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ORIGINAL PAPER

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Biodegradation of seven polychlorinated biphenyls by a newly isolated aerobic bacterium (*Rhodococcus* sp. R04)

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Abstract An aerobic bacterial strain, designated R04, belonging to the genus *Rhodococcus* has been isolated and characerized by 16S rDNA analysis. The capability of this strain to degrade seven different polychlorinated biphenyls (CBs), 500 ppm 3-CB, 3,4-CB, 4,4′-CB, 2,4,6-CB, 2,4′,5-CB, 2,3,4,5-CB and 3,4,3′,4′-CB in liquid medium, was evaluated. After 5 days of incubation, the concentration of chloride increased to 0.35 mM in cultures containing 3-CB and R04, whereas in cultures with 3,4-CB, 2,3,4,5-CB or 3,4,3′,4′-CB plus R04 the chloride content increased to 0.1 mM. However, non-stoichiometric amounts of chloride were produced in cultures with R04 and 4,4′-CB, 2,4,6-CB and 2,4′,5-CB. The spectrum of supernatants from R04 grown on seven PCBs had a UV-visible (UV-VIS) absorption at 200–500 nm, characteristic of biphenyl-derived cleavage products. Gas-chromatographic (GC) analysis showed that R04 was able to transform 100% of 3-CB and 3,4-CB after 1 day of incubation, and 95% of 4,4′-CB, 2,4,6-CB, 2,4′,5-CB, 2,3,4,5-CB and 3,4,3′,4′-CB after 5 days of incubation. The position of the chlorine substituents on the rings strongly influenced the degradation of polychlorinated biphenyls (PCBs) and their intermediate metabolites by *Rhodococcus* sp. R04. The degradation of PCBs was further evaluated by monitoring intermediate metabolites of PCBs.

Keywords *Rhodococcus* sp. R04 · PCB · Degradation · Dehalogenation · Chloride

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Abbreviations PCB: Polychlorinated biphenyl · CB: Chlorinated biphenyl · MM: Minimal medium · YBCP: Yellow biphenyl-derived cleavage product

Introduction

Polychlorinated biphenyls are widespread contaminants in soils, sediments, and landfills. They have been of public and scientific concern for several decades because of their persistence in the environment, their ability to bioaccumulate and their potential carcinogenicity.

Polychlorinated biphenyls are extremely thermostable (up to 350°C) and resistant to oxidation, acids and bases. Therefore, they are difficult to degrade. Several methods for the destruction of PCBs in environmental matrices have been suggested [9]. However, classical remediation techniques (land filling, incineration, etc.) are not only generally expensive and inefficient, but also may produce secondary pollution. As a result, current research has focused on the development of bioremediation processes [10, 11].

Chlorinated biphenyls can undergo microbial transformation under both oxic and anoxic (reductive dechlorination) conditions by different metabolic pathways [15]. PCBs are biodegraded in three general ways: (1) aerobically as a growth substrate, (2) aerobically by cometabolism, and (3) anaerobically by reductive dehalogenation. Under oxic conditions, PCBs are metabolized through the *meta* or *ortho*-cleaveage pathway encoded by the operon described in many gram-negative and, to a lesser extent, in gram-positive bacteria [14, 16]. Under anoxic conditions, highly chlorinated PCB congeners can be dechlorinated to lower chlorinated congeners [1]. However, to date, dehalogenation of highly dechlorinated biphenyls has not been found under oxic conditions.

In this report, a PCB-degrading strain, *Rhodococcus* sp. R04, newly isolated from an oil field in northern

China, and its capability to degrade and dechlorinate PCBs under oxic conditions are described.

Material and methods

Chemicals

Biphenyl was obtained from the Academy of Chinese Army Medicine; 3,4-CB, 2,4,6-CB and 2,3,4,5-CB were purchased from Chem Service (West Chester, Penn., USA) (99% purity). 3-CB, 4,4′-CB, 2,4′,5-CB and 3,4,3′,4′-CB were purchased from RdH Laborchemmikalien (98% purity). None of the PCBs contained biphenyl.

Isolation of bacterium

Strain R04 was isolated by selective enrichment from oil-contaminated soil in northern China. The soil sample was incubated continually in oil leavening for 3 days. Suspensions of enriched mixes were serially diluted in sterile water and plated onto minimal medium (MM) plates containing 0.1% biphenyl, then incubated at 30°C for a week. Single colonies obtained in this manner were incubated on the same plates. After 3 days, the single colonies were transferred to 50 ml of liquid MM medium containing 0.05% biphenyl and incubated for 3–6 days. The resultant yellow cultures were streaked on MM plates. One strain that grew with biphenyl as the sole source of carbon and energy was obtained.

16S rDNA analysis

The 16S rDNA of strain R04 was amplified by PCR using universal 16S rDNA primers 27f (5′-AGA GTTTGATCC/ATGGCTCAG-3′) and 1541r (AAG GAGGTGATCCAGCC-3′) [20]. The 16S rDNA products were sequenced on an Applied Biosystems (Foster, Calif., USA) Automatic Sequencer. 16S rDNA sequences were compared and aligned with sequences deposited in the GenBank database using the BLAST program.

Growth medium

Cells were grown in a basal MM (PAS medium, pH 7.0) containing (in g per l): KH_2PO_4 2.93, $K_2HPO_4{\cdot}3H_2O$ 7.69, $MgSO_4{\cdot}7H_2O$, $FeSO_4{\cdot}7H_2O$ 0.01, $CaSO_4{\cdot}H_2O$ 0.35, $(NH_4)_2SO_4$ 5, and 2 ml of a trace elements solution, which contained (in g per l): $Na_2MO_4{\cdot}H_2O$ 6.7, $ZnSO_4{\cdot}5H_2O$ 28, $CuSO_4{\cdot}5H_2O$ 2, H_3PO_4 4, $MnSO_4{\cdot}5H_2O$ 4, $CoSO_4{\cdot}7H_2O$ 4.7. Succinate was added at a concentration of 1 g/l as the carbon and energy source.

Resting cell incubation

Cells were grown in MM containing 0.2% biphenyl to an optical density at 600 nm of 1.0, then filtered through glass wool to remove biphenyl crystals, and washed twice with 0.05 M sodium phosphate buffer (pH 7.5). Cells were resuspended in the same buffer to an optical density at 615 nm of 1.0. Cell suspensions (1 ml) were incubated in MM (containing 1 $g\,l^{-}$ succinate) with each of the PCBs mentioned above. Each PCB was provided at a final concentration of 500 nmol/ml. In the cell growth and dechlorination assays, controls were incubated in MM containing 1 $g\,l^{-}$ succinate without PCBs. In the PCB degradation studies, controls had perchloric acid (final concentration, 0.7%) added at time zero. Cells were incubated at 30°C at 250 rpm on a gyratory shaker.

Cell growth and dechlorination assay

Cell growth was monitored by measuring the increase in optical density at 600 nm (OD_{600}) with a UV-VIS spectrophotometer. Chloride content was measured with a pH/ISE meter.

Spectroscopic methods

Suspensions of strain R04 incubated with PCBs were filtered through glass cotton, centrifuged and the aqueous supernatant was scanned in a UV-VIS spectrophotometer. MM without PCBs was used as a control.

PCB extractions

Polychlorinated biphenyl were extracted from the cell suspensions with four volume of *n*-hexane, and samples were shaken vigorously for 20–30 min in a horizontal position on a reciprocating platform shaker. The upper solution was removed and prepared for GC analysis.

PCB analysis

Identification and quantitative determination of PCB congeners were based on analysis utilizing a Varian CP-3800 GC gas chromatograph equipped with an electron-capture detector (ECD). A high performance capillary column (Varian CP-Sil 5 CB: 30 m×0.25 mm, 0.25 μm film thickness) was used. A 10-μl portion of each sample was introduced by the splitless injection technique. The initial oven temperature of 50°C was held for 2 min and then programmed to increase by 10°C/min to 200°C, by 20°C/min to 260°C, by 5°C/min to 280°C. The injector temperature was 250°C and the ECD was maintained at 280°C.

Results

Isolation and characterization of bacterium

A bacterium was isolated using biphenyl as the sole carbon and energy source from a soil sample collected from an oil field in northern China, and designated as R04. Bacterium R04 on MM solid medium was typically round, convex with a smooth margin; it produced exocellular pigments, generally from pink to red with time. Colonies were composed of spherical, short rod cells. Strain R04 was gram-positive. After incubation in solid MM containing biphenyl for 30 h at 30°C, the presence of a yellow halo was observed around the colonies. Presumptively, this indicated that the bacterium had a high capacity to degrade biphenyl. In addition, R04 was capable of degrading many aromatic compounds, such as aniline, phenol, naphthalene, and aroclor (data not shown).

16S rDNA gene sequence analysis

The length of the 16S rDNA gene sequence was 1,477 bp. The bacterium R04 had a 16S rDNA gene sequence identical with *Rhodococcus pyridinovorans* sp. [19] and showed, respectively, 97.3, 96.5, and 95.9% 16S rDNA sequence similarity to *Rhodococcus rhodochrous*01, *Rhodococcus* sp. 814 and *Rhodococcus* rubber. Thus, strain R04 was assigned to the genus *Rhodococcus*.

Growth of R04 on seven PCBs and release of chloride

During the 5-day incubation period, the complete or near-complete disappearance of 500 ppm of 3-CB, 3,4-CB, 4,4′-CB, 2,4,6-CB, 2,4′,5-CB, 2,3,4,5-CB and 3,4,3′,4′-CB was observed. Yellow compounds were produced in bottles, except for 2,4,6-CB and 3,4,3′,4′-CB.

Figure 1 shows the optical density of the culture and chlorine ion concentration during growth of strain R04 on 3-CB. The growth curve on 3-CB was similar to that of the control, but biomass on 3-CB was higher than that of the control within the same time. The release of chloride did not parallel growth. The rate of release of Cl^- in the culture with 3-CB began to decrease after the lag period. As biomass increased over the 5-day incubation period , the concentration of chloride increased to 0.35 mM in bottles containing 3-CB and R04, whereas in cultures with 3,4-CB,2,3,4,5-CB or 3,4,3′,4′-CB the chloride content was about 0.1 mM (Fig. 2). However, in the culture of strain R04 with 4,4′-CB, 2,4,6-CB, 2,3,4,5-CB and 2,4′,5-CB, non-stoichiometric amounts of chloride were produced. It is clear that dehalogenation of PCBs from strain R04 is restricted to the number and site of chlorine substituents, and dehalogenation of PCB congeners with *ortho*-chloride substituents is more difficult under oxic conditions by R04.

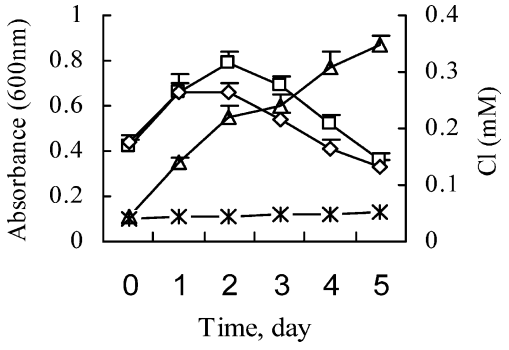

Fig. 1 Growth of strain R04 on 500 ppm of 3-CB. Culture optical density in control bottles (see resting cell incubation) (*open diamonds*), Chloride ion production in control bottles (*asterisks*), Culture optical density in bottles with 3-CB and R04 (*open squares*), Chloride ion production in bottles with 3-CB and R04 (*open triangles*)

Spectral analysis

During incubation of strain R04 with each of the seven PCBs, the supernatants from the incubated solution were scanned at various times. As shown by the Figs. 3 and 4, the scans of supernatants from strain R04 grown on 3-CB, 3,4-CB, 4,4′-CB, 2,4′,5-CB, 2,3,4,5-CB exhibited UV wavelength maxima at 389–435 nm, characteristic of the biphenyl-derived cleavage product (Fig. 3), while supernatants from strain R04 grown on 2,4,6-CB and 3,4,3′,4′-CB did not exhibit characteristic absorption at 389–435 nm. The results suggest that the degradation pathways of the former five PCBs might not be consistent with those of the latter two PCBs.

Figure 5 illustrates changes of the yellow biphenyl-derived cleavage products (YBCPs) formed during the course of incubation with PCBs. After 5 min of incubation, cultures in bottles containing 3,4-CB and R04

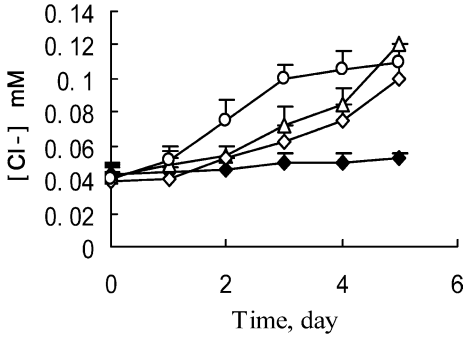

Fig. 2 Chloride ion release during growth of bacterium R04 on 500 ppm 3,4-CB, 2,3,4,5-CB and 3,4,3′,4′-CB. Chloride production in control bottles (see resting cell incubation) (*filled diamonds*), and in bottles with 3,4-CB (*open triangles*), 2,3,4,5-CB (*open diamonds*), or 3,4,3′,4′-CB, and strain R04 (*open circles*)

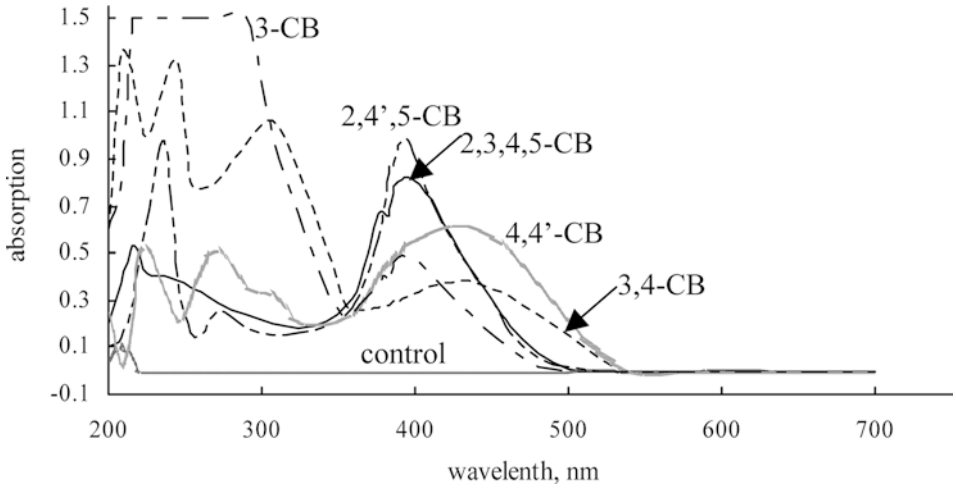

Fig. 3 Spectrum of culture supernatants containing 3-CB, 3,4-CB, 4,4′-CB, 2,4′,5-CB or 2,3,4,5-CB. The spectra of 3-CB and 3,4-CB were recorded after 4 h, and those of 4,4′-CB, 2,4′5-CB and 2,3,4,5-CB after 20 h

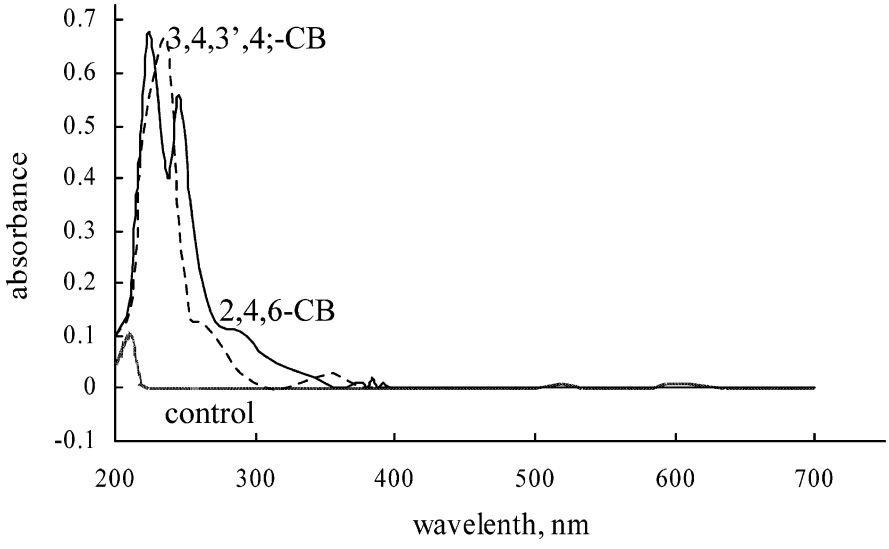

Fig. 4 Spectrum of the supernatants of a culture containing 2,4,6-CB or 3,4,3′, 4′-CB

gradually became yellow, whereas cultures containing 3-CB, 2,3,4,5-CB, 2,4′,5-CB or 4,4′-CB became yellow after 30 min, 4, 10, and 12 h of incubation, respectively (Table 1, Fig. 5). After 10 h of incubation, the yellow color in the culture with 3,4-CB and R04 vanished completely, and the yellow color in the cultures containing 3-CB and 2,3,4,5-CB vanished completely at 14 and 18 h, respectively. However, the YBCPs in the cultures containing 4,4′-CB and 2,4,5-CB were still observed after 5 days. It was clear that during the

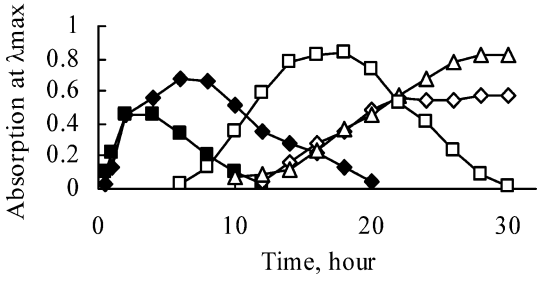

Fig. 5 Time courses of the changes of 2-hydroxy-6-oxo-6-phenylh-exo-2,4-dienoate (HOPDA) derived from PCBs. The ring cleavage products of 3,4-CB (*filled squares*) and 3-CB (*filled diamonds*). Absorption of the ring-fission product of 4,4′-CB (*open diamonds*), 2,4′,5-CB (*open triangles*), and 2,3,4,5-CB (*open squares*)

Table 1 Degradation of 3-CB and 3,4-CB by strain R04 in liquid medium

Duration (h)	Percent remaining[a]	
	3-CB	3,4-CB
0	100	100
4	70 ± 8.1	74 ± 6.8
8	26.6 ± 3.5	52.1 ± 6.6
12	4.1 ± 0.35	42.2 ± 5.3
16	0	27.6 ± 3.1
20	0	7.9 ± 1.1
24	0	0

Note percent degradation was calculated relative to a control (killed cell)

[a]Values shown are mean ± SD for two replicates

Table 2 Degradation of 4,4′-CB, 2,4,6′-CB, 2,4′,5-CB, 2,3,4,5-CB and 3,4,3′,4′-CB by strain R04 in liquid medium

Duration (day)	Percent remaining[a]				
	4,4′-CB	2,4,6′-CB	2,4′,5-CB	2,3,4,5-CB	3,4,3′,4′-CB
0	100	100	100	100	100
1	39.7 ± 4.6	73.7 ± 8.7	56.3 ± 5.9	37.9 ± 4.5	57.2 ± 5.6
2	21.2 ± 3.2	37.3 ± 5.1	32.1 ± 3.1	23.8 ± 3.0	44.8 ± 3.8
3	12.6 ± 1.0	12.9 ± 2.0	17.3 ± 2.0	17.5 ± 1.6	25.8 ± 2.2
4	7.9 ± 1.2	8.0 ± 1.3	11.9 ± 0.9	8.1 ± 1.1	13.1 ± 2.1
5	4.7 ± 0.21	4.7 ± 0.51	5.4 ± 0.41	3.9 ± 0.09	6.3 ± 0.19

[a]Values shown are mean ± SD for two replicates

degradation of PCBs, the formations and transformations of YBCPs were affected by the number and site of chlorine substituents in the PCB rings.

GC analysis

Rsesting cells of strain R04 were incubated with 500 ppm 3-CB or 3,4-CB for 24 h, and 4,4′-CB, 2,4, 6-CB, 2,4′,5-CB, 2,3,4,5-CB or 3,4,3′,4′-CB for 5 days. A sample of culture (1 ml) was taken after various incubation times and extracted with *n*-hexane for analysis of the PCB content by GC.

It was clear that strain R04 isolated from contaminated soil sample was able to degrade 500 ppm of each of the PCBs. The percent degradation of the assayed PCBs by strain R04 are summarized in Tables 1 and 2. After 16 h of incubation, 500 ppm of 3-CB were degraded completely, whereas after 24 h 500 ppm 3,4-CB were completely degraded, and after 5 days about 95% of 500 ppm 4,4′-CB, 2,4,6-CB, 2,4′,5-CB or 2,3,4,5-CB were removed by strain R04.

These data indicate that strain R04 was able to metabolize mono-, di-, tri- and tetra-chlorinated biphenyl. Furthermore, the strain is able to degrade PCB congeners with doubly flanked chlorines. It is noteworthy that the 95% degradation of PCB congeners with chlorines on both rings was achieved by strain R04 after 5 days.

Discussion

To our knowledge, bacterium R04, is a new strain of *Rhodococcus* and is capable of depleting PCBs, similar to *Rhodococcus* sp. RHA1, *Rhodococcus* sp. M5, *R. erythropolis* TA421, *R. erythropolis* BD2, *R. globerulus* P6 [4, 8, 13, 18].

In the study of bacterium growth and dechlorination, the biomass obtained on 3-CB was higher than that of the control at the same time, and the lag period of strain R04 on succinate and 3-CB was longer than that of the control (Fig. 1). This suggests the bacterium can utilize succinate as well as 3-CB, thereby prolonging the lag period. Moreover, 0.35 mM chloride was detected after 5 days of incubation. It was clear that strain R04 dechlorinated 3-CB. It has been reported that many bacteria are aerobically able to remove chlorine from mono-chloro biphenyls and grow on them. [2, 3, 5]. However, to date, the production of chloride during the course of degradation of highly chlorinated PCBs under oxic conditions has not been reported. It is noteworthy that chloride ion was detected in our studies, when strain RO4 was in the presence of 2,3,4,5-CB and 3,4,3′,4′-CB.

Because there are several disadvantages to using aroclor assays to measure degradation of PCBs [6], we chose seven different mono-, di-, tri- and tetra-chlorobiphenyls, representing a variety of structural features, to illustrate PCB transformation. Tables 1 and 2 show the percent degradation of each congener. It is of interest that strain R04 was able to completely remove 500 ppm 3-CB after 16 h of incubation, 500 ppm 3,4-CB after 24 h, and 95% of 500 ppm 4,4′-CB, 2,4′,5-CB, 2,3,4,5-CB and 3,4,3′,4′-CB after 5 days. Compared to previous studies, completely different results were obtained regarding the degradation of congeners with two *ortho* chlorines (2,2′ or 2,6). Most of the bacterial strains previously studied degraded these congeners poorly [7, 17]. Conversely, strain RO4 transformed 95% of 500 ppm 2,4,6-CB after 5 days of incubation. During the degradation of the five PCBs, 3-CB and 3,4-CB were degraded much earlier than 4,4′-CB, 2,4′,5-CB and 2,3,4,5,-CB, perhaps due to the transformation of their intermediate metabolites (YBCPs). During the transformation of PCBs, biphenyl-derived cleavage products from 3-CB and 3,4-CB were detected after 30 and 5 min of incubation, respectively, and there was total transformation within no more than 1 day. However, YBCPs from 4,4′-CB, 2,4′,5-CB and 2,3,4,5,-CB were produced after 4 h of incubation, and disappeared from 2,3,4,5,-CB after 30 h of incubation, whereas the formation of YBCPs from 4,4′-CB, 2,4′,5-CB was accompanied by the appearance of a persistent bright yellow color after 5 days (Fig. 5). Thus, YBCPs were significantly accumulatedand thus restricted the degradation of 4,4′-CB. This suggests that the formation and accumulation of YBCPs from these PCBs was strongly related to with their degradation.

After a day of incubation, 500 ppm 4-CB were completely degraded by the bacterium R04, while the chloride content in the cultures containing 4-CB and R04 was only 0.1 mM, but after 5 days had increased to 0.35 mM (Tables 1 and 2, Figs. 1 and 2). Therefore, the initial degradation occurred most likely prior to chloride release. Sanggo et al. [13] observed that the initial degradation step of 4-CB did not result in chloride release.

In most of the reports on PCB degradation, the transformation of PCBs was determined by the decrease of PCBs. However, we found that, although PCBs were almost completely degraded, there was a signifcant accumulation of intermediate metabolites, such as YBCPs. It is possible that these yellow derivatives and their substrates are harmful to the environment. Therefore, we suggest that further research on PCB degradation be focused not only on PCBs but also on their intermediate metabolites.

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