工學碩士 學位論文

다염화비페닐(polychlorinated biphenyls; PCBs)의 환경정화에 있어서 테르핀(terpenes)에 의한 생분해촉진기작의 분자생물학적·생태학적 규명

Molecular Biological and Ecological Investigation of PCBs Biodegradation Facilitated by Plant Terpenes

指導敎授 高 星 澈

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韓國海洋大學校 大學院

土木環境工學科

鄭 慶 子

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위원장 : 박 상 윤 (인)

위 원 : 고 성 철 (인)

위 원 : 김 인 수 (인)

2001년 12월 14일

韓國海洋大學校 大學院

土木環境工學科 鄭 慶 子

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ABSTRACT

본 연구에서는 천연적으로 식물에서 합성이 이루어지며 자연계에 광범위하게 존재하는 식물 테르핀 (terpenes) 가 PCBs 및 천연 할로겐화 물질이 호기성 상태의 PCBs로 오염된 토양의 환경친화적 처리에 있어서 이러한 추가기질로서 어떤 역할을 하는지를 분자생물학적 및 생태학적 기법을 이용하여 규명하고자 하였다.

우선적으로, 2종의 PCB 분해균주에서 테르핀 (carvone) 에 의해 PCB 분해경로가 유도된다는 사실을 생화학적 및 분자생물학적 기법들을 이용하여 밝혀내었다. Carvone에 의한 *Ralstonia eutropha* H850의 *bphC* 유전자의 발현결과는 H850의 토양내에서의 발현추적에 도움이 될 것이다.

이러한 연구결과를 바탕으로 테르핀 (carvone, limonene, terpinene 등) 를 처리한 토양에 H850 균주를 접종하여 관찰한 결과, 최소한 2개월 이상 존재할 수 있음을 밝혔다. 그리고 처리구별 토양 총 RNA를 추출하여 RT-PCR을 통해 *bphC* 유전자의 mRNA 발현을 조사한 결과 비페닐 처리구 (4일째)에서 그 유전자가 발현됨을 밝혔다. 그러나 테르핀 처리구에서는 유전자의 발현이 나타나지 않은 것으로 보아 계면활성제의 처리를 통한 생물이용율을 증가시킬시 유전자의 발현 촉진, 그리고 지속적 기질 첨가 효과를 검토할 필요가 있었다. 이러한 이유로 계면활성제 sorbitan trioleate의 토양첨가실험을 실시하였다. 그 실험을 통해 sorbitan trioleate는 PCBs 분해균의 성장기질로 사용되어지는 것을 확인할 수 있었으며 또한 그 균주에 대한 독성도 없는 것으로 나타났다. 따라서 본 계면활성제가 분해균의 초기 밀도를 높이고 그 후 테르핀이 PCBs

분해 유전자의 발현을 촉진할 경우 그 분해효과는 극대화될 것으로 판단되며 이는 현장 적용시 상당한 이점으로 작용할 수 있을 것이다.

또한 자연의 성장 기질 또는 유도물질로서 식물 테르핀이 PCB congeners에 어떠한 생분해 영향을 주는가에 대한 연구도 이루어졌다. PCB 분해자인 *Rhodococcus* sp. P166와 *Rhodococcus* sp. T104는 비페닐과 테르핀 ((*S*)-(-)-limonene, *p*- cymene, *α*-terpinene)을 성장기질로 이용함을 밝혀내었다. 그리고 congener 분해시험에서 (*S*)-(-)-limonene, *p*-cymene와 *α*-terpinene에서 키운 strain T104가 비페닐에 성장한 분해율의 50% 에 해당하는 30%까지 4,4'-DCBp를 분해시킬 수 있음을 보여주었다. 더구나 (*S*)-(-) limonene을 이용하여 자란 균주 T104 또한 2,2'-DCBp를 30%까지 분해시킬 수 있음을 확인하였다. 따라서 본 연구는 자연에 널리 존재하는 테르핀이 PCB 분해균의 성장기질로서 사용되어 그 분해유전자가 유도되며 그 결과 PCB의 congener를 기질 특이적으로 분해할 수 있음을 보여주었다. 이것은 기존의 순수 PCBs 분해 균주의 resting cell assay를 통한 *meta* ring fission 과 비교해 볼 때 환경시료(부산항 저질; 자료 제시하지 않음)에서 농화배양으로 분리한 균주의 성장기질로서, 유도체로서 사용됨을 실험으로 확인할 수 있었고 이 연구에서 설정한 가설이 틀리지 않았음을 증명할 수 있었다.

Key words: 다염소화비페닐(polychlorinated biphenyls; PCBs), 생물학적 정화기술, 테르핀, 토양 microcosms, 계면활성제

Ⅰ. INTRODUCTION

PCBs (polychlorinated biphenyls), once a very useful industrial chemical, are now strictly regulated because of their potential toxicity (*e.g.*, mutagenicity and endocrine disruption). They are mostly recalcitrant and persistent in soil and sediments due to their hydrophobicity. Although decontamination may be possible through physicochemical treatment technology (*e.g.*, combustion and photolysis), these techniques are either risky, as they generate other waste products, or are costly. However, bioremediation technologies utilizing PCB degraders, hold great promise for an inexpensive means by which sites polluted with PCBs can be decontaminated. Anaerobic degradation of PCB occurs through dechlorination of highly chlorinated congeners to less chlorinated ones [18]. Then occurs aerobic degradation of less chlorinated congeners to chlorobenzoates. Biphenyl has been conventionally been used as a growth substrate (C-source) with which to isolate and grow bacteria that degrade PCB congeners [10], and to enhance their biodegradation in soil [27] and sediments [32]; this despite the fact that it is not a normal constituent of soil [33]. Unfortunately, use of biphenyl as a soil amendment is not possible given its adverse health effects [42], cost [57], and low water solubility.

Therefore, alternative natural substrates, with structural similarities to biphenyl that exist in nature, should be sought for the purpose of PCB decontamination [25, 26]. Search for a soluble and nontoxic inducer in PCB bioremediation has led to the hypothesis that plant terpenes may be the

"natural" substrates for biphenyl biodegradation enzymes, or for their ancestors, since biphenyl is not naturally abundant [33]. Furthermore, structural similarities exist between plant terpenes and biphenyl (Fig. 1). Plant flavonoids equal to biphenyl in promoting metabolism of PCBs have also been found [22]. Recently, it has been speculated that certain plant compounds, or root exudates, may serve as natural substrates for induction of the *bph* genes, including flavonoids [24], lignin [28, 35], and terpenoids [25, 26]. Carvone, a chemical component of spearmint, may successfully induce the PCB degradative pathway in the Gram-positive bacteria, *Arthrobacter* sp. B1B [30]. L-carvone and other *p*-menthene structure motif compounds (limonene, *p*-cymene and isoprene) induced *Arthrobacter* sp. B1B to cometabolize Aroclor 1242 (Fig. 1) [30]. Limonene is one of the most abundant terpenes found on earth and produced by more than 300 plants [19].

In this study we attempted to examine how the PCB degradative pathway can be induced by using plant terpenes (cymene, limonene and terpinene, etc.) as a sole carbon source, and to elucidate how these terpenes can affect the degradation of PCB congeners with different substrate specificities.

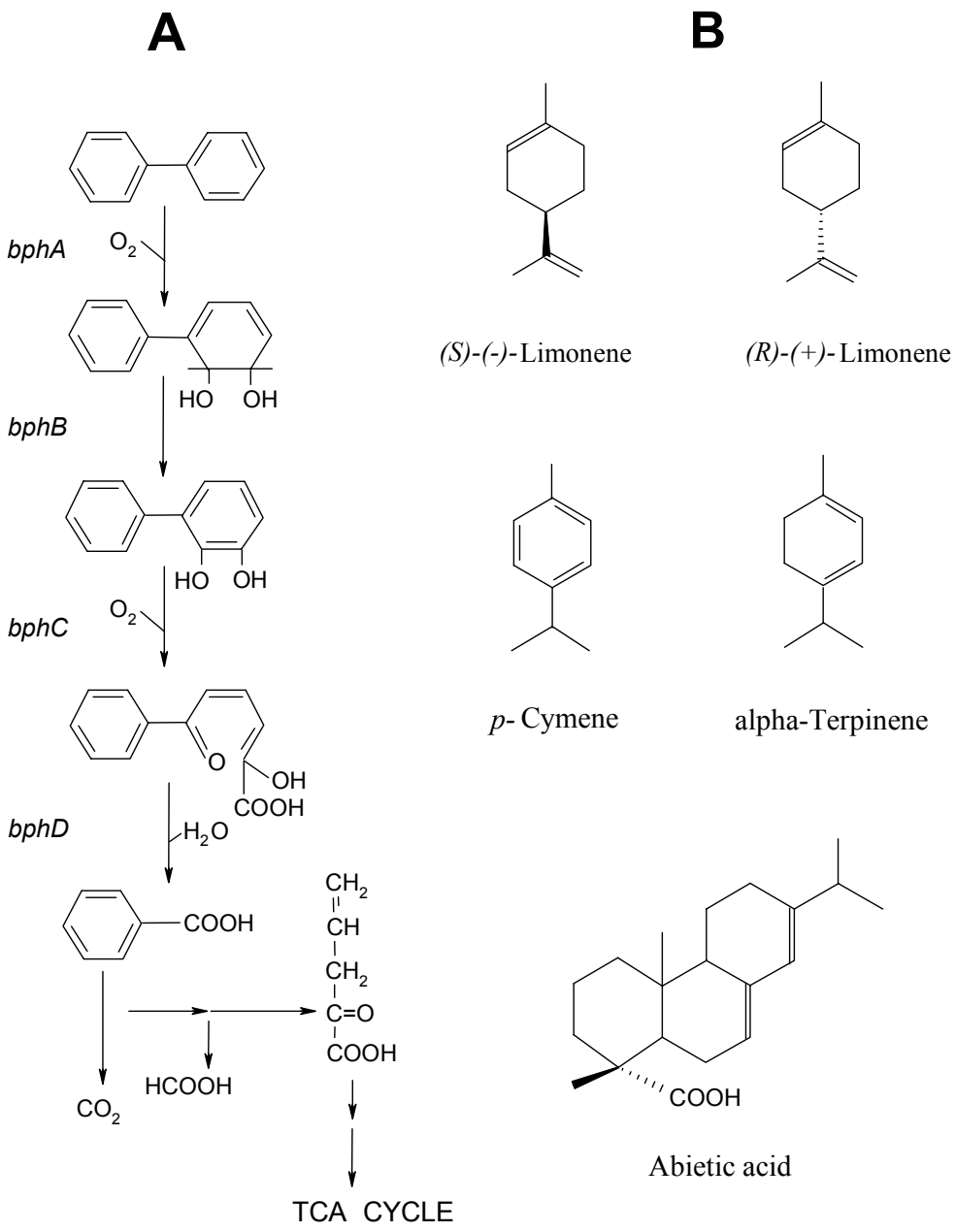


Figure 1. Bacterial metabolic pathway of biphenyl (A)[15] and structural similarity of various terpenes and terpenoids to biphenyl (B).

Ⅱ. LITERATURE REVIEW

Polychlorinated biphenyls (PCBs) are synthesized by direct chlorination of biphenyl, resulting in 209 possible congeners. The physical and chemical nature of PCBs, including lipophilicity, heat resistance, and relative inertness, has resulted in their widespread industrial application. The nature of PCBs has also led to their persistence and bioaccumulation and has caused health problems in contaminated organisms [14, 37]. As a result, the production of most PCBs was banned in the United States in 1976 and in Canada in 1977. However, several locations, including areas in the Canadian Arctic, remain polluted with PCBs. Canadian environmental legislation and aboriginal land settlement agreements require remediation of many of these Arctic sites. Conventional clean-up strategies at such remote locations are very expensive because excavation and transportation of polluted soil to treatment facilities are generally required. Bioremediation of PCBs, then, may be the most cost-effective strategy, since it allows on-site treatment [43].

PCBs (polychlorinated biphenyls) are mixtures of synthetic organic chemicals. Different mixtures can take on forms ranging from oily liquids to waxy solids. Although their chemical properties vary widely, different mixtures can have many common components (Table 2). Table 1-1 shows the overlapping composition of some commercially manufactured mixtures. Because of their inflammability, chemical stability, and insulating properties, commercial PCB mixtures had been used in many industrial applications, especially in capacitors, transformers, and other electrical equipment. These

chemical properties, however, also contribute to the persistence of PCBs after they are released into the environment. Because of evidence that PCBs persist in the environment and cause harmful effects, domestic manufacture of commercial mixtures was stopped in 1977; existing PCBs, however, continue in use. Table 1-2 shows some commercial mixtures as a percentage of domestic production.

Table 1-1. Typical composition (%) of some PCB mixture [6, 61].

	Aroclor					Clophen		Kanechlor		
	1016	1242	1248	1254	1260	A30	A60	300	400	500
Mono-CBs	2	1	-	-	-	-	-	-	-	-
DI-CBs	19	13	1	-	-	20	-	17	3	-
Tri-CBs	57	45	21	1	-	52	-	60	33	5
Tetra-CBs	22	31	49	15	-	22	1	23	44	25
Penta-CBs	-	10	27	53	12	3	16	1	16	55
Hexa-CBs	-	-	2	25	42	1	51	-	5	13
Hepta-CBs	-	-	-	4	35	-	28	-	-	-
Octa-CBs	-	-	-	-	7	-	4	-	-	-
Nona-CBs	-	-	-	-	1	-	-	-	-	-
Deca-CBs	-	-	-	-	-	-	-	-	-	-

Colums may not tatal 100 % due to rounding "-" signifies less than 1 %.
Lot-to-lot variability exists but has not been quantified.
Impurties Include chlorinated dibenzofurans and naphthalenes; see World Health Organization (WHO)(1993) for sample concentrations.
Sources: Adapted from Silterhorn et al. (1990). ATSDR (1995)

Table 1-2. Domestic production (%) of commercial PCB mixture [18].

Mixture	Percent of production
Aroclor 1016	13
Aroclor 1221	1
Aroclor 1232	<1
Aroclor 1242	52
Aroclor 1248	7
Aroclor 1254	16
Aroclor 1260	11
Aroclor 1262	1
Aroclor 1268	<1

Column does not tatal 100 % due to rounding.
Source: Adapted from Brown (1994).

In the environment, PCBs also occur as mixtures of congeners, but their composition differs from the commercial mixtures. This is because after release into the environment, the composition of PCB mixtures changes over time, through partitioning, chemical transformation, and preferential bioaccumulation.

Partitioning refers to processes by which different fractions of a mixture separate into air, water, sediment, and soil. PCBs adsorb to organic materials, sediments, and soils; adsorption tends to increase with chlorine content of the PCBs and organic content of the other material [20]. PCBs can volatilize or disperse as aerosols, providing an effective means of transport in the environment [20]. Congeners with low chlorine content tend to be more volatile and also more soluble in water [20]. Vaporization rates and water solubility of different Aroclors and individual congeners vary over several orders of magnitude (Table 2) [23, 36].

Table 2. Properties of some chlorobiphenyls [9, 31].

IUPAC number	Substitution Pattern	Molecular Weight	Melting Point	Water solubility (㎛ol/l)	GLC Relative Retention Time (OCN = 1.0)
1	2	189	34	23.4	0.1554
4	2,2'	223	60	3.5	0.2245
15	4,4'	223	148	0.2	0.3387
18	2,5,2,'	258	45	0.2	0.3378
28	2,4,4'	258	57	0.3	0.4031
31	2,5,4'	258	67	0.4	0.4024
37	3,4,4'	258	87		0.4858
44	2,3,2',5'	292	48	0.6	0.4832
52	2,5,2',5'	292	88	0.052	0.4557
70	2,5,3',4'	292	104	0.056	0.5407
77	3,4,3',4'	292	178	0.039	0.6295
95	2,3,6,2',5'	326	99		0.5464
101	2,4,5,2',5'	326	77	0.013	0.5816
110	2,3,6,3',4'	326	oil		0.6314
118	2,4,5,3',4'	326	109		0.6693
126	3,4,5,3',4'	326	154		0.7512
128	2,3,4,2',3',4'	361	150	0.0012	0.7761
136	2,3,6,2',3',6'	361	114	0.017	0.6257
153	2,4,5,2',4',5'	361	103	0.0033	0.7036
194	2,3,4,5,2',3',5',5'	430	156	0.00029	0.9620

2.1. PCBs (polychlorinated biphenyls) in the Environment and Their Problems

PCBs have significant ecological and human health effects other than cancer, including neurotoxicity, reproductive and developmental toxicity, immune system suppression, liver damage, skin irritation, and endocrine disruption. Toxic effects have been observed from acute and chronic exposures to PCB mixtures with varying chlorine content.

PCBs can accumulate selectively in living organisms. PCBs are highly soluble in lipids and are absorbed by fish and other animals. Rates of metabolism and elimination are slow and vary by congener [44]. Bioaccumulation through the food chain tends to concentrate congeners of higher chlorine content, producing residues that are considerably different from the original Aroclors [41, 52, 60]. PCB residues in fish and turtles, changed through environmental or metabolic alteration, could not be characterized by Aroclor 1242, 1248, 1254, or 1260 standards [60]. Congener distributions in several species, including humans, do not resemble any Aroclor [46]. Because, in general, some toxic congeners are preferentially retained, bioaccumulated PCBs appear to be more toxic than commercial PCBs [7].

PCBs are widespread in the environment, and humans are exposed through multiple pathways. Levels in air, water, sediment, soil, and foods vary over several orders of magnitude, often depending on proximity to a source of release into the environment [5, 64]. Average daily intake by humans via ambient air is about 100 ng, and about an order of magnitude higher if indoor concentrations are considered [5]. Average daily intake via

drinking water is less than 200 ng [5]. Estimates of average daily intake via diet vary widely depending on geographic area, food habits, and sampling methodology; 5-15 μg is considered a good estimate of average daily intake via diet in industrialized countries [64].

2.2 PCB Biodegradation

Biodegradation transforms the chemical composition of PCB mixtures in the environment. Anaerobic bacteria in sediments selectively remove chlorines from meta and para positions, appearing to reduce the toxicity and bioaccumulation potential of residues; the occurrence and extent of these dechlorinations can be limited by sediment PCB concentrations [1, 16, 40]. (Dechlorination is not synonymous with detoxication, as congeners having carcinogenic activity can be formed through dechlorination.) Anaerobic degradation: dechlorination of highly chlorinated congeners to less chlorinated ones (Fig. 2) [18].

Aerobic bacteria remove chlorines from PCBs with low chlorine content (1-4 chlorines) and break open the carbon rings through oxidation [1]. PCBs with higher chlorine content are extremely resistant to oxidation and hydrolysis [20]. Photolysis can slowly break down congeners with high chlorine content [20]. Overall, dechlorination processes are slow and altered PCB mixtures can persist in the environment for many years. Aerobic degradation: less chlorinated congeners to chlorobenzoates; fortuitously effected by four sequential steps encoded by the *bphABCD* genes of biphenyl degrading bacteria (Figure 1A) [25, 26].

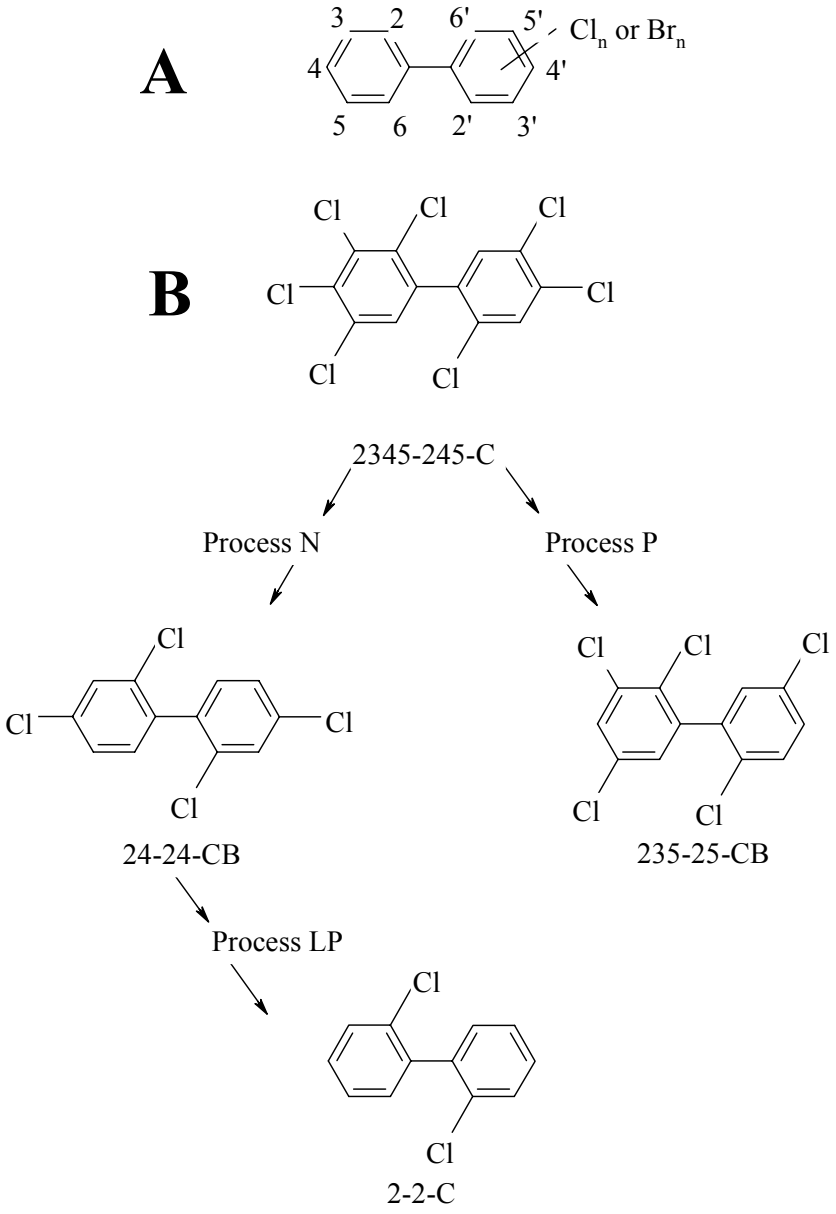


Figure 2. Structure of halogenated biphenyls, showing the numbering scheme (A) and the dechlorination of a major heptachlorobiphenyl by Process N, Process N plus LP, and Process P (B)[12].

2.3. Biphenyl and Natural Substrates as Growth Substrates in PCB Biodegradation

Although the rate of metabolism is slow [49], PCBs may be converted by hepatic enzymes to hydroxylated metabolites. The relative rates of conversion are dependent on the number and placement of the chlorine atoms present.

PCBs with fewer chlorines and with adjacent, unsubstituted carbon atoms are more readily susceptible to metabolic attack. Cytochrome P-450 isozymes may catalyze these hydroxylation reactions via an electrophilic arene oxide intermediate or via direct insertion mechanisms. Evidence for the intermediacy of arene oxides during PCB metabolism is found in the identification of (1) NIH-shift products, (2) dihydrodiol metabolites, (3) mercapturic acid products, and (4) sulfone metabolites [61]. PCB metabolites with multiple hydroxyl groups also have been identified in animals and in microsomal incubations [47]. Dihydroxy metabolites may be oxidized in vitro to *o*- or *p*-quinones by peroxidases. *In vitro* studies have demonstrated that adducts of PCBs and nucleotides (dGp and dAp) or exogenous DNA may be formed during the hydroxylation step (from electrophilic arene oxides) and during the peroxidase-catalyzed oxidation of PCB catechol and hydroquinone metabolites to the respective *o*- and *p*-quinones [48]. Hydroxylated PCB metabolites may have estrogenic activity [35].

2.4. Plant Terpenes and Their Relevance to PCB Biodegradation

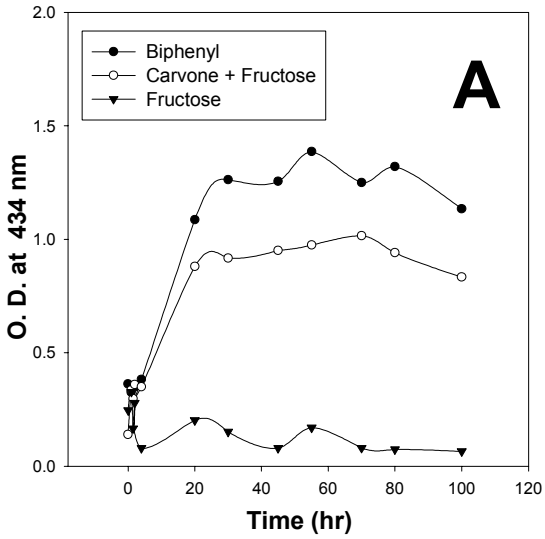
Monoterpenes are branched-chain C_{10} hydrocarbons composed of two isoprene units and are widely distributed in nature with more than 400 naturally occuring monoterpenes identified [21]. *R*-(+)-limonene, *S*-(+)-carvone (caraway-like flavor) and *R*-(-)-carvone (spearmint-like flavor) are important aroma compounds used in foods and beverages. Resin acids, including dehydroabietic acid (DhA), are diterpenoids which are a minor component of wood. All taken, terpenes hold potential practical applications not only in the pharmaceutical and chemical industries but also in the fragrance and flavor industries [38].

An extensive screening of bacteria able to grow on carvone or limonene as the sole carbon source yielded many Gram-positive strains from environmental sources including freshwater sediments, and some of these were able to utilize limonene, carvone and their derivatives. Seven strains of the class Proteobacteria utilizing dehydroabietic acid were able to use a mixture of 12- and 14-chlorodehydroabietic acid (Cl-DhA) [50]. Consistent with the ability of certain bacteria to use DhA as the sole organic substrate, a report indicates that aerobic biological treatment systems are capable of effectively removing resin acids [50]. Recently, it was suggested that monoterpenes play an important role in altering nitrogen and carbon cycling in forest soils [63].

Search for an available and nontoxic inducer in PCB bioremediation has led to the hypothesis that plant terpenes may be the "natural" substrates for

biphenyl biodegradation enzymes, or for their ancestors, since biphenyl is not naturally abundant [33] and structural similarities exist between plant terpenes and biphenyl (Fig. 1). Plant flavonoids may be an alternative to biphenyl in promoting metabolism of PCBs. This hypothesis leads to an interesting question of whether the diterpenoid dioxygenase would be ancestral to biphenyl dioxygenase [54]. *P. abientaniphila* BKME-9 could not grow on biphenyl as a sole organic substrate [50]. However, it might be interesting to test the possible cometabolism of DhA and biphenyl by BKME-9 or the transformation of biphenyl by the enzyme encoded by the cloned gene *ditA*. A structure-function analysis of potential inducers of PCB biodegradation by *Arthrobacter* sp. strain B1B suggested that isoprenoids were able to induce PCB degradation, with the most potent inducer being an aromatic isoprenoid (*p*-cymene) much resembling the aromatic region of the DhA molecule (Fig. 1) [50]. A recent experiment revealed that a monoterpene, carvone, could induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Ralstonia eutropha* H850 (Fig. 3) [54].

The mechanisms by which natural plant products facilitate the degradation of PCBs are not well documented. However, it may be likely that terpenes (and/or their metabolites) can induce PCB-degradative pathway. This hypothesis is based on the structural similarities between plant terpenes and biphenyl (and its metabolites). The use of plant terpenes as an inducer for PCB degradative pathway may have these advantages: good bioavailability, compatibility (nontoxicity) in natural environments, and their ubiquity in the environment.



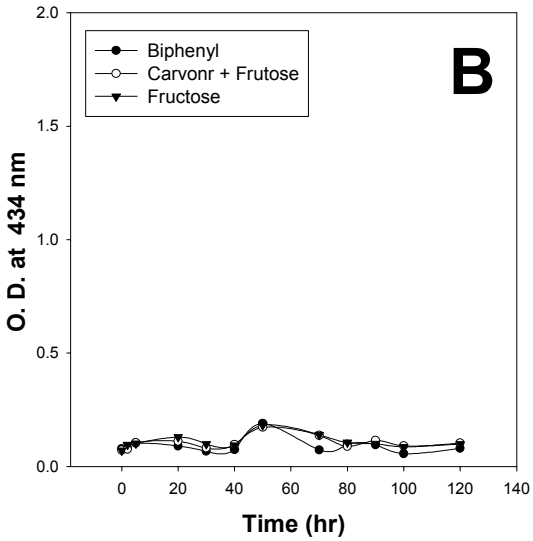


Figure 3. Time course of accumulation of *meta* ring fission product from 4,4'-dichlorobiphenyl(DCBp) by the resting cells of *Arthrobacter* sp. B1B (panel A) and *Ralstonia eutropha* H850 (panel B) grown on biphenyl, carvone+fructose, and fructose [54].

2.5. Soil Microcosms Undergoing PCB Biodegradation

2.5.1. Role of Surfactants in PCB Biodegradation

Surfactants have been successfully used to enhance the apparent solubility of nonpolar organic contaminants (NOC) as well as their subsequent removal from soil. It has been proposed that addition of surfactants to contaminated soil enhances the solubility of target compounds; however, surfactants may simultaneously reduce the adhesion of bacteria to hydrophobic surfaces. If the latter mechanism is important for the biodegradation of virtually insoluble contaminants, then the use of surfactants may not be beneficial.

Industrial chemicals, such as hydrocarbons, have been released into the soil environment as a result of mechanical failure, incineration practices, corrosion, leakage, accidental spillage, and improper disposal practices. Biodegradation is an attractive method for remediating contaminated sites because of its economic viability and environmental soundness. One limitation of biodegradation, however, is that many hydrocarbons are poorly accessible to bacteria. Heavily contaminated soils contain a separate non-aqueous-phase liquid (NAPL), which may be present as droplets or films on soil surfaces. Biodegradation takes place more readily when the target contaminants are dissolved in an aqueous solution, but many hydrocarbons are virtually insoluble in water and remain partitioned in the NAPL. Thus, there have been efforts to improve the bioavailability of hydrocarbons through the use of surfactants [55].

Surfactant-enhanced removal of residual hydrocarbon requires surfactant concentrations greater than the critical micelle concentration (CMC), which is the concentration where the surfactant molecules spontaneously aggregate into micelles or vesicles. Surfactant-enhanced flushing of contaminated soil also requires that the surfactant-mobilized NAPL be collected and treated. An alternative to flushing strategies is to promote the *in situ* biodegradation of entrapped contaminants. There have been several reports showing that biodegradation of hydrocarbons that have low solubility or that may be sorbed by soil particles can be enhanced by the addition of biosurfactants. The effect of biosurfactant addition is not yet well understood [34].

Surfactants were also found to have no negative effect on bacterial survival, as cell numbers were the same or higher after incubation without surfactants [13].

2.5.2. PCR-DGGE Fingerprinting for Analysis of Microbial Community

Application of molecular techniques to ecological studies has disclosed a wide diversity of microorganisms in natural and controlled communities, previously unknown to microbial ecologists. They have also devoted much effort to tracking biological interactions between species in the environment. The molecular techniques, including fatty acid methyl ester (FAME), phospholipid fatty acid ester (PLFA), C0t1/2 curve analysis, sequencing of gene encoding small subunit of ribosomal RNA and denaturing gradient gel electrophoresis (DGGE) have also been applied to analyze microbial communities with or without culturing microbes. Of these, DGGE is one of

the most useful fingerprinting techniques that is culture-independent and can process multiple samples at a time. Virtually, culture-independent methods have revealed more complexity in the microbial populations of particular ecosystems than culture-based methods. Mostly these techniques are real-time based, sensitive, rapid, and effective.

Amplification of total DNA mixture from microbial communities using primers specific for 16S rRNA gene fragments of bacteria generates mixtures of PCR products. These mixtures have different base sequences so that they have different melting temperatures in the polyacrylamide gel containing a gradient of denaturants, typically a mixture of urea and foramide. The amplified DNA products first enter as double-stranded molecules and later they become melted and stop at different positions within the gel where different denaturant concentrations are met (Figure 4).

Numerous studies in microbial ecology have used PCR-DGGE fingerprinting for the analysis of microbial community composition up to now. It has been shown by several studies that the approach is reproducible and sensitive. These beneficial features as well as the simpleness of DGGE techniques will surely attract even more scientists to adapt this relatively inexpensive technique as a new tool in analyzing various unexplored microbial communities in the future. The limitations of the techniques can be overcome by employing conventional microbiological methodologies, compensating molecular techniques, and the microbial genomics techniques including DNA microarray and differential display techniques.

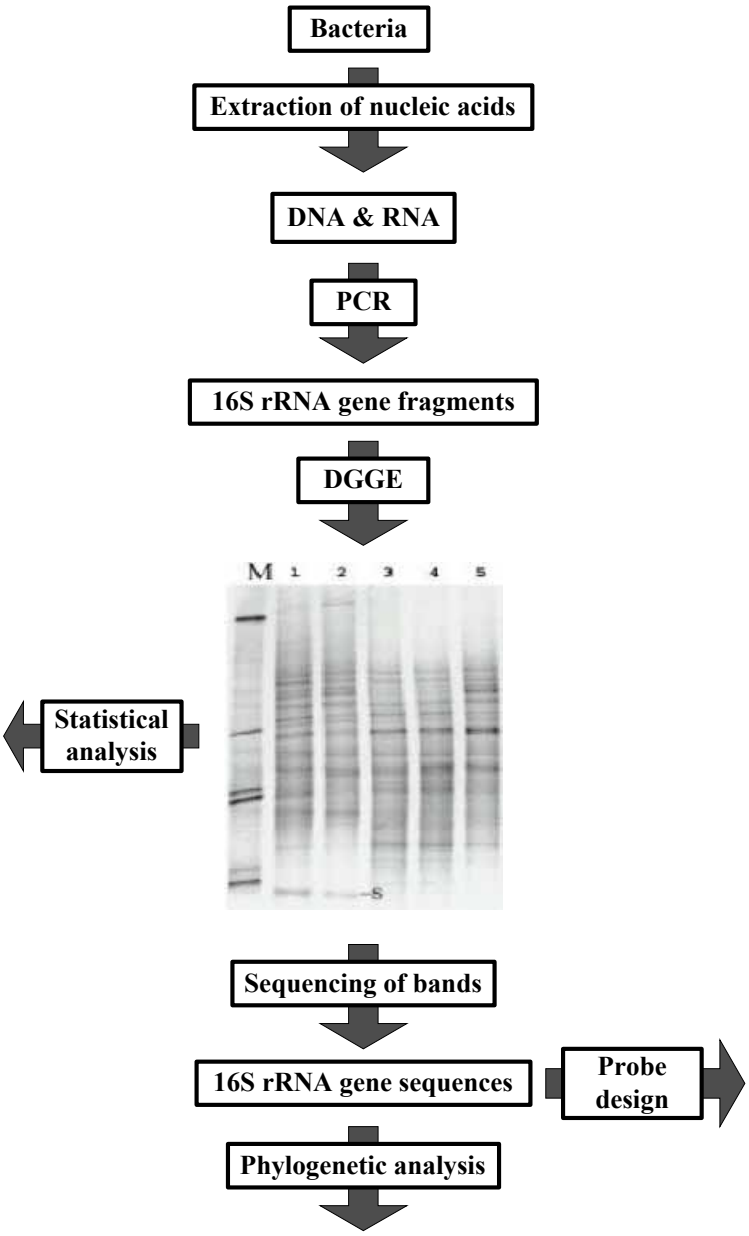


Figure 4. Flow diagram of PCR-DGGE analysis of microbial communities [58].

Ⅲ. MATERIAL AND METHODS

3.1. Chemicals.

(*S*)-(-)-limonene, *p*-cymene, α-pinene, α-terpinene were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). PCBs, including 4,4'-dichlorobiphenyl (4,4'-DCBp), 2,2'-dichlorobiphenyl (2,2'-DCBp) and 2,2'3,3',5,5'- hexachlorobiphenyl (2,2'3,3',5,5'-HCBp), were obtained from Accustandard, Inc. (New Haven, Conn.). Biphenyl and 3-methylcatecol were purchased from Junsei (Tokyo, Japan). All solvents were reagent grade or better.

3.2. Bacterial Strains, Culture Maintenance and Growth Conditions

PCB degraders used in this study were *Pseudomonas* sp. P166 [3], *Arthrobacter* sp. B1B [30], *Cellulomona*s sp. T109 and *Rhodococcus* sp. T104 [33]. The other PCB degraders were isolated from lawnground soil, sediments and a sediment landfill site. Other isolates tested were enriched from environments such as turf soil and coastal sediments. They were grown on mineral salts medium (MSM) containing biphenyl or terpenoid as a sole carbon source. All PCB-degraders were maintained on mineral salts agar using inverted Petri plates with biphenyl crystals placed on the lid. The (MSM) [45] consisted of 10 mM K_2HPO_4, 5 mM $(NH_4)_2SO_4$, 3 mM NaH_2PO_4, 1 mM $MgSO_4$, and 10 mL of chloride-free trace element stock

solution, which contained the following (in milligrams per liter): $CaSO_4$, 200; $FeSO_4 \cdot 7H_2O$, 200; $MnSO_4 \cdot H_2O$, 20; $NaMoO_4 \cdot 2H_2O$, 10; $CuSO_4$, 20; $CoSO_4 \cdot 7H_2O$, 10; and H_3BO_3, 5. The cultures were grown in shaking flasks containing liquid medium (300 ppm biphenyl or terpenoids included) on a rotary shaker at 200 rpm at 26℃.

3.3 Nucleic Acid Extraction From pure cultures

3.3.1 Total DNA extraction

Direct DNA extractions were performed by cell disruption through a modification of the method described by Rainer et al. (1993) [56]. PCBs degraders, 1.5-ml portions of cultures in liquid minimal medium were centrifuged at 13,000 rpm for 5min. And the pellet was washed with in 1.0 ml washing buffer (0.12M Na-P buffer, pH 8.0). The suspension was then centrifuged at 13,000 rpm for 3 min, and the precipitate was resuspended in 0.6 ml of a cell-suspending buffer (0.15 M NaCl, 0.1 mM EDTA, and 1% [wt/vol] lysozyme). After incubation for 3h at 37℃, 10 % [wt/vol] sodium dodecyl sulfate (SDS) was added, and the suspension was incubated at 65℃ for an additional 10 min. The suspension was then incubated for 10 min on ice (-70℃). The freeze and thawing procedures were repeated twice. Next, the mixture was extracted with phenol-chloroform three times and with chloroform once, vortexing for 10 s by inversion, and centrifuging as before. The sample was centrifuged at 12,000 rpm at 4℃ for 15 min. The aqueous layer was collected in to another tube, reextracted with an equal volume of

chloroform-isoamyl alcohol (25:1) and centrifuged to separate the phases, and equal volumes of absolute ethanol (100 %) was added, and after gently mixing, the solution was incubated at -70℃ for 30 min. Nucleic acids were precipitated by centrifugation at 13,000 rpm for 15 min, and, after the preparation was washed with 1 ml of an 70 % ethanol solution. And the pellet to air dried. Finally, the dried pellets of the samples were resuspended in 50 ㎕ TE. The alcohol precipitated DNA was resuspended in TE buffer containing 10 ㎍ per ml of RNase A and incubated at 37℃ for 2 hr to remove residual RNA. The DNA perparations were used for the PCR assays.

3.3.2 RNA extraction

Direct RNA Extractions were performed by cell disruption by a modification of the method described by Ausubel (1989) [8]. The samples were collected at liquids culture (3-ml). After centrifugation at 13,000 rpm for 3min, the pellets was washed with ice-cold AE (20 mM sodium acetate [pH 5.5], 1 mM EDTA) and resuspended in 250 ㎕ of AE. Added 30 ㎕ 10 % SDS, 220 ㎕ AE, 1 ㎖ phenol and incubation at 60℃ shaken for 15 min by every 5min. The emulsion chilled 4 to 0℃ for 15 min. Centrifuged at 13,000 rpm at 4℃ for 5 min, and transferred upper phase to a clean tube. Sodium acetate (3 M, pH 5.2) was added up to the 0.25M. The aqueous layer extracted three times with phenol-chloroform mixture. An equal volume of phenol-chloroform (1:1, vol/vol), equilibrated with pH 5.1 buffer, was added, and the sample was mixed vigorously. The sample was centrifuged at 12,000 rpm at 4℃ for 15 min. The aqueous layer was

removed to another tube, reextracted with an equal volume of chloroform-isoamyl alcohol (25:1) and centrifuged to separate the phases, and RNA present in the aqueous layer was precipitated for 30 min at -70℃ with 2 volumes of absolute ethanol (100 %). The precipitated RNA was pelleted at 12,000 rpm at 4℃ for 15 min. The supernatant was removed, and 1 ml of 70% ethanol at 20℃ was washed along the side of the tube, which was then centrifuged again at 12,000 rpm at 4℃ for 30 sec. The supernatant was removed, and the tube was inverted and allowed to air dried. The RNA samples were combined and digested at 37℃ for 2 h with 100 U of RNase-free DNase I (Ambion, Inc., Austin, Tex.) in 50 ㎕ of TM buffer (40 mM Tris-HCl [pH 7.5], 6 mM $MgCl_2$) and then mixed by gently pipetting up and down. DNase-digested RNA samples were extracted with an equal volume of phenol-chloroform and centrifuged at room temperature for 15 min to separate the phases, and the aqueous layer was reserved. RNA in the aqulous phase was precipitated by the addition of 10 M sodium acetate (final concentration, approximately 0.25 M), 2 volumes of absolute ethanol, and 10 ㎍ of glycogen. The RNA pellets were washed with 70% ethanol and dried on air. Finally, the dried pellets of RNA sample were respended in TE buffer (10 mM Tris HCl, 1 mM EDTA [pH 7.5]) and pooled to give a final volume of 50 ㎕.

3.4. Utilization Test of Terpenes

The experiment was performed following a protocol that was modified from Gilbert and Crowley [30]. Each culture was inoculated into an Erlenmeyer flask containing 100 ml of MSM with (*S*)-(-)-limonene, *p*-cymene, α-pinene, α-terpinene, terpinene or abietic acid (Sigma-Aldrich; 300 ppm) as a sole carbon source, and then incubated at 26℃ at 200 rpm. Cultural growth was monitored spectrophotometrically at 525 nm (Jasco, Model V-550, Tokyo, Japan).

3.5. Induction of PCB-degradative Pathway by Terpenes

Unless stated otherwise, induction of the PCB-degradative pathway was performed by growing on MSM containing various terpenoids as a sole carbon source. The representative PCB degraders tested were *Pseudomonas* sp. P166, *Arthrobacter* sp. B1B and *Rhodococcus* sp. T104. MSM containing biphenyl (500 ppm) and fructose (or succinate; 0.1 %) were used as positive and negative controls, respectively.

3.6. Resting Cell Assay of PCB Cometabolism

Biodegradation was monitored by resting cell assay [30] using 4,4'-dichlorbiphenyl or 2,2'-DCBp as a substrate. Ring fission products for each substrate was monitored using the supernatants at 434 nm (4,4'-DCBp) and 391 nm (2,2'-DCBp), respectively. In order to assay ring fission activity,

cells were grown until the late-log phase, filtered through the glass wool to trap substrate residues, were washed twice, and resuspended in a 50 mM phosphate buffer. The resting cell suspensions (10 ml; adjusted to 1.5 at 525 nm) were aliquoted into 100 ml of Erlenmeyer flasks, with the stock solution, 4,4'-dichlorobiphenyl or 2,2'-dichlorbiphenyl (Allied Signal, Inc., Seelze, Germany) prepared in hexane, added to give a final concentration of 100 μM.

3.7. Analysis of PCB Biodegradation

3.7.1 Extraction of PCBs

Extraction were performed 12 ml vials equipped with screw caps. PCBs were added through a hexane solution, and the solvent was evaporated in a fume hood to give the desired concentration (10 to 100 mg · liter^{-1}). 4 ml of the washed cell suspension was added to the vial and incubated on a platform shaker. The cap was be loosely secured to permit adequate aeration. Because PCBs also could adsorb to glassware and to cells, it was necessary to run a heat-killed control for each culture. Comparison between each culture and its control ensured that a biological degradation occurred. At the end of the incubation, an internal standard was added to the flask in a hexane solution to give a concentration about 1/50 of the initial concentration of PCBs, which would be equivalent to a single congener. This congener (e.g., 2,2',3,3'5,5'- hexachlorobiphenyl) should be one that was not present in Aroclor 1242 or 1254 and was clearly separable from other

congeners in the gas chromatographic analysis. Retention times and characteristics of the congeners of commercial Aroclors could be found elsewhere [35, 51]. After resting cell assay, internal standard, 0.1 ml of 1.5M H_3PO_4, Triton X-100 (1 % final mixture), and equivalent volume of hexane 4ml were added to each sample (4-ml), which was agitated on a shaking plateform for 1hr. Na_2SO_4 is added in excess of its solubility to reduce emulsion. The hexane phase was removed to a vial and remaining aqueous phase was extracted with 4 ml of hexane and shaken for 15 min. The hexane layer was dried over anhydrous Na_2SO_4 and drawn off into a 10-ml volumetric flask. Extraction of aqueous phase was performed two more times, and the volumetric flask was finally topped off with hexane [26].

3.7.2 GC Analysis of PCB Degradation

PCB degradation was confirmed by GC analysis. After the resting cell assay, a PCB extraction was made using Triton X-100 (1% of final mixture) and an equivalent volume of hexane [26]. The extracts were analyzed on a Hewlett Packard 5890 Series Ⅱ gas chromatograph using an automatic sampler, an electron capture detector, and DB-5 capillary column (30m × 0.32 mm ID) [33]. The temperature program ranged from 100℃ to 252℃ with the following ramping conditions: initial temperature 100℃, 9℃ min-1 to 160℃, 3.0℃ min^{-1} to 252℃. The internal standard, 2,2',3,3',5,5'-hexa-chlorobiphenyl (retention time 31 min), was added prior to extraction.

3.7.3 Analysis of Chloride Ions Released from 4,4'-DCBp Degraded

Each culture inducing conditions were centrifuged at 5,000 rpm and the resulting supernatants were subjected to quantitation of chloride ions released, through the degradation of 4.4' DCBp using Dioxenx IC

3.8. Colorimetric Enzymes Assay (agar plates)

Colonies from the highest sample dilutions from biphenyl-enriched microcosms were assayed for the activity of the 2,3-dihydroxybiphenyl dioxygenase enzyme. To this end, plates were sprayed with an aqueous solution containing 3-methylcatechol. 0.1% (wt/vol) dihydroxybiphenyl and 10% (vol/vol) acetone. Appearance of the yellow metabolite 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid, which is formed by *meta* cleavage of 2,3-dihydroxybiphenyl, indicated that 2,3-dihydroxybiphenyl dioxygenase was present and was observed within several minutes. As many single yellow colonies as possible were picked. Isolates were purified by several transfers to MS medium plates. If two or more morphological types of colonies were visible, both were subsequently analyzed.

Purified strains were tested for their ability to degrade biphenyl by (i) growing the organisms in liquid minimal medium containing biphenyl as the sole source of carbon and energy and (ii) assaying for the presence of 2,3-dihydroxybiphenyl dioxygenase.

3.9. Soil Microcosm Experiment

3.9.1 Soil Microcosms Set up

Soil microcosms were set up by treating a grassland soil with biphenyl and terpenoids ((*S*)-(-)-limonene, succinate, *p*-cymene and α-terpinene), and then by inoculating *Ralstonia eutropha* H850 and *Rhodococcus sp*. T104. The microcosms were then incubated at room temperature under a dark condition. One g of soil was taken periodically for analyses of the degraders population density and gene expression using viable count and PCR techniques. The viable cells of PCBs degrader were maintained at least 10E8 (cells/g fresh soil) at all treating conditions for at least two months. The treatment of surfactant as a control was also shown to maintain the degraders population at the similar level. The correlation between the population density of T104 and its the PCB degradation activity monitored by GC analysis and RT-PCR was investigated [33].

Table 3. Scheme of Soil Microcosms (1st trial)

Sample	Amount	Replicate*	Inducer	Inoculum** (10^8 cfu/㎖)
TR-1	**200**	**2**	**biphenyl (500 ppm)**	**20 ㎖**
TR-2	**200**	**2**	**only succinate (0.1%;0.2)**	**20 ㎖**
TR-3	**200**	**2**	**carvone (100 ppm;20㎕) + succinate (0.1%)**	**20 ㎖**
TR-4	**200**	**2**	**limonene (100 ppm;20㎕) + succinate (0.1%)**	**20 ㎖**
TR-5	**200**	**2**	**Orange peel (5g /soil 100g) + succinate (0.1%)**	**20 ㎖**

** Liquid culture of *Ralstonia eutropha* H850 in log phase

Table 4. Scheme of Soil Microcosms (2nd trial)

Sample	Amount	Replicate*	Inducer	Inoculum** (10^8 cfu/㎖)
TR-1	200	2	surfactant	20 ㎖
TR-2	200	2	biphenyl (400ppm;80㎎;2.6×10^3㎛) + surfactant	20 ㎖
TR-3	200	2	only succinate (0.1%;0.2)	20 ㎖
TR-4	200	2	limonene (2.6×10^3㎛;354.2 ppm) + surfactant	20 ㎖
TR-5	200	2	cymene (2.6×10^3㎛;348.9 ppm) + surfactant	20 ㎖
TR-6	200	2	Terpinene (2.6×10^3㎛;354.2 ppm) + surfactant	20 ㎖
TR-7	200	2	Citrus extract + surfactant	20 ㎖

* Cells of *Rhodococcus* sp. T104 in early stationary phase (about 10E8/ml).

3.9.2 Viable Counting of PCB Degraders

(1) Measurement of Population Density of *Ralstonia eutropha* H850

The total number of cultivable bacteria in the soil were determined by the plate count method by using mineral sodium medium. Biphenyl-degrading microorganisms were selectively enumerated on mineral salts medium agar plates. Biphenyl crystals were placed in the lid of each petri-dish which was upside down. To enumerate bacteria, 1-ml aliquots of the slurries were

serially diluted in phosphates buffer (final concentration of 10 mM K_2HPO_4, 3 mM $NaHPO_4$), and two appropriate dilutions of each sample were plated in duplicate. All of the plates were incubated at 28℃ for 1 week (minimal medium).

(2) Measurement of Population Density of *Rhodococcus* sp. T104

Soil samples diluted in P-buffer were plated onto MSM agar and incubated 26℃ for one week before observation of colonies [33].

3.9.3 Total Soil DNA Extraction and PCR

(1) Total soil DNA, RNA extraction

Total bacterial community DNA was extracted to assess the effect of PCBs on bacterial community diversity. Direct DNA Extractions were performed by cell disruption by a modification of the method described by Rainer et al. (1993) [56]. The samples were collected at liquids culture (3ml). After centrifugation for 3min (13,000 rpm), discard the supernatants. And 1.0 ml washing buffer (0.12M Na-P buffer, pH 8.0) to the pellet (cell). After this procedure, centrifuge at 13,000 rpm for 3 min, and discard the supernatant. The pellets were dissolved 5 M NaCl, and 0.5 M EDTA, and supplemented with lysozyme (final concentration should be approximately 0.15 M NaCl, 0.1 M EDTA, and 1% lysozyme) up to 0.6 ml. And place the mixture in a boiling water bath at 37℃ for 3 h and incubated at 37℃ for 3 h. The resulting suspensions were mixed with 60 ㎕ of 10 % SDS solution, and incubated at 65℃ for 10 min ; keep on ice (-70℃) for 10 min. The freeze and thawing procedures were repeated twice. The mixture

was then extracted with phenol-chloroform three times and with chloroform once. DNA was extracted with equal volumes of Phenol and centrifuged at 12,000 rpm for 15 min. The upper phase was transferred to a clean tube, and discard lower phase (twice). Both resulting aqueous phases were further extracted with equal volume of chloroform-isoamyl alcohol (24:1) to the upper phase and mixed. DNA was precipitated for 30 min at -70℃ with 2 volumes of ice-cold 100 % ethanol. The precipitated pellets were to air-dried. Finally, the dried pellets of the samples were resuspended in 50 ㎕ TE. The alcohol precipitated DNA was resuspended in TE buffer containing RNase A and incubated at 37℃ for 2 h to remove residual RNA. The DNA perparations were applied for PCRs.

RNA extraction was carried out by a modification of the method of Orgam (1999) [53] and incorporated procedures from the methods of Ausubel (1989) [8]. Chemicals and reagents were obtained from Sigma (St. Louis, Mo) expect where otherwise noted and were of molecular biology grade.

Seven different samples were evaluated by using two procedures (Table 3). These procedures were designed to combine bead mill homogenization [53] and hot phenol [8] extraction for RNA extraction. Duplicate 500-mg samples of the turf soils were added to 2-ml screw-cap plastic vials containing 0.1 mm diameter zirconium/silica beads (BioSpec Product, Bartlesville, OK).

Pretreatment of the soil samples were carried out according to bead-mill/SDS lysis method. After pretreatment, extraction of total RNA were performed by hot-phenol method.

3.10. Primers Design

(1) *bphC* primers design

Primers were designed from the conserved regions of *bphC* gene of *Arthrobacter sp*. M5. The protein sequence alignment and analysis were accomplished using the sequence databases of Gene Bank and the Blast sequence analysis protocol available at National Center for Biotechnology Information (National Institute of Health).

- *bphC2* : 5'-CTG-CAC-TGC-AAC-GAA-CGC-CAC-3'
- *bphC3* : 5'-GAC-ACC-ATG-TGG-TGG-TGG-GT-3'

(2) *bphA* primers design

Primers were designed from conserved regions of *bphA* gene from both *Pseudomonas pseudoalcaligenes* KF707 and *Rhodococcus psedoalcaligenes* RHA1.

- *bphA1-1* : 5'-AAY-CAR-AGY-AGR-CAY-AGR-3'
- *bphA1-2* : 5'-TGY-AAY-TGG-AAR-TTY-GCN-3'
- *bphA1-3* : 5'-NCC-RTC-RTC-YTG-YTC-RAA-3'

(3) 16S rRNA primers design

The variable V3 region of 16S rRNA was enzymatically amplified in the PCR with the primers from conserved regions of 16 rRNA genes. The

nucleotide sequenced of the primers were as follows :

- 314F-GC (Bacteria) :
  5'-CGC-CCG-CCG-CGC-CCC-GCG-CCC-GTC-CCG-CCG-CCC-CCG-CCC-GCC-TAC-GGG-AGG-CAG-CAG-3'
- 518R (Universal) : 5'-ATT-ACC-GCG-GCT-GCT-GCT-GG-3'

3.11. Reverse Transcription PCR (RT-PCR)

The total RNA purification for RT-PCR was performed according to the method discribed by Ausubel with a slight a modification. Cells grown by following the induction scheme (Table 3, 4) were washed once with an ice cold AE buffer (20 mM sodium acetate, pH 5.5 and 1 mM disodium EDTA) and resuspended in 250 ㎕ of AE. Then 30 ㎕ of 10 % SDS, 200 ㎕ of AE buffer and 1 ml phenol were added and then mixed. The reaction mixture was incubated at 60℃ for 15 min with a brief vortexing every 5 min. The emulsion was chilled on ice at 0~4℃ and centrifuged at 4℃ for 15 min. Aqueous phases were taken and 3 M of the sodium acetate (pH 5.2) was added up to 0.25 M. The solution was then extracted with phenol-chloroform (25:24) 3 times and then ethanol-precipitated. The dried pellet was resuspended in TM buffer (40 mM Tris-HCl, pH 7.5; 6 mM $MgCl_2$) and residual DNA was removed by DNase (Promega). Concentration of RNA was measured by spectrophotometric method.

The primers used in the PCR were designed from a conserved region of the chromosomally located *bphC* gene which encoded 2,3-dihydroxy-

biphenyl dioxygenase of *Arthrobacter* sp. M5, a Gram-positive bacterium. This region turned out to be homologous to the equivalent gene of *Pseudomonas pseudoalcaligenes* KF707. Forward and reverse primers were designed from the gene fragment (182 bp) of the N-terminal sequence, and their sequences were 5'-CTGCACTGCAACGAACGCCAC-3' (primer 1) and 5'-GACACCATGTGGTGGTTGGT-3' (primer 2), which were custom-synthesized by GenoTec, Inc. (Taejon, Korea). One to 3.3 ㎍ of the total RNA was used as a template. For a positive control, *bphC* gene, carried on pKTF18 which was cloned from *Pseudomonas pseudoalcaligenes* KF707, was also employed. Each reaction mixture (50 ㎕) contained the following reagents: 5 ㎕ of Taq DNA polymerase 10X buffer, DTT (10 mM), $MgCl_2$ (1.5 mM), dNTPs (250μM, each), RNasin (10U) (Promega), Primer 1 (310 nM), Primer 2 (310 nM), AMV-RT (15U) (Promega), and *Taq* polymerase (1.3U) (Promega). RT-PCR was performed in a DNA thermocycler (Perkin Elmer model; GeneAmp PCR System 2400). Reverse transcription (RT) and the subsequent DNA amplification were conducted in the same tube. The RT thermal condition was 65℃, 10 min for RNA denaturation and 50℃, and 8 min for reverse transcription. Also, the PCR condition was denaturation (94, 5 min), 25 cycles of the standard PCR (94℃, 1min; 60℃, 30 sec, 72℃, 30 sec), and a final chase reaction of (72℃, 7 min).

3.12. PCR-DGGE for the analysis of microbial community

Total bacterial community DNA was extracted to assess the effects of PCBs on bacterial community diversity. DNA was performed by cell disruption by a modification of the method described by Rainer et al (1993) [56].

PCR was performed using 100 ng of template DNA with primers 341F-GC and 518R, located at the V3 region of the 16S rRNA genes of bacteria. 341F-GC consisted of a region that were conserved among the domain bacteria, and 518R is located at a universal conserved region. PCR mixtures contained Ready-to-use PCR reagent from Bioneer (Seoul, Korea), 20 pmol of each primer, template DNA, and sterile water in a final volume of 20 ㎕. DNA template was added to the reaction mixture after the predenaturing step (95℃, 10min). PCR conditions were 95℃ for 5 min, followed by 30 cycles of 95℃ for 45 s, 55℃ for 1 min, and 72℃ for 1 min and a single final extension at 72℃ for 10 min.

DGGE was performed with 6% (wt/vol) acrylamide gels containing a linear chemical gradient ranging from 30 to 60 % denaturant, with 100% defined as 7M urea and 40% formamide. Gels were run for 5h at 200V with a Dcode Universal Mutation System (Bio-Rad). DNA was visualized by silver staining.

Ⅳ. RESULTS AND DISCUSSION

4.1. Growth of PCB Degraders on Biphenyl and Terpenes

The PCB degraders, *Pseudomonas sp*. P166 and *Rhodococcus sp*. T104, were found to grow on both biphenyl and terpenoids ((*S*)-(-)-limonene, *p*-cymene and α-terpinene; 300ppm), whereas *Arthrobacter sp*. B1B was unable to use these terpenoids as a sole carbon source (Fig. 5). Strains P166 and T104 exhibited a reasonable growth on these terpenes (300 ppm) and reached at least 0.3 O.D. after 7 days. However, the observed growth rates were significantly lower than that achieved using biphenyl. For example, strain T104 reached 1.5 O.D. after 7 days when grown on biphenyl (500 ppm). Growth of strain P116 on α-terpinene was better than the strain T104 as shown in Fig. 5. Furthermore, T104 was also unable to use carvone as a sole carbon source [54]. These findings indicated that growth of the biphenyl degraders was strain-specific and that the terpenoid substrate range for PCB degraders was strain-specific.

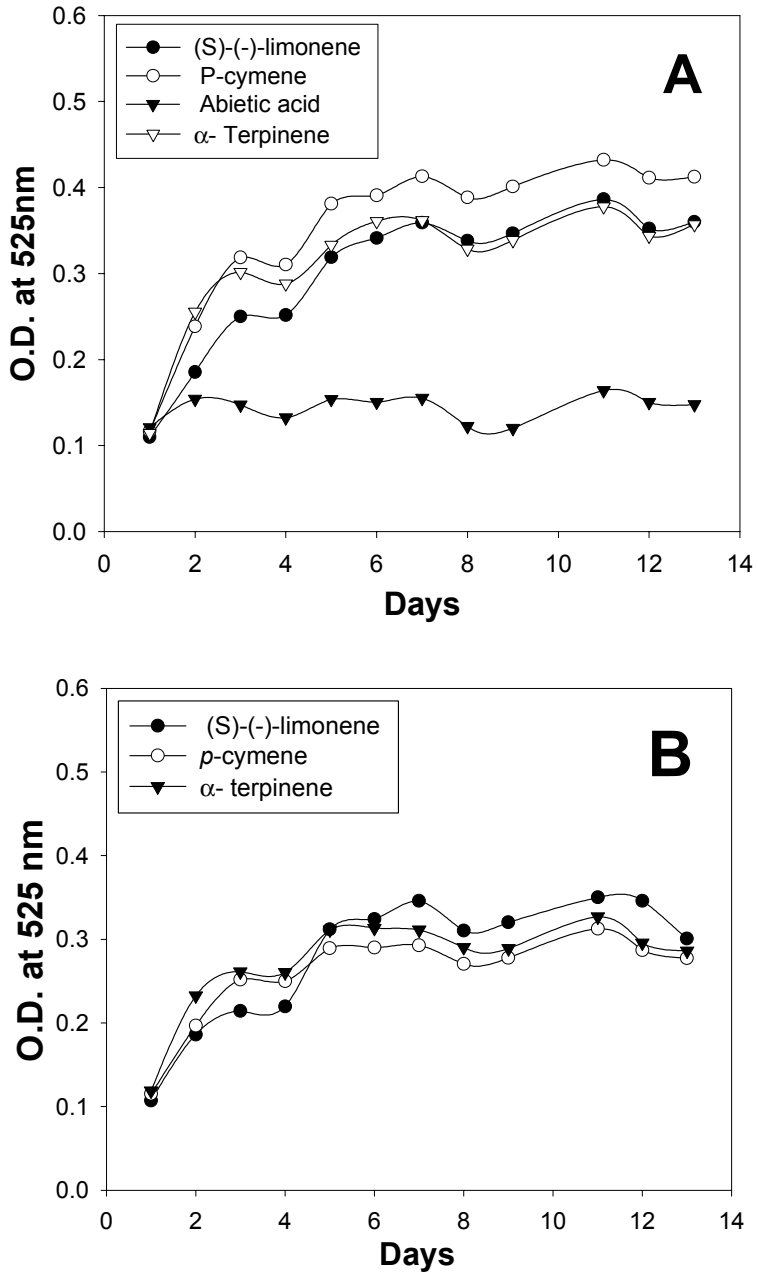


Figure 5. Growth of *Pseudomonas* sp. P166 (A) and *Rhodococcus* sp. T104 (B) on various terpenes and terpenoids (300 ppm).

4.2. Colorimeric a Assay of PCB Degradation under Different Terpenes-inducing Conditions

4.2.1 Resting cell assay

When grown on biphenyl, strain B1B exhibited good degradation activity for 4,4'-DCBp, while strains P166 and T104 attained approximately 25% of B1B's activity (Fig. 6). This means that 4,4'-DCBp would be relatively a poor substrate for T104 and P166. Induction of the PCB degradation pathway by these strains in the presence of cymene, limonene and terpinene was hardly detected through the resting cell assay technique. This appeared to be due to relatively lower induction effect of these terpenoids when compared with biphenyl.

The results of cometabolism of 2,2'-DCBp or 4,4'-DCBp by resting cells of B1B, T109 and T104 are depicted in Figs. 7-9. 2,2'-DCBp, or 4,4'-DCBp (100μM as a final concentration), were added to B1B, T109 and T104 cell suspensions after they were washed twice with the phosphate buffer and adjusted to 1.5 O.D (at 525 nm) in density. The accumulation of 4,4'-DCBp *meta* ring cleavage product in the culture supernatant, a characteristic of *para*-substituted chlorobiphenyls, was monitored spectrophotometrically at 434 nm. The formation of the yellow ring fission product was obvious in B1B cells grown on biphenyl as a sole carbon source (Fig. 7B). The ring fission product of 2,2'-DCBp however, was much less than that of 4,4-DCBp in the strain B1B (Fig. 7A). The formation of the yellow ring fission product was barely noticeable in the T109 and T104 cells grown on biphenyl as a sole

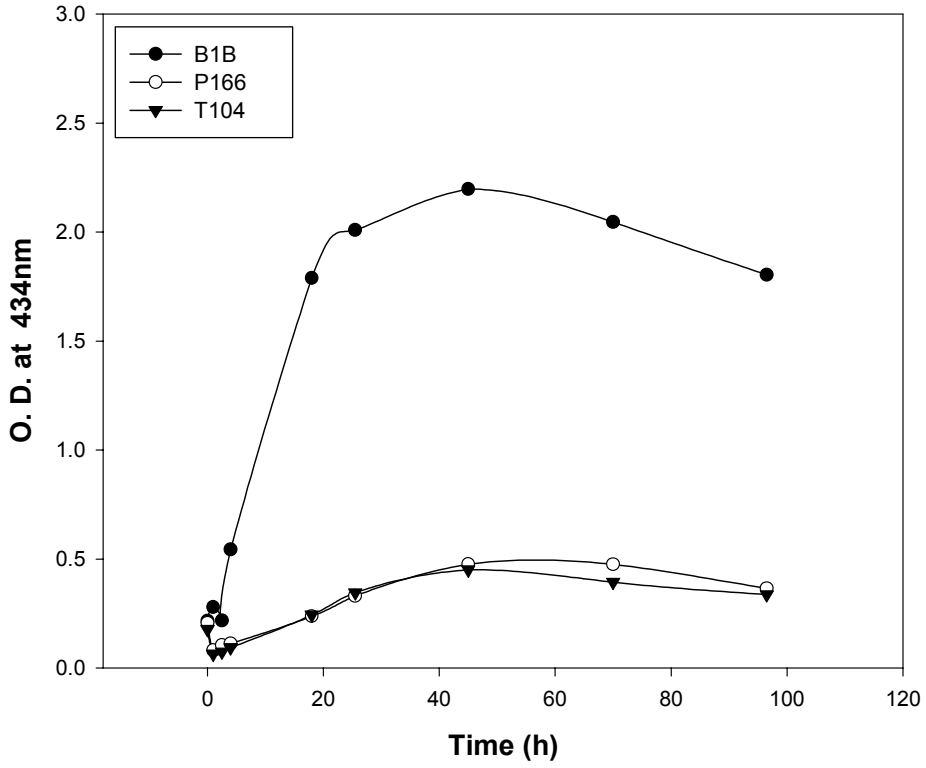


Figure 6. Production of a ring cleavage product (2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acid) from 4,4'-DCBp by resting cells of PCB degraders (*Pseudomonas* sp. P166, *Arthrobacter* sp. B1B and *Rhodococcus* sp. T104) grown on biphenyl.

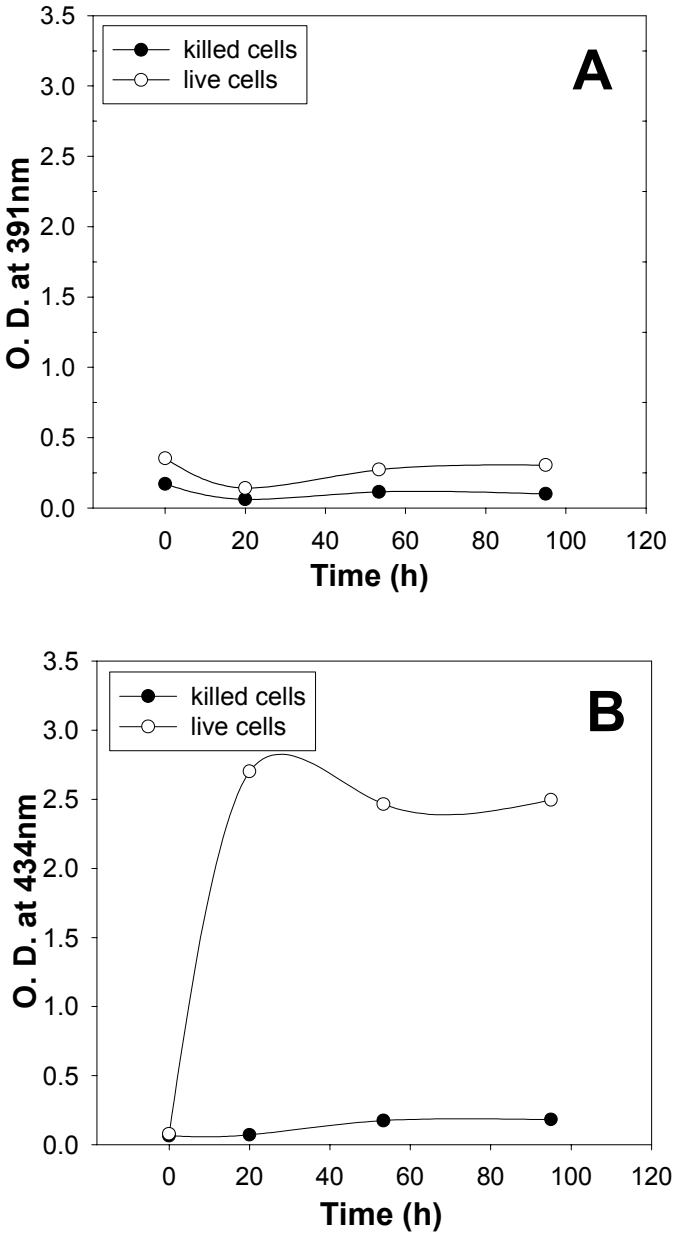


Figure 7. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Arthrobacter* sp. B1B grown on biphenyl.

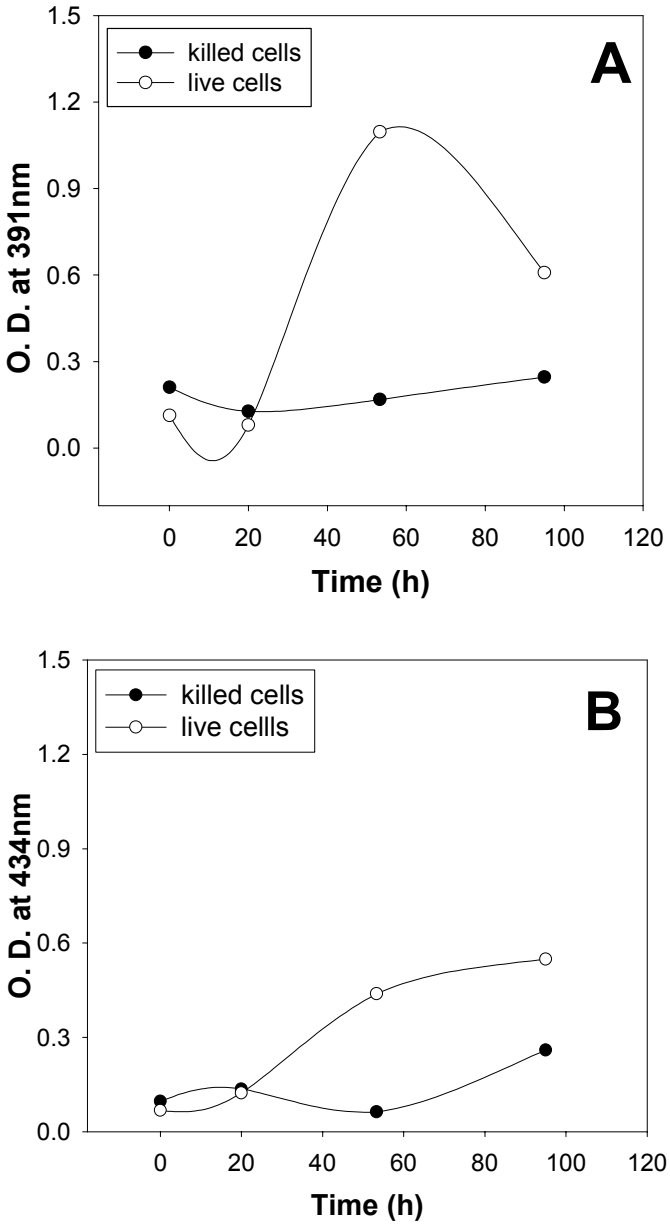


Figure 8. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Cellulomonas* sp. T109 grown on biphenyl.

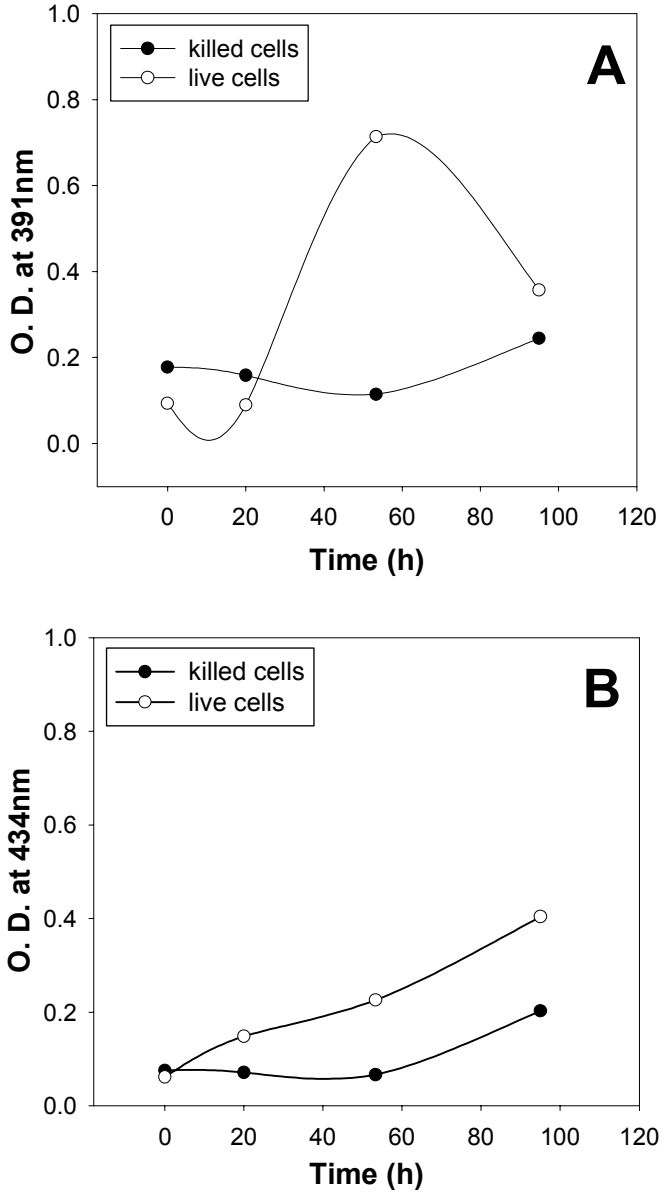


Figure 9. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Rhodococcus* sp. T104 grown on biphenyl.

carbon source (Fig. 8B and Fig. 9B). In contrast to the B1B cells, the amount of ring fission product of 2,2'-DCBp was relatively higher than that obtained for 4,4'-DCBp in both the T109 and T104 cells that prefer 2,2'-DCBp to 4,4'-DCBp as an oxidation substrate (Fig. 8A and Fig. 9A).

4.2.2 Spray Plate Assay of Dihydroxybiphenyl Dioxygenase

The PCB degraders were grown on the MSM agar under biphenyl rapor at least 7 days. The cultures were then subjected to the flooding of 3-methycatechol. The color changes caused by the induced enzyme were observed as described in the Table 5.

Most PCB degraders have shown the presence of the dihydroxybiphenyl dioxygenase activity as observed by color formation. However, some strains like T101 and T109 did not show a specific color reaction. This color formation seemed to be strain-specific.

Table 5. Spray plate assay of various of PCB degraders grown on biphenyl.

Strain sp.	Incubation		
	3 hr	6 hr	24 hr
T101	-	-	-
T104	+	+ (yellow)	-
T109	-	-	-
H850	+	-	-
B1B	+	-	-
LB400	+	+ (yellow)	-
P166	+	+ (yellow)	-
A5	++	+ (yellow)	-

4.2.3 GC analysis of PCB Degradation during Resting Cell Assay

(1) Measurement of PCB degradation activity

To confirm the degradation of PCB during the resting cell assay, the remaining PCB was extracted, their concentrations were measured, and their removal rates were calculated in comparison with heat-killed controls. *Ralstonia eutropha* H850 clearly favored 2,2'-DCBp over 4,4'-DCBp as a substrate. This was also true for *Cellulomonas* sp. T109; a Gram-positive bacterium. This was reflected by the formation of more ring fission product when 2,2'-DCBp, as opposed to 4,4'-DCBp, were used as a substrate (Fig. 8A). In B1B and T104 cells grown on biphenyl however, formation of fission product was not proportional to their PCB removal rates. Strain B1B

was able to degrade 2,2'-DCBp at almost equivalent amount of 4,4'-DCBp. Furthermore, comparative analysis of various PCB degraders grown on biphenyl revealed that there was a stereospecificity in the degradation of PCBs: 2,2'-DCBp was preferred by H850 and T109 and 4,4'-DCBp by T104. Strain B1B exhibited favorable degradation rates for both substrates. Strain T104, grown on (*S*)-(-)-limonene, *p*-cymene and α-terpinene, was able to degrade 4,4'-DCBp up to 30%, equivalent to 50% of biphenyl induction level. Strain T104, grown on (*S*)-(-)-limonene however, could also degrade 2,2'-DCBp up to 30% (Fig. 10B). Strain T104 exhibited a better degradation rate for 4,4'-DCBp than for 2,2'-DCBp; that was not expected in the spectrophotometric monitoring of their ring fission products as shown in Fig. 9. This experiment revealed that monoterpenes are able to induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Ralstonia eutropha* H850 [54]. PCB biodegradation appears to be highly dependent upon the stereospecificity of the target congeners [4, 10, 11]. This was also confirmed by the congener specificity of PCB degradation by the PCB degrader *Ralstonia eutropha* H850 [11, 27]. Here, 4,4-DCBp was a less degradable substrate than 2,2'-DCBp.

Sphingomonas paucimobilis SYK-6 is able to degrade a wide variety of dimeric lignin compounds, including β-aryl ether, biphenyl, and diaryl-propane [2]. SYK-6 could therefore also degrade PCBs. Alternative natural substrates, such as terpenes [33, 54] and flavonoids [30] have been shown to enhance PCB degradation to an equal or greater extent than biphenyl. The mechanisms by which natural plant products enhance degradation of PCBs are unclear. However, it seems likely that terpenes and lignin (or their metabolites) can induce the PCB-degradative pathway. This assumption is

based on the structural similarities between plant terpenes or lignin (and their metabolic products), and biphenyl (and its metabolites) [39].

(2) PCB degradative activity and PCB removal of *Rhodococcus* sp. T104 chloride ion release during the PCB degradation

T104 preferentially degraded 4,4'-DCBp under biphenyl and terpenoid inducing conditions. Terpenoid such as limonene induced the degradation activity up to more than 50 % of that biphenyl. Moreover, the chloride ion release data showed similar pattern to each PCB degradation activity, that is, more than 50 % release rates for various terpenes inducing conditions.

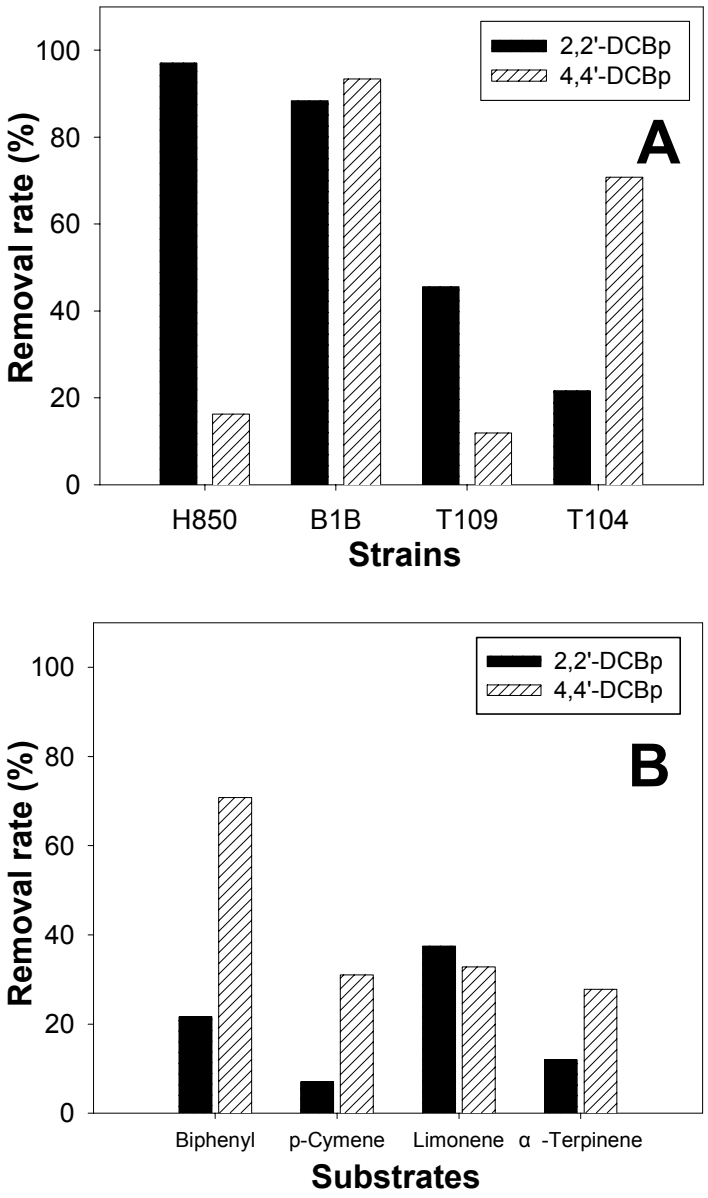


Figure 10. PCB removal rates of *Rhodococcus* sp. T104 grown on biphenyl (A) and various plant terpenes (B).

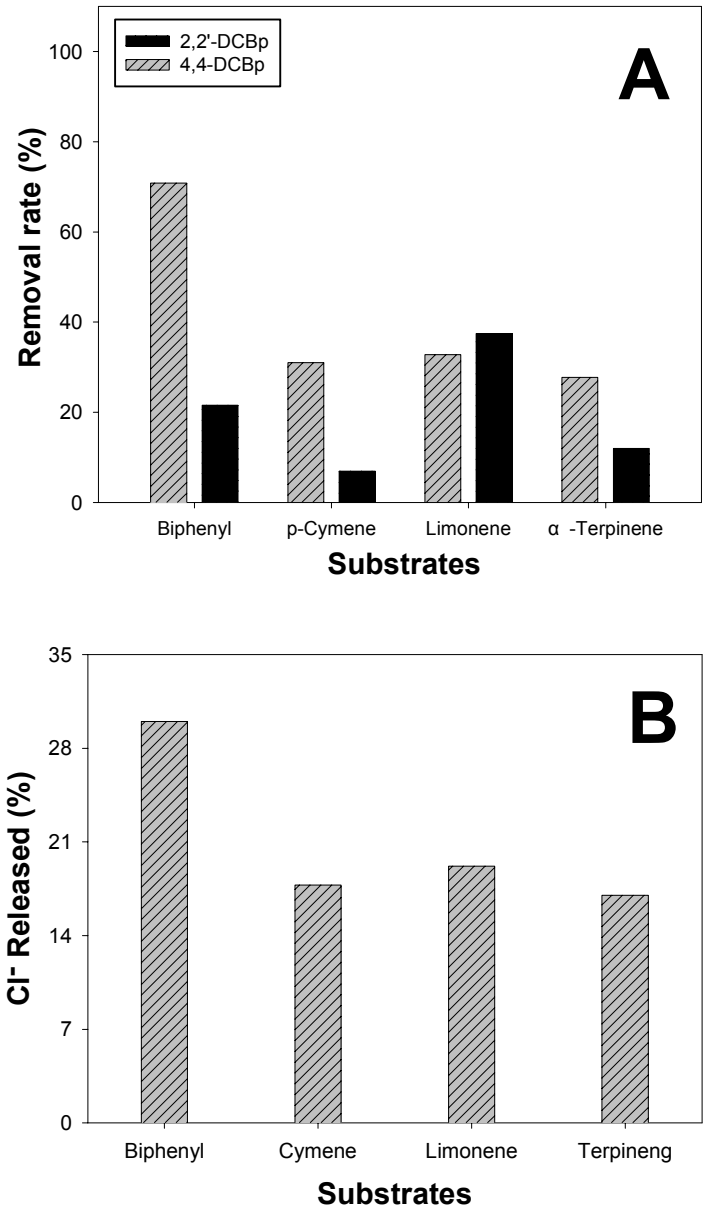


Figure 11. Comparative analysis of PCB degradation activity and the concomitant chloride ion release by *Rhodococcus* sp. T104.

4.3. PCR amplification of *bphC* gene

4.3.1. PCR Monitoring *bphC* Gene of *Rhodococcus* sp. T104 in Pure Culture

The primers (*bphC2* and *bphC3*) used for the amplification of *bphC* gene were first designed from *bphC* gene sequences of a Gram-positive bacterium *Arthrobacter* sp. M5. These primer set was successful in amplifying a homologous gene (182 bp) from a Gram-negative bacterium *Ralstonia eutropha* H850. Many attempts have been made to amplify the corresponding gene from *Rhodococcus* sp. T104 that is a Gram-positive bacterium. Recently it was possible to amplify a putative homologous gene from the strain T104 through modifying a few PCR conditions such as template DNA concentration and initial denaturing of the template DNA. However, PCR specificity was rather low so that several multiple bands were observed (Fig. 12).

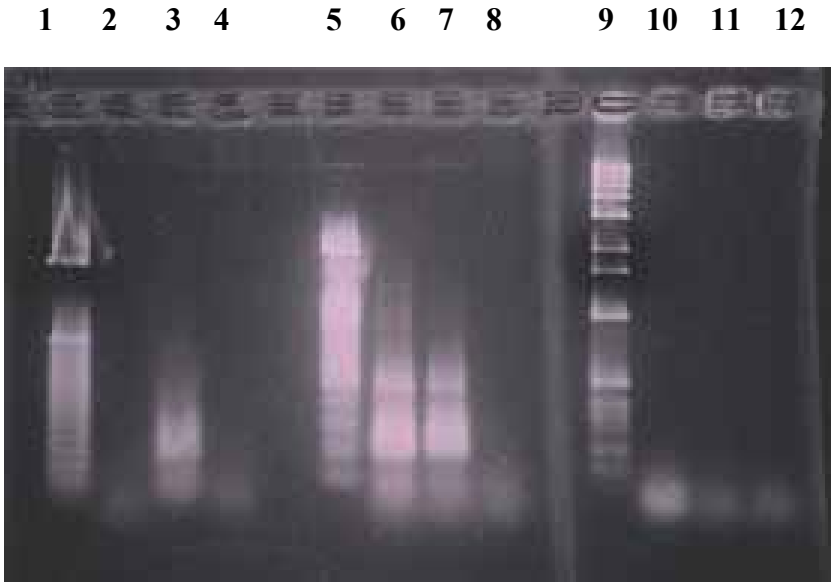


Figure 12. PCR amplification of *bphC* gene using primers specific to Gram positive from *Rhodococcus* sp. T104. Lane 1; DNA marker (50bp), Lane 2, 3, 4; 0.1, 0.25, 0.5 μg (*bphC*), Lane 5; DNA marker (100bp), Lane 6, 7, 8; 0.1, 0.25, 0.5 μg (*bphC*), Lane 9; DNA marker (1kb), Lane 10, 11, 12; 0.1, 0.25, 0.5 μg (*bphC-GP*).

4.4. Monitoring of *bph* Gene Expression in Pure Culture of *Rhodococus* sp. T104 through RT-PCR

Total RNA extraction has been performed for test of the gene expression in T104 grown under different *bph* gene induction conditions. The positive control (RNA from biphenyl grown cells of T104) was first subjected to RT-PCR to find optimal RT-PCR conditions. As expected, it was successful to observe the *bph* gene expression in the positive control (Fig. 13). The gene expression was also justified by the degradation of 4,4'-DCBp in the culture medium. RT-PCR analysis of other treatments will be performed in similar way.

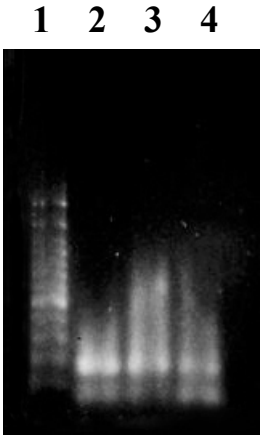


Fig 13. Monitoring *bphC* gene expression through RT-PCR in *Rhodococcus* sp. T104. The lanes show RT-PCR products (182bp) from total RNA template prepared from cells grown on biphenyl. Lane 1; 100 bp DNA ladder; lanes 2, 3, 5; 3$\mu\ell$, 5$\mu\ell$ and 10$\mu\ell$ of RT-PCR product of *bphC* gene, respectively

4.5. Monitoring of PCB Degraders Population and Their Gene Expression in Soil Microcosms

4.5.1 Population Dynamics of PCBs Degrader

(1) *Ralstonia eutropha* H850

The population was maintained at least 10E6 (cells/g fresh soil) at all treating conditions after almost a month. Addition of biphenyl appeared to increase the population density by one order of magnitude in the sterilized soils (Fig. 14).

(2) *Rhodococcus* sp. T104

The population was maintained at least 10E6 (cells/g fresh soil) at all treating conditions after almost a month. Addition of biphenyl appeared to increase the population density by one order of magnitude in the sterilized soils (Fig. 15).

4.5.2 Gene expression in soil microcosms

(1) PCR monitoring of *bphC* gene of *Ralstonia eutropha* H850 in soil

Amount of *bphC* gene amplification of H850 cells was not generally proportional to the cell density observed in soil microcosms (Fig 16).

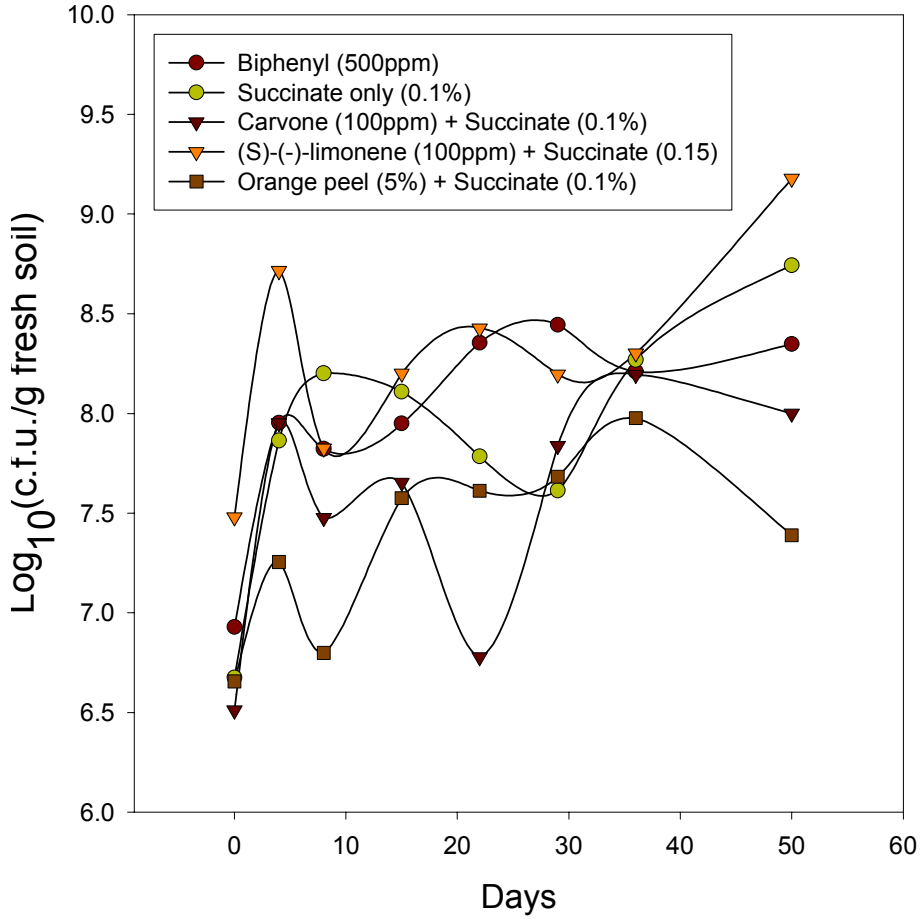


Figure 14. Population dynamics of the viable *Ralstonia eutropha* H850 in soil treated with biphenyl, succinate, carvone + succinate, limonene + succinate and orange peel (1st trial). Medium used for the viable counting was mineral salts agar containing biphenyl.

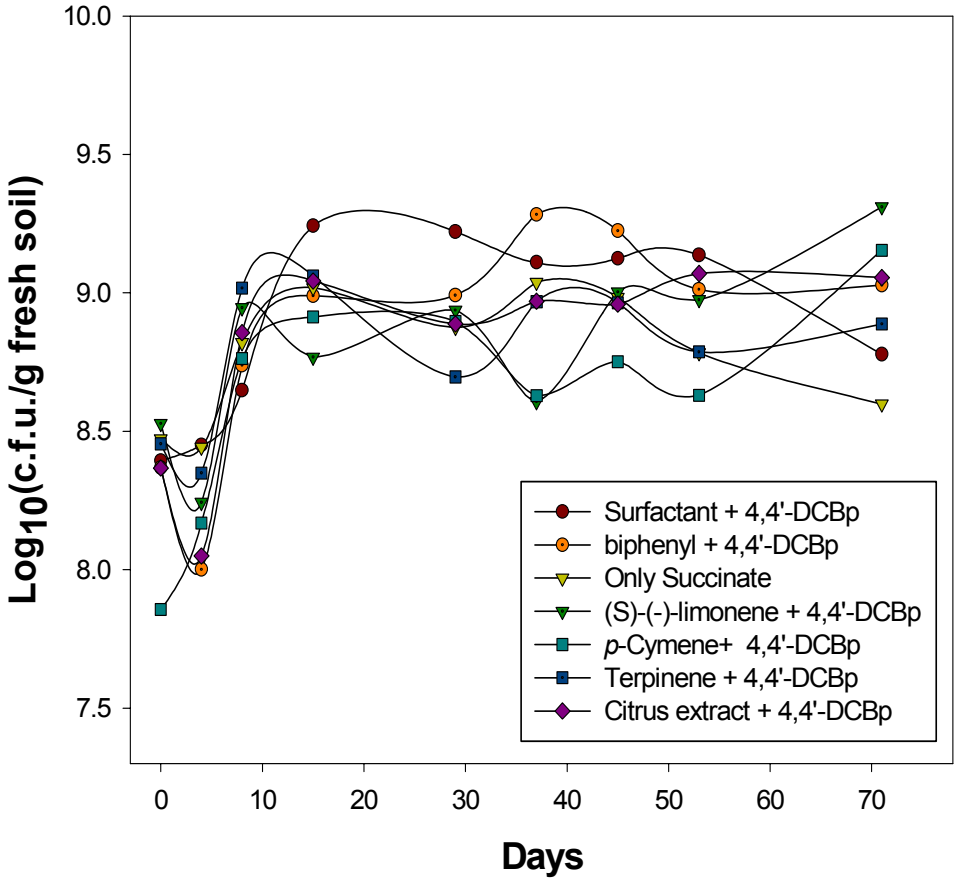


Figure 15. Population dynamics of *Rhodococcus* sp. T104 in soil treated with biphenyl and various terpenes (2nd trial).

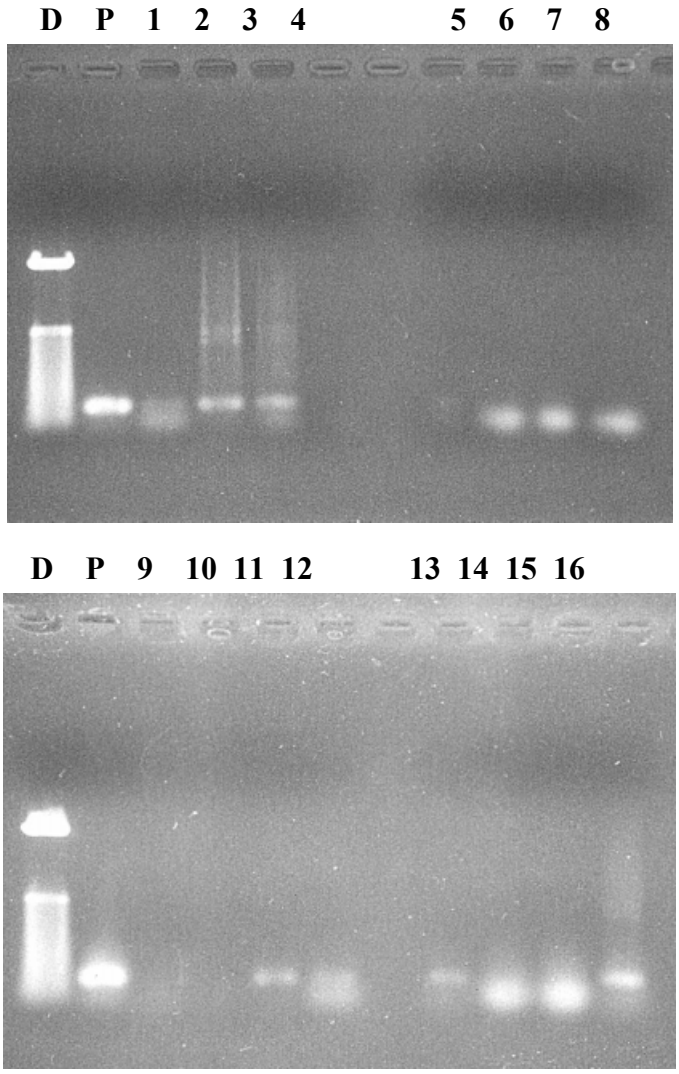


Figure 16. PCR-monitoring of *Ralstonia eutropha* H850 seeded in a pasture soil treated with biphenyl and carvone as inducers for the PCBs-degradative pathway. Lanes D and P, 50 bp ladder (Promega) and a PCR product from pKTF18 (45 ng) carrrying *bphC* gene, respectively. Sampling dates: lanes 1～4 (day 5), lane 5～8 (day 13), lane 9～12 (day 23) and lanes 13～16 (day 59).Inducers: fructose (lanes 1, 5, 9 and 13), carvone+fructose (lanes 2, 6, 10 and 14), biphenyl (lanes 3, 7, 11 and 15), succinate (lanes 4, 8, 11, and 12)(from M.S thesis Y.I. Park).

(2) Expression in pure culture of *Ralstonia eutropha* H850

A successful amplification (182 bp) of mRNA transcribed from *bphC* gene was made in H850 cells, which were grown on biphenyl or induced by carvone. The degree of induction by biphenyl appeared to be higher than by carvone (Fig. 17). The *bphC* gene amplification was authentic because the corresponding primer regions of *bphC* gene in *Alcaligenes eutrophus* A5 had a high DNA sequence homology with those of H850 and KF707 (*e.g.* 95.2 % and 85 % for forward and reverse primers, respectively based on a BLAST search for H850) [54].

(3) Monitoring *bphC* gene (*Ralstonia eutropha* H850) expression in Soil by RT-PCR

The gene expression was observed in soil treated with biphenyl (4 days) but not with succinate, carvone, limonene and orange peel (Fig. 18). This indicated that carvone and limonene would not be inducers in the presence of succinate as a growth substrate in soil. The effective inducers would be natural substrates that can be also utilized as a growth substrate. Their bioavailability in soil would be also important in PCB biodegradation.

The gene expression was only observed until 4 days after inoculation but was not observed at 25th day. This indicates the *bphC* gene could be induced in the presence of biphenyl but the population of H850 was not maintained in the soil environment.

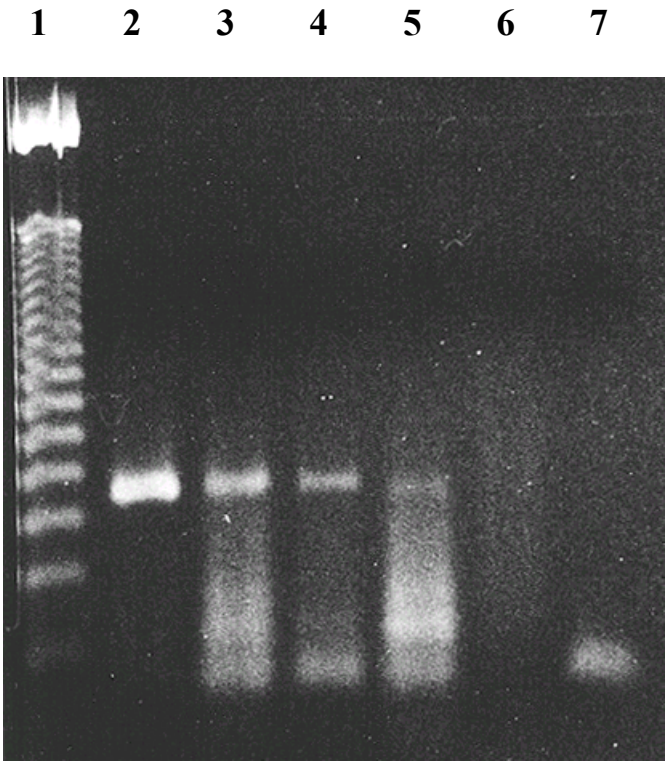


Fig 17. Monitoring *bphC* gene expression through RT-PCR in *Ralstonia eutropha* H850. Lane 1 50 bp DNA ladder; lane 2 PCR product from the template pKTF18 carrying *bphC* gene of *Pseudomonas pseudoalcaligenes* KF707. The subsequent lanes show RT-PCR products (182bp) from total RNA template prepared from cells grown on biphenyl (lane 3), carvone+fructose (lane 4) and fructose only (lane 5), and succinate only (lane 6). A negative control (lane 7): RNA template of lane 4 was treated with RNase and amplified at the same conditions [54].

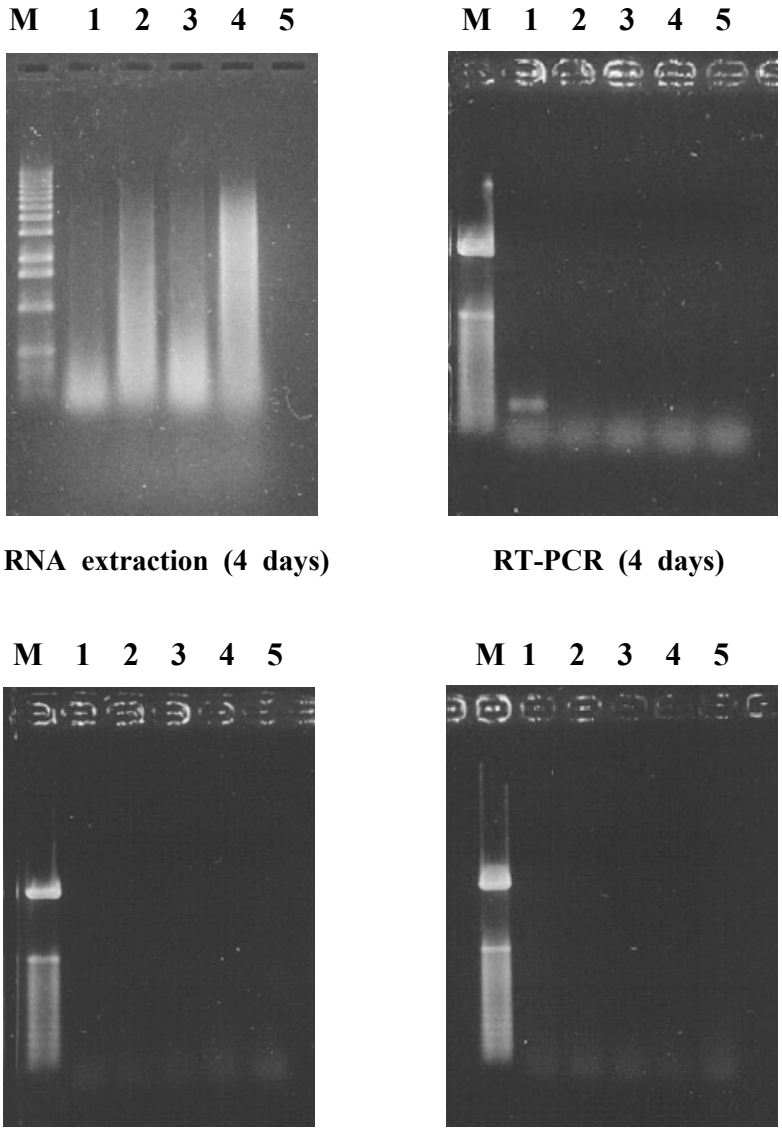


Figure 18. Extraction of total RNA from soil treated with terpenes and the results of RT-PCR. 1-5 : Tr.-1; biphenyl, Tr.-2; only succinate, Tr.-3; carvone+succinate, Tr.-4; limonene+succinate, Tr.-5; Orange peel + succinate (refer to Materials and Methods for detail).

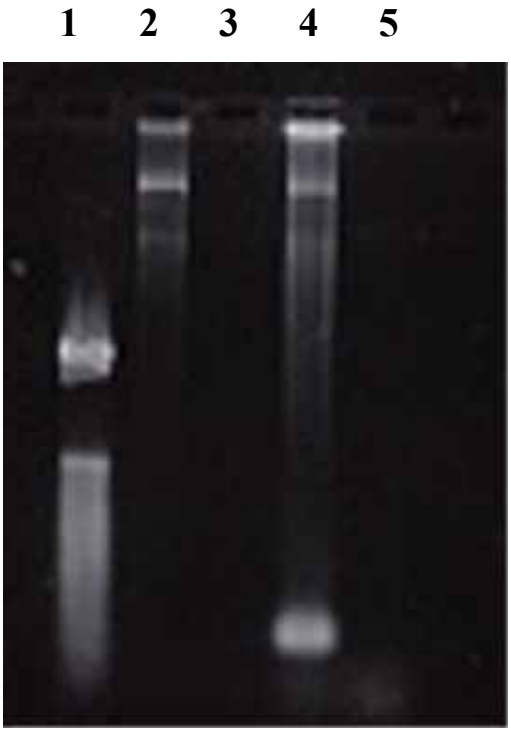


Figure 19. PCR amplification of *bphA* and *bphC* gene from *Rhodococcus* sp. T104 using primers specific to a Gram-positive PCB degrader, *Arthrobacter* sp. M5 Lane 1; DNA marker (50 bp), Lane 2, 3 ; *bphA1-1* and *bphA1-2*; pKTF18, T104 (biphenyl) Lane 4, 5; *bph 2* and *bphC* 3; pKTF18, T104 (biphenyl).

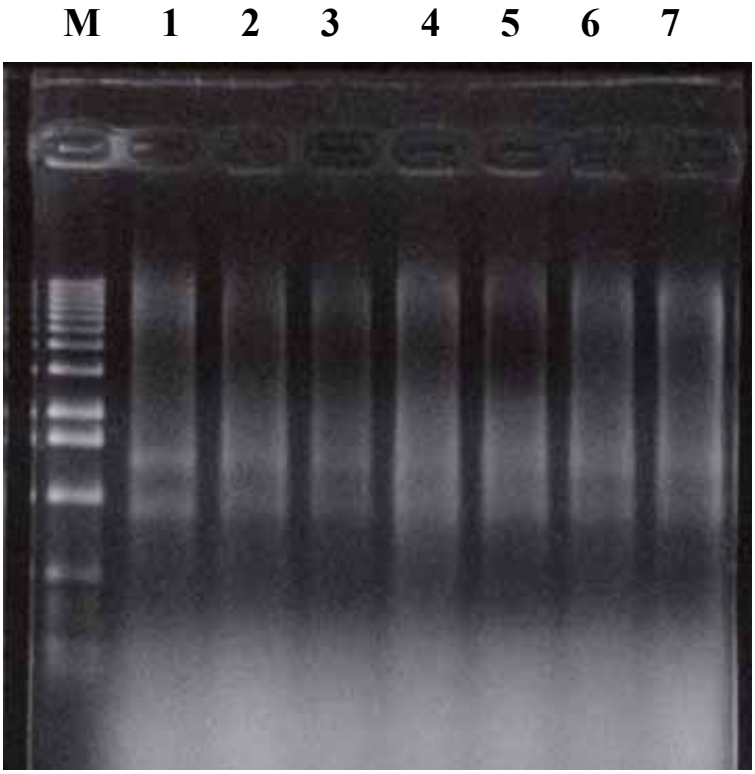


Figure 20. Direct extraction of total RNA from soil treated with various substrates including terpenes. 1-7 : Tr.-1; surfactant, Tr.-2; biphenyl + surfactant, Tr.-3; only succinate, Tr.-4; limonene + surfactant; Tr.-5; cymene + surfactant Tr.-6; Terpinene + surfactant, Tr.-7; Citrus extrac + surfactant (refer to Materials and Methods for detail).

4.5.3 PCR-DGGE Fingerprinting for the aAnalysis of Microbial Community

(1) PCR -DGGE monitoring of population of *Rhodococus* sp. T104 in soil

As a positive control, total DNA of *Rhodococus* sp. T104 was subjected to PCR-DGGE using D-Code System (Bio-Rad). As shown in Fig. 21, the PCR-DGGE pattern of T104 pure culture strain was unique and showed multiple bands. Among these bands, an expected size of the target universal bacterial gene was 200 bp (Fig. 21). Generation of other non-specific bands appeared to result from a non-specific annealing of the PCR primers. Interestingly, PCR-DGGE profile of the total DNA from soil microcosm undergoing PCB biodegradation was quite similar to that of the pure culture. This indicates that the strain T104 would be the most dominant population in the soil microcosm.

For more specific monitoring of T104 or other indigenous microbes in the soil microcosm, PCR conditions needed to be optimized such as primer specificity and target and primer DNA concentrations, and annealing temperature and so forth.

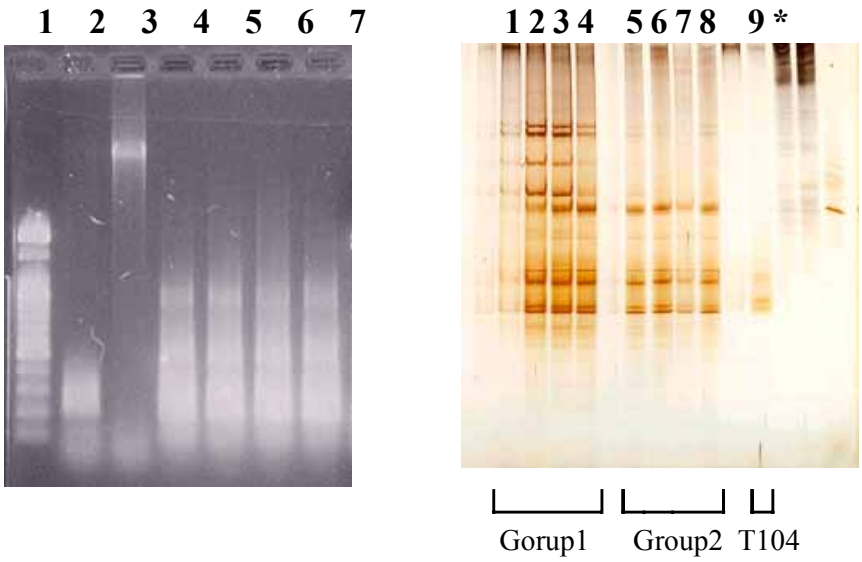


Figure 21. Extraction of total DNA from soil treated with terpenes (A) and the results (B) of PCR-DGGE monitoring the population of *Rhodococcus* sp. T104. Panel A. Lane 1; DNA marker (100m bp), Lane 2; pure culture (predenaturation), Lane 3; pure culture (No predenaturation), Lane 4, 5, 6, 7; Tr-1, 2, 4, 7 (Tr-1; surfactant, Tr-2 ; Surfactant + Biphenyl, Tr-4 ; Surfactant + limonene, Tr-7 ; Surfactant + Citrus extraction); Panel B. PCR-DGGE amplification of 16S rRNA gene from PCB degraders in Soil Microcosm. Lane 1, 5; Orange Peel + Surfactnat, Lane 2, 6 ; Limonine + Surfactnat, Lane 3, 7; Biphenyl + Surfactnat, Lane 4, 8; Surfactant Lane 9; T104 (Group 1; 20m $\mu\ell$, Group 2; 10 $\mu\ell$)

Ⅴ. CONCLUSION

The following conclusions can be drawn based on the results from this study.

1. A known PCB degrader *Ralstonia eutropha* H850 was found to survive in soil treated with terpenes (e.g., carvone, limonene and orange peel) more than 2 months

2. In contrast to liquid culture conditions, *bphC* gene in H850 was barely induced in soil treated with the terpenes except biphenyl.

3. A known PCB degrader *Rhodococcus* sp. T104 was found to survive in soil treated with terpenes (e.g., limonene, cymene, terpinene and tangerine extract) as growth substrates for more than 2 months.

4. Availability of plant terpenes could be a critical factor for the induction of PCB-degradative pathway in the soil systems. So surfactant application would significantly improve the availability and hence the PCB degradation.

5. PCB biodegradation appeared to be grealty affected by stereospecificity of the congeners.

Ⅵ. FURTHER WORKS

The following needs to be considered for a further research.

1. Real time monitoring of population dynamics of PCB degraders and other indigenous organisms using DGGE technique.

2. Real time monitoring of the expression of *bph* genes during soil bioremediation of PCBs through RT-PCR.

3. Correlation of GC analysis data and expression of *bph* genes to develop an efficient monitoring system of PCB biodegradation.

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누군가가 말했습니다.
“논문의 90%는 거짓이다. 다만 10%의 진실만이 존재할 뿐이다.” 라고.
물론 이 말은 제가 아는 분의 지도교수님을 가르치신 어느 대가의 말씀이십니다. 저는 처음에 이 말을 듣고 이해할 수는 없었지만 참 멋진 말이라고 생각하였습니다. 하나의 결과를 얻기 위해 얼마나 많은 노력과 땀을 쏟아내어야 하는지 이제는 어렴풋이 나마 알기 때문입니다. 그리고 조그마한, 아주 사소하게 지나칠 수 있는 하나의 사실로부터 가설을 세우고 그것을 증명하기 위해 반복되어지는 수많은 실험과 그 과정에서 느껴지는 성취감과 좌절감.....그리고 학부, 대학원 모두 합쳐 7 년이라는 짧지 않은 시간을 쏟아부었음에도 지금 제가 얻을 수 있는 것은 얼마 되지 않는 몇 가지 사실들뿐이라는 것을 조금씩 깨달아 갈 뿐입니다. 그 분은 어쩌면 그 10%의 사실과 진실을 얻기 위해 많은 노력과 인내가 필요하다는 것, 시시각각으로 쏟아져 나오는 수평적으로 나열된 지식들을 가려서 받아들일 줄 알아야한다는 것, 그리고 어디에서건 자만하지 않고 늘 겸손한 자세를 잃지 않았으면 하는 바램에서 이제 막 첫발을 내디디려는 후학들에게 하신 말씀이셨는지도 모르겠습니다.

막상 졸업이라는 자리에 서 보니, 지난 2년간의 대학원 생활을 돌아보면 아쉬운 것들과 후회되는 것들이 너무나 많습니다. 그 때 좀 더 열심히 할 것을 그러하지 못했던 제 자신이 부끄러워집니다. 오랜 시간 학교라는 울타리에서만 생활하다보니 아직 미숙한 점도, 부족한 점도 많은 모습입니다. 이러한 제가 학교라는 울타리를 벗어나 지금까지 보다 더 넓고 큰 사회라는 곳으로 나아가려 합니다. 지금의 제 모습은 잔뜩 웅크린 채로 아주 작게 느껴지지만 언젠가의 도약을 위해 항상 준비하고 노력하는 모습의 제가 될 수 있도록 하겠습니다.

이런 미흡한 저를 2 여년의 대학원 생활을 무사히 마칠 수 있도록 도와주신 많은 분들께 감사 드립니다. 언제나 마지막까지 최선을 다할 수 있도록 늘 곁에서 지켜 봐주신 고성철 지도교수님께 먼저 감사드립니다. 교수님께서 몸소 보여주신 학문에 대한 열정과 연구에 대한 열의는 앞으로 저에게 많은 가르침이 될 것 같습니다. 그리고 학부 때부터 많은 관심을 가져주시고 진로결정에 조언을 해주신 박상윤 교수님, 새내기 때부터 함께 하면서 제게 환경공학이란 무엇인지 처음부터 하나하나 가르쳐주신 김인수 교수님, 어렵게만 느껴지는 대학원 수업을 항상 여유 있고 편안한 분위기로 이끌어나가신 송영채 교수님, 이 세 분께 감사의 마음을 전하고 싶습니다.

제게 2년간의 대학원 생활동안 남는 것이 있다면 우리 실험실 식구들..
학교에서 함께 할 땐 몰랐는데 학교를 떠나보니 새삼 그 소중함을 다시 느끼게 됩니다. 늘 거침없이 말하는 모습에 처음에는 상당히 무서웠던, 실험실 짱인 성우오빠, 이제는 그 한마디 한마디가 저를 위한 배려에서 우러나온 마음이었다는 것을 느낍니다. 그리고 학부선배지만 대학원은 후배인 성규, 명현 선배께도 함께 해서 즐거웠다고 전하렵니다. 그리고 마지막으로 동기면서 이 길을 어깨 겨누며 나란히 걸어나갈 병혁이에게 너무나 많은 신세를 지고 있음을, 그래서 더 좋은 연구성과를 가지고 이제 막 시작인 대학원 생활을 잘 꾸려나갔으면 하는 바램입니다. 참, 그리고 우리 실험실 귀염둥이(?) 영이와 건도, 이제는 공학 실험실에 있는 상조까지 같이 한 시간은 얼마 되지 않지만 그나마 단조로운 일상을 심심지 않게 보낼 수 있었다고 전하고 싶습니다.

대학원 동기가 상호선배, 정희 언니, 희경이, 이렇게 저까지 넷밖에 되지 않는데 같이 어울릴 기회는 별로 없었던 것 같아 아쉬움이 남습니다. 부디 앞으로 하는 일에 좋은 일만 생기길 바라겠습니다. 그리고 기기 실험실, 공학 실험실 다른 분들께도 그 동안의 고마움을 전합니다.

지난 7년간의 학교생활에서 동고동락했던 동기들과 지금은 사회라는 큰 울타리 속에서 당당히 제 몫을 하고 있는 미경, 양임, 현숙, 용삼 그리고 아직은 군생활을 하고 있는 성원이와 상규에게 힘들 때마다 함께 해줘서 많은 힘이 되었다고 다시 한 번 얘기해주고 싶습니다. 이제는 동아리 동기라는 사실 보단 친구라는 이름이 더 어울리는 지금은 외국으로 어학연수를 떠난 성호에게 네게 부끄럽지 않은 친구로 남겠다고 말하고 싶습니다.

또 지치고 힘들 때 서로 바빠 만나지는 못해도 늘 마음만은 함께였던 현정이와 후배이면서 친구의 사랑하는 사람인 영철이에게 앞으로도 이쁜 사랑하길 바라며, 저를 항상 목놓아 기다리게 하는 후배 진옥이에게도 승철 오라버니와 올해엔 좋은 결실 맺길 바라겠습니다.

그리고 아직 부족하기 만한 논문이지만 이 결실을 사랑하는 부모님께 바칩니다. 부모님 곁을 떠나 처음으로 하는 타지 생활에 아직도 제 걱정만 하시는 두 분께 이젠 혼자 힘으로 당당히 서는 딸의 모습을 보여드리려 합니다.
사랑하는 동생, 윤!
언제나 누나인 내게 가리어 빛을 발하기 보단 스스로 빛이 약해져야했던 네게 가장 미안한 맘이 드는구나. 곧 다가올 새로운 생활에서도 잘 적응하길 바라며 늘 네 곁에 내가 있음을 잊지 말았으면 한단다. 사랑한다.

CURRICULUM AND VITAE

Name: Kyung-Ja, Jung
Date of Birth: Jan. 10, 1977
e-mail Address : ardeco95@korea.com

Education

M.S. in Division of Civil and Environmental Engineering
Korea Maritime University, Korea
Mar. 2000 - Feb. 2002

B.S. in Department of Environmental Engineering
Korea Maritime University, Korea
Mar. 1995 - Feb. 2000

Publications

1. Thesis:

Molecular Biological and Ecological Investigation of PCBs Biodegradation Facilitated by Plant Terpenes.

2. Journal Papers:

Jung, K. J., E. B. Kim, J. S. So, and S. C. Koh. 2001. Specific bio-degradation of polychlorinated biphenyls (PCBs) facilitated by Plant terpenoids. Biotechnol. Bioprocess Eng. 6(1):61-66.

3. Conference papers:

Jung, K. J., Y. J. Park, and S. C. Koh. 2000. Effect of terpenoids as Natural Growth Substrate on PCB Biodegradation in Soil. Abstr. Of 100th

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Eun-Taex Oh, K. J. Jung. 2001. Monitoring of *Pseudomonas pseudoalcaligenes* KF707 Labeled with gft as a Reporter Gene. The Korean Society for Biotechnology and Bioengineering. June. 20-22. Chonan, Korea.

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5. Research Reports

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