Production and characterization of laccase and manganese peroxidase from the ligninolytic fungus *Fomes sclerodermeus*

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Abstract: ***Fomes sclerodermeus*** **was grown on semi-defined media based on yeast extract, peptone and glucose (YPG). The fungus produced a minimum basal level of laccase activity irrespective of culture medium. The highest laccase production (20 U cm^{-3}) was obtained in cultures supplemented with $CuSO_4$. Manganese peroxidase (MnP) could only be detected when $MnSO_4$ was added to the medium. None of the aromatic compounds tested stimulated further laccase or MnP production. Laccase and MnP stimulated by Cu^{2+} or Mn^{2+} respectively were purified. Two different laccase isoenzymes with the same molecular mass (67 kDa) and N-linked carbohydrate content (3%) and a slight difference in their p*I* values (3.41 and 3.48) were characterized. In addition, two different MnP isoenzymes with the same molecular mass (47 kDa) and N-linked carbohydrate content (4%) and different p*I* values (3.35 and 3.45) were characterized. Both enzymes showed good stability at 25 °C and over a wide range of pH. Both laccases oxidize ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) more efficiently than 2,6-dimethoxyphenol (DMP) with similar efficiency values (K_{cat}/K_m) while the MnP I, the major peroxidase isoenzyme in the studied conditions, oxidizes the Mn^{2+} and Mn-mediated activity on DMP more efficiently than MnP II.**

Keywords: aromatic compounds; *Fomes sclerodermeus*; laccase; manganese peroxidase; purification

INTRODUCTION

White-rot fungi are organisms capable of degrading lignin to CO_2 and H_2O. These fungi have an oxidative and nonspecific system composed of several extracellular oxidoreductases, low molecular weight metabolites and activated oxygen species.[1] To date, the ligninolytic enzymes which have been found to be involved in this degradative process are laccases and peroxidases, which catalyze the one-electron oxidation of lignin units, producing bond cleavage and other subsequent nonenzymatic reactions.[2]

Three peroxidases involved in lignin degradation are produced by white-rot fungi. Lignin and manganese peroxidases (LiP and MnP) were initially described in *Phanerochaete chrysosporium*.[3] For a long time LiP was considered to be a key enzyme in lignin biodegradation due to its ability to catalyze directly the oxidation of compounds with a high redox potential.[4] However, this enzyme has not been detected in most of the white-rot fungi.[5,6] Hybrid peroxidases sharing the catalytic properties of both LiP and MnP have also been shown to occur in species of *Bjerkandera* and *Pleurotus*.[7]

Laccase is an enzyme secreted by most of the lignin-degrading basidiomycetes,[5,6] and it has been reported to be an essential enzyme for lignin degradation in those fungi without ligninolytic peroxidases, such as *Pycnoporus cinnabarinus* and *Coriolopsis rigida*.[8,9] In addition, laccase is associated with other physiological processes, such as pigment development in *Aspergillus nidulans*,[10] formation of fruiting bodies in *Agaricus bisporus*[11] and phytopathogenic processes in *Heterobasidion annosum*.[12] This enzyme may also play a role in the processes of detoxification of phytoalexins.[13] In recent years, the application of ligninases to the degradation of xenobiotics such as polycyclic aromatic hydrocarbons (PAHs),[14] dyes[15] and a wide range of pesticides[16] has been intensively studied.

The white-rot fungus *Fomes sclerodermeus*, isolated from decayed wood in Tucumán (Argentina), is able to degrade and detoxify the fungicide Malachite Green.[17] The ligninolytic system of *F. sclerodermeus* was efficiently induced by copper or manganese,[18] and wheat straw,[19] producing large amounts of laccase and MnP. The aim of this work was to characterize these ligninolytic enzymes to study their kinetic properties and

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compare them with those secreted by other white-rot fungi. Their effect on the degradation of recalcitrant compounds is discussed.

EXPERIMENTAL

Organism and culture conditions

F. sclerodermeus (Léveillé) Cooke BAFC 2752 (culture collection of the Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires) (= IJFM 772) was maintained in agar (2%)/malt extract (1.3%) slants at 4 °C.

To obtain high and rapid enzyme production the cultures were performed in 2 dm^3 Erlenmeyer flasks containing 200 cm^3 of semi-defined medium (YPG) (shaken at 180 rpm) with the following composition: 2% glucose, 0.5% peptone and 0.2% yeast extract. $CuSO_4$ (0.15 mmol dm^{-3}) and $MnSO_4$ (1.0 mmol dm^{-3}) were added to culture media in order to stimulate laccase and MnP production, respectively. The influence of ferulic, vanillic and veratric acids was tested at 1 mmol dm^{-3}. The pH of the media was adjusted to 5.5 by addition of HCl. The inoculation was carried out using homogenized pellets from 7-day-old shaken cultures.

Analysis of proteins, reducing sugars and enzyme assays

Extracellular protein was determined by the Bradford method,[20] using Bio-Rad protein assay. Reducing sugars were assayed by the method of Somogyi and Nelson.[21]

All enzymes were determined spectrophotometrically at 25 °C in a total volume of 1 cm^3. Laccase activity was determined by oxidation of 2,6-dimethoxyphenol (2,6-DMP) to coerulignone (ε_{469} = 27.5 (mmol dm^{-3})$^{-1}$ cm^{-1}). The reaction mixture contained 2,6-DMP (10 mmol dm^{-3}) in 0.1 mol dm^{-3} sodium tartrate buffer, pH 5. MnP activity was determined by oxidation of Mn^{2+} to an Mn^{3+}-tartrate complex (ε_{238} = 6.5 (mmol dm^{-3})$^{-1}$ cm^{-1}). The reaction mixture contained 50 mmol dm^{-3} succinate buffer pH 4.8, 0.1 mmol dm^{-3} $MnSO_4$ and 0.1 mmol dm^{-3} H_2O_2. LiP was determined by the H_2O_2-dependent formation of veratraldehyde from 2 mmol dm^{-3} veratryl alcohol in 100 mmol dm^{-3} sodium tartrate buffer (pH 3); reactions were started by the addition of H_2O_2 (0.4 mmol dm^{-3}). One unit of enzymatic activity was defined as the amount of the enzyme needed to oxidize 1 μmol of substrate per min.

Purification and characterization of laccase and MnP

Laccase was purified from 9-day-old liquid cultures conducted on YPG medium supplemented with 0.15 mmol dm^{-3} $CuSO_4$. MnP was purified from 4-day-old cultures conducted on the same medium with the addition of 1.0 mmol dm^{-3} $MnSO_4$. The liquid cultures were harvested, filtered and concentrated 20-fold by ultrafiltration at 4 °C (5 kDa cut-off membrane).

Crude supernatants with laccase or MnP activity were dialyzed against 10 mmol dm^{-3} sodium phosphate, pH 6.0, and 10 mmol dm^{-3} sodium tartrate, pH 4.5, respectively. The subsequent purification steps for laccase and MnP were the same as those used by Saparrat *et al*.[9] and Martínez *et al*.[22] respectively. The purification factor was calculated as SpA_0/SpA_x where SpA_0 is initial specific activity and SpA_x is specific activity at the X purification step.

SDS–PAGE was performed on 12% polyacrylamide gels using trypsin (21.5 kDa), carbonic anhydrase (31.0 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa) and phosphorylase *b* (97.4 kDa) as molecular mass standards (Bio-Rad, Hercules, CA, USA). The N-carbohydrate content of purified laccase and MnP was determined by the molecular mass of the deglycosylated enzymes with endoglycosylase-H (endo-H, Boehringer, Mannheim, Germany). Isoelectric focusing (IEF) was performed on 5% polyacrylamide gel with a thickness of 1 mm and a pH range of 2.5–5 (prepared with LKB-Pharmacia [Uppsala, Sweden] ampholites) with 0.5 mol dm^{-3} H_3PO_4 and 0.5 mol dm^{-3} NaOH as the catholyte and anolyte, respectively. The pH gradient was measured on the gel, using a contact electrode. Protein bands, after SDS–PAGE and IEF, were stained with Coomassie Blue R-250. The ratios of absorbance at 280 nm to that at 610 nm (A_{280nm} A_{610nm}^{-1}) for laccases and at 410 nm to that at 280 (A_{410nm} A_{280nm}^{-1}) for MnP were calculated as a characteristic parameter of the respective purified enzymes.

Kinetic studies were performed in 1 cm^3 quartz cuvettes with 1 cm path lengths. Reactions were initiated by the addition of the enzyme in the case of laccases and the addition of H_2O_2 in the case of the peroxidases. The initial rates were obtained from the linear portion of the progress curve. The study was carried out in 0.1 mol dm^{-3} sodium tartrate buffer, pH 5, for both laccase and MnP enzymes. K_{cat} was calculated as V_{max} (molecular mass of deglycosylated enzyme)$^{-1}$, then the value K_{cat} K_m^{-1} was considered as the enzyme efficiency. The substrates used for laccase specificity were DMP and ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (ε_{436} = 29.3 (mmol dm^{-3})$^{-1}$ cm^{-1}). The substrates used for MnP specificity studies were H_2O_2 (in the presence of 0.1 mmol dm^{-3} $MnSO_4$), $MnSO_4$ and DMP (in the presence of 0.1 mmol dm^{-3} H_2O_2) and DMP (in the presence of 0.1 mmol dm^{-3} H_2O_2 and 0.1 mmol dm^{-3} $MnSO_4$).[22]

RESULTS

Enzyme production

The production of laccase and MnP by *F. sclerodermeus* growing on YPG medium was investigated (Fig. 1). YPG medium contains a natural source of nitrogen and vitamins. The highest laccase level (20 U cm^{-3}) was observed in cultures with added copper. In

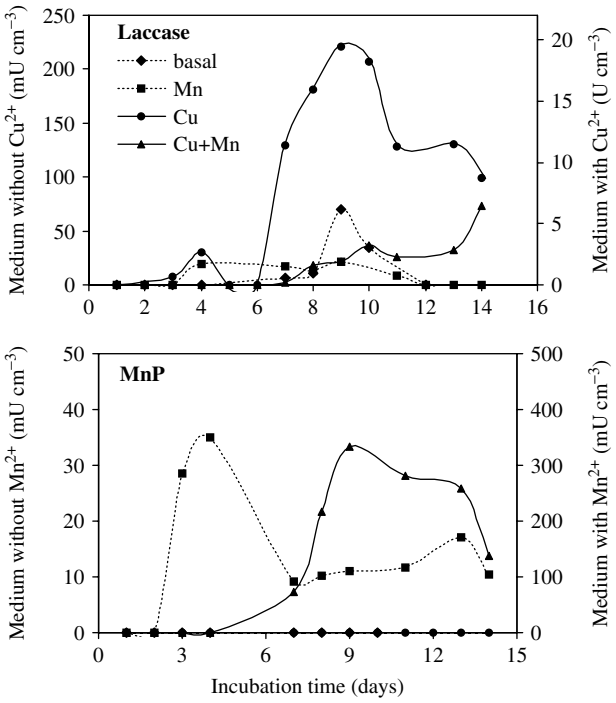

Figure 1. Time course of laccase and MnP activities, in basal medium or media supplemented with $MnSO_4$ (1.0 mmol dm^{-3} (Mn)), $CuSO_4$ (0.15 mmol dm^{-3} (Cu)) or both $MnSO_4$ (1 mmol dm^{-3}) and $CuSO_4$ (0.15 mmol dm^{-3}) (Cu + Mn).

manganese, copper–manganese and control cultures laccase activities were at least four-fold lower.

MnP activities were only observed in manganese- and copper-manganese-supplemented cultures. The highest MnP level (400 mU cm^{-3}) was observed in manganese-supplemented cultures on day 4, while copper–manganese-supplemented cultures produced a similar level on day 9. No MnP activity was detected in copper-supplemented or control cultures.

The effect of aromatic compounds (1 mmol dm^{-3}) on laccase production is shown in Table 1. Laccase production in cultures supplemented with the combination aromatic compound-copper was up to 40 times higher than those cultures containing only added aromatic compound. The highest laccase production was observed with the combination ferulic acid–copper (20.29 U cm^{-3}) on day 11.

Table 1. The influence of aromatic compounds (1 mmol dm^{-3}) and the combination of aromatic compound plus copper (0.15 mmol dm^{-3}) on the production of laccase by *F. sclerodermeus*

Aromatic compound	Laccase (U cm^{-3})
Ferulic acid	0.327 (12)
Vanillic acid	0.109 (12)
Veratric acid	0.445 (11)
Ferulic acid–copper	20.29 (11)
Vanillic acid–copper	6.37 (12)
Veratric acid–copper	8.64 (12)

Values were recorded at day of maximum production (in parenthesis). No MnP was detected under these conditions. Values represent the media of duplicate independent experiments. SEM was less than 10%.

Enzyme purification

Laccase and MnP produced in the YPG medium supplemented with Cu and Mn, respectively, were purified to electrophoretic homogeneity. The purification steps are summarized in Table 2 and Table 3, respectively. Three purification steps (Q-cartridge, Sephacryl S-200 and Mono-Q) were necessary to separate two laccase isoenzymes (Lac I and Lac II), which showed electrophoretic homogeneity after the process. The same chromatographic steps used for laccase were required to purify the MnP from *F. sclerodermeus*. After the last step a major (MnP I) and a minor (MnP II) protein were separated. All the isoenzymes purified showed electrophoretic homogeneity on SDS and IEF gels.

Characterization of isoenzymes

The SDS–PAGE and IEF results for isoenzyme gels are shown in Fig. 2. Both laccase isoenzymes from *F. sclerodermeus* were glycoproteins with a 3% N-linked carbohydrate content and a molecular mass estimated to be around 67 kDa by SDS–PAGE. Analytical IEF showed that both isoenzymes have slightly different p*I* values, 3.41 for Lac I and 3.48 for Lac II. Both MnP isoenzymes from *F. sclerodermeus* were also glycoproteins with a 4% N-linked carbohydrate content and a molecular mass estimated to be around 47 kDa. Analytical IEF showed a slight difference between both isoenzymes, the p*I* being around 3.45 and 3.35 for MnP I and MnP II, respectively.

All of the laccases and MnP studied followed typical Michaelis–Menten kinetics. The UV-visible

Table 2. Scheme of the purification process of laccases from *F. sclerodermeus*

	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Culture liquid	190	20 000	105	100	1.0
Ultrafiltered	123	19 000	154	95	1.5
Q-cartridge	88	15 417	174	77	1.7
Sephacryl	68	15 200	223	76	2.1
Mono-Q MnP I	17	9799	562	49	5.3
Mono-Q MnP II	20	5300	265	27	2.5
Total	37	15 099	403	75	–

Table 3. Scheme of the purification process of MnP from *F. sclerodermeus*

	Protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification factor
Culture liquid	120	350	3	100	1.0
Ultrafiltered	75	325	4	93	1.5
Q-cartridge	47	236	5	67	1.7
Sephacryl	33	230	7	66	2.4
Mono-Q MnP I	12	188	16	54	5.4
Mono-Q MnP II	1	20	15	6	5.3
Total	13	208	16	59	–

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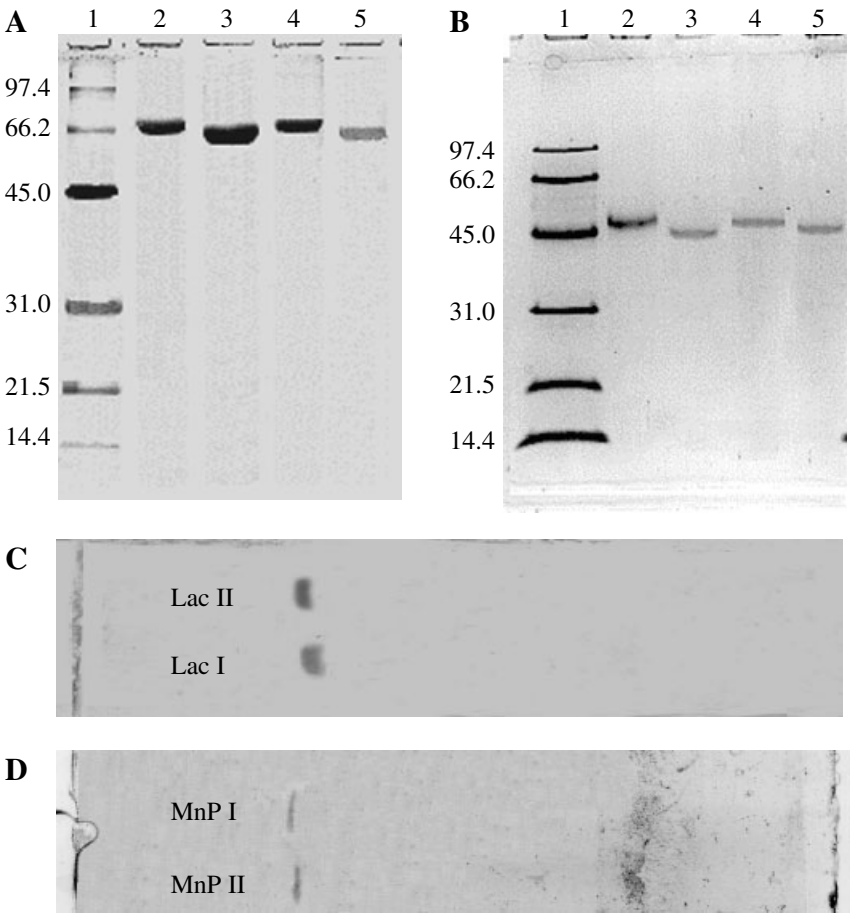

Figure 2. SDS–PAGE (A and C) and IEF (B and D) of laccase (A and B) and MnP (C and D). Lanes in SDS–PAGE: 1, molecular mass standards; 2 and 4, isoenzymes I and II respectively; 3 and 5, deglycosylated isoenzymes I and II respectively.

spectra of native laccase isoenzymes showed the typical characteristics of copper-containing enzymes. Regarding the ratio of absorbances, both laccase isoenzymes showed an $A_{280nm}\ A_{610nm}^{-1}$ value of around 20. On the other hand, $A_{410nm}\ A_{280nm}^{-1}$ values were 4.2 and 3 for MnP I and MnP II, respectively. The kinetic constants of laccases and MnP are summarized in Table 4. The highest efficiency values for both laccases were against ABTS, showing both similar affinity and efficiency on the other substrates assayed. Preliminary studies showed that both laccase isoenzymes degraded benzo[*a*]pyrene in the presence of 1-hydroxybenzotriazole as redox mediator (data not shown). MnP I, the major isoenzyme, showed higher affinity on Mn^{2+} than MnP II and both exhibited a very low Mn^{2+}-independent activity at high DMP concentrations. Both MnP isoenzymes were not saturated even at the highest DMP concentration assayed (Fig. 3).

Both purified enzymes showed activity losses of less than 5% when stored in 50 mmol dm^{-3} citrate phosphate buffer (pH 6) at 4 °C after 1 month. The stability of laccases and peroxidases at room temperature and wide range of pH (2–8.5) were

Table 4. Kinetic constants of laccase and MnP isoenzymes from *F. sclerodermeus*

	K_m		K_{cat}		Efficiency	
	Lac I (mmol dm^{-3})	Lac II (mmol dm^{-3})	Lac I (s^{-1})	Lac II (s^{-1})	Lac I (s^{-1} (mmol dm^{-3})$^{-1}$)	Lac II (s^{-1} (mmol dm^{-3})$^{-1}$)
2,6-DMP	0.177	0.142	206	218	1162.9	1533
ABTS	0.027	0.121	199	182	7382.7	8667
	MnP I (mmol dm^{-3})	MnP II (mmol dm^{-3})	MnP I (s^{-1})	MnP II (s^{-1})	MnP I (s^{-1} (mmol dm^{-3})$^{-1}$)	MnP II (s^{-1} (mmol dm^{-3})$^{-1}$)
H_2O_2 (0.1 mmol dm^{-3} Mn^{2+})	0.011	0.007	415	84	37704.5	12 727
Mn^{2+} (0.1 mmol dm^{-3} H_2O_2)	0.030	0.027	581	285	19375.0	10 556
DMP (0.1 mmol dm^{-3} Mn^{2+} and 0.1 mmol dm^{-3} H_2O_2)	0.029	0.084	257	51	8844.8	607

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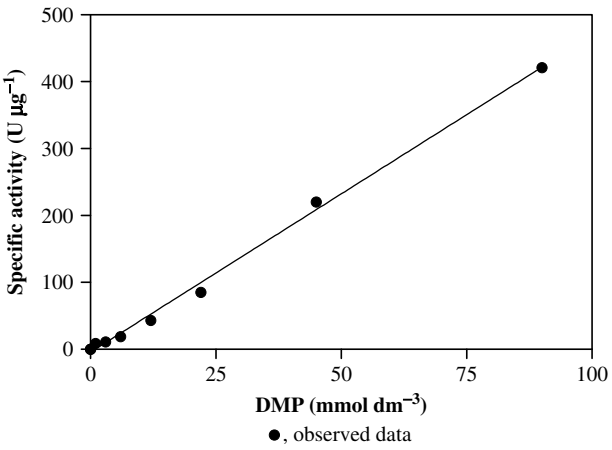

Figure 3. Influence of substrate concentration on specific Mn^{2+}-independent activity of MnP I towards DMP. Observed data were fitted to a straight line by linear regression ($r^2 = 0.99$).

studied. MnP showed complete stability for 24 h, while a drastic reduction in activity occurred at the pH ranges 2–4 and 7–8.5 after 48 h of incubation. Laccase was less stable than MnP at low pH; it lost 90% of activity at pH 2 after 24 h. In the pH range 6–8.5 the laccase was completely stable after 24 h. There was hardly any activity at pH range 2–4 after 48 h, while at pH range 6–8 it was completely stable. Only at pH 6 and after 76 h incubation were both enzymes completely stable.

DISCUSSION

The positive effect of aromatic compounds on ligninases production has been observed in several basidiomycetes,[23,24] including other *Fomes* species.[25] In this study, the addition of aromatic compounds delayed the laccase production by up to 3 days, presumably because of the toxic effects of such compounds on the fungus. The highest level of laccase, in the presence of aromatic compounds, was achieved using the combination of ferulic acid–copper. However, this combination did not further increase the levels obtained in the medium supplemented only with copper. Similar results were observed in *Trametes versicolor* which produced maximal laccase activity in a medium with copper and xylidine.[26] The increased production of fungal laccase by copper has been commonly reported, e.g. *T. pubescens*,[27] *P. chrysosporium*,[28] *T. trogii*[29] and *Coriolopsis rigida*.[9] On the other hand, MnP activity from *F. sclerodermeus* was only detected in cultures supplemented with Mn^{2+}. The requirement for manganese in the culture medium to increase MnP activity is a characteristic common among most of the white-rot fungi.[30] It has been demonstrated that Mn^{2+} regulates the expression of the *mnp* gene.[31] However, the Mn^{2+} repression of an Mn^{2+}-oxidizing peroxidase in *Pleurotus* species has been reported.[22]

The molecular masses of two laccases and two MnP isoenzymes (67 and 47 kDa, respectively) secreted by *F. sclerodermeus* are in the same range as those enzymes reported in the most ligninolytic fungi, such as *P. chrysosporium* BKM-F1767[32] or *Trametes* sp.[33] Purified laccase and MnP can be hydrolyzed by endoglycosidase H, demonstrating that laccase and MnP contain N-glycosylation. MnP are N-glycosylated proteins with a molecular mass between 42 and 53 kDa.[34] The p*I* of laccases and MnP isoenzymes secreted by *F. sclerodermeus* were among the more acid ligninolytic enzymes with a p*I* range of 3.3 to 3.5. The highest p*I* value for MnP has been reported from *P. chrysosporium* (p*I* 4.5–4.9)[35] and the lowest from *T. versicolor* and *Panus tigrinus* (p*I* 2.9).[36] In the case of laccases the greatest difference in p*I* value has been reported in *P. ostreatus* isoenzymes (p*I* 2.9–6.7).[37] Usually laccase isoenzymes, with different molecular masses and p*I* values, have been reported from white-rot fungi, e.g. those from *P. eryngii*.[38] Spectroscopic characteristics of both laccases confirmed that they are members of the group of blue copper oxidases. The $A_{280}\ A_{610}{}^{-1}$ and $A_{410}\ A_{280}{}^{-1}$ values obtained for laccases and MnPs were similar to those obtained for other white-rot fungi.[33,38,39] The catalytic properties were very similar in the Lac I and Lac II from *F. sclerodermeus*, and some differences were detected in MnP I and MnP II, as indicated by the kinetic constants. Both laccases showed the highest affinity and efficiency on ABTS. With respect to MnP, both isoenzymes showed high affinity and efficiency on H_2O_2, on oxidation of DMP and only a very low Mn^{2+}-independent activity on DMP was observed only at very high concentration of substrate, similar to those reported in the case of the MnP from *P. chrysosporium*.[40] The existence of two Mn-oxidizing peroxidases, i.e. those that require Mn^{2+} such as the MnP from *P. chrysosporium*, and those that, showing high Mn^{2+} affinity, also have Mn^{2+}-independent activity, such as those from *Bjerkandera* and *Pleurotus* species, has been reported.[40,41] The present results suggest that MnP from *F. sclerodermeus* requires Mn^{2+} to complete its catalytic cycle and it is similar to *P. chrysosporium* MnP isoenzymes.

Both major ligninases produced in the same strain and their combined production were characterized. *F. sclerodermeus* was shown to produce large amounts of laccase and MnP as its ligninolytic system. Although both enzymes are involved in the degradation of toxic and recalcitrant compounds,[42] most of the studies were performed with laccase, while papers dealing with the production and characterization of both enzymes in the same strain are scarce. Characterization of both ligninolytic enzymes in the same strain contributes to knowledge of the complex process of degradation caused by this group of fungi. In addition, as was demonstrated in *Rigidoporus lignosus*,[43] MnP and laccase may act synergistically, therefore enzymatic supernatants containing both activities could be more efficient in bioremediation processes.

Unlike *P. chrysosporium*, the laccase was produced at a basal level in all media tested; this characteristic in the

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physiology of secretion of ligninases in *F. sclerodermeus* may contribute to the tolerance and detoxification of Malachite Green by this fungus.[17] Copper and manganese were the most efficient inducers of laccase and MnP, respectively. The purification and biochemical characterization of its ligninolytic system showed that it was similar to that found in other white-rot fungi. The role of laccase and MnP in the process of wood degradation is under investigation and these enzymes seem to be new members of the growing families of ligninolytic enzymes for industrial applications.

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