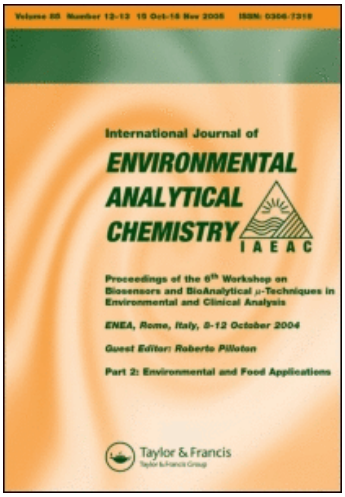

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DEGRADATION OF PCB BY BACTERIA ISOLATED FROM LONG-TIME CONTAMINATED SOIL

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Four bacterial isolates belonging to the genera *Pseudomonas* and *Alcaligenes* were obtained by the enrichment method, using biphenyl as the sole carbon source, from the soil, which underwent long-time contamination with technical mixtures of PCB. Kinetics of PCB degradation by individual isolates was measured using n-hexane extraction of the cultivation media in proper time intervals and analysed by congener specific gas chromatography with electron capture detection. All isolates exhibit interesting biodegradative potential. Specific degradation of individual congeners with respect to the number and position of chlorine substituents is discussed. The influence of glucose, biphenyl and 3-chlorobenzoic acid on the PCB degradation has been assessed.

KEY WORDS: PCB, congeners, microbial degradation, *Alcaligenes, Pseudomonas*, Delor 103, Delor 106, GC-ECD.

INTRODUCTION

PCB rank among the most persistent pollutants. In spite of cessation of their industrial production in eighties, they represent a serious ecological problem due to their low degradability, toxicity, and accumulation in biological systems. This is caused mainly by enormous hydrophobicity and negligible chemical reactivity of these compounds. A possibility for their removal from the environment is represented by the use of selected microorganisms, which are able to transform PCB into less hydrophobic products, both anaerobically and aerobically. Although it is generally accepted that there is a common metabolic pathway for biphenyl and PCB, the pattern and number of chlorine substituents affect the biodegradability of various PCB congeners.[1] Considerable differences in the congener selectivity patterns and ranges of activity of various PCB degrading bacteria have been reported.[2]

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Besides stimulating the indigenous microbial population to degrade organic compounds in the subsurface, another option is to add microorganisms with specific metabolic capabilities to the soil. The suspension of microorganisms can be injected with the infiltrating water at the contaminated site. The objective of this inoculation is to increase the number of adapted microorganisms at the site, in order to accelerate biodegradation. There is much uncertainty about the efficacy of the addition of microorganisms to the subsoil and the possibilities of transporting bacteria through the soil, in order to get them at the spots where they are needed. Generally, 95% of the soil population tends to absorb on soil particles, whereas only 5% can be transported.[3] In spite of this fact, the use of inoculation of selected strains in *in situ* bioremediation is incontestable from ecological and economical standpoints. General aspects of isolation and cultivation of microbes with biodegradative potential were reviewed by Cook *et al.*[4]

The aim of our work was the isolation of microorganisms with enhanced ability to degrade various PCB, including highly chlorinated congeners. Specific degradation of individual congeners and the effect of carbon source on degradation of PCB by the most promising isolate was studied.

EXPERIMENTAL

Chemicals. A commercial mixture of PCB, Delor 103 (equivalent to Aroclor 1242, Chemko Strážske, Slovakia) containing 40–42% (w/v) of bound chlorine, Delor 106 (equivalent to Aroclor 1260, Chemko Strážske, Slovakia) containing 60% (w/v) of bound chlorine, biphenyl and glucose (Lachema, Brno, Czech Republic), 3-chlorobenzoic acid (Merck, Germany), 2,2′,3,3′,4,4′,5,5′,6,6′-decachlorobiphenyl (Institute for Drug Research, Modra, Slovakia), n-hexane UV (Pestiscan, Labscan Ltd, Ireland) and acetone (Microchem Bratislava, Slovakia) with purity grade were used. In order to ensure a wide spectrum of PCB congeners, the mixture consisting of Delor 103 and Delor 106 (2:1 w/w) and decachlorobiphenyl (2% w/w) was used in all experiments.

Microorganisms. Four bacterial isolates from long-time contaminated soil, numbered 1–4, *Pseudomonas putida* CCM 3423, *Alcaligenes eutrophus* CCM 3727 and *Micrococcus varians* CCM 2353 (Czech Collection of Microorganisms Masaryk University, Brno) were tested.

Cultivation conditions. The synthetic DMA medium for bacteria as described by Pirt[5] was used. This is composed of five parts, A to E. Part A is K_2HPO_4, 84.5 g; part B is $MgSO_4.7H_2O$, 20 g; part C is $CaCl_2$, 1.0 g; part D consists of $FeSO_4.7H_2O$, 5.0 g; $ZnSO_4.7H_2O$, 5.0 g; $MnSO_4.5H_2O$, 5.0 g; $CuSO_4.5H_2O$, 1.0 g; $CoCl_2.6H_2O$, 1.0 g; $Na_2B_4O_7$, 1.0 g; $NaMoO_2H_2.H_2O$, 1.0 g; and E is NH_4Cl, 20 g. Parts A, B, C and E were each dissolved in 500 ml of distilled water and autoclaved 20 min at 120 kPa. As for part D, the first three ingredients were each dissolved in 100 ml distilled water, and the other ingredients of part D in 11 each, autoclaved separately (20 min at 20 kPa) and mixed in the volume ratio 10:1:1:10:10:10:10. This solution (52 ml) was mixed with distilled water (448 ml) to give

the final part D solution. The solutions of parts A to E were then mixed in the volume ratio 10:1:1:1:10. To 124 ml of this solution 876 ml of distilled water was added.

PCB extraction. Water matrix samples were extracted with n-hexane, using acetone addition for preventing emulsion formation. To 22 ml of cultivation medium 6 ml of acetone was added and after 15 minute ultrasonic bath extracted by 3 ml of n-hexane. The whole procedure was repeated 3 times. Combined extracts were supplemented with n-hexane to 10 ml and analysed by gas chromatography.

GC analysis. The degradation kinetics of the individual PCB congeners were monitored by GC (HP 5890) of 1 μl samples of the hexane extracts with H_2 as carrier gas (60 kPa, 1.5 ml/min, split-splitless inlet mode), using an electron capture detector (ECD) (280°C, make up gas N_2 at 60 ml/min), and a 50 m × 0.32 mm I.D. fused-silica capillary column with a non polar stationary phase HP 1 (thickness 0.17 μm). Temperature conditions: 45°C (0.5 min)—20°C/min—150°C—2°C/min—250°C (6 min). The detector response was calibrated using the method described by Krupčík *et al.*[6] The reproducibility of the quantitative analysis was controlled using the standard solution of Delor 103 and Delor 106 (c = 7.5 μg/ml). Relative deviations for standard congeners which did not interfere with background were around 3%. GC chromatogram of the mixture Delor 103 and Delor 106 (2:1, w/w) and decachlorobiphenyl (2% w/w) with peak numbering, retention times, IUPAC numbers and chlorine positions are given elsewhere[7].

Isolation of microorganisms. The microorganisms were obtained by preparing an enrichment culture from a long-time PCB contaminated soil sample. The enrichment was set up by adding 10 g of the soil to a 1 l flask containing 500 ml of (a) an cultivation medium[8]: glucose, 0.5 g/l; peptone, 0.5 g/l; KH_2PO_4, 0.5 g/l, pH 7.0 and incubated for 3 days on a rotary shaker (180 rev/min), 30°C; (b) directly in distilled water without cultivation. Other steps were the same for both experiments. Isolation was carried out in a 500 ml flask containing 200 ml of a DMA mineral salt medium[5] with 1 g/l of biphenyl as the sole carbon source. An inoculum of the enrichment culture (4 ml) was transferred to this flask and incubated for 7 days on a rotary shaker. To isolate the organisms an inoculum of the above liquid cultures was streaked on the surface of the agarised DMA medium with excessive biphenyl (5 g/l) in Petri dishes. After incubation a portion of each single colony was picked up and subcultured aerobically in liquid DMA medium with biphenyl and streaked on the solid medium again. Isolates were identified in Czech Collection of Microorganisms, Masaryk University, Brno.

Degradation of PCB by isolates and adapted collection strains of microorganisms. Degradation was carried out in 50 ml flasks containing 20 ml of synthetic DMA medium with PCB (9.5 μg/ml) as the sole carbon source and 2 ml of inoculum (d.w. 0.6 g/l). Before the degradation experiment the microorganisms were adapted to biphenyl (5 g/l) in DMA medium for 4 days. The adapted cells were harvested by centrifugation (4000 rev/min, 10 min), and washed with 0.05 mol/l phosphate buffer (pH 7.0). The total amount of individual PCB congeners remaining in the medium and the total degradation were calculated relative

to the control analysed at the initial time. The total degradation is expressed on the weight basis.

RESULTS

Isolation of PCB degradable bacteria and their characteristics

Four bacterial isolates, obtained from a long-time contaminated soil by the enrichment method using biphenyl as the sole carbon source were identified in Czech Collection of Microorganisms, Masaryk University, Brno, as the bacterial strains of the genus *Alcaligenes*[9] and *Pseudomonas*.[10] The isolate 1 is a mixed culture of *Alcaligenes xylosoxidans* and *Pseudomonas stutzeri*, the isolate 2 is *Alcaligenes xylosoxidans*, the isolate 3 is *Pseudomonas putida* and the isolate 4 is a mixed culture of *Alcaligenes piechaudii* and an unidentified strain.

The degradation of PCB by isolates 1, 2, 3 and 4 was monitored at proper time intervals. Figure 1 shows the spectrum of individual PCB congeners at the initial time (a), after 7 days (b) and 14 days (c) of cultivation *Alcaligenes xylosoxidans* (isolate 2) in the synthetic medium with PCB as the sole carbon source. It is interesting to notice that congener 209—decachlorobiphenyl (retention time 59.92, the last peak on the chromatograms) exhibited negligible changes during the 14 day cultivation. Details (peak numbering, retention times, IUPAC numbers and chlorine position) about PCB congeners eluated in single peaks are given elsewhere.[7]

Congener specific degradation

Our PCB mixture includes congeners ranging from dichloro- to deca-chlorobiphenyls and representing various structural classes[11], e.g. congeners chlorinated on a single ring (2, 3-dichlorobiphenyl), blocked at 2,3 sites (2,5,2′,5′-tetrachlorobiphenyl), blocked at 3,4 sites (4,4′-dichlorobiphenyl), and lacking adjacent unchlorinated sites (2,4,5,2′,4′,5′-hexachlorobiphenyl). We obtained four isolates which differed in PCB degradative ability. The congener assay clearly demonstrates the differences and similarities in the PCB-degradative competence of these strains (Figure 2). The results show that the isolate 1 (mixed culture *Alcaligenes xylosoxidans* and *Pseudomonas stutzeri*) and the isolate 2 (single strain *Alcaligenes xylosoxidans*) not only degraded majority of the congeners to a greater extent than the isolates 3 and 4 but also caused the degradation of the more highly chlorinated congeners (peaks 50–87, including hexa-, hepta- and octa-chlorobiphenyls). The degradative ability of isolates 3 and 4 was comparable. All the isolates degrade preferentially less chlorinated congeners. The remaining amount of low chlorinated congeners in cultivation medium of all isolates ranged from of 10 to 40%, that of high chlorinated congeners was 40–60% of the initial amount. Marked differences between degradation of *ortho*-, meta-, and *para*-chlorinated congeners were not observed with exception of the most persistent congener 79 (2,2′,3,3′,4′,5,6,6′-octachlorobiphenyl). The

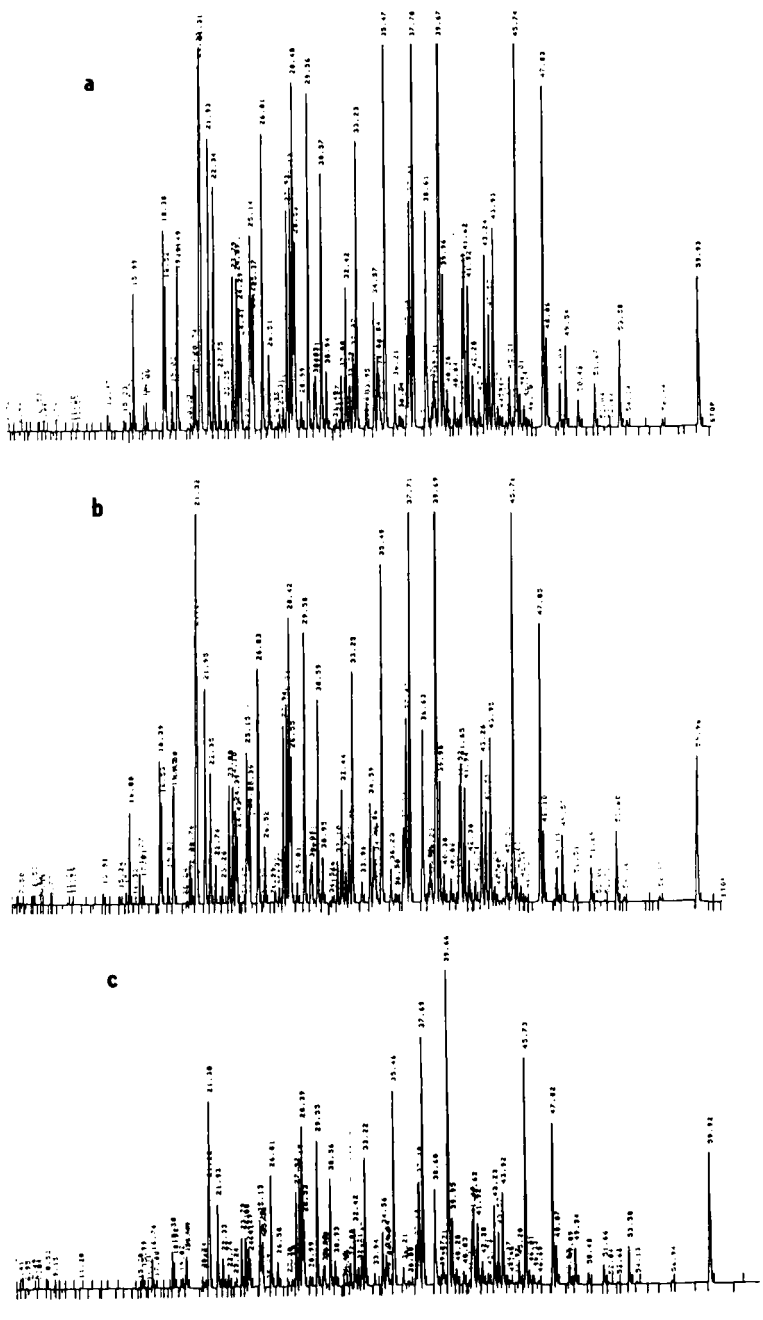

Figure 1 GC chromatograms of PCB after 0 (a), 7 (b) and 14 day (c) cultivation of *Alcaligenes xylosoxidans* (isolate 2) in DMA medium (pH 6.7) with PCB (c = 9.5 μg/ml) as the sole carbon source, on a rotary shaker, 30°C. Congener assignments are given in Haluška *et al.*[7]

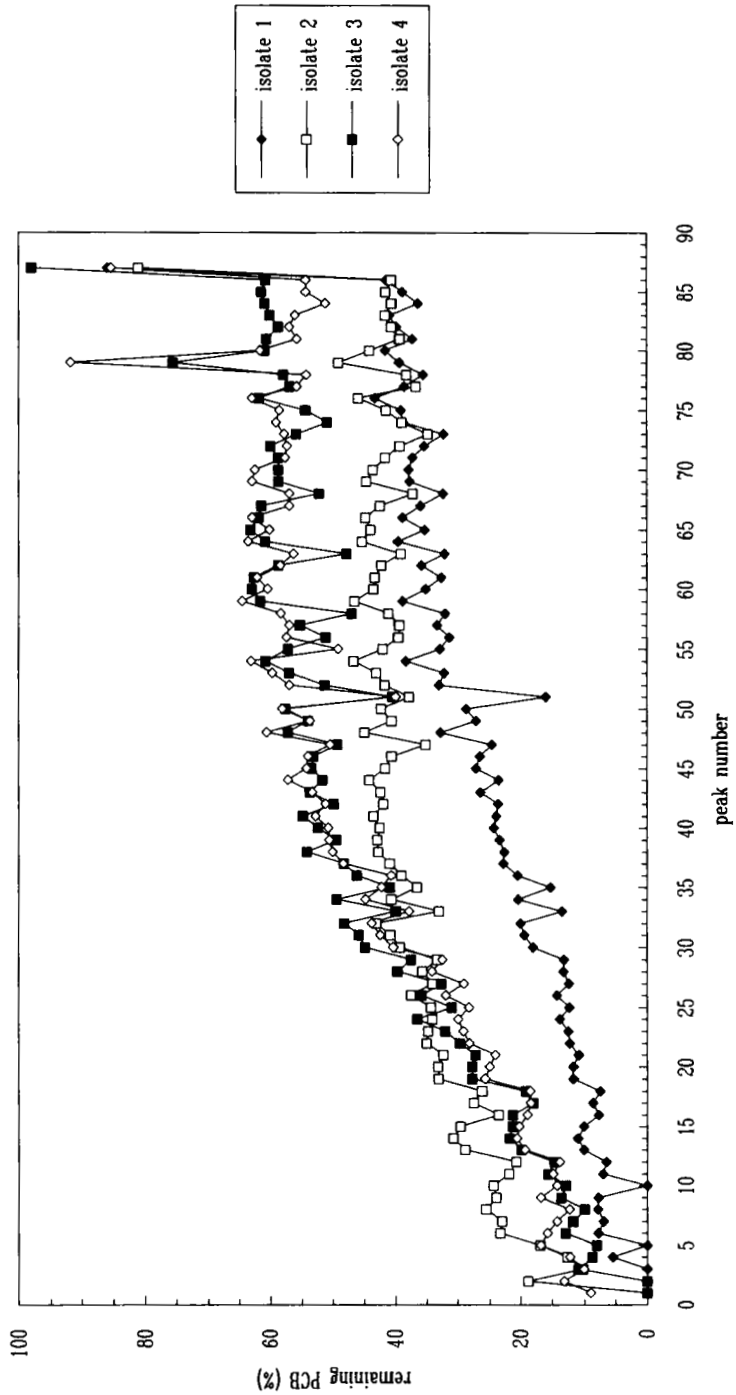

Figure 2 Spectrum of PCB (initial concentration 9.5 μg/ml) congeners remaining in synthetic medium DMA (pH 6.7) after 14 day cultivation of isolate 1, 2, 3 and 4 under aerobic conditions at 30°C. The peaks are assigned as in Haluška *et al.*[7]. —◆— isolate 1, —□— isolate 2, —■— isolate 3, —◇— isolate 4.

congener 95 (2,2′,3,5′,6-pentachlorobiphenyl) is degraded faster than the congeners with similar degree of chlorination by all the cultures. The isolate 1 eliminates well also the congeners 4 and 10 (2,2′; 2,6, peak 1), 7 and 9 (2,4; 2,5, peak 2), 6 (2,3′, peak 3), 19 (2,2′,6, peak 5), and 16 and 32 (2,2′3; 2,4′,6, peak 10). The remaining amounts of PCB in the cultivation medium of the isolates 1 and 2 after 14 day cultivation in dependence on the number of chlorine substituents are shown in Figure 3. While the isolate 2 degrades preferentially di-, tri-, and tetra-CB (ordered according to the decreasing elimination rate), the higher chlorinated congeners being degraded at similar rates, the PCB degradation by the isolate 1 is approximately linearly related to the chlorination degree.

Comparison of isolates with adapted collection strains

Table 1 summarises the results of biodegradation tests of isolates 1, 2, 3 and 4 in comparison with the adapted collection strains *Pseudomonas putida* CCM 3423, *Alcaligenes eutrophus* CCM 3727 and *Micrococcus varians* CCM 2353 after 14 day cultivation. Degradation in the studied bacterial strains ranged from 36 to 78%, 36–52% degradation was observed in the collection strains and 60–78% in isolates. The best single strain *Alcaligenes xylosoxidans* (isolate 2), exhibiting good biodegradative ability and the best growth on biphenyl as the sole carbon source (the results not shown) was selected for the next experiments.

Effect of organic substances on the PCB degradation

Many resistant contaminants can be degraded fortuitously by organisms growing on other C sources (cometabolism) that induce production of the responsible enzymes or because the organisms with degrading capacity use these compounds as substrates[12]. The reports about the influence of various carbon sources on the PCB degradation are rather contradictory: promotion of the biodegradation was observed by Yagi *et al.*[13] and Sugiura[14], Tulp *et al.*[15] and Kong *et al.*[16] reported depression of the process.

Glucose as an easily degradable substrate, biphenyl as an inductor of PCB degrading enzymes and 3-chlorobenzoic acid known as a metabolic inhibitor[17,18], were separately

Table 1 Degradation of PCB (c = 9.5 μg/ml) by isolates and adapted collection strains of microorganisms after 14 day cultivation, aeration, 30°C, synthetic medium DMA, pH 6.7.

Microorganisms	*Degradation (%)*
Isolate 1	78
Isolate 2	63
Isolate 3	60
Isolate 4	60
Pseudomonas putida CCM 3423	52
Alcaligenes eutrophus CCM 3727	46
Micrococcus varians CCM 2253	36

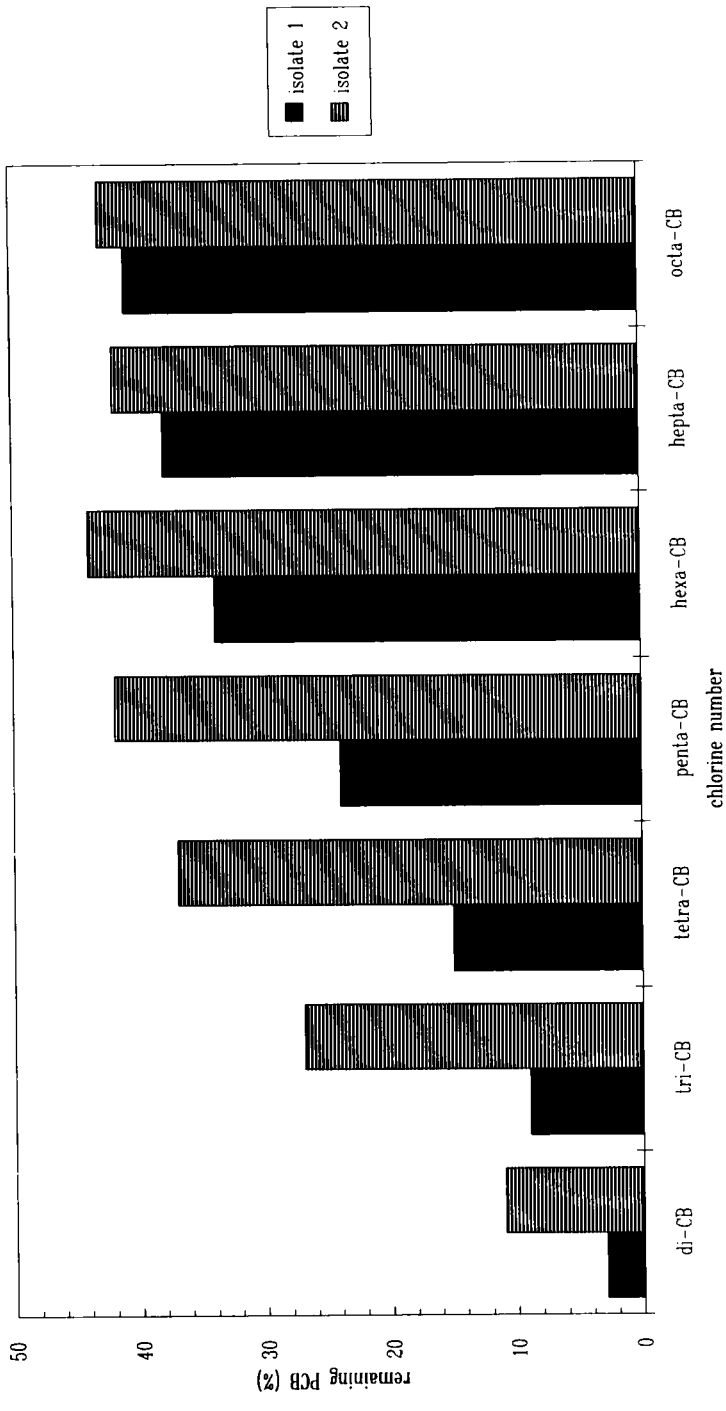

Figure 3 Remaining amount of PCB (initial concentration 9.5 μg/ml) in dependence on chlorine number by isolate 1 and isolate 2 after 14 day cultivation under aerobic conditions at 30°C. ■ isolate 1, □ isolate 2.

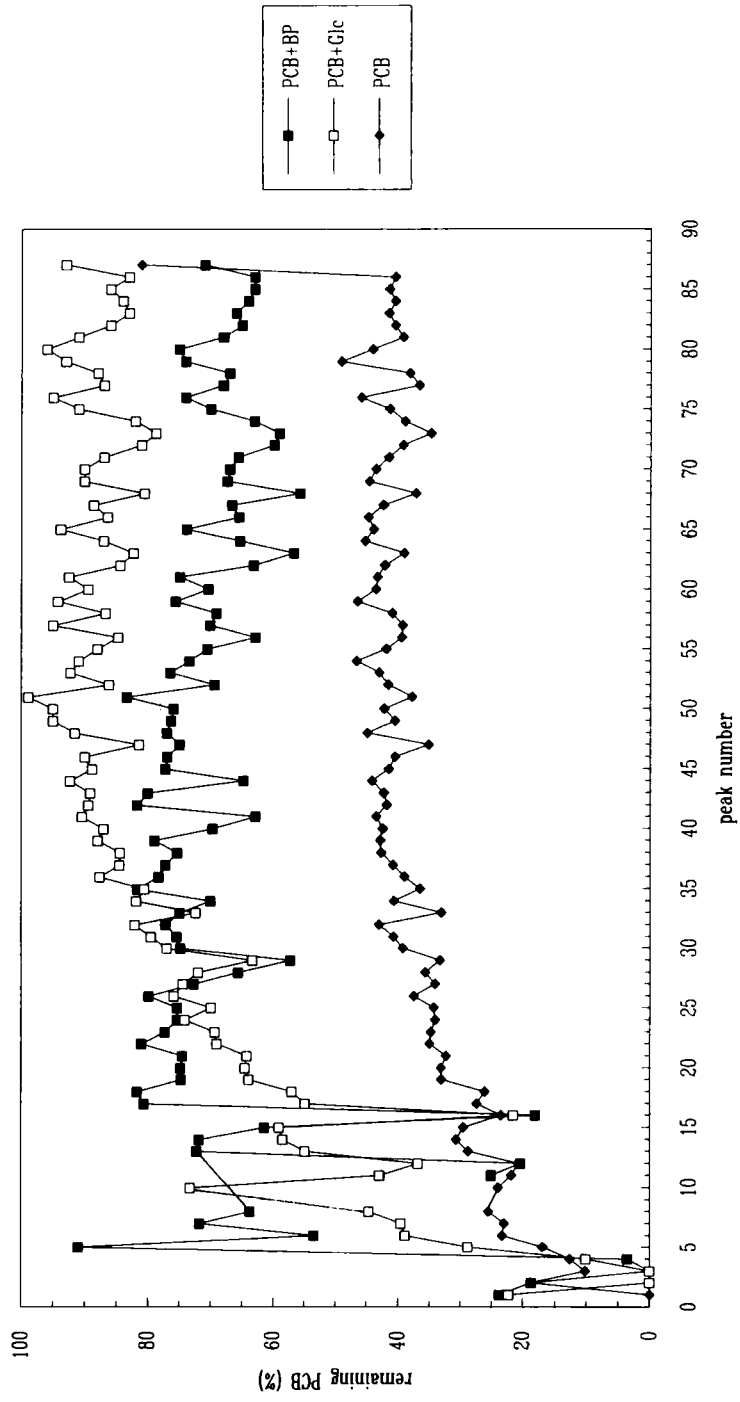

Figure 4 Effect of glucose (3 mg/ml) and biphenyl (3 mg/ml) addition on PCB (9.5 μg/ml) degradation by *Alcaligenes xylosoxidans* in comparison with the degradation of PCB as the sole carbon source. The peak assignment is given in Haluška *et al.*[7]. —■— PCB+BP, —□— PCB+Glc, —◆— PCB.

added to the cultivation medium of *Alcaligenes xylosoxidans* containing PCB with the aim to study the effect of the organic substances on the PCB degradation.

Effect of glucose. The remaining amounts of PCB congeners after 7 day incubation with *Alcaligenes xylosoxidans* with addition of glucose (3 g/l) in comparison with PCB as the sole carbon source are presented in Figure 4. Glucose slows down the PCB degradation significantly. One of the possible reasons for this could be the fact that bacteria preferably take up glucose rather than PCB.

Effect of biphenyl. Figure 4 shows also the spectrum of PCB congeners in the cultivation medium of *Alcaligenes xylosoxidans* as affected by the addition of biphenyl. Biphenyl was added in the concentration (3 g/l) exceeding its aqueous solubility and its undissolved portion served as a depot. The decrease of PCB degradation in the presence of biphenyl may be caused, in principle, by its substrate competition with PCB or by its inhibitory effect. The biphenyl concentration in the medium was not determined, therefore it is not known whether it was utilized or not. Taking into account the published data on inductive ability of biphenyl and its fast degradation[19] it seems plausible that the observed decrease in the PCB degradation in the presence of biphenyl is due to the substrate competition.

Effect of chlorobenzoic acid. Among the six chlorobenzoates (2-CBA, 3-CBA, 4-CBA, 2,4-DBA, 2,5-DBA and 2,6-DBA) previously tested in our experiments (concentration range 1–20 mmol/l) with *Pseudomonas* sp (toxicity decreased in the order 3-CBA, 2-CBA, 2,6-CBA, 4-CBA, 2,5-CBA and 4-CBA), 3-chlorobenzoate was the most effective inhibitor (ID_{50} = 2 mmol/l). It is in accord with the results of Sondossi *et al.*[18] for *Pseudomonas testosteroni* B-356.

The effect of 3-CBA (5 and 100 μg/ml) on degradation of the mixture Delor 103 and Delor 106 (9.5 μg/ml) was studied. Figure 5 shows the remaining amount of PCB congeners after 14 day cultivation with *Alcaligenes xylosoxidans*. The results confirm that 3-CBA is actually an effective inhibitor of PCB degradation already at a concentration of 5 μg/ml.

The studied strains are isolated from a long-time contaminated soil, therefore there is a fair chance that they will degrade PCB effectively also in this milieu. Further experiments with soil samples are needed to confirm this assumption.

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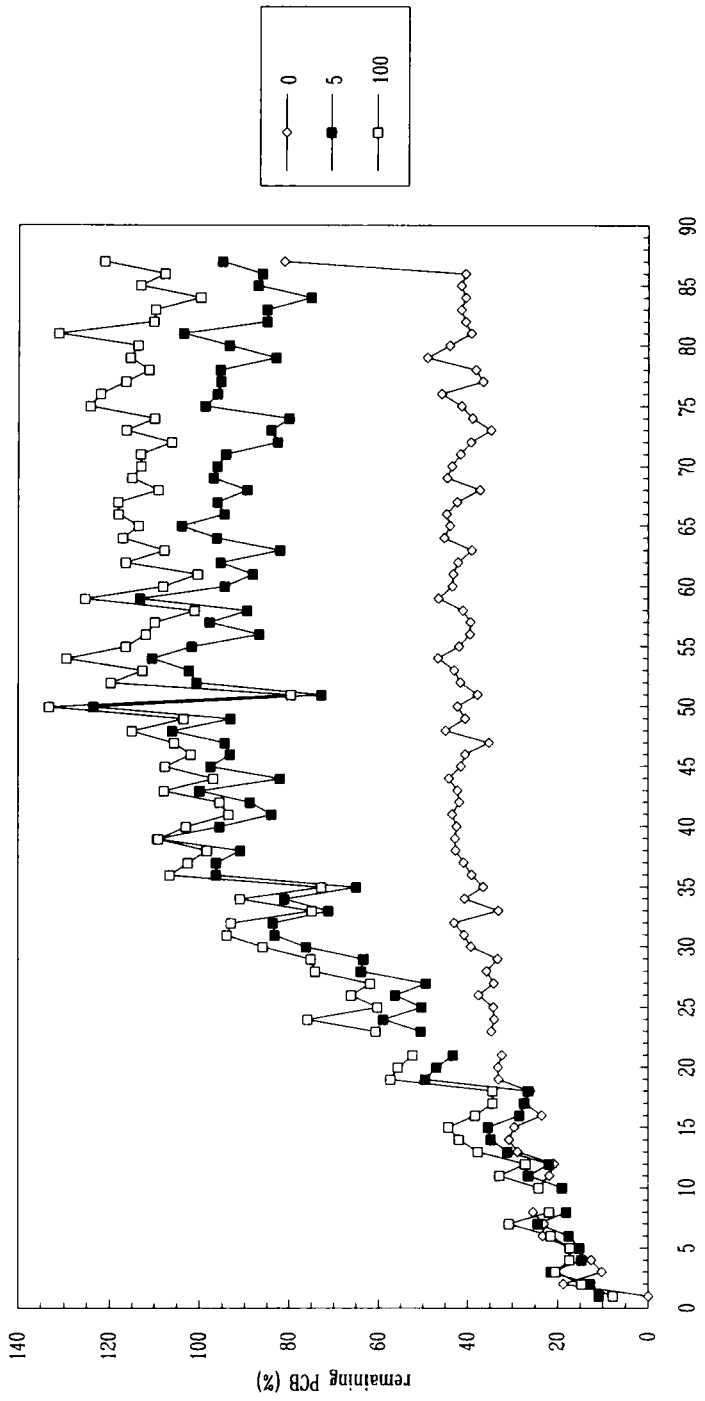

Figure 5 Effect of 3-CBA on PCB degradation by*Alcaligenes xylosoxidans* after 14 day cultivation in the synthetic medium, pH 6.7, at 30°C on a rotary shaker. Initial concentration of PCB 9.5 µg/ml, 3-CBA concentration 5 and 100 µg/ml, respectively. —◇— 0 µg/ml, —■— 5 µg/ml, —□— 100 µg/ml.

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