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Decolorization of textile dyes by whole cultures of *Ischnoderma resinosum* and by purified laccase and Mn-peroxidase

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Abstract

Ischnoderma resinosum produced extracellular ligninolytic enzymes laccase and MnP. The activity of laccase achieved the maximum on day 10 (29.4 U L^{-1}), the MnP on day 14 (34.5 U L^{-1}). Laccase and Mn-peroxidase were purified from the culture liquid using gel permeation and ion-exchange chromatographies. Purified Mn-peroxidase performed decolorization of all textile dyes tested (Reactive Black 5, Reactive Blue 19, Reactive Red 22 and Reactive Yellow 15). Laccase was inactive with Reactive Black 5 and Reactive Red 22, while all dyes were decolorized after addition of the redox mediators violuric acid (VA) and hydroxybenzotriazole (HBT). The culture liquid from *I. resinosum* cultures was also able to decolorize all dyes as well as the synthetic dyebaths in the presence of VA and HBT. The highest decolorization rates were detected in acidic pH (3–4).

Keywords: *Ischnoderma resinosum*; Basidiomycetes; Laccase; Mn-peroxidase; Textile dyes; Ligninolytic enzymes

1. Introduction

Wastewaters from textile industries represents a serious problem all over the world. They contain different types of synthetic dyes, which are mostly toxic, mutagenic and carcinogenic. Moreover, they are very stable to light, temperature and microbial attack, making them recalcitrant compounds. Conventional wastewater treatment systems are often inefficient and existing physical and chemical technologies are expensive, time-consuming and methodologically demanding. Currently, one of the possible alternatives for treatment of textile effluents is the use of ligninolytic fungi, which can oxidize a wide spectrum of organic pollutants including synthetic dyes [1–3].

Biodegradative ability of white-rot fungi is generally assumed to be associated with the production of extracellular ligninolytic enzymes [4,5], among them laccase and manganese peroxidase being the most intensively studied. As the use of whole fungal cultures for decolorization is not easily applicable in a large scale [6], at present, the studies of in vitro decolorization of synthetic dyes by crude or purified enzymes becomes more important [7–11]. The phenol oxidase laccase (*p*-diphenol: oxygen oxidoreductase; EC 1.10.3.2) has been studied for a long time [12–14]. It is a multicopper enzyme which catalyses the oxidation of wide number of phenolic compounds and aromatic amines but its substrate specificity can be extended to non-phenolic compound in the presence of low molecular mass compounds acting as mediators [15,16]. Manganese peroxidase (EC 1.11.1.13) is a heme-containing enzyme catalysing the oxidation of Mn^{2+} to Mn^{3+}, which in turn can oxidise a variety of phenolic substrates [17,18].

Many white-rot fungi (e.g. *Phanerochaete chrysosporium*, *Pleurotus ostreatus*, *Bjerkandera adusta*, *Trametes versicolor*, etc.) and their enzymes have been intensively tested in connection with the decolorization of synthetic dyes [9,19–24]. Recently, we demonstrated both high production of ligninolytic enzymes and decolorization of synthetic dyes Orange G and Remazol Brilliant Blue R by liquid cultures of the white-rot fungus *Ischnoderma resinosum* [25]. The main objectives of this work were: (i) to evaluate the contribution of the ligninolytic enzymes laccase and manganese peroxidase of *I. resinosum* to the degradation of synthetic dyes, commonly used in the textile industry and (ii) to test the possible enhancement of decolorization by the use of laccase redox mediators and the ability of *I.*

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resinosum to decolorize textile dyes contained in the synthetic dyebaths.

2. Material and methods

2.1. Organism and cultivation

I. resinosum (Fr.) P. Karst CCBAS 553 was obtained from the CCBAS collection (Institute of Microbiology ASCR, Prague, Czech Republic). The strain was maintained by serial transfers and kept on wort agar slants at 4 °C.

Cultivation was carried out in 100 mL Erlenmeyer flasks with 20 mL of N-limited (0.2 g L^{-1} of ammonium tartrate) liquid Kirk medium [26]. The flasks were inoculated with two wort agar plugs (2 °C balling, 10 mm diameter), cut from the actively growing part of a colony on a Petri dish, and incubated at 27 °C for 20 days. Enzyme activity was measured in culture filtrates detained after mycelia removal.

Biomass production in liquid media was evaluated by determining the dry mass of mycelia. Mycelia were harvested from the cultivation flasks, washed with distilled water, dried at 105 °C for 24 h and weighed.

2.2. Ligninolytic enzyme assays

Activities of extracellular laccase (EC 1.10.3.2) and manganese peroxidase (EC 1.11.1.13, MnP) were determined spectrophotometrically by monitoring the absorbance increase at 425 nm (laccase) or 590 nm (MnP) in the reaction mixture. Laccase activity was assayed according to [27] by monitoring the oxidation of ABTS. Determination of MnP activity using MBTH and DMAB was based on the method of [28] modified according to [29]. MBTH and DMAB were oxidatively coupled by the action of the enzyme in the presence of added H_2O_2 and Mn^{2+} ions to give a purple indamine dye product. The values were corrected for the activities in the test samples (a non-specific peroxidase activity) without manganese, where manganese sulfate was substitued by ethylenediaminetetraacetate (EDTA) to chelate Mn^{2+} ions present in the extract. All measurements were repeated three times. One unit of enzyme activity (U) was defined as an amount catalyzing the production of 1 μmol of green or purple product per mL per min.

2.3. Purification of laccase and MnP

The culture liquid from a 30-day culture of *I. resinosum* grown on N-limited Kirk medium [26] was filtered and concentrated by ultrafiltration. The concentrate was loaded onto a DEAE–sepharose column (Pharmacia LKB, HR 10/10) equilibrated with 20 mM phosphate buffer, pH 6.0. The proteins were eluted with a gradient of 0–1 M NaCl at a flow rate of 0.5 mL min^{-1}. The active fractions were pooled, desalted and applied to a CIM QA Disk (BIA Separation, Slovenia) equilibrated with 20 mM phosphate buffer, pH 6.0. The fractions were eluted with a gradient of 0–1 M NaCl at a flow rate of 4 mL min^{-1}. Desalted active fractions were applied again to CIM QA Disk (BIA Separation, Slovenia) equilibrated with 20 mM phosphate buffer, pH 7.0 and the proteins were eluted under the conditions described above. After this step the concentrated purified laccase fractions were pooled and kept frozen at −18 °C. MnP purification process continued with two other steps, where active fractions were desalted and again chromatographed on CIM QA Disk (BIA Separation, Slovenia) equilibrated with 20 mM phosphate buffer, pH 7.0. The proteins were eluted with a gradient of 0–1 M NaCl at a flow rate of 4 mL min^{-1}. In the last step the MnP fractions were desalted, chromatographed on CIM QA Disk (BIA Separation, Slovenia), equilibrated with 50 mM acetate buffer, pH 4.5, and the proteins were eluted under the conditions described above. Concentrated purified MnP fractions were pooled and kept frozen at −18 °C. The enzymes were purified until electrophoretic homogeneity (checked by SDS-PAGE). The effect of pH on laccase and MnP activities was examined in the pH range 3.0–8.0 in a 0.1 M citrate–0.2 M phosphate buffer at 25 °C. Small molecular mass fraction of culture liquid was obtained by ultrafiltration using Amicon stirred cell (Millipore, USA) with a 10 kDa cut-off membrane. The small molecular mass fraction did not contain detectable laccase activity.

2.4. Decolorization

Synthetic dyes Reactive Black 5 (RK; azo), Reactive Blue 19 (RB; anthraquinone), Reactive Red 22 (RR; azo), and Reactive Yellow 15 (RY; azo) were used at a concentration of 100 ppm in all experiments. Decolorization was expressed as per cent decrease of absorbance at the respective absorbance maxima: 595 nm for RK, 592 for RB, 519 for RR, and 416 for RY. The decolorization reactions included:

(a) purified laccase from *I. resinosum* (25 mU/mL), with or without the redox mediators violuric acid (VA, 5 mM) and hydroxybenzotriazole (HBT, 5 mM);
(b) purified Mn-peroxidase from *I. resinosum* (115 mU/mL) with 10 mM H_2O_2 and 20 mM $MnSO_4$. 100 mM succinate–lactate buffer, pH 4.5 was used to provide the organic acid for Mn^{2+} chelation;
(c) culture liquid from *I. resinosum* culture diluted with distilled water to contain 25 mU/mL laccase and 115 mU/mL Mn-peroxidase, with or without 5 mM VA, 5 mM HBT, or 10 mM H_2O_2 and 20 mM $MnSO_4$ in 100 mM succinate–lactate buffer, pH 4.5.

The effect of pH on dyes decolorization was examined in the pH range 3.0–8.0 in a 0.1 M citrate–0.2 M phosphate buffer using culture liquid from *I. resinosum* culture diluted with distilled water to contain 25 mU/mL laccase and 115 mU/mL Mn-peroxidase, with or without 5 mM VA, 5 mM HBT, or 10 mM H_2O_2 and 20 mM $MnSO_4$. In the same experiment, culture liquid was also used for the decolorization of dyes dissolved in water (initial pH around 6.1) and within synthetic dyebaths (initial pH around 5.7). The dyebaths contained (per liter): 1200 mg NaCl, 5 mg Na_2CO_3, 5 mg NaOH, 10 mg EDTA, and 10 mM acetic acid. All decolorization reactions were run in three replicates and proceeded at 25 °C in the dark.

3. Results

Under our experimental conditions, *I. resinosum* produced extracellular ligninolytic enzymes laccase and MnP, lignin peroxidase was not detected (data not shown). The activity of laccase achieved the maximum on day 10 (29.4 U L^{-1}), the MnP on day 14 (34.5 U L^{-1}) (Fig. 1). The biomass production was 4–5 g L^{-1} with the maximum on day 14. Laccase and Mn-peroxidase were purified from the culture liquid using gel permeation and ion-exchange chromatographies.

The decolorization of 100 ppm solutions of all textile dyes tested in this study with purified laccase (25 mU/mL) was detected only with PB and PY, the dyes PB and PR were not

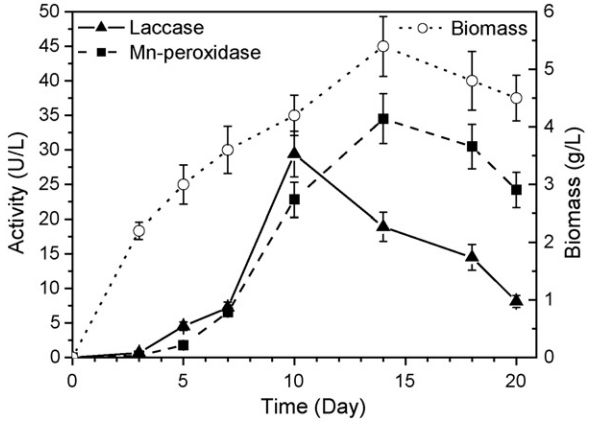

Fig. 1. Activity of ligninolytic enzymes and production of biomass by *I. resinosum* in liquid N-limited medium. The data represent averages and standard deviations ($n = 3$).