



## Application of response surface methodological approach to optimise Reactive Black 5 decolouration by crude laccase from *Trametes pubescens*

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### ABSTRACT

Response surface methodology (RSM) was applied to optimise the decolouration of the diazo dye Reactive Black 5 (RB5) by crude laccase from the white-rot fungus *Trametes pubescens*. The presence of the redox mediator 1-hydroxybenzotriazole (HBT) greatly improved the decolouration levels of RB5 by crude laccase from *T. pubescens*. Central composite design (CCD) using RSM with three variables namely redox mediator (HBT), dye (RB5) and enzyme (laccase) concentrations was used in this study to optimise significant correlation between the effects of these variables on the decolouration of RB5. The optimum concentrations of HBT, RB5 and laccase were 1.17 mM, 150 mg/l and 500 U/l, respectively, for a maximum decolouration of RB5 (about 60% in 20 min). A quadratic model was obtained for dye decolouration through this design. The experimental values were in good agreement with the predicted ones and the model was highly significant, the correlation coefficient being 0.965. Interaction between HBT and RB5 concentrations, HBT and laccase concentrations and RB5 and laccase concentrations were negligible. In addition, when the kinetic parameters for RB5 decolouration were calculated according to Hannes–Wolf plot, the following values were obtained:  $K_M$  of 260.11 mg/l and  $V_{max}$  37.59 mg/l min.

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### 1. Introduction

Synthetic dyes are extensively used in the food, cosmetics, plastics and textile industries among others to colour their products. This results in the generation of large amounts of highly polluted effluents. Approximately 10,000 different dyes and pigments are used in textile and printing industries. About  $8 \times 10^5$  tons of these dyes are produced annually worldwide and at least 10% of the used dyes are discharged into the environment [1,2]. Azo dyes currently represent about 60% of the global market for dyes and are widely used in dyeing of textile fibres [3]. The azo dyes are xenobiotic compounds characterised by the presence of one or more azo bonds ( $-N=N-$ ) and aromatic rings [4]. Some of the azo dyes are known to be very toxic and mutagenic to living organisms [5–7]. Therefore, treatment of azo dye-containing wastewater is essential to prevent ecosystem deterioration.

There are several methods used to remove dyes from wastewater. These methods can be chemical, physical or biological. The

physical and chemical methods have high costs, low efficiency and cannot be used with a great variety of dyes. The use of enzyme-based methods is a good alternative. The potential advantages of the enzymatic treatments compared to the conventional ones include: application to recalcitrant materials, operation at high and low contaminant concentrations over a wide pH, temperature and salinity range, biomass acclimatization is irrelevant and straight forward process control [8].

The main enzymes involved in the decolouration of dyes are ligninolytic which are produced by white-rot fungi and some litter-decomposing fungi. These include lignin peroxidase (LiP; EC 1.11.1.14), manganese peroxidase (MnP; EC 1.11.1.13) and laccase (*p*-diphenol: dioxygen oxidoreductase; EC 1.10.3.2). Laccase-based treatments have received much attention for the degradation of different recalcitrant pollutants, as laccase production is constitutive in most white-rot fungi and can be easily enhanced by the addition of different inducers into the culture medium. In addition, in the presence of certain low molecular weight compounds, named redox mediators, laccases are able to oxidise compounds that are not laccase substrates. The redox mediators are readily oxidised by laccases producing, in some cases, very unstable and reactive cationic radicals, which can oxidise more complex substrates before returning to their original state. The electrons taken by laccases are finally transferred back to oxygen to form water

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[9,10]. 1-Hydroxybenzotriazole (HBT) is one of the most efficient laccase mediators but its high cost and potential toxicity are important drawbacks. Therefore, the optimisation of HBT concentration is important for effective decolouration.

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for analysing the effects of several independent variables [11]. In many processes, the relationship between the response and independent variables is unknown; therefore the first step in RSM is to approximate the function (response) in terms of analysing the independent variables. Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables, which will later be analysed to locate the optimum values of independent variables for the optimum response [12].

The aim of the present study was to optimise the decolouration of the recalcitrant diazo dye Reactive Black 5 (RB5) by crude laccase, obtained from cultivation of the white-rot fungus *Trametes pubescens*, using RSM to optimise the main parameters affecting the decolouration process such as the initial concentrations of enzyme (laccase), dye (RB5) and redox mediator (HBT). The kinetic parameters ( $K_M$  and  $V_{max}$ ) of the RB5 decolouration by laccase were also determined.

## 2. Methods

### 2.1. Laccase production and crude laccase preparation

The white-rot fungus *T. pubescens* (MB 89; Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences, Vienna, Austria) was selected to perform the present study because it has been described as an outstanding laccase producer [13,14]. Laccase was produced under semi-solid-state fermentation conditions using dyed sunflower seed shells as support-substrates as described in Rodríguez Couto et al. [15] because this approach allowed the production of high titres of laccase as the only lignolytic enzyme.

Culture broth was recovered at the maximum laccase activity (day 10), filtered, clarified by centrifugation at  $8000 \times g$  for 15 min, frozen, defrosted and, then, filtered to remove the precipitated polysaccharides. The resulting clear filtrate was concentrated on an Amicon membrane with a molecular cut-off of 10 kDa. The experiments were performed with this concentrated clear filtrate.

### 2.2. Determination of laccase activity

ABTS was used as a substrate for the spectrophotometric determination of laccase activity as described by Niku-Paavola et al. [16]. One activity unit (U) was defined as the amount of enzyme that oxidised  $1 \mu\text{mol}$  of ABTS per min. The activities were expressed in U/l.

### 2.3. Experimental procedure

The dye used was the recalcitrant diazo dye Reactive Black 5 (RB5) purchased from Sigma–Aldrich (St. Louis, MO, USA). RB5 was chosen because it is inexpensive and one of the most widely used dyes in the textile industry [17]. Stock solutions (0.1% w/v in water) were stored in the dark at room temperature and the desired concentrations of the dye were obtained by further dilutions. The reaction mixtures contained citrate phosphate buffer (pH 4.0), different concentrations of crude laccase, redox mediator (HBT) and dye (RB5) in a final volume of 5 ml. All the reactions were incubated at room temperature, without shaking and in darkness. The residual dye concentration was spectrophotometrically measured from 400 to 800 nm and calculated by measuring the area under the plot. This approach takes into account the conversion of the dye

**Table 1**

The range and the levels of the variables.

Factor	Variable	Unit	Range and level of actual and coded values				
			$-\alpha$	$-1$	$0$	$1$	$\alpha$
$X_1$	HBT	mM	0.33	0.5	0.75	1	1.17
$X_2$	Dye	mg/l	65.9	100	150	200	234.1
$X_3$	Enzyme	U/l	163.6	300	500	700	836.4

molecules to other compounds absorbing at different wavelengths and then, the ratio of the area under the visible spectrum is always equal to or lower than the ratio of the absorbances at the peak. A UV–visible spectrophotometer (Thermo Spectronic, model Helios alfa, Cambridge, UK) was used in all the experiments.

Dye decolouration was calculated by means of the formula:

$$D = \frac{(A_{ini} - A_{obs})}{A_{ini}} \times 100 \quad (1)$$

where  $D$  is the decolouration of RB5 (in %),  $A_{ini}$  is the area under the curve of the absorption spectrum from 400 to 800 nm at time zero and  $A_{obs}$  is the area under the curve of the absorption spectrum from 400 to 800 nm at a determined time.

Samples were diluted prior to measurement in order that the absorbance showed values below 1, and hence that the Lambert–Beer law could be applied. A control test containing the same amount of a heat-denatured laccase was also performed in parallel.

### 2.4. Response surface methodology and central composite design

Since the conventional method of optimisation, “one factor at a time” approach is laborious, time consuming and incomplete, response surface methodology (RSM) using central composite design (CCD) was applied to model the decolouration process. This approach involves full factorial search by examining simultaneous, systematic and efficient variation of important components, identifying possible interactions, higher order effects and determining the optimum operational conditions. However, RSM using CCD is useful for a small number of variables (up to five) but it is impractical for a large number of variables due to the high number of experimental runs required [18].

To explore the effect of variables on the response in the region of investigation, a CCD with three variables at three levels was performed. HBT, RB5 and laccase concentrations were considered the most effective independent variables according to previous experiments [19,20]. The percentage of RB5 decolouration was taken as the response. The CCD was applied using Design Expert software (Stat Ease, 7.1 trial version). The range and the levels of the variables under investigation in this study are given in Table 1.

In the regression equation, the test variables were coded according to the equation:

$$X_i = \frac{X_i - X_i^0}{\Delta X_i} \quad (2)$$

where  $X_i$  is the independent variable coded value,  $X_i$  is the independent variable real value,  $X_i^0$  is the independent variable real value on the centre point [21] and  $\Delta X_i$  is the interval,  $i = 1, 2, 3$ .

The response was related to the selected variables by a quadratic model as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (3)$$

where  $Y$  is the predicted response;  $\beta_0$  is a constant coefficient;  $\beta_1, \beta_2, \beta_3$  are the linear coefficients;  $\beta_{11}, \beta_{22}, \beta_{33}$  are the quadratic coefficients;  $\beta_{12}, \beta_{13}, \beta_{23}$  are the interactions of the coefficients and  $X_1, X_2, X_3$  are the coded factors.

**Table 2**  
Experimental conditions of CCD runs of Design Expert 7.1.

Run	Coded values			Actual values		
	$X_1$	$X_2$	$X_3$	HBT (mM)	Dye (mg/l)	Laccase (U/l)
1	-1	-1	-1	0.5	100	300
2	1	-1	-1	1	100	300
3	-1	1	-1	0.5	200	300
4	1	1	-1	1	200	300
5	-1	-1	1	0.5	100	700
6	1	-1	1	1	100	700
7	-1	1	1	0.5	200	700
8	1	1	1	1	200	700
9	-1.68	0	0	0.33	150	500
10	1.68	0	0	1.17	150	500
11	0	-1.68	0	0.75	65.9	500
12	0	1.68	0	0.75	234.1	500
13	0	0	-1.68	0.75	150	163.6
14	0	0	1.68	0.75	150	836.4
15	0	0	0	0.75	150	500
16	0	0	0	0.75	150	500
17	0	0	0	0.75	150	500
18	0	0	0	0.75	150	500
19	0	0	0	0.75	150	500
20	0	0	0	0.75	150	500

Three important parameters: concentration of HBT ( $X_1$ ), concentration of RB5 ( $X_2$ ) and concentration of laccase ( $X_3$ ) were selected as the independent variables and the decolouration percentage ( $Y$ ) was the dependent response variable. Each of the independent variables was studied at five different levels containing two star points ( $\alpha = 1.68$ ) and six replications of the central point with a total of 20 experiments. Percentage of RB5 decolouration corresponding to combined effects of the three variables was studied in their specified ranges, HBT concentration: 0.33–1.17 mM, RB5 concentration: 65.9–234.1 mg/l and laccase concentration: 163.6–836.4 U/l. The other two process variables, pH and temperature were kept constant at 4 and 25 °C, respectively throughout the 20 experiments. The plan of CCD in coded levels of the three independent variables is shown in Table 2.

### 2.5. Software and data analysis

The results of the experimental design were analysed and interpreted using Design Expert software (Stat Ease, 7.1 trial version). The response 3D plots were built using Sigma Plot software 7.1 (Jandel Scientific).

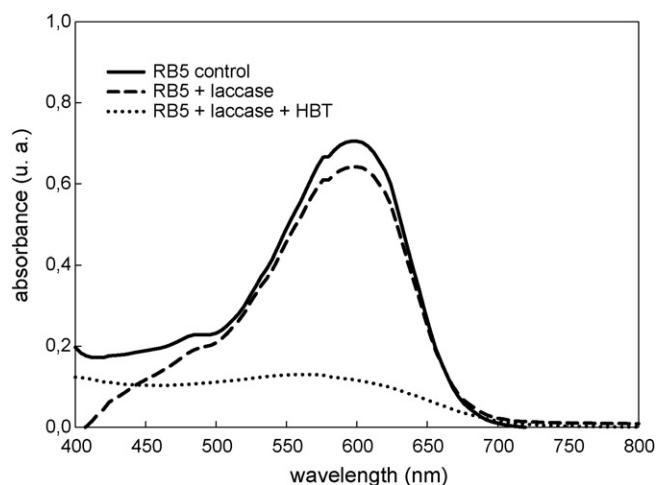
### 2.6. Kinetic study

Substrate specificity towards the dye RB5 was analysed kinetically at room temperature. The reaction mixtures consisted of RB5 solution (at varying concentrations ranging from 50 to 300 mg/l), laccase (700 U/l) and HBT (1.17 mM) in citrate phosphate buffer (pH 4.0) in a final volume of 5 ml. All the reactions were incubated at room temperature (25 °C), without shaking and in complete darkness. Decolouration was spectrophotometrically monitored as described in Section 2.3.

## 3. Results and discussion

### 3.1. Optimisation of RB5 decolouration using RSM

The first step in this study was the identification of the variables that affected decolouration and their range. Thus, initially, we tested the capacity of the crude laccase alone in the decolouration of RB5 at pHs 4 and 5. It was found that the laccase was only able to decolourise the dye RB5 at pH 4 and the decolouration obtained was very low (10% in 24 h). Further, the decolouration



**Fig. 1.** UV-vis absorption spectrum of Reactive Black 5 (RB5) decolouration by crude laccase of *Trametes pubescens*. Spectrum obtained from RB5 (150 mg/l) treated by crude laccase (500 U/l) with and without 0.75 mM 1-hydroxybenzotriazole incubated for 24 h.

of RB5 by crude laccase in the presence of the redox mediator 1-hydroxybenzotriazole (HBT) was studied and was found that HBT had an immediate effect on RB5 decolouration by laccase. As shown in Fig. 1 laccase hardly decolourised the dye RB5 in the absence of HBT. This result is in agreement with those obtained by other researchers [22–27]. Although the redox mediators are effective in some circumstances, over certain concentration they inhibit the laccase activity [28]. Therefore, optimisation of HBT concentration is essential for a successful decolouration. In addition, enzyme and dye concentrations were also selected as variables.

The experimental results were analysed through RSM to obtain an empirical model for the best response. The actual and the predicted dye decolouration percentages are shown in Table 3. Actual values correspond to the measured response data for a particular run and the predicted values were evaluated from the model. The quadratic model was used to explain the mathematical relationship between the independent variables and the dependent response. The mathematical expression of the relationship to RB5 decolouration with the variables  $X_1$ ,  $X_2$  and  $X_3$  is shown below in terms of

**Table 3**

The actual and the predicted dye decolouration percentage.

Run	Response		R-studentised residual
	Observed	Predicted	
1	28.48	24.12	1.581
2	50.02	48.17	0.619
3	11.52	7.96	1.251
4	30.80	32.01	-0.401
5	44.69	41.93	0.944
6	62.15	65.98	-1.359
7	22.91	25.76	-0.979
8	52.16	49.82	0.794
9	14.22	18.03	-1.931
10	59.83	58.48	0.604
11	46.59	48.80	-1.018
12	21.35	21.61	-0.114
13	19.68	23.93	-2.237
14	55.66	53.88	0.809
15	48.50	45.47	0.954
16	44.68	45.47	-0.240
17	42.64	45.47	-0.887
18	42	45.47	-1.105
19	48.52	45.47	0.961
20	46.9	45.47	0.438

**Table 4**  
ANOVA results for the equation of Design Expert 7.1.

Source	Sum of squares	Degree of freedom	Mean square	F-value	Probability > F
Model	4255.49	6	709.25	59.02	<0.0001
$X_1$	1975.10	1	1975.10	164.35	<0.0001
$X_2$	892.43	1	892.43	74.26	<0.0001
$X_3$	1082.74	1	1082.74	90.10	<0.0001
$X_1^2$	93.67	1	93.67	7.79	0.0153
$X_2^2$	189.85	1	189.85	15.80	0.0016
$X_3^2$	77.66	1	77.66	6.46	0.0246
Residual	156.23	13	12.02		
Lack of fit	115.05	8	14.38	1.75	0.2794
Pure error	41.17	5	8.23		
Total	4411.72	19			
R-squared	0.9646	Adj R-squared	0.9482		

**Table 5**  
Decolouration percentages of Reactive Black 5 (RB5) reported by different authors for different RB5, laccase and 1-hydroxybenzotriazole concentrations.

Laccase source	[RB5] (mg/l)	[HBT] (mM)	[laccase] (U/l)	Decolouration (%)	Reference
<i>P. pinsitus</i>	200	2	2400	70% in 16 h	Claus et al. [22]
<i>Trametes modesta</i>	41.7	0.5	12	88% in 3 h	Nyanhongo et al. [23]
<i>Picnoporus cinnabarinus</i>	24.8	0.5	100	50% in 6 h	Camarero et al. [24]
<i>Pleurotus sajor-caju</i>	62.5	1.5	2500	84.4% in 36 h	Murugesan et al. [26]
<i>Ischnoderma resinosum</i>	100	5	25	70% in 1 h	Kokol et al. [27]
<i>T. pubescens</i>	150	1.17	500	60% in 20 min	This study

coded factors:

$$Y = 45.47 + 12.03 \cdot X_1 - 8.08 \cdot X_2 + 8.90 \cdot X_3 + 1.19 \cdot X_1 \cdot X_2 + 0.74 \cdot X_1 \cdot X_3 + 0.55 \cdot X_2 \cdot X_3 - 2.55 \cdot X_1^2 - 3.63 \cdot X_2^2 - 2.32 \cdot X_3^2 \quad (4)$$

Statistical analysis showed that the coefficients  $X_1 \cdot X_2$ ,  $X_1 \cdot X_3$  and  $X_2 \cdot X_3$  were statistically insignificant, being deleted from the equation and added to the lack of fit. As a result, a new regression model was obtained:

$$Y = 45.47 + 1203 \cdot X_1 - 8.08 \cdot X_2 + 8.90 \cdot X_3 - 2.55 \cdot X_1^2 - 3.63 \cdot X_2^2 - 2.32 \cdot X_3^2 \quad (5)$$

The results of analysis of variance (ANOVA) for Eq. (5) are shown in Table 4. The model of the equation was significant at the 1% level and each term was significant at the 5% level. A coefficient of determination ( $R^2$ ) value of 0.965 showed that the equation was highly reliable. The model also revealed a statistically insignificant lack of fit ( $P=0.279$ ) at 5% level.

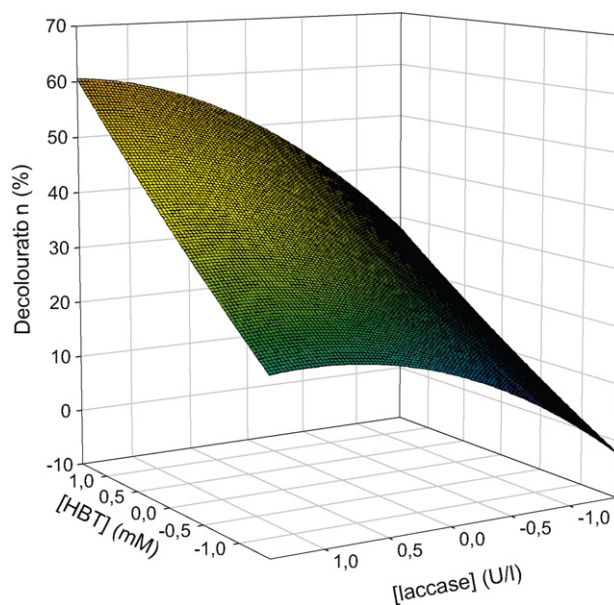
The optimum concentrations of HBT, RB5 and laccase were found to be 1.17 mM, 150 mg/l and 500 U/l, respectively, for a maximum RB5 decolouration of ca. 60% in 20 min. The result obtained is similar or even better than those reported by other authors (Table 5).

The effects of variables on RB5 decolouration are shown through Figs. 2–4. All these figures were obtained at a fixed time of decolouration (20 min) and pH 4. A graph was constructed by plotting the central values of the variables that affect the decolouration (500 U/l of laccase, 0.75 mM HBT and 150 mg/l RB5). The effect of HBT and enzyme concentrations on the response at a fixed dye concentration of 150 mg/l is shown in Fig. 2. Increase in both HBT and laccase concentrations yielded an increase in the response surface. The response value reached its highest value at 1.17 mM HBT and 836.4 U/l laccase. It can be observed that increasing HBT and laccase concentrations over the above-mentioned values does not increase RB5 decolouration. In general, in enzymatic reactions the reaction rate increases with the increase in substrate concentration. When the substrate concentration increases to a certain high value, the

reaction rate reaches a plateau and keeps constant even if more substrate is used [29].

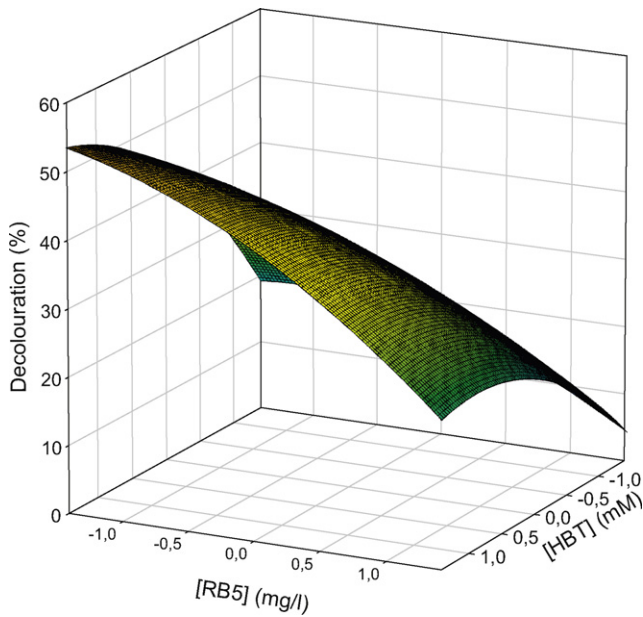
The effect of HBT and dye concentrations on the response at a fixed laccase concentration of 500 U/l was shown in Fig. 3. The response plot revealed that with the increase in HBT concentration and decrease in RB5 concentration dye decolouration increased. Maximum RB5 decolouration was attained at 1.17 mM HBT and 65.9 mg/l RB5.

The effect of enzyme and dye concentrations at a fixed HBT concentration of 0.75 mM is shown in Fig. 4. The plot shows that RB5 decolouration decreased with increase in RB5 concentration and increased with increase in laccase concentration. Maximum decolouration was obtained at concentrations of RB5 between 100 and 125 mg/l and concentrations of laccase between 700 and



**Fig. 2.** 3D surface plot for the removal of Reactive Black 5 (RB5) by laccase as a function of 1-hydroxybenzotriazole (HBT) and enzyme concentrations at a fixed value of 150 mg/l RB5.



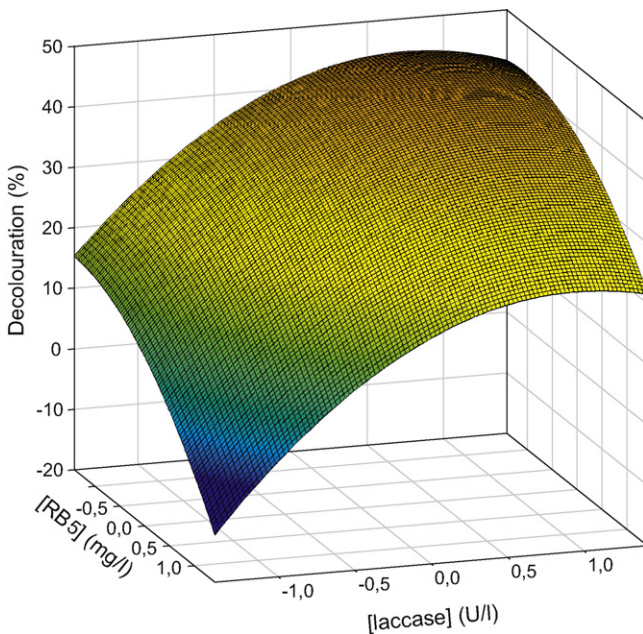


**Fig. 3.** 3D surface plot for the removal of Reactive Black 5 (RB5) by laccase as a function of 1-hydroxybenzotriazole (HBT) and dye concentrations at a fixed value of 500 U/l laccase.

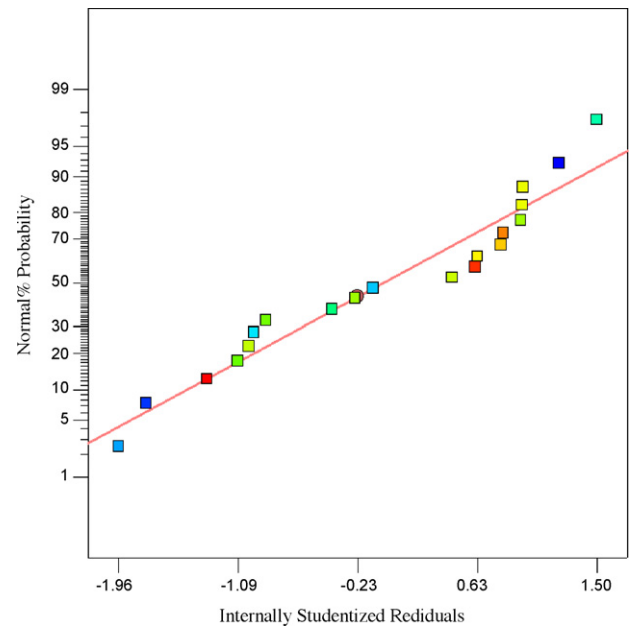
800 U/l. It can be observed that decolouration decreased with increased RB5 concentration even in the presence of high laccase concentrations.

**3.1.1. Adequacy of the model**

Generally, it is important to assess the fitted model to ensure that it gives sufficient approximation of the results obtained in the experimental conditions. A check of the normality assumption can be made by constructing a normal probability plot of the residuals as given in Fig. 5. The normality assumption is satisfied if the residuals plot approximated along a straight line. The coefficient of multiple regression,  $R^2$ , is a global statistic parameter to assess



**Fig. 4.** 3D surface plot for the removal of Reactive Black 5 (RB5) by laccase as a function of dye and enzyme concentrations at a fixed value of 0.75 mM 1-hydroxybenzotriazole (HBT).

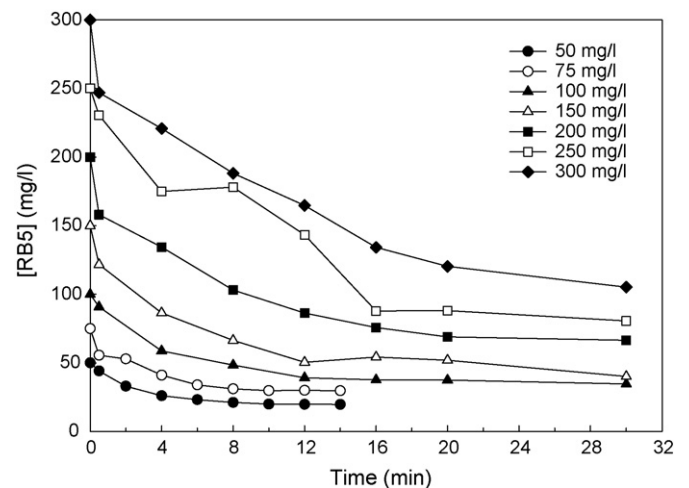


**Fig. 5.** Studentised plot showing normal % probability versus internally studentised residuals.

the fit of a model [11]. In the present model,  $R^2$  was adjudged to be 0.965, which indicates the fitness of the model. For further validation of the model, adjusted  $R^2$  was used for confirming the model adequacy. The adjusted  $R^2$  was calculated to be 0.948, which indicates a good model for using in the field conditions. The residual analysis was also carried out for judging the model adequacy. A rough check of the outliers was performed by examining the internally studentised residuals. The residuals should be approximately normal with mean zero and unit variance [11]. It was observed that none of the studentised residuals had a value higher than 2 (Fig. 5). To validate the model further and check the outliers, the externally studentised residuals were calculated (Table 3). All the values lie within the range of  $-2$  and  $+2$  (values between  $-3$  and  $+3$  being the acceptable limit), thereby validating the model.

**3.2. Kinetic study**

Fig. 6 shows the kinetics of RB5 decolouration by crude laccase form *T. pubescens* corresponding to different initial dye concen-



**Fig. 6.** The residual concentration of Reactive Black 5 (RB5) along time for different initial dye concentrations.

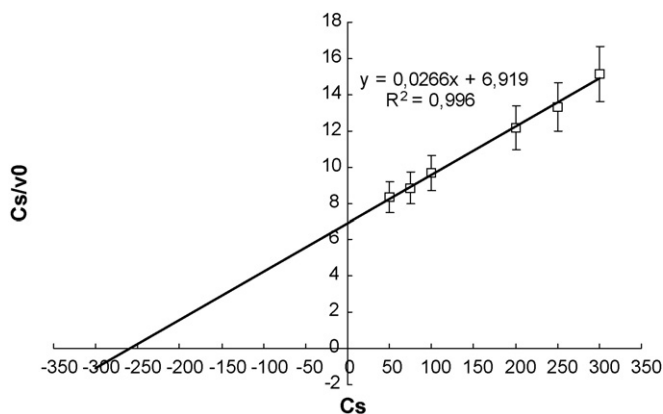


Fig. 7. Hanes–Wolf plot of the kinetics of Reactive Black 5 (RB5) decolouration.

trations. The kinetic parameters of RB5 decolouration by laccase ( $K_M$  and  $V_{max}$ ) were estimated from Hanes–Wolf plot as shown in Fig. 7. The Hanes–Wolf plot gave a  $K_M$  of 260.11 mg/l and  $V_{max}$  of 37.59 mg/l min for the diazo dye RB5 decolouration by laccase. The high  $K_M$  value suggests that RB5 is not susceptible to laccase attack which confirms the necessity of a redox mediator for RB5 decolouration by laccase. The  $K_M$  value was almost 3-fold higher and  $V_{max}$  about 500-fold higher than those obtained by Mazmanci and Ünyayar [30] for cultures of *Funalia trogii* immobilised on *Luffa cylindrica*. So, enzyme systems seem very promising for dye decolouration.

#### 4. Conclusions

Crude laccase enzyme was used as a biocatalyst for the decolouration of the diazo dye Reactive Black 5. Response surface methodology (RSM) was successfully applied to determine the optimal operational conditions for maximum decolouration, which were found to be 150 mg/l RB5, 1.17 mM HBT and 500 U/l laccase. A quadratic model, developed in terms of HBT, RB5 and laccase concentrations, to represent the decolouration percentage and the corresponding coefficients of independent variables was estimated by the application of Design Expert 7.1 trial version. Also, the kinetic parameters of RB5 decolouration by crude laccase were determined. The reaction kinetic fitted well to the Michaelis Menten model and  $K_M$  of 260.11 mg/l and  $V_{max}$  of 37.59 mg/l min were found.

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