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Purification and characterization of catechol 1,2-dioxygenase of *Pseudomonas fluorescens* for degradation of 4-chlorobenzoic acid

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The degradation of 4-chlorobenzoic acid (4-CBA) by *Pseudomonas fluorescens* IST8 was determined. The degradation of 4-CBA proceeded through an oxidative route to yield ortho ring cleavage enzyme, catechol 1, 2-dioxygenase. The cell free extract fractionated by DEAE-cellulose ion-exchange and gel filtration chromatography showed two different fractions of catechol 1,2-dioxygenase with an expected molecular weight of 62 and 48 kDa, respectively. The catechol 1, 2-dioxygenase in the fractions I and II was purified to about 22.3 and 36.5 fold by using purification steps. The *p*H and temperature optima for enzyme activity were 6.5 and 25°C, respectively. The purified protein on SDS-polyacrylamide gel electrophoresis showed molecular weights of 28 and 24 kDa, indicating dimeric nature of the enzyme.

Keywords: catechol-1,2-dioxygenase, 4-chlorobenzoic acid, chlorocatechol, DEAE-cellulose, gel filtration, SDS-polyacrylamide gel electrophoresis

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Introduction

Chlorobenzoic acids are widely used as herbicides and originated from the metabolism of poly-chlorinated biphenyls[1,2]. Although some representatives of the chlorobenzoates are degradable by bacterial strains, but 4-CBA is markedly more refractile to microbial attack[1,3] . The microbial degradation of chlorobenzoate has been reported to occur through chlorocatechol, which is further degraded by the ring cleavage. Chlorocatechol 1, 2-dioxygenase, chloromuconate cycloisomerase, dienelactone hydrolase and maleylacetate reductase are the major enzymes of ortho ring cleavage pathways of chlorobenzoates[1]. The degradation of chlorinated benzoic acid has been a matter of great concern because of lack of genetically potential micro-organisms, which can degrade chlorinated benzoates in the contaminated soil. In view of this problem, the evolution of bacterial strains with complete catabolic enzyme has received special attention for the total degradation of 4-CBA. Hartmann *et al*[4] isolated pseudomonads by continuous enrichment of a mixed population from soil samples and subsequently inoculated them with the toluate degrading *Pseudomonas putida* mt2 and the 3-chlorobenzoate degrading *Pseudomonas* sp. WR912 as carbon source. But bacterial strains developed by these procedures proved unsuccessful for the total degradation of recalcitrant haloaromatics present in heavily contaminated areas or in industrial sewage plants[5-7]. A strategy to develop strains with novel degradation properties has been carried out recently, in which 4-chlorosalicylate degrading bacterial consortium was obtained, comprising *Pseudomonas fluorescens, P. mendocina, P. cichhori* and *Klebsiella pneumonia*, that acquired the ability to utilize chlorinated benzoates[8]. In this paper, the authors report further enrichment and characterization of ring cleavage enzyme responsible for the degradation of 4-CBA by *P. fluorescens*.

Materials and Methods

A 500 ml (effective size) chemostat was set up in a cylindrical glass vessel (total size of the vessel, 22×12

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cm dimension) in continuous mode of cultivation of microorganism, with flow rate of 10 ml/hr, temperature at 28°C, stirred at 150 rev/min, *p*H at 7.2-7.4 and sterile air was introduced. The mineral salt medium containing (per litre): $Na_2HPO_4.2H_2O$, 7.8 g; KH_2PO_4, 6.8 g; $MgSO_4$ .02 g; Fe $(CH_3COO)_3$. NH_4 0.01 g.; $Ca(NO_3)_2.4H_2O$, 0.05 g; $NaNO_3$, 0.085g; trace element solution, 1 ml and 4-CBA (5 m*M*) as carbon and energy source was used for enrichment of bacteria as described by Thakur[8]. The culture medium was inoculated with *P. fluorescens* bacterial cells precultured in nutrient broth. The size of the inoculum was 10%. Sample was removed after the stabilization of growth. The bacterium was served as inoculum for the growth of bacteria in shake flask batch culture in the mineral salt medium with 4-CBA (5 m*M*) for further enrichment of the strain as described above. The flasks were incubated at 29°C on a rotary shaker at 150 rpm and the growth of the bacterial strain was initially measured at 540 nm. The utilization of 4-CBA was monitored at 254 nm by HPLC[9,10] . In this process, the metabolites were extracted with HCl (6 *M*, 1.5 ml) from culture fluid and then extracted twice with equal volumes of diethyl ether. The ether phase was evaporated to dryness in a stream of air, and the extract was dissolved in methanol. For analysis, samples were separated by reverse-phase HPLC with a Bondapack C_{18} column (300×3.0 mm, Water Associates). The mobile phase was methanol-water-acetic acid (ratio 60:40:1 v/v); flow rate, 1.5 ml/min, and detected by UV absorbance at 254 nm[9]. The free chloride ions released in culture broth were determined by the method of Bergmann & Sanik[11]. The culture broth was subjected to ring cleavage test for meta and ortho by Rothera reaction as described by Holding and Collee[12].

The bacterial cells were harvested during exponential growth phase by centrifugation (7650 g, 20 min at 40°C), suspended in phosphate buffer (50 m*M*, *p*H 7.0). The cell suspension was sonicated at 4°C at 2 sec burst in an ultrasonic disintegration unit fitted with a titanium probe of a terminal diam 1 cm operating at 20,000 Hz. The crude extract was centrifuged at 9,180 g for 30 min at 4°C. The catechol 1, 2-dioxygenase activity was assayed as described by Nakazawa and Nakazawa[13] and modified according to Dorn & Knackmuss[14] by the formation of cis,cis-muconic acid and increase in absorbance at 260 nm. The catechol 2, 3-dioxygenase was assayed by measuring the increase in optical density at 375 nm due to conversion of catechol to ∝-hydroxymuconic semialdehyde as described by Nozaki[15]. Protein was estimated by Lowry *et al* (1951) using BSA as the standard[16]. The specific activity of enzyme was expressed as micromoles of substrate utilized per minute per mg of protein at 25°C.

The cell extract was precipitated with ammonium sulphate (80% saturation) by continuous stirring at 10°C. Precipitate was recovered by centrifugation at 10,000 rpm for 20 min, and dissolved in phosphate buffer. The precipitate was dialysed, and preparation was concentrated in dialysis bag against polyethylene glycol molecular weight 6,000. DEAE-cellulose chromatography was used for separation of protein with a solvent pressure generating flow rates of about 20 ml/hr at 4°C. DEAE-cellulose column (size 1.5×20 cm) was equilibrated with Tris-hydrochloride buffer (50 m*M*, *p*H 8.0). The protein was eluted with increasing gradient of NaCl (0 to 0.5 *M*). All the fractions were checked for catechol 1, 2-dioxygenase and catechol 2, 3-dioxygenase. Molecular weight of the enzyme was determined by gel filtration chromatography on Sephadex G-100 column (1.5×50 cm) using known molecular weight standards as described earlier[17]. SDS-PAGE was performed according to the procedure of Laemmli by using 10% acrylamide gels[18]. The enzyme activity was determined at different *p*H (3 to 10) and temperatures (10 to 80°C).

Results and Discussion

Degradation of chlorinated aromatic compounds by bacteria has been reported earlier but application of the strains in bioremediation of contaminated soil has not been studied[1,19]. The microbial community was developed by continuous enrichment of sediment core in a mineral salt medium supplemented with 4-chlorosalicylic acid as the sole source of carbon and energy[8]. Rubio *et al* obtained *Pseudomonas* sp. by continuous enrichment of bacterial strains in the presence of specific carbon source for degradation of chlorosalicylates and chlorobenzoates[7]. This indicated that the substrate can induce synthesis of catabolic enzymes required for the degradation of 4-CBA. In the present study, *P. fluorescens* was isolated by 4-chlorosalicylic acid enrichment in the chemostat.

4-Chlorobenzoic acid is released during degradation of recalcitrant polychlorinated biphenyl and also it is widely used for structural formulation and synthesis of herbicides. Therefore, in the present study bacterial strain was enriched in presence of

4-CBA as sole carbon source in the chemostat to obtain recalcitrant compound degrading bacteria. The growth of the bacterial cells was determined by utilization of 4-CBA, release of chloride and increase in optical density. During initial enrichment in the chemostat the growth of the bacterial cells was monitored up to day 120. The growth of the cells was increased on day 3 and decreased after day 10 and became constant for 45 days. The flow rate of the medium was decreased from 10 to 5 ml/hr. It was observed that growth of the cells was again increased and became constant after 90 days. The culture medium was removed and growth and utilization of 4-CBA was determined by HPLC. It was observed that initially bacteria could not utilize more than 50% of the 4-CBA, but once the bacterial population was stabilized, almost 90% of the 4-CBA was utilized by the organisms.

The bacterial cells of culture medium removed during stable period, on day 120, was further inoculated in shake flask for evaluation of degradation by the strain. Organism grew exponentially with 4-CBA as sole source of carbon and energy in shake flask-batch culture, exhibited doubling time 2-3 hrs. As can be seen in Fig. 1, utilization of 4-CBA determined by HPLC was concomitant to the growth of bacterial cells. In 4-CBA degradation, chloride ions were released in culture medium (Fig. 1). There was increase in chloride release at 6 hrs, which reached to maximum at 18 hrs and decreased after 21 hrs.

Dehalogenation and ring cleavage are important steps in the degradation of 4-chlorobenzoic acid[20]. Mode of ring cleavage (ortho and meta ring fission) was determined. After mixing catechol in cell suspension, no colour was exhibited, indicating the absence of meta ring cleavage. However, a positive Rothera test for ortho ring fission was detected as red-brownish colouration after 8 hrs. Results of this study exhibited the release of chloride followed by the ortho ring fission of 4-CBA. Table 1 indicated that degradation of 4-CBA proceeded through the formation of chlorocatechol, which is similar to earlier reports[4,14]. The data of this study differ to the earlier indicated removal of halogen substituent in early step of degradation by *Acinetobacter* sp and *Arthrobacter* sp.[9,19].

Our results indicated release of significant amount of catechol 1, 2-dioxygenase at 8 hrs in culture filtrate, which reached to maximum at 12 hrs. Release of meta ring cleavage enzyme, catechol 2, 3-dioxygenase, also determined in the culture filtrate of different time periods was not significant (Table 1). The protein content was extracted from the culture filtrate and fractionated by ion exchange chromatography. The crude cell extract showed two different fractions of catechol 1, 2-dioxygenase by DEAE-cellulose ion exchange chromatography. It was observed that both the fractions eluted at a sodium chloride concentration of 0.25 *M* (Fraction I) and 0.5 *M* (Fraction II). Both the fractions were further purified by gel filtration chromatography, and molecular weight was determined. The molecular weights of this protein were found to be 62 and 48 kDa determined by the elution volume of gel filtration chromatography on Sephadex G-100 against the known molecular weight compounds (β-amylase,

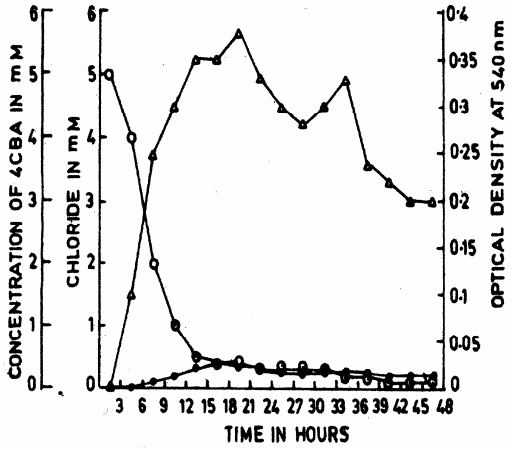

Fig. 1—Growth of *P. fluorescens* IST8 in mineral salt medium supplemented with 4-chlorobenzoic acid. Δ-Δ, indicates as absorbance at 540 nm; ●-●, as chloride release; and ○-○, as utilization of 4-CBA determined by HPLC.

Table 1—Specific activity of catachol 1, 2 dioxygenase in cell free extract of 4-chlorobenzoic acid by *P. fluorescens*

Enzyme	Assay substrate	Specific activity (U/mg protein), time in hrs				
		4	8	12	24	48
Catechol 1, 2 dioxygenase	4-chlorocatechol	0.00	0.12	0.68	0.23	0.14
Catechol 1, 2 dioxygenase	4-chlorobenzoic acid	0.02	0.1	0.61	0.21	0.21
Catechol 2, 3 dioxygenase	4-chlorocatechol	0.00	0.02	0.03	0.01	0.01
Catechol 2, 3 dioxygenase	4-chlorobenzoic acid	0.00	0.01	0.05	0.02	0.03

2,00,000; Bovine Serum Albumin, 66,000; Qvalbumin, 45,000; Carbonic Anhydrase, 29,000; Lysozyme, 14,300 and Cytochrome C, 12,5000) as described by Thakur[17]. Both the fractions of gel filtration were mixed with sample buffer and SDS-PAGE was performed. Single band of molecular weight of 28 and 24 kDa, respectively were indicated on gel surface after staining (Fig. 2). Dorn and Knackmuss[14] have reported two different molecular weight pyrocatechase in 3-chlorobenzoate-degrading *Pseudomonas* sp. B13. The purified enzyme of catechol 1, 2-dioxygenase was tested for its activity on 4-CBA at *p*H varying from 3 to 10. The optimum

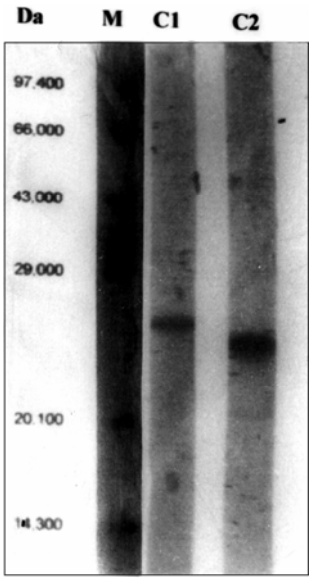

Fig. 2—SDS-polyacrylamide gel electrophoresis of catechol 1, 2-dioxygenase enzyme, fractions I and II indicated as C1 and C2. Molecular weight of the enzymes was determined by log molecular weight of known protein (Banglore Genei, India), containing Phosphorylase b, 97,400; Bovin Serum Albumin, 66,000; Ovalbumin, 43,000; Carbonic Anhydrase, 29,000; Soyabeen Trypsin Inhibitor, 20,100; and Lysozyme, 14,300 and Rf values.

*p*H for enzyme activity was 6.2 (Fig. 3a). The function of both the enzymes was also tested for its activity on various ranges of temperature. It was observed that enzyme was active at optimum temperature of 25°C (Fig. 3b). The rate of enzyme activity was influenced by the substrate concentration of 4-CBA (data not shown). Results of the study indicated similarity with earlier reports, but both the enzymes differ in molecular weight[3].

The purification step adopted in the study is indicated in Table 2. Results of the study indicated

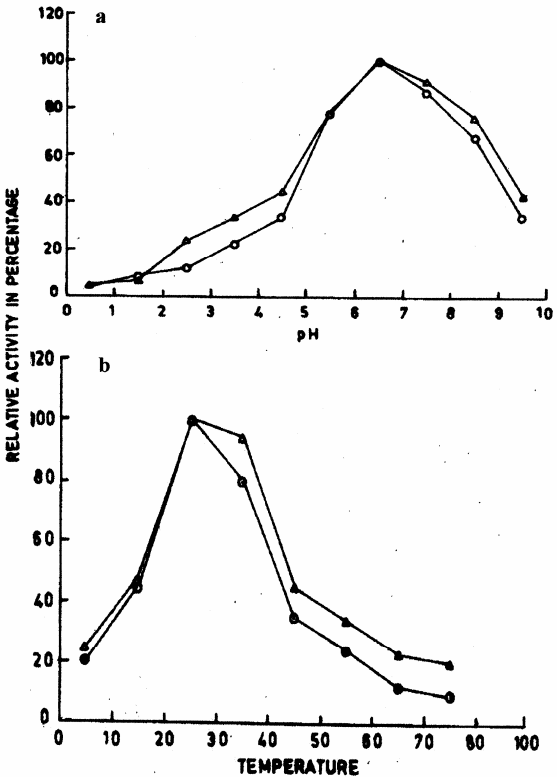

Fig. 3—Percentage changes in enzyme activity of catechol 1,2-dioxygenase, fractions I (Δ--Δ) and II (○--○), (a) in presence of *p*H (3-10) and (b) in presence of temperature (10-80°C).

Table 2— Purification of enzyme, catechol 1, 2-dioxygenase

	Stages of purification	Volume (ml)	Protein (mg)	Activity (U)	Specific activity Umg^{-1}	Recovery	Fold
1	Bacterial cell extract	15	94.5	44.8	0.47	100	1.0
2	Precipitation with ammonium sulphate	10	20.3	18.0	0.88	40.1	1.87
3	DEAE Cellulose-						
	Fraction I	20	2.4	6.0	2.5	13.4	5.3
	Fraction II	20	1.6	8.0	5.0	17.8	11.1
4	Gel filtration						
	Fraction I	5	0.9	4.5	5.0	10.4	10.6
	Fraction II	5	0.7	6.0	8.5	13.4	18.1

significant increase in fold purification reached to 22.3 for fraction I and 36.46 for fraction II of the catechol 1, 2-dioxygenase. By various purification steps it has been possible to increase the specific activity of the purified enzyme with an overall recovery. The possible involvement of gene has been studied in our laboratory, and it has been observed that degradation of 4-CBA is mediated by plasmid[10]. But the authors do not have proper explanation of gene involved in complete degradation of 4-CBA at this stage.

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