Heterologous expression and characterization of a laccase from *Laccaria bicolor* in *Pichia pastoris*

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**ABSTRACT**

Synthetic dyes are known to be highly toxic to mammalian cells and mutagenic and carcinogenic to humans and, therefore, should be detoxified and removed from industrial effluents. Different approaches for removal and detoxication are extensively sought. Biochemical methods are considered the most economical and effective method of dye decolourization. In this research, the laccase gene from *Laccaria bicolor* was modified and expressed in *Pichia pastoris*. The properties of the recombinant laccase and its ability to degrade synthetic dyes were studied. The laccase activity was optimal at pH 2.2 and 50 °C. Its *Km* value was 0.187 mmol/L for ABTS ([2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)]. The laccase obtained was shown to decolorize the synthetic dyes, malachite green, crystal violet and orange G, with ABTS as a mediator. These results indicated that the laccase obtained may be used to treat industrial effluents containing artificial dyes.

**Introduction**

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10.3.2), members of the blue multi-copper oxidase family, catalyse the oxidation of a wide range of organic substrates, including phenols, polyphenols, anilines and even certain inorganic compounds. They were first discovered in plants and have been subsequently identified in fungi, insects and some bacteria. Laccases are involved in lignification, delignification, sporulation and plant pathogenesis. They can catalyse the four-electron oxidation of lignin, a phenolic substrate. The free radical of the substrate is unstable and may undergo a second enzyme-catalysed reaction or a non-enzymatic reaction.

The production of natural laccases is low and cannot meet the requirements of industrial applications. It is feasible to recombine cloned and modified laccase genes into *Pichia pastoris*, which is a well-established host organism, and express them. A lot of heterologous laccases have been reported, for example, in *Fomes lignosus*,[1] *Pycnoporus cinnabarinus*,[2] *Pleurotus sajor-caju* [3] and *Pycnoporus sanguineus*. [4] The expression level of heterologous laccases can be enhanced by using the methanol-inducible alcohol oxidase 1 (AOX1) promoter and applicable vectors in *P. pastoris*, and protein production can also be raised by increasing the copy number of the laccase gene. Besides, *P. pastoris* can grow to high cell density during fermentation, which is extremely useful in industrial fermentation.

Some industries widely use synthetic dyes, which are released into the aquatic environment as a component of waste waters. However, they are cytotoxic agents, mutagens and carcinogens to fish and mammals. In the USA and Europe, although malachite green (MG) and crystal violet (CV), as typical triphenylmethane dyes, are banned in food fish because of significant health risks, these dyes are persistently used in different countries as an effective fungicide, bactericide, ectoparasiticide and antiprotozoan in fish.[5] That is why it is necessary to investigate various methods for the treatment of triphenylmethane dyes. Decolourization and detoxification of triphenylmethane dyes by laccase have been reported.[6] To our knowledge, fungal laccases are normally difficult to use to decolorize azo dyes without a mediator. Taking account of the low cost, high efficiency, simple procedure and reduced generation of secondary pollution, biochemical methods are considered the most

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economical and effective method of dye decolourization and degradation.[7]

In this study, we synthesized a gene according to the protein sequence of LBLCC3I, and expressed it in *P. pastoris*. The enzymatic characteristics and the ability of the enzyme to degrade synthetic dyes were investigated.

**Materials and methods**

**Microorganisms, reagents and culture conditions**

The *Escherichia coli* strain DH5α (Invitrogen, USA) was applied to DNA and plasmid manipulations. *P. pastoris* strain GS115 (Invitrogen, USA) was used for heterologous expression of laccase. pMD18-T (Takara) and modified strain GS115 (Invitrogen, USA) was used for heterologous applied to DNA and plasmid manipulations.

Expression of recombinant protein were prepared follow-

from Shanghai Sangon Co., Ltd. The media used for the synthetic dyes, MG, CV and orange G, were purchased purchased from Shanghai Generay Biotech Co., Ltd. The (Dalian, China). Bradford protein quantitative test kit was manipulation procedures were purchased from TaKaRa (SD, 0.000004% biotin) plates containing ABTS [2,2’-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)]. If the LBLCC3I gene was recombined into yeast genomic DNA and expressed successfully, the medium around the yeast colony would turn blue. The blue colonies were selected and further confirmed by polymerase chain reaction (PCR).

A single colony was picked and inoculated in 50 mL of BMGY (SD, 1% yeast extract, 2% peptone, 1.34% YNB, 0.000004% biotin, 1% glycerol) medium at 28 °C in a shaking incubator until the culture reached to OD[600] of 2–6. The cells were harvested by centrifugation (5000 r/min for 5 min, 4 °C) and resuspended in 100 mL of BMGY, BMM (buffered minimal methanol medium) or MM (minimal methanol medium) medium supplemented with 0.5 mmol/L copper sulphate and returned to the incubator to grow for three days. Methanol was added to a final concentration of 1% every 24 h.

**Purification and characterization of recombinant laccase**

The concentration of purified protein was quantified by the Bradford method protein quantification kit with bovine serum albumin as a standard. The gel was stained with Coomassie brilliant blue. The crude enzyme in the supernatant of the induced culture was precipitated by 40% and 70% ammonium sulphate. Phosphate-buffered saline (PBS; pH 6.0) was used to dissolve and dialyse the precipitate. Then, the desalted solution was purified by nickel-charged iminodiacetic acid column (Sigma-Aldrich), according to the operation manual. The molecular mass of the laccase was estimated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide gel.

The activity measurement was performed with 0.5 mmol/L ABTS in 0.2 mol/L citrate–phosphate buffer at 37 °C in a total of 200 μL. The assay mixture was incubated at 37 °C for 10 min before the enzyme was added. The reaction was terminated by addition of 50 μL 1.0 mol/L NaF. The absorbance of the reactant was measured at 420 nm (Tecan) (ε = 12,000 L/(mol cm)). One unit (U) of laccase activity was defined as the amount of enzyme catalysing the formation of 1.0 μmol of product per minute under the assay conditions. The optimum pH was determined spectrophotometrically with ABTS in 0.2 mol/L citrate–phosphate buffer (pH 2.2–7.8) at 37 °C. The pH stability of LBLCC3I was assessed by enzyme incubation at 4 °C in various buffers for 24 h. The optimum temperature was investigated between 30 and 80 °C at 5 °C increments. Thermostability assays were determined by incubating the enzyme in Mcllvaine buffer (pH
2.2) at 20, 30, 40, 50, 60 or 70 °C for 60 min at 10-min increments. Enzyme activity at starting time was assumed as 100% stability. Enzyme assays were performed in triplicates. The \( K_m \) was done with freshly prepared enzyme mixed with various concentrations of ABTS (from 0.005 to 2 mmol/L) added to McIlvaine buffer (pH 2.2) at 50 °C for 20 min. There were three replicates of each treatment.

**Degradation of synthetic dyes**

The ability of purified laccase to decolourize three synthetic dyes was studied. The reaction mixture for degradation experiments contained a substrate pollutant solution and enzyme preparation in a total of 200 \( \mu \)L of 0.2 mol/L citrate-phosphate buffer (pH 2.2) at 37 °C for each time period of 0, 20, 40, 60, 90 and 120 min, and then subsequently incubated in the dark. ABTS, as the mediator, was added if necessary. Decolourization rate was defined as follows: decolourization (\%) = 100 \times (A_{t0} - A_{tf})/A_{t0}, where \( A_{t0} \) is the absorbance of the assay mixture before incubation and \( A_{tf} \) is the absorbance after incubation. The decolourization of MG, CV and orange G was determined by measuring the absorbance at 620, 590 and 470 nm, respectively.

**Effects of metal ions and inhibitors**

The effect of metal ions was assayed in McIlvaine buffer (pH 2.2) containing 0.5 mmol/L of ABTS with or without 10 mmol/L metal ions (e.g. Mn\(^{2+}\), Na\(^{+}\), Mg\(^{2+}\), K\(^{+}\), Ni\(^{2+}\), Zn\(^{2+}\), Cu\(^{2+}\), Fe\(^{2+}\) and Ca\(^{2+}\)). Inhibitor studies were conducted in McIlvaine buffer (pH 2.2) containing different inhibitors, such as 0.1 and 1 mmol/L of dithiothreitol (DTT), L-cysteine, sodium thiosulphated, sodium sulphite and SDS or 1 mmol/L and 10 mmol/L EDTA.

**Data analysis**

All enzymological assays and dye decolourization tests were performed at least in triplicate. Data presented in figures are mean values from three independent experiments with standard deviation (\( \pm \)SD). Statistical analysis was performed using the SPSS 13.0 statistical software.

**Results and discussion**

**Expression of LBLCC3I gene in P. pastoris**

The LBLCC3I gene was synthesized and sequenced to check its correctness by cloning into the pMD18-T vector. The presence of the expression plasmid for secretion of the recombinant product was verified by restriction enzyme \( Bgl \) II digestion (data not shown). The plasmid and the control vector were digested by \( Bgl \) II and transformed into \( P. \) pastoris GS115. Transformants produced coloured zones on BMMY solid plates containing ABTS. A large number of fungal laccases have been expressed earlier in \( P. \) pastoris by other researchers. But the stability of each different laccase is diverse in culture medium. In order to achieve high levels of foreign protein production, expression conditions need to be determined. In addition, the pH value of the medium has a pivotal role in the yield of protein. In the fermentation process involving \( P. \) pastoris, the pH of the medium often drops to 3 or below. Therefore, the expression medium has to be buffered for better results. In this research, LBLCC3I could not be detected in control group BMM and MM, but in BMGY, which contains yeast extract and peptone. Transformants cultured in BMGY in shake flask at 30 °C for three days produced laccase at an amount of about 498 \( \mu \)g/mL. Yeast extract and peptone were very important for the stability of LBLCC3I during fermentation. It was supposed that LBLCC3I was specifically susceptible to extracellular proteases.

**Characterization of LBLCC3I**

After three consecutive days of fermentation, the enzyme LBLCC3I was purified by affinity chromatography with an Ni\(^{2+}\) column. The purified laccase appeared as a single protein band on the SDS-PAGE gel (Figure 1). As determined by SDS-PAGE, the molecular mass of the purified laccase was 56 kDa. With ABTS as a substrate,
the $K_m$ value of the heterologous laccase was 0.187 mmol/L at pH 2.2 and 50 °C.

The optimum pH value of the catalytic reaction by LBLCC3I was 2.2 (Figure 2(a)), indicating that the recombinant enzyme was more acid-resistant than other laccases from *Lentinula edodes*, *Panus rudis*, and *Albatrellus dispansus*. Furthermore, the enzyme was stable at a wide range of pH values, with most residual activity being up to 95% after incubation at 4 °C for 24 h (Figure 2(b)). This characteristic makes LBLCC3I a good candidate for the potential applications.

The optimum reaction temperature and thermal stability of LBLCC3I were determined with ABTS as the substrate at pH 2.2 (Figure 3(a)). The recombinant laccase had a maximum activity at 50 °C. Apparently, it was not stable at temperatures higher than 60 °C during prolonged incubation (Figure 3(b)). The residual activities were nearly reduced to zero after incubation at 60 and 70 °C for 10 min.

**Degradation of synthetic dyes by LBLCC3I**

In our research, we found that MG, CV and orange G could not be degraded by LBLCC3I unless the redox mediator ABTS was present. However, some laccases could efficiently decolourize dyes without ABTS.[4,11] Notwithstanding, the redox mediator could remarkably improve the decolourization efficiency of other laccases.[12,13] The degradation rate depends on the structure of the dye and the structure and redox-potential of the enzyme.[13] The decolourization rate was proportional to the incubation time. In the presence of ABTS (0.05 mmol/L), the decolourization rates of MG, CV and orange G were nearly 100% after 120 min (Figure 4). As a mediator, ABTS could facilitate the reactions. ABTS can transfer electrons between the laccase and the dyes, and helps the enzyme oxidize the non-substrate dyes.

**Effects of metal ions and inhibitors on laccase activity**

The effect of inorganic salts on the activity of LBLCC3I is shown in Figure 5(a). Inhibition studies on several inorganic salts revealed that LBLCC3I was inhibited by typical inhibitors of laccase, such as $\text{Ca}^{2+}$, $\text{Fe}^{2+}$ and $\text{Mn}^{2+}$. $\text{Fe}^{2+}$ is known to inhibit most laccases, such as laccases from *Ganoderma lucidum*, *G. lucidum 7071-9*, *Monilinia*.
fructigena,[15] Pleurotus ostreatus [11] and Paraconiothyrium variabile.[16] In this study, we found that Fe²⁺ could decrease the activity of LBLCC3I at a low concentration; on the other hand, Fe²⁺ could serve as a terminator for the laccase catalysed reaction. Effluents from textile dyeing facilities contain metals used in dye production technologies or in dye molecules themselves. That is why the effect of metal ions on LBLCC3I is important for its application. The metal ions used in this study are the most common metals present in industrial effluents. Most of the tested had little effect on the activity of LBLCC3I, except Fe²⁺. Therefore, in order to make better use of LBLCC3I, it is necessary to study the possible ways to improve the stability of LBLCC3I in the presence of Fe²⁺. Some typical laccase inhibitors showed a significant effect on the activity of LBLCC3I, except 0.1 mmol/L SDS, 0.1 mmol/L EDTA and 1 mmol/L EDTA (Figure 5(b)). LBLCC3I was strongly inhibited by sodium thiosulphate, sodium sulphite, DTT, L-cysteine and 1 mmol/L SDS. This was assumed to be the result of interference of the copper atoms in the enzyme active site or the disulphide bonds essential for enzyme activity. These findings are in accordance with the general properties of laccase from a diverse range of fungal sources.[17,18]

**Conclusions**

In this research, the laccase gene from *L. bicolor* was modified and expressed in *P. pastoris*. The properties of the laccase and its ability to degrade some synthetic dyes were investigated. The laccase showed high decolourization rates for MG, CV and orange G with ABTS. These results suggest that the recombinant laccase may be used to treat industrial effluents containing synthetic dyes.

**Disclosure statement**

The funders had no role in the study design, data collection and analysis, decision to publish or preparation of this manuscript.

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