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Optimization study for maximizing 2,4-dichlorophenol degradation by *Kocuria rhizophila* strain using response surface methodology and kinetic study

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ABSTRACT

In the present study, the combined effect of four experimental parameters i.e. pH, temperature (°C), inoculum size % (v/v) and ammonium sulphate concentration (g L^{-1}) at different levels on biodegradation of 2,4-dichlorophenol (2,4-DCP) by newly isolated *Kocuria rhizophila* strain 11Y was investigated using the central composite design. The maximum of 98% degradation for 50 mg L^{-1} 2,4-DCP was observed at an optimized condition, i.e. pH 7.45, temperature 36°C, inoculum size 10% (v/v) and $(NH_4)_2SO_4$ concentration 1.6 g L^{-1} which is 15.2% higher than unoptimized condition. Also, the strain was able to utilize high concentration of 2,4-DCP up to 400 mg L^{-1} at an optimized condition which is much higher than at unoptimized condition and the degradation efficiency increased significantly. The biodegradation kinetic of 2,4-DCP at different initial concentrations shows that the degradation rate increases up to 350 mg L^{-1} and after that it started to decrease. The biodegradation kinetic parameters calculated for 2,4-DCP up to 400 mg L^{-1} were maximum rate of degradation (R_m) = 1.17 mg DCP h^{-1} L^{-1}, half-saturation constant (K_s) = 568.1 mg L^{-1} and inhibition constant (K_i) = 215.63 mg L^{-1}.

Keywords: 2,4-dichlorophenol; Biodegradation; Central composite design; Kinetic; *Kocuria rhizophila*; Optimization

1. Introduction

Chlorophenols are regarded as a serious menace to the environment because of their widespread occurrence in the soil, sediments, sludge products, wastewater, surface waters and groundwater [1,2]. Also, chlorophenols have a detrimental effect on the environment due to its physicochemical properties, which results in higher persistent and subsequent bioaccumulation [3,4]. Chlorophenol compounds have been listed as priority pollutants by the US Environmental Protection Agency [5]. The primary sources of chlorophenols are agricultural application, pulp and paper, leather tanning, biocide, dyes, herbicide, chlorination of drinking and wastewater [1,6–9]. Chlorophenol compounds have been reported for their mutagenicity, carcinogenicity, immunogenicity and fatality [10,11]. Therefore, their fate into the environment is important. The different treatment technologies including physicochemical such as adsorption, photodegradation, chemical oxidation and biological treatment have been developed for the treatment of

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chlorophenol contamination. However, the physico-chemical treatments have the disadvantage of high cost, low-energy efficient and production of toxic by-product [1,12]. The biological treatment is eco-friendly and has a promising removal efficiency with complete mineralization of the toxic compounds [7,13].

Kocuria rhizophila strain 11Y was previously isolated by the author from the soil sample collected from the dye industrial effluent-contaminated site. The isolated strain has been able to tolerate 2,4-dichlorophenol (2,4-DCP) up to 300 mg L^{-1} as sole carbon and energy source. However, the optimization of culture condition can significantly improve the degradation efficiency. Chlorophenols are toxic and have an inhibitory effect on the growth of micro-organism and thus limit the degradation capacity of the micro-organism. This inhibitory effect can be diminished to some extent by optimizing the experimental parameters. Different experimental conditions such as pH, temperature, nutrient and substrate concentration affect the degradation capability of an organism by altering its growth and physical properties of the compound, i.e. 2,4-DCP [14,15]. A specific range of pH value is required for the maximum growth of each micro-organism, extreme pH value i.e. acidic or alkaline condition is an inhibition to the growth of bacteria. During 2,4-DCP biodegradation, the value of pH drops due to the release of chloride ion which affects the growth and degradation capacity of bacteria [16,17]. Temperature affects the growth and activity of micro-organism. At higher temperature, enzymes lose their structure and reduce the degradation efficiency. Also at the low temperature, bacterial activity decreases which increases the inhibition effects of toxic compounds. The optimum temperature range mostly used for biodegradation by the micro-organism is 25–35°C [17,18]. The addition of different carbon and nitrogen sources such as glucose, $(NH_4)_2SO_4$, peptone and yeast extract also alters the degradation capacity of the micro-organism [14,19,20]. Thus, optimization of experimental conditions such as pH, temperature, nitrogen source and inoculum size can improve the degradation efficiency of the *K. rhizophila* strain 11Y. Optimization with conventional method i.e. single parameter at once is time consuming and less economical. However, optimization using a statistical method such as response surface methodology (RSM) is more suitable as it simultaneously optimizes the various parameters and their interaction effects at different levels.

RSM is an important statistical and mathematical design used for determining the influence of different factors on desired response and optimizing the desired response [21]. The RSM is more economical and time saving than conventional techniques as the minimum number of experiments were required for getting the desired optimum response. Several studies have been reported that used RSM for the optimization process. In one such study, the effects of environmental parameters, i.e. pH, temperature, time and enzyme concentration were evaluated on biodegradation of 2,4-DCP with laccase from *Pleurotus* sp. using Box–Behnken design of experiment and the maximum 98% degradation of 2,4-DCP was achieved [22]. In another study, the central composite design of RSM was used for optimizing the multiple responses, i.e. maximum 4-CP biodegradation and specific growth rate. The 4-CP biodegradation efficiency was found 23% higher at RSM-optimized conditions than that obtained at unoptimized culture conditions [16]. A fivefold enhancement in secondary carotenoid lutein production by the green microalgae *Auxenochlorella protothecoides* SAG 211–7a was achieved using the central composite rotatable design of RSM [23].

In the present study, the biodegradation of 2,4-DCP was carried out by *K. rhizophila* strain 11Y. The different experimental parameters were optimized at a different level to achieve the maximum biodegradation of 2,4-DCP and biodegradation kinetic parameters were calculated for the same at optimized conditions. This is the first study showing the biodegradation of 2,4-DCP by *K. rhizophila* species and its optimization to the best of our knowledge.

2. Material and methods

2.1. Micro-organism

The organism used in this study was isolated from the soil collected near the dye industries effluent treatment plant and identified as *K. rhizophila* strain 11Y (GenBank Accession Number: KM522854) by 16s rDNA sequence homology and molecular analysis (Fig. 1). For enrichment and acclimation, 10 gm of soil is added to 100 mL of mineral salt medium (MSM) (composition mentioned elsewhere in paper) containing 0.5 g L^{-1} peptone and 2,4-DCP (up to 200 mg L^{-1}) and incubated at 30°C and 120 rpm in rotary shaker for a period of five months. Enriched culture is transferred to fresh MSM at every 15 d with increasing concentration of 2,4-DCP. The 2,4-DCP-degrading bacterial strains were isolated from the final acclimated culture using serial dilution technique and purified by repeated streaking on MSM agar plates containing 1.5% agar and 50 mg L^{-1} 2,4-DCP. The isolated *K. rhizophila* strain was able to tolerate 2,4-DCP up to 300 mg L^{-1}. The strain was maintained on agar slant containing MSM with 1 g L^{-1} peptone, 50 mg L^{-1} 2,4-DCP and 1.5% agar, pH 7.2.

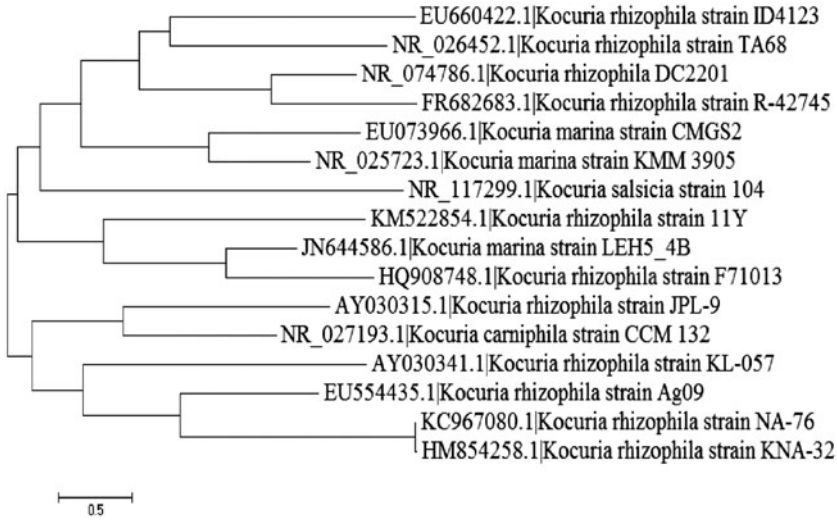

Fig. 1. Phylogenetic tree derived using neighbour-joining method showing an evolutionary relationship of 11 taxa.

2.2. 2,4-DCP degradation study

The degradation of 2,4-DCP was performed in 250-mL Erlenmeyer flask with 50 mL of MSM (modified DSMZ-465) having a composition of (g L^{-1}): $Na_2HPO_4{\cdot}2H_2O$ 3.5, KH_2PO_4 1, $(NH_4)_2SO_4$ 0.5, $MgCl_2{\cdot}6H_2O$ 0.1, $NaNO_3$ 0.05 and 1 mL of trace element solution which contains EDTA 0.5, $FeSO_4{\cdot}7H_2O$ 0.2, $CuCl_2{\cdot}2H_2O$ 0.001, $ZnSO_4{\cdot}7H_2O$ 0.01, $MnCl_2{\cdot}4H_2O$ 0.003, $CoCl_2{\cdot}6H_2O$ 0.02, H_3BO_3 0.03 and $Na_2MoO_4{\cdot}2H_2O$ 0.003. The 2,4-DCP (50 mg L^{-1}) was added to the medium as sole carbon and energy source after autoclaving by filter sterilization with 0.22-μm syringe filter. The inoculum was prepared in MSM with 1 g L^{-1} peptone and 50 mg L^{-1} 2,4-DCP and incubated for 24 h in a rotary shaker. The pH was adjusted using 1 M HCl and 1 M NaOH solution.

2.3. Central composite design

Optimization of experimental parameters to achieve maximum 2,4-DCP biodegradation by *K. rhizophila* strain 11Y was performed by RSM. The experimental parameters selected for optimization were pH, temperature (°C), inoculum size % (v/v) and $(NH_4)_2SO_4$ concentration (g L^{-1}). The central composite design of RSM was used for optimization of experimental parameters. The total number of experimental runs was $n = 2^k + 2k + n_0 = 31$, where k (=4) is the independent variable and n_0 (=7) is the number of centre points used in the design. The factor levels are coded as −1 (low), 0 (central point) and +1 (high). Table 1 represents the coded and actual values of the factor levels used in the experiments. The relationship between the coded and actual values is described by Eq. (1):

$$X_i = \frac{U_i - U_0}{\Delta U} \tag{1}$$

where X_i is the coded level of the independent variable, U_i is the 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 value. The default value of $\alpha = 2$ was taken in the experiment.

Table 1
Independent variables and their corresponding levels used in the optimization study

Factors	Coded Unit	$-\alpha$	−1	0	+1	$+\alpha$
pH	X_1	5	6	7	8	9
Temperature (°C)	X_2	20	25	30	35	40
Inoculum Size[a] % (v/v)	X_3	2	4	6	8	10
$(NH_4)_2SO_4$ (g L^{-1})	X_4	0	0.5	1	1.5	2

[a]one-mL inoculum equals to ~12.78 mg dry biomass.

The second-order polynomial regression model used for fitting the experimental data by response surface method is defined in Eq. (2):

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

where Y = predicted response, k = number factor variables, X_i and X_j = independent variables. The second-order regression model is significant for the optimization process as it counts the interaction effect between variables and surface curvature. The adequacy of the fitted regression model is checked using the analysis of variance (ANOVA) and regression analysis. The robustness of the model can be checked with the help of coefficient of determination (R^2-value) and p-value. All the statistical analyses were performed using the Minitab v16 (trial version), USA.

2.4. Biodegradation kinetic of K. rhizophila

The biodegradation kinetic study of 2,4-DCP was carried out at RSM-optimized condition in 250-mL Erlenmeyer flask with 50 mL of MSM (composition same as above) with different initial 2,4-DCP concentrations of 25, 50, 75, 100, 150, 200, 250, 300, 350 and 400 mg L^{-1}. Peptone having concentration of 0.2 g L^{-1} was added to the medium for boosting the initial growth. The control flask with 50 mg L^{-1} 2,4-DCP in MSM without inoculum was set to check the abiotic loss of 2,4-DCP. The samples are taken at a regular interval for the analysis of residual 2,4-DCP, chloride ion and biomass concentrations.

2.5. Chemicals and reagents

Analytical grade 2,4-DCP (purity 98%) was obtained from the Loba chemie, India. A stock solution of 2,4-DCP is prepared in 0.02 M NaOH and pH was adjusted to 7.4 ± 0.2 by 1 M orthophosphoric acid. All other inorganic chemicals used in the experiments were of analytical grade and obtained from Merck, India. For HPLC analysis, all the reagents were of HPLC grade and obtained from Hi-media, India.

2.6. Analytical methods

Biomass concentration is determined by measuring optical density at 600 nm by UV–visible spectrophotometer (Shimadzu UV-1800, Japan). The residual concentration of 2,4-DCP is determined by HPLC system (Jasco, US) coupled with MD-2015 photodiode array detector and 2080 plus isocratic pump. One mL of sample is centrifuged at 10,000 rpm for 12 min and the supernatant is filtered through a 0.22-μm filter before analysis. The sample volume taken was 20 μL. The column used was Ascentis C18 (15 cm × 4.6 mm); the sample is eluted at a flow rate of 0.75 mL/min with mobile phase consisting of methanol:water:acetic acid (60:38:2%); the detection wavelength is 284 nm where maximum absorbance has occurred. At this condition, the retention time for 2,4-DCP observed is 7.12 min. The 2,4-DCP degradation (%) was calculated by analysing the area under the curve. Electrospray ionization (ESI) mass spectroscopy (Perkin Elmer, Flexar SQ300 MS Detector) was used for identification of dichlorophenol and their biodegradation products. The analysis was carried out in splitless mode; range of mass scan 50–400; with temperatures as follows: injector 260˚C, detector 280˚C; gradient programme: 65–96˚C (4˚C min^{-1}), 96–160˚C (8˚C min^{-1}) and up to 230˚C (12˚C min^{-1}). The MS was equipped with a HP1 column of 30 m length, 0.25 mm internal diameter and 0.25 μm film thickness. Helium was used as the carrier gas at a flow rate of 2.9 ml min^{-1} [12].

The concentration of chloride ions in the samples was analysed colorimetrically by EPA 9215 method [24]. A volume of 0.5 mL of culture supernatant is mixed with 0.5 mL of colour reagent and the mixture is kept for 10 min for colour development. Colour reagent is prepared by equally mixing solution A (saturated solution of mercuric thiocyanate in methanol) and solution B (20.2% ferric nitrate solution in 9 M nitric acid). The absorbance was measured by UV–vis spectrophotometer (Shimadzu UV-1800, Japan) at 460 nm. The chloride ion as quantified against the standard curve of sodium chloride (5–200 mg L^{-1}) [24,25].

3. Result and discussion

3.1. Optimization of experimental parameters using CCD

The optimization of experimental parameters for achieving maximum biodegradation of 2,4-DCP by *K. rhizophila* strain 11Y was successfully performed using RSM. The effect of all four independent variables i.e. pH, temperature (˚C), inoculum size % (v/v) and $(NH_4)_2SO_4$ (g L^{-1}) at different levels was simultaneously analysed on 2,4-DCP biodegradation. The experimental condition and the corresponding per cent degradation obtained as per central composite design are summarized in Table 2. The experimental data were analysed in terms of the second-order polynomial equation and ANOVA.

Table 2
Central composite design of experiments and % biodegradation of 2,4-DCP

Run Order	pH	Temperature (°C)	Inoculum Size % (v/v)	$(NH_4)_2SO_4$ (g L^{-1})	% Degradation	
					Experimental	Predicted
1	0	0	0	0	62	60.14
2	0	2	0	0	70	71
3	0	0	0	0	59	60.14
4	0	0	2	0	90	93.66
5	2	0	0	0	35	38.5
6	0	0	0	0	59	60.14
7	0	0	−2	0	42	49.66
8	1	−1	−1	1	34	33.58
9	0	0	0	0	60	60.14
10	0	−2	0	0	21	31.33
11	−1	−1	1	−1	56	51.41
12	−1	−1	1	1	62	60.25
13	1	1	1	−1	78	77.08
14	1	−1	1	−1	52	49.75
15	1	1	−1	−1	55	52.58
16	1	1	−1	1	63	60.41
17	−1	1	−1	1	55	53.08
18	1	−1	−1	−1	31	25.25
19	0	0	0	0	61	60.14
20	1	1	1	1	85	86.41
21	0	0	0	2	63	66.5
22	0	0	0	0	61	60.14
23	0	0	0	0	58	60.14
24	−1	1	1	−1	68	64.25
25	−1	1	1	1	74	72.58
26	−2	0	0	0	25	32.83
27	−1	1	−1	−1	48	46.25
28	1	−1	1	1	65	59.58
29	−1	−1	−1	1	47	40.75
30	0	0	0	−2	42	49.83
31	−1	−1	−1	−1	39	33.41

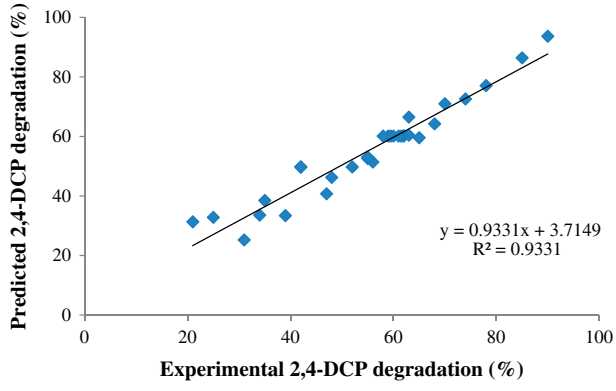

Fig. 2. Linear plot for experimental vs. predicted biodegradation of 2,4-dichlorphenol.

Fig. 2 shows the relationship between the experimental and predicted 2,4-DCP biodegradation. The coefficient of determination (R^2) and adjacent (R^2) obtained was 0.933 and 0.874, respectively, which are closer to 1, which indicates that the model is sufficiently significant and fits the data. The ANOVA and regression coefficient for 2,4-DCP degradation are summarized in Tables 3 and 4, respectively. The *F*-value and *p*-value were used to test the significance of the regression model. Higher *F*-value means that the model significantly explains the relation between the dependent and independent variables. The observed *F*-value for regression model is 15.94 which is higher than critical *F*-value ($F_{0.05,14,16} = 2.33$) at a significant level of $p = 0.05$. This implies that the regression model is significant and explains all the variations. The observed *F*-value for linear and square

Table 3
ANOVA for % biodegradation of 2,4-DCP

Source	DF	Seq SS	Adj SS	Adj MS	F	p
Regression	14	7525.55	7525.55	537.54	15.94	0
Linear	4	5,729	5,729	1432.25	42.46	0
Square	4	1540.55	1540.55	385.14	11.42	0
Interaction	6	256	256	42.67	1.26	0.327
Residual Error	16	539.69	539.69	33.73		
Lack-of-Fit	10	527.33	527.33	52.73	25.6	0
Pure Error	6	12.36	12.36	2.06		
Total	30	8065.24				
$R^2 = 93.31\%$; $R^2_{(adj)} = 87.45\%$						

Notes: DF—degree of freedom; Seq SS—sequential sum of squares; Adj MS—adjusted means square.

Table 4
Regression coefficient for 2,4-DCP biodegradation

Term	Coefficient	SE coefficient	T	p
Constant	−216.649	2.195	27.398	0
X_1	59.9583	1.186	1.195	0.25
X_2	2.34405	1.186	8.365	0
X_3	−9.20536	1.186	9.279	0
X_4	8.03571	1.186	3.515	0.003
X_1^2	−6.11905	1.086	−5.634	0
X_2^2	−0.08976	1.086	−2.066	0.05
X_3^2	0.720238	1.086	2.653	0.017
X_4^2	−1.97619	1.086	−0.455	0.655
$X_1\ X_2$	0.725	1.452	2.497	0.024
$X_1\ X_3$	0.8125	1.452	1.119	0.28
$X_1\ X_4$	0.5	1.452	0.172	0.865
$X_2\ X_3$	−1.14E-16	1.452	0	1
$X_2\ X_4$	−0.05	1.452	−0.086	0.932
$X_3\ X_4$	0.375	1.452	0.258	0.799

terms is greater than critical $F_{0.05,4,16} = 3$ which implies their significant role in the regression model for 2,4-DCP biodegradation. While in case of interaction terms, the critical $F_{0.05,6,16} = 2.74$ is higher than the calculated $F = 1.26$ implying their insignificance in the regression model.

The ANOVA table summarizes the linear, squared and interaction terms. The small p-value for linear and square terms indicates their significant contribution to the regression model. The p-value for interaction terms is $0.327 > 0.05$ indicating their insignificant effect on the regression model. From Table 4, it was concluded that the main effect of temperature, inoculum size and ammonium sulphate concentration is significant at an individual significant level of $p = 0.05$. The small p-value ($p < 0.05$) for quadratic terms pH × pH, Temperature × Temperature and Inoculum size × Inoculum size and also for the interaction terms pH × pH indicate their effects are statistically significant. Thus, the regression model equation (in un-coded form) showing the effect of all four independent variables including interaction effect on 2,4-DCP biodegradation can be presented as below:

$$Y = -216.64 + 2.34X_2 - 9.2X_3 + 8.04X_4 - 6.12X_1^2 - 0.08X_2 + 0.72X_3 + 0.73X_1X_2 \quad (3)$$

where Y = 2,4-DCP biodegradation, X_1 is the pH, X_2 is the temperature (℃), X_3 is the inoculum size % (v/v) and X_4 is the $(NH_4)_2SO_4$ concentration (g L^{-1}).

The interaction effect between independent variables on response was well illustrated using the contour plot and response surface. The interaction effect of the pH and temperature is illustrated using contour plot in Fig. 3(a), while keeping other two factors at middle setting. The contour plot is elliptical in shape showing a significant interaction between the factors. From the plot, it was seen that the % biodegradation increases with temperature and maximum biodegradation obtained in was the range of 35–40℃, whereas the optimum pH condition was around 7.5. However, it was observed that the bacterial growth rate decreases at 40℃. Fig. 3(b) shows the effect of interaction between the pH and $(NH_4)_2SO_4$ concentration while keeping other two factors at middle setting on biodegradation. From the shape of contour plot, it could be observed that the effect of pH is more significant than $(NH_4)_2SO_4$ concentration on the response. Also, the % biodegradation increases with $(NH_4)_2SO_4$ concentration. Using the middle-point setting, the optimum values for maximum 2,4-DCP biodegradation are around 7.3–7.5 for pH and are in the range of 1.5–2 g L^{-1} for $(NH_4)_2SO_4$ concentration. The interaction

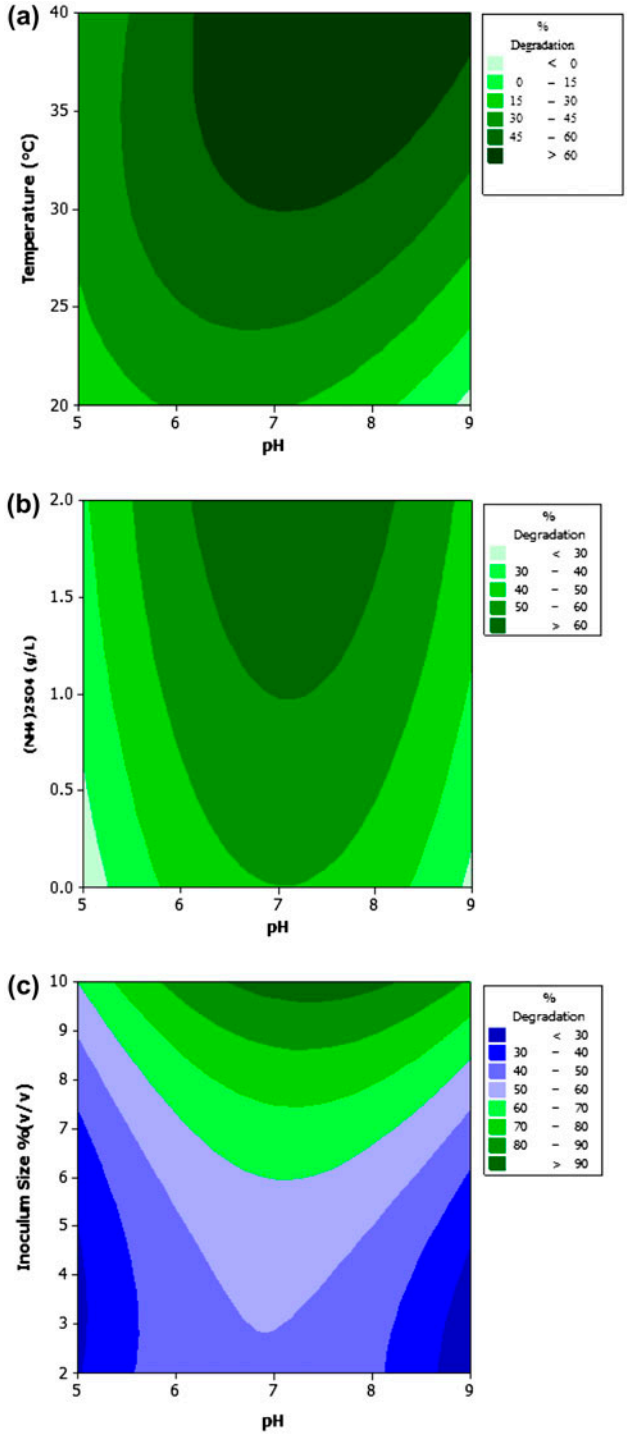

Fig. 3. (a) Contour plot showing the interaction effect of pH and temperature (°C) on 2,4-DCP biodegradation, (b) Contour plot showing the interaction effect of pH and $(NH_4)_2SO_4$ (g L^{-1}) on 2,4-DCP biodegradation and (c) Contour plot showing the interaction effect of pH and inoculum size % (v/v) on 2,4-DCP biodegradation.

effect between the other factors appears non-significant on the response. The effect of inoculum size and pH on % biodegradation, while keeping other factors at middle setting, was shown in Fig. 3(c). From the figure, it can be seen that a high-inoculum size has pronounced effect on biodegradation. This effect diminishes with a decrease in the inoculum size. The internally studentized residuals were analysed to check the model adequacy. The analyses show that all the studentized residuals, except four, have values under two. The normality plot of residuals shows that all the residuals fall along the straight line (Fig. 4).

Using the desirability function, the optimum values of experimental parameters obtained were pH 7.45, temperature 36°C, inoculum size 10% (v/v) and $(NH_4)_2SO_4$ concentration 1.6 g L^{-1}. These optimum values were verified experimentally in batch mode using shake flask culture. The maximum of 98% degradation for 50 mg L^{-1} 2,4-DCP within 20 d was observed which is 15.2% higher than at unoptimized conditions. Also, the strain was able to degrade high concentration of 2,4-DCP up to 400 mg L^{-1} at optimized conditions which is much higher than at unoptimized conditions. The strain was able to degrade up to 300 mg L^{-1} 2,4-DCP at unoptimized conditions after that inhibition effect used to prevail.

There are several reports on optimization of different environmental parameters for improvising the biodegradation of phenolic compounds using RSM. RSM proves effective in terms of economic aspect, time and resource utilization for the optimization process compared to traditional methods. The ammonium sulphate is important as it provides nitrogen source which is significant for bacterial growth and expression of different enzymes. Ammonium sulphate is a cheap and easily available nitrogen source as compared to other nitrogen sources such as amino acids, yeast extract and peptone. Optimum nitrogen concentration is significant for the micro-organism to show maximum enzyme activity. The effect of ammonium sulphate as nitrogen source on biodegradation of various toxic compounds such as phenol, 2,4-DCP and also on enzyme expression has also been reported [20,26,27]. Temperature has an equivalent role as nutrient on micro-organism activity and biodegradation. In the present study, optimum temperature obtained was 36°C. However, the isolate has shown activity up to 40°C temperature, but there was no significant increase in the growth and degradation efficiency observed. Most of the mesophilic micro-organisms reported in the literature have an optimum temperature range from 28 to 35°C for biodegradation [14,16,26]. The optimum pH obtained

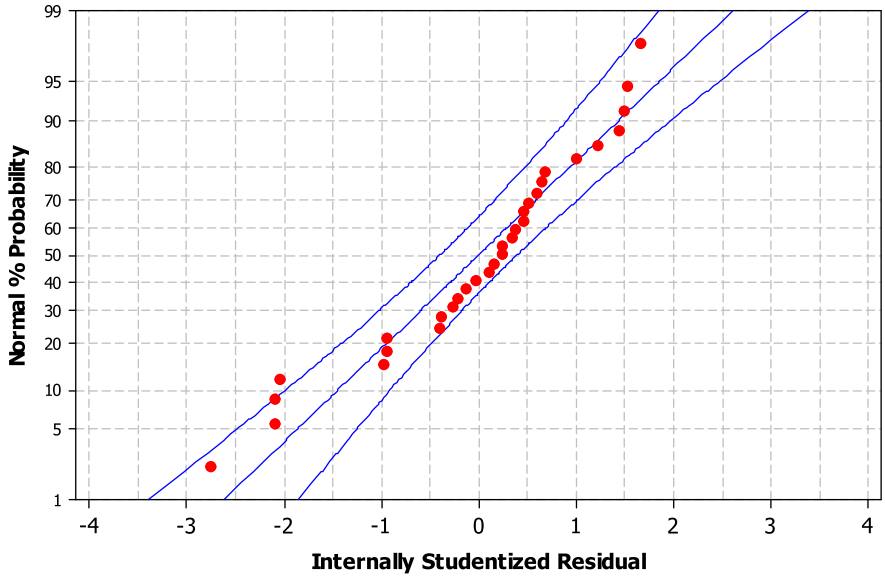

Fig. 4. Normal probability of internally studentized residuals for % biodegradation of 2,4-DCP.

was 7.45 which is in well agreement with the literature. At acidic pH, the toxicity of chlorophenols is higher because at this condition chlorophenols are generally present in their unionized states which are readily absorbed to skin and lipid membrane leading to decreased biomass growth and biodegradation [8,16,28]. The pH above 7.45 has an adverse effect on enzymes leading to decreased biodegradation of 2,4-DCP. The optimum pH for the maximum removal of chlorophenols reported in the literature was in the range of 7.0–7.5 [14,16]. The inoculum size or biomass concentration also has an effect on the biodegradation of chlorophenol compounds. With increasing inoculum size, biodegradation rate also increases. However, it was also reported that after certain inoculum size, an increase in inoculum size does not have a significant effect on growth and biodegradation due to the nutrient limitation condition [29,30].

3.2. Biodegradation kinetic of 2,4-DCP

The biodegradation of 2,4-DCP by *K. rhizophila* strain 11Y was carried out at RSM-optimized conditions. The strain was able to degrade efficiently 2,4-DCP up to 400 mg L^{-1} at optimized conditions. The residual concentration of 2,4-DCP and growth profile was shown in Figs. 5 and 6. The isolated strain was able to degrade up to 98% of 25 and 50 mg L^{-1} 2,4-DCP, whereas around 70–85% degradation was observed for 75 to 350 mg L^{-1} 2,4-DCP. However, the degradation was drastically decreased to 41% for 400 mg L^{-1} 2,4-DCP. In case of unoptimized conditions,

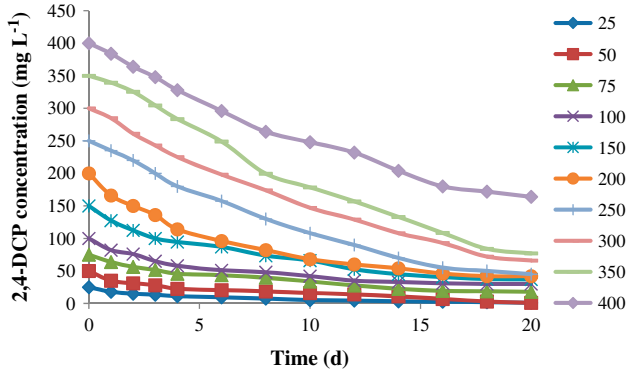

Fig. 5. Residual concentration of 2,4-DCP and its biodegradation profile by *K. Rhizophila* 11Y.

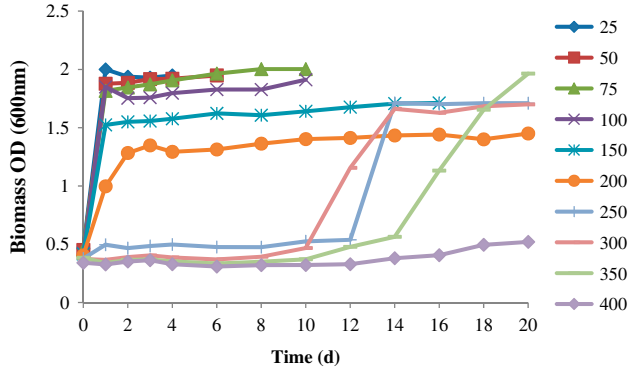

Fig. 6. Biomass growth profile of *K. Rhizophila* 11Y at different initial 2,4-DCP concentrations.

the degradation observed for 300 mg L^{-1} 2,4-DCP was 47% indicating that the degradation capacity of the isolate was greatly increased at optimized conditions. From the growth profile, it was observed that the inhibition effect was greatly prevailed after 200 mg L^{-1} 2,4-DCP which sharply increased for 400 mg L^{-1}. The lag phase was increased after 200 mg L^{-1} 2,4-DCP showing an inhibition effect. It was reported that at higher toxicity level, the micro-organism uses different bioenergetic strategies and gives more energy to biodegradation than biomass growth. While at lower toxicity, the microbes give more energy to biomass growth than biodegradation. So at higher 2,4-DCP concentration, a decreased proliferation of the microbes has been observed. However, at 400 mg L^{-1}, the inhibition effect is large and the prolonged lag phase was observed which can be the reason for lower degradation at 400 mg L^{-1}. The dechlorination of 2,4-DCP was confirmed from the ESI mass spectroscopy and decrease in pH of the medium. Fig. 7 shows the HPLC chromatogram of biodegradation of 300 mg L^{-1} 2,4-DCP at 0 and 20 d. A significant change in the peak area confirms the degradation of 2,4-DCP and production of metabolites. The metabolites were also confirmed using ESI mass spectroscopy analysis which shows the presence of single-chlorinated and non-chlorinated compounds in the degradation products.

The biodegradation kinetic for 2,4-DCP by *K. rhizophila* strain 11Y was performed using the Haldane/Andrew's substrate inhibition model described by Kargi and Eker [31,32].

$$R_s = \frac{R_m S}{K_s + S}\frac{K_{si}}{K_{si} + S} = \frac{R_m}{\left(1 + \frac{K_s}{S}\right)\left(1 + \frac{S}{K_{si}}\right)} \quad (4)$$

where R_s and R_m are the actual and maximum rates of 2,4-DCP degradation (mg DCP L^{-1} h^{-1}); S is the initial 2,4-DCP concentration (mg L^{-1}); K_s is the saturation constant (mg L^{-1}); K_{si} is the 2,4-DCP inhibition constant (mg L^{-1}).

For lower substrate concentration 2,4-DCP < 350 mg L^{-1}, the inhibition constant can be neglected. Hence, the above equation becomes:

$$R_s = \frac{R_m S}{K_s + S} \quad (5)$$

In the linear form:

$$\frac{1}{R_s} = \frac{1}{R_m} + \frac{K_s}{R_m}\frac{1}{S} \quad (6)$$

The plot between the $1/R_s$ vs. $1/S$ was plotted for experimental data (Fig. 8). The plot is linear with the slope of K_s/R_m and the intercept of $1/R_m$. From the best fit line, the following values were obtained for biokinetic parameters:

R_m = 1.17 mg DCP L^{-1} h^{-1} and K_s = 568.1 mg L^{-1} (R^2 = 0.986)

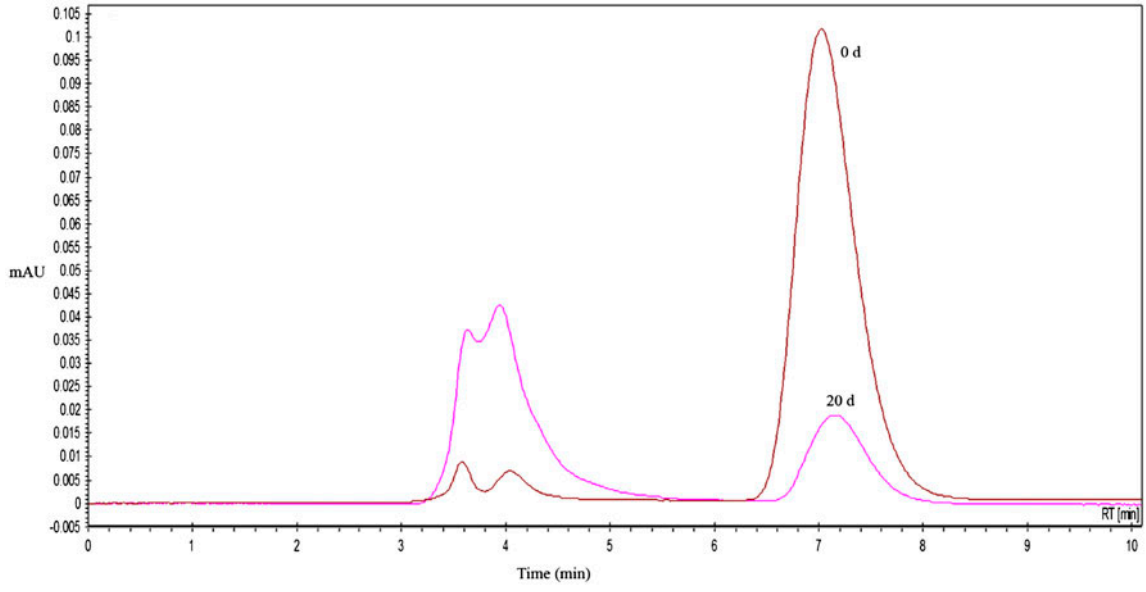

Fig. 7. HPLC chromatogram showing the biodegradation of 300 mg L−1 2,4-DCP within 20 d. The retention time for 2,4-DCP is 7.12 min.

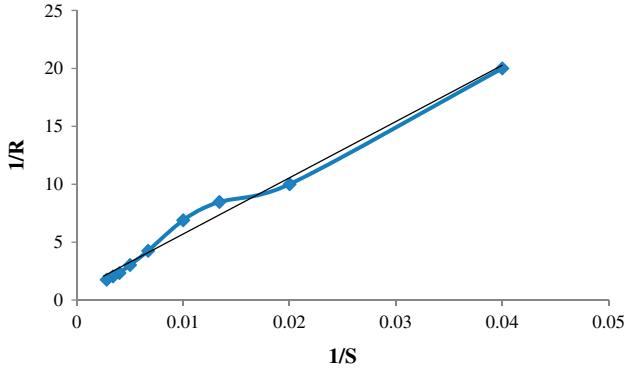

Fig. 8. Double-reciprocal plot between $1/R$ vs. $1/S$.

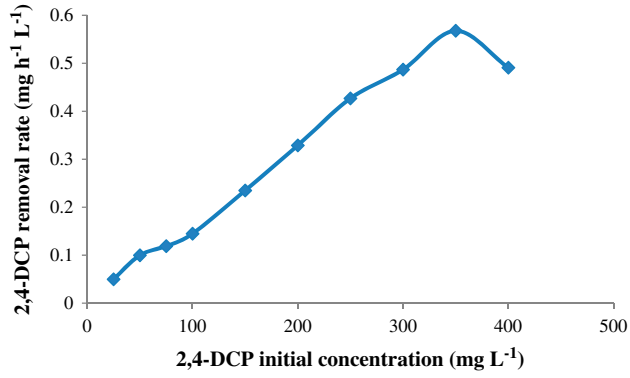

Fig. 9. Relationship between the biodegradation rate at different initial 2,4-DCP concentrations.

2,4-DCP is a toxic compound and after a certain concentration it imposes self-inhibitory effects on the micro-organism. This is called critical substrate concentration above which the removal rate decreases [33,34]. Critical substrate concentration can be obtained by taking the derivation of Eq. (4) with respect to S.

$$\frac{dR_s}{dS} = 0 \tag{7}$$

So, from Eq. (4):

$$S_{max} = \sqrt{K_s K_i} \tag{8}$$

From Fig. 9, it can be observed that the substrate concentration where biodegradation rate is maximum is $S_{max} = 350$ mg L^{-1}. So from Eq. (8), the value of inhibition constant obtained was $K_i = 215.63$ mg L^{-1}.

The biokinetic parameters obtained for the biodegradation of 2,4-DCP by *K. rhizophila* strain 11Y were in agreement with the literature. The maximum biodegradation rate (R_m) obtained in the present study was 1.17 mg DCP L^{-1} h^{-1}. Kargi and Eker [31] and Herrera et al. [12] reported the R_m value of 1.28 and 0.71 mg DCP L^{-1} h^{-1} for the degradation of 2,4-DCP using *P. putida* CP1 and *Bacillus* consortium, respectively. The half-saturation constant indicates the substrate affinity of the micro-organism. Kargi and Eker [31] and Ma et al. [35] reported the K_s value of 427 and 175.2 mg L^{-1} for 2,4-DCP degradation by *P. putida* CP1 and aerobic granules, respectively. A slightly higher value of K_s (568.1 mg L^{-1}) obtained in the present study indicated a bit less affinity of the isolate at low substrate concentration and micro-organism attain its the maximum removal rate at higher substrate concentration. The inhibition constant (K_i) is important as it expresses the inhibition effect of the substrate on the micro-organism. A higher K_i indicates the less inhibition effect of substrate on the micro-organism. The value of K_i obtained in the study was 215.63 mg L^{-1} which is in a good correlation with the literature study [31,35,36]. Goswami et al. [37] and Sahinkaya and Dilek [33] reported the K_i value of 44.46 and 81.34 mg L^{-1}, respectively. The higher value of K_i indicates the higher resistance of *K. rhizophila* 11Y for 2,4-DCP up to 400 mg L^{-1} showing the good potential of the isolate for the removal of 2,4-DCP.

4. Summary

The experimental parameters were successfully optimized using RSM for achieving the maximum biodegradation of 2,4-DCP. The biodegradation efficiency of 2,4-DCP by *K. rhizophila* strain 11Y was increased to 15.2% at RSM-optimized conditions. Also, 2,4-DCP utilization capacity of strain increased from 300 to 400 mg L^{-1} with minimum inhibition effect at the same conditions. So, the optimization of experimental parameters for achieving maximum biodegradation of 2,4-DCP using RSM was proven effective. The biodegradation kinetic of 2,4-DCP at a different initial concentration by *K. rhizophila* strain 11Y shows that the biodegradation rate almost linearly increases up to 350 mg L^{-1} and after that it dropped due to prevailing inhibitory effect. The biokinetic parameters obtained using the experimental data were $R_m = 1.17$ mg DCP L^{-1} h^{-1}, $K_s = 568.1$ mg L^{-1} and $K_i = 215.63$ mg L^{-1}. Overall, the strain *K. rhizophila* 11Y shows good potential for treating the 2,4-DCP contamination in the environment. Also, the information obtained from the study can be useful for effective implementation of treatment technology for biodegradation of 2,4-DCP.

References

[1] A.O. Olaniran, E.O. Igbinosa, Chlorophenols and other related derivatives of environmental concern: Properties, distribution and microbial degradation processes, Chemosphere 83 (2011) 1297–1306.

[2] C.J. Snyder, M. Asghar, J.M. Scharer, R.L. Legge, Biodegradation kinetics of 2,4,6-Trichlorophenol by an acclimated mixed microbial culture under aerobic conditions, Biodegradation 17 (2006) 535–544.

[3] C.T. Chiou, D.W. Schmedding, M. Manes, Improved prediction of octanol−water partition coefficients from liquid−solute water solubilities and molar volumes, Environ. Sci. Technol. 39 (2005) 8840–8846.

[4] Y.G. Lee, S.H. Hwang, S.D. Kim, Predicting the toxicity of substituted phenols to aquatic species and its changes in the stream and effluent waters, Arch. Environ. Contam. Toxicol. 50 (2006) 213–219.

[5] ATSDR, Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), Priority List of Hazardous Substances, (2011).

[6] A.K. Marihal, K.S. Jagadeesh, S. Sinha, Biodegradation of PCP by the Rhizobacteria Isolated from Pentachlorophenol-tolerant Crop Species, Int. J. Civ. Environ. Eng. 1 (2009) 190–194.

[7] J. Field, R. Sierra-Alvarez, Microbial degradation of chlorinated phenols, Rev. Environ. Sci. Biotechnol. 7 (2008) 211–241.

[8] ATSDR, Toxicological profile for Chlorophenols. U.S. Department of Health and Human Services, Atlanta, GA, (1999).

[9] Q. Wang, Y. Li, J. Li, Y. Wang, C. Wang, P. Wang, Experimental and kinetic study on the cometabolic biodegradation of phenol and 4-chlorophenol by psychrotrophic *Pseudomonas putida* LY1, Environ. Sci. Pollut. Res. (2014) 1–9.

[10] V. Daniel, W. Huber, K. Bauer, C. Suesal, J. Mytilineos, A. Melk, C. Conradt, G. Opelz, Association of elevated blood levels of pentachlorophenol (PCP) with cellular and humoral immunodeficiencies, Arch. Environ. Health: An Int. J. 56 (2001) 77–83.

[11] IARC, IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Some Halogenated Hydrocarbons and Pesticide Exposures, vol. 41, International agency for research on cancer, Lyon, France, (1986).

[12] Y. Herrera, A. Okoh, L. Alvarez, N. Robledo, M. Trejo-Hernández, Biodegradation of 2,4-dichlorophenol by a *Bacillus* consortium, World J. Microbiol. Biotechnol. 24 (2008) 55–60.

[13] C.S. Karigar, S.S. Rao, Role of microbial enzymes in the bioremediation of pollutants: A review, Enzyme Res. 2011 (2011) 1–11.

[14] T. Al-Khalid, M.H. El-Naas, Aerobic biodegradation of phenols: A comprehensive review, Cri. Rev. Environ. Sci. Technol. 42 (2012) 1631–1690.

[15] M. Czaplicka, Sources and transformations of chlorophenols in the natural environment, Sci. Total Environ. 322 (2004) 21–39.

[16] N.K. Sahoo, K. Pakshirajan, P.K. Ghosh, A. Ghosh, Biodegradation of 4-chlorophenol by *Arthrobacter chlorophenolicus* A6: Effect of culture conditions and degradation kinetics, Biodegradation 22 (2011) 275–286.

[17] A. Sharma, I.S. Thakur, P. Dureja, Enrichment, isolation and characterization of pentachlorophenol degrading bacterium *Acinetobacter* sp. ISTPCP-3 from effluent discharge site, Biodegradation 20 (2009) 643–650.

[18] S.K. Karn, S.K. Chakrabarty, M.S. Reddy, Pentachlorophenol degradation by *Pseudomonas stutzeri* CL7 in the secondary sludge of pulp and paper mill, J. Environ. Sci. 22 (2010) 1608–1612.

[19] G. Annadurai, R.-S. Juang, D.-J. Lee, Microbiological degradation of phenol using mixed liquors of *Pseudomonas putida* and activated sludge, Waste Manage. 22 (2002) 703–710.

[20] G. Annadurai, L.Y. Ling, J.-F. Lee, Statistical optimization of medium components and growth conditions by response surface methodology to enhance phenol degradation by *Pseudomonas putida*, J. Hazard. Mater. 151 (2008) 171–178.

[21] D.C. Montgomery, Design and Analysis of Experiments, third ed., Wiley, New York, NY, 1991.

[22] S.S. Bhattacharya, R. Banerjee, Laccase mediated biodegradation of 2,4-dichlorophenol using response surface methodology, Chemosphere 73 (2008) 81–85.

[23] S.D. Shinde, S.S. Lele, Statistical media optimization for lutein production from microalgae *Auxenochlorella protothecoides* SAG 211-7A, Int. J. Adv. Biotechnol. Res. 1 (2010) 104–114.

[24] EPA, Method 9251—Chloride—Colorimetric, Auto Ferricyanide AAII. US Environmental Protection Agency, SW-846 ch5, (1986).

[25] R. Chandra, A. Ghosh, R.K. Jain, S. Singh, Isolation and characterization of two potential pentachlorophenol degrading aerobic bacteria from pulp paper effluent sludge, J. Gen. Appl. Microbiol. 52 (2006) 125–130.

[26] A.M. Farag, H.M. Abd-Elnaby, Degradation of phenol by a new-degradable marine halophilic fungus *Fennellia Flavipes* isolated from mangrove sediments, Life Sci. J. 11 (2014) 836–845.

[27] O. Rubilar, M.C. Diez, L. Gianfreda, Transformation of chlorinated phenolic compounds by white rot fungi, Crit. Rev. Environ. Sci. Technol. 38 (2008) 227–268.

[28] WHO, Environmental Health Criteria 93: Chlorophenols other than pentachlorophenol, International Programme on Chemical Safety, WHO, Geneva, Switzerland, (1989).

[29] B. Basak, B. Bhunia, S. Dutta, A. Dey, Enhanced biodegradation of 4-chlorophenol by *Candida tropicalis* PHB5 via optimization of physicochemical parameters using Taguchi orthogonal array approach, Int. Biodeterior. Biodegrad. 78 (2013) 17–23.

[30] J. Balfanz, H.-J. Rehm, Biodegradation of 4-chlorophenol by adsorptive immobilized *Alcaligenes* sp. A 7-2 in soil, Appl. Microbiol. Biotechnol. 35 (1991) 662–668.

[31] F. Kargi, S. Eker, Kinetics of 2,4-dichlorophenol degradation by *Pseudomonas putida* CP1 in batch culture, Int. Biodeterior. Biodegrad. 55 (2005) 25–28.

[32] J.F. Andrews, A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates, Biotechnol. Bioeng. 10 (1968) 707–723.

[33] E. Sahinkaya, F.B. Dilek, Effect of feeding time on the performance of a sequencing batch reactor treating a mixture of 4-CP and 2,4-DCP, J. Environ. Manage. 83 (2007) 427–436.

[34] M.C. Tomei, M.C. Annesini, S. Bussoletti, 4-nitrophenol biodegradation in a sequencing batch reactor: Kinetic study and effect of filling time, Water Res. 38 (2004) 375–384.
[35] J.-Y. Ma, X.-C. Quan, Z.-F. Yang, A.-J. Li, Biodegradation of a mixture of 2,4-dichlorophenoxyacetic acid and multiple chlorophenols by aerobic granules cultivated through plasmid pJP4 mediated bioaugmentation, Chem. Eng. J. 181–182 (2012) 144–151.
[36] Y. Gu, R.A. Korus, Kinetics of pentachlorophenol degradation by a *Flavobacterium* species, Appl. Microbiol. Biotechnol. 43 (1995) 374–378.
[37] M. Goswami, N. Shivaraman, R.P. Singh, Kinetics of chlorophenol degradation by benzoate-induced culture of *Rhodococcus erythropolis* M1, World J. Microbiol. Biotechnol. 18 (2002) 779–783.