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Clean-up and concentration of manganese peroxidases recovered during the biodegradation of *Eucalyptus grandis* by C*eriporiopsis subvermispora*

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Abstract

Ceriporiopsis subvermispora was utilized for the biodegradation of *Eucalyptus grandis* in the presence or absence of co-substrates (glucose and corn steep liquor) during 7, 14 and 28 days. The biodegraded chips were used to prepare enzymatic extracts that, after pre-treatment with activated charcoal, were enriched in activity of manganese peroxidases (MnPs) with anion-exchange chromatography followed by elution with NaCl. Samples of these extracts were then analyzed by denaturing electrophoresis in order to compare the profiles of proteins and MnPs. Depending on the biodegradation period and on the presence or absence of co-substrates in the culture, one or two major proteins (relative molecular weights of 46.8 ± 0.6 and 51.6 ± 1.0 kDa) were identified. The presence of MnPs activity was further confirmed by native electrophoresis followed by phenol red staining.

Keywords: *Ceriporiopsis subvermispora*; *Eucalyptus grandis*; Manganese peroxidases

1. Introduction

Ceriporiopsis subvermispora, a basidiomycete that presents high selectivity for lignin biodegradation [1], has been considered for application in industrial biopulping [2]. Enzymatic extracts obtained during the biodegradation of *Pinus radiata* [3] and *Eucalyptus grandis* [4] by this fungus have shown that the activity of manganese peroxidases (MnPs) prevail over other oxidative enzymes. Thus, it is believed that MnPs play a key role in the biodegradation of lignin by *C. subvermispora*.

The MnPs are heme-proteins expressed as multiple isoenzymes, generally glycosylated [5,6]. The reasons why these proteins are expressed as multiple isoenzymes are not totally understood. In *Phanerochaete chrysosporium*, the isoenzymes are produced only during the secondary metabolism and their expressions are accurately regulated by the age of the culture [7], by the medium composition [8] and by the availability of Mn^{2+} [9]. On the other hand, the MnPs produced by *C. subvermispora* occur during the primary metabolism. In liquid medium, the profile of isoenzymes produced by this fungus, although constant throughout the biodegradation period, depends on the concentration of Mn^{2+} [10]. In solid medium, the profile of isoenzymes depends on the biodegradation period, although different conditions of nutritional supplementation have not been evaluated [10].

In the present study, *C. subvermispora* was used for the biodegradation of *E. grandis* in the presence or absence of glucose and corn steep liquor, co-substrates which maximize the activity of MnPs recovered from the biodegraded wood chips [4]. The biodegraded chips were then used for the preparation of enzymatic extracts that, after pre-treatment with activated charcoal, were enriched in MnPs activity with anion-exchange chromatography followed by elution with NaCl. Such a strategy allowed us to compare the profiles of proteins produced by the fungus, by electrophoresis, in extracts that originally presented very low protein contents and heavy contamination by lignin biodegradation products.

2. Material and methods

2.1. Microorganism, inoculum preparation and wood biodegradation

C. subvermispora (Pilat) Gilbn. & Ryv. (strain SS-3), kept in 20 g/L malt extract agar (MEA) at 4 °C, was used in this study. Erlenmeyer flasks (2 L)

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containing 200 mL of 24 g/L potato dextrose broth (PDB) supplemented with 7 g/L yeast extract were inoculated with 20 discs (8 mm in diameter) of pre-cultured mycelium (MEA). After 12 days of static incubation at 27 °C, the grown mycelium was filtered and washed with 300 mL of sterile water. Mycelia obtained from several PDB cultures were blended with 100 mL of sterile water in three cycles of 15 s, and the resulting suspension was used to inoculate the wood chips.

E. grandis wood chips (2.5 cm × 1.5 cm × 0.2 cm) were immersed in water for 12 h. The surplus water was drained and the wood chips were autoclaved at 121 °C for 15 min inside 20 L reactors. Each reactor was loaded with 2 kg of wood chips and 1 g of blended mycelium (both on dry basis), and supplemented (S) or not (NS) with glucose and corn steep liquor (both at 5 g/kg of wood, on dry basis). After inoculation, the bioreactors were incubated at 27 °C for 7, 14 or 28 days, being aerated at a flow rate of 23 L/h.

2.2. Enzyme extraction

Extracellular enzymes were extracted with 50 mM sodium acetate buffer at pH 5.5 added of 0.1 g/L Tween 60. The mycelium-colonized chips were transferred to 2 L Erlenmeyer flasks in fractions of 200 g (wet weight) and mixed with 500 mL of the aforementioned buffer at 120 rpm and 10 °C for 4 h. Afterwards, the mixture was filtered through porous glass filter for solids removal and the liquid fraction was centrifuged at 3.400 × *g* for 15 min to remove particulate material. The extracts thus obtained were kept in an ultra-freezer at −80 °C.

2.3. Pre-treatment of the extracts and enrichment of MnPs activity

Activated charcoal (*Labsynth Ltda.*) was used to remove aromatic compounds from the enzymatic extracts. The experiments were carried out in 1 L Erlenmeyer flasks, using 500 mL of extract and different amounts of charcoal. After mixing at 100 rpm and 30 °C for 30 min, the liquid fraction was recovered by filtration through porous glass filter. The amount of charcoal used in each extract was defined previously in 2.5 mL Eppendorff tubes, in order to maximize the removal of aromatic compounds with minimal losses in the activity of MnPs.

After the pre-treatment with activated charcoal, the extracts had their pHs adjusted to 4.8 with 2 M acetic acid, being subsequently loaded into a column (10.2 cm × 0.98 cm) packed with the anion-exchange resin DEAE Sepharose CL 6B (*GE Healthcare*). Previously to the loading of the extracts, the column was equilibrated with 50 mM sodium acetate buffer at pH 4.8. Loading and washing operations were carried out at a flow rate of 0.50–0.75 mL/min. Elution afterwards was by 0.2 M buffered NaCl at the same flow rate.

2.4. Determination of manganese peroxidases

MnPs activity was determined by measuring the oxidation of phenol red at 610 nm (ε = 22.000/M cm) [11]. The reaction mixture (5 mL) consisted of 17.5 mM sodium succinate buffer at pH 3.2, 15 mM sodium lactate, 0.1 g/L phenol red, 0.1 mM manganese sulfate, 0.9 g/L bovine serum albumin, enzymatic extract and 0.1 mM hydrogen peroxide. After the beginning of the catalysis, 1 mL samples of the reaction mixture were taken in 1 min intervals and transferred to 65 μL of a 6.5 M NaOH solution, whose absorbance at 610 nm was measured in a spectrophotometer. One international unit (IU) of MnPs activity was calculated as the amount of enzyme that promoted the oxidation of 1 μmol phenol red in 1 min.

2.5. Determination of proteins

The extracts were assayed for protein concentration according to the Coomassie Blue method, using bovine serum albumin as the standard [12].

2.6. Electrophoresis

Denaturing and non-denaturing electrophoresis were performed in 12.5% polyacrylamide gels, using a discontinuous system [13,14]. Proteins were stained with silver nitrate. Bands of MnPs were visualized by incubating the gel in a mixture identical to that used for the determination of its activity.

Table 1
Activities of MnPs and relative 280 nm absorptions determined in the extracts obtained during the biodegradation in the presence (S) or absence (NS) of co-substrates

Extract	Activity (IU/mL)	Relative 280 nm absorption
NS-7 days	0.107	4.73
NS-14 days	0.116	8.71
NS-28 days	0.081	16.22
S-7 days	0.230	7.13
S-14 days	0.405	13.07
S-28 days	0.221	23.46

3. Results and discussion

Enzymatic extracts containing high activities of MnPs were recovered from *E. grandis* wood chips biotreated by *C. subvermispora*. Cultures performed on glucose/CSL supplemented medium (S) provided higher activities of MnPs as compared to the control cultures performed on non-supplemented medium (NS) (Table 1). Similar results were recently reported for the same fungus acting on *E. grandis*, and the activities of MnPs could not be correlated with the extent of lignin degradation [4]. One question emerging from such results is whether different MnPs isoenzymes are produced in such differentiated culture conditions. To evaluate this subject, enzymatic extracts recovered from 7- to 28-day-old cultures were analyzed by polyacrylamide gel electrophoresis. However, they presented very low protein contents and heavy contamination by lignin biodegradation products, which interfered in the detection of the enzymes. To overcome this problem, the extracts were submitted to clean-up and concentration procedures, which enabled their further characterization by gel electrophoresis.

The content of lignin degradation products present in the extracts could be roughly estimated by the absorbance values at 280 nm multiplied by the dilution factors used in each determination. This relative absorption coefficient, named here as relative 280 nm absorption, increased considerably throughout the biodegradation (Fig. 1). Taking this parameter as an approximate measurement of the content of aromatic compounds derived from lignin [15], it can be inferred that, independently of the wood supplementation with co-substrates, the fungus

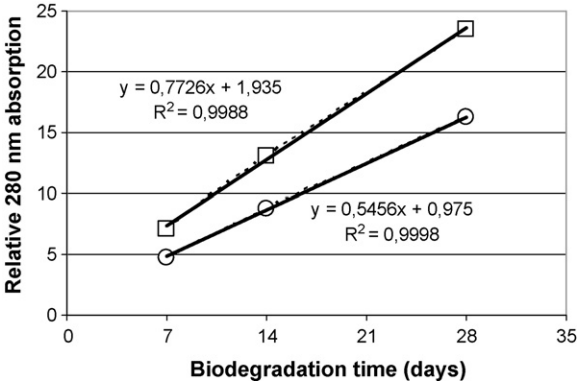

Fig. 1. Relative 280 nm absorption of the extracts obtained during the biodegradation in the presence (□) or absence (◯) of co-substrates.