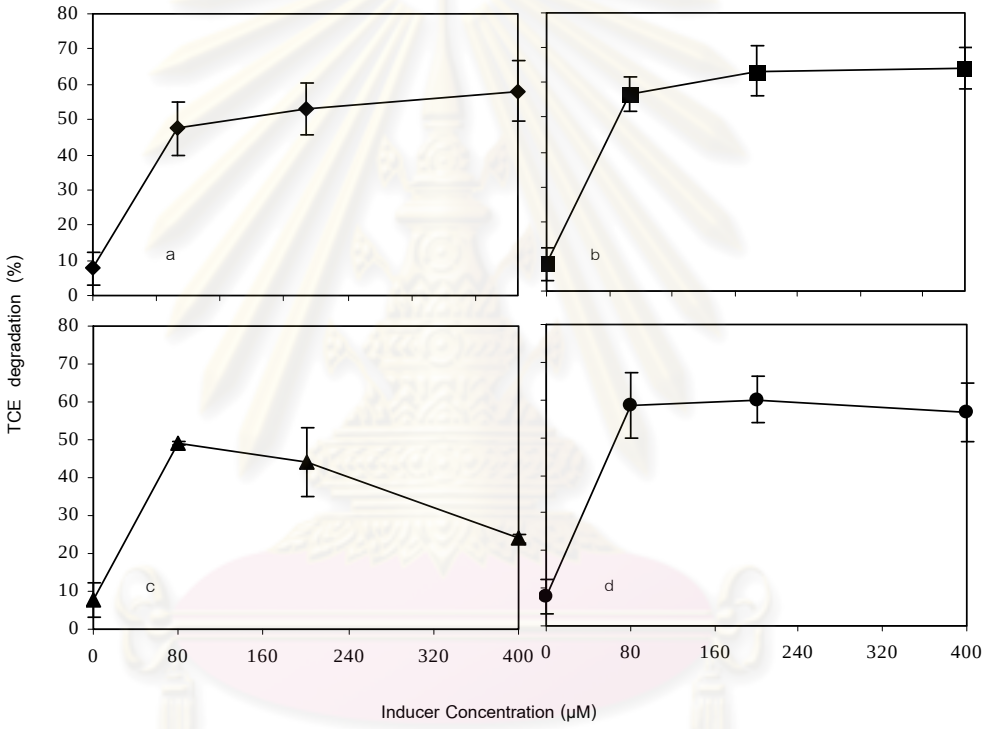


Figure 3.5 TCE degradation after 24-h incubation using cell suspensions of *Rhodococcus* sp. L4 after induction with citral (a), limonene (b), cumin aldehyde (c) and cumene (d) at various concentrations. TCE degradation was calculated by comparison with the efficiency of glucose-grown cells in the same period.

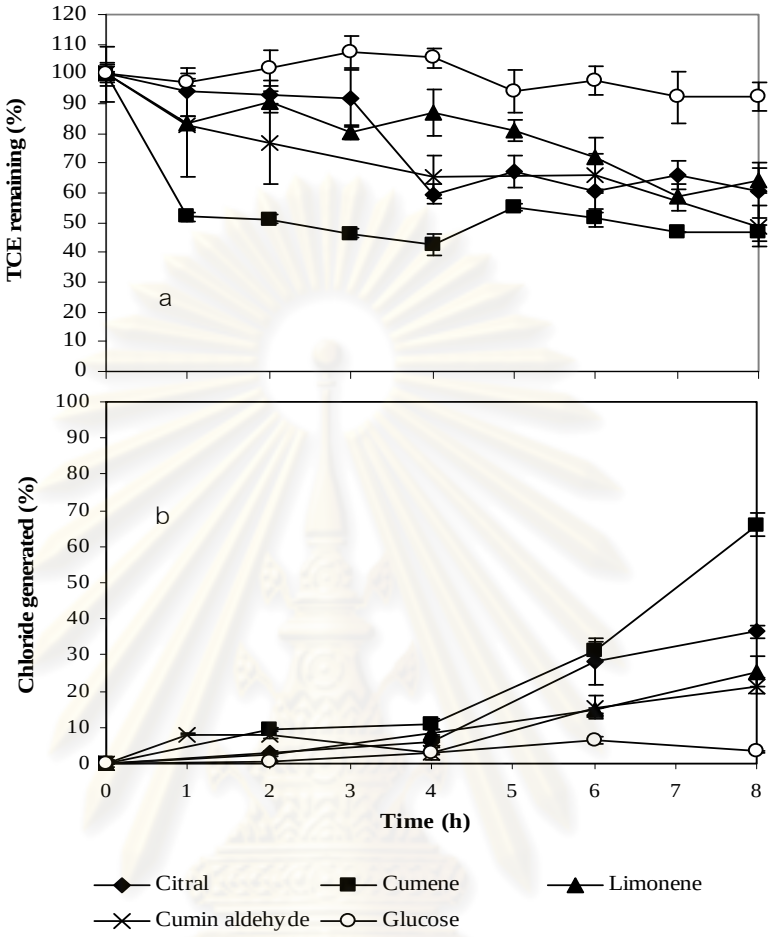


Figure 3.6 Time course of TCE degradation (a) and chloride formation (b) by *Rhodococcus* sp. L4 after induced with various essential oil components. The bacteria were grown in glucose-MS media containing citral, limonene, cumin aldehyde or cumene. Control is the bacteria grown on glucose-MS media without essential oil components. All data are the average of triplicate samples with the standard deviations (error bars) given.

The variation in TCE degradation activity may be explained by the variation of the amounts of inducible enzymes or different types of enzymes may be produced from each inducer. TCE degradation can be initiated by several monooxygenases and dioxygenaseses, which have relaxed substrate ranges (Arp *et al*., 2001). The efficiency of

cumene on inducing TCE degradation is suggested to be due to the bulky isopropyl residue adjacent to the double bond on benzene ring (Fig. 2.2), which may mimic the two chlorines in the TCE molecule (Dabrock *et al*., 1992). This structure is also present in cumin aldehyde, potentially explaining why cumin-aldehyde induced cells contributed to a higher TCE degradation than citral- and limonene-induced cells. The presence of many types of monooxygenase and dioxygenases in Rhodococci is common (Larkin *et al*., 2005). Moreover, the ability to induce multiple aromatic oxygenase genes in *Rhodococcus* sp. T104 has been reported for limonene as well as for other plant terpenes (Kim *et al*., 2003). Consequently, it is possible that *Rhodococcus* sp. L4 might produce other TCE-degrading oxygenases when induced with citral and limonene.

3.4.4 Cumin seeds as alternative inducer for *Rhodococcus* sp. L4 to degrade and dechlorinate TCE

Cumin is commonly used as spice or constituent of household medicines in Asia. Cumin volatile oil and its main component, cumin aldehyde, have antimicrobial activities especially against fungi and yeasts (Shetty *et al*., 1994). In this study, *Rhodococcus* sp. L4 could not utilize cumin oil for growth. However, cumin oil contained oil components including cumin aldehyde and cumene that were effective for induction of TCE-degrading enzymes (Fig. 3.6). The high concentration of essential oils in cumin seeds makes them an interesting candidate for use as alternative inducer for *Rhodococcus* bacteria to degrade TCE. Bacteria induced by cumin oil could degrade TCE from 0.16 µmol to 0.104 µmol (14 µM to 9.1 µM TCE) which corresponded to 36 ± 6% TCE degradation (Fig 3.7). The partial TCE degradation correlated to the generation of chloride to the medium. The molar ratio of TCE degraded to chloride ions generated was approximately 1:3.

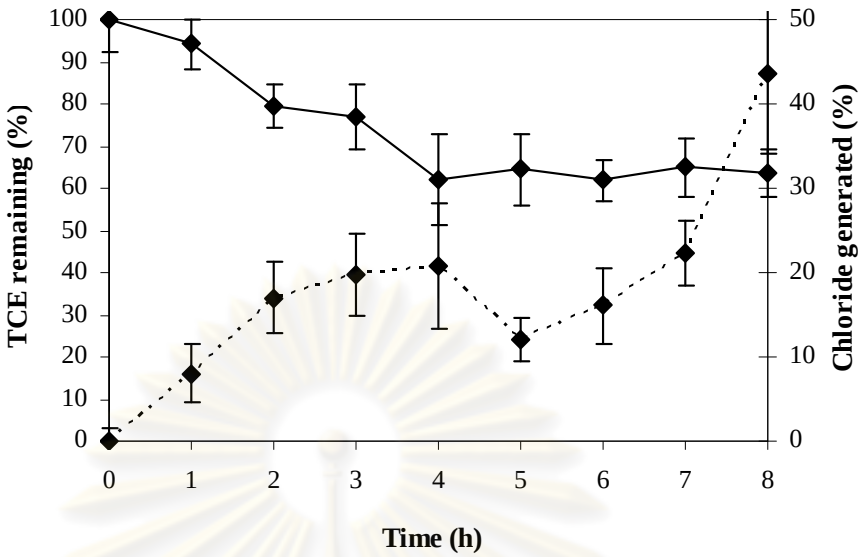


Figure 3.7 Time course of TCE degradation (full line) and chloride formation (dash line) by *Rhodococcus* sp. L4 induced with essential oil of cumin seeds. All data are the average of triplicate samples with the standard deviations (error bars).

The TCE-degrading efficiency of cumin oil-induced cells was higher than lemon- and lemongrass oil-grown cells (Table 3.1). The results suggested that cumin aldehyde and cumene, which are only present in cumin oil, enhanced TCE-degrading enzyme induction. Cumin oil-induced cells had lower TCE-degrading capability than cells induced with the purified oil components (Fig. 3.6). This was probably due to the slow release of essential oil from the seeds or the presence of other components in cumin oil. The application of cumin seeds for TCE biodegradation and bioremediation would be preferred because they are abundant in many countries and are considered non-toxic to humans. However, plant varieties, harvesting period and planting conditions have been shown to affect the quantities of cumin essential oils as well as the types of oil components (Beis *et al*., 2000; El-Sawi and Mohamed, 2002; Jalali-Heravi *et al*., 2007).

This would lead to the variation in TCE-degrading enzyme induction by different batches of the seeds.

3.4.5 Kinetics of TCE degradation by *Rhodococcus* sp. L4

TCE transformation capacity, *Tc*, defined as mass of TCE degraded prior to cells inactivation per mass of biomass added (μg of TCE mg cells^{-1}) and maximum specific rate of TCE degradation, k_c (mg TCE mg cells^{-1} day^{-1}), of *Rhodococcus* sp. L4 were calculated as described by Alvarez-Cohen and McCarty (1991a) (Table 3.2). These two parameters are commonly used for estimating the TCE-degrading ability of microorganisms. T_c is a measure of the cumulative effects of TCE product toxicity in the absence of other substrates (non-growing cells assay) and k_c is a measure of the maximum enzyme efficiency. Glucose-grown cells showed the lowest *Tc* and k_c values. Comparing between induced cells, we could divide the *Tc* values into two groups. One group including citral-, limonene- and cumin oil-induced cells had a moderate *Tc* ranging from 9.4-10.7 μg of TCE mg cells^{-1}. The second group consisted of cumene- and cumin aldehyde-induced cells, which showed the higher *Tc* values of 14.2-15.1 μg of TCE mg cells^{-1}. These values were similar to toluene-grown cells. Neglecting the lag phase, the range of k_c for all induced cells was between 0.09-0.3 mg TCE mg cells^{-1} day^{-1} with the following sequence: cumene > citral > cumin aldehyde > cumin oil > limonene. There was no direct correlation between *Tc* and k_c values, since *Tc* is a measure for the amount of TCE that can be transformed by a given culture prior to inactivation while the maximum specific rate of TCE degradation is a measure for the maximum enzyme efficiency (Chu and Alvarez-Cohen, 1998).

Table 3.2 TCE transformation capacity and rate by *Rhodococcus* sp. L4 compared with other bacteria.

Microorganism	Substrate	Transformation capacity, T_c (μg TCE mg cells^{-1})	Maximum specific degradation rate, k_c (mg TCE mg cells^{-1}day^{-1})
Rhodococcus sp. L4 (This study)	Glucose	2.03 ± 0.063	0.009 ± 0.007
	Toluene	15.06 ± 1.04	0.142 ± 0.001
	Cumene[a]	15.11 ± 0.71	0.303 ± 0.007
	Cumin aldehyde[a]	14.17 ± 0.69	0.110 ± 0.005
	Citral[a]	10.68 ± 0.63	0.203 ± 0.030
	Limonene[a]	9.44 ± 0.81	0.060 ± 0.005
	Cumin oil[a]	9.72 ± 0.59	0.094 ± 0.004
M. trichosporium OB3b (Fitch *et al*., 1996; Oldenhuis *et al*., 1991)	Methane; Formate	150; 290	21; 55
P. putida BH (Futamata *et al*., 2001)	Phenol	NA	0.06
P. putida BCRC 14349 (Chen *et al*., 2007)	Phenol	NA	0.408 (mg TCE mg VSS^{-1}day^{-1})
P. putida (Heald and Jenkins, 1994)	Toluene	5.20	NA
P. cepacia G4 (Folsom *et al*., 1990; Landa *et al*., 1994)	Toluene; Phenol	34; NA	1.5; 0.94
M. Vaccae JOB5 (Wackett *et al*., 1989)	Propane	NA	0.057
Mixed culture (Chang and Alvarez-Cohen, 1995)	Propane	6.50	0.45
N. Europaea (Ely *et al*., 1997)	Ammonia	8.00	1.00

[a] The compound was used as inducer for *Rhodococcus* sp. L4.

NA = Not available

With the exception of methanotrophs, the range of *Tc* and k_c values of all essential oil component-induced cells were similar to other toluene, phenol, propane or ammonia degraders, which have the *Tc* and k_c ranges between 5.2- 34.0 µg of TCE mg cells^{-1} and 0.057-1.5 mg TCE mg cells^{-1} day^{-1}, respectively (Table 3.2). Although these kinetic parameters are specific for the tested bacterial strain, the values are also depended on the test conditions. We did not add substrate and/or inducing compound during TCE biodegradation experiments; thus the bacteria might lose their activity during TCE degradation and could not produce new enzymes. Other possibilities that explain low values of kinetic coefficients in our study might be the relatively high concentration of initial TCE. The published studies conducted TCE cometabolic degradation with initial concentrations between 0.038-60 µM (0.05-8 mg L^{-1}) (Folsom *et al*., 1990; Hopkins and McCarty, 1995; Fitch *et al*., 1996; Chen *et al*., 2007). Chen *et al*. (2007) suggested that the suppression of TCE degradation at higher TCE concentration (i.e. 15 to 150 µM) is probably due to the limitation of oxygenase enzymes. The toxicity resulted from high TCE concentration and the inactivation of oxygenases.

3.4.6 Effect of TCE concentrations on its transformation capacity

Essential oil component-induced cells were tested with various TCE concentrations and the amount of remaining TCE was measured after 8 h. When the initial aqueous concentrations of TCE were increased from 2 µM to 14 µM, TCE transformation capacity (T_c) was increased with all inducers (5 to 15 µg of TCE mg cells^{-1}) (Fig. 3.8). Cumin aldehyde- and limonene-induced cells had a maximum T_c about 13 µg of TCE mg cells^{-1}, when 14 µM and 25 µM TCE were added, respectively. However, their T_c values were lower than 10 µg of TCE mg cells^{-1}, when TCE concentrations were higher than 25 µM. Cumene- and citral-induced cells were more resistant to TCE than

cumin aldehyde- and limonene-induced cells. These cells could tolerate up to 50 µM TCE, which gave a maximum T_c of 20 and 36 µg of TCE mg cells^{-1} (Fig. 3.8).

The initial rate of TCE degradation in cumene-degrading bacterium, *R. erythropolis* BD1, increased when the TCE concentrations were elevated up to 30 µM (Dabrock *et al*., 1992). The variations in TCE degradation activity were probably the results of the different amounts of enzymes produced by each inducer and the different susceptibility of each induced cells towards TCE toxicity. Heald and Jenkins (1994) suggested that there might be a toxicity threshold for the bacteria during TCE degradation, beyond which cells activity will drop remarkably. The knowledge of the concentration dependence is important for the selection of essential oil components as inducer for TCE bioremediation in contaminated sites. For example, our results suggested that citral might be a more suitable inducer for *Rhodococcus* sp. L4 when it is applied to sites with high TCE concentrations.

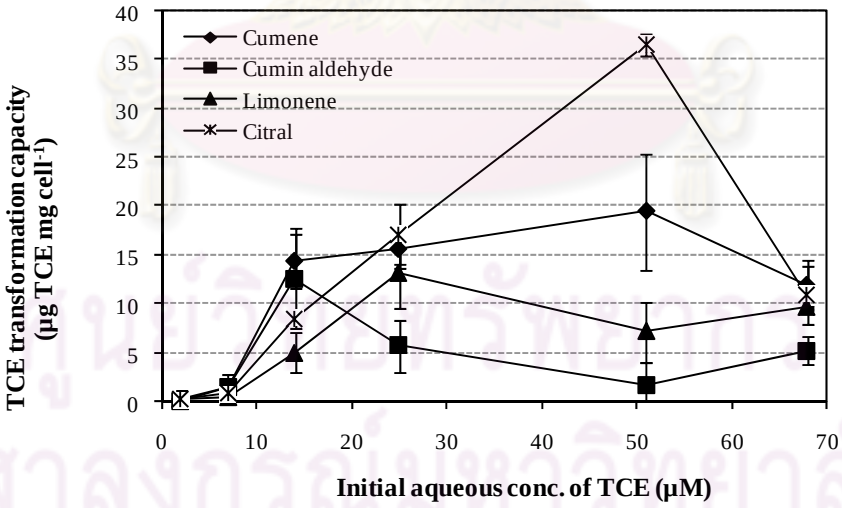


Figure 3.8 TCE degradation by essential oil component-induced *Rhodococcus sp.* L4. The initial aqueous TCE concentrations were varied from 2 µM to 68 µM. TCE

degradation was measured after 8-h incubation. All data represent the average of triplicate samples with the standard deviations (error bars) given.

3.4.7 Repeated addition of essential oil component for enhancing TCE degradation

In the previous study, the efficiency of TCE degradation was only around 35-55%. This was probably due to the loss of bacterial activity during TCE degradation rather than cells death, since the number of bacteria was constant at $3x10^8$ CFU mL^{-1} throughout the study. The addition of essential oil components was therefore suggested to help the bacteria produce new enzymes. In this experiment, each essential oil component was further added to the cells at 80-400 µM either only at the beginning or every hr. When the essential oil was added only at the beginning, the oil was degraded readily and completely within 2-4 hr by all induced cells (Fig. 3.9a-d-2). However, TCE-degrading activity of these bacteria was almost similar to those without oil addition (Fig. 3.9a-d-1).

In the treatment with repeated addition of essential oil, a lag phase about 1-3 hr for TCE degradation was observed in all samples (Fig 3.9a-d-3), while the added essential oil was degraded more readily. The result suggested a competitive inhibition between TCE and essential oil components. There was insignificant reduction of TCE and essential oil component in suspensions of killed-cells (Fig. 3.9a-d-4). This suggested that abiotic processes such as TCE adsorption on the bacteria cells and the interaction between TCE and each essential oil component had no impact on TCE loss.

We could divide the samples into 2 groups based on the pattern of essential oil component degradation. The first pattern was from cumene and limonene, in which both

of them were utilized rapidly within 3 hr while TCE degradation was not detected at that time. Since both cumene and limonene were the growth substrates for *Rhodococus* sp. L4, they might be used prior to TCE. It was also suggested that the enzymes induced earlier might be responsible for the ready cumene or limonene degradation rather than the new enzymes. After 2 hr, the remaining cumene was close to zero even when its addition was repeated, whereas most of TCE was removed from the system (Fig. 3.9a-3). For limonene-induced cells, the amount of limonene and TCE was constant after 4 hr (Fig. 3.9b-3). The results indicated that the enzymes in limonene- and cumene-induced cells were active throughout the study. The addition of cumene and limonene molecules might protect the enzyme from inactivation caused by TCE intermediates and induce new enzymes during this period. Consequently, these enzymes considerably increased the degradation of its own inducer as well as TCE degradation.

The second pattern was from cumin aldehyde and citral, in which the bacteria could degrade these compounds at lower amount than cumene and limonene (Fig. 3.9c-d-3). It was probably because neither of them could serve as growth substrate. Most of TCE was degraded by cumin aldehyde-induced cells after 1 h, while citral-induced cells degraded TCE after 3 hr. At these periods, the degradation rate of cumin aldehyde or citral was slow. These essential oil components were accumulated afterwards.

It was clear that the repeated addition of theses essential oil components enhanced TCE-degrading activity more than single addition (Fig 3.9a-d-2-3). Nonetheless, an accumulation of essential oil components i.e. cumin aldehyde and citral might lead to an enzyme inhibition caused by high concentration of these compounds and

thus decreased the degradation of compounds themselves or decreased the whole degradation processes i.e. in case of citral-induced cells.

.

Due to the advantage of repeated addition of essential oil components toward TCE degradation, we later maintained the presence of essential oil components during TCE degradation assay by immobilizing *Rhodococcus* sp. L4 on plant material rich in essential oil in CHAPTER IV.

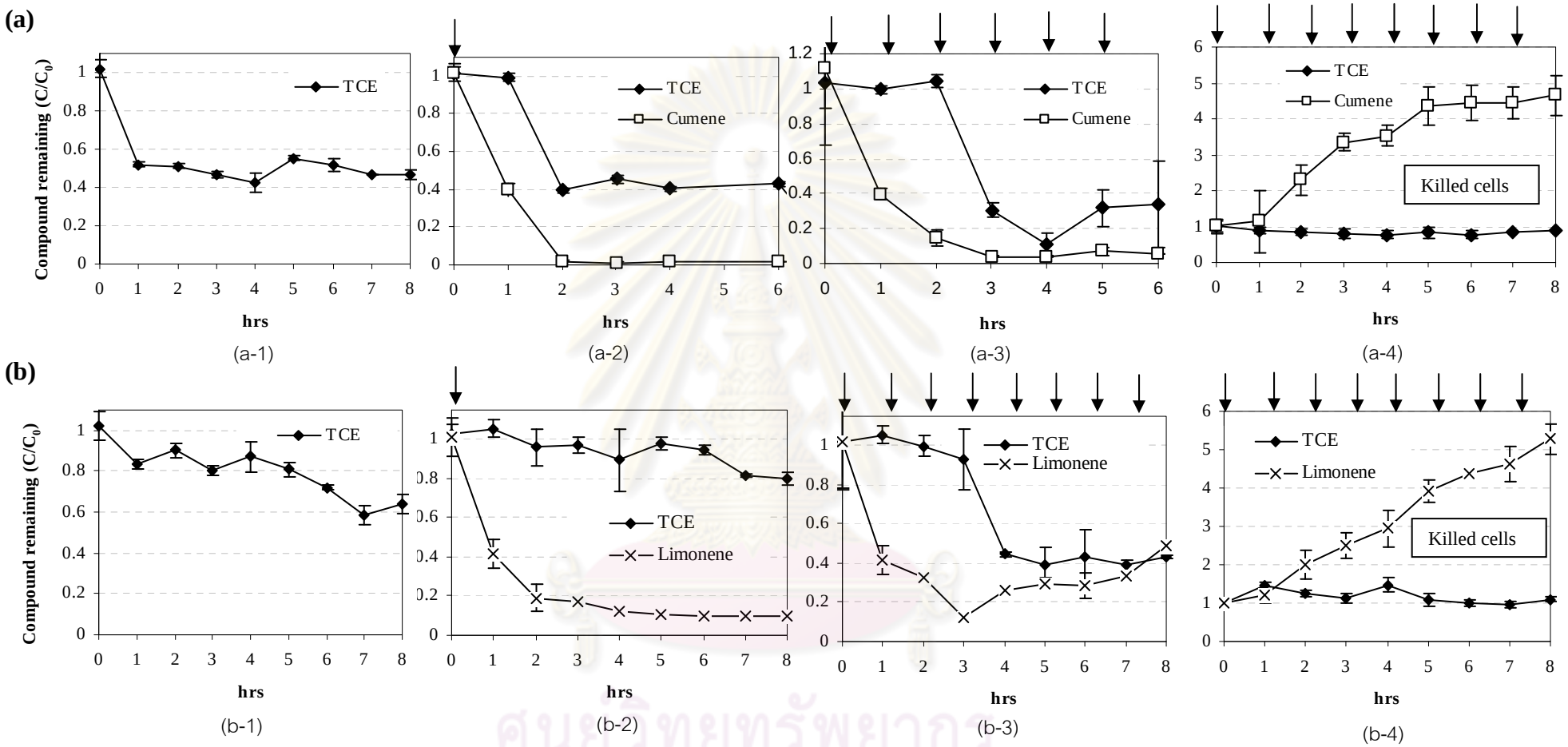


Figure 3.9 Time course of TCE (♦) and essential oil components; cumene (□), limonene (x), cumin aldehyde (Δ), and citral (○) degradation in cell suspensions of induced-(a-d1-3) and killed *Rhodococcus* sp. L4 (a-d4). The arrows show repeated addition of essential oil components to induced- and killed cell samples. Amount of oil component at each time point was measured before repeated addition. TCE was added only at the beginning of study.

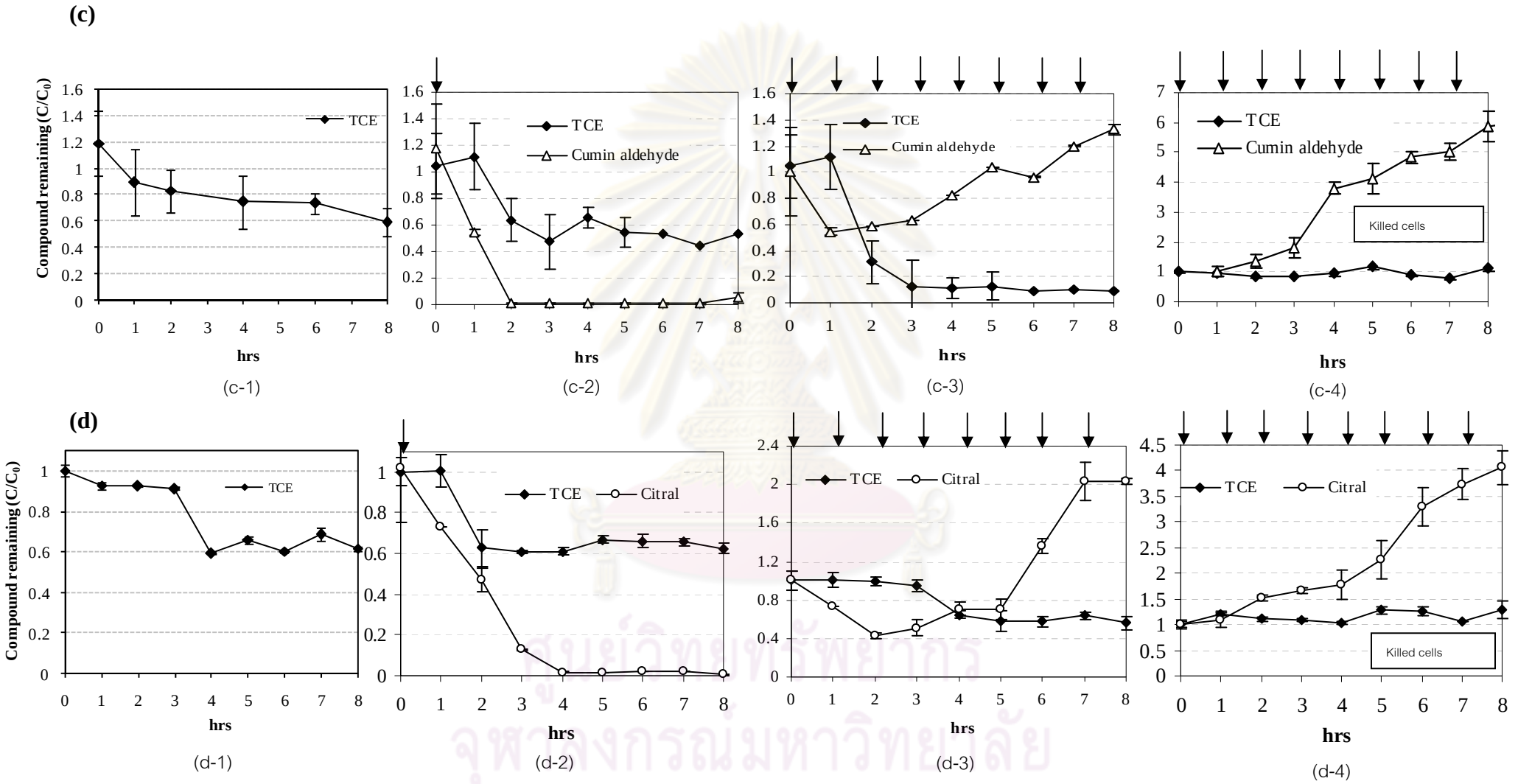


Figure 3.9 (cont.)

CHAPTER IV

IMMOBILIZATION OF *RHODOCOCCUS* SP. L4 ON PLANT MATERIALS RICH IN ESSENTIAL OILS FOR TCE BIODEGRADATION

4.1 Introduction

Because of its extensive use as an industrial solvent, degreasing agent and electronic parts cleaning in manufacturing processes, trichloroethylene (TCE) has become one of the most frequently detected soil and groundwater contaminant in Thailand (TEI, 1998). TCE is toxic and persistence in the environment, thus extensive efforts have been made to remediate this compound. One of the most promising clean-up technologies is bioremediation because of its low cost and potential for complete degradation of the pollutants (Arp *et al*., 2001). In our previous study, we have found that plant essential oil components i.e. citral, limonene, cumene and cumin aldehyde can induce TCE cometabolically degradation in our toluene-degrading bacterium, *Rhodococcus* sp. L4 (Suttinun *et al*., 2008; CHAPTER III). These compounds belong to a group of plant secondary metabolites. Singer *et al*. (2003) suggested that plant metabolites would be acceptable for enhancing bioremediation of subsurface soil and aquifers since these compounds are effective at low concentration and are considered non-toxic.

Meanwhile, the removal of TCE by plant essential oil-induced bacteria in the absence of growth substrate was effective only a short period (Suttinun *et al*., 2008; CHAPTER III). Several researches with *Nitrosomonas europaea* (Hyman *et al*., 1995),

Burkholdria cepacia G4 (Yeager *et al*., 2001a), *Pseudomonas putida* F1 (Morono *et al*., 2004) and butane-oxidizing bacteria i.e. *Pseudomonas butanovora, Mycobacterium vaccae,* and *Nocardioides* sp. CF8 (Halsey *et al*., 2005) also showed an unsustainable process due to cytotoxicity, inhibition, inactivation of TCE-degrading enzymes and rapid disappearance of growth substrate/inducer during TCE cometabolic degradation. Owing to these effects, TCE bioremediation was ultimately limited. To improve bacterial capability for TCE removal, this study aims to develop immobilized *Rhodococcus* sp. L4 on plant material rich in essential oils for TCE biodegradation. The essential oils contained in these plants are expected to be a good source for producing and maintaining TCE–degrading enzyme activities as well as to sustain TCE cometabolic degradation.

Numerous publications have presented the advantage of cells immobilization for degradation of xenobiotic compounds. Immobilized microorganisms could degrade the compounds at a higher initial concentration and for a longer period (Wilson and Bradley, 1996; Obuekwe and Al-Muttawa, 2001). In addition, these cells were also protected from harmful effects of toxic compounds (Quek *et al*., 2006). Pattanapong *et al*. (2004) suggested that carbendazim and 2,4-dichlorophenoxyacetic acid could be simultaneously degraded by an immobilized bacterial consortium on loofa sponge. Chen *et al*. (2004) reported that a high activity for propylene epoxidation was observed in *Methylomonas* sp. Strain GYJ3 entrapped in sol-gel. The effective biodegradation of various petroleum products by cells immobilized in polyvinyl alcohol and polyurethane foam was reported by Cunningham *et al*. (2004) and Quek *et al*. (2006). Obuekwe and Al-Muttawa (2001) found that bacteria immobilized on inert surfaces could encourage crude oil degradation and these immobilized cells could be stored for long periods without losing their degrading ability. However, there were only a few reports on cells immobilization for TCE biodegradation. Radway *et al*. (1998) showed that *Burkholderia cepacia* G4

embedded in polyurethane foam could remove up to 80% of 3 mg L^{-1} TCE after the bacteria were induced with phenol. The ability of *Methylocystis* sp. M immobilized in different matrices i.e. agarose, alginate, carrageenan, polyurethane, photo-crosslinkable resin and polyelectrolyte complex for TCE degradation was studied by Uchiyama *et al*. (1993, 1995). Chen *et al*. (2007) studied chitosan-bead immobilized *Pseudomonas putida* for TCE degradation in the presence of phenol as substrate. They found that the rate of TCE degradation in immobilization system was about 1/2 compared to that of free cells but the immobilized cells could tolerate higher TCE concentrations.

The immobilization of bacteria on plant materials rich in essential oils has never been studied. These plant materials are agricultural products, which can be found in our country and are low cost. The self/natural-attachment of microbes to material was reported to maintain high amount of active cells leading to increased biodegradation activity (Cohen, 2001). During immobilization, the bacteria naturally attached to the material surface, thus eliminated the use of extraneous chemical substances in immobilization process. Consequently, the whole process is simple and inexpensive. In this study, we screened several plant materials rich in essential oils for immobilization and TCE degradation. The immobilized cells on a selected material i.e. cumin seeds were further tested for their degrading ability with various TCE concentrations as well as their reusability. The production of immobilized cells can be beneficial for commercial purposes as well as for emergency responses following TCE contamination. In addition, the research could be used as an example for biodegradation and bioremediation of other hazardous pollutants.

4.2 Methodology Outline

(1) Inoculum preparation for bacteria immobilization

(2) Development of *Rhodococcus* sp. L4 immobilization

(2.1) Screening of plant materials rich in essential oils as immobilizing support

- Three plant materials rich in essential oils: cumin seeds, orange peels, and lemon grass leaves.
- Selection criteria: TCE removal efficiency and stability of immobilized materials after application.

(2.2) Optimizing of bacteria immobilization on selected materials (cumin seeds)

- Optimized parameters: (1) seeds sizes and (2) incubation time based on exopolysaccharides production by bacteria.

(3) TCE degrading ability of bacteria immobilized on cumin seeds

The amount of immobilized materials was varied to obtain the highest TCE removal efficiency.

(3.1) Comparing of TCE transformation efficiency by bacteria immobilized on cumin seeds to that of free cells (CHAPTER III) and the known TCE-degrader

(3.2) Determining the effect of increased TCE concentrations on its transformation capacity

(3.3) Determining the effect of repeated addition of TCE

(3.4) Determining the reusability of immobilized bacteria.

4.3 Materials and Methods

4.3.1 Chemicals, plant materials and media

TCE (99.5%) were obtained commercially from Fluka Chemical Industrial. TCE stock solutions were prepared by dissolving aliquots of TCE in N,N-dimethylformamide (Merck, USA) to obtain the desired concentration. Toluene (99.5%) purchased from Merck was supplied as carbon and energy source for culture maintenance. Five essential oil components (cumene (99.0%), cumin aldehyde (4-isopropyl benzaldehyde) (90.0%), (+)-α-pinene (97.0%), γ-terpinene (95.0%) and *p*-cymene (99.0%)) used as standards for their identities were obtained commercially from Merck, USA. Plant materials were purchased in one batch from their distributors i.e. seeds of cumin (Nguan Soon, Bangkok), lemon grass leaves (OTOP product of Nakhonprathom, Thailand). Other materials, orange peels and loofa sponge, were purchased from local market in Bangkok. Activated carbon (granular form) was purchased from Merck, USA. Alcian blue (S.D. Fine-Chem Limited) solutions were prepared in 3% acetic acid (Fisher Scientific, Inc.) for determination of exopolysaccharides production. Bradford reagent (Bio-Rad) was used for protein analysis with bovine serum albumin (BSA), 2 mg mL^{-1} as a protein standard. All chemicals were analytical grade. The MS medium used for TCE degradation experiments and determination of living toluene/TCE-degrading bacteria was a chloride-free minimal medium prepared according to Focht (1994) with details in Appendix A. All chemicals for MS medium preparation were obtained from Merck. For agar plate, 1.5% agar (HiMedia laboratories Limited) was added.

4.3.2 Bacterial strain and culture maintenance

Rhodococcus sp. L4 is deposited at the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR) and given the accession number TISTR 1542. The partial 16S rRNA gene sequence of *Rhodococcus* sp. L4 is available in GenBank under the accession number EF527237. The bacterium used in this study was maintained by culturing on mineral salts (MS) agar incubated in a glass box equilibrated with toluene vapor (its enrichment substrate) at room temperature. Approximately every 10-14 days, the culture was transferred to a new MS-agar for maintaining the ability of enzyme production by bacteria throughout the course of study.

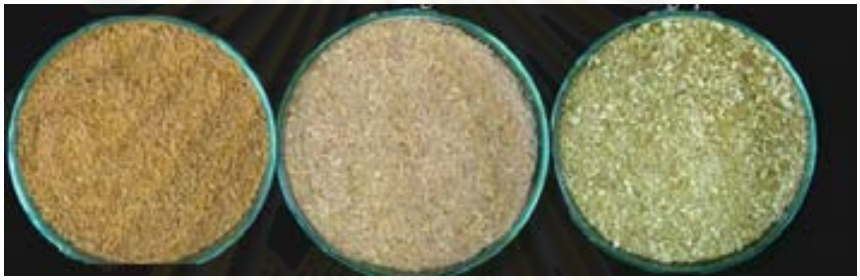
4.3.3 Inoculum preparation for *Rhodococcus* sp. L4 immobilization

To allow the bacteria to acclimatize to the liquid media prior to use in the experiment, the pre-inoculum was prepared by culturing a loopful of *Rhodococcus* sp. L4 (on MS-agar) in a 250-mL Erlenmeyer flask containing 100 mL MS medium that was supplied with growth substrate in the vapor phase by adding 200 µL toluene (12.5 mmol L^{-1} gas phase) in the Eppendorf tube fixed at the top of the flask (Fig. 3.1). After overnight incubation at 200 rpm and room temperature, 10 mL of late-log phase pre-inoculum was transferred into the inoculum flask containing 100 mL of MS medium. The optical density (OD) at 600 nm was about 0.06-0.08 at the beginning. A vapour phase of toluene (growth substrate) was supplied as previously stated. The cultures were incubated on an orbital shaker at 200 rpm, room temperature, for 24 h. Then, the cells were harvested by centrifugation at 2516 x *g* (7,500 rpm) for 10 min. Cells were washed twice and resuspended in MS medium to a OD_{600} of 1.0 before they were used as inocula for immobilization as described below.

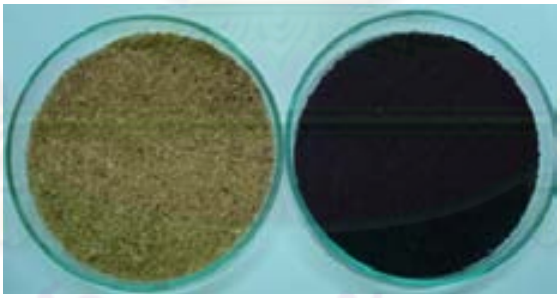
4.3.4 Development of *Rhodococcus* sp. L4 immobilized on plant materials by attachment technique

4.3.4.1 Screening of plant materials for immobilization

Three plant materials rich in essential oils i.e. cumin seeds , orange peels, and lemon grass leaves were screened as immobilizing support for *Rhodococcus* sp. L4. Dried plant materials were ground and put through sieves between 1- 2 mm.



Cumin seeds Lemon grass Orange



Loofa Activated

Figure 4.1 Sieved plant materials rich in essential oils consisting of cumin seeds, lemon grass leaves, and orange peels were used as bacterial immobilizing support compared to the known support materials without essential oil i.e. loofa sponge and activated carbon.

The material surfaces were explored by scanning electron microscope (SEM) before application. The immobilization by attachment technique was initiated by adding 1 g of non-sterilized or sterilized (autoclaved) plant materials into flasks containing 50 mL cell suspensions of *Rhodococcus* sp. L4 (OD_{600}= 1.0). The samples were incubated at 130 rpm and room temperature for 4 days to ensure a formation of well-immobilized cells. Subsequently, the immobilized cultures were washed with mineral salt (MS) medium and filtrated through sterilized filter paper for removing unattached cells. The immobilized cells were air dried in a sterile hood and analyzed for the number of bacterial cells and the efficiency of TCE removal. Two sets of immobilizing supports without essential oil i.e. activated carbon and loofa sponge were employed as positive control materials. Another set of controls consisting of each sterilized material (without cells) and killed immobilized cells were used to indicate the abiotic loss of TCE i.e. TCE adsorbed on bacteria cells and/or on materials**.** The selection of immobilizing support was based on their ability to enhance TCE degradation and stability for repeated application.

Table 4.1 Amount of essential oil and its major component in plant materials used for *Rhodococcus* sp. L4 immobilization

Plant materials	amount of essential oil (%)	Types of oil component (%)
Cumin seeds	1.4-2.8 [a]	Terpinene (24.07), Pinene (21.85),Cymene (19.56), Cumin aldehyde (16.10),Cumene (1.31) [b]
Orange peels	2.5 [c]	Mainly citral and limonene [c]
Lemon grass leaves	0.2-0.5[c]	Mainly citral and limonene [c]

Source: [a] Beis *et al*., 2000; Jalali-Heravi *et al*., 2007; [b] Suttinun *et al*., 2008
[c]http://www-ang.kfunigraz.ac.at/~katzer/engl/and
http://www.tistr.or.th/pharma/RD_anal.htm

4.3.4.2 Immobilization of *Rhodococcus* sp. L4 on cumin seeds

At the end of screening part, we selected cumin seeds as immobilizing support for further experiments since they showed the highest capability for TCE degradation and stability in repeated application. Seeds of cumin were ground and pulverized by passage through a sequence of 2 mm, 1 mm and 500 µm sieves. The sieved seeds were sterilized by autoclaving and were left overnight to allow the viable microorganisms to grow. Then the cycle was repeated twice to remove the viable bacteria. The properties of cumin seeds were determined as described below. The immobilization procedures were optimized by varying (1) sizes of cumin seeds between 500 µm and $\geq$ 2 mm (2) incubation time (1-7

days) for formation of well- attached cells based on exopolysaccharides production (see Alcian Blue assay below) and (3) amount of immobilized culture i.e. 0.02, 0.1 or 0.2 g. Similar to screening part, 1 g of sterilized seeds was incubated with 50 mL cell suspensions of *Rhodococcus* sp. L4 (OD_{600}= 1.0) at 130 rpm and room temperature for a period of time to ensure a formation of well-immobilized cells. Subsequently, the immobilized cultures were washed with mineral salt (MS) medium and filtrated through sterilized filter paper for removing unattached cells. Filtrated culture on plant materials were air dried in a sterile hood and analyzed for the number of bacterial cells, cell dry weight, amount of protein and the attachment of bacteria before TCE biodegradation study. After the optimization process was achieved, the immobilized bacteria were produced in large batch so that they can be used for the next experiment with the same quality.

4.3.5 TCE biodegradation test

(1) The biodegradation experiment consisted of 0.02, 0.1 or 0.2 g of immobilized materials and 0.16 µmol TCE (14 µM initial aqueous concentration) in 22 mL headspace vials containing 2 mL MS medium sealed with Teflon-faced silicone septum and aluminum crimp caps. The aqueous concentrations of TCE in liquid-gas systems at 30 ^{0}C were calculated with a dimensionless Henry's constant of 0.49 (Gossett, 1987). There were re-equilibrations between gas and liquid phases during TCE degradation. The total TCE mass that remained in the system was calculated from the summation of TCE in both aqueous and gaseous phases. The oxygen was provided by leaving 90% of air headspace in the tested vial only at the beginning to minimize the loss of TCE via volatilization. The mixture vials were incubated at 130 rpm and room temperature. Two

sets of triplicate samples were taken by sacrificing at intervals. In one set of samples the reaction was stopped by adding a few drops of 10 M H_2SO_4 to the vials before TCE analysis by headspace GC-FID as stated below. Another set of samples was used to determine the number of toluene/TCE degraders as described below. The control sets consisted of sterilized materials (uninoculated) and killed immobilized cells which represented TCE loss by abiotic process i.e. TCE adsorption on materials and/or on bacteria cells. TCE transformation capacity, T_c (µg of TCE mg cells^{-1}) and initial specific rate of TCE degradation, k_c (mg TCE mg cells^{-1} day^{-1}), were determined and compared with the known TCE degrading bacteria. The optimum amount of immobilized materials of 0.02 g was used for (2), (3), and (4).

(2) To observe the effect of increased TCE concentrations on its biodegradation, further experiment was done which contained 0.02, 0.08, 0.16, 0.3, 0.6 or 0.8 µmol TCE (2 to 68 µM initial aqueous concentration) in separated vials of immobilized cells similar to (1). TCE consumption was monitored at the beginning and at the end of incubation period.

(3) The effect of repeated addition of TCE on immobilized cells was observed. The preparation of immobilized cells and incubation conditions were performed as described above. To test that the immobilized bacteria were active and could degrade TCE continuously, 0.16 µmol of TCE was added repeatedly to the vials after TCE was degraded. The total amount added TCE was 0.64 µmol. The initial aqueous concentration of TCE after repeated addition was maintained at a concentration which did not cause bacterial inhibition (not exceed 50 µM, see Fig. 4.9). The amount of TCE in the vials

prior to and after repeated addition of TCE was determined every 24 h by headspace-GC-FID as stated below.

(4) The reusability and reactivation of immobilized bacteria were studied. Generally, the bacteria/enzymes responsible for TCE degradation were inactivated in the degradation process (see CHAPTER V), thus methods for their reactivation are necessary. The used immobilized bacteria from each vial were washed with MS medium and filtrated through sterilized filter paper, then they were reactivated by putting them in a 250-mL Erlenmeyer flask containing 50 mL MS medium per gram material. The flasks were incubated at room temperature with 130 rpm shaking for 12 h or 24 h before they were reused for TCE biodegradation according to (1). At the end of study, the amount of TCE remaining was determined for each application cycle and compared to that of those without reactivation.

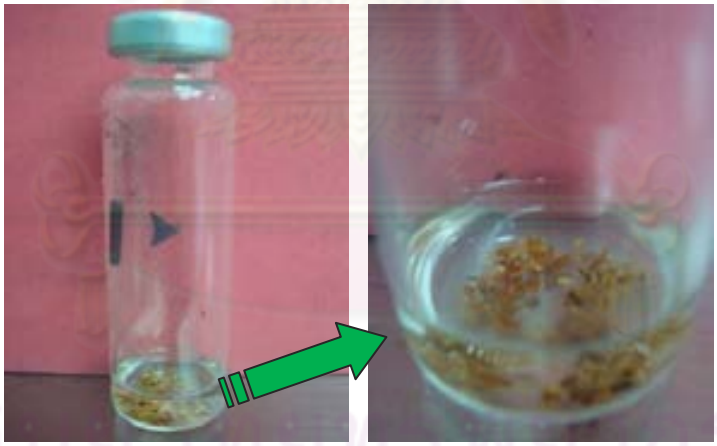


Figure 4.2 A 22-mL headspace vial containing 0.02 g immobilized bacteria on cumin seeds and 2 mL MS medium saturated with TCE.

4.3.6 Analytical methods

4.3.6.1 Cumin seeds properties

The following properties of cumin seeds were determined;

Table 4.2 Selected properties of cumin seeds

Property	Characteristic	Method/Analyzed by
Essential oils Amount (%): Major components (%):	 1.4–2.8 Terpinene (24.07) Pinene (21.85) Cymene (19.56) Cumin Aldehyde (16.10) Cumene (1.31)	 Steam distillation/Beis *et al*., 2000; Jalali-Heraviet *et al*., 2007 Headspace GC-FID (Perkin Elmer)/ Suttinun *et al*., 2008
Available nitrogen NH_4^+ (mg kg^{-1}): NO_3^- (mg kg^{-1}): Phosphorus (mg kg^{-1}):	 91 25.2 15.02	 A method of Attanun and Juncharoensuk, (1999)/ Environmental Research Institute, Chulalongkorn University
TOC (%):	44.96	TOC analyzer (Solid sample module-SSM-5000A, Shimadzu) /NCE-EHWM, Chulalongkorn University
Charge:	Positive	Zeta-meter system 3.0+ (Zeta-Meter, Inc.)/ The author
Total pore volume (cc g^{-1}) Interior-porosity (%)	 0.0018 0.22	Surface area analyzer (Quantachrome, Autosorb-1)/ Petroleum and Petrochemical College, Chulalongkorn University
Surface	see Fig.4.3	Scanning electron microscope (SEM)/Scientific and Technological Research Equipment Centre, Chulalongkorn University.

4.3.6.2 Bacterial attachment on cumin seeds

To view bacterial attachment on cumin seeds, the samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH = 7.2) for 1 h, washed with phosphate buffer, dehydrated by sequential ethanol (30, 50, 70, 90 and 100%) extraction, and dried by a critical point dryer. The samples were put on the stub then coated with gold before scanning electron microscopy (SEM) analysis by the Scientific and Technological Research Equipment Centre**,** Chulalongkorn University.

4.3.6.3 Exopolysaccharide production

The ability of *Rhodococcus* sp. L4 to produce exopolysaccharides on cumin seeds was examined by a modified Alcian Blue adsorption assay of Vandevivere and Kirchman (1993). Firstly, the bacteria were immobilized following the method 4.3.4.2. Three sizes of seeds were used with varying incubation time from 0-7 days. Every 24 h incubation, a replicate of immobilized flasks were taken by sacrificing and the unattached cells were removed through filtration, then 10 mL of MS medium were freshly added into each flask. The assay for exopolysaccharides production was initiated by transferring 100 µL of 1% Alcian Blue solution prepared in 3% acetic acid (pH = 2.5; filtrated) into the flasks. Subsequently, the samples were slowly shaken at 80 rpm for 15 min and filtrated. The absorbance of the supernatant was measured at 606 nm. The decline in the absorbance (after cells with dye bound to exopolysaccharides were removed by filtration) relative to control i.e. cumin seeds without cells plus dye solution was a measure of the amount of exopolysaccharide produced. The production of exopolysaccharides ensured a strong attachment of bacteria to materials during immobilization (Obuekwe and Al-Muttawa, 2001). The compositions of exopolysaccharides are mainly polysaccharides (up to 65%) and proteins (10-15%) (Lazarova and Manem, 1995).

4.3.6.4 Enumeration of bacteria immobilized on support materials

The bacterial extraction from support materials was performed by a modified procedure of Pattanasupong *et al*. 2004. Basically, 0.02 g immobilized material was placed into a vial containing 1.98 mL MS medium and left to rehydrate for 3 min. The bacterial supports were sonicated (Ultrasonic bath, Clifton) for 2 min and shaken vigorously on a vortex mixer for another 2 min to release bacteria from the materials. The process was repeated twice. Subsequently, the supernatants containing the suspended cells were centrifuged at 7,500 rpm, 4^0C for 10 min and the pellet was resuspended in MS medium. The number of toluene/TCE-degrading bacteria on the support material was enumerated by plate count technique. The procedure consisted of diluting the bacteria with a series of sterile MS medium and plating onto MS agar plates. The plates were incubated at room temperature in a glass box supplied with toluene as the sole carbon and energy source for 5 days. Thereafter, the bacterial colonies were counted. Duplicate samples were performed, the results were averaged and a number of viable bacteria per g immobilized materials (CFU g^{-1} material) was estimated.

4.3.6.5 Cell dry weight

Cell dry mass was calculated as milligram of cell dry weight after the bacteria were removed from cumin seeds. Before estimation, 0.4 g immobilized material was placed into a vial containing 9.6 mL MS medium and further procedures to release bacteria from cumin seeds were done as described in 4.3.6.4. After centrifugation, the pellet was resuspended in 20 mL MS medium. Cells dry weight was determined by measuring the mass difference between preweighed dried crucible with 20 mL of mineral salts (MS) medium and those with 20 mL of bacterial culture after both sets were

incubated overnight at 103–105 ^{0}C. Samples were performed in duplicate. The value obtained was 27.5 mg cell dry weight g material^{-1}.

4.3.6.6 Protein determination

The bacteria attached on cumin seeds were extracted as previously stated in 4.3.6.4 for protein analysis. After centrifugation, the pellet was re-suspended in 1 mL MS medium followed by cell solubilization by diluting cell suspension with 2 N NaOH at 1:1 ratio in an Eppendorf tube. The sample was heated at 82-85 ^{0}C for 10 min (Wackett and Gibson, 1988). The heat-killed cells were collected by centrifugation at 13,000 rpm for 10 min. The supernatant was analyzed for amount of protein by Bradford protein assay in which 3 mL of 1x dye reagent (Bio-Rad) were added in disposable cuvettes containing 60 µL standards or unknown sample solution and mixed thoroughly. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 595 nm. MS medium filled with dye reagent was used as a blank reference. Bovine serum albumin (BSA), 2 mg mL^{-1} was used as a protein standard. The protein was assayed in triplicate. The estimated protein concentration was 0.35 mg protein g material^{-1}.

4.3.6.7 Quantitative analysis of TCE and cumin oil components

The amounts of TCE and cumin oil components were analyzed by the PerkinElmer TurboMatrix Automated Headspace Sampler with the Clarus 500 gas chromatography equipped with a flame ionization detector (Headspace GC-FID) and a HP-5 (5% phenyl methyl siloxane) fused-silica capillary column (30 m x 0.32 mm ID; thickness, 0.25 µm). Sample vials were heated to 93 ^{0}C for 30 min for equilibration. The following headspace conditions were applied; the head-space pressure, 20 psi; injection

time, 0.05 min; pressurized time, 0.5 min; withdraw time, 0.2 min. The gas chromatography conditions were as follows; injector temperature, 150 ^{0}C; detector temperature, 250 ^{0}C; initial column temperature, 40 ^{0}C (1.80 min) then, programmed at 40 ^{0}C to 55 ^{0}C at a rate of 45 ^{0}C min^{-1}, and 55 ^{0}C to 135 ^{0}C at a rate of 10 ^{0}C min^{-1}. The carrier gas (nitrogen) pressure was 14.0 psi with a flow rate of 20 mL min^{-1}. The retention time of TCE was 1.78 min. External standard quantitative calibrations were performed for the analysis of TCE concentrations. The essential oil components contained in cumin seeds were identified by comparing their retention times with standard compounds. The retention times of cumene, pinene, cymene, terpinene and cumin aldehyde were 4.22, 4.93, 5.64, 6.16 and 8.9 min, respectively.

4.4 Results and discussion

4.4.1 Screening of plant materials rich in essential oil for *Rhodococcus* sp. L4 immobilization

Three plant materials rich in essential oils including cumin seeds, lemon grass leaves and orange peels were screened for the ability to immobilize *Rhodococcus* sp. L4 and to enhance TCE degradation. The essential oils (ESO) of these plants contain several essential oil components (ESOC) (Table 4.1) that have been found to stimulate microbial degradation of xenobiotic compounds such as polychlorinated biphenyls (PCBs), toluene, phenol and TCE (Crowley *et al*., 2001; Singer *et al*., 2003; Suttinun *et al*., 2008). These selected plants are local agricultural products and inexpensive. Two immobilizing materials without essential oil i.e. loofa sponge and activated carbon were used as control materials. In addition, the uninoculated sterilized materials were used to represent the possibility of TCE loss by abiotic process e.g. TCE adsorption on materials. The selection

criteria were the ability of *Rhodococcus* sp. L4 to immobilize on material and to remove TCE and the stability of immobilized material after application. The surface of these materials by SEM analysis is shown in Fig. 4.3.

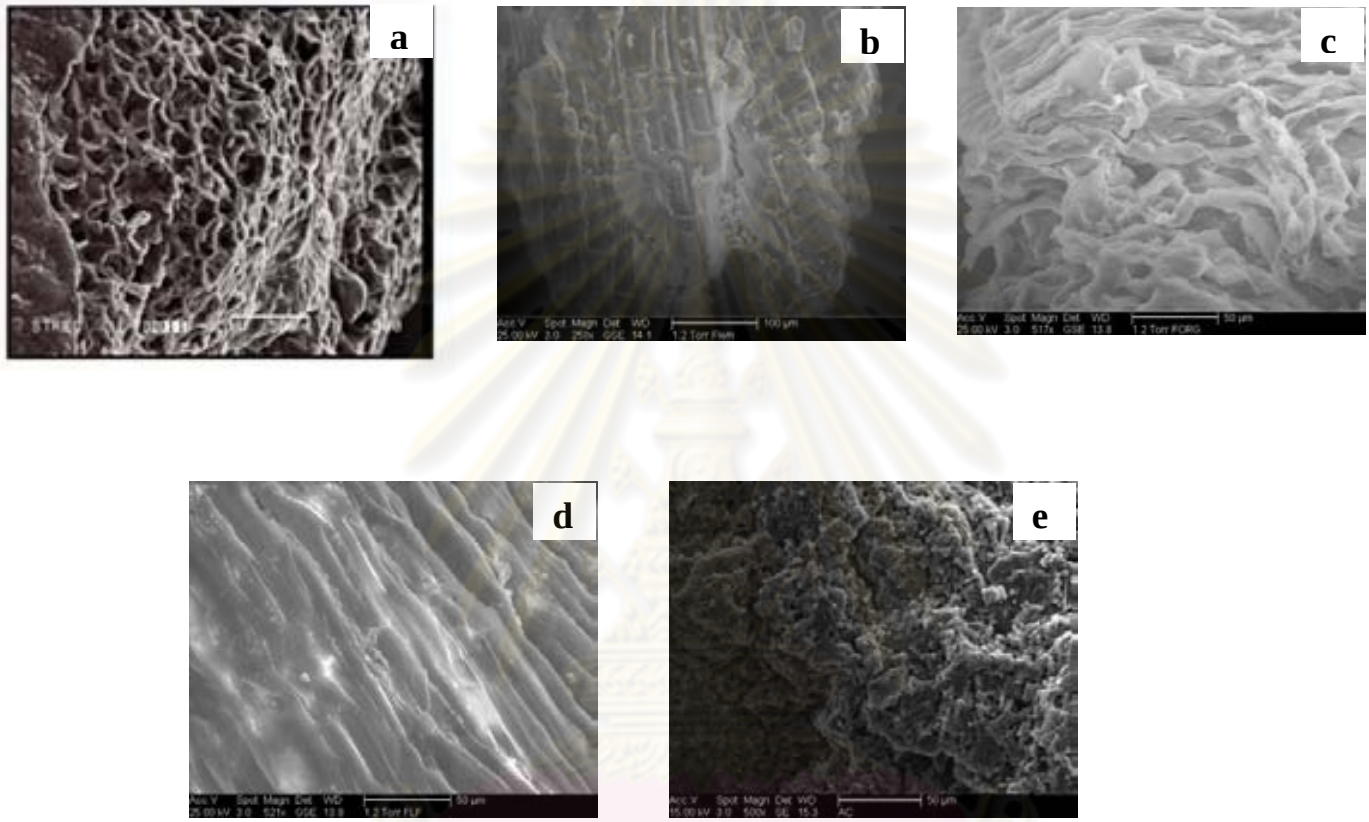


Figure 4.3 Surface of materials explored by scanning electron microscope (SEM); cumin seeds (a), lemon grass leaves (b), orange peels (c), loofa sponge (d) and activated carbon (e).

An attachment technique was developed for immobilization of *Rhodococcus* sp. L4 on these materials in which the bacteria were incubated along with each sieved sterilized material on a shaker. The number of cells on these materials was approximately 10^9 CFU g material^{-1} after 4 days. TCE degradation assay was carried out by adding 0.16 µmol TCE (14 µM initial aqueous concentration) to a 22-mL headspace vial containing

0.02 g immobilized cells and 2 mL MS medium. The samples were incubated and analyzed for the remaining TCE after 24 h and 72 h.

The percentage of TCE removal (% of control) was calculated from the difference between the total mass of TCE in both gas and aqueous phases at the time zero (M_{T0}, as 100% baseline) and the remaining of TCE mass at 24 or 72 h in each immobilized cells material ($M_{T24, 72}$) relative to its control sterilized material ($MCT_{T24, 72}$). The calculation was as follows: TCE removal (% of control) = M_{T0} - ($M_{T24, 72}$/$MCT_{T24, 72}$*100). The percentage of TCE removal by cells immobilized on different sterilized materials and their controls is presented in Fig 4.4(a-b). There was no significantly difference in TCE degradation in all immobilized materials at 24 h when about 6-18% TCE was removed compared to control sterilized materials without cell (uninoculated). This was probably the maximum capacity of each material for TCE sorption. Immobilized cells on support materials containing ESO showed higher TCE degradation than those without ESO after 72 h. The immobilized cells on cumin seeds, orange peels and lemon grass leaves were able to degrade 72%, 76% and 51% TCE while immobilized cells on activated carbon and loofa sponge removed about 9 and 35%TCE after 72 h incubation, respectively. It is suggested that TCE removal after 24 h was due to the degradation by immobilized cells.

The results indicate that ESO and its ESOC in plant materials (Table 4.1) were able to induce TCE biodegradation in the attached bacteria. Similarly, our previous study showed that cell suspensions of *Rhodococcus* sp. L4 were induced for TCE degradation by limonene, citral, and cumin aldehyde, which are the major components in lemon, lemon grass and cumin essential oils (Suttinun *et al*, 2008). Several bacteria grown on

cumene, a trace component in cumin essential oil, could also effectively degrade TCE (Dabrock *et al*. 1992, 1994; Pflugmacher *et al*. 1996; Morono *et al*. 2004, 2006).

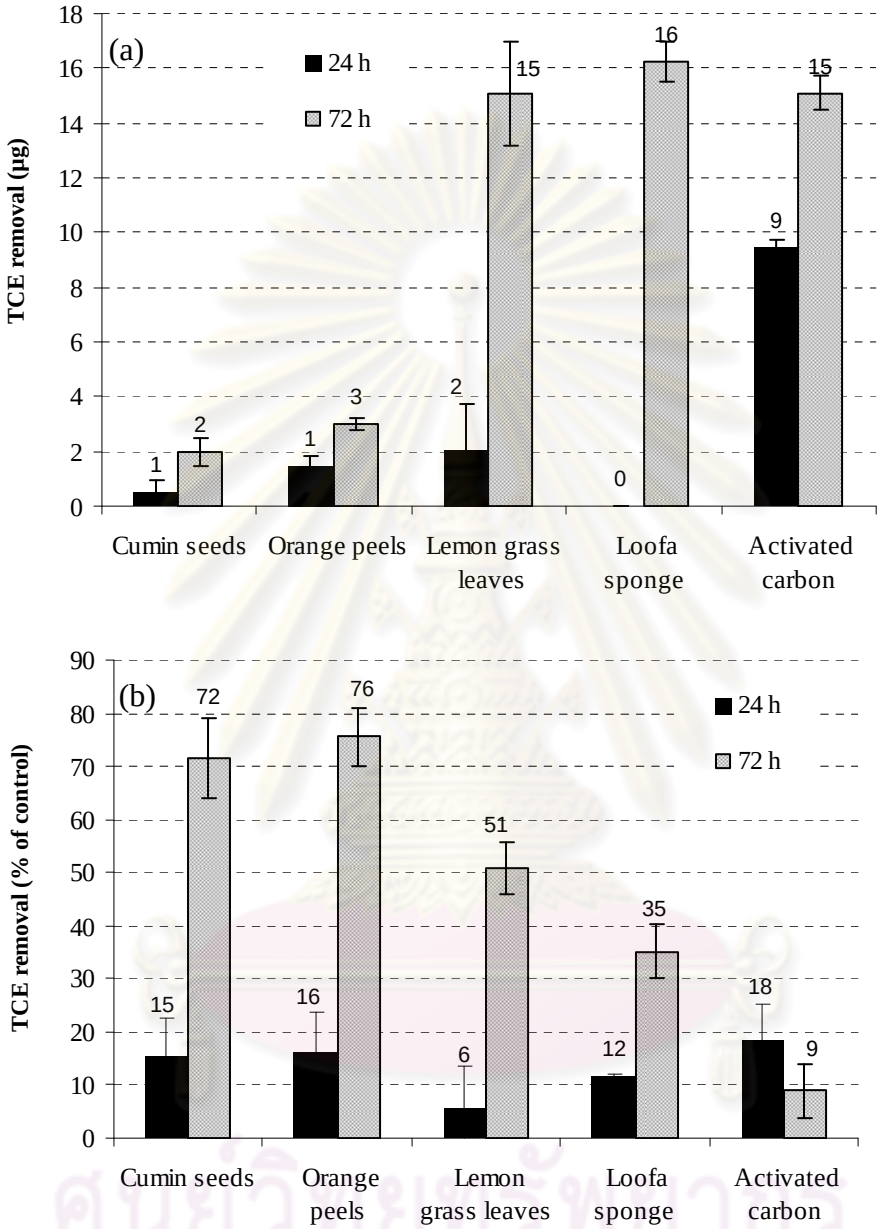


Figure 4.4 TCE removal in control sterilized material without cells (uninoculated) (a) and immobilized *Rhodococcus* sp. L4 on different sterilized materials calculated by comparing with control after 24 h and 72 h incubation (b).

The number of toluene-degrading bacteria originally present on non-sterilized cumin seeds, lemon grass leaves, and orange peels were approximately 10^5 CFU g material^{-1}. These bacteria were representatives of TCE-degrading bacteria since most of toluene-degrading bacteria were capable of TCE degradation (Morono *et al*. 2006). To observe whether these indigenous toluene degraders could support TCE degradation in the presence of *Rhodococcus* sp. L4, an experiment was carried out by non-sterilized materials in comparison with sterilized materials. Similar procedures for cells immobilization were performed as described in materials and methods (4.3.4.1) before TCE analysis. TCE degrading efficiencies of immobilized cells on non-sterilized and sterilized materials are presented in Table 4.3. All samples with immobilized cell on non-sterilized materials were capable of lower TCE degradation than those on sterilized materials. Lower TCE degradation efficiency may result from the competitive inhibition of the microorganisms originally presented on the materials. It was also suggested that not all toluene-degrading bacteria were TCE degraders.

From screening part, the most effective materials for immobilizing cells were sterilized orange peels and cumin seeds, in which the immobilized cells removed TCE up to 70% after 72 h incubation (Fig.4.4 and Table 4.3). Apart from the benefit of essential oil, their porous structure as visualized by SEM (Fig. 4.3) might promote the attachment of bacteria as well as reduce mass transfer limitations. However, only cumin seeds were selected as supporting material for further TCE degradation tests because they were stable after use. Whereas, orange peels were deteriorated rapidly after incubation with the bacteria.

Table 4.3 Averaged percentage TCE removal (% of control) after 72 h incubation with immobilized *Rhodococcus* sp. L4 on either sterilized or non-sterilized materials rich in essential oils

Immobilized cells on various materials	TCE degradation at 72 h [a]
Sterilized cumin seeds	71.7 ± 7.6
Sterilized orange peels	75.6 ± 5.4
Sterilized lemongrass leaves	50.8 ± 4.8
Non-sterilized cumene seeds[b]	35.3 ± 11.1
Non-sterilized orange peels[b]	11.6 ± 4.4
Non-sterilized lemongrass leaves[b]	50.7 ± 0.4

[a] Averaged percentage of TCE degradation was calculated from the changes in GC peak areas in each immobilized culture materials relative to the control with sterilized materials only (uninoculated) representing the effect of TCE adsorption on materials. The total TCE mass added was 0.16 µmol. The number of immobilized cells on these supports was approximately 10^9 CFU g material^{-1}.

[b] Non-sterilized materials were used in order to observe whether toluene/TCE degrader originally available on materials support our immobilized cells in TCE degradation. The number of toluene degrader originally available on these materials was approximately 10^5 CFU g material^{-1}.

4.4.2 Optimization of *Rhodococcus* sp. L4 immobilization on cumin seeds

Cumin seeds were selected as immobilizing support for *Rhodococcus* sp. L4 in this experiment. To prevent an easy detachment of cells from the seeds, we investigated the production of exopolysaccharides/extracellular polysaccharides (EPS) by cells during the immobilization. EPS are carbohydrate polymers that are secreted by a wide variety of bacteria. The attachment of cells to solid surface is facilitated by EPS synthesis (Vandevivere and Kirchman, 1993; Obuekwe and Al-Muttawa, 2001; Olofsson *et al*., 2003). Consequently, the high amount of EPS could minimize the leakage of cells during TCE biodegradation and making them attractive for further repeated application.

In this experiment, cells immobilization was done as described in methods 4.3.4.2, in which only sterilized cumin seeds were used. *Rhodococcus* sp. L4 was incubated with various sizes of cumin seeds (between 500 µm and ≥ 2 mm) for various periods (0-7 days) to determine the optimum incubation time for a stable cell attachment on cumin seeds. EPS were determined in the samples by Alcian Blue adsorption assay (see methods 4.3.6.3). The amount of EPS produced was estimated by the decline in absorbance of the supernatant at 606 nm. The A_{606} at time zero presented the 100% baseline.

There was a slight decline in A_{606} in control sterilized material without cells which might be due to abiotic factors e.g. the dye adhered to tested tubes. However, this was considered insignificant in this study. A small amount of EPS (5-10%) was detected after the first day incubation in all seed sizes (Fig 4.5). After 2 days, the production of EPS of immobilized cells on seed size ≥ 2 mm had stopped which yielded only 15% EPS throughout the course of study. Longer incubation resulted in larger EPS formation in seed sizes 500 µm -1 mm and 1-2 mm in which about 50% and 30% EPS was produced

after 4 days of incubation. After 4 days, the decline in A_{606} had stopped in both 500 µM-1 mm and 1-2 mm seeds size, indicating constant EPS synthesis after this period. The presence of EPS fibers probably led to the attachment of cells on the seeds as shown in SEM analysis (Fig. 4.6).

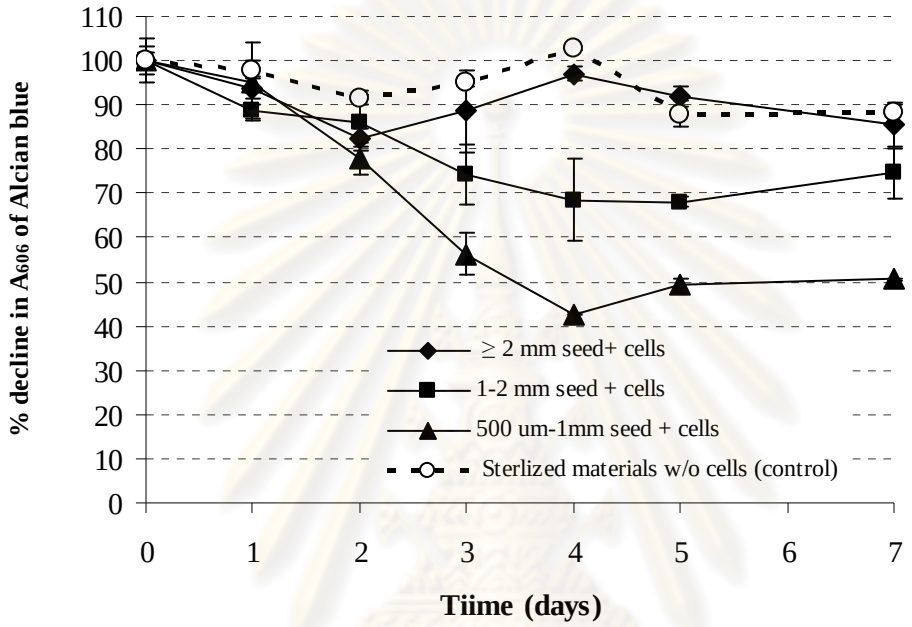


Figure 4.5 Percent decline in absorbance of Alcian Blue at 606 nm (A_{606}) after incubated with immobilized cells on three sizes of cumin seeds (500 µM-1 mm, 1-2 mm and ≥2 mm) separately (solid line) and the A_{606} of sterilized materials without cell as control (dash line). The seeds were ground and put through selected sieves before tested. The seeds size ≥ 2 mm were non-ground seeds.

The results indicated that the production of EPS was increased (seed size 500 µM-1mm > 1-2 mm > 2 mm) when the seed sized was decreased (Fig 4.5). This is probably because the increase of surface area i.e. 500 µM- 1mm > 1-2 mm > 2 mm seed size enabled a larger quantity of cells and consequently more EPS production for the

attachment. ESO and ESOC readily released from ground seeds i.e. size 500 µM-1 mm or 1-2 mm might stimulate EPS formation as well. While the liberation of ESO and ESOC from non-ground seeds (size ≥ 2 mm) were prevented by their outer shell, suggesting that less EPS were produced. Similarly, citronellol and cinnamaldehyde, the main ESOC in *C. nardus* and *C. cassia* extract, stimulated ESP formation in *E. coli* ATCC 33456 and *P. aeruginosa* PAO1, respectively (Niu and Gilbert, 2004). The authors suggested that high concentration of these compounds (greater than 1.75 mM) can activate a stress-induced response possibly by increasing EPS production or by acting as a poly-L-lysine-like adhesive.

Nonetheless, several forces could be involved in the attachment of *Rhodococcus* sp. L4 on cumin seeds surface. The surface charge of our bacteria determined experimentally was negative and the cumin seeds were positively charged (Table 4.2), thus the electrostatic forces could involve the attachment/adsorption process. Hydrophobic interaction and covalent bond formation were also important. Since, hydrophobic group or a variety of reactive groups on the microbial surface could interact with hydrophobic or specific groups on the materials (Cohen, 2001).

At the end of this part, 500 µm – 1 mm ground cumin seeds and 4-day incubation period were chosen for immobilization of *Rhodococcus* sp. L4 to obtain a strong attachment of cells on the seeds before subsequent TCE biodegradation test.

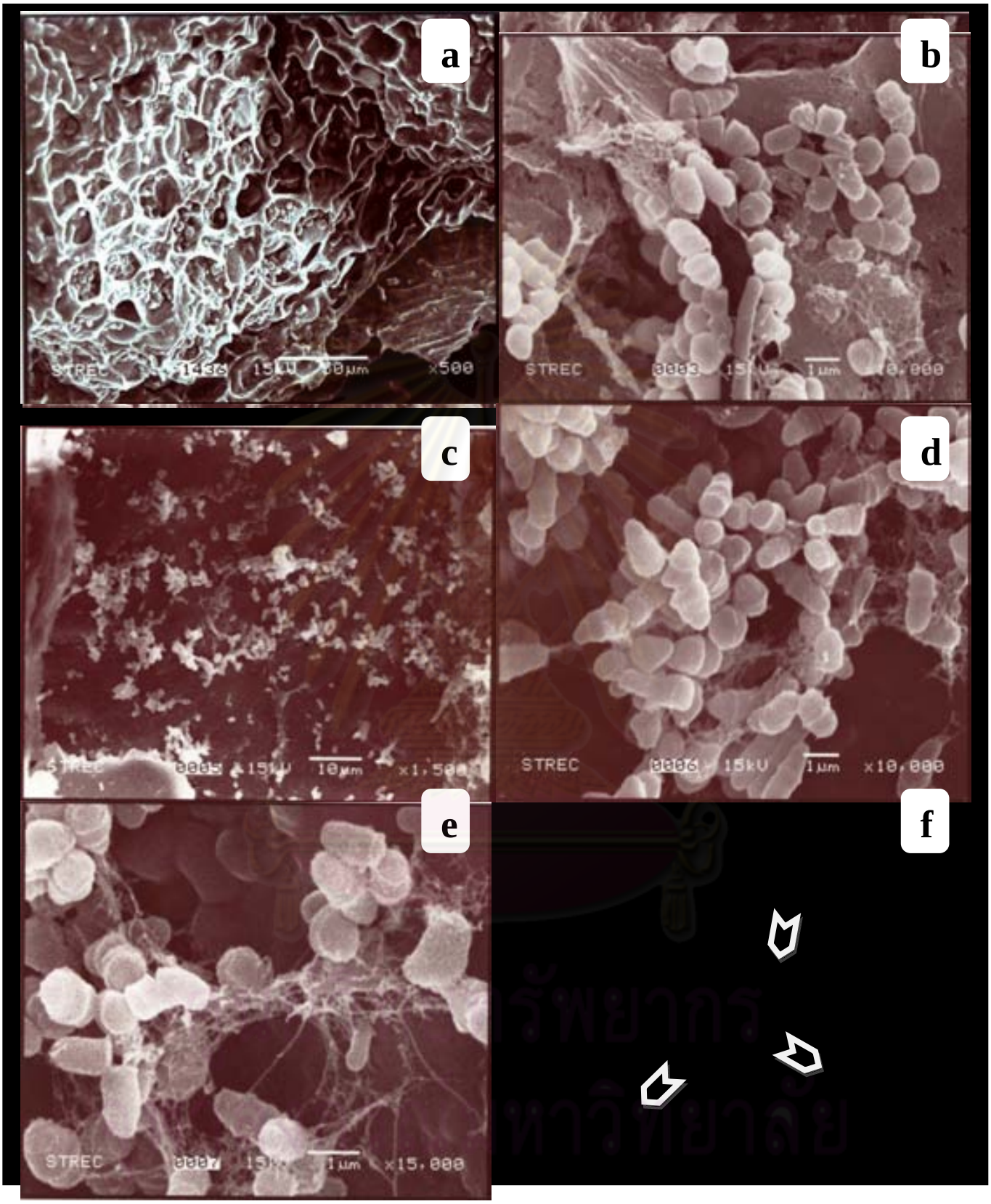


Figure 4.6 SEM (x 500-15,000) showing bacteria cells attached on cumin seeds following 4 days incubation (a)-(f) with a network of cells attached on cumin seeds surrounded by exopolysacccharides as pointed by arrows (f).

4.4.3 TCE biodegradation by immobilized cells on cumin seeds

Rhodococcus sp. L4 immobilized on cumin seeds obtained from the previous experiment was used for TCE biodegradation test here. TCE degradation ability of the immobilized cells was calculated as TCE transformation capacity (T_c) defined as the maximum mass of TCE that could be degraded prior to inactivation per mass of biomass added (μg of TCE mg dry cells^{-1}) and initial specific rate of TCE degradation, k_c (mg TCE mg cells^{-1} day^{-1}) described by Alvarez-Cohen and McCarty (1991a). These two parameters are commonly used for estimating the TCE-degrading ability of microorganisms. T_c is a measure of the cumulative effects of TCE product toxicity in the absence of other substrate (non-growing cells assay) while k_c is a measure of the maximum enzyme efficiency.

4.4.3.1 Effect of the amount of immobilized materials on TCE removal

In the first section, we determined the optimal amount of immobilized materials for TCE removal. The biodegradation experiment consisted of 0.02, 0.1 or 0.2 g of immobilized seeds which yielded 0.55, 2.75 and 5.5 mg of cell dry weight. They were incubated with 0.16 μmol TCE (14 μM initial aqueous concentration) in 22-mL headspace vials containing 2 mL MS medium. This concentration of TCE was used according to the whole-cell study (CHAPTER III) at which our bacteria were capable of high TCE degradation. A set of uninoculated materials was used as control for TCE adsorption on the bacteria and/or the seeds. TCE removal of both immobilized cells and control materials were relative to amount of TCE at time zero. After 4 days incubation, about 50 % of TCE was removed by 0.02 g of immobilized cumin seeds (0.55 mg of cell dry weigh), while 15 % of TCE was adsorbed by control material. There was no evidence of TCE removal when the larger amount of immobilized seeds i.e. 0.1 or 0.2 g was

applied (Fig. 4.7). Moreover, higher amount of TCE was detected at the end of study compared to that of time zero (Fig.4.7). It was implied that TCE might be adsorbed on large amounts of materials/cells i.e. in 0.2 g treatment when the test started, then it dissolved to the liquid medium later. It could be suggested that high amounts of cells and materials might prevent TCE degradation by diffusion limitation. As reported by Uchiyama *et al*. (1995) the TCE degradation efficiency decreased with the increasing cell density from 10 to 70 mg ml gel $^{-1}$, which resulted from the limitation of oxygen and TCE diffusion in *Methylocystis* sp. strain M immobilized in calcium alginate gel.

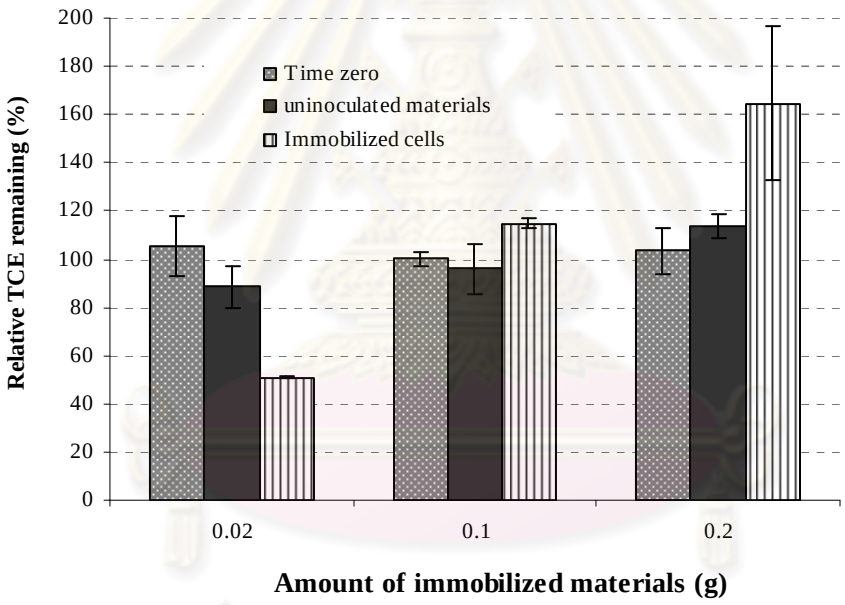


Figure 4.7 TCE remaining after 4 days incubation with different amount of immobilized and uninoculated materials i.e, 0.02, 0.1 and 0.2 g relative to amount of TCE at time zero (%).

After obtained the optimum amount of immobilized materials of 0.02 g for TCE removal, the TCE transformation efficiencies were further determined. With 0.02 g

immobilized materials, we could achieve T_c value of 24.72 ± 0.85 µg TCE mg dry cells^{-1}. This T_c value of immobilized cells was higher than that of free cells induced with cumin essential oil (9.72 ± 0.59 µg TCE mg dry cells^{-1}). It was suggested that the cells immobilized on cumin seeds could be protected from undesired environment such as toxicity of TCE transformation products or high TCE concentration.

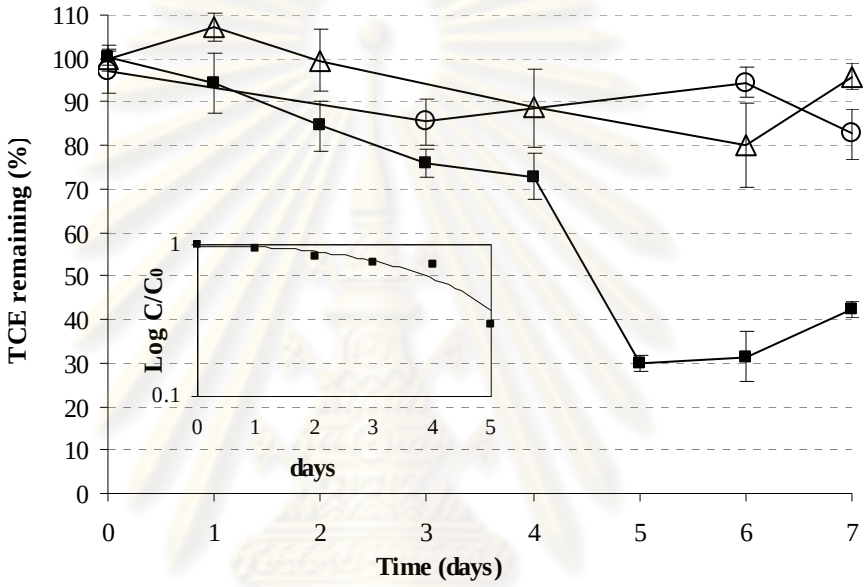


Figure 4.8 Time course of 14 µM TCE degradation with 0.02 g immobilized cell on cumin seeds (0.55 mg dry cells) (■) and controls consisted of sterilized material without cell (Δ) and killed immobilized cells (O) during 7 days incubation. TCE mass was calculated from the summation of TCE mass remained in both gas and aqueous phase. The inset showed a plot of logarithm of C/C_0 as a function of time.

As shown in Fig 4.8, the initial TCE degradation rate, k_c, (between 0-5 days) were given by dC/dt (zero-order kinetic was fitted as a plot of logarithm of C/C_0 as a function of time). The estimated initial specific rate of TCE degradation was about 0.0046 ± 0.0008 mg TCE mg cells^{-1} day^{-1} (1.9 nmol mg of protein^{-1}min^{-1}). The immobilized

bacteria probably need time to become active, thus TCE degradation during the first 4 days was slow. Several investigators reported the mass transfer limited in immobilization system led to a decrease in activity of immobilized cells (Uchiyama *et al*., 1993; Chen *et al*., 2004; Chen *et al*., 2007).

The reports with *Methylocystis* sp. M immobilized in calcium alginate showed high k_c value for TCE (0.0744 mg TCE mg cells^{-1}day^{-1}) (Uchiyama *et al*., 1993). A recent study by Chen *et al*. (2007) found that the rate of TCE degradation by *Pseudomonas putida* immobilized in chitosan-bead was 0.0086 mg TCE mg cells^{-1}day^{-1}. The higher rate of these studies might be due to the presence of primary substrate i.e. methane or phenol during the test. The decrease in essential oils contained in cumin seeds during processes (data not shown) could be the cause of lower degradation rate in our study. Although these kinetic parameters are specific for the tested bacterial strain, the values also depended on the test conditions.

4.4.3.2 Effect of increased TCE concentration on T_c

The maximum transformation capacity of immobilized cell at different TCE concentrations was determined as illustrated in Fig 4.9. The T_c value for free cells increased with TCE concentrations from 2 µM to 25 µM (0-16 µg TCE mg dry cells^{-1}), beyond which T_c decreased sharply (Fig 4.9). Free cells could not further degrade TCE at concentration higher than 50 µM. While immobilized cells could sustain TCE degradation for all TCE concentrations with the maximum T_c of 59 µg TCE mg dry cells^{-1} (Fig 4.9). The much higher T_c value of immobilized cells compared to free cells indicated that the immobilized cells were resistant to toxicity caused by high TCE concentrations. Other known toluene, phenol, propane or ammonia degraders have T_c values between

5.2–34.0l µg of TCE mg cells^{-1} (Heald and Jenkins, 1994; Folsom *et al*. 1990; Chang and Alvarez-Cohen 1995; Ely *et al*. 1997), thus our observed T_c for immobilized cells was relatively high.

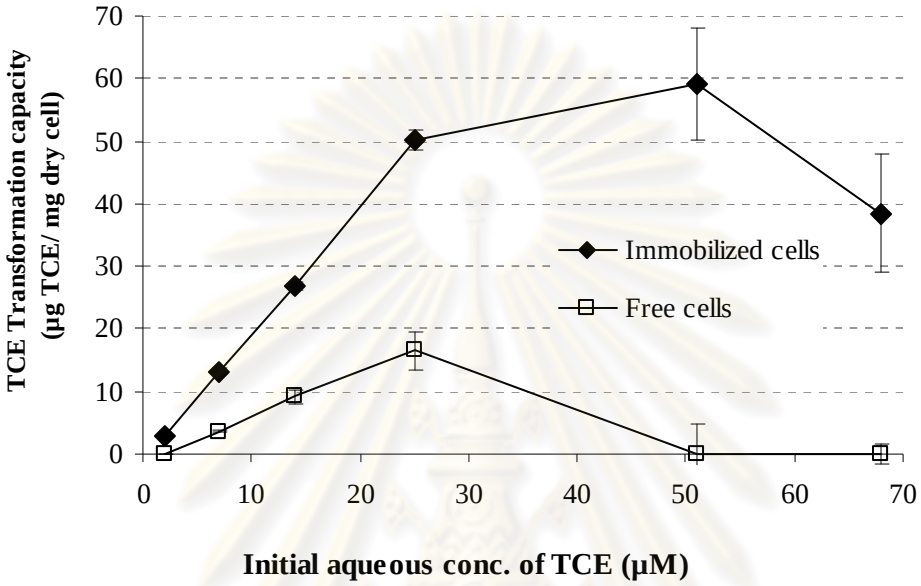


Figure 4.9 TCE transformation capacity (T_c) for suspended and immobilized *Rhodococcus* sp. L4 on cumin seeds as a function of TCE concentration after 5 days incubation.

4.4.3.3 Effect of repeated addition of TCE on its degradation

To determine whether the immobilized cells were able to degrade TCE continuously, TCE was repeatedly added to the immobilization system. The cell immobilization by attachment technique was done as described previously. Briefly, 0.02 g immobilized seeds were incubated with 0.16 µmol of TCE (14 µM initial aqueous concentration). To make sure that the immobilized cells were active and able to degrade TCE, we allowed the bacteria to degrade some TCE before the constant amount of TCE was added repeatedly to the vials in total 4 additions over 7 days (as pointed by arrows).

The initial aqueous concentration of TCE was kept in the range of 50 µM to avoid the possibility of dose-dependent substrate inhibition. Every 24 h, the samples were analyzed for remaining TCE by HS-GC-FID. The killed immobilized cells on cumin seed were used as control for presenting TCE loss by abiotic processes e.g. TCE adsorption on cells and/or on seeds.

In this experiment, TCE loss from abiotic factors was insignificant at the beginning but its loss became larger at the end of study as 20% TCE was removed by abiotic vials (Fig. 4.10). At day 4, nearly 40% of total TCE mass was degraded by immobilized cells while a small amount of TCE (6%) was removed in the control treatment (Fig. 4.10). After 4 days, this system had a twice the amount of TCE at time zero. At day 5, the immobilized cells removed up to 45% of added TCE (from 0.22 µmol to 0.12 µmol), while only 15% of TCE (from 0.32 µmol to 0.27 µmol) was removed by the control treatment. Further addition of TCE resulted in about 40-50% TCE degradation in immobilization vials whereas TCE loss by abiotic control was about 20% at the end of study. As a result of 4 addition cycles, the total amount of TCE transformed (T_c) reached 94.5 µg TCE mg dry cells^{-1}, corresponding to about four times the amount degraded in the first 5 days (24.72 µg TCE mg dry cells^{-1}) (Fig 4.8). This T_c value was in the range of those reported for Methanotrophs (without exogeneous reductant addition) which had T_c between 43-320 µg TCE mg dry cells^{-1}(Alvarez-Cohen and McCarty, 1991a; Tompson *et al*., 1994; Chu and Alvarez-Cohen, 1996; Chang and Alvarez-Cohen, 1995; Smith *et al*., 1997). It was clear that our immobilized cells could maintain TCE-degrading ability during the repeated TCE addition and suggested that the bacteria were protected from the toxicity of TCE/its intermediates. However, the activity of immobilized cells tended to

decrease as indicated by the TCE accumulation after 4 addition cycles. The oxygen might be limiting since we did not provide additional oxygen during the study.

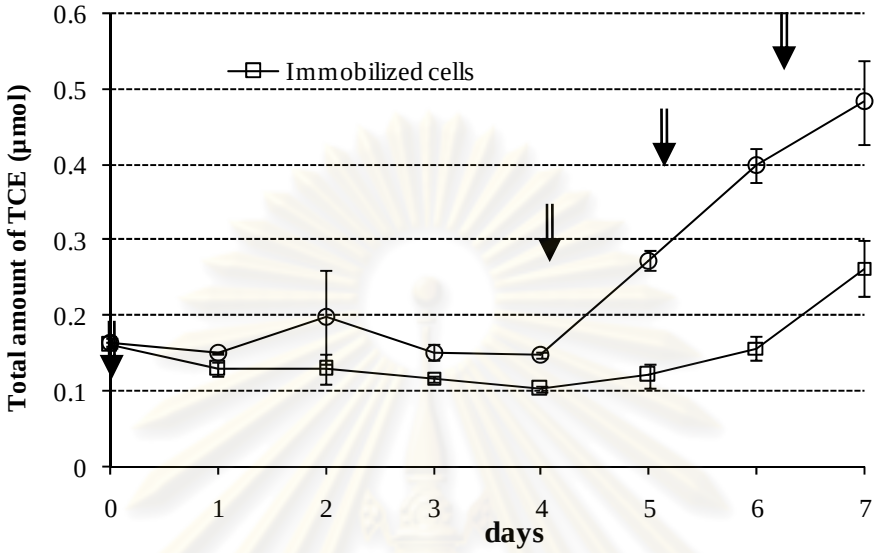


Figure 4.10 Time course of TCE degradation in immobilized cells on cumin seeds (□) and control killed immobilized cells (o). The arrows showed repeated addition of TCE to vials. All data are the average of triplicate samples with the standard deviations (error bars) given.

4.4.3.4 The reusability of immobilized bacteria for TCE degradation.

(1) TCE degradation ability

TCE degrading ability of the immobilized cells on cumin seeds were determined after repeated use. The immobilized cells were prepared as described in materials and methods. The application was started by incubating them with TCE (0.16 µmol), and analyzed for the amount of remaining TCE after 5 days incubation. Generally, the bacteria/enzymes responsible for TCE degradation were inactivated after degradation process (see CHAPTER V), thus methods for their reactivation are necessary. The used immobilized cells were reactivated by putting them in MS medium for 12 h or 24 h before

reusing them in another TCE biodegradation system. Providing the bacteria with the new environment (without TCE) along with their original media, they utilized the residual essential oil for growth and production of new enzymes. At the end of study, the amount of remaining TCE was determined for each application cycle compared to those without reactivation. A set of samples containing killed immobilized cells on cumin seeds were used as controls for TCE removal by abiotic factors.

For the first application, immobilized cells were able to degrade about 64% of TCE compared to control. The TCE degradation ability of all samples was insignificantly different. About 27-37% of TCE was degraded in the second application. However, the samples without the reactivation could not further degrade TCE in the third application, whereas both reactivated samples (12 h and 24 h) were capable of degrading TCE. 62% and 17% TCE was removed, respectively (Fig 4.11). It was suggested that 12 h reactivation time was sufficient for the immobilized bacteria.

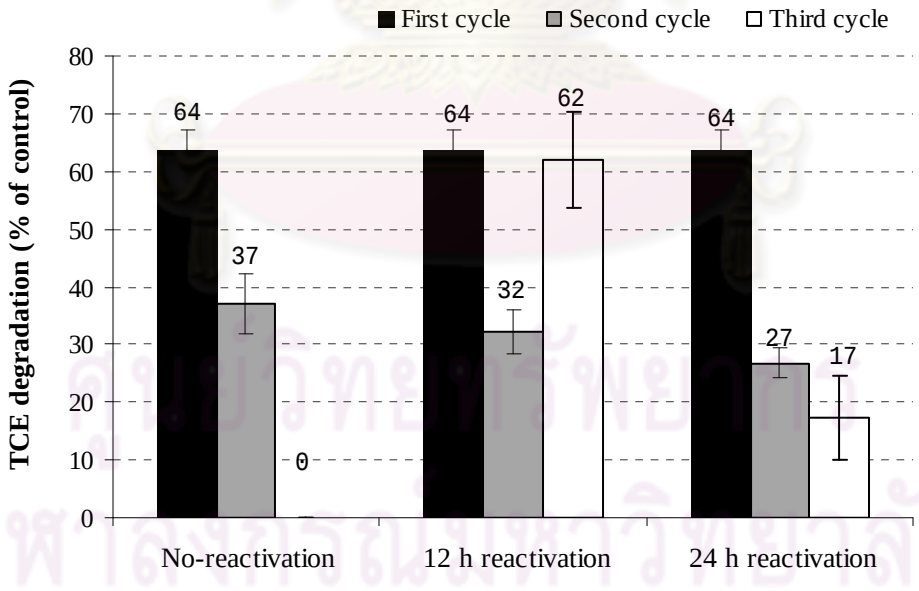


Figure 4.11 Percent of TCE degradation by immobilized cells on cumin seeds in each application cycle compared to control killed immobilized cells after 5 days incubation.

The samples consisted of non-reactivated immobilized cells (1), immobilized cells were reactivated by placing in MS medium for 12 h (2) or 24 h (3).The total mass of TCE in each application cycle was 0.16 µmol (14 µM initial aqueous concentration).

(2) Number of toluene/TCE degrading bacteria

The number of toluene/TCE degraders was used to determine the survival of *Rhodococcus* sp. L4 immobilized on cumin seeds. The data were obtained only from the cells without reactivation. The results showed that toluene populations on the seeds increased from approximately 9.4 x 10^{10} CFU g $seed^{-1}$ at the beginning of study to 2.9 x 10^{11} CFU g $seed^{-1}$ after 5 days of incubation (Table 4.4). This could be a result of essential oils, organic content and/or other nutrients available in the seeds (Table 4.2) that could support bacteria growth and cell multiplication. The effect of essential oil and its components on enhanced bacteria growth was described in CHAPTER III. Similarly, Hernandez et al. (1997) demonstrated that soil enriched with plant materials rich in essential oils i.e. orange peel, ivy leaves, pine needles or eucalyptus leaves resulted in 10^5 times more biphenyl utilizers (10^8 CFU g $soil^{-1}$) than in unamended soil (10^3 CFU g $soil^{-1}$) and simultaneously polychlorinated biphenyls (PCBs) degradation was induced. Moreover, Morono et al. (2006) reported that cumene, an essential oil component in cumin oil, could be a substrate for several toluene degraders.

Although, the number of bacteria had increased after the second and third application cycle (0.9-1.0 x10^{13} CFU g $seed^{-1}$), the TCE degradation efficiency was not proportional to their growth (Fig 4.11). This may be explained by the data in CHAPTER V (Fig. 5.3), which showed that some of enzymes involved in TCE degradation were inactivated by TCE transformation rather than cell death and the recovery of TCE

degradability was suggested to be due to the reactivated cells themselves, not the increase in cell number.

About 2.5-920 x10^6 CFU mL media^{-1} of bacteria was removed from the seeds into the liquid medium (Table 4.4). When compared between the material and media, the densities of bacteria were remarkably lower at all time points, indicating that our bacteria formed a sufficiently strong attachment to the seeds and these immobilized cells were suitable for reuse in TCE biodegradation.

Table 4.4 Number of toluene/TCE degrading bacteria in each application cycle after 5 days incubation.

Application cycle	**Number of bacteria[a]**	
	On materials (X 10^6 CFU g seed^{-1})	**In liquid media (X10^6 CFU mL media^{-1})**
1st cycle	290,000	2.5
2nd cycle	10,300,000	230
3rd cycle	9,050,000	920

[a] The data obtained from the no-reactivation condition. The initial number of bacteria immobilized on cumin seeds before TCE biodegradation test was 9.4 x 10^{10} CFU g seed^{-1}.

CHAPTER V

EFFECT OF TCE ON ACTIVITY AND RECOVERY OF TOLUENE DIOXYGENASE FROM *RHODOCOCCUS* SP. L4

5.1 Introduction

Various nonspecific oxygenase enzymes have been reported to initiate TCE cometabolic degradation such as toluene 2-monooxygenase (Newman and Wackett, 1997), toluene 4-monooxygenase (Leahy *et al*., 1996), toluene dioxygenase (Heald and Jenkins, 1994; Morono *et al*., 2004) and soluble methane monooxygenase (Oldenhuis *et al*., 1991; Chu and Alvarez-Cohen, 1998). These oxidative enzymes generally catalyze a reaction that incorporates one (monooxygenases) or two (dioxygenases) atom(s) of molecular oxygen into the substrates. Most of TCE-degrading oxygenases are not constitutively produced in bacterial cells. Their production is typically induced by the presence of a particular growth substrate such as methane, ammonia, phenol, toluene, isoprene or cumene (isopropylbenzene). Toluene oxygenases (TDO) are one of the most effective TCE-degrading enzymes that have been studied extensively. Leahy *et al*. (1996) reported that bacteria with different toluene oxygenases demonstrated different TCE degrading capabilities.

One of the factors limiting the effectiveness of TCE oxidation is the inactivation of oxygenase enzymes by TCE itself and/or by the diffusible and reactive TCE oxidation products. This inactivation has been previously shown in several whole-cell studies (Oldenhuis *et al*., 1991; Heald and Jenkins, 1994 Yeager *et al*., 2001a,b; Halsey *et al*., 2005) as well as in studies with purified enzymes such as soluble methane

monooxygenase from *M. trichosporium* OB3b, toluene dioxygenase from *P. putida* F1, and toluene 2-monooxygenase from *B. cepacia* G4 (Fox *et al*., 1990; Li and Wackette, 1992; Lange and Wackett, 1997; Newman and Wackette, 1997). However, the toxic effects of TCE with different oxygenases varied (Arp *et al*., 2001). Li and Wackett (1992) suggested that TCE was oxidized to formic and glyoxylic acids in a 2:1 ratio by toluene dioxygenase and enzyme inactivation was observed from reactive acyl chloride intermediate compounds that alkylate proteins. Many reports showed that some bacteria could recover from TCE toxic effects e.g. nitrifying bacterium *N. europaea* (Hyman *et al*., 1995), toluene degrading bacteria *B. cepacia* G4 (Yeager *et al*., 2001b).

Bacteria from genus Rhodococcus exhibit broad substrate specificity for the degradation of many environmental pollutants e.g. aromatic compounds, halogenated hydrocarbon, alkanes, substituted benzenes (Finnerty, 1992; Bell *et al*., 1998). However, only two types of Rhodococcus enzymes i.e. alkene monooxygenase in *R. corallinus* and isopropylbenzene/toluene dioxygenase in *R. erythropolis* BD2 have been reported for TCE degrading ability (Saeki *et al*., 1999; Dabrock *et al*., 1992, 1994). Neither of them was studied for the effect of TCE on the enzyme level or the extent of recovery of oxygenase after exposure to TCE. In this chapter, we focused on studying the effect of TCE on activity and recovery of partially purified TDO from *Rhodococcus sp*. L4 isolated from petroleum contaminated soil. This bacterium could utilize many organic compounds as a sole carbon source such as BTEX, essential oil (lemon, lemon grass or cumin oil) and their components (limonene, cumene or pinene). Its TCE degradation ability was observed while growing on toluene or essential oil/essential oil component (Luepromchai, 2003; Suttinun *et al*., 2008). The enzyme involved in TCE degradation was preliminary characterized as toluene dioxygenase (Luepromchai, 2004).

From the results in our whole-cells study (CHAPTER III), TCE degrading activity of induced *Rhodococcus* sp. L4 was effective only a short period in the absence of substrate/inducer. The repeated addition of essential oil component e.g. cumene could considerably enhance TCE degradation. Dabrock *et al*. (1994) reported that Cumene-grown cells have been reported to stimulate TCE degradation and contained isopropylbenzene dioxygenase genes analogous to toluene dioxygenase. The presence of cumene restored TCE degradation activity of toluene dioxygenase in *P. putida* F1 by displacing inhibitors i.e. TCE molecule or its reactive intermediate from the enzyme active site (Morono *et al*., 2004). It is therefore interesting to study the effect of cumene on TDO activity of *Rhodococcus* sp. L4 during TCE oxidation. Cumene (isopropylbenzene), a trace component in cumin essential oil, was used as a sole carbon source for cultivation of *Rhodococcus* sp. L4 in this study.

5.2 Methodology outline

5.2.1 Growth and preparation of cells

5.2.2 Preparation of cell-free crude extract

5.2.3 Partial purification of crude protein extract by ammonium sulfate precipitation

- Optimizing of ammonium sulfate saturation

5.2.4 Assay for toluene dioxygenase (TDO) enzyme activity

- Effect of TCE on TDO activity

- The recovery of TDO activity after exposure to TCE

- Protection of TDO during TCE oxidation by addition of cumene

5.3 Materials and methods

5.3.1 Chemicals and reagents

Toluene (99.5%) was purchased from Merck. TCE (99.5%) and cumene (99.0%) were obtained commercially from Fluka Chemical Industrial. TCE and cumene stock solutions were prepared by dissolving aliquots of TCE and cumene in N,N-dimethylformamide (Merck, USA) to obtain the desired concentration. Tris (Fisher Scientific, Inc.) was used for preparation of Tris-Cl buffer, pH 7.5 for enzyme activity assay. Indole was obtained from Fluka, Switzerland. Dithiothreitol (DTT) and β-nicotinamide adenine dinucleotide, reduced form (NADH) were purchased from Sigma-Aldrich. Ammonium sulfate and Iron sulfate were purchased from Carlo Erba Reagent. All chemicals used were of the highest purity available. BugBuster Master Mix reagent (Novagen) was used for protein extraction. Bradford reagent (Bio-Rad) was used for protein analysis with bovine serum albumin (BSA), 2 mg mL^{-1} as a protein standard.

5.3.2 Bacterial strain, culture maintenance and growth conditions

Rhodococcus sp. L4 is deposited at the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR) and given the accession number TISTR 1542. The partial 16S rRNA gene sequence of *Rhodococcus* sp. L4 is available in GenBank under the accession number EF527237. The bacterium used in this study was maintained by culturing on mineral salts (MS) agar incubated in a glass box equilibrated with toluene (its enrichment substrate) vapor at room temperature. Approximately every 10-14 days the culture was transferred to a new MS-agar for maintaining the ability of enzyme production by bacteria throughout the course of study.

MS medium is a chloride-free minimal medium prepared according to Focht (1994) with details in Appendix A. To allow the bacteria to acclimatize to the liquid media prior to use in the experiment, the pre-inoculum was prepared by culturing a loopful of *Rhodococcus* sp. L4 (on MS-agar) in a 250-mL Erlenmeyer flask containing 100 mL MS medium that was supplied with growth substrate in the vapor phase by adding 200 µL toluene (12 mmol L^{-1}gas phase) in the Eppendorf tube fixed at the top of the flask (Fig. 3.1). The culture was incubated overnight at room temperature with shaking at 200 rpm.

5.3.3 Bacterial cultivation

Rhodococcus sp. L4 was cultured by transferring 60 mL late-log phase of pre-inoculum into a 2-L Duran bottle containing 540 mL of MS medium. The optical density (OD) at 600 nm was about 0.08-0.1 at the beginning. The growth substrate was supplied as a vapor by adding 600 µL of cumene (3 mmol L^{-1}gas phase) to a sterilized Eppendorf tube fixed at the top of the bottle. The cultures were incubated for 60 h on an orbital shaker at 180 rpm, room temperature. Then, the late-log phase cells were harvested by centrifugation at 2516 x *g* (7,500 rpm), 4^0C for 10 min. Cells were washed, resuspended in MS and centrifuged as before. The pellet was allowed to drain and as much liquid as possible was removed before storage at -20 ^{0}C.

5.3.4 Preparation of cell-free crude extract

In general, TDO is produced intracellularly (inside cells) after bacterial induction. Therefore, the *Rhodococcus* sp. L4 cells were disrupted to release their protein content into the aqueous phase and the cell-free crude extract was used for further analysis. The crude extract enzyme was prepared from 1.5 g of frozen cell paste by resuspending of

thawed cells in 5 mL of BugBuster Master Mix reagent. The cell suspension was incubated on shaker at a slow speed and at room temperature for 25-30 min. The insoluble cell debris was removed by centrifugation at 7558 x *g* (13,000 rpm) for 30 min at 4 ^{0}C. The supernatant (cell-free crude extract) was transferred to a fresh Eppendorf tube, filtered through a microfilter with 0.2 µM pore size to eliminate the remains of the cell debris in the supernatant and stored at -20 ^{0}C until needed.

5.3.5 Partial purification of crude protein extract by ammonium sulfate precipitation

The crude protein extract obtained from 5.3.4 was partially purified by ammonium sulfate precipitation. Ammonium sulfate protein precipitation is a commonly used technique for concentrating dilute solutions of proteins and for fractionating a mixture of proteins because it is highly soluble in water and stabilizes most proteins in solution (Scopes, 1987). This method could remove any nucleic acids, lipoproteins and lipids from the protein solutions. Protein precipitates at specific concentrations of ammonium sulphate relative to their net charge density.

The crude extract was put in a pre-chilled beaker which was placed in a plastic container containing a mixture of sodium chloride and ice. The crude extract protein was precipitated by addition of a solid ammonium sulfate to make 0 to 40%, 40 to 60% and 60 to 80% saturated fractions. The calculated amount of ammonium sulfate (Engel, 1996) was added slowly to the crude extract protein while mixing with a magnetic bar at 4 ^{0}C. After stirring for 1 h, precipitated protein was collected by centrifugation at 7558 x *g* (13,000 rpm) for 30 min at 4 ^{0}C. The pellet was resuspended in 100 mM Tris-Cl buffer (pH 7.5) containing 10% glycerol, 10% ethanol (TEG buffer) and 1mM dithiothreitol

(DTT) with a ratio of 1 gram pellet: 2 mL buffer. The supernatant was kept for subsequent saturation concentrations i.e. 40 to 60% and 60 to 80% where similar procedures were repeated till the precipitated protein was obtained in each precipitation step. Proteins from three fractions were tested for TDO activity based on indoxyl formation as described below. The fraction of protein with highest activity was used for TCE oxidation assay.

5.3.6 Protein analysis

Amount of protein in the crude extract and in each precipitated protein was determined by Bradford assay in which 3 mL of 1x dye reagent (Bio-Rad) was added in disposable cuvettes containing 60 µl standard or unknown sample solution and were mixed thoroughly. The mixture was incubated at room temperature for 30 min and the absorbance was measured at 595 nm. Buffer filled with dye reagent was used as a blank reference. Bovine serum albumin (BSA), 2 mg mL^{-1} was used as a protein standard. The protein was assayed in triplicate.

5.3.7 Toluene dioxygenase (TDO) activity assay

TDO activity in crude extract (see 5.3.4) and partially purified protein (from 5.3.5) was assayed according to a modified method of Jenkins and Dalton (1985), which is based on the measurement of a yellow dye (indoxyl) by spectrophotometry (A_{400}) (Specord 40 plus WinASPECT software, Analytik Jena AG). Indoxyl, a soluble yellow dye is formed via oxidation of indole to indigo by TDO (see Fig 5.1). A small amount of indigo was detected at A_{600} when the reaction was incubated longer. This very small increase in absorbance limited the accuracy and precision of the measurements. Therefore, indoxyl measurement was prefered. The 3 mL reaction assay was performed in

a quartz cuvette consisting of 100 mM Tris-Cl buffer, 0.1 mM $FeSO_4$ (co-factor), 2 mM NADH (electron donor), and protein i.e. crude extract or partially purified protein. The reaction was initiated by the addition of indole to 0.2 mM. This concentration of indole was reported to have no inhibition effect on indole oxidation by toluene dioxygenase (Jenkins and Dalton, 1985). The sample was mixed thoroughly before the absorbance at 400 nm was measured against a blank containing all ingredients except indole.

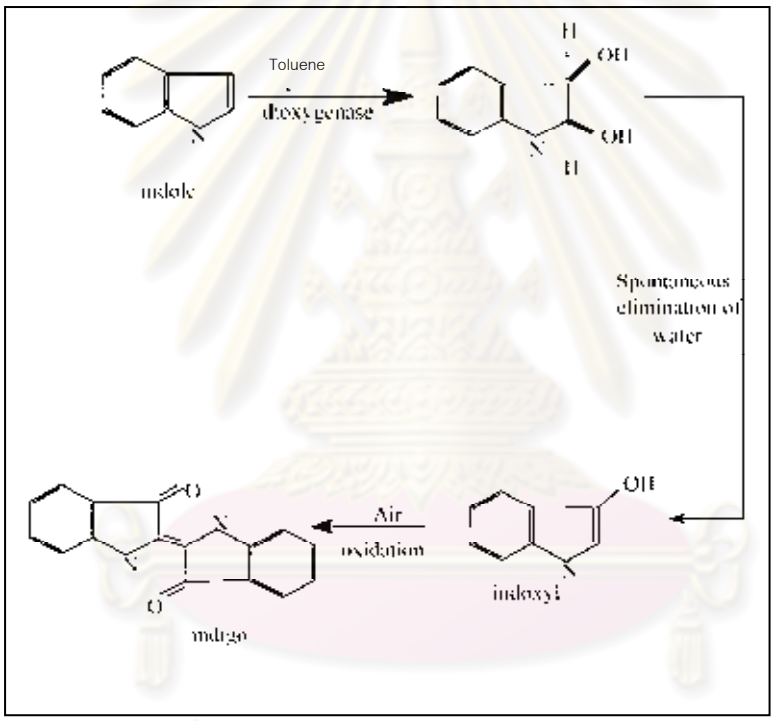


Figure 5.1 A proposed indole-degradative pathway to indoxyl and indigo by toluene dioxygenase (modified from Jenkins and Dalton, 1985).

5.3.8 TCE oxidation by TDO

The study of TCE oxidation by TDO was divided into 3 experiments as follows;

(1) Effect of TCE on TDO activity

To observe whether the TCE-degrading intermediates or TCE itself inactivate TDO enzyme activity, the partially purified protein from 5.3.5 was used for TDO activity assay in the presence of TCE. A 3 mL reaction mixture contained all ingredients as described in TDO assay including TCE at initial concentrations of 10, 20, 40, 60 or 80 µM. The total TCE added was calculated as being in the 3-mL liquid phase. The TDO activity assay was measured by the absorbance at 400 nm. Control is a set of samples without TCE. The sample measurements were made in duplicate.

(2) Recovery of TDO activity exposed to TCE

The recovery of TDO activity after exposure to TCE was determined. First, the activity of TDO in the presence of 80 µM initial aqueous concentration of TCE was measured as described in (1). Then the reaction mixture was incubated in a dialysis bag (typical molecular weight cut-off = 14,000), which has the ability to retain the protein (enzyme) but not TCE. The bag was submerged in 100 mL TEG buffer and stirred continuously by magnetic stirrer. The experiment was carried out in a 250-mL beaker that was put on ice to maintain the enzyme activity. The amount of TCE outside dialysis bag was analyzed by headspace GC-FID (see CHAPTER III for TCE analysis) at designated times to ensure that the equilibrium was reached. After 3 h incubation, the buffer outside the bag was replaced and the incubation was continued for another 3 h. The solution inside dialysis bag was collected and assayed for TDO activity following TCE exposure

as described above. TDO activity was compared to that during TCE exposure. The sample measurements were made in duplicate.

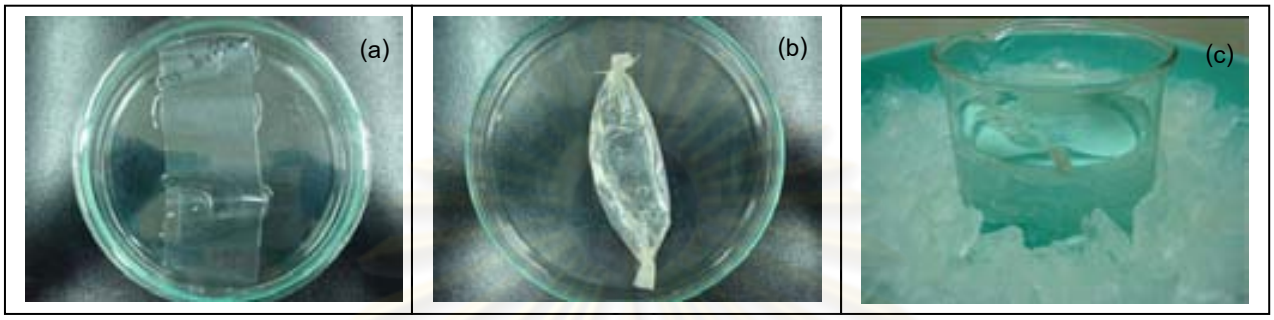


Figure 5.2 The recovery of TDO activity after exposure to TCE was determined in dialysis bag: (a) dialysis bag placed in buffer before used (b) dialysis bag containing 3 mL enzyme assay and TCE and (c) dialysis bag submerged in TEG buffer to remove TCE.

(3) Protection of TDO during TCE oxidation by addition of cumene

The presence of cumene, a primary substrate for TDO could protect TDO inactivation during TCE oxidation. In this experiment cumene was added into the reaction mixtures containing 80 µM TCE and all ingredients ot the TDO assay (5.3.7). The TDO activity was measured as described above. The result was compared to those without cumene addition. The sample measurements were made in duplicate.

5.4 Results and discussion

5.4.1 TDO activity of crude protein extract

(1) TDO activity of partially purified crude extract from various ammonium sulfate saturated fractions

Crude protein extract from *Rhodococcus* sp. L4 was partially purified by various saturated fractions of ammonium sulfate i.e. 0 to 40%, 40 to 60% and 60 to 80% described in 5.3.5. Precipitated protein was collected from each precipitation step and tested for TDO specific activity based on the ability of this enzyme to transform indole to indoxyl. This assay was performed by measuring the rate of increase in indoxyl absorbance at 400 nm (A_{400}) normalized to protein content of the sample. The reference consisted of all ingredients except indole. Since a commercial standard of indoxyl was not available, it was difficult to quantify the product formation. The estimated values of toluene dioxygenase (TDO) enzyme activity, purification fold, and yield were shown in Table 5.1. TDO specific activity of 0.0064 and 0.0036 A_{400} min^{-1} mg $protein^{-1}$ were detected only in crude protein extract precipitated with 0 to 40% and 40 to 60% ammonium sulfate saturated fractions, respectively (Table 5.1). Both of saturated fractions also showed higher purification of protein (3.76 and 2.12 fold) when compared to crude protein extract (non-purified protein), suggesting these steps are appropriate for enrichment of TDO. Yield increased when the ammonium sulfate concentration was increased up to 60%. TDO protein was not detected in fractions with more than 60% saturation. Part of TDO (7%) were precipitated between 0 to 40% saturation and most of the enzyme was precipitated at 40 to 60% (49% yield) saturation. However, to obtain as much as possible of TDO , we increased ammonium sulfate concentration directly from 0 to 60% in the next experiment.

(2) TDO activity of the partially purified crude extract from 0 to 60% ammonium sulfate saturated fractions

To confirm that ammonium sulfate fractions from 0 to 60 % saturation resulted in larger amount of TDO as suggested above, we tested the precipitated protein for TDO specific activity (Table 5.1). It was obvious that crude protein extract precipitated with 0 to 60 % saturation increased TDO activity to 0.0110 A_{400} min^{-1}mg $protein^{-1}$, a 6.47-fold purification and 350% yield. This implies that most of our TDO was precipitated at these optimum conditions. The TDO from this step was used to determine the effect of TCE on its activity and recovery in a further experiment.

Table 5.1 TDO activity, purification fold and yield in crude protein extract and crude protein extract precipitated with different ammonium sulfate saturation fractions

Samples	**Enzyme activity[a]** (A_{400} $min^{-1}mL^{1}$)	**Enzyme Volume** (mL)	**Total activity** (A_{400} min^{-1})	**Protein conc.** (mg mL^{-1})	**Specific activity[b]** (A_{400} min^{-1} mg $protein^{-1}$)	**Purification [c]** (fold)	**Yield[d]** (%)
1st test							
Crude extract	0.0110	3.85	0.0424	6.6267	0.0017	1	100
0-40%	0.0064	0.45	0.0029	1.0017	0.0064	3.76	6.84
40-60%	0.0227	0.92	0.0209	6.3683	0.0036	2.12	49.29
60-80%	ND	0.56	ND	5.9017	ND	ND	ND
2nd test							
Crude extract	0.0110	3.5	0.0385	6.6267	0.0017	1	100
0-60%	0.0900	1.5	0.135	8.2183	0.0110	6.47	350.65

[a] TDO activity was determined from the initial rate of indoxyl formation (in the first 20 min) by plotting the increase in indoxyl absorbance as a function of time ($\Delta A_{400}/\Delta t$).

[b] Specific activity was defined as initial rate of indoxyl formation (TDO activity) per mg of protein.

[c] Purification of protein precipitated with ammonium sulfate when compared to crude protein extract (non-purified protein) as one fold purification.

[d] Yield was determined from total activity of each precipitated protein compared to that of crude protein extract as 100%.

5.4.2 Effect of TCE on activity and recovery of TDO

(1) **Effect of TCE on activity of TDO**

To determine the effect of TCE on activity of TDO, various concentrations of TCE from 10 to 80 µM was added into the reaction mixtures containing all compounds as stated in materials and methods 5.3.7. The absorbance of indoxyl (A_{400}) in all TCE-containing samples was lower than that of the sample without TCE at all time points (Fig 5.3). This implied that TDO enzyme activity was inhibited by TCE. The inhibition depended on TCE concentrations. Sample with 20 µM TCE has a slightly lower amount of indoxyl than the control, while 40 µM TCE concentration decreased indoxyl production by about half. When TCE concentration exceeded 60 µM, the activity of TDO enzyme was completely inhibited and indoxyl was not detected throughout the study. It is possible that at 10 µM TCE, the TDO enzyme degraded TCE and produced reactive intermediates that inactivated the enzyme. On the other hand, the inhibition of TDO at high concentration was mainly due to TCE itself.

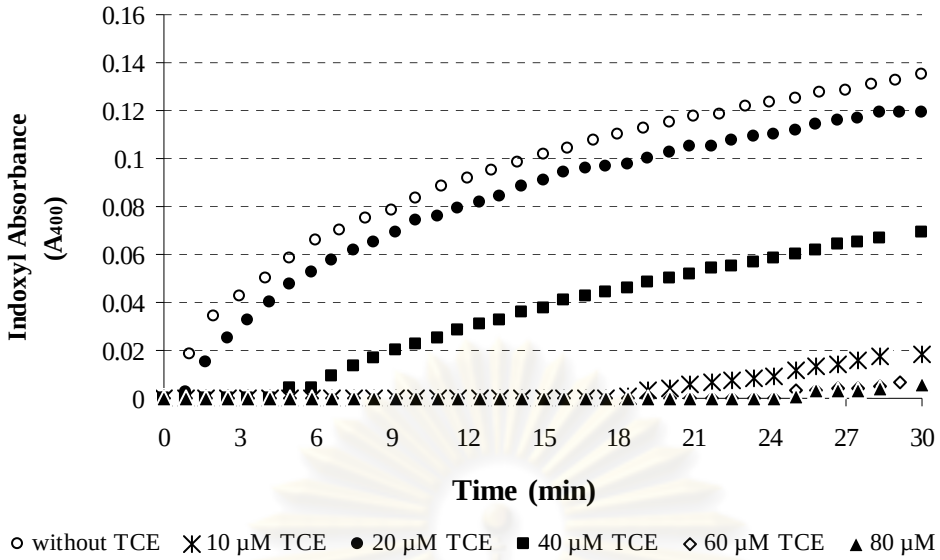


Figure 5.3 Assay of TDO enzyme activity. Absorbance of indoxyl (A_{400}) as a function of time for indole oxidation in the samples with/without TCE. The data were the average of duplicate samples.

In the absence of TCE, TDO achieved a maximum specific activity for indole oxidation about 0.057 A_{400} min^{-1}mg $protein^{-1}$, whereas the activity in the presence of TCE decreased to about 0.004 to 0.049 A_{400} min^{-1}mg $protein^{-1}$ in the first 30 min (Table 5.2). The results suggested that TCE molecules might competitively inhibit TDO activity for indole oxidation because TCE was also served as substrate for this enzyme. Previous investigations also showed a competition between primary substrate such as toluene, phenol or ammonia and TCE, which resulted in an inhibition of primary substrate oxidation as well as an inhibition of overall cometabolism processes (Folsom *et al*., 1990; Hyman *et al*., 1995; Kocamemi and Cecen, 2007). Another possibility might be that TDO was inactivated by TCE reactive product. Similarly, Newman and Wackett (1997) reported that purified toluene 2-monooxygenase was inactivated by reactive TCE-degradation intermediates such as TCE epoxide, formyl chloride, and glyoxyl chloride. The term competitive inhibition was mostly referred to when two substrates are present at

the same time for the enzyme. The competition between the two compounds effects the rate of primary substrate degradation or the whole process degradation. While the term inactivation is generally used for the cellular or protein damage caused by reactive intermediates during compound degradation.

Although, competitive inhibited and/or inactivated TDO enzyme for indole oxidation was observed (Fig 5.3, Table 5.2), the degree of inhibition or inactivation varied when various TCE concentrations were added (Fig. 5.4). The concentration of TCE less than 20 µM caused about 93% TDO activity loss compared to those in the absence of TCE. Increase of TCE concentration from 20 to 40 µM, decreased TDO activity about 15-58%. TCE concentration higher than 40 µM resulted in complete activity loss. The explanation of these results is not clear yet. As TCE also served as substrate for TDO, the measurement of TCE and its degradation pattern are suggested for further discussion how TDO is inhibited and inactivated in the presence of various TCE concentrations.

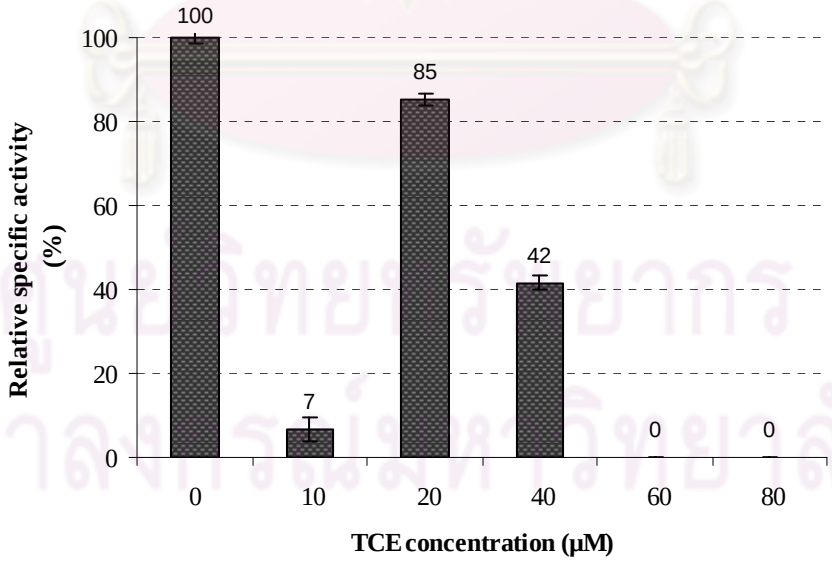


Figure 5.4 TDO activity (A_{400} min^{-1} mg $protein^{-1}$) of samples in the presence of various TCE concentrations relative to those without TCE.

(2) **Recovery of TDO activity after exposure to TCE**

From experiment (1), it is evident that TDO is inactivated by TCE. We therefore investigated its recovery following TCE exposure. The methods for TDO enzyme preparation are described in 5.3.4 and 5.3.5. Generally, after we tested the effect of TCE on TDO activity, the sample with 80 µM TCE was put in a dialysis bag in which TCE was removed from the reaction mixture (see 5.3.8 (2)). An amount of TCE presented in the buffer (outside the bag) indicated that TCE was removed from the dialysis bag. The activity of TDO after TCE removal increased remarkably compared to those containing TCE. TDO activity could be recovered following TCE exposure. This TDO enzyme gave a specific activity of 0.052 A_{400} min^{-1} mg $protein^{-1}$ which was comparable to sample without TCE (0.057 A_{400} min^{-1} mg $protein^{-1}$), corresponding to 90% TDO activity recovery (Fig 5.5, Table 5.2). It was suggested that high TCE concentration (80 µM) might competitively inhibit TDO enzyme activity for indole oxidation rather than inactivate the enzyme or damage the protein. Thus the reaction is reversible. However, further experiment is needed to prove whether it is reversible in the presence of low TCE concentration e.g. 10 µM. This is the first study that reported the recovery of TDO at enzyme level. This result supports our immobilization study, which showed that *Rhodococcus* sp. L4 immobilized on cumin seeds were inactivated during TCE transformation but could be recovered by growing on MS medium for 12 hr (CHAPTER IV). Consequently, those immobilized cells could be reused for TCE degradation.

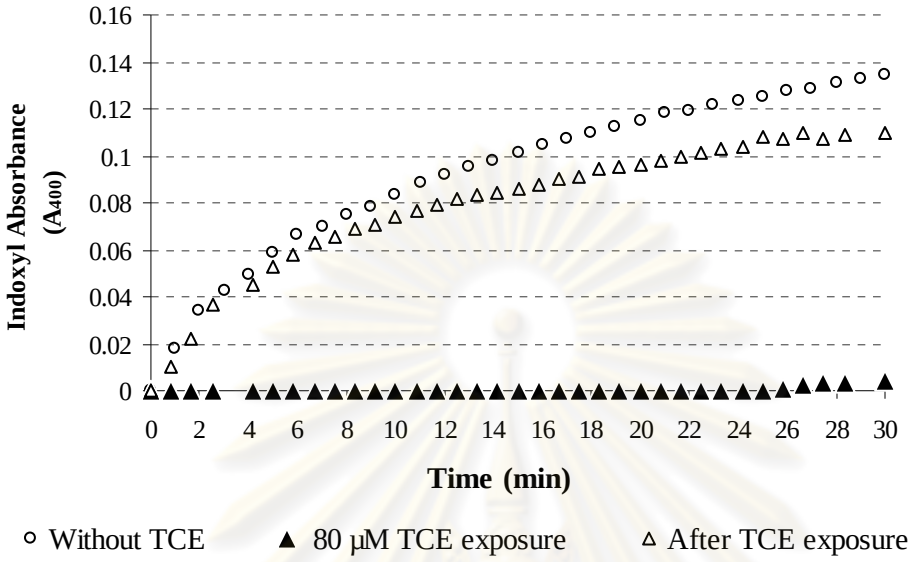


Figure 5.5 Assay for TDO enzyme activity. Absorbance of indoxyl (A_{400}) as a function of time for indole oxidation in the samples without/with TCE and those after TCE was removed by dialysis bag.

(3) **Protection of TDO during TCE oxidation by addition of cumene**

In this experiment, we proved that cumene could protect TDO enzyme during TCE conversion. The preparation of TDO enzyme and the measurement of TDO activity were according to materials and methods 5.3.4, 5.3.5 and 5.3.7. In the presence of 80 µM cumene along with 80 µM TCE, the activity of TDO was maintained and was comparable to those without TCE and cumene, whereas TDO activity was completely lost in the sample which contained only TCE (Fig 5.6).

Morono *et al*. (2004) proposed that the loss of TDO activity in resting cells of *Pseudomonas puti*da F1 during TCE transformation might be pseudoinactivation. They suggested that some of TCE-degrading intermediates, or TCE itself, might remain in the

active site of TDO during TCE degradation and inactivate the enzyme. Aromatic substrates such as toluene, benzene or cumene can displace these inhibitors, thereby restoring enzyme activity. In our experiment, cumene, a true substrate for TDO, was provided simultaneously along with TCE, thus cumene might protect the enzyme against inhibition/inactivation from TCE-degrading intermediates (or TCE itself). This observation corresponded with our previous experiments, which showed that the repeated addition of essential oil components e.g. cumene enhanced TCE degradation in our whole-cell study (CHAPTER III). Moreover, it could explain why the immobilized cells on plant materials rich in essential oils sustained TCE degradation (CHAPTER IV).

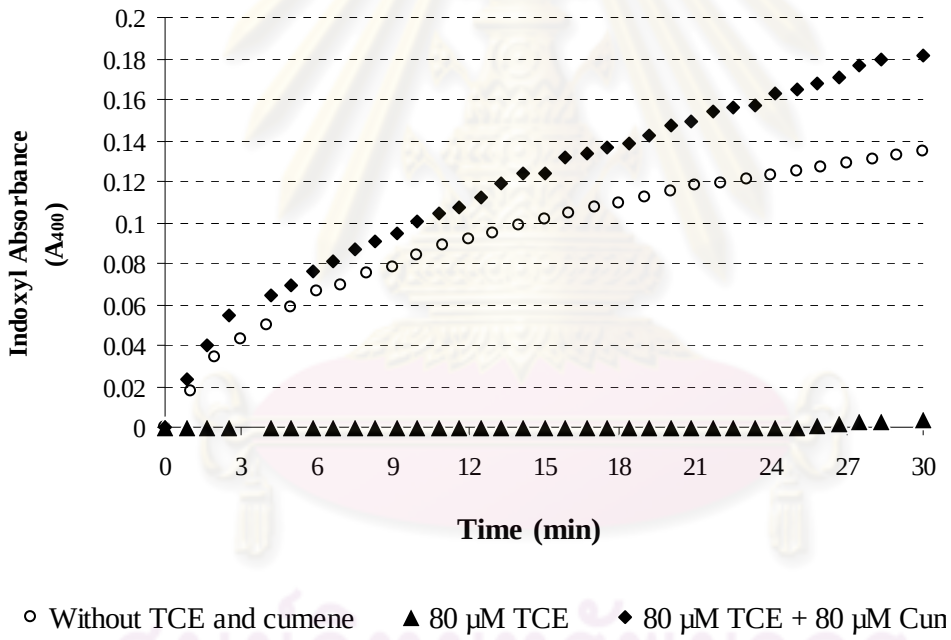


Figure 5.6 Assay for TDO enzyme activity. Absorbance of indoxyl (A_{400}) as a function of time for indole oxidation in the samples containing TCE, TCE plus cumene compared to control sample without TCE and cumene.

Table 5.2 Summary of TDO specific activity in various conditions

Samples	TDO specific activity (A_{400} min^{-1} mg $protein^{-1}$)
Without TCE and cumene	0.057
10 µM TCE	0.004
20 µM TCE	0.049
40 µM TCE	0.024
60 µM TCE	ND
80 µM TCE	ND
After TCE removal	0.052
80 µM TCE plus 80 µM cumene	0.065

ND = not detectable

CHAPTER VI

CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

6.1 Conclusions

Part I: Whole-cell study

Essential oils from cumin, lemon, and lemongrass were found to induce *Rhodococcus* sp. L4, an isolate generally grown on toluene to degrade TCE. Lemon and lemongrass oil could be used as sole carbon source for the bacteria; however cumin oil should only be used in small amounts as an inducer. The induction of TCE-degrading enzymes was suggested to be due to the presence of citral, cumin aldehyde, cumene, and limonene in the essential oils. *Rhodococcus* sp. L4 induced with either of these oil components effectively cometabolized and dechlorinated TCE. Consequently, cumin, lemon, and lemongrass oils or plant materials rich in these essential oils such as cumin seeds are suggested as alternatives to toluene to induce TCE-degrading enzymes.

The effect of TCE concentrations on its transformation capacity (T_c) for each of essential oil component-induced cells varied. This was probably due to the amount of enzyme and/or types of inducible enzymes produced in each induced cell as well as the susceptibility of each induced cell towards TCE toxicity. Citral-induced cells could tolerate higher TCE concentrations than other induced cells thus citral was suggested to be a suitable non-toxic inducer for TCE-degrading bacteria applied to contaminated site.

The repeated addition of essential oil components could enhance TCE degrading activity in *Rhodococcus* sp. L4 by which the added compounds might protect the inactivation of TCE-degrading enzyme from reactive TCE intermediates or TCE itself.

Part II: Immobilization study

Of all plant materials rich in essential oils, cumin seeds were the most effective immobilizing support for *Rhodococcus* sp. L4 and for TCE degradation. Apart from the benefit of essential oil to stimulate TCE degradation, their porous structure and stability after use made them advantageous over orange peels and lemon grass leaves.

The immobilized cells on cumin seeds provided a maximum transformation capacity (T_c) of 59 µg of TCE mg cells^{-1} while free cells were able to degrade only 16 µg of TCE mg cells^{-1} under the same conditions. It was obviously shown that cumin seeds protected bacteria cells from cumulative toxicity of high TCE concentration. Comparing to other reported TCE degraders, our T_c values was in the range of methanotrophs (43-320 µg TCE mg dry cells^{-1}) but higher than the known toluene, phenol, propane or ammonia degraders (5.2–34.01 µg of TCE mg cells^{-1}).

There was an evidence of bacteria/enzymes inactivation after TCE degradation and it could be reactivated by putting the used immobilized cells in MS medium for 12 hr. This reactivated sample could be used for further TCE biodegradation. Only a low number of *Rhodococcus* sp. L4 was leached from cumin seeds during the repeated TCE application indicating a strong attachment between bacteria and materials based on exopolysaccharide production.

Part III: Enzymatic study

The results from enzymatic study showed that toluene dioxygenase (TDO) was inhibited and/or inactivated by TCE. The activity of TDO was found to be recovered after TCE was removed from the reaction mixture. This is the first study that reported the recovery of TDO at enzyme level. This result supported our immobilization study, which showed that *Rhodococcus* sp. L4 immobilized on cumin seeds were inactivated during TCE transformation but it could be reactivated. Consequently, those immobilized cells could be reused for TCE degradation.

The addition of cumene, a true substrate of TDO, along with TCE was suggested to protect enzyme inactivation from TCE or its intermediates. This observation corresponded to our previous experiments, which showed that the repeated addition of aromatic compounds i.e. cumene, cumin aldehyde, limonene or citral enhanced TCE degradation in our whole-cell study. Moreover, it could explain why the immobilized cells on plant materials rich in essential oils sustained TCE degradation.

From the results of these three parts, we suggest that plant essential oil and its component can effectively induce TCE degradation in *Rhodococcus* sp. L4. The immobilization of bacteria on plant material rich in essential oil such as cumin seeds protects our bacteria and sustains TCE degradation. The application of these plant essential oils or plant material is cost-effective and environmentally friendly for TCE bioremediation.

6.2 Suggestions for Future Work

In the whole-cell study, the distribution of essential oil components between liquid and gas phases should be determined. Since there was no Henry's constant reported for all essential oil components, we assume these compounds completely dissolved in the aqueous phase. The concentration of essential oil components as inducers in our study may be overestimated. In addition, it may be possible to screen other essential oils for TCE induction or to apply other plant materials rich in essential oils for immobilization of bacteria and for TCE degradation.

Previous reports on kinetics parameters and inhibitory interactions between TCE and growth substrate/inducer for each bacterium have varied enormously. It is interesting to determine the parameters such as K_s or K_m (a constant that represents the affinity of bacteria for substrate in whole-cell or purified enzyme level, respectively) and V_{max} (maximum reaction rate), thus we could obtain good explanations on interactions between TCE and essential oil component as well as the effect of various TCE concentrations on our bacterial activity.

Larger scale of cells immobilization on cumin seeds for TCE biodegradation should be conducted for application in the real contaminated site. Moreover, the immobilizing conditions should be optimized to provide the highest TCE degradation. Future studies on reactivation process are required for the effective reuse of the immobilized cells. An amount of essential oil contained in plant material was utilized by the bacteria during immobilization and TCE biodegradation, thus it might decrease the rate of new TCE-degrading enzyme production and reduce the ability of enzyme

protection. Consequently, an exogeneous source of essential oils such as vapor phase of cumin seeds (see Fig. 3.2) should be provided along with MS medium during the reactivation period.

6.3 Trends in applications

There are several possibilities to link the know-how from this work and field remediation. In situ treatment approach can be achieved by applying the immobilized cells on plant material rich in essential oils to the subsurface for a sustained transformation of TCE e.g. installing a permeable reactive biobarrier (PRB) containing the immobilized bacteria in the flow path of contaminant plumes (Fig. 6.1). During the treatment, other factors including available oxygen, pH, mixture of other contaminants and so on should be taken into account. Ex-situ bioreactors containing high concentration of immobilized cells to cometabolize TCE could also be made for clean-up TCE contaminated soil and water.

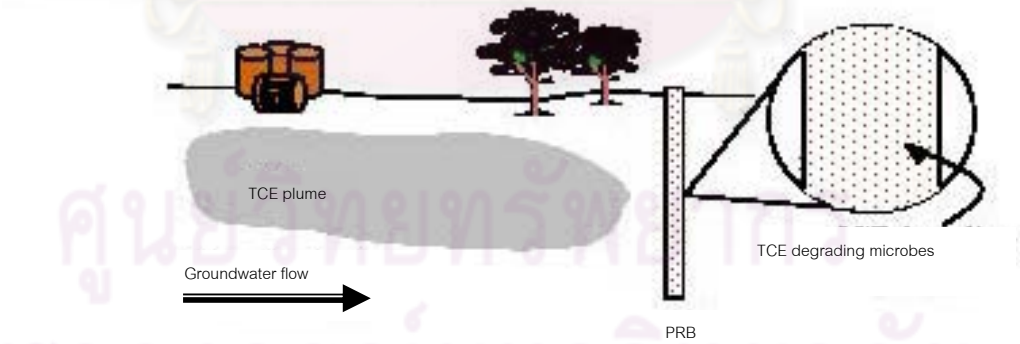


Figure 6.1 Schematic drawing of bioaugmented PRB (Swadley, 2001).

Concerning TCE contaminated soil, rhizosphere bioaugmentation may be carried out by introducing plants that naturally produce effective essential oils through their

rhizosphere and adding the immobilized cells to the sites (Fig. 6.2). Thus, the plant essential oils will enhance the activity of immobilized cells. The supporting material will also increase the survival of bacteria that have been inoculated into the soil. Moreover, plant essential oils may stimulate the indigenous rhizosphere microbial populations to help to detoxify the pollutants. It is also beneficial that plant roots penetrate into the soil and maintain aerobic condition in the rhizosphere, leading to the increased TCE degradation activities of those microorganisms. Further study in enzymatic issue combined with genetic and molecular information would enable us to modify microbes or enzymes for TCE degradation as well as for other pollutants.

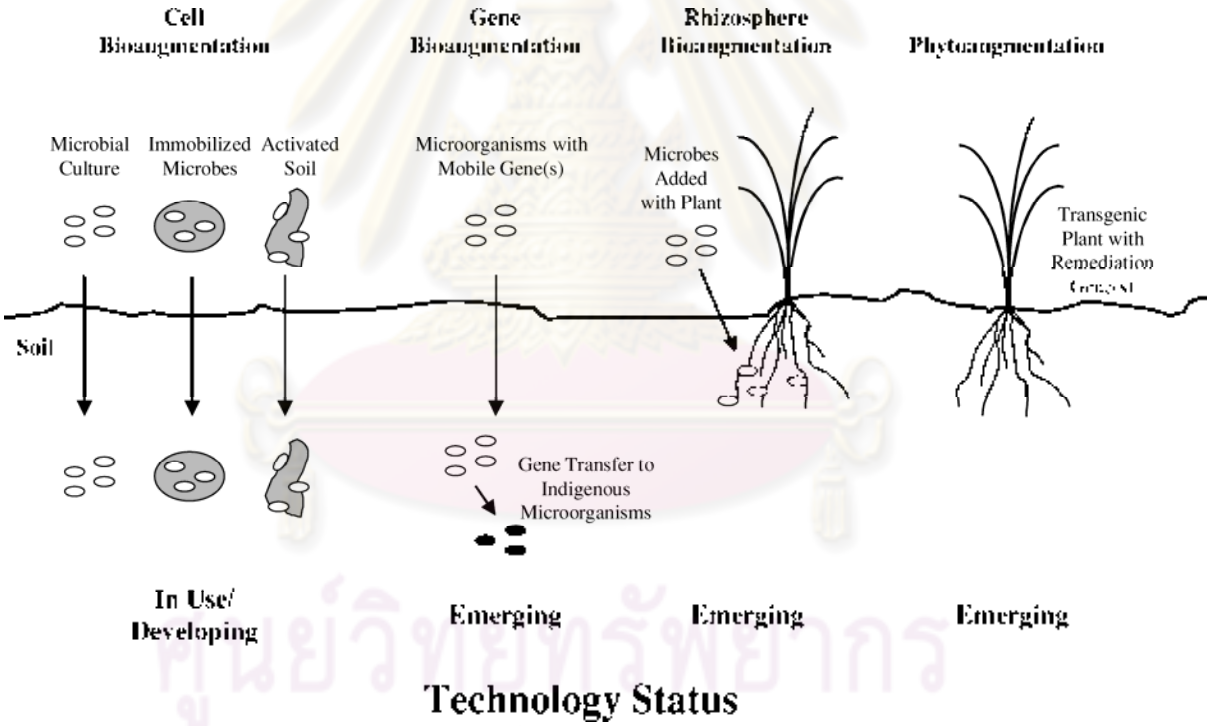


Figure 6.2 Overview of different technologies for delivering remediation microbes/genes to contaminated site (Terry *et al*., 2004).

REFERENCES

Albrechtsen, H.J., Smith, P.M., Nielsen, P., and Christensen, T.H. (1996). Significance of biomass support particles in laboratory studies on microbial degradation of organic chemicals in aquifers. Water Res. 30: 2977-2986.

Alexander, M., ad. (1994). Biodegradation and bioremediation. p 212. San Diego: Academic Press.

Alvarez-Cohen, L., and McCarty, P.L. (1991a). A co-metabolic biotransformation model for halogenated compounds exhibiting product toxicity. Environ. Sci.Technol. 25: 1381-1387.

Alvarez-Cohen, L., and McCarty, P.L. (1991b). Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene transformation by methanotrophic resting cells. Appl. Environ. Microbiol. 57: 1031-1037.

Alvarez-Cohen, L., and Speitel, G.E. (2001). Kinetics of aerobic cometabolism of chlorinated solvents. Biodegradation 12: 105-126.

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].

Arcangeli, J.P., and Arvin, E. (1997). Modeling of the cometabolic biodegradation of trichloroethylene by toluene-oxidizing bacteria in a biofilm system. Environ. Sci. Technol. 31: 3044-3052.

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 .[2007, May 15].

Attanun, T., and Chancharoensuk, J. (1999). Soil and plant analysis (in Thai). Department of geology, Faculty of agriculture, Kasetsart University, Bangkok, Thailand.

Beis, S.H., Azcan, N., Ozek, T., Kara, M., and Baser, K.H.C. (2000). Production of essential oil from cumin seeds. Chem. Nat. Compd. 36: 265-268.

Bell, K.S., Philp, J.C., Aw, D.W.J., Christofi, N. (1998). The genus *Rhodococcus*. J.Appl. Microbiol. 85: 195-210.

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. J. Soil Contam. 7:433-453.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food. Microbiol. 94: 223-253.

Byl T.D., and Williams S.D. (2000). Biodegradation of Chlorinated Ethenes at a Karst Site in Middle Tennessee, U.S. Geological Survey, Water-Resources Investigations Report 99-4285.

Cassidy, M.B., Lee, H., and Trevors, J.T. (1996). Environmental application of immobilized cells: a review. J. Ind. Microbiol. 16: 79-100.

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.

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. [2008, January 15].

Chang, H.L., and Alvarez-Cohen, L. (1995). Transformation capacities of chlorinated organics by mixed cultures enriched on methane, propane, toluene, or phenol. Biotech. Bioeng. 45: 440-449.

Chang, H.L., and Alvarez-Cohen, L. (1996). The biodegradation of individual and multiple chlorinated aliphatics by mixed and pure methane oxidizing cultures. Appl. Environ. Microbiol. 62: 3371-3377.

Chang, I.S., Kim, I.C., and Nam, B.U. (2005). The influence of poly-vinyl-alcohol (PVA) characteristics on the physical stability of encapsulated immobilization media for advanced wastewater treatment. Process Biochem. 40: 3050-3054.

Chen, J., Xu, Y., Xin, J., Li, S., Xia, C., and Cui, J. (2004). Efficient immobilization of whole cells of *Methylomonas* sp. strain GYJ3 by sol-gel entrapment. J. Mol. Catal. B: Enzym 30: 167-172.

Chen, K.C., Lee, S.C., Chin, S.C., and Houng, J.Y. (1998). Simultaneous carbon-nitrogen removal in wastewater using phosphorylated PVA-immobilized microorganisms. Enzyme Microb. Technol. 23: 311-320.

Chen, W-M., Chang, J-S., Wu, C-H., and Chang, S-C. (2004). Characterization of phenol and trichloroethylene degradation by the rhizobium *Ralstonia taiwanensis*. Res. Microbiol. 155: 672-680.

Chen, Y-M., Lin, T-F., Huang, C., Lin, J-C., and Hsieh, F-M. (2007). Degradation of phenol and TCE using suspended and chitosan-bead immobilized *Pseudomonas putida*. J. Harzard. Mater. 148: 660-670.

Chu, K-H., and Alvarez-Cohen, L. (1996). Trichloroethylene degradation by methane-oxidizing cultures grown with various nitrogen sources. Water Environ. Res. 68: 76-82.

Chu, K-H., Alvarez-Cohen, L. 1998. Effect of nitrogen source on growth and trichloroethylene degradation by methaneoxidizing bacteria. Appl Environ Microbiol 64:3451–3457.

Chu, K.H., and Alvarez-Cohen, L. (1999). Evaluation of toxic effects of aeration and trichloroethylene oxidation on methanotrophic bacteria grown with different nitrogen sources. Appl. Environ. Microbiol. 65: 766-772.

Cohen, Yariv. (2001). Biofiltration-the treatment of fluids by microorganisms immobilized into the filter bedding material: a review. Bioresource Technol. 77: 257-274.

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

Cunningham, C.J., Ivshina, I.B., Lozinsky, V.I., Kuyukina, M.S., Philp, J.C. (2004). Bioremediation of diesel-contaminated soil by microorganisms immobilized in polyvinyl alcohol. Int. Biodeterior. Biodegrad. 54: 167-174.

Dalton, H., and Stirling, D.I. 1982. Co-metabolism. Phil. Trans. R. Soc. Lond. B 297: 481-496.

Dabrock, B., Kebeler, M., Averhoff, B., and Gottschaalk, 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., and Gottschalk, G. (1992). Isopropylbenzene (cumene) an new substrate for the isolation of trichloroethene-degrading bacteria. Arch. Microbiol. 158: 9-13.

Dias, J.C., Rezende, R.P., and Linardi, V.R.(2000). Biodegradation of acetonitrile by cells of *Candida guilliermondii* UFMG-Y65 immobilized in alginate, k-carrageenan and citric pectin. Braz.J.Microbiol. 31.

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.

Duetz, W. A., Fjallman, A. H. M., Ren, S., Jourdat, C., and Witholt, B. (2001). Biotransformation of D-limonene to (+) trans-carveol by toluene-grown *Rhodococcus opacus* PWD4 cells. Appl. Environ. Microbiol. 67: 2829-2832.

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.

Ely, R.L., Hyman, M.R., Arp, D.J., Guenther, R.B., and Williamson, K.J. (1995). A cometabolic kinetics model incorporating enzyme inhibition, inactivation, and recovery II trichloroethylene degradation experiments. Biotechnol. Bioeng. 46: 232-245.

Ely, R.L., Williamson, K.J., and Arp, D.J. (1997). Cometabolism of chlorinated solvents by nitrifying bacteria: kinetics substrate interactions, toxicity effects, and bacterial response. Biotech. Bioeng. 54: 520-534.

Engel, P.C. (1996). Enzymology labfax. p 289. Academic Press.

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

Environmental Agency of Japan. (1995). Environmental white paper : Biotechnology in sustaninable environment. New York: Plenum press.

EPA (Environmental Protection Agency). (1996). Technology Transfer Network. Trichloroethylene [online], Available from http://www.epa.gov.th/ttnuatw1/hlthef/tri-ethy.html_[2006, November 5].

Ewers, J., Freier-Schröder, D., and Knackmuss, H-J.(1990). Selection of trichloroethylene (TCE) degrading bacteria that resist inactivation by TCE. Arch Microbiol 154: 410-413.

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

Finnerty, W.R. (1992). The biology and genetics of the genus *Rhodococcus*. Annu. Rev. Microbiol. 46: 193-218.

Focht, D.D. (1994). Microbiological procedures for biodegradation research. In: Weaver RW, Angle JS, Bottomley PS (eds) Methods of soil analysis, part 2 Microbiological and biochemical properties. Soil Science Society of America, Madison, WI.

Folsom, B.R., Chapman, P.J., and Pritchard, P.H. (1990). Phenol and trichloroethylene degradation by Pseudomonas cepacia G4: Kinetic and interactions between substrates. Appl. Environ. Microbiol. 56: 1279-1285.

Fox, B.G., Borneman, J.G., Wackett, L.P., and Lipscomb, J.D. (1990). Haloalkene oxidation by the soluble methane monooxygenase from Methylosinus trichosporium OB3b: Mechanistic and environmental applications. Biochem. 29: 6419-6427.

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. Appl. Environ. Microbiol. 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. Appl. Environ. Microbiol. 63: 1515-1522.

Futamata, H., Harayama, S., Watanabe, K. 2001 .Diversity in kinetics of trichloroethylene-degrading activities exhibited by phenol-degrading bacteria. Appl Microbiol Biotechnol 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.

Gossett, J.M. (1987). Measurement of Henry's law constants for C1 and C2 chlorinated hydrocarbons. Environ. Sci. Technol. 21: 202-208.

Halsey, K.H., Sayavedra-Soto, L.A., Bottomley, P.J., and Arp, D.J. (2005). Trichloroethylene degradation by butane-oxidizing bacteria causes a spectrum of toxic effects. Appl. Microbiol. Biotechnol. 68: 794-801.

Heald, S. and Jenkins, R.O. (1994). Trichloroethylene removal and oxidation toxicity mediated by toluene dioxygenase of *Pseudomonas putida*. Appl. Environ. Microbiol. 60: 4634-4637.

Heo, J., Thomas, K.J., Seong, G.H., and Crooks, R.M. (2003). A microfluidic bioreactor based on hydrogel-entrapped E. *coli*: cell viability, lysis, and intracellular enzyme reactions. Anal Chem. 75: 22-2.

Herman, D.C., Lenhard, R.J., and Miller, R.M. (1997). Formation and removal of hydrocarbon residual in porous media: effects of attached bacteria and biosurfactants. Environ. Sci. Technol. 31: 1290-1294.

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 in Curt Peterson (1999). Aerobic degradation of TCE [Online]. Available from: http://class.et.byu.edu/ce540/termpaper/1999-w/petersonc.pdf. [2008, May 20]

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

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

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

Hunter, W.J. (2005). Injection of innocuous oils to create reactive barriers for bioremediation:laboratory studies, J. Contam. Hydrol. doi:10.1016/j.jconhyd. 2005.06.08.

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.

Jalali-Heravi, M., Zekavat, B., and Sereshti, H. (2007). Use of gas chromatography-mass spectrometry combined with resolution methods to characterize the essential oil components of Iranian *cumin* and *caraway*. J. Chromatogr. A 1143: 215-226.

Jenkins, R.O., and Dalton, H. (1985). The use of indole as a spectrophotometric assay substrate for toluene dioxygenase. FEMS Microbiol. Lett. 30: 227-231.

Junter, A.G., and Jouenne, T. (2004). Immobilized viable microbial cells: from the process to the proteome...or the cart before the horse. Biotechnol. Adv. 22: 633-658.

Kao, C.M., Chen, S.C., and Su, M.C. (2001). Laboratory column studies for evaluating a barrier system for providing oxygen and substrate for TCE biodegradation. Chemosphere 44: 925-934.

Kelly, C.J., Bienkowski, P.R., and Sayler, P.S. (2000). Kinetic analysis of a tod-lux bacterial reporter for toluene degradation and trichloroethylene cometabolism. Biotech. Bioeng. 69: 256-265.

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

Kim, B-H., Oh, E-T., So, J-S., Ahn, Y., and Koh, S-C. (2003). Plant terpene-induced expression of multiple aromatic ring hydroxylation oxygenase genes in *Rhodococcus* sp. Strain T104**.** J. Microbiol. 41: 349-352.

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

Kocamemi, B.A., and Cecen, F. (2007). Kinetic analysis of the inhibitory effect of trichloroethylene (TCE) on nitrification in cometabolic degradation. Biodegradation 18:71-81: 71-81.

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.

Landa, A.S., Sipkema, E.M., Weijma, J., Beenackers, AACM., Dolfing, J., and Janssen, D.B. (1994). Cometabolic degradation of trichloroethylene by Pseudomonas cepacia G4 in a chemostat with toluene as the primary substrate. Appl Environ Microbiol 60: 3368–3374.

Lange, C.C., and Wackett, L.P. (1997). Oxidation of aliphatic olefins by toluene dioxygenase: enzyme rates and product identification. J. Bacteriol. 179: 3858-3865.

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

Larkin, M.J., Kulakov, L.A., and Allen, C.C.R. (2005). Biodegradation and Rhodococcus - masters of catabolic versatility. Curr. Opin. Biotechnol. 16: 282-290.

Lazarova, V., and Manem, J. (1995). Biofilm characterization and activity analysis in water and wastewater treatment. Wat. Res. 29: 2227-2245.

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, E.Y. (2003). Continuous treatment of gas-phase trichloroethylene by Burkholderia cepacia G4 in a two-stage continuous stirred tank reactor/trickling biofilter system. J. Biosci. Bioeng. 96: 572-574.

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. Environ. Toxicol. Chem. 21: 1609-1617.

Lee, S.B., Strand, S. E., and Stensel, H. D. (2000). Sustained degradation of trichloroethylene in a suspended growth gas treatment reactor by an actinomycetes enrichment. Environ. Sci. Technol. 34: 3261-3268.

Leenen, E.J.T.M., Dos Santos, V.A.P., Grolle, K.C.F., Tramper, J., and Wijffels, R,H. (1996). Characteristics of and selection criteria for support materials for cell immobilization in wastewater treatment. Water. Res. 30: 2985-2996.

Li, S. and Wackett, P. L. (1992). Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Commun. 185: 443-451.

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.

Lozinsky, V.I, and Plieva, F.M. (1998). Poly (vinyl alcohol) cryogels employed as matrices for cell immobilization. 3. overview of recent research and developments. Enzyme Microb. Technol. 23: 227-242.

Luepromchai, E. (2004). Utilization of plant terpenes for induction of Rhodococcus pyridinivorans L4 bacteria to degrade trichloroethylene (TCE) and characterization of involving gene. Environmental Research Institute, Chulalongkorn University, Bangkok, Thailand.

Luepromchai, E., and Suttinun, O.(2003). Substrate diversity of a new *Rhodococcus pyridinovorans* and its application for TCE biodegradation. In 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.

Mars, A.E., Houwing, J., and Janssen, D.B. (1996). Degradation of toluene and trichloroethylene by Burkholderia cepacia G4 in growth-limited fed batch culture. Appl. Environ. Microbiol 62: 886-891.

McCarty, P.L., Goltz, M.N., Hopkins, G.D., Dolan, M.E., Allen, J.P., Kawakami, B.T., Carrothers, T.J. (1998). Fullscale evaluation of in situ cometabolic degradation of trichloroethylene in groundwater through toluene injection. Environ. Sci. Technol. 32 (1): 88–100.

Milintawisamai, M., Chanarong, S., Sunthondecha, P., Savesthayothin, V., and Pantachak, C. (2001). Contamination of chlorinated ethylene in soil and groundwater: the case study of Thailand (in Thai). Rsearch Development and Technology Promotion Group, The Environmental Research and Training Center, Department of Environmental Quality Promotion, Bangkok.

Moran, B.N., and Hickey, W.J. (1997). Trichloroethylene biodegradation by mesophilic and psychrophilic ammonia oxidizers and methanotrophs in groundwater microcosms. Appl. Environ. Microbiol. 63: 3866-3871.

Morono, Y., Unno, H., and Hori, K. (2006). Correlation of TCE cometabolism with growth characteristics on aromatic substrates in toluene-degrading bacteria. Biochem. Eng. J. 31: 173-179.

Morono, Y., Unno, H., Tanji, Y., and Hori, K. (2004). Addition of aromatic substrates restores trichloroethylene degradation activity in *Pseudomonas putida* F1. Appl. Environ. Microbiol. 70: 2830-2835.

Mu, D.Y., 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.

Munakata-Marr, J., McCarty, P.L., Shields, M.S., Reagin, M., and Francesconi, S.C. (1996). Enhancement of trichloroethylene degradation in aquifer microcosms bioaugmented with wild type and genetically altered *Burkholderia*(Pseudomonas) *cepacia* G4 and PR1. Environ.Sci.Technol. 30: 2045-2052.

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

Newman, L.M., and Wackett, P.L. (1997). Trichloroethylene oxidation by purified toluene 2-monooxygenase: products, kinetics, and turnover-dependent inactivation. J.Becteriol.179: 90-96.

Niu, C., and Gilbert, E.S. (2004). Colorimetric method for identifying plant essential oil components that affect biofilm formation and structure. Appl. Environ. Microbiol. 70: 6951-6956.

OAIC (Ozone Action Information Clearinghouse). (1996). Terpenes as replacements for chlorinated solvents [online]. Available from: http://es.epa.gov/p2pubs/oaic/992.html/ [2007, September 14]

Obuekwe, C.O., and Al-Muttawa, E.M. (2001). Self-immobilized bacterial cultures with potential for application as ready-to-use seeds for petroleum bioremediation. Biotechnol. Lett. 23: 1025-1032.

Ogbonna, J.C., Liu, Y.C., Liu, Y.K., and Tanaka, H. (1994). Loofa (Luffa cylindrical) sponge as a carrier for microbial cell immobilization. J. Ferment. Bioeng. 78: 437-442.

Ohlen, K., Chang, Y.K., Hegemann, W., Yin, C.-R., and Lee, S.-T. (2005). Enhanced degradation of chlorinated ethylenes in groundwater from a paint contaminated site by two-stage fluidized-bed reactor. Chemosphere 58: 373-377.

Oldenhuis, R., Oedzes, J.Y., van der Waarde, J.J., and Janssen, D.B. (1991). Kinetics of chlorinated hydrocarbon degradation by *Methylosinus trichosporium* OB3b and toxicity of trichloroethylene. Appl. Environ. Microbiol. 57: 7-14.

Olofsson, A-C., Hermansson, M., and Elwing, H. (2003). N-acetyl-l-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces. Appl. Environ. Microbiol. 69: 4814: 4822.

Pattanasupong, A., Nagase, H., Sugimoto, E., Hori, Y., Hirata, K., Tani, K., Nasu, M., and miyamoto, K. (2004). Degradation of Carbendazim and 2-4 dichlorophenoxyacetic acid by immobilized consortium on loofa sponge. J. Biosci. Bioeng. 98: 28-33.

Parsons, F., and Lage, G.B. (1985). Chlorinated organics in simulated groundwater environments. J. Am. Water Works Assoc. 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) .(2004). Information and services, soil quality standards [online], Available from http://www.pcd.go.th/info_serv/en_reg_std_soil01.html [2008, December 11].

Pedrazzani, R., Bertanza, G., Maffezzoni, C., Gelmi, M., Manca, N., and Depero, L.E. (2005). Bacteria enclosure between silica-coated membranes for the degradation of organic compounds in contaminated water. Water Res. 39: 2056-2064.

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.

Quek, E., Ting, Y-P., and Tan, H.M. (2006). *Rhodococcus* sp. F92 immobilized on polyurethane foam shows ability to degrade various petroleum products. Bioresource Technol. 97: 32-38.

Radway, J.C., Domingo, J.W.S., Hazen, T.C., and Wilde, E.W. (1998). Evaluation of biodegradation potential of foam embedded *Burkholderia cepacia* G4. Biotechnol. Lett. 20: 663-666.

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. [2008, September 20]

Saeki, H., Akira, M., Furuhashi, K., Averhoff, B., and Gottschalk, G. (1999). Degradation of trichloroethene by a linear-plasmid-encoded alkene monooxygenase in *Rhodococcus corallinus* (*Nocardia corallina*) B-276. Microbiology 145: 1721-1730.

Salerno, M.B. (2005). A micro- and macro-scale analysis of the molecular details of bacterial adhesion to hydrophilic and hydrophobic surfaces. Ph.D. 's Thesis. Department of chemical engineering, Graduate School College of Engineering , Pennsylvania State University.

Scopes, R.K. (1987). Protein purification : principles and practice. p 329. New York: Springer-Verlage.

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., McCarty, P.L. (1990). A field evaluation of in-situ biodegradation of chlorinated ethenes. Ground Water. 29: 239-250.

Shetty, R.S., Singhal, R.S., and Kulkarni, P.R. (1994). Antimicrobial properties of cumin. World. J. Microbiol. Biotechnol. 10: 232-233.

Shimomura, T., Suda, F., Uchiyama, H., and Yagi, O. (1997). Biodegradation of trichloroethylene by *Methylocystis* sp. strain M immobilized in gel beads in a fluidized-bed bioreactor. Water Res. 31: 2383-2386.

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

Singer, C.A., 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.

Smith, L.H., Kitanidis, P.K., and McCarty, P.L. (1997). Numerical modeling and uncertainties in rate coefficients for methane utilization and TCE cometabolism by a methane-oxidizing mixed culture. Biotech. Bioeng. 53: 320-331.

Sukhapan, J. 2007. The document of seminar in Development of Environmental and Emission Standard of VOCs 2007**.** [online]. Available from: http://www.pcd.go.th/info_serv/air_seminarVOC07.html. [2008, December 8]

Suttinun, O., Lederman, P. B., and Luepromchai, E. (2004). Application of terpene-induced cell for enhancing biodegradation of TCE contaminated soil. Songklanakarin J. Sci. Technol. 26 (Suppl. 1): 131-142.

Suttinun, O., Muller, R., and Luepromchai, E. (2008). Trichloroethylene cometabolic degradation by *Rhodococcus sp. L4* induced with plant essential oils. Biodegradation. Doi.10.1007/s10532-008-9220-4.

Swadley, L.A. (2001). Bioaugmented porous ceramics for degradation of trichloroethylene in groundwater: characterization and upscaling. NFF End-of-the-year report, preliminary results and planning AY 2000-2001.

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.

Terry, G.J., Rensing, C., and Pepper, L.L. (2004). New approaches for bioaugmentation as a remediation technology. Crit. Rev. Env. Sci. Technol. 34 (5): 447-494.

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

Tom Kuo, M.C., Liang, K.F., Han, Y.L., and Fan, K.C. (2004). Pilot studies for in-situ aerobic cometabolism of trichloroethylene using toluene-vapor as the primary substrate. Water Res. 38: 4125-4134

Tompson, A.F., Knapp, R.B., Hanna, M.L., and Taylor, R.T. (1994). Simulation of TCE migration and biodegradation in a porous medium under conditions of finite degradation capacity. Adv. Water Resour. 17: 241-249.

TRI (Toxic Chemical Release Inventory). 1995. Data contained in the Toxic Chemical Release Inventory (TRI) [online]. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/html/gen?TRI & search 79-01-6 [2008, January 13].

Uchiyama, H., Yagi, O., Oguri, K., and Kokufuta, E. (1994). Immobilization of tricholoroethylene-degrading bacterium, *Methylocystis* sp. strain M in different matrices. J. Ferment. Bioeng. 77: 73-177.

Uchiyama, H., Oguri, K., Nishibayashi, M., Kokufuta, E., and Yagi, O. (1995). Trichloroethylene degradation by cells of a methane-utilizing bacterium, *Methylocystis* sp. M, immobilized in calcium alginate. J. Ferment. Bioeng. 79: 608-613.

Vandevivere, P., and Kirchman, D.L. (1993). Attachment stimulate exopolysaccharide synthesis by a bacterium. Appl. Eviron. Microbiol. 59: 3280-3286.

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.

Verpoorte, R., and Memelink, J. (2002). Engineering secondary metabolite production in Plants. Curr. Opin. Biotechnol. 13: 181-187.

Vogel, R.M., and McCarty, P.L. (1985). Biotransformation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49: 1080-1083.

Wackett, L.P., and Gibson, D.T. (1988). Degradation of trichloroethylene by toluene dioxygenase in whole-cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol. 54: 1703-1708.

Wackett, L.P., and Householder, S.R. (1989). Toxicity of trichloroethylene to Pseudomonas putida F1 is mediated by toluene dioxygenase. Appl. Environ. Microbiol 55: 2723-2725.

Wilson, N.G., and Bradley, G. (1996). Enhanced degradation of petroleum (Slovene diesel) in an aqueous system by immobilized *Pseudomonas fluorescens*. J. Appl. Microbiol. 80: 99-104.

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

Woo, H-J., Sanseverino, J., Cox, C.D., Robinson, K.G., and Sayler, G.S. (2000). The measurement of toluene dioxygenase activity in biofilm culture of *Pseudomonas putida* F1. J. Microbiol. Meth. 40: 181-191.

Yaithavorn, P., and Charnnarong, S. (1998). Use of isotope techniques to trace the transformation of volatile organic compounds in polluted ground water in the area of northern region industrial estate, Lampoon province. Environmental Research and Training Center, Department of Environmental Quality Promotion, Pathumthani.

Yang, L., Chang, Y-F., and Chou, M-S. (1999). Feasibility of bioremediation of trichloroethylene contaminated sites by nitrifying bacteria through cometabolism with ammonia. J. hazard. mater. 69: 111-126.

Yeager, C.M., Bottomley, P.J., and Arp, D.J. (2001a). Cytotoxicity associated with trichloroethylene oxidation in *Burkholderia cepacia* G4. Appl. Environ. Microbiol. 67: 2107-2115.

Yeager, C.M., Bottomley, P.J., and Arp, D.J. (2001b). Requirement of DNA repair mechanisms for survival of *Burkholderia cepacia* G4 upon degradation of trichloroethylene. Appl. Environ. Microbiol. 67: 5384-5391.

Yeager, C.M., Bottomley, P.J., Arp, D.J., and Hyman, M.R. (1999). Inactivation of toluene 2-monooxygenase in *Burkholderia cepacia* G4 by alkynes. Appl. Environ. Microbiol. 65: 632-639.

APPENDICES

APPENDIX A

A.1 The mineral salts (MS) medium

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

Table A.1.1 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 ^{o}C for 15 min before use.

A.2 Growth curve determination

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

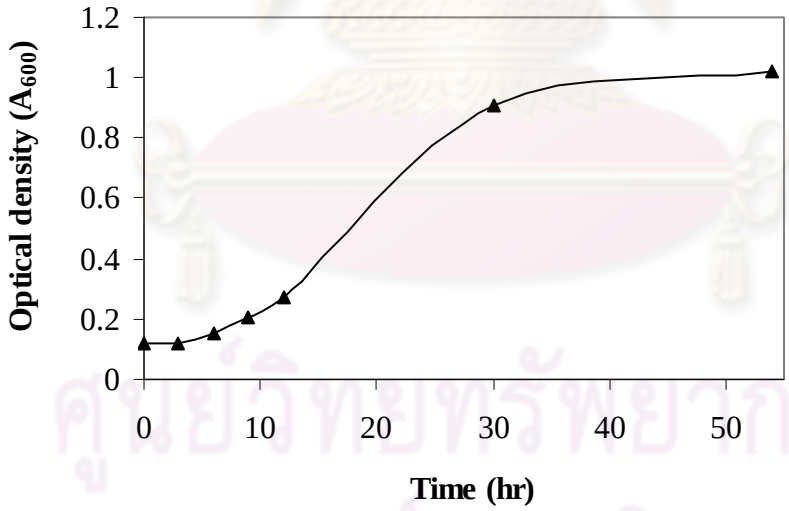


Figure A.2.1 Growth curve determination for the culture of *Rhodococcus* sp.L4 growing in a-250 ml Erlenmeyer flask containiing100 ml MS medium with toluene supplied as vapor phase.

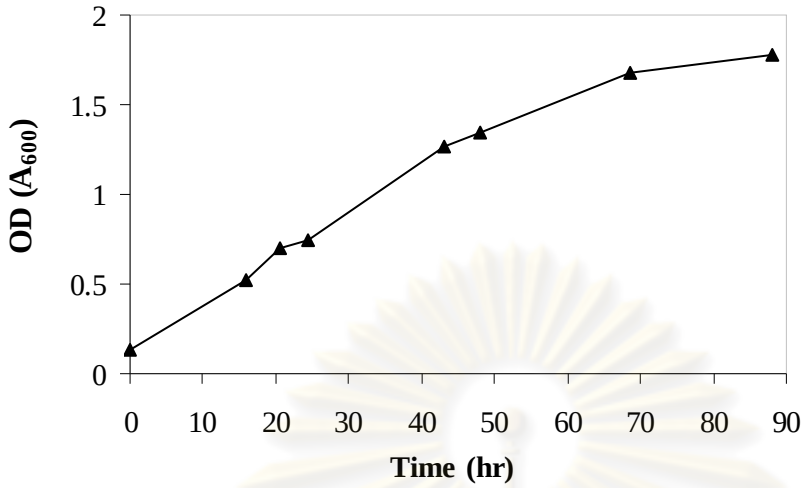


Figure A.2.2 Growth curve determination for the culture of *Rhodococcus* sp. L4 growing in a-2 L Duran bottle containing 600 ml MS medium with cumene supplied as vapor phase.

APPENDIX B

B.1 Calculation for an initial aqueous concentration of TCE in two-phase system

- Using a dimensionless Henry's constant (H_c) at 30 ^oC equals 0.49

(Gossett, 1987), we can calculate an initial aqueous concentration of TCE according to

$$H_c = C_g/C_w$$

- For example, the total moles (M_t) of TCE added to 22- ml vial containing

2 ml liquid culture are equal to 0.16 µmol (10µl of 16 mM TCE stock solution added). The TCE is partitioned between air-aqueous phases at equilibrium as

$$Mt = M_g + M_w$$

$$Mt = C_gV_g+C_wV_w$$

According to $C_g = H_cC_w$

$$M_t = H_cC_wV_g + C_wV_w$$

$$M_t = C_w (H_cV_g+V_w)$$

$$C_w = M_t/(H_cV_g+V_w)$$

Where H_c = Henry's constant (dimensionless), C_g = concentration of compound in the gas phase (mol/L), C_w = concentration of compound in the aqueous phase (mol/L), M_t = total mass of compound (moles), M_g= mass of compound in the gas phase (moles), M_w = mass of compound in the aqueous phase (moles), V_g = volume of headspace in the vial (L) , V_w = volume of aqueous in the vial (L)

- Solving for an initial aqueous concentration of TCE (C_w) is shown as

follows;

C_w (µ mol/L) = 0.16 (µ mol) / ((0.49*0.020) (L) + 0.002 (L))

C_w (µ mol/L) = 0.16 (µmol) / 0.0118 (L) = 13.56

- We can estimate the concentration of TCE in the gas phase according

To

$$C_g = Hc*Cw$$

C_g (µ mol/L) = 0.49 * 13.56 = 6.64

- Confirmation of TCE mass in gas and aqueous phase with calculated TCE

concentration;

(1) 13.56 µmol/L TCE in aqueous phase = (13.56 µmol/L) * 0.002 L = 0.0271 µmol or 3.56 µg

(2) 6.64 µmol/L TCE in gas phase = (6.64 µmol/L) * 0.020 L = 0.1328 µmol or 17.45 µg

(3) Total TCE mass (aqueous and gas phase) = 0.0271+ 0.1328 µmol = 0.16 µmol or 21.01 µg

B.2 Calculation for an initial aqueous concentration of toluene in two-phase system

- Using a dimensionless Henry's constant (H_c) at 30 ^oC equals 0.34 (Yeager,

1999), the total moles (M_t) of toluene added to 22- ml vial containing 2 ml liquid culture were equal to 0.4 µmol (10µl of 40 mM toluene stock solution added). We can calculate an initial aqueous concentration of toluene as described above

$$C_w\ (\mu\ mol/L) = 0.4\ (\mu\ mol) / ((0.34*0.020)\ (L) + 0.002\ (L))$$

$$C_w\ (\mu\ mol/L) = 0.4\ (\mu mol) / 0.0088\ (L) = \quad 45.5$$

B.3 Determination of amount of oxygen during TCE degradation test

The amount of oxygen was analyzed by Gas Chromatography (GC) with a Hewlett-Packard 6890 equipped with thermal conductivity detector (TCD) and a HP-Molesieve fused-silica capillary column (30 m x 0.32 mm ID; thickness, 12 µm). The following operating conditions were used: Injector temperature 80 ^oC, detector temperature 150 ^oC, initial column temperature 40^oC (1 min) then, programmed at 40 ^oC to 55 ^oC at a rate of 5 ^oC/min (2 min). The carrier gas was helium with gas flow of 55 ml/min, and a splitless mode The make up gas was N_2 at 2.2 ml/min. Oxygen had a retention time of 1.714 min under these conditions. The ambient air was used as standard for the identity of oxygen.

Table B.3.1 An amount of oxygen remaining in tested samples

Sample	O_2 remaining (%)		
	0 hr	4 hr	8 hr
Toluene-grown cells	100	35.14	13.55
Cumin aldehyde-induced cells	100	64.24	28.85

APPENDIX C

C.1 Results of Whole-cells study

The calibration standards were prepared and analyzed similar to sample procedures. Calibration curve of TCE, chloride and essential oil components were shown below.

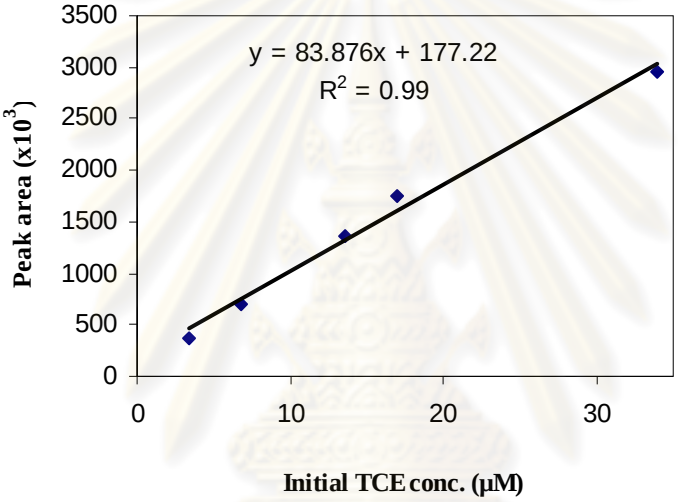


Figure C.1.1 Calibration curve of TCE

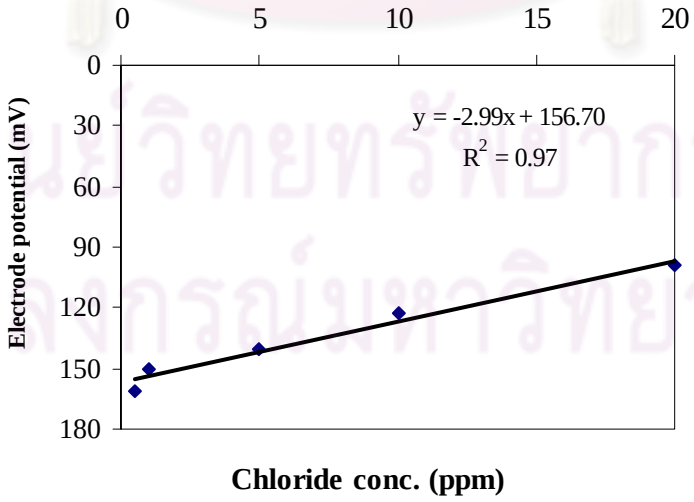


Figure C.1.2 Chloride electrode calibration curve

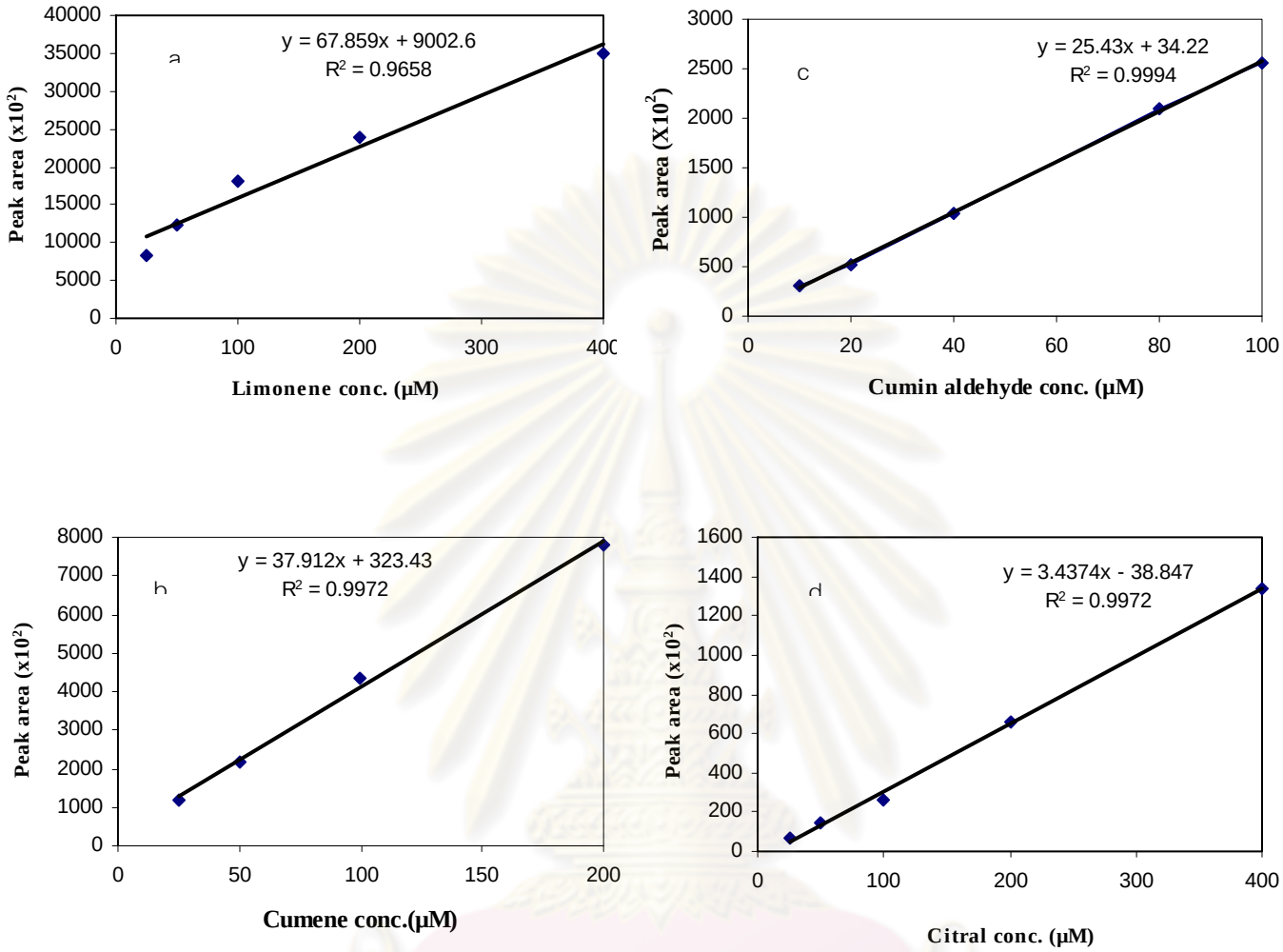


Figure C.1.3 Essential oil component calibration curve; limonene (a), cumene (b), cumin aldehyde (c), citral (d).

Table C.1.1 Percentage of TCE remaining by toluene grown *Rhodococcus* sp. L4 and controls (killed-cells and glucose grown cells) after 8 h incubation

Sample	Time	TCE remaining (%) Sample No.		AVG (%)	SD
		1	2		
Killed-cells	0	96.19	103.96	100.08	5.49
	1	111.01	107.13	109.07	2.74
	2	110.12	106.46	108.29	2.59
	3	96.00	100.28	98.14	3.03
	4	102.40	109.01	105.70	4.67
	5	98.88	109.95	104.42	7.83
	6	110.29	110.59	110.44	0.21
	7	97.62	98.25	97.94	0.44
	8	102.57	99.18	100.87	2.39
Glucose-grown cells	0	100.00	105.00	102.50	3.54
	1	96.20	97.97	97.09	1.25
	2	97.88	106.07	101.97	5.80
	3	103.83	111.40	107.62	5.35
	4	103.04	107.82	105.43	3.38
	5	89.10	99.11	94.11	7.08
	6	94.46	101.06	97.76	4.67
	7	85.94	98.44	92.19	8.84
	8	88.90	95.55	92.23	4.70
Toluene-grown cells	0	96.00	102.00	99.00	4.24
	1	85.58	78.10	81.84	5.29
	2	82.25	79.37	80.81	2.04
	3	75.43	74.98	75.21	0.31
	4	53.89	53.14	53.52	0.53
	5	60.05	61.33	60.69	0.90
	6	62.48	57.73	60.11	3.36
	7	44.55	42.28	43.42	1.61
	8	46.54	39.21	42.87	5.18

Table C.1.2 Percentage of TCE remaining by essential oil component-induced *Rhodococcus* sp. L4 after 8 h incubation

Sample	Time	TCE remaining (%) Sample No.			AVG (%)	SD
		1	2	3		
Cumene	0	100.00	100.00	92.00	97.33	4.62
	1	51.25	53.81	51.08	52.05	1.53
	2	50.50	52.88	50.21	51.20	1.46
	3	47.68	46.89	44.92	46.50	1.42
	4	37.13	45.95	44.93	42.67	4.82
	5	56.37	54.83	53.93	55.04	1.23
	6	50.14	49.69	55.31	51.71	3.12
	7	46.69	46.73	46.71	46.71	0.02
	8	44.60	45.63	49.67	46.64	2.68
Limonene	0	100.00	101.00	91.00	97.33	5.51
	1	84.03	85.62	81.25	83.63	2.21
	2	90.99	86.65	93.66	90.43	3.54
	3	81.74	81.40	77.70	80.28	2.24
	4	78.59	89.48	93.22	87.10	7.60
	5	78.02	84.74	79.77	80.84	3.49
	6	70.71	72.63	72.53	71.96	1.08
	7	57.71	54.59	63.69	58.66	4.63
	8	63.18	60.19	69.17	64.18	4.57

Table C.1.2 (Cont.)

Sample	Time	TCE remaining (%) Sample No.			AVG (%)	SD
		1	2	3		
Citral	0	103.56	96.00	108.00	102.52	6.07
	1	96.45	91.23	94.10	93.93	7.94
	2	91.34	97.80	89.80	92.95	4.57
	3	90.10	95.30	89.54	91.67	9.46
	4	54.15	65.22	59.10	59.54	3.16
	5	66.12	63.10	72.50	67.08	5.35
	6	56.90	62.10	61.40	60.57	0.34
	7	65.18	66.40	66.50	66.14	4.95
	8	56.13	75.80	50.40	60.81	9.43
Cumin aldehyde	0	100.00	89.29	111.99	100.43	11.35
	1	120.75	59.96	81.96	87.56	30.78
	2	101.26	62.83	75.41	79.83	19.59
	4	97.14	53.01	58.83	69.66	23.98
	6	76.49	64.49	61.92	67.63	7.77
	8	63.16	43.20	45.93	50.76	10.83
Cumin seeds	0	100.00	100.00	100.00	100.00	0.00
	1	95.30	98.60	89.00	94.14	5.76
	2	78.13	76.30	84.35	79.42	5.18
	3	76.50	80.40	73.30	76.86	7.79
	4	60.75	69.50	58.40	62.13	10.78
	5	64.32	73.43	58.40	64.48	8.49
	6	60.12	58.20	66.46	61.81	4.77
	7	64.34	70.90	60.32	65.02	6.83
	8	65.13	68.13	58.10	63.66	5.55

Table C.1.3 Amount of chloride generated during TCE degradation in *Rhodococcus* sp. L4 grown on toluene compared to control (killed-clles and glucose grown cells) after 8 h incubation

Sample	Time	Chloride generated (ppm) Sample No.		AVG (%)	SD
		1	2		
Killed-cells	0.00	0.10	0.07	0.08	0.02
	2.00	0.10	0.15	0.14	0.04
	4.00	0.01	0.02	0.02	0.01
	6.00	0.03	0.02	0.02	0.01
	8.00	0.04	0.03	0.04	0.01
glucose	0.00	0.00	0.00	0.00	0.00
	2.00	0.05	0.06	0.06	0.01
	4.00	0.26	0.21	0.24	0.04
	6.00	0.50	0.60	0.55	0.07
	8.00	0.25	0.30	0.28	0.04
toluene	0.00	0.00	0.00	0.00	0.00
	2.00	1.00	1.20	1.10	0.14
	4.00	1.46	1.50	1.48	0.03
	6.00	3.50	4.00	3.75	0.35
	8.00	4.32	4.10	4.21	0.16

Table C.1.4 Amount of chloride generated during TCE degradation in *Rhodococcus* sp. induced with various inducers.

Sample	Time	Chloride generated (ppm) Sample No.		AVG (%)	SD
		1	2		
Cumene					
	0	0.00	0.00	0.00	0.00
	2	0.80	0.82	0.81	0.01
	4	0.82	1.00	0.91	0.13
	6	2.80	2.50	2.65	0.21
	8	5.41	5.80	5.61	0.28
Citral					
	0	0.00	0.00	0.00	0.00
	2	0.22	0.27	0.25	0.04
	4	0.42	0.61	0.52	0.13
	6	2.00	2.80	2.40	0.57
	8	3.00	3.20	3.10	0.14
Limonene					
	0	0.00	0.00	0.00	0.00
	2	0.20	0.26	0.23	0.04
	4	0.69	0.78	0.74	0.06
	6	1.30	1.20	1.25	0.07
	8	2.42	1.90	2.16	0.37
Cumin aldehyde	0	0.73	0.62	0.69	0.08
	2	0.66	0.64	0.66	0.01
	4	0.267	0.27	0.27	0.00
	6	1.32	1.29	1.32	0.02
	8	1.80	1.84	1.82	0.03
Cumin seeds	0	0.00	0.00	0.00	0.00
	1	0.64	0.73	0.69	0.06
	2	1.43	1.46	1.45	0.02
	3	1.62	1.72	1.67	0.07
	4	1.70	1.83	1.76	0.09
	5	1.05	1.05	1.03	0.00
	6	1.45	1.25	1.37	0.14
	7	1.89	1.93	1.90	0.03
	8	3.30	4.12	3.70	0.58

Table C.1.5 TCE Transfromation capacity of essential oil component-induced *Rhodococcus* sp. L4 after incubation with various TCE concentrations for 8 h. (Cell density = 0.4 mg dry cells mL^{-1})

Cumene						
TCE conc. (µM)	**Sample No.**	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	1	2.23	0.18	0.23		
	2	2.63	0.16	0.20		
	3	2.71	0.14	0.18	0.20	0.02
7	1	10.31	1.77	2.22		
	2	10.51	0.14	0.18		
	3	12.88	1.78	2.23	1.54	1.18
14	1	20.71	10.04	12.56		
	2	21.02	10.40	13.01		
	3	24.85	14.02	17.52	14.36	2.75
25	1	37.30	11.18	13.98		
	2	39.42	11.96	14.94		
	3	44.06	14.19	17.73	15.55	1.95
51	1	58.32	7.14	12.00		
	2	78.84	12.25	15.31		
	3	89.24	19.00	23.75	19.53	5.96
68	1	99.49	7.16	8.95		
	2	105.12	11.00	13.75		
	3	105.12	10.39	12.99	11.90	2.58

Table C.1.5 (Cont.)

			Cumin aldehyde			
TCE conc. (µM)	**Sample No.**	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	1	2.23	0.00	0.00		
	2	2.63	0.00	0.00		
	3	3.13	0.00	0.00	0.00	1.17
7	1	9.69	1.74	2.18		
	2	10.51	0.92	1.16		
	3	10.62	0.76	0.95	1.43	0.66
14	1	20.54	12.97	16.21		
	2	21.02	7.22	9.02		
	3	21.92	data loss*	data loss*	12.62	5.09
25	1	30.43	1.58	1.97		
	2	39.42	4.58	5.73		
	3	43.76	0.00	0.00	5.70	2.66
51	1	77.13	1.33	1.66		
	2	78.84	1.22	1.53		
	3	79.48	0.00	0.00	1.59	2.41
68	1	92.81	4.97	6.21		
	2	105.12	3.35	4.19		
	3	105.12	0.00	0.00	5.20	1.43

* Sample vial was damaged during GC analysis

Table C.1.5 (Cont.)

Limonene						
TCE conc. (µM)	**Sample No.**	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	1	2.48	0.10	0.13		
	2	2.63	0.29	0.36		
	3	2.65	0.00	0.00	0.24	0.16
7	1	10.19	0.49	0.61		
	2	10.51	0.23	0.28		
	3	10.62	0.29	0.37	0.42	0.17
14	1	17.56	2.86	3.57		
	2	21.02	5.22	6.52		
	3	21.23	4.34	5.42	5.97	0.77
25	1	36.48	12.18	15.23		
	2	39.42	12.29	15.36		
	3	41.14	7.18	8.97	13.19	3.65
51	1	70.94	4.01	5.02		
	2	78.84	7.47	9.34	7.18	3.06
68	1	106.23	7.73	9.67		
	2	105.12	7.81	9.76	9.72	0.07

Table C.1.5 (Cont.)

Citral						
TCE conc. (µM)	**Sample No.**	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	1	2.61	0.18	0.22		
	2	2.63	0.11	0.13		
	3	3.26	0.00	0.00	0.18	0.06
7	1	10.34	1.06	1.32		
	2	10.51	0.20	0.25		
	3	11.49	0.00	0.00	0.79	0.76
14	1	22.02	6.70	8.38		
	2	21.02	6.99	8.74		
	3	22.51	7.71	9.64	9.19	0.64
25	1	39.01	13.14	16.42		
	2	39.42	11.61	14.51		
	3	41.35	16.40	20.50	17.14	3.06
51	1	77.23	28.71	35.88		
	2	78.84	29.97	37.46		
	3	81.27	33.81	42.26	38.53	3.3
68	1	104.21	10.36	12.95		
	2	105.12	5.98	7.48		
	3	112.96	9.98	12.48	10.97	3.03

C.2 Results of immobilization study

Table C.2.1 Percent decline in A_{600} of Alcian Blue after incubation with immobilized cells on three sizes of cumin seeds and control (uninoculated material)

Control materials (500 µm-1mm seeds)				
Time (d)	**A_{600}**	**%decline**	**AVG**	**SD**
0	0.56	101.43		
	0.55	98.59	100.01	2.00
1	0.56	99.34		
	0.54	96.63	97.98	1.92
2	0.51	90.55		
	0.51	92.60	91.58	1.45
3	0.54	95.91		
	0.52	94.57	95.24	0.95
4	0.58	103.02		
	0.57	102.45	102.74	0.40
5	0.48	85.91		
	0.50	90.07	87.99	2.95
7	0.49	86.76		
	0.50	89.33	88.05	1.82

Table C.2.1 (cont.)

Non-ground seeds (≥ 2mm seeds)				
Time (d)	**A_{600}**	**%decline**	**AVG**	**SD**
0	0.56	95.43		
	0.59	99.29		
	0.59	105.53	100.09	5.10
1	0.54	95.64		
	0.54	92.19	93.91	2.44
2	0.48	81.30		
	0.50	83.92	82.61	1.85
3	0.46	82.01		
	0.56	95.25	88.63	9.36
4	0.56	95.86		
	0.58	98.32	97.09	1.74
5	0.53	93.68		
	0.53	90.44	92.06	2.29
7	0.46	82.22		
	0.53	89.15	85.69	4.90

Table C.2.1 (Cont.)

1-2 mm seeds				
Time (d)	**A_{600}**	**%decline**	**AVG**	**SD**
0	0.56	98.47		
	0.57	98.05		
	0.58	103.58	100.03	3.08
1	0.49	87.41		
	0.51	89.71	88.56	1.63
2	0.46	81.54		
	0.51	90.40	85.97	6.27
3	0.39	69.28		
	0.46	78.96	74.12	6.84
4	0.35	62.02		
	0.43	75.09	68.56	9.24
5	0.38	68.73		
	0.38	67.24	67.98	1.06
7	0.45	78.65		
	0.41	70.64	74.64	5.67

Table C.2.1 (Cont.)

500µm-1mm seeds				
Time (d)	**A_{600}**	**%decline**	**AVG**	**SD**
0	0.58	99.50		
	0.58	99.69		
	0.58	100.82	100.00	0.71
1	0.51	89.12		
	0.59	101.45	95.29	8.72
2	0.44	75.29		
	0.47	80.43	77.86	3.64
3	0.30	52.90		
	0.34	59.64	56.27	4.76
4	0.25	42.58		
	0.25	42.97	42.77	0.28
5	0.28	48.28		
	0.29	50.34	49.31	1.46
7	0.29	50.28		
	0.29	50.80	50.54	0.37

Table C.2.2 TCE transformation capacity by cumin seed-immobilized cells and free cells after incubation with various TCE concentrations for 5 days.

Immobilization					
TCE Conc. (µM)	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	2.54	1.51	2.75		
	2.63	1.43	2.60		
	3.12	1.56	2.83	2.73	0.12
7	10.13	7.12	12.94		
	10.51	7.16	13.02		
	11.02	7.56	13.75	13.24	0.45
14	20.74	14.99	27.25		
	21.02	14.44	26.25		
	21.44	14.89	27.08	26.86	0.53
25	37.24	27.76	50.48		
	39.42	28.42	51.67		
	41.68	26.56	48.29	50.15	1.71
51	74.83	33.65	61.19		
	78.84	36.69	66.72		
	79.36	27.20	49.45	59.12	8.82
68	98.55	17.81	32.39		
	105.12	18.49	33.63		
	105.12	27.18	49.41	38.48	9.49

Table C.2.2 (Cont.)

Free-cells					
TCE Conc. (µM)	**Total TCE mass (µg)**	**TCE degraded (µg)**	***Tc*** **µg TCE /mg cells**	**AVG *Tc***	**SD**
2	2.45	0.01	0.02		
	2.63	0.00	0.00		
	2.62	0.00	0.00	0.006	0.14
7	9.60	2.71	3.39		
	10.51	2.92	3.65		
	10.76	2.93	3.67	3.57	0.15
14	19.38	7.21	9.01		
	21.02	8.35	10.44		
	19.33	6.48	8.10	9.18	1.18
25	34.89	10.80	13.50		
	39.42	13.06	16.33		
	45.30	15.65	19.57	16.46	3.04
51	83.02	0.00	0.00		
	78.84	0.00	0.00		
	76.35	0.00	0.00	0.00	-
68	87.71	0.00	0.00		
	105.12	0.00	0.00	0.00	-

Table C.2.3 Amount of TCE remaining by immobilized cells and control (killed immobilized cells) when TCE was added repeatedly during the test.

Immobilized cells			
Time (d)	**TCE remaining (μmol)**	**AVG**	**SD**
0*	0.16		
	0.16		
	0.16	0.16	0.003
1	0.12		
	0.13		
	0.13	0.13	0.009
2	0.11		
	0.12		
	0.15	0.13	0.020
3	0.12		
	0.11		
	0.12	0.12	0.004
4*	0.09		
	0.10		
	0.11	0.10	0.004
5*	0.11		
	0.11		
	0.14	0.12	0.016
6*	0.14		
	0.16		
	0.17	0.16	0.015
7	0.24		
	0.25		
	0.31	0.26	0.037

***** TCE was repeatedly added to the sample vial.

Table C.2.3 (Cont.)

Control-killed immobilized cells			
Time (d)	**TCE remaining (µmol)**	**AVG**	**SD**
0*	0.16		
	0.16		
	0.17	0.163	0.007
1	0.15		
	0.15		
	0.15	0.151	0.001
2	0.16		
	0.16		
	0.27	0.190	0.062
3	0.14		
	0.15		
	0.16	0.151	0.011
4*	0.14		
	0.15		
	0.15	0.15	0.004
5*	0.26		
	0.27		
	0.29	0.273	0.013
6*	0.38		
	0.40		
	0.42	0.400	0.022
7	0.43		
	0.48		
	0.54	0.484	0.056

***** TCE was repeatedly added to the sample vial.

Table C.2.4 Percent of TCE degradation by immobilized cells on cumin seeds in each application cycle after 5 days incubation.

Application cycle	Reactivation time (hr)	TCE degradation (% of control)	AVG	SD
1st		67.28		
		65.06		
	0	65.49	65.94	1.18
2nd	0	44.36		
		28.84		
		39.79	37.67	7.98
	12	35.90		
		26.57		
		30.73	31.06	4.68
	24	26.06		
		20.98		
		29.45	25.49	4.26
3rd	0	0.00		
		0.01		
		0.00	0.00	0.01
	12	71.94		
		60.79		
		51.33	61.35	10.32
	24	22.66		
		17.41		
		15.86	18.64	3.56

C.3 Results of enzymatic study

Table C.3.1 Absorbance at 400 nm of indoxyl formation in the sample (1) without/with TCE

Enzyme without TCE			
Time	**A_{400}**		
(s)	**1**	**2**	**AVG**
0	0.0034	0.0472	0.0253
60	0.0271	0.0594	0.0433
120	0.0446	0.0742	0.0594
180	0.0558	0.0803	0.0681
240	0.0635	0.0865	0.0750
300	0.0720	0.0958	0.0839
360	0.0808	0.1020	0.0914
420	0.0846	0.1053	0.0950
480	0.0896	0.1104	0.1000
540	0.0949	0.1123	0.1036
600	0.0994	0.1186	0.1090
660	0.1022	0.1254	0.1138
720	0.1070	0.1272	0.1171
780	0.1106	0.1300	0.1203
840	0.1134	0.1337	0.1236
900	0.1160	0.1372	0.1266
960	0.1189	0.1407	0.1298
1020	0.1221	0.1433	0.1327
1080	0.1251	0.1447	0.1349
1140	0.1279	0.1475	0.1377
1200	0.1317	0.1494	0.1406
1260	0.1343	0.1521	0.1432
1320	0.1340	0.1541	0.1441
1380	0.1364	0.1569	0.1467
1440	0.1376	0.1590	0.1483
1500	0.1394	0.1610	0.1502
1560	0.1416	0.1636	0.1526
1620	0.1427	0.1649	0.1538
1680	0.1446	0.1677	0.1562
1740	0.1459	0.1696	0.1578
1800	0.1468	0.1730	0.1599

Table C.3.1 (cont.)

Enzyme with 10 µM TCE			
Time	**A_{400}**		
(s)	**1**	**2**	**AVG**
0	0.0000	0.0000	0.0000
50	0.0000	0.0000	0.0000
100	0.0000	0.0000	0.0000
150	0.0000	0.0000	0.0000
200	0.0000	0.0000	0.0000
250	0.0000	0.0000	0.0000
300	0.0000	0.0000	0.0000
350	0.0000	0.0000	0.0000
400	0.0000	0.0000	0.0000
450	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000
550	0.0000	0.0000	0.0000
600	0.0000	0.0000	0.0000
650	0.0000	0.0000	0.0000
700	0.0000	0.0000	0.0000
750	0.0000	0.0000	0.0000
800	0.0000	0.0000	0.0000
850	0.0000	0.0000	0.0000
900	0.0000	0.0000	0.0000
950	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000
1050	0.0000	0.0000	0.0000
1100	0.0000	0.0000	0.0000
1150	0.0036	0.0042	0.0039
1200	0.0050	0.0051	0.0051
1250	0.0064	0.0072	0.0068
1300	0.0078	0.0079	0.0079
1350	0.0084	0.0086	0.0085
1400	0.0097	0.0089	0.0093
1450	0.0108	0.0098	0.0103
1500	0.0123	0.0133	0.0128
1550	0.0137	0.0147	0.0142
1600	0.0149	0.0153	0.0151
1650	0.0162	0.0172	0.0167
1700	0.0173	0.0189	0.0181
1800	0.0187	0.0195	0.0191

Table C.3.1 (cont.)

Enzyme with 40 µM TCE			
Time	**A_{400}**		
(s)	**1**	**2**	**AVG**
0	0.0000	0.0000	0.0000
50	0.0000	0.0000	0.0000
100	0.0000	0.0000	0.0000
150	0.0048	0.0000	0.0048
200	0.0099	0.0000	0.0099
250	0.0128	0.0000	0.0128
300	0.0183	0.0000	0.0183
350	0.0220	0.0000	0.0220
400	0.0261	0.0000	0.0261
450	0.0303	0.0038	0.0171
500	0.0332	0.0079	0.0206
550	0.0358	0.0119	0.0239
600	0.0384	0.0151	0.0268
650	0.0411	0.0176	0.0294
700	0.0436	0.0202	0.0319
750	0.0460	0.0228	0.0344
800	0.0486	0.0250	0.0368
850	0.0510	0.0278	0.0394
900	0.0528	0.0306	0.0417
950	0.0553	0.0336	0.0445
1000	0.0571	0.0359	0.0465
1050	0.0586	0.0378	0.0482
1100	0.0604	0.0400	0.0502
1150	0.0624	0.0425	0.0525
1200	0.0645	0.0442	0.0544
1250	0.0663	0.0457	0.0560
1300	0.0676	0.0483	0.0580
1350	0.0694	0.0491	0.0593
1400	0.0710	0.0510	0.0610
1450	0.0726	0.0526	0.0626
1500	0.0741	0.0543	0.0642
1550	0.0757	0.0558	0.0658
1600	0.0770	0.0586	0.0678
1650	0.0783	0.0601	0.0692
1700	0.0797	0.0613	0.0705
1800	0.0814	0.0645	0.0730

Table C.3.1 (cont.)

Enzyme with 80 µM TCE			
Time	**A_{400}**		
(s)	**1**	**2**	**AVG**
0	0.0000	0.0000	0.0000
50	0.0000	0.0000	0.0000
100	0.0000	0.0000	0.0000
150	0.0000	0.0000	0.0000
200	0.0000	0.0000	0.0000
250	0.0000	0.0000	0.0000
300	0.0000	0.0000	0.0000
350	0.0000	0.0000	0.0000
400	0.0000	0.0000	0.0000
450	0.0000	0.0000	0.0000
500	0.0000	0.0000	0.0000
550	0.0000	0.0000	0.0000
600	0.0000	0.0000	0.0000
650	0.0000	0.0000	0.0000
700	0.0000	0.0000	0.0000
750	0.0000	0.0000	0.0000
800	0.0000	0.0000	0.0000
850	0.0000	0.0000	0.0000
900	0.0000	0.0000	0.0000
950	0.0000	0.0000	0.0000
1000	0.0000	0.0000	0.0000
1050	0.0000	0.0000	0.0000
1100	0.0000	0.0000	0.0000
1150	0.0000	0.0000	0.0000
1200	0.0000	0.0000	0.0000
1250	0.0000	0.0000	0.0000
1300	0.0000	0.0000	0.0000
1350	0.0000	0.0000	0.0000
1400	0.0000	0.0000	0.0000
1450	0.0000	0.0000	0.0000
1500	0.0010	0.0010	0.0010
1550	0.0023	0.0043	0.0033
1600	0.0032	0.0052	0.0042
1650	0.0034	0.0050	0.0042
1700	0.0047	0.0057	0.0052
1800	0.0054	0.0084	0.0069

Table C.3.1 (cont.)

Enzyme containing TCE		
Time	**20 µM TCE**	**60 µM TCE**
(s)	**A_{400}**	**A_{400}**
0	0.0000	0.0000
50	0.0021	0.0000
100	0.0153	0.0000
150	0.025	0.0000
200	0.0327	0.0000
250	0.0396	0.0000
300	0.0472	0.0000
350	0.0522	0.0000
400	0.0579	0.0000
450	0.0615	0.0000
500	0.0654	0.0000
550	0.0695	0.0000
600	0.0739	0.0000
650	0.076	0.0000
700	0.0793	0.0000
750	0.0816	0.0000
800	0.0842	0.0000
850	0.0881	0.0000
900	0.0905	0.0000
950	0.0941	0.0000
1000	0.0957	0.0000
1050	0.0967	0.0000
1100	0.0977	0.0000
1150	0.0999	0.0000
1200	0.1029	0.0000
1250	0.1049	0.0000
1300	0.1049	0.0000
1350	0.1079	0.0000
1400	0.1089	0.0000
1450	0.1099	0.0000
1500	0.1119	0.0030
1550	0.1139	0.0033
1600	0.1159	0.0040
1650	0.1169	0.0043
1700	0.1189	0.0052
1800	0.1189	0.0067

Table C.3.2 A_{400} of indoxyl formation in the sample after TCE exposure

Enzyme after TCE was removed			
Time	**A_{400}**		
(s)	**1**	**2**	**AVG**
0	0.0000	0.0000	0.0000
50	0.0212	0.0000	0.0106
100	0.0461	0.0198	0.0330
150	0.0627	0.0314	0.0471
200	0.0700	0.0417	0.0559
250	0.0771	0.0489	0.0630
300	0.0832	0.0534	0.0683
350	0.0894	0.0578	0.0736
400	0.0924	0.0599	0.0762
450	0.0952	0.0635	0.0794
500	0.0976	0.0654	0.0815
550	0.0994	0.0695	0.0845
600	0.1023	0.0723	0.0873
650	0.1049	0.0751	0.0900
700	0.1073	0.0777	0.0925
750	0.1086	0.0788	0.0937
800	0.1094	0.0798	0.0946
850	0.1118	0.0811	0.0965
900	0.1135	0.0837	0.0986
950	0.1152	0.0856	0.1004
1000	0.1164	0.0871	0.1018
1050	0.1198	0.0899	0.1049
1100	0.1205	0.0909	0.1057
1150	0.1219	0.0923	0.1071
1200	0.1228	0.0945	0.1087
1250	0.1238	0.0967	0.1103
1300	0.1257	0.0978	0.1118
1350	0.1281	0.0998	0.1140
1400	0.1283	0.1012	0.1148
1450	0.1293	0.1080	0.1187
1500	0.1298	0.1062	0.1180
1550	0.1333	0.1080	0.1207
1600	0.1333	0.1030	0.1182
1650	0.1343	0.1045	0.1194
1700	0.1349	0.1052	0.1201
1750	0.1354	0.1069	0.1212
1800	0.1368	0.1075	0.1222

Table C.3.3 A_{400} of indoxyl formation in the sample containing TCE and cumene

Enzyme with TCE and cumene			
Time	A400		
(s)	1	2	AVG
0	0.2441	0.1534	0.1988
50	0.2718	0.1726	0.2222
100	0.2898	0.1886	0.2392
150	0.3051	0.2008	0.2530
200	0.3163	0.2098	0.2631
250	0.3196	0.2160	0.2678
300	0.3264	0.2236	0.2750
350	0.3321	0.2277	0.2799
400	0.3345	0.2372	0.2859
450	0.3376	0.2406	0.2891
500	0.3443	0.2431	0.2937
550	0.3496	0.2497	0.2997
600	0.3514	0.2543	0.3029
650	0.3531	0.2598	0.3065
700	0.3592	0.2635	0.3114
750	0.3634	0.2716	0.3175
800	0.3679	0.2767	0.3223
850	0.3652	0.2798	0.3225
900	0.3765	0.2845	0.3305
950	0.3748	0.2898	0.3323
1000	0.3793	0.2914	0.3354
1050	0.3780	0.2959	0.3370
1100	0.3834	0.2995	0.3415
1150	0.3862	0.3065	0.3464
1200	0.3881	0.3081	0.3481
1250	0.3895	0.3158	0.3527
1300	0.3901	0.3187	0.3544
1350	0.3923	0.3195	0.3559
1400	0.3965	0.3259	0.3612
1450	0.3982	0.3299	0.3641
1500	0.3999	0.3327	0.3663
1550	0.4005	0.3388	0.3697
1600	0.4075	0.3424	0.3750
1650	0.4095	0.3475	0.3785
1700	0.4109	0.3499	0.3804
1750	0.4131	0.3545	0.3838
1800	0.4165	0.3581	0.3873

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 degree (second class honors) from Thammasat University in the faculty of science and technology in 2001. Her major study was Agricultural Technology with minor in Biotechnology. She pursued her Master degree in the International Postgraduate Programs in Environmental Management, Chulalongkorn University, Bangkok, Thailand since 2002 to 2004. After that she started her Ph.D. in the same program and completed her Doctoral degree in Environmental Management in 2008.

Presentations and Posters

- **Suttinun, O**., Müller, R., and Luepromchai, E. (2008) "*Induction of Rhodococcus sp. L4 to Cometabolize Trichloroethylene by Plant Residues*". The XII International Congress of Bacteriology and Applied Microbiology, the Meetings of the Three Divisions of the International Union of Microbiological Societies (IUMS) 2008, Istanbul, Turkey, August 5 –15, 2008.
- **Suttinun, O**., Müller, R., and Luepromchai, E. (2007) *"Effect of various terpenes on inducing Rhodococcus sp. L4 to cometabolize trichloroethylene"* .The Sixth Princess Chulabhorn International Science Congress, Bangkok, Thailand, November 25-29, 2007.
- **Suttinun, O**., and Luepromchai, E. (2005). "*Bioremediation of trichloroethylene contaminated soil by the addition of terpene-induced Rhodococcus and plant residues*". The XI International Congress of Bacteriology and Applied Microbiology, the Meetings of the Three Divisions of the International Union of Microbiological Societies (IUMS) 2005, San Francisco, California, USA. July 23-28, 2005.
- **Suttinun, O.**, and Luepromchai, E. (2004). "*Application of terpene induced cell for enhancing biodegradation of TCE contaminated soil*". 3rd National Environmental Conference, Songkla, Thailand, 28-30 January 2004.
- **Suttinun, O.,** and Luepromchai, E. (2003). "*Plant terpene as an inducer for trichloroethylene (TCE) biodegradation and bioremediation*". EMECS 2003: 6th International Conference on the Environmental Management of Enclosed Coastal Seas, Bangkok, Thailand, 18-21 November 2003.

Publications

- **Suttinun, O**., Müller, R., and Luepromchai, E. (2008). Trichloroethylene cometabolic degradation by *Rhodococcus* sp. L4 induced with plant essential oils. Biodegradation: DOI 10.1007/s10532-008-9220-4.
- **Suttinun, O**., Lederman, P. B., and Luepromchai, E. (2004). Application of terpene-induced cell for enhancing biodegradation of TCE contaminated soil. Songklanakarin J. Sci. Technol. 26 (Suppl. 1): 131-142.