Dehalogenation of 4 – Chlorobenzoic Acid by *Pseudomonas* isolates

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Abstract Twenty three bacterial isolates either pure or consortium were initially screened on the basis of their ability to degrade as well as dechlorinate 4 – chlorobenzoic acid (4–CBA). Based on comparative growth response, three pure isolates *Pseudomonas putida* GVS-4, *Pseudomonas aeruginosa* GVS-18 and *Pseudomonas aeruginosa* GWS-19 and a consortium SW-2 was finally selected for further studies. The enzyme studies performed with cell free extracts revealed that dehalogenase activity was substrate specific with maximum activity at 300 µgml⁻¹ substrate concentration. Catechol 1,2 dioxygenase activity was found to be present in cell free extracts suggesting that 4 – chlorobenzoic acid (4–CBA) is catabolized by ortho-ring cleavage pathway. The dehalogenase enzyme profile showed single enzyme band in case of GVS-4 (Rm 0.76), GVS-18 (Rm 0.84), GWS –19 (Rm 0.85) and two bands in SW-2 (Rm 0.71 & 0.10).

Keywords 4-chlorobenzoic acid · Dehalogenase · Degradation · *Pseudomonas putida* · *Pseudomonas aeruginosa*

Introduction

Extensive use of chemical insecticides triggered discoveries of compounds effective in the control of plant diseases, weeds, rodents etc. With increase in their use, large quantities of these chemicals have entered the environment and other food commodities and are subject to bioaccumulation and biomagnification^{1–4}. In addition, the industrial development has been accompanied by a new quality of environmental pollution, namely the production and dispersal of chemicals never or rarely seen before in the biosphere xenobiotics^{5–6}. Xenobiotics frequently include unusual chemical bonds or substitution with halogens or other functional groups that can render a molecule highly resistant to degradation⁷.

Luckily, by means of exploiting the metabolic versatility and catabolic abilities of microorganism, it has been possible to transform the contaminants into less harmful end products, which are then integrated into natural biogeochemical cycles^{8–9}. Bioremediation offers a potentially more effective and economical cleanup technique than conventional physicochemical methods. Among the various biodegrading bacteria investigated, *Pseudomonas* sp. has been found to be the most versatile because of the vast array of their plasmid and chromosomal encoded degradative genes. Halogenated aromatic compounds exhibits steric hindrance, plus the effect of halogen/ chlorine atoms on benzene ring interferes with their enzymatic breakdown⁷.

Chlorobenzoates originate as intermediates of microbial degradation of Polychlorobenzoates (PCBs) and are the major sources of environmental pollution. Resistance of these compounds to biodegradation is due to their xenobiotic nature and substitution by halogen atom. The degree of recalcitrance depends on number and location of halogen atom. Thus, bacterial strains isolated from nature are able

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to degrade chlorinated benzoates¹⁰. Majority of microorganisms capable of degradation proceed *via* the corresponding halocatechol and the dehalogenation occurs after the ring cleavage¹¹. So, it is assumed that the microorganisms having catabolic function can produce appropriate enzymes by adaptation in a closed system with substrate specificity to degrade structural analogs of halogenated benzoates.

Dechlorinase/dehalogenase is the key enzyme in degradation of chlorinated compounds. The carbon-halogen bond is cleaved either by enzymatic dehalogenation, where the biocatalysis of the carbon – halogen bond is achieved by specific enzymes – the dehalogenases, or by spontaneous chemical dehalogenation of unstable intermediates. In addition, there are enzymes which, because of their relaxed substrate specificity, catalyze the conversion of halogenated analogs of the corresponding unsubstituted substrates or of related compounds, which might lead to ‘fortuitous’ dehalogenation of the substrate analog^{10,12}. Dehalogenases, normally inducible enzymes enable the microorganisms to grow on halogenated aliphatic and aromatic compounds, as their sole carbon and energy source^{13–14}. The bacterial degradation of Chlorobenzoates (CBAs) involve most of the time a rate limiting step of inorganic chloride removal which is species specific and is carried out by a group of enzymes referred to as dehalogenase.

Material and Methods

Growth Kinetics

The buffered salt solution medium¹⁵ supplemented with 500 µg/ml of 4-chlorobenzoic acid (4-CBA), as a sole source of carbon and energy, was used for studying the growth kinetics. Periodic increase in optical density (540 nm) and chloride ion accumulation were measured as function of growth of the organism. The degradation of 4-CBA in the culture medium was monitored spectrophotometrically by measuring chloride ion release in the culture filtrate at 460 nm by mercuric thiocyanate method described by Bergmann and Sainik (1957)¹⁶ and decrease in 4-CBA concentration at 260 nm. Based on comparative growth response, three pure isolates GVS-4, GVS-18 and GWS-19 and three consortia SW-1, SW-2 and SW-3 were finally selected for further studies. GVS-4, GVS-18 and GWS-19 were identified as *Pseudomonas putida*, *P. aeruginosa* and *P. aeruginosa* respectively at MTCC, IMTECH, Chandigarh. The dechlorinase/ dehalogenase activity of all the cultures was estimated in cell free crude extracts by reacting with the different concentration (100 µg/ml – 500 µg/ml) of 4-CBA in the reaction mixture. Enzyme kinetics of dehalogenase

enzyme of *Pseudomonas* pure strains and consortium were studied.

Dehalogenase Activity

The activities of pyrocatechase (catechol 1, 2 dioxygenase), metapyrocatechases (catechol 2, 3 dioxygenase) and dehalogenase were measured according to methods of Dorn and Knackmuss¹⁷ and Nozaki¹⁸.

Preparation of Crude Extract: The cells were grown in 1.5 litres of synthetic Dorn’s medium for 24 hrs under shake conditions and harvested by cold centrifugation at 10,000 rpm for 15 min at 5°C. The cells were washed twice with 10 mM TrisSO₄ (pH 7.6) buffer containing 1 mM β-mercaptoethanol and suspended in the same buffer at 4°C. The cell mass obtained was disrupted by sonication at 0°C using ice bath for eight to ten minutes in 30 sec bursts. The sonicated sample was again centrifuged at 10,000 rpm for 10 min at 5°C and the supernatant obtained was used for enzyme assay and total protein estimation. The proteins in the crude extract were measured by the method described by Lowry *et al.*¹⁹. Bovine serum albumin was used as standard.

Pyrocatechase Assay (EC 1.13.11.1): For pyrocatechase (catechol 1,2 dioxygenase) assay, the reaction mixture of 2 ml in the cuvette had following composition: Catechol, 100 µg; TrisSO₄ buffer (pH 7.6), 100 mM; EDTA, 2 mM; 2 mercaptoethanol, 100 µM; Crude extract, 100 µl. The formation of cis-cis muconate during the reaction was measured by the change in absorbance at 260 nm for 6 min. One unit of enzyme activity was defined as the amount of enzyme that caused an absorbance change of 0.1 per minute.

Metapyrocatechase Assay (EC 1.13.11.2): Metapyrocatechase (catechol 2,3 dioxygenase) assay reaction mixture in the final volume of 2 ml in the cuvette had the following composition : Catechol, 100 µg; TrisSO₄ buffer (pH 7.6), 100 mM; Crude extract, 100 µl. The enzyme activity was assayed spectrophotometrically by measuring the rate of 2-hydroxy semialdehyde formation at 375 nm during 6 min of reaction period. One unit of enzyme activity was defined as the amount of enzyme that caused an absorbance change of 0.1 per minute.

Dahalogenase Assay: The dehalogenase activities were assayed in 50 mM TrisSO₄ buffer (pH 7.6) containing 100–500 µg/ml substrate and 100 µl of crude extract in the final volume of 3 ml with incubation time of 1 hr using Mercuric thiocyanate method¹⁶. Specific activity was defined as mg/l of chloride/mg protein.

Electrophoresis: Polyacrylamide disc-gel electrophoresis was used for separation and detection of dehalogenase enzyme according to method by Davis²⁰.

Fig. 1 Catechol 1,2 dioxygenase (C 1,2 O) & Catechol 2,3 dioxygenase (C 2,3 O) activities of *Pseudomonas* isolates.

Results and Discussion

Dehalogenase Assay: Dechlorinase/dehalogenase is the key enzyme in degradation of chlorinated compounds. The activities of dechlorinase enzyme in cell free extracts of *Pseudomonas* isolates were compared by growing cells in 4-CBA as sole source of carbon and energy, with view to assess their relative potentialities. Maximum specific activity of 3.0 was observed in case of SW-2 and 2.4 in case of isolates GVS-4 and GWS-19 while it is 2.2 in case of GVS-18 (Table1).

Enzyme Substrate Kinetics Studies: The dechlorinase/dehalogenase activity of all the cultures was estimated in cell free crude extracts by reacting with the different concentration (100 µg/ml – 500µg/ml) of 4-CBA in the reaction mixture. Enzyme kinetics of dehalogenase enzyme of *Pseudomonas* pure strains GVS-4, GVS-18, GWS-19 and consortium SW-2 were studied. A comparatively lower de-

halogenase activity at 100µg/ml of 4-CBA concentration was observed and the activity was found to increase with increase in concentration upto 300µg/ml while constancy was observed on further increase in concentration. Earlier studies also suggested that high substrate concentration in enzyme systems results in low affinities for the substrate as the presence of residual substrate, after blockage of avail-

Table 1 Specific dehalogenase activity of *Pseudomonas* isolates in cell free extracts.

Culture	O. D at 520 nm	Conc. of Proteins (mg/ml)	Enzyme activity µg/ml/min	Specific activity
GVS-4	0.862	8.5	32.7	2.4
GVS-18	0.622	6.4	23.6	2.2
GWS-19	0.914	9.0	34.6	2.4
SW-2	1.211	11.9	41.7	3.0

able enzyme active sites did not influence the enzyme activity^{21, 22}.

Characterization of Dehalogenase Enzyme: Cell free extracts were subjected to Polyacrylamide Disc Gel Electrophoresis (PAGE). Dechlorinase enzyme proteins were identified by reacting with specific substrates. The gels were removed from the tubes and incubated in 0.02 M TrisSO₄ buffer (pH 7.6) containing the specific substrate at a concentration of 200µgml⁻¹ for 30 min at 28 ± 2°C followed by reaction with silver nitrate which resulted in silver chloride precipitation followed by the formation of white coloured bands at the site of reaction on the gels²³.

A single enzyme band was observed in all cases while in consortium SW-2, two enzyme bands appeared. The bacterial strain GVS-4 showed single band with Rm value 0.76, GVS-18 with Rm value 0.84, GWS-19 again with a single band Rm 0.85 and two bands in case of SW-2 with Rm values 0.71 and 0.10 respectively, with 4-CBA as substrate. The presence of enzyme bands with Rm value 0.81 with different substrates has also been observed by Hardman and Slater²⁴. Sandhu²² indicated the presence of more than one dehalogenase in *Pseudomonas putida* PP3 acting against different substrates. Koch²⁵ postulated gene duplication resulting in strains with two or more enzymes with similar activities towards different substrates. Dehalogenase enzyme bands with similar Rm values with 4-CBA were observed in all isolates indicating that these different isolates may carry identical dechlorinases. Hardman and Slater²⁴ reported similar results and suggested that a single gene is distributed among different organisms.

Dioxygenase Activities of Cell Free Extracts: Activities of pyrocatechase (catechol 1,2 dioxygenase) and metapyrocatechases (catechol 2,3 dioxygenase) were compared in cell free extracts of *Pseudomonas* strain GVS-4, GVS-18, GWS-19 and SW-2. The results presented in Figure 1 show that the cells grown on 4-CBA indicate a low catechol 1,2 dioxygenase activity initially with an increase between three to six minutes and then again decreased with increase in time. However, no catechol 2,3 dioxygenase activity was observed with any of the strain. Catechol 1,2 dioxygenase activity, in case of GVS-4, Fig. 1a shows an increase in optical density from 1.87(0min), 2.36(3min) to 2.72(5min) and decreased to 2.00 at 10min of incubation whereas there is a constant catechol 2,3 dioxygenase activity i.e. 2.12, 2.38, 2.44 at 0, 5 and 10 min respectively. Similar is the case in GVS-18 (Fig. 1b) and GWS-19 (Fig. 1c). Catechol 1,2 dioxygenase activity in case of SW-2 consortium (Fig. 1d), was found to be 1.94, 2.41, 2.50, 1.69 at 0, 3, 5 and 10 min, respectively, with no/constant catechol 2,3 dioxygenase activity from 0 to 10 min of incubation. When the

activities of these strains were compared, it was found that GVS-19 among pure isolates and SW-2 consortium show significantly high catechol 1,2 dioxygenase activity followed by GVS-18 and GVS-4.

So, the dioxygenase assay of the *Pseudomonas* isolates revealed the presence of catechol 1,2 dioxygenase but no catechol 2, 3 dioxygenase activity suggesting that 4-CBA is degraded by ortho ring fission. In fact, a large number of workers have reported degradation of 4-CBA by the formation of catechols as intermediates that are formed by spontaneous removal of chlorine from chlorocatechols as a result of 1,2 dioxygenase attack as also confirmed^{26–29}.

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