Biodegradation of the mixture of 2,4,6-trichlorophenol, 4-chlorophenol, and phenol by a defined mixed culture

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Two new strains, *Pseudomonas* sp. TCP114 degrading 2,4,6-trichlorophenol (TCP) and *Arthrobacter* sp. CPR706 degrading 4-chlorophenol (4-CP), were isolated through a selective enrichment procedure. Both strains could also degrade phenol. The degradability of one component by a pure culture was strongly affected by the presence of other compounds in the medium. For example, when all three components (TCP, 4-CP, and phenol) were present in the medium, a pure culture of CPR706 could not degrade any of the components present. This restriction on degradability could be overcome by employing a defined mixed culture of the two strains. The mixed culture could degrade all three components in the mixture through cooperative activity. It was also demonstrated that the mixed culture could be immobilized by using calcium alginate for the semi-continuous degradation of the three-component mixture. Immobilization not only accelerates the degradation rate, but also enables reuse of the cell mass several times without losing the cells' degrading capabilities.

Key Words—*Arthrobacter* sp. CPR706; 4-chlorophenol; degradation; immobilization; phenol; *Pseudomonas* sp. TCP114; 2,4,6-trichlorophenol

Chlorinated phenols have been widely used as herbicides, insecticides, fungicides, and wood preservatives. They are also found in Kraft pulp mill effluents in large quantities (Lindström and Nordin, 1976). The inadequate handling of chlorophenol-treated materials, accidental spills, and leaching from dumping sites have resulted in the serious contamination of soil and groundwater (Kitunen et al., 1987). Chlorophenols released into the natural environment are known to have toxic and carcinogenic properties.

One of the chlorophenols widely used as a wood preservative, 2,4,6-trichlorophenol (TCP), can be partially dechlorinated to 4-chlorophenol (4-CP) by anaerobic microorganisms (Armenante et al., 1992; Madsen and Aamand, 1992; Mohn and Kennedy, 1992) that are normally present in nature. Since 4-CP is stable against biological degradation in anaerobic conditions (Woods et al., 1989), TCP-contaminated sites tend to contain a mixture of TCP, 4-CP, and other compounds such as phenol.

The biological degradation of chlorophenols has been regarded as an attractive means of treating contaminated regions because many soil microorganisms can convert chlorophenols into cell mass and harmless products such as CO₂ and chloride. Many different types of microorganisms are known to use chlorophenols as their sole carbon and energy source, which include *Pseudomonas* (Knackmuss and Hellwig, 1978; Radehaus and Schmidt, 1992), *Arthrobacter* (Stanlake and Finn, 1982), *Rhodococcus* (Apajalahti et al., 1986), *Flavobacterium* (Saber and Crawford, 1985), and *Azotobacter* (Li et al., 1991).

A single chlorophenolic compound can be degraded using one of the above mentioned strains. However, in a practical sense, the pure culture approach has serious limitations for the bioremediation of such mixtures of TCP, 4-CP, and phenol. The presence of a compound that cannot be degraded by a microorganism can severely inhibit the growth and/or degradation abilities of the microorganism (Klecka and Maier, 1988). There have been no reports that investigate biodegradation of the mixture of TCP, 4-CP, and phenol.

Recently, Brunsbach and Reineke (1995) reported that a defined mixed culture of four specialized strains could treat a soil slurry contaminated with 13 different chloroaromatic compounds. Likewise, Bouchez et al.

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(1995) studied the cooperative activities of a defined mixed culture in the degradation of a polycyclic aromatic hydrocarbon mixture. These studies showed the possibility of using defined mixed cultures to remove complex and toxic aromatic mixtures.

In this study, we examined the possibility of employing a defined mixed culture in the degradation of a mixture of TCP, 4-CP, and phenol as a model system.

Materials and Methods

Isolation and characterization of chlorophenol-degrading bacteria. Chlorophenol-degrading bacteria were isolated by a selective enrichment procedure described by Lee et al. (1991). The isolates were identified on the basis of their morphological, physiological, and chemotaxonomical properties. Gram staining, catalase, oxidase, oxidation fermentation, and poly- β -hydroxybutyrate accumulation were tested according to the procedures outlined in "Manual of Methods for General Bacteriology" (Smibert and Krieg, 1981). The DNA base composition was determined after enzymatic hydrolysis of the DNA into nucleosides (Tamaoka and Komagata, 1984) using reverse-phase HPLC (Waters Associates, MA, U.S.A.). Cellular fatty acid composition was determined according to a method described by Sasser (1990). The diamino-pimeric acid isomer was identified by the method of Becker et al. (1964). Menaquinone was determined by the method of Kroppenstedt (1985). Cellular morphology and flagella were observed using a transmission electron microscope (Philips CM 20, The Netherlands) at 80 kV after staining with 2% ammonium molybdate.

Media. Medium I for suspension cultures consisted of the following components per liter: 1 g K_2HPO_4 , 0.6 g NaH_2PO_4 , 1 g NH_4NO_3 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 0.002% yeast extract, and 1 ml trace elements solution (Lee et al., 1991). Medium II for immobilized cell cultures contained per liter: 0.1 g K_2HPO_4 , 0.2 g $MgSO_4 \cdot 7H_2O$, 0.2 g KCl, 1 ml trace elements solution, and 0.002% yeast extract. The pH of the media was adjusted to 7.2 with 2 N NaOH before autoclaving (121°C, 20 min). Chlorophenols (Aldrich Chemical Co., Milwaukee, WI, U.S.A.) and phenol were added to the culture medium after autoclaving.

Immobilization. Cells were cultivated at 30°C in 3-l Erlenmeyer flasks containing 1 l Bacto nutrient broth (Difco Laboratories, Detroit, MI, U.S.A.). Exponentially growing cells were harvested by centrifuging the culture broth at 6,000 rpm for 20 min and washing with a 0.9% (w/v) NaCl solution. The washed cells (1.4 g dry wt./l) were immobilized in gel beads of calcium alginate, as described in a previous report (Ferschl et al., 1991).

Culture conditions. One or two colonies of cells grown on Bacto nutrient agar plates were suspended in 100–300 ml of Medium I in 1,000-ml Erlenmeyer flasks and shaken at 120 rpm and 30°C overnight (about 16 h). The overnight-suspended cultures were divided into 500-ml Erlenmeyer flasks. All batch degradation experiments were initiated by adding suitable carbon sources to the overnight-suspended cultures (50 ml per 500-ml flask) with the approximate initial cell concentration.

For the defined mixed cell experiments, the overnight-suspended culture (25 ml) of TCP114 was mixed with that of CRP706 and suitable carbon sources were added. The cell densities of both cultures were adjusted to 51 mg cells (dry wt.) per liter before mixing.

The immobilized cell cultures were carried out in 1-l Erlenmeyer flasks containing 200 ml of Medium II and suitable carbon sources.

Analytical methods. The growth of freely suspended cells was determined by measuring the optical density of culture media at 600 nm. The absorbance was converted to dry weight using a standard curve. In order to estimate cell concentration in gel beads, the beads were dissolved with a 0.2 M potassium phosphate buffer solution (Keweloh et al., 1989). The concentrations of chlorophenols and phenol in the supernatant of the culture media were determined by reverse-phase HPLC equipped with a Nova pack C_{18} column (3.9×150 mm) and a UV detector at 280 nm. The injection volume of samples was 15 μ l. The mobile phase consisted of methanol–water–acetic acid (100:100:2, v:v:v) pumped at a flow rate of 1 ml/min. Chloride ion concentration was determined using a pH meter (Orion, no. 701A, Boston, MA, U.S.A.) with a chloride-specific electrode. The standard chloride concentration was calibrated with NaCl in minimal salts medium before measurement.

Results and Discussion

Characterization of chlorophenol-degrading bacteria

A TCP-degrading bacterium (TCP114) and a 4-CP-degrading bacterium (CPR706) were isolated from soil samples serially enriched with TCP and 4-CP, respectively. Their morphological, physiological and chemotaxonomical characteristics are summarized in Table 1.

TCP114 was Gram-negative, accumulated poly- β -hydroxybutyrate, and did not grow at 41°C. It displayed a strict aerobic growth as a chemoheterotroph. Most tested characteristics of strain TCP114 were consistent with those of *Pseudomonas solanacearum*, listed as section II of the genus *Pseudomonas* (Palleroni, 1984). In this study, we designated this

Table 1. Characteristics of chlorophenol-degrading bacteria TCP114 and CPR706.

Characteristic	Strain	
	TCP114	CPR706
Morphological		
Flagella	>1	—
Spore	—	—
Size (μm)	0.7–1.0	0.6–1.4
Shape	Rod	Rod
Physiological		
Catalase	+	+
Oxidase	+	—
Oxidative/Fermentative	Inert	Inert
$\text{NO}_3 \rightarrow \text{N}_2$	+	—
Arginine dihydrolase	—	—
β -Galactosidase	—	+
Indole production	—	—
Urease	+	—
PHB ^a formation	+	—
Gelatin liquefy	—	—
Starch hydrolysis	—	—
Tryptophan deaminase	—	ND ^b
Esculin hydrolysis	—	+
Growth at 41°C	—	ND
Fluorescence pigment	—	—
Chemotaxonomical		
G+C content	68.5	63.5
Major menaquinone (MK)	ND	MK-9(H ₂)
Cell wall diamino acid	ND	L-Lys
Fatty acid types ^c	nC14, 2OH-C14, 3OH-C14, nC16, C16:1, C18:1, Δ C17	a-C13, i-C14, nC14, i-C15, a-C15, i-C16, nC16, i-C17, a-C17

^a PHB, poly- β -hydroxy butyrate.

^b ND, not determined.

^c i, iso branched; a, anteiso branched; n, straight chain; 2OH, 2 hydroxylated; 3OH, 3 hydroxylated; Δ , cyclized; C16:1, hexadecenoic acid; C18:1, octadecenoic acid.

strain as *Pseudomonas* sp. TCP114.

The 4-CP degrading bacterium (CPR706) was positive for gram staining and for the catalase test but negative for the oxidase test. CPR706 was identified as *Arthrobacter* sp. according to the description of the genus *Arthrobacter* (Keddie et al., 1986).

These isolates were deposited at the Korean Collection for Type Cultures (KCTC) at the Korea Institute of Science and Technology as *Pseudomonas* sp. TCP114 KCTC 0106BP and *Arthrobacter* sp. KCTC 3614, respectively.

Degradability of chlorophenols and phenol by the isolates

In order to determine the degradability of chlorophenols, two isolates were cultivated in Medium I containing one of the following chlorophenols (10 mg/l): 2-, 3-, 4-chlorophenol; 2,4-, 2,5-, 2,6-, 3,4-, 3,5-dichlorophenol; 2,3,4-, 2,3,5-, 2,3,6-, 2,4,5-, 2,4,6-trichlorophenol; or pentachlorophenol. Among the tested chlorophenols, TCP114 degraded only TCP

and CPR706 only 4-CP. However, both strains showed high degrading activity with phenol (10 mg/l).

Single-component degradation in pure cultures

In order to see the degradation of chlorophenol or phenol as a single substrate, isolates were cultivated in Medium I containing one substrate component with different initial concentrations (Fig. 1).

TCP114 could degrade TCP up to the initial concentration of 3.6 mM, above which level the degradation of TCP was completely inhibited (Fig. 1A). The duration of the lag phase increased as initial TCP concentration increased due possibly to the toxic effects of the substrate at higher concentrations. TCP degradation accompanied cell growth when the initial TCP concentration was less than 4.5 mM. In this range, the growth yield (Y) of TCP was 0.34 g dry cells per gram TCP.

Figure 1B shows the degradation of 4-CP by CPR706 in shake-batch cultures. Like the case of TCP114 cultures, the increase in initial concentration increased the time required for degradation of both

Fig. 1. Degradation of a single component by isolates in shake-batch cultures: A) TCP degradation by TCP114, B) 4-CP degradation by CPR706, C) phenol degradation by TCP114, D) phenol degradation by CPR706.

□, TCP; ○, 4-CP; ●, phenol.

substrates due to the extended lag phase and decreased degradation rate. The maximal degradable concentration of 4-CP was 1.5 mM, and the growth yield was about 0.32 g dry cells per gram 4-CP.

During chlorophenol degradation by both strains, a stoichiometric amount of chloride ions accumulated in the culture broth (data not shown), indicating complete dechlorination.

Figure 1C and D show phenol degradation in the shake-batch cultures of TCP114 and CPR706, respectively. Like chlorophenol degradation, the increase in initial phenol concentration increased the lag period. However, the tolerances of phenol by both strains were better than those of chlorophenols, as indicated by the fact that both strains could degrade phenol up to the concentration at which chlorophenol degradation was completely inhibited. The degradation of phenol was also faster than that of chlorophenol at the same initial concentration. TCP114 degraded phenol at the rate of 180–200 $\mu\text{M}/\text{h}$, whereas it degraded TCP at the rate of 62–79 $\mu\text{M}/\text{h}$; CPR706 degraded phenol at the rate of 160–210 $\mu\text{M}/\text{h}$, whereas it degraded 4-CP at the rate of 50–54 $\mu\text{M}/\text{h}$. The higher tolerance and degradation rate of phenol than those of TCP or 4-CP indicate the high toxicity of chlorophenols as compared to phenol in the degradation process. The growth yields (Y) of phenol in the cultivation of TCP114 and CPR706 were calculated to be about 0.77 and 0.61 g dry cells per gram phenol, respectively. Phenol's higher growth yield is due to the fact that it has a higher carbon content per unit mass

Fig. 2. Degradation of a two- or three-component mixture in shake-batch cultures of TCP114 or CPR706.

Culture conditions were described in MATERIALS AND METHODS section. □, TCP; ○, 4-CP; ●, phenol.

than chlorophenol.

Two- or three-component mixtures in pure cultures

In the previous sections, we showed that each isolate can degrade two different components separately: phenol and one of the chlorophenols. In order to find out if a mixture of both components can also be degraded by each pure culture, we cultivated the isolates in the presence of the two components. As shown in Fig. 2A, TCP114 could degrade both TCP and phenol in the mixture. TCP was completely degraded before phenol degradation started. A long stationary phase (about 60 h) was present at the moment when the degradation of TCP stopped and degradation of phenol began. This biphasic degradation in the TCP and phenol mixture indicates that two different enzyme systems are required for the degradation of phenol and TCP. The phenol-degrading enzyme system may be newly induced during the stationary interval after the complete depletion of TCP. When the TCP and phenol mixture was re-supplied with the culture broth, phenol was degraded prior to TCP by the newly induced phenol-degrading enzymes.

On the other hand, CPR706 could degrade 4-CP but could not degrade phenol when both 4-CP and phenol were added (Fig. 2B). It is not clear why CPR706 could not degrade phenol in the 4-CP and phenol mixture. One possible explanation is that the phenol-degrading ability of CPR706 may be destroyed by the presence of 4-CP because of the incompatibility of two different ring-fission mechanisms catalyzing

4-CP and phenol degradation.

Most environmental contaminants are found as a mixture of several compounds. To eliminate them, we need to find out how the presence of non-degradable compounds affects the degradation activities of the isolated strains. In order to examine such effects, we cultivated the isolates in a mixture of phenol, 4-CP, and TCP (Fig. 2C and D).

TCP114 could degrade TCP but could not degrade 4-CP and phenol in the mixture (Fig. 2C). The inability of TCP114 to degrade phenol was quite unexpected because the strain could degrade phenol in the absence of 4-CP. Compared to the mineralization of TCP in TCP alone (about $62 \mu\text{M/h}$) or in the mixture of two degradable components (about $33 \mu\text{M/h}$), the TCP degradation rate was also decreased to about $17 \mu\text{M/h}$ by the presence of 4-CP. Cell concentration increased slightly during the uptake of TCP and gradually decreased afterward. When living cells were counted, cell concentration decreased from the initial 10.5×10^{11} to 3.9×10^{10} (living cells/l) during the 200 h of culture time, showing the toxic effect of 4-CP on cells.

In the case of CPR706 (Fig. 2D), none of the three components were degraded. The degradability of 4-CP and phenol by CPR706 was completely eliminated the presence of TCP. This inability of CPR706 to degrade 4-CP and phenol was demonstrated to be caused by cell death resulting from the addition of TCP. The viable cell counts revealed that all CPR706 cells were dead after 200 h of cultivation.

Mixed culture for the three-component mixture

When a three-component mixture of TCP, 4-CP, and phenol was added to a pure culture of the two strains, TCP114 could only degrade TCP, and CPR706 could not degrade any of the three components (Fig. 2). This suggests that nondegradable components can inactivate enzyme systems required for the degradation of otherwise degradable components. Furthermore, the addition of nondegradable toxic components inhibited cell growth and caused cell death. Such toxicity of chlorophenols has been well documented in several types of microorganisms (Kiyohara et al., 1992). These inhibitory effects of nondegradable components show the limitations of the use of pure cultures for the bioremediation of a mixture of contaminants, which prompted us to test a new approach: the use of a defined mixed culture of two strains.

Figure 3 shows the degradation patterns of TCP, 4-CP, and phenol by defined mixed cultures of two strains, TCP114 and CPR706. All three components were degraded when the initial concentrations of the three components were 0.15, 0.3, 0.5, or 0.7 mM. Among the three components, TCP was first degraded by the strain TCP114, after which 4-CP and

Fig. 3. Degradation of a three-component mixture by a defined mixed culture of TCP114 and CPR706 in shake-batch cultures.

The concentrations of each component added into overnight-suspended cultures were 0.15 mM (●), 0.3 mM (○), 0.5 mM (■), 0.7 mM (□).

phenol were degraded. The total degradation of the mixture is the result of cooperative activity by the two isolates. First, TCP114 removed TCP from the mixture, which provided a TCP-free environment so that CPR706 could degrade 4-CP. Phenol might be degraded by TCP114 because CPR706 could not degrade phenol in the two-component mixture (Fig. 2B).

When the initial concentrations of the three components were increased to 0.7 mM, the mixed cells only removed TCP during the incubation period of 200 h, after a dramatically extended lag phase (about 80 h).

Immobilized cell culture for the three-component mixture

In order to speed up the degradation process and reuse active cells, we immobilized a high concentration of mixed culture using calcium alginate. Cell immobilization has been reported to increase the tolerance of cells against phenolic compounds (Keweloh et al., 1989). Balfanz and Rehm (1991) reported also that cultures with high cell concentrations could degrade higher concentrations of 4-CP than those with low cell concentrations.

Figure 4 shows the degradation patterns during the semi-continuous operation of immobilized mixed cells with calcium alginate. During the first semi-continuous operation, the mixed cultures degraded all three components in a pattern identical to that of the free cells

Fig. 4. Semi-continuous operation of an immobilized defined mixed culture of TCP114 and CPR706 in a three-component mixture.

□, TCP; ○, 4-CP; ▲, phenol; ●, freely-suspended cell concentration; △, immobilized cell concentration.

(Fig. 3). However, the time required for complete degradation of the three components (0.3 mM each) decreased to 55 h as opposed to the 105 h required for the free-cell culture, which may be due in part to the increase of the degradation rate by means of increased cell concentration and also in part to the reduction of the toxic effects of the components. Furthermore, when the medium was drained and recharged for the second semi-continuous run, the time needed for degradation of the three components was shortened further. This was possibly due to the increased degradation rate and shortening of the intermediate lag phase before 4-CP and phenol were degraded. The average time required for complete degradation was about 20 h for all semi-continuous runs except the first one.

Figure 4 also shows the changes of cell concentration in the immobilized matrix and in the medium. The former stayed constant during semi-continuous operation, whereas the latter increased as the newly born cells were released from or leaked out of the immobilized matrix. Despite the fact that cells leaked out, the physical integrity of the immobilized beads was stable in this culture system.

When the concentration of each component increased to 0.5 mM, the immobilized cells degraded in a pattern similar to when the concentrations were at 0.3 mM (data not shown). Like the free-cell culture, the immobilized cells did not degrade all three mixtures whenever the concentration increased to 0.7 mM.

In conclusion, we demonstrated that mixtures of hazardous chemicals can be bioremediated using a mixture of several different strains. This is an alternative approach to the traditional mixed culture technique that employs undefined mixtures of environmental bacteria. Uncharacterized mixed cultures such as traditional activated sludge may frequently produce undesirable toxic metabolites that severely inhibit the growth of the chlorophenol-utilizing microorganisms in

the mixture.

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