JOURNAL OF PURE AND APPLIED MICROBIOLOGY, FEBRUARY 2014. Vol. **8**(1), p. 11-19

Non-Mediated Oxidation of Non-Phenol Dye Methyl Red with Purified Laccase from *Trametes hirsuta* lg-9

Haibo Zhang[3], Youshuang Zhu[2], Yinglong Zhang[4] and Feng Huang[1]*

[1]State Key Laboratory of Microbial Technology, Shandong University, Jinan 250100, China.
[2]Department of Biological Science, Jining Medical University, Jining 272067, China.
[3]CAS Key Laboratory of Biobased Materials, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, Qingdao 266071, China.
[4]Biotechnology Department, Shandong Institute of Commerce and Technology, Jinan 250103, China.

(Received: 28 July 2013; accepted: 16 September 2013)

A strain that was capable of producing an atypical laccase had been isolated and identified as *Trametes hirsuta* lg-9. Non-phenolic azo dye methyl red was oxidized by the atypical laccase. UV-Vis spectrophotometry was used for process monitoring of the oxidation products of methyl red. High performance liquid chromatography (HPLC) was used for detecting the products. Additionally, the structures of products were elucidated with electrospray injection mass spectroscopy. The products indicated the atypical laccase could directly attack non-phenolic azo dye methyl red. Probably degradation mechanism of the azo dye methyl red oxidized by the atypical laccase directly had been proposed. Factorial design, steepest ascent design and central composite design were successfully applied to optimize the decolorization conditions of methyl red. The optimum decolorization of methyl red was approximately 80% at enzyme concentration of 1.8 U l^{-1}, pH 4.93, time 130.23 min.

Key words: Decolorization, Laccase, Mediator, Methyl red, Response surface methodology.

Laccases [benzenediol: oxygen oxidoreductases (EC1.10.3.2)] play an important part in fungal ligninolytic systems, and they are a class of proteins that belongs to the multicopper oxidase family[1, 2]. Laccases can catalyze the oxidation of a wide range of substrates (typically mono-, di-, and polyphenols, aromatic amines, methoxyphenols and ascorbate), and they can be used in the paper industry, the food industry, in dye or stain bleaching, bioremediation, plant fibre modification, ethanol production, biosensors, biofuel cells, organic synthesis, and drug synthesis[3-, 8]. The redox potential of laccase is low and in the 0.5-0.8 V *vs*. NHE range[9]. Laccase can directly oxidize phenol and other small molecular low redox potential compounds. Laccase also can indirectly oxidize non-phenol compounds and high molecular compounds with the help of mediators. However, the need for mediators is still a barrier to the utilization of laccases because of the cost of the mediators. Laccase with atypical spectra and oxidative characteristics was found to oxidize some non-phenol compounds and polyaromatic hydrocarbons directly[10,-12]. Some laccases that lack the characteristic T1 site absorption spectra have been isolated and proved to have atypical characteristics. The atypical laccases can be divided into yellow laccase and white laccase. These atypical laccases were expected to be promising enzymes in biotechnology.

* To whom all correspondence should be addressed. Haibo Zhang and Youshuang Zhu contributed equally to this work.
Tel.: +86-0531-88364384; Fax: +86 531 88565234;
E-mail: lignin302304@yahoo.cn

Synthetic dyes are widely used in various industrial dyeing and printing processes. Synthetic dyes can be mainly divided into azo dyes, anthraquinone dyes and indigo dyes, according to their major chromophores. The reactive group azo dyes are mostly used in textile dyeing due to their stability and superior fastness to the applied fabric[13]. The azo dyes possess about 50% of the industrial dyes. Most of the synthetic dyes are toxic, mutagenic and carcinogenic[14-15]. Several physical and chemical methods have been attempted to treat dye-contaminated wastewater[16-18]. Biological and enzymatic methods have been considered as the promising methods in dye degradation and color removal in recent years. Laccase is one of the promising enzymes [19, 20, 21].

In our previous work, a laccase with atypical characters was purified, but its oxidation mechanism was not further researched[12]. In this study, UV-Vis spectrophotometry was used for process monitoring of the oxidation products of methyl red. High performance liquid chromatography (HPLC) was used for detecting the products. Additionally, the structures of products were elucidated with electrospray injection mass spectroscopy. The mechanism of transformation was tried to understand using the experimental approaches. As the laccase can directly oxidize non-phenol methyl red, it has great potential in biotechnology, especially in decolorization. We tried to optimize the decolorization conditions of methyl red, using factorial design, steepest ascent design and central composite design.

MATERIALS AND METHODS

Chemicals

Methyl red, 2, 2' -azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was purchased from Sigma-Aldrich (Shanghai, China).The other chemicals were of chromatographic purity.

Laccase and Enzyme assays

Fungal strain *Trametes hirsuta* lg-9 (CGMCC No. 2422) used in this study was isolated by our laboratory from Mengshan Mountain. The laccase was purified to be homogeneous based on SDS-PAGE as well as native PAGE. The physical and chemical properties of laccase were characterized as previous reported[12]. Laccase activity was assayed at room temperature (25°C) using ABTS as substrates. The assay mixture contained 1 mM ABTS, 20 mM sodium acetate buffer (pH 4.8) and a suitable amount of enzyme in a total volume of 2.0 ml. The oxidation of ABTS was followed by an absorbance increase at 420 nm (ε = 36,000 $M^{-1} cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme that oxidized 1µM of ABTS per minute. All the measurements were performed in triplicate on a spectrophotometer (UV-3100, Shimadzu).

UV-Vis detection, reaction dynamics

Methyl red was dissolved with pure ethanol, and then diluted with 40 mM sodium acetate buffer (pH 4.8). Finally, the reaction system contained 25 µM methyl red, 1% ethanol and 0.5 U l^{-1} laccase. The reactions were stopped by adding 10 mM NaF at intervals[22]. Control samples were measured by adding 10 mM NaF at the beginning of the reaction. The absorption spectra of all the samples were measured from 200 to 700 nm. All the measurements were performed in triplicate on a spectrophotometer (UV-3100, Shimadzu).

HPLC analysis, detection of products

The products of the samples were analyzed by high performance liquid chromatography (HPLC, Class-VP Ver.6.1, Shimadzu); compounds were monitored by UV absorption at 280 nm (SPD-10A, Shimadzu). The HPLC column was 4.6 × 150 mm Shim-pack VP-DOS. The injected volume was 20 µl. A mixture of methanol and tridistilled water (50:50, vol/vol) served as solvent at a flow rate of 1 ml/min under isocratic conditions.

ESI-MS analysis, identification of oxidation products

Mass spectroscopy (LTQ-Orbitrap XL, Thermo Fisher, Germany) with electrospray injection (ESI) was used, coupled to the HPLC apparatus. The source voltage was maintained at -4.5 kV, the electrospray injection N_2 sheath gas flow pressure was set at 50 arb, the auxiliary gas flow pressure was 10 arb, and the capillary temperature was 275°C.

Decolorization optimization

Decolorization determination was performed in phosphate-citric acid buffers, and they were assayed with the UV-Vis spectrophotometer. The other decolorization

conditions will be described in the following text according to the design of the experiment.

Factorial design

Factorial design is the first experiment of the series. Two level factorial design is a design for 2 to 21 factors where each factor is varied over two levels. In our experiment, the ranges of the variables tested were enzyme 0.5-1.5 U l^{-1} (step length: 0.5 U l^{-1}), pH 3.8-5.0 (step length: 0.6), temperature 25-35 °C (step length: 5 °C) and time 60-120 min (step length: 30 min). Four centre points were added to check for curvature. For a 2^{4-1} factorial design with four factors at two levels, twelve experimental runs were required. Table 1 shows the four independent variables and the different coded levels of the factorial design experiments. Design Expert software (Version 7.0.0, Stat-Ease Inc.) was used to analyze the data that were obtained from the experiment. A fitted first-order model was obtained based on the analysis of the data (Eqs. 1). The quality of fit of the first-order model equation was expressed by the coefficient of determination R^2, and its statistical significance was determined by an F-test.

$$Y = k_0 + k_1X_1 + k_2X_2 + k_3X_3 + k_4X_4 + k_{12}X_1X_2 + k_{13}X_1X_3 + k_{14}X_1X_4 + k_{23}X_2X_3 + k_{24}X_2X_4 + k_{34}X_3X_4 \quad ..(1)$$

Y is the predicted response; k_0 is intercept;
k_1, k_2, k_3 and k_4 are first-order linear coefficients;
k_{12}, k_{13}, k_{14}, k_{23}, k_{24} and k_{34} are the interaction coefficients.

Table 1. Result of Factorial Design

Run	X_1 Enzyme (U l^{-1})	X_2 pH	X_3 Temp. (°C)	X_4 Time (min)	Response Decolorization (%)
1	0.5	3.8	25	60	23.59
2	1.5	3.8	25	120	52.45
3	0.5	5	25	120	47.32
4	1.5	5	25	60	52.43
5	0.5	3.8	35	120	38.46
6	1.5	3.8	35	60	32.47
7	0.5	5	35	60	42.41
8	1.5	5	35	120	78.40
9	10	4.4	30	90	54.43
10	1.0	4.4	30	90	55.05
11	1.0	4.4	30	90	55.50
12	1.0	4.4	30	90	54.59

Steepest ascent path

The step lengths of the steepest ascent path were based on the results obtained from the factorial design. The direction of steepest ascent is the direction in which Y increases most rapidly. This direction is parallel to the normal to the fitted response surface. The steps along the path are proportional to the regression coefficients k_1, k_2, k_3, and k_4. The coefficients of the model can be used to construct the steepest ascent path. The step length can be calculated from the Eqs. 2. Table 2 shows the design and experimental results along the path of steepest ascent.

$$S = e\Delta_j k_j \quad ...(2)$$

S is the step length;
e is a factor determined by experimenter based on practical consideration;
Δ_j is the step length of Factorial Design;
k_j is the regression coefficients of first-order model equation.

Response surface methodology

In this study, we used Box-Behnken response surface methodology for the optimization of decolorization. Box-Behnken response surface methodology was applied using the Design Expert software to our study with three variables at three levels (-1, 0, +1). The different concentrations of enzyme, different incubation pH and times were the variables and designated as X_1, X_2 and X_4, respectively. The range of variables investigated is given in Table 3. The variables can be approximated by the quadratic model Eqs. 3.

$$Y = k_0 + k_1X_1 + k_2X_2 + k_4X_4 + k_{11}X_1^2 + k_{22}X_2^2 + k_{44}X_4^2 + k_{12}X_1X_2 + k_{14}X_1X_4 + k_{24}X_2X_4 \quad ...(3)$$

Table 2. Experimental results along the path of steepest ascent

Experiment No.	Enzyme (U l^{-1})	pH	Time (min)	Decolorization (%)
1	1.5	5	120	78.06
2	1.95	5.6	150	63.07
3	2.4	6.2	180	39.9
4	2.85	6.8	210	9.9
5	3.3	7.4	240	5.68

Y is the predicted response;
k_0 is intercept, k_1, k_2 and k_4 are first - order linear coefficients;
k_{11}, k_{22} and k_{44} are second - order quadratic coefficients;
k_{12}, k_{14} and k_{24} are the interaction coefficient.

The three-dimensional response surface plots were plotted to find the impact of each factor. A total number of 17 experiments were necessarily carried out to estimate the coefficients. Data were analyzed using Design Expert programs including analysis of variance (ANOVA) to find out the interaction between the variables and the response.

RESULTS AND DISCUSSION

UV-Vis detection, reaction dynamics

Spectrophotometric analysis is a convenient method to detect the degradation process. The UV-Vis spectra of the samples treated with the laccase from *T. hirsuta* lg-9 is shown in Fig. 1. The observed reduction in absorbance at 525 nm indicated that methyl red was oxidized. The increases in absorbance at about 250 nm and 350 nm indicated that new compounds were produced. The result indicated the atypical laccase from T. *hirsuta* lg-9 could directly oxidize methyl red without the help of mediators.

HPLC analysis, detection of production

HPLC had been used to detect the products of methyl red which had been treated with laccase from *T. hirsuta* lg-9. The result was shown in Fig.2. Results of HPLC analysis indicated that at least three products were produced. The retention times of the three products were about 145 (Fig.2, fraction 1), 214 (Fig.2, fraction 2) and 280 s (Fig.2, fraction 3). The other chemical products either were not formed or were formed only in trace quantities.

ESI-MS analysis, identification of oxidation products

ESI-MS was used to identify the products of methyl red. MS analysis of the products of methyl red which had been treated with the atypical laccase showed the following *m/z* values for the negative nano ESI mode (in parenthesis the most probable structure): 149 (2-hydrazinobenzoic acid, N_2H-Ph-COO^-, Fig.3a), 139 (*4*-nitrophenol, O_2N-Ph-OH^-, Fig.3b). The two products (*m/z* 149 and 139) corresponded to fraction 2 and fraction 3, respectively. The fraction 1 (Fig.2) was not detected by the ESI-MS.

Direct decolorization of azo dye methyl red by yeast, crude laccase and partly purified laccase have been studied[23, 24], but they cannot reveal the mechanism because of the presentation

Table 3. Result of response surface methodology of methyl red decolorization

Run	X_1 Enzyme (U l^{-1})	X_2 pH	X_4 Time (min)	Decolorization Actual (%)	Decolorization Predicted (%)	Residual (%)
1	1.05	4.4	120	62.08	62.58	-0.50
2	1.95	4.4	120	67.05	66.39	0.66
3	1.05	5.6	120	56.12	56.77	-0.66
4	1.95	5.6	120	62.35	61.85	0.50
5	1.05	5	90	68.34	67.73	0.61
6	1.95	5	90	73.99	74.53	-0.55
7	1.05	5	150	75.71	75.16	0.55
8	1.95	5	150	76.65	77.26	-0.61
9	1.5	4.4	90	57.19	57.30	-0.11
10	1.5	5.6	90	58.03	57.99	0.05
11	1.5	4.4	150	68.20	68.24	-0.05
12	1.5	5.6	150	57.31	57.20	0.11
13	1.5	5	120	78.37	78.09	0.28
14	1.5	5	120	77.43	78.09	-0.66
15	1.5	5	120	77.90	78.09	-0.19
16	1.5	5	120	78.84	78.09	0.75
17	1.5	5	120	77.90	78.09	-0.19

of other lignin modifying enzymes, manganese peroxidase and lignin peroxidase. Laccases have been demonstrated to oxidize phenolic azo dyes. The common laccases and lignin peroxidases can both oxidize the phenolic ring of the azo dyes by one electron to generate a phenolic radical. The phenolic radical can be oxidized by the enzymes again to produce a carbonium ion where the charge is localized on the phenolic ring carbon bearing the azo linkage. Then, the nucleophilic attack by water caused cleavage of the azo dyes[25, 26]. So far, laccase directly oxidize non-phenolic azo dyes has not reported. The study of the oxidation mechanism of the atypical laccase is of good use to the application of the laccase in decolorization industry.

As methyl red cannot be oxidized to generate a phenolic radical, the oxidation mechanism of the atypical laccase to oxidize non-phenolic methyl red may be also different from the oxidation mechanism of phenolic azo dyes with common laccase. UV-Vis spectrophotometry was used for process monitoring of the oxidation products of methyl red. The results of UV-Vis spectrophotometry (Fig. 1) indicated the productions of methyl red increased with the oxidation time running. HPLC was used for detecting the products. The results of HPLC indicate the quantities of the products are no less than three (Fig. 2). Additionally, the structures of products were elucidated with electrospray injection mass spectroscopy. The structures of products indicate the oxidation mechanisms of the atypical laccase and common laccase are similar but different (Fig.3). The cleavage sites of them are the same, while the products are different[25]. There were no quinones detected. We speculate the atypical laccase can directly attack the dye, which causes the *N*-demethylation of the dye. Similar reactions have been reported in decolorization by laccase-mediator system[27] and laccase-ultrasound treatment[2]. The formed amino-group can be further oxidized by laccase[28]. Then, the atypical laccase attack the azo linkage, which induces a variety of subsequent reactions. Leontievsky et al. proposed that yellow laccases

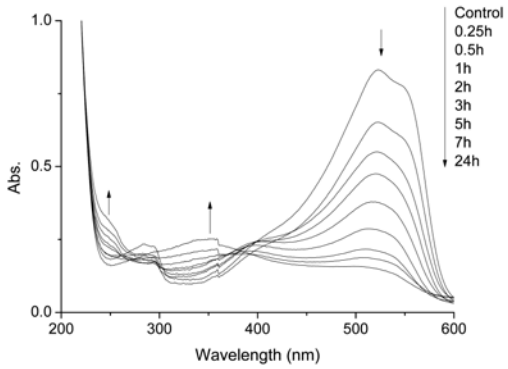

Fig. 1. UV-Vis absorption spectra of methyl red decolorization by purified laccase of *T. hirsuta* lg-9

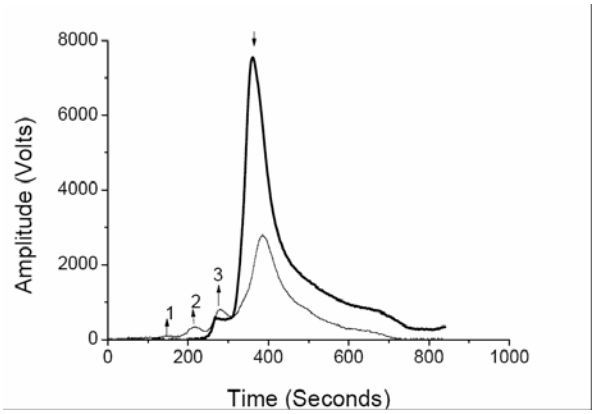

Fig. 2. HPLC analysis of methyl red decolorization by purified laccase of *T. hirsuta* lg-9. Sample (fine line), Control (bold line)

Table 4. ANOVA results for the quadratic equation of Design Expert 7.0.0 for decolorization

Source	Sum of Squares	Dgree Freedom	Mean Square	F Value	p-value Prob > F	
Model	1198.29	9	133.14	240.33	< 0.0001	significant
Residual	3.88	7	0.55			
Lack of Fit	2.73	3	0.91	3.17	0.15	not significant
Pure Error	1.15	4	0.29			
Cor Total	1202.16	16				

were formed by modification of blue laccases by mediators, so they can directly oxidize non-phenolic compound and hydroxy polyaromatic dye[10, 11] The white laccases are formed by the replace of T_1 Cu with other transition metals[29, 30]. The T1 site functions as the primary electron acceptor and is important to the redox potential of laccase[31]. The laccase from *T. hirsuta* lg-9 may belong to white laccase which may be formed by the replace of T_1 Cu with Mn[12]. This is probably the main reason of direct oxidation of methyl red.

Decolorization optimization

Results of factorial design

The experimental results of methyl red decolorization by a four-factor-two level factorial (2^{4-1}) experiment design are shown in Table 1. A first-order model was best fit to the data (Eqs. 4). We could predict the concentration of enzyme, incubation pH and time could remarkably influence the decolorization of methyl red. The value of k_1, k_2 and k_4 (+8.00, +9.20 and +8.22) indicated that increasing the concentration of enzyme, incubation pH and time could enhance the decolorization. A first-order model is limited to predict the product of laccase, but it is instructful to the steepest ascent path design.

$$Y = +45.94 + 8.00X_1 + 9.20X_2 + 1.99X_3 + 8.22X_4 + 2.88X_1X_2 + 3.27X_1X_4 \quad ...(4)$$

Results of steepest ascent path

We obtained the steepest ascent path design based on the first-order model. The value of *e* was 0.115 based on practical consideration. So the step length of steepest ascent path design was 0.4598 (X_1), 0.6 (X_2) and 28.34 (X_4) according to Eqs.2. We chose the step lengths as 0.45 U l^{-1}

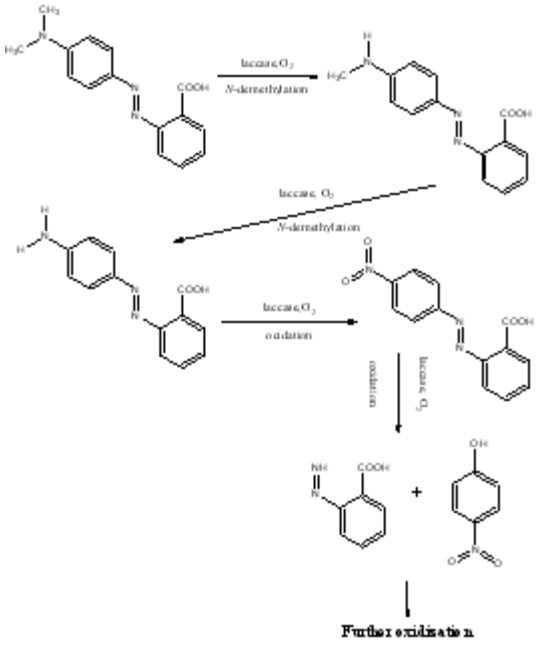

Fig. 3. The probably reaction scheme of methyl red directly oxidized by the atypical laccase from *T. hirsuta* lg-9

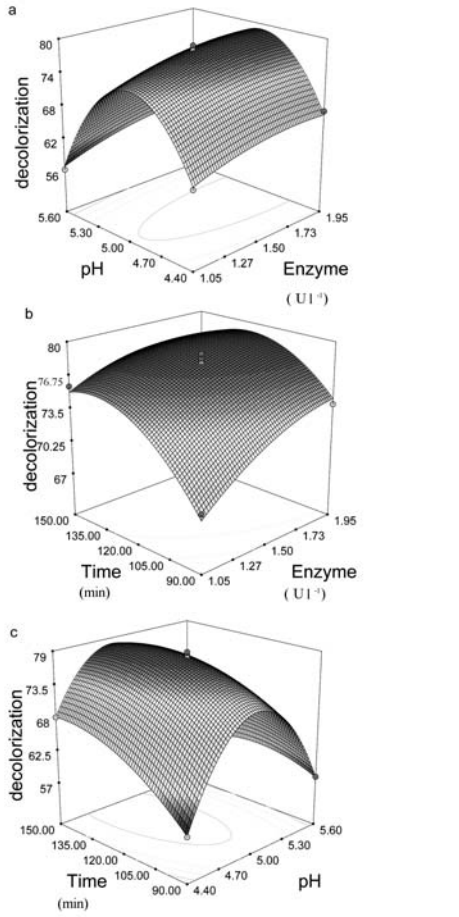

Fig. 4(a). The response surface plot showing the effect of different concentrations of enzyme and different pH on methyl red decolorization (Time = 120 Min). 4(b). The effect of enzyme and time on methyl red decolorization (pH = 5.0). 4(c). The effect of time and pH on methyl red decolorization (Enzyme concentration = 1.5 U l^{-1})

(X_1), 0.6 (X_2) and 30 min (X_4) to make the experiment more convenient (Table 2). The design and experimental results along the path of steepest ascent are shown in table 2. It is shown that the decolorization of methyl red is about 78.06%, at the point of No.1 (X_1: 1.5 U l^{-1}; X_2: 5.0; X_4: 120 min). The No. 1 point was chosen as the centre point of the central composite design to optimize the decolorization of methyl red.

Results of response surface analysis

The experimental data were first analyzed in order to determine second-order equations including terms of interaction between the variables with Design Expert. The equations (Eqs. 5) given below are based on the statistical analysis of the experimental data. The figure shows the predicted data of the response from the empirical model are in good agreement with the experimentally obtained data (Table 3).

$$Y = +78.09 + 2.22X_1 - 2.59X_2 + 2.54X_4 + 0.32X_1X_2 - 1.18X_1X_4 - 2.93X_2X_4 - 1.35X_1^2 - 14.84X_2^2 - 3.07X_4^2 \quad ...(5)$$

The Eqs.5 can be converted into the uncoded unit where

$$X_1 = \frac{C_{enzyme} - 1.5}{0.45}, \quad X_2 = \frac{pH - 5}{0.6}, X_4 = \frac{Time - 120}{30} \quad ...(6)$$

The results of analysis of variance (ANOVA) are summarized in Table 4. The Model F-value of 240.33 implies the model is significant. There is only a 0.01% chance that a "model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case X_1, X_2, X_4, X_1X_4, X_2X_4, X_1^2, X_2^2 and X_4^2 are significant model terms. The "lack of Fit F-value" of 0.15 implies the lack of fit is not significant relative to the pure error.

In the present model, R^2 was adjudged to be 99.68% [32]. The residual analysis was also carried out for judging model adequacy. The residuals should be approximately normal with mean zero and unit variance[33]. The externally studentized residuals were calculated (Table 3). There were no signs of any problems in our data, and the model was validated.

The 3-D response surface plot (Fig. 4) and the negative values of second order quadratic coefficients (Eqs.5) indicate the applicability of maximization methyl red decolorization. Maximum and minimum principle of differential calculus was used to maximize the equation (Eqs. 5) with respect to individual tested variables. The partial differential equations obtained are:

$$\partial Y / \partial X_1 = -2.22X_1 + 0.32X_2 - 1.18X_4 + 2.22 \quad ...(7)$$

$$\partial Y / \partial X_2 = 0.32X_1 - 29.68X_2 - 2.93X_4 - 2.59 \quad ...(8)$$

$$\partial Y / \partial X_4 = -1.18X_1 - 2.93X_2 - 6.14X_4 + 2.54 \quad ...(9)$$

When the decolorization of methyl red is maximization, $(\partial Y / \partial X_1)_{X2,\ X4}$, $(\partial Y / \partial X_2)_{X1,\ X4}$ and $(\partial Y / \partial X_4)_{X1,\ X2}$ are equal to zero. We can calculate the value of Y was maximum when the algebraic solutions were , $X_2 = -0.11$ and $X_4 = 0.34$. These values correspond to the uncoded value of $C_{Enzyme} = 1.8$ U l^{-1}, $pH = 4.93$ and $Time = 130.23$ min. The maximum predicated decolorization of methyl red was 79.41%. These optimum values were checked with experiments. The actual values of the methyl red decolorization were at an average of 80%.

$X_1 = 0.66$

CONCLUSION

In summary, this study demonstrates the atypical laccase from T. *hirsuta* lg-9 is capable of directly attacking non-phenolic azo dye methyl red, and it can cause the degradation of methyl red. Such laccase can be used in the oxidative degradation of azo dyes in the environment. The decolorization conditions have been optimized using factorial design, steepest ascent design and central composite design. The direct oxidation of non-phenolic compound is also significant to reveal the role of laccase in lignin degradation.

ACKNOWLEDGEMENTS

This study was supported by the National Science Foundation of China (Grant Nos. 30371136 and 30871985) and the Doctoral Scientific Research Foundation of Jining Medical University (Grant Nos. 094201).

REFERENCES

1. Camarero, S., Ibarra, D., Martínez , M. J., MartínezÁ. T. Lignin-Derived Compounds as

Efficient Laccase Mediators for Decolorization of Different Types of Recalcitrant Dyes. *Appl. Environ. Microbiol.*, 2005; **71**: 1775-1784.

2. Tauber, M.M., Guebitz, G.M., and Rehorek, A. Degradation of Azo Dyes by Laccase and Ultrasound Treatment. *Appl. Environ. Microbiol*., 2005; **71**: 2600-2607.
3. Singh, G., Ahujab, N., Batisha, M., Capalashc, N., Sharma, P. Biobleaching of wheat straw-rich soda pulp with alkalophilic laccase from *gamma-proteobacterium* JB: Optimization of process parameters using response surface methodology. *Bioresour. Technol.*, 2008; **99**: 7472-7479.
4. Baldrian, P. Fungal laccases - occurrence and properties. *FEMS Microbiol. Rev*., 2006; **30**: 215-42.
5. Riva, S. Laccases: blue enzymes for green chemistry. Trends Biotechnol., 2006; **24**: 219-226.
6. Couto, S. R. , Herrera, J. L. T. Industrial and biotechnological applications of laccases: A review. *Biotechnol. Adv.*, 2006; **24**: 500-513.
7. Weihua, Q., Hongzhang, C. An alkali-stable enzyme with laccase activity from entophytic fungus and the enzymatic modification of alkali lignin. *Bioresour. Technol*., 2008; **9**: 5480-5484.
8. Moldes, D., Vidal, T. Laccase-HBT bleaching of eucalyptus kraft pulp: Influence of the operating conditions. *Bioresour. Technol*., 2008; **99**: 8565-8570.
9. Yaver, D.S., Xu, F., Golightly, E.J., Brown, K.M., Brown, S.H., Rey, M.W., Schneider, P., Halkier, T., Mondorf, K., Dalboge, K. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete Trametes villosa. *Appl. Environ. Microbiol*., 1996; **62:** 834-841.
10. Leontievsky, A., Myasoedova, N., Pozdnyakova, N., Golovleva, L. 'Yellow' laccase of Panus tigrinus oxidizes non-phenolic substrates without electron-transfer mediators. *FEBS Lett.*, 1997; **413**: 446-448.
11. Pozdnyakova, N.N., Rodakiewicz-Nowak, J., Turkovskaya, O.V. Catalytic properties of yellow laccase from *Pleurotus ostreatus* D1. *J. Mol. Catal. B: Enzym.* 2004; **30**: 19-24.
12. Zhang, H.B., Zhang ,Y.L., Huang, F., Gao P.J., Chen, J.C. Purification and characterization of a thermostable laccase with unique oxidative characteristics from *Trametes hirsuta*. *Biotechnol. Lett.*, 2009; **31**: 837-843.
13. Wong, Y., Yu, J. Laccase-catalyzed decolorization of synthetic dyes. *Water Res.*, 1999; **33**: 3512-3520.
14. Chung, K.-T., Stevens, S.E., Cerniglia, C.E. *The Reduction of Azo Dyes by the Intestinal Microflora. Crit. Rev. Microbiol*., 1992; **18**: 175 - 190.
15. Wesenberg, D., Kyriakides, I., Agathos, S.N. White-rot fungi and their enzymes for the treatment of industrial dye effluents. *Biotechnol. Adv.*, 2003; **22**: 161 -187.
16. Körbahti, B.K., Tanyolaç, A. Electrochemical treatment of simulated textile wastewater with industrial components and Levafix Blue CA reactive dye: Optimization through response surface methodology. *J. Hazard. Mater.*, 2008; **151**: 422-431.
17. Shu, H.-Y., Chang, M.-C. Decolorization effects of six azo dyes by O3, UV/O3 and UV/H2O2 processes. Dyes and Pigments, 2005. **65**: 25-31.
18. Niladevi, K.N., Prema, P. Effect of inducers and process parameters on laccase production by Streptomyces psammoticus and its application in dye decolourization. *Bioresour. Technol*., 2008; **99**: 4583-4589.
19. Kim, S.-Y., An, J.-Y., Kim, B.-W. The effects of reductant and carbon source on the microbial decolorization of azo dyes in an anaerobic sludge process. *Dyes and Pigments*, 2008; **76**: 256-263.
20. Cristovao, R.O., Tavares, A.P.M., Ferreira, L.A., Loureiro, J.M., Boaventura, R.A.R., MacedoE, A. *Modeling the discoloration of a mixture of reactive textile dyes by commercial laccase. Bioresour. Technol*., 2009; **100**: 1094-1099.
21. Jiang, M., Ten , Z., Ding, S.J. Decolorization of Synthetic Dyes by Crude and Purified Laccases from *Coprinus comatus* Grown Under Different Cultures: The Role of Major Isoenzyme in Dyes Decolorization. *Appl. Biochem. Biotechnol.* , 2013; **169**: 660-672.
22. Xu, F., Shinb, W., Browna, S.H., Wahleithnera, J.A., Sundaramb, U.M., SolomonE.I. A study of a series of recombinant fungal laccases and bilirubin oxidase that exhibit significant differences in redox potential, substrate specificity, and stability. *Biochim. Biophys. Acta.*, 1996; **1292**: 303-311.
23. Jadhav, J.P., Parshetti, G.K., Kalme, S.D. , Govindwar, S.P. Decolourization of azo dye methyl red by *Saccharomyces cerevisiae* MTCC 463. *Chemosphere*, 2007; **68**: 394-400.
24. Couto, S.R. Decolouration of industrial azo dyes by crude laccase from Trametes hirsuta. *J. Hazard. Mater.*, 2007; **148**: 768-770.
25. Chivukula, M., Renganathan, V. *Phenolic Azo Dye Oxidation by Laccase from Pyricularia oryzae. Appl. Environ. Microbiol.*, 1995; **61**:

4374-4377.

26. *Chivukula, M., Spadaro, J. , Renganathan, V.* Lignin peroxidase-catalyzed oxidation of sulfonated azo dyes generates novel sulfophenyl hydroperoxides. *Biochemistry*, 1995; **34**: 7765-7772.
27. Murugesan, K., Yang, I.H., Kim, Y.M., Jeon, J.R., Chang Y.S. Enhanced transformation of malachite green by laccase of Ganoderma lucidum in the presence of natural phenolic compounds. *Appl. Microbiol. Biotechnol.*, 2009; **82**: 341-350.
28. Xu, F. Oxidation of Phenols, Anilines, and Benzenethiols by Fungal Laccases: Correlation between Activity and Redox Potentials as Well as Halide Inhibition. *Biochemistry*, 1996; **35**: 7608-7614.
29. Palmieri, G., Giardina, P., Bianco, C., Scaloni, A. Capasso, A., Sannia, G A novel white laccase from Pleurotus ostreatus. *J. Biol. Chem*., 1997; **272**: 31301-31307.
30. Min, K.-L., Kimb, Y.H.,Kimb, Y.W.,Jungb, H.S., Hahb, Y.C. Characterization of a Novel Laccase Produced by the Wood-Rotting Fungus Phellinus ribis. *Arch. Biochem. Biophy*., 2001. **392**: 279-286.
31. Ducros, V., Brzozowski, A.M., Wilson, K.S., Brown,S.H., Østergaard, P., Schneider, P., Yaver, D.S., Pedersen, A.H., and DaviesG.J. Crystal structure of the type-2 Cu depleted laccase from Coprinus cinereus at 2.2 A resolution. *Nat. Struct. Biol*., 1998; **5**: 310-316.
32. Bhattacharya, S.S., Banerjee, R. Laccase mediated biodegradation of 2,4-dichlorophenol using response surface methodology. *Chemosphere*, 2008; **73**: 81-85.
33. Ceylan, H., Kubilaya, S., Aktasa, N., Sahiner, N. An approach for prediction of optimum reaction conditions for laccase-catalyzed bio-transformation of 1-naphthol by response surface methodology (RSM). *Bioresour. Technol.*, 2008; **99**: 2025-2031.