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Original Research Paper

Optimization of the production, purification and characterization of a laccase from the native fungus *Xylaria* sp.

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ABSTRACT

The production of a laccase enzyme secreted by the Ascomycota fungus *Xylaria* sp. was improved under submerged fermentation using a combination of the one factor-at-a-time method for different carbon and nitrogen sources, a Taguchi orthogonal array and inducers. The laccase activity of the optimized culture was $20{,}535 \pm 1405$ U L^{-1}, which is 10 times higher than the activity of the control culture (1929 ± 44 U L^{-1}) and a high value compared with other fungi. From this culture, a laccase was purified through diafiltration and anion exchange and size exclusion chromatography, with a purification factor of 7.45 and a 0.51% yield. According to a two-dimensional (2D) electrophoretic analysis, the molecular mass of the protein was 38 kDa and had a pI of 4.9. Analysis of the protein using mass spectrometry revealed the presence of the peptides GPASAPYDEDK and LVNTAIDTMFK, which coincide with other reported laccases. The purified enzyme had an optimal activity at pH 3.0–4.0 and 50–66 °C. Using 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as substrate, a K_m of 297 μM and V_{max} of 581.4 μM min^{-1} were determined. The enzyme was stable at temperatures between 4–30 °C and pH values of 6–8, and it was inhibited by the Fe^{2+} ion but induced by the Cu^{2+} ion. Additionally, the enzyme was slightly inhibited by ethylenediaminetetraacetic acid (EDTA) but strongly inhibited by sodium azide, dithiothreitol (DTT) and potassium cyanide (KCN). The observed biochemical characteristics found indicate that *Xylaria* sp. laccase has the potential for use in biotechnological processes related to bioremediation.

1. Introduction

Laccases (benzenediol-oxygen oxidoreductases; EC 1.10.3.2) are oxidases with multiple copper atoms that catalyze the oxidation, by one electron, of phenolic, aromatic, amino, and other substrates rich in electrons. Consequently, O_2 is reduced to H_2O by a free radical mechanism (Baldrian, 2006). The use of these enzymes has been extensively reported in many fields, such as the degradation of phenolic contaminants, the avoidance of undesired food component oxidation in the food industry, and the fabrication of biosensors for the determination of phenolic compounds (Kudanga et al., 2011).

Laccases are widely distributed in nature and are present in fungi (largest producers), higher plants, bacteria, and insects (Riva, 2006). In fungi, these enzymes have been isolated from Ascomycetes, Deuteromycetes, and Basidiomycetes (Brijwani et al., 2010), which secrete laccases that contribute to lignin degradation and play an essential role in the carbon cycle by degrading lignocellulosic material (Leonowicz et al., 2001). The production of laccases has been documented for many fungi, such as *Pleorotus ostreatus*, *Trametes pubescens*, *Pycnoporus cinnabarinus*, among others (Eggert et al., 1996; Palmieri et al., 2000; Galhaup et al., 2002).

In this work, we used a fungus strain from the *Xylaria* genre, which was isolated from decomposing wood obtained from Chicaque Natural Park. This park is located in the High Andean Forest ecosystem, along the Chicaque trail, in the municipality of San Antonio del Tequendama, Cundinamarca, Colombia. Fungi of the Xylariaceae family cause a special type of decomposition in wood known as type-II soft rot that is similar to the decomposition caused by brown-rot fungi, which produces strong cellulose and hemicellulose degradation and moderate lignin alteration (Blanchette, 1995). Unlike the effect of white-rot fungi, which produce strong lignin and cell wall polymer degradation (Blanchette, 1995; Schilling et al., 2012).

The production of fungi laccases in submerged cultures has been widely reported (Stajic et al., 2006; Osma et al., 2007; Elisashvili et al., 2009). However, the constitutive expression of

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laccases has been low when fungi are cultured following this strategy, even using the natural substrate (Galhaup et al., 2002). Larger amounts of the enzyme can be induced by the addition of aromatic compounds, such as 2,5-xylidine, guaiacol, veratryl alcohol, lignin, or ferulic acid, into the culture media (Xiaoyu et al., 2013). A larger inductive effect in laccase yield has been observed for 2,5-xylidine (Eggert et al., 1996; Revankar and Lele, 2006). The optimal inducer concentration depends on the fungal species. The carbon and nitrogen sources used for the microbial culture are also important factors that affect the enzyme yield (Stajic et al., 2006; Elsayed et al., 2012; Xiaoyu et al., 2013).

This study aimed to improve the production of laccases in the fungus *Xylaria* sp. using submerged fermentation by combining the one factor-at-a-time method for different carbon and nitrogen sources, orthogonal design and different aromatic inducers. Few studies have investigated this approach, although good results have been obtained (Revankar and Lele, 2006; Nandal et al., 2013). In addition, the purification and characterization of laccase secreted by this organism were performed as the initial step towards the formulation of a biotechnological application.

2. Materials and methods

2.1. Sample collection and isolation

Laccase positive ascomycete fungus of the *Xylaria* sp. used in this study was isolated from woody decay on forest soil in Chicaque Natural Park, Cundinamarca, Colombia (N 4°36′32″ and W 74°18′25″; 2410 altitude; 21 °C temp; and 75% relative humidity) and deposited in a public culture collection belonging to the WDCM (Pontificia Universidad Javeriana-148-CMPUJ register). The fruit body was cut into pieces that were immersed in 96% (v/v) ethanol for 30 s and washed several times with sterilized water. The samples were dried and inoculated onto potato dextrose agar media (PDA) with added chloramphenicol (200 mg L^{-1}) and kanamicine (50 mg L^{-1}) to avoid bacterial contamination. They were subcultured on PDA at 25 °C until pure fungal isolates were obtained. After purification, the fungi were maintained on agar plates that contained a medium with the following composition: wheat bran 50 g L^{-1}, $(NH_4)_2SO_4$ 1.4 g L^{-1}, KH_2PO_4 2.0 g L^{-1}, $CaCl_2 \cdot 2H_2O$ 0.4 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.3 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 5 mg L^{-1}, $MnSO_4 \cdot H_2O$ 1.18 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 1.4 mg L^{-1}, $CuSO_4 \cdot 5H_2O$ 3 mg L^{-1}, and bacteriological agar 15 g L^{-1} at 4 °C. The samples were stored in glycerol stocks at −80 °C for long-term storage and were sub-cultured every three months.

2.2. Culture preparation and submerged fermentation

The cultures were carried out in Erlenmeyer flasks (100 ml) covered with aluminum paper, which contained 30 mL of a medium with the following modified composition: Wheat bran 50 g L^{-1}, $(NH_4)_2SO_4$ 1.4 g L^{-1}, KH_2PO_4 2 g L^{-1}, $CaCl_2.2H_2O$ 0.4 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.3 g L^{-1}, $FeSO_4 \cdot 7H_2O$ 5 mg L^{-1}, $MnSO_4 \cdot H_2O$ 1.18 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 1.4 mg L^{-1}, and $CoCl_2 \cdot 6H_2O$ 2.6 mg L^{-1} before the sterilization pH was adjusted to 5.6. The media were inoculated with three agar plugs (diameter, 5 mm) from an actively growing fungus on a wheat bran agar plate. Flasks were grown at 26 ± 2 °C with continuous shaking at 150 rpm for 16 days in the dark. The samples were measured every two days to determine the laccase activity.

2.3. Effects of carbon and nitrogen sources in the media culture using the one factor-at-a-time method

To investigate the effects of the carbon and nitrogen sources on laccase production using the *Xylaria* sp., the one factor-at-a-time method was used. In the culture media, the carbon source was substituted with five carbohydrate substrates, including fructose (10 g L^{-1}), starch (10 g L^{-1}), mandarin peels (40 g L^{-1}), and empty fruit bunches of oil palm previously cut at 1 mm size fiber (50 g L^{-1}) and 10 mm size fiber (50 g L^{-1}). The nitrogen source $(NH_4)_2SO_4$ was then replaced with three different sources, including yeast extract, peptone, and KNO_3, at the same concentration (1.4 g L^{-1}) using the best carbon source selected previously.

Table 1
Results of Taguchi orthogonal array L_9 (3^4) for production of laccase from *Xylaria* sp. fungus.

(a) Design and results for the orthogonal array experiments carried out.

Experiment	Concentration levels*				Laccase activity (U L^{-1})
	Wheat bran	**KNO_3**	**$CuSO_4$**	**KH_2PO_4**	
1	1	1	1	1	1013.6 ± 39
2	1	2	2	2	1201.3 ± 149
3	1	3	3	3	3030.1 ± 120
4	2	1	2	3	14,218.1 ± 1512
5	2	2	3	1	11,369.7 ± 764
6	2	3	1	2	9665.6 ± 1008
7	3	1	3	2	11,250.0 ± 1457
8	3	2	1	3	9125.51 ± 561
9	3	3	2	1	9809.67 ± 726

(b) Analysis of the results from the Taguchi orthogonal array

Factors	Level 1	Level 2	Level 3	Range
	(U L^{-1})	(U L^{-1})	(U L^{-1})	(U L^{-1})
Wheat bran	1748.0	11,716.3	10,061.2	9968.3
KNO_3	8792.4	7231.8	7501.3	1560.6
$CuSO_4 \cdot 5H_2O$	6601.1	8375	8549.4	1948.3
KH_2PO_4	7397.3	7371.8	8756.4	1359.1

* The numbers 1–3 refers to different concentration levels described in each case within material and methods section.

2.4. Optimization of the media composition using the Taguchi orthogonal matrix method

A L_9 (3^4) orthogonal array was designed to evaluate four components at three different concentration levels. The four components were wheat bran (40, 50 and 60 g L^{-1}); KNO_3 (1.4, 2.5 and 5 g L^{-1}); $CuSO_4 \cdot 5H_2O$ (0.002, 0.004 and 0.006 g L^{-1}) and KH_2PO_4 (0.5, 1.0 and 2.05 g L^{-1}). The arrangement of each experimental combination is shown in Table 1a. The experiments were carried out at 26 ± 2 °C and continuous shaking at 150 rpm in the dark. The laccase activity was assayed at eighth day, which was the best enzymatic production time found. The results were analyzed by Qualitek-4 (17.1.0) software (Nutek Inc, Bloomfield Hills, Michigan USA), using the option *Bigger is better*. This software was also used to predict the theoretical laccase activity using the best concentrations that were found for each component of the media.

2.5. Laccase activity

Laccase activity was determined by measuring the oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) according to Gomes et al. (2009). One unit (U) was defined as 1 μmol of ABTS oxidized per minute, and the activity was expressed in U L^{-1}. The enzymatic activity was expressed as the mean of three biological replicates.