

# Biodegradation of 4-chlorobenzoic acid by *Pseudomonas aeruginosa* PA01 NC

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**Abstract** A bacterial consortium capable of degrading chloroaromatic compounds was isolated from pulp and paper mill effluents by selective enrichment on 4-chlorobenzoic acid as sole source of carbon and energy. The four different bacterial isolates obtained from bacterial consortium were identified as *Pseudomonas aeruginosa* AY792969 (A), *P. aeruginosa* PA01 NC (B), *Pseudomonas* sp. ZZ5 DQ113452 (C) and *Pseudomonas* sp. AY762360 (D) based on their morphological and biochemical characteristics and by phylogenetic analysis based on 16S rRNA gene sequences. These bacterial isolates were found to be versatile in degrading a variety of chloroaromatic compounds including fluoro- and iodobenzoic acids. *P. aeruginosa* PA01 NC utilized 4-chlorobenzoic acid at 2 g/l as growth substrate. Biodegradation studies have revealed that this organism degraded 4-chlorobenzoic acid through 4-chlorocatechol which was further metabolized by *ortho*-cleavage pathway and the dechlorination occurred after the ring-cleavage.

**Keywords** Biodegradation · Bacterial consortium · Chloroaromatics · *Pseudomonas aeruginosa* PA01 NC · 4-Chlorocatechol

## Introduction

Chlorinated aromatic compounds are the major chemical pollutants because they enter the environment in large quantities and are toxic and resistant to degradation and bioaccumulate (Chaudhry and Chapalamadugu 1991). The chlorinated aromatics are produced in several industries including pulp and paper manufacture, which has led to their widespread release into the environment and contamination of both soil and ground water. A large number of these chemicals have been designated as priority pollutants by the US Environmental protection agency (EPA). Chlorobenzoates are produced as dead-end metabolites in the biodegradation of polychlorinated biphenyls (PCBs), DDT and some herbicide (Bedard et al. 1987; Furukawa et al. 1983; Hartman et al. 1979; Ilori et al. 2007; Kamanavalli and Ninnekar 2004). Microorganisms play an important role in the biodegradation of such hazardous chemicals in the environment. It has been established that contaminated environments harbour a wide range of unidentified pollutant-degrading microorganisms that have crucial role in bioremediation (Margesin et al. 2003). For instance, bleached kraft pulp mill effluents (BKME) is a suitable place to search for new

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strains with catabolic activity towards chloroaromatic pollutants (Fulthorpe et al. 1993). BKME contains chloroorganic compounds such as chlorophenols, chlorobenzoates, chlorocatechols and chloroguaiacols generated in the chlorine bleaching process (Kringstad and Lindstroem 1984; Leuenberger et al. 1985). There are few reports on characterization of bacterial communities capable of degrading chloroaromatic pollutants (Greene et al. 2000; Kubicek et al. 2003; Watanabe et al. 2002). In this paper, we describe the isolation and characterization of bacterial consortium capable of degrading chloroaromatic compounds and the pathway for the degradation of 4-chlorobenzoic acid by *Pseudomonas aeruginosa* PA01 NC.

## Materials and methods

### Bacterial cultures and growth conditions

The pulp and paper mill effluents and sludge samples collected from the West Coast Paper Mills Ltd., Dandeli, India were used for isolation of bacterial cultures. The bacteria were grown in Seubert's mineral salts medium (Seubert 1960) containing 0.2% (w/v) 4-chlorobenzoate (4-CB) as sole carbon source in 500 ml Erlenmeyer flasks on a rotary shaker (150 rpm) at room temperature. Growth was measured turbidometrically at 660 nm. The cultures were maintained on 4-CB-mineral salts agar slants.

### Isolation and identification of 4-CB degrading bacteria

A bacterial consortium capable of degrading 4-CB was isolated from a pulp and paper mill effluents after continuous enrichment in mineral salts medium containing 4-CB as sole source of carbon for a period of 4 months. The bacterial community obtained after enrichment was spread over mineral salt medium 4-CB agar plate and purified by repeated streaking. The bacterial colonies appeared on 4-CB-mineral salts agar plates were morphologically characterized and purified by repeated culturing. The four different bacterial isolates obtained from bacterial consortium were identified on the basis of their morphological and biochemical characteristics and by phylogenetic analysis based on 16S rRNA gene sequences.

### Identification of bacterial isolates by 16S rRNA gene sequencing

The bacterial 16S rRNA gene was amplified from the total genomic DNA using universal eubacteria-specific primers 16F27 (5' CCA GAG TTT GAT CMT GGC TCA G 3') and 16R1525XP (5' TTC TGC AGT CTA GAA GGA GGT GWT CCA GCC 3'), which yielded a product of approximately 1,500 base pairs. The polymerase chain reaction (PCR) conditions were 35 cycles of 95°C denaturation for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min and in addition one cycle of extension at 72°C for 10 min. The PCR product was purified by PEG-NaCl precipitation (Sambrook et al. 1989). Briefly, the PCR product was mixed with 0.6 volumes of PEG-NaCl solution (20% PEG 6000, 2.5 M NaCl) and incubated for 10 min at 37°C. The precipitate was collected by centrifugation at 12,000 rpm for 10 min. The pellet was washed twice with 70% ethanol and dried under vacuum, which was then resuspended in glass distilled water at a concentration of >0.1 pmol/ml. The purified product was directly sequenced using a Big Dye terminator kit (Applied Biosystems, Inc., Foster City, CA) (Pidiyar et al. 2004). The sequencing reactions were run on ABI-PRISM automated sequencer (ABI-3730 DNA analyser). The nucleotide sequence analysis was done at Blast-n site at NCBI server ([www.ncbi.nlm.nih.gov/BLAST](http://www.ncbi.nlm.nih.gov/BLAST)). The alignment of the sequences were done using CLUSTALW program V1.82 at the European Bioinformatics site ([www.ebi.ac.uk/clustalw](http://www.ebi.ac.uk/clustalw)). The analysis of 16S rRNA gene sequence was done at Ribosomal Database Project (RDP) II (<http://rdp.cme.msu.edu>). The sequence was refined manually after crosschecking with the raw data to remove ambiguities. The phylogenetic tree was constructed using the aligned sequences by the neighbour-joining method using Kimura-2-parameter distances in MEGA 2.1 software (Kumar et al. 2001). The sequences of the 16S rRNA gene of the isolated strains of *Pseudomonas* are available under the Gen Bank accession numbers AY762360, ZZ5DQ113452, PA01 NC and AY792969.

### Analytical methods

The metabolites were isolated from culture filtrate of the organism grown on 4-chlorobenzoic acid by extraction with diethyl ether and analysed by thin layer

chromatography (TLC) on silica gel G plates using the solvent systems: A) Benzene-acetic acid (85:15, v/v) and B) Benzene-toluene-acetic acid (2:2:1, v/v). The metabolites were visualized under ultraviolet (UV) light (at 254 nm) or by exposure to iodine vapours and also by spraying with 1%  $\text{FeCl}_3$ – $\text{K}_3\text{Fe}(\text{CN})_6$  solution in water. Phenolic compounds gave their characteristic colour on spraying with diazotized *p*-nitroaniline or with Gibbs reagent (2% solution of 2,6-dichloroquinone-4-chlorimide in methanol). *ortho*-Dihydroxy compounds were detected by spraying with Arnow's reagent (Arnow 1937). The metabolite were analysed by reversed-phase HPLC, using a 5- $\mu$  Spherisorb-ODS ( $\text{C}_{18}$ ) column (25 cm  $\times$  4.6 mm) and water–methanol (30:70, v/v) solvent system, at a flow rate of 1 ml/min. Peaks were detected at 254 nm or scanned at 220–400 nm in a spectrophotometer. UV–Visible spectra were obtained by using spectrophotometer (Hitachi 3100). The Infra red (IR) spectra were recorded with Nicolet Impact 410 FT-IR spectrometer. Chloride ions released during 4-CB degradation was estimated spectrophotometrically by using sodium chloride as standard (Bergmann and Sanik 1957).

### Enzyme assays

Cell-free extracts were prepared from washed cells suspended in three volumes of 50 mM phosphate buffer (pH 7.0) containing 15% glycerol (v/v) and 2 mM dithiothreitol by sonication (Ultrasonic processor model XL 2010) for 5 min and centrifugation at  $12,000\times g$  for 30 min at 4°C. The clear supernatant was used as crude extract for enzyme assays. 4-Chlorobenzoate 1,2-dioxygenase was assayed spectrophotometrically by measuring the decrease in absorbance at 340 nm due to NADH. The reaction mixture (1 ml) contained 50 mM phosphate buffer (pH 7.0), 0.2 mM NADH and 0.2 ml cell extract. The reaction was started by addition of 0.5 mM 4-chlorobenzoate. The reaction rate was corrected by subtracting NADH oxidation rate in the absence of 4-chlorobenzoate. 4-Chlorocatechol 2,3-dioxygenase was assayed spectrophotometrically by measuring the increase in absorbance at 378 nm due to the formation of 2-chloro-5-hydroxy muconic semialdehyde (CHMS). 4-Chlorocatechol 1,2-dioxygenase was assayed by measurement of substrate-dependent oxygen uptake using oxygen electrode. The oxygen uptake rates were corrected for those obtained in the absence of substrate. 4-Chlorobenzoate-4-hydroxylase

was assayed spectrophotometrically by measuring the increase in absorbance at 255 nm due to formation of 4-hydroxybenzoate. 4-Hydroxy-3-hydroxylase was assayed as described by Karegoudar et al. (1999). Protocatechuate-3,4-dioxygenase was assayed as described by Fujisawa and Hayaishi (1968). Protein was determined by the method of Lowry et al. (1951). One unit of enzyme activity was defined as the amount required to catalyse the formation or consumption of 1  $\mu$ mol of product or substrate per minute. Specific activities were expressed as units per mg of protein.

## Results and discussion

### Characterization of organisms

An indigenous bacterial consortium was isolated from a pulp and paper mill effluents by enrichment on 4-chlorobenzoate as sole source of carbon and energy. The four different bacterial isolates obtained from a bacterial consortium were characterized by their morphological and biochemical properties as shown in Table 1. All the four isolates were gram-negative, aerobic, rod-shaped, non-sporulating motile bacteria. Isolates A and B produced green and bluish-green pigments respectively. All the isolates except C grew in medium containing 5% NaCl. The optimum temperature and pH for the growth of isolates were 35°C and 7.0 respectively. According to Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) the isolates were identified as *Pseudomonas* species. This was further confirmed by phylogenetic analysis based on 16S rRNA gene sequences.

The complete sequence of 16S ribosomal RNA gene of the four bacterial isolates (A, B, C and D) were determined. Analysis of sequences were done at RDP II and NCBI, where relevant sequences from these data bases were downloaded for further analysis (Cole et al. 2005). The isolates showed 99.9% sequence similarities with *Pseudomonas* species. In the phylogenetic analysis, all the isolates clustered with *Pseudomonas resinovorans*, *Pseudomonas alcaligenes*, *Pseudomonas nitroreducens*, *Pseudomonas citronellolis*, *Pseudomonas azelaica* strain DS, *Pseudomonas oleovorans*, *Pseudomonas anguilliseptica* st, *Pseudomonas flavescens* and *Pseudomonas straminea* thus, confirming their identities as belonging to *Pseudomonas* species. The phylogenetic relationship has been

**Table 1** Morphological and biochemical characteristics of the bacterial isolates capable of degrading chloroaromatic compounds

| Characteristics                   | Observations<br>Bacterial isolates |              |              |              |
|-----------------------------------|------------------------------------|--------------|--------------|--------------|
|                                   | A                                  | B            | C            | D            |
| Morphological                     |                                    |              |              |              |
| Cell shape                        | Rod                                | Rod          | Rod          | Rod          |
| Gram reaction                     | –ve                                | –ve          | –ve          | –ve          |
| Motility                          | Motile                             | Motile       | Motile       | Motile       |
| Endospores                        | Absent                             | Absent       | Absent       | Absent       |
| Pigment formation                 | Green                              | Bluish-green | Absent       | Absent       |
| Biochemical                       |                                    |              |              |              |
| Catalase                          | +                                  | +            | +            | +            |
| Urease                            | –                                  | –            | –            | –            |
| Arginine dihydrolase              | +                                  | +            | +            | +            |
| Starch hydrolysis                 | –                                  | –            | –            | –            |
| Gelatin hydrolysis                | +                                  | +            | –            | –            |
| H <sub>2</sub> S production       | –                                  | –            | –            | –            |
| Nitrate reduction                 | +                                  | +            | +            | +            |
| Growth on 5% NaCl                 | +                                  | +            | –            | +            |
| MR reaction                       | –                                  | –            | +            | +            |
| Oxidation/fermentation of glucose | Oxidation                          | Oxidation    | Fermentation | Fermentation |
| Citrate utilization               | +                                  | +            | +            | +            |

+ Present; – absent

shown in Fig. 1. The isolate ‘A’ was identified as *P. aeruginosa* AY792969, ‘B’ as *P. aeruginosa* PA01 NC, ‘C’ as *Pseudomonas* sp. ZZ5 DQ113452 and ‘D’ as *Pseudomonas* sp. AY762360.

#### Growth on various halogenated aromatic compounds

The ability of the four different bacterial isolates obtained from bacterial consortium to utilize various halogenated aromatic compounds (0.2%, w/v) as sole source of carbon and energy is given in the Table 2. These bacterial isolates utilized most of the chloroaromatic compounds and also *p*-fluorobenzoate and *o*-iodobenzoate as growth substrates. The growth of *P. aeruginosa* PA01 NC on 4-chlorobenzoic acid (0.2%, w/v) as sole source of carbon is shown in Fig. 2. The organism utilized 4-chlorobenzoic acid at 2 g/l as sole source of carbon and energy.

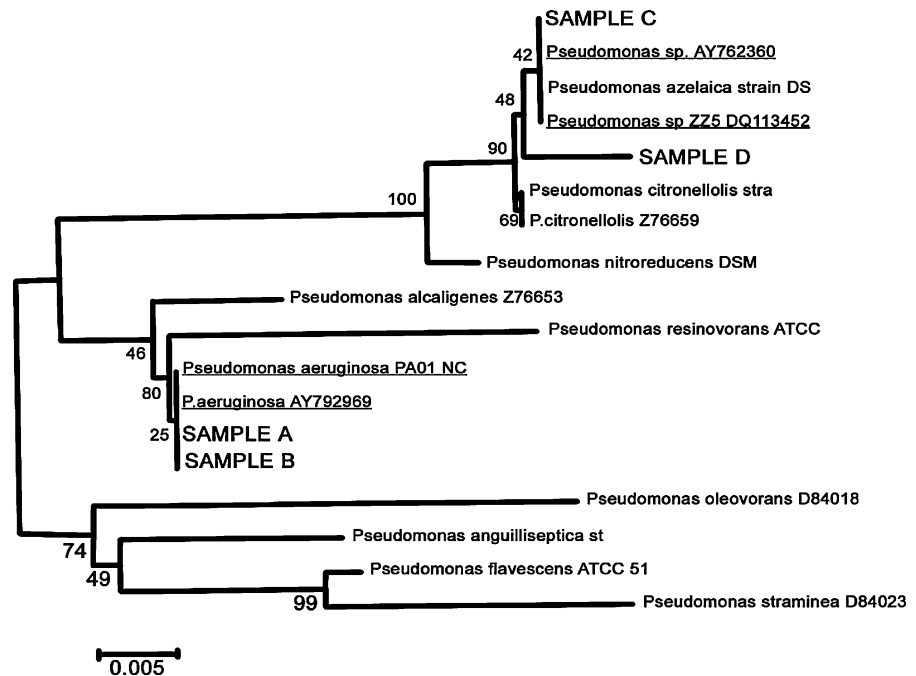
#### Identification of metabolites

The analysis of the culture extracts of *P. aeruginosa* PA01 NC grown on 4-chlorobenzoic acid by TLC

revealed the presence of a compound, whose  $R_f$  value corresponded with that of authentic 4-chlorocatechol (Table 3). HPLC analysis of isolated metabolite showed the retention time of 3.91 min identical to that of authentic 4-chlorocatechol. IR spectrum showed characteristic absorption bands of –OH stretching at  $3,425.1\text{ cm}^{-1}$ , aromatic CH stretching at  $2,924.7\text{ cm}^{-1}$ ,  $\text{>C=C<}$  stretching at  $1,513.3$  and  $1,464.9\text{ cm}^{-1}$  and C–Cl stretching vibration at  $800.2\text{ cm}^{-1}$ . The ring cleavage product of 4-chlorocatechol was identified as 3-chloro-*cis,cis*-muconate that showed  $\lambda_{\text{max}}$  at 267 nm (Dorn and Knackmuss 1978). It was transiently accumulated in the culture supernatants of inoculated samples but not in the controls. There was a release of  $0.901 \times 10^{-3}\text{ mM}$  of chloride ion in the culture supernatants after ring-cleavage of 4-chlorocatechol.

The cell free extracts of *P. aeruginosa* PA01 NC grown on 4-chlorobenzoic acid contained the activities of 4-chlorobenzoate 1,2-dioxygenase, and 4-chlorocatechol 1,2-dioxygenase (Table 2). 4-Chlorobenzoate-4-hydroxylase, 4-hydroxybenzoate-3-hydroxylase, 4-chlorocatechol 2,3-dioxygenase and protocatechuate-3,4-dioxygenase activities were not detected in the

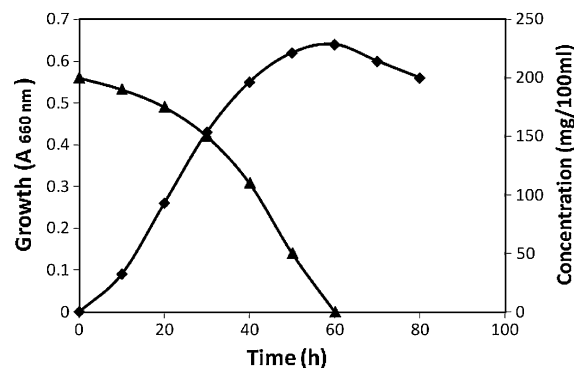
**Fig. 1** Phylogenetic tree based on 16S rRNA gene sequences of bacterial isolates (samples A, B, C and D) and related species



**Table 2** Utilization of various halogenated aromatic compounds by the isolated bacterial strains

| Compounds                 | Growth of bacterial strains |    |    |    |
|---------------------------|-----------------------------|----|----|----|
|                           | A                           | B  | C  | D  |
| 2-Chlorobenzoic acid      | ++                          | ++ | ++ | ++ |
| 4-Chlorobenzoic acid      | ++                          | ++ | ++ | ++ |
| <i>o</i> -Dichlorobenzene | ++                          | +  | ++ | +  |
| <i>p</i> -Dichlorobenzene | ++                          | ++ | ++ | ++ |
| 4-Chlorobenzophenone      | +                           | +  | +  | ++ |
| 2-Chlorophenol            | ++                          | +  | ++ | ++ |
| <i>p</i> -Chlorophenol    | –                           | –  | –  | +  |
| 2,4-Dichlorophenol        | –                           | –  | –  | +  |
| <i>p</i> -Chlorobiphenyl  | ++                          | +  | +  | +  |
| Benzene hexachloride      | ++                          | ++ | ++ | ++ |
| 4-Chloroacetophenone      | +                           | +  | +  | ++ |
| <i>o</i> -Iodobenzoate    | ++                          | +  | +  | ++ |
| <i>p</i> -Fluorobenzoate  | ++                          | ++ | ++ | ++ |
| 4-Chlorocatechol          | ++                          | ++ | ++ | ++ |

++ Good growth; + moderate growth; – no growth



**Fig. 2** Utilization of 4-chlorobenzoic acid (filled triangles) during growth (filled squares) of *P. aeruginosa* PA01 NC

cell-free extracts grown on 4-chlorobenzoate. Relative activities of 4-chlorocatechol 1,2-dioxygenase with catechols showed lower activities for catechol and 3-chlorocatechol than for 4-chlorocatechol (Table 4).

Cell-free extracts of glucose grown cells did not contain any of these enzyme activities. These results have indicated that the degradative enzymes were induced by growth of organism on 4-chlorobenzoate.

It is evident from these results that the organism degraded 4-chlorobenzoic acid by initial hydroxylation to yield 4-chlorocatechol, which was further metabolized through *ortho*-cleavage pathway and the dechlorination had occurred after the ring cleavage (Fig. 3). Microbial degradation of 4-chlorobenzoate have been shown to occur by dehalogenation either

**Table 3** Chromatographic and spectral properties of metabolite of 4-chlorobenzoic acid by *P. aeruginosa* PA01 NC

| Property                                                | Isolated metabolite | Authentic 4-chlorocatechol |
|---------------------------------------------------------|---------------------|----------------------------|
| TLC: R <sub>f</sub> values in different solvent systems |                     |                            |
| A. Benzene:toluene:acetic acid (2:2:1, v/v)             | 0.74                | 0.74                       |
| B. Benzene:acetic acid (85:15, v/v)                     | 0.41                | 0.42                       |
| UV absorption of λ <sub>max</sub> in ethanol (nm)       | 284                 | 284                        |
| HPLC retention time (min)                               | 3.91                | 3.91                       |

HPLC high-performance liquid chromatography

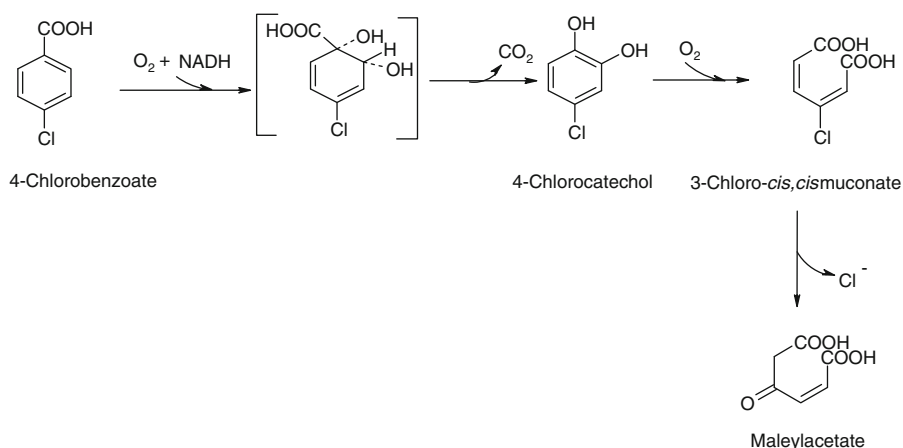
prior to or after the ring-cleavage (Arendorf and Focht 1995; Marks et al. 1984; Reineke and Knackmuss 1988). There are reports of dehalogenation of 4-chlorobenzoate to 4-hydroxybenzoate, which was further degraded through protocatechate pathway by *Arthrobacter* sp. (Marks et al. 1984; Ruisinger et al. 1976; Shimao et al. 1989), *Nocardia* sp. (Klages and Lingens 1979), *Acinetobacter* sp. (Kobayashi et al. 1997; Tobita and Iyobe 1992) and *Aspergillus niger* (Shailubhai et al. 1983). The oxidation of 4-chlorobenzoate to 4-chlorocatechol and its dehalogenation after the ring-cleavage have been shown to occur in *Pseudomonas* sp. B13 (Reineke and Knackmuss 1980), *Pseudomonas cepacia* P166 (Arendorf and Focht 1995), *P. aeruginosa* 3mT (Ajithkumar and Kunhi 2000) and *Ralstonia eutropha* JMP134

**Table 4** Specific activities of enzymes in the cell-free extracts of *P. aeruginosa* PA01 NC grown on 4-chlorobenzoic acid

| Enzyme                           | Substrate        | Specific activities (units/mg protein) |
|----------------------------------|------------------|----------------------------------------|
| 4-Chlorobenzoate 1,2-dioxygenase | 4-Chlorobenzoate | 0.182                                  |
| 4-Chlorocatechol 1,2-dioxygenase | Catechol         | 0.112 <sup>a</sup> (64)                |
|                                  | 4-Chlorocatechol | 0.173 <sup>a</sup> (100)               |
|                                  | 3-Chlorocatechol | 0.122 <sup>a</sup> (70)                |
| 4-Chlorocatechol 2,3-dioxygenase | Catechol         | 0.002                                  |
|                                  | 4-Chlorocatechol | 0.001                                  |
|                                  | 3-Chlorocatechol | 0.000                                  |

<sup>a</sup> Relative activities given in parentheses are expressed as percentages of that for 4-chlorocatechol as 100%

(Ledger et al. 2002). The pathway of degradation of 4-chlorobenzoate by *P. aeruginosa* PA01 NC appears to be similar to that reported in *Pseudomonas* sp. B13 and *R. eutropha* JMP134 but differs from that in *Acinetobacter* sp., *Arthrobacter* and *A. niger*. Since catechol 2,3-dioxygenase was not detected in *P. aeruginosa* PA01 NC, the degradation of chlorocatechols by dead-end *meta*-pathway was absent. 4-Chlorobenzoate was completely degraded by *ortho*-cleavage pathway. Thus the indigenous bacterial isolates from pulp and paper mill effluents were found to versatile in degrading a variety of chloroaromatic compounds, which are reported to be priority pollutants.

**Fig. 3** Proposed pathway for the degradation of 4-chlorobenzoate by *P. aeruginosa* PA01 NC

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