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ORIGINAL PAPER

Response surface methodology mediated optimization of Remazol Orange decolorization in plain distilled water by *Pseudomonas aeruginosa* BCH

S. B. Jadhav · S. N. Surwase · S. S. Phugare · J. P. Jadhav

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Abstract The bacterium *Pseudomonas aeruginosa* BCH decolorized and degraded the sulphonated azo dye Remazol Orange in plain distilled water. The effects of different parameters, i.e. pH, temperature and cell mass concentration on the biodegradation of dye in aqueous phase was evaluated using response surface methodology. Optimization was carried out using three-level Box–Behnken design. Predicted values were found to be in good agreement with experimental values (R^2 0.9997 and pred R^2 0.9984), which indicated suitability of the employed model and the success of response surface methodology. Optimum dye decolorization was maximized and the favourable conditions were pH 7.43, temperature 29.39 °C and cell mass concentration 2.88 g l^{-1}, which resulted in 96.01 % decolorization within 5 h. It was validated from the predicted response (97.37 %). According to the analysis of variance results, the proposed model can be used to navigate the design space. 3D plot analysis disclosed the significant interaction between all three tested factors on decolorization process. The combinations of the three variables predicted during response surface methodology were confirmed through confirmatory experiments. Observations indicated that higher cell mass accelerated the decolorization process. Degradation of the dye was verified through high performance liquid chromatography analysis. Phytotoxicity studies carried out with dye and dye metabolites using *Phaseolus mungo*, *Triticum aestivum* and *Sorghum vulgare* indicated the detoxification of dye.

Keywords Biodegradation · Box–Behnken design · High performance liquid chromatography · Phytotoxicity

S. B. Jadhav · S. S. Phugare · J. P. Jadhav
Department of Biochemistry, Shivaji University, Kolhapur 416004, India

S. N. Surwase
Department of Microbiology, Shivaji University, Kolhapur 416004, India

J. P. Jadhav (✉)
Department of Biotechnology, Shivaji University, Kolhapur 416004, India
e-mail: jpjbiochem@gmail.com

Introduction

Azo dyes are the most widely and diversely used textile dyes. Besides their negative aesthetic effects, these substances may show toxicity to aquatic life, can be responsible for allergenic effects and are often potentially carcinogenic or mutagenic to humans (Brown and Hamburger 1987). Generally, it is assumed that the first step in the biodegradation of azo compounds is the reduction to the corresponding amines, a reaction catalyzed by azoreductase. Then, the resultant aromatic amines are further degraded aerobically (Sandhya et al. 2005). Moreover, conventional aerobic wastewater treatment processes, such as activated sludge, cannot usually efficiently remove the colour of azo dyes, since these compounds are often recalcitrant aerobically (Chang et al. 2001). So, the need to develop a novel biological process leading to a more effective cleanup of azo dye contamination and degradation of azo dyes by microorganisms with a high metabolizing capacity is greatly desirable.

Biodegradation of azo dyes has been extensively studied in the last decades. Various microbial species, individually or in consortium, have been demonstrated for their activity to degrade azo dyes (Asad et al. 2007; Fang et al. 2004; Gou et al. 2009; Patil et al. 2010). Even mutant strains also have been constructed to increase the efficiency for azo dye degradation (Gopinath et al. 2009). However, degradation of azo

dye in plain distilled water has been reported only for yeast species *Saccharomyces cerevisiae* (Jadhav et al. 2007).

A number of statistically designed experimental models have been applied to optimize the culture parameters in biological research. Response surface methodology (RSM) is an experimental approach to identify the optimum conditions for a multivariable system. This methodology has been successfully applied in optimization of the enzyme production, dye and other pollutants degradation, media optimization for dye decolorization and other pollutants degradation (Bae and Shoda 2005; Fan et al. 2004; Nyanhongo et al. 2002; Singh et al. 2010; Trupkin et al. 2003; Wang et al. 2004; Zhou et al. 2011). However, herein we applied RSM for textile dye degradation in plain distilled water. There is no such report on the decolorization of textile dye in the absence of any kind of nutrients by a bacterial species. In this study, a three-level Box–Behnken design was employed to optimize and maximize the decolorization and degradation of textile azo dye Remazol Orange (RmO) by *Pseudomonas aeruginosa* BCH. Further dye degradation and detoxification was assessed with high performance liquid chromatography (HPLC) and phytotoxicity studies, respectively.

Materials and methods

Dye and chemicals

Azo dye RmO used in this study was obtained from textiles industries, Ichalkaranji, Maharashtra, India. Medium components for bacterial growth, *o*-tolidine, veratryl alcohol and L-ascorbic acid were purchased from Hi-Media Pvt. Ltd. India. Catechol was obtained from Thomas-Baker. Solvents, ethyl acetate and dichloromethane were bought from Merck, India and HPLC grade methanol and water was from Sigma-Aldrich, India.

Bacterial strain and culture conditions

Bacterial strain *P. aeruginosa* BCH used in this study was previously isolated in our laboratory from dye contaminated soil (Jadhav et al. 2010). The pure culture was maintained at 4 °C on yeast extract agar medium having composition (g l^{-1}): yeast extract 5, NaCl 5 and agar 25. The medium for bacterial growth was composed of (g l^{-1}): yeast extract 2, NaCl 5.

Biodegradation assay

A single colony of bacterium was inoculated in 250 ml Erlenmeyer flasks containing 100 ml growth medium and grown for 8 h at 30 °C at shaking condition (120 rpm). From this inoculum (log phase), 1 ml of culture was then inoculated in the 100 ml fresh medium and grown for 24 h as mentioned above. Cells were harvested by centrifugation at 6,000 rpm for 10 min at 4 °C (dry cells weight was 30 ± 2 mg). Centrifuged cells were washed twice with sterile distilled water and re-suspended in distilled water at a required concentration (1.0 or 2.0 or 3.0 g l^{-1} dry weight). Dye was added at a concentration of 50 mg l^{-1} from the stock solution prepared in sterile distilled water. Adjustment of the pH was done with 0.1 M NaOH and 0.1 M HCl prior to re-suspension of the cells in water. For studies under various temperatures, flasks with cell suspension in water were incubated at respective temperatures for 15 min. (to attain the temperature) prior to dye addition. Then after dye addition, incubation was continued at respective temperatures. Aliquots of 1 ml from this decolorization assay sample were withdrawn immediately after dye addition (zero time) and, after dye decolorization, centrifuged at 5,500 rpm for 15 min at room temperature and supernatants were analyzed at 495 nm on spectrophotometer (Shimadzu, UV-1800). The decolorization activity was calculated by using the formula:

$$\text{Decolorization}\,(\%) = \frac{[(\text{Initial absorbance}) - (\text{Observed absorbance})]}{(\text{Initial absorbance})} \times 100 \quad (1)$$

All experiments were performed in triplicates in aseptic conditions.

Response surface methodology (RSM)

RSM is an empirical modelling technique used to assess the correlation between a set of controllable experimental factors and observed results. This optimization technique involves three major steps: (i) performing statistically designed experiments, (ii) estimating the coefficients in a mathematical model and (iii) predicting the response and checking the adequacy of the model (Box and Behnken 1960). The Box and Behnken design was applied using Design-Expert (Stat-Ease) software trial version 8.0.6.1 to our study with three variables at three levels. The three parameters of pH, temperature and cell mass concentration were chosen based on the results from the preliminary experiments. These parameters were chosen as the critical variables to maximize dye decolorization and designated as *A*, *B*, and *C* and the boundary conditions for each parameter with actual design are depicted in Table 1. The significance of the model equation and model terms was evaluated by *F* test. The quality of the quadratic model equation was expressed by the determination coefficient R^2 and adjusted R^2. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. The optimal values

Table 1 The Box–Behnken design matrix with variables along with actual and predicted responses

Std. order	Factor *A* (pH)	Factor *B* (temp., °C)	Factor *C* (cell mass, g l^{-1})	Actual response (*Y*, %)	Predicated response (Y_1, %)	Externally studentized residual
1	6	20	2	56.98	57.25	−1.065
2	8	20	2	45.89	46.10	−0.811
3	6	40	2	55.53	55.32	0.811
4	8	40	2	42.01	41.74	1.065
5	6	30	1	71.02	70.88	0.534
6	8	30	1	43.97	43.88	0.318
7	6	30	3	89.89	89.98	−0.318
8	8	30	3	92.10	92.25	−0.534
9	7	20	1	30.97	30.84	0.463
10	7	30	3	41.31	41.67	−1.521
11	7	20	3	78.91	78.55	1.521
12	7	30	3	61.30	61.43	−0.463
13	7	30	2	86.99	87.61	−1.463
14	7	30	2	86.06	87.61	0.965
15	7	30	2	88.32	87.61	1.745
16	7	30	2	87.21	87.61	−0.862
17	7	30	2	87.49	87.61	−0.251

were obtained by solving the regression equation and analyzing the response surface plot.

Enzyme analysis

Preparation of cell-free extract

Bacterial cells grown for 24 h, as mentioned above, were centrifuged at 6,000 rpm for 10 min at 4 °C (control). Centrifuged cells (from 100 ml broth) were washed and re-suspended in 20 ml potassium phosphate buffer (50 mM, pH 7.2). Further, these cells were homogenized in a glass homogenizer and sonicated (Sonics-vibracell ultrasonic processor) keeping sonicator output at 60 amplitudes and giving 7 strokes each of 30 s with 2-min interval at 4 °C. The homogenate was centrifuged at 6,000 rpm for 10 min at 4 °C and the supernatant was used as a source of crude enzyme. To quantify the enzyme activities after dye decolorization, cells were harvested from decolorization flask by centrifugation. Similar procedure was followed to get the cell-free extract as an enzyme source. Supernatant was used to quantify the extracellular enzyme activities.

Enzyme assays

Activities of laccase, veratryl alcohol oxidase and NADH-DCIP reductase and tyrosinase were assayed spectrophotometrically by using Shimadzu UV–vis spectrophotometer (UV 1800). Enzyme assay for laccase was similar as mentioned in our previous report (Jadhav et al. 2011). Briefly, activity was monitored with *o*-tolidine (50 mM) in 2.1 ml reaction mixture containing 1.8 ml buffer (acetate buffer 0.1 M and pH 4.8), 0.2 ml *o*-tolidine and 0.2 ml enzyme. NADH-DCIP reductase was carried out as mentioned previously (Salokhe and Govindwar 1999). For veratryl alcohol oxidase assay, the 2 ml reaction mixture contained 4 mM veratryl alcohol in 0.05 M citrate phosphate buffer, pH 3, and 0.2 ml enzyme to start the reaction (Jadhav et al. 2009). For tyrosinase assay, protocol mentioned by Surwase and Jadhav (2011) was followed. The final assay mixture (3 ml) contained 50 mM potassium phosphate (pH 7.4), 0.17 mM catechol and 0.070 mM L-ascorbic acid equilibrated. The ΔA_{265nm} was monitored until constant, and then 0.1 ml of the supernatant from the reaction mixture was added. The decrease in the ΔA_{265nm} was recorded for 1 min. The ΔA_{265nm} was obtained using the maximum linear rate for both the test and control. One unit of tyrosinase activity was equal to a ΔA_{265nm} of 0.001 per min at pH 7.4 in a 3.0 ml reaction mixture containing L-catechol and L-ascorbic acid. All enzyme assays were carried out at room temperature; reference blanks contained all components except the enzyme.

HPLC analysis

Sample preparation

Decolorized sample was centrifuged at 9,000 rpm for 20 min at room temperature and the supernatant obtained was used to extract metabolites firstly with equal volume of ethyl acetate and then with equal volume of dichloromethane. Both the extracts were combined, dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator.

This dried residue was redissolved in small volume of HPLC grade methanol and used for HPLC analysis.

Analytical method

High performance liquid chromatography analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector. Column specifications were—C_{18} column with symmetry 4.6 mm × 250 mm. HPLC grade methanol:water (80:20) was used as mobile phase with flow rate 1 ml min^{-1} and HPLC run was carried out for 10 min.

Phytotoxicity

The biodegradation metabolites of RmO extracted in solvents were dried and dissolved in water to form the final concentration of 500 ppm for phytotoxicity studies. Phytotoxicity of the dye (500 ppm) and its extracted degradation products was carried out using *Sorghum vulgare*, *Phaseolus mungo* and *Triticum aestivum* (10 seeds of each) by watering separately 10 ml sample of dyes and its degradation products per day. Control set was carried out using water at the same time. Germination (%) and the length of the plumule and radical were recorded after 8 days.

Results and discussion

Dye decolorization

Pseudomonas aeruginosa BCH was found to have the potential for the decolorization of textile azo dye RmO in plain distilled water. There is no such report on bacterial dye decolorization in plain distilled water. However, yeast species *S. cerevisiae* MTCC 463 was shown to degrade triphenylmethane dyes in plain distilled water (Jadhav and Govindwar 2006). In that study, the decolorization of the dye was occurred through both biosorption and biotransformation. In order to check whether bacterium *P. aeruginosa* BCH in the present study followed similar mechanism, heat-killed cell suspension was checked for the decolorization activity. No dye biosorption was observed visually as well as in solvent desorption experiments with various solvents (Phugare et al. 2010). Dye desorption experiments were also carried out with live cell culture after dye decolorization, and no dye adsorption was observed. However, dye adsorption was observed only initially. It was also important to check the stability of dye over various pH ranges. We carried out UV–vis spectral analysis of dye at different pHs. It was observed that there was no shift or change in the absorbance maxima of the dye, when scanned in visible range, at different pHs (figure not shown). It indicates that dye was stable at various pHs and also signify that the decolorization was mediated by bacterial activity only. In order to optimize this bacterial decolorization performance in plain distilled water, we employed RSM technique.

Experimental design, significance analysis and adequacy of the model

RSM with Box–Behnken design was applied to optimize the decolorization performance of the bacterium. During preliminary dye decolorization studies system, variables and their levels were identified. To accomplish a more realistic model, prior knowledge gained from previous studies for understanding the process and the process variables under investigation is necessary for the RSM optimization method (Sharma et al. 2009a). Each run was performed in triplicate and mean values for % decolorization was putted in the software. The design matrix of the variables in actual units is given in Table 1 along with the predicted and experimental values of response. By applying multiple regression analysis methods, the predicted response, Y (% decolorization) for can be obtained and given in terms of coded factors as:

$$Y = +87.61 - 6.18 \times A - 1.57 \times B + 16.87 \times C - 0.61 \times A \times B + 7.32 \times A \times C - 6.99 \times B \times C - 8.19 \times A^2 - 29.32 \times B^2 - 5.17 \times C^2 \quad (2a)$$

or in terms of actual factors as:

$$\%\ \text{Decolorization} = -548.97500 + 88.41925 \times \text{pH} + 19.95420 \times \text{Temperature} + 35.34150 \times \text{Cell mass} - 0.060750 \times \text{pH} \times \text{Temperature} + 14.63000 \times \text{pH} \times \text{Cell mass} - 1.39750 \times \text{Temperature} \times \text{Cell mass} - 8.19450 \times \text{pH}^2 - 0.29317 \times \text{Temperature}^2 - 20.69800 \times \text{Cell mass}^2 \quad (2b)$$

The statistical significance of model was evaluated by F test and the ANOVA for response surface quadratic model is summarized in Table 2.

ANOVA of regression model demonstrates that the model is highly significant, as it is evident from the Fisher's F test with a very low probability value [(Pmodel > F) = 0.0001]. The lack-of-fit test measures the failure of the model to represent data in the experimental domain at points, which are not included in the regression. This test is desired to be non-significant to signify the model (Sharma et al. 2009b). In this study, the non-significant value of lack of fit (F value 0.62) exposed that the quadratic model is statistically significant for the response, and therefore it can be used for further analysis.

In general, it is imperative to substantiate the fitted model to make sure that it gives sufficient approximation to the actual test. Model should represent a satisfactory fit,

Table 2 Analysis of variance (ANOVA) for the fitted quadratic polynomial model of RmO decolorization

Source	Sum of squares	*df*	Mean square	*F* values	*P* value (Prob > *F*)	
Model	7,254.46	9	806.0511	3,049.107	<0.0001	Significant
pH (*A*)	305.6628	1	305.6628	1,156.253	<0.0001	
Temperature (*B*)	19.845	1	19.845	75.0691	<0.0001	
Cell mass (*C*)	2,275.763	1	2,275.763	8,608.692	<0.0001	
AB	1.476225	1	1.476225	5.584222	0.0501	
AC	214.0369	1	214.0369	809.6527	<0.0001	
BC	195.3006	1	195.3006	738.7777	<0.0001	
A^2	282.7361	1	282.7361	1,069.526	<0.0001	
B^2	3,618.89	1	3,618.89	13,689.44	<0.0001	
C^2	112.7387	1	112.7387	426.4649	<0.0001	
Residual	1.850495	7	0.264356			
Lack of fit	0.585175	3	0.195058	0.616629	0.6398	Not significant
Pure error	1.26532	4	0.31633			
Cor total	7,256.31	16				

R^2 0.9997; adj R^2 0.9994; pred R^2 0.9984; CV 0.76; PRESS 11.34; adeq precision 155.706

df degrees of freedom

otherwise proceeding with investigation and optimization of the fitted response surface is likely to give poor or misleading results (Murugesan et al. 2007). Generally multiple correlation coefficient, normal probability plot and 'adequate precision' can be analysed to judge the model adequacy. To ensure the normality assumption, a normal probability plot of the residuals was analysed (Fig. 1).

Mean zero and unit variance should be approximately normal with residuals. It was observed that none of the internally studentized residuals had a value over 2. The normality assumption was fulfilled as the residual plot approximated along a straight line. Externally studentized residuals were further employed to validate the model. As evident from Table 1, no outliers were found and all the values ranged well within −2 to +2. Acceptable limit is −3 to +3 (Bhattacharya and Banerjee 2008). The integrity of the model can be checked by the determination coefficient R^2 and the multiple correlation coefficient *R*. It measures the proportion of variation explained by the model relative to mean (Anderson and Whitcomb 2005). The closer the values of *R* (multiple correlation coefficient) to 1, the better is the correlation between the experimental and predicted values (Pujari and Chandra 2000). Here, the value of R^2 (0.9997) indicates good relation between the experimental and predicted values of the response. "Predicted R^2" of 0.9984 is in good agreement with the "adjusted R^2" of 0.9994 indicating that this response surface design can be used for modelling the design space. The R^2 is a global statistic to access the fit of a model (Bhattacharya and Banerjee 2008). Also, "adeq precision" measures the signal nose ratio. In our study, this ratio of 155.706 represents an adequate signal and indicates that the model can be used to navigate the design space.

Figure 2 shows the Box–Cox plot which provides guideline for selecting the correct power law

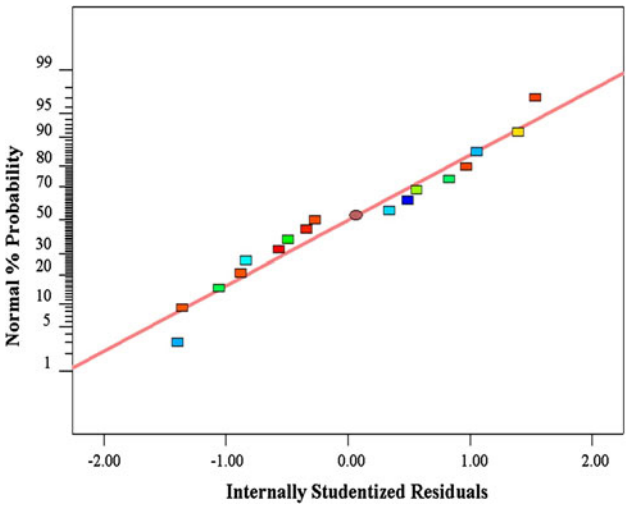

Fig. 1 The studentized residual and normal % probability plot of decolorization of RmO

transformation. Typically, most of the parameters estimate might appear to be significant outside the region of the optimum, but near it only a few will be highly significant. Our study model showed that the minimum confidence interval value is 0.84 and the maximum value is 1.16. The current point of confidence interval ($\lambda = 1$) matches to model design value (best = 1); therefore, no transformation of the model was required as recommended by the Box–Cox analysis.

Effect of interactive variables

To investigate the interaction between the two parameters by keeping the third parameter constant, three-dimensional surface plots and the contour plots were analysed.

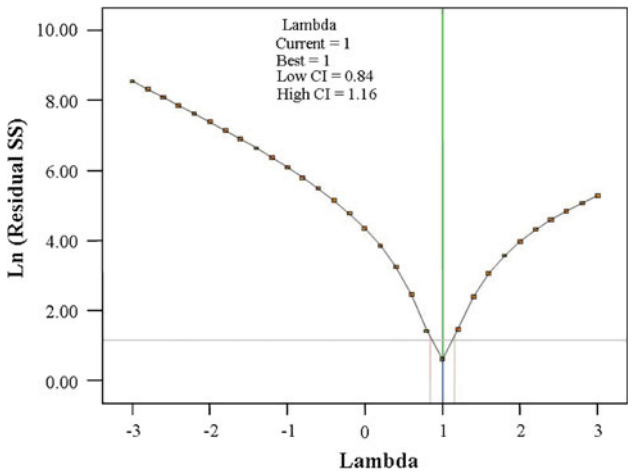

Fig. 2 Box–Cox plot of model transformation for the decolorization of RmO by *P. aeruginosa* BCH

The 3D response surface plots are the graphical representations of the regression equation. These three-dimensional plots, for the interaction of tested variables, are given in Fig. 3a–c along with contour plots. The main aim of this response is to mark efficiently the optimum values of the variables in order to maximize the goal (% decolorization).

Interactive effect of temperature and PH

Analysis of Fig. 3a clearly suggests that as temperature increased, increase in % decolorization occurred with increasing pH. There was increase in dye decolorization from 20 to 30 °C, but further increase from 30 to 40 °C leads to the decrease in decolorization activity of the bacterium. For pH, there was increase in % decolorization with increasing pH from 6.0–7.0 units. However, the influence of pH was less as compared to that of temperature. This indicates that the bacterium is at its optimum activity over a range of pH and at temperature 30 °C. The shapes of the contour plots (circular or elliptical) designate whether the communal interactions between the variables are significant or not. If the nature of contour is circular, then it indicates that the interactions between the corresponding variables are negligible. An elliptical type of the contour plots demonstrates that the interactions between the corresponding variables are significant (Muralidhar et al. 2001). The elliptical nature of the contour plot can be seen in our study for all parameter interactions. For instance from Fig. 3a (at temperature 20 °C and pH 6.0), dye removal efficiency was about 59 % which increases up to 90 % as temperature increases to temperature 30 °C and pH range 6.3–7.3 units.

Interactive effect of cell mass and pH

Figure 3b shows the interactive effect of cell mass concentration and pH. When cell mass was increased from 1.0 to 3.0 g l^{-1}, linear increase in decolorization from 43.97 to 92.10 % was evidenced. This response clearly demonstrates that pH had less influencing effect as compared to cell mass concentration. Also, at lower cell mass concentration, i.e. 1.0 g l^{-1} influence of pH was higher; in that case at pH 8.0 only 43.97 % decolorization was achieved and at pH 6.0 it was 71.02 %. Cell or enzyme stability can be affected by the pH. Similar effect of pH can be seen here, where there was high effect of pH observed when cells were present at low concentration as compared to cell concentration of 3 g l^{-1}.

Interactive effect of cell mass and temperature

Similarly when interaction of cell mass and temperature was investigated, temperature was found to be affecting parameter in this analysis also, where similar type of effect can be observed as in Fig. 3a. Cell mass concentration showed increasing decolorization rate with increasing cell mass (Fig. 3c). Temperature was found to be affecting most at the cell concentration 1 g l^{-1}, where 30.97 % decolorization can be seen at 20 °C and 41 % decolorization can be seen at 40 °C. Maximum decolorization can be observed at a temperature of 30 °C and cell mass concentration of 3 g l^{-1}. This designate that when there were fewer cells present to utilize the available dye molecules, the overall % decolorization appeared to be less, but when the number of cells increased, maximum of the available dye molecules were utilized within the same time, therefore exhibiting higher % decolorization.

Model validation and confirmation

To validate the optimum combination of variables, confirmatory experiments were carried out. When command was given to maximize the goal (% decolorization), numbers of combinations and their responses were predicted as solution. Few such combinations were chosen and confirmatory experiments were run in triplicate. Table 3 represents these solutions along with observed responses. It can be seen that 96.01 % decolorization response was observed from the predicted optimum combination (pH 7.43, temperature 29.39 and cell mass 2.88). Also, all the observed results were well accorded with the predicted results which reflected the applicability and correctness of RSM. Moreover, it was also evident from Table 3 that higher cell mass concentrations were required to get the maximum dye decolorization. As an outcome, the model developed was considered to be accurate and reliable.

Enzymatic inspection

Enzymatic analysis of various bioremediation enzymes was carried out to check their role during RmO degradation

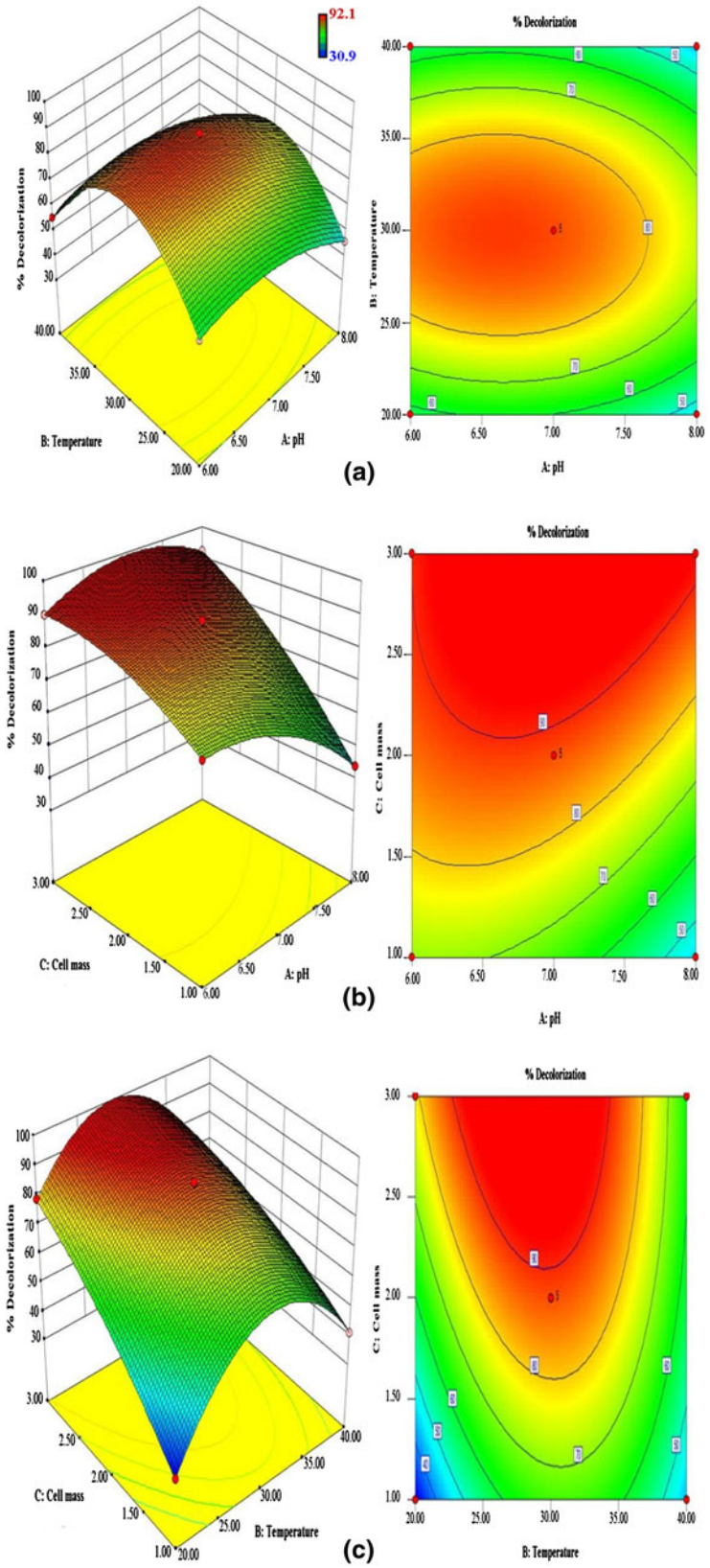

Fig. 3 **a** 3D response surface plot and contour plot of interactions of pH and temperature for RmO decolorization. **b** 3D response surface plot and contour plot of interactions of cell mass and pH for RmO decolorization. **c** 3D response surface plot and contour plot of interactions of effect of temperature and cell mass on decolorization of RmO decolorization

Table 3 Predicted solutions for model validation and confirmation of Box–Behnken design matrix

Solution	pH units	Temp. (°C)	Cell mass (g l^{-1})	Predicted response (%)	Observed response (%)
1	6.66	25.46	2.92	95.07	94.24
2	6.31	30.51	2.49	92.27	90.86
3	6.46	27.83	2.97	96.57	95.01
4	6.19	27.55	2.99	93.27	91.98
5	7.43	29.39	2.88	97.37	96.01

Table 4 Bioremediation enzymes analysis of before and after decolorization of dye

Enzyme	Intracellular		Extracellular	
	Before decolorization	After decolorization	Before decolorization	After decolorization
Laccase[a]	0.735 ± 0.029	0.914 ± 0.043**	ND	ND
VAO[a]	1.161 ± 0.055	1.241 ± 0.040	ND	ND
Tyrosinase[a]	245.0 ± 31.83	709.8 ± 25.5***	ND	1,500.0 ± 63.3***
NADH-DCIP reductase[b]	61.69 ± 2.756	108.5 ± 4.73***	ND	205.26 ± 9.09***
Azoreductase[c]	ND	ND	ND	ND

Values are mean of three experiments ± SD. Significantly different from control (before decolorization) at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey Kramer comparison test

ND not detected

[a] Units mg^{-1} protein min^{-1}

[b] µg of DCIP reduced mg^{-1} protein min^{-1}

[c] µmol of methyl red reduced mg^{-1} protein min^{-1}

and also to understand the mechanism of dye degradation. From Table 4, it is apparent that the intracellular activities of laccase, veratryl alcohol oxidase, NADH-DCIP reductase and tyrosinase were induced after dye decolorization. Besides this, no activities of all these enzymes were detected in extracellular samples before dye decolorization (zero time), but activities of tyrosinase and NADH-DCIP reductase were significantly induced after dye decolorization. Whereas laccase and veratryl alcohol oxidase were absent in the extracellular crude enzyme sample. This implies that there was an involvement of these enzymes in the degradation of RmO and their communal action was important for dye degradation; also, the dye degradation was mediated intracellularly and extracellularly. The role and mode of action of these bioremediation enzymes are well known. The activity of azoreductase was not detected in intracellular as well as extracellular sample.

HPLC analysis

Often the decolorization of dye is due to its structural degradation. To confirm this, HPLC of dye (control) and its decolorized metabolites was carried out. Single peak was observed for RmO at retention time of 2.370 min. (Fig. 4a).

Generally, textile dyes may be either of high purity or may contain some impurities. This can be confirmed through HPLC or other chromatographic analysis. In some of our previous studies (Jadhav et al. 2011), there was more than one peak appeared for control/parent dyes in HPLC analysis which indicates that there were little impurities present. However, in the case of RmO, there was only single peak observed in HPLC analysis, which indicate that the dye is of highest purity. Chromatogram for metabolite sample showed disappearance of this control peak and appearance of the three new major peaks and two minor peaks with altered retention times viz.—1.952, 2.868, 3.285, 2.275 and 3.456 min, respectively (Fig. 4b). Therefore, from HPLC analysis, it can be concluded that the dye was degraded by bacterial decolorization activity.

Phytotoxicity studies

When the improperly treated effluents are discharged in the natural environment, these can directly affect the plant ecosystem. So, the toxicity of RmO towards the seedlings of commercially important plants, such as *S. vulgare*, *T. aestivum* and *P. mungo*, was assessed. The dye was found to be toxic at the concentration 500 ppm for both plants during preliminary studies, so the experiments were carried out using the concentration 500 ppm for dye as well as metabolites. Strong inhibition in % germination was observed in the case of all the plants (Table 5).

It was 40, 30 and 30 % for *P. mungo*, *T. aestivum* and *S. vulgare*, respectively, in dye-treated samples as compared to 80, 90 and 80 % for water-treated (control) *P. mungo*, *T. aestivum* and *S. vulgare* samples, respectively.

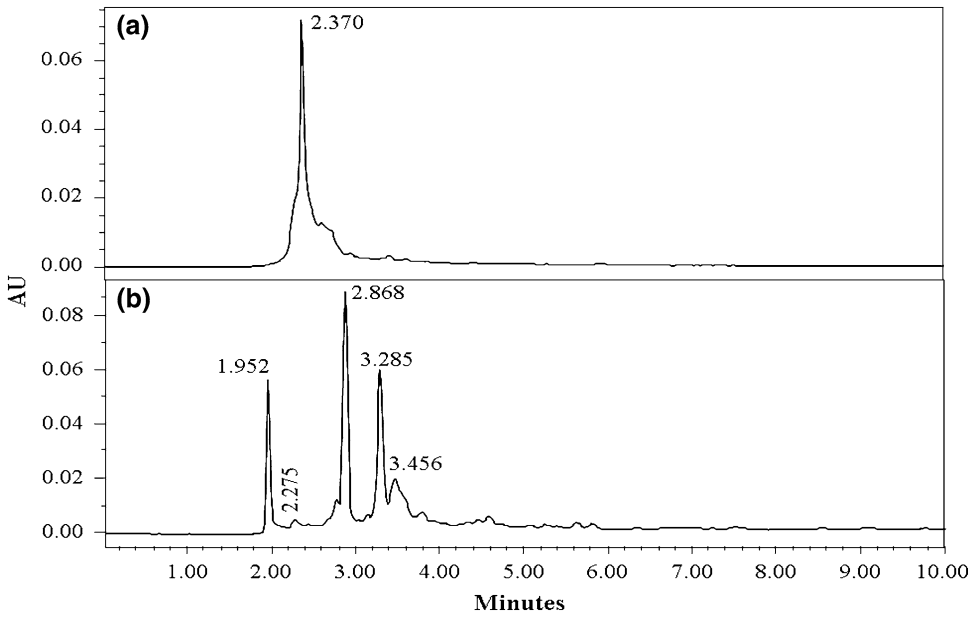

Fig. 4 HPLC elution profile of **a** dye and **b** its biodegradation metabolites

Table 5 Phytotoxicity studies of Remazol Orange and its biodegraded metabolites

Plants	Parameters		
	Germination (%)	Plumule (cm)	Radical (cm)
Phaseolus mungo			
Control[a]	80	6.35 ± 0.56	4.83 ± 0.63
Dye[b]	40**	2.96 ± 0.24***	1.98 ± 0.82**
Metabolite[a]	70	5.41 ± 0.78	4.51 ± 0.82
Triticum aestivum			
Control	90	10.0 ± 1.25	5.13 ± 0.62
Dye	30***	4.74 ± 1.72***	3.03 ± 0.74*
Metabolite	70	8.73 ± 1.02	4.01 ± 0.12
Sorghum vulgare			
Control	80	8.28 ± 1.93	5.01 ± 0.73
Dye	30***	5.03 ± 0.92*	2.43 ± 0.51**
Metabolite	80	7.56 ± 0.73	3.98 ± 0.14

Values are mean of three experiments ± SEM, significantly different from the control (seeds germinated in water) at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ by one-way analysis of variance (ANOVA) with Tukey Kramer comparison test

[a] Water-treated sample

[b] 500 ppm

Whereas metabolite-treated samples showed % germination as 70, 70 and 80, respectively. In all tested parameters of phytotoxicity, the metabolites showed almost negligible effect on all tested plants as compared to the dye. It gives clear indication that toxicity of dye was reduced after its treatment with *P. aeruginosa* BCH. Such phytotoxicity studies have been applied successfully to evaluate the toxicity of other azo dye (Ayed et al. 2010, 2011; Dhanve et al. 2008; Patil et al. 2008). In our study also, RmO dye was found to affect the overall growth of the tested plants. Therefore, from the phytotoxicity studies, it is apparent that the metabolites formed after dye degradation were less toxic.

Conclusion

The bacterium *P. aeruginosa* BCH possesses the ability to decolorize the textile azo dye RmO in the absence of organic and inorganic nutrients. This application of bacterial decolorization in plain distilled water seems to be a practical approach. RSM was found to be appropriate technique to optimize the bacterial decolorization performance. Observed and predicted results reflected were very close indicating the success of RSM. The value of determination coefficient ($R^2 = 0.9997$) point out that a very negligible of the total variations were elucidated by the model. The value of adjusted determination coefficient (adj $R^2 = 0.9994$) was very much close to 1, suggesting very high significance of model. Through the application of Box–Behnken design, the process parameters were optimized to achieve 97 % decolorization within 5 h. HPLC analysis indicated the bacterial-mediated structural degradation of dye lead to decolorization. Overall, *P. aeruginosa* BCH can be a strong applicant for the real-scale application in decolorization of textile azo dye RmO present in textile effluents. Further pilot scale studies are required with this strain for real-scale industrial applications.

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