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Batch Biodegradation of Para-Nitrophenol Using *Arthrobacter chlorophenolicus* A6

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Abstract The present study reports the kinetics of p-nitrophenol (PNP) biodegradation by *Arthrobacter chlorophenolicus* A6 in batch shake flasks for initial PNP concentrations in the range of 25–225 mg l^{-1}. Results of batch growth kinetics of *A. chlorophenolicus* A6 at various initial PNP concentrations revealed that the culture followed substrate inhibition kinetics with estimated decay coefficient value of 0.0132 h^{-1}. Biokinetic constants involved in the process were estimated by fitting the experimental data to several substrate inhibition kinetics models available from the literature. Among the models tested, Webb model fitted the experimental data best with the least root mean square error value, and the estimated model constants values were μ=0.161 h^{-1}, K_i=128 mg l^{-1}, K_s=60.15 mg l^{-1}, and K=100 mg l^{-1}. In addition, observed and theoretical yield coefficients, maintenance energy, and specific growth rate of the culture at various initial PNP concentrations were also investigated in the study.

Keywords p-Nitrophenol · *Arthrobacter chlorophenolicus* A6 · Biokinetic constants · Substrate inhibition kinetics · Webb model

Introduction

The presence of substituted groups in phenols, particularly the NO_2 group, increases the toxic effects of phenols on the environment as well as on the human health owing to carcinogenic and recalcitrant properties of these compounds [1]. The U.S. Environmental Protection Agency has listed p-nitrophenol (PNP) as a priority pollutant and recommended

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its maximum permissible concentrations in natural waters to be below 10 ng l^{-1} [2] and monthly average effluent concentrations in industrial discharges not exceeding 162 μg l^{-1} [3]. PNP is probably the most important among the mono-nitrophenols in terms of the quantities used which is currently 20 million kilograms per year [4]. The major sources of wastes that discharge PNP are the industries mainly involved in the management of explosives, drugs, dyes, insecticide, pesticides, and leather. PNPs are also formed in aqueous matrices during pesticide formulation, distribution, and field application [5]. In addition, PNPs are also detected in rainwater due to the photochemical reaction between benzene and nitrogen monoxide in the atmosphere [6]. It may also have the potential to leach through soil and enter groundwater, where it remains hardly degraded and hence persist in the environment [7]. These aspects warrant a high-efficiency treatment of wastewaters contaminated with PNP prior to their final discharge into the environment. Although several physical and chemical methods such as volatilization, photodegradation, photo-catalysis, and advanced oxidation have been tested to treat wastewaters containing nitrophenol [8], biodegradation seems to be an ultimate solution to the problem [4]. However, the presence of nitro groups enhances the resistance of the aromatic ring against biodegradation [1], and therefore, only selective bacteria species belonging to *Pseudomonas*, *Rhodococcus*, *Corynebacterium*, and *Microalgae* are known to metabolize PNP as a sole source of carbon or/and nitrogen for biomass growth [9–12]. Among these microbial species, *Arthrobacter chlorophenolicus* A6 has been demonstrated to degrade a number of toxic-substituted phenols including 4-chlorophenol (4-CP) as high as 300 mg l^{-1} within 24 h even in simple batch shake flasks [13]. However, its performance on biodegradation of PNP in neither batch shake flasks or in any reactor system has been investigated so far. Furthermore, for design of a suitable biological treatment system, knowledge of growth and degradation kinetics of such pollutants is essential in order to optimize the operational conditions and to meet the effluent quality. Hence, the present study investigated the kinetics of biomass growth and PNP degradation by *A. chlorophenolicus* A6 in batch shake flasks at its various initial concentrations.

Materials and Methods

Chemicals and Reagents

Analytical grade PNP was obtained from HiMedia (India). All other chemicals and reagents used were also of analytical grade and obtained from HiMedia, (Mumbai, India) and Merck (India).

Microorganism and its Maintenance

A. chlorophenolicus A6 used in the study was kindly gifted by Prof. Janet K. Jansson, Department of Biochemistry, Stockholm University, Sweden. The culture was maintained on slants containing mineral salt media as described by Westerberg et al. [13] with 0.3% yeast extract and 2% agar, pH 7.4.

Microorganism and Culture Conditions

The media used for developing the seed culture contained mineral salt media with 3% yeast extract and 100 mg l^{-1} PNP [13]. One hundred milliliters of the aforementioned seed culture medium taken in a 250-ml Erlenmeyer flask was inoculated with a loop full of the

culture freshly grown on agar slants and incubated in an incubator shaker for 40 h at 30 °C and 210 rpm. Obtained cells were then centrifuged (5,000×*g*, 20 min at 22 °C), washed in sterile phosphate buffer (pH 7.4), and regrown overnight in optimized minimal salts medium [14] with 200 mg l^{-1} of PNP as the sole source of carbon and energy. The active cells were again centrifuged (5,000×*g*, 20 min at 22 °C), washed with 1× phosphate buffer saline (pH 7.4), and suspended in 250-ml biodegradation flask containing 100 ml of optimized mineral salt media [14] with initial PNP concentration in the range of 12.5–225 mg l^{-1} so as to give an initial inoculum concentration of 0.1 OD_{600}. Samples were taken at regular interval of time during the experiments and were analyzed for biomass and residual PNP concentrations. All the experiments in this study were performed in triplicate and results reported are average with standard deviation ±2.5%.

Analytical Methods

Biomass concentration in samples was determined by measuring optical density at wavelength 600 nm (OD_{600}) using a UV–visible spectrophotometer (Model lambda-45, Perkin Elmer, USA). The absorbance values were expressed as dry cell weight using a calibration curve of optical density (OD_{600}) versus mixed liquor suspended solids (MLSS) of the sample. One unit of absorbance was found equivalent to 235 mg l^{-1} of MLSS. PNP concentration in biomass free samples was estimated using reverse phase HPLC (Varian Prostar 210) equipped with an Onsphere 5-pesticides C-18 column (Varian) using methanol–water and acetic acid (50:49.1 *v*/*v*) as the mobile phase. The retention time of PNP was found to be 3.21 min at a flow rate of 0.4 ml min^{-1}. PNP concentration was measured in the HPLC equipped with a UV–Vis detector at wavelength 280 nm.

Results and Discussion

Cell Growth and Degradation of PNP

Figure 1 shows the time profile of PNP degradation by the *A. chlorophenolicus* A6 at its various initial concentrations in the medium, which clearly reveals that the time taken by the microorganism to degrade the compound was dependent upon its initial concentration.

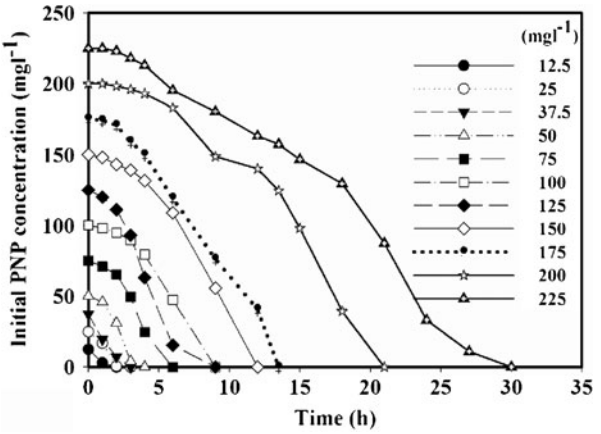

Fig. 1 Time profile of PNP degradation by *A. chlorophenolicus* A6

For instance, to degrade 125 mg l^{-1} of PNP, the culture took about 9 h, but for 225 mg l^{-1}, it took a higher time of 30 h for complete degradation of the compound. The results also showed that a maximum degradation rate was achieved at 125 mg l^{-1} of PNP and concentrations both below and above this concentration yielded low degradation rates, which indicated a strong influence of PNP concentration on its degradation rate [15]. The maximum PNP concentration studied so far in the literature for its degradation in a bioreactor system is only 320 mg l^{-1} [5], whereas in batch shake flasks it is 400 mg l^{-1}; however, in both of these cases, a very high initial MLSS of 1.5 gm l^{-1} was used in comparison with only 20 mg l^{-1} used in the present study [16]. Further specific degradation rate by *A. chloropehnolicus* A6 obtained in the present study is found to be very high, i.e., 0.829 h^{-1} for an initial PNP concentration of 225 mg l^{-1} in comparison with literature reported value of only 0.009 h^{-1} using *Nocardioides* sp. NSP41 [16].

Similar to time taken by the culture to degrade PNP at its various initial concentrations, the culture followed a similar pattern for its growth. This is illustrated in Fig. 2 where biomass growth ($OD_{600\ nm}$) of the culture is plotted against time for various initial PNP concentrations in the media. It could be seen from Fig. 2 that PNP concentration between 25 and 175 mg l^{-1} did not show any significant repression on the biomass output without any lag phase, but at concentrations greater than 175 mg l^{-1}, a lag phase in its growth was evident and correlated well with the delay in the utilization of PNP by the culture. Thus, it could be inferred that PNP at concentration above 175 mg l^{-1} exerted toxic effect on the culture growth. Moreover, at high initial PNP concentration in the media the culture took more time for complete utilization of the compound (9 h at 125 mg l^{-1} versus 30 h at 225 mg l^{-1}).

A typical growth curve shows a decline in cell population following complete consumption of substrate. During this declining phase, some part of the cell population becomes food for the rest of the cell population. This part of the growth curve in a batch reactor can be modeled using the following equation [17]:

$$\frac{dx}{dt} = -k_d X \tag{1}$$

where X is biomass concentration (in milligrams per liter) at time t (in hours) and k_d is decay constant, whose value correlates with biomass growth rate but inversely and is also

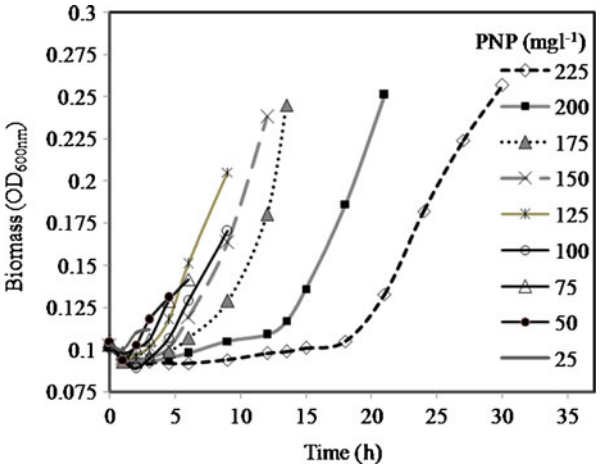

Fig. 2 Time profile of biomass growth ($OD_{600\ nm}$) for different initial PNP concentration

independent of substrate initial concentration. Thus, the batch growth experiment with 225 mg l^{-1} initial PNP concentration in the present study was continued further for another 3 days even after complete consumption of the substrate, and decay constant value was estimated from negative slope of the curve obtained by plotting ln (optical density) versus time. The value obtained (k_d=0.0132 h^{-1}) was found to be very much in agreement with several literature reported values, which ranges from 0.1 to 0.6 day^{-1} [18, 19].

Modeling the Growth Kinetics of *A. chlorophenolicus* A6 in the Presence of PNP

In order to establish the effect of PNP concentration on growth of *A. chlorophenolicus* A6, specific growth rates of the culture at different PNP concentrations were calculated as per the following relationship:

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{2}$$

where X is biomass concentration (in milligrams per liter) at time t (in hours) and μ is the specific growth rate (in hours) [20]. For each batch culture with a certain initial PNP concentration, the specific growth rate was estimated by performing a linear least squares regression on the semilogarithmic plot of biomass concentration over cultivation time in the exponential growth phase. Figure 3 depicts the variation of specific growth rate of the culture with the initial PNP concentrations, which shows that the culture-specific growth rate increased to 0.12 h^{-1} with the initial PNP content up to 125 mg l^{-1} and it decreased to 0.076 h^{-1} from 125 to 225 mg l^{-1}. This result clearly indicated the inhibitory effect of PNP at concentrations above 125 mg l^{-1}. This type of growth behavior by *A. chlorophenolicus* A6 due to higher PNP concentration demonstrated substrate inhibition pattern that has been studied by other authors, as well, for other substrates using different microorganisms [21, 22]. In order to predict the patterns of PNP degradation and culture growth in the system, kinetics of these two phenomena were analyzed by fitting the data to substrate inhibition models found in the literature. Table 1 presents the model equations tested in the study [23–28]. For solving the model equations, nonlinear regression method was applied using MATLAB 7.0. The aforementioned Fig. 3 also shows the model-predicted specific growth rate along with the experimentally obtained values. From this figure, it could be seen that

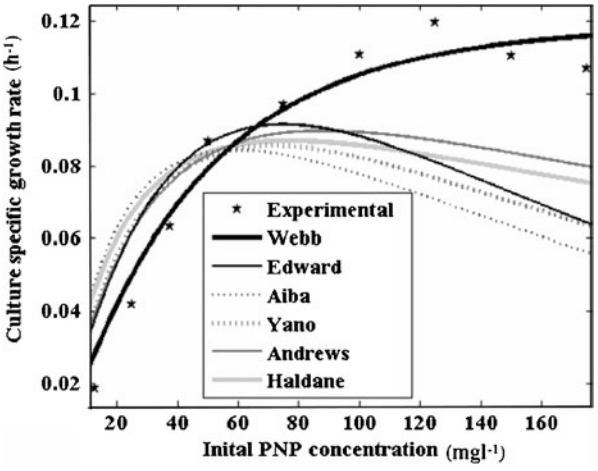

Fig. 3 Experimental and model predicted specific growth rate of the culture for different initial PNP concentrations

Table 1 Various literature models on biomass growth with substrate inhibition tested in the study

Author(s)	Model	References
Edward	$\mu_g = \mu_{max}[\exp(-S/K_i) - \exp(-S/K_S)]$	Edward [23]
Aiba et al.	$\mu_g = \mu_{max}S[\exp(-S/K_i)]/(S + K_S)$	Aiba et al. [24]
Yano et al.	$\mu_g = \mu_{max}S/[S + K_s + S^2/K_i(1 + S/K)]$	Yano et al. [25]
Andrews	$\mu_g = \mu_{max}/(1 + K_S/S + S/K_i)$	Andrews [26]
Haldane	$\mu_g = \mu_{max}S/(S + S^2/K_i + K_S + SK_S/K_i)$	Haldane [27]
Webb et al.	$\mu_g = \mu_{max}S(1 + S/K)/(S + K_S + S^2/K_i)$	Webb et al. [28]

μ_{max} maximum specific growth rate, K_s half saturation constant, K_i substrate inhibition constant, K Yano constant, S substrate concentration, μ_g predicted specific growth rate

among the six models tested, Webb model was found to fit the data quite accurately. The kinetics parameters estimated from these six models are presented in Table 2 along with root mean square error (RMSE) between model predicted and experimental-specific growth rate of the culture. These results further confirmed the accuracy of Webb type model in explaining the experimental data (RMSE=0.006 and R^2=0.97). All the models adopted in this study have generally been used in the literature to describe substrate inhibition on the growth of microbial cultures. Therefore, it is more likely that the models fitted the experimental data obtained in the present study to a reasonable level of accuracy. However, some models showed slight deviation in the values of bio-kinetic constants, such as μ_{max}, K_s, and K_i, probably due to their differences in the origin of development. For example, Edward model mainly concerns with the effect of a metabolite that may be formed during degradation on the growth of microbial culture, whereas Haldane model is valid only at constant biomass yield [29]. Although predicted values using the Webb model equation correlated well with the experimental data with R^2=0.97, the fit was valid for PNP concentration only up to 175 mg l^{-1}; above this concentration, the overall prediction accuracy of the model was slightly poor. Similar discrepancy in model prediction of experimental-specific growth rate of microorganisms has been noted in the literature [15].

An attempt was made to compare the model parameter values obtained in the present study with the literature and only a very few literature reports were found for PNP degradation using pure as well as mixed culture. The value of maximum specific growth rate (μ_{max}=0.161 h^{-1}), estimated from the best fitted model of Webb, correlated well with

Table 2 Estimated values of kinetic parameters on PNP degradation using the various models tested in the study

Model	Estimated value of parameters				RMSE	R^2
	μ_{max} (h^{-1})	K_s (mg l^{-1})	K_i (mg l^{-1})	K (mg l^{-1})		
Edward	0.24	38.83	205	–	0.028	0.37
Aiba	0.10	30.88	61	–	0.036	0.21
Haldane	0.27	34.91	164.2	–	0.026	0.45
Yano	0.10	44.53	177	300	0.030	0.25
Andrews	0.17	47.2	134.9	–	0.0201	0.62
Webb	0.16	60.15	128	100	0.0062	0.97

RMSE root means square error

the value found in literature on PNP degradation by pure culture of *Ralstonia eutropha* [30]. When compared with μ_{max} value (0.049 h^{-1}) obtained by Goswami et al. [31] for 4-CP degradation using *Rhodococcus erythropolis* M1, the value obtained in the present study is significantly high indicating that the substrate is more readily utilized by *A. chlorophenolicus* A6 for its growth. The larger μ_{max} value may also be due to high initial inoculum size and initial concentration of PNP used in the experiments. Furthermore, a high μ_{max} value obtained in this study may also be attributed to the channelling of energy gained from substrate utilization more towards biomass formation than for its maintenance. In addition, *A. chlorophenolicus* A6 biomass may be shunting very less percentage of the electrons for the regeneration of NADPH which is generally used to activate the monoxygenase enzyme involved in substituted phenol biodegradation compared to *R. erythropolis* M1, an aspect, however, requiring further investigations to confirm. Half saturation constant (K_s) in the model is a measure of affinity between biomass and substrate [32] and the value (60.15 mg l^{-1}) estimated in the study is found to correlate well with those found in literature on PNP and phenol degradation by mixed cultures [33, 34]. The model parameter value is also found to be greater than the K_s value of 13.33 mg l^{-1} obtained by Bhatti et al. [35] and Ray et al. [36] for the degradation of PNP using a mixed culture which indicates a high affinity of *A. chlorophenolicus* A6 towards the substrate. The value of the kinetic parameter K_i signifies the degree of resistance of the microorganism to toxic effects of PNP and, in general, a larger K_i value reveals that the biomass is highly resistant to inhibition by its substrate. The K_i value of the actinomycetes in the present study (128 mg l^{-1}) was found higher than the value obtained for 4-CP degradation by *R. erythropolis* M1 and for PNP degradation using mixed culture [31, 35, 36] indicating good tolerance of *A. chlorophenolicus* A6 towards growth and degradation of PNP in treating contaminated wastewater. A high resistance of *A. chlorophenolicus* A6 as observed from the estimated K_i value may be due to the production of micro colonies during its growth in culture media. It has been reported that some actinomycetes form hyphae and large micro colonies to enable protection of the inner cell mass thus favoring easy degradation and tolerance to toxic substrates [37].

Yield Coefficient

From the experimental result on biomass growth and PNP degradation, yield coefficient (Y) was calculated using the following equation [17]:

$$Y = \frac{X - X_0}{S_0 - S} \tag{3}$$

where X_0 and X are the initial and final biomass concentration at the end of the culture with corresponding substrate concentrations S_0 and S. Figure 4 is a plot of the cell mass yield coefficient versus initial PNP concentration. It can be seen that the yield coefficient varied from 0.16 g g^{-1} to a maximum of 0.295 g g^{-1} as initial PNP concentration varied from 12.5 to 225 mg l^{-1}. However, the value remained almost the same for PNP concentration lower than 128 mg l^{-1} which is due to the fact that the substrate below this concentration did not inhibit the growth of the culture. In the literature, several authors have also observed that biomass yield coefficient remains nearly constant for substrate concentrations below its inhibition level [17, 38, 39] probably due the requirement of lower maintenance energy at sub-inhibitory concentration than at higher concentration of the substrate [38]. At PNP concentration above 125 mg l^{-1}, considerable decrease in the value of yield coefficient was observed indicating the stronger inhibitory effect wherein the energy expended to maintain

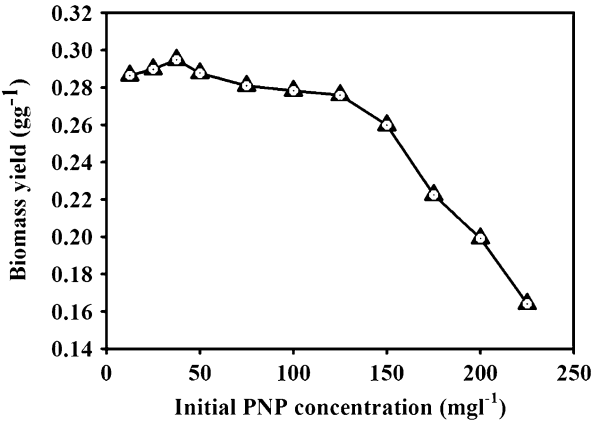

Fig. 4 Effect of initial PNP concentration on biomass yield

the cell activity is more. Therefore, the effect of maintenance energy requirements on substrate consumption and biomass production cannot be avoided.

The variation of cell mass yield with PNP concentration can be further rationalized by a material balance on PNP consumption. Because PNP is consumed mainly for assimilation into biomass and for growth and maintenance energy, Eq. 3 can be used to described this aspect [40]:

$$\frac{1}{Y} = \frac{1}{Y_C} + \frac{1}{Y_E} \tag{4}$$

where, Y_C represents the theoretical yield of cell mass on PNP, Y_E represents the yield of cell mass on PNP consumed for energy, and Y is the observed cell yield on PNP. Y_C can be calculated theoretically to be 1.15 g g^{-1} based on the assumption that the carbon content of dry cells is about 45% [41]. Y_E can, therefore, be determined according to Eq. 3. The percentage of the total substrate carbon converted to energy for cell growth and maintenance can be obtained as Y/Y_E, and the percentage of the total substrate carbon assimilated into biomass can be obtained as Y/Y_C. Figure 5 plots Y/Y_C and Y/Y_E versus initial PNP concentration. It can be clearly seen that Y/Y_E initially remained almost steady up to PNP concentration of 128 mg l^{-1}. Later, the value increased with a raise in PNP concentration. Also, the relative proportion of the substrate consumed for energy (Y/Y_E) was drastically exceeded that for assimilation into cell mass (Y/Y_C) for PNP concentrations

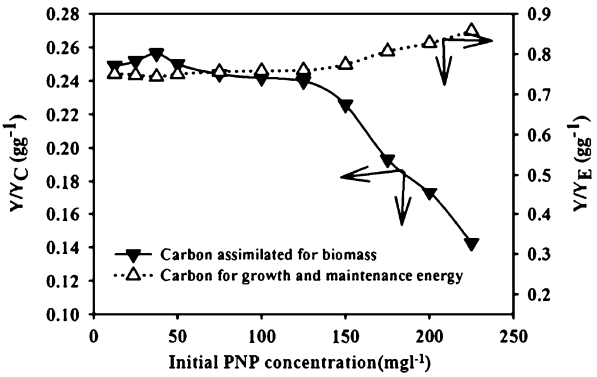

Fig. 5 Effect of initial PNP concentration on the proportion of substrate carbon for cell mass (Y/Y_C) and for energy (Y/Y_E)

greater than 128 mg l^{-1}, which could be due to the requirement of high-maintenance energy for overcoming the effect of substrate inhibition at high PNP concentrations. All these results of biodegradation kinetics of PNP by *A. chlorophenolicus* establish its potential in treating phenolic wastewaters.

Conclusions

The present study showed that synthetic wastewater containing PNP could be treated effectively using *A. chlorophenolicus* A6 with complete degradation of the compound within a short period of time at the entire concentration range studied. However, PNP concentration higher than 125 mg l^{-1} inhibited the biomass growth of the culture. The results of substrate inhibition kinetic values were better described by the Webb inhibitory growth kinetics model. The values of endogenous decay and yield coefficients for PNP were also determined and were in agreement with those found in the literature. The estimation of such kinetic constants will invariably prove helpful in the proper design of suitable reactor systems for treatment of phenolic wastewaters.

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