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Effect of chlorobenzoates on the degradation of polychlorinated biphenyls (PCB) by *Pseudomonas stutzeri*

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Chlorobenzoic acids (CBA) are frequently dead-end products of partial aerobic biodegradation of polychlorinated biphenyls (PCB). When CBA produced from PCB accumulate in the growth medium, they can inhibit the bacterial growth and consequently, slow down PCB biodegradation. In this study, the effects of seven mono- and dichlorinated CBA on growth of *Pseudomonas stutzeri* on different substrates and on the PCB degradation by this strain in a liquid mineral medium were tested. 3-CBA was the strongest growth inhibitor for *P. stutzeri* growing on glucose, benzoate and biphenyl. It was found to inhibit heavily the elimination of some di- and trichlorinated biphenyls. In contrast, its influence on the elimination of more chlorinated congeners was much less significant.

Key words: Chlorobenzoic acids, degradation, polychlorinated biphenyls, *Pseudomonas*.

Polychlorinated biphenyls (PCB) are co-metabolized by biphenyl-degrading bacteria to chlorinated benzoates (CBA) which differ in substitution and configuration from the original congeners (Fava & Marchetti 1991). The soil bacteria that co-metabolize PCB tend to accumulate CBA because they are unable to grow on these substrates (Sondossi *et al.* 1992). Complete mineralisation of PCB requires the presence of two sets of genes, one for the bioconversion of PCB to CBA and the other for the degradation of CBA (Shields *et al.* 1985; Sondossi *et al.* 1992). Sondossi *et al.* (1992) confirmed that CBA inhibit biphenyl and PCB transformation in *Pseudomonas testosteroni* B-356. On the basis of their observations, it appears that when CBA produced from PCB accumulate in the growth medium, they are converted into unproductive metabolites that reduce the flux through the biphenyl and PCB degradation pathway. The presence of CBA degraders in bacterial consortia is, therefore, essential for complete mineralisation of PCB (Sondossi *et al.* 1992).

In this study, the effect of added CBA on the elimination rate of a commercial mixture of PCB by a biphenyl-degrading strain *Pseudomonas stutzeri* and on the growth of the bacterium in a liquid mineral medium was tested.

Materials and Methods

Chemicals

Commercial mixtures of PCB, Delor 103 (equivalent to Aroclor 1242, containing 40–42% (w/v) of bound chlorine) and Delor 106 (equivalent to Aroclor 1260, containing 60% (w/v) of bound chlorine) are ex-products of Chemko Strážske, Slovakia. Biphenyl (Lachema Brno, Czech Republic), 2-chlorobenzoic acid (2-CBA), 3-chlorobenzoic acid (3-CBA), 4-chlorobenzoic acid (4-CBA), 2,4-dichlorobenzoic acid (2,4-dCBA), 2,5-dichlorobenzoic acid (2,5-dCBA), 2,6-dichlorobenzoic acid (2,6-dCBA), 3,5-dichlorobenzoic acid (3,5-dCBA) (all from Merck, Germany), n-hexane UV (Pestiscan, Labscan Ltd, Ireland), acetone p.a. (Mikrochem Bratislava, Slovakia), dimethylsulphoxide (DMSO) (Lachema Brno, Czech Republic) and chemicals for mineral media (Lachema Brno, Czech Republic) were used.

Toxicity of CBA

The growth curves were determined in 20-ml test tubes containing 5 ml of the synthetic DMA medium (Pirt 1967) with different carbon sources: glucose (15 mM), benzoate (10 mM) and biphenyl (5 mM). CBA was added as a solution in DMSO (2M) to the medium. The concentration of DMSO did not exceed 1% (v/v). At this concentration DMSO does not influence bacterial growth. The final concentrations of CBA ranged from 0 to 20 mM. The initial biomass concentration of the inoculum was 0.1 g of dry wt/l

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of medium. Test tubes were incubated 3 days on rotary shaker (180 rev/min) at 28°C without illumination. Optical density readings were taken periodically with a UK-1 colorimeter (Karl Zeiss Jena, Germany) at 650 nm. The growth curve was measured on each substrate for several CBA concentrations. The CBA inhibition of growth on glucose, benzoate and biphenyl respectively has been characterised by ID_{50} value (the concentrations of CBA causing 50% decrease in the specific growth rate) after 24 h of exposure.

Addition of PCB to Liquid Media
The stock solution of PCB was prepared by dissolving Delor 103 and Delor 106 (2:1, w/w) in acetone (4 mg/ml). For contamination studies 50 μl of the stock solution was added to 20 ml DMA medium in Erlenmeyer flasks and the acetone was allowed to evaporate. When biodegradation of PCB in the presence of CBA was tested, the stock solution of CBA in DMSO (2 M) was added to the medium (final concentration of CBA 100 μg/ml, 1% (v/v) DMSO).

Sorbent Column
To determine the loss of PCB by evaporation during incubation, the incubation flasks were fitted with a column filled with the sorbent SILIPOR C18 (0.5 g creating about a 1 mm thick layer) as described by Vrana *et al.* (1995). The top of the glass column was closed with a cotton wool stopper.

Microorganisms
A bacterial isolate obtained by the enrichment method in DMA medium with biphenyl as the sole carbon source from a long-term contaminated soil (Dercová *et al.* 1995), identified as *Pseudomonas stutzeri* in the Czech Collection of Microorganisms, Masaryk University Brno, was used. The bacterium is able to grow on biphenyl and benzoate, but it cannot metabolize CBA as the sole carbon source.

Inoculum
Pseudomonas stutzeri was incubated for 3 days in DMA medium, pH 6.7 with biphenyl (2.5 g/l) at 28°C to 1.5 g dry wt/l. The inoculum was used in both biodegradation experiments and toxicity tests.

Incubation of PCB-Contaminated Samples
Biodegradation of PCB was carried out in Erlenmeyer flasks (100 ml), containing DMA medium (20 ml) without carbon source, closed with the sorbent column. The apparatus for biodegradation experiments was described in detail by Vrana *et al.* (1995). The PCB stock solution (final concentration 10 μg/ml) and bacterial inoculum (final concentration 0.5 g dry wt/l) were added to the incubation flasks. The flasks were incubated for 15 days at 28°C without illumination on a rotary shaker (180 rev/min). The samples (whole flasks) for PCB analysis were taken periodically every 3 days. The amount of PCB in the liquid medium and on the sorbent were analysed.

Extraction of PCB from the Aqueous Phase
After incubation, the flask was filled with 10 ml n-hexane:acetone (9:1 v/v) and put into an ultrasonic bath (15 min). The contents of the vessel were transferred to a separation funnel and intensively shaken (1 min). The hexane layer was collected into a 25 ml volumetric flask. The aqueous layer was returned to the original vessel and the procedure repeated. The hexane layers were combined and the volume increased to 25 ml with n-hexane. The recovery rate of PCB from the aqueous phase was 90 ± 5%.

Desorption of PCB from Sorbent
After incubation the evaporated PCB were eluted from the sorbent directly from the original glass column by n-hexane (10 ml). The volume of the eluent was adjusted to 10 ml with n-hexane and analysed by GC. The recovery rate of PCB from the sorbent was 100%.

PCB Analysis
Samples were analysed by GC (HP 5890) with H_2 as a carrier gas (60 kPa, 1.5 ml/min, split-splitless inlet mode), using an electron capture detector (280°C, make up gas N_2 at 60 ml/min), and a fused-silica capillary column (50 m × 0.32 mm internal diameter) with a non-polar stationary phase HP 1 (thickness 0.17 μm). Temperature conditions: injector 250°C, column 45°C (0.5 min) increased by 20°C/min to 150°C and 2°C/min to 250°C (6 min). Identification of peaks and their calibration was made according to Krupčík et al. (1992). The reproducibility of the quantitative analysis was checked using the standard solution of Delor 103 and Delor 106 (20 μg/ml). Relative deviations for congeners that did not interfere with the background were around 3%. Individual congeners with the corresponding peak number, IUPAC number and the chlorine substitution pattern are given in Haluška *et al.* (1995).

Kinetics of Evaporation and Elimination
During the biodegradation experiments two processes take place simultaneously: elimination and evaporation of PCB. It is assumed that both evaporation and elimination of PCB can be described by the first-order kinetics.

The temporal decrease of the concentration of each PCB congener in the medium (c_l) caused by evaporation and elimination (characterised by the rate constants k with the subscripts *ev* and *el*, respectively) can be described as:

$$c_l = c_0 e^{-(k_{ev} + k_{el})t} \quad (1)$$

where c_0 is the initial concentration of the PCB congener.

The amount of each PCB congener sorbed after evaporation on the sorbent (m_s) can be described as:

$$c_{ev} = \frac{m_s}{V_l} = \frac{k_{ev} c_0}{k_{ev} + k_{el}} (1 - e^{-(k_{ev} + k_{el})t}) \quad (2)$$

where c_{ev} is concentration evaporated from the medium.

Equations (1) and (2) can be fitted by nonlinear regression analysis to the experimentally determined time courses of the concentrations of each PCB congener in the aqueous phase (c_l) and on the sorbent (c_{ev}). Regression parameters were used for calculation of the evaporation and elimination rate constants for individual PCB congeners as described in Vrana *et al.* (1996).

Results and Discussion

Effect of CBA on Bacterial Growth
In order to get information on how CBA and their metabolites affect bacterial growth, the effect on the growth of *Pseudomonas stutzeri* on different growth substrates (glucose, benzoate and biphenyl) was measured. The inhibitory effect of CBA on the growth of *P. stutzeri* was quantified using the ID_{50} value. As shown in Figure 1, among all CBA tested, 3-CBA is the strongest growth inhibitor for *P.*

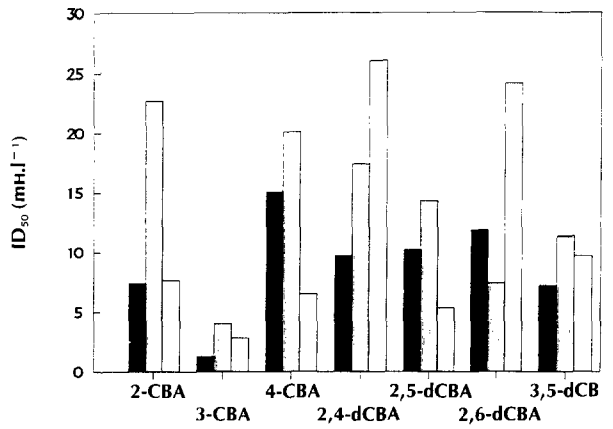

Figure 1. ID_{50} values of CBA for the growth of *Pseudomonas stutzeri* on: ■ —glucose (15 mM); ⁞⁞ —benzoate (10 mM) and □ —biphenyl (5 mM) as the sole carbon source in DMA medium (pH 7) at 28°C without illumination on a rotary shaker (180 rev/min).

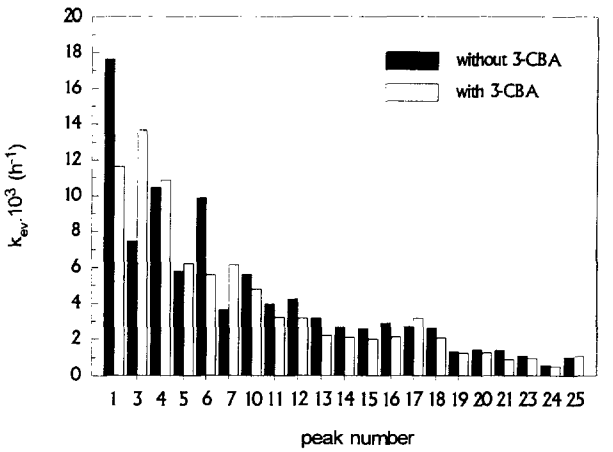

Figure 2. The PCB evaporation rate constants (k_{ev}) in the presence ■ and absence □ of 3-CBA (100 μg/ml) in the biodegradation experiment with *Pseudomonas stutzeri*. The composition of individual peaks, IUPAC numbers and the chlorine substitution patterns are given in Haluška *et al.* (1995).

stutzeri growing on glucose, benzoate and biphenyl. When benzoate is used as the growth substrate, monochlorobenzoates are less toxic than when using biphenyl. This fact is in agreement with the proposal of Sondossi *et al.* (1992), that monochlorobenzoates inhibit biphenyl-induced oxygenases that are not present in the benzoate-grown cells. Toxicity of dichlorobenzoates varies depending on both the substitution pattern and the growth substrate.

When *P. stutzeri* is growing on biphenyl in the presence of 2-CBA (up to 10 mM) or 3-CBA (up to 5 mM), black metabolites are excreted into the medium; 2-CBA and 3-CBA are transformed in biphenyl-grown cells. Transformation of 2,4-dCBA, 2,5-dCBA and 2,6-dCBA by the biphenyl-grown cells, produced a yellow product (visually detected).

Effect of 3-CBA on the PCB Elimination Rate

The influence of CBA on PCB elimination was evaluated by comparison of the elimination rate constants of PCB congeners with and without addition of CBA. In these experiments only the influence of 3-CBA was tested. This acid was reported to have the strongest inhibitory effect on the PCB degradation (Sondossi *et al.* 1992) and had also the lowest ID_{50} value for the growth inhibition of *P. stutzeri* among the CBA tested. The concentration of CBA added to the medium was chosen as half of the ID_{50} value when growing on glucose (1 mM). The concentration of CBA possibly liberated to the medium by the partial degradation of some low chlorinated PCB congeners is negligible in comparison with the added CBA concentration.

The values of the evaporation (k_{ev}) and elimination (k_{el}) rate constants for individual PCB congeners with and without addition of 3-CBA, are given in Figure 2 and Figure 3, respectively. The values of k_{ev} decrease approximately with the increasing number of chlorines except the peaks 1, 3, 6, 13 and 21. CBA seems to have no significant effect on the PCB evaporation. When comparing the elimination rate constants of PCB (Figure 3) in the presence and in the absence of 3-CBA it can be seen that the influence of this acid is much more pronounced in the case of low chlorinated congeners (two and three chlorines, peaks 1 to 17). It comprises inhibition (most affected are 2,3'-dichlorobiphenyl [peak 3], 2,3-dichlorobiphenyl and 2,4'-dichlorobiphenyl [peak 4], 2,2',6-trichlorobiphenyl [peak 5], 4,4'-dichlorobiphenyl and 2,2',4-trichlorobiphenyl [peak 7]), but also stimulation: 2,2',5-trichlorobiphenyl [peak 6], 2,3',5-trichlorobiphenyl [peak 11], 2,3',4-trichlorobiphenyl [peak 12] and 2,4',5-trichlorobiphenyl [peak 13]. CBA has no significant effect on the elimination of tetrachlorinated and more chlorinated congeners (peaks 18 to 25, data not shown). However, these compounds are eliminated more slowly than di- or trichlorobiphenyls in the absence of CBA. If the inhibitory mechanism of CBA results from the inhibition of some PCB degradation pathway enzymes, the elimination of tetra- and more chlorinated biphenyls seems to be catalysed by enzymes unaffected by CBA or alternatively the elimination of these compounds is a nonenzymatic process. This assumption is supported by the fact that the k_{el} values do not depend on the substitution pattern of chlorines on both aromatic rings of biphenyl.

Acknowledgements

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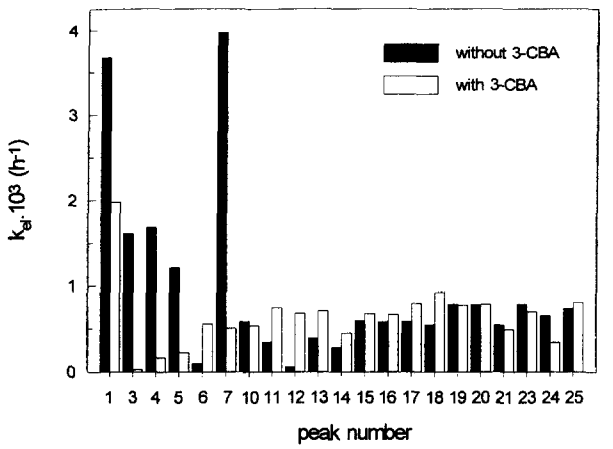

Figure 3. The PCB elimination rate constants (k_{el}) in the presence ■ and absence □ of 3-CBA (100 μg/ml) in the biodegradation experiment with *Pseudomonas stutzeri*. Other details as in Figure 2.

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