Evaluation of Catalytic Efficiency of *Coriopsis caperata* DN Laccase to Decolorize and Detoxify RBBR Dye

Ajit M. Patel¹ · Vanita M. Patel² · Juhi Pandya³ · Ujjval B. Trivedi³ · Kamlesh C. Patel³

Received: 9 March 2017 / Revised: 31 July 2017 / Accepted: 1 August 2017 / Published online: 13 August 2017
© Springer Nature Singapore Pte Ltd. 2017

Abstract Application of enzymes for the removal of environmentally hazardous synthetic dyes from waste water has been considered eco-friendly and economic as compared with non-enzymatic techniques. In the present study, response surface methodology has been applied to decolorize and detoxify a recalcitrant and toxic anthraquinone dye Remazol Brilliant Blue R (RBBR) using *Coriopsis caperata* DN laccase. Optimum concentrations of laccase, pH, and temperature for decolorization of RBBR dye (100 to 500 ppm) were 0.5 U ml⁻¹, 3.5, and 40 °C, respectively. Result of RSM showed that optimum value of tested variables for maximum dye decolorization was 1 U ml⁻¹ enzyme, 1000 ppm dye, and 60 min. Result of kinetic study showed that the K_m , V_{max} , K_{cat} , and K_{cat}/K_m values for RBBR decolorization were 1.06 mM, 0.226 mM U⁻¹ min⁻¹, 135 S⁻¹ and 1.27×10^5 S⁻¹ M⁻¹, respectively. The thermodynamic studies (E_a , 10.87 kJ M⁻¹; ΔH , 7.408 kJ M⁻¹; and ΔS , 80.06 J M⁻¹ K) follow first-order kinetics, and the reaction is endothermic with a negative value of ΔG , suggesting spontaneous nature of reaction. The results of HPTLC, FTIR, and LC-MS analysis confirmed oxidative cleavage of N–H bond of RBBR dye by laccase. Treatment of laccase significantly reduced toxicity (phyto-, cyto-, and micro-) of RBBR dye. The present investigation confirmed *C. caperata* DN laccase as an efficient biocatalyst for bioremediation of textile dyes.

Keywords Bioremediation · Kinetics · Pollution · Response surface methods · Toxicity · Wastewater

Introduction

One of the major and serious problems of the modern world is environmental pollution. Highly colored waste water generated by industries like textile, paper and pulp, dyestuff, distillery, tanneries, printing, cosmetic, plastic, and pharmaceuticals is a major contributor to pollution problem. Many different types of synthetic dyes having complex organic structures including acidic, basic, disperse, azo, diazo, anthraquinone based, and metal complex are used by these industries. Every year, at least 10% of the used dyestuff enters in the environment out of 800,000 tons of world production [1]. Anthraquinone dye Remazol Brilliant Blue R (RBBR) is frequently used for the production of polymeric dyes but has toxicity and recalcitrance due to the anthracene derivative [2] and is possibly carcinogenic to humans (group 2B) (<https://monographs.iarc.fr/ENG/Monographs/vol101/mono101-001>). Presence of dyes in water can cause diseases like hemorrhage, ulceration of skin, nausea, severe irritation of skin, and dermatitis. It also hampers the growth of photoautotrophic organisms and increases the biochemical oxygen demand of the water [3]. Due to complex chemical structure and synthetic origin, dyes are usually resistant to decolorization by exposure to light, water, and various chemicals [4]. The strong persistent color, high biological oxygen demand (BOD), and carcinogenicity are environmentally unacceptable [5] which direct to develop efficient and economical processes for the degradation of synthetic dyes.

Enzymatic removal of synthetic dyes is more acceptable as compared with conventional physicochemical techniques because of its simplicity, low cost, effectivity over a wide range

✉ Kamlesh C. Patel
comless@yahoo.com

¹ J. & J. College of Science, Nadiad, Gujarat 388120, India

² V. P & R.P.T.P Science College,
Vallabh Vidyanagar, Gujarat 388120, India

³ P. G. Department of Biosciences, Sardar Patel University,
Vallabh Vidyanagar, Gujarat 388120, India

of pollutant concentration and physiological condition, minimal impact on ecosystem and less sludge production. The pathway used by different microorganisms/enzymes for the degradation of dyes depends on dye structure. It is necessary to identify the products after biodegradation to determine the mechanistic pathways involved and assess the toxicity of the intermediates or degraded product compared with original dyes [6].

Laccases can decolorize broad range of synthetic dyes by catalyzing mono-electronic oxidation of molecules to corresponding reactive radicals with conversion of oxygen to water [7]. Hence, laccase and laccase-mediated system (LMS)-assisted dye decolorization may provide a reasonable basis for the development of biotechnological processes for removal of color and aromatic compounds from various industrial effluents at large scale [8–10]. For development of sustainable technology, it is necessary to isolate new microorganisms and enzymes, with broader substrate specificity and higher activity to control pollution [6]. For making the overall process cost effective and time saving, statistical approaches like response surface methodology (RSM) can be applied to optimize enzymatic dye decolorization process. Recently, the potential application of RSM in the laccase-mediated decolorization of various dyes has been published [11].

The present research work has been undertaken to evaluate the potential of laccase from *Coriolopsis caperata* DN to decolorize RBBR dye using statistical design (response surface methodology) where RBBR dye concentration, laccase concentration, and time were considered important parameters. Kinetic and energetic study of RBBR decolorization by laccase, analysis of biotransformation products using high-performance thin-layer chromatography (HPTLC), Fourier transforms infrared spectroscopy (FTIR), and liquid chromatography-mass spectroscopy (LC-MS), as well as the assessment of the toxicity of degraded RBBR dye products have also been carried out.

Materials and Methods

Chemicals

RBBR and 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS) were from Sigma-Aldrich (St. Louis, MO, USA). The chemical formula of RBBR dye is $C_{22}H_{16}N_2Na_2O_{11}S_3$, and its molecular weight is 626.54 g M^{-1} .

Production and Partial Purification of Laccase

White rot fungus isolated from natural source was identified as *C. caperata* DN (NCBI, accession no. KF564288). Laccase enzyme was produced by *C. caperata* DN in statistically

optimized medium under submerged condition. The fermented broth obtained after separation of mycelial mass from the medium was subjected to fractional precipitation by ammonium sulfate. The enzyme protein precipitated between 40 and 60 g% (w/v) was collected and then suspended in 100 mM sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight at 4 °C with three changes of equal intervals. The dialyzed enzyme solution was used for the decolorization of RBBR dye.

Laccase Enzyme Assay

Extracellular laccase activity (E.C.1.10.3.2) was measured at 30 °C by the oxidation of ABTS. The reaction mixture contained 500 μl of 1.0 mM ABTS, 2400 μl of 20 mM Na-acetate buffer (pH 3.5), and 100 μl of appropriately diluted enzyme sample. Increase in absorbance for 5 min was measured spectrophotometrically using SPECORD 200 PLUS Spectrophotometer against a suitable blank at 436 nm ($\epsilon = 29,300 \text{ cm}^{-1} \text{ M}^{-1}$) [12]. One enzyme unit (U) was defined as 1.0 μM of product (ABTS^+) formed per minute under the assay conditions.

Optimization of RBBR Dye Decolorization

Effect of Physicochemical Parameter on Dye Decolorization

Stock solution of RBBR dye (1000 ppm) was prepared in 100 ml deionized water. The decrease in the spectral absorbance during decolorization of RBBR dye (592 nm) by laccase was recorded using UV-visible spectrophotometer. Effect of pH was studied at different pH ranges (pH 2.5–7.0). Influence of temperature towards decolorization of dyes was studied at optimum pH at different temperature ranges (10–50 °C). Effect of temperature on the rate of dye decolorization was expressed in terms of temperature quotient (Q_{10}) value which is a measure of the temperature sensitivity of an enzymatic reaction rate by 10 °C [13].

$$Q_{10} = \text{antilog } e \left(E_{ax} 10 / RT^2 \right) \quad (1)$$

A control test containing the same amount of a heat-denatured laccase was performed in parallel. The percentage of dye decolorization was calculated according to Xin and Geng [14] by using the formula:

$$D = 100 \times \left(A_{ini} - A_{obs} / A_{ini} \right) \quad (2)$$

where D is the percent decolorization, A_{ini} is the initial absorbance, and A_{obs} is the observed absorbance. Decolorization percentage of RBBR dye was converted to milligrams per milliliter and finally in millimolar concentration.

Statistical Optimization of RBBR Dye Decolorization

Central composite design of RSM was performed to optimize the concentration of RBBR dye, laccase enzyme, and time in minutes. The range of coded and uncoded variables is given in Table 1. A five-level three-factor CCD requiring 20 experiments was used in the study (Table 2). The CCD consist of a 2^K factorial runs with 2k axial runs and X_0 number of center points (six replicates). The number of experimental run was calculated as:

$$N = 2^k + 2K + X_0 \quad (3)$$

where N is total experimental run (20), K is number of variables (3), and X_0 is the number of central points (6). The behavior of the system was explained by the following quadratic equation.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ii} X_i^2 \quad (4)$$

where Y is predicted response, β_0 is constant, β_i is linear term coefficient, β_{ii} is quadratic term coefficient, β_{ij} is interaction coefficient, and X_i is dimensionless-coded value of X_i [15]. Quadratic multiple regression equation and the response surface contour plots (Design Expert software (version 9) from Stat-Ease, Inc., Minneapolis, MN, USA) were used to analyze and interpret the data of RBBR degradation. After optimization, an experiment was run using the optimum values for variables to validate the predicted and experimental value of RBBR degradation.

Kinetics and Thermodynamics of RBBR Decolorization

Kinetics of Decolorization

To optimize the concentration of laccase for decolorization of RBBR dye, 0.2 to 2.0 U ml⁻¹ of enzyme and 100 ppm RBBR dye was used under optimized condition. Optimized enzyme concentration was used for decolorization of the dye (0.079–0.79 mM) at different time intervals, and the velocity was determined by using the slope at each

Table 1 Experimental range and levels of the independent variables of selected components used for response surface central composite design

	$-\alpha$	-1	0	$+1$	$+\alpha$
Laccase (U ml ⁻¹)	-0.41	0.2	1.1	2.0	2.61
RBBR dye (mg l ⁻¹)	-273.85	50.0	525.0	1000	1323.85
Time (min)	-13.74	5.0	32.5	60.0	78.74

Table 2 Central composite design with actual and coded level of variables and their response

Std	Run	Factor 1 A–A laccase (U ml ⁻¹)	Factor 2 B–B RBBR dye (mg l ⁻¹)	Factor 3 C–C time (min)	Response (mg l ⁻¹)	Predicted value (mg l ⁻¹)
11	1	1.1	00.0	32.5	0.000	3.57
8	2	2.0	1000	60.0	674.58	679.99
1	3	0.2	50.0	5.0	3.701	-0.82
2	4	2.0	50.	5.0	32.46	30.87
13	5	1.1	525.0	00.0	0.000	3.51
18	6	1.1	525.0	32.5	261.42	261.50
12	7	1.1	1323.85	32.5	557.25	552.44
16	8	1.1	525.0	32.5	259.28	261.50
7	9	0.2	1000.0	60.0	335.76	338.24
15	10	1.1	525.0	32.5	267.85	261.50
20	11	1.1	525.0	32.5	258.21	261.50
19	12	1.1	525.0	32.5	262.39	261.50
9	13	00.0	525.0	32.5	0.000	2.75
5	14	0.2	50.0	60.0	31.562	30.60
4	15	2.0	1000.0	5.0	374.11	375.96
10	16	2.61	525.0	32.5	320.76	316.77
3	17	0.2	1000.0	5.0	19.52	18.62
6	18	2.0	50.0	60.0	44.90	46.69
14	19	1.1	525.0	78.74	290.34	285.59
17	20	1.1	525.0	32.5	259.60	261.50

dye concentration (Fig. 1c). The Michaelis-Menten curve was then drawn by plotting the obtained initial velocity ($1/V_0$) against dye concentrations ($1/S$). The kinetic constants ($V_{\max}$, K_m , K_{cat} , and K_{cat}/K_m) were determined by Michaelis-Menten kinetics.

Thermodynamics of RBBR Degradation

The effect of temperature on decolorization of RBBR was evaluated by performing the dye decolorization (0.1–0.5 mM) experiment at 10–50 °C temperature, and the obtained velocities (for each temperature) were plotted against initial dye concentrations. The apparent first-order rate constant (K) for each temperature was calculated based on the slope of each trend line. The Arrhenius curve achieved by drawing the $\ln(K)$ versus $1/T$ ($\times 10^3 \text{ K}^{-1}$) (T is the absolute temperature (K)) was used to calculate the value of activation energy (E_a). The slope of Arrhenius plot indicates $-E_a/R$ where E_a is activation energy and R is the gas constant (8.3145 J/mol K). K_{eq} is the apparent equilibrium constant and was calculated from the difference of initial dye concentration and residual dye concentration at equilibrium state [16]. The Van't

Fig. 1 Kinetics study of RBBR dye decolorization. **a** Optimization of concentration of laccase. **b** The time course study of RBBR (0.0798 to 0.798 mM) decolorization by laccase. **c** Straight line graph was obtained from (b) to find out V_0 (slope of trend line) for each dye concentration. **d** The Lineweaver-Burk plot was drawn using V_0 values obtained from (c) V_s $1/S$

Hoff plot was drawn by plotting the $\ln(K_{eq})$ against $1/T$ ($\times 10^3$ K⁻¹) and used to estimate the enthalpy (ΔH) and entropy (ΔS) while the Gibbs free energy (ΔG) was calculated using the equation:

$$\Delta G = \Delta H - T\Delta S. \quad (5)$$

Analysis of Metabolites of RBBR Degradation

Spectral Scanning

After decolorization of RBBR dye (100–500 ppm) by laccase, the spectral scan (190–1100 nm) of each solution was carried out using UV spectrophotometer and changes in its absorption spectrum were recorded.

High-Performance Thin-Layer Chromatography

HPTLC chromatogram was obtained by spotting metabolites obtained after decolorization on the silica gel-coated glass plate. The composition of mobile phase was water:butanol:glacial acetic acid:pyridine (2:4:1:1, v/v). The control dye and postdegradation metabolites were analyzed by scanning at 200–800 nm using TLC scanner

(CAMAG), and the results were generated by using HPTLC software WinCATS 1.4.4.6337.

Fourier Transforms Infrared Spectroscopy

FTIR analysis was done in the mid-IR region of 400–4000 cm⁻¹ with 16 scan speed. The pellets prepared using spectroscopic pure KBr (5:95) were fixed in a sample holder to carry out analysis.

Liquid Chromatography-Mass Spectroscopy

Identification of postdegradation metabolites of RBBR was carried out using a LC 8030 gas chromatography coupled with mass spectroscopy (Shimadzu, Japan). The ionization voltage was 35 eV, and nitrogen was used as a carrier gas with a flow rate 0.5 ml min⁻¹ and 35 min run time. Gas chromatography was conducted in temperature programming mode with a C-18 column (4.6 $\times$ 150 m; particle size is 5 μ m). The mobile phase was composed of acetonitrile and distilled water (65:35) while the temperature of column oven was 280 °C and of injection was 200 °C. Temperature was hold at 40 °C for 1 min then rose to 280 °C at 10 °C rise min⁻¹ [2].

Toxicity Test

Phytotoxicity Assay

Phytotoxicity test was carried out in order to assess the toxicity of RBBR before and after laccase treatment using *Phaseolus mungo* (dicot), pulses normally cultivated in India. Phytotoxicity was conducted at room temperature using different concentrations of control dye (100, 50, 25, and 12.5 ppm) and laccase-treated dye. Twenty seeds of *P. mungo* were soaked in 10 ml solution of control RBBR and its degradation products for 24 h while distilled water was used as control. After 24-h incubation, the seeds were placed in a glass petriplates, containing a filter paper soaked in 2 ml of each dye concentration. The plates were incubated in the dark for 3 days. Germination percentage of seeds as well as the length of radical was recorded after incubation, and the germination index (GI) was calculated by Eq. (5) while the inhibitory concentration that causes 50% inhibition of growth of radicals (IC_{50}) was also calculated.

$$GI = (GP \times I_a) / I_c \quad (6)$$

where GP is germinated seeds expressed as percentage of control value and I_a and I_c were average root length (mm) in dye solution and in the control, respectively.

Acute Toxicity Assay

For acute toxicity test, the freshwater copepod *Cyclops* was used according to the methodology developed by Marus et al. [17]. The adult *Cyclops* was transferred to each set of tubes (ten per tube) with different concentrations of control dye (100, 50, 25, and 12.5 ppm) and enzyme-treated dye. Negative control (aerated distilled water) was kept in parallel. The number of surviving *Cyclops* was counted after 24 h of incubation, and the lethal concentration (LC_{50}) was calculated.

Microtoxicity Assay

To determine the microtoxicity of RBBR dye before and after laccase treatment, two Gram-negative (*Escherichia coli*, *Enterobacter aerogenes*) and three Gram-positive (*Micrococcus luteus*, *Staphylococcus aureus*, and *Bacillus subtilis*) bacterial cultures were used. A culture of each bacterial strain was prepared in Muller-Hinton broth, and 0.2 optical densities were set at OD_{600} . Filter-sterilized RBBR dye solution (laccase-treated and untreated) with 100 ppm concentration was separately added to each culture medium and incubated at 37 °C for 10 h. Change in OD_{600} was recorded after every 2 h for each bacterial strain, and the results were analyzed as percentage of growth inhibition (GI%) which was calculated as

$$GI\% = \left[\left(1 - OD_{600S} / OD_{600C} \right) \times 100 \right] \quad (7)$$

where OD_{600S} is the OD_{600} of sample and OD_{600C} is the OD_{600} of control.

Result and Discussion

Ligninolytic white rot fungi effectively degrade recalcitrant synthetic dyes due to the production of extracellular ligninolytic enzymes, mainly laccases [11, 18–20]. In the present study, we used laccase enzyme produced by indigenous fungal isolate *C. caperata* DN, under submerged condition, to decolorize and detoxify anthraquinone dye RBBR.

Physiological Optimization of RBBR Decolorization

The effect of pH and temperature were studied at pH values between 2.5 and 7.0 and temperature between 10 and 50 °C using 100 ppm dye and 0.5 U ml⁻¹ laccase. Optimum pH for decolorization of RBBR was 3.5, and the range of pH was 3.0 to 6.0 while the optimal temperature for decolorization was 40 °C. Fungal laccases decolorize all applied dyes maximally at acidic pH 5.0 [21]. The enzyme activity is decreased at higher pH due to the binding of OH⁻ ion to T2/T3 coppers of laccase which interrupt the internal electron transfer from T1 to T2/T3 centers of laccase [22]. The rate of reaction of dye decolorization was enhanced by increasing temperature, and the optimum temperature for dye decolorization was 40–60 °C [23] which was in agreement with the obtained result. Temperature quotient (Q_{10}) value which gives an increase of rate of reaction due to rise in temperature by 10 °C was 1.49. The effect of laccase concentration on the rate of RBBR decolorization was studied by using laccase concentration from 0.2 to 2.0 U ml⁻¹ with dye concentration at 100 mg l⁻¹. The rate of RBBR decolorization linearly increased up to 0.5 U ml⁻¹ of enzyme (Fig. 1a); hence, 0.5 U ml⁻¹ concentration of laccase was selected for further study. The result of RBBR (0.079 to 0.79 mM) decolorization after treatment with the laccase at different time intervals is shown in Fig. 1b, which shows that the RBBR decolorization rate is linear with increasing concentration of dye up to 6.0 min and all the concentration of RBBR was decolorized up to 75.0% within 45.0 min under optimized condition. The spectral scan (190–1100 nm) result of RBBR dye (100 ppm) before and after laccase treatment to various concentrations of RBBR dye (100–500 ppm) indicates (Fig. 2 (b)) that the peak of parent dye RBBR disappears and a new peak is obtained, suggesting oxidation of dye.

Optimization of RBBR Decolorization Using RSM

Substrate concentration affects the velocity of reaction, while enzyme concentration affects the cost of bioprocess. The responses generated by performing a total of 20 runs which include concentration of each component at two levels were used to obtain quadratic model presented in Table 2. The value of lack of fit, model F value, and model p value were found to be 2.66, 3589.38, and <0.0001 , respectively, indicating that the model was significant. In addition, Table 3 indicates laccase activity (A), dye concentration (B), time (C), and their interactions; AB , BC , A^2 , and C^2 as significant model terms (p value <0.0001). The coefficient of determination R^2 is also applied to analyze the fitness of model, and in the present

study, it was found to be 0.998 indicating that only less than 0.20% of the total variance could not be explained by the model. The “pred r -squared” of 0.99 is in reasonable agreement with the “Adj r -squared” of 0.99. “Adeq Precision” measures the signal-to-noise ratio, and it is 204.61 which is greater than 4.0 and indicates adequate signals. The higher significance of squared terms (A^2 , C^2) shown in Table 3 indicates that optimum values of variables used for dye decolorization are within the range of selected values [15]. The low coefficient variation (2.21%) confirms the precision and reliability of the model experiment performed. The quadratic model explains the statistical relationship between the selected variables, and the response in terms of coded factors was best fitted with the following equation.

$$\begin{aligned} \text{Dye decolorization (R1)} = & (-37.96) - (106.56 \times A) + (-0.07 \times B) + (3.88 \times C) - \\ & (0.19 \times A \times B) - (-0.15 \times A \times C) + (5.51 \times B \times C) + \\ & (-44.40 \times A^2) + (2.58 \times B^2) + (-0.05 \times C^2) \end{aligned} \quad (8)$$

Fig. 2 Analysis of RBBR degradation products by TLC (a). Spectral scan result of RBBR dye before and after laccase treatment (b). HPTLC analysis of RBBR dye (c1) and its product (c2). FTIR analysis of control dye (d1) and degraded product (d2)

Table 3 Analysis of variance of quadratic model for decolorization of RBBR dye

Source	Sum of squares	df	Mean squares	F value	p value	
Model	7.15E+005	9	79,471.08	3589.38	< 0.0001	Significant
A–A laccase	1.19E+005	1	1.190E+005	5376.06	< 0.0001	Significant
B–B RBBR dye	3.63E+005	1	3.637E+005	16,424.89	< 0.0001	Significant
C–C time	96,048.53	1	96,048.53	4338.11	< 0.0001	Significant
AB	53,024.77	1	53,024.77	2394.91	< 0.0001	
AC	121.60	1	121.60	5.49	0.0411	
BC	41,531.13	1	41,531.13	1875.79	< 0.0001	
A ²	18,645.84	1	18,645.84	842.15	< 0.0001	
B ²	490.70	1	490.70	22.16	0.0008	
C ²	24,637.28	1	24,637.28	1112.76	< 0.0001	
Residual	221.41	10	22.14			
Lack of fit	160.92	5	32.18	2.66	0.1533	Not significant

Coefficient of variance, 2.21; predicted *R*-squared, 0.99; adjusted *R*-squared, 0.99; and adequate precision, 204.61
df degree of freedom, *p* probability

where R1 is the response (yield of decolorization) and A, B, and C are laccase activity, dye concentration, and time, respectively.

In order to investigate the relation between independent variables and their responses, contour plots were generated by RSM using the Design Expert software. The response surface plots (Fig. 3) presents the decolorization of RBBR dye as function of two variables while the third one is kept at a constant level. Surface plot demonstrates an increase in dye decolorization with rise in the enzyme concentration. Figure 3a,

c clearly shows that even small alterations of the enzyme concentration influence the decolorization rate. On the basis of the result of R1 and three-dimensional response surface graphs (Fig. 3), the optimal levels for enzyme activity, dye concentration, and time were found to be 1 U ml^{−1}, 1000 mg l^{−1}, and 60 min, respectively. Validation was carried out by solving Eq. (7) and analyzing response surface graphs. As shown in Table 4, the maximum decolorization efficiency is 542.77 ± 9.21 mg l^{−1} implying a strong similarity between experimental and predicted values. Osma et al. [24] reported

Fig. 3 Contour plots showing the interaction effect between **a** laccase and RBBR dye concentration, **b** laccase and time in minutes, and **c** RBBR dye concentration and time in minutes

Table 4 Validation of RSM results using statistically optimal condition

No.	Laccase (U ml ⁻¹)	RBBR dye (mg l ⁻¹)	Time (min)	Experimental result ^a (mg l ⁻¹)	Predicted result (mg l ⁻¹)
1	1	1000.00	60.0	542.77 ± 9.21	525.65
2	1	270.79	18.0	115.86 ± 5.52	122.842

^a Mean ± standard deviation

that 5 U ml⁻¹ immobilized laccase of *Trametes pubescens* decolorize 44% of RBBR (133.0 mg l⁻¹) within 42 h. Kunamneni et al. [25] observed that 20 U ml⁻¹ of immobilized *Myceliophthora thermophila* laccase in the presence of 1 mM HBT decolorize 31% of RBBR (200.0 mg l⁻¹) within 24 h. Murugesan et al. [26] used 25 U ml⁻¹ of *Ganoderma lucidum* laccase in the presence of HBT to obtain 92% of RBBR (50 mg l⁻¹) decolorization within 2 h. Laccase from *Trametes trogii* at 0.2 U ml⁻¹ decolorized 72% of RBBR (200 mg l⁻¹) within 1 h [27]. Palmieri et al. [1] reported that *Pleurotus ostreatus* laccase at 1 U ml⁻¹ decolorize 86% of RBBR (30 mg l⁻¹) within 1.7 h. Soares et al. [28] reported that commercial laccase at 10 U ml⁻¹ decolorize RBBR (20 mg l⁻¹) up to 72% within 1 h. Sathishkumar et al. [11] applied RSM to optimized RBBR (52.90 mg l⁻¹) decolorization using *Pleurotus florida* laccase and achieved 82% of decolorization within 7.17 min by 1.87 U ml⁻¹ of laccase in the presence of 0.85 mM HBT. After statistical optimization, the rate of RBBR decolorization by *C. caperata* DN laccase at 1 U ml⁻¹ is 542 mg l⁻¹ (1000 mg l⁻¹) within 1 h, which is better than those reported by other authors in previous studies (Table 5).

Kinetic and Energetic Study of RBBR Dye Decolorization

As shown in Fig. 1c, the linear relationship between the initial velocity and dye concentration proved that RBBR decolorization by enzyme is a first-order reaction. The K_m and V_{max} values of laccase determined through Lineweaver–Burk plot were 1.06 mM and 0.226 mM U⁻¹ min⁻¹, respectively (Fig. 1d). The number of RBBR molecules turned over per laccase molecule (K_{cat} value) was 135.0 S⁻¹. The second-order constant (K_{cat}/K_m) was 1.27×10^5 S⁻¹ M⁻¹, indicating high catalytic power of enzyme. Sathishkumar et al. [11] reported that the K_m and V_{max} values of *P. florida* laccase for RBBR dye decolorization was 145.82 mg l⁻¹ and 24.86 mg l⁻¹ min⁻¹, respectively, in the presence of laccase mediator HBT.

Arrhenius equation was used to study the effect of temperature on enzyme kinetics (Fig. 4b), and the calculated activation energy (E_a) for RBBR dye degradation was 10.87 kJ M⁻¹, which was similar to the activation energy (14.25 kJ M⁻¹) required for decolorization of acid blue 80 by laccase of *Lentinus polychrous* Lev. [32]. The E_a is the energy difference between the reactants and the activated complex, also known as transition state. The lower

Table 5 Application of different enzymes for decolorization of RBBR dye

Enzyme	Source	RBBR dye (ppm)	Laccase (U ml ⁻¹)	Mediator (mM)	Time	Decolorization (mg)	Reference
Laccase	<i>Pleurotus ostreatus</i>	31.30	1.0	—	1.7 h	26.91	[1]
Laccase	<i>Pleurotus florida</i>	52.90	1.87	0.85	7.17 min	43.46	[11]
Laccase	<i>Trametes pubescens</i>	133.0	0.5 ^a	—	42.0 h	58.52	[24]
Laccase	<i>Myceliophthora thermophila</i>	200.0	20.0 ^a	1.0	24.0 h	62.0	[25]
Laccase	<i>Ganoderma lucidum</i>	50.0	25.0	1.0	2.0 h	46.0	[26]
Laccase	<i>Trametes trogii</i>	100.0	0.2	—	1.0 h	97.0	[27]
Laccase	Commercial laccase	20.0	10.0	—	1.0 h	14.4	[28]
Soyabean peroxidase	Soyabean seed hull	40.0	70.4	—	13.0 min	34.40	[29]
Horseradish peroxidase	<i>Armoracia rusticana</i>	60.0	3.5	—	5.0 min	57.60	[30]
Bilirubin Oxidase	<i>Myrothecium verrucaria</i>	100.0	10.0	—	4.0 h	40.0	[31]
Laccase	<i>Coriolopsis caperata</i> DN	1000.0 200.0	1.0 1.0	— —	1.0 h 6.0 min	542.0 103.78	Present work

^a Immobilized laccase enzyme used for decolorization of RBBR dye

Fig. 4 Energetic studies of RBBR dye decolorization. **a** The velocities (V_0) at temperature range 10–40 °C was plotted against initial dye concentration, **b** Arrhenius plot, **c** Van't Hoff plot, and **d** the Gibb's free energy (ΔG) changes plot

activation energy (10.87 kJ M^{-1}) and Q_{10} value (1.49) suggested that the reaction rate is faster and is less sensitive to a change in temperature. The calculated values for ΔH and ΔS were 7.408 kJ M^{-1} and $80.06 \text{ J M}^{-1} \text{ K}$, respectively. The lower enthalpy (ΔH) value showed that the formation of transition state or activated complex between enzyme and substrate was more efficient. The positive sign of ΔH and ΔS or the negative slope of Van't Hoff plot (Fig. 4c) implies that the reaction is endothermic and spontaneous at higher temperature. The feasibility and extent of a chemical reaction is best determined by measuring change in ΔG for substrate catalysis. The negative value of ΔG suggested that the reaction is spontaneous (Fig. 4d).

Analysis of Metabolites

It is necessary to identify the metabolites produced from RBBR dye after laccase treatment in order to understand the mechanism of oxidative degradation of its chemically stable complex structure. Figure 2 shows the analysis result of RBBR dye degradation and metabolites formed after its treatment with laccase. The result of HPTLC analysis showed that the R_f value of RBBR dye is 0.58 at 592 nm, while after laccase treatment, two new bands were obtained with R_f values 0.34 at 401 nm and 0.40 at 511 nm (Fig. 2 (a)). Similar results (Fig. 2 (c1, c2)) were observed in which a single major peak was obtained with control RBBR dye and

two new peaks were obtained after laccase treatment. The results of FTIR analysis showed that 1634 peaks present in RBBR dye were absent in laccase-treated RBBR dye (Fig. 2 (d1, d2)), indicating that the primary amine bond (N–H, 1650–1590) disappeared and two new products were produced. LC-MS analysis was carried out to investigate the degradation products of RBBR dye after laccase treatment. Figure 5 represents the chromatogram (a1) and mass spectral (b1) data of RBBR dye which showed a major peak with a retention time of 22.16 min and m/z value 627.60 Da. The chromatogram data of laccase-treated RBBR dye showed new peaks with retention time of 19.48 min and 21.06 (Fig. 5 (a2)). Mass spectral (Fig. 5 (b2)) data showed presence of intermediates having m/z values 337.20 and 343.30 Da. The present study proposed that laccase oxidizes RBBR dye and produced two subproducts (sodium 1-amino-9,10-dioxo-9,10-dihydroanthracene-2-sulphonate and sodium 2-((3-aminophenyl) sulfonyl) ethyl sulfate) (Fig. 6), which is similar to the result reported by Osma et al. [24] and Hadibarata et al. [2].

Toxicity Test

Phytotoxicity Test with *P. mungo*

Effluent from dyeing industries may cause environmental and health hazard. Water after effluent treatment can be used for

Fig. 5 LC-MS analysis of RBBR dyes (*a1*, *b1*) before and after laccase treatment (*a2*, *b2*)

agriculture, so it is necessary to assess the toxicity of dye and its degradation products. Zucconi et al. [33] used germination index (GI) to evaluate the phytotoxicity which combines relative seed germination and relative root elongation in presence of phytotoxic compound. *P. mungo* (dicot), normally cultivated in India was used to assess the toxicity of RBBR and metabolites formed after its decolorization. As shown in

Table 6, root elongation, percentage of seed germination, and the GI of seeds grown at 200 ppm concentration of RBBR dye were found to be 12.5 ± 8.4 mm, 75%, and 40.5%, respectively. Root elongation, percentage of seed germination, and the GI of seeds grown in the presence of laccase-treated RBBR dye were found to be 24.65 ± 4.7 , 100%, and 79.9%, respectively. The IC_{50} value of RBBR

Fig. 6 Product produced after laccase treatment to RBBR dye

Table 6 Toxicity study of RBBR dye before and after laccase treatment using *Phaseolus munga*

Phytotoxicity test	RBBR concentration (mg l ⁻¹)	Seed germination number	Root elongation ^a (mm)	Seed germination (%)	Germination index (%)
Control	00.0	20.0	30.85 ± 6.6	100.0	100
RBBR dye	200.0	15.0	12.5 ± 8.4	100.0	40.51
	100.0	17.0	18.75 ± 8.8	75.0	60.77
	50.0	19.0	21.55 ± 6.9	85.0	69.85
	25.0	20.0	30.75 ± 4.9	95.0	99.67
Laccase-treated RBBR dye	200.0	20.0	24.65 ± 4.7	100.0	79.90
	100.0	20.0	29.15 ± 4.1	100.0	94.48
	50.0	20.0	31.00 ± 3.3	100.0	100.48
	25.0	20.0	31.05 ± 4.8	100.0	100.64
Laccase control	00.0	20.0	31.40 ± 4.6	100.0	101.78

The *t* score and *p* value for phytotoxicity (GI%) were 5.87 and 0.0042, respectively. The result is significant at *p* ≤ 0.01

^a Mean ± standard deviation

dye at 200 ppm concentration is 160 ppm (Fig. 7a). According to Zucconi et al. [33], less than 50% GI value indicates high phytotoxicity, values between 50 and 80% mean moderate phytotoxicity, and more than 80% indicates no phytotoxicity. Thus, the toxicity of metabolites produced after the treatment of laccase from *C. caperata* DN to RBBR dye is much lower than that of RBBR dye, and similar result was reported by Osma et al. [24] and Sathishkumar et al. [11].

Acute Toxicity Test with Cyclops

The acute toxicity test was carried out using common water copepod Cyclops. After 24 h of incubation, the living Cyclops was counted and the calculated 50% lethal concentration (LC₅₀) was 40 mg l⁻¹ (Fig. 7b). Verma [34] used *Daphnia magna* as an aquatic experimental animal and reported that the 48-h EC (50) value for the azo dyes, Remazol Parrot Green, and Remazol Golden Yellow was 46.84 and

55.32 mg l⁻¹, respectively. As shown in Table 7, the mortality percentage of Cyclops was related to the concentrations of RBBR dye and enzyme-treated dye as well as duration of exposure. The result showed that the acute toxicity of RBBR dye was significantly reduced after laccase treatment.

Microtoxicity Test

Many protocols have been used to determine the toxicity of chemical compounds based on the inhibition of growth of bacterial or animal cells. As shown in Table 8, the GI% of RBBR dye at 100 ppm for *E. coli*, *E. aerogenes*, *M. luteus*, *S. aureus*, and *B. subtilis* was found to be 35.06 ± 3.06%, 36.84 ± 2.17%, 70 ± 3.57%, 85 ± 2.71%, and 53.24 ± 4.34%, respectively, while the GI% of laccase-treated RBBR dye was 2.59 ± 1.05%, 5.26 ± 1.87%, 4.21 ± 0.97%, 10 ± 2.32%, and 2.59 ± 2.51%, respectively. The result of the present study revealed that the toxicity of

Fig. 7 **a** Percentage of root growth inhibition of *Phaseolus mungo* and **b** percent mortality rate of Cyclops according to the concentration of RBBR dye before and after laccase treatment

Table 7 Toxicity study of RBBR dye before and after laccase treatment using Cyclops

Cytotoxicity test	RBBR concentration (mg l ⁻¹)	Number of Cyclops	Mortality rate ^a		Mortality rate (%)	
			24 h	48 h	24 h	48 h
Control	00.0	10.0	00.0	00.0	00.0	00.0
RBBR dye	100.0	10.0	7.0 ± 1.5	10.0 ± 0.0	70.0	100.0
	50.0	10.0	6.0 ± 0.5	10.0 ± 0.0	60.0	100.0
	25.0	10.0	5.0 ± 1.0	10.0 ± 0.5	50.0	100.0
	12.5	10.0	3.0 ± 1.0	9.0 ± 1.0	30.0	90.0
Laccase-treated RBBR dye	100.0	10.0	00.0	2.0 ± 0.5	00.0	20.0
	50.0	10.0	00.0	00.0	00.0	00.0
	25.0	10.0	00.0	00.0	00.0	00.0
	12.5	10.0	00.0	00.0	00.0	00.0
Laccase control	00.0	10.0	00.0	00.0	00.0	00.0

The *t* score and *p* value for cytotoxicity (24 h) were 9.39 and 3.2E–05, respectively. The result is significant at *p* ≤ 0.01

^a Mean ± standard deviation

RBBR dye significantly reduces after laccase treatment, and the metabolites produced are less toxic. Palmieri et al. [1] used laccase of *Pleurotus ostreatus* to decolorize RBBR dye and reported 95% viability of *Bacillus cereus* after laccase treatment of RBBR dye, which is similar to our results. Increased toxicity of enzyme-treated RBBR dye was reported by Champagne and Ramsay [35] who used laccase and Maria et al. [29] using soyabean peroxidase.

Conclusions

This is the first report which statistically evaluates the potential use of laccase enzyme from *C. caperata* DN to decolorize anthraquinone dye RBBR which represents an important class of toxic and recalcitrant organopollutants. The kinetic study

follows Michaelis-Menten model and thermodynamic parameters of the laccase enzyme suggests that this enzyme has great potential to be used commercially for large-scale treatment of industrial effluents. The analysis of laccase-treated RBBR dye solution showed that laccase oxidizes RBBR dye and degrades it into two end products. The toxicity tests showed that after laccase treatment, the toxicity of RBBR dye was significantly reduced. Laccase enzyme (1 U ml⁻¹) from *C. caperata* DN efficiently decolorize and detoxify 51.89% of 200 ppm RBBR dye within 6.0 min and 54.2% of 1000 ppm RBBR dye within 60.0 min without mediator, suggesting higher catalytic efficiency of laccase as compared with the ones reported. Thus, present study evaluates laccase produced by *C. caperata* DN as green catalyst to replace conventional oxidation processes generally applied for bioremediation of environmental pollutants.

Table 8 Toxicity study of RBBR dye before and after laccase treatment using microorganisms

Microtoxicity test	Percentage inhibition of bacterial strain ^a				
	Test organisms				
	<i>Escherichia coli</i>	<i>Enterobacter aerogenes</i>	<i>Micrococcus luteus</i>	<i>Staphylococcus aureus</i>	<i>Bacillus subtilis</i>
Control	00.0	00.0	00.0	00.0	00.0
RBBR dye (100 mg ⁻¹)	35.06 ± 3.06	36.84 ± 2.17	70.00 ± 3.57	85.0 ± 2.71	53.24 ± 4.34
Laccase-treated RBBR dye	2.59 ± 1.05	5.26 ± 1.87	4.21 ± 0.97	10.0 ± 2.32	2.59 ± 2.51

The *t* score and *p* value for microtoxicity were 5.87 and 0.0042, respectively. The result is significant at *p* ≤ 0.01

^a Mean ± standard deviation

Acknowledgments Ajit M. Patel received financial support from University Grant Commission, Government of India, New Delhi, as minor research project. (UGC approval Letter No. and Date: File No. 47-508/12 (WRO) 7th March 2013).

Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interest.

References

- Palmieri G, Cennamo G, Sannia G (2005) Remazol Brilliant Blue R decolourisation by the fungus *Pleurotus ostreatus* and its oxidative enzymatic system. *Enzym Microb Technol* 3:17–24
- Hadibarata T, Yusoff ARM, Kristanti RA (2011) Decolorization and metabolism of anthraquinone-type dye by laccase of white-rot fungi *Polyporus* sp. S113. *Water Air Soil Pollut* 223:933–941
- Ghaly, A.E., Ananthashankar, R., Alhattab, M., & Ramakrishnan, V.V. (2014). Production, characterization and treatment of textile effluents: a critical review. *J Chem Eng Process Technol*, 5. Doi: [10.4172/2157-7048.1000182](https://doi.org/10.4172/2157-7048.1000182)
- Buddolla V, Bandi R, Avilala J, Arthala PK, Golla N (2014) Fungal laccases and their applications in bioremediation. *Enzyme Res* 2014:1–22
- Wang XJ, Gu XY, Lin DX (2007) Treatment of acid rose dye containing wastewater by ozonizing-biological aerated filter. *Dyes Pigments* 74:736–740
- Luciana P, Madalena A (2012) Dyes—environmental impact and remediation. In: Malik A, Grohmann E (eds) *Environmental protection strategies for sustainable development*. Springer Science+ Business Media, pp 111–162
- Arora DS, Sharma RK (2010) Ligninolytic fungal laccases and their biotechnological applications. *Appl Biochem Biotechnol* 160: 1760–1788
- Mohammadian FM, Mesdaghinia AR, Naddafi K, Nasser S, Yunesian M, Assadi MM et al (2010) Optimization of reactive blue 19 decolorization by *Ganoderma* sp. using response surface methodology. *Iran J Environ Health Sci Eng* 7:35–42
- Sharma YC, Singh BU (2009) Fast removal of malachite green by adsorption on rice husk activated carbon. *Open Environ Pollution Toxicol J* 1:74–78
- Upadhyay P, Shrivastava R, Agrwal PK (2016) Bioprospecting and biotechnological applications of fungal laccase. *3 Biotech* 6:15. doi: [10.1007/s13205-015-0316-3](https://doi.org/10.1007/s13205-015-0316-3)
- Sathishkumar P, Balan K, Palvannan T, Kamala-Kannan S, Oh BT, Rodríguez-Couto S (2013) Efficiency of *Pleurotus florida* laccase on decolorization and detoxification of the reactive dye Remazol Brilliant Blue R (RBBR) under optimized conditions. *Clean-Soil Air Water* 41:665–672
- Niku-Paavola ML, Karhunen E, Salola P, Raunio V (1988) Ligninolytic enzymes of the white-rot fungus *Phlebia radiata*. *Biochem J* 254:877–884
- Prajapati VS, Trivedi UB, Patel KC (2014) Kinetic and thermodynamic characterization of glucoamylase from *Colletotrichum* sp. KCP1. *Indian J Microbiol* 54:87–93
- Xin F, Geng A (2011) Utilization of horticultural waste for laccase production by *Trametes versicolor* under solid-state fermentation. *Appl Biochem Biotechnol* 163:235–246
- Maniknandan M, Pasic L, Kannan V (2009) Optimization of growth medium for obtaining high-cell density cultures of halophilic archaea (family Halobacteriaceae) by response surface methodology. *Bioresour Technol* 100:3107–3112
- Annuar MSM, Adnan S, Vikineswary S, Chisti Y (2009) Kinetics and energetics of azo dye decolorization by *Pycnoporus sanguineus*. *Water Air Soil Pollut* 202:179–188
- Marus EM, Elphick JR, Bailey HC (2015) A new toxicity test using the freshwater copepod *Cyclops vernalis*. *Bull Environ Contam Toxicol* 95:357–362
- Erkurt EA, Unyayar A, Kumbur H (2007) Decolorization of synthetic dyes by white rot fungi, involving laccase enzyme in the process. *Process Biochem* 42:1429–1435
- Faraco V, Pezzella C, Miele A, Giardina P, Sannia G (2009) Bioremediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes. *Biodegradation* 20:209–220
- VinothKumar V, Sathyaselvabala V, Premkumar MP, Vidyadevi T, Sivanesan S (2012) Biochemical characterization of three phase partitioned laccase and its application in decolorization and degradation of synthetic dyes. *J Mol Catal B Enzym* 74:63–72
- Mirzadeh SS, Khezri SM, Rezaei S, Forootanfar H, Mahvi AH, Faramarzi MA (2014) Decolorization of two synthetic dyes using the purified laccase of *Paraconiothyrium variabile* immobilized on porous silica beads. *J Environ Health Sci Eng*. doi:[10.1186/2052-336X-12-6](https://doi.org/10.1186/2052-336X-12-6)
- Baldrian P (2006) Fungal laccases—occurrence and properties. *FEMS Microbiol Rev* 30(215–2):42
- Ashrafi SD, Rezaei S, Forootanfar H, Mahvi AH, Faramarzi MA (2013) The enzymatic decolorization and detoxification of synthetic dyes by the laccase from a soil-isolated ascomycete, *Paraconiothyrium variabile*. *Int Biodeter Biodegr* 85:173–181
- Osma JF, Toca-Herrera JL, Rodríguez-Couto S (2010) Transformation pathway of Remazol Brilliant Blue R by immobilised laccase. *Bioresour Technol* 101:8509–8514
- Kunamneni A, Ghazi I, Camarero S, Ballesteros A, Plou FJ, Alcalde M (2008) Decolorization of synthetic dyes by laccase immobilized on epoxy-activated carriers. *Process Biochem* 43: 169–178
- Murugesan K, Nam IH, Kim YM, Chang YS (2007) Decolorization of reactive dyes by a thermostable laccase produced by *Ganoderma lucidum* in solid state culture. *Enzym Microb Technol* 40:1662–1672
- Mechichi T, Mhiri N, Sayadi S (2006) Remazol Brilliant Blue R decolorization by the laccase from *Trametes troglia*. *Chemosphere* 64:998–1005
- Soares GMB, Pessoa-de Amorim MT, Costa-Ferreira M (2001) Use of laccase together with redox mediators to decolorize Remazol Brilliant Blue R. *J Biotechnol* 89:123–129
- Silva MC, Torres JA, Vasconcelos de Sa LR, Chagas PMB, Ferreira-Leitão VS, Corrêa AD (2013) The use of soybean peroxidase in the decolorization of Remazol Brilliant Blue R and toxicological evaluation of its degradation products. *J Mol Catal B Enzym* 89:122–129
- Reis da Silva M, Vasconcelos de Sa LR, Russo C, Scio E, Ferreira-Leitão VS (2010) The use of HRP in decolorization of reactive dyes and toxicological evaluation of their products. *Enzyme Res*. doi:[10.4061/2010/703824](https://doi.org/10.4061/2010/703824)
- Liu Y, Huang J, Zhang X (2009) Decolorization and biodegradation of Remazol Brilliant Blue R by bilirubin oxidase. *J Biosci Bioeng* 108:496–500

32. Ratanapongleka K, Phetsom J (2014) Decolorization of synthetic dyes by crude laccase from *Lentinus polychrous* Lev. IJCEA 5:26–30
33. Zucconi F, Monaco A, Forte M, Bertoldi MD (1985) Phytotoxins during the stabilization of organic matter, in composting of agricultural and other wastes. In: Gasser JKR (ed) Commission European communities. Elsevier, London, pp 73–86
34. Verma Y (2008) Acute toxicity assessment of textile dyes and textile and dye industrial effluents using *Daphnia magna* bioassay. Toxicol Ind Health 24:491–500
35. Champagne PP, Ramsay JA (2010) Dye decolorization and detoxification by laccase immobilized on porous glass beads. Bioresour Technol 101:2230–2235