



Ligninolytic enzymes production by *Dichostereum sordulentum* cultures in the presence of *Eucalyptus* bark as a natural laccase stimulator

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ABSTRACT

A strain of a fungal species, *i.e.*, *Dichostereum sordulentum*, not yet isolated from either Uruguay or decayed *Eucalyptus* spp. wood, was investigated for its ability of producing extracellular ligninolytic enzymes. To this aim, the strain was grown on a N-rich liquid medium added with either bark or wood *Eucalyptus dunnii* sawdust. The former additive was more effective than the latter instimulating laccase leading to a production of 4.78 IU mL⁻¹ which was further enhanced by the concomitant presence of 80 ppm CuSO₄ (6.56 IU mL⁻¹). The two order of magnitude higher proanthocyanidin content in bark than in wood suggests that this fraction might be responsible for the higher stimulatory effect of the former on laccase production.

Indexing terms/Keywords

Bark; *Dichostereum sordulentum*; *Eucalyptus*; laccase; ligninolytic enzymes.

Academic Discipline And Sub-Disciplines

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INTRODUCTION

In Uruguay, the forestry industry has grown in the last 20 years. Recently planted area has reached 1.812.000 ha, and the zone destined for *Eucalyptus* plantation raised 694.000 ha in 2012 [1].

Eucalyptus wood is used to produce cellulose pulp and significant amounts of bark are generated as byproducts. *Eucalyptus* bark composition is well known to be rich in extractives. In recent years, lipophilic fractions have been shown to contain triterpenic acids [2] and around twenty phenolic compounds were identified from methanolic extracts of different *Eucalyptus* species [3].

In nature, lignin is efficiently decomposed by Basidiomycetes belonging to the ecological group of white-rot fungi. Due to the structural complexity of lignin and its random phenylpropanoic polymeric structure, enzymes involved in its degradation rely on radical-based reactions and exhibit a very low substrate specificity [4]. Three are the most important extracellular enzymes involved in this process, lignin peroxidases, manganese peroxidases and laccases. In general, laccases and Mn-peroxidases are more widely distributed among white-rot fungi than lignin peroxidases [5].

Manganese peroxidase (MnP, EC 1.11.1.13) is one of the main oxidoreductases involved in lignin degradation [6, 7]. It is an extracellular glycosylated heme protein produced by almost all wood-colonizing white-rot fungi. MnP catalyzes the H₂O₂-dependent oxidation of Mn²⁺ to Mn³⁺, which, in turn, acts as a non-specific oxidant that attacks phenolic lignin structures by a one-electron abstraction [8]. As MnP, lignin peroxidase (LiP, EC 1.11.1.14) is an extracellular glycoprotein able to catalyze the oxidation of a large number of aromatic compounds by H₂O₂ [9]. The catalytic mechanism for lignin peroxidase is similar to that of other peroxidases [10, 11].

Fungal laccases (LAC, EC 1.10.3.2) are multi-copper phenol oxidases that oxidize many phenolic compounds and aromatic amines using molecular oxygen as the terminal electron acceptor [12].

The degradation pattern of these enzymes has high potential for the delignification of wood and non-wood plant materials in biopulping and bioethanol production applications [13, 14].

The main aim of this study was to evaluate the ability of a fungal isolate collected for the first time from both bark and decayed wood in Uruguayan *Eucalyptus* plantations, to produce ligninolytic enzymes (i.e., LAC, MnP and LiP). This capacity has not been previously studied in this fungus. Bearing in mind the isolation medium of this strain, the possible stimulatory effect of *Eucalyptus dunnii* wood and bark on ligninolytic enzyme production was assessed.

MATERIALS AND METHODS

Wood rotting fungi collection, isolation and identification

Fruit bodies present on bark and white rotted wood from *Eucalyptus globulus* plantations in Palmar de Quebracho, Paysandú, Uruguay (31°55'12" S, 57°42'48" W) were collected during studies of the wood rotting Basidiomycetes of *Eucalyptus* species from Uruguay. Specimens were hand collected and transferred to the laboratory in paper bags. For those species previously unrecorded on *Eucalyptus* in Uruguay [15, 16], were obtained isolates and voucher specimens were deposited at the herbarium of the Facultad de Ciencias, Montevideo, Uruguay (MVHC). Isolation was performed by aseptically excising small pieces of context which were plated onto Petri dishes containing 2% Malt extract agar (MEA; 2% malt extract, 1.5% agar, Difco). Pure cultures were transferred onto slants containing fresh 2% MEA and deposited in the culture collection of the Mycology Laboratory of Facultad de Ciencias, Universidad de la República. Fungal identification was performed according to micro- and macro-morphological characteristics of the fruit bodies, as described previously [16] (Martínez and Nakasone, 2010). A preliminary assessment of the possible occurrence of ligninolytic enzyme was performed on fungal cultures grown on MEA using the Stalpers' enzyme test, viz. α -naphthol (on laccase), 1% pyrogallol 0.4% hydrogen peroxide (on peroxidase) [17]. The strain was kept at the Collection of the Biocatalysis and Biotransformation Laboratory, Facultad de Química (Montevideo, Uruguay) as DS1488.

Analytical determinations of *Eucalyptus dunnii* wood and bark.

Healthy *E. dunnii* bark and wood of 18 year-old trees were collected from the Estación Experimental Prof. Bernardo Rosengurtt, Cerro Largo, Uruguay (32°21'57"S, 54°26'25" W). Samples were dried to constant weight at 100 °C, and then ground prior to analysis. Lignin determination was performed according to NREL method [18].

All samples (30 g) were sequentially extracted in a Soxhlet apparatus with 300 mL dichloromethane and then with 300 mL methanol for 25 cycles each. The solvents were rota-evaporated to dryness. The results were expressed on percent of dry wood and bark. The extracts were analyzed for their total phenolic and proanthocyanidins contents (please, see subsections 2.1 and 2.2, respectively). All determinations were performed in triplicate and the respective mean values \pm standard deviations were calculated.

Total phenol content

Total phenol content was determined by the Folin-Ciocalteu method [19, 20]. In particular, 2.5 mL of Folin-Ciocalteu reactive, previously diluted with water (1:10, v/v), and 2.0 mL of an aqueous solution of sodium carbonate (75 g L⁻¹) were added to 0.5 mL of a properly diluted aqueous solution of the extract. The mixture was kept for 5 min at 50 °C and, after cooling, the absorbance at 760 nm was measured. The total phenol content was expressed as gallic acid equivalent from a calibration curve of gallic acid standard solutions (15-100 μ g mL⁻¹).



Proanthocyanidin content

The proanthocyanidin determination was carried out by the vanillin-HCl method [21] with some modifications. In particular 1.0 mL of aqueous extract was mixed with 2.5 mL of a freshly prepared 1% vanillin solution (w/v) in methanol and 2.5 mL of 9 mol L⁻¹ HCl in methanol were then added. The reaction mixture was incubated for 30 min at room temperature and the absorbance measured at 500 nm. The results were expressed as mg of proanthocyanidins gr⁻¹ of extract [22]. The analyses were done in triplicate and the mean value calculated.

Enzyme production

The fungal strain DS-1488 was maintained on potato dextrose agar (PDA) slants at 4 °C and routinely sub-cultured every six months to maintain its viability. A preliminary approach to detect enzymes production was performed using Stalpers methodology [17].

The pre-inoculum was prepared by adding with five mycelial plugs (1 cm diameter), obtained from actively growing PDA cultures, to 500 mL Erlenmeyer flasks containing a liquid medium (100 mL) with the following composition (%): malt extract, 5; bacto-peptone, 1; *E. dunnii* bark/wood sawdust, 0.25 (MEPWS medium). The flasks were incubated at 28 °C for 7 days under orbital shaking (130 rpm). At the end of the incubation, cultures were then homogenized with the UltraTurrax T18 (IKA Labortechnik, Staufen, Germany) and used as the inoculum.

The evaluation of ligninolytic enzyme production were performed in liquid cultures under slurry conditions. In particular, the MEPWS medium was modified by adding either bark or wood *E. dunnii* sawdust at 5% (w/v) and from here onwards referred to as MMEPWS. One of the following enzyme inducers (i.e., CuSO₄, 80 ppm, MnSO₄, 24 ppm) were added to this modified basal production medium. Cultures were performed in 500 mL Erlenmeyer flask containing 100 mL MMEPWS medium either in the presence or in the absence of the above mentioned inorganic inducers and incubated at 28 °C for 16 days under orbital shaking (130 rpm). Fungal cultures, grown in parallel on malt extract peptone medium, were used as the control. Samples were collected at start and after 4, 8, 12 and 16 days incubation.

Enzyme determination

Laccase activity was assayed spectrophotometrically at 35 °C using 2 mM 2,6-dimethoxyphenol as the substrate in 0.1 M acetate buffer pH 4.5 and monitoring the formation of 3,3',5,5'- tetramethoxy-*p*-diphenoquinone at 477 nm ($\epsilon = 14,600 \text{ M}^{-1}\text{cm}^{-1}$). Total oxidase activity was determined with the same substrate solution added with 0.5 mM MnSO₄ and 0.2 mM H₂O₂.

Manganese peroxidase activity was determined by subtracting laccase activity from total oxidase activity. Lignin peroxidase was measured by monitoring veratryl alcohol (VA) oxidation at 25°C in reaction mixtures containing 2 mM VA in 0.1 M sodium tartrate buffer (pH 3.0) and 10 μL of culture filtrate in a total volume of 1 mL. The reaction was started by adding H₂O₂ to a final concentration of 0.2 mM and the increase in absorbance at 310 nm ($\epsilon = 9,300 \text{ M}^{-1}\text{cm}^{-1}$) was monitored over time.

All activities were expressed as international units (IU), defined as the amount of enzyme which produces 1 μmol of product per minute under the assay conditions.

RESULTS AND DISCUSSION

Fruit bodies of the white rot fungus studied were identified as *Dichostereum sordulentum* (Cooke and Masee) Boidin and Lanquetin based on macro- and microcharacteristics of the basidiocarps based on descriptions of Boidin and Lanquetin (1980) [23]. Voucher specimen was deposited at the herbaria of Facultad de Ciencias, Montevideo, Uruguay, with the number MVHC5370, being the the first record in Uruguay of this species, which had been previously collected in other South American countries and USA [23, 24, 25]. In addition, to the best of our knowledge, isolation from fruit bodies and decaying wood was performed. Oxidative tests, conducted by adding α -naphthol to PDA growing cultures, showed the presence of laccase activity. Conversely, the same tests for peroxidase were negative. Although these are semiquantitative tests, the high positive laccase reaction observed and the absence of information regarding the ligninolytic enzyme production by this species prompted us to perform further experiments in liquid cultures in the presence of sawdust derived from the same medium from which *D. sordulentum* had been isolated, namely *E. dunnii* wood and bark. The combined production of MnP and LAC has been reported to commonly occur among selective lignin-degrading white-rot fungi [6]. In the present study, regardless of the medium composition, this enzyme production pattern was evident for *D. sordulentum* DS1488. Both enzyme activities were even detected in fungal cultures performed in the malt extract and peptone control medium (LAC 0.72 IU mL⁻¹ and MnP 0.21 IU mL⁻¹ on day 16) (Figures 1 and 2). In both non-supplemented and supplemented cultures, the highest MnP and LAC activities were observed at the end of the incubation. For this reason, comparisons reported in Figure 1 and 2 regard 16-day-old cultures.

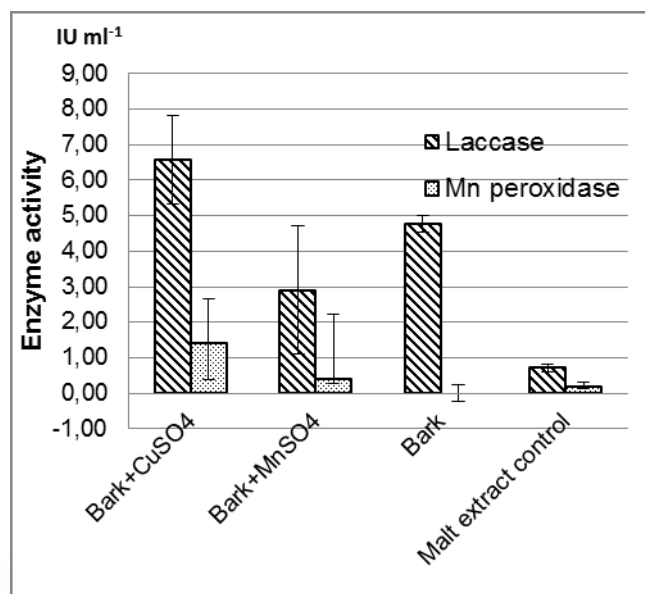


Figure 1 – LAC and MnP activity using *Eucalyptus* bark: Laccase and Manganese peroxidase activities in 16-day-old *D. sordulentum* culture broths from control cultures grown on the malt extract-peptone control medium and in the presence of 5% (w/v) *E. dunnii* bark sawdust alone or added with either 80 ppm CuSO₄ or 24 ppm MnSO₄

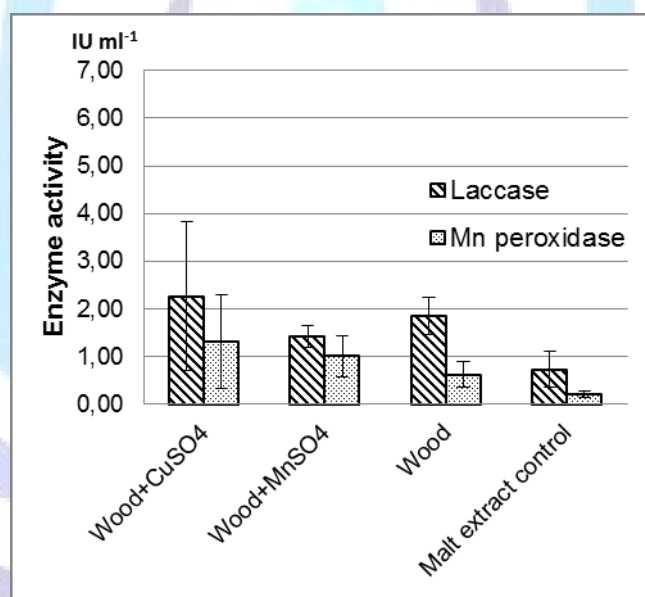


Figure 2 – LAC and MnP activity using *Eucalyptus* wood: Manganese peroxidase activities in 16-day-old *D. sordulentum* culture broths from control cultures grown on the malt extract-peptone control medium and in the presence of 5% (w/v) *E. dunnii* wood sawdust alone or added with either 80 ppm CuSO₄ or 24 ppm MnSO₄.

The addition of either bark or wood sawdust had a marked stimulatory effect on laccase production leading to a 6.6- and 2.6-fold increase, respectively, as compared to that found in control cultures. Conversely, MnP production was totally suppressed in the bark sawdust-supplemented cultures while a 3-fold increase was found in wood sawdust-supplemented ones.

In order to enhance ligninolytic enzyme production, two widely known inorganic inducers, cupric and manganous ions, were added to cultures containing either bark or sawdust. A further increase in LAC production was only observed in cultures that underwent concomitant supplementation of bark and Cu²⁺, reaching an activity peak of 6.56 IU mL⁻¹. Conversely, in wood-supplemented cultures, LAC activity was lower in the presence of Cu²⁺ than in its absence (2.27 vs. 4.78 IU mL⁻¹). In this respect, transcriptional activation of laccase genes by copper has been shown for several white-rot fungi [26, 27, 28, 29, 30] and the presence of putative metal response elements in the promoter of *P. ostreatus* laccase genes has been shown [31].



The stimulatory effect of Mn^{2+} on MnP production is well established [32, 33]. In the present study, a MnP activity of 0.42 IU mL⁻¹ was observed in cultures that underwent concomitant addition of bark and Mn^{2+} . Thus, the presence of Mn^{2+} appeared to counteract the suppression of this activity that had been observed in cultures supplemented with bark alone. In wood-supplemented cultures, instead, the concomitant presence of Mn^{2+} led to a moderate increase from 0.62 U to 1.0 IU mL⁻¹ with respect to cultures supplemented with wood sawdust alone. Mn^{2+} exerted a depressive effect on laccase production in cultures supplemented with either wood or bark with respect to cultures supplemented with these additives alone (2.91 vs. 4.78 IU mL⁻¹, respectively, and 1.42 vs. 1.85 IU mL⁻¹, respectively).

In summary, best results were obtained in cultures undergoing concomitant bark and Cu^{2+} addition, where laccase and MnP activities were 9 and 7 times higher than those found in the control medium. Bark was more effective than wood in stimulating laccase production. Table 1 shows that the methanol extracts from *E. dunnii* wood and bark had very similar total phenolic contents (321.4 vs. 398.4 mg GAE g⁻¹ d.w.) while they differed in their respective proanthocyanidins contents by two orders of magnitude (3.4 vs. 321.4 mg g⁻¹ dw). In this respect, this is in line with Pinto and collaborators suggesting that the most significant differences between *Eucalyptus* bark and wood stemmed from their contents in polar compounds [34]. It has been reported that the main polar extractives in the *Eucalyptus* bark are tannins, polyphenolic compounds and flavonoids [35]. Results of the present study suggest that *E. dunnii* bark sawdust might be a valuable stimulator of laccase production in liquid cultures.

Table 1– Soluble and Insoluble lignin, Total phenol and Proanthocyanidins content of *Eucalyptus dunnii* bark and wood

| <i>E. dunnii</i> sawdust from | Insoluble lignin (%) | Soluble lignin (%) | Total phenol (mg GA† g ⁻¹ d.w.) | Proanthocyanidins (mg g ⁻¹ d.w.) |
|-------------------------------|----------------------|--------------------|--|---|
| Bark | 16,40 ± 0,21 | 3,89 ± 0,03 | 398,2 ± 9,3 | 229,1 ± 5,3 |
| Wood | 20,0 ± 0,55 | 2,53 ± 0,05 | 321,4 ± 7,5 | 3,4 ± 0,2 |

†GA: gallic acid

CONCLUSION

A species of the white-rot Basidiomycete present in *Eucalyptus* plantations and previously not recorded in Uruguay was isolated and identified as *D. sordulentum* [15, 16]. To the best of our knowledge, pure cultures obtained from fruit bodies of this species was carried on for regarding the *D. sordulentum* ability for producing extracellular ligninolytic enzymes.

The results showed that this species produces both Laccase and Manganese peroxidase with the former enzyme being predominant; under the tested conditions, no Lignin peroxidase activity was observed.

The better efficacy of bark in stimulating LAC production than wood might stem from its higher proanthocyanidins content.

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