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Enrichment and Characterization of PCB-Degrading Bacteria as Potential Seed Cultures for Bioremediation of Contaminated Soil

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Summary

The main objective of our study was to obtain seed cultures for enhancing the transformation of polychlorinated biphenyls (PCBs) in contaminated soil of the transformer station in Zadar, Croatia, damaged during warfare activities in 1991. For enrichment, six soil samples were collected from different polluted areas and microcosm approach, stimulating the growth of biphenyl-degrading bacteria, was employed. Enrichment experiments resulted in the selection of two fast growing mixed cultures TSZ7 and AIR1, originating from the soil of the transformer station and the airport area, respectively. Both cultures showed significant PCB-degrading activity (56 to 60 % of PCB50 mixture was reduced after a two-week cultivation). Furthermore, the cultures displayed similar PCB-degrading competence and reduced di- to tetrachlorobiphenyls more effectively than penta- to heptachlorobiphenyls. Strain Z6, identified as *Rhodococcus erythropolis*, was found to be the only culture member showing PCB-transformation potential similar to that of the mixed culture TSZ7, from which it was isolated. Based on the metabolites identified in the assay with the single congener 2,4,4'-chlorobiphenyl, we proposed that the strain Z6 was able to use both the 2,3- and 3,4-dioxygenase pathways. Furthermore, the identified metabolites suggested that beside these pathways another unidentified pathway might also be active in strain Z6. Based on the obtained results, the culture TSZ7 and the strain Z6 were designated as potential seed cultures for bioremediation of the contaminated soil.

Key words: PCB biodegradation, PCB degrading bacteria, 2,3-dioxygenase activity, 3,4-dioxygenase activity, *Rhodococcus erythropolis*

Introduction

Polychlorinated biphenyls (PCBs) have been known as persistent and widespread contaminants which nowadays represent one of the most serious environmental problems. However, despite the stability, toxicity and poor availability of PCBs, a number of bacteria have been isolated that can cometabolize PCBs through the biphenyl

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catabolic pathway. Research on aerobic bacteria isolated so far has mainly focused on Gram-negative strains belonging to the genera *Pseudomonas*, *Burkholderia*, *Ralstonia*, *Achromobacter*, *Sphingomonas* and *Comamonas* (*1–8*). However, several reports about PCB-degrading activity and characterization of the genes that are involved in PCB degradation indicated PCB-degrading potential of some Gram-positive strains as well (genera *Rhodococcus*, *Janibacter*, *Bacillus*, *Paenibacillus* and *Microbacterium*) (*9–15*). Aerobic catabolic pathway for PCB degradation seems to be very similar for most of the bacteria and comprises four steps catalysed by the enzymes BphA, BphB, BphC and BphD. The pathway is initiated by the insertion of two oxygen atoms at the carbon positions 2,3 of one aromatic ring by 2,3-dioxygenase, followed by dehydrogenation, *meta*-cleavage and hydrolysis, resulting in the production of the corresponding benzoic acid and five-carbon fragment (*3,6,16*). This sequence of reactions forms the so-called biphenyl catabolic pathway. Some bacteria are also assumed to possess biphenyl 3,4-dioxygenase enzyme, which attacks the molecule at the positions 3,4. Although this pathway has not yet been defined, the formation of chloroacetophenones has been proposed (*1–3,16,17*).

PCBs have not been produced in Croatia, but they have been used as coolants and lubricants in transformers, capacitors, and other electrical equipment. Despite the reduction in PCB inventories since the implementation of regulatory controls, releases of PCBs to the environment through spills and fires continue to occur. The most recent contamination with PCBs in Croatia was a result of warfare activities in the period 1991–1995 when many military vehicles, condensers and transformers were damaged or destroyed, which caused leaking of PCB-containing oils in the ground. Our experimental field that should be subjected to bioremediation is the contaminated soil of the transformer station in Zadar.

The overall objective of our work was to select the most active PCB-degrading cultures to be used as seed cultures in future bioremediation experiments. The specific objectives were to enrich PCB-degrading bacterial cultures from different PCB-contaminated sites in Croatia and to study their capacity to degrade different PCBs under aerobic conditions. In the present study, we have examined the congener-specific degradation of PCB50 mixture and Aroclor 1248 by two selected enriched mixed cultures, TSZ7 and AIR1, originating from different PCB-polluted areas, and by *Rhodococcus erythropolis* strain Z6, isolated from the culture TSZ7.

Materials and Methods

Soil samples

Soil samples (depth 0–20 cm) used in this study were taken from different PCB-contaminated sites in Croatia (two samples from airport areas, and four samples from the experimental field area in the vicinity of the transformer station in Zadar, TS 110/35 kV). Transformer station was damaged during warfare operations in 1991. Soil samples were analysed for PCB content and the obtained results are summarized in Table 1. Gas chromatographic-mass spectrometric (GC-MS) analysis of the experimental field soil samples showed contamination with 21 dominant PCB congeners (IUPAC numbers: 16, 17, 18, 28, 31, 32, 40, 41, 42, 44, 47, 48, 49, 52, 60, 64, 66, 70, 71, 74, 95) mostly tri- and tetrachlorobiphenyls.

Chemicals

Biodegradation experiments were performed by using two mixtures of PCB congeners (PCB50 and Aroclor 1248). PCB50 mixture, containing di- to heptachlorinated congeners, was obtained by courtesy of the Environmental Protection Institute, Maribor, Slovenia, origin unknown. The composition of PCB50 mixture, analysed and characterized by using GC-MS, is presented in Table 2. The PCB congeners in the chromatogram of the PCB50 mixture were identified by comparison with the chromatograms of commercially available standard Aroclor PCB mixtures published in the literature (*18*) as well as with the chromatogram of a standard mixture of 20 individual PCB congeners (IUPAC numbers: 28, 52, 60, 74, 77, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 170, 180, 189). Commercial mixture Aroclor 1248, containing di- to heptachlorinated congeners, was purchased from Supelco, Bellefonte, USA. Pure PCB congener (2,4,4'--trichlorobiphenyl, PCB-28) was purchased from LGC Promochem, Wesel, Germany; biphenyl was obtained from Merck, Hohenbrunn, Germany; dihydroxybiphenyl from Fluka, Sigma-Aldrich, Deisenhofen, Germany and *n*--hexane (p.a. purity) for organic trace analysis from E. Merck, Darmstadt, Germany. All other chemicals of p.a. purity were products of Kemika, Zagreb, Croatia.

Culture media

The same phosphate-buffered mineral salts medium (PAS) supplemented with 50 mg/L of yeast extract was used as basal medium for all experiments. It was prepared according to Bedard *et al.* (*19*) with the addition of

Table 1. Sampling locations of the contaminated soil from which mixed cultures were enriched

Enriched culture	Origin	PCB mass fraction/(mg/kg)	Determined against
TSZ1	TS	0.19	a mixture of 20 PCBs*
TSZ2	TS	0.42	a mixture of 20 PCBs*
TSZ6	TS	48.00	Aroclor 1248+1254
TSZ7	TS	6044.00	Aroclor 1248+1254
AIR1	airport 1	2.21	Aroclor 1242+1260
AIR2	airport 2	2.25	Aroclor 1242+1260

TS – transformer station; * PCB congeners' IUPAC numbers: 28, 52, 60, 74, 77, 101, 105, 114, 118, 123, 126, 138, 153, 156, 157, 167, 169, 170, 180, 189

Table 2. Degradation of PCB50 mixture expressed as the reduction of chromatographic peak areas in GC-MS chromatograms of culture extracts after 2-week shake-flask cultivation of mixed cultures TSZ7 and AIR1 and pure culture Z6 in the presence of biphenyl

			Reduction*/%		
Peak no.	IUPAC number	PCB congener	TSZ7	AIR1	Z6
1	NA	di-CB	100	100	100
2	NA	di-CB	100	100	96
3	NA	tri-CB	47	50	–
4	18, 15, 17	4,4'; 2,2',4; 2,2',5	99	98	97
5	27	2,3',6	95	93	85
6	16, 32	2,2',3; 2,4',6	74	74	67
7	26, 25	2,3',4; 2,3',5	98	97	95
8	28	2,4,4'	99	99	99
9	33, 53, 20,21	2,3,3'; 2,3,4; 2',3,4; 2,2',5,6'	70	70	71
10	NA	tetra-CB	–	–	–
11	22, 51	2,3,4'; 2,2',4,6'	83	82	70
12	45	2,2',3,6	–	–	–
13	52	2,2',5,5'	42	43	36
14	49, 43	2,2',3,5; 2,2',4,5'	–	–	
15	47, 48, 75	2,2',4,4'; 2,2',4,5; 2,4,4',6	56	54	57
16	44, 37	2,2',3,5'; 3,4,4'	99	93	97
17	42, 59	2,2′,3,4′; 2,3,3′,6	73	67	59
18	64, 71, 41	2,3,4',6, 2,3',4',6; 2,2',3,4	–	–	
19	40	2,2',3,3'	75	70	61
20	67, 100, 57, 103	2,3',4,5; 2,2',4,4',6; 2,3,3',5; 2,2',4,5',6	88	87	83
21	63	2,3,4',5	36	36	32
22	74, 94	2,4,4',5; 2,2',3,5,6'	91	81	88
23	70	2,3',4',5	98	87	96
24	66, 76, 95, 102, 93	2,3',4,4'; 2',3,4,5; 2,2',3,5',6; 2,2',4,5,6'; 2,2',3,5,6	66	60	64
25	91, 55	2,2',3,4',6; 2,3,3',4	–	–	–
26	60, 56	2,3,4,4'; 2,3,3',4'	40	42	41
27	101, 90	2,2',4,5,5'; 2,2',3,4',5	–	–	–
28	99	2,2',4,4',5	–	–	–
29	83	2,2',3,3',5	–	–	–
30	97, 152	2,2',3',4,5; 2,2',3,5,6,6'	52	52	54
31	87, 117, 125	2,2',3,4,5'; 2,3,4',5,6; 2',3,4,5,6'	–	–	–
32	85	2,2',3,4,4'	–	–	–
33	77, 136	3,3',4,4'; 2,2',3,3',6,6'	–	–	–
34	151	2,2',3,5,5',6	–	–	–
35	123, 149, 139, 118	2',3,4,4',5; 2,2',3,4',5',6; 2,2',3,4,4',6; 2,3',4,4',5	42	46	45
36	153	2,2',4,4',5,5'	35	40	41
37	105, NA	2,3,3',4,4'; hexa-CB	–	–	–
38	141, 179	2,2',3,4,5,5'; 2,2',3,3',5,6,6'	–	–	–
39	138, 163, 164	2,2',3,4,4',5'; 2,3,3',4',5,6; 2,3,3',4',5',6	–	–	–
40	NA	hepta-CB	–	–	–
41	167	2,3',4,4',5,5'	–	–	–
42	NA	hepta-CB	–	–	–
43	180	2,2',3,4,4',5,5'	–	–	–

*Data values are averages of duplicate experiments; NA = IUPAC number not assigned
CB = chlorobiphenyl; – = peak area reduction less than 25 % (not considered significant degradation)

sterile molten biphenyl. To prepare PAS agar plates Bacto Agar (Difco Laboratories, USA) was added to the basal medium (3.75 g/L).

Microcosm enrichment

Duplicate microcosms were prepared by mixing 2 g of soil and 18 mL of PAS medium in 100-mL Erlenmeyer flasks, with the addition of 100 mg/L of biphenyl. Every 7–10 days 10 % of enriched culture was transferred into 300-mL Erlenmeyer flasks containing 100 mL of fresh medium supplemented with 1 g/L of biphenyl. Flasks were shaken on a rotary shaker (200 rpm, 28 °C). During culturing the colour in microcosms was monitored in order to observe yellow metabolite 2-hydroxy-6-oxo-6-phenyl--hexa-2,4-dienoic acid.

Characterization of bacterial communities

In order to determine the composition of mixed cultures during the growth in microcosms (expressed as CFU per mL of sample), colony counts were performed by spreading appropriate dilutions onto PAS agar plates. Molten biphenyl was placed in the lid of Petri dish and plates were incubated at the temperature of 30 °C for 14 days. After incubation, morphologically similar colonies were grouped into different morphotypes and members of each group were counted and expressed as percentage of total bacterial population.

Isolation of bacterial strains expressing dioxygenase activity

To test the activity of the 2,3-dihydroxybiphenyl dioxygenase enzyme, colonies grown on appropriate plate dilutions were sprayed with aqueous solution containing 1 mg/mL of dihydroxybiphenyl and 0.1 mL/mL of acetone (*20*). Positive colonies, recognized as the colonies surrounded by a yellow zone, were further purified by respreading a single colony several times on PAS agar plates until the purity was confirmed.

16S rDNA analysis

Sequence analyses of 16S ribosomal DNA (rDNA) were performed on strains by amplifying the 16S rRNA genes by PCR using universal primers (27f and 1492r) (*21,22*) in 50-μL volume using a TGradient thermal cycler (Biometra, Göttingen, Germany) and following the standard protocol (*23*). PCR products were separated in 0.8 % agarose gel and purified with a Qiagen QIAquick gel extraction kit according to the manufacturer's instructions. Sequence analyses were performed with automatic sequence analyzer »ABI PRISM® 3100-Avant Genetic Analyser« (Applied Biosystem, USA) with internal primers 27f, 1492r, 38f, 338f and 518r. 16S rDNA sequences were analysed using GenBank NCBI database and the Ribosomal Database Project (Sequence Match and Classifier Programs) to obtain the most closely matched species. Also, using Clustal X program, multiple sequence alignments were performed.

PCB biodegradation assay

For biodegradation assays with PCB50 mixture, 5 mL of inoculum (three-day-old shaken-flask culture grown in PAS medium supplemented with biphenyl) were added into 300-mL Erlenmeyer flasks containing 50 mL of PAS medium and supplemented with biphenyl (1 g/L) and PCB50 mixture (50 mg/L). For the assay with Aroclor 1248, 2 mL of pure culture inoculum (prepared as described above) were added into 50-mL Erlenmeyer flasks containing 20 mL of PAS medium and supplemented with biphenyl (0.7 g/L) and Aroclor 1248 (45 mg/L). Control experiments without the addition of bacterial cells were performed as well. All experiments, including the control, were done in duplicates. In both assays, flasks were incubated for 2 weeks on a rotary shaker (200 rpm, 28 °C). After incubation, the whole flask was submitted to the extraction as follows: heating (90 °C, 10 min); sonication (15 min); addition of 20 mL of *n*-hexane; shaking on rotary shaker (2 h, 250 rpm, 18 °C); addition of anhydrous sodium sulphate (5 g) and centrifuging (4000 × *g*, 4 min). The obtained *n*-hexane layer was collected and GC-MS analyses of the extracts were performed by using a Varian Saturn II GC-MS system (Varian, Walnut Creek, CA, USA) consisting of a Varian 3400 gas chromatograph and ion trap detector (ITD). Separation of PCB congeners was performed on an Rtx-5MS fused silica column, dimensions 60 m × 0.25 mm (i.d.), film thickness 0.25 μm (Restek, Bellefonte, PA, USA). The column temperature was programmed from 60 °C (1 min hold) to 200 °C at 40 °C/min, then to 240 °C at 2 °C/min, and then to 260 °C at 40 °C/min (20 min hold). The injector temperature was programmed from 100 °C (0.1 min hold) to 270 °C at 200 °C/min (3 min hold). The carrier gas was helium. Column head pressure was 103 Pa. The GC-MS (ITD) was operating in the electron impact ionization mode (70 eV) at the filament emission current of 20 μA. Transfer line and manifold temperatures were 240 and 220 °C, respectively. The spectra of PCB congeners were recorded in the full scan acquisition mode (mass range 45–500 *m/z*) and the scan rate of 1 scan/s. Areas of specific peaks in chromatograms of the samples were measured and compared to those in chromatograms of control samples. The peak areas in chromatograms of the culture extracts after incubation period were expressed as a percentage of the corresponding peak areas in chromatograms of the control sample extracts.

Biodegradation assay with single PCB congener

Assay with a single congener PCB-28 was performed with pure culture Z6 isolated from mixed culture TSZ7. Inoculum (2 mL of three-day-old culture grown in PAS medium supplemented with biphenyl) was added into 20 mL of PAS medium. Flasks were supplemented with biphenyl (250 mg/L) and 5 mg/L of the congener. The experiment was performed in duplicates. Culture was incubated for 7 days under the same conditions as previously described for biodegradation assays. After incubation, the whole flask was submitted to the extraction performed on a rotary shaker by the addition of dichloromethane (25 mL) and the organic layer was collected for further analysis. Extraction was repeated but this time the culture was acidified by the addition of 100 μL of concentrated hydrochloric acid (to give pH=2–3). Both organic layers from neutral and acid extractions were joined, dried with anhydrous sodium sulphate and concentrated under the stream of nitrogen (to the final volume of 250 μL). The extracts were subjected to GC-MS analyses using a GC/MS HP AGILENT instrument (Palo Alto, CA, USA) equipped with DB-5MSITD column (J&W Scien-

tific, Folsom, USA). The column temperature was programmed from 35 °C (1 min hold) to 300 °C at 5 °C/min (15 min hold). The mass spectra of metabolites were recorded in the full scan acquisition mode (mass range 33–650 *m/z*) and the scan rate of 1 scan/s. The carrier gas was helium.

Nucleotide sequence accession number for strain Z6

The nucleotide sequence described in this article is deposited in the NCBI/Gen Bank nucleotide sequence database under the accession no.: DQ397663 (16S rDNA).

Results

Enrichment and selection of biphenyl degrading bacterial cultures

Microcosm experiments presented in this work resulted in the enrichment of six mixed bacterial cultures (derived from soil samples of different PCB contamination levels) within 1–5 months (*i.e.* after 4–20 subculturings) in mineral medium with biphenyl as the only carbon source (Table 1). The fastest enrichment was achieved for the culture TSZ7. During enrichment, formation of the yellow metabolite 2-hydroxy-6-oxo-6-phenyl-hexa-2,4-dienoic acid (HOPDA), indicating 2,3-dihydroxybiphenyl dioxygenase activity (2,3-DHBD), was observed in all cultures except in the TSZ1. At the beginning of the enrichment, HOPDA appeared within 3 days from the onset of incubation. This period was shortened for cultures AIR1 and TSZ7 as the enrichment continued, to be finally observed within 24 hours. Biphenyl consumption was monitored likewise, and its total exhaustion with the cultures AIR1 and TSZ7 was observed within 7 days. Furthermore, growth was detected in all cultures, although the highest turbidity was observed with the mixed cultures AIR1 and TSZ7. Therefore, in our further work we concentrated on the characterization of these two fastest growing mixed cultures which originated from the polluted soils collected at the airport area (culture AIR1) and at the experimental field of the transformer station (TS) area (culture TSZ7).

Characterization of the selected mixed bacterial cultures AIR1 and TSZ7

The study of the structure of the selected mixed cultures suggested that cultures AIR1 and TSZ7 each contained eight morphologically different colony types. Morphological similarity was observed between the colony types T6 and Z6 originating from mixed cultures AIR1 and TSZ7, respectively. After incubation on PAS agar plates under biphenyl atmosphere, the presence of a yellow halo around the colonies of these two members was observed, as an indication of dioxygenase activity. To verify this assumption, colonies were sprayed with dihydroxybiphenyl, and the appearance of yellow colour within a few minutes confirmed 2,3-DHBD activity of both members. None of the other grown colonies of the selected cultures showed this activity. Colony morphology of biphenyl degraders was as follows: round, convex, smooth with wave margins and creamy brown coloured.

During the enrichments the selected mixed cultures went through two phases of growth: the first »yellow phase« occurred within 3 days following each transfer in fresh PAS medium and was characterized by the accumulation of HOPDA; the second »turbid phase« lasted until the exhaustion of biphenyl and was characterized by an increase in bacterial biomass and disappearance of the yellow colour. The analysis of the grown colonies on PAS agar plates suggested that during these phases the structure of both mixed cultures changed significantly. Surprisingly, colony types Z6 and T6, expressing 2,3-DHBD activity and characterized as biphenyl degrading strains, were present in small proportions (approximately 1 %) of total population in both mixed cultures. Both strains Z6 and T6 were submitted to 16S rDNA nucleotide sequence analysis, which showed their phylogenetic similarity, *i.e.* sequence identity of 95–98 % to *Rhodococcus erythropolis* strains. In further experiments we chose the strain Z6, which originated from the polluted soil of the TS area.

Screening of enriched mixed cultures for their PCB-degrading activity

Screening of PCB-degrading activity of the selected mixed cultures AIR1 and TSZ7 was performed in shake-flasks by using PCB50 mixture, which contained di- to heptachlorobiphenyls (di- to hepta-CBs). The average reduction percentage of chromatographic peak areas in the chromatograms of culture extracts after 2-week incubation of the selected cultures is presented in Table 2. As evident, the cultures expressed similar PCB-degrading activities. They reduced 19 out of 43 peaks in the PCB50 mixture chromatogram with the efficiency of 50 to 100 %. Among them the reduction higher than 80 % was obtained for 11 peaks corresponding to di- to tetra-CBs and between 50 and 80 % for 8 peaks corresponding to tri- to penta-CBs. The remaining peaks, corresponding mainly to penta- and hexa-CBs were reduced between 25 and 50 % and hepta-CBs less than 25 % (the latter was not considered a significant degradation).

The results presented in Table 2 revealed as well that the selected cultures are capable of degrading tri-CBs regardless of different chlorine substitution. In contrast, selected cultures showed different degradation activity toward different substituted tetra-CBs (from none to 97 % reduction). For 2,2′,5,5′-CB (peak no. 13) and 2,2′,3,3′-CB (peak no. 19), both di(*ortho*)- and di(*meta*)-substituted congeners, lower reduction was achieved for the congener with the only free positions 3,4 for dioxygenase attack (41 *vs*. 72 %, respectively). Reduction was not observed for the tri(*ortho*)-substituted 2,2′,3,6-CB (peak no. 12). The congener 2,3,4′,5 (peak no. 21) with blocked both *meta* positions on one ring was degraded less efficiently than the congeners 2,3′,4′,5 (peak no. 23) and 2,2′,3,3′-CB (peak no. 19) with blocked one *meta* position on each ring (36 % compared to 92 and 72 %, respectively).

PCB-degrading activity of the strain Z6

To screen PCB-degrading activity of the strain Z6, the only member of the mixed culture TSZ7 which showed 2,3-DHBD activity, the first biodegradation experiment was performed under the same conditions as described for the mixed cultures. The obtained results (Table 2) revealed similarities in the degradation potential of this strain and the mixed culture TSZ7 from which it was isolated.

For further analysis of PCB-degrading activity of the strain Z6 the second shake-flask biodegradation experiment was performed with Aroclor 1248. The results presented in Table 3 revealed that reduction of di- and tri-CBs was higher than 80 %, except for 2,2′,6-CB (peak no. 4) (51 %). Furthermore, tetra-CBs were reduced up to 100 %, with average reduction of 67 %, while the reduction for higher chlorinated congeners was weak. Thus, from penta-CBs, only 2,2′,3′,4,5-CB (peak no. 34) and 2,3′,4,4′,5-CB (peak no. 42) were considered degraded and the remaining hexa- and hepta-CBs non degraded by the strain Z6.

Furthermore, degradation from 40 to 96 % was achieved for both congeners with the only free positions for 2,3-dioxygenase attack (2,3,4,4′-CB; 2,4,4′,5-CB; 2,3′,4,4′, 5-CB) and the congeners with the only free positions 3,4 (2,2′,5,5′-CB). However, when comparing structurally similar congeners (2,2′,3,5′-CB and 2,2′,3,3′-CB *vs*. 2,2′,5,5′--CB), higher reduction was obtained for congeners with free positions for both dioxygenase attacks. Furthermore, strain Z6 efficiently degraded di(*ortho*)-CBs (2,2′-CB; 2,2′,4-CB; 2,2′,5-CB), di(*para*)-CBs (2,4,4′-CB; 2,4,4′,5-CB) and di(*meta*)-CBs (2,2′,3,5′-CB; 2,2′,3,3′-CB; 2,3′,4′,5-CB).

Table 3. Degradation of PCB congeners in Aroclor 1248 after 2-week shake-flask cultivation of *Rhodococcus erythropolis* strain Z6 in the presence of biphenyl

Peak no.	IUPAC number	PCB congener	Concentration/(μg/mL)		
			initial	after 2 weeks	degraded*/%
1	4	2,2'	0.029	0.000	100
3	8	2,4'	0.185	0.000	100
4	19	2,2',6	0.067	0.033	51
5	18+17	2,2',4; 2,2',5	2.281	0.044	98
6	27	2,3',6	0.042	0.000	100
7	32+16	2,4',6; 2,2',3	0.952	0.206	78
10	28+31	2,4,4'; 2,4',5	6.116	0.355	94
15	52	2,2',5,5'	4.112	1.605	61
16	49	2,2',4,5'	2.783	1.673	40
17	48+47	2,2',4,5; 2,2',4,4'	2.250	0.867	61
18	44	2,2',3,5'	3.939	0.016	100
19	42	2,2',3,4'	1.232	0.228	81
20	71+41+64	2,3',4',6; 2,2',3,4; 2,3,4',6	3.857	2.681	30
22	40	2,2',3,3'	0.739	0.106	86
25	74	2,4,4',5	2.698	0.119	96
26	70	2,3',4',5	5.461	0.132	98
27	66+95	2,3',4,4'; 2,2',3,5',6	6.214	3.725	40
28	91	2,2',3,4',6	0.410	0.353	-
29	60	2,3,4,4'	1.649	0.866	47
30	101	2,2',4,5,5'	1.540	1.240	–
31	99	2,2',4,4',5	1.220	1.042	–
32	119	2,3',4,4',6	0.038	0.039	–
33	83	2,2',3,3',5	0.170	0.139	–
34	97	2,2',3',4,5	0.856	0.298	65
35	87	2,2',3,4,5'	0.977	0.838	–
41	123	2',3,4,4',5	0.049	0.061	–
42	118	2,3',4,4',5	1.768	1.066	40
43	114	2,3,4,4',5	0.101	0.078	–
45	153	2,2',4,4',5,5'	0.273	0.234	–
46	105	2,3,3',4,4'	1.200	1.077	–
49	138	2,2',3,4,4',5'	0.329	0.308	–
52	167	2,3',4,4',5,5'	0.008	0.006	–
55	180	2,2',3,4,4',5,5'	0.096	0.086	–
56	170	2,2',3,3',4,4',5	0.029	0.034	–

*Data values are averages of duplicate experiments
– = reduction less than 25 % (not considered significant degradation)

Also, lower reduction was obtained for 2,2′,6-CB, with tri(*ortho*)-substituents. Different reduction was obtained as well for structurally similar PCB-congeners such as 2,2′,4,5′-CB (peak no. 16) *vs.* 2,2′,3,4′-CB (peak no. 19), or 2,3,4,4′-CB (peak no. 29) *vs.* 2,4,4′,5-CB (peak no. 25), and among penta-CBs between 2,2′,3,4′,5′-CB (peak no. 34) and 2,2′,4,5,5′-CB (peak no. 30).

During the biodegradation experiment with the congener PCB-28 (2,4,4′-CB), metabolites formed were also monitored by GC-MS analyses. The total ion current chromatogram of culture extract after 7-day incubation is presented in Fig. 1 and the main metabolites that are presumed to be formed by the strain Z6 are listed in Table 4. Chlorobenzoic acids were identified by comparing

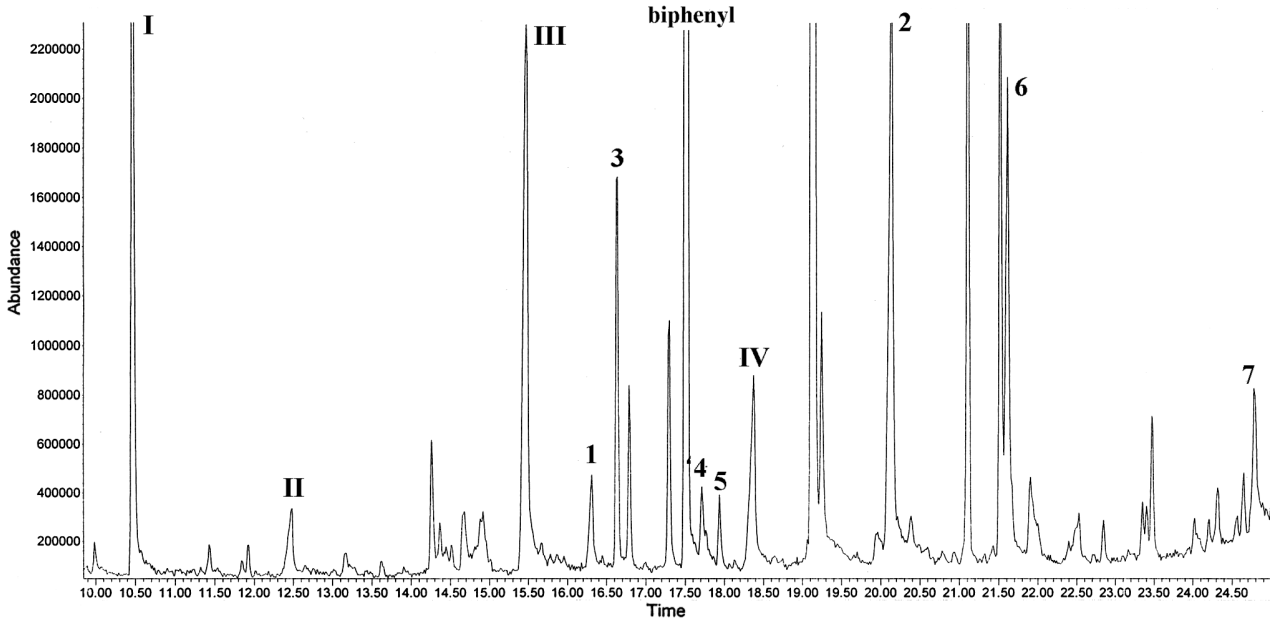
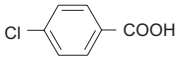
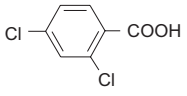
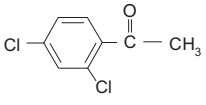
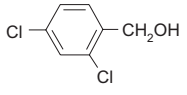
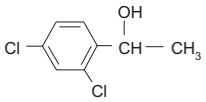
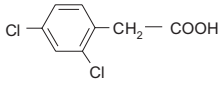
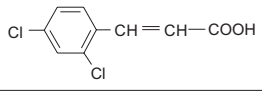


Fig. 1. Total ion current chromatogram of metabolites detected in extracts of the assay with 2,4,4′-trichlorobiphenyl (PCB-28) by using *Rhodococcus erythropolis* strain Z6
1 to 7 = metabolites presumed to be generated from PCB-28 transformation (structure shown in Table 4)
I to IV = metabolites presumed to be generated from biphenyl transformation (I = acetophenone; II = benzoic acid; III = 2-hydroxybenzoic acid, and IV = 3-phenyl-2-propenoic acid)

Table 4. Main metabolites detected in single PCB-congener assay with *Rhodococcus erythropolis* strain Z6 during transformation of PCB–28

No.	Metabolite	Structure	*m/z* (relative intensity/%)
1	4-chlorobenzoic acid		156 (70) 139 (100) 111 (55) 75 (36) 50 (19)
2	2,4-dichlorobenzoic acid		190 (55) 173 (100) 145 (26) 109 (17) 74 (36)
3	2,4-dichloroacetophenone		188 (17) 173 (100) 145 (29) 109 (15) 75 (17)
4	2,4-dichlorophenyl methanol		176 (39) 141 (70) 113 (68) 77 (100)
5	α-methyl-2,4-dichlorophenyl methanol		190 (17) 175 (100) 147 (14) 111 (62) 75 (15)
6	2,4-dichlorophenyl acetic acid		204 (24) 171 (17) 159 (100) 125 (37) 89 (28)
7	3-(2,4-dichlorphenyl)-2-propenoic acid		216 (10) 181 (100) 171 (30) 136 (20)

mass spectra and the retention times of the compounds to available standards, while the identification of other metabolites was based on their mass spectra and data suggested by MS computer program library, since authentic standards of these compounds were not available. As can be seen from the presented results, in addition to chlorobenzoic acids, known to be formed within biphenyl pathway (Fig. 1, metabolites no. 1 and 2), some other unexpected chlorinated aromatic metabolites were also identified in the culture extracts (Fig. 1, metabolites no. 3–7). Furthermore, unchlorinated metabolites presumed to be generated from biphenyl transformation were identified as well.

Discussion

The enrichment approach employed in this work was based on growth stimulation of biphenyl-degrading bacteria, which are considered as potential PCB-degraders (*8*,*12*–*14*). From six soil samples collected at different locations with long-time PCB contamination history, two mixed cultures (TSZ7 and AIR1) were chosen as potential PCB-degrading cultures since they: (*i*) established stable population densities within 1–5 months, (*ii*) showed the highest growth on biphenyl, and (*iii*) expressed 2,3-DHBD activity. The fastest enrichment of the culture TSZ7, originating from the experimental field of the TS Zadar, could be due to high level of PCB contamination in the soil.

Morphological characterization of the selected mixed cultures grown on PAS agar plates under the atmosphere of biphenyl suggested that both selected mixed cultures are very complex communities. From each culture a member that produced yellow metabolite HOPDA was isolated, which indicated the presence of at least one bacterial strain that was capable of opening the biphenyl ring. They were present in small proportions of the total population but the detected dioxygenase activity suggested their crucial role in PCB degradation. Sàágua *et al.* (*11*) also reported about a Gram-positive strain which supported PCB degradation and was not the dominant strain in the mixed culture.

Comparison of PCB-degrading activity of the selected mixed cultures TSZ7 and AIR1

Our two-week biodegradation experiment with the selected mixed cultures confirmed their substantial PCB--degrading activity (56 to 60 % of the initial PCB50 mixture was reduced). The obtained results are in agreement with the fact that enzymes expressed during growth on biphenyl are involved in PCB catabolism. Furthermore, despite the fact that the selected cultures originated from the soils with different PCB contamination history, both of them contained PCB-degrading strains belonging to the same species (*Rhodococcus erythropolis*) and showed similar PCB-degrading activity. Similarly, after 6-month enrichment with biphenyl, Wagner-Döbler *et al.* (*20*) found few taxonomic differences between PCB-degrading mixed cultures derived from seven different samples and almost all the cultures contained bacteria belonging to the genus *Rhodococcus*. This suggested that *Rhodococcus* species are widespread in different contaminated soils. The detailed analysis of GC-MS peaks during biodegradation experiments revealed that our cultures reduced less chlorinated biphenyls more efficiently than the highly chlorinated ones (transformed majority of di- to tetra-CBs with the efficiency higher than 50 %; a few peaks containing penta- and hexa-CBs and none of hepta-CBs were considered to be reduced). Similar degrading activity toward less chlorinated congeners has also been reported by other authors (*6*,*7*). Complete disappearance of 2,4,4′-CB, the congener with blocked positions 3,4 for dioxygenase attack, and a significant degradation of 2,2′,5,5′-CB, with blocked positions 2,3 for dioxygenase attack, implied the activity of both 2,3- and 3,4-biphenyl dioxygenase enzyme systems (BDO) in both cultures. However, degradation was higher for the congener with free positions for 2,3-dioxygenase attacks, which further indicated that positions 2,3 were oxidized more easily by our bacteria. The cultures readily degraded di- and tri-CBs with very small differences toward chlorine substitution (di(*ortho*)-CBs (2,3′,6; 2,2′,3; 2,4′,6), di(*meta*)-CB (2,3′,5) or di(*para*)-CB (2,4,4′)). For tetra-CBs, both cultures were efficient in degrading di(*ortho*)-substituted congeners, although it has been suggested that these congeners are more resistant to microbial degradation (*24*,*25*). However, they were not capable of reducing the tri(*ortho*)-CB, but they were capable of degrading di(*para*)-CBs and di(*meta*)-CBs, although better degradation was observed for the congeners with *meta* substitution distributed on both rings. It is necessary to note that the above discussion relates only to the peaks in GC-MS chromatograms representing a single PCB congener.

Congener-specific degradation by Rhodococcus erythropolis strain Z6

Based on 16S rDNA analysis, strain Z6, biphenyl-degrading member of the selected culture TSZ7, was identified as *Rhodococcus erythropolis*. As previously mentioned, most of the research on PCB-degradation reported so far was concentrated on Gram-negative strains, while a few studies were focused on Gram-positive bacteria including *Rhodococcus* species (*13*,*26*–*28*). Furthermore, it is important to note that catabolically active rhodococci have been isolated from different environments and there is a general belief that these bacteria play a crucial role in biodegradation of different pollutants in the soil (*29*,*30*). These facts and the following characteristics make the bacteria of the genus *Rhodococcus* promising candidates for bioremediation of PCB-contaminated soils: (*i*) their cells are hydrophobic, enabling the degradation of hydrophobic pollutants such as PCBs by allowing cells to adhere to the oil/water interfaces (*31*), (*ii*) they can persist in soil even in starvation conditions (*30*), (*iii*) rhodococci usually produce surfactants which decrease the interfacial tension between the phases, making it easier for hydrophobic compound to enter the microbial cell (*29*), and (*iv*) these bacteria exhibit multiple homologues of enzymes, thus enhancing their metabolic versatility (*32*).

GC-MS analyses of the culture extracts of both biodegradation experiments, carried out with PCB50 and Aroclor 1248, confirmed substantial PCB-degrading activity of the strain Z6, comparable to that of the mixed culture TSZ7 from which it was isolated. More detailed analysis of GC-MS chromatograms revealed no marked differences in congener specificity either in degrading congeners with different degree of chlorination or with different chlorination pattern. This further supported our hypothesis

that strain Z6, the only member expressing 2,3-biphenyl dioxygenase activity, is crucial for PCB-degrading activity of the mixed culture TSZ7.

Further analyses of its degradation capability toward congeners with three blocked positions on one ring suggested that strain Z6 preferred 2,5,4-substitution over 2,3,4-substitution. When analyzing di(*ortho*)-CBs, with one *meta* substitution on each ring, degradation seems to follow the rule: 3,5′ is preferred over 3,3′, which is preferred over 5,5′. Also, if *meta* position is switched with *para*, congener is less degradable, while *ortho* position switched with *para* position does not affect the degradation. In comparison with well characterized PCB degrading bacteria *Pseudomonas pseudoalcaligenes* KF707, exhibiting low activity toward *ortho*-substituted congeners and *Burkholderia* sp. LB400, which showed low activity toward *para*-substituted congeners (*5,7,10*), our strain Z6 expressed wider substrate specificity and degraded different *ortho*, *meta* and *para* substituted CBs. Similar substrate specificity was also suggested for some other Gram-positive strains: *Microbacterium* sp. B51 (*10*), *Rhodococcus* sp. R04 and *Rhodococcus* sp. RHA1 (*13,15*).

Catabolic versatility of strain Z6

Further experiment with single congener PCB-28 (2,4,4′-CB) was conducted in order to better elucidate PCB-degrading potential of the strain Z6. As reported so far, most bacteria degrade PCBs *via* a major biphenyl pathway that is initiated by the attack of 2,3-biphenyl dioxygenase (2,3-BDO) and followed by a *meta*-1,2 fission. In that case the corresponding chlorobenzoic acids (CBAs) were detected as main metabolites (*3,6,16*). The metabolites 2,4-CBA and 4-CBA, identified in the culture extracts of our assay with 2,4,4′-CB, suggested that transformation was initiated by 2,3-BDO enzyme and that this congener was attacked on both rings. However, beside these metabolites, 2,4-chloroacetophenone (2,4-CA), as well as some chlorinated benzyl or benzoyl metabolites with shorter aliphatic side-chain (Table 4), which were not expected as part of PCB-degradation pathway, were also identified. In similar investigations with 2,4,4′-CB, CBAs were the only detected metabolites during transformation (*16,33*).

Formation of chloroacetophenones during PCB transformation has been proposed in the studies with Gram-negative bacteria such as *Pseudomonas* sp. 2 and *Pseudomonas* sp. MB86 (*16,17*), *Burkholderia* sp. LB400 (*24*) and *Alcaligenes eutrophus* H850 (*1*). Although the pathway is not unequivocally known, it has been suggested that PCB-degradation was initiated by 3,4-biphenyl dioxygenase (3,4-BDO) attack. Following this assumption, identification of 2,4-CA in our culture extracts suggested that besides 2,3-BDO pathway strain Z6 also used the 3,4-BDO pathway and, due to chlorine substitution, dechlorination has been proposed. However, possible formation of 2,4-CA through some alternative novel pathway involving dioxygenase attack on 2,3- free positions should not be excluded.

Benzyl and benzoyl acidic metabolites with shorter aliphatic side-chain, similar to those we detected in culture extracts, had also been identified in the studies with *Achromobacter* sp. B-218, *Bacillus brevis* B-257 and *Pseudomonas putida* DA2 (*34,35*). The authors suggested that these metabolites were generated from HOPDA through biotransformation involving the genes which were not part of the biphenyl pathway. In their research, Seah *et al.* (*36*) suggested that HOPDAs, the metabolites of 2,4,4′-CB, 4,4′-CB and 2,5-CB transformation, were poor substrates for 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoate hydrolase (BphD) and thus inhibited further transformation of the congener. Applying the latter assumption to our case, detected benzyl and benzoyl acidic metabolites could be a result of produced HOPDA which initiated an alternative pathway for further transformation of the formed metabolite. Alternative pathway(s) could act as parallel catabolic pathway(s) alongside the biphenyl pathway or could be activated by an intermediate metabolite that inhibited further transformation. Without further discussion about potential metabolic pathways used by strain Z6, since our experiments on this subject are in progress, the detected metabolites in the culture extracts undoubtedly suggested that in addition to the major biphenyl pathway, strain Z6 degraded PCBs through other alternative pathway(s). Also, the corresponding unchlorinated phenylpropenoic acids, detected in the culture extracts presumed to be generated from biphenyl transformation, further supported the existence of one or more alternative degradation pathways in the strain Z6. This further points to the catabolic versatility of the strain Z6 in PCB transformation.

Conclusions

This study resulted in the selection of the mixed culture TSZ7 and the pure culture *Rhodococcus erythropolis* strain Z6, isolated from the mixed culture, both of which showed substantial PCB-degrading activity toward a wide range of different substituted di- to tetra-CBs. Compared to well-characterized PCB-degraders, strain Z6 is considered to degrade *para-* and *ortho*-CBs and to use some alternative PCB degrading pathway(s) beside the known biphenyl pathway, which suggests its catabolic versatility. Furthermore, it is important to note that the mixed culture TSZ7 originates from the contaminated soil, thus representing indigenous bacterial community, and that catabolically active culture member Z6 belongs to the genus *Rhodococcus*, which is generally recognized as degrader of various pollutants in the soil. The aforementioned and the fact that the soil is mainly contaminated with di- to tetra-CBs allows the presumption that the selected cultures are promising seed cultures for further bioaugmentation experiments at the experimental field of the transformer station.

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