ECRONICON OPEN ACCESS

EC MICROBIOLOGY
Research Article

Laccase Enzyme from *Trametes versicolor* with a High Decolorizing Ability on Malachite Green

Zhijie Qin, Qiulin Yue, Orlando Borras-Hidalgo and Xinli Liu*

Shandong Provincial Key Laboratory of Microbial Engineering, Qi Lu University of Technology, Jinan, China

***Corresponding Author**: Xinli Liu, Shandong Provincial Key Lab. of Microbial Engineering, School of Bioengineering, Qi Lu University of Technology, Jinan, China.

Received: July 24, 2017; **Published:** August 04, 2017

Abstract

Laccases enzyme provide a promising future in terms of its use for the treatment of Malachite green (MG), which is highly toxic residue for aquatic animals. The objectives of this study were the production, purification and characterization of a laccase from *Trametes versicolor* 50001 with a degrading ability on malachite green. *Trametes versicolor* 50001 was used to produce laccase enzyme under liquid-state fermentation. The maximum enzyme activity was of 1071 ± 21 U l^{-1} after 9 days of fermentation. The enzyme was purified 31-fold by employing DEAE-Sepharose FF chromatography with a specific activity of 165.5 U mg^{-1} and a molecular mass about 50 - 60 kDa. The optimal pH and temperature were 2 and 35°C, respectively. Decolorization efficiency of laccase was evaluated against malachite green and the decolorization rate was 95.67% without added any mediator after 12h of treatment. The data suggested that the laccase from *Trametes versicolor* might has a great potential to decolorize malachite green in highly toxic residue.

Keywords: *Decolorization; Laccase; Malachite Green; Trametes versicolor; White-Rot Fungi*

Abbreviations

MG: Malachite Green

Introduction

Laccase belongs to the multicopper oxidase family, which catalyses the oxidation of a wide range of phenolic and non-phenolic compounds using molecular oxygen as the electron acceptor and producing water as the only by-product. Laccase is widely distributes in plants, fungi, bacteria and particularly abundant in fungi [1]. Among them, white-rot fungi is the major laccase producer, and *Trametes versicolor* is an important representative of white-rot fungi. Many fungal laccases have been purified and characterized, such as *Trametes* sp. LAC-01, *Pleurotus* sp. MAK-II laccase and *Cerrena* sp. Laccase [1-3]. The purified laccases have been widely used in many industrial fields [4]. Degrading dye is one of the most important applications. As enzymatic method is environmentally friendly and cost-effective, it is widely used nowadays [5].

Malachite green (MG) is a triphenylmethane dye used for coloring textiles and aquaculture [6]. However in aquatic animals, MG is highly toxic residue. Both of MG and its derivatives are accumulated in fish tissues. For this reason, some human carcinogenesis and mutagenesis might be induced after the public consume of MG-treated fish. Therefore, many countries have banned and regulated the use of malachite green. Nevertheless, the MG is illegally used in aquaculture for its low price and good effects [3]. Since Trupkin., *et al*. used laccase from *Trametes trogii* to degrade MG, many kinds of laccases from white-rot fungi have been evaluated for their ability to degrade MG, such as laccase enzyme from *Pycnoporus cinnabarinus, Pleurotus pulmonarius, Stereum hirsutum, Ganoderma lucidum* [7-10]. In this work, a laccase enzyme from *Trametes versicolor* 50001 was purified, characterized, and evaluated the decolorization efficiency on malachite green. This laccase enzyme showed a great potential to decolorize malachite green, making it a good candidate for potential applications in the aquaculture industry.

Materials and Methods

Fungal strain and laccase enzyme production in liquid culture

Trametes versicolor 50001 was obtained from China Center of Industrial Culture Collection. *Trametes versicolor* mycelia stored at 4°C were inoculated on potato dextrose agar (PDA) plates and incubated at 28°C. After 6 days, three mycelia plugs (1 cm in diameter) were excised from the plates and used to inoculate at a 300 ml Erlenmeyer flask containing 100 ml of potato dextrose broth (PDB). The liquid cultures were then cultured at 28°C on rotary shaker incubators operated at 150 rpm for 4 days on natural light condition and pH6. Cultures were homogenized by glass beads, and 5% (v/v) of the homogenate were inoculated in the fermentation medium (200g potato l^{-1}, 20 g glucose l^{-1}, 3g KH_2PO_4 l^{-1}, 1.5g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.01g vitamin B1 l^{-1}, pH6). And then $CuSO_4$ solution was added into the fermentation medium with the final concentration of 1 mM at the second day. Laccase activity of fermentation liquid was evaluated every 24 hours according to the procedure mentioned below.

Purification of laccase

After 9 days of fermentation, the culture liquid was filtered to remove mycelia, followed by centrifugation at 6000×g and 4°C for 10 min. Subsequently, the proteins isolated from the supernatant were fraction precipitated overnight with ammonium sulfate to a final concentration of 30 to 80% (w/v), followed by centrifugation at 10000 × g and 4°C for 10 min. The precipitate was also dissolved in 0.01 M potassium phosphate buffer (pH 5) and dialyzed against the same buffer using a cellulose membrane for 24h. The dialyzed enzyme solution was loaded onto a DEAE-Sepharose FF column (10×150 mm) and pre-equilibrated with 0.01 M potassium phosphate buffer (pH 5). The column was washed two time with 0.01 M potassium phosphate buffer (pH 5) to remove unbound protein. Finally, the bound protein was eluted with buffer solutions containing 0.1 - 0.5 M NaCl at a flow rate of 0.5 ml min^{-1} and enzyme active fractions were pooled and concentrated.

Enzyme assay

Laccase activity was determined with 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as the substrate using a spectrophotometric method. The absorbance was measured at 420 nm (ε_{420} = 36000 M^{-1} cm^{-1}). The reaction mixture contained 200 µl of 1 mM ABTS, 790 µl of 0.1 M Na-acetate buffer (pH 5) and 10 µl of enzyme. The reaction was monitored by measuring the change of A420 for 3 min at room temperature (25°C). One enzyme activity unit was defined as the amount of enzyme that oxidized 1 µmol of substrate per minute [11]. Protein content was estimated by the Bradford method with bovine serum albumin as the standard.

Gel electrophoresis

The purification of the laccase enzyme was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), with a 5% (w/v) stacking gel and a 12% (w/v) separating gel using a vertical gel electrophoresis system. Proteins were stained with Coomassie brilliant blue G-250. The molecular weight of laccase was determined by comparison of its electrophoretic mobility with a low-molecular-mass protein marker (TaKaRa).

Effects of pH and temperature on the activity and stability of purified laccase

Laccase activity was examined at a pH range from 2 to 7 using 100 mM citrate-phosphate buffer at room temperature with ABTS as substrate. The effect of temperature on the activity was measured by incubating enzyme solution from 25 to 85°C at the optimal pH. The enzyme activity was determined according to the assay method described above and the maximum activity of purified laccase was defined as the 100%.

To determine the pH stability, the purified laccase was incubated in 100 mM citrate/phosphate buffer (pH 2.0 - 4.0) for 100 min at room temperature, and the residual laccase activity was measured with ABTS as substrate. The thermal stability of purified laccase was evaluated by incubating the laccase at different temperatures ranging from 25°C to 85°C for 50 min in absence of substrate. The enzyme activity was estimated with standard assay every 10 minutes. The activity of the sample assayed at 0 min was defined as 100%.

Degradation tests

Decolorization efficiency of laccase was evaluated against malachite green. The degradation of MG was determined by monitoring the decrease of the absorbance value at 617 nm using a spectrophotometric method. The reaction was performed in a 1 ml mixture solution containing laccase (0.657 U ml^{-1}) and MG (25 mg l^{-1}) in 100 mM citrate-phosphate buffer (pH 2). The reaction mixtures were incubated at room temperature for 12h.

The absorbance of the mixture at the maximum wavelength was measured in a UV-visible spectrophotometer. Decolorization was defined as: decolorization (%) = [(A0−At)/A0] × 100%, where A0 and At represent the initial and observed absorbance after incubation with the laccase, respectively. All experiments were preformed in triplicate.

Statistical Analysis

The comparisons between experimental groups were performed using One-Way ANOVA, a P value less than 0.05 were considered statistically significant.

Results and Discussion

Laccase enzyme production

The ratio between glucose and nitrogen sources and the inducer are critical factors to the laccase production. Copper atoms which serve as cofactors in the catalytic core of laccase is one of the most effective and commonly used inducer of laccase in fungi [12]. As shown in figure 1, the maximum laccase activity reached 1071 U l^{-1} at the 9th day when the fermentation medium was supplemented with 1 mM of copper ion. Compared with the fermentation medium without copper ion, laccase production with 1 mM Cu^{2+} showed a increasing of 121%. The behavior was similar in *Coriolus versicolor* MTCC 138 and *Trametes hirsuta* BT 2566, which might also be induced to improve laccase production by addition of 1 mM of copper ion [12].

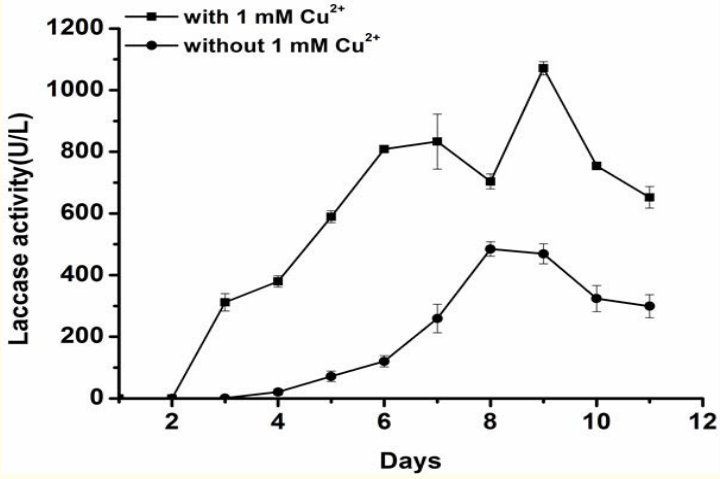

Figure 1: *Laccase activity in Trametes versicolor 50001. The bar represent the mean ± S.D. Data obtained from triplicate assays. One-Way ANOVA was used and P < 0.05.*

Purification of laccase enzyme

For fungal laccases, purification procedures were determined by species, laccase properties and the purity level required for subsequent applications. Column chromatography methods are normally used as purification procedures [12]. In this study, samples were concentrated by ammonium sulfate precipitation, followed by dialysis, and then DEAE-Sepharose FF chromatography. After sequential puri-

fication steps, the laccase was purified 31-fold with a final yield of 18.1%. The specific activity of the laccase was 165.5 U mg-1 with ABTS as the substrate (Table 1). The appearance of a single band in SDS-PAGE indicated that the laccase was purified using DEAE-Sepharose FF chromatography (Figure 2). The molecular weight of this laccase was about 55 - 60 kDa, similar to laccases with regular three domains [5].

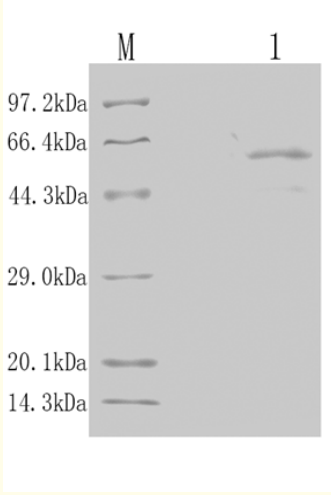

Figure 2: *SDS-PAGE of purified laccase. Lane M, a low-molecular-mass protein marker; Lane 1, purified laccase.*

Purification	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yields (%)	Purification (fold)
Crude culture filtrate	18.289	3.384	5.405	100	1
$(NH_4)_2SO_4$ (30 - 80%)	6.945	0.319	21.771	38	4
DEAE-Sepharose FF	3.31	0.02	165.5	18.1	31

Table 1: *Purification of the laccase from Trametes versicolor 50001.*

Effect of pH on activity and stability of laccase enzyme

In general, the optimum pH of fungal laccase was meta-acid at a range of 3.0 to 6.0 [13]. As shown in figure 3a, the optimum pH of the laccase from *Trametes versicolor* 50001 was 2, in accordance with laccases reported previously [14,15]. The purified laccase was strong stable at a meta-acid range of pH from 2.0 to 4.0, with at least 80% enzyme activity remained after 2 h (Figure 3b).

Effect of temperature on activity and stability of laccase enzyme

As shown in figure 3c, the optimum temperature for the purified laccase in this study was at 35°C, lower than most fungal laccases [13]. The thermostability of the purified laccase in this study was easily affected by temperature. The purified laccase showed strong stability at room temperature, but the enzyme was almost completely inactived when incubated for 10 min at 55°C (Figure 3d). In a recently published study, the laccase thermostability from *Colletotrichum lagenarium* was affected by the temperature [16].

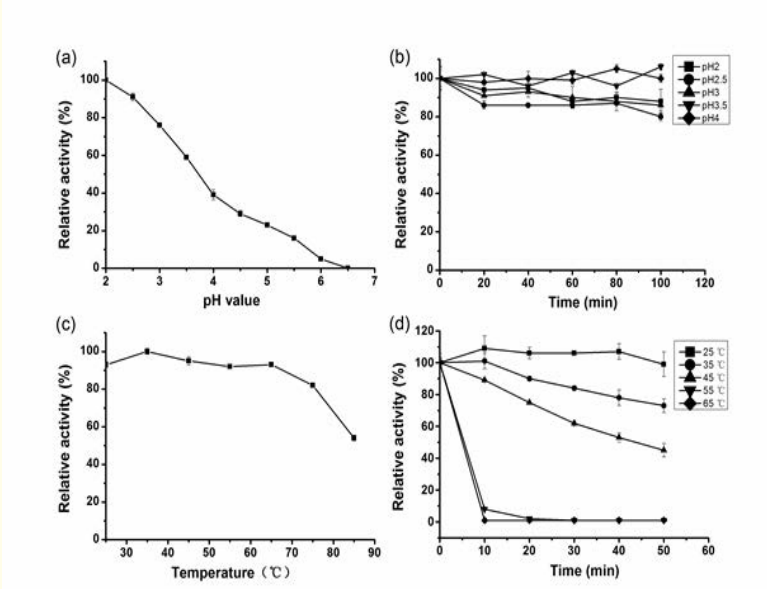

Figure 3: *Effect of pH and temperature on the activity and stability of purified laccase. (a) Effect of pH on the activity of purified laccase. (b) Effect of pH on the stability of purified laccase. (c) Effect of temperature on the activity of purified laccase. (d) Effect of temperature on the stability of purified laccase. One-Way ANOVA was used and P < 0.05.*

Degradation tests

The degradation of MG by Laccase has been evaluated in others studies. For example, Zhang., *et al.* showed that the degradation of malachite green was apparently improved, with an efficiency of 79.6% when the laccase from *B. vallismortis* fmb-103 and ABTS were combined [17]. On the other hand, the decoloration rates of MG in the laccase-only and laccase-acetylacetone systems were 15 and 90%, respectively, where acetylacetone was a mediator for laccase in pollutant detoxification process [18]. Indeed, the preheated *Cereus pterogonus* and *Opuntia vulgaris* plant laccase enzyme forms (60 - 90°C) in the presence of 1 mM Cu^{2+} achieved 97% of Malachite green dye decolorization in 12h according to Kumar and Srikumar [19]. Meanwhile, the use of mediators increase the cost of application, and potential toxicity of their by-products [12]. Herein, the laccase from *Trametes versicolor* 50001 showed an effective decoloration of MG dye with a decoloration rate of 95.67% without any added mediator after 12h of treatment with laccase (Figure 4a). According to Jadhav and Govindwar, reductases were responsible for the leuco-metabolites of malachite green [20]. Laccase belongs to the multicopper oxidase family, it won't turn malachite green into leucomalachite green (LMG). The UV-visible absorbance spectrum of MG before and after decolorizing was shown in figure 4b, none characteristic peak of LMG was observed.

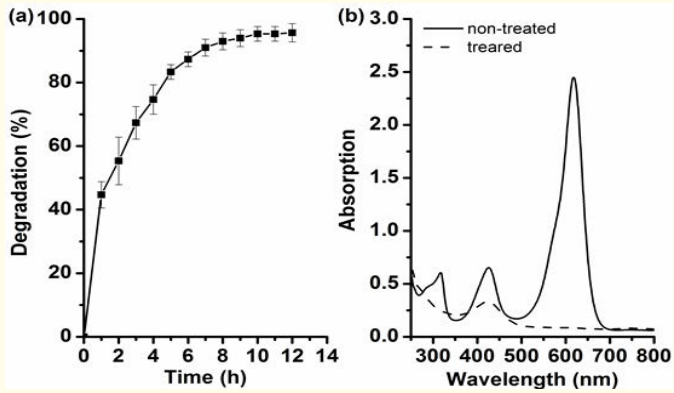

Figure 4: *Kinetics and spectrum scan of malachite green degradation. (a) Kinetics of malachite green degradation by purified laccase. (b) Spectrum scan of malachite green before and after decolorizing. One-Way ANOVA was used and P < 0.05.*

Conclusions

A fungal laccase was produced from *Trametes versicolor* 50001 for the first time. The laccase activity was of 1071 U l^{-1} after induction with 1 mM Cu^{2+}. The laccase was purified, characterized, and exhibited acidic stability and malachite green decoloration ability. The decoloration rate of malachite green (25 mg l^{-1}) was around 95.67%, suggesting that the laccase from *Trametes versicolor* has a great potential to decolorize malachite green.

Acknowledgments

This work was supported by Science and Technology Major Project of Shandong Province (2015ZDXX0403B01) and Natural Science Foundation of Shandong Province (ZR2016CQ28).

Bibliography

1. Manavalan A., *et al*. "Characterization of a Solvent, Surfactant and Temperature-Tolerant Laccase from Pleurotus Sp. Mak-Ii and Its Dye Decolorizing Property". *Biotechnology Letters* 37.12 (2015): 2403-2409.

2. Ling ZR., *et al*. "An Extracellular Laccase with Potent Dye Decolorizing Ability from White Rot Fungus Trametes Sp. Lac-01". International Journal of Biological Macromolecules 81 (2015): 785-793.

3. Yang J., *et al*. "Laccase-Catalyzed Decolorization of Malachite Green: Performance Optimization and Degradation Mechanism". *PLoS One* 10.5 (2015): e0127714.

4. Pezzella C., *et al*. "How to Enjoy Laccases". *Cellular and Molecular Life Sciences* 72.5 (2015): 923-940.

5. Giardina P., *et al*. "Laccases: A Never-Ending Story". *Cellular and Molecular Life Sciences* 67.3 (2010): 369-385.

6. Jasinska A., *et al*. "Malachite Green Decolorization by the Filamentous Fungus Myrothecium Roridum--Mechanistic Study and Process Optimization". *Bioresource Technology* 194 (2015): 43-48.

7. Murugesan Kumarasamy., *et al*. "Enhanced Transformation of Malachite Green by Laccase of Ganoderma Lucidum in the Presence of Natural Phenolic Compounds". *Applied Microbiology and Biotechnology* 82.2 (2009): 341-350.

8. Trupkin S., *et al*. "Optimization of a Culture Medium for Ligninolytic Enzyme Production and Synthetic Dye Decolorization Using Response Surface Methodology". *Journal of Industrial Microbiology and Biotechnology* 30.12 (2003): 682-690.

9. Jayasinghe Chandana., *et al*. "Degradation of Three Aromatic Dyes by White Rot Fungi and the Production of Ligninolytic Enzymes". *Mycobiology* 36.2 (2008): 114-120.

10. Mouso Nora, *et al*. "Acción De Stereum Hirsutum (Wild) Pers. En La Degradación De Colorantes". *Revista Iberoamericana de Micología* 24.4 (2007): 294-298.

11. Chen L., *et al*. "A Novel Ethanol-Tolerant Laccase, Tvlac, from Trametes Versicolor". *Biotechnology Letters* 38.3 (2016): 471-476.

12. Majeau JA., *et al*. "Laccases for Removal of Recalcitrant and Emerging Pollutants". *Bioresource Technology* 101.7 (2010): 2331-2350.

13. Yan Jinping., *et al*. "Purification and Characterization of a Thermotolerant Laccase Isoform in Trametes Trogii Strain and Its Potential in Dye Decolorization". *International Biodeterioration and Biodegradation* 93 (2014): 186-194.

14. Zouari-Mechichi Héla., *et al*. "Laccase Purification and Characterization from Trametes Trogii Isolated in Tunisia: Decolorization of Textile Dyes by the Purified Enzyme". *Enzyme and Microbial Technology* 39.1 (2006): 141-148.

15. Chmelova D and M Ondrejovic. "Purification and Characterization of Extracellular Laccase Produced by Ceriporiopsis Subvermispora and Decolorization of Triphenylmethane Dyes". *Journal of Basic Microbiology* 56.11 (2016): 1173-1182.

16. Wang KF., *et al*. "Scale-up Laccase Production from Trametes Versicolor Stimulated by Vanillic Acid". *Bioprocess and Biosystems Engineering* 39.7 (2016): 1041-1049.

17. Zhang C., *et al*. "Purification and Characterization of a Temperature- and Ph-Stable Laccase from the Spores of Bacillus Vallismortis Fmb-103 and Its Application in the Degradation of Malachite Green". *Journal of Agricultural and Food Chemistry* 61.23 (2013): 5468-5473.

18. Yang H., *et al*. "Potential of Acetylacetone as a Mediator for Trametes Versicolor Laccase in Enzymatic Transformation of Organic Pollutants". *Environmental Science and Pollution Research International* 22.14 (2015): 10882-10889.

19. Kumar GN and K Srikumar. "Characterization of Xerophytic Thermophilic Laccase Exhibiting Metal Ion-Dependent Dye Decolorization Potential". *Applied Biochemistry and Biotechnology* 167.3 (2012): 662-676.

20. Jadhav JP and SP Govindwar. "Biotransformation of Malachite Green by Saccharomyces Cerevisiae Mtcc 463". *Yeast* 23.4 (2006): 315-323.

Volume 10 Issue 3 August 2017