African Journal of Microbiology Research Vol. 6(14), pp. 3501-3509, 16 April, 2012
Available online at http://www.academicjournals.org/AJMR
DOI: 10.5897/AJMR12.026
ISSN 1996-0808 ©2012 Academic Journals

Full Length Research Paper

Decolourization characterizations of crude enzymes from a novel basidiomycete *Mycena purpureofusca*

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Accepted 29 February, 2012

A *Mycena purpureofusca* strain was investigated in submerged fermentation for its high production of laccase and very low mycelium biomass, as a novel dye-decolourizing white-rot fungus. Laccase was the only lignolytic enzyme produced by this strain during LSF. The extracellular crude enzyme from *M. purpureofusca* showed characteristic activity in synthetic dye colour removal, decolourizing 70.7% Bromothymol blue (500 mg/L), 58.8% Remazol Brilliant Blue R (RBBR 100 mg/L), and 22.2% Crystal violet (100 mg/L) without the addition of redox mediators. The effects of laccase inhibitor on decolourization corroborated laccase as the major enzyme involved in the decolourization of dyes. The decolourization of RBBR, an anthraquinonic dye extensively used in the textile industry was further studied. Box-Behnken design was used to evaluate the effects of three parameters on the RBBR decolourization yield. Results clearly indicated that the significant effect of incubation period as well as enzyme concentration. High efficiency decolourization of RBBR by crude laccase was gained by low laccase concentration without redox mediators. The selected optimal conditions allowed 97.03% of RBBR decolourization versus 94.21% for the predicted value. These results showed a promising future for application of the laccase system for industrial wastewater bioremediation.

Key words: *Mycena purpureofusca,* laccase, Remazol Brilliant Blue R (RBBR), Box-Behnken design.

INTRODUCTION

Synthetic dyes are being extensively used in textile dyeing and printing processes. On the basis of dying process, the decolourization of textile dye effluent is complicated and poses a serious environmental problem. Dyes are generally very difficult to break down biologically, due to their highly structured organic compounds (da Fonseca Araujo et al., 2007). Hence, satisfactory results from the treatment of this wastewater are difficult to obtain by traditional biological processes. This has impelled the search for alternative methods, such as the use of oxidative enzymes produced by white-rot fungi, which are to date the best-known dye-decolourising microorganisms (Osma et al., 2010). White rot fungi are well known organisms for the decolourization of a wide range of synthetic dyes due to their non-specific extracellular ligninolytic enzyme system consisting of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Khlifi et al., 2010; Mechichi et al., 2006). The broad substrate specificity of these enzymes in the ligninolytic systems of white rot fungi makes it possible for them to degrade a variety of recalcitrant compounds and even complex pollutants including dyes. Laccases are oxidoreductases that belong to the multinuclear copper-containing oxidases and are able to decolourize and detoxify industrial dyes (Khlifi et al., 2010; Mechichi et al., 2006).

Previous studies have shown that the efficiency of dye decolourization by laccase depends on many factors such as the reaction time, the concentration of the enzyme and the structure and concentration of the dye, and the redox mediator (Murugesan et al., 2007a;

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Abbreviations: RSM, Response surface methodology; **LSF**, liquid submerged fermentation; R^2, determination coefficient; **RBBR,** Remazol Brilliant Blue R.

Tavares et al., 2009). RSM is a collection of mathematical and statistical techniques useful for analyzing the effects of several independent variables (Myers and Montgomery, 2002). These conventional methods are time consuming and incapable of detecting the true optimum (Czitrom, 1999). RSM has been successfully applied in the optimization of the experimental conditions of dye decolourization with fungal laccases (Neifar et al., 2011; Tavares et al., 2009). Remazol Brilliant Blue R (RBBR) is an industrially important dye that is frequently used as a starting material in the production of polymeric dyes. RBBR is an anthracene derivative and represents an important class of toxic and recalcitrant organopollutants (Osma et al., 2010). There are several reports on decolourization of RBBR by laccases (Hu et al., 2009; Mohorcic et al., 2006; Osma et al., 2010; Palmieri et al., 2005; Soares et al., 2001a), but none has been decolorized by *Mycena* genus. The white-rot fungus *Mycena purpureofusca* is well characterized in liquid fermentation for its high production of laccase and very low mycelium generation.

The present work focuses on applying laccase from *M. purpureofusca* without mediators in order to decolourize synthetic dyes especial RBBR. The main objectives of this work were to gain a better understanding of the relationship between the decolourization variables (enzyme concentration, dye concentration, and incubation time) and the response (RBBR decolourization yield), and to obtain the optimum experimental conditions for high efficient decolourization using a three-level Box-Behnken design and RSM. All of the results obtained in this study provide a sound basis for further exploration.

MATERIALS AND METHODS

Chemicals and fungal strains

The dyes used were of industrial grade and obtained from Sigma Chemical, USA. All of the other chemicals used were of analytical grade. The *M. purpureofusca* strain was obtained from the Fujian General Station of Technology Popularization for Edible Fungi (Fuzhou, China) and maintained on potato dextrose agar (PDA; potato 200 g/L, glucose 20 g/L, agar 20 g/L) at 25°C with a periodic transfer.

Enzyme production

The liquid medium contained (per litre) 4.26 g sucrose, 15 g yeast powder, 2.7 g KH_2PO_4, 4.83 g $MgSO_4{\cdot}7H_2O$, 5.625 mg $CuSO_4{\cdot}5H_2O$, and 0.1 g Vitamin B_1. All experiments were carried out in triplicate and each data point was the mean of three independent scorings. The liquid seed was inoculated with mycelia mat (ca. 50 mm^2) and cultivated at 24°C on a rotary shaker at 110 rpm for 8 days. The broth was filtered through a nylon mesh, the filtrate further was clarified by centrifugation at 8000 rpm for 10 min at 4°C, and the supernatant were retained and stored at 4°C for further experimentation.

Enzyme assays

Laccase activity assay is conducted in 3 mL reaction mixtures consisting of 2.7 mL of 0.1 M sodium acetate buffer (pH 4.5), 0.2 mL of 1 mM 2, 2-azino-bis-3- ethylbenzothiazoline-6-sulphonic acid (ABTS) solution, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{420} for 3 min at 40°C. One unit of enzyme activity is defined as the amount of enzyme that oxidized 1 µM ABTS per min. The extinction coefficient of 3.6×10^4 mol^{-1} cm^{-1} was used for oxidized ABTS (Niladevi et al., 2009). The reaction mixtures used to determine MnP activity contained 2.7 mL of 50 mM sodium lactate (pH 4.5), 0.1 mL of 1.6 mM manganese sulfate, 0.1 mL of 2.0 mM H_2O_2, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{240} for 3 min at 40°C (Glenn et al., 1986). The reaction mixtures used to determine LiP activity contained 2.7 mL of 0.5 M sodium tartrate (pH 4.0), 0.1 mL of 1.2 M methylene, 0.1 mL of 2.0 mM H_2O_2, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{664} for 3 min at 40°C. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 µM of substrate per min (Sedlak and Ho, 2004).

Dye decolourization experiments

Crude enzyme was obtained after 8 days of fermentation by removing fungal mycelium through centrifugation at 8000 rpm for 10 min. The decolourization mixture contained 0.1 M sodium acetate buffer (pH 4.5), the dyes Bromothymol blue (triphenylmethane-type dye) 500 mg/L, Crysal violet (triphenylmethane-type dye) 100 mg/L, RBBR (anthraquinonic dye) 100 mg/L and crude enzyme (0.1 U/mL). Experiments were performed in a shaking incubator (40°C, 120 rpm). The decolourization processes were monitored for 480 min by measuring the absorbance of the reaction mixture at the following time intervals; 0 min, 60 min, 120 min, 240 min and 480 min at 420 nm for Bromothymol blue, 595 nm for RBBR and 590 nm for Crystal violet.

The maximum absorbance of each dye was obtained by scanning the UV/VIS absorbance spectrum. The amount of every component varied with each optimization experiment. The decolourization of dye, expressed as dye decolourization (%), was calculated according to the formula:

$$\text{Decolourization (\%)} = [(Ci - Ct)/Ci] \times 100,$$

Where Ci: initial concentration of the dye and Ct: dye concentration along the time (Lorenzo et al., 2006).

A control test containing the same amount of a heat-denatured crude enzyme was performed in parallel. The RBBR concentration of the dyes was calculated using the following equation obtained by the standard curve

$$Y = 125.15X - 1.3883,$$

$R^2=0.9989$, where Y is dye concentration, X is OD value.

The influence of the parameters namely; temperature (30 to 80°C), pH (2.2 to 8) and time (30 to 180 min) on decolourization process was closely investigated. Furthermore, to confirm whether laccase was involved in the dye decolourization process, the kojic acid of the laccase inhibitors was added to the culture supernatants of *M. purpureofusca* at a final concentration of 100 mM and was used to investigate their effects on dye decolourization.

Optimization conditions for optimal decolourization efficiency

In this work, a Box-Behnken design was set up in order to seek the best experimental conditions for the three independent factors affecting the efficiency of the decolourization of RBBR namely;

Table 1. Experimental conditions of Box-Behnken design in coded and natural variables and the corresponding experimental and theoretical responses.

Run No.	X_1	X_2	X_3	X_1: Time (h)	X_2: Enzyme (U/mL)	X_3: dye concentration (mg/L)	Measured decolourization (%)	Estimated Decolourization (%)
1	1	1	0	3	0.25	200	91.37	90.62
2	1	-1	0	3	0.05	200	66.67	66.44
3	0	-1	-1	2	0.05	100	58.45	58.19
4	0	1	1	2	0.25	300	89.98	90.23
5	-1	-1	0	1	0.05	200	38.29	39.04
6	-1	0	-1	1	0.15	100	81.84	81.35
7	-1	1	0	1	0.25	200	84.51	84.74
8	-1	0	1	1	0.15	300	61.34	60.85
9	1	0	1	3	0.15	300	81.52	82.01
10	0	-1	1	2	0.05	300	55.18	54.92
11	0	1	-1	2	0.25	100	92.50	92.76
12	1	0	-1	3	0.15	100	92.98	93.47
13	0	0	0	2	0.15	200	83.48	83.65
14	0	0	0	2	0.15	200	84.08	83.65
15	0	0	0	2	0.15	200	83.40	83.65
16	0.73	0.67	-0.58	2.73	0.22	142	97.03	94,21

incubation time (X_1), enzyme concentration (X_2), and dye concentration (X_3) (Table 1). The relationship between the response (RBBR decolourization yield) and the three quantitative variables was approximated by a regression equation (Equation 1), where *Y* is the predicted response, β_0 the offset term, β_i the linear effect, β_{ii} the squared effect, β_{ij} the interaction effect, and X_i the dimensionless coded value of the independent variables under study (Murat, 2004). Data were processed to attain Equation (1) indicating the interaction between the process variables and laccase production.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_{ji} \quad (1)$$

In the regression equation, the test variable was coded according to Equation (2), where X_i is the independent variable coded value, U_i the independent variable value, and U_i^0 the independent variable real value on the centre point and the step change value (Murat, 2004).

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

A three-variable Box-Behnken design with 15 experiments (Table 1) was used to estimate the model coefficients. The experimental points are located in the middle of a cube ridge (12 experiments: runs no. 1 to 12) and at the centre of the cube (3 experiments: runs no. 13 to 15), the experiment was performed under the optimized conditions and at the maximal decolourization yield point to verify the reliability of this prediction model (run no. 16).

The significance of the fitted model was tested by mean of the analysis of variance (ANOVA). The fitted model was used to study the relative sensitivity of the response to the variables in the whole domain and to seek the optimal experimental conditions. The relationship between the response and the experimental variables was illustrated graphically by plotting the response surfaces (Ghorbel et al., 2010). In this study, the generation and the data treatment of the Box-Behnken design were performed by Design-Expert 7.1.6.

RESULTS

Decolourization experiment

In this paper, the decolourization of dyes with two structural patterns was investigated using culture supernatant. As shown in Figure 1a, anthraquinone dyes (RBBR) could be decolourized directly utilizing the crude enzyme produced by *M. purpureofusca*, while the decolourization of triphenylmethane-type dye (Bromothymol blue and Crystal violet) differed.

Within 2 h, Bromothymol blue (500 mg/L), RBBR (100 mg/L) and Crystal Violet (100 mg/L) could be decolourized to 64.17, 48.07 and 13.09%, respectively, and to 70.67, 58.82, and 22.20%, respectively within 8 h. Bromothymol blue was decolourized most efficiently as 1 h reaction could decompose the dye up to 42.29%.

Detection of enzyme(s) involved in dye decolourization

Laccase was the main enzyme involved in the decolourization since there were no other ligninolytic enzymes detected in the culture supernatant. The involvement of laccase in decolourization was also demonstrated by employing laccase inhibitors. Kojic acid, a specific inhibitor of fungal laccase (Kim et al., 2008; Yang et al., 2011), was used to confirm that the

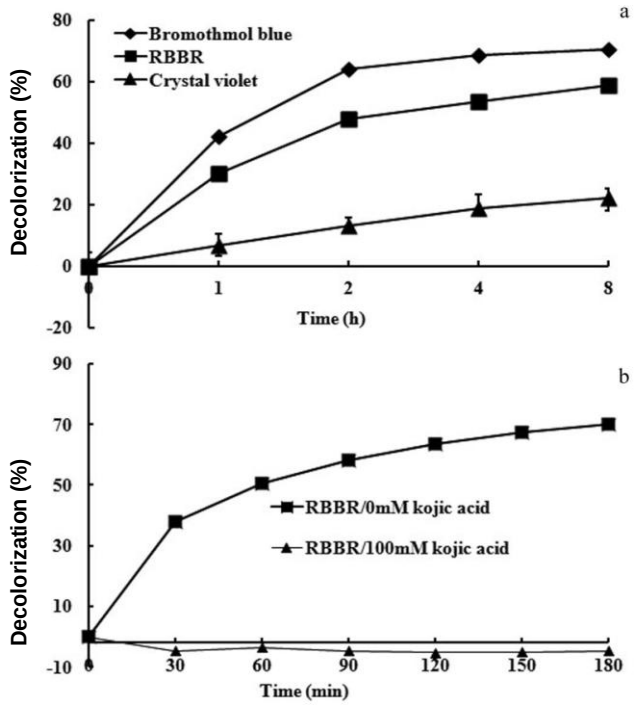

Figure 1. Decolourization changes of the reaction mixture using crude enzyme from *M. purpureofusca.* (a) Decolorization of RBBR (100 mg/L), Bromothmol blue (500 mg/L) and Crystal violet (100 mg/L) by crude enzyme. (b) Decolourization of RBBR (100 mg/L), RBBR/100 mM kojic acid means that 100 mM kojic acid was added to the culture supernatants of *M. purpureofusca* and then Decolourization was perfumed. RBBR/0 mM kojic acid means that no kojic acid added.

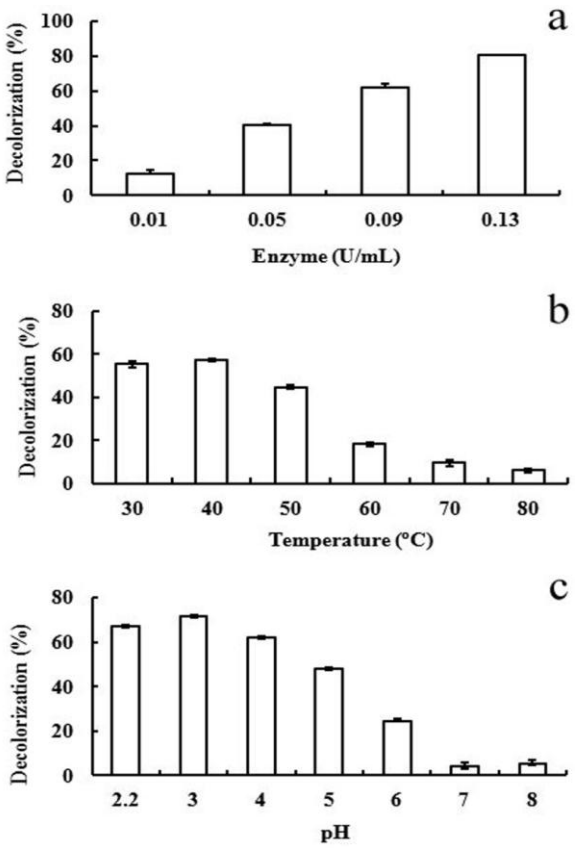

Figure 2. Effects of laccase activity (a), temperature (b), and PH (c) on Decolourization of RBBR by the crude laccase. Incubation conditions: (a) RBBR (100 mg/L), laccase (0.01-13 U/Ml), pH4.5, 40˚C. (b) RBBR (100 mg/L) laccase (0.1 U/Ml), pH 4.5, 30-80˚C. (c) RBBR (100 mg/L), laccase (0.1 U/Ml), pH (2.2-8.0), 40˚C.

decolourization of dyes was dependent on laccase. Kojic acid was added to the culture supernatants of *M. purpureofusca*. Results shown in Figure 1b reveal that after adding laccase inhibitor, the decolourization ratios changed at the various times of 30, 60, 90, 120 and 180 min, respectively. The decolourization of RBBR was inhibited when the concentration of kojic acid was 100 mM.

Furthermore, 100 mM kojic acid concentration was enough to completely inhibit laccase activity, compared with control (516.56 U/L). During decolourization, only laccase activity was detected in the culture filtrates whereas MnP and LiP activities were absent. Laccase was also detected from the first day of incubation and was found to increase steadily with maximum activity on the twelfth day (673.39 U/L).

Thus, the above results demonstrate that the capability for decolourizing RBBR was positively related to laccase activity. Therefore, this result from using kojic acid as the specific inhibitor of fungal laccase also suggests that laccase was the main enzyme of the crude enzyme involved in decolourization. This is consistent with the recent finding for *Ganoderma lucidumand* and *Trametes trogii* which secreted only laccase contributing to decolourizing RBBR in solid substrate fermentation (Murugesan et al., 2007b; Zeng et al., 2011).

Effect of various parameters on RBBR decolourization

The parameters of the RBBR dye decolourization process affect the dye-removal reaction directly. The effect of laccase activity on decolourization was performed by utilizing crude enzyme (laccase activity 0.01 to 0.13 U/mL). The optimum laccase activity for the decolourization of RBBR was 0.09 U/mL, and the decolourization was increased with the increasing laccase activity (Figure 2a). Therefore, the subsequent experiments were carried out using 0.1 U/mL laccase. Temperature affected the reaction rates as well as laccase stability. In this study, dyes were decolourized to a maximum of 40°C, and a sharp reduction in decolourization occurred at temperatures above 60°C (Figure 2b).

This result was in agreement with that of Zeng et al.

Table 2. ANOVA results for the equation of Design-Expert 7.1.6 for the response.

Source	Sum of squares	df	Mean square	F value	P-value (Prob > F)
Model	3765.33	10	376.53	552.64	< 0.0001
Residual	2.73	4	0.68		
Lack of Fit	2.45	2	1.23	8.91	0.1009
Pure Error	0.28	2	0.14		
Cor Total	3768.06	14			
R-square	0.9993	R_{adj}- square	0.9975	R_{pred}-square	0.9738

(2011). The pH values played a key role in decolourization by crude enzyme. As shown in Figure 2c, the maximum decolourization rates for RBBR occurred at 3.0. When pH was 7.0, the decolourization could almost be omitted. An explanation might be that laccase achieves optimum activity at pH 2.2 to 5.0 and no activity at pH 7.0. Figure 1b showed the effect of time on dye decolourization, and the decolourization yield increased with the extension of time, but the increase in decolourization yield was not significant after 2 h.

Optimization conditions for high efficient decolourization

Estimated model

Fifteen experiments were carried out. The experimental conditions, shown in Table 1, were arranged according to the three-variable Box-Behnken design. The corresponding observed values of the decolourization yield are indicated in the last column of Table 1. The observed responses were used to compute the model coefficients. The relationship between the response (RBBR decolourization yield) and the three quantitative variables was approximated by the second-order polynomial function (Equation 1). The results of the variance analysis showed the model *F*-value of 23.16 implies that the model is significant. There is only a 0.15% chance that a "Model F-Value" this large could occur due to noise, but the "Lack of Fit F-value" of 213.23 implies that the lack of fit is significant. The "Pred R-Squared" of 0.6262 is less close to the "Adj R-Squared" of 0.9344 as one might normally expect, which may indicate a large block effect or a possible problem with our model. In this case, X_1, X_2, X_3, X_2^2 are significant model terms, and so the polynomial function was optimized by adding the highest order interaction variables. This allowed us to write the following estimated model:

$$Y = 83.65 + 8.32*X_1 + 17.47*X_2 - 1.45*X_3 - 4.02*X_1*X_1 - 5.38*X_1*X_2 + 2.26*X_1*X_3 - 9.41*X_2*X_2 + 0.19*X_2*X_3 - 0.21*X_3*X_3 - 0.21*X_1*X_1*X_3 \quad (3)$$

Statistical analysis and validation of the model

The relationship between the response (RBBR decolourization yield) and the three quantitative variables was approximated by the high order polynomial function (Equation 3). The analysis of variance for the fitted model (Table 2) showed that the regression sum of the squares was statistically significant at the 99.9% and that the Lack of Fit was not significant. The "Pred R-Squared" of 0.9738 is as close to the "Adj R-Squared" of 0.9975 as one might normally expect.

Thus, we concluded that the model represented well the measured data. In addition, in order to verify the reliability of this prediction model, the experiment was performed with the optimized parameters and the maximal percentage of decolourization was found to be 97.03, which was in close agreement with the model's prediction.

Interpretation of the RSM

The relationship between the response and the experimental variables can be illustrated graphically by plotting three dimensional response surface plots (Figure 3a, b, c). In these plots, each figure represented the effect of two variables on the production, while the other variable was held at the zero level. Such plots are helpful in studying the effects of the variation of the factors in the domain studied and consequently, in determining the optimal experimental conditions (Ghorbel et al., 2010). The incubation time and enzyme concentration effects on the response at a fixed optimum RBBR content of 200 mg/L are illustrated in Figure 3a. It clearly shows that the decolourization yield increases with the incubation time and enzyme concentration. The effect of the incubation time and enzyme concentration is significant. The response value reached its lowest level of 38.29% while the enzyme concentration showed a minimum at 0.05 U/mL for 1 h.

High decolourization yields above 83% can be reached when using an enzyme concentration > 0.15 U/mL and an incubation time in the range of 2.0 to 3 h. Figure 3b represents the effect of the incubation time (X_1) and dye concentration (X_3) on RBBR decolourization at constant

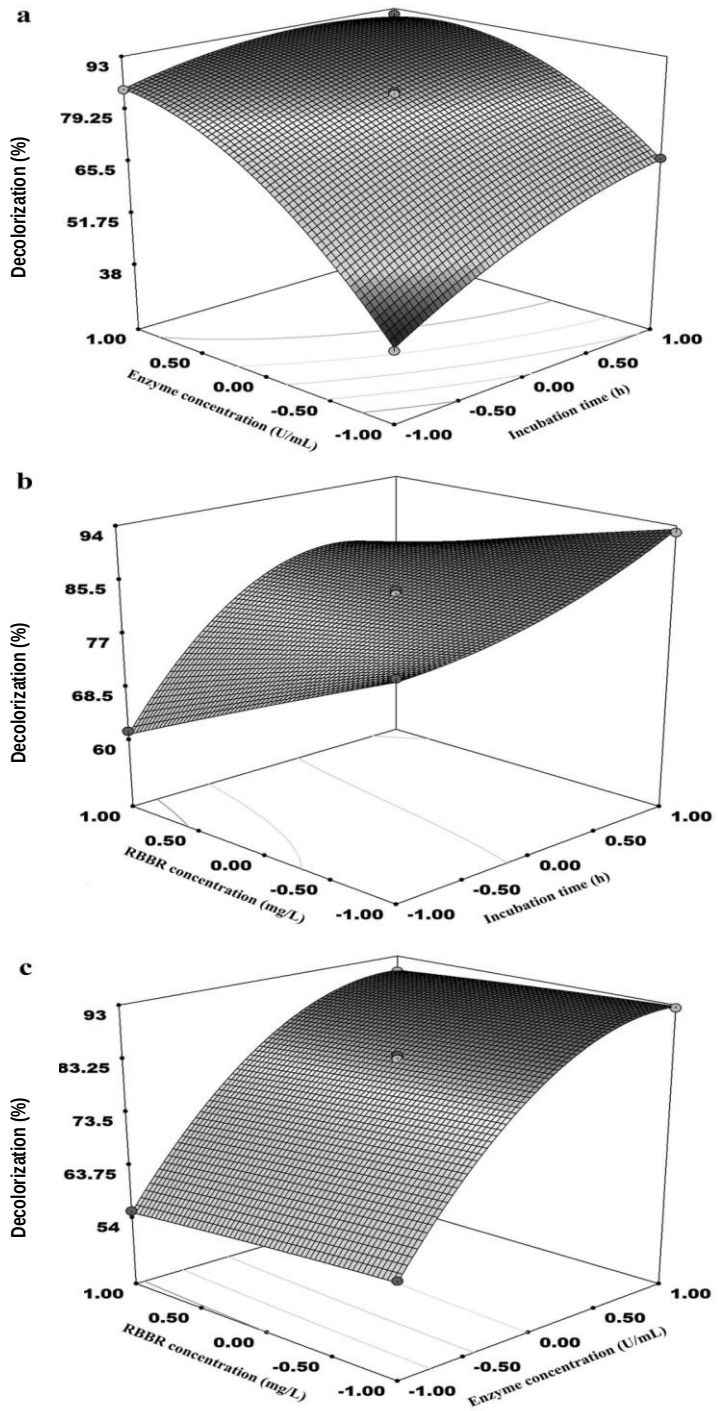

Figure 3. Three-dimensional response surface plots for the effect of two variables on the decolourization of RBBR, another variable is maintained at zero level. (a) Effect incubation time and enzyme concentration at a fix value of 200 mg/L dye on the decolourization of RBBR. (b) Effect incubation time and RBBR concentration at a fix value of 0.15 U/mL laccase on the decolourization RBBR. (c) Effect dye and laccase concentrations at constant incubation time (2 h) on the decolourization of RBBR.

enzyme concentration (0.15 U/mL). The contour plots of Figure 3b also support the important role of the incubation time. The positive effect of enzyme concentration is also demonstrated in Figure 3c. The

decolourization yields increased with the increase in enzyme concentration, whereas it decreased with the increase in dye concentration when the incubation time was fixed at 2 h (X_1 = 0), improving the RBBR decolourization yield.

The selection of the optimal conditions was based on the determination of the experimental conditions leading simultaneously to the maximization of RBBR decolourization and the minimization of the process cost. The optimal conditions are an enzyme concentration of 0.22 U/mL, an RBBR concentration of 142 mg/L, and time 2 h 43.8 min. Under these conditions, the value of the RBBR decolourization yield was 97.03% which was in close agreement with the predicted value (94.21%).

DISCUSSION

Laccases and their decolourization are proportional to enzyme activity (Wong and Yu, 1999). Moreover, high potential laccases have been shown to decolourize anthraquinone dyes more efficiently than other classes of dye (Champagne and Ramsay, 2005). In this paper, the decolourization of dyes with two structural patterns was investigated using crude extracellular enzyme. Although Bromothymol blue and Crystal violet are triphenylmethane-type dyes, their decolourization efficiencies were different. The concentration of Bromothymol blue was five-fold as that of Crystal violet, but the crude laccase from *M. purpureofusca* was showed to decolourize Bromothymol blue more efficiently than Crystal violet and RBBR. The change in log (*k*cat/*K*m) was found to be proportional to the one-electron redox potential difference between the laccase's type 1 copper site and the substrate.

This correlation indicated that the first electron transfer from substrate to laccase was governed by the "outersphere" mechanism (Xu, 1996). Phenols are typical substrates, because their redox potentials are low enough to allow electron by the T1 Cu (II) that, although a relatively modest oxidant is the electron-acceptor in laccases. The efficiency of oxidation was found to increase with the decrease in redox potential of the substrates (Kawai et al., 1988; Tadesse et al., 2008). Bromothymol blue may have lower redox potential than that of Crystal violet as phenolic substrates. However, Bourbonnais and Paice (1990) reported that laccases can catalyze the oxidation of non-phenolic benzylalcohols in the presence of a redox mediator. Decolourization of the Crystal violet may be in the presence of a redox mediator with the crude laccase from *M. purpureofusca*, and so the dye decolourization yield depended upon the dye structure, the enzymes used, and the system conditions. The decolourization yield of the laccase from *M. purpureofusca* is correlated with the chemical structure of the dyes.

The predicted values were evaluated from the model and generated by using the approximating function. The regression model gives poor or misleading results unless it is an adequate fit (Myers and Montgomery, 2002). If there are many insignificant model terms (not counting those required to support the hierarchy), model reduction may bring about improvements, but the method will increase the number of poor predictions or estimates of function (Korbahti and Rauf, 2009). The "Pred R-Squared" of 0.6262 is less close to the "Adj R-Squared" of 0.9344 as one might normally expect in this study. R^2 is a measure of the amount of reduction in the variability of the response obtained by using the independent factor variables in the model. There is a good chance that insignificant terms have been included in the model when R^2_{pred} and R^2_{adj} differ dramatically (Montgomery, 1996; Myers and Montgomery, 2002). The sum of squares for regression always increases with the addition of a variable to the regression model. Therefore, the sufficiency of the increase in the regression sum of squares must be decided.

Furthermore, adding an unimportant variable to the model can actually increase the mean square error (Montgomery, 1996; Myers and Montgomery, 2002). The highest order polynomial was selected where the additional terms are significant and the model is not aliased, so the polynomial function was optimized by adding the highest order interaction variables. The main purpose of this study was to find a suitable approximating function in order to predict and determine the response, and to obtain the operating conditions for high decolourization yields. The highest order interaction of $X_1^2X_3$ was added to optimize the function. The optimized function improved the fit of the model. The experimental values were in good agreement with the predicted ones and the model was highly significant, the correlation coefficient being 0.9993. The effect of the incubation time and enzyme concentration is significant. Previously, Soares et al. (2001b) reported that increasing concentration of laccase increased decolourization yield for RBBR in the presence of HBT. The interaction between RBBR and enzyme concentrations was consistent with the result that decolourization of reactive black 5 by laccase produced by *Pleurotus sajor-caju* (Murugesan et al., 2007a).

Laccases have been reported in many other white rot fungi, which are involved in the degradations of diverse recalcitrant compounds such as dyes (Minussi et al., 2002; Palonen and Viikari, 2004), coloured industrial wastewater (Li et al., 1999) and endocrine-disrupting chemicals (Xu, 1996). Most laccases required redox mediators to decolourize RBBR. In the present study, the purified laccase from *Pycnoporus sanguineus* was able to decolourize RBBR efficiently in the absence of any redox mediators. Eggert et al. (1996) proposed that the laccase from *Pycnoporus cinnabarinus* might have a broader substrate range than the other white rot fungal laccases as the lignolytic system of *P. cinnabarinus* lacked both

lignin peroxidase and manganese peroxidase, because the structural and/or redox potential differences of the individual enzymes played a major role in decolourization (Nyanhongo et al., 2002). Laccase from *M. purpureofusca* was the sole phenoloxidase in the culture medium. The crude laccase from *M. purpureofusca* showed active decolourizing activity even without a mediator. The decolourization yield was very high (97.03%) and higher efficiency than the values were obtained by using purified laccase from other fungi under the optimal conditions (Baldrian, 2004; Nagai et al., 2002; Rodriguez-Couto, 2011). The results are supported by Eggert's point and was consistent with the conclusion that laccase produced by white rot fungus which secreted laccase as the sole or major lignolytic enzyme, might have more advantages in dye decolourization than other fungal laccases (Lu et al., 2007). This indicates that *M. purpureofusca* laccase has potential for use in the decolourization of industrial dyes. However, further research is required to study colour removal by the purified laccase in order to explore the mechanisms at work.

Conclusion

The crude enzyme from *M. purpureofusca* in LSF showed good activity in synthetic dye color removal without redox mediators. The results demonstrated that the laccase plays the major role in the decolourization of dyes. The decolourization was increased with the increasing laccase activity. Temperature affected the reaction rates as well as laccase stability. In this study, the highest dye decolourization yield was obtained at 40°C and pH 3.0. When pH was 7.0, the decolourization could almost be omitted.

Under the optimized conditions of 0.22 U/mL enzyme concentration, 142 mg/L RBBR concentration, and time 2 h 43.8 min, the experimental values were in accordance with the predicted ones, indicating suitability of the model and the success of RSM in optimizing the conditions of RBBR decolourization of laccase. In the optimization, R^2_{pred} and R^2_{adj} correlation coefficients for polynomial model were evaluated quite satisfactorily as 0.9738 and 0.9975, respectively.

ACKNOWLEDGEMENTS

This work was financially supported by the Key Project of Fujian Province of China (No.2010N0004) and the General Program of the Provincial Education Department of Fujian Province of China (No. JA11079).

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