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Assessment of *Heteroporus biennis* secretion extracts for decolorization of textile dyes

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ABSTRACT

In the present study, the dye-decolorizing properties of soluble extracts from the indigenous fungus *Heteroporus biennis* IOC4581 were determined. Decolorizing activity was associated with the secretion of a 45-kDa laccase. Laccase expression was extremely sensitive to carbon and nitrogen contents in culture media. Supplementation with metal ions and aromatic compounds resulted in extremely variable protein secretion profiles, although enzyme yields were not improved. Kinetic parameters of the soluble crude extract were promising, destaining seven out of nine textile dyes without the need of redox mediators. The extract was insensitive to Ca^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} ions, and catalysis was enhanced in the presence of Cu^{2+} and Al^{3+} ions. The soluble fungal extract retained its maximal decolorizing activity at a wide range of pH (2.0–9.0) and temperature (4–50 °C), indicating potential application to dye biodegradation.

1. Introduction

The textile dyeing industry consumes large volumes of water, and subsequently disposes of colored effluents containing complex mixtures of dyes, organic compounds, and salts into water reservoirs (Adinew, 2012). Among the several strategies for the treatment of these wastewaters, biological approaches such as enzyme-mediated decolorization are increasingly gaining attention because of reduced costs, decreased consumption of reagents, and biodegradability of the resulting by-products (Rauf and Salman, 2012). Several enzymes that display dye-decolorizing properties have been described in the literature, and most of these correspond with the so-called lignin-modifying enzymes (LMEs). These are generally secreted by fungi associated with decaying wood and litter (Wesenberg et al., 2003). The enzymes involved in this process are mainly laccases (Lacs) (EC 1.10.3.2) and peroxidases (EC 1.11.1), which possess relatively low specificity. Nonetheless, LMEs degrade a broad range of man-made xenobiotics that share structural similarities with the building blocks of the natural substrate lignin (Pointing, 2001).

Although several reports describe enzymes with potential applications to dye decolorization and treatment of colored effluents, these enzymes are often sensitive to variable pH, temperature, salt concentrations, and dye contents. Thus, novel robust enzymes with desirable catalytic properties are required.

In this study, the dye-decolorizing activity of a soluble secretion extract from a *Heteroporus biennis* isolate was investigated. The identity of the enzyme involved in the process and the effects of several culture parameters, such as carbon, nitrogen, metal ions, and aromatic contents, on its expression were also investigated. In addition, the performance and stability of the crude enzyme in the presence of wide ranges of pH, salt concentration, and temperature were described. Finally, the effects of redox mediators on decolorization of several substrates were assessed.

2. Materials and methods

2.1. Dyes and chemicals

All test dyes were kindly supplied by Dystar (São Paulo, Brazil) and Siderquímica (Curitiba, Brazil). Test batches had a minimum dye concentration of 85% (w/w). 2,2′-Azino-bis-(3-

Abbreviations: PAGE, Polyacrilamide gel electrophoresis; SDS, Sodium dodecyl sulfate; ABTS, 2,2′-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid); RB220, Reactive blue 220; 2,6 DMP, 2,6-Dimethoxyphenol; LMM, Liquid minimal medium; SMM, Solid minimal medium; EDTA, Ethylenediaminetetraacetic acid; MnP, Manganese peroxidase; MiP, Manganese-independent peroxidase.

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ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,6-dimethoxyphenol (2,6 DMP), guaiacol, and redox reagents were obtained from Sigma Chemical Company (St. Louis, MO, USA). All other chemicals were of the highest available purity and analytical grade.

2.2. Organisms and culture conditions

Fresh fruiting bodies of the white rot fungus *H. biennis* were collected from a tree stump at the campus of the Federal University of Paraná (Curitiba, Brazil) and maintained in potato dextrose agar plates using serial cultivation at 28 °C in the dark. The isolate was identified according to morphology and then deposited at the Institute Oswaldo Cruz culture collection as *H. biennis* IOC4581. Prior to experiments, the fungus was cultured on plates of solid minimal medium (SMM) containing (per liter): 6.0 g $NaNO_3$, 1.5 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4$, 0.01 g $FeSO_4 \cdot 7H_2O$, 0.02 g $ZnSO_4 \cdot 7H_2O$, 10.0 g glucose, and 10.0 g bacteriological agar at pH 6.8. Liquid minimal medium (LMM) had identical contents but lacked agar. After 7–10 days of growth on SMM, 4-mm-diameter plugs from active culture borders were used to inoculate 5 mL replicates of sterilized liquid culture media. The cultures were then incubated at 28 °C prior to enzyme assays.

2.3. Preparation of soluble culture-derived extracts

Liquid culture samples were centrifuged at 1500 rpm for 5 min, the supernatants were collected for further analysis, and biomass was determined in mycelia pellets. Solid culture samples were washed two times in 2 mL of salt solution or glucose- and $NaNO_3$-free LMM for 30 min in an orbital shaker at 150 rpm. Washed samples were then collected and centrifuged, and the supernatants were used for direct analysis or stored at −10 °C. Mycelial biomass was calculated after filtration through previously dried and tared Whatman No. 1 filter papers, washing in distilled water, and drying at 50 °C to a constant weight.

2.4. Decolorization assays

Decolorizing activities of fungus-derived samples were assessed using reactive blue 220 (RB220) as a standard substrate. To 1 mL of the corresponding secretion extract, 0.1 mL of 10 × RB220 stock was added, giving a final dye concentration of 0.1 g L^{-1}. The absorbance of the reaction mixture was monitored at 280 and 600 nm on a Shimadzu UV-160A UV–Vis spectrophotometer for 90 min at 28 °C. Percentage decolorization was calculated as follows:

$$\text{decolorization}(\%) = \frac{(A_0 - A_{90})x100}{A_0} \quad (1)$$

where A_{90} is the absorbance (at 600 or 280 nm) after 90 min incubation, and A_0 is the initial absorbance (at the same wavelength) of the assay mixture.

2.5. Effects of carbon and nitrogen contents, and metal ion and aromatic compound supplementation on decolorizing activity

Six carbon (glucose, maltose, fructose, starch, sucrose, and glycerol) and six nitrogen (sodium nitrate, ammonium chloride, ammonium tartrate, ammonium oxalate, urea, and peptone) sources were introduced to culture media, and decolorizing activities of soluble extract supernatants and soluble extracts from fungal cultures were determined. The concentrations used for each source were 5, 10, and 15 g L^{-1}. Alternatively, cultures were supplemented with copper sulfate or manganese sulfate (0.1 and 0.25 mM, respectively) and the aromatic compounds veratryl alcohol, veratraldehyde, vanillic acid, 2,5-xylidine, ferulic acid, vanillin, p-anisidine, or gallic acid. Each aromatic compound was diluted in deionized water or 50% ethanol, sterilized by filtration, and added to culture media at a final concentration of 1 mM. The cultures were incubated for 15 days at 28 °C in the dark, and supernatants and mycelia were collected as described above.

2.6. LMEs

The activity of manganese peroxidase (MnP), manganese-independent peroxidase (MiP), and Lac were spectrophotometrically determined in supernatants or soluble culture extracts using ABTS, as described previously (Jordaan and Leukes, 2003). One unit of enzyme was defined as the amount of sample that oxidizes 1 μmol of substrate per min.

2.7. Effects of pH, temperature, and salt concentration on decolorizing activity

Supernatants and soluble extracts derived from 15-day-old cultures were tested for decolorizing activity under optimized conditions (carbon and nitrogen sources and metal ion supplementation) and under varying conditions of pH, temperature, and salt concentration. Adjustments of pH from 2.0 to 12 were performed using 50 mM citrate-phosphate buffer. Reaction mixtures were also assayed at temperatures from 20 to 70 °C and NaCl concentrations of 0.05–0.6 M. In all experiments, UV–Vis absorbance was determined after incubation for 90 min at 28 °C. The stability of culture extracts was determined by preincubation of samples under the conditions of pH, temperature, and salt concentration described above at intervals of 0, 3, 6, 9,and 24 h. Decolorizing activity was measured at the end of each incubation period. Residual decolorization was calculated as a percentage of the highest decolorizing value observed under each condition of temperature, salt concentration, and pH.

2.8. Effects of redox mediators on decolorizing activity

The decolorizing ability of culture supernatants was verified using several commercial dyes (Supplementary Table 1) in the presence and absence of the redox mediators p-coumaric acid, vanillin, acetosyringone, or syringaldehyde. The reaction mixture contained 1 mL of culture supernatant, a dye (0.1 g L^{-1}), and a redox mediator (1 mM) in a final volume of 1.1 mL. The absorbance of the mixture at the λ_{max} of each dye was measured after 30 and 90 min and after 24 h of incubation at 28 °C, and percentage decolorization was calculated.

2.9. Effects of metal ions on decolorizing activity

The effects of several metal ions and chelating agents on decolorizing activity was determined by adding $CaCl_2$, $MgCl_2$, $FeCl_2$, $MnSO_4$, $ZnSO_4$, $CuSO_4$, $Al_2(SO_4)_3$, $MgSO_4$, or ethylenediaminetetraacetic acid (EDTA) to the decolorization assay at 6–50 mM.

2.10. Kinetic parameters

Secretion extracts from 15-day-old liquid cultures were used to assess kinetic parameters using ABTS (2.5–250 μM) and 2,6 DMP (5.5–550 μM)as substrates. Assays were performed in 0.18 M citrate buffer (pH 4.5) at 20 °C, and substrate oxidation was monitored at 420 and 470 nm for ABTS and DMP, respectively. Michaelis–Menten constant (K_m) and maximum reaction velocity (V_{max}) were calculated using Hanes–Woolf plots.