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การใช้สารเทอร์ปีนจากพืชเพื่อชักนำแบคทีเรีย *Rhodococcus pyridinivorans* L4 ให้ย่อยสลายสารไตรคลอโรเอธิลีน (ทีซีอี) และการจำแนกยีนที่เกี่ยวข้อง

โดย

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ชื่อโครงการวิจัย การใช้สารเทอร์ปีนจากพืชเพื่อชักนำแบคทีเรีย *Rhodococcus pyridinivorans* L4 ให้ย่อยสลายสารไตรคลอโรเอธิลีน (ทีซีอี) และการจำแนกยีนที่เกี่ยวข้อง

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บทคัดย่อ

ไตรคลอโรเอธิลีน (ทีซีอี) เป็นสารมลพิษสำคัญที่พบปนเปื้อนในดินและน้ำอย่างแพร่หลาย การย่อยสลายทีซีอีทางชีวภาพมีแนวโน้มที่จะใช้เป็นเทคโนโลยีบำบัดที่มีราคาถูก การศึกษานี้เน้นการประยุกต์ใช้แบคทีเรียท้องถิ่นชื่อ *Rhodococcus pyridinivorans* L4 ซึ่งปกติโคเมตาบอไลซ์ทีซีอีเมื่อเลี้ยงด้วยโทลูอีน โดยตรวจสอบสารเทอร์ปีนจากพืชหลายชนิด เพื่อใช้เป็นทางเลือกของการชักนำเอนไซม์ย่อยสลายทีซีอี สารซิทรัล คิวมีน และไลโมนีนที่ 25-50 พีพีเอ็ม สามารถชักนำแบคทีเรียให้ย่อยสลายทีซีอี 15 พีพีเอ็ม ภายใน 30 ชั่วโมง เซลล์ที่ชักนำด้วยเทอร์ปีนย่อยสลายทีซีอีอย่างสมบูรณ์ (mineralization) ได้มากกว่าเซลล์ที่ไม่ถูกชักนำ *R. pyridinivorans* L4 กับใบตะไคร้ เมล็ดยี่หร่า หรือเปลือกส้ม ส่งเสริมการย่อยสลายทีซีอีในชุดดินทดลองขนาดเล็กอย่างมีประสิทธิภาพ ต่อมาได้ทำการจำแนกเอนไซม์ย่อยสลายทีซีอีขั้นต้นและยีนที่เกี่ยวข้อง โดยวิธีวิเคราะห์ดีเอ็นเอและเอนไซม์ ผลการทดลองพบว่า *R. pyridinivorans* L4 มีจีนสำหรับโทลูอีนไดออกซิจีเนส ซึ่งเหมือนกับแบคทีเรียที่ย่อยสลายทีซีอีและโทลูอีนชนิดอื่น ลำดับเบสของจีน (100%) เหมือนกับ toluene inducible dioxygenase large subunit ใน *Rhodococcus* sp. I24 ซึ่งพบในประเทศสหรัฐอเมริกา การวิเคราะห์ RT-PCR พบว่าเซลล์ที่ชักนำด้วยเทอร์ปีนแสดงออกจีนสำหรับโทลูอีนไดออกซิจีเนส มากกว่าเซลล์ที่ไม่ถูกชักนำ อย่างไรก็ดีเซลล์ที่เลี้ยงด้วยโทลูอีนมีปริมาณ mRNAของไดออกซิจีเนสมากที่สุด

Project Title: Utilization of plant terpenes for induction of *Rhodococcus pyridinivorans* L4 bacteria to degrade trichloroethylene (TCE) and characterization of involving gene

Investigator: Ekawan Luepromchai, Ph.D.

Year: December 2004

ABSTRACT

Trichloroethylene (TCE) is a priority pollutant that causes widespread contamination of water and soil. Biodegradation of TCE has the potential for being a cost-effective remediation technology. This study focused on the application of a local bacterium, *Rhodococcus pyridinivorans* L4 that normally cometabolize TCE while growing on toluene. Various plant terpenes were investigated as alternative TCE degrading enzyme inducers. Citral, cumene, and limonene at 25-50 ppm induced the bacterium to degrade 70% of 15 ppm TCE within 30 hrs. Terpene induced cells were also mineralized TCE more than non-induced cells. *R. pyridinivorans* L4 along with lemon grass leaves, cumin seeds, or orange peels were effectively enhanced TCE degradation in soil microcosms. Characterization of the initial TCE degrading enzyme and its encoding gene were later carried out by enzyme and DNA assay. The results suggested that *R. pyridinivorans* L4 contained toluene dioxygenase encoding gene, which is also found in other TCE/toluene degrading bacteria. DNA sequences of the gene were 100% homology to toluene inducible dioxygenase large subunit in *Rhodococcus* sp. I24, an isolate from USA. RT-PCR analysis revealed that terpene induced cells expressed higher amount of dioxygenase gene than non-induced cells. Meanwhile, toluene grown cells contained the highest amount of dioxygenase mRNA.

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I would like to thank Assist. Prof. Dr. Pornpimol Rongnoparut, Mahidol University and Dr. Onruthai Pinyakong, Chulalongkorn University, for their helpful suggestions regarding gene characterization. Support from Ms. Oramas Sutthinun is greatly appreciated. Finally, I am grateful to the Environmental Research Institute, Chulalongkorn University and the National Research Center for Environmental and Hazardous Waste Management (NRC-EHWM), Chulalongkorn University for their support on scientific instruments and reagents.

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1. INTRODUCTION

1.1 Background and Motivation

The increase industrial activities nowadays have led to an extensive use of hazardous chemicals and thereby resulting in widespread contamination of water and soil. Contamination of chlorinated solvent, especially trichloroethylene (TCE) has become an important environmental problem because of its toxicity and persistent in the environment. TCE is infiltrated and accumulated in soil and groundwater from inappropriate disposal of dry cleaning agents, degreasing solvents, and paint strippers. In Thailand, TCE consumption has been increased annually thus its contamination is likely to occur.

Bioremediation is one of the most promising technologies to clean-up hazardous chemicals like TCE since it is cost effective, easy to implement, and environmental friendly. During bioremediation, microorganisms convert hazardous organic chemicals to environmentally benign products such as water, carbon dioxide, biomass, and salts. However, the success of bioremediation is site specific. It depends on the types and behavior of microbial populations present at the site, as well as physical and chemical factors that control the microbial activities against the pollutants (Head, 1998). To develop a bioremediation technology suitable for Thailand environment, we therefore require information of the local microorganisms such as type, degrading activities, genetic makeup, and growth condition.

A new *Rhodococcus pyridinivorans* strain L4 was recently isolated from petroleum contaminated soil collected from Bangkok area (Luepromchai and Suttinun, 2003). *Rhodococcus*, a group of Gram positive soil bacteria, is known for their ability to degrade wide variety of toxic compounds such as aromatic and aliphatic hydrocarbons, insecticides, explosives, and chlorinated hydrocarbons. Consequently, it

has gained considerable interest for use in bioremediation of the environment and biotransformation of chemicals in reactor based system (Dabrock *et al.*, 1994).

Similar to other *Rhodococcus* species, *R. pyridinivorans* L4 can utilize diverse organic compounds as a sole carbon source; for example BTEX (benzene, toluene, xylene, and ethylbenzene) and plant terpenes such as limonene, cumene, and pinene. It also cometabolizes TCE while growing on toluene. Cometabolism is a special form of microbial metabolism that occurred when a microorganism grown on certain substrate (inducer) indirectly oxidizes a hazardous chemical into nontoxic compounds. The phenomenon of TCE cometabolism and substrate diversity in *R. pyridinivorans* L4 suggested that the bacteria would be useful for TCE biodegradation and for bioremediation of polluted sites. Meanwhile, genetic information of this bacteria as well as an approach to improve its ability are required to assure the success of application.

This study is consisted of two parts; 1) the utilization of plant terpenes as an alternative TCE inducer for *R. pyridinivorans* L4 and 2) the characterization of genes involved in TCE cometabolism. An alternative TCE inducer other than toluene is of interest because toluene is a classified hazardous substance that should not be used as soil or water amendments. To prevent this problem, alternative compound that is non-toxic, environmentally friendly, and cheap is required for TCE cometabolism. Terpenes are a group of plant volatile organic compound. which generally associated with plant characteristic fragrances for example limonene from lemon oil, carvone from peppermint oil, and pinene from pine oil. Plant terpenes have been reported to stimulate microbial degradation of xenobiotic compounds such as polychlorinated biphenyls (PCB), toluene, and phenol (Singer et al., 2003). however the utilization of

terpenes for TCE cometabolism is limited and its application for soil bioremediation has never been studied.

The proposed study will investigate the ability of various terpenes on induction of TCE co-metabolism by *R. pyridinivorans* L4, and then develop a bioremediation treatment for TCE contaminated soil using terpene and the bacteria. The acquired knowledge would open the prospect of applying plant terpenes and local bacteria isolates for biodegradation of TCE as well as for development of a low cost, environmental friendly bioremediation process. Furthermore, the utilization of plant terpenes would increase the value of local agricultural products, since we grow many terpene producing plants in Thailand.

The second part of this study is to characterize genes involved in TCE cometabolism from *R. pyridinivorans* L4. Aerobic TCE cometabolism is initiated by various oxygenase enzymes, a class of enzyme that catalyze the incorporation of one (monooxygenases) or two (dioxygenases) atom of dioxygen into substrates. The common feature among TCE degrading oxygenases is that each enzyme has relaxed substrate range and found at the beginning of a catabolic pathway used to harvest growth substrate i.e. an uncharged, hydrophobic molecule such as toluene, phenol, methane, and ammonia. Since, there are high diversities of TCE degrading enzymes, the impact of each enzyme on TCE cometabolism in the environment is not well understood and the predictions about their reactivity, reaction rates, and product distributions are difficult to make (Arp *et al.*, 2001).

The study of enzyme coding genes in *R. pyridinivorans* L4 would therefore increase the understanding of TCE degrading process as well as facilitate the application of this bacterium for TCE bioremediation. With the genes in hand,

application of genetic engineering is become feasible, for example, to design gene probes for monitoring *R. pyridinivorans* L4 and other TCE degrading populations during bioremediation, to develop a TCE sensing bacteria for detection of its contamination, and to improve degrading activity and survival rate of TCE degrading bacteria. In addition, the bacterial gene will be a model for study TCE degrading mechanism carried out by local bacteria. Finally, the acquired knowledge will be a foundation for study other native TCE degrading bacteria as well as for develop an appropriate bioremediation strategy to clean-up our polluted environment.

1.2 Objectives

The main objective of the proposed study is to develop a low cost, environmental friendly approach for TCE bioremediation and to acquire genetic information of the local bacteria isolate that involve in TCE biodegradation. The specific objectives are:

1.2.1 To investigate the ability of various plant terpenes as TCE inducer for *R. pyridinivorans* L4.

1.2.2 To develop a bioremediation treatment for TCE contaminated soil using plant terpenes and *R. pyridinivorans* L4.

1.2.3 To determine probable initial TCE degrading enzyme in *R. pyridinivorans* L4.

1.2.4 To characterize initial TCE degrading enzyme encoding gene in *R. pyridinivorans* L4.

1.3 Scope of work

TCE inducing activity of plant terpenes was studied from purified solutions, essential oil, and plant materials. The induction was carried out by growing *R. pyridinivorans* L4 in liquid media that supplemented with terpene solutions or plant essential oil. The tested terpene solutions were consisted of carvone, citral, cumene, limonene, and pinene, while essential oil samples are lemon, lemon grass, mint, and pine oil. TCE biodegradation was detected from the decreasing of TCE and the production of chloride ion after incubating terpene induced cells with TCE. During bioremediation development, we studied TCE induction ability of terpene solutions as well as dry plant materials including orange peels, cumin seeds, and lemon grass leaves. The application of terpene was combined with the addition of *R. pyridinivorans* L4 to enhance TCE degradation. To characterize initial TCE degrading gene, we focused on toluene oxygenase enzyme family since the bacteria is originally utilize toluene as TCE inducer. The type of toluene oxygenase and its encoding gene in *R. pyridinivorans* L4 was identified based on enzyme activities and gene sequences of known bacteria isolates from other countries.

2. SURVEY OF RELATED LETERATURE

2.1 Environmental problems of TCE

2.1.1 Physical and Chemical properties

TCE is produced from chlorination of ethylene and sold under trade names such as Algylen, Benzinol, Chlorylen, Dow-Tri, Petzinol, Threthylen, Triasol, Trichloran, Triclene, and Vitran. It is widely used for degreasing of fabric and metal parts such as airplane, engines, and circuit boards for more than 30 years. It is also used as an extraction solvent for greases, oils, fats, waxes, and tars; a chemical intermediate in the production of other chemicals; and as a refrigerant. In consumer products, TCE is found in typewriter correction fluids, paint removers/strippers, adhesives, spot removers, and rug-cleaning fluids.

TCE is belong to a class of chlorinated aliphatic hydrocarbons (CAHs), which generally contains one or two carbon atoms and one to six chlorine atoms. CAHs are consisted of tetrachloroethylene (PCE), trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), 1,2 trans-dichloroethylene (trans-DCE), 1,2-cis-dichloroethylene (cis-DCE), vinyl chloride and 1,1,1-dichloroethane (1,1,1-TCA). Most chlorinated solvents, including TCE are classified as dense non-aqueous phase liquids (DNAPLs), which tend to sink and accumulate at the bottom of a confined aquifer. TCE and other CAHs are highly resistant to chemical and biological breakdown, which is also a major reason for the widespread commercial use.

TCE is a nonflammable colorless solvent with a sweet odor similar to ether or chloroform and has a sweet burning taste. The synonyms of TCE are acetylene trichloride, ethylene trichloride, trichlororide, and trichloroethene. The density of TCE is 1.465 g/ml, whereas its solubility in water at 25°C is 1.366 g/l. TCE is miscible with many common organic solvents such as ether, alcohol, and chloroform. The vapor

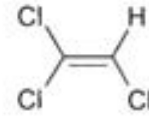
pressure and Henry's law constant at 25°C for TCE is 74 mm Hg and 0.011 atm-m^3/mol, respectively. Thus, TCE is very mobile and also classified as volatile organic compounds (VOCs). Some physical and chemical properties of TCE are showed in Table 2.

2.1.2 Sources and Environmental Fate

The widespread use of TCE has caused environmental contamination due to the release of TCE either deliberately or accidentally into soil and groundwater. TCE and various CAHs are the most commonly detected pollutants at hazardous waste sites and in municipal groundwater supplies in the United States (Steingraber, 1998). TCE can also enter the air and water after accidental spills or when it is disposed of at chemical waste sites. The spent solvent containers eventually corroded and their contents leaked onto the soil. Later, TCE will sink 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. TCE is also formed by the transformation of tetrachloroethane (TeCA) and perchloroethylene (PCE), other chlorinated solvents that contaminated soil and groundwater (Ferguson and Pietari, 2000).

Table 2.1 Physical and chemical properties of TCE

(Adapted from http://www.atsdr.cdc.gov/toxprofiles/tp19-c3.pdf)

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

In Thailand, TCE is widely used in industry for metal degreasing and electronic parts cleaning as well as in plastic processing. In 1998, Thailand Environment Institute (TEI) surveyed 476 factories and found that 16% of the factories used high amount of TCE and stored the spent chemical in metal or plastic containers before sent to the waste treatment company (TEI, 1998). TCE was mainly imported from United Kingdom, EU countries, Japan, and China for cleaning and degreasing operations and for substitution of chlorofluorocarbon (CFC). The consumption of TCE has been increased annually from 3,523 tons in 2001 to 7,364 tons in 2002 (Thai Customs Department). This year (2004), 6,267 tons of TCE has been imported during January to October.

The study of TCE contamination is limited in our country. Only Milintawisamai et al. (2001a) had studied the level of TCE contamination in three Thai factories that utilized TCE in their production process. TCE was found in soil and groundwater from all factories at the concentration ranges from 0.03 - 970 ppm. Furthermore, small amount of TCE was transformed by soil microorganisms to 1, 2 DCE, which is less toxic but still accumulated. The results suggested that the current handling, disposal, and treatment methods are probably ineffective or inappropriate to prevent TCE contamination at the factories. It is also implied that TCE may contaminate other factories in Thailand as well. Therefore, TCE would be a major environmental contaminant and may cause long term health effects in the near future.

2.1.3 Health Effects and Regulations

TCE has been found to trigger both cancinogenic and non-cancinogenic effects in human. Recent epidemiological studies reported that people exposed to high levels

of TCE in drinking water or in workplace air over long periods, have shown evidence of increased cancer incidents (ATSDR, 1997). There are several types of human cancers associated with TCE, for example kidney, liver, cervix, and lymphatic system. In addition, some studies with mice and rats have suggested that high levels of TCE may cause liver or lung cancer(ATSDR, 1997). Acute (short-term) and chronic (long-term) inhalation exposure to TCE in humans results in central nervous system (CNS) effects, with symptoms such as dizziness, headaches, confusion, euphoria, facial numbness, and weakness. Liver, kidney, immunological, endocrine, and developmental effects have also been reported in human.

The most common sources of high level TCE exposure are occupational especially in factories where it is manufactured or used. 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).

Low level exposure is through the contaminated water, use of products containing TCE, and from evaporation and leaching from waste disposal site. WHO drinking water quality guideline for TCE was established at 0.070 mg/l (70 ppb), while the US EPA and Canadian standard is 0.005 mg/l (5 ppb) and 0.050 mg/l (50 ppb), respectively. There is no TCE standard for drinking water quality in Thailand; however, the groundwater quality standards regulated TCE at 0.005 mg/l (5 ppb). In

addition, soil quality standard draft has been established and no more than 28 ppm and 61 ppm TCE should be found in residential soil and industrial soil, respectively (PCD, 2000).

2.2 TCE biodegradation and bioremediation

Biodegradation of TCE can occur under both aerobic and anaerobic conditions. Under aerobic conditions, TCE can be biodegraded by organisms expressing a variety of oxygenase enzymes after induction with numerous primary substrates. The process is known as cometabolism. In this process, 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 (Alvarez-Cohen and McCarty, 1991, Madsen, 1991). On the other hand, anaerobic biodegradation of TCE occurs by reductive dechlorination, which TCE serves as an electron acceptor and one chlorine atom is replaced with a hydrogen atom. Anaerobic 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. Consequently, TCE bioremediation research is recently focused on aerobic cometabolitm process, which resulting in the mineralization of TCE and production of carbon dioxide, water, and chloride ions.

2.2.1 Co-metabolism of TCE

Cometabolism of TCE was firstly investigated by Wilson and Wilson (1985). They studied 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).

In the soil, methanotrophic populations were increased and TCE was degraded extensively to carbon dioxide. Without exposure to methane, TCE degradation was insignificant. Therefore, it can be suggested that TCE does not serve as a primary (growth) substrate for microorganisms and is biodegraded under aerobic conditions only through the process known as co-metabolism or co-oxidation (Little *et al.* 1988).

Recently, more work on TCE biodegradation has been carried out. A number of studies demonstrated that TCE is transformed co-metabolically by a group of aliphatic and aromatic hydrocarbon-degrading bacteria. Generally, co-metabolism of TCE requires compounds such as benzene, toluene, ethylbenzene, xylene, phenols or methane to produce energy as well as to induce for TCE degrading enzymes. In aerobic condition, these microorganisms were reported to completely mineralize TCE to CO_2 and H_2O (Semprini, 1997). Example of TCE concentrations used for the biodegradation study in liquid cultures were 0.4 mg/l (Little *et al.*, 1988), 3 - 24 mg/l (Hyman *et al.*, 1991; 1995), 1 mg/l (Fries *et al.*, 1997), and 1 mg/l (Vannelli *et al.*, 1990), while the amounts of TCE in soil studies were 0.1-10 mg of TCE/l in soil solution (Fan and Scow, 1993), 50 to 60 mg of TCE/l in soil solution (Mu and Scow, 1994).

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

Hopkins *et al.* (1993) investigated the ability of aerobic groundwater microorganisms, especially phenol oxidizing bacteria to co-metabolically degrade

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

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

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. Futamata *et al.* (2001) compared the whole-cell kinetics between phenol and TCE-degrading activities, indicating that phenol was a much preferred substrate for the degradative enzymes.

rather than TCE. This suggested that TCE is not efficiently degraded in the presence of phenol and they also reported that the phenol concentration should be carefully determined in phenol amended biostimulation. However, phenol addition may be questioned since chlorination of groundwater containing phenol produces chlorinated phenols that cause taste and odor problems (Hopkins *et al.*, 1993). To prevent this problem, alternative compound that is non-toxic, environmentally friendly, and cheap is required.

Understanding the importance of TCE cometabolism has led to the development of several TCE bioremediation processes. For example; growing indigenous microbes in situ, injecting bacteria grown in reactor into the soil subsurface, and adding soluble substrate (i.e. phenol and toluene) into contaminated aquifer. In some cases, direct addition of gaseous substrate (i.e. propane and methane) was used for TCE treatments. Ex situ bioremediation may be implemented to treat soil and groundwater as well.

2.2.2 TCE Bioremediation

At the present, remediation of TCE contaminated groundwater and soil relies primarily on physical and chemical processes such as air stripping and carbon adsorption. However, these methods are typically expensive and just transferred the contaminant from one part of the environment to another. Bioremediation is an alternative approach that has been recognized as a cost-effective and environmentally sound treatment for TCE cleanup (Luu *et al.*, 1995). The objective of bioremediation is to exploit biological processes to clean up contaminated site, however the success of bioremediation is site specific and depends on the types and behavior of microbial

populations present at the site, as well as physical and chemical factors that control the microbial activities against the pollutants (Head, 1998). To develop a bioremediation technology suitable for Thailand environment, we therefore require information of the local degrading microorganisms such as type, degrading activities, genetic makeup, and growth condition.

TCE bioremediation may employ variety of strategies including bioaugmentation, biostimulation, and natural attenuation. Bioaugmentation is the inoculation of specialized microorganisms into contaminated site to degrade specific target compounds that are not easily degraded by the indigenous microflora (Forsyth *et al.*, 1995). Biostimulation is the adjustment of nutrients, pH, water, and oxygen levels to enhance the degrading activity of indigenous microorganisms (Bollag and Bollag, 1995). Bioaugmentation appears to be a promising technique for in-situ bioremediation schemes since it eliminates long startup periods, ensures the presence of an active, efficient TCE-degrading population, and increase substantially the degree of TCE degradation (Munakata-Marr *et al.*, 1996). Natural attenuation relies on the intrinsic (i.e., naturally occurring) supplies of electron acceptors, nutrients, and other necessary materials to develop a biologically active zones and prevent the migration of contamination away from its source (Rittmann and McCarty, 2001).

Actual application of TCE bioremediation is still limited in practice but is rapidly gaining popularity. Different methods of application have been studied in both field and laboratory scale, for example growing indigenous microbes in situ, injecting bacteria grown in reactor into the soil subsurface, and adding soluble substrate (i.e. phenol and toluene) into contaminated aquifer. In some cases, direct addition of

gaseous substrate (i.e. propane and methane) was used for TCE treatments. Ex situ bioremediation may be implemented to treat soil and groundwater as well.

2.2.3 Example of TCE bioremediation strategies

The effects for bioaugmentation on the aerobic cometabolism of TCE in groundwater were investigated using small-column aquifer microcosms (Munakata-Marr *et al.*, 1996, 1997). They found that 60-250 μg/l TCE was cometabolized after the introduction of wild type and genetically altered *Burkholderia cepecia* G4 and PR1 along with the bacteria primary substrate i.e. phenol or lactate to the microcosms. However, the requirement of a primary substrate to induce TCE-degrading enzymes complicates in situ bioremediation, as many of the primary substrates shown to effectively support TCE-transforming cultures pose mass transfer and/or potential regulatory problems with their addition to groundwater systems.

Bioaugmentation of TCE contaminated groundwater under anaerobic condition was also studied. Major *et al.* (2002) demonstrated that addition of a dechlorinating microbial consortium stimulated complete dechlorination of TCE and PCE at Kelly Air Force Base. The site groundwater contained about 1 mg/l of PCE and lower amounts of TCE and DCE. They concluded that bioaugmentation offer a solution for contaminated sites where dechlorination is not occurring naturally (where the appropriate organisms are lacking) or where it is too slow to be practical (very low numbers of dechlorinating organisms).

For contaminated soil, TCE could be removed by a combination of phytoremediation and bioaugmentation under aerobic condition. The treatment was operated by inoculating wheat rhizosphere with root-colonizing bacteria that

genetically engineered to degrade TCE e.g. *Pseudomonas fluorescens* 2-79TOM and *Rhizobium* sp 35645A (Yee *et al.*, 1998, Shim *et al.*, 2000). Consequently, the bacteria are able to express TCE degrading enzyme constitutively while colonize plant roots stably and competitively. In these studies wheat roots provide a large surface area for bacterial growth and can transport the bacteria to pollutants deep in the soil by soil penetrating. The results showed that an average of 63% of the initial TCE was degraded in 4 days compared to the 9% of the initial TCE removed by uninoculated wheat and sterile soil.

The biostimulation trials included the injection of methane (Semprini *et al.*, 1990), toluene (Hopkins and McCarty, 1995) and phenol (Hopkins *et al.*, 1993; Hopkins and McCarty, 1995) into aquifers to stimulate indigenous bacteria. Example of bioaugmentation trial was studied by Duba *et al.* (1996). They used methanotrophic bacteria suspended in groundwater and injected into an aquifer. After withdrawal of the contaminated groundwater, 98% TCE decreased during the first 50 hours incubation. For contaminated soil, Fan and Scow (1993) reported that toluene supported the degradation of TCE in silt loam soils and was not degraded in the absence of toluene. About 60 to 75% of the initial TCE (1 mg/l of soil solution) was degraded during treatment, while 100% of the toluene was disappeared. The addition of phenol also stimulated the degradation of TCE, while glucose did not induce for TCE degradation. Temperature and soil moisture also strongly influenced the degradation of TCE in soil. The initial rates of TCE degradation at 32, 25, 18 °C were similar, at 10 °C the rate of TCE degradation were lower than other temperatures. There was virtually no degradation of TCE or toluene at soil moisture levels of 2.5-

5%, whereas there was little difference in the rate and extent of toluene or TCE degradation at levels of 16, 25, and 30%.

Lee *et. al.* (2002) studied the effect of sorption and desorption resistance on aerobic TCE biodegradation in aqueous and soil-slurry phase. In aqueous phase, the TCE degradation rates were clearly increased in microcosms when toluene was added multiple times and related to the increased toluene dioxygenase (TOD) enzyme activity over time. The results indicated that TCE co-metabolism occurred by the microbial population. The TCE biodegradation rate was approximately 0.31 mg of TCE/l/h for the first 2 h with 27mg/l toluene addition. After toluene was depleted from the system, the TCE degradation rate slowed considerably to 0.035 mg of TCE/l/h. In soil-slurry experiments, freshly contaminated soil, TCE degradation rate of approximately 0.15 mg TCE/kg/h was observed during 39 hours of incubation and then TCE degradation rate decreased considerably to 0.00059 mg TCE/kg/h. The TCE degradation rates in soil-slurry microcosms containing the desorption-resistant fraction of TCE-contaminated soil were approximately 0.00032 mg TCE/kg/h. It is clear from these results that mass transfer into the aqueous phase limited bioavailability of TCE in the contaminated soil.

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

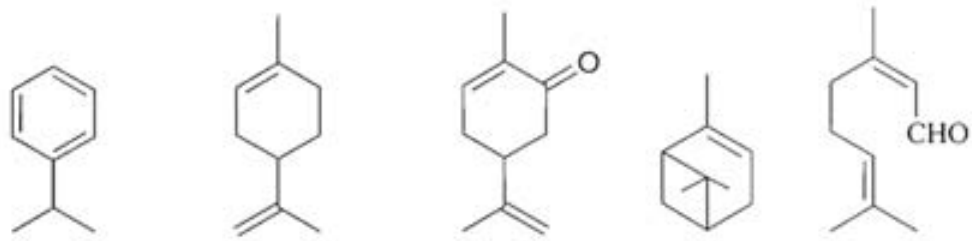
at Robins Air Force Base in Georgia, USA, which contains a mixture of trichloroethylene TCE and chlorobenzene (CB). Results from the field investigation suggest that an intrinsic bioremediation process is occurring, which caused the decrease in TCE and CB concentrations, and increase in TCE degradation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

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.

2.3 Plant terpenes

Terpenes are the largest class of natural products that are widely distributed throughout vegetation types (Amaral *et al.*, 1998). They function in plant as membrane

sterols, carotenoids, pigments, biocides, insect attractants, growth hormones, and signal compounds. Terpenes are based on polymerization of 5-carbon isoprene units that polymerized into compounds with 10, 15, 20 or more carbons. To date, several thousand of these compounds have been identified, of which more than one hundred terpenes are known. Essential oils from plants are the main source of terpenes. For example, cumene from cumin seeds, limonene from orange peels, and carvone from mint leaves are important aroma compounds for use in foods and perfume industries (Figure 2.1).



Terpene:	Cumene	Limonene	Carvone	Pinene	Citral
Plant:	Cumin seed	Citrus	Spearmint	Pine	Lemon grass

Figure 2.1 Example of terpene and its source.

2.3.1 Biotransformation of terpenes

All of the terpenes synthesized by plants are broken down through natural processes. Several bacteria can use terpenes as sole carbon source under aerobic condition. The earliest study was carried out by Joglekar and Dhavlikar (1969). They found that *Pseudomonas aeruginosa* isolated from soil can grow on citral as well as lemon-grass oil. The metabolites of citral were geranic acid (62%), 6-methyl-5-heptanoic acid (0.5%), 3-methyl-2-butenoic acid (1%), and 1-hydroxy-3,7-dimethyl-6-

octen-2-one (0.75%). Misra *et al.* (1996) reported that limonene, pinene, terpinene, and terpinolene are readily degraded when using forest-soil extract and enriched cultures as inocula. Terpene disappearance was the result of biodegradation accompanied by microbial growth and mineralization as seen by the increase in biomass and headspace CO_2 concentrations and the depletion of monoterpenes.

Biotransformation of terpenes is of great commercial interest since microbial enzymes can specifically transform low cost terpenes into more expensive products. For example, limonene, a by-product from orange juice industry, can be transformed into several flavor and fragrance compounds such as perillyl alcohol, α-terpineol, carveol, carvone and menthol (Duetz *et al.*, 2003, de Carvalho and da Fonseca, 2003, Chatterjee and Bhattacharyya, 2001). Enzymes that involve in the transformation processes are epoxide hydrolase (Barbirato *et al.*, 1998, Habe *et al.*, 1996), dioxygenase (Pflugmacher *et al.*, 1996), and monooxygenase (Duetz *et al.* 2003). Moreover, one of the enzymes from a toluene degradation pathway in *Rhodococcus opacus* PWD4 is responsible for the biotransformation of limonene to trans-carveol and carvone (Duetz *et al.*, 2001). The authors suggested that toluene 2,3-dioxygenase is a likely candidate since it has been found to be capable of monooxygenations (e.g. indene to 1-indenol).

2.3.2 Terpenes as inducer for xenobiotic biodegradation

Many studies have showed that plant terpenes can stimulate co-metabolic degradation of xenobiotic compounds. This is probably because they shares similar skeletal structure with the pollutant (Singer *et al.*, 2003). They suggested that chemicals of similar form are more likely to fit within the active site of the same enzyme, whereas

dissimilar structures will only share the same active site if it is a broad-substrate enzyme. In addition, plant terpenes probably affect xenobiotic degradation by enrichment for degrader organisms, enhancement of bacterial growth, and induction of cometabolism in certain microorganisms that carry degradative genes and plasmids (Crowley *et al.* 2001).

In 1997, Gilbert and Crowley screened several terpene compounds, including carvone, cumene, carvacrol, thymol, limonene, cymene and cinnamic acid for their ability to induce polychlorinated biphenyls (PCB) biodegradation in *Arthrobacter* sp. Carvone, the principal component prepared from spearmint extract, was able to induce the biotransformation of 62% of Aroclor 1242 at concentration as low as 50 mg/l. Carvone had higher solubility among other substrates, thus increasing its bioavailability to utilize by microorganisms. The authors also suggested that no growth was detected in *Arthrobacter* sp. when carvone was provided as the sole carbon source at 100, 200, 300, 450, 500 mg/l or greater, suggesting that the induced metabolism of carvone by *Arthrobacter* sp. B1B was principally a detoxification mechanism.

A subsequent study by the same researchers (1998) investigated the efficacy of repeated applications of carvone-induced *Arthrobacter* sp. strain B1B onto Aroclor-1242-contaminated soil, resulting in the removal of 27 ± 6 % of PCB after 17 applications (9 weeks). Later, Singer *et al.* (2000) developed an effective treatment for decontamination of PCB-contaminated soils using two carvone-induced bacteria species, *Arthrobacter* sp. strain B1B and *Rasstonia eutruphus* H850 and sorbitan trioleate, a non-toxic surfactant added to improve bioavailability of PCB. The results showed over 60% removal of PCBs from the contaminated soil.

Koh *et al.* (2000) showed that a monoterpene, carvone, could induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Alcaligenes eutrophus*. Tandlish *et al.* (2001) evaluated the effect of two terpenes, carvone and limonene, as potential inducers of the PCB degradative pathway in *Pseudomonas stutzeri* in the presence of glucose, biphenyl, glycerol and xylose as a sole carbon and energy sources. Interesting results were obtained for PCB biodegradation when xylose was used as the sole carbon source and carvone as a possible inducer. In this case, 7-37% of the individual PCB congeners were degraded from the system without carvone addition, while 30-70% of congeners were removed after supplementing with carvone.

2.3.3 Application of terpenes for bioremediation

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

The utilization of terpenes for TCE degradation is limited and the only terpenes 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 cumene enrichment, were able to cometabolize TCE. 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. The two bacteria exhibited pH-optimum ranging from 5.5-8.0. The effect of temperature was varied between 10-40 °C and found that at 20-30 °C was the optimum temperature.

Nevertheless, the utilization of terpenes for degradation of TCE in contaminated soil has never been studied, and therefore prevented the development of TCE bioremediation treatment. Since terpenes are often effective at very low concentrations and have natural origin, there would be fewer concerns about introducing them into subsurface soil or aquifer and the use of these compounds to remediate the environment is therefore a promising technology (Singer *et al.*, 2003). The application of terpene or plant materials would also increase the value of agriculture produces in our country.

2.4 Enzymes and genes involving in TCE biodegradation

Many bacteria isolates are reported for their TCE degrading activity but the most prevalent are Gram (-) bacteria such as *Pseudomonas*, *Burkholderia*, *Methylosinus*, *Alcaligenes*, and *Acinetobacter* (Arp *et al.*, 2001, Milintawisamai *et al.*,

2001b). On the other hand, Gram (+) TCE degrading bacteria is rare, only actinomycetes i.e. *Rhodococcus* and other filamentous bacteria are reported (Dabrock et al., 1994, Saeki *et al.*, 1999, Lee *et al.*, 2000, Luepromchai and Suttinun, 2003). These bacteria utilize various oxygenase enzymes to aerobically degrade TCE.

2.4.1 Enzymes

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. Meanwhile, 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.

Oxygenases are a group of enzymes that play a significant role in microbial catabolic pathways by incorporating one (monooxygenases) or two (dioxygenases) atom of dioxygen into substrates. In general, oxygenases are not constitutively produced in bacterial cells. Its production is induced by the presence of a particular growth substrate e.g. toluene, methane, and isopropylbenzene. While some oxygenases are very specific for particular substrates, others have remarkably relaxed substrate ranges (Arp *et al.*, 2001). For example, Shim and Wood (2000) studied a monooxygenase from *Pseudomonas stutzeri* OX1 and found that it has a relaxed regiospecificity (hydroxylates toluene in the ortho, meta, and para positions and o-

xylene in both the 3 and 4 positions) as well as a broad substrate range as it oxidizes o-xylene, m-xylene, p-xylene, toluene, benzene, ethylbenzene, styrene, and naphthalene as well as TCE, 1,1-DCE, and CF (chloroform).

During TCE degradation, various intermediates were produced using different oxygenase enzymes (Figure 2.2). The end products from these processes are carbon dioxide, formate, glyoxylate, and oxalate. Enzymatic TCE degradation is limited by several mechanisms e.g. enzyme inactivation, competition between TCE and the inducing substrate, and cell damage from toxic intermediates (Newman and Wackett, 1997, Yeager *et al.*, 2001). However, different oxygenases have different effects on the cells after transformed TCE (Arp *et al.*, 2001). Morono *et al.* (2004) reported that TCE degrading activity in *Pseudomonas putida* F1 could be restored by the addition of benzene and cumene. They suggested that some of the TCE degradation products, or TCE itself, might remain in the active site of TDO during TCE degradation process 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.

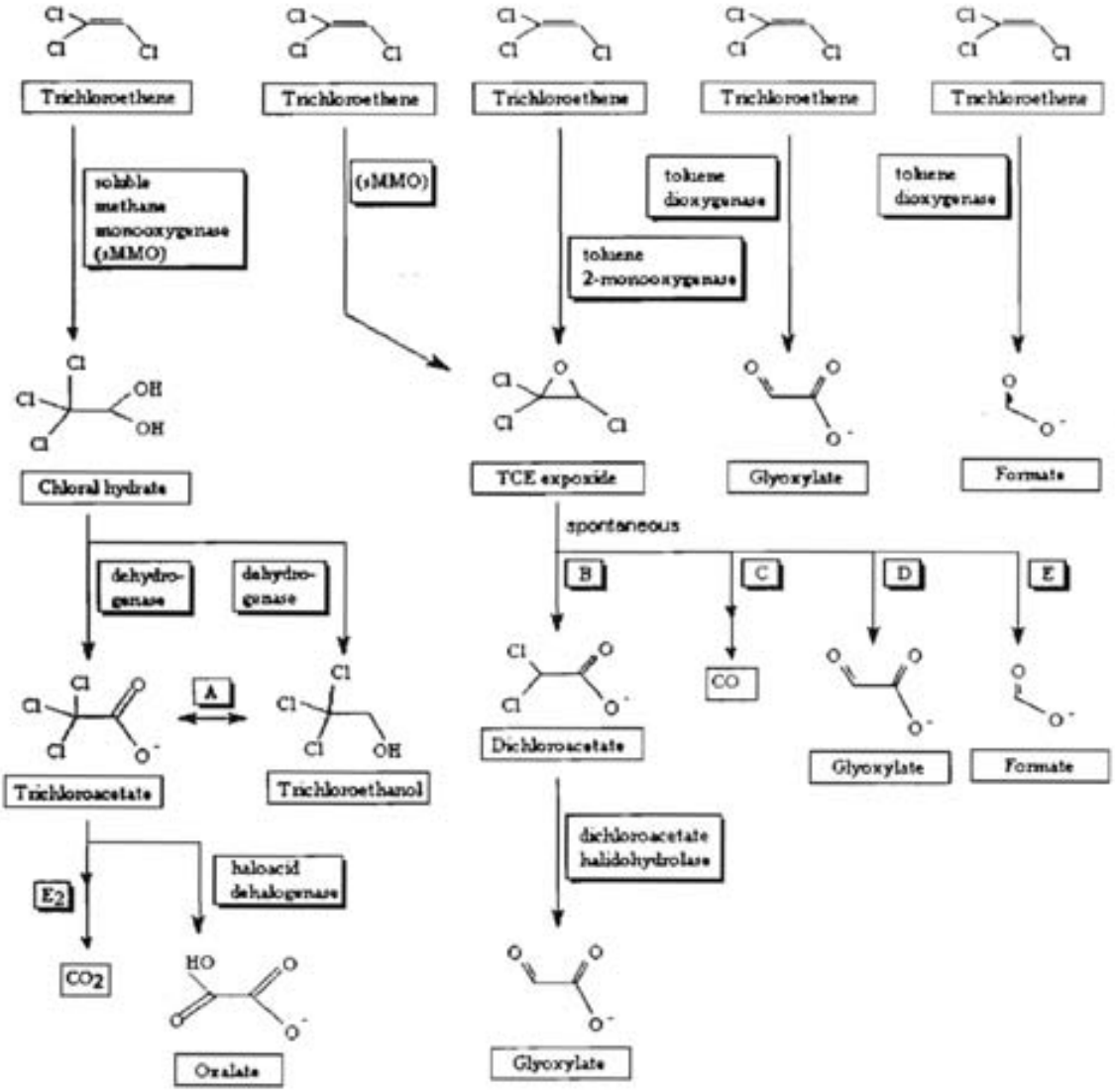


Figure 2.2 Biodegradation pathways of TCE

(Source: http://umbbd.ahc.umn.edu/tce/tce_image_map.html)

2.4.2 Genes

The ability of bacteria to degrade TCE affects by diversities of the genes that code for mono- and dioxygenases as well as their induction process (Arp, 1995). The genes that code for a given oxygenase are usually clustered together in a single operon and often share homology with counterparts that code for the subunits of related oxygenases in other bacteria (Arp *et al.*, 2001). For example, a gene cluster of TOD endcoded by *todC1C2BA* of *Pseudomonas putida* F1 and *bphA1A2A3A4* coding for biphenyl dioxygenase of *Pseudomonas pseudoalcaligenes* KF707 are similar in gene organization and nucleotide sequences (Furukawa, 2003)

Recently, Baldwin *et al.* (2003) aligned DNA and amino acid sequences of the large subunit of various aromatic oxygenase genes and grouped them into families. They found that aromatic oxygenases can be divided into 4 families, namely naphthalene dioxygenase (type N), biphenyl and monoaromatic dioxygenases (type D), ring-hydroxylating monooxygenases (type R), and alkyl group-hydroxylating monooxygenase (type T) (Figure 2.3A and B). Later, PCR primer sets were designed from the conserved region unique to each family and used for detection and enumeration of known aromatic degraders.

Genetic information of TCE degradation process is important for the development of new degradation pathways and the changing of substrate ranges, reactivitiy, and etc. For example, the modification of oxygenases by molecular biology technique can enhance their effectiveness. Studies from Japan showed that hybrid enzymes between TDO and biphenyl oxygenase (TodC1-BphA2-BphA3-BphA4) showed higher affinity for TCE and degraded TCE more efficiently (Furukawa, 2003).

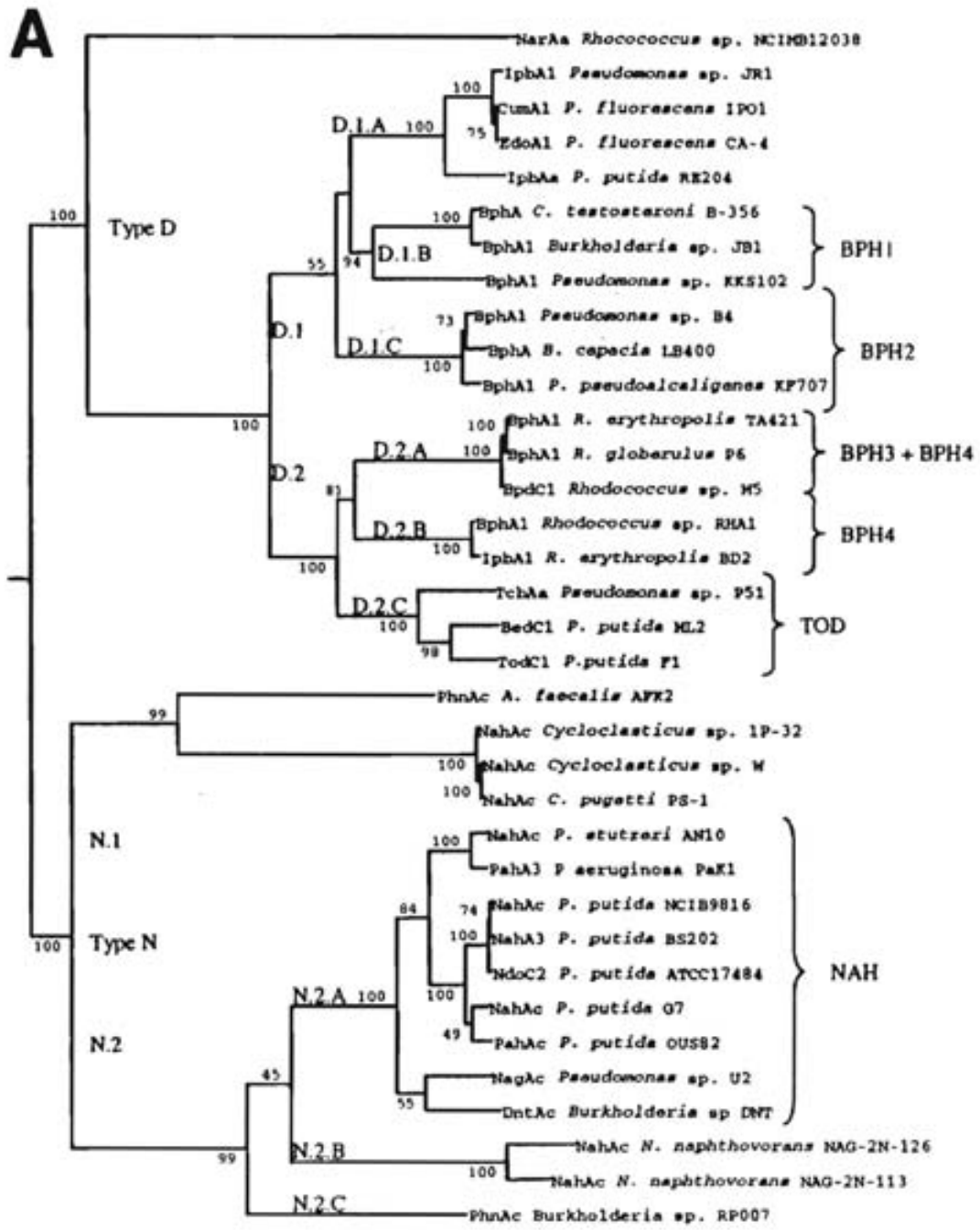


Figure 2.3A. Phylogenetic analysis of the large subunits of aromatic dioxygenase genes. Designations at branch points, e.g., D.1.A, indicate type (D), family (2), and subfamily (A). Subfamilies of genes were used to perform alignments leading to the identification of PCR primer sets. (Source: Baldwin *et al.*, 2003)

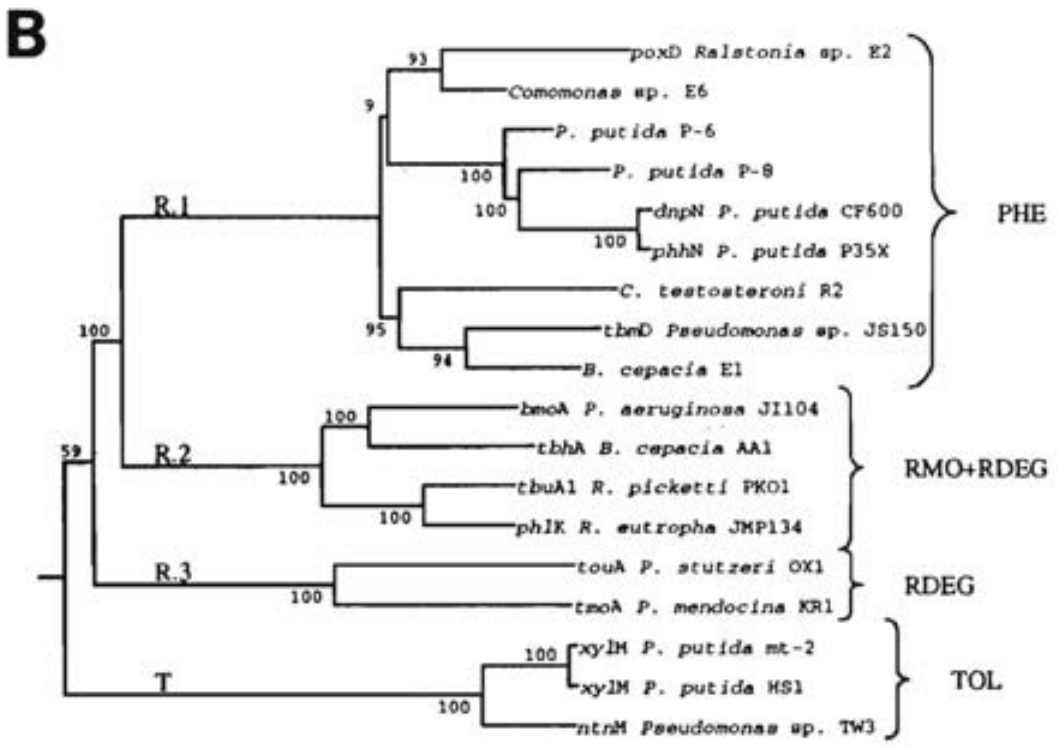


Figure 2.3B. Phylogenetic analysis of the large subunits of ring-hydroxylating monooxygenase genes. (Source: Baldwin et al., 2003)

Since, there are high diversities of TCE degrading enzymes, the impact of each enzyme on TCE cometabolism in the environment is not well understood and the predictions about their reactivity, reaction rates, and product distributions are difficult to make (Arp *et al.*, 2001). The study of enzyme coding genes in *Rhodococcus pyridinivorans* L4, a new Gram (+) TCE degrading bacteria isolated locally, would therefore increase the understanding of TCE degrading process as well as facilitate the application of this bacteria for TCE bioremediation.

2.4.3 Transformation of Toluene

TCE degrading activities in *Rhodococcus pyridinivorans* L4 are originally induced by toluene and the process occur under aerobic condition. We therefore assume that an enzyme analogous to toluene oxygenase would be responsible for TCE degradation. Five toluene aerobic degradation pathways have been reported from various bacteria (Figure 2.4). Each pathway initiates by specific oxygenase enzyme, that are toluene 2-monooxygenase (T2MO), toluene 3-monooxygenase (T3MO), toluene 4-monooxygenase (T4MO), toluene-o-xylene monooxygenase (ToMO), and toluene dioxygenase (TDO).

Gene sequences of toluene oxygenases usually came from the study of Gram (-) bacteria. For example, TDO from *Pseudomonas putida* F1 encoded by todC1C2BADE, T2MO of *Burkholderia cepacia* G4 encoded by tomA012345, and ToMO of *Pseudomonas stutzeri* OX1 encoded by touABCDEF (Zylstra and Gibson, 1989, Li and Wackett, 1992, Luu *et al.*, 1995). ToMO has greatest similarity to the aromatic monooxygenases of *Burkhoderia pickettii* PKO1 and *Pseudomonas mendocina* KR1 (Bertoni *et al.*, 1998).

Toluene degradation pathways can be differentiated by both enzymatic and molecular approaches. Toluene-degrading bacteria produce variety of pigments after culturing in the present of indole. The different colors have been used to preliminary identify three toluene degrading enzymes, namely a dark green-black for toluene dioxygenase, a light blue for toluene 4-monooxygenase, and a vivid red-orange for toluene 2-monooxygenase (Luu *et al.*, 1995). In addition, Keener *et al.* (2001) used chromogenic substrates (e.g. α,α,α-Trifluoro-m-cresol, catechol, naphthalene, and indole) complemented with oxygenase inhibitors (e.g. phenylacetylene and 1-pentyne)

to differentiate known strains expressing the five known toluene degradation pathways. PCR primers were also used for detecting genes initiating toluene degradation pathways. For example, TOD, TOL, and RMO primers were designed for the amplification of toluene dioxygenase, xylene monooxygnase, and toluene monooxygenase, respectively (Baldwin *et al.*, 2003).

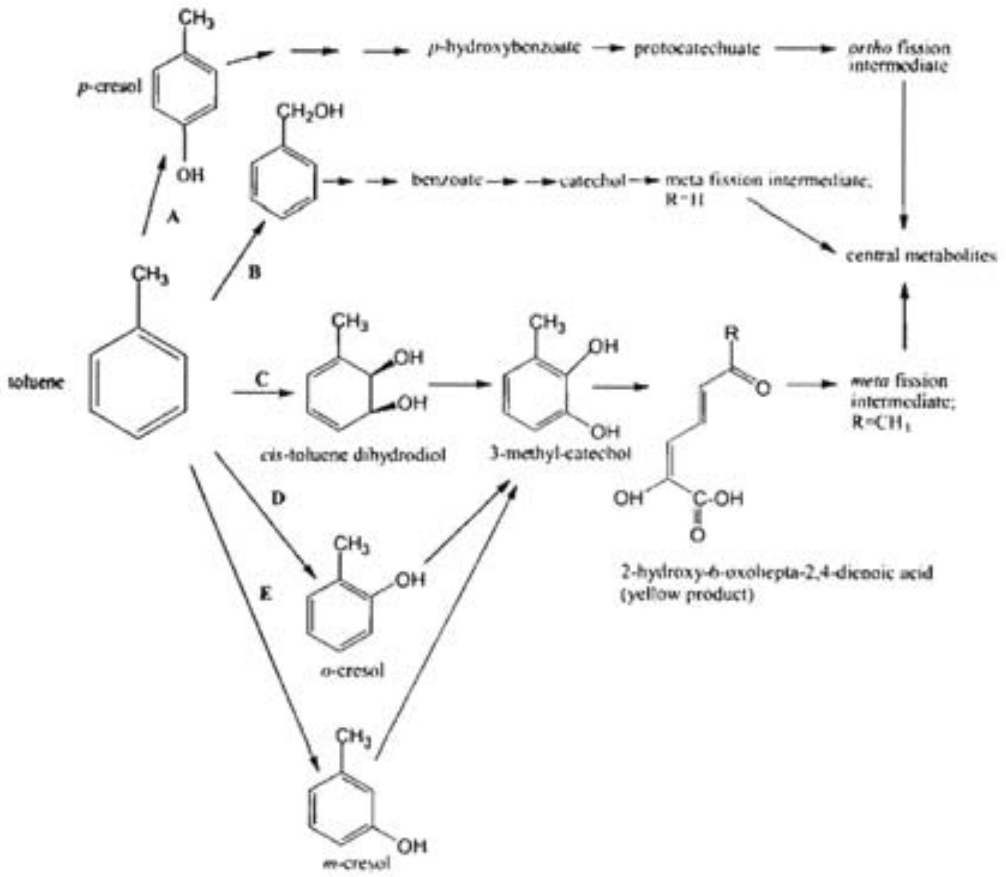


Figure 2.4 Toluene-degradative pathways in known strains (Keener *et al.*, 2001). (A) T4MO initiating the pathway in *Pseudomonas mendocina* KR1. (B) ToMO initiating the TOL pathway in *P. putida* mt-2. (C) TOD initiating the pathway in *P. putida* F1, *P. fluorescens* CFS215, and *Burkholderia* sp. strain JS150. (D) T2MO initiating the pathway in *B. cepacia* G4 (E) Toluene 3-monooxygenase initiating the pathway in *B. pickettii* PKO1.

2.5 Xenobiotic degradation by genus *Rhodoccoccus*

Rhodococcus belongs to the group of high G+C Gram positive actinomycetes bacteria that generally isolated from soil. Actinomycetes have shown primary biodegradative activity, secreting a range of extracellular enzymes, and exhibiting the capacity to metabolize recalcitrant molecules (McCarthy and Williams, 1992). Similarly, *Rhodococcus* is known for their ability to degrade many organic compounds, their ability to produce surfactants and their environmental persistence (Larkin *et al.*, 1998). Xenobiotic compounds that can be degraded by *Rhodococcus* species include aromatic and aliphatic hydrocarbons, insecticides, explosives, and chlorinated hydrocarbons. Consequently, it has gained considerable interest for use in bioremediation of the environment and biotransformation of chemicals in reactor based system (Dabrock *et al.*, 1994). However, only few *Rhodococcus* have been studied in details. Earlier biodegradation studies were focus on Gram negative bacteria such as *Pseudomonas*, *Acinetobactor*, and *Alcaligenes*.

2.5.1 TCE degradation

Only two types of *Rhodococcus* were reported for the TCE degrading ability, they are *R. corallinus* and *R. erythropolis*. The bacteria use different enzymes for initiating TCE degradation. *R. corallinus* B-276 utilize alkene monooxygenase to catalyses TCE oxidation, while growing on both glucose and propene (Saeki *et al.*, 1999). This bacterium is the first TCE-degrading strain that oxidize TCE in a growth-phase-dependent manner but independent of inducer substrates. Genes coding for alkene monooxygenase of *R. corallinus* B-276 is located on a linear megaplasmid. The

authors therefore suggested that linear plasmids may play a major role in acquiring novel catabolic capabilities by horizontal gene transfer between *Rhodococcus* species.

In 1992, Dabrock *et al.* isolated *R. erythropolis* from enrichment cultures containing isopropylbenzene (cumene) and found that the bacteria degrade TCE while using isopropylbenzene as a sole carbon and energy source. TCE degradation process is reported to initiate by an isopropylbenzene dioxygenase analogous to toluene dioxygenase. The enzymes are encoded by a conjugative linear plasmid, pBD2, in which the plasmid-deficient strains had lost both the isopropylbenzene degradation and TCE degradation (Dabrock *et al.*, 1994). The complete nucleotide sequences of this plasmid (*ipb* operon) have high similarities to nucleotide sequences of *bph* operons in *Rhodococcus* sp. RHA1, a polychlorinated biphenyl degrader (Stecker *et al.*, 2003).

2.5.2 Toluene degradation

Several *Rhodococcus* species can utilize toluene as carbon and energy sources. The process is interested for 1) the bioremediation of toluene contaminated sites and 2) the production of trans-carveol and carvone that use as flavor and fragrances for food and perfume industries. It was found that *R. opacus* PWD4 and *R. globerulus* PWD8 are able to hydroxylate limonene exclusively at the 6-position, yielding enantiomerically pure trans-carveol and traces of carvone (Duetz *et al.*, 2001, de Carvalho and da Fonseca, 2003). These bacteria were isolated from soil extract as toluene degraders and then screened for the ability to transform limonene. The information is important for the biocatalytic production of trans-carveol and carvone.

Toluene is one of priority pollutants that found in air, water, and soil from leaking storage tanks, spilling solvent, and burning fuel. For toluene bioremediation, *R.*

rhodochrous (Deeb and Alvarez-cohen, 1999), *R. pyridinovorans* PYJ-1 (Jung and Park, 2004), and *Rhodococcus* sp. DK17 (Kim *et al.*, 2002) were isolated. The bacteria are able to degrade toluene as well as benzene, ethylbenzene, and xylene. Another strain is *R. rhodochrous* OFS, which has both dioxygenase and monooxygenase activities during toluene catabolism (Vanderberg *et al.*, 2000). In addition, it transformed wide array of aromatic groundwater pollutants including styrene, ethylbenzene, and chlorobenzene when grown on toluene. The authors suggested that the bacterium probably possesses a very relaxed substrate specificity enzyme for the oxidation of aromatic compounds or diverse pathways that operate simultaneously. The regulation of these pathways would help us elucidate the process; unfortunately there is no further publication following this work. Continued study of these *Rhodococcus* species will lead to new insights in the xenobiotic degrading process as well as facilitate the application of this bacterium for bioremediation.

3. PROCEDURE

3.1 Utilization of plant terpenes as an alternative TCE inducer

3.1.1 Materials and Media

TCE (99.5% purity), toluene, and four types of purified terpene solutions (96.0% limonene, 99.0% cumene, 95% citral, and 97% pinene) were obtained from Merck & Co. Inc. Essential oils including lemon, lemon grass, pine, and mint oil were purchased from aromatherapy stores, while plant materials including dried lemon grass leaves, cumin seeds, and orange peels were obtained from markets. TCE solutions were prepared by dissolving aliquots of TCE in N,N-dimethylformamide (Merck & Co. Inc) to obtained the desired concentration. Hexane (Fisher Scientific, Inc.) and triton x-100 (Fluka Chemical Industrial) were used for extraction of TCE from liquid cultures and soil samples. All solvents were reagent grade. Mineral salts (MS) medium used to supply essential nutrients for bacteria was prepared according to Focht (1994). For agar plate, 1.5% agar (HiMedia laboratories Limited) was added.

3.1.2 Microorganism and culture maintenance

R. pyridinivorans L4 is a Gram positive bacterium isolated from petroleum contaminated soil collected from Bangkok area (Luepromchai and Suttinun, 2003). The bacterium is deposited at the Microbiological Resources Center, Thailand Institute of Scientific and Technological Research (TISTR) and its accession number is TISTR 1542. *R. pyridinivorans* L4 generally utilize toluene as sole carbon source. In the study, the bacterium was maintained by culturing on mineral salts (MS) agar and incubated in toluene equilibrated glass box at room temperature. Approximately every two to three weeks, a single colony was transferred to a new MS medium.

3.1.3 Terpene-induced cell preparation

Terpene induction was carried out by growing *R. pyridinivorans* L4 culture in a 250-ml Erlenmeyer flask containing 100 ml glucose-MS medium (4 g/l) with terpene solution in the Eppendorf tube hanging above the medium (Figure 3.1). Otherwise, stock solution of terpene (diluted in N,N-dimethylformamide) was added directly to the flask to give a desired concentration. The culture was incubated on orbital shaker at 200 rpm, room temperature for 24 hrs, before harvested by centrifuging at 6,500 rpm for 10 minutes. The supernatant was discarded, and terpene induced cells were washed and resuspended in fresh medium.

Figure 3.1 Erlenmeyer flask with the Eppendorf tube suspended at the top of the flask used for bacterial culture.

3.1.4 TCE Biodegradation assay

TCE biodegradation assay was carried out in liquid bacterial culture by a method adapted from Nelson *et al.* (1987) and Luu *et al.* (1995). TCE biodegradation samples were made by placing 5 ml terpene induced-cells into 50-ml serum bottles

sealed with Teflon-lined rubber septum and an aluminium crimp seal (Figure 3.2). In growing cell assay, induced-cells was resuspended in glucose-MS medium to the concentration of 0.25 OD_{600} and then terpene in N,N-dimethylformamide was added to the serum bottles. This way, the bacteria was growing and producing enzymes during the assay. We also studied the degradation of TCE by resting cell assay, which used concentrated induced-cells (OD_{600} = 1.0) in MS medium without terpene added.

TCE stock solution was prepared in N,N-dimethylformamide, and aliquot was added with gas-tight syringe to give a desired concentration. The bottles were shaken at 200 rpm, room temperature. The experiment was done in triplicate. After incubation, the samples were analyzed for the remaining of TCE in the aqueous phase by hexane extraction and gas chromatography as described below. The 100% baseline used to determine the extent of TCE degradation was determined from the average of the triplicate, first-hour samples.

Figure 3.2 50-ml serum bottles containing 5 ml liquid media capped with Teflon-lined rubber septum and aluminum crimp caps.

3.1.5 TCE Bioremediation assay

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

Bioremediation experiments were consisted of bioaugmentation, biostimulation, and control (no treatment). Terpene was added as terpene solutions or plant materials (Figure 3.3). Dried plant materials consisting of cumin seeds, lemon grass leaves, and orange peels were ground and sieved through a 2 mm sieve before application. Soil microcosm was consisted of 5 g soil in 50 ml serum bottles. In bioaugmentation treatment, induced-cell suspension was added to the soil microcosm to give a final concentration of 10^8 CFU/g soil. Terpene was further supplied to continue the induction of TCE degrading enzymes. In biostimulation treatments, terpene was added directly to the soil microcosms. Soil moisture content of each microcosm was adjusted to 30% by adding cell suspensions or fresh medium. Excess oxygen was provided at the beginning of study. The microcosms were sealed with Teflon-lined rubber septums before spiking TCE using gas-tight syringe. The soil microcosms were incubated at room temperature under dark condition. Each treatment

was done in triplicate. After incubation, the samples were analyzed for the remaining of TCE in soil by hexane extraction and gas chromatography as described below. The 100% baseline used to determine the extent of TCE degradation was determined from the first-day samples.

Figure 3.3 Dried plant materials after grinding and sieving through a 2 mm sieve.

3.1.6 TCE Extraction and analysis

TCE extraction and analysis procedure was modified from Leahy *et al.* (1996). For liquid culture, 5 ml hexane and 1 ml 15% Triton x-100 solution (or 10 ml hexane and 3 ml 15% Triton x-100 solution for soil microcosm) were added to the sacrificed bottle with gas-tight syringe and shaken for 2 hrs at 200 rpm. For soil sample, 10 ml hexane and 3 ml 15% Triton x-100 solution were added directly to the sacrificed soil microcosm with gas-tight syringe and shaken for 2 hrs at 200 rpm. After shaken, the bottle was frozen at -4 ^{0}C to solidify the lower aqueous layer, and then the solvent fraction was transferred with Pasteur pipette to an 8 ml vial where 2-3 g of anhydrous sodium sulfate was added to dewater the sample. TCE dissolved in solvent fraction

was transferred to auto sampler vial for analysis using gas chromatography (GC). The extraction efficiency during the experiment in liquid culture and soil microcosm were 90 ± 10%. Recovery rates were consistent for all samples.

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

3.1.7 Chloride ion analysis

Concentration of chloride ions generated from TCE mineralization was analyzed by an ion-sensitive chloride combination electrode (model 94-17B, Thermo Electron Formerly Orion Research, Inc., USA). Chloride ion standard (1000 ppm sodium chloride) was used for calibration from concentration of 0.5 to 20 ppm chloride. 2% (v/v) ionic strength adjustor ($NaNO_3$) was added to the samples before chloride measuring in a well-stirred 25-ml beaker.

3.2 Characterization of initial genes involved in TCE cometabolism

3.2.1 Enzyme assay

Indole/agar indicator plates were used to roughly determine the initial TCE degrading enzymes, especially toluene oxygenases. Around 5 mg indole crystals (Fluka Chemical Industrial) were added directly to the lid of agar plate containing overnight grown *R. pyridinivorans* L4 culture. The plate was further incubated for 24 hr or until the color of bacterial colony changed from the control plate without indole.

The results were confirmed using two selective irreversible oxygenase inhibitors, phenylacetylene and 1-pentyne according to Keener *et al.* (2001). Phenylacetylene and 1-pentyne were obtained from Fluka Chemical Industrial and diluted in toluene to 3.5 and 10% v/v, respectively. Each reagent was supplied as vapor to *R. pyridinivorans* L4 culture by soaking a 1.5x1.5 cm filter paper in the reagents and placing on the lid of agar plate. The inhibition was measured by comparing the extent of *R. pyridinivorans* L4 growth to the control plate without any inhibitor after 5 day-incubation.

3.2.2 Genomic DNA assay

3.2.2.1 Genomic DNA isolation

Genomic DNA was extracted from *R. pyridinivorans* L4 using Sabai Kit (Bioservice Unit, Thailand) with some modifications. Briefly, 0.1 g bacterial cells were added to 2-ml screw cap tube containing 0.4 g zirconia/silica beads (0.1 mm diameter) and Sabai kit reagents. Then, the tube was processed in FP120 FastPrep cell disruptor (Savant Instruments, Inc.) for 20 sec to grind the cells. The mixture was incubated at 65°C for 10 min, followed by sequential extraction with equal volume of

phenol/chroform/isoamyl alcohol. Genomic DNA was recovered by precipitating with 1 volume of cold isopropanol. DNA pellet was washed with 80% cold ethanol before dissolved in 50 µl TE containing RNAse (20 µl/ml). The DNA samples were incubated at 37°C for 1 hr to digest RNA and then electrophoresed on a 0.5% agarose gel to check for their sizes. High-molecular weight genomic DNA with minimal smearing was used for genome walking process. The concentration of DNA was quantified by Turner Quantech Digital Filter Fluorometer (Barnstead International) after reacted with Hoechst 33258 dye (Sigma-Aldrich) according to the manufacturer.

3.2.2.2 PCR amplification of oxygenase gene fragments

Polymerase chain reaction (PCR) was conducted using primers from Baldwin *et al.* (2003) and *R. pyridinivorans* L4 genomic DNA. The primers have been designed for the detection and enumeration of toluene and biphenyl oxygenase genes (Table 3.1). Toluene oxygenase primers were selected to amplify enzymes initiating five toluene aerobic degradation pathways, including toluene 2-monooxygenase (T2MO), toluene 3-monooxygenase (T3MO), toluene 4-monooxygenase (T4MO), toluene-o-xylene monooxygenase (ToMO), and toluene dioxygenase (TDO). In the study, biphenyl dioxygenase primers were also chosen because of their ability to amplify oxygenase genes in several *Rhodococcus* species and the high similarity between biphenyl and toluene dioxygenase gene sequences.

Table 3.1 PCR primers for amplification of oxgenase genes*.

Target	Primer	Primer Sequences	T_m (°C)
Toluene dioxygenase	TOD-F	ACCGATGA(A/G)GA(C/T)CTGTACC	53
	TOD-R	CTTCGGTC(A/C)AGTAGCTGGTG	
Toluene monooxygenase	RMO-F	TCTC(A/C/G)AGCAT(C/T)CAGAC(A/C/G)G ACG	53
	RMO-R	TT(G/T)TCGATGAT(C/G/T)AC(A/G)TCCCA	
Toluene monooxygenase (Degenerated primers)	RDEG-F	T(C/T)TC(A/C/G)AGCAT(A/C/T)CA(A/G)AC (A/C/G)GA(C/T)GA	52
	RDEG-R	TT(A/G/T)TCG(A/G)T(A/G)AT(C/G/T)AC(A/ G)TCCCA	
Toluene-o-xylene monooxygenase	TOL-F	TGAGGCTGAAACTTTACGTAGA	55
	TOL-R	CTCACCTGGAGTTGCGTAC	
Biphenyl dioxygenase	BPH4-F	AAGGCCGGCGACTTCATGAC	62
	BPH3-R	TGCTCCGCTGCGAACTTCC	

*Source: Baldwin *et al.* (2003)

PCR reaction contained 100 ng DNA, 20 pmol of each primer, 15 µl Taq PCR Master Mix (Qiagen Inc.), and distilled water to a final volume of 30 µl. Amplification was performed on a Gene Amp® PCR System 2700 thermocycler (Applied Biosystem). The temperature program was carried out according to Baldwin *et al.* (2003) as follows: (i) 10 min at 95 °C; (ii) 30 cycles, with 1 cycle consisting of 1 min at 95 °C, 1 min at the optimum annealing temperature (T_m), and 2 min at 72 °C, and (iii) a final extension step of 10 min at 72 °C. PCR products were visualized by agarose gel electrophoresis. The expected products were ranged from 452-757 bp. Sequences of PCR products were determined by automated sequencer at the BioService Unit. After acquiring DNA sequences, BLAST software (GenBank, www.ncbi.nlm.nih.gov.) was used to determine its identity and nearest gene neighbor.

3.2.2.3 Genome walking process

Genome walking technique was used to find genomic DNA sequences adjacent to the oxygenase gene fragment from 3.2.2.2 (Figure 3.4). In the study, BD GenomeWalker™ Universal Kit (BD Biosciences Clontech) was used according to user manual. Briefly, *R. pyridinivorans* L4 genome libraries were constructed by digesting genomic DNA with various restriction enzymes to leave blunt ends. The digested DNA was then ligated separately to GenomeWalker Adaptor fragments supplied from the kit. Next, nested PCR amplification was performed on the genome libraries with gene specific and adaptor primers.

Adaptor primers (AP1 and AP2) were provided in the kit, while oxygenase gene specific primers were designed using sequences of PCR product acquired in 3.2.2.2 and Fast PCR program (Institute of Biotechnology, University of Helsinki,

Finland). These primers annealed to sequences beyond 3'end of the oxygenase PCR product and were design to extend in both upstream and downstream directions. Primers sequences are in Table 3.2. Consequently, PCR products from the walking process were expected to start from the oxygenase gene specific primers and extend into the surrounding genomic DNA that coding for oxygenase enzyme.

```
1    TAACCTACANTGGTGCTGAGGATCCGNTNATGGTCGTTCGTCAGAAGAAC   50
                                        ← GSU 2
51   GGGGAGATCCGCGNTCTTCCTCAACCAGTGTCGCCACCGCGGAATGCGGA   100
101  TCTGCCGCGCGGACGGGGGCAATGCCAAGTCATTCACCTGCAGCTATCAC   150
                    ← GSU 1
151  GGNCTGGGCCTACGATACGGGCGGCAACTTGGTGAGTGTGCCTTTCGAGG   200
201  AGCAGGCCTTCCCCGGGCTGAGGAAAGAAGATTGGGGCCCGCTACAG GCT  250
                        → GSD1
251  CGCGTCGAGACCTACAAGGGCCTG ATTTTCGCAAACTGGGACGCTGACGC  300
301  CCCGGACCTGGACACCTATCTGGGTGAAGCGAAGTTCTACATGGACCACAT  350
351  GTTGGATAGAACCGAAGCGGGCACCGAAGCGATCCCGGGGATTCAGAAGT   400
          → GSD2
401  GGGTCATTCCCTGCAACTGGAAGTTCGCAGCGGAACAACT             441
```

Figure 3.4 Regions of primers for upstream and downstream extension of the oxygenase gene fragment from 3.2.2.2. The gene specific primers for upstream extension were GSU1 and GSU2, whereas those for downstream extension were GSD1 and GSD2.

Table 3.2 Oxygenase gene specific PCR primers for genome walking process.

Primer	Direction	Primer Sequences	Application
GSD1	Sense	GCTCGCGTCGAGACCTACA AGGGCCTG	First downstream extension
GSD2	Sense	GGGATTCAGAAGTGGGTC ATTCCCTGC	Secondary upstream extension
GSU1	Anti-sense	AAGTTGCCGCCCGTATCGT AGGCCCAG	First upstream extension
GSU2	Anti-sense	CGCATTCCGCGGTGGCGAC ACTGGTTG	Secondary upstream extension

PCR reaction contained 100 ng DNA, 20 pmol of each primer, 10X PCR reaction buffer, 100 μM dNTP, 50X Advantage Genomic Polymerase mix (Clontech Laboratory, Inc) and distilled water to a final volume of 25 μl. The PCR cycles are showed in Table 3.3. The first or primary PCR used the outer adaptor primer (AP1) and an outer gene specific primer (GSD1 or GSU1). The primary PCR mixture was then diluted and used as a template for the secondary PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSD2 or GSU2).

Table 3.3 PCR reactions for genome walking process.

Application	Primer	Type of Amplification	PCR cycles
	AP1	Primary Amplification	7 cycles: 94°C 2 sec, 72°C 3 min
	GSD1		32 cycles: 94°C 2 sec, 67°C 3 min
Downstream			1 cycle: 67°C 4 min
Extension	AP2	Secondary Amplificatior	5 cycles: 94°C 2 sec, 72°C 3 min
	GSD2		20 cycles: 94°C 2 sec, 67°C 3 min
			1 cycle: 67°C 4 min
	AP1	Primary Amplification	7 cycles: 94°C 2 sec, 72°C 3 min
	GSU1		32 cycles: 94°C 2 sec, 67°C 3 min
Upstream			1 cycle: 67°C 4 min
Extension	AP2	Secondary Amplificatior	5 cycles: 94°C 2 sec, 72°C 3 min
	GSU2		20 cycles: 94°C 2 sec, 67°C 3 min
			1 cycle: 67°C 4 min

3.2.2.4 Cloning and sequencing of PCR products

PCR products from genome walking process were electrophoresed on a 1.5% agarose gel. The major bands were excised and purified by gel extraction kit (Qiagen Inc.) before cloning into pGEM T-easy vector (Promega Corporation) according to the manufacturers. The insert DNA was then transformed into *E. coli* DH5α. Recombinant plasmid DNA was isolated from white colonies on LB/ampicillin/IPTG/X-Gal plate and then digested with *Eco*R I enzyme to check for the insert. Plasmid with the correct size of insert was sent to BioService Unit for sequencing. The DNA sequences were transformed into amino acid sequences by Fast PCR program. Identities and similarities between oxygenase gene sequences from *R. pyridinivorans* L4 and other microorganisms were studied by blastp program (protein-protein BLAST). Further alignment of *R. pyridinivorans* L4 oxygenase sequences with other related proteins was carried out by Clustal W program, a multiple sequence alignment program (http://www.ebi.ac.uk/ clustalw/). Nucleotide and protein sequences of other organisms were retrieved from http://www.ncbi.nlm.nih.gov/entrez/. Phylogenetic tree was later constructed on a phylogram using Clustal W program.

3.2.3 Expression of *R. pyridinivorans* L4 oxygenase gene

3.2.3.1 Total RNA extraction

Ability of terpene for inducing oxygenase gene was studied by comparing the amount of mRNA in 24-hr *R. pyridinivorans* L4 induced cells. RNA stabilization was carried out by mixing cell suspensions (OD_{600} = 1.0) with RNAprotect Bacteria Reagent (Qiagen Inc.) following the manufacturer's protocol. The protected cells were later added to a 2-ml screw cap tube containing 0.1 g zirconia/silica beads (0.1 mm

diameter) and processed in FP120 FastPrep cell disruptor (Savant Instruments, Inc.) for 20 sec to grind the cells. Total RNA was extracted from the disrupted cells using RNeasy Mini kit combined with RNase-Free DNase set (Qiagen Inc.) according to the manufacturer.

3.2.3.2 Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR was conducted using QIAGEN OneStep RT-PCR Kit. The reaction contained 5 μl total RNA, 20 pmol of each primer (BPH4-F and BPH3-R), 5 μl 5x QIAGEN OneStep RT-PCR buffer, 1 μl dNTP Mix, 1 μl QIAGEN OneStep RT-PCR Enzyme Mix, 5 μl Q-Solution, and distilled water to a final volume of 25 μl. Amplification was performed on a Gene Amp® PCR System 2700 thermocycler (Applied Biosystem). The temperature program was carried out as follows: (i) 30 min at 50 °C; (ii) 15 min at 95 °C; (iii) 30 cycles, with 1 cycle consisting of 1 min at 95 °C, 1 min at 62 °C, and 2 min at 72 °C, and (iv) a final extension step of 10 min at 72 °C. PCR products were visualized by agarose gel electrophoresis.

4. RESULTS

4.1 Utilization of plant terpenes as an alternative TCE inducer

4.1.1 TCE degradation by *R. pyridinivorans* L4 cells that utilize terpene solutions or essential oil as carbon source

R. pyridinivorans L4 was tested for their ability to degrade TCE while utilizing terpenes as well as essential oils as carbon sources. Substrate utilization was determined by growing the bacteria in 10 ml MS medium with terpene solution in the Eppendorf tube hanging above the medium and shaken at 200 rpm for 48 h. Bacterial growth was determined from the increased turbidity of cell culture. From 10 substrates, *R. pyridinovorans* L4 was able to utilize five substrates including toluene, limonene, lemon oil, lemon grass oil, and cumene (Table 4.1). The growth substrates were then selected to determine their TCE inducing capability by resting cell assay.

R. pyridinovorans L4 was grown in MS medium supplied with the selected substrate for 24 hr. The culture was then harvested and resuspended in MS medium to the concentration of 1.0 OD_{600}. TCE resting cell assay was carried out by adding 10 ppm TCE to the cells and further incubated for 30 hr. Initial amount of TCE from the triplicate samples was used as 100% baseline. Other than toluene, cumene was the best substrate for *R. pyridinivorans* L4 to degrade TCE (Table 4.1). About 60% of 10 ppm TCE was degraded by cumene grown cells. The results also showed that bacteria grown on lemon oil and lemon grass oil were able to degrade 19-24% of 10 ppm TCE. Although, limonene is the major terpene in essential oil of lemon, limonene grown bacteria were able to degrade only a small amount of TCE (2%). The results suggested that other terpenes in the essential oil might involve in TCE degradation. Otherwise, the induction of TCE degrading enzymes might require lower concentration of terpene because high amount of the inducing compound might inhibit enzyme production.

Table 4.1 Growth and percent TCE degradation of *R. pyridinivorans* L4 after culturing in various substrates.

Substrates	Growth[1]	% TCE Degradation[2]
Toluene	+++++	69.3±3.9
Terpenes		
Carvone	-	ND
Cumene	++	60.1±4.3
Limonene	++++	2.4±8.9
Pinene	+	ND
Citral	-	ND
Essential oil		
Lemon oil	+++++	19.1±0.6
Lemon grass oil	++	24.0±5.4
Mint oil	-	ND
Pine oil	-	ND

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

[2] Percent TCE degradation from the resting cell assay. Values given are average of three replicates ± standard deviation. ND = Not determined

4.1.2 TCE degradation by *R. pyridinivorans* L4 cells that utilize terpene solutions as enzyme inducer

To prevent the toxic effects of terpene that might occur, *R. pyridinivorans* L4 was grown in glucose-MS medium that contained low amount of terpene solutions. The bacteria therefore utilized glucose as carbon source and produced TCE degrading enzymes after induction by terpene. The induced bacterial cells were harvested after 24 hr and tested for TCE degrading activities in growing cell assay. The optimum TCE degrading period was preliminary determined by monitoring the amount of TCE in liquid bacterial culture for 30 consecutive hrs (Figure 4.1). The results showed that percent TCE degradation from both induced and non-induced *R. pyridinivorans* L4 cells was increased gradually and reached a steady state after 24-hr incubation. Consequently, we selected 30 hrs as the optimum TCE degrading period.

The tested terpene solutions were consisted of citral, limonene, and cumene at 10, 25, and 50 ppm. Citral was selected for the study since it is the major terpene in lemon grass oil and also found in the essential oil of lemon. From Table 4.2, 50 ppm citral, 25 ppm cumene, and 25-50 ppm limonene were able to induce *R. pyridinivorans* L4 cells to degrade TCE at the level significantly more than non-induced cells (no terpene). After 30 hrs incubation, these induced bacteria were able to degrade 65-72% of 15 ppm TCE, while 54% of TCE was decreased in non-induced cells treatment. The results indicated that each terpene solution has different capability to induce TCE degradation in *R. pyridinivorans* L4. Furthermore, the amount of terpene should be optimized accordingly.

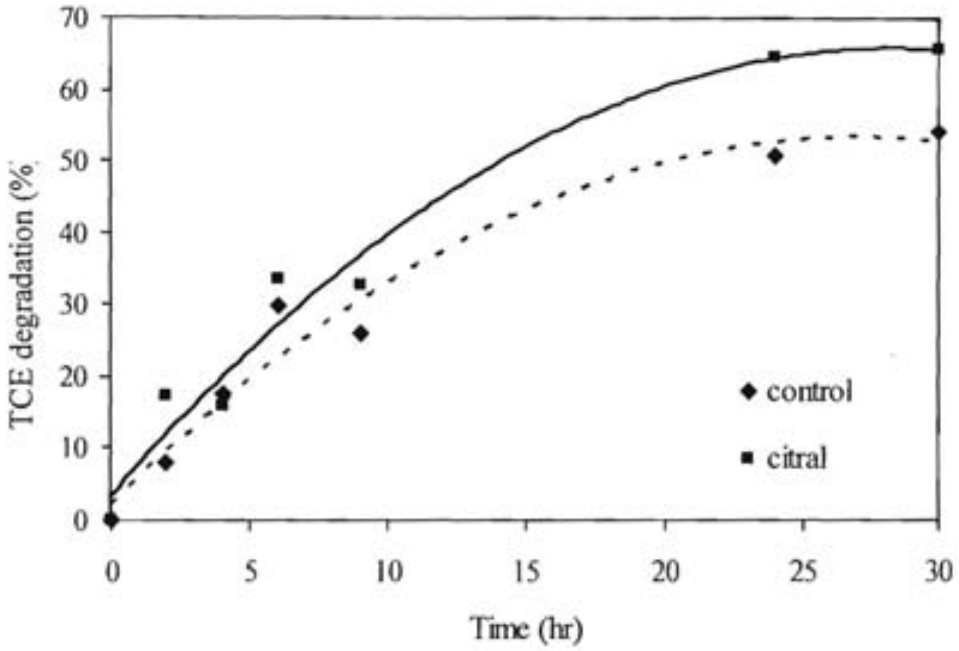


Figure 4.1 Comparison of citral induced *R. pyridinivorans* L4 and control non-induced cells on TCE degradation. Solid and dashed lines indicate nonlinear regression of the data.

Table 4.2 Degradation of 15 ppm TCE by terpene-induced *R. pyridinivorans* L4 cells after 30-hr incubation.

Type of terpenes	Amount of terpene (ppm)	% TCE Degradation[1]
None	0	54.1±7.9
Citral	10	55.2±7.6
	25	60.8±7.2
	50	65.9±8.7*
Cumene	10	66.1±8.7
	25	67.7±6.2*
	50	64.4±7.8
Limonene	10	64.4±4.8
	25	71.1±7.0*
	50	71.9±6.1*

[1] Values given are average of three replicates ± standard deviation. Percent TCE degradation is significantly higher than non-induced cells (no terpene) if marked.

4.1.3 TCE mineralization by *R. pyridinivorans* L4 cells that utilize terpene solutions as enzyme inducer

TCE mineralization is the completely destruction of TCE into carbon dioxide and chloride ions. To study TCE mineralization, chloride ions generated during TCE degradation process was measured by ion-sensitive chloride combination electrode. *R. pyridinivorans* L4 was induced by adding terpene solutions at the concentrations of 25 ppm cumene, 50 ppm citral, or 50 ppm limonene. Suspensions of induced cells were tested with 10 ppm TCE in growing cell assay. The results showed that all terpene-induced cells produced more chloride ions than non-induced cells after incubation (Figure 4.2). There were 1.5 ppm chloride ions in the treatment with non-induced cells. Of all terpenes, citral was the best inducer for TCE mineralization. The amount of chloride ions were rapidly increased in citral-induced cell treatment to 4.2 ppm at the end of study. The highest amount of chloride ions in this treatment was 6.1 ppm after 4 hr incubation (Figure 4.2). On the other hand, the amount of chloride ions in cumene and limonene were slowly increased to 3.2 and 2.0 ppm after 24 hr incubation, respectively. Meanwhile, citral-induced cells degraded TCE at the same extent as cumene- and limonene-induced cells (Table 4.2).

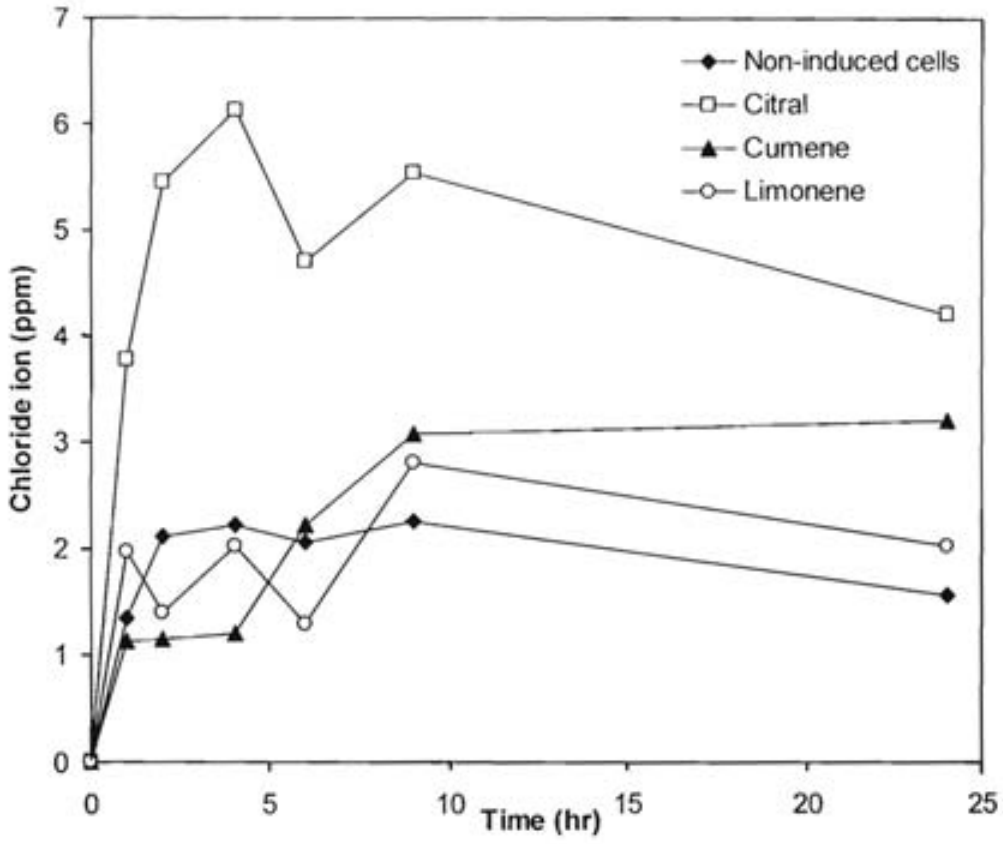


Figure 4.2 Chloride ion generated during TCE mineralization by terpene induced *R. pyridinivorans* L4 and control non-induced cells.

4.1.4 Development of a bioremediation treatment for TCE contaminated soil using terpene solutions and *R. pyridinivorans* L4.

Biodegradation of TCE contaminated soil was studied in microcosm containing 5 g spiked soil with 30% moisture content. All treatments were performed in the same manner and the amount of remaining TCE was determined by extracted the whole microcosm at once. Bioremediation treatments were consisted of bioaugmentation, biostimulation, and control. Initial amount of TCE from the triplicate samples was used as 100% baseline. Differences in the amount of remaining TCE were used to determine the losses of TCE due to biodegradation and any other loss mechanisms that may be associated with the treatment.

4.1.4.1 Effect of *R. pyridinivorans* L4 and terpene solutions

Citral, cumene, and limonene had comparable TCE degrading activities but citral was mineralized more TCE. We therefore selected citral as a modeled terpene for the development of bioremediation strategy. In the experiment, bioaugmentation was the addition of citral-induced *R. pyridinivorans* L4 cells, while biostimulation was the addition of citral. The concentrations of citral were 50 ppm and MS medium was used to adjust moisture content in both treatments. The control treatments were consisted of untreated soil and MS medium amended soil. The initial amount of TCE in soil microcosms was 100 ppm. Bioaugmentation and biostimulation treatments significantly enhanced TCE degradation within the first 14 days. In the microcosms with citral-induced cells and citral solution, percent TCE remaining were decreased rapidly to less than 10% at day 14, while there were still more than 35% of TCE remaining in the control treatments (Figure 4.3). The results indicated that citral-

induced *R. pyridinivorans* L4 cells degraded TCE after application and citral solution was able to induce soil indigenous bacteria to degrade TCE. Nonetheless, the differences between TCE degradation in bioremediation and control treatments were not significance after 21-day incubation. Reduction of TCE in untreated soil microcosms indicated the effect of abiotic process, whereas MS medium microcosms represented the effects of intrinsic bioremediation that might have occurred.

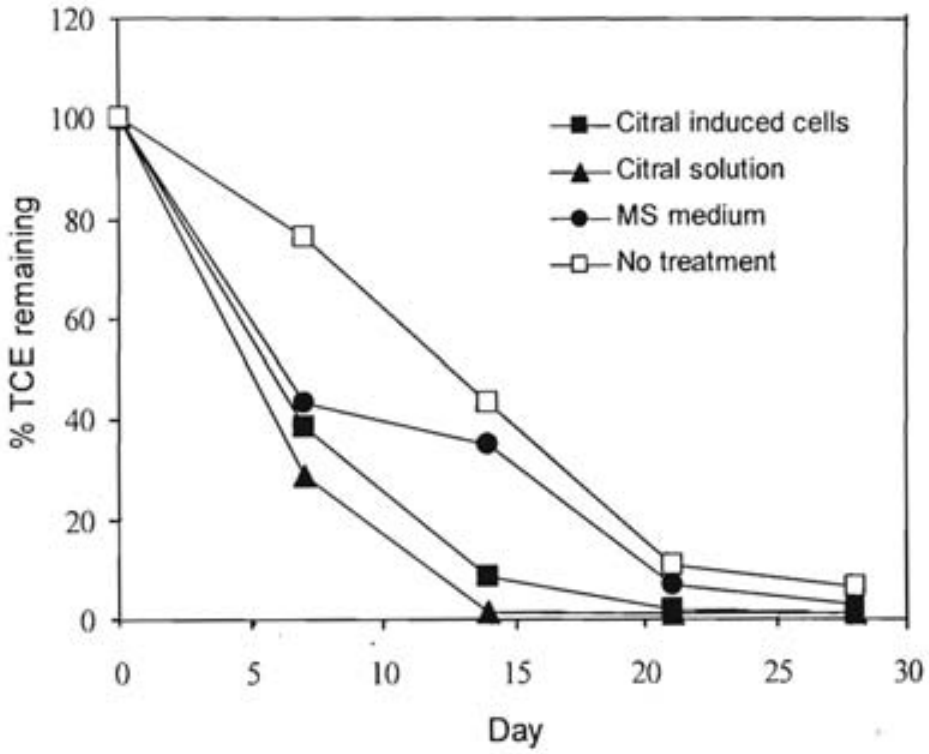


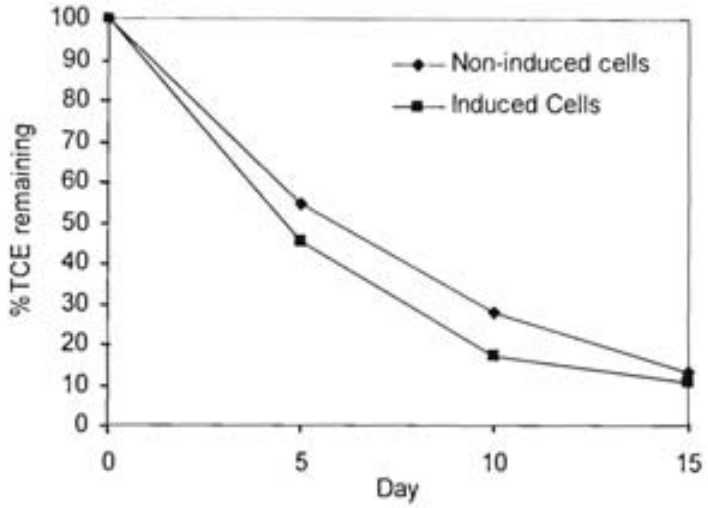
Figure 4.3 Comparison of bioremediation treatments in soil microcosms that initially spiked with 100 ppm TCE. The treatments included bioaugmentation (addition of citral induced *R. pyridinivorans* L4), biostimulation (addition of citral solution), and control (addition of MS medium and no treatment).

4.1.4.2 Effect of *R. pyridinivorans* L4, terpene solutions, and carbon source

TCE degradation is carried out by cometabolism, thus the degrading bacteria do not gain energy from this process. To improve TCE bioremediation strategy, another organic substrate may be applied as bacterial carbon source. We therefore studied the effect of glucose on enhancing TCE degradation in soil. Glucose was added at 4% concentration along with MS medium to the 150 ppm TCE spiked soil microcosms. The compound would support the growth of *R. pyridinivorans* L4 as well as other indigenous bacteria.

Bioaugmentation treatment was done by adding citral induced *R. pyridinivorans* L4 cells along with glucose-MS medium to the soil microcosms. Citral solutions were further added to give a final concentration of 50 ppm. TCE was decreasing rapidly after treatment. Percent TCE remaining in the microcosms contained citral induced cells and non-induced cells after 15-day incubation were 11 and 13%, respectively (Figure 4.4A). Meanwhile, percent TCE remaining in the microcosms contained non-induced cells was much higher than the microcosms contained citral-induced cells on day 5 and 10. The result showed that citral-induced *R. pyridinivorans* L4 cells increased the degradation of TCE at higher extent than non-induced cells. Biostimulation treatments were conducted by adding 50 ppm citral along with glucose-MS medium to the soil microcosms. After 15-day incubation, percent TCE remaining in the microcosms contained citral-glucose-MS medium and glucose-MS medium only were 24 and 29%, respectively (Figure 4.4B). The degradation of TCE in citral-glucose-MS medium microcosms was more rapid than in the microcosms with glucose-MS medium only. The result showed that citral was able to stimulate the degradation of TCE by indigenous bacteria.

A. Bioaugmentation



B. Biostimulation

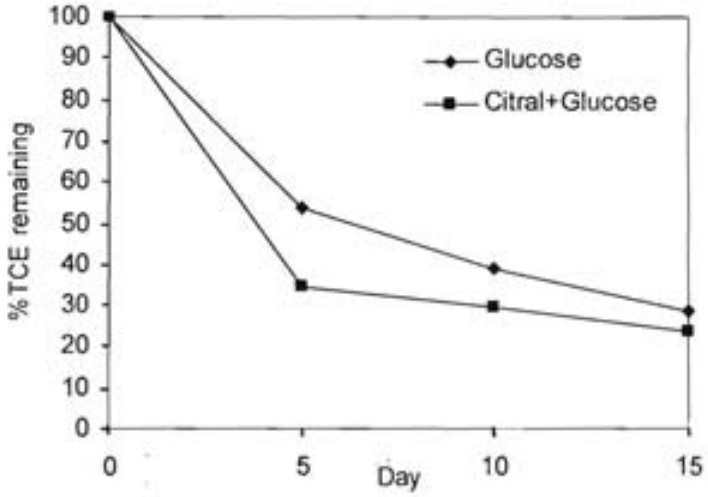


Figure 4.4 Effect of carbon source on bioremediation treatments in soil microcosms that initially spiked with 150 ppm TCE. The bioaugmentation treatments consisted *R. pyridinivorans* L4 (induced cells or non-induced cells) and 4% glucose-MS medium, while biostimulation treatments consisted of citral and 4% glucose-MS medium or 4% glucose-MS medium only.

Both bioaugmentation and biostimulation results confirmed that citral is necessary for the induction of TCE degradation enzymes. In addition, carbon source was also important. We found that the amount of TCE degradation in citral-induced cells/glucose-MS and glucose-MS treatments (Figure 4.4A and B) was higher than the non-glucose treatment (Figure 4.3). Percent TCE remaining in these microcosms were comparable but the initial amount of TCE in glucose treatment was higher (150 ppm). Meanwhile, the combination for citral and glucose-MS medium may promote the growth of indigenous bacteria that could not degrade TCE. Percent TCE remaining in citral/glucose-MS treatment (Figure 4.4B) was higher than the non-glucose citral treatment (Figure 4.3).

4.1.5 Development of a bioremediation treatment for TCE contaminated soil using plant materials and *R. pyridinivorans* L4.

We selected three plant materials for the development of TCE bioremediation strategy, including lemon grass leaves, cumin seeds, and orange peels. The essential oils of these plants contain several terpenes that have been found to induce the production of TCE degrading enzymes in *R. pyridinivorans* L4 (Table 4.3). Grounded plant materials were added to give a final concentration of 50 ppm terpene in the 150 ppm TCE spiked soil microcosms. Glucose-MS medium was further supplied to adjust soil moisture and enhance bacterial growth. The bioremediation treatments were consisted of bioaugmentation (terpene induced *R. pyridinivorans* L4 cells + plant materials) and biostimulation (plant materials) treatments. Citral-, cumene-, and limonene-induced cells were used in treatments with lemon grass leaves, cumin seeds,

and orange peels, respectively. Control treatments were consisted of untreated soil and glucose-MS medium amended soil.

Table 4.3 Terpene in plant materials used for the induction of TCE degrading enzymes in *R. pyridinivorans* L4 and soil bacteria.

Plant Materials	**Amount of essential oil[1] (%)**	**Type of terpene in essential oil[1]**	**Amount of added material (g)[2]**
Lemon grass leaves	0.2-0.5	Mainly citral	0.12
Cumin seeds	2.5-4.0	25-35% Cumene	0.04
Orange peels	2.5	Mainly citral and limonene	0.02

[1] Source: http://www-ang.kfunigraz.ac.at/~katzer/engl/ and http://www.tistr.or.th/pharma/RD_anal.htm

[2] The final concentration of terpene in the soil microcosm was approximately 50 ppm.

The amounts of TCE in both bioaugmentation and biostimulation treatments were decreased to less than 10% of the initial amount after 15 days (Table 4.4). The difference between bioaugmentation and biostimulation was not significance. However, the extents of TCE degradation in bioremediation treatments were significantly higher than the control treatment. Around 30% of TCE was remained in the untreated and glucose amended soil. When compared between types of plant materials, percent TCE remaining in soil with cumin seeds was slightly lower than soil with lemon grass leaves and orange peels. After 15-day incubation, percent TCE

remaining was lower in microcosms with plant materials than with terpene solutions (Figure 4.4). The results suggested that several terpenes in plant materials may responsible for the induction of various TCE degrading enzymes in the soil microcosms.

Table 4.4 Percent TCE remaining in soil microcosms amended with *R. pyridinivorans* L4 and plant materials after 15-day incubation.

Treatment	Plant materials	% TCE remaining*
Control		
No treatment	None	30.65±11.58
Glucose	None	28.77±12.12
Bioaugmentation		
(*R. pyridinivorans* L4 +	Lemon grass leaves	5.10±0.82
plant materials + glucose)	Cumin seeds	3.81±0.83
	Orange peels	4.21±0.89
Biostimulation		
(plant materials + glucose)	Lemon grass leaves	6.28±0.59
	Cumin seeds	3.70±1.03
	Orange peels	6.38±2.58

* Values given are average of three replicates ± standard deviation. The initial amount of TCE was 150 ppm.

4.2 Characterization of initial genes involved in TCE cometabolism

4.2.1 Enzyme assay

Determination of probable initial TCE degrading enzyme in *R. pyridinivorans* L4 was based on Luu et al. (1995) and Keener *et al.* (2001). We monitored the transformation of indole (white color) to indigo (blue color) and other products as well as the difference of bacterial growth after addition of enzyme inhibitors, phenylacetylene and 1-pentyne. The assays gave different results when using different substrates to grown *R. pyridinivorans* L4 (Table 4.5). Toluene grown cells was able to transform indole to dark green compound and their growth was not inhibited by both inhibitors. On the other hand, glucose grown cells could not transform indole and their growth was partially inhibited by 1-pentyne. The results suggested that toluene grown cells probably contained toluene dioxygenase enzyme, while glucose grown cells did not produce this enzyme. Meanwhile, the activity of *R. pyridinivorans* L4 on phenylacetylene were slightly different from the known toluene dioxygenase containing strains such as *P. putida* F1, *Burkholderia cepacia* JS150, and *P. fluorescens* CFS215. Keener *et al.* (2001) reported the changes of colony color from beige to yellow after testing these bacteria with phenylacetylene. However, the beige color of *R. pyridinivorans* L4 colonies was not changed with similar test.

Table 4.5 Appearance of *R. pyridinivorans* L4 colonies in the presence of indole, phenylacetylene, and 1-Pentyne.

Test Compound	Reaction	
	Toluene grown cells	**Glucose grown cells**
Indole	Dark green to gray	No color change
Phenylacetylene	No growth inhibition	No growth inhibition
1-Pentyne	No growth inhibition	Partial growth inhibition

4.2.2 Genomic DNA assay

4.2.2.1 PCR amplification of oxygenase gene fragments

Polymerase chain reaction (PCR) was conducted to confirm the present of toluene dioxygenase in *R. pyridinivorans* L4 as well as to acquire short DNA fragment for further extension by genome walking technique. The primers have been designed by Baldwin *et al.* (2003) for the detection and enumeration of aromatic oxygenase genes (Table 3.1). However, only BPH4-F and BPH3-R primers could amplify *R. pyridinivorans* L4 genomic DNA (Table 4.6 and Figure 4.5). The size of PCR product was about 450 bp, which corresponded to the expected product in Baldwin *et al.* (2003). The amplified fragment was then sequenced by automated sequencer at the BioService Unit. Sequences of this PCR product were in Figure 3.4. From blastn program, we found that *R. pyridinivorans* L4 PCR product had high similarity to toluene inducible dioxygenase of *Rhodococcus* sp. I24, isopropylbenzene 2,3-dioxygenase of *R. erythropolis* BD2, and biphenyl dioxygenase of *Rhodococcus* sp. RHA1 (Table 4.7).

Table 4.6 PCR amplification of oxygenase gene in *R. pyridinivorans* L4

Primer	Target	Result
TOD-F, TOD-R	Toluene dioxygenase	No amplification
RMO-F, RMO-R	Toluene monooxygenase	No amplification
RDEG-F, RDEG-R	Toluene monooxygenase (Degenerated primers)	No amplification
TOL-F, TOL-R	Toluene-o-xylene monooxygenase	No amplification
BPH4-F, BPH3-R	Biphenyl dioxygenase	PCR product about 450 bp long

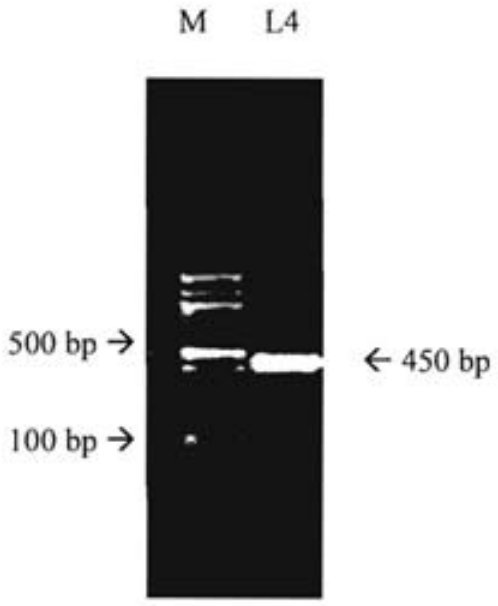


Figure 4.5 Amplification of *R. pyridinivorans* L4 genomic DNA by BPH4-F/BPH3-R primers. Lane M is 100 bp molecular weight marker.

Table 4.7 Homology of *R. pyridinivorans* L4 oxygenase PCR product to protein deposited in the GenBank/EMBL data bank.

Microorganism	Protein	Accession No.	Identity (% Similarity)
Rhodococcus sp. I24	Putative toluene inducible dioxygenase	AF452376	417/418 (99%)
R. erythropolis	Isopropylbenzene 2,3-dioxygenase	U24277	416/418 (99%)
Rhodococcus sp. RHA1	Biphenyl dioxygenase	D32142	415/418 (99%)
R. opacus	Biphenyl 2,3-dioxygenase	AJ544524	144/144 (100%)
R. erythropolis TA421	Biphenyl terminal dioxygenase	D88020	302/371 (81%)
Pseudomonas sp.	Chlorobenzene dioxygenase	U152981	154/176 (87%)
Rhodococcus sp. M5	Biphenyl dioxygenase	U27591	301/371 (81%)
Burkholderia sp. PS12	Chlorobenzene dioxygenase	U78099	149/170 (87%)
P. putida	Toluene dioxygenase	Y18245	145/170 (85%)

4.2.2.3 Genome walking

Genome walking technique was used to find genomic DNA sequences adjacent to the *R. pyridinivorans* L4 oxygenase PCR product in 4.2.2.2. *R. pyridinivorans* L4 genomic DNA were first digested with various restriction enzymes from the BD GenomeWalker™ Universal Kit. We found that the genomic DNA was digested by *Pvu* II and *Stu* I enzymes (Figure 4.6) but not by *Dra* I and *Eco*R V (data not showed). *R. pyridinivorans* L4 genome libraries was then constructed by ligating the *Pvu* II and *Stu* I digested DNA separately to GenomeWalker Adaptor fragments. Nested PCR amplification was performed on the genome libraries with gene specific (GSU and GSD) and adaptor (AP) primers.

Primary PCR products were obtained from the downstream extension of *Pvu* II library and upstream extension of *Stu* I library (Figure 4.7). Meanwhile, there was no amplification from upstream extension of *Pvu* II library and downstream extension of *Stu* I library. The primary PCR products were diluted 50 folds and subjected to secondary PCR amplification. There were several major bands from the downstream extension of *Pvu* II library, however only band A (1Kb) was corresponding to the primary PCR product (Figure 4.7). On the other hand, the major band from upstream extension of *Stu* I library was 600 bp and designated as band B (Figure 4.7). Both PCR fragments were later excised and cloned into pGEM T-easy vector. Plasmids with the correct size of insert were sent to BioService Unit for sequencing.

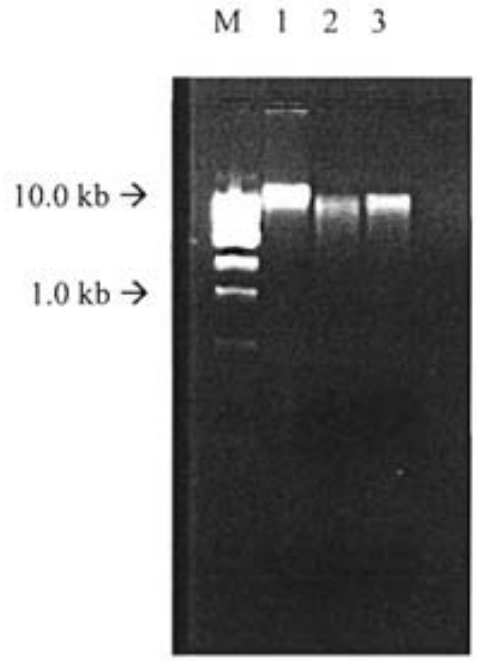


Figure 4.6 *R. pyridinivorans* L4 genomic DNA on 0.5% agarose gel. Lanes: M, 1 Kb molecular weight marker; 1, purified genomic DNA; 2 and 3 are the genomic DNA samples after digested with *Pvu* II and *Stu* I, respectively.

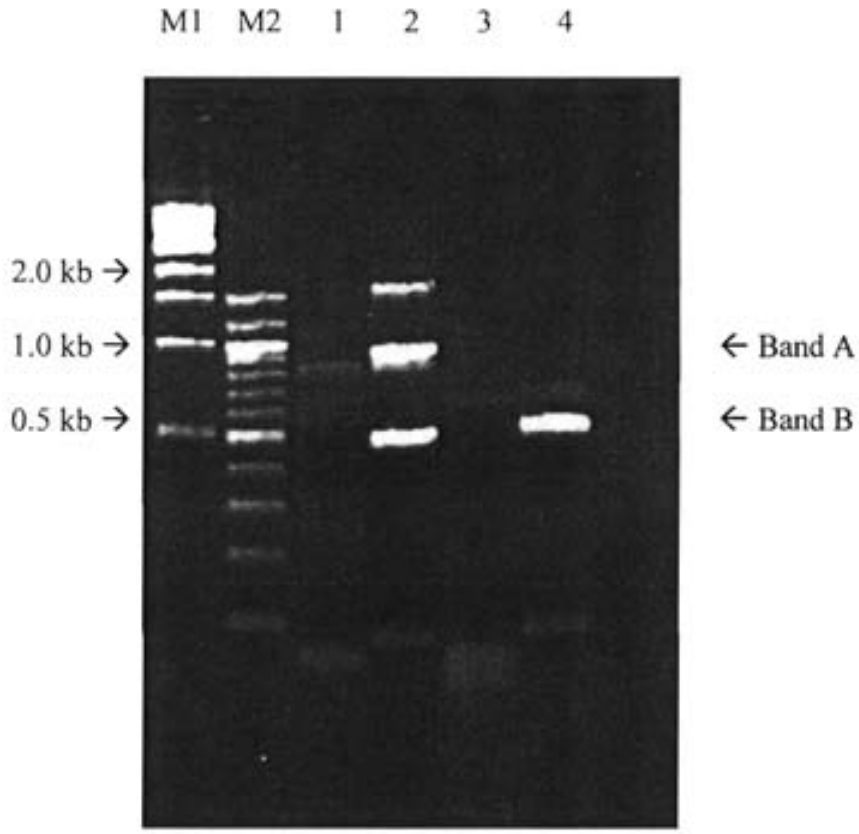


Figure 4.7 PCR products from *R. pyridinivorans* L4 DNA libraries on 1.5% agarose gel. M1 and M2 are molecular weight markers, 1 Kb and 100 bp ladder. Downstream extensions of the oxygenase PCR product from *Pvu* II library are in lane 1 and 2, while upstream extensions from *Stu* I library are in lane 3 and 4. In this gel, lane 1 and 3 are primary PCR, while lane 2 and 4 are secondary PCR. Band A and B from the secondary PCR were excised and subjected to cloning and sequencing.

Full DNA sequences of dioxygenase corresponding gene were later constructed by connecting the overlapped area of oxygenase PCR product (Figure 3.4) and band A and B (Figure 4.7). After removed the sequences of pGEM T-easy vector, we had a 1,747 bp DNA fragment (Figure 4.8). The identity of this fragment was analyzed by blastn program and found to have 100% similarity with the sequences of *Rhodococcus* sp. I24 cosmid 2G11 (AF452376.1) from bases 2947 to 4693. The DNA sequences were later transformed into amino acid sequences by Fast PCR program and checked for any conserved domains (CD) and protein homology by blastp program (protein-protein BLAST). The fragment contained 582 amino acids with a predicted start codon on residue 71 (Figure 4.9). From Entrez database of conserved domains, the corresponding dioxygenase of *R. pyridinivorans* L4 contained five conserved domains (Figure 4.9). Details of these conserved domains were provided according to Marchler-Bauer *et al.*, (2003).

1) Cd00680: Ring_hydroxylating_alpha

This CD includes the catalytic domain (alpha) of aromatic-ring-hydroxylating dioxygenase systems of eubacteria. Eubacterial ring hydroxylating dioxygenases are multicomponent 1,2-dioxygenase complexes that convert closed-ring structures to non-aromatic cis-diols. The complex has both hydroxylase and electron transfer components. The hydroxylase component is itself composed of two subunits: an alpha subunit of about 50 Kd, and a beta subunit of about 20 Kd. The electron transfer component is either composed of two subunits: a ferredoxin and a ferredoxin reductase or by a single bifunctional ferredoxin/reductase subunit. Sequence analysis of hydroxylase subunits of ring hydroxylating systems (including toluene, benzene and

napthalene 1,2-dioxygenases) suggests they are derived from a common ancestor. The alpha subunit binds both a Rieske-like 2Fe-2S cluster and an iron atom.

2) Pfam00848: Ring_hydroxyl_A

This family is the catalytic domain of aromatic-ring- hydroxylating dioxygenase systems. The active site contains a non-heme ferrous ion coordinated by three ligands.

3) Pfam00355: Rieske

The Rieske domain has a [2Fe-2S] centre. Two conserved cysteines that one Fe ion while the other Fe ion is coordinated by two conserved histidines. Conserved Cys and His residues in the N-terminal region may provide 2Fe-2S ligands, while conserved His and Tyr residues may coordinate the iron. The active site contains a non-heme ferrous ion coordinated by three ligands.

4) COG4638: HcaE

The domain is phenylpropionate dioxygenase and related ring-hydroxylating dioxygenases, large terminal subunit. It is predicted to involve in inorganic ion transport and metabolism.

5) COG2146: {NirD}

The domain is ferredoxin subunits of nitrite reductase and ring-hydroxylating dioxygenases. It is predicted to involve in inorganic ion transport and metabolism.

1	GGTTCCGTAGTTTTCCCGGATGTTCGCCGATGAGATCTGGATCACTATGG	50
51	ATGCCAGCGAGTGAGCACGGCGAATGGATCGGAAGTTTTTCCGTCGGGCC	100
101	GTGGTTCACCGTCGGCGACGTAGCCGAAGGCATTTACGACGCAGACCTGA	150
151	GTTCCATTGAACGCACTAACGACCGTACAAGTGTGATTTGCCGACGAGAA	200
201	AGGAAAGCCGCATGACTGACGTGCAATGTGAACCCGCGCTTGCGGGGAGA	250
251	AAGCCCAAGTGGGCCGACGCGGACATCGCTGAACTCGTAGACGAAAGGAC	300
301	CGGCCGGCTAGACCCGCGGATCTACACCGACGAGGCGCTGTACGAACAGG	350
351	AACTGGAGCGGATCTTCGGTCGCTCGTGGTTGCTGATGGGCCACGAGACG	400
401	CAGATTCCGAAGGCCGGCGACTTCATGACGAACTACATGGGCGAGGATCC	450
451	CGTGATGGTCGTTCGTCAGAAGAACGGGGAGATCCGCGTCTTCCTCAACC	500
501	AGTGTCGCCACCGCGGAATGCGGATCTGCCGCGCGGACGGGGGCAATGCC	550
551	AAGTCATTCACCTGCAGCTATCACGGCTGGGCCTACGATACGGGCGGCAA	600
601	CTTGGTGAGTGTGCCTTTCGAGGAGCAGGCCTTCCCCGGGCTGAGGAAAG	650
651	AAGATTGGGGCCCGCTACAGGCTCGCGTCGAGACCTACAAGGGCCTGATT	700
701	TTCGCAAACTGGGACGCTGACGCCCCGGACCTGGACACCTATCTGGGTGA	750
751	AGCGAAGTTCTACATGGACCACATGTTGGATAGAACCGAAGCGGGCACCG	800
801	AAGCGATCCCGGGGATTCAGAAGTGGGTCATTCCCTGCAACTGGAAGTTC	850
851	GCAGCGGAGCAATTCTGCAGCGACATGTACCACGCGGGCACCACATCCCA	900
901	TCTTTCCGGCATTCTCGCGGGCCTGCCTGATGGCGTCGATCTGTCGGAGC	950
951	TCGCGCCCCCCACGGAAGGTATCCAGTACCGCGCAACCTGGGGCGGGCAC	1000
1001	GGTAGCGGCTTCTACATCGGCGATCCCAACCTGTTGCTCGCCATCATGGG	1050

Figure 4.8 DNA sequences of dioxygenase corresponding gene and flanking regions from *R. pyridinivorans* L4 genome walking.

1051	GCCGAAGGTCACCGAGTACTGGACTCAGGGCACTGCCGCAGAGAAGGCTT	1100
1101	CCGAGCGCCTGGGAAGCACAGAGCGTGGCCAGCAACTAATGGCGCAGCAC	1150
1151	ATGACCATCTTCCCAACCTGTTCGTTCCTGCCAGGCATCAACACCATCCG	1200
1201	AGCGTGGCACCCTCGCGGGCCGAACGAGATCGAGGTCTGGGCCTTCACCG	1250
1251	TCGTTGATGCCGACGCACCCGAGGAGATGAAAGAGGAATACCGCCAGCAG	1300
1301	ACACTGCGAACCTTCTCGGCAGGTGGTGTCTTCGAGCAGGACGACGGCGA	1350
1351	GAACTGGGTTGAGATCCAGCAGGTGCTGCGCGGACACAAGGCCCGCAGTC	1400
1401	GGCCGTTCAACGCGGAGATGGGACTCGGCCAGACCGACACGGACAATCCC	1450
1451	GATTACCCCGGCACGATCAGCTACGTCTACAGCGAAGAAGCGGCGCGTGG	1500
1501	GCTGTATACGCAATGGGTCCGGATGATGACTTCGCCGGACTGGGCTGCAC	1550
1551	TGGACGCCACCCGACCCGCAGTGTCCGAGTCGACCCACACGTGACCGCGG	1600
1601	CGGGGCGAGCGGACCGCGGTGGAACCATCGCCGGGTCTCCACCTGAACAG	1650
1651	AACTCCCGACTGATTAGGAAGAATTATGATTGACGCCGAATCGCCAACCA	1700
1701	CGGCATTTCGAACAAAACCCGCACCAGTAGACCCCAGCCTGCAGCAC	1747

Figure 4.8 *(continued)*

A.

1	vp*fsrmfadeiwitmdase*arrmdrkffrravvhrrrrsrrhlrrrpefh*th*rpyk	60
	Predicted start codon	
61	cdlptrkesrmtdvqcepalagrkpkwadadiaelvdertgrldpriytdealyeqeler	120
	Rieske [2Fe-2S] domain	
121	ifgrswllmghetqipkagdfmtnymgedpvmvvrqkngeirvflnqcrhrgmricradg	180
181	gnaksftcsyhgwaydtggnlvsvpfeeqafpglrkedwgplqarvetykglifanwdad	240
	Ring hydroxylating alpha subunit (catalytic domain)	
241	apdldtylgeakfymdhmldrteagteaipgiqkwvipcnwkfaaeqfcsdmyhagttsh	300
301	lsgilaglpdgvdlselapptegiqyratwgghgsgfyigdpnlllaimgpkvteywtqg	360
361	taaekaserlgstergqqlmaqhmtifptcsflpgintirawhprgpneievwaftvvda	420
421	dapeemkeeyrqqtlrtfsaggvfeqddgenwveiqqvlrghkarsrpfnaemglggtd	480
481	dnpdypgtisyvyseeaarglytqwvrmmtspdwaaldatrpavsestht*prrgertav	540
541	epspglhlnrtpd*lgrimidaespttafrtkpapvdpslqh	582

B.

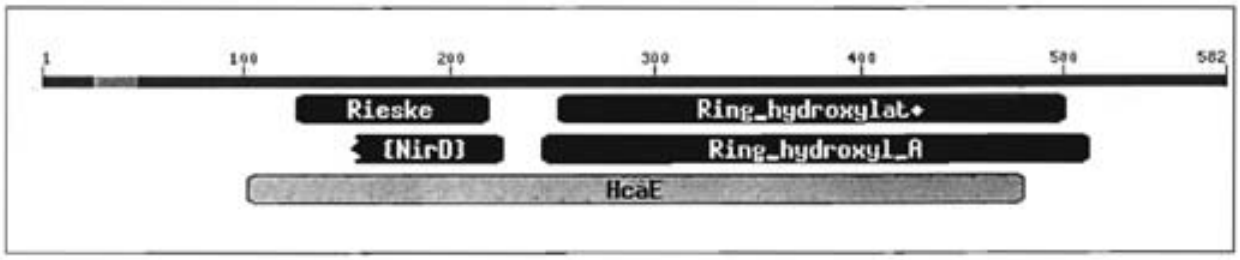


Figure 4.9 Deduced amino acid sequences of dioxygenase gene in *R. pyridinivorans* L4 (Panel A). Panel B showed diagram of conserved domains (CD) from the alignment of *R. pyridinivorans* L4 dioxygenase amino acid sequences with Entrez database of conserved domains.

Blastp program also compared the sequences of *R. pyridinivorans* L4 dioxygenase with other deposited proteins in the GenBank/EMBL data bank. The results showed 100% similarity between 460 deduced amino acid residues of *R. pyridinivorans* L4 dioxygenase and *Rhodococcus* sp. I24 toluene inducible dioxygenase large subunit (*TidA*) (Table 4.8). In addition, 99% of amino acid sequences of *R. pyridinivorans* L4 dioxygenase were similar to isopropylbenzene 2,3-dioxygenase of *R. erythropolis* BD2 and biphenyl dioxygenase of *Rhodococcus* sp. RHA1. Other dioxygenase protein with more than 65% similarity to *R. pyridinivorans* L4 dioxygenase were bnzene 1,2-dioxygenase of *P. putida* ML2, biphenyl dioxygenase of *P. pseudoalcaligenes* KF707, cumene dioxygenase of *P. fluorescens* IP01, alkylbenzene dioxygenase of *P. putida* 01G3, and toluene dioxygenase of *P. putida* F1.

Later, we selected 460 amino acid residues from *R. pyridinivorans* L4 sequences that matched perfectly with toluene inducible dioxygenase to align with full sequences of other dioxygenase large subunit using CLUSTAL W multiple sequence alignment program. Figure 4.10 showed conserved amino acid sequences among the dioxygenase large subunit proteins. An average distance tree using PID was later constructed (Figure 4.11). The results from CLUSTAL W suggested that aromatic dioxygenase gene sequences in *Rhodococcus* species were highly conserved and probably came from the same ancestor. In conclusion, the genomic DNA assay indicated that *R. pyridinivorans* L4 contained *TidA* gene homologue. *TidA* is coding for toluene inducible dioxygenase large subunit and responsible for oxygenating indene to indandiols after *Rhodococcus* sp. I24 grown on toluene (Priefert *et al.*, 2004).

Table 4.8 Homology of *R. pyridinivorans* L4 deduced amino acid sequences to dioxygenase large subunit deposited in the GenBank/EMBL data bank.

Gene	Protein/Microorganism	Accession No.	Identity (% Similarity)
BedC1	Benzene 1,2-dioxygenase/ *P. putida* ML2	AAA17758.1	335/439 (76%)
Bpha1a2	Biphenyl dioxygenase/ *Rhodococcus* sp. RHA1	BAA06868.1	458/460 (99%)
BphA1	Biphenyl dioxygenase/ *P. pseudoalcaligenes* KF707	AAF22429.1	303/438 (69%)
CumA1	Cumene dioxygenase/ *P. fluorescens* IP01	BAA07074.1	296/438 (67%)
EbdAa	Alkylbenzene dioxygenase/ *P. putida* 01G3	CAB99196.1	290/438 (66%)
IpbA1	Isopropylbenzene dioxygenase/ *R. erythropolis* BD2	AAP74038.1	457/460 (99%)
TidA	Toluene inducible dioxygenase/ *Rhodococcus* sp. I24	AAL61663.2	460/460 (100%)
TodC1	Toluene dioxygenase/ *P. putida* F1	AAA26005.1	342/436 (78%)

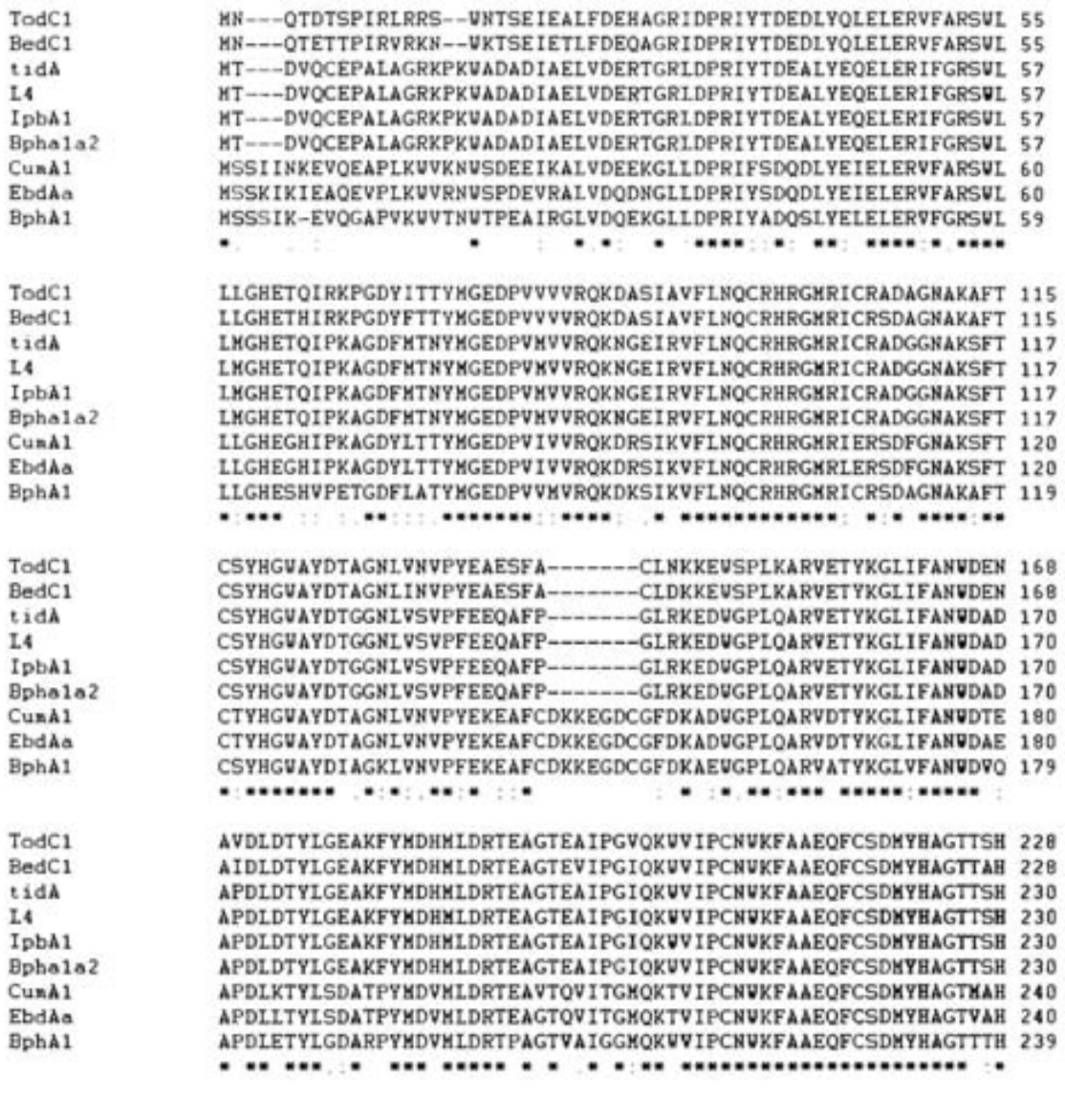


Figure 4.10 Comparison of deduced amino acid sequences of *R. pyridinivorans* L4 (bold letter) and those of other dioxygenase large subunits including *TodC1* of *P. putida* F1, *BedC1* of *P. putida* ML2, *TidA* of *Rhodococcus* sp. I24, *IpbA1* of *R. erythropolis* BD2, *Bpha1a2* of *Rhodococcus* sp. RHA1, *CumA1* of *P. fluorescens* IP01, *EbdAa* of *P. putida* 01G3, and *BphA1* of *P. pseudoalcaligenes* KF707. The conserved substitution, semi-conserved substitution, and identical or conserved residues in all sequences are present as ., :, and *, respectively.

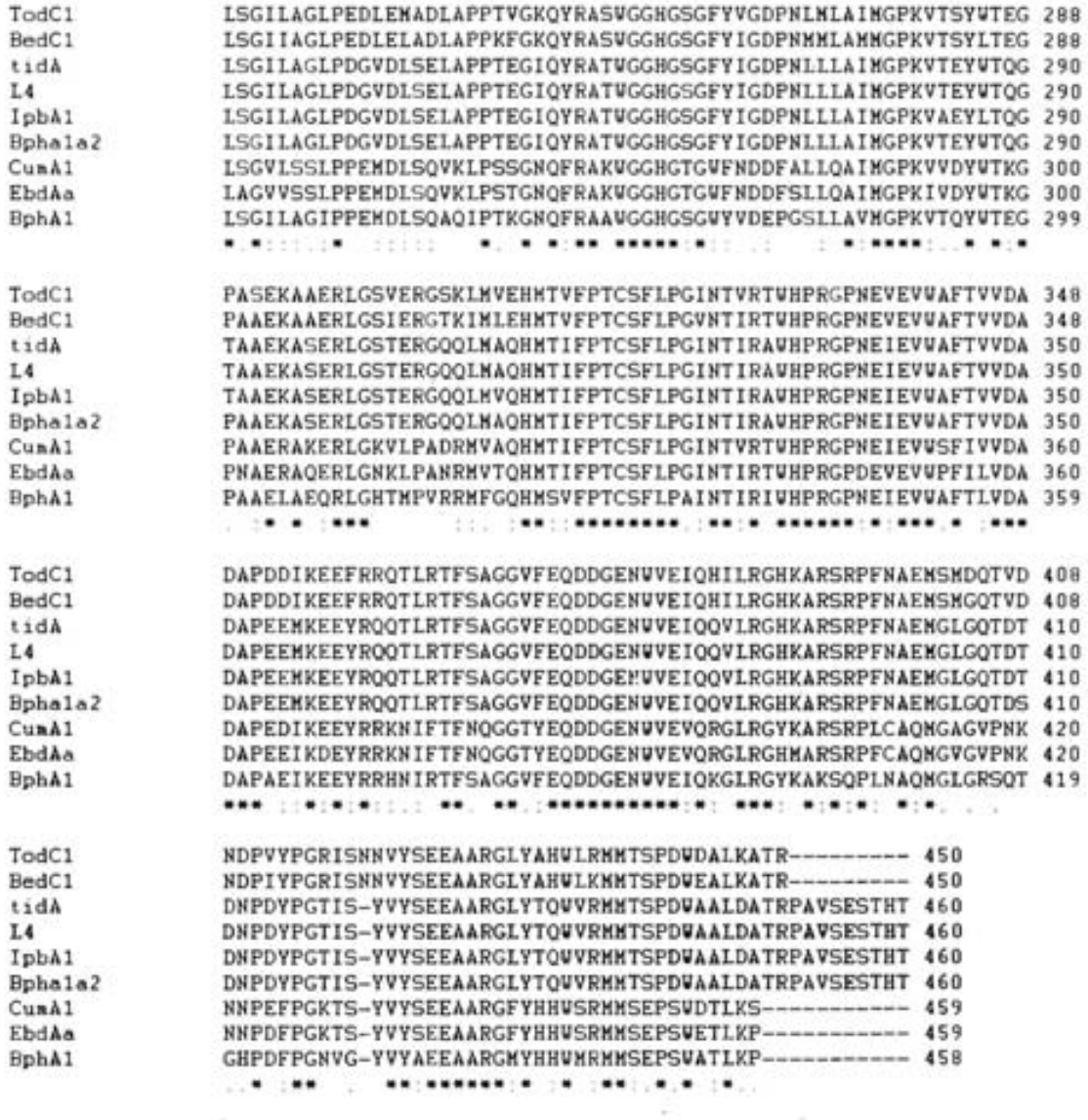


Figure 4.10 *(continued)*

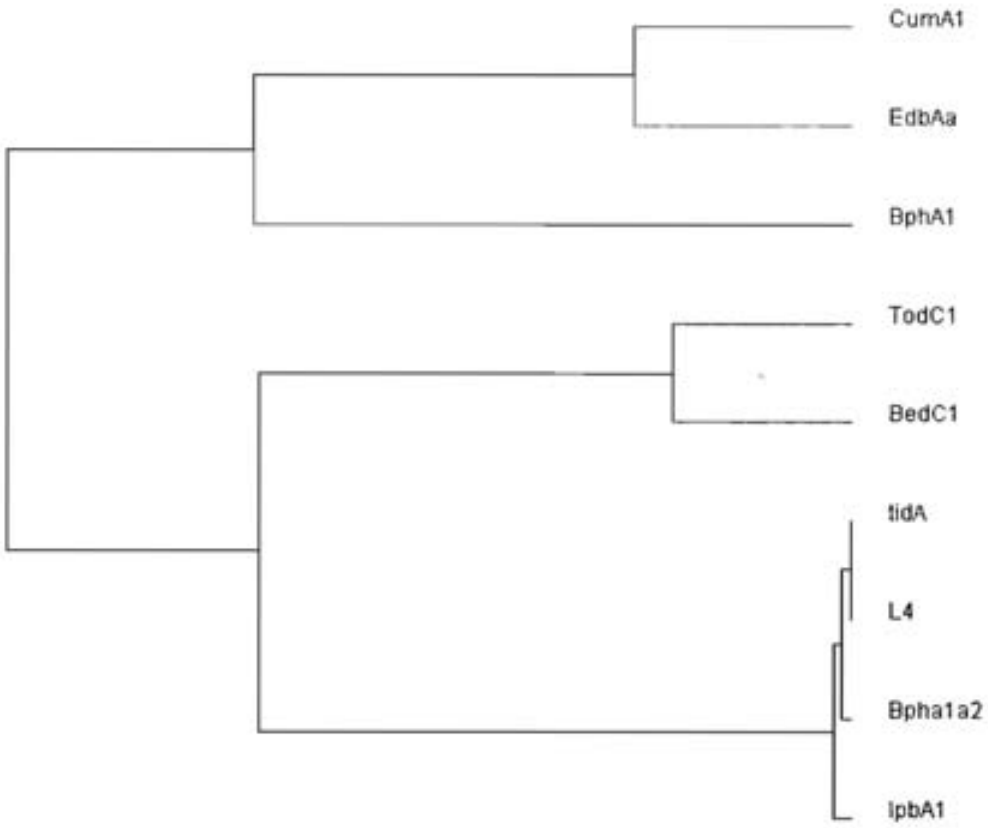


Figure 4.11 Average distance tree of deduced amino acid sequences of *R. pyridinivorans* L4 and those of other dioxygenase large subunit including *TodC1* of *P. putida* F1, *BedC1* of *P. putida* ML2, *TidA* of *Rhodococcus* sp. I24, *IpbA1* of *R. erythropolis* BD2, *Bpha1a2* of *Rhodococcus* sp. RHA1, *CumA1* of *P. fluorescens* IP01, *EbdAa* of *P. putida* 01G3, and *BphA1* of *P. pseudoalcaligenes* KF707.

4.2.3 Expression of *R. pyridinivorans* L4 oxygenase gene

Ability of terpene for inducing oxygenase gene was studied by comparing the amount of dioxygenase mRNA after RT-PCR of total RNA, which extracted from 24-hr *R. pyridinivorans* L4 cells. The size of PCR products from every RNA samples were about 450 bp (Figure 4.12). The result was corresponded to the product from *R. pyridinivorans* L4 genomic DNA amplification by similar BPH4-F/BPH3-R primers (Figure 4.5). However, these PCR products had different band intensities. The most intense DNA band was from toluene grown cells, which indicated the highest amount of dioxygenase mRNA. Citral-, cumene-, and limonene-induced cells expressed higher amount of dioxygenase gene than non-induced cells (glucose grown cells). When compared between terpene solutions, limonene had lowest ability to induce dioxygenase gene. The result was consistent with the lowest ability of limonene-induced cells on TCE mineralization (Figure 4.2). Meanwhile, the present of DNA band from glucose grown cells suggested that the bacterium constitutively produced low amount of dioxygenase enzyme.

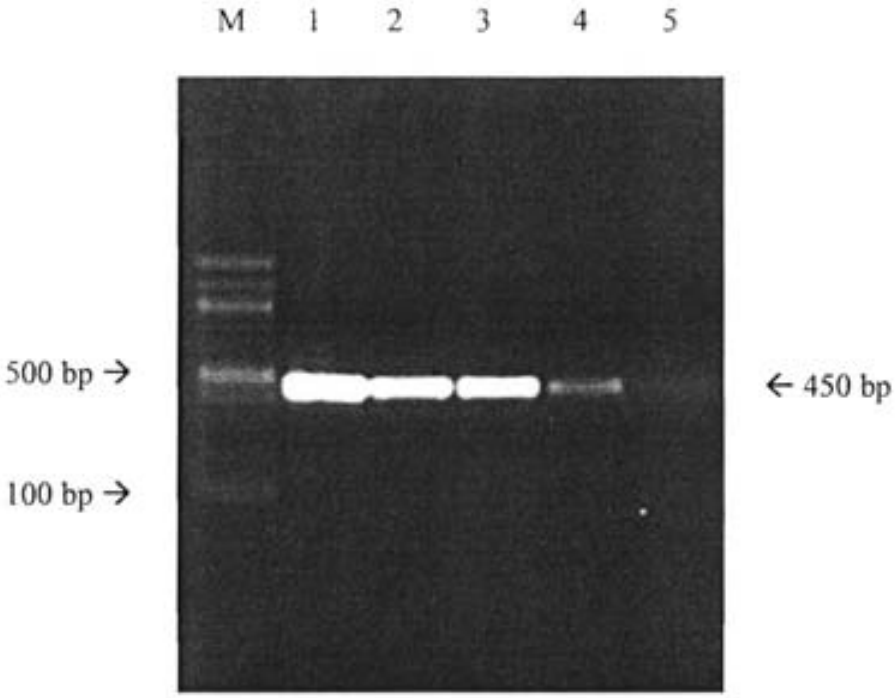


Figure 4.12 Amplification of *R. pyridinivorans* L4 total RNA by BPH4-F/BPH3-R primers. Lanes: M, 100 bp molecular weight marker; 1, toluene grown cells; 2, citral-induced cells; 3, cumene-induced cells; 4, limonene-induced cells; and 5, glucose grown cells.

5. DISCUSSION

5.1 Utilization of plant terpenes as an alternative TCE inducer

The results showed that *R. pyridinivorans* L4 degraded more TCE when terpene was present in the system. However, the extent of TCE degradation depended on the amount and type of plant terpenes. Citral, cumene, and limonene at 25-50 ppm induced the bacteria to degrade 65-72% of 15 ppm TCE within 30 hrs. TCE degrading activities of *R. pyridinivorans* L4 were less effective when using these terpenes as bacterial carbon source. The ability of cumene on TCE induction was similar to previous reports by Dabrock *et al.* (1992, 1994) and Pflugmacher *et al.* (1996). They found that cumene induce the production of dioxygenase in *R. erythropolis* BD2 and the induced bacteria cells degraded 71% of 50 μM TCE after 20 hrs incubation. Earlier report from our laboratory also showed that cumene induced *R. gordoniae* P3 to degrade 75% of 10 ppm TCE within 24 hrs (Sutthinun *et al.*, 2004). Cumene is able to induce TCE degradation probably because the bulky isopropyl residue adjacent to the double bound on benzene ring may mimic the two chlorines in the TCE molecule (Dabrock *et al.*, 1992). This structure may be an important element for inducing TCE degrading enzyme.

Meanwhile, the ability of citral and limonene on TCE induction has never been reported. It is not known how limonene or citral induced for TCE degradation; however the enzymes involved in the limonene degradation process may be toluene degrading enzymes. Duetz *et al.* (2001) reported that *R. opacus* PWD4 is able to transform limonene to carveol after growing on toluene. The authors suggested that toluene 2,3-dioxygenase is a likely candidate since it has been found to be capable of monooxygenations (e.g. indene to 1-indenol). Similarly, *R. pyridinivorans* L4 probably produced toluene degrading enzyme after induction by limonene and then

used the enzymes to degrade TCE. Limonene also induces the production of dioxygenase enzyme in *P. stutzeri* and consequently enhances polychlorinated biphenyls (PCB) biodegradation (Tandlich *et al.*, 2001). This is interesting because we may use limonene and other terpenes to induce for the degradation of TCE as well as other hazardous materials.

Besides TCE degradation, terpene-induced cells mineralized TCE and resulted in the production of chloride ions. Of all terpenes, citral was the best inducer for TCE mineralization. The activity of terpene on inducing TCE mineralization may be different from TCE degradation, since citral-induced cells degraded TCE at the same extent as cumene- and limonene-induced cells but mineralized TCE at higher amount. Otherwise, cumene and limonene may bind to TCE degrading intermediates and then prevent the releasing of chloride ions from those intermediates. Luu *et al.* (1995) also reported the difference of TCE mineralization and degradation in *P. cepacia* G4 PR1 after grown on different media. *P. cepacia* G4 PR1 grown on chloride-free and chloride-ion-containing minimal medium is able to degrade similar amount of TCE (85% of 75 μM TCE) but the extent of mineralization is slightly superior in the chloride-free minimal medium (77% compared to 60% of 75 μM TCE).

To apply terpene for clean-up TCE contaminated soil, bioremediation treatments were developed using *R. pyridinivorans* L4, terpene solution, glucose, and plant materials, including lemon grass leaves, cumin seeds, and orange peels. Bioaugmentation was carried out by adding terpene-induced *R. pyridinivorans* L4 cells along with terpene solutions or plant materials to maintain the TCE degrading activities in soil. For biostimulation, terpene solutions or plant materials was added to enhance TCE degrading activity of soil indigenous microorganisms. Both

bioaugmentation and biostimulation results confirmed that terpene was necessary for the induction of TCE degradation in soil. The amount of TCE in treated soil was decreased more rapidly than untreated soil. From our results, terpenes may be added to the contaminated soil as purified solutions and plant materials. However, the extent of TCE degradation in treatment with plant materials was higher than with purified terpene solutions. Plant materials such as orange peels and cumin seeds contained several terpenes, may responsible for the induction of various TCE degrading enzymes in the soil microcosms.

Our earlier study on the biodegradation of TCE contaminated soil found that the inoculation of cumene-induced *R. gordoniae* P3 and the addition of cumene to contaminated soil accelerated TCE reduction only at the beginning of study (Sutthinun *et al.*, 2004). Although, the type of adding bacteria and terpene were different from this study, the results here showed a similar trend. The amount of TCE in the untreated soil was slowly reduced after incubation. This indicated the possibility for intrinsic bioremediation of TCE contaminated soil. Similarly, Kho and Prosser (1999) reported that an intrinsic bioremediation process is occurring at a TCE and chlorobenzene (CB) contaminated site in Georgia, USA. They found the decrease in TCE and CB concentrations, and increase in TCE degradation by-products e.g., dichloroethylene isomers (DCEs), and vinyl chloride (VC) concentrations. However, this technology may not suitable to all contaminated sites and we still need bioaugmentation or biostimulation for a rapid clean-up.

TCE does not serve as a primary (growth) substrate for microorganisms and is biodegraded under aerobic conditions only through the process known as co-metabolism or co-oxidation (Little *et al.* 1988). In our study, terpene was added at low

amount for the induction purpose, thus it was probably not enough to support bacterial growth. To further enhance TCE degradation, glucose was added as carbon source. We found that the amount of TCE degradation in citral-induced cells/glucose-MS and glucose-MS treatments was higher than the treatments without glucose. The important of bacteria substrate was also reported by Munakata-Marr *et al.* (1996, 1997). They found that 60-250 µg/l TCE was cometabolized after the introduction of wild type and genetically altered *Burkholderia cepecia* G4 and PR1 along with the bacteria primary substrate i.e. phenol or lactate to the microcosms.

Citral and limonene are the major terpenes in lemon grass and lemon/orange essential oil, respectively. Both compounds have been used as flavor and fragrance additive in food, household products, and perfumes. Cumene is found in a variety of essential oils from plants and foodstuffs such as cumin seed and curry. The compound is also a constituent of crude oil and finished fuels. From Hazardous Substances Data Bank (HSDB, http://toxnet.nlm.nih.gov/index.html), citral, cumene, and limonene are not classified as human carcinogen. Once exposed, these compounds are metabolized and readily excreted out of the body. If released to soil, these compounds are expected to volatilize and undergo considerable biodegradation in soil environments.

Citral, cumene, and limonene were the most effective terpenes that could induce TCE co-metabolic pathway of *R. pyridinivorans* L4. When compared between each terpene, cumene is considered to be more hazardous than citral and limonene. The compound has been regulated in several USA environmental standards, for example RCRA Requirements, Atmospheric standards, and State drinking water guideline of Florida (0.8 ug/l), Minnesota (300 ug/l), and New Hampshire (280 ug/l) (HSDB). Since, the efficiency of all terpenes on enhancing TCE bioremediation was

almost the same, the best alternative to toluene for TCE biodegradation would be citral and limonene due to their benign and non-persistent nature. Future research on the application of citral and limonene as well as their corresponding plant materials should be carried out to develop a low cost, environmental friendly approach for TCE bioremediation. From their ability to induce oxygenase enzymes, citral and limonene may be applied to degrade other organic hazardous chemicals as well, for example chlorinated solvents (e.g. dichloroethylenes, vinyl chloride, and tetrachloroethylene), polyaromatic hydrocarbons (PAHs), and organochlorine pesticides.

5.2 Characterization of initial genes involved in TCE cometabolism

Aerobic TCE cometabolism is initiated by various oxygenase enzymes, a class of enzyme that catalyze the incorporation of one (monooxygenases) or two (dioxygenases) atom of dioxygen into substrates. From enzyme assay, *R. pyridinivorans* L4 showed characteristics corresponding to toluene dioxygenase. However, genomic DNA from *R. pyridinivorans* L4 could not be amplified by toluene dioxygnease primers, TOD-F/TOD-R from Baldwin *et al.* (2003). Only BPH4-F and BPH3-R, the primers targeting *Rhodococcus* biphenyl dioxygenase could amplify *R. pyridinivorans* L4 DNA. Luepromchai (2004) reported that *R. pyridinivorans* L4 can utilize diverse organic compounds as a sole carbon source; for example BTEX (benzene, toluene, xylene, and ethylbenzene) and plant terpenes such as limonene, cumene, and pinene, but not biphenyl. The DNA sequences from genome walking later indicated that *R. pyridinivorans* L4 contained gene analogous to *TidA*, which coding for toluene inducible dioxygenase large subunit in *Rhodococcus* sp. I24 (Priefert *et al.*, 2004). Other enzymes with high homologous sequences were isopropylbenzene 2,3-

dioxygenase of *R. erythropolis* BD2 (Kesseler *et al.*, 1996), biphenyl dioxygenase of *Rhodococcus* sp. RHA1 (Masai *et al.*, 1995) and toluene dioxygenase of *P. putida* F1 (Zylstra and Gibson, 1989).

Rhodococcus sp. I24, the bacterium with 100% similarity to *R. pyridinivorans* L4 dioxygenase sequences, was isolated from a toluene contaminated aquifer in USA (Chartain *et al.*, 1998). After grown on toluene and naphthalene, the bacterium can oxygenate indene to indandiols, the key precursors for HIV protease inhibitor Crixivan® (Indinavir) production (Treadway *et al.*, 1999, Chartain *et al.*, 1998). Genes encoding toluene inducible dioxygenase of *Rhodococcus* sp. I24 are located on a ~340 kb extrachromosomal element in the I24 strain and the expression of *tid* operon (*tidABCD*) in induced by toluene but not by naphthalene (Priefert *et al.*, 2004). The *tid* operon shows sequence homology and gene organization that are consistent with other toluene-type dioxygenases such as *ipb* of *R. erythropolis* BD2 and *tod* of *P. putida* F1 (Priefert *et al.*, 2004). *Rhodococcus* sp. I24 has never been tested for TCE degradation, but *R. erythropolis* BD2 and *P. putida* F1 were able to oxidize TCE after induction by cumene and toluene, respectively (Dabrock *et al.*, 1994, Wackett and Gibson, 1988).

Toluene dioxygenase (TDO) is a versatile enzyme that can catalyze oxidative dehalogenation of various halogenated substrates such as TCE, 1,1-dichloro-1-propene, and 1,1-dichloroethene (Arp *et al.*, 2001). The active site of TDO is similar to other aromatic dioxygenases, which contains both a Riske-type [2Fe-2S] center and a mononuclear iron center (Harayama *et al.*, 1992). The sequences conserved for Rieske domain and the catalytic domain (alpha) of aromatic-ring-hydroxylating dioxygenase systems were also found in *R. pyridinivorans* L4. Meanwhile, the organization of *tid* operon in this bacterium and their corresponding functions should be studied to

confirm its identity. Since, dioxygenase sequences of *R. pyridinivorans* L4 were highly similar to other *Rhodococcus* species; they probably share a common ancestor or involve in the transfer of genetic elements. Several catabolic genes such as *ipb* and *tid* are located on megaplasmids in *Rhodococcus* species. These plasmids can be transferred by conjugation to derivatives of the parent strain or to closely related strains. (Larkin *et al.*, 1998). The occurrence of *R. pyridinivorans* L4 and its dioxygenase in Thailand suggested that the wide distribution of similar bacteria and genes in the environment.

Two types of *Rhodococcus*, including *R. corallinus* and *R. erythropolis* were previously reported for the TCE degrading ability. The bacteria use different enzymes for initiating TCE degradation. *R. corallinus* B-276 utilize alkene monooxygenase to catalyses TCE oxidation after growing on both glucose and propene (Saeki *et al.*, 1999). On the other hand, *R. erythropolis* BD2 degrade TCE while using isopropylbenzene (cumene) as a sole carbon and energy source (Dabrock *et al.*, 1992). The bacterium contains an isopropylbenzene dioxygenase analogous to toluene dioxygenase, which are encoded by a conjugative linear plasmid, pBD2 (Dabrock *et al.*, 1994).

The expression of toluene dioxygenase gene in *R. pyridinivorans* L4 was studied by RT-PCR analysis. The bacterium produced highest amounts of dioxygenase mRNA when grown on toluene. This result suggested that toluene is probably the true substrate for this enzyme. Meanwhile, terpene could also induce dioxygenase gene in *R. pyridinivorans* L4. The amounts of dioxygenase gene were in citral-, cumene-, and limonene-induced cells were much higher than non-induced cells. Based on the evidences presented, the initial TCE degrading enzyme of *R. pyridinivorans* L4 was

analogous to toluene dioxygenase and it could be induced by toluene and various terpenes. To our knowledge, *R. pyridinivorans* L4 is the third TCE degrading *Rhodococcus* that have been reported.

6. CONCLUSIONS

From the study, we concluded that:

1) Purified terpene solutions, including citral, cumene, and limonene at 25-50 ppm concentrations were able to induce TCE degradation in *R. pyridinivorans* L4.

2) Bioremediation treatments for TCE contaminated soil could be carried out by adding *R. pyridinivorans* L4, carbon source, and terpene solutions or plant materials. Lemon grass leaves, cumin seeds, and orange peels are low cost plant materials that effectively reduced the amount of TCE in the soil microcosms.

3) The probable initial TCE degrading enzyme in *R. pyridinivorans* L4 was toluene dioxygenase. The enzyme was also reported from other known TCE degrading bacteria such as *R. erythropolis* BD2 and *P. putida* F1.

4) The initial TCE degrading enzyme encoding gene in *R. pyridinivorans* L4 was analogous to *TidA*, which coding for toluene inducible dioxygenase large subunit in *Rhodococcus* sp. I24. Previous reports showed that the *tid* operon have sequence homology and gene organization consistent to other toluene-type dioxygenases.

5) The expression of toluene dioxygenase in *R. pyridinivorans* L4 could be induced by terpene solutions i.e. citral, cumene, and limonene.

7. SUGGESTIONS FOR FURTHER WORK

7.1 Utilization of plant terpenes as an alternative TCE inducer

7.1.1 Biochemistry of terpene induction

Based on the results of this study, further research is needed to extend the basic knowledge on how terpenes induce TCE degrading enzymes and whether the compounds affect other bacterial functions. Morono *et al.* (2004) recently reported that TCE degrading activity in *P. putida* F1 could be restored by the addition of benzene and cumene. They suggested that some of the TCE degradation products, or TCE itself, might remain in the active site of toluene dioxygenase during TCE degradation process and then inactivate the enzyme. Aromatic substrates such as toluene, benzene, and cumene can displace these inhibitors from the active site of toluene dioxygenase, thereby restoring enzyme activity. These phenomena may also take place in *R. pyridinivorans* L4.

To elucidate terpene induction process, we may characterize intermediates produced during TCE and terpene degradation, compare the effects of different terpene on toluene dioxygenase induction, and study the regulation of toluene dioxygenase and relevant enzymes. Further information may be acquired from the study of *R. pyridinivorans* L4 genome. The advances in genomics and high-throughput analytical technologies such as proteomics, transcriptomics, and metabolomics would enable us to gain global insights into the metabolic potential and activity of microorganisms, and on how the genome is utilized by an organism under various conditions (Pieper *et al.*, 2004). The acquired information is important for the optimization of enzyme induction process and consequently the development of TCE bioremediation.

7.1.2 TCE Bioremediation

Examples of future research topics that can improve the efficiency of bioaugmentation and biostimulation program are optimization of the terpene concentration for TCE induction, optimization of physical, chemical, and biological parameters during the clean-up of polluted soil, and study bacterial types and diversity involving TCE degradation and bioremediation in Thailand. The knowledge from these researches will be useful for further study concerning other organic hazardous chemicals such as volatile organic compounds (VOCs), petroleum compounds, polyaromatic hydrocarbons (PAHs), and organochlorine pesticides. Moreover, the effect of terpene on TCE in liquid and solid phase should be studied. For example, the distribution of terpenes between liquid and gas phases, fate and transport of terpene in soil, interactions between terpene and TCE, and etc. It may be possible to apply terpenes by continuous feeding using water sprinkler or growing plants that produce terpenes through their rhizosphere for enhancing TCE and other xenobitic compounds degradation.

Bioaugmentation using effective bacterial strains, both engineered and naturally occurring, may hold potential for enhancing both in situ and ex situ treatment. We may later modify the genetic of *R. pyridinivorans* L4 to make it more effective. Additionally, use of carrier material may increase the potential for successful bioaugmentation by enhancing the survival of bacteria that have been inoculated into the soil because of their favorable characteristics for field application (Swadley, 2001). The survival and activities of added bacteria in soil are important for the degradation of pollutants. Powerful molecular methods such as metagenomic libraries and microarrays may be used to explore simultaneously the taxonomic and functional

variety of indigenous microorganisms as well as the applied bacteria (Eyers *et al.*, 2004). Finally, larger scale bioremediation system should be conducted before applied the technology to the real contaminated site.

8.2 Characterization of initial genes involved in TCE cometabolism

8.2.1 Characterization of *R. pyridinivorans* L4 genes

Since, genes encoding toluene dioxygenase are usually organized in operon , for example *todC1C2BADE* from *P. putida* F1 and *tidABCD* from *Rhodococcus* sp. I24. Characterization of other genes in *tid* operon will confirm the present of toluene dioxygenase in *R. pyridinivorans* L4. With the genes in hand, application of genetic engineering is become feasible, for example, to design gene probes for monitoring the added bacteria and other TCE degrading populations during bioremediation, to develop a TCE sensing bacteria for detection of its contamination, and to improve degrading activity and survival rate of TCE degrading bacteria. In addition, the bacterial gene will be a model for study TCE degrading mechanism carried out by local bacteria.

Like most other bacteria, members of the genus *Rhodococcus* have been showed to possess a variety of plasmid DNA molecules such as small cryptic closed circular plasmids to large linear plasmids (Larkin *et al.*, 1998). Pulsed-field gel electrophoresis should be carried out to check whether *tid* operon in *R. pyridinivorans* L4 is located on linear plasmids. Catabolic genes on linear plasmids have been showed to be transferred by conjugation to derivatives of the parent strain or to closely related strains (Larkin *et al.*, 1998). Horizontal transfer of such elements may be the basis of the biocatalytic diversity seen among the genera (Priefert, 2004).

8.2.2 Application of *R. pyridinivorans* L4 and its genes

The remarkable ability of members of the genus *Rhodococcus* to degrade many organic compounds, their ability to produce surfactants and their environmental persistent, make them ideal candidates for enhancing the bioremediation of contaminated sites (Larkin *et al.*, 1998). In addition to applications in bioremediation, oxygenases and other biodegradative enzymes are benign catalysts that can be used for the production of industrially useful compounds (Parales and Haddock, 2004).

Biotransformation of terpenes is of great commercial interest since microbial enzymes can specifically transform low cost terpenes into more expensive products. For example, limonene, a by-product from orange juice industry, can be transformed into several flavor and fragrance compounds such as perillyl alcohol, α-terpineol, carveol, carvone and menthol (Duetz et al., 2003, de Carvalho and da Fonseca, 2003, Chatterjee and Bhattacharyya, 2001). One of the enzymes from a toluene degradation pathway in *R. opacus* PWD4 has been reported for the biotransformation of limonene to trans-carveol and carvone (Duetz et al., 2001). Besides the transformation of terpenes, *R. pyridinivorans* L4 may have some functions similar the *Rhodococcus* sp. I24, the bacterium with 100% homology dioxygenase sequences. This bacterium is able to oxygenate indene to indandiols, the key precursors for HIV protease inhibitor Crixivan® (Indinavir) production. Meanwhile, more detailed studies are required.

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