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Kinetics of simultaneous degradation of 4-bromophenol and 4-chlorophenol by *Arthrobacter chlorophenolicus*A6

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ABSTRACT
Biodegradation of p-bromophenol (4-BP) along with p-chlorophenol (4-CP) was investigated in batch shake flasks using a actinomycetes strain of *Arthrobacterchlorophenolicus* A6. A Two level full factorial design at two different levels (low and high) was applied to perform the biodegradation of 4-CP and 4-BP in the mixed substrate system. Result reveals that the presence of 4-CP in low concentration range in the mixture (20–60 mgl^{-1}) did not inhibit 4-BP biodegradation by the actinomycetes. However, at high concentration range of 4-CP (100-200 mgl^{-1}), the 4-BP biodegradation was inhibited. Further, 4-BP degradation was faster than 4-CP. In order to study variation in the specific degradation rate of these pollutants and their interaction effect exist between them, the experimental data were fitted to a sum kinetics model. The experimental data have been fitted with the model with a high correlation coefficient value (R^2> 0.94). The model fitted data reveals a strong negative interaction effect of 4-CP on biodegradation of 4-BP by the *A. chlorophenolicus*A6.
Keywords:*4-bromophenol; 4-chlorophenol; Arthrobacterchlorophenolicus A6; biodegradation; substrate inhibition kinetics.*

1. INTRODUCTION
The presence of halogen substituted groups in phenol (chloro and bromo) enhances its toxicity effect and causes significant human health hazard and environmental damage owing to its carcinogenic and persistence nature [1 2,3]. The lethal concentration 50 of chlorophenol is in the range of 3–4 mgl^{-1} [4] Therefore, both the chlorophenol and bromophenol are listed as priority pollutants by the U.S. EPA [5]. There are many industries associated with discharge 4-CP and 4-BP into the receiving environment such as; pharmaceutical, phosphorganic insecticides, pesticides, paper pulp and combustion of leaded petrol [6,7]. Chloro-phenols are also produced during the disinfection of water and bleaching of pulp by chlorine gas. Thus, it is very crucial to treat these phenolic contaminated wastewater before releasing into the receiving environment. Several methods have been reported in the degradation of phenolic pollutants from contaminated wastewater, for instance; adsorption, volatilization, advanced oxidation, photo-catalytic degradation electrochemical methods [8,9,10,11,12]. However, in complete removal, generation of toxic intermediate products, high cost and low efficiency are the major drawback of these conventional techniques. Therefore, the eco-friendly biodegradation method emerges as the most promising technology for removal of toxic phenolic compounds from the contaminated environment. Although the halogen substituents on phenol enhance the resistance of the aromatic ring against biodegradation [13], however, many bacteria have the ability to degrade 4-CP and 4-BP [3,14,15,16]. In the recent few decades, several microorganism species have been reported to degrade 4-CP and 4-BP successfully as sole source of carbon and energy such as; *Flavobacterium*, *Moraxella*, *Nocardia*, *Acaligenes*, *Pseudomonas, Ochrobactrum*, and *Arthrobacter species* [15,16]. Among these microorganisms, actinomycetes found to be the most promising candidates in degradation of substituted phenol due to its ability to secrete both extracellular and intracellular enzymes. Westerberg et al., [17] demonstrated the ability of *A. chlorophenolicus*A6 in degradation of a wide variety of toxic substituted phenols in batch shake flasks culture. Though aerobic biodegradation of chlorinated phenol has been widely reported in the literature however, most of the cases it is limited to a single substrate system and very rarely in a mixed substrate system [18]. This is very important because wastewater generally contaminated with a mixture of different pollutants rather than single. However, in mixed substrate system competition among substrate as well as crossed inhibition particularly co-metabolic degradation, synergistic and antagonistic interaction could severely affect the toxicity profile of the pollutants on the microbial cells and consequently on the microbial growth and biodegradation rates [9]. Further, these phenomenons are more prominent when the pollutants are structurally related like 4-CP and 4-BP. For instance; interference in the catabolite repression, induction of enzyme substrate competition for its active sites and toxicity effect of the co-substrate and generation intermediate products are commonly occurs consequently alter the biodegradation efficiency of the microorganism. All these aspects necessitate investigation on biodegradation of halogenated phenol in mixed substrate system (chloro and bromo), which are generally the major constituents of pesticide, paper pulp, fungicide industrial effluent [6,19,20]. However, biodegradation of a mixture of halogenated phenol is very scanty in literature. In microbial treatment, knowledge of biomass growth and kinetics of pollutant degradation plays a vital role in the prediction of industrial wastewater effluent quality. Further, bio-kinetic parameters immensely facilitate in optimization of the operational conditions

of the bioreactor to meet the environmental discharge limits [21]. Statistically valid two level full factorial design of experiments were effectively applied to perform experimental investigations for the simultaneous biodegradation of 4-CP and 4-BP by the *A. chlorophenolicus*A6. The statistical based factorial design of experiment offers better significant information on the main and interaction effects among these factors involved in biodegradation of halogenated phenol [22]. Moreover, such type of statistical technique offers the added advantage in the form of a reduced number of experiments to be performed. Therefore, in the present study, biomass growth and substrate degradation kinetics of *A. chlorophenolicus* A6 for biodegradation of 4-CP and 4-BP in mixed substrate system were performed to improve the simultaneous biodegradation of these halogenated phenolic compounds.

2. MATERIALS AND METHODS

2.1. Chemicals and Reagents.

Analytical grade p-chlorophenol (4-CP) and p-bromophenol (4-BP) were procured from Sigma Aldrich (Germany) and HiMedia (India) respectively. All other analytical grade reagents and chemicals employed in the present study were purchased from either Merck (India) and or HiMedia, (Mumbai, India).

2.2. Inoculum Preparation.

All the 4-CP and 4-BP biodegradation experiments ware performed using minimum salts medium (MSM) (g l^{-1}): KH_2PO_4 0.4, NH_4NO_3 0.58, K_2HPO_4 2.6, $CaCl_2$ 0.038, $MgSO_4$ 0.17 and $FeCl_3$ 0.002) with 300 mgl^{-1} 4-CP and 0.3 yeast extract as described by Sahoo et al., [23]. The over night grown seed culture was centrifuged at 8000g at 10 ºC for 15 min and then rinsed in sterile PBS solution of pH 7.5 (phosphate buffer solution). Then the *A. chlorophenolicus* A6 cells were again grown in 300 mgl^{-1} of 4-CP as sole source of carbon and energy and the centrifuged cells were rinsed in PBS solution of pH 7.5. The PBS washed cells were used as the inoculum for the biodegradation of halogenated phenolic mixture. The initial concentration of biomass in the biodegradation flasks was kept at 0.2 OD_{600nm}.

2.3. Mixed Substrate Degradation.

Batch biodegradation experiments were carried out with 4-CP and 4-BP added together in 100 ml of the previously optimized mineral salt media in 250 ml Erlenmeyer flasks. Different combinations of concentration were chosen as per the two level full factorial design in this mixed substrate degradation experiments. After inoculating with the culture, the flasks were incubated at 207 rpm and 30˚C in a rotating orbital incubator shaker [21]. During the experiment, two ml of samples were collected at regular interval of time. After analysis of biomass profile at OD_{600nm}, the samples were centrifuged at 8000 ×*g* for 10 min and the supernatants were used to estimate the remaining concentration of 4-BP and 4-CP. For choosing the concentration combinations of 4-CP and 4-BP in the mixed substrate degradation study, two different concentrations range were selected. (I) low concentration range (20 - 100 mgl^{-1} each of 4-CP and 4-BP) and (II) high concentration range (100 - 200 mgl^{-1} each). These two concentration ranges of the pollutants were selected in such a way that each represent either positive or negative effect on the specific degradation rate of the culture, 2^2 full factorial design with the two halogenated phenolic pollutants as the factors at two different levels was adopted for performing the experiment in their two concentration ranges. Table 1 represent the details of the design matrix used in the present investigation.

Table 1. Full factorial design employed for simultaneous biodegradation of 4-CP and 4-BP in the mixed substrate system.

Experimental Run No	4-CP	4-BP	Symbolic Designation
1	+1	+1	RO-1
2	-1	-1	RO-2
3	0	0	RO-3
4	0	0	
5	+1	-1	RO-5
6	-1	+1	RO-6

Zero stands for center point of the substituted phenol, –1 represent low concentration and +1 represent high concentration of phenolic compounds.

In the design, two center point replicates were also chosen to find out the experimental error. In total, six number of combinations of initial concentration of 4-CP and 4-BP were chosen for biomass growth and biodegradation of halogenated phenol in both at the low and high concentration ranges implemented in the present investigation.

2.4. Analytical Methods.

Biomass profiles of the samples were analyzed by estimating the optical density (OD_{600nm}) by employing a UV-visible spectrophotometer (Perkin Elmer U.S.A, Model lambda-45). The obtained biomass (OD_{600}) was then expressed in terms of dry cell weight by plotting a calibration curve between dry weights of biomass versus OD_{600nm}. High performance liquid chromatography (Varian prostar 210) was employed to quantify concentrations of 4-CP and 4-BP in the centrifuged supernatant samples. The analysis was carried out by using a Onsphere 5- pesticides C-18 column (Varian) with a mobile phase of methanol-water and acetic acid (50:49.1, v/v). The retention time of 4-CP is monitored at 4.8 min whereas, for 4-BP it was 6.41 min for a flow rate of 0.4 $mlmin^{-1}$ and at 28°C. The identification of these phenolic compounds was made employing a UV detector set at 280 nm.

3. RESULTS

3.1. Simultaneous Biodegradation of 4-BP and 4-CP.

The biodegradation patterns of the *A. chlorophenolicus* A6 both at low and high initial concentration ranges of these mixtures of substituted phenol are presented in Fig.1 and Fig. 2 respectively. From Fig. 1 it is clearly understood that for a particular pollutant concentration, the actinomycetes culture required more time for complete degradation of 4-CP compared to 4-BP. Further, a significant lag period was observed in degradation of 4-CP particularly when its initial concentration was at higher ranges as shown in Fig. 2.

On the other hand, no such lag period was observed for 4-BP biodegradation at its higher initial concentration. However, preferential uptake of 4-BP over 4-CP occurred for a given concentration of the substrates, which may be due to their bioavailability to the *A. chlorophenolicus* A6. This preferential

uptake of halogenated phenol was detected both in its low and high concentration range of the halogenated phenol.

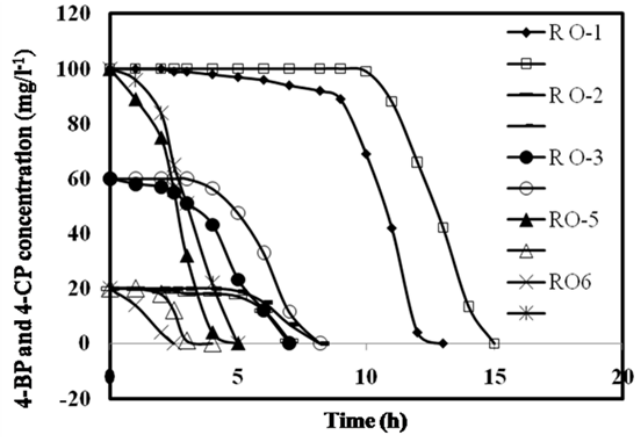

Figure 1. Biodegradation profile of *A. chlorophenolicus* A6 at low initial concentration ranges of 4-CP and 4-BP. Dark legend denote 4-BP and light for 4-CP.

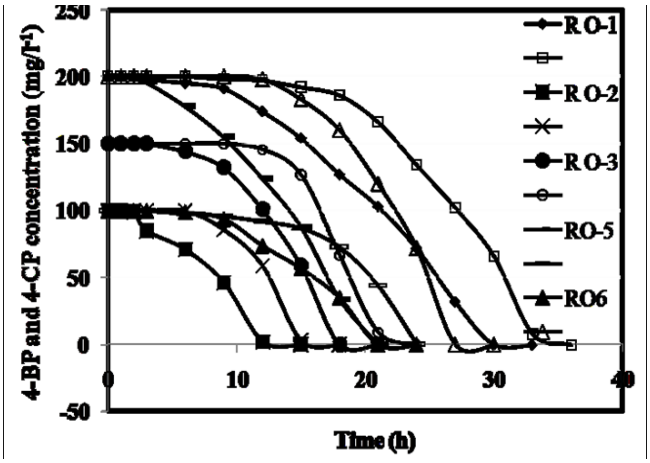

Figure 2. Biodegradation profiles of *A. chlorophenolicus* A6 at high initial concentration ranges of 4-CP and 4-BP.

The reason for slower degradation rates of substituted phenol in a mixed substrate system compared to single substrate system [21] may be due to enhanced toxicity effect exerted on the microbial cells by the phenolic mixture. For instance, phenolic compounds exert their toxicity by uncoupling the mitrochondrial oxidative phosphorylation and the uncoupling activity is enhanced by formation of dimmers between two differently halogenated phenols [24]. In addition, formation of different intermediates in a mixed substrate degradation system may inhibit enzymes vital for degradation, or its active binding site. The abiotic loss of the phenolic compounds was also evaluated by performing control experiments without the microorganism and was found negligible.

3.2. Biomass yield and specific degradation rate profile of *A. chlorophenolicus* A6 in degradation of mixture of halogenated phenol.

The experimental data on biodegradation of 4-CP and 4-BP at different combinations of initial concentration were used for estimation of biomass yield of the culture and specific degradation rates of the substituted phenolic mixture as given below.

$$Y_{X/S} = \frac{X_F - X_0}{S_0 - S_F} \quad (1)$$

$$q = -\frac{1}{x}\frac{ds}{dt} \quad (2)$$

Where, q represents thespecific degradation rate of the halogenated phenol, the biomass concentration of the culture at time t (h) is denoted by X (mgl^{-1}), $Y_{X/S}$ stands for the biomass yield of the microorganism. X_0 and X_F represent the initial and final dry weight of the microbial culture respectively. Similarly, S_0 and S_F represent the corresponding initial and final concentrations of substituted phenol (4-CP + 4-BP). The biomass yield profile obtained in the present study at different concentrations combination ranges of the substituted phenolic mixture is presented in Table 2. From Table 2 it is observed that values of biomass yield are found to be higher particularly at low initial concentration combinations of these substituted phenols.

Table 2 Biomass yield profile of the culture in degradation of 4-CP and 4-BP in mixed substrate system.

Initial Conc. (High Conc. Range)			Initial Conc. (Low Conc. Range)		
4-CP (mgl^{-1})	**4-BP (mgl^{-1})**	**Biomass Yield (g/g)**	**4-CP (mgl^{-1})**	**4-BP (mgl^{-1})**	**Biomass Yield (g/g)**
100	100	0.257	20	20	0.13
100	150	0.18	20	60	0.169
100	200	0.15	20	100	0.201
150	100	0.176	60	20	0.19
150	150	0.17	60	60	0.21
150	200	0.15	60	100	0.219
200	100	0.17	100	20	0.245
200	150	0.15	100	60	0.237
200	200	0.11	100	100	0.26

Abuhamed et al., [25] reported a maximum biomass yield of 0.75 gg^{-1} by *Pseudomonas putida* strain at an initial benzene concentration of 700 mgl^{-1}. Simmilarly, Wilson and Kim, (26) reported a yield coefficient value of 0.67 g-COD g-COD^{-1} for aerobic microorganisms. In the present investigation, the maximum biomass yield value of 0.2607 gg^{-1} was obtained at a low concentrations combination of 100 mgl^{-1} each of 4-CP 4-BP; the least value of 0.1127 was obtained at a combination of 200 mgl^{-1} each of these pollutants. These values clearly indicated that both 4-CP and 4-BP concentrations influence the biomass yield in the experiments. Further, it was observed that high concentrations combination reduced the culture biomass yield. The specific degradation rate of the substituted phenolic mixture (*q)* for both 4-CP and 4-BP is shown in Table 3. From the table it is clearly understood that at higher concentration range combinations of these pollutants, 4-BP specific degradation rates are always higher than that of the specific degradation rate of 4-CP except in run order 1 and 6.

Table 3 Specific degradation rates of 4-CP and 4-BP in mixed substrate system by the *A. chlorophenolicus*A6.

Run No	Initial Conc. Level		4-CP Specific Degradation Rate (h^{-1})		4-BP Specific Degradation Rate (h^{-1})	
	4-CP	4-BP	Low Range	High Range	Low Range	High Range
1	+1	+1	0.8	0.13	0.79	0.08
2	-1	-1	0.98	0.65	0.66	0.79
3	0	0	0.53	0.32	0.60	0.35
4	0	0	0.54	0.3	0.58	0.32
5	+1	+1	0.55	0.39	0.77	0.45
6	-1	-1	0.77	0.4	0.89	0.39

Moreover, high values of q were obtained in the low concentration range of the substrates. However, in the low concentration combination ranges of the substituted phenolic mixture, the values

of specific degradation rate for both 4-CP and 4-BP varied largely with the initial concentration combination of the pollutants. The experimental error in the study was also negligible as noted from Table 3, and was almost found equal q values of 4-CP and 4-BP at experiments performed at their center point levels (0).

3.3. Sum kinetics model fitting to the specific degradation rates of 4-BP and 4-CP.

In order to assess the interaction effects exist between the two substituted phenolic mixtures on their individual specific degradation rates, the experimental data obtained in the present study were fitted to a sum kinetic model as described by Abuhamed et al., [25], where the specific growth rate (μ) was substituted with specific degradation rate (*q*) as shownin Eq. (3). This model has been applied to find out the interaction effects between the halogenated phenolic compounds in the biodegradation process.

$$q = \frac{q_{\max 1} S_{1L}}{K_{S,1} + S_{1L} + \frac{S^2_{1L}}{K_{1i}} + I_{2,1} S_{2L}} + \frac{q_{\max 2} S_{2L}}{K_{S,2} + S_{2L} + \frac{S^2_{2L}}{K_{21}} + I_{1,2} S_{1L}} \quad (3)$$

In the above equation, the interaction parameter $I_{1,2}$ reveals the extent of the substrate *1* affects on the biodegradation of substrate *2*. In general a large negative value of the parameter reveals the existence of strong inhibition effect of one substrate to other in their biodegradation process [25]. The other kinetic parameters half-saturation constant (K_s), maximum specific degradation rate (q_{max}), inhibition constant (K_i) in the equation are the same as reported for single substrate system [21,27]. To solve the model equation a non-linear regression technique with constraints for positive integer value of the parameters was applied using MATLAB 7. Very high determination coefficient value was achieved (R^2 = 0.94) by fitting the model equation to the obtained *q* values on biodegradation of 4-CP and 4-BP in mixed substrate system at their high concentration combination ranges. By solving the model equations the obtained interaction parameters ($I_{1,2}$) was estimated to be : $I_{1,2}$ (4-CP, 4-BP) = -0.2015 and $I_{2,1}$ (4-BP, 4-CP) = -0.087. (The interaction parameter *I 4-CP*, *4-BP* represents the effect of 4-CP on 4-BP degradation, the interaction parameter ($I_{2,1}$ *4-BP*, 4-CP) represents the effect of 4-BP on 4-CP degradation by the culture). Thus based on the estimated values of interaction effects, it is clearly understood that 4-CP showed significant inhibition of 4-BP degradation at its high concentration range. Similar interaction effects have been reported by Abuhamed et al., [25] for the effect of toluene on biodegradation of benzene by a strain of *Pseudomonas putida*. Hence, it can be interpreted that, 4-CP showed considerable inhibition or interaction on 4-BP biodegradation at their high concentration combination range.

4. CONCLUSIONS

A. chlorophenolicus A6 showed complete removal of 4-CP and 4-BP at a maximum concentration combination of 200 mgl^{-1} each. 4-CP degradation was quicker however preceded after 4-BP degradation. Halogenated phenol specific degradation rates and biomass yield profiles of the actinomycetes indicate that at higher concentration combinations of 4-CP and 4-BP inhibit the growth of the culture. The interaction effect between the two substituted phenol obtained by fitting the experimental specific degradation rates of the phenolic compounds with a sum kinetic model revealed that 4-CP showed a considerable inhibitory effect on degradation of 4-BP at higher concentration combination ranges. The culture growth, substituted phenol degradation kinetic and the interaction parameters obtained in the present study facilitate to interpret the role of the individual pollutant in the biodegradation process under a mixed substrate system.

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