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KINETICS OF GROWTH AND BIODEGRADATION OF P-NITROPHENOL AND P-CHLOROPHENOL BY *ARTHROBACTER CHLOROPHENOLICUS A6*

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Abstract: *Simultaneous biodegradation of mixed substrate of p-nitrophenol (PNP) with p-chlorophenol (PCP) as mixed substrate was investigated in batch shake flasks using a pure actinomycetes strain of Arthrobacter chlorophenolicus A6. A 2^2 full factorial design with the two substrates at two different levels and different initial concentration ranges (low and high) was employed to carry out the biodegradation study. Result showed that in the mixed substrate system, strong interaction between the compounds was obtained on the culture growth and their degradation. The biodegradation results also showed that the presence of PCP in low concentration range (20–60 mg/L) did not inhibit PNP biodegradation by the actinomycetes, however the compound at its high concentrations (100-200 mg/L) PNP biodegradation was inhibited by the culture. Further, PNP was found degraded quickly than PCP and the biomass yield was observed to be largely due to PCP concentration in the study. A sum kinetics model was used to describe the variation in the substrate specific degradation rates, which gave a high coefficient of determination value ($R^2 > 0.90$) at the high concentration range of the substrates. From the estimated interaction parameter values obtained from this model, the inhibitory effect of PCP on PNP degradation by the culture was found to be more pronounced compared to that of PNP on PCP. Overall in this study showed a good potential of A. clorophenolicus A6 in degrading phenolics substrates together.*

Key words: *p-nitrophenol; p-chlorophenol; Arthrobacter chlorophenolicus A6; biodegradation; substrate inhibition kinetics*

INTRODUCTION

The presence of substituted groups in phenols (i.e. in nitro and chloro-phenols) increases the toxic effects exerted on the environmental life as well as on the human health owing to the carcinogenic and persistence nature of the chemical compound (Uberoi et al, 1997).

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Both the p-nitrophenol (PNP) and p- chlorophenol (PCP) are listed as priority pollutants by the U.S. environmental protection agency of their concentrations in natural waters are recommended to be below 10 ng l^{-1} and 1µg l^{-1} respectively (Wild et al, 1993; Crosby 1981; Federal Register 1984). The major sources of wastes that discharge PNP and PCP are the industries mainly involved in the management of explosives, drugs, dyes, phosphororganic insecticides (methyl parathion), pesticides and wood preservatives. PNP are also formed in aqueous matrices during formulation, distribution and field application of pesticides (Concetta Tomeia et al, 2003), in addition, PNP can also formed in the atmosphere through the photochemical reaction between benzene and nitrogen monoxide (EPA, 1980). Similarly chlorophenols are also formed as a by-product when chlorine is used for bleaching of pulp and for disinfection of water; therefore, a high efficiency treatment of wastewater contaminated by these compounds is required prior to its discharge into the environment. There exist several available techniques such as volatilization, photo-decomposition, physical adsorption, solvent extraction, chemical oxidation and electrochemical methods for the removal of phenol and phenolic compounds from wastewaters [Ra et al, 2008]. However, high cost, low efficiency and generation of toxic by-products are some of the limiting factors of these conventional remediation strategies. The eco-friendly biodegradation process has gained maximum attention due to its many advantages over the traditional methods. Although the nitro and chloro groups enhance the resistance of the aromatic ring against biodegradation (Uberoi et al, 1997) many bacteria have the ability to degrade PNP and PCP (Ferraroni et al, 2006; Radehaus and Smith, 1992). Owing to their efficient enzyme system Several PNP and PCP degrading microorganisms have been successfully isolated from contaminated sites during the recent few decades, of which few microorganisms, including *Flavobacterium*, *Moraxella*, *Nocardia*, *Acaligenes* sp., *Pseudomonas* and *Arthrobacter* can metabolize PNP as a source of carbon or/and nitrogen for growth (Ferraroni et al, 2006; Radehaus and Smith 1992). Among these microbial species, actinomycetes secrete both extracellular and intra cellular enzymes and have thus shown good potential in degrading PNP and PCP. Westerberg et al, (2000) demonstrated the ability of *Arthrobacter chlorophenolicus A6* an aerobic actinomycete, in degrading a wide variety of toxic substituted phenols in batch shake flasks culture. Pollutants usually occur in mixtures in the environment and biodegradation of a single compound is often influenced either positively or negatively by other compounds due to substrate interactions (Timmis et al, 1994). PCP often exerts inhibition effects on the degradation of other phenols, due mainly to the incompatibility of different pathways (Saez & Rittmann, 1988)

Knowledge of microbial growth and substrate utilization kinetics is important for the purpose of prediction of effluent quality is biological treatment processes (Ellis and Anselm, 1999). Biokinetic parameters also help in optimizing the operational conditions to meet the discharge requirements (Ellis and Anselm, 1999).These aspects of growth kinetics of *A. chlorophenolicus A*6 for PNP and PCP biodegradation were, therefore, performed in the present study for enhancing the simultaneous biodegradation of the compounds simultaneously and to estimate the biokinetic parameters involved in the process.

Statistically valid 2^2 full factorial design of experiments was successfully employed to

carry out experimental investigations in order to study the kinetics of simultaneous degradation of PNP and PCP by *A. chlorophenolicus A*6. Compared to conventional one factor at a time experiments, statistical based factorial design of experiments give more meaningful information on the effects, main and interaction, of the factors involved in a process (Montgomery, 2004). Moreover, such type of statistical techniques offer the added advantage in the form of reduced number of experiments to be performed.

1 MATERIALS AND METHODS

1.1 Chemicals and Reagents

Analytical grade p-chlorophenol (PCP) and p-nitrophenol (PNP) was obtained from Sigma Aldrich (Germany) and Himedia (India) respectively. All other chemicals and reagents used were also of analytical grade and obtained from either HiMedia, (Mumbai, India) or Merck (India).

1.2 Preparation of Inoculums

All the PNP and PCP biodegradation experiments ware performed using minimum salts medium (MSM) (g l^{-1}: K_2HPO_4 2.6242, KH_2PO_4 0.4, NH_4NO_3 0.5797, $MgSO_4$ 0.173, $CaCl_2$ 0.0377 and $FeCl_3$ 0.002) with 300 mg l^{-1} PCP as the sole source of carbon and energy. The seed culture cells were, washed in sterile phosphate buffer (pH 7.5) and were grown overnight in PCP at 300 mg l^{-1} as the sole source of carbon and energy to induce the PCP degrading enzymes. After this adaptation period to grow in PCP the cells were centrifuged (6000g, 20 min at 22ºC), washed in 1X phosphate buffer saline (pH 7.4), and were subsequently used as the inoculums in the biodegradation experiments. The final biomass concentration in the inoculums was 0.1 OD_{600}.

1.3 Mixed Substrate Degradation

Batch biodegradation experiments were carried out with PNP and PCP added together in 100 ml of the previously optimized mineral salt media using Erlenmeyer flasks (250 ml). Different combinations of concentrations were chosen as per the 2^2 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. During the experiments, a sample volume of exactly two ml was withdrawn at regular time intervals until complete degradation of the substrate(s). After analysis for cell density, the samples were centrifuged at 6000 ×*g* for 10 min and the resulting supernatant was analyzed for residual PNP and PCP concentrations. Control experiments without the culture were also performed, and the initial concentrations of the substrates were found to remain unchanged indicating that abiotic loss of the substrates in the study was negligible. For choosing the concentration combinations of PNP and PCP in the mixed substrate degradation study, two different concentration ranges for PNP and PCP were selected. (I) low concentration range (25 - 75 mg l^{-1} PNP, 20 - 100 mg l^{-1} PCP) and (II) high concentration range (75 -125 mg l^{-1} PNP, 100 - 200 mg l^{-1} PCP). These two concentration ranges of the compounds were selected in such a way that each represented either positive or negative effect on the culture specific degradation rate, 2^2 full factorial design with the two substrates as the factors at two different levels was adopted for performing the experiments in their two concentration ranges. Table 1 shows the design matrix employed

in the study. Two center point replicates in the design were also included to check experimental error in the experiments. In total, six combinations (experimental runs) of PNP and PCP initial concentrations were investigated for studying their biodegradation and the culture growth in the low and high initial concentration ranges adopted in this study.

Table 1. 2^2 Full Factorial Design Adopted for Studying Simultaneous Biodegradation of PNP and PCP at Both Low and High Initial Concentration ranges

	Factor and Level	
Experimental Run No	**PCP**	**PNP**
1	+1	+1
2	-1	-1
3	0	0
4	0	0
5	+1	-1
6	-1	+1

–1: low level; +1: high level; 0: center point or middle level of the factors.

1.4 Analytical Methods

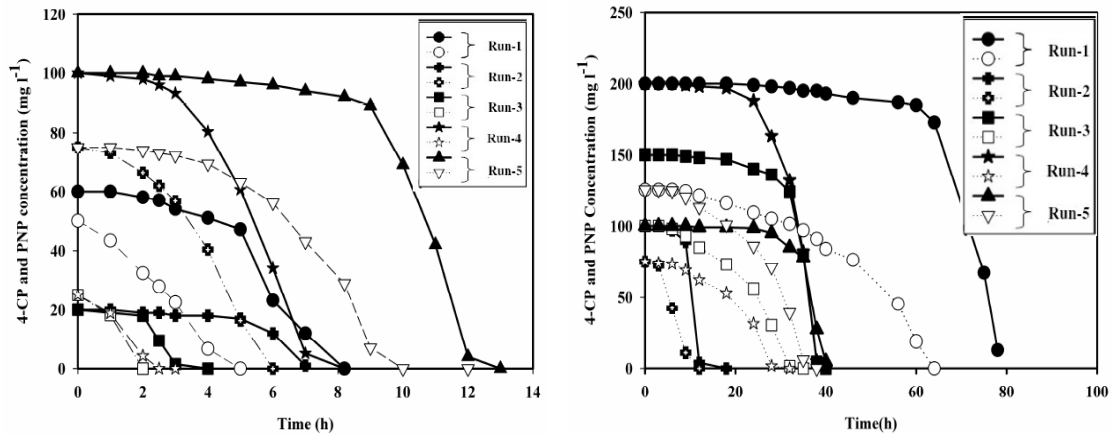
Cell density in the samples was estimated by with UV-visible spectrophotometer (Model lambda-45 Perkin Elmer U.S.A) by measuring its absorbance (OD) at 600 nm wavelength, OD600 was then converted to dry cell weight using a calibration curve, which was obtained by plotting dry weight of biomass per milliliter vs. OD600. High performance liquid chromatography (Varian prostar 210) was employed to quantify PNP and PCP concentrations in the biomass free samples. The analysis was performed with Onsphere 5-pesticides C-18 column (Varian) with methanol-water and acetic acid (50:49.1, v/v) as the mobile phase. The retention time of PNP was found to be 3.1 minute whereas for PCP it was 4.8 minute for a flow rate of 0.4 ml/min and at 28˚C. Detection of these compounds were made possible in the system using a UV detector set at wavelength 280 nm.

2 RESULTS AND DISCUSION

2.1 Simultaneous Degradation of PNP and PCP in the Mixed Substrate System

The biodegradation patterns due to the actinomycetes culture for low and high initial concentration ranges of these compounds are presented in Fig.1a and Fig1b, respectively. It could be observed that for a given concentration of the substrates, the culture took longer duration for complete utilization of PNP compared to PCP. Also, while PNP, in either low or high initial concentration range, did not show any lag phase in its degradation, lag phase in PCP degradation was found to be significant, particularly in its high concentration range. It should be noted here that always each experimental run in the study contained both the substrates, the concentration of PNP was low compared to PCP

degradation, the combination of PCP and PNP as substrates exhibited different degradation patterns of the substrate in the study.



(a) **(b)**

Fig. 1 PCP and PNP biodegradation patterns shown by the culture in the low (a) and high (b) initial concentration ranges of the substrates.
* solid lines indicated PCP concentration and dashed line for PNP

However preferential uptake of PNP over PCP for a given concentration of the substrates may be due to its bioavailability to the degrading microorganism. This preferential uptake of phenol was observed both in the low and high concentration range of the substrates. Similar to this present study, Westberg et al, (2008) also observed that PNP was preferentially degraded first over PCP, and PCP uptake was only towards depletion of PNP in the media.

2.2 Biomass Yield and Specific Degradation Rate of the Substrates

The experimental data on the substrate degradation at various combinations of initial concentrations of PNP and PCP were utilized for calculating biomass yield of the microorganism and specific degradation rates of the substrates according to the following equation

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

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

In the above expression for specific degradation rate (q) of substrate S (PNP or PCP), X (mg/L) is the biomass concentrations at time t (h), and in the expression for biomass yield ($Y_{X/S}$), X_F and X_0 are the respective maximum and initial dry cell concentrations corresponding to the total substrate (PNP + PCP) concentrations for initial (S_0) and final (S_F) concentration. The calculated total biomass yield values for the both concentrations ranges are presented in Table 2. It could be seen that high values of yield coefficients were obtained in the study, especially at low initial concentration combination of the

substrates. However, irrespective of the combinations of initial PNP and PCP concentrations, the biomass yield decreased with an increase in the initial PNP concentration at a fixed PCP concentration in the mixture. However, increase in PCP concentration seems to form higher biomass yield of the culture. Abuhamed et al, (2004) observed a maximum biomass yield value of 0.75 g/g due to benzene at 700 mg/L initial concentration on the growth of a *Pseudomonas putida* strain.
In the present study, a highest yield value of 0.2307g/g was obtained at low combination of concentrations 100 mg/L PCP and 25 mg/L PNP; the least value of 0.139 was obtained at a

Table 2. Estimated Biomass Yield of the Culture for Low and High Initial Concentration Ranges of the Substrates

Initial Conc. (High Conc. Range)			Initial Conc. (Low Conc. Range)		
PCP (mg l-1)	**PNP (mg l-1)**	**Biomass Yield (g/g)**	**PCP (mg l-1)**	**PNP (mg l-1)**	**Biomass Yield (g/g)**
100	75	0.2279	20	25	0.1392
100	100	0.1611	20	50	0.1591
100	125	0.1442	20	75	0.1924
150	75	0.1921	60	25	0.1811
150	100	0.1595	60	50	0.213
150	125	0.1511	60	75	0.2091
200	75	0.1712	100	25	0.2307
200	100	0.1565	100	50	0.2287
200	125	0.1527	100	75	0.2317

combination of 20 mg/L PCP and 25 mg/L PNP. These values clearly indicated that PCP concentration particularly influence the biomass yield in the experiments. Further it was observed that high concentrations combination reduced the culture biomass yield, and saturated at above a concentration combination of 150 mg/L of PCP and 100 mg/L of the yield PNP. Table 3 presents the calculated *q* values for both PCP and PNP obtained in this mixed substrate degradation study,

Table 3. Calculated Specific Degradation Rates of PCP and PNP Obtained in Low and High Initial Concentration Ranges of the Substrates

Experimental Run No	Initial Conc. Level		PCP Specific Degradation Rate (h^{-1})		PNP Specific Degradation Rate (h^{-1})	
	PCP	**PNP**	**Low**	**High**	**Low**	**High**

			Range	Range	Range	Range
1	+1	+1	0.533	0.1306	0.311	0.0526
2	-1	-1	0.978	0.503	1.303	0.287
3	0	0	0.481	0.285	0.452	0.092
4	0	0	0.434	0.281	0.447	0.091
5	+1	-1	0.749	0.175	0.975	0.1192
6	-1	+1	0.971	0.307	0.493	0.094

which shows that in high concentration range combinations of the substrates, PCP specific degradation rates were found to be always higher than the q values for PNP moreover, high values of q were obtained in the low concentration range of the substrates. However, within a given concentration range, particularly in the low range, the *q* values (of both PCP and PNP) varied largely depending upon the concentration combination of the substrate in each experiment. The experimental error in the study was also negligible as noted from table 3, and was almost found equal q values of PCP and PNP at experiments performed at their center point levels (0). In order to find any interaction between the two substrates on their individual specific uptake rates, the result obtained were fitted to a sum kinetic model proposed by Yoon et al, (1977).

2.3 Sum Kinetics Model Fitting of Specific Degradation Rates of PNP and PCP

Sum kinetics model proposed by Yoon et al, (1977) was slightly modified to fit the experimental data on specific degradation rates of the substrates obtained in the present study, where in the specific growth rate (μ) in the original equation was replaced with specific degradation rate (*q)* as represented in Eq.(3). This model was applied to evaluate and estimate the relative interaction effects on the individual degradation rates.

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

In the above equation, the interaction parameter $I_{1,2}$ indicates the degree to which substrate *1* affects the biodegradation of substrate *2*. In general a large negative value of the parameter indicates a strong inhibition on substrate uptake by another substrate in the biodegradation system (Yoon et al, 1977). The other kinetic parameters q_{max}, *K*s, K_i in the equation are the same as those for any single substrate system, which are respectively maximum specific degradation rate, half-saturation constant and inhibition constant due to the single substrate in the medium. To solve the model equation a non-linear regression technique involving constraints for positive integer values of the parameters was employed using the software MATLAB 7. Very high determination coefficient (R^2) value of 0.90 was thus obtained by fitting the model equation to the obtained *q* values of PCP and PNP observed from the experiments with different combinations of the two substrates in their high concentration range. The following interaction parameters ($I_{1\,2}$) values were thus obtained by solving the model equations: $I_{PCP,\,PNP} = -0.4095$ and $I_{PNP,\,PCP} = -0.1187$. (The interaction parameter $I_{PCP,\,PNP}$ represents the effect of PCP on PNP degradation, the interaction parameter ($I_{PNP,\,PCP}$) represents the effect of PNP on PCP degradation by the culture). Hence from the obtained interaction values of these parameters, it could be said

that PCP showed a significant inhibition on PNP degradation at its high concentration range. In literature, a maximum value of 5.16 for the parameter has been reported for the effect of toluene on benzene degradation by a *Pseudomonas putida* strain (Abuhamed et al, 2004). Considering the fact that the microorganism was well expected to degrade PCP, a large value of its inhibitory effect on PNP degradation by the microorganism followed in the present study is quite unlikely. Hence obtained interaction values of the parameters, it could be said that PCP showed strong inhibition on PNP degradation at its high concentration range. Furthermore, this is strongly supported in Fig. 2 (a), which revealed that two lines were going to cross each other at high concentrations of PNP that confirm there exists a significant interaction of PCP on PNP degradation. In addition, Fig. 2 (a) also showed that at 100 mg/L PCP inhibited PNP degradation by the actinomycetes more than at 200 mg/L in the media. On the other hand, Fig.2 (b) indicated that the two lines were found to be relatively parallel to each other which confirm that there exists least interaction of PNP on PCP degradation. Similar observations were made by Pakshirajan et al, (2007) for the interaction of glucose on m-cresol degradation rates.

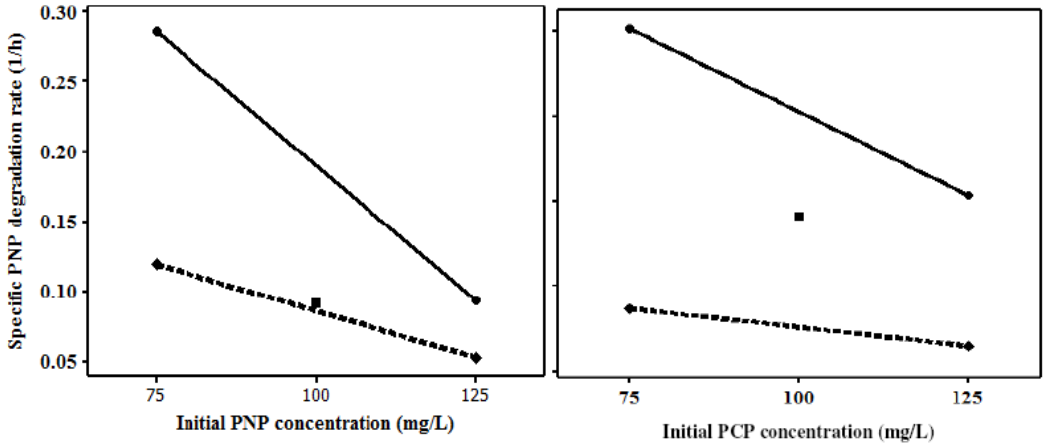
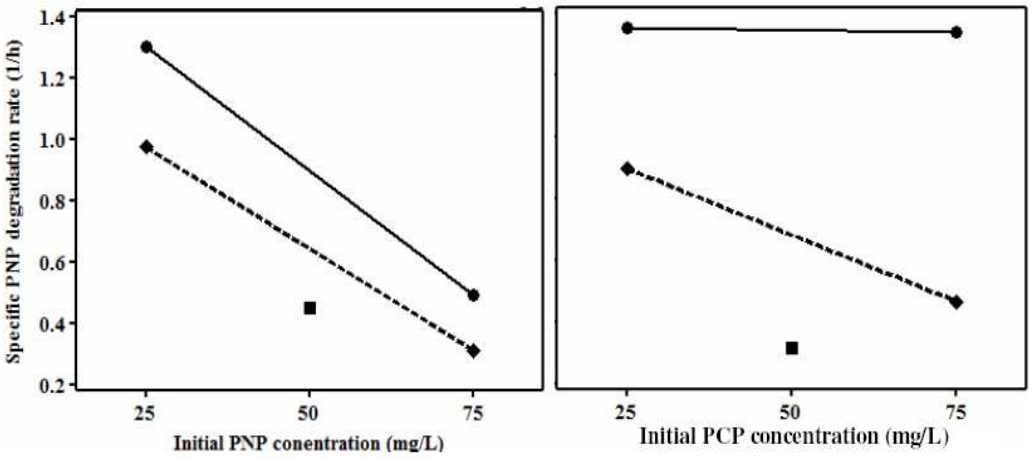


Fig. 2 Interaction effect between PCP and PNP in high concentration ranges combination on their specific degradation rate. (a) Interaction effect of PCP on PNP specific degradation rate (b) Interaction effect of PNP on 4-CP specific degradation rate.



(a) **(b)**

Fig. 3 Interaction effect plot between PCP and PNP on each other specific degradation rate (a) Interaction effect of PCP on PNP specific degradation rate (b) Interaction effect of PNP on PCP specific degradation rate.

However it was observed that the sum kinetic model failed or did not fit accurately the degradation kinetics of the substrates in their low concentration range combination. Moreover the interaction effect plot depicted in Fig. 3 (a) and 3 (b) also shown lack of proper interaction between the two substrate on each other degradation in this low concentration range as revealed by non interacting nature of the two lines (Montgomery, 2004).

3 CONCLUSIONS

A pure culture of *Arthrobacter chlorophenolicus A6* showed complete removal of PCP and PNP at a maximum combination of 200 mg/L and 125 mg/L respectively. PCP degradation was, quick however preceded after PNP degradation. Total biomass yields and specific degradation rates of the substrates revealed that the PNP and PCP combination with a certain high concentration inhibited the culture growth, and PCP yielded higher biomass than PNP. The interaction parameter between the two substrates, obtained by fitting a sum kinetic model to the experimental specific degradation rates of the substrate revealed that PCP showed a significant inhibitory effect on PNP degradation at higher concentration ranges. Kinetic analysis of the results and the estimated sum kinetics model parameters helped in good interpretation role of the individual substrate in the mixed substrate system.

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