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Degradation of Chlorophenols and Phenol Mixtures by Cooperative Activities of Chlorophenol-degrading Strains

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Three strains capable of degrading a chlorophenol were isolated by selective enrichment from soils contaminated with industrial wastewater. A *Pseudomonas solanaccarum* TCP114 could use 2,4,6-trichlorophenol (TCP) as sole carbon and energy source, while two strains of *Pseudomonas testosteroni* CPW301 and *Arthrobacter ureafaciens* CPR706 could use 4-CP. All isolates also grew well on phenol. The degradation of one component by a pure strain was strongly affected by the presence of other compounds in the medium. CPW301 and CPR706 entirely lost the ability to degrade 4-CP and phenol in the presence of TCP. TCP114 also lost the ability to degrade phenol when 4-CP was added to the culture medium. These restrictions on the degradability could be overcome by employing defined mixed cultures (TCP114 and one strain of 4-CP degrading strains). All three components were successfully degraded by defined mixed cultures through their cooperative activities. It was also demonstrated that defined mixed cultures could be immobilized by using calcium alginate for the semi-continuous degradation of the three component mixture. Immobilization could not only accelerate the degradation rate, but also allowed the reuse of the cell mass several times without loss of the cells' degrading capabilities.

Polychlorinated phenols have been released into the environment because of their wide usage as biocides, wood preservatives, and organic precursors of chlorophenoxyacetate herbicides. In natural environments they are often converted to various lower chlorinated phenols or phenol by anaerobic microorganisms (6, 8, 19). Therefore, polychlorophenol contaminated sites can also contain monochlorophenols or phenol as a mixture.

The biological degradation of chlorophenols has been regarded as an attractive means of treating contaminated regions because many soil microorganisms have the potential to use a chlorophenol as their sole carbon and energy source (1, 14, 15, 18, 21, 24). However, in a practical sense, the pure culture approach has serious limitations for the bioremediation of mixtures of polychlorophenols, monochlorophenols, and phenol. The limitation of a pure culture approach may actually lie in the different degrading mechanisms that are used for degrading these compounds. Polychlorinated phenols are degraded via chlorinated *para*-hydroquinone while mono- and dichlorophenols are degraded via chlorocatechol and *ortho* ring-fission (11). On the other hand, phenol is frequently degraded via a *meta*-cleavage pathway (9). There have been no reports that investigate the biodegradation of the mixture of polychlorophenol, monochlorophenol and phenol.

Recently, some studies showed the possibility of using defined mixed cultures to remove complex and toxic aromatic mixtures. Brunsbach and Reineke (7) reported that a defined mixed culture of four specialized strains could treat soil slurry contaminated with 13 different chloroaromatic compounds. Likewise, Bouchez *et al.* (5) have studied the cooperative activities of a defined mixed culture in the degradation of a polycyclic aromatic hydrocarbon mixture.

In the present study, we examined the possibility of employing defined mixed cultures in the degradation of a polychlorophenol, monochlorophenol, and phenol mixture using 2,4,6-trichlorophenol (TCP), 4-chlorophenol (4-CP), and phenol as model compounds.

MATERIALS AND METHODS

Isolation and Characterization of Chlorophenol-degrading Bacteria

Bacterial strains capable of degrading chlorophenols were isolated by a selective enrichment procedure as described by Lee *et al.* (17). The isolates were identified on

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the basis of their morphological, physiological and chemotaxonomical characteristics. Gram staining, catalase, oxidase, oxidation-fermentation, and poly-β-hydroxybutyrate accumulation were tested as described in the manual by Smibert and Krieg (23). The DNA base composition was determined after enzymatic hydrolysis of the DNA into nucleosides (25) by using reverse-phase HPLC (Waters Associates, Massachusetts). Cellular fatty acid composition was determined according to a method described by Sasser (22). The diaminopimelic acid isomer was identified by the method of Becker *et al.* (4). Menaquinone was determined by the method of Kropenstedt (16). Cellular morphology and flagella were observed using a transmission electron microscope (Philips CM 20, Netherlands) at 80 kV after staining with 2% ammonium molybdate.

Media and Culture Conditions

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 (17). Medium II for immobilized cell cultures contained the following 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., Ltd., Milwaukee, Wisconsin) and phenol were added to the culture medium after autoclaving.

Colonies of cells grown on Bacto nutrient agar plates (Difco Laboratories, Detroit, MI) were suspended in 50 ml of Medium I in 500-ml Erlenmeyer flasks and shaken at 120 rpm and 30°C for an overnight (about 16 h). All batch degradation experiments were initiated by adding suitable carbon sources (50 g/l stock solution) to the overnight suspended culture with the initial cell concentration of 30 mg dry cells per liter. For the defined mixed cell experiments, the overnight suspended culture (25 ml) of two strains of isolates were mixed and suitable substrates were added. The cell densities of both cultures were adjusted to 51 mg cells (dry wt.) per liter before mixing.

The immobilized cell cultures (see following section) were carried out in 1-liter Erlenmeyer flasks containing 200 ml of Medium II and suitable carbon sources.

Immobilization

Cells were cultivated at 30°C in 3-liter Erlenmeyer flasks containing 1 liter Bacto nutrient broth (Difco Laboratories, Detroit, Michigan). Exponentially growing cells were harvested by centrifuging the culture broth at 6,000 rpm for 20 min and washing with 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 the previous report (10).

Analytical Methods

The growth of freely suspended cells was determined by measuring the optical density of culture media at 600 nm. Absorbance was converted to dry weight by using a standard curve. In order to estimate cell concentrations in gel beads, the beads were dissolved with 0.2 M potassium phosphate buffer solution (13). 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 μl. The mobile phase consisted of methanol-water-acetic acid (100 : 100 : 2) that was pumped with the flow rate of 1 ml/min. Chloride ion concentration was determined by using a pH meter (Orion, no. 701A, Boston, MA) with a chloride specific electrode. The standard chloride concentration was calibrated with NaCl in minimal salts medium before measurement.

RESULTS AND DISCUSSION

Isolation and Identification of Chlorophenol-degrading Bacteria

A TCP-degrading bacterium (TCP114) and two strains of 4-CP-degrading bacteria (CPW706 and CPW301) were isolated from soil samples serially enriched with TCP and 4-CP, respectively. Each isolate was purified by repeated subculture on solid medium I (18% agar added) with TCP or 4-CP as the sole carbon source. Characteristics of each strain are summarized in Table 1. The characteristics of the strain TCP114 were consistent with those of *Pseudomonas solanacearum* listed in section II of the genus *Pseudomonads* (20). On the other hand, two 4-CP-degrading strains, CPR706 and CPW301, were identified as *Arthrobacter ureafaciens* and *Pseudomonas testosteroni*, respectively, according to Bergey's Manual (12, 20).

Degradation of Chlorophenols and Phenol by the Isolates

To determine the degradability of chlorophenols, isolates were cultivated in Medium I containing one of 15 chlorophenols (50 mg/l). As shown in Table 2, all isolates have a strong substrate specificity for chlorophenols. TCP114 could degrade only TCP. When TCP was used as a single substrate, the strain TCP114 could degrade TCP up to the initial concentration of 3.5 mM, above which concentration the degradation of TCP was completely inhibited due to cell death (data not shown). Spectrophotometric scanning (UV range) and HPLC analysis of the medium supernatant revealed that the TCP degradation process did not produce any intermediate compounds detectable by the methods used.

On the other hand, CPR706 and CPW301 could not degrade any other chlorophenols except for 4-CP. The maximal degradable concentrations of 4-CP by CPR706

Table 1. Characteristics of chlorophenol-degrading bacteria.

Characteristics	CPW301	CPR706	TCP114
Gram stain	–	+	–
Flagella	one polar	–	>1
Spore	–	–	–
Size (μm)	0.5 – 1.7	0.6 – 1.4	0.7 – 1.0
G+C content	61.3	63.48	68.5
MK type	ND	MK-9 (H_2)	ND
DAP type	ND	L-Lysine	ND
Catalase test	+	+	+
Oxidase test	+	–	+
O/F test	inert	inert	inert
$NO_3 \rightarrow N_2$	–	–	+
Arginine dihydrolase	–	–	–
β-galactosidase	–	+	–
Indole production	–	–	–
Urease test	+	–	+
PHB accumulation	–	–	+
Gelatine liquefy	–	+	–
Starch hydrolysis	–	–	–
T.D.A	–	ND	–
Esculine hydrolysis	–	+	–
Fluorescence pigment	–	–	–
Fatty acid type	C12:0, C17:Δ, 2OHC12:0, C16:0, C16:1, 3OHC12:0, C18:1, C19:Δ	a-C13:0, i-C14:0, C14:0, i-C15:0, a-C15:0, i-C16:0, C16:0, i-C17:0, a-C17:0	C14:0, C16:0, 2OHC14:0, C16:1, 3OHC14:0, C18:1, C17:Δ

PHB, poly-β-hydroxybutyrate; MK, menaquinone type; DAP, diaminopimelic acid; ND, not determined; T.D.A., tryptophan deaminase; O/F, Oxidative/Fermentative. i, iso branched; a, anteiso branched; n, straight chain; 2OH, 2 hydroxylated; 3OH, 3 hydroxylated; Δ, cycled; C16:1, hexadecenoic acid; C 18:1, octadecenoic acid.

Table 2. Degradation of phenol and chlorinated phenlos by isolates.

Compound	Degradation[a] by strains of		
	CPW301	CPR706	TCP114
Phenol	+	+	+
2-CP	–	–	–
3-CP	–	–	–
4-CP	+	+	–
2,3-DCP	–	–	–
2,4-DCP	–	–	–
2,5-DCP	–	–	–
2,6-DCP	–	–	–
3,4-DCP	–	–	–
3,5-DCP	–	–	–
2,3,4-TCP	–	–	–
2,3,5-TCP	–	–	–
2,3,6-TCP	–	–	–
2,4,5-TCP	–	–	–
2,4,6-TCP	–	–	+
pentachlorophenol	–	–	–

[a]Degrading ability was determined by the disappearance of added substrates and the increase of cell turbidity in culture medium.

and CPW301 were 1.5 and 0.6 mM, respectively. During the degradation of 4-CP, CPW301 excreted a yellow intermediate with the absorption maximum at 378 nm while strain CPR706 produced a metabolite with the absorption maximum at 286 nm. The production of different intermediates from 4-CP indicated that these two strains degraded 4-CP through different catabolic pathways.

All isolates showed high degrading activity with phenol without the production of a detectable intermediate. The tolerances of phenol by isolates was better than that of chlorophenols. TCP114 could degrade phenol up to the concentration of 7 mM. On the other hand, CPR706 and CPW301 could also degrade phenol up to the concentration of 5 and 1.7 mM, respectively.

Two Component Mixture in Pure Cultures

In order to find out if each isolate could also degrade chlorophenol (TCP or 4-CP) in the presence of phenol, we cultivated the isolates in a mixture of both components. As shown in Fig. 1, CPW301 could degrade both components. The strain simultaneously degraded 4-CP and phenol, which is attributable to the fact that it degrades 4-CP and phenol through a single catabolic pathway, meta-cleavage pathway (unpublished data).

On the other hand, another 4-CP degrading strain, CPR706, could degrade 4-CP but could not degrade phenol when both 4-CP and phenol were added (Fig. 1). It was not clear why CPR706 could not degrade phenol

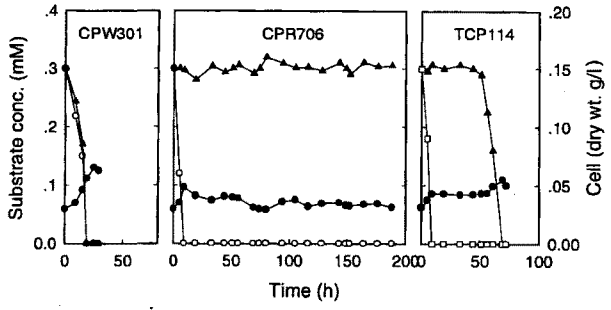

Fig. 1. Degradability and cell growth during a pure culture in a two-component mixture.
●, cell growth; ○, 4-CP; ▲, phenol; □, TCP.

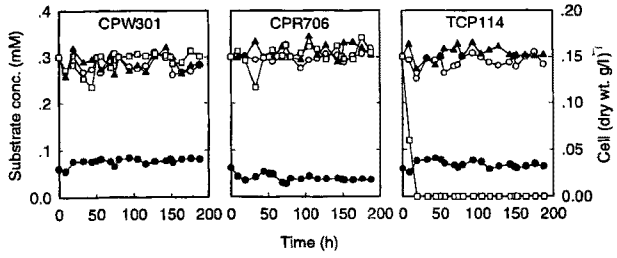

Fig. 2. Degradability and cell growth during a pure culture in a three-component mixture.
●, cell growth; ○, 4-CP; ▲, phenol; □, TCP.

in the presence of 4-CP. 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 both 4-CP and phenol degradation. This incompatibility of two different pathways has been demonstrated in the metabolism of methylated aromatic compound by some organisms (3).

TCP114 could degrade both TCP and phenol in the mixture (Fig. 1). TCP was completely degraded before phenol degradation was started showing a biphasic growth curve. A long stationary phase (about 60 h) was present between the two exponential growth phases from the moment when degradation of TCP stopped and degradation of phenol began. Both biphasic growth indicates that two different enzyme systems are required for the degradation of phenol and TCP. The phenol-degrading enzyme system may be activated or newly induced during the stationary interval after the complete depletion of TCP.

Three Component Mixture in Pure Cultures

Most environmental contaminants are found as a mixture of several aromatic compounds. In order to remove them efficiently, it is required to demonstrate how non-degradable compounds affects the degradation activities of a pure strain. To examine such effects, the isolates were cultivated in a three component mixture (TCP, 4-CP and phenol). As shown in Fig. 2, CPW301 and CPR706 did not degrade either phenol or 4-CP in the presence of TCP. This result indicates that TCP inactivates enzyme systems required for the degradation of otherwise degradable components. Furthermore, the addition of TCP caused the death of CPR706 after 200 hours of cultivation. Such toxicity to chlorophenols has been well documented in several types of microorganisms (14).

Unlike the complete loss of degrading property of the 4-CP degrading strains (CPW301 and CPR706), TCP114 degraded TCP in the presence of the nondegradable component, 4-CP. Compared to the degradation of TCP in a mixture of two degradable components (Fig. 1), TCP degradation rate was decreased due to the increase of the added concentration of toxic components. TCP114 also could not degrade phenol in the mixture (Fig. 2) probably due to the lack of needed enzyme induction. The number of living cells was also decreased from the initial 10.5×10^{11} to 3.9×10^{10} (cells/l) by cultivating the strain in the mixture for 200 h showing the toxic effect of 4-CP.

These inhibitory effects of nondegradable components show the limitations of the use of pure cultures for the bioremediation of a mixture of contaminants, this prompted us to test a new approach: the use of a defined mixed culture of two strains.

Mixed Culture for the Three Component Mixture

Fig. 3 shows the degradation patterns of three components during the cultivation of defined mixed cultures of two strains: CPW301+TCP114 and CPR706+TCP114. In both cases, the defined mixed cultures could degrade all three components within 220 h.

In the mixed culture of CPW301+TCP114 (Fig. 3A), TCP was degraded first by TCP114, and then the remaining 4-CP and phenol were simultaneously degraded about 150 h later by CPW301. The simultaneous degradation pattern of 4-CP and phenol was similar to that shown in Fig. 1 for the pure culture.

In the mixed culture of CPR706+TCP114 (Fig. 3B), TCP was also degraded first by TCP114, then 4-CP by CPR 706 about 80 h later. Phenol started to be degraded after about 200 h, which may be by TCP114, because CPR706 could not degrade phenol in the two component mixture (Fig. 1).

The total degradation of three components by defined mixed cultures is the result of the cooperative activity of two isolates. First, TCP114 removed TCP from the mixture, which provided a TCP-free environment so that CPR706 and CPW301 could degrade 4-CP. The removal of 4-CP was also thought to assist the degradation of phenol by TCP114 or CPR706 because both strains did not degrade phenol in the presence of 4-CP.

Immobilized Cell Cultures for the Three Component Mixture

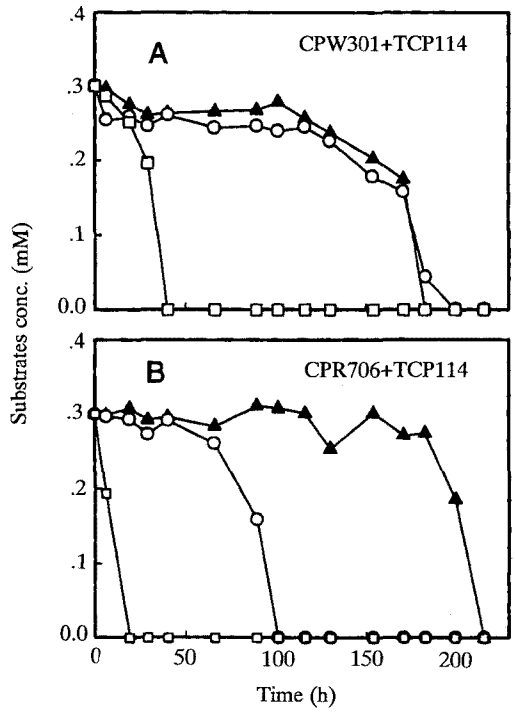

Fig. 3. Degradation of a three-component mixture during defined mixed cultures.
○, 4-CP; ▲, phenol; □, TCP.

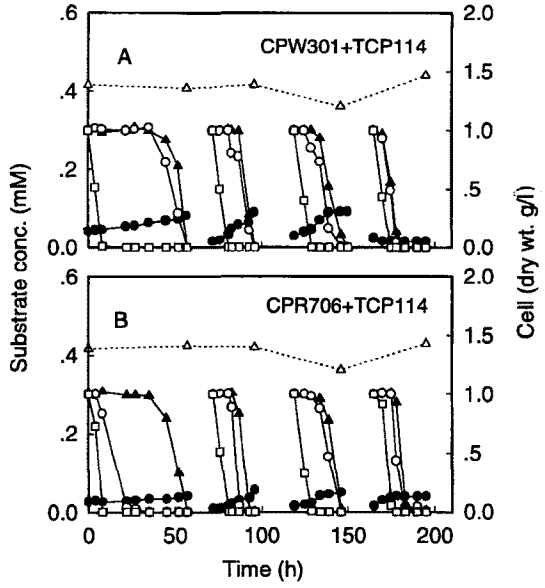

Fig. 4. Semi-continuous operation of immobilized defined mixed cultures in a three-component mixture.
●, free cells; △, immobilized; ○, 4-CP; ▲, phenol; □, TCP.

The mixed cultures of free cells showed that the cooperative activity of the TCP-degrading and 4-CP-degrading strains made the total degradation of the three components possible. However, due to the slow growth rate of cells in the presence of the three components, it took over 220 h for complete degradation. In order to speed up the degradation process, we immobilized a high concentration of the mixed culture using calcium alginate. Immobilized cells have been reported to have high tolerance against phenolic compounds (12). Furthermore, high cell concentration was also known to increase the degradable concentration of phenolic compounds (2).

Fig. 4 shows the degradation patterns during the semi-continuous operation of immobilized mixed cells. During the first semi-continuous operations, both mixed cultures degraded all three components with the same pattern as the free cells (Fig. 3). However, the time required for the complete degradation was reduced to 55 h instead of 220 h for the pure culture. This dramatic reduction of the time required for the complete degradation may be in part due to the increase of the degradation rate by the increased cell concentration and in part due to the reduction of the toxic effects of the components.

When the medium was drained and recharged for the second semi-continuous run, the time needed for degradation of the three components was further shortened, which was possibly due to the increased degradation rate and the shortening of the lag phase proceeding the degradation of 4-CP and phenol. The shortened lag period may be attributable to the facts that the tolerance of cells to toxic substrates increased, and that the enzyme induction becomes easy in the second semi-continuous operation. The average time required for complete degradation was about 20 h for all semi-continuous runs except the first one.

Fig. 4 also shows the changes of cell concentration in the immobilized matrix and in the medium as free cells. The former stayed constant during the semi-continuous operation, whereas the latter was increased as the newly born cells are released or leaked out from the immobilized matrix.

In a conclusion, we demonstrated that a mixture of hazardous chemicals can be bioremediated by 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. The uncharacterized mixed culture such as traditional activated sludge may frequently produce undesirable toxic metabolites that severely inhibit the growth of the chlorophenol utilizing microorganisms in the mixture. The concentration of toxic contaminants is a significant factor to decide the degradation capacity of applied microorganisms. Since the concentration of toxic contaminants exceeding the capacity of a defined mixed culture can spoil the degradation activity, the loading concentration of them have to be carefully controlled in the range of

optimal level.

Fig. 4 shows the effect of the loading concentration on the degradation patterns of the three components. TCP 114 and two 4-CP-degrading strains were immobilized after inducing with TCP and 4-CP, respectively. This immobilized cells repeatedly degraded the 0.3 mM of the three components within average 22 h. It took 60-88 h for the degradation of the increased concentration up to 0.5 mM. The elongated time was due to the decreased degradation rate caused by the inhibitory effect of the higher concentration. The further increasing the concentration up to 0.8 mM completely inhibited the degradation of chlorophenols, though did not the degradation of phenol.

The immobilization of cells has been reported to improve the tolerance against toxic phenolic compounds (13).

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