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

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Abstract

Brominated phenols are listed as priority pollutants, and are the key components of paper pulp wastewater together with nitrophenol and chlorophenol. 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 mgl^{-1}and4-NP from 50–100 mgl^{-1}. Almost complete degradation of this mixture of substituted phenols was achieved at an initial concentration combination of 125, 125, and 100 $mgl^{-1}$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 both degradations of 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. Further, 4-BP biodegradation rates were found to be higher than that of 4-CP followed by 4-NP. Besides, the variation of 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 prospective of *A. chlorophenolicus A6* for the degradation of 4-BP in mixed substrate systems.

Introduction

Bromophenols are widely discharged from the effluent of different industries such as; brominated flame retardants, dyes, paints, photographic, pulps, polymer and resin intermediates, pesticides, herbicides, and wood preservatives (Saeed et al. 2016; Xu etal. 2017; Li et al. 2015). Besides, the combustion of leaded petrol produces a considerable quantity of bromophenol (Sahoo et al. 2013). The annual global production of bromophenol flame retardant (BFRs) has been raised from 0.13 million tons in 2002 to over 0.2 million tons in 2013 (Xiong et al. 2015).The world wide annual production of bromophenol isover 23,000 tons (Liang et al. 2017). The concentration of bromophenol rises to 187 mgl^{-1} in the effluent of photographic industrial wastewater (Sahoo et al. 2014a). Phenolic pollutants undergo active transformation inside the living organisms by cytochrome P450 enzyme system and generate more toxic electrophilic metabolites. These active metabolites may bind and impair the DNA or other vital enzymes system of the living organisms, consequently causes mutagenicity and carcinogenicity (Dayana and Bakthavatsalam 2019). Owing to its acute genotoxic, hepatocytes, thyrotoxic, neurotoxic and carcinogenic properties, phenolic compounds are considered as priority pollutants by USEPA (Acosta et al. 2018; Zou et al. 2016; Sharma and Dutta 2018; Liang et al. 2019; Peng et al. 2017).

Over the past decades, several conventional methods have been studied for the treatment of phenolic wastewater 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 is 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 (Panigrahy et al. 2020a; Jiang et al. 2016c; Zhou and Nemati 2018). However, the presence of halogenated substitute groups in phenolic ring offers resistance to microbial cleavage of the aromatic ring, which consequently inhibits its biodegradation activity (Sahoo et al. 2011a; 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, Bacillus sp.* GZT, *Clostridium sp*. Ma13, *Desulfatiglans parachlorophenolica* DS, *Dehalobacter sp. Phylotype* FTH1, *Sphingomonas* sp. strain TTNP3 and *Achromobacter piechaudii* strain TBPZ (Li et al. 2015; Li et al. 2016; Zu et al. 2012; Li et al. 2015; Sahoo et al. 2014a).Though anaerobic treatment of brominated phenol has been well reported in the literature, however, fastidious nutritional requirements, a prerequisite of 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 is very popular and has proven to be a promising tool. Furthermore, bromophenol along with nitrophenol and chlorophenol typically occurs in paper pulp, pesticide, insecticide, herbicide and fungicide, chemical-synthesis factories, and plasticindustrial 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 the enzyme stimulation, competition for active sites of enzymes, catabolite repression, and toxicity of the co-pollutants over and above dead-end products are usually influencing 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 effect and preferential utilization of substrate. Further, when substrates inhibit microbial growth or degrade co-metabolically, the microbial population dynamics and the degradation kinetics could turn very complex. Thus, it is highly essential to study the simultaneous biodegradation of these phenolics mixture.

Understanding microbial growth and biodegradation kinetics are very essential to optimize the operational process, design bioreactor system, and its scale-up for the prediction of effluent quality of microbial wastewater remediation processes (Panigrahy et al. 2020a; Sahoo et al. 2011b). 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, however, the investigation on the treatment of brominated phenols in mixed substrate systems is very few (Sharma and Dutta 2018). Thus, in this study, the prospective of *A. chlorophenolicus A6* for the degradation of 4-BP in a mixed substrate system has been evaluated. Though many mechanistic models have been studied in the literature, however, application of statistical design of experiment and its analysis is more practical and offers a clear perceptive of the main and interaction effects that exist among the different variables (Mohanty and Jena 2018; Panigrahy et al. 2019). Thus, a 2^2 factorial design of experiments was applied in the present study. The application of factorial design and CCD has been successfully applied in many fields (Farag et al. 2018; Khanpour-Alikelayeh et al. 2020). A factorial design is a promising statistical

technique to evaluate their relative significance as well as interactional effects that exist among numerous factors on the responses at diverse levels (Khatoon and Rai 2020; Elhalil et al. 2016). As compared to other techniques FD and RSM have numerous advantages like it saves time, saves energy, and resources with a minimum number of trial runs and evaluate multiple parameters (Khatoon and Rai 2020; Nam et al. 2017; Mohammed-Ridha 2019). The specific objectives of the present investigation were to (i) study the co-metabolic degradation of 4-BP in a mixed substrate system (ii) evaluate the microbial growth, biomass yield and pollutant degradation kinetics, and (iii) explicate 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 cultureconditionsof *A. chlorophenolicus* A6

In the present study, the actinomycetes strain was obtained from Prof. Janet K. Jansson, Lawrence Berkeley National Laboratory, Sweden. The *A chlorophenolicus* A6 seed culture was developed using 0.3% yeast extract medium as described by (Sahoo et al. 2010). The seed culture cells were centrifuged (8,000rpm 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 mgl^{-1} of 4-BP as the only source of carbon.

Modelling of phenol degradation

The statistical experimental design is a very popular and potent tool to predict the effect of different variables and their interactions very precisely with fewer numbers of experimental runs (Khatoon and Rai 2020; Nam et al. 2017). The 2-level factorial design of experiments is a prospective and broadly accepted tool for biochemical processes persuaded by multiple variables (Mohammed-Ridha 2019; Panigrahy et al. 2019). The factorial design of experiment analysis associated with 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. $A2^3$ full factorial design 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 level. The effect of each factor was computed as follows:

$$E_{(xi)} = \frac{2(\sum M_{i+} - M_{i-})}{N} \quad (1)$$

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

$$x_i = \frac{U_i - U_0}{\Delta U} \quad (2)$$

Where, X_i symbolizes the coded level (−1, 0, +1) of these substituted phenols. ΔU is the step change and U_0 is the uncoded level of these phenolics 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:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad (3)$$

Where *Y* stands for the predicted response, while the number of variables represented by *k*. X_i and X_j represent independent variables, β_0 is the constant term while β_i, $\beta_{ii,}$ and β_{ij} denote the coefficient for linear, squared and interaction terms, respectively. The experimental results were statistically analyzed by ANOVA and the Students 't' test employing a 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 degrees of the fit of the experimental result to the polynomial model were evaluated by the estimated values of correlation coefficient (R^2). When the computed *F*-value is greater than the theoretical-value, the effect of the independent factors is found significant (Khatoon and Rai 2020).Whereas, the lower value of *P*- signifies the appropriateness of rejecting the null hypothesis. The significance of the tested variable was analyzed using Student's *t*-test.

The mixture of phenolics biodegradation study was planned as per 2^2 factorial design of experiments. In this study, the various concentrations of these phenolic compounds were chosen (75-125 mgl^{-1} for both 4-CP and 4-BP whereas 50-100 mgl^{-1} in case of 4-NP). 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 error three-center point replicates were chosen in the design matrix. For the experimental set-

up the above-mentioned freshly growing actinomycetes cells were centrifuged (8,000rpm 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 optimized MSM (Sahoo et al. 2010) with varying concentrations of substituted phenols as per the 2-level factorial design 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's 't' test by employing MINITABs oftware (Version 12.2 PA, USA).

Specific phenolics degradation rate and biomass yield of *A. chlorophenolicus* A6

The experimental data on degradation of these three substituted phenols were employed for the determination of specific degradation rates of the phenolics as shown below.

$$q = -\frac{1}{x}\frac{ds}{dt} \qquad (4)$$

Where q is the specific biodegradation rate of the substituted phenol, X represents the biomass concentration (mgl^{-1}) 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:

$$Y_{X/S} = \frac{X_F - X_0}{S_0 - S_F} \qquad (5)$$

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 employing a UV-visible spectrophotometer by estimating the optical density of the culture sample at wavelength 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 mgl^{-1} of MLSS. The collected culture samples were centrifuged at 8000 rpm followed by filtration

through a Millipore syringe filter of 0.22 µm (Pall USA). Concentrations 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 wavelength 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^{-1} 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 And Discussion

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 2^2 full factorial design were recorded. Fig. 1 shows their biodegradation patterns at low concentration ranges (Fig. 1 (a), run 1 and 5) and high concentration ranges (Fig. 1 (b), run 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 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 slow 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 mgl^{-1} (Sahoo et al. 2011b) compared with a minimum culture period of 18 h in a mixed substrate system. The possible reason for slower biodegradation rates in a mixed substrate system might be due to the enhanced toxicity effect exerted by the phenolic compounds on the microbial cells. The phenolic compounds exert their toxicity by uncoupling oxidative phosphorylation (Xie et al. 2018). The formation of dimers between two different substituted phenolics compounds can further aggravate the uncoupling activity (Panigrahyet al. 2018; Escher et al. 2001). Besides, the formation of different intermediates in a mixed substrate degradation system may inhibit enzymes vital for degradation, or its active binding site (Sahoo et al. 2014b). The degradation patterns of individual substrates in the mixed substrate system also differed considerably from those obtained in their respective single substrate systems. 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 phenolics pollutants (100 mgl^{-1}) over free cyanide (>2.5 mgl^{-1}) has been reported by Sharma and Philip (2014). Between 4-BP and 4-CP, 4-BP degradation was found quicker than the other; however, the difference was less significant. 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 enzyme in the biodegradation pathway has a higher affinity towards 4- NP than those to 4-BP and 4-CP, otherwise, there could be transport-level interactions influencing the biodegradation of the phenolics 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 7.2–7.5 of the *A.*

chlorophenolicus A6 culture media, almost 65% of the 4-NP might have dissociated to phenolate ion which usually enters into the microbial cells. On the other hand, it is less than 2% in the case of 4-BP and 4-CP. Therefore, due to the different pKa values, 4-NP degraded preferentially over 4-CP and 4-BP. Further, concurrent biodegradation of 4-BP and 4-CP mixture was possible due to their almost alike pKa values of 9.17and 9.3, respectively. Also, this observation was correlated well with the results achieved under these individual substituted phenolic degradation systems. For instance, the values of half-saturation constants (K_s) for 4-CP and 4-BP were nearly the same i.e 30.83 and 30.77 mgl^{-1}, respectively; whereas, the values for 4-NP was lower (20.15 mgl^{-1}) (Sahoo et al. 2011a; Sahoo et al. 2011b; Sahoo et al. 2014a). Many researchers in literature have reported similar observations on the preferential biodegradation of one aromatic pollutant over another in mixed substrate systems, even though the same degradation pathway was followed for these compounds by different microorganisms. For example, the rate of benzene biodegradation was faster than o-xylene by fungal species 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 *Cupriavidusnecator* JMP134, *Pseudomonas* sp. WBC-3 and *Alcaligenes* sp. NyZ2015, 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 preferentially degraded over 3-NP and 4-NP by the *Pseudomonas* sp. WBC-3. Dey and Mukherjee (2013) investigated the mixture of phenol and resorcinol biodegradation under an aerobic batch reactor. They reported complete degradation of phenol and resorcinol at an initial concentration of 400 mgl^{-1} each within 58 h. Further, the inhibition effect of resorcinol on specific substrate degradation rate is larger than the inhibition effect caused by phenol. Bai et al. (2007) evaluated the performance of *Alcaligenes faecalis* in the degradation of *m*-cresol and phenol in a mixed substrate system. The researchers reported that faster biodegradation of *m*-cresol occurred towards the exhaustion of the phenol in the culture medium. Zou et al. (2018) have investigated the competition for molecular oxygen (O_2) and an electron donor (2H) in 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., (2019) studied the degradation of cresol and phenol by *Chlorella vulgaris.* They observed that low concentrations of initial phenol (60 – 100 mgl^{-1}) improved the rate of *p*-cresol degradation. In the present study, a similar finding was observed; where 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 mgl^{-1} of 4-BP or 4-CP takes about 16 h for completely degradation in single substrate systems (Sahoo et al. 2011a: Sahoo et al. 2014a) whereas, in mixed substrate systems, 125 mgl^{-1} 4-BP and 125 mgl^{-1} 4-CP took only 12 h in presence of 50 mgl^{-1} 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 required more time to grow when a higher concentration of 4-NP (> 100 mgl^{-1}) was present in the medium (together with

4-CP and 4-BP); along with a poor biomass yield. The observation on the biomass profile of the culture in the mixture of pollutants' system differed considerably with the patterns observed in the single substrate system (Sahoo et al. 2011a; Sahoo et al. 2011b; Sahoo et al. 2014a). This phenomenon is obvious owing to a difference in the accessibility of carbon source in the culture medium along with increased toxicity. These observations were in close agreement with that of Bai et al. (2007), who studied similarly mixed substrate effect on the growth of *Alcaligenes faecalis*. Moreover, a lag phase was observed beyond certain concentrations combination of 100 mgl^{-1} for 4-BP, 4-CP, each, and 75 mgl^{-1} of 4-NP, probably due to their 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 microorganism and phenolics degradation were used for calculating the pollutant degradation rates of these substrates in the mixture. Fig. 3 shows the degradation rates of these substituted phenols at different concentration combinations (run order number) as per the 2^2-level full factorial design of 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+50 mgl^{-1} 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 may be due to high initial biomass produced during the 4-BP and 4-CP degradation process. Further, these values were very low when the concentration ranges of the substrates were high. For instance, degradation of 4-NP at lower concentration range (75+75+50 $mgl^{-1}$of 4-BP, 4-CP, and 4-NP, respectively) was found to be 0.139h^{-1}; on the contrary, the value was 0.043h^{-1} at higher concentration range (125+125+100 mgl^{-1} of 4-BP, 4-CP, and 4-NP, respectively). This finding confirmed the fact that the growth of the culture, as well as its phenolics degradation rates, was inhibited at higher concentration ranges of these pollutants.

Statistical analysis of biodegradation of phenolics mixture using ANOVA and Student's t-test

Based on the above-obtained results were the statistical analyzed in terms of ANOVA and Student's '*t*' test. This statistical analysis was performed primarily to interpret the roles of individual variables played on phenolic biodegradation. Tables 2 (a), (b), and (c) present the results of ANOVA of 4-CP, 4-BP, and 4-NP degradation rates in the study. From this statistical analysis of the result, it can be seen that both the main (individual) and two-way interaction terms for these phenolics pollutants were very much significant

on the biodegradation activity at greater than 97.3% confidence level (P<0.027). On the other hand, except 4-NP degradation rate, the ANOVA results presented in Table 2 (a) and (b) revealed that the three-way interaction term was insignificant (P>0.05).The ANOVA table for each pollutants degradation rate also represents an error term, which revealed that the experimental error in this study was quite insignificant. Further, the higher values of the determination coefficient (R^2>99) indicate that the polynomial model is highly accurate in predicting phenolics biodegradation. Further, 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. Fig. 4 (c) demonstrates that the negative main effect of 4-NP on its degradation was comparatively lesser than that of 4-CP. Further, the interaction effect between 4-NP and 4-CPon 4-NP biodegradation activity and that between 4-CP and 4-BP on 4-BP degradation was considerably negative than that of between 4-BP and 4-CP on 4-CP degradation (Fig. 4 a, b, c). To analyze the main and interaction effects that exist among these substituted phenols 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 a 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 was concluded that 4-NP degradation performance 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 (X_1 X_2) were found insignificant (P = 0.613) on 4-NP degradation but was negatively significant on their degradation performance (P =.0.004 and 0.017). This analysis demonstrated that 4-NP was significantly inhibited by 4-CP concentration in the mixture whereas the vice-versa was not correct (P = 0.757). Other interaction effects except 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 biodegradation of different pollutants (Mohanty and Jena 2018; Farag et al. 2018; Khanpour-Alikelayeh et al. 2020; Nam et al. 2017; Khatoon and Rai 2020).

Biomass yield on biodegradation of mixture of substituted phenols

The calculated biomass yield values for different concentration ranges in the mixed substrate system are presented in Table 4. Though 4-NP was biodegraded before 4-BP and 4-CP, the biokinetic data revealed that 4-NP had a large negative effect on the microbial cells than the other two pollutants. Similarly, the actinomycetes degraded 4-NP faster; however, the biomass yield was very poor. This might be due to the presence of a nitro group on the phenolic ring impart greater toxicity to the microorganism compared with the other two phenolic pollutants (Tian et al. 2020). This phenomenon revealed that the microbial cells are in a more harsh condition when cultured in 4-NP than that of 4-BP and 4-CP. 4-NP possibly a more intoxicating mitochondrial uncoupling agent compared to 4-BP and 4-CP and hence affects the microbial cells more negatively. 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 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 lower biomass yield due to 4-BP, 4-CP, and 4-NP at 125, 125, and 100 mgl^{-1} may be attributed due

to high initial concentration combination exerted elevated toxicity level on the microbial cells as a consequence of which most of the carbon sources is diverted to maintenance energy rather than biomass growth (Sahoo et al. 2011b). Panigrahy et al. (2020b) reported that the biomass yield coefficient increases from 0.57 -0.73 g dry cell mass g^{-1} of cresol with a change in the initial concentration of cresol from 100–1200 mgl^{-1} 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 gg^{-1} using paracetamol than that of phenol (0.20 gg^{-1} COD). Similar biomass yield coefficient ranged from 0.293 to 0.64 gg^{-1} of phenol has been reported in the literature using *Alcaligenes strain* TW1 and *Bacillus brevis* (Essam et al. 2010). In this study, the highest yield value of 0.2152 gg^{-1} was obtained at a higher concentration combination of 4-CP and 4-BP with a low level of 4-NP (125+125+50 $mgl^{-1}$of 4-BP, 4-CP, and 4-NP, respectively). On the other hand, a very low yield value of 0.1673 was achieved at the same range of 4-BPand 4-CP but a higher 4-NP concentration of 100 mgl^{-1}. This value indicates that the concentration of 4-BP and 4-CP particularly influenced the biomass yield in the experiments. The lower biomass yield achieved in this study may be attributed to the elevated toxicity effect of substituted phenol on microbial cells, as the aromatic ring of the phenolic compounds with its shared resonance electrons offers higher stability and resistance to the microbial enzymatic attack. Halogen substituents on the aromatic ring further improved the stabilization, as the halogens promote electron-withdrawing effect and create a steric hindrance to enzymes (Uberoi and Bhattacharya 1997; 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 researcher observed the substrate to biomass ratio for 2-CP, 2-NP (1.5 and 5.5 mg COD_{eq}/mg MLSS) is higher than that of phenol (8.5 mg COD_{eq}/mg MLSS), which indicating 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.

Conclusion

The experimental data on the mixture of substituted phenols biodegradation was fitted to a second-order polynomial regression model to analyze the effects of the three independent variables and their mutual interactions exist among them. The accuracy of the model was verified by ANOVA and student's t-test. Among the three variables, 4-NP had the most prominent influence on the degradation of both 4-CP and 4-BP. 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. Further, the 4-BP degradation rate was higher than that of 4-CP followed by 4-NP. The specific biodegradation rates and biomass yield profiles of the actinomycetes were inhibited at higher concentration combinations of these phenolics mixture. Particularly, at an elevated level of 4-NP, the biomass yield was significantly decreased. Almost complete degradation of this mixture of substituted phenols was achieved at an initial concentration combination of 125, 125, and

100 mgl^{-1} of 4-CP, 4BP, and 4-NP, respectively in 68 h, which is found superior to many-reports in literature. This statistical analysis technique proved as a useful and powerful tool and the obtained interaction effect on the culture growth as well as the kinetics of phenolic degradation facilitate to interpret of the role of the individual pollutant on the mixture of phenolics biodegradation. This investigation demonstrated the prospective of *A. chlorophenolicus* A6 in the treatment of wastewater contaminated with 4-BP along with 4-CP and 4-BP mixture.

Declarations

- Data Availability: All data generated or analysed during this study are included in this manuscript
- Animal Research (Ethics): Not applicable
- Consent to Participate (Ethics): Not applicable
- Consent to Publish (Ethics): Not applicable
- Plant Reproducibility: Not applicable
- Author Contribution: MMS: Conduct experiments and collected data, Analysed sample and Data, Writing draft manuscript, & Editing. SR: Formal analysis, Visualization, Review & Editing. AD: Conceptualization, Supervision, Review & Editing. NKS: Conceptualization, Supervision, Writing-review and editing, Funding acquisition, project administration, Funding resources.
- Conflict of Interest: None
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Tables

Table 1 2^2 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

Table 2 (a) ANOVA of 4-BP degradation in the mixed substrate system

Source	df	SS	Adj MS	*F*	*P*	R^2
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				

Table 2 (b) ANOVA of 4-CP degradation in the mixed substrate system

Source	Df	SS	Adj MS	*F*	*P*	R^2
Main effect	3	4923.3	1641.12	1641.1	0.001	99.96
2-way interaction	3	322.37	107.46	107.46	0.009	
3-way interaction	1	15.12	15.12	15.12	0.060	
Residual error	2	2	1			
Pure error	2	2	1			
Total	10	5262.8	5262.8			

Table 2 (c) ANOVA of 4-NP degradation in the mixed substrate system

Source	Df	SS	Adj MS	*F*	*P*	R^2
Main effect	3	520.375	173.458	520.37	0.002	99.9
2-way interaction	3	83.375	27.792	83.37	0.012	
3-way interaction	1	28.125	28.125	84.38	0.012	
Residual error	2	0.667	0.333			
Pure error	2	0.667	0.333			
Total	10	632.54				

Df is the degrees of freedom; SS represents sum of squares; MS is the mean sum of squares. F is the Fisher's F value; P is the probability of error.

Table 3 Student's t test of degradation of substituted phenol in the mixed substrate system

Terms	4-BP degradation		4-CP degradation		4-NP degradation	
	T	P	T	P	T	P
Constant	96.37	0.000	172.18	0.000	452.54	0.000
4-CP (X_1)	-8.15	0.015	-57.63	0.000	-37.35	0.001
4-BP (X_2)	-15.4	0.004	-37.83	0.001	0.61	0.603
4-NP (X_3)	-7.02	0.020	-13.08	0.006	-12.86	0.006
X_1 X_2	-7.59	0.017	-15.20	0.004	0.61	0.613
X_1 X_3	0.84	0.488	0.35	0.757	-2.86	0.063
X_2X_3	-7.02	0.020	-2.55	0.071	4..2	0.0700
X_1 X_2X_3	0.84	0.488	3.89	0.060	-9.19	0.012

Table 4 Estimated biomass yield of the *A. chlorophenolicus A6* in the mixture study

Run Order	Initial Conc. (mgl^{-1})			
	4-CP	4-BP	4-NP	Yield (gg^{-1})
1	75	75	50	0.2076
2	125	75	50	0.2016
3	75	125	50	0.1929
4	125	125	50	0.2152
5	75	75	100	0.1824
6	125	75	100	0.2000
7	75	125	100	0.1936
8	125	125	100	0.1673
9	100	100	75	0.2024
10	100	100	75	0.2029
11	100	100	75	0.2011

Figures

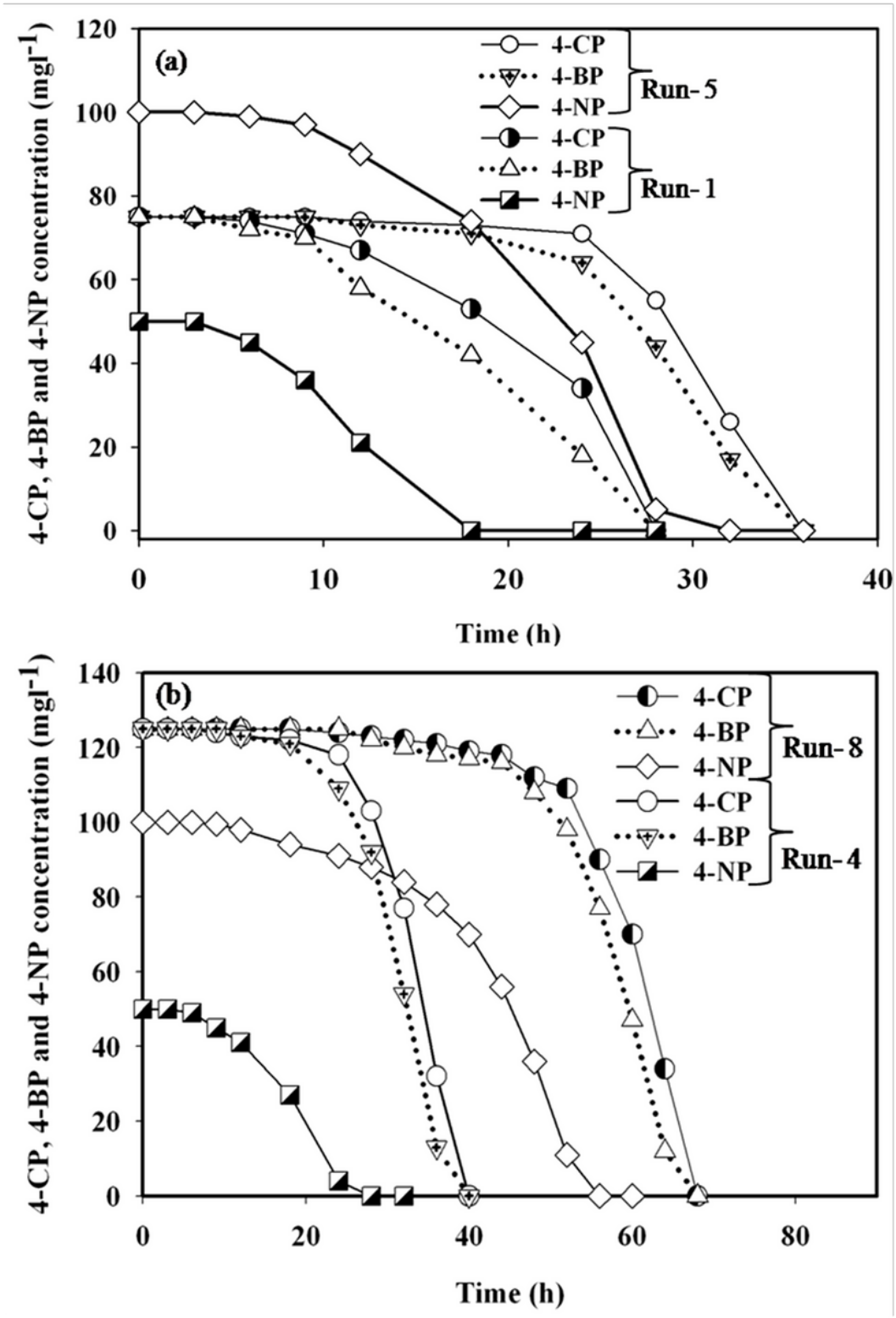

Figure 1

Biodegradation of 4-BP, 4-CP and 4-NP by A. chlorophenolicus A6 in a mixed substrate system for different initial substrate concentration ranges. (a) At relatively low concentration range (75+75+50 mgl-1, run-1, and 75+75+100 mgl-1, run-5) (b) at relatively high concentration range (125+125+50 mgl-1, run 4 and 125+125+100 mgl-1, run- 8)

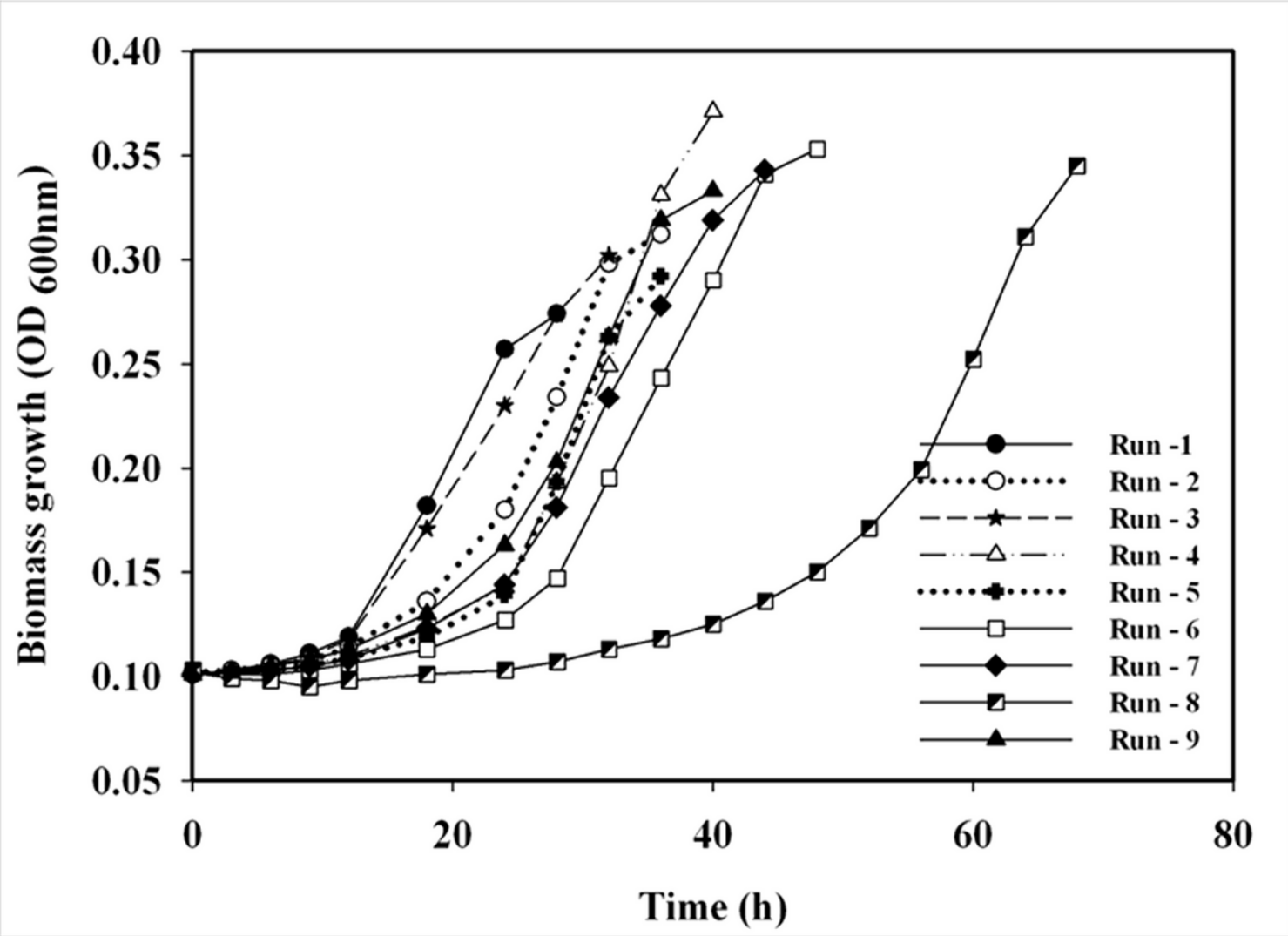

Figure 2

Biomass profile of A. chlorophenolicus A6 when grown in medium containing 4-BP, 4-CP, and 4-NP

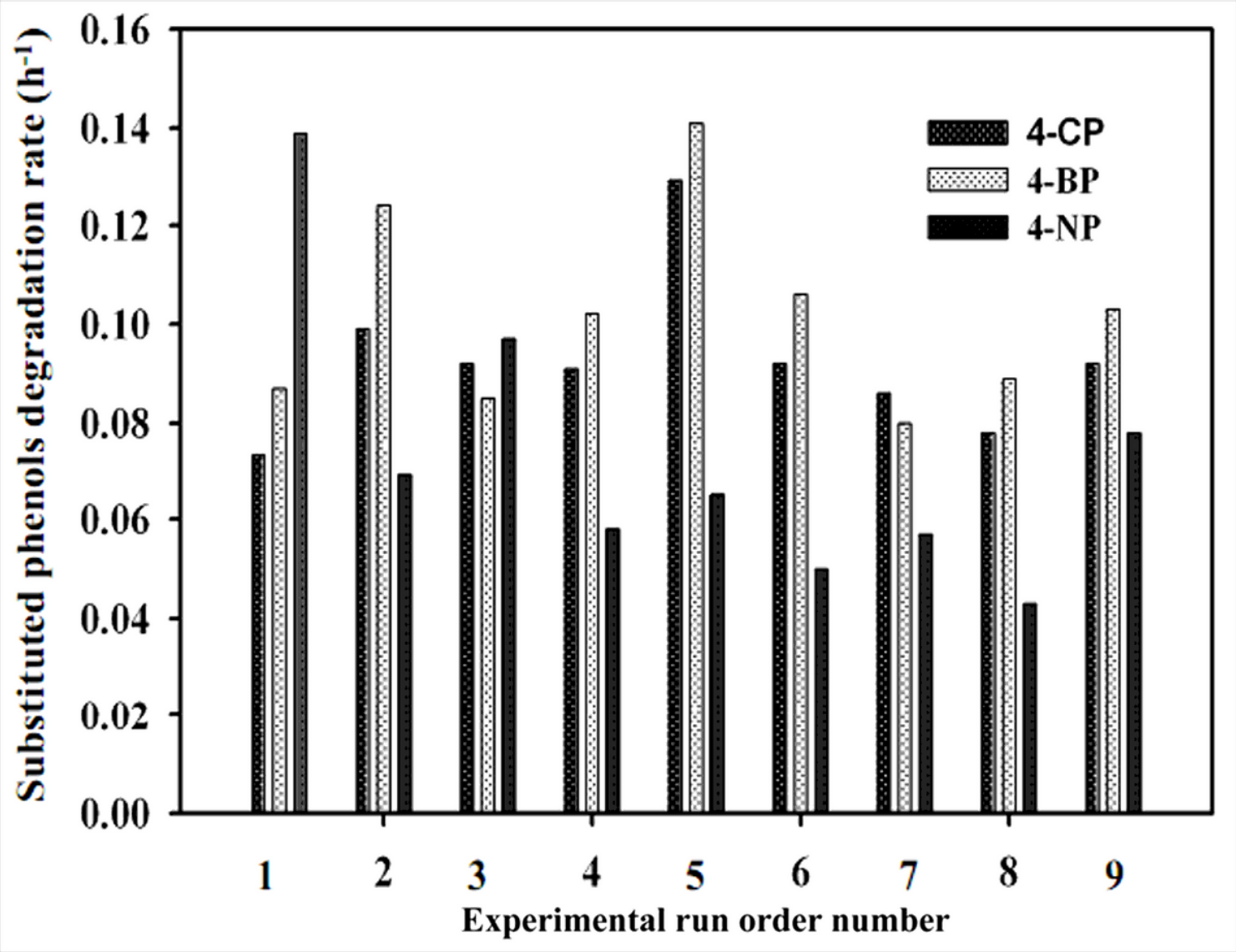

Figure 3

Degradation rates of 4-CP, 4-BP, and 4-NP mixture obtained at different initial concentration ranges of these substrates

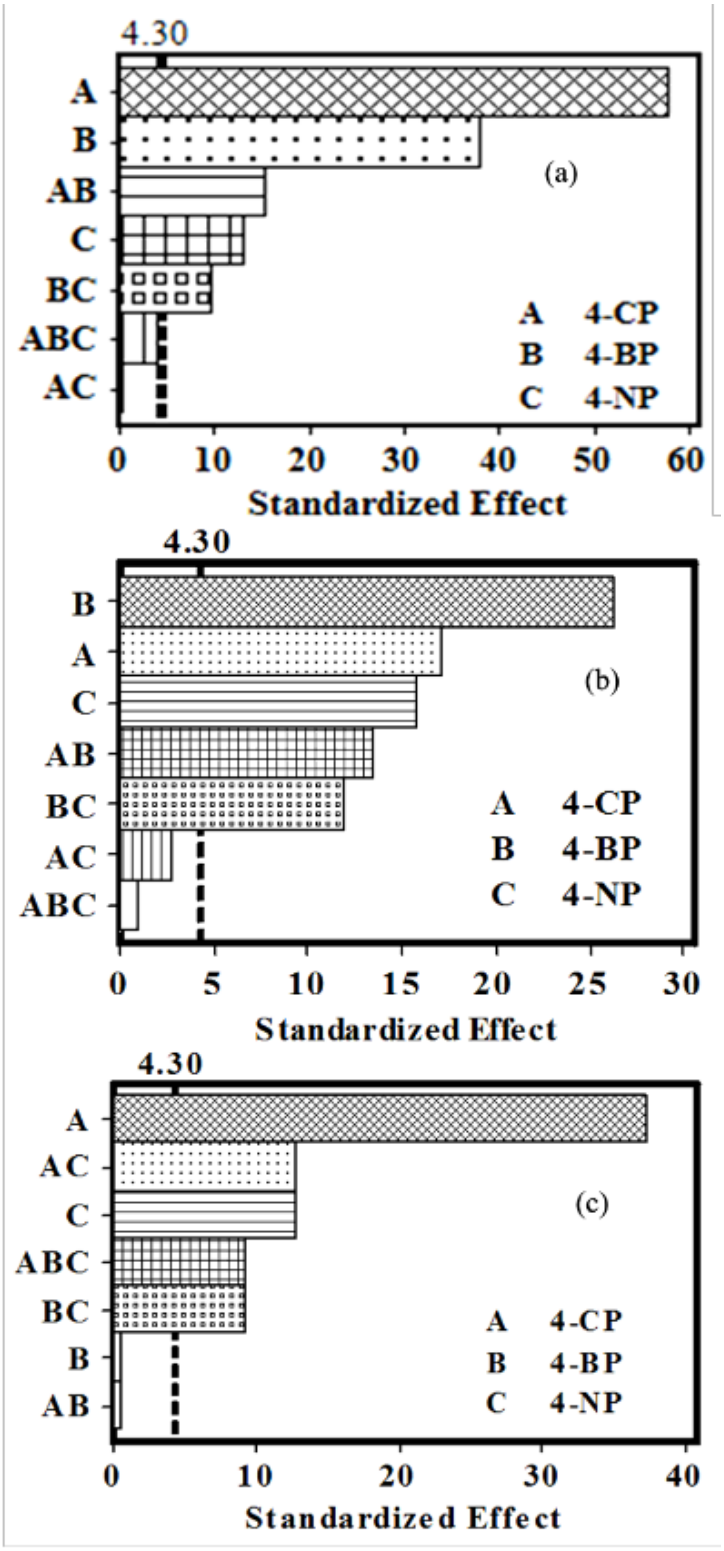

Figure 4

Pareto chart of the standardized effects of the factors on (a) 4-CP biodegradation rate (b) 4-BP degradation rate (c) 4-NP biodegradation rate in the mixture study (α=0.05)