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Biodegradation of 3-chlorobenzoate and 3-hydroxybenzoate by polyurethane foam immobilized cells of *Bacillus* sp. OS13

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ABSTRACT

Due to widespread use, persistence and toxicity, 3-chlorobenzoate (3-CBA) is well-known as an environmental pollutant. *Bacillus* sp. OS13, isolated as a 2-nitrotoluene tolerant, has the ability to utilize 3-CBA (4 mM) as a sole source of carbon and energy. Nevertheless, this organism has also simultaneously utilized the 4-chlorophthalate, 3-hydroxybenzoate, protocatechuate and 2-chlorobenzoate as a growth substrate. In order to achieve our study objective, firstly we followed the response surface methodology (RSM), and assessed the optimal growth conditions of pH (7.20) and temperature (33 °C) for *Bacillus* sp. OS13 to degrade the 3-CBA. We also determined the two major degradation products (i.e., 3-hydoxybenzoate and protocatechuate) of 3-CBA by using HPLC and GC–MS. Additionally, the activities of 3-hydroxybenzoate-4-hydroxylase and protocatechuate 3,4-dioxygenase enzymes in the cell-free extract were also observed. Thus, in *Bacillus* sp. OS13, 3-CBA was transformed into protocatechuate via 3-hydroxybenzoate with the release of chloride ions and thereby enter into the *ortho*-cleavage pathway. In addition, there was complete degradation of 3-CBA (4 mM) and 3-hydroxybenzoate (4 mM) by the polyurethane foam (PUF)-immobilized cells of *Bacillus* sp. within 60 h. Furthermore, when the initial concentrations of both substrates were increased to 10 mM, the same PUF-immobilized cells were degraded approximately 98% of both substrates within 60 h. Our findings would be very useful towards the environmental management of 3-CBA in the surrounding contaminated environment.

1. Introduction

Chloroaromatics have been widely reported group of environmental pollutants due to their ubiquitous distribution, toxicity, persistence and bioaccumulation properties [1,2]. Monochlorobenzoate such as 3-chlorobenzoate (3-CBA) has been widely used into the production of various synthetic chemicals like dyes, pharmaceuticals, fungicides and also as a preserving agent for adhesives and paints [3,4]. Several studies reported that 3-CBA as the end product of polychlorinated biphenyls (PCBs), chlorotoluenes and 4-chlorophthalic acid [5–8]. There are reports of widespread contamination of industrial effluents, rivers and groundwaters by 3-CBA [4]. Hence, extensive distribution of 3-CBA into the environment has raised a great concern on a global scale, due to its high toxicity and recalcitrance [9]. There are reports on the degradation of 3-CBA by microorganisms [4,10–12]. Few studies [7] reported that genus of *Bacillus* such as *Bacillus* sp. strain FO can transform the 4-chlorophthalate into 3-CBA, whereas in *Bacillus mucilaginosus* MAM-24, 3-CBA can also be transformed into benzoate and further reduce into four more metabolites [13].

In recent years, due to eco-friendly and cost-effectiveness, several bioremediation approaches has emerged as one of the most promising tool for the removal of toxic/hazardous pollutants (i.e., chloroaromatics, nitroaromatics, pesticides, phenolic compounds, etc.). Few studies [14–17] reported that the degradation of

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Table 1
Degradation of 3-CBA by bacterial cells were immobilized in various matrices.

3-CBA concentration (mM)	% of removal (3-CBA)	Bacteria	Matrices used for the immobilization of bacteria	Reference
~0.63 mM	91.7	*Pseudomonas putida*	Hollow cylinders of PVC conduit	[14]
~1.28 mM	100	*Rhodococcus erythropolis* strain S-7	PVA-calcium alginate and chitosan-calcium alginate	[15]
10 mM	78	*Pseudomonas* sp.US1 ex	Calcium alginate	[16]
10 mM	100	*Pseudomonas* sp. CP4 and *P. aeruginosa* 3mT	Calcium alginate	[17]

3-CBA by immobilized microorganisms (Table 1). However, for the degradation of 3-CBA, most of the previous studies used calcium alginate entrapment alone or combination with other entrapments like polyvinyl alcohol and/or chitosan for the immobilization of bacterial cells. Given that thick layer and weak strength of calcium alginate as a substrate may hinder the metabolic activities and those findings have several limitations. Nevertheless, the microbial gel cubes/beads prepared using mixers of calcium alginate with other substrates, i.e., chitosan and/or PVA/bentonite clay-powdered activated charcoal (PAC), were found to be highly effective to degrade various toxic/hazardous pollutants. Though, these bacterial immobilized beads are still dependent on the toxicity of chemical pollutants in fed-batch degradation experiments. However, the efficacy of microbe-substrate system can also be further improved, by using the polyurethane foam (PUF), which has been reported to exhibit most effective entrapment for the immobilization of microorganisms due to its high porosity, adsorbing capability as well as its strong mechanical strength [18,19]. Keeping in view the scenario, there is dire need to explore more bacterial strains to degrade the 3-CBA and to evaluate the efficiency of better substrate systems. In the current study, we investigated the metabolic pathway of 3-CBA by using *Bacillus* sp. OS13. In addition, the results of our studies on the degradation of 3-CBA (4 mM and 10 mM) and 3-hydroxybenzoate (4 mM and 10 mM) by freely suspended cells and PUF-immobilized cells of *Bacillus* sp. OS13 have also been evaluated.

2. Materials and methods

2.1. Chemicals

3-Chlorobenzoic acid, 4-chlorophthalic acid, 2-chlorobenzoic acid, protocatechuic acid, 3-hydroxybenzoic acid, catechol with 99% purity were purchased from Merck, Sigma–Aldrich, Fluka. All other chemicals of analytical grade.

2.2. Organism and optimal conditions evaluation by response surface methodology (RSM)

The *Bacillus* sp. OS13 used in this study was isolated and identified in our laboratory from the pesticide-contaminated soil samples by selective enrichment on 2-nitrotoluene as a sole source of carbon and energy [20]. This organism utilized 3-CBA (4 mM) as a sole source of carbon and energy. The organism growth was measured turbidometrically at 600 nm using photoelectric colorimeter (Systronics, India) [21]. *Bacillus* sp. OS13 was maintained on slants containing 3-CBA-mineral salts agar and sub-cultured thrice in a month.

The bacterial culture conditions favoring 3-CBA degradation by *Bacillus* sp. OS13 was determined by response surface methodology (RSM) [22]. The preliminary one-factor-at-a-time experiments have been conducted for optimal ranges of three main factors such as temperature (20–40 °C), pH (5–9) and bacterial inocula size (0.2–0.6 g L^{-1}). RSM using Box–Behnken design (as factorial experimental design) was applied to optimize the above said key growth factors and their interface which considerably influenced the ability of sp. OS13 to degrade the 3-CBA [22,23]. The organism was inoculated into mineral salts medium (MSM 1) containing 3-CBA (4 mM), and the samples were collected after 72 h to quantify the residual concentration of 3-CBA. A three-factor Box-Behnken design consisting of 15 experimental runs with 3 duplicates at the midpoint was applied in the present study. The experiment design was described in Table 2. Eq. (1) shows the quadratic polynomial equation.

Table 2
Box–Behnken experimental design with three independent factors.

Run	X_1	X_2	X_3	Responses of 3-CBA degradation (%)
1	0	1	1	77
2	0	0	0	99
3	−1	−1	0	34
4	0	−1	1	50
5	−1	1	0	56
6	1	−1	0	72
7	−1	0	−1	48
8	1	0	1	89
9	0	−1	−1	64
10	1	1	0	62
11	0	0	0	100
12	0	1	−1	68
13	0	0	0	100
14	−1	0	1	50
15	1	0	−1	88

X_1 refers pH; X_2 refers to temperature; X_3 refers to bacterial inocula size. The data was analysed by statistical analysis system (SAS) software. All the values were averages of triplicates.

$$Y_i = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where Y_i is the expected response, b_0 is the constant, b_i is the linear coefficient, b_{ii} is the quadratic coefficient, X_i and X_j are factors and b_{ij} is the interaction coefficient.

2.3. Utilization of various chloroaromatic compounds by Bacillus sp. OS13

The utilization of different chloroaromatic compounds by *Bacillus* sp. OS13 as sole carbon source was determined by measurement of growth in MSM 1 containing different chloroaromatic compounds (4 mM). The utilization of 3-CBA during growth of *Bacillus* sp. was determined by HPLC. Uninoculated (with 3-CBA) controls were used to determine any transformation of 3-CBA affected by physical factors. The effect of 3-CBA concentration (1–7 mM) on the yield of *Bacillus* sp. was determined after 96 h of the incubation period.