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Simultaneous degradation of phenol and 3-chlorobenzoate by a mixture of separately immobilized cells of two strains of *Pseudomonas* – A preliminary study

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ABSTRACT

Simultaneous degradation of halogenated and non-halogenated aromatic compounds that are catabolised through different cleavage pathways is generally difficult due to biochemical incompatibility. However, free cells of a mixed culture of *Pseudomonas* sp. CP4 that degrades phenol through *meta*-pathway and *Pseudomonas aeruginosa* strain 3mT that degrades 3-chlorobenzoate through *ortho*-pathway was shown, earlier, to degrade mixtures of phenol/cresols and 3-chlorobenzoate in shake flasks. In the present study the degrading efficiency of these strains when immobilized (separately) in Ca-alginate gel beads was tested using a fluidized bed reactor. Complete mineralization of up to 5 mM equimolar mixture of phenol and 3-chlorobenzoate was observed when the beads were used at 1:1 ratio. From a 10 mM equimolar mixture although phenol was completely mineralized 3-chlorobenzoate degradation was not complete as evidenced by accumulation of some intermediary metabolites and the release of only 86% of inorganic chloride (Cl^-). Degradation rates of both the compounds by the immobilized cells, in general, were far better than that by the free cells. With further studies to select suitable gel matrices and optimization of bioreactor conditions these strains can be effectively deployed for treatment of heterogeneous aromatic wastes.

Keywords: immobilized *Pseudomonas* cells; phenol; 3-chlorobenzoate, co-degradation

INTRODUCTION

Phenols, chlorobenzoates and other aromatics are generated by various chemical and pharmaceutical industries as well as through partial degradation of certain insecticides and herbicides and also occur naturally. They contribute a formidable bulk to the environmental pollution and have tremendous adverse effects on the biota and need to be eliminated (Chaudhry and Chapalamadugu, 1991; Ajith-Kumar and Kunhi, 1997; Agarry *et al*., 2008; Basha *et al*., 2010). Processes based on microbial degradation are generally preferred for elimination of these toxic chemical pollutants because of their lower operational costs and the possibility of complete mineralization as against conventional treatment technologies (Vidali, 2001; Brar *et al*., 2006). The biodegradation pathways of most of the aromatic compounds generally converge at a dihydroxy ring compound such as catechol, protocatechuate, gentisate or their derivatives which is then cleaved by a dioxygenase (Dorn and Knackmuss, 1978; Bartels *et al*., 1984; Ajith-Kumar and Kunhi, 1997). Generally, chlorocatechols are cleaved by catechol 1, 2-dioxygenase (C12D, pyrocatechase II) through a modified *ortho*-mode (Chaudhry and Chapalamadugu, 1991; Fetzner, 1998; Ajith-Kumar and Kunhi, 2000), though a few exceptions have been reported (Kaschabek *et al*., 1998). Non-haloaromatics such as phenol, benzoate, cresols, methyl benzoates etc., on the other hand, are usually degraded by bacteria through a *meta*-fission pathway involving catechol 2, 3-dioxygenase (C23D, *meta*-pyrocatechase) (Agarry *et al*., 2008; Basha *et al*., 2010) although there have been reports, in recent years, on the involvement of C12D in the catabolism of phenol (Ahamad and Kunhi, 1996; Agarry *et al*., 2008; Basha *et al*., 2010).

Most bacteria are not capable of degrading chloro- and non-chloroaromatics simultaneously due to metabolic incompatibility. Inhibition of the *meta*-ring cleaving enzyme C23D (Bartels *et al*., 1984) or the reduction in the rate of C12D reaction in the presence of chlorocatechols, particularly of 3-chlorocatechol (3-CC) (Dorn and Knackmuss, 1978) has been shown to be the reason for this failure. Various techniques such as use of hybrid and mutant strains, and cloned organisms have been tried to circumvent these metabolic bottlenecks for degrading mixtures of halo- and non-haloaromatics simultaneously (Chaudhry and Chapalamadugu, 1991; Franck-Mokroß and Schmidt, 1998). All these strains, which operate through a modified *ortho*-pathway, have limitations such as relatively low rates of degradation and inability to degrade relatively high concentrations of the substrates. More efficient degradation of high concentrations of mixtures of chloro- and non-chloroaromatics by mixed cultures of microbial strains that can degrade a high concentration of at least one of the compounds requires the use of mutually compatible strains. We have, earlier, demonstrated efficient degradation of 3-CBA-phenol/cresols mixtures simultaneously by free cells of a two-membered mixed culture, in shake flasks, when added at appropriate inoculum and substrate ratios (Babu

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et al., 1995a). This mixed culture contained *Pseudomonas* sp. strain CP4 (CP4), which could degrade up to 1.5 g/L of phenol (Babu *et al*., 1995b) and up to 2.0 g/L of cresols (Ahamad and Kunhi, 1999; Ahamad *et al*., 2001) through *meta*-cleavage pathways and *P. aeruginosa* strain 3mT (3mT) capable of degrading 8.0 g/L of 3-CBA and 12 g/L of 4-chlorobenzoate (4-CBA) *via* a modified *ortho*-pathway (Ajith-Kumar and Kunhi, 2000). A mixed culture of the strain 3mT and an *ortho*-pathway-following phenol degrader, *Pseudomonas stutzeri* strain SPC-2 (Ahamad and Kunhi, 1996) also degraded 3-CBA-phenol mixtures efficiently when used as free suspended cells in shake flasks (Jayachandran and Kunhi, 2009).

It has been well established that immobilization of the degrading organisms improves their efficiency and such systems have been exploited, during the last few decades, for elimination of a variety of pollutant chemicals (Keweloh *et al*., 1989; Cassidy *et al*., 1996). There have been several reports on degradation of phenol individually by several immobilized microbial systems (Agarry *et al*., 2008; Basha *et al*., 2010). Recently, we also have shown that tolerance to and degradation rate of phenol could be increased several-fold by immobilizing the cells of *Pseudomonas* sp. CP4 in both Ca-alginate and agar gel beads (Ahamad and Kunhi, 2011). However, there have been hardly few reports on degradation of chlorobenzoates using immobilized cell systems (Sahasrabudhe *et al*., 1991; Yun *et al*., 2009) and there seems to be no report at all on simultaneous degradation of phenol and chlorobenzoates by immobilized microbial systems. In the present communication we present some preliminary data on simultaneous degradation of equimolar mixtures of phenol and 3-CBA, in a fluidized bed reactor, by a 1:1 mixture of immobilized cells of strains CP4 and 3mT that were entrapped separately in Ca-alginate gel beads.

MATERIALS AND METHODS

Chemicals

Phenol (AR grade) procured from Qualigens Fine Chemicals, Bombay, India, was purified by distillation and was used immediately. 3-CBA was purchased from Sigma Chemical Co., Mo, U.S.A. Sodium alginate was purchased from Allied and Company, Bombay, India. Agar powder and the chemicals used in the culture media and reagents were of high purity and were procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India.

Microorganisms

The two bacterial strains viz., *Pseudomonas* sp. CP4 (Babu *et al*., 1995b; Ahamad and Kunhi, 1999; Ahamad *et al*., 2001) and *Pseudomonas aeruginosa* 3mT (Babu *et al*., 1995a; Ajith-Kumar and Kunhi, 2000; Jayachandran and Kunhi, 2009) used in this study were the ones isolated in the laboratory previously.

Preparation of cell biomass and entrapment in calcium alginate gel

Cell biomass of strain CP4 was prepared by growing it in a 10 L fermenter ('Digi-Ferm fermenter', locally fabricated) as described previously (Ahamad and Kunhi, 2011) in a mineral medium (M3 medium) (Babu *et al*., 1995b) containing 500 mg/L of phenol. M3 medium contained: (g/L) KH_2PO_4, 2.72; Na_2HPO_4, 5.0; NH_4NO_3, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $Ca(NO_3)_2$, 0.1; trace minerals solution, 1.0 mL [consisting of (g/L) $FeSO_4 \cdot 7H_2O$, 1.0; $MnSO_4 \cdot H_2O$, 1.0; $Na_2MoO_4 \cdot H_2O$, 0.25; H_3BO_3, 0.1; $CuCl_2 \cdot 2H_2O$]. The pH of the medium was maintained at 7.0. Strain 3mT was also grown similarly in the same mineral medium but with 3-CBA (500 mg/L) as carbon source.

The cells of both the strains were harvested and washed and then immobilized by entrapment in Ca-alginate gel separately as described previously (Ahamad and Kunhi, 2011). (The cells of the two strains were not co-immobilized in the same gel beads to avoid the likely problem of biochemical incompatibility that might arise due to close proximity). The cell-entrapped beads containing 2% alginate had an average diameter of 3 mm in both the cases.

Degradation studies with Ca-alginate entrapped cells

All the degradation experiments were carried out in a Gallenkamp modular fermenter (A. Gallenkamp and Co., London, UK) fitted with a 1 L capacity fermenter jar (bioreactor), air sparger, peristaltic pumps for drawing samples and for adding alkali or acid for maintaining pH, if required (the setup of the bioreactor system is shown in Figure 1). M3 medium devoid of the buffering salts KH_2PO_4 and Na_2HPO_4 was used in all the experiments as these phosphates caused extensive disruption of the Ca-alginate beads as described earlier (Ahamad and Kunhi, 2011). In the fermenter jar 500 mL medium was taken and 20 g (wet wt.) each of the Ca-alginate-entrapped cells of the two strains were suspended in it. Phenol and 3-CBA were added as the mixed substrates in required quantities

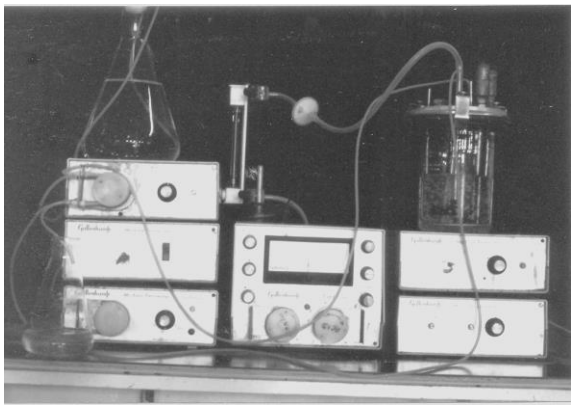

Figure 1: The experimental setup of the Gallenkamp modular fermenter used in the studies.

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such as 2, 5, and 10 mM each. Sterile air was sparged from the bottom of the reactor jar through a sparger at a rate of 500 mL/min (i.e. at 1 vvm) in all the experiments. Studies on the degradation of higher concentrations of the substrates i.e. 5 and 10 mM equimolar levels were carried out in consecutive batches using the same beads that were inoculated to the previous batch of 2 mM substrates, after washing them with the medium. Degradation of the substrates was followed by collecting samples of the culture broth at different periods and analyzing them for residual phenol, 3-CBA, free cell content, inorganic Cl^- released and intermediary metabolites.

Quantitative determinations

Determination of cell biomass

Cell biomass in the gel beads and the cells leached out to and grown in the medium were estimated in terms of total cell protein by the method of Lowry *et al*. (1951) as described previously (Ahamad and Kunhi, 2011).

Determination of phenol concentration

Phenol was estimated by the modified 4-aminoantipyrene colorimetric method of Lacoste *et al*. (1959).

Determination of 3-CBA

3-CBA was estimated by HPLC (Shimadzu LC10A) with a C18, reverse-phase column (150 x 4.6 mm) using methanol/water/acetic acid (40:60:1, by vol.) at a flow rate of 1 ml/min and was detected by UV absorbance at 235/275 nm (Jayachandran and Kunhi, 2009).

Determination of inorganic chloride

Inorganic chloride (Cl^-) released to the medium was estimated by the procedure of Bergmann and Sanik (1957) based on the principle of displacement of thiocyanate ion from mercuric thiocyanate by chloride ion, in the presence of ferric ion, as described by Jayachandran and Kunhi (2009).

Determination of 3-chlorocatechol

3-Chlorocatechol (3-CC) accumulated in the medium was qualitatively determined as described previously (Ajith-Kumar and Kunhi, 2000).

RESULTS

Ca-alginate beads [20 g (wet wt.) each] entrapping separately the cells of strains CP4 and 3mT were inoculated into 500 mL M3 medium (devoid of phosphates) containing phenol and 3-CBA at 2, 5, or 10 mM levels each. Both the gel beads had a cell population of the respective strain equivalent to a cell protein content of about 2.8 mg/g beads (wet wt.) to start with. Aeration was kept at 1 vvm in all the experiments.

In the case of 2 mM each of the substrates complete disappearance of phenol and 3-CBA occurred at 6 and 7 h, respectively (Figure 2). Release of stoichiometric amounts of Cl^- was observed within 8 h. Leaching of cells to the medium was minimal, the total free cells observed being equivalent of 0.06 mg protein/ml at 8 h. The medium containing 5 mM each of phenol and 3-CBA was inoculated with the same beads used in the case of 2 mM batch, after washing them with the medium. Now, the beads contained 2.4 mg cell protein/g beads (wet wt.). There was an initial steep decrease in concentrations of both the substrates until 6 h, followed by a slackening until about 20 h. After that the disappearance was faster and complete degradation of phenol occurred within 25 h, while 3-CBA disappeared in 28 h (Figure 3). Release of stoichiometric amounts of Cl^- from 3-CBA was noticed at 30 h. Leaching of cells was minimal and the maximum amount of free cells observed in the medium was equivalent to 0.11 mg protein/mL. The beads recovered from the 5 mM batch containing 2.1 mg cell protein/g beads (wet wt.) were inoculated into the 10 mM batch. With 10 mM each of the substrates the pattern of their disappearance was similar to that of lower concentrations and complete disappearance of phenol and 3-CBA occurred at 66 and 72 h, respectively (Figure 4). The medium started turning slightly brownish after about 8 h and the colour deepened slightly with time and remained until the end of the experiment (not shown in the Figure). This colour formation was due to the accumulation and auto-oxidation of 3-CC, the intermediary metabolite of 3-CBA degradation (Ajith-Kumar and Kunhi, 2000; Jayachandran and Kunhi, 2009). The Cl^- released at 24 h was about 40%, which increased to only 86% even after 78 h and did not show any further increase. The cells leached and grown in the medium at 78 h was equivalent of about 0.145 mg protein/mL.

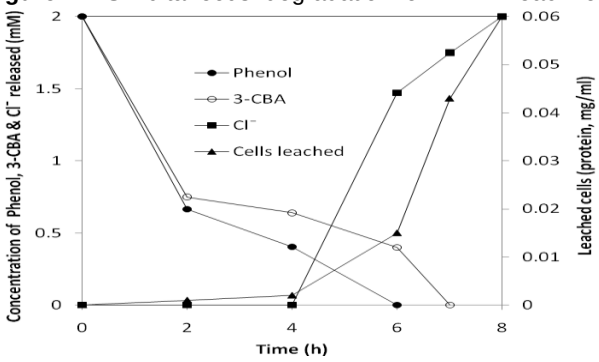

Figure 2: Simultaneous degradation of 2 mM each of phenol and 3-CBA by a mixture of 20 g each of gel beads of Ca-alginate-entrapped cells of *Pseudomonas* sp. CP4 and *P. aeruginosa* 3mT in 500 mL phosphate-free M3 mineral medium in a fluidized bed bioreactor aerated by sparging sterile compressed air at 1 vvm. Other details are as in "Methods".

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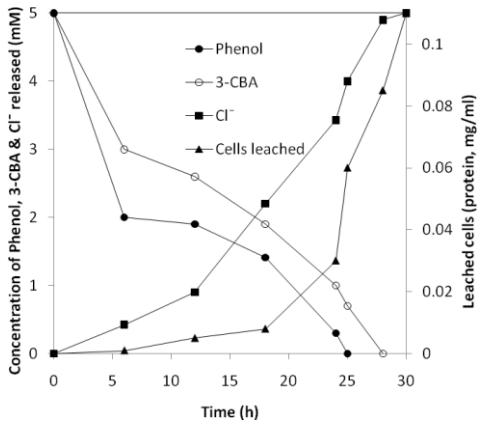

Figure 3: Degradation of a mixture of 5 mM each of phenol and 3-CBA simultaneously by a mixture of Ca-alginate-entrapped cells of *Pseudomonas* sp. CP4 and *P. aeruginosa* 3mT (20 g each of gel beads) in 500 mL phosphate-free M3 mineral medium in a fluidized bed bioreactor aerated by sparging sterile compressed air at 1 vvm. Other details are as in "Methods".

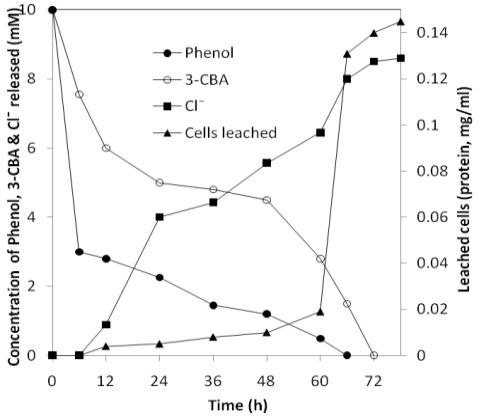

Figure 4: Degradation pattern of 10 mM each of phenol and 3-CBA mixture by a mixture of Ca-alginate-entrapped cells of *Pseudomonas* sp. CP4 and *P. aeruginosa* 3mT (20 g each of beads) in 500 mL phosphate-free M3 mineral medium in a fluidized bed bioreactor aerated by sparging sterile compressed air at 1 vvm. Other details are as in "Methods".

With all the 3 concentrations an initial sharp decline in both the substrates was observed (Figures 2, 3 and 4) which could be due to the adsorption or biosorption of the substrates to the gel or the bacterial cells. Such a phenomenon of reaching the adsorption/biosorption equilibrium at early hours of incubation has been demonstrated in several cases (Aksu, 2005). In our previous studies on simultaneous degradation of phenol/3-CBA mixture by a mixed culture of free cells of strains of SPC-2 and 3mT in shake flasks also such a phenomenon was observed (Jayachandran and Kunhi, 2009).

DISCUSSION

Simultaneous degradation of chlorinated- and non-chlorinated aromatic compounds generally is not effective due to the biochemical incompatibility. However, we have reported earlier that a mixed culture containing free cells of strains CP4 and 3mT could efficiently degrade a phenol-3-CBA mixture (Babu *et al*., 1995a). Similarly, a mixed culture of strains 3mT and *P. stutzeri* SPC-2, another phenol-degrader with an *ortho*-pathway also was shown to degrade both these substrates simultaneously (Jayachandran and Kunhi, 2009). However, there seems to be no report on degradation of these two compounds simultaneously by immobilized cells of any organisms, although there have been several reports of degradation of phenol (Agarry *et al*., 2008; Ahamad and Kunhi, 2011) and a few reports on catabolism of 3-CBA (Sahasrabudhe *et al*., 1991; Yun *et al*., 2009) separately by immobilized microbes. In the present study, the complete disappearance of 2 mM each of phenol and 3-CBA occurred within 6 and 7 h, respectively with the release of stoichiometric amounts of Cl^- whereas with free cells the disappearance of the same amounts of substrates took more than 9 h and the complete mineralization with the release of stoichiometric amount of Cl^- took about 24 h (Babu *et al*., 1995a). Degradation of same amounts of the substrates by free cells of a mixed culture of strains 3mT and SPC-2 also took more than 24 h (Jayachandran and Kunhi, 2009). Both those studies, however, were conducted in shake flasks and the inoculum levels were lower than that were used in the present immobilized system. Nevertheless, much improved tolerance and degradation of phenol (as a single substrate) was observed when immobilized cells of strain CP4 were used, as compared to the free cells of the same, in a fluidized bed bioreactor under similar conditions (Ahamad and Kunhi, 2011). Free cells could degrade only 1000 mg/L of phenol in the bioreactor whereas the Ca-alginate entrapped cells were able to degrade 1500 mg/L. Agar gel entrapped CP4 was far superior in tolerating and degrading phenol at much higher concentrations up to 3000 mg/L. The time taken for complete degradation of phenol (as a single substrate) at 500, 1000, 1500, 2000, 2500, and 3000 mg/L by agar-entrapped cells were 14, 34, 35, 72, 90, and 135 h, respectively (Ahamad and Kunhi, 2011). In the present case 5 and 10 mM (i.e. 470.55 and 941.1 mg/L) phenol in the equimolar mixture was degraded within 25 and 66 h, respectively (Figures 3 and 4) whereas the mixed culture in free suspended form in shake flasks took 48 and 90 h to reach maximum growth indicating complete utilization of 5 and 10 mM phenol in equimolar mixtures (Babu *et al*., 1995a).

3-CBA from a 5 mM equimolar mixture disappeared in 28 h and complete mineralization occurred at 30 h as indicated by 100% release of Cl^- (Figure 3). In the case of 10 mM equimolar mixture, although 3-CBA completely disappeared from the medium at 72 h, complete mineralization did not occur as indicated by accumulation of 3-CC and release of only 86% of Cl^- (Figure 4). There

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are only a few reports on degradation of chlorobenzoates by immobilized systems. Sahasrabudhe *et al*. (1991) have reported dehalogenation of mixtures of monochlorobenzoates and 2, 4-dichlorophenoxyacetic acid by Ca-alginate entrapped cells of *Pseudomonas* sp. US1 ex, the rates of dehalogenation being comparable to that of free cells. Recently, Yun *et al*. (2009) have made a comparative study of degradation of 3-CBA by free and polymer-entrapped cells of *Rhodococcus erythropolis* strain S-7. They used Ca-alginate (CA), polyvinyl alcohol (PVA), and combinations of PVA-CA and chitosan-CA for entrapment of the cells. CA alone or PVA alone were found to be not suitable as the beads disintegrated in the former case and the cells got disrupted in the latter case. However, PVA-CA and chitosan-CA gels were found to be stronger and the former being better in terms of oxygen transfer. 200 mg/L of 3-CBA was degraded within 60 h by free cells as well as by the cells entrapped in these combination gels, PVA-CA being slightly better. Disruption of the beads in media containing phosphates is well known and that was the reason why M3 medium devoid of phosphates was used in the present study as described previously (Ahamad and Kunhi, 2011). We have also observed earlier that agar gel was far superior as a cell-entrapment matrix in the case of phenol degradation, in terms of gel strength, tolerance to higher concentration of the substrate and the rate of degradation (Ahamad and Kunhi, 2011). In the present study, being a preliminary one, alginate was chosen because it entraps whole cells under mild conditions. It would be worthwhile making a comparative study of different gels for entrapment so as to improve the degradation of phenol/3-CBA mixtures simultaneously.

This seems to be first study where 2 different organisms following 2 different ring-cleavage modes were separately immobilized and used as a mixed culture for simultaneous degradation of phenol and 3-CBA. There have been, however, a few reports on simultaneous degradation of phenol and other chloroaromatics such as chlorophenols by immobilized systems (Menke and Rehm, 1992). They immobilized the cells of *Alcaligenes* sp. A7-2 as a biofilm on lava that was used as a filling material in the packed-bed reactors. Degradation patterns obtained with immobilized cells were comparable with that of free cells in batch culture. The continuous cultures remained stable despite increasing the input rates of chlorophenol and phenol mixtures up to 1.16 mM/L/h for several weeks.

Repeated usability is one of the important advantages of immobilized systems. In the present case after repeated use with increasing equimolar concentrations of the substrates the cell-immobilized beads were found to be active, although there was a slight decrease in the cell populations in the beads i.e. the initial 2.8 mg protein/g beads decreased to less than 2.0 mg protein/g beads after the third run with 10 mM each of substrate. This could be because the medium was devoid of phosphates which are required for the active growth of the cells. Similar phenomenon was observed with Ca-alginate entrapped CP4 when used repeatedly with increasing concentrations of phenol in M3 medium without phosphates (Ahamad and Kunhi, 2011). On the contrary, a slight gain in the cell content was observed, even after 6 passages through increasing concentration of 500 through 3000 mg/L phenol, when agar beads were used (Ahamad and Kunhi, 2011). Mukherjee-Dhar *et al*. (1998) have reported 3 successive uses of Ca-alginate immobilized cells of *Rhodococcus opacus* strain TSP203 in batch cultures for the degradation of trichloro-, tetrachloro-, and pentachlorobiphenyls with decreasing degrees of efficiency while free cells in solution lysed after the first use itself. Although Ca-alginate afforded protection against toxicity of PCBs, they were also unable to obtain a stable regeneration of the biocatalyst, probably, due to the absence of phosphate in the medium that they also used. It is possible that entrapment in agar gel or PVA-CA, which provides better gel strength and makes it possible to use in phosphate-containing medium, may improve the degrading ability of both CP4 and 3mT strains, but needs to be verified experimentally.

In conclusion it could be said that *Pseudomonas* sp. CP4 (that degrades phenol/cresol isomers through *meta*-pathways) and *P. aeruginosa* 3mT (that degrades 3-CBA/4-CBA through a modified *ortho*-pathway) immobilized separately by encapsulation in Ca-alginate when used as a mixture exhibited more effective degradation of phenol-3-CBA mixtures than by their free cells. However, use of a medium devoid of phosphates to avoid the disintegration of the gel beads seems to be a drawback with respect to their repeated usability. This could be overcome by using alternate gel matrices such as agar gel or modified alginate gel such as PVA-CA which has been shown to be superior. Both the strains CP4 and 3mT have been shown to degrade other aromatic compounds besides being very efficient degraders of their respective substrates. Strain CP4 could effectively mineralize all 3 isomers of cresol, creosote, aniline, benzoate, 3-hydroxy and 4-hydroxybenzoates, catechol, 4-methyl catechol, gentisate, protocatechuate etc. besides degrading phenol at high concentrations (Babu *et al*., 1995a; 1995b; Ahamad and Kunhi, 1999; Ahamad *et al*., 2001, Ahamad and Kunhi, 2011). Strain 3mT is capable of degrading various aromatic compounds such as benzoates, 3- and 4-hydroxybenzoates, catechol, 3- and 4-CC etc. besides being able to degrade as much as 8 g/L of 3-CBA and 12 g/L of 4-CBA (Ajith-Kumar and Kunhi, 2000). This strain has also proved to be an effective partner with both a *meta*-cleaving phenol degrader strain CP4 and an *ortho*-cleaving phenol degrader strain SPC-2 for simultaneous degradation of phenol-3-CBA mixtures (Babu *et al*., 1995a; Jayachandran and Kunhi, 2009). This strain also was shown to bioremediate effectively soils contaminated with 3-chloro and 4-chlorobenzoates, thus eliminating their toxicity and helping the germination of tomato seeds (Ajith-Kumar *et al*., 1998). All these positive attributes and the results of this study indicate a clear possibility of deploying immobilized cells of these strains for simultaneous degradation of heterogeneous mixtures of phenol/cresol isomers, 3-CBA/4-CBA and other aromatics. However, further detailed studies are needed to select

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suitable gel matrices and optimization of cultural and bioreactor conditions, before a viable treatment technology could be developed.

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