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The Degradation Pathway of 4-Chlorobenzoic Acid by Genetically Modified Strain of *Pseudomonas aeruginosa*

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Abstract: ***Pseudomonas aeruginosa a genetically modified strain degrading 4-chlorobenzoic acid was isolated from soil. This bacterium could degrade 15 mM of 4-chlorobenzoic acid rapidly with stoichiometric formation of chloride ion. The metabolites such as catechol and cis, cis-muconic acid was detected in the grown medium. Further, ring cleavage enzyme catechol 1, 2-dioxygenase was detected and isolated in crude cell extract. Thus, the biodegradation pathway of 4-chlorobenzoic acid by this bacterium was proposed involve the catechol as intermediate compound and further this converted into cis, cis-muconic acid by the process ortho ring cleavage pathway.***

Keywords: 4-chlorobenzoic, *Pseudomonas aeruginosa*, 4-chlorocatechol, catechol, *cis*, *cis*-muconic acid

1. Introduction

The chlorobenzoates constitute one of the important classes of recalcitrant compound polluting this biosphere. These are introduced into the environment by used as herbicides and as metabolic products of polychlorinated biphenyl. The biodegradation of polychlorinated biphenyl by soil microorganisms was found as given rise to the accumulation of 4-chlorobenzoic acid in the environment (Arensdorf and Focht 1995, Kim and Picard mm, 2000). There has been report that 4-chlorobenzoic acid could be utilized as a carbon source by microorganisms such as *Alcaligenes* sp. (Dorn *et al.1*985) and *Arthrobacter* sp. (Schmitz *et al.*1992). The degradation of 4-chlorobenzoic acid in aerobic bacteria commonly proceeds via 4-chlorocatechol, which is a common intermediate in the biodegradation of various halogenated aromatics compounds (Chatterjee & Chakrabarty 1982). The ring cleavage through a mechanism known as *ortho* cleavage pathway proceeds the spontaneous removal of chlorine ion. There are several workers reported that the removal of chlorine occurs after the ring cleavage by the process hydrolytic dehalogenation reactions (Chae *et al.*1999). The *Pseudomonasaeruginosa* dehalogenation occur before the ring cleavage by the process reductive dehalogenation. Although reductive dehalogenation normally seen in anaerobic condition. The dehalogenation before ring cleavage is important for the enhancement of the efficiency of benzene ring cleavage enzyme. This report describes the degradation pathway of 4-chlorobenzoic acid and characterization of metabolites.

2. Materials and Methods

2.1 Microorganism

The strain of *Pseudomonas aeruginosa* was isolated from soil in aerobic condition and genetically modified in artificial habitat. It was selected for its capacity to grow in a medium containing 4-chlorobenzoic acid as a sole source of carbon and energy.

2.2 Culture Condition

The minimal salt medium used in this study was containing gl^{-1}, $Na_2HPO4.2H_20$, 7.8; KH_2PO4, 6.8; MgSO4, 0.2; $NaNO_3$, 0.085; Ca $(N0_3)$ $2.4H_20$, 0.05; ferrous ammonium citrate, 0.01 and 1 ml of trace elements (Pfennig & Lippert 1986). The 15 mM of 4-chlorobenzoic acid was added in the medium (pH 7.0) and sterilized by autoclaving at 15 lb for 20 min. The cultivation of culture in 250 ml Erlenmeyer flask was carried out at 30 ^{0}C with shaking at 150 revolution min^{-1}. The cell growth was determined by measuring optical density at 600 nm using Spectrophotometer.

2.3 Estimation of Chloride

The culture medium (2 ml) was mixed with 0.2 ml ferric ammonium sulphate (0.025 M) dissolved in HNO_3 (3 M). The mixture was again mixed with 0.2 ml of saturated solution of mercuric thiocyanate dissolve in ethanol. The optical density of orange color was determined at 460 nm against blank (Bergman & Sanik 1957). The different concentrations of NaCl were used to plot the standard curve for the determination chloride released.

2.4 Preparation of Cells Extract

The cells of *Pseudomonas aeruginosa* was harvested by removing liquid culture after 24 h of incubation and centrifuged at 7000 revolution min^{-1} for 30 min at 4 ^{0}C. The pellets ware washed in a solution of chilled phosphate buffer (0.05 M, pH 7.0). The suspension was subjected to ultrasonic irradiation for 4 min at 4 ^{0}C and recentrifuged at 12000 revolution min^{-1} for 20 min at 4 ^{0}C. The pellets ware discarded and supernatants used for protein and enzymatic estimation.

2.5 Estimation of Enzyme

The assay of catechol 1, 2-dioxgygenase is based a measurement of the rate of formation of *cis, cis*-muconic acid at 260 nm (Nakazawa *et al.1*988). The assay mixture

consisted 4 µm EDTA, 100 µl catechol (10 mM), 2.7 ml phosphate buffer (0.05 M, pH 7.0) and the reaction was initiated by adding 100 µl of enzyme extract. One unit of enzyme activity is defined as that amount which catalyze the formation of 1 µm of *cis, cis*-muconic acid per min at 24 ^{0}C. The conversion of 1 µm catechol in to *cis, cis*-muconic acid causes an increase in absorbance of 5.66 units at 260 nm. The protein content was estimated according to the method of Lowery *et al*. (1951), using bovine serum albumin as standard.

2.6 Detection of Metabolites

The 10 ml culture fluid was taken from 6, 9, 12, 15 and 18 hours of incubation period and centrifuged at 1500 rpm for 30 min. The pellets were discarded and supernatants which contain metabolites separated and an equal volume of diethyl ether was added in to it. The ether phase was evaporated by a stream of air and extract was redissolved in methanol.

2.7 Thin Layer Chromatography

TLC was performed using pre-coated TLC plates with silica gel 60 F-254. Thefive µl of metabolites and standards were placed on the silica plate. The solvent system containing toluene: ethyl acetate: acetic acid: water by volume in the ratio of 60: 30: 5: 5 was used for separation of metabolites. The plate was dried at room temperature for two hours and then sprayed with fluorescein dye followed by Folinciocateu's reagent, ammonia solution and ferrous sulphate solution (10%) serially. The gray spot of metabolites appeared on the plate was detected by UV-absorbance at 270 nm. The metabolites traveled on chromatogram were determined by Rf value for each compound.

2.8 High Pressure Liquid Chromatography

HPLC was performed with 4.0 x 250 mm ODS C-18 column. The column temperature was 30 ^{0}C and the peaks were detected by using UV-Vis-detector. The acid (10 mM) containing 50% acetonitrile was used as the mobile phase with a flow rate of 1 mlmin^{-1} and the peak was detected at 278 nm. The catechol and *cis, cis*-muconic acid determined from prepared cell extract with a mobile phase of 50% methanol containing 40 mM acetic acid and detection wave length at 284 nm and 265 nm respectively. The retention times of metabolites and standard peaks were compared in different mobile phase by both separate and coinject methods.

3. Results

3.1 Identification of Strain

The 10 g of each sample was resuspended in 20 ml of distilled water and filtered through Whatman filter paper no.1. The 4 ml aliquots were then inoculated in 125 ml Erlenmeyer flasks containing 50 ml minimal salt medium supplemented with 15 mM of 4-chlorobenzoic acid and shaking at 30 ^{0}C. All turbid cultures were subcultures 5 to 10 times by transferring 50 ml of turbid culture broth to 50 ml of fresh medium. Finally, broth from each turbid culture was streaked on minimal salt agar medium supplemented with 4-chlorobenzoic acid to obtain pure cultures. The isolate which showed higher growth yield in presence of 4-chlorobenzoic acid was selected further for their ability to degrade 4-chlorobenzoic acid. This isolate was identified by morphological, physiological and biochemical tests. This was confirmed as *Pseudomonas aeruginosa* on the basis of identified scheme in Berger's Manual of Determinative Bacteriology (Holt *et al*.1994).

3.2 Utilization of 4-chlorobenzoic Acid

Figure 1 show that the initially 15 mM of 4-chlorobenzoic acid is completely utilizes by *Pseudomonas aeruginosa*up to 18 hours of incubation period. This shows the degradation of 4-chlorobenzoic acid for the utilization of carbon and energy source. The rate of 4-chlorobenzoic acid utilization was approximately proportional to the cell mass of *Pseudomonasaeruginosa*. This growth is represented the sigmoid curve and the exponential phase of growth occur at 15 hours and after this growth become at stationary phase. The chloride concentration release in the culture medium is increase as the incubation period increase and become maximum 15 mM at 18 hours.

3.3 Characterization of Metabolites

The metabolites were characterized by thin layer chromatography. The TLC plat indicates that after 12 hours and15 hours of incubation period the *Rf* value of metabolites were 0.69 and 0.70 respectively. This *Rf* value is identical with the authentic *Rf* value of catechol and *cis, cis*-muconic acid. The characterization of *cis, cis*-muconic acidin the culture medium is the identification of *ortho* ringcleavage pathway. The metabolites were further characterized by HPLC for this we observed the peaks with retention time equivalent with authentic catechol and *cis, cis*-muconic acid as shown in figure 2 and 3 respectively. The result was confirmed by observation of higher peak area when samples were co-injected with authentic compound.

4. Discussion

The *Pseudomonas aeruginosa* was able to utilize 4-chlorobenzoic acid as sole source of carbon and energy. The degradation is associated with microbial growth and metabolism and therefore any of the factors affecting microbial growth will influence degradation. The aerobic degradation is considerably faster than the anaerobic process (Hollinger& Zehnder 1996). To maintain the aerobic condition supply of oxygen will be needed for rapid degradation. Temperature effects the microbial growth and pH of the medium affected both growth and solubility of the compound. The temperature and pH of the medium was standardized. This strain has capability to degrade 4-chlorobenzoic acid via formation of 4-chlorocatechol as in intermediate compound. After this the dehalogenation occurs and this is converted into catechol which is further metabolizing into *cis, cis*-muconic acid as shown in figure 4. The formation of *cis, cis*-muconic acid is the clear evidence for the degradation of 4-chlorobenzoic acid by *ortho* ring cleavage pathway. The microorganisms which degrade 4-

chlorobenzoic acid via catechol so far have not been isolated by classical enrichment techniques, probably because the benzoate 1, 2-dioxygenase of ordinary benzoate degrader not active on 4-chlorobenzoic acid. Hartman *et al*. (1979) isolated *a Pseudomonas* sp. by continuous enrichment which degraded 4-chlorobenzoic acid via the corresponding chlorocatechol. Reinecke and Knack muss (1980) constructing in *vitro* a 4-chlorobenzoic acid degrader by combining the genes of 3-chlorobenzoic acid pathway of *Pseudomonas* sp. strain B-13 and some genes of the TOL plasmid of *Pseudomonas* sp. strain mt-2. Further, Hempel *et al. (*1998) investigated *a Pseudomonas* sp. B13 FR1pFRC20 P by continuous culture technique which is mineralizing the 4-chlorobenzoic acid but the plasmid stability of this strain decreases with increasing dilution rate.

The majority of haloaromatic compounds are degraded aerobically by hydrolytic dehalogenation and under an aerobic condition these are reductive dehalogenation, (Cho *et al*.2001). The formation of catechol in the reaction containing 4-chlorocatechol and crude extract was shown to require NADPH. This supports the evidence of a reductive dichlorination of 4-chlorobenzoic acid by *Pseudomonas aeruginosa* which is aerobic bacterium. To our knowledge this is the first report for aerobic metabolism of halogenated compounds involving reductive dehalogenation. The reductive dichlorination should be important for the biodegradation of many chlorinated aromatic compounds. This solved the problem for inefficiency of ring cleavage enzymes in halo catechol transformation. Chae *et al*. (1999) reported that the efficiency of ring cleavage enzyme maximum for catechol in comparison to the halo catechol.

The metabolites were analyzed by HPLC. It was found that the metabolite peak of the HPLC corresponded to authentic catechol. The result indicated that catechol in the culture broth could have resulted from the activity 4-chlorobenzoic acid degrading enzyme. To further characterized pathway, the supernatant was analyzed by HPLC and result indicated that the metabolite peak was identical to the authentic *cis, cis*-muconic acid. This is clear evidence for the *ortho* ring cleavage pathway. Arensdorf & Foch (1995) reported that the key metabolites of *meta* and *ortho* ring cleavage product is 5-chloro-2 hydroxy muconic semialdehyde and *cis, cis*-muconic acid respectively. The presence of *cis, cis*-muconic acid could have resulted from the activity of enzyme catechol 1, 2-dioxygenase. This indicated that the 4-chlorobenzoic acid was degraded by newly isolated *Pseudomonas aeruginosa* through *ortho* ring cleavage pathway. These are evidence that this strain has much more potential for the degradation of xenobiotic compounds.

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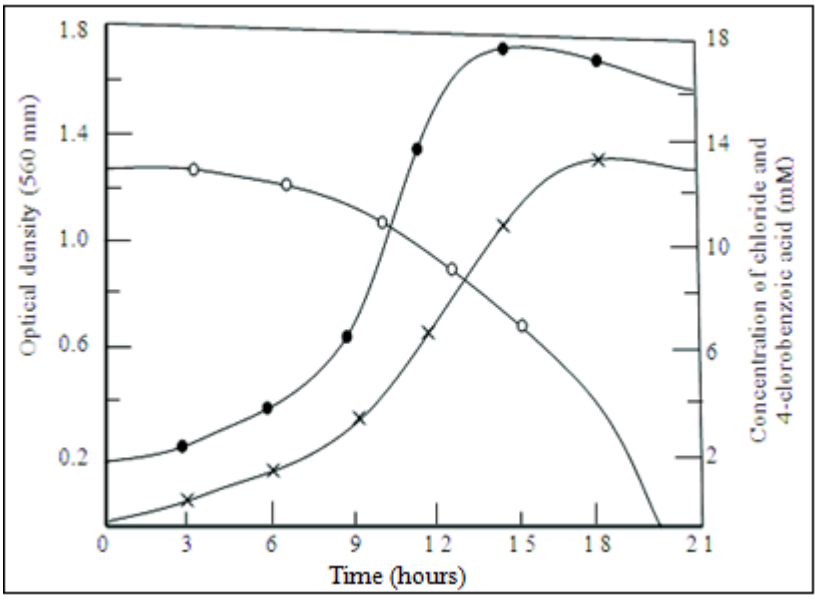

Figure 1: Growth curve of *Pseudomonas aeruginosa* in minimal salt medium supplemented with 4-chlorobenzoic acid as carbon source (●); utilization of 4-chlorobenzoic acid by the above strain (○); concentration of chloride released in the culture medium (.).

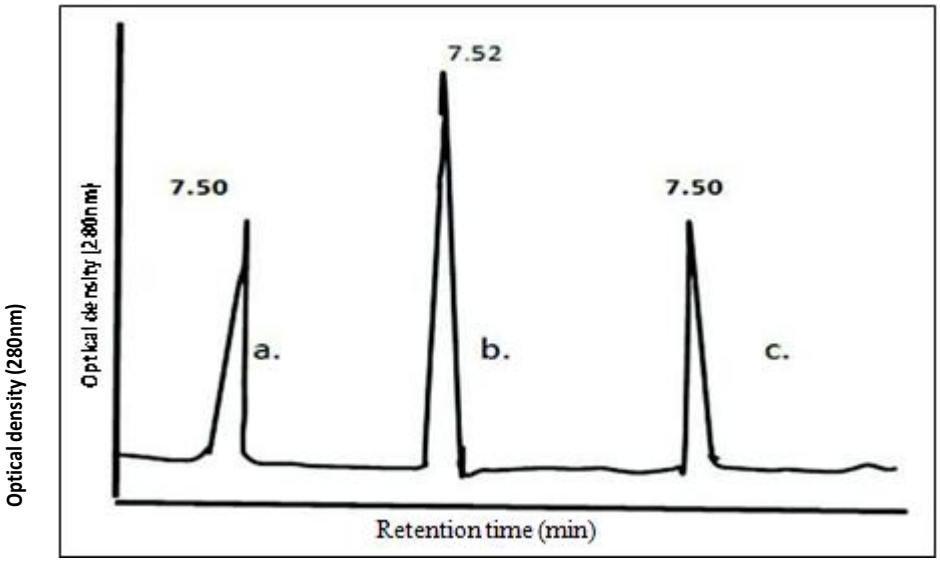

Figure 2: HPLC chromatogram and retention times showing the presence of catechol in the culture filtrate of *Pseudomonas aeruginosa* grown in 4-chlorobenzoic acid containing medium. (a) authentic 4-catechol, (b) co-injection of extract and authentic catechol, (c) extract only.

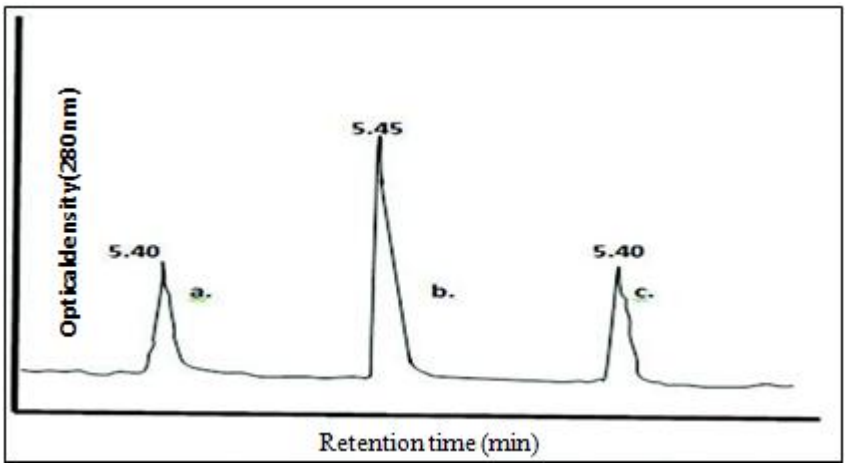

Figure 3: HPLC chromatograms and retention times showing the presence of *cis, cis*-muconic acid in the culture filtrates of *Pseudomonas aeruginosa* grown in 4-chlorobenzoic acid containing medium. (a) authentic *cis, cis* – muconic acid, (b) co-injection of extract and authentic *cis, cis*-muconic acid, (c) extract only.

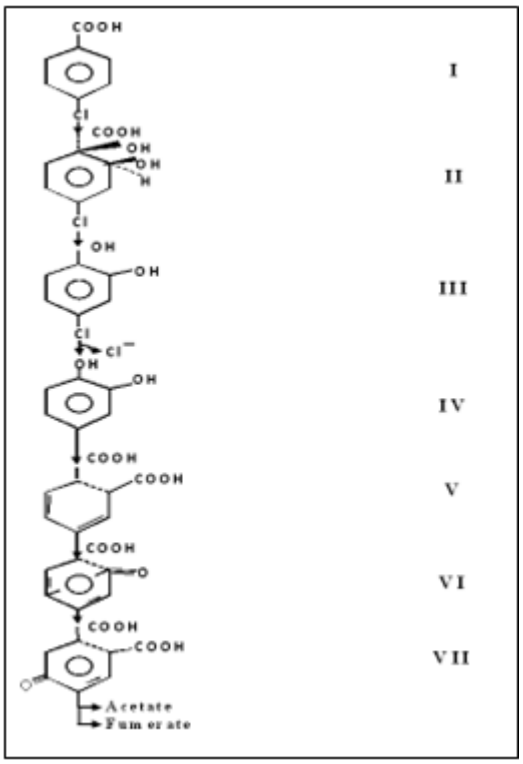

Figure 4: Proposed pathway for degradation of 4-chlorobenzoic acid by *Pseudomonas aeruginosa* I: 4-chlorobenzoic acid, II: 4-chlorodihydroxy benzoic acid, III: 4-chlorocatechol, IV: catechol, V: *cis*, *cis*-muconic acid, VI: dine lactone, VII: malylacetate.