농학석사학위논문

농업토양에서 분리된 *Pseudaminobacter* sp. SP1a와 *Nocardioides* sp. SP1b의 영양공생을 통한 Propoxur의 생분해

Syntrophic Biodegradation of Propoxur by *Pseudaminobacter* sp. SP1a and *Nocardioides* sp. SP1b Isolated from Agricultural Soil

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ABSTRACT

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The *N*-methylcarbamate insecticide propoxur, 2-isopropoxyphenyl *N*-methylcarbamate, is one of commonly applied insecticides all around the world. Propoxur was applied to agricultural fields and in-and –around industrial, commercial, and residential facilities for public health. Propoxur has an adverse effect on the brain and central nervous system in human by inhibiting cholinesterase. Moreover, the U.S. EPA classified propoxur as a probable human carcinogen. In addition to direct impacts on human, propoxur has toxicity to non-target organisms including not only fish and other aquatic organisms, birds, bees, and mammals but also soil microbial community. Therefore, cleanup of propoxur residues from the environment is important. In this study, three syntrophic pairs of propoxur degraders

were isolated from agricultural soils. Based on 16S rRNA gene sequence analysis and repetitive extragenic palindromic PCR (REP-PCR), the isolates consisting of syntrophic pairs were related to members of genera *Pseudaminobacter*, *Mesorhizobium*, and *Nocardioides*. All pairs were capable of degrading and utilizing propoxur as a sole carbon and energy source. Among these pairs, SP1 syntrophic pair degrading propoxur most rapidly was chosen for further characterization studies. Propoxur degradation rate of SP1 pair increased when the pair was incubated at moderate temperature. Moreover, SP1 pair degrade propoxur more quickly with shorter lag period in the presence of glucose or peptone. The syntrophic pair appeared to transform propoxur to 2-isopropoxyphenol (2-IPP) and further mineralize the intermediate when analyzed by gas chromatography-mass spectrometry (GC-MS). When analyzed with PCR amplification using previously-reported carbamate hydrolase genes, strain SP1 showed homology with *cehA* gene. This is first time that bacteria involved in propoxur mineralization have been isolated. Considering complete degradation ability of these syntrophic pair, they might be useful for bioremediation purposes in fields contaminated with propoxur.

Keywords: Propoxur, Syntrophy, Biodegradation, 2-Isopropoxyphenol, Biomineralization, Bacteria, Hydrolysis

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I. INTRODUCTION

Carbamate pesticides are a group of organic compounds derived from carbamic acid and distinguished by carbamate ester functional group. Chemicals belonging to this group are propoxur (2-isopropoxyphenyl N-methylcarbamate), aldicarb (2-methyl-2-(methylthio) propanal *O*-(*N*-methylcarbamoyl) oxime), carbaryl (1-naphthyl methylcarbamate), carbofuran (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate), fenoxycarb (ethyl *N*-[2-(4-phenoxyphenoxy) ethyl] carbamate), and fenobucarb (2-*sec*-butylphenyl-*N*-methylcarbamate). They act as the inhibitor of acetylcholinesterase, resulting in excessive accumulation of acetylcholine at the synapse (Casida, 1963). This inhibition causes paralysis and muscle spasm for insects, mammals, and fish (WHO, 2005). Among these carbamate pesticides, propoxur (2-isopropoxyphenyl *N*-methylcarbamate) has been applied to control pests such as leafhoppers, aphids, mosquitoes, mites, and cockroaches across the world due to its rapid knockdown and long residual effect. Although propoxur has an effective insecticidal property, several studies have showed the applied pesticides are transported to surface water and are also highly toxic to humans and other organisms such as birds, honey bees and aquatic organisms (WHO, 2005). Moreover, microbial processes such as nitrification and production of carbon dioxide are hindered as soil microbial communities are affected by propoxur (Gupta et al., 1975). Therefore, it has been important to reveal the fate of propoxur residues and investigate the abiotic and biotic factors involved in degradation of propoxur in terrestrial and aquatic ecosystems.

Among technologies for eliminating pesticide remains, biodegradation has been

referred to as an environmentally safe and cost-effective method. Several bacterial strains isolated from soils and municipal wastes such as *Pseudomonas* sp., *Neisseria subflava*, *Staphylococcus aureus*, *Aeromonas* sp., *Bacillus pasteurii*, and *Corynebacterium kutsheri* have been reported to degrade propoxur to the phenol derivative known as 2-isopropoxyphenol (Anusha et al., 2009; Gupta et al., 1975; Kamanavalli and Ninnekar, 2000). In order to curtail adverse effects of residual propoxur on the ecosystems in the area of application, the detoxification of the pesticide is needed. However, little information is available on the biodegradation of propoxur and its derivatives. To date, there has been no reports on bacterial mineralization and metabolic pathway of propoxur through microbial activities.

In this study, three syntrophic pairs of bacteria able to completely mineralize propoxur were isolated from agricultural soils. Species diversity was investigated by 16S rRNA gene sequence analysis and extragenic palindromic PCR (REP-PCR) genomic DNA fingerprinting method, and a degradative pathway was proposed on propoxur biodegradation by the SP1 syntrophic pair.

II. MATERIALS AND METHODS

1. Media and culture condition

All isolated bacteria were cultivated on mineral medium (Park and Ka, 2003) containing propoxur at a concentration of 100 μg ml^{-1}. Peptone-tryptone-yeast extract-glucose (PTYG) medium containing (per liter) 0.25 g of peptone (Difco Laboratories, Detroit, USA), 0.25 g of tryptone (Difco), 0.5 g of yeast extract (Difco), 0.5 g of glucose, 0.03 g of magnesium sulfate, and 0.003 g of calcium chloride was used to isolate strains and cultivate colonies for the repetitive REP-PCR (Table 1). All cultures were incubated at 28 °C and liquid cultures were aerated by shaking at 150 rpm on a rotary shaker (Vision Co., Korea).

2. Chemicals

Analytical grade of propoxur (2-isoprpoxyphenyl *N*-methylcarbamate), 2-isopropoxyphenol, aldicarb (2-methyl-2-(methylthio) propanal *O*-(*N*-methylcarbamoyl) oxime), carbaryl (1-naphthyl methylcarbamate), carbofuran (2,2-dimethyl-2,3-dihydro-1-benzofuran-7-yl methylcarbamate), 4-nitrophenol, 2-*sec*-butylphenol, and catechol (1,2-dihydroxybenzene) were obtained from Sigma Chemical Co., St. Louis, Mo.

Table 1. The composition of bacterial culture media

Medium		Composition (g L^{-1})	
PTYG		Peptone	**0.25**
		Tryptone	**0.25**
		Yeast extract	**0.5**
		Glucose	**0.5**
		$MgSO_4$	**0.03**
		$CaCl_2$	**0.003**
MMO	**Sol A**	Na_2HPO_4	**0.71**
		KH_2PO_4	**0.68**
	Sol B	$(NH_4)_2SO_4$	**0.3**
	Sol C	$MgSO_4 \cdot 7H_2O$	**0.05**
	Sol D	$CaCl_2 \cdot H_2O$	**0.001**
	Sol E	$FeSO_4 \cdot 7H_2O$	**0.006**
		$ZnSO_4 \cdot 7H_2O$	**0.0028**
		$MnSO_4 \cdot 7H_2O$	**0.0012**
		$Co(NO_3)_2 \cdot 6H_2O$	**0.0017**
		$CuSO_4 \cdot 5H_2O$	**0.0004**
		$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	**0.0002**

3. Enrichment and isolation of propoxur-degrading bacteria

Agricultural soil samples were collected at various locations in South Korea where crops has been cultivated with propoxur application for several years (Fig. 1). Samples from the top 15 cm of soil were taken, sieved through 2-mm-pore size mesh and kept at 4 °C prior to use. A 15 g amount of each soil sample was transferred to each 50 ml sterilized beaker, treated with propoxur dissolved in distilled water to a final concentration of 100 μg g^{-1} soil, and completely mixed. The treated soil was incubated with periodic mixing at room temperature. Five weeks after the propoxur application, a 1 g of soil sample from each beaker was homogenized in 9.5 ml of a sterilized 0.85 % saline solution by shaking the preparation at 150 rpm on a rotary shaker. Samples (0.1 ml) of appropriate 10-fold dilutions were transferred to test tubes containing 3 ml of propoxur medium (100 μg ml^{-1}). The tubes were incubated at 28 °C for four weeks on a rotary shaker at 150 rpm and degradation of propoxur was analyzed by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and high-performance liquid chromatography (HPLC) (Shimadzu, Japan). The culture of the terminal positive tube showing substantial cell growth and less than 20 % of the propoxur remaining was enriched by two additional transfers into fresh medium. Each enriched culture was streaked onto PTYG agar medium and combinations of single colonies were then tested for propoxur degradation in fresh propoxur medium before strain purification (Fig. 2).

Fig. 1. Soil sampling sites in South Korea.

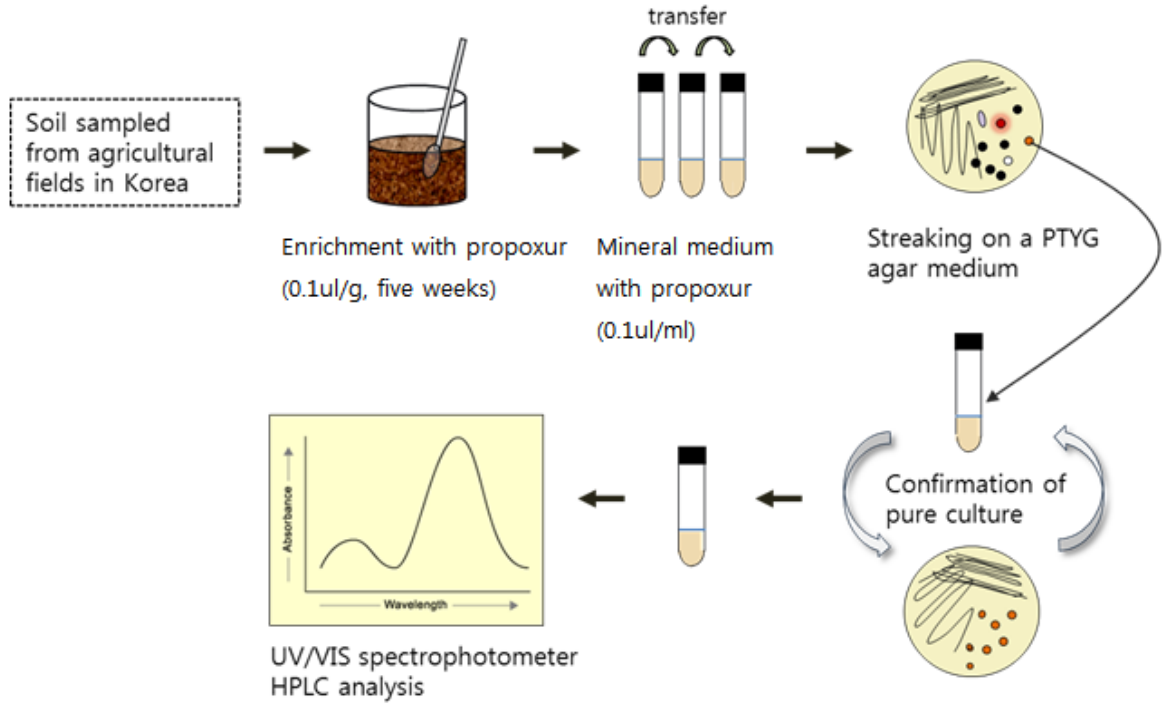

Fig. 2. Isolation of propoxur-degrading bacteria

4. Identification of isolates by 16S rRNA gene sequence analysis

Total genomic DNAs were extracted from the isolates and PCR amplification of 16S rRNA genes was performed with 27mf and 1492r as previously described (Weisburg et al., 1991). The amplified 16S rRNA genes were sequenced using an ABI Prism BigDye Terminator Cycle Sequencing Ready Kit according to the manufacturer's instruction (Perkin-Elmer) with the sequencing primers 515r, 926f and 1055r (Goodfellow and Stackebrandt, 1991). Approximately 1,400 obvious nucleotide positions were used for comparison to the data in EzTaxon server (Chun et al., 2007). Sequences from nearest relatives were identified from the Ribosome Database Project (RDP) using the SIMILARITYRANK program of the RDP (Maidak et al., 2000). The sequences were aligned by using CLUSTRALW. A phylogenetic tree was constructed using MEGA, Version 6 (Tamura et al., 2013).

Table 2. PCR conditions for 16S rRNA gene amplification

PCR reaction mixture		
10× buffer		5.0 µl
dNTP (2.5mM)		5.0 µl
27mf primer (20pmole μl^{-1})		5.0 µl
1492r primer (20pmole μl^{-1})		5.0 µl
Tag polymerase (5 U μl^{-1})		0.5 µl
Distilled water		28.5 µl
Template DNA		1.0 µl
PCR reaction condition		
Step 1	94 °C	5.0 min
Step 2	94 °C	1.0 min
Step 3	55 °C	1.0 min
Step 4	72 °C	1.0 min
Step 2, 3, 4: 29 cycles		
Step 5	72 °C	10 min
Step 6	4 °C	

5. Colony REP-PCR

To distinguish the isolates at subspecies level using genomic fingerprints of the isolates, colony REP-PCR was performed using BOXA1R primer (5'-CTACGGCAAGGCGACGCTGACG-3'), as described previously (Debruijn, 1992). Each isolate was grown on PTYG agar medium for 48 h, and a small amount of cells was resuspended in 25 µl of PCR mixture containing Gitschier buffer [1 M $(NH_4)_2SO_4$, 1M Tris–HCl (pH 8.8), 1 M $MgCl_2$, 0.5M EDTA (pH 8.8), 14.4 M β-mercaptoethanol], 0.1 % bovine serum albumin, 100 % dimethyl sulfoxide, each deoxynucleotide triphosphate at a concentration of 2.5 mM, and 50 pmole μl^{-1} of DNA. The cycles used were as follows: 1 cycle at 93 °C for 7 min, 35 cycles at 92 °C for 1 min, 52 °C for 1 min, and at 65 °C for 8 min; 1 cycle at 65 °C for 16 min; and a final soak at 4 °C (Table 3). After the reactions, PCR products were separated by electrophoresis on 1.2 % agarose gels. After electrophoresis, the image was photographed with UV trans-illumination (306 nm).

Table 3. Colony REP-PCR conditions

PCR reaction mixture		
Gitschier buffer		5.0 µl
BSA (0.1 %)		0.4 µl
DMSO (100 %)		2.5 µl
dNTP (2.5 mM)		5.0 µl
BOXA1R primer (50 pmole μl^{-1})		5.0 µl
Tag polymerase (5 U μl^{-1})		0.8 µl
Distilled water		1.8 µl
Template DNA		1.0 µl
PCR reaction condition		
Step 1	93 °C	7.0 min
Step 2	92 °C	1.0 min
Step 3	52 °C	1.0 min
Step 4	65 °C	8.0 min
Step 2, 3, 4: 35 cycles		
Step 5	65 °C	16 min
Step 6	4 °C	

6. Analysis of bacterial growth and degradation of propoxur

The isolates were cultivated in PTYG medium for 24 hours. Cells were collected by centrifugation at 19,000 g for 10 min at room temperature and washed two times with mineral medium. Aliquots of resuspended cells were inoculated into flasks containing 200 ml of mineral medium supplemented with propoxur (100 μg ml^{-1}) as the sole carbon source at a final density of OD_{600} = 0.01. All cultures were incubated at 28 °C in the dark on a rotary shaker (150 rpm). At routine intervals, aliquots of the cultures were taken out and used to measure cell growth and the concentration of propoxur residues. Cell growth was determined at optical density 600 nm with spectrophotometer. For quantification of propoxur, 3 ml of cultures were filtered using a PTFE syringe filter. Filtered culture was used for the measurement of residual propoxur using reverse-phase HPLC, and the concentration of propoxur was calculated using standard curve prepared from the known concentrations of propoxur in mineral medium.

7. Analysis on effects of temperature on propoxur biodegradation

The inoculums were prepared in the same manner as described above. Five cultures were incubated at 15, 20, 25, 28, and 37 °C in the dark on a rotary shaker (150 rpm), respectively. At routine intervals, aliquots of the cultures were taken out and used to measure cell growth and the concentration of propoxur. Cell growth was determined at optical density 600 nm with spectrophotometer. For quantification of propoxur, 3 ml of cultures were filtered using a PTFE syringe filter. Filtered culture was used for the measurement of residual propoxur using reverse-phase HPLC. The concentration of propoxur was calculated using standard curve prepared from the known concentrations of propoxur in the same medium.

8. Analysis on effects of extra carbon and nitrogen sources on propoxur biodegradation

The inoculums were prepared in the same manner as described above. Glucose was used as an external carbon source, and peptone and ammonium nitrate were added to propoxur mineral medium as additional nitrogen sources, respectively. Mineral medium containing propoxur were supplemented with extra nutrients dissolved in mineral medium to a final concentration of 100 µg ml^{-1}. Cultures containing glucose, peptone, and ammonium nitrate were incubated at 28 °C and 150 rpm in the dark on a rotary shaker. At routine intervals, aliquots of the cultures were taken out and used to measure cell growth and the concentration of propoxur. Cell growth was determined at optical density 600 nm with spectrophotometer. For quantification of propoxur, 3 ml of cultures were filtered using a PTFE syringe filter. Filtered culture was used for the measurement of residual propoxur using reverse-phase HPLC. The concentration of propoxur was calculated using standard curve prepared from the known concentrations of propoxur in the same medium.

9. Chemical analysis and identification of intermediates

Incubation conditions were same as above. The 30 mL of culture samples were filtered with PTFE syringe filters with a pore size of 0.2 μm (Pall Corporation), and compounds in the filtered samples were extracted and concentrated using solid phase extraction. Solid phase extraction was conducted with Sep-Pak Plus C18 cartridges (Waters Corporation) using a Visiprep 24^{TM} SPE vacuum manifold (Supelco) which enables to simultaneously extract up to 24 samples. The cartridges were activated by passing 10 mL of acetonitrile and 10 mL of distilled water, successively. The prepared samples were poured and percolated into the cartridges at a flow rate of 1 mL min^{-1}. The cartridges were washed with 10 mL of distilled water and vaccum-dried for 5 min to eliminate remaining water. Lastly, the retained target compounds were eluted with 2 mL of acetonitrile. Concentrated samples were analyzed by HPLC and GC-MS. Residual propoxur was analyzed by HPLC on a Luna 5u C18 column (4.6 mm × 250 mm). Propoxur was detected with SPD-10A VP UV–Vis detector (Shimadzu) at 280 nm. The mobile phase was acetonitrile/water (70 : 30, v/v), and the flow rate was 1 mL min^{-1}. Metabolites of propoxur were determined by GC-MS analysis. The GC-MS analyses were achieved in electron ionization mode (70 eV) with Perkin-Elmer clarus 680 GC equipped with DB-5MS column (30 m × 0.25 mm id; 0.25 μm film thickness). The column temperature system was programed from 120 °C (1 min hold) to 240 °C at 20 °C min^{-1} and then held for 8 min. The helium was used as the carrier gas at a constant flow of 2 mL min^{-1}. The samples were analyzed in split mode (1 : 20) at an injection temperature of 230 °C and detected in the mass range from m/z 30 to 650.

10. PCR amplification of the genes involved in the hydrolysis of carbamates

The partial gene sequences specific to the propoxur degradation pathway were amplified by PCR with specific primers targeted for the carbofuran-hydrolase gene *mcd*, and the carbaryl-hydrolase genes *cahA*, and *cehA*. The primers for the *mcd* gene were designed based on the conserved gene sequence found in carbofuran hydrolase genes of *Achromobacter* sp. WM111 (Parekh et al., 1996): mcdL1-For primer, 5'-CAAGAACTCAAATCCATCTACCTTGCCC-3' and mcdR1-Rev primer 5'-ATCCTTCCCTCGGAATGA ATCGTCTCG-3'. The primer sequences for the *cahA* gene have been reported in *Arthrobacter* sp. RC100 (Hashimoto et al., 2006): cahA-For primer, 5'-CAGTGCCGCCAYCGTGGYATG- 3' and cahA-Rev primer, 5'-GCCACTTCCATGYCCRCCCCA-3'. The primer sequences for the *cehA* gene have been reported in *Arthrobacter* sp. AC100 (Hashimoto et al., 2002): cehA-For primer, 5'-GGACCAACCATTCAAACCAG-3' and cehA-Rev primer, 5'-GCGGATCAAACTGTGCTTCT-3'. The amplification of the *mcd*, *cahA*, and *cehA* genes with the corresponding primers was expected to produce 590, 1,498, and 2,295 bp DNA fragments, respectively (Table 4).

Table 4. PCR primers and product sizes

Primer	Target	Sequences	size	Source of reference
mcdL1-F mcdL1-R	Methylcarbamate-degrading gene (*mcd*)	5’-CAAGAACTCAAATCCATCTACCTTGCCC-3’ 5’-ATCCTTCCCTCGGAATGA ATCGTCTCG-3’	590 bp	Parekh et al., 1996
cahA-F cahA-R	Carbaryl hydrolase gene (*cahA*)	5’-CAGTGCCGCCAYCGTGGYATG- 3’ 5’-GCCACTTCCATGYCCRCCCCA-3’	1498 bp	Hashimoto et al., 2006
cehA-F cehA-R	Carbaryl hydrolase gene (*cehA*)	5’-GGACCAACCATTCAAACCAG-3’ 5’-GCGGATCAAACTGTGCTTCT-3’	2295 bp	Hashimoto et al., 2002

III. RESULTS

1. Isolation of propoxur-degrading bacteria

During enrichment process three syntrophic bacterial pairs were isolated from agricultural soils collected in different locations. 112 out of 120 soil samples did not show any degradation of propoxur during incubation on mineral medium containing propoxur for five weeks. Among 8 samples which showed detectable degradation of propoxur, 5 samples were partially capable of degrading propoxur. Only three samples could completely mineralized propoxur as a source of carbon and energy. During strain purification steps on these 3 samples, there were no single strains able to mineralize propoxur. When combinations of the different colony types from each of cultures were inoculated on propoxur mineral medium, three combinations consisting of two bacterial strains (denoted by a and b) capable of degrading propoxur without any dead end products were isolated from three different soils (Table 5).

Table 5. Nearest relatives of the propoxur-degrading isolates based on 16S rRNA gene sequence analysis

Isolates	Accession number	Soil sites	Nearest relative[a]	Similarity (%)
SP1a	KX518600	Seogwipo, Jeju-do	*Pseudaminobacter salicylatoxidans*	97.61
SP1b	KX518601	Seogwipo, Jeju-do	*Nocardioides nitrophenolicus*	99.93
SP2a	KX518602	Seogwipo, Jeju-do	*Pseudaminobacter salicylatoxidans*	97.68
SP2b	KX518603	Seogwipo, Jeju-do	*Nocardioides nitrophenolicus*	99.93
UR1a	KX518604	Uiryeong, Gyeongsangnam-do	*Mesorhizobium huakuii*	99.79
UR1b	KX518605	Uiryeong, Gyeongsangnam-do	*Nocardioides kongjuensis*	98.21

[a] Based on nearly fully sequences of the 16S rRNA gene.

2. Strain identification by 16S rRNA gene sequence analysis

When analyzed by 16S rRNA gene sequences, strains SP1a and SP2a showed 97.61 % and 97.68 % degree of similarities with *Pseudaminobacter salicylatoxidans* BN12^T (AF072542), respectively (Table 5). Their colonies were circular, convex, orange colored on the center, and whitish on the edge of the colonies. Strain UR1a was closely related to *Mesorhizobium huakuii* IAM 14158^T (D12797). Its colonies were circular, raised, mucilaginous, and white. These isolates were found to be belonged to Gram-negative bacteria. On the other hand, strains SP1b, SP2b, and UR1b were found to be Gram-positive and belonged to members of genus *Nocardioides*. Strains SP1b and SP2b showed 99.93 % degree of 16S rRNA gene sequence similarities with *Nocardioides nitrophenolicus* NSP 41^T (AF005024), respectively, and their colonies were flat, smooth, and yellowish-white with irregular edges. Strain UR1b showed 98.21 % similarity with *Nocardioides kongjuensis* A2-4^T (DQ218275). Morphological characteristics of the colonies of strain UR1b were similar to those of strains SP1b and SP2b. Figure 3 shows phylogenetic tree of three syntrophic pairs and their related species based on the 16S rRNA sequences. Although strains were isolated from different soils, the isolates were phylogenetically related. Colony REP-PCR experiment was conducted by PCR amplification with the BOXA1R primer in order to investigate genomic relatedness among the strains. One identical REP-PCR pattern was obtained from strain SP1b and SP2b and four different DNA fingerprinting patterns were obtained (Fig. 4). This result means that strains SP1a and SP2a were different and strain SP1b and SP2b were identical strains.

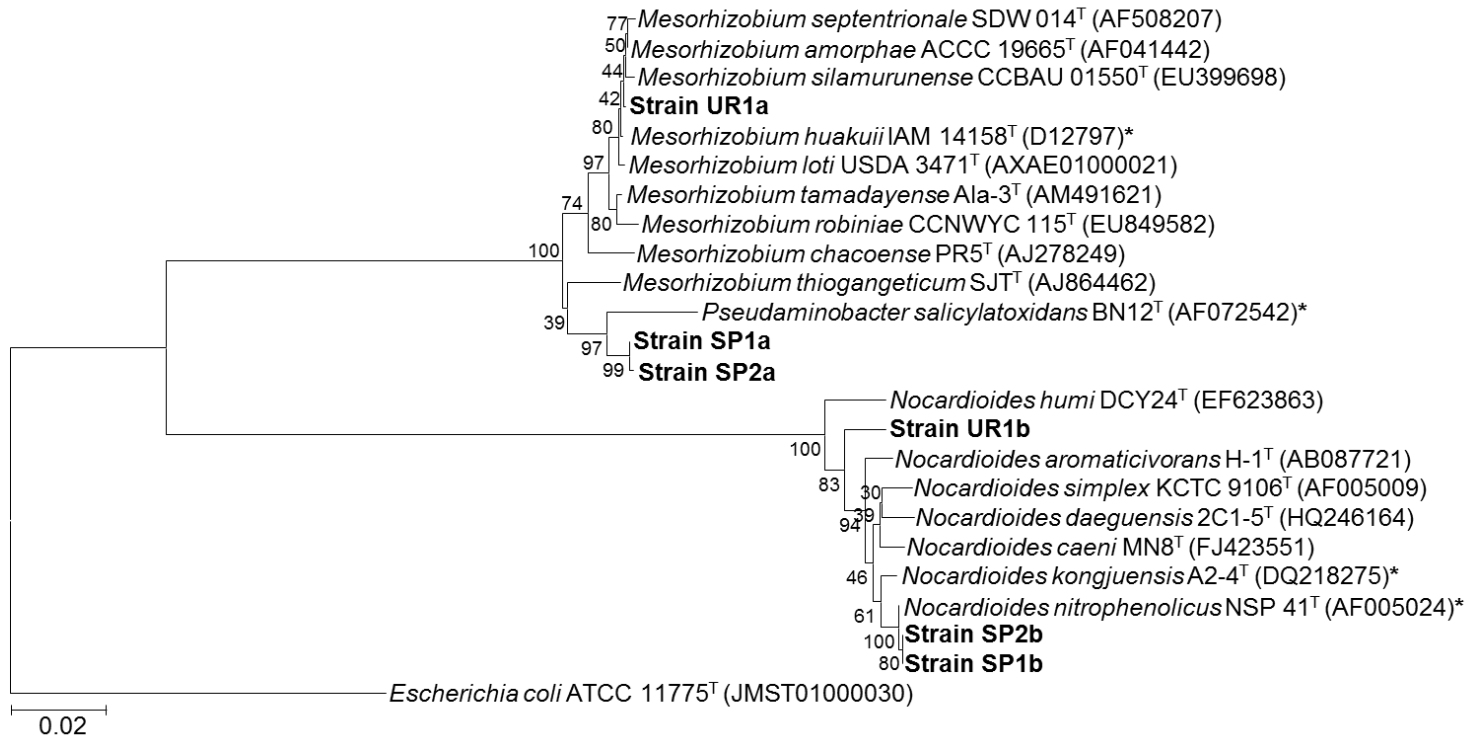

Fig. 3. Phylogenetic relationships constructed by the neighbor-joining method based on 16S rRNA gene sequences of the isolates and closely related type strains (*). Numbers on braches indicate bootstrap confidence estimates obtained with 1,000 replicates. Scale bar represents an evolutionary (Knuc) of 0.02.

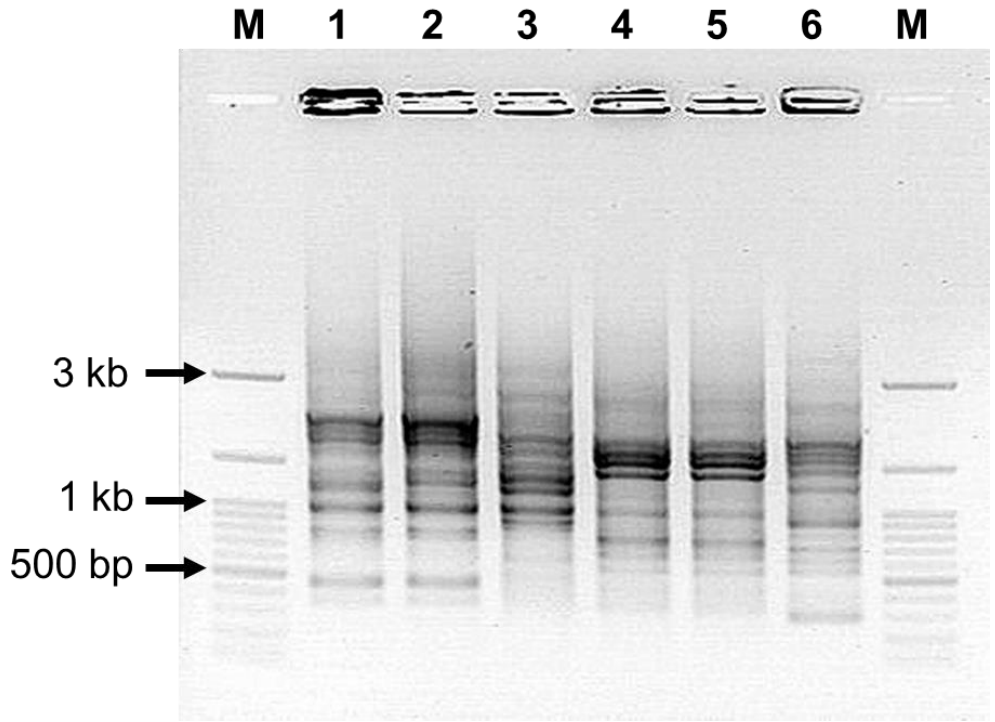

Fig. 4. REP-PCR patterns of the isolates. Lanes: 1, SP1a; 2, SP2a; 3, UR1a; 4, SP1b; 5, SP2b; 6, UR1b; M, DNA size marker.

3. Syntrophic biodegradation of propoxur by SP1 pair

All syntrophic pairs were able to fully degrade propoxur with no remains when propoxur was supplemented as a sole carbon and energy source (Fig. 5). Among these pairs, SP1 pair showing the fastest degradation activity was further investigated. To comprehend growth patterns of the syntrophic pair SP1, each of the pairs was inoculated into mineral medium containing propoxur (100 μg ml^{-1}). SP1 pair grew in propoxur medium, degrading propoxur completely without any residual compounds (Fig. 5a; Fig. 6; Fig. 7b). When each of the strains which consisting of SP1 pair was inoculated separately, strain SP1a could grow slightly on propoxur mineral medium but strain SP1b could not grow on the same medium (Fig. 6). When measured with HPLC, the concentration of an intermediate increased as that of propoxur decreased in propoxur mineral medium inoculated with strain SP1a (Fig. 7a). Through HPLC assay and GC-MS, the residual compound was identified as 2-isopropoxyphenol. However, the concentration of propoxur remained steady during entire incubation period on medium inoculated with strain SP1b (Fig. 7a). These results indicate that propoxur was utilized as a carbon and energy source by not strain SP1b but strain SP1a. To verify that strain SP1b utilize 2-isopropoxyphenol produced by strain SP1a, strain SP1b was inoculated on mineral medium containing 2-isopropoxyphenol as a sole carbon source. Strain SP1b could degrade and utilize 2-isopropoxyphenol, indicating that propoxur was completely degraded by syntrophic metabolism of the two strains (Fig. 8).

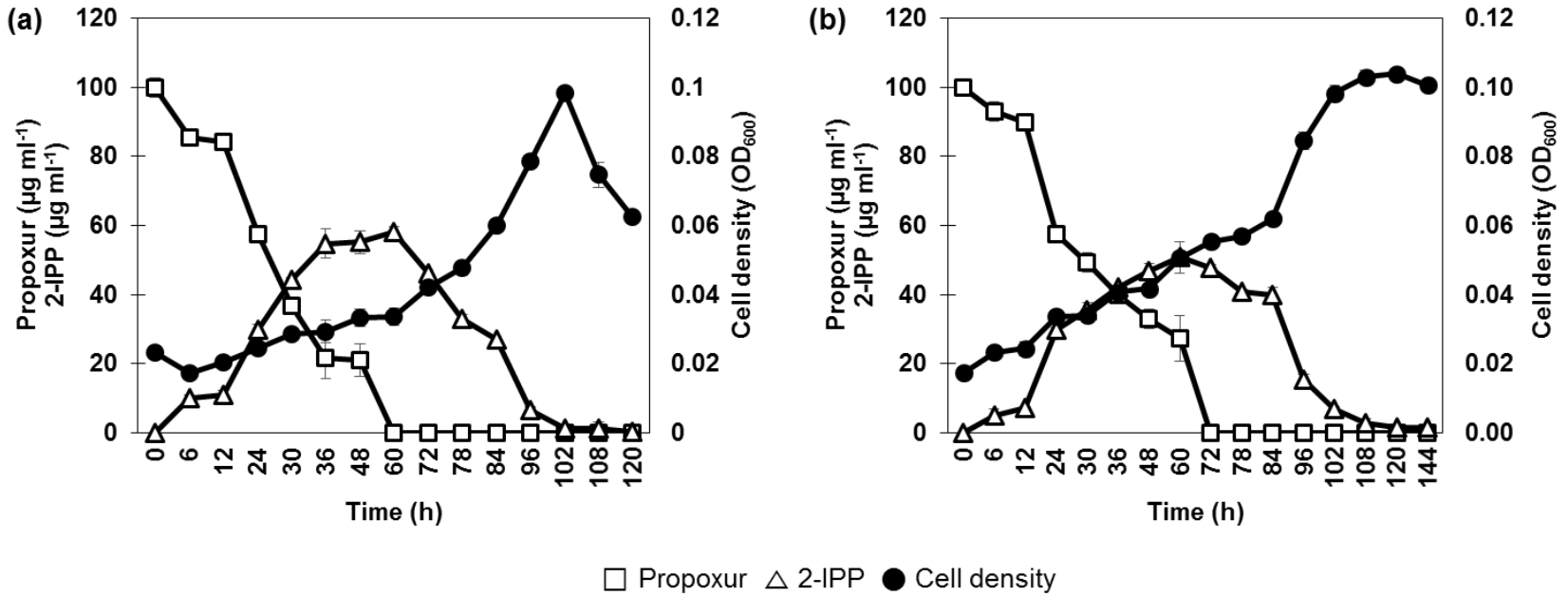

Fig. 5. Degradation of propoxur and cell growths of syntrophic pairs. (a) Syntrophic pair SP1; (b) Syntrophic pair UR1. (□) concentration of propoxur resiudes; (△) concentration of 2-isopropoxyphenol residues; (●) OD 600 nm values of syntrophic pairs in propoxur mineral medium.

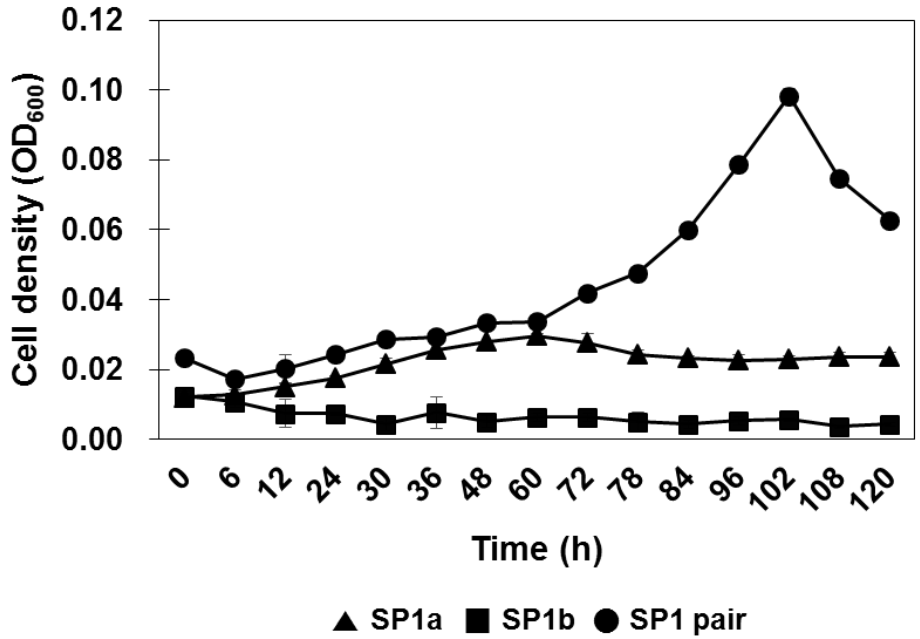

Fig. 6. Growth profiles of strains SP1a and SP1b in propoxur mineral medium. (▲) slight growth of strain SP1a; (■) no growth of strain SP1b; (●) syntrophic growth of strains SP1a and SP1b.

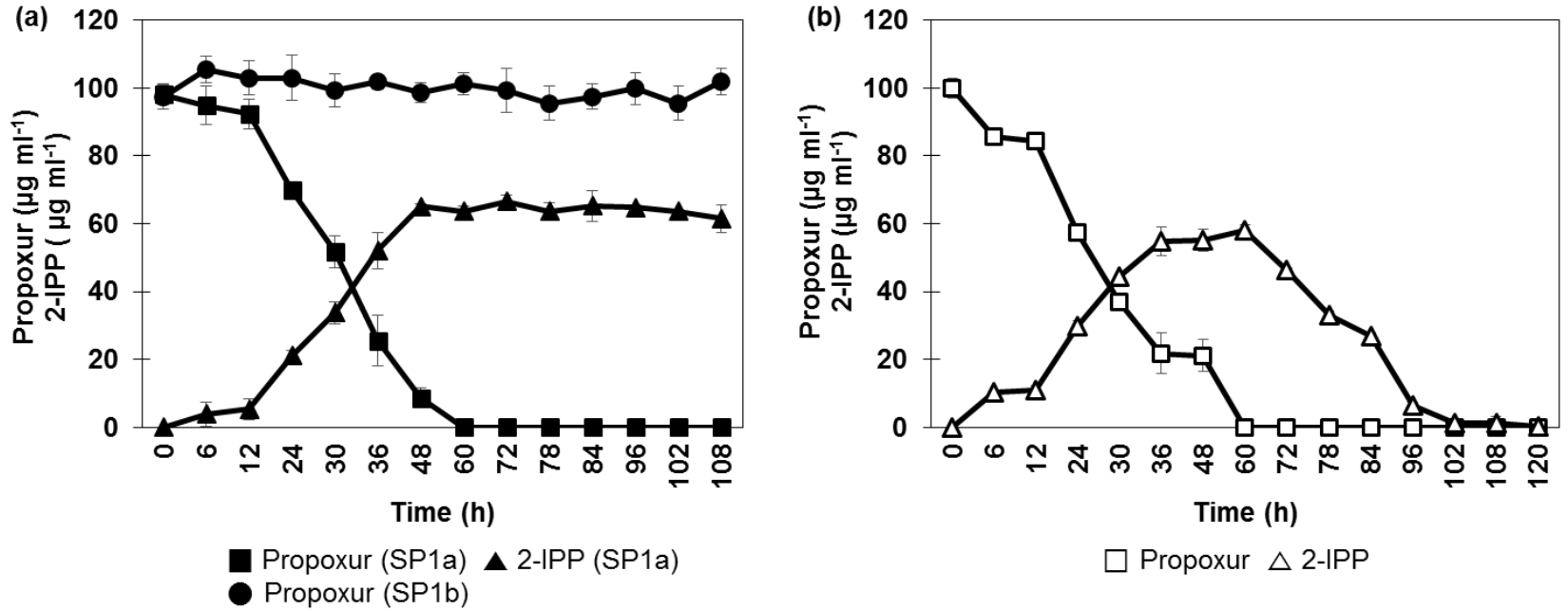

Fig. 7. Degradation profiles of SP1 pair and its members. (a) Degradation profiles by single strains; (■) concentration of propoxur residues in propoxur mineral medium inoculated with strain SP1a; (▲) concentration of 2-isoporpoxyphenol residues in propoxur mineral medium inoculated with strain SP1a; (●) concentration of propoxur residues in propoxur mineral medium inoculated with strain SP1b. (b) Degradation profile by syntrophic pair SP1; (□) concentration of propoxur resiudes in propoxur mineral medium inoculated with SP1 pair; (△) concentration of 2-isopropoxyphenol residues in propoxur mineral medium inoculated with SP1 pair.

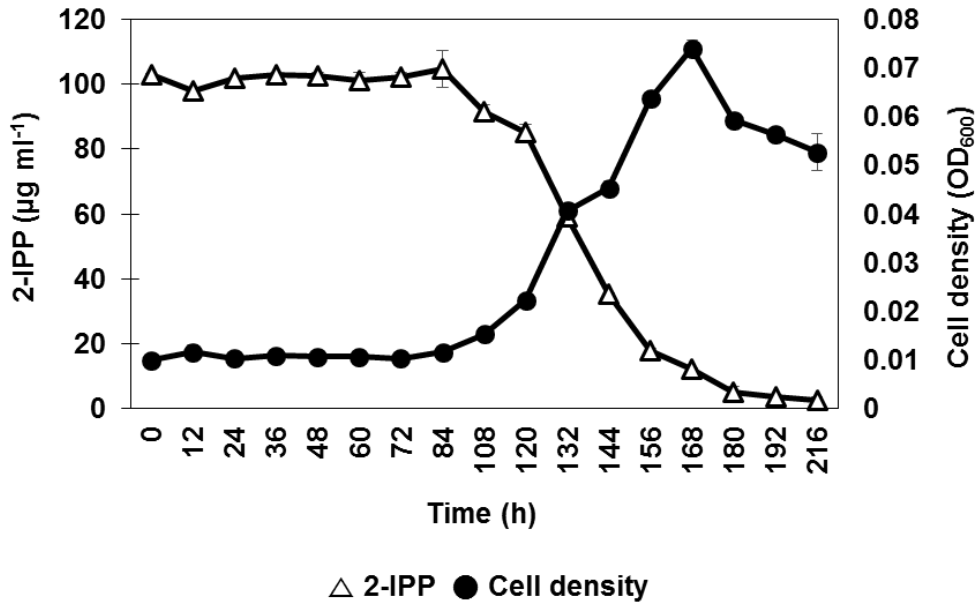

Fig. 8. Growth and degradation profiles of strain SP1b in 2-isopropoxyphenol mineral medium. (△) concentration of 2-isopropoxyphenol residues; (●) OD 600 nm values of strain SP1b in 2-isopropoxyphenol mineral medium.

4. Effect of temperature on propoxur biodegradation

To investigate changes in propoxur degradation ability in diverse temperature conditions, five combinations of strain SP1a and SP1b were inoculated into propoxur mineral medium and were incubated at 15, 20, 25, 28, and 37 °C, respectively. Figure 9 shows the growth patterns of SP1 pair incubated at different temperature. Except the pair incubated at 37 °C, the pairs grown in relatively cold or moderate temperature conditions degraded and utilized propoxur for their growth. In particular, the cell density of SP1 pair incubated at 25 °C increased most quickly during the same period (Fig. 9).

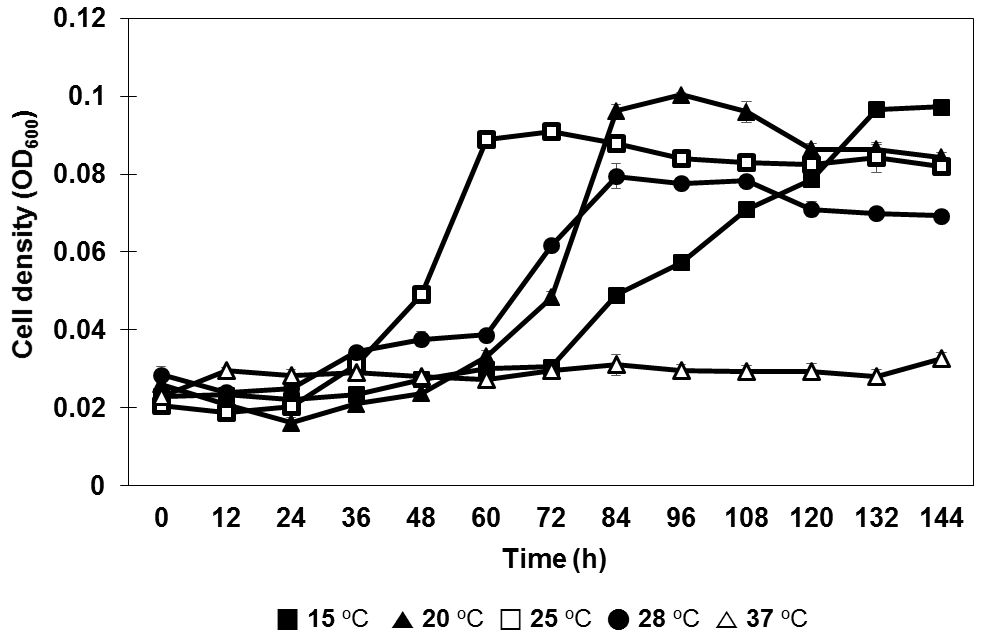

Fig. 9. Growth profiles of syntrophic pair SP1 at different temperatures. (■) growth at 15 °C; (▲) growth at 20 °C; (□) growth at 25 °C; (●) growth at 28 °C; (△) growth at 37 °C.

5. Effect of extra carbon and nitrogen sources on propoxur biodegradation

In order to evaluate change in propoxur degradation ability of SP1 pair when other nutrients exist with propoxur, glucose, ammonium nitrate, and peptone were used as additional carbon, fixed nitrogen, and proteinaceous and peptidic nitrogen, respectively. Cell density of SP1 pair increased sharply when SP1 pair was inoculated into propoxur medium with peptone or glucose. Although additional fixed nitrogen source was supplemented into propoxur medium, cell density of the culture into which fixed nitrogen was supplemented was similar to that of SP1 pair incubated into propoxur mineral medium (Fig. 10b). On the other hand, in addition to increase in cell density, propoxur was degraded faster when extra glucose or peptone were present, showing a shorter lag period after propoxur was transformed to 2-isopropoxyphenol (Fig. 10c and d). This results indicate that glucose and peptone as readily available resources may provide energy and materials needed for enzyme synthesis with the syntrophic pair.

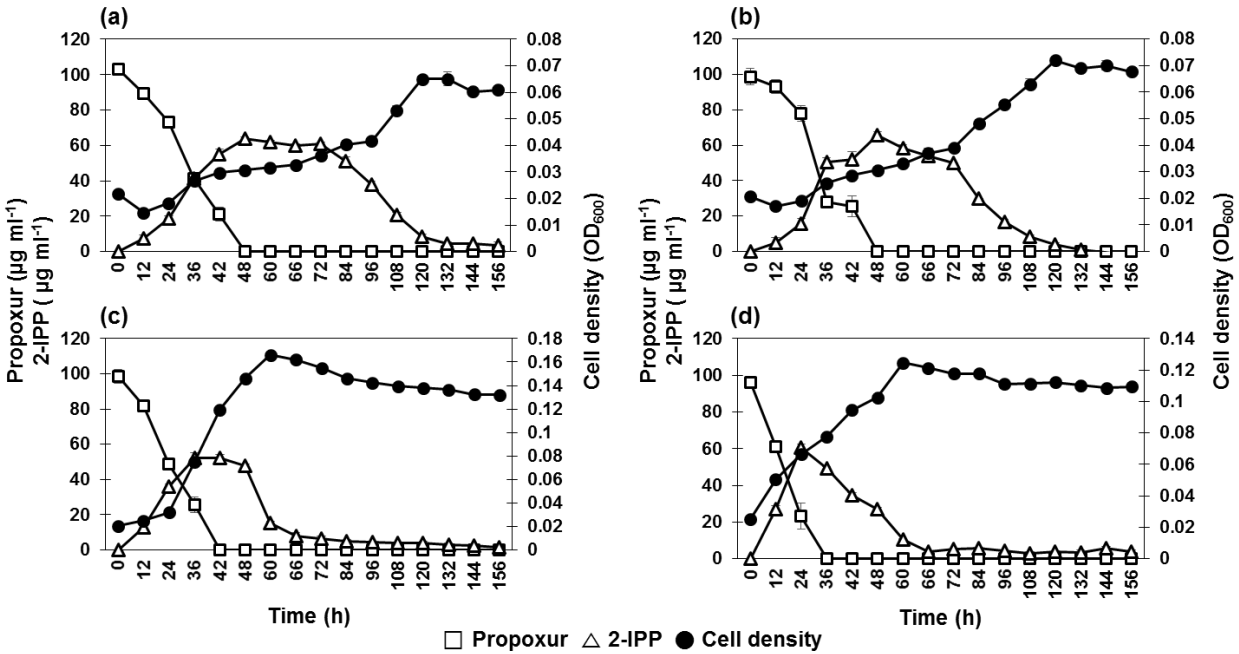

Fig. 10. Degradation of propoxur and cell growth of SP1 pair with extra carbon and nitrogen sources. (a) Degradation of propoxur and cell growth of SP1 pair without any supplements; (b) Degradation of propoxur and cell growth of SP1 pair with ammonium nitrate; (c) Degradation of propoxur and cell growth of SP1 pair with glucose; (d) Degradation of propoxur and cell growth of SP1 pair with peptone. (□) concentration of propoxur resiudes; (△) concentration of 2-isopropoxyphenol residues; (●) OD 600 nm values of SP1 pair in propoxur mineral medium. All sources were supplemented at a concentration of 100 µg ml^{-1}.

6. Substrate utilization diversity analysis

Strains SP1a and SP1b were grown on the PTYG medium to investigate whether these strains are capable of using compounds which are structurally related to propoxur or 2-isopropoxyphenol. Each strain was inoculated separately and together on the mineral medium containing carbamate pesticides or phenolic compounds shown in table 6. Strain SP1a was able to fully degrade carbaryl, but hardly use other carbamates and phenolic compounds. On the other hands, strain SP1b grew on the mineral medium containing phenolic compounds such as 2-isopropoxyphenol, 4-nitrophenol, and catechol. However, this strain did not use 2-*sec*-butylphenol and the carbamate pesticides as a carbon and energy source (Table 6). This means that strain SP1a could degrade a common functional group in *N*-methyl carbamates and strain SP1b play a role in mineralizing phenolic compounds which possess two oxygen atoms for the cleavage of a benzene ring structure in their molecules.

Table 6. Substrate utilization patterns by SP1 pair and its members

Isolate[a]	Substrate[b]								
	Carbamate pesticides					Phenols			
	Propoxur	**Aldicarb**	**Carbaryl**	**Carbofuran**	**Fenobucarb**	**2-IPP**	**2-*sec*-Butylphenol**	**4-Nitrophenol**	**Catechol**
SP1a	+	+	++	+	-	-	-	-	-
SP1b	-	-	-	-	-	++	-	++	++
SP1 pair	++	+	++	+	-	++	-	++	++

[a] The isolates were grown on PTYG before the test of substrate utilization.

[b] ++: Over 95% reduction in peak height as determined by UV scanning and substantial growth (OD_{600} >0.05), +: 40 to 60% reduction in peak height as determined by UV scanning and substantial growth (OD_{600} >0.025), -: below 10% reduction in peak height and scant growth (OD_{600}<0.007).

7. Identification of intermediates and degradation pathway

To investigate the syntrophic degradation pathway of propoxur, strain SP1a which was capable of degrading propoxur was inoculated into propoxur mineral medium. After 24 hours incubation with strain SP1a, the intermediate was purified and identified with GC-MS analysis, and then, two large peaks A and B were detected (Fig. 11a). Peak A [R_t (min) = 17.13] was identified as propoxur, which was originally contained in propoxur mineral medium (Fig. 12), and peak B [R_t (min) = 11.26] was identified as 2-isopropoxyphenol (Fig. 13). This result indicated that strain SP1a transformed propoxur to 2-isopropoxyphenol. After 84 hours incubation with SP1 pair, metabolites in the culture filtrate was purified, concentrated, and analyzed in the same manner. And then two peaks, peak B [R_t (min) = 11.23] and peak C [R_t (min) = 9.28], were detected in the GC profile (Fig. 11b). Through mass spectrometry analysis, the large peak B [R_t (min) = 14.35] was identified as 2-isopropoxyphenol (Fig. 13). On the other hand, there was no reasonable mass spectrum matched with that of the peak C in NIST mass spectrometry database (Fig. 14). The structure of this peak could be determined with NMR spectroscopy in further research. From the results described above, syntrophic degradation pathway of propoxur by SP1 pair was suggested in figure 15. It was assumed that propoxur was degraded through 2-isopropoxyphenol and methylamine by strain SP1a, and strain SP1b mineralized 2-isopropoxyphenol subsequently.

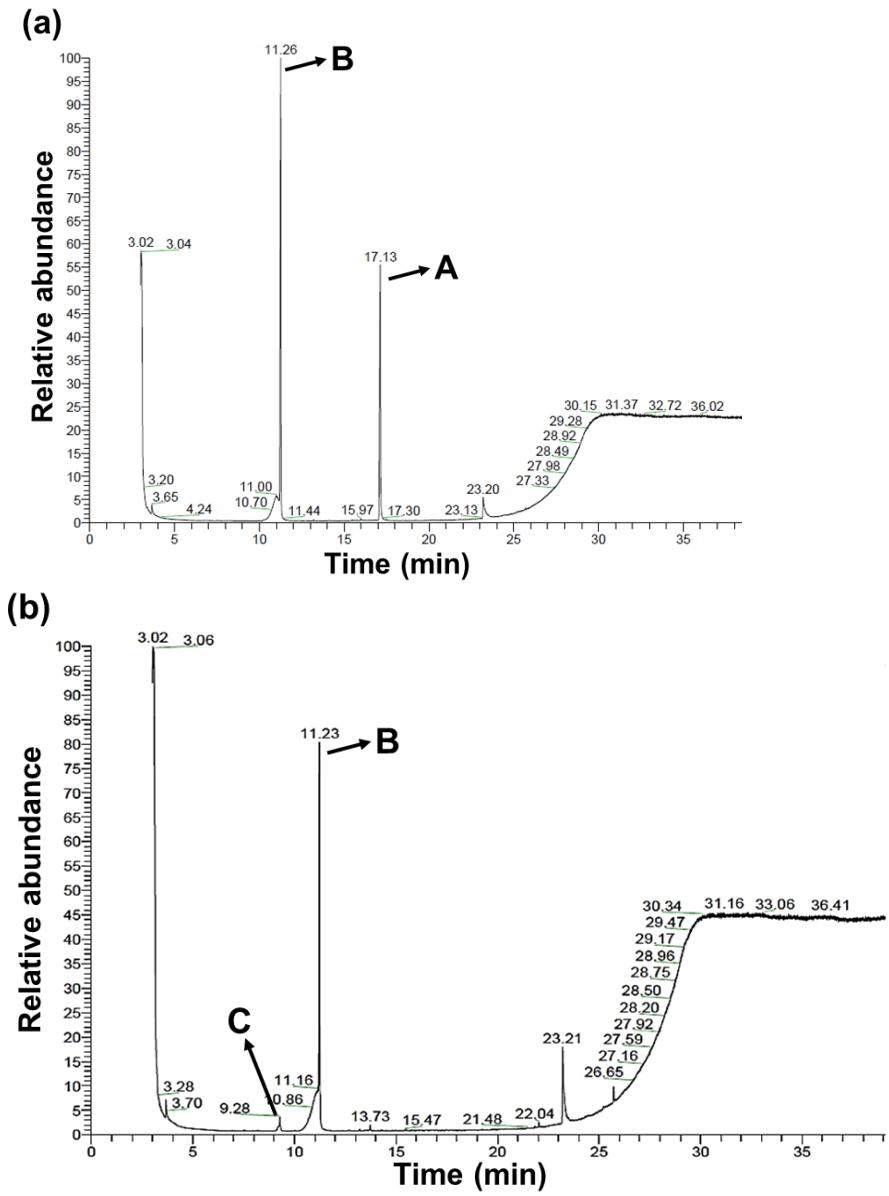

Fig. 11. Gas chromatogram of the culture filtrate for propoxur mineral medium. (a) Gas chromatogram of the culture filtrate for propoxur mineral medium after 24 h inoculation of strain SP1a; (b) Gas chromatogram of the culture filtrate for propoxur mineral medium after 84 h inoculation of SP1 pair. (A) propoxur with a retention time 17.13 min; (B) the hydrolysis product of propoxur with a retention time 11.26 and 11.23 min; (C) the unknown metabolite with a retention time 9.28 min.

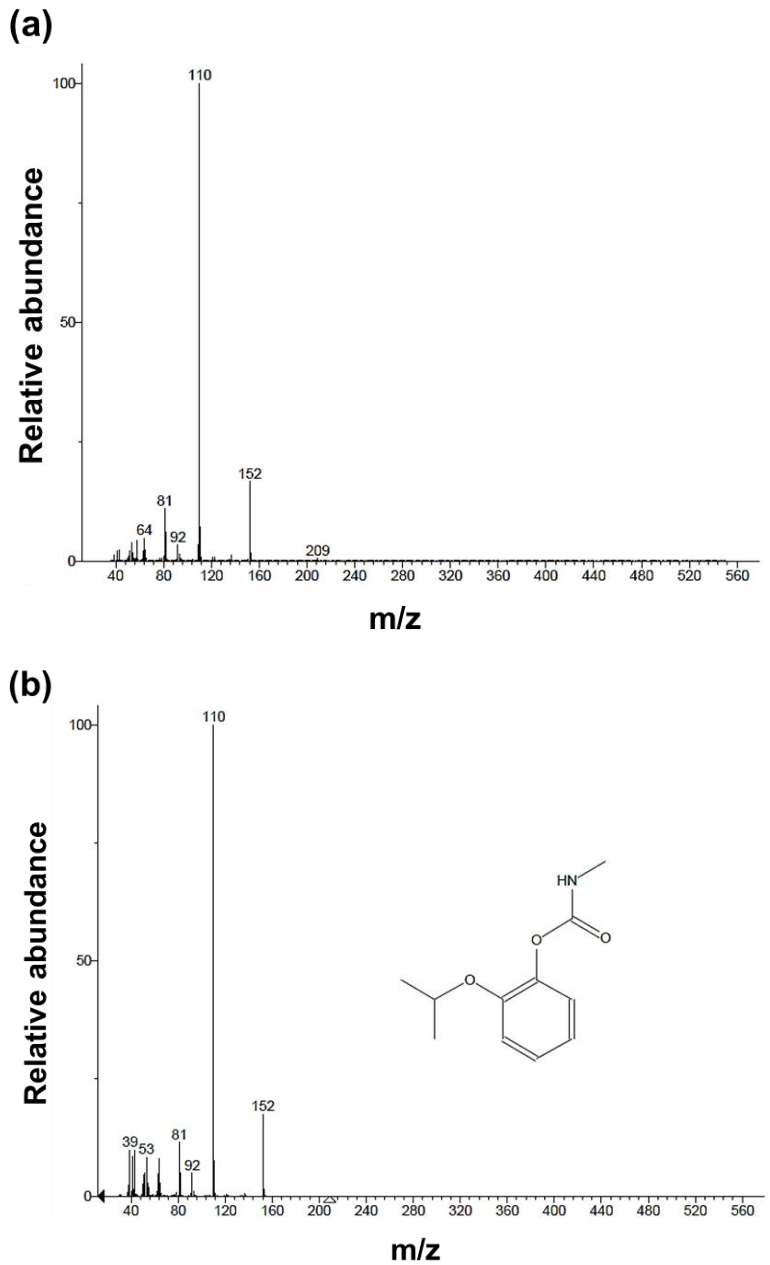

Fig. 12. Mass spectrum of gas chromatogram fragmentation and most related standard peak at NIST library. (a) Fragment patterns of the peak A with a retention time 17.13 min; (b) Standard peak of propoxur in NIST library.

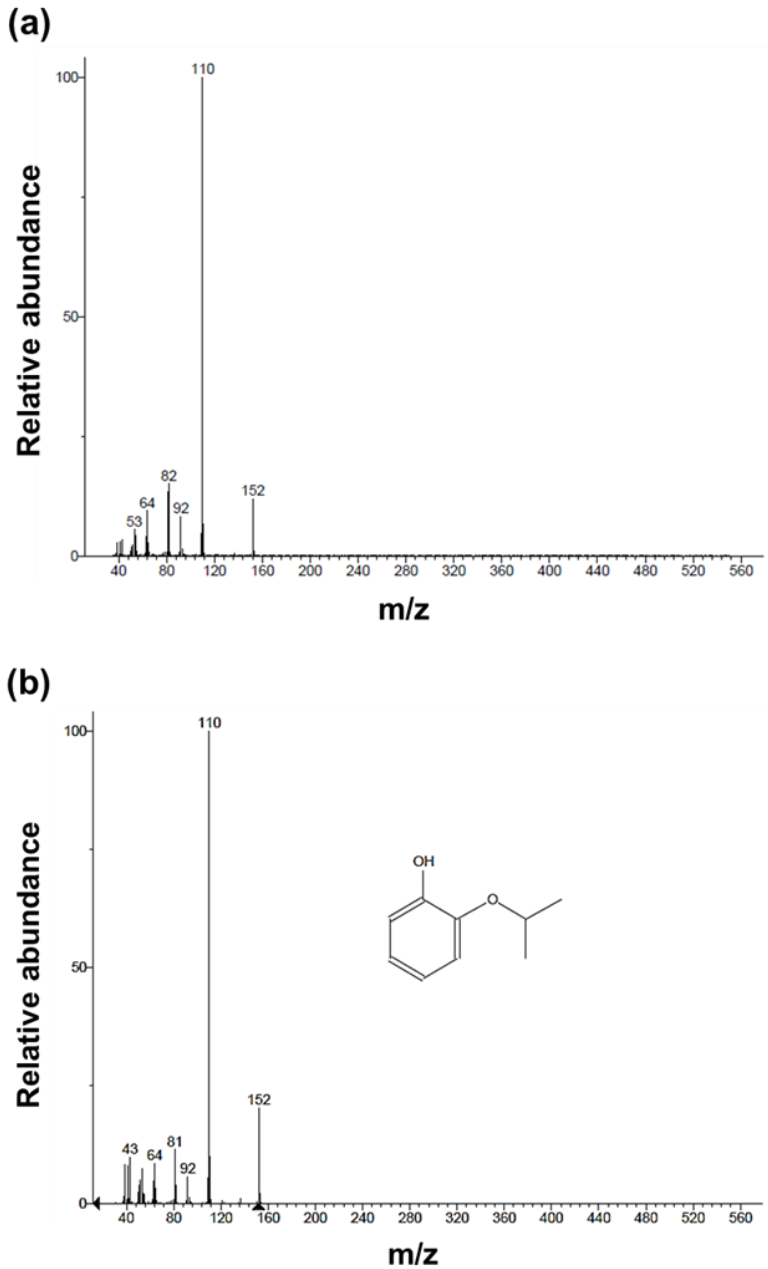

Fig. 13. Mass spectrum of gas chromatogram fragmentation and most related standard peak at NIST library. (a) Fragment patterns of the peak B with a retention time 11.26 and 11.23 min; (b) Standard peak of 2-isopropoxyphenol in NIST library.

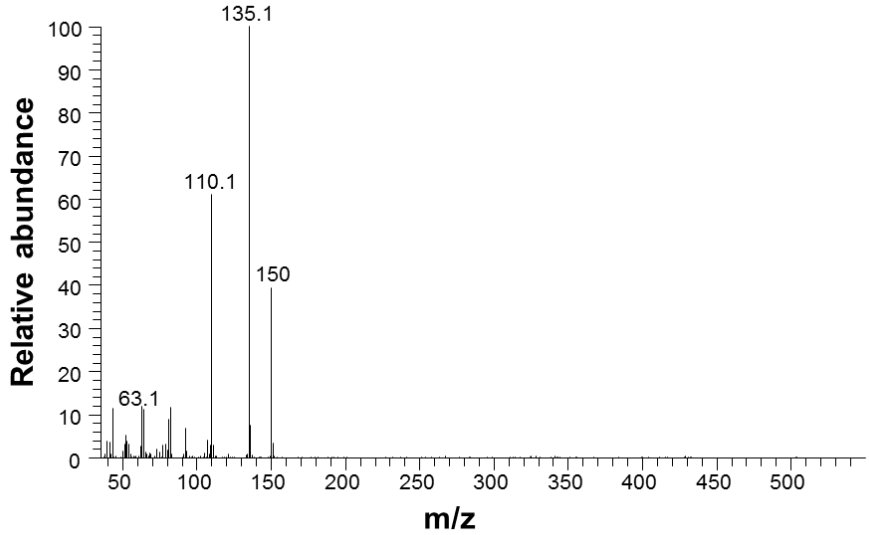

Fig. 14. Fragment patterns of the peak C with a retention time 9.28 min.

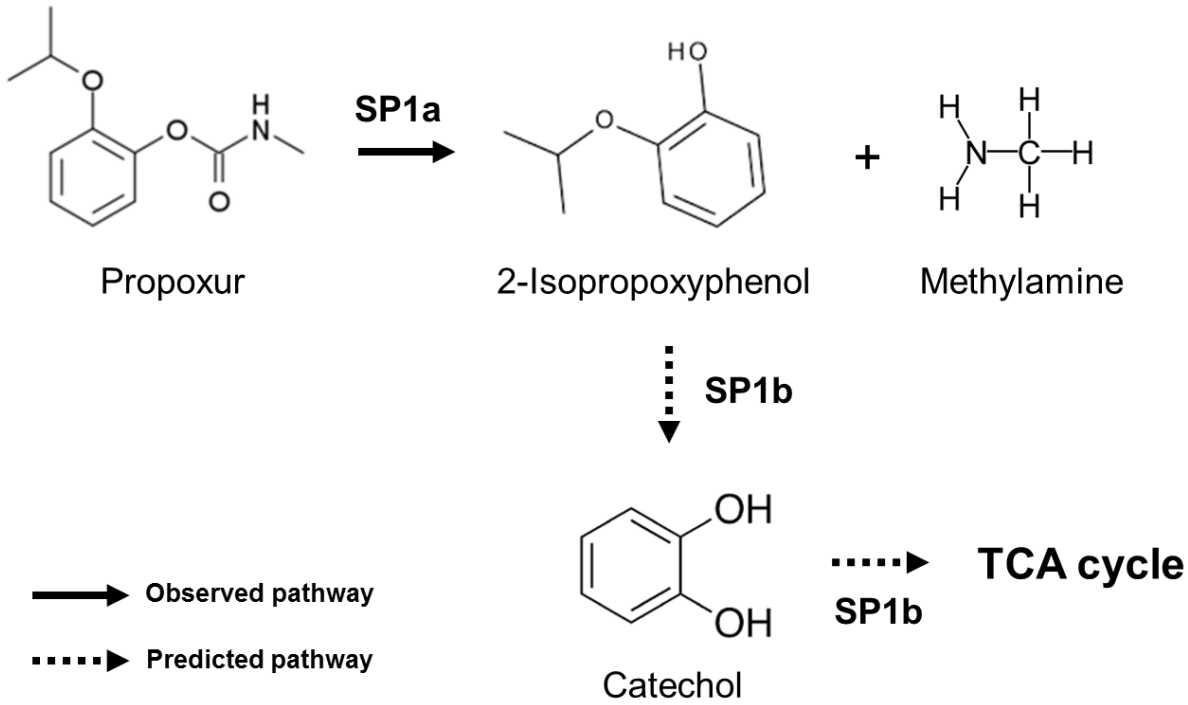

Fig. 15. Proposed metabolic pathway of propoxur by syntrophic pair SP1.

8. PCR amplification of the degradation genes involved in hydrolysis of propoxur

In order to investigate genes related to the degradation of propoxur, PCR amplification was conducted using PCR primers targeting carbamate hydrolase and phenol hydroxylase genes which were previously reported in other bacterial strains. All the isolates did not show any positive bands of methyl carbamate degradation gene (*mcd*) and carbaryl hydrolase gene (*cahA*) (Fig. 16a and b). On the other hand, when PCR amplification was performed using the other carbaryl hydrolase gene (*cehA*), strain SP1a showed a positive DNA band of *cehA* gene (Fig. 16c).

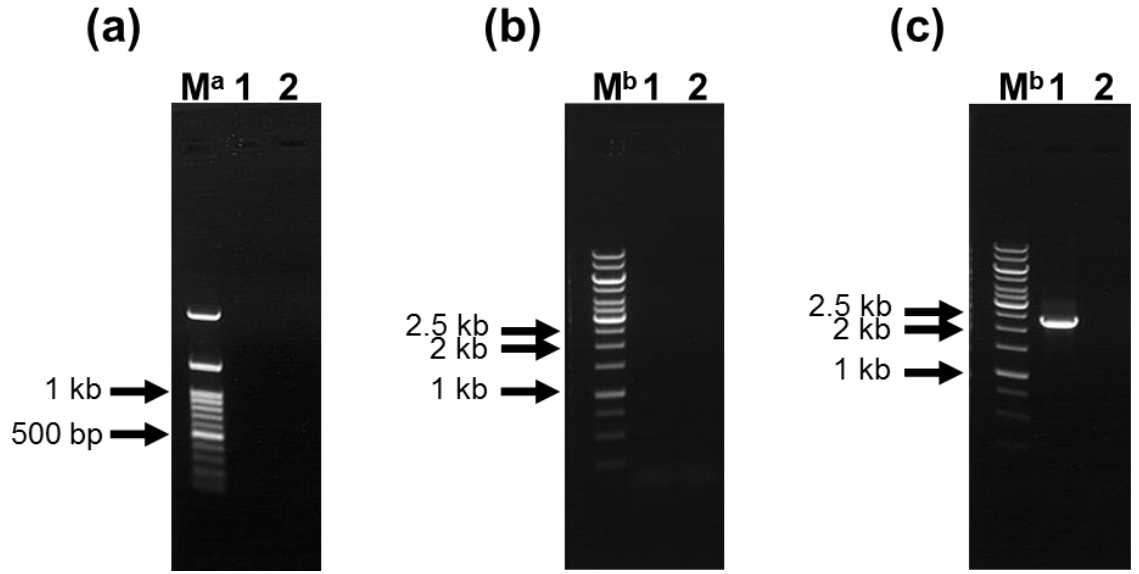

Fig. 16. PCR amplification with the primers specific for the genes involved in degradation of carbamates. (a) PCR amplification with the primers specific for the *mcd* gene; (b) PCR amplification with the primers specific for the *cahA* gene; (c) PCR amplification with the primers specific for the *cehA* gene. Lane: 1, strain SP1a; 2, strain SP1b; M, DNA size marker.

[a] 100 bp DNA ladder

[b] 1 kb DNA ladder

IV. DISCUSSION

Three syntrophic pairs consisting of Gram-negative and Gram-positive bacteria able to degrade propoxur and its phenolic derivative, 2-isopropoxyphenol were isolated from agricultural soil in Korea. 16S rRNA gene sequence analysis revealed that the isolates involved in syntrophic pairs were related to *Pseudaminobacter* sp., *Mesorhizobium*, and *Nocardioides* sp (Table 5; Fig. 4). In previous studies, members of *Pseudaminobacter* have been reported to be able to degrade substituted salicylates, substituted naphthalene, atrazine, and methyl-parathion (Kampfer et al., 1999; Topp et al., 2000). In addition, members of *Mesorhizobium* played an important role in promoting plant growth through symbiotic relationships with legume plants (Laranjo et al., 2014). Some were involved in biodegradation of pesticides or other contaminants. Osborn et al. (2010) isolated *Mesorhizobium* sp. capable of degrading oxamyl from agricultural soils in United Kingdom. Jabeen et al. (2015) also reported that *Mesorhizobium* sp. HN3 was able to degrade chloropyrifos and 3, 5, 6-trichloro-2-pyridinol as a sole carbon and energy source. *Nocardioides* spp. were known to have a role in degradation of pollutants such as *N*-acylhomoserine lactone, alkanes, pyridine, phenanthrene, phenols such as 4-nitrophenol, and pesticides including herbicides (Yoon et al., 1999; Yoon and Park, 2006; Yoon et al., 2006). These reports suggest that the members of three genera have an excellent ability to effectively adapt and degrade xenobiotics or new compounds in ecosystem. Previous studies have explained that members of *Mesorhizobium* and *Aminobacter* which are phylogenetically related to genera *Pseudaminobacter* and *Mesorhizobium* could degrade *N*-methylcarbamate insecticides including carbofuran (Desaint et al., 2000; Ortiz-Hernández et al., 2011). Propoxur has been widely used for pest control in

agriculture and public health but it has adverse effects on not only human but also structures and functions of ecosystems. For this reason, removal of propoxur residues in environment is important. Previous studies on biodegradation of propoxur revealed that single strains such as *Arthrobacter* sp., *Pseudomonas* spp., *Neisseria subflava*, *Staphylococcus aureus*, *Corynebacterium kutscheri*, *Bacillus pasteurii*, and *Aeromonas* sp. and microbial consortia were able to degrade propoxur to 2-isopropoxyphenol and methyl amine (Anusha et al., 2009; Dewi et al., 2015; Gupta et al., 1975; Kamanavalli and Ninnekar, 2000; Ou et al., 1992). However, these studies did not show complete degradation of propoxur and its degradation pathway. To our knowledge, this study is the first report on isolation and characterization of bacterial syntrophic pair able to fully degrade propoxur.

Three propoxur-degrading syntrophic pairs showed similar growth and degradation patterns (Fig. 5). Among three syntrophic pair, when syntrophic pair SP1 were incubated on propoxur mineral medium (100 μg ml^{-1}), lag periods were observed in 12 hours and between 48 and 72 hours after inoculation with syntrophic pair SP1 (Fig. 5; Fig. 10a). A lag period was also observed in mineral medium supplemented with 2-isopropoxyphenol inoculated with strain SP1b (Fig. 8). When strains SP1a and SP1b were inoculated and incubated separately on propoxur mineral medium, strain SP1a could degrade propoxur with residual compounds and strain SP1b could degrade and utilize the metabolite produced by strain SP1a (Fig. 7a and b). From this, the initial lag period might be the time that strain SP1a produced enzymes needed to hydrolyze propoxur and second lag period was shown during enzyme synthesis by strain SP1b. In particular, duration of the lag period appeared after 48 hours was prolonged when strain SP1b was inoculated into mineral medium

containing 2-isopropoxyphenol (100 μg ml^{-1}) which concentration was more than the concentration of the intermediate produced by the hydrolysis of 100 μg ml^{-1} of propoxur (Fig. 8; Fig. 10a). This means that strain SP1b is sensitive to the concentration of 2-isopropoxyphenol.

When syntrophic pair SP1 was incubated at various temperature conditions on propoxur mineral medium (100 μg ml^{-1}), the pair was able to utilize propoxur as a source of carbon and energy at 15, 20, 25, and 28 °C (Fig. 9). Additionally, lag periods observed in cell growth were shortened when the pair was incubated at moderate temperature (Fig. 9). At 37 °C, the pair did not grow on mineral medium containing propoxur but was able to grown on PTYG medium (data not shown). This indicates that enzymes involved in propoxur degradation are optimized at moderate temperature.

Syntrophic pair SP1 showed increased cell density and rapid degradation of propoxur in the presence of additional nutrients (Fig. 10). Especially, glucose and peptone accelerated biodegradation of propoxur with shorter lag period. In previous studies on enhanced degradation of pesticides, *Pseudomonas* sp., Quinalphos-degrading bacterium, which utilized 38.2 % of the pesticide without glucose was able to degrade 90.4 % of the pesticides in the presence of glucose in the same period (Pawar and Mali, 2014). Karpouzas et al. (2005) reported that cadusafos-degrading bacteria, *Flavobacterium* sp. and *Sphingomonas paucimobilis* degraded cadusafos in 72 hours when cultures were supplemented with glucose while two isolates took 124 hours to completely degrade the pesticide. These results indicate that glucose and peptone provided energy and materials for synthesis of enzymes involved in pesticide degradation and bacterial growth with pesticide-degrading bacteria.

However, some reports have demonstrated that biodegradation of pesticide was inhibited in the presence of extra nutrients. Gebendinger and Radosevich (1999) reported that degradation of atrazine by *Ralstonia* sp. M91-3 was inhibited when exogenous nitrogen sources existed. Glucose could also impede degradation of pesticides (Sreenivasulu and Aparna, 2001). Thus, it is significant to investigate which nutrients are available to bacteria and which concentration of nutrients is needed in order to stimulate degradation of pesticides. In pesticide-contaminated fields, proper application of extra carbon or nitrogen sources may help pesticide-degrading bacteria to adapt effectively and to degrade xenobiotics more rapidly.

In this study, although 2-isopropoxycatechol and catechol were not detected as intermediates of propoxur except 2-isopropoxyphenol with GC-MS, 2-isopropoxyphenol appeared to be further degraded to catechol (Fig. 15). Degradation pathways of propoxur have been proposed in previous studies. Mahalakshmi et al. (2009) detected 2-isopropoxyphenol, 2-isopropoxycatechol (2-hydroxy-3-(1-methylethoxy)-phenol), and catechol as photocatalytic degradation products of propoxur with GC-MS. In plant, propoxur was degraded subsequently through 2-isopropoxyphenol and catechol by hydrolysis and O-dealkylation (Roberts and Hutson, 1998). Degradation of chemicals by O-dealkylation was also reported in bacteria. Karlson et al. (1993) reported that *Rhodococcus* sp. 116 could degrade 2-ethoxyphenol to catechol through O-dealkylation caused by enzymatic activity of cytochrome P450. Similarly, 7-ethoxycoumarin was transformed to 7-hydroxycoumarin by O-dealkylation activity of *Rhodococcus* sp. NCIMB 9784 (Roberts et al., 2002). Thus, O-dealkylation is one of the ways to transform O-alkylated chemicals to hydroxylated ones. In degradation diversity analysis, it was

observed that strain SP1b could utilize catechol as a sole carbon and energy source on catechol mineral medium (50 μg ml^{-1}) (Table 6) and completely degrade catechol in 24 hours (Data not shown). From this result, we presumed that the concentration of catechol might stay at a hardly detectable level as catechol produced from 2-isopropoxyphenol was immediately utilized as a carbon and energy source for strain SP1b.

Hydrolysis of the carbamate ester group is an initial step in which phenolic derivatives are produced during biodegradation of methyl carbamate pesticides (Laveglia and Dahm, 1977). This reaction is catalyzed by carbamate hydrolases, and some carbamate hydrolase genes encoding the enzymes such as carbofuran hydrolase and carbaryl hydrolase have been cloned and identified from bacterial strains including *Achromobacter* sp. WM111, *Arthrobacter* sp. AC100, and *Arthrobacter* sp. RC100 (Hashimoto et al., 2002; Hashimoto et al., 2006; Parekh et al., 1996). In this study, strain SP1a exhibited a positive band with carbaryl hydrolase gene (*cehA*) when PCR amplification was conducted with specific primers for partial sequences of *mcd* and full-length sequences of *cahA* and *cehA* (Fig. 16). Strain SP1a could degrade not only carbaryl but also aldicarb, carbofuran, and propoxur which have an ester of carbamate group in common (Table 6). This indicated that the carbaryl hydrolase of strain SP1a has a relatively wide substrate range. On the other hand, strain SP1b did not show any bands with three genes, and could only metabolize phenolic compounds which have one or two hydroxyl groups and oxygens linked with other atoms or functional groups such as 2-isopropoxyphenol, 4-nitrophenol, and catechol (Fig. 16; Table 6). However, strain SP1b was not able to degrade 2-*sec*-butylphenol. Previous studies reported that 2-*sec*-butylphenol could

be metabolized to 3-*sec*-butylcatechol through hydroxylation (Toyama et al., 2010; van der Maarel and Kohler, 1993). Strain SP1b may not have any hydroxylases for further phenol degradation but may have a catechol dioxygenase for ring cleavage of catechol and a cytochrome P450 enzyme for O-dealkylation. Further studies are needed in order to investigate how 2-isopropoxyphenol is dealkylated and which enzymes are involved in O-dealkylation and to identify whether strain SP1b possesses catechol 1, 2-dioxygenase or catechol 2, 3-dioxygenase for transforming a benzene ring to a chain structure. Thus, syntrophic pair SP1 degraded propoxur through syntrophic interaction between strain SP1a capable of hydrolyzing an ester group of *N*-methylcarbamates and strain SP1b able to cleave a ring structure of phenolic compounds.

Propoxur is able to bring about a shift in the composition and function of ecosystems as it has deleterious effects on non-target organisms and microbial communities in applied fields. Accordingly, removal of propoxur residues presented in environment is needed. In this study, syntrophic pairs capable of mineralizing propoxur was isolated and characterized in laboratory condition. These syntrophic pairs may be used as microbial resources for bioremediation purposes in propoxur-contaminated sites.

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농업토양에서 분리된 *Pseudaminobacter* sp. SP1a와 *Nocardioides* sp. SP1b의 영양공생을 통한 프로폭서의 생분해

김현

초록

Propoxur는 *N*-메틸카바메이트계 농약으로 주로 살충제로써 농업해충 및 상업시설과 주거지 등의 위생해충을 방제하기 위해 전세계적으로 쓰이고 있다. 이 농약은 살충효과가 뛰어나지만 인간 및 생태계의 구성원에게 부정적인 영향을 끼친다. 인간이 propoxur에 노출될 경우 뇌를 비롯한 중추신경계에 존재하는 cholinesterase가 억제됨에 따라 급성중독증세가 나타나며 미국의 EPA는 propoxur를 잠정적인 발암원으로 규정하고 있다. 인체에 대한 유해성과 더불어 생태계에 유출된 propoxur는 생태계 내 비표적 생물에게 강한 독성을 나타내며 토양미생물군집에도 영향을 주어 생태계의 구성과 기능에 부정적인 영향을 미친다. 이러한 propoxur의 유해성을 줄이는 방법 중 하나는 미생물을 이용한 잔여물질의 생분해이다. propoxur의 생분해에 대한 이전 연구에서는 propoxur가 미생물의 활동에 의해 2-isopropoxyphenol로 가수분해된다는 것이 밝혀졌으나 이 중간물질이 미생물의 탄소 및 에너지원으로써 완전히 분해되는 것은 보고되지 않았다. 본 연구에서는 propoxur를 완전히 분해할 수 있는 두 종의 세균으

로 구성된 세 쌍의 syntrophic pair가 농업토양에서 분리되었다. 16S rRNA 유전자 염기서열 분석 및 repetitive extragenic palindromic PCR (REP-PCR)을 통해 syntrophic pair를 구성하는 세균들이 *Pseudaminobacter* 및 *Mesorhizobium*, *Nocardioides*와 연관이 있는 것을 확인하였다. 세 쌍의 syntrophic pair 중 가장 빠르게 propoxur를 분해하는 SP1 pair에 대해 연구한 결과, SP1 pair의 분해활동은 중온 조건에서 가장 활발하였으며, 추가적인 영양분으로 glucose와 peptone을 propoxur와 동일한 농도로 같이 첨가하여 배양할 경우 더욱 빠르게 농약을 분해하였다. Propoxur 이외의 다른 농약에 대한 기질 다양성 실험을 통해 SP1 pair가 propoxur만을 영양공생을 통해 완전히 분해할 수 있다는 것을 확인하였다. Gas chromatography-mass spectrometry (GC-MS) 분석을 통해 SP1 pair를 구성하는 균주 SP1a에 의해 propoxur가 2-isopropoxyphenol로 가수분해되며 이것이 균주 SP1b에 의해 추후 완전히 분해됨을 보았다. 기존 보고된 메틸카바메이트 분해 유전자의 primer를 이용해 PCR증폭실험을 실시한 결과 propoxur의 가수분해가 균주 SP1a가 가지는 carbaryl hydrolase를 인코딩하는 cehA 유전자에 의한 것임을 확인하였다. 이 연구는 propoxur가 완전히 분해될 수 있다는 것을 확인한 첫 연구이며 이 연구를 통해 밝혀진 propoxur를 완전히 분해할 수 있는 syntrophic pair를 propoxur에 오염된 토양의 정화를 위한 미생물 자원으로 이용할 수 있을 것으로 기대된다.

주요어 : Propoxur, 영양공생, 생분해, 2-Isopropoxyphenol, 생물학적 광물화, 세균, 가수분해

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