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ORIGINAL PAPER

Biodegradation of 4-chlorophenol by *Arthrobacter chlorophenolicus A6*: effect of culture conditions and degradation kinetics

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Abstract Among known microbial species, *Arthrobacter chlorophenolicus* A6 has shown very good potential to treat phenolic wastewaters. In this study, the levels of various culture conditions, namely initial pH, agitation (rpm), temperature (°C), and inoculum age (h) were optimized to enhance 4-chlorophenol (4-CP) biodegradation and the culture specific growth rate. For optimization, central composite design of experiments followed by response surface methodology (RSM) was applied. Results showed that among the four independent variables, i.e., pH, agitation (rpm), temperature (°C), and inoculum age (h) investigated in this study, interaction effect between agitation and inoculum age as well as that between agitation and temperature were significant on both 4-CP biodegradation efficiency and culture specific growth rate. Also, at the RSM optimized settings of 7.5 pH, 207 rpm, 29.6°C and 39.5 h inoculum age, 100% biodegradation of 4-CP at a high initial concentration of 300 mg l^{-1} was achieved within a short span of 18.5 h of culture. The enhancement in the 4-CP biodegradation efficiency was found to be 23% higher than that obtained at the unoptimized settings of the culture conditions. Results of batch growth kinetics of *A. chlorophenolicus A6* for various 4-CP initial concentrations revealed that the culture followed substrate inhibition kinetics. Biokinetic constants involved in the process were estimated by fitting the experimental data to several models available from the literature.

Keywords 4-Chlorophenol · *Arthrobacter chlorophenolicus A6* · Biodegradation · Optimization · Substrate inhibition kinetics · Edward model

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Introduction

Chlorophenols are listed as priority pollutants by the U.S. Environmental Protection Agency due to their acute toxicity and carcinogenic properties (Crosby 1981; Federal Register 1984; Wild et al.1993). The major sources of chlorophenol discharging wastes are industries such as pesticides, leather, pharmaceutical and wood preservatives. Chlorophenols are also formed as a by-product when chlorine is used for bleaching of pulp and for disinfection of water; therefore, effluent from these industries poses a serious threat to the receiving 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. Several reports are available on biodegradation of chlorophenols by *Flavobacterium* sp., *Pseudomonas* sp., *Sphingomonas* sp., *Acaligenes* sp., *Rhodococcus* sp., *Arthrobacter* sp. and fungal species like white-rot basidiomycetes sp. Among these microbial species, *Arthrobacter* sp. secrete both extracellular and intracellular enzymes and have thus shown good potential in degrading chlorophenols.

Arthrobacter chlorophenolicus A6 is an aerobic microorganism that has been demonstrated to degrade wide different types of toxic substituted phenols and is also reported to be one of the most efficient strains that completely mineralize 4-chlorophenol (4-CP) within 24 h of culture even at an initial concentration of 300 mg l^{-1} (Westerberg et al. 2000). It is also reported that among the aerobic chlorophenol degrading microorganisms, *A. chlorophenolicus A6* degrade the compound by a novel route via hydroxyquinol pathway with reductive dechlorination being one of the key intermediate steps in the process (Nordin et al. 2005). However, considering the fact that the microorganism degrades 4-CP aerobically as well as anaerobically it is essential to optimize the culture conditions, particularly the agitation speed as a higher value of the parameter affects dissolved oxygen concentration in the culture media, which may hinder anaerobic reductive dechlorination step in effective degradation of the compound and a lower value is detrimental to the microorganism growth (Sharma and Pant 2001). It has also been reported that the *A. chlorophenolicus A6* strain can degrade 4-CP over a wide range of temperature from 5 to 28°C; however, while the degradation rate at 28°C is found to be higher than at 5°C, viability of the microorganisms at 5°C after complete degradation of 4-CP is better than at 28°C (Backman and Jansson 2004). It is also found that during the course of 4-CP degradation, pH of the culture medium drops down from its initial value of 7.4 to a final value of 6.9. Inoculum age of microorganisms is another parameter that has been reported to play a vital role in biodegradation of such xenobiotic compounds (Van and Ward 2001). Hence, optimization of the culture conditions agitation speed, temperature, pH, inoculum age, can plausibly enhance in efficiency of 4-CP biodegradation by *A. chlorophenolicus A6*, which has however not been addressed so far in the literature. The traditional "one-variable-at-a-time approach" for medium optimization disregards the complex interactions among various components (Abdel-Fattah et al. 2005). On the other hand, statistically based experimental designs such as response surface methodology (RSM) can be effectively used to study the effects of several factors and their interaction by varying the factor levels simultaneously (Montgomery 1991; Elibol 2004; Piyushkumar et al. 2007). This type of statistical design techniques have been successfully applied in studies, such as optimization of caffeine degradation media by *Pseudomonas sp*, where by the caffeine degradation rate was increased from 0.1 to 0.18 g $l^{-1}h^{-1}$ at the RSM optimized settings (Dash and Sathyanarayana 2007). Similarly, by optimizing the conditions for reactive azo dye degradation by an integrated treatment process involving UV/H_2O_2 and aerobic biological treatment, removal efficiency of the dye was enhanced from 20 to 86% (Sudarjanto et al. 2006). Similar sequential designs of experiments have been carried out on diesel oil degradation by *Rhodococcus erythropolis* (Huang et al. 2008) and biodegradation of lindane by *Pleurotus ostreatus* (Rigas et al. 2007).

Knowledge of microbial growth and substrate utilization kinetics is important for the purpose of prediction of effluent quality by biological treatment processes (Ellis et al. 1996a, b; Grady et al. 1996; Ellis and Anselm 1999). Biokinetic parameters also help in optimizing the operational conditions to meet the discharge requirements (Ellis and Anselm 1999). These two aspects, i.e., optimization of culture conditions and growth kinetics of *A. chlorophenolicus A6* for 4-CP biodegradation were therefore investigated in the present study for enhancing the biodegradation of the compound and to estimate the biokinetic parameters involved in the process.

Materials and methods

Chemicals and reagents

Analytical grade 4-CP was obtained from Sigma–Aldrich (Germany). All other chemicals and reagents

used in the study were also of analytical grade and obtained from either HiMedia (Mumbai, India) or Merck (India).

Microorganism and its maintenance

A chlorophenolicus A6 used for 4-CP biodegradation was a kind gift from Prof. Janet K. Jonson, Department of Biochemistry, Stockholm University, Sweden. The culture was maintained on slants containing mineral salt media (Westerberg et al. 2000) with 0.3% yeast extract and 2% agar, pH 7.4.

Seed culture medium

The media used for developing the seed culture for use in biodegradation experiments contained mineral salt media (Alexandar and Lustigman 1996) with 0.1% yeast extract and 4-CP at a concentration of 150 mg l^{-1}. The seed culture medium (100 ml) taken in a 250 ml Erlenmeyer flask was inoculated with a loop full of the culture freshly grown on agar slants and incubated for 48 h at 28°C and 180 rpm.

4-CP degradation medium

All 4-CP biodegradation experiments in the study were performed with minimal salt media having the composition (g l^{-1}): K_2HPO_4 2.62, KH_2PO_4 0.4, NH_4NO_3 0.58, $MgSO_4$ 0.17, $CaCl_2$ 0.038, $FeCl_3$ 0.002, and containing 300 mg l^{-1} 4-CP as the sole source of carbon and energy. Fresh culture of the microorganism grown for 48 h using the above mentioned seed culture media was collected by centrifugation (5,000 *g*, 20 min at 22°C), washed in sterile phosphate buffer (pH 7.4) and were re-grown overnight using the biodegradation media containing 4-CP at 300 mg l^{-1} as the sole source of carbon and energy. These cells were subsequently used as the inoculum in the biodegradation experiments.

Optimization of culture conditions using RSM

For determining the best set of culture conditions for maximizing both 4-CP biodegradation and specific growth rate due to *A. chlorophenolicus* A6 experiments were performed by simultaneously varying the levels of culture conditions as per the central composite design.

Culture conditions chosen for optimization were pH, agitation (rpm), temperature (°C), and inoculum age (h); the total number of treatment combinations (experiments) was $31 = 2^k + 2k + n_0$, where 'k' is the number of independent variables and $n_0 = 7$ the number of replicates performed at center point of the variables. Table 1 presents the range and levels of the four variables tested in the study. The levels –1, 0 and +1 of the culture conditions were chosen in such a way that center point (0) values first represented the factor levels used conventionally for growing *A. chlorophenolicus* A6 (Westerberg et al. 2000); and, accordingly the other levels of the variables were determined using the following relationship:

$$X_i = \frac{U_i - U_0}{\Delta U} \quad (1)$$

where X_i is the coded level ($-\alpha$, –1, 0, +1 and $+\alpha$) of any independent variable, U_i is the uncoded/actual level of the independent variable, U_0 is the uncoded level of the independent variable at its centre point and ΔU is the step change. In the present case, as per the design, the default α value was taken to be 2.

For fitting the experimental results by response surface regression procedure the following second order polynomial equation was used:

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

where Y is the predicted response, k is the number of factor variables. X_i *and* X_j are independent variables, β_0 is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient and β_{ij} is the ijth interaction coefficient.

The statistical software package, MINITAB Release 15.1, PA, USA, was used for regression analysis of the experimental data. Since 4-CP biodegradation by *A. chlorophenolicus A6* is a growth associated process, optimum levels of each variables

Table 1 Experimental range and levels of the variable used in the optimization study

Independent variables (gl^{-1})	$-\alpha$	−1	0	+1	$+\alpha$
pH	6.6	7.0	7.4	7.8	8.2
Agitation (rpm)	120	150	180	210	240
Temperature (°C)	20	24	28	32	36
Inoculum age (h)	24	36	48	60	72

affecting both the responses, viz. 4-CP degradation and the microorganism specific growth, were determined by method of desirability function, and for which the following equation was solved (Derringer and Suich 1980; Harrington 1965):

$$d_i(\hat{y}_i) = \begin{cases} \left(\dfrac{\hat{y}_i - {}^{0} L_i}{T_i - {}_{1} L_i}\right)^{r_i} & \hat{y} < L_i \\ & L_i \le \hat{y} \le T_i \\ & \hat{y} > T_i \end{cases} \quad (3)$$

where $d_i(\hat{y}_i)$ is desirability function of a response, L_i and T_i are the lower and target values of response measured from experimental data. In the present study, while L_i for the two responses (4-CP degradation and specific growth rate) were 0% and 0 h^{-1}, respectively, T_i values were set at 100% and 0.0937 h^{-1}, respectively. $\hat{y}_i$ is the value of a response predicted by the second order polynomial equations generalized before; r is the weight of desirability function of a response. In this study, both the responses were given equal weight. The overall desirability function D in turn was computed as shown below:

$$D = \left(\prod d_i^{wi}\right)^{1/W} \quad (4)$$

where d_i is individual desirability for the ith response, w_i = importance of the ith response, and $W = \sum w_i$. In the present study w_i was taken to be one for each of the responses. For solving the desirability function, the statistical software package MINITAB Release 15.1, PA, USA, was used.

Growth kinetics of *A. chlorophenolicus A6* for 4-CP biodegradation

At the optimized settings of the culture conditions, batch 4-CP degradation experiments were carried out in Erlenmeyer flasks (250 ml) containing 100 ml of the mineral salt media with different initial 4-CP concentrations of 25, 50, 100, 150, 200, 250, 300, and 350 mg l^{-1}. Samples were taken at regular interval of time during the experiments and were analyzed for biomass and residual 4-CP concentrations. All the experiments in this study were performed in duplicate.

Analytical methods

Biomass in the samples was determined by measuring its optical density at wavelength 600 nm in UV–visible spectrophotometer (Model lambda-45 Perkin Elmer U.S.A). 4-CP concentration in the samples was estimated by reverse phase HPLC (Varian Prostar 210) using an onsphere C-18 column with acetonitrile–water (80:20, v/v) as the mobile phase. The retention time of 4-CP was found to be 5.6 min at a flow rate of 0.8 ml/min and at 28°C. Detection of 4-CP was made possible in the system using a UV detector at wavelength 280 nm

Results and discussion

Optimization of culture conditions using RSM

For maximizing 4-CP biodegradation efficiency and the culture specific growth rate, the levels of the four important variables, i.e., pH, agitation (rpm), temperature (°C) and inoculum age (h) were varied using the central composite design of experiment, and the results were analyzed in the form of analysis of variance (ANOVA). Table 2a and b presents the results of ANOVA of 4-CP biodegradation and specific growth rate of the culture, respectively. The Fisher's F value (8.2) for 4-CP biodegradation in the model owing to regression is found to be higher than the critical F value ($F_{0.05}$ 14, 6 = 2.54; Table 2a); the large F value indicates that most of the variations in the response could be explained by the regression model equation for 4-CP biodegradation in the study. Generally, a large F value with a corresponding small P-value indicates a high significance of the respective coefficient (Tanyildizim et al. 2005). The associated P value is used to judge whether F is large enough to indicate statistical significance or not. The linear and square terms of both the regression models for 4-CP biodegradation and specific growth rate were found to be highly significant at P less than 0.02. In the present study the model F-values of 8.2 and 7.48 for 4-CP biodegradation and specific growth rate, respectively, indicate that the respective regression models could explain most of the variation in the responses. Further, P values of lack of fit term in the ANOVA Table 2a and b showed that the second-order polynomial models for 4-CP biodegradation and specific growth rate were adequate in predicting the responses. These regression model equations are presented below.

$$Y_1 f(x) = -2874.87 + 490.65X_1 + 54.46X_3 + 12.66X_4 - 34.23X_1^2 - 1.01X_3^2 - 0.04X_4^2 - 0.04X_2X_4 - 0.11X_3X_4 \quad (5)$$

$$Y_2\ f(x) = -2.78 + 0.46X_1 + 0.05674X_3 + 0.01098X_4 - 0.03190X_1^2 - 0.001104X_3^2 - 0.00004X_4^2 - 0.00003X_2X_4 - 0.00011X_3X_4 \quad (6)$$

where Y_1 = 4-CP biodegradation, Y_2 = specific growth rate, X_1 is pH, X_2 is agitation (rpm), X_3 temperature (°C) and X_4 is inoculum age (h).

Further, to determine significance of the regression coefficients in the two models, the results were subjected to student's *t*-test and are presented in Table 3. From Table 3, it could be seen that the regression coefficients of linear and quadratic terms for temperature in both the models for 4-CP biodegradation and specific growth rate were found to be highly significant ($P < 0.003$) whereas the coefficient due to pH and inoculum age indicated less significance on the responses. On the other hand, agitation did not show any significance ($P > 0.5$). From Table 3, the regression coefficient terms for interaction between agitation and inoculum age were found to be highly important ($P < 0.005$); however, interaction effects between temperature and inoculum age revealed slightly less significance. Other coefficient terms in the models did not seem to be of considerable significance ($P > 0.3$) on 4-CP biodegradation as well as on specific growth rate of the culture. It should be noted here that such observations on significance of interaction effects between the variables would have been lost if the experiments were carried out by conventional optimization methods (Ravi Kumar et al. 2005) Table 4.

To illustrate the above mentioned interaction effects between the variables in the study, two dimensional contour diagrams were plotted between any of the four independent variables and the responses by maintaining the other variables at their middle (zero) levels. The contour plot between inoculum age and temperature, and that between agitation and inoculum age are depicted in Fig. 1a and b. In general, the contours in such plots help in proper identification of the type of interactions between test variables; the surface confined in the

Table 2 (a) ANOVA of 4-CP biodegradation in the optimization study; (b) ANOVA of the culture specific growth rate in the optimization study

(a)

Source	df	SS	Adj MS	*F*	*P*	R^2
Regression	14	14559.8	1039.98	8.20	0.000	94.48
Linear	4	3022.4	592.98	4.68	0.011	
Square	4	8496.2	2124.04	16.76	0.000	
Interaction	6	3041.2	506.87	4.00	0.012	
Lack of fit	10	48.463	4.8463	0.3241	0.134	
Error	6	89.7	14.95			
Total	30	16587.9				

(b)

Source	df	SS × 10^{-3}	MS × 10^{-3}	*F*	*P*	R^2
Regression	14	14.31	1.022	7.48	0.000	86.8
Linear	4	2.99	0.567	4.15	0.017	
Square	4	8.437	2.109	15.43	0.000	
Interaction	6	2.884	0.481	3.52	0.021	
Lack of fit	10	0.05255	0.00525	0.375	0.118	
Error	6	0.085	0.014			
Total	30	0.016498				

SS sum of squares, *df* degrees of freedom, *MS* mean sum of squares. *F* Fisher's *F* value (calculated by dividing the MS owing to the model by that due to error), *P* probability of incorrectly rejecting the null hypothesis when it is actually true

Table 3 Result of Student's *t*-test for 4-CP biodegradation and the culture specific growth rate in the optimization study

	4-CP degradation		Specific growth rate	
Term	*t*	*P*	*t*	*P*
Constant	−3.083	0.007	−2.872	0.011
pH (X_1)	2.367	0.031	2.141	0.048
Agitation (rpm) (X_2)	0.549	0.590	0.643	0.529
Temperature (°C) (X_3)	3.446	0.003	3.457	0.003
Inoculum age (h) (X_4)	2.507	0.023	2.093	0.053
X_1^2	−2.602	0.019	−2.335	0.033
X_2^2	0.026	0.979	−0.219	0.829
X_3^2	−7.668	0.000	−7.600	0.000
X_4^2	−2.860	0.011	−1.900	0.076
$X_1 \times X_2$	0.132	0.896	0.068	0.946
$X_1 \times X_3$	0.354	0.728	0.261	0.798
$X_1 \times X_4$	0.001	0.999	0.056	0.956
$X_2 \times X_3$	0.322	0.751	0.368	0.718
$X_2 \times X_4$	−4.497	0.004	−4.208	0.005
$X_3 \times X_4$	−1.877	0.079	−1.783	0.094

Table 4 Available literature models on biomass growth with substrate inhibition

Author(s)	Model	References
Edward	$\mu_g = \mu_{\max}[\exp(-S/K_i) - \exp(-S/K_S)]$	Edwards (1970)
Aiba et al.	$\mu_g = \mu_{\max} S[\exp(-S/K_i)]/(S + K_S)$	Aiba et al. (1968)
Yano et al.	$\mu_g = \mu_{\max} S/(S + K_s + S^2/K_i(1 + S/K))$	Yano et al. (1966)
Andrews	$\mu_g = \mu_{\max}/(1 + K_S/S + S/K_i)$	Andrews (1968)
Haldane	$\mu_g = \mu_{\max} S/(S + S^2/K_i + K_S + SK_S/K_i)$	Haldane (1965)
Webb	$\mu_g = \mu_{\max} S(1 + S/K)/(S + K_S + S^2/K_i)$	Webb (1963)

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

smallest curve of such contour diagram can also be used to predict optimum response of the system. Hence, from the given plot in Fig. 1a, the corresponding coordinates in the region of the contour diagram gave the optimum values of the respective factors. Also, the response surface contour plots of mutual interaction between the variables inoculum age and temperature (Fig. 1a) was found to be elliptical indicating significant interaction between the two. In Fig. 1b, which is a typical saddle point contour plot, the optimum values were obtained at the point of intersection of the lines that are formed by joining the locus (Murthy et al. 2000). Besides the two contour plots showing interaction between the variables, response surface contours drawn between other factors were circular indicating non-significant nature of their interactions. Figure 2a and b represent linear plots of measured versus predicted values of 4-CP degradation and culture specific growth rate, respectively, which clearly reveals that both the experimental and predicted values were in close agreement with each other.

In order to determine the optimal levels of each variable for maximizing both 4-CP biodegradation and the culture specific growth rate, the method of desirability function was applied. The overall desirability functions for 4-CP biodegradation and specific growth rate were close to 1 indicating the fact that the function increases linearly towards the desired target values of the two responses (Derringer and Suich 1980; Jahani et al. 2008). In addition, individual desirability values of the two responses were calculated; while the value for specific growth rate was computed to be 1 with a maximum predicted response of 0.0943 h^{-1}, the value for 4-CP biodegradation was also found to be 1 with maximum predicted value of 102.66%. Thus, using the

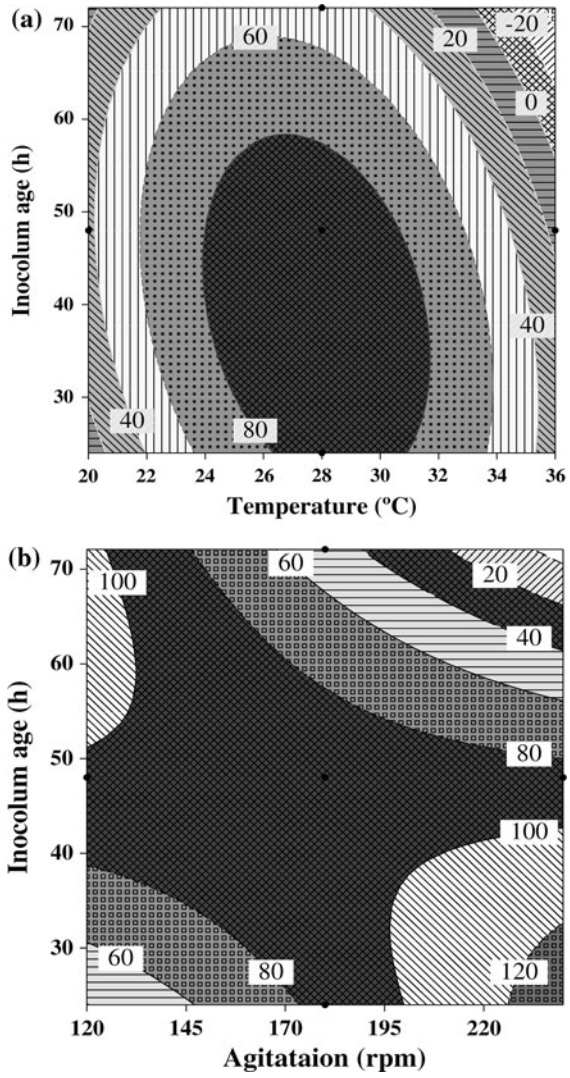

Fig. 1 **(a)** Contour plot showing the interaction between temperature and inoculum age on 4-CP biodegradation. **(b)** Contour plot showing the interaction between inoculum age and agitation speed on 4-CP biodegradation

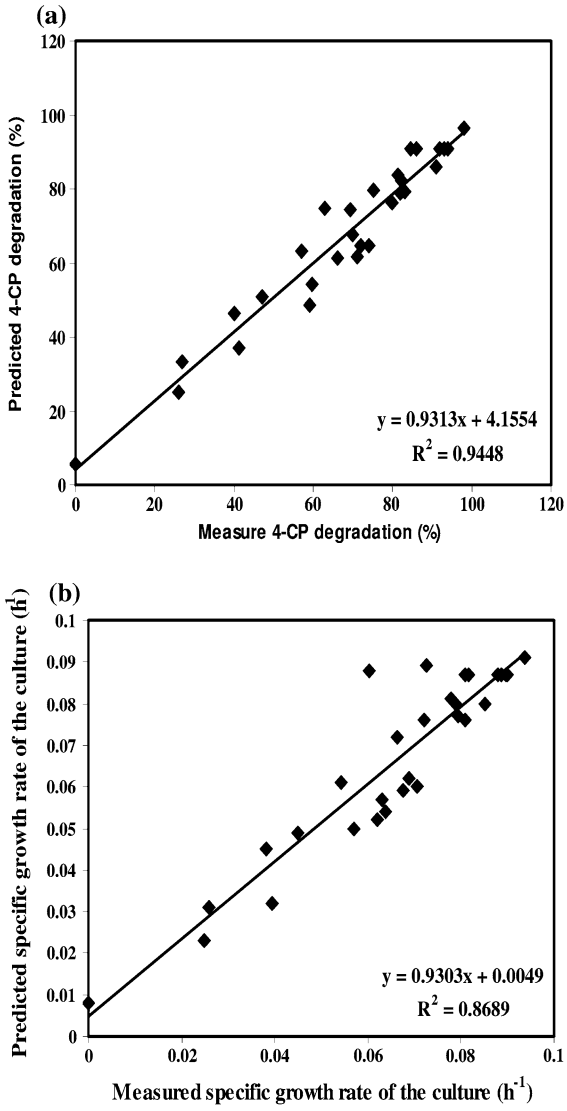

Fig. 2 **(a)** Linear plot of predicted versus measured values of 4-CP degradation in the optimization study. **(b)** Linear plot of predicted versus measured values of specific growth rate of the culture in the optimization study

desirability function method for optimizing both the responses (discussed earlier) optimum values of the culture conditions were estimated to be: pH 7.5; agitation 207 rpm; temperature 29.6°C and inoculum age 39.5 h. These optimum values were experimentally verified in batch shake-flask and the corresponding maximum 4-CP biodegradation efficiency was found to be 100% for an initial 4-CP concentration of 300 mg l^{-1} within 18.5 h of its culture.

Agitation speed is important for maintaining homogenous chemical and physical conditions, dispersion of dissolved oxygen into smaller bubbles for increased interfacial area and oxygen mass transfer rate, and all these factors play a crucial role in enhancing both substrate utilization and growth of microbial cultures (Sharma and Pant 2001). In this study the agitation speed was found to be optimum at 207 rpm, which is in agreement with the literature reports (Sharma and Pant 2001; Khaled and Khleifat 2006). However, any further increase in the agitation rate to 250 rpm did not improve the 4-CP degrading ability by the organism in the present study, which may be attributed to induced shear stress on the cells leading to the cell loss or reduced biomass concentration (Hoq et al. 1995). Moreover, since this particular microorganism utilizes both aerobic and anaerobic metabolic pathway for 4-CP degradation (Nordin et al. 2005) higher agitation speeds may hinder the anaerobic reductive dechlorination step in effective degradation of the compound. Mesophilic temperature range is reported to have a strong impact on microbial degradation of aromatic compounds and plays an equivalent or larger role than nutrient availability in the degradation media (Margesin and Schinner 1997). In the present study it is observed that an optimum culture temperature of 29.58°C was required for complete degradation of 4-CP. Similar observations on biodegradation of phenol, endosulfan and crude oil due to temperature effect are reported by other authors as well (Bandyopadhyay et al. 1998; Sharma and Pant 2001; Hussain et al. 2007). But, temperature above 36°C, in the current study, ceased both the growth and 4-CP degradation rate by the microorganism probably due to inhibition of multi-enzyme complex system of the cell (Bandyopadhyay et al. 1998). pH is another important factor for growth and degradation of chlorophenols by microorganisms, and the optimum pH in this study was found to be 7.5. It is known that at acidic pH values chlorophenols remain in its unionized hydrophobic state, and its toxicity to microorganisms in this state is generally high as it can readily penetrate into the lipid cell membranes of the microorganisms (Penttinen 1995; Leeuwen and Vermeire 2007). Therefore an optimum pH of 7.5 in the study for better degradation of the compound and growth of *A. chlorophenolicus* A6 is quite likely. However, pH values above 7.5 inhibited the growth and chlorophenol degradation due to its negative effect on the activities of phenol oxidase and peroxidase enzymes (Tabatabai 1994; Sinsabaugh

et al. 2008). Similar finding on phenol biodegradation was reported by Khaled and Khleifat (2006). Since the biodegradation media used in the present study contained only 4-CP as the sole source of carbon and energy, inoculum age plays a direct role on the rate and extent of lag phase of the culture. From the optimization results, inoculum age was found to be optimum at 39.5 h, a value which is well supported in literature for phenol degradation (Bandyopadhyay et al. 1998).

At the optimum settings of the culture conditions, the organism was capable of completely degrading (100%) 4-CP for an initial concentration of 300 mg l^{-1} within 18.5 h of its culture. The time required to degrade 4-CP was also found to be much less (by 5.5 h) compared to that required at the unoptimized culture conditions reported earlier by Westerberg et al. (2000). Furthermore, the 4-CP degradation efficiency obtained at the optimum levels of the culture conditions was observed to be 22.99% higher than that obtained using the unoptimized culture conditions. Overall, the results of the study clearly showed very good enhancement in the 4-CP biodegradation efficiency by *A. chlorophenolicus A6* by optimizing the culture conditions employing the non-conventional statistical based design technique.

Growth kinetics of *A. chlorophenolicus A6* for 4-CP biodegradation

Figure 3 shows the time profile of *4-CP* degradation by the *A. chlorophenolicus A6* at its various initial concentrations. It is clear from the profile that the time taken by the organism to degrade the compound mainly depended on its initial concentration. For instance, to degrade 100 mg l^{-1} of 4-CP the culture took about 6 h, but for 350 mg l^{-1} it took a long time of 36 h for complete degradation of the compound. The results also showed that maximum degradation rate achieved was at 100 mg l^{-1} of 4-CP; and low degradation rates were obtained both below and above this concentration thus indicating strong influence of 4-CP concentration on its degradation rate (Hao et al. 2002). It is also observed that towards the end of the substrate consumption curve (Fig. 3) a region of relatively less rate of substrate removal existed in each concentration. Possible explanations towards this phenomenon may be given based on a fall in pH (from 7.5 to 7.1) and depletion of oxygen in

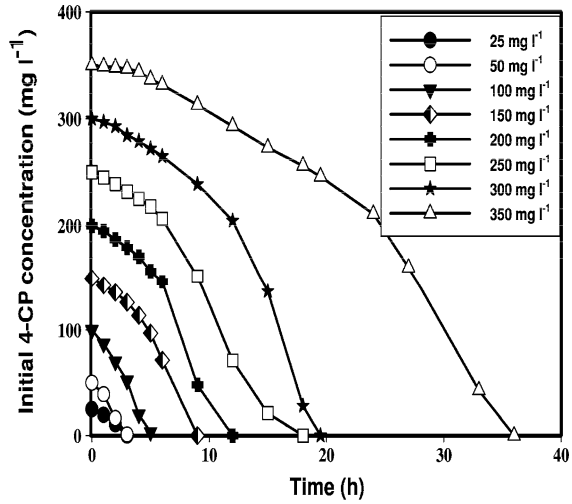

Fig. 3 Time profile of 4-CP degradation by *A. chlorophenolicus A6*

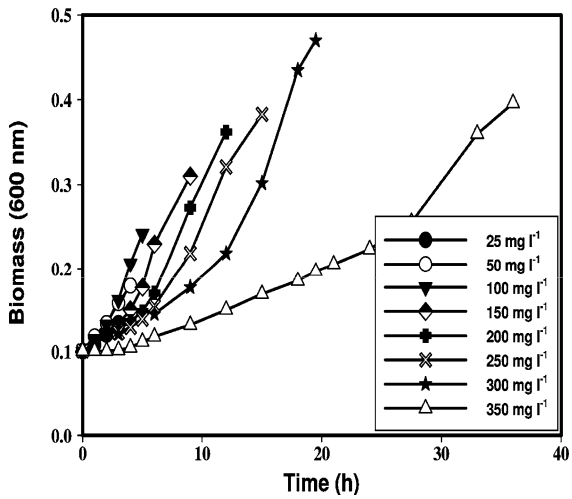

Fig. 4 Time profile of biomass growth (OD 600 nm) at different 4-CP concentration

the culture medium (Yang and Humphrey 1975; Lallai and Mura 1989; Blanch and Clark 1996).

Similar to time taken by the culture to degrade 4-CP at its various initial concentrations, the culture growth also followed a similar pattern. This is illustrated in Fig. 4 where biomass growth (OD 600 nm) of the culture is plotted against time for various 4-CP concentrations in the media. It could be seen from Fig. 4 that 4-CP concentration between 25 and 300 mg l^{-1}did not show any significant repression on the biomass output, but at concentration greater than 300 mg l^{-1}, a lag

phase in the growth was evident. The lag phase observed in its utilization (degradation) and therefore the culture growth could be easily attributed to a highly toxic nature of the compound above a certain level, which in this case was 300 mg l^{-1}. However, at concentrations below 300 mg l^{-1}, no such lag phase was observed (Fig. 4). Moreover, as the 4-CP concentration was increased in the media the culture took more time for complete utilization of the compound (6 h at 100 mg l^{-1} vs. 36 h at 350 mg l^{-1}).

In order to establish the effect of 4-CP concentration on growth of *A. chlorophenolicus A6*, specific growth rates of the culture at different 4-CP concentrations were calculated as per the following relationship:

$$\mu = \frac{1}{X}\frac{dX}{dt} \tag{7}$$

where X is biomass concentration (mg l^{-1}) at time t (h) and μ is the specific growth rate (h^{-1}) (Monod 1949; Kovari and Elgi 1998).

Figure 5 depicts the variation of initial 4-CP specific growth rates with the initial 4-CP concentrations, which shows that the culture specific growth rate increased to 0.163 h^{-1} with the initial 4-CP content up to 100 mg l^{-1} whereas the value decreased to 0.047 h^{-1} from 100 to 350 mg l^{-1}. This clearly indicated inhibitory effect of 4-CP at concentrations above 100 mg l^{-1} on the culture growth. Similarly the 4-CP degradation rate was found to be decreased from 21.16 to 10.43 mg l^{-1} h^{-1} when initial 4-CP concentration in the media was raised from 100 to 350 mg l^{-1}. This type

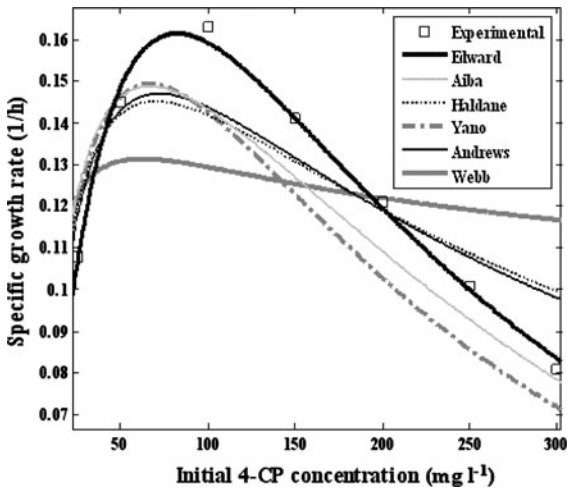

Fig. 5 Experimental and model predicted specific growth rate of the culture at different 4-CP concentrations

of growth behavior by *A. chlorophenolicus A6* due to a high 4-CP concentration clearly indicated substrate inhibition pattern that has been studied by other authors as well (Yan et al. 2006; Bai et al. 2007). In order to predict the patterns of 4-CP 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.

Modeling the growth kinetics of *A. chlorophenolicus A6* in presence of 4-CP

Since the specific growth rate (μ) of the culture was subjected to substrate inhibition due to 4-CP, variation of μ with respect to the 4-CP concentrations were modeled using suitable deterministic models reported in the literature (Edwards 1970; Singh et al. 2008). These model equations (shown in Table 4) were solved using nonlinear regression method using MATLAB 7.0. The aforementioned Fig. 5 shows the experimental specific growth rate and the model predicted ones. From this figure it could be seen that among the six models tested, Edward model was found to fit the data quite accurately. The kinetics parameters estimated from these six models are shown in Table 5 along with root mean square (RMS) error between the substrate inhibition model predicted and experimental specific growth rate of the culture at various 4-CP concentrations. From the Table 5, it is clear that Edward model yielded the least RMS value of 0.003 with a very high correlation coefficient (R^2) value of 0.99 confirming that Edward model best fitted the experimental data.

All the models adopted in this study have generally been used in the literature to describe substrate

Table 5 Estimated values of parameters in the various kinetic models

Model	Model parameters				RMSE	R^2
	μ_{max} (h^{-1})	K_s (mg/l)	K_i (mg/l)	K (mg/l)		
Edward	0.22	30.83	275	–	0.0031	0.99
Aiba et al.	0.25	21.3	275	–	0.0161	0.78
Haldane	0.26	21.91	224.2	–	0.0149	0.77
Yano et al.	0.26	24.53	290	250	0.0160	0.68
Andrews and Noack	0.26	25.2	204.9	–	0.0134	0.81
Webb	0.16	6.8	150	250	0.026	0.30

inhibition on growth of microbial cultures. Therefore, it is more likely that these models fitted the experimental data in the present study to a reasonable level of accuracy. However, some models showed slight deviation in the values of biokinetic constants, such as μ_{max}, K_s and K_i, probably due to their differences in 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). Further, predicted values using the Edward equation correlated well with the experimental data with $R^2 = 0.99$, the fit was valid for 4-CP concentration only up to 300 mg l^{-1}, beyond this concentration the overall prediction of the model was slightly poor. Similar discrepancy in model prediction of experimental specific growth rate due to microorganism was also noted in the literature (Hao et al. 2002).

It is to be mentioned here that while attempting to compare the model parameter values obtained in the present study with the literature, although large number of relevant kinetic studies on phenol were available, only very few literature reports were found on chlorophenol degradation using pure cultures. In the present study maximum specific growth rate (μ_{max}), as per the best fitted model of Edward, was found to be 0.22 h^{-1}. In the literature on 4-CP degradation, Sahinkaya and Dilek (2005) and Goswami et al. (2002) reported μ_{max} values of 0.049 and 0.256 h^{-1} using *Rhodococcus erythropolis M1* and unacclimatized activated sludge, respectively. A larger μ_{max} value (0.22 h^{-1}) obtained in the present study as compared to that obtained using *Rhodococcus erythropolis M1* (0.049 h^{-1}) indicates 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 4-CP used in the experiments. Furthermore, a high μ_{max} value obtained with *A. chlorophenolicus A6* may be attributed to the energy gained from substrate utilization being channelled towards biomass formation rather than for its maintenance. In addition, *A. chlorophenolicus A6* biomass may be shunting very less percentage of the electrons for regeneration of NADPH which is generally used to activate the monoxygenase enzyme involved in 4-CP biodegradation compared to *Rhodococcus erythropolis M1*. However, this aspect needs further investigations to confirm. The value of half saturation coefficient (K_s), which indicates the affinity of biomass to the substrate (Juang and Tsai 2006), was estimated from Edward model (30.83 mg l^{-1}) and was found to correlate well with those found in literature on phenol and *p*-cresol degradation by mixed and pure cultures (Kumaran and Paruchuri 1997; Kumar et al. 2005). However, the higher K_s value obtained in the present study was higher compared with that obtained for 2,4 dichlorophenol (2,4 DCP) degradation by an acclimated mixed culture (Sahinkaya and Dilek 2007); considering the fact that the substrate 2,4 DCP is different than 4-CP any difference in the K_s values is not unlikely. The degree of resistance of the microorganism to toxic effects of 4-CP is indicated by the value of the kinetic parameter K_i, and, in general, a large K_i value reveals that the biomass is highly resistant to inhibition by the substrate. In the present study, the K_i value was estimated to be 275 mg l^{-1} which is larger than the K_i value of 194.4 mg l^{-1} obtained by Sahinkaya and Dilek (2005) for 4-CP degradation using unacclimated activated sludge. 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 microorganisms form hyphae and large micro colonies to enable protection of the inner cell mass thus favoring easy degradation and tolerance to toxic substrates (Golovleva et al. 1992). Also the K_i value in the present study was higher than the value obtained for *Rhodococcus erythropolis M1* (Goswami et al. 2002) indicating good tolerance of *A. chlorophenolicus A6* towards growth and degradation of 4-CP in treating contaminated water. Overall, the study revealed good potential of the microorganism in degrading 4-CP even at very high concentrations.

Conclusions

At the RSM optimized levels of pH, agitation, temperature, and inoculum age, 23% enhancement in the 4-CP biodegradation efficiency by the *A. chlorophenolicus A6* was achieved within 18.5 h of culture. The culture could also degrade 4-CP even at high initial concentration of 375 mg l^{-1} within 40 h, which is superior compared to other literature reports on 4-CP biodegradation.The use of nonconventional,

statistically based design techniques allowed good interpretation of the results obtained, and, therefore, proved useful for enhancing the 4-CP biodegradation efficiency. Further substrate inhibition due to 4-CP on growth of the microorganism was explained using suitable models found from the literature. Among the various models tested, Edward model gave the best fit to the experimental data and the model biokinetic constants evaluated. Overall, the study revealed very good potential of *A. chlorophenolicus A6* culture in treating wastewaters containing highly recalcitrant compounds such as 4-CP.

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