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Lignin degradation by selected fungal species

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HIGHLIGHTS

- *Pleurotus* spp. were the best laccase producers, but the weakest lignin degraders.
- MnIP was reported in *Lenzites betulinus*, *Fomitopsis pinicola* and *Dichomytus squalens*.
- *Dichomytus squalens* is promising lignin degrader.
- Key role in lignin degradation is attributed to Mn-oxidizing peroxidases.

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ABSTRACT

As biological decomposition of plant biomass represents a popular alternative environmental-friendly and economically justified process, screening of ligninolytic enzyme systems of various fungal species is a topical study area. The goal of the study was to obtain clear insight into the dynamics of laccase, Mn-dependent peroxidase, and Mn-independent peroxidase activity and levels of wheat straw lignin degradation in seven wood-rotting fungi. The best laccase producers were *Pleurotus ostreatus* and *Pleurotus eryngii*. *Lenzites betulinus* and *Fomitopsis pinicola* were the best Mn-dependent peroxidase producers, and *P. ostreatus* the weakest one. The peak of Mn-independent peroxidase was noted in *Dichomytus squalens*, and the minimum value in *P. ostreatus*. The profiles of the three enzymes, obtained by isoelectric focusing, were variable depending on the species and cultivation period. *D. squalens* was the best lignin degrader (34.1% of total lignin amount), and *P. ostreatus* and *P. eryngii* the weakest ones (7.1% and 14.5%, respectively).

1. Introduction

Permanent intensive development of agriculture and industry causes production of various plant raw materials in enormous amount, as well as presence of pesticides, toxic xenobiotics, polycyclic aromatic hydrocarbons, chlorophenols, metal, metalloids and other heavily degradable compounds (Limón-Pacheco and Gonsebatt, 2009). Only 3% of annual production of agricultural residues (approximately, 170–200 billion tons) is used directly and the rest becomes environmental ballast (Zechendorf, 1999). Resistance of plant biomass biodegradation is related directly to the presence of lignin, one of the most recalcitrant natural compounds (Martínez et al., 2005). According to the authors, in nature, lignin mineralization is an enzyme-dependent process, which is catalyzed by complex ligninolytic enzyme systems composed of extracellular oxidoreductases, such as laccase and peroxidases. Wood-rotting fungi are the only eukaryotic organisms producing these enzymes and they have a crucial role in degradation of plant raw materials, as well as numerous phenolic pollutants and therefore, in bioremediation of soil and industrial waters (Baldrian and Šnajdr, 2006). However, efficiency of the complex degradation processes depends on the potential of the degrading organism, its oxidative mechanisms, and culture conditions (Wan and Li, 2010). Although study of ligninolytic enzyme systems has received much attention, fewer studies have attempted to access the relationship between enzyme production and lignin degradation (Arora et al., 2002; Martínez et al., 2005; Robertson et al., 2008).

Wheat straw is one of the abundant and cheapest crop residues in European countries. Contrary to its chemical and physical degradation, which is an expensive and inefficient process, biological decomposing is more acceptable and feasible, and represents a popular alternative environmental-friendly and economically justified process (Croan, 2000). Due to its appropriate chemical composition [around 0.5% total nitrogen, 29–42% α-cellulose, 26–32% hemicellulose, 16–23% lignin, 1130–8230 ppm Ca, 3–6 ppm Cu, 21–175 ppm Fe, 9–128 ppm Mn, and 7–25 ppm Zn (Salvachúa et al., 2011)], wheat straw could be a prospective substrate for

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bioconversion into fungal biomass and ligninolytic enzymes, and in such a way become the main useful raw material for food, feed, paper, and biofuel production (Tabka et al., 2006).

Previous data influenced formulation of the aims of the study, comparative analysis of ligninolytic enzyme activity of selected fungal species, their ability to degrade lignin, as well as relation between the activity and the degradation rate.

2. Methods

2.1. Organisms

Cultures of the fungal species were isolated from fruiting bodies collected from Serbia and Russia or obtained from the culture collection of the Institute of Evolution, University of Haifa, Israel (HAI). They were maintained on malt agar medium in the culture collection of the Institute of Botany, Faculty of Biology, University of Belgrade (BEOFB) (Table 1).

2.2. Substrate and growth conditions

The inoculum preparation had several steps: (i) inoculation of 100 mL of synthetic medium (glucose, 10.0 g L^{-1}; NH_4NO_3, 2.0 g L^{-1}; K_2HPO_4, 1.0 g L^{-1}; $NaH_2PO_4 \times H_2O$, 0.4 g L^{-1}; $MgSO_4 \times 7H_2O$, 0.5 g L^{-1}; yeast extract, 2.0 g L^{-1}; pH 6.5) with 25 mycelial discs (Ø 0.5 cm, from 7-day-old culture from malt agar); (ii) incubation at room temperature (22 ± 2 °C) on a rotary shaker (100 rpm) for 7 days; (iii) washing of the resultant biomass (3 times) with sterile distilled water (dH_2O); (iv) biomass homogenization with 100 mL of sterile dH_2O in a laboratory blender.

Ligninolytic enzyme production was studied after solid-state fermentation of wheat straw, which was washed, dried at 50 °C and chopped up to 0.5–1.0 cm in a blender.

Solid-state cultivation was carried out at 25 °C in 100-mL flasks containing 2.0 g of wheat straw as the carbon source and 10 mL of the modified synthetic medium (without glucose and with NH_4NO_3 in a previously determined optimal nitrogen concentration of 25 mM). Flasks were then inoculated with 3 mL of homogenized inoculum. Medium composed of wheat straw and dH_2O was used as the control.

Samples from flasks were harvested after 7, 10, and 14 days of cultivation. Synthesized ligninolytic enzymes were extracted by stirring samples with 50 mL of dH_2O on a magnetic stirrer at 4 °C for 10 min. The resultant extracts were separated from straw and mycelial debris by centrifugation (3000*g*, 10 min, 4 °C) and the supernatants were used for measuring the activities of laccase (EC 1.10.3.2) and Mn-oxidizing peroxidases (EC 1.11.1.13), as well as total protein content. Five replicate flasks for each sampling occasion were analyzed.

Table 1
Studied fungal species.

Scientific name of species	Code of strain	Origin of strain
Dichomytus squalens (P. Karst.) D.A. Reid	BEOFB 700	Verkhnyaya Kvazhva, Russia
Fomitopsis pinicola (Sw.:Fr.) P. Karsten	BEOFB 600	Verkhnyaya Kvazhva, Russia, from *Pinus silvestris*
Ganoderma lucidum (Leyss.:Fr.) Karst.	BEOFB 432	Belgrade, Serbia
Lenzites betulinus (L.:Fr.) Fr.	BEOFB 500	Verkhnyaya Kvazhva, Russia, from *Populus tremula*
Pleurotus eringii (DC.:Fr.) Quél. var. *eryngii*	HAI 507	Cultivated strain, Hawaii, Nextlab
Pleurotus ostreatus (Jacq.: Fr.) Kumm.	HAI 592	KW, A. S. Buchalo. Coll. Russia, Sochi
Trametes versicolor (L.:Fr.) Lloyd	BEOFB 320	Iverak brdo, Loznica, Serbia

2.3. Enzyme activity assays

Activity of the selected ligninolytic enzymes was determined spectrophotometrically.

Laccase activity was estimated by monitoring the A_{436} change related to the rate of oxidation of 50 mM 2,2′-azino-bis-[3-ethylthiazoline-6-sulfonate] (ABTS) (ε_{436} = 29,300 M^{-1} cm^{-1}) in 0.1 M phosphate buffer (pH 6.0) at 35 °C. The reaction mixture (V_{tot} = 1 mL) contained buffer, ABTS, and sample.

Activities of Mn-oxidizing peroxidases [Mn-dependent peroxidase (MnP) and Mn-independent peroxidase (MnIP)] were determined with 3 mM phenol red (ε_{610} = 22,000 M^{-1} cm^{-1}) as a substrate, in a buffer with the following content: succinic acid disodium salt, albumin from bovine serum, and DL-lactic acid sodium salt (pH 4.5). The reaction mixture (V_{tot} = 1 mL) contained: buffer, sample, 2 mM H_2O_2, and phenol red, with or without 2 mM $MnSO_4$ (for MnP and MnIP, respectively). The reaction was stopped by adding 2 M NaOH.

Enzymatic activity of 1 U is defined as the amount of enzyme that transforms 1 μmol of substrate min^{-1}. Enzyme activities presented correspond to the mean ± S.E. of five replicates.

2.4. Determination of total proteins

The amount of total proteins was determined according to the method of Silva et al. (2005). The reaction mixture, comprising 800 μL of sample supernatant and 200 μL of Bradford's reagent, was incubated at room temperature for 5 min and after that absorbance was measured at 595 nm. Total protein content is presented as mg mL^{-1}.

2.5. Electrophoresis

The profiles of laccase and Mn-oxidizing peroxidases are screened at all sampling times for all species.

Enzyme isoforms and their isoelectric points (pI) were determined by isoelectric focusing (IEF), which was performed using the Multiphor II electrophoresis system (GE Healthcare). Focusing was carried out in 7.5% polyacrylamide gels with 5% ampholyte on a pH gradient from 3.0 to 10.0, at 7 W constant power, and temperature of 10 °C for 1.5 h. An IEF marker for the pI range from 3.6 to 9.3 (Sigma–Aldrich) was used. Bands with laccase activity were located by incubating the gel in a mixture composed of 10 mM ABTS and 200 mM phosphate buffer (pH 5.0). MnP zymogram detection was performed as follows: (i) 2 × 5-min washing of the gel with dH_2O; (ii) 2 × 5-min equilibration with 0.1 M sodium acetate buffer (pH 4.5); (iii) immersion into visualization solution [4-Cl-alpha-naphthol (0.1 mg mL^{-1}), H_2O_2 (0.05 mM), and $MnCl_2 \times 4H_2O$ (0.1 mM) dissolved in 0.1 M sodium acetate buffer (pH 4.5)], and incubation at 25 °C until the appearance of dark-brown bands indicating MnP activity (approximately 10 min). Zymogram detection of laccase and MnIP was carried out using above described procedure with different visualization solution (for laccase, 1.0 mg mL^{-1} in 0.1 M phosphate buffer, pH 6.0; for MnIP, 0.5 mg mL^{-1} in 0.1 M sodium acetate buffer, pH 4.5).

2.6. Determination of lignin content

A quantitative procedure for lignin determination in untreated and fungal-treated wheat straw was carried out using the Klason or 72% H_2SO_4 method (Kirk and Obst, 1988). Wheat straw was air-dried, ground to pass a 20-mesh screen, and then dried in an oven at 50 °C. A sample of known weight (1.0 g) was placed in a