Co-metabolic biodegradation of 4-bromophenol in a mixture of pollutants system by *Arthrobacter chlorophenolicus* A6

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Accepted: 19 November 2021 / Published online: 21 January 2022 © The Author(s), under exclusive licence to Springer Science+Business Media, LLC, part of Springer Nature 2021

Abstract

Brominated phenols are listed as priority pollutants together with nitrophenol and chlorophenol are the key components of paper pulp wastewater. However, the biodegradation of bromophenol in a mixed substrate system is very scanty. In the present investigation, simultaneous biodegradation kinetics of three substituted phenols 4-bromophenol (4-BP), 4-nitrophenol (4-NP), and 4-chlorophenol (4-CP) were investigated using *Arthrobacter chlorophenolicus* A6. A 2^3^ full factorial design was applied with varying 4-BP and 4-CP from 75–125 mg/L and 4-NP from 50–100 mg/L. Almost complete degradation of this mixture of substituted phenols was achieved at initial concentration combinations of 125, 125, and 100 mg/L of 4-CP, 4-BP, and 4-NP, respectively, in 68 h. Statistical analysis of the results revealed that, among the three variables, 4-NP had the most prominent influence on the degradation of both 4-CP and 4-BP, while the concentration of 4-CP had a strong negative interaction effect on the biodegradation of 4-NP. Irrespective of the concentration levels of these three substrates, 4-NP was preferentially biodegraded over 4-CP and 4-BP. Furthermore, 4-BP biodegradation rates were found to be higher than those of 4-CP, followed by 4-NP. Besides, the variation of the biomass yield coefficient of the culture was investigated at different initial concentration combinations of these substituted phenols. Although the actinomycetes consumed 4-NP at a faster rate, the biomass yield was very poor. This revealed that the microbial cells were more stressed when grown on 4-NP compared to 4-BP and 4-CP. Overall, this study revealed the potential of *A. chlorophenolicus* A6 for the degradation of 4-BP in mixed substrate systems.

Keywords *Arthrobacter chlorophenolicus* A6 · Biomass yield · 4-Bromophenol · Interaction effect · Mixed substrate · Statistical analysis

Introduction

Bromophenols are widely discharged from the effluent of different industries, such as brominated flame retardants, dyes, paints, photography, pulps, polymer and resin, pesticides, herbicides, and wood preservatives (Saeed et al. 2016; Xu et al. 2017; Li et al. 2015). Besides, the combustion of leaded petrol releases a considerable quantity of bromophenol (Sahoo et al. 2014a). The annual global production of bromophenol as flame retardant (BFRs) has been raised from 0.13 million tonnes in 2002 to over 0.2 million tonnes in 2013 (Xiong et al. 2015). The worldwide annual production of bromophenol is over 23,000 tonnes (Liang et al. 2017). The concentration of bromophenol rises to 187 mg/L in the effluent of photographic industrial wastewater (Sahoo et al. 2014a). Phenol is an extensively used chemical with increasing ecological damage, especially for aquatic creatures (Duan et al. 2017; Ruan et al. 2018). Inside living organisms, the cytochrome P450 enzyme system actively transforms phenolic pollutants, producing more dangerous electrophilic intermediates. These active metabolites have the potential to bind to and disrupt the DNA or other essential enzyme systems of living organisms, resulting in mutagenicity, carcinogenicity, and endocrine-disrupting activities, as well as causing serious environmental damage (Dayana and Bakthavatsalam 2019;
Darbre 2019; Spataro et al. 2019; Acosta et al. 2018). Owing to their acute genotoxic, hepatocytes, thyrotoxic, neurotoxic and carcinogenic properties, the United States Environmental Protection Agency (USEPA) classifies phenolic contaminants as priority pollutants (Acosta et al. 2018; Zou et al. 2016; Sharma and Dutta 2018; Liang et al. 2019; Peng et al. 2017). The toxicity of phenolics to aquatic creatures is much more likely to be a direct impact than a result of food chain transmission (Rocha et al. 2016). Oxidative stress caused by phenol has been observed in a variety of taxa, including fish (Sayed et al. 2016), microalgae (Cho et al. 2016), and yeast (Khan et al. 2015). In general, the median lethal concentration (LC50) or median effect concentration (EC50) values reveal that the phenol is virtually non-toxic to slightly toxic to algae, non-toxic to bacteria, slightly toxic to moderately toxic to fish, non-toxic to slightly toxic to rotifera, literally nearly non-toxic to highly toxic to crustaceans, and practically non-toxic to molluscs (Duane et al. 2018). It’s worth noting that phenol is amongst the most commonly found compounds in marine accidents (Cunha et al. 2015). For instance, during the explosion catastrophe on August 12, 2015, massive quantities of phenol were released into the Tianjin Port Basin (Duan et al. 2017). When large amounts of phenol are spilled in port areas or at sea, it poses a serious hazard to aquatic life. Ruan et al. (2018) evaluated the phenolics’ median effective dose (ED50) values in the sea species of E. vanus and the freshwater species Paramecium multimicronucleatum. They observed that when the time of exposure was prolonged, the ED50 of P. multimicronucleatum declined more rapidly than that of E. vanus. Another study found that after a 21-day exposure to phenolics, metabolic stress induction, branchial function, and tissue damage in Oreochromis mossambicus can occur even at very low concentrations of phenolics (1/10 96-h- LC50) (Varadarajan et al. 2014). Cirrhisimus Mnmirigala showed the highest phenol sensitivity among freshwater organisms, with a 96-h-LC50 value of 1.555 mg/L (Duan et al. 2018). Similarly, Archaeomysis kokuboi has the highest sensitivity among marine organisms, with a 96-h LC50 of 0.26 mg/L. BPP (bisphenols) has a 48 h EC50 of 1.6 mg/L, showing stronger acute toxicity against D. magna than BPA, which has an EC50 of 10 mg/L (Noszczyńska and Piotrowska-Seget 2018). Because of these factors, there is an increasing need to find technologies for removing phenolic pollutants from aquatic habitats.

Several conventional approaches for the treatment of phenolic wastewater have been studied over the last few decades, such as adsorption, Fenton reagent, electrochemical processing, ozonization, photocatalysis, and biodegradation (Sahoo et al. 2020; Eslami et al. 2018; Muntathir et al. 2019). Among these remediation techniques, biodegradation has proven to be the most promising and popular due to its eco-friendly nature, cost-effectiveness, and potential to degrade the pollutant completely without the production of toxic secondary metabolites (Jiang et al. 2016; Zhou and Nemati 2018). However, the presence of halogenated substitute groups in the phenolic ring offers resistance to microbial cleavage of the aromatic ring, which consequently inhibits its biodegradation activity (Xu et al. 2017; Li et al. 2015). Therefore, only a few microorganisms have been reported in the literature with the capability to degrade bromophenol, such as Ochrobactrum sp. T, Bacillussp. GZT, Clostridium sp. Ma13, Desulfa-tiglans parachlorophenolica DS, Dehalobacter sp. Phylotype FTH1, Sphingomonas sp. strain TTNP3, and Achromobacter piechaudii strain TBPZ (Li et al. 2015; Li et al. 2016; Liang et al. 2019; Li et al. 2015). Though anaerobic treatment of brominated phenol has been well reported in the literature, fastidious nutritional requirements, a prerequisite of the electron donor, slow growth rates, low biomass yield, and sensitivity to oxygen have been the major limiting factors of this technique (Jugder et al. 2015). Thus, the aerobic treatment of brominated phenol has proven to be a promising tool. Furthermore, bromophenol, nitrophenol, and chlorophenol are commonly found in paper pulp, pesticide, insecticide, herbicide, fungicide, chemical-synthesis plants, and plastic industrial effluent (Arya et al. 2011; Fernández et al. 2013; Panigrahy et al. 2018). However, in a mixed substrate system, the occurrence of competition for substrate and crossed inhibition could seriously influence the growth of the microorganism and the rate of pollutant biodegradation. Mostly, when the pollutants are structurally correlated, phenomena such as interference in enzyme stimulation, competition for active sites of enzymes, catabolite repression, and toxicity of the co-pollutants over and above dead-end products, usually influence the efficiency of the degradation of these contaminants. It is also quite likely that in a mixed substrate system, the culture may exhibit various other phenomena, such as interaction effects and preferential utilization of the substrate. Furthermore, when substrates inhibit microbial growth or degrade co-metabolically, the microbial population dynamics and degradation kinetics become extremely complicated. Thus, it is highly essential to study the simultaneous biodegradation of these phenolic mixtures.

Understanding the kinetics of microbial growth and biodegradation is critical for optimizing the operational process, designing a bioreactor system, and scaling it up for the prediction of effluent quality in microbial wastewater remediation processes (Panigrahy et al. 2020b). However, studies on the kinetics of bromophenol biodegradation are very limited in the literature. Though many approaches have been developed for the degradation of phenolic pollutants, in contrast, the investigations on the treatment of brominated phenols in mixed substrate systems are very few
(Sharma and Dutta 2018). Thus, in the present investigation, the potential of \textit{A. chlorophenolicus} A6 for the degradation of 4-BP in a mixed substrate system has been evaluated. Though different mechanistic models have been adopted in literature, the statistical design of experiments appears to be a more practical alternative offering clear insight into the main and interaction effects among the process variables (Mohanty and Jena 2018). Thus, a $2^3$ factorial design (FD) of experiments was applied in the present study. The applications of FD and central composite design (CCD) have been successfully applied in many fields (Farag et al. 2018; Khanpour-Alikelayeh et al. 2020). FD is a promising statistical technique to evaluate their relative significance as well as interactional effects that exist between numerous factors on the responses at diverse levels (Khatoon and Rai 2020; Elhalil et al. 2016). When compared to other techniques, FD and response surface methodology (RSM) have numerous advantages, including the ability to save time, energy, and resources while evaluating multiple parameters with a small number of trial runs (Khatoon and Rai 2020; Nam et al. 2017; Mohammed-Ridha 2019). Therefore, the specific objectives of the present investigation were to (i) study the co-metabolic degradation of 4-BP in a mixed substrate system by applying $2^3$ FD of experiments, (ii) evaluate the microbial growth, biomass yield, and pollutant degradation kinetics, and (iii) elucidate the individual functions governed by these substituted phenolics and their interaction effects on each other’s biodegradation using the actinomycetes species (i.e., simultaneous or preferential, synergistic and antagonistic).

**Materials methods**

**Chemicals and reagents**

Analytical grade 4-bromophenol (4-BP), 4-chlorophenol (4-CP), and 4-nitrophenol (4-NP) were purchased from Himedia (India). All other reagents and chemicals employed were also of analytical grade and purchased from either Merck (India) or Himedia (Mumbai, India).

**Maintenance and culture conditions of \textit{A. chlorophenolicus} A6**

In the present study, the actinomycetes strain was obtained from Prof. Janet K. Jansson, Lawrence Berkeley National Laboratory, Sweden. The \textit{A. chlorophenolicus} A6 seed culture was developed using a 0.3% yeast extract medium as described by (Sahoo et al. 2010). The seed culture cells were centrifuged (6000 g for 20 min at 22 °C) and washed in a sterilized phosphate buffer solution of pH 7.4 (PBS). Then the microbial cells were re-grown overnight in a previously optimized mineral salt medium (MSM) medium (Sahoo et al. 2010) with 300 mg/L of 4-BP as the only source of carbon.

**Modeling of phenol degradation**

The factorial design of experiments is a popular and potent tool to predict the effects of different variables and their interactions very precisely with a small number of experimental runs (Khatoon and Rai 2020; Nam et al. 2017). The 2-level FD of experiments is a prospective and broadly accepted tool for biochemical processes persuaded by multiple variables (Mohammed-Ridha 2019; Panigrahi et al. 2019). The analysis of FD can delineate which variables exhibit significant effects on the response and how the effect of one variable differs with a change in the level of other variables. A $2^3$ full FD of experiments was applied with three substituted phenols (4-BP, 4-CP, and 4-NP) as variables at two different levels. The principal effects of each factor on the biodegradation of these substituted phenols were analyzed as the variation between both average values at the higher and lower levels. The effect of each factor was computed as follows:

$$E_{(x)} = \frac{2(\sum M_{+i} - M_{-i})}{N}$$  \hspace{1cm} (1)

Where $E_{(x)}$ represents the concentration effect of the tested variable. $M_{+i}$ and $M_{-i}$ are the phenolic biodegradation efficiencies from the trials at high and low levels of the respective concerned variable ($X_i$), in different N numbers of trials. The levels $-1$, $0$, and $+1$ of these phenolic pollutants were selected based on the phenolic biodegradation profiles under a single substrate system by \textit{A. chlorophenolicus} A6 (Sahoo et al. 2014a; Sahoo et al. 2011b; Sahoo et al. 2011a). The coded values of these variables were estimated as described by Khatoon and Rai (2020) as follows:

$$X_i = \frac{U_i - U_0}{\Delta U}$$  \hspace{1cm} (2)

Where $X_i$ represents the coded level of these substituted phenols ($-1, 0, +1$). $\Delta U$ is the step change and $U_0$ is the uncoded level of these phenolic pollutants at the center point. $U_i$ represents the uncoded level of these independent parameters. The obtained experimental results were fitted to the second-order polynomial model with linear, quadratic, and interaction terms as shown below:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_i X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j$$  \hspace{1cm} (3)
Where \( Y \) represents the predicted response and \( k \) represents the number of variables. \( X_i \) and \( X_j \) represent independent variables, \( \beta_0 \) is the constant term, while \( \beta_i \), \( \beta_{ii} \), and \( \beta_{ij} \) denote the coefficients for linear, squared, and interaction terms, respectively. ANOVA and the student’s \( t \)-test were used to statistically analyze the experimental results using MINITAB statistical software (15.1 PA, USA). The statistical significance of the model terms in the polynomial model was analyzed by Fisher’s \( F \)-test. The agreement of the fit of the experimental results to the polynomial model were evaluated by the estimated values of the correlation coefficient \( (R^2) \). When the computed \( F \)-value is greater than the theoretical value, the effect of the independent factors is found to be significant (Khatoon and Rai 2020). Whereas, the lower \( P \)-value signifies the appropriateness of rejecting the null hypothesis. The significance of the tested variables was analyzed using the Student’s \( t \)-test.

The mixture of phenolics biodegradation study was planned as per \( 2^3 \) FD of experiments (Table 1). In this study, the various concentrations of these phenolic compounds (75–125 mg/L for both 4-CP and 4-BP, whereas 50–100 mg/L in the case of 4-NP) were chosen. Table 1 presents various combinations of the initial concentrations of phenolics, where \(-1 \) and \(+1 \) represent the low and high concentration levels, respectively, and zero designates for the center point value of these three variables. To determine the experimental errors, three-center point replicates were chosen in the design matrix. For the experimental set-up, the above-mentioned freshly growing actinomycete cells were centrifuged (6000 g for 20 min at 22°C) and then washed with PBS buffer (pH 7.4). The PBS-washed cells were suspended in a 250 mL Erlenmeyer flask carrying 100 mL of the previously depicted MSM at pH 7.5 with varying concentrations of substituted phenols as per the 2-level FD as shown in Table 1. The initial inoculum size in the biodegradation flasks was maintained at 0.1 OD\(_{600} \) nm. The biodegradation flasks were incubated in an orbital shaker incubator at 207 rpm and 30°C for 40 h. Samples were removed from the flasks at regular time intervals and estimated for biomass and phenolic concentrations. Results of biomass growth and pollutant biodegradation profiles were statistically examined in the terms of ANOVA and Student \( \text{'}t\text{'} \) test by employing MINITAB software (Version 12.2 PA, USA).

### Specific phenolics degradation rate and biomass yield of \( A. \) chlorophenolicus A6

The experimental data on the degradation of these three substituted phenols were employed for the determination of specific degradation rates of the phenolics, as shown below (Dey and Mukherjee 2013).

\[
q = -\frac{1}{x} \frac{ds}{dt}
\]  

Where \( q \) is the specific biodegradation rate of the substituted phenol, \( X \) represents the biomass concentration (mg/L) of the culture at time \( t \) (h). The specific degradation rate was computed based on the experimental data obtained in the exponential growth phase by constructing a semi-logarithmic plot of the substituted phenol concentration versus microbial cultivation time. The biomass yield of the microbial culture was estimated using the following equation (Panigrahy et al. 2020b):

\[
Y_{X/S} = \frac{X_F - X_0}{S_0 - S_F}
\]

Where \( Y_{X/S} \) represents the biomass yield of the actinomycetes species. \( X_0 \) and \( X_F \) stand for the initial and final dry weight of the \( A. \) chlorophenolicus A6, respectively. Likewise, \( S_0 \) and \( S_F \) stand for initial and final concentrations of phenolics, respectively.

### Analytical methods

The concentration of biomass was determined through UV-Vis spectrophotometry by estimating the optical density of the culture sample at a wavelength of 600 nm (OD\(_{600}\)) (Evolution 220, Thermo Fisher Scientific, Waltham, MAUSA). The values of optical density of the culture were expressed in terms of dry biomass weight by constructing a calibration curve plotted between optical densities (OD\(_{600}\)) versus mixed liquor suspended solids (MLSS) of the microbial culture. One absorbance unit was equivalent to 240 mg/L of MLSS. The collected culture samples were centrifuged at 6000 g followed by filtration through a Milipore syringe filter of 0.22 μm (Pall USA). Concentrations

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**Table 1** \( 2^3 \) full factorial design of experiment adopted in the mixture of phenolics degradation

| Experimental run no. | Factors and their levels |
|----------------------|-------------------------|
|                      | 4-CP        | 4-BP        | 4-NP        |
| 1                    | −           | −           | −           |
| 2                    | +           | −           | −           |
| 3                    | −           | +           | −           |
| 4                    | +           | +           | −           |
| 5                    | −           | −           | +           |
| 6                    | +           | −           | +           |
| 7                    | −           | +           | +           |
| 8                    | +           | +           | +           |
| 9                    | 0           | 0           | 0           |
| 10                   | 0           | 0           | 0           |
| 11                   | 0           | 0           | 0           |
of 4-BP, 4-NP, and 4-CP were estimated by employing a reverse phase HPLC with an Onsphere C-18 column and UV-Vis detector (Varian Prostar 210, USA) at a wavelength of 280 nm. A mixture of methanol-water and acetic acid (50:49.1 v/v/v) at a flow rate of 0.4 mL/min was used as the mobile phase in the HPLC analysis. The retention times of 4-BP, 4-NP, and 4-CP in the HPLC column were 6.41, 3.1, and 5.4 min, respectively.

Results

Simultaneous degradation profiles of substituted phenols

The biodegradation patterns of the phenolic substrates (4-BP, 4-NP, and 4-CP) in different experimental runs of $2^3$ full FD were recorded. The phenolic biodegradation patterns are depicted in Fig. 1 at low concentration ranges [Fig. 1(a), runs 1 and 5] and high concentration ranges [Fig. 1(b), runs 4 and 8]. It is observed that the microbial culture of A. chlorophenolicus A6 took a longer duration to completely degrade 4-BP and 4-CP as compared to 4-NP in all cases. A prominent lag phase was observed during 4-BP and 4-CP degradation. Further, the lag phase for 4-BP and 4-CP degradation was increased with an increase in the initial 4-NP concentration. A similar pattern of lag phase for 4-NP degradation was observed with an increase in 4-CP concentration in the culture media. 4-NP biodegradation was slowed down with an increase in the concentration of 4-CP in the culture medium. For instance, in a single substrate system, the culture took 8 h to degrade 4-NP at an initial concentration of 100 mg/L (Sahoo et al. 2011a), compared with a minimum culture period of 18 h in a mixed substrate system. Irrespective of the concentration levels of these three substrates, 4-NP was preferentially biodegraded over 4-CP and 4-BP (Fig. 1). Between 4-BP and 4-CP, 4-BP degradation was found quicker than the other. However, the difference was less significant. Further, the rates of 4-BP and 4-CP degradation were improved with a low concentration of 4-NP and towards its depletion in the culture medium. For instance, 250 mg/L of 4-BP or 4-CP takes about 16 h for complete degradation in single substrate systems (Sahoo et al. 2011b; Sahoo et al. 2014a), whereas, in mixed substrate systems, 125 mg/L 4-BP and 125 mg/L 4-CP took only 12 h in the presence of 50 mg/L 4-NP.

Biomass growth of the culture in the mixed substrate system

Biomass profiles obtained at different concentration combinations of these phenolics are presented in Fig. 2 in the form of $OD_{600nm}$ of the culture. It is clear from Fig. 2 that the actinomycetes took more time to grow when a higher
concentration of 4-NP (>100 mg/L) was present in the medium (together with 4-CP and 4-BP), and resulted in a poor biomass yield. The observation of the biomass profile of the culture in the mixture of pollutants’ system differed considerably from the patterns observed in the single substrate system (Sahoo et al. 2011b; Sahoo et al. 2011a; Sahoo et al. 2014a). This phenomenon is obvious due to a variation in the actinomycete’s accessibility of the toxic phenolic carbon sources from the culture media. These observations were in close agreement with those of Surkatti and El-Naas (2017). They reported that the biodegradation of o-cresol in wastewater was hampered by the presence of m-cresol and/or p-cresol in a mixed substrate system. Moreover, a lag phase was observed beyond certain concentration combinations of 100 mg/L for 4-BP, 4-CP, each, and 75 mg/L of 4-NP, probably due to the combined toxicity effect of the phenolics. On the other hand, as discussed earlier, 4-BP and 4-CP were degraded simultaneously. In the present study, the growth of the actinomycetes did not reveal any plateau phase in the transition point of the different substituted phenols, indicating no diauxic growth as depicted in Fig. 2. Unell et al. (2008), employed a mutant strain of A. chlorophenolicus A6 (T99) carrying a transposon in a hydroxyquinol 1, 2-dioxygenase gene (T99 mutant) which seriously impaired the microbial growth on 4-CP. They reported that the T99 mutant behaved the same way when 4-BP or 4-NP was used as the only source of carbon. This phenomenon revealed that the same enzyme system was used by the actinomycetes for biodegradation of 4-CP and thus confirmed no diauxic growth.

Statistical analysis of a mixture of substituted phenol biodegradation

Experimental results obtained on the growth of the microorganisms and phenolic degradation were used for calculating the pollutant degradation rates of these substrates in the mixture. Figure 3 shows the degradation rates of these substituted phenols at different concentration combinations (run order number) as per the $2^3$-level full FD of the experiment (Table 2). The figure reveals that the degradation rates of 4-BP and 4-CP were higher than that of 4-NP except at their lower (experimental run number-1) concentration combination (75, 75, and 50 mg/L of 4-BP, 4-CP, and 4-NP, respectively). In general, rates of 4-BP biodegradation were higher than that of 4-CP followed by 4-NP. Moreover, higher biodegradation rates of 4-CP and 4-BP were obtained in the higher concentration range of 4-NP, which might be due to the fact that high initial biomass was available during the start of 4-BP and 4-CP degradation process. Further, these values were very low when the concentration ranges of the substrates were high.

### Table 2: ANOVA of 4-BP degradation in the mixed substrate system

| Source         | Df | SS     | Adj MS  | F       | P    | R²   |
|----------------|----|--------|---------|---------|------|------|
| Main effect    | 3  | 2245.50| 748.500 | 118.18  | 0.008| 99.57|
| 2-way interaction | 3  | 681.50 | 227.167 | 35.87  | 0.027|
| 3-way interaction | 1  | 4.50   | 4.500   | 0.71   | 0.488|
| Residual error | 2  | 12.67  | 6.333   |        |      |
| Pure error     | 2  | 12.67  | 6.333   |        |      |
| Total          | 10 | 2944.17|         |        |      |

**Statistical analysis of a mixture of substituted phenol biodegradation**

**Table 2:** a ANOVA of 4-BP degradation in the mixed substrate system. b ANOVA of 4-CP degradation in the mixed substrate system. c ANOVA of 4-NP degradation in the mixed substrate system.

**Fig. 3** Degradation rates of 4-CP, 4-BP, and 4-NP mixture obtained at different initial concentration ranges of these substrates.
instance, degradation of 4-NP at a lower concentration range (75, 75, and 50 mg/L of 4-BP, 4-CP, and 4-NP, respectively) was found to be 0.139 h$^{-1}$; on the contrary, the value was 0.043 h$^{-1}$ at a higher concentration range (125, 125, 100 mg/L of 4-BP, 4-CP, and 4-NP, respectively). This finding confirmed the fact that the growth of the culture, as well as its phenolic degradation rates, was inhibited at higher concentration ranges of these pollutants.

Table 2a–c presents the results of the ANOVA of 4-CP, 4-BP, and 4-NP degradation rates in the study. From this statistical analysis, it can be seen that both the main (individual) and two-way interaction terms for these phenolic pollutants were significant in the biodegradation activity at a confidence level of greater than 97.3% (P < 0.027). On the other hand, except for the 4-NP degradation rate, the ANOVA results presented in Table 2a, b revealed that the three-way interaction term was insignificant (P > 0.05). Further, the higher values of the determination coefficient (R2 > 99) indicate that the polynomial model is highly accurate in predicting phenolic biodegradation. While the Pareto charts illustrated in Fig. 4(a), (b), and (c) revealed a significant negative (inhibitory) individual effect of 4-CP on 4-NP as well as on its degradation performance. Figure 4(c) shows that the negative main effect of 4-NP on its degradation was less than that of 4-CP. Further, the interaction effect between 4-NP and 4-CP on 4-NP biodegradation activity and that between 4-CP and 4-BP on 4-BP degradation was considerably negative than that between 4-BP and 4-CP on 4-CP degradation (Fig. 4a, b, c). To analyze the main and interaction effects that exist among these substituted phenols, a student’s t-test was executed. Table 3 represents the calculated coefficients of individual and interaction terms along with the associated t and P values. Generally, a larger t value with a smaller P-value of a variable designates the higher significance of the respective model term. The coefficient of ‘t’ value for 4-NP degradation reveals strong inhibition mainly due to 4-CP (P = 0.001), followed by 4-BP (P = 0.015). Hence, it is concluded that 4-NP degradation was considerably inhibited compared to 4-BP due to the presence of 4-CP. On the other hand, the interaction between the other two factors, viz. 4-CP and 4-BP (X1 X2) was found to be insignificant (P = 0.613) on 4-NP degradation but was negatively significant for their degradation performance (P = 0.004 and 0.017). This analysis revealed that the presence of 4-CP in the mixture significantly inhibited 4-NP, whereas the converse was incorrect (P = 0.757). Other interaction effects, except for three-way interaction effects, were found to be significant at a 95% confidence level. Similar statistical analysis and interpretation have been reported in the literature for the biodegradation of different pollutants (Mohanty and Jena 2018; Farag et al. 2018; Khanpour-Alilakeyeh et al. 2020; Nam et al. 2017; Khatoon and Rai 2020).

The calculated biomass yield values for different concentration ranges of these substituted phenols in the mixed substrate system are presented in Table 4. Despite the fact that 4-NP degraded faster than 4-BP and 4-CP, biokinetic data revealed that 4-NP had a more negative effect on microbial cells than...
the other two pollutants. Similarly, the actinomycetes degraded 4-NP faster. However, the biomass yield was poor. Further, irrespective of the different combinations of initial 4-BP, 4-CP, and 4-NP concentrations, the biomass yield increased with an increase in the initial 4-BP and 4-CP concentration at a fixed 4-NP concentration in the mixture, except in the case of experimental run number 8. The lowest biomass yield was obtained at 125, 125, 100 mg/L of 4-BP, 4-CP, and 4-NP, respectively. In this study, the highest biomass yield value of 0.2152 g g⁻¹ was obtained at a higher concentration combination of 4-CP and 4-BP with a low level of 4-NP (125 + 125 + 50 mg/L of 4-BP, 4-CP, and 4-NP, respectively). On the other hand, a low yield value of 0.1673 was achieved at the same range of 4-BP and 4-CP but with a higher 4-NP concentration of 100 mg/L. This value indicates that the concentration of 4-BP and 4-CP particularly influenced the biomass yield in the experiments.

### Discussion

Because the physical and chemical properties of these halogenated substituted phenolic compounds are nearly identical, *A. chlorophenolicus* A6 could degrade 4-CP and 4-BP by a similar pathway, namely the formation of hydroxyquinol metabolic pathway (Nordin et al. 2005). Further, *A. chlorophenolicus* A6 could degrade 4-NP, 4-BP, and 4-CP as the sole source of carbon and energy, which indicated that all these phenolic compounds could efficiently induce their corresponding degradation enzymes. This phenomenon has been well corroborated with literature reports that the enzymes associated with biodegradation of halogenated phenolic pollutants do not differentiate between brominated and chlorinated compounds (Aranda et al. 2003). However, further research is needed to confirm the pathway. Varadarajan and Philip (2016) found significant increases in malonaldehyde (MDA) concentration in the gills and liver of Mozambique tilapia (*Oreochromis mossambicus*) exposed to sub-lethal levels of m-cresol (1/10 96 h-LC50, i.e., 2.2 mg/L) and phenol for 21 days (1/1096 h-LC50, i.e., 3.12 mg/L). In the present study, almost complete degradation of this mixture of substituted phenols was achieved at an initial concentration combination of 125, 125, and 100 mg/L of 4-CP, 4-BP, and 4-NP, respectively, in 68 h, which is superior compared to many reports in the literature under batch shake flask mode. Thus, it is understood that the environmental ecotoxicity due to a mixture of substituted phenolic pollutants can be minimized by using the *A. chlorophenolicus* A6. However, *A. chlorophenolicus* A6 biodegraded phenolics at a slower rate in a mixed substrate system than in a single substrate system. This might be due to the synergistic toxicity effect of the phenolic compounds on the microbial cells. Phenolic contaminants exhibit their microbial toxicity by uncoupling mitochondrial oxidative phosphorylation (Xie et al. 2018). The formation of dimers between two dissimilar substituted phenolic pollutants further aggravated the uncoupling activity (Panigrahy et al. 2018). Further, the rate of phenolic biodegradation primarily depends on the types and position of substituents present in the phenolic ring structure (Fernández et al. 2013). The degradation of phenolics can often produce more harmful intermediate chemicals, which can increase the toxicity of live cells and tissue. The toxicity to an organism depends on the structure of the chemical and the bioavailability of the test organisms (Backhaus 2014). Besides, the formation of different toxic intermediate products, such as chlorocatechol, hydroxyquinol, and nitrocatechol might have inhibited the growth of the actinomycetes as well as enzymes vital for the biodegradation process at their higher initial concentration combinations (Sahoo et al. 2014b). Fernández et al. (2013) reported the inhibition of...
4-NP biodegradation due to the accumulation of intermediate products like hydroquinone by *Acinetobacter* and *Arthrobacter* sp. A similar finding was also reported on co-metabolic biodegradation of carbazole by *Arthrobacter* sp. W1 using phenol as a growth substrate (Shi et al. 2015). The degradation patterns of individual substrates and the toxicity of a mixture of pollutants in the mixed substrate system also differed considerably from those obtained in their respective single substrate systems. When two or three substances are combined, synergistic and antagonistic effects are more likely to occur (Backhaus 2014). Irrespective of the concentration levels of these three substrates, 4-NP was preferentially biodegraded over 4-CP and 4-BP. Similar preferential degradation of aromatic hydrocarbons and phenolic pollutants (100 mg/L) over free cyanide (>2.5 mg/L) has been reported by Sharma and Philip (2014). Unell et al. (2008) reported that although the same enzyme system is responsible for biodegradation of 4-BP, 4-CP, and 4-NP by *A. chlorophenolicus* A6, one compound is preferentially biodegraded over the other. For example, in the present case, 4-NP was degraded preferentially over others (Fig. 1). A possible reason could be the fact that the enzyme in the biodegradation pathway has a higher affinity for 4-NP than those for 4-BP and 4-CP. However, this needs further investigation. Otherwise, there could be transport-level interactions influencing the biodegradation of phenolic pollutants. This might be due to the differences in pKa values of these substituted phenols. For example, the pKa value of 4-NP is 7.1, while it is 9.3 for 4-CP and 9.17 for 4-BP. Thus, at pH 7.5 of the *A. chlorophenolicus* A6 culture medium, most of the 4-NP might have dissociated into phenolate ions which usually enter the microbial cells. On the other hand, it is comparatively less in the case of 4-BP and 4-CP. Therefore, due to the different pKa values, 4-NP is degraded preferentially over 4-CP and 4-BP. Further, concurrent biodegradation of the 4-CP and 4-BP mixture was possible due to their almost identical pKa values of 9.17 and 9.3, respectively. This observation is also well correlated with the results obtained using these individual substituted phenolic degradation systems. For example, the half-saturation constants (*K*<sub>s</sub>) for 4-CP and 4-BP were nearly the same, at 30.83 and 30.77 mg/L, respectively, while 4-NP had a lower value (20.15 mg/L) (Sahoo et al. 2011b; Sahoo et al. 2011a; Sahoo et al. 2014a). In the literature, many researchers have reported similar observations on the preferential biodegradation of one aromatic pollutant over another in mixed substrate systems, even though the same degradation pathway is followed for these compounds by different microorganisms. For example, the rate of benzene biodegradation was faster than o-xylene by fungal species, which might be because benzene is the preferred substrate in a benzene-xylene mixture (Khoramfar et al. 2020). Fu et al. (2017) have reported the simultaneous biodegradation of 3-NP, 2-NP, and 4-NP by the microbial consortium of *Cupriavidus necator* JMP134, *Pseudomonas* sp. WBC-3 and *Alcaligenes* sp. NyZ 2015, in a sequential batch reactor. Their results showed that almost complete degradation of this phenolic mixture at 0.5 mM each was attained by the microbial consortium in 84 h. Interestingly, 4-NP is preferentially degraded over 3-NP and 4-NP by *Pseudomonas* sp. WBC-3. 

Though 4-NP was biodegraded before 4-BP and 4-CP, the biokinetic data revealed that 4-NP had the most prominent influence on the degradation of both 4-CP and 4-BP. Similar negative interaction effects have been reported by many authors in the literature using different mixed substrates and microbial systems. For example, Dey and Mukherjee (2013) investigated the mixture of phenol and resorcinol biodegradation in an anaerobic batch reactor. They reported complete degradation of phenol and resorcinol at an initial concentration of 400 mg/L each within 58 h. Further, the inhibition effect of resorcinol on the specific substrate degradation rate is larger than the inhibition effect caused by phenol. It has been reported that in a mixed substrate system, the biodegradation of o-cresol is hampered by the presence of m-cresol and/or p-cresol in the wastewater system (Surkatti and El-Naas 2017). Zou et al. (2018) have investigated the competition between molecular oxygen (*O*₂) and an electron donor (2H) in the concurrent degradation of quinoline and phenol using a vertical baffled bioreactor (VBBR). They reported the existence of mutual inhibition between quinoline and phenol, which competed for electron donor (2H) and molecular oxygen (*O*₂) during the simultaneous degradation process. Xiao et al. (2019a) studied the degradation of cresol and phenol by *Chlorella vulgaris*. They observed that low concentrations of initial phenol (60–100 mg/L) improved the rate of p-cresol degradation. Xiao et al. (2019b) evaluated the effect of hydroxypropyl-β-cyclodextrin on co-metabolic degradation of phenanthrene from coal chemical wastewater using phenol as a growth substrate by a novel *Chryseobacterium* sp. According to the researchers, the presence of phenol accelerates the biodegradation of phenanthrene, whereas phenanthrene inhibits the biodegradation of phenol. Furthermore, 50 mg/L hydroxypropyl-β-cyclodextrin increases the biodegradation rate of phenol and phenanthrene by 55% due to its ability to improve apparent solubility and reduce phenanthrene toxicity. In the present study, the rates of 4-BP and 4-CP degradation were improved with a low concentration of 4-NP and towards its depletion in the culture medium. This could be due to the faster consumption of 4-NP by *A. chlorophenolicus* A6, as well as the production of NADH and FADH<sub>2</sub> cofactors as electron donors, which stimulated the secretion of enzymes involved in the growth and biodegradation of 4-BP and 4-CP. It is well known that co-metabolic substrates offer
adequate carbon and energy sources for the growth of microorganisms; synthesize NADH and FADH₂ cofactors as electron donor, and stimulate the secretion of enzymes associated with the growth and degradation of pollutants. In addition, their metabolic products can also contribute to the degradation of recalcitrant pollutants (Wang et al. 2017; Shi et al. 2018).

In the present study, the actinomycetes degraded 4-NP at a faster rate than 4-BP and 4-CP. However, the biomass yield was poor. This might be due to the presence of a nitro group on the phenolic ring, which imparts greater toxicity to the microorganism compared with the other two phenolic pollutants (Tian et al. 2020). A similar lower biomass yield at higher concentrations of 2-chloro-4-nitrophenol has been reported by Min et al. (2018). The authors hypothesized that this phenomenon may be explained by the need for greater energy to overcome the inhibitory effects of 2-chloro 4-nitrophenol on the microorganism at higher initial concentrations (Min et al. 2018). In addition, the accumulation of nitrite was also responsible for the decreased biomass yield profile (Min et al. 2018). Thus, it is clearly understood that the microbial cells were in a harsher condition due to the presence of 4-NP than that of 4-BP and 4-CP in the mixed substrate system. 4-NP is possibly a more intoxicating mitochondrial uncoupling agent compared to 4-BP and 4-CP and hence affects microbial cells more negatively. Further, 4-NP uncouples mitochondrial oxidative phosphorylation and decreases the biomass growth yield as it dissociates anabolism from catabolism (Fernández et al. 2013). The energy generated by the catabolism of 4-NP degradation is larger than the anabolism prerequisite, which results in excessive energy spilling (Fernández et al. 2013). However, irrespective of the different combinations of initial 4-BP, 4-CP, and 4-NP concentrations, the biomass yield was increased with an increase in the initial concentration of 4-BP and 4-CP at a fixed concentration of 4-NP in the mixture, except in the case of the experimental run number 8. The lower biomass yield might be attributed to the high initial concentration combination of 4-BP (125 mg/L), 4-CP (125 mg/L), and 4-NP (100 mg/L), which exerted an elevated toxicity level on the microbial cells. As a consequence, a major portion of the carbon source is diverted to maintenance energy rather than to biomass growth (Panigrahy et al. 2020b). In this study, the highest biomass yield value of 0.215 g/g was obtained at a concentration combination of 125, 125, and 50 mg/L of 4-BP, 4-CP, and 4-NP, respectively, which is comparatively lower than many reports in the literature. For instance, Panigrahy et al. (2020a) reported that the biomass yield coefficient increased from 0.57–0.73 g dry cell mass/gm of cresol with a change in the initial concentration of cresol from 100–1200 mg/L using an indigenous Pseudomonas citronellolis NS1. In another study, Dionisi and Etteh (2019) investigated a mixture of paracetamol and phenol biodegradation by a mixed microbial culture. They reported a higher biomass yield of 0.51 g/g using paracetamol than that of phenol (0.20 g/g COD). The lower biomass yield obtained in this study may be attributed to the toxic effect caused by these substituted phenols on microbial cells, as the aromatic ring of the phenolic compounds with its shared resonance electrons offers higher stability and resistance to microbial enzymatic attack. Halogen substituents on the aromatic ring further improved the stabilization, as the halogens promote an electron-withdrawing effect and create a steric hindrance to enzymes (Panigrahy et al. 2018).

Similar, higher microbial toxicity of substituted phenols has been reported in the literature. For instance, Aktaş (2012) investigated a mixture of 2-CP, 2-NP, and phenol biodegradation using activated sludge. The researchers observed the substrate to biomass ratio for 2-CP and 2-NP (1.5 and 5.5 mg CODₑₑ/mg MLSS) is higher than that of phenol (8.5 mg CODₑₑ/mg MLSS), which indicates the higher microbial toxicity of substituted phenol compared to phenol. Therefore, the necessity of higher maintenance energy to overcome the toxic effect of these substituted phenols cannot be ignored. This is well supported by many reports in the literature that the addition of o-CP to a mixture of 4-NP and o-cresol causes complete failure of the sequencing batch reactor system (Fernández et al. 2013).

**Conclusion**

The experimental data on the biodegradation of a mixture of substituted phenols were statistically analyzed, and 4-NP had the relevant influence on the degradation of both 4-CP and 4-BP among the three variables. On the other hand, the concentration of 4-CP had a strong negative interaction effect on the biodegradation of 4-NP. Irrespective of the concentration levels of these three substrates, 4-NP was preferentially biodegraded over 4-CP and 4-BP. The 4-BP degradation rate was higher than that of 4-CP, followed by 4-NP. Further, at an elevated level of 4-NP, the biomass yield significantly decreased. Almost complete degradation of this mixture of substituted phenols was achieved at an initial concentration combination of 125, 125, and 100 mg/L of 4-CP, 4BP, and 4-NP, respectively, within 68 h, which is found superior to many reports in the literature. Thus, it can be inferred that the environmental ecotoxicity induced by a mixture of substituted phenolic contaminants can be minimized by utilizing A. chlorophenolicus A6. The applied statistical analysis technique has proven to be a useful and powerful tool and the obtained interaction effects on culture growth as well as the kinetics of phenolic degradation aid in the interpretation of individuals’ roles in the phenolic biodegradation mixture.
Data availability

All data generated or analysed during this study are included in this manuscript.

Author contributions

MMS: conduct experiments and collected data, analysed sample and data, writing the draft manuscript and editing. SR formal analysis, visualization, review and editing. AD: conceptualization, supervision, writing, review, and editing. NKS: conceptualization, supervision, review, and editing, funding acquisition, project administration, funding resources.

Funding

This study was funded by the financial support received from the Department of Biotechnology Government of India, New Delhi (SAN No. 102/IFD/SAN/3610-3612/2016-2017), for carrying out this research work.

Compliance with ethical standards

Conflict of interest

The authors declare no competing interests.

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