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Dechlorination of chlorobenzoates by an isolated bacterial culture

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A range of chlorobenzoates is produced as dead-end metabolites during the microbial degradation of chlorinated aromatic compounds, including polychlorinated biphenyls (PCBs) and some herbicides. Hence studies on the microbial degradation of composites of chlorobenzoates are important for developing an effective bioremediation technology. An isolated bacterial culture had the ability to tolerate and grow in the presence of 2-chlorobenzoate (2-cba), 3-chlorobenzoate (3-cba), 4-chlorobenzoate (4-cba), a mixture of these chlorobenzoate isomers, 4-chloro,2-nitrobenzoate (4cnb) and chlorobenzene (cbz) using benzoate as the growth substrate. The microbial culture was able to dechlorinate all the above-mentioned chloroaromatics as well as their mixture to the extent of 50–75% under aerobic conditions. The rate of dechlorination followed the

order mixture >3-cba >4-cba >2-cba, with maximal release of 60, 75, 60 and 50 ppm of free chloride respectively, in the presence of 0.6% benzoate. Dechlorination appeared to begin at the end of the exponential growth phase and followed a linear course until the end of the lytic phase. The dechlorinating property of the isolated bacterial culture enabling it to act on mixtures of chlorinated benzoates in the presence of growth substrates such as benzoate may be useful for remediation of sites contaminated with PCB. To our knowledge the isolated bacterial culture is one of the few versatile strains to biotransform a variety of chlorinated aromatic compounds and their mixture through initial dechlorination.

Keywords: Bacterial culture, biodegradation, chlorobenzoates, dechlorination.

CHLOROBENZOATES are produced as metabolites during the microbial degradation of chlorinated herbicides and polychlorinated biphenyls (PCBs), and persist in contaminated areas as dead end-products¹. Reports have shown that the efficiency of biodegradation of monochlorinated biphenyls can be increased by the presence of bacteria that can degrade chlorobenzoates directly or by co-metabolism^{2,3}. Thus enhancing the efficiency of chlorobenzoate degraders and enrichment of their population is the key to successful bioremediation of PCB under *in situ* or *ex situ* conditions. Biodegradation of some individual mono- and dichlorobenzoates has been studied using pure or mixed microbial cultures. For example, *Pseudomonas aeruginosa* JB2 and *Pseudomonas putida* p111 have been shown to degrade 2-chloro, 3-chloro, 2,3-dichloro, 2,5-dichloro and 2,3,5-trichlorobenzoates via chlorinated catechols as central intermediates^{4,5}. However, few reports are available on the degradation of mixtures containing isomers/classes of chlorobenzoates. Commercial PCBs are available only as mixtures of several congeners thereby leading to the formation of range of a chlorobenzoates in PCB-contaminated zones through biotransformation activities. Thus the study of factors influencing the rates and extent of biodegradation of a mixture of chlorobenzoates is relevant.

In the present investigation we have evaluated the capability of an isolated aerobic bacterial culture to dechlorinate 2-, 3- and 4-chlorobenzoates (2-cba, 3-cba and 4-cba respectively), 4-chloro,2-nitrobenzoate (4cnb) and chlorobenzene (cbz) either singly or as mixtures, since these are the simplest model compounds for investigating the degradation of chloroaromatics in soil or water. The rate and extent of dechlorination of individual monochlorobenzoates or their mixtures were monitored in the presence and absence of growth-supportive substrate, benzoate. Also, the study involved relating the position of chlorine atom on the ring and effects of other substituents on the rate and extent of release of halogen from mono-chloroaromatic compounds.

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Experiments related to enrichment of microbial culture, growth and dechlorination were conducted in batch mode using Erlenmeyer flasks (250 ml capacity) containing target chlorinated compounds dissolved in 100 ml of growth medium.

A bacterial culture was isolated from garden soil by enrichment technique using mixture of 2-, 3- and 4-chlorobenzoates (130 ppm of each isomer) and 0.5% of benzoate. Individual monochlorobenzoates were added into the cooled autoclaved medium from their respective sterile stock solutions. The mineral salts (MS) medium comprised of 2.5 g KH_2PO_4 , 1.0 g $(\text{NH}_4)_2\text{SO}_4$, 0.55 g NaOH, 0.5 g nitrilotriacetic acid, 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g sodium benzoate and 1 ml of Bauchop and Elsdon⁶ trace metal solution added to 1l of deionized water. Following inoculation, cultures were maintained on a rotary platform shaker at 200 rpm and 30°C.

Efficiencies of three growth substrates, namely vanillate (0.5%), benzoate (0.5%) and glucose (1%) for mediating dechlorination of individual monochlorobenzoates (390 ppm) and their mixture (130 ppm of each isomer) by isolated bacterium were compared using the growth conditions described above.

Let us now consider the growth and dechlorination at various benzoate concentrations. The bacterial culture was grown in MS growth medium containing 0.2%, 0.4% or 0.6% benzoate, and 390 ppm of 2-cba, 3-cba or 4-cba or a mixture containing 130 ppm of each of the isomers. Abiotic control experiments were set up using killed inoculum. Aliquots of the growth medium were withdrawn at suitable time intervals and analysed for cell density by recording optical density (OD) at 600 nm using a Spectronic 20 colorimeter. The cell suspension was suitably diluted to obtain an OD of less than 0.5. An OD of 0.1 corresponded to $\sim 10^8$ cells per ml. Aliquots of cell-free supernatant were analysed for free chloride concentration using an ion selective combination chloride electrode (model 96/17, Orion Research Inc., Cambridge, Massachusetts, USA).

Now let us compare the growth and dechlorination profiles using monochloroaromatics with various functional groups. The microbial culture was grown on MS medium containing 0.6% benzoate as the growth-supportive substrate and 260 ppm of either 4-cba, 4-cnb, cbz or their mixture. The target compounds were added in three installments, the first at time zero and subsequent ones when constant rates of dechlorination were achieved. The cumulative concentration of compounds after final dosing was 780 ppm (5 mM). Samples were withdrawn at various intervals of time to measure growth and dechlorination. Abiotic control experiments were set up using killed inoculum.

The isolated bacterial cells were seen growing on agar plates as light yellowish colonies. Examination of the culture under light microscope revealed single, double or multiple coccoid forms. The culture was unable to utilize

2-cba, 3-cba or 4-cba or a mixture of monochlorobenzoates as the sole source of carbon and energy. However, the culture was able to dechlorinate individual monochlorobenzoates or their mixture in the presence of growth-supportive substrates such as benzoate, vanillate or glucose (Tables 1 and 2). Benzoate was the most effective growth substrate, inducing $\sim 68\%$ dechlorination after 192 h of incubation. The extent of chloride released increased with increasing concentration of benzoate, as seen in Table 2. The results presented in Tables 1 and 2 imply that: (a) transformation (dechlorination) of chlorobenzoates by the microbial culture had an obligate requirement for the presence of an easily degradable growth-supportive substrate such as benzoate and (b) the extent of dechlorination seems to be related to the overall (net) growth yield of bacterial isolate, which in turn is dependent upon the total concentration of the primary substrate (benzoate) provided in the MS medium. Stimulation of microbial cultures for faster metabolism of monochlorobenzoates through addition of growth-supportive substrates such as acetate has been reported⁷. As an example, *Rhodococcus* species co-oxidized monochlorinated isomers of anilines and phenols in the presence of growth substrates such as glucose and acetate⁸. Stimulation of dehalogenation of chlorinated benzoates by organic and inorganic growth supplements has also been reported by Fava *et al.*⁹.

The rates of dechlorination followed the trend: mixture > 3-cba > 4-cba > 2-cba, being 1.17, 1.07, 0.94 and 0.76 ppm per h respectively (Figure 1). The maximum

Table 1. Dechlorination of mixture containing 2- 3- and 4-chlorobenzoates (130 ppm of each) using different growth substrates

Growth substrate (concentration, %)	Per cent dechlorination after 192 h
Benzoate (0.5)	68 ± 5
Vanillate (0.5)	51 ± 5
Glucose (1.0)	39 ± 5

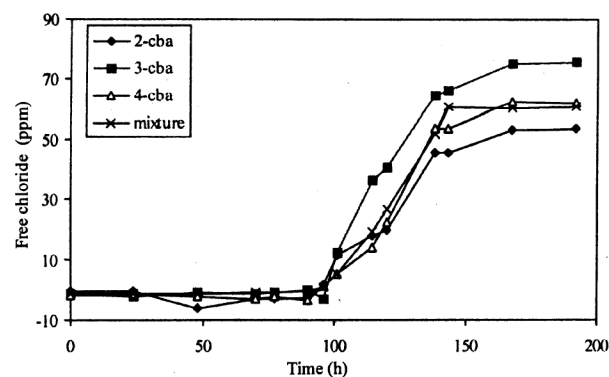


Figure 1. Profiles of bacterial dechlorination of 2-, 3- and 4-chlorobenzoates and their mixture in medium supplemented with 0.6% benzoate as the primary substrate.

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Table 2. Total free chloride release* in growth medium containing 2-, 3- or 4- chlorobenzoates (390 ppm each) or a mixture (130 ppm of each isomer) after 192 h using various initial concentrations of benzoate

Benzoate % (w/v)	Chloride (ppm) from 2-cba	Chloride (ppm) from 3-cba	Chloride (ppm) from 4-cba	Chloride (ppm) from mixture of cbas	Total yield of biomass (dry wt, g/l)
0.6	53	76	62	60	2.0
0.4	52	36	60	47	1.2
0.2	33	23	45	33	0.7
None	BDL	BDL	BDL	BDL	Growth not detected

*Standard error of deviation from the values reported here was $\pm 5\%$; cba, Monochlorobenzoate; BDL, Below detectable limit.

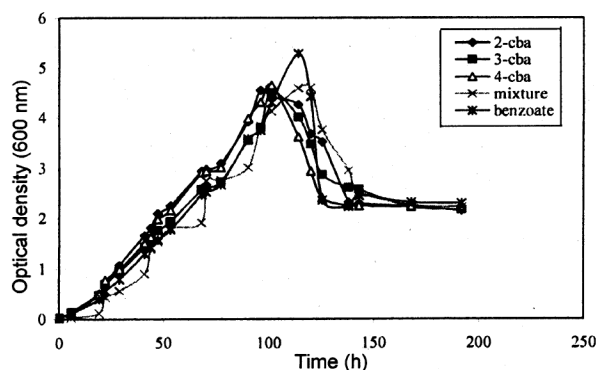


Figure 2. Growth profiles of bacterial culture in 0.6% benzoate in the presence of individual monochlorobenzoates or their mixture.

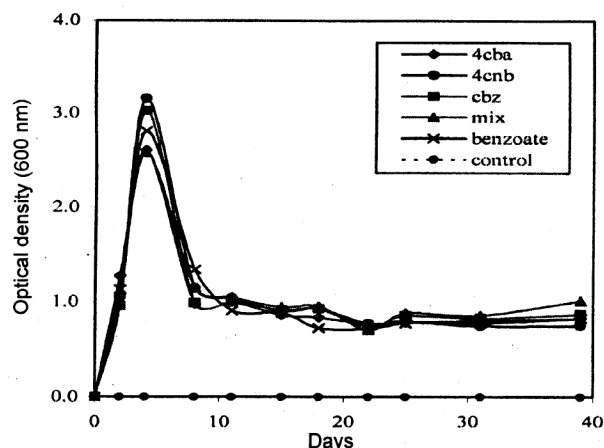


Figure 3. Growth profile of bacterial culture in the presence of various monochloroaromatic compounds using 0.6% benzoate as the primary substrate.

chloride release, as seen in Figure 1 and Table 2, was 50 ppm with 2-cba, 60 ppm with 4-cba and the mixture to as much as 75 ppm with 3-cba in the presence of 0.6% benzoate as the carbon source. Differences in the rate and extent of dechlorination of the three isomers may stem from differences in toxicity or the position of the chlorine

atom in relation to the carboxyl group of the aromatic ring. Alternatively, accumulation of specific metabolites formed from a chlorinated isomer may inhibit further dehalogenation and metabolism of the parent compound.

The microbial culture was able to efficiently dehalogenate the mixture containing all the three monochlorobenzoate isomers, as seen in Figure 1. For example, nearly 60% of the organically bound chlorine was released as free chloride in the medium containing a mixture of monochlorobenzoates with 0.6% benzoate for supporting growth. Brunsbach and Reineke⁷ reported the dechlorination of mono- and dichlorobenzoates as single compounds or as a mixture in soil and soil slurry through addition of specialized bacteria. However, dechlorination seemed to be facilitated through a consortium of bacteria (including soil bacterial population), rather than individual bacterial strains. The present investigation reports the combined dehalogenation capacity in a mixture containing all the three monochlorobenzoates by an isolated bacterial strain. Inhibition and co-metabolic effects using various combinations of mono- and dichlorobenzoates have been studied by Stratford *et al.*¹⁰

Growth of the isolated bacterial culture in the medium containing 0.6% benzoate and individual chlorobenzoates or their mixture followed an exponential curve until 100 h, reaching a maximum OD of 4.5, following which it declined (lytic phase) to 2.5 at 120 h and remained constant thereafter (Figure 2). Distinct transitions in the colour of the spent medium were noticed. The culture medium turned blue at the start of the exponential phase, possibly due to the formation of catechols/chlorocatechols. It subsequently changed to greenish-yellow towards the end of the exponential phase, probably due to the accumulation of ring cleavage products^{11,12}.

Comparison of chloride release and growth profiles (Figures 1 and 2) indicated that dechlorination was initiated towards the end of the exponential growth phase, following a linear course until the end of the lytic phase. However, during the lytic stage, depletion of internal energy and electron supply of the cell and/or product toxicity may limit the efficacy of cells to degrade chlorobenzoates. Thus dechlorination is gradually halted. Dehalogenation of 4-cba by *Arthrobacter* sp. strain SB8 and

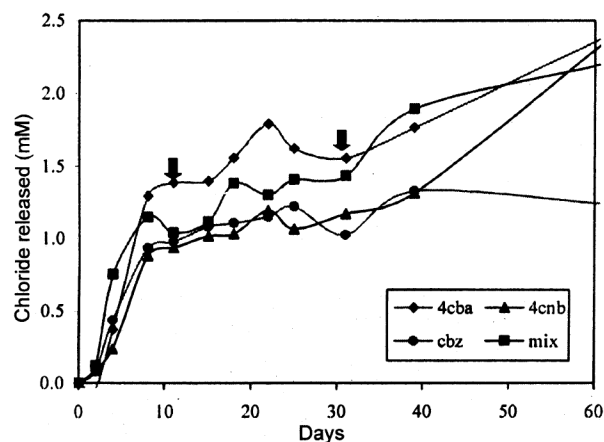


Figure 4. Dechlorination of monochlorinated aromatic compounds by bacterial culture grown in 0.6% benzoate. Arrows indicate time points at which further additions of target compounds were made.

4-chloro, 4-bromo and 4-iodobenzoates by *Alcaligenes denitrificans* NTB-1 in stoichiometric amounts during the exponential growth phase, have been reported by Shimao *et al.*¹³ and van den Tweel *et al.*¹⁴ respectively.

The isolated bacterial culture was also able to dechlorinate 4-cnb, cbz, and a mixture containing 4-cnb, cbz and 4-cba during growth on 0.6% benzoate. Exponential growth was observed until the fourth day followed by lytic phase up to the tenth day (Figure 3). The trend observed with respect to initial rates (based on data up to 11 days) of dechlorination was: mixture and 4-cba (0.17 mM/day) > cbz (0.13 mM/day) > 4-cnb (0.12 mM/day; Figure 4). Progressive dechlorination was noticed until the eighth day when a constant concentration (1.0–1.25 mM) of free chloride ion was attained (Figure 4). Further release of chloride was noticed following two additional dosings (260 ppm each) of all target chloroaromatic compound(s), except chlorobenzene. This result suggests that the culture was capable of dechlorinating higher concentrations of the target chlorinated compounds, if they are supplied in two or three installments.

The isolated bacterial culture had the potential to dechlorinate 2-cba, 3-cba and 4-cba and their mixture in the presence of benzoate. Also, the culture was able to act upon substituted chloro-aromatic compounds, namely 4-cnb and cbz. To our knowledge the isolated bacterial culture is one of the few versatile strains to biotransform a variety of chlorinated aromatic compounds and their mixture through initial dechlorination. However, further investigations focused on evaluating the capabilities of this culture for transforming dichlorinated and trichlorinated benzoates and PCB congeners are required for field-scale application.

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