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

Walter Carvalho, André Ferraz, Adriane M.F. Milagres*

Escola de Engenharia de Lorena, Departamento de Biotecnologia Estrada Municipal do Campinho, Lorena, CEP 12.602-810, S.P., Brazil Received 12 July 2007; received in revised form 1 October 2007; accepted 13 October 2007

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.

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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 Mn²⁺ [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)

^{*} Corresponding author. Tel.: +55 12 553 3165.

E-mail address: adriane@debiq.eel.usp.br (A.M.F. Milagres).

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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 precultured mycelium (MEA). After 12 days of static incubation at 27 $^{\circ}$ 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 $(2.5 \text{ cm} \times 1.5 \text{ cm} \times 0.2 \text{ cm})$ 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 $3.400 \times g$ 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 \times 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 μ L 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 μ mol 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 cosubstrates

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 (\Box) or absence (\bigcirc) of co-substrates.



Fig. 2. Percentages of relative 280 nm absorption left in the extracts after adsorption in activated charcoal (assays in Eppendorff tubes): S-7 days (\blacksquare); S-14 days (\blacksquare); S-28 days (\blacksquare); NS-7 days (\blacksquare); NS-14 days (\blacksquare); NS-28 days (\blacksquare); NS-7 days (\blacksquare); NS-28 days (\blacksquare).



Fig. 3. Percentages of activities of MnPs left in the extracts after adsorption in activated charcoal (assays in Eppendorff tubes): S-7 days (\blacksquare); S-14 days (\blacksquare); S-28 days (\blacksquare); NS-7 days (\square); NS-14 days (\blacksquare); NS-28 days (\blacksquare).

was able to degrade this complex macromolecule. However, as demonstrated by the regression equations shown in Fig. 1, the addition of co-substrates led to an increase of about 40% in the rate of biodegradation.

The extracts from the different biodegradation periods were pre-treated with activated charcoal in order to reduce the concentration of the compounds that absorb light at 280 nm. This strategy is commonly used for the detoxification of hemicellulosic hydrolysates and presents a high efficiency in the removal of aromatic compounds [15].

Preliminary experiments were carried out in Eppendorff tubes in order to adjust the amount of charcoal to be used in the pretreatment of each extract (Figs. 2 and 3). As shown in Fig. 2, the concentrations of aromatic compounds could be reduced substantially in all the extracts. However, the amount of charcoal necessary to achieve high efficiencies varied according to the biodegradation period and to the presence or absence of cosubstrates in the cultures. For instance, an efficiency higher than 80% was observed when using only 2% activated charcoal to pre-treat the NS-7 days extract, while 7% activated charcoal was necessary to achieve the same efficiency when pre-treating the S-28 days extract. MnPs activities also decreased during the pretreatment with activated charcoal (Fig. 3). The higher the content of non-proteic compounds in the extracts, the higher was the selectivity for removal of these undesirable compounds during the adsorption onto activated charcoal. For instance, a loss of 11% in the activity of MnPs was necessary to reduce the relative 280 nm absorption of NS-7 days extract in 66% (1% charcoal), while a loss of only 9% in the activity of MnPs occurred when the relative 280 nm absorption of the S-28 days was decreased by 84% (8% charcoal).

Table 2 shows the percentual values of MnPs activity and relative 280 nm absorption left in the enzymatic extracts after

optimal pre-treatments with activated charcoal in larger scale (Erlenmeyer flasks). As can be observed, depending on the extract and on the amount of charcoal used, it was possible to remove 40-70% of the initial relative 280 nm absorption with only small losses in the MnPs activities (less than 15%).

Previous studies conducted to identify and characterize cellulases and hemicellulases produced by *C. subvermispora* during the colonization of wood chips pointed out that certain proteins were produced in very small amounts, posing additional difficulty and even preventing their characterization [16–18]. In the present study, anion-exchange chromatography was used as a technique to increase the titers of MnPs in the extracts pre-treated with activated charcoal.

As can be seen in Table 3, the activities of MnPs were increased considerably by the chromatographic procedure, reaching values up to 23.5 IU/mL. Although the relative 280 nm absorption of the concentrated extracts had been superior to those of the original raw extracts, the increases in the activity of MnPs (more than 30 times) were more expressive than the increases in the relative 280 nm absorption (less than 5

Table 2

Percentages of MnPs activity and of relative 280 nm absorption left in the extracts after pre-treatment with activated charcoal (assays in Erlenmeyer flasks)

Extract	Charcoal (%, w/v)	Activity (%)	Relative 280 nm absorption (%)
NS-7 days	0.5	90.7	59.4
NS-14 days	1.5	93.1	35.8
NS-28 days	3.0	85.2	26.9
S-7 days	1.0	92.6	40.3
S-14 days	2.0	98.5	35.3
S-28 days	3.0	102.7	30.3

Table 3

Extract	MnPs (IU/mL)	Relative 280 nm absorption	Relative 405 nm absorption	Proteins (mg/mL)
NS-7 days	3.632	15.02	0.85	0.162
NS-14 days	12.270	12.74	1.08	0.170
NS-28 days	7.809	40.55	1.50	0.218
S-7 days	14.386	31.80	1.89	0.350
S-14 days	23.500	34.30	2.69	0.241
S-28 days	20.023	50.35	2.43	0.394

Activities of MnPs, relative 280 and 405 nm absorptions, and concentrations of proteins determined in the extracts pre-treated with activated charcoal and concentrated with anion-exchange resin



Fig. 4. Relative absorption spectra of the extracts after pre-treatment with activated charcoal and concentration with anion-exchange resin.

times). Despite this considerable relative increase in the activity of MnPs, defined peaks of absorbance at 405 nm, typical for purified heme-peroxidases [19], were detected only in the NS-14, S-14 and S-28 days extracts (Fig. 4). The increases in the absorbances at 405 nm observed after the pre-treatment and concentration of the extracts (1.9–4.5 times) were much lower than those expected considering the increases in the activity of MnPs (33.9–105.8 times). In this context, it is important to emphasize that the mass balances for the activities of MnPs along the pretreatment and concentration resulted in values higher than 100% for the extracts NS-14, NS-28 and S-28 days, thus evidencing gain of activity (Table 4). Lobos et al. [10] also observed a similar behavior when using an anion-exchange resin (Q-Sepharose) to concentrate MnPs produced by the same fungus during the biodegradation of *P. radiata*. They assigned the gain of activity to the removal of inhibitors from the extracts during the chromatographic procedure.

The clean-up and concentration procedures allowed to evaluate the profiles of proteins in the extracts by electrophoresis. As can be observed in Figs. 5 and 6, two major bands with relative molar masses (M_R) of 46.8 ± 0.6 and 51.6 ± 1.0 kDa were detected in the concentrated extracts of both supplemented and



Fig. 5. Profiles of proteins and MnPs in polyacrylamide gels. Extracts obtained in the absence of co-substrates, after pre-treatment and concentration. Values of M_R in kDa.



Fig. 6. Profiles of proteins and MnPs in polyacrylamide gels. Extracts obtained in the presence of co-substrates, after pre-treatment and concentration. Values of $M_{\rm R}$ in kDa.

non-supplemented cultures. In the non-supplemented cultures, these two proteins were neatly visualized in the extracts obtained after 28 days of biodegradation (NS-28 days). On the other hand, among the extracts obtained during the biodegradation in the presence of co-substrates, these two bands were clearly visualized at 14 days of biodegradation (S-14 days). The presence of MnPs activity was further confirmed by electrophoresis under non-denaturing conditions followed by staining with phenol red, one very diffuse band being observed in all the extracts. As expected, the native proteins went through longer distances in the gel than their denatured counterparts.

It would be incorrect to speculate about the effect of the nutritional supplementation on the profile of the proteins produced by the fungus along the biodegradation. To obtain defined bands in the polyacrylamide gels after silver staining, free of artifacts, the extracts had to be pre-treated and concentrated, and thus had their compositions altered. In spite of this, considering that the two bands with different $M_{\rm R}s$ were observed in both the supplemented and the non-supplemented cultures, it can be inferred that the nutritional supplementation did not lead to the production of different major proteins.

Table 4

Recovery of MnPs along the steps of pre-treatment with activated charcoal and concentration with anion-exchange resin

Extract	Recovery (%)			
	Initial	Pre-treated	Concentrated	
NS-7 days	100	87.1	18.2	
NS-14 days	100	89.7	130.0	
NS-28 days	100	70.4	108.0	
S-7 days	100	89.1	40.1	
S-14 days	100	90.4	73.7	
S-28 days	100	88.8	130.5	

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References

- Akhtar M, Blanchette RA, Myers GE, Kirk K. An overview of biomechanical pulping research. In: Young R, Akhtar M, editors. Environmentally Friendly Technologies for the Pulp and Paper Industry. New York: John Wiley & Sons; 1998. p. 309–83.
- [2] Akhtar M, Scott GM, Swaney RE, Shipley DF. Biomechanical pulping: a mill-scale evaluation. Resour Conserv Recycl 2000;28:241–52.
- [3] Cruz PBS, Freer J, Siika-Aho M, Ferraz A. Extraction and determination of enzymes produced by *Ceriporiopsis subvermispora* during biopulping of *Pinus taeda* wood chips. Enzyme Microb Technol 2004;34:228–34.
- [4] Vicentim MP, Ferraz A. Enzyme production and chemical alterations of *Eucalyptus grandis* wood during biodegradation by *Ceriporiopsis subvermispora* in cultures supplemented with Mn²⁺, corn steep liquor and glucose. Enzyme Microb Technol 2007;40:645–52.
- [5] Hattaka A. Lignin-modifying enzymes from selected white-rot fungi: production and role in lignin degradation. FEMS Microbiol Rev 1994;13:125–35.
- [6] Hofrichter M. Review: lignin conversion by manganese peroxidases. Enzyme Microb Technol 2002;30:454–66.
- [7] Pease EA, Tien M. Heterogeneity and regulation of manganese preoxidases from *Phanerochaete chrysosporium*. J Bacteriol 1992;174:3532–40.
- [8] Leisola MSA, Kozulic B, Meussdoerffer F, Fiechter A. Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium*. J Biol Chem 1987;262:419–24.
- [9] Bonnarme P, Jeffries TW. Mn(II) regulation of lignin peroxidases and manganese-dependent peroxidases from lignin-degrading white rot fungi. Appl Environ Microbiol 1990;56:210–7.
- [10] Lobos S, Larrain J, Salas L, Cullen D, Vicuña R. Isoenzymes of manganese-dependent preoxidase and laccase produced by the lignindegrading basidiomycete *Ceriporiopsis subvermispora*. Microbiology 1994;140:2691–8.
- [11] Lundell T, Leonowicz A, Rogalski J, Hatakka A. Formation and action of lignin-modifying enzymes in cultures of *Phlebia radiata* supplemented with veratric acid. Appl Environ Microbiol 1990;56:2623–9.

- [12] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal Biochem 1976;72:248–54.
- [13] Davis BJ. Disc electrophoresis. II. Method and application to human serum proteins. Ann NY Acad Sci 1964;121:404–27.
- [14] Ornstein L. Disc electrophoresis. I. Background and theory. Ann NY Acad Sci 1964;121:321–49.
- [15] Parajó JC, Dominguez H, Dominguez JM. Biotechnological production of xylitol: operation in culture media made from lignocellulose hydrolysates. Bioresour Technol 1998;66:25–40.
- [16] Milagres AMF, Magalhães PO, Ferraz A. Purification and properties of a xylanase from *Ceriporiopsis subvermispora* cultivated on *Pinus taeda*. FEMS Microbiol Lett 2005;253:267–72.
- [17] Magalhães PO, Ferraz A, Milagres AMF. Enzymatic properties of two βglucosidases from *Ceriporiopsis subvermispora* produced in biopulping conditions. J Appl Microbiol 2006;101:480–6.
- [18] Heidorne FO, Magalhães PO, Ferraz AL, Milagres AMF. Characterization of hemicellulases and cellulases produced by *Ceriporiopsis subvermispora* grown on wood under biopulping conditions. Enzyme Microb Technol 2006;38:436–42.
- [19] Rubia T, Linares A, Pérez J, Muñoz-Dorado J, Romera J, Martinez J. Characterization of manganese-dependent peroxidase isoenzymes from the ligninolytic fungus *Phanerochaete flavido-alba*. Res Microbiol 2002;153:547–54.