*Biotechnol. Bioprocess Eng.* 2001, 6: 61-66

# Specific Biodegradation of Polychlorinated Biphenyls (PCBs) Facilitated by Plant Terpenoids

Kyung-Ja Jung[1], Eungbin Kim[2], Jae-Seong So[3], and Sung-Cheol Koh[1]*

[1] Division of Civil and Environmental Engineering, Korea Maritime University, Pusan 606-791, Korea
[2] Department of Biology, Yonsei University, Seoul 120-749, Korea
[3] Department of Biological Engineering, Center of Advanced Bioseparation Technology, Inha University, Incheon 402-751, Korea

**Abstract** The aim of this study was to examine how plant terpenoids, as natural growth substrates or inducers, would affect the biodegradation of PCB congeners. Various PCB degraders that could grow on biphenyl and several terpenoids were tested for their PCB degradation capabilities. Degradation activities of the PCB congeners, 4,4'-dichlorobiphenyl (4,4'-DCBp) and 2,2'-dichlorobiphenyl (2,2'-DCBp), were initially monitored through a resting cell assay technique that could detect their degradation products. 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) whereas *Arthrobacter sp.* B1B could not grow on the terpenoids as a sole carbon source. The B1B strain grown on biphenyl exhibited good degradation activity for 4,4'-DCBp and 2,2'-DCBp, while the activity of strains P166 and T104 was about 25% that of the B1B strain, respectively. Concomitant GC analysis, however, demonstrated that strain T104, grown on (S)-(-) limonene, *p*-cymene and α-terpinene, could degrade 4,4'-DCBp up to 30%, equivalent to 50% of the biphenyl induction level. Moreover, strain T104 grown on (S)-(-) limonene, could also degrade 2,2'-DCBp up to 30%. This indicates that terpenoids, widely distributed in nature, could be utilized as both growth and/or inducer substrate(s) for PCB biodegradation in the environment.

*Keywords*: *Arthrobacter* B1B, bioremediation, *bph* genes, terpenoids, *Pseudomonas* sp. P166, PCBs, resting cell assay, *Rhodococcus* sp. T104

## 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 [1]. 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 [2], and to enhance their biodegradation in soil [3] and sediments [4]; this despite the fact that it is not a normal constituent of soil [5]. Unfortunately, use of biphenyl as a soil amendment is not possible given its adverse health effects [6], cost [7], 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 [8]. Search for a soluble and nontoxic inducer in PCB bioremediation has led to the hypothesis that plant terpenoids may be the "natural" substrates for biphenyl biodegradation enzymes, or for their ancestors, since biphenyl is not naturally abundant [5]. Furthermore, structural similarities exist between plant terpenoids and biphenyl (Fig. 1). Plant flavonoids equal to biphenyl in promoting metabolism of PCBs have also been found [9]. 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 [10], lignin [11,12], and terpenoids [8]. Carvone, a chemical component of spearmint, may successfully induce the PCB degradative pathway in the Gram-positive bacteria, *Arthrobacter* sp. B1B [13]. L-carvone and other *p*-menthene structure motif compounds (limonene, *p*-cymene and isoprene) induced *Arthrobac-*

*Corresponding author
Tel: +82-51-410-4418 Fax: +82-51-410-4415
e-mail: skoh@hanara.kmaritime.ac.kr

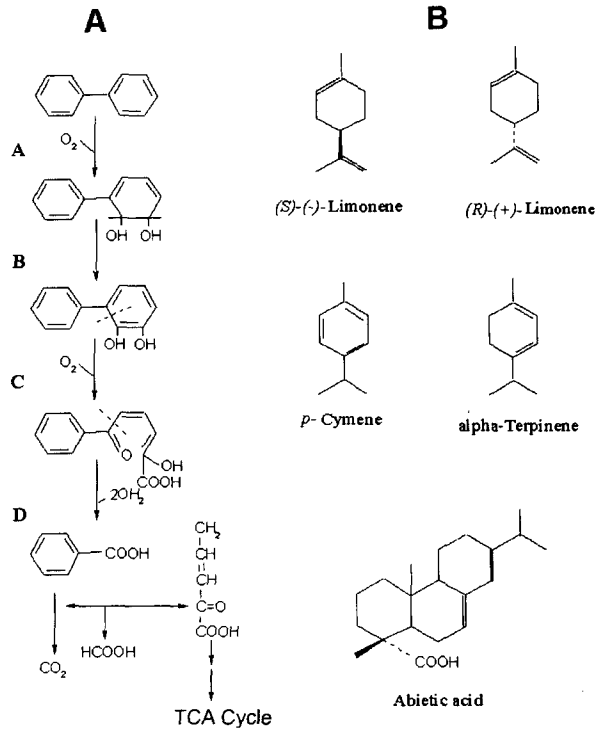


Fig. 1. Bacterial metabolic pathway of biphenyl (A) and structural similarity of various terpenoids to biphenyl (B).

ter sp. B1B to cometabolize Aroclor 1242 (Fig. 1) [13]. Limonene is one of the most abundant terpenoids found on earth and produced by more than 300 plants [14].

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

## MATERIALS AND METHODS

### Bacterial Strains, Culture Maintenance and Growth Conditions

PCB degraders used in this study were *Pseudomonas* sp. P166 [15], *Arthrobacter* sp. B1B [13], *Cellulomonas* sp. T109 and *Rhodococcus* sp. T104 [5]. 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) [16] 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°C.

### Utilization Test of Terpenoids

The experiment was performed following a protocol that was modified from Gilbert and Crowley [13]. 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°C at 200 rpm. Cultural growth was monitored spectrophotometrically at 525 nm (Jasco, Model V-550, Tokyo, Japan).

### Induction of PCB-degradative Pathway by Terpenoids

Unless otherwise stated, 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.

### Resting Cell Assay of PCB Cometabolism

Biodegradation was monitored by resting cell assay [13] 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.

### 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 [17]. 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 (30 m × 0.32 mm ID) [5]. The temperature program ranged from 100°C to 252°C with the following ramping conditions: initial temperature 100°C, 9°C $min^{-1}$ to 160°C, 3.0°C $min^{-1}$ to 252°C.

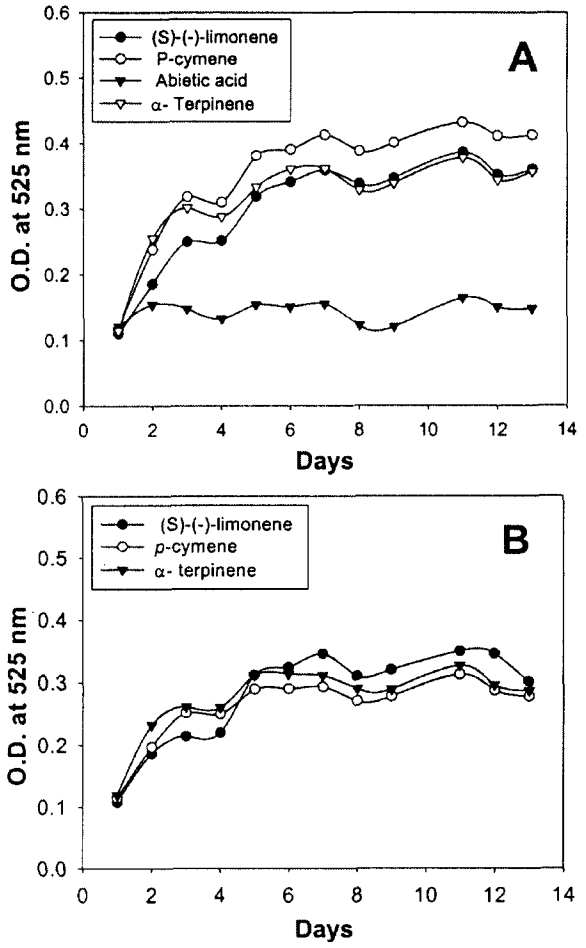


Fig. 2. Growth of *Pseudomonas* sp. P166 (A) and *Rhodococcus* sp. T104 (B) on various terpenoids (300 ppm).

The internal standard, 2,2', 3,3', 5,5'-hexachlorobiphenyl (retention time 31 min), was added prior to extraction.

## RESULTS AND DISCUSSION

### Growth of PCB Degraders on Biphenyl and Terpenoids

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; 300 ppm), whereas *Arthrobacter* sp. B1B was unable to use these terpenoids as a sole carbon source (Fig. 2). Strains P166 and T104 exhibited reasonable growth on these terpenoids (300 ppm) and reached at least 0.3 O.D. after 7 days. However, the observed growth rateswere significantly lower than that achieved using biphenyl. By way of 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. 2. Furthermore, T104 was also unable to use carvone as a sole carbon source [18]. These findings indicated that growth of the biphenyl degraders was strain-specific and that the terpenoid substrate range for PCB degraders was strain-specific.

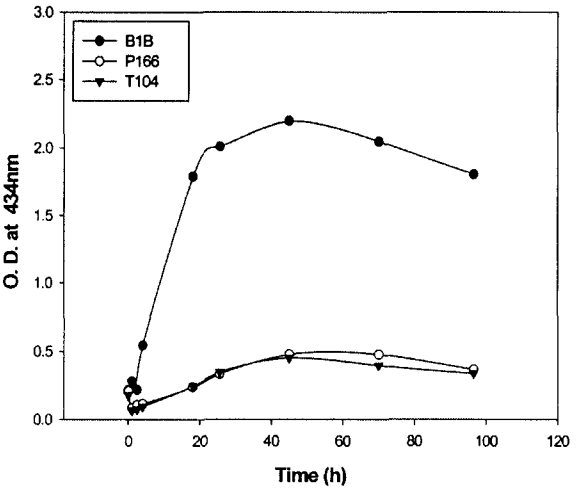


Fig. 3. Production of a ring cleavage product (2-hydroxy-6-oxo-phenylhexa-2,4-dienoic acid) from 4,4'-DCBp by resting cells of PCB degraders (*Pseudomonas* sp. P166, *Arthrobacter* sp. B1B and *Rhodococcus* sp. T104) grown on biphenyl.

### Cometabolism of 2,2'-DCBp and 4,4'-DCBp by the Resting Cells of PCB Degraders under Different Inducing Conditions

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. 3). 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. 4-6. 2,2'-DCBp, or 4,4'-DCBp (100 μM 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 [19]. The formation of the yellow ring fission product was obvious in B1B cells grown on biphenyl as a sole carbon source (Fig. 4B). The ring fission product of 2,2'-DCBp however, was much less than that of 4,4'-DCBp in the strain B1B (Fig. 4A). The formation of the yellow ring

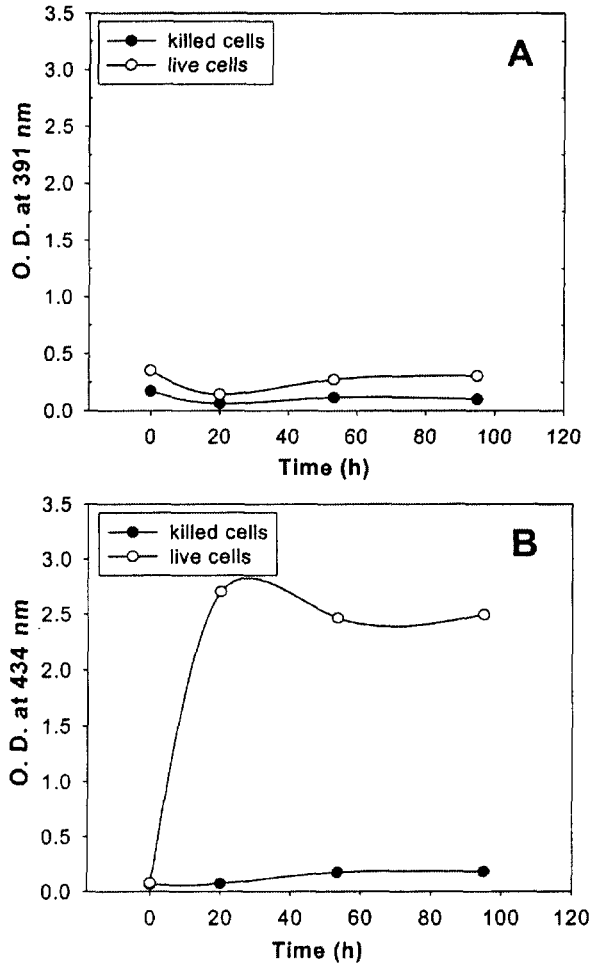


Fig. 4. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Arthrobacter* sp. B1B grown on biphenyl.

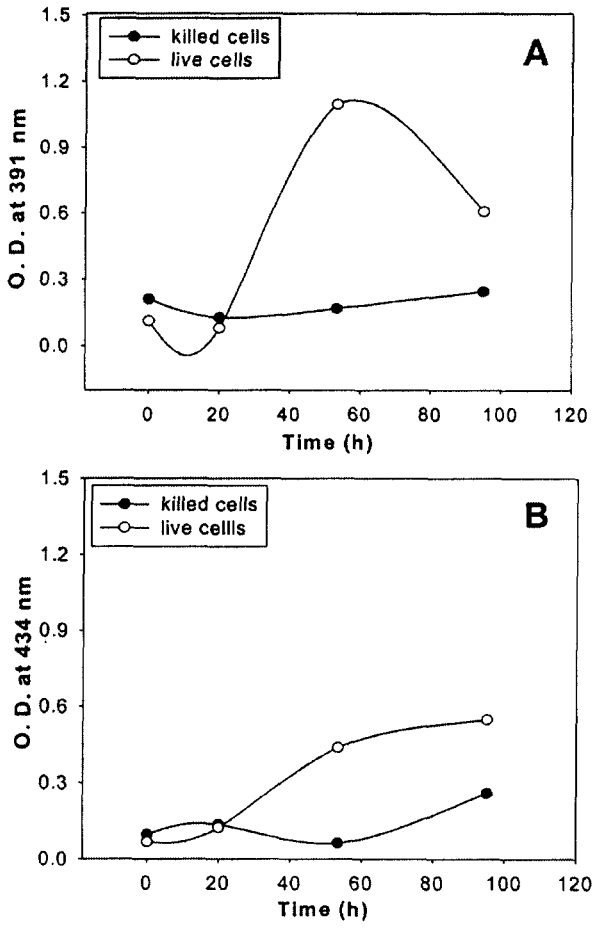


Fig. 5. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Cellulomonas* sp. T109 grown on biphenyl.

fission product was barely noticeable in the T109 and T104 cells grown on biphenyl as a sole carbon source (Fig. 5B and Fig. 6B). 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. 5A and Fig. 6A).

## GC analysis of PCB Degradation during Resting Cell Assay

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. *Alcaligenes eutrophus* 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. 5A). 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. 7B). 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. 6. This experiment revealed that mono-terpenoids are able to induce the PCB degradative pathway in *Arthrobacter* sp. B1B and *Alcaligenes*

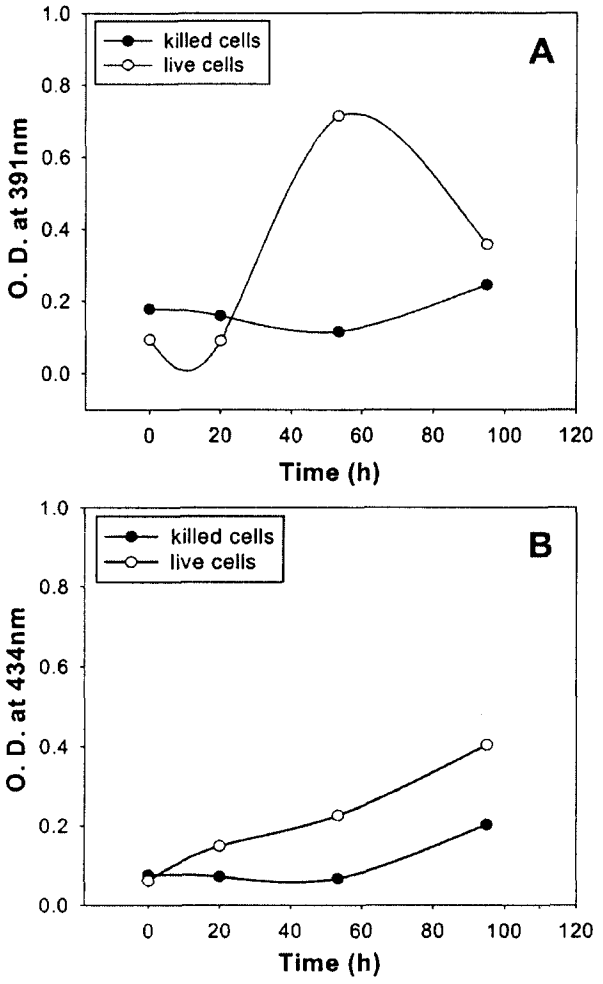


Fig. 6. Production of ring cleavage product from 2,2'-DCBp (A) and 4,4'-DCBp (B) by resting cells of *Rhodococcus* sp. T104 grown on biphenyl.

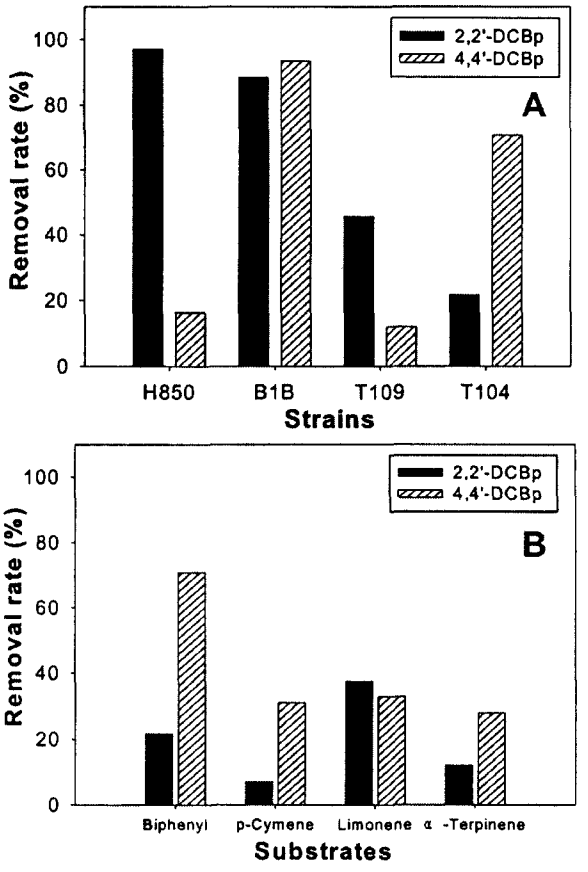


Fig. 7. PCB removal rates of *Rhodococcus* sp. T104 grown on biphenyl (A) and various plant terpenoids (B).

*eutrophous* H850 [18]. PCB biodegradation appears to be highly dependent upon the stereospecificity of the target congeners [2,20,21]. This was also confirmed by the congener specificity of PCB degradation by the PCB degrader *Alcaligenes eutrophous* H850 [3,21]. 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 diarylpropane [22]. SYK-6 could therefore also degrade PCBs. Alternative natural substrates, such as terpenes [5,18] and flavonoids [13] 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) [23].

## CONCLUSION

Strain T104, utilizing (S)-(-)-limonene, *p*-cymene and α-terpinene as a sole carbon source, could degrade 4,4'-DCBp up to 30%, equivalent to 50% of biphenyl induction level. The strain T104 grown on (S)-(-)-limonene however, was also able to degrade 2,2'-DCBp up to 30%. PCB biodegradation appears to be affected by stereospecificity of the congeners. Availability of plant terpenoids could be a critical factor for the induction of the PCB-degradative pathway in an environment such as soil. The use of plant terpenoids as an inducer for the degradative pathway has clear advantages: good bioavailability, compatibility (nontoxicity) in natural environments, and their ubiquity in the environment.

**Acknowledgements** We are very grateful to Dr. D. Focht at the University of California, Riverside for his generous gift of the PCB degraders used in this study. We also thank Dr. O-Seob Kwon for helping us with the GC analysis. This study was supported by an academic research fund of the Korean Research Foundation

(#1999-015- DP0321).

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[Received January 30, 2001; accepted February 15, 2001]