J Ind Microbiol Biotechnol (2003) 30: 157–160
DOI 10.1007/s10295-003-0025-5

ORIGINAL PAPER

V.L. Papinutti · L.A. Diorio · F. Forchiassin

Production of laccase and manganese peroxidase by *Fomes sclerodermeus* grown on wheat bran

Received: 3 September 2002 / Accepted: 17 December 2002 / Published online: 4 March 2003

Abstract The aim of this work was to study the growth and production of ligninolytic enzymes by *Fomes sclerodermeus* using a natural medium based on wheat bran as the principal substrate in a solid-state fermentation. Growth was monitored by measuring the chitin content in the substrate. The maximum rate of growth was observed between days 7 and 18. A 38% total dry-weight loss of the substrate was measured after 28 days of cultivation. Differential hydrolysis of the substrate revealed that cellulose was more extensively degraded than lignin. In the 28-day incubation period, the losses of cellulose and lignin were 38 and 15%, respectively. No lignin peroxidase activity was found in any of the media tested. The maximum manganese-dependent peroxidase activity recorded was 6.3 U g^{-1} at 14 days, while the maximum laccase activity was 270 U g^{-1} at 28 days post-inoculation. Addition of commonly used inducers such as copper or manganese did not produce a further increase in the enzyme activities, nor did addition of glucose, asparagine, or malt extract.

Keywords Fermentation · *Fomes sclerodermeus* · Ligninases · Lignocellulosic

Introduction

Important quantities of lignocellulosic materials are produced annually. These are available for exploitation as potential sources of food or as substrate for the production of metabolites by microorganisms. The organisms able to degrade efficiently the major components of wood, cellulose, hemicellulose and lignin, are white-rot fungi. These fungi possess the hydrolytic enzymes, cellulases and xylanases, that typically are induced by their substrates [15]. Lignin is degraded by a ligninase complex (LC) composed of at least three enzymes: lignin peroxidase (LiP) [28], manganese-dependent peroxidase (MnP) [9] and laccase. LiP is a heme protein with high oxidation potential that oxidizes phenolic and nonphenolic substrates [1,17]. MnP is considered unable to oxidize non-phenolic substrates, although it can depolymerize synthetic [29] or natural [10] lignins in vitro. Laccase belongs to a family of multicopper oxidases that has a wide range of reducing substrates like polyphenols and methoxy-substituted phenols [27]. Appropriate primary substrates like 2,2′-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) or 1-hydroxybenzotriazole (1-HBT) can act as cooxidants, extending its range of substrates [2]; natural cooxidants have also been identified [11]. MnP and laccase from *Rigidoporus lignosus* act synergistically in lignin degradation [7]. Therefore, organisms able to produce both enzymes are interesting in view of their potential importance in processes such as bioremediation, biobleaching of pulp paper, and degradation and detoxification of recalcitrant substances. The production of MnP and laccase by solid-state fermentation in a low-cost medium instead of the chemically defined liquid media that are widely used would thus be advantageous.

Fomes sclerodermeus BAFC 2752 is a white-rot basidiomycete that was isolated in Tucumán, Argentina. Previously, the production of both laccase and MnP by this fungus in defined liquid medium was demonstrated [19]. *F. sclerodermeus*, besides degrading lignin in wood [21], was able to grow and detoxify the fungicide malachite green [20]; therefore, high-level production of its ligninolytic enzymes in a low-cost medium would have many biotechnological applications. The aim of this work was to study the growth and production of MnP and laccase by the

V.L. Papinutti · L.A. Diorio · F. Forchiassin (✉)
Laboratorio de Micología Experimental,
Departamento de
Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires,
Ciudad Universitaria Pab.II,
1428 Buenos Aires, Argentina
E-mail: fla@bg.fcen.uba.ar
Fax: +54–11–4576–3384

white-rot fungus *F. sclerodermeus* in natural low-cost medium containing wheat bran as the substrate. The effect of an additional carbon source, e.g., glucose, as well as the addition of nutrients such as Mn^{2+}, Cu^{2+}, and nitrogen was evaluated.

Materials and methods

Organism and culture conditions

Fomes sclerodermeus (Léveillé) Cooke BAFC 2752 (Science Faculty Collection at Buenos Aires University), was maintained in malt-extract agar (malt extract 1.2%, glucose 1%, agar 2%,) at 4 °C.

The organism was cultivated in a basal medium containing 4 g wheat bran and 16 ml distilled water. The effect of additives was evaluated by replacement of the distilled water with an aqueous solution of the additive to be tested at the following concentrations: 0.15 mM $CuSO_4$, 1 mM $MnSO_4$, 10 g l^{-1} glucose, micronutrients [14] and 4 g l^{-1} asparagine. The pH of the media was around 6. The cultures were inoculated aseptically by using three agar cubes (25 mm^2) obtained from the advancing margin of a colony grown on malt-extract agar.

Weight losses were determined by drying the content of each flask to constant weight at 80 °C. Dried samples were ground in a mortar and stored until they were used for chitin, cellulose and lignin determinations. Crude extract was obtained by adding 100 ml of 50 mM acetate buffer, pH 4.8, to the contents of each flask, stirring for 20 min, followed by filtration and centrifugation. The supernatant was stored at −20 °C until needed. For all experiments, measurements were carried out in triplicate parallel cultures. The values are reported as the mean ± S.E.M.

Analysis of proteins, reducing sugars, chitin, cellulose, and lignin

Soluble proteins in the crude extract were determined by the method of Bradford [4] using BSA as the standard. The chitin content of dried samples and mycelium from malt-extract liquid cultures was determined by measuring *N*-acetylglucosamine (NAGA) released from chitin after hydrolysis of with 6 N HCl [23]. Reducing sugars in the crude extract were assayed by the method of Somogyi and Nelson [18] using glucose as the standard. Cellulose and lignin in the dried samples were determined by the TAPPI method [25].

Enzyme assays

All enzyme activities were determined spectrophotometrically at 30 °C in a total volume of 1 ml. Laccase activity was determined at 420 nm ($\epsilon_{420} = 36\ mM^{-1}\ cm^{-1}$) using 5 mM ABTS [2,2′-azino-*bis* (3-ethylbenzthiazoline-6-sulfonic acid] as substrate [3]. Measurements were made in 0.1 M sodium acetate buffer, pH 3.6. Manganese peroxidase was determined using phenol red as a substrate. The reaction product was measured at 610 nm ($\epsilon_{610} = 22\ mM^{-1}\ cm^{-1}$) [9]. The reaction mixture contained 50 mM succinate buffer, pH 4.5, 0.01% phenol red and the appropriate volume of enzyme. The addition of H_2O2 (0.1 mM final concentration) initiated the reaction. LiP activity was determined by oxidation of veratryl alcohol to veratryl aldehyde ($\epsilon_{310} = 9{,}300\ M^{-1}\ cm^{-1}$). The reaction mixture contained 0.1 M sodium tartrate buffer, pH 3, 2 mM veratryl alcohol, 0.4 mM H_2O2 and the appropriate volume of enzyme. One enzyme unit (U) was defined as 1.0 μmol of product formed per minute under the assay conditions. In terms of production, the activity was defined as U per g dry wheat bran (U g^{-1}).

Results

Weight loss, soluble proteins, reducing sugars, pH, and chitin content

Time courses of weight loss, soluble proteins, reducing sugars, pH, and chitin content are shown in Fig. 1. Growth was monitored by measuring the chitin content in the dried substrate. The maximum rate of growth was observed between days 7 and 18. As mycelium of *F. sclerodermeus* contains 35 μg NAGA mg^{-1}, actual biomass could be estimated as a maximum of 136 mg (g dry substrate)$^{-1}$. Data for reducing sugars showed that the weight loss due to minor carbohydrates was negligible, with an initial value of 45 μg (g dry wheat bran)$^{-1}$. A 38% loss in total dry weight in the medium was measured after 28 days of cultivation, with most of the loss occurring between days 7 and 18. Differential acid hydrolysis of the dried samples showed that the weight losses were due to cellulose and lignin degradation (Fig. 2). Cellulose was more extensively degraded, with the highest rate of loss between days 7 and 14. During the 28-day incubation period, the losses of cellulose and lignin were 38 and 15%, respectively.

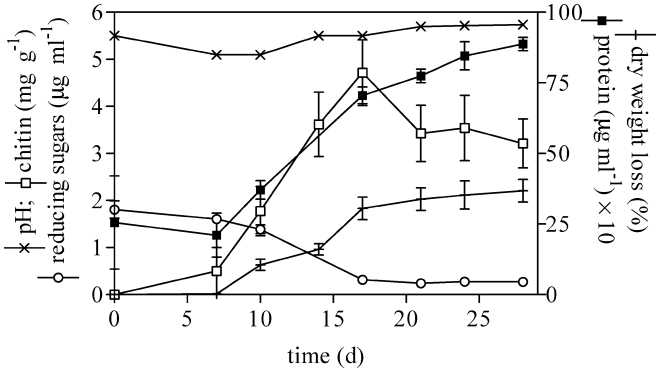

Fig. 1 Time course of chitin, pH, reducing sugars, dry-weight loss, and soluble protein from *Fomes sclerodermeus* grown in wheat bran. Means of three replicates and S.E.M. are shown

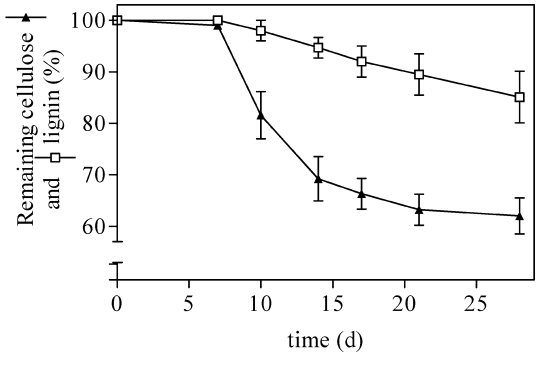

Fig. 2 Time course of percentages of remaining cellulose and lignin in the wheat bran. Means of three replicates and S.E.M. are shown

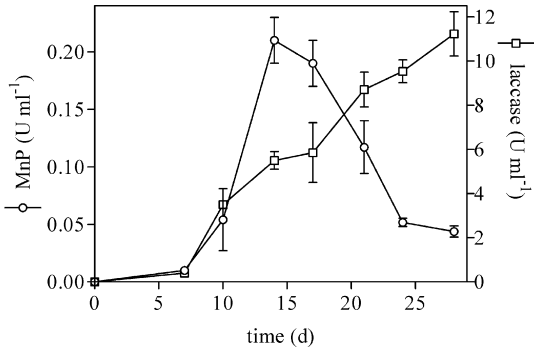

Fig. 3 Time course of MnP and laccase production by *F. sclerodermeus* grown in wheat bran. Means of three replicates and S.E.M. are shown

Table 1 Effect of the addition of nutrients to wheat-bran medium on laccase, MnP, and dry-weight loss. Standard deviations were $<0.05\%$ of the means of triplicate cultures

Treatment	Laccase (U ml^{-1})	MnP (U ml^{-1})	Dry weight loss (%)
Control	9.9	0.12	32
Malt extract 6 g l^{-1}	7.0	0.03	28
Malt extract 20 g l^{-1}	5.8	0.11	30
Glucose 15 g l^{-1}	4.4	0.10	25
SO_4NH_2 4 g l^{-1}	5.4	0.11	29
$CuSO_4$ 150 μM	8.5	0.12	33
$MnSO_4$ 1 mM	7.6	0.11	33

Laccase and MnP activities

No LiP activity was measured in any medium tested. Time courses of MnP and laccase activities are shown in Fig. 3. The maximum MnP activity was measured at 14 days post-inoculation (0.21 U ml^{-1}), corresponding to 6.3 U g^{-1}, and then decreased. The maximum laccase activity production by *F. sclerodermeus* was at 28 days post-inoculation; Fig. 3 shows a linear increase of this activity until the last sampling day. In terms of productivity, the value at 28 days was 270 U g^{-1}.

Table 1 shows the effect of different additives on weight loss of the substrate and both MnP and laccase activities at 21 days post-inoculation. *F. sclerodermeus*, under the conditions studied in this work, did not produce increased laccase or MnP activities in response to any of the additives tested. The maximum activity reached by each enzyme was comparable to that obtained by other fungi cultured in chemically defined media.

Discussion

F. sclerodermeus did not produce LiP activity under the conditions tested. The production of LiP is an exceptional event among the white-rot fungi [22,26]. As LiP and MnP appear only during the stationary phase, ligninolytic enzymes in *P. chrysosporium* are associated with secondary metabolism. In *F. sclerodermeus* in solid-state fermentation, MnP and laccase activities increased during trophophase until day 18 in parallel with growth, showing that these enzymes are not secondary metabolites but seem to be growth-dependent. The MnP and laccase activities obtained were higher than those obtained in other fungi. The activity of MnP in *Lentinula edodes* strains cultured under similar conditions reached 3 mU g^{-1} [16] while MnP production by *F. sclerodermeus* was 2,100 times higher. With regard to laccase activity, the white-rot fungus *Pleurotus sajor-caju* cultured in solid-substrate fermentation not only produced a lower titer than obtained in *F. sclerodermeus* but the medium was supplemented with aromatic compounds to further increase the activity [13]. *F. sclerodermeus* cultured in liquid medium without the addition of inducers produced 0.17 and 0.34 U ml^{-1} of MnP and laccase activity, respectively [19]. Addition of inducers such as copper and aromatic compounds is routinely used in order to increase laccase and MnP activities. The highest laccase titers were obtained with copper induction. Positive effects have been reported for laccase production by *Trametes pubescens* [6], *Trametes trogii* [14] and several isoenzymes secreted by *Pleurotus ostreatus* [8]. Manganese added as an inorganic salt is widely used as a MnP inducer [24,26], but negatively affects growth of certain organisms [5]. Due to their biomass dependence, these enzyme activities might be increased by adding glucose, nitrogen, or other nutrients favoring growth [12]. In this work, we used a natural culture medium that probably contained laccase and MnP inducers due to the fact that none such activities could be increased by the addition of inducers. Thus, because of its low cost and the resulting high levels of enzyme production, the unsupplemented medium could be used to scale up the production of both laccase and MnP. The fact that *F. sclerodermeus* produces high levels of both laccase and MnP is a further advantage with respect to their possible synergism [7] in bioremediation processes.

Acknowledgements This work was partly supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas) Argentina, and Universidad de Buenos Aires.

References

1. Banci L, S Ciofi-Baffoni, M Tien (1999) Lignin and Mn peroxidase-catalyzed oxidation of phenolic lignin oligomers. Biochemistry 38:3205–3210
2. Bourbonnais R, MG Paice, B Freiermuth, E Bodie, S Borneman (1997) Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 63:4627–4632
3. Bourbonnais R, MG Paice, ID Reid, P Lanthier, M Yaguchi (1995) Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2,2′-azino-bis (ethylbenzothiazoline-6-sulfonic acid) in kraft lignin depolimerization. Appl Environ Microbiol 61:1876–1880

4. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
5. Buswell JA, YJ Cai, Y Chang (1995) Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinula* (*Lentinus*) *edodes*. FEMS Microbiol Lett 128:81–88
6. Galhaup C, H Wagner, B Hinterstoisser, D Haltrich (2002) Increased production of laccase by the wood-degrading basidiomycete *Trametes pubescens*. Enzyme Microb Technol 30: 529–536
7. Galliano H, G Gas, JL Seris, AM Boudet (1991) Lignin degradation by *Rigidoporus lignosus* involves synergistic action of two oxidizing enzymes: Mn peroxidase and laccase. Enzyme Microb Technol 13:478–482
8. Giardina P, G Palmieri, A Scaloni, B Fontanella, V Faraco, G Cennamo, G Sannia (1999) Protein and gene structure of a blue laccase from *Pleurotus ostreatus*. Biochem J 341:655–663
9. Glenn JK, MH Gold (1985) Purification and characterisation of an extracellular Mn(II)-dependent peroxidase from the lignin degrading basidiomycete *Phanerochaete chrysosporium*. Arch Biochem Biophys 242:329–341
10. Hatakka A, T Lundell, A Hatakka (2001) Conversion of milled pine wood by manganese peroxidase from *Phlebia radiata*. Appl Environ Microbiol 67:4588–4593
11. Johannes C, A Majcherczyk (2000) Natural mediators in the oxidation of polycyclic aromatic hydrocarbons by laccase mediator systems. Appl Environ Microbiol 66:524–528
12. Kanunfre CC, GT Zancan (1998) Physiology of exolaccase production by *Telephora terrestris*. FEMS Microbiol Lett 16:151–156
13. Kumaran S, CA Sastry, S Vikineswary (1997) Laccase, cellulase and xylanase activities during growth of *Pleurotus sajor-caju* on sago hampas. World J Microbiol Biotechnol 13:43–49
14. Levin L, F Forchiassin, AM Ramos (2002) Copper induction of lignin modifying enzymes in the white-rot fungus *Trametes trogii*. Mycologia 94:377–383
15. Magnelli P, F Forchiassin (1999) Regulation of the cellulase complex production by *Saccobolus saccoboloides*: induction and repression by carbohydrates. Mycologia 91:359–364
16. Mata G, JM Savoie (1998) Extracellular enzyme activities in six *Lentinula edodes* strains during cultivation in wheat straw. World J Microb Biotechnol 14:513–519
17. Mester T, K Ambert-Balay, S Ciofi-Baffoni, L Banci, AD Jones, M Tien (2001) Oxidation of a tetrameric nonphenolic lignin model compound by lignin peroxidase. J Biol Chem 276:22985–22990
18. Nelson NJ (1944) A photometric adaptation of the Somogyi method for the determination of glucose. J Biochem 153:375–380
19. Papinutti VL, F Forchiassin (2000) Enzimas de hongos de pudrición blanca involucradas en la degradación de lignina. Rev Arg Microbiol 32:83–88
20. Papinutti VL, LN Levin, F Forchiassin (2001) Decoloración y detoxificación del colorante Verde de Malaquita por *Fomes sclerodermeus*. In: Abstracts of the 4th meeting of the Society of Environmental Toxicology and Chemistry Latin America, Buenos Aires
21. Papinutti VL, N Mouso, F Forchiassin (2001) Degradación de celulosa y lignina en madera de álamo por *Fomes sclerodermeus*. In: Abstracts of the 9th Congreso Argentino de Microbiología, Buenos Aires
22. Peláez F, MJ Martínez, AT Martínez (1995) Screening of 68 species of basidiomycetes for enzymes involved in lignin degradation. Mycol Res 99:37–42
23. Plassard CS, DG Mousain, LE Salsac (1982) Estimation of mycelial growth of basidiomycetes by means of chitin determination. Phytochemistry 21:345–348
24. Ruttimann-Johnson C, D Cullen, RT Lamar (1994) Manganese peroxidases of the white rot fungus *Phanerochaete sordida*. Appl Environ Microbiol 60:599–605
25. Tappi methods (1983) Acid insoluble lignin in wood and pulp. T222 om-83
26. Tekere M, R Zvauya, JS Read (2001) Ligninolytic enzyme production in selected sub-tropical white rot fungi under different culture conditions. J Basic Microbiol 41:115–129
27. Thurston CF (1994) The structure and function of fungal laccases. Microbiology 140:19–26
28. Tien M, TK Kirk (1983) Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. Science 221:661–663
29. Wariishi H, K Valli, MH Gold (1991) In vitro depolymerization of lignin by manganese peroxidase of *Phanerochaete chrysosporium*. Biochem Biophys Res Commun 176: 269–275