

Decolorization of SN4R Dye by a Recombinant Laccase from *Pichia pastoris*

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This study is the first report on synthetic dye decolor by recombinant *Pleurotus djamor* laccase. A cDNA encoding for a laccase was isolated from the white-rot fungus *P. djamor* was isolated by RT-PCR and expressed in the *P. pastoris* strain SMD1168H under the control of the alcohol oxidase (AOX1) promoter. The laccase native signal peptide efficiently directed the secretion of the recombinant laccase in an active form. Factors influencing laccase expression, such as pH, cultivation temperature, copper concentration and methanol concentration, were investigated. The recombinant enzyme was purified to electrophoretic homogeneity, and was estimated to have a molecular mass of about 62.5 kDa. The purified enzyme behaved similarly to the native laccase produced by *P. djamor* and efficiently decolorized four synthetic dyes including azo, anthraquinone, heterocyclic and triphenylmethane dyes, without the addition of redox mediators. The decolorization capacity of this recombinant enzyme suggests that it could be a useful biocatalyst for the treatment of dye-containing effluents.

Key words: Dye decolorization, *Pichia pastoris*, Purification, Recombinant laccase.

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2) belong to the family of blue multicopper oxidases (MCO) that catalyze the oxidation of an array of aromatic substrates by the reduction of dioxygen to water¹. Laccases are widely distributed in many bacteria, fungi and plants². Many true fungal "sensu stricto" laccases belong to the group of "blue" oxidases which contain four copper atoms located in two metal centers (T1, T2/T3) within the three domains. The typical metal content of laccases includes one type-1 (T1, Cu1), one type-2 (T2, Cu2) and two type-3

(T3, Cu3) copper ions, with Cu2 and Cu3 arranged in a trinuclear cluster (TNC). The Cu1 is located in domain 3, whilst the TNC cluster is embedded between domains 1 and 3 with both domains providing residues for copper coordination. The structure of basidiomycete laccases is stabilized by two disulfide bridges between domains 1 and 3, and 1 and 2³.

The substrate spectrum of laccase is very broad and can be expanded considerably in the presence of appropriate oxidation-reduction mediators⁴. Therefore, laccases possess promising potential in many industrial and biotechnological applications⁵. The successful applications of laccases in these areas require the production of large quantities of laccases at low-cost.

Pleurotus djamor is a white rot fungi. In nature, white rot fungi produce high levels of

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laccases, and white rot fungi laccase activity is relatively high⁶. Nevertheless, in most fungi laccases are produced at levels that are too low for commercial use. Furthermore, white rot fungi often express multiple laccase genes encoding isozymes with a highly similar primary structure but different physicochemical characteristics². This complexity makes it difficult to purify individual isozymes for analysis. Instead, heterologous expression in an alternative host can provide an effective mechanism of recombinant laccase production. Many laccase genes or cDNAs have been cloned and some have been expressed in yeasts (*Saccharomyces cerevisiae* and *Pichia pastoris*) and in filamentous fungi (*Trichoderma reesei* and *Aspergillus oryzae*)^{7,8,9}. Although filamentous fungi are generally good hosts for protein secretion, they are more time consuming to work with than yeasts. The heterologous expression of laccase would not only generate large quantities of enzyme, but also allow site-directed mutagenesis or in vitro evolution for design of improved laccases for biotechnical applications^{10,11}. The methylotrophic yeast *Pichia pastoris* is particularly suited to foreign protein expression for a number of reasons, including high expression levels, efficient secretion of extracellular protein, post-translational modifications and growth to high cell densities on defined minimal medium. In addition methods for molecular manipulation of *P. pastoris* are rapid and well developed, and purification of secreted recombinant proteins is easily achieved due to the relatively low levels of native secreted proteins¹².

For industrial and biotechnological purposes, laccases were among the first fungal oxidoreductases to be employed in large-scale applications such as removal of polyphenols from wine and beverages, conversion of toxic compounds and textile dyes in wastewaters, and in bleaching and removal of lignin from wood and non-wood fibers¹³. Synthetic dyes are increasingly used in the textile, leather, paper, cosmetics and pharmaceutical industries. These compounds, chemically classified as anthraquinone, azo, heterocyclic, triphenylmethane or phthalocyanine dyes, cause serious environmental pollution¹⁴. Most synthetic dyes are toxic and highly resistant to degradation. Laccases and laccase mediator systems can efficiently decolorize a variety of synthetic dyes¹⁵. So far a number of studies have

focused on the ability of white rot fungi to decolorize and biodegrade these dyes¹⁵. Laccase dye decolorization is an efficient method, and is attracting increasing interest⁵, however only limited research into purified recombinant laccase dye decolorization has been carried out^{16,17,18}. In this study, we describe the heterologous production of *P. djamor* laccase via cDNA gene expression in *P. pastoris*. The recombinant enzyme was purified and we investigated its ability to decolorize several chemically different synthetic dyes.

MATERIALS AND METHODS

Microbial strains, media and vectors

Pleurotus djamor was maintained on potato dextrose agar (PDA) slants at 4°C, Reserve in Heilongjiang China (125° 532 E and 50° 102 N). *E. coli* JM109 was grown in Low Salt Luria-Bertani (LB) medium. *Escherichia coli* JM109 (Tiangen, Beijing, China) were used for subcloning. *Pichia pastoris* SMD1168H (Invitrogen, Carlsbad, CA, USA) was used for expression of laccase. Minimal methanol (MM), buffered minimal methanol (BMM), buffered glycerol-complex (BMGY) and yeast extract-peptone-dextrose (YPD) media were prepared according to the EasySelect *Pichia* Expression Kit manual (Invitrogen). The pMD20-T simple vector (TaKaRa, Dalian, China). In addition, the *Pichia* expression vector pPICZ B (Invitrogen, USA) was used to clone the entire *P. djamor* laccase cDNA.

Vector construction

All enzymes used for cloning and restriction analysis were obtained from TaKaRa. The laccase cDNA was isolated and amplified by RT-PCR as described previously. The forward primer (5'-CGGAATTC ATGACTCTTCTGACTCTCTC -3') and reverse primer (5'-GCTCTGATTATGGTTGAAGATCGTTGGT -3') were used to generate an *EcoR* I and an *Xba* I restriction site, respectively (in italics). The PCR amplification program was initiated at 95°C for 5 min, followed by 30 cycles of 95°C for 45 s, 58°C for 30 s and 72°C for 3 min, and a final extension at 72°C for 15 min. The PCR products were purified using the DNA Gel Extraction Kit (TaKaRa), and cloned into the pMD18-T simple vector. The expression vector pPICZB and the recombinant pMD18-T simple vector containing the entire laccase cDNA were

digested with *EcoRI* and *XbaI*, and the resulting fragments were purified as described above. The purified laccase cDNA was ligated with the digested pPICZB fragment using T4 DNA ligase overnight at 4°C. The ligation mixture was transformed into *E. coli* JM109 and selected on Low Salt LB medium supplemented with 25 µg/ml Zeocin (Invitrogen). Transformants were isolated and the recombinant pPICZB/lacI vector was verified by restriction enzyme digestion, agarose gel electrophoresis and sequence analysis.

Transformation of *P. pastoris*

The pPICZB-Lg-lacI and the control vector pPICZB were linearized by *Bam*HI digestion, respectively, and transformed into *P. pastoris* SMD1168H (Invitrogen) by the electroporation method described in the instruction of the Multi-Copy *Pichia* Expression Kit (Invitrogen), respectively. The electroporated cells were plated onto YPD agar plates for selecting the 100 µg ml⁻¹ Zeocin⁺ transformants. The selected transformants were transferred to MM agar plates containing 0.2 mM 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma, St Louis, MO, USA) and 0.1 mmol l⁻¹ CuSO₄. The plates were incubated inverted at 28°C and 100 µl of methanol was added to the lid of the plate each day to compensate for loss by evaporation. Laccase-producing transformants were identified by the presence of a dark green halo around the *Pichia* colonies.

Assay of laccase activity

Laccase activity was determined at 25°C using 0.5 mg ml⁻¹ ABTS as the substrate. The samples were centrifuged at 5,000×g for 5 min at 4°C to obtain supernatants. Laccase activity was measured by the ABTS method as described by Eggert et al¹⁹. There were three replicates of each treatment and standard deviation did not exceed 5% of the average values.

Optimization of laccase production in *P. pastoris*

All chemicals were analytical grade, unless stated otherwise. Positive transformants were grown in BMGY liquid medium at 28°C with orbital shaking at 260 rpm for 1.5-2d to OD_{600nm} of 3-5. Cells were harvested by centrifugation at 1,500×g for 4 min under sterile conditions at 4°C. The cell pellets were resuspended in 100 ml BMMY medium to an OD_{600nm} of about 1.0 in 500 ml baffled shake flasks. The flasks were cultivated at 28°C in

a shaking incubator with orbital shaking at 240 rpm, and filter-sterilized methanol was added to a final concentration of 0.5% (v/v) daily. Samples were withdrawn at intervals for the determination of laccase activity and cell growth. For laccase activity, the samples were centrifuged at 5,000×g for 5 min at 4°C to obtain supernatants. The cell pellets were washed and suspended in lysis buffer (50 mM sodium phosphate, pH 7.4, 1 mM PMSF, 1 mM EDTA and 5% glycerol), and were then disrupted by sonication in ice bath in the presence of glass beads (JYD-150L, Zhisun, Shanghai, China). The sonicator was programmed to provide 5 s pulses with 5 s pauses for a total period of 10 min at 99% output power. The cell extract was obtained by centrifugation at 12,000 g for 20 min at 4°C and was used for determination of intracellular laccase activity. The transformant with highest laccase-secretion was selected for investigating the effects of temperature, pH, copper concentration and methanol concentration on the recombinant laccase production. There were three replicates of each treatment.

Purification and characterization of recombinant laccase

The purification of heterologous laccase was carried out as follows: the supernatant (550 ml) was filtered and concentrated to 50 ml in a Labscale TFF system (Millipore, Bedford, MA, USA) using a 30-kDa filter cassette. The sample was applied to a DEAE-Sepharose Fast Flow column (1.6×50 cm, Dingguo, Beijing, China) equilibrated with 20 mM sodium phosphate buffer (pH 6.5). The column was washed with the same buffer, and absorbed proteins were eluted by a linear concentration gradient of NaCl (150 ml, 0-1M) at a flow rate of 1 ml min⁻¹. SDS-PAGE was performed according to the protocol of Laemmli with 5% stacking gel and 15% resolving gel using a Hoefer mini VE vertical electrophoresis system (Amersham Biosciences, San Francisco, CA, USA). The molecular mass of the recombinant laccase was estimated by calculating the relative mobility of standard protein markers (TaKaRa, Dalian, China). The optimal pH and temperature, Km, Vmax, and the extent of glycosylation of the purified enzyme were also determined. There were three replicates of each treatment.

Dye decolorization

The ability of the recombinant laccase to

decolorize four synthetic dyes of different classes was assessed. The reaction mixture contained a final concentration of 0.1M citrate-phosphate buffer (pH 3.4), different dyes (each 50 mg l⁻¹), and 500 µl culture supernatant as enzyme solution (containing 0.005 U laccase) in a total volume of 3 ml. The reactions were initiated with enzyme and incubated at 40°C under mild shaking conditions. The time course of decolorization was detected at 0 h, 0.5 h, 1 h, 4 h, 6 h, 10 h, 14 h and 18 h by measuring the absorbance at 467 nm for SN4R, 465 nm for neutral red, 466 nm for congo red, and 502 nm for crystal violet. The decolorization of dye, expressed as dye decolorization (%), was calculated with the formula: decolorization (%) = [(C_i-C_t)/C_i] \times 100, where, C_i: initial concentration of the dye, C_t: dye concentration along the time²⁰. Samples were assayed in parallel, without enzyme under identical conditions, and all measurements were made in triplicates.

RESULTS

Heterologous expression of laccase in *P. pastoris*

The *P. djamora* laccase cDNA, including the native signal sequence, was cloned into the vector pPICZB and transformed into electro-competent *P. pastoris* SMD 1168H cells. Transformants were tested for laccase expression by two days growth on MM plates supplemented with ABTS and CuSO₄ (Fig. 1). An intense green zone halo formed around the *P. pastois* containing the recombinant plasmid, pPICZB-Lg-lac1, while no halo was observed around the colony containing empty vector, pPICZB. Subsequent experiments using liquid cultures also included control transformants. Laccase activity was found in the supernatant of the culture medium, indicating that laccase was secreted from yeast cells in an active form. Laccase activity was detected neither in the cell extract nor in the supernatant of control

culture.

Optimization of the recombinant laccase production

The transformant with high laccase-secreting ability was selected to optimize the heterologous expression of laccase under various cultivation conditions. The culture supernatants were assayed daily for seven consecutive days. The culture medium (BMM) was supplemented with 0.1 mM CuSO₄ to support copper incorporation into the laccase.

The pH of the culture broth was only maintained for a short period and then declined gradually during cultivation (data not shown). To stabilize the pH of the cultures, 0.6% alanine was added into the BMM growth medium. Cultures containing 0.6% alanine were set up with initial pH values of 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0 to study the effect of pH on laccase production. Higher laccase activity was observed in cultures with initial pH 6.0, as measured on day five (Fig. 2a). The pH control produced by alanine was successful at pH 6.5 and 7.0 during the cultivation, while there was a slight increase of pH in the media with initial pH of 4.0 and 5.0 after two days.

The effect of growth temperature on laccase expression was analyzed at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C, using BMM medium (pH 6.0) containing 0.2 mM CuSO₄ and 0.6% alanine. Increasing the growth temperature led to an enhancement of laccase activity (Fig. 2b). The laccase activity of culture supernatants was measured daily for seven consecutive days. Laccase secretion peaked at day five. After five days cultivation, the laccase activity of the culture maintained at 30°C was 1.96 times higher than that maintained at 20°C. Subsequent tests were performed at a cultivation temperature of 30°C.

The recombinant *P. pastoris* culture laccase activity was enhanced by altering the copper concentration in the BMM medium (pH

Table 1. Purification of the recombinant laccase from *p. pastoris*

Purification step	Total protein(mg)	Total activity(U)	Specific activity (U mg ⁻¹)	Purification fold	Yield (%)
Culture supernatant	32.58	898.34	37.03	1.03	100
Ultrafiltration	13.84	484.58	49.42	1.53	59.04
DEAE-Sepharose Fast Flow	3.67	354.47	132.67	4.37	42.79
Sephadex G-75	1.53	235.86	232.56	5.97	32.48

6.0). If copper was absent from the medium, the laccase activity level was very low. Increasing copper concentrations enhanced laccase activity, peaking at 0.5 mM CuSO₄ (Fig. 2c).

Variation of methanol concentration between 0.5% and 1.5% was used to induce the expression of laccase in BMM (pH 6.0) supplemented with 0.6% alanine and 0.5 mM CuSO₄. The laccase activity decreased with increasing

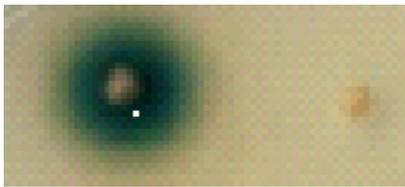


Fig. 1. Detection of the expression of lac1 gene in yeast transformants. The BMM agar plates containing CuSO₄ and ABTS were used to screen the transformants producing active laccase. Dark green zones were present around the colonies of GS115(pPICZB-lac1) after 2 days growth

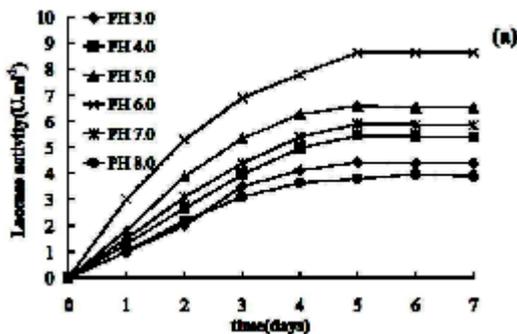


Fig. 2. Effects of pH on laccase activity of the transformant pPICZB/Lac1 in BMMY medium supplemented with 0.1 mM CuSO₄ (a)

methanol concentration (Fig. 2d). A methanol concentration of 0.5% produced the highest level of laccase activity. Under the optimal conditions, laccase activity reached 3245 U l⁻¹ on day seven (Fig. 3), and the highest laccase output was 5006 U l⁻¹ after 14 days of cultivation. The pH of the culture was successfully maintained at 5.8-6.2 for ten days. **Purification and characterization of recombinant laccase**

The purification process is summarized in Table 1. The recombinant laccase was purified from 550 ml culture broth following seven days growth in BMM medium. The recombinant laccase was obtained with an overall yield of 36.5% and a specific activity of 375.71 U mg⁻¹. The purified enzyme appeared on SDS-PAGE as a single band with a molecular mass of 62.5 kDa (Fig. 4).

The activity profile of isolated laccase was determined over the pH range of 2.2 - 6.0. The heterologous laccase activity peaked at pH 2.4. The optimal temperature of the enzyme was 55°C.

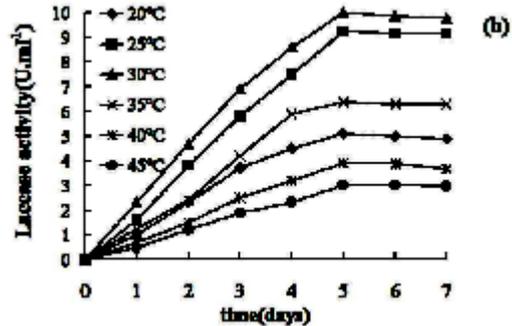


Fig. 2. The effect of temperature on laccase activity of the transformant pPICZB/Lac1 in BMMY medium supplemented with 0.1 mM CuSO₄ (b)

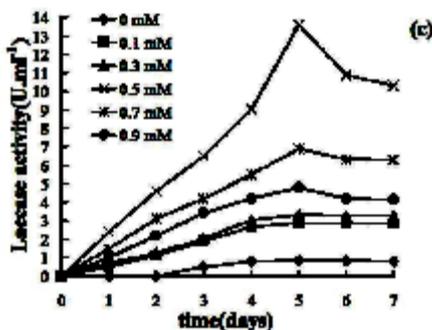


Fig. 2. Laccase activity level of transformant pPICZB/Lac1 incubated at 28°C in BMMY medium at different copper concentrations (c)

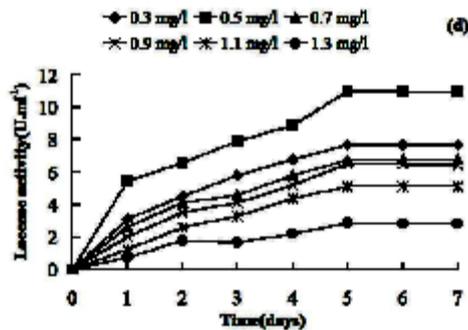


Fig. 2. Laccase activity level of transformant pPICZB/Lac1 incubated at 28°C in BMMY medium at different methanol concentrations (d)

The heterologous laccase exhibited K_m and V_{max} values of 168 mM and 24.25 mmol min^{-1} , respectively, for ABTS at pH 2.4 and 25°C.

The ability of the purified laccase to decolorize synthetic dyes differed, as demonstrated in Fig. 2c. All the tested dyes were decolorized by the recombinant laccase without the addition of any mediators. The decolorization of SN4R occurred rapidly within 30 min, and remained unchanged (98.85%) after one hour. In contrast, decolorization of the other three dyes proceeded more slowly and required more than one hour to decolorize 50% of the dye (Fig. 5). After 18 hours, decolorization of methyl orange (70.55%), neutral red (60.38%), and malachite green oxalate (50.78%) was achieved.

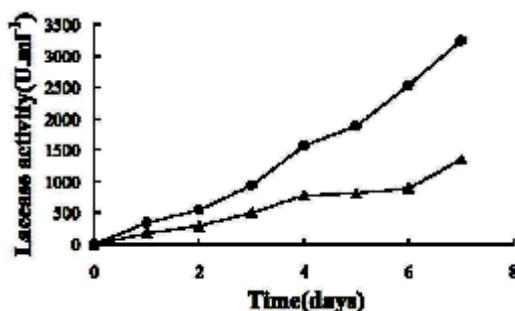


Fig. 3. The activity of recombinant laccase in BMM medium under different cultivation conditions. Before optimization: pH 6.5, 28°C, supplemented with 0.1 mM CuSO_4 and 0.4% methanol (2%). After optimization: pH 6.0, 30°C, supplemented with 0.5% alanine, 0.5 mM CuSO_4 and 0.5% methanol (1%). The values are mean of triplicates (standard deviation <5%)

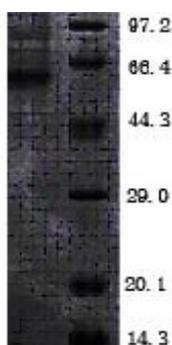


Fig. 4. SDS-PAGE of the recombinant laccase from *P. pastoris*. Lane 1: purified laccase; M: molecular weight markers

DISCUSSION

The *P. pastoris* expression system has been used to produce a wide variety of heterologous proteins. The recombinant proteins can be expressed at a high level under the control of the alcohol oxidase I (AOX1) promoter, one of the strongest and tightly regulated promoters induced by methanol. The laccase cDNA from *P. djamor* was successfully expressed and secreted in a functional form by *P. pastoris*, and the secreted protein was correctly processed and its productivity was optimized by the tailored expression conditions. However, the laccase activity of the *P. pastoris* transformant was relatively low, which may be further enhanced by optimization of the heterologous expression condition.

The molecular weight of the recombinant laccase (62.5 kDa) was similar to the native enzyme (62.4 kDa). The molecular weight of the recombinant laccase may have been increased by *P. pastoris* glycosylation²¹.

Four synthetic dyes that could be classified as azo, anthraquinone, triphenylmethane and heterocyclic dyes were decolorized by the purified laccase to different extents (Fig. 4). The variation in the efficiency of decolorization could be attributed to the difference in the molecular structures of the dyes¹⁴. Despite considerable research into azo dyes, limited information exists on the biotransformation of anthraquinone dyes²². There is no available data on the decolorization efficiency of anthraquinone dye SN4R, but the

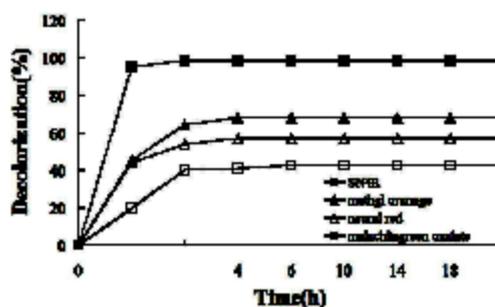


Fig. 5. Decolorization of synthetic dyes by the purified recombinant laccase (3 U ml^{-1}) at pH 3.0, 40°C. SN4R (1%); methyl orange (2%); neutral red (3%); malachite green oxalate (1%). The values are mean of triplicates (standard deviation <5%)

decolorization efficiency of anthraquinone dye in RBBR has been reported. The purified recombinant laccase from *P. methanolica* requires 16 h to decolorize about 90% of RBBR under a higher laccase activity (25 U ml⁻¹)¹⁸. Among the four dyes tested, heterocyclic carmine was the most recalcitrant to decolorization, indicating it was not a suitable substrate for the purified enzyme, but in the presence of mediators, the decolorization of heterocyclic carmine by laccase might be greatly enhanced¹⁷. Industrially, azo dyes are usually decolorized to aromatic amines by bacterial azoreductases. These colorless products may be toxic, mutagenic and carcinogenic to humans and animals²³. The oxidation mechanism of laccase may avoid the formation of these hazardous aromatic amines. It has previously been reported that laccases alone cannot efficiently decolorize azo dyes. In this case laccase activity depends on the presence of some small redox mediators⁴. However, we found that the purified recombinant laccase could efficiently decolorize the anthraquinone dye SN4R in the absence of any mediators. These results suggest a potential use of this recombinant laccase in decolorizing and detoxifying industrial synthetic dyes.

pH control is usually needed during laccase production, as some acidic proteases may be activated at a lower pH and cause a detrimental effect on laccase production²⁴. It has been reported that the metabolism of alanine by *P. pastoris* could stabilize the culture pH²⁵. We used 0.6% alanine in the BMM growth medium to stabilize the pH around 6.0 during the cultivation. The activities of several heterologous laccases expressed by yeasts were observed to be improved by lowering the cultivation temperature to 20°C²⁶. However, the expression of *P. djamor* laccase in *P. pastoris* was not improved by decreasing the cultivation temperature. Laccase activity in *P. pastoris* shake-flask cultures was much higher at 30°C than at 20°C; this might be ascribed to the lower growth speed of the *P. pastoris* when cultivated at 20°C. Laccases are copper-containing enzymes, and the copper atoms are very important for the activity of laccases. The heterologous expression of *Pycnoporus coccineus* laccase in *Aspergillus oryzae* and *Saccharomyces cerevisiae* has demonstrated that copper is necessary for the correct folding and assembly of laccase at the post-

transcriptional step²⁷. The activity of the recombinant laccase was directly proportional to the concentration of copper in the growth medium, indicating the requirement of the enzyme for copper. The increasing of methanol concentration caused a negative effect on laccase production. The lower laccase activity obtained at higher methanol concentrations may be attributed to the accumulation of the toxic products of methanol metabolism, which would retard the growth and decrease the observed biomass yield of *P. pastoris*²⁸.

In conclusion, we have demonstrated the use of the *P. pastoris* expression system to produce heterologous laccase, which could be used in the removal of dyes from industrial effluents. *P. pastoris* laccase efficiently decolorized SN4R without additional redox mediators. This enzyme also catalyzed decolorization of other synthetic dyes in the absence of redox mediators.

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