Optimization of Fermentation Conditions for Laccase Production by Recombinant *Pichia pastoris* GS115-LCCA Using Response Surface Methodology and Its Application to Dye Decolorization

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In this work, recombinant *Pichia pastoris* GS115-lccA was cultured and expressed with the highest laccase activity of 2357 U/L with ABTS (2, 2'-nazinobis-(3-ethylbenzthiazoline-6-sulphonate)) as reacting substrate. To achieve the higher laccase activity, multiple key factors were screened (using a Plackett-Burman design of experiments methodology) related to the fermentation conditions for producing GS115-lccA. Subsequently, a Box-Behnken response surface methodology was employed for further optimization. The optimum fermentation conditions of the fermentation were obtained as follows: 0.603% methanol added into the culture every 24 h, medium optimal initial pH 7.1, and liquid medium volume of 20.4% provided the highest enzyme activity of 5235 U/L. The decolorization experiments of dyes (Reactive Blue KN-R and Acid Red 35) were carried with the laccase cultured by recombinant *P. pastoris* GS115-lccA. This purified enzyme showed excellent decolorization capacity. After 24 h, the decolorization of Reactive Blue KN-R with 100 mg/L at 50 °C, pH 4.5 using 20 mM acetate buffer with 2 U/mL purified enzyme was 91.33%, and for Acid Red 35, the decolorization was 78.96%. All results suggested that this laccase may be suitable for the wastewater treatment of similar azo and anthraquinone dyes from the deinking and dyeing industries.

Keywords: Laccase; Response surface methodology; Dye decolorization; Reactive Blue KN-R; Acid Red 35

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INTRODUCTION

Laccases, which belong to the group of enzymes called the multi-copper oxidases, can catalyze the four-electron reduction of O_2 to H_2O with the concomitant oxidation of phenolic compounds (Solomon *et al.* 1996; Cusano *et al.* 2009). During the past decades, vast amount of laccases have been isolated and characterized from various sources such as fungal species, certain types of bacteria, and even some insects (Heinzkill *et al.* 1998; Diamantidis *et al.* 2000; Dittmer and Kanost 2010). Laccases derived from fungi are found to be efficient for degrading toxic compounds and for decoloring synthetic dyes, as well as for other biotechnological applications. Among these fungal species, *Trametes versicolor* has received considerable attention for its bioremediation potential (Madzak *et al.* 2006). The successful applications of laccase require large amounts production at low cost and short time. However, the production of laccase from original strains obtained by the optimization of culture medium and cultivation conditions does not meet modern-day

demands of industry. One of the possible solutions is to produce the recombinant protein in a heterologous host. Consequently, various studies have been carried out to investigate the characteristics and cultivation conditions of the recombinant laccase production from *Trametes versicolor* from the yeast *Pichia pastoris* system for the expression of heterologous proteins, which are seen as the most cost-effective and green strategy of laccase production (He *et al.* 2011). For any heterologous protein, expression conditions need to be optimized in order to achieve high levels of protein production.

The biological process optimization, which aims to improve the activity of final product, is an indispensable phase of work for the biotechnological industry. Optimization techniques for these biological processes include two major categories, *i.e.* non-statistical optimization techniques (Sigal and Whyne 2010) and statistical optimization techniques (Box and Wilson 1951; Tanyildizi *et al.* 2005; Jo *et al.* 2008). In recent years, the statistical methods have become a powerful and effective approach for screening key factors rapidly from a multivariable system to optimize fermentation conditions. Several common optimization techniques were compared with each other, and the response surface method was introduced and applied (Box and Wilson 1951; Vasconcelos *et al.* 2000; Muralidhar *et al.* 2001; Liu *et al.* 2003). Optimization of media components for the production of laccase by response surface methodology has been reported in the case of different fungal strains (Trupkin *et al.* 2003; Nyanhongo *et al.* 2002). Therefore, in this present study, fermentation conditions for the recombinant yeast GS115-lccA were optimized by applying different statistical experimental designs and data analysis methods. Fermentation conditions were considered as independent variables, and laccase enzyme activity was the primary response value, to achieve the highest activity and provide the effective enzyme for the industrial application.

It is known that azo, anthraquinone, and indigo dyes are the major chromophores found in commercial dyes (Thurston 1994; Abadulla *et al.* 2000). Over 10,000 different dyes are in common use with an annual production of over 0.7 million tons of dyestuff being manufactured and 10 to 20% of dyes lost in the industrial effluents. Reactive dyes, including Reactive Blue KN-R and Reactive Red 7, form covalent bonds with cellulose during a typical dyeing process; such dyes are commonly used in cotton dyeing industries. It is evident that textile dyes in discharge effluents can have negative impacts on the entire ecosystem. Decolorization of these dyes by physical and chemical methods (coagulation-flocculation, adsorption, oxidation, ion-exchange, or electron chemistry) is prohibitively expensive, time-consuming, mostly inefficient and even unsafe (Sheng and Chi 1996; Mechichi *et al*. 2006). By contrast, as the highly free-radical-mediated and non-specific processes, the biodecolorization processes used the extracellular oxidative enzyme laccase which come from the *Trametes versicolor* are environmental-friendly and cost-competitive. Remazol Brilliant Blue R, as a starting material in the production of polymeric dyes, was studied on decolorization using purified laccase (Schliephake and Lonergan 1996). The dye was added on day three of the fermentation, and more than 48 h were required to get about 75% decolorization in the fungal culture. Previous researchers have reported that the laccase derived from *P. ostreatus* could decolorize 70% of malachite green for 24 h of incubation (Yan *et al.* 2009). Bromophenol blue could be decolorized by the laccase from *Trametes* sp.*420*. But the decolorization efficiency was relatively low. The redox mediator, such as ABTS, could increase the decolorization efficiency to 45% (Tong *et al.* 2007). Thus, laccase as a kind of valuable enzyme has potential to degrade a wider range of compounds.

The aim of this present investigation was to study the optimal fermentation conditions for laccase production in a shake flask using the response surface method and to purify the enzyme by ultrafiltration and ammonium sulfate precipitation. This research will lay the foundation for further applications of *Trametes versicolor* for the biodegradation of industrial dyes.

MATERIALS AND METHODS

Fungus Strains and Growth Media

The recombinant strain *Pichia pastoris* GS115-lccA was preserved by Microbial Technology Research Laboratory, Nanjing Forestry University (NJFU).

YPD (yeast extract-peptone-dextrose) agar plates (+Zeocin): yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L, and agar 20 g/L adding Zeocin (100 mg/L).

BMGY medium (buffered glycerol-complex medium) was prepared according to the manual of the EasySelect Pichia Expression Kit (Invitrogen, USA): 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34 % YNB, 4×10-5% biotin, and 2% glycerol.

Expression of the recombinant Laccase

The recombinant strain *P. pastoris* GS115 was incubated in 30 mL of BMGY medium for 24 h at 28 ^{o}C with constant shaking at 180 rpm. When OD_{600} reached values between 3.0 and 8.0, the cultures were harvested, resuspended in 30 mL of BMGY containing 0.3 mM Cu^{2+} with the initial pH 6.0, and incubated in the same conditions until an OD_{600} of 1.0 was reached. 100% methanol was added daily (final concentration 0.5% (v/v)) to maintain induction. The samples were centrifuged at 8,000 × g for 5 min. The supernatant was stored at 4 ^{o}C until used to determine enzyme activity.

Assay of Laccase Activity and Protein

The laccase activity assay system contains 1 mL of 1 mM ABTS (ε_{420} = 36,000 $M^{-1}cm^{-1}$) and 1980 μL 50 mM sodium citrate buffer (pH 4.5). The change in the absorbance at 420 nm of the mixture (at 50 °C) for 5 min was recorded every minute using a spectrophotometer. One enzyme activity unit (1 U) is defined as the amount of enzyme that oxidizes 1 μmol of substrate per minute (Childs and Bardsley 1975).

Protein concentrations were determined using the Bradford method with bovine serum albumin as a standard (Bradford 1976).

Plackett-Burman Design of Experiments (Plackett and Burman 1946)

The Plackett-Burman design of experiments, an efficient technique for medium-component optimization (Li *et al.* 2008), was used to select the key factors that significantly influenced the laccase activity. Laccase activity was the measured response variable; nine independent variables were examined to determine the key fermentation components significantly affecting the activity of laccase. Based on the Plackett-Burman design, each factor was performed at two levels: -1 for the low level and +1 for high level. Table 1 lists the levels of the independent variables and Table 2 illustrates the combinations used for each run in the statistical design of experiment.

In the present study, nine assigned variables were screened in 12 experimental runs in addition with three runs at their center points. Laccase production was carried out

in triplicate, and the factors significant at 95% level (P<0.05) were considered to have significant effect on laccase activity, and thus, used for the further optimization by response surface methodology. The analysis of variance for the data and the model coefficients were computed with the software Design-Expert® 7.1.6 (Stat-Ease, Inc., Minneapolis, MN, USA).

Table 1. Levels of the Variables in Plackett-Burman Design

Variables		Parameter	Level	
			-1	+1
Physical parameter	X_1	Rotate speed (r/min)	180	210
Chemical parameter	X_2	Methanol concentration (%)	0.6	1.0
Chemical parameter	X_3	Biotin concentration (%)	0.02	0.04
Chemical parameter	X_4	Initial OD_{600}	0.6	1.0
Chemical parameter	X_5	Cu^{2+} concentration (mM)	0.2	0.5
Blank	X_6	Contrast	-1	+1
Chemical parameter	X_7	Tween-80 concentration (%)	2	4
Chemical parameter	X_8	Liquid medium volume (%)	20	28
Physical parameter	X_9	Initial pH	6	7
Blank	X_{10}	Contrast	-1	+1
Chemical parameter	X_{11}	Peptone concentration (%)	2	4

Table 2. Plackett-Burman Design for the Laccase Production

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1	+1
2	-1	-1	+1	+1	-1	+1	+1	+1	+1	-1	-1
3	+1	+1	-1	+1	+1	-1	+1	+1	-1	-1	-1
4	-1	-1	-1	-1	+1	-1	+1	+1	+1	+1	+1
5	-1	+1	-1	+1	+1	+1	-1	-1	+1	+1	-1
6	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1
7	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1
8	+1	-1	+1	-1	+1	+1	-1	+1	-1	+1	-1
9	+1	+1	+1	-1	-1	-1	+1	-1	+1	+1	-1
10	+1	-1	+1	+1	-1	+1	+1	-1	-1	+1	+1
11	-1	+1	-1	-1	+1	+1	+1	-1	-1	-1	+1
12	+1	-1	+1	+1	+1	-1	-1	-1	+1	-1	+1

Box-Behnken Response Surface Methodology (Box and Behnken 1960)

A response surface methodology was used to optimize the three key factors for enhanced laccase production based on Box-Behnken design. For statistical calculations, the relation between the coded values and real values are described as follows. The levels of the independent variables for each experimental run are shown in Table 3. In this paper, a three-factor and three-level second-order regression was performed; the Box-Behnken statistical design used 17 trial runs and the value of the dependent response variable was the average of three measurements. The polynomial coefficients were calculated and analyzed using the software Design-Expert® 7.1.6. Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA).

Purification of Recombinant Laccase

The supernatant (200 mL) was harvested from a culture of the recombinant *P. pastoris* GS115-lccA (16 days old). The supernatant was obtained from the cultured sample by centrifugation at a speed 6,000 g for 10 min; afterwards, the centrifuged

sample was purified by the ultrafiltration using a CXA-50 50000 ultrafiltration membrane (Shanghai Yadong Nuclear Grade Resins Limited Company, China). The resulting filtrate was precipitated by adding $(NH_4)_2SO_4$ to the final mass concentration of 80%. The protein was dissolved in 20 mM sodium citrate buffer (pH 4.5) and dialyzed 12 h against the same buffer.

Table 3. Box-Behnken Design with Three Independent Variables

Run	X_1 Methanol concentration (%)	X_2 Liquid medium volume (%)	X_3 Initial pH
1	0.6 (0)	28 (+1)	6 (-1)
2	0.6 (0)	20 (0)	7 (0)
3	0.8 (+1)	12 (-1)	7 (0)
4	0.8 (+1)	20 (0)	6 (-1)
5	0.6 (0)	20 (0)	7 (0)
6	0.4 (-1)	20 (0)	6 (-1)
7	0.4 (-1)	28 (+1)	7 (0)
8	0.4 (-1)	20 (0)	8 (+1)
9	0.6 (0)	12 (-1)	8 (+1)
10	0.6 (0)	20 (0)	7 (0)
11	0.6 (0)	20 (0)	7 (0)
12	0.8 (+1)	28 (+1)	7 (0)
13	0.8 (+1)	20 (0)	8 (+1)
14	0.6 (0)	28 (+1)	8 (+1)
15	0.6 (0)	20 (0)	7 (0)
16	0.4 (-1)	12 (-1)	7 (0)
17	0.6 (0)	12 (-1)	6 (-1)

Decolorization of Dye by Recombinant Laccases

The purified laccase which was produced by recombinant GS115-lccA was used to decolorize two dyes: Reactive Blue KN-R and Acid Red 35 (Zhejiang Runtu Chemical Group Limited Company, China). The structures are shown in Fig. 1. The involvement of *Trametes versicolor* laccases in dye decolorization was investigated with the purified enzymes. Decolorization was determined by measuring the decrease of the dye's absorbance as its maximum visible wavelength and results are expressed in terms of a percentage. Laccase-catalyzed decolorization of synthetic dyes was carried out in 10 mL samples containing buffer (20 mM), a certain amount of purified enzyme and 100 mg/L dyes (Reactive Blue KN-R or Acid Red 35) were added. And the reaction system was reacted statically under the corresponding temperature for more than 24 h. During incubation, the time of decolorization was detected every 6 h by measuring the absorbance. Control samples without the enzyme were processed in parallel with the test samples. Then the decolorization of the two dyes was performed triple as described above.

In order to select the suitable decolorization conditions, the effects of main factors were investigated. The main factors and the variation ranges were as follows: buffers (citric acid-sodium citrate buffer, succinic acid - NaOH buffer, acetic acid-sodium acetate buffer, tartaric acid-sodium tartrate buffer), initial pH values (3.0 to 6.0), temperatures (30 to 70 ^oC), and enzyme dosage (1 to 5 U/L).

The absorption wavelengths of the two dyes were 610 nm for Reactive Blue KN-R and 505 nm for Acid Red 35. The decolorization efficiency of each dye was shown as dye decolorization (%), as given by Eq. 1,

The decolorization of dyes was calculated by means of the equation:

$$\text{Decolorization (\%)} = [(C_i - C_t)/C_i] \times 100 \quad (1)$$

where C_i is the initial concentration of the dye, and C_t is the dye concentration at time t (Lorenzo *et al.* 2006).

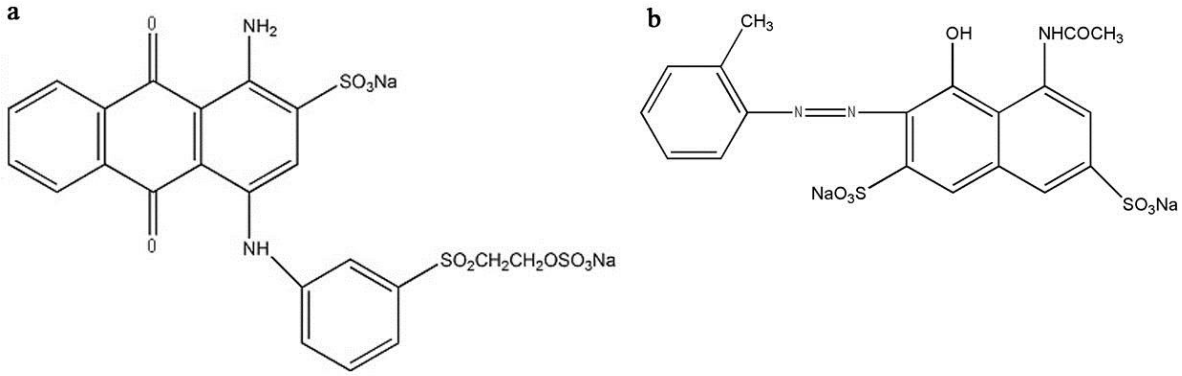

Fig. 1. The structures of the two dyes: (a) Reactive Blue KN-R (b) Acid Red 35

The concentration of the dye was calculated according to the value of absorbance. Figure 2 indicated the linear relationship between concentration of the dye and the value of absorbance.

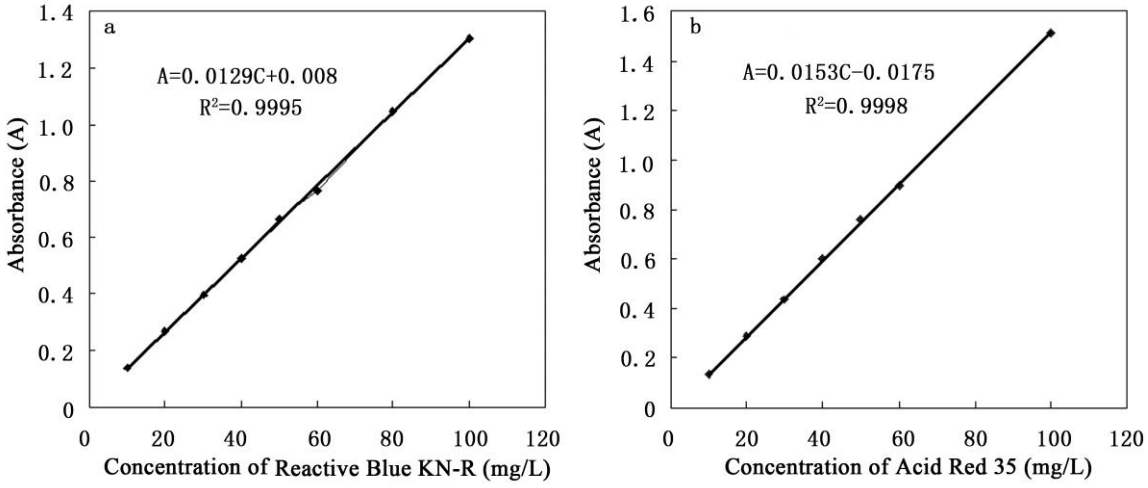

Fig. 2. The standard curve of the two dyes: (a) Reactive Blue KN-R (b) Acid Red 35

RESULTS AND DISCUSSION

Evaluation of Important Parameters Affecting Laccase Activity

Although extensive studies (Bourbonnais *et al.* 1995; Hess *et al.* 2002) have been conducted with laccases from *Trametes versicolor* for potential industrial applications, *Pichia pastoris* GS115 is still the main host and preference for laccase production. The

yield of the heterologous enzymes produced by the new laccase gene *lccA* of *Pichia pastoris* GS115 was lower than expected; the cultural conditions may have played an important role in the production yields of laccase. To improve the expression system, multiple cultural condition factors (rotate speed, methanol concentration, biotin concentration, initial OD_{600} (microbial concentration), Cu^{2+} concentration, Tween-80 concentration, liquid medium volume, initial pH and peptone concentration) were screened using a Plackett-Burman design of experiments. The identified key factors from these experiments were then optimized using a Box-Behnken response surface methodology.

The predicted regression equation from the Plackett-Burman design of experiments was as follows:

$$R_1 = 2880.83+72.50X_1-1055.83X_2+150.83X_3+235.83X_4-25.83X_5+ 152.50X_7-850.83X_8+747.50X_9+204.17X_{11} \quad (2)$$

where R_1 is the predicted laccase enzyme activity (U/L) and X_1-X_{11} are the coded independent variables as shown in Table 1.

Table 4. Evaluation of Significance of Various Factors

Parameter	Prob>F	Significance
X_1	0.0801	
X_2	0.0055	significant
X_3	0.0386	
X_4	0.0247	
X_5	0.2171	
X_7	0.0382	
X_8	0.0069	significant
X_9	0.0078	significant
X_{11}	0.0286	

As can be seen in Table 4, the main effect of each variable upon laccase activity was estimated as the difference between both averages of measurement made at the high level (+1) and the low level (-1) of that factor. The activity of the laccase was strongly affected by the three key factors, which were methanol concentration, initial pH, and liquid medium volume; the other six factors had a negligible effect on laccase production. The coefficient of determination (R^2) of the model was calculated to be 0.999, which indicated that 99.9% of the variability in the response could be explained by the model. The variables with confidence levels greater than 95% (P<0.05) were considered as significantly influencing laccase activity and were selected for the further optimization studies.

Optimization of Fermentation Conditions using Response Surface Methodology

Response surface methodology has been successfully applied in many recent biotechnological studies, such as improving the activity of laccases (Niladevi *et al.* 2009). In this paper, the optimal level of three key factors (methanol concentration, initial pH, and liquid medium volume) and their interactions on the laccase activity were further

explored by the Box-Behnken design of experiments; the other six factors (rotation speed, biotin concentration, initial OD_{600}, Cu^{2+} concentration, Tween-80 concentration, and peptone concentration) were held constant at their zero value (Table 1). By analyzing the experimental data, the following second-order polynomial equation was established to explain the laccase activity:

$$R_1=2978.80-42.62X_1+66.00X_2+221.38X_3-300.50X_1X_2-135.75X_1X_3 -119.50X_2X_3-409.78X_1^2-717.03X_2^2-413.27X_3^2 \quad (3)$$

where R_1 is the predicted laccase enzyme activity (U/L), and X_1, X_2, and X_3 are the coded values of methanol concentration, liquid medium volume, and initial pH, respectively.

Table 5. ANOVA for Response Surface Quadratic Model for Laccase Enzyme Activity

Source	F	Prob > F
Model	4.211651	0.0356
X_1	0.112373	0.7473
X_2	0.269414	0.6197
X_3	3.031024	0.1252
X_1X_2	2.792487	0.1386
X_1X_3	0.569878	0.4749
X_2X_3	0.441609	0.5276
X_1X_1	5.465998	0.0520
X_2X_2	16.73583	0.0046
X_3X_3	5.55977	0.0505

The analysis of the variance (ANOVA) was conducted to test the significance of the fit of the second-order polynomial equation to the experimental data as shown in the Table 5. The model's F-value of 4.21 indicated the model is significant, and the p-value for "lack of fit" was 0.13, which indicated that the "lack of fit" was not significant at the 95% level of significance. The p-values were used as a tool to check significance of each independent variable (Montgomery 1996). The smaller the p-value becomes, the higher is the significance of the corresponding variable. The p-values in this study were less than 0.1000, which indicated the model's terms were significant. In this experiment, the quadratic terms X_1^2, X_2^2, and X_3^2 were significant in the model. The p-values that were greater than 0.1000 indicated model terms that are not significant. Here, the R^2 value was 0.998, which could explain 99.8% of the variability of the response. The results also indicated that the model could accurately predict the highest activity of laccase based on the condition values used during fermentation.

From Equation 3, the optimal coded values of X_1, X_2, and X_3 were estimated to be 0.015, 0.004, and 0.1, respectively. The uncoded values were: methanol concentration 0.603%, liquid medium volume 20.4%, and initial pH 7.1. The model predicted that the maximum laccase activity to be 5360 U/L. To confirm these results, a validation experiment was performed under these optimized conditions in triplicate. The observed experimental values of laccase activity were 4916, 5458, and 5332 U/L with the average of 5235 U/L. The excellent correlation between predicted and measured values verifies the model's predictability of production process.

Three-dimensional response surface plot described by the regression model (Eq. 3) can be used to further explain the results of the statistical analysis. The response surface plots shown in Fig. 3 indicated that there is a maximum value for the laccase enzyme activity, which depended on the levels of the factors in the medium. In Fig. 3a the initial pH was invariable, whereas the methanol concentration and liquid medium volume were changed as shown in Table 3. Similarly, as shown in Fig. 3b and 3c, the constant value was liquid medium volume and methanol concentration, respectively. Consequently, Fig. 3 showed the maximum laccase enzyme activity as 5360 U/L with the three key factors under the optimal conditions and other variables at their zero levels.

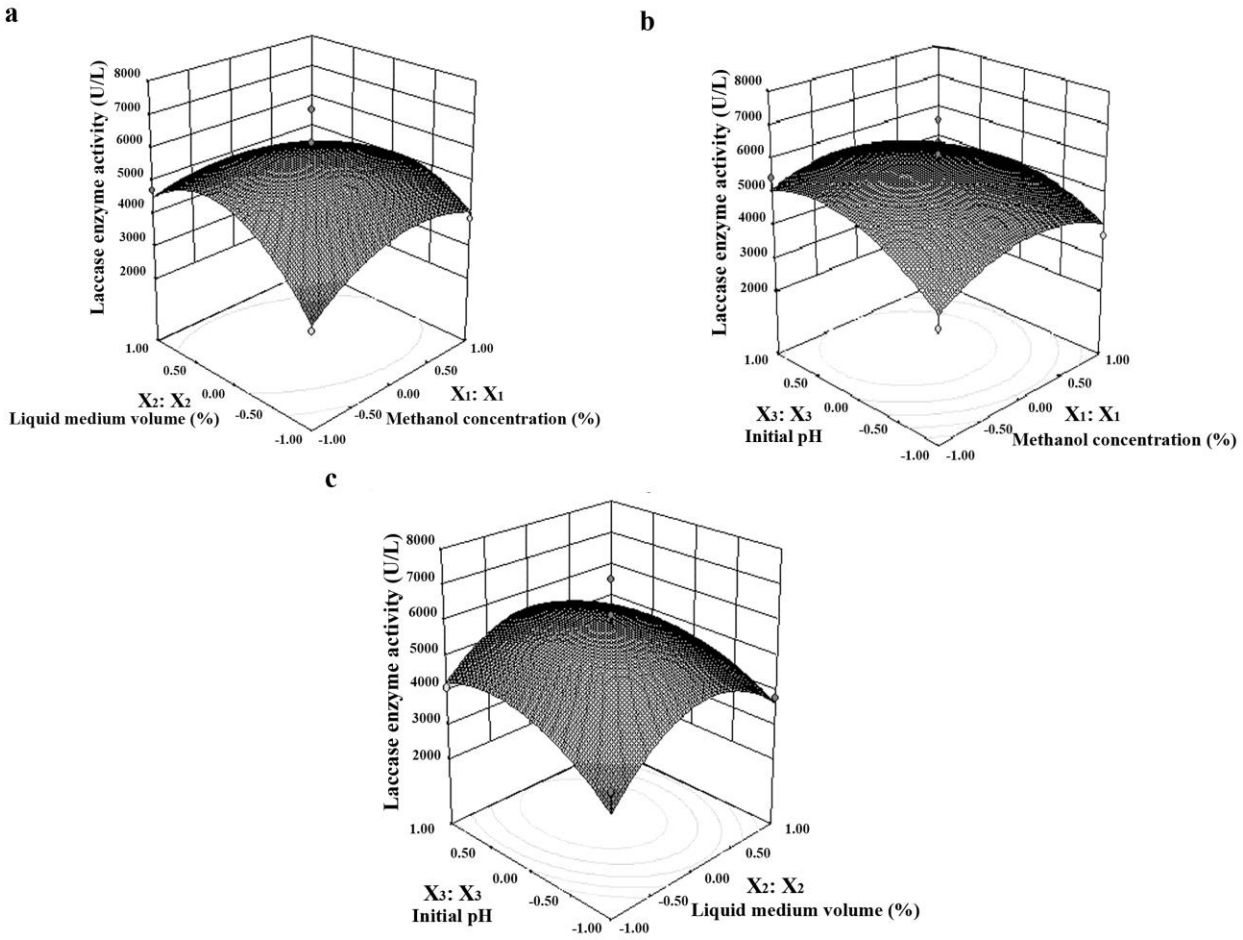

Fig. 3. Response surface plot for the laccase enzyme activity in terms of the effects: (a) X_1 (Methanol concentration) and X_2 (Liquid medium volume), (b) X_1 (Methanol concentration) and X_3 (Initial pH), and (c) X_2 (Liquid medium volume) and X_3 (Initial pH)

Purification and Characterization of Recombinant Laccase

The supernatant from fermentation was harvested from a culture of recombinant *P. pastoris* GS115-lccA (16 days old) by centrifugation; the resulting supernatant was purified by ultrafiltration and ammonium sulfate precipitation. The purification steps and relevant details, such as laccase activity, specific activity, and fold purification, are shown in Table 6. The results showed a 2.7-fold purification, which corresponded to a final enzyme yield of 31.8%.

The homogeneity of the purified laccases was checked on SDS-PAGE (Fig. 4). The enzyme showed a single band of approximately 58 kDa. So the production cost, the main bottleneck for application of laccase, will be reduced dramatically because of the simple purification steps.

Table 6. Purification of Recombinant *P. pastoris* GS115-lccA

Purification step	Total volume (mL)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Culture supernatant	500	2617	239.7	10.9	100	1
Ultrafiltration (30 kDa)	40	1568	88.3	17.8	59.9	1.6
Ammonium sulfate precipitation	5	833	28.0	29.8	31.8	2.7

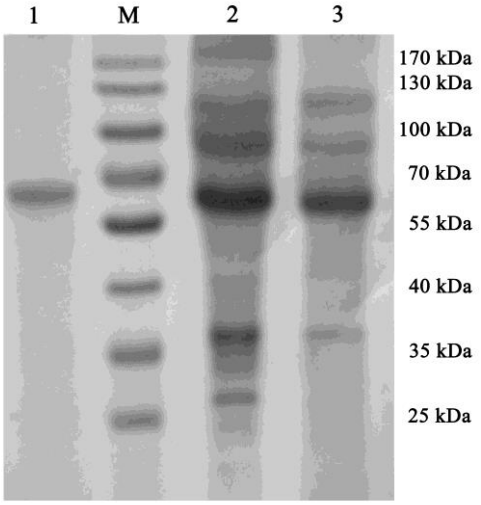

Fig. 4. SDS-PAGE of the recombinant laccase GS115-LccA: Lane1: purified laccase by adding $(NH_4)_2SO_4$ to the final mass concentration of 80% for only 12 h; Lane M: Molecular weight markers (10-170 kDa); Line 2: purified laccase by the ultrafiltration using a CXA-50 50000 ultrafiltration membrane; Line 3: Crude laccase

Decolorization of Dyes by Recombinant GS115-lccA

Laccase is known to have an ability to decolorize dyes of various classes (Knapp and Newby 1999). Different laccase isozymes from various sources are known to be diverse with respect to their efficiency to decolorize certain dyestuffs. For the practical application, the use of purified laccase is more economical and cost-effective. In this paper, the capability of the purified recombinant laccases, LccA, from the recombinant *P. pastoris* was assessed by using two dyes (Reactive Blue KN-R and Acid Red 35). To improve the decolorization of the Acid Red 35 and the Reactive Blue KN-R, conditions of the reaction were optimized by the single factor experiments.

Effect of Different Buffers on Decolorization

The effect of different pH buffer media (Citric acid-sodium citrate buffer, Succinic acid-NaOH buffer, acetic acid-sodium acetate buffer, tartaric acid-sodium

tartrate buffer) on decolorization of dyes (100 mg/L) was studied. As shown in Fig. 5a, after 12 h, it was observed that different buffer media could influence the decolorization of dyes, which is useful to accelerate the decolorization of dyes. Among these buffers, the succinic acid-NaOH buffer was the best for Reactive Blue KN-R, and the citric acid-sodium citrate buffer was the best for the Acid Red 35. In the following experiment, the succinic acid-NaOH buffer and citric acid-sodium citrate buffer were chosen in decolorizing the two dyes respectively.

Effect of pH on Decolorization

Different pH values of 3.0, 4.0, 4.5, 5.0, and 6.0 where obtained by adjusting succinic acid-NaOH buffer solution and tartaric acid-sodium tartrate buffer for Reactive Blue KN-R and Acid Red 35, respectively. As shown in Fig. 5b, increasing the pH caused the decolorization of Reactive Blue KN-R to decrease. Meanwhile, the decolorization at the pH value of 3.0 and 4.0 were both good. The pH stability property of purified recombinant laccase is favorable for the potential applications of the enzyme. Consequently, additional experiments (pH stability of this recombinant enzyme) indicated that a pH value of 4.0 was the best for the decolorization of Reactive Blue KN-R. However, the decolorization of Acid Red 35 increased as the pH increased to 4.5; pH values greater than 4.5 resulted in lower decolorization of Acid Red 35. Therefore, the pH value of 4.5 was determined to be the best for the decolorization of Acid Red 35 by the laccase. Consequently, the pH values for the following experiments of decolorizing Reactive Blue KN-R and Acid Red 35 were 4.0 and 4.5, respectively.

Effect of Temperature on Decolorization

In previous studies, the optimal temperature of the recombinant laccase was 50 ^{o}C. Meanwhile, thermostability assays indicated that its residual activity was more that 50% after being incubated at 60 ^{o}C for 1 h. In practical applications, the high thermostability of the enzyme is desired because the longer active life means less consumption of the enzyme. The laccase was stable after incubation at 30, 40, and 50 ^{o}C for 2 h but residual activity dropped fast at 70 ^{o}C. Temperature stability properties of purified recombinant laccase were found to be favorable for the potential applications of the enzyme. The decolorization of the two dyes (Reactive Blue KN-R and Reactive Blue KN-R) were conducted at different temperatures (30, 40, 50, 60, and 70 °C), after 12 h, as shown in Fig. 5c. The optimum temperatures for decolorizing the Reactive Blue KN-R and the Acid Red 35 were 50 °C.

Increasing the temperature above 50 °C caused the decolorization of the two dyes to decrease, which was consistent with the temperature stability of laccase.

Effect of Enzyme Dosage on Decolorization

The higher the laccase activity, the shorter is the reaction time required for dye decolorization. According to the above conditions, the decolorization of the two dyes (100 mg/L) was accomplished with different enzyme dosages (1, 2, 3, 4, and 5 U/mL), as shown in Fig. 5d. As the enzyme dosage increased, the decolorization rate increased and the curve tended to level out after 24 h. The enzyme dosage in decolorizing Reactive Blue KN-R (anthraquinonic dye) and Acid Red 35 (azo dye) were 2 U/mL and 4 U/mL, respectively; in addition, GS115-lccA was better at decolorizing Reactive Blue KN-R than the Acid Red 35. The good decolorization rates further enabled laccase to decolorize

the real textile industry wastewater containing synthetic dyes of different chemical structures such as anthraquinone, azo, *etc*.

In summary, the final maximum decolorization efficiency for 24 h of incubation was determined to be 91.33% for Reactive Blue KN-R and 78.96% for Acid Red 35, under their respective optimum conditions with 2 U/mL and 4 U/mL of enzyme dosage, respectively (Fig. 6). The different chemical structures of dyes might explain these differences in the decolorization efficiencies (Moldes *et al.* 2003). The described results demonstrated that the capability of decolorizing different dyes was positively related to the laccase activity. The ability of decolorizing these two dyes was dependent on laccase.

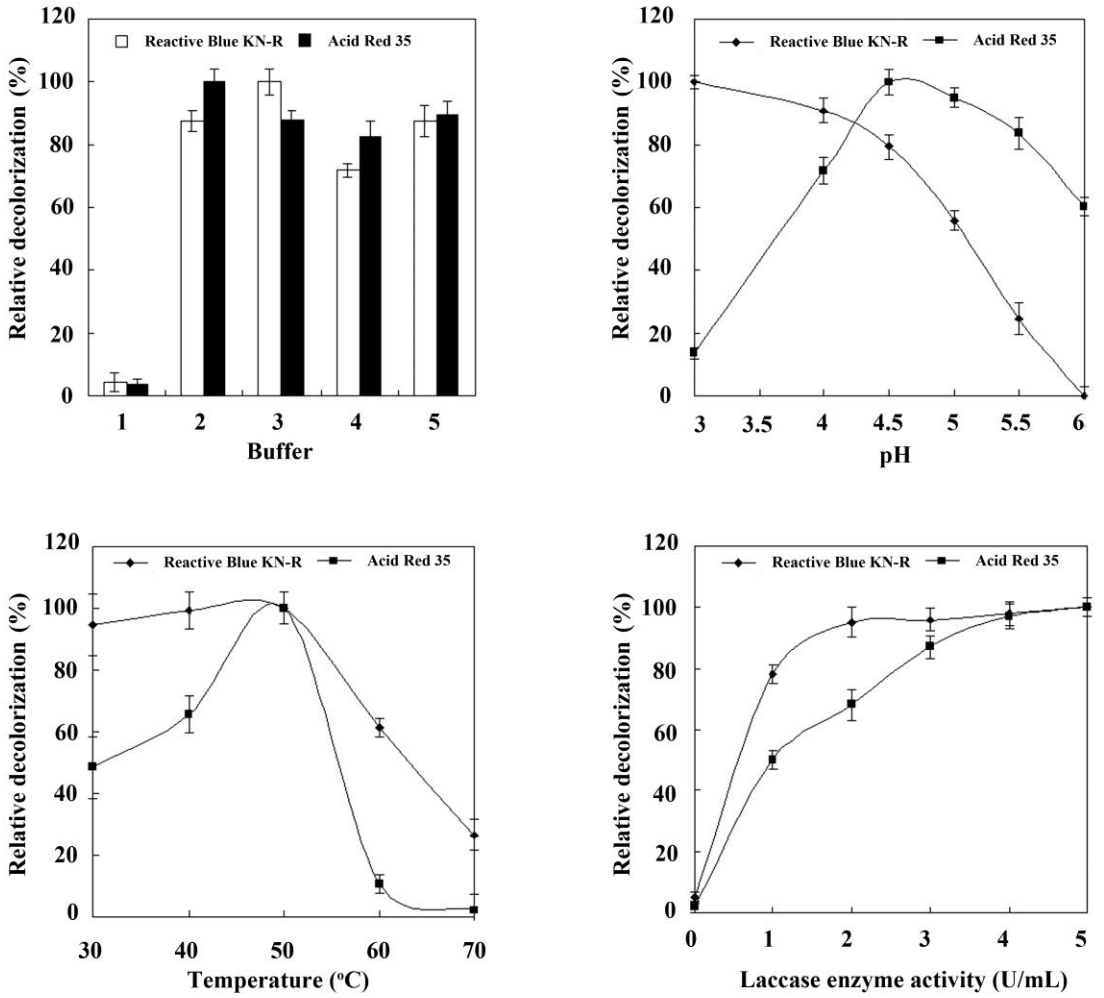

Fig. 5. Effect of conditions of the decolorization of the two dyes (the time of decolorization was 24 h): (a) Buffer of the decolorization reaction (1, Blank control 2, Citric acid-sodium citrate buffer 3, Succinic acid - NaOH buffer 4, acetic acid-sodium acetate buffer 5, tartaric acid-sodium tartrate buffer) (b) pH of the decolorization reaction (c) Temperature of the decolorization reaction, and (d) Laccase enzyme activity of the decolorization reaction

The superior points for the recombinant strain *P. pastoris* GS115-lccA derived from *Trametes versicolor* in comparison with some other laccases reported previously are summarized below: firstly, the capability of recombinant GS115-lccA for decolorizing Reactive Blue KN-R and Acid Red 35 was relatively good; Secondly, a high decolorization efficiency could be obtained by the recombinant laccase from *Trametes*

versicolor without adding any mediator. That is, a redox mediator was not required for efficient decolorization of dyes by the recombinant laccase. This is more economical and cost-effective for its practical application. Thus, lccA derived from *Trametes versicolor* exhibits great potential and promising application for decolorizing and detoxifying industrial dyes in waste effluents. This work further provided strong evidence for the efficient function of laccase for decolorizing industrial dyes. Therefore, a further study will focus on assessing the phytotoxicity of the dyes before and after decolorization.

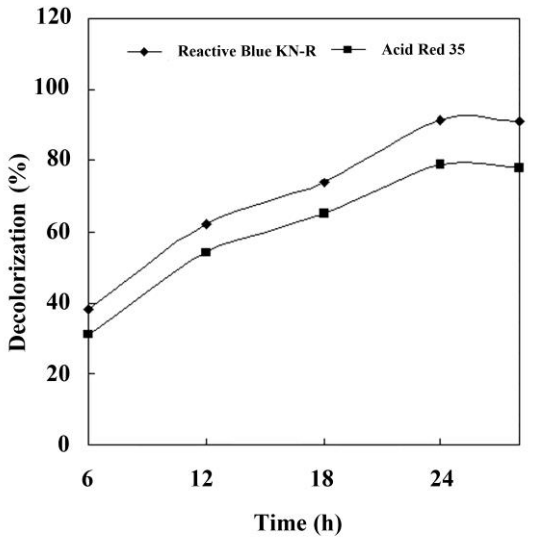

Fig. 6. Effect of time on the decolorization rate of the two dyes by recombinant GS115-lccA

CONCLUSIONS

1. The recombinant strain *Pichia pastoris* GS115-lccA could be used to produce heterologous laccase with high enzymatic activity by optimizing the fermentation conditions. The optimum fermentation conditions were: methanol additive amount 0.601%, medium initial pH 7.1, and liquid medium volume 20.4%. The highest enzyme activity of 5235 U/L was achieved, which was 2.2 times the original laccase activity (2357 U/L).
2. This enzyme showed excellent decolorization capacity for different dyes. The optima for the decolorizing systems for each dye were as follows: decolorization of Reactive Blue KN-R was achieved by reacting with 2 U/mL enzyme dosage at 50 °C using succinic acid-NaOH buffer (pH 4.0) for 24 h, and the decolorization efficiency for Reactive Blue KN-R of incubation was 91.33%; and the decolorization of Acid Red 35 was achieved with 4 U/mL enzyme dosage at 50 °C using tartaric acid-sodium tartrate buffer (pH 4.5) for 24 h, and the decolorization efficiency for Acid Red 35 was 78.96%, which was still relatively lower compared to Reactive Blue KN-R.
3. In summary, our work indicated that this laccase may be used in decolorizing and decontaminating wastewater treatment of similar dyes such as azo, anthraquinones, *etc*. This work also suggested that laccase has great potential application for color removal of industrial wastewater and for reducing the impact of industrial wastewater on the environment.

ACKNOWLEDGEMENTS

This work was supported by the International Advanced Forestry Science and Technology Project Imported by State Forestry Administration (Grant No. 2011-4-15, 2010-4-19), and the project was funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions. Ordinary university graduate student research innovation projects of Jiangsu province (Grant No. CXLX12_0529 and No. CXZZ12_0537) also funded the project.

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Article submitted: March 19, 2013; Peer review completed: May 27, 2013; Revised version received: May 30, 3013; Accepted: May 31, 2013; Published: June 13, 2013.