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ORIGINAL PAPER

Degradation of 3-chlorobenzoate and phenol singly and in mixture by a mixed culture of two ortho-pathway-following *Pseudomonas* strains

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Abstract The compatibility and efficiency of two ortho-cleavage pathway-following pseudomonads viz. the 3-chlorobenzoate (3-CBA)-degrader, *Pseudomonas aeruginosa* 3mT (3mT) and the phenol-degrader, *P. stutzeri* SPC-2 (SPC-2) in a mixed culture for the degradation of these substrates singly and simultaneously in mixtures was studied. Another phenol-degrading strain, *Pseudomonas* sp. SoPC-5 (SoPC-5) that utilizes a meta-cleavage mode also was tried in co-culture with 3mT. The former combination was found to be a better degrader of both the substrates when present alone. But, with inoculum levels of 0.15 mg cell dry wt each of 3mT/SPC-2 or 3mT/SoPC-5 growth with 2 mM each of 3-CBA and phenol was slow with a lag of 24 h and degradation being incomplete. However, with higher inocula in the ratios 1:1, 1:2, and 2:1, i.e., 0.3 + 0.3, 0.3 + 0.6, and 0.6 + 0.3 mg cell dry wt of 3mT and SPC-2, respectively complete degradation of both the substrates occurred. Degradation of 3-CBA was complete with the release of stoichiometric amounts of chloride (Cl^-) when concentrations of phenol/3-CBA were varied as 2:2, 2:4, and 4:2 mM, i.e., even when the concentration of the more toxic co-substrate 3-CBA was higher than phenol effective simultaneous degradation occurred at the inoculums ratio of 1:1 (0.3 mg dry cell wt. of each strain). These studies clearly indicated the better suitability of ortho-cleavage-utilizing strains as partners in a mixed culture than those follow different modes.

Keywords 3-Chlorobenzoate · Phenol · *P. aeruginosa* · *P. stutzeri* · Mixed culture · Simultaneous degradation

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Introduction

Phenols, chlorobenzoates and other benzene derivatives in waste effluents from various chemical and pharmaceutical industries contribute a formidable bulk to the environmental pollution and have tremendous adverse effects on the biota [16]. In environmental remediation, biological methods have the advantage of reduced capital and operating costs compared with other methods, besides being eco-friendly [28]. Contemporary research underscores the significance of microbial bioconversion as a better strategy for remediation and environmental conservation [40]. The usage of whole cells for bio-catalysis offers added advantages over enzymes such as, easy culturing of cells, reduction of expenditure for extraction and purification of enzymes and the low cost and feasibility to obtain biomass [28].

As a general rule, the biodegradation pathways of most of the aromatic compounds by bacteria converge at a dihydroxy ring compound such as, catechol, protocatechuate, gentisate or their derivatives [12, 19]. This key intermediary metabolite is then cleaved by a dioxygenase. Generally,

chlorocatechols are cleaved through a modified ortho-mode by pyrocatechase II (catechol 1,2-dioxygenase, C12D) [6, 16, 20, 34, 36], although a few exceptions have been reported [8, 23, 25, 27]. Non-haloaromatics such as, phenol, benzoate, cresols, methyl benzoates, and so on, on the other hand, are usually degraded by bacteria through a meta-fission pathway involving meta-pyrocatechase (catechol 2,3-dioxygenase, C23D) [4, 11, 13, 17, 18, 24, 31, 32] although there have been several reports, in recent years, on the involvement of C12D in the catabolism of non-haloaromatics [1, 15].

Most bacteria are not capable of degrading chloro- and non-chloroaromatics simultaneously because of metabolic incompatibility. Inhibition of the meta-ring cleaving enzyme C23D [12, 29] or the reduction in the rate of C12D (pyrocatechase II) reaction [19] in the presence of chlorocatechols, particularly 3-chlorocatechol (3-CC) has been shown to be the reason for this failure. Various techniques have been tried to overcome these metabolic bottlenecks. Mixed culture systems, hybrid and mutant strains, and cloned organisms have been developed for degrading simultaneously mixtures of phenol, cresol, and chlorophenols [37], chloro- and methyl aromatics [35], chlorobenzene and toluene [33], 3-CBA and 3-methyl benzoate [39], and *m*-toluate and chlorocatechol [41]. Franck-Mokroß and Schmidt [21] have tested the competitiveness of *Pseudomonas strain* possessing both ortho- and meta-pathways and the ortho-pathway exclusively, for simultaneous degradation of chloro- and methyl-benzoate mixtures. However, all these systems had the limitations of the inability to degrade higher concentrations of the substrates and low rates of degradation. In another approach, we have demonstrated efficient degradation of phenol or cresols/3-CBA mixtures by a defined mixed culture of two strains of *Pseudomonas* at appropriate inoculum and substrate ratios [10]. *Pseudomonas* sp. strain CP4 (CP4) that can degrade up to 1.5 g/l of phenol [11] and up to 2.0 g/l of cresols [3] *via* meta-pathway 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) through a modified ortho-pathway [6] constituted the mixed culture. Strain 3mT also exhibited the ability to degrade 3-CBA and 4-CBA efficiently from soil, thus eliminating their inhibitory effect on germination of tomato seeds [5]. Strain 3mT cannot utilize phenol or cresols and CP4 cannot utilize 3- and 4-CBAs, though it can convert them to 3-CC and 4-CC, their respective intermediary metabolites. The mixed culture degraded up to 10 mM equimolar mixture of 3-CBA and phenol [10]. CP4/3mT inoculum ratios of 1:1 or 1:2 gave the most effective degradation of both the substrates in the mixture. However, the degradation of 3-CBA was complete only when its concentration was equal or less than that of phenol.

We have also isolated, for the first time, a *Pseudomonas stutzeri* strain SPC-2 which can degrade up to 1.2 g/l of phenol through an ortho-pathway [1]. This strain cannot utilize chlorobenzoates. Nevertheless, as it follows an ortho-pathway, it was thought to be interesting to study the compatibility of this strain with the ortho-pathway-following 3mT for an effective and simultaneous degradation of phenol and 3-CBA. Another bacterial isolate, *Pseudomonas* sp. SoPC-5 which degrades phenol through meta-pathway [2] was also used in combination with 3mT for a comparison. The results of these studies are presented here. 3-CBA and phenol were taken as substrates in this study because this is a follow up of our previous experiments with another mixed culture, 3mT/CP4 using the same as model pollutant chemicals [10].

Materials and methods

Chemicals

Phenol of analytical grade procured from Qualigens Fine Chemicals, Bombay, Maharashtra, India, was purified by distillation and used immediately. 3-CBA was purchased from Sigma Chemical Co., Mo, USA. All bacteriological media components and other chemicals were of the analytical grade and of the highest purity.

Microorganisms and culture conditions

P. aeruginosa 3mT [5, 6, 10], *Pseudomonas* sp. CP4 [3, 4, 11], *Pseudomonas* sp. SoPC-5 [2], and *P. stutzeri* SPC-2 [1] used in the study were all laboratory isolates.

The cultures were grown in a mineral medium (MM) containing (g/l) KH_2PO_4, 0.675; Na_2HPO_4, 5.455; NH_4NO_3, 0.25; $MgSO_4{\cdot}7H_2O$, 0.200, $Ca(NO_3)_2$, 0.100, 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; $Co(NH_3)_2{\cdot}6H_2O$, 0.25; $NiSO_4{\cdot}6H_2O$, 0.1; Conc. H_2SO_4, 5 ml] and the carbon source in required amounts. The pH of the MM was maintained at 7.0.

The bacterial strains were maintained on MM agar slants containing 200 ppm of phenol or 3-CBA as required.

The inocula of the cultures were prepared by growing them on the respective substrates (1.0 g phenol or 3-CBA/l) separately, in conical flasks on a rotary shaker (150 rev/min) at 30°C. After reaching late exponential phase, which takes about 48 h, the cells were harvested under sterile conditions by centrifugation at 6,000 *g*, washed and re-suspended in small amounts of MM so as to obtain a thick cell suspension. The inoculants were added in to the experimental flasks (50 ml medium, taken in each 250 ml conical flasks), in different amounts to give a 3mT/SPC-2 ratio of

1:1, 1:2 or 2:1 (details are provided in the legends of the respective figures and in "Results and discussion"). 3-CBA and phenol were added at 2 + 2 mM, 2 + 4 mM, and 4 + 2 mM, respectively. SoPC-5 and 3mT were added at 1:1 ratio and the substrates were added at 2 + 2 mM levels. The cultures in triplicate were incubated at 30°C on a rotary shaker (150 rev/min) for the required durations. At required intervals three flasks each were removed and various analyses were carried out immediately.

Quantitative determinations

The growth of the organisms was measured turbidimetrically by determining the absorbance of the culture broth at 600 nm using a spectrophotometer (Shimadzu UV-160A).

Residual phenol in the cell-free culture broth was estimated by a 4-aminoantipyrene colorimetric method based on the procedure of Lacoste et al. [30].

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

Catechol and 3-CC in the cell-free broth were quantified by the method of Arnow [9] as follows: to 1 ml of the sample solution (appropriately diluted culture filtrate) 1 ml 0.5 N HCl was added followed by the addition of 1 ml of nitrite-molybdate reagent (10 g sodium nitrite and 10 g sodium molybdate in 100 ml distilled water) and 1 ml of 1 N NaOH, mixing well after each addition. Volume was made up to 5 ml and the absorbance at 510 nm was measured against a reagent blank in a spectrophotometer (Shimadzu UV-60A). The values were computed from a standard graph prepared using catechol or 3-CC. 3-CC was prepared by shaking 1 mM 3-CBA with an excess amount of *Pseudomonas* sp. CP4 cells, which converted 3-CBA to 3-CC stoichiometrically [10, 11]. The absorption spectrum of 3-CC was similar to that of catechol with an absorption peak at 510 nm. Equimolar concentrations of the two showed same values and hence, catechol was used routinely as a standard so as to avoid the inconsistency that might arise due to the highly unstable nature of 3-CC. Phenol that gives a faint yellow-brown color with Arnow's reagents slightly interfered with the catechol(s) estimation and hence, the values contributed by phenol present in the respective culture broths were deducted from the total and computed the actual catechol(s) concentration. However, it was not possible to obtain the concentrations of catechol and 3-CC separately by this method.

The concentration of Cl^- released from 3-CBA to the medium was estimated by the colorimetric method of Bergmann and Sanik [14] based on the principle of displacement of thiocyanate ion from mercuric thiocyanate by chloride ion, in the presence of ferric ion, which was first described by Iwasaki et al. [26]. To 2 ml of appropriately diluted culture filtrate 0.2 ml of 0.25 M ferric ammonium sulfate solution in 9 M HNO_3 and 0.2 ml of saturated solution of mercuric thiocyanate in ethanol were added and mixed well. Allowed to stand for 10 min and absorbance was measured against a distilled water blank at 460 nm in a spectrophotometer (Shimadzu UV-160A). Chloride concentration was computed from a standard plot prepared using NaCl.

Results and discussion

A *P. stutzeri* strain SPC-2 that degrades phenol (1,200 ppm) through ortho-pathway was reported by Ahamad and Kunhi [1]. *Pseudomonas* sp. strain SoPC-5, another laboratory isolate could degrade up to 1,000 ppm of phenol through meta-fission pathway [2]. Both these strains cannot utilize chloroaromatics, including 3-CBA as carbon sources. In the present study, it was intended to see whether *P. aeruginosa* 3mT, a potent 3-CBA-degrader that follows a modified ortho-pathway [6] and *P. stutzeri* SPC-2, a phenol degrader that also utilizes an ortho-pathway could be better suited as partners in a mixed culture for simultaneous degradation of 3-CBA and phenol, as compared to a combination of strains 3mT and CP4, reported earlier [10]. A combination of strain 3mT and *Pseudomonas* sp. SoPC-5 also was tested for its efficiency. Both these combinations were also tested for their ability to degrade either phenol or 3-CBA when they were present alone.

Degradation of phenol by mixed cultures

Degradation of 2 mM phenol by mixed cultures 3mT/SPC-2 and 3mT/SoPC-5 was tested by inoculating with 0.15 mg cell dry wt of each strain. Although 3mT cannot degrade phenol as such it can very well utilize catechol, the intermediary metabolite of phenol catabolism [6]. Hence, it was intended to see the interaction of 3mT with the phenol-degrading SPC-2 and SoPC-5. Growth of both the mixed cultures was almost similar on phenol, though the initial lag was shorter (6 h) in the former than in the latter (12 h) (Fig. 1a, b). Growth in both cases increased with time up to 48 h. Phenol disappeared in both the cases by 24 h. By 3 h, 50 and 67% of phenol disappeared in flasks containing 3mT/SPC-2 (Fig. 1a) and 3mT/SoPC-5 (Fig. 1b), respectively, indicating that the early induction of the first enzyme, facilitating the early disappearance of a good quantity of phenol. *P. stutzeri* SPC-2 that uses an ortho-pathway for the degradation of phenol has been catabolically compatible with 3-CBA-utilizing 3mT. Catechol was detected in the medium, the concentration of which increased gradually in both the cases reaching a maximum

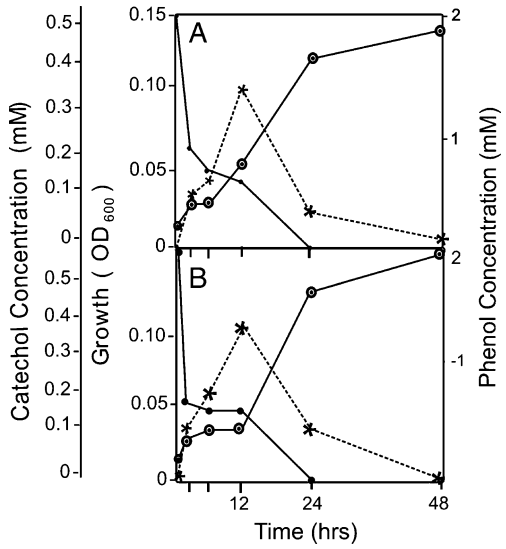

Fig. 1 Degradation of phenol (2 mM) by mixed cultures of **a** *P. aeruginosa* 3mT + *P. stutzeri* SPC-2 and **b** *P. aeruginosa* 3mT + *Pseudomonas* sp. SoPC-5. To 50 ml medium taken in 250 ml conical flasks were added induced cells of the respective strains at 0.15 cell dry wt each. Other details are as given in "Methods" [*line with bulleted circle* growth (OD_{600}), *line with closed circle* residual phenol, *dotted line with star* catechol]

level of about 0.35 and 0.4 mM at 24 h in 3mT/SPC-2 (Fig. 1a) and 3mT/SoPC-5 (Fig. 1b), respectively. Even after the complete disappearance of phenol at 24 h about 0.1 mM of catechol was detected in both the cultures. But, the medium turned slightly brownish in the case of 3mT/SoPC-5 indicating the accumulation and auto-oxidation of catechol. Hence, the zero value of catechol obtained at 48 h cannot be the correct value as the auto-oxidized and polymerized form of catechol does not react with Arnow's reagent. Auto-oxidation of catechols and formation of black/brown pigments of the polymerized products have been reported by several workers [10, 12, 22, 42]. It could be observed in the case of 3mT/SoPC-5 that catechol increased while the growth (OD_{600}) and phenol concentration remained constant between 6 and 12 h (Fig. 1b). This may be because the induction levels of the respective dioxygenase, i.e., C12D in SPC-2 and C23D in SoPC-5 may be different, the former being higher, whereas the levels of phenol oxidase, the first enzyme of the pathway may be similar in both. However, this cannot be ascertained in the absence of enzymological data. The culture broth of 3mT/SPC-2 did not show any brownish coloration indicating complete degradation of catechol within 48 h.

Degradation of 3-CBA by mixed cultures

Both the mixed cultures (3mT/SPC-2 and 3mT/SoPC-5) were inoculated with 0.15 mg cell dry wt levels in to the medium containing 2 mM 3-CBA. The growth of mixed culture 3mT/SPC-2 on 3-CBA was very low (almost half) (Fig. 2a) as compared to its growth on phenol (Fig. 1a). Same is true with 3mT/SoPC-5 (Fig. 2b). Growth did not pick up till 12 h of incubation in both the combinations of strains. Similar growth pattern was observed earlier also when mixed cultures of 3mT/CP4 was used, the biomass build up of 3mT on 3-CBA being far less than that of CP4 on equimolar concentration of phenol [10]. The initial steep drop in the substrate concentrations at early hours of incubation in all the cases (Fig. 1 through 5) may also be due to the adsorption or biosorption of the substrates to the bacterial cells. Such a phenomenon of reaching the adsorption/biosorption equilibrium at early hours of incubation has been well established [7]. In the case of Fig. 2b also the decrease in 3-CBA, without consequent increase in 3-CC and OD_{600} may be due to an enhanced adsorption of 3-CBA to strain SoPC-5 cells making it unavailable for strain 3mT to act upon, whereas the adsorption of 3-CBA to SPC-2 may not be as pronounced as that to SoPC-5. However, further studies are required before any conclusion could be drawn. 3-CBA did not disappear completely in cultures with both the combinations. Small amounts, viz., 0.04 and 0.063 mM of the substrate were still found even after 48 h incubation in the cases of 3mT/SPC-2 and 3mT/SoPC-5, respectively (Fig. 2a, b). The disappearance of 3-CBA with both the combinations of strains was rather slow in contrast to that of phenol (Fig. 1a, b). A maximum concentration of 3-CC was observed when the 3-CBA concentration was only 0.047 mM at 24 h of growth of 3mT/SPC-2 and it decreased by 48 h (Fig. 2a). A 3-CC concentration of about 0.12 mM and a residual 3-CBA concentration of 0.076 mM at 24 h remained without any change up to 48 h of growth of 3mT/SoPC-5. The strain 3mT has been shown to be a very efficient degrader of 3-CBA through 3-CC formation, the turnover rate of 3-CC being high resulting in no accumulation of 3-CC in the medium at any time [6]. However, in the present case when strain 3mT was used along with the phenol degrader SPC-2 or SoPC-5 accumulation of 3-CC occurred. This may be because of the low inoculum of 3mT used in this experiment.

Degradation of equimolar mixture of phenol and 3-CBA by mixed cultures

The growth patterns of the mixed cultures of both 3mT/SPC-2 and 3mT/SoPC-5 were almost similar with 2 mM each of phenol and 3-CBA when the inoculum size of each strain was 0.15 mg cell dry wt. Growth increased steadily after a lag which extended up to 24 h (Fig. 3a, b). It could be noted that the growth (OD_{600}) after 24 h on phenol alone (Fig. 1a, b) was much more than that on phenol+3-CBA (Fig. 3a, b), while the growth on 3-CBA (Fig. 2a, b) was almost same as that on the mixed substrate (Fig. 3a, b). The

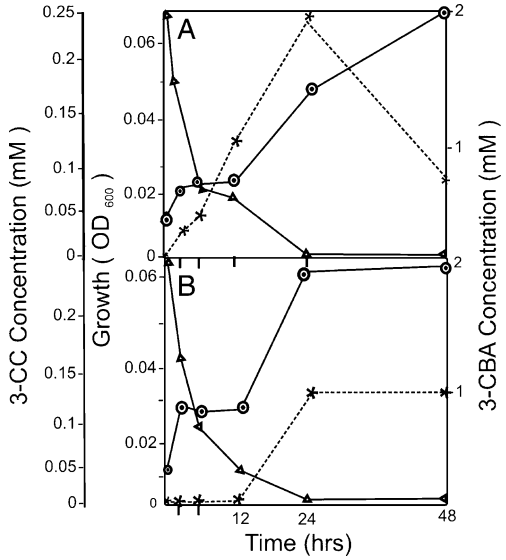

Fig. 2 Degradation of 3-CBA (2 mM) by mixed cultures of **a** *P. aeruginosa* 3mT + *P. stutzeri* SPC-2 and **b** *P. aeruginosa* 3mT + *Pseudomonas* sp. SoPC-5. Other details are as in the legends of Fig. 1 [*line with open triangle* residual 3-CBA, *line with bulleted circle* growth (OD_{600}), *dotted line with star* 3-CC]

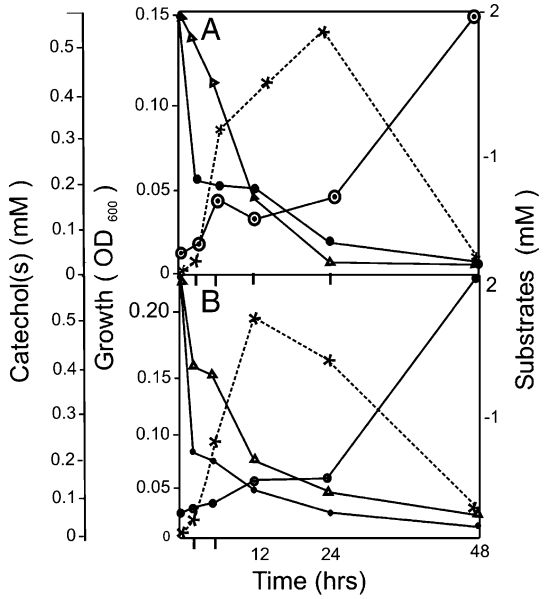

Fig. 3 Degradation of equimolar mixtures of phenol and 3-CBA (2 mM each) by mixed cultures of **a** *P. aeruginosa* 3mT + *P. stutzeri* SPC-2 and **b** *P. aeruginosa* 3mT + *Pseudomonas* sp. SoPC-5 (*dotted line with star* catechol(s). Other details and the symbols used are as given in the legends to Figs. 1 and 2

biomass build up of strain 3mT on 3-CBA has been shown to be low, even in our earlier experiments as compared to the growth of the phenol-degraders on equivalent amount of phenol [1, 2, 10, 11]. Moreover, in the present case, the sum total of growth was less in both the cases (Figs. 1, 3). The slow pick up of growth and low biomass build up may not be because the inoculums was not properly induced before adding to the medium, as could be suspected, since the cells were pre-grown to late exponential phase on their respective substrates. It may be because of the lower inoculum levels. The cells may not, probably, be able to withstand the substrate concentrations used. However, with higher inoculum levels (Figs. 4, 5) the growth picked up early and more biomass was formed.

In the mixed culture 3mT/SPC-2, the disappearance of phenol took 48 h (Fig. 3a) as compared to 24 h when phenol was used as a single carbon source (Fig. 1a). Even though, there was an early drop in phenol concentration at 3 h of incubation, (64% had disappeared), it became slower with time and completely disappeared only by 48 h. Almost similar pattern was observed with the mixed culture 3mT/SoPC5, which also needed 48 h for complete removal of phenol (Fig. 3b). Degradation of both the compounds occurred at a faster rate by the culture containing 3mT and SPC-2 as compared to that with 3mT and SoPC-5. In both the combinations of organisms, 3-CBA did not completely disappear even by 48 h. With increase in growth, the catechol(s), although apparently disappeared, it may not be the correct value, as it was not possible to estimate catechol(s) after they got auto-oxidized and polymerized as indicated by a slight brown coloration of the medium [22, 42]. However, the accumulation of catechol(s) in the medium was not as much as that was seen in the medium containing 3-CBA alone (Fig. 2a, b). The brown coloration of the medium was very much less in the case of 3mT/SPC-2 as compared to that with 3mT/SoPC-5. Whether it is due to the fact that in the former case both the strains follow an ortho-pathway cannot be ascertained without further enzymological studies. In our earlier studies with the mixed culture 3mT/CP4 with equimolar concentrations of 3-CBA and phenol no accumulation of catechol(s) was observed [10]. In that case higher levels of inocula (1.5 mg cell dry wt of each strain per 50 ml medium) was used, whereas in the present case only one tenth of it was added. That could be the main reason for the slow degradation as well as a slight accumulation of catechol(s). In the later studies with strains 3mT and SPC-2 higher inoculum levels were used. The mixed culture 3mT/SoPC-5 was not taken for further studies as it was found to be inferior to 3mT/SPC-2 in the degradation of 3-CBA and phenol alone and in combination.

Effect of varying concentrations of phenol and 3-CBA on their degradation by mixed culture

In this experiment, the mixed culture 3mT/SPC-2 was used. The concentration of the substrates phenol and 3-CBA was varied in such a way as to get a ratio of 1:1, 1:2, and 2:1, i.e., 2 + 2, 2 + 4, and 4 + 2 mM of phenol and 3-CBA, respectively in 50 ml medium. Each flask was inoculated

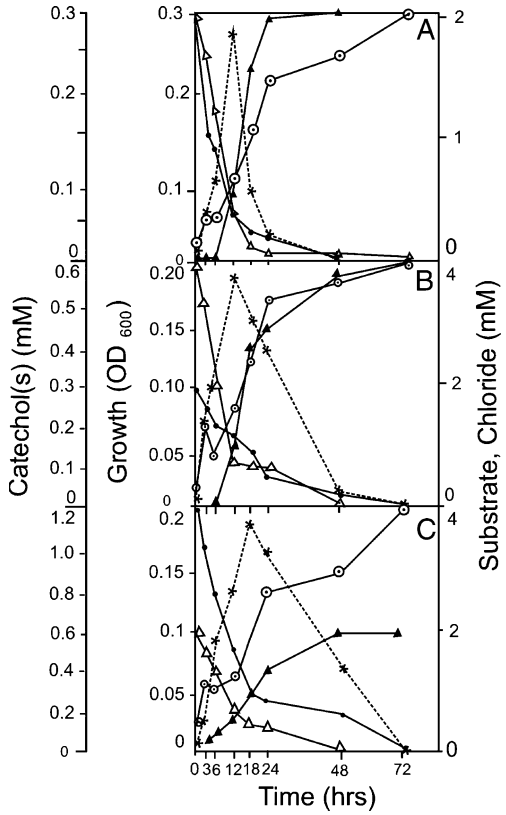

Fig. 4 Effect of varying concentrations of phenol and 3-CBA viz. **a** 2 + 2 mM, **b** 2 + 4 mM, and **c** 4 + 2 mM, respectively on their degradation by the mixed cultures of *P. aeruginosa* 3mT + *P. stutzeri* SPC-2 contains of inoculums of 0.3 mg dry cell wt of each strains (*line with closed triangle* chloride released). Other details and symbols are as in Figs. 1, 2, and 3

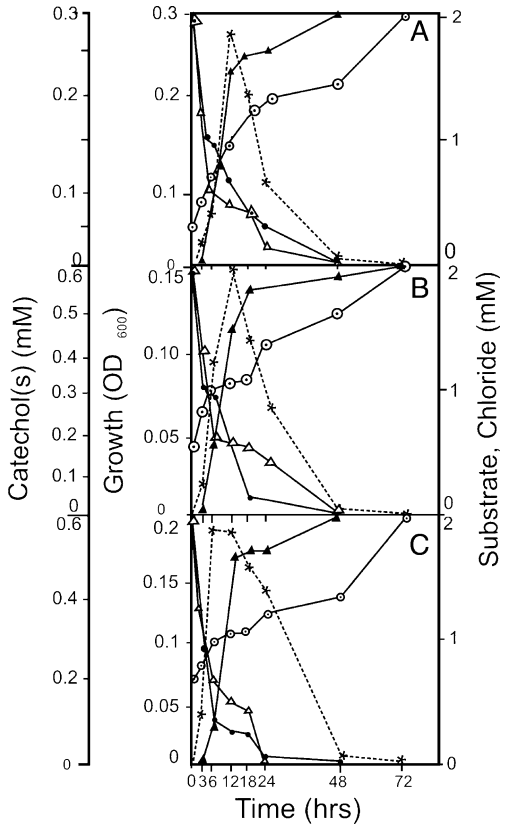

Fig. 5 Effect of varying innoculums ratio of *P. aeruginosa* 3mT and *P. stutzeri* SPC-2 at 1:1, 1:2, and 2:1 viz. **a** 0.3 + 0.3, **b** 0.3 + 0.6, and **c** 0.6 + 0.3 mg dry cell wt, respectively on the degradation of equimolar mixture (2 mM each) of phenol and 3-CBA. Other details and symbols are as in Figs. 1, 2, 3, and 4

with induced biomass of SPC-2 and 3mT equivalent of 0.3 mg cell dry wt each. The cultures with all three substrate ratios showed a short lag of about 6 h and then the growth increased steadily up to 72 h. With 1:1 ratio a growth of OD_{600} of 0.3 was obtained (Fig. 4a) which was double as much that was observed in the previous experiment where the inoculum size was half (Fig. 3a). When the concentration of 3-CBA was doubled, i.e., the phenol/3-CBA ratio was kept at 1:2 (2 + 4 mM) the growth was comparatively less (OD_{600} = 0.2) (Fig. 4b) than that with the substrate ratio of 1:1 (2 + 2 mM) (OD_{600} = 0.3) (Fig. 4a) though the total substrate concentration was more. When the concentration of phenol was double that of 3-CBA (i.e., phenol/3-CBA ratio of 2:1) (Fig. 4c), also the growth was less (OD_{600} of 0.2). In the case of the substrate ratio of 1:1 phenol was degraded completely by 48 h and more than 96% of 3-CBA also disappeared during that period, the complete degradation occurring by 72 h (Fig. 4a). When the concentration of 3-CBA was double, i.e., the phenol/3-CBA ratio was 1:2 (2 + 4 mM) more than 95% of 3-CBA disappeared within 48 h as against the expectations (Fig. 4b). However, the degradation of phenol was slower in this case as compared to that with a ratio of 1:1 though in both these cases phenol concentration was 2 mM. Complete disappearance of phenol occurred at 72 h. Similarly, with a phenol/3-CBA ratio of 2:1 (i.e., 4 + 2 mM) phenol disappeared completely by 72 h (Fig. 4c), though the concentration was higher than that of the two previous cases.

With 2 mM phenol and 4 mM 3-CBA or 4 mM phenol and 2 mM 3CBA, relatively high concentrations of catechol(s) (up to about 0.55 and 1.1 mM, respectively) were observed at 12 h, probably, due to the availability of more substrates. Though the concentration of catechol(s) receded drastically thereafter, a slight accumulation, as indicated by brown coloration of medium due to auto-oxidation and polymerization, was observed where concentration of phenol was double. In contrast to this, in our earlier study with the mixed culture of 3mT/CP4, a phenol/3-CBA concentration of 2 + 4 mM turned brown while with the ratio of 4 mM: 2 mM was colorless [10]. This was because of the reason that the strain CP4 was capable of converting 3-CBA in to 3-CC, thus enhancing the release of 3-CC which probably could not be handled by strain 3mT.

In all, the three substrate combinations stoichiometric amounts of Cl^- was released indicating the complete degradation of 3-CBA. With the phenol/3-CBA ratio of 1:1 and 2:1 100% Cl^- was released within 48 h, whereas in the case of 1:2 it took 72 h, though more than 95% was released in 48 h. Hence, it is evident that the catechol that got accumulated in the flasks containing phenol/3-CBA at 2:1 ratio was actually due to incomplete degradation of phenol, may be because of insufficient SPC-2 inoculum. On the contrary, in the case of simultaneous degradation of phenol/3-CBA at 1:2 ratio by mixed culture of 3mT/CP4 the brown coloration was shown to be due to the accumulation of 3-CC, as only 46% Cl^- release was observed [10]. It also points to the importance of using strains that do not interfere with each other's substrate, like in the present case. All these data also highlight the importance of using the right proportions of chloroaromatic and non-chloroaromatic compounds if both are to be effectively eliminated. Schmidt et al. [38] reported an effective degradation of a mixture of 4-chlorophenol and phenol by a mixed culture only when they were present at 1 mM: 5 mM ratio. Babu et al. [10] obtained an effective degradation of 3-CBA and phenol by mixed culture 3mT/CP4 only when the concentration of 3-CBA was equal in amount to or less than that of phenol. However, in the present case, the two substrates were degraded effectively when present in a phenol/3-CBA ratio of 1:1 and 1:2 and when the inocula of the two strains were in equal amounts. This seems to be a more advantageous situation.

Effect of inoculum ratio on the degradation of phenol and 3-CBA

Effect of the inoculum ratio of the two strains was studied by adding, in to 50 ml medium containing 2 mM each of phenol and 3-CBA, the induced cell biomass at varying amounts in such a way as to obtain 3mT/SPC-2 ratios of 1:1, 1:2, and 2:1, i.e., 0.3: 0.3, 0.3: 0.6, and 0.6: 0.3 mg cell dry wt of 3mT and SPC-2, respectively. In all the three cases, there was no apparent lag, but a better growth (OD_{600} of 0.3 at 72 h) was observed when the inoculums ratio was 1:1 (Fig. 5a). At the same period, the OD_{600} of 3mT/SPC-2 ratio of 1:2 and 2:1 were 0.15 and 0.2, respectively (Fig. 5b, c). The complete disappearance of both the substrates in all the three cases was observed at 48 h (Fig. 5a, b, c), though the rate of degradation of phenol was higher and degradation rate of 3-CBA was slightly lower in the flasks with double the amount of SPC-2 cells (Fig. 5b). Higher amounts of catechol(s) were detected after 12 h in flasks containing 3mT/SPC-2 at 1:2 and 2:1 ratios (about 0.6 mM each, probably because of the enhanced turnover rate in the presence of higher cell biomass), whereas it was almost half in the flasks with equal amounts of inocula. Stoichiometric amounts of Cl^- was released within 48 h in the cases of equal amounts of inocula and double the amount of 3mT (Fig. 5a, c). Where the inoculum of SPC-2 was double it was about 95%, complete release taking place at 72 h (Fig. 5b). This could be due to the higher production of catechol from phenol, because of the presence of higher amounts of SPC-2 cells, which can be metabolized by 3mT also [6], thus diluting its C12D activity. However, the hindrance was not as pronounced as what happens when double the amount of phenol was present in the medium, where accumulation of catechol occurred (Fig. 4b).

In the present case, the phenol degrading strain SPC-2 [1] though not as efficient in degrading phenol as strain CP4 [11], its other attributes such as its non-interference with the co-substrate 3-CBA makes it better suited to be a partner with the very efficient chlorobenzoate-degrader strain 3mT. A higher inoculum of SPC-2 provided a better degradation rate of phenol. This is in contrast to what was observed in the case of 3mT/CP4 combination, where double the amount of the phenol degrader CP4 actually hindered the degradation of 3-CBA [10]. The more important and a very advantageous fact in the present case is that better degradation of 3-CBA occurs even when its concentration is double than that of phenol, which again is in contrast to the earlier observation with 3mT/CP4, where not even half the amount of the 4 mM 3-CBA was mineralized [10].

Elimination of aromatic compounds from industrial effluents and sewage by microbial degradation usually is not very effective because of the chemical heterogeneity that leads to biochemical incompatibility of native microorganisms. The intermediary metabolite, chlorocatechol of the chlorobenzoate degradation pathway irreversibly inhibit meta-cleaving enzyme, C23D and also decelerate the reaction of the ortho-pathway enzyme, pyrocatechase II [12, 19, 29]. Hence, simultaneous degradation of chlorobenzoates and phenol is usually not very effective. However, mixed cultures containing strains that can degrade one or the other of the mixed substrates have been shown to be effective in eliminating both the chemicals, but only when the substrates are present in such ratios as the concentration of the chloroaromatic compound is equal or less than the non-chloroaromatic co-substrate [10, 37, 38]. In studies of competitiveness between a strain possessing both ortho-and meta-pathways viz. *P. putida* WR201 and strains that degraded methyl- and chloro-substituted benzoates exclusively via ortho-pathway viz. *Pseudomonas* sp. D7-4 and *Pseudomonas* sp. B13 FR1(pFRC20P), the former was outcompeted even by an acetate-pre-grown *Pseudomonas* D7-4 in a batch culture, even when 3-methyl and 3-chlorobenzoate were present in 1:1 mixture [21]. Even in chemostat cultures, a low dilution rate was required for the culture to be competent. Simultaneous degradation of 3-CBA/phenol mixture by the mixed culture 3mT/SPC-2 occurred more

efficiently even when the more toxic 3-CBA was in higher concentrations. Whether it is due to the fact that the phenol-degrader SPC-2 also follows an ortho-pathway as the 3-CBA-degrader 3mT cannot be ascertained unless detailed enzymological studies are carried out.

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