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Ligninolytic Ability and Potential Biotechnology Applications of the South American Fungus *Pleurotus laciniatocrenatus*

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ABSTRACT. The extracellular ligninolytic enzyme system of *Pleurotus laciniatocrenatus*, grown under different culture conditions, was characterized and the ability of this strain to degrade different components of *Eucalyptus globulus* wood was determined. In shaken liquid cultures grown on a C-limited medium supplemented with yeast extract (0.1 %) and peptone (0.5 %), the fungus produced extracellular aryl-alcohol oxidase (Aao), laccase (Lac), manganese-dependent peroxidase (MnP) and manganese-independent peroxidase (MiP) activities, their maximum levels being, respectively, about 600, 50, 1360, and 920 pkat/mL. The supplementation of 1 mmol/L vanillic acid and 150 μmol/L $CuSO_4$ produced an increase of Lac activity levels up to 4-fold and 68.3-fold, respectively. No significant differences were found in the levels of the other ligninolytic enzyme activities when compared to the basal medium. Solid-state fermentation cultures on *E. globulus* wood chips revealed Lac and MiP activities. These cultures showed degradative activity on lignin and lipophilic wood extractives.

Biodiversity, defined as the variety and richness of life forms on Earth, includes genetic pools, species, ecosystems and the ecological processes of which they are a part (Hawksworth 2001). Even though South American biodiversity is well known, this knowledge is mainly based on the study of vascular plants and vertebrates. At the moment, there is no comprehensive study on the wood-rotting fungi from South America, which play a fundamental role in nutrient cycling and are potential tools for biotechnological applications. However, the available evidence suggests that the fungal diversity in this continent is high (Hawksworth 2001; Ryvarden 1996). Therefore, a contribution to the knowledge of fungal diversity from South America would be important for building a better theoretical background, as well as for applied uses in solving real-world problems. In this sense, several white-rot fungi and their extracellular ligninolytic enzymes are currently being studied (Abdullah and Zafar 1999; Cing *et al*. 2003; Johannes and Majcherczyk 2000; Kahraman and Yeşilada 2003; Martínez *et al*. 1994, 1999; Saparrat *et al*. 2002; Tomšovský and Homolka 2003; Wong and Yu 1999).

The genus *Pleurotus* is well known for the production of edible mushrooms and includes strongly ligninolytic species. Different *Pleurotus* species are being extensively studied due to their ability to degrade lignin and other aromatic pollutants causing environmental problems (Cohen *et al*. 2002; Eichlerová *et al*. 2003; Kahraman and Yeşilada 2003; Martínez *et al*. 1994; Novotný *et al*. 1999; Rodríguez *et al*. 2004). These fungi have developed a nonspecific oxidative system to degrade aromatic recalcitrant compounds, including extracellular oxidases and peroxidases, low-molar-mass metabolites and activated oxygen species (Sannia *et al*. 1986, 1991; Bourbonnais and Paice 1989; Guillén *et al*. 1994, 2000; Martínez *et al*. 1996; Muñoz *et al*. 1997; Giardina *et al*. 2000).

Pleurotus laciniatocrenatus (SPEG.) SPEG. (*Agaricales*, *Basidiomycetes*) is a South American fungus, largely found in warm and temperate regions of Argentina, Brazil, Paraguay and Uruguay, which has been relatively little studied compared to other *Pleurotus* species. *P. laciniatocrenatus* has shown capability to colonize different lignocellulosic substrates (Salusso and Moraña 1997) and ABTS-oxidizing activity in solid medium (Saparrat *et al*. 2000). However, no information about the enzyme system of this fungal species has been reported. The aim of our work was to provide knowledge on the enzyme composition of the ligninolytic system of *P. laciniatocrenatus* (CLPS 39 strain), grown under different culture conditions, as well as its ability to degrade different components from *Eucalyptus globulus* LABILL. wood. This approach

would contribute to explore autochthonous fungal species for biotechnological processes and increase our knowledge about the enzyme characteristics and ecophysiology of South American fungi.

MATERIALS AND METHODS

Fungal strain. *Pleurotus laciniatocrenatus* CLPS 39 (*Culture Collection of the La Plata Spegazzini Institute*, Argentina) was isolated from the tissue of a fruiting-body collected from the trunk of a *Taxodium* sp. living tree in an urban forest area (Parque Pereyra woodland; Buenos Aires Province, Argentina). Stock cultures were maintained on slants containing malt extract agar supplemented with 0.4 % yeast extract and *Populus nigra* L. wood chips at 4 °C.

Liquid cultures. The production of extracellular ligninolytic enzymes was carried out on a basal C-limited medium supplemented with 0.1 % yeast extract and 0.5 % peptone (Saparrat *et al*. 2002). The effect of $CuSO_4 \cdot 5H_2O$ (150 µmol/L) and vanillic acid (1 mmol/L) on the extracellular ligninolytic enzyme system was tested by adding them on the 3rd day of incubation to cultures grown on basal medium. The strain was cultivated in 1-L Erlenmeyer flasks with 200 mL medium, under shaking (2.5 Hz) at 25 ± 1.5 ºC; a mycelial suspension was used as inoculum (Saparrat *et al.* 2002). The cultures were done in three replicates. Aliquots of 5 mL were taken periodically from each replicate flask. The mycelium was separated from the culture liquid by centrifugation (10 000 ***g***, 4 ºC, 30 min). The supernatant of the liquid culture was collected to measure reducing sugars, protein, pH and enzyme activity.

Solid-state fermentation (SSF). The CLPS 39 strain was cultivated in 100-mL Erlenmeyer flasks containing 2 g (dry mass) of *E. globulus* wood chips (1–2 × 10–20 mm) and 5 mL water (sterilized at 121 ºC for 2 periods of 30 min) that were inoculated with two mycelium plugs (diameter 5 mm) from cultures on 2 % malt extract agar. The inoculated flasks were incubated at 28 ± 1.5 ºC under stationary conditions and constant humidity. Six replicates were done in parallel. Uninoculated sterilized flasks containing wood were incubated under the same conditions as control. After 15 and 30 d, three replicate flasks were analyzed for the presence of ligninolytic enzyme activity (expressed as pkat/g wood). The extraction of enzymes from wood treated with the fungus was carried out by adding 10 mL of 50 mmol/L sodium tartrate buffer (pH 5) to SSF cultures and shaking (0.83 Hz, 2 h, 4 ºC); the enzyme extracts were obtained by filtration and dialysis against the same extraction buffer. Simultaneously, the other three cultures were analyzed in order to estimate wood mass loss and the degradation of Klason lignin and acetone extractives of eucalypt wood treated with the fungus.

Analytical methods. Extracellular protein was determined according to Bradford (1976), using *Bio-Rad* protein assay and bovine serum albumin as standard. Reducing sugars were assayed by the Somogyi and Nelson method (Somogyi 1945), using glucose as standard. Dry mass of wood was determined at 60 ± 5 ºC in an aerated oven for 12 h. Lipophilic wood-acetone extract compounds were obtained *via* Soxhlet extraction of sawdust (<0.4 mm), from control wood and from wood treated with the fungus, with acetone for 6 h (Martínez *et al*. 1999). The acetone extracts were evaporated to dryness for mass estimation. Klason lignin content was determined according to *TAPPI* (1993). The determinations were done in triplicates.

Enzyme assays. Aryl-alcohol oxidase (EC 1.1.3.7; Aao) activity was estimated through veratraldehyde formation from 5 mmol/L veratryl alcohol (*Fluka*) in 0.1 mol/L phosphate buffer, pH 6 (Guillén *et al*. 1994). Laccase (EC 1.10.3.2; Lac) activity was measured using 5 mmol/L 2,6-dimethoxyphenol (*Fluka*) in 0.1 mol/L sodium tartrate buffer, pH 3 (Martínez *et al*. 1996). Lignin peroxidase (EC 1.11.1.14; LiP) activity was determined by H_2O_2-dependent veratraldehyde formation from 2 mmol/L veratryl alcohol in 0.1 mol/L sodium tartrate buffer, pH 3; all reactions were started by the addition of 0.4 mmol/L H_2O_2 (Saparrat *et al*. 2002). Manganese-dependent peroxidase (EC 1.11.1.13; MnP) activity was estimated by the formation of Mn^{3+}-tartrate complex during the oxidation of 0.1 mmol/L Mn^{2+} (as $MnSO_4$) in 0.1 mol/L sodium tartrate buffer (pH 5) in the presence of 0.1 mmol/L H_2O_2 (Martínez *et al*. 1996). Manganese-independent peroxidase (EC 1.11.1.7, MiP) activity was assayed as Lac activity in the presence of 0.1 mmol/L H_2O_2. All the oxidation rates were determined at 25 ºC, using a *Beckman* DU 640 UV–VIS spectrophotometer. The enzyme activity is expressed in pkat (picokatals; 1 pkat is the enzyme activity releasing 1 pmol of oxidized product per s).

Statistical analysis. The effect of vanillic acid and $CuSO_4$ on extracellular ligninolytic enzyme activity levels, compared to cultures grown on basal medium, was analyzed statistically using one-way ANOVA with Tukey's honestly significant difference contrasts.

RESULTS AND DISCUSSION

P. laciniatocrenatus grown under shaking in a C-limited medium with 0.1 % yeast extract and 0.5 % peptone was found to be in trophophase until the 6th day of incubation, exhibiting an idiophase under C-limited conditions during the rest of the incubation (Fig. 1). A slight increase in the initial pH (5.8) was observed during the trophophase, followed by an acidification at the beginning of idiophase and a further increment of pH to values close to neutrality. Extracellular proteins exhibited a pattern of net consumption during the trophophase, followed by a production peak (121 ± 9.6 μg/mL) after 9 d. Shaken cultures of P. *laciniatocrenatus* on C-limited peptone–yeast extract medium revealed different ligninolytic enzyme activities. Peptone has been reported to contain peptides that induce the secretion of peroxidases in *Pleurotus* species and other basidiomycetes (Kaal *et al*. 1993; Martínez *et al*. 1996). Whereas MnP activity with maximum levels ≈1360 pkat/mL was detected during the trophophase, Aao activity, which attained a peak of 600 pkat/mL, was only detected after glucose depletion. However, Lac activity, which revealed low levels (50 pkat/mL), and MiP activity, whose maximum was about 920 pkat/mL, were detected in relation to both the trophophase and under C-limited conditions. No LiP activity was detected in the supernatant of liquid cultures under our working conditions. In this sense, it is necessary to remark that a Mn^{2+}-oxidizing peroxid-

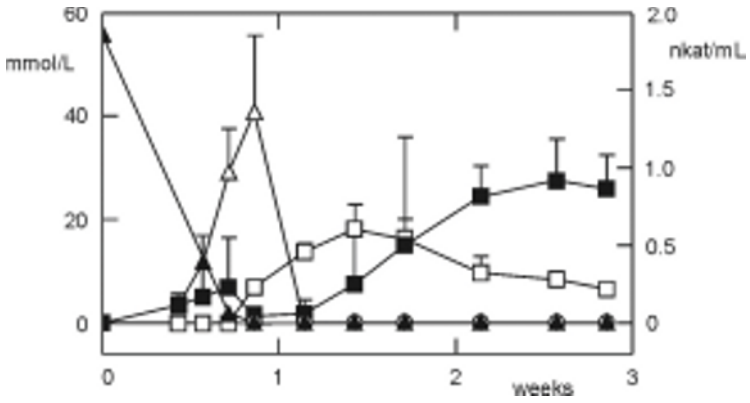

Fig. 1. Time course of enzyme activities (nkat/mL; Aao, *open squares*; Lac, *circles*; MiP, *closed squares*; MnP, *triangles*) and reducing sugars (mmol/L; *closed triangles*) in the extracellular fluid of shaken *Pleurotus laciniatocrenatus* cultures grown in C-limited peptone–yeast extract medium; means of three replicates ± SD.

ase, sharing catalytic properties with LiP and MnP, was first reported for *P. eryngii* (Martínez *et al*. 1996). This enzyme, called afterwards versatile peroxidase (VP) and reported in other *Pleurotus* and *Bjerkandera* species (Camarero *et al*. 1996; Sarkar *et al.* 1997; Heinfling *et al*. 1998; Mester and Field 1998), showed a high activity on Mn^{2+} but also on phenolic and nonphenolic aromatic compounds without Mn^{2+}. The MnP and MiP activities detected in *P. laciniatocrenatus* could correspond to the VP enzyme. However, the differences in the production pattern of MnP and MiP in this fungus suggest that the MnP activity could be related to the Mn^{2+}-oxidizing enzyme reported for other *Pleurotus* species, whereas the MiP activity could correspond to a different type of peroxidase in these fungi. Such activity had been reported only in *Bjerkandera* sp. (de Jong *et al*. 1992).

The addition of $CuSO_4$ or vanillic acid to the basal medium did not produce significant differences in the patterns and levels of extracellular Aao and MnP activities, and in the pH, proteins and reducing sugars under our experimental conditions. However, the presence of both compounds affected the Lac and MiP activities (Table I). Lac activity was significantly increased by the addition of vanillic acid to a maximum level of 200 pkat/mL on the 5th day ($p \leq 0.05$). This value represents a 4-fold increase, compared to cultures grown on basal medium. The increase in Lac activity levels by this compound was previously reported for other white-rot basidiomycetes, including *Pleurotus* species (Hatakka 1994; Muñoz *et al*. 1997; Farnet *et al*. 1999). The Lac activity of *P. laciniatocrenatus* was also increased by the addition of $CuSO_4$, showing a peak titer after 14 d of incubation (3420 pkat/mL, $p \leq 0.01$). In this case, the resulting activity level was 68 times higher than that found in the basal medium. A strong induction of Lac activity by copper had been also described in the basidiomycetes *Coriolopsis rigida*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Trametes versicolor* (Collins and Dobson 1997; Dittmer *et al*. 1997; Palmieri *et al*. 2000; Saparrat *et al*. 2002). No significant differences were found in MiP activity levels in the supplemented cultures in relation to control ones, although the presence of $CuSO_4$ revealed the maximum activity earlier than in the basal medium, which was significantly different from the results obtained with vanillic acid ($p \leq 0.05$). Although no increase of peroxidase activity has been found in *P. laciniatocrenatus* cultures with the above

compounds, it has been reported that they are effective inducers of these enzymes in other basidiomycetes (Rogalski *et al*. 1991; Hatakka 1994; Schlosser *et al*. 1997; Levin *et al*. 2002).

Table I. Effect of 150 μmol/L $CuSO_4$ (Cu) and 1 mmol/L vanillic acid (Va) on the levels of extracellular Lac and MiP activities (pkat/mL; mean ± SD of three replicates) in liquid cultures of *P. laciniatocrenatus* grown on a C-limited peptone–yeast extract medium.

Culture time d	Lac activity			MiP activity		
	control	+ Cu	+ Va	control	+ Cu	+ Va
4	20 ± 0	50 ± 30	180 ± 20	170 ± 240	290 ± 230	240 ± 0
5	40 ± 10	160 ± 60	200 ± 10	320 ± 230	820 ± 220	360 ± 310
6	20 ± 20	670 ± 220	40 ± 10	40 ± 60	360 ± 40	0 ± 10
8	30 ± 0	1300 ± 350	30 ± 10	60 ± 90	300 ± 140	10 ± 10
10	30 ± 10	1480 ± 570	30 ± 10	250 ± 360	570 ± 180	140 ± 70
12	40 ± 10	2910 ± 470	20 ± 10	500 ± 330	460 ± 230	330 ± 120
15	50 ± 0	3420 ± 680	30 ± 10	810 ± 200	330 ± 120	1300 ± 70
18	50 ± 10	3120 ± 100	40 ± 0	920 ± 270	260 ± 110	1250 ± 70
20	30 ± 0	3040 ± 110	30 ± 10	870 ± 210	180 ± 130	1200 ± 160

The existence of different ligninolytic enzymes depends on the fungal species, though it has been reported that differences in the enzyme expression could be a consequence of the culture conditions (Rogalski *et al*. 1991; Hatakka 1994; Ruiz-Dueñas *et al*. 1999). The ligninolytic enzymes of *P. laciniatocrenatus* on eucalypt wood SSF cultures, as well as the degradation of lignin and lipophilic wood extracts, are shown in Table II. This wood was chosen since it is currently used as raw material for paper pulp manufacturing in certain Latin American countries, *e.g*., Brazil, Argentina and Uruguay. In spite of the inducing effect of lignin and/or lignocellulose on the activity and expression of the ligninolytic enzyme system in different basidiomycetes (Rogalski *et al*. 1991; Muñoz *et al*. 1997; Schlosser *et al*. 1997), only extracellular Lac and MiP activities were found in *P. laciniatocrenatus* SSF enzyme extracts. Similar results were reported for other *Pleurotus* species under similar conditions (Rodríguez *et al*. 2004). In *P. laciniatocrenatus* SSF cultures the mass loss of eucalypt wood was also determined, as well as the degradation of lignin and acetone wood extractives after 15 and 30 d of incubation (Table II). The low mass loss of the wood treated with *P. laciniatocrenatus* is a prerequisite for wood pretreatment in paper pulp manufacture (to avoid yield loss in the process). The percentage of lignin and acetone wood extractive degradation was increased about twice after 30 d compared to those obtained after 15 d. The capacity of different *Pleurotus* species and other basidiomycetes to degrade lignin has been related to ligninolytic enzyme production (Hatakka 1994; Camarero *et al*. 1996). It is necessary to emphasize that basidiomycetes have been described as the most adequate fungi for pith biocontrol in eucalypt wood SSF cultures (Martínez *et al*. 1999) and the use of laccases and peroxidases seems to be also involved in the transformation of wood extractives (Buchert *et al*. 2001; del Río *et al*. 2002).

Table II. *Eucalyptus globulus* wood treatment with *Pleurotus laciniatocrenatus*

Incubation time, d	Enzyme activity[a], pkat/g wood		Wood mass loss[a,b] %	Lignin degradation[a,b,c] %	Acetone-extractive removal[a,b], %
	Lac	MiP			
15	470 ± 120	150 ± 60	1.8 ± 0.5	1.9 ± 0.6	11.7 ± 3.6
30	480 ± 160	160 ± 50	2.4 ± 1.0	3.6 ± 1.1	21.6 ± 4.1

[a]Means ± SD of three replicates. [b]Degradation in relation to the content of uninoculated sterilized wood chips (control).
[c]Analyzed by the Klason method.

The genus *Pleurotus* and/or its enzymes are being studied for applications in different industries (edible protein production, applications in paper and pulp production and bioremediation studies). Our work provides preliminary information on the ability of a South American *Pleurotus* strain to produce enzymes that are currently being tested for biotechnological applications from other wood fungi, and to degrade lignin and extractives from *E. globulus* wood. Since a thorough knowledge of the biodiversity of a given area

permits the exploration of potential uses of autochthonous biological resources for the implementation of new environmentally-sound methods and technology, the relevance of studies focusing on our fungal diversity must be emphasized.

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