Available online at www.sciencedirect.com
ScienceDirect
Dyes and Pigments 75 (2007) 176–184
DYES and PIGMENTS
www.elsevier.com/locate/dyepig

Decolourization of reactive black 5 by laccase: Optimization by response surface methodology

Kumarasamy Murugesan [a], Ankur Dhamija [b], In-Hyun Nam [a], Young-Mo Kim [a], Yoon-Seok Chang [a,*]

[a] *School of Environmental Science and Engineering, Pohang University of Science and Technology, San 31, Hyojadong, Namgu, Pohang 790-784, Republic of Korea*
[b] *Department of Chemical Engineering, Indian Institute of Technology, Kanpur, India*

Received 7 November 2005; received in revised form 21 January 2006; accepted 30 April 2006
Available online 8 August 2006

Abstract

Response surface methodology (RSM) was applied for the decolourization of the azo dye reactive black 5 (RB-5) using purified laccase from a white rot fungus *Pleurotus sajor-caju*. We observed that the presence of 1-hydroxybenzotriazole (HBT) is essential for decolourization of RB-5 by purified laccase from *P. sajor-caju*. Box–Behnken design using RSM with four variables namely dye (25–100 mg l^{-1}), enzyme (0.5–2.5 U ml^{-1}), redox mediator (0.5–1.5 mM) concentrations and incubation time (24–48 h) was employed in this study to optimize significant correlation between the effects of these variables on the decolourization of RB-5. The optimum concentrations of dye, enzyme, HBT, and time were found to be 62.5 mg l^{-1}, 2.5 U ml^{-1}, 1.5 mM and 36 h, respectively, for maximum decolourization of RB-5 (84.4%). A quadratic model was obtained for dye decolourization through this design. The experimental values were in good agreement with predicted values and the model was highly significant, the correlation coefficient being 0.999. Increased decolourization was observed with increase in enzyme concentration at lower dye concentration. Interaction between HBT and dye concentrations was negligible. The optimization of HBT is independent of dye concentration.

Keywords: Laccase; Reactive black 5; Dye decolourization; Response surface methodology

1. Introduction

Synthetic dyes are being extensively used in various industrial dyeing and printing processes. The textile industry is the largest user of synthetic dyes consuming about 56% of the total annual world production (7×10^5 tons) [1,2]. Among the available dyes, about 50% of the industrial dyes produced in the world are azo dyes [3]. Reactive group azo dyes are mostly used in textile dyeing due to their superior fastness to the applied fabric, high photolytic stability, and resistance to microbial degradation. However, reactive dyes are particularly problematic, because the dyes exhibit low levels of fixation with the fiber and about 10–20% of total dye used in dyeing process remain left in the spent dye bath with accessory chemicals [4]. Treatment of the textile dye containing effluent is difficult and ineffective with conventional biological (activated sludge) processes [5,6] because many synthetic dyes are very stable to light, temperature and resistant to microbial attack. Several physico-chemical methods have been used, including coagulation/flocculation, membrane filtration, and activated carbon adsorption, for colour removal from effluent. Unfortunately, these methods of effluent treatment have high operating costs and of limited applicability [7]; further these treatment methods produce large quantities of sludge which again create a problem in waste disposal. In recent years, biological decolourization method has been considered as an alternative and eco-friendly method to dye degradation and colour removal [8–12].

* Corresponding author. Tel.: +82 54 279 2281; fax: +82 54 279 8299.
E-mail address: yschang@postech.ac.kr (Y.-S. Chang).

0143-7208/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2006.04.020

White rot fungi are a heterogeneous group of organisms but have in common the capacity to degrade lignin as well as other wood components and wide variety of recalcitrant compounds including synthetic dyes [10,13–16]. The dye degrading ability of the white rot fungi is due to its lignolytic enzyme system consisting of lignin peroxidase, manganese dependent peroxidases, and laccases [17–19] as well as H_2O_2 producing oxidases [20]. A number of white rot fungi have been explored for decolourization of various industrial dyes and treatment of dye effluent [9,10,12,14,21]. Majority of these studies were conducted with fungal mycelia. One of the major disadvantages of using fungal cultures to decolourization is the accumulation of biomass, which would cost the wastewater treatment in industrial scale. To overcome this disadvantage the application of isolated enzymes for dye decolourization has increased in recent years [19,22–24].

Laccases [(benzenediol:oxygen oxidoreductase, (E.C. 1.10.3.2)] are multi copper-containing enzymes that catalyze the oxidation of a wide variety of aromatic compounds, with concomitant reduction of oxygen to water [25]. This oxidative enzyme is particularly abundant in white rot fungi and has been purified and characterized extensively from many white rot fungi [19,21,26]. In recent years, laccase based treatment method has received much attention in treatment of various recalcitrant pollutants because the laccase production is constitutive in most of the white rot fungi and it can be easily enhanced by chiefly available laccase inducers. Many studies have been demonstrated for dye decolourization using both crude and purified forms of laccase [17,19,21–23,27,28]. Some of the chemicals serving as redox mediators facilitate the dye degrading activity of laccase and enhance its specificity to wide range of dyes [22,27] and addition of redox mediator is essential for certain dyes [21,27]. 1-hydroxybenzotriazole (HBT) is one of the most efficient laccase mediators, but the major drawbacks of HBT are its high cost, high dose, limited biodegradability, and potential toxicity [29]. Hence, the optimization of HBT is important for effective decolourization.

A number of statistically designed experimental models have been applied to optimize the culture parameters in biological research. Response surface methodology, first described by Box and Wilson [30], is an experimental approach to identify the optimum conditions for a multivariable system. This methodology has been successfully applied in optimization of the culture parameters in lignolytic enzyme production and dye decolourization with fungal cultures [31–33]. Recently, RSM has been applied for the optimization of redox mediator in laccase mediated pulp bleaching [29]. In enzymatic dye decolourization, optimization of the concentrations of redox mediator, dye and enzyme is an important criterion for successful decolourization. Understanding the combined interactions between these factors is also important. To our knowledge there has been no study for the optimization of parameters in enzymatic dye decolourization using RSM. The aim of this study was to optimize the concentrations of HBT, enzyme, and dye and the incubation time in order to obtain best possible results in reactive azo dye decolourization by purified laccase. In this study we selected reactive black 5 (RB-5) (Fig. 1), a widely used reactive diazo textile dye, as a model and purified laccase from a white rot fungus *Pleurotus sajor-caju*.

2. Materials and methods

2.1. Chemicals and organism

Reactive black 5 (RB-5), DEAE cellulose, Sephadex G-100 and ABTS (2,2′-azinobis-3-ethylbenzothiazoline-6-sulfonic acid) were purchased from Sigma–Aldrich Co. HBT (1-hydroxybenzotriazole) was obtained from ICN Biochemicals Inc, Germany and all other chemicals were of analytical grade obtained from Junsei Chemical Co, Japan. The white rot fungus, *P. sajor-caju* described earlier [21] was obtained from Centre for Advanced Studies in Botany, University of Madras, India, and used for enzyme production.

2.2. Enzyme preparation

Laccase production from *P. sajor-caju* was carried out in solid-state fermentation using wheat bran as described earlier [21]. The extracellular enzymes from 12 days old wheat bran culture was separated by filtration through nylon cheese cloth and the supernatant was centrifuged $8000 \times g$ for 20 min twice to remove fine particles. The proteins present in the culture filtrate were precipitated by 70% ammonium sulfate saturation. Then the protein precipitate was dissolved in 50 mM sodium tartrate buffer (pH 5.0) and dialyzed overnight against the same buffer with low concentration (10 mM). Further purification through ion exchange and gel filtration chromatography was carried out as described previously [21]. The purity of purified laccase was checked by SDS-PAGE.

2.3. Response surface methodology

Response surface methodology is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results. This optimization process 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 [34]. A class of three level complete factorial design for the estimation of the parameters in a second-order model was developed by Box–Behnken [35]. In this study we selected Box–Behnken design for the optimization of laccase mediated reactive dye decolourization. This design was applied using Design Expert 6.0 to our study with four variables at three levels. Three different concentrations of dye (25, 62.5, 100 mg l^{-1}), enzyme (0.5, 1.5, 2.5 U ml^{-1}), HBT (0.5, 1.0, 1.5 mM), and different time intervals (24,36,48 h) were chosen as the critical variables and designated as A, B, C, and D, respectively, as shown in Table 1. The four significant

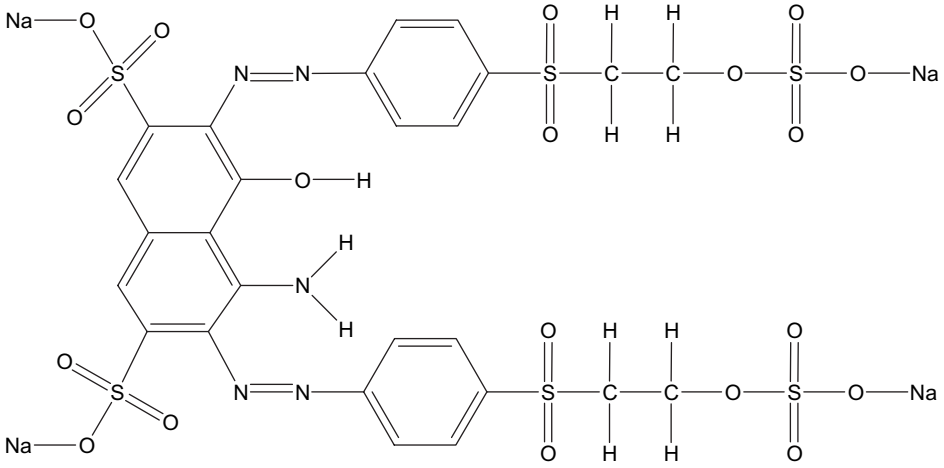

Fig. 1. The chemical structure of reactive black 5 dye (RB-5) (C.I. 20505; M.W = 991 Da).

variables can be approximated by the quadratic model equation as follows:

$$Y = k_0 + k_a A + k_b B + k_c C + k_d D + k_{aa} A^2 + k_{bb} B^2 + k_{cc} C^2 + k_{dd} D^2 + k_{ab} AB + k_{ac} AC + k_{ad} AD + k_{bc} BC + k_{bd} BD + k_{cd} CD \quad (1)$$

where Y is the predicted response; k_0 is a constant; k_a, k_b, k_c, k_d are the linear coefficients; k_{ab}, k_{ac}, k_{ad}, k_{bc}, k_{bd}, k_{cd} are the cross-coefficients; k_{aa}, k_{bb}, k_{cc}, k_{dd} are the quadratic coefficients. This response is preferred because a relatively few experimental combinations of the variables are adequate to estimate potentially complex response function. A total number of 29 experiments were necessarily carried out to estimate the 15 coefficients for the decolourization of RB-5. Data were analyzed using Design Expert 6.0 program including ANOVA to find out the interaction between the variables and the response. The quality of the fit of this model was expressed by the coefficient of determination (R^2) in the same program.

2.4. Dye decolourization test

Dye decolourization experiments were carried out in 2 ml Eppendorf tube. Total reaction mixture (1 ml) containing 100 mM sodium acetate buffer (pH 5.0), purified laccase at various concentrations (0.5, 1.5 and 2.5 U ml^{-1}), HBT (0.5, 1.0, 1.5 mM) and reactive black dye (25, 62.5, 100 mg l^{-1}) was prepared as described in Table 2. The reaction tubes were incubated at 25 °C under dark and the decolourization was monitored spectrophotometrically at different incubation hours (24, 36, 48 h) by recording the absorbance at the λ_{max} of the dye (596 nm). Before starting the actual designed experiments preliminary tests were conducted using 1.0 U ml^{-1} laccase, 25 mg l^{-1} dye and 1 mM HBT. The reaction mixture was incubated at 24 h and monitored for colour reduction. The control sample received heat-killed enzyme. In order to find

Table 1
The level and range of independent variables chosen for RB-5 decolourization

Factor	Variable	Unit	Range and level of actual and coded values		
			−1	0	+1
A	Dye	mg l^{-1}	25	50	100
B	Enzyme	U ml^{-1}	0.5	1.5	2.5
C	HBT	mM	0.5	1.0	1.5
D	Hours	h	24	36	48

Table 2
The actual design of experiments and response for RB-5 decolourization

Observations	Factor 1	Factor 2	Factor 3	Factor 4	Response
	A: dye	B: enzyme	C: HBT	D: hours	
1	25	0.5	1.0	36	44.9
2	100	0.5	1.0	36	35.9
3	25	2.5	1.0	36	80.0
4	100	2.5	1.0	36	56.5
5	62.5	1.5	0.5	24	22.3
6	62.5	1.5	1.5	24	56.9
7	62.5	1.5	0.5	48	36.2
8	62.5	1.5	1.5	48	71.8
9	25	1.5	1.0	24	63.4
10	100	1.5	1.0	24	33.8
11	25	1.5	1.0	48	62.4
12	100	1.5	1.0	48	61.0
13	62.5	0.5	0.5	36	20.2
14	62.5	2.5	0.5	36	37.2
15	62.5	0.5	1.5	36	42.6
16	62.5	2.5	1.5	36	84.5
17	25	1.5	0.5	36	40.0
18	100	1.5	0.5	36	24.5
19	25	1.5	1.5	36	75.4
20	100	1.5	1.5	36	59.9
21	62.5	0.5	1.0	24	26.7
22	62.5	2.5	1.0	24	62.1
23	62.5	0.5	1.0	48	47.2
24	62.5	2.5	1.0	48	70.1
25	62.5	1.5	1.0	36	60.8
26	62.5	1.5	1.0	36	61.3
27	62.5	1.5	1.0	36	62.4
28	62.5	1.5	1.0	36	61.7
29	62.5	1.5	1.0	36	61.4

the effect of HBT, experiments were also conducted without addition of HBT.

2.5. Analytical methods

Extracellular protein was estimated by the method of Bradford [36] using bovine serum albumin as standard (Sigma–Aldrich, USA). Laccase activity was determined spectrophotometrically as described by Niku-Paavola et al. [37]. One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of ABTS per minute. Dye decolourization was monitored by measuring the absorbance decrease at the maximum absorbance wavelength of the dye (596 nm) and by scanning in UV–vis spectrophotometer (Varion, Australia).

3. Results and discussion

In the present study we used the purified laccase from white rot fungus *P. sajor-caju*. It has been reported that this fungus efficiently degrades wide range of synthetic dyes in nitrogen-limited medium which was mainly due to laccase [21,38]. Purified laccase of this fungus was also found to degrade the synthetic dyes [21]. The reactive dye RB-5 is widely used for textile dyeing process which is resistant to biodegradation. In the present study we have tested the ability of purified laccase to decolourize the azo dye RB-5 and found that purified laccase was unable to decolourize the dye, however, in the presence of 1 mM HBT the dye was transformed from blue colour to reddish pink colour and then turned colourless. The absorbance spectrum of the RB-5 and its decolourization by laccase is shown in Fig. 2 which revealed that HBT is essential for the decolourization of RB-5. This result shows consistency with the results of other researchers reported for RB-5 decolourization by laccase [39,40]. Bourbonnais and Paice [25] first described the oxidation of non-phenolic compounds by laccase in the presence of low molecular weight redox mediators. The mechanism of action of laccase-mediator system has been studied and it was commercially applied for pulp and paper bleaching [41]. Although the laccase-mediator systems are effective, over certain concentration it inhibits the laccase activity [29]. Therefore optimization of HBT concentration is essential for the successful decolourization. In the present study we have monitored the combined effects of enzyme, dye, and HBT concentrations, and the incubation time at various levels for the decolourization of RB-5 using RSM.

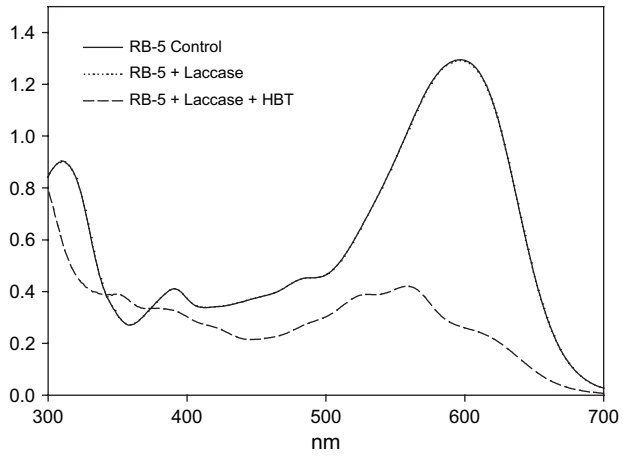

Fig. 2. UV–vis absorption spectrum of RB-5 decolourization by purified laccase of *P. sajor-caju*. Spectrum obtained from RB-5 (25 mg l^{-1}) treated by purified laccase with and without 1 mM HBT incubated for 24 h.

Response surface methodology has been successfully applied for optimizing conditions for dye degradation in fungal cultures and laccase catalyzed catechol polymerization. [29,31,42]. Box–Behnken design was chosen in this study and the levels of (coded–actual) four significant variables are presented in Table 1. Table 2 shows the data resulting from the experiment of the effect of four variables dye (A), enzyme (B), HBT (C) concentrations and incubation time (D) on decolourization of RB-5. The experimental results were analyzed through RSM to obtain an empirical model for the best response. The results of theoretically predicted responses are shown in Table 3. The estimated response seems to have a functional relationship only in a local region or near the central points of the model. The quadratic model was used to explain the mathematical relationship between the independent variables and dependent responses. The mathematical expressions of relationship to the decolourization of RB-5 with variables like A, B, C and D are shown below as in terms of coded factors:

$$\begin{aligned}\text{Decolourization} = {} & +61.52 - 7.88 \times A + 14.41 \times B + 17.56 \\ & \times C + 6.96 \times D - 1.69 \times A^2 - 5.42 \times B^2 \\ & - 9.97 \times C^2 - 4.67 \times D^2 - 3.62 \times A \times B \\ & + 7.05 \times A \times D + 6.22 \times B \times C - 3.13 \\ & \times B \times D + 0.25 \times C \times D \end{aligned} \quad (2a)$$

and in terms of actual factors:

$$\begin{aligned}\text{Decolourization} = {} & -69.44 - 0.48 \times \text{dye} + 33.63 \times \text{enzyme} \\ & + 94.68 \times \text{HBT} + 2.28 \times \text{hours} - 1.2\,E\text{-}03 \\ & \times \text{dye}^2 - 5.41 \times \text{enzyme}^2 - 39.87 \times \text{HBT}^2 \\ & - 0.03 \times \text{hours}^2 - 0.09 \times \text{dye} \times \text{enzyme} \\ & + 0.01 \times \text{Dye} \times \text{hours} + 12.45 \times \text{enzyme} \\ & \times \text{HBT} - 0.26 \times \text{enzyme} \times \text{hours} + 0.04 \\ & \times \text{HBT} \times \text{hours} \end{aligned} \quad (2b)$$

The results of analysis of variance (ANOVA) are shown in Table 4 which indicates that the predictability of the model is at 95% confidence interval. The predicted response fit well with those of the experimentally obtained response. A coefficient of determination (R^2) value of 0.999 showed that the equation is highly reliable. Further the computed F value (1942.04) is much greater than that of the tabular $F_{0.01\ (14,14)}$ value (3.70) suggesting that the treatment is highly significant. A P value less than 0.01 indicates that the model is statistically significant. The model also revealed statistically insignificant lack of fit, as is evident from the lower computed F value

Table 3
Comparison of experimentally obtained and theoretically predicted values for decolourization of RB-5

Observations	Response – decolourization		Residual
	Actual value	Predicted value	
1	44.90	44.25	0.65
2	35.90	35.75	0.15
3	80.00	80.32	−0.32
4	56.50	57.32	−0.82
5	22.30	22.62	−0.32
6	56.90	57.23	−0.33
7	36.20	36.03	0.17
8	71.80	71.65	0.15
9	63.40	63.13	0.27
10	33.80	33.28	0.52
11	62.40	62.94	−0.54
12	61.00	61.29	−0.29
13	20.20	20.39	−0.19
14	37.20	36.76	0.44
15	42.60	43.06	−0.46
16	84.50	84.33	0.17
17	40.00	40.18	−0.18
18	24.50	24.43	0.075
19	75.40	75.29	0.11
20	59.90	59.54	0.36
21	26.70	26.94	−0.24
22	62.10	62.01	0.092
23	47.20	47.11	0.092
24	70.10	69.68	0.42
25	60.8	61.52	−0.72
26	61.3	61.52	−0.22
27	62.4	61.52	0.88
28	61.7	61.52	0.18
29	61.4	61.52	−0.12

(0.9) than that of the tabular F 0.05 (10,4) value (5.96) even at 5% level. The model was found to be adequate for prediction within the range of variables chosen. Fig. 3 shows observed decolourization versus those from the statistical model (Eqs. (2a) and (2b)). The figure explains that the predicted data of the response from the empirical model is in good agreement with the experimentally obtained data.

Using RSM the combined effect of four variables can be predicted which is difficult to observe in conventional methods. The effects of variables on RB-5 decolourization are shown through Figs. 3–6. Fig. 3 shows the 3-D response surface plot of interactions between varying concentrations of enzyme and dye on decolourization at 1 mM HBT and 36 h. The surface plot shows the decrease in

Table 4
ANOVA results for the quadratic equation of Design Expert 6.0 for decolourization of RB-5

Source	Degree of freedom	Sum of squares	Mean square	F-value	P
Model	14	8732.05	623.72	1942.04	<0.0001
Residual	14	4.5	0.32		
Lack of fit	10	3.11	0.31	0.9	Not significant
Pure error	4	1.39	0.35		
Total	28	8736.55			

Std. dev.: 0.57; R^2: 0.9995; adj R^2: 0.999.

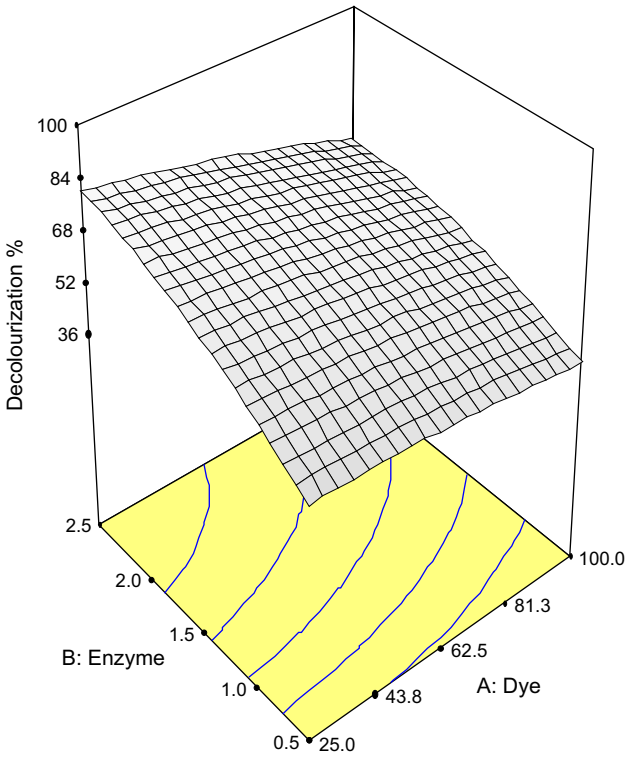

Fig. 3. Response surface plot showing the effect of different concentrations of enzyme and dye on RB-5 decolourization at 1.0 mM HBT concentration and 36 h.

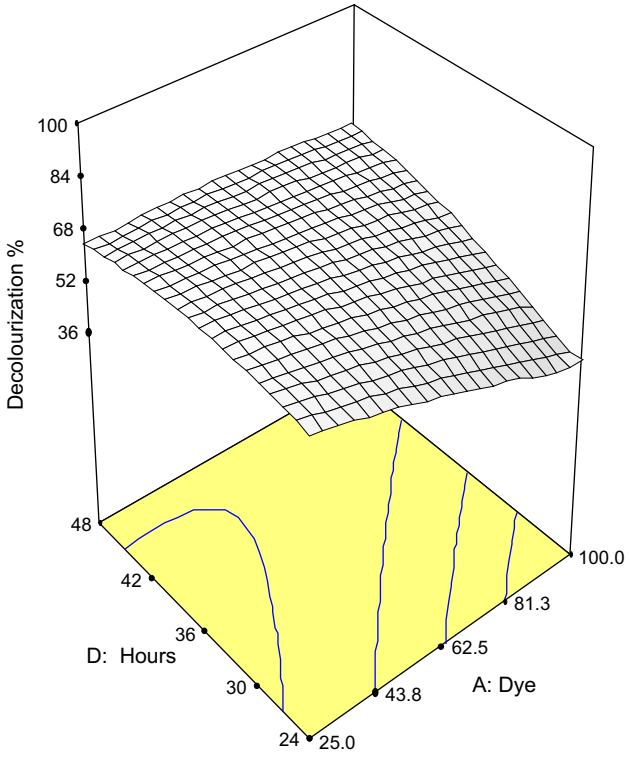

Fig. 4. Response surface plot showing the effect of dye concentration and incubation time on RB-5 decolourization at fixed concentration of enzyme and HBT 1.5 U ml^{-1} and 1.0 mM, respectively.

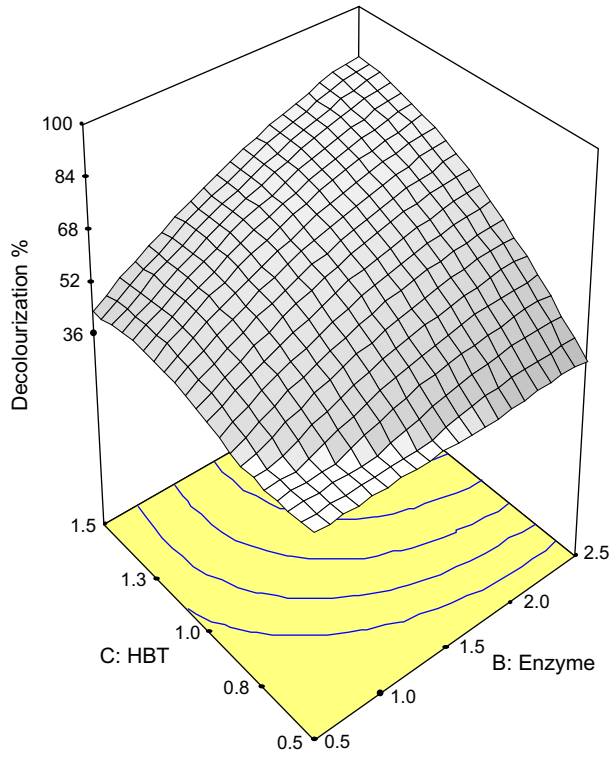

Fig. 5. Response surface plot showing the effect of enzyme and HBT concentrations at 36 h and fixed dye concentration 62.5 mg l^{-1}.

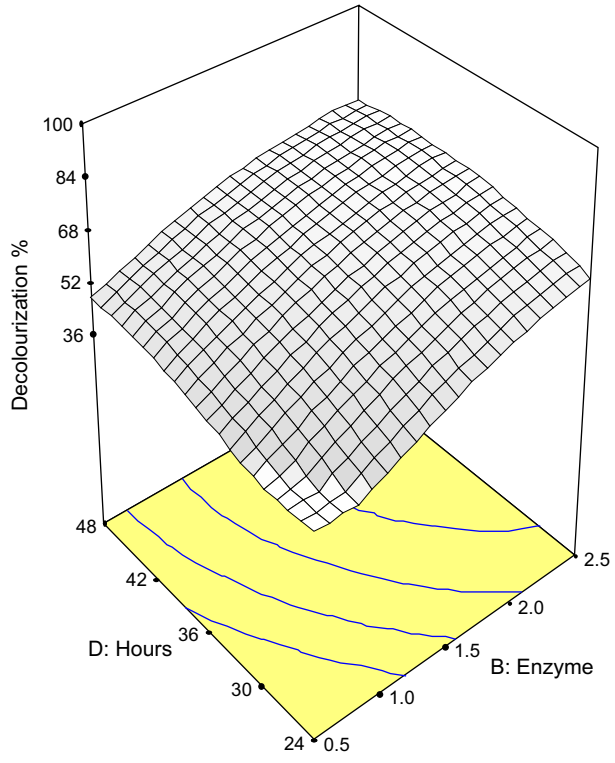

Fig. 6. Response surface plot showing the effect of enzyme concentration and incubation time at fixed concentrations of dye and HBT 62.5 mg l^{-1} and 1.0 mM, respectively.

dye decolourization with increase in dye concentration while the decolourization increased with increase in enzyme concentration. Maximum decolourization was observed at low dye and high enzyme concentration (2.5 U ml^{-1}). Decolourization was decreased with increase in dye content even in the presence of high enzyme concentration (2.5 U ml^{-1}). At high dye concentration the efficiency of enzyme reduced but did not completely diminish. The surface plot also shows the best decolourization (80.32%) obtained at 25 mg dye and 2.5 U ml^{-1} which is corresponding with the experimentally obtained response (80%) under the conditions used. Previously, Soares et al. [43] had also reported that increasing concentration of laccase from 0 to 25 U ml^{-1} increased the rate of decolourization for remazol brilliant blue R in the presence of 0.06% HBT.

Fig. 4 represents the effect of varying dye concentration and incubation time at fixed concentrations of enzyme and HBT. Maximum decolourization was 63.2% for 25 mg l^{-1} dye at 24 h when keeping the enzyme and HBT concentrations at 1.5 U ml^{-1} and 1.0 mM, respectively. It can be seen from Fig. 4 that increasing the dye concentration, keeping of all other factors constant, decreases the decolourization. However, increasing the incubation time enhances the dye decolourization. At 100 mg l^{-1} dye concentration the decolourization was 33.5% at 24 h, however, it was doubled when incubating for additional 24 h. To achieve higher decolourization, optimization of other factors, such as enzyme and HBT concentrations is important.

HBT, a potential redox mediator, plays a critical role in enhancing the rate of laccase mediated dye degradation, bleaching of pulp and other environmental pollutants [29,44]. HBT or other laccase-mediator is essentially required for the decolourization of some dyes [27]. In this study, HBT plays an important role in decolourization of RB-5 by purified laccase. The dosage of HBT is an important factor for the enzyme activity and reduction of kappa number and in paper pulp bleaching [29]. The past studies conducted on laccase mediated dye decolourization focused only on the effect of HBT [22,29,43]. There has been no study focused on the combined effects of enzyme and HBT concentrations on dye decolourization. Fig. 5 shows the combined effect of varying concentration of enzyme and HBT on RB-5 decolourization. The response plot revealed that an increase in enzyme concentration, keeping all other factors at fixed level, increased the decolourization level. The rate of decolourization increased with the increase in HBT concentration, however, at lower enzyme concentration (0.5 U ml^{-1}) HBT started to inhibit the decolourization rate at the concentration above 1.4 mM suggesting that HBT is toxic to laccase beyond this concentration. But increasing the concentration of enzyme diminishes the effect of HBT at the tested range. Maximum response observed on response plot was 84.44% at 2.5 U ml^{-1} and 1.5 mM concentrations of enzyme and HBT, respectively, for 62.5 mg l^{-1} RB-5 at 36 h that coincided well with experimentally obtained response.

Recent report of Zille et al. [23] showed that decolourization of RB-5 was achieved with laccase of *Trametes villoso*

without addition of laccase-mediator system. Li et al. [45] compared fungal laccase in combination with different redox mediators and found that the redox potential of laccase varied depending on the source of laccase. Past study showed that HBT is essential for decolourization of remazol brilliant blue R (RBBR) for *Aspergillus* laccase [27] whereas it is not required for laccase from *Pycnoporus cinnabarinus* laccase [46].

Stability of enzyme over a period of time and the concentration of enzyme are more important for enzymatic dye decolourization. Fig. 6 represents the effect of varying concentration of enzyme at different incubation times on RB-5 decolourization under 62.5 mg l^{-1} dye and 1 mM HBT concentrations. The results indicate that the response increased when increasing the enzyme concentration as well as the incubation time. The best decolourization value 71.2% was observed on surface plot at 42 h with 2.5 U ml^{-1} enzyme; however, RB-5 decolourization would increase when the concentration of HBT is increased more than 1 mM. Our results indicate that the purified laccase from *P. sajor-caju* is stable over 48 h at the conditions employed. Many laccases have been identified as thermostable and are even more active even under high stress conditions [26,47].

3.1. Adequacy of the model

Generally, it is important to confirm the fitted model to make sure that it gives sufficient approximation to the actual test. Unless the model shows a satisfactory fit, proceeding with investigation and optimization of the fitted response surface likely gives poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy. By constructing a normal probability plot of the residuals, a check was made for the normality assumption as shown in Fig. 7. The normality assumption was satisfied as the residual plot approximated along a straight line. The plot of studentized residuals versus the run order was tested and the residuals scattered randomly on the display suggesting that the variance of the original observations was constant for all values or response [48].

From the experimental responses we have calculated the optimum HBT concentration for better decolourization by performing the stationary point analysis on the 3-D surface using partial differential calculus. If the HBT concentration corresponds to the maximum value of the decolourization response function at fixed values of dye concentration, enzyme concentration, and reaction hours, then the corresponding point on the surface graph corresponds to a stationary point, that is, no change in the response function occurs in the direction of the HBT concentration axis, then

$$(\delta Y/\delta C)_{A,B,D} = 0 \tag{3}$$

The solution to this equation gives the optimal concentration of HBT (C*) beyond which it starts inhibiting the reaction. Applying the partial derivative condition (Eq. (3)) to the response function defined in Eqs. (2a) and (2b), considering only the significant terms, we can get

$$C^* = (17.56 + 6.22B + 0.25D)/19.94 \text{ as in terms of coded factors} \tag{4a}$$

and

$$\text{HBT concentration} = (94.68 + 12.45 \times \text{enzyme} + 0.04 \times \text{hours})/79.74 \tag{4b}$$

as in terms of actual factors. The values obtained from Eqs. (4a) and (4b) are well fitted with optimization tools of design experiment. Although HBT is essential for enhancing decolourization of RB-5, it starts inhibiting the reaction with increase in dye concentration. Using the higher HBT values than C* would lead to lower decolourization and at the same time wastage of chemical. Thus, using Eqs. (4a) and (4b) we can determine the concentration of HBT required for optimization of the decolourization of RB-5 by including the enzyme concentration and incubation time. The optimization of HBT is independent of dye concentration. This further verifies the negligible interaction between the two factors.

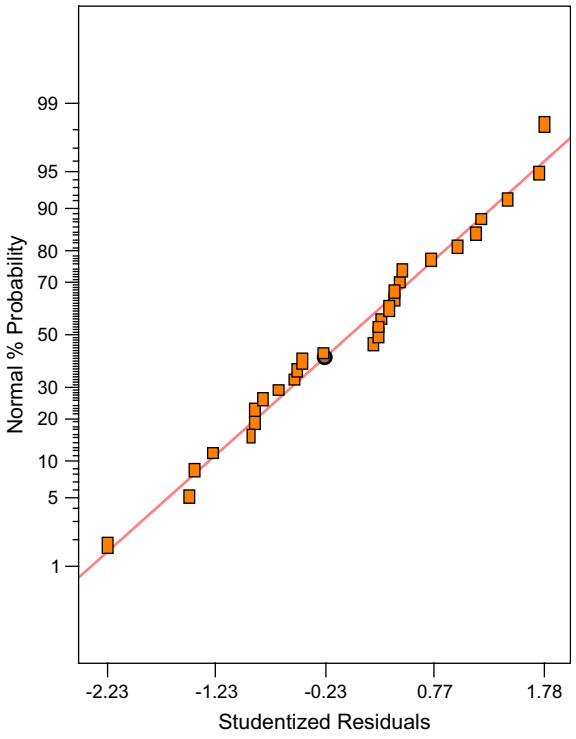

Fig. 7. Normal probability plot of studentized residuals.

4. Conclusions

Purified laccase from *P. sajor-caju* was tested for decolourization of RB-5. The results clearly showed that the redox mediator HBT is essentially required for decolourization of RB-5. The concentrations of dye, enzyme, redox mediator

and the reaction time are the most important factors for decolourization. All these factors showed combined effects on decolourization of RB-5. Response surface methodology was successfully applied to find out the optimum level of the above factors using Box–Behnken design. The optimum concentrations of dye, enzyme, HBT and, time were found to be 62.5 mg l^{-1}, 2.5 U ml^{-1}, 1.5 mM and 36 h, respectively, for maximum decolourization of RB-5 (84.33%). A quadratic model was obtained for this design using Design Expert 6.0. The model employed provided good quality of predictions for the above variables in terms of effective dye decolourization, and a good correlation coefficient of R^2 0.999 was obtained. By this model we can predict the response for the above variables at any point. To our knowledge, this is the first report on the demonstration of RSM to optimize the laccase mediated enzymatic dye decolourization.

Acknowledgments

The authors wish to thank Stat-Ease, Inc. for using Design Expert 6.0 (Trial version) in our study and Brain Korea 21 for financial support.

References

[1] Hutzinger O [Part A]. The handbook of environmental chemistry, vol. 3. Berlin, Heidelberg: Springer-Verlag; 1980. 188 p.
[2] Vaidya AA, Datye KV. Environmental pollution during chemical processing of synthetic fibres. Colourage; 1982:3–10.
[3] Kulkarni SV, Blackwell CD, Blackard AL, Stackhocese CW, Alexander MW. Textile dyes and dyeing equipment, classification, properties and environmental aspects. Research Triangle Park, NC: U.S. Environmental Protection Agency; 1985. EPA – 6002/2-85/010.
[4] Gouvea CAK, Wypych F, Moraes SG, Duran N, Nagata N, Peralta-Zamora P. Semiconductor-assisted photocatalytic degradation of reactive dyes in aqueous solution. Chemosphere 2000;40:433–40.
[5] Peralta-Zamora P, Kunz A, de Moraes SG, Pelegrini R, Moleiro PD, Reyes J, et al. Degradation of reactive dyes—I. A comparative study of ozonation, enzymatic and photochemical processes. Chemosphere 1999;38:835–52.
[6] Arslan I, Balcioglu IA, Bahnemann DW. Advanced chemical oxidation of reactive dyes in simulated dyehouse effluents by ferrioxalate-Fenton/UV-A and TiO2/UV-A processes. Dyes Pigments 2000;47:207–18.
[7] Cooper P. Removing colour from dye house wastewater – a critical review of technology available. J Soc Dyers Colour 1993;109:97–108.
[8] O'Neill C, Lopez A, Esteves S, Hawkes F, Hawkes DL, Wilcox S. Azo dye degradation in anaerobic–aerobic treatment system operating on stimulated textile effluent. Appl Biochem Biotechnol 2000;53:249–54.
[9] Wesenberg D, Buchon F, Agathos SN. Degradation of dye containing textile effluent by the agaric white-rot fungus *Clitocybula dusenii*. Biotechnol Lett 2002;24:989–93.
[10] Kashinath A, Novotny CK, Kamalesh KS, Patel C, Vaclava A. Decolourization of synthetic dyes by *Irpex lacteus* in liquid culture and packed bed bioreactor. Enzyme Microb Technol 2003;32:167–73.
[11] Senan RC, Abraham TE. Bioremediation of textile azo dyes by aerobic bacterial consortium. Biodegradation 2004;15:275–80.
[12] Ramsay JA, Goode C. Decoloration of a carpet dye effluent using *Trametes versicolor*. Biotechnol Lett 2004;26:197–201.
[13] Ollika P, Alhonmaki K, Leppanen VM, Glumoff T, Rajola T, Suominen I. Decolourization of azo, triphenyl methane, heterocyclic and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. Appl Environ Microbiol 1993;59:4010–6.
[14] Moreira MT, Mielgo I, Feijoo G, Lema JM. Evaluation of different fungal strains in the decolourisation of synthetic dyes. Biotechnol Lett 2000;22:1499–503.
[15] Murugesan K, Kalaichelvan PT. Synthetic dye decolourization by white rot fungi. Indian J Exp Biol 2003;41:1076–87.
[16] Novotny C, Svobodova K, Erbanova P, Cajthamal T, Kasinath A, Lang E, et al. Lignolytic fungi in bioremediation: extracellular enzyme production and degradation rate. Soil Biol Biochem 2004;36:1545–51.
[17] Chivukula M, Renganathan V. Phenolic azo dye oxidation by laccase from *Pyricularia oryzae*. Appl Environ Microbiol 1995;61:4374–7.
[18] Heinfling A, Martinez MJ, Martinez AT, Bergbauer M, Szewzyk U. Transformation of industrial dyes by manganese peroxidase from *Bjerkandera adusta* and *Pleurotus eryngii* in a manganese-independent reaction. Appl Environ Microbiol 1998;64:2788–93.
[19] Abadulla E, Tzanov T, Costa S, Robra KH, Cavaco-Paulo A, Gubitz GM. Decolourization and detoxification of textile dyes with a laccase from *Trametes hirsuta*. Appl Environ Microbiol 2000;66:3357–62.
[20] Kim SJ, Shoda M. Purification and characterization of a novel peroxidase from *Geotrichum candidum* Dec.1 involved in decolourization of dyes. Appl Environ Microbiol 1999;65:1029–35.
[21] Murugesan K. Studies on production, purification, characterization and crystallization of laccase from a white rot fungus *Pleurotus sajor-caju* and its application in bioremediation of textile dye effluent and dye contaminated soils. PhD thesis. Madras University; 2002.
[22] Reyes R, Pickard M, Vazquez-Duhalt R. Hydroxybenztriazole increase the range of textile dyes decolourized by immobilized laccase. Biotechnol Lett 1999;21:875–80.
[23] Zille A, Tzanov T, Gübitz GM, Cavaco-Paulo M. Immobilized laccase for decolourization of reactive black 5 dyeing effluent. Biotechnol Lett 2003;25:1473–7.
[24] Kandelbauer A, Maute O, Kessler RW, Erlacher A, Gubitz GM. Study of dye decolorization in an immobilized laccase enzyme-reactor using on-line spectroscopy. Biotechnol Bioeng 2004;87:552–63.
[25] Bourbonnais R, Paice MG. Oxidation of non-phenolic substrates. An expanded role for laccase in lignin biodegradation. FEBS Lett 1990;267:99–102.
[26] Jordaan J, Pletschke BI, Leukes WD. Purification and partial characterization of a thermostable laccase from an unidentified basidiomycete. Enzyme Microb Technol 2004;34:635–41.
[27] Soares GMB, Amorim MTP, Costa-Ferreira M. Use of laccase together with redox mediators to decolourize remazol brilliant blue R. J Biotechnol 2001;89:123–9.
[28] Baldrian P. Purification and characterization of laccase from the white-rot fungus *Daedalea quercina* and decolorization of synthetic dyes by the enzyme. Appl Microbiol Biotechnol 2004;63:560–3.
[29] García O, Camarero S, Colom JF, Martínez ÁT, Martínez MJ, Monje R, et al. Optimization of a laccase-mediator stage for TCF bleaching of flax pulp. Holzforschung 2003;57:513–9.
[30] Box GEP, Wilson KN. On the experimental attainment of optimum conditions. J R Stat Soc Ser B 1951;13:1–45.
[31] Nagarajan G, Annadurai G. Biodegradation of reactive dye (Verofix Red) by the white rot fungus *Phanerochaete chrysosporium* using Box–Behnken experimental design. Bioprocess Eng 1999;20:435–40.
[32] Nyanhongo GS, Gomes J, Gubitz G, Zvauya R, Read JS, Steiner W. Production of laccase by a newly isolated strain of *Trametes modesta*. Bioresour Technol 2002;84:259–63.
[33] Trupkin S, Levin L, Forchiassin F, Viale A. Optimization of a culture medium for ligninolytic enzyme production and synthetic dye decolorization using response surface methodology. J Ind Microbiol Biotechnol 2003;30:682–90.
[34] Box GEP, Hunter JS. Multifactor experimental design for exploring the response surfaces. Ann Math Stat 1957;28:195–242.
[35] Box GEP, Behnken DW. Some new three level design for study of quantitative variables. Technometrics 1960;2:455–75.

[36] Bradford MM. A rapid and sensitive method for the quantification of protein utilizing the principle of protein–dye-binding. Anal Biochem 1976;72:248–54.
[37] Niku-Paavola ML, Raaska L, Itavaara M. Detection of white rot fungi by non-toxic stain. Mycol Res 1990;94:27–31.
[38] Chagas EP, Durrant LR. Decolorization of azo dyes by *Phanerochaete chrysosporium* and *Pleurotus sajorcaju*. Enzyme Microb Technol 2001;29:473–7.
[39] Ramsay JA, Nguyen T. Decoloration of textile dyes by *Trametes versicolor* and its effect on dye toxicity. Biotechnol Lett 2002;24:1757–61.
[40] Mazmanci MA, Ünyayar A. Decolourisation of Reactive Black 5 by *Funalia trogii* immobilised on *Luffa cylindrica* sponge. Process Biochem 2005;40:337–42.
[41] Call HP, Muke I. History, overview and applications of mediated lignolytic systems, especially laccase-mediator systems (lignozyme-process). J Biotechnol 1997;53:163–202.
[42] Atkas N. Optimization of biopolymerization rate by response surface methodology (RSM). Enzyme Microb Technol 2005;37:441–7.
[43] Soares GMB, Pessoa-de-Amorim MT, Costa-Ferreira M. Use of laccase together with redox mediators to decolourize remazol brilliant blue R. J Biotechnol 2001;89:123–9.
[44] Cho SJ, Park SJ, Lim JS, Rhee YH, Shin KS. Oxidation of polycyclic aromatic hydrocarbons by laccase of *Coriolus hirsutus*. Biotechnol Lett 2002;24:1337–40.
[45] Li K, Xu F, Eriksson KEL. Comparison of fungal laccases and redox mediators in oxidation of non-phenolic lignin model compound. Appl Environ Microbiol 1999;65:2654–60.
[46] Schliephake K, Mainwaring DE, Lonergan GT, Jones IK, Baker WL. Transformation and degradation of the disazo dye Chicago sky blue by a purified laccase from *Pycnoporus cinnabarinus*. Enzyme Microb Technol 2000;27:100–7.
[47] Niku-Paavola ML, Fagerström R, Kruus K, Viikari L. Thermostable laccases produced by a white-rot fungus from *Peniophora* species. Enzyme Microb Technol 2004;35:100–2.
[48] Myers RH, Montgomery DC. Response surface methodology. USA: Wiley; 2002. p. 43.