Biocatalysis and Agricultural Biotechnology ∎ (∎∎∎∎) ∎∎∎–∎∎∎

Contents lists available at ScienceDirect

Biocatalysis and Agricultural Biotechnology

journal homepage: www.elsevier.com/locate/bab

Original Research Paper

# Biodegradation of chloroaromatic pollutants by bacterial consortium immobilized in polyurethene foam and other matrices

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ARTICLE INFO

*Article history:*
Received 24 July 2013
Received in revised form
31 October 2013
Accepted 2 March 2014

*Keywords:*
Biodegradation
*Pseudomonas* sp. AY762360
2-Chlorobenzoic acid
Catechol
Immobilization
Bacterial consortium

ABSTRACT

Chlorinated aromatic compounds are the major pollutants of the environment because of their extensive use and toxicity. A bacterial consortium isolated from Pulp and paper mill effluents was shown to degrade various chloroaromatic compounds as a sole source of carbon and energy. The bacterial consortium and *Pseudomonas* sp. AY762360 were immobilized in various matrices such as polyurethane foam (PUF), sodium alginate (SA), polyacrylamide and agar. The rate of degradation of 2-chlorobenzoic acid (2-CBA), 4-chlorobenzoic acid (4-CBA), 1,2-dichlorobenzene (1,2-DCB) and 1,4-dichlorobenzene (1,4-DCB) at a concentration of 0.2 and 0.6% by the immobilized cells of bacterial consortium in batches with shaken cultures were compared with that of freely suspended cells and immobilized cells of *Pseudomonas* sp. AY762360. There was complete degradation of these compounds by the PUF-immobilized bacterial consortium when compared to that by freely suspended cells, *Pseudomonas* sp. AY762360 immobilized in various matrices and the cells of bacterial consortium immobilized in polyacrylamide, SA and agar. The PUF-immobilized cells of bacterial consortium showed more tolerance to pH and temperature changes than freely suspended cells. *Pseudomonas* sp. AY762360, an isolate from the bacterial consortium, was shown to degrade 2-CBA through catechol by a *meta*-cleavage pathway. Thus these bacterial strains could potentially be useful in the bioremediation of chloroaromatics contaminated sites.



## 1. Introduction

There is a widespread contamination of the environment by chloroaromatic compounds because of their large scale manufacture and extensive use. These chemicals reported to be are highly toxic to mammals and other organisms (Niedan and Scholer, 1997; Deweard and Bedard, 1999; Field and Alvarez, 2008). Hence, it is necessary to investigate the metabolic fate of these chemicals in the environment and their remediation. Chlorobenzoic acids, chlorobenzenes and chlorophenols are released into the environment as a degradation by-product of polychlorinated biphenyls (PBCs), chlorinated pesticides and herbicides by the microorganisms (Monferran et al., 2005; Banta and Kahlon, 2007; Field and Alvarez, 2008; Sunday et al., 2008). The degradation of chlorinated benzene ring is very essential for the complete mineralization of PCBs by microorganisms (Tarawneh et al., 2010). The chlorinated aromatic compounds such as mono-chlorobenzoic acids, mono-chlorophenols and dichlorobenzenes are considered to be highly resistant to degradation, because the chlorine atom(s) attached to benzene ring interfere with the enzymatic reaction (Field and Alvarez, 2008). Chlorine atom(s) in these compounds were found to exhibit steric hindrance and render them more resistant towards the degradation by microorganisms. There are reports on the degradation of chlorobenzoic acids, chlorophenols and dichlorobenzenes by *Acinetobacter* sp., *Alcaligenes* sp., *Burkholderia* sp., *Pseudomonas* sp., *Rodococcus* sp., and *Xanthobacter flavus* 14p1 (Arensdorf and Focht, 1995; Spiess et al., 1995; Zaitsev et al., 1995; Krooneman et al., 1996; Tross et al., 1996; Tsoi et al., 1999; Hoskeri et al., 2011; Mutharasaiah et al., 2012).

The various approaches have been developed for the treatment of industrial effluents contaminated with chloroaromatic pollutants (Cespedes et al., 1996). The constraints are the availability of the suitable microorganisms that can overcome their culturing limitations from their natural habitats to the effluent conditions. The microbial immobilization technique seems to be promising in the bioremediation of chloroaromatics contaminated effluents (Manikandan et al., 2007; Suneetha et al., 2008). Since the entrapped cells remain viable for a considerable duration, they would be better than freely suspended cells for the biodegradation of a variety of toxic chemicals from the effluents (Wang and Hu, 2007; Tallur et al., 2009; Mulla et al., 2012). In this paper, we report the degradation of various chloroaromatic compounds by

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http://dx.doi.org/10.1016/j.bcab.2014.03.001
1878-8181/

the bacterial consortium and *Pseudomonas* sp. AY762360 immobilized in PUF and other matrices. The results of our studies on biodegradation of 2-chlorobenzoic acid (2-CBA) by *Pseudomonas* sp. AY762360 have also been reported in this paper.

## 2. Materials and methods

### 2.1. Chemicals

2-Chlorobenzoic acid (2-CBA), 4-chlorobenzoic acid (4-CBA), 1,2-dichlorobenzene (1,2-DCB), 1,4-dichlorobenzene (1,4-DCB), 4-chlorobiphenyl, benzene hexachloride, 2,3-dihydroxybenzoic acid, gentisic acid, catechol, and 3-chlorocatechol with 99% purity were purchased from Sigma-Aldrich chemicals (St. Loui, MO, USA). All other chemicals were of analytical grade obtained from commercial sources. 2-CBA and 4-CBA were individually dissolved in methanol at a concentration of 3000 mg/10 ml. About 2.5 ml of 1,2-DCB was dissolved in 7.5 ml of acetone and 1,4-DCB was dissolved in acetone at a concentration of 2500 mg/10 ml. The stock solutions of different chloroaromatic compounds were sterilized by membrane filtration and rationed into a medium to get the desired concentrations.

### 2.2. Organisms and growth conditions

The bacterial consortium used in this study were previously isolated and identified in our laboratory from pulp and paper mill effluents by enrichment on 4-chlorobenzoic acid as a sole carbon source (Hoskeri et al., 2011). The organisms were grown in 100 ml of mineral salts medium (MM 1) containing 0.2% (w/v) of 2-CBA as a sole source of carbon in 500 ml Erlenmeyer flasks on a rotary shaker (150 rpm) at 35 °C as described previously (Hoskeri et al., 2011). Growth was measured turbidometrically at 660 nm using a colorimeter (Systronics). The cultures were maintained on 2-CBA-mineral salts agar slants.

### 2.3. Utilization of various chloroaromatic compounds by bacterial consortium

The ability of the bacterial consortium to utilize chloroaromatic compounds as a sole source of carbon and energy was determined by the measurement of growth in MM 1 containing 0.2% (w/v) of the compound. The utilization of 2-CBA during growth of *Pseudomonas* sp. AY762360 was determined spectrophotometrically by the measurement of decrease in UV absorbance at 236 nm (Tarawneh et al., 2010). To monitor the concentration of residual 2-CBA and other chloroaromatic compounds in the culture medium, 5-ml aliquots were removed from each experimental flask over time and the biomass was separated by filtration through membrane filters. The biomass on the membrane filters was washed twice with diethyl ether to collect any adsorbed or bioaccumulated compounds. The filtrates collected were extracted with diethyl ether (1:1, v/v). The diethyl ether layers were dried over anhydrous $Na_2SO_4$ and evaporated in *vacuo*. The residues were dissolved in methanol and subjected to a UV–vis spectrophotometer. Uninoculated controls were used to determine any transformation of 2-CBA affected by physical factors. The effect of 2-CBA concentration (0.05–0.3%, w/v) on the growth of *Pseudomonas* sp. AY762360 was measured after 60 h of incubation.

### 2.4. Isolation and identification of metabolites

The metabolites were isolated from culture filtrate of the organism grown on 2-CBA by extraction with diethyl ether and analyzed by thin layer chromatography (TLC) on silica gel G plates using the solvent systems: (A) benzene–dioxan–acetic acid (74:2:2, vol/vol), (B) toluene–dioxan–acetic acid (90:20:4, v/v) and (C) chloroform–acetic acid (95:5, v/v). The metabolites were visualized under ultraviolet (UV) light (at 254 nm) or by exposure to iodine vapors and also by spraying with 1% $FeCl_3$–$K_3Fe(CN)_6$ solution in water. Phenolic compounds gave their characteristic color 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). Aldehydes were detected by spraying with a solution of 2,4-dinitrophenylhydrazine (0.1%) in 2 M HCl. Isolate was tested for the mode of ring cleavage by *ortho* or *meta* pathway as described by Stanier et al. (1966). GC–MS analysis was performed using a Shimadzu QP2010 Plus. GC–MS apparatus equipped with a quadruple mass filter Rtx-5 MS capillary column (30 m × 0.25 mm), scan interval 0.5 s and mass range 40–500 *m/z*. The temperature program was held at 50 °C for 1 min with 20 °C increase $min^{-1}$ to a final temperature of 280 °C for 14.5 min and the injector temperature was kept at 250 °C. The injection volume was 1 μl and the carrier gas was helium. The mass spectrometer was operated at an electron ionization energy of 70 eV. The metabolite was analyzed by HPLC (Shimadzu, Japan) equipped with a SPD-10AVP UV-Detector using Silica gel-packed $C_{18}$ column (4.6 × 250 mm) of particle size (5 μm) (Phenomenex) with the mobile phase consisting of a mixture of methanol–40 mM acetic acid (1:1) applied at a flow rate of 1 ml $min^{-1}$. Nuclear magnetic resonance (NMR) spectra were recorded using a 300 MHz spectrometer (Bruker AMX) of metabolites with tetramethylsilane (TMS) as an internal standard at room temperature. UV–visible spectra were recorded with a spectrophotometer (Hitachi, 3100) and the infra red (IR) spectra with a Nicolet 5700 FT-IR spectrometer (USA make). Chloride ions released during 2-CBA degradation were estimated spectrophotometrically using sodium chloride as a standard (Bergmann and Sanik, 1957).

### 2.5. Enzyme assays

Cell-free extracts were prepared from washed cells suspended in three volume of 50 mM phosphate buffer pH 7.0 by sonication (Ultrasonic processor, model XL 2010) for 5 min and centrifugation at 12,000*g* for 45 min at 4 °C. The clear supernatant was used for further enzymatic studies. 2-Chlorobenzoate-1,2-dioxygenase activity was assayed spectrophotometrically for the determination of the product catechol at 275 nm. Catechol 1,2-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 260 nm due to the formation of *cis,cis*-muconic acid according to Hayaishi et al. (1957). Catechol 2,3-dioxygenase activity was assayed spectrophotometrically by measuring the increase in absorbance at 375 nm due to the formation 2-hydroxymuconic semialdehyde according to Kim et al. (1992). Protein was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard. One unit of enzyme activity was defined as the amount required to catalyze the formation or consumption of 1 μmol of product or substrate per minute.

### 2.6. Immobilization of bacterial consortium and Pseudomonas sp. AY762360

The mineral salts medium (MM 2) used for the degradation studies contained $K_2HPO_4$, 6.30; $MgSO_4.7H_2O$, 0.20; $CaCl_2.2H_2O$, 0.20; $NH_4NO_3$, 1.0 and $FeCl_3$, 0.05 g $L^{-1}$(Mulla et al., 2012). The pH of the medium was adjusted to 7.0 and different concentrations of 2-CBA, 4-CBA, 1,2-DCB and 1,4-DCB were added after sterilization of the medium.

Please cite this article as: Hoskeri, R.S., et al., Biodegradation of chloroaromatic pollutants by bacterial consortium immobilized in polyurethene foam and other matrices. Biocatal. Agric. Biotechnol. (2014), http://dx.doi.org/10.1016/j.bcab.2014.03.001