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Purification and characterization of a thermotolerant laccase isoform in *Trametes trogii* strain and its potential in dye decolorization

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ABSTRACT

Using response surface methodology, the maximum laccase activity of 122.9 U ml^{-1} was obtained in *Trametes trogii* S0301. The major isoform of the laccase secreted in the optimized medium was purified by 4-fold to a specific activity of 352.1 U mg^{-1} protein. The laccase (a molecular mass of 56 kDa), acted optimally at pH 3.0 and exhibited an optimum temperature of 45 °C using ABTS as substrate, with the half-life at 60 °C and 75 °C for 3 h and 10 min, respectively. The purified laccase was highly resistant to Co^{2+}, Cu^{2+}, Zn^{2+} and Mn^{2+} (100 mM). In addition, the purified laccase was effective to decolorize malachite green, bromophenol blue, crystal violet and acid red without the addition of redox mediators. Peptide-mass fingerprinting analysis by MALDI-TOF MS showed the purified laccase of *T. trogii* S0301 was a typical laccase isoform, which shared 99.3% identity with a laccase from *Coriolopsis gallica*. Further, the full-length DNA of the laccase was cloned based on the highly conservative copper-binding domains using degenerate PCR and TAIL-PCR, and the deduced amino acid sequence of the matured protein matched exactly with the peptides of the purified laccase.

1. Introduction

Laccase (EC1.10.3.2) is a group of copper-containing polyphenol oxidases which belongs to multi-copper oxidases family (Solomon et al., 1996; Hakulinen et al., 2002). The enzyme can non-specifically oxidize various phenolic and non- phenolic compounds, using molecular oxygen as the final electron acceptor (Baldrian, 2006; Hildén et al., 2009; Polak and Jarosz-Wilkolazka, 2012). Thus, laccase has many kinds of actual and potential applications in various fields, including pulp and paper industries, textile and dye industries, bioremediation (Couto and Herrera, 2006; Kunamneni et al., 2008; Dwivedi et al., 2011), organic synthesis, nanobiotechnology (Couto and Herrera, 2006; Dwivedi et al., 2011), cosmetics (Couto and Herrera, 2006), cross-linking of polysaccharides, medical applications, enzymatic and immunochemical assays (Dwivedi et al., 2011).

The first laccase was found in the Japanese lacquer tree *Rhus vernicifera* by Yoshida (1883). Nowadays, laccases have been found in plants, insects, bacteria and fungi (Dwivedi et al., 2011), but white rot fungi (WRF) are the major producers (Palonen et al., 2003; Baldrian, 2006; Dwivedi et al., 2011; Schückel et al., 2011). Interestingly, there are several isoforms with different kinetic or physicochemical features even in the same fungus strain, and those isoforms can express under different cultivation conditions or at different stages during the fungal life cycle (Baldrian, 2006; Kunamneni et al., 2008).

The activity of fungal laccases usually drops rapidly when the temperature is above 60 °C (Hildén et al., 2009; Dwivedi et al., 2011). Exceptionally, most laccases from the *Trametes* (synonym *Coriolous*) species show highly thermal stability (Hildén et al., 2009). Moreover, the *Trametes* species are among the most important sources for laccases with other attractive properties such as higher resistance to high alkalinity, extreme acidity, organic solvents and heavy metals (Boonlue et al., 2003; Hildén et al., 2009; Grassi et al., 2011; Daâssi et al., 2013). As a result, laccases from the *Trametes* strains, such as *Trametes trogii*, *Trametes pubescens* and *Trametes versicolor* have attracted much more attention (Guan et al., 2011; Zhu et al., 2011; Si et al., 2013).

The ability of *T. trogii* strains and the efficiency of their laccases to decolorize and degrade industrial dyes have been confirmed by several studies (Levin et al., 2003, 2005; Chakroun et al., 2009; Khlifi et al., 2010; Zeng et al., 2011). And some laccase isoforms of

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T. trogii strains have been purified and characterized (Vares and Hatakka, 1997; Levin et al., 2002; Deveci et al., 2004; Patrick et al., 2009; Grassi et al., 2011; Guan et al., 2011). However, those studies reported that the half-life of laccase activity ($T_{1/2}$) of the purified laccase from *T. trogii* only was 30 min at 60 °C (Patrick et al., 2009), and *T. trogii* 463 with $T_{1/2}$ of 2 h at 60 °C (Levin et al., 2002; Grassi et al., 2011). In addition, only two different laccase genes (*lcc1*, CAC13040 and *lcc2*, CAL23367) have been cloned in *T. trogii* until now (Colao et al., 2003). *T. trogii* is a thermotolerant species preferring sun-exposing habitat, and the properties of the laccases are usually correlated with the temperature range of the growth of the source organism (Hildén et al., 2009). Therefore, it is evident that there are other laccase isoforms with attractive properties in *T. trogii* strain.

T. trogii Berk S0301 strain was obtained in our previous studies, and the crude laccase of that strain showed efficient decolorization of malachite green at high temperatures and ionic concentrations (Yan et al., 2014). Based on those results, the main objectives of this study were (i) to enhance the laccase production in *T. trogii* S0301 by response surface methodology; (ii) to purify and characterize the major isoform of laccase secreted in the optimized medium; (iii) to assess the decolorization ability of the purified laccase without the addition of redox mediators, and to (iv) identify the gene of the laccase.

2. Materials and methods

2.1. Chemicals and fungal strain

2, 2′-azino bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) is from Sigma (USA), and Malachite Green (MG), Gentian Violet and Bromophenol Blue from Merck (USA). Acid Red is supplied by the Environmental Monitoring Station of Dali, Yunnan province, China. *T. trogii* S0301 strain is stored at the strain collection of Biotechnology Research Center of Life Science and Technology College, Kunming University of Science and Technology. Strain is routinely maintained on GYP slant at 4 °C (Fan et al., 2011).

2.2. Culture condition

The mycelia from the slant were transferred to the GYP plates and incubated at 30 °C for 5 d. Inocula were prepared in 250 ml Erlenmeyer flask containing 50 ml of GYP starting from 4 mycelial plugs (1 cm in diameter). Cultures grow for 5 days at 30 °C were homogenized by beaded glasses (0.3 mm in diameter), and 5% (v/v) aliquots of the mycelia suspension were used as inocula for the further study.

2.3. Optimization of laccase production by response surface methodology (RSM)

In this study, the production optimization medium contained glucose, yeast extract/peptone (1/1), $FeSO_4$, $CaCl_2$, $MgSO_4$, $MnSO_4$, VB_1, $CuSO_4$, phenol, Tween-80 and PEG 4000. $CuSO_4$, phenol and PEG 4000 were considered the most effective independent variables based on the results of the earlier Plackett–Burman experiment (Design-Expert 8.0.5 b). Using the laccase production as the response variable, further optimization was conducted by the central composite design (CCD) of RSM, and $CuSO_4$, phenol and PEG 4000, at five different levels ($-\alpha$, −1, 0, +1 and $+\alpha$), were chosen in this study. The levels of these variables and twenty runs were designed by Design-Expert 8.0.5 b (Table 1). The proof tests were carried out under the optimum medium contained glucose 28 g L^{-1}, yeast extract/peptone 10 g L^{-1}, $FeSO_4$ 0.4 mM, VB_1 0.2 g L^{-1}, $CuSO_4$ 1.65 mM, phenol 30.54 mg L^{-1}, Tween-80 6 g L^{-1} and PEG4000 45 mg L^{-1} according to the fitted equation below (X_1-$CuSO_4$, X_2-phenol and X_3 -PEG4000). Variance (ANOVA) and regression analysis, and contour plots were drawn by Design-Expert 8.0.5 b.

Table 1
Experimental design and results of CCD.

Std	$CuSO_4$ (mM)	Phenol (mg l^{-1})	PEG4000 (mg l^{-1})	Response for activity (U ml^{-1})		Residual value (U ml^{-1})
	X_1	X_2	X_3	Actual	Predicted	
1	1.15	24.50	45.00	63.95	66.15	−2.20
2	1.65	24.50	45.00	93.92	98.45	−4.53
3	1.15	31.50	45.00	49.88	54.04	−4.17
4	1.65	31.50	45.00	115.42	117.32	−1.90
5	1.15	24.50	75.00	82.04	83.75	−1.72
6	1.65	24.50	75.00	77.33	76.78	0.55
7	1.15	31.50	75.00	56.96	56.04	0.92
8	1.65	31.50	75.00	78.63	80.05	−1.42
9	0.98	28.00	60.00	33.61	31.09	2.52
10	1.82	28.00	60.00	81.03	78.43	2.59
11	1.40	22.11	60.00	80.13	77.18	2.95
12	1.40	33.89	60.00	71.91	69.75	2.16
13	1.40	28.00	34.77	125.32	119.46	5.86
14	1.40	28.00	85.23	102.16	102.92	−0.75
15	1.40	28.00	60.00	94.36	91.87	2.49
16	1.40	28.00	60.00	85.70	91.87	−6.17
17	1.40	28.00	60.00	94.43	91.87	2.57
18	1.40	28.00	60.00	98.34	91.87	6.48
19	1.40	28.00	60.00	86.60	91.87	−5.27
20	1.40	28.00	60.00	90.89	91.87	−0.98

$$Y = -665.95 + 553.32X_1 + 21.18X_2 + 1.77X_3 + 8.85X_1X_2 - 2.62X_1X_3 - 0.074X_2X_3 - 209.90X_1^2 - 0.53X_2^2 + 0.030X_3^2$$

2.4. Laccase purification

The supernatants of 9-day-old liquid culture of *T. trogii* S0301 were obtained by centrifugation (9000 rpm, 5 min at 4 °C), and then the total protein was precipitated from the supernatants with ammonium sulfate (80% saturation). The precipitates formed was dissolved in buffer A (50 mM Tris–HCl, pH 6.0) and dialyzed against the same buffer overnight at 4 °C (8 kDa cut off). The dialyzed enzyme solution was loaded onto a Q SepharoseTM ion-exchange chromatography column (GE Healthcare) pre-equilibrated with 1 M NaCl in buffer A, and then the enzyme was eluted with a linear salt gradient in 0–1 M NaCl in buffer A at the flowing rate of 2 ml min^{-1}. The fractions with laccase activity were collected, concentrated by lyophilization and dissolved in 3 ml buffer A. And then, the concentrated enzyme solution was loaded onto a Sephadex G-75 Medium chromatography (Biotopped) column pre-equilibrated with buffer A, and then eluted with buffer A at the flowing rate of 0.4 ml min^{-1}. The purified laccase was collected and stored at −20 °C until use.

2.5. Enzyme assay

Laccase activity was determined with ABTS ($\varepsilon 420 = 36 (mM\,cm)^{-1}$) as substrate. Mixture of 0.5 ml appropriately diluted crude or purified enzyme and 1.1 ml of 2 mM ABTS in phosphate citrate buffer (100 mM, pH 4.0) was used to determine the activity. The increase in absorbance was monitored at 420 nm for 3 min (Bourbonnais and Michael, 1990). One unit of the enzyme activity was defined as the amount of the enzyme that oxidized 1 μmol of the ATBS per minute. Protein concentration was estimated by the Bradford method (Bradford, 1976), with bovine serum albumin as the standard. All experiments were